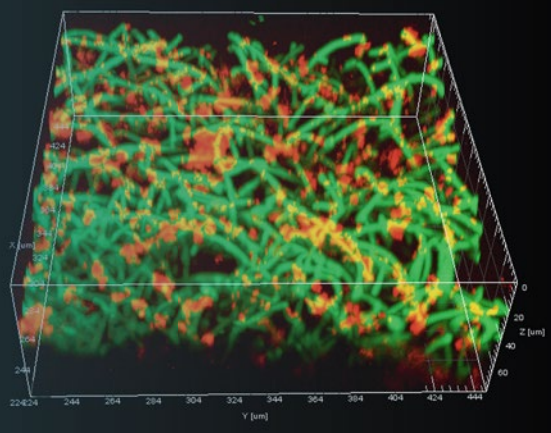
Methods in Molecular Biology 1356

Springer Protocols



Richard Calderone
Ronald Cihlar *Editors*

Candida Species

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springer.com/series/7651

Candida Species

Methods and Protocols

Edited by

Richard Calderone and Ronald Cihlar

Department of Microbiology & Immunology, Georgetown University Medical Center, Washington, DC, USA

Humana Press

Editors
Richard Calderone
Department of Microbiology & Immunology
Georgetown University Medical Center
Washington, DC, USA

Ronald Cihlar
Department of Microbiology & Immunology
Georgetown University Medical Center
Washington, DC, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-3051-7 ISBN 978-1-4939-3052-4 (eBook)
DOI 10.1007/978-1-4939-3052-4

Library of Congress Control Number: 2015944317

Springer New York Heidelberg Dordrecht London

Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

Preface

It has been almost 10 years since we were first asked to edit a volume on *Candida albicans* methods for the Methods in Molecular Biology series. Since that time, many significant developments have been reported in genetic, molecular, and diagnostic investigations concerning *C. albicans* and related species. Of course, these studies also emphasized the use of novel methods that proved indispensable to the particular investigations. More important, many of the protocols are of general interest for use by researchers studying *Candida species*. Thus, upon being approached again to edit a new volume describing recent methodological advances in the field, we were delighted to proceed. New contributions to the current volume provide detailed discussion of a variety of important techniques that researchers use to study fungal molecular biology and pathogenesis, as well as those interested in diagnostics and antifungal drug discovery, will find both interesting and important to their research goals.

Washington, DC, USA ***Richard Calderone***
Ronald Cihlar

Contents

Contributors

Deepu Alex • *Department of Pathology, GU-MedStar Hospital, Georgetown University Medical Center, Washington, DC, USA*

Martinna Bertolini • *Department of Oral Health and Diagnostic Sciences, University of Connecticut Health Center, Farmington, CT, USA*

Jonathan Binkley • *Department of Genetics, Stanford University Medical School, Stanford, CA, USA*

Stéphane Bretagne • *Institut Pasteur, Unité de Mycologie Moléculaire, Centre National de Référence Mycoses Invasives et Antifongiques, Paris cedex 15, France; CNRS URA3012, Paris, France; Laboratoire de Parasitologie-Mycologie, Groupe hospitalier Lariboisière-Saint Louis, Assistance Publique-Hôpitaux de Paris (APHP), Paris, France*

Richard Calderone • *Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, DC, USA*

Jyotsna Chandra • *Center for Medical Mycology and Department of Dermatology, University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH, USA*

Gautam Chatterjee • *Molecular Mycology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India*

Athanasios Chatzimoschou • *Laboratory of Infectious Diseases, 3rd Department of Pediatrics, Aristotle University of Thessaloniki, Hippokration Hospital, Thessaloniki, Greece*

Cornelius J. Clancy • *VA Pittsburgh Healthcare System, Pittsburgh, PA, USA; Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA; Division of Infectious Diseases, University of Pittsburgh, Pittsburgh, PA, USA*

Albert D. de Boer • *Regional Center for Biomedical Research, Albacete Science & Technology Park, University of Castilla–La Mancha, Albacete, Spain*

Piet W.J. de Groot • *Regional Center for Biomedical Research, Albacete Science & Technology Park, University of Castilla–La Mancha, Albacete, Spain*

Henk L. Dekker • *Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands*

Marie Desnos-Ollivier • *Institut Pasteur, Unité de Mycologie Moléculaire, Centre National de Référence Mycoses Invasives et Antifongiques, Paris cedex 15, France; CNRS URA3012, Paris, France*

Patricia Diaz • *Department of Oral Health and Diagnostic Sciences, University of Connecticut Health Center, Farmington, CT, USA*

Anna Dongari-Bagtzoglou • *Department of Oral Health and Diagnostic Sciences, University of Connecticut Health Center, Farmington, CT, USA*

Dea Garcia-Hermoso • *Institut Pasteur, Unité de Mycologie Moléculaire, Centre National de Référence Mycoses Invasives et Antifongiques, Paris cedex 15, France; CNRS URA3012, Paris, France*

Françoise Gay-Andrieu • *Department of Pathology, GU-MedStar Hospital, Georgetown University Medical Center, Washington, DC, USA*

Mahmoud Ghannoum • *Center for Medical Mycology and Department of Dermatology, University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH, USA*
Emilia Gómez-Molero • *Regional Center for Biomedical Research, Albacete Science & Technology Park, University of Castilla–La Mancha, Albacete, Spain*
Kearney T.W. Gunsalus • *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
Gregor Jansen • *Biochemistry Department, McGill University, Montreal, QC, Canada*
Bastiaan P. Krom • *Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Free University Amsterdam, Amsterdam, The Netherlands*
Carol A. Kumamoto • *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
Randal J. Kuykendall • *Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA*
Dongmei Li • *Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, DC, USA*
Martin J. Lizak • *National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA*
Shawn R. Lockhart • *Fungal Reference Laboratory, Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA*
Jaideep Mallick • *Biology Department, Concordia University, Montreal, QC, Canada*
Sreyoshi Mitra • *Molecular Mycology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India*
Pranab K. Mukherjee • *Center for Medical Mycology and Department of Dermatology, University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH, USA*
Jeeva Munasinghe • *National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA*
Dhammika H. Navarathna • *Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA*
M. Hong Nguyen • *VA Pittsburgh Healthcare System, Pittsburgh, PA, USA*
Laxmi Shanker Rai • *Molecular Mycology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India*
Mauricio Retuerto • *Center for Medical Mycology and Department of Dermatology, University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH, USA*
David D. Roberts • *Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA*
Emmanuel Roilides • *Laboratory of Infectious Diseases, 3rd Department of Pediatrics, Aristotle University of Thessaloniki, Hippokration Hospital, Thessaloniki, Greece*
Kaustuv Sanyal • *Molecular Mycology Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India*
Nina Scheres • *Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Free University Amsterdam, Amsterdam, The Netherlands*

Gavin Sherlock • *Department of Genetics, Stanford University Medical School, Stanford, CA, USA*
Maria Simitsopoulou • *Laboratory of Infectious Diseases, 3rd Department of Pediatrics, Aristotle University of Thessaloniki, Hippokration Hospital, Thessaloniki, Greece*
Marek S. Skrzypek • *Department of Genetics, Stanford University Medical School, Stanford, CA, USA*
Takanori Sobue • *Department of Oral Health and Diagnostic Sciences, University of Connecticut Health Center, Farmington, CT, USA*
Malcolm Whiteway • *Biology Department, Concordia University, Montreal, QC, Canada*
Hubertine M.E. Willems • *Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Free University Amsterdam, Amsterdam, The Netherlands*
Cunle Wu • *National Research Council of Canada, Montreal, QC, Canada; Division of Experimental Medicine, McGill University, Montreal, QC, Canada*
Hongbin Xu • *Department of Oral Health and Diagnostic Sciences, University of Connecticut Health Center, Farmington, CT, USA*

Part I

Molecular Analyses of Pathogenesis

Chapter 1

How to Use the *Candida* Genome Database

Marek S. Skrzypek, Jonathan Binkley, and Gavin Sherlock

Abstract

Studying *Candida* biology requires access to genomic sequence data in conjunction with experimental information that provides functional context to genes and proteins. The *Candida* Genome Database (CGD) integrates functional information about *Candida* genes and their products with a set of analysis tools that facilitate searching for sets of genes and exploring their biological roles. This chapter describes how the various types of information available at CGD can be searched, retrieved, and analyzed. Starting with the guided tour of the CGD Home page and Locus Summary page, this unit shows how to navigate the various assemblies of the *C. albicans* genome, how to use Gene Ontology tools to make sense of large-scale data, and how to access the microarray data archived at CGD.

Key words *Candida*, Genome database, Expression analysis, Gene ontology, GO slim

1 Introduction

The *Candida* Genome Database (CGD; http://www.candidagenome.org) started in 2004 as an online compendium of genomic, genetic, and molecular biology information about *Candida albicans*. At its inception, CGD was based on the then newly assembled genomic sequence of strain SC5314 [1]. The primary goal was to couple the sequence data with the literature-derived experimental data in a single, easy-to-navigate Web-based resource. Since then, the expanding scope of *Candida* research, facilitated by the progress in sequencing of genomes from other strains and species, has prompted the incorporation of similar data for *C. glabrata* CBS138, *C. parapsilosis* CDC317, and *C. dublinensis* CD36. CGD has also become an archive that provides access to genomic sequences for other related strains and species, including *C. albicans* WO-1, *C. guilliermondii* ATCC_6260, *C. lusitaniae* ATCC_42720, *C. orthopsilosis* Co 90-125, *C. tropicalis* MYA-3404, *Debaryomyces hansenii* CBS767, and *Lodderomyces elongisporus* NRLL YB-4239.

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_1,

At the core of CGD lies human curation, a process that involves manually extracting gene-specific experimental information from the published, peer-reviewed literature and associating those annotations with the relevant genomic features. Genes and their annotations are organized in such a fashion that the information is easily browsable, searchable, and retrievable for further analysis and perusal. CGD curators also make sure that every piece of information is traceable to its original source, usually a publication in a scientific journal, thus providing access to all available experimental details and their interpretations. CGD also includes a rigorous analysis of orthology between species [2] and of protein domain structure, which allows consistent predictions of functions for genes that have not been experimentally characterized [3]. Thus, CGD provides a structured, unbiased, and continuously updated collection of a large variety of experimental results and computational predictions that has become indispensible for *Candida* researchers.

In order to ensure a uniform representation of biological information across different organisms, most genome databases use controlled vocabularies to annotate various attributes of genes and gene products. The most widely used vocabulary for capturing the key aspects of gene product biology is the Gene Ontology (GO; http://www.geneontology.org/; [4]). GO is a system of standardized terms with defined relationships that describe a primary activity of the gene product (Molecular Function), a broader cellular role the gene product is involved in (Biological Process), and the predominant localization, such as a protein complex, a subcellular structure, or an organelle (Cellular Component). CGD uses GO as the main vocabulary to annotate genes. Another data type that CGD captures, mutant phenotypes, is curated using Ascomycete Phenotype Ontology (APO), a vocabulary developed at *Saccharomyces* Genome Database (SGD) [5] that we adapted to the specific needs of *Candida* biology.

The information in CGD is organized in a system of interlinked web pages, designed with the goal of making them intuitive, easily navigable, and user friendly. However, the sheer complexity of the data presented in CGD can make it difficult for a newcomer to find the right information. This chapter provides help for navigating the site and highlights some new features of CGD. We present an overview of the main entry point, the Home Page, and the central organizing principle of the database, the Locus Summary Page. We show how to navigate between the current assembly of the *C. albicans* genome and the many historical assemblies that are still widely in use and need to be accessed frequently. We also explain how to perform some of the most common types of analysis that utilize GO annotations. Finally, we show how to access and browse large-scale datasets collected at CGD.

2 Methods

2.1 Exploring CGD Home and Locus Summary Pages

The CGD home page (http://www.candidagenome.org) serves as a place for database-related announcements, community news, and upcoming meetings of interest to the *Candida* community. It also provides a starting platform for many of the features and tools available at CGD. The search box is the primary gateway to a variety of data types available in the database and is present on most CGD pages (*see* **Note 1**). The Locus Summary page (Fig. 1) offers an up-to-date summary of what is known about a particular gene (*see* **Note 2**).

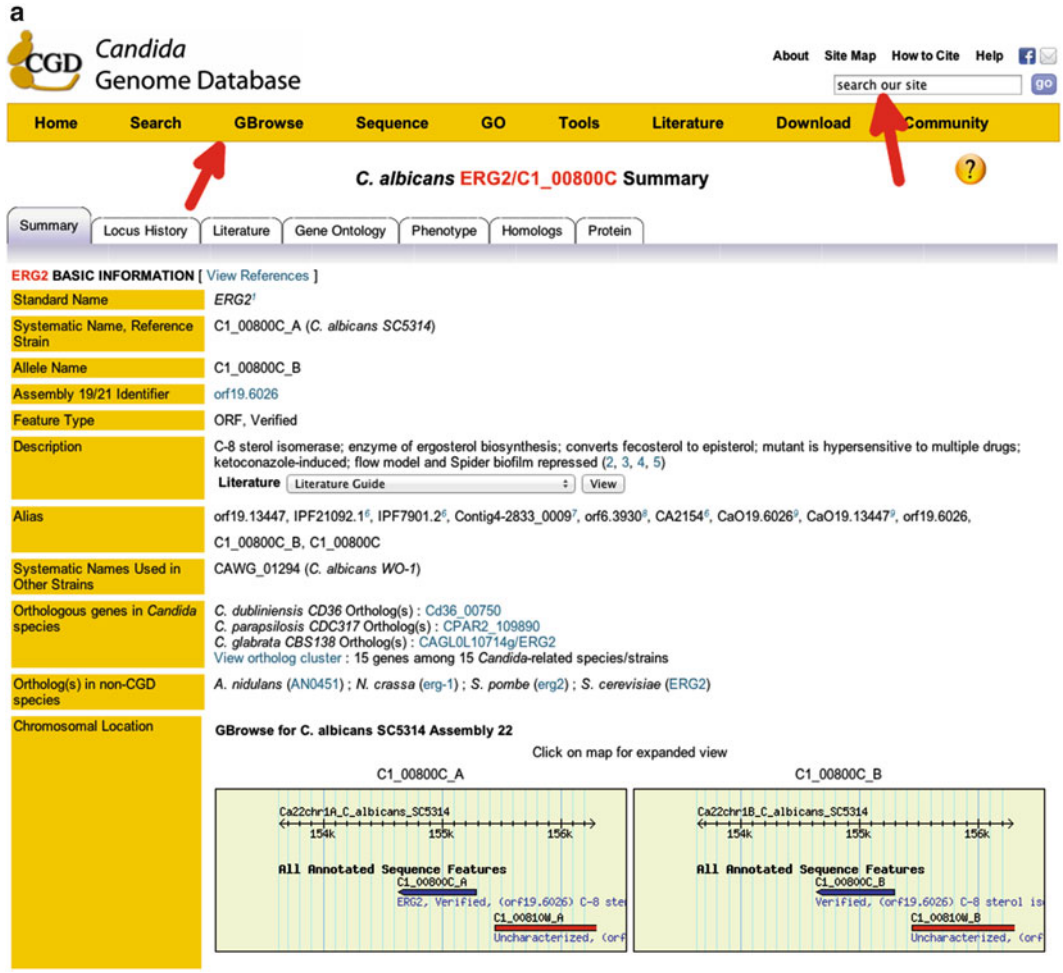


Fig. 1 Locus Summary page for *ERG2*. (**a**) Basic Information section; *red arrows* point to the Banner and search box present on most CGD pages. (**b**) GO Annotations and Mutant Phenotype sections. (**c**) Sequence Information section; *red arrow* indicates sequence retrieval and analysis tools

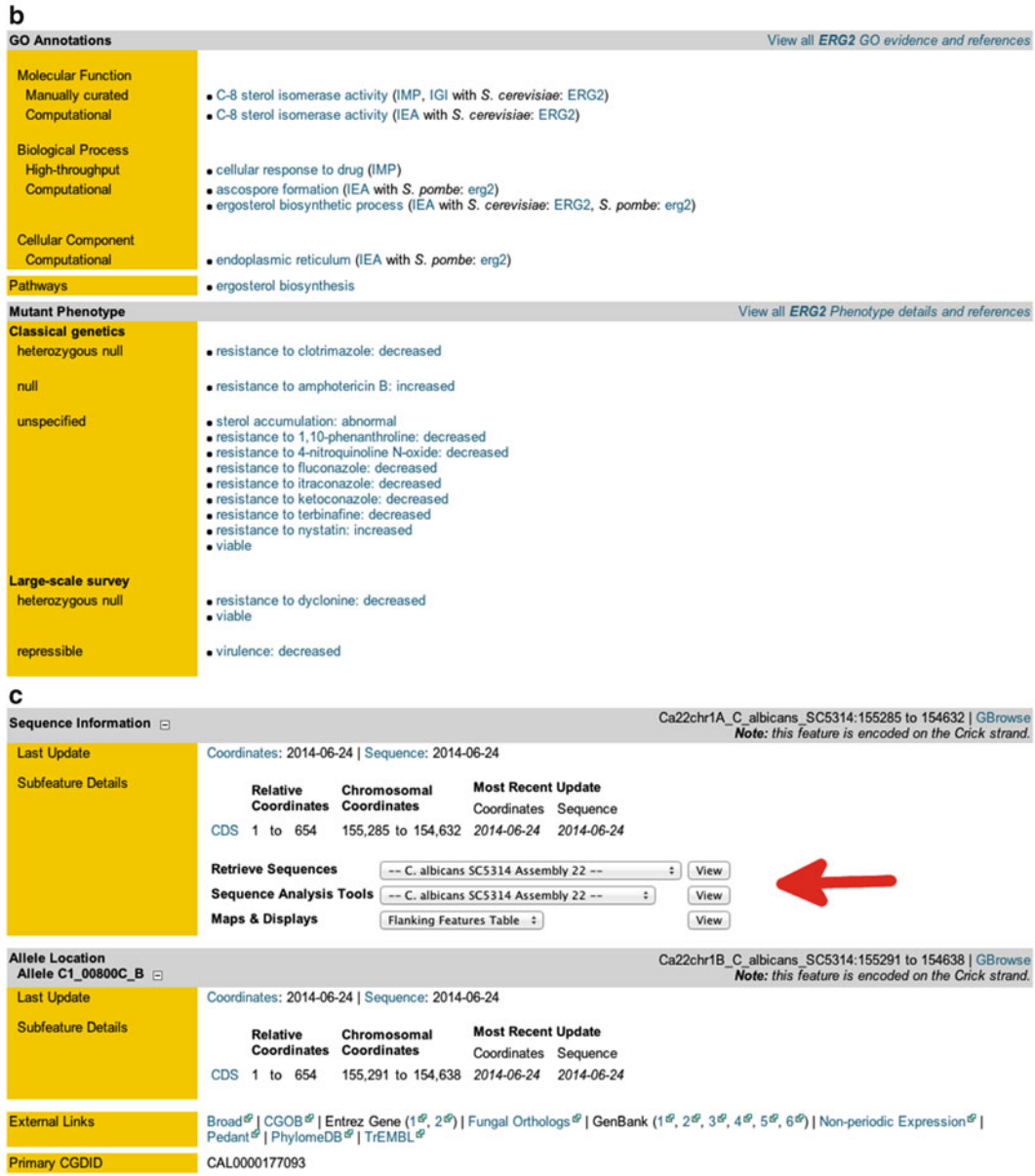


Fig. 1 (continued)

1. Open the home page (http://www.candidagenome.org) and explore the Banner at the top of the page (*see* Fig. 1a). This Banner, present on most CGD pages, provides quick links to multiple data search and analysis tools, literature tools including Textpresso, a full-text searching tool [6], as well as bulk download tools and various community-related information. Hover the mouse over each item to reveal a drop-down menu with available options.

2. Enter your query into the "Search our site" box above the Banner. If you enter a unique gene name, such as a systematic name, clicking on the result will jump directly to the Locus Summary page for that gene (*see* **Note 3**). If your query produces multiple hits, for instance, a gene name that is used in several *Candida* species represented in CGD, you will get a "CGD Quick Search Result" page that lists the type and number of hits, general and broken down by species. Positive hits are hyperlinked to either their respective Locus Summary pages, or to an intermediate list of individual hits. Select an individual hit to open its Locus Summary page.
3. The Locus Summary page contains several tabs, with the Summary tab open by default. Typically, for a protein-coding gene, the other tabs are Locus History, Literature, Gene Ontology, Phenotype, Homologs, and Protein. Flip back and forth through the tabs to see specific types of information they include.
4. The Basic Information section at the top of the Summary tab (*see* Fig. 1a) contains a description that summarizes the most significant features of the locus in a concise, headline-like format. It also lists all the names associated with the locus including the standard name—typically the genetic name under which the gene was first published. If there are other names by which the gene has been referred to in the literature, they are listed as aliases. The identifier assigned during genome sequencing is listed as Systematic Name along with the name of the reference strain used in the sequencing project (*see* **Note 4**). The Basic Information section allows easy navigation to genes in other organisms. Click on any hyperlinked name listed among "Orthologous genes in *Candida* species" to open its Locus Summary page in CGD. You can also click on the "View ortholog cluster" link, which will show the orthologs from 15 *Candida* species in their genomic context, a report produced by the Candida Gene Order Browser [2]. In order to explore orthologs from other fungal species, select a gene from "Ortholog(s) in non-CGD species" and you will access information at other resources: AspGD, Broad Institute, PomBase, and SGD, for genes from *A. nidulans, N. crassa*, *S. pombe*, and *S. cerevisiae*, respectively (*see* **Note 5**). At the bottom of this section, there is a thumbnail showing the chromosomal location of the gene, hyperlinked to GBrowse2 [7], which provides a graphical interface to inspect the genomic context of the gene.
5. The GO Annotations and Mutant Phenotypes sections (Fig. 1b) show current information about the function of the gene. For more detailed information, including the references on which these annotations are based, open the Gene Ontology

or Phenotype tab, respectively. GO annotations on the Summary tab are divided by the GO aspect (Molecular Function, Biological Process, Cellular Component) and by the annotation method. Manually curated annotations are assigned by a CGD curator on the basis of published, experimental (in most cases) results. Computational annotations are produced by transferring experiment-based GO annotations from orthologous genes in other species, or by predictions based on domain structure. Each annotation is accompanied by an evidence code that indicates the reason behind the annotation; for instance, IMP means Inferred from Mutant Phenotype. Click on any evidence code to see a table of all the evidence codes and their definitions. Annotations based on sequence similarity and genetic or physical interactions also list the source gene(s) and the organism. Click on any gene name to see a report for that gene in CGD or in an external database. Each GO term itself is hyperlinked to another CGD page that provides more information, including term definitions, a diagram depicting the relevant segment of the ontology, and a list of other genes in CGD that are also annotated with that term. Similarly, each mutant phenotype is hyperlinked to a page that lists all other genes in CGD that display the same phenotype.

6. The Sequence Information section (Fig. 1c) shows the basic data about the gene (chromosomal coordinates, intron-exon structure), but also provides easy access to sequences and sequence analysis tools. Open the drop-down menu next to Retrieve Sequences to see available options that include retrieval of DNA sequence in several configurations and, for protein-coding genes, for the predicted protein sequence as well. Similarly, open the Sequence Analysis Tools drop-down menu to start BLAST searches, restriction analysis, or primer design tools. More analysis tools are available from the Banner on top of the page; click on Search, Sequence, Tools, or Download to see the options. In addition, at the bottom of the Sequence Information section there are links to sequence data available from external resources, such as GenBank, UniProt, and others.

2.2 Navigating between the Current *C. albicans* Assembly and Historical Assemblies

Clinical isolates of *C. albicans* (including the sequenced reference strain, SC5314) are diploids, and exhibit a high level of heterozygosity: on average, a single-nucleotide polymorphism (SNP) occurs once every 237 bases between homologous chromosomes [1]. The first genome sequence published for *C. albicans* (Assembly 19) included both haplotypes—that is, the sequence from both members of every homologous chromosome pair was represented. However, full chromosome sequences were not available: rather, the assembly consisted of 1,213 contigs of 2kb or greater

(contiguous segments inferred from overlapping sequencing reads). The contigs were not mapped to or ordered along their parent chromosomes. The next major sequence release for the reference strain (Assembly 21) was assembled to the full chromosome level [8]. However, this assembly presented a pseudo-haploid genome (also known as reftigs), consisting of ordered, non-redundant sequences, but with any given contiguous region not necessarily derived from the same haplotype. The latest *C. albicans* assembly (Assembly 22; [9]) addresses both issues: it includes both haplotypes, and is assembled to the chromosome level. Since much research was conducted that referred to the older assemblies, it is important to be able to easily navigate between the current and past assemblies, and to relate them to each other. CGD provides tools and mapping files to accomplish this. Here we describe two ways to relate information between chromosomal features from different *C. albicans* assemblies.

2.2.1 Navigate between Current and Historic Features Using GBrowse

1. From the Locus Summary Page of a feature of interest, scroll down to the section entitled "Chromosomal Location." Two small map images are displayed, showing the immediate genomic region around the feature in each of the two Assembly 22 haplotypes. Click on one of the images to open the GBrowse tool.
2. A new window opens, displaying a browser with a map of the feature plus the regions 10,000 bases upstream and downstream on the chromosome. Above the browser window on the left side, click on the tab labeled "Select Tracks." Choosing this tab reveals several groups of checkboxes, which control what is displayed in the browser. One group is titled "Historic assemblies." Choose to display features from Assemblies 21 and 19 by checking "Assem21 ORF" and "Assem19 ORF (AWG)," respectively, and then click "Back to Browser" above the checkboxes to return to the browser window.
3. The browser now displays the same Assembly 22 region and features as before, with the selected historic tracks displayed above. Most of the historic features will correspond to current features, but the comparison may reveal features that have been added or deleted in the current assembly relative to the older assemblies. Clicking on the icons for the historic features opens a page from which files in GFF format, containing location information for the historic features, can be downloaded.

2.2.2 View the Assembly 21 Locus Summary Page

1. From the Locus Summary Page of a feature of interest, scroll down to the section entitled "Assembly 19/21 Identifier." Click on the link to the right, which is labeled with the old identifier (beginning with "orf19").

2. A new page opens, displaying a summary of information for the same feature in the context of Assembly 21. Much of the information on this page is redundant with or superseded by information on the current (Assembly 22) page, but of interest are the sections labeled "Chromosomal Location" and "Sequence Information."

   (a) The Chromosomal Location section shows several small maps. The top map shows the feature and its immediate genomic region for Assembly 21. Clicking on the map opens a GBrowse window, from which the user may navigate to other Assembly 21 features. The lower maps, if available, show the feature for both haplotypes in Assembly 19.

   (b) The Sequence Information section has two pull-down menus. From the "Retrieve Sequences" menu the user can display or download sequences for the feature: genomic DNA (with or without flanking DNA), coding DNA (introns removed), or protein sequence (*see* Fig. 1c). Sequences are also available from this menu for the "allele"—the feature from Assembly 19 corresponding to the opposite haplotype. The second pull-down menu, labeled "Sequence Analysis Tools," allows users to perform BLAST searches, design primers, or create restriction-fragment maps using sequences from the Assembly 21 feature or the allele.

2.3 GO Term Finder and GO Slim Mapper, Tools for GO Analysis

The GO vocabularies are constructed as hierarchies, where more general terms, so-called parents, encompass more specific "child" terms. When making annotations, curators are required to assign the most specific (granular) term that is supported by the evidence presented in the publication. Within the hierarchical structure of the ontology, annotation to a child term implies that annotation to its parent term is also correct. This feature of GO has to be taken into account during analysis of large-scale data, when it is necessary to identify common biological features in a set of genes that are, for instance, co-regulated in a microarray experiment. Finding statistically significant similarities in GO annotations for multiple genes requires a tool, GO Term Finder [10], that is able to determine if there are any GO terms that annotate genes in a list (either directly or indirectly via the GO hierarchy) at a rate greater than would be expected by chance. Given the complexity of GO, it can also be desirable to group genes into broad categories using only high-level terms. The GO Slims are such lists of high-level terms from each ontology branch (Molecular Function, Biological Process, and Cellular Component), carefully selected to cover most of the curated GO information in CGD. The tool, GO Slim Mapper, is able to categorize (map) a large set of genes to user-selected GO Slim terms.

2.3.1 Using GO Term Finder

1. Open the GO Term Finder page by selecting it from the options in the Search or GO pull-down menus in the Banner (*see* **Note 6**).
2. Select the species in step 1; the default species is *Candida albicans*, but you can run this analysis for other CGD-curated species: *C. glabrata*, *C. parapsilosis*, or *C. dublinensis*.
3. In step 2, enter a list of gene names. You can either type the name of the genes in the input box or upload a file that contains the list. Either genetic names (CGD Standard Names, e.g., "AAF1") or systematic names ("C3_06470W_A," or orf19 identifiers, e.g., "orf19.7436") may be used (*see* **Note 7**).
4. In step 3, select one of the three branches of GO (biological process, molecular function, or cellular component) by checking the boxes. The tool only searches one of the three branches at a time.
5. Click the Search button after step 2 to use the default settings or go further down to steps 3 and 4 to specify and customize your background set and/or refine the types of annotations in your background set.
6. You may change your background set in step 4. The default background set includes all the genes in the database that have at least one GO annotation. You can also customize the background set by choosing which feature type(s) it should include.
7. In step 5 you can deselect specific types of GO annotations that should not be used for calculations. By default, annotations collected by all methods and with all types of evidence are included.
8. The results page displays the significant shared GO terms (or their parents) in both graphic and table form, within the set of genes entered on the previous page.
9. The graphic shows the GO tree that includes terms used directly or indirectly in annotations for the genes in your list. The terms are color-coded to indicate their statistical significance (*p*-value score). Genes associated with the GO terms are shown in gray boxes, with links to their respective Locus Summary pages.
10. The table below the graph lists each significant GO term, the number of times the GO term is used to annotate genes in the list, and the number of times that the term is used to annotate genes in the background set (*see* **Note 8**).
11. Additional columns list the *p*-value, the false discovery rate (FDR), and a list of all the genes annotated, either directly or indirectly, to the term. FDR is an estimate of the percent chance that a particular GO term might actually be a false positive. It represents the fraction of the nodes with *p*-values as good or better than the node with this FDR that would be expected to be false positives.

12. The statistical significance of the association of a particular GO term with a group of genes in the list is indicated by the *p*-value: the probability of seeing at least *x* number of genes out of the total *n* genes in the list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO term. The closer the *p*-value is to zero, the more significant the particular GO term association with the group of genes is (i.e., the less likely to occur by chance).

2.3.2 Using GO Slim Mapper

1. Select GO Slim Mapper from the options in the Search or GO pull-down menus in the Banner. In step 1 you can type or paste your list of genes or upload them as a file.
2. In step 2 use the pull-down menu to select the GO Set Name: GO Slim Component, GO Slim Function, or GO Slim Process. The list of terms from the selected set appears in the window in step 4.
3. In step 4 you can specify which particular term, or terms, you want to use. The default setting is "Select ALL Terms," but you can highlight only terms you are interested in.
4. In optional step 5 you can exclude certain types of annotations, e.g., computational and high throughput.
5. Click Search to start the process; note that long lists of genes will take significant time to analyze.
6. Results appear in a table with three columns: the GO Slim terms chosen, with a link to graphical depiction of that branch of GO, the percentage of genes in your list annotated to each term, and the genes from your list that are annotated to that term. You can also download the results in a tab-delimited file.

2.4 Browsing Large-Scale Data and GeneXplorer

CGD has been collecting large-scale datasets from its inception. The datasets from authors' websites or from supplementary materials that accompany publications are archived in the Datasets page accessible from the Download menu in the Banner, thus offering a convenient one-stop shop for datasets pertaining to *Candida* that can be then imported into one's own tools for analysis. The microarray results are displayed by GeneXplorer [11], a Web tool that allows browsing and searching of the expression data at the CGD site, with the benefit of an instant access to functional annotations.

1. To access the archived datasets, click on the Download pull-down menu in the top Banner and select Datasets. The resulting page lists all the publications from which the datasets are archived in CGD, ordered by year and author. All the references have icons that lead to the abstract, full-text articles and to the original data website, as well as to the download directory at CGD. The microarray-containing references have a pair

Fig. 2 GeneXplorer page. (**a**) Example entries in the Dataset Archive page; *red arrow* points to icons that lead to the GeneXplorer browser or pcl file download. (**b**) GeneXplorer page: *red arrows* indicate the zoom buttons, the gene search box, and the individual expression profile to click on to see genes similarly expressed to *SAP4*

(or pairs) of green-blue icons. Click the one on the right that is labeled ".pcl" and you will get the entire datasets in the pcl format, a standard format that makes the dataset amenable to analysis by many popular software suites. Find a publication of interest and click the icon on the left to see the data in GeneXplorer (Fig. 2a).

2. The GeneXplorer page (Fig. 2b) shows a heat map image of clustered expression profiles from the selected publication, where increased or decreased gene expression is shown in shades of red or green, respectively. Zoom in and out using the + or – buttons above the cluster. Select any particular region by dragging over it. Click on a profile for a particular gene to see the expression patterns of the most similarly or dissimilarly expressed genes. Find a gene of interest by entering its name into the "Search for" box. Click on any gene name to go to its Locus Summary page.

3 Notes

1. The query entered into the search box may be a *Candida* gene or protein name (standard or systematic name, or an alias), author or colleague name, PubMed ID, or any keyword (such as a functional term or phenotype). It can even be a name of an ortholog from one of the non-CGD species. When there are multiple hits, a list of matches is displayed.
2. In addition to protein-coding genes, several other entities have their Locus Summary pages in CGD, including various RNA genes (tRNA, rRNA, snRNA, etc.), as well as a variety of chromosomal features, such as centromeres, telomeres, repeated sequences, and others.
3. "Search our site" box accepts a wildcard character *****. For example, enter "act*****" to retrieve any piece of data starting with "act." Also, the search box has an autocomplete feature, which provides suggestions when you start typing your query.
4. For *C. albicans*, the systematic name shown on the Locus Summary page is always the name of the haplotype A allele, as denoted by an "A" suffix, with the corresponding haplotype B allele listed below. Since the systematic names from previous assemblies of *C. albicans* genome (so-called orf19 names) continue to be widely used, the Assembly 19/21 identifier is also shown.
5. The ortholog mappings among *Candida* strains, and between *Candida* strains and *S. cerevisiae*, are derived from the curated syntenic groupings at the Candida Gene Order Browser (CGOB) [12]. The ortholog mappings between *Candida* strains and *S. pombe*, *A. nidulans*, and *N. crassa* are made by pairwise comparisons using the InParanoid software [13].
6. Links to both GO Term Finder and GO Slim Mapper appear at the bottom of many pages with results of searches that produce a list of genes. Selecting those links open the respective tool with the gene list already pre-loaded into the input box.
7. When a name is entered that is an alias for one gene or feature, the program will map the name to that gene. If the name is an alias for more than one gene but not a standard or systematic name for any genes, the program will present a list of possible mappings. The user can decide which gene was intended and edit the input.
8. Because the frequency of any given annotation within the background set is compared against the frequency of the annotation within the query set (input), the choice of background set affects the significance of the results that are returned by

the tool. Please note that the specific background set of genes that was used in the absence of any user-defined set (the default background set) has changed over time.

Acknowledgements

This work was supported by National Institute of Dental and Craniofacial Research at the US National Institutes of Health (grant no. R01 DE015873).

References

1. Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, Scherer S (2004) The diploid genome sequence of Candida albicans. Proc Natl Acad Sci U S A 101:7329–7334
2. Fitzpatrick DA, O'Gaora P, Byrne KP, Butler G (2010) Analysis of gene evolution and metabolic pathways using the Candida Gene Order Browser. BMC Genomics 11:290
3. Inglis DO, Arnaud MB, Binkley J, Shah P, Skrzypek MS, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G (2012) The Candida genome database incorporates multiple Candida species: multispecies search and analysis tools with curated gene and protein information for Candida albicans and Candida glabrata. Nucleic Acids Res 40:D667–D674
4. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–29
5. Engel SR, Balakrishnan R, Binkley G, Christie KR, Costanzo MC, Dwight SS, Fisk DG, Hirschman JE, Hitz BC, Hong EL, Krieger CJ, Livstone MS, Miyasato SR, Nash R, Oughtred R, Park J, Skrzypek MS, Weng S, Wong ED, Dolinski K, Botstein D, Cherry JM (2010) Saccharomyces Genome Database provides mutant phenotype data. Nucleic Acids Res 38:D433–D436
6. Muller HM, Kenny EE, Sternberg PW (2004) Textpresso: an ontology-based information retrieval and extraction system for biological literature. PLoS Biol 2:e309
7. Stein LD (2013) Using GBrowse 2.0 to visualize and share next-generation sequence data. Brief Bioinform 14:162–171
8. van het Hoog M, Rast TJ, Martchenko M, Grindle S, Dignard D, Hogues H, Cuomo C, Berriman M, Scherer S, Magee BB, Whiteway M, Chibana H, Nantel A, Magee PT (2007) Assembly of the Candida albicans genome into sixteen supercontigs aligned on the eight chromosomes. Genome Biol 8:R52
9. Muzzey D, Schwartz K, Weissman JS, Sherlock G (2013) Assembly of a phased diploid Candida albicans genome facilitates allele-specific measurements and provides a simple model for repeat and indel structure. Genome Biol 14:R97
10. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G (2004) GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20:3710–3715
11. Rees CA, Demeter J, Matese JC, Botstein D, Sherlock G (2004) GeneXplorer: an interactive web application for microarray data visualization and analysis. BMC Bioinformatics 5:141
12. Maguire SL, OhEigeartaigh SS, Byrne KP, Schroder MS, O'Gaora P, Wolfe KH, Butler G (2013) Comparative genome analysis and gene finding in Candida species using CGOB. Mol Biol Evol 30:1281–1291
13. Ostlund G, Schmitt T, Forslund K, Kostler T, Messina DN, Roopra S, Frings O, Sonnhammer EL (2010) InParanoid 7: new algorithms and tools for eukaryotic orthology analysis. Nucleic Acids Res 38:D196–D203

Chapter 2

Transcriptional Profiling of *Candida albicans* in the Host

Kearney T.W. Gunsalus and Carol A. Kumamoto

Abstract

Gene expression profiling has become an important tool for determining gene functions, performing phenotypic analysis, and quantifying the differential expression of individual transcripts under various conditions. *Candida albicans* gene expression is highly responsive to environmental conditions and rapidly adapts to various niches within the host. Here, we describe a mouse model of gastrointestinal colonization with *C. albicans*, the measurement of colonization in fecal pellets, and the collection of samples for transcriptional profiling. We describe how to extract and purify RNA suitable for analysis via RT-qPCR or microarray.

Key words *Candida albicans*, RNA, Transcriptional profiling, Gene expression, Host

1 Introduction

Transcriptional profiling provides a valuable tool for studying changes in gene expression in response to environmental factors, genetic manipulation, and other experimental conditions. Previous studies have shown that *Candida albicans* gene expression in the gastrointestinal tract differs from that observed during laboratory growth [1, 2]. We describe the extraction of RNA from *C. albicans* in the host using a mouse model of gastrointestinal colonization. Alternative mouse models of gastrointestinal colonization have been used (reviewed in ref. 3).

A variety of techniques have been used to measure *C. albicans* gene expression in the host (reviewed in refs. 4, 5). In vivo expression technology [6] and reverse transcriptase-PCR (RT-PCR) [7, 8] have been used to study the expression of individual *C. albicans* genes during infection. Quantitative (or real-time) reverse transcription PCR (RT-qPCR) is a sensitive and highly reproducible method for measuring individual transcripts. GFP fusion constructs have been used to measure the expression of individual genes in

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_2,

single-*C. albicans* cells [9] in infected tissue. However, neither RT-qPCR nor GFP fusion constructs are well suited to profiling the expression of large numbers of genes. Microarray expression profiling enables the simultaneous and relatively inexpensive measurement of many transcripts. Although early studies using microarrays to measure *C. albicans* gene expression in the host required additional amplification steps in order to increase hybridization signals from the relatively small fungal biomass recovered from infected host tissues [10, 11], a method for enriching fungal cells from host tissues eliminated the need for this amplification step [12], and the methodology for transcriptional profiling of *C. albicans* has been described in detail [13]. However, microarray profiling is limited to the detection of known genes/splice variants, and it has a limited dynamic range of detection. mRNA-seq is becoming the preferred technology for transcriptional profiling, as it is highly accurate [14, 15] and reproducible [16], has a wide dynamic range [15], and provides data for both known and novel transcripts [17].

Transcriptional profiling of *C. albicans* in the host gastrointestinal tract poses a unique set of challenges. Although the fungal biomass in the gastrointestinal tract is small compared to that obtainable *in vitro*, yield is not a major limitation of the technique (unlike measuring *C. albicans* gene expression from infected host tissues). The primary challenge when measuring *C. albicans* gene expression in the host gastrointestinal tract is the presence of biological and chemical contaminants. Biological "contaminants" include non-*C. albicans* species in the microbiota; contamination by host RNA was found to be minimal [1]. The contribution of bacterial RNAs is minimized first by using an oligo(dT) primer for first-strand cDNA synthesis to select for polyadenylated RNAs. Then a second, technique-specific step can be used to further enrich for *C. albicans*-specific transcripts. Microarrays are particularly well suited for use with this type of impure sample, as *C. albicans-specific* hybridization probes reduce the contribution from other species. The use of *C. albicans*-specific primers serves a similar function during RT-qPCR. While RNA-seq lacks this second enrichment step, sequencing reads from other species can be filtered out during analysis by aligning reads to the *C. albicans* genome.

Chemical impurities from the diet and the digestive process may include lipids, bile acids, and other substances that could interfere with downstream applications. In order to remove chemical contaminants, RNA is extracted using a two-step purification process: guanidinium thiocyanate-phenol-chloroform extraction (using TRIzol Reagent) followed by silica-membrane column purification. This produces RNA of sufficient purity for use in RT-qPCR and microarray experiments. Although RNA-seq experiments have not been performed using RNA prepared according to the method described here, the yield is typically sufficient: a typical yield from the contents of one mouse cecum using this method is between 1 and 6 μg of RNA (*see* Table 1).

Table 1
Typical RNA yield from the contents of one mouse cecum

CFU/g[a]	Sample weight (mg)[b]	RNA concentration (ng/μl)	RNA yield (μg)
10^6–10^7	100–300	50–300	1–6

[a]Measured from fecal pellets or cecum contents
[b]Weight of centrifuged pellet following filtration through polypropylene mesh (i.e., as measured in **step 10** of Subheading 3.2)

2 Materials

Prepare all solutions using deionized water and analytical grade reagents unless otherwise specified.

2.1 Animal Models

1. Five- to 7-week-old female BALB/c mice (*see* **Note 1**): House in pathogen-free conditions in autoclaved enclosed filter-top cages with sterile food and water.
2. Antibiotic-containing drinking water for mice: 1 g/l tetracycline, 2 g/l streptomycin, 0.1 g/l gentamicin. Weigh antibiotics; mix and make up to appropriate volume. Filter sterilize. Store at room temperature protected from light. Make fresh at least once per week (*see* **Notes 2** and **3**).
3. Sterile phosphate-buffered saline (PBS).
4. Autoclaved plastic 1 l beakers or other sterile containers of similar size (one per mouse) for collecting fecal pellets.
5. For collecting fecal pellets: forceps and 70 % ethanol for sterilization.
6. Microtube mixer (such as Tomy MT-360) or shaking tissue homogenizer (such as Qiagen TissueLyser) (*see* **Note 4**).
7. 1000× streptomycin/ampicillin solution (for making YPD-SA plates): Weigh 1 g streptomycin and 0.5 g ampicillin. Make up to 10 ml with water. Filter sterilize. Store 1 ml aliquots at –20 °C.
8. YPD-SA plates (yeast extract-peptone-dextrose agar plus 100 μg/ml streptomycin and 50 μg/ml ampicillin): Add about 700 ml ultrapure water (deionized water purified to 18 MΩ) to a 1-l graduated cylinder or flask. Weigh 10 g yeast extract, 20 g peptone, and 20 g glucose. Mix until no powder can be seen, and then add 20 g agar. Make up to 1 l with water. Autoclave to sterilize. Cool to 50 °C and add 1 ml of 1000× streptomycin/ampicillin solution (from **item 7**, above). Carefully pour into sterile petri dishes.
9. Fresh YPD made with ultrapure molecular biology-grade water: 1 % yeast extract, 2 % peptone, 2 % glucose. Weigh 1 g

yeast extract and 2 g peptone. Make up to 90 ml with ultrapure molecular biology-grade water (*see* **Note 5**) and sterilize. Weigh 2 g of glucose, make up to 10 ml with ultrapure molecular biology-grade water, and filter sterilize. For a 10 ml overnight culture, mix 9 ml of sterile yeast extract/peptone solution and 1 ml of sterile glucose solution.

10. Hemocytometer or other cell counter.
11. Sterile 1 ml disposable syringes and gavage needles (*see* **Note 6**).
12. RNA stabilization solution such as RNAlater (Ambion/Life Technologies, Carlsbad, CA; *see* **Note 7**).
13. Liquid nitrogen or dry ice.

2.2 RNA Extraction

1. Mechanical homogenizer (such as a Mini-Beadbeater from BioSpec Products).
2. Vials for mechanical homogenization (2 or 7 ml polypropylene vials, depending on sample volume; *see* **Note 8**).
3. 0.5 mm diameter zirconia/silica beads (BioSpec Products, catalog no. 11079105z) for mechanical homogenization: Transfer the beads to a small glass beaker, cover with aluminum foil, and bake at 200 °C for 2 h to eliminate RNase contamination.
4. 250 μm polypropylene mesh filter (Small Parts, Inc., Miami Lakes, FL; *see* **Note 9**).
5. TRIzol Reagent (Ambion/Life Technologies, Carlsbad, CA).
6. Chloroform and 70 % ethanol.
7. PureLink RNA Mini Kit (Ambion/Life Technologies, Carlsbad, CA) or a similar silica-membrane spin column kit for RNA purification with an on-column DNase I digestion step; *see* **Note 10**.
8. NanoDrop spectrophotometer.

2.3 Reverse Transcription for qPCR

1. SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA).
2. Oligo$(dT)_{20}$ (50 μM).
3. dNTP mix: 10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH.
4. RNase inhibitor.

3 Methods

3.1 Animal Models

1. Upon arrival, house mice three to four per cage in autoclaved, enclosed, filter-top cages under pathogen-free conditions with sterile food and water. Let the mice acclimate for 7 days prior to inoculation.

2. Four days prior to inoculation, streak strains from glycerol stocks onto YPD agar plates. Incubate at 25 °C for 3 days or 30 °C for 4 days.
3. Four days prior to inoculation, replace the drinking water in mouse cages with antibiotic-containing water (1 g/l tetracycline, 2 g/l streptomycin, 0.1 g/l gentamicin). To ensure adequate antibiotic consumption, also give moist food daily (*see* **Note 11**).
4. One or two days prior to inoculation, mark mice and verify that mice are not colonized with fungi (**steps 5–11**, below).
5. Prepare and weigh 1.5 ml tubes containing 1 ml sterile PBS (one tube per mouse).
6. In a laminar flow hood, transfer each mouse to a sterile 1 l plastic beaker to collect fecal pellets. Once the mouse has produced at least two fecal pellets, return the mouse to its cage and, using sterile forceps, transfer two fresh fecal pellets to the prepared tube of PBS. Sterilize the forceps in 70 % ethanol between mice.
7. Reweigh the tubes containing the fecal pellets.
8. Homogenize fecal pellets in PBS by shaking for 1 min with a microtube mixer or by vortexing. If pellets are not broken up after 1 min of mechanical homogenization, use a pipette tip to break them up. Shake for 5 more minutes.
9. Vortex for 5 s, let settle for 3 s, and pipette from the top using pipette tips with the tip cut off to avoid clogging.
10. Divide a YPD-SA plate into quadrants; in each, spread 50 µl of fecal homogenate.
11. Incubate YPD-SA plates at 37 °C overnight (*see* **Note 12**). Prior to inoculation, there should not be any colonies (*see* **Note 13**).
12. The morning before you plan to inoculate the mice, pick a colony from the freshly streaked YPD plate (from **step 2**) into 10 ml fresh YPD (*see* **Note 5**) and incubate on a rotating wheel for 24 h at 37 °C (*see* **Note 14**).
13. Harvest the 24-h culture by transferring it to a 50 ml conical tube; centrifuge at $3220 \times g$ for 6 min.
14. Discard the supernatant. Wash the pellet with 20 ml sterile PBS. Centrifuge at $3220 \times g$ for 6 min.
15. Repeat **step 14** once.
16. Discard the supernatant. Resuspend the pellet in 3 ml sterile PBS.
17. Dilute 5 µl of cell suspension in 1 ml PBS (1:200 dilution). Count the cells and prepare 5 ml of cell suspension at a concentration of 5×10^8 cells/ml (in sterile PBS).

18. Inoculate mice via oral gavage: administer 0.1 ml (5×10^7 cells) per mouse.
19. Starting 1 day post-inoculation, measure colonization by collecting fecal pellets and plating homogenates on YPD-SA plates as described above (**steps 5–11**; *see* **Note 15**).
20. Prepare tubes prior to sacrificing the mice. For each mouse, prepare tubes as follows for whichever samples you wish to collect (*see* **Note 16**):

    To quantify colonization, prepare and weigh a tube containing 1 ml sterile PBS for each sample to be collected.

    To harvest RNA, prepare 15 ml conical tubes containing 5–7 ml RNAlater (one tube per mouse for cecum contents; for ileum or stomach contents, one tube for two to four mice; *see* **Note 17**).
21. Upon sacrificing the mice, collect all samples in a laminar flow hood using sterile technique.
22. To quantify colonization, collect the desired samples in the prepared tubes (from **step 20**) as follows: first collect the tongue; both kidneys; and/or a piece of the liver. For stomach or cecum contents, collect a small pea-sized drop of contents. For ileum colonization, collect a 1 cm piece of ileum (tissue with contents). Homogenize the tissue and/or GI contents in PBS and plate to determine CFU/g (*see* **Note 18**).
23. For RNA extraction, collect the contents of desired GI tract organs into prepared tubes of RNAlater. Let sit at room temperature for ~5 min, and then put on dry ice or flash freeze in liquid nitrogen. Store at −80 °C.

3.2 RNA Extraction

1. Thaw samples on ice.
2. Prepare vials for mechanical disruption (*see* **Note 8**): label the vials and fill ¾ full with zirconia/silica beads. Chill the vials at −20 °C while preparing the samples.
3. Weigh an empty 50 ml conical tube for each sample.
4. Fold a sterile polypropylene 250 μm mesh filter into a cone (*see* **Note 19**) and push snugly into the top of a pre-weighed 50 ml conical tube. With one hand, hold the mesh filter in the tube to keep it folded.
5. With the other hand, vortex the thawed sample, pour it into the mesh filter, and let it drip into the 50 ml conical tube (*see* **Note 20**). Rinse the tube of thawed sample with 1 ml RNAlater to resuspend any remaining material and add to the mesh filter.
6. Once most of the liquid has dripped through the filter, carefully fold down the top of the cone over the outside of the

conical tube and firmly screw on the cap over the filter (*see* **Note 21**).

7. Centrifuge at 3220 × *g* for 10 min at 4 °C.
8. Remove the mesh filter from the tube and aspirate off the supernatant.
9. Centrifuge at 3220 × *g* for 10 min at 4 °C. Aspirate off all the remaining liquid (*see* **Note 22**).
10. Weigh the tube and determine the sample weight.
11. Resuspend the pellet in TRIzol (~1 ml TRIzol per 50–100 mg sample) and add to the pre-chilled homogenization vials (prepared in **step 2**). Rinse the sample tube with additional TRIzol and add to the homogenization vial; there should only be a small amount of air left at the top of the vial (*see* **Note 23**).
12. Shake the samples with the mechanical homogenizer for 30 s, and then chill samples on ice for 1 min or until cool to the touch (*see* **Note 24**). Repeat five times (for a total of 3 min of homogenization per sample).
13. Transfer the liquid homogenate to sterile RNase-free 1.5 ml tubes (*see* **Notes 25** and **26**).
14. Centrifuge at 12,000 × *g* for 10 min at 4 °C (*see* **Note 27**).
15. Transfer the cleared supernatant to a new sterile RNase-free 1.5 ml tube (*see* **Note 28**).
16. Incubate samples at room temperature for 5 min.
17. Add 1/5 volume chloroform (i.e., 0.2 ml chloroform per 1 ml TRIzol).
18. Shake the tubes vigorously by hand for at least 30 s.
19. Incubate the samples at room temperature for 3 min.
20. Centrifuge at 12,000 × *g* for 15 min at 4 °C.
21. Transfer the upper aqueous phase to a new RNase-free tube (*see* **Note 29**).
22. Add an equal volume of 70 % ethanol. Mix well by vortexing and/or inverting the tube.
23. Next perform a silica-gel membrane purification with on-column DNase treatment (using a PureLink RNA Mini Kit) according to the manufacturer's instructions (**steps 24–40**, below [18]; *see* **Note 10**).
24. Prepare the PureLink DNase mixture (80 µl per sample): in a sterile, RNase-free microcentrifuge tube, mix 10× DNase I reaction buffer (8 µl per sample), resuspended DNase (10 µl per sample), and RNase-free water (62 µl per sample).
25. Transfer up to 700 µl of sample to a PureLink spin cartridge with a collection tube (*see* **Note 30**).

26. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
27. Repeat **steps 25** and **26** until the entire sample has been loaded onto the PureLink column.
28. To the spin column, add 350 µl wash buffer I.
29. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and the collection tube. Insert the spin cartridge into a new collection tube.
30. Add 80 µl of PureLink DNase mixture directly onto the surface of the spin cartridge membrane.
31. Incubate at room temperature for 15 min.
32. To the spin column, add 350 µl wash buffer I.
33. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and the collection tube. Insert the spin cartridge into a new collection tube.
34. To the spin column, add 500 µl wash buffer II.
35. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
36. Repeat **steps 34** and **35** once.
37. Centrifuge the spin cartridge at 12,000 × *g* for 1 min to dry the membrane. Discard the collection tube and insert the spin cartridge into a recovery tube.
38. Add 32 µl of RNase-free water to the center of the spin cartridge.
39. Incubate at room temperature for 1 min.
40. Centrifuge the spin cartridge and recovery tube at ≥12,000 × *g* for 1 min at room temperature.
41. Determine the RNA concentration with a NanoDrop spectrophotometer (*see* **Note 31**). Store at –80 °C. (For use in microarray experiments, *see* ref. 13.)

3.3 Reverse Transcription for qPCR

1. Determine how much RNA template to use (*see* **Note 32**).
2. For each sample, add the following to a nuclease-free 0.2 ml tube (20 µl reaction volume): 1 µl oligo$(dT)_{20}$ (50 µM), 1 µl dNTP mix, 10 pg–5 µg RNA (*see* **Note 32**), and sterile nuclease-free water to 12 µl (*see* **Note 33**).
3. Incubate at 65 °C for 5 min.
4. Incubate on ice for at least 1 min.
5. Make reverse transcription master mix. Per 20 µl reaction: 4 µl of 5× first-strand buffer, 1 µl of DTT (0.1 M), 0.5 µl of RNase inhibitor, and 0.5 µl of SuperScript III RT.

6. Briefly centrifuge the tubes from **step 4** to collect the contents; add 6 µl of reverse transcription master mix to each reaction. Mix gently by pipetting up and down.
7. Incubate at 50 °C for 60 min.
8. Inactivate the reaction by incubating at 70 °C for 15 min.
9. The cDNA is now ready to be diluted (*see* **Note 34**) and used as a template for qPCR. Make 20–40 µl aliquots of cDNA diluted 1:10 or 1:20 in nuclease-free water. Store at −20 °C. Use 1–2 µl of diluted cDNA per 20 µl qPCR reaction.

4 Notes

1. Alternative mouse strains have been used, such as 5–7-week-old female C57BL/6 [1] or 19–20 g female Swiss Webster [19] mice. For experiments in an immunocompromised host, 5–7-week-old BALB/c *nu/nu* mice have been used [20]. (Mouse models of gastrointestinal colonization by *C. albicans* are reviewed in ref. 3.)
2. Antibiotic water is made up fresh weekly; otherwise, antibiotics begin to precipitate.
3. Alternative antibiotic treatments have been used (reviewed in ref. 21). For example, for antibiotic-containing drinking water without tetracycline: 1 g/l bacitracin, 2 g/l streptomycin, and 0.1 g/l gentamicin [22].
4. A mechanical tissue homogenizer will be required for homogenizing tissue (tongue, kidney, liver, or ileum); for measuring colonization from fecal pellets or stomach/ileum/cecum contents, a microtube mixer or other shaking device will make homogenization much easier, but it could be accomplished by vortexing.
5. We use ultrapure molecular biology-grade water to make YPD for the inoculum because we have had variable growth in medium made from deionized water supplied from our central deionized water source. Make the YPD fresh for each experiment (use within 1–2 weeks).
6. After use, do not bleach gavage needles, as it destroys stainless steel. When inoculating the mice, have ready a 15 ml tube of water; after gavaging the mice, immediately flush the gavage needle thoroughly with water, and then 70 % ethanol, by repeatedly filling and emptying the syringe. The needles are then ready to be autoclaved for the next use.
7. RNAlater is a high-salt solution; the salt may interfere with some downstream applications. For techniques that are highly sensitive to salt, an alterative method of RNA preservation may be preferable.

8. For cecum contents, use 7 ml polypropylene screw-cap vials (available from BioSpec Products, catalog no. 3205). For the contents of two to four ilea/stomachs, use 2 ml screw-cap polypropylene vials with an o-ring seal (available from BioSpec Products or other suppliers).
9. Cut the polypropylene mesh into 3″ squares and trim the corners to make octagons. Sterilize in 70 % ethanol and air-dry. After use, immediately wash thoroughly with deionized water and re-sterilize.
10. If substituting a different column purification kit, substitute the manufacturer's instructions for **steps 24–40** of the RNA extraction. Perform an on-column DNase treatment and elute with 32 μl of RNase-free water.
11. Some, but not all, mice will readily drink antibiotic-containing water. BALB/c mice tend not to like antibiotic-containing water, but will readily consume it when it is used to moisten food pellets. Mice will eventually adjust and accept antibiotic-containing water. Fresh moist food can also be given ~30 min prior to sacrifice if stomach contents will be collected.
12. To be certain that there are no slow-growing fungal colonies, incubate the pre-inoculation plates for an extra day or 2.
13. Calculate CFU/g fecal pellets using the weights of the 1.5 ml tubes containing PBS before and after adding fecal pellets.
14. The expected density of a 24-h culture is $\sim 3 \times 10^8$ cells/ml.
15. For day 1 post-inoculation, dilute the fecal pellet homogenate 10^{-1} and 10^{-3}. Spread 10 μl each of the 10^{-1} and 10^{-3} dilutions and 50 μl of the 10^{-3} dilution on YPD-SA agar. Over the first several days post-inoculation, expect to see ~10^6–10^8 CFU/g. As colonization levels change over time, adjust the volume and dilution of homogenates plated.
16. Colonization can be quantified at many points throughout the GI tract (i.e., stomach, ileum, cecum, colon). We have found that colonization measured in cecum contents correlates well with fecal pellet measurements. Additionally, we typically check for, but do not see, colonization in tongue, kidney, and liver homogenates.
17. For microarray experiments, the cecum contents of three mice were typically pooled and three to four biological replicates were used (i.e., 9–12 mice).
18. Typically, to measure colonization in stomach, ileum, cecum, or colon, plate homogenates at the same dilutions you have been using for fecal pellets. Plate 50 μl of undiluted homogenate for tongue, kidney, and liver tissue.
19. To fold the polypropylene mesh, fold in half and then fold over a third. Open the large portion as the cone.

20. It is often helpful to gently "stir" the sample in the filter with a sterile 1000 μl pipette tip, as solids in the sample can clog the filter and prevent the liquid from passing through.
21. If the cap is not screwed on securely it may pop off, allowing the filter to unfold and causing loss of sample.
22. RNAlater is a high-salt solution. The salt may interfere with downstream applications, so it is important to remove as much as possible. This is typically sufficient for RT-qPCR.
23. Extra TRIzol is added to nearly fill the vial because the sample will foam during homogenization if the tube contains too much air.
24. To keep the samples cold, homogenize the samples in short bursts, resting the samples on ice in between rounds of homogenization. Adjust the length of each burst of homogenization as necessary to keep samples from getting hot; homogenize each sample for a total of 3 min.
25. Homogenized cecum contents will typically have to be split into three or four 1.5 ml tubes per sample; homogenized ileum/stomach contents usually fit into one tube per sample.
26. Transfer the homogenate using a 1000 μl micropipette; firmly put a sterile 10 μl pipette tip (do not use a filter tip) on over the 1000 μl tip (the beads are too small to easily pass through the opening of the 10 μl tip) and pipette homogenate from the bottom of the vial to avoid transferring beads. After sucking the beads dry, vortex the vial and repeat; vortexing allows additional homogenate to settle to the bottom of the vial.
27. In high-fat samples, a layer of fat may collect above the supernatant; carefully remove and discard this layer.
28. According to the manufacturer, homogenized sample can be stored at −80 °C for at least a month [18].
29. Be careful not to disturb the interphase. It is better to leave 1–2 mm of aqueous phase than to risk contamination with material from the interphase.
30. We typically use a single PureLink column for the contents of one cecum even though the amount of RNA typically exceeds the amount recommended by the manufacturer [18].
31. Do not expect absorbance curves with a single, "clean" peak at 260 nm. There will often be a second peak at 230 nm, or a single, broad peak from 230 to 260 nm. We typically obtain concentrations of 50–300 ng/μl (or a total yield of ~1–6 μg of RNA from the contents of 1 cecum), but it is not uncommon for some samples to have poor yields (≤10 ng/μl, or ≤300 ng total).
32. Typically the amount of RNA template that can be used is limited by the concentration of the sample with the lowest yield.

Based on this limitation, we typically use 80–600 ng of RNA in a 20 µl reaction.

33. Add water to the reaction first, then oligo(dT), and RNase inhibitor; add RNA last. Use oligo(dT) instead of random primers because this selects for eukaryotic mRNAs, and eliminates much of the contamination from bacterial RNAs.
34. Dilute cDNA 1:10 or 1:20 in nuclease-free water; do not use undiluted cDNA, as it typically contains inhibitors that will interfere with efficient qPCR amplification. If aliquots are prepared in 8-well strip tubes, a multichannel pipette can be used to add cDNA to qPCR reaction tubes.

Acknowledgements

K.T.W.G. is supported by Award Number K12GM074869 (TEACRS) from the National Institute of General Medical Sciences. Research in the Kumamoto lab is supported by grant R01AI081794 from the National Institutes of Health (to C.A.K.).

Kearney T.W. Gunsalus is supported by Training in Education and Critical Research Skills (TEACRS) Program (Award Number K12GM074869 from the National Institute of General Medical Sciences)

References

1. Rosenbach A, Dignard D, Pierce JV, Whiteway M, Kumamoto CA (2010) Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. Eukaryot Cell 9(7):1075–1086. doi:10.1128/EC.00034-10
2. Pande K, Chen C, Noble SM (2013) Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. Nat Genet 45(9):1088–1091. doi:10.1038/ng.2710
3. Koh AY (2013) Murine models of *Candida* gastrointestinal colonization and dissemination. Eukaryot Cell 12(11):1416–1422. doi:10.1128/EC.00196-13
4. Brown AJP, Odds FC, Gow NAR (2007) Infection-related gene expression in *Candida albicans*. Curr Opin Microbiol 10(4):307–313, doi:http://dx.doi.org/10.1016/j.mib.2007.04.001
5. Hube B (2004) From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. Curr Opin Microbiol 7(4):336–341, doi:http://dx.doi.org/10.1016/j.mib.2004.06.003
6. Staib P, Kretschmar M, Nichterlein T, Hof H, Morschhäuser J (2000) Differential activation of a *Candida albicans* virulence gene family during infection. Proc Natl Acad Sci U S A 97(11):6102–6107
7. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schafer W, Hube B (2002) *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. Infect Immun 70(7):3689–3700
8. Schofield DA, Westwater C, Warner T, Nicholas PJ, Paulling EE, Balish E (2003) Hydrolytic gene expression during oroesophageal and gastric candidiasis in immunocompetent and immunodeficient gnotobiotic mice. J Infect Dis 188(4):591–599
9. Barelle CJ, Manson CL, MacCallum DM, Odds FC, Gow NAR, Brown AJP (2004) GFP as a quantitative reporter of gene regulation in *Candida albicans*. Yeast 21(4):333–340
10. Andes D, Lepak A, Pitula A, Marchillo K, Clark J (2005) A simple approach for estimating gene expression in *Candida albicans* directly from a systemic infection site. J Infect Dis 192(5):893–900. doi:10.1086/432104
11. Thewes S, Kretschmar M, Park H, Schaller M, Filler SG, Hube B (2007) *In vivo* and *ex vivo* comparative transcriptional profiling of invasive

and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion. Mol Microbiol 63(6):1606–1628. doi:10.1111/j.1365-2958.2007.05614.x

12. Walker LA, Maccallum DM, Bertram G, Gow NA, Odds FC, Brown AJ (2009) Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. Fungal Genet Biol 46(2):210–219. doi:10.1016/j.fgb.2008.10.012
13. Nantel A, Rigby T, Hogues H, Whiteway M (2006) Microarrays for studying pathogenicity in *Candida albicans*. In: Kavanagh K (ed) Medical mycology: Cellular and Molecular Techniques. Wiley
14. Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M, Snyder M (2008) The transcriptional landscape of the yeast genome defined by RNA sequencing. Science 320(5881): 1344–1349. doi:10.1126/science.1158441
15. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5(7):621–628. doi:10.1038/nmeth.1226
16. Marioni JC, Mason CE, Mane SM, Stephens M, Gilad Y (2008) RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 18(9):1509–1517. doi:10.1101/gr.079558.108
17. Wilhelm BT, Marguerat S, Watt S, Schubert F, Wood V, Goodhead I, Penkett CJ, Rogers J, Bahler J (2008) Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. Nature 453(7199): 1239–1243. doi:10.1038/nature07002
18. PureLink RNA Mini Kit User Manual. Version B edn. Invitrogen
19. White SJ, Rosenbach A, Lephart P, Nguyen D, Benjamin A, Tzipori S, Whiteway M, Mecsas J, Kumamoto CA (2007) Self-regulation of *Candida albicans* population size during GI colonization. PLoS Pathog 3(12):e184. doi:10.1371/journal.ppat.0030184
20. Pierce JV, Kumamoto CA (2012) Variation in *Candida albicans* EFG1 expression enables host-dependent changes in colonizing fungal populations. MBio 3(4):e00117–e00112. doi:10.1128/mBio.00117-12
21. Kumamoto CA (2011) Inflammation and gastrointestinal *Candida* colonization. Curr Opin Microbiol 14(4):386–391. doi:10.1016/j.mib.2011.07.015
22. Wiesner SM, Jechorek RP, Garni RM, Bendel CM, Wells CL (2001) Gastrointestinal colonization by *Candida albicans* mutant strains in antibiotic-treated mice. Clin Diagn Lab Immunol 8(1):192–195. doi:10.1128/CDLI.8.1.192-195.2001

Chapter 3

SRYTH: A New Yeast Two-Hybrid Method

Jaideep Mallick, Gregor Jansen, Cunle Wu, and Malcolm Whiteway

Abstract

Many biological processes are regulated by protein-protein interactions, and the analysis of these interactions has been a productive endeavor contributing to our understanding of cellular organization and function. The yeast two-hybrid technique is a widely used, powerful method of analyzing protein-protein interactions. The currently used formats, however, have inherent limitations, providing an opportunity to develop new alternatives that extend our ability to detect protein-protein interactions of biological relevance. Here we present a two-hybrid system named SRYTH (*S*te11p/Ste50p *r*elated *y*east *t*wo-*h*ybrid) based on the Ste11p/Ste50p interaction that uses the activation of the HOG pathway of *Saccharomyces cerevisiae* as a reporter for interactions. The system is suitable for detecting cytoplasmic protein interactions in their natural subcellular environment, and has been successfully used to investigate protein-protein interactions, including transcription factor associations, in *Candida albicans*.

Key words Yeast two-hybrid, Protein-protein interaction, *Candida albicans*, HOG pathway

1 Introduction

Ever since Fields and Song [1] reported the first yeast two-hybrid system, this technique has been a very popular method for studying protein-protein interactions. The underlying principle of this method is very simple; most protein domains are modular, and can be combined to create new functionalities. The specific protein used in the first yeast two-hybrid system was the Gal4p transcription activator protein of *Saccharomyces cerevisiae*, which has two functionally independent domains termed the DNA-binding domain and the transcriptional activator domain, both of which are required to switch on the transcription of a reporter gene regulated by Gal4p. To test whether Protein A interacted with Protein B, each was expressed as a fusion protein, with A fused to the DNA-binding domain of Gal4p and B fused to the transcription activator domain. If A and B interacted, this would bring together the two domains of Gal4p, creating a functional transcription factor that switches on a reporter gene. This method works efficiently for

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_3,

many interacting proteins but had limitations in analyzing protein-protein interactions among transcription factors, many of which were capable of switching on the transcription of the reporter by themselves without the need of an interacting partner. Another potential limitation was that the detection of the protein-protein interaction took place in the nucleus of the yeast cell. If the interaction in question was cytosolic in nature, especially in the case of membrane-bound proteins, the conventional yeast two-hybrid system might not reflect the interaction properly. These deficiencies of the conventional yeast two-hybrid system led to the development of alternative strategies of using protein fusions to detect protein-protein interactions (reviewed in ref. 2).

One such method was based on the Ste11p/Ste50p interaction that is required for the activation of the HOG pathway of *S. cerevisiae*; the activation in turn is used as the reporter of the protein-protein interaction. Normally, the interaction of Ste11p and Ste50p, through their sterile alpha motif (SAM) domains, is required for the HOG pathway activation and osmo-adaptation; this interaction is critical for the survival of yeast under hyperosmotic stress in the absence of the cell's two-component osmosensing pathway (Fig. 1) [3]. In the current assay two putative interacting proteins (or protein domains), named the "bait" and "prey," respectively, are fused to Ste11p and Ste50p in place of the protein's original SAM domains. If bait and prey interact they bring together Ste50p and Ste11p, leading to activation of the

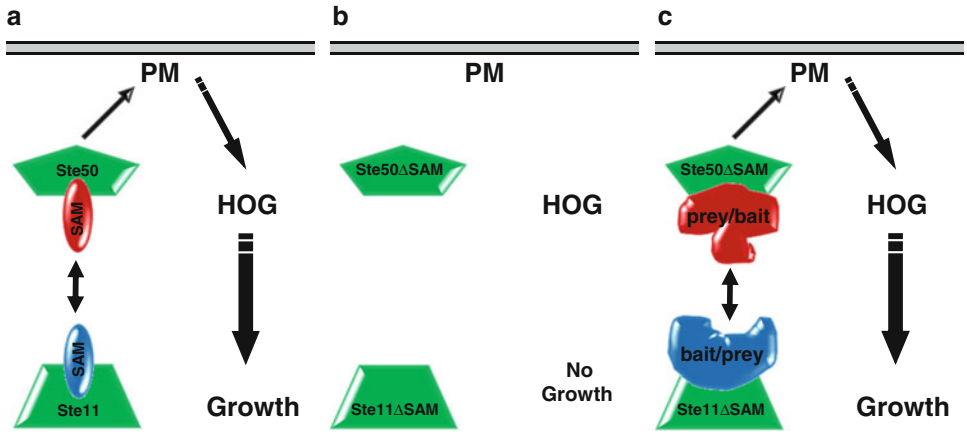


Fig. 1 How the cytoplasmic yeast two-hybrid works: (**a**) The interaction of Ste11p and Ste50p via their SAM domains, together with the plasma membrane association of Ste50p, is essential for the HOG pathway activation in the *SH01* signaling branch of osmo-adaptation. (**b**) Ste11p and Ste50p lacking their SAM domains are unable to activate the HOG pathway. (**c**) Detecting protein-protein interactions in the cytoplasm using SRYTH: The two-hybrid system is based on the interchangeability of the Ste50p and Ste11p interaction domains. Replacing these domains with a pair of interacting bait-prey proteins restores the HOG pathway activation. Interaction of bait and prey is monitored by the ability to enable osmo-sensitive yeast *ste50*Δ*ssk2*Δ*ssk22*Δ to grow on high-osmolarity medium

HOG pathway and allowing the cell to survive in hyperosmotic media.

The key features of this system are as follows:

(a) The use of a metabolic ability of the cells as the readout.

(b) The cellular compartment of the protein-protein interaction is the cytoplasm as opposed to the nucleus in a conventional system.

(c) The system has been designed in a way that no sub-cloning steps are required to prepare the bait and prey plasmids.

This method has been successfully used to study protein-protein interactions in the human pathogen *Candida albicans*. Côte et al. [4] used this method to analyze the interactions among components of the pheromone pathway while investigating how the pheromone pathway scaffold proteins had changed during evolution of the Ascomycetes. Later Mallick and Whiteway [5] looked at the interaction of transcription factors Ifh1p, Fhl1p, and Tbf1p while studying how the control of ribosomal proteins gene transcription has changed from *S. cerevisiae* to *C. albicans*. These studies of circuit rewiring were easily done using this unique yeast two-hybrid system by producing *C. albicans* proteins in *S. cerevisiae*, in spite of the fact that there is a different codon usage in the two organisms (in *C. albicans* CTG gets translated to serine instead of the almost universal leucine). This suggest that either the CTG-encoded serine residues did not have critical contributions to the interactions or the interacting surfaces being analyzed here were big enough that the occasional serine-to-leucine change was not enough to abolish the interaction.

2 Materials

1. Yeast (*S. cerevisiae*) strains required:

   yCW1476 (*MAT* **a** *ura3 leu2 his3 ssk2*Δ*::LEU2 ssk22*Δ*::LEU2 ste50*Δ*::TRP1*).

   yCW1477 (*MAT*α *ura3 leu2 his3 ssk2*Δ*::LEU2 ssk22*Δ*::LEU2 ste50*Δ*::TRP1*).

2. Plasmids required:

   Plasmid #1: "pCW778" (markers: *HIS3*, *URA3*), deletes the stuffer *URA3* marker upon *Sma*I digest.

   Plasmid #2: "pYL040" (markers: *URA3*, *HIS3*), deletes the stuffer *HIS3* marker upon *Sma*I digest.

   Figure 2a, b (Plasmid Maps).

   The plasmid pYL40 contains the fragment of *STE11* encoding Ste11p lacking its SAM domain (aa 105–717) at the *Sal*I site

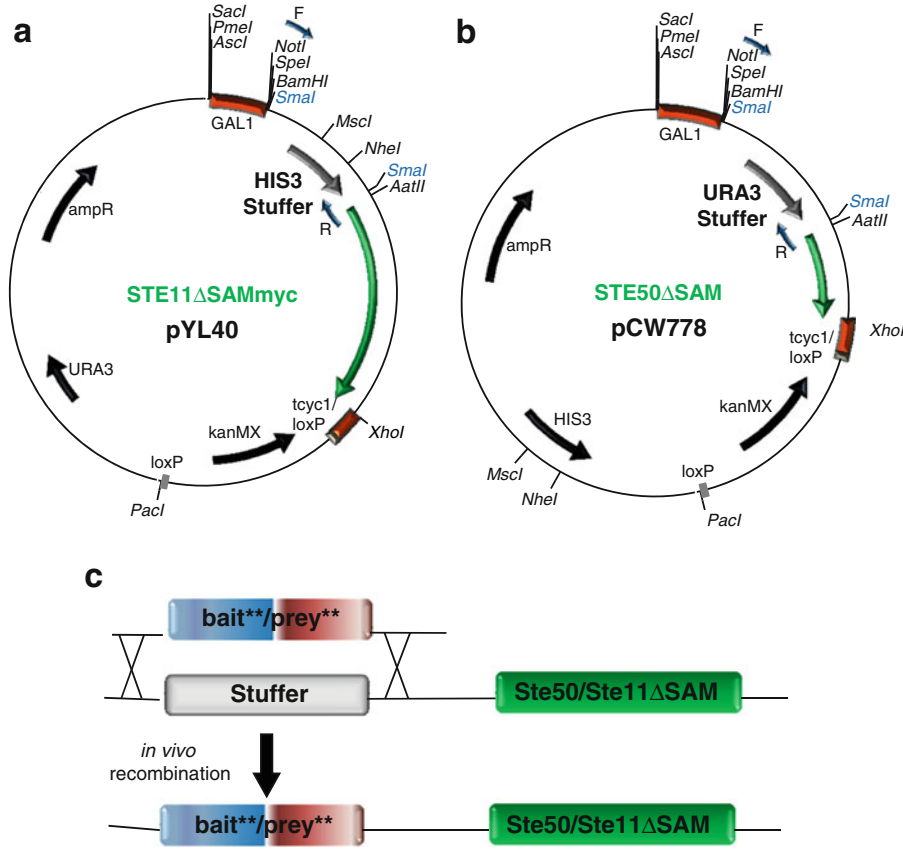


Fig. 2 Plasmid maps of (**a**) pYL040 and (**b**) pCW778 with their respective stuffer markers: *Blue arrows* indicate IVR primers forward "F" and reverse "R." (**c**) The stuffer fragments of pYL040 and pCW778 are replaced by bait and prey using in vivo homologous recombination

of pGREG506 [6], and a *HIS3* stuffer marker inserted at the *Sma*I site in front of Ste11ΔSAM. Similarly, pCW778 contains the fragment of *STE50* encoding Ste50p without its SAM domain (aa 115–346) at the *Sal*I site of pGREG503 [6], and a *URA3* stuffer marker inserted at the *Sma*I site in front of Ste50ΔSAM.

3. The water used in the procedure should be sterilized, double-distilled, or reverse osmosis water or equivalent.
4. Media required for bacteria and yeast growth:
   - Luria Broth liquid media and Luria Broth agar plates for growing *E. coli* cells.
   - YPD liquid and YPD agar plates for growing *S. cerevisiae*.
   - SD^{-his}, SD^{-ura}, $SD^{-his\ -ura}$ plates: Synthetic dropout media plates required for selection.
   - Hyperosmotic stress media plates YP Gal (2 %) + hyperosmotic stress inducer (from 0.5 to 1 M NaCl or 1.2 to 1.5 M Sorbitol).

5. Solutions, enzymes, and buffers required for yeast transformation:
   - *Sma*I (restriction enzyme used to digest plasmid to remove the stuffer marker) and appropriate buffer for the enzyme.
   - Boiling-denatured salmon sperm DNA (ssDNA) at 10 μg/μl (10 min at 95 °C then chill on ice for 1 min).
   - 50 % PEG solution (sterilized by autoclaving).
   - 1 M LiAC (lithium acetate) pH 7.4.
   - Sterile water (sterilized by autoclaving).
6. Primers for in vivo recombination (IVR):
   - Forward primer F:

     5′-ATTCTAGAGCGGCCGCACTAGTGGATCCCCC GGG-gene-specific sequence (20–25 bases in frame) (starting with ATG)-3′.
   - Reverse primer R:

     5′-TCGATAAGCTTGATATCGAATTCCTGCAGCCC GGG-gene-specific sequence (reverse complement of 20–25 bases in frame) (no STOP codon)-3′.

   All candidate ORFs or their fragments were PCR amplified using the above primer pair (for both plasmids) and cloned into the vector plasmids pYL40 and pCW778 at the *Sma*I sites through IVR in yeast strains YCW1476 and YCW1477. The primers used for the PCR amplification reactions contain gene-specific sequences and common sequences used for IVR in a layout as shown above.
7. Primers for sequencing and PCR confirmation of the presence of cloned inserts:
   - GAL1 primer for forward sequencing: (5′-AATATACCTCTATACTTTAACGTC-3′).
   - OCW168, annealing to *STE11* for reverse sequencing of pYL40-based constructs: (5′-GTGGCTAATAATTCATGTGGC-3′).
   - OCW172, annealing to *STE50* for reverse sequencing of pCW778-based constructs: (5′-GCGAATTCTTCA TTACGTCCAAGAC-3′).

3 Methods

To query the protein-protein association conventionally referred to as the "bait-prey interaction" in the yeast two-hybrid parlance, the IVR-positive clones (stuffer marker negative with correct inserts, Fig. 2c) of the baits in one of the two yeast strains were crossed to the IVR-positive clones of the preys in the other yeast

strain. The mating products were selected by means of their ability to grow on double-selection plates ($SD^{-his\ -ura}$) as the mated cells contain both the plasmids bearing the two different selection markers, and then the interaction of the bait and prey is examined by their ability to activate the HOG pathway measured by the ability to grow on hyperosmolarity media.

For convenience the experimental procedure is laid out according to the day on which it needs to be done.

3.1 Day 1

- Streak for single colony on YPD plate yCW1476 *MAT* **a** and yCW1477 *MAT*α cells.
- Incubate for 24–48 h at 30 °C.

Plasmid preparation (*E. coli*).

- Streak for single colony on LB + amp plates *E. coli* containing pCW778 and pYL040 plasmids.
- Incubate overnight (O/N) at 37 °C.

PCR out the fragments of genes of the proteins being tested for interactions from a genomic DNA template. The primers used for this PCR are the ones for IVR as described above.

3.2 Day 2

Plasmid preparation (*E. coli*) (*contd.*).

- Start 5 ml LB-amp primary liquid culture of single colony (for plasmid preparation).
- Incubate secondary culture diluting from the primary culture 1:100 in LB-Amp media overnight or for a maximum of 16–18 h at 37 °C with 220 rpm shaking. Store the *E. coli* cells at −80 °C (*see* **Note 1**).

3.3 Day 3

Plasmid preparation (*E. coli*) (*contd.*).

- Plasmid DNA is purified from the stored *E. coli* cells using a standard kit.
- The purified DNA is both checked on a 0.8 % agarose gel and is quantified spectrophotometrically.

The purified plasmid is digested with *Sma*I in the following way: Remove 3 μl of each plasmid for checking the uncut DNA.

- 37 μl of Plasmid DNA is mixed with 1 μl *Sma*I and 4.3 μl NEB buffer 4 (10×).
- This mix is incubated at 25 °C for 4 h in a water bath.
- The reaction is stopped by heat inactivating the enzyme mix at 65 °C for 20 min.
- The results of the digestion are run on an agarose gel (0.8 %) using 3 μl of cut plasmid; add 7 μl ddH_2O and 1.6 μl 6× DNA-loading dye.

– Use uncut plasmid DNA as a control to check the digestion efficiency (*see* **Note 2**).
– Cut plasmid can be stored at –20 °C for a month.

S. cerevisiae cells are grown in the following way:

– Prepare two sets of 27.5, 25, and 22.5 ml of YPD in 50 ml screw-cap tubes.
– Name them 1/10, 1/100, and 1/1000 YCW1476 (*MAT*a) or YCW1477 (*MAT*α).
– For both mating types, resuspend a single colony in 1 ml of sterile water.
– Take 100 µl and inoculate the first 27.5 ml YPD tube of each series.
– Vortex well and transfer 2.5 ml to the 1/100 dilution.
– Vortex well and transfer 2.5 ml to the 1/1000 dilution.
– Finally all tubes contain 25 ml of YPD.
– Incubate overnight with shaking at 30 °C.

3.4 Day 4

S. cerevisiae transformation protocol:

– Prepare 50 ml of fresh 0.1 M LiAc (use 50 ml screw-cap tube).
– Prepare the transformation mix as follows:

Reagent	Final concentration	Volume (for 10 reactions)
50 % PEG	40 %	1.4 ml
LiAC 1 M pH 7.4	0.1 M	200 µl
ssDNA (boiled)	10 µg/µl	10 µl
Sterile H_2O	–	250 µl

– Choose between *1/10, 1/100, or 1/1000* dilution the one that is the closest to an OD_{600nm} ~0.6 (check that by eye or by spectrophotometer).
– Spin cells down at room temperature at 2500 × *g* for 5 min.
– Wash the cell pellet with sterile 25 ml of 0.1 M LiAc.
– Mix well and spin cells down (2500 × *g* for 5 min).
– Remove as much as possible of the supernatant without damaging the cell pellet.
– Resuspend the cell pellet with the 150 µl transformation mix (*see* **Note 3**).
– Next, in a sterile 2 ml screw-cap tube, add the following:

  100–500 ng of cut plasmid.

  10–20 µl of PCR-amplified DNA (fragments of the proteins being tested).

100–150 μl of *S. cerevisiae* cells in the transforming mix (*see* **Note 4**).

The IVR work is as follows: yeast cells are transformed simultaneously with the cut vector and the PCR-amplified product of the insert one wants to put into the vector.

- Incubate the mixture for 30 min at 30 °C (in an incubator, no water bath required).
- Heat shock the suspended cells at 42 °C for 20 min.
- Wait for 2 min for the cells to cool to room temperature. Centrifuge the mixture at 4000 × *g* for 1 min at room temperature, remove the supernatant, and resuspend the cells in sterile distilled water (optional).
- Plate on the appropriate selective synthetic media plates. (pCW778 transformants are plated on His plate and pYL040 transformants on SD^{-ura} plates.)
- Incubate the plates at 30 °C for 48–72 h.

3.5 Day 6

As soon as colonies appear, replica-plate them on the same velvet for the two plates.

- First, the selection medium (which will also clean up any background growth).
- Second, the counter selection medium.
- For pCW778 transformants, the selection medium is a SD^{-his} and the counterselection media is SD^{-ura} while for a pYL040 transformant the selection medium is a SD^{-ura} and the counterselection medium is SD^{-his}.
- The correct transformants should grow on the selection media but not on the counterselection media.
- Incubate the plates at 30 °C for 24 h.

3.6 Day 7

Colonies are streaked for pre-mating.

- On a new selective plate, streak isolated colonies to make pre-mating plate on selective media (e.g., pCW778 transformants on SD^{-his}, pYL040 transformants on SD^{-ura}) (*see* **Note 5**).
- Incubate at 30 °C for 24 h (Fig. 3a).

3.7 Day 8

Yeast two-hybrid mating cross to bring the bait and prey together.

- The plates with the *MAT* **a** and *MAT* α transformants are crossed on a YPD plate for mating. This is done by replica-plating both strains onto the same velvet with the lines of growing cells at right angles to each other as shown in Fig. 3a.
- Incubate at 30 °C overnight (*see* **Note 6**) (Fig. 3a).

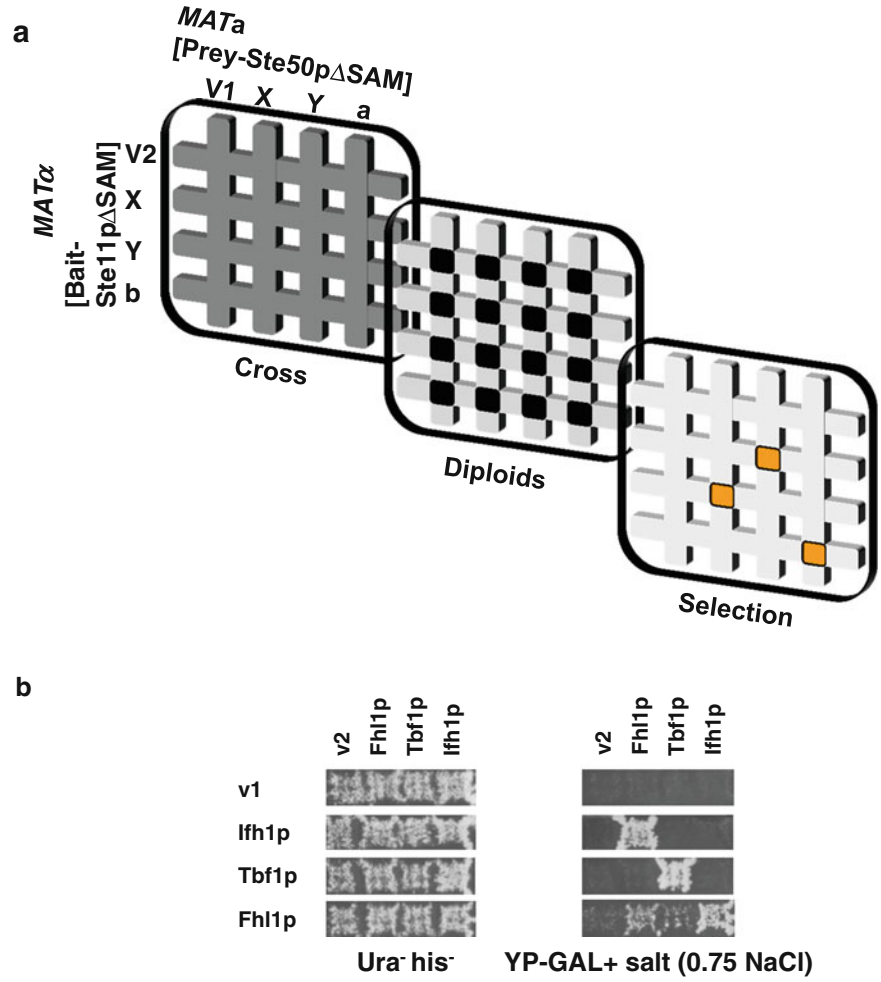


Fig. 3 Schematic analysis of protein-protein interaction: (**a**) Candidate proteins are cloned in both bait and prey configurations through in vivo recombination in two isogenic, osmo-sensitive yeast strains (*ste50*Δ *ssk2*Δ *ssk22*Δ of opposite mating types (yCW1476 and yCW1477)). Stuffer marker negative strains are selected to ensure that there is a candidate insert. The recombinants of bait and prey were crossed and diploid cells containing both bait and prey were selected to assay whether bait and prey interact by testing their ability to activate the HOG pathway as scored for growth on hyperosmotic media. A typical assay will include both negative (V1 and V2) and positive (**a** and **b**) controls. (**b**) An experimental example showing interactions between interacting domains of *Candida* proteins Ifh1p, Fhl1p, and Tbf1p (4). V1 and V2 are empty vectors (Ste11pΔSAM) and (Ste50pΔSAM) used as negative control

3.8 Day 9

Yeast two-hybrid mating product selection for diploid cells that contain both bait and prey.

- Replica-plate the mating plate on double-selection plate ($SD^{-his\ -ura}$).
- Incubate at 30 °C for 48 h (*see* **Note 7**) (Fig. 3a).

3.9 Day 11

Yeast two-hybrid hyperosmotic stress selection:

- Replica plate the growing cells on the double-selection plate onto the stress media (hyperosmolarity media of choice; we routinely use SD + 0.75 M NaCl + 2 % galactose) (*see* **Note 8**).
- Incubate at 30 °C for 5–7 days (*see* **Note 9**) (Fig. 3b).

To confirm that the interaction observed is real in nature, the plasmids from each yeast strain need to be recovered and sequenced to confirm correct cloning and the absence of mutations in the bait and prey queries. The primers for that purpose are given in Subheading 2.

4 Notes

1. Do multiple 5 ml culture preparations per plasmid; usually five to six are enough. Alternatively, larger preparations could be done to maximize quantity.
2. The *Sma*I digestion removes the stuffer marker in each plasmid. Therefore one should expect two bands per digestion. pCW778: 8.0 and 1.0 kb stuffer. pYL40: 9.1 and 1.1 kb stuffer.
3. The volumes of transformation mix in which cell pellets will be resuspended are variable; resuspending the cell pellet for 12 up to 35 reactions has worked well consistently. Because of the viscosity of PEG, it is recommended to give an extra 20 % to cover pipetting errors. Final concentration of 100 mM DTT may be added to the transformation mix to improve transformation efficiency if needed.
4. It should include an "empty vector control." This strain will be used as the mating positive control and hyperosmotic stress negative control.
5. Make a minimum of two lines for each construction (for a total of five colonies).
6. Do not leave the mating plate in 30 °C for longer than overnight.
7. The empty vector control should grow just as well as all other constructions. A positive control of two previously known interacting proteins may be added if available.
8. If instead of mating patches a continuous line is visible, this clone (or one of the five if it is a mixture of colonies) carries both markers and the hyperosmotic stress test will not work.
9. Growing patches should be visible after 3 days. The stronger the interaction is the earlier the patches will grow. To remove background growth, re-replicate the plate on the same media, or on media with higher osmotic stress.

References

1. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340(6230):245–246
2. Bruckner A et al (2009) Yeast two-hybrid, a powerful tool for systems biology. Int J Mol Sci 10(6):2763–2788
3. Wu C et al (2006) Adaptor protein Ste50p links the Ste11p MEKK to the HOG pathway through plasma membrane association. Genes Dev 20(6):734–746
4. Cote P et al (2011) Evolutionary reshaping of fungal mating pathway scaffold proteins. MBio 2(1):e00230-10
5. Mallick J, Whiteway M (2013) The evolutionary rewiring of the ribosomal protein transcription pathway modifies the interaction of transcription factor heteromer Ifh1-Fhl1 (interacts with forkhead 1-forkhead-like 1) with the DNA-binding specificity element. J Biol Chem 288(24):17508–17519
6. Jansen G et al (2005) Drag&Drop cloning in yeast. Gene 344:43–51

Chapter 4

Chromatin Immunoprecipitation (ChIP) Assay in *Candida albicans*

Sreyoshi Mitra, Laxmi Shanker Rai, Gautam Chatterjee, and Kaustuv Sanyal

Abstract

Chromatin immunoprecipitation (ChIP) is a widely used technique which can determine the in vivo association of a specific protein on a particular DNA locus in the genome. In this method cross-linked chromatin is sheared and immunoprecipitated with antibodies raised against a target protein of interest. The end result of this process is the enrichment of DNA fragments associated with the desired protein. Thus, interactions between proteins and genomic loci in cellular context can be determined by this technique. Here, we are describing a ChIP protocol that is optimized for *Candida albicans*. The protocol requires 4–5 days for completion of the assay and has been used to produce robust ChIP results for diverse proteins in this organism and its related species including *Candida dubliniensis* and *Candida tropicalis*.

Key words Protein–DNA interaction, Antibody, Affinity purification, *Candida dubliniensis*, *Candida tropicalis*, Polymerase chain reaction (PCR), Microarray, Next-generation sequencing

1 Introduction

Chromatin immunoprecipitation (ChIP) is a useful technique that has become indispensable over the last decade for studying the in vivo interactions of proteins with specific regions of a genome. The first ChIP assay was developed for analyzing the binding of RNA polymerase II over transcribed and poised genes in *Escherichia coli* and *Drosophila* [1–3]. Over the years, this technique has found its use in diverse cellular processes ranging from the identification of transcription factor binding sites to post-translational modifications (PTMs) of histones associated with expressed or silent chromatin [4]. The basic principle behind this technique involves the use of a small, easily diffusible and reversible cross-linking agent (like formaldehyde) for capturing in vivo protein–DNA interactions. Following cross-linking, the protein bound DNA is sheared by sonication or digested by enzymes to generate suitably smaller

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_4,

fragments which are then immunoprecipitated (IP) with antibodies specific to the protein of interest. During immunoprecipitation, the DNA sequences associated with the protein are preferentially pulled down from the sheared chromatin, thereby giving a realistic approximation of the genomic regions associated with the protein. Following elution of protein–DNA complexes from Protein-A or Protein-G agarose resins, chromatin is de-cross-linked, treated with proteases, and DNA fragments are purified. The technique described above is commonly known as the X-ChIP (Fig. 1). On the other hand, in native ChIP (N-ChIP), proteins remain in the native state, i.e., they are not cross-linked to DNA by formaldehyde. In the N-ChIP technique, shearing of chromatin is performed by micrococcal nuclease (MNase) digestion that fragments chromatin in mononucleosomes as compared to the random fragmentation obtained by sonication. The N-ChIP protocol is suitable for proteins that can remain stably associated during chromatin processing and immunoprecipitation (such as histones) [5]. There are several methods of analysis by which the enriched protein binding sites on the genome can be detected. In the initial studies the IP DNA was immobilized onto a nitrocellulose membrane and hybridized with a radioactive probe from the region of interest to detect binding [3]. However, because of its convenience and to facilitate fast and accurate detection, polymerase chain reaction (PCR), both in its conventional semi-quantitative end-point form [6] and the more sophisticated quantitative real-time PCR (qPCR) [7], is more commonly used for determining enrichment of proteins at specific genomic regions. Subsequently, the applicability of ChIP assays widened significantly as genome-wide association of proteins can be studied by hybridizing ChIP DNA to a DNA microarray (ChIP on chip or ChIP-chip) [8], or can be sequenced by the next-generation sequencing technique (ChIP-sequencing or ChIP-seq) [9, 10].

In this chapter, we describe a ChIP protocol that is followed in our laboratory to study in vivo protein–DNA interactions in the pathogenic budding yeast *Candida albicans* [11–15]. Given the prominence of *C. albicans* as a prolific opportunistic pathogen [16, 17], understanding the global distribution of proteins such as transcription factors and histone PTMs is important for constructing regulatory networks controlling virulence and associated attributes in this organism. Apart from that, *C. albicans* is important as an alternative model system for studying the evolutionary transitions in the mechanism of basic biological processes such as cell division due to the atypical architecture of well-known chromatin landmarks such as centromeres [11, 12]. Thus, an optimized ChIP protocol for *C. albicans* will provide a robust tool for studying these relevant problems.

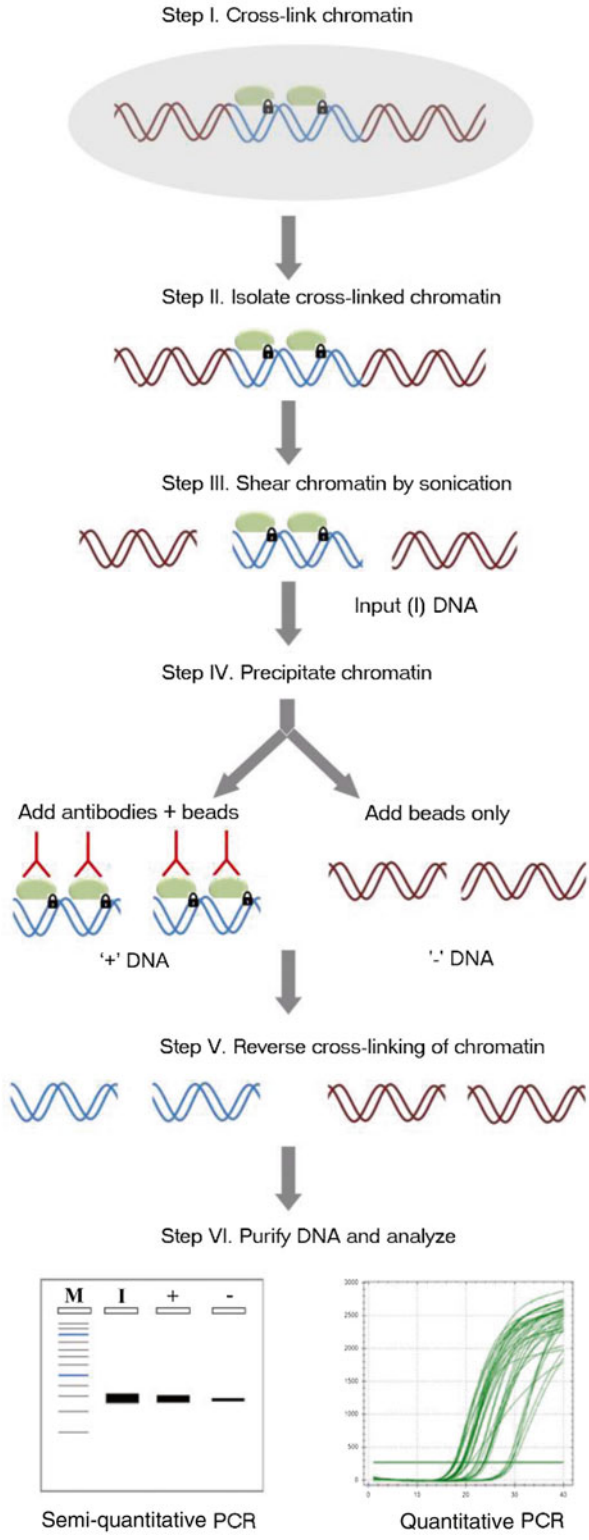


Fig. 1 Schematic of major steps of the chromatin immunoprecipitation assay

2 Materials

Prepare all buffers and other solutions using autoclaved double distilled water and analytical grade reagents. Prepare and store all the solutions at room temperature unless stated otherwise. Strictly follow the appropriate disposal regulations when disposing waste materials.

2.1 Reagents

1. Growth media.
2. Formaldehyde (37 %) (*see* **Note 1**).
3. 2.5 M glycine.
4. 10 % Na-dodecyl sulfate (SDS) (*see* **Note 2**).
5. Lyticase (Sigma Cat. No. L2524).
6. Protease Inhibitor Cocktail (Sigma Cat. No. P8215).
7. Antibodies to the protein of interest (ChIP grade).
8. Protein-A Sepharose beads (Sigma Cat. No. P9424) (*see* **Note 3**).
9. RNase A (Sigma Cat. No. R4875).
10. Proteinase K (Thermo Scientific Cat. No. EO0492).
11. Phenol:chloroform:isoamyl alcohol (Tris saturated) (25:24:1) (Sigma Cat. No. P2069) (*see* **Note 4**).
12. Ethanol.
13. 4 M LiCl.
14. Agarose.
15. PCR reagents.
16. Autoclaved distilled water.

2.2 Buffers

1. Resuspension buffer: 0.1 mM Tris–HCl (pH 9.4), 10 mM Dithiothreitol (Sigma Cat. No. D0632). Make the buffer just before use.
2. Spheroplasting buffer: 20 mM Na-HEPES (pH 7.4), 1.2 M Sorbitol.
3. Post-spheroplasting buffer: 20 mM Na-PIPES (pH 6.8), 1.2 M Sorbitol, 1 mM $MgCl_2$.
4. PBS (1×): 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.10 mM Na_2HPO_4.
5. Wash buffer I: 0.25 % Triton X-100, 10 mM EDTA (pH 8.0), 0.5 mM EGTA, 10 mM Na-HEPES (pH 6.5).
6. Wash buffer II: 200 mM NaCl, 1 mM EDTA (pH 8.0), 0.5 mM EGTA, 10 mM Na-HEPES (pH 6.5).
7. Extraction buffer I: 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1 % Triton X-100, and 0.1 %

Na-deoxycholate. Mix well to dissolve detergent, filter sterilize, and store at 4 °C.

8. Extraction buffer II: 50 mM HEPES-KOH (pH 7.5), 500 mM NaCl, 1 mM EDTA (pH 8.0), 1 % Triton X-100, and 0.1 % Na-deoxycholate. Mix well to dissolve detergent, filter sterilize, and store at room temperature.
9. LiCl wash buffer: 10 mM Tris–HCl (pH 8.0), 250 mM LiCl, 1 mM Igepal CA-630, 0.5 % Na-deoxycholate, and 1 mM EDTA (pH 8.0). Mix well to dissolve detergent, filter sterilize, and store at room temperature.
10. Elution buffer I: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 % SDS.
11. Elution buffer II: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.67 % SDS.
12. IP Dilution buffer: 167 mM Tris–HCl (pH 8.0), 167 mM NaCl, 1.1 mM EDTA (pH 8.0), 1.1 % Triton X-100.
13. TE buffer (1×): 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).
14. TAE buffer (1×): 40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0).
15. Protein-A Sepharose beads suspension: Transfer 1 ml of Protein-A Sepharose beads (shipped as ethanol slurry) into a 15 ml disposable conical tube using a cut-off tip. Wash beads twice in ten volumes of 1× TE (pH 8.0) stored at 4 °C. Briefly, add 10 ml of 1× TE (pH 8.0) and gently rotate on a rotator mixer at 4 °C for 10 min. Spin down beads at 4 °C, 2750×*g*, 10 min. Aspirate supernatant. Repeat wash and spin. Aspirate supernatant. Estimate pellet volume and add an equal volume of 1× TE (pH 8.0) to generate 50 % slurry. Store the beads at 4 °C.

2.3 Equipment

1. Conical flasks.
2. Centrifuge (benchtop).
3. Gel electrophoresis apparatus.
4. Shaker-incubators.
5. Microcentrifuge.
6. Micropipettor with tips.
7. PCR thermocycler.
8. Rotator.
9. Sonicator.
10. Spectrophotometer.
11. Tubes (conical, 15 ml, 50 ml).
12. Tubes (microcentrifuge, 1.5 ml).
13. Vortex mixer.

3 Method

Inoculate an isolated colony of *C. albicans* from a freshly streaked plate into 5 ml of suitable growth media. Grow in a rotating shaker-incubator at 30 °C (or desired temperature), 180 rpm overnight. Next day, inoculate 100 ml media with adequate preculture volume necessary to bring culture to 1.000 OD_{600} units by 5–6 h. Grow in a rotating shaker-incubator at 30 °C (or desired temperature), 180 rpm for 5–6 h (*see* **Note 5**).

3.1 Cross-Linking and Chromatin Preparation

1. Harvest cells at $OD_{600} = 1.000$ (~2×10^7 cells/ml)
2. Fix cells with 2.7 ml of 37 % formaldehyde solution per 100 ml of culture for 15 min at room temperature with occasional gentle swirling (*see* **Note 6**). Henceforth, all the reagent volumes are indicated for a 100 ml ChIP experiment.
3. Quench the cross-linking reaction with 5.4 ml of 2.5 M glycine and incubate at room temperature for 5 min. Store the flask in an ice bath prior to centrifugation (if doing more than one cross-linking at a time). Spin down cells in pre-chilled falcon tubes at 4 °C, 2750 × *g* for 10 min. Pour off the supernatant into a formaldehyde waste bottle (*see* **Note 7** and Table 1).
4. Wash cell pellet with 10 ml of ice-cold resuspension buffer. Incubate on a rotating shaker at 30 °C, 150 rpm for 15 min. Pour resuspended cells into a 50 ml conical tube and spin down cells at room temperature, 2750 × *g* for 10 min. Pour off supernatant.
5. Wash cells with 20 ml of spheroplasting buffer. Spin down cells at room temperature, 2750 × *g* for 10 min. Resuspend pellet in 5 ml of spheroplasting buffer. Remove 50 µl to 1 ml of 5 % SDS (0 min time point) and read OD_{800}.
6. Spheroplast cells by adding 50–70 µl of lyticase (10 mg/ml) to the tube. Incubate the cells at 30 °C, 65 rpm for ~45 min to obtain >90 % spheroplasts (*see* **Note 8** and Table 1).
7. Next, add 20 ml of post-spheroplasting buffer to the tube and spin at room temperature, 2750 × *g* for 5 min. Keep spheroplasts on ice hereafter.
8. Wash the spheroplasts sequentially with 15 ml of ice-cold 1× PBS, 15 ml of buffer I, and 15 ml of buffer II. Pellet cells at 4 °C, 2750 × *g* for 5 min, between each wash.
9. Resuspend the pellet in 2 ml of ice-cold extraction buffer I and add 10 µl of protease inhibitor cocktail to the solution. Distribute 330 µl of the lysate, thus prepared, to 1.5 ml Eppendorf tubes. Keep the tubes on ice (*see* **Note 9**).

Table 1
Troubleshooting tips for ChIP protocol

Problem	Possible reason	Solution
1. Yield of input DNA is low	1. Too few cells 2. Loss of spheroplasts during washing	1. Proper cell number (as mentioned in the protocol) should be monitored while harvesting the cells 2. Supernatant should be decanted carefully while washing the spheroplasts or reduce the time of spheroplasting (if the pellet is loose)
2. Chromatin fragments are too large (>900 bp)	1. Cells were over cross-linked 2. Foaming during sonication 3. Insufficient sonication 4. Improper spheroplasting	1. Cross-linking time has to be reduced 2. If using a probe sonicator, the sonicator tip should be kept centered and away from the bottom of the tube 3. Number of cycles of sonication has to be increased 4. Ensure proper spheroplasting by monitoring at OD_{800} during lyticase treatment and also confirm by microscopic observation
3. No specific signal/enrichment is observed in IP	1. Protein of interest did not cross-link efficiently to chromatin 2. Sheared DNA fragments are too large or too small 3. Antibodies did not efficiently bind to protein from cross-linked chromatin 4. Protein epitope recognized by antibody is masked by cross-linking 5. Loss of signal during beads washing 6. Protein of interest is expressed in vivo at a low level	1. Increase the time of formaldehyde cross-linking 2. Adjust the sonication time to get the DNA fragment size between 200 and 700 bp 3. Antibody specificity can be indirectly gauged by western blot and immunoprecipitation. However, it may be necessary to generate another batch of antibodies (preferably polyclonal) 4. This is a problem specific to monoclonal/peptide antibodies. It may be necessary to generate polyclonal antibodies against the protein Cross-linking time needs to be optimized so that over cross-linking is prevented 5. Washing stringency to be reduced with longer washing times being avoided 6. Expression of the protein of interest can be artificially increased under regulatory promoter such as *PCK1* [20] and *TDH3* [21]
4. High background signal in negative control	1. Nonspecific binding of protein to the IP beads 2. Genomic or plasmid DNA contamination in the buffers or PCR reagents	1. Pre-clear lysates with beads before adding antibodies [22] 2. Perform control PCR without chromatin DNA to check for contamination

10. Sonicate lysate to shear DNA. Sonication can be performed with 12 rounds of 10 s bursts at 30 % amplitude using a sonicator (Sonics Vibra-Cell). Keep samples on ice at all times and wait 1 min between bursts. Alternatively, sonication can be done in a bath sonicator such as Bioruptor (Diagenode) using 16 rounds of 15 s on and 15 s off cycle at "High" amplitude setting. After every four cycles, change the water of the bath to maintain 4 °C ambient temperature. These conditions will generate an average chromatin size between 200 and 700 bp (*see* **Notes 10** and **11**, Fig. 2a, and Table 1).
11. Spin down cell debris in a microcentrifuge at 4 °C, 19200 × *g* for 15 min. Collect the supernatant (*see* **Note 12**).

3.2 Chromatin Immunoprecipitation

1. Input (I): Remove 500 µl of cleared lysate to a 15 ml disposable conical tube as input (I). Add 1.5 ml elution buffer I, and incubate at 65 °C overnight to reverse cross-links.
2. Immunoprecipitate (IP): Divide remaining lysate in half between two 15 ml disposable conical tubes. Add 5.7 volumes IP dilution buffer. Leave one as "without antibody" or "beads only" control ("–"). To the "with antibody" ("+") sample add the requisite antibody (typically 5–10 µg is sufficient). Incubate on a rotator at 4 °C, overnight.

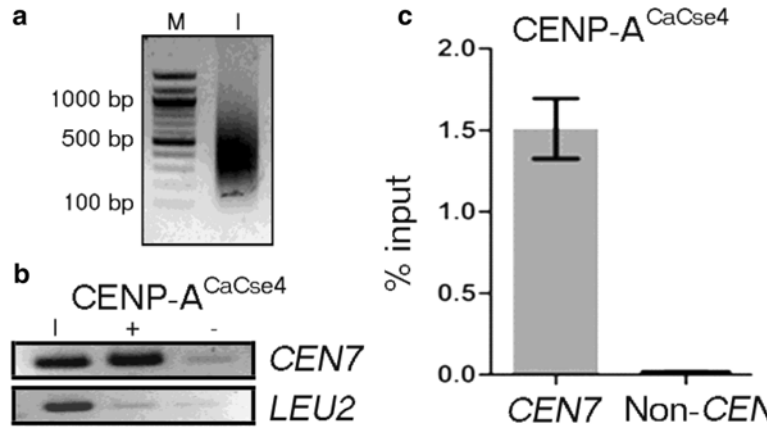


Fig. 2 Analysis of ChIP DNA. (**a**) A 2 % agarose gel image showing sheared and purified input (I) DNA of *C. albicans* along with 100 bp ladder (M). The number of sonication cycles used in this experiment was 16 (Bioruptor, Diagenode). (**b**) The ChIP-PCR profile of centromere 7 (*CEN7*) region of *C. albicans* showing the enrichment of CENP-A^{CaCse4}. Input (I), total chromatin; +, precipitation with antibodies (anti-CENP-A^{CaCse4} - Prot A); –, precipitation without antibodies. The *LEU2* locus serves as a noncentromeric negative control region. (**c**) The quantitative real-time PCR (qPCR) profile of centromere region of *C. albicans* showing enrichment of CENP-A^{CaCse4} at the centromere (unpublished)

3.3 Immunoprecipitate (IP) Capture

1. Next day, add 50 µl of washed Protein-A-linked Sepharose beads (50 % slurry in 1× TE pH 8.0) per ml of IP to each IP reaction. Incubate on a rotator at 4 °C for 10–12 h (*see* **Note 13**).

3.4 IP Washes and Elution

1. Spin down captured complex/beads at room temperature, 2750 × *g* for 3 min, and aspirate supernatant.
2. Wash beads sequentially using the following protocol: Twice for 5 min each in 12.5 ml extraction buffer I, once for 5 min in 12.5 ml extraction buffer II, once for 5 min in 12.5 ml LiCl wash buffer, and twice for 5 min in 12.5 ml 1× TE (pH 8.0). Incubate tubes on a rotator at room temperature for 5 min between each wash. After each wash spin down beads at room temperature, 2750 × *g* for 3 min, and aspirate the supernatant carefully with a micropipette (*see* **Note 14**).
3. Elute IP in 1/10 volume of elution buffer I. Incubate the tubes at 65 °C for 15 min. Spin down beads at room temperature, 2750 × *g* for 3 min and transfer eluate to a fresh 15 ml tube.
4. Elute a second time with 1/6.7 volume of elution buffer II. Incubate at 65 °C for 5 min. Spin down beads at room temperature, 2750 × *g* for 3 min and pool eluates I and II. Incubate at 65 °C overnight to reverse cross-links (*see* **Note 15**).

3.5 IP DNA Purification

1. Add 1.56 ml of 1× TE (pH 8.0) and 5 µl RNase A (10 mg/ml) to the de-cross-linked eluate. Incubate at 37 °C for 3 h.
2. Add 46 µl of Proteinase K (20 mg/ml). Incubate at 37 °C for 3 h.
3. Add 405 µl of 4 M LiCl and 4.05 ml phenol:chloroform:isoamyl alcohol and vortex for 10 s. Spin down phenol extraction at room temperature, 2750 × *g* for 5 min. Pool aqueous layer and distribute 500 µl into 1.5 ml Eppendorf tubes. Add 1 ml of ice-cold 100 % ethanol and precipitate DNA at –20 °C, overnight (*see* **Note 16**).

3.6 Input (I) DNA Purification

1. To the input, add 665 µl of 1× TE (pH 8.0) and 5 µl RNase A (10 mg/ml). Incubate at 37 °C for 1 h.
2. Add 25 µl Proteinase K (20 mg/ml). Incubate at 37 °C for 4 h.
3. Add 230 µl of 4 M LiCl and 2.3 ml of phenol:chloroform:isoamyl alcohol and vortex for 10 s.
4. Pool aqueous layer and distribute 500 µl into 1.5 ml Eppendorf tubes.
5. Add 1 ml of 100 % ethanol per tube and vortex. Precipitate DNA at room temperature for ≥15 min.
6. Spin down the precipitate in a microcentrifuge at room temperature, 19200 × *g* for 20 min.

7. Wash DNA pellet with 1 ml of 70 % ethanol. Spin down the precipitate in a microcentrifuge at room temperature, 19200 × *g* for 5 min.
8. Dry the pellet in speedvac for 10 min.
9. Resuspend DNA pellet in 50 µl of 1× TE (pH 8.0) and store at 4 °C.
10. Check size of sheared input DNA: Run 5 µl input DNA on a 1 % agarose 1× TAE gel at 100 V for 35 min. Include 0.5 µg 100 bp DNA ladder. Use 0.5× bromophenol blue tracking dye. The average fragment size of the sheared DNA should be ideally between 200 and 700 bp in range (Fig. 2a) (*see* **Note 17** and Table 1).

3.7 IP DNA Precipitation

1. Spin ethanol-precipitated IP DNA in microcentrifuge at 4 °C, 19200 × *g* for 30 min. Wash each DNA pellet with 1 ml of ice-cold 70 % ethanol and spin down in a microcentrifuge at 4 °C, 19200 × *g* for 15 min.
2. Dry pellet in speedvac for 10 min.
3. Resuspend DNA in 100 µl 1× TE (pH 8.0) by pipetting up and down and transferring until the last tube is completed. Store at 4 °C (short term) or at −20 °C (long term).

3.8 PCR

1. Set up 25 µl reactions with three units of Taq DNA polymerase and 50 pmol of each primer. Initially try 2 µl of IP material (both +DNA and −DNA samples) and 1/2000 of input (I) (2 µl of 1:40 dilution).
2. Amplify with a customized PCR program.
3. Electrophorese on 2 % agarose 1× TAE gels and visualize bands after staining with ethidium bromide (*see* **Note 18**) (Fig. 2b). Digitally photograph the gels and determine densitometry values with Quantity One software. The relative enrichment can be computed as (+DNA) − (−DNA)/I DNA of the test (e.g., centromere) divided by (+DNA) − (−DNA)/I DNA of the control (e.g., noncentromeric locus) (*see* **Note 19** and Table 1).
4. Alternatively perform quantitative real-time PCR reaction (qPCR) with the IP and input DNA (Fig. 2c). The qPCR enrichment is usually determined by the percent input method (% input) [18]. In brief, the C_t values for input (I) are adjusted for the dilution factor and then the percent of the input chromatin immunoprecipitated by antibodies is calculated as $100 \times 2^{(\text{Adjusted Input } C_t - \text{IP } C_t)}$ (*see* **Note 20**).

3.9 Controls

1. Negative control: Use a "without antibody" sample as a negative control to determine the background of the assay. Alternatively, for an epitope-tagged protein, ChIP DNA

obtained from an untagged strain can be used as a negative control. ChIP DNA obtained by using a control immunoglobulin (nonspecific antibodies of similar isotype or pre-immune serum) can also be used as a negative control.

2. Positive and negative control loci: A genomic locus where the protein of interest is known to bind and a locus where it does not bind can be used as positive and negative control loci respectively for conventional semi-quantitative PCR as well as qPCR analysis. These controls confirm the specificity of the protein binding.
3. Non-template control: A non-template control should be included in the PCR or qPCR reactions for checking PCR contamination.
4. Positive control antibodies: ChIP with well-established ChIP-grade antibodies against proteins such as histones provides a positive control to test whether all the steps of the protocol are working. However, it does not ensure the success of another ChIP experiment being performed.

4 Notes

1. Formaldehyde is flammable. It is highly toxic on inhalation, swallowing, or in contact with skin. Formaldehyde should be used with adequate safety measures such as protective gloves, clothing, and glasses in a sufficiently ventilated area. The formaldehyde waste should be disposed of as per regulations for hazardous waste.
2. SDS causes irritation on contact with eye and skin. It causes respiratory tract irritation on inhalation, nausea and vomiting on ingestion. SDS should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation.
3. Alternatively Protein-G sepharose beads can be used, depending on the subtype of the primary antibody.
4. Old phenol:chloroform:isoamyl alcohol solution or solutions with low pH causes DNA degradation. Phenol is toxic and causes burns on the skin. It is toxic when swallowed. Further, it is irritating to the eyes, respiratory system, and skin. The phenol:chloroform:isoamyl alcohol solution should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation. The phenol:chloroform:isoamyl alcohol waste should be disposed of as per regulations for hazardous waste.

5. The culture should not be harvested beyond 1 OD_{600} (mid-log phase) as cultures grown beyond this density tend to produce lower ChIP DNA yield.
6. Cross-linking time has to be optimized for each protein analyzed. For example, 15 min cross-linking time is sufficient for histones whereas for proteins that transiently bind to the DNA or associate with the DNA through another protein/protein complex, the cross-linking time can be extended to 90 min or more. Over cross-linking can interfere with the chromatin shearing and lead to denaturation of the protein or even can cross-link nonspecific DNA–protein interaction. Insufficient cross-linking leads to reduced recovery of regions associated with the protein of interest.
7. Be careful while decanting the supernatant as the pellet may be loose. After pelleting at this step, the cell pellet can be stored at –20 °C for a few days, alternatively.
8. The time for spheroplasting varies between different strains in *C. albicans* and also depends on the morphological states (yeast, pseudohyphae, and hyphae). Thus, the time and the amount of lyticase to be used for spheroplasting have to be optimized for the above conditions. It has also been observed that the spheroplasting efficiency varies with the batch of lyticase used. Over-spheroplasting results in a very loose pellet leading to a loss of chromatin in subsequent washes. Insufficient spheroplasting can reduce the efficiency of chromatin shearing.
9. Use cut micropipette tips to resuspend the spheroplasts gently by pipetting up and down. Be careful while decanting the supernatant between each wash so as not to lose spheroplasts. Take care that the pellet is dissolved uniformly in the extraction buffer.
10. The sonication time has to be optimized for different strains and morphological states being studied. The sonication efficiency is also reduced by over cross-linking and improper spheroplasting. The size of the sheared chromatin obtained should be checked during optimization, as discussed later.
11. Avoid foaming and heating of the samples during sonication as it can result in reduction in sonication efficiency and degradation of chromatin, respectively.
12. The cleared lysate can be stored at 4 °C for a few days.
13. Make sure that the bead slurry is completely resuspended before adding to the IP. Cut off the ends of regular micropipette tips and use them to transfer beads.
14. Be careful not to aspirate any beads while taking the supernatant.
15. The minimum time for reverse cross-linking is 6 h. Extending the de-cross-linking step overnight or up to 15 h does not cause a significant damage to the DNA.

16. Glycogen can be used during IP DNA precipitation for increasing the yield of IP DNA. Alternatively, DNA can be purified by QIAGEN DNA purification kits.
17. The size of the sheared chromatin is to be optimized with the desired spacing and length of the PCR probes. For example, an average fragment size of 400 bp is optimum for probes spaced ~1 kb apart and an amplicon length of 200–300 bp.
18. Ethidium bromide is a potent mutagen and can be absorbed through skin. It is an irritant to the eyes, skin, mucous membranes, and upper respiratory tract. Ethidium bromide should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation. The ethidium bromide waste should be disposed of as per regulations for hazardous waste.
19. For increasing the reliability, replicates of the PCR reaction can be run with serial dilutions of input and IP DNAs in order to ensure that the reactions are in a linear range of detection. Further, a combination of test and control primers can be used for amplification so as to obtain test and control bands in the same agarose lane.
20. The amount of both IP and input DNAs has to be standardized for qPCR experiment. The C_t value between 20 and 25 cycles is most desirable. At least three technical replicates of each qPCR reaction are required to ensure accuracy of the results.

5 Applications

The ChIP technique is a robust and reliable method to study in vivo binding patterns of proteins. The applicability of this technique is enhanced dramatically by inclusion of ChIP-chip and ChIP-seq assays. In the ChIP-chip technique, a reference and test IP DNA is purified, amplified by ligation-mediated PCR (LM-PCR), and differentially labeled with fluorescent dyes. Next, they are hybridized to a DNA tiling array comprised of oligonucleotide probes covering the entire genome, individual chromosomes or specific genomic loci. The relative fluorescent intensity of the test versus reference channels gives the degree of enrichment of the protein of interest. The end result of this process is the generation of high-resolution genome-wide maps of protein occupancy. However, with the development of large-scale next-generation sequencing techniques, ChIP-seq has largely replaced ChIP-chip as the method of choice for genome-wide studies of protein–DNA interactions. In this method, the IP DNA is purified, processed, and analyzed by massively parallel DNA sequencing [10]. The major advantages of ChIP-seq over ChIP-chip are higher resolution

and coverage. The high-resolution genome-wide profiles of protein binding generated by ChIP-chip and ChIP-seq analyses help in delineating expression and regulatory domains on chromatin. These techniques are also indispensable for finding genome-wide binding of evolutionarily conserved proteins in relatively less worked out organisms, whose whole genome has been sequenced. A detailed protocol of ChIP-seq assay for *C. albicans* has been described elsewhere [15].

Further, the protocol described here has also been used for performing ChIP and ChIP-seq in the related *Candida* species viz. *Candida dubliniensis* [19, 15] and *Candida tropicalis* (unpublished). Thus, this procedure can be used as a standard ChIP protocol for many *Candida* sp.

6 Limitations

Despite their immense utility, ChIP and its associated techniques have several limitations. First, success of this assay depends primarily on the choice of the antibodies. Although, several antibodies can perform well in applications such as western blot, they often fail to recognize the presented epitope in a cross-linked chromatin. Further, under in vivo conditions, they can also show cross-reactivity with other chromatin proteins. These problems manifest themselves in the form of low signal to noise ratio in the output data. Second, the resolution of the traditional ChIP assays is not enhanced enough to identify precise binding sites of a protein. Although, the resolution is significantly increased in ChIP-chip and ChIP-seq assays, the enhanced sensitivity leads to increased probability of obtaining false positive hits. Therefore, whenever possible, the ChIP-seq and ChIP-chip hits should be validated by conventional PCR. Finally, binding of proteins to a genomic locus by ChIP does not, by itself, demonstrate the functional significance of the binding. Thus, additional experiments must be performed to determine the implications of the binding.

Acknowledgements

The ChIP method for *Candida albicans* was first developed by KS in John Carbon's laboratory and further modified. The work was supported by a grant from the Department of Biotechnology, Government of India, to KS. The intramural support from JNCASR is also acknowledged. SM, LSR, and GC were supported by senior research fellowships from Council of Scientific and Industrial Research (CSIR), Government of India.

References

1. Gilmour DS, Lis JT (1984) Detecting protein-DNA interactions in vivo: distribution of RNA polymerase on specific bacterial genes. Proc Natl Acad Sci U S A 81(14):4275–4279
2. Gilmour DS, Lis JT (1985) In vivo interactions of RNA polymerase II with genes of *Drosophila melanogaster*. Mol Cell Biol 5(8):2009–2018
3. Gilmour DS, Lis JT (1986) RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in *Drosophila melanogaster* cells. Mol Cell Biol 6(11):3984–3989
4. Rundlett SE, Carmen AA, Suka N, Turner BM, Grunstein M (1998) Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature 392(6678): 831–835
5. O'Neill LP, Turner BM (2003) Immunoprecipitation of native chromatin: NChIP. Methods 31(1):76–82
6. Hecht A, Strahl-Bolsinger S, Grunstein M (1996) Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature 383(6595):92–96
7. Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. Plant Methods 3:11
8. Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E, Volkert TL, Wilson CJ, Bell SP, Young RA (2000) Genome-wide location and function of DNA binding proteins. Science 290(5500):2306–2309
9. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129(4):823–837
10. Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T, Euskirchen G, Bernier B, Varhol R, Delaney A, Thiessen N, Griffith OL, He A, Marra M, Snyder M, Jones S (2007) Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. Nat Methods 4(8):651–657
11. Sanyal K, Baum M, Carbon J (2004) Centromeric DNA sequences in the pathogenic yeast *Candida albicans* are all different and unique. Proc Natl Acad Sci U S A 101(31):11374–11379
12. Baum M, Sanyal K, Mishra PK, Thaler N, Carbon J (2006) Formation of functional centromeric chromatin is specified epigenetically in *Candida albicans*. Proc Natl Acad Sci U S A 103(40):14877–14882
13. Roy B, Burrack LS, Lone MA, Berman J, Sanyal K (2011) CaMtw1, a member of the evolutionarily conserved Mis12 kinetochore protein family, is required for efficient inner kinetochore assembly in the pathogenic yeast *Candida albicans*. Mol Microbiol 80(1): 14–32
14. Thakur J, Sanyal K (2012) A coordinated interdependent protein circuitry stabilizes the kinetochore ensemble to protect CENP-A in the human pathogenic yeast *Candida albicans*. PLoS Genet 8(4), e1002661
15. Thakur J, Sanyal K (2013) Efficient neocentromere formation is suppressed by gene conversion to maintain centromere function at native physical chromosomal loci in *Candida albicans*. Genome Res 23(4):638–652
16. Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJ, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PW, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KA, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MP, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NA, Lorenz MC, Birren BW, Kellis M, Cuomo CA (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459(7247):657–662
17. Mavor AL, Thewes S, Hube B (2005) Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. Curr Drug Targets 6(8):863–874
18. Mukhopadhyay A, Deplancke B, Walhout AJ, Tissenbaum HA (2008) Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*. Nat Protoc 3(4):698–709
19. Padmanabhan S, Thakur J, Siddharthan R, Sanyal K (2008) Rapid evolution of Cse4p-rich centromeric DNA sequences in closely related pathogenic yeasts, *Candida albicans* and *Candida dubliniensis*. Proc Natl Acad Sci U S A 105(50):19797–19802
20. Leuker CE, Sonneborn A, Delbruck S, Ernst JF (1997) Sequence and promoter regulation of the PCK1 gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen *Candida albicans*. Gene 192(2):235–240
21. Perez JC, Kumamoto CA, Johnson AD (2013) *Candida albicans* commensalism and pathogenicity are intertwined traits directed by a tightly knit transcriptional regulatory circuit. PLoS Biol 11(3), e1001510
22. Harlow E, Lane D (2006) Immunoprecipitation: preclearing the lysate. CSH Protoc2006(4)

Chapter 5

Assessing Mitochondrial Functions in *Candida albicans*

Dongmei Li and Richard Calderone

Abstract

This chapter is designed to present methods for characterizing the annotation of genes associated with mitochondrial functions in *Candida* spp. Methods include drop plate assays for evaluating inhibitors of the respiratory electron transport system complexes as well as measuring the enzyme activity of complex I-V enzyme activities. Assays are also presented to measure toxic ROS production that accompanies gene mutations or gene loss and chronological aging that often is shortened in Complex I dysfunction. Also presented are methods to isolate mitochondria, visualize mitochondria, and extract mitochondrial proteins.

Key words Mitochondria, Electron transport chain, Complexes, Life span, Isolation, Gel electrophoresis

1 Introduction

Mitochondria are the major energy producing organelles of most microorganisms, including fungal pathogens. The many roles of mitochondria in cell processes are critical to both their growth and survival under stress conditions. Among these cell processes of which mitochondria are essential in *Candida* spp. include chronological life-span, filamentation, cell wall synthesis, prevention of apoptosis, membrane synthesis, and virulence [1–12]. Several studies have documented the requirement of mitochondria for virulence [2, 9, 11]. Peroxisomes and mitochondria are known to reversibly shuttle acetyl-CoA, and gene deletions of acetyl-CoA transferases result in decreases in virulence [13]. Energy production by mitochondria is associated with the electron transport system (ETS) composed of four complexes (CI-CIV) and CV which generates ATP via CI, CIII, and CIV reactions. The CI complex of diverse eukaryotes has been reported [14]. Of importance, mitochondria vary among mammals and fungi, in the number and specificity of complex I (CI) subunit proteins [10] and mitochondrial respiratory pathways [1]. At least two CI subunit proteins are fungal specific, suggesting their exploitation in antifungal drug discovery (submitted).

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_5,

There are several reasons why *Candida* species may serve as an alternative example to that of the model yeast, *S. cerevisiae*. Studies of *Candida* species mitochondria include those of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* [1]. While these species have three respiratory pathways, the classical, alternative, and PAR, *S. cerevisiae* lacks at least the alternative and PAR pathways. The alternative and PAR pathways are presumed to be backups to a defective classical pathway. Further, the multi-subunit Complex 1 (CI) of the classical respiratory pathway is nonexistent in *S. cerevisiae*, while it is fully developed among the *Candida* species just mentioned [14]. There are mitochondrial proteins of CI that are fungal specific as mentioned above. Gene knockout strains in CI, for example, result in profound loss of cell functions (submitted). The exquisite specificity of mitochondrial proteins in the CTG clade of the Ascomycotina deserves mentioning. The CTG clade of organisms includes most *Candida* species except *C. glabrata*. The *C. albicans* Goa1p was first identified in a *goa1*Δ mutant with hypersensitivity to reactive oxidants [2]. Further pursuit of this protein revealed its critical role in CI complex enzyme activity as well as a profound upregulation of peroxisomal genes [4].

2 Materials

1. *Strains of C. albicans and growth media.*
   (a) YPD broth—1 % yeast extract, 2 % peptone, 2 % glucose, w/wo 1.7 % agar.
   (b) SC minimal medium. [4, 16] Synthetic complete, including 2% glucose
   (c) Non-glucose media: 2 % Glycerol-YP; 2 % lactate-YP; 2 % ethanol-YP.
2. *Drop-plate assays; ROS compounds*; H_2O_2 (2–6 mM); menadione (0.125 mM) in YPD; respiratory pathway inhibitors (1 μM rotenone, 6 μM TTFA, 8 μM antimycin A, 1 or 10 mM KCN, 20 μM oligomycin, and 2 mM SHAM [final concentrations]).
3. *ROS measurements:* 50 μM DCFDA (final concentration); FACScan flow cytometer (488 nm; Becton Dickinson).
4. *Oxygen uptake by fungal cells:* 125 mM sucrose, 65 mM KCl, 10 mM HEPES (pH 7.2), 2.5 mM KH_2PO_4, 1 mM $MgCl_2$.
5. *Chronological life-span (CLS).* YPD agar medium; synthetic SC medium (synthetic glucose); 5 mM H_2O_2.
6. *Mitochondrial membrane potential*: Green fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide) (stock 5 μM JC-1 in 2 % DMSO); JC-1 (monomer $\lambda_{em} = 527$ nm); JC-1 forms red fluorescent J-aggregates ($\lambda_{em} = 590$ nm).

7. *Spheroplast preparation:* 10 mM dithiothreitol (DTT) in 100 mM Tris–HCl, pH 9.0 (*Buffer A*); Zymolyase 100 T (Seikagaku Biobusiness, Inc.) 0.25 mg/ml in 1 M sorbitol, 20 mM MOPS pH 7.4.
8. *Isolation of mitochondria*: *Buffer B*, 0.6 M sorbitol, 20 mM MOPS, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF); *Buffer C*, 0.6 M mannitol, 20 mM MOPS, pH 7.0, 2 mM $MgCl_2$, and 1 mM EDTA; *Buffer D*, 0.6 M mannitol, 20 mM MOPS pH 7.0.
9. *Mitochondria-enriched isolation: Buffer E* (0.3 M mannitol, 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], 0.1 % [wt/vol] BSA, pH 7.5) containing 30 % (vol/vol) Percoll; *Buffer F* (0.3 M sucrose, 10 mM TES, 0.1 % [wt/vol] BSA, pH 7.5) containing 20 % Percoll (vol/vol).
10. *Extraction of semi-purified respiratory complex proteins CI-CIV: cold S1 buffer* (272 mM sucrose, 40 mM HEPES, 150 mM KCl, pH 7.5); *S2 buffer* (40 mM HEPES, 150 mM KCl, pH 7.5); *S3 buffer* (solubilization medium; 750 mM 6-aminohexanoic acid [ε-amino-*n*-caproic acid], 50 mM bis-Tris–HCl, 0.5 mM EDTA, pH 7.0). A concentration of 2 mg of protein/ml was used in assays.
11. *Blue native (BN) polyacrylamide gel electrophoresis* of *CI-CIV respiratory complexes:* 2× BN sample buffer (1.5 M 6-aminohexanoic acid, 0.05 M bis-Tris (pH 7.0), 65 µl of 10 % DMM, 20 µl of proteinase inhibitor mixture, and 100 µl of glycerol); cathode buffer (10× stock of 500 mM Tricine), 150 mM bis-Tris (pH 7.0; or 75 mM imidazole), and 0.02 % Serva Blue G-250, +0.02 % DDM. Anode buffer, 50 mM bis-Tris, pH 7.0; *In-gel enzyme assay for CI*; MilliQ water, equilibrated in 0.1 M Tris–HCl, pH 7.4 (reaction buffer), for 20 min; reaction buffer with 0.2 mM NADH—0.2 % nitro blue tetrazolium (NBT) for 1 h; fixing gels, 45 % methanol—10 % (vol/vol) acetic acid; destain overnight in the same solution. Image J software, processing [4, 16].
12. *Enzyme assays of CI-CIV proteins (performed with 20 µg semi-purified mitochondrial proteins)*

    These assays are described in other refs. [4, 15].

    (a) *Complex I* (NADH:ubiquinone oxidoreductase): protein added to 0.2 ml of a solution containing 50 mM Tris, pH 8.0, 5 mg/ml BSA, 0.24 mM KCN, 4 µM antimycin A, and 0.8 mM NADH (*substrate*), *electron acceptor*, 50 µM DB (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4 benzoquinone).

    (b) *Complex II* (succinate:ubiquinone oxidoreductase): protein added to 10 mM KH_2PO_4 (pH 7.8), 2 mM EDTA, 1 mg/ml BSA, 80 µM DCPIP (acceptor; 2,6-dichlorophenolindophenol), 4 µM rotenone,

0.24 mM KCN, 4 μM antimycin A, and 0.2 mM ATP (total of 1 ml); *substrate*, 10 mM succinate, CII-specific activity, 1 mM TTFA (thenoyltrifluoroacetone) is added.

(c) *Complexes II and III (succinate:cytochrome* c *reductase):* 40 μM oxidized cytochrome *c*, followed by the addition of 1 mM TTFA; *substrate*, 10 mM succinate.

(d) *Complex IV (oxidation of reduced cytochrome* c): 5 mg of L-ascorbic acid + 100 mg of cytochrome *c* in 8 ml of 10 mM potassium phosphate buffer, pH 7.0; dialyzed cytochrome *c* solution, dialyzed against 10 mM potassium phosphate buffer, pH 7.0, for 20 h at 4 °C with three changes of buffer. Cytochrome c (10 mM) also can be reduced using 0.1 mM DTT, which is adjudged as a color change from dark orange-red to pale purple red after a 15 min treatment at

RT. Reduced cytochrome *c* in 10 ml with 0.1 M phosphate buffer (pH 6.8). In a 1-ml reaction medium, 20 μg of mitochondrial protein was added to a buffer containing 10 mM KH_2PO_4 (pH 6.5), 0.25 M sucrose, 1 mg/ml BSA, and 10 μM reduced cytochrome *c*. 2.5 mM lauryl maltoside is used to permeabilize mitochondria.

13. *Visualizing mitochondria* [2]. Mid-log phase cells; DAPI (5 μg/mL); Mitotracker Red. Far-red-fluorescent dye (abs/em ~581/644 nm) that stains living cells/mitochondria [2, 11].
14. *TCA cycle and associated enzymes.* (a) α-ketoglutarate dehydrogenase (KGDDH): 25 mM MOPS, 0.05 % Triton X-100, pH4; 2.5 mM NAD, 4 mM pyruvate 0.13 mM CoASH, 0.13 mM thiamine pyrophosphate; 8 μM rotenone (to prevent oxidation of NADH). (b) Citrate synthase: 100 mM Tris–HCl, 0.25 mM DTNB, 0.2 mM oxaloacetate, 0.1 mM acetyl-CoA. (c) Isocitrate lyase (ICL) activity: 100 mM potassium phosphate buffer, pH 7.0 + 4 mM phenylhydrazine, 2.5 mM cysteine, 2.5 mM $MgCl_2$, 2 mM isocitrate [16, 17].

3 Methods

1. *Strains and their growth.* All strains were maintained as frozen stocks and propagated on yeast extract-peptone-dextrose (YPD) agar, or YPD broth overnight at 30 °C when needed (1 % yeast extract, 2 % peptone, 2 % glucose, w/wo 2 % agar). Methods can apply to the testing of multiple sets of strains which usually consist of (1) the parental cells from which genetic manipulations have been done to construct, (2) a null strain, lacking both alleles of a specific gene, and [2] a gene-reconstituted strain that is heterozygous for the gene under investigation. Thus, the relationship of gene dosage to a particular phenotype

can be determined [2]. Most assays described below are easily used with a set of these mutants. If growth (generation time) varies among strains, especially a null compared to WT or a gene-reconstituted strain, it may be necessary to use incubation times that reflect similar optical density measurements.

2. *Drop-plate assays.* Growth inhibition is visualized by plating 5 μl of tenfold serial dilutions of a 5×10^5 cell stock suspension onto YPD agar plates containing hydrogen peroxide, menadione, or respiratory complex inhibitors (see Subheading 2, **item 2** and Subheading 4, *see* **Notes 1–3**). Plates are photographed and evaluated after 48 h of incubation at 30 °C. For non-glucose utilization, glucose in yeast extract-peptone agar is replaced with 2 % citrate, glycerol, lactate, oleic acid, or ethanol, and drop plates are done as described above.
3. *Reactive oxidant species measurements (ROS).* Intracellular ROS production is detected by staining cells with the ROS-sensitive fluorescent dye DCFH-DA (2,7-dichlorofluorescein diacetate; Sigma). Cells from 25-ml cultures grown at 30 °C overnight are centrifuged. The cell pellets are washed twice with PBS (pH 7.0), suspended to 10^6 cells in 10 ml of PBS plus 2 % glucose, and treated with or without DCFH-DA for 30 min at 37 °C in the dark (*see* **Note 1**). Cells from each sample are collected, washed twice after staining, and fluorescence is measured using a FACScan flow cytometer (Becton Dickinson). To measure dead cells prior to DCFH-DA assays, propidium iodide (PI) is added to each sample so that ROS is measured only in live cells.
4. *Mitochondria membrane potential.* Mitochondrial membrane potential is measured by staining cells with the JC-1 reagent. Cells from 25-ml cultures grown at 30 °C overnight in YPD medium are pelleted by centrifugation, then washed twice with YPD medium, and suspended in YPD broth (final concentration, 10^6 cells/ml). The cells are treated with or without 0.5 μM JC-1 at 37 °C in the dark (*see* **Note 1**). Cells from each sample are collected, washed twice after staining, and fluorescence is measured using a FACScan flow cytometer (Becton Dickinson).
5. *Oxygen uptake.* Oxygen consumption is measured polarographically using a Clark-type electrode (model 5300; Yellow Springs Instruments, OH). Cells of each strain are collected from 3 to 4 h cultures (exponential growth in YPD broth at 30 °C), washed in PBS, and suspended to 2.5×10^8 cells in 1 ml of YPD broth or 125 mM sucrose broth. The oxygen consumption rate is determined at 1-min intervals for a total of 5 min including a 0 time control.
6. *Mitochondria isolation* [4]. Overnight-grown cells (YPD) are collected, washed, and suspended in 25-ml *Buffer A (containing*

DTT) at 30 °C, 100 rpm for 15 min. The protoplasts are prepared by treatment with Zymolyase 100 T (0.25 mg/ml) at 30 °C, 100 rpm for 1 h or until ~90 % spheroplast formation occurs [4]. After spheroplasts are collected and suspended in ice-cold *Buffer B* (0.6 M mannitol-EDTA), the pellet is lysed using a French Press (1000 psi). Following low-speed centrifugation (1000 × *g* for 10 min), the pellet is discarded and the supernatant is then subjected to high-speed centrifugation (6,100 × *g* for 10 min) and the pellet is suspended in ice-cold *Buffer C*. The crude cell membranous portion is washed twice with ice-cold Buffer C and then suspended in *Buffer D*. Mitochondria are isolated by a two-step Percoll gradient method. *Buffer E* (30 % Percoll) is placed at the bottom of a centrifugation tube. An equal volume of 20 % Percoll in *Buffer F* is layered on top of Buffer E. A semi-pure mitochondrial preparation is then overlaid at the top of the centrifuge tube. Following centrifugation (40,000 × *g* for 45 min at 4 °C), a whitish band (purified mitochondria) is collected. Mitochondrial protein is determined by the biuret method.

7. *Assays of mitochondria ETC C I-IV enzyme activity.* Details of assays for CI-IV complex assays are available [4, 15]. For assays of each complex, a complex-specific inhibitor should be used to determine the specificity of the CI-IV enzyme data. For example, rotenone (CI inhibitor) should abolish nearly all CI activity in the presence of other complex inhibitors.

   CI-CIV assay substrates, electron acceptors, and specific inhibitors are described in Table 1. The assays described directly below indicate the assay readouts. Enzyme activities of the mitochondrial electron transport chain (ETC) CI, CII/III, and CIV are measured spectrophotometrically in 96-well plates. 10–20 μg of mitochondrial protein is used per assay.

   CI assay. (NADH:ubiquinone oxidoreductase). The decrease in absorbance at OD_{600} of (NADH → NAD) is measured immediately after the addition of substrate (340 nm minus that at 380 nm using an extinction coefficient of 5.5 mM^{-1} cm^{-1}). Readings are taken at 30-s intervals for 5 min at 37 °C. As mentioned above, assays are also measured in the presence of 0.1 mM rotenone (CI inhibitor).

   CII assay. (succinate:ubiquinone oxidoreductase). Following the addition of the substrate (succinate), the absorbance at OD_{600} is measured at 1-min intervals for 5 min at 37 °C as a decrease in absorbance at 600 nm due to the reduction of DCPIP (acceptor) (extinction coefficient of 13 mM^{-1} cm^{-1}). The specificity of CII activity is verified in the presence of 1 mM TTFA.

   CII and CIII assay. (succinate:ubiquinone oxidoreductase). Activity is determined at OD_{550} by measuring an increase in absorbance that results from reduction of oxidized cytochrome

Table 1
Assays for mitochondrial C1-IV showing substrates, electron acceptors, and complex-specific inhibitors

Complex	Substrate	Electron acceptor	Assay output	Complex-specific inhibitors[a]
CI—NADH:ubiquinone oxidoreductase	0.8 mM NADH	50 mM DB[b]	NADH: decreased absorbance (340–380 nm)	4 mM Rotenone
CII—succinate:ubiquinone oxidoreductase	10 mM succinate	80 mM DCIP[c]	Reduction of DCPIP	1 mM TTFA
CII and III—succinate: cytochrome C reductase 5 mg ascorbic acid[d]	10 mM succinate	Oxidized cytC	Reduction of oxidized cytC	1 mM TTFA
CIV	40 mM cytC 5 mg ascorbic acid	Oxidized cytC	Decrease in absorbance	0.24 mM KCN

[a]TTFA CI, II, III, and IV assays are done with specific inhibitors or are used as a cocktail to measure so that activity reflects the specific complex being assayed. CI assays would include KCN (CIV inhibitor) and antimycin A
[b]DB: 2,3-dimethoxy-5-methyl-6-n-decyl-1,4 benzoquinone
[c]DCPIP: 2,6-dichlorophenolindophenol
[d]Ascorbic acid is used to reduce cytC

C (*cytC*). The donor substrate for this assay is 10 mM succinate again. The reduction of *cytC* is determined by changes in absorbance at OD_{550} at 30-s intervals for a total of 3 min at 37 °C.

CIV assay (cytochrome c oxidation). The oxidation of reduced cytochrome c is measured at 550 nm as a decrease in absorbance. Cytochrome c was first reduced using ascorbic acid or DTT, which is adjudged as a color change from dark orange-red to pale purple red. Oxidation assays were conducted after the addition of lauryl maltoside and reduced cytC. 0.24 mM KCN was used to determine the specificity of the assay for CIV.

8. *TCA and accessory enzyme assays*. The following assays are described in detail by others [16, 17]. Preparation of mitochondria for these assays is described in Subheading 2, **item 13**, and Subheading 3, **step 6**.

   KGDH. Mitochondria are suspended in MOPS-Triton X-100, pH 7.4; α-KG activity is assayed spectrophotometrically as the conversion of NAD → NADH following the addition of α-KG and MgCl2, CoASH, thiamine pyrophosphate. The reaction mixture also should include rotenone, since NADH oxidation by CI will influence assay data. Results are expressed in nmol of reduced NADH/min/mg protein.

   Citrate synthase. The reaction is initiated with freshly prepared mitochondria. Citrate synthase catalyzes the reaction between

acetyl-CoA and oxaloacetic acid (OAA). The thioester of acetyl-CoA is hydrolyzed forming CoA-SH (thioester) which reacts with the DTNB reagent to form TNB (thio-2-nitrobenzoic acid). The yellow product of the reaction is measured by absorbance at 412 nm [17].

Isocitrate lyase. Isocitrate lyase cleaves isocitrate to glyoxylate and succinate while malate synthase condenses glyoxylate and acetyl-CoA to form malate [16]. This reaction enables cells to conserve CO_2 for assimilation. In this assay, the reaction is started by the addition of 10 mM isocitrate. Glyoxylate is detected upon the addition of 40 mM phenylhydrazine. The reaction is measured at A_{324}/min for 5 min.

4 Notes

1. DCFDA incubation with cells for the determination of cell ROS (or for any fluorescence microscopy) must be carried out in darkness. We use aluminum foil to avoid degradation.
2. Hydrogen peroxide may break down over time and stored samples should not be used after 30–45 days if opened. Menadione should be stored in tightly closed and light-resistant containers.
3. Enzymatic CI, II, III, and IV assays are done with specific inhibitors alone or are used as a cocktail so that activity reflects the specific complex being assayed. CI assays would include KCN (CIV inhibitor) and/or antimycin A.

References

1. Alex D, Calderone R, Li D et al (2012) Back to the future: *Candida* mitochondria and energetics. In: Calderone R, Clancy CJ (eds) Candida and candidiasis, 2nd edn. ASM Press, Inc., Washington, DC, pp 331–343
2. Bamback A, Fernandes MP, Ghosh A et al (2009) Goa1p of *Candida albicans* localizes to the mitochondria during stress and is required for mitochondrial function and virulence. Eukaryot Cell 8:1706–1720
3. Sun N, Fonzi W, Chen H et al (2013) Azole susceptibility and transcriptome profiling in the *Candida albicans* mitochondrial electron transport chain complex I mutants. Antimicrob Agents Chemother 57:532–542
4. Li D, Chen H, Florentino A et al (2011) Enzymatic dysfunction of mitochondrial complex I of the *Candida albicans goa1* mutant is associated with increased reactive oxidants and cell death. Eukaryot Cell 10:672–682
5. Chen U, Calderone R, Sun N et al (2012) Caloric restriction restores the chronological life span of the *goa1*Δ null mutant of *Candida albicans* in spite of high levels of ROS. Fungal Genet Biol 49:1023–1032
6. She X, Zhang L, Chen H et al (2013) Cell changes in the *Candida albicans* mitochondria mutant *goa1*Δ are associated with reduced recognition by innate immune cells. Cell Microbiol 15:1572–1584
7. Khamooshi K, Sikorski P, Sun N et al (2014) The Rbfi, HFl1 and Dpb4 of *Candida albicans* regulate common as well as transcription factor-specific mitochondrial and other cell activities. BMC Genomics 15:56. doi:10.1186/1471-2164-15-56
8. Calderone R, Sun N, Gay-Andrieu F et al (2014) Antifungal drug discovery: the process and outcomes. Future Microbiol 9: 791–805

9. Shingu-Vazquez M, Traven A (2011) Mitochondria and fungal pathogenesis: drug tolerance virulence and potential for antifungal therapy. Eukaryot Cell 10:1376–1383
10. Dagley M, Gentile I, Beilharz T et al (2011) Cell wall integrity is linked to mitochondrial and phospholipid homeostasis in *Candida albicans* through the activity of the post-translational regulator Ccr4-Pop2. Mol Microbiol 79:968–989
11. Qu Y, Jelicic B, Pettoloni F et al (2012) Mitochondrial sorting and assemble machinery subunit Sam37 in *Candida albicans*: insight into the roles of mitochondria and fitness, cell wall integrity and virulence. Eukaryot Cell 11: 532–544
12. Hewitt V, Heinz E, Shingu-Vazquez M et al (2012) A model system for mitochondrial biogenesis reveals evolutionary rewiring of protein import and membrane assembly pathways. Proc Natl Acad Sci U S A 109: 3358–3366
13. Ramirez MA, Lorenz MC (2007) Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. Eukaryot Cell 6:280–290
14. Gabaldon T, Rainey D, Huynen MA (2005) Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (Complex I). J Mol Biol 348:857–870
15. Barrientos A (2002) *In vivo* and organelle assessment of OXPHOS activities. Methods 26:307–316
16. Guedouari H, Gergondey R, Bourdais A et al (2014) Changes in glutathione-dependent redox status and mitochondrial energetic strategies are part of the adaptive response during the filamentation process in *Candida albicans*. Biochim Biophys Acta 1842:1855–1869
17. Faloona G, Srere P (1969) *Escherichia coli* citrate synthase. Purification and the effect of potassium on some properties. Biochemistry 8:4497–4503

Chapter 6

Imaging Candida Infections in the Host

Dhammika H. Navarathna, David D. Roberts, Jeeva Munasinghe, and Martin J. Lizak

Abstract

Disseminated fungal infections caused by *Candida* species are associated with homing of the pathogen to specific organs in human and murine hosts. Kidneys are a primary target organ of *Candida albicans*, and invasion into the kidney medulla can lead to loss of renal function and death. Therefore, development of noninvasive methods to assess kidney infections could aid in the management of disseminated candidemia. We describe a magnetic resonance imaging method utilizing iron oxide-based contrast agents to noninvasively assess recruitment of phagocytes and kidney inflammation. *C. albicans* also colonizes the brain and can cause meningoencephalitis. We describe additional imaging methods to assess loss of the blood–brain barrier function that initiates brain infections.

Key words *Candida albicans*, Noninvasive imaging, Magnetic resonance imaging, Blood–brain barrier, Kidney, Brain, Phagocytes, Inflammation

1 Introduction

Several aspects of human disseminated candidiasis can be effectively modeled in mice and have contributed much to our present understanding of the pathogenesis of *Candida albicans* infections and host immune responses to infection [1]. Kidney and brain are the primary target organs of this organism during disseminated blood-borne infections. Within the kidney, massive fungal invasion and growth can occur, resulting in inflammatory reactions that lead to tissue necrosis [2]. *C. albicans* also invades the brain during acute infections and causes meningoencephalitis [3]. Fifty percent of patients with disseminated candidiasis have central nervous system (CNS) fungal invasion, which is associated with a mortality rate reaching 90 % [4, 5]. *Candida* is the second most common contaminant cause of brain abscess formation as a result of hematopoietic stem cell transplantation [6]. *C. albicans* has also been reported to cause meningoencephalitis without systemic infection in healthy individuals [7]. Brain infection results in micro-abscesses,

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_6,

mostly in the junctional area between white and gray matter. The basal ganglia and other cerebral regions are frequently involved [4]. These micro-abscesses are surrounded by inflammatory cells. Therefore, monitoring inflammatory changes and breaching of the blood–brain barrier (BBB) is an important element of *C. albicans* pathogenesis studies. Breaching of the BBB and inflammatory cell infiltration due to *Candida* infections can be effectively monitored using magnetic resonance imaging (MRI) [8].

Many biologically active nuclei (e.g., ^{31}P, ^{13}C, ^{23}Na) satisfy the conditions to perform MRI and among them, protons (^{1}H) are the main component of biological water, and due to its higher natural abundance and MR sensitivity ^{1}H is the default choice for biological MRI. The MRI signal is firstly a function of the separation of the nuclear energy levels in the presence of the main magnetic field of the scanner. Secondly, the signal depends on the complex interplay of the excitation of protons using external energy and also how efficiently those levels are depopulated and relaxed once excitation energy is ceased.

Inherently small separations in nuclear energy states, therefore, result in weak signal-induced relaxation (depopulation of those energy levels), thus yielding low sensitivity of the MRI technique. Therefore, higher fields will yield more MR signal, and thus the signal per unit volume is larger and enables higher resolution to be achieved. However, hardware limitations and power depositions on tissue limit the field strengths and size of clinical scanners (7 or 11 Tesla), but small animal investigations can utilize even higher fields (14–17 Tesla) and improved electronics to achieve higher spatial resolution.

The natural abundance of water in microstructures in tissue helps circumvent the low MR signal sensitivity, and furthermore, alteration to the local cellular/tissue architecture is likely to reflect changes in the properties of water that can be detected by MRI. The relaxation of the excited protons is assessed by two characteristic times: spin–spin (T_2) and spin–lattice (T_1) relaxation times. Furthermore, the behavioral variations of tissue water protons in a magnetic field (e.g., diffusion of water) are also a function of molecular interactions (e.g., water and macromolecular interactions) within that environment. As the mechanisms of the T_1 and T_2 relaxation processes and other associated interactions are different, probing the desired properties during the MR acquisition time scale, whether it is by variation of timing parameters or other manipulations, enables the weighting of one or more chosen characteristic into the image.

MRI provides noninvasive insights into physiological and anatomical characteristics of a tissue. The wide array of inherent parameters and external conditions that can be varied makes

MRI a flexible modality not only to delineate anatomical details but also to probe functional behavior. The MR image, which primarily originates from tissue water, is dependent on the microenvironment of tissue, and changes in the integrity or the architecture might in turn affect the intra- and extracellular water, thereby altering qualitative or quantitative information depicted in the image.

The inherent spin–spin relaxation (T_2) is a function of the local tissue structure that could show changes once *Candida* has infiltrated the brain tissue. Among the array of timing parameters available in generating an MR image during data acquisition, parameters that affect the inherent T_2 values could be used to weigh the MR signal in the tissue to varying degrees to subsequently yield a spatial map differentiating possible damage that otherwise is hard to identify. The rate of random diffusion of water within brain tissue reflects neuronal architecture. Pathogenesis resulting from *Candida* infections can change the diffusional properties in the infected regions and is measured as diffusion coefficient. Diffusion contrast imaging can assess these changes and adds an extra dimension to understanding disease progression.

Intravascular MRI contrast agents, such as Gd-DTPA, will extravasate selectively into regions where the BBB is breached, and therefore, the measured recovery is slow in comparison to normal areas with an intact BBB. As the contrast agent affects the inherent spin–lattice (T_1) relaxation times, an appropriate T_1 weighted MRI can map the areas of damage as changes in image contrast under the influence of an appropriate imaging method. Alternatively, fast dynamic MRI scans could help map the movement of a contrast agent, which also can image changes in intensity reflecting recovery of contrast due to Gd-DTPA infusion when the BBB is breached.

The ideal evolution of the MR experiment is expected to be in an environment as homogeneous as possible. Under such condition, the loss of the MRI signal in the detecting plane is characterized by spin–spin relaxation (T_2). However, biological tissues are far from homogenous, and due the microscopic variations of magnetic fields experienced, known as magnetic susceptibility, the MRI signal detected has a different decay constant denoted as T_2^*, generally leading to image artifacts. Iron oxide (FeO) exerts paramagnetic properties in an otherwise homogeneous biological system, thus generating higher susceptibility variations. Imaging with timing parameters to enhance T_2^* effects will accentuate the susceptibility artifact that the iron oxide induces in targeted tissue. Therefore, iron oxide-based contrast agents can be employed to induce changes that affect the T_2 values. T_2^* imaging will accentuate the susceptibility artifact that the iron oxide induces in the tissue.

2 Materials

The experimental protocol, housing, and care of the mice should be in accordance with approved guidelines of the Institutional Animal care and use committee.

2.1 Mouse Infection Model

1. *C. albicans* wild-type (WT) strain SC5314 or any clinical strain that has shown virulence in mice can be used for mouse model studies. Genetically modified strains of *C. albicans* can be compared with WT strains that have identical auxotrophies to evaluate how potential virulence genes influence trafficking to specific target organs [9]. Alternatively, standard virulent strains of *C. albicans* can be administered to transgenic mice and WT mice to examine the role of specific host genes in pathogenesis and organ colonization.
2. For the challenge of mice, *C. albicans* cells are grown overnight in 50 mL of yeast peptone dextrose (YPD) medium at 30 °C with aeration. Higher temperatures and inclusion of media components that induce hyphal differentiation should be avoided for achieving reproducible imaging.
3. *Candida* yeast cells are harvested by centrifugation at 4200 × *g* for 10 min, washed twice with 50 mL of sterile nonpyrogenic normal saline and resuspended in 10 mL of sterile nonpyrogenic saline before quantification of the cell numbers using a counting chamber (Petroff–Hausser or hemocytometer).
4. The cell suspensions are adjusted to the final concentration for parenteral administration using nonpyrogenic sterile saline.
5. Inbred, outbred, or transgenic mouse strains can be used for these methods. Several variables must be controlled to minimize inter-animal variability in responses. Mice should be matched for age and sex, and mice from commercial vendors should be acclimated to the institutional vivarium for at least 2 weeks prior to use for imaging to minimize variation in response to *C. albicans* secondary to differences in their microbiomes.
6. An institutionally certified animal facility that can provide the required animal care and maintenance should be available.
7. Groups of five animals are placed in polycarbonate cages with stainless steel wire tops, using aspen shavings as bedding material (e.g., Harlan Teklad Laboratory Grade Sano28 Chips®, Madison, WI), and maintained on a 12-h light/dark cycle in heated, thermostatically controlled rooms for the duration of the studies.
8. The mice are fed a commercial rodent diet (e.g., 4 % Mouse/Rat Diet 7001, Harlan Teklad, Madison, WI) ad libitum. Filtered water should be provided in glass bottles fitted with stainless steel nipples mounted in rubber corks.

2.2 MRI Components

1. MRI experiments are performed on 7-T, horizontal Bruker Avance (brain) and Bruker Pharmascan (kidney) scanners (BrukerBiospin Inc., Billerica, MA, USA).
2. MagnevistW is a Food and Drug Administration-approved gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) contrast agent for MRI.
3. Macrophage and other phagocyte infiltration of the kidneys and brain are observed using ultrasmall particles of iron oxide, USPIO (Molday ION™, BIOPaL, Worcester, MA, USA).
4. The contrast agents are administered via a tail vein catheter consisting of PE10 tubing and a 30 gauge needle.
5. The anesthesia chamber consists of isoflurane (Matrx™ VIP3000 Isoflurane, Orchard Park, NY, USA) which is delivered to animals and controlled as required (*see* **Note 1**).
6. The in-house air mixture of medical gas (20 % O_2 and 80 % N_2) and O_2 (100 %) is delivered to the anesthesia equipment.
7. The Isoflurane/air mixture is controlled via a stop-cock assemble to first pass anesthetic to a Plexiglas setup chamber for initial knock down and subsequently to the nose cone fitted on a stereotactic cradle for MRI setup.
8. The air mixture is passed through a Capnocheck, Dual Stream EtCO2 (BCI International. Waukesha, WI, USA), and a SAR-830 AP small animal ventilator (CWE Inc., Ardmore, PA, USA).
9. The body core temperature is maintained at 37 ± 2 °C with a water circulating heating pad. The water heater is a Poly stat 6.5 L heated bath (Cole Palmer, IL, USA), and the rectal temperature is monitored via a Digi Sense Temp controller unit (Cole Palmer).
10. A respirator monitoring unit (Small Animal Instruments, NY, USA) consisting of a pressure transducer that lays under the abdominal cavity is used to monitor the breathing rate during Brain scans. For kidney MRI, breathing is synchronized with the MR acquisition window to minimize motion artifacts by data sampling on the ramp of each breath.
11. The radiofrequency coils used to obtain MRI images consist of two units. A larger 72 mm *i.d.* volume coil is used to induce the MR signal in the brain. The induced signal is detected with a 25 mm surface coil laid in proximity to the brain. The two coils are electronically isolated (decoupled) to avoid interactions during the role of each coil.
12. The radiofrequency coil used for kidney MRI is a 35 mm *i.d.* volume coil, which could accommodate an anesthetized mouse and plays the dual role of transmission and acquisition of the RF energy.

3 Methods

3.1 Mouse Infection with C. albicans

1. 8- to 12-week-old (18–20 g) mice are used for all animal experiments. Mice are randomly allocated to groups of five to six animals and housed and cared for with access to filtered water and provided standard mouse chow ad libitum. The handling and care of the animals are conducted in compliance with the guidelines established by the Animal Care and Use Committee of the Institute.
2. Each group of mice is inoculated intravenously in the lateral caudal tail vein using a 30-gauge needle with a volume of 0.1 mL containing 5×10^5 *C. albicans* cells [8]. This level of infection induces subacute candidiasis in mice with meningoencephalitis and kidney inflammation at 1, 3, and 5 days post-inoculation (dpi). Lower dose should be administered for longer term monitoring.
3. At least five mice per group at days 0, 1, 3, and 5 dpi are used to assess the level of BBB breach and inflammatory changes.
4. As the disease progresses, sick mice lose weight and show slight dehydration. Clinical signs of illness in each mouse should be evaluated three times daily, and mice that display severe signs must be euthanized immediately by CO_2 inhalation or using other euthanasia methods approved for use in the host vivarium.
5. After MRI examinations mice should be euthanized immediately by CO_2 inhalation and processed for complete necropsy and collection of tissues for histopathological examination of tissue inflammation and colonization to permit comparative studies with MRI images.

3.2 BBB Integrity Study

1. Mice are anesthetized in an induction chamber with a 30 % oxygen/70 % nitrogen (oxygen-enhanced air) gas mixture containing 5 % isoflurane. After anesthesia is induced (indicated by loss of righting reflex, decreased respiratory rate, and nonresponsiveness to toe pinch), isoflurane is reduced to 1.5–2 %, and the animals are maintained via a nose cone.
2. The catheter is introduced into the lateral tail vein, consisting of PE10 tubing and a 30 gauge needle. This facilitates administration of Gd-DTPA while in the MRI scanner.
3. Take care to allow for chest expansion to facilitate breathing. A respiratory sensor pillow is placed under the mouse or attached above to monitor the respiratory rate and pattern.
4. Mouse respiratory rate is monitored using the pressure transducer, which in turn is coupled to the acquisition software via the scanner consol. A nominal breath rate of about 60 bpm allows data acquisition with breath/data synchronization during MRI and, furthermore, seems to cause minimal stress to the animal.

5. The mouse's head needs to be centered in a 72/25-mm transmit/receive coil ensemble, and three mutually perpendicular scout images should be acquired. These pilot scans enable the positioning of the subsequent imaging slices to achieve spatial integrity.
6. T_1-weighted axial images (TR/TE = 200/5.9 ms; slice thickness = 1 mm; number of averages = 8; matrix size = 256 × 256; field of view (FOV) = 1.92 cm) encompassing the whole brain are acquired using a gradient echo (GE) sequence, before and 5 min after the administration of a bolus of Gd-DTPA (0.2 cm^3/kg body weight).
7. In order to assess the relative breaching of the BBB, analysis can be simplified by investigating selected multiple regions of interest (ROIs) in the brain placed in different regions, including the cortices of both hemispheres (Fig. 1).

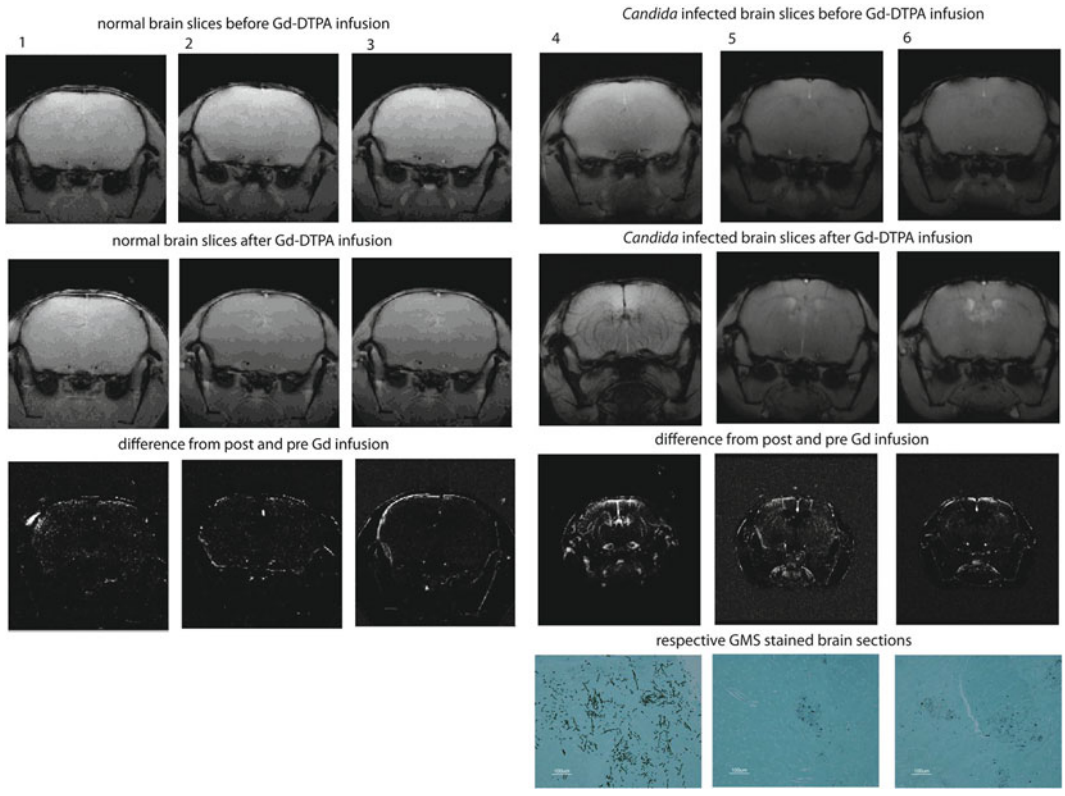


Fig. 1 MRI scans of mouse brains before (*upper panels*) and after *i.v.* administration of Gd-DTPA (*second row panels*). Gd does not penetrate the BBB in uninfected mouse brains, and thus limited changes are visible in the difference (pre-post) images (*panels 1–3, lower panels*). *C. albicans* infected mouse brains at 3 days post-inoculation show hyper-intense regions indicating BBB breaching (*panels 4–6*). GMS staining of sections obtained from the brains of the infected mice post-imaging confirms *C. albicans* colonization in the respective brains (*panels 4–6, lower panels*)

8. Further visualization of BBB integrity is achieved by dynamic contrast imaging employing an echo planar imaging (EPI) gradient echo sequence (spectral width = 350 kHz; TE = 22 ms; temporal resolution = 2 s; matrix size = 128 × 128; FOV = 1.92 cm) before, during, and after a bolus of Gd-DTPA (*see* **Note 2**).

3.3 Quantitative MRI

1. Average spin–spin relaxation times (T_2) and apparent diffusion coefficients (ADCs) are analyzed using a separate set of six mice per group at required intervals.
2. Nine contiguous T_2 (TE = 12 ms; 16 echoes; matrix size = 128 × 128; FOV = 1.92 cm) and diffusion (TE = 15 ms; Δ = 20 ms; b = 0,1000 mm/s along read gradient axes; matrix size = 128 × 128; FOV = 1.92 cm) weighted axial images, originating 2 mm posterior to the olfactory bulb, should be acquired. The data are transferred offline and processed.
3. The scanned animals are then perfused under anesthesia to remove blood from the circulatory system, and the brain histology should be studied. Unless otherwise mentioned, all the MRI experimental procedures are reproduced at required intervals, utilizing at least five mice per group.

3.4 Kidney Inflammation Study Using MRI

1. Mice are anesthetized in an induction chamber with a 30 % oxygen/70 % nitrogen (oxygen-enhanced air) gas mixture containing 5 % isoflurane. After anesthesia is induced (indicated by loss of righting reflex, decreased respiratory rate, and nonresponsive to toe pinch), isoflurane is reduced to 1.5–2 %, and the animals are maintained via a nose cone.
2. For MR imaging of the region of interest, the body of the anesthetized mouse is restrained in a plastic holder with either vet wrap or tape (*see* **Note 3**).
3. Macrophage and other phagocyte infiltration of the kidneys and brain can be observed using USPIO (Molday ION™, BIOPaL, Worcester, MA, USA).
4. Each mouse is given 0.015 mL of Molday ION dissolved in 0.1 mL of sterile saline, 24 h before the scan.
5. T_2^*-weighted images of the kidneys need to be acquired using a Fast Low-Angle SHot (FLASH) sequence for six mice per group at required time point and 24 h following the administration of the USPIO contrast agent, compared with control mice.
6. Thereafter, five mice representing each experimental replicate at the required time points are used for quantitative analysis at each time point to compare the progression of kidney and brain pathology.

7. After anesthesia is induced, each mouse should be positioned on a holder with its face in an anesthesia mask and its legs extended to the front and back.
8. The mice need to be centered in a 35 mm linear birdcage coil, and twelve 1 mm slices are acquired through the length of the body using a GE sequence (FOV = 5.0×3.2 cm^2; TE = 10 ms; flip angle = 30; matrix size = 256×256; number of averages = 4).
9. In order to reduce motion artifacts, acquisition is synchronized with the breathing sensor. This yields an effective TR of approximately 1500 ms.
10. Loading with ultrasmall particles of iron oxide (USPIO) serves as a contrast agent to label phagocytes. Thus, more iron indicates more phagocytes, which in turn indicates greater inflammatory reactions in infected kidneys.

3.5 Brain Inflammation Study Using MRI

1. A separate cohort of mice is scanned on a second 7-T Bruker scanner.
2. The setup is identical to the BBB Gd study (Subheading 3.2), where the mouse's head is centered in a 72/25-mm transmit/receive coil ensemble, and three mutually perpendicular scout images are acquired.
3. T_1-weighted axial images (TR/TE = 200/5.9 ms; slice thickness = 1 mm; number of averages = 8; matrix size = 256×256; FOV = 1.92 cm) encompassing the whole brain were acquired using a GE sequence.

3.6 Data Analysis

1. Data from T_2, ADC, post-Gd signal variation and Dynamic contrast are analyzed using in-house software (Mathworks Inc., Natick, MA, USA).
2. T_2 and ADC contrast images are fitted to appropriate equations to evaluate respective maps, where each pixel corresponds to the local variations of the entity.
3. The blood–brain barrier breaching is computed as relative contrast ($[(\text{pre\_Gd} - \text{post\_Gd})/\text{pre\_Gd}] \times 100$) for a series of selected anatomical areas (ten ROIs per region in the cortex and two ROIs per region in the hippocampus, with 10 pixel areas of 5.625×10^{-4} cm^2) from images obtained before and after Gd-DTPA infusion. Alternate visualization of the BBB integrity could be done by monitoring the temporal variation of the signal in dynamic contrast images. In this study two ROIs (10 pixel areas of 5.625×10^{-4} cm^2) are selected in the cortex and hippocampus.

4 Notes

1. Note that isoflurane concentrations must be adjusted for some mouse strains to maintain viability and proper blood pressure.
2. Gd reduces the T1 values of mainly blood water, which yields hypo-intensities at the ROIs as the Gd flows through a given MRI slice and recovers as a sigmoid function when the BBB is intact. However, when the BBB is breached, the leaked Gd continues to yield a reduced MRI signal because the washout from those affected regions is comparatively slow.
3. Care is taken to allow for chest expansion to facilitate breathing. A respiratory sensor pillow is placed under the mouse or attached above to monitor respiratory rate and pattern. MRI may be performed for up to 3 h; however a typical anatomical scan is approximately 1 h or less.

Acknowledgements

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research (DDR, Z01 SC 009173), and National Institute of Neurological Disorders and Stroke (MJL).

References

1. Ashman RB (2008) Protective and pathologic immune responses against *Candida albicans* infection. Front Biosci 13:3334–3351
2. Navarathna DH, Nickerson KW, Duhamel GE, Jerrels TR, Petro TM (2007) Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. Infect Immun 75:4006–4011
3. Witek-Janusek L, Shareef MJ, Mathews HL (2002) Reduced lymphocyte-mediated antifungal capacity in high-risk infants. J Infect Dis 186:129–133
4. Pendlebury WW, Perl DP, Munoz DG (1989) Multiple microabscesses in the central nervous system: a clinicopathologic study. J Neuropathol Exp Neurol 48:290–300
5. Sanchez-Portocarrero J, Perez-Cecilia E, Corral O, Romero-Vivas J, Picazo JJ (2000) The central nervous system and infection by *Candida* species. Diagn Microbiol Infect Dis 37:169–179
6. Hagensee ME, Bauwens JE, Kjos B, Bowden RA (1994) Brain abscess following marrow transplantation: experience at the Fred Hutchinson Cancer Research Center, 1984–1992. Clin Infect Dis 19:402–408
7. Borha A, Parienti JJ, Emery E, Coskun O, Khouri S, Derlon JM (2009) Candida albicans cerebral granuloma in an immunocompetent patient. A case report. Neurochirurgie 55:57–62
8. Navarathna DH, Munasinghe J, Lizak MJ, Nayak D, McGavern DB, Roberts DD (2013) MRI confirms loss of blood–brain barrier integrity in a mouse model of disseminated candidiasis. NMR Biomed 26:1125–1134
9. Navarathna DH, Lionakis MS, Lizak MJ, Munasinghe J, Nickerson KW, Roberts DD (2012) Urea amidolyase (DUR1,2) contributes to virulence and kidney pathogenesis of *Candida albicans*. PLoS One 7, e48475

Chapter 7

Identification of Secreted *Candida* Proteins Using Mass Spectrometry

Emilia Gómez-Molero, Henk L. Dekker, Albert D. de Boer, and Piet W.J. de Groot

Abstract

Analysis of fungal secretomes using mass spectrometry is a useful technique in cell biology. Knowledge of the secretome of a human fungal pathogen may yield important information of host–pathogen interactions and may be useful for identifying vaccines candidates or diagnostic markers for antifungal strategies. In this chapter, with a main focus on sample preparation aspects, we describe the methodology that we apply for gel-independent batch identification and quantification of proteins that are secreted during growth in liquid cultures. Using these techniques with *Candida* and other yeast species, the majority of the identified proteins are classical secretory proteins and cell wall proteins containing N-terminal signal peptides for secretion, although dependent on sample preparation quality and the mass spectrometric analysis also usually, a number of nonsecretory proteins are identified.

Key words *Candida*, Secretome, Signal peptide, Mass spectrometry, LC/MS/MS, Trypsin

1 Introduction

Antigens of human pathogenic fungi that are secreted into the growth environment during fungal colonization are considered as ideal candidates for development of novel antifungal vaccines or diagnostic tools [1, 2]. Recently, gel-independent methodologies based on mass spectrometric analysis have been developed to identify proteins in the spent medium of liquid cultures, commonly called the secretome, of *Candida albicans* (Table 1) [1, 3–6] and other yeast species [7–12].

For secretome analysis, it is necessary to perform culturing in defined liquid medium devoid of proteins (see Fig. 1). After culturing, as the concentration of secreted proteins is generally rather low (in *C. albicans* estimated to be 0.1–0.2 % of the total biomass) [1, 13], a necessary step in the process to identify these proteins is to concentrate the proteins. Here, we describe the use of centrifugal filters; however, concentration may also be achieved using

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_7,

Table 1
Results of different *C. albicans* secretome studies using gel-independent approaches

Study	Number of SignalP-positive proteins (% of total)	Number of different growth conditions analyzed	Number of biological replicates	Runs per growth condition	Quantitative analysis	Analytical MS equipment used
Hiller et al. [5]	13 (92 %)	2	Unknown	Unknown	No	nanoLC coupled to ion trap
Sorgo et al. [1]	47 (61 %)	4	Unknown	12–18	PC	nanoLC coupled to Q-TOF
Sorgo et al. [6]	50 (59 %)	2	5	15	PC	nanoLC coupled to Q-TOF
Ene et al. [3]	39 (nd[a])	3	3	6	PC	nanoLC coupled to Q-TOF
Gil-Bona et al. [4][b]	EV[c]: 45 (60 %); EV-free: 55 (90 %)	1	3	Unknown	No	nanoLC coupled to ion trap

PC—relative semi-quantitative analysis by peptide counting
[a]Details concerning intracellular protein identifications are not given in this paper
[b]Listed proteins were identified in at least two replicates with >2 peptides in at least one of them
[c]An extracellular vesicle (EV) fraction was obtained by ultracentrifugation

protein precipitation protocols [14, 15]. The final preparation steps of the protein samples include reduction and *S*-alkylation of cysteine residues, and endoproteolytic digestion, usually by trypsin, to obtain smaller-sized peptides that are within the range of the mass spectrometric equipment. Finally, samples are desalted (and can be further concentrated) using pipette tip filters.

For mass spectrometric analysis of secretome samples, MS/MS based on partial peptide fractionation and peptide sequencing rather than peptide mass fingerprinting should be applied. The most important reason is that due to abundant *O*-glycosylation onto serine and threonine residues, and less frequent *N*-glycosylation onto asparagine residues, many of the tryptic peptides of secretory glycoproteins have different masses than calculated based on their amino acid sequences. The peptide fractionation spectrum of a single unglycosylated peptide may already yield enough sequence information for a solid protein identification; however, in recent years the tendency in proteomic research is to have at least two

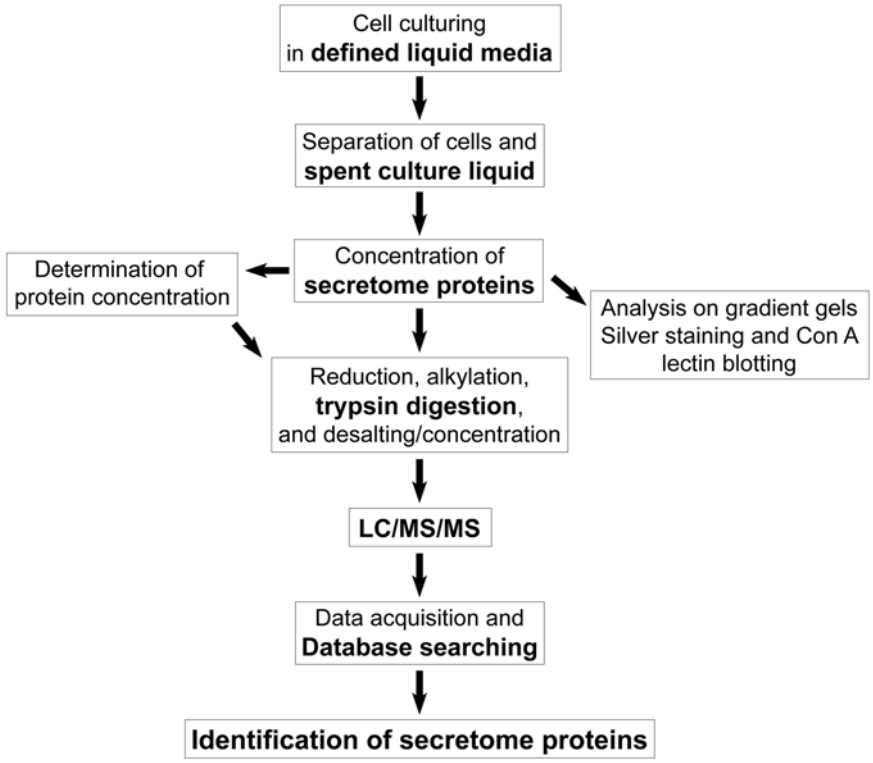


Fig. 1 Flow chart of sample preparation for secretome analysis. Culturing is performed in defined liquid media. After culturing, cells and spent medium are separated, after which the samples can be directly prepared for MS/MS analysis. Database searching of fragmentation spectra against a proteome database using Mascot yields identifications of secreted proteins. Optional during sample preparation is determination of the protein concentration and analysis on gel

peptide identifications with reliable scores to reach a solid protein identification.

Depending on the research question and equipment available, several different types of mass spectrometers can be used for analysis of the peptide mix, for instance, electrospray ionization time-of-flight (ESI-TOF), ion trap, or Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers. In each case, the mass spectrometer is usually coupled to a reversed-phase (RP) liquid chromatography (LC) column to fractionate the peptides before entering the mass spectrometer (see for instance Table 1).

In this chapter, we describe the use of LC-ESI-MS/MS for a qualitative secretome analysis. For use of other equipment we refer to the earlier mentioned literature (see also Table 1). For quantitative analysis, several labeling techniques are available, and in this chapter we have included some aspects of sample preparation using metabolic labeling (Fig. 2). For quantitative mass spectrometric details we refer to papers that have performed such analysis on very similar cell wall samples using LC-FT-MS [6, 16–18]. Several

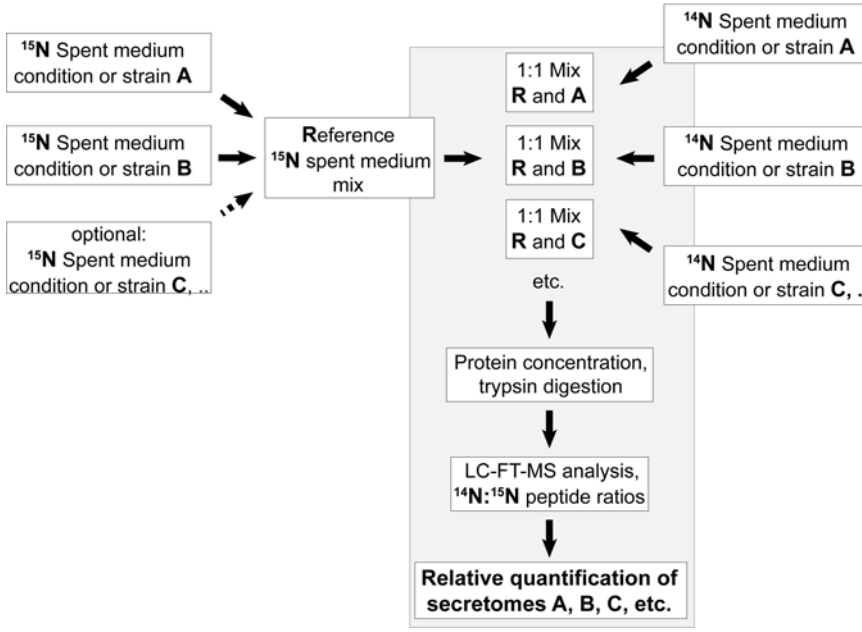


Fig. 2 Experimental strategy for the relative quantification of query secretomes versus a mixed ^{15}N-labeled reference secretome. A ^{15}N-labeled reference is prepared by mixing spent media from a few different ^{15}N-labeled cultures, optimally to contain as many as possible of the proteins in the query cultures at detectable levels. The reference is mixed in a 1:1 ratio with spent medium of query cultures (based on the amount of biomass at the time of harvest). After protein concentration and further sample preparation, the secretome samples are analyzed by LC-FT-MS. Data acquisition, calculation of ^{14}N:^{15}N peptide ratios, and relative protein abundances are described in [16]

publications on secretome analysis have presented a less precise but label-free semi-quantitative analysis by counting the total number of peptides identified for each protein and relating that directly to protein abundance [1, 3, 6, 7, 16, 17].

We advise to use gel separation techniques only for analytical purposes and not as preparative step(s) for mass spectrometric analysis of secreted proteins. As most of the secreted proteins are glycoproteins, their sizes and pI values on 1D or 2D gels tend to be heterogenous, and the glycan additions may also hamper their staining. As a consequence, enhanced staining (e.g., silver) of the protein gels may actually most of all lead to visualization of more easily stainable intracellular proteins that may be present at low concentrations in the spent medium. These proteins are often very highly expressed, suggesting that they may be the result of a small percentage of cell lysis during the cell culturing and harvesting procedures. Interestingly, two recent papers showed that most of the intracellular proteins found in extracellular fractions of *C. albicans* and *Saccharomyces cerevisiae* were associated with membranes in a vesicle-enriched fraction [4, 12]. However, to date, it is still not very clear whether these vesicles are formed from healthy living

cells and have a biological function or that they are artifacts as a consequence of the unnatural way of growing yeasts in thoroughly shaken flasks in liquid media. In any case, because the secretome is a small subproteome consisting of a limited number of different proteins, protein fractionation using gel separation techniques is not necessary and this step can simply be skipped in the sample preparation process.

Nevertheless, especially in case many biological and technical replicates are analyzed or when using highly sensitive advanced mass spectrometry equipment, it is not uncommon to find some intracellular proteins in secretome samples. Therefore, as a useful extra step, enrichment of glycoproteins in the secretome can be achieved for instance by applying concanavalin A affinity chromatography or ultracentrifugation to spin down non-classical secretory proteins associated with extracellular vesicles (see refs. [4, 11]). Another approach to focus on genuine secretome proteins is to list the proteotypic peptides, i.e., peptides that are observed in at least 50 % of all protein identifications [1]. Furthermore, identified proteins can be analyzed using freely available software for the presence or absence of specific protein features such as the presence of a canonical N-terminal signal peptide for secretion using SignalP [19].

2 Materials

In proteomics research it is crucial to work keratin-free. Human keratin is among the most common contaminants in mass spectrometric analysis of protein samples, and if present in large amounts may hamper identification of proteins of interest in biological samples. Therefore, work very clean (always wear gloves), use ultrapure chemicals and disposable plastics where possible, and prepare fresh solutions. Any glassware that is used for culturing should be rinsed very carefully prior to use.

2.1 Cell Culturing and Harvesting Components

1. YEPD medium. Dissolve 50 g YEPD (Pronadisa) in 1 L water. Sterilize by autoclaving.
2. YNB-S: 1.7 g/L yeast nitrogen base (YNB, Pronadisa), 5 g/L ammonium sulfate, 20 g/L sucrose (*see* **Notes 1** and **2**).
3. ^{15}N YNB-S: Like normal YNB-S but with 5 g/L ^{15}N-labeled ammonium sulfate (Spectra Stable Isotopes; ^{15}N content 99 %) instead of normal ammonium sulfate.
4. Cooled tabletop centrifuge with swinging bucket rotor (5804 R, Eppendorf).
5. 10 mM Tris–HCl, pH 7.5.
6. Cellulose acetate 0.45 μm syringe filters (*see* **Note 3**).

2.2 Preparation of Samples for Mass Spectrometric Analysis

1. Centrifugal filter devices. We use filters of two different volumes (15 ml and 0.5 ml) and a nominal molecular weight limit (NMWL) of 10,000 (Amicon) (*see* **Note 4**).
2. BCA (bicinchoninic acid) protein assay kit (Pierce). This kit contains reagents A and B as well as 2.0 mg/mL albumin standard solutions. Fifty parts of reagent A is mixed with one part of reagent B. 1 ml of this mix is added to 5 μl of protein sample and incubated at 37 °C for 30 min. The OD_{562} is determined and compared to the albumin standard (0–25 μg) taken through the same reactions (*see* **Note 5**).
3. For reduction and alkylation the following solutions are prepared from fresh 1 M stock solutions:
   (a) Ammonium bicarbonate (NH_4HCO_3) solution: 100 mM NH_4HCO_3.
   (b) Reducing solution: 100 mM NH_4HCO_3, 10 mM DTT.
   (c) Alkylating solution: 100 mM NH_4HCO_3, 65 mM Iodoacetamide.
   (d) Quenching solution: 100 mM NH_4HCO_3, 55 mM DDT.
4. Trypsin Gold, Mass Spectrometry Grade (Promega) (*see* **Note 6**).
5. OMIX C-18 (Agilent Technologies) tips (80 μg capacity) for desalting and concentration of samples.
   (a) Wetting solution: 50 % acetonitrile (ACN).
   (b) Equilibration and washing solution: 0.1 % trifluoroacetic acid (TFA).
   (c) Elution solution: 60 % ACN, 0.1 % TFA.

2.3 Analytical Gel Separation and ConA Lectin Blotting Components

1. NuPAGE® Novex 3–8 % gradient Tris-acetate protein gels (Invitrogen) (*see* **Note 7**).
2. XCell SureLock® Mini-Cell running tank.
3. Tris-acetate running buffer (20×): 89.5 g Tricine, 60.6 g Tris base, 10 g SDS in 500 ml water.
4. Sample buffer (4×): NuPAGE® LDS sample buffer (Invitrogen).
5. Silver staining kit (Bio-Rad).
6. Protein blotting:
   (a) Hybond™-P PVDF membrane (Amersham) (*see* **Note 8**).
   (b) Blotting buffer: 14.4 g/l glycine, 3 g/l Tris, 200 ml/l methanol.
   (c) Blotting tank. TE22 Mini Tank Transfer Unit (Hoefer) (*see* **Note 9**).

7. Concanavalin A lectin staining:
   (a) PBS: 80.1 g/l NaCl, 17.8 g/l $Na_2HPO_4{\cdot}2H_2O$, 2.7 g/l K_2HPO_4, 2.0 g/l KCl, pH 7.4.
   (b) Blocking solution: 6 % bovine serum albumin (BSA) in PBS.
   (c) Con A solution: 0.5–1.0 μg/ml Concanavalin A-HRP conjugate (Sigma), 3 % BSA, 25 mM $MnCl_2$, 25 mM $CaCl_2$ in PBS.
   (d) ECL or ECL Prime (Amersham).

2.4 LC-ESI-MS/MS

1. Control peptide mix. Pierce Retention Time Calibration Mixture, 5 pmol/μl (Thermo Scientific). This stock solution is diluted 200× with 0.1 % TFA in water.
2. 0.1 % TFA in water.
3. Ultimate 2000 nano-HPLC (LC Packings), packed with a PepMap C18 reversed-phase column (75 μm id, 25 cm length; Dionex).
4. Quadrupole time-of-flight mass spectrometer (Q-TOF MS; Micromass).

2.5 Data Processing and Analysis

1. MassLynx ProteinLynx software (Micromass) for data processing. The MaxEnt3 algorithm of ProteinLynx is used for spectra deconvolution and generation of monoisotopic $(M+H)^+$ peak lists (.pkl files).
2. Mascot software (Matrixscience). Mascot software is available online; however, we prefer the use of a licensed version of Mascot to be able to perform searches against custom species-specific protein databases.
3. Proteome files containing all translated ORFs of a given *Candida* species can be downloaded from the Candida Genome database (CGD; http://www.candidagenome.org/) or the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).
4. Probability of extracellular localization of the identified proteins can be verified using prediction software.
   (a) SignalP (http://www.cbs.dtu.dk/services/SignalP/) is a good program for identifying N-terminal signal peptides [19].
   (b) The fungal-specific Big-PI algorithm (http://mendel.imp.ac.at/gpi/fungi_server.html) is a good program for recognition of GPI-anchoring signal peptides [20] (*see* **Note 10**).

3 Methods

3.1 Culturing in Semi-defined Media and Harvesting

1. Grow precultures of cells overnight in liquid YEPD.
2. Grow cultures in YNB-S (*see* **Note 11**). Preferably, cultures are started at a low cell density (*see* **Note 12**) and grown overnight (about 18 h) until they reach log phase ($OD_{600} = 1–2$). In our experience, about 100 μg of protein is a good amount to be prepared for mass spectrometric analysis. Following the method below, on average we isolate 5–6 μg protein/ml spent medium. This means that about 20 ml of spent culture liquid should yield sufficient protein for mass spectrometric analysis, but of course this may depend on the *Candida* strain to be analyzed and on the applied growth conditions. As cultures in volumes up to 200 ml can easily be processed in the concentration steps detailed below, it is advisable to culture cells in 100–200 ml medium to ensure isolating enough protein material.
3. In case a relative quantitative mass spectrometric analysis of the secretome is required, also a reference culture is required in which amino acids are labeled by metabolic labeling (*see* **Note 13** and Fig. 2). To this end, perform a culture with ^{15}N-labeled YNB-S (*see* **Note 14**). Make sure to prepare a large enough volume of the reference culture(s) so that all samples of interest can be spiked in a 1:1 ratio with this reference (*see* **Note 15**).
4. Separate cells and spent culture medium by centrifugation at 4500 × *g* for 10 min. We use a cooled tabletop centrifuge set at 4 °C and 50 ml sterile disposable conical centrifuge tubes (*see* **Note 16**). The cell pellet after this centrifuge step can be washed with 10 mM Tris–HCl pH 7.5 and stored for other research purposes (e.g., biomass determination).
5. Carefully pour the supernatant into a fresh tube and repeat the centrifugation.
6. To remove the last traces of cells, filter the supernatant through sterile 0.45 μm pore size syringe filters.

3.2 Concentration of Proteins from the Culture Medium

Mass spectrometric analysis of proteins in the spent medium requires the proteins to be concentrated to reach detectable levels. Following the procedure described below, secreted proteins can easily be concentrated up to 500-fold, which should be sufficient for bringing their concentrations (at least the more abundant proteins) within detectable levels of most mass spectrometers. All following steps during the process of concentrating proteins from the culture medium are performed under cold conditions; samples are kept on ice as much as possible and are centrifuged in the cooled tabletop centrifuge with swinging bucket rotor at 4 °C.

1. Cell-free spent medium is poured into 15 ml centrifugal filters with a 10 kDa Mw cut-off (*see* **Note 3**), and spun for 15 min at 4500 × *g*. After spinning, the concentrated sample (about 0.2 ml) is poured into a new disposable tube. The collection tube containing the protein-less flow-through is emptied, and the filter unit is refilled with unconcentrated spent medium and centrifuged again.
2. This process can be repeated with the same filter unit until all the spent medium is concentrated but making sure that the orientation of the filter unit in the centrifuge is changed at each spinning step. In this way, the same filter can be used for concentrating proteins from volumes up to 200 ml spent medium.
3. After all spent medium has been filtered, the concentrated and pooled protein sample is poured back into the centrifugal filter unit and spun again. The required spinning time now depends on the volume of the sample and may be influenced by the protein concentration in the sample.
4. To remove medium components, the sample is washed by adding sterile milliQ water (or an appropriate buffer) to the concentrated sample in the centrifugal filter unit and spinning again until the desired volume is reached. A volume of about 2 ml is fine for continuing the concentration of the samples with smaller-sized centrifugal filter units.
5. Using centrifugal filters with a maximum volume of 0.5 ml, the samples can be further concentrated. With these smaller filters, centrifugation is performed in an Eppendorf centrifuge (14000 × *g*, 10 min) with fixed angle rotor. Collection of concentrated samples from these smaller filters is done by putting them up-side-down in a clean collection tube and spinning for 1 min at 1000 × *g*. Finally, the samples should be about 500 times concentrated.
6. At this point, it is good to determine the protein concentration in the samples; however, as the same 0.5 ml filter units can be used during the reduction and alkylation procedure, it is advisable to continue with these steps (Subheading 3.3). Determination of protein concentration can be achieved quickly using standard methods such as the BCA protein assay kit.

3.3 Preparation of Concentrated Secreted Proteins for Mass Spectrometric Analysis

About 100 μg protein (or protein concentrate derived from about 20 ml spent medium) is prepared for mass spectrometric analysis in a procedure that involves reduction and alkylation of cysteine residues in the proteins, followed by endoproteolytic digestion to create smaller-sized peptides whose sizes are within the range of commonly used mass spectrometers. The reduction on alkylation

procedure is based on a protocol that was developed for in-gel digestion of proteins by Shevchenko and colleagues [21, 22].

1. Prepare fresh 100 mM NH_4HCO_3, reducing solution (100 mM NH_4HCO_3, 10 mM DTT), alkylating solution (100 mM NH_4HCO_3, 65 mM iodoacetamide), and quenching solution (100 mM NH_4HCO_3, 55 mM DDT).
2. Pipette about 100 μg protein (or protein concentrate derived from about 20 ml spent medium) back into the 0.5 ml centrifugal filter unit. Fill the unit up to 0.5 ml with 100 mM NH_4HCO_3. Spin 10 min at 14,000 rpm and dispose the flow-through.
3. Fill the filter with reducing solution (10 mM DTT, 100 mM NH_4HCO_3). Leave the samples at 55 °C for 1 h.
4. Centrifuge, dispose flow-through, and fill the filter with alkylating solution. Incubate the samples for 45 min in the dark at RT.
5. Centrifuge, dispose flow-through, and fill the filter with quenching solution. Leave the samples for 5 min at RT.
6. Wash the samples by centrifuging the filters, disposing flow-through, and refilling the filters with 100 mM NH_4HCO_3. Repeat this step three times.
7. Collect the concentrated samples in a fresh collection tube as before.
8. Trypsin digestion is performed overnight at 37 °C in a 50 mM NH_4HCO_3 solution with trypsin added in a 50:1 (protein:trypsin) ratio. For instance, to 100 μg protein sample (in 100 mM NH_4HCO_3), 100 mM NH_4HCO_3 solution is added to a volume of 50 μl, after which 2 μg trypsin (usually delivered in a 1 μg/μl concentration) and water are added to a final volume of 100 μl.
9. After trypsin digestion, the sample is desalted and concentrated using OMIX C-18 tips (80 μg capacity) following the manufacturer's protocol. Briefly:
   (a) Place an OMIX C-18 tip with a capacity of 80 μg on a pipette and wet the tip by aspirating and dispensing a 50 % ACN solution three times.
   (b) Equilibrate the column by pipetting up and down three times in a 0.1 % TFA solution.
   (c) Pipette sample into the tip. Dispense and aspirate up to ten times to improve binding.
   (d) Rinse the tip by pipetting and dispensing three times in 0.1 % TFA solution.
   (e) Elute the peptides by pipetting and dispensing three times in 10–20 μl 60 % ACN, 0.1 % TFA solution.

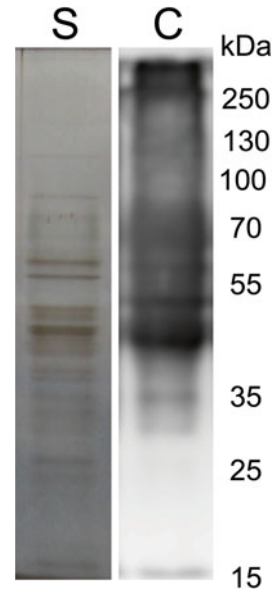


Fig. 3 Analysis of *C. glabrata* (strain HTL; ref. [30]) secretome proteins on gel. *S* Silver staining; *C* Concanavalin A lectin blotting. About 15 μg protein was loaded on gel

3.4 Analysis of Secreted Proteins by SDS-PAGE and Subsequent Visualization

Although we advise not to combine gel separation techniques with mass spectrometric analysis of secreted glycoproteins, running an SDS-PAGE gel and subsequent silver staining or Concanavalin A (Con A) lectin blotting may be quite informative to validate the quality of the concentrated secretory protein samples before applying them to mass spectrometric analysis (Fig. 3). Especially Con A lectin blotting is a very sensitive method for specific visualization of glycoproteins. Below, the methodology for protein separation, blotting, and staining techniques is mentioned briefly. For more details, we refer to existing protocols [23].

1. Mix 10–20 μg protein samples with 4× sample buffer. Heat samples for 10 min at 70 °C.
2. Load samples on a 3–8 % gradient Tris-acetate NuPAGE® gel. Run the gel at 100 V (*see* **Note 17**) following the manufacturer's instructions.
3. Silver staining of SDS-PAGE gels can be performed with a commercially available kit such as the Silver staining kit from Bio-Rad (*see* **Note 18**) following the manufacturer's instructions.
4. A very sensitive alternative method for specific visualization of glycoproteins is to perform a Con A lectin blotting experiment, as described in detail by [23]. Briefly, proteins are blotted onto PVDF membrane. The blot is blocked (6 % BSA in

PBS), incubated with Concanavalin A-HRP conjugate, washed with PBS, and developed with chemiluminescent detection reagents (ECL Prime).

3.5 Mass Spectrometric Analysis

Several types of mass spectrometry instruments can be employed for analysis of secretome samples. Here we describe secretome analysis using an LC-ESI-MS/MS setup consisting of an Ultimate 2000 nano-HPLC system coupled to a Q-TOF mass spectrometer.

1. The amount of peptides in the secretome samples is estimated by comparing with 250 fmol of a peptide mix control sample, which generates 2–3 $mAU_{214\ nm}$ upon injection into the nano-HPLC system. Normally, samples contain about 2.5–5 pmol peptides/μl.
2. 1 μl of sample containing 2.5–5 pmol is mixed with 10 μl of 0.1 % TFA solution (to lower the ACN concentration), and from this dilution 10 μl is injected into the Ultimate nano-HPLC equipped with a PepMap100 C18 reversed-phase column (*see* **Notes 19** and **20**).
3. Peptides are eluted with a linear gradient of 0–50 % ACN over 45 min using a flow rate of 0.3 μl/min.
4. Eluting peptides are directly ionized by electrospray in a Q-TOF. Survey scans are acquired in an *m/z* range of 350–1200.
5. Every few seconds the MS can automatically select the most intense ions above a set threshold of BPI (base peak intensity) for collision-induced dissociation (MS/MS).

3.6 Data Processing and Analysis

1. MS/MS ion spectra are processed with MassLynx ProteinLynx software, yielding peak lists (.pkl files).
2. The .pkl files are submitted to Mascot software for protein identifications through searches against species-specific proteome files (*see* **Note 21**).
3. Typical Mascot search parameters are the following: allow one missed cleavage, fixed carbamidomethyl modification of cysteine, and an error tolerance of 0.3 Da for peptides and MS/MS fragments.
4. Probabilistic Mascot scoring is used to evaluate the peptide identifications and a *p* value of less than 0.05 is considered significant.
5. If all identified peptides of a protein have a score close to 0.05, they are checked manually by analyzing their raw MS/MS spectra using MassLynx software before inclusion in the protein identification list.
6. Extracellular localization of the identified proteins is verified using prediction software such as SignalP and Big-PI.

4 Notes

1. In case of strains with amino acid auxotrophies, this medium should be supplemented with the appropriate amino acid(s).
2. *Candida* cells are known to acidify their environment [24]. Therefore, if a stable pH during culturing is required, a buffer that is not or poorly metabolized by *Candida* cells should be added to the medium. For instance, addition of 75 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), for buffering at neutral pH, or 75 mM tartaric acid, for buffering at acidic pH, has been applied successfully [1].
3. Avoid the use of filters with high protein binding.
4. Although we typically use filters with a NMWO of 10,000, filters with a lower Mw limit of 3000 might be useful in case proteins of low molecular weight are expected among the secreted proteins.
5. Before adding BCA reagents, all protein samples including albumin standards, are adjusted to a volume of 20 µl (by adding water).
6. Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The specificity of trypsin is essential for protein identification. Native trypsin is subject to autolysis, generating pseudotrypsin, which exhibits a broadened specificity and would result in additional peptide fragments that could interfere with database analysis of fragments detected by mass spectrometry. Trypsin Gold is manufactured to provide maximum specificity.
7. Although standard SDS-PAGE gels can be used, for optimal separation of glycoproteins it is advisable to use a gradient gel system. We prefer the pre-cast 3–8 % acrylamide gradient NuPAGE® Tris-acetate gels (Invitrogen).
8. The PVDF membrane needs to be activated by a 30 s incubation in 100 % methanol.
9. For blotting glycoproteins, we prefer a wet blotting system using a blotting tank (Hoefer) filled with blotting buffer and blotting at low Voltage (20 V) overnight.
10. Many secretome proteins are known as GPI-modified cell wall proteins. Their presence in the extracellular environment may result from incomplete incorporation into the wall or release into the medium during growth-related remodeling of the cell wall [2]. Therefore, it may be useful to analyze identified proteins for the presence of a C-terminal GPI-anchoring signal peptide.
11. Analysis of proteins from spent liquid medium requires the use of a growth medium that is devoid of proteins or peptides such

as present in for instance yeast extract. For instance, YNB-S [1, 6] as described in this chapter.

12. Prior to performing cultures in YNB-S, especially when using isotope labeling, it may be advisable to determine the growth rate of the cells in the same medium to find the optimal inoculation density.
13. For incorporation of stable isotopes by metabolic labeling, we use ^{15}N ammonium sulfate. A well-described alternative method for metabolic labeling is stable isotope labeling with amino acids in cell culture (SILAC) [25–28]. As both methods are applicable to *Candida* as well as host models, they allow quantitative proteomic analyses of both organisms during host–pathogen encounters.
14. To maximize the number of expressed secreted proteins in the reference sample, it may be beneficial to mix spent culture liquids from different cultures that were grown under different conditions in ^{15}N YNB-S (Fig. 2).
15. Ultimately, relative quantification is achieved by mixing equivalent amounts of proteins or peptides from unlabeled cultures-of-interest with those of the labeled reference "culture," and determining the ratios of corresponding $^{14}N/^{15}N$ peptide pairs by mass spectrometry (Fig. 2), in the same way as has been described for cell wall proteome analysis [16, 17].
16. Prior to centrifugation, cool the culture by placing the flasks on ice water for 20 min.
17. Running at 150 V is recommended by the manufacturer; however, we prefer running at lower voltage to optimize the separation of the proteins.
18. To enhance the staining of glycoproteins, a periodic acid treatment may be performed after fixation by incubating the protein gel in a solution of 50 mM periodic acid, 100 mM sodium acetate for 30 min at room temperature (RT). The periodic acid treatment will oxidize hydroxyl groups on adjacent carbon atoms of carbohydrates, thereby creating aldehydes that react with ammoniacal silver solutions [23].
19. Before applying secretome samples, the system is equilibrated by running a tryptic digest of a standard protein.
20. It may be useful to run technical replicates. Furthermore, to exclude overflow of abundant peptides between samples, each biological sample is followed by at least one blank run before applying a new biological sample.
21. For pipeline analysis of large numbers of sequences, we recommend the use of ProFASTA (http://www.bioinformatics.nl/tools/profasta/), which allows quick analysis of output files generated by SignalP and Big-PI [29].

Acknowledgments

PdG is recipient of an INCRECyT fellowship from Parque Científico y Tecnológico de Albacete. This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) [grant SAF2013-47570-P] and the Junta de Comunidades de Castilla-La Mancha [grant PPII-2014-007-P].

References

1. Sorgo AG, Heilmann CJ, Dekker HL et al (2010) Mass spectrometric analysis of the secretome of *Candida albicans*. Yeast 27: 661–672
2. Sorgo AG, Heilmann CJ, Brul S et al (2013) Beyond the wall: *Candida albicans* secret(e)s to survive. FEMS Microbiol Lett 338:10–17
3. Ene IV, Heilmann CJ, Sorgo AG et al (2012) Carbon source-induced reprogramming of the cell wall proteome and secretome modulates the adherence and drug resistance of the fungal pathogen *Candida albicans*. Proteomics 12:3164–3179
4. Gil-Bona A, Llama-Palacios A, Parra CM et al (2014) Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in *Candida albicans*. J Proteome Res. doi:10.1021/pr5007944
5. Hiller E, Heine S, Brunner H et al (2007) *Candida albicans* Sun41p, a putative glycosidase, is involved in morphogenesis, cell wall biogenesis, and biofilm formation. Eukaryot Cell 6:2056–2065
6. Sorgo AG, Heilmann CJ, Dekker HL et al (2011) Effects of fluconazole on the secretome, the wall proteome, and wall integrity of the clinical fungus *Candida albicans*. Eukaryot Cell 10:1071–1081
7. Buerth C, Heilmann CJ, Klis FM et al (2011) Growth-dependent secretome of *Candida utilis*. Microbiology 157:2493–2503
8. Madinger CL, Sharma SS, Anton BP et al (2009) The effect of carbon source on the secretome of *Kluyveromyces lactis*. Proteomics 9:4744–4754
9. Mattanovich D, Graf A, Stadlmann J et al (2009) Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. Microb Cell Fact 8:29
10. Swaim CL, Anton BP, Sharma SS et al (2008) Physical and computational analysis of the yeast *Kluyveromyces lactis* secreted proteome. Proteomics 8:2714–2723
11. Stead DA, Walker J, Holcombe L et al (2010) Impact of the transcriptional regulator, Ace2, on the *Candida glabrata* secretome. Proteomics 10:212–223
12. Giardina BJ, Stanley BA, Chiang HL (2014) Glucose induces rapid changes in the secretome of *Saccharomyces cerevisiae*. Proteome Sci 12:9
13. Klis FM, de Koster CG, Brul S (2014) Cell wall-related bionumbers and bioestimates of *Saccharomyces cerevisiae* and *Candida albicans*. Eukaryot Cell 13:2–9
14. Koontz L (2014) TCA precipitation. Methods Enzymol 541:3–10
15. Englard S, Seifter S (1990) Precipitation techniques. Methods Enzymol 182:285–300
16. Heilmann CJ, Sorgo AG, Siliakus AR et al (2011) Hyphal induction in the human fungal pathogen *Candida albicans* reveals a characteristic wall protein profile. Microbiology 157:2297–2307
17. Sorgo AG, Brul S, De Koster CG et al (2013) Iron restriction-induced adaptations in the wall proteome of *Candida albicans*. Microbiology 159:1673–1682
18. Sosinska GJ, de Koning LJ, De Groot PWJ et al (2011) Mass spectrometric quantitation of the adaptations in the wall proteome of *Candida albicans* in response to ambient pH. Microbiology 157:136–146
19. Petersen TN, Brunak S, Von Heijne G et al (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786
20. Eisenhaber B, Schneider G, Wildpaner M et al (2004) A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. J Mol Biol 337:243–253
21. Shevchenko A, Wilm M, Vorm O et al (1996) Mass spectrometric sequencing of proteins

silver-stained polyacrylamide gels. Anal Chem 68:850–858

22. Shevchenko A, Tomas H, Havlis J et al (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860
23. Klis FM, De Groot P, Brul S (2007) Identification, characterization, and phenotypic analysis of covalently linked cell wall proteins. In: Stansfield I, Stark MJR (eds) Methods in microbiology, Vol. 36. Yeast gene analysis, 2nd edn. Academic Press, San Diego, pp 281–301
24. Tsuboi R, Matsuda K, Ko IJ et al (1989) Correlation between culture medium pH, extracellular proteinase activity, and cell growth of *Candida albicans* in insoluble stratum corneum-supplemented media. Arch Dermatol Res 281:342–345
25. Reales-Calderon JA, Sylvester M, Strijbis K et al (2013) *Candida albicans* induces pro-inflammatory and anti-apoptotic signals in macrophages as revealed by quantitative proteomics and phosphoproteomics. J Proteomics 91:106–135
26. de Godoy LM, Olsen JV, Cox J et al (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature 455:1251–1254
27. de Godoy LM, Olsen JV, de Souza GA et al (2006) Status of complete proteome analysis by mass spectrometry: SILAC labeled yeast as a model system. Genome Biol 7:R50
28. Gruhler S, Kratchmarova I (2008) Stable isotope labeling by amino acids in cell culture (SILAC). Methods Mol Biol 424:101–111
29. De Groot PWJ, Brandt BW (2012) ProFASTA: a pipeline web server for fungal protein scanning with integration of cell surface prediction software. Fungal Genet Biol 49:173–179
30. Schwarzmüller T, Ma B, Hiller E et al (2014) Systematic phenotyping of a large-scale *Candida glabrata* deletion collection reveals novel antifungal tolerance genes. PLoS Pathog 10, e1004211

Chapter 8

In Vitro Models for *Candida* Biofilm Development

Bastiaan P. Krom and Hubertine M.E. Willems

Abstract

Development of *Candida* spp. biofilms on medical devices such as catheters and voice prosthesis has been recognized as an increasing clinical problem. Different in vitro models are presented with increasing complexity. Each model system can be utilized for analysis of new active compounds to prevent or treat *Candida* biofilms as well as to study molecular processes involved in biofilm formation. Susceptibility studies of clinical isolates are generally performed in a simple 96-well model system similar to the CLSI standard. In the present chapter, optimized conditions that promote biofilm formation within individual wells of microtiter plates are described. In addition, the method has proven useful in preparing *C. albicans* biofilms for investigation by a variety of microscopic and molecular techniques. A more realistic and more complex biofilm system is presented by the Amsterdam Active Attachment (AAA) model. In this 24-well model all crucial steps of biofilm formation: adhesion, proliferation, and maturation, can be simulated on various surfaces, while still allowing a medium throughput approach. This model has been applied to study susceptibility, complex molecular mechanisms as well as interspecies (*Candida*–bacterium) interactions. Finally, a realistic microfluidics channel system is presented to follow dynamic processes in biofilm formation. In this Bioflux-based system, molecular mechanisms as well as dynamic processes can be studied at a high time-resolution.

Key words Biofilm, Microtiter plate assay, Susceptibilitytesting, Amsterdam Active Attachment model, Bioflux

1 Introduction

Microbial biofilms are structured communities of cells surrounded by a self-produced matrix, of extracellular polymeric substance (EPS). Biofilms usually develop at the interface of an aqueous medium and solid surface, and are of particular interest due to their contribution to microbial pathogenesis, as well as the clinical problems presented by the increased resistance of resident cells to antimicrobial agents. In order to determine the susceptibility of bacteria and fungi to antimicrobial drugs, standard assays have been cataloged by the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_8,

Standards (NCCLS)), and in most cases, these have been developed exclusively for planktonically growing cells.

It is clear, however, that similar to bacteria, many *C. albicans* infections are directly or indirectly due to biofilm formation ([1, 2]). As a consequence, results of currently used susceptibility assays for *C. albicans* may not be a true reflection of in vivo realities and thus the use of suitable assays that measure the sensitivity of *C. albicans* cells growing in biofilms is desirable. In addition, prevention of *C. albicans* biofilm formation requires insight into the molecular mechanisms governing biofilm formation. Such insight requires more advanced and technically challenging model systems. This chapter describes methods for *C. albicans* biofilm formation in microtiter plates [3] as well as in the more advanced Amsterdam Active Attachment (AAA) model [4] and in microfluidics channels of the Bioflux system [5]. Biofilms prepared using the 96-well high throughput model can easily be used for susceptibility assays (sessile minimal inhibitory concentration; SMIC) or other types of investigations that require concurrent development of independent biofilms. Biofilms prepared in the AAA-model allow detailed analysis of molecular mechanisms as well as interspecies interactions at a physical and metabolic level. Biofilms prepared in the Bioflux allow analysis of dynamic processes at short and long time-intervals.

2 Materials

2.1 Strains and Media

1. *Candidaalbicans* SC5314 [6].
2. Yeast Peptone agar plates with 2 % glucose (YPD) or Trypticase Soy Agar (TSA) plates.
3. RPMI1640 (GIBCO BRL) buffered with 0.165 M 3-[*N-morpholino*]propanesulfonic acid (MOPS) (Sigma-Aldrich, USA) was prepared according to NCCLS 27-A2 standard [7].
4. Difco™ yeast nitrogen base w/o amino acids (YNB) (Becton, Dickinson Co., USA) was prepared according to the manufacturers instructions and supplemented with 0.5 % w/v glucose [3].
5. The pH of the medium was adjusted to 7.0 with 1 M KOH.
6. The medium was filter-sterilized (*see* **Note 1**).
7. Brain heart infusion (BHI) was prepared by dissolving 37 g/L of Difco™ BHI (BD Biosciences, USA) in distilled water and sterilized by autoclaving.

2.2 Biofilm Development in 96-Well Plates

1. Phosphate-buffered saline (PBS) (10 mM potassium phosphate, 150 mM NaCl, pH 7.0).
2. Flat bottom, low-evaporation tissue culture 96-well microtiter plate (Falcon™, Becton, Dickinson Co., USA).

Fig. 1 The AAA model. *Left image*, a stainless steel lid containing 24 holes to fit pins. Glass coverslips (*top-right*) or HA disks (*bottom right*) can be snap-fixed. Alternatively, thin surfaces might need gluing (President, Coltene, Switzerland)

3. Fetal calf serum (FCS) (Sigma-Aldrich, USA), alternatively, fetal bovine serum (Sigma-Aldrich, USA) can be used with identical results.

2.3 Biofilm Susceptibility Assay

1. Amphotericin B (AmpB) and ketoconazole were obtained from Sigma-Aldrich (USA), and stock solutions were prepared in DMSO as described in NCCLS 27-A2.
2. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is reduced by metabolically active cells to an insoluble dark purple crystal (Abs_{max} at 550 nm) (Sigma-Aldrich, USA).
3. MTT (0.5 mg/mL) was dissolved in PBS containing 1 % glucose and 10 μM menadione (dissolved in acetone) (*see* **Note 2**).
4. Acid isopropanol (5 % 1 M HCl in isopropanol).
5. Tecan platereader, Model: GENios, or other appropriate reader (optional).

2.4 AAA-Biofilm Model

1. Stainless steel, custom-made lid on which 24 clamps are fixed (Fig. 1).
2. Glass coverslips (diameter 12 mm; Menzel, Braunschweig, Germany) and hydroxyapatite (HA) disks (diameter 12 mm, 2 mm thick, HIMED Inc., USA).
3. Standard polystyrene 24-well plates (multiwell plates; Greiner Bio One, Alphen aan den Rijn, The Netherlands).

4. Tip-probe sonicator (for instance: Vibracell VCX130 sonicator with a maximum of 130 W and 20 kHz (Sonics & Materials, Newtown, USA)).

2.5 Bioflux Biofilm Model

1. Bioflux 1000Z setup (Fluxion Biosciences, San Francisco, USA) with an automated microscope (for example, Zeiss Z1, Jena, Germany) (*see* **Note 3**).
2. 48-well microfluidics plate (Fluxion Biosciences, USA).
3. Image analysis software (for instance ImageJ (1.46r) http://imagej.nih.gov/ij/).
4. Prewarming of all solutions to the required temperature is critical to prevent air bubbles from forming during the incubation.

3 Methods

Candida biofilm formation occurs in a well-orchestrated fashion, and is described in more detail by Chandra and coworkers [8]. Initial transient adhesion of yeast cells is followed by permanent adhesion. Adhesion involves physicochemical interactions and specific protein-protein interactions. In the method described here, the latter is achieved by preparing a conditioning layer of serum proteins on the surface of the wells. Adhesion is followed by germ-tube formation and further cellular differentiation into hyphae and pseudohyphae. During maturation, EPS is produced and the biofilm obtains its slimy appearance and more importantly, induces resistance to AmpB [9].

The use of colorimetric assays, such as XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamine) carbonyl]-2*H*-tetrazolium hydroxide) [10], or MTT, (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) [3] to determine cell viability and thereby drug susceptibility of *Candida* cells in biofilms is widely accepted. MTT is reduced to insoluble purple formazan crystals, while XTT is reduced to water-soluble formazan. When using the MTT assay, it is required to solubilize the crystals prior to the measurement (*see* **Note 4**).

Comparison of MTT reduction as determined using the described method and plating have shown an excellent correlation between both MTT reduction and plating (Fig. 2).

3.1 Biofilm Growth

1. Overnight (ON) cultures of *C. albicans* grown in YPD are harvested by centrifugation (5 min $6000 \times g$).
2. Cells are washed with PBS and standardized to an optical density of 1×10^7 CFU/mL as determined by OD_{600} determination (*see* **Note 5**).
3. The wells are conditioned with 50 % FCS in PBS for at least 30 min at room temperature.

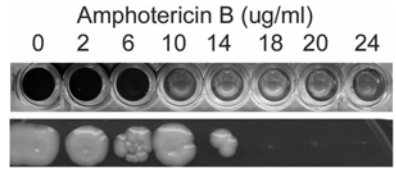


Fig. 2 Verification of the use of MTT reduction to assay viability. Prior to solubilization with acid isopropanol 5 μL of biofilm (resuspended in residual liquid) was transferred to a YPD agar plate and incubated at 30 °C for 24 h. In all our experiments there was very good agreement between the MTT results and the plating

4. The excess FCS is aspirated and wells are rinsed once with 200 μL PBS.
5. 100 μL of the cell suspension is added to each well.
6. The plate is incubated statically at 37 °C for 90 min.
7. Non-adherent cells are removed by aspiration and wells are washed with PBS twice to remove loosely associated cells.
8. Biofilm growth is initiated by addition of 200 μL YNB to each well and subsequently incubated at 37 °C for the desired amount of time (generally 24–48 h) (*see* **Note 6**).

3.2 Biofilm Susceptibility Assay

1. Biofilms are washed once with 200 μL PBS.
2. To each well containing a washed biofilm, 190 μL buffer or medium is added.
3. 10 μl of a 20-fold stock solution of AmpB or ketoconazole is added.
4. Biofilms are incubated at 37 °C for an additional 24–48 h.

3.3 Detection of Biofilm Viability

1. After incubation, the biofilms are washed once with 200 μL PBS.
2. 100 μL of MTT is added to each well.
3. The plate is incubated at 37 °C for 30 min.
4. The MTT reaction is terminated by aspiration of all solution.
5. The crystals are solubilized in 100 μL acid isopropanol. Efficient solubilization can be achieved by vigorous pipetting, or shaking the plate for 10–15 min.
6. Killing efficacies can be monitored by eye or quantitatively by measuring the Abs at 550 nm (after transferring the solution to new 96-well plates).

In the standard susceptibility assay (the minimal inhibitory concentration (MIC) is determined), the inhibitor is included in the growth medium. To determine the MIC for biofilms, also referred to as the sessile minimal inhibitory concentration (SMIC),

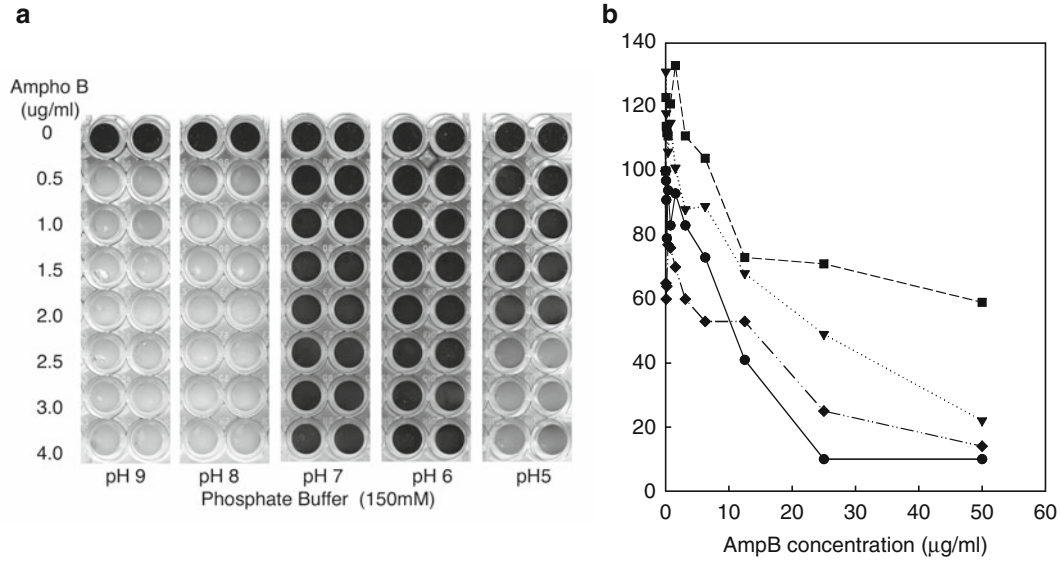


Fig. 3 Panel **a** shows the results of the MTT assay on *Candida* biofilms exposed to AmpB in 150 mM potassium phosphate buffer with different pH values. Panel **b** Residual MTT reduction ability (% of no inhibitor) for biofilms exposed to increasing AmpB concentrations for 24 h at 37 °C in YNB of different pH (*Filled diamond* = pH 5, *Filled square* = pH 6, *Filled down triangle* = pH 7 and *Filled circle* = pH 8)

biofilms have to develop in the absence of inhibitor. After washing, the biofilms are incubated in different buffers, or media, with inhibitor. This allows us to investigate the influence of different parameters on the killing process. Biofilms were grown for 48 h, washed with PBS and incubated in different media and buffers with different pH and buffer composition. The SMIC for AmpB was determined after 24 h incubation at 37 °C. No striking differences were observed when comparing YNB, MOPS, YNB MOPS and RPMI MOPS, all at pH 7 (not shown). Interestingly, the killing by AmpB appears to be very dependent on pH: lower pH yields lower SMIC values. Striking is that in 150 mM potassium phosphate buffer, at both pH 8 and pH 9 the SMIC is extremely low to below 1.0 µg/mL (Fig. 3a). In YNB the pH effect is less dramatic, but still present (Fig. 3b). This increased sensitivity at higher and lower pH was not observed when biofilms were incubated with ketoconazole (data not shown). Since ketoconazole is a cytostatic rather then a cytotoxic drug, we tested the efficacy of another cytotoxic drug, Nystatin. Incubation of biofilms with increasing concentrations of nystatin at increasing pH resulted in a pattern similar to the two azoles tested; there is no effect of pH on the SMIC value. It is therefore likely that the observed pH effect is specific for AmpB.

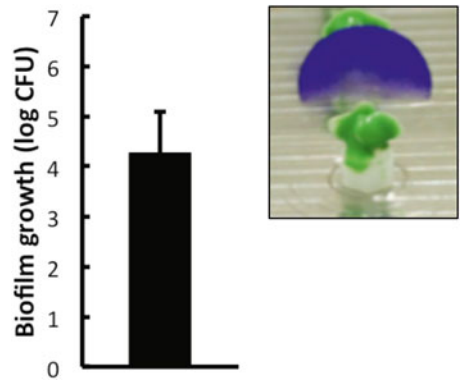


Fig. 4 Biofilm formation of *C. albicans* on a glass slide using the AAA model. A biofilm was grown for 48 h and biomass was estimated using CFU plating (*left panel*). Biofilm growth can also be estimated after staining with crystal *violet* (*right panel*)

3.4 Candida albicans Biofilms in the AAA- Model

1. The model is inoculated using a diluted ON culture ($OD_{600} = 0.1$) in BHI medium containing 10 % fetal bovine serum.
2. Six hours after inoculation the medium is replaced with sterile BHI medium (*see* **Note 7**).
3. Medium is refreshed every 24 h after initial inoculation up to a total of 72 h of biofilm formation.
4. Biofilm formation can be quantified using crystal violet staining or CFU plating (Fig. 4).
5. For CFU plating, the biofilm-covered surface is removed from the lid and placed in a sterile container with 2 mL PBS.
6. The biofilm is dislodged by sonication.
7. A serial dilution in sterile PBS can be prepared and plated.

3.5 Candida albicans Biofilms in the Bioflux

1. An ON culture of *C. albicans* is harvested and resuspended to a final OD_{600} of 0.1 in PBS.
2. Prewarmed PBS is used to fill the channels of the Bioflux plate from the inlet-side using a flow rate of 1–2 dyne/cm^2 (*see* **Note 8**).
3. The pre-warmed inoculum is placed into the outlet well and is pushed into the channel using a flow-rate of 0.2 dyne/cm^2. Take care not to flow the inoculum past the end of the channel, as this will result in clogging of the channel inlet.
4. Allow cells to adhere to the glass surface for 15–30 min without flow (*see* **Note 9**).
5. Flow pre-warmed YNB with and without a compound of interest from the inlet well through the system at 0.2–0.5 dyne/cm^2 for 20–42 h. Images can be taken every 5 or 10 min. For example, the effect of arginine on the dynamics of *C. albicans* biofilms can be studied (Fig. 5).

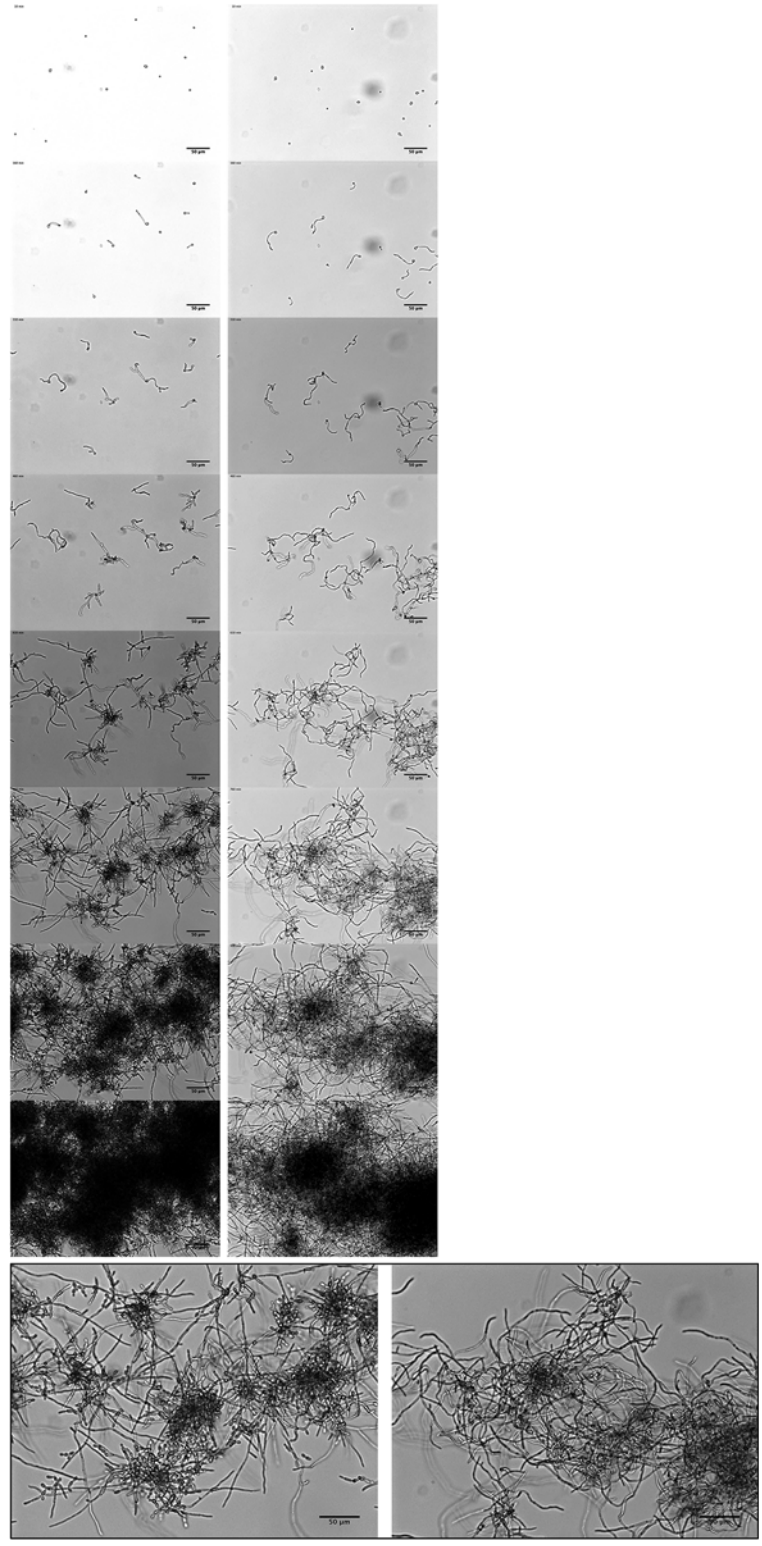


Fig. 5 The effect of arginine on biofilm formation of *C. albicans*. Biofilms were grown in the bioflux system for 20 h in medium without (*top panel*, *left*) or with 1.6 % arginine (*top panel*, *right*). A clear difference in number of yeast cells (present in control, absent in arg-grown biofilms) can be observed in these stills taken after 800 min of growth (*bottom panel*)

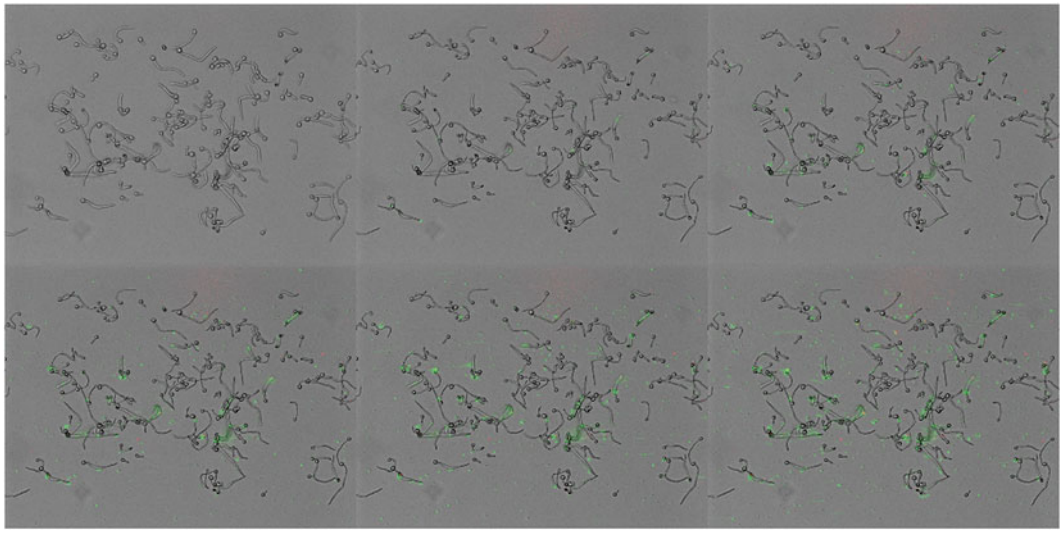


Fig. 6 Adhesion of *Aggregatibacter actinomycetemcomitans* to hyphae of *C. albicans*. *A. actinomycetemcomitans* was stained with Syto9 prior to adhesion. A time-lapse series taking images every 1 min was captured using the Bioflux Z1000 system

6. In addition, by adding bacteria to the flow, adhesion of bacteria to hyphae of *C. albicans* can be visualized (as described previously [10, 11]). Bacteria can be stained with SYTO9 and visualized while adhering to the surface or to hyphae (Fig. 6).

4 Notes

1. Alternatively, the medium without glucose can be sterilized in an autoclave after which the sterile glucose can be added from a sterile 20 % stock solution.
2. For optimal results, 0.5 mg/mL of MTT is dissolved in PBS + glucose and this solution should be prewarmed to 37 °C.
3. More information on the various options with the Bioflux system can be found on http://fluxionbio.com/bioflux/. Several application notes and protocols can be downloaded here (http://support.fluxionbio.com/categories/20124307-BioFlux).
4. Kuhn and coworkers [12] have optimized the use of the XTT assay and showed that although XTT reduction results in a water soluble formazan. It is recommended to solubilize the biofilm with DMSO prior to absorbance measurements. For both assays it is important to notice that it is difficult to compare results from different isolates directly because the rate of MTT or XTT metabolism shows strain-to-strain variation. However, for susceptibility studies this is not relevant since a drug free control is included for each individual strain.

5. For *C. albicans* strain SC5314 $OD_{600} = 1$ represents approximately 10^7 CFU/mL. Although the density at which adhesion is allowed matters to some extent, for susceptibility assays this is not as important. In our experience this relation between OD_{600} and CFU is a good estimate for cell density of other clinical isolates.
6. Biofilm formation is influenced by agitation; especially EPS production is induced by agitation [13] and consequently elevated resistance might be observed due to penetration differences [14], therefore the influence of static versus shaken incubation should be taken into account. No distinct effects of static versus shaken incubation was observed when the biofilms were grown in the 96-well plates, possibly because not enough medium flow is established in these small wells. However, by using wells with larger surface area, shear-induced EPS formation can cause heterogeneity in biofilm behavior within wells.
7. This refreshment is incorporated to ensure the use of substrate attached microorganisms.
8. If a coating of the glass bottom is desired, this can be incorporated in this step. Coating with FBS, collagen and fibronectin have been used successfully. For up-to-date information see the application notes at http://support.fluxionbio.com/categories/20124307-BioFlux.
9. To ensure that gravity does not induce a flow through the channel, the inlet and outlet wells should be filled with equal volumes of liquid.

Acknowledgements

The authors thank Dr Huajun Li and Kevin Kos for practical assistance. Purchase of the Bioflux Z1000 was made possible through a grant from the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO). This work was funded with financial support of the Dutch Burns Foundation (12.102). BPK is also supported by a grant from the University of Amsterdam for research into the focal point "Oral Infections and Inflammation."

References

1. Douglas LJ (2002) Medical importance of biofilms in *Candida* infections. Rev Iberoam Micol 19(3):139–143
2. Kojic EM, Darouiche RO (2004) *Candida* infections of medical devices. Clin Microbiol Rev 17(2):255–267
3. Krom BP et al (2007) Optimized candidal biofilm microtiter assay. J Microbiol Methods 68(2):421–423
4. Exterkate RA et al (2010) Different response to amine fluoride by *Streptococcus mutans* and polymicrobial biofilms in a novel high-throughput active attachment model. Caries Res 44(4):372–379
5. Nance WC et al (2013) A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. J Antimicrob Chemother 68(11):2550–2560

6. Fonzi WA, Irwin MY (1993) Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134(3):717–728
7. NCCLS (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts: approved Standard—Second Edition. NCCLS document M27-A2 (ISBN 1-56238-469-4). NCCLS, 940 west valley road, suite 1400, Wayne, Pennsylvania 19087-1898 USA
8. Chandra J et al (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 183(18):5385–5394
9. Al-Fattani MA, Douglas LJ (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol 55(8):999–1008
10. Bachtiar EW et al (2014) AI-2 of *Aggregatibacter actinomycetemcomitans* inhibits *Candida albicans* biofilm formation. Front Cell Infect Microbiol 4:94
11. Schlecht LM et al (2015) Systemic *Staphylococcus aureus* infection mediated by *Candida albicans* hyphal invasion of mucosal tissue. Microbiology 161:168–181
12. Kuhn DM et al (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. J Clin Microbiol 41(1): 506–508
13. Hawser SP et al (1998) Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol 47(3):253–256
14. Baillie GS, Douglas LJ (2000) Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother 46(3):397–403

Chapter 9

The Fungal Biome of the Oral Cavity

Jyotsna Chandra, Mauricio Retuerto, Pranab K. Mukherjee, and Mahmoud Ghannoum

Abstract

Organisms residing in the oral cavity (oral microbiota) contribute to health and disease, and influence diseases like gingivitis, periodontitis, and oral candidiasis (the most common oral complication of HIV-infection). These organisms are also associated with cancer and other systemic diseases including upper respiratory infections. There is limited knowledge regarding how oral microbes interact together and influence the host immune system. Characterizing the oral microbial community (oral microbiota) in health and disease represents a critical step in gaining insight into various members of this community. While most of the studies characterizing oral microbiota have focused on bacterial community, there are few encouraging studies characterizing the oral mycobiome (the fungal component of the oral microbiota). Our group recently characterized the oral mycobiome in health and disease focusing on HIV. In this chapter we will describe the methods used by our group for characterization of the oral mycobiome.

Key words Mycobiome, Sequencing, Fungus, Amplification, Fungal DNA extraction, Oral microbiota

1 Introduction

The oral cavity is a major entry point for microbes (bacteria, fungi and viruses) into the body, and microorganisms in this milieu have been associated with oral gingivitis, periodontitis and candidiasis. These microbial communities are also associated with systemic diseases including respiratory infections [1] and cancer [2]. Moreover, there is a limited understanding of how these organisms interact together and whether these interactions influence the host immune status in a synergistic, antagonistic, or indifferent manner. Therefore, characterizing the oral microbial community in health and disease represents a critical step in gaining insight into how various members of this community influence each other and how they impact host-immune response. To date, most of the studies characterizing the oral microbiota have focused on identifying the

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_9,

bacterial community [3, 4]. Fortunately, this is changing and recent studies, using deep sequencing, have characterized the oral mycobiome (the fungal component of the oral microbiota) in healthy individuals [5, 6] as well as in the setting of HIV-infection [7]. Our group showed that the oral mycobiome of healthy individuals comprises 74 culturable and 11 non-culturable fungal genera [5]. In addition, the total number of culturable species detected was 101, with each individual having between 9 and 23 species. In a separate study, our group characterized the oral microbiota (bacteriome and mycobiome) in HIV infected patients and matched uninfected controls, and identified antagonistic interaction between *Candida* and *Pichia* [7]. These studies are encouraging and point to the need that investigations aimed at characterizing the mycobiome in different diseases are promising and may allow us to discover new ways to manage these diseases.

The differences between bacteria (prokaryotic organism) and fungi (eukaryotic microbes) necessitate the use of different methods to characterize them. In this chapter we will describe the method our group follows to characterize the oral mycobiome. This is our attempt to standardize the methods used to study the fungal community. Such standardization is critical since using common methods by various investigators will allow comparison of results generated by different working groups. This chapter does not include bioinformatics analyses of the generated sequencing data, which is a critical step, but beyond the scope of this chapter. For bioinformatics analyses of microbiome data, the reader is referred to publications from our group and others [7–10].

2 Materials

Prepare all reagents using nuclease-free, PCR grade water stored at –80 °C. Prepare single use 1.5 mL aliquots prior to storage.

2.1 Fungal DNA Extraction

1. Fast Prep-24 (MP, Biomedical, Inc.).
2. QIAamp DNA Mini kit, (Qiagen), Kit reagents: (AL-Lysis buffer, AW1-Wash Buffer, AW2-Wash buffer, AE-Elution Buffer, Proteinase K, collection tubes and columns).
3. Glass beads, 425–600 μm size (Sigma-Aldrich).
4. Microcentrifuge Tubes, 2 mL (Fisher).
5. Fast-prep screw caps for tubes (MP bio).
6. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).
7. Salmon sperm DNA solution (10 mg/mL stock, Invitrogen).
8. Microcentrifuge.

2.2 Fungal Internal Transcribed Spacer (ITS) Amplification

1. ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS2 (5′-GCTGCGTTCTTCATCGATGC)—20 μM final concentration in PCR grade water.
2. 2× Phusion® High-Fidelity PCR Master Mix with GC Buffer (Thermo Fisher Scientific, Waltham, MA, USA).
3. 100 % Dimethylsulfoxide (DMSO, Thermo Fisher Scientific, Waltham, MA, USA).
4. Bio-Rad 96-well iCycler thermal cycler (Bio-Rad Inc, Hercules, CA, USA).

2.3 Amplicon Purification

1. Agencourt® AMPure® XP Reagent (Agencourt Bioscience Corp., Beverly, MA,USA).
2. 96-well Supermagnet (Alpaqua Engineering, Beverly, MA, USA).
3. 200 proof HPLC grade Ethanol (Thermo Fisher Scientific, Waltham, MA, USA).
4. LowTE (Life Technologies, Grand Island, NY, USA).
5. 96-Well PCR Plates (Axygen Scientific, Tewksbury, MA, USA).
6. Eppendorf LoBind Micro-centrifuge Tubes (Eppendorf, Hauppauge, NY, USA).
7. MicroAmp® Clear Adhesive Film (Life Technologies, Grand Island, NY, USA).

2.4 Qubit DsDNA Quantitation

1. Qubit fluorometer (Life Technologies, Grand Island, NY, USA).
2. Qubit dsDNA BR Buffer (Life Technologies, Grand Island, NY, USA).
3. Qubit Assay Tube (Life Technologies, Grand Island, NY, USA).
4. Qubit dsDNA BR Reagent (Life Technologies, Grand Island, NY, USA).

2.5 DNA Fragmentation

1. Ion Shear Plus Reagent Kit (Life Technologies, Grand Island, NY, USA).
   (a) Ion Shear Plus 10× Reaction Buffer.
   (b) Ion Shear Plus Enzyme Mix II.
   (c) Ion Shear Plus Stop Buffer.

2.6 Barcode/Adapter Ligation

1. Ion Xpress Plus Fragment Library Kit (Life Technologies, Grand Island, NY, USA).
   (a) LowTE.
   (b) 10× Ligase Buffer.
   (c) dNTP Mix.

(d) DNA Ligase.

(e) Nick Repair Polymerase.

2. Ion Xpress Barcode 1-96 (Life Technologies, Grand Island, NY, USA).

(a) Barcode X.

(b) Ion PI Adapter.

2.7 Targeted Size Selection

1. Pippen Prep Gel Based Size Selection Device (Sage Scientific, Beverly, MA, USA).

(a) Calibration pad.

(b) Dye-free Cassette 2DF.

(c) Marker L.

(d) Electrophoresis Pippin Buffer.

2.8 DNA Library Quantitation

1. Ion Library Quantitation Kit (Life Technologies, Grand Island, NY, USA).

(a) *E. coli* DH10B Control Library (68 pM).

(b) Ion Library TaqMan qPCR Mix (2×).

(c) Ion Library TaqMan Quantitative Assay (20×).

2.9 Ion Sphere Particle Templating via Emulsion PCR

1. Ion PGM™ Template OT2 Reaction 400 Kit (Life Technologies, Grand Island, NY, USA).

(a) OneTouch Plus Reaction Filter Assembly.

2. Ion PGM™ Template OT2 Supplies 400 Kit (Life Technologies, Grand Island, NY, USA).

(a) Ion OneTouch™ Reagent Tubes.

(b) Ion OneTouch™ Recovery Routers.

(c) Ion OneTouch™ Recovery Tubes.

(d) Ion OneTouch™ Sipper Tubes.

(e) Ion OneTouch™ 2 Amplification Plates.

3. Ion PGM™ Template OT2 Reagents 400 Kit (Life Technologies, Grand Island, NY, USA).

(a) Ion PGM™ Template OT2 400 Reagent Mix.

(b) Ion PGM™ Template OT2 400 Enzyme Mix.

(c) Ion PGM™ Template OT2 400 Ion Sphere™ Particles.

(d) Ion PGM™ Template OT2 400 Reagent X.

4. Ion PGM™ Template OT2 Solutions 400 Kit (Life Technologies, Grand Island, NY, USA).

(a) Ion PGM™ Template OT2 400 PCR Reagent B.

(b) Ion OneTouch™ Oil.

(c) Ion OneTouch™ Reaction Oil.
(d) Nuclease-free water.
(e) Ion PGM™ OT2 Recovery Solution.

2.10 ISP Enrichment

1. One Touch™ Enrichment System (Life Technologies, Grand Island, NY, USA).
   (a) Ion PGM™ Enrichment Beads.
   (b) MyOne™ Beads Wash Solution.
   (c) Neutralization Solution.
   (d) Tween® Solution I.
   (e) Wash Solution.

2.11 Ion Torrent PGM Sequencing

1. Ion Personal Genome Machine® (PGM™) System (Life Technologies, Grand Island, NY, USA).
2. Torrent Server (Life Technologies, Grand Island, NY, USA).
3. Tank of compressed nitrogen (Airgas Inc, Cleveland OH, USA).
4. Ion Chip MiniFuge (Life Technologies, Grand Island, NY, USA).
5. Ion 314™ Chip Kit v2 or Ion 316™ Chip Kit v2 or Ion 318™ Chip Kit v2 (Life Technologies, Grand Island, NY, USA).
6. 1 N Sodium Hydroxide (NaOH) molecular biology grade (Thermo Fisher Scientific, Waltham, MA, USA).
7. Ion PGM™ Sequencing Supplies 400 Kit (Life Technologies, Grand Island, NY, USA).
   (a) Wash Bottle Sipper Tubes.
   (b) Reagent Bottle Sipper Tubes.
   (c) Reagent Bottles w/labels (50 mL).
   (d) Wash 1 Bottle w/label (250 mL).
   (e) Wash 2 Bottle w/label (2 L).
   (f) Wash 3 Bottle w/label (250 mL).
8. Ion PGM™ Sequencing Reagents 400 Kit (Life Technologies, Grand Island, NY, USA).
   (a) Ion PGM™ Sequencing 400 dGTP.
   (b) Ion PGM™ Sequencing 400 dCTP.
   (c) Ion PGM™ Sequencing 400 dATP.
   (d) Ion PGM™ Sequencing 400 dTTP.
   (e) Ion PGM™ Sequencing 400 Polymerase.
   (f) Sequencing Primer.
   (g) Control Ion Sphere™ Particles.

9. Ion PGM™ Sequencing Solutions 400 Kit (Life Technologies, Grand Island, NY, USA).
   (a) Ion PGM™ Sequencing 400 W2 Solution.
   (b) Ion PGM™ Cleaning Tablet.
   (c) Annealing Buffer.
   (d) Ion PGM™ Sequencing 400 1× W3 Solution.

3 Methods

Steps described below outline the use of kits from various manufacturers. Although readers can obtain instructions about how to use these kits from the respective companies, we decided to include the details as described in the kits with modifications, so that the reader will have easy access to the information in a "cookbook" format.

3.1 Fungal DNA Extraction (According to Manufacturer's Instructions, QIAamp DNA Mini Kit from Qiagen)

1. Centrifuge all samples at high speed for 5–10 min in microcentrifuge tubes.
2. After centrifugation in **step 1**, slowly aspirate all the solution from the pellet.
3. To the pellet, add 500 μL AL lysis buffer from QIAamp DNA Mini kit (Qiagen).
4. Leave it in lysis buffer for 2 h to overnight at 4 °C.
5. Next day, add 500 μL glass beads in a MP Bio tube and add 15 μL Proteinase K to tube with beads. These are the "Matrix Tubes."
6. Sonicate samples in AL buffer (from **step 4**) for 5 min and then transfer to the matrix tube with glass beads and proteinase K.
7. Incubate above tubes at 56 °C for 1 h (vortex every 15–20 min for few seconds while tubes are incubating).
8. Place Matrix Tubes in Fast Prep-24 device and vortex for three rounds at velocity of 6 m/s for 30 s.
9. Put Matrix Tubes on ice between rounds.
10. Centrifuge the above tubes at 12,000 × *g* for 10 min.
11. Transfer DNA supernatant (approximately 200 μL) slowly and carefully to a new tube containing 200 μL of 100 % ethanol.
12. Pulse vortex to mix and add the solution to QIAmp mini spin column.
13. Centrifuge at 6000 × *g* for 1 min, discard the flow through.
14. Wash the spin column with 500 μL AW1 buffer.
15. Centrifuge at 6000 × *g* for 1 min, Discard the flow through.

16. Wash the spin column with 500 μL AW2 buffer.
17. Centrifuge at 6000 × *g* for 5 min, discard the flow through.
18. Transfer column to a new labeled microcentrifuge tube.
19. Elute DNA from the column using 100 μL AE elution buffer. Wait for 1 min after adding 100 μL elution buffer to the column.
20. Centrifuge at 6000 × *g* for 1 min.
21. Repeat the above step by taking 100 μL of eluted DNA and adding back to the spin column. Wait for 1 min.
22. Centrifuge at 6000 × *g* for 1 min.
23. Quantitate DNA using NanoDrop 2000 spectrophotometer.

3.2 Quantitating Extracted DNA Using NanoDrop 2000 Spectrophotometer

1. Open NanoDrop 2000 program (pre-installed on a computer), select "Nucleic acids," then "DNA."
2. Close the lid of NanoDrop spectrophotometer to initialize the machine for a few seconds.
3. Add 1 μL AE buffer (which is used for eluting DNA) very carefully on NanoDrop.
4. Press Blank.
5. Wipe NanoDrop with a wet wipe, and add 1 μL Salmon sperm DNA (Validation stock 20–25 ng/μL). Press Measure.
6. NanoDrop will give the readings (ng/μL or μg/mL can be selected from the drop down button), (Note: 1 μg = 1000 ng).
7. Wipe NanoDrop between samples and add 1 μL for each sample.
8. After analyzing samples, add blank and press "Measure" to ensure that readings for blank are still negative or zero.
9. Press Report to generate a report for all samples measured.
10. Export the report to a spreadsheet and save it.

QC step:

- Measure DNA concentration in 1 μL AE diluted Salmon sperm DNA (Validation stock 20–25 ng/μL).
- Measure DNA concentration in Blank at the start and end of all sample measurements. The readings for Blank should be ≤0.

3.3 DNA Amplification

1. For polymerase chain reaction (PCR) mixture, add 25 μL 2× Phusion® High-Fidelity PCR Master Mix with GC Buffer, 1.5 μL DMSO, 1 μL (0.4 μM) each of ITS1 and ITS2 primers, and 5 ng fungal DNA extracted above.
2. Run the thermal cycler using the following cycling instructions:

No hot start PCR conditions			
Cycle steps	**Temp (°C)**	**Time**	**Cycles**
Initial denaturation	98	30 s	1×
Denaturation	98	8 s	
Annealing	59	10 s	40×
Extension	72	15 s	
Final extension	72	5 min	
	4	Hold	1×

3.4 Validating PCR Product (Fig. 1)

1. Prepare 1.5 % agarose gel.
2. Heat in a microwave oven on high for 90 s or until agarose is completely dissolved.
3. Cool agarose gel solution for 10 min at room temperature, ensure solution does not begin to solidify.
4. Add 7 μL of ethidium bromide for every 100 mL of cooled liquid gel preparation, swirl gently to mix. (Avoid forming air bubbles.).
5. Pour gel into gel electrophoresis system containing the combs.
6. Allow gel to completely solidify before continuing to next step.
7. Dislodge gel cassette and orient the cassette so the comb is closest to the black negative polarity probe.
8. Add fresh Tris-buffer to chamber until gel is completely submerged.
9. Carefully and with both hands remove the comb from the gel cassette being careful not to damage the well generated by the comb mold.
10. Load 7 μL Mid-range DNA Ladder to first available well.
11. Load 7 μL PCR Product (add 1 μL loading dye to 50 μL PCR product before loading), to each well.
12. Run gel at 100 V, 60 min at room temperature (RT) (*see* **Notes 1** and **2**).
13. Turn off power source and disconnect probes from power source.
14. Rinse gel with distilled water and take the picture using an imaging device (Fig. 1).

3.5 Long Amplicon (PCR amplified DNA) Purification Protocol

[*Protocol adapted from USER BULLETIN ("Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit from Ion Torrent") by Life Technologies* [11]].

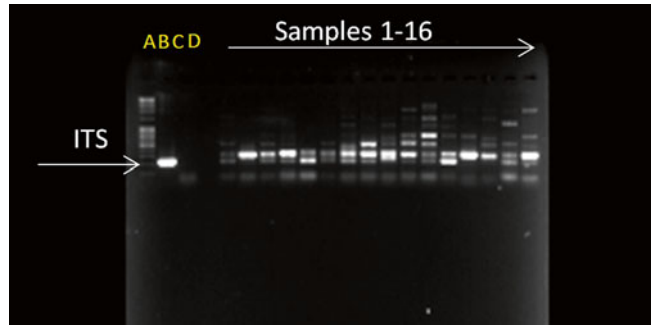


Fig. 1 Representative gel picture showing PCR amplified fungal DNA: (*A*) Mid-range DNA ladder, (*B*) Positive control, (*C*) Negative control, (*D*) Blank, Samples 1–16, PCR amplified DNA showing signals for fungal specific primers ITS1 and ITS2

1. Resuspend the Agencourt® AMPure® XP Reagent, and allow the mixture to come to room temperature (~30 min).
2. Prepare 70 % ethanol: 300 μL per amplicon (*see* **Note 3**).
3. In each well or tube, add equal volume Agencourt® AMPure® XP reagent to PCR amplified product to get 1:1 ratio. Pipet up and down five times to thoroughly mix the bead suspension with the PCR-amplified DNA and incubate the mixture at room temperature for 5 min.
4. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 3 min or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.
5. Without removing the samples from the magnet, dispense 150 μL of freshly prepared 70 % ethanol into each well or tube. Incubate the samples at room temperature for 30 s. After the solution clears, remove and discard the supernatant without disturbing the pellet.
6. Repeat **step 5** for a second wash with 150 μL 70 % ethanol.
7. To remove residual ethanol, keep the sample on the magnet and carefully remove any remaining supernatant with a 20-μL pipette without disturbing the pellet.
8. Keeping the sample on the magnet, air-dry the beads at room temperature for 3–5 min (*see* **Note 4**).
9. Remove the plate or tubes from the magnet, and add 40 μL of nuclease-free water (PCR high grade) directly to each bead pellet to disperse the beads. Pipet the mixture up and down five times to mix thoroughly (*see* **Note 5**).
10. Place the plate or tubes on a magnet for at least 1 min. After the solution clears, transfer the supernatant containing the

purified long amplicons to a new plate or tube without disturbing the pellet (*see* **Note 6**).

STOPPING POINT (Optional) Store the DNA at –10 to –30 °C. Can go directly to the next quantitation step.

3.6 Quantitate DNA ("Long Amplicon") Using Qubit Fluorometer

1. Prepare Qubit "Working solution": Dilute Qubit dsDNA BR reagent 1:200 with Qubit dsDNA BR buffer.
2. Dispense 197 μL of Qubit Working Solution in pre-labeled Qubit assay tubes (labeled for each "long amplicon" sample, purified in subheading 3.5, above).
3. Add 3 μL of each amplicon sample to the corresponding labeled Qubit assay tubes (prepared in **step 2**). Mix well. (Final volume in each assay tube will be 200 μL.).
4. Turn the Qubit 2 fluorometer ON, press "DNA," select 3 μL volume.
5. Press "Read" and change units to ng/μL. Save data as a spreadsheet.

3.7 Fragment 100 ng Long Amplicon DNA with Ion Shear Plus Reagents

[*Protocol adapted from USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit, (page 15) from Ion Torrent by Life Technologies*], [11]:

1. Prepare a fresh PCR plate with 100 ng amplicon DNA in 30 μL volume.
2. Vortex Ion Shear Plus 10× Reaction Buffer and Ion Shear Plus Enzyme Mix II each for 5 s, pulse spin to bring the contents to the bottom of the tubes, and place on ice. (Thoroughly mix Ion Shear Plus 10× Reaction Buffer and Ion Shear Plus Enzyme Mix II individually before dispensing them in the next steps.)
3. Put the PCR plate with amplicon DNA (from **step 1**, above) on thermal cycler, and hold it at 4 °C.
4. Add the following reagents in the indicated order to the above PCR plate:

Ion Shear Plus 10× Reaction Buffer	3.5 μL
Ion Shear Plus Enzyme Mix II	2.5 μL

5. Mix the reaction by rapidly pipetting up and down eight to ten times. Do not mix by vortexing and avoid creating bubbles.
6. Stop 4 °C program and immediately open second thermal cycler program.

7. Run the program on thermal cycler which runs at 37 °C for 15 min as follows:

Cycle 1: (1×)	Step 1	37 °C for 15 min
Cycle 2: (1×)	Step 1	4 °C hold

8. Add 2.5 μL of Ion Shear Stop Buffer immediately after incubation for 15 min, and mix thoroughly by mixing wells for at least 5 s. Store the reaction tube on ice (*see* **Note** 7).

3.8 Purification of Fragmented DNA and Ligating Adapters, Nick-Repairing/ Barcoding to Fragmented DNA

[*Protocol adapted from USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit, (pages 16–18) from Ion Torrent by Life Technologies*], [11]:

1. Resuspend Agencourt® AMPure® XP Reagent, and allow the mixture to come to room temperature (~30 min).
2. Prepare 70 % ethanol: Need 300 μL per fragmented DNA.
3. In each well or tube, add equal volume Agencourt® AMPure® XP reagent to fragmented DNA in a 1:1 ratio. For example, for 38.5 μL fragmented DNA, add ~38.5 μL bead reagent, pipet up and down five times to mix thoroughly, and incubate the mixture at room temperature for 5 min.
4. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 3 min or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.
5. Without removing the samples from the magnet, dispense 150 μL of freshly prepared 70 % ethanol into each well or tube. Incubate the samples at room temperature for 30 s. After the solution clears, remove and discard the supernatant without disturbing the pellet.
6. Repeat **step 5** for a second wash with 150 μL 70 % ethanol.
7. To remove residual ethanol, keep the sample on the magnet and carefully remove any remaining supernatant with a 20-μL pipette without disturbing the pellet.
8. Keeping the sample on the magnet, air-dry the beads at room temperature for 3–5 min (ensure that the pellet does not dry out completely).
9. Remove the plate or tubes from the magnet, and add 49 μL ligation solution (prepared as below) directly to each bead pellet to disperse the beads. Pipet the mixture up and down five times to mix thoroughly:

Ligation solution	Vol of reagents per well (μL)
Nuclease-free water (PCR high grade)	7
LowTE	25
10× Ligase Buffer	5
dNTP Mix	1
DNA Ligase	1
Nick repair polymerase	4
Ion PI adapter (add at the end)[a]	1

[a]Add Ion.5 PI Adapter at the end, after adding all other reagents in the ligation solution

10. Place the plate or tubes on a magnet for at least 1 min. After the solution clears, transfer the supernatant containing the ligated DNA to a new plate containing 1 μL Ion Xpress Barcode X per well.
11. Place the plate with adapter-ligated and Barcode reagents on thermal cycler and run the following program:

Cycle 1: (12×)	Step 1	10 °C for 30 s
	Step 2	24 °C for 5 min
	Step 3	30 °C for 30 s
Cycle 2: (1×)	Step 1	25 °C for 60 min

3.9 Purification of Adapter-Ligated and Nick-Translated-(Barcoded) DNA Library

[*Protocol adapted from USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit, (page 18) from Ion Torrent by Life Technologies*], [11]:

1. In each well or tube, add equal volume Agencourt® AMPure® XP reagent to ligated DNA in a 1:1 ratio. Pipet up and down five times to thoroughly mix the bead suspension with ligated/barcoded DNA, and incubate the mixture at room temperature for 5 min.
2. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 3 min or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.
3. Without removing the samples from the magnet, dispense 150 μL of freshly prepared 70 % ethanol into each well or tube. Incubate the samples at room temperature for 30 s. After the solution clears, remove and discard the supernatant without disturbing the pellet.

4. Repeat **step 3** for a second wash with 150 μL of 70 % ethanol.
5. To remove residual ethanol, keep the sample on the magnet and carefully remove any remaining supernatant with a 20-μL pipet without disturbing the pellet.
6. Keeping the sample on the magnet, air-dry the beads at room temperature for 3–5 min.
7. Remove the plate or tubes from the magnet, and add 40 μL low TE directly to the pellet to disperse the beads. Pipet the mixture up and down five times, then vortex the sample for 10 s, to mix thoroughly.
8. Place the plate or tubes on a magnet for at least 1 min. After the solution clears, transfer the supernatant containing the ligated/barcoded DNA library to a new plate.

   Stopping Point: (Optional) Store the DNA at –10 to –30 °C.
9. Proceed to size-select the ligated/barcoded library, as described below.

3.10 Size-Select the Ligated/Barcoded Library

[*Protocol adapted from USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit (pages 21–22), from Ion Torrent by Life Technologies*], [11]:

1. *Pool ligated barcoded library*: Take 5 μL from each sample well and mix thoroughly in a reservoir.
2. After **step 1**, all samples are pooled together.
3. Transfer everything from the reservoir into a microcentrifuge tube.
4. Transfer 150 μL from the pooled library from **step 3** into a new microcentrifuge tube and add 50 μL of Marker L.
5. Turn ON Pippin prep machine and calibrate it with calibration pad.
6. Add the dye-free cassette 2DF to the machine.
7. Remove two adhesive sealing tapes from left (loading wells) and right (elution wells) of the cassette.
8. Replace all 50 μL elution buffer from right (elution wells) in the cassette with 50 μL Electrophoresis Pippin Buffer.
9. Press run on the machine after selecting the correct cassette 2DF.
10. Machine will initialize and check for errors, if it shows pass, then proceed to next steps.
11. Remove the solution from the loading wells of the cassette as much as possible carefully without touching the gel.
12. Add 40 μL from **step 4** (pooled ligated library with Marker L) to the loading wells on the cassette.

13. Run Pippin instrument by selecting the template and saving with new project name.
14. Pippin instrument runs for approximately 90 min.
15. After 90 min, remove 60 μL sample from all five elution wells on the right of the cassette (Total sample for size-select ligated DNA library is approximately 300 μL).

3.11 Purification of Size-Selected DNA Library

[*Protocol adapted from USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit (page 23) from Ion Torrent by Life Technologies*], [11]:

1. Add equal volume Agencourt® AMPure® XP reagent to size-select ligated DNA library (from **step 15** above), to get 1:1 ratio (size-select DNA is 300 μL, so add 300 μL bead reagent, gives total sample = 600 μL). Pipet up and down five times to thoroughly mix the bead suspension with size-select DNA and incubate the mixture at room temperature for 10–20 min.
2. Separate 150 μL of the above bead mixed sample into four PCR tubes each.
3. Place all four tubes on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 5 min or until the solution clears. Remove and discard the clear supernatant as much as possible from each tube without disturbing the bead pellet.
4. Without removing the samples from the magnet, dispense 200 μL of freshly prepared 70 % ethanol into each tube. Incubate the samples at room temperature for 30 s. After the solution clears, remove and discard the supernatant without disturbing the pellet.
5. Repeat **step 4** for a second wash with 200 μL of 70 % ethanol.
6. To remove residual ethanol, keep the four PCR tubes with sample on the magnet and carefully remove any remaining supernatant with a 20-μL pipet without disturbing the pellet.
7. Keeping the sample on the magnet, air-dry the beads at room temperature for 3–5 min. Do not dry the beads completely.
8. Remove the four PCR tubes with the dried bead from the magnet (from **step 7**), and add 50 μL of low TE directly to each tube to disperse the beads. Pipet the mixture up and down five times, then vortex the sample for 10 s, to mix thoroughly.
9. Place the tubes on a magnet for at least 5 min. After the solution clears, transfer the supernatant containing the purified size-select DNA library to one microcentrifuge tube (to pool everything together).

10. Proceed to qPCR steps as below, for quantitating purified size-select DNA library (from **step 9** above).

3.12 Quantitation of Size-Select DNA Library Using qPCR

[*Protocol adapted from Ion Library Quantitation Kit USER GUIDE (pages 7–9), TaqMan assay quantitation of Ion Torrent Libraries, from Ion Torrent by Life Technologies*], [12].

3.12.1 Prepare Serial Dilutions of the E. coli DH10B Control Library

1. Thaw the *E. coli* DH10B Control Library on ice.
2. Prepare five sequential tenfold dilutions from the control library (~68 pM stock concentration) in nuclease-free water, as shown in the table below. Mix the diluted solutions by gently pipetting up and down at least ten times. Take care not to vortex the diluted solutions. Use of non-stick microcentrifuge tubes is highly recommended for preparation of the serial dilutions.
3. Prepare sufficient volume of each dilution for the size of your qPCR reactions and the number of replicates (for example, for a 20-μL qPCR reaction volume, prepare 5 μL of diluted standard per reaction or 15 μL per triplicate plus a small overage for pipetting loss).

Standard	Control library	Water[a] (μL)	Fold dilution	Concentration (pM)
1	5 μL (undiluted)	45	0.1	6.8
2	5 μL (Std 1)	45	0.01	0.68
3	5 μL (Std 2)	45	0.001	0.068
4	5 μL (Std 3)	45	0.0001	0.0068
5	5 μL (Std 4)	45	0.00001	0.00068

[a]Nuclease-free water (not DEPC water)

3.12.2 Dilute the Size-Select Sample Library

1. Prepare an initial 1:20 dilution of the sample library in nuclease-free water in a non-stick microcentrifuge tube on ice.
2. Prepare dilutions of the sample library that target a concentration within the serial dilutions of the control library, as described in the following table. It is best to prepare three independent dilutions for qPCR. At a minimum, prepare three technical replicate qPCR reactions of each individual dilution.

   For a standard 20-μL qPCR reaction, prepare 5 μL of each library dilution per reaction.

Dilution	Library input	Water[a] (μL)
1:200	1 μL of 1:20	9
1:2000	1 μL of 1:20	99
1:20,000	5 μL	45

[a]Nuclease-free water (not DEPC water)

3.12.3 Set Up the PCR Reactions

1. Thaw frozen components on ice. Gently but thoroughly mix each thawed component, then briefly centrifuge to bring the contents to the bottom of the tube. *Do not vortex the Ion Library TaqMan qPCR Mix.*

   Prepare a master mix of the following components on ice, as described in the following table. Scale the volumes based on the number and volume of your qPCR reactions (At a minimum, prepare sufficient volume for three technical replicates of each control dilution, library dilution, and no-template control).

Component	Volume per 20 μL reaction
Ion library TaqMan qPCR Mix, 2×	10 μL
Ion library TaqMan quantitative assay, 20×	1 μL
Nuclease-free water	to 15 μL

2. For each reaction, transfer 15 μL of the master mix into a well of the PCR plate.
3. Add 5 μL of the diluted control or sample library to each appropriate well.

   Add 5 μL of nuclease-free water to the no-template control (NTC) wells.
4. Seal the plate and centrifuge the plate briefly to spin down the contents and eliminate air bubbles. Run the qPCR program as follows:

3.12.4 Running the Real-Time qPCR Instrument

1. Program your real time instruments according to the manufacturer's instructions. Enter the concentrations for the standards of the control library from the table above. ROX passive reference dye is included in the Ion library TaqMan qPCR mix. TaqMan probe reporter/quencher: FAM dye/MGB. Use the cycling program for StepOnePlus System as follows:

Applied biosystem real-time PCR system	Stage	Temp (°C)	Time
Step One Plus System	Hold (UDG incubation)[a] Cycle (40 cycles)	50	2 min
	Hold (polymerase activation)[b]	95	20 s
	Cycles (40 cycles)	95	1 s
	Cycles (40 cycles)	60	20 s

[a]Required for optimal UDG activity
[b]Required for AmpliTaq DNA Polymerase activation and template denaturation

2. Place the plate in the real-time PCR instrument, run the reactions, and collect the real-time data.

3.12.5 Determine the Concentration for the Sample Library

The real-time PCR instrument software calculates the diluted library concentration. The undiluted library concentration is calculated by multiplying the concentration determined with qPCR by the sample dilution (e.g., 2000 or 20,000).

1. Choose the Ct data from the appropriate library dilution.
   - For many libraries, both dilutions of the sample library will fall within the standard curve. Use the Ct values from the most concentrated sample dilution to calculate the library concentrations.
   - For very concentrated or very dilute libraries, use the Ct values from the dilution that falls within the standard curve.
2. Calculate the undiluted sample library concentration:

   Undiluted library concentration = (Concentration determined by qPCR) × (Library dilution).

   Example: The diluted library concentration determined by qPCR is 6.11 pM for a 1:2000 library dilution.

   Undiluted library concentration = (6.11 pM) × (2000) = 12,220 pM.
3. Usually need 20–26 pM library concentration for template preparation next, so dilute the library to get this concentration. Refer to the appropriate Ion library preparation user guide for dilution recommendations for template preparation.

3.13 Preparing Template

[*Protocol adapted from Ion PGM™ Template OT2400 kit USER GUIDE, (pages 31–38), for use with: the Ion One Touch™ 2 system, from Ion Torrent by Life Technologies*], [13]:

1. Dilute the library as shown in the table below:

	gDNA fragment or amplicon library
Library concentration	26 pM
Volume of library	25 µL
Volume of nuclease-free water	0
Total volume of diluted library to add to the amplification solution	25 µL

   (a) Vortex the diluted library for 5 s, then centrifuge for 2 s.

   (b) Place the diluted library on ice.
2. In a 1.5 mL LoBind microcentrifuge tube at 15–50 °C, add the following components in the designated order. Add each component, then pipet the amplification solution up and down to mix:

Order	Reagent	Cap color	Volume (μL)
1	Ion PGM™ template OT2 400 Reagent Mix	Violet	500
2	Ion PGM™ template OT2 400 Reagent B	Blue	285
3	Ion PGM™ template OT2 400 Enzyme Mix	Brown	50
4	Ion PGM™ template OT2 400 Reagent X	White	40
5	Diluted library (not stock library)	–	25
–	Total	–	900

3. Vortex the solution prepared in **step 2** at maximum speed for 5 s, then centrifuge the solution for 2 s.
4. Prepare the Ion PGM™ Template OT2 400 Ion Sphere Particles (ISPs):
   (a) Vortex the Ion PGM™ Template OT2 400 Ion Sphere Particles at maximum speed for 1 min to resuspend the particles.
   (b) Centrifuge the ISPs for 2 s.
   (c) Pipet the Ion PGM™ Template OT2 400 Ion Sphere Particles up and down to mix.
   (d) Immediately proceed to the next step.
5. Add the Ion PGM™ Template OT2 400 Ion Sphere Particles to the amplification solution:

Order	Reagent	Cap color	Volume (μL)
1	Amplification solution *without* Ion PGM™ Template OT2 400 Ion Sphere Particles (from **step 2** of this procedure)	–	900
2	Ion PGM™ template OT2 400 Ion Sphere Particles	Black	100
–	Total	–	1000

6. Vortex the complete amplification solution prepared in **step 5** at maximum speed for 5 s.
7. Proceed immediately to "Fill the Ion PGM™" One Touch Plus Reaction Filter Assembly.
8. Run the Ion One Touch™ 2 Instrument for ~8 h (*see* **Note 8**).

3.14 Recover the Template-Positive Ion PGM™ Template OT2 400 Ion Sphere™ Particles (ISPs) and Proceed for Enriching the ISPs

[*Protocol adapted from Ion PGM™ Template OT2400 kit USER GUIDE, (pages 39–41), for use with: the Ion One Touch™ 2 system, from Ion Torrent by Life Technologies*], [13]:

1. At the end of the run, follow the screen prompts to centrifuge the sample. If you removed the reaction tubes at the end of the run *before* the Ion OneTouch™ 2 Instrument had spun the sample or have not processed the sample within 15 min, centrifuge the sample on the instrument:
   (a) On the home screen of the instrument, touch Open Lid, then insert the two filled Ion OneTouch™ Recovery Tubes from the run in the centrifuge rotor. Close the lid until it locks.
   (b) Touch Options, then touch Final Spin, then follow the screen prompts (touch Next on the next two screens) until the centrifugation begins. Centrifugation of the samples takes 10 min.
   (c) Immediately proceed to **step 2**.
2. Immediately after the centrifuge has stopped, on the instrument display, touch Open Lid, wait until the lid clicks open, then remove and discard the Ion OneTouch™ Recovery Router (*see* **Note 9**).
3. *Carefully* remove both Ion OneTouch™ Recovery Tubes from the instrument. And put the two recovery tubes in a tube rack. You may see some cloudiness in the tube, which is normal.
4. Remove excess Ion PGM™ OT2 Recovery Solution from the Ion PGM™ Template OT2 400 Ion Sphere™ Particles:
   (a) Use a pipette to remove all but 100 μL of the recovery solution from each recovery tube. Withdraw the supernatant from the surface and on the opposite side from the pellet. Remove any white flocculent material. Do not disturb the pellet of Ion PGM™ Template OT2 400 Ion Sphere™ Particles:
   (b) With a new tip and using the same tip for both tubes, resuspend the Ion PGM™ Template OT2 400 Ion Sphere™ Particles in the remaining Ion PGM™ OT2 Recovery Solution. Pipet the pellet up and down until each pellet disperses in the solution.
5. Process the Ion PGM™ Template OT2 400 Ion Sphere™ Particles (ISPs):
   (a) Label a new 1.5-mL Eppendorf LoBind® Tube for the ISPs.
   (b) Add 500 μL of Ion OneTouch™ Wash Solution to the first recovery tube.

(c) Pipet the ISPs up and down to disperse the ISPs, then transfer suspension from first to the second recovery tube, pipet up and down and transfer everything into new labeled 1.5-mL Eppendorf LoBind® Tube.

(d) Add 500 μL again to first recovery tube and repeat as in **step 5c**.

(e) Heat the ISPs at 50 °C for 5 min.

(f) Centrifuge the ISPs for 2.5 min at 15,500 × *g*.

(g) Use a pipette to remove all but 100 μL of the wash solution from the tube. Withdraw the supernatant from the surface and on the opposite side from the pellet.

6. Obtain an 8-well strip from the Ion OneTouch™ ES Supplies. Ensure that the square-shaped tab of an 8-well strip is on the *left* which corresponds to well 1.
7. Pipet the ISPs up and down ten times to mix, then transfer the entire volume (100 μL) of the suspension (**step 5** of this procedure) to Well 1 of the 8-well strip. Retain an aliquot of the unenriched Ion PGM Template OT2 400 Ion Sphere Particles from Well 1 for quality assessment (Check the USER GUIDE One touch 2 Instrument).(*see* **Note 10**) for handling barcoded adapters/templates.

3.14.1 Prepare Reagents to Fill the 8-Well Strip

1. Prepare Melt-Off Solution by combining the components in the following order (*see* **Note 11**):

Order	Component	Volume (μL)
1	Tween solution	280
2	1 M NaOH	40
	Total	320

2. Add melt-off solution to No. 7 well of the 8-well strip.
3. Vortex the tube containing the Dynabeads® MyOne™ Streptavidin C1 Beads for 30 s to thoroughly resuspend the beads, then centrifuge the tube for 2 s.
4. Open the tube, then use a new tip to pipet up and down the dark pellet of beads until the pellet disperses. *Immediately* proceed to the next step.
5. Transfer 13 μL of Dynabeads® MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind® Tube.
6. Place the tube on a magnet such as a DynaMag™-2 magnet for 2 min, then *carefully* remove and discard the supernatant without disturbing the pellet of Dynabeads® MyOne™ Streptavidin C1 Beads.

7. Add 130 μL of MyOne™ Beads Wash Solution (green cap) to the Dynabeads® MyOne™ Streptavidin C1 Beads.
8. Add the resuspended Dynabeads® MyOne™ Streptavidin C1 Beads in the 130 μL MyOne™ Beads Wash Solution to Well 2 of the 8-well strip.
9. Remove the tube from the magnet, vortex the tube for 30 s, and centrifuge.
10. Fill wells 3, 4, and 5 with 300 μL Ion One touch wash solutions, kit 400.
11. Also add 10 μL (Red Cap) neutralizing solution into a new PCR tube.
12. Insert the opened 0.2-mL PCR tube with the neutralization solution into the hole at the base of the Tip Loader, Confirm that a new tip and opened 0.2-mL PCR tube with the neutralization solution have been loaded and that the 8-well strip is correctly loaded.
13. Ensure that Well 1 (ISP sample) is the left-most well and that the 8-well strip is pushed to the far-right position within the slot 2.
14. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.
15. Press Start/Stop. The screen displays "run" during the run. The run takes ~45 min. (If necessary to stop a run, press Start/Stop). The instrument completes the current step, then stops the run and displays "End." Press Start/Stop again to return the Tip Arm to the home position. It is not possible to restart (where you left off) after stopping a run.
16. At the end of the run, the instrument displays "End" and beeps every 60 s. Press the Start/Stop button to silence this alarm and reset the Ion OneTouch™ ES for the next run. The instrument can be left on between runs.
17. Immediately after the run, securely close and remove the PCR tube containing the enriched ISPs.
18. Mix the contents of the PCR tube by gently inverting the tube five times. Store it at 4 °C till further use or till sequencer is getting ready (*see* **Note 12**).
19. Remove the used tip: While you are standing above the Tip Arm, and with the Tip Arm in its cradle, twist the tip *counterclockwise* and pull it downward to remove and discard the tip.

3.15 Sequencing Machine Steps

[*Protocol adapted from Ion PGM™ Sequencing 400 Kit User Guide, For use with the Ion Personal Genome Machine (PGM™) System and the Ion 314™ Chip v2, Ion 316™ Chip v2, and Ion 318™ Chip v2, from Ion Torrent by Life Technologies*], [14].

3.15.1 Prepare Wash 1, 2 and 3 Solutions

For the following steps, label the Wash 1 and Wash 3 Bottles to avoid confusion.

Cleaning Steps: Turn the sequencing machine ON (front orange button), open N_2 gas tank, then follow machine prompts. Press "Clean" and add 250 mL chloride solution (prepared by adding one pouch of chloride into 1000 mL Milli-Q (MQ) water, then filter-sterilizing it) in the Chloride bottle. The Chloride bottle goes in W1 position, facing the user. Follow machine prompts. After cleaning is complete, replace Chloride bottle with 250 mL MQ water bottle. Clean again using machine prompts. Cleaning steps are completed using old chip.

Replace old chip with the new one. (We used Chip 318 v2.).

Follow steps below to prepare wash solutions (*see* **Note 13**):

1. Rinse Wash 1 and Wash 3 Bottles three times with 50 mL of 18 MQ water and Wash 2 Bottle (2 L) three times with 200 mL of 18 MQ water.
2. *Wash 1 Bottle*: Add 350 μL of freshly prepared 100 mM NaOH to the Wash 1 Bottle and cap the bottle.
3. *Wash 3 Bottle*: Add Ion PGM™ Sequencing 400 1× W3 Solution (stocks stored at 4 °C) to the 50-mL line marked on the Wash 3 Bottle and cap the bottle.
4. *Wash 2 Bottle*: Fill the bottle to the mold line with MQ water. Volume of water will be ~2 L. Flush nitrogen to remove the air in wash 2 bottle.
5. *Add entire* contents of bottle containing Ion PGM™ Sequencing 400 W2 Solution to the Wash 2 Bottle. (Retain the Ion PGM™ Sequencing 400 W2 Solution bottle to scan the barcode during the initialization procedure below).
6. Add 130 μL 100 mM NaOH to the wash 2 bottle and mix it gently.
7. Cap the bottle and invert five times to mix, and immediately proceed through the rest of the initialization procedure.

3.15.2 Begin Initialization

8. Confirm that the chip (318 v2) is in place in the Ion PGM™ System.
9. On the Main Menu, press Initialize (*see* **Note 14**).
10. In the next screen, scan or enter the barcode on the Ion PGM™ Sequencing 400 W2 Solution bottle (from **step 5** above). Alternatively, select Ion PGM™ Sequencing 400 Kit from the dropdown list.
11. Press "Next" and confirm that new chip is on the instrument and the Reagent Bottle sipper tubes and collection trays are in place. Press "Next" again.

12. The system will verify gas pressure. If gas pressure is sufficient, press "Next" to begin the initialization. If gas pressure is low, press "Yes" to retry gas-pressure verification. If the gas pressure remains low, contact Technical Support.
13. Wearing clean gloves, firmly attach a new sipper tube (long gray end) to the cap in the W2 position. Immediately attach the prepared Wash 2 Bottle in the W2 position (slot 2) and tighten the cap. Press Next.
14. Change gloves and firmly install new sipper tubes (short gray end) in the caps in the W1 and W3 positions.
15. Immediately attach the prepared Wash 1 (slot 1 towards user) and 3 (extreme right, slot 3 on the machine) Bottles and tighten the caps. Press Next.
16. The Ion PGM™ System will test the bottles for leaks, fill Wash 1 Bottle, and then adjust the pH of W2 Solution. This procedure takes ~30 min (*see* **Note 15**).

3.15.3 Prepare 50-mL Reagent Bottles (Part of the Kit) with dNTP Solutions

17. After each dNTP stock solution has thawed, vortex to mix and centrifuge to collect the contents. Keep dNTP stock solutions on ice throughout this procedure.
18. Use the labels provided with the kit to label four new Reagent Bottles (50 mL falcon tubes) as dGTP, dCTP, dATP, and dTTP.
19. Using filtered pipette tips and clean gloves, carefully transfer 20 μL of each dNTP stock solution into its respective Reagent Bottle.

3.15.4 Attach the Sipper Tubes and Reagent Bottles (Comes with the Kit)

20. After the wash solutions have initialized, follow the touchscreen prompts to remove the used sipper tubes and collection trays from the dNTP ports in front of the machine. Change gloves.
21. Using new gloves, firmly insert a new sipper tube (blue end) into each dNTP port. Do not let the sipper touch any surfaces (*see* **Note 16**).
22. Attach each Reagent Bottle to the correct dNTP port and tighten firmly until snug. The correct order of the Reagent Bottles on the Ion PGM™ System is dGTP, dCTP, dATP, and dTTP (left to right when facing the instrument) (*see* **Note 17**).
23. Follow the touchscreen prompts to complete initialization. The Ion PGM™ System will fill each Reagent Bottle with 40 mL of W2 Solution.

    You can create Planned Runs and/or prepare the ISPs while the Ion PGM™ System is initializing. See the following sections.

24. At the end of initialization, the Ion PGM™ System will measure the pH of the reagents:
    - If every reagent is in the target pH range, a green Passed screen will be displayed.
    - If a red failure screen appears, see Appendix B. Troubleshooting, USER GUIDE [14].
25. Press Next to finish the initialization process and return to the Main Menu.
26. Proceed to the appropriate sequencing protocol for your chip type.

3.15.5 Sequencing Protocol: Ion 318™ Chip v2

Before Starting

- Thaw the Sequencing Primer and sequencing polymerase on ice.
- Make sure that the Torrent Suite™ and Ion PGM™ System software is updated (Version 3.6 or later).

Prepare Enriched, Template-Positive ISPs

1. Vortex the Control Ion Sphere™ Particles for 1 min and centrifuge for 2 s before taking aliquots.
2. Add 5 μL of Control Ion Sphere™ Particles directly to the entire volume of enriched, template-positive ISPs (**step 18** from subheading 3.14.1, above) in a 0.2 mL non-polystyrene PCR tube.
3. Proceed to Anneal the Sequencing Primer.

Anneal the Sequencing Primer

1. Mix the contents of the tube by thoroughly pipetting up and down. Centrifuge for 2 min at 15,500×*g*.
2. Carefully remove the supernatant without disturbing the pellet, leaving 5 Ion PGM™ Sequencing 400 in the tube (visually compare to 5 μL of liquid in a separate tube) (*see* **Note 18**).
3. Add Annealing Buffer 10 μL.
4. Add 12 μL of the Sequencing Primer (white cap) and confirm that the total volume is 27 μL.
5. Pipet the sample up and down thoroughly to disrupt the pellet.
6. Program a thermal cycler for 95 °C for 2 min and then 37 °C for 2 min, using the heated lid option.
7. Place the tube in the thermal cycler and run the program. After cycling, the reaction can remain in the cycler at room temperature.

Bind Sequencing Polymerase to the ISPs

1. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler and add 3 μL of Ion PGM™ Sequencing 400 Polymerase to the ISPs, for a total final volume of 30 μL.

2. Pipet the sample up and down to mix, and incubate at room temperature for 5 min and it is ready to load on the chip.

Chip Check and Running Samples

Chip Check tests the chip and ensures that it is functioning properly prior to loading the sample.

1. Add the new chip to the machine after cleaning the machine with chloride and MQ water and before adding the wash buffers 1, 2 and 3 as described above (*see* **Notes 19** and **20**).
2. Machine will check the chip and test for any errors.
3. Remove chip from the machine, empty the solution from the chip ports (injection/loading and elution) using pipet and remove all the bubbles.
4. Pass nitrogen for 30 s through the chip ports to dry it completely.
5. Add fresh isopropanol (100 μL of 100 % isopropanol) to the chip from injection port. Leave it for few seconds.
6. Set pipet to 32 μL and empty all isopropanol from the chip to dry it completely.
7. Dry again using nitrogen for 30 s.
8. Load the sample [sample from above, **step 2** ("Bind Sequencing Polymerase to ISPs")] into the chip from the injection port or loading port.

   (For loading the above sample into the chip, set 100 μL pipet to 30 μL, then use TURN PIPET METHOD: insert the pipet tip with 30 μL sample all the way into the loading port on the chip, rotate the top of the pipet clockwise till everything is loaded into the chip, then take out the pipet slowly).
9. Spin the chip with the sample for 30 s with tab inside (sample will bind onto the chip).
10. After spinning remove all the unbound sample from the elution port of the chip to make it completely dry, make sure there are no air bubbles.
11. Spin the chip again for 30 s with tab out this time.
12. Put the chip on the Ion Torrent sequencing machine, hit plan run, then "Next." Machine will start to run.
13. At the end of the run after 8–10 h, the sequencing machine will generate a summary report for the run (Fig. 2).
14. Save the data and analyze it. Also clean the machine with chloride solution and with MQ water, follow the prompts on the computer.
15. At the end of the cleaning steps, close nitrogen gas tank and shut down the Ion Torrent sequencing platform.

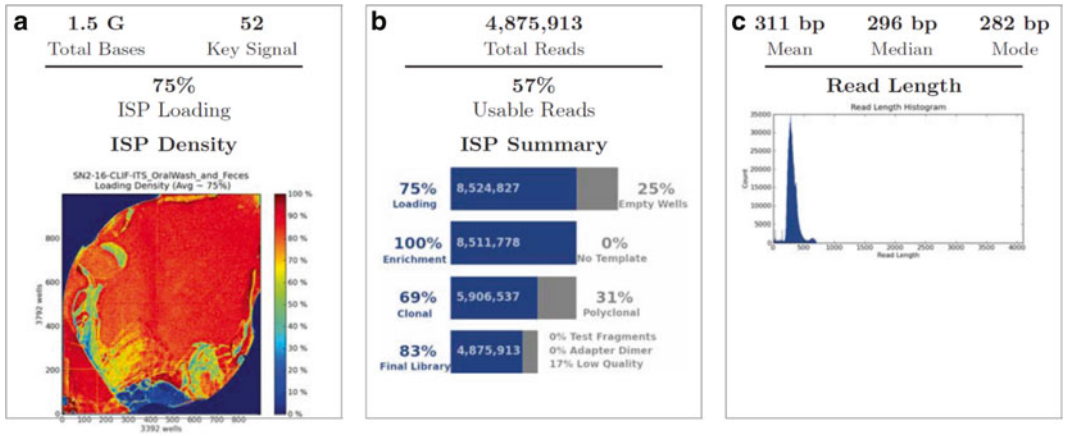


Fig. 2 Representative summary report generated after a typical run through the Ion Torrent platform. (**a**) Image showing the loading density of Ion Sphere particles, ISPs, (**b**) Number of reads detected on ISP (**c**) Length of reads generated during the sequencing run

4 Notes

1. Do not reverse the electrodes during electrophoresis. DNA is negatively charged molecule and will migrate towards the positive red electrode.
2. For electrophoresis of PCR product there is no need to add loading dye if master mix already contains the dye.
3. Use freshly prepared 70 % ethanol for all purification/washing steps. A higher percentage of ethanol could cause inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.
4. After each purification/washing step, ensure that the pellet does not dry out completely. It is hard to resuspend the dried pellet.
5. For amplicons to be fragmented using the Ion Shear™ Plus reagents, it is important to elute the amplicon DNA in nuclease-free water and not in EDTA. EDTA can significantly interfere with the Ion Shear™ reaction.
6. After washing the amplicon, do not discard the supernatant as it contains the purified amplicon.
7. The ion shear reaction is very sensitive to sample integrity and operator handling method. Care should be taken while handling it.
8. Start the run on the Ion One Touch™ 2 Instrument within 15 min after preparing the amplification solution.

9. Wait until rotation stops for template formation and spinning, then open the lid. Rotating parts can cause injury.
10. When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.
11. Prepare Melt-Off Solution as needed for filling 8-well strip, but appropriately dispose off the solution after 1 day. The final composition of the Melt-Off Solution is 125 mM NaOH and 0.1 %. (*Ion PGM™ Template OT2 400 Kit User Guide* Chapter 4 Enrich the Template-Positive Ion PGM™ Template OT2 400 Ion Sphere™ Particles).
12. For enriching ISPs step, ensure that the 0.2 mL PCR tubes have >200 of solution containing the enriched ISPs. After a successful run on the instrument, the sample is in ~230 μL of Melt-Off Solution, Ion One Touch™ Wash Solution, and Neutralization Solution. If a tube has <200 μL of solution containing the enriched ISPs, contact Life Technologies Technical support.
13. For sequencing steps prepare wash 1, 2 and 3 buffers fresh and do not store these solutions as it may cause oxidation of the solutions. Also load the bottles as quickly as possible to prevent atmospheric CO_2 from reducing the pH of the Wash 2 Bottle solution.
14. If a wash buffer leaks or if an error occurs during the automatic pH process, see Appendix B, Trouble shooting in the USER GUIDE [14]. If the error message indicates problems adjusting the pH of the prepared W2 Solution, see Initialization: Auto pH errors on page 51, User Guide [14], in the Trouble shooting section.
15. The Ion PGM™ System checks the pressure of the Reagent Bottles and Wash Bottles. If a bottle leaks, you are prompted to check that it is tightly attached to the instrument. If it continues to leak, it should be replaced. If the instrument still does not pass the leak check, contact Technical Support.
16. Precautions should be taken while changing new sippers. Change gloves frequently and do not let new sipper tubes touch any surface. Be careful to firmly push each sipper onto the port. Loosely attached sippers may adversely affect results. Do not put sippers in the dNTPs ports unless prompted for that.
17. Handle nucleotides with care so that there is no cross-contamination, change gloves frequently and make sure that the right dNTP is installed in the right port.
18. For each initialization, run should be started within 1 h after initialization. The ISPs are difficult to see. To avoid aspirating

the particles in the following steps, orient the PCR tube the same way each time when centrifuging so that it is easy to know where the pellet has formed, and remove the supernatant from the top down.

19. To avoid damage due to electrostatic discharge (ESD), do not place the chip directly on the bench or any other surface. Always place the chip either on the grounding plate on the Ion PGM™ System or in the custom Ion centrifuge adapter/rotor bucket.
20. To avoid ESD damage, do not wear gloves when transferring chips on and off the instrument.

Acknowledgement

Funding support is acknowledged from the NIH/NIDCR [RO1DE17846] and the Oral HIV AIDS Research Alliance (BRS-ACURE-S-11-000049-110229 and AI-U01-68636) to MAG; NIH/NIDCR [R01DE024228] to MAG and PKM, NIH/NEI and NIH/NIAID (R21EY021303 and R21AI074077), pilot funding from the Infectious Diseases Drug Development Center (IDDDC, Case), the National Eczema Association (Research Grant), and the National Psoriasis Foundation (Lozick Discovery Research Grant) to PKM, and the CWRU/UH Center for AIDS Research (CFAR, NIH grant number P30 AI036219).

References

1. Li Q, Pan C, Teng D, Lin L, Kou Y, Haase EM, Scannapieco FA, Pan Y (2014) Porphyromonas gingivalis modulates Pseudomonas aeruginosa-induced apoptosis of respiratory epithelial cells through the STAT3 signaling pathway. Microbes Infect 16(1):17–27
2. Dietz A, Wichmann G (2011) Head and neck cancer: effective prevention in youth and predictive diagnostics for personalized treatment strategies according to biological differences. EPMA J 2(2):241–249
3. Mason MR, Preshaw PM, Nagaraja HN, Dabdoub SM, Rahman A, Kumar PS (2015) The subgingival microbiome of clinically healthy current and never smokers. ISME J 9:268–272
4. Zaura E, Nicu EA, Krom BP, Keijser BJ (2014) Acquiring and maintaining a normal oral microbiome: current perspective. Front Cell Infect Microbiol 4:85. doi:10.3389/fcimb.2014.00085, eCollection 2014
5. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, Gillevet PM (2010) Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog 6(1):e1000713
6. Dupuy AK, David MS, Li L, Heider TN, Peterson JD, Montano EA, Dongari-Bagtzoglou A, Diaz PI, Strausbaugh LD (2014) Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. PLoS One 9(3):e90899. doi:10.1371/journal.pone.0090899, eCollection 2014
7. Mukherjee PK, Chandra J, Retuerto M, Sikaroodi M, Brown RE, Jurevic R, Salata RA, Lederman MM, Gillevet PM, Ghannoum MA (2014) Oral mycobiome analysis of HIV-infected patients: identification of *Pichia* as an antagonist of opportunistic fungi. PLoS Pathog 10(3):e1003996
8. Morgan XC, Huttenhower C (2012) Chapter 12: Human microbiome analysis. PLoS Comput Biol 8(12):e1002808
9. McMurdie PJ, Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis

and graphics of microbiome census data. PLoS One 8(4):e61217

10. Drell T, Lillsaar T, Tummeleht L, Simm J, Aaspõllu A, Väin E, Saarma I, Salumets A, Donders GG, Metsis M (2013) Characterization of the vaginal micro- and mycobiome in asymptomatic reproductive-age Estonian women. PLoS One 8(1):e54379
11. USER BULLETIN, Preparing Long Amplicon Libraries (>400 bp) using the Ion Xpress Plus Fragment Library Kit, from Ion Torrent by Life Technologies, Publication Part Number MAN0007044, Revision 3.0, user bulletin as provided by the manufacturer
12. Ion Library Quantitation Kit USER GUIDE, TaqMan assay quantitation of Ion Torrent Libraries, from Ion Torrent by Life Technologies, Catalog Number 4468802, Publication Part number 4468986, Revision E, user guide as provided by the manufacturer
13. Ion PGM™ Template OT2400 kit USER GUIDE, for use with: the Ion One Touch™ 2 system, from Ion Torrent by Life Technologies, Catalog Number 4479878, Publication Part Number MAN0007218, Revion 3.0, user guide as provided by the manufacturer
14. Ion PGM™ Sequencing 400 Kit User Guide, For use with the Ion Personal Genome Machine Delete R (PGM™) System and the Ion 314™ Chip v2, Ion 316™ Chip v2, and Ion 318™ Chip v2, from Ion Torrent by Life Technologies, Catalog Number 4482002, Publication Part Number MAN0007242, Revision 2.0, user guide as provided by the manufacturer

Chapter 10

Experimental Models of *C. albicans*-Streptococcal Co-infection

Takanori Sobue, Patricia Diaz, Hongbin Xu, Martinna Bertolini, and Anna Dongari-Bagtzoglou

Abstract

Interactions of *C. albicans* with co-colonizing bacteria at mucosal sites can be synergistic or antagonistic in disease development, depending on the bacterial species and mucosal site. Mitis group streptococci and *C. albicans* colonize the oral mucosa of the majority of healthy individuals. These streptococci have been termed "accessory pathogens," defined by their ability to initiate multispecies biofilm assembly and promote the virulence of the mixed bacterial biofilm community in which they participate. To demonstrate whether interactions with Mitis group streptococci limit or promote the potential of *C. albicans* to become an opportunistic pathogen, in vitro and in vivo co-infection models are needed. Here, we describe two *C. albicans*-streptococcal co-infection models: an organotypic oral mucosal tissue model that incorporates salivary flow and a mouse model of oral co-infection that requires reduced levels of immunosuppression compared to single fungal infection.

Key words *Candida albicans*, *Streptococcus*, Organotypic culture, Flow cells, Oral infection, Mouse model

1 Introduction

Oropharyngeal candidiasis (OPC) is an opportunistic infection afflicting humans in a variety of immunosuppressed states, which may also predispose them to invasive infection [1]. Although *Candida albicans* is the primary etiologic pathogen in OPC, the microbial ecology of this infection is complex since it contains members of the endogenous mucosal bacterial flora [2–4]. Interactions of *C. albicans* with co-colonizing bacteria at mucosal sites can be synergistic or antagonistic in disease development, depending on the bacterial species and mucosal site [5]. Synergistic interactions in biofilm development between *C. albicans* and oral streptococci have been demonstrated by our group and others in vitro [6–10]. More recently we examined the outcome of these interactions in a mouse oral co-infection model with *C. albicans*

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_10,

and *S. oralis* [11]. In this model *S. oralis* promoted the virulence of *C. albicans* in the oral mucosa by enhancing the inflammatory response to infection, without significantly affecting fungal burdens.

Here we describe our in vitro mucosal flow cell system and mouse oral co-infection models which can be used to further characterize the role of the interaction between *C. albicans* and oral streptococci in the pathogenesis of mucosal infection.

2 Materials

2.1 In Vitro Salivary Flow Three-Dimensional Mucosal Model

2.1.1 Plates/Inserts

1. Plates: six-well Transwell Carrier (Organogenesis, TS01-001, Canton, MA).
2. Corning Costar Transwell 3414 (24 mm diameter inserts, 3.0 μm pore size, Tewksbury, MA).

2.1.2 Fibroblast Line

1. 3T3 cells (ATCC).

2.1.3 Fibroblast Culture Media: DMEM-10 % FBS-1 % P/S

1. DMEM: Dulbecco's Modified Eagle Medium (DMEM) (1×) liquid (high glucose) without pyruvate: DMEM (−) (Gibco #11965-084).
2. FBS: fetal bovine serum (HyClone).

2.1.4 Epithelial Cells

OKF6/TERT2 cells (floor of the mouth epithelium, obtained from J. Rheinwald, Harvard University) (*see* **Note 1**).

2.1.5 Epithelial Cell Culture Media

KSFM (Invitrogen, #17005-042) with Bovine Pituitary Extract (BPE) (1 tube/each bottle), hEGF (0.2 ng/ml), $CaCl_2$ (0.4 mM), and Pen/Strep (1:100).

2.1.6 Matrix and Cell Culture Media Components

1. DMEM (−) (Gibco #11965-084).
2. Ham's F12 (Gibco #11765-054).
3. 10× EMEM (BioWhittaker #12-684F).
4. 7.5 % Na-Bicarbonate (BioWhittaker #17-613E).
5. FBS (HyClone).
6. L-Glutamine (Cellgro #25-005CI).
7. Type I Collagen (Organogenesis #200/50).
8. Matrigel (BD Biosciences #354234).
9. Hydrocortisone (Sigma #H0888): MW = 362.46.
   - Dissolve 0.0269 g in 2.5 ml EtOH.
   - Add into 97.5 ml DMEM (−): 0.74 mM.
   - Filter-sterilize, dispense into aliquots, and store at −20 °C.

10. ITES—Insulin, Transferrin, Ethanolamine, and Selenium (BioWhittaker #17839Z).
11. *O*-phosphorylethanolamine (Sigma #P0503).
    - Dissolve 0.705 g in 100 ml DMEM (−): 50 mM.
    - Filter-sterilize, dispense into aliquots, and store at −20 °C.
12. Adenine (Sigma #A9795).
    - Dissolve 1.55 g in 100 ml warm (37 °C) ddH_2O: 0.09 M.
    - Filter-sterilize, dispense into aliquots, and store at −20 °C.
13. Progesterone (Sigma #P8783).
    - Dissolve 1 mg in 1 ml ETOH.
    - Add 14.7 ml ddH_2O.
    - Dilute 1 ml of that in 100 ml DMEM (−): 2.0 μM.
    - Filter-sterilize, dispense into aliquots, and store at −20 °C.
14. Triiodothyronine (Sigma #T5516).
    - Dissolve 1 mg in 1 ml 1N NaOH.
    - Add 19 ml of DMEM (−).
    - Dilute 4 μl of that in 31 ml plain DMEM (−): 1 nM.
    - Filter-sterilize, dispense into aliquots, and store at −20 °C.
15. Newborn Calf Serum (HyClone #SH 3011802).
16. Gentamicin, 50 mg/ml (Cellgro #MT30-0005-CR).
17. Saliva, collected from systemically healthy volunteers (IRB approval is required). Whole stimulated saliva is collected in polypropylene tubes on ice, pooled, and treated with 2.5 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) for 10 min to reduce salivary protein aggregation. The saliva is then centrifuged at 7500 × *g*, at 4 °C, for 20 min, and supernatants are diluted with Dulbecco's phosphate-buffered saline (D-PBS) to obtain a 25 % (vol/vol) saliva/D-PBS solution. Diluted saliva is then filtered through a 0.22-μm-pore-size polyethersulfone low-protein-binding filter (Nalgene; Thermo Fisher Scientific, Rochester, NY), divided into aliquots, and frozen at −80 °C until further use in culture media.

2.2 In Vivo Oral Infection Mouse Model

Mice: 6–8 weeks old female C57BL/6 mice are purchased from the Jackson Laboratory (Maine, US). Animals are maintained in BSL-2 biocontainment during the infection process.

2.3 Microorganisms Used and Microbiological Media

Organisms that can form mixed mucosal biofilms in both in vitro and in vivo models are *Candida albicans* SC5314 [12], *Streptococcusoralis* 34 (kindly provided by P. E. Kolenbrander), and *Streptococcus gordonii* Challis CH1 (kindly provided by J. M. Tanzer) [8, 11]. *Streptococcus sanguinis* SK36 (ATCC BAA-1455)

can also form a biofilm with *C. albicans* under flow in vitro [8]. *C. albicans* is routinely maintained in yeast extract-peptone-dextrose (YPD) agar and grown in YPD medium, aerobically, at room temperature, in a rotor shaker. YPD medium consists of 5 g of yeast extract (Fisher Scientific, Pittsburgh, PA) liter^{-1}, 10 g of peptone (Fisher Scientific) liter^{-1}, and 20 g dextrose (Fisher Scientific) liter^{-1}. Streptococci are routinely grown in brain heart infusion (BHI) medium (Oxoid, Ltd., Cambridge, UK) under static conditions at 37 °C, 5 % CO_2.

3 Methods

3.1 In Vitro Flow Three-Dimensional Mucosal Model

3.1.1 Preparation of Oral Mucosa Tissue Analogue [13–15]

Day 0

Thaw Matrigel overnight (one vial for 2.5 OTC plates) at 4 °C.

Day 1

15–30 min prior to matrix preparation:

Place FBS, L-Glutamine, 10× EMEM and 7.5 % Na-bicarbonate, Matrigel, and type I collagen on ice.

Place transwell inserts into each well in six-well Transwell Carrier plate.

3.1.2 Making Acellular Layer

1. Add 10× EMEM, FBS, L-Glutamine, Na-bicarbonate, and type I collagen in this order in 50 ml tube on ice (Table 1).
2. Mix gently using a 25 ml pipette in a 50 ml tube on ice (*see* **Note 2**).
3. Pour 1 ml per insert using a 10 ml pipette.
4. Leave inside the tissue culture hood while preparing fibroblasts and cellular layer (Table 1).

Table 1
Acellular layer media

Acellular layer	1 plate	2 plates
10× EMEM	690 µl (×1)	690 µl (×2)
FBS	700 µl (×1)	700 µl (×2)
L-Glutamine	60 µl (×1)	120 µl (×1)
Na-bicarbonate	140 µl (x 1)	280 µl (×1)
Type I collagen	5.6 ml (×1)	11.2 ml (×1)

3.1.3 Making Cellular Layer

1. Prepare 6×10^5/ml 3T3 cell suspension.
2. Add 10× EMEM, FBS, L-Glutamine, Na-bicarbonate, type I collagen, Matrigel, and fibroblasts in this order into a new 50 ml tube on ice (Table 2).
3. Mix gently using a 25 ml pipette in 50 ml tube on ice (*see* **Note 2**).
4. Pour 3 ml per insert using a 10 ml pipette.
5. Incubate for 30–45 min in the tissue culture incubator (37 °C, 5 % CO_2).
6. Add DMEM-10 % FBS-1 % P/S: 10 ml into the bottom of the wells, 2 ml into the insert.

Day 2—Dislodge matrix and add 2 ml of DMEM-10 % FBS-1 % P/S into the insert.

1. Use a sterile glass Pasteur pipette to go around (two to three times) the matrix along the inner wall of the insert. Feel the friction at the tip on the transwell membrane, but try not to pierce it (*see* **Note 3**).
2. No need to change medium. Wait at least 4 days before seeding epithelial cells.

Day 5 or later—Seeding epithelial cells.

1. Make DMEM (−)/F12 (Table 3) to pre-saturate the OTC matrix.
2. Remove old medium from the OTC plates/inserts.
3. Add DMEM+F-12. Add 10 ml to the bottom well and 2 ml into the insert.
4. Incubate for 1 h to equilibrate the matrix.
5. Prepare 1×10^7/ml epithelial cell suspension in the regular medium used to grow epithelial cells (e.g., KSFM for OKF6/TERT2) (*see* **Note 4**).

Table 2
Cellular layer media

Cellular layer	1 plate	2 plates
10× EMEM	1.8 ml (×1)	3.6 ml (×1)
FBS	2 ml (×1)	4 ml (×1)
L-Glutamine	160 µl (×1)	320 µl (×1)
Na-bicarbonate	380 µl (×1)	760 µl (×1)
Type I collagen	11.4 ml (×1)	11.4 ml (×2)
Matrigel	3.8 ml (×1)	7.6 ml (×1)
6×10^5/ml fibroblasts*	1.6 ml (×1)	3.2 ml (×1)

Table 3
DMEM/F12 media

DMEM/F12 media (3:1)	2 plates (144 ml+)	3 plates (216 ml+)
DMEM(−)	120 ml	180 ml
Ham's F12	40 ml	60 ml

Table 4
EP2 media

EP2	2 plates (300 ml)	3 plates (450 ml)
DMEM (−)	218 ml	327 ml
F12	72 ml	108 ml
L-Glutamine (LQ)	6 ml	9 ml
Hydrocortisone (H)	600 µl	900 µl
ITES	600 µl	900 µl
O-phosphorylethanolamine (O)	600 µl	900 µl
Adenine (A)	600 µl	900 µl
Progesterone (P)	600 µl	900 µl
Triiodothyronine (T)	600 µl	900 µl
Newborn calf serum (NBCS)	300 µl	450 µl
Gentamycin	300 µl	450 µl

6. Remove DMEM+F-12.
7. Seed epithelial cells by adding 50 µl (5×10^5) of cell suspension per well onto the center of the surface of contracted matrix.
8. Incubate for 2 h in the tissue culture incubator without medium.
9. Make EP2 (Table 4). Store at 4 °C.
10. Add EP2 to plate—10 ml into the bottom well and 2 ml into the insert.

Day 7 (*or 2 days after seeding epithelial cells*)—Medium change with EP2.

Day 9 (*or 4 days after seeding epithelial cells, Air Lifting*)—Medium change with EP3.

1. Make EP3 (Table 5). Store at 4 °C.
2. Remove old medium from both inserts and the bottom well.
3. Add 7.5 ml of EP3 into the bottom wells only.

Table 5
EP3 media

EP3	2 plates (200 ml)	3 plates (300 ml)
DMEM (–)	95 ml	142.5 ml
F12	95 ml	142.5 ml
L-Glutamine (LQ)	4 ml	6 ml
Hydrocortisone (H)	400 µl	600 µl
ITES	400 µl	600 µl
O-phosphorylethanolamine (O)	400 µl	600 µl
Adenine (A)	400 µl	600 µl
Triiodothyronine (T)	400 µl	600 µl
Newborn calf serum (NBCS)	4 ml	6 ml
Gentamycin	200 µl	300 µl

Day 11 (*or 6 days after seeding epithelial cells*)—Medium change with EP3, prepared on day 9.

1. Remove old medium from both inserts and the bottom well (*see* **Note 5**).
2. Add 7.5 ml of EP3 into the bottom wells only.

Day 13 (*or 8 days after seeding epithelial cells*)—Medium change with EP4 (infection medium).

1. Make EP4 (Table 6). Store at 4 °C. This will be used for the rest of the experiment as well. Remove old medium from both inserts and the bottom well.
2. Transfer inserts to six-wells dish (Corning 3516) with tweezers.
3. Add 1.5 ml of EP4 into both inserts and the bottom well.

Day 14 (*or 9 days after seeding epithelial cells*)—Infection.

Three-dimensional mucosal organotypic culture is now ready to use in flow cell assembly and infection process.

3.1.4 Fabrication and Assembly of Tissue Harboring Flow Cells (Day 13)

In order to grow mucosal biofilms under an environment that resembles the upper GI tract, a flow cell system was designed to harbor the mucosal tissue analogue, pre-grown in a porous membrane, with saliva flowing over the apical epithelial surface and cell culture medium flowing basally under the tissue [8]. The flow cell apparatus was custom-built by Sirois Tool Company (Berlin, CT) and consists of two discrete pieces that can be assembled to form two flow chambers separated by a membrane. The body of the upper and lower flow cell components was milled from a block of

Table 6
EP4 media

EP4 (infection medium)	3 plates (196 ml)
DMEM (−)	95 ml
F12	95 ml
L-Glutamine (LQ)	4 ml
Hydrocortisone (H)	400 µl
ITES	400 µl
O-phosphorylethanolamine (O)	400 µl
Adenine (A)	400 µl
Triiodothyronine (T)	400 µl

polytetrafluoroethylene (MSC Industrial Direct, Inc.). The connectors used between the components and the tubing were 0.0625-in. hose barb connectors with a #10–32 threaded port made of Kynar polyvinylidene fluoride (Small Parts, Inc.). The flow cell apparatus is assembled as illustrated in Fig. 1 by connecting two medium reservoirs to the upper and lower components of the flow cell via silicone manifold tubing (Watson-Marlow, Inc., Wilmington, MA). For convenient visualization, a window was built into the upper flow cell chamber and is sealed by attaching a 22-by-22-mm glass coverslip to the outside flow cell surface. The flow cell is assembled with an O-ring placed between the membrane and the upper component in order to seal the system. The upper and lower components are held in place by screws. One medium reservoir is filled with saliva-supplemented medium, which flows through the upper chamber. The second medium reservoir is filled with a 3:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, and EPES buffer to a final concentration of 15 mM. This medium flows through the lower chamber. A peristaltic pump model 205S/CA (Watson-Marlow Inc., Wilmington, MA) is connected downstream of the flow cells to establish flow (Fig. 1).

Day 13

1. Assemble flow cells.
2. Glue glass slides to flow cells with silicone.
3. Autoclave medium reservoirs with appropriate tubing, grease, twisters, distilled water, and beakers.
4. Inoculate bacteria or yeast for overnight growth in BIH or YPD broth, respectively.
5. Place mucosal tissues to be used in infection medium and incubate overnight at 37 °C with 5 % CO_2 (Fig. 1).

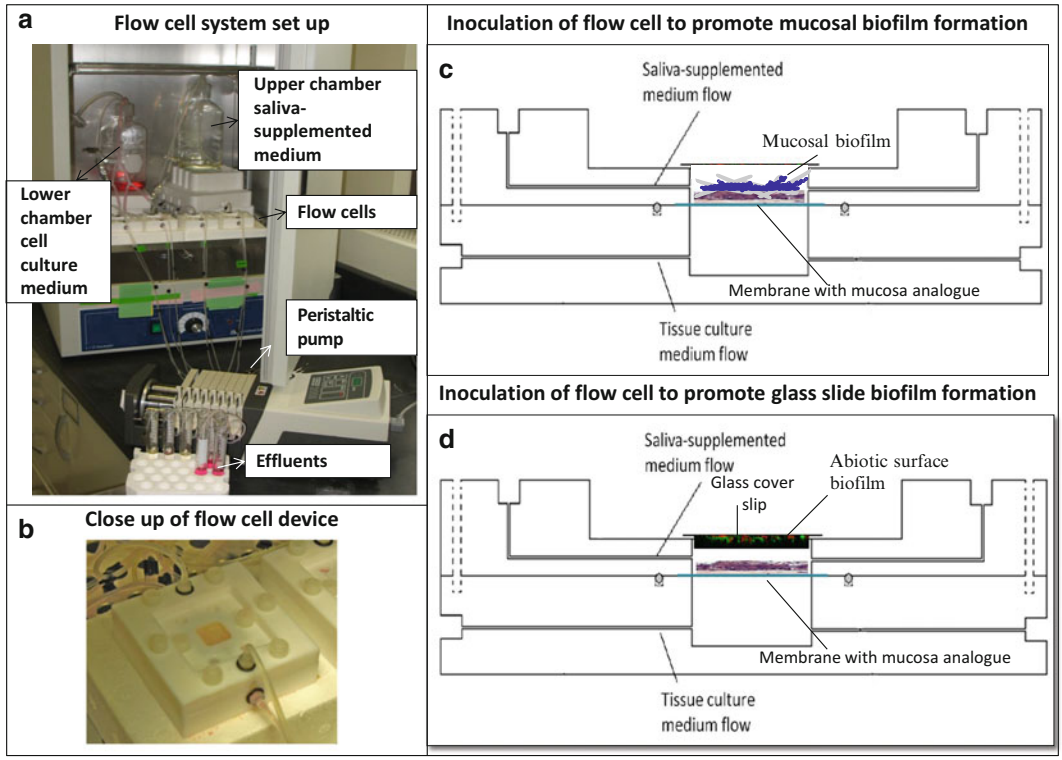


Fig. 1 Mucosal flow cell system. Panels (**a**) and (**b**) depict the flow cell apparatus and setup. Panel (**c**) is a schematic representation of the flow cell system with organisms inoculated on the mucosa to promote biofilm formation. In panel (**d**) the flow cell was inoculated on the glass coverslip to promote biofilm formation on an abiotic surface in close proximity to the mucosa

3.1.5 Mucosal Biofilm Growth in Tissue Harboring Flow Cells

Day 14

1. Clean flow cell with 0.1 M HCl and 10 % hypochlorite 1 and 2 h, respectively.
2. Cut membranes out of PET inserts with sterile twisters and blade. Place on a petri dish. Assemble flow cell with PET membrane in the middle. Make sure this is done under sterile conditions.
3. Check flow rate at this point, 4.5 rpm gives around 95 μl/min, which is around 6 ml per track.
4. Calculate volume of saliva/10 % BHI and DMEM: F12 (3:1) needed (*see* **Note 6**).
5. Condition flow cell surface with saliva/10 % BHI in upper track and DMEM: F12 (3:1) in the lower chamber for 15 min (*see* **Note 7**).
6. Cut out tissue-harboring inserts under the hood and connect to flow cell chambers.
7. Prepare microorganism inoculum with 10^5 yeast and 10^6 bacteria in 500 μl total volume. Inoculate microorganisms on

the mucosal surface or glass slide using a sterile syringe. Incubate under static conditions for 30 min. After 30 min adhesion period, start flow with 4.5 rpm.

8. Incubate flow cell overnight at 37 °C with 5 % CO_2 (*see* **Note 6**).
9. After overnight culture, stop flow and remove tissue samples from flow cell chambers. For histology, place samples in 4 % paraformaldehyde for fixation at least 2 h at 4 °C (*see* **Note 8**). After fixation, process for paraffin embedding.

3.1.6 In Vivo Murine Oral Co-infection Model

After arrival of mice at the animal facility, allow them to adapt to the new housing environment for 10 days (*see* **Note 9**).

Day 1—Animal setup and immunosuppression.

Organize animal cages by infection group, and set up individual mouse identification system by ear punch. Immunosuppress by subcutaneous injection with cortisone acetate (225 mg/kg) dissolved in 200 μl PBS containing 0.5 % Tween-80.

Days 1 to 4—Overnight culture of microorganisms.

Prepare fresh overnight cultures of *C. albicans* SC5314 in YPD broth (aerobically, at room temperature, on a rotor shaker) and *S. oralis* 34 or *S. gordonii* Challis CH1 culture in BHI medium (aerobic, static conditions, at 37 °C) for each day of infection (days 2–4).

Day 2—Oral infection under anesthesia.

Anesthetize mice with intraperitoneal (IP) injection of ketamine: xylazine (90–100 and 10 mg/kg of body weight, respectively). Prepare small cotton pads soaked with 100 μl of a *C. albicans* cell suspension (6×10^8 yeast/ml), or 100 μl of streptococcal cell suspension (2.5×10^9 bacteria/ml), or 50 μl of *C. albicans* cell suspension (1.2×10^9 yeast/ml) combined with 50 μl of streptococcal cell suspension (5×10^9 bacteria/ml). Place two cotton pads into oral cavity of anesthetized mice and swab the entire oral cavity (*see* **Note 10**). The cotton pads are left for 2 h under the tongue and are removed before the animals awake. Add fresh microorganism cultures daily in drinking water (*C. albicans* 6×10^8 yeast organisms/ml, or streptococci 2.5×10^9 bacteria/ml) or the combination of the two, to maintain high oral carriage loads throughout the experimental period [16].

Day 3—Repeat cortisone injection and oral infection through drinking water.

Day 4—Continue oral infection through drinking water.

Day 5—Sacrifice animals.

Sacrifice animals after CO_2 inhalation by cervical translocation and excise tongues. Immediately photograph dorsal surface digitally (1:1 lens) for Image J analysis of the surface area covered with lesions, as we previously described [16] (Fig. 2a). For histology cut ½ tongue and place in 4 % paraformaldehyde for fixation at least 2 h at 4 °C (*see* **Notes 8** and **11**). Process for paraffin embedding.

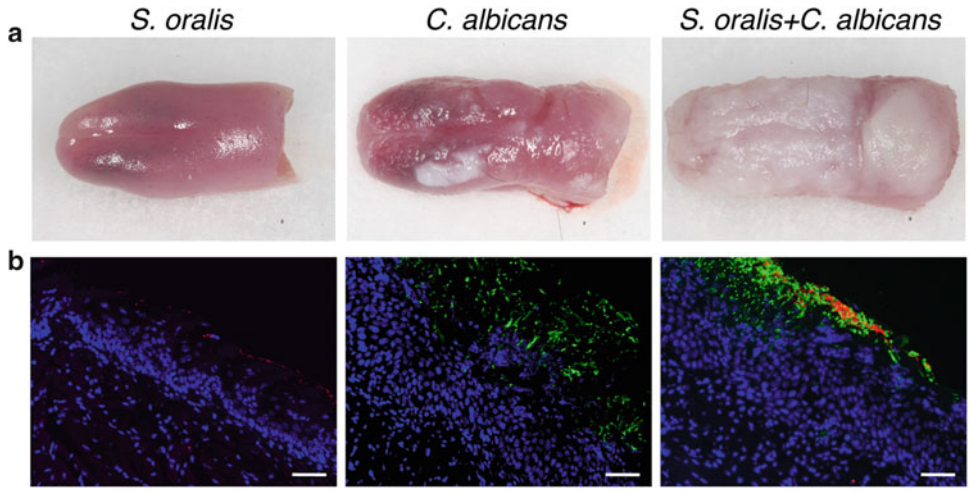


Fig. 2 Pathogenic synergy between *C. albicans* and *S. oralis* in a mouse oral co-infection model. (**a**) Tongues of mice were excised after 5 days of infection and the dorsal aspect was digitally photographed. Representative pictures are shown from one mouse in each group. Note the extensive biofilm lesion forming on the tongues of co-infected animals. (**b**) Overlay images of tongue tissue sections stained with a FITC-labeled anti-*Candida* antibody (*green*), followed by FISH with an Alexa 546-labeled *S. oralis*-specific probe (*red*), and counterstained with the nucleic acid stain Hoechst 33258 (*blue*). The FISH signal was completely absent in biofilms formed by *C. albicans* only, and the FITC signal was completely absent in animals infected with *S. oralis* only, showing staining specificity. Bars = 50 μm

3.2 Quantification of Fungi and Bacteria in Vitro and In Vivo

3.2.1 Molecular Method of Streptococcal Quantification in Murine Fecal Samples

1. Bacterial genomic DNA from mouse fecal samples collected daily is isolated and purified by QIAGEN DNA Stool mini kit according to the manual. DNA quantity and quality is assessed using the NanoDrop device (ND-1000 spectrophotometer, NanoDrop Technologies).
2. To calculate the *S. oralis 34* cell numbers in stools, *S. oralis 34* cultures are used to generate pure genomic DNA. To create a standard curve, pure gDNA is prepared for a tenfold serial dilution of known amounts of gDNA: 0.32 ng, 0.032 ng, 0.0032 ng, and 0.00032 ng. According to genomic size and mass of *S. oralis*, the corresponding genomic copy numbers of the single copy glucosyltransferase R (GtfR) gene, or cell numbers (for this single copy gene) are: 1.45×10^5, 1.45×10^4, 1.45×10^3, and 1.45×10^2, respectively.
3. Primers for the GtfR gene (gtf-F: TCCCGGTCAGCA ACTCCAGCC, gtf-R: GCAACCTTTGGATTTGCAAC) are used to target *S. oralis 34* DNA in the samples and the cell numbers of *S. oralis 34* are calculated according to the standard curve [15] (*see* **Note 12**).

4. Real-time PCR is performed with BIO-RAD CFX96 cycler real-time PCR detection system. All PCR reaction mixtures contain the following: 10 μl 2 × iQTM SYBR® Green Supermix (for cDNA) or Soso Advanced SYBR green Mix (DNA) (Bio-Rad, Hercules, CA), 1 μl of first-strand cDNA or DNA, 0.1 μM of primers and H_2O to bring the final volume to 20 μl (*see* **Note 13**).
5. The program for cDNA amplification is 95 °C incubation for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s.
6. The program for gDNA amplification is 98 °C incubation for 5 min, followed by 40 cycles of 98 °C for 10 s and 67 °C for 50 s.
7. Data are analyzed by the CFX96 cycler system software (Bio-Rad) (Fig. 3) [11].

3.2.2 Confocal Microscopy/ Immunohistochemistry: Immuno-FISH Staining for Mucosal Analogues and Tongue Tissue

1. After sectioning, deparaffinize sections by immersion in xylene and dehydration in a series of ethanol washes.
2. Stain with anti-*C. albicans*-FITC antibody (300 μl/slide, dilute 1:20 in PBS, Meridian B65411F). Incubate at room temperature for 2 h (*see* **Note 11**).
3. Wash three times with PBS (200–300 μl/slide).
4. Permeabilize bacterial cells with lysozyme (200–300 μl/slide, 70,000 U/ml in 20 mM Tris–HCl, 5 mM EDTA pH 7.5.

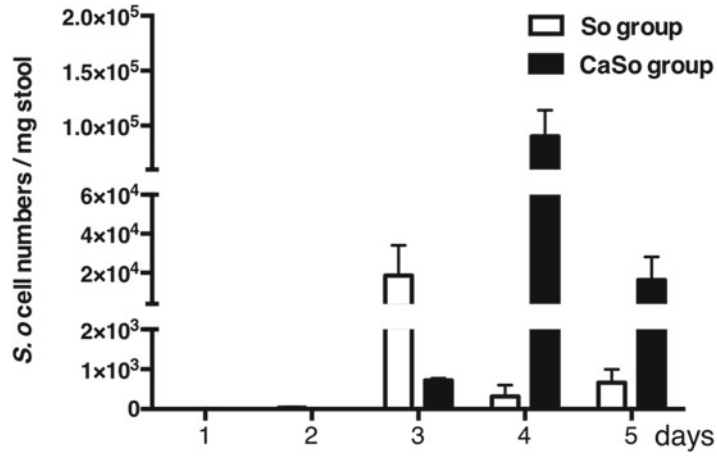


Fig. 3 Following infection stool samples were collected daily for five days, pooled from each group and analyzed by qPCR in triplicate. qPCR quantification of *S. oralis* in fecal samples. *S. oralis 34* genomic DNA in stool samples of mice infected *S. oralis* alone (So), or co-infected with *C. albicans* and *S. oralis* (CaSo) were analyzed by qPCR using primers specific for the *S. oralis-species-specific gtfR* gene and the cell numbers were calculated by a standard curve. $^{*}P < 0.05$. Linear regression software in Excel® is used to calculate cell numbers from standard curve

Lysozyme Sigma-Aldrich L6876) for 9 min at 37 °C in a humid atmosphere (*see* **Note 12**).

5. Rinse with PBS and dehydrate with a series of ethanol washes (50, 80, and 100 %) 2–3 min each wash.
6. Expose cells to 200 µl hybridization buffer, 10 ng/ml *Streptococcus*-specific oligonucleotide probe STR405, labeled with Alexa 546 in 0.9 M NaCl, 20 mM Tris–HCl (pH 7.5), 0.01 % SDS, 25 % formamide. Incubate slides at 46 °C for 90 min in a humid atmosphere (*see* **Note 13**).
7. Wash slides with pre-warmed washing buffer (20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.01 % SDS, and 159 mM NaCl) for 15 min at 46 °C.
8. Rinse with ice-cold water.
9. Counterstain with Hoechst 33258 (2 µg/ml in PBS, Invitrogen H3569) for 30 min at room temperature.
10. Rinse with water, air dry, and mount with coverslip. Observe under epifluorescence microscope (Fig. 2b, 4a, and 5a).

3.2.3 Biovolume Quantification Using Confocal Microscopy

1. After immuno-FISH staining as described above, confocal images of biofilms are analyzed for biovolume quantification using the IMARIS 7.0 software package (Bitplane AG).
2. 3D reconstructions of biofilm must be generated using the "Surpass" volume rendering option and a new "Surface" must be created for each channel used on confocal microscopy (Fig. 4) (*see* **Note 14**).

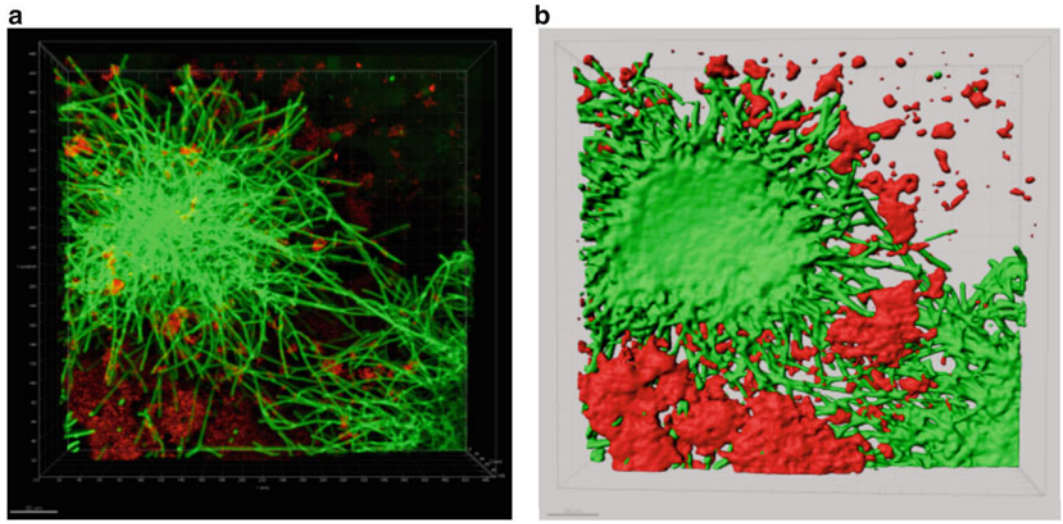


Fig. 4 Imaris® image visualizations. (**a**) Original confocal image after 3D reconstruction; (**b**) the software-created "Surface" (*green* for *C. albicans* and *red* for *S. oralis*) as displayed in the viewing area

3. To start the "Surface" creation, click on the Surface button (blue icon at the top left) and it will automatically appear at your object list.
4. Four steps are necessary to create a "Surface" for biovolume measurement: selection of a "Region of Interest," "Source Channel," "Threshold" adjustment, and "Surface Classification," when working with more than one channel/organism (*see* **Notes 15** and **16**). Once "Surface" creation is finished, it now has the same dimensions of the original confocal image, displayed as a "Solid Surface" (Fig. 4a, b).
5. At the Object properties area, several tabs are now available. Go to "Statistics" tab, select "Detailed"—"Specific Values"—"Volume" to measure the volumes of the created "Surfaces" and save as an excel file for further analysis (Fig. 5a, b).

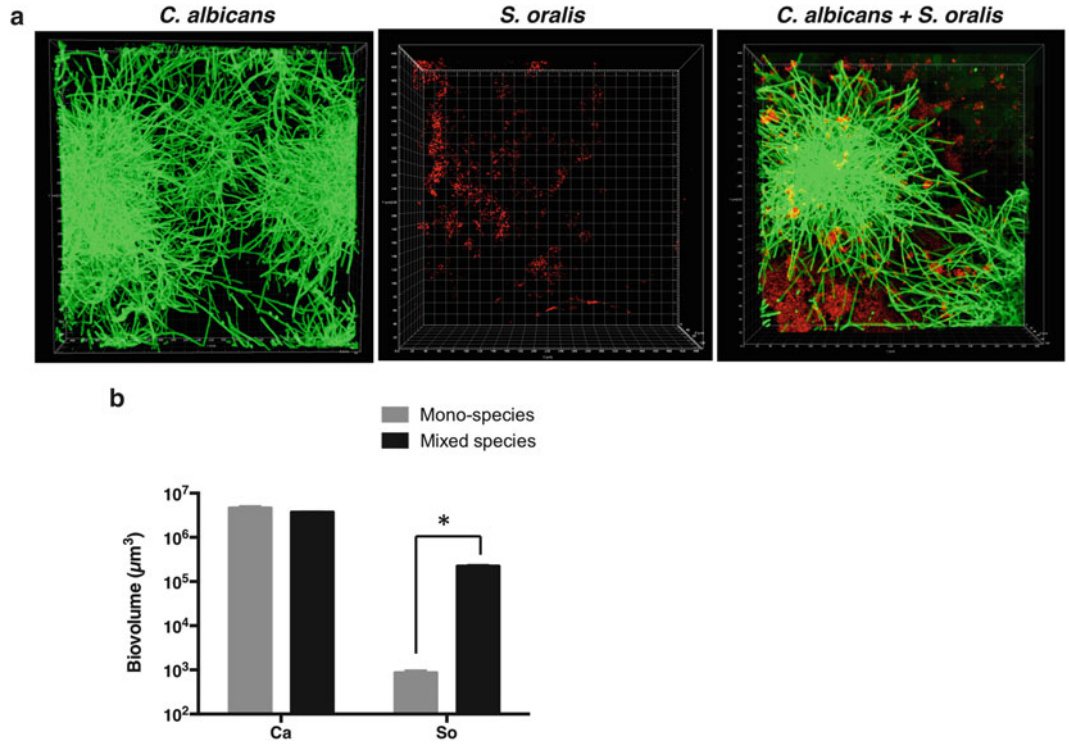


Fig. 5 *C. albicans* and *S. oralis* biofilms forming on an oral mucosa analogue. (**a**) Confocal microscopy images used for 3D reconstructions and biovolume estimates of monospecies and mixed-species mucosal biofilms of *C. albicans and S. oralis* after 16 h of biofilm growth. *C. albicans* was stained with a FITC-labeled anti-*Candida* antibody (*green*), and *S. oralis* was visualized with an Alexa 546-labeled Streptococcus-specific probe (*red*). (**b**) Biovolume quantifications from CLSM image stacks of *C. albicans* and *S. oralis* in monospecies or mixed-species mucosal biofilms. $^{*}P < 0.05$

4 Notes

1. Use freshly thawed vial of OKF6/TERT2 cells from Liquid N2. Subculture when cells are 70–80 % confluent. Subculture 1 week after starting culture.
2. When making acellular and cellular layers, mix components gently on ice to avoid bubbles.
3. The matrix will contract over next few days.
4. 5×10^5 epithelial cells are seeded per insert/well. One ~80 % confluent 75-cm^2 flask yields about $2–3 \times 10^6$ OKF6/TERT2 cells.
5. After air lifting, further significant contraction will be seen. Contraction can be better assessed during medium change.
6. Prepare extra media, to avoid drying the tissues during overnight culture after inoculation.
7. Check for any media leakage. If leakage is found, tighten all screws. It may be necessary to disassemble flow cell and add grease between upper and lower chambers.
8. Do not over-fix. It may mask certain antigens. 2–3 h is sufficient for fixation with 4 % paraformaldehyde.
9. If animals are bred in animal facility, the 10-day adjustment period is not necessary.
10. Some animals may awake during this period; it may be necessary to inject additional anesthetic solution to maintain cotton pads in the mouth.
11. Some animals may have visible thrush on their tongues at day 4. Tongues may be checked daily with ring forceps (Fine Science Tools 11101-09).
12. gtfR primers are specific for *S. oralis* species and target all *S. oralis* strains. If more specificity is needed, new primers need to be designed. For *S. oralis 34*, we designed strain-specific wefA-wefH primers to confirm the data with gtfR [11].
13. Both iQTM SYBR® Green Supermix and Soso Advanced SYBR green Mix can be used to amplify gDNA, but Soso Advanced SYBR green Mix works better.
14. Histology slides should be kept in the dark in plastic boxes with some water inside to avoid evaporation.
15. A new "Surface" must be created for *Candida* and *S. oralis* stains, separately, since they will be analyzed separately by selection of two different channels.
16. During "Surface" creation all parameters can be set interactively at the "Object Properties Area." "Threshold" adjustments will cover the entire confocal image.

Acknowledgements

This work was supported by NIH/NIDCR grant RO1 DE013986.

References

1. Villar CC, Dongari-Bagtzoglou A (2008) Immunodefence mechanisms and immunoenhancement strategies in oropharyngeal candidiasis. Expert Rev Mol Med 10, e29
2. Dongari-Bagtzoglou A, Kashleva H, Dwivedi P et al (2009) Characterization of mucosal *Candida albicans* biofilms. PLoS One 4, e7967
3. Nett JE, Marchillo K, Spiegel CA et al (2010) Development and validation of an *in vivo Candida albicans* biofilm denture model. Infect Immun 78:3650–3659
4. Nobile CJ, Fox EP, Nett JE et al (2012) A recently evolved transcriptional network controls biofilm development in *Candida albicans*. Cell 148:126–138
5. Morales DK, Hogan DA (2010) *Candida albicans* interactions with bacteria in the context of human health and disease. PLoS Pathog 6, e1000886
6. Bamford CV, d'Mello A, Nobbs AH et al (2009) *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. Infect Immun 77:3696–3704
7. Silverman RJ, Nobbs AH, Vickerman MM et al (2010) Interaction of *Candida albicans* cell wall Als3 protein with *Streptococcus gordonii* SspB adhesin promotes development of mixed-species communities. Infect Immun 78:4644–4652
8. Diaz PI, Xie Z, Sobue T et al (2012) Synergistic interaction between *Candida albicans* and commensal oral streptococci in a novel *in vitro* mucosal model. Infect Immun 80:620–632
9. Ramsey MM, Rumbaugh KP, Whiteley M (2011) Metabolite cross-feeding enhances virulence in a model polymicrobial infection. PLoS Pathog 7, e1002012
10. Whitmore SE, Lamont RJ (2011) The pathogenic persona of community-associated oral streptococci. Mol Microbiol 81:305–314
11. Xu H, Sobue T, Thompson A et al (2013) Streptococcal co-infection augments *Candida* pathogenicity by amplifying the mucosal inflammatory response. Cell Microbiol 16:214–231
12. Gillum AM, Tsay EY, Kirsch DR (1984) Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae* ura3 and *E. coli* pyrF mutations. Mol Gen Genet 198:179–182
13. Dongari-Bagtzoglou A, Kashleva H (2006) Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat Protoc 1:2012–2018
14. Kalabis J, Wong GS, Vega ME et al (2012) Isolation and characterization of mouse and human esophageal epithelial cells in 3D organotypic culture. Nat Protoc 7:235–246
15. Xie Z, Thompson A, Kashleva H et al (2011) A quantitative real-time RT-PCR assay for mature *C. albicans* biofilms. BMC Microbiol 11:93
16. Dwivedi P, Thompson A, Xie Z et al (2011) Role of Bcr1-activated genes Hwp1 and Hyr1 in *Candida albicans* oral mucosal biofilms and neutrophil evasion. PLoS One 6, e16218

Chapter 11

Staphylococcus–Candida Interaction Models: Antibiotic Resistance Testing and Host Interactions

Nina Scheres and Bastiaan P. Krom

Abstract

The fungus *Candida albicans* and bacterium *Staphylococcus aureus* can coexist in polymicrobial biofilms. *S. aureus* attaches strongly to hyphae, but not to the yeast form, of *C. albicans* with important consequences for virulence. Hyphae-associated *S. aureus* is less susceptible to antibiotic treatment. Furthermore, co-inoculation of *C. albicans* and *S. aureus* causes more severe and widespread infection than either microorganism alone. In this chapter, a basic in vitro model for studying the interaction between *C. albicans* hyphae and *S. aureus* is presented, which makes use of a fluorescently labeled *S. aureus* strain. Furthermore, two protocols are described that allow investigation of the effect of *C. albicans* and *S. aureus* interaction on antibiotic susceptibility or on interactions with the host. The latter focuses on phagocytosis of *C. albicans*-adhered *S. aureus* by macrophages. The protocols presented here may serve as a starting point to study the interaction of *C. albicans* with various other bacterial species.

Key words *Candida albicans*, *Staphylococcus aureus*, Antibiotic treatment, Fungus, Macrophages

1 Introduction

In nature, and in a host, *Candida albicans* usually does not exist in planktonic form, but rather in biofilms that often consist of more than one microbial species. Such polymicrobial biofilms present an important clinical problem [1]. Within the polymicrobial biofilm, the different microorganisms closely interact with each other, and this can have a strong influence on their behavior and virulence. *C. albicans* is often found together with the opportunistic pathogen *Staphylococcus aureus* [2], a bacterium that commonly colonizes human hosts and that can cause infections when the immune system is compromised. *S. aureus* can strongly adhere to the hyphae, but not to the yeast form, of *C. albicans* [3, 4]. This interaction is mediated by the hyphal-specific protein Als3 [5], and both *C. albicans* and *S. aureus* may benefit from this close physical

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_11,

interaction. For instance, *S. aureus* becomes less susceptible to vancomycin when growing in biofilms with *C. albicans* [6]. The mechanism of this decreased susceptibility is presently unknown and in vitro model systems, such as the one described here, are required to study the molecular mechanisms involved.

The interaction between *C. albicans* and *S. aureus* may also influence their interaction with the host. For instance, when *C. albicans* and *S. aureus* are used together to induce a combined infection in mouse model, the production of pro-inflammatory cytokines that are involved in the innate immune response was increased more compared to an infection with either microorganism alone, and more neutrophils were attracted [7]. *C. albicans*–*S. aureus* interaction thus seems to have an effect on the rapid innate host response against an infection. However, the immunological mechanisms behind this remain largely unknown, and an in vitro model such as described in this chapter could be a tool to study this.

The present chapter first presents a basic in vitro model to study *Candida*–bacteria interactions (*e.g.*, adhesion and biofilm growth) followed by two specific experiments to study the effect of *Candida*–bacterium interactions on antibiotic susceptibility and host interactions. We illustrate this model using the interaction between *S. aureus* and *C. albicans*; however, this basic model can be used and adapted to investigate various effects of the interaction of various other bacteria with *C. albicans*.

2 Materials

2.1 Strains and Media

1. *C. albicans* SC5314 [8]. When required, *C. albicans* can be visualized using fluorescence with calcofluor white (Fluorescent Brightener 28, Sigma-Aldrich, USA).
2. GFP-labeled *S. aureus* ATCC 12600 [9] (*see* **Note 1**).
3. Brain-Heart-Infusion medium (BHI) (*see* **Note 2**) and BHI supplemented with 10 μg/mL tetracycline. BHI was prepared by dissolving 37 g/L of Difco™ BHI (BD Biosciences, USA) in distilled water. Tetracycline was added to *S. aureus*-GFP cultures to ensure maintenance of the GFP-containing plasmid pMV158-GFP [9]. Should a different mutant strain be used, the appropriate antibiotic must be added.

2.2 C. albicans and S. aureus Incubations

1. Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, USA) (*see* **Note 3**).
2. Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA).
3. Sterile 12-well cell culture plates with lid (Greiner Bio-one, Austria) (*see* **Note 4**).

2.3 Antibiotics

1. Vancomycin (1 mg/ml final concentration).
2. Gentamicin (50 μg/ml final concentration).

2.4 Macrophages

1. J774A.1 murine macrophage cell line (ATCC® TIB-67™).
2. Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA), supplemented with 10 % FBS.
3. T75 cell culture flasks (Cellstar®, Greiner Bio-one, Austria).
4. Optional: Propidium Iodide (PI) staining for dead cells, 1.0 mg/mL in water (Molecular Probes®, Life Technologies, USA). PI is a red fluorescent dye that cannot permeate the membrane of live cells and therefore stains only dead or damaged cells. Alternatively, other dyes with similar working mechanisms could be used.

2.5 Time-Lapse Microscopy

1. Axio Observer Z1 automated microscope with heated plate holder (Zeiss, Jena, Germany). This microscope is computerized and allows for the selection and memorizing of fixed stage positions, so that multiple positions on a plate can be followed in a single experiment. In addition, this setup can simultaneously acquire visible light and multiple fluorescence images.
2. GFP filter settings: 475/40 bandpass excitation filter, a 500 nm beamsplitter, and a 530/50 bandpass emission filter.
3. PI filter settings: 545/25 bandpass excitation filter, a 570 nm beamsplitter, and a 605/70 bandpass emission filter.
4. DAPI/Calcofluor filter settings: 365/50 nm excitation filter, 395 nm beamsplitter, and a 445/50 bandpass emission filter.

3 Methods

3.1 C. albicans Adhesion

1. Cultures of *C. albicans*, grown overnight in BHI at 30 °C, shaken and at an oblique angle are harvested by centrifugation (2 min at 5000 × *g*).
2. *C. albicans* pellets are thoroughly resuspended in HBSS and diluted to an OD_{600} of 0.01 in HBSS (*see* **Note 5**).
3. 1 ml of *Candida* suspension is added into wells of a 12-well tissue culture-treated plate (*see* **Note 4**) and incubated at 37 °C for ~3 h without aeration, to induce hyphal growth.

3.2 Serum Coating

1. Non-adhered *C. albicans* are removed by aspiration and wells are carefully washed with HBSS.
2. Wells are coated with 1 ml of 50 % FBS (in HBSS) for 30–60 min at 37 °C.
3. FBS is removed and wells are washed two times with HBSS.

Serum coating is included in this protocol to ensure optimal adhesion of *S. aureus* to hyphae of *C. albicans*, and to minimize attachment of *S. aureus* to the bottom of the polystyrene wells [10]. In addition, serum is well-known to induce/enhance hyphal growth; however, serum coating can be skipped if it is not favorable, for instance, due to comparisons with other conditions. Part of *S. aureus* will then adhere to the plastic rather than to hyphae, and hyphae in general will be shorter (see Fig. 1). A longer incubation period for hyphal growth before addition of *S. aureus* is recommended in this situation.

3.3 S. aureus Attachment to C. albicans Hyphae

1. Cultures of *S. aureus*-GFP, grown overnight in BHI with 10 μg/ml tetracycline (see Subheading 2.1) at 37 °C and shaken, are harvested by centrifugation (2 min, 5000 × *g*).
2. *S. aureus* pellets are resuspended in HBSS and diluted to an optical density measured at 600 nm (OD_{600}) of 0.01 in HBSS (*see* **Note 5**).
3. 1 ml of *S. aureus* suspension is added to wells with *C. albicans* and incubated for at least 15 min at 37 °C with agitation.
4. Wells are washed thoroughly with HBSS to remove non-adherent *S. aureus*.

Initial attachment of *S. aureus* to hyphae can occur after 15 min, but longer incubations, up to 1 h, are also possible and may lead to more adhered *S. aureus*. It is recommended to check attachment of *S. aureus* to hyphae by microscopy before washing.

After adherence of *S. aureus* to hyphae, the *C. albicans*–*S. aureus* biofilms can be experimentally treated as required.

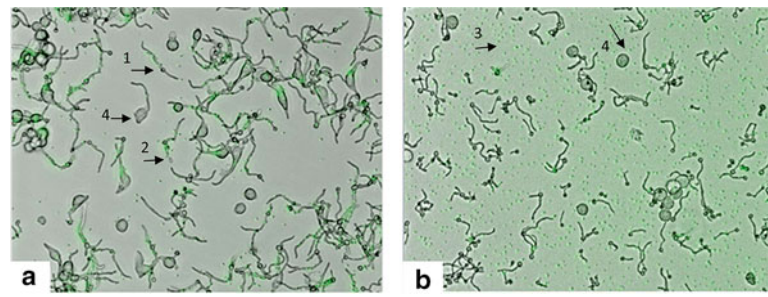


Fig. 1 Micrographs (20×) of *C. albicans* SC5314 with adhered *S. aureus*-GFP (ATCC 12600) and J774 murine macrophages, after 3–5 h of hyphal growth, and 1 h incubation with *S. aureus*. The *black arrows* indicate *C. albicans* with hyphae (1), *S. aureus* (*green*) attached to hypha (2), *S. aureus* (*green*) attached to plastic (3), macrophage on hypha (4), and macrophage on plastic (5). In panel (**a**), a 50 % serum coating has been applied before incubation with *S. aureus*. >90 % of *S. aureus* is adhered to *C. albicans* hyphae. In panel (**b**), no serum coating was applied. Without the serum coating, a large part of *S. aureus* is adhered to the well bottom, and hyphae are shorter

Below, an example is given on how to test antibiotic susceptibility (3A), and on how to visualize phagocytosis of *C. albicans*-adhered *S. aureus* by macrophages (3B).

3.4 Antibiotic Susceptibility of Candida-Adhered S. aureus

1. The antibiotic to be tested is added in the appropriate concentration(s) and incubated for 1 h at 37 °C.
2. Medium containing antibiotic is removed and wells are washed two times with HBSS (*see* **Note** PBS).
3. Viability of GFP-labeled *S. aureus* can be followed using fluorescence microscopy (Fig. 2) or using a fluorescence-enabled spectrophotometer (Fig. 3). Microscopically, dead *S. aureus* stain red upon staining with PI. This is probably caused by a combination of loss of GFP fluorescence and increased PI binding to the DNA. Red staining is absent in viable bacteria resulting in clearly visible green cells. The effect of antibiotics can be quantified using a fluorescence-enabled spectrophotometer. Increased *S. aureus* biomass results in increased GFP-derived fluorescence.

3.5 Preparation of Macrophages for Phagocytosis Assays

1. Macrophages are best prepared during the last minutes of *C. albicans*–*S. aureus* incubation (**step 3.3**). J774 macrophages, grown until 80–90 % confluence in a T75 culture flask in DMEM with 10 % FBS, are washed once with DMEM and removed from flask bottom with a cell scraper.
2. Loosened macrophages are resuspended in 10 ml DMEM containing 10 % FBS, removed from flask, and further diluted 10× in DMEM with 10 % FBS.

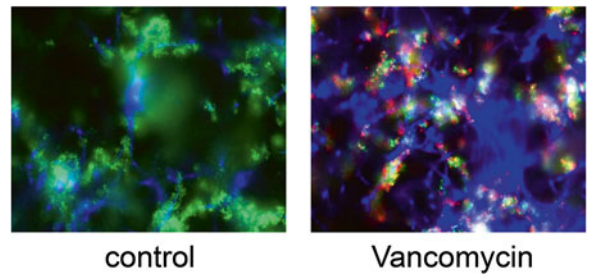


Fig. 2 Effect of the presence of *C. albicans* SC5314 on the susceptibility of *S. aureus*-GFP to vancomycin. *C. albicans* (stained fluorescent blue with calcofluor) with adhered *S. aureus*-GFP (*fluorescent green*) was allowed to adhere for 1 h at 37 °C. The samples were washed, and growth medium without (*left*) and with (*right*) vancomycin (1 mg/ml) was added. The plates were incubated for an additional 1 h at 37 °C. The plates were subsequently washed with PBS to remove non-adherent cells and stained with PI to visualize dead cells. Treatment of *C. albicans*-adhered *S. aureus* killed only part of the bacterial population (Ovchinnikova ES and Krom BP, unpublished data)

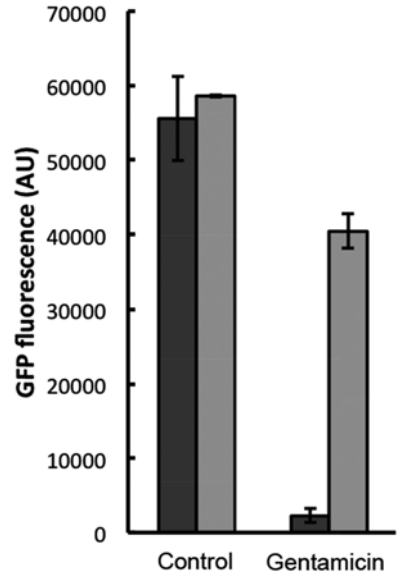


Fig. 3 Effect of the presence of *C. albicans* SC5314 on the susceptibility of *S. aureus*-GFP to gentamicin. *S. aureus*-GFP alone (*dark grey*) or together with *C. albicans* (*light grey*) were incubated at 37 °C for 3.5 h, the plates were washed, and growth medium without (*left*) and with (*right*) gentamicin (50 μg/ml) was added. The plates were incubated for an additional 18 h at 37 °C. The plates were subsequently washed with PBS to remove non-adherent cells. Growth was determined in a Fluostar OPTIMA spectrophotometer using 488 nm excitation and 530 nm emission wavelengths. Presence of gentamicin resulted in a clear reduction in *S. aureus*-GFP growth, which was absent in the presence of *C. albicans* (Ovchinnikova ES and Krom BP, unpublished data)

3. Optional: PI staining is added to diluted macrophage suspension in a 5000× dilution (0.2 μ/mL final concentration).

PI staining can be included to visualize cell death or damage of macrophages during the phagocytosis assay. PI staining will label macrophages directly after cell death. PI staining is not macrophage-specific and may also label dead *C. albicans* or *S. aureus*.

3.6 Addition of Macrophages: Start of Phagocytosis

1. 1 ml of macrophage suspension (approximately 10^5 cells/ml) is added to each well.

Phagocytosis rapidly starts after addition of macrophages, and it is recommended to proceed to time-lapse visualization of phagocytosis as soon as possible. Preferably, part of the installation of the proper microscope settings (see Subheading 3.7, **steps 2** and **3**) has already been performed before addition of macrophages. Depending on the type of microscopy and the number of selected positions, this process may take a significant amount of time. Fixed plate positions could already be set approximately, so that only small adjustments to the settings might be needed after addition of macrophages.

3.7 Visualization of Phagocytosis by Time-Lapse Microscopy

1. Plates are placed under an inverted microscope at 37 °C (*see* **Note 6**), covered with lid (*see* **Note 6**).
2. Fixed positions on plate are determined and programmed into microscope for retrieval of images.
3. For detection of unstained *C. albicans*, *S. aureus*-GFP, and unstained macrophages, the microscope is set to use both bright-field illumination and GFP fluorescence. GFP fluorescence is detected here using a filter block containing a 475/40 bandpass excitation filter, a 500 nm beamsplitter, and a 530/50 bandpass emission filter.
4. Optional: PI fluorescence can be detected using a filter block containing a 545/25 bandpass excitation filter, a 570 nm beamsplitter, and a 605/70 bandpass emission filter.
5. Time-lapse is programmed to take images of fixed positions on plate, every 10 min, for a maximum duration of 12 h (*see* **Note 6**).

The use of fixed positions allows for multiple sites on the plate to be monitored, and multiple conditions to be tested in a single run. It is recommended to include at least two different positions per well. Optimally, positions are selected in which free hyphae with several adhered *S. aureus* are clearly visible. Positions that are "crowded" with hyphae will hamper sufficient vision of macrophage activity.

Naturally, the optimal settings for image retrieval depend on the type of microscope and software used. Similarly, image processing after time-lapse microscopy depends on the available or preferred software. The images in Fig. 4 were obtained from a 3 h phagocytosis assay, using the microscope, software, and settings as described above. Overlay images of the bright-field and green fluorescent images were created using Montage software (version 7.7.11.0, Molecular Devices). Further processing of images, such as the selection, enlargements, and cropping was performed with Image J software (http://imagej.nih.gov/ij/).

4 Notes

1. Non-GFP-labeled bacteria might be stained with membrane permeable nontoxic nucleic acid stains such as SYTO9 (Molecular Probes®, Life Technologies, USA). Staining procedures should be optimized per species and strain to prevent toxicity effects and background staining of *C. albicans* nuclei.
2. The medium choice is irrelevant for the experiment. Any medium that facilitates *C. albicans* growth in the yeast morphology can be used.

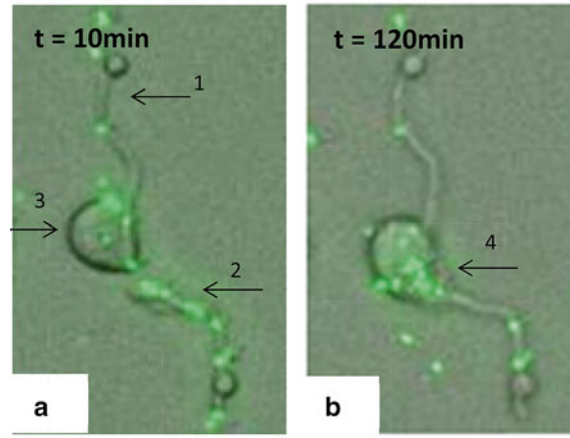


Fig. 4 Details from micrographs of *C. albicans* SC5314 with adhered *S. aureus*-GFP (ATCC 12600) and J774 murine macrophages, at the beginning stage (panel **a**) and end stage (panel **b**) of a 3 h phagocytosis assay. The *black arrows* indicate *C. albicans* with hyphae (1), *S. aureus* (*green*) attached to hypha (2) and a macrophage on hypha (3, 4). In panel (**a**), a macrophage has attached to a hypha and starts to phagocytose *S. aureus*. In panel (**b**), the macrophage has ingested several *S. aureus* that were first present on the lower hypha. These *S. aureus* can be seen as *green spots* inside the macrophage (*arrow 4*)

3. HBSS is used as a neutral condition for *C. albicans* and *S. aureus* interaction so that cells remain viable/active, but do not multiply. Also, the absence of nutrients in HBSS, along with incubation at 37 °C, promotes hypha formation in *C. albicans*. Instead of HBSS, any sterile, culture-grade PBS solution with neutral pH could be used.
4. 12-well plates were chosen here due to their compatibility with a temperature-regulated plate holder for microscopy. The recommended volumes and dilutions of microorganisms in the protocol can be adapted for other plate types.
5. For *C. albicans* strain SC5314, OD_{600} of 1 represents approximately 1×10^7 CFU/mL. A suspension of OD_{600} 0.01 results in an incomplete coverage of the well bottom by *Candida*. This is optimal for microscopic images in which free-grown hyphae with adhered *S. aureus* and active macrophages can be clearly observed. Less or more coverage by *Candida* can be obtained by adjusting the dilution. *C. albicans* at OD_{600} 0.05 will give >90 % coverage.

   For *S. aureus* strain ATCC 12600, OD_{600} of 1 represents approximately 1 x 10^9 CFU/mL. A ratio of *C. albicans*:*S. aureus* of approximately 1:100 is optimal for *S. aureus* interaction with hyphae.
6. If placed at 37 °C, macrophages will remain active for longer incubation times than at room temperature. During a 12 h

assay, up to 75 % of macrophages will remain viable at 37 °C. After 12 h, *C. albicans* may start to re-form yeast bodies from hyphae. With incubation at 37 °C, condense formation in the lid should be taken into account. If assays are performed at RT, shorter incubations of 3 h or 4 h are recommended.

Acknowledgements

The authors thank Carolien Bode for practical assistance. Purchase of the Bioflux Z1000 was made possible through a grant from the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO). This work funded with financial support of the Dutch Burns Foundation (12.102). BPK is also supported by a grant from the University of Amsterdam for research into the focal point "Oral Infections and Inflammation."

References

1. Harriott MM, Noverr MC (2011) Importance of *Candida*-bacterial polymicrobial biofilms in disease. Trends Microbiol 19(11):557–563
2. Klotz SA et al (2007) Polymicrobial bloodstream infections involving *Candida* species: analysis of patients and review of the literature. Diagn Microbiol Infect Dis 59(4):401–406
3. Harriott MM, Noverr MC (2009) *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob Agents Chemother 53(9): 3914–3922
4. Ovchinnikova ES et al (2012) Evaluation of adhesion forces of *Staphylococcus aureus* along the length of *Candida albicans* hyphae. BMC Microbiol 12:281
5. Peters BM et al (2012) *Staphylococcus aureus* adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p. Microbiology 158(Pt 12):2975–2986
6. Harriott MM, Noverr MC (2010) Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. Antimicrob Agents Chemother 54(9): 3746–3755
7. Peters BM, Noverr MC (2013) *Candida albicans-Staphylococcus aureus* polymicrobial peritonitis modulates host innate immunity. Infect Immun 81(6):2178–2189
8. Fonzi WA, Irwin MY (1993) Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134(3):717–728
9. Li J et al (2011) Analysis of the contribution of sedimentation to bacterial mass transport in a parallel plate flow chamber: part II. Use of fluorescence imaging. Colloids Surf B Biointerfaces 87(2):427–432
10. Ovchinnikova ES et al (2013) Exchange of adsorbed serum proteins during adhesion of *Staphylococcus aureus* to an abiotic surface and *Candida albicans* hyphae—an AFM study. Colloids Surf B Biointerfaces 110: 45–50

Part II

Drug Discovery and Susceptibility Testing

Chapter 12

Genetic Screens for Determination of Mechanism of Action

Françoise Gay-Andrieu, Deepu Alex, and Richard Calderone

Abstract

The search for new antifungal drugs and cell targets continues. During the discovery process, mechanism-of-action (MOA) studies are critical to the continued progress of the compound through the pipeline. There are many approaches that can be utilized in understanding the MOA. One of these approaches is a genetic screen utilizing the availability of *Saccharomyces cerevisiae* mutant libraries. Both null and heterozygous library mutants covering the entire genome of this model yeast are available. The desired phenotype when screening the new compound is either resistance (null mutants) or haploinsufficiency or loss of fitness (heterozygote mutants). Both types of mutants can be clustered by software into common targets that provide clues as to a pathway or other cell process. Below, methods are described for genetic screens.

Key words Genetic screen, MIC, Susceptibility, Haploinsufficiency, MOA

1 Introduction

The determination of mechanism of action (MOA) of novel compounds is probably the most challenging aspect of drug discovery. While target identification is not absolutely essential for drug development, it facilitates the optimization of a compound [1].

Several approaches can be used to identify drug targets, but over the last few years there have been an increasing number of studies based on the use of genetic tools. Following the sequencing of the *Saccharomyces* genome in 1986, the launch of the Saccharomyces Genome Deletion Project allowed the creation of genome-wide yeast mutant collections, which offered a very powerful tool to the scientific community in order to explore the fitness of the mutants under specific conditions [2–4].

Thus, one approach to identifying gene targets is compound screens based on the availability of fungal mutant libraries and especially those of *Saccharomyces cerevisiae* that cover its entire genome. A critical yet obvious requirement before one ventures into this approach to target identification is that the compound(s) should inhibit *S. cerevisiae* parental cells. But, even then, if it does

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_12, © Springer Science+Business Media New York 2016

not, still the screen would provide negative data that might be useful. We have not observed gene-wide negative screens thus far. In this chapter we will describe screens based on two different yeast libraries, each consisting of ~4,700 to 6000+ mutants representing the entire genome of *S. cerevisiae* constructed in a diploid background (BY4743), including (1) null strains lacking both copies of genes, (2) strains that are hemizygotic and display a haploinsufficiency (HI) [1, 5]. The basic concept behind this assay is that HIP (Haploinsufficiency Profiling) identifies genes in which one functional copy, compared to two, confers hypersensitivity to inhibition by the compound. This indicates pathways directly affected by the compound. HOP (Homozygous Profiling) indicates synthetic lethality and identifies compensating pathways directly affected by the compound. Mutants that displayed an alteration of the "fitness" need to be verified by standardized tube dilution assays to obtain MIC values and to be compared to MICs of parental cells [6–11]. Two types of procedures can be applied to these screens. First, we describe in detail in this chapter a manual method. Every mutant in the library is screened in 96-well microtiter plates or agar plate assays with the "hit" compound incorporated. Since each strain is specifically barcoded, screening of mutants can also be achieved in batch cultures followed by isolation of genomic DNA, PCR amplification of the barcodes, and array hybridization: it enables identification of sensitive (absence) or resistant (survival) strains after treated cultures are compared to untreated cultures [12].

The manual protocol that we describe has disadvantages, most important of which is that the procedure is quite time-consuming and that reproducibility is difficult to achieve without much practice. However, there are advantages, especially since the method is based on commonly used mycology culture procedures and can be performed in even instrument lacking laboratories. It is suitable for screening of one or two collections against a limited number of compounds. But larger studies would of course require more automated procedures [12]. Whatever the procedure that has been chosen, a major advantage of HIP includes the comprehensive analysis of the entire genome. Virtually every encoded gene mutant is assayed against compounds by screening for a reduced fitness in all mutants. Additionally, the approach is unbiased, generally applicable to any compound that is able to enter yeast cells or other living target organism, and of importance, yields a cluster of potential targets. Then, these genes can be functionally grouped using algorithms such as FunSpec [13]. The genetic approach provides strong evidence of a mechanism of action of a compound. However, still the hypothesis needs to be confirmed by phenotypic assays that correlate with the screening data and clustering of genes by FunSpec [13–15].

2 Materials

2.1 Reagents

1. Frozen glycerol stock plates of the Saccharomyces deletion collection, in 96-well plates. Four different mutant collections have been generated: haploids of mating types MATa and MATα, homozygous diploids for nonessential genes, and heterozygous diploids, which contain the essential and nonessential ORFs (*see* **Note 1**). For the experiments that we describe in this protocol, we used two of these collections: YSC Homozygous Diploid collection (YSC1056, GE Healthcare) and YSC Yeast Heterozygous Collection (YSC1055, GE Healthcare) (*see* **Notes 1** and **2**). The background is the BY4743 wild-type strain.
2. YPD liquid: yeast extract (10 g), peptone (20 g), dextrose (20 g), H_2O (1 l). Weigh and mix yeast extract, peptone, and dextrose in H_2O. Stir gently until dissolution, split in 2×500 ml glass bottle, and autoclave (120 °C, 20 min). Allow media to cool and store at 4 °C.
3. YPD agar: yeast extract (10 g), peptone (20 g), dextrose (20 g), agar (20 g), H_2O (1 l). Weigh and mix yeast extract, peptone, and dextrose in H_2O to a large flask (2 l). Add agar after complete dissolution. Autoclave (120 °C, 20 min). Allow medium to cool to approximately 50 °C before adding the drug at the appropriate concentration (*see* **Note 3**).
4. Compound: stock solution and work solution have to be prepared and stored according to the specific requirement of the compound (DMSO for example).
5. Ethanol 70 % (EtOH) for sterilizing the pin tool.
6. Glycerol 40 %: prepare a 40 % solution of glycerol and autoclave (120 °C, 20 min). Glycerol is used for cryopreservation of yeasts (final concentration 20 %, freezing at −80 °C).

2.2 Equipment

1. 96-well pin tool. Transfer volume of the pin tool is 5 μl per well.
2. 96-well flat bottom plates.
3. 150 mm culture plates.
4. Filter paper to dry the pin tool (*see* **Note 4**).
5. Adhesive seals for 96-well plates.
6. Flame for sterilizing the pin tool.
7. Incubator to 30 °C for growing plates.

3 Methods

3.1 Procedure for Sterilizing the Pin Tool

1. This procedure is the same for all the experiments performed with the pin tool (transfer for duplication of the stock plates, transfer to prepare working plates, transfer of the yeasts from working plates to agar plates). This protocol has been slightly adapted from the procedure described by Pierce and collaborators [12].
2. Prepare two sterilized water baths and two ethanol 70 % baths. Use culture plate tops or sterile pipette tip tops.
3. Dip the pin replicator in the first water bath to rinse away the remaining cells, followed by two dips in 70 % ethanol baths.
4. Carefully flame the pin replicator.
5. Dip the pin replicator in the second water bath for a few seconds to cool it down and allow it to dry and cool for one more minute on sterile filter paper.
6. Make sure that all the levels in the different baths allow an appropriate destruction of the remaining cells on the tool (*see* **Note 5**).

3.2 Replication of the Collection (See Note 6)

1. Remove the plates to duplicate from the freezer and allow them to thaw very slowly and completely (put them on ice at the beginning). Work with a manageable number of plates in order to avoid leaving too many plates at room temperature for an extended period.
2. Prepare the appropriate number of 96-well plates by dispensing 100 µl of glycerol 40 % + 100 µl of YPD liquid into each well (final glycerol concentration = 20 %).
3. Carefully sterilize the pin tool (see above).
4. When the pin replicator is cool enough, insert it carefully into the first thawed 96-well glycerol stock plate, swirl gently during at least 20 s to suspend the yeasts, and then transfer carefully the pin replicator to the new glycerol plate and swirl gently at least 5 s to allow the cells to dilute in the fresh medium.
5. Rinse and sterilize the pin, and let it cool on the filter paper for about 1 min.
6. During this time cover both plates with a new sterile adhesive plate seal.
7. Repeat the procedure for all the plates to be transferred.
8. Put the plates on ice before −80 °C freezing.

3.3 Collection Screening for Hypersensitive Mutants with Compound (Fig. 1)

The screening for hypersensitive mutants does not require large amounts of compounds and can be performed on agar plates.

1. On day 1, transfer individual deletion strains (5 μl) from frozen stocks to 200 μl of YPD for overnight growth (working plates). The procedure to transfer the strains is the same as the procedure described above to duplicate the collection except the fact that the plate contains 200 μl of YPD instead of YPD/Glycerol.
2. On day 2, repeat the same procedure to transfer the mutants from the working plates (5 μl) into fresh YPD in 96-well plates (200 μl) to achieve a 1:40 dilution of cells (dilution plates) (*see* **Note 7**).
3. Print each mutant with the pin tool onto YPD agar plates with or without compound (*see* **Note 8**). The concentration of the compound in agar plates has to be determined by preliminary experiments performed on a subset of five to ten plates of the collection. According to our experience and previously

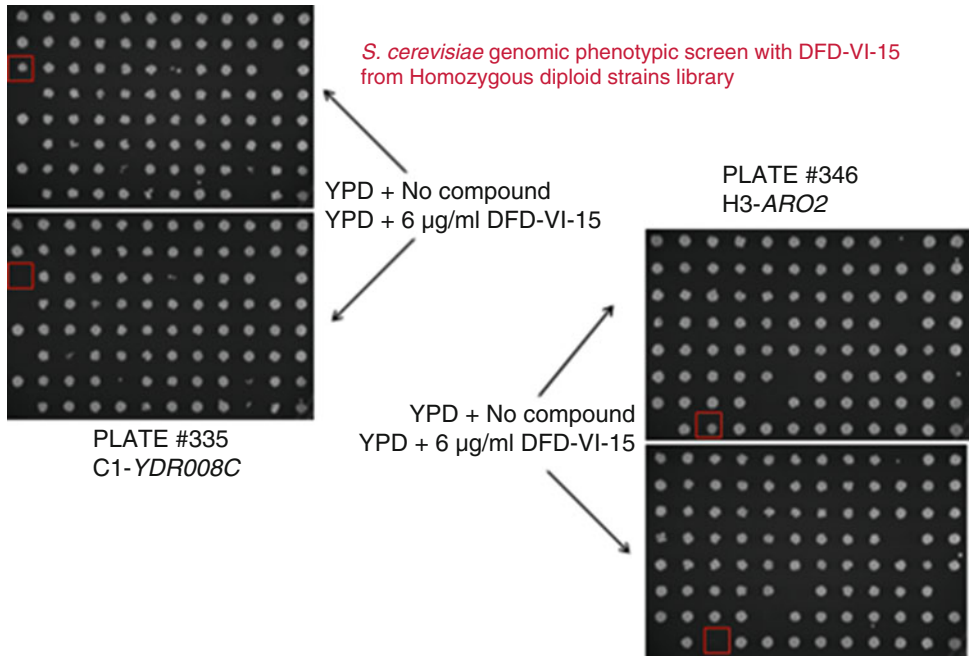


Fig. 1 Two sets of 96-well plates are shown, each well inoculated with a different coded mutant, from a library of *S. cerevisiae* (2N) homozygous mutants [1, 5]. Plates are numbered as a reference to a specific set of mutants. Alternatively, a heterozygous library of the same organism (about 6200 mutants) is available also; the homozygous library (nulls) does not include essential gene mutants. The upper plate of both sets is used as a no compound control and the lower plate of each set is treated with a compound that is inhibitory to test fungal organisms but the compound MOA has not been identified. The compound is named DFD-VI-15 and its concentration indicated. Plates are incubated at 30 °C and inhibited mutants are boxed in *red*. Growth is measured at 24 and 48 h. Growth inhibited or resistant mutants can be verified for their phenotype doing tube dilutions. Homozygous mutants (2N) that are resistant could represent the compound target

published protocols an accurate concentration would yield approximately 1–5 % mutants per 96-well plate with a hypersensitive phenotype, according to previously published protocol and preliminary data [1, 7].

4. Allow the printed agar plates to dry on the bench for a few minutes before transporting them to the 30 °C incubator.
5. Growth is evaluated at 24, 48, and 72 h (*see* **Note 9**). The growth of each deletion mutant is determined visually at 48 and 72 h and compared to the same mutant grown in the absence of the compound. Mutants that display hypersensitivity after 48 h and confirmed after 72 h have to be considered as hypersensitive (*see* **Note 10**).
6. It is recommended to make pictures of the agar plates in order to be able to double check the difference of growth later on.
7. The hypersensitivity of all the mutants detected by the screen(s) has to be confirmed with MIC determinations.

Collection screening for resistant mutants with compound

1. The same methodology can be used for identifying resistant mutants but one obstacle is that working on agar requires large amounts of compound. As the available quantity of a new product may be limited, solid agar plates can be replaced by 96-well plates with YPD broth containing, or not, the compound. The concentration of the compound has to be fungicidal for *S. cerevisiae* BY4743.
2. Except this modification, the procedure is exactly the same as the procedure described above for detection of hypersensitive mutants.
3. Growth is determined at 24 and 48 h, and the resistant mutants are identified visually as those that grew in the presence of the compound.

Interpretation

1. The mutants that are sensitive or resistant to the compound are compiled.
2. Each hypersensitive/resistant mutant is identified by its position on the plate (*see* **Note 2**) related to its ORF. The corresponding gene can be obtained from online databanks like Saccharomyces Genome Database [15] (http://yeastmine.yeastgenome.org).
3. Then the genes are grouped into functional categories to identify a target or pathway (or both) using algorithms such as FunSpec [13] (http://funspec.med.utoronto.ca/) or Gene Ontology (GO) (http://www.geneontology.org/).

4. As this stage, one can devise a hypothesis concerning a potential target/pathway. The following step consists of phenotypic analyses to confirm (or change) the hypothesis based on the screens.

4 Notes

1. A helpful practical manual providing details on the collections can be found online at http://dharmacon.gelifesciences.com/uploadedfiles/resources/yeast_knockout_manual.pdf.
2. Excel files denoting the plate locations of each particular knock-out strain in the collection can be found online at http://dharmacon.gelifesciences.com.
3. An excessive temperature may alter the compound. In order to avoid the risk of contamination of the medium with a nonsterile thermometer, we simply checked the temperature manually. We assumed that the temperature was not too high when we were able to keep the hands on the hot flask during several seconds (around 10 s).
4. In order to carefully cool and dry the pin tool after sterilization, we used sterilized filter papers. We cut pieces of large sheets of filter paper, adapted to the size of the pin tool, and sterilized them in aluminum sheets.
5. Working near the flame was safe enough to avoid airborne contaminations. Each plate displays two empty wells: one is specific of the collection (*e.g.*, H1 for the homozygous library, H4 for heterozygous library) and the other one is specific of the plate within the collection. These empty wells, useful in case of misidentification of the plate, also work as sterility controls.
6. The main risk of the collection screening is cross contamination between wells/mutants. In order to be able to go back to the original plate, in case of contamination, it is essential to carefully duplicate the collection before the beginning of any experiment.
7. This step of dilution is not mandatory on a theoritical point of view but may be needed to decrease the number of yeasts that are printed on the agar and then, decrease the ratio between yeasts and compound, in order to detect hypersensitive mutants while not using too high amounts of compound. The 1/40 dilution is convenient (5 µl into 200 µl) but may be adapted according to the MIC of the compound.
8. For each working/dilution plate transferred, it is absolutely mandatory to have a control agar plate without compound. The number of transferred yeasts cannot be equal between different wells; then growth reduction of each mutant has to be appreciated in comparison to control plate without compound.

Moreover a set of two or three different concentrations of compound may be used to observe a dose-dependent effect.

9. Difference in fitness can be observed as soon as 24 h, but the difference of growth between mutants is clearer at 48 h and better to be confirmed at 72 h.
10. As the reproducibility of this manual technique is quite challenging to reach, it is recommended to screen the whole collection twice. The mutants that are identified as hypersensitive in both screens are included for further analyses.

References

1. Alex D, Gay-Andrieu F, May J et al (2012) Amino-acid derived 1,2-benzisothiazolinone derivatives as novel small molecule antifungal inhibitors: identification of potential genetic targets. Antimicrob Agents Chemother 56(9):4630–4639
2. Giaever G, Chu AM, Ni L et al (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418:387–391
3. Goffeau A, Barrell BG, Bussey H et al (1996) Life with 6000 genes. Science 274(546):563–567
4. Winzeler EA, Shoemaker DD, Astromoff A et al (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285:901–906
5. Gay-Andrieu F, May J, Li D, Sun N, Hui C, Calderone R, Alex D (2012) Insights in antifungal drug discovery. In: Clancy CJ, Calderone RA (eds) Candida and candidiasis. ASM Press, Washington, DC, pp 387–402
6. Arita A, Zhou X, Ellen TP et al (2009) A genome-wide deletion mutant screen identifies pathways affected by nickel sulfate in *Saccharomyces cerevisiae*. BMC Genomics 10:524
7. Batova M, Klobucnikova V, Oblasova Z et al (2010) Chemogenomic and transcriptome analysis identifies mode of action of the chemosensitizing agent CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine). BMC Genomics 11:153
8. Hillenmeyer ME, Fung E, Wildenhain J et al (2008) The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science 320:362–365
9. Hillenmeyer ME, Ericson E, Davis RW et al (2010) Systematic analyses of genome-wide fitness in yeast reveals novel gene function and drug action. Genome Biol 11:R30
10. Hoepfner D, Karkare S, Helliwell SB et al (2012) An integrated approach for identification and target validation of antifungal compounds active against Erg11p. Antimicrob Agents Chemother 56:4233–4240
11. Smith AM, Ammar R, Nislow C et al (2010) A survey of yeast genomic assays for drug and target discovery. Pharmacol Ther 127: 156–164
12. Pierce SE, Davis RW, Nislow C et al (2007) Genome-wide analysis of barcoded *Saccharomyces cerevisiae* gene-deletion mutants in pooled cultures. Nat Protoc 2:2958–2974
13. Robinson MD, Grigull J, Mohammad N et al (2002) FunSpec: a web-based cluster interpreter for yeast. BMC Bioinformatics 3:35
14. Giaever G, Nislow C (2014) The yeast deletion collection: a decade of functional genomics. Genetics 197:451–465
15. Cherry JM, Adler C, Ball C et al (1998) SGD: saccharomyces genome database. Nucleic Acids Res 26:73–79

Chapter 13

Microbroth Dilution Susceptibility Testing of *Candida* species

Randal J. Kuykendall and Shawn R. Lockhart

Abstract

Antifungal susceptibility testing for *Candida* species is now widely accepted as a methodology to predict the success or failure of antifungal therapy for some antifungal/*Candida* species combinations. There are many different ways to perform susceptibility testing of antifungal agents, but broth microdilution has become the most popular over the last 10 years. This chapter describes in detail two methods for antifungal susceptibility testing of *Candida* species using the commercially available microbroth dilution tray (YeastOne®) and a commercially available gradient agar diffusion technique (Etest®) for isolates that appear resistant.

Key words Susceptibility testing, Antifungal, Broth microdilution, Etest, MIC

1 Introduction

Antifungal susceptibility testing is a useful protocol for mutation discovery, for epidemiology of clinical failure, and for drug discovery. However, the major purpose of antifungal susceptibility testing is to determine the minimum inhibitory concentration (MIC) of antifungal drugs against clinical isolates in order to predict clinical outcome. This latter use is the basis for a difficult determination because an MIC is an in vitro measurement of susceptibility and clinical outcome is dependent upon the health and immune status of the host, the pharmacodynamics/pharmacokinetics of the antimicrobial at the particular dosage/site of infection, and the overall sensitivity of the infecting organism to the particular antifungal. Nevertheless, methodologies for antimicrobial susceptibility testing have been developed that allow the rapid and relatively reliable determination of MIC values.

There are four major methods for manual susceptibility testing: broth macrodilution, broth microdilution, disk diffusion, and microgradient diffusion. Broth macrodilution involves inoculation of the test organism into a set of test tubes containing growth

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_13,

medium and increasing concentrations of an antimicrobial agent. The endpoint is read as the concentration in the tube in which microbial growth is decreased to a prespecified degree. Broth microdilution is similar, but instead of using test tubes, microbial growth takes place in a 96-well microtiter plate. Again, the endpoint is read as the concentration in the well in which microbial growth is decreased to a prespecified degree. Disk diffusion is performed by placing a paper disk saturated with an antimicrobial onto an agar plate inoculated with a test organism. The antimicrobial diffuses into the agar and forms a gradient around the disk. The endpoint is read as the diameter of the clearing around the disk in which there is no microbial growth. This methodology requires that the zone diameter is related to susceptibility or resistance, so that the diameter obtained with the test organism can be put into context. Microgradient diffusion uses a paper or plastic strip impregnated with various amounts of antimicrobial in a continuous concentration gradient. The strip is placed on an agar plate inoculated with a test organism. The antimicrobial diffuses into the agar and forms an elliptical gradient centered on the strip. The endpoint is read as the concentration where the microbial growth intersects the strip. Each of these mechanisms has their good and bad points but broth microdilution has become the standard for susceptibility testing in Europe and the United States. In Europe, a standardized protocol for testing yeasts by broth microdilution has been established by the European Committee for Antimicrobial Susceptibilitytesting (EUCAST) as document EDef 7.1 [1]. In the United States, the standardized protocol for yeasts has been established by the Clinical and Laboratory Standards Institute (CLSI) in document M27-A3 [2].

In this chapter, the CLSI methodology for broth microdilution testing of yeasts will be described. As preparation of the antifungal dilutions and the 96-well microtiter plates is described quite succinctly in the CLSI document M27-A3, this chapter will concentrate on using a commercially available microbroth dilution tray (YeastOne®) and a commercially available gradient agar diffusion technique (Etest®). Both of these methodologies compare favorably to the CLSI M-27 and EUCAST methodologies [3–6].

2 Materials

Sabouraud's Dextrose agar or other fungal growth medium.

Inoculating loops.

Sterile water—2 ml in a tube that will fit the spectrophotometer.

Vortex.

Spectrophotometer or nephelometer.

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate).

MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer.

NaOH (1 mol/l).

Bacto Agar.

D-Glucose.

15 ml screw cap test tubes.

15 × 100 mm plastic petri dishes.

Inoculum reservoir.

12 channel pipette.

Manual mirror viewer.

CLSI documents M27-A3 and M27-S4.

Quality control isolates *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258.

Seed trough.

YeastOne® susceptibility plate (Thermo Fisher Scientific, Oakwood Village, OH).

Etest strips:

Amphotericin B (BioMerieux, cat# 526348).

Fluconazole (BioMerieux, cat# 510858).

Itraconazole (BioMerieux, cat# 525858).

Voriconazole (BioMerieux, cat# 532840).

3 Methods

3.1 Media

RPMI 1640 broth

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate) 10.4 g/l (Sigma, #1383).
MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer 34.5 g/l.
NaOH (1 mol/l).
15 ml screw cap test tubes.

1. Dissolve RPMI, MOPS, and glucose into 900 ml of distilled H_2O. Adjust to pH 7.0 at 25 °C using 1 mol/l NaOH. Adjust the final volume to 1 l.
2. Filter sterilize using a 0.2 μm filter.
3. Dispense into 11 ml aliquots in test tubes and store at 4 °C.

RPMI Agar Plates

RPMI 1640 powder (with glutamic acid and phenol red, without bicarbonate) 10.4 g/l (Sigma, #1383).

MOPS (3-[*N*-morpholino] propanesulfonic acid) buffer 34.5 g/l.

NaOH (1 mol/l).

Bacto Agar 15 g/l.

D-Glucose 20 g/l.

15 × 100 mm plastic petri dishes.

1. Dissolve RPMI, MOPS, and glucose into 450 ml of distilled H_2O. Adjust to pH 7.0 at 25 °C using 1 mol/l NaOH.
2. Filter sterilize using a 0.2 μm filter.
3. Sterile filter (0.2 μM) and place in water bath to increase temperature to 50 °C.
4. Add 15 g of agar to 400 ml of H_2O. Autoclave for 20 min to sterilize.
5. Add the sterile agar to the RPMI broth, adjust the final volume to 1 l with sterile distilled H_2O and stir to mix.
6. Aseptically dispense 25 ml per petri dish and store at 4 °C for up to 6 months.

3.2 Sample Preparation and Inoculation

1. Starting from a frozen stock or a fresh culture, streak the test isolate on a SAB agar plate or another fungal growth medium and allow it to grow for 24 h at 37 °C. If taken from a frozen stock, re-streak on a SAB agar plate and allow additional growth for 24 h. Include a fresh culture of the two quality control isolates ATCC 22019 (*C. parapsilosis*) and ATCC 6258 (*C. krusei*). Streak all of the isolates for colony isolation.
2. Prepare a stock solution from each isolate by picking two to three small isolated colonies from the fresh culture and suspending them in 2 ml of sterile water, vortexing well. The amount picked will depend on the size of the colony and can be adjusted according to the subsequent density readings.
3. Vortex resulting suspension for 15 s, then adjust cell density with sterile water to a spectrophotometer transmittance reading of 80–82 % at a wavelength of 530 nm. This will yield a yeast stock suspension of $1–5 \times 10^6$ cells per ml. Alternatively, use a nephelometer and adjust the turbidity to 0.5 McFarland units.
4. Transfer 20 μl of the cell suspension to the 11 ml RPMI broth tubes and vortex well. Pour this suspension in the seed trough and use for primary inoculation of the plates.

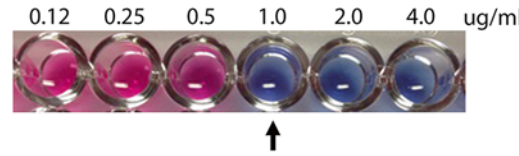


Fig. 1 Sample wells of *Candida albicans* and fluconazole following 24 h of incubation. The arrow indicates the well at which the MIC should be read. The MIC of this isolate against fluconazole is 1.0 μg/ml

5. Using the 12 channel pipette, dispense 100 μl of the suspension into each well of the YeastOne plates (*see* **Note 1**).
6. A colony count to ensure correct inoculum should be performed by taking 10 μl out of the positive control growth well and plating it on Sabouraud's dextrose agar. If the inoculum is correct, and depending on how the density was adjusted, 50–500 colonies should grow on the plate.
7. Seal the YeastOne plates and incubate at 35 °C for 24 h in a non-CO2 incubator.
8. Plates are read after 24 h of incubation.

3.3 Reading the Plates and Assigning an Interpretation

1. Plates are read visually using normal room lighting. Set the plate on top of the manual mirror viewer. You will be looking at the bottom of the plate in the mirror.
2. With the YeastOne colorimetric system, all of the wells start out as blue. As growth appears, the wells turn red due to a change in the colorimetric indicator dye. Make sure that the positive control well has turned red. If not, the plate can be reincubated for up to another 24 h.
3. The MIC is read as the lowest concentration where the well has remained blue (indicating a lack of growth) (Fig. 1) (*see* **Notes 2** and **3**).
4. If there is no color change in any of the wells for a given antifungal agent, the MIC is read as ≤ the lowest concentration of the antifungal tested. If all of the wells have turned red, the MIC is read as > the highest concentration of the antifungal tested.
5. Before interpretation of the breakpoints is made, the MIC values for the two quality control isolates should be determined to make sure that they are in range (Table 1). If the quality control isolates are not in the established quality control range, the test is invalid.
6. Species-specific interpretive breakpoints are available for *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* against fluconazole, micafungin, caspofungin, and anidulafungin; for *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C.*

Table 1
Quality control ranges for the two quality control isolates

	MIC in μg/ml	
Antifungal	*C. parapsilosis* ATCC 22019	*C. krusei* ATCC 6258
Fluconazole	0.5–4	8–64
Voriconazole	0.015–0.12	0.06–0.5
Itraconazole	0.12–0.5	0.12–1
Posaconazole	0.06–0.25	0.06–0.5
Micafungin	0.5–2	0.06–0.25
Caspofungin	0.25–1	0.12–1
Anidulafungin	0.25–2	0.03–0.12
Flucytosine	0.5–4	4–16
Amphotericin B	0.25–2	0.5–2

krusei against voriconazole; and *C. guilliermondii* against micafungin, caspofungin, and anidulafungin [7]. There are no breakpoints for any other species of *Candida* for any other antifungal agents and there are no breakpoints for any *Candida* species for itraconazole, posaconazole, isavuconazole, amphotericin B, or flucytosine. As the breakpoints are not static, the latest antifungal document from the Clinical and Laboratory Standards Institute (http://clsi.org) should be checked to determine the most recent breakpoints.

7. True resistance to the azole antifungals among *C. albicans* and *C. tropicalis* isolates is rare. Quite often it is the result of a misinterpretation of a trailing isolate (*see* **Note 3**). These isolates should have their MIC values confirmed by Etest to determine whether they are truly resistant or just show an in vitro trailing effect as described in **Note 3**.

3.4 Etest Susceptibility Testing

The Etest is based upon a continuous concentration gradient infused on a plastic non-porous strip. The antifungal agent diffuses into an agar plate and allows an accurate determination of MIC values based on elliptical growth around the antifungal gradient.

1. Grow the isolate to be tested and the quality control isolates as described in Subheading 3.1.
2. Remove RPMI agar plates from refrigerator and allow them to reach room temperature.
3. Following the cell dilution technique from above Subheading 3.1 [2, 3], plate approximately 100 μl of the initial

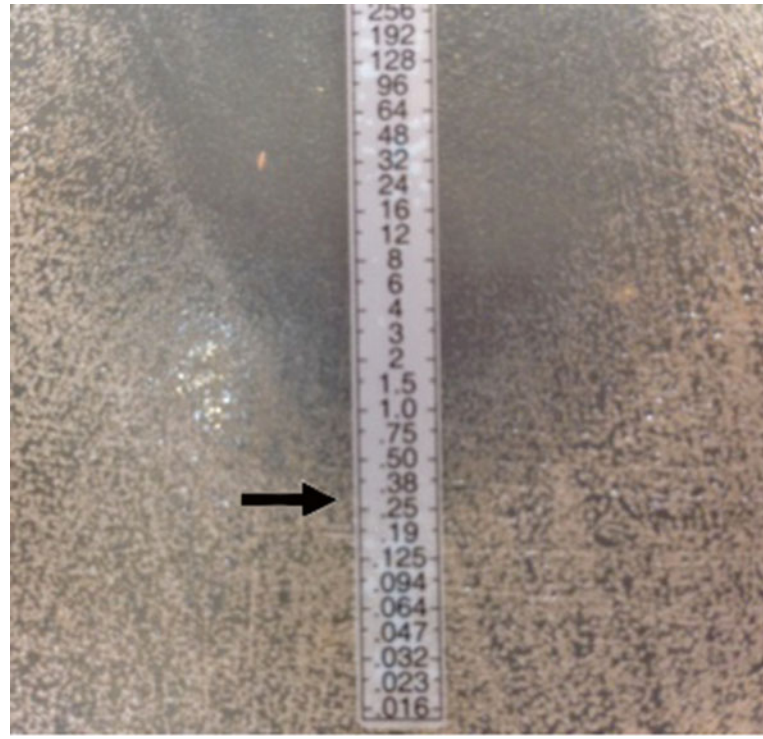


Fig. 2 Etest of an isolate with trailing growth. This is an Etest read after 20 h of growth. The trailing growth can clearly be seen inside the ellipse. The MIC is read as the first point in the ellipse without microcolonies. In this case it would be 0.38 μg/ml

sterile water cell suspension on the RPMI plate. Use a sterile cotton swab to streak the plate completely in three directions to make sure the entire plate is covered. Allow the plate to sit for at least 15 min so that the added moisture can dry.

4. Take the appropriate number of Etest strips out of the −20 °C freezer. Extract the appropriate number of test strips and allow them to come to room temperature. Using a forceps, apply the Etest strips to plates with the MIC scale facing upward, being careful to not allow bubbles under the strip. If there are bubbles, remove them by gently pressing down with the forceps, being careful not to slide the strip.
5. Incubate the plate at 35 °C for 24 h or until a confluent lawn of growth is seen.
6. The interpretation of the MIC value is dependent upon the antifungal agent. For susceptible isolates, an ellipse will be seen with the growth concentrated near the MIC endpoint. For azoles and echinocandins the MIC is read at the MIC value where the first significant decrease in the density is seen (interpreted as an 80 % decrease in growth) (*see* **Note 4**) (Fig. 2).

Always read the value on the side of the strip with the highest growth. For amphotericin B, it is interpreted as the value where there is 100 % growth inhibition. If growth is inhibited between MIC values, use the higher of the two values as the MIC.

4 Notes

1. There are alternative ways to inoculate the tray. It can be performed manually using a single pipette and inoculating all 96 wells individually. Sensititre® and a few other companies manufacture an auto inoculator that allows the user to insert the suspension tube so that each well is inoculated individually. This device ensured uniformity and avoids carpal tunnel syndrome for the laboratorian.
2. Occasionally there will be a skipped well (a blue well in between two red wells). In the case of a skipped well, if there is growth on either side, ignore the skipped well and record the highest growth. If there are two skipped wells in a row, the test is invalid. If there are two skipped wells in a single column, all of the tests on the tray are invalid.
3. Occasionally with the azole antifungals and *C. albicans*, *C. tropicalis*, and *C. glabrata*, there will be growth in the wells above what would normally be the MIC value; this is called trailing growth and the isolate is called a trailer [8]. In the case of the YeastOne plates, this is usually manifested as a purple color (in between the negative blue and the positive red). YeastOne recommends that the MIC is read as the first well that shows a less intense color change compared to the positive control well (Fig. 3). This is a very subjective interpretation. The authors recommend that trailing isolates have their MIC values confirmed by Etest.
4. For trailers, there may be fairly confluent growth on the plate but a visible ellipse can be seen when the plate is either very young or when it is held at an angle. The inside of the ellipse will be made up of microcolonies. If the isolate is resistant, no ellipse will be seen.

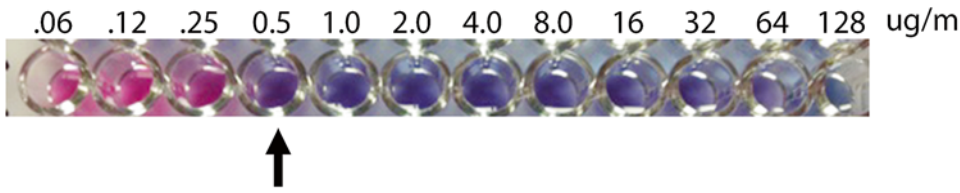


Fig. 3 Sample wells of trailing growth of *Candidaalbicans* and fluconazole following 24 h of incubation. Only in the well with 128 μg/ml of fluconazole can the authentic blue color signifying no growth be seen. The MIC is read as the first well with a less intense red color which is 0.5 μg/ml in this case

Acknowledgement

The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

References

1. Rodriguez-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M et al (2008) EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect 14:398–405
2. Clinical and Laboratory Standards Institute. 2008. Reference method for Broth dilution antifungal susceptibility testing of yeasts; approved standard—Third Edition, CLSI Document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA
3. Alexander BD, Byrne TC, Smith KL, Hanson KE, Anstrom KJ, Perfect JR et al (2007) Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. J Clin Microbiol 45:698–706
4. Bertout S, Dunyach C, Drakulovski P, Reynes J, Mallie M (2011) Comparison of the Sensititre YeastOne® dilution method with the Clinical Laboratory Standards Institute (CLSI) M27-A3 microbroth dilution reference method for determining MIC of eight antifungal agents on 102 yeast strains. Pathol Biol 59:48–51
5. Espinel-Ingroff A, Pfaller M, Messer SA, Knapp CC, Holliday N, Killian SB (2004) Multicenter comparison of the Sensititre YeastOne colorimetric antifungal panel with the NCCLS M27-A2 reference method for testing new antifungal agents against clinical isolates of *Candida* spp. J Clin Microbiol 42:718–721
6. Cuenca-Estrella M, Gomez-Lopez A, Alastruey-Izquierdo A, Bernal-Martinez L, Cuesta I, Buitrago MJ et al (2010) Comparison of the Vitek 2 antifungal susceptibility system with the clinical and laboratory standards institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Broth Microdilution Reference Methods and with the Sensititre YeastOne and Etest techniques for in vitro detection of antifungal resistance in yeast isolates. J Clin Microbiol 48:1782–1786
7. Clinical and Laboratory Standards Institute (2012) M27-S4 reference method for Broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. Clinical and Laboratory Standards Institute. Wayne, PA
8. Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG et al (1998) Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. J Clin Microbiol 36:153–156

Chapter 14

Biofilms and Antifungal Susceptibility Testing

Maria Simitsopoulou, Athanasios Chatzimoschou, and Emmanuel Roilides

Abstract

Yeasts and filamentous fungi both exist as single cells and hyphal forms, two morphologies used by most fungal organisms to create a complex multilayered biofilm structure. In this chapter we describe the most widely used assays for the determination of biofilm production and assessment of susceptibility of biofilms to antifungal agents or host phagocytes as various methods, the most frequent of which are staining, confocal laser scanning microscopy, quantification of extracellular DNA and protein associated with extracellular matrix and XTT metabolic reduction assay. Pathway-focused biofilm gene expression profiling is assessed by real-time reverse transcriptase polymerase chain reaction.

Key words Biofilm, Fungi, *Candida*, *Aspergillus*, Extracellular matrix quantitation, Antifungal susceptibility testing

1 Introduction

1.1 Clinical Importance of Fungal Biofilms

Both yeasts and filamentous fungi exist as single cells and hyphal forms, two morphologies used by most fungal organisms to create a complex multilayered biofilm structure. Fungal organisms can switch to the biofilm mode of life cycle in order to survive in hostile environmental conditions and thus contribute to disseminated life-threatening infections. *Candida albicans*, a commensal of human mucosal surfaces, can behave as a pathogen when the immune system or other commensal organisms are unable to confine its growth, leading to superficial and systemic infections. The frequent use of a broad range of medical implant devices in hospitalized individuals is one of the most important risk factors for candidemia [1]. Because catheters serve as substrates for biofilm formation, the risk of biofilm development on catheters can reach up to 30 % depending on the catheter implantation site [2]. The oral cavity is another site that offers a suitable environment for biofilm growth mostly by *C. albicans* but also by other important non-*albicans Candida* species such as *C. glabrata*, *C. parapsilosis*,

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_14, © Springer Science+Business Media New York 2016

C. tropicalis or *C. krusei*. Denture stomatitis is characterized by *Candida* species growing between denture prosthesis and the surface close to the oral mucosa [3, 4].

The expanded clinical importance of fungal biofilms is reflected by a growing number of reports on other yeasts and filamentous fungi including *Cryptococcus neoformans*, *Candida lusitaniae*, *Aspergillus fumigatus*, *Fusarium* species [5, 6]. For example, the lower respiratory tract of cystic fibrosis patients, because of the presence of excessive mucous on bronchial epithelial cells, is prime candidate for airborne filamentous fungi to adhere, colonize and form biofilms [7]. Most frequently, conidia of *A. fumigatus*, a ubiquitous filamentous mold, can be inhaled and reach the alveoli where, depending on the immune status of individual, can cause aspergilloma, allergic bronchopulmonary aspergillosis or invasive pulmonary aspergillosis leading to multiple organ dissemination. Histological examination of bronchopulmonary lavage samples of these individuals reveals the presence of complex multicellular structures embedded in a dense hyphal mesh, which is typical of a biofilm phenotype [8]. *Aspergillus* species have also been implicated in infections of cardiac pacemakers, joint replacements and breast augmentation implants [9–11].

1.2 Life Cycle of Fungal Biofilms

In general, fungal biofilm formation is characterized by four stages: (1) Adherence of fungal cells to a surface is mediated through fungal cell wall proteins; this stage is induced by several environmental signals, such as changes in nutrient concentrations, pH, flow velocity of surrounding body fluids (urine, blood, saliva), temperature, oxygen concentration, osmolality or iron. (2) Growth of the attached cells into a micro-colony. (3) Maturation of biofilm, which involves the development of pseudohyphae and hyphae as well as secretion of extracellular hydrophobic matrix (ECM). ECM is composed of carbohydrates, proteins, chitin, and DNA. (4) The last phase of biofilm formation is cell detachment, which is required for the dissemination of single or clustered cells through the bloodstream to other organ systems and is dependent on nutrient availability and environmental pH [12].

1.3 Antifungal Susceptibility of Biofilms

One of the characteristic phenotypic traits of biofilms is the reduced susceptibility to conventional antifungal agents, although drug susceptibility can differ among clinical strains [13–15]. The drug concentrations needed to reduce the metabolic activity of biofilms by 50 % is five to eight times higher compared to the corresponding planktonic cells and minimum inhibitory concentrations (MIC) increase more than 30-fold. Factors that contribute to drug resistance include drug efflux pumps, differential cell membrane composition, growth rate, ECM, increased cell density and differential expression of biofilm genes. The increased levels of biofilm resistance underline the importance of developing assays to test biofilm antifungal susceptibilities.

1.4 Evaluation of Biofilm Formation

In this chapter we describe the most widely used assays for the determination of biofilm production and assessment of antifungal efficacy against biofilms as outlined below:

1. Crystal violet staining.
2. Safranin staining.
3. Adhesion test.
4. Quantification of ECM associated extracellular DNA (e-DNA).
5. Quantification of ECM associated protein.
6. Microscopic visualization of biofilm structure by Confocal Laser Scanning Microscopy (CLSM).
7. Assessment of antifungal efficacy by:
   (a) XTT reduction assay.
   (b) Pathway-focused gene expression profiling by real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

2 Materials

2.1 Preparation of Fungal Isolates

1. Clinical isolates or strains from a culture bank (i.e., ATCC, CBS, etc.).
2. Mycological media including potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA).
3. Hanks' balanced salt solution without calcium and magnesium ($HBSS^-$).
4. Stock solution: 25 % glycerol, 75 % peptone.
5. Phosphate buffered saline buffer (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4 (pH 7.4), supplemented with 0.05 % Tween 80.
6. Yeast nitrogen base (YNB) supplemented with 0.25 % glucose.
7. RPMI-1640 culture medium supplemented with L-glutamine buffered to pH 7.2 with 0.165 M 3-[*N*-Morpholino]-propanesulfonic acid (MOPS).
   - RPMI-1640 supplemented with L-glutamine and 10 % fetal calf serum (FCS).
8. Sterile water.
9. 12 mL polypropylene conical tubes.
10. Neubauer plaques.
11. 96-well sterile polystyrene flat-bottomed microtiter plates.

2.2 Staining

1. 0.5 % aqueous crystal violet solution.
2. 95 % absolute ethanol.
3. 1 % safranin solution.

2.3 Quantification of the ECM Material

1. 0.2 M EDTA.
2. 0.45 μm syringe filter.
3. Phenol–chloroform–isoamyl alcohol (25:24:1, v/v).
4. Ice-cold isopropanol.
5. Ice-cold 70 % ethanol.
6. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5.
7. 10 mM Tris–HCl, pH 8.0.

2.4 CLSM

1. Plastic Thermanox coverslip disks, 15-mm in diameter.
2. 12-well cell culture plates.
3. 10 mM FUN-1 cell stain.
4. 5 mM Calcofluor White M2R.
5. Anhydrous dimethylsulfoxide (DMSO).

2.5 XTT Reduction Assay

1. 0.25 mg/mL 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide (XTT) prepared in PBS.
2. 5 mg/mL 2,3-dimethoxy-5-methyl-*p*-benzoquinone (coenzyme Q_0) prepared in acetone or 10 mM menadione prepared in acetone.

2.6 Multiple Gene Expression Profiling by Real-Time RT-PCR

1. AE buffer: 50 mM sodium acetate, pH 5.2, 10 mM EDTA.
2. 25 % SDS.
3. Acid phenol.
4. Isopropanol.
5. 2 M sodium acetate, pH 5.0.
6. 70 % absolute ethanol.
7. Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT).
8. SYBR green master mix.

3 Methods

3.1 Preparation of Fungal Isolates

3.1.1 Preparation of Blastoconidia of Yeasts (See Note 1)

1. Take a small aliquot of yeast isolate stock maintained in a solution containing 25 % glycerol and 75 % peptone at –20 °C.
2. Inoculate an SDA plate and incubate at 37 °C overnight.
3. Next day, pick two to three colonies from the plate and scrape them off the agar surface with a sterile inoculating loop.
4. Suspend the colonies in a 20-mL flask containing YNB medium supplemented with 2 % glucose and incubate at 37 °C overnight on a rocking table.

5. Next morning, centrifuge the grown culture at 1200 × *g* for 10 min.
6. Wash pellet once with PBS and resuspend in 5 mL of RPMI-1640, pH 7.2.
7. Determine the concentration of suspension by counting blastoconidia on a Neubauer hemocytometer.

3.1.2 Preparation of Conidia of Filamentous Fungi (See Note 2)

1. Take a small aliquot of filamentous isolate stock maintained on SDA agar slants at −20 °C.
2. Inoculate a PDA plate and incubate at 37 °C for 3 days (*Aspergillus* spp.; *see* **Note 3**).
3. Harvest conidia by flooding the plate with PBS containing 0.05 % Tween 80 and scraping the surface of grown media with a sterile cotton swab.
4. Filter the conidial suspension through a sterile gauze to remove the hyphae.
5. Centrifuge conidia at 1200 × *g* for 10 min.
6. Wash pellet once with PBS and resuspend in 5 mL of RPMI-1640, pH 7.2, supplemented with 10 % FCS.
7. Determine the concentration of suspension by counting conidia on a Neubauer hemocytometer.

3.2 Biofilm Formation

1. Adjust fungal suspension to a final concentration of 10^6 cells/mL (yeasts) or 5×10^5 cells/mL (filamentous fungi).
2. Add 200 μL of a standardized cell suspension to each well of a 96-well flat-bottomed microtiter plate.
3. Incubate the microtiter plate containing the organism at 37 °C with shaking (yeasts) or statically (filamentous fungi) for 24–72 h (*see* **Notes 4** and **5**).
4. At the end of the incubation period, wash three times with 200 μL sterile water or PBS to remove non-adherent cells.

3.3 Crystal Violet Staining

1. After the washing step, let the biofilm-coated wells of the microtiter plate to air-dry for 45–90 min (*see* **Note 6**).
2. Stain each well with 150 μL 0.5 % aqueous crystal violet solution for 45 min.
3. Wash three to five times with sterile water.
4. Destain the wells by adding 200 μL of 95 % ethanol for 45 min.
5. Transfer 100 μL to a new microtiter plate and quantitate the level of crystal violet present using a spectrophotometer and reading the optical density (OD) at 595 nm (*see* **Note 7**).

3.4 Safranin Staining

1. After the washing step, let the biofilm-coated wells of the microtiter plate to air-dry for 45–90 min. Alternatively, place the microtiter plate without the lid in a dry oven set at 50 °C for 30 min.
2. Stain each well with 200 μL 1 % safranin for 2–3 min.
3. Wash three times with sterile water.
4. Let the microtiter plate to air-dry for 20–30 min.
5. Quantitate the level of safranin present using a spectrophotometer and reading the OD at 492 nm.

3.5 Adhesion Test

1. Grow the organism as instructed in Subheading 3.1 and adjust final concentration to 10^7 cells/mL.
2. Add 100 μL of 10^7 cells/mL to the wells of a microtiter plate.
3. Incubate for 90 min at 37 °C with shaking (yeasts) or statically (filamentous fungi).
4. Wash two times with 200 μL PBS to remove non-adherent cells.
5. Quantitate the presence of adherent cells by the XTT reduction assay (*see* Subheading 3.8).

3.6 Quantification of ECM Material

3.6.1 Isolation of ECM Material

1. Inoculate a standardized conidial suspension in a 12-well culture plate containing RPMI-1640 medium (yeasts) or RPMI-1640 supplemented with 10 % FCS (filamentous fungi).
2. Incubate at 37 °C for 48 h with shaking (yeasts) or under static conditions (filamentous fungi).
3. Remove the biofilm by means of a sterile cell scraper and transfer material in a 50 mL conical tube.
4. Wash twice with PBS and treat disaggregated biofilm with 0.2 M EDTA. Alternatively, after washing, vortex biofilm cells for 1 min, sonicate in a sonicator bath for 30 min, and vortex a second time for 2 min.
5. Centrifuge the mixture at 10,000 × *g* for 20 min at 15 °C.
6. Recover the supernatant fraction and filter using a 0.45 μm syringe filter.

3.6.2 Quantification of ECM Associated e-DNA

7. Isolate e-DNA from supernatant using a DNA extraction kit following the manufacturer's suggested protocol (*see* **Note 8**).
8. Quantify e-DNA using the Qubit dsDNA BR assay kit (Invitrogen) according to the supplier's instructions and measure the emitted fluorescence at 485 nm (*see* **Note 9**).
9. Quantitation range of the assay kit: 2 ng–1 μg of DNA.

3.6.3 Quantification of ECM Associated Protein

10. Protein content of the ECM material is quantified with the Bradford procedure using a commercial kit from Thermo Scientific, as recommended by the manufacturer (*see* **Note 10**).

11. Calculate the unknown protein sample using the values obtained from the standard curve as follows:
    (a) Make a graph with the standard curve values using an Excel spreadsheet. Add the dependent variable (protein concentration) on the *X* axis and the independent variable (Abs at 595 nm) on the *Y* axis.
    (b) Without connecting the dots, perform a linear regression on the data. Show the regression "r" value on the graph (the closer the number to 1 the more fit the data are).
    (c) Calculate the unknown protein concentration from the equation given.
    (d) If samples are diluted, multiply the concentration by the dilution factor.
12. Quantitation range of the assay kit: 1–25 μg/mL of protein.

3.7 Visualization of Biofilm Structure by CLSM

For examination by CLSM, untreated or drug-treated *Candida* or *Aspergillus* biofilms are formed on plastic Thermanox coverslip disks.

1. Grow fungal cells as outlined in Subheading 3.1 and adjust fungal suspension at 1×10^6 cells/mL in RPMI-1640.
2. Add the standardized suspension onto coverslip disks within 12-well culture plates and incubate the plates at 37 °C for 48 h.
3. After washing with PBS, add the desired antifungal and incubate the plates at 37 °C for an additional 24 h (control biofilms are incubated in RPMI-1640 culture medium only).
4. Wash biofilms with PBS and stain using the LIVE/DEAD FUN 1–Calcofluor White fluorescent stain kit (Molecular Probes) as per manufacturer's instructions (*see* **Notes 11** and **12**).
5. Wash biofilms with PBS and mount sample on a slide.
6. Visualize using a confocal microscope. Perform the imaging with a Zeiss LSM 510 META confocal microscope equipped with 40× C-Apochromat (numerical aperture 1.2) objective lens.
7. Take depth measurements at regular intervals across the width of the device. To determine the structure of the biofilms, a series of horizontal (*xy*) optical sections with a thickness of 0.9 μm, at 0.44-μm intervals, must be taken throughout the full length of the biofilm.
8. Analyze three-dimensional images using computer software.

3.8 Assessment of Antifungal Efficacy

3.8.1 XTT Reduction Assay

XTT is a slightly yellow compound that is reduced via the electron-coupling agent coenzyme Q_0 to a formazan product with a brightly orange color. The oxidation–reduction process is initiated by mitochondrial dehydrogenases of metabolically active cells. Percent fungal damage induced by an antifungal agent is assessed colorimetrically.

The colorimetric change is proportional to the number of living cells and can be quantified spectrophotometrically. In order to assess the antifungal efficacy and determine the minimum inhibitory concentration at which 50 % of biofilm cells or planktonic cells have been inactivated by the antifungal agent, the protocol as outlined below may be followed:

Day 1

- Revive frozen stock of the organism by plating on SDA plates.

Day 3

- Transfer an inoculum to a 20 mL flask containing 10 mL YNB supplemented with 0.25 % glucose and grow the fungal organism at 37 °C overnight.

Day 4

- Add 100 μL of a standardized solution to a 96-well microtiter flat-bottomed plate and incubate at 37 °C for 48 h for biofilm production.
- Prepare a range of twofold double dilutions (e.g., 0.007–256 μg/mL) of the antifungal drug:
  (a) Prepare 4× the highest drug concentration (e.g., 256 × 4 = 1024 μg/mL) in RPMI-1640.
  (b) Add 150 μL of 1024 μg/mL drug to five wells of the first row containing 150 μL RPMI-1640 and, after mixing two to three times the contents of each well with the multichannel micropipette, transfer 150 μL from the five wells of the first row to the corresponding five wells of the second row. Repeat the process for all 16 rows of the 96-well microtiter plate (Fig. 1a).
  (c) Store drug plate at −20 °C.

Day 5

1. Repeat procedure of *Day 3* to make a fresh culture for planktonic cells.

Day 6

1. Add 100 μL of a standardized solution prepared on *Day 5* to a 96-well microtiter flat-bottomed plate (Fig. 1b).
2. Add another 100 μL of twofold double dilutions of the drug plate prepared on *Day 4* (Fig. 1b).
3. Centrifuge the biofilm plate prepared on *Day 4* at 4000 rpm for 20 min and wash twice with sterile distilled water.
4. Add 100 μL RPMI-1640 to the biofilm plate and 100 μL of twofold dilutions of the drug plate prepared on *Day 4*.

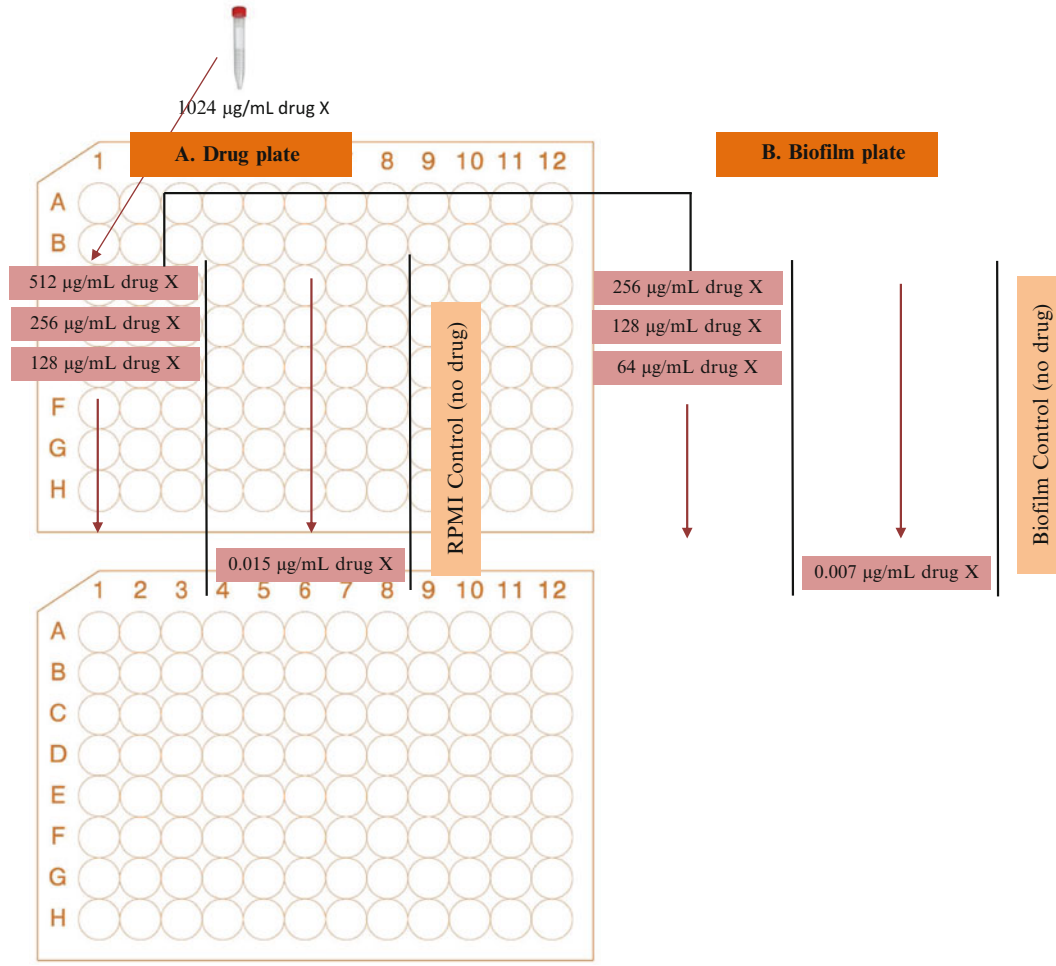


Fig. 1 Proposed experimental design for the assessment of biofilm antifungal susceptibility by XTT reduction assay

5. Incubate both plates (biofilm and planktonic) at 37 °C for a desired time period.
6. At the end of the incubation period, centrifuge both plates and wash once with sterile distilled water.
7. Add 150 µL XTT-coenzyme Q_0 solution (final concentration: 0.25 mg/mL XTT and 40 µg/mL coenzyme Q_0) and incubate at 37 °C until the control wells (without drug) change to a bright orange color. Alternatively, add 150 µL XTT-menadione solution (final concentration: 0.1 mg/mL XTT and 1 µM menadione) and incubate at 37 °C for a desired time period.
8. Transfer 100 µL from each well to a new 96-well plate.
9. Measure absorbance at 450 nm (for XTT-coenzyme Q_0 solution) or at 490 nm (for XTT-menadione solution) with

reference wavelength set at 690 nm. Include a blank well containing 100 μL of XTT-coenzyme Q_0 or XTT-menadione solution.

10. % fungal damage = $(1 - X/C) \times 100$, where X is the absorbance of test wells and C is the absorbance of control wells with organism alone.

3.8.2 Multiple Gene Expression Profiling by Real-Time RT-PCR

Real-time RT-PCR is a powerful tool for studying a focused panel of genes, because it requires small amounts of RNA, offers the option of analyzing multiple genes at the same time and compares relative gene expression levels among several experimental conditions. Gene expression responses of mature biofilms to antifungal drug exposure or antifungal efficacy against different stages of biofilm development can also be studied at the molecular level by analyzing the transcriptional profile of a focused array of genes. The real-time RT-PCR gene microarray method outlined below includes RNA isolation from biofilm cells, conversion of total RNA to cDNA, SYBR green labeling, amplification of amplicons in real-time PCR instrument and critical points to take into consideration for data analysis. Helpful tips for the successful completion of all steps of the method are also pointed out.

3.8.3 RNA Isolation from Biofilm Cells

Wear gloves throughout the procedure, use fresh reagents and labware.

1. Wash biofilms in ice-cold sterile PBS and detach cells from the bottom of a 12-well plate with a cell scraper.
2. Pellet cells by centrifugation at 3000 rpm for 15 min and count resuspended cells in PBS. A concentration of 5×10^7–7×10^7 cells/mL is preferred to account for partial RNA losses due to phenol–chloroform extraction steps that follow.
3. Following a second centrifugation step, resuspend pellet in 500 μL AE buffer and add 30 μL 25 % SDS and 500 μL acid phenol.
4. Incubate cell lysate for 10 min at 65 °C, then vortex for 1 min and subsequently cool on trimmed ice for 1 more min for a total of 5 min. Repeat this step three times.
5. Centrifuge at 12,000 × *g* for 15 min and transfer the upper aqueous layer to a new tube.
6. Add 700 μL chloroform, mix the two layers and centrifuge at 200 × *g* for 1 min.
7. Transfer the upper aqueous layer to a new tube, add 1 volume of ice-cold isopropanol and 0.1 volume of 2 M sodium acetate (pH 5.0).
8. Centrifuge at 18,000 × *g* for 30 min at 4 °C.
9. Wash pellet in 0.8 mL 70 % ice-cold ethanol.

10. Centrifuge at 18,000 × *g* for 20 min at 4 °C to collect the RNA.
11. Air-dry pellet and dissolve in 50 μL sterile ultrapure water.
12. Prior to concentration assessment, heat to 65 °C for 3 min and leave at RT for 5 min.
13. Measure RNA concentration and purity spectrophotometrically (*see* **Note 13**).
14. Check for RNA integrity on a 1.2 % denaturing agarose gel.
15. Store at −80 °C, if not used immediately.
16. Starting RNA amounts for multiple gene expression profiling by real-time RT-PCR range from 0.5 to 2 μg of total RNA.

3.8.4 DNase Treatment of Total RNA

1. Remove probable DNA contamination by treating RNA samples with DNase, an enzyme that selectively degrades DNA. Use a DNase treatment kit from Ambion, Quiagen, or Invitrogen as per manufacturer's instructions.
2. Measure 1 μL of DNase-treated RNA samples using a NanoDrop spectrophotometer.

3.8.5 cDNA Synthesis

1. Use 1 μg of RNA in a reaction of 20 μL.
2. Add 1 μL of random primers and *x*μL of sterile water. Denature RNA and primers at 65 °C for 5 min and transfer samples on ice.
3. Add 10 μL of reverse transcriptase (RT) mix that consists of *x*μL RT buffer and 2 μL M-MLV RT (Moloney Murine Leukemia Virus RT).
4. Incubate at 42 °C for 15 min and stop the reaction (inactivation of M-MLV enzyme) by incubating at 95 °C for 5 min.
5. Place the reactions on ice and prepare reagents to amplify the cDNA amplicons.

3.8.6 Real-Time RT-PCR Controls

1. A positive PCR control that consists of an artificial DNA sequence that tests the reagents used for RT-PCR as well as the PCR program chosen.
2. A negative PCR control that consists of all reagents with sterile distilled water as DNA template to test probable contamination of PCR reagents.
3. A reverse transcription control that tests the efficiency of the cDNA synthesis reaction.

3.8.7 Primer Design and Validation

1. Select up to 84 genes of interest based on published literature indicating pathway specificity and importance in biofilm development and antifungal susceptibility.
2. Find DNA sequences using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and design the corresponding primer pairs.

3. To maximize amplification efficiency, select primer pairs with a melting temperature of 58–60 °C, an amplicon size of 120 bp and the last five nucleotides at the 3′ end should contain three A's or T's.
4. To validate primer pair specificity, the expected single PCR product should be verified by gel electrophoresis, bidirectional DNA sequencing and BLAST search the resulting DNA sequence to verify the correct gene target sequence.
5. A final validation of primer specificity is to conduct dissociation (melting) curve analysis for each primer pair in order to detect the presence of primer dimers or decreases in SYBR Green fluorescence due to dissociation of PCR products. For cycler-specific melting curve analysis settings, refer to the Instrument Setup Guide of your cycler (*see* **Note 14**).

3.8.8 Real-Time RT-PCR

Physically separate operations for PCR setup and post-PCR processing.

1. Cycler conditions are: 1 cycle, 95 °C for 10 min; 40 cycles, 95 °C for 15 s; and 60 °C for 1 min. The number of cycles may differ depending on the type of cycler at hand.
2. A 96-well plate is prepared with a forward and reverse primer pair in each well.
3. Perform real-time RT-PCR in a total volume of 25 μL containing *x*μL of cDNA sample, *x*μL of SYBR green PCR master mix (buffer, dNTPs, Hot Start thermostable DNA polymerase, and SYBR green dye) and *x*μL of sterile RNase-free water.
   - Set the baseline two cycles before the earliest visible amplification.
4. Determine the threshold cycle (C_T) for each well using the cycler's software; choose a threshold value above the background signal.
5. Export the C_T values for each well to an Excel spreadsheet to analyze the data using a Web-based software.

3.8.9 Data Analysis

1. Normalize the data by subtracting the C_T value of a gene of interest (GI) from the average C_T value of the housekeeping genes (HG): $\Delta C_T = C_T^{GI} - C_T^{AVG\ HG}$.
2. Calculate the fold change between the experimental well (GI) and the control well (untreated control) as 2^(ΔC_T GI – ΔC_T untreated control).
3. Significant changes in gene expression are then assessed by comparing each normalized gene with other normalized gene in the data set.

4. If the fold change is greater than 1, the result is reported as a fold up-regulation. If the fold change is less than 1, the result is reported as a fold down-regulation.

Specific instrument setup, kits for all steps of pathway-focused gene expression by real-time RT-PCR, kinds of controls, data analysis Excel templates, software and informatics are available from any real-time RT-PCR provider (e.g., Applied Biosystems, Qiagen/SABiosciences).

4 Notes

1. Yeasts are unicellular cells called blastoconidia that grow into pseudohyphae (e.g., *Candida* spp.). Because cells tend to aggregate, vortex at high speed for 10 min prior to counting of cells for determining the concentration of the cell suspension by hemocytometer.
2. Filamentous fungi are composed of multiple conidia that develop into branching hyphal structures (*e.g.*, *Aspergillus* spp.). Vigorous vortexing is also recommended as in **Note 1**.
3. Different filamentous spp. need different incubation time to observe plate confluence.
4. Duration period for biofilm formation depends on the fungal organism under study.
5. For biofilm formation, use an angle rotator/shaker, not an orbital one, and set the digital display unit to 6–8, equivalent to 60–80 rpm.
6. To facilitate the drying process, remove the lid from the microtiter plate and place it in an upside-down position.
7. The OD values of the control (wells without biofilm) should be subtracted from the values of the experimental wells to minimize background inconsistencies.
8. An inexpensive way to isolate e-DNA from the supernatant fraction is the following:
   (a) Extract DNA with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v).
   (b) Centrifuge at 4000 × *g* at 15 °C for 2 min.
   (c) Transfer the upper layer to another tube and precipitate DNA with ice-cold isopropanol (1:1, v/v).
   (d) Centrifuge at 10,000 × *g* and 15 °C for 10 min.
   (e) Discard the supernatant and rinse the pellet with 1 mL of 70 % ethanol.

(f) Centrifuge at 10,000 × *g* and 15 °C for 5 min and let the pellet to air-dry for 10 min.

(g) Dissolve the dried pellet in 20 μL of TE buffer.

9. Fluorescent labeling of e-DNA is also achieved with the ds DNA binding dye SYBR-green I which is used at a ratio of 1:1 (dye–DNA) and DNA levels are measured by a fluorescence 96-well plate reader [16].
10. If plate reader is not available with 595 nm filter, the blue color can also be measured at any wavelength between 570 and 610 nm.
11. Intact plasma membrane and metabolic function of fungi are required to convert the yellow-green-fluorescent intracellular staining of FUN 1 into red-orange fluorescence. Calcofluor White M2R labels cell-wall chitin with blue-fluorescence regardless of metabolic state.
12. DMSO should be stored frozen at −20 °C. Because calcofluor dye solution may precipitate after several times of freezing-thawing, centrifuge the solution for 3 min at 10,000 × *g* before use.
13. Measure RNA concentration in 10 mM Tris–HCl, pH 8.0 at 260 nm. An absorbance of 1 corresponds to 40 μg/mL. A260:A280 ratio should be 1.8–2.0, but not <1.7 for RNA purity.
14. If there is no default melting curve program in the cycler, use the following program: 95 °C, 1 min; 65 °C, 2 min; 65–95 °C at 2 °C/min.

References

1. Kim J, Sudbery P (2011) *Candida albicans*, a major human fungal pathogen. J Microbiol 49:171–177
2. Ramage G, Martinez JP, Lopez-Ribot JL (2006) *Candida* biofilms on implanted biomaterials: a clinically significant problem. FEMS Yeast Res 6:979–986
3. Williams DW, Kuriyama T, Silva S et al (2011) *Candida* biofilms and oral candidosis: treatment and prevention. Periodontol 2000 55:250–265
4. Pereira-Cenci T, Del Bel Cury AA, Crielaard W et al (2008) Development of *Candida*-associated denture stomatitis: new insights. J Appl Oral Sci 16:86–94
5. Hasan F, Xess I, Wang X et al (2009) Biofilm formation in clinical *Candida* isolates and its association with virulence. Microbes Infect 11:753–761
6. Martinez LR, Fries BC (2010) Fungal biofilms: relevance in the setting of human disease. Curr Fungal Infect Rep 4:266–275
7. Chotirmall SH, McElvaney NG (2014) Fungi in the cystic fibrosis lung: bystanders or pathogens? Int J Biochem Cell Biol 52:161–173
8. Muller FM, Seidler M, Beauvais A (2011) *Aspergillus fumigatus* biofilms in the clinical setting. Med Mycol 49(Suppl 1):S96–S100
9. Yilmaz M, Mete B, Ozaras R et al (2011) *Aspergillus fumigatus* infection as a delayed manifestation of prosthetic knee arthroplasty and a review of the literature. Scand J Infect Dis 43:573–578
10. Clark D III, Ahmed MI, Sungur A et al (2013) Cardiac device-related invasive aspergilloma assessed by live/real time three-dimensional transthoracic echocardiography. Echocardiography 30:719–722

11. Wright PK, Raine C, Ragbir M et al (2006) The semi-permeability of silicone: a saline-filled breast implant with intraluminal and pericapsular *Aspergillus flavus*. J Plast Reconstr Aesthet Surg 59:1118–1121
12. Ramage G, Mowat E, Jones B et al (2009) Our current understanding of fungal biofilms. Crit Rev Microbiol 35:340–355
13. Seidler MJ, Salvenmoser S, Muller FM (2008) *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. Antimicrob Agents Chemother 52:4130–4136
14. Chatzimoschou A, Katragkou A, Simitsopoulou M et al (2011) Activities of triazole-echinocandin combinations against *Candida* species in biofilms and as planktonic cells. Antimicrob Agents Chemother 55:1968–1974
15. Simitsopoulou M, Peshkova P, Tasina E et al (2013) Species-specific and drug-specific differences in susceptibility of *Candida* biofilms to echinocandins: characterization of less common bloodstream isolates. Antimicrob Agents Chemother 57:2562–2570
16. Leggate J, Allain R, Isaac L et al (2006) Microplate fluorescence assay for the quantification of double stranded DNA using SYBR Green I dye. Biotechnol Lett 28:1587–1594

Chapter 15

Typing *Candida* Species Using Microsatellite Length Polymorphism and Multilocus Sequence Typing

Dea Garcia-Hermoso, Marie Desnos-Ollivier, and Stéphane Bretagne

Abstract

To gain more insight into the epidemiological relationships between isolates of *Candida* spp. obtained from various origins, several molecular typing techniques have been developed. Two methods have emerged in the 2000s as soon as enough knowledge of the *Candida* spp. genomes was available to choose adequate loci and primers, namely microsatellite length polymorphism (MLP) and multilocus sequence typing (MLST). To contrast with previous PCR-based methods, specific amplifications with stringent conditions easily reproducible are the basis of MLP and MLST. MLST relies on Sanger sequencing to detect single-nucleotide polymorphisms within housekeeping genes. MLP needs a first in silico step to select tandemly repeated stretches of two to five nucleotides. One of the two primers used to amplify a microsatellite locus is labeled and fragment sizing is automatically performed using high-resolution electrophoresis platforms. MLST provides results easily comparable between laboratories and active MLST schemes are publicly available for the main *Candida* species. For comparative studies, MLP needs standards to compensate for the electrophoretic variations depending on the platforms used. Both methods can help us gain insight into the genetic relatedness of fungal isolates, both with advantages and drawbacks, and the choice of one method rather than the other depends on the task in question.

Key words ITS sequencing, Mold identification, Non-sporulating molds, Respiratory specimens, Genotyping, Multilocus sequence typing, Microsatellite polymorphic marker, Short tandem repeat, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*

1 Introduction

Biotyping must be understood here as variation within a given species to address issues related to this species. The biotyping methods can be phenotypic or genotypic. The genotyping methods have been widely accepted because of their non-subjective interpretation. These genotyping methods are well adapted to some fundamental issues such as population genetics, reproduction modes, sexual recombination, phenotype–genotype relationships but also to more practical issues such as epidemics investigation, nosocomial acquisition, route of transmission, emergence of antifungal agent-resistant strains, infection reoccurrence in a given patient

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_15,

versus reinfection, or quality control of reference strains to detect drift after repeated subcultures.

The main desirable features of a genetic marker are (1) to have a mean mutation rate able to generate enough polymorphism to distinguish isolates but not too high to allow isolate grouping, (2) not to be under selective pressure, (3) to have a weak reversion rate, (4) to avoid homoplasia, and (5) to be independent. From the practical point of view, these markers must be accurate, successful for every isolate of a given species, reproducible, easy to interpret, rapid with a high throughput, and cheap. However, instead of searching for an ideal genotyping method with the highest level of discrimination, the objective should be to use the method which answers the question. For investigating *Candida* transmission between two patients, a unique marker able to discriminate two isolates can be sufficient, knowing that when identical genotypes even with multiple markers are observed, a well designed temporal study must be undergone to prove transmission between these two patients. For population genetics, several markers are mandatory, if possible well spread all over the genome to get the most reliable view of the entire genome. The numerical index of discriminatory power (D) is often used to synthetize the quality of the genetic marker based on the formula [1]:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j (x_j - 1)$$

where s is the number of profiles, xj is the number of the population falling into the jth type, and N is the size of the population.

The DNA-based methods developed during the 1980s were diverse. They included pulsed fields, restriction enzyme analysis, and restriction fragment length polymorphism (RFLP) associated with probe hybridization after Southern blotting [2]. These techniques were technically demanding, had a low throughput, and generated results difficult to standardize. They were replaced in the 1990s by PCR-based methods such as randomly amplified polymorphic DNA (RAPD) [2], single-strand conformation polymorphism analysis [3], and amplified fragment length polymorphism [4]. These methods could be used without any previous knowledge of the genome of the studied microorganism, but yielded fingerprint profiles that consist of complex banding patterns that were difficult to reproduce in different settings [2, 5]. For instance, PCR reaction with short and nonspecific primers and low temperature of hybridization as used in RAPD is impossible to reproduce when dealing with complex genomes. In the late 1990s and the early 2000s, two methods overcoming most of the previous limitations of PCR based methods have emerged, namely microsatellite length polymorphism (MLP) [6, 7], and multilocus sequence typing (MLST) [8].

In contrast to RAPD, MLP and MLST need previous knowledge of the DNA sequences of the targeted microorganisms to design primers in conserved regions to be able to amplify all the isolates of the species. To retrieve repeated sequences, several software are needed to select appropriated loci (e.g., http://c3.biomath.mssm.edu/trf.html) [9]. For MLST targeting housekeeping genes, preliminary comparative studies with a model organism such as *Saccharomyces cerevisiae* are also needed [10]. This has obvious pitfalls when dealing with a rare *Candida* species with no genome sequenced. On the other hand, the PCR reactions in MLP and MLST rely on very specific primers and reaction conditions, which assume reproducible PCR results.

MLST methods for *C. albicans* and *C. glabrata* have been described elsewhere [10, 11] but are described here since we report only the methods routinely used in our laboratory to genotype isolates of *C. albicans*, *C. glabrata*, *C. parapsilosis* (MLP) and *C. tropicalis*, *C. krusei* (MLST).

1.1 Microsatellites

The microsatellites, also named short tandem repeats, were first investigated for eukaryotic genomes [12]. They are part of the numerous DNA repeated fragments found in eukaryotic genomes mainly in noncoding regions, hence less susceptible to selective pressure. Microsatellites are defined as tandem stretches of two to five nucleotides repeated 5–100 times and are classically opposed to minisatellites which consist in longer repeats (8–100 bases) repeated 2 to >100 times often clustered in telomeric regions. The polymorphism of these markers relies on variation in the number of tandem repeats in a short core sequence according to the different isolates. The loci retained for genotyping are those where several alleles are empirically observed. The discriminatory power of the locus depends on the number of alleles observed.

MLP genotyping is based on the labeling of one of the two primers used to amplify a microsatellite locus and measurement of the amplicon length (Fig. 1). Fragment sizing is performed automatically using high-resolution electrophoresis platforms. Microsatellite alleles are often expressed as DNA fragments of different sizes obtained after PCR amplification with primers flanking the microsatellite region. The final data can be used as such in bp or converted into the corresponding number of repeats by comparison to reference strains. If several primer sets labeled with different dyes are used, multiplex PCR can be used to save time and increase throughput. Moreover, since microsatellite markers test the presence of different alleles at a given loci, distinguishing heterozygotes in diploid organisms such as *C. albicans* is easy, which is impossible with RFLP or RAPD methods (Fig. 2).

Based on the variations in repeat numbers, genetic relatedness between different isolates can be assessed. MLP genotyping has already been reported for *C. albicans* [13–16], *Candida glabrata*

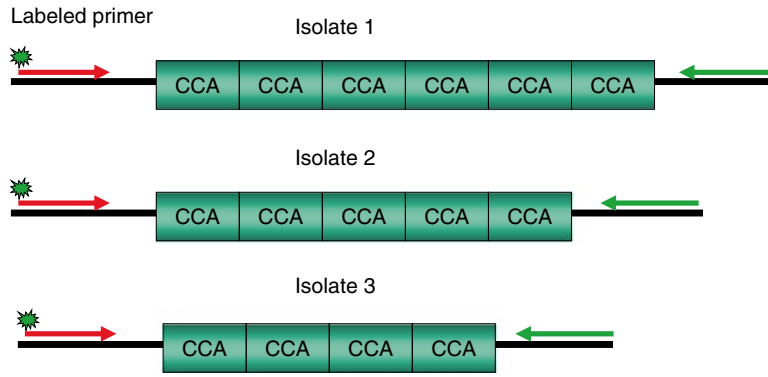


Fig. 1 Example of a polymorphic CCA microsatellite (or Short Tandem Repeats) at a given locus between three isolates. Thanks to the labeling of one primer, the PCR products can be easily differentiated on a sequencing electrophoretic gel according to the number of CCA repeats

[17, 18], *Candida tropicalis* [19], or *Candida parapsilosis* [20, 21]. Microsatellites provide high-resolution analysis that is consistent with MLST analysis [22].

Although the digital format is very attractive for exchanging results between laboratories, the present limitation of microsatellite typing is transferability. The size of the DNA fragments is calculated according to their electrophoretic mobility in capillary electrophoresis platforms. This calculation can be influenced by multiple factors such as exact base composition, separation matrix, presence of denaturing compounds, temperature, and fluorescent labels. Even the size standard and the polymerase that is used for amplification may affect the calculated size of an allele [23]. Thus, careful calibration of the different platforms should be established [23, 24]. A straightforward and universally applicable method to achieve such a calibration is through the use of allelic ladders [25]. An allelic ladder consists of a well-defined mixture of alleles with predetermined repeat numbers and can be used to create reference positions for the interpretation of typing results (Fig. 2).

MLP is also subject to homoplasia, which can hamper the accuracy of the results. High-resolution DNA melting (HRM) analysis and SNaPshot minisequencing after a single amplification can help to investigate this possibility, but can also on the other hand add polymorphism and increase the discriminatory power of a single MLP marker [26].

1.2 Multilocus Sequence Typing

MLST is widely used in bacteriology and relies on DNA sequence analysis of housekeeping genes MLST [27]. The starting point is selecting genes with enough single-nucleotide polymorphism to differentiate isolates but with enough common sequences to design

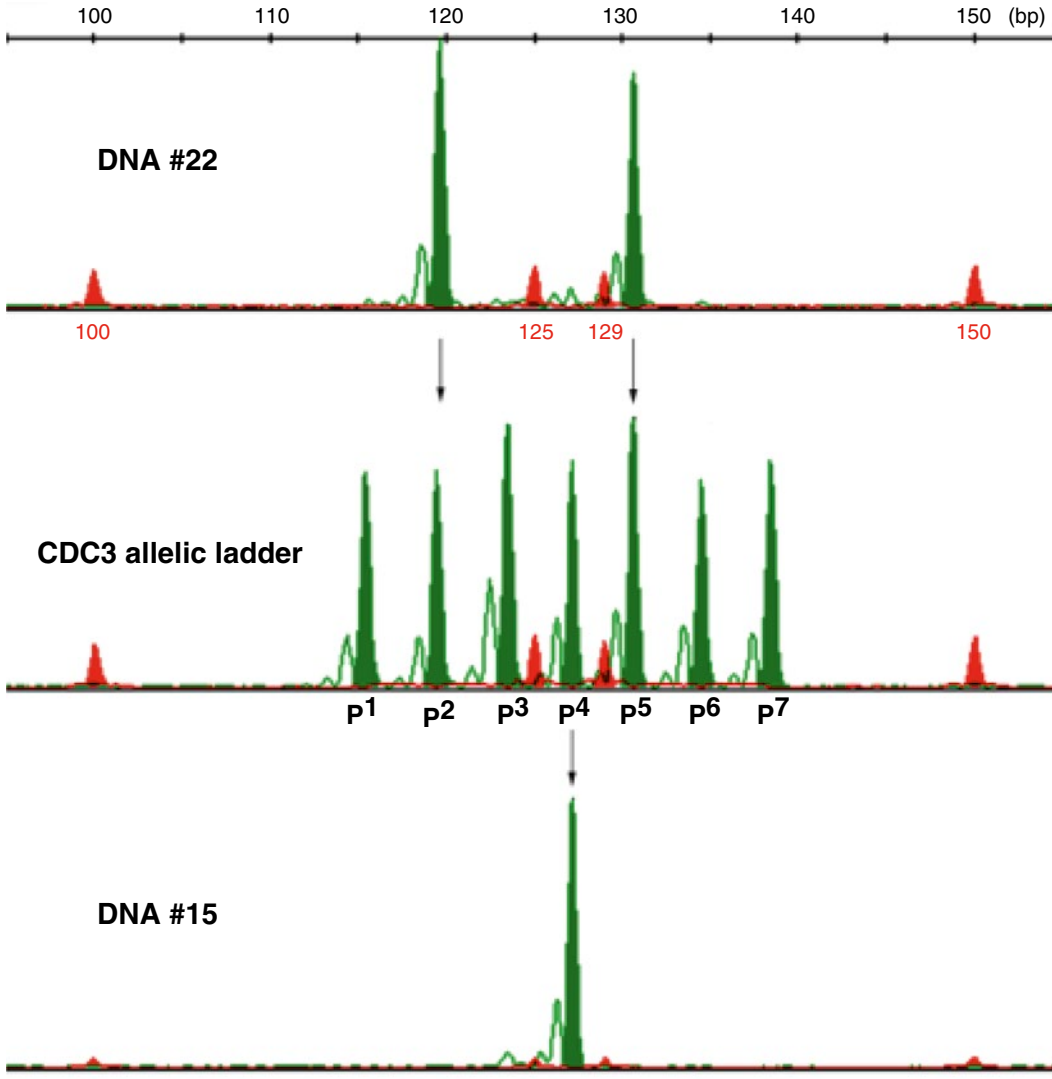


Fig. 2 Example of allele assignment using the CDC3 allelic ladder for two isolates, numbers 22 and 15. Allele peaks in the ladder (*green peaks*) are marked as p1–p7. GeneFlo 625 internal size standards (*red peaks*) with sizes in bp are shown beneath each peak. Isolate 15 is p2–p5 heterozygous, and isolate 15 is p4 homozygous [after ref. [25]]

primers able to amplify every isolate of the studied species. For this purpose, housekeeping genes fulfill the prerequisite conditions. MLST measures the DNA sequence variations in the selected genes and characterizes strains by their unique allelic profiles. Approximately 450–500 bp internal fragments of each gene are used, which is the length of the PCR product providing readable chromatograms upon Sanger sequencing. For each gene, the different sequences are assigned as distinct alleles (number) and, for each isolate, the alleles at each of the loci define the allelic profile

or sequence type (ST) or diploid sequence type (DST) for diploid microorganisms. The main advantage of MLST is the ability to provide indisputable data based on sequencing with Sanger methodology and the possibility to compare the results with those deposited in data banks (http://calbicans.mlst.net/).

This method was first developed for *Candida albicans* in sequencing five to seven housekeeping genes [10] and a consensus on the methodology was reached [8]. Similar MLSTs were developed for *C. glabrata* [11], *C. tropicalis* [28], and *Candida krusei* [29]. Beside *C. albicans*, active MLST schemes are publicly available for *C. glabrata* (http://cglabrata.mlst.net/), *C. tropicalis* (http://pubmlst.org/ctropicalis/), and *C. krusei* (http://pubmlst.org/ckrusei/). There are few technical limits to MLST when the Sanger sequencing provides readable chromatograms. Sequencing of both stands is recommended to avoid reading mistakes.

1.3 Comparison of MLST and MLP

Both methods are useful tools for genotyping of *Candida* isolates with high typeability, discriminatory power and good reproducibility. MLST data are exportable using online databases whereas MLP data can only be exportable when results are normalized with an allelic ladder. MLST method is more time and money consuming compared to MLP. Comparison of MLST using seven genes of *C. albicans* with three microsatellite markers showed a similar discriminatory power [22]. As MLP, MLST can easily give access to study heterozygosity in diploid microorganisms.

2 Materials

This section should list the composition of all buffers, media, solutions, specialist equipment, etc., that are necessary for carrying out the method described in Subheading 3. Suppliers are not needed for routine reagents (the reader will use his/her own local supplier) and catalog numbers are not required at all for reagents. All buffers, solutions and media should be presented in the same format, i.e., name, colon then composition on one continuous line, with components separated by commas not semicolons.

The five major yeast species responsible of invasive human infection are ascomycetes belonging to the order of *Saccharomycetales*: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (currently named *Pichia kudriavzevii*). *Candida albicans*, *C. parapsilosis*, and *C. tropicalis* belong to *Lodderomyces/Candida albicans* clade in the family *Debaryomycetaceae* [30]. *Candida glabrata* has been reclassified in the genus *Nakaseomyces* in the family

Saccharomycetaceae and *P. kudriavzevii* belong to the family *Pichiaceae* [30, 31]. See also; Mycobank at http://www.mycobank.org/.

1. Genotype for *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are determined after purity check by using BBLChromagar and species identification by carbon assimilation profile determination and/or ITS sequencing for *C. parapsilosis* or duplex ITS/actin PCR for *C. albicans* [32].
2. Reference strains used as positive controls (PCR and allelic size control): *C. albicans* (B311, diploid), *C. glabrata* (ATCC2001, haploid), *C. parapsilosis* (ATCC22019, diploid), *C. tropicalis* (ATCC750, diploid), *C. krusei* (ATCC6258, diploid).
3. Yeasts are subcultured on Sabouraud dextrose agar plates for 24 h at 30 °C. DNA is extracted using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions and stored at −20 °C until use.
4. For PCR reactions: Amplitag Gold DNA polymerase, $MgCl_2$, PCR buffer 10× (Roche, Applied Biosystems); deoxynucleotide (dNTP) solution mix at 25 mM; one primer of each pair (forward or reverse) used in the microsatellite assays are labeled in 3′, with one of the following fluorochromes:

   HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein).

   6FAM (6-carboxyfluorescein).

   NED (2′-chloro-5′-fluoro-7′,8′-fused phenyl-1.4-dichloro-6-carboxyfluorescein).

   TET (6-carboxy-2′,4,7,7′-tetrachlorofluorescein).
5. PCRs are performed on an iCycler thermocycler (Bio-Rad, Hercules, CA).
6. Hi-Di Formamide and Geneflo 625 DNA ladder Rox labeled (6-carboxy-X-rhodamine, succinimidyl ester, Eurx) are used for the microsatellite mix.
7. Conical V-bottom 96-well microplates are used for microsatellites assays.
8. Double strand sequencing and capillary electrophoresis are performed on an ABIPrism 3730 XL (Applied Biosystems).
9. For MLP analysis, the determination of allelic sizes is performed using GeneMapper or PeakScanner software (Applied Biosystems).
10. For MLST analysis, the chromatogram analysis is performed using Geneious software or any other sequence editing software (Chromas, Sequencher, etc.) (*see* **Note 1**).

3 Methods

3.1 Microsatellites (MLP)

To assign a specific length to a PCR fragment, we systematically test the reference strain in all the PCR runs. To observe stutter peaks is normal due to artifacts of the DNA polymerase when encountering short tandem repeats [23]. The last highest peak is to be considered. Therefore, each allele is named according to the length in bp of the amplified fragment after alignment with the reference strain. In the case of diploid species (*C. albicans* and *C. parapsilosis*), for each marker and for a given isolate, one or two peaks can be observed. Then each peak observed is assigned to an allele. When we observe electropherograms harboring one signal for a given locus, we consider the isolates to be homozygous for this locus [33]. For each species, microsatellite repeat types, primer sequences, gene products and chromosomic locations are listed in Tables 1, 2, and 3. For *C. albicans*, an allelic ladder for CDC3 marker is used to determine allelic size [25].

3.1.1 Candida albicans

Five microsatellites markers are amplified in duplex (loci CDC3/EF3) or uniplex (loci HIS3, CDR1, and STPK) PCR using the following conditions;

(a) Reaction volume of 20 μL contained 2 μL of genomic DNA, 1.25 U of DNA polymerase, 2 μL of 10× PCR Buffer, 5 mM of $MgCl_2$, a 0.25 mM concentration of dNTP, 10 pmol (for EF3), 5 pmol (for HIS3), and 2 pmol (for CDC3, CDR1, and STPK) primers.

(b) The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 30 cycles (or 27 cycles for CDR1 and STPK loci) of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension step of 30 min at 72 °C.

3.1.2 Candida glabrata

Five microsatellites markers are amplified by duplex (Cg4 and Cg6) or multiplex (RPM2/ERG3 and MTIA) PCR using the following conditions:

(a) Reaction volume of 20 μL contained 1 μL of genomic DNA, 1.25 U of DNA polymerase, 2 μL of 10× PCR Buffer, 5 mM of $MgCl_2$, a 0.25 mM concentration of dNTP, 10 pmol (for ERG3, MTIA, Cg4), 5 pmol (for RPM2 and Cg6) primers.

(b) The PCR program for the duplex PCR, consisted of an initial denaturation step at 95 °C for 5 min, followed by 27 cycles of 30 s at 95 °C, 30 s at 52 °C, and 45 s at 72 °C, with a final extension step of 10 min at 72 °C.

(c) The PCR program for the multiplex PCR, consisted of an initial denaturation step at 95 °C for 10 min, followed by 27 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a inal extension step of 5 min at 72 °C.

Table 1
Description of the microsatellites markers used to genotype isolates of *Candida albicans*

Locus, chromosome	Gene product	Primer	Sequence 5′–3′	5′Dye	Repeat type	Size range	Reference
EF3, chr 5	Elongation factor 3	EF3a EF3b	TTTCCTCTTCCTTTCATATAGAA GGATTCACTAGCAGCAGACA	6FAM	TTTC-TTC	122–143	[6]
CDC3, chr 1	Cell division cycle protein	CDC3a CDC3b	CAGATGATTTTTTGTATGAGAAGAA CAGTCACAAGATTAAAATGTTCAAG	HEX	AGTA	112–140	[7]
HIS3, chr 2	Imidazole glycerol phosphate dehydratase	HIS3F HIS3R	TGGCAAAAATGATATTCCAA TACACTATGCCCCAAACACA	NED	ATTT	144–184	[7]
CDR1, chr3	Multidrug resistance protein	CDR1a CDR1b	GACATGGTGGTATCTCAAACG AGAGTCGGCATAATCCAATC	HEX	TTG	224–245	–
STPK, chr 5	Serine/threonine protein kinase	STPKf C4STPKB	CAAGACGAGGTTGACACTGG TGTCACTATCATCCCCTTGG	6FAM	AAC	209–226	–

Table 2
Description of the microsatellites markers used to genotype isolates of *Candida glabrata*

Locus, chromosome	Gene product	Primer	Sequence 5′–3′	5′Dye	Repeat type[a]	Size range	Reference
RPM2, chr I	Mitochondrial RNAseP precursor	RPM2f RPM2r	ATCTCCCAACTTCTCGTAGCC ACTTGAACGACTTGAACGCC	6FAM	AGCACA	144	[17]
ERG3, chr F	5,6 sterol desaturase	ERGf ERGr	AGTGCGAGTGTATGTAAAGAATG CGTATACCTTATCTCCGTTCAA	HEX	TACAAGTAG	183	[17]
MTI, chr D	Metallothionein I	MTIF MTIR	CAGCAATAATAGCTTCTGACTATGAC GACAGGAGCAACCGTTAGGA	6FAM	ACAACAA	251	[17]
chr E	Noncoding region	Cg4f Cg4r	AATGCGTGTGTGTGCGTAGT AAAAATTTAGGCCCCATCG	HEX	$(GT)_{19}$	228–252	[18]
chr E	Noncoding region	Cg6f Cg6r	AGCAAGAGGGAGGAGGAAAC AAATCCGGGGATAGATGAGG	6FAM	$(CA)_{12}$	301–345	[18]

[a]Based on ATCC2001 sequences

Table 3
Description of the three microsatellites loci used to genotype isolates of *Candida parapsilosis sensu stricto* [21]

Locus	Primer	Sequence 5′–3′	5′ Dye	Annealing temperature (°C)	Repeat type	Size range
Contig 005807	CP1f CP1r	AAAGTGCTACACACGCATCG GGCTTGCAATTTCATTTCCT	TET	58	$(AAG)_{27}$	213–297
Contig 006372	CP4f CP4r	CAAATCATCCAGCTTCAAACC CATCAAACAAGAATTCGATATCAC	HEX	60	$(AAC)_{29}$	276–447
Contig 005809	CP6f CP6r	CAGGAACAGGACAATGGTGA TCTGGAGCCTCTAGGACGTTT	6FAM	60	$(AAC)_{48}$	183–327

3.1.3 *Candida parapsilosis*

Three microsatellites markers (CP1, CP4, and CP6) are amplified in uniplex PCR using the following conditions:

(a) Reaction volume of 25 µL contained 2 µL of genomic DNA, 1.25 U of DNA polymerase, 2.5 µL of 10× PCR Buffer, 1.5 mM of $MgCl_2$, a 0.2 mM concentration of dNTP, 2.5 pmol for each primer.

(b) The PCR program consisted of an initial denaturation step at 95 °C for 10 min, followed by 28 cycles of 30 s at 95 °C, 30 s at 58 °C (for CP1) or at 60 °C (for CP4 and CP6), and 30 s at 72 °C, with a final extension step of 7 min at 72 °C.

3.1.4 *Microsatellite Mix*

Following PCR, 2 µL of the amplification product is added to 13 µL of HiDi-Formamide and to 0.5 µL of Geneflo 625 ladder (*see* **Note 2**).

3.2 *MultiLocus Sequence Typing (MLST)*

Consensus sequences are edited by comparison of both DNA strands using the one-letter code for nucleotides from the International Union of Pure and Applied Chemistry (IUPAC) for heterozygous nucleotides. Fragmented sequences are then obtained after delimitation by start and end-sequences (Tables 4 and 5).

For each gene, distinct alleles were identified and numbered using the MLST database. The Sequence Type (ST) is the result of combination of the alleles at the different loci. For each species primers sequences are listed in Tables 4 and 5.

3.2.1 *Candida tropicalis*

Six loci (ICL1, MDR1, SAPT2, SAPT4, XYR1, and ZWF1a) are amplified by PCR according to Tavanti *et al.* [28] with slight modifications:

(a) Reaction volume of 50 µL contained 3 µL of genomic DNA, 2.5 U of DNA polymerase, 5 µL of 10× PCR Buffer, 2.5 mM of $MgCl_2$, a 0.25 mM concentration of dNTP, 10 pmol for each primer.

Table 4
Description of the MLST markers to genotype isolates of *Candida tropicalis* [28]

Locus	Primer	5′–3′ Sequence	Amplification size (bp)	Sequence start	Sequence end	Sequenced fragment length (bp)
ICL1	ICL1f ICL1r	CAACAGATTGGTTGCCATCAGAGC CGAAGTCATCAACAGCCAAAGCAG	737	CGAAGCTG	TGGCAATT	447
ZWF1α	ZWF1f ZWF1r	GGTGCTTCAGGAGATTTAGC ACCTTCAGTACCAAAAGCTTC	647	TGCCTTGTTT	ATTGTTCAGT	520
SAPT2	SAPT2f SAPT2r	CAACGATCGTGGTGCTG CACTGGTAGCTGAAGGAG	658	CTGGTGTC	TXTTCCAA	525
SAPT4	SAPT4f SAPT4r	AGTTGGTTTCGGATGTTG TCGTAAATCAAAGCACCAGT	483	CATXATTA	CAACAATT	390
XYR1	XYR1f XYR1r	TGCTTCTCCTACAACTTCACCTCC ATTCCCATGACTCCCTGAGCAACA	479	TCTACAAT	AAATTGGT	370
MDR1	MDR1f MDR1r	TGTTGGCATTCACCCTTCCT TGGAGCACCAAACAATGGGA	663	TGATGGTG	GCCYTTAT	425

Table 5
Description of the MLST markers to genotype isolates of *Candida krusei* [29]

Locus	Primer	5′–3′ sequence	Amplification size (bp)	Sequence start	Sequence end	Sequenced fragment (bp)
ADE2	CK-ADE2f CK-ADE2r	GTCACTTCTCAGTTTGAAG ACACCATCTAAAGTAGAGCC	600	AAACAAAT	CTCATTTA	470
LYS2	CK-LYS2f CK-LYS2r	ATCTGAGAAGCAGTTGGCGC AGACTTGTAAGAATTATCCC	631	AAAGATTG	TCTGAACT	441
LEU2	CK-LEU2f CK-LEU2r	CTGTGAGACCAGAACAGGGG GCAGAGCCACCCAAGTCTCC	802	GTAACTTT	AAGCTCTC	619
HIS3	CK-HIS3f CK-HIS3r	GGAGGGGACATATCACTGCC AATCTTTAATTGCCAAAGCC	534	AATCCCAA	GTTGATTG	400
TRP1	CK-TRP1f CK-TRP1r	AGCTATGTCGAGCAAAGAGG ACATCAACGCCACAACACCC	503	ATATGAGG	CAGGTGGG	380
NMT1	CK-NMT1f CK-NMT1r	CTGATGAAGAAATCACCG GCTTGATATCATCTTTGTCC	738	GCTTCATTT	GAAGTGAA	537

(b) The PCR program consisted of an initial denaturation step at 94 °C for 7 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min 5 s at 72 °C, with a final extension step of 10 min at 72 °C.

(c) Sequences compared to the database online (http://pubmlst.org/ctropicalis/) developed by Jolley and sited at the University of Oxford [34].

3.2.2 *Candida krusei*

Six loci (ADE2, HIS3, LEU2, LYS2, NMT1, and TRP1) are amplified by PCR according to Jacobsen *et al.* [29] with slight modifications:

(a) Reaction volume of 50 μL contained 3 μL of genomic DNA, 1.5 U of DNA polymerase, 5 μL of 10× PCR Buffer, 2.5 mM of $MgCl_2$, a 0.25 mM concentration of dNTP, 10 pmol for each primer.

(b) The PCR program consisted of an initial denaturation step at 94 °C for 7 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C.

(c) Sequences compared to the database online (http://pubmlst.org/ckrusei/) developed by Jolley and sited at the University of Oxford [34].

4 Conclusion and Perspectives

Genotyping based on MLST or MLP can help us gain insight into the genetic relatedness of fungal isolates. Both have advantages and drawbacks depending on the task in question. Beside these well-established typing methods, other technologies are appearing in mycology, whereas they are now well used in bacteriology. Next-generation sequencing (NGS) technology is probably revolutionizing the way of genotyping microorganisms although the size of the fungal genomes makes NGS more expensive than for bacteria. The use of such advanced methods is currently restricted to specialized laboratories, but wider applications are possible in the near future.

5 Notes

1. When no DNA is amplified upon MLP or MLST, one can check for the identification of the isolate, since one of the main advantages of MLP and MLST is to be specific for the species studied.

2. When more alleles than expected are observed with MLP (*e.g.*, three peaks for a diploid organism), this suggests a mixture of isolates. Checking purity can be performed to exclude this possibility. This last point is hardly investigatable using MLST since Sanger sequencing is not able to detect minority allele under 40 %.

References

1. Hunter PR (1991) A critical review of typing methods for *Candida albicans* and their applications. Crit Rev Microbiol 17:417–434
2. Soll DR (2000) The ins and outs of DNA fingerprinting the infectious fungi. Clin Microbiol Rev 13:332–370
3. Graser Y, Volovsek M, Arrington J, Schonian G, Presber W, Mitchell TG, Vilgalys R (1996) Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. Proc Natl Acad Sci U S A 93: 12473–12477
4. Tavanti A, Hensgens LA, Ghelardi E, Campa M, Senesi S (2007) Genotyping of *Candida orthopsilosis* clinical isolates by amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients. J Clin Microbiol 45:1455–1462
5. Gil-Lamaignere C, Roilides E, Hacker J, Muller FM (2003) Molecular typing for fungi—a critical review of the possibilities and limitations of currently and future methods. Clin Microbiol Infect 9:172–185
6. Bretagne S, Costa JM, Besmond C, Carsique R, Calderone R (1997) Microsatellite polymorphism in the promoter sequence of the elongation factor 3 gene of *Candida albicans* as the basis for a typing system. J Clin Microbiol 35:1777–1780
7. Botterel F, Desterke C, Costa C, Bretagne S (2001) Analysis of microsatellite markers of *Candida albicans* used for rapid typing. J Clin Microbiol 39:4076–4081
8. Bougnoux ME, Tavanti A, Bouchier C, Gow NA, Magnier A, Davidson AD, Maiden MC, D'Enfert C, Odds FC (2003) Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. J Clin Microbiol 41:5265–5266
9. Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 27:573–580
10. Bougnoux ME, Morand S, d'Enfert C (2002) Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. J Clin Microbiol 40:1290–1297
11. Dodgson AR, Pujol C, Denning DW, Soll DR, Fox AJ (2003) Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. J Clin Microbiol 41:5709–5717
12. Bently DR, Dunham I (1995) Mapping human chromosomes. Curr Opin Genet Dev 5:328–334
13. Eloy O, Marque S, Botterel F, Stephan F, Costa JM, Lasserre V, Bretagne S (2006) Uniform distribution of three *Candida albicans* microsatellite markers in two French ICU populations supports a lack of nosocomial cross-contamination. BMC Infect Dis 6:162
14. Stephan F, Bah MS, Desterke C, Rezaiguia-Delclaux S, Foulet F, Duvaldestin P, Bretagne S (2002) Molecular diversity and routes of colonization of *Candida albicans* in a surgical intensive care unit, as studied using microsatellite markers. Clin Infect Dis 35:1477–1483
15. Albano L, Bretagne S, Mamzer-Bruneel MF, Kacso I, Desnos-Ollivier M, Guerrini P, Le Luong T, Cassuto E, Dromer F, Lortholary O (2009) Evidence that graft-site candidiasis after kidney transplantation is acquired during organ recovery: a multicenter study in France. Clin Infect Dis 48:194–202
16. Sampaio P, Gusmao L, Correia A, Alves C, Rodrigues AG, Pina-Vaz C, Amorim A, Pais C (2005) New microsatellite multiplex PCR for *Candida albicans* strain typing reveals microevolutionary changes. J Clin Microbiol 43:3869–3876
17. Foulet F, Nicolas N, Eloy O, Botterel F, Gantier JC, Costa JM, Bretagne S (2005) Microsatellite marker analysis as a typing system for *Candida glabrata*. J Clin Microbiol 43:4574–4579
18. Grenouillet F, Millon L, Bart JM, Roussel S, Biot I, Didier E, Ong AS, Piarroux R (2007) Multiple-locus variable-number tandem-repeat analysis for rapid typing of *Candida glabrata*. J Clin Microbiol 45:3781–3784
19. Desnos-Ollivier M, Bretagne S, Bernede C, Robert V, Raoux D, Chachaty E, Forget E,

Lacroix C, Dromer F (2008) Clonal population of flucytosine-resistant *Candida tropicalis* from blood cultures, Paris, France. Emerg Infect Dis 14:557–565

20. Lasker BA, Butler G, Lott TJ (2006) Molecular genotyping of *Candida parapsilosis* group I clinical isolates by analysis of polymorphic microsatellite markers. J Clin Microbiol 44:750–759
21. Sabino R, Sampaio P, Rosado L, Stevens DA, Clemons KV, Pais C (2010) New polymorphic microsatellite markers able to distinguish among *Candida parapsilosis* sensu stricto isolates. J Clin Microbiol 48:1677–1682
22. Garcia-Hermoso D, Cabaret O, Lecellier G, Desnos-Ollivier M, Hoinard D, Raoux D, Costa JM, Dromer F, Bretagne S (2007) Comparison of microsatellite length polymorphism and multilocus sequence typing for DNA-based typing of *Candida albicans*. J Clin Microbiol 45:3958–3963
23. de Valk HA, Meis JF, Klaassen CH (2007) Microsatellite based typing of *Aspergillus fumigatus*: strengths, pitfalls and solutions. J Microbiol Methods 69:268–272
24. Pasqualotto AC, Denning DW, Anderson MJ (2007) A cautionary tale: lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J Clin Microbiol 45:522–528
25. Garcia-Hermoso D, MacCallum DM, Lott TJ, Sampaio P, Serna MJ, Grenouillet F, Klaassen CH, Bretagne S (2010) Multicenter collaborative study for standardization of *Candida albicans* genotyping using a polymorphic microsatellite marker. J Clin Microbiol 48:2578–2581
26. Costa JM, Garcia-Hermoso D, Olivi M, Cabaret O, Farrugia C, Lecellier G, Dromer F, Bretagne S (2010) Genotyping of *Candida albicans* using length fragment and high-resolution melting analyses together with minisequencing of a polymorphic microsatellite locus. J Microbiol Methods 80:306–309
27. Enright MC, Spratt BG (1999) Multilocus sequence typing. Trends Microbiol 7:482–487
28. Tavanti A, Davidson AD, Johnson EM, Maiden MC, Shaw DJ, Gow NA, Odds FC (2005) Multilocus sequence typing for differentiation of strains of *Candida tropicalis*. J Clin Microbiol 43:5593–5600
29. Jacobsen MD, Gow NA, Maiden MC, Shaw DJ, Odds FC (2007) Strain typing and determination of population structure of *Candida krusei* by multilocus sequence typing. J Clin Microbiol 45:317–323
30. Daniel HM, Lachance MA, Kurtzman CP (2014) On the reclassification of species assigned to *Candida* and other anamorphic ascomycetous yeast genera based on phylogenetic circumscription. Antonie Van Leeuwenhoek 106:67–84
31. Lachance MA, Boekhout T, Scorzetti G, Fell JW, Kurtzman CP (2011) *Candida* Berkhout (1923). In: Kurtzman CP, Fell JW, Boekhout T (eds) The Yeasts, a taxonomic study. Elsevier, Amsterdam, pp 987–1278
32. Donnelly SM, Sullivan DJ, Shanley DB, Coleman DC (1999) Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of ACT1 intron and exon sequences. Microbiology 145(Pt 8):1871–1882
33. Costa JM, Eloy O, Botterel F, Janbon G, Bretagne S (2005) Use of microsatellite markers and gene dosage to quantify gene copy numbers in *Candida albicans*. J Clin Microbiol 43:1387–1389
34. Jolley KA, Chan MS, Maiden MC (2004) Mlstdbnet—distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics 5:86

Chapter 16

Diagnostic Methods for Detection of Blood-Borne Candidiasis

Cornelius J. Clancy and M. Hong Nguyen

Abstract

β-D-glucan (Fungitell) and polymerase chain reaction-based (T2Candida) assays of blood samples are FDA-approved adjuncts to cultures for diagnosing candidemia and other types of invasive candidiasis, but their clinical roles are unclear. In this chapter, we describe laboratory protocols for performing Fungitell and T2Candida assays. We then discuss step-by-step methods for interpreting test results at the bedside using a Bayesian framework, and for incorporating assays into rational patient management strategies. Prior to interpreting results, clinicians must recognize that test performance varies based on the type of invasive candidiasis being diagnosed. In general, the type of invasive candidiasis that is most likely in a given patient can be identified, and the pretest likelihood of disease estimated. From there, positive and negative predictive values (PPV, NPV) for an assay can be calculated. At a population level, tests can be incorporated into screening strategies for antifungal treatment. NPV and PPV thresholds can be defined for discontinuing antifungal prophylaxis or initiating preemptive treatment, respectively. Using the thresholds, it is possible to assign windows of pretest likelihood for invasive candidiasis (and corresponding patient populations) in which tests are most likely to valuable. At the individual patient level, tests may be useful outside of the windows proposed for screening populations. The interpretive and clinical decision-making processes we discuss will be applicable to other diagnostic assays as they enter the clinic, and to existing assays as more data emerge from various populations.

Key words Candidemia, Invasive candidiasis, Intra-abdominal candidiasis, β-D-glucan, Fungitell, Polymerase chain reaction (PCR), T2Candida, Diagnosis, Bayesian

1 Introduction

The development of new diagnostic tests for candidemia and other forms of invasive candidiasis is among the top priorities in infectious diseases [1, 2]. Blood and deep tissue cultures, the gold standard tests, have sensitivity of only 50 %, turnaround times of several days, and turn positive late in the course of disease [1]. Tissue cultures are further limited by need for invasive sampling procedures, which are often dangerous and infeasible in patients at-risk for candidiasis. Non-culture diagnostics like β-D-glucan and polymerase chain reaction (PCR) assays are directed against constituents

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4_16,

of Candida cells rather than viable organisms [1]. At present, two assays of blood samples are approved by the U.S. Food and Drug Administration as adjuncts to cultures for the diagnosis of invasive candidiasis. The older and more widely employed test is the Fungitell serum β-D-glucan assay (Associates of Cape Cod, East Falmouth, MA), which is cleared for diagnosing a variety of invasive fungal infections. The assay does not provide Candida speciation nor distinguish between Candida, Aspergillus and certain other fungi. The more recently approved T2Candida assay (T2 Biosystems, Lexington, MA) uses a self-contained instrument to amplify detect Candida DNA within whole blood by PCR and T2 magnetic resonance, respectively. Used thoughtfully as adjuncts to cultures, these tests may identify more patients with invasive candidiasis, at earlier stages of disease. Despite growing literature on β-D-glucan and PCR-based assays, there is much confusion about test performance in the clinic, and how to interpret and best utilize results when caring for patients [3].

In this chapter, we describe methods for using Fungitell β-D-glucan and T2Candida assays in the laboratory and clinic. First, we provide detailed protocols for performing the assays (Subheadings 3.1 and 3.2). Then, we describe step-by-step methods for interpreting results at the bedside using a Bayesian framework (Subheadings 3.3 and 3.4), and for incorporating tests into rational patient management strategies (Subheadings 3.5 and 3.6). The interpretive and clinical decision-making processes that we describe will be applicable to other diagnostic assays as they enter clinical practice, and to existing assays as more data emerge from various patient populations. Our discussion focuses on candidemia and intra-abdominal candidiasis, the most common types of invasive candidiasis.

2 Materials

2.1 Fungitell Assay (See Note 1*)*

2.1.1 Materials Supplied with the Kit (See Note 2*)*

1. Fungitell Reagent, a lyophilized (1,3)-β-D-Glucan specific Limiulus Amebocyte Lysate (LAL; two vials).
2. Pyrosol Reconstitution Buffer, Tris–HCl 0.2 M pH 7.4 (two vials).
3. Glucan standard (two vials).
4. Reagent grade water (RGW) (two bottles).
5. Flat-bottom, 96-well, uncoated microplates, with lids (two).
6. KCl 1.2 M and KOH 0.25 M (one vial each).

2.1.2 Materials Not Supplied with the Kit (See Note 3*)*

1. Pipette tips* (250 μL) (*see* **Note 4**).
2. Pipettors capable of delivering 5–25 μL and 100–1000 μL volumes.

3. Stepper pipettor, with syringe tips, capable of delivering 100 µL.
4. Test tubes* (13 × 100 mm borosilicate glass).
5. Incubating (37 °C) plate reader capable of dual wavelength monitoring at 405 and 490 nm, with a dynamic range up to ≥2.0 Absorbance Units, coupled with appropriate computer-based kinetic assay software.
6. Sterile, glucan-free, screw-cap storage tubes for aliquotting samples (most tubes that are certified to be RNAse, DNAse, and pyrogen-free are free of interfering levels of (1,3)-β-D-Glucan).
7. Parafilm.

2.2 T2Candida Assay (See Note 5)

1. T2Candida Panel, comprised of the T2Candida Cartridge and T2Candida Reagent Pack (*see* **Note 6**).

2.2.1 Materials Supplied with the Kit

2. T2Candida Cartridge includes T2Candida Base (calcium hypochlorite and lysis reagent comprised of a detergent mix and 0.09 % sodium azide in an aqueous buffer solution) and T2Candida Sample Inlets (12 single-use bases and inlets per box).
3. T2Candida Reagent Pack includes internal control (aqueous buffered solution containing internal control DNA, carrier DNA and 0.09 % sodium azide preservative), reagent A (aqueous buffered solution containing dNTPs, *Candida* primers, and 0.09 % sodium azide preservative), enzyme solution (polymerase), *Candida albicans/C. tropicalis* particles, *C. parapsilosis* particles, *C. krusei/C. glabrata* particles, and Candida internal control particles (probe-coupled superparamagnetic particles that hybridize to amplicons of given spp. or internal control sequence, in an aqueous buffered solution containing 0.09 % sodium azide preservative).

2.2.2 Materials Not Supplied with the Kit

1. T2Dx Instrument and Barcode Scanner (*see* **Note 7**).
2. Bleach (household bleach at 5 % sodium hypochlorite, such as Clorox diluted 1:10), Bleach-Rite Disinfecting Spray or equivalent.
3. 70 % Isopropyl Alcohol.
4. Powderless disposable gloves, lint-free wipes, absorbent pads.
5. Distilled or deionized water.
6. Biohazard waste bags.
7. APG External Positive Control, TPK External Positive Control, Negative External Control (*see* **Note 8**).

3 Methods

3.1 Performing the Fungitell Assay

3.1.1 Sample Collection (*See* Note 9)

1. Collect serum samples in sterile vacuum tubes (red tops) or serum separator tubes (SST), and allow them to clot. Separate serum from clot and decant to a suitable container that is free of interfering levels of (1,3)-β-D-Glucan.
2. Store serum samples at 2–8 °C before assay, or freeze at ≤−20 °C. Conduct testing promptly to minimize the possibility of sample degradation (*see* **Note 10**).

3.1.2 Testing Procedure (*See* Note 11)

1. Set plate reader software to collect data in the Vmean mode. Ensure proper settings such that the value calculated is the mean rate of optical density change for all data points gathered (*see* **Note 12**). The curve fit setting should be "linear/linear" or equivalent.
2. Set the interval between reads to the minimum allowed over the 40 min test period.
3. Set software wavelength as 405 nm minus the background at 490 nm. If dual wavelength reading is not available, read at 405 nm.
4. Set incubation temperature at 37 °C.
5. Shake the plate for 5–10 s prior to the start of reading. Perform reading without lag time.
6. Plan the microtiter plate lay-out to include (in duplicate) standards (St; 25 μL/well), negative controls (Neg) and unknowns (Uk) (Fig. 1). Outside wells may be used if it has been demonstrated that performance is comparable to that of internal wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		St1	St1		Uk1	Uk4	Uk7	Uk10	Uk13	Uk16	Uk19	
C		St2	St2		Uk1	Uk4	Uk7	Uk10	Uk13	Uk16	Uk19	
D		St3	St3		Uk2	Uk5	Uk8	Uk11	Uk14	Uk17	Uk20	
e		St4	St4		Uk2	Uk5	Uk8	Uk11	Uk14	Uk17	Uk20	
f		St5	St5		Uk3	Uk6	Uk9	Uk12	Uk15	Uk18	Uk21	
G		Neg	Neg		Uk3	Uk6	Uk9	Uk12	Uk15	Uk18	Uk21	
H												

Fig. 1 Fungitell β-D-Glucan plate layout. Example of a typical layout, in which samples are plated in duplicate

7. Dissolve one vial of the Glucan standard with the volume of RGW stated on the vial, to make a 100 pg/mL solution. Vortex for at least 30 s to resuspend (creating solution 1). Store the glucan solution at 2–8 °C, and use within 3 days.
8. Prepare a standard curve by first mixing 500 µL RGW and 500 µL of solution 1 in a glucan-free tube (solution 2; 50 pg/mL). Repeat dilution scheme through solution 5 (6.25 pg/mL). Vortex for at least 10 s between each step (*see* **Note 13**).
9. Prepare the alkaline serum pretreatment reagent by combining equal volumes of 0.25 M KOH and 1.2 M KCl, and vortexing well. Recommended volumes are up to 900 µL of each reagent, permitting two preparations. Cover the vials with Parafilm for use with the second plate. Cover the vial with Parafilm using the side of the Parafilm that faced the paper backing.
10. Add serum and pretreatment reagent to plates, after thawing and vortexing frozen serum samples at room temperature (*see* **Note 14**). Transfer 5 µL of each serum sample to the designated wells (labeled as Uk), and add 20 µL of the serum pretreatment reagent to each well containing serum (*see* **Note 15**). Agitate the plate for 5–10 s to mix well contents, and incubate for 10 min at 37 °C.
11. While pretreatment incubation is in progress, reconstitute one vial of Fungitell reagent by adding 2.8 mL of RGW and 2.8 mL of Pyrosol Reconstitution buffer using the 1000 µL pipettor. Cover the vial with Parafilm using the side of Parafilm that faced the paper backing. Swirl the vial gently to dissolve completely—do not vortex.
12. At the end of serum pretreatment incubation, remove the plate and add standards and negative controls to wells (25 µL).
13. Use the stepper pipettor to add 100 µL of Fungitell reagent to each well containing negative controls, standards and samples, insert the plate into the microplate reader (equilibrated to 37 °C) with the lid on, and shake for 5–10 s.
14. Read the plate without the lid at 405 nm minus 490 nm, for 40 min at 37 °C. If background subtraction (at 490 nm) is unavailable, read at 405 nm. If a plate shaking function is unavailable with the microplate reader, use an external microplate shaker.
15. Collect data and analyze by examining optical density plots of test samples and checking for kinetic trace patterns other than a smooth increase comparable to those of standards. Invalidate plots indicating optical interference.
16. Calculate the mean rate of optical density change (milliabsorbance units per minute) for all points between 0 and 40 min.

17. Results (expressed in pg/mL of serum) range from non-detectable (<31 pg/mL) to >500 pg/mL, and are printed out by the software or read from the standard curve. Accurate values >500 pg/mL require that the sample be diluted in RGW and retested. Results are interpreted as follows: <60 pg/mL—negative; 60–79 pg/mL—possible infection, for which additional sampling and testing are recommended; ≥80 pg/mL—positive (*see* **Note 16**).

3.2 Performing the T2 Assay (See Note 17*)*

3.2.1 Sample Collection

1. Collect blood (≥3 mL) in 4 mL plastic Vacutainer plastic K2EDTA Venous Blood Collection Tubes 13 mm (lavender top), and invert eight to ten times to thoroughly mix with the anticoagulant (*see* **Note 18**).
2. Store whole blood in the K2EDTA collection tube at 15–25 °C for no longer than 12 h before analysis. Specimens should be tested as soon as possible after collection. Specimens held for longer than 3 days at 2–8 °C may result in a decrease of viable organisms in the specimen. If stored at 2–8 °C, ensure that the sample has equilibrated to room temperature before analysis.

3.2.2 Work Area Preparation

1. Wearing fresh gloves, spray Bleach-Rite (or equivalent) onto a new lint-free wipe. Wipe the prep area bench top in a unidirectional motion and discard the wipe.
2. Using a new wipe for each, repeat the same procedure to clean the T2Dx Instrument touch screen, Barcode Scanner, drawer panel, and the bench top surrounding the T2Dx Instrument. Allow the bleach solution to sit for at least 3 min.
3. Wearing fresh gloves, repeat cleaning procedure using 70 % isopropyl alcohol to wipe all surfaces. Should gloves become soiled during any of the steps of this procedure, remove and replace with a clean pair of gloves following standard lab procedures.

3.2.3 Testing Procedure (See Note 19*)*

1. Wearing fresh gloves, place a disposable absorbent pad on the work surface. Remove specimens or controls from storage and ensure that there is sufficient sample volume (≥3 mL) in the K2EDTA Vacutainer and that the specimen or control barcode is legible and undamaged. If there is any blood or fluid present on the exterior of the sample tube, clean the Vacutainer using standard lab practices.
2. Wearing fresh gloves, obtain the required number of T2Candida Reagent Packs and T2Candida Cartridges from storage. Open the packaging without touching the inner contents. Since the outer packaging is considered potentially contaminated, do not remove from the package at this time (*see* **Note 20**).

3. Wearing fresh gloves, take out one T2Candida Sample Inlet, one T2Candida Base and one T2Candida Reagent Pack and place them on the clean absorbent pad. Do not touch the outside of the packaging or the foil on top of the T2Candida Reagent Pack. Check the labels and barcodes for integrity.
4. Holding the Reagent Pack on the sides and being careful not to touch the foil on top, briefly mix by agitating in a horizontal motion for 3–5 s. Visually inspect the contents to ensure all solutions are homogeneous. Gently tap on the bench top to displace any trapped air bubbles, and visually confirm that air bubbles are removed.
5. Insert the T2Candida Reagent Pack onto the T2Candida Base using the orientation notch of the T2Candida Reagent Pack to assist in properly aligning the two components. While taking care to avoid the seal over the wells, push down on the T2Candida Reagent Pack at the site of the barcode until an audible snap-in sound is heard, indicating that the T2Candida Reagent Pack is fixed onto the T2Candida Base.
6. Assure that the specimen is at room temperature (~20 min for refrigerated samples). Resuspend the patient sample in a capped blood collection tube by inverting the blood collection tube a minimum of eight to ten times. A blood specimen rocker may also be used to ensure sample homogeneity. Do not use the blood sample if the mixture is not homogenous after resuspension.
7. Uncap the blood collection tube following standard laboratory procedures. Exercise care to not spill or aerosolize the sample. Dispose of cap as biohazard waste.
8. Invert the T2Candida Sample Inlet and use it to re-cap the blood collection tube, using a push and twist motion to secure the blood collection tube. It is critical to ensure that a good seal has formed between the tube and the Sample Inlet blood collection tube seal (the soft portion of the inlet). Ensure that the blood collection tube is seated firmly in the Sample Inlet before proceeding to the next step.
9. Invert the Sample Inlet assembly and blood collection tube and assure that the sample level in the blood collection tube drops as the blood transfers from the collection tube to the T2Candida Sample Inlet. If the blood collection tube's liquid level does not drop after 1 min, invert back to the original position; ensure that there is at least 3 mL of specimen remaining in the blood collection tube and repeat **step 8**.
10. Once it is verified that sample is flowing into the Sample Inlet, place the T2Candida Base on a flat surface and snap the T2Candida Sample Inlet onto it. Taking care to avoid contact with the foil seal, push the T2Candida Sample Inlet down until

an audible snap-in sound is heard, indicating that the T2Candida Sample Inlet is correctly attached to the T2Candida Base. Do not place the assembled cartridge in front of the instrument to avoid accidental drops due to automated drawers.

11. Wearing fresh gloves, press "Load" on the touch screen of the T2Dx Instrument.
12. Using the Barcode Scanner on the T2Dx Instrument, scan the patient sample barcode, the T2Candida Reagent Tray barcode and the T2Candida Base barcode, according to the instrument prompts. When necessary, enter a unique sample identifier using the touch screen keyboard instead of scanning the blood collection tube barcode.
13. The system will open an available drawer and prompt loading of the fully assembled T2Candida Cartridge with specimen into the T2Dx Instrument drawer. Ensure that the T2Candida Cartridge is level when seated in the drawer and fully in contact with the metal rails and the Location Pins.
14. Once the cartridge is properly positioned in the T2Dx Instrument drawer, press "Next". When prompted by the instrument to "Tear Off Label", gently remove the top seal from the cartridge by pulling on the tab while holding down the cartridge assembly (*see* **Note 21**).
15. Press "Confirm" on the T2Dx Instrument touch screen. The locking mechanism will engage to hold the T2Candida Cartridge in place and the drawer will close (*see* **Note 22**).
16. The T2Dx Instrument automatically selects the next available drawer for use (1 through 7). Drawer 7 (orange STAT drawer) may be used at any time to process priority samples.
17. After changing gloves, repeat **steps 3–16** for each additional sample if there are available drawers in the T2Dx Instrument. The T2Dx touch screen "Drawer Status" display will indicate whether drawers are available.
18. Once the sample is finished, the "Run Complete" indicator will appear on the display screen. All the used and unused disposables along with reagents, sample and liquid waste are contained in the T2Candida Cartridge.
19. To remove the used T2Candida Cartridge from the T2Dx Instrument, put on a fresh pair of gloves and press "Unload" on the T2Dx Instrument touch screen. Follow the T2Dx Instrument prompts to open the drawer.
20. With one hand inside a biohazard bag, remove the used T2Candida Cartridge from the T2Dx Instrument and pull the biohazard bag over the cartridge, as an additional precaution to limit the risk of inadvertent cross-contamination (*see* **Note 23**).

Table 1
Interpretation of results as reported by T2Dx instrument

T2Dx instrument result	Interpretation
A/T: Positive and IC: Valid	*Candida albicans* and/or *Candida tropicalis* detected.
A/T: Negative and IC: Valid	*Candida albicans* and/or *Candida tropicalis* not detected. Internal Control is valid.
P: Positive and IC: Valid	*Candida parapsilosis* detected.
P: Negative and IC: Valid	*Candida parapsilosis* not detected. Internal Control is valid.
K/G: Positive and IC: Valid	*Candida glabrata* and/or *Candida krusei* detected.
K/G: Negative and IC: Valid	*Candida glabrata* and/or *Candida krusei* not detected. Internal Control is valid.
IC: Invalid and all species detection results: Negative	Internal Control is invalid and the specimen result cannot be determined.

21. Seal or tie up the bag, and then discard in a biohazardous waste receptacle. Dispose of gloves immediately into biohazard waste.
22. Wearing fresh gloves, press "Next" twice more in the T2Dx Instrument touch screen and the drawer will close. Repeat for all other drawers that display a "Run Complete" indication, using a fresh pair of gloves for each cartridge.
23. Each valid T2Candida Panel will yield three total results (Positive or Negative) for (1) *C. albicans/C. tropicalis* (A/T), (2) *C. parapsilosis* (P) and (3) *C. glabrata/C. krusei* (K/G) (Table 1). In addition, an Internal Control (IC) result (Valid or Invalid) will be reported (*see* **Note 24**).

3.3 Prior to Interpreting Test Results: Understanding Invasive Candidiasis, Test Performance Characteristics, and Pretest Likelihoods of Invasive Candidiasis

1. Recognize that invasive candidiasis comprises the distinct, but overlapping diseases of candidemia and deep-seated candidiasis (*see* **Note 25**).
2. Understand the sensitivity and specificity of tests for diagnosing different types of invasive candidiasis (*see* **Note 26**).
3. Assess the most likely type of invasive candidiasis in the patient being tested (Table 2) (*see* **Note 27**).
4. Estimate the pretest likelihood of invasive candidiasis in the patient being tested (Table 2) (*see* **Note 28**).

3.4 Interpreting Test Results at the Bedside: A Bayesian Framework

1. Understand positive and negative predictive values (PPV, NPV) in various clinical settings (Table 3) (*see* **Note 29**).
2. Understand the impact of blood culture results on the interpretation of non-culture test results (Table 3) (*see* **Note 30**).
3. Calculate PPV and NPV of the result in the patient being tested (*see* **Note 31**).

Table 2
Incidence of the most common types of invasive candidiasis in various populations

Patient population	Definition	Most common type of IC	Incidence of IC[a] (%)	References
Low-risk hospitalized patients[b]	Any patient in whom a blood culture is collected	Candidemia with or without deep-seated candidiasis	<1	[10]
Low-risk ICU patients	Point prevalence Post-cardiothoracic surgery	Candidemia with or without deep-seated candidiasis	<1	[14, 15]
Low-to-moderate risk hospitalized patient	Patients in septic shock	Candidemia with or without deep-seated candidiasis	~3–4	[17]
Low-to-moderate risk liver transplant recipients	Absence of risk factors for fungal infections	Intra-abdominal candidiasis	~3–4	[20]
Low-to-moderate risk peritoneal dialysis patients	Patients with peritonitis	Intra-abdominal candidiasis	~3–6	[8]
Low-to-moderate risk ICU patients	≥4 d in ICU	Candidemia with or without deep-seated candidiasis	~3–7	[16, 19]
Moderate-risk liver transplant recipients	Presence of various risk factors identified in retrospective studies	Intra-abdominal candidiasis	~5–20	[20]
Moderate-risk ICU patients	≥4 d in ICU and positive clinical predictive criteria	Candidemia with or without deep-seated candidiasis	~10–15	[16, 19]
High-risk GI disease	Severe acute or necrotizing pancreatitis	Intra-abdominal candidiasis	~20–30	[22]
High risk liver transplant recipients	Presence of various risk factors and post-transplant bile leak	Intra-abdominal candidiasis	~30	[20]
High-risk GI surgery	Recurrent GI tract leak	Intra-abdominal candidiasis	~30–40	[5, 6]

IC invasive candidiasis, *ICU* intensive care unit, *GI* gastrointestinal, *d* days
[a]Data are selected from representative publications. Incidence in comparable patient populations may differ by center. In order to best interpret and utilize non-culture test results, clinicians should be aware of the approximate incidence of invasive candidiasis in various settings at their centers
[b]For descriptive purposes, the follow definitions of level of risk are used in this paper: Low: <3 %; Low-to-moderate: ~3–10 %; Moderate: ~10–20 %; High: >20 %

Table 3
Performance of non-culture tests for invasive candidiasis in various populations

Most common type of IC	Pretest likelihood of IC[a] (%)	Corresponding patient populations	β-D-glucan[b]		PCR[c]		Ideal assay[d]	
			PPV (if BCx (–))	NPV (if BCx (–))	PPV (if BCx (–))	NPV (if BCx (–))	PPV (if BCx (–))	NPV (if BCx (–))
Candidemia ± DSC[e]	<1	Low-risk hospitalized patients Low-risk ICU patients	<4 % (<<1 %)	>99.7 % (>99.7 %)	<8 % (<<1 %)	>99.8 % (>99.8 %)	<8 % (<<1 %)	>99.8 % (>99.8 %)
	3	Low-to-moderate risk ICU patients Low-to-moderate risk hospitalized patients (septic shock)	11 % (2 %)	99 % (>99.3 %)	22 % (2 %)	>99.6 % (>99.6 %)	22 % (3 %)	>99.6 % (>99.6 %)
	10	Moderate-risk ICU patients	31 % (8 %)	97 % (98 %)	50 % (8 %)	99 % (99 %)	50 % (10 %)	99 % (99 %)
Primary IAC[f]	3	Low-to-moderate risk liver transplant[g]	7 % (6 %)	98 % (99 %)	8 % (6 %)	99 % (99 %)	22 % (18 %)	>99.6 % (>99.7 %)
	5	Low-to-moderate risk peritoneal dialysis with peritonitis	11 % (9 %)	97 % (98 %)	12 % (10 %)	99 % (99 %)	32 % (27 %)	>99.4 % (>99.5 %)
	10	Moderate-risk liver transplant	21 % (17 %)	94 % (96 %)	23 % (20 %)	97 % (98 %)	50 % (44 %)	99 % (99 %)
	20	High-risk severe acute or necrotizing pancreatitis	37 % (31 %)	88 % (91 %)	40 % (34 %)	93 % (95 %)	69 % (63 %)	97 % (98 %)
	30	High-risk liver transplant High-risk GI surgery	51 % (43 %)	78 % (86 %)	53 % (46 %)	89 % (92 %)	79 % (74 %)	95 % (97 %)

The data in the table assume that testing is performed in the absence of antifungal treatment. Conclusive data on the impact of antifungal treatment on β-D-glucan or PCR performance are lacking. Antifungal agents can certainly attenuate β-D-glucan levels and PCR signal threshold. In a recent study, however, the performance of β-D-glucan and PCR in identifying patients with candidemia or deep-seated candidiasis was not impacted by prior or ongoing antifungal treatment. Nevertheless, it is reasonable to assume that test performance will be adversely affected as treatment durations are prolonged. Antifungal treatment clearly reduces the sensitivity of blood cultures [4, 35]

PCR polymerase chain reaction, *IC* invasive candidiasis, *DSC* deep-seated candidiasis, *IAC* intra-abdominal candidiasis, *PPV* positive predictive value, *NPV* negative predictive value, *BCx* blood culture, ± with or without, *ICU* intensive care unit, *GI* gastrointestinal

[a]Pretest likelihoods are approximated from incidence data in Table 2. Pretest likelihoods will vary by center, which will impact anticipated PPVs and NPVs. However, the principles for interpreting and utilizing test results as outlined in the paper remain unchanged

[b]Sensitivity/specificity for candidemia (with or without DSC) and primary DSC are assumed to be 80 %/80 % and 60 %/75 %, respectively

[c]Sensitivity/specificity for candidemia (with or without DSC) and primary DSC are assumed to be 90 %/90 % and 80 %/70 %, respectively

[d]Sensitivity/specificity for candidemia (with or without DSC) and primary DSC are assumed to be 90 %/90 % and 90 %/90 %, respectively. In this paper, T2Candida performance is assumed comparable to published data for PCR assays

[e]Calculations of PPVs and NPVs assume that 1/3 of patients will have blood culture-negative DSC. For these cases, sensitivity/specificity of the non-culture test are the same as for primary DSC

[f]Calculations of PPVs and NPVs assume that all patients with invasive candidiasis in these settings have DSC

[g]Data are more limited in liver transplant recipients than general ICU populations, but support the sensitivity/specificity used here [34]

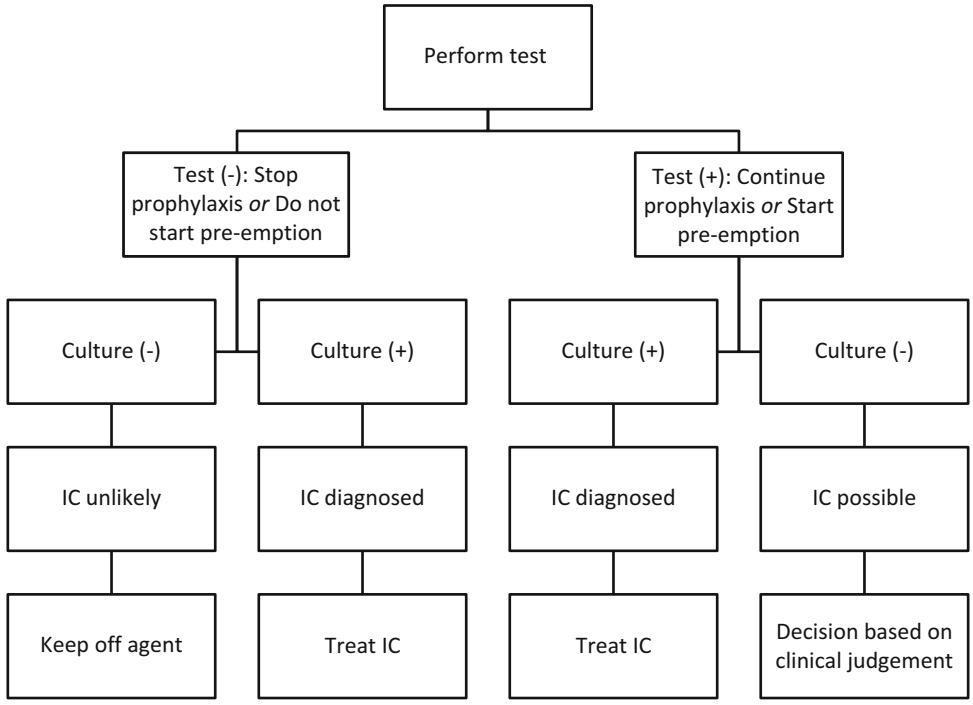


Fig. 2 Paradigm for incorporating non-culture tests into prophylactic or preemptive antifungal strategies against invasive candidiasis. Treatment decisions are made at two stages, in response to non-culture test results and non-culture results combined with blood cultures, respectively. The viability of the paradigm depends upon NPVs and PPVs at each stage. By applying data from Table 3, clinical settings in which non-culture test-driven strategies are likely to be useful can be identified (Table 4). Strategies such as these that use non-culture diagnostics to direct antifungal treatment require validation in clinical trials

3.5 Making Treatment Decisions: Using Non-culture Diagnostics as Screening Tools for Antifungal Treatment (See* Note 32*)

1. Understand a paradigm that uses non-culture diagnostics and blood cultures to guide prophylactic or preemptive antifungal treatment (Fig. 2) (*see* **Note 33**).
2. Define NPV and PPV thresholds to discontinue prophylaxis or initiate preemptive treatment (*see* **Note 34**).
3. Assign windows of pretest likelihoods in which non-culture tests are most likely to be valuable in guiding antifungal therapy (Table 4) (*see* **Note 35**).

3.6 Making Treatment Decisions: Using Non-culture Diagnostics in the Management of Individual Patients

1. Understand that non-culture tests may be useful in the management of individual patients, even if performed outside of the windows proposed for screening populations (*see* **Note 36**).

Table 4
Windows of pretest likelihoods in which non-culture tests are predicted to be useful in guiding antifungal treatment

Predominant type of IC	Test	Windows[a]		Comments
		Pretest likelihood (%)	Corresponding populations[b]	
Candidemia (± DSC)	β-DG[d]	~5–40 %[c]	Low-to-moderate- to high-risk ICU patients	NPVs are ≥~85 % at pretest likelihoods as high as the upper limits of these ranges, suggesting that prophylactic or preemptive strategy would remain viable. Compared to β-D-glucan, T2Candida or a comparable assay would broaden the window for treatment to include lower-risk patients, including those in septic shock. Based on the particulars of a case, clinicians may decide to stop treatment if blood cultures are negative despite positive non-culture test results. With negative blood cultures, β-D-glucan or T2Candida PPVs are <15 % in all settings with pretest likelihood ≲15 %.
	T2Candida (PCR)[g,h]	~2–60[c]	Low- to high-risk ICU patients, and patients in septic shock	
	Ideal assay[e]	~2–60[c]		
IAC	β-DG[f]	~7–20	Moderate-risk liver transplant recipients, and patients with severe pancreatitis	Compared to β-D-glucan, T2Candida or other PCR-based assays (if validated) would broaden the window for prophylactic or preemptive treatment to include high-risk surgery patients with GI leaks, or high-risk liver transplant recipients with bile leaks. An ideal assay for intra-abdominal candidiasis would further broaden the window to include lower-risk patients. Using negative blood culture results to justify discontinuing prophylaxis or withholding preemptive treatment among patients with negative β-d-glucan results may broaden the window of pretest likelihoods to ~7–30 %. Otherwise, negative blood cultures do not significantly impact antifungal strategies.
	T2Candida (PCR)[f,h]	~7–40	Moderate to high-risk GI surgery and liver transplant patients, and patients with severe pancreatitis	
	Ideal assay[e]	~2–60	Low-to-moderate risk liver transplant recipients and peritoneal dialysis patients with peritonitis, in addition to groups above	

IC invasive candidiasis, *DSC* deep-seated candidiasis, *IAC* intra-abdominal candidiasis, *β-DG* β-D-glucan, *PCR* polymerase chain reaction, *ICU* intensive care unit, *NPV* negative predictive value, *PPV* positive predictive value, *GI* gastrointestinal

[a]Windows defined by PPV ≥15 % and NPV ≥85 %

[b]As indicated in the footnote to Table 2, the follow definitions of level of risk are used: Low: <3 % pretest likelihood; Low-to-moderate: ~3–10 %; Moderate: ~10–20 %; High: >20 %

[c]In general, clinical prediction models have identified patients with incidence of candidemia up to ~16 % [16, 19]

[d]Sensitivity/specificity: 80 %/80 %

[e]90 %/90 %

[f]60 %/75 %

[g]80 %/70 %

[h]As throughout this paper, T2Candida performance is assumed comparable to published data for PCR assays

4 Notes

1. The reader is referred to highly detailed instructions provided with the Fungitell kit. Note that the assay detects fungi in addition to Candida spp. A positive result does not distinguish between fungi. Common medically relevant fungi that are not detected include Cryptococcus spp., Zygomycetes such as Rhizopus, Mucor and Absidia, and (in most instances) the yeast morphology of *Blastomyces dermatitidis*.
2. Materials supplied with each kit are sufficient to assay 110 wells on two microtiter plates (55 wells on each). Store all reagents at 2–8 °C in the dark. Use reconstituted Fungitell within 2 h, or freeze at −20 °C for up to 20 days, thaw once and use.
3. Glassware must be dry-heat depyrogenated at ≥235 °C for 7 h (or a validated equivalent) to be considered suitable for use. Glass pipettes with cotton plugs are a potential source of glucan contamination.
4. Materials marked by asterisks are certified free of interfering glucans and available from Associates of Cape Cod, Inc.
5. The reader is referred to highly detailed instructions provided with the T2Candida panel and T2Dx Instrument. Note that T2Candida results are reported for three spp. groups: (1) *C. albicans/C. tropicalis*; (2) *C. parapsilosis;* and (3) *C. glabrata/C. krusei*. The assay is not quantitative, nor does it detect other Candida spp. or fungi.
6. Store T2Candida Cartridge at 15–30 °C. Store T2Candida Reagent Packs at 2–8 °C.
7. Assays can only be run with the T2Dx Instrument.
8. Available from T2 Biosystems.
9. Use powder-free gloves when handling patient specimens.
10. Off-color or turbid samples such as those that are grossly hemolyzed, lipemic, or contain excessive bilirubin may cause interference. If such samples are tested, results should be examined for evidence of optical interference and/or unusual kinetic trace patterns.
11. The Fungitell assay requires rigorous attention to technique and testing environment. The assay should be performed by personnel who are thoroughly trained in the methods and in the avoidance of contamination.
12. Settings may vary with different instruments and software.
13. When plotting the standard curve, multiply the concentration of the standards by five so that the range is from 500 to 31 pg/mL. Enter the standards into the software settings as 500, 250, 125, 62.5, and 31 pg/mL, respectively.

14. To avoid accidental contamination when manipulating the plate, replace the cover after adding samples and reagents to the wells. Remove the cover before reading to avoid optical interference from condensation.
15. The steps in this sentence can be conducted in reverse order.
16. There are several quality control measures that should be assessed. (1) The correlation coefficient (r) of the standard curve should be >0.980. (2) Negative control wells (25 μL of RGW only) should have actual optical density rate (Vmean) values <50 % of the lowest standard. If values are not in this range, the assay should be repeated using all new reagents. (3) If optical density kinetics are unusual in testing a sample that is cloudy, off-color, or turbid, the sample must be diluted in RGW and retested. The dilution must be accounted for in the reporting of results by multiplying the result by the dilution factor. Typically, the dilution factor is entered in the software setup for the sample and the correction is automatically applied. (4) Control samples at cut-off and highly positive levels may be run to verify that reagents and the assay are performing properly. Each user of the test should establish a quality control program to assure proficiency in the performance of the test.
17. The assay should only be processed by technicians who have been trained on use of the T2Candida Panel and the T2Dx Instrument.
18. Blood should be collected using aseptic technique, similar to blood culture sample acquisition. A minimum blood specimen volume of 3 mL is required to assure optimal T2Candida Panel performance. Specimens should not be drawn from a central line or port through which antifungal therapy has been administered.
19. As for any nucleic acid amplification technology, avoidance of contamination is crucial. Pay scrupulous attention to maintaining a clean environment, changing gloves as specified, etc.
20. Excellent figures for steps throughout the procedure are provided in the T2Candida manual.
21. Ensure that all tray components are present before proceeding.
22. Watch the locking mechanism to ensure that it engages the T2Candida Cartridge as the drawer closes.
23. Be careful when removing the used T2Candida Cartridge to avoid spillage of reagents, samples and disposables. Do not tip or invert the used T2Candida Cartridge. Ensure that the T2Candida Cartridge remains upright while unloading.
24. If the Internal Control is invalid and the specimen result is reported as negative, “Invalid” will be displayed as the Internal

Control result. In this case, repeat the T2Candida Panel from a different sample from the patient. T2 Biosystems provides positive and negative T2Candida External Controls separately, formulated in whole blood, to be used for periodic quality control checks with the T2Candida Panel reagents and the T2Dx Instrument. It is recommended that a single Positive (APG or TPK) Control Tube and a single Negative Control Tube from the respective External Controls kits are run at least once every 30 days. Users should alternate the multiplex blend between APG and TPK Positive Control Tubes with each QC check. Positive APG, Positive TPK and Negative Control Tubes should be run when a new reagent lot is received into the laboratory, or significant maintenance (including software upgrades) is performed on the T2Dx Instrument.

25. It is impossible to interpret non-culture test results without considering the type of invasive candidiasis that is being diagnosed. Candidemia and deep-seated candidiasis (submucosal tissue and organ infections) may occur concurrently or independently. Approximately 50 % of primary candidemia causes secondary deep-seated candidiasis due to hematogenous seeding [1]. Primary deep-seated candidiasis results from non-hematogenous introduction of Candida into sterile sites, most commonly the abdominal cavity following disruption of the gastrointestinal (GI) tract or via an infected peritoneal catheter or drain. Studies suggest that 5–20 % of primary deep-seated candidiasis is associated with secondary candidemia [4–6]. Therefore, to be most useful, diagnostic tests must identify three clinical entities: (1) candidemia without deep-seated candidiasis; (2) candidemia with deep-seated candidiasis; (3) deep-seated candidiasis without candidemia. Studies suggest that roughly one-third of patients with invasive candidiasis fall into each group [7]. Best estimates are that blood cultures will identify the majority of patients in group 1 during active candidemia, approximately 50 % of patients in group 2, and none of the patients in group 3. The power of non-culture tests lies in their potential for identifying the so-called "missing 50 %"—patients in the latter two groups who are currently not diagnosed by blood cultures. The identification of these patients is possible through detection of Candida cellular components that persist in the bloodstream after elimination of viable cells or that are released from deep tissue sites.

26. In well-performed meta-analyses of β-D-glucan studies, pooled sensitivity and specificity for invasive candidiasis (the vast majority of which was candidemia) were 75–80 % and 80 %, respectively [8, 9]. T2Candida was approved by the FDA based on data from 1500 control patients with Candida-negative blood cultures, and 250 contrived blood specimens spiked

with *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, or *C. krusei* at concentrations ranging from 1 to 100 CFU/mL [10]. The overall sensitivity and specificity of T2Candida were 91 % and 98 %, respectively. The performance of the assay (in particular, its sensitivity) needs to be corroborated, as there are limited published data on whole blood specimens from patients with invasive candidiasis [11, 10]. Nevertheless, the preliminary findings are in keeping with data from a meta-analysis of Candida PCR studies, in which pooled sensitivity and specificity for invasive candidiasis (vast majority candidemia) were 95 % and 92 %, respectively [12].

In two recent studies, Fungitell β-D-glucan was 53–65 % sensitive and 73–78 % specific for diagnosing deep-seated Candida infections, almost all of which was intra-abdominal candidiasis [4, 5]. Blood cultures were only 7–21 % sensitive. Sensitivity and specificity of Candida PCR for deep-seated candidiasis were 89 % and 70 %, respectively, in one of the studies [4]; PCR was significantly more sensitive than β-D-glucan. For purposes of this chapter, sensitivities and specificities of Fungitell and PCR-based assays for diagnosing candidemia are assumed to be 80 %/80 % and 90 %/90 %, respectively. The respective values for intra-abdominal candidiasis are assumed to be 60 %/75 % and 80 %/75 %, respectively. Since clinical data are much less extensive for T2Candida than Fungitell β-D-glucan, we assume that performance characteristics for the former assay are comparable to published data for PCR assays.

27. Candidemia and intra-abdominal candidiasis are believed to occur with equal frequency [1, 7]. The predominant type of invasive candidiasis is dictated by the clinical setting. When clinicians order a diagnostic test in a given patient, the most likely type of invasive candidiasis should be apparent. For the most part, candidemia is a low-incidence disease among relatively large at-risk populations. Risk factors are nonspecific and common in hospitalized patients, including broad-spectrum antibiotics, intravenous access devices, total parenteral nutrition, mechanical ventilation, renal insufficiency and replacement therapy, diabetes mellitus, corticosteroids, neutropenia or neutrophil dysfunction, and Candida colonization [13]. In contrast, intra-abdominal candidiasis is a relatively high-incidence disease among more narrowly defined populations. While patients often have some of the risks above, the disease does not occur in the absence of predisposing intra-abdominal factors such as liver transplantation, severe pancreatitis, or disruption of GI tract or peritoneal cavity integrity by disease or medical intervention.
28. In most instances, it should be possible to estimate the pretest likelihood of invasive candidiasis in the patient being tested.

Based on published data, the incidence of candidemia increases from <1 to ~10 % as one moves from any hospitalized patient in whom blood cultures are collected, low-risk intensive care unit (ICU) patients or patients undergoing non-gastrointestinal (GI) surgery [14, 15], to more moderate-risk patients who are ICU residents for ≥4 days or who are in septic shock [16, 17], to higher-risk ICU patients identified by clinical prediction rules [16, 18, 19] (Table 2). The incidence of intra-abdominal candidiasis increases from ~3 to ≥30 % as one moves from low-to-moderate risk liver transplant recipients or peritoneal dialysis patients with peritonitis [20, 21], to moderate-risk liver transplant recipients, to high-risk patients with severe acute or necrotizing pancreatitis, recurrent leaks of the GI tract, or bile leaks following liver transplantation [6, 13, 22] (Table 2). Using data such as those in Table 2 as a starting point, the estimated pretest likelihood of invasive candidiasis can be adjusted based on the context of a particular case. "Context" encompasses factors such as presenting complaints, underlying conditions, medications, physical findings, imaging studies and laboratory data, and the likelihood or exclusion of alternative etiologies. Each factor can be considered a "result" in its own right, which adjusts the probability of invasive candidiasis [23]. As an example, moderate-risk ICU patients who fulfill clinical predictive criteria may have different pretest likelihoods of candidemia at the time of testing, even though criteria by themselves assign comparable risk (~10 % incidence).

29. Before interpreting a test result and making treatment decisions in a patient, it is imperative to understand the Bayesian nature of non-culture diagnostics. No matter how sensitive or specific a non-culture assay for invasive candidiasis may be, clinicians must accept a level of uncertainty when interpreting results. By definition, a positive blood culture or tissue culture obtained in a sterile manner establishes the diagnosis of invasive candidiasis. In contrast, non-culture tests are biomarkers that assign a probability of disease, which is shaped by the pretest likelihood, and sensitivity and specificity of the test. In Table 3, PPVs and NPVs of non-culture tests are calculated for various clinical settings in which candidemia or primary intra-abdominal candidiasis is the predominant disease. At low pretest likelihoods of either disease, PPVs and NPVs are extremely low and extremely high, respectively. As likelihoods increase, PPVs improve at the expense of NPVs. For candidemia, β-D-glucan NPVs remain exceptional (≥97 %) in each of the clinical settings; PPV is 31 % in the high-risk ICU setting. The superior sensitivity and specificity of PCR-based assays improve PPVs for candidemia compared to β-D-glucan, but the impact on NPVs is negligible. PPVs and NPVs are lower for intra-abdominal candidiasis than candidemia at a given pretest

likelihood of disease, due to lower sensitivities and specificities. β-D-glucan NPV for intra-abdominal candidiasis is strong (≥97 %) in low-risk settings, but values drop below 90 or 80 % in higher-risk settings (*e.g.*, severe acute or necrotizing pancreatitis, high-risk GI surgery or liver transplantation). β-D-glucan PPV rises to 51 % among the highest-risk patients. The superior performance of a PCR-based assay impacts NPVs in the higher-risk settings, but has lesser impact on PPVs.

30. In clinical practice, non-culture tests will be used in conjunction with cultures. It is important to understand that cultures are very sensitive at recovering viable Candida from the bloodstream, with median limits of detection (~1 CFU/mL) that are at or below those of PCR [24, 25]. For this reason, cultures should be positive during the vast majority of ongoing Candida bloodstream infections. If cultures are negative due to extremely low-level candidemia, Fungitell and PCR assays are unlikely to make the diagnosis reliably. At present, it is unknown whether T2Candida is more sensitive than conventional Candida PCR. Even if non-culture tests are not more sensitive than cultures for diagnosing active candidemia, their shorter turnaround times should facilitate more rapid treatment decisions. Perhaps more importantly, non-culture diagnostics have the potential to identify blood culture-negative, primary or secondary deep-seated candidiasis.

    In one study, the combination of either a positive Candida blood culture or Fungitell β-D-glucan was 79 % sensitive for diagnosing all types of invasive candidiasis; a positive blood culture or positive PCR was 98 % sensitive [4]. The interpretation of positive non-culture tests in the setting of negative blood cultures is difficult, since the latter make active candidemia extremely unlikely but do not preclude deep-seated candidiasis. Indeed, the likelihood ratio of primary intra-abdominal candidiasis given negative blood cultures is ~0.8 [4, 5]; in other words, the odds of having the disease are reduced ~20 % if blood cultures are negative. If it is assumed that ~50 % of candidemia results in target organ infection, the likelihood ratio of secondary deep-seated candidiasis despite negative blood cultures may be as high as 0.4. Using these data, PPVs and NPVs of β-D-glucan and PCR for invasive candidiasis can be readjusted for negative blood cultures (Table 3).

31. Clinicians can use the estimated pretest likelihood of invasive candidiasis in the patient being tested to calculate PPV and NPV, along the lines of the examples in Table 3. If blood cultures are drawn concurrently with the non-culture test, PPV and NPV are assessed in two stages (based on the non-culture test alone, and then adjusted for culture results). Of course, positive blood cultures diagnose invasive candidiasis conclusively, regardless of the non-culture result. Just as clinicians

may adjust the pretest likelihood of invasive candidiasis in response to the context of a particular case, they may modify PPV and NPV based on the magnitude or number of test results. For example, two highly positive results are more compelling than a single borderline result, and multiple negative results are more compelling than a single negative result. It is infeasible for clinicians to calculate precise running tallies of likelihood ratios as they estimate pretest likelihoods or interpret test results in each patient. Nevertheless, they can conceptualize results qualitatively prior to making treatment decisions [23]. Examples of useful qualitative evaluations include "the patient is reasonably likely to have invasive candidiasis, and a positive result significantly increases that likelihood", or "in this low-risk patient, a positive result does not help me, but a negative result essentially excludes the disease."

32. The management strategies presented in this chapter are based largely on data from antifungal prophylaxis studies in various patient populations. These strategies (or others that use non-culture diagnostics to direct antifungal treatment) have not been validated in clinical trials. Therefore, the methods we propose may be a basis for designing future research studies, as well as a guide for clinicians struggling to make use of non-culture tests in their practices. As mentioned earlier, the interpretive and clinical decision-making processes we describe can be applied to other assays as they enter the clinic, and to Fungitell and T2Candida assays as more data emerge.

33. Broadly speaking, non-culture tests may be used to screen populations for invasive candidiasis or to manage individual patients. At the population level, screening may be incorporated into prophylactic or preemptive antifungal strategies. In the former, an antifungal agent is initiated in a population at risk for invasive candidiasis, and a negative test result is used to discontinue treatment. In preemptive strategies, antifungal treatment is initiated in response to a positive test result. Several studies suggest that prophylaxis is beneficial in preventing invasive fungal infections in various settings with baseline rates of disease ≥~15 % [26–32]. Preemptive strategies are attractive conceptually, but they have not been validated conclusively [18, 33]. A paradigm that uses non-culture diagnostics and blood cultures to guide prophylaxis or preemptive treatment is presented in Fig. 2. The feasibility of the strategy in a given setting will depend upon PPVs and NPVs in the particular clinical setting.

34. PPV and NPV thresholds to trigger or discontinue antifungal treatment are not established conclusively. Based on the prophylaxis data cited above, however, PPV≥~15 % and NPV≥~85 % are reasonable thresholds to consider initiating

preemptive antifungal treatment and withholding antifungal treatment, respectively. NPVs <85 % are likely to leave too high a posttest probability of invasive candidiasis for antifungals to be deferred comfortably. In order for non-culture screening to be viable, a positive or negative result must provide marginal value beyond simply knowing the pretest likelihood. In other words, clinicians should ask: Do results sufficiently change the probability of invasive candidiasis such that threshold PPV and NPV is now achieved?

In certain low- and high-risk settings, the answer to this question is no, and screening with non-culture tests will not be useful. At very low pretest likelihoods of either candidemia or intra-abdominal candidiasis, the practical value of negative test results is negligible. For example, a negative β-D-glucan in an ICU setting that is low-to-moderate risk for candidemia reduces disease likelihood from ~3 to ~1 % (Table 3). At the same time, a β-D-glucan PPV of ~11 % is likely to be too low to justify preemptive treatment in this population, in the absence of a positive culture or other evidence of disease. At some high-end pretest likelihood, NPVs become too low to be useful clinically. The NPV of β-D-glucan among high-risk GI surgery or liver transplant patients (pretest likelihood ~30 %) is only 78 %, meaning clinicians must be willing to forego treatment despite a >20 % chance that intra-abdominal candidiasis is present. Likewise, it is not clear that the 51 % PPV of β-D-glucan in these settings has greater practical value than simply knowing the pretest likelihood. At some point, the pretest likelihood of invasive candidiasis may be sufficient to justify antifungal treatment regardless of non-culture test results. Indeed, universal prophylaxis is beneficial among groups such as bone marrow transplant recipients, surgical patients with recurrent GI leaks, and high-risk liver transplant recipients with bile leaks (≥30 % incidence of Candida infection) [5, 20].

35. By applying data such as those in Table 3 to the paradigm of Fig. 2, it is possible to assign windows of pretest likelihoods in which non-culture tests are most likely to valuable in guiding preemptive treatment (Table 4). If validated, the improved performance of T2Candida (or other PCR-based assay) over β-D-glucan should expand the patient populations for prophylactic or preemptive treatment. T2Candida would have greatest impact among those at risk for candidemia, as antifungal strategies become viable for lower-risk ICU patients (e.g., ICU resident for ≥4 days) and patients in septic shock. Such an assay would also extend prophylactic or preemptive treatment to patients at highest-risk for intra-abdominal candidiasis. An ideal non-culture test would diagnose both candidemia and intra-abdominal candidiasis with sensitivity/specificity of ~90 %/90 %. A test

with this performance for intra-abdominal candidiasis, rather than 80 %/70 % sensitivity/specificity suggested by the PCR literature, would expand the window to include lower-risk surgical ICU populations and peritoneal dialysis patients with peritonitis.

36. The use of non-culture tests in caring for individuals is more nuanced than when screening populations as part of prophylactic or preemptive antifungal strategies. Clinicians must make decisions about when to order tests based on the context of the particular case, without strict regard to the windows assigned above. For example, a positive non-culture test result with predictive value that is ≤15 % nevertheless may justify antifungal treatment if a patient is sick and there is no alternative diagnosis. Likewise, there is often clinical value in excluding invasive candidiasis in a patient for whom the cause of illness is not apparent, even if the pretest likelihood of invasive candidiasis is beneath the threshold for screening populations.

Financial Disclosures/Acknowledgements

Drs. Clancy and Nguyen have served as site PIs for diagnostic clinical trials funded by T2 Biosystems. Dr. Clancy has received financial support for investigator-initiated research projects from Pfizer, Merck, and CSL Behring. Dr. Nguyen has received financial support for investigator-initiated research projects from Pfizer, Merck, Astellas, and Vira-Cor IBT. The authors received no funding or other assistance for the preparation of this manuscript.

References

1. Clancy CJ, Nguyen MH (2013) Finding the "missing 50%" of invasive candidiasis: how nonculture diagnostics will improve understanding of disease spectrum and transform patient care. Clin Infect Dis 56(9):1284–1292. doi:10.1093/cid/cit006
2. Andes DR, Safdar N, Baddley JW, Playford G, Reboli AC, Rex JH, Sobel JD, Pappas PG, Kullberg BJ (2012) Impact of treatment strategy on outcomes in patients with candidemia and other forms of invasive candidiasis: a patient-level quantitative review of randomized trials. Clin Infect Dis 54(8):1110–1122. doi:10.1093/cid/cis021
3. Wheat LJ (2009) Approach to the diagnosis of invasive aspergillosis and candidiasis. Clin Chest Med 30(2):367–377, viii. doi:10.1016/j.ccm.2009.02.012
4. Nguyen MH, Wissel MC, Shields RK, Salomoni MA, Hao B, Press EG, Shields RM, Cheng S, Mitsani D, Vadnerkar A, Silveira FP, Kleiboeker SB, Clancy CJ (2012) Performance of Candida real-time polymerase chain reaction, beta-D-glucan assay, and blood cultures in the diagnosis of invasive candidiasis. Clin Infect Dis 54(9):1240–1248. doi:10.1093/cid/cis200
5. Tissot F, Lamoth F, Hauser PM, Orasch C, Fluckiger U, Siegemund M, Zimmerli S, Calandra T, Bille J, Eggimann P, Marchetti O (2013) Beta-glucan antigenemia anticipates diagnosis of blood culture-negative intraabdominal candidiasis. Am J Respir Crit Care Med 188(9):1100–1109. doi:10.1164/rccm.201211-2069OC
6. Calandra T, Bille J, Schneider R, Mosimann F, Francioli P (1989) Clinical significance of Candida isolated from peritoneum in surgical patients. Lancet 2(8677):1437–1440, S0140-6736(89)92043-6 [pii]

7. Leroy O, Gangneux JP, Montravers P, Mira JP, Gouin F, Sollet JP, Carlet J, Reynes J, Rosenheim M, Regnier B, Lortholary O (2009) Epidemiology, management, and risk factors for death of invasive Candida infections in critical care: a multicenter, prospective, observational study in France (2005–2006). Crit Care Med 37(5):1612–1618. doi:10.1097/CCM.0b013e31819efac0
8. Karageorgopoulos DE, Vouloumanou EK, Ntziora F, Michalopoulos A, Rafailidis PI, Falagas ME (2011) Beta-D-glucan assay for the diagnosis of invasive fungal infections: a meta-analysis. Clin Infect Dis 52(6):750–770. doi:10.1093/cid/ciq206
9. He S, Hang JP, Zhang L, Wang F, Zhang DC, Gong FH (2014) A systematic review and meta-analysis of diagnostic accuracy of serum 1,3-beta-d-glucan for invasive fungal infection: focus on cutoff levels. J Microbiol Immunol Infect. doi:10.1016/j.jmii.2014.06.009
10. Mylonakis E, Clancy CJ, Ostrosky-Zeichner L, Garey KW, Alangaden GJ, Vazquez JA, Groeger SJ, Judson MA, Vinagre YM, Heard SO, Zervou FN, Zacharioudakis IM, Kontoyiannis DP, Pappas PG (2015) T2 magnetic resonance assay for the rapid diagnosis of candidemia in whole blood: a clinical trial. Clin Infect Dis 60:892–899
11. Neely LA, Audeh M, Phung NA, Min M, Suchocki A, Plourde D, Blanco M, Demas V, Skewis LR, Anagnostou T, Coleman JJ, Wellman P, Mylonakis E, Lowery TJ (2013) Magnetic resonance enables nanoparticle-mediated rapid detection of candidemia in whole blood. Sci Transl Med 5(182):182ra154. doi:10.1126/scitranslmed.3005377
12. Avni T, Leibovici L, Paul M (2011) PCR diagnosis of invasive candidiasis: systematic review and meta-analysis. J Clin Microbiol 49(2):665–670. doi:10.1128/JCM.01602-10
13. Delaloye J, Calandra T (2014) Invasive candidiasis as a cause of sepsis in the critically ill patient. Virulence 5(1):161–169. doi:10.4161/viru.26187
14. Michalopoulos AS, Geroulanos S, Mentzelopoulos SD (2003) Determinants of candidemia and candidemia-related death in cardiothoracic ICU patients. Chest 124(6):2244–2255
15. Kett DH, Azoulay E, Echeverria PM, Vincent JL (2011) Candida bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. Crit Care Med 39(4):665–670. doi:10.1097/CCM.0b013e318206c1ca
16. Ostrosky-Zeichner L, Sable C, Sobel J, Alexander BD, Donowitz G, Kan V, Kauffman CA, Kett D, Larsen RA, Morrison V, Nucci M, Pappas PG, Bradley ME, Major S, Zimmer L, Wallace D, Dismukes WE, Rex JH (2007) Multicenter retrospective development and validation of a clinical prediction rule for nosocomial invasive candidiasis in the intensive care setting. Eur J Clin Microbiol Infect Dis 26(4):271–276. doi:10.1007/s10096-007-0270-z
17. Hadley S, Lee WW, Ruthazer R, Nasraway SA Jr (2002) Candidemia as a cause of septic shock and multiple organ failure in nonimmunocompromised patients. Crit Care Med 30(8):1808–1814
18. Ostrosky-Zeichner L, Shoham S, Vazquez J, Reboli A, Betts R, Barron MA, Schuster M, Judson MA, Revankar SG, Caeiro JP, Mangino JE, Mushatt D, Bedimo R, Freifeld A, Nguyen MH, Kauffman CA, Dismukes WE, Westfall AO, Deerman JB, Wood C, Sobel JD, Pappas PG (2014) MSG-01: a randomized, double-blind, placebo-controlled trial of caspofungin prophylaxis followed by preemptive therapy for invasive candidiasis in high-risk adults in the critical care setting. Clin Infect Dis 58:1219–1226. doi:10.1093/cid/ciu074
19. Paphitou NI, Ostrosky-Zeichner L, Rex JH (2005) Rules for identifying patients at increased risk for candidal infections in the surgical intensive care unit: approach to developing practical criteria for systematic use in antifungal prophylaxis trials. Med Mycol 43(3):235–243
20. Eschenauer GA, Kwak EJ, Humar A, Potoski BA, Clarke LG, Shields RK, Abdel-Massih R, Silveira FP, Vergidis P, Clancy CJ, Nguyen MH (2015) Targeted versus universal antifungal prophylaxis among liver transplant recipients. Am J Transplant 15:180–189. doi:10.1111/ajt.12993
21. Matuszkiewicz-Rowinska J (2009) Update on fungal peritonitis and its treatment. Perit Dial Int 29(Suppl 2):S161–S165
22. Hall AM, Poole LA, Renton B, Wozniak A, Fisher M, Neal T, Halloran CM, Cox T, Hampshire PA (2013) Prediction of invasive candidal infection in critically ill patients with severe acute pancreatitis. Crit Care 17(2):R49. doi:10.1186/cc12569
23. Gill CJ, Sabin L, Schmid CH (2005) Why clinicians are natural bayesians. BMJ 330(7499):1080–1083. doi:10.1136/bmj.330.7499.1080
24. Pfeiffer CD, Samsa GP, Schell WA, Reller LB, Perfect JR, Alexander BD (2011) Quantitation of Candida CFU in initial positive blood cultures. J Clin Microbiol 49(8):2879–2883. doi:10.1128/JCM.00609-11

25. Telenti A, Steckelberg JM, Stockman L, Edson RS, Roberts GD (1991) Quantitative blood cultures in candidemia. Mayo Clin Proc 66(11):1120–1123
26. Playford EG, Webster AC, Sorrell TC, Craig JC (2006) Antifungal agents for preventing fungal infections in non-neutropenic critically ill and surgical patients: systematic review and meta-analysis of randomized clinical trials. J Antimicrob Chemother 57(4):628–638. doi:10.1093/jac/dki491
27. Shorr AF, Chung K, Jackson WL, Waterman PE, Kollef MH (2005) Fluconazole prophylaxis in critically ill surgical patients: a meta-analysis. Crit Care Med 33(9):1928–1935, quiz 1936
28. Eggimann P, Francioli P, Bille J, Schneider R, Wu MM, Chapuis G, Chiolero R, Pannatier A, Schilling J, Geroulanos S, Glauser MP, Calandra T (1999) Fluconazole prophylaxis prevents intra-abdominal candidiasis in high-risk surgical patients. Crit Care Med 27(6):1066–1072
29. Garbino J, Lew DP, Romand JA, Hugonnet S, Auckenthaler R, Pittet D (2002) Prevention of severe Candida infections in nonneutropenic, high-risk, critically ill patients: a randomized, double-blind, placebo-controlled trial in patients treated by selective digestive decontamination. Intensive Care Med 28(12):1708–1717. doi:10.1007/s00134-002-1540-y
30. Pelz RK, Hendrix CW, Swoboda SM, Diener-West M, Merz WG, Hammond J, Lipsett PA (2001) Double-blind placebo-controlled trial of fluconazole to prevent candidal infections in critically ill surgical patients. Ann Surg 233(4):542–548
31. Goodman JL, Winston DJ, Greenfield RA, Chandrasekar PH, Fox B, Kaizer H, Shadduck RK, Shea TC, Stiff P, Friedman DJ et al (1992) A controlled trial of fluconazole to prevent fungal infections in patients undergoing bone marrow transplantation. N Engl J Med 326(13):845–851. doi:10.1056/NEJM199203263261301
32. Slavin MA, Osborne B, Adams R, Levenstein MJ, Schoch HG, Feldman AR, Meyers JD, Bowden RA (1995) Efficacy and safety of fluconazole prophylaxis for fungal infections after marrow transplantation—a prospective, randomized, double-blind study. J Infect Dis 171(6):1545–1552
33. Muldoon EG, Denning DW (2014) Editorial commentary: Prophylactic echinocandin: is there a subgroup of intensive care unit patients who benefit? Clin Infect Dis 58(9):1227–1229. doi:10.1093/cid/ciu080
34. Akamatsu N, Sugawara Y, Kaneko J, Tamura S, Makuuchi M (2007) Preemptive treatment of fungal infection based on plasma (1→3)beta-D-glucan levels after liver transplantation. Infection 35(5):346–351. doi:10.1007/s15010-007-6240-7
35. Jaijakul S, Vazquez JA, Swanson RN, Ostrosky-Zeichner L (2012) (1,3)-Bta-D-glucan as a prognostic marker of treatment response in invasive candidiasis. Clin Infect Dis 55(4):521–526. doi:10.1093/cid/cis456

INDEX

Richard Calderone and Ronald Cihlar (eds.), *Candida Species: Methods and Protocols*, Methods in Molecular Biology, vol. 1356, DOI 10.1007/978-1-4939-3052-4, © Springer Science+Business Media New York 2016

K

L

M

N

O

P

R

S

T

Y