General Protocol for End-Point PCR

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20.1 Introduction and Purpose

This protocol provides a general workflow for PCR amplification of DNA or cDNA obtained from formalin-fixed and paraffin-embedded samples (FFPE) [1, 2]. The protocol is dedicated not to quantitative, but to qualitative, PCR analysis. Prerequisites for successful PCR include the design of optimized primer pairs, the use of appropriate primer concentrations, and optimization of the PCR conditions [3].

PCR products can be detected by using different tools, ranging from gel electrophoresis to capillary electrophoresis and Southern blot, depending on the analysis requisites.

PCR setup should be performed in a separate area from PCR analysis to ensure that reagents used for PCR do not become contaminated with PCR products (carry-over). Similarly, pipettes used for analysis of PCR products should never be used to set up PCR.

The major problem with FFPE tissues is the extent of degradation of the extracted nucleic acids. In routinely fixed samples, the available fragments of DNA are usually about 300 bases long, while for RNA, (refer to this value if the starting material is cDNA) they are about 100 bases long.[1] In any case, we recommend the designing of amplicons[2] no longer than 200–250 bases for DNA and about 60–80 bases for

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[1] Shorter amplicons are recommended in extracts from autopsy tissues, because of the higher degradation level. For DNA, 90 bases amplicons and for cDNA 70 bases fragments are detectable, on average, in autopsy tissues.

[2] Check homology of the sequences by BLAST analysis: http://www.ncbi.nlm.nih.gov/BLAST/.

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cDNA. Shorter amplicons increase the efficiency of the molecular amplification.

20.2 Protocol

20.2.1 Reagents

- *10× PCR buffer with $MgCl_2$* [3]: We report the standard composition of PCR buffer: 150 mM $MgCl_2$, 500 mM KCl, 100 mM Tris pH 8.3 at 25°C[4]
- *Commercial 100 mM stock solution of each dNTP (pH 8)*
- *dNTPs:* Dilute the dNTP stock solution to prepare a 10 mM solution of each dNTP in sterile water or DEPC-treated water
- *Primers*[5]: Lyophilized primers should be dissolved in a small volume of distilled water or 10 mM Tris pH 8 to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10–30 pmol/µl to avoid repeated thawing and freezing. Store all primer solutions at – 20°C. Primer quality can be checked on a denaturing polyacrylamide gel; a single band should be seen
- *Taq DNA Polymerase*
- *Positive and negative controls*: Inclusion of control reactions in the PCR assay is essential to PCR interpretation. A positive control should be included to check if the used PCR conditions can successfully amplify the target sequence. As PCR is extremely sensitive, requiring only a few copies of target template, a negative control containing no template DNA should always be included to ensure that the solutions used for PCR have not become contaminated with template DNA

20.2.2 Equipment

- *Disinfected*[6] *adjustable pipettes,* range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*1.5, 0.5, and thin walled 0.2 ml tubes[7] (autoclaved)
- *Centrifuge* suitable for centrifugation of 1.5 ml tubes at 13,200 or 14,000 rpm
- *Laminar Flow Cabinet equipped with a UV lamp*
- *Thermal Cycler*: A large number of programmable thermal cyclers marketed by different companies are available for use in PCR. The choice of the commercial instruments depends on the investigator's inclination, budget, etc… The main requisite is that the instrument must have the license for use in PCR. The thermal cycler must be equipped with a heated lid in order to prevent internal contamination during the amplification process

20.2.3 Method

Preparation of the reaction mixture should be performed under a laminar flow cabinet. Fresh gloves should be worn.

- In a sterile 0.2 ml tube, add, in the following order, the 10× amplification buffer, dNTPs (10 mM), primers, Taq Polymerase, and finally, the template DNA. The suggested final volume for PCR reaction is 25–50 µl. Each reaction sample must contain the proper amount of target DNA,[8] 50 mM KCl, 10 mM Tris pH 8.3, 1.5–2.0 mM $MgCl_2$, 200 µM of each dNTP, 15–25 pmol of forward primer, 15–25 pmol of reverse primer,[9] and 1–5 units of Taq Polymerase.[10]

[3]There are commercial PCR premixed solutions for convenient PCR setup, containing PCR buffer and dNTPs. Usually, Taq Polymerase is provided with its dedicated 10× 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.

[4]Because of the high temperature dependence of the pKa of Tris, the pH of the reaction will drop to about 7.2 at 72°C.

[5]Primer design could be performed using a particular kind of software, please refer to http://molbiol-tools.ca/PCR.htm for on line available software.

[6]Clean the pipettes with alcohol or another disinfectant and leave them under the UV lamp for at least 10 min. Alternatively it is possible to autoclave the pipette depending on the provider instructions.

[7]Micro titer plates could also be used to run PCR.

[8]The amount of target DNA required varies according to the complexity and level of target sequence. Usually, it ranges from 1 pg to 1 µg.

[9]The primer and Mg^{2+} concentration in the PCR buffer and the annealing temperature of the reaction may need to be optimized for each primer pair for a more efficient PCR.

[10]In order to prevent artifacts, a hot start PCR using a thermostable Taq Polymerase (e.g., AmpliTaq Gold by Applied Biosystems) is suggested.

- For every PCR run, include a positive control, a bystander DNA that does not contain the sequence of interest and a blank negative control (H_2O or better the extraction obtained from a paraffin block without tissue).[11]
- Displace the tubes in the thermal cycler and amplify the target DNA using the proper PCR program made up by denaturation, annealing, and elongation steps. For small stretches, the time required in every step of the PCR could be shorter, ranging from 30 s to 1 min, with a final 5–7 min extension step at 72°C. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For DNA extracted from FFPE, usually at least 35–40 cycles are performed.[12]
- Programme the instrument in order to maintain the amplified samples at 4°C at the end of amplification, before analysis or storing.
- Check the amplification by agarose electrophoresis stained with ethidium bromide,[13] by means of Southern blot or dot blot (using an internal probe) (see Chap. 17, Sect. 17.1 and Appendix A for agarose gel preparation). Store the PCR products at –20°C avoiding repeated thawing and freezing.

20.2.4 Troubleshooting

- If the bands of the desired product are sharp but faint, both in positive controls and samples, inefficient priming or inefficient extension may have occurred. To solve this problem, set up a series of PCRs containing different concentrations of the two primers (e.g., from 5 to 30 pmol each/reaction). Once the optimal primer concentration is found, set up a series of PCRs containing different concentrations of Mg^{2+} (e.g., ranging from 1 to 4.5 mM) to determine the optimal concentration.
- If there are bands in the negative controls, contamination of solutions or plasticware with template DNA may have occurred. In such case, make up new reagents and operate in separate spaces to prepare the PCR solution and to analyze the products of amplification.
- If distinct bands of unexpected molecular weight appear, they are the result of nonspecific priming by one or both primers. To eliminate this problem, decrease the annealing time and/or increase the annealing temperature. Furthermore, it is possible to perform a hot start by utilizing a Taq polymerase requiring thermal activation.
- If a generalized smear of amplified DNA is present, too much template DNA was introduced in the reaction. Try the analysis with a scaling down of the target amount.
- If the amplification is weak or nondetectable, there could be different possible causes: defective reagents, defective thermal cycler, or programming error. If you are setting up the PCR system, the cause could also be a suboptimal extension of annealed primers, an ineffective denaturation, or you may have chosen too long stretches for your PCR analysis.

[11] If the thermal cycler is not fitted with a heated lid, overlay the reaction mixture with one drop (about 20 µl) of light mineral oil to prevent evaporation and cross contamination.

[12] Too many PCR cycles could promote aspecific amplifications, and/or PCR product carry-over.

[13] EtBr is a potentially carcinogenic compound. Always wear gloves and use it under a laminar hood. Used EtBr solutions must be collected in containers for chemical waste and discharged according to the local hazardous chemical disposal procedures.

References

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