

Cel I Purification

Obtain CEL1 activity using this protocol. A 1.5 L extract yields 150 ml crude, whole-protein extract.

Obtain the Following Supplies and Equipment:

- 4° C Cold-room bench space
- Celery (4 bunches ≈ 2.5 kg trimmed stalks ≈ 1.5L Juice)
- Purified water (reverse osmosis filtered or double-distilled)
- Buffer component stocks:
 - 2M Tris, pH 7.7
 - 2M KCl
 - 0.1M PMSF solvated in isopropanol (cannot be stored in aqueous solution)
 - 20% Triton X-100
- 10 or 25 ml pipettes, Pasteur pipettes, and pipette bulbs or pumps
- 500 ml graduated cylinder
- Large glass or plastic 5+ L beakers
- Balance and weigh boats (300 gram capacity or larger)
- Stir plate and stir-bars (2 or 3 inch stir-bars generate high torque at low speeds)
- Juicer (pulp ejecting preferred over centrifugal models for ease of use)
- Centrifuge and Rotor capable of spinning 250 ml centrifuge bottles at ≥ 15,000 g
 - (Sorvall RC 5 series and SLA-1500 rotor)
- 18-36 250 ml centrifuge bottles rated for ≥ 25,000 g
 - (Fisher catalog # 14-375-353)
- Dialysis tubing with 10,000 Dalton molecular weight cut-off (MWCO)
 - (Spectra/Por Biotech Regenerated Cellulose membrane, catalog #128616)
- Closures for dialysis tubing (20-50 closures)

Observe the following precautions:

- Perform all steps and store all solutions, supplies, and equipment at 4 °C
- Chill centrifuge and rotor to 4 °C
- Use purified water for all solutions (reverse osmosis processed or double distilled)

Overview of Steps:

- Produce 1.5 L buffered celery juice
- Centrifuge to remove cell debris
- Precipitate protein at 25% ammonium sulfate
- Centrifuge and discard pellet (more debris)
- Precipitate protein at 80% ammonium sulfate
- Centrifuge and save pellet (whole protein extract)
- Accumulate pellets in storage (optional)
- Resuspend pellets in a volume of **Buffer B** equivalent to 1/10th the initial juice volume
- Resuspend pellets in **Buffer B** (0.1 M Tris pH 7.7, 0.5 M KCl, 100 μM PMSF, 0.01% Triton X-100)
 - Use a total resuspension volume equivalent to 1/10th the initial juice volume
- Dialyze thoroughly against **Buffer B**
- Aliquot and store protein solution

Juicing Celery and Precipitating Proteins

Note the Following:

- Have ready all PMSF and Tris-Buffer solutions
- Thaw aliquots of PMSF solution immediately before use
- Use a sieve to remove floating debris

- 1) Prepare celery for juicing:
 - a. Cut off the bottom from each bunch to separate stalks
 - b. Trim off tops, leaves, and tough, white bottoms from each stalk
 - c. Rinse with water and dry with paper towels
- 2) Juice celery at 4° C to obtain 1.5 L juice
- 3) Make a 0.1 M Tris-HCL, pH 7.7, 100 µM PMSF solution in celery juice
 - a. per L juice add: 50 ml 2M Tris pH 7.7
 - b. per L juice add: 1 ml 0.1 M PMSF
- 4) Centrifuge the juice solution at 15,000 g for 20 minutes, 4 °C
- 5) Decant and **save supernatant**, discard pellet
 - a. Pellet: fibrous and sticky, bright green
 - b. Supernatant: cloudy greenish-yellow
- 6) Bring supernatant to 25% ammonium sulfate
 - a. per L supernatant add: 144 g (NH₄)₂SO₄
 - b. Volume will increase marginally (3%)
- 7) Mix solution gently for 1 hour, 4 °C
- 8) Centrifuge solution at 15,000 g for 45 minutes, 4 °C
- 9) Decant and **save supernatant**, discard pellet
 - a. Pellet: small and sticky, green or light gray
 - b. Supernatant: cloudy brownish-yellow
- 10) Bring supernatant to 80% ammonium sulfate
 - a. Per L supernatant add: 390 g (NH₄)₂SO₄
 - b. Volume will increase significantly (25%)
- 11) Mix solution gently for 1 hour or overnight, 4 °C
- 12) Centrifuge solution at 15,000 g for 90 minutes, 4 °C
- 13) **Save pellet**, decant and discard supernatant
 - a. Pellet: large compared to previous pellets, yellowish-brown or beige
 - b. Supernatant: transparent brownish-yellow
- 14) Store protein-precipitate pellet at 4 °C (weeks) or -80 °C (indefinite)

Dialysis

Precautions:

- Use Gloves in All Steps, Note that PMSF is TOXIC.
- PMSF will recrystallize at 4 °C – thaw and use immediately
- PMSF will degrade over time in aqueous solutions – do not allow to sit in solution

- 1) Cut dialysis tubing – use a length sufficient for sample, clamps, and airspace
- 2) Prepare dialysis tubing following manufacturer directions
- 3) Have the following ready to use:
 - a. Cleaned dialysis tubing clamps
 - b. 25 ml pipette and bulb
 - c. Glass Pasteur pipette and pipette bulb or pump
- 4) Prepare 5 L Buffer B:

	For a 5 L Solution:
a. 0.1 M Tris pH 7.7	add 50 ml 2 M stock
b. 0.5 M KCl	add 250 ml 2 M stock
c. 0.01% Triton X-100	add 5 ml 20% stock
d. 100 µM PMSF	add 5 ml 0.1 M stock
- 5) Resuspend pellets in a volume of **Buffer B** equivalent to 1/10th the initial volume of celery juice
 - a. For a 1.5 L prep, resuspend in a 150 ml solution of protein in **Buffer B**
 - b. Scrape pellet from wall of centrifuge bottle using a metal spatula
 - c. Smear or break down large chunks of pellets for easier solvation
 - d. Vortex gently by hand and avoid over-foaming solution
 - e. Transfer buffer and pellets from tube to tube until all pellets are combined
 - f. Resuspended pellet should be homogeneous and free of debris
- 6) Load dialysis tubing with resuspended protein
 - a. Squeegee excess buffer from dialysis tubing
 - b. Clamp bottom of dialysis tubing
 - c. Open the membrane using a plastic toothpick or Pasteur pipette tip
 - d. Load resuspended protein using a 10 to 25 ml pipette
 - e. Introduce an airspace large enough to float dialysis tubing vertically in solution
 - f. Clamp the top of the dialysis tubing and place into dialysis buffer
- 7) Dialyze in 5 or 6 changes of 5 L Buffer B
 - a. Stir solution slowly to avoid drawing dialysis tubes down into the stir bar
 - b. Allow last dialysis to proceed for at least 4 hours or overnight
- 8) Collect the dialyzed protein solution and aliquot
 - a. Squeegee excess liquid from top of dialysis tubing
 - b. Unclamp tubing and pour contents into a glass beaker
 - c. Aliquot into labeled and dated 1.5 or 2.0 ml tubes
- 9) Store aliquots at -80 °C

Testing of CEL 1 Activity

Test each batch of CEL 1 enzyme to determine the appropriate amount needed. This is done by comparing results as visualized on a polyacrylamide gel between different treatments of CEL 1 enzyme. The new preparation is compared at different concentrations to whatever is considered to be standard for the current preparation. This procedure is simply a regular TILLING PCR reaction with a modified CEL 1 digestion setup. The different CEL 1 treatments (original, none, new batch) are striped across 96-well plate in the same direction as the samples are loaded. This ensures that the different treatments are grouped together in the gel image for easier interpretation of results.

Have Ready:

- Primer pair amplifying a fragment with known polymorphisms
 - Example: **opn4_1** amplified with primers **ztil 312** and **ztil 315**.
- TILLING Pool plate (5 μ L, 0.5 ng/ μ L DNA) or Tilling Mutation Plate (see *mutationPlate.doc*)
- CEL 1 reagent, both from the current and new preparation
- PCR reagents, thawed

Assay Setup

- 1) Following standard procedures for TILLING, amplify the fragment (see *document x*).
- 2) Perform CEL 1 digestion of samples, using multiple conditions
 - a. Determine the number of CEL 1 conditions to be tested (4 or 6)
 - b. Assay conditions should include:
 - i. Positive control: original CEL 1 prep at usual volume
 - ii. Negative control: no CEL 1 at all
 - iii. Experimental: new CEL 1 at the same volume as the original CEL 1
 - iv. Other: new CEL 1 at $\frac{1}{4}x$, $\frac{1}{2}x$, $2x$, or other fold change in volume
 - c. Make half-volume mixes for each CEL 1 condition to be tested
 - d. Aliquot 30 μ L CEL 1 mix to the appropriate wells
 - e. Incubate at 45°C for 30 minutes
 - f. See **Appendix 1** for more lay out information.
- 3) Continue to completion with the remaining TILLING protocol

Analysis

- 1) Use GelBuddy to view the gel images
- 2) Look for the following:
 - a. See that there is full length product across the gel
 - b. Look to see that a polymorphism is present across the gel
- 3) Choose a condition where
 - a. Digestion fragments are clearly visible
 - b. Background is light enough that mutations/polymorphisms stand out
 - c. Background is dark enough to ensure correct land counting
 - d.

I really should have example images here of what I had done previously... ☹ fill this in yourselves. See the presentation from 2005 (Cecilia's HHMI Review retreat).

