Multiplex PCR Guidelines for Multiplex PCR 5X Master Mix

Overview

Guidelines

1. **Reaction Setup:** Non-specific primed synthesis during reaction setup and first heating cycle have been identified as a source of undesired products in some reactions. This can often be avoided by doing a manual “hot-start”. To carry out a manual hot-start, assemble reaction components except for the master mix and heat up to 95°C for 2 minutes, pause, add the master mix to the tube, and then immediately start PCR cycling.

   An alternative convenient manual “hot-start” can be carried out as follows: assemble all reaction components on ice, add the PCR Master Mix last and immediately transfer the reactions to a thermocycler preheated to the denaturation temperature (95°C).
2. **Template:** The quality of the DNA template has a great impact on the PCR amplification. For DNA samples of high complexity such as human genomic DNA, we recommend 10 ng to 1 µg template DNA in a 50 µl reaction. For DNA of low complexity, such as lambda DNA or plasmid DNA, we recommend 10/pg to 10 ng template DNA in a 50 µl reaction.
3. **Primers:**
   1. Primer Design
      Primer design is critical to successful multiplex PCR. Primers are generally 24-35 nucleotides in length and ideally have a GC content of 40-60% (preferably 50-60%). Try to avoid complementary sequences at the 3´ end of all primers, runs of three or more G/C at the 3´ end, and secondary structures within primers. Computer programs such as PrimerSelect™ (DNAStar Inc., Madison, MI) and Primer3 can be used to design or analyze single primer pairs.

      The melting temperature of all primers for mPCR should be more than 60°C according to the formula T_m (°C) = 2 x (nA+nT) + 4 x (nG + nC). Primers with T_m higher than 68°C are preferred because the difference in T_m of primer pairs does not affect performance as much.
   2. Primer Quality and Molar Concentration
      Primers should be purchased desalted or HPLC-purified as the primer quality is a critical factor for good multiplex PCR. Primers should be dissolved in 0.5X TE buffer (5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) and the concentration should be accurately measured by a spectrophotometer. The molar concentration should be calculated using the molar extinction coefficient (ε_{260}) and absorbance at 260 nm.
      Molar conc. of primers (M) = A_{260}/ ε_{260}
      ε_{260} = 0.89 x (nA x 15480 + nC x 7340 + nG x 11760 + nT x 8850) where n is the number of respective bases.
      For example, if a primer with 6A, 7C, 8G, and 9T, then its ε_{260} is 283,011 [0.89 x (6 x 15480 + 7 x 7340 + 8 x 11760 + 9 x 8850)].
      Adjust the concentration of the primer stock to 50 µM (store at -20°C to -80°C). Mix all primers at equimolar concentration to 1 µM in 0.5X TE buffer and store in small aliquots at -20°C or -80°C. Repeated freeze-thaw cycles should be avoided since they may lead to primer degradation.
      The final concentration of each primer in a typical mPCR is between 0.05-0.4 µM. In most cases, a final concentration of 0.15 µM gives satisfactory results. Increasing the primer concentration up to 0.4 µM may increase the yield.
      The Multiplex PCR 5X Master Mix is used at a final concentration of 1X in most cases; however, in some cases, the Multiplex PCR 5X Master Mix can be used as low as 0.8X or up to final 1.5X to increase product yields.
   3. Annealing temperature
      Single-plex PCR should be first performed for each pair of primers, testing a gradient of annealing temperature to determine the optimal conditions. If the single-plex PCR gives non-specific PCR bands or very low yield, the primers should be re-designed since they are unlikely to perform well in mPCR. In multiplex PCR, choose an annealing

temperature that allows all the single-plex reactions to give specific products.

If you cannot perform temperature-gradient PCR reactions, 60°C is a good starting point. If some bands are missing, lower annealing temperature in 1.5°C steps. If non-specific bands exist, increase annealing temperature in 1.5°C steps.

4. **Reaction Parameters:**
    1. Mg^{++} and additives
       The Mg^{++} concentration in the Multiplex PCR 5X Master Mix is 12.5 mM, which gives a final 2.5 mM at 1X in PCR reactions. This gives satisfactory amplification of most amplicons. However, Mg^{++} can be further optimized in 0.5-1.0 mM increments using $MgCl_2$.

       For some difficult targets such as GC-rich sequences, additives such as, DMSO (3), may be included to improve amplification.
    2. Denaturation
       An initial 1-2 min denaturation at 95°C is recommended prior to PCR cycling to fully denature the DNA.

       Subsequent denaturation cycles should be 5-30 sec.
    3. Annealing
       The annealing step is typically 30 sec to 1 minute. Annealing temperature can be optimized by doing a temperature gradient PCR starting 5°C below the calculated T_m. We recommend using NEB's Tm Calculator to determine appropriate annealing temperatures for PCR.
    4. Extension
       The recommended extension 68°C. Extension time is based on the size of the largest amplicon in the mPCR reaction. An extension rate of 1 minute per kb is recommended for multiplex PCR reactions with five pairs of primers or less and 2 minutes per kb for multiplex PCR reactions with more than six pairs of primers. A final extension of 5 minutes at 68°C is recommended.
    5. Cycle number
       Generally, 30-35 cycles yield sufficient product. Up to 40 cycles may be required to detect low-copy-number.
    6. 2-step PCR
       When primers with annealing temperatures above 60°C are used, a 2-step PCR protocol is possible.

Initial Denaturation	95°C	1 minute
30-40 Cycles	95°C 60-68°C	20 seconds 1-2 minutes/kb
Final Extension	68°C	5 minutes
Hold	4-10°C	