Troubleshooting

QUESTIONS	SOLUTIONS
1. I get (many) longer unspecific products. What can I do	**D**ecrease annealing time. **I**ncrease annealing temperature. **D**ecease extension time. **D**ecrease extension temperature to 62-68^{o}C. **I**ncrease KCI (buffer) concentration to 1.2x-2x, but keep $MgCI_2$ concentration at 1.5-2mM **I**ncrease $MgCI_2$ concentration up to 3- 4.5 mM but keep dNTP concentration constant. **T**ake less primer. **T**ake less DNA template. **T**ake less Taq polymerase **I**f none of the above works: check the primer for repetitive sequences (BLAST align the sequence with the databases) and change the primer (s) **C**ombine some/all of the above.
2. I get (many) shorter unspecific products. What can I do	**I**ncrease annealing temperature. **I**ncrease annealing time. **I**ncrease extension time. **I**ncrease extension temperature to 74 - 78 ^{o}C **D**ecrease KCI (buffer) concentration to 0.7-0.8x, but keep $MgCI_2$ concentration at 1.5-2mM. **I**ncrease $MgC1_2$ concentration up to 3-4.5 mM but keep dNTP concentration constant **T**ake less primer. **T**ake less DNA template. **T**ake less Taq polymerase **I**f none of the above works: check the primer for repetitive sequences. (BLAST align the sequence with the databases) and change the primer (s) **C**ombine some/all of the above.

3. Reaction was working before, but now I can't get any product.	**M**ake sure all PCR ingredients are taken in the reaction (buffer, template, Taq, etc). **C**hange the dNTP solution (very sensitive to cycles of thawing and freezing, especially in multiplex PCR). **I**f you just bought new primers, check for their reliability (bad primer synthesis?). **I**ncrease primer amount. **I**ncrease template amount. **D**ecrease annealing temperature by 6-10^{o}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. **C**ombine some/all of the above
4. My PCR product is weak. Is there a way to increase the yield?	**G**radually decrease the annealing temperature to the lowest possible. **I**ncrease the amount of PCR primer. **I**ncrease the amount of DNA template. **I**ncrease the amount of Taq polymerase. **C**hange buffer (KCI) concentration (higher if product is lower than 1000bp or lower if product is higher than 1000bp). **A**dd 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. **C**heck primer sequences for mismatches and/or increase the primer length by 5 nucleotides. **C**ombine some/all of the above
5. My two primers have very different melting temperatures (Tm) but I cannot change their locus. What can I do to improve PCR amplification?	**A**n easy solution is to increase the length of the primer with low Tm. 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.

6. I have a number of primer pairs I would like to use together. Can I run a multiplex PCR with them?. How?	**V**ery likely, yes. **T**ry amplify all loci separately using the same PCR program. If one of the primer pairs yield 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 ^{o}C. **I**f some of the loci are weak or not amplified, read bellow!!
7. How many loci can I amplify in multiplex PCR at the same time?	**D**ifficult to say. The author has routinely amplified from 2 to 14 loci. Literature describes up to 25 loci or so.
8. One or a few loci in my multiplex reaction are very weak or invisible. How can amplify them?	**T**he 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.
9. Short PCR products in my multiplex reaction are weak. How can I improve their yield?	**I**ncrease KCI (buffer) concentration to 1.2x-2x, but keep $MgCI_2$concentration at 1.5-2mM. **D**ecrease denaturing time. **D**ecrease annealing time and temperature **D**ecrease extension time and temperature **I**ncrease amount of primers for the "weak" loci while decreasing the amount for the " strong" loci. **A**dd 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. **C**ombine some/all of the above).

10.Longer PCR products in my multiplex reaction are weak. How can I improve their yield?	**D**ecrease KCI (buffer) concentration to 0.7-0.8x, but keep $MgCI_2$ concentration at 1.5-2mM. **I**ncrease $MgCI_2$ concentration up to 3- 4.5 mM but keep dNTP concentration constant. **I**ncrease denaturing time. **I**ncrease annealing time. **D**ecrease annealing temperature. **I**ncrease extension time and temperature **I**ncrease amount of primers for the "weak" loci while decreasing the amount for the "strong" loci **A**dd 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. **C**ombine some/all of the above.
11. All products in my multiplex reaction are weak. How can I improve the yield?	**D**ecrease annealing time in small steps (2°C). **D**ecrease extension temperature to 62- 68°C. **I**ncrease extension time. **I**ncrease template concentration. **I**ncrease overall primer concentration. **A**djust Taq polymerase concentration. **C**hange KCI (buffer) concentration, but keep $MgCI_2$ concentration at 1.5- 2mM. **I**ncrease $MgCI_2$ concentration up to 3-4.5mM but keep dNTP concentration constant. **A**dd 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.**C**ombine some/all of the above.
12.Unspecific products appear in my multiplex reaction. Can I get rid of them somehow?	**I**f long: increase buffer concentration to 1.2-2x, but keep $MgCI_2$ concentration at 1.5-2mM **I**f short: decrease buffer concentration to 0.7-0.9x, but keep $MgCI_2$ concentration at 1.5-

	2mM. **G**radually increase the annealing temperature. **D**ecrease amount of template. **D**ecrease amount of primer. **D**ecrease amount of enzyme. **I**ncrease $MgCI_2$ concentration up to 3-4.5mM but keep dNTP concentration constant. **A**dd 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. **I**f nothing works: run PCR reactions for each (multiplexed) locus individually, using an annealing temperature lower than usual. **C**ompare 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. **C**ombine some/all of the above (Note: primer-primer interactions in multiplex PCR are usually translated into lack of some amplification products rather than the appearance of unspecific products).