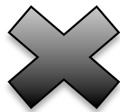
PCR Protocol

Master Mix Preparation

To expedite the process of making PCR solution a master mix is to be made with all of the reagents except our organisms DNA. The exact components of the master mix will depend on the method being employed. The CRUSTOMICS Lab currently uses the easy to use and effective GoTaq Green Master Mix for most PCR applications. This master mix for this method is to be prepared as follows:

For 25µl reactions:

12.5 µl GoTaq Green
8.5 µl Molecular Grade Water
1 µl Forward Primer
1 µl Reverse Primer



Number of Samples (including controls and an extra volume for buffer)

This results in 23 µl of master mix per sample, that are to be aliquoted to individual previously labeled PCR tubes. Once aliquoted, 2 µl of sample DNA are added to each tube (except the negative control) changing the pipette tips every time to avoid contamination.

Thermocycling

The PCR tubes are ready to be transferred to the thermal cyclers. Make sure the lids are closed properly to avoid evaporation, and that the thermal cycler lid is secured according to the individual machine requirements.

Programming the thermal cycler protocols is fairly simple. A basic PCR protocol contains the following:

1. Initial Denaturation Step
The samples are heated up (to 94ºC / 95ºC) for 3 minutes to separate the two DNA strands and allow for subsequent primer annealing. This initial step ensures the DNA strands can separate and are not all "tangled up".

2. Denaturation Step
The samples are heated up (to 94ºC / 95ºC) for 30 seconds to separate the two DNA strands and allow for subsequent primer annealing.

3. *Annealing Step*
The temperature is dropped to allow for primer annealing to the target sequence. The temperature chosen here is critical for a proper reaction and depends entirely on the nucleotide composition of the primer pair. This temperature should be just under the melting temperature of the primers, so these exclusively bind to the target sequence to be amplified. A lower temperature would permit mismatches in the primer and target

sequences, which might be useful in cases where the primers are non-taxa specific. However, this greatly increases the chances of non-specific priming and amplification of non-target sequences (e.g. pseudogenes and other paralogs). Although it depends on the specific primer sequences, a general rule of thumb is to keep the annealing temperature above 50ºC to avoid non-specific priming. Anything lower than this greatly increases the chances of amplifying other loci resulting in unsequenceable PCR products. Often, the use of different primer pairs is preferable to lowering the annealing temperature. Ensuring the proper amplification of our target sequence (and not paralogs or erroneous loci) is of paramount importance for any kind of genetic study. The length of time for this step also has implications for primer specificity. The longer this step is, the more time that is allowed for primer annealing, and the higher the chances of non-specific priming to occur. A length of 30 seconds is perfectly adequate for most applications.

4. Extension
The temperature is increased to 72ºC to activate the Taq Polymerase and have it elongate our sequences starting from our primers. The length of time here depends on the processivity of our Taq polymerase and the sequence length of our amplicon. An extension time of 1 minute is suitable for most applications and enough for any amplicons to be sequenced using Sanger methods.

5. Repeat Steps 2-4
The number of cycles will depend on how much starting DNA we have and how well our samples are being amplified with our protocol settings. 35 cycles is a good starting point and should produce enough amplicons using standard amounts of DNA. For samples with very low DNA concentrations this can be increased to 40 cycles. However, increasing the number of cycles also magnifies any problems with non-specific amplification that our reactions might have and can create noise and/or unsequenceable PCR products.

6. Final Extension
Once the cycles are completed, the samples are kept at 72ºC for a final extension step. This ensures that every amplicon has been extended to its total length before stopping the reaction. Seven to ten minutes usually is sufficient for this purpose.

7. Hold at 4ºC
This step simply tells the thermal cycler to hold the samples at a cool temperature to keep DNA stable until the PCR products are taken out of the machine.

An example version of the thermal cycling protocol would therefore look like the following:

1. 94° C 3:00
2. 94° C 0:30
3. *52° C 0:30 * The temperature will vary according to primers employed
4. 72° C 1:00
5. Repeat (2-4) for 35 cycles
6. 72° C 7:00
7. Hold 4° C

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