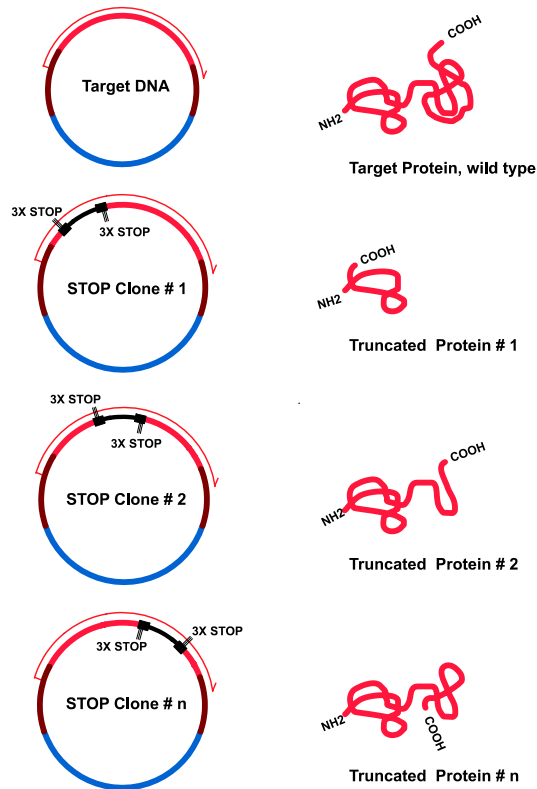


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## Introduction

The Stop Generation System™ is a transposon-based tool for functional analysis of proteins. The kit contains components for performing transposition reactions in which MuA Transposase inserts a copy of an artificial DNA transposon, Entranceposon, into target plasmid DNA. The Entranceposon included in the STOP™ kit contains translational stop codons in all three reading frames within the terminal portion of the transposon sequence\*. The proprietary sequence modification makes it possible to generate a saturated C-terminal deletion library virtually from any target protein.



Protein translation from the DNA clones that contain an Entranceposon (STOP) inside the coding region terminates at the site of the insertion. As a result, truncated versions of the target protein are formed.

\*Patent pending

## Strategy Outline









1. Purify your target DNA *i.e.* a plasmid carrying the gene in which you plan to insert the translational stop codons.

*Note:* The Stop Generation System™ kit can be used also to insert Entranceposons into linear target DNA (e.g. restriction fragment or PCR product). A slightly modified reaction protocol is available at [www.finnzymes.fi](http://www.finnzymes.fi).

2. Perform the transposition reaction.
3. Transform competent *E. coli* cells with the transposition reaction mixture using either chemical transformation or electroporation.
4. Grow transformants on selection plates.
5. *Optional:* Map the Entranceposon insertion sites in the clones by colony-PCR.  
*Note:* The PCR products from the mapping reactions can also be used directly as templates for DNA sequencing reactions.
6. *Optional:* Use primers SeqE and SeqW for DNA sequencing in order to map the insertion sites at the base pair level.
7. Make plasmid preps from the clones (either individual mapped or unmapped clones or pooled clones) and analyze them in an expression system that is specific to your target protein.

## Kit Components

The F-703 Stop Generation System™ kit contains sufficient materials for 10 reactions.

|  |        |   |
|--|--------|---|
| Entranceposon (STOP-Kan <sup>R</sup> )   | 10 µl  |    |
| See Appendix II (page 12) or < <a href="http://www.finnzymes.fi">www.finnzymes.fi</a> > for a detailed map and sequence. |        |   |
| MuA Transposase  | 10 µl  |    |
| 5X Reaction Buffer for MuA Transposase   | 100 µl |    |
| DMSO 100%  | 500 µl |   |
| Control Target DNA   | 10 µl  |  |
| Primers:   |        |   |
| MuEnd-2 Primer   | 50 µl  |  |
| SeqE Primer  | 250 µl |  |
| SeqW Primer  | 250 µl |  |

Store the components at -20°C.

## User Supplied Materials

Target DNA:

Use 60 fmoles of target DNA per a 20- $\mu$ l reaction.

The amount equals approximately 40 ng DNA per kb of target.

*Example:* Your target DNA plasmid consists of a 3-kb insert cloned into a 2.8-kb vector. The size of the target plasmid is 3 kb + 2.8 kb = 5.8 kb.

The optimal amount of target DNA per reaction is: 5.8 kb  $\times$  40 ng/kb = 232 ng

Therefore you should use 200-300 ng of the target plasmid per reaction.

Purify target DNA using standard methods such as alkaline lysis or commercial DNA purification kits. Target DNA should be in a low-salt buffer such as 1X TE buffer or in deionized water.

*Important:* Make sure that your target DNA plasmid does not contain a kanamycin selection marker gene.

*Note:* Linear DNA molecules (e.g. restriction fragments or PCR products) serve also efficiently as transposition target DNA. A slightly modified protocol for linear target DNA is available at:

[http://www.finnzymes.fi/products/transposon\\_products/applications\\_linear\\_target\\_dna.htm](http://www.finnzymes.fi/products/transposon_products/applications_linear_target_dna.htm)

Competent cells:

Any standard *E. coli* strain that is suitable for DNA cloning can be used as a transformation host.

Both electrocompetent and chemically competent *E. coli* cells may be used.

*Important:* Make sure that the *E. coli* strain is not resistant to kanamycin.

*Important:* Make sure that the *E. coli* strain is compatible with the target DNA plasmid. The transformation efficiency must be at least  $10^6$  cfu/  $\mu$ g target DNA plasmid in order to obtain thousands of insertion clones from transposition reaction.

Thermal cycler or heat blocks, 30°C and 75°C

SOC medium and LB agar plates with antibiotics. See Appendix V (page 15) for the media recipes

*Optional:* Reagents and equipment for PCR mapping

*Optional:* Reagents and equipment for DNA sequencing

## Transposition Reaction

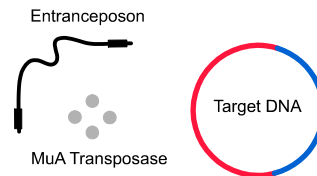
1. Set up the following reaction:

*Important.* MuA Transposase should be added last.

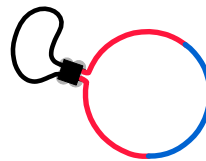
| Reagent                                    | Volume (μl)  |
|--|--------------|
| Target DNA (see "User Supplied Materials") | 1-12         |
| Deionized water                            | <i>ad</i> 12 |
| 5X Reaction Buffer for MuA Transposase     | 4            |
| DMSO 100%                                  | 2            |
| Entranceposon (STOP-Kan <sup>R</sup> )     | 1            |
| MuA Transposase                            | 1            |
|  | <hr/>        |
|  | 20           |

For a control reaction, use 1 μl (370 ng) of Control Target DNA supplied with the kit.

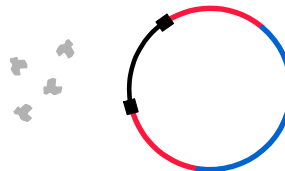
2. Mix the reagents.



3. Incubate 1 hour at 30°C.



4. Heat inactivate at 75°C for 10 minutes.



5. Transform.

## Transformation

Please read the important remarks concerning the choice of competent cells in the section: "User Supplied Materials" on page 5 before starting.

### Chemical transformation:

Use 1-10  $\mu\text{l}$  of the reaction mixture to transform 50-100  $\mu\text{l}$  chemically competent *E. coli* cells.

Follow the standard transformation protocols or proceed as instructed by the manufacturer of your competent cells.

### Electroporation:

Dilute the reaction mixture 10-fold in deionized water.

Use 1-5  $\mu\text{l}$  of the dilution per electroporation shot (typically 25-50  $\mu\text{l}$  of electrocompetent *E. coli* cells).

Before plating on selective plates, it is necessary to grow the cells in 1 ml SOC medium for one hour at 37°C to ensure expression of the *npT* marker gene from the Entranceposon.

Plate aliquots of the transformation mixture (e.g. 5, 50 and 500  $\mu\text{l}$  of 1 ml) on LB+kanamycin (20  $\mu\text{g}/\text{ml}$ ) selection plates.

*Note:* In order to reduce the number of Entranceposon insertions in the vector backbone of your target plasmid, supplement the selection plates additionally with the antibiotic that selects the target clone.

Incubate the plates overnight at 37°C.

### **Optional: Insertion Mapping by Colony-PCR**

If you plan to analyze only certain parts (e.g. domains) of your target protein, it is rational to focus only on the clones that contain the Entranceposon insertion in the corresponding DNA region. The most convenient way to map the insertion site in a given clone is to run a PCR amplification reaction using a bacterial colony from the transformation plate as reaction template.

DNA amplification:

Perform two separate 20- $\mu$ l PCR reactions per an insertion clone to ensure reliable mapping of Entranceposon insertion sites.

1. Prepare two PCR master mixes containing the MuEnd-2 primer, one with a vector-binding forward primer and the other with a reverse primer.

| <b>Reagent</b>                                       | <b>Final conc.</b> | <b>Volume (<math>\mu</math>l)</b> |
|--|--------------------|-----------------------------------|
| Deionized water                                      |                    | 14.8                              |
| 10X Optimized DyNAzyme™ EXT buffer                   | 1X                 | 2                                 |
| dNTPs (10 mM each)                                   | 200 $\mu$ M each   | 0.4                               |
| Vector forward <b>OR</b> reverse primer (25 $\mu$ M) | 0.5 $\mu$ M        | 0.4                               |
| Mu End-2 Primer (25 $\mu$ M)                         | 0.5 $\mu$ M        | 0.4                               |
| DyNAzyme™ EXT DNA Polymerase (1U/ $\mu$ l)           | 0.05 U/ $\mu$ l    | 1                                 |
|  |                    | <hr/> 19                          |

*Note:* The reaction conditions above have been optimized for the DyNAzyme™ EXT DNA polymerase. Efficient amplification of most GC-rich sequences can be achieved by supplementing the reaction mixture with 5% DMSO and by decreasing the annealing temperature 2-3°C.

2. Aliquot 19  $\mu$ l of the PCR reaction master mixes into reaction tubes on ice.
3. Touch a colony on a selection plate with a pipet tip. Dip the pipet tip into 50  $\mu$ l deionized water to suspend the cells.

4. Pipet 1  $\mu$ l of the cell suspension into two separate reaction tubes. Also transfer a small amount of the dilution on an appropriate selection plate to “replica-plate” the colonies picked for the PCR mapping reactions.
5. Use the following thermal cycling protocol for DNA amplification:

|                               |      |                            |
|-------------------------------|------|----------------------------|
| Step 1                        | 95°C | 1 min 30 s                 |
| Step 2                        | 95°C | 30 s                       |
| Step 3                        | *    | 15 s                       |
| Step 4                        | 72°C | 1 min/1.3 kb of insert DNA |
| Repeat the steps 2-4 30 times |      |                            |

Adjust the annealing temperature according to your vector-binding primers,  $T_m$  for MuEnd-2 65.7°C

#### Agarose gel electrophoresis:

Analyze the PCR products by standard agarose gel electrophoresis.

Length of the PCR product obtained from a given clone with the primer pair MuEnd-2 plus a vector primer equals to the distance between the Entranceposon insertion site and the vector-binding primer.

*Note:* The Entranceposon insertion sites in target DNA can also be mapped using restriction enzymes. There are several unique restriction enzyme sites for that purpose in the Entranceposon sequence (see Appendix II, page 12).

## Optional: DNA Sequencing

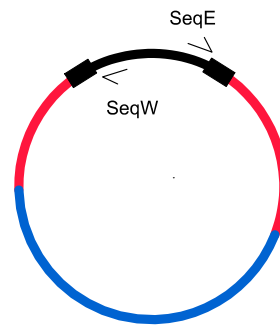
In order to locate the insertion site in a given clone at base pair level, perform DNA sequencing reactions with the primers SeqE and SeqW.

Sequencing from plasmid DNA:

Prepare plasmid DNA from the insertion clones of interest using standard techniques.

*Important:* Use the primers SeqW or SeqE for sequencing from plasmid DNA, not MuEnd-2. The primer MuEnd-2 anneals on the terminal repeat sequences present at each end of the Entranceposon.

The recommended annealing temperature for the primers SeqW and SeqE in a cycle sequencing reaction is 50°C.

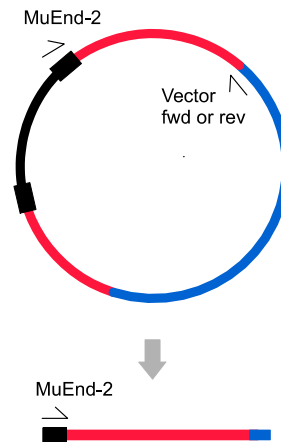


Sequencing from PCR products:

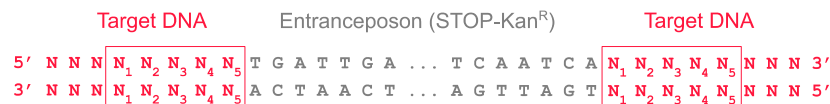
Purify PCR-amplified DNA using standard techniques, such as gel filtration, if that is required for your DNA sequencing system.

*Important:* Use the primer MuEnd-2 for sequencing directly from PCR-amplified DNA. A linear PCR product amplified with MuEnd-2 and a vector primer does not contain binding sites for the DNA sequencing primers SeqE and SeqW.

The recommended annealing temperature for the primer MuEnd-2 in a cycle sequencing reaction is 55°C.



*Important:* The transposition reaction generates a 5-bp sequence duplication at the Entranceposon insertion site.



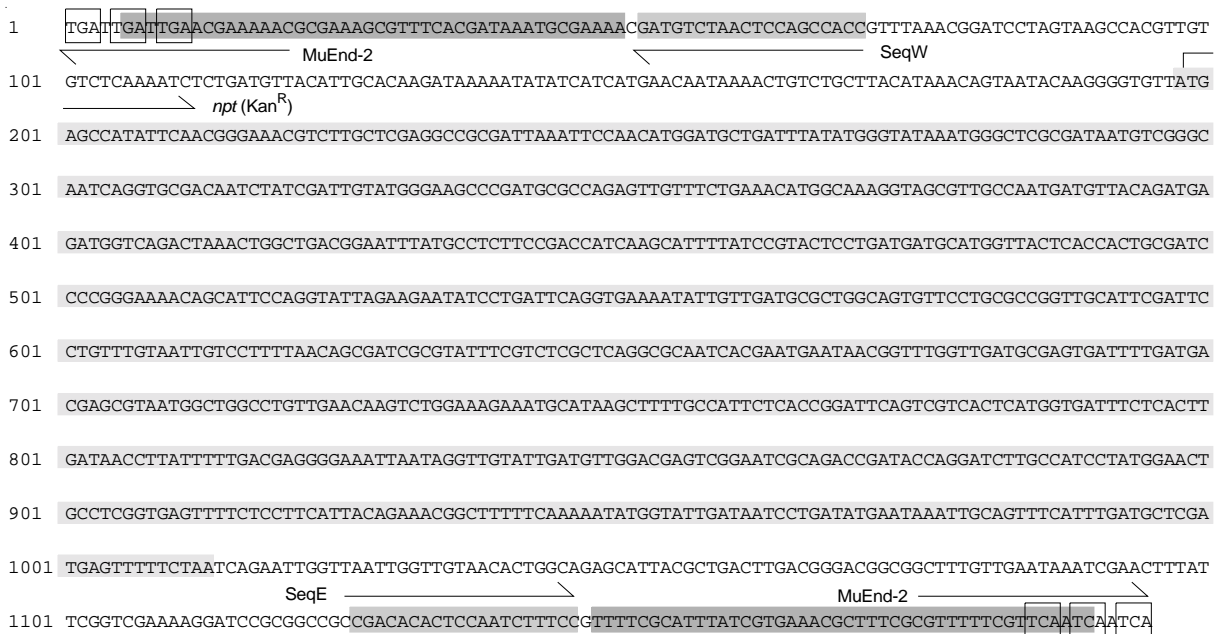
## Appendix I: Description of Kit Components

|  |        |
|--|--------|
| Entranceposon (STOP-Kan <sup>R</sup> )<br>100 ng/μl in TE, pH 8.0  | 10 μl  |
| The Entranceposon is composed of the proprietary modified sequences from the right end of the bacteriophage Mu genome flanking the kanamycin marker gene, <i>npt</i> .   |        |
| MuA Transposase<br>0.22 μg/μl in MuA Storage Buffer  | 10 μl  |
| 5X Reaction Buffer for MuA Transposase   | 100 μl |
| DMSO 100%  | 500 μl |
| Control Target DNA<br>370 ng/μl in TE, pH 8.0  | 10 μl  |
| A 6.6 kb <i>HindIII</i> fragment of bacteriophage lambda DNA in pUC19.   |        |
| MuEnd-2 Primer<br>25 μM in dd water<br>5' GCGTTTTTCGTTCAATCAATCA 3'<br>T <sub>m</sub> 65.7°C (0.5 μM)*   | 50 μl  |
| <i>Important:</i> The binding site of the MuEnd-2 primer is present at each end of the Entranceposon. Therefore MuEnd-2 can not be used for sequencing from plasmid DNA that contains an intact copy of the Entranceposon. |        |
| SeqE Primer<br>10 μM in dd water<br>5' CGACACACTCCAATCTTTCC 3'<br>T <sub>m</sub> 59.1°C (0.1 μM)   | 250 μl |
| SeqW Primer<br>10 μM in dd water<br>5' GGTGGCTGGAGTTAGACATC 3'<br>T <sub>m</sub> 58.1°C (0.1 μM)   | 250 μl |

\*The T<sub>m</sub> calculations were done essentially as described by Breslauer *et al.* (*PNAS* 83, 3746-50) using the Oligonucleotide T<sub>m</sub> determination program at the Virtual Genome Center (<http://alces.med.umn.edu/rawtm.html>).

## Appendix II: Map and Sequence of Entranceposon

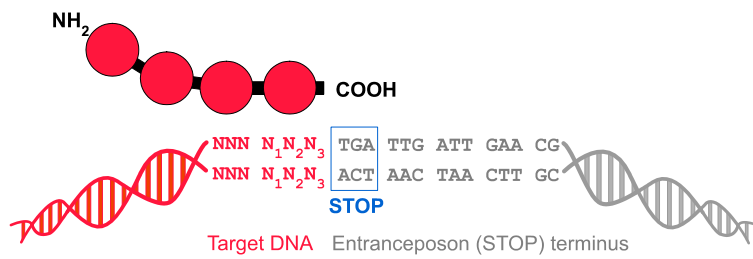
Entranceposon (STOP-Kan<sup>R</sup>), 1195 bp:



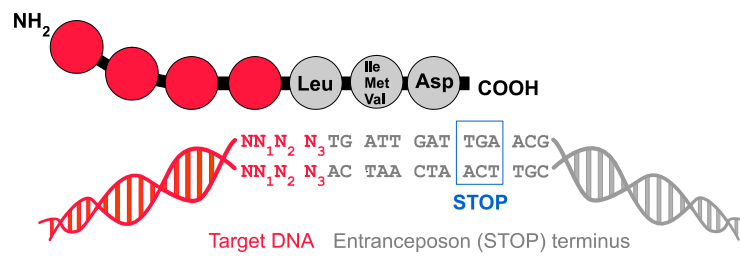
### Appendix III: STOP Codons in Three Reading Frames

Insertion site on DNA and protein level:

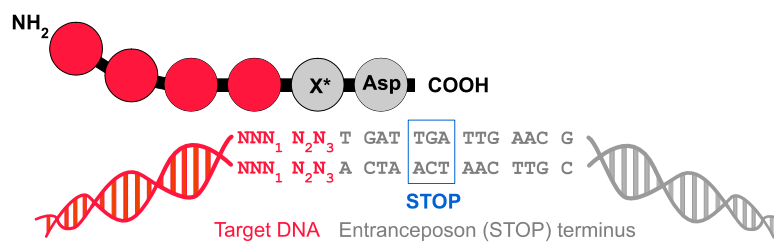
Frame 1:



Frame 2:



Frame 3:



x\* Any amino acid except Gln, Glu, Lys, Met or Trp

## Appendix IV: Transposition Troubleshooting

*Problem: No, or only a few colonies on LB+kanamycin plates.*

Prepare a control transposition reaction using the Control Target DNA supplied with the kit to determine whether the problem occurs in the transposition or transformation step.

Plate aliquots of the transformation mixture (total vol. 1 ml) on two different kinds of selective plates:

- a.) 0.1-1  $\mu$ l on an LB+ampicillin (100  $\mu$ g/ml) plate
- b.) 10-100  $\mu$ l on an LB+kanamycin (20  $\mu$ g/ml) plate

Possible outcomes of the control reaction:

*# 1 Colonies appear both on the LB+amp and LB+kan plates.*

Suggestions for scoring more colonies with your target DNA:

Make sure that the competent cells are compatible with your target DNA clone. If not, try another strain of *E. coli*.

If you are using a chemical transformation method, consider using electroporation.

To increase electroporation efficiency, desalt and precipitate the transposition reaction mixture using the following protocol\*:

Add 30  $\mu$ l dH<sub>2</sub>O to a 20- $\mu$ l transposition reaction (ad 50  $\mu$ l)

Add 500  $\mu$ l *n*-butanol

Vortex for 20 s

Centrifuge at 14 000 rpm for 15 min

Discard supernatant, dry pellet

Resuspend pellet in 10  $\mu$ l dH<sub>2</sub>O

Use 1-10  $\mu$ l per one electroporation shot

Decrease the kanamycin concentration on plates to 10  $\mu$ g/ml especially if your target DNA clone is a low copy number replicon.

*# 2 Colonies appear on the LB+amp plate but not on the LB+kan plate.*

Suggestions for scoring more colonies with your target DNA:

Make sure that the incubations are performed at correct temperatures.

Extend the transposition reaction incubation at 30° up to 4 h.

Make sure that the kanamycin concentration on the selective plates does not exceed 20  $\mu$ g/ml.

\*Thomas, M. R. 1994. *BioTechniques* 16, 988.

## Appendix V: Media Recipes

### LB agar with antibiotics, per liter

|               |      |
|---------------|------|
| Tryptone      | 10 g |
| Yeast Extract | 5 g  |
| NaCl          | 10 g |
| Agar          | 15 g |

Adjust pH to 7.0 with NaOH  
Autoclave

Cool to 55°C and add :

|                       |       |
|-----------------------|-------|
| Kanamycin (per liter) | 20 mg |
|-----------------------|-------|

Optional: supplement the medium with the antibiotic that selects for the target DNA replicon.

### SOC medium, per liter

|                    |         |
|--------------------|---------|
| Tryptone           | 20 g    |
| Yeast Extract      | 5 g     |
| NaCl               | 0.5 g   |
| KCl (final 2.5 mM) | 0.186 g |

Adjust pH to 7.0 with NaOH  
Autoclave

Before use add sterile solutions:

|                       |       |
|-----------------------|-------|
| 1 M MgCl <sub>2</sub> | 10 ml |
| 1 M MgSO <sub>4</sub> | 10 ml |
| 1 M Glucose           | 20 ml |

## Appendix VI: Related Products

### Stand-alone enzymes:

- F-750 MuA Transposase
- F-750L MuA Transposase, large
- F-750C MuA Transposase, 5X conc.

### Ready-to-use Entranceposons:

*Note:* These products contain wild-type Mu ends, no STOP modification

- F-778 Entranceposon (Cam<sup>R</sup>-3)
- F-779 Entranceposon (Kan<sup>R</sup>-3)
- F-784 Entranceposon (Tet<sup>R</sup>-3)
- F-771 Entranceposon (*supF*)
- F-774 Entranceposon (*lacZ*)

### Plasmid Entranceposons:

*Note:* These products contain wild-type Mu ends, no STOP modification

- F-765 pEntranceposon (Cam<sup>R</sup>)
- F-766 pEntranceposon (Kan<sup>R</sup>)
- F-767 pEntranceposon (Tet<sup>R</sup>)
- F-773 pEntranceposon (*supF*)
  
- F-701 Mutation Generation System™, MGS™
- F-702 Template Generation System™ II, TGS™ II

### DyNAzyme™ EXT DNA Polymerase products:

- F-505S 200 U
- F-505L 1000 U
- F-552XS Kit for 25-50 reactions
- F-552S Kit for 100-200 reactions
- F-552L Kit for 500-1000 reactions

More information available at: [www.finnzymes.fi](http://www.finnzymes.fi).

Template Generation System™, Mutation Generation System™, Stop Generation System™, TGS™, MGS™, STOP™ and DyNAzyme™ are trademarks of Finnzymes Oy.

## Appendix VII: Mu Transposition References

Mizuuchi, K. (1992). Transpositional recombination: mechanistic insights from studies of Mu and other elements. *Annu. Rev. Biochem.* 61, 1011-1051.

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Lamberg, A., Nieminen, S., Qiao, M. & Savilahti H. (2002). Efficient insertion mutagenesis strategy for bacterial genomes involving electroporation of *in vitro*-assembled DNA transposition complexes of bacteriophage mu. *Appl. Environ. Microbiol.* 68, 705-12.

Vilen, H., Aalto, J.-M., Kassinen, A., Paulin, L. & Savilahti, H. (2003). A direct transposon insertion tool for modification and functional analysis of viral genomes. *J. Virol.* 77, 123-134.

## Appendix VIII: Warranty

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

1. That this warranty is in lieu of all other warranties, express or implied;
2. That **ALL WARRANTIES OF MERCHANT ABILITY OR OF FITNESS FOR A PARTICULAR PURPOSE ARE HEREBY EXCLUDED AND WAIVED;**
3. That the buyer's sole remedy shall be to obtain replacement of the product free of charge from Finnzymes Oy; and
4. That this remedy is in lieu of all other remedies or claims for damages, consequential or otherwise, which the buyer may have against Finnzymes Oy.

### **Exclusive terms of sale:**

Finnzymes Oy does not agree to and is not bound by any other terms or conditions, unless those terms and conditions have been expressly agreed to in writing by a duly authorised officer of Finnzymes Oy.

### **Prices are subject to change without notice.**

### **Recommended Guidelines for Safe Use of the Products:**

Finnzymes Oy recommends that the buyer and other persons using the products follow the N.I.H. guidelines published in the Federal Register, Volume 41, No. 131, July 7, 1976, and any amendments thereto. Finnzymes Oy disclaims any and all responsibility for any injury or damage which may be caused by the failure of the buyer or any other person to follow said guidelines.

### **Research Use Only:**

Since these products are intended for research purposes by qualified persons, the Environmental Protection Agency does not require us to supply Premanufacturing Notice.

### **Notice to User:**

The information presented here is accurate and reliable to the best of our knowledge and belief, but is not guaranteed to be so. Nothing herein is to be construed as recommending any practice or any product in violation of any patent or in violation of any law or regulation. It is the user's responsibility to determine for himself or herself the suitability of any material and/or procedure for a specific purpose and to adopt such safety precautions as may be necessary.