A Protocol for Electrophoretic Mobility Shift Assay (EMSA) from Primary Neuron

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[Abstract] The interaction of transcriptional or co-transcriptional factors with DNA is crucial for changes of neuronal gene expression during normal brain development as well as neurodegeneration. The electrophoretic mobility shift assay (EMSA) is a very powerful technique for studying changes of neuronal gene expression and determining protein: DNA interactions. EMSA can be used qualitatively to identify specific transcriptional or co-transcriptional factors in brain crude lysates or primary neurons and, in conjunction with mutagenesis, to identify the important binding sequences within a given gene. An advantage of studying protein: DNA interaction by an electrophoretic assay provides a better understanding of epigenetic changes during normal brain development and neurodegenerative process.

Materials and Reagents

1. Neuronal cell pellet
2. Biotin 5' end-labeled and non-labeled DNA probes (competition) (Integrated DNA Technologies)

   Table S- sequences of EMSA probes

Probe name	sequence
Cre-up	-\5BiosG \agagattgcctgacgtcagagagctag
Cre-down	-\5BiosG \ctagctctctgacgtcaggcaatctct
Cre-mutant-up	-\5BiosG \agagattgcctgagacgggagagctag
Cre-mutant-down	-\5BiosG \ctagctctcccgtctcaggcaatctct
MEF2-up	-\5BiosG\cgctctaaaaataaccct
MEF2-down	-\5BiosG\agggttatttttagagcg
MEF2-mutant-up	-\5BiosG\cgctctaaggctaaccct

3. Positively charged nylon membrane (Sigma-Aldrich, catalog number: Z670197)

4. Tris base
5. Boric acid
6. EDTA
7. BSA
8. Poly (dIdC) (0.5 μg/μl) (Pierce Antibodies, catalog number: 20148)
9. Antibody (ab1437)
10. 5x loading buffer (QIAGEN, catalog number: 1037650)
11. X-ray film
12. High-quality blotting paper (Whatman, catalog number: 3030-931)
13. Polyacrylamide gel in 0.5x TBE
14. Cytoplasmic extract buffer (NE-PER Nuclear and Cytoplasmic Extraction Kit) (Pierce Antibodies, catalog number: 78835)
15. Washing buffer (LightShift Chemiluminescent EMSA Kit) (Pierce Antibodies, catalog number: 20148)
16. Nuclear extraction buffer (NE-PER Nuclear and Cytoplasmic Extraction Kit) (Pierce Antibodies, catalog number: 78835)
17. 2x reaction buffer (LightShift Chemiluminescent EMSA Kit) (Pierce Antibodies, catalog number: 20148)
18. Acrylamide
19. Bis-acrylamide
20. TBE buffer
21. TEMED
22. Ammonium persufate
23. Phosphatase inhibitors
24. 6% non-denature PAGE gel (see Recipes)
25. 5x TBE (pH 8.3) (see Recipes)

Equipment

1. Centrifuges
2. UV lamp or crosslinking device equipped with 254 nm bulbs or 312 nm transilluminator
3. Electrophoresis apparatus
4. Electroblotter or capillary transfer apparatus
5. 1.5 ml microcentrifuge tube

Procedure

A. Prepare the nuclear protein extract from neuron
   1. Collect 1-5 x 10^{6-7} neurons pellet in 1.5 ml microcentrifuge tube (DIV 14 Primary cultural neuron was from mouse E16.5 cortex).
   2. Resuspend neurons in 200 µl cytoplasmic extract buffer with protease and phosphatase inhibitors, and keep on ice for 10 min to break the cell membrane.
   3. Spin cells at 16,000 *x g* for 5 min at 4 °C to separate nuclei with cytoplasmic component (quality of fraction was tested by nuclear/cytoplasmic protein markers-HDAC1 and HSP90).
   4. Remove supernatant as cytoplasmic extract.
   5. Wash the nuclear pellet with 300 µl washing buffer.
   6. Spin cells at 16,000 *x g* for 10 min at 4 °C to pellet the nuclei.
   7. Resuspend the nuclear pellet in 100 µl nuclear extraction buffer and aliquot the lysate into10 µl/tube.
   8. Freeze the nuclear extracts in -80 °C.
   9. Measure protein concentration and adjust it to 1 µg/µl with the extraction buffer for use in gel shift assay.

B. Prepare and pre-run gel
   1. Prepare a native PAGE gel in 0.5x TBE. The appropriate polyacrylamide percent depends on the size of the target DNA and the binding protein. Most systems use a 4 -6% PAGE gel in 0.5x TBE.
   2. Place the gel in the electrophoresis unit. Fill the inner chamber with 0.5x TBE to a height several millimeters above the top of the wells. Fill the outside of the tank with 0.5x TBE to just above the bottom of the wells, which reduces heat during electrophoresis. Flush wells and pre-electrophorese the gel for 30-60 min at 100 V.

C. Perform binding reactions
   1. 2x reaction buffer 12 µl.
   2. BSA (1 µg/µl) 3 µl.
   3. Poly (dIdC) (0.5 µg/µl) 2 µl.
   4. Nuclear extract (1 µg/µl) 3 µl (cytoplasmic extract as control).
   5. dH_2O 3 µl.
   6. Keep at room temperature or on ice for 10 min without Antibody, 20 min with Antibody; Keep rotation (option).
   7. Add in Biotin-labeled DNA probe (20 fmol/ reaction).

8. Keep at room temperature for 20 min.

D. Electrophorese binding reactions
   1. Add 5 µl of 5x loading buffer to each 20 µl binding reaction, pipetting up and down several times to mix. Run gel at 200 V for 1-1.5 h. Use DNA loading buffer in lane 1 as indicator of free probe. Free probe usually run at the same mobility as the blue dye of the DNA loading buffer. Stop the gel when the dye runs at 3 cm to the bottom.

E. Electrophoretic transfer of binding reactions to nylon membrane
   1. Soak nylon membrane in 0.5x TBE for 15 min.
   2. Sandwich the gel, nylon membrane and blotting paper in a clean electrophoretic transfer unit.
   3. Transfer at 380 mA (~100 V) for 30 min.
   4. When the transfer is complete, place the on a dry paper towel for 1-3 min.

F. Crosslink transferred DNA to membrane and detection
   1. 10-15 min with the membrane face down on a transilluminator equipped with 312 nm bulbs.
   2. After the membrane is crosslinked, proceed directly to the LightShift Chemiluminescent detection kit. Alternatively, the membrane may be stored dry at room temperature for several days.

Recipes

1. 6% non-denature PAGE gel
   For example, to make 50 ml volumes gel:
   7.5ml 40% acrylamide
   5ml 2% Bis-acrylamide
   2.5ml 10x TBE buffer
   50 µl TEMED
   0.5 ml 10% ammonium persufate
   34.5 ml dH_2O
2. 5x TBE (pH 8.3)
   450 mM Tris
   450 mM boric acid
   10 mM EDTA

Acknowledgments

This protocol is adapted from Li *et al*. (2012).

References

1. Li, J., Chen, J., Ricupero, C. L., Hart, R. P., Schwartz, M. S., Kusnecov, A. and Herrup, K. (2012). Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia. *Nat Med* 18(5): 783-790.