INSTRUCTIONS

# LightShift® Chemiluminescent RNA EMSA Kit

## 20158

2179.1

| Number | Description |
|---|---|
| **20158** | **LightShift Chemiluminescent RNA EMSA (REMSA) Kit**, contains components for 100 binding reactions and sufficient detection reagents for approximately 1,000cm$^2$ of membrane |

**Kit Contents:**

**LightShift RNA EMSA Optimization and Control Kit (20158X):**

**RNA and Extract Component (store components at -80°C)**

- **Biotinylated IRE Control RNA,** 35µL, 125nM
  - 5′ – UCCUGCUUCAACAGUGCUUGGACGGAAC – 3′-Biotin
- **Unlabeled IRE Control RNA,** 25µL, 10 µM
  - 5′ – UCCUGCUUCAACAGUGCUUGGACGGAAC – 3′
- **Cytosolic Liver Extract,** 50µL, 2mg/mL
- **tRNA,** 100µL, 10 mg/mL

**REMSA Binding Buffer (10X)**, 1mL, 100mM HEPES (7.3), 200mM KCl, 10mM $MgCl_2$, 10mM DTT; store at -20°C

**Glycerol**, 50%, 500µL, store at -20°C

**KCl**, 2M, 500µL, store at -20°C

**$MgCl_2$**, 1M, 500µL, store at -20°C

**DTT,** lyophilized; reconstitute in 100µL nuclease-free water to 500mM; store at -20°C

**Nuclease-Free Water,** 1.5mL, store at -20°C

**REMSA Loading Buffer (5X)**, 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

**Nucleic Acid Detection Blocking Buffer**, 500mL, store at 4°C

**Wash Buffer (4X)**, 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 20158X is shipped with dry ice. Box 89880 is shipped with an ice pack.

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## Introduction

RNA:protein interactions are crucial for normal cell function and regulation. Measurement of these interactions often requires performing radioactive electrophoretic mobility gel shift assays (EMSA) or 2D protein gel electrophoresis with radiolabeled RNA probes. The Thermo Scientific LightShift Chemiluminescent RNA EMSA Kit contains reagents for performing non-radioactive EMSAs. The kit includes a biotinylated RNA probe (positive control), an unlabeled RNA, cell extract (source of RNA-binding protein), 10X binding buffer and accessory binding buffer components for optimization. The kit also contains reagents for chemiluminescent detection of the biotin-labeled RNA.

To demonstrate a functional EMSA, the LightShift Chemiluminescent RNA EMSA Kit includes the positive control system IRE (iron-responsive element)/IRP (iron-responsive protein), which responds to cellular iron status. This system is ubiquitous, yields a robust bandshift, and uses a cytosolic liver extract (containing abundant IRP protein) with biotinylated RNA in the binding reaction. This reaction is subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled RNA is detected using the Streptavidin Horseradish Peroxidase Conjugate and a highly sensitive chemiluminescent substrate.

## Additional Materials Required

- Biotin-labeled RNA sequence: RNA may be prepared by *in vitro* transcription or synthetically synthesized, incorporating the biotin into the 3′ or 5′-end of the RNA, or using a biotinylated nucleotide (such as biotin-11-UTP) in the *in vitro* transcription reaction
- 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
- Electrophoresis apparatus
- Electroblotter or capillary transfer apparatus
- High-quality blotting paper
- Circulating water bath
- Plastic forceps
- Polyacrylamide gel in 0.5X TBE
- Nuclease-free pipettes, pipette tips, and tubes

## Procedure for Electrophoretic Mobility Shift Assay (EMSA)

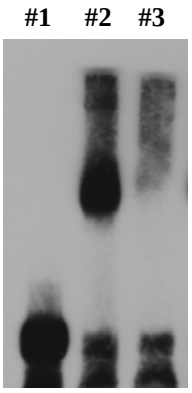
This kit has been optimized for use with polyacrylamide mini (8 × 8 × 0.1cm) gels. For larger gels, adjust electrophoresis conditions and detection reagent volumes accordingly. Use nuclease-free precautions when handling RNA and reagents to prevent RNA degradation.

### A. Plan Binding Reactions

#### Control System

Include a complete set of three reactions each time an EMSA is performed. These reactions and expected results for the Control IRE/IRP 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-IRE Control RNA | No protein extract for RNA to bind; therefore, no shift is observed. Establishes the position of an unshifted probe band. |   |
| #2 | Biotin-IRE Control RNA + Cytosolic liver extract | Contains sufficient target protein to effect binding and shift of the Biotin-IRE RNA. Shift detected by comparison to band position in #1. | |
| #3 | Biotin-IRE Control RNA + Cytosolic liver extract + 200-fold molar excess of unlabeled IRE RNA | Demonstrates that the signal shift observed in #2 can be prevented by competition from excess non-labeled RNA (i.e., the shift results from specific protein:RNA interaction). | |

The Control IRE/IRP System results reported in Table 1 were generated using binding reactions prepared according to Table 2. Each 20µL binding reaction contains 6.25nM of Biotin-IRE Control RNA. 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 IRE/IRP System reactions to verify that the kit components and overall procedure are working properly.

**Table 2. Binding reactions for the Control IRE/IRP System.**

| Component | Final Amount | Control Reactions | | |
|---|---|---|---|---|
| | | #1 | #2 | #3 |
| Nuclease-Free Water (µL) | ---- | 14.8 | 12.8 | 10.8 |
| 10X REMSA Binding Buffer (µL) | 1X | 2 | 2 | 2 |
| 50% Glycerol (µL) | 5% | 2 | 2 | 2 |
| tRNA (10mg/mL) (µL) | 2µg | 0.2 | 0.2 | 0.2 |
| Unlabeled IRE RNA (µL) | 1µM | ----- | ----- | 2 |
| Cytosolic Liver Extract (2mg/mL) (µL) | 4µg | ----- | 2 | 2 |
| Biotin–IRE Control RNA (µL) | 6.25nM | 1 | 1 | 1 |
| **Total Volume (µL)** | ---- | 20 | 20 | 20 |

**Test System**

As with the Control IRE/IRP 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 tRNA, together with the biotin-labeled RNA, protein extract and unlabeled RNA of the Test System).

Optimization of the Test System can be achieved by adding other components supplied in the kit such as KCl, glycerol, $MgCl_2$, DTT and tRNA and determining their effects on the shift. Increasing DTT concentrations might enhance binding, but too much DTT will increase the background. Too much glycerol in the binding reactions can cause vertical streaks along the edges of the lanes. Additionally, heating the RNA for 5-10 minutes at 80°C and then placing it on ice before use in the binding reaction might help relax RNA folding.

tRNA, which is included in the kit, is the nonspecific competitor RNA. The amount of tRNA may be titrated from 0.1 µg to 10 µg per reaction, depending on the RNA:protein interaction. As an alternative, heparin may also be used as a nonspecific competitor.

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

**Table 3. Binding reactions for the Test System.**

| Component | Final Amount | Reaction | | |
|---|---|---|---|---|
| | | #1 | #2 | #3 |
| Ultrapure Water | ----- | | | |
| 10X Binding Buffer (µL) | 1X | 2 | 2 | 2 |
| **Optional**: 50% Glycerol | | | | |
| **Optional**: 500mM DTT | | | | |
| **Optional**: 2M KCl | | | | |
| **Optional**: 1M $MgCl_2$ | | | | |
| **Optional:** tRNA (10 mg/mL) | 0.1-10µg | | | |
| Unlabeled Target RNA | 1-10µM | ----- | ----- | |
| Protein Extract | system-dependent | ----- | | |
| Biotin-labeled Target RNA | 0.5-2nM | | | |
| **Total Volume (µL)** | ---- | 20 | 20 | 20 |

**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 RNA and the binding protein. Most systems use a 4-6% polyacrylamide gel in 0.5X TBE.
2. Fill an electrophoresis unit with 0.5X TBE to just above the bottom of the wells. (This reduces heat generated during electrophoresis.) Flush wells and pre-electrophorese the gel for 30-60 minutes. Apply 100 V for an 8 × 8 × 0.1cm gel.
3. Proceed to Section C while gel is pre-electophoresing.

### C. Prepare and Perform Binding Reactions

**Note**: Include controls in the assay to ensure the system is working properly (see Procedure, Section A). Maintain a nuclease-free environment while performing the binding reactions. Always wear gloves, perform work in a clean area and use nuclease-free tubes, pipette tips and other disposable plasticware for reagent preparations and binding reactions.

1. Thaw all binding reaction components, RNA and extract component, and Test System samples, and place them on ice. Avoid excessive warming of RNA probes and the cytosolic liver extract. Avoid multiple freeze-thaws of RNA and liver extract component by freezing in working aliquots and storing at -80°C after the initial thaw.
2. Prepare complete sets of 20µL binding reactions for the Control IRE/IRP System and/or the Test System according to Procedure Section A, Tables 2 and 3.
3. Incubate binding reactions at room temperature for 20-30 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 100 V 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-IRE Control RNA migrates just above 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 and nylon membrane in a clean electrophoretic transfer unit according the manufacturer's instructions. Use 0.5X TBE cooled to ~10ºC with a circulating water bath. Use 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 400mA (~35V) for 30 minutes. Typical transfer times are 30-45 minutes at 400mA 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.

   **Note:** Transfer may also be performed using a semi-dry transfer apparatus. The Control Reaction was successfully tested using the Thermo Scientific Pierce Fast Semi-Dry Blotter (Product No. 88217) using the Nucleic Acid Transfer Technique.

### F. Crosslink Transferred RNA to Membrane

Two 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 3-5 minutes with a hand-held UV lamp equipped with 254nm bulbs. Excessive exposure to UV will cause degradation of the RNA.

  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 RNA by Chemiluminescence

The indicated 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 Nucleic Acid Detection 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 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 other intense light can harm the Working Solution. For best results keep the Working Solution in an amber bottle and avoid prolonged exposure to any 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 RNA side down onto a puddle of the Substrate 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 1-5 minutes. Develop the film according to manufacturer's instructions. Exposure time may be adjusted to obtain the desired signal.

## Troubleshooting

| Problem | Cause | Solution |
|---|---|---|
| High background | Particulate in Blocking Buffer or Wash Buffer | Gently warm until no particulate remains |
| | Excess free biotin in biotinylated RNA preparation | Remove excess biotin by extracting with chloroform or using a Sephadex® Column |
| | Excess biotin in extract preparation | Remove endogenous biotin using streptavidin agarose (Product No. 20357) to pre-clear extract |
| | Contaminants in the TBE | Use high-quality reagents or filter TBE through a 0.2 µm filter before use |
| | The transfer unit or sponges used were not clean | Use clean equipment and sponges that were not previously used for Western blotting |
| Speckling/spots | Precipitate in HRP conjugate | Filter the conjugate through a 0.2 µm 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 RNA without a biotin label | Use target RNA labeled with biotin |
| | Not enough biotin target RNA used | Increase target RNA concentration |
| | Inefficient biotin incorporation into RNA | Optimize labeling method; test for biotin labeling efficiency before assay. |
| | Target RNA degraded | Check integrity of target RNA |
| | Poor transfer to membrane | Check transfer protocol |
| | Wrong type of membrane used | Use Biodyne® B positively charged nylon membrane |
| | Blot dried out during detection steps | Cover membrane completely during incubations |
| | Did not crosslink/poor crosslinking | Check crosslinking efficiency |
| | 4X wash buffer not diluted | 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; for example: KCl, glycerol, NP-40, $Mg^{2+}$ |
| All RNA shifted to top of gel | Did not use nonspecific competitor RNA | Use a nonspecific competitor RNA such as tRNA or heparin |

## Related Thermo Scientific Products

**20159** **tRNA,** 10 mg/mL, 100µL

**20357** **High Capacity Streptavidin Agarose Resin,** 2mL of settled resin (4mL total volume)

**77016** **Biodyne B Nylon Membrane**, 8 cm × 12 cm, 0.4 µm 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

**89880** **Chemiluminescent Nucleic Acid Detection Module**

**89880A** **Nucleic Acid Detection Blocking Buffer,** 500mL (component of 89880)

**88217** **Pierce Fast Semi-Dry Blotter**

## References


1. Starzynski, R., *et al*. (2004). A characterization of the activities of iron regulatory protein 1 in various farm animal species. *Cell Mol Biol Lett* **9:**651-64.
2. Samaniego, F., *et al*. (1994). Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. *J Biol Chem* **269:**30904-10.
3. Rouault, T., *et al*. (1989). The iron responsive-binding protein: a method for the affinity purification of a regulatory RNA-binding protein. *Proc Natl Acad Sci USA* **86:**5768-72.
4. Coulson, R. and Cleveland, D. (1993). Ferritin synthesis is controlled by iron-dependent translational derepression and by changes in synthesis/transport of nuclear ferritin RNAs. *Proc Natl Acad Sci USA* **90:**7613-7.




Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.