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Dear dr. Vineeta Bajaj,

Thank you very much for handling of our manuscript and for the very positive and constructive referee comments. We have addressed all issues raised by the reviewers and thoroughly checked manuscript and introduce changes according to editorial comments. We hope that you will find our revised manuscript suitable for publication.

With best regards

Krzysztof J. Skowronek

TITLE:

In Vitro Directed Evolution of a Restriction Endonuclease with More Stringent Specificity

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KEYWORDS:

directed evolution, in vitro compartmentalization, restriction endonuclease, sequence specificity, split-and-mix oligonucleotide synthesis, in vitro selection

SUMMARY:

Restriction endonucleases with new sequence specificity can be developed from enzymes recognizing a partially degenerate sequence. Here we provide a detailed protocol that we successfully used to alter the sequence specificity of NlaIV enzyme. Key ingredients of the protocol are the in vitro compartmentalization of the transcription/translation reaction and selection of variants with new sequence specificities.

ABSTRACT:

Restriction endonuclease (REase) specificity engineering is extremely difficult. Here we describe a multistep protocol that helps to produce REase variants that have more stringent specificity than the parental enzyme. The protocol requires the creation of a library of expression selection cassettes (ESCs) for variants of the REase, ideally with variability in positions likely to affect DNA binding. The ESC is flanked on one side by a sequence for the restriction site activity desired and a biotin tag and on the other side by a restriction site for the undesired activity and a primer annealing site. The ESCs are transcribed and translated in a water-in-oil emulsion, in conditions that make the presence of more than one DNA molecule per droplet unlikely. Therefore, the DNA in each cassette molecule is subjected only to the activity of the translated, encoded enzyme. REase variants of the desired specificity remove the biotin tag but not the primer annealing site. After breaking the emulsion, the DNA molecules are subjected to a biotin pulldown, and only those in the supernatant are retained. This step assures that only ESCs for variants that have not lost the desired activity are retained. These DNA molecules are then subjected to a first PCR reaction. Cleavage in the undesired sequence cuts off the primer binding site for one of the primers. Therefore, PCR amplifies only ESCs from droplets without the undesired activity. A second PCR reaction is then carried out to reintroduce the restriction site for the desired specificity and the biotin tag, so that the selection step can be reiterated. Selected open reading frames can be overexpressed in bacterial cells that also express the cognate methyltransferase of the parental REase, because the newly evolved REase targets only a subset of the

methyltransferase target sites.

INTRODUCTION:

Sequence specificity engineering is extremely challenging for class II REases. In this class of endonucleases, sequence recognition and catalysis are closely intertwined, most probably as an evolutionary safeguard against creation of an endonuclease of broader specificity than its cognate methyltransferase, which would damage host DNA. Directed evolution of new specificities in cells is further complicated by the need to protect host DNA against the newly engineered endonuclease activity. Therefore, there are only a few successful attempts of REase engineering reported and all of them exploit the unique features of a particular enzyme[1-7].

Here we provide a detailed protocol for specificity engineering that can be used to generate endonuclease variants that have narrower specificity than a parental enzyme that is based on our successful engineering of a NlaIV endonuclease[8]. For any such enzyme with an arbitrary recognition sequence, extra specificity can be introduced for bases in the flanks. For parental enzymes that recognize partially degenerate sequences (such as NlaIV with its GGNNCC target), additional specificity can also be introduced within the recognition sequence. As extra specificity will likely require protein-DNA contacts, the newly recognized bases should lie within the footprint of the parental endonuclease on DNA. In principle, selection schemes can be set up for any desired specialization of the recognition sequence. However, most REases that recognize palindromic and nearly palindromic target sequences are functional dimers that recognize only a half-site of the palindrome. Hence, selection of new specificities that violate the symmetry of protein nucleic interactions is unlikely to work. For the dimeric NlaIV, for example, the GGNNCC sequence can theoretically be narrowed down to GGATCC but narrowing the specificity down to GGAACC is expected to be more difficult. Our scheme involves both positive and negative selection.

The process is more efficient when negative selection is also used to remove the specificities able to cleave all sequences other than the preferred narrower specificity. For example, selection for GGATCC could be combined with antiselection against GGBVCC (where B is any base other than A, and V is any base other than T). When some of the possible target sequences are not covered, the outcome of the selection experiment depends on the effectiveness of positive and negative selection. In our NlaIV work, we selected for GGATCC, and against GGSSCC (where S is G or C), and obtained a specificity that, ignoring symmetry breaking targets, could be described as GGWWCC (where W is A or T), suggesting that in this particular case, negative selection was more important than positive selection.

Our approach starts with the creation of an expression selection cassette (ESC). The ESC is structured in sections. On the inside core section, there are variants of the open reading frame (ORF) of the REase, under T7 promoter control. This core section of the ESC cannot contain any cognate site for the engineered REase. The core is sandwiched between two cognate sites for wild type REase: a cleavage site for the undesired activity (counter selected sequence, GG**SS**CC in this example) and a cleavage site for the desired activity (selected sequence, GG**AT**CC in the example). The final step of the preparation of the ESC in PCR adds biotin close to the desired

activity at the 5' end and creates a variety of counter selected sequences (GGSSCC in the example). The selection strategy relies on the use of carefully designed primers at the ESC reamplification protocol after an in vitro transcription/translation/selection protocol (**Figure 1A**). The ESC library is expressed in an in vitro compartmentalized transcription translation water-in-oil emulsion[9-11]. Within each droplet, the specificity of the expressed enzyme affects the state of the ESC (**Figure 1B**, step I). For the described arrangement, the desired cleavage activity of the translated protein removes the DNA's biotin tag but does not affect the other ESC end with the counter selected sequence. When the emulsion is broken, biotinylated fragments are removed by streptavidin affinity pulldown, so that only fragments from droplets with the desired activity remain (**Figure 1B**, step II). This step removes inactive REase variants. The supernatant fraction of the pull-down step is then amplified by PCR. In the first PCR reaction primers F2 and R1 are used (**Figure 1A**,**B**, step III). Primer F2 binds to the ESC section between the counter selected sequence and the molecule end. Therefore, ESCs expressing variants that are capable of cleaving the counter selected sequence (and, therefore, separate the binding sites for primers F2 and R1 into two different DNA molecules) are not amplified and are thus removed from the library. The primer R1 binds between the selected site and the core of the ESC so that it is not affected by the cleavage status of the selected site and restores the cleavage site for the desired activity (GGATCC). The cycle is closed by a second PCR (with primers F1 and R2) that adds biotin at the 5' end close to the selected site and restores designed variation at the counter selected site close to the opposite end of the ESC (**Figure 1B**, step IV). The resulting DNA mixture is ready for another round of selection.

The success of the selection protocol depends strongly on the proper choice of the new, more stringent target recognition sequence and on careful design of the mutagenesis strategy and its effective implementation. Because it is much easier to improve upon slight preexisting preferences of the REase than to overcome them, we recommend starting with a kinetic study of any preexisting preferences. The necessity of careful mutagenesis design results from the limited size of a mutant library that can be processed by the presented protocol (10^9 clones in a single experiment). Therefore all 20 possible amino acid substitutions can be effectively tested in only a few positions (see Discussion). Random mutagenesis, such as error-prone PCR (EP-PCR) presented as an alternative method, will lead to profound undersampling of existing complexity. If any information concerning potential amino acid positions involved in contacts with DNA (or even located in a close proximity to the degenerate nucleotides in a cognate sequence) is available, it certainly should be used to select a few amino acids for oligonucleotide guided saturation mutagenesis (protocol steps 1.6–3.10).

PROTOCOL:

1. Preparation of ESCs

1.1. Clone methyltransferase of the restriction-modification system to be engineered in a low copy number plasmid (e.g., pACYC184 or pACYC174 or their derivatives).

NOTE: The bacterial host strain must be able to tolerate methylation introduced by the cloned

enzyme and provide inducible expression of T7 RNA polymerase. Use of the ER2566 strain (carrying McrA, McrBC, and Mrr mutations) is recommended.

1.2. Confirm that the recombinant plasmid DNA is protected against cleavage by the cognate endonuclease by treating 0.5 µg of plasmid DNA with 10 units of cognate REase in buffer and temperature recommended by the enzyme supplier for 2 h.

1.3. Prepare competent cells of this strain.

NOTE: Any method can be used. The NlaIV engineering project used a simple calcium chloride method[12].

1.4. Construct recombinant plasmid with the ORF for the REase under control of the T7 promoter from a different exclusion group and with a different selection marker than the one containing the methyltrasferase gene in step 1.1. Vectors pET28 and pET30 can be used.

1.5. Remove all recognition sites for the engineered enzyme from the section of the recombinant plasmid between the T7 promoter and the stop codon of the enzyme ORF by introducing silent mutations (**Figure 2**, **Table 1A**).

NOTE: If more than one such site must be removed, multiple mutation rounds will be necessary (steps 1.5.1–1.5.7).

1.5.1. Use an inside-out PCR reaction that amplifies the full-length plasmid with designed variations introduced at the 5' ends of the primers (**Table 2A**).

1.5.2. Remove the template DNA, add 10 units of DpnI endonuclease to the 50 µL of the PCR reaction, and incubate for 2 h at 37 °C.

1.5.3. Resolve the products by agarose gel electrophoresis. Cut out the band corresponding to the full-length plasmid and purify it with a commercial kit.

1.5.4. Add 10x ligation buffer (to a 1x concentration) and supplement with ATP (to 1 mM). Add 10 units of T4 polynucleotide kinase and incubate for 20 min at 37 °C. Inactivate the enzyme by heating at 70 °C for 10 min.

1.5.5. Add PEG 4000 to 5%, supplement again with ATP (to 1 mM), and add 5 units of T4 DNA ligase. Incubate for 2 h at room temperature (RT).

1.5.6. Transform into a competent bacterial strain carrying cognate methyltransferase (step 1.1).

1.5.7. Isolate the plasmid DNA in small scale and confirm the introduction of sequence changes by dideoxy sequencing.

1.6. Introduce unique restriction sites in close to the sequence(s) targeted by oligonucleotide guided mutagenesis (**Figure 2**, **Table 1B**). Follow steps 1.5.1–1.5.7 for each site.

NOTE: This step is performed only when a targeted mutagenesis is used. If doing random mutagenesis, skip steps 2–3 and proceed to section 3 instead. In the presented example all sites were introduced upstream of the targeted regions, but they can be introduced downstream as well.

1.7. Design primers for the amplification of the ESC (**Table 1C**).

1.7.1. Design a reverse primer binding downstream of the endonuclease ORF that will introduce the selected recognition site (R1) and its shorter version (R2) that binds outside the selected NlaIV sequence and contains biotin at the 5' end (see **Figure 1**).

1.7.2. Design a forward primer (F1) binding to the ESC upstream of the T7 promoter. This primer should also introduce counterselected variant(s) of the original recognition sequence (i.e., the maximum of sequence variations recognized by the original enzyme with the exception of the selected reverse sequence).

NOTE: A shorter version of this primer (F2) that covers the sequence distal to the counterselected sequence will be used later in the selective PCR (step 5.9).

2. Split-and-mix synthesis of mutagenic primers

NOTE: This step is used only for projects that require subsaturation mutagenesis at more than one site. A synthesizer with multiple synthesis columns is required. Assign columns for synthesis of randomized NNS codon triplets and wild type codon triplets according to the mutagenesis frequencies. For example, if seven equal volume synthesis columns are available, and a mutagenesis rate of 0.3 is desirable at a given site, add randomized NNS codons in ~0.3 x 7 or two columns, and wild type codons in ~0.7 x 7 or five columns (**Figure 3**).

2.1. Decide about sites for subsaturation mutagenesis. Choose mutagenesis frequencies according to the hypothetical importance of the sites (i.e., the more important the site, the higher the frequency), keeping limits on the overall library complexity in mind (see Discussion).

2.2. Synthesize oligonucleotides in all columns, up to the triplet immediately preceding the second subsaturation mutagenesis site counting from the 3'-end. At this step, it is not necessary to remove the 5'-trityl protecting group (use the trityl-on option on the synthesizer). The protecting group will be removed at the beginning of the next synthesis cycle (step 1 in **Figure 3**).

2.3. Open the synthesis columns. Collect controlled pore glass (CPG) synthesis support into a dry 1.5 mL tube and mix by vortexing. Repartition the mixed CPG resin into new synthesis columns. Avoid introducing humidity, because it will decrease the overall yield (steps 2 and 4 in **Figure 3**).

2.4. Continue synthesis, starting from the subsaturation mutagenesis site triplet. Assign columns to randomized NNS triplets or wild type triplets according to the desired mutagenesis frequency (see note above). If additional subsaturation sites are present, proceed only to the triplet preceding the next subsaturation mutagenesis site. Again, leave a 5'-trityl group on at the end (5'-trityl-on option on the synthesizer) (step 3 in **Figure 3**). Then continue with step 2.3.

2.5. If no more subsaturation sites are present downstream, complete the synthesis, leaving a 5'-trityl group at the end (5'-trityl-on option on the synthesizer) (step 5 in **Figure 3**).

2.6. Deprotect and purify the oligonucleotide library according to the purification cartridge manufacturer's instructions.

NOTE: Oligonucleotides released by deprotection from the CPG can also be purified in the reverse phase high performance liquid chromatography (HPLC) with trityl-on followed by a manual trityl group removal (1 h treatment with 80% acetic acid at RT) and a second HPLC purification.

2.7. Check the oligonucleotide library quality in a urea-PAGE gel.

3. Generating variant libraries

NOTE: Use the recombinant plasmid from step 1.6.

3.1. Generate the libraries by oligonucleotide directed mutagenesis.

NOTE: Alternatively, use the EP-PCR protocol (step 3.2).

3.1.1. Amplify a section from the T7 promoter to the unique restriction enzyme site flanking the sequence targeted with mutagenesis (in case of NlaIV: SalI, EcoRI, or Eco52I) (**Table 1B–C**, **Table 2B**, **Figure 4**). Amplify the second part from the unique restriction enzyme site to the 3' end of the ESC.

3.1.2. Mix separately 5 µL of the PCR reactions (from step 3.1.1) with 8 µL of water, 1.5 µL of 10x restriction enzyme buffer, and 5 units of the appropriate restriction enzymes (SalI, EcoRI, or Eco52I) and incubate at the appropriate temperature for 2 h.

3.1.3. Resolve the products of both reactions using agarose gel electrophoresis. Cut out the expected size bands and purify with a commercial kit.

3.1.4. Run up to 1/3 of the purified products in an agarose gel and measure the concentration of each purified band by densitometry.

3.1.5. Set up the ligation of two parts of the ESCs in a 1:1 molar ratio with 1x ligase buffer and 1 unit of T4 DNA ligase and incubate for 2 h at RT.

3.1.6. Resolve the reaction products in the agarose gel. Cut out the expected size products and purify with a commercial kit.

3.1.7. Amplify the purified ligation products in a PCR reaction with primers F1 and R2 (**Table 1C** and **Table 2A**). Do not run more than 20 amplification cycles.

3.1.8. Fractionate the PCR reactions in an agarose gel. Cut out the products and purify with a commercial kit.

3.1.9. Run a 5 µL aliquot of the purified library from the previous step in the agarose gel and measure the concentration by densitometry.

3.1.10. Clone a small sample of the library (up to 5 µL) and sequence >15 clones to check the mutation frequency and distribution (**Table 3**). Proceed to step 4.

NOTE: Alternatively, high throughput sequencing of the small sample of the ESCs can be used.

3.2. Perform EP-PCR.

3.2.1. Amplify the ESC from the plasmid obtained in step 1.5.7 with primers F1 and R1. Run 20 cycles with Taq I polymerase (**Table 1B**).

3.2.2. Gel purify the PCR product.

3.2.3. Set up EP-PCR with 2 ng of purified PCR product from the previous step and run 15 cycles of EP-PCR (**Table 1C**) with F1 and R1 primers.

3.2.4. Gel purify the product and quantify it by gel densitometry.

NOTE: Due to the low concentration of the purified EP-PCR product use about 1/3 for quantification.

3.2.5. Clone a small sample of the library (up to 1/5) and sequence >15 clones to check mutation frequency and distribution (**Table 4**).

NOTE: Alternatively, perform high-throughput sequencing of the small sample of the ESCs.

4. Performing compartmentalized in vitro transcription-translation reaction

4.1. Test endonuclease expression and enzymatic activity in in vitro transcription-translation.

4.1.1. Prepare a short (200–500 bp) substrate with a single recognition site for the endonuclease located close to the center of the molecule so the cleavage reaction can be easily detected.

NOTE: The easiest way to prepare the substrate is by PCR amplification of an appropriate fragment of any DNA molecule. The substrate can be radiolabeled or fluorescently labeled to simplify cleavage detection.

4.1.2. Set up 50 µL of a transcription-translation reaction with 0.5 µg of wild type ESC according to manufacturer's recommendations. Add magnesium salt ($MgCl_2$, $MgSO_4$, and magnesium acetate can be tested) to 1.5 mM and the appropriate amount of substrate from the previous step (at least 0.5 µg in case of unlabeled DNA).

NOTE: Any transcription/translation kit that does not contain nuclease activated by magnesium can be used. Some kit vendors use nucleases to remove DNA contamination during production and then add chelators as nuclease inhibitors. Such kits are not compatible with this method.

4.1.3. Incubate the transcription-translation reaction according to the manufacturer's instructions. Then transfer the reaction mixture to the optimal temperature for the restriction enzyme for 2 h.

4.1.4. Analyze cleavage of the substrate in an agarose gel followed by appropriate detection (e.g., DNA staining, fluorescence visualization, or autoradiography) (**Figure 5**).

NOTE: At least partial cleavage of the substrate is necessary before proceeding with the compartmentalization. If this is not achieved, further optimization of the magnesium chemical or its concentration is necessary.

4.2. Prepare an oil-surfactant mixture by adding 225 µL of Span 80 and 25 µL of Tween 80 to 5 mL of mineral oil in a 15 mL conical tube. Mix thoroughly by gentle inverting the tube 15x.

4.3. For each library transfer 950 µL of the oil-surfactant mixture to a 2 mL round bottom cryogenic vial, label with a library name, and transfer to ice. Put one small cylindrical stirring bar (5 x 2 mm) into each vial.

4.4. Prepare an in vitro transcription-translation reaction mixture (50 µL for each library) according to the manufacturer's suggestions. Supplement the mixture with magnesium chloride to a final concentration of 1.5 mM (see step 4.1.4).

4.5. Dispense 50 µL aliquots into 1.5 mL tubes on ice.

4.6. Add 1.7 fmole of the library (from section 3) to the reaction mixture on ice.

NOTE: Do not use a higher amount of expression library for selection efficiency. It is crucial to minimize the frequency of aqueous droplets containing more than one DNA molecule.

4.7. Prepare water-in-oil emulsion consecutively for each library.

4.7.1. Put a small beaker (or large bottle cup) filled with ice on a magnetic stirrer with the stirring speed set at 1,150 rpm.

4.7.2. Transfer a cryogenic vial with 950 µL of oil-surfactant mixture and a small stirring bar from step 4.3 to an ice-cold beaker on the magnetic stirrer. Check that the stirring bar is spinning.

4.7.3. Add five 10 µL aliquots of the in vitro library-transcription-translation mixture over a 2 min period in 30 s intervals and continue stirring for an additional minute. Transfer the vial with the emulsion to an ice container. Proceed with the next library starting with step 4.7.2.

4.7.4. After all the libraries are processed start the incubation of all the libraries according to the kit manufacturer's recommendations.

4.8. Transfer the vials to the temperature optimal for the engineered endonuclease for an additional 2 h and then put them on ice for at least 10 min.

5. Continued processing of libraries and selection

5.1. Transfer the emulsions from the cryogenic vials into cold 1.5 mL tubes and centrifuge them at 13,000 x *g* for 5 min at 4 °C.

5.2. Remove the upper oil phase with a pipette. If an oil-water interphase is not visible, incubate the tube for at least 5 min at -20 °C to freeze the aqueous phase, then immediately pipet out the liquid oil phase.

5.3. Immediately perform extraction with 50 µL of phenol:chloroform (1:1 v/v) by short vortexing followed by phase separation by 30 s centrifugation at 13,000 x *g*. Collect the upper aqueous phase.

5.4. Precipitate the DNA by adding 0.1 vol (5 µL) of 3 M sodium acetate (pH = 5.2) and 2.5 vol (125 µL) of ethanol in 2.5–5 µg of glycogen. Incubate at -20 °C for 1 h and centrifuge for 15 min at 13,000 x *g*, 4 °C. Discard the supernatant and briefly wash the pellet with 1 mL of cold 70% ethanol.

5.5 Dry the DNA/glycogen pellet in a speedvac or air dry for >5 min.

5.6. Dissolve the pellet in 50 µL of 10 mM Tris-HCl (pH = 7.5). Add 5 µL of streptavidin magnetic beads prepared according to the manufacturer’s instructions and mix for 1 h at RT, preferably in a carousel mixer or by gentle vortexing.

5.7. Separate the beads on a magnetic stand and collect the liquid enriched in DNA without biotin.

5.8. Concentrate the DNA by ethanol precipitation (steps 5.4–5.5).

5.9. Dissolve the concentrated DNA from the previous step in 5 µL of water and use as a template in a PCR reaction with F2 and R1 primers (**Table 1A**).

NOTE: To avoid problems with template contamination and minimize PCR artifacts use Taq polymerase (not Pfu or Phusion) and run 18–20 cycles with the extension time proportional to the template size (1 kb = 1 min) (see **Table 2B**).

5.10. Fractionate the PCR product in an agarose gel and cut out the expected size product. Some smearing indicates that there are products of different sizes (see **Figure 6**). Purify the DNA from the gel slab with a commercial kit.

5.11. Run a second PCR reaction with up to 50 ng of DNA from step 5.10 and primers F1 and R2 using the same protocol as in step 5.9. Proceed with product purification as described in 5.10. Purified DNA after quantification by agarose gel densitometry (not UV spectroscopy) can be used in the next round of in vitro selection (step 4.6).

6. Screen variants for altered sequence specificity

6.1. Clone selected variants.

6.1.1. Digest the product from step 5.10 for 2 h with 10 units of restriction enzymes appropriate for cloning of the ORF into the expression vector (for NlaIV: NcoI and XhoI) in the temperature and buffer recommended by the enzyme vendor. Resolve the products with agarose gel electrophoresis and isolate the expected size fragment.

6.1.2. Prepare the plasmid vector (e.g., pET28) by double cleavage with the same enzymes as in step 6.1.1 and gel purify the product with a commercial DNA gel purification kit.

6.1.3. Estimate the concentrations of vector and insert by densitometry with agarose gel electrophoresis.

6.1.4. Set up a ligation with 1–5 units of T4 DNA ligase and vector:insert molar ratio 1:3–1:5 in 1x ligase buffer recommended by the enzyme vendor. Incubate for 2 h at RT and introduce into appropriate host bacteria (from step 1.3) by transformation or electroporation[12].

6.1.5. Select extransformants on LB plates containing the appropriate antibiotic (50 µg/mL of kanamycin for pET28 or pET30 vectors) and 1% glucose.

6.2. Express protein variants.

6.2.1. Inoculate single colonies from the transformation (up to 24 clones can be easily processed in a single run) into 2 mL of LB with kanamycin (50 µg/mL) and 1% glucose and grow overnight at 37 °C with shaking.

6.2.2. Inoculate 15 mL of warm (37 °C) LB containing 100 µg kanamycin and no glucose with 0.75 mL of the overnight culture and incubate at 37 °C with vigorous shaking.

NOTE: Either 50 mL centrifuge tubes or 100 mL Erlenmayer flasks can be used.

6.2.3 Add 176 µL of glycerol to 1 mL of overnight culture (final concentration of glycerol = 15%) mix thoroughly and freeze at -70 °C.

6.2.4 After 2–3 h supplement 15 mL of the culture (from step 6.2.1) with IPTG to 1 mM and culture for an additional 5 h.

6.2.5. Collect the bacterial pellet by centrifugation (10,000 x *g*, 4 °C, 10 min) and freeze at -70 °C.

6.3. Purify the protein variants.

6.3.1. Transfer 20 µL of nickel affinity resin suspension into 200 µL of B1 buffer in a 1.5 mL tube with a wide bore pipette tip, mix gently, and centrifuge (5,000 x *g*, 30 s, 4 °C). Remove the supernatant by pipetting and leave the tube on ice.

6.3.2. Resuspend the bacterial pellet from step 6.2.5 in 300 µL of B1 by vigorous vortexing. Transfer the suspension into a 1.5 mL tube.

6.3.3. Add 3 µL of 100x protease inhibitor cocktail and lysosome solution in B1 (final concentration of 1 mg/mL). Disintegrate the cells by sonication with a tip equipped probe. Use six 10 s bursts per sample with >15 s tip cooling time in ice in between. Keep cell suspensions on ice all the time.

6.3.4. Pellet cell debris by centrifugation (2 min, 12,000 x *g*, 4 °C) and transfer 250 µL of supernatant to the resin aliquot from step 6.3.1.

6.3.5. Mix for 15 min in a cold room, preferably in a carousel mixer or by gentle vortexing.

6.3.6. Centrifuge (5,000 x *g*, 30 s, 4 °C) and aspirate the supernatant with a pipette.

6.3.7. Add 500 µL of W buffer and gently resuspend the resin. Centrifuge (5,000 x *g*, 30 s, 4 °C) and aspirate the supernatant with a pipette.

6.3.8. Repeat step 6.3.7.

6.3.9. Add 20 µL of buffer E, gently resuspend the resin, and leave the sample on ice for 2–5 min. Centrifuge (5,000 x *g*, 30 s, 4 °C) and collect the supernatant.

6.3.10. Repeat step 6.3.9. Pool supernatants.

6.3.11. Analyze protein samples in by SDS-PAGE (5–10 µL) (**Figure 7**).

6.4. Screen for variants with the altered specificity.

6.4.1. Assay cleavage activity on bacteriophage lambda DNA. The protein sample can constitute up to 10% of the final reaction volume. A total of 2 µL of protein sample per 0.5 µg of DNA and 2 h reaction time is a good starting point.

6.4.2. Analyze the reaction products by agarose gel electrophoresis along with the products generated by the wild type enzyme. Select the clones generating cleavage patterns clearly distinguishable from the one generated by the wild type enzyme for further analysis (**Figure 8**).

REPRESENTATIVE RESULTS:

This protocol is just a tool to increase the frequency of desired variants of an engineered REase by depleting (but not eliminating) two unwanted classes: inactive enzymes and endonucleases with unchanged wild type sequence specificity. On the other hand, because changing REase specificity is extremely difficult, finding even one such variant producing a cleavage pattern that is different from the wild type enzyme in a single screening of 24 clones should be considered a success. In our hands the best screens could identify up to 20% of promising variants (**Figure 8A**).

The positive outcome strongly depends on a library quality (i.e., limited frequency of substitutions and their random distribution) and efficient capture of the biotinylated population of library members (steps 3.6–3.7). Both problems can be detected. The library quality should be checked prior to the selection by sequencing as many clones as possible (>15) or by direct sequencing of the library by high throughput sequencing (step 3.10, **Table 3**). If a majority of the selected clones are not active, this is a clear indication of failure of the streptavidin capture selection. A similar effect is observed in the case of libraries that undergo many selection cycles, because such libraries are most probably dominated by inactive variants that escaped the streptavidin capture selection step **(Figure 8B**). Therefore, it is advisable to run screening after every selection cycle and further develop manually selected promising variants rather than to depend on selection iteration.

FIGURE AND TABLE LEGENDS:

Figure 1: In vitro selection of a new sequence specificity based on NlaIV engineering. (**A**) The organization of the expression/selection cassette (ESC) includes two recognition sites for REase, 1) the selected sequence (GGATCC) close to the right end and 2) the counter selected sequence (GGSSCC) close to the left end, as well as the T7p and T7t–T7 promoter and T7 terminator. The primer binding sites are shown below. Cleavage by wild type and selected NlaIV variants are shown as red and green triangles respectively. (**B**) Selection cycle steps: I) Emulsification of transcription-translation-cleavage reaction mixes with the ESC library; II) All biotinylated DNA is captured on magnetic particles coated with streptavidin and removed, thus removing encoding inactive variants; III) ESCs encoding REases with wild type activity (i.e., those able to cleave the GGSSCC sequence) are eliminated because cleavage of the sequence separates the binding sites for the forward and reverse primers. Therefore, no amplification of these ESCs occurs; IV) Input

for the next selection round is created by addition of biotin on the right end and reintroducing variation of the counter selected sequences on the left end. Reprinted from Czapinska et al.[8] with permission from Elsevier.

Figure 2: Preparation of ESC. Fragment derived from the original construct in an expression vector containing NlaIV ORF under control of the T7 promoter was modified to be suitable for expression/selection. The NlaIV site downstream from the NlaIV ORF was removed and unique sites (SalI, EcoRI and Eco52I) that were used to mutagenize selected positions were introduced in the NlaIV ORF as silent mutations. The final construct was amplified with flanking primers that introduced two flanking NlaIV sites: The counter selected sequence (GG**SS**CC) on the left and selected sequence (GG**AT**CC) on the right. The reverse primer also introduced biotin. Primers used in creation of mutated ECS are shown as blue arrows and labeled below (see **Table 1B**,**C**).

Figure 3: Scheme of split and mix synthesis. The example refers to MutB primer synthesis where an NNS sequence was introduced at 0.8 frequency at four positions (see also **Table 3**). Note that chemical synthesis is carried out from 3' to 5' but all sequences are shown in canonical 5'-3' orientation (i.e., it proceeds from left to right in this scheme). Wild type sequences at mutagenized positions are shown in green while NNS mutagenic sequences are in red. The SalI recognition site that is later used to introduce mutations in ESCs is underlined. Points of mixing and splitting steps (2 and 4) are indicated.

Figure 4: Use of unique restriction enzyme sites in oligonucleotide targeted mutagenesis. The strategy of mutation introduction is shown on an example of the construction of libraries A-C (see steps 3.1–3.7). Reprinted from Czapinska et al.[8] with permission from Elsevier.

Figure 5: Endonucleolytic cleavage in in vitro transcription-translation. (**A**) Cleavage of a test substrate in optimal REase buffer: 1) Substrate, 612 bp PCR product with a single NlaIV recognition site; 2) Cleavage products, 355 bp and 257 bp. (**B**) Cleavage in an in vitro transcription-translation reaction (containing 0.5 µg of ESC): 1) 2–15 µL aliquots of in vitro transcription translation without substrate; 2) Reaction supplemented with 1.5 mM $MgCl_2$; 3) 4–15 µL aliquots of in vitro transcription-translation with 1 µg of test substrate; 4) Reaction supplemented with 1.5 mM $MgCl_2$. S–DNA size marker (pBR322 digested with MspI). Samples were resolved in 6% native PAGE. DNA was stained with ethidium bromide.

Figure 6: Products of the first PCR in the selection cycle. See **Figure 1B**, step III; protocol step 5.10. Column sets 1 and 2 are aliquots of two different libraries loaded in triplicate. S–DNA size standard (lambda DNA digested with HindIII and EcoRI). Arrow indicates position of the full-length ESC (1,050 bp).

Figure 7: NlaIV variants purified for further screening in mini scale. See step 6.3.11. Each line contains a 10 µL aliquot of a different variant. S–protein molecular weight standard. Molecular mass of NaIV REase subunit is 29.9 kDa.

Figure 8: Examples of screening of NlaIV variants for sequence specificity alteration. See step

6.4.2. (**A**) Successful screening with high frequency of promising variants. S=DNA size marker, lambda DNA cleaved with HindIII and EcoRI; wild type (wt)=lambda DNA cleaved with wild type NlaIV; λ=lambda DNA substrate, not cleaved; other columns=variants with very low activity. Variants are labeled ! = promising variants that produce a cleavage pattern distinct from the wild type enzyme; ? = variants that also might have altered sequence preference. (**B**) Unsuccessful screening, with a majority of variants inactive and one variant with apparently unaltered cleavage pattern.

Figure 9: Alternative selection by ligation. This alternative can be used for all REases generating sticky ends. Here we present an example protocol for a selection scheme for MwoI enzyme (unpublished). I) Selected sequence (located at the right end of the ESC) with defined residues shown in red and selected variation of the cognate sequence shown in blue. In parentheses below the counter selected sequence to be placed at the left end of the ESC is shown; II) Product of MwoI cleavage; III) After terminating in vitro transcription/translation, products are purified and ligation is performed with excess adapter. Only the cleavage products that were cleaved in the selected sequence can participate in ligation. Therefore, inactive variants are eliminated, and the pulldown step is unnecessary. The cleavage product in the counter selected sequence (on left end of the ESC, not shown) cannot participate in this ligation because the protruding end of the adaptor is not complementary to the counter selected sequence; IV) Selective PCR uses the same strategy as in the main protocol to eliminate variants with the wild type degenerate sequence specificity (F1 primer binding distal to the counter selected site) whereas inactive variants are eliminated by the selective reverse primer that cannot bind to the uncleaved (and therefore not modified by adapter ligation) right end. In the next cycle the process can be iterated by using adaptor that is identical to the cleavage product of the preceding step (i.e., the “cleaved cassette” in panel III), and an appropriate selective reverse primer.

Table 1: Primers used in NlaIV engineering. Sequences of the restriction sites mentioned in the comments are underlined. Small letters indicate sequences that do not have complements in the DNA templates.

Table 2: Conditions of PCR reactions to be used in the protocol. T_m= primer melting temperature (if T_m is different for the primers, the lower T_m should be used).

Table 3: Results of quality check of two mutagenic primers synthesized with split-and-mix strategy. Mutagenized codons are indicated with [XXX]. A lower index number indicates the position of an encoded amino acid. Adapted from Czapinska et al.[8] with permission from Elsevier.

Table 4: Results of EP-PCR. Main parameters derived from sequence analysis of 22 clones of ECS.

DISCUSSION:

The selection protocol described here was tested for NlaIV[8], a dimeric PD-(D/E)XK fold recognition sequence that recognizes a palindromic target site with central NN bases and catalyzes a blunt end cut between the NN bases. NlaIV was picked because cleavage between the NN bases suggests that these bases are close to the protein in the complex. In principle, the

protocol could be used for any sequence specific restriction endonuclease, monomeric or dimeric, of any fold group, catalyzing double strand breaks of any stagger, irrespective of whether catalytic and specificity domains coincide (as in the NlaIV example) or are separate (e.g., FokI). Moreover, the protocol in principle is useful not only for the generation of new, more narrow enzyme specificity, but could also be used to eliminate star activities, or to create high fidelity endonucleases. However, all this has not been tested yet. In particular, targeted elimination of star activity may be complicated, because the same amino acid residues could be involved in binding to the desired and undesired bases. The in vitro steps described in this protocol are not limited to the selection of narrowed down specificities but could also be used to select otherwise altered specificities. However, there is then a problem with variant endonucleases: if the spectrum of substrates includes novel targets not cleaved by the parental endonuclease, there is in general no good way to protect cells from the harmful effects of this activity. In contrast, if endonuclease specificity is only narrowed down, the targets are a subset of the wild type targets, and hence the already available cognate methyltransferase should be fully protective.

Our protocol differs in several respects from many directed evolution protocols. Open reading frame diversity is generated once at the beginning of the experiment, not in every iteration. Moreover, it is created by split-and-mix synthesis, rather than by EP-PCR. For NNS substitutions of codons, as used in this work, there are $(4 \times 4 \times 2)^6 \sim 1.07 \times 10^9$ combinations for six positions. Therefore, any given variant is present on average once in 1.7 fmoles of ESC. This capacity can be increased to seven positions by using synthesis with a mixture of 20 trinucleotide precursors that is offered by Glen Research or by decreasing mutation frequency in less promising positions with split-and-mix oligonucleotide synthesis. If possible, it is recommended to limit the extent of variation to six positions. Obviously, such mutagenesis targeting requires some preexisting knowledge about at least the regions of the REase involved in substrate binding. The split-and-mix protocol to generate diversity has clear advantages in comparison to EP-PCR. Using EP-PCR, we obtained unchanged variants and sequences carrying eight substitutions for NlaIV ESCs in the same EP-PCR (**Table 4**). The library from EP-PCR contains a substantial fraction of clones that should be avoided (wild type sequences, multiple substitutions, frameshift and nonsense mutations, and mutations in places unlikely to affect sequence specificity).

Our protocol also differs from many other directed evolution protocols by the presence of two sequential selection steps. Positive selection makes sure that the desired activity is retained, otherwise the biotin tag is not removed, and the coding sequence can be removed by pull-down. It is technically possible that the fortuitous emergence of a novel, non-overlapping specificity (e.g., GCATGC) could lead to severing of the biotin tag as well, if a suitable cleavage site is present near the desired cleavage, but not elsewhere. However, this should be highly unlikely. Negative selection removes open reading frames that code for enzymes that still have the undesired activity. This step is not strictly mandatory, because the protocol will still enrich the output library with variants that are able to cleave the selection sequence but not able to cleave elsewhere in the ESC, therefore rendering it unsuitable for PCR amplification. However, selection effectiveness is expected to be lower because enzymes with the original sequence specificity will not be removed from the output and will outcompete promising variants with altered specificity but also decreased enzymatic activity. Note that at the population level, both desired and undesired

target sequences can, but need not be, degenerate. In the NlaIV example, the anti-target was degenerate and the target non-degenerate. Even when there is degeneracy at the population level, in a single droplet only one (non-degenerate) target or anti-target is present. In our protocol, target and anti-target sequences are reintroduced at every repetition of the selection steps. Therefore, an open reading frame must encode an enzyme capable of cleaving all possible targets, and unable to cleave any of the anti-targets, to survive multiple selection rounds. Notice that the need to reintroduce the antiselection target at each iteration of the protocol enforces two sequential PCRs. The first PCR uses a primer that anneals outside the anti-target, so that cleavage of the anti-target prevents the PCR reaction. The second PCR requires a primer that reaches beyond the anti-target, and reintroduces anti-target, to make sure that during multiple rounds of selection, each open reading frame is tested against all variants of the anti-target.

For enzymes that generate sticky ends, a related alternative protocol based on a previously described method for isolation of REase ORF[10] can be used. The depletion of inactive variants by biotin capture that is used in our experiments is replaced in the alternative protocol by ligation of the compatible adapter with a sequence that is used as a primer binding site in a selective PCR (**Figure 9**). Only ESCs that produce enzymes with the selected specificity generate ligation-capable ends and will therefore be selected. The sequence of the sticky end of the counter eselected sequence must be designed in such a way that it cannot participate in ligation with adapters. Iteration of the selection process can be easily achieved by switching between two different adapters and consequently two different reverse primers in selective PCR.

Even with new protocols, the task of engineering novel specificities in vitro is still very challenging. For typical type II REases, sequence specificity and endonucleolytic activity depend on the same protein regions. It is therefore difficult to alter one without affecting the other. Success is made more likely by a strategy that takes into account the footprint of the enzyme, respects the symmetry of protein-DNA interactions, and builds on preexisting enzymatic preferences, which should be determined upfront in biochemical experiments, as was done for the NlaIV example[8].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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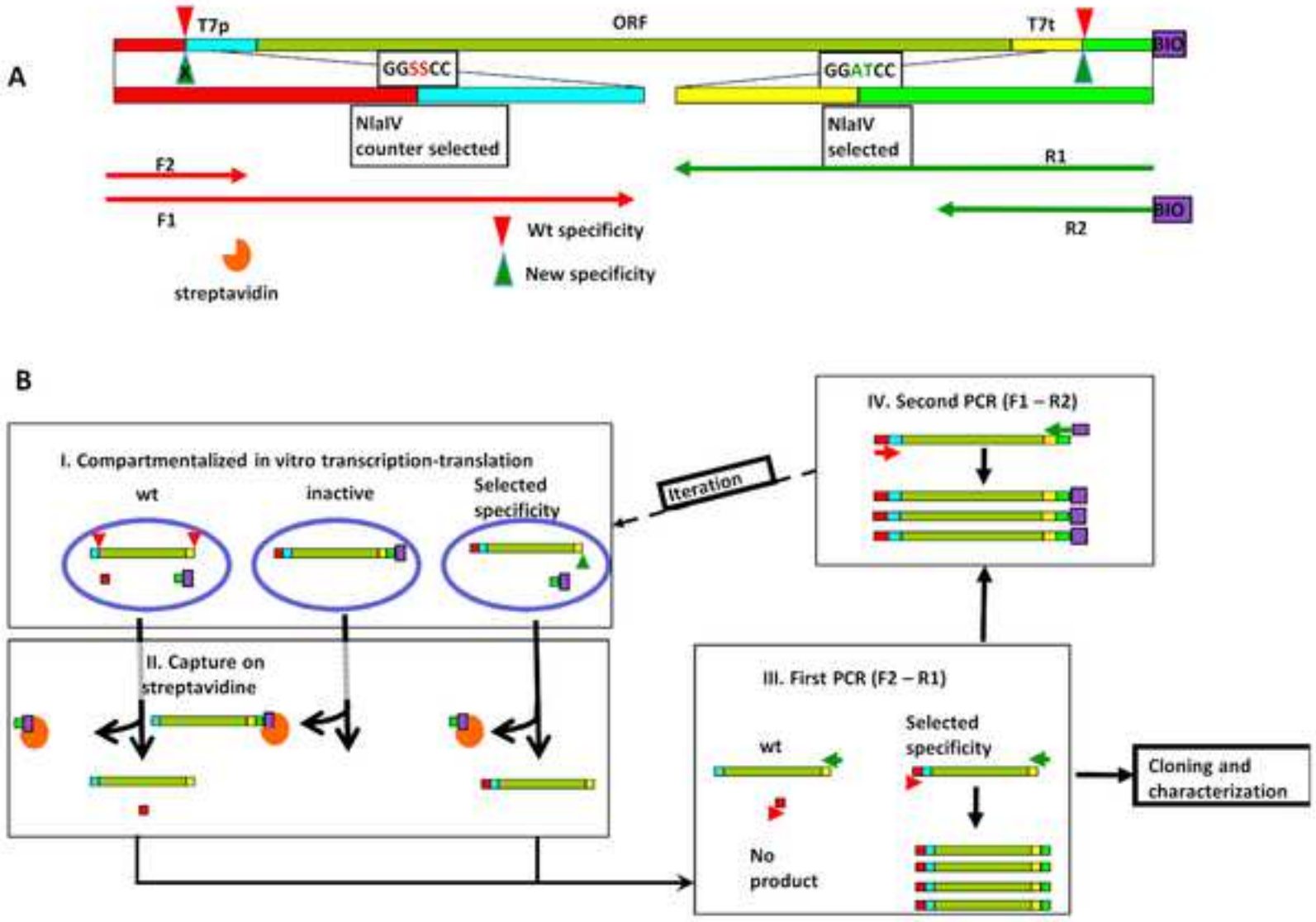


Figure 2

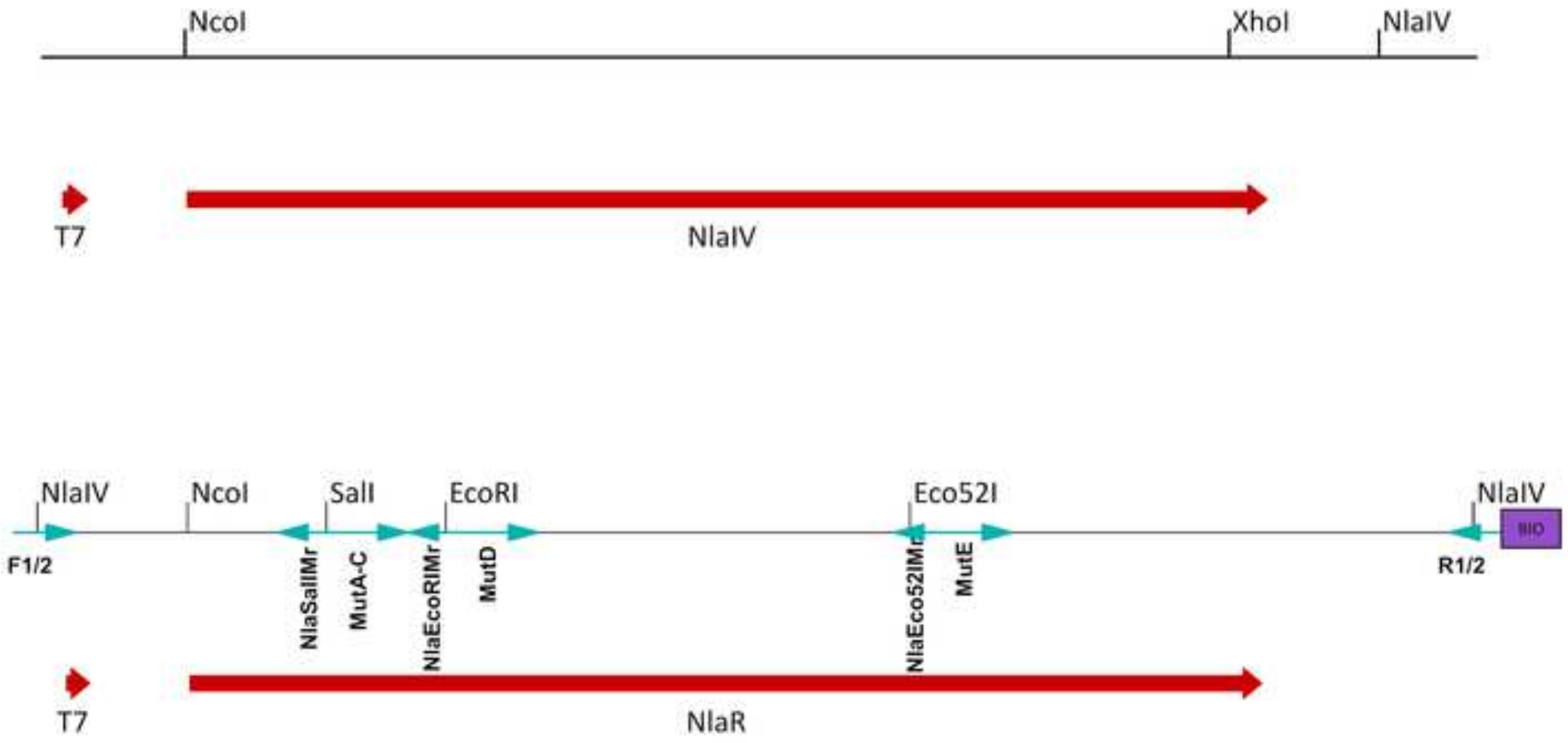
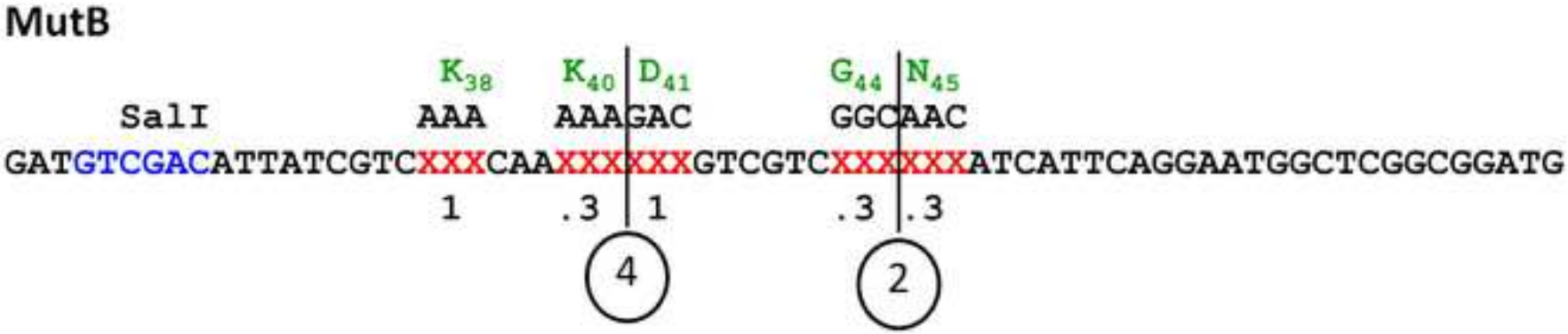


Figure 3



1. Synthesis of **NNS**ATCATTCAGGAATGGCTCGGCGGATG (2 of 7 columns) and **AAC**ATCATTCAGGAATGGCTCGGCGGATG (5 of 7 columns)
2. Mix and spilt
3. Synthesis of **NNS**GTCGTC**NNS** (2 of 7 columns) and **NNS**GTCGTC**GGC** (5 of 7 columns)
4. Mix and split
5. Synthesis of GAT**GTCGAC**ATTATCGTC**NNS**CAA**NNS** (2 of 7 columns) and GAT**GTCGAC**ATTATCGTC**NNS**CAA**AAA** (5 of 7 columns)

Figure 4

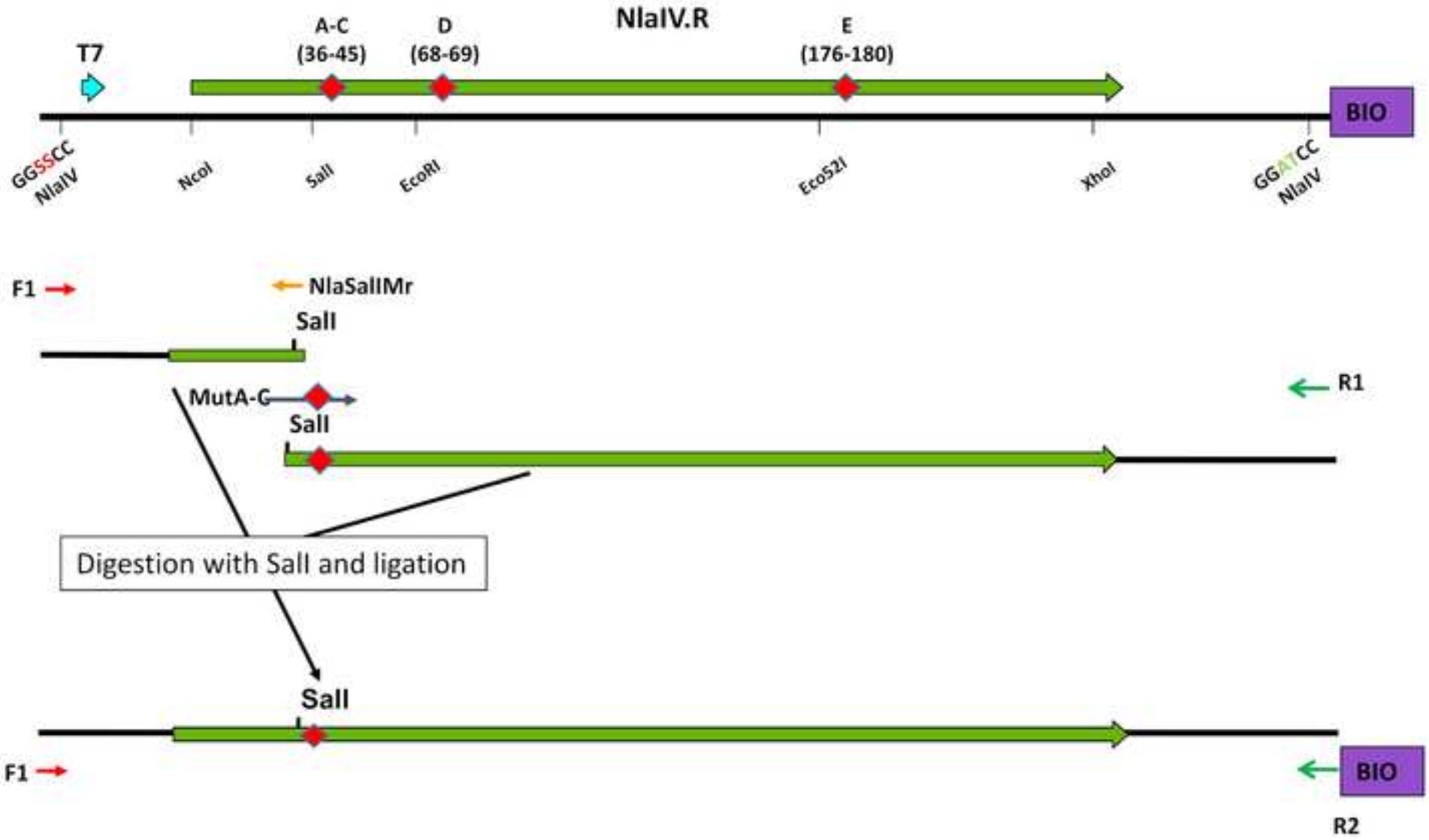


Figure 5

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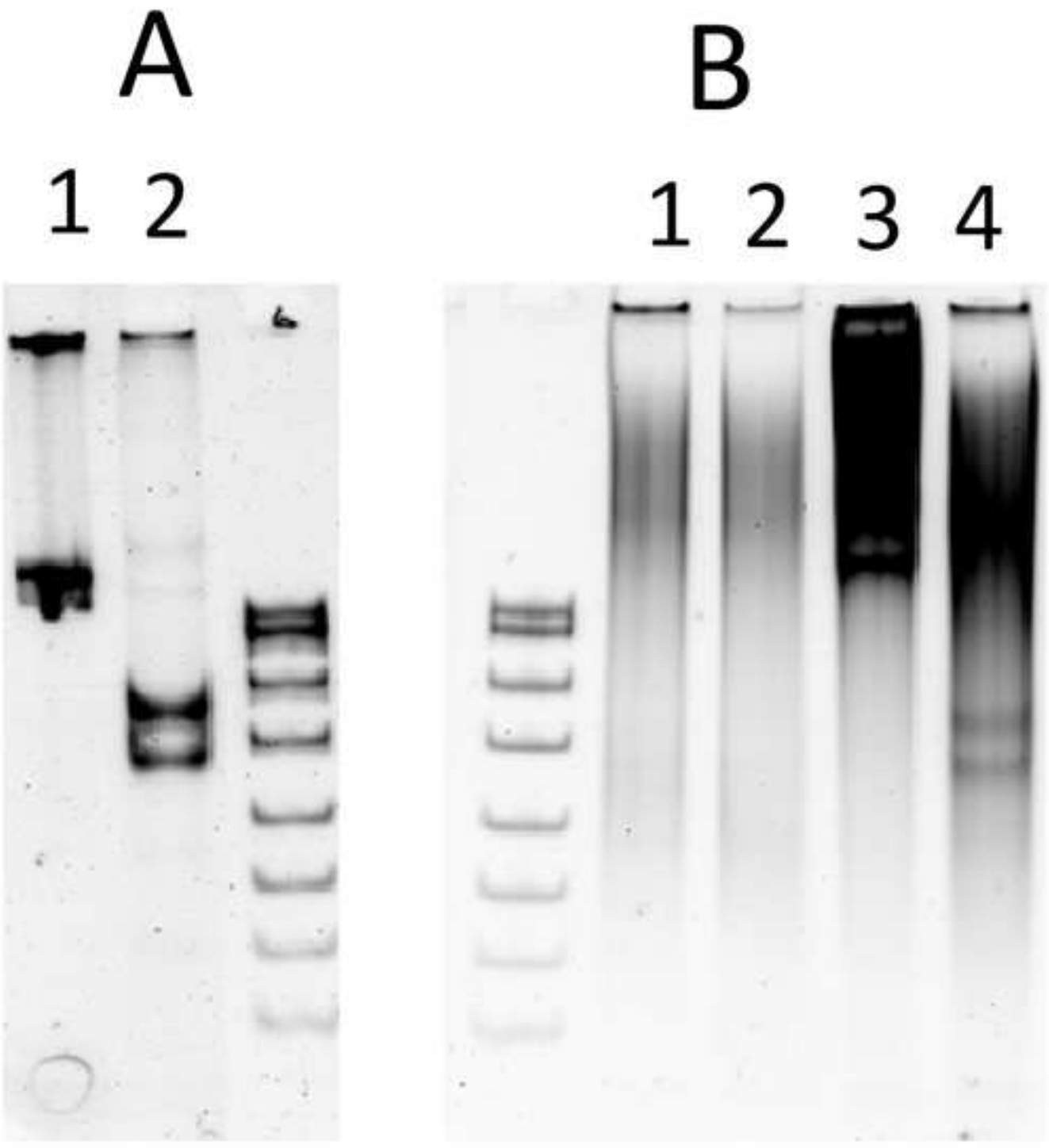

Figure 6

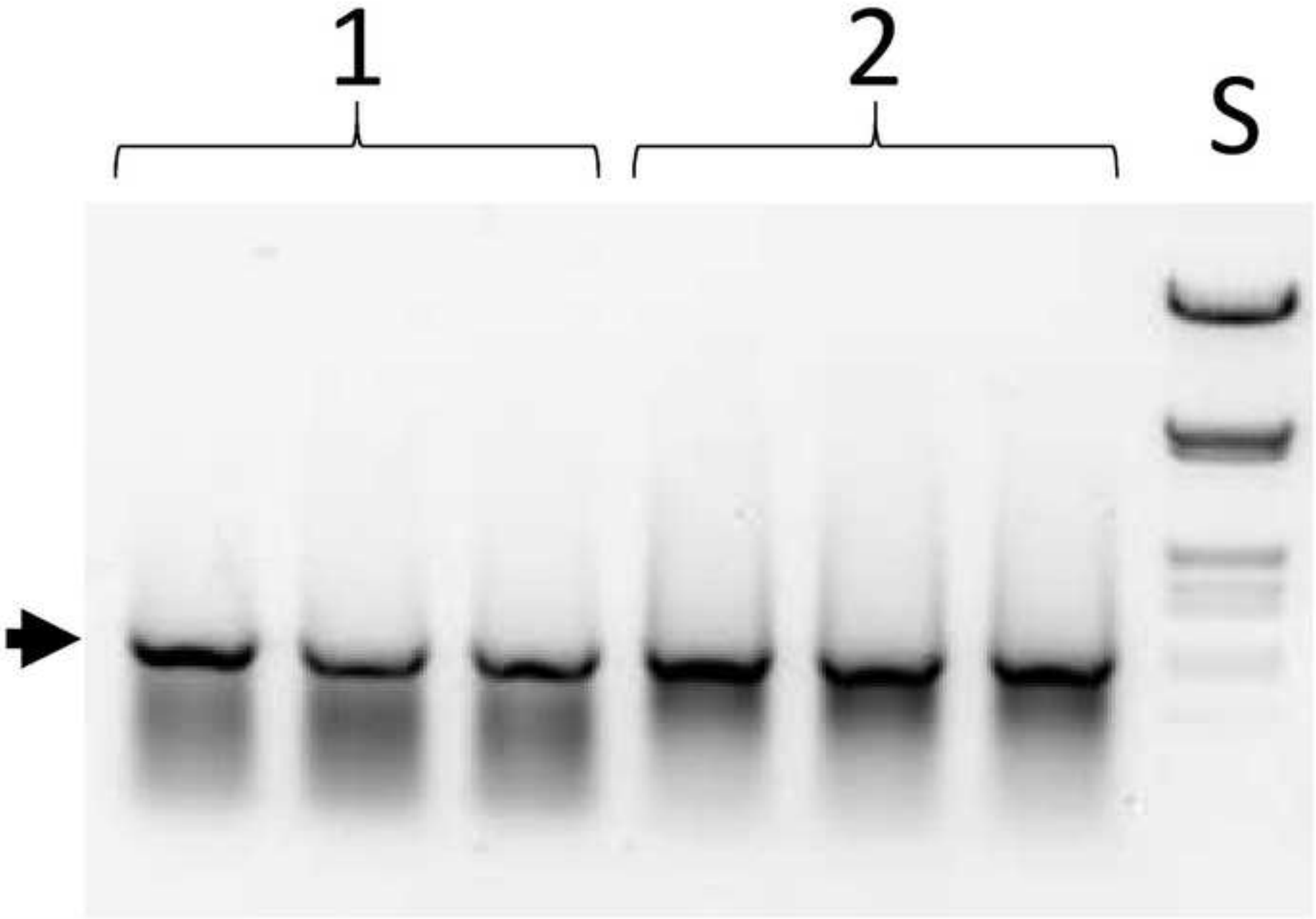

Figure 7

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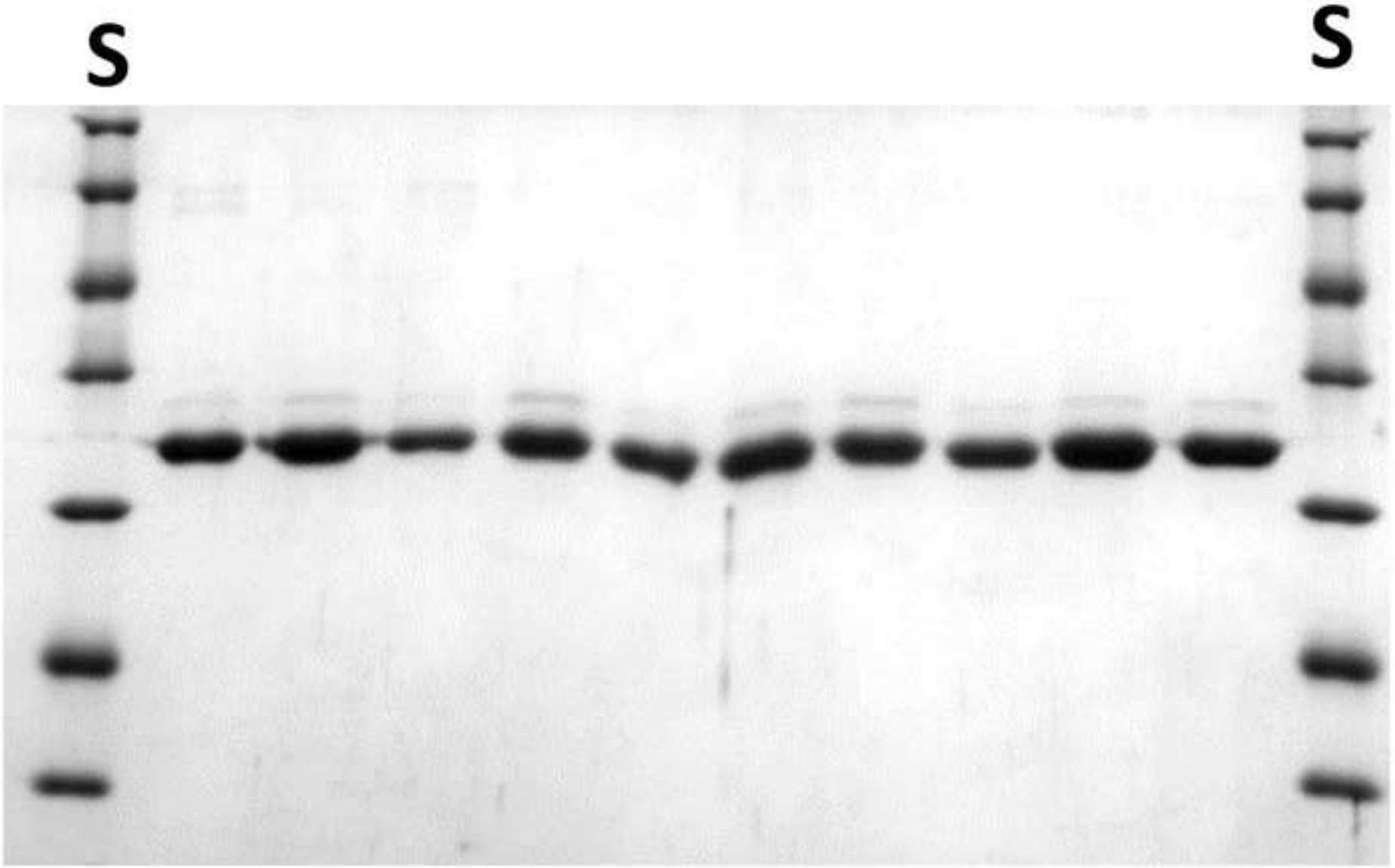

Figure 8

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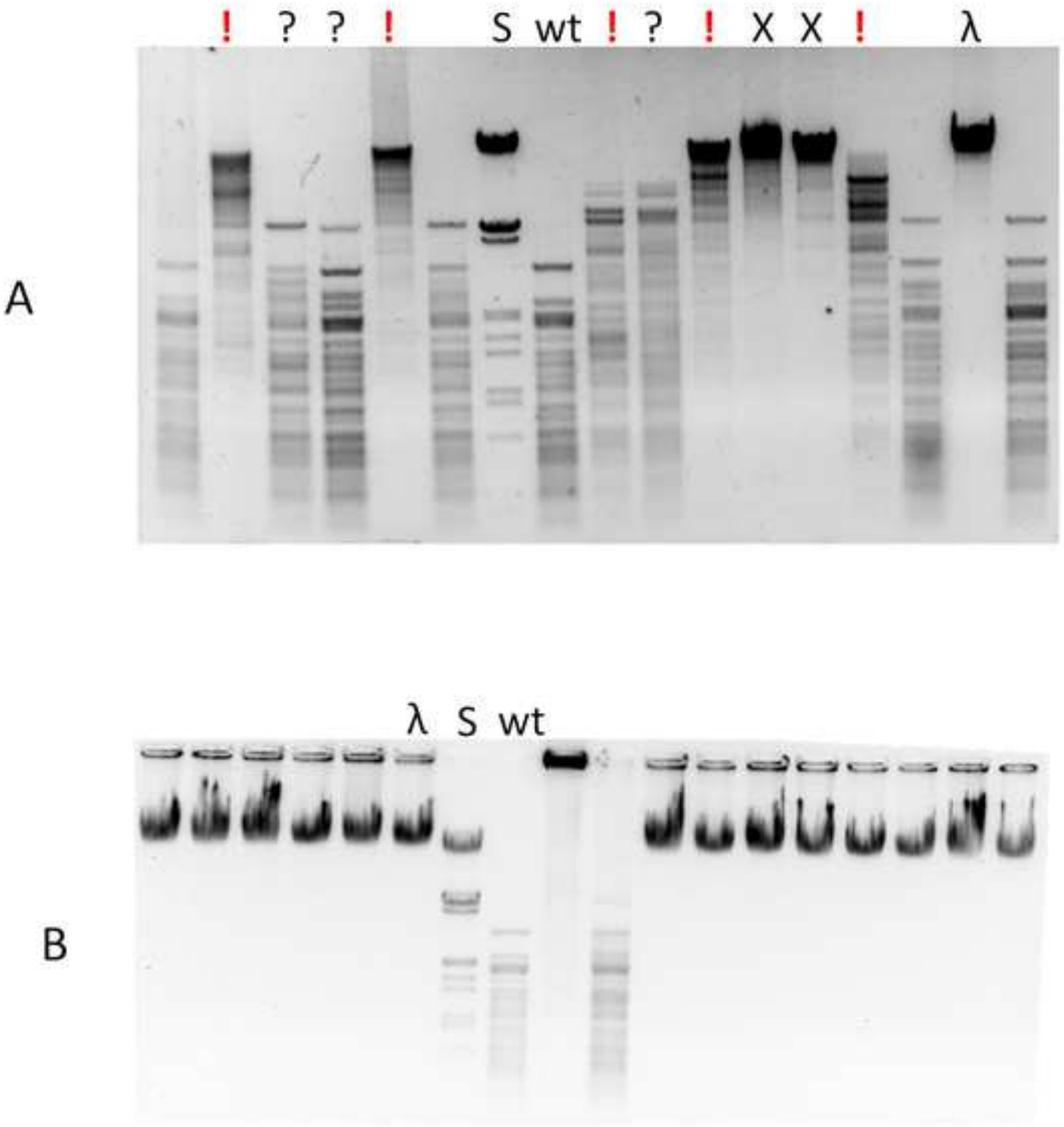

Figure 9

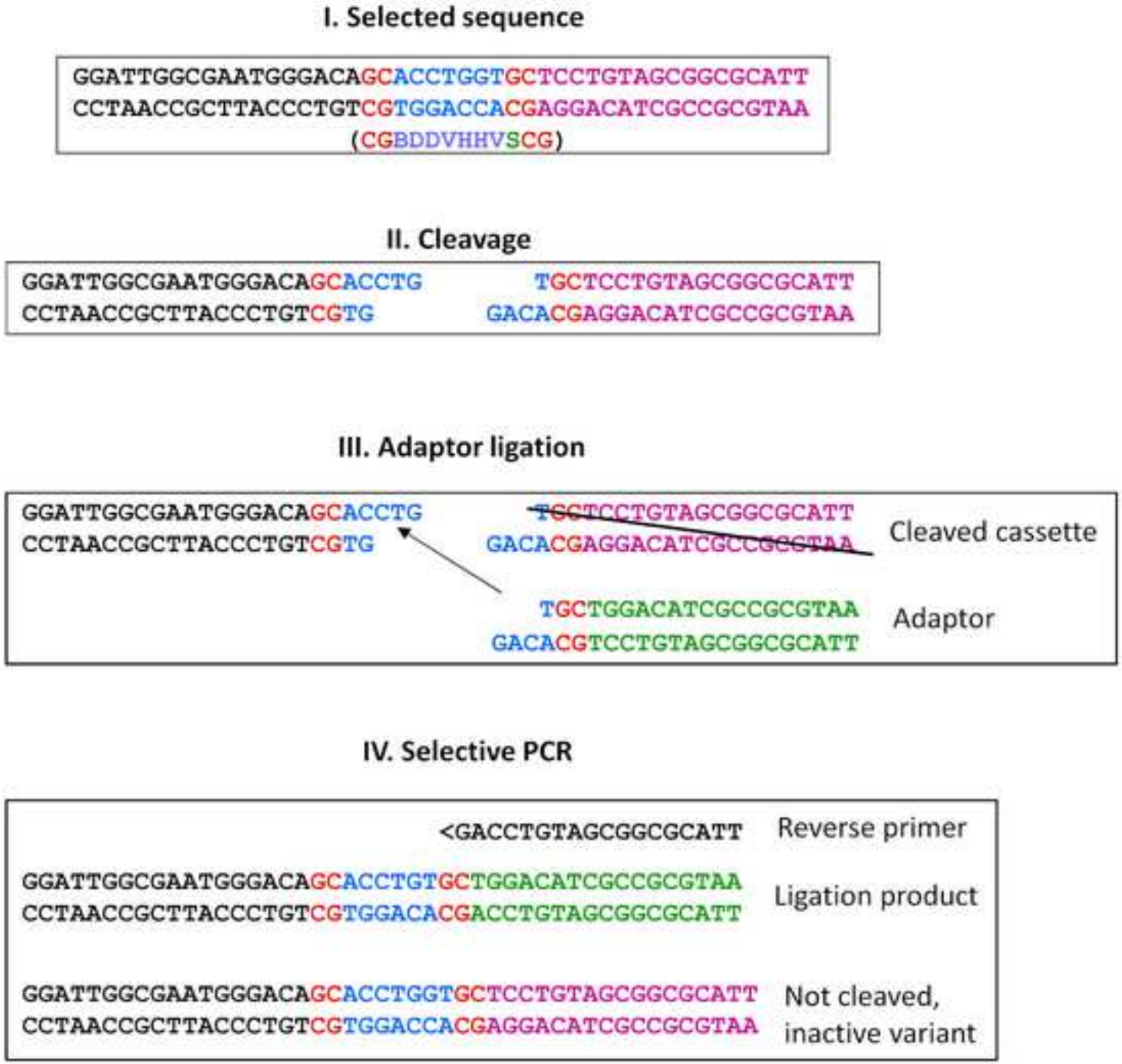

Table 1

Primer name	Primer sequence	Comments
A. Preparation of the expression cassette		
NlaSalIF	acATTATCGTCAAACAAAAAGAC	Silent mutation introducing unique SalI site preceding mutagenesis region A-C
NlaSalIR	cgACATCGCCCAAGAAAAATC	
NlaEcoRIF	cGATGTTTCAACCAATACC	Silent mutation introducing unique EcoRI site preceding mutagenesis region D
NlaEcoRIR	AATTCAATTTCCCTTTTTCTC	
NlaEco52IF	CCGGGTGTTTGGTAC	Silent mutation introducing unique Eco52I site preceding mutagenesis region E
NlaEco52IR	cCGGATTTTATGCACCAC	
NlaNlaF	tCTCTAAACGGGTCTTG	Mutation replacing NlaIV site 79 bp downstream of NlaIV ORF with Eco31I site
NlaNlaR	CCCCAAGGGGTTATG	
B. Mutagenesis primers		
MutA	GATGTCGACATT[**nns**]GTC[**AAA/nns**]CAA[**AAA/NNS**]GACGTCGTCGGCAACATCATTCAG	Mutagenic primer for library A (with SalI site)
MutB	GATGTCGACATTATCGTC[**nns**]CAA[**AAA/NNS**][**nns**]GTCGTC[**GGC/nns**][**AAC/nns**]ATCATTCAGGAATGGCTCGGCGGATG	Mutagenic primer for library B (with SalI site)
MutC	GATGTCGACATTATCGTCAAACAAAAA[**nns**]GTCGTC[**GGC/nns**][**AAC/nns**]ATCATTCAGGAATGGCTCGGCGGATG	Mutagenic primer for library C (with SalI site)
MutD	TGAATTCGATGTTTCA[**nns**][**nns**][**NNS**]TGGGTCATGCCTCCCGATTTCTTTTTAAATAAAAAAGACCGCAG	Mutagenic primer for library D (with EcoRI site)
MutE	TCCGGCCGGGTGTTTGGTAC[**nns**]ATC[**nns**][**AAA/nns**][**AAG/nns**]AATATGCCTATGTTTGAATGCTTGGAAGATTTTGTTTCC	Mutagenic primer for library E (with Eco52I site)
C. Other primers for expression cassette construction		
NlaSalIMr	AATGTCGACATCGCCCAAGAAAAATCTAATTTGACCATTAGC	Primer for amplification of cassette upstream of mutagenized region for libraries A-C (with SalI site)
NlaEcoRIMr	ATTGGTTGAAACATCGAATTCAATTTCCCTTTTTCTCAACCATCC	Primer for amplification of cassette upstream of mutagenized region for library D (with EcoRI site)
NlaEco52IMr	GTACCAAACACCCGGCCGGATTTTATGC	Primer for amplification of cassette upstream of mutagenized region for library E (with Eco52I site)
F1	GTCCGGCGTAGAGGATCGgsscCTCGATCCCGCGAAATTAATAC	Forward primer for expression cassette amplification with counter selected NlaIV site. Used in libraries construction to produce wild type part of it upstream of the mutagenized region (together with primers NlaSalIMr, NlaEcoRIMr or NlaEco52IMr). Also used in the second PCR of the selection protocol (step IV on Figure S2, panel B) in pair with primer R2.
F2	GTCCGGCGTAGAGGATCG	Forward primer for the expression cassette amplification preceding counter selected NlaIV site. Used in libraries construction to produce the final library of the mutagenized expression cassettes in pair with primer R2. Also used in the first PCR of the selection protocol (step III on Figure S2, panel B) in pair with primer R1.
R1	aatgcgccgctacagggaTCCTCCCATTCGCCAATCC	Reverse primer for the expression cassette amplification with selected NlaIV site. Used in libraries construction to produce mutagenized part of it starting from the mutagenized region (in combination with primers MutA, MutB, MutC, MutD or MutE). Also used in the first PCR of the selection protocol (step IV on Figure1B) in combination with primer F2.
R2	[**BIO**]AATGCGCCGCTACAGG	Reverse primer for the expression cassette amplification preceding selected NlaIV site. Used in libraries construction to produce the final library of the mutagenized expression cassettes in pair with primer F1. Also used in the second PCR of the selection protocol (step III on Figure 1B) in combination with primer R1. Contains biotin on the 5' end

Table 2

Reaction Compontents (final concentrations)	Initial denaturation	Numer of cycles	Amplification cycle	Final extension
A. Inside-ot PCR, step 1.5.1.				
Buffer: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM TrisHCl pH 8.8, 2 mM $MgSO_4$, 0.1 mg/ml BSA, 0.1% Triton X-100 primers, 1µM each Pfu polymerase, 2.5 u dNTPs, 0.35 mM Water (to 50 µl)	95°C, 1 min.	25	95°C, 30 sec. Tm-5°C, 30 sec. 72°C, 2 min./kb of product	72°C, 5 min.
B. Regular PCR, steps 3.1.1, 3.1.2,				
Buffer: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM TrisHCl pH 8.5, 2 mM $MgSO_4$, 0.1% Triton X-100 Primers, 1µM each Taq DNA polymerase, 1 u dNTPs, 0.2 mM Water (to 50 µl)	94°C, 1 min.	18-25	94°C, 30 sec. Tm-5°C, 30 sec. 72°C, 1 min./kb of product	72°C, 5 min.
C. Error-prone PCR, step 3A.3				
Buffer: 50 mM KCl, 7 mM $MgCl_2$, 10 mM TrisHCl, pH 8.3, 0.5 mM $MnCl_2$ Primers, 2 µM each Taq DNA polymearase, 5 u 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP Water to 100 µl	94°C, 1 min.	15	94°C, 30 sec. Tm-5°C, 30 sec. 72°C, 3 min.	None

Table 3

Position	Actual (designed) mutation frequency	Substitutions
Primer MutA: GATGTCGACATT**[XXX]**$_{36}$GTC**[XXX]**$_{38}$CAA**[XXX]**$_{39}$GACGTCGTCGGCAACATCATTCAG (n=18)		
I36	1 (1)	S,T,W_2,C,M_3,Q, A_2,L_2,R,$stop_3$
K38	0.56 (0.5)	M,S_2,E,R,C_2,T,A_2
K40	0.28 (0.5)	L,A,R,H,G
Primer MutE: TCCGGCCGGGTGTTTGGTAC**[XXX]**$_{176}$ATC**[XXX]**$_{178}$**[XXX]**$_{179}$**[XXX]**$_{180}$AATATGCCTATGTTTG, n=24		
S176	0.75 (0.8)	A_2,R_5,P_2,L_2,G_3,$stop_2$,I,D
N178	0.75 (0.8)	A_3,V_2,T_2,I,K,L,E,Y,C_2,R,P,S
K179	0.88 (0.8)	N,A_3,Q,P_3,Y_3,stop,S_2,G_3,L,R_2,W
K180	0.79 (0.8)	T,A_3,I,R_2,S,G_2,E_2,Q_2,N,L,W_2,Y

Table 4

Number of clones sequenced	22
Number of amino acids in ORF	251
Maximum number of affected codons in one clone	8
Number of clones with no amino acid changes	4 (18.1%)
Average number of amino acids substitutions per clone	3.36
Total number of affected codons	74
Frame shifts	3 (4.2%)
Stop codons (nonsense mutations)	8 (11.1%)
Synonimous codons (silent mutations)	6 (8.3 %)
Amino acid substitutions	57 (77.0%)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials Skowronek_Bochtler.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1000Å CPG Support (dA, dT, dC, dG)	Biosset	45-1000-050	Other vendors can be used as well
ASM-800 DNA/RNA	Biosset	800-001-000	
GeneJET Gel Extraction Kit	Thermo Scientific	K0691	Any other kit can be used
Glen-Pak DNA purification cartridge	Glen Research	60-5200	
HIS-Select Nickel Affinity Gel	Sigma	P6611	
pET 28a vector			Any other vector with T7 promoter upstream of plycloning site can be used instead
Phusion High-Fidelity DNA Polymerase	Thermo Scientific	F530S	Any other high fidelity and highly processive thermophilic polymearse can be used instead
Porous steel foil	Biosset	40-063	
Rapid Translation System RTS 100, E.coli HY Kit	Roche	3 186 148	
Restriction endonucleases	Thermo Scientific		Obviously other vendors, enzymes can be used
Streptavidin Magnetic Beads	New England Biolabs	S1420S	Other vendors can be used as well. We have positively tested beds form Sigma
Synthesis chemicals including phosphoramidities	Carl Roth		Other vendors can be used as well
Synthesis columns (different sizes)	Biosset		
T4 DNA ligase	Thermo Scientific	EL0011	Any other ligase can be used

Response to Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

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2. 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.
For example: CPG synthesis tubes, Eppendorf, Roche RTS 100 kit, HIS-Select, etc.

Done as suggested except for CPG. CPG stands for controlled pore glass and is not a trademark or company name but general type of support resin for oligonucleotide synthesis.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.”

Checked

4. The Protocol should contain only action items that direct the reader to do something.

Checked

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.
6. 1.1, 1.2, 1.4, 1.5.1: Please describe how is this done in brief or provide a citation

We have added all details that can be specified keeping the protocol general enough as different restriction enzymes and different vectors can be used in these steps.

7. Please include the size of the products wherever applicable?

We have added this information in Figure 6 and 7 legends. It was already present in Figure 5 legend.

8. There is a 10-page limit for the Protocol (including headings and spacings), but there is a 2.75-page limit for filmable content (including headings and spacings). 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.

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9. Please ensure that you describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. , e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

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We included proper citation of Figures 1 and 4 and Table 3 in the original submission. In the revised deposition we include permission from publisher of the source.

11. As we are a methods journal, please ensure that the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

Checked

12. Please sort the materials table in alphabetical order.

Done as suggested.

Response to referee comments:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a recent approach to specificity engineering of type II restriction endonucleases. The manuscript is well written, provides a detailed and effective protocol for a specificity altering and will be interesting for all molecular biologists, working with DNA-binding proteins. The protocol was confirmed, using as an example NlaIV restriction-modification system. My suggestion is to accept this article for publication without any changes. Only some typos should be corrected.

Major Concerns:

No

Minor Concerns:

Only some typos were observed.

Reviewer #2:

Manuscript Summary:

The manuscript by Skowronek and Bochtler entitled "In vitro directed evolution of a restriction endonuclease with altered specificity" describes a laborious but logical and effective strategy to increase the specificity of a restriction endonuclease be selecting enzyme variants that effectively cleave only a defined subset (generally a single desired target sequence) of the enzyme's initial group of related target sequences. The protocol appears to be described largely in terms of tightening the specificity of a symmetric type II restriction endonuclease, although it is not entirely clear if or why it could not also be applied towards asymmetric type II REases (type IIS, type IIG?) or even some type I or III systems.

The broader applicability of the approach is now discussed in the first paragraph of the Discussion. We are unsure of the applicability of the method to type I or type III systems, and therefore would prefer not to speculate about this point. All other aspects of broader applicability are now covered.

Major Concerns:

Overall this is a well-written manuscript that clearly describes the necessary steps of the protocol in sufficient detail to reproduce or adopt in the lab with experience and skill in restriction-modification systems; I could envision working up and succeeding with this protocol without undue problems beyond normal troubleshooting. I actually have no specific concerns or suggestions for improvement of the protocol itself (or its corresponding figures and tables) as they were easy to understand both in terms of their rationale and their execution at the bench. Therefore, the remainder of this evaluation is limited to questions regarding the presentation of the system in text that precedes the actual protocol.

1. The title, in my opinion, is misleading. I believe instead of 'altered specificity' it should read 'tightened specificity' or 'increased specificity'. To me, 'altered specificity' corresponds to a broader description that includes applications wherein an REase is altered in a manner such that a target site that cannot initially be cleaved by the enzyme becomes a valid substrate, either via a broadening of the enzyme's specificity (i.e. it still cleaves its original cognate targets) or via a clean shift of its specificity. Several of the citations (#'s 1 to 7) in the reference list that correspond to prior studies actually do achieve such alterations (in particular, the Mme system), whereas the current protocol is really limited to elimination of one or more initially recognized target site options and thereby reducing the enzyme's available substrates to few sequences than it initially could tolerate.

The title has been changed to “In vitro directed evolution of a restriction endonuclease with more stringent specificity.”

2. The introduction largely revolves around the tightening of specificity for an enzyme that normally recognizes a target site in a manner where multiple bases are tolerated in an entirely promiscuous fashion (i.e. turning NlaV from an enzyme that can cleave any sequence corresponding to 5' - GGNNCC - 3' into an altered enzyme that can cleave only 5' GGATCC - 3'), and also focuses largely on applying the strategy to homodimeric REases that provide the advantage of naturally maintaining a preference for palindromic targets. This is fine, but I would like to know whether the system could be used (for example) to eliminate of star activities (thereby creating 'high fidelity' REases) and/or employed towards REases that display recognition of asymmetric target sites via binding of enzyme monomeric subunits (for example, a type IIS enzyme such as FokI, or a type IIG enzyme such as BpuSI).

We agree with the referee that the general applicability of the protocol was insufficiently covered in the previous version of the manuscript. However, we also feel that to appreciate this outlook, the reader needs to first know the protocol. We have therefore decided to discuss the

broader applicability of the protocol not in the introduction, but in the first paragraph of the Discussion.

Minor Concerns:

1. The authors cite one study (reference 9, Miller et al. Nature Methods) to illustrate prior applications of in vitro translation and compartmentalization for REase engineering. That's ok, but I believe that it would be an improvement to cite two additional studies that have used IVC for restriction endonuclease or meganuclease engineering (Zheng and Roberts (2007) "Selection of restriction endonucleases using artificial cells" Nucleic Acids Res and Takeuchi et al. (2014) "Redesign of extensive protein-DNA interfaces of meganucleases using iterative cycles of in vitro compartmentalization" PNAS).

We have added these references as suggested.

2. In the second paragraph of the introduction, I think a paragraph break would be advisable at line 67, starting the new paragraph with "The outcome is easiest to predict…". I also feel that the text after that line is confusing, although I don't have a good suggestion for how to improve it (partly because I don't fully understand it). The authors should examine that section carefully, and perhaps have some colleagues read it and offer suggestions for improved clarity.

We rephrased this section from:

The outcome is easiest to predict when negative selection removes the complement of those sequences covered under the more narrow specificity. For example, selection for GGATCC could be combined with antiselection against GGBVCC (where B is any base other than A, and V is any base other than T). When the union of positively selected and negatively selected target sequences is smaller than the original set of target sequences, the outcome of the selection experiment depends on the effectiveness of positive and negative selection.

into:

The process is more efficient when negative selection is also used to remove the specificities able to cleave all sequences other than the preferred more narrow specificity. For example, selection for GGATCC could be combined with antiselection against GGBVCC (where B is any base other than A, and V is any base other than T). When some of the possible target sequences are not covered neither by positive nor by negative selection, the outcome of the experiment depends on the relative effectiveness of positive and negative selection.

We hope that now this section is more clear

3. In the first line of the third paragraph of the introduction (line 78) the authors should re-define 'ESC' upon its first use in the main text of the paper.

Done as suggested.

4. In the fourth line of the third paragraph of the introduction, (line 81) a colon or some other punctuation is needed after "WT REase". I suggest "The core is sandwiched between two cognate sites for WT REase: a cleavage site for the undesired activity……."

Done as suggested.

5. In the same paragraph two lines further down (line 83), a pair of 'the' are needed. I suggest "The final step of preparation of the ESC…."

Done as suggested.

6. "In vivo" and/or "in vivo" should be italicized throughout the text and consistently.

Done as suggested.

7. On line 90, 'seleted' is a misspelled word and should instead be 'selected'.

Done as suggested.

8. Line 93 states "The step removed inactive REase variants." This is true. However, if somehow a construct in the library actually had a fully altered target specificity (for example, shifting somehow from 5' - GGNNCC - 3' to 5' - GCNNGC -3') that would also be removed at this step, I believe. Perhaps this is worth mentioning?

Yes, but we feel that it would interrupt the flow of the argument in the Introduction. Instead, we prefer to discuss this point in the Discussion, in the third paragraph that is now dedicated to selection.

9. In the final paragraph of the introduction (lines 107 to 109) the authors recommend 'starting with a kinetic study of any pre-existing preferences'. I see their general point, but find this statement vague and not particularly useful. I would recommend that the authors provide a

simple example of the type of observation from such kinetic studies that would influence fundamental decisions about the type of experiment that they would actually choose to carry out with this protocol.

The use of a kinetic study to detect pre-existing preferences was demonstrated in our original NlaIV paper. We now reference this paper to make the recommendation more concrete.

Reviewer #3:

Manuscript Summary:

Skowronek et. al. describe a method to engineer restriction endonucleases exhibiting new specificities entitled "In vitro directed evolution of a restriction endonuclease with altered specificity." In the example described by the report, variants of the NlaIV restriction enzyme are evolved that acquire a more narrow specificity (although not a unique specificity) than that of the parental enzyme. A strength of the method is that it is sufficiently general that it can also be used to generate enzymes with unique specificities. Overall, the paper does a very good job of providing enough detail to replicate the selection of NlaIV variants, and it also provides some general directions for how to apply the method to other selection procedures that might be implemented. There are several steps to the method, and the authors describe tests that can be done to confirm that the intermediates that are being produced at various steps satisfy the necessary criteria required in order to have a successful final outcome.
At several places in the paper the writing was awkward and difficult to follow and further polishing would improve the manuscript. Only the most significant language issues and typographical errors are pointed out below. In addition, one figure is accidentally substituted with another.

Major Concerns:

1. Abstract and elsewhere: The authors explain that the selection step is being "reiterated." The term "reiterate" is not precisely correct as it is used here. In many other directed evolution studies, there are selections that are being reiterated since additional mutagenesis steps are being performed during each iteration of the selection. However, in this study, the second PCR reaction is actually being used to introduce a new selection, one against a different negative target. It is not being used to repeat the first selection. In essence, this protocol performs the selections in series rather than in parallel, as is often done. The word "reiteration" suggests that the same selection is being repeated, but that is not the case here.

The Discussion has been rewritten to make this point clearer. We now emphasize in the second paragraph that open reading frame diversity is generated only once, at the outset of the experiment. We draw attention to the fact that PCR does not play a role in generating open reading frame diversity. Moreover, we now discuss open reading frame diversity generation and selection in separate paragraphs. We hope that this way of presenting the protocol also helps to make it clearer that only the selection steps are repeated.

2. page 2, line 94-103 and page 4, line 187: The primers that are used to perform the selection are incorrectly cited in several places. The sentence in line 94 should read "In the first PCR reaction primers F2 and R1 are used.." In line 95 it should read "Primer F2 binds to the ESC section…" In line 98, "The primer R1 binds between the selected…" In line 101, it should read "(with primers F1 and R2)…" On page 4, it should read "Shorter version of this primer (F2) that covers…"

We are particularly thankful for this comment. We corrected this error in the revised text.

3. Figure 8: Figure 6 has been accidently used as Figure 8.

Actually it was Figure 5 that was deposited again as Figure 8. We have corrected this error in the revision.

Minor Concerns:

1. page 1, line 57 Indicate that "W" comprises "A" or "T" bases

Done as suggested.

2. page 1, line 78 Define ESC when it is first used

Done as suggested.

3. There is no reference in the text to Figure 6.

Figure 6 is referenced in step 5.10 of the Protocol.

4. page 10, line 473: "Distinguishable" should be used instead of "discernible"

Done as suggested.

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