Alternative enzymology for mismatch cleavage for TILLING and Ecotilling: extraction of enzymes from weedy plants.

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1. OBJECTIVE

A crude celery extract containing the single-strand-specific nuclease CELI has been widely used in TILLING and Ecotilling projects around the world. Yet, celery is hard to come by in some Member States. Based on previous studies and bioinformatic analysis suggestion homologies exist to CELI in all plants. Therefore, we developed a protocol for extraction of active enzyme from plants common across the world: weeds. We isolated weed plants from the grassland around the Seibersdorf laboratories and isolated a crude enzyme extract (in parallel to the enzyme extracts from celery). Since, there was no or only very low mismatch digestion activity in the crude extract, we applied a centrifuge-based filter method to concentrate the enzyme extract.

2. MATERIALS

MATERIALS / BUFFERS FOR ENZYME EXTRACTIONS	Notes
hand-held mixer (or juicer)	From any supplier
STOCK: 100mM PMSF (stock in isopropanol)	To prepare an aqueous solution of 100µM PMSF (for buffers A and B), add 1 ml 0.1M PMSF per liter of solution immediately before use.
STOCK: 1M 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, pH7.7, 0.5 M KCl, 100 µM PMSF.	
Dialysis tubing with a 10,000 Dalton molecular weight cut off (MWCO)	e.g. Spectra/PorR Membrane MWCO: 10,000, Spectrum Laboratories, Inc.
$(NH_4)_2SO4$ (Ammonium sulphate)	
Sorvall Centrifuge	Or equivalent centrifuge/rotor combination to achieve needed gravitational force
MATERIALS FOR CONCENTRATION OF ENZYME EXTRACTS	
Amicon Ultra Centrifugal Filters (0.5mL, 10K)	Millipore Amicon Ref.No. UFC501024 24Pk
Refrigerated (4°C) Microcentrifuge	e.g. Eppendorf 5415R
TILLING-PCR	
Thermocycler	e.g. Biorad C1000 Thermal cycler
PCR tubes	Life Science No 781340
TaKaRa Ex Taq™ Polymerase (5U/ul)	TaKaRA
10X Ex Taq™ Reaction Buffer	TaKaRa
dNTP Mixture (2.5mM of each dNTP)	TaKaRa
Agarose gel equipment	

3. METHODS

3.1 Enzyme extraction

1. Collect approximately 200 grams of mixed monocot and dicot weedy plants were collected that were growing on the periphery of our sorghum field.

2. Wash material 3x in water and then ground using a hand-held mixer and by adding about 300 mls of water to facilitate tissue disruption (or optional in a juicer)

3. Add 1M Tris-HCl (pH=7.7) and 100mM PMSF to a final concentration of Buffer A (=0.1M Tris-HCl and 100µM PMSF) (NOTE: Stocks and water should be kept at 4C, perform subsequent steps at 4C)

4. Centrifuge for 20 min at 2600 x g in Sorvall GSA rotor to pellet debris. Save supernatant.

5. Bring the supernatant to 25% ammonium sulphate (add 144 g per liter of solution). Mix gently at 4°C (cold room) for 30 min.

6. Centrifuge for 40 min at 4ºC at ~14,000 x g in sorvall GSA rotor (~9000 rpm). Discard the pellet.

7. Bring the supernatant to 80% ammonium sulphate (add 390 g per liter of solution). Mix gently at 4°C for 30 min.

8. Centrifuge for 1.5 hours at 4ºC at ~14,000 x g in sorvall GSA rotor. SAVE the pellet. Discard the supernatant (be careful in decanting the supernatant!) The pellet can be stored at –80°C for at least two weeks.

9. OPTIONAL: Pellets can be frozen at -80C for months.

10. Resuspend the pellets in ~ 1/10 the starting volume with Buffer B (Frozen pellets of the weed juice extract were suspended in 15mL Buffer B and pellets of the celery juice extract in 10 mL Buffer B). Ensure the pellet is thoroughly resuspended.

11. Dialyze against Buffer B at 4ºC (2 Liters per 10mls of resuspended solution).. Use e.g. Spectra por 7 MWCO 10000 tubing. (NOTE: Soak the dialysis tubing in nanopure water for 30 min. before use.)

12. Dialyze for 1 hour against Buffer B at 4°C

13. Repeat for a total of 4 dialysis steps with a minimum of 4 hours dialysys. (NOTE: Longer dialysis is better, it is often convenient to perform the third dialysis overnight).

14. Remove liquid from dialysis tubing.. It is convenient to store ~75% of the liquid in a single tube at -80C and the remainder in small aliquot 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 5 limits the chance of reduced enzyme activity

15. Perform activity test (step 3.3, or proceed immediately to enzyme concentration, step 3.2)

Figure 1. Mixture of different plant species (weedy plants) from the grassland around the Seibersdorf laboratories used for the isolation of a enzyme extract for mismatch cleavage.

3.2 CONCENTRATION OF ENZYME EXTRACTS of weed and celery enzyme extracts using Amicon Ultra 10K centrifugal filter devices (for 0.5mL starting volume; in 1.5-mL tubes)

1. Perform with 600uL of protein extract after dialysis
2. Clear extract by centrifugation at 30 min / 10.000 x g / 4^0C (to pellet plant material) in refrigerated microcentrifuge
3. Transfer 500 uL of the (cleared) supernatant to a filter device (keep the rest of the supernatant as control "before concentration").
4. Centrifuge the filter device with a collection tube inserted per manufacturers instructions for 30 min / 14.000 x g / 4^0C
5. Remove filter device, invert and place in new collection tube.
6. Centrifuge for 2 min / 1.000 x g / 4^0C
7. Measure the recovered volume. This is your concentrated protein. Calculate the concentration factor with the following formula: Starting volume/Final folume = concentration factor

3.3 TEST OF MISMATCH CLEAVAGE ACTIVITY

1. Produce TILLING-PCR products for mismatch cleavage tests with the concentrated enzyme extracts. The exmple below is for barley.

GENES/PRIMER: nb2-rdg2a (1500bp-PCR product)

nb2-rdg2a_F2	TCCACTACCCGAAAGGCACTCAGCTAC
nb2-rdg2a_R2	GCAATGCAATGCTCTTACTGACGCAAA

TILLING PCR REACTIONS (TaKaRa ExTaq enzyme): total volume: 25uL

10x ExTaq buffer (TaKaRa)	2.5 uL
dNTP mix (2.5 mM)	2.0 uL
Primer forward (10 uM)	0.3 uL
Primer reverse (10 uM)	0.3 uL
TaKaRa Taq (5U /ul)	0.1 uL
Barley genomic DNA (5 ng/uL)	5.0 uL
H2O (to 25 uL)	14.8 uL

TILLING PCR cycling program for TILLING ("PCRTM70")

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.

2. Mix 10uL of PCR product with 10uL weed digestion mix to a volume of 20uL

3. Incubate at 45^0C for 15 min

4. Add 2.5uL of 0.5M EDTA (pH=8.0) – to stop reaction

4. Load a 10uL aliquot on an agarose gel

4. Example results

Concentrations of protein extracts:

Table 1. Calculations of concentration factors after centrifugation with Amicon Ultra 10K – Starting volume: 500 uL

("Before" centrifugation = considered as 1x concentrated)

	Recovered volume	**Concentration factor** (calculated from 500 uL starting volume)
Weed	~42 uL	**11.9x**
Cell	~33 uL	**15.2x**

Mismatch digestions using celery and weed enzyme extracts:

Table 2. Set-up of mismatch digestions using celery and weed enzyme before and after centrifugation with Amicon Ultra 10K. The enzyme concentration in the extracts were calculated using the calculated concentration factors from Table 1.

	1 - BEFORE	**2 - after**	**3 - after**	**4 – after**
Enzyme	3.5 uL (1x)	0.5 uL	3 uL	6 uL
Cell buffer	1.5 uL	1.5 uL	1.5 uL	1.5 uL
H2O	5 uL	8.0 uL	5.5 uL	2.5 uL
Tot.Volume	10 uL	10 uL	10 uL	10 uL
Celery enzyme concentration in relation to extract before centrifugation (3.5uL – before = 1x)	**1x**	7.6 uL **2.2x**	45.6 uL **13.0x**	91.2 uL **26.1x**
Weed enzyme concentration in relation to extract before centrifugation (3.5uL - before = 1x)	**1x**	5.95 uL **1.7x**	35.7 uL **10.2x**	71.4 uL **20.4x**

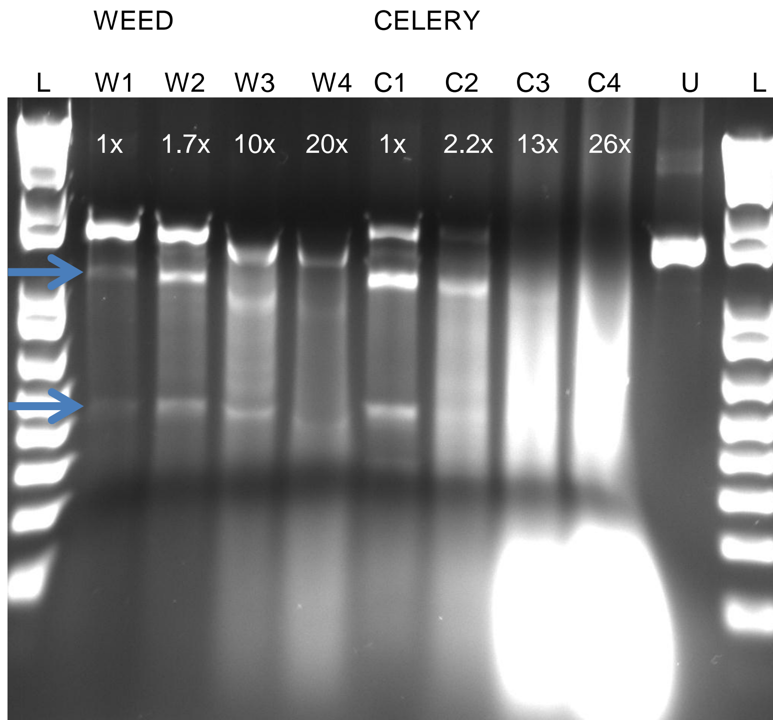

Figure 2. Mismatch cleavage with celery and weed enzyme extracts. TILLING-PCR products of the target gene nb2-rdg2a (1500bp-PCR product) were produced from genomic DNA of barley. The PCR products were digested with weed and celery enzyme extracts before and after concentration by centrifugation with Amicon Ultra 10K. 10 uL of the digested PCR products were separated on a 1.5% agarose gel. Position of SNPs are marked with blue arrows. Concentrations of Weed (W) and Celery (c) extracts are listed above the lanes. A 1kb ladder is loaded on either side of the samples.

5. CONCLUSIONS

Crude enzyme extracts of weeds show a similar activity to that of celery extract for the cleavge of single nucleotide polymorphisms. The per unit activity, however, was lower than than for CEL I, likley owing to the co-precipitation of other plant proteins in weeds, presumably including a larger amount of RUBISCO. This limitation can be overcome through the use of a simple centrifugation based protein concentration step. 150 mls of weed extract produces enough enzyme for approximately 2000 reactions with this protocol.