Thermo Scientific
Stop Generation System Kit

Technical Manual

F-703 10 reactions

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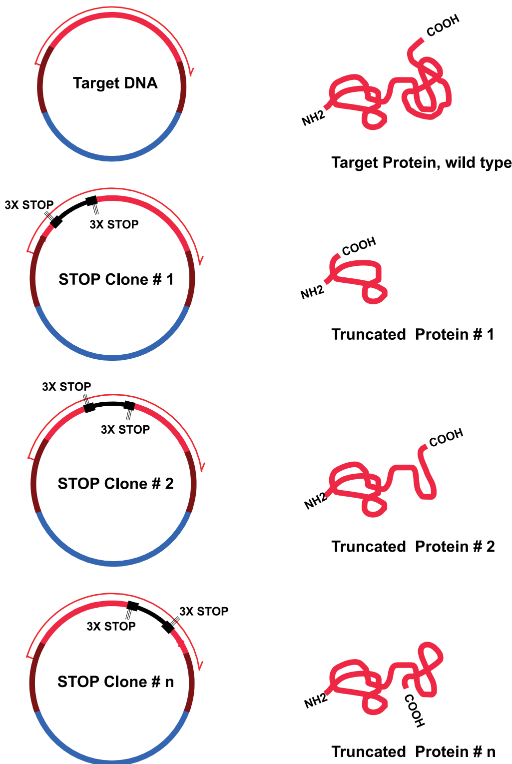
1. Introduction

The Thermo Scientific Stop Generation System (STOP) 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.

2. 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 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.thermoscientific.com/stopkit.
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 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.

3. Kit components

The STOP kit contains sufficient materials for 10 reactions.

Product	Size
Artificial transposons:	
Entranceposon* (STOP-Kan^R)	10 µl
MuA Transposase	10 µl
5X Reaction Buffer for MuA Transposase	100 µl
DMSO 100%	500 µl
Control Target DNA	10 µl
Primers for insertion mapping:	
MuEnd-2 Primer	50 µl
Primers for DNA sequencing:	
SeqE Primer	250 µl
SeqW Primer	250 µl

* See Appendix II for the complete sequences of the Entranceposons.

Store the components at -20°C.
Material safety data sheet (MSDS) is available at www.thermoscientific.com/fzmsds.

4. User-supplied materials

Target DNA

Use 60 fmoles of target DNA per a 20 µl 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 × 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) also serve efficiently as transposition target DNA. A slightly modified protocol for linear target DNA is available at: www.thermoscientific.com/stop.

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/µg target DNA plasmid in order to obtain thousands of insertion clones from transposition reaction.

Thermal cyclers

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

Media

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

Optional: Reagents and equipment for PCR mapping
Optional: Reagents and equipment for DNA sequencing

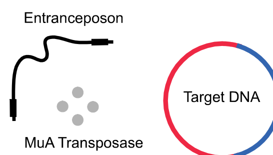
5. Transposition reaction protocol

1. Set up the following reaction:
   Important: MuA Transposase should be added last.

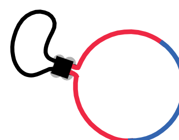
Reagent	Volume
Target DNA (see "User-Supplied Materials")	1 to 12 µl
H_2O	add to 20 µl
5X Reaction Buffer for MuA Transposase	4 µl
DMSO 100%	2 µl
Entranceposon (STOP-Kan^R)	1 µl
MuA Transposase	1 µl
	20 µl

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

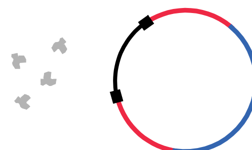
2. Mix the reagents. Do not vortex.



3. Incubate 1 hour at 30°C.



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



5. Transform.

6. Transformation

Please read the impotant remarks concerning the choice of competent cells in the section "User-Supplied Materials" before starting.

Chemical transformation

Use 1-10 µl of the reaction mixture to transform 50-100 µl 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 µl of the dilution per electroporation shot (typically 25-50 µl 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 µl of 1 ml) on LB+kanamycin (20 µg/ml) selection plates.

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

Incubate the plates overnight at 37°C.

7. 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.

7.1 DNA amplification

Perform two separate 20 µl 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.

Reagent	Final conc.	Volume
H_2O		14.4 µl
5X Phire Reaction Buffer	1 ×	4 µl
dNTPs (10 mM each)	200 µM each	0.4 µl
Vector forward OR reverse primer (25 µM)	0.5 µM	0.4 µl
Mu End-2 Primer (25 µM)	0.5 µM	0.4 µl
Phire Hot Start II DNA Polymerase		0.4 µl
		20 µl

Note: The reaction conditions above have been optimized for the Thermo Scientific Phire Hot Start II 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 µl 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 µl deionized water to suspend the cells.
4. Pipet 1 µl 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	Temperature	Time
Step 1	98°C	30 s
Step 2	98°C	5 s
Step 3	X°C*	5 s
Step 4	72°C	10-15 s/kb
Step 5	Go to the step 2 for 29 times	

* As a basic rule, for primers >20 nt, anneal for 5 seconds at a Tm +3°C of the lower Tm primer. For primers ≤20 nt, use an annealing temperature equal to the Tm of the lower Tm primer. If necessary, use a temperature gradient to find the optimal annealing temperature for each template-primer pair combination. Use the Tm calculator and instructions on website www.thermoscientific.com/pcrwebtools to determine Tm for your vector-binding primer.Adjust the annealing temperature according to your vector-binding primers, Tm for MuEnd-2 is 66.1°C.

7.2 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.

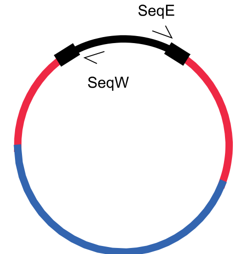
Note: The Entranceposon insertion sites in target DNA can also be mapped using restriction enzymes. There are several unique restriction enzyme sites for that purpose in the Entranceposon sequence (see Appendix II).

8. Optional: DNA sequencing

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

8.1 Sequencing from plasmid DNA

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

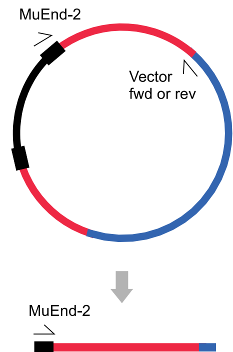


Important: Use the primers SeqW or SeqE for sequencing from plasmid DNA, not MuEnd-2. The primer MuEnd-2 anneals on the terminal repeat sequences present at each end of the Entranceposon.

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

8.2 Sequencing from PCR products

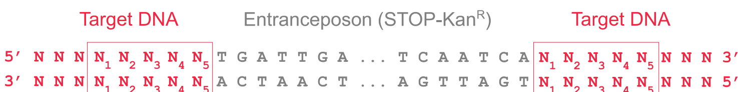
Purify PCR-amplified DNA using standard techniques, such as gel filtration, if that is required for your DNA sequencing system.



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^R)

10 µl 100 ng/µl in TE, pH 8.0

The Entranceposon is composed of the proprietary modified sequences from the right end of the bacteriophage Mu genome flanking the kanamycin marker gene, *npt.*

MuA Transposase

10 µl 0.22 µg/µl in MuA Storage Buffer

5X Reaction Buffer for MuA Transposase

100 µl

DMSO 100%

500 µl

Control Target DNA

10 µl 370 ng/µl in TE, pH 8.0

A 6.6 kb *Hin*dIII fragment of bacteriophage lambda DNA in pUC19.

MuEnd-2 Primer

50 µl 25 µM in H_2O

5′ GCGTTTTTCGTTCAATCAATCA 3′

Tm 66.1°C (0.5 µM)

Important: 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

250 µl 10 µM in H_2O

5′ CGACACACTCCAATCTTTCC 3′

Tm 59.1°C (0.1 µM)

SeqW Primer

250 µl 10 µM in H_2O

5′ GGTGGCTGGAGTTAGACATC 3′

Tm 58.1°C (0.1 µM)

Appendix II: Map and sequence of entranceposon

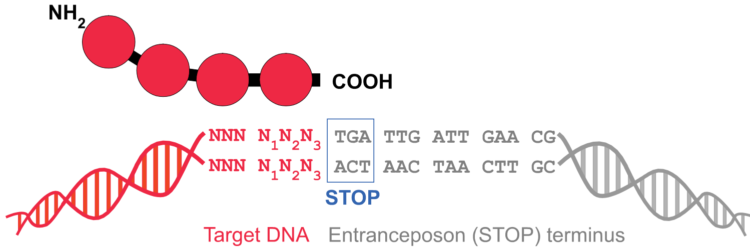
Entranceposon (STOP-Kan^R), 1195 bp:

```
1     TGATTGATTGAACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACGATGTCTAACTCCAGCCACCGTTTAAACGGATCCTAGTAAGCCACGTTGT
                 MuEnd-2                                              SeqW
101   GTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATG
                 npt (KanR)
201   AGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGC
301   AATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGA
401   GATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATC
501   CCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTC
601   CTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGA
701   CGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCTCACTT
801   GATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACT
901   GCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGA
1001  TGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTTAT
                      SeqE                                         MuEnd-2
1101  TCGGTCGAAAAGGATCCGCGGCCGCCGACACACTCCAATCTTTCCGTTTTCGCATTTATCGTGAAACGCTTTCGCGTTTTTCGTTCAATCAATCA
```

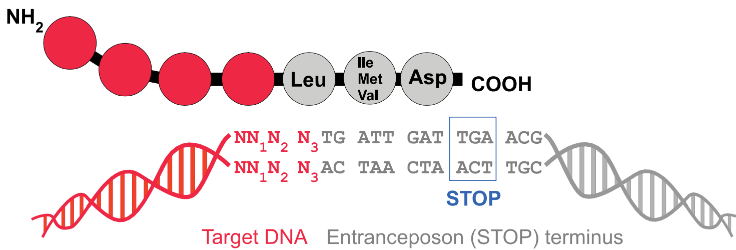
Appendix III: STOP sodons in three reading frames

Insertion site on DNA and protein level:

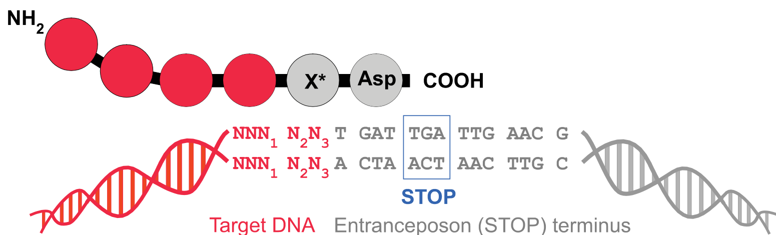
Frame 1:



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 µl on an LB+ampicillin (100 µg/ml) plate
b.) 10-100 µl on an LB+kanamycin (20 µg/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 µl dH_2O to a 20-µl transposition reaction (ad 50 µl)
  - Add 500 µl n-butanol
  - Vortex for 20 s
  - Centrifuge at 14000 rpm for 15 min
  - Discard supernatant, dry pellet
  - Resuspend pellet in 10 µl dH_2O
  - Use 1-10 µl per one electroporation shot
- Decrease the kanamycin concentration on plates to 10 µg/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 µg/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 1 M 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 $MgCl_2$	10 ml
1 M $MgSO_4$	10 ml
1 M Glucose	20 ml

Appendix IV: Related products

Stand-alone enzymes

F-750 MuA Transposase

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^R-3)

F-779 Entranceposon (Kan^R-3)

F-784 Entranceposon (Tet^R-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^R)

F-766 pEntranceposon (Kan^R)

F-767 pEntranceposon (Tet^R)

F-773 pEntranceposon (*supF*)

F-701 Mutation Generation System (MGS)

F-702 Template Generation System II (TGS II)

PCR products

F-122S/L Thermo Scientific Phire Hot Start II DNA Polymerase

Appendix VII: Mu Transposition References

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Taira, S., Tuimala, J., Roine, E., Nurmiaho-Lassila, E.-L., Savilahti, H. & Romantschuk, M. (1999). Mutational analysis of the Pseudomonas syringae pv. tomato hrpA gene encoding Hrp pilus subunit. *Mol. Microbiol.* 34, 737-744.

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Product use limitation

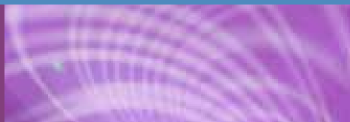
This product has been developed and is sold exclusively for research purposes and in vitro use only. This product has not been tested for use in diagnostics or drug development, nor is it suitable for administration to humans or animals.

Patent notices; label licenses

The STOP Generation System Kit is covered by patent US7172882B2

The quality system of Finnzymes Oy, now part of Thermo Fisher Scientific, is certified according to standard SFS-EN ISO9001:2008.

Thermo Scientific Stop Generation System Kit
Technical Manual



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