Chapter 19

PERMutation Using Transposase Engineering (PERMUTE): A Simple Approach for Constructing Circularly Permuted Protein Libraries

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Abstract

Rearrangements that alter the order of a protein's sequence are used in the lab to study protein folding, improve activity, and build molecular switches. One of the simplest ways to rearrange a protein sequence is through random circular permutation, where native protein termini are linked together and new termini are created elsewhere through random backbone fission. Transposase mutagenesis has emerged as a simple way to generate libraries encoding different circularly permuted variants of proteins. With this approach, a synthetic transposon (called a permuteposon) is randomly inserted throughout a circularized gene to generate vectors that express different permuted variants of a protein. In this chapter, we outline the protocol for constructing combinatorial libraries of circularly permuted proteins using transposase mutagenesis, and we describe the different permuteposons that have been developed to facilitate library construction.

Key words Circular permutation, Library, Protein engineering, Transposase, Transposon

1 Introduction

Mutational processes that alter protein length (fission and domain insertion) and contact order (circular permutation) are frequently used to develop biotechnologies for systems and synthetic biology [1, 2]. However, our understanding of sequence–structure–function relationships is not yet sufficient to predict the effects of these mutational lesions on protein structure and function [3]. Because it is hard to anticipate how the structure of marginally stable proteins responds to mutational lesions, combinatorial libraries of vectors encoding mutants are typically generated and screened (or selected) to discover proteins with the desired functional properties [4]. Libraries of vectors encoding circularly permuted proteins have traditionally been generated by manipulating protein-coding sequences using nonspecific nucleases, such as DNAse I [5, 6]. While these efforts have led to the discovery of functional proteins, nuclease-based protocols can be arduous to learn and implement

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because nuclease incubation times must be optimized, and low efficiency blunt cloning is used within the library construction workflow. In addition, vectors created in these libraries express protein variants that often have deletions and duplications of DNA sequence [7–9], which may not be desired. One way to avoid deletions and duplications is to use methods that leverage transposases [10]. Unlike nucleases, transposases generate well-defined DNA modifications that do not result in sequence deletions.

Transposase methods have emerged as a simple strategy to create libraries encoding proteins with random amino acid deletions [11], backbone cleavages [12–14], peptide and domain insertions [15, 16], affinity tag insertions [17], and truncations [18]. With these library construction approaches, the MuA transposase is used to insert synthetic transposons at different locations within a gene encoding the protein of interest. Recently, a transposase approach was described for creating circularly permuted protein libraries that is called PERMutation Using Transposase Engineering, PERMUTE [19]. In PERMUTE, a new class of transposons (called *permutetransposons*) are used with MuA transposase to generate an expression vector library. A permuteposon is linear DNA that contains all of the attributes of an expression vector (*see* Fig. 1a), including an origin of replication (ori), a selectable marker (Ab^R-1), and regulatory sequences required to initiate the transcription and translation of permuted proteins. Permuteposons additionally contain MuA transposase binding sites located at the ends of the transposon (R1R2 and R2R1), which allow for MuA recognition and insertion into the protein coding sequence being permuted [10]. In the first step of constructing a circularly permuted protein library, MuA is used to insert a permuteposon into a target vector (*see* Fig. 1b) that contains the gene of interest (GOI), i.e., the gene being permuted. To isolate gene–permuteposon hybrids from the resulting MuA library, the product of the MuA reaction is incubated with a restriction enzyme (RE1) that cuts at sites flanking the gene of interest in the target vector. The gene–permuteposon hybrids are then separated from genes lacking a permuteposon using gel electrophoresis, and those genes containing a single permuteposon are purified and circularized by ligation to yield the final library of vectors that express different permuted variants of a protein [19]. PERMUTE creates well-defined sequence diversity equal to the gene length times two, since permuteposons can be inserted at any location and in two orientations.

2 Materials

All reactions should use molecular biology grade water that is DNAse- and protease-free and has been filtered through a 0.2 μm filter and autoclaved (*see* **Note 1**).

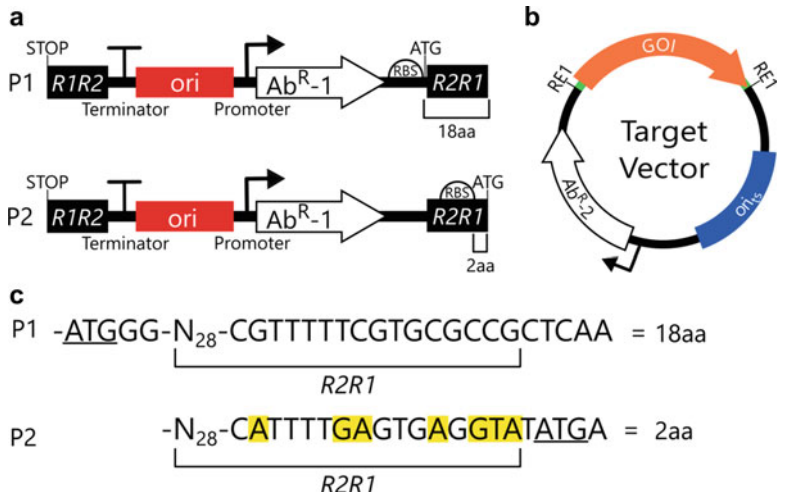

Fig. 1 DNA constructs required to perform PERMUTE. (**a**) The permuteposon serves as an expression vector for permuted proteins in the final PERMUTE libraries. This linear DNA must contain an origin of replication (ori) that functions at any *E. coli* growth temperature and a selectable marker (Ab^R-1) that is distinct from the target vector. Additionally, the permuteposon must contain MuA transposase recognition sequences (R1R2 and R2R1) at each end and regulatory sequences that allow for transcription and translation of the permuted ORFs created by PERMUTE, including a promoter, RBS, and terminator. The RBS can precede R2R1 as shown for permuteposon 1 (P1) or be embedded within R2R1 as illustrated for permuteposon 2 (P2). (**b**) The gene of interest (GOI) encoding the protein being permuted is cloned into a target vector, which must contain a temperature-sensitive origin of replication (ori_{ts}). In this vector, the GOI should be flanked on both sides by the same restriction site (RE1) and should lack a stop codon. Extra base pairs should be included between RE1 and the GOI, which encode the linker used for permutation. The number of base pairs in the linker can vary, but the number of base pairs in the linker and restriction site must add up to a number that is divisible by 3. The target vector must also contain a selectable marker (Ab^R-2), which differs from that in the permuteposon. (**c**) R2R1 sequences can be used that lack or contain an RBS, which differ in the location of the first codon used for translation initiation (ATG). When the RBS sequence precedes R2R1 (P1), the number of residues added to the termini of permuted proteins is large. However, this design allows flexibility in RBS sequence. In contrast, R2R1 containing mutations (*highlighted*) that incorporate an RBS into the transposase binding site minimize peptide additions (P2)

2.1 Nucleic Acids

1. Temperature-sensitive target vector containing the gene being permuted (*see* **Note 2**): 600 ng (*see* **Note 3**). As illustrated in Fig. 1b, the gene being permuted must be flanked by the sequence that encodes a peptide linker and restriction sites (RE1) that allow for gene fragment ligation in the last step of PERMUTE (*see* **Note 4**). In the final library, the linker encodes the region that covalently connects the original N- and C-termini of the protein.

2. Permuteposon: 200 ng (*see* **Note 5**). As shown in Fig. 1a, permuteposons require an origin of replication (*see* **Note 6**), a selectable marker (*see* **Note 7**), two transposase binding sites (*see* **Note 8**), a promoter for transcribing permuted genes (*see* **Note 9**), a stop codon followed by a transcriptional terminator for permuted genes (*see* **Note 10**), and a ribosome binding site (RBS) for initiating translation of permuted proteins (*see* **Note 11**).

2.2 Transposase Reaction

1. PCR tube: one sterile tube.
2. Target vector: 600 ng per 40 μL reaction (*see* **Note 12**).
3. Linearized permuteposon: 200 ng per 40 μL reaction (*see* **Note 13**).
4. MuA transposase: 0.44 μg per 40 μL reaction (*see* **Note 14**).
5. MuA reaction buffer: 8 μL of a 5× stock containing 125 mM Tris–HCl, pH 8.0, 50 mM $MgCl_2$, 550 mM NaCl, 0.25% Triton X-100, and 50% glycerol.
6. Thermal cycler (*see* **Note 15**).
7. DNA purification kit: Zymo DNA Clean and Concentrator kit or equivalent kit from another manufacturer that yields high-quality, purified DNA (*see* **Note 16**).

2.3 Cellular Materials

1. Electrocompetent *Escherichia coli*: 50 μL of cells (*see* **Note 17**) and 1 mL of recovery medium (*see* **Note 18**).
2. Electroporation cuvette and electroporator.
3. Culture tube: sterile 14 mL tube.
4. Luria broth (LB) agar plates: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl containing 1.5% agar. Adjust to pH 7.0 using 5 N NaOH. Five petri plates (70 mm diameter) containing 25 mL LB-agar and two antibiotics, one that selects for the permuteposon and one that selects for the target vector (*see* **Note 19**).
5. Shaking incubator: set to 37 °C and 250 rpm (*see* **Note 20**).
6. Gravity incubator: set to 37 °C or higher (*see* **Note 21**).

2.4 Vector Harvesting

1. LB medium: 10 mL for harvesting cells from five LB-agar plates (*see* **Note 22**).
2. Sterile spreader.
3. Tubes: sterile 14 mL tube (*see* **Note 23**) and a 0.5 mL PCR tube.
4. DNA purification kit such as the Qiagen Miniprep Kit (*see* **Note 24**).
5. Restriction enzyme (RE1) and appropriate buffer (*see* **Note 25**).

6. Agarose gel and casting unit. Typically run agarose gels in Tris–acetate–EDTA (TAE) buffer (*see* **Note 26**) and 1 μg/mL ethidium bromide (*see* **Note 27**).
7. Gel loading dye: 4 μL of 6× stock containing 15% Ficoll®-400, 66 mM EDTA, 20 mM Tris–HCl, pH 8.0, 0.1% SDS, and 0.09% bromophenol blue (*see* **Note 28**).
8. Gel box and transilluminator (*see* **Note 29**).
9. DNA recovery kit such as Zymo Gel DNA Recovery kit (*see* **Note 30**).
10. Incubator or heat-block: set to 37 °C.

2.5 DNA Ligation

1. PCR tube: one sterile tube.
2. Gene–permuteposon hybrids: 100 ng for 20 μL ligation reaction (*see* **Note 31**).
3. T4 DNA ligase: 400 U per 20 μL reaction (*see* **Note 32**).
4. T4 DNA ligase buffer: 2 μL of 10× stock containing 500 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP (*see* **Note 33**).
5. Thermal cycler: set to 16 °C.
6. DNA purification kit such as Zymo DNA Clean and Concentrator kit (*see* **Note 16**).

2.6 Quality Control

1. Electrocompetent *Escherichia coli*: 50 μL of cells (*see* **Note 17**) and 1 mL of recovery medium (*see* **Note 18**).
2. Electroporation cuvette and electroporator.
3. Culture tube: sterile 14 mL tube.
4. LB agar plates: five petri plates (70 mm diameter) containing 25 mL LB-agar and antibiotic (Ab^R-1) that selects for the final library.
5. Shaking incubator: set to 37 °C and 250 rpm.
6. Gravity incubator: set to 37 °C.
7. LB medium: 10 mL for harvesting cells from five LB-agar plates (*see* **Note 22**).
8. Sterile spreader.
9. Culture tube: sterile 14 mL tube (*see* **Note 23**).
10. DNA purification kit such as the Qiagen Miniprep kit (*see* **Note 24**).
11. Two restriction enzymes, which cut at sites RE1 and RE2 and buffers (*see* **Note 34**).
12. Agarose gel: cast using TAE buffer and 1 μg/mL ethidium bromide.
13. Gel-loading dye: 12 μL of 6× stock.
14. Gel box and transilluminator.

3 Methods

The protocol described below requires 2 days to produce a library of vectors that expresses the different possible circularly permuted variants of a protein (*see* Fig. 2).

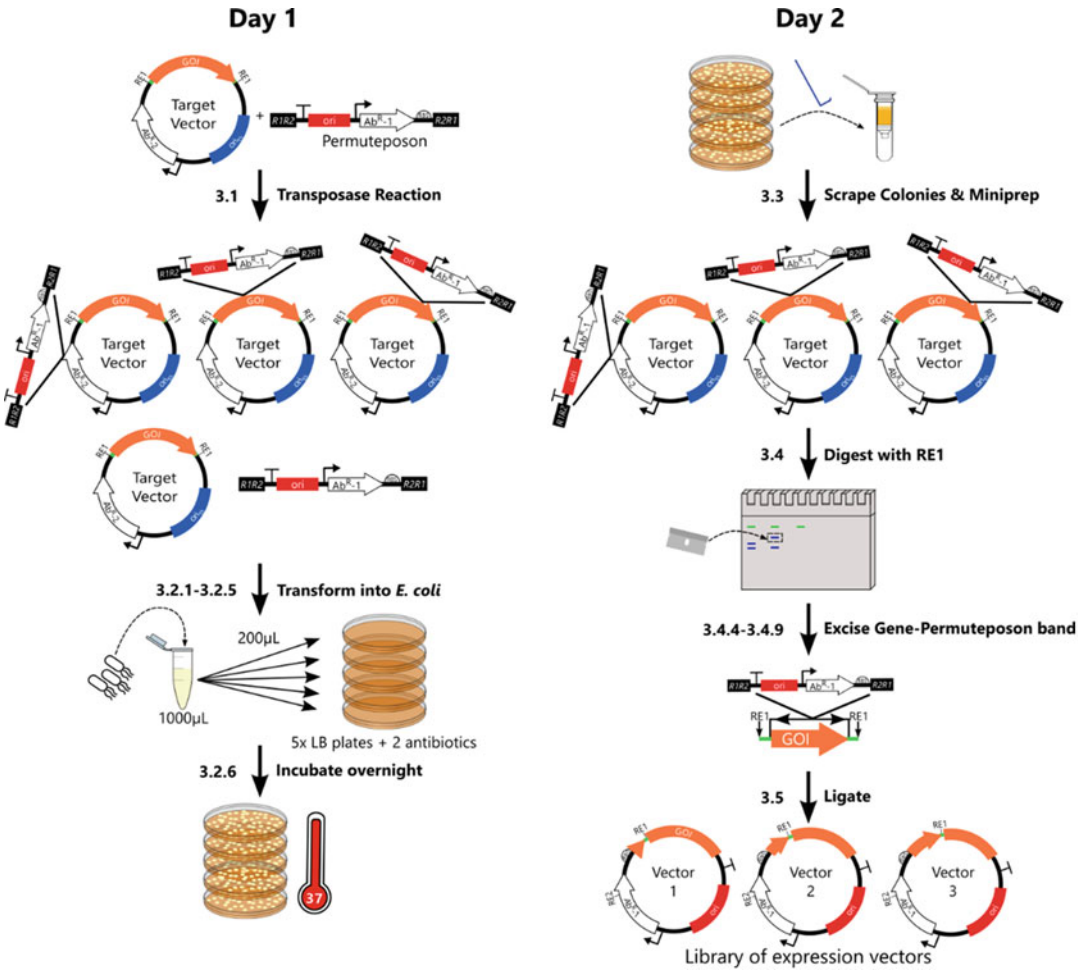

Fig. 2 Scheme for PERMUTE. First, a permuteposon that will ultimately serve as an expression vector for each permuted protein is inserted into a temperature-sensitive target vector containing a gene of interest (GOI) using MuA transposase. The vectors generated by this reaction have the permuteposon inserted at different locations. Second, the DNA product of the MuA reaction (which includes permuteposon, target vector and vector–permuteposon hybrids) is transformed into bacteria and plated on LB-agar containing a pair of antibiotics (Ab-1 and Ab-2) that select for cells containing a permuteposon and target vector. Third, to amplify cells with the vector–permuteposon hybrids, these plates are incubated at a high temperature where the target vector origin of replication (ori_{ts}) cannot function. Fourth, colonies are harvested from plates after a day of growth, and plasmid DNA is purified from this cell mixture. Fifth, the purified DNA is digested using an enzyme (RE1) that cuts at sites adjacent to the gene of interest. Gene–permuteposon hybrids are separated by agarose gel electrophoresis and the gene–permuteposon hybrids are purified using a DNA recovery kit. Finally, the gene–permuteposon hybrids are circularized through ligation to generate the final library of vectors that express different circularly permuted variants

3.1 MuA Transposase Reaction

1. In the PCR tube, make up a 38 µL reaction using 8 µL 5× MuA Reaction Buffer, 600 ng target vector, 200 ng linear permuteposon, and water.
2. Add 0.44 µg MuA Transposase (2 µL) and mix gently by pipetting (*see* **Note 35**).
3. Incubate the reaction in a thermal cycler for 16 h at 37 °C followed by 10 min at 75 °C to inactivate the enzyme.
4. Purify the DNA from the reaction using a DNA cleanup kit (e.g., Zymo DNA Clean and Concentrator kit) and elute with 6 µL of water (*see* **Note 16**).

3.2 Vector–Permuteposon Hybrids

1. Add all of the DNA purified from the MuA reaction from **step 4** in Subheading 3.1 to 50 µL *E. coli* cells on ice and mix gently by pipetting.
2. Transfer the cell-DNA mixture to a chilled electroporation cuvette and electroporate using 2 kV (*see* **Note 36**).
3. Add 1 mL of recovery medium (e.g., SOC medium) to the cuvette, mix by pipetting, and transfer the slurry to a sterile 14 mL culture tube.
4. Incubate culture for 1 h at 37 °C while shaking at 250 rpm (*see* **Note 20**).
5. Spread 200 µL aliquots of the cells on each LB-agar plate (*see* **Note 37**).
6. Incubate plates overnight at 37 °C to obtain colonies (*see* **Note 21**).

3.3 Vector–Permuteposon Library

1. Add 1 mL of LB medium to each agar plate (*see* **Note 22**).
2. Generate a bacterial slurry by gently scraping plates with a sterile spreader.
3. Angle plates so that the cell slurry pools on one side (*see* **Note 38**).
4. Pool the cell slurries from each plate into a 14 mL culture tube (*see* **Note 39**).
5. Cap the 14 mL tube containing the cell slurry and invert to mix.
6. Purify vectors from the pooled cells using a Qiagen Miniprep kit. Only 20% of the recommended culture volume (400 µL slurry) should be used in each miniprep (*see* **Note 40**).
7. Determine the concentration of purified DNA (*see* **Note 41**).

3.4 Gene–Permuteposon Hybrids

1. Digest 500 ng of the purified DNA from **step 6** in Subheading 3.3 in a PCR tube using 1 unit of a restriction enzyme (RE1) that cuts at sites flanking both sides of the GOI (*see* **Note 42**). This reaction should be performed in a 20 µL volume using water and the buffer provided with the restriction enzyme.

2. Incubate the restriction digest reaction at 37 °C for 1 h.
3. Heat-inactivate the restriction enzyme as recommended by the manufacturer.
4. Add 4 μL of 6× gel loading dye to the digested product.
5. Separate the DNA products of the restriction digest using agarose gel electrophoresis (*see* **Note 43**).
6. Visualize the gel using a transilluminator.
7. Use a clean razor blade to excise the band corresponding to the size of the gene–permuteposon hybrid.
8. Recover the gene–permuteposon DNA using a DNA clean up kit such as the Zymo Gel DNA Recovery kit and elute the DNA using 6 μL sterile water two times.
9. Quantify the gene–permuteposon hybrid concentration (*see* **Note 44**).

3.5 Circularize Gene–Permuteposon Hybrids

1. In a sterile PCR tube, make a 19 μL reaction containing 2 μL 10× T4 DNA ligase buffer, all of the DNA recovered from the gel in **step 8** in Subheading 3.4, and water to 19 μL.
2. Mix the reaction thoroughly and then add 1 μL of T4 DNA ligase (400 U/μL).
3. Incubate the ligation reaction at 16 °C for 16 h.
4. Purify the DNA with a DNA purification kit such as Zymo DNA Clean and Concentrator kit and elute with 6 μL of water (*see* **Note 45**).

3.6 Quality Control

1. Add 6 μL of the ligated DNA from **step 4** in Subheading 3.5 into an 1.5 mL Eppendorf tube containing 50 μL electrocompetent *E. coli* cells on ice and mix by pipetting.
2. Transfer the cell-DNA mixture to a chilled electroporation cuvette and electroporate at 2 kV (*see* **Note 36**).
3. Add 1 mL of recovery medium (e.g., SOC medium) to the cuvette, mix by gently pipetting, and transfer the slurry to a sterile 14 mL culture tube.
4. Incubate the culture for 1 h at 37 °C while shaking at 250 rpm.
5. Spread 200 μL aliquots of the transformation mix onto each LB-agar plate.
6. Incubate plates overnight at 37 °C to obtain colonies.
7. Use the protocol outlined in Subheading 3.3 above to harvest the final plasmid library, which encodes expression vectors for different permuted variants of the gene of interest.
8. Digest 200 ng of the final library with a restriction enzyme that cuts the restriction site (RE1) that was ligated to create the final library using a 20 μL reaction (*see* **Note 46**).

9. Digest 200 ng of the final library with a restriction enzyme (RE2) that cuts at a single site within the permuteposon using a 20 μL reaction (*see* **Note 47**).
10. Digest 200 ng of the final library with the two restriction enzymes (RE1 and RE2) in parallel using a 20 μL reaction (*see* **Note 48**).
11. Add 4 μL of 6× gel-loading dye to each reaction.
12. Separate the DNA products of the restriction digests using agarose gel electrophoresis (*see* **Note 43**).
13. Visualize the gel using a transilluminator.

4 Notes

1. Commercial molecular biology grade water is recommended.
2. The gene of interest must lack a stop codon so that permuted open reading frames can be fully translated.
3. A vector with a temperature-sensitive origin of replication (ori_{ts}) allows for selection of vector–permuteposon hybrids at high temperatures where the ori_{ts} cannot function. A method for using a circularized gene as an alternative was recently described [20].
4. Structural information should be used to estimate a linker length that is sufficiently long enough to enable proper folding of permuted proteins. In addition, glycine/serine-rich sequences encoding flexible linkers are recommended [2].
5. Permuteposon sequence information was previously described [19]. All aspects of a permuteposon can be customized, provided that functional transposase binding sites are included in the design (*see* Fig. 1a).
6. Avoid using the same origin of replication in the permuteposon and target vector to minimize undesired recombination events.
7. Use distinct selectable markers in the permuteposon and target vector to allow for selection of vector–permuteposon hybrids using two antibiotics (Ab^R-1 and Ab^R-2).
8. Two sequences of transposase binding sites have been reported (*see* Fig. 1c). In the first transposon described [19], the RBS used to initiate translation is separated from the open reading frame of permuted proteins by the transposase binding site. This separation results in the addition of a large 18 amino acid peptide (MGFRIYRETLSRFSCAAQ) at the N-terminus of each permuted variant, which is encoded by the transposase binding site. More recently, a transposase binding site was described that allows for translation initiation closer to the gene sequence encoding the protein variant [13]. With this

transposon, only two extra residues are amended to the N-terminus of proteins expressed from the transposon after insertion into the gene of interest, a methionine followed by a residue whose identity varies depending on the sequence at the site of permuteposon insertion.

9. Regulated or constitutive promoters can be used to control transcription of the permuted genes.
10. A terminator should be incorporated into the region of the permuteposon that follows the stop codon. This terminator reduces the plasmid burden on cell growth caused by unnecessary transcription.
11. When using transposase binding sites (*see* Fig. 1c) that contain mutations that introduce an RBS [13], it is important to use a strong promoter because translation initiation will vary with each permuted protein due to changes in the genetic context of the RBS.
12. Temperature-sensitive vectors frequently acquire mutations, so it is important to perform all cloning at temperatures that avoid selective pressure on this phenotype. Additionally, it is important to run controls to verify that the vector preparation used for cloning retains the temperature sensitive phenotype.
13. Permuteposons can be amplified like vectors. This can be achieved by flanking the terminal transposase binding sites with a unique restriction enzyme [19] and circularizing the permuteposons through ligation. However, circular permuteposons must be linearized by restriction digestion to serve as a substrate for MuA.
14. MuA transposase can be purchased with the 5× reaction buffer from Thermo Scientific (Catalog No. F-750).
15. A thermal cycler is recommended to provide fine control over the reaction temperature and the heat inactivation of the enzyme.
16. To elute DNA from the spin columns, molecular biology grade water is recommended rather than the DNA elution buffer provided with the kit. This allows for electroporation of the eluted DNA without further manipulation.
17. Library-quality competent cells (Invitrogen MegaX DH10B; Catalog No. C6400-03) are recommended to ensure that sequence diversity is not limited by the transformation.
18. Recovery medium provided with commercial cells is recommended to maximize transformation efficiency.
19. Multiple plates are recommended to allow for sufficient separation of the colonies and counting. The DNA sequence diversity depends on the number of transformants obtained at each step and can be limited by poor transformations.

20. The shaking incubator should be set to a temperature that selects against the temperature-sensitive vector.
21. The gravity incubator temperature must be sufficient to select against a temperature-sensitive vector lacking an inserted permuteposon. Control experiments that examine the colony counts obtained from transforming a target vector alone are recommended to verify the temperature is sufficiently high to select against the temperature-sensitive vector.
22. Prior to scraping colonies from plates, sterile liquid should be added to each plate. The addition of liquid makes it easier to remove the scraped cells by pipetting. If cells are not harvested immediately after the incubation, colonies can harden and become challenging to scrape off plates.
23. Cells harvested from each plate should be pooled into one tube.
24. Water should be used rather than the buffer provided with the kit to minimize the salt concentration in the eluted DNA, since high salt levels can inhibit the activity of some restriction enzymes.
25. A restriction enzyme (RE1) is needed that cuts at sites adjacent to the 5′ and 3′ ends of the gene being permuted. An enzyme should be used that does not cut anywhere else in the target vector, gene, or permuteposon.
26. An agarose percentage is recommended that enables separation of the different DNA products generated by a digest with the restriction enzyme (RE1).
27. Alternative dyes can be used, such as SYBR® Stains.
28. Commercial gel loading dye is recommended (New England Biolabs; Catalog No. 7021S).
29. Gel visualization tools should be used that are compatible with the DNA visualization dye.
30. Water is used rather than the buffer provided by the manufacturer to minimize the salt concentration in the eluted DNA, which can affect the ligation reaction.
31. Lower concentrations of gene–permuteposon hybrids can be used, but this could limit the sequence diversity sampled.
32. T4 DNA ligase from New England Biolabs (Catalog No. M0202S) is recommended, which is provided at a concentration of 400 U/μL.
33. To maximize ligation efficiency, a ligase buffer should be used that has not been subjected to multiple freeze-thaw cycles.
34. Two enzymes are required for quality control (*see* Fig. 3): (1) RE1, which cuts at a single unique site in the gene being

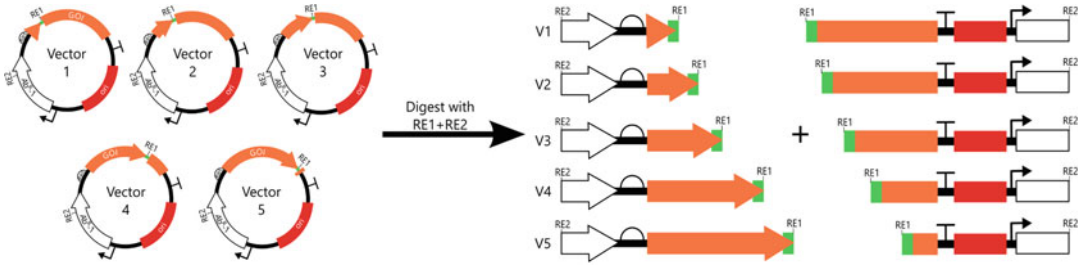

Fig. 3 A scheme illustrating how to assess PERMUTE library quality. All of the variants contain two restriction sites: (1) RE1, which is found at a unique location within each of the permuted genes, and (2) RE2, which is found at one location within the permuteposon backbone. RE2 should be identified before beginning the library construction. A double digest of the final PERMUTE library with RE1 and RE2 will yield DNA smears that vary by a length that corresponds to the number of base pairs in the GOI. In contrast, digestion of the final PERMUTE library with each restriction site individually will yield linear DNA, which has a size corresponding to the length of the GOI plus the length of the permuteposon

permuted, and (2) RE2, which cuts at a single unique site in the permuteposon.

35. The activity of MuA can be calibrated by analyzing the activity using a commercially available transposon (Entranceposons from Thermo Scientific) with a selectable marker that is not present in the target vector.
36. The kV setting on the electroporator should match the value recommended by the electrocompetent cell manufacturer.
37. LB agar plates should be dried sufficiently to remove any excess water on the surface, which can cause colony smearing upon incubation.
38. Elevating one end of the plate 1 cm above the other end should be sufficient.
39. If cells are not fully removed by the protocol, then add 1 mL of LB medium to each plate, scrape, and remove additional liquid.
40. Scraped cells are typically more concentrated than cells from liquid cultures, so cell volumes should be reduced to those as recommended by the manufacturer.
41. DNA concentration can be quantified using a spectrophotometer (e.g., a NanoDrop) or by gel electrophoresis.
42. Permuteposons are randomly inserted into the target vector. In some vectors, they will be found within the GOI (*see* Fig. 2), while other vectors will contain a permuteposon within the vector backbone. Because transposons are randomly inserted, restriction enzyme digestion using RE1 will yield four DNA products: (1) a gene lacking a permuteposon, (2) a gene containing a permuteposon, (3) a target vector backbone lacking a

permuteposon, and (4) a target vector backbone containing a permuteposon.

43. A DNA ladder should be used to provide a frame of reference when performing the size-selection step. The goal of this step is to purify the band corresponding to the gene–permuteposon hybrid.
44. The desired yield is 100 ng of gene–permuteposon.
45. The DNA obtained from this purification can be screened or selected for variants with desired functional properties, although quality control is recommended to ensure the desired sequence diversity was generated.
46. Any restriction enzyme can be used for RE1, provided that it only cuts at one unique site within the gene that was permuted. This digest should yield a single product.
47. Any restriction enzyme can be used for RE2, provided that it cuts at one unique site within the permuteposon and does not cut within the gene that was permuted. This digest should yield a single product.
48. The double digest at RE1 and RE2 should yield products of varying size, resulting in a smear on the gel, because the distance between the two restriction sites will vary with each of the permuted genes (*see* Fig. 3).

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References

1. Ostermeier M (2009) Designing switchable enzymes. Curr Opin Struct Biol 19:442–448
2. Yu Y, Lutz S (2011) Circular permutation: a different way to engineer enzyme structure and function. Trends Biotechnol 29:18–25
3. Liberles DA, Teichmann SA, Bahar I, Bastolla U, Bloom J, Bornberg-Bauer E, Colwell LJ, de Koning APJ, Dokholyan NV, Echave J et al (2012) The interface of protein structure, protein biophysics, and molecular evolution. Protein Sci 21:769–785
4. Zhao H, Arnold FH (1997) Combinatorial protein design: strategies for screening protein libraries. Curr Opin Struct Biol 7:480–485
5. Graf R, Schachman HK (1996) Random circular permutation of genes and expressed polypeptide chains: application of the method to the catalytic chains of aspartate transcarbamoylase. Proc Natl Acad Sci U S A 93:11591–11596
6. Hennecke J, Sebbel P, Glockshuber R (1999) Random circular permutation of DsbA reveals segments that are essential for protein folding and stability. J Mol Biol 286:1197–1215
7. Guntas G, Ostermeier M (2004) Creation of an allosteric enzyme by domain insertion. J Mol Biol 336:263–273
8. Hida K, Won SY, Di Pasquale G, Hanes J, Chiorini JA, Ostermeier M (2010) Sites in the

AAV5 capsid tolerant to deletions and tandem duplications. Arch Biochem Biophys 496:1–8

9. Judd J, Wei F, Nguyen PQ, Tartaglia LJ, Agbandje-McKenna M, Silberg JJ, Suh J (2012) Random insertion of mCherry into VP3 domain of adeno-associated virus yields fluorescent capsids with no loss of infectivity. Mol Ther Nucleic Acids 1:e54
10. Haapa S, Taira S, Heikkinen E, Savilahti H (1999) An efficient and accurate integration of mini-Mu transposons in vitro: a general methodology for functional genetic analysis and molecular biology applications. Nucleic Acids Res 27:2777–2784
11. Jones DD (2005) Triplet nucleotide removal at random positions in a target gene: the tolerance of TEM-1 beta-lactamase to an amino acid deletion. Nucleic Acids Res 33:e80
12. Segall-Shapiro TH, Nguyen PQ, Dos Santos ED, Subedi S, Judd J, Suh J, Silberg JJ (2011) Mesophilic and hyperthermophilic adenylate kinases differ in their tolerance to random fragmentation. J Mol Biol 406:135–148
13. Segall-Shapiro TH, Meyer AJ, Ellington AD, Sontag ED, Voigt CA (2014) A 'resource allocator' for transcription based on a highly fragmented T7 RNA polymerase. Mol Syst Biol 10:742
14. Pandey N, Nobles CL, Zechiedrich L, Maresso AW, Silberg JJ (2014) Combining random gene fission and rational gene fusion to discover near-infrared fluorescent protein fragments that report on protein-protein interactions. ACS Synth Biol. 4:615–624. doi:10.1021/sb5002938
15. Poussu E, Vihinen M, Paulin L, Savilahti H (2004) Probing the alpha-complementing domain of *E. coli* beta-galactosidase with use of an insertional pentapeptide mutagenesis strategy based on Mu *in vitro* DNA transposition. Proteins 54:681–692
16. Edwards WR, Busse K, Allemann RK, Jone DD (2008) Linking the functions of unrelated proteins using a novel directed evolution domain insertion method. Nucleic Acids Res 36:e78
17. Hoeller BM, Reiter B, Abad S, Graze I, Glieder A (2008) Random tag insertions by Transposon Integration mediated Mutagenesis (TIM). J Microbiol Methods 75:251–257
18. Poussu E, Jäntti J, Savilahti H (2005) A gene truncation strategy generating N- and C-terminal deletion variants of proteins for functional studies: mapping of the Sec1p binding domain in yeast Mso1p by a Mu in vitro transposition-based approach. Nucleic Acids Res 33:e104
19. Mehta MM, Liu S, Silberg JJ (2012) A transposase strategy for creating libraries of circularly permuted proteins. Nucleic Acids Res 40:e71
20. Pandey N, Kuypers BE, Nassif B, Thomas EE, Alnahhas RN, Segatori L, Silberg JJ (2016) Tolerance of a Knotted Near-Infrared Fluorescent Protein to Random Circular Permutation. Biochem 55:3763–3773