Insertional mutagenesis in *Medicago truncatula* using *Tnt1* retrotransposon.

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1. Insertional mutagenesis in *M. truncatula*

Gene function discovery is generally done by altering the expression or by disrupting the gene under study in a given organism. Such insertional mutagenesis technology is a powerful tool to inactivate genes in many organisms and mutants created by this technique can in turn be used to elucidate gene functions and to contribute for example to the elucidation of the function of many genes revealed by sequencing the genome of a model organism.

In legume plants, large scale T-DNA mutagenesis necessary for the construction of large mutant collections is not feasible principally because of the absence of an efficient transformation technology (Schauser et al., 1998, Scholte et al., 2002). Thus, alternative routes for mutagenesis had to be explored and developed. Insertion mutagenesis with heterologous transposable elements is one alternative to identify legume symbiotic genes as well as genes of agronomic importance. However, insertion mutagenesis tools developed for other plants, for example maize transposable elements, were not applicable to *M. truncatula.* Previously, we demonstrated that maize *En/Spm* and *Arabidopsis thaliana Tag1* transposable elements are either inactive or poorly active in *M. truncatula* (d'Erfurth et al. 2003b and 2006). In contrary, the tobacco retrotransposon *Tnt1* is an efficient mutagen and is active in both *M. truncatula* ssp. *tricycla* line R108 (d'Erfurth et al., 2003a) and the Jemalong line (*M. truncatula* ssp. *truncatula*) used for molecular studies worldwide (www.medicago.org; www.eugrainlegumes.org/).

Tnt1 is an autonomous 5.3 kb long copia-like LTR element from tobacco that creates a target site duplication of 5 bp upon insertion (Grandbastien *et al.*, 1989). *Tnt1* transposition was induced in cultures of tobacco protoplasts (Pouteau *et al.*, 1991) and during *in vitro* transformation of *A. thaliana* (Courtial *et al.*, 2001). Its integration seems to be random as no site-specificity can be observed. In addition, genes are frequently disrupted upon insertion, indicating preferential insertion in euchromatic regions. The insertions are stable and frequently unlinked due to the replication cycle of LTR retrotransposons (Courtial *et al.*, 2001). All these characteristics make *Tnt1* a versatile tool for insertion mutagenesis in different plants.

In *M. truncatula*, *Tnt1* transposition generates from 4 to up to 40 insertions per transformed plant. These insertions are stable during the life cycle of *M. truncatula* and most of them are genetically independent and can be separated by recombination. In addition, *Tnt1* seems to transpose preferentially into open reading frames (d'Erfurth et al., 2003a). *Tnt1* multiplication by transposition can be re-induced by tissue culture, making this element an attractive candidate to set up a large scale insertion mutagenesis in *M. truncatula* (d'Erfurth et al., 2003a). The efficiency of *Tnt1* as a mutagen allowed initiating several international large scale mutagenesis programs using this transposable element (www.noble.org/medicago/; www.eugrainlegumes.org/). The aim of the produced collections is to make them available to the scientific community.

M. truncatula mutants generated in these programs will be used for forward as well as reverse genetics which can be conducted by PCR screening on DNA pools or by sequencing *Tnt1* insertion sites in the transgenic lines. Forward genetics screen of *Tnt1* insertion lines will be a non destructive assay to save the lines for seed collection. Screens are done in batches of 1,000 lines/year, where interested people get together and screen for various phenotypes of interest at the same time. PCR based screening of DNA pools can be performed for any favoured gene at any time without a need for community screen. In the future, the development of a sequence database corresponding to the majority of the *Tnt1* inserts in the population may represent a valuable tool for the scientific community. We have initiated to build a flanking sequence database at the Noble Foundation which will be publicly available. We already have a limited number (~1000) of *Tnt1* flanking sequence tags (FSTs). Similarly

1,000 *Tnt1 Medicago* FSTs should be available from the GLIP-FP6 program by the end of 2007.

Recovering all the *Tnt1* flanking sequences from a mutant line is less efficient due to the inherent multicopy nature of *Tnt1* and inability to use plasmid rescue protocols. Efforts are underway to improve the efficiency of flanking sequence recovery.

2. Specificity of the *Tnt1* mutant collections.

The *Tnt1* mutant collections can be used for phenotypic screen (forward genetics) or through PCR based reverse genetic approaches. The phenotypic screen used will depend on the biological question asked (see chapter "Mutant screening / Phenology key"). The efficiency of *Tnt1* transposition during the regeneration process results in mutant *Medicago* lines carrying multiple *Tnt1* inserts (d'Erfurth et al., 2003a) and by consequence possibly multiple mutations. This has led us to develop new approaches for mutant plant analysis and isolation of the tagged loci. In order to propagate one mutant line with its different inserts, it is important to grow several plants of the progeny as a pool, in order to maintain the different inserts in this progeny. In order to correlate the presence of one particular insertion to any observed phenotype (genetic linkage) the mutant lines should be analyzed in details and it is first reasonable to backcross these mutant lines to a wild-type one in order to decrease the *Tnt1* copy number and facilitate further analysis. If it is necessary to cross a R108 line to Jemalong, our experience indicates that it might be better to use the Jemalong line as a female parent.

When a forward genetic approach was taken and an interesting phenotype (for example, Altered Leaf Development; ALD) was observed in a mutant line not yet characterized, one possibility is, using the PCR techniques described below, to isolate various insertion sites in this line. It might be better to first do backcrosses in order to reduce the *Tnt1* copy number because the lower the *Tnt1* copy number is, greater is the chance to isolate the flanking sequence. Once these loci are sequenced, the genetic linkage between the mutant loci and the ALD phenotype can be analyzed by PCR in a segregating population. Alternatively, transposon display technology (Melayah et al., 2001) can be applied on a segregating population in order to find the *Tnt1* border linked to the phenotype. This technique might be more time consuming but also more powerful for the characterization of the tagged locus.

When a reverse genetic approach was taken and a mutant line has been identified, containing Your Favourite Gene (YFG) disrupted, the original line should be back-crossed to wild-type plants (see chapter "Vernalizations, crossings and testing for pollen viability") and the progeny analyzed for co-segregation of a putative phenotype with the insertion. The disrupted region can be easily followed in the progeny by PCR, using one oligonucleotide corresponding to the *Tnt1* end and one oligonucleotide in the sequence of YFG. Using one oligonucleotide on each side of the insertion site will in addition indicate for each plant if the YFG locus is wild-type, heterozygous or homozygous for the insertion. Using this simple PCR analysis several successive backcrosses can be performed and the disrupted locus detected in the plants independently of their phenotype. Genetic linkage between phenotype and homozygous mutant loci can then be confirmed in a segregating population.

The mutant collection developed in the different laboratories are based on two plant lines, the *M. truncatula* ssp. *tricycla* line R108 (Trinh et al. 1998) and the Jemalong line (*M. truncatula* ssp. *truncatula*). The later correspond to the EU developed collection (FP6-GLIP). These two lines have different growth behaviour as well as different plant-microbe specificities. The Gif-CNRS original collection was obtained mainly by transformation using the pTnk23 vector. Thus, these lines contain at least one T-DNA copy different in each line. One should keep in mind that these T-DNA copies can also act as insertional mutagen. The

regenerated lines from the Noble foundation and from the CNRS do not differ in the T-DNA insert and there is no new mutation due to T-DNA insertion. There are 3 *Tnt1* inserts in the progenitor line used for regeneration at Noble which are probably common to all regenerated lines and all of the newly introduced inserts are obtained by transposition and contain no T-DNA.

The lines produced in the GLIP-FP6 program are Jemalong based but were obtained either by regeneration of starter lines or by transformation using either the ptnk23 vector (Lucas et al., 1995) or a pCAMBIA 1381XC (www.cambia.org) hygromycin based vector (d'Erfurth unpublished) that can both act as additional insertional mutagen. We observed that *Tnt1* was less reactivated in multicopy Jemalong lines when compared to R108 lines.

Once a disrupted gene was characterized it might be important, in order to confirm the tagging, to complement the mutation. As *Tnt1* transposition is reactivated by tissue culture (somatic embryogenesis) it might be better to use a protocol based on organogenesis for the introduction of the transgene. We have tested the cotyledon transformation protocols (Ding et al. 2003; Zhou et al. 2004) and found that *Tnt1* transposition is not reactivated in both R108 and A17 lines. A combination of other hormonal, chemical and stress treatments also failed to activate transposition (Ratet, Tadege and Mysore unpublished observations). Only if a desired shoot phenotype results in absolute sterility, there may be a need to resort to somatic embryogenesis for complementation in which case one has to deal with the additional inserts that will be generated by *Tnt1* reactivation and could complicate further analyses. Nevertheless, additional phenotypes appear to be not as big a problem because the new *Tnt1* inserts will be heterozygous in the rescued parent and will segregate in progenies. We have done such complementation by somatic embryogenesis and it works well. For root based phenotypes, hairy root (see chapter "*Agrobacterium rhizogenes*-mediated transformation") complementation can be used.

3. Molecular description of the *Tnt1* retroelement:

The *Tnt1* sequence Accession number is: X13777. In the T-DNA plasmids the sequence surrounding the *Tnt1* element is coming from the tobacco *nia-2* gene (nitrate reductase). The 5334 bp *Tnt1* sequence is composed of two 610 bp long LTR surrounding the 4114 bp reverse transcriptase region (see below). Generally the oligonucleotides are designed on the LTR sequence. However, for the first PCR reaction it might be convenient to use oligonucleotides designed on the non LTR sequence.

General structure of the tobacco *Tnt1* retroelement. The LTR region are indicated by red arrows and the reverse transcriptase region in green. Note that the two LTR are identical in sequence.

oligo name	sequence	position in *Tnt1*	orientation
LTR3	AGTTGCTCCTCTCGGGGTCGTGGTT	436-460 / 5160-5184	foward
LTR31	GCTCCTCTCGGGGTCGTGG	440-458 / 5164-5182	foward
LTR4	TACCGTATCTCGGTGCTACA	534-553 / 5258-5277	forward
LTR5	GCCAAAGCTTCACCCTCTAAAGCCT	203-179 / 4927-4903	reverse
LTR6	GCTACCAACCAAACCAAGTCAA	99-78 / 4823-4802	reverse
LTR7	TATTATTCCGCTTTATTACCGTGA	555-578 / 5279-4302	forward
Tntail1	TATGCAAAGAACTTGTCGGCATGC	4644-4667	forward
Tntail2	CAAACTAGAAGACAGTGCTACCTC	4671-4694	forward
Tntail3	TCTGGATGAATGAGACTGGAGG	4696-4717	forward

Table1: *Tnt1* oligonucleotides used in the PCR protocols described below. When relevant, the position in the two LTR is given.

4. Southern blot analysis of *Tnt1* containing lines:

Southern blots can be used to analyse the *Tnt1* containing lines. This technique is however tedious and not very informative in lines containing more then 10 *Tnt1* copies. The choices of the probe and the restriction enzymes used to digest the genomic DNA are also important. It is better to choose the probe in the internal part of the *Tnt1* element because both LTR are identical (see the figure with *Tnt1* general structure) and a probe including them or part of them will reveal the two flanking sequences and complicate the analysis. Best results will be obtained using restriction enzymes cutting quite often in the *Medicago* genome like *Dra*I for example. Digesting the genomic DNA overnight with restriction enzyme that cuts the *Tnt1* sequence only once and using a probe from only one half of the cut *Tnt1* fragment excluding LTR, results at least theoretically in detecting all *Tnt1* inserts and each insert only once. We use NcoI for digestion, and a 600-950 bp probe from the right side of NcoI site, and get modest results for lower copy *Tnt1* lines. As the *Tnt1* copy number increases, separating fragments especially larger ones becomes difficult and many fragments seem to migrate at the same size. In order to get a good fragment separation we used 1% agarose gel in TAE buffer run at 35 Volt/cm for 20 hours.

5. PCR based technologies for flanking site characterization:

Mutant lines can be analyzed and characterized using PCR based technology. This allows the rapid characterization of a large number of lines. We have been using three types of protocols; IPCR and AFLP in the Gif and Perugia laboratories or IPCR and TAIL PCR at the Noble Foundation as described below.

5.1 Inverse PCR.

This protocol allows the isolation of the two sides (flanking sequences) of *Tnt1* copies inserted in the genome. It is based on the restriction of the genomic DNA by restriction enzymes not cutting in the *Tnt1* sequence but in the *Tnt1* flanking sequences and self ligation of these restriction fragments. It does not requires an adaptor so that various restriction enzymes (or RE combinations) can be tested. The only limitation is that when using two RE the cohesive ends should be compatible or blunt. In the later case the self ligation efficiency

will be reduced. It is a nested PCR protocol that will amplify the flanking sequences using oligonucleotides reading outward from the transposable element.
We have observed that the choice for the restriction enzyme is important because large fragments (more than 1 Kb) will be difficult to amplify or will generate rearranged structures difficult to interpret. The frequency of a restriction site in the *Medicago* genome will depend on its recognition sequence. Ase1 (AT'TAAT) or the combination of EcoRI+MfeI (EcoR1: G'AATTC and Mfe1: C'AATTG have the same cohesive ends) generates fragments with an average size below 1 Kb that can be easily amplified. Doing two I-PCRs, one based on Ase1 digestion and another based on EcoRI+MfeI digestion followed by cloning and sequencing of 48 colonies from each will be more efficient than doing 96 colonies from one I-PCR.

Genomic DNA digestion:

- Digest genomic DNA (200 ng) in 50µL minimum 2 h at 37°C (or 1 night at 16°C). Digestion for *Tnt1* I-PCR can be performed with *Ase*1 or with *Eco*RI+*Mfe*I.

  5 µL DNA
  + 5 µL Buffer 10x
  + 2 µL Enzyme
  + 38 µL H_2O

- Restriction enzyme inactivation 20 min at 65°C

Genomic DNA self ligation:

- Ligation in 200 µL 1 night at 16°C

  50 µL Digestion
  + 20 µL Buffer 10x
  + 2 µL Ligase
  + 128 µL H_2O

PCR step 1:

IPCR 1 of the ligation mix by using the Takara kit (Takara Ex Taq™ ; ref. RR001A ; www.takara-bio.co.jp)

10 µL Ligation
+ 2 µL Buffer 10x
+ 1,6 µL dNTP
+ 0,5 µL LTR3 oligo (10 µM stock)
+ 0,5 µL LTR5 oligo (10 µM stock)
+ 0,1 µL Takara Ex Taq
+ 5,3 µL H_2O

94°C	2 min	
94°C	20 sec	x 30
72°C	3 min	
72°C	5min	

PCR step 2:

- IPCR 2 with Takara kit using the IPCR 1 reaction
  2 µL IPCR 1 diluted 100x
  + 2 µL Buffer 10x
  + 1,6 µL dNTP
  + 0,5 µL LTR4 oligo (10 µM stock)
  + 0,5 µL LTR6 oligo (10 µM stock)
  + 0,1 µL Takara
  + 13,3 µL H_2O

94°C	2 min	
94°C	20 sec	x 30
60°C	20 sec	
72°C	3 min	
72°C	5min	

Fragment characterization:
Use 5µL of the PCR2 reaction.
Use 1.5 % agarose gel in order to have a good separation of the fragments.

5.2 TAIL PCR for recovering *Tnt1* flanking plant sequences.

We have optimized the Thermal Assymetric Inter Laced (TAIL) PCR protocol for *M. truncatula*, with some minor modifications of TAIL PCR protocol used for Arabidopsis. TAIL PCR uses one arbitrary primer and three gene specific nested primers, and the reaction requires three PCR runs: the first reaction using the genomic DNA of interest as template, the second using diluted first PCR product and the third using diluted second PCR product as templates. Each gene specific nested primer is used in combination with the same arbitrary primer and the final PCR product is then cloned into pGEM-T vector for sequencing. We use the first *Tnt1* specific primer outside but close to the LTR region near the 3' end to avoid amplification of *Tnt1* alone. The subsequent two nested primers are from the LTR region. Digestion of genomic DNA with NcoI seems to improve the efficiency of junction recovery by enriching for *Tnt1* sequences and avoiding *Tnt1* amplification. Generally, TAIL PCR gives more inserts and longer fragments than the IPCR. It is certainly faster than IPCR and the absence of a ligation step before the PCR reaction helps to avoid rearranged artifacts associated with IPCR mentioned above. Sequencing using the 3rd TAIL PCR primer always shows the insert in one direction making it easy to determine the orientation of the insert and design other primers for genotyping. Generally both IPCR and TAIL PCR suffer from the fact that short PCR products have a higher chance of being preferentially amplified and cloned into the vector in a mixed population and hence one has to sequence more colonies to get representative inserts. We usually sequence 48 or 96 colonies from one line and obtain approximately 5 – 20 non redundant inserts. Doubling the number of colonies from 48 to 96 gives slightly more inserts but far below from doubling the number of inserts. Doing one TAIL PCR and one IPCR or two separate TAIL PCR reactions with different primers and sequencing 48 colonies from each, on the other hand, appears to double the number of inserts and recovers nearly all inserts from a given line.

TAIL PCR protocol

1. Genomic DNA should be pure enough to be digested by restriction enzyme. Use the CTAB method or Qiagen kit for DNA extraction. Digest 1 µg DNA with enzyme o/n and test on 1% agarose gel to see if digestion is complete. CTAB and Qiagen mini plant DNase kit usually give clean DNA that can be completely digested. If you use other methods and the DNA is not restricted, it is best to purify it once more with phenol/chloroform before proceeding to TAIL PCR.
2. Digest ~200 ng of genomic DNA with NcoI O/N (can also use undigested DNA) to use as a template and amplify using *Tnt1* specific primer (Tntail1, 2, 3, or other outside the LTR) and one of the arbitrary primers. The more degenerate the arbitrary primer, the higher the concentration required in the PCR reaction.
3. Run the first PCR reaction as specified below.
4. Test 10 µl of the PCR product on 1% gel. Most of the strong bands at this stage correspond to unspecific amplification.
5. Dilute the PCR product 100X and use 2µl of the diluted sample as a template in the second PCR reaction.
6. The second PCR reaction is exactly the same as the first PCR reaction except that the template is different and *Tnt1* specific primer 1 is replaced by *Tnt1* specific primer 2. If you started with AD1 in the first reaction, you should use AD1 for all the reactions. The same applies if you started with AD2 , AD3 or any other arbitrary primer.
7. Test 5 µl of the second PCR product on 1% agarose gel. If there are strong bands that were not clearly visible in the first reaction, the reaction could be stopped here as final and the products could be cleaned through a PCR purification kit and ligated to pGEM –Teasy vector o/n at 16 ^{O}C. but most commonly one more step is performed.
8. Dilute the second PCR product 100X and use 1 µl of the diluted sample as template in the third PCR reaction.
9. Perform the tertiary PCR reaction replacing *Tnt1* specific primer 2 with *Tnt1* specific primer 3.
10. Test 5µl of the product on gel, clean the product with PCR purification kit, and clone.
11. Transform *E.coli* with the ligation product using blue/white selection (see below 1.5.4).
12. Miniprep 48 white colonies in a microtiter plate and sequence. Repeat the whole procedure with new set of *Tnt1* specific or arbitrary primers or both if more inserts are required.

Arbitrary primers

AD1 NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT
AD2 NGTCGA(G/C)(A/T)GANA(A/T)GAA
AD3 (A/T)GTGNAG(A/T)ANCANAGA

PCR Reaction 1 (primary):

4 µl 5x buffer
2 µl NcoI digested or undigested (~200 ng dig in 20 µl) genomic DNA
1.6 µl (2.5 mM stock) dNTPs
1.0 µl (10 µM stock) Tntail3
3 µl AD2 (20 µM stock)
0.3 µl Ex Taq
X µl water to 20µl final volume.

PCR Reaction 2 (secondary):

4 µl 5X buffer
2 µl 100X diluted PCR product 1
1.6 µl l (2.5 mM stock) dNTPs
1.0 µl (10 µM stock) LTR4
2.0 µl AD2 (20 µM stock)
0.3 µl Ex taq
X µl water to 20 µl final volume.

PCR Reaction 3 (tertiary):

4 µl 5x buffer
1 µl 100X diluted secondary PCR product
1.6 µl (2.5 mM stock) dNTPs
1 µl (10 µM stock) LTR7
1.6 µl AD2 (20 µM stock)
0.3 µl Ex Taq
X µl water to 20µl final volume.

TAIL PCR Conditions:

Reaction	**file no.**	**cycle no.**	**thermal settings**
Primary	1	1	93 ^{O}C, 1min, 95 ^{O}C, 1 min
	2	5	94 ^{O}C, 30 sec; 62 ^{O}C, 1 min; 72 ^{O}C, 2.5 min
	3	1	94 ^{O}C, 30 sec; 25 ^{O}C, 3 min; ramping to 72 ^{O}C, over 3 min; 72 ^{O}C, 2.5 min
	4	15	94 ^{O}C, 30 sec; 68 ^{O}C, 1 min; 72 ^{O}C, 2.5 min 94 ^{O}C, 30 sec; 68 ^{O}C, 1 min; 72 ^{O}C, 2.5 min 94 ^{O}C, 30 sec; 44 ^{O}C, 1 min; 72 ^{O}C, 2.5 min
	5	1	72 ^{O}C, 5 min.
Secondary	6	12	94 ^{O}C, 30 sec; 64 ^{O}C, 1 min; 72 ^{O}C, 2.5 min 94 ^{O}C, 30 sec; 64 ^{O}C, 1 min; 72 ^{O}C, 2.5 min 94 ^{O}C, 30 sec; 44 ^{O}C, 1 min; 72 ^{O}C, 2.5 min
	5	1	72 ^{O}C, 5 min.
Tertiary	7	20	94 ^{O}C, 60 sec ; 44 ^{O}C 1 min ; 72 ^{O}C, 2.5 min
	5	1	72 ^{O}C, 5 min.

5.3 AFLP type PCR protocol for Tnt1 border characterization.

This protocol allows the amplification of the *Tnt1* borders using an oligonucleotide adaptator. This is an AFLP type protocol co-developed by Andrea Porcedu, (CNR-Perugia, Italie), and Pascal Ratet (CNRS-Gif sur Yvette, France). Briefly the genomic DNA from a *Tnt1* line is digested with EcoR1 plus Mfe1, ligated to an oligonucleotide adaptor and the borders are amplified through a nested (two steps) PCR. EcoR1 (G'AATTC) and Mfe1 (C'AATTG) have the same cohesive ends compatible with the oligonucleotide adaptor. This enzyme combination gives us good results when the *Tnt1* copy number is not very high. Note that these enzymes can be used separately to reduce the PCR complexity if needed.

The structure of the double stranded oligonucleotide adaptor is:

```
CCCCTCGTAGACTGCGTACC        (Adapt + strand)
     gCATCTgACgCATggTTAA    (Adapt - strand)
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Oligonucleotides:

Adapt + strand	5' CCC CTC GTA GAC TGC GTA CC 3'
Adapt - strand	5' AAT TGG TAC GCA GTC TAC G 3'
Eco1	5' CTC GTA GAC TGC GTA CCA A 3'
Eco2	5' CGT AGA CTG CGT ACC AAT T 3'

To construct 50 mM (10X) double strand adaptor, mix equal volume of the 100 mM Adaptor 1 and Adaptor 2 oligonucleotide solutions, heat the mix at 95°C for 10 mn and then let the mix cool at room temperature.
The use of a high quality enzyme (Takara ExTaq enzyme, www.cambrex.com) for the PCR (see below) seems to be very important.

Protocol:

Genomic DNA digestion:
Double digest 2 to 3 micrograms of genomic DNA with 10u EcoR1 and 10u Mfe1 in 50 µL final volume for 3h at 37°C.

Inactivate the enzymes 20 mn at 65°C.

Ligation:

5 µL digested DNA
1 µL double stranded adaptator (1X, 5mM)
2 µL 10X ligation buffer
0,1 µL ligase (Biolabs ref: M02025, 400u/µL)
11,9 µL H2O (QSP 20 µL)

Ligation overnight at room temp or in a 16°C bath. We have also done it 5H at 16°C followed by 2H at 37°C.

Border amplification:

This is a nested (2 steps) PCR.

PCR1:

ligated DNA ……	2 µL
10X Taq buffer……...2	µL
dNTP mix……...1.6	µL
10 µM LTR31 oligo …	0.5 µL
10 µM Eco1 oligo ……	0.5 µL
Takara ExTaq	0.08 µL
H2O	13.32 µL

PCR1 program:

94°C 2mn 1X
94°C 20s, 60°C 20s, 72°C 2mn 5X
94°C 20s, 58°C 20s, 72°C 2mn 5X
94°C 20s, 56°C 20s, 72°C 2mn 20X

PCR2:
!!!!!!!Dilute PCR1 100 times!!!!!!!

Diluted DNA from PCR1	2 µL
10X Taq buffer	2 µL
dNTP mix	1.6 µL
10 µM LTR4 oligo	0.5 µL
10 µM Eco2 oligo	0.5 µL
Takara ExTaq	0.08 µL
H2O	13.32 µL

PCR2 program:

94°C 2mn 1X
94°C 20s, 55°C 20s, 72°C 2mn 10X
94°C 20s, 52°C 20s, 72°C 2mn 25X

Fragment characterization:
Use 5µL of the PCR2 reaction.
Use 1.5 % agarose gel in order to have a good separation of the fragments.

5.4 Cloning the PCR amplified FST for sequencing:

If it is necessary to clone and sequence the borders, we generally bulk clone the PCR fragments representing the *Tnt1* insertion sites using the pGEM-T based technology (www.promega.com). The ligation mix (see below) is then transformed in *E. coli* using a blue/white selection and the plasmids sequenced.

- Cloning the PCR 2 product in pGEM-T : 1 night at 16°C
  7 µL IPCR 2
  + 2 µL Buffer 10x
  + 0,5 µL pGEM-T
  + 1 µL Ligase
  + 9,5 µL H_2O

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