Biochemicals, Ready-to-Use Buffers and Reagents
Troubleshooting Guide for PCR Reagents

Axil Scientific Pte Ltd
41 Science Park Road, #01-22 / 23 The Gemini, Singapore Science Park II Singapore 117610 T: +65-6775 7318 F: +65-6775 7211 E: info@axilscientific.com

No.	Observations	Possible Causes	Recommended Actions
1	**Low yield/ No amplicon**	**Insufficient template quantity or poor quality**	**Template integrity** Template DNA should be of high molecular weight. Evaluate template size and quality by agarose gel electrophoresis. Apply DNA isolation methods that minimize shearing and nicking of DNA. Resuspend isolated DNA in 1X TE buffer, pH 8.0 (BUF-3024-1X), or in Water, nuclease-free (BUF-1180).
			Template purity Trace amounts of certain agents (e.g. phenol, EDTA, Proteinase K) may inhibit thermostable DNA polymerases. High ionic concentrations like K and Mg may lead to suboptimal reaction conditions for the enzymes. For such cases, the template should be re-purified using a spin-column or gel extraction.
			Template quantity Increase the amount of template or enzymes, however note that increased enzyme concentrations sometimes lead to non-specific products. For most assays, the optimum amount of DNA polymerase is between 1 - 2.5U per 50µl reaction. Alternatively, use enzymes or master mixes with higher sensitivity than Taq DNA Polymerase.
		Poor primer design	Avoid using primers with self-complementary sequences. Extension of primer duplexes will consume reaction components, resulting in lower yields of the target PCR product. Verify that primers and the correct strand of DNA template are complementary. Several primer design software programs are available for convenience, they can be used to ensure the primer sequence have the basic, recommended general characteristics.
		Suboptimal thermal cycling conditions	**Annealing Temperature** Optimize annealing temperature stepwise within 1-2°C increments. Annealing temperature should be 5℃ lower than the primer mel ting temperature (T_m). Alternatively, use a gradient cycler to optimize the annealing temperature of a specific primer pair (±10℃). with addition of addi tives that change the melting temperature of the primer-template duplex (such as DMSO, glycerol, betaine, formamide and TMANO), adjust annealing temperature accordingly. The formula can be used to give an approximation of the T_m for primers: $T_m = 81.5 + 16.6 * (\log_{10}[Na^+]) + 0.41 * (\%GC) - 675 / n$ Where $[Na^+]$ stands for molar salt concentration and n = no. of bases in the primer.
			Extension The recommended reaction temperature is 72℃ for Taq DNA polymerases (BIO-5110) and REDiant Taq DNA Polymerase (BIO-5115). The extension step with Taq DNA Polymerase takes 1min/kb. For amplification of longer templates (>3 kb) the extension temperature may be reduced to 68℃.
			Cycle Numbers Cycle numbers varies with the amount of template DNA in the PCR mixture and the expected yield of the PCR product. 25 - 35 cycles are usually sufficient in most cases to produce an adequate yield of PCR product. If not more than 10 copies of template DNA are present in the reaction, extend the number of cycles to 40.

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		Suboptimal reaction conditions	**Insufficient DNA polymerase** For a 50 µl PCR mixture, 1 - 1.5U of *Taq* DNA Polymerase is recommended. It may be necessary to increase the amount of DNA Polymerase if the PCR mixture contains inhibitors due to contamination of the template DNA. Pipetting errors are frequent cause of insufficient enzyme concentrations due to inaccurate dispensing of submicroliter volumes of enzymes in viscous glycerol. It is strongly recommended to use PCR master mixes (BIO-5180) and REDiant PCR master mixes (BIO-5185) as this will increase the initial pipetting volume of reactants hence reducing pipetting errors.
			Insufficient primer Low primer concentrations may result in exhaustion before the reaction is complete, leading to lower yield of desired product. Generally the PCR reaction is successful with wide range of PCR primer concentrations (0.1 - 1 µM) and the optimal conditions varies depending on specific primer/template pair. A primer concentration of 0.4 µM is a good starting point for optimization. PCR primers may degrade due to the 3'→5' exonuclease activity, therefore, PCR mixtures should be kept on ice during the reaction set-up and the polymerase or master mix should be the last component added to the reaction mixture.
			Insufficient Mg^{2+} concentration Low Mg^{2+} concentration may reduce the yield of PCR product. If there is inadequate presence of free magnesium ions, *Taq* DNA polymerase is inactive. Mg^{2+} bind to dNTPs, primers and DNA template, therefore, it's concentration often needs to be optimized for maximal PCR yields. The recommended concentration range for optimizations is between 1 - 3 mM. 1.5mM would be a good starting $MgCl_2$ concentration for standard PCRs. If template DNA contains EDTA, citrate 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+}). Some PCR applications require higher concentrations of dNTP. Since dNTPs bind to Mg^{2+}, Mg^{2+} concentration has to be increased accordingly. 1st BASE's *Taq* DNA polymerase (BIO-5110) and REDiant *Taq* DNA polymerase (BIO-5115) is magnesium-free and comes with a separate vial of $MgCl_2$ which allows researcher to optimize the concentration for each reaction.
		Difficult template	For GC-rich template, additive can be used to enhance DNA denaturation. However annealing temperature might needs to be lowered and enzyme concentration might need to be increased, depending on type of additives being used.
2	**Non-specific PCR products**	**Poor primer design**	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. Avoid direct repeats in the primers to limit the appearance of large PCR products compared to the target amplicon. Avoid complementary sequences within a primer sequence and between the primer pair.
		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 soon as all reactants are mixed. As a result, non-specific priming events lead to generation of unexpected amplification products during PCR. To avoid this, when using *Taq* DNA polymerase, the PCR reaction set-up should always be performed on ice and to always add the enzyme last.

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		Suboptimal thermal cycling conditions	Optimized annealing temperature stepwise within 1-2℃ increments. Annealing temperature should be 5℃ lower than the primer-template melting temperature. If available, use a gradient cycler to optimize the annealing temperature of a specific primer pair (±10℃). When additives that change the melting temperature of the primer-template duplex (such as DMSO, glycerol, betaine, formamide and TMANO) are added, adjust annealing temperature accordingly.
		Suboptimal reaction conditions	**Excess DNA polymerase** For a 50 µl PCR mixture, 1 - 1.5U of *Taq* DNA Polymerase is recommended. It may be necessary to increase the amount of DNA Polymerase if the PCR mixture contains inhibitors due to contamination of the template DNA. However, excess of enzymes sometimes lead to decrease specificity.
			Excess primer Generally the PCR reaction is successful with wide range of PCR primer concentrations (0.1 - 1 µM) and the optimal conditions varies depending on specific primer/template pair. A primer concentration of 0.4 µM is a good starting point for optimization. Higher primer concentrations may promote mispriming which results in accumulation of non-specific products.
			Excess Mg^{2+} Concentration If the Mg2+ concentration is too high, non-specific PCR products may appear due to reduced enzyme fidelity. The recommended concentration range for optimizations is 1 - 3 mM. For standard PCR, 1.5mM would be a good starting $MgCl_2$ concentration. 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 some PCR applications, higher dNTP concentrations are required. dNTPs also binds to Mg^{2+}, therefore, Mg^{2+} concentration has to be increased accordingly. Higher amount of template increases the risk of generation of non-specific PCR products too.
			Unbalanced dNTP concentration It is important to have equal concentrations of all the nucleotides (dATP, dCTP, dGTP and dTTP, (BIO-5125)) in the reaction. If the nucleotide concentrations are not balanced, the PCR error rate may be dramatically increased. dNTP Mixes (BIO-5120) contain 10 mM of each nucleotides. The concentrations of all four dNTPs are perfectly balanced to provide fidelity and to increase the yield of PCR products.
3	**Product in negative control**	**Cross contamination**	General recommendations to lower the risk of DNA or RNA contamination in the working environment as follows: • Preparation of samples, PCR mixtures, performing thermal cycling and analyzing of PCR products should be done in separate areas. • PCR mixture could be prepared on ice and in a laminar flow cabinet to prevent contamination. • Change a fresh pair of gloves for DNA purification and reaction set up. • Use positive displacement pipettes, or use pipette tips with aerosol filters to prepare DNA samples and set up PCR. • Use certified and high quality reagents.
		Carry-over contamination	PCR can be contaminated by amplicons from previous experiment. If the same amplicon is to be generated multiple times, use technique to prevent such contamination. One way to avoid carry-over contamination is to apply dUTP-UDG method.
		Poor primer design	Primer multimers will generate unexpected product. Avoid Self-complementarities, direct repeats, and complementarities in between primer pairs.
4	**Insufficient PCR cloning**	**Incorrect choice of polymerase**	Different type of DNA polymerase should be selected depending on the type of cloning required.

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		Insufficient cleavage	**Poor primer quality** When oligonucleotides are synthesized in the 3'→5' direction, inconsistencies may appear in the 5' end.
			Presence of DNA polymerase Remove active thermophilic DNA polymerases before digestion of a PCR product by spin column purification or phenol/chloroform extraction and subsequent ethanol precipitation. DNA polymerase may alter the ends of cleaved PCR products.
			Restriction enzyme is sensitive to PCR components Purify the PCR product by spin column or phenol/chloroform extraction and subsequent ethanol precipitation.
		Inefficient dA-tailing	**Insufficient time for final extension** To ensure a high efficiency of dA-tailing of PCR product, final extension for PCR cycling can be prolonged to 20-30 minutes. This will result in 3-4 fold higher numbers of recombinant clones.
			Poor primer design 3'-end extension activity of *Taq* DNA polymerase exhibits template specificity with respect to the 3'-terminal nucleotide. For efficient TA cloning of PCR products it is important to look at the 5'-end nucleotide of the primers. According to dA-tailing efficiency, 5'-end nucleotides is in order: G > C > T > A.
		Nuclease contamination	DNA nucleases contamination during extraction procedures or in the digestion reaction mixture may alter the ends of the PCR product.