Troubleshooting Guide for PCR

PCR or RT-PCR problem

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Table 3.2. Troubleshooting Guide for PCR.

Problem	Possible cause and recommended solution
1. Low yield or no PCR/RT-PCR product	**1.1. Low template quality or quantity.** **1.1.1. Poor template integrity.** DNA templates. Evaluate template integrity by agarose gel electrophoresis. Use DNA isolation methods that minimize shearing and nicking of DNA. Resuspend isolated DNA in TE buffer, pH 8.0, or in Water, nuclease-free (#R0581). RNA templates. RNA purity and integrity is essential for synthesis of full-length cDNA, which results in high quality RT-PCR products. Always assess the integrity of RNA prior to cDNA synthesis. For example. if sharp bands of both the human 18S rRNA (runs at approx. 1.9 kb) and the 28S rRNA (runs at approx. 5 kb) are formed during denaturing agarose gel electrophoresis of total human RNA, the mRNA in the sample is intact. Follow general recommendations to avoid RNase contamination (p.313). **1.1.2. Low template purity.** DNA templates. Generally most commercially available DNA purification methods yield template suitable for PCR, e.g. Genomic DNA Purification Kit (#K0512) or GeneJET™ Plasmid Miniprep Kit (#K0502). However, trace amounts of certain agents used in home-made DNA purification protocols, such as phenol, EDTA, and Proteinase K may inhibit thermostable DNA polymerases. In addition, high ionic concentrations (e.g. K^+, Mg^{2+}, etc.) may lead to suboptimal reaction conditions for the DNA polymerase. In cases such as this, the template should be re-purified using using a spin-column or re-precipitated and washed with 70% ethanol. RNA templates. Trace amounts of agents used in RNA purification protocols may remain in solution and inhibit reverse transcriptases, e.g. SDS, EDTA, guanidine salts, phosphate, pyrophosphate, polyamines, spermidine. Precipitate the RNA with ethanol and wash the pellet with 75% ethanol. **1.1.3. Low quantity of template.** DNA templates. Increase the amount of template or use hot start enzymes or master mixes, e.g. TrueStart™ *Taq* DNA Polymerase (#EP0611), Maxima™ Hot Start *Taq* DNA Polymerase (#EP0601) or PyroStart™ Fast PCR Master Mix (2X) (#K0211). Alternativley, use enzymes or master mixes with higher sensitivity than that of *Taq* DNA Polymerase, e.g. DreamTaq™ DNA Polymerase (#EP0701) or High Fidelity PCR Enzyme Mix (#K0191). RNA templates. Increase the amount of template to the recommended level. After DNaseI treatment, terminate the reaction by heat inactivation in the presence of EDTA (*see* protocol on p.313). Heat inactivation in the presence of divalent cations degrades RNA.
	1.2. Faulty primer design. DNA templates. Use REviewer™ primer design software at www.fermentas.com/reviewer or follow general recommendations for PCR primer design in p.306. Make sure primers are not self-complementary and avoid between primer complementary sequences. Extension of primer duplexes will consume reaction components and result in lower yields of the target PCR product. Verify that the primers are complementary to the correct strands of template DNA. RNA templates. Use the correct primer for the type of RNA template used for reverse transcription (*see* p.313). Do not use the oligo$(dT)_{18}$ primer for bacterial RNA or RNA without poly(A) tail. In such cases the random hexamer primer is recommended. If using sequence-specific primer, ensure it is complementary to 3'-end of the template RNA.
	1.3. Suboptimal thermal cycling conditions. Follow the general PCR cycling recommendations for the specific DNA polymerase you use (*see* Protocols and Recommendations for PCR, p.306). **1.3.1. Annealing.** The annealing temperature should be 5°C lower than the primer-template melting temperature (Tm). The annealing temperature may be optimized stepwise in 1-2°C increments. If available, use a gradient cycler to optimize the annealing temperature of a specific primer pair (±10°C). The annealing temperature also has to be adjusted when additives that change the melting temperature of the primer-template duplex are used, e.g. glycerol, DMSO, formamide, betaine, TMANO (trimetylamine-N-oxide). **1.3.2. Extension.** The recommended reaction temperature is 72°C for *Taq* and *Pfu* DNA polymerases. As a general rule, the extension step with *Taq* DNA Polymerase takes 1min/kb. As *Pfu* DNA Polymerase has a lower extension rate, allow 2 min/kb for extension step with this enzyme. For fast cycling PCR, e.g. using Fermentas PyroStart™ Fast PCR Master Mix (2X) (#K0211), the extension time can be shortened to 25 s/kb and lower. For amplification of longer templates (>3 kb) the extension temperature can be reduced to 68°C. Long PCR Enzyme Mix (#K0181) and High Fidelity PCR Enzyme Mix (#K0191) are ideal for such applications. In addition to the standard extension time (1 min/kb) an auto-extension per cycle is recommended for later cycles when amplifying longer templates *see* table on p.312. **1.3.3. Number of Cycles.** The number of cycles varies depending on the amount of template DNA in the PCR mixture and the expected yield of the PCR product. Generally 25-35 cycles are sufficient to produce an adequate yield of PCR product. If less than 10 copies of the template DNA are present in the reaction, extend the number of cycles to 40. For calculation of the template copy number, refer to the formula given in the Appendix on p.484

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Table 3.2. Troubleshooting Guide for PCR.

Problem	Possible cause and recommended solution
1. Low yield or no PCR/RT-PCR product	**1.4. Suboptimal reaction conditions.** **1.4.1. Insufficient amount of DNA polymerase.** For a 50 µl PCR mixture, we recommend adding 1-1.5 u of *Taq* DNA Polymerase or 1.25-2.5 u of *Pfu* DNA Polymerase. However, it may be necessary to increase the amount of DNA Polymerase, if the PCR mixture contains inhibitors, due to contamination of the template DNA. **1.4.2. Insufficient amount of primer.** Generally the PCR reaction is successful with wide range of PCR primer concentrations (0.1-1 µM) and the optimal conditions will vary depending on specific primer/template pair. A primer comcentration of 0.4 µM is a good starting point for optimization. For long PCR and PCR with degenerate primers a minimum of 0.5 µM is recommended. Also, assay primer degradation on a denaturing polyacrylamide gel. PCR primers may degrade due to the 3'→5' exonuclease activity of *Pfu* DNA Polymerase or PCR Enzyme Mixes. Therefore, PCR mixtures should be kept on ice during the reaction set-up and the polymerase or mix should be the last component added to the reaction mixture. Alternatively, phosphorothioate primers can be used to avoid primer degradation by *Pfu* DNA Polymerase. **1.4.3. Insufficient Mg^{2+} concentration.** If the Mg^{2+} concentration is too low, the yield of PCR product may be reduced. Due to the binding of Mg^{2+} to dNTPs, primers and DNA template, Mg^{2+} concentration often needs to be optimized for maximal PCR yields. The recommended concentration range for optimizations is 1-4 mM. For standard PCR with 0.2 mM dNTP and Fermentas *Taq* DNA Polymerase, a good starting $MgCl_2$ concentration is 1.5 mM (for *Taq* buffer with KCl) and 2.0 mM (for *Taq* buffer with $(NH_4)_2SO_4$). For *Pfu* DNA Polymerase we recommend a starting concentration of 2 mM of $MgSO_4$ and for DreamTaq™ DNA Polymerase – 2 mM of $MgCl_2$. If template DNA contains EDTA or other metal chelators, the Mg^{2+} ion concentration in the PCR mixture should be increased accordingly (1 molecule of EDTA binds 1 molecule of Mg^{2+}). In certain PCR applications higher dNTP concentrations are required. dNTPs also complex Mg^{2+}, therefore the Mg^{2+} concentration has to be increased accordingly. **1.4.4. dUTP or modified nucleotides in reaction mix.** Proofreading polymerases or enzyme mixes containing such proofreading polymerases may incorporate dUTP with much less efficiency compared to standard dNTPs. If possible, use non-proofreading polymerases like *Taq* DNA Polymerase to incorporate dUTP into the PCR product. Alternatively, use a higher ratio of dUTP:dNTP to achieve the desired yield of labeled PCR product.
	1.5. Complex template. **1.5.1. GC-rich template.** DNA templates. If the template has high GC content, and/or forms a complex secondary structure, we recommend using our Long PCR Enzyme Mix (#K0181). Alternatively DNA denaturation can be enhanced by the addition of either 10-15% glycerol, 10% DMSO or 5% formamide. When any of these additives are used, the annealing temperature has to be lowered. Since DMSO and formamide inhibit polymerases by approximately 50%, the amount of the enzyme in the PCR mix also has to be increased. RNA templates. If the RNA template is GC rich or known to contain secondary structures, use reverse transcriptases with high thermostability, e.g. AMV Reverse Transcriptase (#EP0641) or RevertAid™ H Minus Reverse Transcriptase (#EP0451), and increase the temperature of the reverse transcription reaction. **1.5.2. Long template.** Use appropriate long PCR enzymes for templates longer than 3 kb, e.g. High Fidelity PCR Enzyme Mix (#K0191) or Long PCR Enzyme Mix (#K0181). The extension temperature can be reduced to 68°C. In addition to the standard extension time (1 min/kb) an auto-extension per cycle is recommended for later cycles when amplifying long templates *see* table on p.312.
2. Non-specific PCR products	**2.1. Faulty primer design.** Use REviewer™ primer design software at www.fermentas.com/reviewer or follow the general recommendations for PCR primer design in p.306. Verify that the primers are complementary to correct strands of template DNA. Verify that the primers are specific to the template region selected for amplification and have no complementarity with other regions in the template DNA. Otherwise, primers will anneal nonspecifically and generate unexpected PCR products. Ensure primers are not self-complementary contain between primer complementary sequences. Otherwise extension of primer duplexes will generate unexpected products. Avoid direct repeats in the primers to limit the appearance of large PCR products compared to the target amplicon.

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Table 3.2. Troubleshooting Guide for PCR.

Problem	Possible cause and recommended solution
2. Non-specific PCR products	**2.2. Reaction set up at room temperature.** When a PCR reaction is set up at room temperature, *Taq* DNA polymerase exhibits low but noticeable activity during the reaction set-up. As a result non-specific priming events may lead to generation of unexpected amplification products during PCR. To avoid this, when using *Taq* polymerase the PCR reaction set-up should always be performed on ice. Alternatively, use hot start PCR enzymes that have no activity at room temperature and are activated only at high temperatures during PCR cycling, e.g. TrueStart™ *Taq* DNA Polymerase (#EP0611), Maxima™ Hot Start *Taq* DNA Polymerase (#EP0601) or PyroStart™ Fast PCR Master Mix (2X) (#K0211). In hot start PCR non-specific amplification is minimized and target yield is increased. PCR can be set-up at room temperature with hot start enzymes.
	2.3. Suboptimal reaction conditions. **2.3.1. Excess Mg^{2+} concentration.** If the Mg^{2+} concentration is too high, non-specific PCR products may appear. The recommended concentration range for optimizations is 1-4 mM. For standard PCR with 0.2 mM dNTP and Fermentas *Taq* DNA Polymerase, a good starting point of $MgCl_2$ concentration is 1.5 mM (for *Taq* buffer with KCl) and 2.0 mM (for *Taq* buffer with $(NH_4)_2SO_4$). For DreamTaq™ DNA Polymerase or PCR Enzyme Mixes – we recommend a starting concentration of 1.5 mM $MgCl_2$. For *Pfu* DNA Polymerase – 2 mM $MgSO_4$ and DreamTaq™ DNA Polymerase – 2 mM $MgCl_2$ is a good starting point. **2.3.2. Template amount too high.** Optimal amounts of template DNA in a 50 µl reaction volume are in the 0.01-1 ng range for both plasmid and phage DNA, and in the 0.1-1 µg range for genomic DNA. Higher amounts of template increase the risk of generation of nonspecific PCR products. **2.4. Suboptimal thermal cycling conditions.** The optimal annealing temperature normally is about 5°C lower than the primer-template melting temperature (Tm). The annealing temperature may be optimized stepwise in 1-2°C increments. If available, use a gradient cycler to optimize the annealing temperature of a specific PCR (±10°C). The annealing temperature also has to be adjusted when additives that change the melting temperature of the primer-template duplex are used (e.g. glycerol, DMSO, formamide, betaine, TMANO (trimetylamine-N-oxide) or hydroxy-ectoine).
3. Sequence errors in PCR product	DNA sequence errors detected after cloning and sequencing of PCR product can be a result of procedures used for cloning, therefore also refer to the troubleshooting given in the chapter Molecular Cloning (p.340). **3.1. Sequence errors within a PCR product.** **3.1.1. Low fidelity thermostabile DNA polymerase used in PCR.** For downstream applications such as cloning or site-directed mutagenesis, high fidelity thermostabile DNA polymerases, such as *Pfu* DNA Polymerase (#EP0501, #EP0571) or High Fidelity PCR Enzyme Mix (#K0191) are recommended. **3.1.2. Sub-optimal reaction conditions.** Excess Mg^{2+} concentration. If the Mg^{2+} concentration is too high the fidelity of the PCR decreases. The recommended Mg^{2+} concentration range for PCR optimizations is 1-4 mM. For standard PCR with 0.2 mM dNTP and Fermentas *Taq* DNA Polymerase, a good starting point of $MgCl_2$ concentration is 1.5 mM (for *Taq* buffer with KCl) and 2.0 mM (for *Taq* buffer with $(NH_4)_2SO_4$). For DreamTaq™ DNA Polymerase, 2 mM $MgCl_2$. For PCR Enzyme Mixes we recommend a starting concentration of 1.5 mM $MgCl_2$. For *Pfu* DNA Polymerase a good starting point is 2 mM $MgSO_4$. Suboptimal template concentration. Optimal amounts of template DNA in the 50 µl reaction volume are in the 0.01-1 ng range for both plasmid and phage DNA, and in the 0.1-1 µg range for genomic DNA. Lower amounts of template reduce the accuracy of the amplification. Imbalanced dNTP concentration. It is very important to have equal concentrations of all the nucleotides (dATP, dCTP, dGTP and dTTP) in the reaction. If the nucleotide concentrations are not balanced, the PCR error rate may dramatically increase. Fermentas dNTP Mixes contain either 2 mM (#R0241) or 10 mM (#R0191), or 25 mM (#R1121) of each nucleotide. The concentrations of all four dNTPs are perfectly balanced to provide fidelity and to increase the yield of PCR products. **3.1.3. Exposed to UV PCR product.** Use a long UV wavelength (360 nm) light-box when analyzing and excising PCR products from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit exposure to UV to several seconds. Keep the gel on a glass plate or on a plastic plate during illumination with UV. Alternatively, use dyes visible in ambient light to visualize PCR products in standard agarose gels (1, 2). **3.1.4. Sequencing error.** To verify the reliability of sequencing results, sequence both DNA strands.

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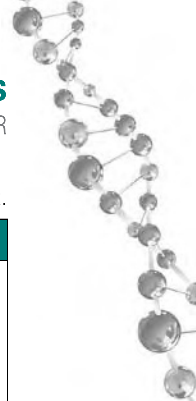

Table 3.2. Troubleshooting Guide for PCR.

Problem	Possible cause and recommended solution
3. Sequence errors in PCR product	**3.2. Sequence errors at PCR product termini.** DNA sequence errors at the end of the PCR product can only be identified after subsequent cloning of the PCR product, therefore please also refer to the troubleshooting given in the chapter Molecular Cloning (p.340). **3.2.1. Faulty primer design.** Avoid direct repeats in primers as multiple repeats may appear at the ends of the PCR product. **3.2.2. Low primer quality.** As oligonucleotides are synthesized in the 3'→5' direction near the 5'-end sequence inconsistencies may appear. Reorder primers from a reliable supplier. **3.2.3. Alteration of PCR product ends during cloning.** Nuclease contamination. DNA nucleases present during extraction procedures or in the digestion or ligation reaction mixture may alter the ends of the PCR product. Please refer to the troubleshooting in chapter Molecular Cloning (p.340). DNA polymerase present in digestion reaction mixture. Remove active thermophilic DNA polymerases before digestion of a PCR product by spin column purification or phenol/chloroform extraction and subsequent ethanol precipitation (*see* protocol on p.356). DNA polymerase may alter the ends of cleaved PCR products. PCR product damaged by exposure to UV light. Use a long wavelength UV (360 nm) light-box when analyzing and excising PCR products from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit exposure to UV to several seconds. Keep the gel on a glass plate or on a plastic plate during illumination with UV. Alternatively, use dyes visible in ambient light to visualize PCR products in standard agarose gels (1, 2). **3.2.4. Sequencing error.** To verify the reliability of sequencing results, sequence both DNA strands. Ensure sequencing primers are located in a distance of at least 20 nucleotides from the insertion site on cloning vector.
4. PCR/RT-PCR product in negative control	**4.1. Cross-over contamination.** PCR was contaminated by DNA or RNA present in the working environment. Avoid contamination by following general working recommendations described in p.306.
	4.2. Carry-over contamination. PCR contaminated by amplicons from previous reactions. If the same amplicon is to be generated multiple times, use carryover contamination control techniques. A common method used to avoid carry-over contamination is to incorporate dUTP into PCR products generated in the working environment followed by treatment with UDG (4).
	4.3. Faulty primer design. DNA templates. Avoid direct repeats, self-complementarities and complementarities in between primer pairs, as primer multimers generate unexpected product. RNA templates. To avoid amplification of genomic DNA, design PCR primers on exon-intron boundaries. Remove gDNA from RNA using DNase I, RNase-free (*see* protocol on p.313).
	4.4. RNA template contamination with genomic DNA. PCR product in the negative control denotes contamination with genomic DNA. Perform DNase I digestion prior reverse transcription (*see* protocol on p.313).

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Table 3.2. Troubleshooting Guide for PCR.

Problem	Possible cause and recommended solution
5. Inefficient PCR cloning	**5.1. Incorrect choice of polymerase.** For generation of PCR products suitable for direct blunt-end cloning use a proofreading DNA polymerase such as *Pfu* DNA Polymerase (#EP0501, #EP0571). For TA cloning use DreamTaq™ DNA Polymerase (#EP0701), Maxima™ Hot Start *Taq* DNA Polymerase (#EP0601), TrueStart™ *Taq* DNA Polymerase (#EP0611), *Taq* DNA Polymerase (#EP0401) or other non-proofreading polymerases. For efficient cloning with any DNA polymerase, use the CloneJET™ PCR Cloning Kit (#K1231).
	5.2. Inefficient cleavage of PCR product. **5.2.1. Faulty primer design.** When introducing restriction endonuclease sites into primers for subsequent digestion and cloning of a PCR product, refer to Table 1.9 on p.162 to define the number of extra bases required for efficient cleavage by conventional Restriction Enzymes. For efficient digestion of PCR product follow the protocol on p.165. For FastDigest™ restriction enzymes follow the recommendations given in Table 1.3 on p.44 and in the protocol for fast digestion of PCR products on p.43. **5.2.2. Low primer quality.** As oligonucleotides are synthesized in the 3'→5' direction inconsistencies may appear in the 5' end. Reorder primers from a reliable supplier. **5.2.3. DNA polymerase is present in digestion reaction mixture.** For cloning purposes remove active thermophilic DNA polymerase before digestion of a PCR product by spin column purification or phenol/chloroform extraction and subsequent ethanol precipitation (*see* protocol on p.356). DNA polymerase may alter the ends of cleaved PCR products and reduce the ligation efficiency. **5.2.4. Restriction enzyme is sensitive to PCR mixture components.** For efficient digestion of PCR product by conventional restriction enzymes follow the digestion protocol on p.165. For FastDigest™ restriction enzymes follow the recommendations in the protocol for fast digestion of PCR products on p.43. In case digestion of larger amounts of PCR products is desired scale up the above mentioned protocols according to the given recommendations. If digestion in smaller volumes is necessary, purify the PCR product by spin column or phenol/chloroform extraction and subsequent ethanol precipitation (*see* protocol on p.356).
	5.3. Inefficient dA-tailing of PCR product for TA cloning. **5.3.1. Final extension step is too short.** This step can be prolonged to 20-30 minutes in the PCR cycling protocol to ensure a high efficiency of dA-tailing of PCR product, which can result in up to 3-4 fold higher numbers of recombinant clones. **5.3.2. Faulty primer design.** The terminal transferase (3'-end extension) activity of *Taq* DNA polymerase exhibits template specificity with respect to the 3'-terminal nucleotide. Therefore, for efficient TA cloning of PCR products it is important to consider the 5'-end nucleotide of the primers. 5'-end nucleotides can be listed in the following order (according to dA-tailing efficiency): G> C >T >A (3).
	5.4. Nuclease contamination. DNA nucleases present during extraction procedures or in the digestion or ligation reaction mixture may alter the ends of the PCR product. Please refer to the troubleshooting in chapter Molecular Cloning (p.340).

References

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