INSTRUCTIONS

LightShift® Chemiluminescent EMSA Kit

20148

0919.7

Number	Description
20148	**LightShift Chemiluminescent EMSA Kit**, contains components for 100 binding reactions and sufficient detection reagents for approximately 1000cm^2 of membrane

Kit Contents:

LightShift EMSA Optimization and Control Kit (20148X):

10X Binding Buffer, 1mL, 100mM Tris, 500mM KCl, 10mM DTT; pH 7.5, store at -20°C

Biotin–EBNA Control DNA, 50µL, 10fmol/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

The 60 bp biotin end-labeled duplex contains the following binding site:

```
5' BIOTIN-…TAGCATATGCTA…-3'
       3'-…ATCGTATACGAT…-BIOTIN 5'
```

Unlabeled EBNA DNA, 50µL, 2pmol/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

The ~25 bp duplex contains the following binding site:

```
5'-…TAGCATATGCTA…-3'
3'-…ATCGTATACGAT…-5'
```

Epstein-Barr Nuclear Antigen (EBNA) Extract, 125µL, store at -20°C

Poly (dI•dC), 125µL, 1µg/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

50% Glycerol, 500µL, store at -20°C

1% NP-40, 500µL, store at -20°C

1 M KCl, 1mL, store at -20°C

100mM $MgCl_2$, 500µL, store at -20°C

200mM EDTA pH 8.0, 500µL, store at -20°C

5X Loading Buffer, 1mL, store at -20°C

Chemiluminescent Nucleic Acid Detection Module (89880):

Stabilized Streptavidin-Horseradish Peroxidase Conjugate, 1.5mL, store at 4°C

Chemiluminescent Substrate, stable for 6 months at room temperature or 1 year at 4°C

- **Luminol/Enhancer Solution**, 80mL
- **Stable Peroxide Solution**, 80mL

Blocking Buffer, 500mL, store at 4°C

4X Wash Buffer, 500mL, store at 4°C

Substrate Equilibration Buffer, 500mL, store at room temperature or 4°C

Storage: Upon receipt store individual components as indicated above. Box 20148X is shipped with dry ice. Box 89880 is shipped with an ice pack.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

Table of Contents

Introduction

The electrophoretic mobility shift assay (EMSA) has been used extensively for studying DNA-protein interactions.[1-3] This technique is based on the fact that DNA-protein complexes migrate slower than non-bound DNA in a native polyacrylamide or agarose gel, resulting in a "shift" in migration of the labeled DNA band.

The Thermo Scientific LightShift Chemiluminescent EMSA Kit uses a nonisotopic method to detect DNA-protein interactions. Biotin end-labeled DNA containing the binding site of interest is incubated with a nuclear extract or purified factor. This reaction is then subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA is detected using the Streptavidin-Horseradish Peroxidase Conjugate and the Chemiluminescent Substrate.

Additional Materials Required

- Biotin 3' or 5' end-labeled DNA target. Use existing end-biotinylated DNA targets or prepare them using a biotin end-labeling kit (see Related Thermo Scientific Products). Do not use probes with internal biotin labels (i.e., targets biotinylated at sites other than the 3' or 5' end, such results from random prime labeling methods) because the internal labels may inhibit binding of the DNA binding protein.
- Positively charged nylon membrane (see Related Thermo Scientific Products)
- 5X TBE (450mM Tris, 450mM boric acid, 10mM EDTA, pH 8.3)
- X-ray film (see Related Thermo Scientific Products) or CCD camera
- UV lamp or crosslinking device equipped with 254nm bulbs or 312nm transilluminator
- Electrophoresis apparatus
- Electroblotter or capillary transfer apparatus
- High-quality blotting paper
- Circulating water bath
- Plastic forceps
- Polyacrylamide gel in 0.5X TBE

Procedure for Electrophoretic Mobility Shift Assay (EMSA)

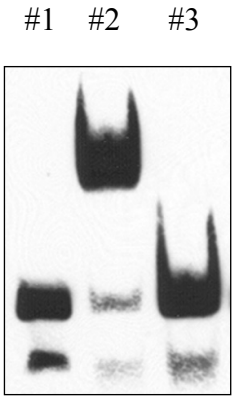
This kit has been optimized for use with polyacrylamide mini (8 × 8 × 0.1cm) gels. For larger gels, adjust electrophoresis conditions and detection reagent volumes accordingly.

A. Plan Binding Reactions

- Understanding the Control EBNA System

Include a complete set of three reactions each time an EMSA is performed. These reactions and expected results for the Control Epstein-Barr nuclear antigen (EBNA) System, which is included with the kit, are described in Table 1.

Table 1. Description of control reactions and expected results.

Reaction	Contents of Reaction	Description	Result
#1	Biotin-EBNA Control DNA	No protein extract for DNA to bind; therefore, no shift is observed. Establishes the position of an unshifted probe band.	
#2	Biotin-EBNA Control DNA + EBNA extract	Contains sufficient target protein to effect binding and shift of the Biotin-EBNA DNA. Shift detected by comparison to band position in #1.	
#3	Biotin-EBNA Control DNA + EBNA extract + 200-fold molar excess of unlabeled EBNA DNA	Demonstrates that the signal shift observed in #2 can be prevented by competition from excess non-labeled DNA, i.e., the shift results from specific protein:DNA interaction.	

The Control EBNA System results reported in Table 1 were generated using binding reactions prepared according to Table 2. Each 20µLbinding reaction contains 20 fmol of Biotin-EBNA Control DNA. Reactions were electrophoresed, transferred and detected according to the steps in Sections B-G of this protocol. If the kit is being used for the first time, perform the Control EBNA System reactions to verify that the kit components and overall procedure are working properly.

Table 2. Binding reactions for Control EBNA System.

Component	Final Amount	Control Reactions		
		#1	#2	#3
Ultrapure Water	----	12µL	11µL	9µL
10X Binding Buffer (20148A)	1X	2µL	2µL	2µL
50% Glycerol (20148F)	2.5%	1µL	1µL	1µL
100mM $MgCl_2$ (20148I)	5mM	1µL	1µL	1µL
1µg/µL Poly (dI•dC) (20148E)	50 ng/µL	1µL	1µL	1µL
1% NP-40 (20148G)	0.05%	1µL	1µL	1µL
Unlabeled EBNA DNA (20148C)	4 pmol	-----	-----	2µL
EBNA Extract (20148D)	1 Unit	-----	1µL	1µL
Biotin–EBNA Control DNA (20148B)	20 fmol	2µL	2µL	2µL
Total Volume	----	20µL	20µL	20µL

- Planning and optimizing the Test System

As with the Control EBNA System, a complete set of three reactions should be performed with the Test System. Use Table 3 as a guide for planning the Test System binding reactions. If specific binding conditions are not already known, use only minimal reaction components; e.g., 10X binding buffer and Poly (dI•dC), together with the biotin-labeled target DNA, protein extract and unlabeled DNA of the Test System.

Nuclear protein extracts prepared using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (see Related Thermo Scientific Products) are an excellent source of target protein. Use 2-3µL of NE-PER® Nuclear Extract per 20µL binding reaction. If a greater volume of NE-PER Extract is required, remove excess salts in the extract by dialyzing into a buffer containing 200mM salt (use a Slide-A-Lyzer® MINI Dialysis Unit; see Related Thermo Scientific Products) before use in the LightShift EMSA Kit.

Optimization of the Test 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] and determining their effects on the shift. Bovine serum albumin and basic peptides have also been shown to enhance some DNA-protein interactions.[8-10] Too much glycerol in the binding reactions may cause vertical streaks along the edges of the lanes.

Poly (dI•dC), which is included in the kit, is the nonspecific competitor DNA of choice for most systems. However if the Test System target DNA sequence is GC-rich, try Poly (dA•dT), sonicated calf thymus, salmon sperm or *Escherichia coli* DNA.

The order of addition of the nuclear extract and biotin-labeled target DNA may affect the specificity of the DNA-protein complexes. Always add the binding reaction components in the order listed in Table 3. To overcome strong nonspecific interactions, a short pre-incubation may be required before adding the biotin-labeled target DNA.

Table 3. Binding reactions for the Test System.

Component	Final Amount	Reaction		
		#1	#2	#3
Ultrapure Water	-----			
10X Binding Buffer (20148A)	1X	2µL	2µL	2µL
1µg/µL Poly (dI•dC) (20148E)	50ng/µL	1µL	1µL	1µL
Optional: 50% Glycerol (20148F)				
Optional: 1% NP-40 (20148G)				
Optional: 1M KCl (20148H)				
Optional: 100mM $MgCl_2$ (20148I)				
Optional: 200mM EDTA (20148J)				
Unlabeled Target DNA	4pmol	-----	-----	
Protein Extract (e.g., 2-3µLNE-PER Reagent extract)	system-dependent	-----		
Biotin End-Labeled Target DNA	20fmol			
Total Volume	----	20µL	20µL	20µL

B. 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. Place the gel in the electrophoresis unit, and clamp it to obtain a seal. 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 minutes. Apply 100V for an 8 × 8 × 0.1cm gel.
3. Proceed to Section C while gel is pre-electophoresing.

C. Prepare and Perform Binding Reactions

Notes:

- Include controls in the assay to ensure the system is working properly (see Procedure, Section A).
- Do not vortex the Control DNA or the EBNA extract.

1. Thaw all binding reaction components, EBNA Control System components and Test System samples, and place them on ice. Do not thaw the EBNA Extract until immediately before use. Thaw the EBNA Extract at room temperature. DO NOT heat the EBNA Extract, which includes thawing in your hand.
2. Prepare complete sets of 20 binding reactions for the Control EBNA System and/or the Test System according to Procedure Section A, Tables 2 and 3; add the reagents in the order listed in the tables. Do not vortex tubes at any time during this procedure.
3. Incubate binding reactions at room temperature for 20 minutes.
4. Add 5µL of 5X Loading Buffer to each 20µL binding reaction, pipetting up and down several times to mix. DO NOT vortex or mix vigorously.

D. Electrophorese Binding Reactions

1. Switch off current to the electrophoresis gel.
2. Flush the wells and then load 20µL of each sample onto the polyacrylamide gel.
3. Switch on current (set to 100V for 8 × 8 × 0.1cm gel) and electrophorese samples until the bromophenol blue dye has migrated approximately 2/3 to 3/4 down the length of the gel. The free biotin-EBNA Control DNA duplex migrates just behind the bromophenol blue in a 6% polyacrylamide gel.

E. Electrophoretic Transfer of Binding Reactions to Nylon Membrane

1. Soak nylon membrane in 0.5X TBE for at least 10 minutes.
2. Sandwich the gel, nylon membrane and blotting paper in a clean electrophoretic transfer unit according the manufacturer's instructions. Use 0.5X TBE cooled to ~10ºC with a circulating water bath. Use very clean forceps and powder-free gloves, and handle the membrane only at the corners.

   Note: Use clean transfer sponges. Avoid using sponges that have been used in Western blots.
3. Transfer at 380mA (~100V) for 30 minutes. Typical transfer times are 30-60 minutes at 380mA using a standard tank transfer apparatus for mini gels (8 × 8 × 0.1cm).
4. When the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. (There should be no dye remaining in the gel.) Allow buffer on the membrane surface to absorb into the membrane. This will only take a minute. Do not let the membrane dry. Immediately proceed to Section F.

F. Crosslink Transferred DNA to Membrane

Three options are available for crosslinking:

- **Option 1**: Crosslink at 120mJ/cm^2 using a commercial UV-light crosslinking instrument equipped with 254nm bulbs (45-60 second exposure using the auto crosslink function).
- **Option 2**: Crosslink at a distance of approximately 0.5 cm from the membrane for 5-10 minutes with a hand-held UV lamp equipped with 254nm bulbs.
- **Option 3**: Crosslink for 10-15 minutes with the membrane face down on a transilluminator equipped with 312nm bulbs.

After the membrane is crosslinked, proceed directly to Section G. Alternatively, the membrane may be stored dry at room temperature for several days. Do not allow the membrane to get wet again until ready to proceed with Section G.

G. Detect Biotin-labeled DNA by Chemiluminescence

The recommended volumes are for an 8 × 10cm membrane. If larger gels are used, adjust volumes in Section G accordingly. Perform all blocking and detection incubations in clean trays or in plastic weigh boats on an orbital shaker.

1. Gently warm the Blocking Buffer and the 4X Wash Buffer to 37-50°C in a water bath until all particulate is dissolved. These buffers may be used between room temperature and 50°C as long as all particulate remains in solution. The Substrate Equilibration Buffer may be used between 4°C and room temperature.
2. To block the membrane add 20mL of Blocking Buffer and incubate for 15 minutes with gentle shaking.
3. Prepare conjugate/blocking buffer solution by adding 66.7µL Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 20mL Blocking Buffer (1:300 dilution).

   Note: This conjugate/blocking buffer solution has been optimized for the Nucleic Acid Detection Module and should not be modified.
4. Decant blocking buffer from the membrane and replace it with the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 minutes with gentle shaking.
5. Prepare 1X wash solution by adding 40mL of 4X Wash Buffer to 120mL of ultrapure water.
6. Transfer membrane to a new container and rinse it briefly with 20mL of 1X wash solution.
7. Wash membrane four times for 5 minutes each in 20mL of 1X wash solution with gentle shaking.
8. Transfer membrane to a new container and add 30mL of Substrate Equilibration Buffer. Incubate membrane for 5 minutes with gentle shaking.
9. Prepare Substrate Working Solution by adding 6mL Luminol/Enhancer Solution to 6mL Stable Peroxide Solution.

   Note: Exposure to the sun or any intense light can harm the Working Solution. Keep the Working Solution in an amber bottle and avoid prolonged exposure to intense light. Short-term exposure to typical laboratory lighting will not harm the Working Solution.
10. Remove membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean container or onto a clean sheet of plastic wrap placed on a flat surface.
11. Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Alternatively, the membrane may be placed DNA side down onto a puddle of the Working Solution. Incubate membrane in the substrate solution for 5 minutes without shaking.
12. Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.
13. Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles.
14. Expose 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 manufacturer's instructions. Exposure time may be adjusted to obtain the desired signal.

Additional Information Available from our Website

- Tech Tip: Anneal complementary pairs of oligonucleotides
- Frequently Asked Questions (FAQ) for the LightShift Chemiluminescent EMSA Kit

Troubleshooting

Problem	Cause	Solution
High background	Particulate in Blocking Buffer or Wash Buffer	Gently warm until no particulate remains
	Contaminants in the TBE	Use high-quality reagents or filter TBE through a 0.2µm filter before use
	The transfer unit or sponges used were dirty	Use clean equipment and sponges that were not previously used for Western blotting
Speckling/spots	Precipitate in HRP conjugate	Filter the conjugate through a 0.2µm filter or centrifuge 1 minute at maximum speed
	Air bubbles	Eliminate bubbles between gel and membrane before transfer
No bands detected/low signal	Used target DNA without a biotin label	Use target DNA with end-labeled biotin
	Not enough biotin target DNA used	Increase target DNA concentration
	Target DNA degraded	Check integrity of target DNA
	Poor transfer to membrane	Check transfer protocol
	Wrong type of membrane used	Biodyne® B positively charged nylon membrane (see Related Thermo Scientific Products)
	Blot dried out during detection steps	Cover membrane completely during incubations
	Did not crosslink/poor crosslinking	Check efficiency of crosslinker
	4X wash buffer not diluted to 1X	Dilute 4X wash buffer to 1X
	Insufficient film exposure	Increase exposure time
No shift detected	Disrupted the complex by vortex mixing or heating	Try running the gel with cold buffer
	Not enough extract	Use more extract
	Extract degraded	Try using protease inhibitors
	System not optimized	Determine effects of additives on the system;[12] for example: KCl, glycerol, NP-40, Mg^{2+}, Zn^{2+}
All DNA shifted to top of gel	Did not use nonspecific competitor DNA	Use a nonspecific competitor DNA such as Poly (dI•dC)

Related Thermo Scientific Products

89818 **Biotin 3' End DNA Labeling Kit**, components for 20 labeling reactions

78833 **NE-PER Nuclear and Cytoplasmic Extraction Reagents**

77016 **Biodyne B Nylon Membrane**, 8cm × 12cm, 0.4µm pore size, 25 sheets per package

34090 **CL-Xposure™ Film (5" × 7" sheets)**, 100 sheets per package

21065 **Pierce® Background Eliminator Kit,** for eliminating background from X-ray film

69550 **Slide-A-Lyzer MINI Dialysis Unit**, 10-100µL capacity, 3.5K MWCO, 50 per package

89880 **Chemiluminescent Nucleic Acid Detection Module**

20158 **LightShift Chemiluminescent RNA EMSA (REMSA) Kit**

Cited References

1. Fried, M. and Crothers, D.M. (1981). Equilibria and kinetics of lac repressor-operator interactions by polyacylamide gel electrophoresis. *Nucl. Acids Res.* **9:**6505-25.
2. Revzin, A. (1989). Gel electrophoresis assays for DNA-protein interactions. *BioTechniques* **7:**346-54.
3. Hendrickson, W. (1985). Protein-DNA interactions studied by the gel electrophoresis-DNA binding assay. *BioTechniques* **3:**198-207.
4. Winston, R.L., *et al.* (1999). Characterization of the DNA binding properties of the bHLH domain of deadpan to single and tandem sites. *Biochemistry* **38:**5138-46.
5. Triplett, B. (1992). Salt-dependent formation of DNA-protein complexes *in vitr*o, as viewed by the gel mobility shift assay. *BioTechniques* **13:**354-5.
6. Szczelkun, M.D. and Connolly, B.A. (1995). Sequence-specific binding of DNA by the EcoRV restriction and modification enzymes with nucleic acid and cofactor analogues. *Biochemistry* **34:**10724-33.
7. Hodgson, J. and Enrietto, P.J. (1995). Constitutive and inducible kappa B binding activities in the cytosol of v-Rel-transformed lymphoid cells. *J. Virol.* **69:**1971-9.
8. Zhang, X.Y., *et al*. (1992). Increasing the activity of affinity-purified DNA binding proteins by adding high concentrations of nonspecific proteins. *Anal. Biochem*. **201:**366-74.
9. Kozmik, Z., *et al*. (1990). Albumin improves formation and detection of some specific protein-DNA complexes in the mobility shift assay. *Nucl. Acids Res*. **18:**2198.
10. Bannister, A. and Kouzarides, T. (1992). Basic peptides enhance protein-DNA interaction *in vitro*. *Nucl. Acids Re*s. **20:**3523.
11. Sambrook, J., *et al.* (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press.
12. Kironmai, K.M., *et al*. (1998). DNA-binding activities of Hop1 protein, a synaptonemal complex component from *Saccharomyces cerevisia*e. *Mol. Cell Biol.* **18:**1424-35.

Product References

Cornelussen, R.N.M., *et al.* (2001). Regulation of prostaglandin A_1-induced heat shock protein expression in isolated cardiomyocytes. *J. Mol. Cell Cardiol*. **33:**1447-54.

Ishida, A., *et al.* (2002). Transforming growth factor-β induces expression of receptor activator of NF-κB ligand in vascular endothelial cells derived from bone. *J. Biol. Chem*. **277(29):**26217-24.

MacLachlan, T.K. and El-Deiry, W.S. (2002). Apoptotic threshold is lowered by p53 transactivation of caspase-6. *PNAS*. **99(14):**9492-7.

Matata, B.M. and Galinanes, M. (2002). Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-κB DNA binding activity. *J. Biol. Chem*. **277(3):**2330-5.

Sauzeau, V., *et al.* (2003). RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. *J. Biol. Chem.* **278(11):**9472-80.

Tarumi, T., *et al.* (2002). Cloning and characterization of the human factor XI gene promoter. *J. Biol. Chem*. **277(21):**18510-16.