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ProductInformation

MOBILITY SHIFT OPTIMIZATION KIT
Product Number **SHIFT-1**

TECHNICAL BULLETIN

Product Description
The mobility shift assay (also called gel shift, gel retardation or band shift assay), is a useful tool for identifying proteins which interact with DNA and mediate cell functions such as gene expression, DNA repair and DNA packaging. The assay can also be used to determine the affinity, abundance, binding constants, and binding specificity of DNA-binding proteins.[1-3]

When a protein binds specifically to a double stranded (ds) DNA sequence the resulting complex migrates more slowly than the non-protein bound dsDNA in a non-denaturing gel electrophoresis. This results in discrete bands corresponding to the individual protein-DNA complexes and is the basis of the Shift-1 procedure.[4,5]

The mobility shift assay is performed by incubating dsDNA, the probe, with the protein(s) of interest (purified or crude extract). The mixture is separated on a non-denaturing polyacrylamide gel and the shifted bands corresponding to the DNA-protein complexes are visualized relative to the unbound dsDNA.

Traditionally, the DNA sequences are dsDNA oligonucleotides end-labeled with ^{32}P and the bands become visible on exposure to autoradiography film. Oligonucleotides can be also labeled with non-radioactive tags such as fluorescein, and detected with an appropriate scanner.

The specificity of the mobility shift assay can be assessed in two ways:

a. Sequence specificity: Binding is specific if unlabeled specific dsDNA consensus (cold probe) added to the protein-DNA reaction mixture competes with the labeled dsDNA consensus (labeled probe) for binding to the protein. The labeled probe released from the complex will migrate to the bottom of the gel and a detectable retarded band is eliminated.

b. Protein specificity (optional): The identity of the protein bound to the DNA can be determined by adding a specific antibody (if available) to the protein-DNA mixture. This will result in a heavier complex which will migrate even more slowly than the original shifted band in electrophoresis.

Sigma's Mobility Shift Optimization Kit is designed to optimize the binding of purified or crude preparations of proteins to DNA in studies such as gene expression, DNA repair, or DNA packaging where DNA-protein complexes are formed. The mobility shift assay depends on multiple factors that may vary between different proteins and the DNA sequences they recognize.

The kit includes "ready-to-use" buffers and additives for optimizing the Mobility Shift Assay, SP1 consensus oligonucleotide for a positive control, and a basic protocol for performing and optimizing the assay. The kit does not include the radiolabeled DNA consensus and the DNA-binding proteins.

Reagents
Reagents Provided
The kit is designed to perform at least 75 reactions

- 5X Binding Buffer A, 1 ml
  Product No. B 1550
  60 mM HEPES, pH 8.0, 20 mM Tris-HCl, 300 mM KCl, 5 mM EDTA, 5 mM EGTA, 60% glycerol
- 5X Binding Buffer B, 1 ml
  Product No. B 1675
  100 mM Tris-HCl, pH 8.0, 300 mM KCl, 5 mM EDTA, 60% glycerol
- 5X Binding Buffer C, 1 ml
  Product No. B 1800
  100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 60% glycerol
- Poly (dI-dC)•(dI-dC), 150 µl
  Product No. P 0976
  1 µg/µl in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA

- Poly (dG-dC)•(dG-dC), 150 µl
  Product No. P 1226
  1 µg/µl in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA
- Poly (dA-dT)•(dA-dT), 150 µl
  Product No. P 1351
  1 µg/µl in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA
- 1% Bovine serum albumin (BSA), 1 ml
  Product No. B 1925
- 100 mM Dithiothreitol (DTT), 1 ml
  Product No. D 6059
- 25 mM Magnesium chloride, 1 ml
  Product No. M 3802
- 1 M Sodium chloride, 1 ml
  Product No. S 0556
- 1% Igepal CA-630, 1 ml
  Product No. I 4526
- 10X Loading Buffer with Tracking Dye, 1 ml
  Product No. L 2660
  250 mM Tris-HCl, pH 7.5, 40% glycerol,
  0.2% bromophenol blue
- 10X Loading Buffer without Tracking Dye, 1 ml
  Product No. L 2535
  250 mM Tris-HCl, pH 7.5, 40% glycerol
- Water, for molecular biology, 1 ml
  Product No. W 4502
- Sp1 consensus oligonucleotides, 1 vial
  Product No. S 0431
  Double stranded, annealed, 35 pmole/vial
  5'- ATT CGA TCG GGG CGG GGC GAG C - 3'
  3'- TAA GCT AGC CCC GCC CCG CTC G - 5'

Reagents and Equipment Required But Not Provided
(Sigma product numbers are given where appropriate)

Reagents for Non Denaturing Polyacrylamide Gel Preparation
- Water, for molecular biology, Product No. W 4502
- Desired buffer for electrophoresis
- 10X TAE (Tris-acetate-EDTA) buffer, Product No. T 9650, T 8280 or T 4038

or

- 10X TBE (Tris-borate-EDTA) buffer, Product No. T 4415

or

- Trizma® base, Product No. T 6066, glycine, Product No. G 8790, and EDTA, Product No. E 5134
- Acrylamide/bis-acrylamide (19:1), 40% solution, Product No. A 2917
- Ammonium persulfate, Product No. A 9164 or A 3426
- TEMED, Product No. T 7024
- Gel electrophoresis equipment

Reagents for DNA Consensus Oligonucleotide Labeling
- γ-^{32}P-ATP (3000 Ci/mmol at 10 mCi/ml)
- 0.5 M EDTA, Product No. E 7889
- 100X TE (Tris-EDTA) buffer, Product No. T 9285
- 1X TE buffer (Dilute 100X TE buffer 1:100 with water)
- Stop reaction buffer (Add 10 µl of 0.5 M EDTA to 890 µl 1X TE buffer)
- 10X Polynucleotide kinase buffer (w/o ATP)
  (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$,
  10 mM dithiothreitol, 1 mg/ml BSA,
  12 µg/µl micrococcal-nuclease-treated salmon DNA)
- Polynucleotide kinase (5-10 units/µl), Product No. P 4390
- Water, for molecular biology, Product No. W 4502
- Oligonucleotide (double stranded) with dephosphorylated 5'-end
- Microspin G-25 column (Amersham Pharmacia Code 27-5325-01)

Reagents for Mobility Shift Assay
- 5% polyacrylamide gel from Procedure Section I
- γ-^{32}P-labeled Sp1 oligonucleotides from Procedure Section II
- HeLa cell nuclear extract.
- Non-relevant consensus DNA, AP-1, Product No. A 9590 or any appropriate non-relevant consensus DNA
- Blotting paper, Product No. P 6664, P 6914 or P 9039

Storage
Store the kit at –20 °C

Preparation Instructions

1. dsDNA Consensus Probe
   Labeled dsDNA fragments ranging in size from 20-300 bp long may be used as probes. Longer fragments may be used, but they are likely to contain several binding sites for different proteins which may complicate the interpretation of the results. Labeling is performed by one of two labeling methods:
   a. By the filling-in reaction using the Klenow fragment of *E. coli* DNA polymerase and [α-^{32}P]-labeled nucleotide
   b. By end-labeling using polynucleotide kinase (PNK) and [γ-^{32}P-ATP]-labeled nucleotide.[6] For end-labeling procedure with γ-^{32}P-ATP, see Procedure, Section B.

   Note: PNK End-labeled probes are not recommended when using crude protein preparations because the protein preparations may contain phosphatases that can dephosphorylate the label from the probe.
2. Controls
   a. Unlabeled dsDNA consensus ("cold" probe)
   b. Unlabeled non-relevant dsDNA: dsDNA consisting of different, unrelated sequence, but about the same size as the "cold" probe. To demonstrate that the protein binding to the dsDNA consensus is specific, excess "cold" probe is added to the reaction mixture. This sequence should compete with the labeled probe and eliminate the detected shifted band. A "cold" non-relevant dsDNA should not interfere with the protein-DNA interaction and therefore will not influence the shifted band.
3. DNA Binding Proteins (pure/crude) - The source of protein for the band shift assay can be either a purified known protein or a soluble extract from isolated cells or nuclei.[7]

Discussion

Formation and stability of DNA-protein complexes are affected by a number of factors such as ion concentration, pH, non specific proteins or DNA fragments, chelators and detergents.

- pH, salt and ion concentrations
  The mobility of the DNA-protein complex is determined primarily by the size and charge of the protein bound to the dsDNA fragment and by the conformation of the complex. Salts and pH are important factors that can significantly alter the mobility of a given DNA-protein complex through the non-denaturing gel. Novel DNA-protein complexes may be formed by varying the pH and the ions in the binding buffer. This kit provides three different commonly used reaction buffers (5X concentrates) that contain either KCl or NaCl are at two different pH values. It is recommended to try both a high and a low ionic strength buffer system for each putative DNA-protein interaction.
- Reducing agents
  A reducing reagent like DTT is important for keeping the protein in a reduced form.
- Bovine serum albumin (BSA)
  BSA seems to improve the stability of some complexes during electrophoresis.[8]
- Divalent cation
  $MgCl_2$ is commonly used and is needed for some DNA-protein complexes to form.
- Monovalent cations
  DNA-protein interaction is affected by the concentration of monovalent ions. This kit provides 1M NaCl which can be added to the reaction mixture to increase the monovalent cation concentration above that of the KCl or NaCl already present in the 5X binding buffers included in the kit.
- DNA carrier
  Binding of non-specific proteins to the labeled DNA may result in non-specific bands that are also retarded. The inclusion of natural or synthetic DNA polymers in the reaction mixture is sufficient to prevent non-specific proteins from binding to the labeled DNA, but not to prevent the binding of specific proteins. This kit provides three different synthetic polymers: Poly(dI-dC)•(dI-dC), Poly(dG-dC)•(dG-dC), and Poly(dA-dT)•(dA-dT).
- Detergents
  Detergents like Igepal CA-630 can increase the stability of the protein and prevent possible aggregation, contributing to a more stable DNA-protein complex. A 1% Igepal CA-630 solution is included in the kit. The final working concentration should be 0.033% (1:30 dilution).
- Temperature
  Optimal incubation temperature for individual proteins can vary from 4 °C to 37 °C depending on the DNA-protein complex heat lability.
- Gel concentration and acrylamide:bis-acrylamide ratio
  Typical gels for mobility shift assays contain 4-6% acrylamide with an acrylamide:bis-acrylamide ratio of 40:1 or 19:1. For preparation, see Procedure, Section I.

Procedure

I. Non-Denaturing Polyacrylamide Gel Preparation

Different conditions can be used to run the non-denaturing gel which include different acrylamide concentrations and different buffers. Typical gels used in this assay are in the range of 4% to 6% acrylamide.

Many gel buffers have been successfully used for mobility shift assays. The stability of DNA-protein complexes can be strongly affected by the choice of buffer, so it is worth trying different systems for the protein of interest. The most common buffers used are Tris-acetate-EDTA (TAE), Tris-glycine-EDTA (TGE), and Tris-borate-EDTA (TBE). The electrophoresis buffers are typically used at 0.5X, 1.0X or 2.0X concentrations. The gel running buffer should match the buffer used to prepare the gel. The table below may be used to prepare a 5% acrylamide/bis-acrylamide gel; the buffer may be changed in order to optimize of the mobility shift assay.

See Reagents and Equipment Required but Not Provided section for list of required reagents**.**

Preparation of 5% non-denaturing gel mix

	0.5X Buffer	1X Buffer
40% solution acrylamide/bis-acrylamide (19:1)	12.5 ml	12.5 ml
10X buffer*	5 ml	10 ml
Water	81.5 ml	76.5 ml
10% Ammonium persulfate	1 ml	1 ml
TEMED	125 µl	125 µl
Total volume	100 ml	100 ml

*use TAE, TBE or TGE.

To prepare 10X TGE (Tris-glycine-EDTA) buffer, dissolve

30.28 g	Trizma base	(0.25 M)
142.7 g	Glycine	(1.9 M)
3.92 g	EDTA	(10 mM)

in water to 1 liter and adjust pH to approx. 8.3

II. DNA Consensus Oligonucleotide Labeling

Use the following procedure for γ-^{32}P 5'-end labeling of the supplied SP1 consensus oligonucleotide.
See Reagents and Equipment Required but Not Provided section for list of required reagents.

1. Phosphorylation Reaction

   b. Combine the following reagents in a sterile microcentrifuge tube in an ice bath:

   2 µl oligonucleotide (dephosphorylated) (1.75 pmole/µl)
   or
   2 µl SP1 consensus oligonucleotide (1.75 pmole/µl)
   1 µl Polynucleotide kinase 10X buffer
   1 µl Polynucleotide kinase
   1-3 µl γ-^{32}P-ATP (see Notes 1 and 2 below)
   3-5 µl water for molecular biology

   10 µl Total volume

Note 1: Use 1 µl γ-^{32}P-ATP within the first week of receipt and 2-3 µl within the second week.
Note 2: Add γ-^{32}P-ATP to the reaction mixture last.

   b. Incubate at 37 °C for 30 minutes.

   c. Stop the reaction by adding 90 µl of stop reaction buffer.

2. Removal of Unincorporated Label by G-25 Spin Chromatography

   a. Follow procedure according to manufacturer instructions. Expected incorporation is 40-70%.

   b. Store the radiolabeled reagent at –20 °C. The material is stable for a maximum of two weeks at –20 °C.

III. Basic Protocol for Mobility Shift Assay

For uncharacterized DNA-protein complexes, begin with Procedure III to observe the shifted bands of newly formed DNA-protein complexes. With the basic protocol the $MgCl_2$ can be optimized and the sequence specificity of the formed complexes can be determined by competitive assay. Based on these results, further optimization steps may be recommended as outlined below.

See Reagents and Equipment Required but Not Provided section for list of required reagents.

1. Remove the comb from the previously prepared 5% gel (Procedure, Section I) and set the gel in an electrophoresis unit.

2. Pre-run the gel without sample for 30-60 minutes at 10 V/cm.

3. Thaw the kit components.

4. For each labeled consensus DNA prepare 6 marked tubes. Note: Tubes 1-3 have no added $MgCl_2$ and tubes 4-6 have added $MgCl_2$:

   Tubes 1 and 4: Basic reaction to observe shifted bands
   Tubes 2 and 5: Competition with unlabeled ("cold") consensus DNA to observe the interference or elimination of the specific shifted band
   Tubes 3 and 6: Competition with unlabeled ("cold") nonrelevant DNA to confirm there is no interference with the specific shifted band

Note: As a positive control for correct assay performance it is recommended to concurrently run a parallel set of reactions (3 tubes, 7-9) using labeled Sp1 consensus, supplied in the kit unlabeled. The optimal conditions for Sp1 consensus mobility shift are 5X Binding Buffer A, HeLa nuclear extract and addition of $MgCl_2$.

5. Combine the reagents as directed in Table 1:

Table 1

	Tube Number					
Components	#1 (+)	#2 (-)	#3 (+)	#4 (+)	#5 (-)	#6 (+)
5X Binding buffer (A, B or C) Note: Buffer A is most commonly used	6 µl in each tube					
100 mM DTT	0.5 µl in each tube					
1% BSA	2 µl in each tube					
Poly(dI.dC)•(dI,dC), 1 µg/µl	2 µl in each tube					
DNA-binding protein or HeLa nuclear extract, 5-10 mg/ml	2 µl in each tube					
Unlabeled consensus DNA probe, 1.75 pmole/µl	-----	2 µl	----	-----	2 µl	____
Unlabeled nonrelevant DNA probe, 1.75 pmole/µl	-----	-----	2 µl	-----	-----	2 µl
25 mM $MgCl_2$	**-----**	**-----**	**-----**	3 µl	3 µl	3 µl
Water, for molecular biology	15.5 µl	13.5 µl	13.5 µl	12.5 µl	10.5 µl	10.5 µl
Incubate 10 minutes at room temperature, then add:						
γ-^{32}P labeled consensus DNA probe (See Note 1)	1-3 µl in each tube					

Note 1: Use dsDNA consensus that has been radiolabeled not more than two weeks before the test is performed. Add 1 µl within the first week after labeling, 2-3 µl within the second week after labeling.

6. Mix and incubate the reaction for 20 minutes at room temperature. Mix gently by tapping the bottom of the tube with a finger. Avoid introducing bubbles in the mix. Spin the tubes in a microcentrifuge for a few seconds.

7. To each tube add ~3 µl of loading buffer. Note: Loading buffer is supplied in two separate vials, with tracking dye (Product No. L 2660) and without tracking dye (Product No. L 2535). For most systems, use the loading buffer with tracking dye. If it is suspected that the dye may interfere with the complex formation, use the loading buffer without tracking dye and add an additional 3 µl of loading buffer with dye to an extra lane, to monitor the migration of the bands during the electrophoresis.

8. Stop the pre-run of the gel.

9. Load the contents of each tube into separate wells on the gel.
   There is no stacking gel in the system, so precise loading with minimum mixing of the buffer is necessary to obtain sharp bands. Washing the wells with running buffer before loading the samples is recommended; empty the wells by inverting the gel on blotting paper. Allow the sample (~30 µl) to fall along one side of the well to prevent dilution of the sample and avoid bubbles in the well.

10. Carefully add running buffer to the wells with a pipet and then add buffer to the upper electrophoresis chamber.

11. Run the gel at 10 V/cm to give clear separation of the free labeled DNA consensus and the DNA-protein complexes. Stop the gel run when the tracking dye is 2/3 of the way through the gel.

12. Remove the gel from the gel box and carefully remove the side spacers.

13. Using a spatula, slowly separate the glass plates, allowing air to get between the gel and the upper glass plate.

14. Lay the lower glass with the gel facing up on a hard surface. Place three sheets of blotting paper cut to size on top of the gel.

15. Peel the blotting paper with the gel attached to it from the plate.

16. Cover the gel with a nylon membrane and dry under vacuum (~1.5 hours at 80 °C).

17. Expose the dried filter to autoradiography film overnight at room temperature (or overnight at –70 °C, with an intensifying screen).

IV. Binding Buffer and DNA Carrier Optimization of Mobility Shift Assay

This step optimizes the results obtained with the Basic Protocol for Mobility Shift Assay (Section III) and selects the most effective binding buffer (A, B or C) and DNA carrier (Poly(dI-dC)•(dI-dC), Poly(dG-dC)•(dG-dC), or Poly(dA-dT)•(dA-dT)).

Follow steps 1-17 as in Procedure, Section III but in step 5 use Table 2:

Table 2

	Tube Number											
	Buffer A				Buffer B				Buffer C			
Components	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
5X Reaction buffer A	6 µl in each tube				----	----	----	---	----	----	----	----
5X Reaction buffer B	----	----	----	----	6 µl in each tube				----	----	----	----
5X Reaction buffer C	----	----	----	----	----	----	----	----	6 µl in each tube			
Poly(dI-dC)•(dI-dC) 1 µg/µl (See Note 1)	----	2 µl	----	----	----	2 µl	----	----	----	2 µl	----	----
Poly(dA.dT)•(dA.dT) 1 µg/µl (See Note 1)	----	----	2 µl	----	----	----	2 µl	----	----	----	2 µl	----
Poly(dG.dC)•(dG.dC) 1 µg/µl (See Note 1)	----	----	----	2 µl	----	----	----	2 µl	----	----	----	2 µl
1% BSA	1 µl in each tube				1 µl in each tube				1 µl in each tube			
100 mM DTT	0.5 µl in each tube				0.5 µl in each tube				0.5 µl in each tube			
DNA-binding protein 5-10 mg/ml (See Note 2)	1-3 µl in each tube				1-3 µl in each tube				1-3 µl in each tube			
25 mM $MgCl_2$ (See Note 3)	Either none or 3 µl per tube				Either none or 3 µl per tube				Either none or 3 µl per tube			
Molecular biology grade water	X µl (to bring total reaction volume to 30 µl)				X µl (to bring total reaction volume to 30 µl)				X µl (to bring total reaction volume to 30 µl)			
Mix thoroughly then add:												
γ-^{32}P labeled consensus DNA probe (See Note 4)	1-3 µl in each tube				1-3 µl in each tube				1-3 µl in each tube			
Mix gently (See Note 5) and incubate the reaction for 20 minutes at the appropriate temperature (See Note 6)												

Note 1: The appropriate amount of DNA carrier is determined empirically.
Note 2: The amount of DNA-binding protein depends on the amount of the potential protein within the extract.
Note 3: Add 25 mM $MgCl_2$ to the reaction if the results from the mobility shift assay (Procedure, Section III) show improved resolution in Tubes 4-6. Omit the 25 mM $MgCl_2$ if the resolution in Tubes 4-6 is comparable to that of Tubes 1-3.
Note 4: Use dsDNA consensus which has been labeled not more than two weeks before the test is performed. Add 1 µl within the first week after labeling, 2-3 µl within the second week after labeling.
Note 5: Mix gently by tapping the bottom of the tube with a finger. Avoid introducing bubbles in the mix. Spin the tubes in a microcentrifuge for a few seconds.
Note 6: Optimal incubation temperature depends on the specific protein and DNA consensus, and can vary from 4 °C to 37 °C with incubation periods of 10-30 minutes. If unsatisfactory results are obtained when incubating at room temperature, try incubations at 4 °C or at 37 °C.

V. Divalent Cations and Nonionic Detergents Optimization of Mobility Shift Assay

This step is designed to further optimize the DNA-protein complex formation by checking the influence of different concentrations of divalent cations such as $MgCl_2$ and the influence of non-ionic detergents such as Igepal CA-630 while using the optimal binding buffer and DNA carrier selected in Procedure Section IV .

Follow steps 1-17 as in Procedure, Section III, but in step 5 use Table 3:

TABLE 3

	Tube Number						
	#1	#2	#3	#4	#5	#6	**#7**
5X Binding buffer	6 µl in each tube						
DNA carrier, 1 µg/µl (See Note 1)	1-2.5 µl	1-2.5 µl	1-2.5 µl	-----	1-2.5 µl	1-2.5 µl	1-2.5 µl
1% BSA	1 µl in each tube						
100 mM DTT	0.5 µl in each tube						
DNA-binding protein 5-10 mg/ml (See Note 2)	1-3 µl in each tube						
Unlabeled consensus DNA probe, 1.75 pmole/µl	-----	2 µl	-----	-----	------	-----	-----
Unlabeled nonrelevant DNA probe, 1.75 pmole/µl	-----	-----	2 µl	-----	-----	-----	-----
25 mM $MgCl_2$ (See Note 1)	-----	-----	-----	------	1-6 µl	------	1-6 µl
1% Igepal CA-630	-----	-----	-----	-----	1 µl	1 µl	-----
Molecular biology grade water	X µl (to bring total reaction volume to 30 µl)						
Mix thoroughly. Incubate 10 minutes at room temperature, then add:							
γ-^{32}P labeled consensus DNA probe (See Note 3)	1-3 µl	1-3 µl	1-3 µl	1-3 µl	1-3 µl	1-3 µl	1-3 µl
Mix gently (See Note 4) and incubate the reaction for 20 minutes at the appropriate temperature (See Note 5)							

Note 1: The appropriate amounts of DNA carrier and $MgCl_2$ are determined empirically.
Note 2: The amount of DNA-binding protein depends on the amount of the potential protein within the extract.
Note 3: Use dsDNA consensus which has been labeled not more than two weeks before the test is performed. Add 1 µl within the first week after labeling, 2-3 µl within the second week after labeling.
Note 4: Mix gently by tapping the bottom of the tube with a finger. Avoid introducing bubbles in the mix. Spin the tubes in a microcentrifuge for a few seconds.
Note 5: Optimal incubation temperature depends on the specific protein and DNA consensus, and can vary from 4°C to 37°C with incubation periods of 10-30 minutes. If unsatisfactory results are obtained when incubating at room temperature, try incubations at 4 °C or at 37 °C.

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