

# Journal of Visualized Experiments

## Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58996R3
Full Title:	Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli
Keywords:	Mutagenesis; Mutagenic analysis; site-directed; EMSA; Western blot; RNA-RNA interactions; RNA-protein interactions; oligonucleotide-directed mutagenesis; dual plasmid
Corresponding Author:	Mikkel Jørgensen, PhD Syddansk Universitet Odense M, Fyn DENMARK
Corresponding Author's Institution:	Syddansk Universitet
Corresponding Author E-Mail:	mikkelj@bmb.sdu.dk
Order of Authors:	Patrick Rosendahl Andreassen, MS Jens Sivkær Pettersen Mikkel Girke Jørgensen
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Odense, 5230, Denmark

**TITLE:**

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

**AUTHORS& AFFILIATIONS:**

Patrick Rosendahl Andreassen<sup>1</sup>, Jens Sivkær Pettersen<sup>1</sup>, Mikkel Girke Jørgensen<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

**Corresponding Author:**

Mikkel Girke Jørgensen  
mikkelj@bmb.sdu.dk

**Co-authors' e-mail addresses**

prandreassen@bmb.sdu.dk  
jenspettersen94@gmail.com

**KEYWORDS:**

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

**SUMMARY:**

Site-directed mutagenesis is a technique used to introduce specific mutations in deoxyribonucleic acid (DNA). This protocol describes how to do site-directed mutagenesis with a 2-step and 3-step polymerase chain reaction (PCR) based approach, which is applicable to any DNA fragment of interest.

**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<sup>1,2</sup> or protein<sup>3,4</sup>. 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<sup>2,4,5</sup>. 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**

1.1. Choose a vector to perform downstream experiments with. Any vector is applicable for this 2- and 3-step PCR method.

1.2. Based on choice of vector, choose appropriate restriction enzymes for cloning.

### **2. Primer design for site directed mutagenesis**

2.1. 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.

2.2. Design primers for 2-step PCR.

2.2.1. 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.

2.2.2. 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.

2.3. Design primers for 3-step PCR.

2.3.1. 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.



2.3.2. 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, see<sup>6</sup>.

3.1. Perform PCR<sup>6</sup> 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**.

3.2. Validate PCR by agarose gel electrophoresis.

3.2.1. 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.

3.2.2. 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.

3.3. 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**).

3.4. Store the purified DNA at -20 °C (in Tris-EDTA (TE) buffer – long term storage) or 4 °C (in dH<sub>2</sub>O – short term storage) until used in step 5.

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

4.1. PCR for 2-step PCR (see step 4.2 for 3-step PCR)

4.1.1. Perform PCR<sup>6</sup> 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).

4.1.2. Validate PCR by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.

4.1.3. 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**).

4.1.4. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH<sub>2</sub>O) until used in step 5.

4.1.5. Perform PCR<sup>6</sup> 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).

4.1.6. Validate PCR by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.

4.1.7. Purify PCR product with a gel extraction kit (**Table of Materials**) and measure concentration of purified DNA with a spectrophotometer (**Table of Materials**).

4.1.8. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH<sub>2</sub>O) until step 5.

## 4.2. PCR for 3-step PCR

4.2.1. Perform PCR<sup>6</sup> 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).

4.2.2. Validate PCRs by agarose gel electrophoresis as in step 3.2.1 and 3.2.2.

4.2.3. Purify PCR products with a gel extraction kit (**Table of Materials**) and measure the concentration of purified DNA with a spectrophotometer (**Table of Materials**).

4.2.4. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH<sub>2</sub>O).

4.2.5. Perform PCR<sup>6</sup> 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).

4.2.6. 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.

4.2.7. 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**).

4.2.8. Store purified DNA at -20 °C (in TE buffer) or 4 °C (in dH<sub>2</sub>O) until step 5.

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

NOTE: For details on following steps, see<sup>7</sup>.

5.1. Digest purified PCR products I and III (from 2-step PCR) and/or IV (from 3-step PCR) using the relevant restriction enzymes.

5.1.1. In 20  $\mu\text{L}$  of 1x digestion buffer with 1  $\mu\text{L}$  of each restriction enzyme, digest 200 ng of PCR product at 37 °C for 30-60 min.

5.2. 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 dH<sub>2</sub>O) until used for step 5.6.

5.3. 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.

5.3.1. In 30  $\mu\text{L}$  of 1x digestion buffer with 1  $\mu\text{L}$  of each restriction enzyme and 1  $\mu\text{L}$  of alkaline phosphatase, digest 1,000 ng of the vector at 37 °C for 30-60 min.

5.4. 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.

5.5. 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 dH<sub>2</sub>O) until used for step 5.6.

5.6. Ligate digested PCR products into digested vector with the reactions specified in **Table 2**.

5.7. Incubate at room temperature for 2 h or overnight at 16 °C.

5.8. Transform recipient strain (e.g., *E. coli* K12) with ligation reactions.

5.8.1. Grow strain to OD<sub>600</sub>=0.3-0.5 and transfer a 1 mL culture to as many 1.5 mL tubes as ligation reactions.

5.8.2. Spin at 3,500 x *g* for 5 min and discard supernatant.

5.8.3. Resuspend cells in 200  $\mu\text{L}$  of transformation buffer (10 mL of lysogeny broth (LB) with 0.1 g/mL polyethylene glycol 3350, 5% dimethyl sulfate and 20 mM MgCl<sub>2</sub>).

5.8.4. Add ligation reaction, and place the tubes on ice for 30 min.

5.8.5. Heat-shock for 2 min at 42 °C.

5.8.6. 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.

5.8.7. Spin the cells at 3,500 x *g* for 5 min, discard 1 mL of supernatant, and resuspend the cells in the remaining supernatant.

5.8.8. Plate the cells on plates with appropriate antibiotics and incubate overnight at 37 °C.

5.9. Identify transformants harboring vectors with successful integration of DNA insert (e.g., by PCR using vector- and insert-specific primers).

5.10. 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**

6.1. 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, see<sup>8</sup>.

6.1.1. Grow *E. coli* K12 strains with constructed vectors in appropriate medium and induce expression if required. Harvest samples by centrifugation.

6.1.2. 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)**.

6.1.3. Load 10<sup>7</sup> cells of each sample in separate wells (include a protein ladder), and run gel at 200 V until proteins are separated (approximately 45 min).

6.1.4. Blot the proteins onto a cellulose-membrane by semi-dry transfer at 80 mA for 1 h.

6.1.5. Block the membrane with a mixture of proteins (e.g., 5% milk-powder dissolved in 1x Tween 20-Tris-buffered saline (TTBS) buffer).

6.1.6. 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.

6.1.7. Wash membrane in 1x TTBS for 10 min to remove unbound antibodies. Repeat twice more.

6.1.8. 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.

6.1.9. Wash the membrane in 1x TTBS for 10 min to remove unbound antibodies. Repeat twice more.

6.1.10. 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).

## 6.2. 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, see<sup>9</sup>.

6.2.1. Make in vitro transcripts using a T7 in vitro transcription kit (**Table of Materials**) and vectors from step 5 as templates.

6.2.2. 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**).

6.2.3. Label RNA (e.g., radiolabeling with  $\gamma$ -<sup>32</sup>P-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.

6.2.4. 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 MgCl<sub>2</sub>, 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  $\mu$ M Hfq protein (monomer concentration).

6.2.5. 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.

6.2.6. Visualize the gel with a technique compatible with the labeling from step 6.1.3. (e.g., by phosphoimaging if radiolabeling was applied).

6.2.7. 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  $\beta$ -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*<sup>FLAG</sup> and pBAD-*csgD*<sup>63-66FLAG</sup> (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-mcaS<sup>42-45</sup> (mutated at position 42-45 relative to transcriptional start site).

To assay the effect of the mutations, *E. coli* strains harboring the pBAD-*csgD*<sup>FLAG</sup> or pBAD-*csgD*<sup>63-66FLAG</sup> and pNDM220, pNDM-mcaS or pNDM-McaS<sup>42-45</sup> were grown in M9 minimal medium supplemented with 0.2% glycerol to OD<sub>450</sub> 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 from<sup>2</sup>). 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<sub>d</sub>-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 from<sup>4</sup>). 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.

#### FIGURE AND TABLE LEGENDS:

##### Table 1: PCR program

##### Table 2: Ligation reactions

##### 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).

**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+IIIC: 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, IIC-G: mutated PCR products amplified using primer 3A with PCR products IA and IIC-G as templates).

**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*).  $\alpha$ -FLAG antibodies were used to target the FLAG-tagged CsgD and  $\alpha$ -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 from<sup>2</sup>.

**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  $\mu$ 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 (Kd) values were determined using SigmaPlot. This figure has been modified from<sup>4</sup>.

## 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, see<sup>3</sup>. 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 PCR<sup>6</sup>), but with the right conditions even difficult PCRs are usually successful.

Several alternative methods for site-directed mutagenesis are available, such as Kunkel's method<sup>10</sup>, whole plasmid mutagenesis<sup>11</sup>, cassette mutagenesis<sup>12</sup>, de novo gene synthesis and CRISPR<sup>13,14</sup>.

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.

## **ACKNOWLEDGMENTS:**

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

#### DISCLOSURES:

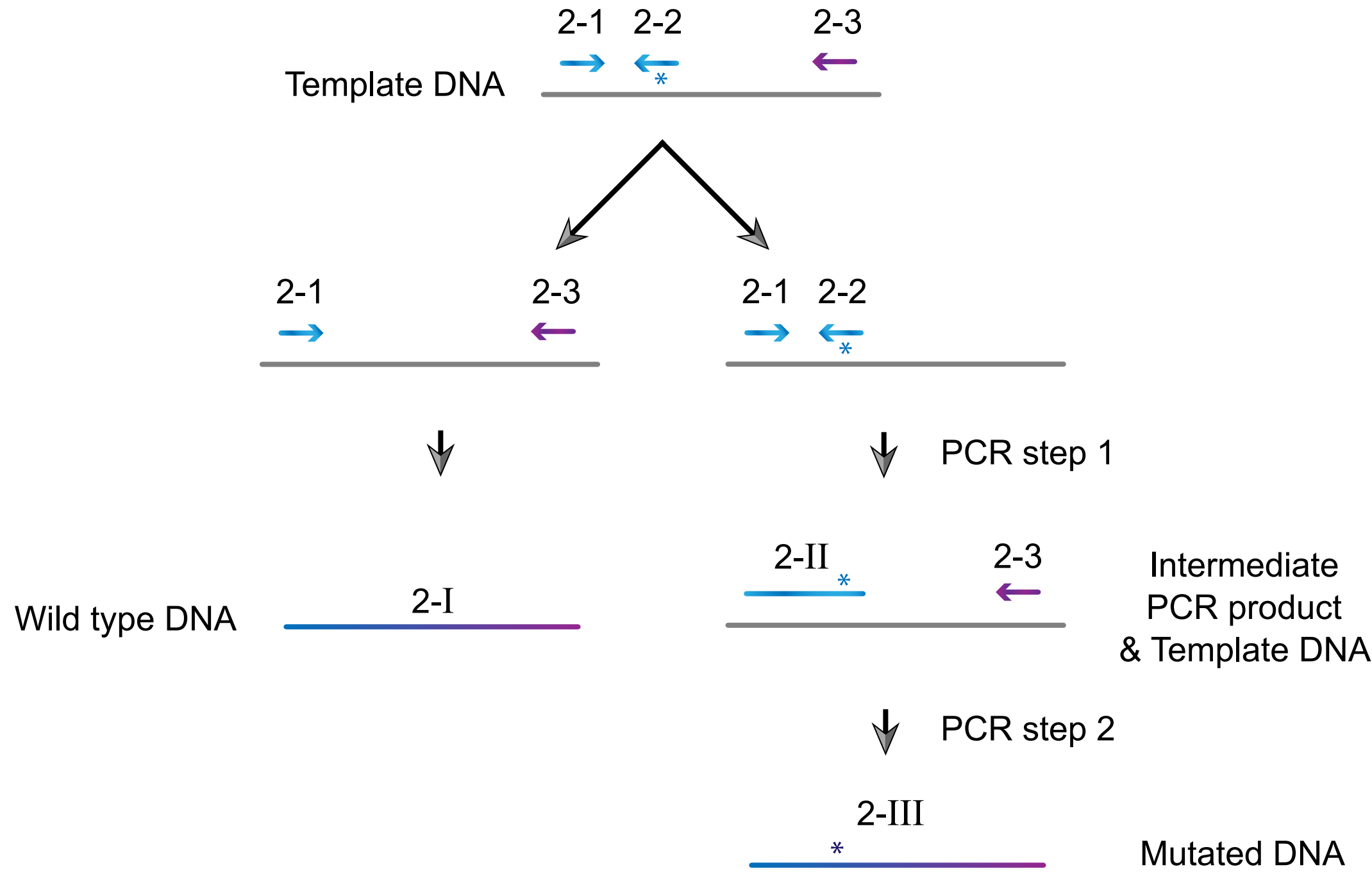
The authors declare no competing interests.

#### 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*. 10.1093/nar/gky479 (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 *Journal of Visualized Experiments* Science Education Database. Basic Methods in Cellular and Molecular Biology. Molecular Cloning. JoVE, 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).

A

# 2-step PCR



B

# 3-step PCR

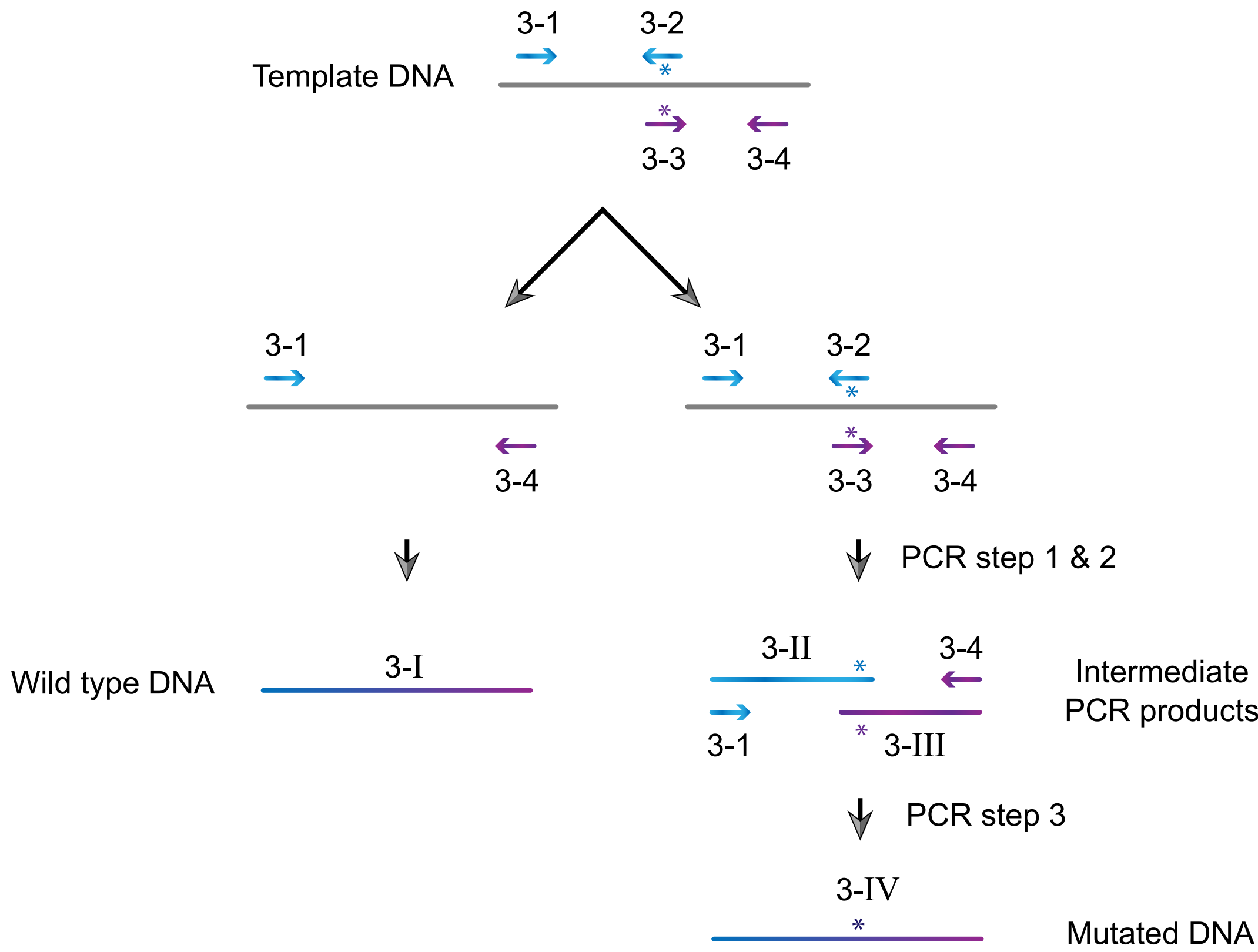
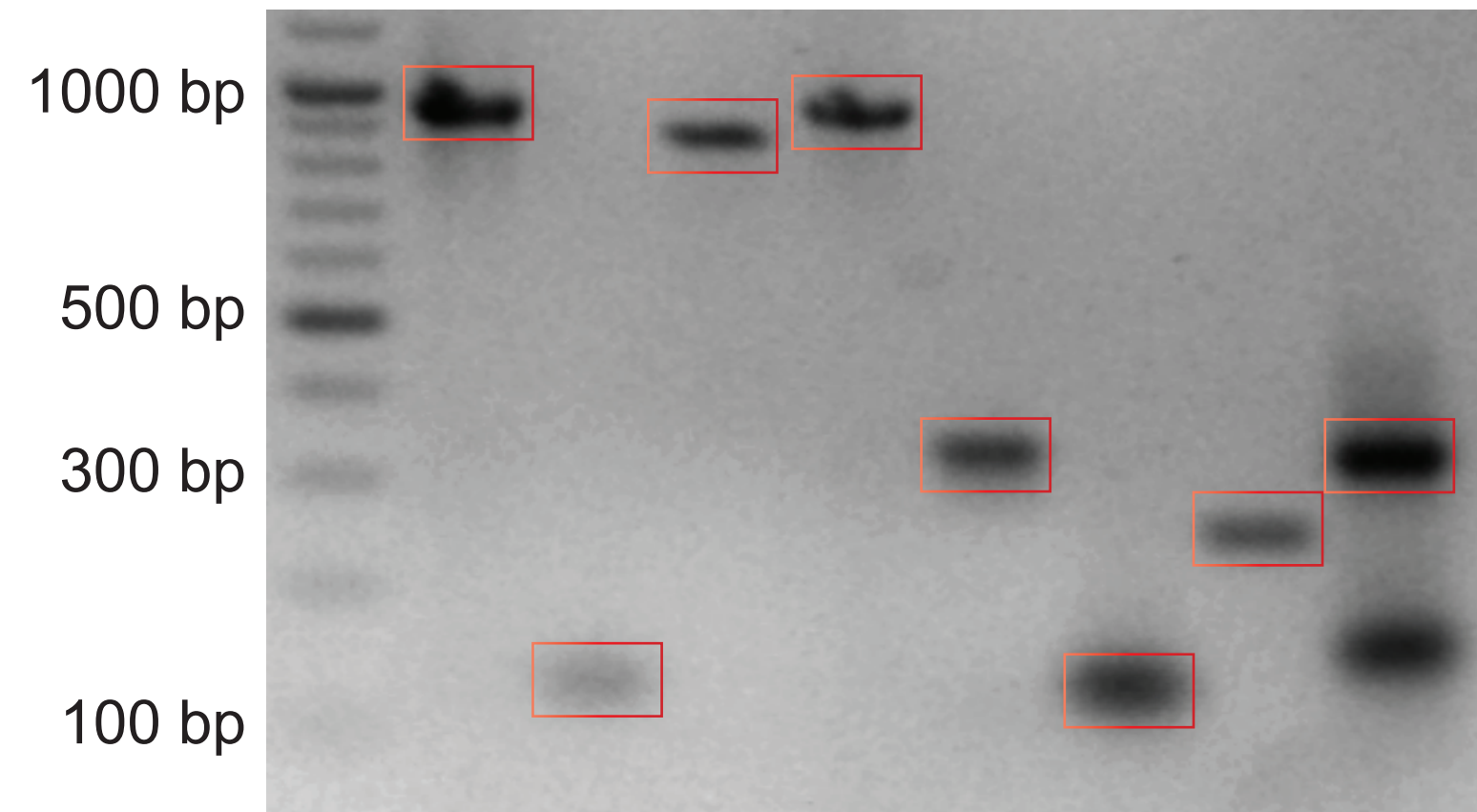


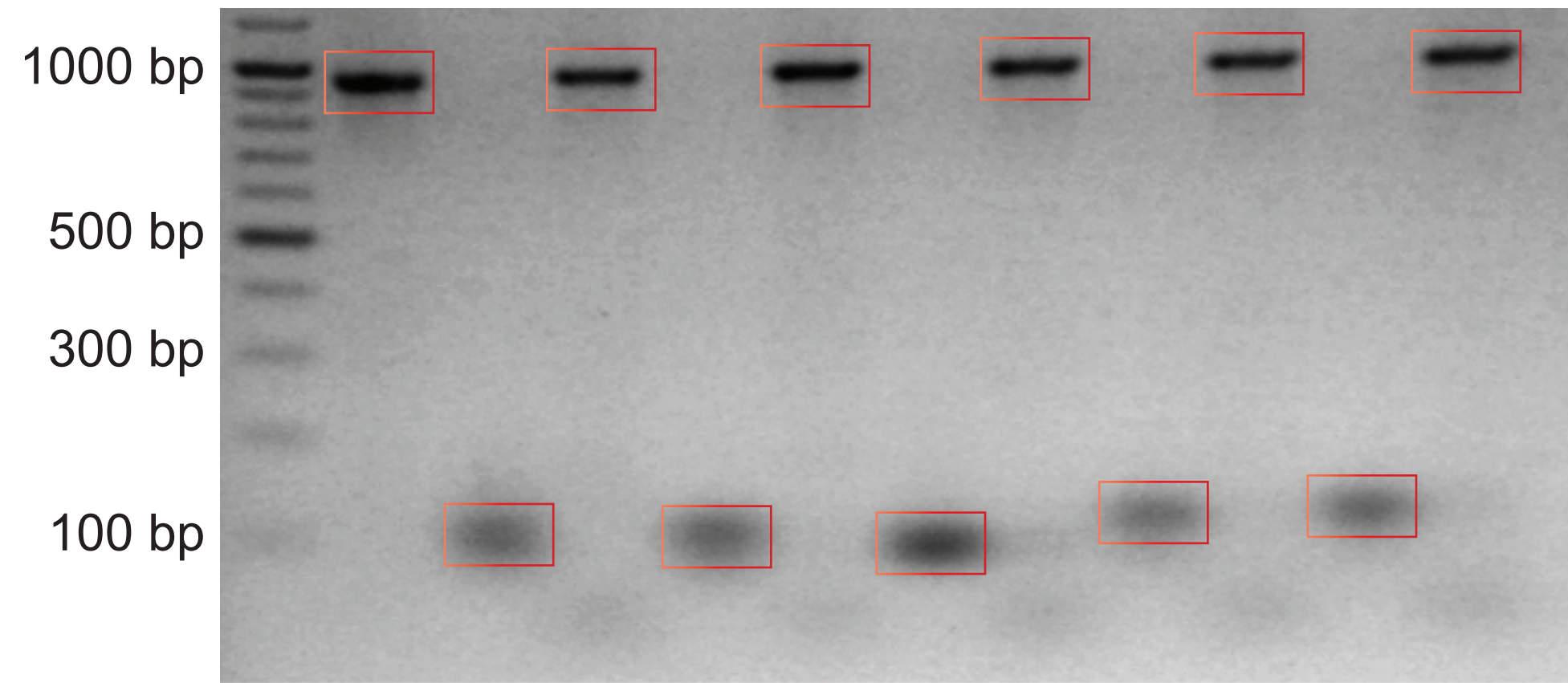
Figure 2

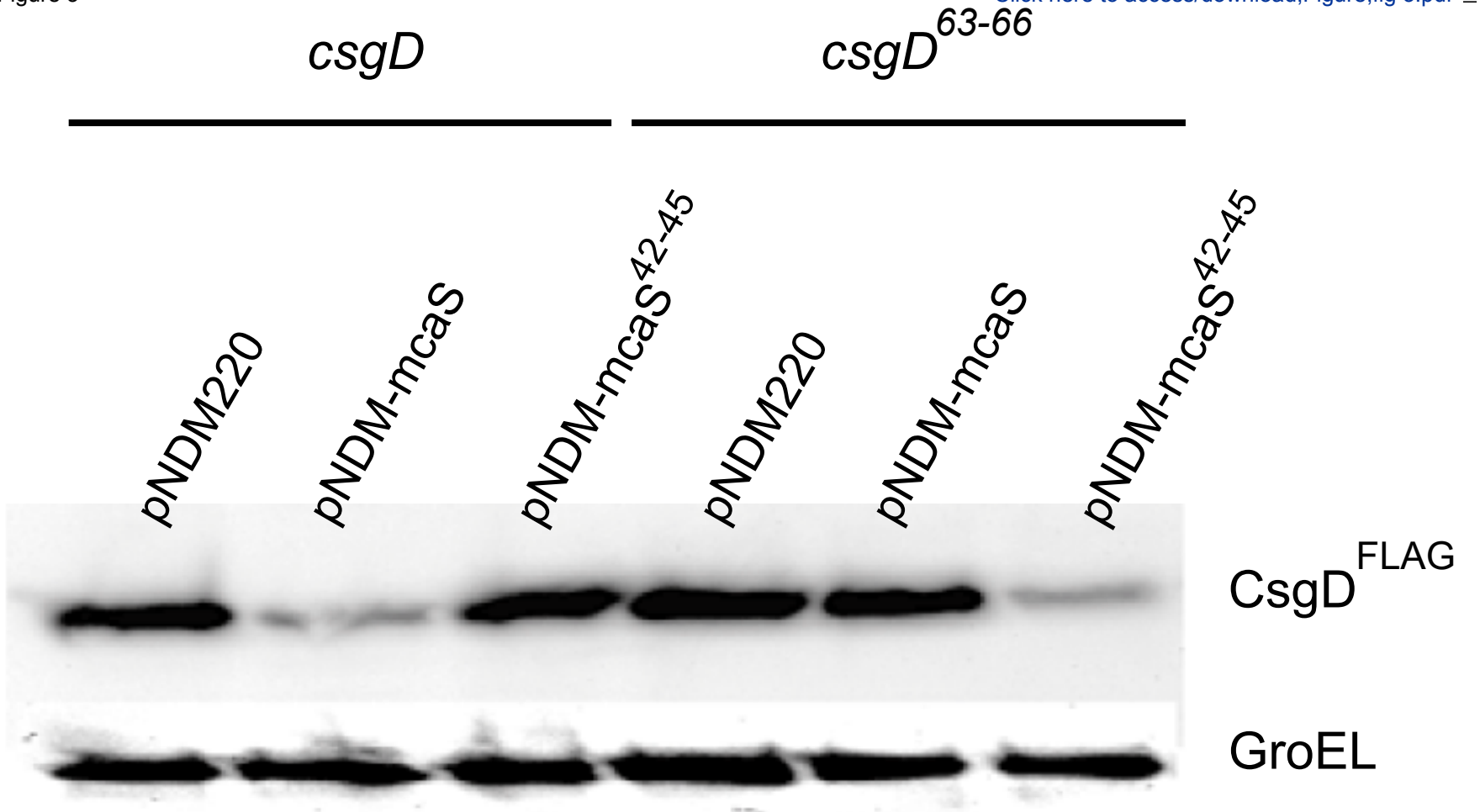
**A**

IA IIA IIIA IVA IB IIB IIIB IVB

**B**

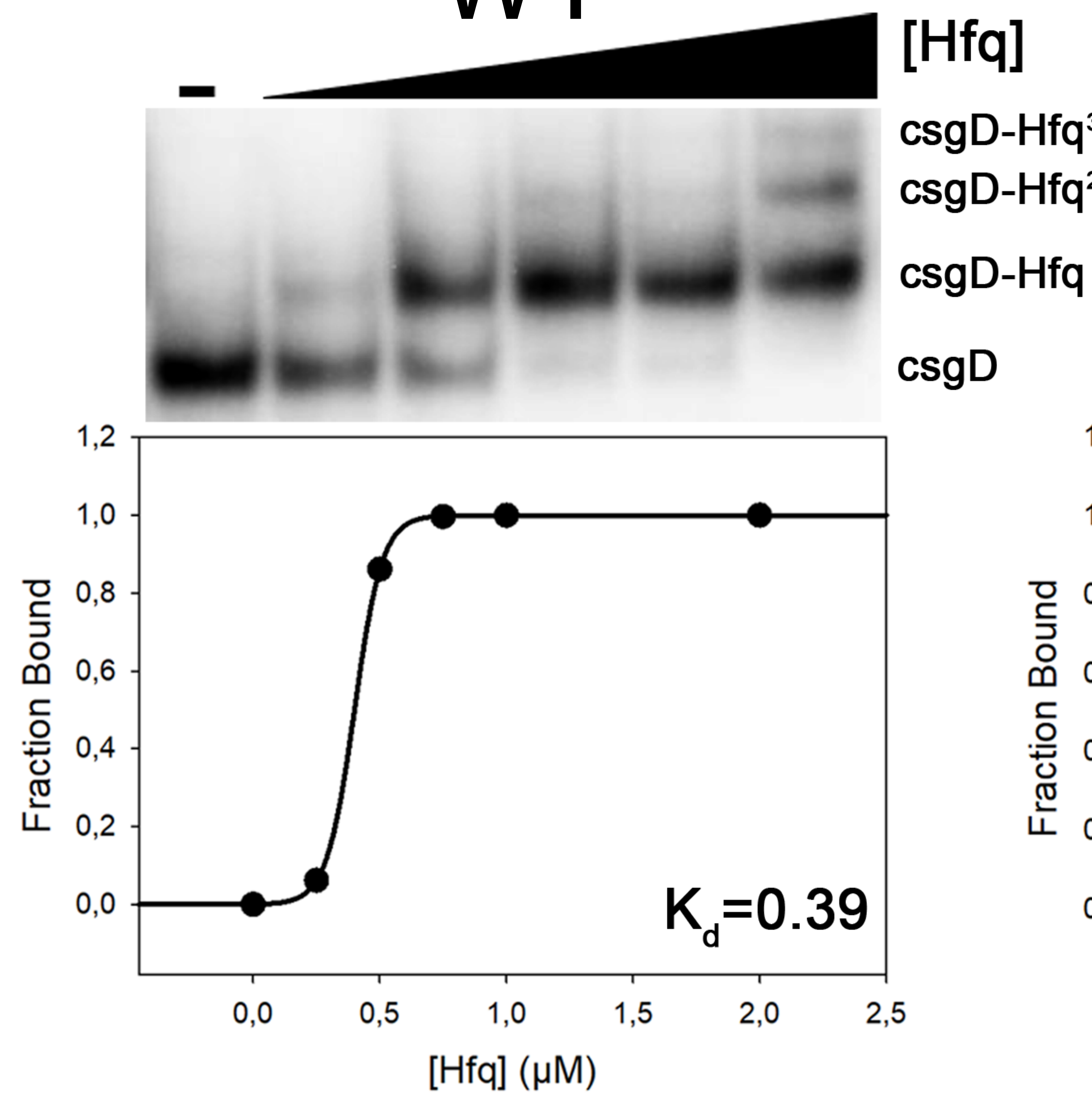
IA IIC IIIC IID IIID IIE IIIE IIF IIIF IIG IIIG



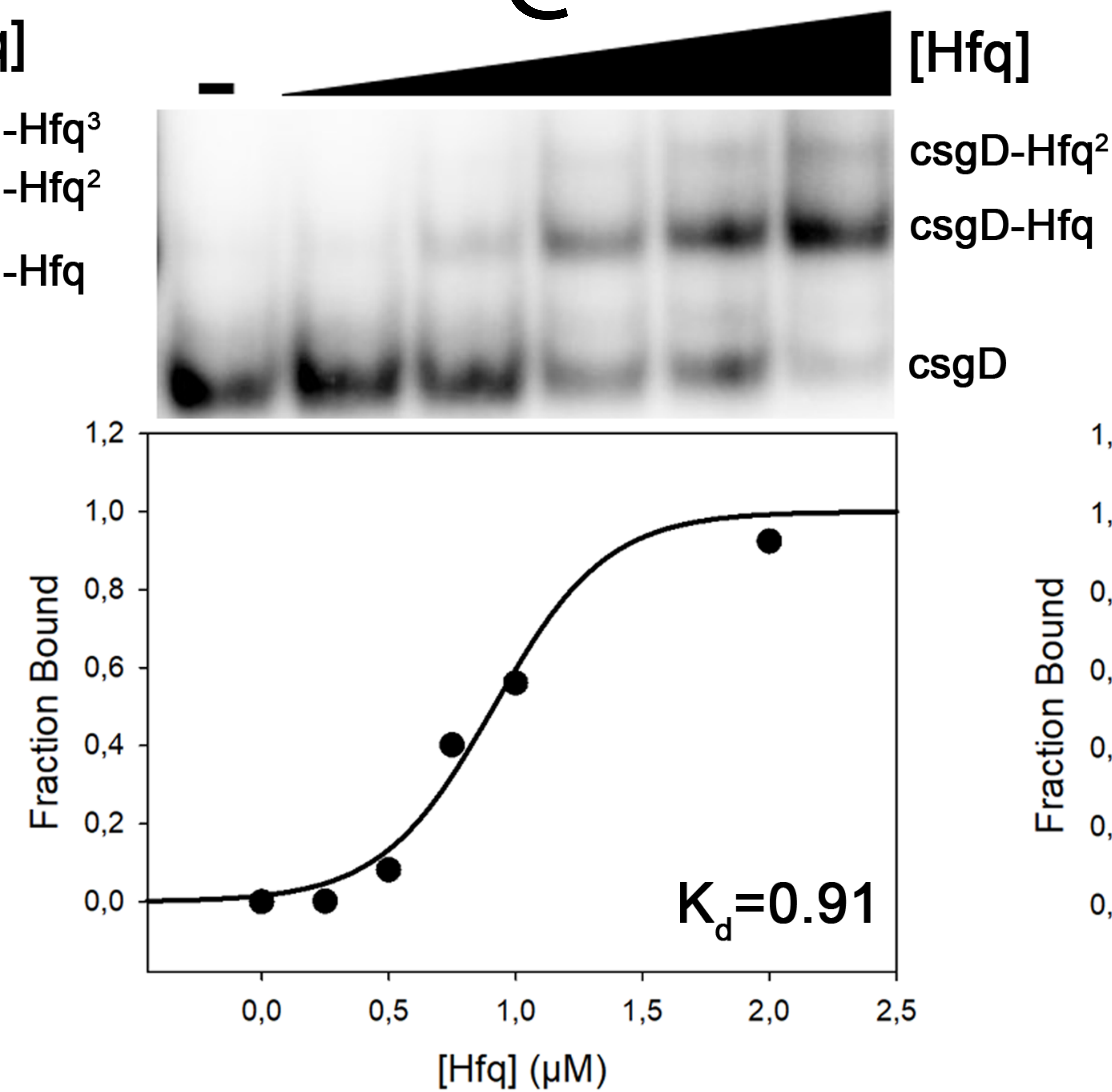




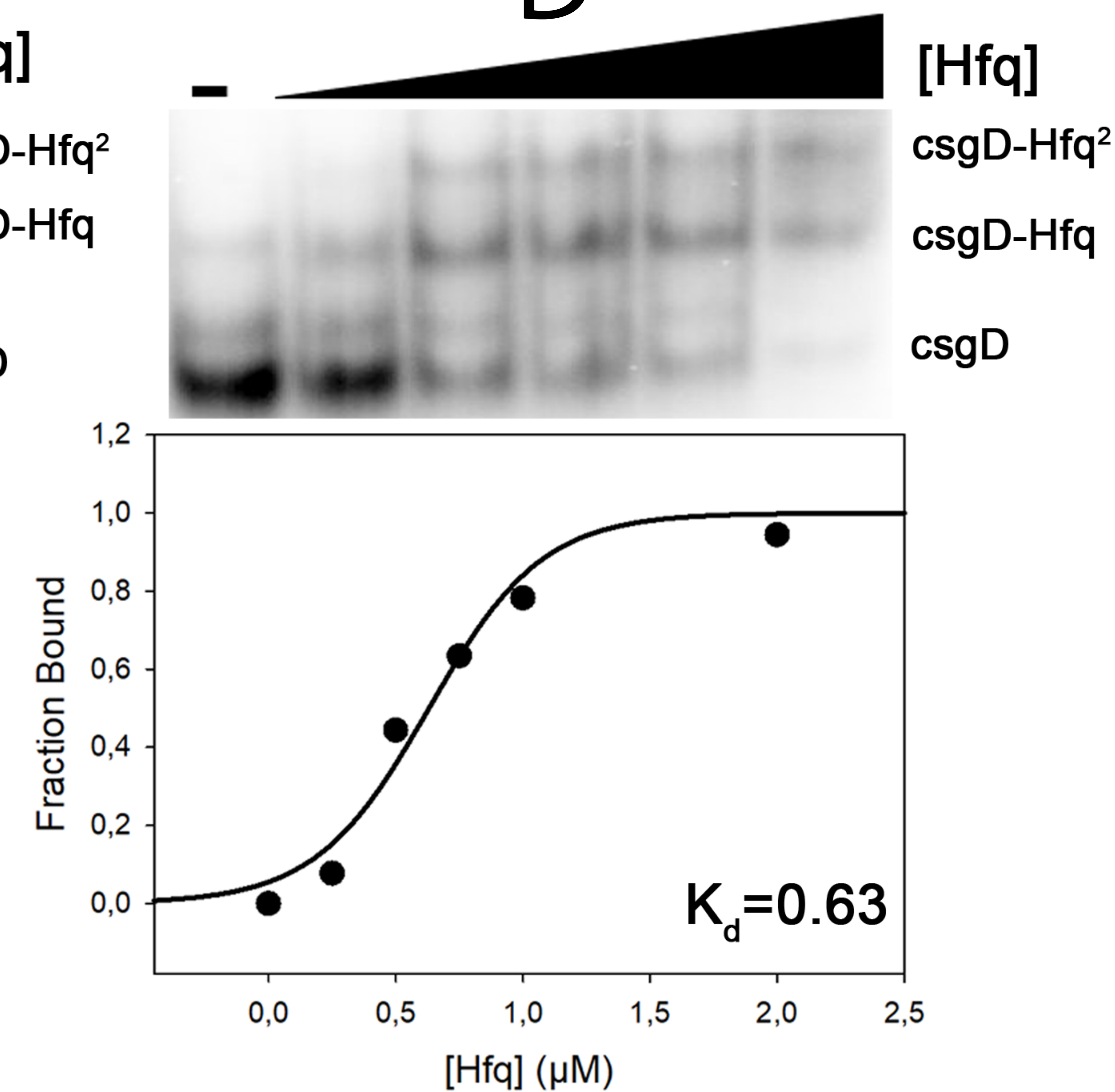
WT



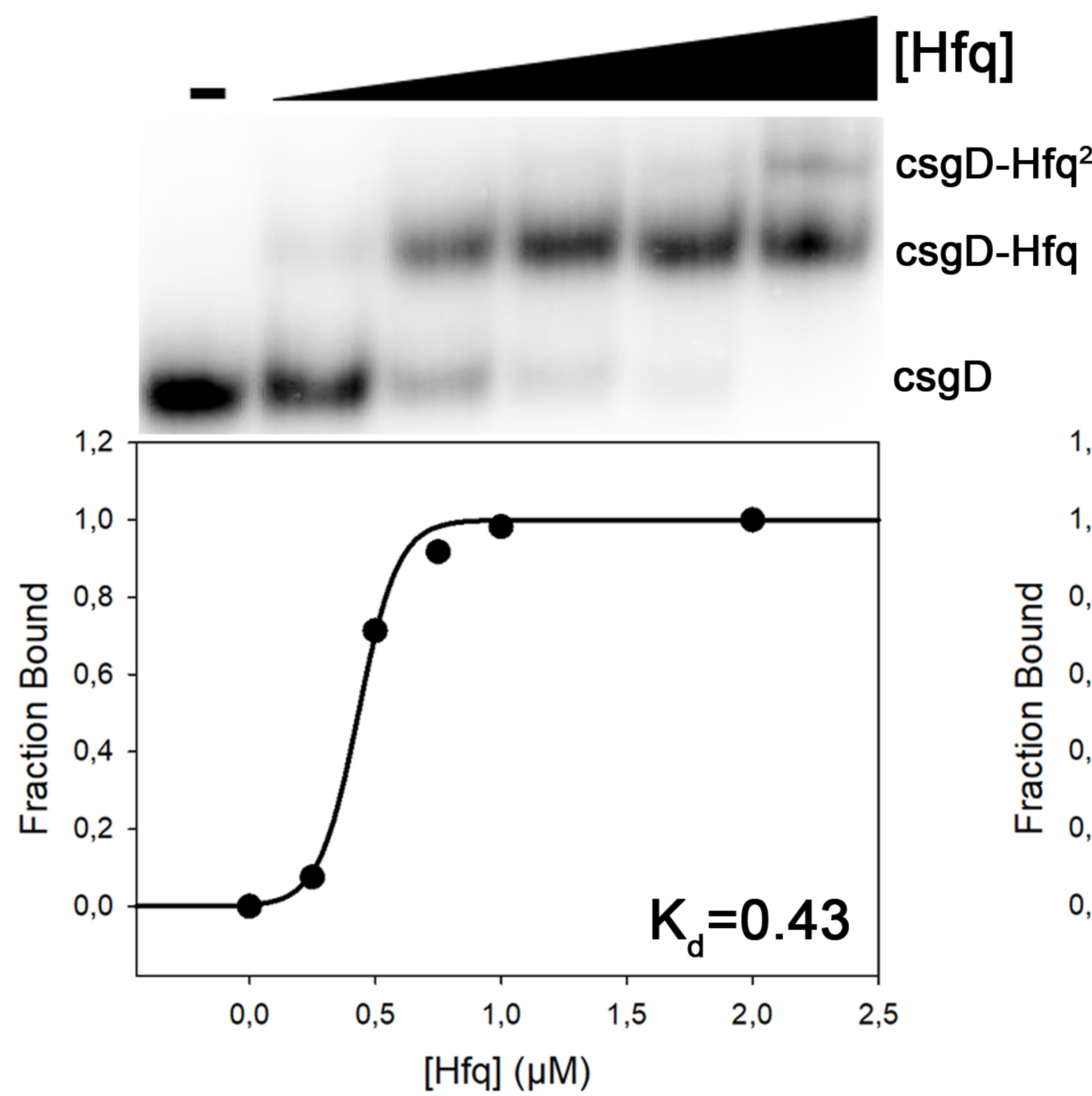
C



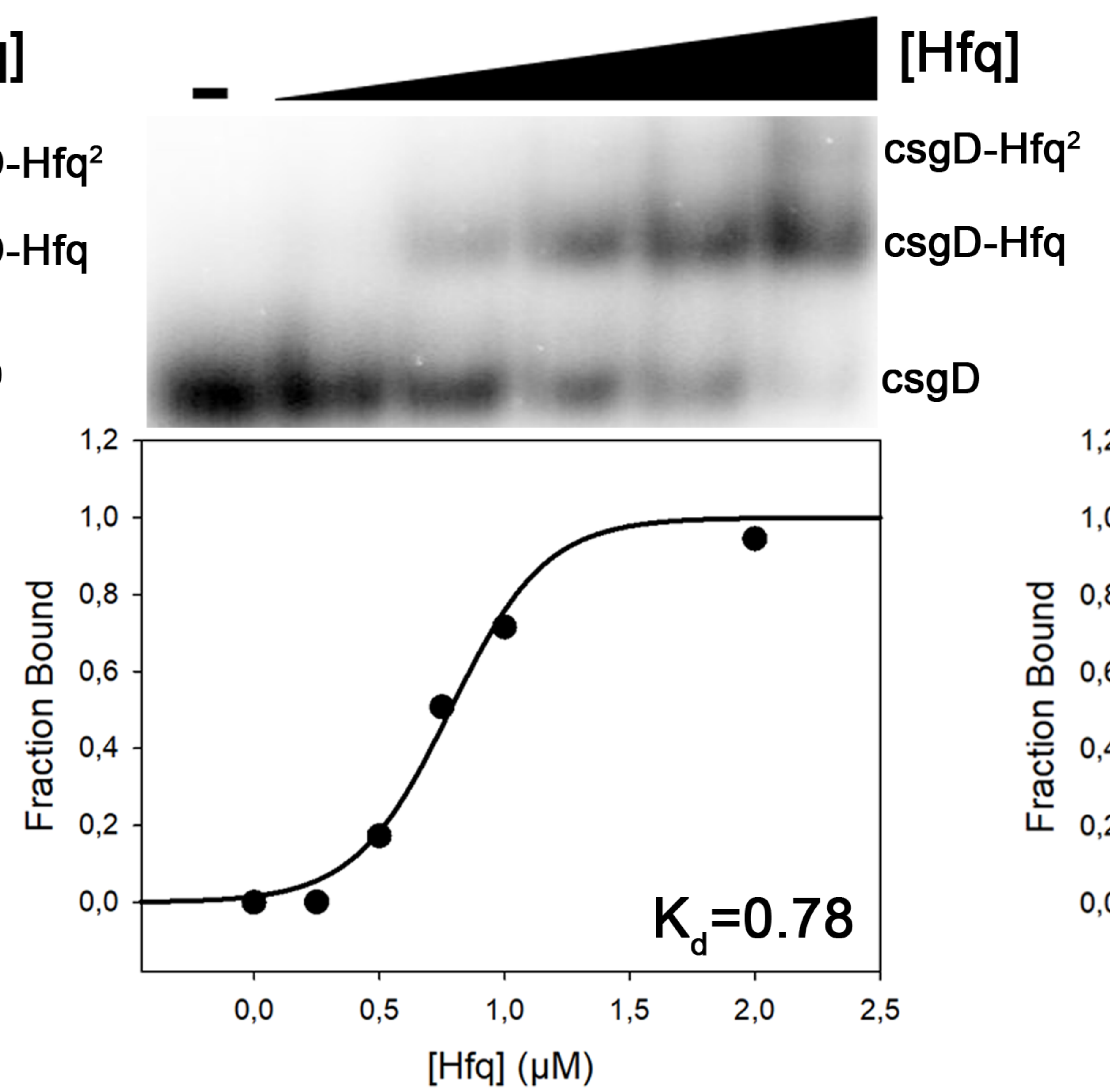
D



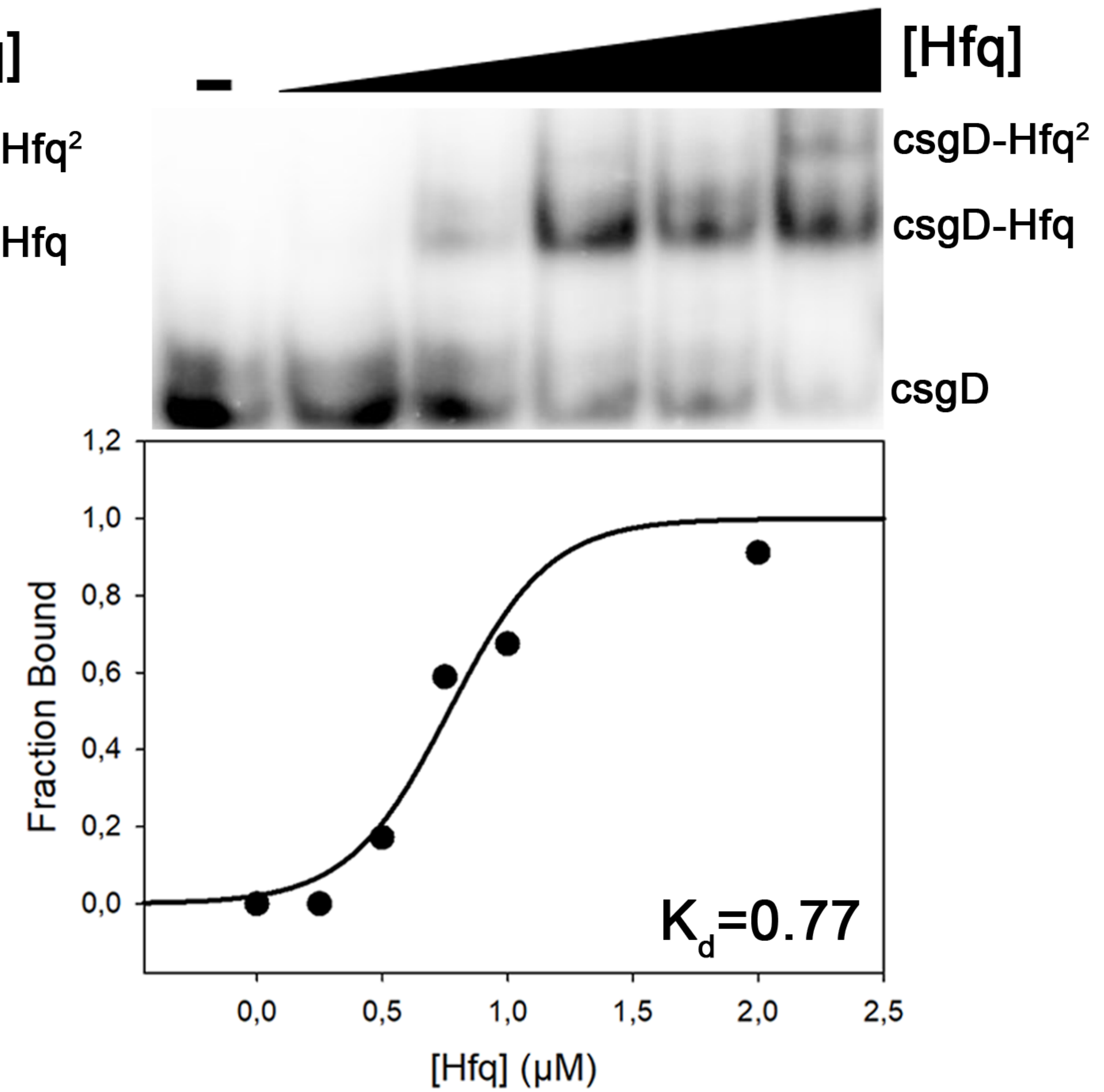
E



F



G





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	

Primer name
2-1A or 3-1A
2-3A or 3-4A
3-2A
3-3A
3-1B
3-4B
3-2B
3-3B
2-2C
2-2D
2-2E
2-2F
2-2G
5A
5C
5D



5E

5F

5G

6

---

## Sequence

---

GCGC**GGATCC**TACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCA  
TCAGATGTAATCCATTAGT

CCGC**CTGCAG**AAAAAAACCCCGCAGCAGCGGGGTTTTCTACCAGACGAG  
AAC

CAGAAGTACTGACAGATGTTGGTGAGCTGTGTGTAGTAATAAATC

GATTTATTACTACACACAGCTCACCAACATCTGTCAGTACTTCTG

CGCC**TGACGT**CGGC AAAAAGAGTGTTGACTTGTGAGCGGATAACAATGAT  
ACTTAGATTCACCGGCGCAGAGGAGACAATGCC

AATT**GGATCC**AAAAAAATAGAGTCTGTGCGACATC

CTCTACAGTACACACAGCTCACCATCCGCGTCTTAAATC

GATTTAAGACGCGGATGGTGAGCTGTGTGTACTGTAGAG

CAGCCCTAAATGGGTCTAATGGATTACATCTG

CAGCCCTAAATGGGTAAACCCCAACTAATGGATTACATCTG

CTGTGTGTAGTAATAAATCAGTAAAATATAAACTAATGGATTACATCTG

GTAATAAATCAGCCCTACTACTAGAATAATGGATTACATCTG

CTGCTGTGTGTAGTAATCCATCAGCCCTATGACTAAACTAATGGATTAC  
ATCTG

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGTTTTATATTTTACCC

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGACCCATTTAGG

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGTTTGGGGTTTAC

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGTTTTATATTTTACTGATTTATTAC

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGTTCTAGTAGTAG

GAAATTAATACGACTCACTATAGGCAGATGTA  
ATCCATTAGTTTTAGTCATAGG

GTATGACCATGAATACTATGG

---

**Used for - mutations**

---

2- and 3-round PCR on *csgD*

2- and 3-round PCR on *csgD*

3-round PCR on *csgD* – substitutes 4 nt

3-round PCR on *csgD* – substitutes 4 nt

3-round PCR on McaS

3-round PCR on McaS

3-round PCR on McaS – substitutes 4 nt

3-round PCR on McaS – substitutes 4 nt

2-round PCR on *csgD* – deletes 11 nt

2-round PCR on *csgD* – substitutes 4 nt

2-round PCR on *csgD* – deletes 11 nt

2-round PCR on *csgD* – deletes 9 nt and substitutes 7 nt

2-round PCR on *csgD* – deletes 9 nt and substitutes 7 nt

T7 PCR

T7 PCR

T7 PCR

T7 PCR

T7 PCR

T7 PCR

T7 PCR

Reagent	Control reaction	20 fmol reaction	100 fmol
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 <sub>2</sub> O	To 19 µL	To 19 µL	To 19 µL
Ligase	1 µL	1 µL	1 µL

<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
Anti-GroEL antibody produced in rabbit	Merck	G6532
Azure c200	Azure	NA
Custom DNA oligo	Merck	VC00021
DeNovix DS-11	DeNovix	NA
DNA Gel Loading Dye (6X)	Thermo Scientific	R0611
Ethidium bromide solution 1 %	Carl Roth	2218.1
GeneJET Gel Extraction Kit	Thermo Scientific	K0691
GeneRuler DNA Ladder Mix	Fermentas	SM0333
Gerard GeBAflex-tube Midi	Gerard Biotech	TO12
MEGAscript T7 Transcription Kit	Invitrogen	AM1334
Mini-Sub Cell GT Cell	Bio-Rad	1704406
Monoclonal ANTI-FLAG M2 antibody produced in mouse	Merck	F3165
Mouse Immunoglobulins	Dako Cytomation	P0447
NucleoSpin miRNA	Macherey Nagel	740971
NuPAGE 4-12% Bis-Tris Protein Gels	Thermo Scientific	NP0323BOX
Phusion High-Fidelity PCR Master Mix with HF Buffer	New England Biolabs	M0531S
PowerPac HC High-Current Power Supply	Bio-Rad	1645052
Rabbit Immunoglobulins	Dako Cytomation	P0448
SeaKem LE Agarose	Lonza	50004
SigmaPlot	Systat Software Inc	NA
T100 Thermal Cycler	Bio-Rad	1861096
T4 DNA ligase	New England Biolabs	M0202
T4 Polynucleotide Kinase	New England Biolabs	M0201S
TAE Buffer (Tris-acetate-EDTA) (50X)	Thermo Scientific	B49

### **Comments/Description**

Primary antibody

Gel imaging workstation

Spectrophotometer for nucleic acid measurements

Dialysis tubes for electro elution

Horizontal electrophoresis system

Primary antibody

HRP conjugated secondary antibody

RNA purification

Bis-Tris gels for protein separation

DNA polymerase

HRP conjugated secondary antibody

Graph and data analysis software tool

PCR machine

Ligase





1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli

Author(s):

Patrick R. Andreassen, Jens S. Pettersen, Mikkel G. Jørgensen

Item 1 (check one box): The Author elects to have the Materials be made available (as described at

<http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):



The Author is NOT a United States government employee.



The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.



The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to Sections 4 and 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in Item 1 above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.



## ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have



## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's


expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name:	Mikkel Girke Jørgensen	
Department:	Biochemistry and molecular biology	
Institution:	University of Southern Denmark	
Article Title:	Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli	
Signature:		Date: 27/8-18

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

## Response to editor and referees

Your manuscript, JoVE58996 "Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

*Response: We are happy to submit a revised manuscript and we are grateful for the referee comments. We have addressed all the concerns raised by the reviewers and rewritten parts of the manuscript to improve clarity. Below is a detailed response to each editorial and referee comment.*

### Editorial comments:

Changes to be made by the author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Response: The manuscript has been thoroughly proofread and large parts of the manuscript was rewritten.**

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

**Response: Links to publisher's editorial policy is provided in a separate file.**

3. Figure 2: Please explain different lanes in the figure legend.

**Response: Added for fig. 2A+B**

4. Figure 4: Please describe different panels in the figure legend.

**Response: Added to the figure legend**

5. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

**Response: Trademark (™) and registered (®) symbols are now removed from the table.**

6. Please expand the Long Abstract (150-300 words). It should include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate.

**Response: The long abstract has been modified**

7. Please define all abbreviations before use.

**Response: All abbreviations are now defined before use.**

8. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

**Response: Alternative method has been described with reference to the literature. The advantage of this method has also been described. A statement regarding the applications of the method has been added.**

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Generuler™, Fermentas, etc.

**Response: Commercial names are now removed from the manuscript.**

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

**Response: Use of language not written in imperative tense is removed and changed in section 2.2.1 and 2.3.1. In addition, we have included a comment on safety precautions when working with radioactivity.**

11. 1, 2, and sub-steps: Please note that these vague design steps cannot be adequately filmed. Please provide specific actions being performed, otherwise please consider un-highlighting them.

12. 3.1, 4.1.1, 4.1.5, 4.2.1, 4.2.5, etc.: Please specify PCR conditions throughout.

**Response: Added, see table 1.**

13. 3.2: Please describe how to perform agarose gel electrophoresis.

**Response: Added, see section 3.2.1**

14. 3.3: Please describe how to purify the PCR product and measure DNA concentration.

**Response: Added, see section 3.3**

15. Much of the protocol is very abstract. We cannot film a generalized protocol; we need more specifics (such as strains and specific conditions used in step 6) in order to film.

**Response: Specifics has been added.**

16. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

**Response: Highlighting has been updating.**

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Response: Highlighting has been updating.**

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Response: Highlighting has been updating.**

19. References: Please do not abbreviate journal titles.

**Response: Journal titles have been extended**

## Reviewers' comments:

### Reviewer #1:

#### Manuscript Summary:

The authors described the general molecular technique, site-directed mutagenesis based on PCR tool which approaches for protein-RNA and RNA-RNA interaction studies. This manuscript is well written and arranged in suitable organization; however, some scientific concerns were raised and more suitable references must be added.

**Response:** We thank the reviewer for their comments on the manuscript and tried to address all the points raised by the reviewer.

#### Overall Concerns:

##### Methods

Section 1.1 A good vector should be concerned for an appropriated regulation based on promoter activity (high toxic protein requires tightly regulation or gaining high yield required strong promoter).

**Response:** Upon revision of the manuscript, we have removed the part regarding choice of vector as it extends beyond the scope of this method. The method can be applied to any vector, which is now stated instead.

Section 2.2 (Both .1 and .2) Most of the criteria were varies depending on the polymerase enzyme efficiency and template characteristics.

**Response:** We thank the reviewer for this observation. As general oligo design is not a part of this protocol we have removed the section. However, we have kept the details of primer design introducing specific point mutations.

Section 3.3/4.1.3/4.1.7/4.2.3/4.2.7 please briefly describe or give references how to purify the PCR product and measure the concentration

**Response:** Description on PCR purification has been added, see section 3.3/4.1.3/4.1.7/4.2.3/4.2.7

Section 3.4/4.1.4/4.1.8/4.2.4/4.2.8/5.5 Store at 4C or -20C depending on the elution of PCR products and a time kept. If 4C, it should be kept in DW but if -20C, it should be kept in TE buffer or cryoprotecting agent.

**Response:** Solvents added, see section 3.4/4.1.4/4.1.8/4.2.4/4.2.8/5.5

Section 5.3 Treat RE-digested PCR product with AP enzyme can be allowed only using blunt-end generated RE, not common in sticky-end generated RE.

**Response:** It is correct that phosphatase treatment of a RE-digested product PCR product is not necessary for sticky ends. However, AP treatment catalyzes the release of 5' and 3' phosphate groups on sticky DAN ends as well. We routinely dephosphorylate our cloning vector DNA to eliminate recircularization during ligation.

Section 5.4 Agarose gel usually cause a loss of DNA during experiment, using directly PCR purification column will give a better yield of DNA product (but only the contaminated DNAs are less than 100 bp).

**Response:** While this is correct, our lab has experience with fewer colonies on the negative control plate when separating the digested vector on agarose gels as compared to purifying it directly.

Section 5.6 Ligation protocol could be described otherwise references must be stated.

**Reponse:** Ligation protocol added.

Section 5.7 Transformation protocol could be described otherwise references must be stated. Heat shock transformation is normally used for E. coli transformation.

Reponse: Heat shock protocol for transformation added

Section 5.8 Methods for checking the correct insertion must be listed i.e. PCR using primers one in vector and another one in an inserted DNA. Transforming an expression vector or an integration into chromosome must be concerned and using different techniques, please give a caution in this section.

Reponse: Method for checking the correction insertion is now mentioned in this section (5.8). Caution statement has been added. We use vector specific primers to check correct insertion and sequencing the entire fragments.

Section 6.1.2 Preparation of SDS-PAGE or native PAGE (which one is suitable?) should be mentioned in more details or give references in term of cell preparation (boiling or not) and gel electrophoresis set up.

Reponse: SDS-PAGE preparation is now elaborated.

Section 6.1.4-6.1.10 Western blot technique is applied in these sections, it would be great if the author give in details otherwise references must be states.

Reponse: More details on the western blot technique are now added throughout this section.

Section 6.2.1 In vitro transcription, it would be great if the author give in details otherwise references must be states.

Reponse: More details on *in vitro* transcription are now added to this section.

Section 6.2.3-6.2.6 Radioactive labelling used in this step, the caution and information of the radioactive using regulation must be provided.

Reponse: Type of radioactive labelling is now elaborated. Caution on radioactive lab-work has been added. We cannot directly specify how to work with radioactivity as safety precautions vary between regions and countries. We have added a statement to follow the guidelines issued by the local radiation protection officer.

## Results

The results presented were clear and well described; however, description of band intensity analysis to construct the kinetic curve must be well written in the method with statistical analysis applied.

Reponse: A section shortly describing the method for analyzing the band intensities and determining dissociation constant values are now added.

Moreover, some statements below should be clarified:

1. In Figure 2 legend, please state a final concentration of ethidium bromide and DNA ladder instead of volume.

Reponse: Corrected

2. In Figure 3 legend, fold of dilution using a full-stop symbol for a thousand unit may confuse some readers, please use comma sign instead, otherwise using text writing description.

Reponse: Corrected

3. In Figure 4, number showing the Hfq concentration with concentration unit must be presented in the figure.

Reponse: Concentrations (with concentration unit) are shown in the graph below the gel images and stated in the figure legend.



## Discussion

The discussion presented were well written and organized. More details on pros/cons or comparison between this technique and other methods, and some successful applications in term of RNA interactions could help to improve this section.

Reponse: Pros/cons has been added to the discussion regarding alternative methods for site-directed mutagenesis. Many successful applications of RNA interactions are found in the literature. A statement about STM being the golden standard for RNA interactions studies has been added to the beginning of the discussion.

## Reviewer #2:

### Manuscript Summary:

Andreassen et al. described a two and three step PCR technique to incorporate specific mutations into DNA and evaluate how target mRNA's and small non-coding RNA's interact throughout the PCR process. The authors also assessed how site-directed mutagenesis can be utilized to understand where the protein binding sites are in RNA. Although each technique was described in some detail, both techniques and the protocol need additional information in order for the work to be reproducible. This manuscript is valuable for others conducting PCR studies however, major revisions are needed to fully understand how this study was done and to be able to reproduce the results.

Response: We thank the reviewer for the comments on the manuscript and we have tried to address all the points raised by the reviewer.

### Major Concerns:

The authors seem to be hoping to appeal to a very broad audience but the protocol they are sharing is far too vague in its current form to be helpful to someone trying to follow it.

Response: We have rewritten large parts of the manuscript and specified many protocol points.

- The authors present model data and specific primer sequences to perform these reactions without describing the parameters of their PCR reactions. For example, they do not mention duration, temperature, primer T<sub>m</sub>, copy number of initial template, etc. and state only that the parameters can change. They reference other papers that have more details protocol but this tendency this would be unhelpful to anyone trying to use this paper as it is to perform the reactions they describe.

Response: Details has been added.

As it is, the protocol is very often too vague by using phrases like "perform PCR", "wash membrane", "visualize with a method", "identify transformants", "ligate products" without describing any details on how to perform these steps. The authors do sometimes reference other papers that describe these details steps but these steps should be included in this text because in its current form it would require this paper and at least three or four papers that are referenced to successfully get through one of the reactions they describe.

Response: Details have been added to most steps throughout the manuscripts.

They state that the 2 step PCR strategy is applicable if the mutation is <200bp from the end of the DNA of interest. However, in the 2 step PCR strategy in the protocol (line 51) - The authors state ("2 step is only for mutation >200bp from either end of DNA of interest"). Which one is correct? (Line 103).

Response: We thank the reviewer for noticing this error. <200 is correct, and the typo has been corrected in the manuscript.



-How do the authors plan to confirm that no errors were incorporated into the PCR products during the process? (Lines 60-66)

Response: Section 5.9 states that the sequence is validated by sanger sequencing

- Although the authors say they have chosen an appropriate vector, they don't specify or give guidelines on how they or anyone would go about choosing a vector. Do these two PCR strategies not work for certain plasmids, what are the caveats for choosing a vector?(line 87).

Response: It has been clarified in the text that the approach is not dependent on a specific vector.

There are other parts of the protocol that seem to be lacking details such as in Line 153 where the authors state "store purified DNA at -20°C or -4°C". Is one for long term storage compared to short term storage? What is the length of time the DNA can be stored at either temperature or does the DNA degrade and become unusable after a certain amount of time? More details are needed throughout the entirety of the protocol.

Response: It is now mentioned that -20°C is for long term storage and 4°C is for short term storage in section 3.4.

-Again we are missing information about what type of label can be used and how is the DNA actually being purified? Are they using a specific kit or spin columns etc. (Line 270)

Response: The labeling method is now elaborated in the section (6.2.3)

The description of Figure 1 is explained well however, the layout of the actual diagram could be improved. For example, having panels that separate out each set of primers individually then adding step 1 and step 2 after those panels may provide clarity and be less confusing to readers (line 340, Figure 1).

Response: We have changed the figure to improve clarity and reduce confusion.

Figure 1B: This figure is also a bit confusing and the layout/presentation of the information could be improved.

Response: We have changed the figure to improve clarity and reduce confusion.

Figure 3: Should there be a control when running out the western blot? Answer..yes.. sloppy work not to automatically have one.

Response: We respectfully invite the reviewer to look at the figure again. The experiment examines a mutant *mcaS* allele in which we had disrupted the putative *csgD* binding site in the sRNA by changing four consecutive loop residues. In addition, we introduced compensatory mutations into the *csgD* 5'-UTR to restore interaction with the McaS42-45 mutant sRNA. As shown, regulation of CsgD<sup>63-66FLAG</sup> synthesis was lost with wild-type McaS but was restored with the McaS42-45 mutant. Collectively, the results indicate that McaS mediated downregulation of CsgD synthesis requires an antisense interaction between sRNA and the target RNA. Included controls are the empty vector and induction of the individual mutants as well as probing GroEL as loading control. What control does the reviewer feel is missing?

-The authors state "it might be necessary to do more optimization of the PCR conditions.." what conditions did they use for each step of the experiment? Did they have to run optimizations; the specifics of each step of the PCR should be included (line 402).

Response: The specific PCR program used throughout the protocol is now added in section 3.1. Suggestions for optimizations has been added.

How are some methods more costly than others? They seem to be very generalized/broad in terms of cost. Is there more planning or reagents required in other methods vs. PCR that was shown here? Is there a way to check that the correct targets were achieved with the 2 or 3 step PCR method without error?(Line 413-

420).

Response: Cost has been added to the discussion regarding *de novo* synthesis being costlier than 2- or 3-step PCR. Sanger sequencing is mentioned in the protocol, which checks that the target was amplified without error.

In Figure 2: It might be helpful to highlight the correct sized bands in each of the gels so the reader can identify those immediately.

Response: Corrected

Lastly, is there a way for the formatting to be changed to allow the primer names to match up with their sequences? Again, this lack of detail reflects a sloppy presentation.

Response: We use a monospaced font for DNA sequences. This is, in general, used throughout the scientific community and across journals. When we write primer names, we consistently use the same format as the rest of the manuscript.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. **Manuscript has been proofread**
2. Please provide more references in Introduction to support your manuscript. **3 references have been added to the introduction.**
3. Please use standard SI unit symbols and prefixes such as  $\mu\text{L}$ , mL, L, g, m, etc., and h, min, s for time units. **ml has been corrected to mL.**
4. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **personal pronouns have been removed**
5. Please define all abbreviations before use. **Definitions added**
6. Line 121-126, 138-143: Please ensure that all text in protocol steps are written in complete sentences and in imperative tense. **Bullet points have been removed.**
7. The highlighted protocol steps are over 2.75 page limit. Please highlight fewer steps for filming. **Highlighting has been reduced to 1.75 page.**
8. Step 3.1: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Reference has been added to the step**
9. 4.1.1: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Reference has been added to the step**
10. 4.1.2: Please add more details to your protocol steps. How to validate PCR by agarose gel electrophoresis? **Reference to previous step has been added**
11. 4.1.5: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Reference has been added to the step**
12. 4.1.6: Please add more details to your protocol steps. How to validate PCR by agarose gel electrophoresis? **Reference to previous step has been added**
13. 4.2.1: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Reference has been added to the step**
14. 4.2.2: Please add more details to your protocol steps. How to validate PCR by agarose gel electrophoresis? **Reference to previous step has been added**
15. 4.2.5: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Reference has been added to the step**
16. 5.1: Please add more details to your protocol steps. How to digest PCR products? Please provide detailed procedures. **Details have been added**
17. 5.2: How to purify digested PCR products? **Details have been added**
18. 5.3: How to digest purified vector? **Details have been added**
19. 5.4: How to separate digested vector from waster DNA? **Reference to previous step has been added**
20. Eppendorf is commercial language. Please remove it from the manuscript. **Eppendorf has been removed**
21. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. **Symbols have been removed**

## Copyright policies regarding republication

### Nucleic acid research

“As part of the licence agreement, authors may use their own material in other publications provided that the Journal is acknowledged as the original place of publication and Oxford University Press as the Publisher. Information about the **New Creative Commons licence** can be found [here](#).”

<https://academic.oup.com/nar/pages/Policies#Copyright>

### Molecular microbiology

“In the copyright transfer agreement signed by an author, ASM grants to that author (and coauthors) the right to republish discrete portions of his/her (their) article in any other publication (print, CD-ROM, and other electronic forms) of which he/she is (they are) the author(s) or editor(s), on the condition that appropriate credit is given to the original ASM publication.”

<https://mmb.asm.org/content/editorial-policy>