Nested-PCR

22

Serena Bonin and Isabella Dotti

Contents

22.1 Introduction and Purpose

This section aims to provide the general guidelines with a specific protocol, as an example of how to apply nested-PCR to DNA or cDNA obtained from formalin-fixed and paraffin-embedded (FFPE) samples [1–3]. For general requisites for PCR reaction, please refer to the related section in Chap. 20. The goal of this document is to provide a standard workflow for nested amplification, along with a non-isotopical system for PCR product detection. Nested-PCR is related to the reamplification of a first product of PCR by using a second set of primers within the first amplification product. The utilized primers should be different in both reactions. This methodology increases both the sensitivity and specificity of the assay. On the other hand, the risk of contamination increases significantly because of the greater amount of amplification products and working steps involved. In the traditional workflow of nested-PCR, the primers for the second-round PCR were added directly after the first step PCR. Nowadays, only a small amount (a few micro liters) of the first-round PCR product is amplified in a second PCR reaction. Alternatively, to avoid the first-round product contamination, it is also possible to perform a single-tube two-round PCR in single closed reaction tubes [4]. In addition, single-tube two-round PCR could be performed by mixing immediately the primers for the first- and the second-round PCR if they show marked differences in their annealing temperatures. The selected high annealing temperature of the first-round primers results in high specificity in the first-round PCR. This approach has also been described by the use of real-time PCR [5].

The time required for the whole procedure is 3 days.

The extremely high sensitivity of the nested-PCR system could often result in contaminations from

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_22, © Springer-Verlag Berlin Heidelberg 2011

previous reactions, then resulting in false-positive analyses. To check the validity of the assay, we strongly recommend using negative controls and taking wide routine precautions. Positive controls are also recommended in the nested-PCR, but to avoid contamination, we suggest using highly diluted controls. We recommend making at least two different sample aliquots and storing them in a laboratory room outside the PCR space. Furthermore, reagent preparation and extractions should be performed in a separate flow-laminar cabinet. First and second nested-PCR rounds should be performed in different areas, e.g. in two different laminar flow hoods, which should be distinct from the extraction ones. In these different spaces, we also suggest employing different supplies, pipette tips, eppendorf tubes, and any common reagent such as deionized water and PCR buffer. Finally, the detection process should be carried out in a dedicated laboratory. Operators are encouraged to wear disposable gloves in each PCR space, changing them frequently, especially when moving from one dedicated area to others.

For its high sensitivity, nested-PCR systems are commonly used in the detection and genotyping of pathogenic viruses and bacteria. Hereafter, we are going to describe a system of nested-PCR to detect borrelial DNA, the causative agent for Lyme disease, in DNA from FFPE specimens. The method presented here has been previously described by Wienecke et al. [6].

22.2 Protocol

22.2.1 Reagents

Reagents from specific companies are here reported, but reagents of equal quality purchased from other companies may be used.

- *10× PCR buffer with $MgCl_2$[1] (stock solution):* 150 mM $MgCl_2$, 500 mM KCl, 100 mM Tris pH 8.3 at 25°C[2]
- *Commercial 100 mM stock solution of each dNTP (pH 8)*
- *dNTPs:* Dilute the dNTPs stock solution to prepare a 10-mM solution of each dNTP in sterile water or DEPC-treated water
- *Primers*[3]: Oligonucleotide primers and the method here described for Borrelia detection were designed by Wienecke et al. [6]. The outer primer pair (Bb1 and Bb2) flanks a 171-bp fragment, while the inner primer pair (Bb3 and Bb4) gives a 92-bp product. An internal hybridization control (Bb-hyb) is used. Primer sequences are reported in Table 22.1
- 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*
- *Agarose powder electrophoresis grade*
- *Agarose gel loading buffer (6×):* 100-mM EDTA pH 8, 0.25% (w/v) Bromo phenol blue (BMP), 50% (v/v) glycerol in H_2O
- *Positively charged nylon membrane* for nucleic acid transfer (e.g., Hybond-N+, GE Healthcare)
- *5× TBE (stock solution):* 225-mM Tris base, 225-mM Boric acid, 5-mM EDTA pH 8
- *Ethidium Bromide, 10 mg/ml (stock solution)*[4] Stock solution of EtBr should be stored at 4°C in a dark bottle
- *0.5-M NaOH, 1.5-M NaCl solution*

Table 22.1 Primer sequences

Label	Sequence (5′–3′)
Bb1	AAACGAAGATACTCGATCTTGTAATTGC
Bb2	TTGCAGAATTTGATAAAGTTGG
Bb3	TCTGTAATTGCAGAAACACCT
Bb4	GAGTATGCTATTGATGAATTATTG
Bb-Hyb	TTGAATTAAATTTTGGCTT

[1]There are commercial PCR premixed solutions for convenient PCR setup, containing PCR buffer and dNTPs. Taq Polymerase is usually 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.

[2]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.

[3]Primer design could be performed using a specific software; please refer to http://molbiol-tools.ca/PCR.htm for online available software.

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

- *20× SSC:* 3-M NaCl, 0.3-M Na_3 citrate·$2H_2O$. Adjust to pH 7 with HCl 1 M
- *N-laurylsarcosine* (30% stock solution-Fluka)
- *Poly dATP*
- *10% SDS (w/v) (stock solution)*[5]
- *DIG Oligonucleotide Tailing Kit, second Gen.* (Roche, cat. No. 03 353 583 910)
- *DIG Nucleic Acid Detection Kit* (Roche, cat. No. 11 175 041 910)
- *3-MM Paper*

22.2.2 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 among the commercial instruments depends on the investigator's inclination, budget, etc. The main requisite is that the instrument must have the licence for use in PCR. For diagnostic purpose, the thermal cycler must be equipped with a heated lid in order to prevent contamination.
- *Horizontal electrophoresis devices*
- *UV transilluminator*
- *Hybridization oven*
- *UV Crosslinker*

22.2.3 Method

- In a sterile 0.2-ml tube, mix 10 µl of extracted DNA and 40 µl of PCR reaction mixture for a final volume of 50 µl. Each reaction contains 10-mM Tris-HCl pH 8.3, 50-mM KCl, 1.5-mM $MgCl_2$, 200-µM each dNTP, one unit of Taq DNA Polymerase, 25 pmol each of the outer primers Bb1 and Bb2. For every PCR run, include a diluted positive control, a bystander DNA that does not contain the sequence of interest (DNA of another bacterial pathogen), a blank control (H_2O).[8]
- Displace the tubes in the thermal cycler. After the initial denaturation for 5 min at 95°C, run 30 amplification cycles as follows: 95°C/1 min; 50°C/1 min ; and 72°C/1 min. Program the instrument in order to maintain the amplified samples at 4°C before storing.
- Samples and controls derived from the first PCR run are submitted to the nested step.[9] In a sterile 0.2-ml tube, mix 2 µl of first-round PCR product and 23-µl of PCR reaction mixture for a final volume of 25 µl. Each reaction contains 10-mM Tris-HCl pH 8.3, 50-mM KCl, 1.5-mM $MgCl_2$, 200-µM each dNTPs, one unit of Taq DNA Polymerase, 25 pmol each of the inner primers Bb3 and Bb4.
- Displace the tubes in the thermal cycler and amplify the target DNA using the previous PCR programme. Set the instrument in order to maintain the amplified samples at 4°C before storing.
- Analyze 10 µl of the final products by nondenaturing 2.5% agarose gel electrophoresis. In a 100-ml pirex bottle, weigh 1.25 g of agarose powder (electrophoresis grade). Add 50 ml of 1× TBE buffer. Melt the powder in the microwave oven, avoiding loss of material. Cool the gel solution at about 50°C, and add 3 µl of EtBr stock solution. Pour the melted agarose solution into the gel chamber, and let the gel thicken.
- Load 10 µl of amplification product using 2 µl of loading buffer 6×. Separate the PCR products at 75 V in 1× TBE buffer until the dye reaches ¾ of the gel length.
- Check the gel at the UV light.
- Denature the separated PCR products by soaking the gel for two times in the 0.5-M NaOH, 1.5-M NaCl solution for 15 min at room temperature with gently swirling.

[5]Weigh the proper amount of SDS powder under a fume hood, because it is harmful.

[6]Clean the pipettes with a disinfectant (e.g., Meliseptol®rapid) and leave them under the UV lamp for almost 10 min. Alternatively it is possible to autoclave the pipette depending on the provider instructions.

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

[8]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.

[9]Special precautions should be taken in this step for the possibility of carry-over (false-positive results due to contamination among different tubes). It is recommended to work in a flow hood with samples cooled in ice and with anti-aerosol pipette tips.

- Prepare the following sandwich for Southern transfer (bottom-up): three sheets of paper 3 MM presoaked in 0.5-M NaOH and 1.5-M NaCl, gel upside down, positively charged membrane over the gel surface avoiding bubble formation,[10] one sheet of paper 3 MM wet with deionized water, three dry sheets of paper 3 MM, some paper towels, finally a glass with a weight on the top. Allow the DNA to transfer onto the membrane for at least 6 h.[11]
- Wash the membrane in 2× SSC for 5′, put the membrane between two sheets of paper to dry it, and then store it at room temperature until it is used for hybridization.
- Before hybridization, crosslink blotted nucleic acids to the membrane at the UV crosslinker twice with face up (each crosslinking takes about 1′ and 18″ and goes from 0.25 to 0 J).
- Perform probe hybridization in 5× SSC, 0.1%-*N*-laurylsarcosine, 0.02%-SDS, 5-µg/ml poly-dATP, 10-ng/ml of detection oligonucleotide Bb-Hyb, which has been tailed with digoxigenin-dUTP, according to the protocol of the manufacturer (Roche, Dig Oligonucleotide Tailing Kit). Incubate overnight in a hybridization oven at 41°C, swirling gently.
- Wash the membrane three times with 0.1× SSC, 0.1%-SDS, at 28°C. Detect the system according to the DIG nucleic acid detection kit that uses an enzyme immunoassay and enzyme-catalyzed colour reaction with NBT/BCIP, contained in the DIG Nucleic Acid Detection Kit (Roche).

22.2.4 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. Find the optimal primer concentration and then set up a series of PCRs containing different concentrations of Mg^{2+} to determine the optimal concentration (see Chap. 20, Sect. 20.2.4).
- If there are bands in the negative controls, contamination of solutions or plastic ware with template DNA may have occurred. In this case, make up new reagents and operate in separate spaces to run PCR and to analyze the products of amplification.
- If distinct bands of unexpected molecular weight are present, they are the result of nonspecific priming by one or both primers. To solve this problem, decrease the annealing time and/or increase the annealing temperature.
- If a generalized smear of amplified DNA is present, too much template DNA was introduced.
- If the amplification is weak or non-detectable, there could be different possible causes: defective reagents, defective thermal cycler, or programming errors. If you are setting up the PCR system, the cause could also be a suboptimal extension of annealed primers, an ineffective denaturation, or the stretches of DNA you have chosen to amplify may be too long.
- For troubles in hybridization detection, refer to the DIG nucleic acid detection kit data sheet instructions (http://www.roche-applied-science.com/pack-insert/1175041a.pdf).

[10] Bubbles can be removed by rolling a Pasteur pipette on the surface.

[11] Alternatively to Southern blot, it is possible to transfer directly the nested PCR products onto the membrane by dot-blot apparatus without electrophoretic separation (See Chap. 23 for details). In this case, after the dot-blot transfer, go directly to the UV cross linking of the membrane and follow the protocol for membrane hybridization.

References

1. Cseke L, Kaufman P, Podila G, Tsai C (2004) Handbook of molecular and cellular methods in biology and medicine, 2nd edn. CRC, Boca Raton
2. Sambrook J, Russel DW (2001) Molecular cloning. A laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press, New York
3. Tarrago-Asensio D, Avellon-Calvo A (2005) Nested PCR and multiplex nested PCR. In: Fuchs J, Podda M (eds) Encyclopedia of diagnostic genomics and proteomics. Marcel Dekker, New York, pp 906–910
4. Olmos A, Cambra M, Esteban O, Gorris MT, Terrada E (1999) New device and method for capture, reverse transcription and nested PCR in a single closed-tube. Nucleic Acids Res 27(6):1564–1565
5. Berg J, Nagl V, Muhlbauer G, Stekel H (2000) Rapid-cycle PCR in temporarily compartmentalized capillaries: two-round PCR in a single capillary prevents product carry-over. Biotechniques 29(4):680–684
6. Wienecke R, Neubert U, Volkenandt M (1993) Molecular detection of borrelia burgdorferi in formalin-fixed, paraffin-embedded lesions of Lyme disease. J Cutan Pathol 20(5):385–388