In Vitro NER Assay

Reagents:

Water
YPD
Yeast extraction Buffer (200 ml): 0.2 M Tris-acetate (pH 7.5) (40 ml), 0.39 M $(NH_4)_2S0_4$ (78 ml), 10 mM $MgSO_4$ (2 ml), 20% Glycerol (40 ml), 1mM EDTA (pH8.0) (0.4 ml), and water (39.6 ml). Store at 4°C. **Add DTT to 0.1 mM and protease inhibitors before use**.
Protease Inhibitor Stocks: 100X PMSF (100 mM in ethanol); 1000X Benzamidine hydrochloride (Ben) (1M in water); 1000X Pepstatin A (Pep) (3.5 mg/ml in DMSO); 1000X Leupeptin (Leu) (1mg/ml in water); 200X Aprotinin (Apo) (2 mg/ml in water). Store all stocks at -20°C.
Solid Ammonium Sulfate
Dialysis Buffer (500 ml): 20 mM HEPES-KOH (pH 7.5) (10 ml), 20% Glycerol (100 ml), 10 mM $MgSO_4$ (5 ml), 10 mM EGTA (10 ml), and water (367.5 ml). Store at 4°C. **Add DTT to 5mM and 100X PMSF before use**.
Plasmid DNA: pGEM-3Z (2.8 kbp) and pGEM-3Zf(-) (3.2 kbp). Purified by Qiagen DNA purification kit.
N-acetoxy-2-acetylaminofluorene (NAAAF) 1 mM in ethanol. Store at -20°C.
100 mM dNTP stocks and 100 mM rATP(pH7.5)
4x Repair Biuffer A (RBA) (10 ml aliquot as 1 ml): 180 mM HEPES-KOH (pH 7.8) (1.8 ml), 280 mM KCl (2.8 ml), 29.6 mM $MgCl_2$ (148 μl), 3.6 mM DTT (36 μl), 1.6 mM EDTA (pH 8.0) (32 μl), 13.6% Glycerol (1.36 ml), and water (3.824 ml). Store at -20°C.
4Xrepair Buffer B (RBB) (10 ml aliquot as 1 ml): 8 mM rATP (800 μl), 0.08 mM dGTP (8 μl), 0.08 mM dATP (8 μl), 0.08 mM dTTP (8 μl), 0.032 mM dGTP (3.2 μl), and water (9.1728 ml). Store at -20°C.
10 mg/ml BSA: dissolve in water, aliquot and store at -20°C.
1M Disodium phosphocreatine: dissolve in water aliquot and store at -20°C.
Creatine phosphokinase: 2.5 mg/ml in 50 mM HEPES-KOH (pH 7.6), 20 mM magnesium acetate, and 50% glycerol. Store at -20°C.
[α-^{32}P]dCTP (3000 Ci/mmol.
10% SDS: 10% (w/v) DSD in water. Store at room temperature.
Proteinase K: Make 20 mg/ml in water. Store in 50-μl aliquots at -20°C.
10 mg/ml RNase A: make in water. Boil for 10 minutes to inactivate DNase. Store at -20°C.
Phenol:Chloroform:Isoamyl alcohol (25:24:1). Store at 4°C.
7.5 M Ammonium acetate.
Ethanol: 100% and 70%. Store at -20°C.
1X TBE: 89 mM Tris-base 89 mM boric acid, and 2mM EDTA (pH 8.0).

Agarose
5-20% sucrose (for gradient): dissolve sucrose in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), and 0.5 M NaCl. **Prepare fresh and cool to 4°C.**
Ethidium Bromide stock: Dissolve 10 mg/ml in water. Wrap in foil and store at 4°C.
TE buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Prepare using autoclaved stock solutions.

Protocol:

Yeast Growth and Harvesting:

1. Inoculate yeast strain into 10 ml of YPD and grow to saturation at 30°C with shaking.
2. Inoculate 2-L YPD in a 4-L Flask with the 10 ml starter culture. Incubate at 30°C with shaking overnight (24 hours). $O.D._{600}$ of 2-4.
3. Chill flask in ice water. Transfer to centrifuge bottles (500 ml) **(weigh empty bottles to determine wet weight!)** KEEP CULTURES COLD FROM THIS POINT ON!!
4. Centrifuge for 5 minutes at 4°C at 5,000 rpm in the GS-3 rotor. Keep repeating spin until all the cells have been collected from the 4-L flask.
5. Discard supernatant. Add 100 ml of ice-cold sterile water, resuspend, and repeat step 4. Discard supernatant.
6. Resuspend cells in 100 ml cold yeast extraction buffer with DTT and protease inhibitors. Then repeat step 4.
7. Discard supernatant and remove residual supernatant with pipet. Measure the wet weight of the cells.
8. Using a spatula scoop yeast paste into cooled 10-ml syringe with plunger removed (do not put a needle on it).
9. Extrude yeast paste into small plastic 250-ml beaker filled with liquid nitrogen. Make sure yeast “noodles” remain submerged under nitrogen. (**Break noodles with spatula to help keep volume down)**

10. Cover container with foil and allow nitrogen to evaporate. Then frozen yeast can be stored at -80°C until the day of extract preparation.

Preparation of Whole Cell Extract:

1. Transfer container with yeast (step 10 above) from freezer into bucket of dry ice.
2. Weigh out frozen yeast noodles in precooled small beaker (use 10 grams) and place back on dry ice. **All following steps to be done in cold room!**
3. Take the cooled mortar and pestle to cold room and fill 3/4 full with liquid nitrogen (evaporates), then fill again.
4. Pour yeast noodles into mortar.
5. Crush frozen yeast under liquid nitrogen (reduce to chunks or pellets).
6. Replenish liquid nitrogen.
7. Grind yeast pellet by driving the pestle in a circular path around the mortar applying pressure to the walls.
8. Keep refilling with liquid nitrogen (wait for nitrogen to boil off to initiating grinding). Make slurry with yeast pellet.
9. Grind until yeast shows a powdery smooth consistency.
10. Pour more liquid nitrogen into the mortar to wash yeast powder to bottom
11. Pour yeast/liquid nitrogen suspension into a 1-L plastic beaker (cooled with nitrogen). Scrape remaining yeast powder into beaker with spatula.
12. CAN STOP!! (store at -80°C). Or let nitrogen boil off completely from the beaker in the cold room to do extraction preparation.
13. Add Yeast Extraction Buffer (1 ml/1 g yeast) **supplemented with DTT and protease inhibitors.** No need to pre-cool buffer.
14. Allow Yeast to thaw slowly in the cold room (~15-20 min.) to obtain a fluid suspension. Disperse clumps by pipeting up and down.

15. Transfer thawed extract to a centrifuge tube on ice. Centrifuge at 120,000g for 2 hours at 4°C (33,000 rpms in 70Ti rotor).
16. Remove cleared supernatant and place in 100-ml beaker with stir bar (measuring volume of supernatant)
17. Weigh out 337 mg solid ammonium sulfate/ ml of lysates and add to beaker with lysates slowly over the course of an hour while stirring. Then allow to stir for another 30 minutes
18. Transfer to a centrifuge tube and precipitate protein by centrifugation at 40,000g (20,000 rpm in 70Ti rotor) for 15 minutes at 4°C.
19. Discard supernatant. Resuspend pellet in 50μl/g (500 μl) of dialysis buffer with DTT and protease inhibitors.
20. Transfer to dialysis tubing and dialyze overnight (12-16 hours) against 1L of dialysis buffer.
21. After dialysis, transfer to a microfuge tube and centrifuge 1-2 minutes to remove precipitated proteins.
22. Transfer supernatant to microfuge tubes in small aliquots (100μl) and quick-freeze and store at –80°C ; also determine protein concentration using Bradford reagent and BSA standards.

Preparation of Damaged DNA Substrates:

1. Incubate 100 μg of pGEM-3Z at 30°C for 3 hours in 1 ml of TE Buffer containing 3μM N-acetoxy-2-acetylaminofluorene (NAAAF) (**Negative control: pGEM-3Zf(-) with no NAAAF**).
2. Layer the DNA sample onto a linear 5-20% sucrose gradient in 10mM Tris-HCl, pH 7.5, 1mM EDTA, and 0.5M NaCl.
3. Centrifuge at 28,000 rpm in a SW41 rotor (135,000g) for 17 hours at 4°C.
4. Collect 1 ml fractions from the bottom of the tube.
5. Check 3μl of each fraction on a 1% Agarose gel.

6. Identify fractions containing closed circular, supercoiled DNA.
7. Pool these fractions and recover by ethanol precipitation.
8. Resuspend the DNA in TE buffer to give a concentration of 300ng/μl and store at –20°C.

In Vitro **DNA Repair Synthesis:**

1. To a 1.5-ml microfuge tube, add 12.5 μl of 4x repair buffer A, 12.5 μl of 4x repair buffer B, 1.8 μl of 10 mg/ml BSA, 2 μl of disodium phosphocreatine, 1 μl creatine phophokinase, 300 ng (1 μl) of pGEM-3Z (NAAAF treated) and pGEM-3Zf(-) (non-treated) DNA, and 2 μCi (0.2μl) of [α-^{32}P]dCTP. **(Total 32*µl/* reaction)**
2. Add 250 μg of yeast extract and water to 50μl final volume.
3. Incubate at 28°C for 2 hours.
4. Stop reaction by adding 2 μl of 0.5 M EDTA. Add 0.5 μl of ribonuclease A, mix and incubate at 37°C for 10 minutes.
5. Add 2.5 μl of 10% SDS and 0.5 μl of proteinase K. mix and incubate at 37°C for 30 minutes.
6. Extract with equal volume of phenol/chloroform. Centrifuge for 10 minutes at room temp.
7. transfer aqueous layer to new microfuge tube and repeat step 6 one more time.
8. add ammonium acetate to 2.5 M (1/2 volume of 7.5 M stock) and precipitate DNA with 2 volumes of ethanol at –80°C for a minimum of 10 minutes.
9. Recover DNA by centrifugation at top speed for 10 minutes. Then wash pellet with 100 μl 70% ethanol and recentrifuge.
10. Remove ethanol and allow to air dry at least 10 minutes.
11. Digest DNA overnight with HindIII (20U) in a 20-μl final volume.

12. Add gel loading buffer and run on a 1% agarose gel with ethidium bromide.
13. Photograph gel under UV light and then transfer to blotting paper and vacuum dry at 80°C for 1 hour.
14. expose dried gel to x-ray film and phosphoimager screen.