# Protocol: PCR for site-directed mutagenesis

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**Note:**

- Always work on ice.
- Wear gloves.
- Use nuclease-free water and sterile tubes.
- Completely thaw and thoroughly mix all reagents before use.
- Avoid repeated freeze-thaw cycles of reagents.
- **Important:** The first thing you do should be reading through the protocol and thawing all reactants on ice. This will take some time so the earlier the better.

**Materials and reactants:**

- Pfu Hotstart Master Mix
- Forward Primer
- Reverse Primer
- Template DNA (or colonies)
- Sterile 0.2 ml PCR tubes
- 10 µL sterile pipette tips
- Micropipette 10 µL
- Thermal cycler

# Part A. PCR setup

1. Thaw Pfu Hotstart Master Mix, primer solutions and template DNA. It is important to thaw the solutions completely and mix thoroughly before use to avoid localized concentrations of salt.
2. Below is a scheme of how to make a PCR reaction mix for a recommended final volume of 20 µL. In every PCR tube you should have a total volume of 20 µL.

    - Note that the different genes use different primers; make sure that you pick the correct primers for the gene.
    - Make sure that you dilute the primer solutions to the correct concentration (0.5 µM).

| Component | Amount per reaction (µL) | Final concentration |
|---|---|---|
| Pfu Hotstart Master Mix (2x) | 25 | 1x |
| Primer Forward (10 µM) | 2.5 | 0.5 µM |
| Primer Reverse (10 µM) | 2.5 | 0.5 µM |
| Plasmid DNA (x ng/µL) | x | 10 ng |
| MilliQ H2O | x | Fill up to 50 µL |
| Total volume | 50 | - |

3. Mix the reaction mix thoroughly and dispense 50 µl into each PCR tube. Mix gently by pipetting up and down.
4. Program the thermal cycler as shown in Table 2. Adjust annealing temperature based on primer melting temperature (typically 3–5°C below Tm). Program the thermal cycler according to Table 2.

**Table 2.**

| Segment | Number of cycles | Temperature | Duration |
|---|---|---|---|
| Initial Denaturation | 1 | 95°C | 2 minutes |
| Denaturation | 30 | 95°C | 30 seconds |
| Anealing | | Primer Tm – 5°C = 62 - 5 = 57 °C | 30 seconds |
| Elongation | | 72°C | 8 minutes* |
| Final elongation | 1 | 72°C | 10 minutes |
| Holding | - | 4°C | Infinite |

* - Recommended time is 1 minute per kb of plasmid, but can be increased to 2 minute per kb of plasmid, if it is larger than 10 kb

**Note:** For optimal specificity and amplification an individual optimization of the recommended parameters may be necessary for each new template DNA and/or primer pair.

6. Place tubes in the thermal cycler and start the PCR.

# Part B. DpnI Digestion

After cycling is finished, add 1 µL of DpnI restriction endonuclease to the reaction mixture to remove the original plasmid DNA. Incubate at 37 °C for 30 min.