DNA Sequencing from PCR Products

26

Giorgio Basili, Serena Bonin, and Isabella Dotti

Contents

26.1 Introduction and Purpose

Direct DNA sequencing can give important information about the role of genetic alterations in the pathogenesis of diseases. DNA mutations are at the origin of many cancer types, such as the germline mutations in BRCA1 and BRCA2 in breast cancer [1] or the activating mutations in the oncogene BRAF in melanoma [2]. Moreover, the detection of mutations can be used to address the choice of the therapy. For example specific tumour-associated somatic mutations in the tyrosine kinase domain of the EGFR can predict sensitivity to tyrosine kinase inhibitors (e.g. Gefitinib), while mutations in K-Ras gene predict response to monoclonal therapy against EGFR (e.g. Cetuximab) [3]. This section provides a procedure and some advice for direct sequencing of DNA extracted from formalin fixed and paraffin embedded (FFPE) tissues. Mutational analysis requires the following main steps: DNA extraction from FFPE tissues (see chapter "DNA Extraction from FFPE Tissues" and other specific chapters dedicated to DNA extraction), PCR amplification, PCR product purification and sequencing.

26.2 Precautions for Detection of Somatic Mutations

Single nucleotide polymorphisms (SNPs) and germline mutations involve all the cells of the organism. Conversely, somatic mutations appear only in tumoral cells. In order to detect the presence of somatic mutations in tumour tissues by DNA sequencing, it is necessary to limit the non-mutated DNA component coming from non-tumoral cells (like tumour infiltrating

G. Basili (✉), S. Bonin, and I. Dotti
Department of Medical, Surgical and Health Sciences,
University of Trieste, Cattinara Hospital,
Strada di Fiume 447, Trieste, Italy

G. Stanta (ed.), *Guidelines for Molecular Analysis in Archive Tissues*,
DOI: 10.1007/978-3-642-17890-0_26, © Springer-Verlag Berlin Heidelberg 2011

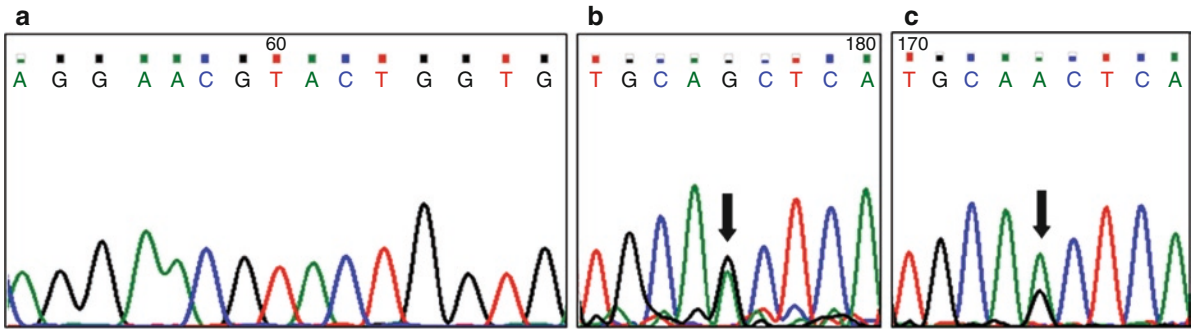

Fig. 26.1 Examples of electropherograms obtained by direct DNA sequencing. (**a**) Electropherogram of a portion of exon 21 of the EGFR gene obtained from a normal tissue without mutations. This is an ideal condition where no background is detected. (**b**) Electropherogram of a portion of exon 20 of the EGFR gene obtained from one tumoral tissue with 100% of cells bearing the sequence alteration in heterozygosis. The alteration is well visible with a double peak despite some background. (**c**) Electropherogram of the portion of exon 20 in another tumoral tissue. Here, although 100% of cells bear the mutation in heterozygosis, the size of the two peaks is not similar and this is a problem when a high background is present

lymphocytes, stromal cells, and endothelial cells). Such component could give electropherograms in which the tumour-associated mutations are not visible because the signal of the non-mutated DNA is prevalent. Usually tumour-associated mutations are present in heterozygosis, resulting in a double peak: one is due to the normal gene and the other one to the mutated gene (Fig. 26.1) [4].

The following limitations and precautions should be considered when using FFPE for DNA mutational analysis:

- Formalin causes a strong DNA degradation [5] and this can result in difficult PCR amplifications that would require higher amounts of starting material.
- PCR amplification on DNA extracted from formalin fixed specimens is usually unsuccessful for fragments longer than 400 bases [6]. Subsequently, the design of short amplicons is essential also in sequence analysis. For a successful DNA amplification, we recommend designing amplicons no longer than 200–250 base pairs. In any case, in establishing the sequencing fragment it must be considered that 20 bases after the primers are not usually correctly read. To bypass this inconvenience we strongly recommend sequencing the PCR product twice using both the forward and reverse primer.
- Sequencing of PCR products obtained by isolating DNA from FFPE can give a number of artifacts four times higher than those detected in DNA from fresh frozen samples[1] [4, 5, 7]. Due to the risk of artifacts, the mutation should be confirmed either by repeating PCR and sequencing or by other means (e.g. use of digestion enzymes capable to discriminate the specific nucleotidic alterations).

In order to overcome these problems, the use of alcoholic fixatives should be taken into consideration as a good alternative to formalin fixation because of their higher preservative properties on nucleic acids (see chapter "Formalin Free Fixatives").

Once these precautions are followed, FFPE can be used as a good source for DNA sequencing. Moreover, FFPE are the ideal material when an enrichment of the tissue sample in specific cell component (e.g.tumoral area) is required because, in comparison to fresh frozen tissues, they allow performing a more precise microdissection and their manipulation is easier.

[1]Artifacts are *ex novo* sequence alterations introduced by the Taq polymerase during the PCR reaction that become visible in the electropherogram when the enzyme amplifies a single strand of damaged DNA. This situation appears when few nanograms of DNA template are used (i.e. 10 ng or less) or when DNA template has high degradation levels (such as DNA from formalin-fixed samples). The expected frequency of artifacts can be considered around 1/4,000 in fresh frozen samples and 1/1,000 bp in formalin fixed samples

26.3 Protocol

26.3.1 Reagents

- *10x PCR buffer with $MgCl_2$*[2] (standard composition: 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris pH 8.3 at 25°C)
- *10 mM dNTPs* prepared from the stock dNTP solution
- *Primers*[3,4]: Lyophilized primers should be dissolved in sterile ddH_2O or 10 mM Tris pH 8 to make a concentrated stock solution. Use sterile ddH_2O to prepare small aliquots of working solutions containing 10–30 pmol/µl primer to avoid repeated thawing and freezing. Store all primer solutions at –20°C
- *Taq DNA Polymerase*
- *Positive and negative controls*: A positive control (e.g. DNA containing the target sequence) should be included to check that the used PCR conditions successfully amplify the target sequence. Negative controls could be water or extracts from a paraffin block without included tissues
- *DNA purification with silica resin or similar.* The columns with silica membrane, e.g. provided by the "QIAquick Gel Extraction Kit" (QIAGEN), are suitable for this purpose.
- *Low melting agarose*
- *40% Polyacrilamide 29:1*
- *Ethidium Bromide, 100 mg/ml*
- *High degree deionized water (ddH_2O)* is recommended for the elution steps. The use of Milli-Q water (Millipore) is suitable for this purpose

26.3.2 Equipment

See Chap. 20 and 21

26.3.3 Method[5]

26.3.3.1 Sample Preparation

- Cool the paraffin blocks in order to properly cut the sections. Cut 5–8 sections of 5 µm thickness from the tissue block, depending on the size of the available sample, discarding the first section and lay the others on glass slides. Stain a section with H&E and use it to evaluate at the microscope if the tissue sample is suitable for microdissection.
- If microdissection is required, mark the area of interest with a pen-marker directly on the coverglass of the H&E stained section. Deparaffinize the corresponding unstained slides (See chapters 4 and 6 for details on microdissection). Fit the unstained sections on the marked H&E slide and scratch away the unnecessary portion of tissue[6] using a scalpel or

[2]There are commercial PCR premixed solutions for convenient PCR setup, containing PCR buffer and dNTPs. Usually Taq Polymerase is provided with its dedicated 10x buffer. Check the composition for the presence of $MgCl_2$. Different commercial PCR buffers are $MgCl_2$ free but a 25 mM or 50 mM $MgCl_2$ solution is supplied with buffer and enzyme. $MgCl_2$ content could be incremented in cases of multiplex PCR or decremented in cases of high fidelity PCR

[3]Primer design could be performed using specific software, please refer to http://molbiol-tools.ca/PCR.htm for the on line available software. Since the first 20–40 bases of the electropherograms are not usually reliable, it is necessary to design primers that match about 40–50 bases before the region of interest

[4]Primer design depends on the quality of starting material. For sub-optimal quality of material (for example DNA from FFPE), shorter PCR products are recommended. However, using higher amounts of starting DNA, the probability to have some intact template molecule is higher and products up to 400 bp can be obtained from FFPE or even longer from alcoholic fixatives. When working with highly damaged DNA, we suggest designing primers for amplicons of around 150 base pairs even if it would cover a short region of interest

[5]In the case of cDNA sequencing, see chapters "RNA Extraction from FFPE Tissues", "DNase treatment of RNA" and "Reverse Transcription (RT), then use the obtained cDNA to proceed with PCR. Use 120 ng of cDNA as template for PCR. If necessary, increase up to 200 ng for difficult amplifications. Primers for cDNA amplification should be intron skipping in order to avoid genomic DNA amplification

[6]In the analysis of tumour associated mutations, the selected region of the tissue slide should not contain more than 20–30% of normal cells in order to obtain a reliable result in the sequencing analysis. In particular, consider the contamination from lymphocytes infiltrating the tumour

the tip of a needle. With the needle collect the tissue of interest in an Eppendorf tube.
- If microdissection is not necessary, cut the whole sections and directly collect them in the Eppendorf tube. Proceed with deparaffinization steps as described in the protocol dedicated to DNA extraction from FFPE.

26.3.3.2 DNA Extraction

- Perform tissue sample digestion following the steps suggested in chapters on DNA extraction from FFPE tissues (Chaps. 7, 8 and 9).
- Resuspend the extracted DNA in a suitable volume (usually15 μl) of sterile ddH_2O.
- Immediately quantify the extracted DNA[7] (see chapter 16, section 16.1).
- Prepare the DNA dilutions in order to use the proper amount of template for the PCR reaction.[8]

26.3.3.3 PCR Procedure

- In a sterile 0.2 ml tube prepare the mastermix including, in the following order, 1X PCR buffer, 200 μM each dNTP, 0.22–0.3 μM of forward and reverse primers,[9] 0.03–0.04 U/μl Taq Polymerase,[10] considering a final PCR reaction volume of 25 μl.[11] The amount of target DNA in each reaction should range between 5 and few hundreds of nanograms.[12] For every PCR run include a positive and a negative control.
- Place the tubes in the thermal cycler and amplify the target DNA using the proper PCR programme (with the annealing temperature required by the primers in use). In the case of DNA extracted from FFPE, a suitable PCR programme consists of: 5 min denaturation step at 95°C, five amplification cycles made up by denaturation, annealing and elongation steps of 1 min. each, 40–45 amplification cycles with steps of 30 s. each. The indicated times are suitable for amplicons shorter then 600–700 bp. Set the final holding step at 4°C. The PCR products can be stored at 4°C until sequencing.[13]

26.3.3.4 PCR Product Analysis

- Load 2 μl of the PCR products on an 8% polyacrylamide gel to evaluate the amount of PCR product and therefore its suitability for sequencing (to prepare the gel follow the procedure described in Appendix B).
- If the expected band is well detected, proceed to step 26.3.3.6.
- If the presence of primer dimers, aspecific amplification products and low amount of specific products is detected,[14] choose one of the following options:

[7] A very accurate quantification of DNA solution is recommended because the PCR for the sequencing analysis requires proper DNA template amounts. When a very little amount of DNA is expected, quantification with a Nanodrop spectrophotometer is recommended because it allows a reliable quantification using only 1 μl of sample

[8] In order to prevent further degradation, whatever the source of the extracted DNA, multiple DNA freezing and thawing should be avoided. For this reason, after quantification, the concentrated DNA sample should be divided into aliquots suitable for the following PCR amplification. The dilutions can be stored at 4°C for a short time (3–4 weeks). The quantification of the concentrated DNA solution should be repeated after each thawing

[9] It is important to reduce the primers to optimal concentration because an excess could result in primer dimers formation and thus interfere with sequencing giving a higher background in the electropherograms

[10] Lowering the amount of Taq polymerase can reduce the presence of aspecific products

[11] Amplifying nanograms of target DNA is troublesome with smaller volumes

[12] A good suggestion is to start with 5–20 ng of DNA template (usually the high DNA degradation caused by formalin does not allow amplifying less than 5 ng). If no PCR products are obtained, try to increase the quantity (do not exceed 500 ng; in case design primers for shorter amplicons)

[13] Freeze-thawing of the PCR products causes their degradation and consequently result in electropherograms with high background. For this reason, it is suggested to store the amplified DNA at 4°C. However, if the PCR products have been frozen repeatedly, good sequencing results may be still obtained by using a higher amount of PCR product (50–100 ng) in the sequencing reaction without changing the amount of primers

[14] Do not confuse polyacrylamide artifacts with aspecific products: the former consist in thin and weak bands of high molecular weight (far more than 1 kbp) and do not interfere with the sequencing reaction

– Try to repeat the PCR reaction using different amplification conditions and/or designing more efficient primers ex novo.
– Go to the optional step 26.3.3.5.

26.3.3.5 Optional: PCR Product Purification from Gel

Load all the PCR product on a 1.5–2% low melting agarose gel stained with ethidium bromide (see Appendix A), and run up to one third of the gel (a longer run can reduce the visibility of the band of interest). Excise the band containing the specific PCR product and purify it using a silica resin or equivalent (e.g. the columns provided by the "QIAquick Gel Extraction Kit" - QIAGEN). Elute the DNA in 25–30 µl. Use 5 µl of the DNA solution as template for a second PCR reaction and check again 2 µl of PCR product on an 8% polyacrylamide gel (repeat steps 26.3.3.3. and 26.3.3.4 of this protocol).

26.3.3.6 PCR Product Purification from the Solution

- Once the PCR product is free of aspecific DNA, purify it (all the available volume) by using the specific silica resin or equivalent (e.g. the columns provided by the "QIAquick Gel Extraction Kit" - QIAGEN). This purification step is intended to separate the PCR product from primers, dNTPs and enzyme. After this step, we strongly recommend eluting the DNA with high degree deionized water (ddH_2O) in order to avoid the presence of salts that could interfere with the following sequencing reaction. The use of 25 µl ddH_2O for a complete elution is suggested.
- Concentrate the DNA by partial evaporation to a final volume of about 5 µl, using a thermoblock at 60°C with open tubes.[15]
- Quantify DNA with a spectrophotometer (see chapter 16, section 16.1) and dilute the DNA sample to a concentration suitable for the sequencing reaction (see the following step).

[15]Since the DNA is highly diluted, this step is necessary to allow an accurate spectrophotometric DNA quantification

26.3.3.7 Sequencing

There are two possibilities for sequencing:

- Performing the sequencing reaction yourself (in this case the procedure will depend on the sequencing system in use in the laboratory).
- Sending the DNA to a sequencing service. For PCR product sequencing, the sequencing primers correspond to the amplification primers. In this case, prepare two tubes each containing the purified PCR product, then add the forward primer to one tube and the reverse primer to the other one. For the analysis of each sequence both the "forward" lecture and the "reverse" lecture are needed in order to confirm all the detected signals. Follow the sequencing service instruction for the proper quantities to be sent. Usually, to sequence 200 bases 4–8 ng of purified PCR product plus 6.4 pmoles of each primer are enough. In this case, before sending DNA + primers are dehydrated in order to preserve them until the sequencing reaction is performed.[16]

26.3.3.8 Electropherogram Analysis

The results can be visualized as electropherograms using specific software such as "Chromas Lite" (www.technelysium.com.au/chromas_lite.html) easily available on line. The corresponding sequence can be easily exported as a text file. Every sequence alteration visualized in the electropherogram can be considered a real mutation only if detected both in the forward and in the reverse lecture. However, in order to exclude PCR artifacts, even the nucleotidic substitution detected in both directions should be confirmed by repeating PCR amplification and sequence analysis.[17] Nucleotidic alterations in heterozygosis are immediately visible in the electropherogram as double peaks, whereas those in homozygosis can be highlighted by aligning the text file of the sequence with the corresponding consensus

[16]The Dehydration step can be performed at 60°C in a thermoblock with open tube caps

[17]The probability that the artifacts detected in the first round of PCR appear in a second independent PCR amplification is extremely low

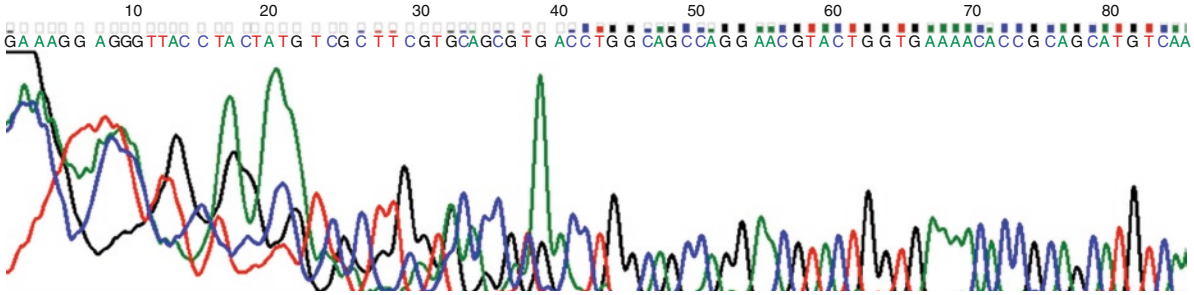

Fig. 26.2 Electropherogram of the first codons of exon 21 of the EGFR gene obtained from a formalin fixed non-tumour sample**.** In this case the electropherogram is reliable after the first 39–40 nucleotides

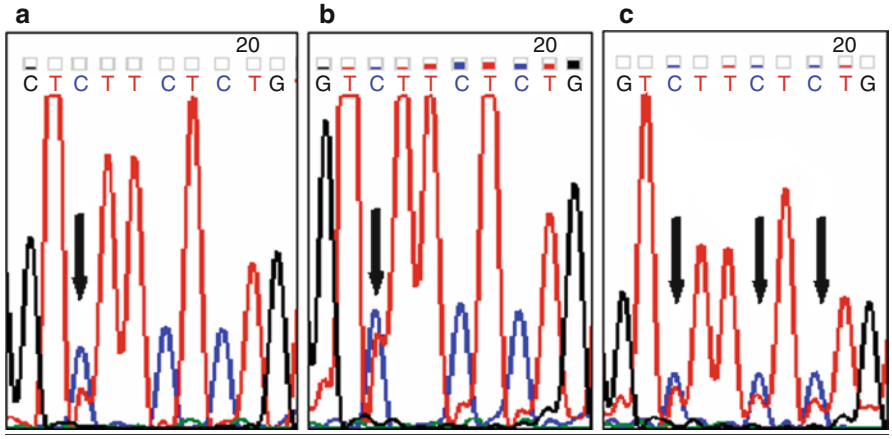

Fig. 26.3 Terminal sections of electropherograms from three different specimens (**a**, **b**, **c**). False peaks are evident (*arrows*) when the real peaks at the same position are short, resulting in double peaks

"wild-type" sequence obtained from a database.[18] Consider that usually the first 20–40 nucleotides in the electropherograms are not readable (Fig. 26.2).

Sometimes, recurrent short "background peaks" can appear in the extremities of the electropherogram, reducing the reliability of the sequencing analysis. These background peaks become evident when the peaks of the DNA sequence are short (Fig. 26.3). They can appear again in the same positions when amplification and sequencing are repeated.

26.3.4 Troubleshooting

- The presence of primer dimers and aspecific products will result in low quality electropherograms characterised by high background. In this case, increase the annealing temperature in the PCR programme, reduce the amount of primers used in the PCR reaction or design more efficient primers ex novo.
- If you don't have successful PCR amplifications, try to increase the number of cycles in the PCR programme (55 cycles at most) or, if needed, increase the amount of template up to 200–500 nanograms.
- If you have neither primer dimers nor aspecific products but still you have bad quality electropherograms, be sure that your PCR product has not been frozen too many times or that the sequencing primers and purified PCR products do not contain salts. Alternatively, reduce the amount of Taq polymerase (around 0.03 U/μl).

[18]On-line free software, such as "ClustalW" (www.ebi.ac.uk/Tools/clustalw2/index.html) can be used to perform the alignment

References

1. Gerhardus A, Schleberger H, Schlegelberger B, Gadzicki D (2007) Diagnostic accuracy of methods for the detection of BRCA1 and BRCA2 mutations: a systematic review. Eur J Hum Genet 15(6):619–627

2. Spittle C, Ward MR, Nathanson KL, Gimotty PA, Rappaport E, Brose MS, Medina A, Letrero R, Herlyn M, Edwards RH (2007) Application of a BRAF pyrosequencing assay for mutation detection and copy number analysis in malignant melanoma. J Mol Diagn 9(4):464–471
3. van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, Quirke P, Flejou JF, Plato Hansen T, de Hertogh G, Jares P, Langner C, Hoefler G, Ligtenberg M, Tiniakos D, Tejpar S, Bevilacqua G, Ensari A (2008) KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch 453(5):417–431
4. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350(21):2129–2139
5. Srinivasan M, Sedmak D, Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 161(6):1961–1971
6. Stanta G, Mucelli SP, Petrera F, Bonin S, Bussolati G (2006) A novel fixative improves opportunities of nucleic acids and proteomic analysis in human archive's tissues. Diagn Mol Pathol 15(2):115–123
7. Quach N, Goodman MF, Shibata D (2004) In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during pcr. BMC Clin Pathol 4(1):1