

EMSA (Electrophoretic Mobility Shift Assay)

Key Steps:

- 1) Prepare protein to be studied by either:
 - 1) In vitro translation using standard protocol
 - 2) Prepare protein from nuclear extract
- 2) Annealing and labeling of the probe(s)
- 3) In vitro binding of probe and NE
- 4) Run native PAGE

Preparing Nuclear Extracts*

*see buffer section for reagents

- 1) Collect between 20 – 100 million cells (Depends on specific experiment) and centrifuge at 1800-2000 rpm for 10 mins at 4C.
- 2) Aspirate media and wash once in cold PBS. ***If pellet is larger than 250ul divide sample into multiple tubes.
- 3) Spin at 1800-2000 rpm for 10 mins at 4C.
- 4) Aspirate PBS and resuspend in 4 times the volume of the pellet in NE Buffer A. (500ul – 1ml).
- 5) Incubate on ice for 1 hour. Put one douncer for every sample on ice.
- 6) Transfer to douncer and homogenize with 25 strokes.
- 7) Transfer homogenized sample to 1.7ml eppendorf tubes and centrifuge at 2000rpm for 5mins at 4C.
- 8) Aspirate supernatant and resuspend the pellet in 1ml of NE Buffer A. Centrifuge at 2000rpm for 5mins at 4C.
- 9) Aspirate supernatant and resuspend the pellet in 2 times it volume of NE Buffer B.
- 10) Incubate on ice for 30mins. Centrifuge at 13000rpm for 20mins at 4C.
- 11) Transfer supernatant to fresh 1.7ml eppendorf tube and add an equal volume of NE Buffer C.
- 12) Aliquot into small volumes (100-200ul).
- 13) Save 1 tube of each sample for Western Blot and Bradford Assay. Snap freeze all other samples in a dry ice/ isopropanol bath. Store at -80C.

Bradford Assay

Mix:

795ul H₂O
5ul Protein (NE)
200ul Bradford Reagent

Measure OD 595 or 600

Need to do standard curve with BSA to determine concentration of protein

Western

*** Do western to determine the amount of your desired protein in the NE***

Annealing and Labeling Probe*

*see buffer section for reagents

I. Annealing

- 1) Resuspend received oligos in 200ul water and measure conc. at OD260.
- 2) Add equal amount of both sense and antisense strand of probe in an eppendorf tube.
- 3) Add 0.5M NaCl and heat the tube at 95C for 10mins.
- 4) Turn off heat block leaving sample in until heat block reaches room temp (2-3 h).
- 5) Purify the double-stranded annealed probe in native PAGE (follow the protocol for oligo purification).

II. Labeling

- 1) Mix 100ng/ul concentration double stranded DNA probe with the following reagents
 - 34.5ul H₂O
 - 2.5ul universal buffer
 - 3ul DNA probe
 - 5ul dNTPs minus dCTP
 - 4ul ³²P dCTP
 - 1ul Klenow
- 2) Add 20ul STE buffer
- 3) Wet Nuntrap filter column with 70ul STE collecting in a 1.7ml eppendorf tube.
- 4) Load probe mix into the same column and collect in the 1.7ml tube from the previous step.
- 5) Wash column with 70ul STE and collect in the same tube from the previous two steps.
- 6) Count probe and then put in the radioactive storage box at -20C.

Binding Reaction*

*see buffer section for reagents

- 1) Pour Medium or large size native gel of appropriate concentration.
- 2) Start pre-running the gel at the voltage the loaded gel will be run at for a minimum of 30mins.
- 3) Mix the binding reaction in a 1.7ml eppendorf tube.
 - 12.5ul Binding Buffer
 - 250ul 4x Binding Buffer
 - 5.0ul BSA 20mg/ml
 - 1.25ul PMSF 100mM
 - 1.0ul DTT 1.0 M
 - 2.0ul 1M KCl
 - 2.0ul dI dC 100ng/ul
 - 1.0ul-6.0ul Nuclear Extract (3-5ug of total protein)**
 - Appropriate amount of Cold Competitor Probe**
 - Appropriate amount of H₂O to bring the final volume to 50ul.
 - Appropriate amount of antibody for supershift.

50ul total volume

**Can run titration of amount of Nuclear Extract used to find best shift in the mobility of the probe.

**Generally 200x to 300x of Cold Competitor to Hot Probe is used

4) Leave binding reaction at RT for 5mins.

5) Add 3.0-5.0ul of probe (100,000 cpm), amount depends on how well labeled the probe is.

6) Incubate the mixture at RT for 15mins.

7) Load the binding reaction mix into the gel using 6x glycerol loading dye (without SDS and xylene cynol).

8) Resolve the gel in 0.5xTBE buffer.

9) Dry gel for at least 45mins and expose to either x-ray film or Phosphor Imager plate (Molecular Dynamics) for desired period of time.

Reagents

NE Buffer A (add Protease Inh.)

10mM HEPES pH7.9 – 100ul 1M
 10mM KCl – 100ul 1M
 1.5mM MgCl₂ – 15ul 1M
 ddH₂O – to 10 mls

NE Buffer B (add Protease Inh.)

20mM HEPES pH 7.9 – 200ul 1M
 10% glycerol – 1 ml 100%
 420mM NaCl – 840ul 5M
 1.5mM MgCl₂ – 15ul 1M
 0.2 mM EDTA – 4ul 0.5M
 ddH₂O – to 10 mls

NE Buffer C (add Protease Inh.)

20mM HEPES pH 7.9 – 200ul 1M
 30% glycerol – 3 ml 100%
 1.5mM MgCl₂ – 15ul 1M
 0.2 mM EDTA – 4ul 0.5M
 ddH₂O – to 10 mls

Universal Buffer

unknown

4x Binding Buffer

80 mM HEPES pH 7.5 – 0.8mls 1M
 0.04% NP40 – 4.0ul 1%
 20% glycerol – 2mls 100%
 10mM MgCl₂ – 0.4mls 1M

STE, pH 7.5

100mM NaCl – 2ml 5M NaCl
 10 mM Tris – 1ml 1M Tris, pH 7.5
 1 mM EDTA – 0.2ml 0.5M EDTA
 H₂O to 100 ml

Native Gel 6%

8.33ml 30% Acrylamide
 5.0ml x10 TBE
 500ul 10% APS
 40ul TEMED
 ddH₂O to 40ml

Protease Inhibitors for 20ml Buffer

Aprotinin 40ul
 Pepstatin A 20ul
 Leupeptin 4ul
 PMSF 200ul