

Video Article

Site-Directed Mutagenesis for In Vitro and In Vivo Experiments Exemplified with RNA Interactions in *Escherichia Coli*

Patrick Rosendahl Andreassen¹, Jens Sivkær Pettersen¹, Mikkel Jørgensen¹

¹Department of Biochemistry and Molecular Biology, University of Southern Denmark

Correspondence to: Mikkel Jørgensen at mikkelj@bmb.sdu.dk

URL: <https://www.jove.com/video/58996>

DOI: [doi:10.3791/58996](https://doi.org/10.3791/58996)

Keywords: Mutagenesis, Mutagenic analysis, site-directed, EMSA, western blot, RNA-RNA interactions, RNA-protein interactions, oligonucleotide-directed mutagenesis, dual plasmid

Date Published: 11/16/2018

Citation: Andreassen, P.R., Pettersen, J.S., Jørgensen, M. Site-Directed Mutagenesis for In Vitro and In Vivo Experiments Exemplified with RNA Interactions in *Escherichia Coli*. *J. Vis. Exp.* (), e58996, doi:10.3791/58996 (2018).

Abstract

Site-directed mutagenesis is a technique used to introduce specific mutations in DNA to investigate the interaction between small non-coding ribonucleic acid (sRNA) molecules and target messenger RNAs (mRNAs). In addition, site-directed mutagenesis is used to map specific protein binding sites to RNA. A 2-step and 3-step PCR based introduction of mutations is described. The approach is relevant to all protein-RNA and RNA-RNA interaction studies. In short, the technique relies on designing primers with the desired mutation(s), and through 2 or 3 steps of PCR synthesizing a PCR product with the mutation. The PCR product is then used for cloning. Here, we describe how to perform site-directed mutagenesis with both the 2- and 3-step approach to introduce mutations to the sRNA, McaS, and the mRNA, *csgD*, to investigate RNA-RNA and RNA-protein interactions. We apply this technique to investigate RNA interactions; however, the technique is applicable to all mutagenesis studies (e.g., DNA-protein interactions, amino-acid substitution/deletion/addition). It is possible to introduce any kind of mutation except for non-natural bases but the technique is only applicable if a PCR product can be used for downstream application (e.g., cloning and template for further PCR).

Introduction

DNA is often referred to as the blueprint of a living cell since all structures of the cell are encoded in the sequence of its DNA. Accurate replication and DNA repair mechanisms ensure that only very low rates of mutations occur, which is essential for sustaining correct functions of coded genes. Changes of the DNA sequence can affect successive functions at different levels starting with DNA (recognition by transcription factors and restriction enzymes), then RNA (base-pair complementarity and secondary structure alterations) and/or protein (amino acid substitutions, deletions, additions or frame-shifts). While many mutations do not affect gene function significantly, some mutations in the DNA can have huge implications. Thus, site-directed mutagenesis is a valuable tool for studying the importance of specific DNA sites at all levels.

This protocol describes a targeted mutagenesis approach used to introduce specific mutations. The protocol relies on two different PCR strategies: a 2-step or a 3-step PCR. The 2-step PCR is applicable if the desired mutation is close to either the 5' end or the 3' end of the DNA of interest (<200 base pairs (bp) from the end) and the 3-step PCR is applicable in all cases.

In the 2-step PCR approach, 3 primers are designed, in which one set of primers is designed to amplify the DNA of interest (primers 1 and 3, forward and reverse, respectively), and a single primer is designed to incorporate the mutation. The mutation introducing primer (primer 2) should have a reverse orientation if the mutation is close to the 5' end and a forward orientation if the mutation is close to the 3' end. In the first PCR step, primer 1+2 or 2+3 amplifies a small fragment close to the 5' end or 3' end, respectively. The resulting PCR product is then used as a primer in step two with primer 1 or 3, thus resulting in a PCR product with a mutation in the DNA of interest (**Figure 1A**).

In the 3-step PCR, 4 primers are designed, in which one set of primers is designed to amplify the DNA of interest (primers 1 and 4, forward and reverse, respectively) and one set of primers is designed to incorporate specific mutations with overlapping complementarity (primers 2 and 3, reverse and forward, respectively). In step one and two, primers 1+2 and 3+4 amplify the 5' and 3' end. In step three, the resulting PCR products from step one and two are used as templates and amplified with primers 1+4. Thus, the resulting PCR product is the DNA of interest with the desired mutation (**Figure 1B**).

While the mutated DNA can be used for any downstream application, this protocol describes how to re-combine the DNA into a cloning vector. The use of cloning vectors has several advantages such as ease of cloning and specific experimental applications depending on features of the vector. This feature is often used for RNA interaction studies. Another technique for RNA interaction studies is structural probing of the RNA in complex with another RNA^{1,2} or protein^{3,4}. However, structural probing is only performed in vitro whereas site-directed mutagenesis and subsequent cloning allow for interaction studies in vivo.

Site-directed mutagenesis has been extensively used for RNA interaction studies as presented here. However, the key method regarding 2- or 3-step PCR is applicable to any piece of DNA, and thus not only limited to RNA-interaction studies.

To exemplify the technique and its possible uses, characterization of regions important for post-transcriptional regulation of the mRNA, *csgD*, of *Escherichia coli* (*E. coli*) is used. In *E. coli*, *csgD* is targeted by the small non-coding RNA, McaS, in cooperation with a protein, Hfq, to repress

protein-expression of CsgD^{2,4,5}. The technique is used to introduce mutations to the base-pairing region between *csgD* and McaS, and to the Hfq binding site of *csgD*. The obtained DNA is then cloned into a vector suitable for subsequent experiments. Downstream applications of the technique include both in vivo and in vitro experiments. For illustration, example 1 is characterized in vivo using a western blot assay and example 2 is characterized in vitro using an electrophoretic mobility shift assay (EMSA). In both cases, it is illustrated how site-directed mutagenesis can be used in combination with other techniques to make biological conclusions about a gene of interest.

Protocol

1. Vector selection

1. Choose a vector to perform downstream experiments with. Any vector is applicable for this 2- and 3-step PCR method.
2. Based on choice of vector, choose appropriate restriction enzymes for cloning.

2. Primer design for site directed mutagenesis

1. Decide between either the 2-step or 3-step PCR strategy (2-step is only for mutations <200 bp from either end of the DNA of interest). For the 2-step PCR, go to step 2.2 and for the 3-step PCR go to step 2.3.
2. Design primers for 2-step PCR.
 1. Design primer 1 and 3 to amplify the DNA of interest and with a 5' overhang that contains 4 nucleotides (e.g., ATAT or AGCT) followed by the relevant restriction recognition site necessary to clone into the chosen vector.
 2. Design primer 2 to introduce mutation(s) at the desired site(s) and flank the mutation with 10-15 complementary nucleotides on both sides. Make the primer reverse if the mutation is introduced at the 5' end or forward if the mutation is introduced at the 3' end.
3. Design primers for 3-step PCR.
 1. Design primer 1 and 4 to amplify the DNA of interest and with a 5' overhang that contains 4 nucleotides (e.g., ATAT or AGCT) followed by the relevant restriction recognition site necessary to clone into the chosen vector.
 2. Design primer 2 and 3 to introduce mutation(s) at desired site(s) and flank the mutation by 10-15 complementary nucleotides on both sides. Primer 2 and 3 are reverse complementary.

3. PCR amplification of wild type DNA for cloning

NOTE: For details on PCR, see⁶.

1. Perform PCR⁶ using primers 1+2 (2-step PCR) or 1+4 (3-step PCR) and use wild type DNA as template to obtain PCR product I. Use the PCR program in **Table 1**.
2. Validate PCR by agarose gel electrophoresis.
 1. Make an agarose gel solution (2%) by adding 2 g of agarose per 100 mL of 1x Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. Dissolve the agarose by boiling in a microwave oven. Add the DNA-staining dye ethidium bromide (to a final concentration of ~ 0.5 µg/mL) to the agarose gel solution for visualization.
 2. Cast agarose gel, place it in an electrophoresis unit, and load PCR samples (mixed with DNA loading dye) and a DNA ladder of known size. Run samples at 75 W for 45 min, or until bands are separated adequately, and visualize bands at an ultra-violet (UV) table or with a gel imaging system.
3. Purify the PCR product with a gel extraction kit (**Table of Materials**) and measure the concentration of purified DNA with a spectrophotometer (**Table of Materials**).
4. Store the purified DNA at -20 °C (in Tris-EDTA (TE) buffer – long term storage) or 4 °C (in dH₂O – short term storage) until used in step 5.

4. PCR to introduce site-directed mutations in the DNA

1. PCR for 2-step PCR (see step 4.2 for 3-step PCR)
 1. Perform PCR⁶ using primers 1+2 if mutations are in the 5' end or 2+3 if the mutations are in the 3' end and use wild type DNA as a template to obtain PCR product II (see **Table 1** for PCR program).
 2. Validate PCR by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.
 3. Purify the PCR product with a gel extraction kit (**Table of Materials**) and measure the concentration of purified DNA with a spectrophotometer (**Table of Materials**).
 4. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH₂O) until used in step 5.
 5. Perform PCR⁶ using PCR product II as the primer together with primer 3 if mutations are in the 5' end or primer 1 if the mutations are in the 3' end and use wild type DNA as template to obtain PCR product III (see **Table 1** for PCR program).
 6. Validate PCR by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.
 7. Purify PCR product with a gel extraction kit (**Table of Materials**) and measure concentration of purified DNA with a spectrophotometer (**Table of Materials**).
 8. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH₂O) until step 5.
2. PCR for 3-step PCR
 1. Perform PCR⁶ using primers 1+2 and 3+4 in separate reactions and use wild type DNA as template to obtain PCR product II and III (see **Table 1** for PCR program).

2. Validate PCRs by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.
3. Purify PCR products with a gel extraction kit (**Table of Materials**) and measure the concentration of purified DNA with a spectrophotometer (**Table of Materials**).
4. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH₂O).
5. Perform PCR⁶ using primers 1+4 and use 2-5 ng of both PCR products II and III as the templates (in the same reaction) to obtain PCR product IV (see **Table 1** for PCR program).
6. Validate PCR by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.
NOTE: It is not unusual to get several incorrect bands (can sometimes be reduced by using less template). However, the incorrect PCR products can be ignored if the correctly sized band is excised and gel-extracted.
7. If correct, purify PCR product with a gel extraction kit (**Table of Materials**) and measure concentration of purified DNA with a spectrophotometer (**Table of Materials**).
8. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH₂O) until step 5.

5. Recombination of wild type and mutant version(s) of DNA into the chosen vector

NOTE: For details on following steps, see⁷.

1. Digest purified PCR products I and III (from 2-step PCR) and/or IV (from 3-step PCR) using the relevant restriction enzymes.
 1. In 20 µL of 1x digestion buffer with 1 µL of each restriction enzyme, digest 200 ng of PCR product at 37 °C for 30-60 min.
2. Purify digested PCR product by gel-extraction using a gel extraction kit (**Table of Materials**), measure the DNA concentration of purified DNA with a spectrophotometer (**Table of Materials**), and store at -20 °C (in TE buffer) or 4 °C (in dH₂O) until used for step 5.6.
3. Digest purified vector with relevant restriction enzymes and treat with alkaline phosphatase to decrease vector re-ligation events. Do not treat PCR products with alkaline phosphatase.
 1. In 30 µL of 1x digestion buffer with 1 µL of each restriction enzyme and 1 µL of alkaline phosphatase, digest 1,000 ng of the vector at 37 °C for 30-60 min.
4. Separate digested vector from waste DNA (e.g., uncut vector and cut-out DNA) using agarose gel electrophoresis as in step 3.2.1 and 3.2.2.
5. Purify digested vector by gel-extraction using a gel extraction kit (**Table of Materials**), measure DNA concentration of purified DNA with a spectrophotometer (**Table of Materials**), and store at -20 °C (in TE buffer) or 4 °C (in dH₂O) until used for step 5.6.
6. Ligate digested PCR products into digested vector with the reactions specified in **Table 2**.
7. Incubate at room temperature for 2 h or overnight at 16 °C.
8. Transform recipient strain (e.g., *E. coli* K12) with ligation reactions.
 1. Grow strain to OD₆₀₀=0.3-0.5 and transfer a 1 mL culture to as many 1.5 mL tubes as ligation reactions.
 2. Spin at 3,500 x g for 5 min and discard supernatant.
 3. Resuspend cells in 200 µL of transformation buffer (10 mL of lysogeny broth (LB) with 0.1 g/mL polyethylene glycol 3350, 5% dimethyl sulfate and 20 mM MgCl₂).
 4. Add ligation reaction, and place the tubes on ice for 30 min.
 5. Heat-shock for 2 min at 42 °C.
 6. Add 1 mL of LB to the 1.5 mL tubes, and allow phenotypic expression of antibiotic resistance for at least 45 min at 37 °C.
 7. Spin the cells at 3,500 x g for 5 min, discard 1 mL of supernatant, and resuspend the cells in the remaining supernatant.
 8. Plate the cells on plates with appropriate antibiotics and incubate overnight at 37 °C.
9. Identify transformants harboring vectors with successful integration of DNA insert (e.g., by PCR using vector- and insert-specific primers).
10. Validate sequence of DNA by sanger sequencing.
CAUTION: Do not use the same primers for sequencing as used for step 5.9.

6. Using constructed vectors for in vitro and/or in vivo experiments

1. In vivo experiment
NOTE: This is an example of using a vector to express wild type/mutated RNA to characterize post-transcriptional regulation. For further details on western blotting, see⁸.
 1. Grow *E. coli* K12 strains with constructed vectors in appropriate medium and induce expression if required. Harvest samples by centrifugation.
 2. Prepare samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by dissolving cell pellets in 1x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5% SDS, 0.002% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol) and boil at 95 °C for 5 min.
NOTE: It is possible to cast a gel or use a commercially available precast gel. The latter was used in the results presented in **Figure 3** (**Table of Materials**).
 3. Load 10⁷ cells of each sample in separate wells (include a protein ladder), and run gel at 200 V until proteins are separated (approximately 45 min).
 4. Blot the proteins onto a cellulose-membrane by semi-dry transfer at 80 mA for 1 h.
 5. Block the membrane with a mixture of proteins (e.g., 5% milk-powder dissolved in 1x Tween 20-Tris-buffered saline (TTBS) buffer).
 6. Add primary antibody (dissolved in 1x TTBS buffer) that target the protein of interest (e.g., GFP-, FLAG-, or HIS-tagged protein) and incubate for 1 h with gentle agitation.
 7. Wash membrane in 1x TTBS for 10 min to remove unbound antibodies. Repeat twice more.
 8. Add secondary antibody (dissolved in 1x TTBS buffer) that targets the primary antibodies and allow for detection (e.g., horseradish peroxidase (HRP)-conjugated secondary antibodies. Incubate for 1 h with gentle agitation.

9. Wash the membrane in 1x TTBS for 10 min to remove unbound antibodies. Repeat twice more.
 10. Visualize the membrane with a technique compatible with the secondary antibodies (e.g., by imaging after incubation with a luminol-derived chemiluminescence, if a HRP-conjugated secondary antibody was used).
2. In vitro experiment
- NOTE: This is an example of using the vector as a template for in vitro transcription of RNA to characterize RNA-protein interactions. For further details on EMSA, see⁹.
1. Make in vitro transcripts using a T7 in vitro transcription kit (**Table of Materials**) and vectors from step 5 as templates.
 2. Separate RNA transcripts by PAGE on a 4.5% 7 M urea denaturing gel, and extract RNA directly from the gel by electro elution with dialysis tubes (**Table of Materials**).
 3. Label RNA (e.g., radiolabeling with γ -³²P-ATP using T4-polynucleotide kinase (**Table of Materials**) and purify again with columns (**Table of Materials**).
CAUTION: Before working with radioactive material, consult with the local radiation safety officer.
 4. Mix labelled-RNA with increasing concentrations of protein in separate reactions in a 1x binding buffer (20 mM Tris, pH 8, 100 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol (DTT)).
NOTE: In the presented results (**Figure 4**), 2 nM of radiolabeled *csgD* mRNA was mixed with a gradient of 0 to 2 μ M Hfq protein (monomer concentration).
 5. Allow protein and RNA to hybridize before loading hybridization mix on a non-denaturing polyacrylamide gel and run the gel for 1.5 h at 200 V.
 6. Visualize the gel with a technique compatible with the labeling from step 6.1.3. (e.g., by phosphorimaging if radiolabeling was applied).
 7. Quantify the relative intensity of the shifted bands with an imaging processing program and fit a curve (sigmoidal) to the data by using a graph and data analysis software (**Table of Materials**). Based on the fitted curve, dissociation constant (K_d) values can be determined automatically with the software.

Representative Results

To investigate RNA interactions regarding post-transcriptional regulation of *csgD*, a double vector setup was chosen: one to express the *csgD* mRNA and another to express the small non-coding RNA, McaS. *csgD* was cloned into pBAD33, which is an arabinose inducible medium-copy plasmid with chloramphenicol resistance and McaS was cloned into mini R1 pNDM220, which is an isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible low copy plasmid with ampicillin resistance. Wild type *csgD* was PCR amplified using primers 1A and 4A (4A includes a FLAG-sequence) to yield PCR product IA, while wild type McaS was PCR amplified using primers 1B and 4B to yield PCR product IB. The 3-step PCR strategy was used to introduce substitutions in the predicted base-pairing region of *csgD* and McaS. In the two first steps, PCR products IIA and IIIA were synthesized using primers 1A+2A, and 3A+4A, respectively. In the third step, PCR product IVA was synthesized using PCR products IIA and IIIA as template and 1A and 4A as primers (**Figure 2A**). Similarly, complementary site-directed mutations were introduced in McaS, using primers 1B, 2B, 3B and 4B to yield PCR products IIB, IIIB in the first two steps and IVB in the third step (**Figure 2A**). pBAD33 and PCR products IA and IVA were digested with both BamHI and PstI and the digested IA and IVA were ligated into digested pBAD33. Resulting constructs were named pBAD-*csgD*^{FLAG} and pBAD-*csgD*^{63-66FLAG} (mutated at position 63-66 relative to the transcriptional start site). pNDM220 and PCR products IB and IVB were digested with AatII and BamHI and digested IB and IVB were ligated into digested pNDM220. Resulting constructs were named pNDM-mcaS and pNDM-mcaS⁴²⁻⁴⁵ (mutated at position 42-45 relative to transcriptional start site).

To assay the effect of the mutations, *E. coli* strains harboring the pBAD-*csgD*^{FLAG} or pBAD-*csgD*^{63-66FLAG} and pNDM220, pNDM-mcaS or pNDM-mcaS⁴²⁻⁴⁵ were grown in M9 minimal medium supplemented with 0.2% glycerol to OD₄₅₀ of 0.4. Expression from the pNDM-vectors was then induced for 10 min by addition of 1 mM IPTG followed by 5 min induction of the pBAD-vectors by addition of 1 mM arabinose. At this point, samples were harvested and western blot analysis was performed with the harvested samples. While expression of wild type McaS prevents translation of wild type CsgD, introduction of mutations in either CsgD or McaS alleviates the observed repression. However, when the mutations are complemented in both CsgD and McaS translational repression of CsgD is restored (**Figure 3**; modified from²). Thus, the site-directed mutational approach supports the hypothesis that McaS and *csgD* base-pairs at this region.

The pBAD33 vector was chosen for the in vivo experiment, and was also used for introducing site-directed mutation to investigate Hfq-binding to the *csgD* mRNA in vitro. Site-directed mutations were introduced with the 2-step PCR strategy to generate *csgD* mutant RNAs with altered primary and/or secondary structures when transcribed. Primers 1 + 2C, 2D, 2E, 2F or 2G were used to amplify and introduce mutations to the 5' end of *csgD*. The resulting PCR products (IIC, IID, IIE, IIF and IIG) were used as templates with primer 3 to amplify and introduce mutations to the entire *csgD* DNA (**Figure 2B**). The resulting PCR products (IIIC, IIID, IIIE, IIIF and IIIG) were cloned into pBAD33 as described above. In vitro transcripts were transcribed with the T7 RNA-polymerase: first, PCR products were synthesized with primers 5A, 5C, 5D, 5E, 5F or 5G + 6 using the constructed vectors as template. The resulting PCR products were used as templates for the T7 RNA-polymerase. In vitro transcribed *csgD* wild type and mutant RNAs were purified, radiolabeled and mixed with increasing concentrations of purified Hfq. The hybridization reactions were run on non-denaturing gels and visualized. The increased K_d -values of the mutant alleles proves that Hfq binds less efficiently to the mutants. Furthermore, Hfq has several binding sites on *csgD* which is shown by the 3 shifts observed for the wild type RNA. However, only 2 binding sites are observed for the different mutant RNAs (**Figure 4**; modified from⁴). Thus, the site-directed mutational approach identifies primary and/or secondary structures of the *csgD* mRNA that are important for complete binding of Hfq.

Taken together, it is possible to perform site-directed mutagenesis with a 2- or 3-step PCR approach in combination with downstream assays to make biological conclusions about gene regulation at the post-transcriptional level as well as protein-RNA interactions.

Step	Temperature	Time
Initial denaturation	98°C	2 min
Denaturation	98°C	10 s
Annealing	55°C	10 s
Extension	72°C	15 s
(30 cycles)		
Final extension	72°C	5 min
Hold	4 °C	

Table 1: PCR program

Reagent	Control reaction	20 fmol reaction	100 fmol reaction
10x ligase buffer	2 µL	2 µL	2 µL
Digested vector DNA	10 fmol	10 fmol	10 fmol
Digested PCR product	0 fmol	20 fmol	100 fmol
H ₂ O	To 19 µL	To 19 µL	To 19 µL
Ligase	1 µL	1 µL	1 µL

Table 2: Ligation reactions

Primer name	Sequence	Used for - mutations
2-1A or 3-1A	GCGC GGATCC TACCTGACGCTTTTATCGCAACTCTCTACTGTTTCTCCATCAGA TGTAATCCATTAGT	2- and 3-round PCR on <i>csgD</i>
2-3A or 3-4A	CCGC CTGCAG AAAAAACCCCGCAGCAGCGGGGTTTTCTACCAGACGAGAAC	2- and 3-round PCR on <i>csgD</i>
3-2A	CAGAAGTACTGACAGATGTTG <u>GTG</u> AGCTGTGTGTAGTAATAAATC	3-round PCR on <i>csgD</i> – substitutes 4 nt
3-3A	GATTTATTACTACACACAGCTC <u>ACCA</u> ACATCTGTCAGTACTTCTG	3-round PCR on <i>csgD</i> – substitutes 4 nt
3-1B	CGCCT GACGT CGGCAAAAAGAGTGTTGACTTGTGAGCGGATAACAATGATACTTA GATTCACCGGCGCAGAGGAGACAATGCC	3-round PCR on McaS
3-4B	AATT GGATCC AAAAAATAGAGTCTGTCGACATC	3-round PCR on McaS
3-2B	CTCTACAGTACACACAGCTC <u>ACCA</u> TCCGCGTCTTAAATC	3-round PCR on McaS – substitutes 4 nt
3-3B	GATTTAAGACGCGGATGGT <u>GAG</u> CTGTGTGTACTGTAGAG	3-round PCR on McaS – substitutes 4 nt
2-2C	CAGCCCTAAATGGGTCTAATGGATTACATCTG	2-round PCR on <i>csgD</i> – deletes 11 nt
2-2D	CAGCCCTAAATGGGTAAA <u>ACCC</u> AACTAATGGATTACATCTG	2-round PCR on <i>csgD</i> – substitutes 4 nt
2-2E	CTGTGTGTAGTAATAAATCAGTAAATATAAACTAATGGATTACATCTG	2-round PCR on <i>csgD</i> – deletes 11 nt
2-2F	GTAATAAATCAGCCCTACTACTAGAACTAATGGATTACATCTG	2-round PCR on <i>csgD</i> – deletes 9 nt and substitutes 7 nt
2-2G	CTGCTGTGTGTAGTAAT <u>CCAT</u> CAGCCCTAT <u>GACT</u> AAAACTAATGGATTACATCTG	2-round PCR on <i>csgD</i> – deletes 9 nt and substitutes 7 nt
5A	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGTTTTATATTTTACCC	T7 PCR
5C	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGACCCATTTAGG	T7 PCR
5D	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGTTTGGGGTTTTAC	T7 PCR
5E	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGTTTTATATTTTACTGATTTATTAC	T7 PCR
5F	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGTTCTAGTAGTAG	T7 PCR
5G	GAAATTAATACGACTCACTATAGGCAGATGTA ATCCATTAGTTTGTAGTCATAGG	T7 PCR
6	GTATGACCATGAATACTATGG	T7 PCR

Table 3: Primers used for site-directed mutagenesis and T7 template synthesis

Bold: restriction enzyme recognition sites (BamHI, PstI and AatII)

Underlined: nucleotide mutations

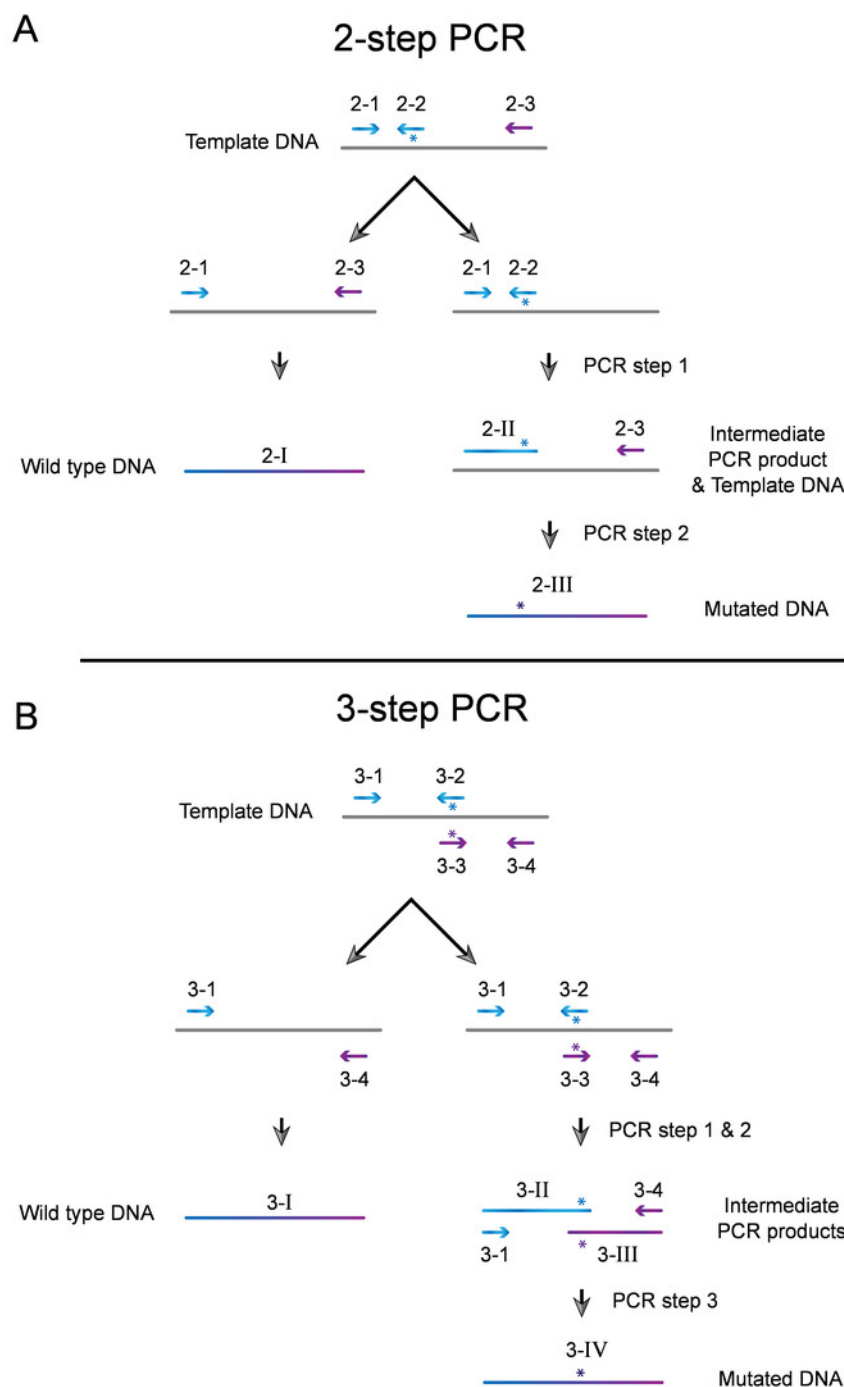


Figure 1: PCR strategy for site-directed mutagenesis. A) Three primers are used in the 2-step PCR approach to introduce site-directed mutations to a gene of interest. Primers 1 and 3 amplifies the gene (PCR product I), while primer 2 introduces specific mutations (*). Primer pairs 1 + 2 amplifies a small fragment at either end of the DNA of interest to synthesize PCR product II (step 1). The resulting PCR product is then used as a primer together with primer 3 to synthesize PCR product III with the site directed mutation incorporated (step 2). B) Four primers are used in the 3-step PCR approach to introduce site-directed mutations to a gene of interest. Primers 1 and 4 amplifies the gene (PCR product I), while primers 2 and 3 introduces specific mutations (*). Primer pairs 1+2 and 3+4 are used in the two first steps of PCR to synthesize PCR product II and III (step 1 & 2). In the third step, PCR products II and III are used as template with primer pair 1+4 to synthesize PCR product IV with the site-directed mutation incorporated (step 3). [Please click here to view a larger version of this figure.](#)

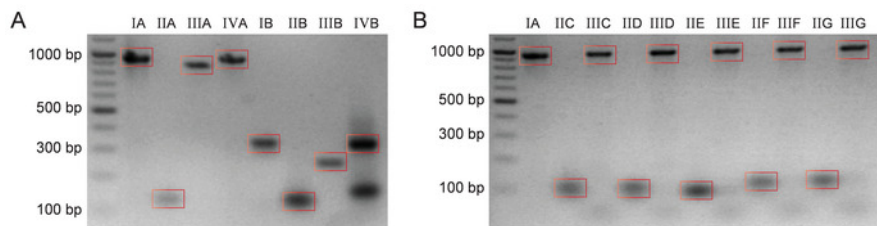


Figure 2: PCR products from site-directed mutagenesis. PCRs were performed with primers and templates as described in the text. PCR products were run on a 2% agarose gel with ethidium bromide (~ 0.5 µg/mL) with 1 µg of DNA ladder mix (**Table of Materials**). Correct size bands are marked with a red square. A) In most PCR reactions only one band of the correct size is visible. However, PCR reaction IVB has two visible bands of which the top band (just above 300 bp) has the correct length. As expected, the sum of the length of PCR products II and IIIC equal to the length of PCR products I and IV (A: *csgD* PCR products, B: *McaS* PCR products, I: wild type *csgD*/*McaS* amplified using primers 1A/B+4A/B, II+III: intermediate PCR products amplified using primers 1A/B+2A/B and 3A/B+4A/B, respectively, IV: mutated PCR product amplified using primers 1A/B+4A/B with the intermediate PCR products II and III as templates). In all PCR reactions only one band of the correct size is visible. In this case, almost all molecules of PCR products II added to PCR reactions III were used to synthesize PCR products III. Only for PCR IIIE is PCR product IIE still visible (IA: wild type *csgD* PCR product amplified using primer 1A+3A, IIC-G: intermediate PCR products amplified using primer 1A+2C-G, IIIC-G: mutated PCR products amplified using primer 3A with PCR products IA and IIC-G as templates). [Please click here to view a larger version of this figure.](#)

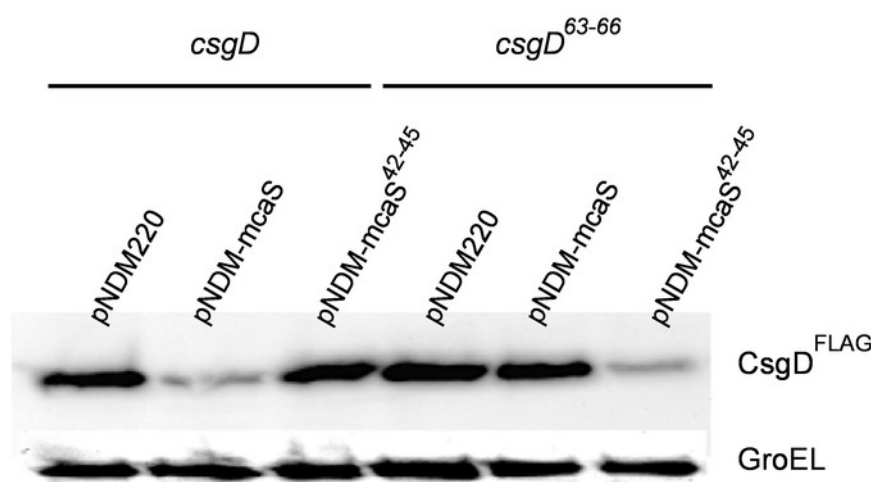


Figure 3: In vivo experiment with site-directed *csgD* and *McaS* mutants. Western blot analysis of strains harboring indicated vectors. Strains were grown to exponential phase and induced for 10 min with 1 mM IPTG (*McaS*) followed by 5 min induction with 1 mM arabinose (*csgD*). α-FLAG antibodies were used to target the FLAG-tagged CsgD and α-GroEL antibodies were used to target the housekeeping protein GroEL (diluted 10,000 and 50,000 times, respectively). Mouse and rabbit HRP-conjugated antibodies were used as secondary antibodies (diluted 2,000 times). This figure has been modified from². [Please click here to view a larger version of this figure.](#)

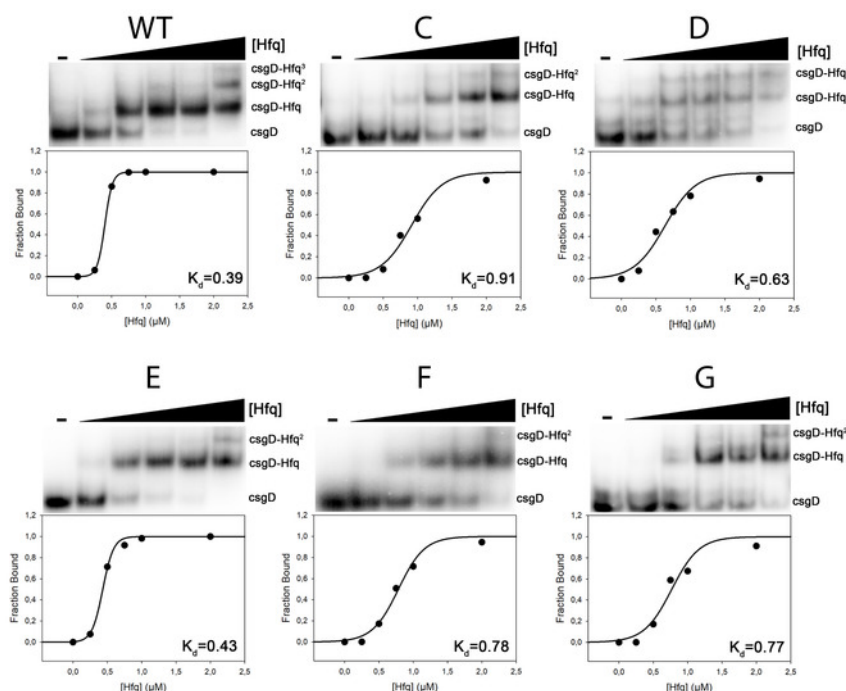


Figure 4: *In vitro* experiment with site-directed *csgD* mutants. EMSA of in vitro transcribed *csgD* wild type (WT) and mutant (Panel C-G) RNAs with respect to Hfq binding. The *csgD* alleles (WT and mutant C-G) were radiolabeled and mixed with 0, 0.25, 0.5, 1 or 2 μ M monomeric Hfq. Hybridization reactions were run on non-denaturing polyacrylamide gels. The relative intensity of the shifted bands was quantified and a sigmoid curve was fitted to the data. Dissociation constant (K_d) values were determined using SigmaPlot. This figure has been modified from⁴. Please click here to view a larger version of this figure.

Discussion

Site-directed mutagenesis has a broad array of different applications, and here, representative results from an *in vivo* and an *in vitro* experiment were included as examples of how to make biological conclusions using the technique. Site-directed mutagenesis has for long been the golden standard for RNA interaction studies. The strength of the technique lies in the combination of introducing relevant mutations with downstream assays and experiments (e.g., western blot or EMSA) to draw conclusions about specific DNA sites and their importance in functions of the gene-products in question. When deciding to do site-directed mutagenesis, the design of primers should be carefully planned to gain the most from the technique (e.g. which sites to mutate); be sure to include the correct overhangs and restriction sites and pay attention the primer/template characteristics.

The actual mutagenesis described in this protocol relies on PCR. The most crucial part of the protocol is therefore the conditions for this. There are several ways of optimizing PCR, including gradients of Mg^{2+} concentrations, DMSO concentration and temperatures of the annealing step. For further details, see³. Besides optimization of the PCR reaction itself, two things are worth making sure when doing site-directed mutagenesis: high-quality templates and carefully designed primers.

Having a high-quality template for PCR often makes the difference between a failed and successful PCR. While it is possible to use a cell lysate to provide the DNA template, purified DNA (genomic-, vector- or PCR-DNA) is always preferable. Furthermore, when it comes to the amount of template, often less is more; especially in the last step of the 3-step PCR (step 4.2.5). For instance, try a 10x dilution series of template to find the optimum if necessary.

Optimal primer design depends on characteristics of the DNA of interest and polymerase. Whenever possible, always try to design primers accordingly. However, in many cases primers must be designed at a specific site and might therefore not be designed according to specific criteria. In those cases, it might be necessary to do more optimization of the PCR conditions instead (see above and protocol on PCR⁶), but with the right conditions even difficult PCRs are usually successful.

Several alternative methods for site-directed mutagenesis are available, such as Kunkel's method¹⁰, whole plasmid mutagenesis¹¹, cassette mutagenesis¹², de novo gene synthesis and CRISPR^{13,14}.

Kunkel's method and whole plasmid mutagenesis relies on the DNA of interest already being in a plasmid (vector) and uses primers to synthesize a single strand or double strand of mutated DNA, respectively. In both techniques, the entire plasmid is being synthesized and used for transformation, whereas 2- or 3-step PCR only synthesizes the DNA piece of interest. A disadvantage of the 2- or 3-step PCR, is that the DNA of interest must be synthesized twice, increasing the risk of mutations therein. On the other hand, the plasmid does not have to be synthesized, thus lowering the risk of mutations here instead. Furthermore, using the 2- or 3-step PCR, a wild type variant does not need to be cloned into a vector beforehand, thus lowering the cloning time by several days.

Cassette mutagenesis does not rely on the use of primers or polymerases. Instead, a small DNA fragment is de novo synthesized and incorporated into the DNA of interest with the use of restriction enzymes. This method, however, relies on the presence of suitable restriction

sites near the targeted site, which is not always present. This approach also requires the wild type variant to be cloned into the vector beforehand, increasing cloning time compared to the 2- or 3-step PCR method.

With the decreasing cost of de novo gene synthesis (large DNA fragments), it is becoming increasingly affordable to introduce mutations by ordering the desired sequence commercially. This method is, however, still costly compared the other methods mentioned. At time of writing de novo synthesis is approximately 3 times more expensive than the method presented here.

Another emerging option is the highly anticipated CRISPR method for genome alterations. This method is highly efficient and adaptable compared to other techniques used for eukaryotic cells. However, with the relative ease and many available techniques for cloning in the simpler organism as bacteria, CRISPR is rarely more suitable than conventional cloning. Thus, the usefulness of CRISPR mostly depends on the organism being studied.

When choosing to do site-directed mutagenesis, it is important to design it carefully; both with regards to downstream applications as well the actual technique used. The 2- and 3-step PCR method described here is applicable to almost any mutation study and with its low cost it is suitable to any laboratory budget.

Disclosures

The authors declare no competing interests.

Acknowledgements

The authors would like to thank University of Southern Denmark open access policy grants.

References

1. Bouvier, M. et al. Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Molecular Cell*. **32** (6), 827-837 (2008).
2. Jorgensen, M. G. et al. Small regulatory RNAs control the multi-cellular adhesive lifestyle of Escherichia coli. *Molecular Microbiol.* **84** (1), 36-50 (2012).
3. Antal, M. et al. A small bacterial RNA regulates a putative ABC transporter. *The Journal of Biological Chemistry*. **280** (9), 7901-7908 (2005).
4. Andreassen, P. R. et al. sRNA-dependent control of curli biosynthesis in Escherichia coli: McaS directs endonucleolytic cleavage of csgD mRNA. *Nucleic Acids Research*. (2018).
5. Thomason, M. K. et al. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. *Molecular Microbiol.* **84** (1), 36-50 (2012).
6. Lorenz, T. C. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*. (63), e3998 (2012).
7. JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. Molecular Cloning. *Journal of Visualized Experiments*. Cambridge, MA (2018).
8. JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. The Western Blot. *Journal of Visualized Experiments*. Cambridge, MA (2018).
9. JoVE Science Education Database. Biochemistry. Electrophoretic Mobility Shift Assay (EMSA). *Journal of Visualized Experiments*. Cambridge, MA (2018).
10. Kunkel, T. A. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proceedings of the National Academy of Sciences of the United States of America*. **82** (2), 488-492 (1985).
11. Laible, M., Boonrod, K. Homemade site directed mutagenesis of whole plasmids. *Journal of Visualized Experiments*. (27) (2009).
12. Wells, J. A., Vasser, M., Powers, D. B. Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites. *Gene*. **34** (2-3), 315-323 (1985).
13. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. **339** (6121), 819-823 (2013).
14. Mali, P. et al. RNA-guided human genome engineering via Cas9. *Science*. **339** (6121), 823-826 (2013).