22 April 2010

EMSA
(Ildem Akerman)

**Preparation of Nuclear Extracts for EMSA (10 cm diameter dish):**

1. Wash cells 1x with 10 mls of PBS
2. Add 1 ml ice cold PBS, scrape off with rubber policeman.
3. Collect cells in an eppendorf and centrifuge 1200g, 2 min, $4^{o}C$
4. Remove supernatant, resuspend the cells in 5x volumes (of pellet) of solution A (usually 250ul)
5. Incubate 15 mins on ice
6. Centrifuge 1200g, 2 min
7. Resuspend pellet in 5x volumes Solution A with 0.05% NP40
8. Dounce gently with 20x strokes
9. Centrifuge at 1200g, 2 mins
10. Remove supernatant (Cytoplasmic)
11. Resuspend the pellet with 1x volume of Solution C in a dropwise fashion while vortexing gently
12. Incubate on ice 30 min, on tilting board (gentle agitation)
13. Centrifuge 20 min, 11 000g.
    Transfer SN to an eppendorf, freeze in liq N2. Store at -80C

| **Solution A** | **Solution C** |
|---|---|
| 10 mM Hepes, pH 7.9 | 20 mM Hepes, pH 7.9 |
| 10 mM KCl | 400 mM KCl |
| 0.1 mM EDTA | 10 mM EDTA |
| 0.1 mM EGTA | 10 mM EGTA |
| 1.5 mM MgCl2 | 1.5 mM MgCl2 |
| | 20% Glycerol |
| 1 mM DTT | |
| 0.5mM PMSF | 1 mM DTT |
| + protease inhibitor tablet | 0.5mM PMSF |
| | + protease inhibitor tablet |

**Design of EMSA oligos**:

Since we use Klenow reaction to radio-label oligos, please remember that oligos need GATC at each end such that:

Oligo:
5'- gatcGACGTGGGGGTCCAAC - 3'
Complementary oligo:
5' - gatcGTTGGACCCCCACGTC - 3'

**Preparation of Oligos:**

1. Resuspend oligos at 100 pmol/ul in TE
   (TE: pH 7.4 , 10 mM Tris.HCl, 1 mM EDTA)

2. Mix complementary oligos at 10 pmol/ul in a final volume of 100 ul of TE
3. Incubate oligos ay 95 C for 5 minutes followed by 37 degrees for 1 hr. (Preferred method by me: the heating block could be switched off so the oligos come to room temperature very slowly)
4. Radiolabel 0.5 ul of 10 pmol/ul of annealed oligo using a kit for Klenow reaction (Amersham, GE Helathcare-rediprime II RPN 1633) *

   i) Add to the labeling mix :
   0.5 ul of 10 pmol/ul of annealed oligo
   46 ul of TE
   3 ul of α32P-CTP *
   ii) Incubate at 37 C for 10 minutes.
   iii) Stop the reaction by adding 5 μl of 0.2 M EDTA.

5. Purify the reaction using a G-50 column (illustra MicroSpin™ G-50 Columns, 27-5330-01)

**Prepare the column:**

1. Re-suspend the resin in the column by vortexing. Loosen the cap one-quarter turn and twist off the bottom closure. Place the column in the supplied Collection tube for support.
2. spin for 1 minute at 735 × g.
3. Place the column into a fresh DNase-free1.5 ml microcentrifuge tube
4. Slowly apply the labelled nucleotide mix to the top center of the resin (not touching the resin!!)

**Elution:**

For removal of labeled nucleotides from DNA labeling reactions, spin for 2 minutes at 735 × g.

**Preparation of the Non-denaturing PAGE Gel:**

Acrylimide Gel (5%): 50 ml volume:
6.3 mls of 37.5:1 acrylimide:bis acrylimide mix
5 ml 5X TBE
0.3 ml 10% APS
0.1 ml TEMED
Let the gel set for at least half an hour, pre-run at 250 V for 20 minutes.

**Binding Reaction and loading the gel:**

1. Set up the binding reaction, and incubate for 20 minutes at room temperature. Suggestion: We generally make a mastermix for components that are in each tube to minimize pipetting errors.
   Remember that the competitor oligo should always be added after the labeled oligo.

**Binding reaction (10 ul final volume):**

| Reagent | Stock | Final | Use (microliters) (per 10 ul reaction) |
|---|---|---|---|
| Binding buffer † | 5x | 1x | 2 |
| BSA | 10 mg/ml | 1 mg/ml | 1 |
| Glycerol | 25% | 5% | 2 |
| DTT | 100 mM | 1 mM | 0.1 |
| PolydI.dC | 1 mg/ml | 100 ng/ul | 1 |
| ZnCl | 10 mM | 0.1 mM | 0.01 |
| Nuclear extracts | X | 2 ug | 2 |
| Labelled oligo | See above | | 1 |
| Competitor* | 10 pmol/ul | 10 pmol | 1 |

H2O to a final volume of 10 ul.
† Binding buffer (5X): 100 mM HEPES pH 7.9, 25 mM MgCl2, 0.25% NP40, 450 mM KCl in H2O.
*can vary (3 pmol OR 10 pmol of annealed, unlabelled competitor oligo)
** OPTIONAL: 1 ul of antibody
1 ul of control (IgG) and experimental antibody can also be added

1. Load onto the gel and run for 100 minutes at 250 V. OPTIONAL: Fix the gel in 20% methanol, 10% acetic acid for 15 mins (Recommended, this fixes the DNA and proteins in the gel).
2. Dry the gel with a vacuum pump at 80 C for 2 hrs.
3. Expose overnight at -80C using hypersensitive film. Bring the cassette to room temperature before developing.

Amersham, GE Helathcare-rediprime II and G-50 columns protocols

http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/8924A216C96365CCC1257628001CC635/$file/RPN1633PL_Rev_C_04-2008_web.pdf

http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/140671AAE1886F2FC1257628001D3444/$file/28977288AD.pdf