Site-directed mutagenesis

Background: Site-directed mutagenesis (SDM) as described here is used to introduce a mutation in the sequence of a plasmid of interest by PCR-based methods. Several different approaches have been previously reported, and also the generation of multiple mutations in a single step is possible. This protocol deals with the simplest version of SDM for production of a small mutation at a single position of a plasmid, *e.g.* for generation of a protein variant or elimination of a restriction site.

Principle: Two primers that are complementary to each other and carry the mutation in the center are used in a PCR reaction with the plasmid as template. However, it is important to note that the product in this PCR is not amplified in an exponential manner, but in a linear manner. The product of one cycle cannot be used as template in the next round of amplification; only the initial template can serve as such. Therefore, the proper equilibrium between template (plasmid) and oligonucleotides used in the reaction is important. A polymerase without strand-displacing activity is used, and will amplify, originating from the primer, around the entire plasmid. After the PCR reaction, the template (the plasmid not carrying the desired mutation) is eliminated by digestion with *Dpn*I. *Dpn*I cuts only methylated DNA, and will therefore cut the template (most laboratory strains of *E. coli* have *Dam* and *Dcm* methylases; this protocol will not work with template DNA produced in a methylation-deficient strain) DNA, but not the PCR product. The PCR product is then directly transformed into *E. coli* competent cells. The residual nicks in the molecules are repaired by endogeneous repair mechanisms. The plasmid containing the mutation can be re-isolated from *E. coli*, and has to be confirmed by DNA sequencing.

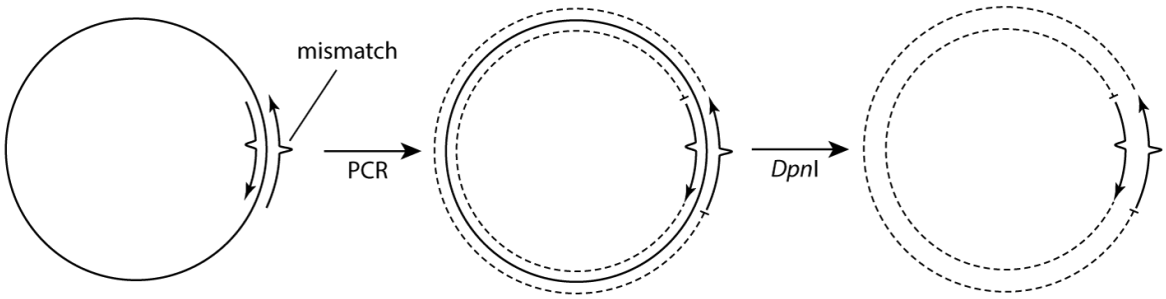
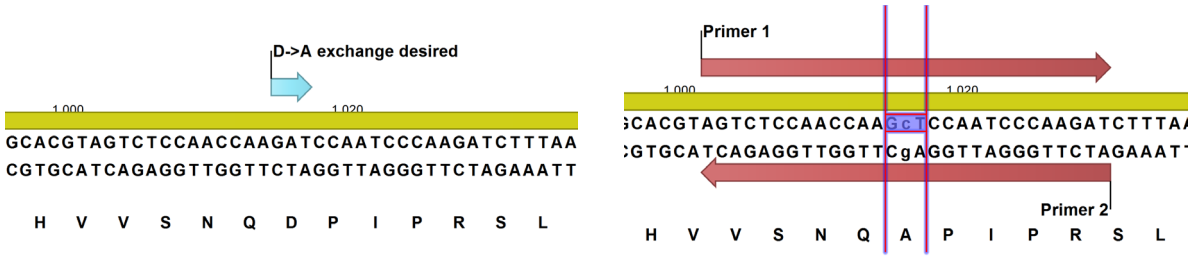


Figure 1: Principle of PCR-based site directed mutagenesis.

PCR primers containing the desired exchange at their center are used in a PCR reaction, and bind to the template plasmid during annealing. In the elongation cycle, the entire plasmid will be amplified. After the PCR, the initial template is eliminated by *Dpn*I digestion.

Primer Design: here examplified by an amino acid exchange within a coding sequence

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Primer 1: AGTCTCCAACCAAGcTCCAATCCCAAGAT
Primer 2: ATCTTGGGATTGGAgCTTGGTTGGAGACT
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- the mismatch = desired exchange should be in the middle of the oligo
- there are rules / calculators for primer length and annealing temperature; for example https://depts.washington.edu/bakerpg/primertemp/
- but a rule of thumb normally also does the job:
  exchange of a single nt: design primers of 27 nt length
  exchange of 2-3 nt: design primers of 29-30 nt length
  exchange of more 4-7 nt: 15-18 nt on each side
- for more extensive modifications, things may get complicated
- primers may also be designed to produce insertions or deletions… but this may require more careful design

Critical points:

- Ratio template : primers. Let's consider that a normal plasmid preparation has a concentration around 100 ng/µl, and plasmids are of 3 – 10 kb in size. A good starting point will be to use a 1:5 dilution of your plasmid in the PCR reaction (2 µl in 20 µl reaction), together with 1 µl of the primers @ 5 µM.
- The polymerase. Not every polymerase works well for SDM, and one may work for one reaction, while the other one will perform better in the next reaction. I advise using a Pfu polymerase (e.g. PfuPlus! DNA Polymerase from Roboklon) and one of the "chimeric" DNA polymerases (Phusion Polymerase, Q5 (both NEB), Polymerase X (Roboklon) or similar) in parallel, as one will normally do the job on a given molecule.

Protocol, for PfuPlus! and Hybrid (Polymerase X) DNA polymerases:

PfuPlus! Polymerase

2 µl (5 – 50 ng)	template DNA
1 µl	Primer 1 (5 µM)
1 µl	Primer 2 (5 µM)
2 µl	dNTPs (2mM)
2 µl	10 x PCR buffer
0.3 µl	*Pfu* Polymerase
11.7 µl	H_2O
20	Total

Hybrid Polymerase

2 µl (5 – 50 ng)	template DNA
1 µl	Primer 1 (5 µM)
1 µl	Primer 2 (5 µM)
1 µl	dNTPs (2mM)
2 µl	10 x PCR buffer
0.3 µl	Hybrid polymerase
12.7	H_2O
20	Total

Conditions:

95 °C	**1 min**	
95 °C	30 sec	18 cycles
56 °C	30 sec	
68 °C	1 min / kb	
68 °C	6 min	

98 °C	**30 sec**	
98 °C	10 sec	18 cycles
58 °C	20 sec	
72 °C	30 sec / kb	
72 °C	4 min	

Expected PCR results: If the amplification works well, a clear band as shown in the picture below will be visible (5µl of PCR loaded on a 1 % agarose gel). A weak band will also be sufficient to obtain clones. Finally, even if no product at all is visible, there may be a few positive clones after all. However, a general recommendation would be in this case to start all over again… and to test a few different concentrations of the template, or yet another PCR enzyme.

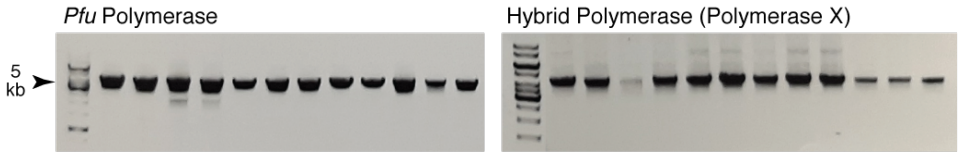

Figure 2: Results from PCR amplification (identical templates / primers) using Pfu or Hybrid DNA polymerases. Note that Pfu will not always perform best, as was the case here.

***Dpn*I digestion:** After verification of the reactions by Agarose gel electrophoresis, simply add 0.3 – 0.5 µl of *Dpn*I directly into the remaining PCR. Incubate 1h to over-night at 37°C. Inactivation of the enzyme is not required; directly proceed to transformation. If using chemically competent cells, there is no harm in transforming the entire reaction for simplicity.

Bacterial transformation: Linear DNA is transformed into *E. coli* at this step, which reduces the efficiency in comparison to transformation of (circular) plasmid DNA. Plating 10 % of the transformation will normally be sufficient to obtain 10 – 1000 clones. Pick 2 single colonies, inoculate small liquid cultures, re-isolate the mutagenized plasmid and verify by sequencing! In some cases, the primer from mutagenesis can become integrated in several copies at its binding site. Also, 2nd site mutations are possible, or the template may have escaped *Dpn*I digestion. Careful verification of sequences is mandatory!