2nd PCR (Template PCR)

Materials:

1. RNase-free tubes and filter tips
2. QIAGEN® PCR 10x Buffer
3. QIAGEN® Q-Solution 5x
4. dNTP Mix (Invitrogen 10 mM; 18427-013) diluted to **2mM**
5. QIAGEN® *Taq* DNA Polymerase (5 units/µl)
6. Gene specific Forward Primer - T7 flanked (5 pmol)[1]
7. Gene specific Reverse Primer – Sp6 flanked (5 pmol)[1]
8. cDNA with concentration of at least 10ng/µL for high expressed genes and more for low expressed genes
9. Ice[2]
10. PCR machine

Methods:

This step should be done when you have confirmed the best temperature for annealing of the primers with the temperature gradient PCR. Use the temperature at which the band on the electrophoresis gel was most sharp (i.e. without non-specific bands).

1. Master mix for 2 genes (again we will prepare master mix for 3 because of pipetting mistakes). The final reaction volume is 100 µL separated into 5x 20 µL.

    1. H_20: 112.5 µL
    2. 10xbuffer: 30 µL
    3. Q solution: 60 µL
    4. 2mM dNTPs: 30 µL
    5. *Taq*: 2.4 µL

2. Mix well by pipetting up and down and transfer 78 µL to Eppendorf tube.
3. Add 20 µL of Primer mix (forward and reverse primers) at concentration 5 pmol/ µL.
4. Add 2 µL of 1st PCR product at concentration 20-50ng.
5. Mix well and transfer to PCR strip 20 µL into 5 wells.
6. The PCR machine should be adjusted to the following guidelines for designing your program.
7. After the end of this PCR run an agarose gel with 5 µL of the product from the PCR. The rest should be purified with DNA purification kit (QIAquick PCR Purification Kit or equivalent).

PCR program:

Temperature (°C)	Time	Cycles	Notes
94°C	2 min	1	Initial denaturation
94°C	25 sec	35	
Temperature from 1st PCR	25 sec	35	
72°C	1 min 20 sec	35	Elongation Time: ~1 min/kb of expected product
72°C	9 min	1	Final elongation
4°C	infinity		

This is how it looks on your PCR machine:

Step	**Temperature (°C)**	**Time (min)**
1.	94°C	2 min
2.	94°C	25 sec
3.	Ideal T from 1st PCR	25 sec
4.	72°C	1 min 20 sec
5.	Go to step 2, 35x	
6.	72°C	9 min
7.	4°C	infinity