



# **Visualizer™ Western Blot Detection Kit**

## **Instruction Manual**

Catalog #s 64-201SP and 64-202SP

For 250 cm<sup>2</sup> of Membrane,  
5 Mini-Gel-Sized Blots

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## I. Introduction and Principle

The Visualizer™ Western Blot Detection Kit was designed to make Western detection of proteins easier by providing the highest level of sensitivity with the least amount of background in a chemiluminescent reagent system. Western blotting has become one of the most common protein analysis techniques used in biomedical research today. It provides a non-radioactive, direct method for identifying proteins in a mixture as well as determining the relative amount of specific proteins in different samples.

Variations on standard Western blotting techniques are numerous. In general, a complex protein mixture (such as a cell lysate, cell extract or purified protein preparation) is fractionated on a gel (e.g. SDS-PAGE, native PAGE, IEF or 2-D PAGE) by electrophoresis. After separation, proteins are transferred to a polyvinylidene fluoride (PVDF) or nitrocellulose membrane. Proteins on the membrane are probed with antibodies and then identified using detection reagents. The Visualizer™ detection reagent works equally well with both PVDF and nitrocellulose membranes. However, due to PVDF's superior protein binding properties as compared to nitrocellulose, greater sensitivity can be obtained by using PVDF in conjunction with the Visualizer™ detection reagent.

Enzyme-linked detection utilizing secondary antibodies (that can recognize virtually any primary antibody) covalently conjugated to enzymes such as horseradish peroxidase (HRP), provided a significant advance in western blotting. The Visualizer Western blot detection reagents in the presence of HRP will go through a catalyzed reaction that results in the steady and stable emission of luminescence. Imaging is performed on standard X-ray film or by using an image documenting station that is fitted with a chemiluminescent-sensitive camera. This provides the capability of making multiple exposures of a blot, securing valuable data, extending the dynamic range of detection and making quantitation easier and more accurate.

Upstate's Visualizer™ Western Blot Detection Kit utilizes a superior version of the chemiluminescent HRP substrate luminol that results in the fastest and most sensitive detection of an antigen while providing a long lasting signal and the highest signal to noise ratio of any competing product on the market. These kits include the chemiluminescent substrates paired with an HRP-Conjugated Secondary Antibody to either rabbit or mouse IgG. The kit has been carefully designed to provide users with the most convenient and highest quality western blot detection reagents available today. Combined with Upstate's array of primary antibodies, the Visualizer™ Western Blot Detection Kit will provide you with the results your research has always needed.

## II. System Components

### A. Reagents Supplied

**Note:** Catalog # 12-349C is supplied with Kit # 64-201SP, and Catalog # 12-348C is supplied with Kit # 64-202SP.

| Component  | Amount | Conc.  | Storage | Cat. No.  |
|--|--------|--------|---------|-----------|
| <b>Visualizer™ Western<br/>Detection Reagents:</b> |        |        |         |           |
| <i>Detection Reagent A</i>                         | 5ml    | -      | 4°C     | 20-275aSP |
| <i>Detection Reagent B</i>                         | 10ml   | -      | 4°C     | 20-275bSP |
| <b>Secondary Antibody<br/>HRP-Conjugates:</b>      |        |        |         |           |
| Goat anti-Mouse IgG                                | 100µg  | 1µg/µl | COA*    | 12-349C   |
| Goat anti-Rabbit IgG                               | 100µg  | 1µg/µl | COA*    | 12-348C   |

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\* For proper storage conditions, please refer to product Certificate of Analysis (COA).

### B. Materials Required but not Supplied

SDS-PAGE Reagents and Apparatus  
 Western Transfer Reagents and Apparatus  
 PVDF, Nitrocellulose, or Other Membrane  
 Rocker or Shaker  
 Membrane Incubation Containers  
 Forceps  
 Paper Towels or Wipes  
 Primary Antibody  
 Wash Buffer  
 Blocking Buffer  
 X-Ray Film  
 Plastic Wrap (i.e. Saran Wrap)  
 Dark Room or Digital Imaging Station  
 X-Ray Film Development Reagents and Equipment  
 Ponceau Stain (optional)  
 Stripping Buffers (optional)

### C. Preparation of Reagents

Wash Buffer may be water, TBS or PBS. Some users prefer to use water or TBS, but PBS may result in a reduced background. Many blotting procedures utilize PBS or TBS containing Tween®-20, from 0.05% to 0.5%; Tween®-20 may reduce nonspecific background on the membrane, but too much may interfere with primary antibody binding resulting in loss of signal. The optimum Wash Buffer formulation for a particular antibody should be empirically determined.

**PBS - Phosphate-Buffered Saline**

PBS formulations may vary from user to user; one formulation is:

|  | To Prepare 1X Reagent: |                  | To Prepare 10X Reagent: |                  |
|--|------------------------|------------------|-------------------------|------------------|
|  | Amount (g)             | Final Conc. (mM) | Amount (g)              | Final Conc. (mM) |
| KH <sub>2</sub> PO <sub>4</sub>                    | 0.144                  | 1.06             | 1.44                    | 10.6             |
| K <sub>2</sub> HPO <sub>4</sub> •7H <sub>2</sub> O | 0.795                  | 2.96             | 7.95                    | 29.6             |
| NaCl   | 9.00                   | 155              | 90.0                    | 1550             |

Dissolve potassium phosphate and NaCl in 800ml water, adjust pH to 7.4, and then dilute to 1L with water. Autoclave if reagent will be stored for more than a few days.

**TBS - Tris-Buffered Saline**

TBS formulations may vary from user to user; one formulation is:

|           | To Prepare 1X Reagent: |                  | To Prepare 10X Reagent: |                  |
|-----------|------------------------|------------------|-------------------------|------------------|
|           | Amount (g)             | Final Conc. (mM) | Amount (g)              | Final Conc. (mM) |
| Tris Base | 3.03                   | 25               | 30.3                    | 250              |
| NaCl      | 9.00                   | 155              | 90.0                    | 1550             |

Dissolve Tris and NaCl in 800ml water, adjust pH to 7.4, and then dilute to 1L with water. Autoclave if reagent will be stored for more than a few days.

**Tween®-20 Formulations in PBS or TBS**

| Amount of Tween®-20 (ml)             | To 1L of 1X PBS or TBS, add: |      |       |      |
|--------------------------------------|------------------------------|------|-------|------|
|                                      | 0.5                          | 1    | 2.5   | 5    |
| For a final Tween®-20 percentage of: | 0.05%                        | 0.1% | 0.25% | 0.5% |

Blocking Buffer - 3% Blocking Reagent (Catalog # 20-200) in Wash Buffer. Prepare just prior to use. Dissolve 0.9g of Blocking Reagent in 30ml of Wash Buffer.

**Stripping Buffer I**

|                   | Amount            | Final Conc. |
|-------------------|-------------------|-------------|
| Tris              | 3.1ml, (1M stock) | 62.5mM      |
| SDS               | 1.0g              | 2%          |
| β-mercaptoethanol | 0.34ml            | 100mM       |

Dissolve SDS in 40ml water, add the Tris, adjust pH to 6.8, add β-mercaptoethanol, and then dilute to 50ml with water.

**Stripping Buffer II**

|           | Amount | Final Conc. |
|-----------|--------|-------------|
| Glycine   | 1.5g   | 0.4M        |
| SDS       | 0.1g   | 0.2%        |
| Tween®-20 | 1ml    | 2%          |

Dissolve Glycine and SDS in 40ml water, adjust pH to 2.2, add Tween®-20, and then dilute to 50ml with water.

## III. Immunoblotting, Detection, & Miscellaneous Procedures

Please read all instructions thoroughly before proceeding. There are many variations on the general procedure outlined below. Although Upstate endeavors to provide specific instructions for each of our antibodies intended for use in Western blotting, the optimal conditions for use of primary antibodies from other suppliers may need to be empirically determined. If the specific instructions provided with a primary antibody deviate from those outlined below, always default to the specific instructions.

### A. Immunoblotting Procedure

1. Prepare samples and perform SDS-PAGE following instructions provided with your specific electrophoresis system.
2. Transfer the gel to a PVDF (recommended for greatest sensitivity) or nitrocellulose membrane following all instructions provided with your specific blotting device and membrane. Note: PVDF must be pre-soaked with methanol for about 1 minute prior to use.
3. Rinse the membrane twice with Wash Buffer to remove transfer buffer and gel particles. If desired, the membrane may be stained with Ponceau S to visualize protein bands. (i.e., to examine protein loading before proceeding with the detection, see section C on page 8).
4. Incubate the membrane in freshly prepared Blocking Buffer, with agitation, for 20 to 30 minutes at room temperature (or overnight at 4°C).
5. Incubate the membrane 1-2 hours at room temperature (or overnight at 4°C) in the appropriate primary antibody diluted in freshly prepared Blocking Buffer with agitation during the incubation. Dilute as recommended for the particular antibody.
6. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
7. Incubate the membrane for 1 hour at room temperature (or overnight at 4°C) in the appropriate secondary antibody HRP conjugate diluted in freshly prepared Blocking Buffer with agitation during in the incubation. Dilute as recommended for the particular conjugate (see the Certificate of Analysis).
8. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
9. (Optional) Rinse the membrane 4-5 times with water.

## B. Chemiluminescent Detection

1. Prepare Visualizer™ Working Solution prior to use by combining in a 1:2 ratio *Detection Reagent A* and *Detection Reagent B*, respectively. You will need approximately 3ml of Working Solution for a single mini-gel sized blot (50cm<sup>2</sup> of membrane). Mix the solution well and protect from excessive exposure to light. The Visualizer™ Working Solution is stable at room temperature for several hours. *For best results, allow the Visualizer™ Working Solution to warm to room temperature before use.*
2. Transfer the membrane(s) from the last wash to a fresh tray or dish. Weigh boats, pipette tip boxes, or other glass or plastic dishes work well. It is best if the tray or dish is only slightly larger than the membrane and is not made of polystyrene plastic.
3. Pipette the Visualizer™ Working Solution onto the blotted membrane and incubate 5 minutes at room temperature. The membrane should be completely covered with Visualizer™ Working Solution.
4. Remove the membrane(s) from the Visualizer™ Working Solution with forceps (never touch the membrane with ungloved fingers), drain excess Visualizer™ Working Solution, and then gently touch the corner of the membrane to a paper towel or filter paper to remove the last drop. DO NOT allow the membrane to become dry, as the enzyme and substrate require moisture to function.
5. Place the membrane(s) on a firm surface such as a small glass plate and seal it with plastic wrap being careful not to have any air bubbles trapped between neither the plate and membrane nor the membrane and plastic wrap.
6. Develop an image for the Western blot using either a dark room and X-ray film or a digital imaging station.
7. Exposure time may vary from a few seconds to a few minutes (or longer), depending upon the amount of antigen being detected.
8. After all desired images have been obtained; membranes may be stripped and reprobed or stained with Ponceau S and/or dried as desired. Dried membranes may be placed within a clean, safe bag which can then be sealed with most common impulse heat sealers for long term storage. The stored membranes can be rehydrated and then reprobed at a later date.

### C. Optional Procedure - Ponceau Staining

Membranes may be stained with Ponceau S either before Immunoblotting or after Detection. If staining is done before Immunoblotting, the membrane must be destained before proceeding. DO NOT allow the membrane to become dry before Immunoblotting and Detection. If staining is performed after Detection, the membrane may be destained to the point where protein bands are distinct, then dried and sealed within plastic as a permanent record.

1. Incubate the membrane in Ponceau S working solution for 5 - 10 minutes at room temperature with agitation. (10X stock is 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid; dilute 1:10 with water for use; Sigma, Catalog # P7767)
2. Remove the Ponceau S solution (the solution may be saved and reused several times).
3. Rinse in water until protein bands are distinct. Change water as necessary. Destaining will be rapid, requiring only 1-2 minutes. If the Immunodetection is complete, the membrane may be dried for storage at this point.
4. If Immunodetection has yet to be performed, the position of molecular weight markers or lanes may be marked with a pencil at this time.
5. The membrane may be rinsed a few more times with water to remove most of the residual stain from the bands. The last of the stain will be removed during blocking of the membrane (Step A.4)

### D. Optional Procedure - Stripping and Reprobing

Chemiluminescent detection is fully compatible with stripping blots and reprobing with different antibodies. Some antigen may be lost with each strip/reprobe cycle, so it is recommended that detection of antigens expected to be present in the least amounts be performed first before any membrane stripping. There are two commonly used stripping procedures. Neither has a strong advantage or disadvantage; the best method to use for a particular antigen/antibody must be empirically determined. If a membrane becomes dry, it will be difficult to strip. It is imperative that membranes to be stripped are kept moist. Generally, if stripping and reprobing is likely, it is a good idea to put membranes into PBS immediately after the final X-ray film exposure has been obtained.

#### Method # 1: Hot SDS/ $\beta$ -mercaptoethanol

1. Incubate the membrane in Stripping Buffer I (20ml per blot or more) for 30 min at 70°C.
2. Wash the membrane with PBS until the odor of  $\beta$ -mercaptoethanol can no longer be detected.

#### Method #2: Acid Glycine

1. Incubate the membrane in Stripping Buffer II (20ml per blot or more) 2 x 30 min at room temperature.
2. Wash the membrane with 3 x 5 minutes with PBS.  
After either stripping procedure, block the membrane and proceed with detection using a new primary antibody.

## E. Abbreviated Immunoblotting and Detection Procedure for Experienced Users

Note: This abbreviated procedure is provided as a convenience to outline and facilitate completion of the assay. As a step is completed, it may be checked off in the appropriate box using a marker. After completion of the assay, the page can be wiped clean with alcohol.

### Immunoblotting

- 1. Prepare samples and perform SDS-PAGE.
- 2. Transfer the gel to a membrane (PVDF is recommended for greatest sensitivity).
- 3. Rinse the membrane twice with Wash Buffer and stain with Ponceau S if desired (Section III, part C).
- 4. Block the membrane by incubating it for at least 20-30 minutes at room temperature.
- 5. Incubate the membrane 1-2 hours at room temperature (or overnight at 4°C) with the appropriate primary antibody.
- 6. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
- 7. Incubate the membrane for 1 hour at room temperature (or overnight at 4°C) with the appropriate secondary antibody HRP conjugate and dilution.
- 8. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
- 9. Rinse the membrane 4-5 times with water.

### Detection

- 1. Prepare Visualizer™ Working Solution (a 1:2 ratio of *Detection Reagent A* to *Detection Reagent B*) and allow solution to come to room temperature. You will need 3ml Visualizer™ Working Solution per 50cm<sup>2</sup> of membrane.
- 2. Transfer the membrane(s) from the last water rinse to a fresh tray or dish.
- 3. Add Visualizer™ Working Solution and incubate 5 minutes at room temperature.
- 4. Remove the membrane(s) from the Working Solution and drain excess.
- 5. Place the membrane(s) on a glass plate and wrap with plastic (i.e. Saran Wrap).
- 6. In a dark room, expose X-Ray film to the membrane(s) and develop film or use digital imaging station in place of film.
- 7. Strip and reprobe the membrane, if desired.
- 8. Stain the membrane with Ponceau S, dry it, and seal it within plastic, if desired for storage.

## IV. Troubleshooting

The following tips address most problems encountered during western blotting:

### **Smear Pattern or Distorted Bands**

- Uneven contact between gel and membrane: cassettes used should allow a tight fit, leading to even pressure over the entire surface of the gel and membrane.
- For Tris-Glycine gels, gel not equilibrated in buffer prior to transfer: the gel should be soaked in Towbin transfer buffer containing methanol for 15 to 30 minutes before assembling the transfer sandwich. For precast gels, make sure to follow the manufacturer's instructions for gel preparation for transfer.

### **"Bald Spots"**

- Bubbles between gel and membrane: bubbles create areas of low transfer efficiency. Bubbles should be completely removed when putting together the transfer sandwich.

### **Incomplete Transfer**

- Incomplete protein transfer: this often occurs with high molecular weight proteins, especially when using a methanol-based transfer buffer. One way to prevent this is by using a nylon membrane, which does not require methanol in the transfer buffer. Adding SDS to the transfer buffer and using higher field strengths also improve protein transfer.
- Proteins transferred through membrane: this may occur when working with proteins of very low molecular weight. Optimizing/shortening transfer times and using a double layer of membrane usually enhances retention of small proteins. PVDF membranes are superior to nitrocellulose for preventing proteins from going through the membrane.
- Inappropriate transfer buffer used: the most stable and commonly used buffers are Tris-Glycine based (Towbin Transfer Buffer).
- Impurities in the transfer buffer: this will lead to a pattern on the membrane that mirrors the holes in the transfer cassette. Fresh buffer should be prepared for each transfer.
- The wrong transfer buffer was used based on the isoelectric point (pI) of the protein being detected. The pH of the transfer buffer used and the pI of the protein being detected will determine the direction of the protein transfer. If the pH of the buffer and the pI of the protein are near equal, the protein will remain in the gel. If the pH of the buffer is lower than the pI of the protein, the protein will have a net negative charge and migrate towards the positive electrode. And if the pH of the buffer is higher than the pI of the protein, the protein will have a net positive charge and migrate towards the negative electrode. Adding SDS to the buffer is one way to make sure all proteins in the gel have a net negative charge and therefore migrate towards the positive electrode. For pre-cast, neutral pH gels, consult the manufacturers instructions.

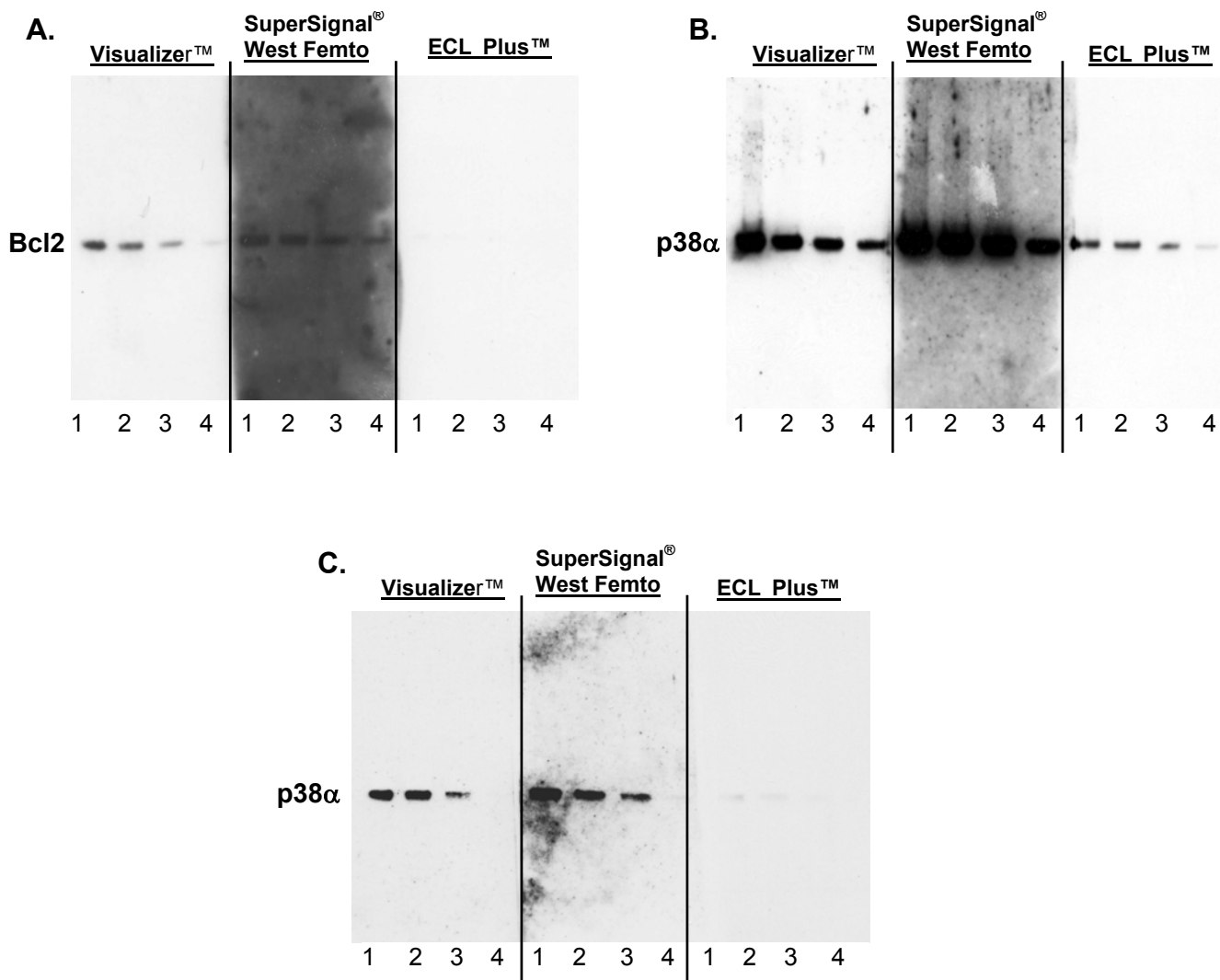
### High Background

- Cross-reactivity between blocking agent and primary antibody: this will result in overall membrane background. Usually, the addition of detergent (Tween®-20) to the Washing Buffer will eliminate the problem. If background persists, changing the blocking agent is recommended.
- Concentration of either primary and/or secondary antibody too high or incubation time too long: the higher the antibody concentration and the longer the incubation time, the greater the non-specific binding. Raising the incubation temperature (e.g. to 37°C) is recommended over lengthening the incubation time. Also, many short washing steps are better than a few long ones.
- Membrane drying during incubation process: care should be taken to keep membrane from drying out during incubation.

### Little or No Signal

- Antigen is not recognized by primary antibody: this can occur especially with monoclonal antibodies that were raised against a native protein. In some cases, a non-reducing gel system may need to be used.
- Inhibition of secondary antibody conjugate: HRP-labeled antibodies should not be used in conjunction with sodium azide or hemoglobin.
- Detergent is too harsh: SDS, Nonidet P-40, and Triton X-100 disrupt binding between proteins. Tween®-20 is the most commonly used and recommended detergent for washing and incubation solutions.

## V. Sample Data



### Western Detection Reagent Product Comparison

Four different quantities of a whole-cell HeLa lysate (Catalog # 12-501) were run on an Invitrogen 4-12% NuPAGE Bis/Tris gel in MES buffer and transferred to a PVDF (Panels A and B) or nitrocellulose (Panel C) membrane. Panels A and B (lane 1: 100ng, lane 2: 75ng, lane 3: 50ng and lane 4: 25ng HeLa lysate). Panel C (lane 1: 1000ng, lane 2: 700ng, lane 3: 400ng and lane 4: 100ng HeLa lysate). The blots were blocked for 1 hour in 5% non-fat milk TBST and then incubated with primary antibody in blocking buffer:

- A.** PVDF with anti-Bcl2 (Catalog # 05-729) at 1μg/ml and incubated overnight at 4°C.
- B.** PVDF with anti-p38α (Catalog #05-454) at 1μg/ml and incubated overnight at 4°C.
- C.** Nitrocellulose with anti-p38α (Catalog #05-454) at 1μg/ml and incubated for one hour at room temperature.

The membranes were washed and then treated with sheep, anti-mouse IgG-HRP at 25ng/ml in blocking buffer at room temperature for 2 hours. After washing, the membranes were then cut into three sections. Each section was placed into its respective western detection reagent for 5 minutes and then brought back together for exposure to film. Panel A. was exposed for 1 minute; Panel B., 2 minutes; Panel C., 5 minutes.

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