Gene Expression Analysis in *Arxula adeninivorans*: A Nested Quantitative Real Time PCR Approach

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Abstract During the preparation of RNA traces of genomic DNA are usually co-isolated which might influence downstream applications. We tested several protocols, commercial kits and DNA hydrolysis procedures to remove the DNA contamination and found them to be insufficient. This can raise problems when it comes to gene expression analysis especially when working with intronless genes. Hence, we used a nested quantitative real time PCR approach to avoid amplification from genomic DNA by the use of an artificial anchor sequence introduced at the cDNA synthesis stage. This anchor sequence cannot be found in the genome of *A. adeninivorans* and a first round of amplification using a gene specific oligo in combination with an oligo for the anchor generates fragments which can emerge only from the cDNA. The second PCR step with nested oligos for the gene of interest and the anchor, respectively, significantly increases gene specificity which is crucial particularly when analysing the gene expression status among highly conserved members of a gene family. This second round of amplification represents the actual quantitative real time PCR assay.

Keywords Real-time PCR · RNA isolation · cDNA synthesis · *Arxula adeninivorans*

1 Introduction

Quantitative real-time polymerase chain reaction (qRT-PCR) is an overall accepted tool for detecting and quantifying transcription patterns of desired target genes. This technology is not only used for quantitative genotyping but has been adapted for a large field of applications like diagnosis of disease in human, animals and plants as well as in taxonomy, forensic science and food safety (Deepak et al. 2007).

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These days high throughput techniques for large scale gene expression analyses like microarrays (Maskos and Southern 1992), SAGE studies (Velculescu et al. 1995) or RNA sequencing (Morin et al. 2008) gain increasing significance and qRT-PCR has proven to be a good confirmatory tool to verify the results from such experiments (Puthoff et al. 2003). However, the entire qRT-PCR assay derives from several handling steps beginning with sampling to RNA extraction and purification, cDNA synthesis and analytical methods. All these steps are crucial but not fail-safe and the individual contribution to the overall conclusion seems sometimes neglected. Here we discuss the case when genomic DNA (gDNA) may become a problem as during the preparation of RNA a certain amount of gDNA is usually co-isolated which might result in inaccuracies in quantification. When working with intron-containing genes, residual gDNA is not a big issue because usually the primers for qRT-PCR are located in different exons or even over exon-exon boundaries to avoid amplification from genomic template. Higher eukaryotes tend to have more introns, in human e.g. only 3% of the genes have no intron (Grzybowska 2012). Considering that the majority of *Arxula adeninivorans* genes (88%) are intronless (Kunze et al. 2014) and since enzymatic removal of gDNA is not 100% sufficient we have developed a nested qRT-PCR approach to overcome the problem of contaminating gDNA.

2 Method

It is known that the common RNA isolation procedures yield RNA with significant amounts of gDNA and there are several protocols to remove these contaminations. Besides purification steps like cesium-chloride centrifugation (Glisin et al. 1974) or oligo (dT) chromatography (Aviv and Leder 1972) the enzymatic removal with DNaseI is the most widespread. Even if the different purification attempts may finally succeed none of these treatments will increase the quality of the isolated RNA and if certain transcripts are lost during these procedures the final results will be inaccurate anyway and all the efforts were useless. We tested different approaches and commercial kits to isolate RNA free of gDNA and exemplarily shown in Fig. 2 is a trial with DNaseI and the frequently cutting restriction enzyme CviKI-1 under different conditions (refer to Fig. 2D). According to the supplier (NEB) CviKI-1, derived from CA-1A, a *Chlorella* virus, has 4 expected recognition sites as well as up to eleven relaxed non-cognate sites (star sites) and DNA can be digested to small oligos under “star” conditions. The treatments were performed either “on the column” during the RNA preparation or after the RNA isolation procedure with subsequent purification. Such processed RNA samples were used to synthesize cDNA by reverse transcription reaction with the oligo (dT)-anchor primer (left hand site in Fig. 2A–C). As a control where no cDNA can be generated, the same reactions were set up without the oligo (dT)-anchor primer (right hand site in Fig. 2A–C). The cDNA samples as well as the controls were evaluated by PCR afterwards. Entirely gene specific PCR primers, located within the same exon

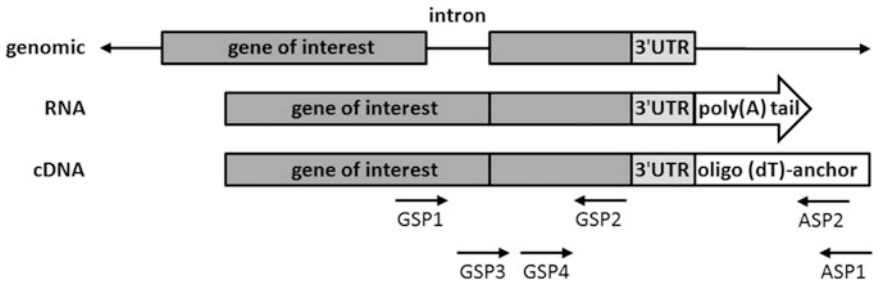

Fig. 1 Principle of the nested quantitative real time PCR approach with positions of gene specific primers (GSP) and anchor specific primers (ASP)

(e.g. GSP2 and GSP4 in Fig. 1), yielded product not only with the cDNA but also with the controls as template indicating that it is virtually impossible to get rid of every last strand of gDNA (Fig. 2A).

Our approach to prevent gDNA emerged products is based on the rapid amplification of cDNA ends (RACE) technique (Frohman 1993). We isolate intact total RNA in as few steps as possible and avoid genomic amplification products during qRT-PCR by the insertion of an artificial anchor sequence at the first strand cDNA synthesis stage. This anchor sequence is part of the oligo (dT)-anchor primer used for cDNA synthesis and cannot be found in the genome of *A. adeninivorans*. Thus, amplification using one gene specific primer (e.g. GSP1 in Fig. 1) in combination with one oligo for the anchor sequence (e.g. ASP1 in Fig. 1) generates fragments which can emerge only from the cDNA. Visible in Fig. 2B is that, in contrast to entirely gene specific primers, PCR products are only gained with cDNA template (left hand site) but no amplification occurs in the controls (right hand site). But since only one of the two primers is designed for the gene of interest unspecific amplification products may occur especially when analysing the gene expression status among highly conserved members of a gene family (Fig. 2B). To increase the specificity of the test a second round of amplification using nested primers (e.g. GSP4 and ASP2 in Fig. 1) is executed subsequently producing a single fragment (Fig. 2C). This second round is performed as the actual qRT-PCR assay while the first round is a standard PCR method. To avoid too many unspecific products during PCR round 1 the number of cycles should be kept as small as possible. As a rule of thumb perform as many as necessary but as few as possible. The optimal number depends on the expression status of the desired target gene and needs to be ascertained for every individual experiment. We usually cycle 5 times in round 1 and use a dilution series of that reaction as template in round 2.

For the design of a nested qRT-PCR assay the contribution of the 3'UTR and the oligo (dT)-anchor primer to the overall PCR product size should be considered. Since the fragments in qRT-PCR assays with intercalating dyes should not be larger than 150 bp we recommend cloning and sequencing the PCR products to gain precise information on the 3' UTR and even on possible alternative polyadenylation events. In case of a large UTR the gene specific primers can be designed to bind to

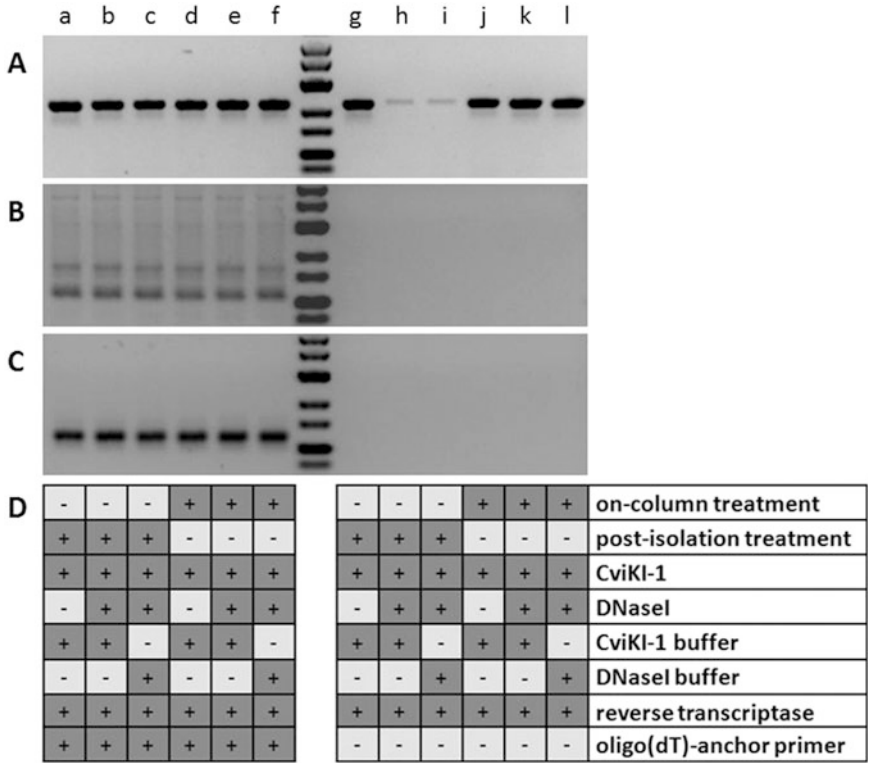

Fig. 2 Differently treated RNA samples (refer to **D**) used for cDNA synthesis with the oligo (dT)-anchor primer (a–f) and control reactions without this oligo (g–l). Subsequently reverse transcription-PCR was performed with entirely gene specific oligos (**A**) and with one gene specific and one anchor oligo (**B**). Shown in (**C**) is the reamplification of (**B**) with nested oligos. RNA was isolated using a spin column Kit and remaining gDNA was hydrolysed after the isolation procedure or meanwhile on the column. For hydrolysis the frequently cutting restriction enzyme CviKI-1 was used alone or in combination with DNAseI in two buffer systems. The different treatments are summarised in (**D**) and marked with (+) when applied and (−) when not

the untranslated region of the transcript which is, furthermore, usually not as conserved as the open reading frame. In addition, the cloned PCR products may serve as templates for the estimation of the primer efficiency.

To demonstrate the influence of residual gDNA in a conventional qRT-PCR assay, we selected an *A. adeninivorans* gene which contains an intron and designed two oligos which are gene specific with one oligo being located on an exon-exon boundary (e.g. GSP2 and GSP3 in Fig. 1) or both oligos being placed within the same exon (e.g. GSP2 and GSP4 in Fig. 1). As a result the qRT-PCR with the latter pair of primers showed a clearly lower C_t value which is due to additional PCR template provided by gDNA (Fig. 3a). The difference in C_t compared to the assay with one exon-exon boundary located oligo is in the shown case 1.45 suggesting

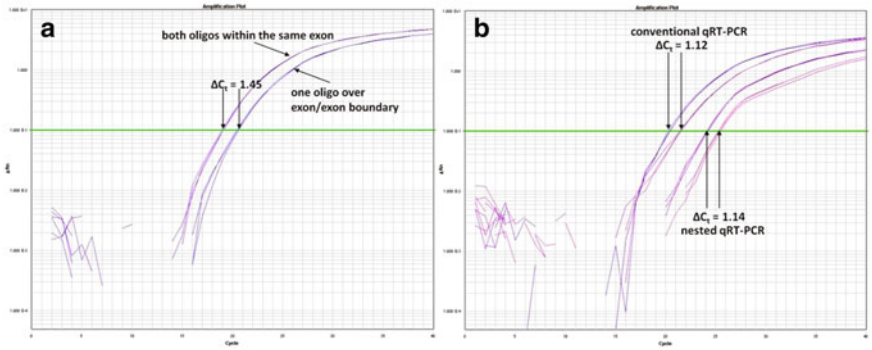

Fig. 3 Amplification plot for a single *A. adeninivorans* gene with oligos which can discriminate cDNA from gDNA (over exon/exon boundary) and those oligos which cannot (within the same exon). In the latter case a significantly lower C_t value indicates the presence of additional template in gDNA form (**a**). Shown in (**b**) is an amplification plot for two *A. adeninivorans* genes with the conventional qRT-PCR compared to the nested qRT-PCR. Similar ΔC_t values indicate that both approaches detect identical gene expression patterns

approximately 3 times more available template for the pair of primers which cannot discriminate cDNA from gDNA.

To validate our experimental design we tested two *A. adeninivorans* genes which do contain introns with a gene specific exon spanning pair of primers (e.g. GSP2 and GSP3 in Fig. 1) and compared the results of this gene expression analysis to a nested qRT-PCR using GSP1 and ASP1 in the first PCR and the nested oligos GSP4 and ASP2 in the qRT-PCR assay. As shown in the amplification plot in Fig. 3b it turned out that the ΔC_t values between two genes was nearly the same with the conventional as with the nested real-time PCR method indicating that both approaches yield similar results in relative gene expression analyses. The difference in C_t values is due to dilution effects after the first PCR and does not affect the relative quantification.

3 Conclusion

The nested qRT-PCR approach with inserted anchor sequence is a suitable method to specifically amplify target cDNAs regardless of contaminating gDNA and yields similar results as the conventional qRT-PCR. Hence, in distinct cases it might supplement other gene expression procedures in terms of plausibility. For example, this method was already successfully applied to verify microarray based data on gene expression during *A. adeninivorans* cultivation with 1-butanol as sole carbon source. Rauter et al. (2016) used nested qRT-PCR to confirm the significant induction of alcohol dehydrogenase 2 (*AADH2*) gene expression upon shift to 1-butanol and revealed its major role in *A. adeninivorans* 1-butanol metabolism.

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