Low-cost TILLING with pooled samples using denaturing agarose gels stained with ethidium bromide.

Version 1.4, Plant Breeding and Genetics Laboratory, February 28th, 2013. Prepared by Owen Huynh and Brad Till

1. Objective

Denaturing gels are used to check the integrity of single-stranded nucleic acids, both DNA and RNA. In the context of mutation discovery, denaturing gels could detect both single strand breaks (nicks) and double strand breaks that are caused by the CEL1 enzyme, potentially providing higher signal-to-noise ratio to improve the low-cost mutation discovery method. Here we attempted to adapt the low-cost TILLING method using denaturing gels.

2. Reagents & Equipment

Material	Supplier and catalogue number (where appropriate)
Ex-Taq hot start version (PCR buffer, dNTP and HS Ex-Taq polymerase)	TaKaRa
$MgCl_2$ (25 mM stock)	
Forward primer	
Reverse primer	
Sodium hydroxide (NaOH)	
Standard agarose	Sigma A9539
1kb DNA ladder	Invitrogen 10787-018
Ethidium bromide (stock 10mg/mL)	Sigma E1510
Thermal cycler	
Agarose Gel Electrophoresis Apparatus	

3. Methods

3.1 Reagents

50× alkaline gel buffer (1.5M NaOH and 50mM EDTA)

1. Dissolve 30g NaOH in 300mL distilled water.
2. Add 100mL of 0.25M EDTA.
3. Make volume up to 500mL with distilled water.

2× alkaline loading dye (60mM NaOH, 2mM EDTA, 20% Ficoll, 0.06% Bromocresol Green)

1. To a 15mL falcon tube, carefully weigh 6mg Bromocresol Green.
2. Add 2g Ficoll.
3. Add 0.4ml 50× alkaline buffer.
4. Make volume up to 10mL with distilled water.

3.2 PCR

1. Mix equal volumes of mutant and wild-type DNA, enough for reactions (5µL required for each reaction). A control sample of just wild-type DNA is also required.
2. Prepare PCR mastermix enough for the number of samples. The following protocol is calculated for a 96 well assay.

water	320
10× Ex-Taq buffer	57
$MgCl_2$ (25mM)	68
dNTP (2.5mM each)	92
Forward primer (100µM stock)	2
Reverse primer (100µM stock)	2
HS Ex-Taq	6

3. Add 5µL of mixed DNA to wells of a 96 well PCR plate.
4. Add 5µL of PCR mastermix to each well.
5. Centrifuge for 2 minutes at 1000g.
6. Run plate with PCR program:

Step 1	Initial denaturation	95°C	2 minutes
Step 2	Denaturation	94°C	20 seconds
Step 3	Primer annealing	73°C (-1°C/cycle)	30 seconds
Step 4	Ramp	0.5°C per second to 72°C	
Step 5	Primer extension	72°C	1 minute
Step 6	Cycling	repeat steps 2-5 for 7 cycles (8 cycles in total)	
Step 7	Denaturation	94°C	20 seconds
Step 8	Primer annealing	65°C	30 seconds
Step 9	Ramp	0.5°C per second to 72°C	
Step 10	Primer extension	72°C	1 minute
Step 11	Cycling	repeat steps 7-10 for 44 cycles (45 cycles in total)	
Step 12	Final extension	72°C	5 minutes
Step 13	Denaturation	99°C	10 minutes
Step 14	Cooling	72°C	20 seconds
Step 15	Cycling & Touchdown	repeat step 14 for 69 cycles (-0.3°C/ cycle)	
Step 16	Hold	4°C	forever

At high temperatures, alkaline conditions will hydrolyze the Agarose polysaccharide chains. To prepare an alkaline gel, the agarose is first melted in water and cooled to near gelling temperature. Buffer concentrate is then added and the gel is poured.

3.3 Denaturing Gel

1. Add 1.2g of agarose to 98mL of distilled water.
2. Microwave until all the agarose is dissolved
3. Cool the gel under water or by leaving on the bench. You should be able to hold the flask which indicates that it is cool enough.
4. Add 2mL of 50× alkaline gel buffer.
5. Add ethidium bromide to the warm gel solution to obtain a final concentration of 0.5µg/mL in the gel. Mix by swirling.
6. Pour gel solution into gel apparatus, according to the instructions of the manufacturer.

7. To 10µL PCR product, add 10µL 2× alkaline loading dye.
8. Run gel in 1× alkaline gel buffer alongside a molecular weight marker until the gel has run a reasonable distance (this will depend on the size of the gel apparatus being used). This is about 60 minutes at 60V on a small gel rig (low voltage required due to buffering capacity of the gel buffer).
9. Visualise the gel under UV light (i.e. using a GelDoc system).

4. Example results

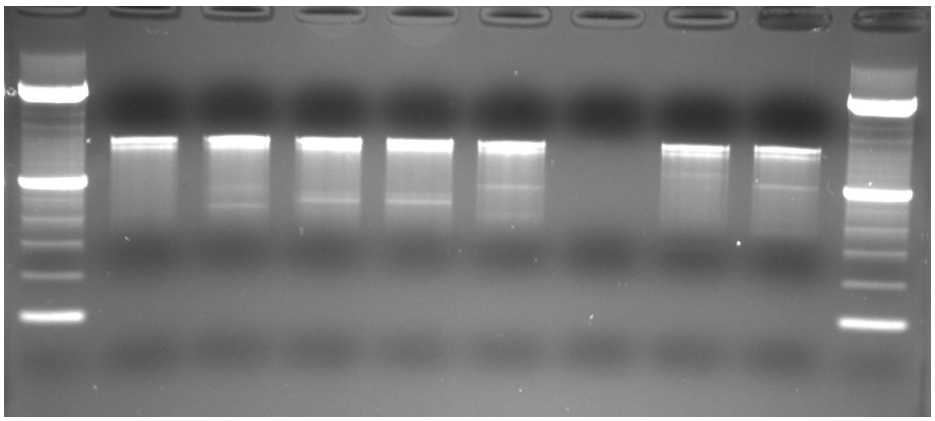

Figure 1. Mismatch cleavage visualised on a denaturing agarose gel. TILLING-PCR products of the target OXI1 gene (1,000bp PCR product) were produced from genomic DNA of Arabidopsis. The PCR products were digested with celery juice extracts and 10µL if the digested products were separated on a 1.2% denaturing agarose gel stained with ethidium bromide. A 1kb ladder was loaded on either sides of the samples.

5. Conclusions

In theory, the products of enzymatic mismatch cleavage should be a collection of DNA that is either nicked or harbors a double strand break. Therefore, use of denaturing gels should provide a higher signal of cleavage products than native gels. However, in this example, on a qualitiative level, we do not observe a major increase in signal. It may be enough of an increase for increased sample pooling and therefore we advise testing both methods with the target species before adopting a specific platform.