Gelshift™ Chemiluminescent EMSA Kit

(version A2)

Catalog No. 37341

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Overview

Electrophoretic mobility shift assays (EMSA), also known as gel shifts, gel retardation assays or mobility assays can be used to study DNA-protein interactions[1-3]. The principle behind EMSA relies on the fact that DNA-protein complexes migrate slower than DNA alone in a native polyacrylamide or agarose gel. This difference in electrophoretic separation of DNA-protein complexes can be visualized as a "shift" in migration of the labeled DNA band.

The Gelshift™ Chemiluminescent EMSA Kit provides a non-radioactive method to detect DNA-protein interactions. In this method, cell extracts or purified factor are incubated with a biotin 3' or 5' end-labeled DNA probe containing the consensus binding site of interest. Samples are then resolved by electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA probe is detected using streptavidin conjugated to horseradish peroxidase (HRP) and a chemiluminescent substrate. The assay can be complete in approximately 5 hours.

product	format	catalog no.
Gelshift™ Chemiluminescent EMSA	100 rxns	37341

Kit Components and Storage

Gelshift Chemiluminescent EMSA Kits are for research use only. Not for use in diagnostic procedures. We recommend storing each component at the temperature indicated in the table below. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
10X Binding Buffer	1 ml	-20°C
Biotin Control DNA	50 µl	-20°C
Unlabeled Control DNA	50 µl	-20°C
Control Nuclear Extract	125 µl	-20°C
Poly d(I-C)	125 µl	-20°C
50% Glycerol	500 µl	-20°C
1% NP-40	500 µl	-20°C
1M KCl	1 ml	-20°C
100 mM $MgCl_2$	500 µl	-20°C
200 mM EDTA, pH 8.0	500 µl	-20°C
5X Loading Buffer	1 ml	-20°C
Streptavidin HRP-conjugate	1.5 ml	4°C
Chemiluminescent Reagent	80 ml	4°C
Reaction Buffer	80 ml	4°C
Blocking Buffer	500 ml	4°C
4X Wash Buffer	500 ml	4°C
Substrate Equilibration Buffer	500 ml	4°C

Additional materials required

- Biotin 3' or 5' end-labeled DNA target. Do not use DNA probes with internal biotin labels as the internal label may inhibit the protein binding to DNA.
- Unlabeled DNA target for use in competition experiments to verify specificity of the DNA-protein complex
- Nuclear extracts containing the target protein of interest. (Nuclear extracts can be prepared using Active Motif's Nuclear Extract Kit Catalog Nos. 40010 & 40410)
- Polyacrylamide gel in 0.5X TBE (most systems use a 4-6% polyacrylamide gel in 0.5X TBE) Commercially available gels such as Life Technologies 6% DNA Retardation Gel 1.0 mm, 10 well (Cat # EC6365BOX) can also be used
- Electrophoresis apparatus
- Positively charged nylon membrane
- Electroblotter or capillary transfer apparatus
- High quality blotting paper
- Clean sponges for electrophoretic transfer
- Circulating waterbath
- Plastic forceps
- Cross-linking device such as a UV lamp or commercial cross-linking device with 254 nm bulbs or a transilluminator equipped with 312 nm bulbs
- Clean plastic trays or weigh boats for detection
- Orbital shaker
- X-ray film or CCD camera

Read the entire protocol before use.

The Gelshift Chemiluminescent EMSA kit includes biotin-labeled and unlabeled control DNA as well as a control nuclear extract. If the kit is being used for the first time, we recommend to run the control system reactions to verify the kit components and procedure are working properly. The control samples are from the Epstein-Barr Nuclear Antigen (EBNA) system. A description of control reactions and expected results are shown in Step 1.

Do not thaw the Control Nuclear Extract provided in the kit until immediately before use. Thaw the remaining binding reaction components and place them on ice. Take care to avoid excessive warming of DNA probes.

Biotin 5' or 3' end-labeled target DNA and unlabeled target DNA can be ordered directly from your oligo supplier. Internal biotin labels, such as those that arise from random prime labeling methods, are not recommended because the internal biotin labels may inhibit binding of the protein to DNA. A typical target duplex DNA is 20-35 bp for use in EMSA. The actual protein:DNA binding sequence is frequently 10-15 bp. Longer probes can be used if the binding sequence is unknown or if multiple regulatory regions are being studied.

Sample nuclear extracts can be prepared using Active Motif's Nuclear Extract Kit (Catalog Nos. 40010 & 40410). The amount of protein required for the binding reactions depends on the amount of active protein in the sample. If the protein being studied is abundant, 0.25 µg of lysate may be sufficient for each binding reaction. However, if the protein is less abundant we recommend using 10 µg or more. Using a large excess of extract may lead to high background signal and nonspecific bands. Some optimization may be required to determine the best conditions for your sample system. The final volume of the binding reaction is 20 µl. If a large volume of nuclear extract is required, remove excess salts in the extract by dialyzing into a buffer containing 200 mM salt before using in the Gelshift Chemiluminescent EMSA Kit.

This kit has been optimized for use with polyacrylamide mini (8 x 8 x 0.1 cm) gels. Please adjust electrophoresis conditions and detection reagent volumes if using larger gels.

Step 1: Plan the Experiment

1. Control DNA and Control Nuclear Extract are included in the Gelshift Chemiluminescent EMSA. It is recommended to perform a set of three control reactions as described in Step 3 each time an EMSA is performed.
2. It is also recommended to perform a set of three binding reactions for each sample system to verify the appropriate shift and protein:DNA specificity of the interaction. Optimization of the sample system can be achieved by adding other components supplied in the kit such as KCl[4,5], glycerol, $MgCl_2$[4,6] and detergent[7,8] to determine their effects on the shift. Please read Step 4 to determine the binding reactions required for the sample system.

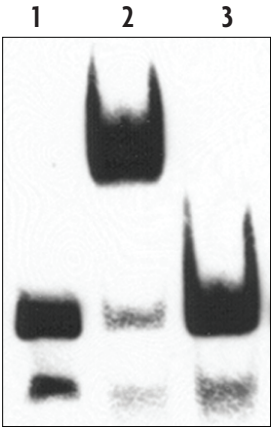
Step 2: Prepare and Pre-Run Gel

1. Prepare a native polyacrylamide gel in 0.5X TBE or use a pre-cast DNA retardation gel. The appropriate polyacrylamide percent depends on the size of the target DNA and the binding protein. Most systems use a 4-6% polyacrylamide gel in 0.5X TBE.
2. Add 0.5X TBE to fill the electrophoresis unit to just below the bottom of the wells. Flush each well with 0.5X TBE. Pre-run the gel for 30-60 minutes at 100 V for an 8 x 8 x 0.1 cm gel.
3. During this pre-run of the gel, proceed to Step 3.

Step 3: Control DNA Binding Reactions

1. Perform a set of three control reactions each time an EMSA is performed.

Reaction	Contents	Expected Result
#1	Biotin Control DNA	No shift. Without the control nuclear extract, the reaction lacks protein to bind DNA and cause a shift.
#2	Biotin Control DNA + Control Nuclear Extract	DNA shift. The target protein present in the control nuclear extract will bind to the biotin-labeled control DNA and cause a shift as compared to reaction #1.
#3	Biotin Control DNA + Control Nuclear Extract + Unlabeled Control DNA	No shift. The excess of unlabeled control DNA competes for binding of the target protein in the control nuclear extract. This verifies the signal observed in reaction #2 is the result of specific DNA-protein interaction.



2. To set up the control reactions, add components in the order shown below to each tube.

Reagent	Final Concentration	Reaction #1	Reaction #2	Reaction #3
Ultrapure Water	–	12 µl	11 µl	9 µl
10X Binding Buffer	1X	2 µl	2 µl	2 µl
50% Glycerol	2.5%	1 µl	1 µl	1 µl
100 mM $MgCl_2$	5 mM	1 µl	1 µl	1 µl
1 µg/µl Poly d(I-C)	50 ng/µl	1 µl	1 µl	1 µl
1% NP-40	0.05%	1 µl	1 µl	1 µl
Unlabeled Control DNA	4 pmol	–	–	2 µl
Control Nuclear Extract	1 unit	–	1 µl	1 µl
Biotin Control DNA	20 fmol	2 µl	2 µl	2 µl
Total Volume	**–**	**20 µl**	**20 µl**	**20 µl**

3. Incubate control binding reactions at room temperature for 20 minutes.
4. Add 5 µl of 5X Loading Buffer to each 20 µl binding reaction. Pipette up and down several times to mix, do not vortex or mix vigorously.
4. To set up Sample DNA Binding Reactions Proceed to Step 4, otherwise proceed to Step 5 on page 7 and continue with the protocol for electrophoresis, transfer, cross-linking and detection of control samples.

Step 4: Sample DNA Binding Reactions

1. Perform a set of three reactions for each sample system to verify the appropriate shift and protein:DNA specificity of the interaction. (See Step 3.1 of Control DNA Binding Reactions for more details)
2. If specific binding conditions are not already known, it is recommended to use minimal reaction components. To further optimize sample binding, the addition of optional reagents such as glycerol, detergent, KCl, $MgCl_2$ or EDTA can be included in the binding reactions. Bovine serum albumin and basic peptides have also been shown to enhance some DNA-protein interactions[8-10].

   Poly d(I-C) is provided as a nonspecific competitor DNA. This is suitable for most sample systems, but if the target DNA sequence is GC-rich, try Poly d(A-T), salmon sperm or *E.coli* DNA.

   Nuclear protein extracts can be prepared using Active Motif's Nuclear Extract Kit (Catalog Nos. 40010 & 40410)
3. Add components in the order shown below to each tube. To overcome strong nonspecific interactions, a short pre-incubation may be required before adding the biotin-labeled sample DNA.

Reagent	Final Conc.	Reaction #1	Reaction #2	Reaction #3
Ultrapure Water	–			
10X Binding Buffer	1X	2 µl	2 µl	2 µl
1 µg/µl Poly d(I-C)	50 ng/µl	1 µl	1 µl	1 µl
Optional: 50% Glycerol*				
Optional: 1% NP-40				
Optional: 1 M KCl				
Optional: 100 mM MgCl2				
Optional: 200 mM EDTA				
Unlabeled Sample DNA	4 pmol	–	–	
Nuclear Extract		–		
Biotin-labeled Sample DNA	20 fmol			
Total Volume	**–**	**20 µl**	**20 µl**	**20 µl**

* Too much glycerol in the binding reaction may cause vertical streaks along the edges of the lanes.

4. Incubate sample binding reactions at room temperature for 20 minutes.
5. Add 5 µl of 5X Loading Buffer to each 20 µl binding reaction. Pipette up and down several times to mix, do not vortex or mix vigorously.

Step 5: Electrophoresis of Binding Reactions

1. Once the pre-run of the polyacrylamide gel is finished (Step 2), turn off current to the unit.
2. Flush each well with 0.5X TBE buffer. Then load 20 µl of sample containing loading buffer onto the polyacrylamide gel.
3. Run the gel at 100 V (for 8 x 8x 0.1 cm gel) until the bromophenol blue dye has migrated approximately 2/3 to 3/4 down the length of the gel. The free Biotin Control DNA duplex migrates just behind the bromophenol blue when using a 6% gel.

Step 6: Transfer of Binding Reactions to Nylon Membrane

1. Soak a nylon membrane in 0.5X TBE buffer for a minimum of 10 minutes.
2. Follow the manufacturer's instructions for your electrophoretic transfer unit. Use a clean transfer unit and clean transfer sponges. Avoid using sponges that have been used in Western blots. Use 0.5X TBE cooled to approximately 10°C with a circulating waterbath for the transfer. Handle the nylon membrane only at the corners using clean forceps and powder-free gloves.
3. Transfer at 380 mA (~100 V) for 30 minutes for mini gels (8 x 8 x 0.1 cm). Typical transfer times are 30-60 minutes at 380 mA using a standard tank apparatus.

4. Once the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. The gel should not contain any visible dye if the transfer was complete. Allow buffer on the nylon membrane surface to absorb into the membrane (~ 1 minute), but do not let the membrane dry. Proceed immediately to Step 7.

Step 7: Cross-link Transferred DNA to Membrane

1. There are three methods to choose from for cross-linking the DNA to the membrane.

   Commercial UV-light cross-linker: Cross-link at 120 mJ/cm^2 using a commercial UV-light cross-linker instrument equipped with 254 nm bulbs (45-60 second exposure using the auto cross-link function).

   Hand-held UV lamp: Cross-link at a distance of approximately 0.5 cm from the membrane for 5-10 minutes with a hand-held UV lamp equipped with 254 nm bulbs.

   Transilluminator: Cross-link for 10-15 minutes with the membrane face down on a transilluminator equipped with 312 nm bulbs.

2. After cross-linking, the membrane may be stored dry at room temperature for several days or else immediately detected in Step 8. If the membrane is stored, do not allow it to get wet until ready to proceed with Step 8.

Step 8: Detect Biotin-labeled DNA by Chemiluminescence

This kit has been optimized for use with polyacrylamide mini (8 x 8 x 0.1 cm) gels. Please adjust electrophoresis conditions and detection reagent volumes if using larger gels. Perform all blocking and detection incubations in clean trays or in plastic weigh boats on an orbital shaker.

Preparation of Blocking Buffer

Warm the Blocking Buffer in a 37-50°C water bath until all particulate is dissolved. Once the particulate is completely dissolved. The buffer may be used between room temperature and 50°C as long as all the particulates remain in solution

Preparation of Streptavidin Conjugate/Blocking Buffer Solution

Prepare a 1:300 dilution of streptavidin conjugate/blocking buffer solution by adding 66.7 µl Streptavidin-HRP conjugate to 20 ml Blocking Buffer. Mix prior to use.

Note: The streptavidin conjugate/blocking buffer solution has been optimized to work at a 1:300 dilution and should not be modified.

Preparation of 1X Wash Buffer

Warm the 4X Wash Buffer in a 37-50°C water bath until all particulate is dissolved. Once the particulate is completely dissolved, prepare the amount of 1X Wash Buffer required for the assay as follows: Dilute 40 ml 4X Wash Buffer with 120 ml ultrapure water and mix prior to use.

1. Prepare the Blocking Buffer, Streptavidin Conjugate/Blocking Buffer Solution and 1X Wash Buffer as stated above.
2. Take the cross-linked membrane from Step 7 and place in a clean tray or plastic weight boat. Add 20 ml Blocking Buffer and incubate for 15 minutes at room temperature with gentle shaking on an orbital shaker.
3. Decant the blocking buffer from the membrane and replace it with the Streptavidin Conjugate/Blocking Buffer solution. Incubate for 15 minutes at room temperature with gentle shaking on an orbital shaker.
4. Transfer the membrane to a new container and rinse briefly with 20 ml 1X Wash Buffer.
5. Wash the membrane four times for 5 minutes each in 20 ml 1X Wash Buffer with gentle shaking on an orbital shaker.
6. Transfer the membrane to a new container and add 30 ml of Substrate Equilibration Buffer. Incubate for 5 minutes with gentle shaking on an orbital shaker.
7. Prepare Chemiluminescent Working solution by adding 6 ml of Chemiluminescent Reagent to 6 ml Reaction Buffer. The Chemiluminescent Working Solution is light sensitive, therefore we recommend keeping this working solution in an amber bottle and avoiding direct exposure to intense light. Short-term exposure to laboratory lighting will not affect the working solution.
8. Remove the membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Place the membrane in a clean container or onto a clean sheet of plastic wrap. Make sure the membrane is on a flat surface.
9. Add the Chemiluminescent Working Solution to the membrane making sure the entire surface of the membrane is covered. Alternatively, the membrane can be placed DNA side down into a puddle of the Chemiluminescent Working Solution. Incubate the membrane with the substrate for 5 minutes at room temperature without shaking.
10. Remove the membrane from the substrate solution, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Do not allow the membrane to become dry.
11. Wrap the moist membrane in plastic wrap. Avoid creating bubbles or wrinkles with the plastic.
12. Expose the membrane to an appropriately equipped CCD camera, or place the membrane in a film cassette and expose to X-ray film for 2-5 minutes. Develop the film according to the manufacturer's instructions. Exposure time may be adjusted to obtain the desired signal.

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Appendix

Section A. Preparation of Nuclear Extract

For your convenience, Active Motif offers a Nuclear Extract Kit (Cat. Nos. 40010 & 40410). If you prefer to make your own buffers, please refer to the following protocol.

This procedure can be used for a confluent cell layer of 75 cm^2 (100-mm dish). The yield is approximately 0.15 mg of nuclear proteins for 9 x 10^6 cells.

1. Wash cells with 10 ml ice-cold PBS/PIB. Discard PBS/PIB.
2. Add 10 ml ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer cells into a pre-chilled 15 ml tube and spin at 300 x *g* for 5 minutes at 4°C. Do not use Trypsin to remove cells as it may alter transcription factor activation states.
3. Resuspend the pellet in 1 ml ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
4. Allow the cells to swell on ice for 15 minutes.
5. Add 50 µl 10% Nonidet P-40 (0.5 % final) and vortex the tube vigorously for 10 seconds. Check the cells under a microscope to monitor the cell lysis. The cell membrane should be completely lysed, while the nuclear membrane remains intact.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge. Remove the supernatant (cytoplasmic fraction) and, if you wish to save this for other uses, transfer it into a pre-chilled microcentrifuge tube. (Store the cytoplasmic fraction at –80°C.)
7. Resuspend the nuclear pellet in 50 µl Complete Lysis Buffer and rock the tube gently on ice for 30 minutes on a shaking platform.
8. Centrifuge for 10 minutes at 14,000 x *g* at 4°C and save the supernatant (nuclear extract). Aliquot and store at –80°C. Avoid freeze/thaw cycles. Discard the debris pellet.
9. Determine the protein concentration of the extract by using a Bradford-based assay. It is recommended to perform a 1:50 dilution of your samples for protein determination. A blank sample consisting of Complete Lysis Buffer diluted 1:50 should be run as control. Standard controls should also be generated in Complete Lysis Buffer diluted 1:50.

Preparation of Buffers for Nuclear Extract

10X PBS	**For 250 ml, mix:**
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	0.5 g

Adjust to 250 ml with distilled water. Prepare a 1X PBS solution by adding 10 ml 10X PBS to 90 ml distilled water. Sterilize the 1X PBS by filtering through a 0.2 µm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

PIB (Phosphatase Inhibitor Buffer)	**For 10 ml, mix:**
125 mM NaF	52 mg
250 mM β-glycerophosphate	0.55 g
250 mM p-nitrophenyl phosphate (PNPP)	1.15 g
25 mM $NaVO_3$	31 mg

Adjust to 10 ml with distilled water. Mix the chemicals by vortexing. Incubate the solution at 50°C for 5 minutes. Mix again. Store at –20°C.

PBS/PIB

Prior to use, add 0.5 ml PIB to 10 ml 1X PBS.

HB (Hypotonic Buffer)	**For 50 ml, mix:**
20 mM Hepes, pH 7.5	0.24 g
5 mM NaF	12 mg
0 µM Na_2MoO_4	5 µl of a 0.1 M solution
0.1 mM EDTA	10 µl of a 0.5 M solution

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 ml with distilled water. Sterilize by filtering through a 0.2 µm filter. Store the filter-sterilized solution at 4°C.

Section B: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
No signal or weak signal	Used sample DNA without a biotin label	Make sure the sample DNA for the target of interest is 5' or 3' end-labeled with biotin
	Not enough biotin sample DNA used	Increase the amount of biotin sample DNA used per reaction
	Sample DNA is degraded	Verify the integrity of the sample DNA by spectrophotometry, or visualization on an agarose gel
	Poor transfer to nylon membrane	Follow the transfer protocol and manufacturer's instructions for your transfer unit. If an agarose gel is used, a capillary transfer may perform better. For polyacrylamide gels either capillary or electrical transfers can be used.
	Wrong membrane used	Use a positively charged nylon membrane
	Blot dried out during the detection steps	Make sure the solutions cover the entire membrane during detection and the membrane stays moist
	Poor cross-linking or no cross-link	Check the efficiency of the cross-linker or ensure that the correct wavelength bulb was used for the method of cross-linking
	4X Wash Buffer was not diluted to 1X	Dilute the 4X Wash buffer to 1X in ultrapure water
	Insufficient film exposure	Increase the exposure time
Spots or speckling	Air bubbles	Eliminate air bubbles between the gel and the membrane during transfer. Also eliminate air bubbles when placing the membrane in plastic for detection
	Precipitate in the HRP conjugate	Filter the conjugate through a 0.2 µm filter or centrifuge for 1 minute at maximum speed to pellet the precipitate
High background	Transfer unit or sponges were dirty	Use clean equipment for the transfer. Use sponges that had not previously been used for Western blotting
	Particulate in Blocking or Wash Buffer	Follow the recommendations to warm the solutions to 37-50°C to ensure particulate has dissolved. Do not dilute 4X Wash Buffer until particulate is completely dissolved
No shift detected	System not optimized	Try to optimize the system using the additional reagents provided: glycerol, KCl, $MgCl_2$, NP-40, EDTA
	Extract degraded	Do not thaw Control Nuclear Extract until immediately before use. Use Active Motif's Nuclear Extract Kit to prepare samples or include protease inhibitors during sample preparation
	Not enough extract	If the protein being studied is abundant, 0.25 µg of extract may be sufficient. For less abundant proteins, use 10 µg or more. Some optimization may be required for your sample system
	Disrupted protein:DNA complex by vortexing, mixing or heating	Follow the protocol for the binding reactions. Try running the gel with cold buffer
All DNA shifted	Did not use nonspecific competitor DNA	Include poly d(I-C), poly d(A-T), salmon sperm or *E.coli* DNA as a nonspecific competitor. Add a short pre-incubation time before adding the biotin-labeled sample DNA to the binding reactions

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
E-mail: tech_service@activemotif.com

Active Motif Europe

UK Free Phone: 0800/169 31 47
France Free Phone: 0800/90 99 79
Germany Free Phone: 0800/181 99 10
Direct: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan

Direct: +81 (0)3 5225 3638
Fax: +81 (0)3 5261 8733
E-mail: japantech@activemotif.com

Active Motif China

Direct: (86)-21-20926090
Cell Phone: 18521362870
E-mail: techchina@activemotif.com

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