

# ReliaBLOT<sup>®</sup>

## Western Blot Kit

10 x 10 cm, 4-12%, 12 well  
Cat No. WB101-41212K  
User Manual



The Polyclonal Antibody Specialists

[www.bethyl.com](http://www.bethyl.com)  
1-800-338-9579

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## Introduction

The ReliaBLOT<sup>®</sup> Western Blot Kit from Bethyl Laboratories conveniently provides the major reagents needed to perform standard western blot assays. The kit provides enough reagents to run five mini-gels and western blot assays. The components provided for SDS-PAGE and gel transfer are compatible with most mini-gel electrophoresis and blotting modules that accept an external gel cassette size of 10 X 10 cm (e.g. Invitrogen XCell Mini-Cell and Blot Module). For western blot, an HRP-conjugated Anti-rabbit secondary antibody is provided for the detection of primary antibodies made in rabbit hosts.

## Kit Components

The ReliaBLOT<sup>®</sup> Kit components, quantities, and storage conditions are as follows:

Component	Quantity	Storage
ReliaBLOT <sup>®</sup> Precast SDS-PAGE Gel Cassettes (4-8%, 4-12%, 4-20% gradient, or 12%, 12-well, 10 X 10 cm)	5 each	2 - 8°C
ReliaBLOT <sup>®</sup> LDS Sample Buffer (4x)	2.0 ml	Room Temperature
ReliaBLOT <sup>®</sup> DTT Reducing Agent	0.15 g	2 - 8°C OR -20°C (after reconstitution)
ReliaBLOT <sup>®</sup> Pre-stained Protein Marker	0.1 ml	-20°C (long-term) OR 2 - 8°C (up to 3 mos.)
ReliaBLOT <sup>®</sup> Running Buffer (20X)	125 ml	Room Temperature
ReliaBLOT <sup>®</sup> Transfer Buffer (10X)	125 ml	Room Temperature
ReliaBLOT <sup>®</sup> Nitrocellulose Membrane/Filter Sandwiches (0.2 µm)	5 each	Room Temperature
ReliaBLOT <sup>®</sup> HRP-conjugated Anti-rabbit Secondary Antibody	0.05 ml	2 - 8°C
ReliaBLOT <sup>®</sup> Chemiluminescent HRP Substrate (A and B)	6.0 ml each	2 - 8°C

## Other Reagents Needed

- Methanol
- Primary Antibodies (made in rabbit hosts)
- Blocking Buffer (e.g. non-fat dried milk or BSA in TBS with Tween)
- Wash Buffer – Tris Buffered Saline with Tween (TBST)
- Ultrapure Water
- Distilled or Deionized Water

## Component Specifications

### ReliaBLOT<sup>®</sup> Precast SDS-PAGE Gel Cassettes

- External size of 10 cm (W) X 10 cm (L) X 1 cm (gel matrix thickness)
- Full length resolving gradient gel (4-8%, 4-12%, 4-20% or 12%)
- Red stacking gel for easy well identification
- High capacity 12-well; load up to 25 µl per well

### ReliaBLOT<sup>®</sup> LDS Sample Buffer (4X)

ReliaBLOT<sup>®</sup> LDS sample buffer (4X) is formulated according to the table below:

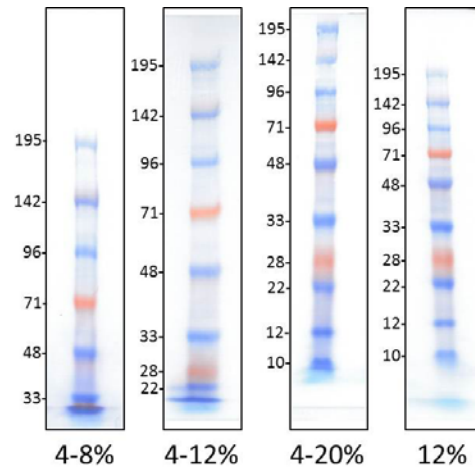
	[4X]
Tris-HCL	424 mM
Tris-base	564 mM
LDS	8 %
Glycerol	40%
EDTA	2.04 mM
Coomassie Brilliant Blue G250	0.88 mM
Phenol Red	0.72 mM

### ReliaBLOT<sup>®</sup> DTT Reducing Agent

- Used as a reducing agent for preparation of protein samples for SDS-PAGE.
- Final concentration after reconstitution is 1.0 M Dithiothreitol (10X solution)

## ReliaBLOT<sup>®</sup> Pre-stained Protein Marker

- 10 recombinant proteins with apparent molecular weights ranging from 7.6 kDa to 195 kDa
- The 28 kDa and 71 kDa markers appear orange for easy identification.
- The marker is ready to load; no need to boil.
- The recommended load volume is 5 – 8  $\mu$ l of the protein marker per well.



The average apparent molecular weights (kDa) for the ReliaBLOT<sup>®</sup> Pre-stained Protein Markers in the ReliaBLOT<sup>®</sup> SDS-PAGE (Tris-Glycine) system are shown. 8  $\mu$ l of ReliaBLOT<sup>®</sup> Pre-stained Protein Marker was loaded on the indicated ReliaBLOT<sup>®</sup> SDS gel, electrophoresed for 1 hour at 150 volts, and transferred to nitrocellulose for 2 hours at 20 volts.

## ReliaBLOT<sup>®</sup> Running Buffer (20X)

ReliaBLOT<sup>®</sup> Running Buffer (20X) is formulated according to the table below:

	20X
Tricine (free base)	0.8 M
Tris (free base)	1.2 M
SDS	2.0%
Sodium Bisulfite	50 mM

## ReliaBLOT<sup>®</sup> Transfer Buffer (10X)

ReliaBLOT<sup>®</sup> Transfer Buffer (10X) is formulated according to the table below:

	10X
Glycine	1.92 M
Tris (free base)	0.25 M
SDS	1%

## ReliaBLOT<sup>®</sup> Nitrocellulose Membrane/Filter Sandwiches

- 100 % nitrocellulose membrane
- Pre-cut and assembled into a membrane and filter sandwich.
- 0.2 um pore size
- 8.3 X 7.3 cm dimensions

## ReliaBLOT<sup>®</sup> HRP-Conjugated Anti-Rabbit Secondary Antibody

- HRP (horseradish peroxidase) conjugated goat immunoglobulin G (IgG) protein (1mg/ml)
- Supplied in phosphate buffered saline (PBS) containing 0.2% BSA and 0.1% Pro-Clean 400
- For use with primary antibodies made in rabbit.
- Reacts specifically with rabbit IgG and with light chains common to other rabbit immunoglobulins.
- Recommended dilutions for western blot and detection by chemiluminescence are in the range of 1:10,000 to 1:20,000.

## ReliaBLOT<sup>®</sup> Chemiluminescent HRP Substrate

- A two-component enhanced chemiluminescent substrate for detecting HRP on immunoblots (components “A” and “B”).
- Working Solution is prepared by mixing equal parts of component “A” and “B”.
- Only 2 ml of Working Solution needed per membrane
- Working Solution is stable for 24 hours at room temperature.
- After incubation of blot with Working Solution, chemiluminescent signal may continue for up to 8 hours but will decrease with time.

# Suggested Protocols for SDS-PAGE and Western Blot

## Buffer and Reagent Preparation

### *ReliaBLOT<sup>®</sup> Running Buffer*

About 300 ml of Running Buffer is needed to run one gel or a pair of gels in a mini-gel system such as Invitrogen's XCell SureLock<sup>®</sup> Mini-Cell.

20X Running Buffer	15 ml
<u>Distilled water</u>	<u>285 ml</u>
Total volume	300 ml

\*Store for up to 1 week at 2-8°C.

### *ReliaBLOT<sup>®</sup> Transfer Buffer*

NOTE: It is recommended to prepare the 1X Transfer Buffer the same day of the transfer. About 250 ml is needed to transfer one gel or a pair of gels in a semi-wet system (e.g. Invitrogen XCell II<sup>™</sup> Blot Module).

10X Transfer Buffer	25 ml
Methanol	50 ml
<u>Distilled Water</u>	<u>175 ml</u>
Total volume	250 ml

\*Chill to 2-8°C before use.

### *ReliaBLOT<sup>®</sup> DTT Reducing Agent*

Reconstitute the DTT by adding 1.0 ml of ultrapure water. Aliquot (100 µl each) into microcentrifuge tubes and store at -20°C.

## ***TBST***

Tris (free base)	6.1 g	[50 mM]
NaCl	8.1g	[138 mM]
Tween-20	500 $\mu$ l	[0.05 %]
Distilled water to	1.0 L	

-Adjust pH to 8.0 with HCL

-Store at 4-25° C.

## ***Blocking Buffer***

NOTE: Blocking buffer should be made fresh and dissolved well.

Carnation non-fat dry milk	2.5 g
TBST	50 ml
Total volume	50 ml

## **Preparation of Protein/Lysate Sample**

For each well, sample volume should not exceed 25  $\mu$ l. The mass of sample required for detection by western blot should be empirically determined.

Typically 10 to 50  $\mu$ g of cell lysate in sample buffer is loaded per well.

1. For each sample, aliquot 10 to 50  $\mu$ g of cell lysate in a sterile microfuge tube (5-25  $\mu$ l).
2. Add 4X LDS sample buffer to the sample to achieve a 1X concentration of LDS sample buffer.
3. Add 10X DTT to achieve a final concentration of 1X DTT.
4. Mix well.
5. Heat samples at 95° C for 5 minutes.
6. Quick spin condensate if needed.
7. Load immediately on ReliaBLOT® SDS-PAGE gel as described below.

## **Electrophoresis in ReliaBLOT® SDS-PAGE Gel**

1. Cut open the package that contains the gel cassette and drain away the buffer.
2. Rinse the wells with distilled water.
3. Place the gels on the buffer core and assemble the electrophoresis cell according to the manufacturer's directions (e.g. Invitrogen X-Cell II™ Blot Module).

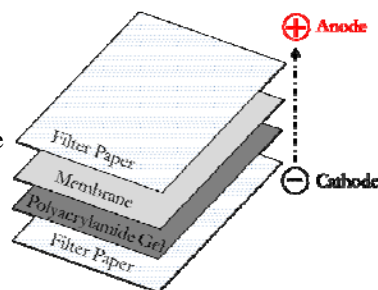


4. Fill the inner core chamber with fresh 1X running buffer to cover the sample wells (about 200 ml). If there are no leaks, fill the outer chamber with the remaining running buffer.
5. Using a pipette (1 ml volume) flush the wells using the 1x running buffer from the inner chamber.
6. Load the prepared samples using a Hamilton syringe or a pipettor fitted with gel loading tips.
7. Run the gels at 150V until the dye front reaches the bottom of the gel (approximately 60 minutes).

## Transfer to Nitrocellulose

1. Reserve and set aside 50 ml of the chilled, freshly prepared 1X Transfer Buffer.
2. Use gloves and forceps to handle the nitrocellulose membranes. For each gel to be transferred, remove and separate a membrane/filter paper sandwich from the blue interleaf paper. Discard the blue interleaf paper.
3. In a shallow tray, pre-wet the nitrocellulose membrane and blotting filter paper in 1X Transfer Buffer for at least 5 minutes.
4. In a shallow tray, soak blotting pads in 1X Transfer Buffer
5. Open the gel cassette by inserting a small metal spatula or gel knife into the gap between the plates and gently twisting the plates apart. The gel will stick to one plate.
6. Note the orientation of the gel and assemble the blotting sandwich as described in the manufacturer's instructions for the blotting module (e.g. Invitrogen X-Cell II™ Blot Module) or according to figure 1.

**Figure 1. Assembly of the Gel Sandwich.** Proteins will migrate toward the anode; therefore, in the sandwich, the membrane should be closest to the anode.



7. Assemble the sandwich into the inner core and place in the buffer chamber.
8. Fill the inner core chamber with the reserved 1X Transfer Buffer.
9. Fill the outer chamber with cold distilled water.
10. Transfer for 2 hours at 20V.
11. When the transfer is complete, remove the membrane from the blotting module/sandwich and place the membrane in a dish of blocking buffer (5% non-fat dry milk in TBST; the buffer should sufficiently cover the membrane).
12. Discard the filter paper.

## **Western Blot**

1. Incubate the membrane in blocking buffer for 1 hour on a rocking platform shaker.
2. Dilute the primary antibody in 15 ml of blocking buffer (5% non-fat dry milk in TBST). For best results, the optimal dilution of antibody should be empirically determined.
3. Pour off the blocking buffer from the membrane and replace with the diluted primary antibody mixture.
4. Incubate the membrane in diluted primary antibody for two hours to overnight with gentle rocking at room temperature.
5. Wash the membrane three times, 10 minutes each time in TBST.
6. Dilute the HRP-conjugated Anti-rabbit Secondary Antibody in 15 ml of 5% non-fat dry milk in TBST. For best results, the optimal concentration of the secondary HRP conjugated antibody should be empirically determined. The recommended range for dilution is 1:10,000 to 1:20,000.
7. Incubate the membrane in diluted HRP-conjugated Anti-rabbit Secondary Antibody for 60 minutes on a rocker platform.
8. Wash as directed in step 5.

## **Development**

1. Make a Working Solution of the Chemiluminescent Substrate by mixing 1 ml of component "A" and 1 ml of component "B". Two mls of Working Solution will be needed for each membrane. Use clean serological pipettes to pipette the components into a 15 ml conical tube.
2. Using forceps remove the membrane from the last wash and blot the edge on a paper towel to remove excess wash buffer, and place the membrane on a clean surface.

3. Pipette 2 ml of the activated substrate solution onto the entire surface of the membrane and incubate at room temperature for 5 minutes.
4. Using forceps lift the membrane and drain off substrate. Blot the edge of the membrane on a paper towel to remove excess substrate.
5. Place the membrane in plastic membrane protector (e.g. plastic film wrap or a page protector). Smooth out bubbles between the membrane and the plastic protector.
6. Expose the membrane to film or a charged-coupled device (CCD) camera. Exposure times will vary in length and will need to be empirically determined.

## Troubleshooting

Problem	Cause	Solution
No Signal	The primary antibody may not be compatible with the secondary antibody provided in the kit.	Primary antibodies must be made in rabbit hosts.
	Primary antibody is too dilute.	<ol style="list-style-type: none"> <li>Increase concentration (lower the dilution) of the primary antibody.</li> <li>Titrate the primary antibody to empirically determine optimal antibody dilution to achieve the best signal/noise ratio.</li> </ol>
	Insufficient binding time	Incubate primary antibody overnight at 2-8°C or room temperature.
	Insufficient antigen	Load at least 20-50 ug of protein.
	The lysate/protein sample is degraded.	<ol style="list-style-type: none"> <li>Store lysates and protein samples at -80 °C</li> <li>Avoid multiple freeze-thawing.</li> <li>Keep protein samples on ice.</li> <li>Check lysate integrity by probing the blot with a control antibody (e.g. anti-actin) or staining the membrane with Ponceau S.</li> </ol>
	Inadequate expression of the protein target in the lysate/sample	<ol style="list-style-type: none"> <li>Use a positive control lysate in which the endogenous target is known to be expressed at relatively abundant levels.</li> <li>Enrich the target by isolating nuclear, membrane, or mitochondrial extracts.</li> <li>Enrich the target by performing an immunoprecipitation.</li> <li>Examine the literature for treatments that may induce endogenous expression of the target.</li> </ol>
	Poor transfer of protein to membrane	<ol style="list-style-type: none"> <li>Check that all of the colored markers of the protein standard have been transferred to the membrane.</li> <li>Monitor transfer efficiency by staining the gel with Coomassie® blue or staining the membrane with Ponceau S.</li> <li>Use only fresh transfer buffer.</li> </ol>

Problem	Cause	Solution
No Signal (continued)	Poor transfer of protein to membrane (continued)	<ol style="list-style-type: none"> <li>4. Small proteins (&gt;20 kDa) may transfer through the membrane. Shorten transfer time or re-evaluate the gel percentage and buffer system used.</li> <li>5. Large proteins (&gt; 200 kDa) may require an overnight transfer at low voltage/ 2-8°C.</li> <li>6. Exceptionally large proteins (&gt;300 kDa) may require the use of a tris-acetate gel and buffer system. Re-evaluate the gel percentage and buffer system used.</li> </ol>
	Target is masked by the blocking solution	<ol style="list-style-type: none"> <li>1. Experiment with alternative blocking buffers (e.g. BSA).</li> <li>2. Lower the percentage of milk (e.g. 1%)</li> <li>3. Block for less time.</li> </ol>
	Chemiluminescent detection	<ol style="list-style-type: none"> <li>1. Confirm that the working solution was made properly.</li> <li>2. Use freshly made working solution.</li> <li>3. Sodium azide is an inhibitor of HRP. Do not use sodium azide as a preservative in buffers.</li> </ol>
High membrane background or "dirty" blot	Insufficient blocking	<ol style="list-style-type: none"> <li>1. 5% non-fat dry milk in TBS or PBS with Tween20 (0.05%) works well to block membrane background.</li> <li>2. Block membranes for at least 1 hr at room temperature.</li> <li>3. Use blocking buffer as the diluent for the primary antibody.</li> <li>4. Ensure good coverage of the membrane with blocking solution during blocking and antibody incubation.</li> <li>5. Ensure that the dried milk is fully dissolved in the blocking buffer</li> <li>6. Blocking buffer should be fresh.</li> </ol>
	Primary antibody	<ol style="list-style-type: none"> <li>1. Decrease the concentration (increase the dilution) of the primary antibody.</li> <li>2. Titrate the primary antibody to empirically determine the</li> </ol>

Problem	Cause	Solution
High membrane background or “dirty” blot (continued)	Primary antibody (continued)	<p>optimal antibody dilution to achieve the best signal/noise ratio.</p> <ol style="list-style-type: none"> <li>3. The nature of some primary antibodies may always result in slight membrane background.</li> <li>4. The stock solution of the primary or secondary antibodies contains aggregates. Microfuge the antibodies at 14,000 X G for 10 minutes at 4°C.</li> </ol>
	Insufficient washing	<ol style="list-style-type: none"> <li>1. After primary and secondary antibody incubation, perform at least three 10-minute washes.</li> <li>2. Include detergent (0.05% Tween20) in the TBS or PBS wash buffer.</li> <li>3. Increase number of washes.</li> </ol>
Multiple bands or “lane background”	Insufficient blocking	<ol style="list-style-type: none"> <li>1. 5% non-fat dry milk in TBS or PBS with Tween20 (0.05%) works well to block non-specific bands and lane background.</li> <li>2. Block membranes for at least 1 hr at room temperature.</li> <li>3. Use blocking buffer as the diluent for the primary antibody.</li> </ol>
	Primary antibody	<ol style="list-style-type: none"> <li>1. Decrease the concentration (increase the dilution) of the primary antibody.</li> <li>2. Titrate the primary antibody to empirically determine the optimal antibody to achieve the best signal/noise ratio..</li> <li>3. Use affinity purified antibody.</li> </ol>
	Secondary antibody	<ol style="list-style-type: none"> <li>1. Decrease the concentration (increase the dilution) of the secondary antibody.</li> <li>2. Incubate secondary antibody for 1 hour.</li> <li>3. Titrate the secondary antibody to empirically determine the optimal antibody dilution to achieve the best signal/noise ratio.</li> </ol>

Problem	Cause	Solution
Multiple bands or “lane background” (continued)	Too much lysate/protein sample loaded into the lane.	Empirically determine the optimal amount of lysate to load per lane to achieve the best signal to noise/ratio.
	The lysate is degraded	<ol style="list-style-type: none"> <li>1. Store lysates and protein samples at -80 °C</li> <li>2. Avoid multiple freeze-thawing.</li> <li>3. Keep protein samples on ice.</li> </ol>
	Cross-reacting proteins	Primary antibodies may cross-react with off-target proteins, even under optimal conditions.
	Modified proteins	The target protein may be present in multiple modified forms (e.g. phosphorylation, ubiquitination, glycosylation) or as different splice variants or isoforms.
	Protein multimers	The protein target may form multimers. Boil samples in SDS or LDS sample buffer before loading.
Ghost bands (reverse/white bands)	Primary antibody concentration too high.	Titrate the primary antibody to empirically determine the optimal antibody dilution to achieve the best signal/noise ratio.
	Secondary antibody concentration too high.	Titrate the secondary antibody to empirically determine the optimal antibody dilution to achieve the best signal/noise ratio.
Uneven bands or “smiling” bands across gel	Gel was run too hot or too fast.	<ol style="list-style-type: none"> <li>1. Run the gel in the cold room or on ice.</li> <li>2. Slow down the run by lowering the voltage.</li> </ol>

## Warranty

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to six months from date of sale. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc.

## Related Products

Description	Size	Catalog No.
ReliaBLOT® Western Blot Kit 4-8%, 10 x 10 cm	1 kit	WB101-40812K
ReliaBLOT® Western Blot Kit 4-12%, 10 x 10 cm	1 kit	WB101-41212K
ReliaBLOT® Western Blot Kit 4-20%, 10 x 10 cm	1 kit	WB101-42012K
ReliaBLOT® Western Blot Kit 12%, 10 x 10 cm	1 kit	WB101-01212K
ReliaBLOT® Western Blot Kit 4-8%, 10 x 8 cm	1 kit	WB102-40812K
ReliaBLOT® Western Blot Kit 4-12%, 10 x 8 cm	1 kit	WB102-41212K
ReliaBLOT® Western Blot Kit 4-20%, 10 x 8 cm	1 kit	WB102-42012K
ReliaBLOT® Western Blot Kit 12%, 10 x 8 cm	1 kit	WB102-01212K
ReliaBLOT® SDS-PAGE Gels 4-8%, 10 x 10 cm	10 gels	WB101-40812G
ReliaBLOT® SDS-PAGE Gels 4-12%, 10 x 10 cm	10 gels	WB101-01212G
ReliaBLOT® SDS-PAGE Gels 4-20%, 10 x 10 cm	10 gels	WB101-42012G
ReliaBLOT® SDS-PAGE Gels 12%, 10 x 10 cm	10 gels	WB101-41212G
ReliaBLOT® SDS-PAGE Gels 4-8%, 10 x 8 cm	10 gels	WB102-40812G
ReliaBLOT® SDS-PAGE Gels 4-12%, 10 x 8 cm	10 gels	WB102-01212G
ReliaBLOT® SDS-PAGE Gels 4-20%, 10 x 8 cm	10 gels	WB102-42012G
ReliaBLOT® SDS-PAGE Gels 12%, 10 x 8 cm	10 gels	WB102-41212G
ReliaBLOT® LDS Buffer (4X)	10 ml	WB104-10
ReliaBLOT® Running Buffer (20X)	500 ml	WB105-500
ReliaBLOT® Transfer Buffer (10X)	500 ml	WB106-500
ReliaBLOT® Nitrocellulose Membrane Filter Sandwiches (7.5 x 8.3 cm)	20/pk	WB107-20
ReliaBLOT® DTT Reducing Agent 10X	1 ml	WB108
ReliaBLOT® Prestained Protein Marker	600 ul	WB103-600
ReliaBLOT® Chemiluminescent HRP Substrate	110 ml	WB110
ReliaBLOT® IP/Western Blot Reagents	20 blots	WB120
Goat anti-Rabbit IgG-h+I HRP	1 ml at 1 mg/ml	A120-101P
Goat anti-Mouse IgG-h+I HRP	1 ml at 1 mg/ml	A90-116P