Positive control kit for enzymatic mismatch cleavage and agarose gel visualization (version 2.4)

Plant Breeding Unit – Brad Till and Owen Huynh
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Kit Contents:

- Genomic DNA from *Arabidopsis thaliana* (40 µl at 0.075 ng/µl)

Sample	Heterozygous mutation in OXI1 target (bp position from forward primer)	Expected bands on agarose gel (bp)
A	624	624, 368
B	566	566, 426
C	477	515,477
D	Wild-type, no mutation	None

- 1 tube (~ 1000 µl) 10x CJE nuclease buffer
- 1 tube (20 µl at 10 µM) OXI1L forward primer, Tm 70°C
- 1 tube (20 µl at 10 µM) OXI1R reverse primer, Tm 70°C
- 1 tube containing a small aliquot of CJE enzyme mix for comparing cleavage activities.

Primer sequences, description of mutant samples, buffer composition and the protocol for extracting CJE are described in (TILL *et al.* 2004; TILL *et al.* 2006).

Appendix 1 contains a protocol for evaluating and optimizing enzymatic activity from crude extracts.

Overview:

The OXI1 primers amplify a 992 base pair target in the Arabidopsis genome. Three mutant samples are provided that harbour a single nucleotide mutation in the target region. A wild-type sample containing no mutations is included as a control. Using the samples provided, you should be able to reproduce the data shown in figure 1. Genomic DNAs and primers can be used for your optimizations including buffer composition, PCR conditions, and single-strand specific nuclease used for enzymatic mismatch cleavage. Note that the included buffer is optimized for crude celery extract containing CEL I, CEL I and related enzymes, and may not be suitable for other enzymes as described in the 2004 reference cited above.

1: PCR

Prepare the following PCR mix (contains excess volume, can be scaled down accordingly):

H_2O	55 µL
10x Ex Taq buffer	10 µL
2.5 mM dNTP mix	8 µL
10 µM L primer	1 µL
10 µM R primer	1 µL
TaKaRa HS taq (5 U/µL)*	0.25 µL

* The choice of Taq may be important, especially when using different single-strand specific nucleases (TILL *et al.* 2004)

Add 10 µl of PCR mix to each DNA sample (10 µl). Mix sample by pipetting up and down three times.

Place your set of 4 samples in the thermal cycler and run the following PCR cycling program: 95°C for 2 min; loop 1 for 8 cycles (94°C for 20 s, 73°C for 30 s, reduce temperature 1°C per cycle, ramp to 72°C at 0.5°C/s, 72°C for 1 min); loop 2 for 45 cycles (94°C for 20 s, 65°C for 30 s, ramp to 72°C at 0.5°C/s, 72°C for 1 min); 72°C for 5 min; 99°C for 10 min; loop 3 for 70 cycles (70°C for 20 s, reduce temperature 0.3°C per cycle); hold at 8°C. This program is designed specifically for amplification

with fluorescently labelled primers and after success with this program you may test cycling programs of reduced complexity.

2: Single-strand specific nuclease digestion

Prepare the following mix on ice (calculated for 5 samples):

81.5 µl water
15 µl 10x CEL I TILLING buffer *
3.5 µl CJE nuclease #

NOTES:

*10X CEL I buffer is:
5 ml 1M $MgSO_4$
100 µl 10% Triton X-100
5 ml 1M Hepes pH 7.5
5 µl 20 mg/ml bovine serum albumen
2.5 ml 2M KCl
37.5 ml water

The amount of enzyme required will vary depending on nuclease source or possibly from batch to batch of the same enzyme from the same source.

Mix components on ice. Add 20µl of mix to the PCR product and mix by pipetting 2-3 times. Incubate at 45°C for 15min (in thermal cycler). Cool to 8°C and stop reaction by adding 10µl 0.25M EDTA to each sample.

3. Agarose gel analysis

Prepare a 1.5% agarose gel in 0.5x TBE containing (0.2 µg/ml Ethidium Bromide).

Combine 10 µL of sample with 2 µL loading dye (60% glycerol plus bromophenol blue, or equivalent). Load samples along with 4 µL low mass DNA ladder (Invitrogen). Electrophorese samples at 100V for 90 minutes (Figure 1).

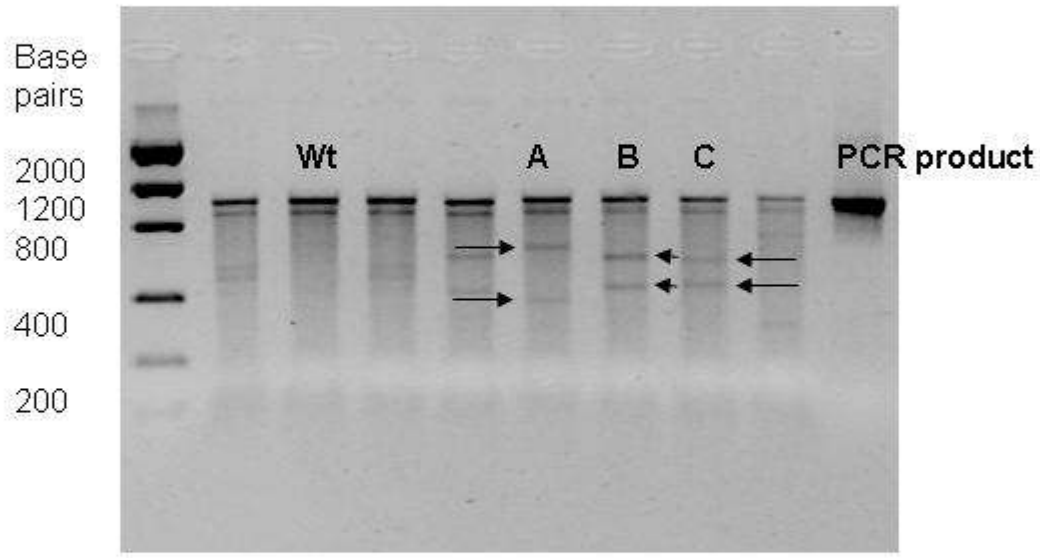

Figure 1 Gel image of mutation discovery using crude celery juice extract for enzymatic mismatch cleavage followed by visualization by agarose gel eletrophoresis. Test samples included in this kit are marked above image as is an example of undigested PCR product. Molecular weights of ladder bands are listed. Bands representing cleavage products at the site of mutation are marked by arrows. Two bands are produced upon double strand cleavage at the site of a mutation. The sizes of the cleaved fragments sums to the size of the full length PCR product. This image was produced at the 2009 FAO/IAEA International Training Course on Novel Biotechnologies for Enhancing Mutation Induction Efficiency by Mr. Saad Alzahrani of Saudi Arabia, and Mr Azhar Bin Mohamad of Malaysia

Appendix:

Celery Juice Extract enzyme activity test

A: PCR amplification

Perform PCR as in Step 1 of the main protocol

B. Single-strand specific nuclease digestion

Prepare the following reaction mixes:

	0 ×	0.1 ×	1 ×	10 ×
water	85	84.5	84.5	80
buffer	15			
enzyme	0	0.5*	0.5	5
total vol	100 µL			

*For the 0.1× reaction, dilute enzyme 1:10 in 1× buffer (buffer diluted to 1× in water).

Combine 10µL PCR product with 20 µL of nuclease reaction mixture (for a total of 16 reactions). Incubate at 45C for 15min. Stop reaction with 5 µL 0.25M EDTA.

C. Agarose gel analysis

Perform agarose gel analysis as in step 3 of the main protocol.

D. Data analysis

Enzyme activity is observed as the degradation of full-length PCR product upon the incubation of increasing amounts of nuclease (Figure 2). If samples with heterozygous polymorphisms are tested, unit enzyme activity for the Li-Cor TILLING assay can be approximated as the amount used on the agarose gel assay that maintains some full-length product while producing cleaved fragments due to

mismatches in heteroduplexed amplicons (defined as 1X in this protocol). Note that enzyme activities may vary dramatically depending on the method of preparation. You may need to adjust volumes to produce the digestion patterns shown in figure 2.

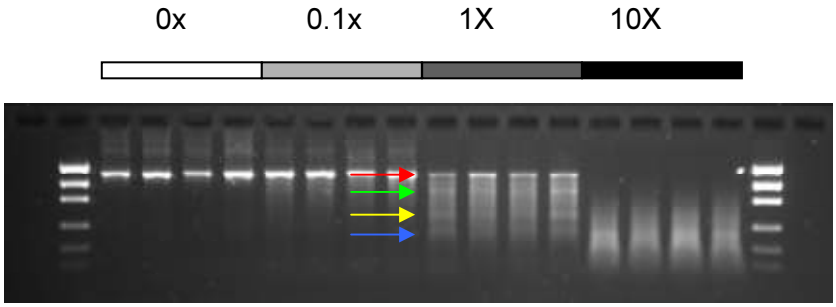

Figure 2 Digested PCR product was run through a 1.5% agarose gel at 100V for 90 minutes. Cut bands can be seen (green, yellow and blue) in samples digested with 1× CJE enzyme and complete digestion of full length product (red) is observed with 10× concentration of enzyme.

References:

TILL, B. J., C. BURTNER, L. COMAI and S. HENIKOFF, 2004 Mismatch cleavage by single-strand specific nucleases. Nucleic Acids Res **32:** 2632-2641.

TILL, B. J., T. ZERR, L. COMAI and S. HENIKOFF, 2006 A protocol for TILLING and Ecotilling in plants and animals. Nat Protoc **1:** 2465-2477.