thermo scientific

PRODUCT INFORMATION

Mutation Generation System Kit

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#F-701

Store at -20°C

www.thermofisher.com

For Research Use Only. Not for use in diagnostic procedures.

Rev.3

Contents

APPLICATIONS

The MGS kit has been designed for inserting fifteen basepairs randomLy into target DNA for:

- Fast generation of in-frame five amino acid insertion libraries of any protein for functional analyses.
- Rapid and random mutagenesis of cloned promoters and other regulatory DNA regions.
- Insertion of a *Not*I restriction enzyme recognition site into target DNA clone.

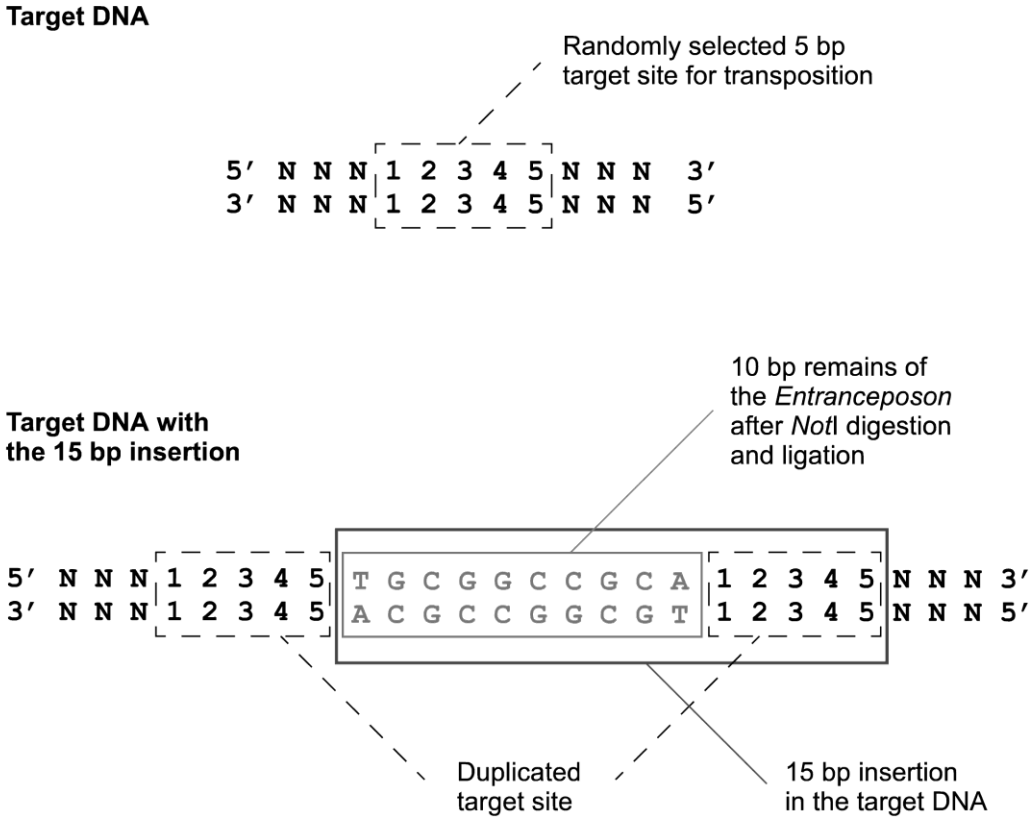


Figure 1. Structure of the 15 bp insertion generated by the MGS.

COMPONENTS OF THE KIT

The MGS kit contains sufficient materials for 10 reactions. See Appendix I for detailed descriptions of the kit components.

Mutation Generation System Kit	**#F-701**
Entranceposon* (M1-Cam^R)	10 µL
Entranceposon* (M1-Kan^R)	10 µL
MuA Transposase	10 µL
5X Reaction Buffer for MuA Transposase	100 µL
Control Target DNA	10 µL
*Not*I Miniprimer	50 µL

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

STORAGE

Store the components at -20°C.

DESCRIPTION

The Mutation Generation System (MGS) Kit has been designed for rapid and efficient construction of linker scanning libraries for functional analyses of proteins and regulatory DNA regions. The system uses the highly efficient transposition machinery of the bacteriophage Mu to insert an artificial transposon, designated as Entranceposon, virtually into any target plasmid in random sites. The simple *in vitro* transposition reaction is catalyzed by a single purified enzyme, MuA Transposase.The insertion clones generated in the transposition reaction are digested with the rare-cutting restriction enzyme *Not*I to remove the body of the Entranceposon. Closure of the *Not*I digested clones by self-ligation results in a 15 bp insertion in the target DNA. In case the Entranceposon insertion occurs in the coding region of the target gene, the 15 bp insertion is translated into five extra amino acids.

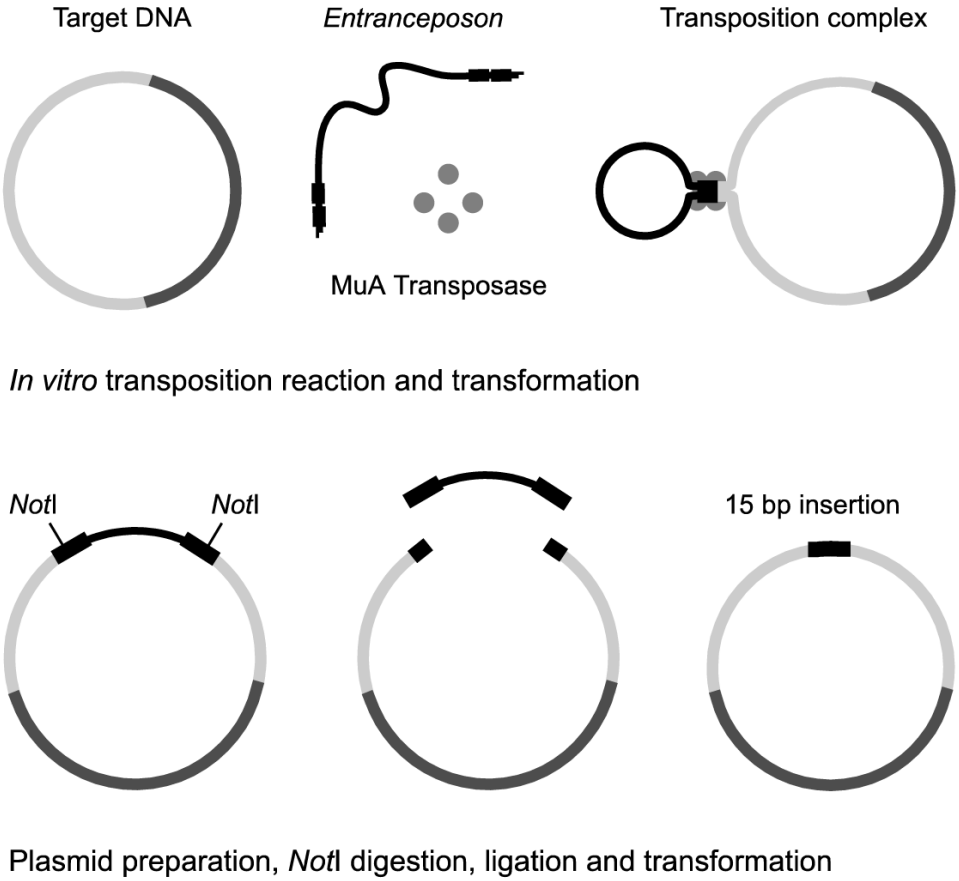


Figure 2. Flowchart of the MGS protocol.

4. USER-SUPPLIED MATERIALS

Target DNA

The *in vitro* transposition reaction of the MGS kit is not affected by minor impurities present in the target DNA preparation. The target DNA should be purified using standard methods, such as alkaline lysis method, or commercial plasmid DNA purification kits. Resuspend the target DNA in low-salt buffer such as TE, pH 8.0 or deionized water. The optimal amount of the target DNA per reaction depends on the size of the plasmid and can be calculated as follows:

Amount of the target DNA (ng) per reaction = Size of the target plasmid (kb) × 40 ng

Example: Your target DNA plasmid consists of a 1.3 kb insert cloned into a 2.8 kb vector. The size of the target plasmid is 1.3 kb + 2.8 kb = 4.1 kb, and the optimal amount per reaction is: 4.1 × 40 ng = 164 ng.
Use minimum of 150 ng of the target DNA per transposition reaction.

Important: Make sure that your target DNA clone does not contain the same selectable marker gene that is present in the Entranceposon that you are using (either CamR or KanR).

Important: The target DNA clone should not contain recognition sites for the restriction enzyme *Not*I (5'-GCGGCCGC-3'). Note: Sometimes it is possible to destroy an extra *Not*I site by a fill-in reaction with a suitable DNA polymerase, such as the Klenow fragment of the *E. coli* DNA polymerase I.

The transposition reaction of the MGS has no target sequence preference. Therefore, the Entranceposon insertions occur at random locations in the target DNA clone. However, in some applications it is preferred that the Entranceposon insertions occur in the region of the DNA of interest ("insert") rather than in the vector backbone of the target clone.That can be achieved by transferring the DNA of interest with an Entranceposon insertion into a fresh cloning vector using standard DNA cloning techniques. In order to perform this, one should release the DNA of interest from the vector with specific restriction enzymes that do not cut inside the Entranceposon sequence. A selection of the non-cutting restriction enzymes for each Entranceposon is listed in Appendix II. Additionally, there should be sufficient difference in the sizes of the cloning vector and the insert ("DNA of interest") to allow separation of the restriction fragments by agarose gel electrophoresis.

Competent *E. coli* cells

Any standard chloramphenicol- or kanamycin-sensitive laboratory strain of *E. coli* that is suitable for high-efficiency DNA cloning can be used as a transformation host. It is important that the efficiency of the transformation is high (>108 cfu/µg pUC19 for electrocompetent cells) in order to maximize the number of independent clones in the insertion scanning libraries. Therefore, electroporation is the recommended transformation method.
Note: Entranceposons contain 50 bp inverted terminal repeats. To avoid potential homologous recombination between the repeats, the use of a *recA– E. coli* strain is recommended.

Thermal cycler:	For transposition reaction (alternatively: heat blocks, 30°C and 75°C) and colony-PCR reactions
Enzymes:	*Not*I, to cut out body of the Entranceposon *Optional:* restriction enzymes that cleave the DNA of interest out of the vector backbone DNA ligase
Media:	LB or SOC medium LB agar with antibiotics
Other reagents:	Reagents for plasmid DNA isolation *Optional:* reagents for extraction of DNA from agarose gel

5. REACTION PROTOCOL

Perform the in vitro transposition reaction:

1. Set up the following reaction in a microcentrifuge tube.
   Important: The MuA Transposase should be added last.
   See the flowchart of the *in vitro* transposition reaction in figure 1.

Reagent	**Volume**
H_2O	add to 20 µL
Target DNA (see "User Supplied Materials")	1 to 14 µL
5X Reaction Buffer for MuA Transposase	4 µL
Entranceposon (M1-Cam^R) OR (M1-Kan^R), 100 ng	1 µL
MuA Transposase	1 µL
	20 µL

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

2. Mix the components gently.
3. Allow the transposition reaction to proceed for one hour at 30°C.
4. Inactivate the MuA Transposase by incubating at 75°C for 10 minutes.

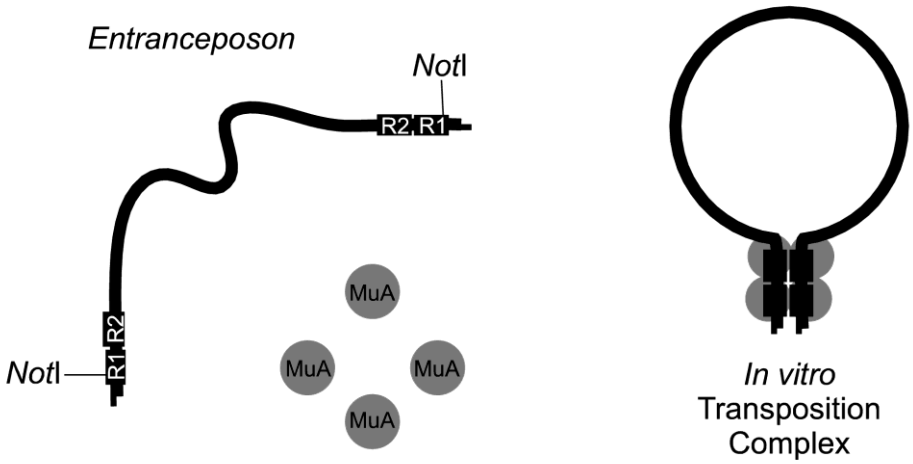


Figure 3 a. The *in vitro* Mu transposition complex is assembled as four monomers of the MuA Transposase protein are bound to the R1 and R2 sites in the ends of the Entranceposon.

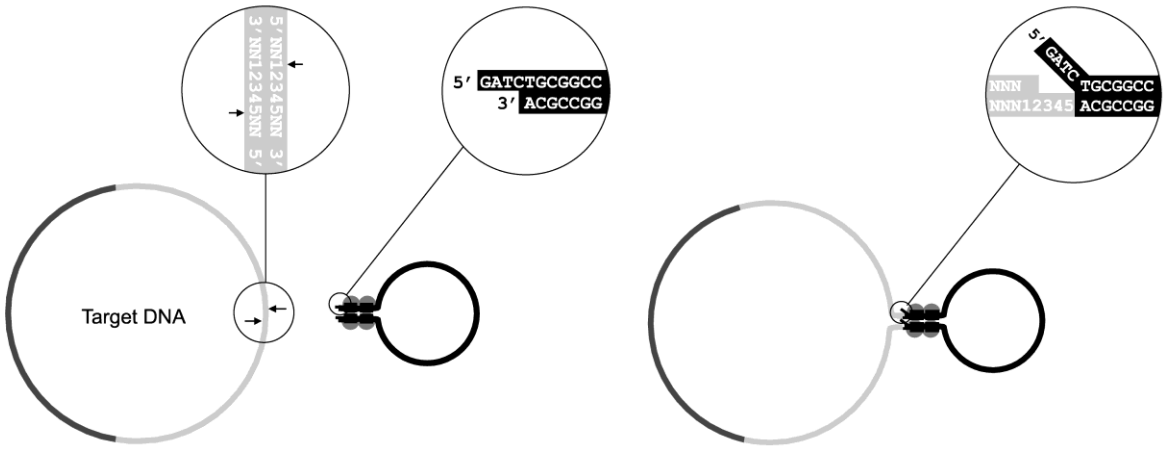


Figure 3 b. The MuA Transposase makes 5 bp staggered cuts in the target DNA and, at the same time, the 3' ends of the Entranceposon are covalently joined to the 5' phosphates in the target site.

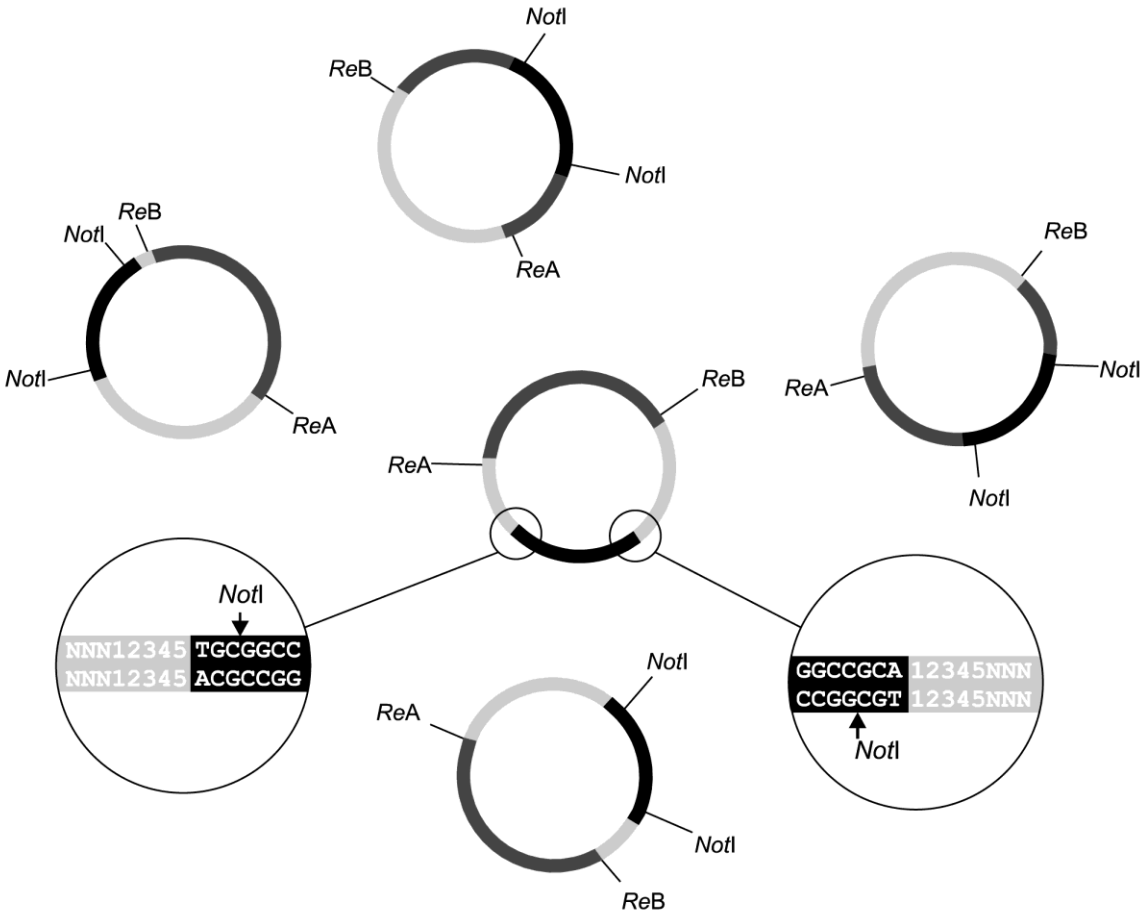


Figure 3 c. The single-stranded gaps are repaired in *E. coli*, resulting in the duplication of the 5 bp target site.

5. Proceed to the transformation step (see Appendix IV for details).
   Dilute the reaction mixture ten-fold in deionized water and use maximum of 10 µL per single electroporation. Alternatively, precipitate the DNA in the reaction mixture, resuspend in deionized water and use aliquots for electroporation. For chemical transformation use 5-10 µL of reaction per transformation.
6. Plate out on several LB agar plates supplemented with 10 µg/mL chloramphenicol OR 10 µg/mL kanamycin and additionally with the antibiotic that selects for the target DNA clone (Appendix V).

Important: The Entranceposon insertions occur in the vector backbone of your target DNA plasmid as well as in the insert ("DNA of interest"). For some applications it is necessary that there are no Entranceposon insertions in the vector. To transfer the DNA of interest with an Entranceposon insertion into a "fresh" cloning vector, continue through the steps 7-11. If the cloning step is not necessary for your application, proceed directly into the step 12.

7. Scrape the transformants from the plates, pool bacteria and prepare plasmid DNA using the standard alkaline lysis method or any commercial DNA preparation kit (Appendix IV).
8. Digest the plasmid preparation with the restriction enzymes that release the DNA of interest from the cloning vector. A selection of the non-cutting restriction enzymes for each Entranceposon is listed in Appendix II. **Note:** In case the cloning vector and the DNA of interest are of the same size, it may be necessary to digest the plasmid additionally with a restriction enzyme that cuts the vector backbone in smaller pieces to be able to separate the fragments by agarose gel electrophoresis.

Figure 3 d. The fragments resulting from the digestion of the plasmid DNA pool with the restriction enzymes that cut out the DNA of interest from the vector.

9. Separate the resulting fragments by standard agarose gel electrophoresis. Extract the DNA of interest with an Entranceposon insertion (Appendix IV).
10. Clone the extracted DNA fragment into "fresh" cloning vector.
11. Transform and plate out on several LB agar plates supplemented with either 10 µg/mL chloramphenicol OR 10 µg/mL kanamycin and additionally with the antibiotic that selects for the cloning vector.
12. Scrape the transformants from the plates, pool the bacteria and prepare plasmid DNA using the standard alkaline lysis method or a commercial plasmid DNA isolation kit.

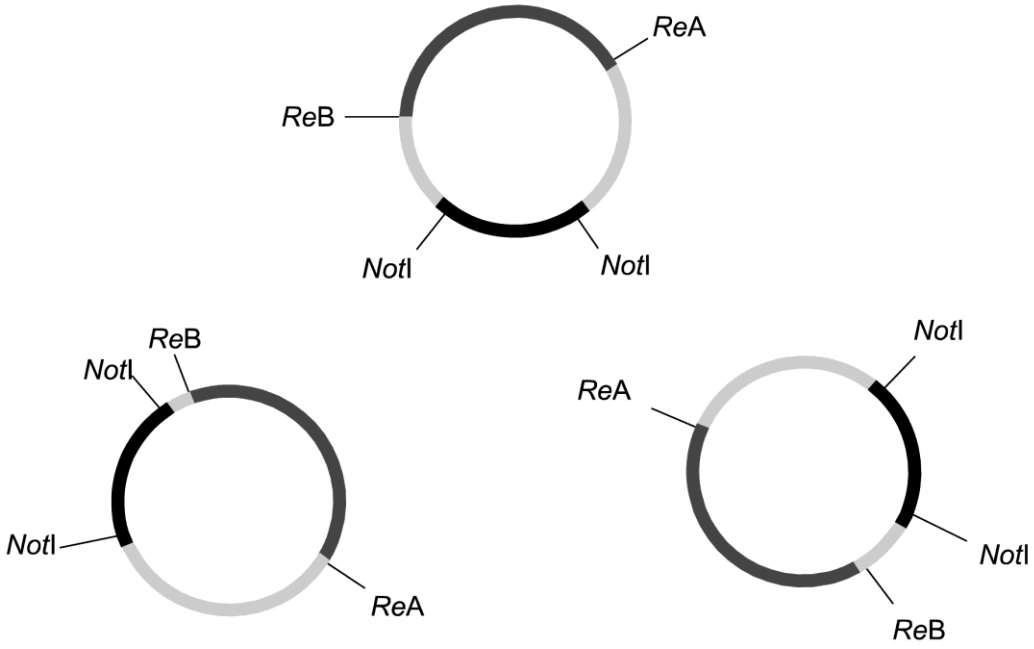


Figure 3 e. Pool of target DNA clones carrying an Entranceposon insertion at random location.

13. Digest the plasmid preparation with *Not*I to cut out body of the Entranceposon.
    Optional: Separate the resulting fragments by standard agarose electrophoresis.
    Extract the fragment with the size of your cloning vector and the DNA of interest.

Figure 3 f. *Not*I digestion cuts out body of the Entranceposon.

14. Use an aliquot of the *Not*I digestion mixture for re-ligation reaction. To favor self-ligation of the *Not*I digestion fragments, increase the volume of the ligation reaction to adjust the DNA concentration of the mixture to 1-5 ng/µl. This is especially important if the digestion fragments are not separated by agarose gel electrophoresis.
15. Transform into competent *E. coli* cells.
16. Plate on LB agar plates supplemented only with the antibiotic that selects for the cloning vector of the plasmid. **Important!** Do not select with chloramphenicol or kanamycin after the Entranceposon has been cut out with *Not*I digestion.
17. Scrape the transformants from the plates, pool the bacteria and prepare plasmid DNA. The insertion library is now ready for your specific application. Alternatively, map the 15 bp insertions in the individual clones by performing a colony-PCR analysis on the transformants. Prepare plasmid DNA only from the clones that carry the 15 bp insertion in the desired region and proceed according to your application.

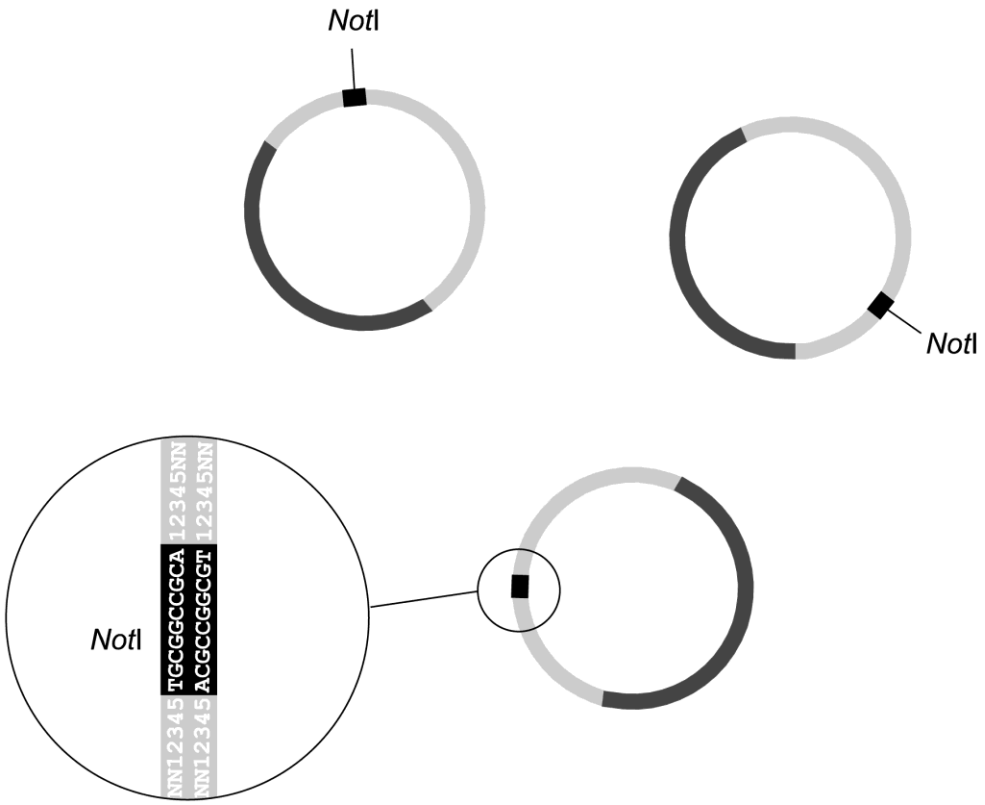


Figure 3 g. Fifteen basepair insertion in the target DNA.

6. 15 bp INSERTION MAPPING BY COLONY PCR

6.1 Reaction conditions

For reliable mapping of the 15 bp insertion in the target DNA, perform two separate 20 µL PCR reactions per insertion clone. In addition to the *Not*I Miniprimer included in the kit, use primers that anneal to the sequence flanking the DNA of interest at opposite sides (see figure 4).

1. Prepare two PCR reaction mixtures, one with the *Not*I miniprimer and vector-binding forward primer (Fwd mix) and the other with *Not*I miniprimer and vector-binding reverse primer (Rev mix). **Note:** If other DNA polymerase than Thermo Scientific Phire Hot Start II DNA Polymerase is used, modify the reaction conditions according to the manufacturer's instructions.

	Final conc.	**Volume**
H_2O		14.4 µL
5X Phire Reaction Buffer	1X	4 µL
dNTPs (10 mM each)	200 µM each	0.4 µL
Vector forward OR reverse primer	0.5 µM	0.4 µL
*Not*I miniprimer	0.5 µM	0.4 µL
Phire Hot Start II DNA Polymerase		0.4 µL
		20 µL

Note: If using Thermo Scientific Phire Hot Start II DNA Polymerase for insertion mapping, difficult PCR templates, such as a GC-rich target DNA, can be managed simply by supplementing the reaction mixture with 5% DMSO and by decreasing the annealing temperature 2-3°C.

2. Place the required number of reaction tubes on ice.
3. Using a toothpick or a pipet tip, transfer bacteria from a single chloramphenicol- or kanamycin-resistant colony into 50 µL of sterile water and vortex the solution. Transfer 1 µL of the cell dilution into two separate reaction tubes, one labeled for the Fwd mix and the other for the Rev mix. Also, at this point be sure to make a new replicate plate on each colony picked.
4. Add 19 µL of the mixtures into the PCR tubes on ice.
5. Amplify according to the following thermal cycling protocol.

6.2 Thermal cycling protocol for 15 bp insertion mapping

Step	**Temperature**	**Time**
Step 1	98ºC	30 s
Step 2	98ºC	5 s
Step 3	XºC*	5 s
Step 4	72ºC	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. Tm for the *Not*I miniprimer 57.5°C

6.3 Agarose gel electrophoresis

Analyze the PCR products by standard agarose gel electrophoresis. The length of a PCR product equals to the distance between the 15 bp insertion site and the vector-binding primer in a given clone.

Note: Since the palindromic *Not*I miniprimer anneals to each strand of the template DNA, a DNA fragment with the length of the whole target plasmid may also be produced in the PCR reaction.

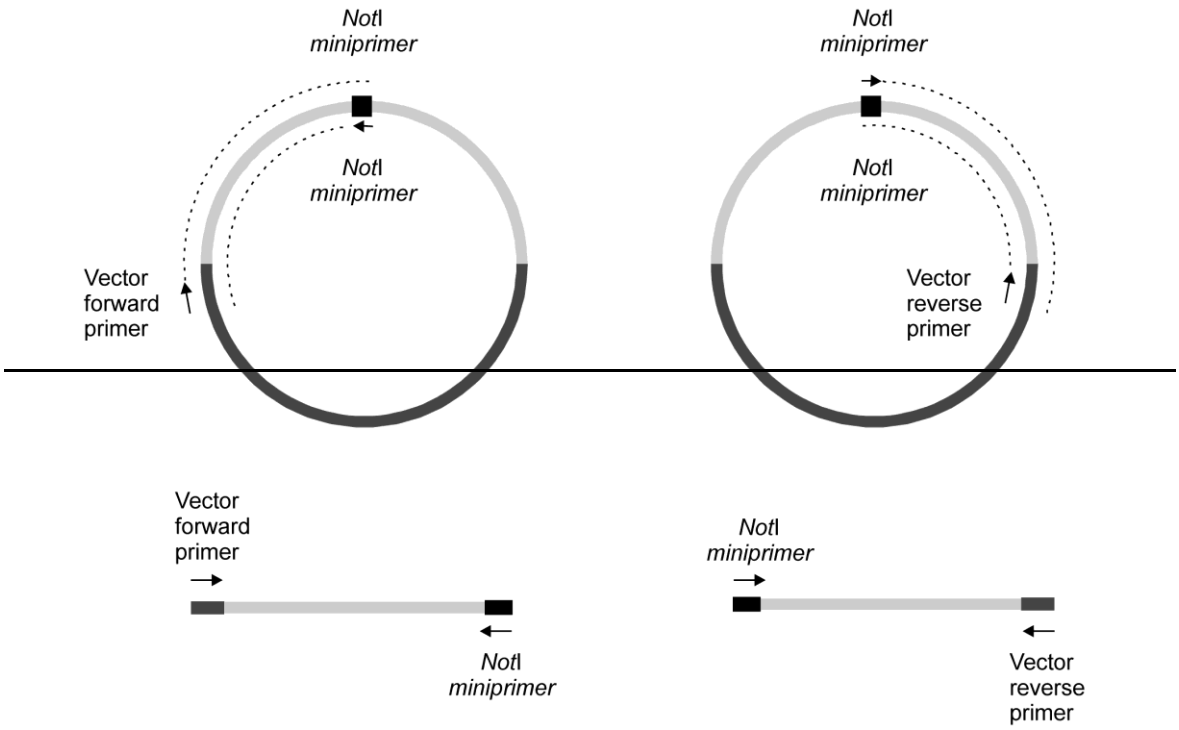


Figure 4. Colony-PCR mapping of the 15 bp insertion in the target DNA clone using the *Not*I miniprimer pairwise with primers that anneal to the vector sequence.

Appendix I: Descriptions of kit components

Entranceposon (M1-Cam^R)
10 µL 100 ng/µL in TE, pH 8.0

Entranceposon (M1-Kan^R)
10 µL 100 ng/µL in TE, pH 8.0
Entranceposon (M1-Cam^R) and (M1-Kan^R) are composed of inverted terminal repeats of modified bacteriophage Mu right end sequence with a *Not*I site, flanking the selectable marker gene, either *cat* (Cam^R) or *npt* (Kan^R). The marker genes code for resistance to the antibiotics chloramphenicol and kanamycin, respectively (see Appendix II for complete sequences).

MuA Transposase
10 µL 0.22 µg/µL in MuA
Storage Buffer A single purified polypeptide that catalyzes the in vitro transposition reaction. Isolated from an *E. coli* strain carrying the cloned bacteriophage MuA gene.

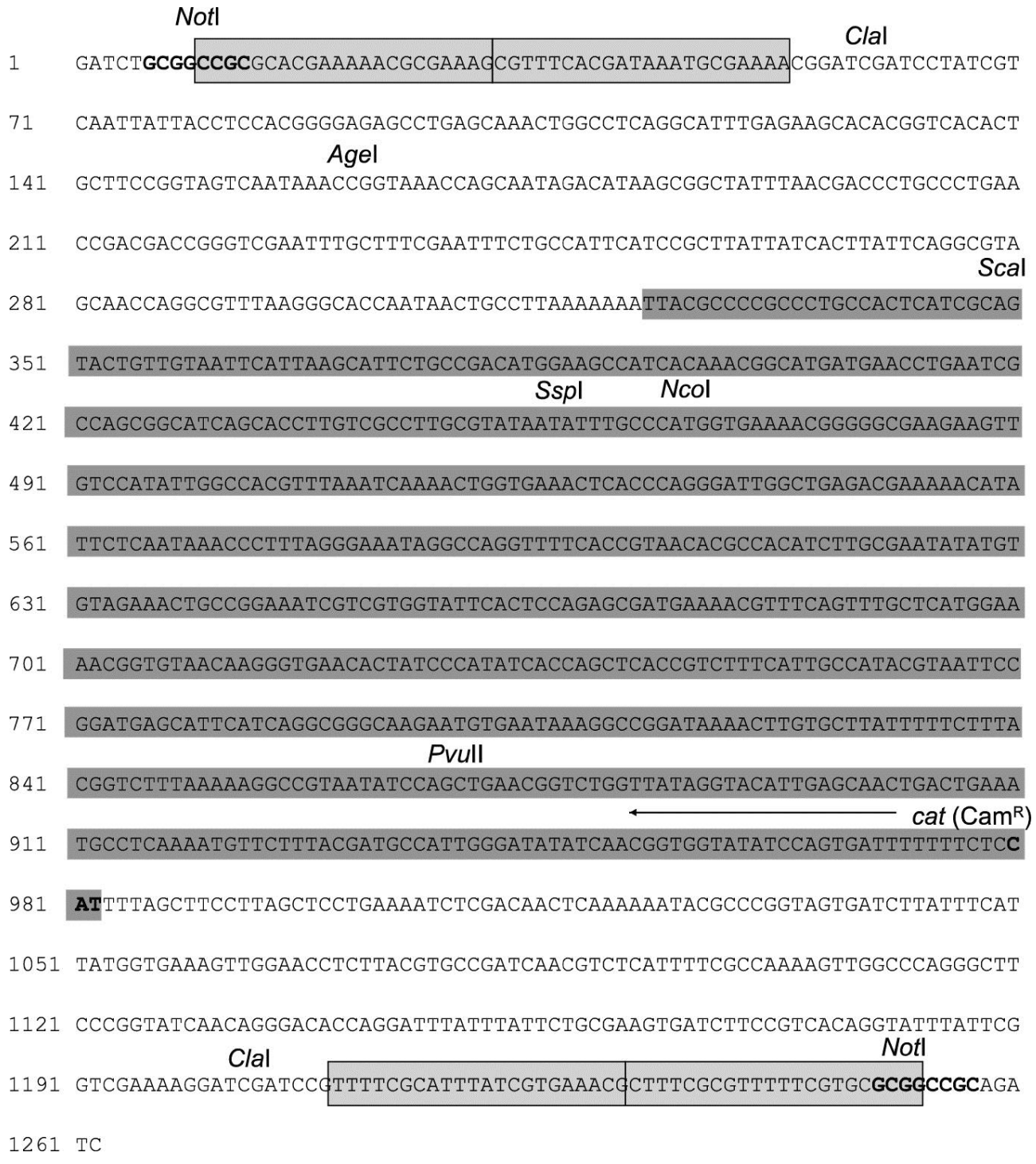
5X Reaction Buffer for MuA Transposase
100 µL Component 5X conc.

Control Target DNA
10 µL 370 ng/µL in TE, pH 8.0
A 6.6 kb *Hin*dIII fragment of bacteriophage lambda DNA cloned into the *Hin*dIII site of the vector pUC19.

***Not*I miniprimer**
50 µL 25 µM in H_2O
5'-TGCGGCCGCA-3' Tm 57.5°C
The miniprimer anneals to each DNA strand at the 15 bp insertion site generated by the MGS. Due to the small size of the primer some unspecific amplification products may appear in the colony-PCR.

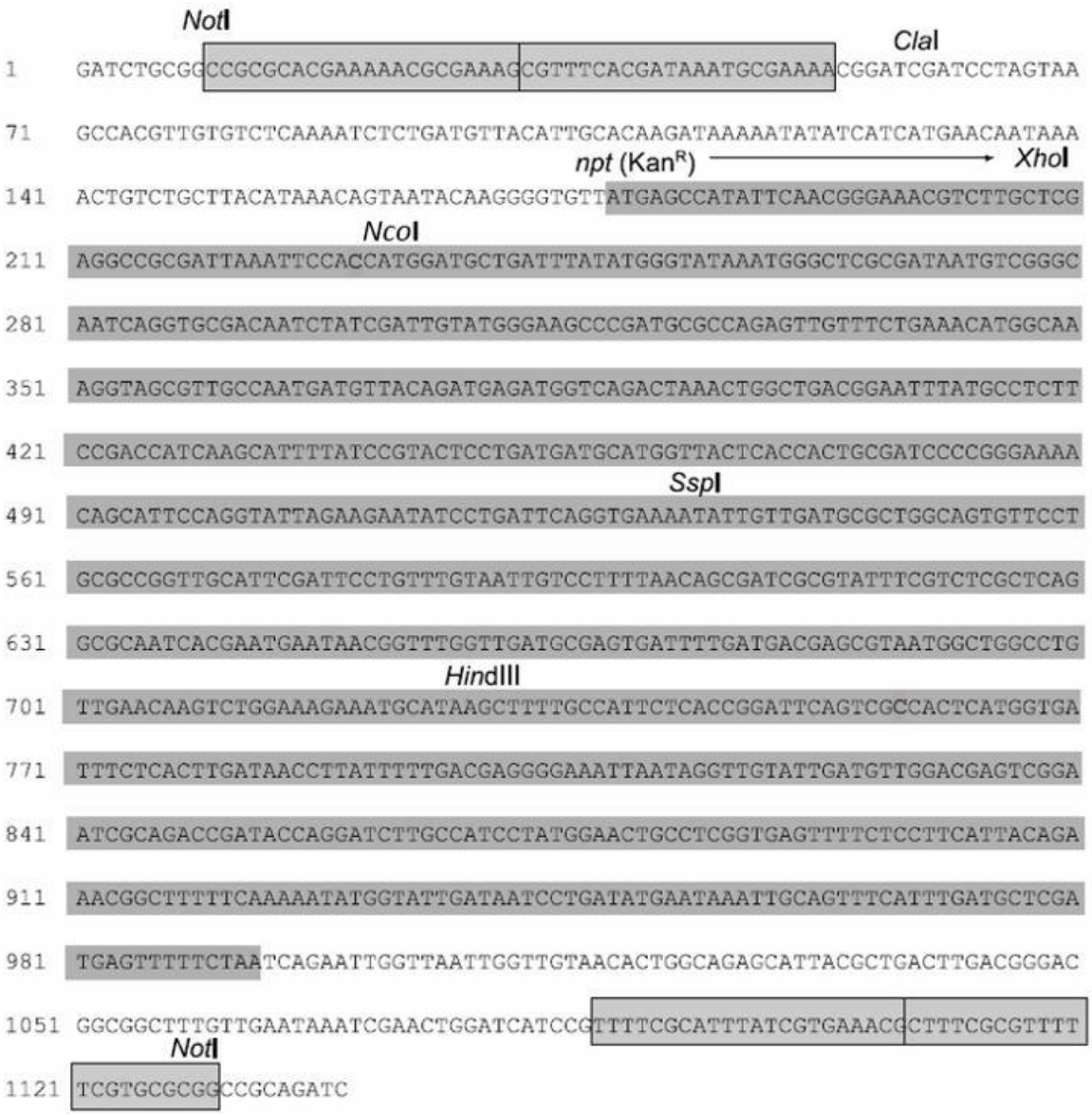
Appendix II: Maps of Entranceposons

a. Sequence of Entranceposon (M1-CamR), 1 254 bp



Entranceposon (M1-Cam^R) and (M1-Kan^R) are composed of inverted terminal repeats of the modified bacteriophage Mu right end sequence with a *Not*I site, flanking the selectable marker gene, either (a.) *cat* (Cam^R) or (b.) *npt* (Kan^R).

b. Sequence of Entranceposon (M1-Kan^{R}), 1 131 bp



Entranceposon (M1-Cam^R), non-cutting enzymes:

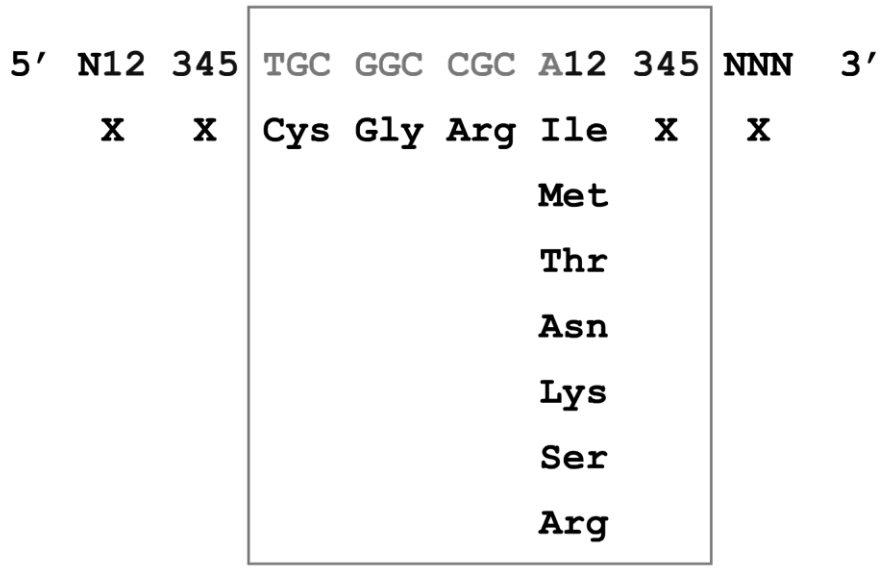
*Aat*II	*Acc*I	*Acc*65I	*Afl*III	*Alw*NI
*Apa*I	ApaLI	*Asc*I	*Ase*I	*Ava*I
*Ava*II	*Avr*II	*Bam*HI	*Ban*II	*Bbs*I
*Bbv*I	*Bcg*I	*Bci*VI	*Bcl*I	*Bfa*I
*Bgl*I	*Bgl*II	*Blp*I	*Bmr*I	*Bsa*I
*Bsa*BI	*Bse*RI	*Bsg*I	*Bsi*HKAI	*Bsi*WI
*Bsp*HI	BspMI	*Bsr*BI	*Bsr*GI	*Bss*HII
*Bss*SI	*Bst*API	*Bst*EII	*Bst*XI	*Dra*III
*Ear*I	*Eco*NI	*Eco*O109I	EcoRI	*Eco*RV
*Fse*I	FspI	*Hae*II	*Hga*I	*Hin*cII
HindIII	*Hpa*I	*Kas*I	*Kpn*I	*Mfe*I
*MLu*I	*Nae*I	*Nar*I	*Nde*I	*Nhe*I
*Nru*I	*Nsi*I	*Nsp*I	*Pac*I	*Ple*I
*Pme*I	*Ppu*MI	*Pst*I	*Pvu*I	*Sac*I
*Sac*II	*Sal*I	*Sap*I	*Sex*AI	*Sfc*I
*Sfi*I	*Sgr*AI	*Sma*I	*SmL*I	*Spe*I
*Sph*I	*Stu*I	*Swa*I	*Tse*I	*Xba*I
*Xcm*I	*Xho*I	*Xma*I	*Xmn*I	

Entranceposon (M1-Kan^R), non-cutting enzymes:

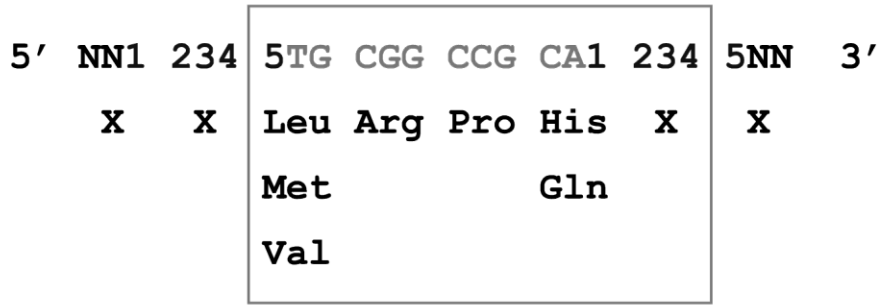
*Aat*II	*Acc*I	*Acc*III	*Acc*65I	*Afl*III
*Age*I	AlwNI	*Apa*I	*Apa*LI	*Asc*I
*Ava*II	*Avr*II	*Bal*I	*Bam*HI	*Ban*I
*Bbs*I	*Bbv*I	*Bcg*I	*Bcl*VI	*Bcl*I
*Bgl*I	*Bgl*II	*Blp*I	*Bmr*I	*Bpm*I
*Bsa*I	*Bsa*AI	*Bsa*BI	*Bse*RI	*Bsg*I
*Bsl*HKAI	*Bsl*WI	*Bsp*MI	*Bsr*BI	*Bsr*GI
*Bss*HII	*Bss*SI	*Bst*API	*Bst*EII	*Bst*XI
*Bsu*36I	*Dra*I	*Drd*I	*Eco*O109I	*Eco*RI
*Eco*RV	*Fau*I	*Fse*I	*Fsp*I	*Hae*II
*Hga*I	*Hin*cII	*Hpa*I	*Kas*I	*Kpn*I
*Mfe*I	*MLu*I	*Msc*I	*Msl*I	*Msp*A1I
*Nae*I	*Nar*I		*Nde*I	*Nhe*I
*Nla*IV	*Nsp*I	*Pac*I	*Pme*I	*Ppu*MI
*Pst*I	*Pvu*II	*Sac*I	*Sac*II	*Sal*I
*Sap*I	*Sau*96I	*Sca*I	*Sex*AI	*Sfc*I
*Sfl*I	*Sgr*AI	*Sna*BI	*Spe*I	*Sph*I
*Stu*I	*Sty*I	*Swa*I	*Tse*I	*Tth*111I
*Xba*I	*Xcm*I	*Xmn*I		

Appendix III: Translation of the 15 bp insertions

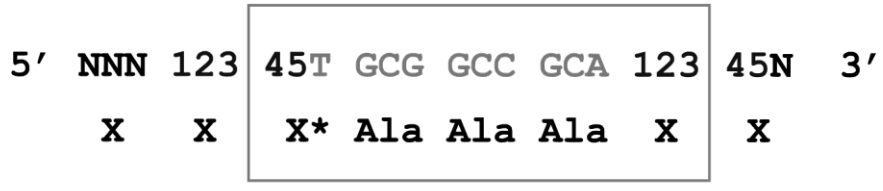
Frame 1:



Frame 2:



Frame 3:



X = Any amino acid

X* = Any amino acid except Gln, Glu, Lys, Met, Trp.

Appendix IV: DNA techniques

DNA precipitation

Precipitate the DNA in the reaction mixture by using the standard techniques.

1. Add 2 µl 3 M NaAc pH 5.5 (1:10 vol) and 50 µl 94% ethanol (2.5 vol).
2. Incubate on ice for 20 minutes and centrifuge 13000 rpm for 30 minutes at 4°C.
3. Remove the supernatant carefully and rinse the DNA pellet with 500 µl 75% ethanol.
4. Spin briefly, remove the ethanol supernatant and air-dry the pellet.
5. Resuspend the DNA pellet in deionized water for electroporation.

Transformation

Electroporation: 1X Reaction Buffer for MuA Transposase contains 125 mM NaCl. To avoid arcing that may result from excessive salt dilute the reaction mixture ten-fold in deionized water and use maximum of 10 µl per single electroporation (typically 40-50 µl electrocompetent cells, transformation efficiency >108 cfu/µg pUC19). If it is necessary to use more than 1 µl of the reaction mixture per single electroporation, precipitate the DNA in the reaction mixture as instructed above, resuspend in deionized water and use aliquots for transforming electrocompetent cells. Use the pulse settings instructed by the manufacturer of your electroporation system.

Chemical transformation: For chemical transformation use 5-10 µl of the reaction mixture per 50-100 µl chemically competent cells (transformation efficiency >10^7 cfu/µg pUC19). Before plating on selective LB agar plates, it is necessary to grow the cells in 1 mL SOC medium for one hour at 37°C to ensure expression of the marker gene present in the Entranceposon.

Preparation of plasmid DNA from transformation plates

1. Scrape small bacterial colonies from transformation plates in TE, pH 8.0. Adjust the cell density to equal an overnight liquid culture.
2. Transfer 1.5-3 mL of the suspension to a fresh tube and pellet the cells by spinning briefly in a microcentrifuge.
3. Discard the supernatant and resuspend the bacteria in 250 µl of 15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A.
4. Add 250 µl of 0.2 M NaOH, 1% SDS to lyse the cells. Mix thoroughly but do not vortex. Incubate 5 min at RT.
5. Add 350 µl of 3 M KAc, pH 5.5 and mix by turning the tubes vigorously immediately after KAc addition.
6. Immediately centrifuge 13000 rpm for 15 minutes at 4°C to pellet the chromosomal DNA and cell depris.
7. Transfer 400 µl aliquot of the supernatant into fresh microcentrifuge tube.
8. Add 1 volume of phenol:chloroform (1:1), vortex briefly and centrifuge at full speed for five minutes to separate the aquous and organic phases.
9. Extract the aquous phase twice with chloroform to remove the traces of phenol.
10. Transfer the aquous phase into a fresh tube and precipitate the DNA as instructed above.
11. Resuspend the DNA pellet in TE, pH 8.0.

DNA extraction from agarose gel

1. Separate the fragments resulting from a restriction enzyme digestion by standard agarose gel electrophoresis.
2. Cut out the desired band from the agarose gel with a clean scalpel.
3. Melt the gel slice at 65°C for 10 min. Add TE, pH 8.0 to decrease the agarose concentration to >0.4%.
4. Add an equal volume of buffered phenol and mix vigorously for 5 min.
5. Centrifuge 13000 rpm for 10 min in a microcentrifuge.
6. Remove the aqueous phase to a fresh eppendorff tube and re-extract the phenol phase with an equal volume of TE, pH 8.0.
7. Combine the aqueous phases and extract again with an equal volume of buffered phenol.
8. Extract the aquous phase twice with chloroform to remove the traces of phenol.
9. Transfer the aquous phase into a fresh tube and precipitate the DNA as instructed above.
10. Resuspend the DNA pellet in deionized water for ligation reaction.

Appendix V: 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:

Chloramphenicol (per liter) **OR**	20 mg
Kanamycin (per liter) **OR**	20 mg
Tetracycline (per liter)	10 mg

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

SOC medium

Tryptone	20 g
Yeast Extract	5 g
NaCl	0.5 g
KCl	0.186 g

Adjust pH to 7.0 with 1 M NaOH
Autoclave
Before use add sterile solutions:

1 M $MgCl_2$	10 mL
1 M $MgSO_4$	10 mL
1 M Glucose	20 mL

TE

10 mM Tris-HCl, pH 8.0
1 mM EDTA

Appendix VI: Related products

Transposon products

F-750 MuA Transposase, 20 µL (0.22 µg/µL)
F-750C MuA Transposase, conc., 20 µL (1.1 µg/µL)
F-760 Entranceposon (M1-CamR)
F-762 Entranceposon (M1-KanR)
F-702 Template Generation System II Kit
F-703 Stop Generation System Kit
F-764 Entranceposon (TetR)
F-771 Entranceposon (*supF*)
F-774 Entranceposon(*lacZ*)
F-765 pEntranceposon (CamR)
F-766 pEntranceposon (KanR)
F-767 pEntranceposon (TetR)
F-773 pEntranceposon (*supF*)

PCR products

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

Appendix VII: References

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