Chapter 7
Alternative Enzymology for Mismatch Cleavage for TILLING and Ecotilling: Extraction of Enzymes from Common Weedy Plants

Abstract A crude celery extract containing the single-strand-specific nuclease CEL I, has been widely used in TILLING and Ecotilling projects around the world. Yet, celery is hard to come by in some countries. Sequences homologous to CEL I can be found in different plant species. Previous work showed that similar mismatch cleavage activities could be found in crude extracts of mung bean (Till BJ, Burtner C, Comai L, Henikoff S. Nucleic Acids Res 32:2632–2641, 2004). It is likely that the same activity can be recovered in many different plant species. Therefore, a protocol for the extraction of active enzyme was developed that uses plants common across the world, namely weeds. Monocotyledenous and dicotyledenous weedy plants from the grassland, field and waste grounds around crop fields are suitable for this protocol. Due to lower recovery of enzymatic activity compared to celery-based extractions, a centrifuge-based filter method is applied to concentrate the enzyme extract.

7.1 Materials

Extraction of single-strand-specific nuclease from weedy material is performed using standard laboratory equipment and consumables (Table 7.1). Concentration of enzyme extracts is accomplished using specialized centrifugation filters (Table 7.2), and testing of enzyme activity relies on standard materials for PCR (Table 7.3).

7.2 Methods

7.2.1 Enzyme Extraction

1. Collect approximately 200 g of mixed monocot and dicot weedy plants. Wash material 3× in water and then grind using a hand-held mixer and by adding about 300 ml of water to facilitate tissue disruption.

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Table 7.1 Chemicals, enzymes, and equipment for extraction of enzymes from common weedy plants

Material description	Comment
Hand-held mixer (or juicer)	From any supplier
STOCK: 100 mM phenylmethylsulfonyl fluoride (PMSF; stock in isopropanol)	To prepare an aqueous solution of 100 μM PMSF (for buffers A and B), add 1 ml 0.1 M PMSF per liter of solution immediately before use
STOCK: 1 M Tris-HCl, pH 7.7	
Buffer A: 0.1 M Tris-HCl, pH 7.7, 100 μM PMSF	
Buffer B: 0.1 M Tris-HCl, pH 7.7, 0.5 M KCl, 100 μM PMSF	
Dialysis tubing with a 10,000 Da molecular weight cut off (MWCO)	E.g., Spectra/Por® Membrane MWCO: 10,000, Spectrum Laboratories, Inc.
$(NH_4)_2SO_4$ (ammonium sulfate)	
Sorvall centrifuge	Or equivalent centrifuge/rotor combination to achieve the required gravitational force

Table 7.2 Chemicals, enzymes, and equipment for concentration of enzyme extracts

Material description	Comment
Amicon ultra centrifugal filters (0.5 ml, 10 kDa MWCO)	Millipore Amicon Ref. No. UFC501024 24Pk
Refrigerated (4°C) microcentrifuge	E.g., Eppendorf 5415R

Table 7.3 Chemicals, enzymes, and equipment for the test of mismatch cleavage activity

Material description	Comment
Thermocycler	E.g., Biorad C1000 Thermal cycler
PCR tubes	Life Science No 781340
TaKaRa Ex Taq™ polymerase (5 U/μl)	TaKaRa
10 Ex Taq™ reaction buffer	TaKaRa
dNTP mixture (2.5 mM of each dNTP)	TaKaRa
Agarose gel equipment	Horizontal electrophoresis from any general laboratory supplier

2. Add 1 M Tris-HCl (pH 7.7) and 100 mM PMSF to a final concentration of buffer A (0.1 M Tris-HCl and 100 μM PMSF) (NOTE: stocks and water should be kept at 4 °C, perform subsequent steps at 4 °C).
3. Centrifuge for 20 min at 2,600 × g in Sorvall GSA rotor or equivalent to pellet debris and transfer the supernatant to a clean beaker.
4. Bring the supernatant to 25 % ammonium sulfate (add 144 g/l of solution). Mix gently at 4 °C (cold room) for 30 min.

5. Centrifuge for 40 min at 4 °C at ~14,000 × *g* in Sorvall GSA rotor (~9,000 rpm) or equivalent. Discard the pellet.
6. Bring the supernatant to 80 % ammonium sulfate (add 390 g/l of solution). Mix gently at 4 °C for 30 min using a magnetic stir bar and plate.
7. Centrifuge for 1.5 h at 4 °C at ~14,000 × *g*. SAVE the pellet. Discard the supernatant (NOTE: Be careful not to disturb pellet while decanting the supernatant).
8. OPTIONAL: Pellets can be frozen at −80 °C for months.
9. Resuspend the pellets by vortexing in ~1/10 the starting volume with Buffer B (frozen pellets of the weed juice extract were suspended in 15 ml Buffer B and pellets of the celery juice extract in 10 ml Buffer B). Ensure that the pellet is thoroughly resuspended by pipetting it up and down or by vortexing.
10. Place the suspension into treated dialysis tubing use e.g. Spectra/Por® 7 10 kDa MWCO tubing (NOTE: follow manufacturer's guidelines for treatment of tubing before use).
11. Dialyze for 1 h against Buffer B at 4 °C with constant agitation of the buffer using a magnetic stir bar and plate. Use at least 2 l of buffer per 10 ml of suspended solution.
12. Repeat for a total of four steps with a minimum of 4 h dialysis for each step (NOTE: Longer dialysis is better, and it is often convenient to perform the third treatment overnight).
13. Remove the liquid from dialysis tubing. It is convenient to store ~75 % of the liquid in a single tube at −20 or −80 °C and the remainder in small aliquots for testing. This protein mixture does not require storage in glycerol and remains stable through multiple freeze–thaw cycles; however, limiting freeze–thaw cycles to five reduces the chance of diminished enzyme activity.
14. Perform activity test (Step 7.2.3, or proceed immediately to enzyme concentration, Step 7.2.2).

7.2.2 Concentration of Enzymes Using Amicon Ultra 10 kDa MWCO Centrifugal Filter Devices (for 0.5 ml Starting Volume; in 1.5-ml Tubes)

1. Perform with 600 µl of protein extract after dialysis.
2. Clear extract of plant material by centrifugation for 30 min at 10,000 × *g*, 4 °C.
3. Transfer 500 µl of the (cleared) supernatant to a filter device and keep the rest of the supernatant as the "before concentration" control.
4. Centrifuge the filter device with a collection tube inserted, as per the manufacturer's instructions for 30 min at 14,000 × *g*, 4 °C.
5. Remove the filter device, invert, and place in a new collection tube.
6. Centrifuge for 2 min at 1,000 × *g*, 4 °C.
7. Measure the recovered volume. This is your concentrated protein. Calculate the concentration factor with the following formula: concentration factor = starting volume/final volume.

7.2.3 Test of Mismatch Cleavage Activity

1. Produce TILLING-PCR products for mismatch cleavage tests with the concentrated enzyme extracts. The example below is for barley.
   GENES/PRIMER: nb2-rdg2a (1,500-bp PCR product)

nb2-rdg2a_F2	TCCACTACCCGAAAGGCACTCAGCTAC
nb2-rdg2a_R2	GCAATGCAATGCTCTTACTGACGCAAA

   TILLING PCR REACTIONS (TaKaRa ExTaq enzyme):
   Total volume: 25 µl

10× Ex Taq buffer (TaKaRa)	2.5 µl
dNTP mix (2.5 mM)	2.0 µl
Primer forward (10 µM)	0.3 µl
Primer reverse (10 µM)	0.3 µl
TaKaRa Taq (5 U/µl)	0.1 µl
Barley genomic DNA (5 ng/µl)	5.0 µl
H_2O (to 25 µl)	14.8 µl

2. TILLING 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.
3. Mix 10 µl of PCR product with 10 µl weed digestion mix to a volume of 20 µl.
4. Incubate at 45 °C for 15 min.
5. Add 2.5 µl of 0.5 M EDTA (pH 8.0)—to stop the reaction.
6. Load a 10 µl aliquot on an agarose gel.
7. Analyze the samples by electrophoresis using a 1.5 % agarose gel. See Chap. 8 for example data.

Reference

Till BJ, Burtner C, Comai L, Henikoff S (2004) Mismatch cleavage by single-strand specific nucleases. Nucleic Acids Res 32:2632–2641