Gel Shift/ EMSA Protocol

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References:

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2. **Ye J**, Zhang X, Dong Z. Characterization of the human GM-CSF promoter: AP1 and a Sp1-related protein activate the promoter activity that is suppressed by YY1. *Molecular and Cellular Biology* 1996;16(1):157-167.
3. **Ye J**, Cippitelli M, Dorman L, Ortaldo JR, Young HA. The nuclear factor YY1 suppresses the human IFN-γ promoter through two mechanisms: inhibition of AP1 binding and activation of a silencer element. *Molecular and Cellular Biology* 1996;16(9):4744-4753.

Electrophoresis System

Vertical gel electrophoresis apparatus (BIBCO BRL model V16-2)

Cast non-denature PAGE Gel:

	50 ml			100 ml		
Stock Solution	3.5%	5%	6%	3.5%	5%	6%
40% acrylamide	4.38ml	6.25ml	7.5ml	8.76ml	12.5ml	15ml
2% Bis-acrylamide	2.91ml	4.15ml	5ml	5.82ml	8.3ml	10ml
10 x TBE buffer	2.5ml	2.5ml	2.5ml	5ml	5ml	5ml
TEMED	50 µl	50 µl	50 µl	100 µl	100 µl	100 µl
10% ammonium persufate	0.6ml	0.6ml	0.6ml	1.2ml	1.2ml	1.2ml
dH_2O	39.6ml	36.5ml	34.4ml	79.1ml	72.9ml	68.7ml

Prepare 0.5 x TBE running buffer 1800ml:

10 x TBE buffer 90ml
dH_2O 1710ml

10 x TBE formula:

Tris base 108 g (89 mM)
Boric acid 55 g (89 mM)
0.5 M EDTA (pH 8.0) 40 ml
Use dH_2O to bring total volume to 1000 ml

Prerun the gel:

Run the gel at 200 V for 30 min

(Prepared by Jianping Ye, M.D.)

Label DNA probe with T4 kinase:

DNA probe	5µl (100 - 500 ng)
5 x Forwarding buffer	4 µl
^{32}P-γ-ATP	5 µl
dH_2O	5 µl
T4 kinase (10 Unit/µl)	1 µl

Incubate at 37 ^{o}C for 30 min

Label DNA probe with Ready-To-Go kit (Pharmacia Biotech: Cat# 27-5335-01):

1. Use dH_2O to dissolve reagent: 40 µl/vial
2. Add in DNA probe (100 - 500 ng): 5 µl
3. Add in 32P-α-dCTP 5 µl
4. Incubate at 37 ^{o}C for 15 min

Purification of labeled probe with G50 micro column (Pharmacia Biotech 27-5335-01)

1. Spin 3000 rpm in 5415C eppendorf centrifuge for 1 min to get ride of buffer.
2. Load probe onto the resin
3. Collect purified probe by spinning the loaded column at 3000 rpm in 5415C eppendorf centrifuge for 2 min: use 1.5 ml microcentrifuge tube to collect probe.
4. Check probe activity with scintillation counter: 1 µl probe/4ml scintillation solution

(Prepared by Jianping Ye, M.D.)

Prepare the nuclear protein extract:

1. 1 - 5 x 10^7 cell pellet in 1.5 ml microcentrifuge tube
2. Resuspend cells in 300 µl lysis buffer, and keep on ice for 4 min to break the cell membrane
3. Spin cells at 10000 rpm for 1 min at 4 ^oC to separate nuclei with cytoplasmic component
4. Remove supernatant as cytoplasmic extract
5. Wash the nuclear pellet with 300 µl washing buffer
6. Spin cells at 10000 rpm for 1 min at 4 ^oC to pellet the nuclei
7. Resuspend the nuclear pellet in 100 µl (for 1 x 10^7 cells) or 200 µl (for 5x 10^7 cells) extraction buffer
8. Freeze the tube in -70 ^oC or continue the extraction.
9. Pipette the nuclear resuspension.
10. Spin cells at 14000 rpm for 5 min at 4 ^oC to collect supernatant as the nuclear extract.
11. Measure protein concentration and adjust it to 1 µg/µl with the extraction buffer for use in gel shift assay.

(See the attached formula for each buffer).

(Prepared by Jianping Ye,

M.D.)

2 x gel shift reaction buffer

Stock Solution	15 ml Buffer	Final Concentration
50% Glycerol	3.6 ml	12%
1 M HEPES (pH 7.9)	360 ul	24 mM
1 M Tris-HCl (pH 8.0)	120 ul	8 mM
0.5 M EDTA (pH 8.0)	60 ul	2 mM
100 mM DTT	150 ul	1 mM
dH_2O	10.7 ml	

Prepare reaction mixture for gel shift assay

1. 2 x reaction buffer 12 µl
2. BSA (1 µg/µl) 3 µl
3. Poly (dI-dC) (0.5 µg/µl) 2 µl
4. Nuclear extract (1 µg/µl) 3 µl
5. dH2O 3 µl
6. Keep at Rt or on ice for 10 min without Ab, 20 min with Ab
7. Add in DNA probe (4000 cpm/µl) 1 µl
8. Keep at Rt for 20 min
9. Load the gel and run at 200 V for 1 - 1.5 hr. 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.
10. Dry the gel and expose the dried gel to X-ray film at -70 ^{o}C over night.
11. Develop the film.

(Prepared by Jianping Ye, M.D.)

Nuclear Protein Preparation Buffer

Lysis Buffer

Stock Solution	5 ml	10 ml	Final Concentration
1 M KCl	250 ul	500 ul	50 mM
IGEPAL CA-630 (Sigma)	25 ul	50 ul	0.5%
1 M HEPES (pH 7.8)	125 ul	250 ul	25 mM
1 mg/ml Leupeptin (Sigma)	50 ul	100 ul	10 ug/ml
1 mg/ml Aprotinin (Sigma)	100 ul	200 ul	20 ug/ml
250 mM DTT	2.5 ul	5 ul	125 uM
100 mM PMSF	50 ul	100 ul	1 mM
dH_2O	4.4 ml	8.8 ml	

Washing Buffer

Stock Solution	5 ml	10 ml	Final Concentration
1 M KCl	250 ul	500 ul	50 mM
1 M HEPES (pH 7.8)	125 ul	250 ul	25 mM
1 mg/ml Leupeptin	50 ul	100 ul	10 ug/ml
1 mg/ml Aprotinin	100 ul	200 ul	20 ug/ml
250 mM DTT	2.5 ul	5 ul	125 uM
100 mM PMSF	50 ul	100 ul	1 mM
dH_2O	4.4 ml	8.8 ml	

Extraction Buffer

Stock Solution	5 ml	10 ml	Final Concentration
1 M KCl	2.5 ml	5 ml	500 mM
1 M HEPES (pH 7.8)	125 ul	250 ul	25 mM
50 % Glycerol	1 ml	2 ml	10%
1 mg/ml Leupeptin	50 ul	100 ul	10 ug/ml
1 mg/ml Aprotinin	100 ul	200 ul	20 ug/ml
250 mM DTT	2.5 ul	5 ul	125 uM
100 mM PMSF	50 ul	100 ul	1 mM
dH_2O	1.2 ml	2.4 ml	

(Prepared by Jianping Ye, M.D.)

Prepare double-stranded DNA probe

1. Measure OD_{260} of single-stranded DNA (5 ul DNA + 995 ul dH_2O)

2. Calculate the DNA concentration with formula:
   OD260 x dilution fold (200) x 33 ÷ 1000 = (ug/ul)

3. Adjust DNA concentration to 1 ug/ul with dH_2O

4. Mix equal amount of the complementary DNA (such as Sense: Antisense = 50 ug: 50 ug)

5. Heat the mixture to 95 ^oC for 5 min in heating block

6. Leave the heating block at RT to let it cool down slowly

7. Measure OD_{260} of the annealed DNA (5 ul DNA + 995 ul dH_2O)

8. Calculate the DNA concentration with formula:
   OD_{260} x dilution fold (200) x 50 ÷ 1000 = ug/ul

9. Dilute some double-stranded DNA to 0.1 ug/ul with dH_2O

10. Keep double and single-stranded DNA in -20 ^oC, keep the diluted DNA at 4 ^oC.

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(Prepared by: Jianping Ye, M.D.)