Positive control kit for enzymatic mismatch cleavage using LI-COR and agarose gel visualization (version 2.5)

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Kit Contents:

- Genomic DNA from *Arabidopsis thaliana* (60 µl at 0.075 ng/µl)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heterozygous mutation in OXI1 target (bp position from forward primer)</th>
<th>Expected bands on gel (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>624, 368</td>
<td>624, 368</td>
</tr>
<tr>
<td>B</td>
<td>566, 426</td>
<td>566, 426</td>
</tr>
<tr>
<td>C</td>
<td>477, 515, 477</td>
<td>515, 477</td>
</tr>
<tr>
<td>D</td>
<td>Wild-type, no mutation</td>
<td>None</td>
</tr>
</tbody>
</table>

- 1 tube (~ 1000 mls) 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 (40 µl) primer mixture (contains unlabelled and IRDye labelled primers).
- 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 figures 1 and 2. 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):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>55 µL</td>
</tr>
<tr>
<td>10x Ex Taq buffer</td>
<td>10 µL</td>
</tr>
<tr>
<td>2.5 mM dNTP mix</td>
<td>8 µL</td>
</tr>
<tr>
<td>10 µM primer mixture1,2</td>
<td>2 µL</td>
</tr>
<tr>
<td>TaKaRa HS taq (5 U/µL)3</td>
<td>0.25 µL</td>
</tr>
</tbody>
</table>

1 If only using agarose gel analysis for mutation detection, conserve IRDye primer mixture and substitute 1 µL OXI1L and 1 µL OXI1R primer in PCR reaction.
2 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.
NOTES:

2 The primer cocktail was made in advance as follows:

3 μl forward primer labeled with IRD700 dye (100μM)
2 μl unlabeled forward primer (100μM)
4 μl reverse primer labeled with IRD800 dye (100μM)
1 μl unlabeled reverse primer (100μM)

This mix was stored at −80°C. Prior to shipment, the mix was thawed on ice, diluted 1:10 with TE (10 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.4).

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 MgSO₄
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).

! Remove 10 µL of sample from reaction tube for agarose gel analysis. Store the remaining volume at -20°C to save for step 4.

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 electrophoresis. 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.
4. Sample purification for LI-COR gel analysis

Prior to loading nuclease digested samples onto the denaturing polyacrylamide (LI-COR) gel, salts must be separated from the DNA and sample volume reduced to 1.5 µl. There are several methods that can be used to accomplish this. Our standard method is size exclusion chromatography using Sephadex G50 medium beads. An alternative is alcohol precipitation. In our experience, Sephadex columns are much faster than alcohol precipitation and provide consistent and high recovery of DNA. 96-well plates containing hydrated Sephadex can be prepared up to one week in advance. The Sephadex method can be found in (TILL et al. 2006), along with references for alcohol precipitation.

5. Preparing, loading, and running Li-Cor gels

Clean and assemble glass plates according to manufacturer’s instructions. Prepare the following mixture:

- 20 ml acrylamide gel mix (6.5%)
- 15 µl TEMED
- 150 µl fresh 10% ammonium persulfate

Fill a 20 ml syringe with acrylamide solution. Dispense along the top, avoiding bubbles by rapping just above the liquid edge whenever it appears one might get trapped. If any bubbles appear, remove them quickly after the gel is poured with a thin wire tool. Leaving a little excess at the well, insert the top spacer all the way and centered. Insert the Plexiglas pressure plate between the glass plate and casting rails. Tighten the top screws as soon the comb^* is inserted, compressing the rubber pads on the pressure plate a little. Add acrylamide to the top glass edge where the comb is inserted and on the edges to assure that polymerization is not inhibited within the gel. Let the gel set at least 30 min before putting it into the gel box. Gels can be poured in advance and stored wrapped in a damp paper towel at 4 °C for several days.
Type of comb depends on sample volume. We typically use 100 tooth membrane combs for high-throughput TILLING and Ecotilling assays.

Once the gel is polymerized, put on powder-free gloves and wash the gel assembly with warm di-water. Lean the top of the gel against the bottom of the sink as you gently remove the comb spacer, so that loose polyacrylamide does not wash into the well. Use the comb spacer or your fingers to remove excess polyacrylamide at the top edge of the front plate. The well must be clear of excess acrylamide. Dry the plates and wipe with isopropanol, making sure the back plate is spotless where the laser shines through.

Put the gel in the LI-COR so the back plate is resting against the heater plate and the bottom edge of the gel is in the empty lower buffer reservoir. After verifying that the back plate is spotless and free of dust where the laser shines through, insert the top buffer reservoir between the glass plate and the casting rails. Tighten the screws a little and fill the upper reservoir. Rinse the well gently with buffer, using a 50 cc syringe without a needle. The slot must be clean, as any loose acrylamide will inhibit insertion of the comb. Put the lids on the buffer reservoirs and close the LI-COR.

6. Electrophoresis

Start the prerun (20 min, 1500 V, 40 mA, 40 W, 50°C.).

After the prerun, clean the sample well with pipettor or syringe.

Load samples (typically 0.25 – 0.5 µl depending on the comb used) and run gel (for a 1 kb fragment, enter the following LI-COR settings: collect time 3 h 45 min run at 1500 V, 40 mA, 40 W, 50°C.).

7. Data analysis

Proper cleavage of mutations produces one new band in the IRDye 700 channel image and one band in the IRDye 800 channel image, whose sizes sum to the size of the full length PCR product (figure 2). Gel analysis can be aided by the use of
specially designed software such as the freely available GelBuddy (http://www.gelbuddy.org). Information on optimization and troubleshooting can be found in (TILL et al. 2006).

**Figure 2** IRDye700 (left) and IRDye800 (right) LI-COR gel image of positive control samples. Arrows and letters mark the location and identity of cleavage products found in mutants included in the kit. Asterisk (*) marks a homopolymeric region of adenine residues. Homopolymeric regions are subject to template breathing and subsequent CJE digestion. Such background bands are common in all samples. This image was produced at the 2009 FAO/IAEA International Training Course on Novel Biotechnologies for Enhancing Mutation Induction Efficiency by Ms Estrellita Ponce Terashima of Peru and Ms Nawal Abu Q. Alhajaj from Jordan.
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:

<table>
<thead>
<tr>
<th></th>
<th>0 x</th>
<th>0.1 x</th>
<th>1 x</th>
<th>10 x</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>85</td>
<td>84.5</td>
<td>84.5</td>
<td>80</td>
</tr>
<tr>
<td>buffer</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>enzyme</td>
<td>0</td>
<td>0.5*</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>total vol</td>
<td></td>
<td></td>
<td></td>
<td>100 μL</td>
</tr>
</tbody>
</table>

*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.

![Digestion Pattern](image)

**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:**
