Nidhi Rawat, WGGRC, Kansas State University

TILLING PROTOCOL

Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetics tool used to scan for mutations in genes of interest in a population of chemically induced mutants. Essentially TILLING needs the following resources at hand:

1. Plant mutant population: Ethyl-methane sulphonate (EMS), methyl methane sulphonate (MMS), hydrogen fluoride (HF), sodium azide, *N*-methyl-*N*-nitrosourea, and hydroxylamine are some of the most widely used chemical mutagens for plants. Consult the literature for selection of the mutagen and size of the population required. The dosage and time of treatment should first be optimized on a small sample size to give a mortality rate of at least 50% in the treated individuals. Once optimized, the same treatment should then be used for large-scale treatment of the entire set. One seed/M_1 plant should be planted to get the M_2 population.
2. Primers: Primer development is the most critical step for TILLING. Primers should be designed such that they give a single, bright band. For agarose-based detection, a product size of 500 bp or higher is recommended. In fact, the greater the product size the better. Generally, it is a good idea to design primers based on exonic sequence of the genes to avoid irrelevant intronic SNPs.
3. Celery juice extract (CJE): Cel-*I* is the enzyme that detects SNPs in PCR products, and a crude CJE does equally as well as the expensive, commercially prepared Cel-*I*.

   Protocol for CJE preparation is based on Till et al. (2004), and is as follows:

   All the steps are at 4 °C

   i. Wash ~1 lb (one bunch) of celery in cool water. Remove any leafy material to aid in juicing. Pass the celery through a juicer. Approximately 400 ml of juice will be produced.
   ii. Centrifuge the juice for 20 min at 2,600 *g* to pellet debris. Transfer the supernatant to a new tube.
   iii. Add 1 M Tris and phenylmethanesulfonyl fluoride (PMSF) to the cleared celery juice so that the final concentration of the solution is 0.1 M Tris-HCl, pH 7.7, 100 µM PMSF. PMSF is hazardous; wear gloves and avoid direct contact with skin or inhalation.
   iv. Bring the supernatant to 25% $(NH_4)_2SO_4$ by adding 144 g/L of solution. Mix gently at 4°C for 30 min. Centrifuge the supernatant at 13,000–16,200 *g* at 4°C for 40 min. Discard the pellet.
   v. Bring the supernatant from 25 to 80% $(NH_4)_2SO_4$ by adding 390 g/L of solution. Mix gently at 4°C for 30 min. Spin at 13,000–16,200 *g* for 1.5 h. Save the pellet and discard the supernatant, being careful in decanting the supernatant. his pellet can be stored at –80°C for at least 2 weeks.

vi. Suspend the pellets in ~1/10 the starting volume (e.g., 40 mL) with Tris/KCl/PMSF buffer, ensuring the pellet is completely dissolved.
vii. Dialyze thoroughly against Tris/KCl/PMSF buffer at 4°C for 1 h. Use dialysis membrane with a 10,000 kDa cutoff size. Use at least 4 L of buffer per 20 mL of enzyme suspension. It may be easiest to split the suspension into several dialysis tubes. After 1 h, replace buffer with fresh Tris/KCl/PMSF buffer. Repeat two more times for a total of at least four buffer changes.

- After 2 buffer changes, dialysis can be left overnight. A minimum of 4 hours of dialysis is required.

viii. If desired, clear the dialyzed solution by spinning at 10,000 *g* for 30 min. More than 90% of the enzyme activity should be retained in the soluble fraction.

- Aliquot into small volumes (~1 mL) and store at –80°C. This protein mixture does not require storage in glycerol and remains stable through multiple freeze-thaw cycles. When activity is determined, working stock aliquots (e.g., enough for 1 week) can be stored at –20°C. One pound of celery should produce enough enzyme for approximately 500,000 reactions.

4. Extract DNA from the M_2 mutant population, normalize, and pool (4X, 8X, or 16 X) depending upon the detection system. Optimize the PCR conditions for bright, neat, single bands, and use these conditions to set up PCR with the pooled DNA. The PCR products are then subjected to heteroduplex formation by denaturing followed by re-annealing.

   The following protocol can be used for heteroduplex formation:

   i. Incubate at 95°C for 2:00
   ii. Incubate at 95°C for 0:01
   iii. Decrease 2°C per cycle
   iv. Cycle to step ii, 5 more times
   v. Incubate at 85°C for 0:10
   vi. Decrease by 1°C per cycle
   vii. Cycle to step 4, 60 more times
   Hold at 4°C

5. Add CJE to the heteroduplexed samples followed by incubation at 45°C for 45 minutes.
6. To stop the enzyme activity, add 0.25 M EDTA to the samples.
7. Add loading dye to the products and electrophorese on a 2% agarose gel.

The mutants will give cleavage bands in addition to the main product bands and the cleaved bands should add up to the main band. Figure 1 gives an example of how the mutant will look like among all the pools.

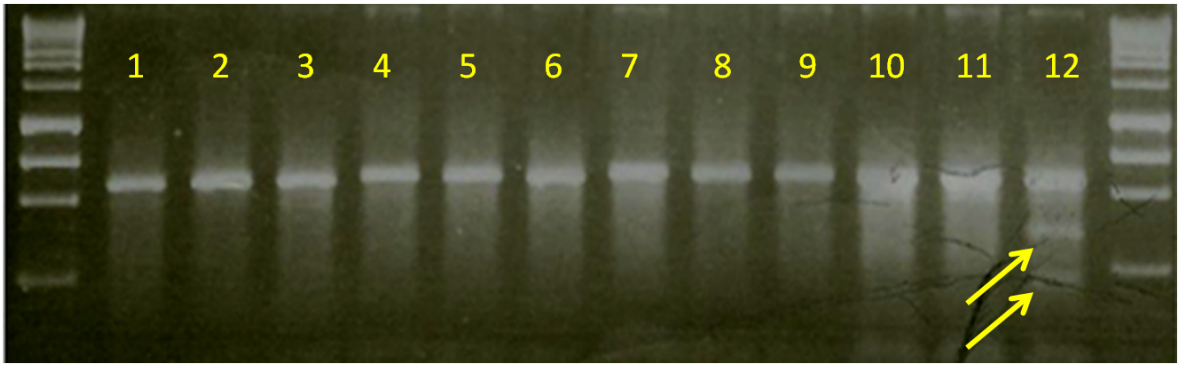

Fig. 1. Selecting a mutant pool among the pooled population.

8. The identified mutant pool is then deconvoluted to get the individual mutant plant. For that, PCR of the individual members of that pool is done in two tubes, one with and the other without wild type (wt) DNA. If an individual shows cleaved products in both the tubes, it is a heterozygous mutant, whereas if the pool contains only wt DNA, it's a homozygous mutant. Figure 2 shows the deconvolution of a pool having a homozygous mutant.

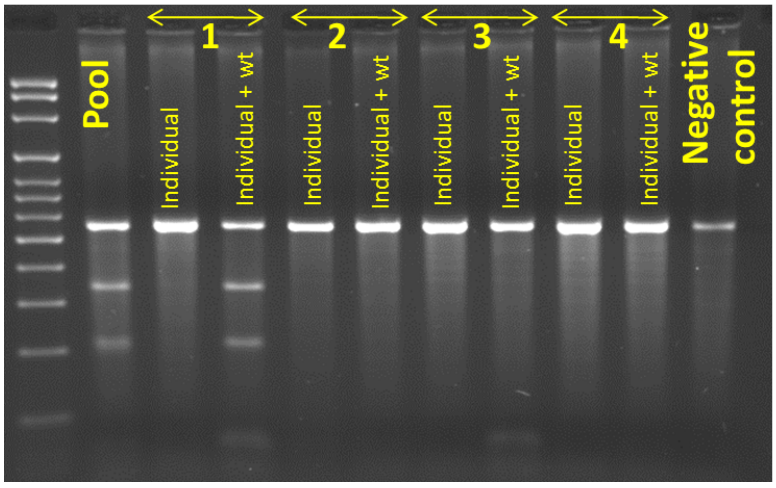

Fig. 2. Deconvolution of pool to identify mutant individual.

9. The SNP is characterized by sequencing. Sequencing can be done immediately if the mutant is homozygous, otherwise, heterozygous individuals need to be selected from the M_3 generation of the mutant.
10. The phenotype arising out of the mutation finally is determined. Because the M_2 plants have a lot of background mutations, 1 or 2 backcrosses are needed to purify the background and examine the phenotypic effect of the particular gene of interest.

This procedure relates a genotype to a phenotype using TILLING, making it a powerful reverse genetics tool.

Reference.

Till BJ, Zerr T, Comai L, and Henikoff S. 2006. A protocol for TILLING and EcoTILLING in plants and animals**.** *Nature Protocols* 1:2465-2477.