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For a complete description of primers, PCR programs and a discussion of the PCR conditions please consult: *Andrologia* **26**: 97-106 (1994) and *Biotechniques* **23**: 504-511 (1997). Click [here](#) to get the Biotechniques paper in PDF format.

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## Troubleshooting for PCR and multiplex PCR

Troubleshooting discussion is based on the PCR protocol as described in the table below. All reactions are run for 30 cycles.

COMPONENT	VOLUME	FINAL CONCENTRATION
1. autoclaved ultra-filtered water (pH 7.0)	20.7 $\mu$ L	-
2. 10x PCR Buffer*	2.5 $\mu$ L	1x
3. dNTPs mix (25 mM each nucleotide)	0.2 $\mu$ L	200 $\mu$ M (each nucleotide)
4. primer mix (25 pmoles/ $\mu$ L each primer)	0.4 $\mu$ L	0.4 $\mu$ M (each primer)
5. Taq DNA polymerase (native enzyme)	0.2 $\mu$ L	1 Unit/25 $\mu$ L
6. genomic DNA template (100 ng/ $\mu$ L)	1.0 $\mu$ L	100 ng/25 $\mu$ L

\* The 10x PCR buffer contains: 500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM MgCl<sub>2</sub> (the final concentrations of these ingredients in the PCR mix are: 50 mM KCl; 10 mM Tris-HCl; 1.5 mM MgCl<sub>2</sub>).

### QUESTIONS

### SOLUTIONS

**1. I get (many) longer unspecific products. What can I do?**

Decrease annealing time  
 Increase annealing temperature  
 Decrease extension time

Decrease extension temperature to 62-68° C  
 Increase KCl (buffer) concentration to 1.2x-2x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM.  
 Increase MgCl<sub>2</sub> concentration up to 3-4.5 mM but keep dNTP concentration constant.  
 Take less primer  
 Take less DNA template  
 Take less Taq polymerase  
 If none of the above works: check the primer for repetitive sequences (BLAST align the sequence with the databases) and change the primer(s)  
 Combine some/all of the above

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**2. I get (many) shorter unspecific products. What can I do?**

Increase annealing temperature  
 Increase annealing time  
 Increase extension time  
 Increase extension temperature to 74-78° C  
 Decrease KCl (buffer) concentration to 0.7-0.8x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM  
 Increase MgCl<sub>2</sub> concentration up to 3-4.5 mM but keep dNTP concentration constant  
 Take less primer  
 Take less DNA template  
 Take less Taq polymerase  
 If none of the above works: check the primer for repetitive sequences (BLAST align the sequence with the databases) and change the primer(s)  
 Combine some/all of the above

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**3. Reaction was working before, but now I can't get any product.**

Make sure all PCR ingredients are taken in the reaction (buffer, template, Taq, etc)  
 Change the dNTP solution (very sensitive to cycles of thawing and freezing, especially in multiplex PCR)  
 If you just bought new primers, check for their reliability (bad primer synthesis ?)  
 Increase primer amount  
 Increase template amount  
 Decrease annealing temperature by 6-10° C and check if you get any product. If you don't, check all your PCR ingredients. If you do get products (including unspecific ones) reaction conditions as described above.  
 Combine some/all of the above

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**4. My PCR product is weak. Is there a way to increase the yield?**

Gradually decrease the annealing temperature to the lowest possible.  
 Increase the amount of PCR primer  
 Increase the amount of DNA template  
 Increase the amount of Taq polymerase  
 Change buffer (KCl) concentration (higher if product is lower than 1000bp or lower if product is higher than

1000bp)

Add adjuvants. Best, use BSA (0.1 to 0.8  $\mu\text{g}/\mu\text{L}$  final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol.

Check primer sequences for mismatches and/or increase the primer length by 5 nucleotides

Combine some/all of the above

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**5. My two primers have very different melting temperatures ( $T_m$ ) but I cannot change their locus. What can I do to improve PCR amplification?**

An easy solution is to increase the length of the primer with low  $T_m$ . If you need to keep the size of the product constant, add a few bases at the 3' end. If size is not a concern, add a few bases at either the 3' or the 5' end of that primer.

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**6. I have a number of primer pairs I would like to use together. Can I run a multiplex PCR with them?. How?**

**Very likely, yes.**

Try amplify all loci separately using the same PCR program. If one of the primer pairs yields unspecific products, keep the cycling conditions constant and change other parameters as mentioned above (#1 and #2).

Mix equimolar amounts of primers and run the multiplex reaction either in the same cycling conditions or by decreasing only the annealing temperature by 4° C.

If some of the loci are weak or not amplified, read below !!

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**7. How many loci can I amplify in multiplex PCR at the same time?**

Difficult to say. The author has routinely amplified from 2 to 14 loci.

Literature describes up to 25 loci or so.

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**8. One or a few loci in my multiplex reaction are very weak or invisible. How can amplify them?**

The first choice should be increasing the amount of primer for the "weak" loci **at the same time** with decreasing the amount of primer for all loci that can be amplified. The balance between these amounts is more important than the absolute values used !!

Check primer sequences for primer-primer interactions

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**9. Short PCR products in my multiplex reaction are weak. How can I improve their yield?**

Increase KCl (buffer) concentration to 1.2x-2x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM

Decrease denaturing time

Decrease annealing time and temperature

Decrease extension time and temperature

Increase amount of primers for the "weak" loci while decreasing the amount for the "strong" loci.

Add adjuvants. Best, use BSA (0.1 to 0.8  $\mu\text{g}/\mu\text{L}$  final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol

Combine some/all of the above

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**10. Longer PCR products in my multiplex reaction are**

Decrease KCl (buffer) concentration to 0.7-0.8x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM

<b>weak. How can I improve their yield?</b>	<p>Increase MgCl<sub>2</sub> concentration up to 3-4.5 mM but keep dNTP concentration constant.</p> <p>Increase denaturing time</p> <p>Increase annealing time</p> <p>Decrease annealing temperature</p> <p>Increase extension time and temperature</p> <p>Increase amount of primers for the "weak" loci while decreasing the amount for the "strong" loci</p> <p>Add adjuvants. Best, use BSA (0.1 to 0.8 µg/µL final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol</p> <p>Combine some/all of the above</p>
<b>11. All products in my multiplex reaction are weak. How can I improve the yield?</b>	<p>Decrease annealing temperature in small steps (2° C)</p> <p>Decrease extension temperature to 62-68° C</p> <p>Increase extension time</p> <p>Increase template concentration</p> <p>Increase overall primer concentration</p> <p>Adjust Taq polymerase concentration</p> <p>Change KCl (buffer) concentration, but keep MgCl<sub>2</sub> concentration at 1.5-2mM</p> <p>Increase MgCl<sub>2</sub> concentration up to 3-4.5 mM but keep dNTP concentration constant.</p> <p>Add adjuvants. Best, use BSA (0.1 to 0.8 µg/µL final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol</p> <p>Combine some/all of the above</p>
<b>12. Unspecific products appear in my multiplex reaction. Can I get rid of them somehow?</b>	<p>If long: increase buffer concentration to 1.2-2x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM</p> <p>If short: decrease buffer concentration to 0.7-0.9x, but keep MgCl<sub>2</sub> concentration at 1.5-2mM</p> <p>Gradually increase the annealing temperature</p> <p>Decrease amount of template</p> <p>Decrease amount of primer</p> <p>Decrease amount of enzyme</p> <p>Increase MgCl<sub>2</sub> concentration up to 3-4.5 mM but keep dNTP concentration constant</p> <p>Add adjuvants. Best, use BSA (0.1 to 0.8 µg/µL final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol</p> <p>If nothing works: run PCR reactions for each (multiplexed) locus individually, using an annealing temperature lower than usual. Compare the unspecific products for each locus tested with the unspecific products seen when running the multiplex PCR. This may indicate which primer pair yields the unspecific products in the multiplex reaction.</p> <p>Combine some/all of the above</p> <p><b>(Note: primer-primer interactions in multiplex PCR)</b></p>

**are usually translated into lack of some amplification products rather than the appearance of unspecific products)**