# 1st PCR (Gradient 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 with option for temperature gradient

**Method:**

Temperature gradient PCR with three temperatures 53°C, 57°C and 61°C should be tested.

1. Prepare the master mix for two genes (we will prepare master mix for three because of pipetting mistakes).
    1. $H_20$: 20.52 µL
    2. 10x buffer: 6 µL
    3. Q solution: 12 µL
    4. 2mM dNTPs: 6 µL
    5. *Taq* Polymerase: 0.16 µL

2. Mix it well by pipetting up and down and place 14 µL of the mix into a PCR strip. Add 5 µL of primer mix and 1 µL of cDNA[3]. Mix well again by pipetting up and down. Centrifuge the PCR strip briefly and put it into the PCR machine. The final reaction volume is 20 µL.

3. The PCR machine should be adjusted to the following guidelines for designing your program.

| Temperature (°C) | Time | Cycles | Notes |
|---|---|---|---|
| 94°C | 3 min | 1 | Initial denaturation |
| 94°C | 30 sec | 35 | |
| Temp gradient (10°C) | 30 sec | 35 | |
| 72°C | 1 min 30 sec | 35 | Time: ~1 min/kb of expected product |
| 72°C | 10 min | 1 | |
| 4°C | infinity | | |

This is how it will look on your PCR machine:

| **Step** | **Temperature (°C)** | **Time (min)** |
|---|---|---|
| 1. | 94°C | 3 min |
| 2. | 94°C | 30 sec |
| 3. | 45 – 65°C | 30 sec |
| 4. | 72°C | 1 min 30 sec |
| 5. | Go to step 2, 35x | |
| 6. | 72°C | 10 min |
| 7. | 4°C | infinity |

**N.B. If some of the bands in the gel shows a desired band, this PCR product is used in the subsequent template generation 2$^{nd}$ PCR.**

Notes:

1. To prepare 5 pmol primers mix solution: Add 5 **μl** forward primer, 5 **μl** reverse primer and add 90 **μl** $H_2O$.

2. Prepare everything on ice to avoid degradation of your *Taq* polymerase and non-specific primer annealing.

3. The amount of cDNA that you are going to use in the PCR reaction depends on the concentration of the cDNA. We are looking for a concentration of at least 10ng/μL. If your cDNA is less that 10ng/μL add more in the reaction.

**Please look at the attached protocol below.**

**Temperature Gradient PCR**
**(for preparation of 1 gene with @three temperatures)**

96-Well-Plate Setup:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | 1 | | | 1 | | | 1 | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |
| | 49°C | 49,3°C | 50,2°C | 51,69°C | 53,2°C | 54,7°C | 56,3°C | 57,8°C | 59,4°C | 60,8°C | 61,7°C | 62,0°C |

| No. | Name | Expected Size of your GENE | Is the Band OK? |
|---|---|---|---|
| 1 | **Your GENE NAME** | 682bp | |

**Master Mix by factor of 4**

| Factor | $H_2$0(µl) | 10xBuffer(µl) | Q (µl) | dNTPs (µl) | *Taq* Pol.(µl) |
|---|---|---|---|---|---|
| 1 x | 6.84 | 2 | 4 | 2 | 0.16 |
| 4x | 27.36 | 8 | 16 | 8 | 0.64 |
| | [ ] | [ ] | [ ] | [ ] | [ ] |

**NB.** Check the boxes if you have added the reagent so you don't forget it.

**Divide the master mix into three by adding 16 µl and add to each 1-2 µl cDNA and 2 µl of Primer mix.**

| Temperature (°C) | | # of Cycles |
|---|---|---|
| 94°C | Pause | |
| 94°C | 2min | 1 cycle |
| 94°C | 25" | 35cycles |
| Gradient temperatures | 25" | 35cycles |
| 72°C | 1min 30" | 35cycles |
| 72°C | 10min | 1 cycle |
| 4°C | pause | Infinite |