PROTEIN EXTRACTION
AND
ESTIMATION

PREPARATION OF ANIMAL TISSUE FOR 2D GEL ELECTROPHORESIS

SOLUTIONS:

Lysis buffer: 10 mM Tris, pH 7.5, 1.0% CHAPS (add protease inhibitors)

10X nuclease stock: 100mM Tris, pH 7.0, 50 mM MgCl2, RNase (500 ugm/ml), DNase (1000ugm/ml) (Ribonuclease A from Bovine Pancreas Type IIIA, Sigma R5125; Deoxyribonuclease I, Type II from bovine pancreas, Sigma D4527).

Urea Sample Buffer: 9.5M urea, 2% CHAPS, 18 mM DTT, 0.5% Ampholines (select range according to strip used) **(note: high ampholyte concentration will increase focusing time. May use 2% ampholyte. May also use IPG Buffer from Amersham Biosciences).**

100x Protease Inhibitor (PI) Stock Solution: contains 20 mM AEBSF (Calbiochem 101500), 1 mg/mL leupeptin (Sigma L2884), 0.36 mg/mL E-64 (Sigma E3132), EDTA (Calbiochem 34103), and 5.6 mg/mL benzamidine (9, Sigma B6506). This stock solution should be added to samples at a final concentration of 1%. **ALTERNATE: COMPLETE TABLETS FROM BOEHRINGER MANNHEIM.**

PROCEDURE:

1. Place frozen sample (~150 mgm) in a cold ceramic mortar with ~100 ml liquid nitrogen
2. Crush sample under Liquid Nitrogen to fine powder
3. Transfer frozen sample to1.5 ml Eppie tube with screw cap
4. Add 0.5 ml Lysis Buffer (with 1X protease inhibitors); vortex
5. Set on ice minimum 10 minutes
6. Add 50 ul of 10X nuclease stock; incubate on ice minimum 10 minutes
7. Vortex occasionally
8. Spin, 13,000 x g, 15 minutes, 4C
9. Remove and store supernatant materials on ice or, –20C; save pellets –20C.
10. Estimate protein with MicroBCA assay. Use 20, 50, 100 & 200 fold dilutions for assay. See separate protocol.

PREPARATION OF TISSUE CULTURE CELLS FOR 2D GEL ELECTROPHORESIS

SOLUTIONS:

Lysis buffer: 10 mM Tris, pH 7.5, 1.0% CHAPS (add protease inhibitors)

10X nuclease stock: 100 mM Tris, pH 7.0, 50 mM MgCl2, RNase (500 ugm/ml), DNase (1000 ugm/ml) (Ribonuclease A from Bovine Pancreas Type IIIA, Sigma R5125; Deoxyribonuclease I, Type II from bovine pancreas, Sigma D4527).

Urea Sample Buffer: 9.5M urea, 2% CHAPS, 18 mM DTT, 0.5% Ampholines (customize according to range selected) **(note: high ampholyte concentration will increase focusing time. May use 2% ampholyte. May also use IPG Buffer from Amersham Biosciences).**

100x Protease Inhibitor (PI) Stock Solution: contains 20 mM AEBSF (Calbiochem 101500), 1 mg/mL leupeptin (Sigma L2884), 0.36 mg/mL E-64 (Sigma E3132), EDTA (Calbiochem 34103), and 5.6 mg/mL benzamidine (Sigma B6506). This stock solution should be added to samples at a final concentration of 1%. **ALTERNATE: COMPLETE TABLETS FROM BOEHRINGER MANNHEIM.**

PROCEDURE:

NOTE: This procedure is designed for a 100 mm dish (1-5E106 cells per plate; 40,000 cells/cm2)

1. Aspirate medium
2. Wash cells 3 x 5 ml ICE COLD Dulbeccos PBS
3. Add 300 ul of Lysis Buffer (containing 1X PROTEASE INHIBITORS) to plate
4. Scrape cells with rubber policeman (note high viscosity of nucleic acids)
5. Transfer lysed cells to 1.5 ml eppie on ice
6. Add 30 ul 10X nuclease stock solution
7. Incubate 30 min on ice (viscosity gone in 15 minutes)
8. Spin microfuge, 10 min, 4C; decant and SAVE SUPERNATANT
9. Save 30 ul supernatant aside for protein assay (note: no reducing agents present)
10. Set aside remaining sample (supernatant) on ice or, -20C (short storage) or –80C (long storage)
11. Estimate protein with a MicroBCA assay. Use 20, 50, 100 & 200 fold dilutions for assay. See separate protocol.

NOTE: PROTEIN CONCENTRATING STEP MAY BE REQUIRED.

PREPARATION OF E. COLI CELLS FOR 2D GEL ELECTROPHORESIS

SOLUTIONS:

Lysis buffer: B-Per™ Extraction Buffer (Pierce Chemical Co.) (add protease inhibitors)

Urea Sample Buffer: 9.5M urea, 2% CHAPS, 18 mM DTT, 0.5% Ampholines (customize according to range selected)**(note: high ampholyte concentration will increase focusing time. May use 2% ampholyte. May also use IPG Buffer from Amersham Biosciences).**

10X nuclease stock: 100mM Tris, pH 7.0, 50 mM MgCl2, RNase (500 ugm/ml), DNase (1000ugm/ml) (Ribonuclease A from Bovine Pancreas Type IIIA, Sigma R5125; Deoxyribonuclease I, Type II from bovine pancreas, Sigma D4527).

100x Protease Inhibitor (PI) Stock Solution: contains 20 mM AEBSF (Calbiochem 101500), 1 mg/mL leupeptin (Sigma L2884), 0.36 mg/mL E-64 (Sigma E3132), EDTA (Calbiochem 34103), and 5.6 mg/mL benzamidine (9, Sigma B6506). This stock solution should be added to samples at a final concentration of 1%. **ALTERNATE: COMPLETE TABLETS FROM BOEHRINGER MANNHEIM.**

NOTE: ALL PROCEDURES PERFORMED ON ICE OR AT 4C.

PROCEDURE 1:

1. Grow culture overnight to saturation
2. Spin 10 ml of cells in 15 ml Corex tubes, 13,000 x g, 15 minutes, 4C
3. Discard the supernatant
4. Add 300 ul of B-Per Extraction Buffer (with 1X protease inhibitors); vortex 1 minute
5. Transfer to a 1.5 ml Eppie tube
6. Centrifuge cells at 13,000 rpm for 5 minutes, 4C
7. Collect supernatant (soluble fraction); save pellet (Optional; insoluble fraction)
8. Add 1/10 volume Nuclease cocktail; incubate on ice, 15-30 min. Check for viscosity by eyeball inspection.
9. Estimate protein concentration using colorimetric assay (MicroBCA, MicroBradford); use 20, 50, 100 & 200 fold dilutions for assay.

PROCEDURE 2:

1. All steps as above.
2. Resuspend cells in buffer of choice (preferably low salt; ie, 10 mM Tris HCl, pH 7.5)
3. Sonicate using a MicroTip (5 minutes, 30% duty, Output #2)

4. Centrifuge cells at 13,000 rpm for 5 minutes, 4C
5. Collect supernatant (soluble fraction); save pellet (Optional; insoluble fraction)
6. Add 1/10 volume Nuclease cocktail; incubate on ice, 15-30 min. Check for viscosity by eyeball inspection.
7. Estimate protein concentration using colorimetric assay (MicroBCA, MicroBradford); same dilutions as above.

PROCEDURE 3: French Press

1. All steps as above
2. Resuspend cells in 1/10 to 1/100 volume of original volume using buffer of choice (preferably low salt; ie, 10 mM Tris HCl, pH 7.5)
3. French Press 2 to 3 times
4. Centrifuge the material at 13,000 rpm, 5 min, 4C
5. Estimate protein concentration using colorimetric assay (MicroBCA, MicroBradford); same dilutions as above.

Samples may be diluted directly in Urea Sample Buffer if they are concentrated enough and contain low salt. Typically we mix sample:USB at 1:4 in a total of 250 ul (ie., 50 ul sample:200 ul USB). The buffer in which the sample is dissolved MUST BE LOW IN SALT (<50 mM total salt; don't forget to count buffer salts, too.) Typically a concentration of 10 mg/ml will be high enough to load ~500 ugm of protein on IPG strip, an amount that is good for Coomassie staining of a complex sample.

If protein is not concentrated enough or contains too high a concentration of salts, the proteins must be concentrated. This can be accomplished by several means, including precipitation (see handouts), microdialysis, or Molecular Weight Cutoff membrane concentrators (available from a variety of commercial suppliers). **REMEMBER, ALL TECHNIQUES WILL USUALLY RESULT IN SOME DEGREE OF SAMPLE LOSS AND, POSSIBLY, REDUCED PROTEIN REPRESENTATION.**

PROTOCOL FOR PLANT PROTEIN EXTRACTION FOR 2D ELECTROPHORESIS

SOLUTIONS:

Solution A:

90% acetone
10% TCA
0.07% beta mercaptoethanol

Suolution B:

100% acetone
0.07% beta mercaptoethanol
2 mM EDTA

PROCEDURE: (500 MGM OF TISSUE)

1. Pipet 1.5 ml of Solution A into a 1.5 ml Eppie tube.
2. Grind tissue in liquid nitrogen until powder.
3. Transfer the ground material (quickly) to the COLD 1.5 ml of Solution A. Vortex.
4. Let stand in –20C for 60 minutes.
5. Spin at 13, 000 x g, 15 minutes, RT.
6. Wash pellet with 1 ml COLD Solution B.
7. Repeat previous step until pellet is colorless (may need sonication with microtip).
8. Vacuum dry the samples for 5 – 10 minutes.
9. Resuspend the pellet in 250 ul of USB or USB.
10. Sonicate with microtip if necessary.
11. Spin 13, 000 x g, 15 minutes, RT.
12. Bradford protein assay on dilutions (20, 40, 80X with water).

PREPARATION OF YEAST, *S. AUREUS* AND OTHER DIFFICULT-TO-LYSE CELLS FOR 2D GEL ELECTROPHORESIS

SOLUTIONS:

Lysis buffer: 10 mM Tris, pH 7.5, 1.0% CHAPS

10X nuclease stock: 100mM Tris, pH 7.0, 50 mM MgCl2, RNase (500 ugm/ml), DNase (1000ugm/ml) (Ribonuclease A from Bovine Pancreas Type IIIA, Sigma R5125; Deoxyribonuclease I, Type II from bovine pancreas, Sigma D4527).

Urea Sample Buffer: 9.5M urea, 2% CHAPS, 18 mM DTT, 0.5% Ampholines (customize according to range selected)**(note: high ampholyte concentration will increase focusing time. May use 2% ampholyte. May also use IPG Buffer from Amersham Biosciences).**

100x Protease Inhibitor (PI) Stock Solution: contains 20 mM AEBSF (Calbiochem 101500), 1 mg/mL leupeptin (Sigma L2884), 0.36 mg/mL E-64 (Sigma E3132), EDTA (Calbiochem 34103), and 5.6 mg/mL benzamidine (Sigma B6506). This stock solution should be added to samples at a final concentration of 1%. **ALTERNATE: COMPLETE TABLETS FROM BOEHRINGER MANNHEIM.**

PROCEDURE:

1. To each sample of gently pelleted cells, add 500 uL of Lysis Buffer (with 1X protease inhibitors) and 1X Nuclease and 100 mg of washed glass beads (Sigma G9268, mesh size 425–6000 microns) per 50–100 uL washed cell pellet.
2. Vortex thoroughly, freeze, centrifuge and repeat until the size of the pellet has been reduced substantially.
3. Add 400 uL of SDS Boiling Buffer minus BME, vortex, and freeze again.
4. Place the tube in a boiling water bath for 5 min and then centrifuge. Lyophilize the supernatant; remember to reserve an aliquot for protein determination.
5. Dissolve the resulting residue in 1:1 diluted SDS Boiling Buffer to at least 5.0 mg/mL for Coomassie blue-stained gels or 1.0 mg/mL for silver-stained gels.

Method provided by Kendricks Labs, Inc. (http://www.kendricklabs.com/index.htm)

PREPARATION OF DROSOPHILA EMBRYOS FOR 2D GEL ELECTROPHORESIS

SOLUTIONS:

Lysis buffer: 10 mM Tris, pH 7.5, 1.0% CHAPS

10X nuclease stock: 100mM Tris, pH 7.0, 50 mM MgCl2, RNase (500 ugm/ml), DNase (1000ugm/ml) (Ribonuclease A from Bovine Pancreas Type IIIA, Sigma R5125; Deoxyribonuclease I, Type II from bovine pancreas, Sigma D4527).

Urea Sample Buffer: 9.5M urea, 2% CHAPS, 18 mM DTT, 0.5% Ampholines (customize according to selected range)**(note: high ampholyte concentration will increase focusing time. May use 2% ampholyte. May also use IPG Buffer from Amersham Biosciences).**

100x Protease Inhibitor (PI) Stock Solution: contains 20 mM AEBSF (Calbiochem 101500), 1 mg/mL leupeptin (Sigma L2884), 0.36 mg/mL E-64 (Sigma E3132), EDTA (Calbiochem 34103), and 5.6 mg/mL benzamidine (Sigma B6506). This stock solution should be added to samples at a final concentration of 1%. **ALTERNATE: COMPLETE TABLETS FROM BOEHRINGER MANNHEIM.**

PROCEDURE:

1. Add 100 ul of ICE COLD Lysis Buffer (with 1X protease inhibitors) to ~100 ul of frozen embryo pellet
2. Homogenize with a plastic pestle in the eppie tube
3. Freeze on dry ice
4. Thaw
5. Add 10 ul of 10X nuclease stock
6. Incubate on ice 20 min
7. Spin 5, RT, 13,000 rpm
8. Remove cloudy supernatant to fresh tube (pellet is heterogenous, layers)
9. Add 1.5 ml chloroform/methanol (2:1) to extract lipids
10. Vortex (ppt forms; floaty)
11. Spin 10 min, 13,000 rpm, RT
12. Remove supernatant, discard
13. Air dry pellet overnight, RT (whitish/brownish)
14. Dissolve in 100 ul Urea Sample buffer, 10 min, RT, vortex, pipet up & back
15. Sonicate, 30 min, 30C (still not completely dissolved; rubbery)
16. Spin 13,000 x rpm, RT
17. Remove 10 ul of supernatant to SDS PAGE analysis (12% acrylamide)(note: contains reducing