PCR TROUBLESHOOTING GUIDE

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Problem	Possible Cause	Potential Solutions
No PCR product	Poor primer design	Ensure that primers are non-complementary, both internally and to each other Increase length of primer Avoid GC-rich 3´ ends Confirm that primer sequences are correct and complementary to the template. Use a primer design program like Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to avoid repetitive sequences, regions with high complementarity, etc. Perform a BLAST search to avoid primers that could amplify pseudogenes or that might prime unintended regions. Use Primer3 with the default salt concentration and 0.1–1 µM primer (depending on your reaction conditions) to calculate Tm. Use an annealing temperature that is 5°C lower than the Tm of the primer.
	Poor primer specificity	Confirm that oligos are complementary to proper target sequence
	Missing component	Check reaction set-up and volumes used
	Insufficient primer concentration	Primer concentration can range from 0.1–1 µM in the reaction. Please see protocols for ideal conditions

Problem	Possible Cause	Potential Solutions
No PCR product	Poor template quality	Examine DNA integrity via gel electrophoresis Check 260/280 ratio of DNA template to determine levels of impurities like salts Template may be sheared or may contain PCR inhibitors. If inhibitors are suspected, dilute existing template; otherwise, use fresh template and increase cycles. Do a positive control reaction in which you use a pure plasmid as template to determine if any inhibitory effects exist in the template you are working with.
	Not enough template was in the reaction	Insufficient amplification can result if the initial amount of template is too low. Increase the number of amplification cycles in increments of 5, or, if possible, increase the amount of template.
	Inhibitors in reaction	Further purify starting template by alcohol precipitation, dialysis or PCR clean up kit Reduce sample volume
	Insufficient number of cycles	Using too few PCR cycles can lead to insufficient amplification. Use 25 – 40 cycles. Use fewer cycles when template concentration is high, and use more cycles when template concentration is low.
	Incorrect thermocycler programming	Check program, verify times and temperatures
	Complex template	For GC-rich templates, use Hifi-App Polymerase (ARP041)
		For longer templates, use Mega-App Polymerase (ARP031)

Problem	Possible Cause	Potential Solutions
No PCR product	Extension time was too short	If the extension time is too short, there will be insufficient time for complete replication of the target. Use an extension time of 15 sec / kb.
	Annealing time was too short	If the annealing time is too short, primers do not have enough time to bind to the template. Use an annealing time of at least 15 sec / kb.
	Annealing temperature was too high	If the annealing temperature is too high, primers are unable to bind to the template. Use an annealing temperature that is 5°C lower than the Tm of the primer. To calculate the primer Tm, use the tool at http://bioinfo.ut.ee/primer3-0.4.0/primer3/ with the default salt concentration and 0.1–1 µM primer. For greater accuracy, optimize the annealing temperature by using a thermal gradient. The annealing temperature should not exceed the extension temperature.
	Denaturation temperature was too low	If the denaturation temperature is too low, the DNA will not completely denature and amplification efficiency will be low. Use a denaturation temperature of 95°C.
	Denaturation time was too long	If the denaturation time is too long, DNA might be degraded. For the initial denaturation, use a maximum of 3 min at 95°C; for denaturation during cycling, use 30 sec at 95°C.

Problem	Possible Cause	Potential Solutions
No PCR product	Denaturation time was too short	If the denaturation time is too short, the DNA will not completely denature and amplification efficiency will be low. For the initial denaturation, use up to 3 min; to denature the template during cycling, use 15 sec.
	PCR target sequence has high GC content (>65%)	GC-rich templates are difficult to amplify. To improve amplification, increase the annealing temperature. For greater accuracy, optimize the annealing temperature by using a thermal gradient. Try using HiFi-App Polymerase (ARP041) which has been designed specifically for GC-rich complex templates.
	Primers contain impurities	Contaminants in primers may inhibit PCR. Use desalted primers or more highly purified primers. Dilute the primers to determine if inhibitory effects exist, but do not add less than 0.1µM of each primer.
	Primer concentration was too high	Excessive concentrations of primers increase the chance of primers binding nonspecifically to undesired sites on the template or to each other. Use well-designed primers at 0.1–1 µM in the final reaction. In addition, verify that the correct concentration was supplied by the manufacturer.
	Primer concentration was too low	Annealing may be inefficient due to too low primer concentration. Use well-designed primers at 0.1–1 µM in the final reaction. In addition, verify that the correct concentration was supplied by the manufacturer.

Problem	Possible Cause	Potential Solutions
No PCR product	Enzyme concentration was too low	Incomplete PCR product replication will occur if the polymerase concentration is too low. The optimal enzyme concentration depends on the length and difficulty of the template. Use between 1.25 - 5.0 units of polymerase per 50µl reaction.
Non-specific products	Excessive cycling	Decrease the number of cycles. Excessive cycling increases the opportunity for non-specific amplification and errors. Use 20–35 cycles. Use fewer cycles when template concentration is high, and use more cycles when template concentration is low.
	Extension time too long	Decrease the extension time. Excessive extension time can allow nonspecific amplification. Generally, use an extension time of 15sec /kb.
	Annealing temperature too low	Increase the annealing temperature
		If the annealing temperature is too low, primers may bind non-specifically to the template. Use an annealing temperature that is 5°C lower than the Tm of the primer. To calculate the optimum primer Tm, use the tool at http://bioinfo.ut.ee/primer3-0.4.0/primer3/ with the default salt concentration and 0.1–1 µM primer. For greater accuracy, optimize the annealing temperature by using a thermal gradient.

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Non-specific products	Extension during set-up	Make sure all reactions are set-up on ice. Run reaction as quickly as possible
		Use a hot start polymerase, such as Hot AppTaq Polymerase
		Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Contamination	Replace each component in order to find the possible source of contamination Contaminants in primers may inhibit PCR. Use desalted primers or more highly purified primers. You can try to dilute the primers to determine if inhibitory effects exist, but do not add less than 0.1µM of each primer. - Setup the PCR and analyse the PCR product in separated areas.
		Re-do the reaction with a negative control (no template). The non-specific bands could be from contamination of one of your stocks with foreign DNA If this is a problem, use new stocks, always use autoclaved PCR vials and wear gloves and a lab coat.
	Poor primer design	Ensure that primers are non-complementary, both internally and to each other
		Increase length of primer
		Avoid GC-rich 3´ ends
		Confirm that primer sequences are correct and complementary to the template. Use a primer design program like Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to avoid repetitive sequences, regions with high complementarity, etc. Perform a BLAST search to avoid primers that could amplify pseudogenes or that might prime unintended regions. Use Primer3 with the default salt concentration and 0.1–1 µM primer (depending on your reaction conditions) to calculate Tm. Use an annealing temperature that is 5°C lower than the Tm of the primer.

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Non-specific products	Incorrect template concentration	For low complexity templates (i.e. plasmid, lambda, BAC DNA), use below 100ng of DNA per 50 µl reaction
		For higher complexity templates (i.e. genomic DNA), use 5ng– 500ng of DNA per 50 µl reaction
		If the template concentration is too high, the polymerase can be inhibited due to carryover of inhibitors or inefficient denaturation. Reduce the number of cycles, reduce the template concentration, and/or increase denaturation time/temperature.
	Annealing time was too long	Excessive annealing time may increase non-specific priming. Use an annealing time of 15 sec during cycling.
	Thermal cycler ramping speed is too slow	If the ramp speed of the cycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for non-specific binding. If ramping speed is not set at the maximum speed for the cycler, increase to maximum ramp rate.
	Calculated primer Tm was inaccurate	If the primer concentration is calculated incorrectly, the calculated annealing temperature will also be incorrect. To calculate the primer Tm, use the tool at http://bioinfo.ut.ee/primer3-0.4.0/primer3/ with the default salt concentration and 0.1–1 µM primer. Use an annealing temperature that is 5°C lower than the Tm of the primer.

Problem	Possible Cause	Potential Solutions
Non-specific products	Primer concentration was too high	Excessive concentrations of primers increase the chance of primers binding non-specifically to undesired sites on the template or to each other. Use well-designed primers at 0.1–1 µM in the final reaction. In addition, verify that the correct concentration was supplied by the manufacturer.
	Impure water was used	Water could have been contaminated during prior pipetting events. Use fresh nuclease-free water.
	Template contained an exonuclease or was degraded	Template may be sheared or contain exonuclease. Use a fresh template.