ReliaBLOT®

IP/Western Blot Reagents
Cat. No. WB120T

Introduction:

ReliaBLOT® IP/Western Blot Reagents and Procedures (patent pending) provide an improved method for the detection of immunoprecipitated proteins assayed via Western Blot.

IMPORTANT

Rabbit Antibodies For use with antibodies raised in rabbits.

Reduction For optimal results, complete reduction of the sample is required. We recommend the use of 0.1 M DTT in SDS-PAGE sample buffer and immediately heating samples and loading and running gels.

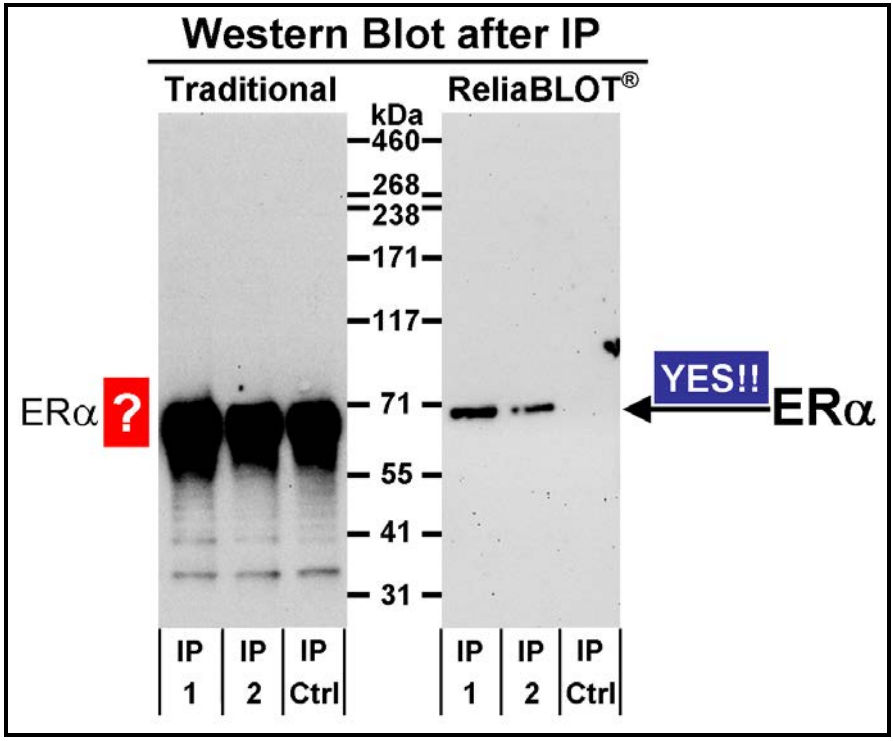
Heavy Chain When the ReliaBLOT® Reagents and Procedures are properly used, background from heavy chain should be reduced to less than 1% of that seen with standard procedures.

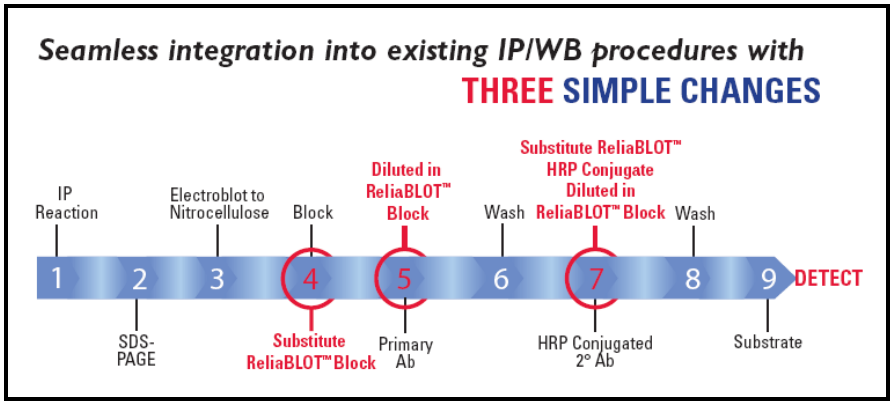
Sensitivity Sensitivity is correlated to the concentration of primary antibody used in the western blot. When performing western blots on immunoprecipitated samples, it is recommended to use the primary antibody 10-25 times more concentrated than that used in western blots on lysates. For example an antibody used at a dilution of 1:5000 in western blots on lysates might be used at a dilution of 1:500 in western blots on immunoprecipitated samples.

Reagents Both the ReliaBLOT® Block and the ReliaBLOT® HRP Conjugate must be used.

The product is sold under a Limited Label License Agreement. See back page for details.

Bethyl Laboratories, Inc., www.bethyl.com
Phone: 800-338-9579 • Fax: 866-597-6105


Western Blot after IP
Traditional
ReliaBLOT®
kDa
460
268
238
171
117
71
55
41
31
ERα ?
YES!!
ERα
IP 1
IP 2
IP Ctrl
IP 1
IP 2
IP Ctrl


Seamless integration into existing IP/WB procedures with
THREE SIMPLE CHANGES
IP Reaction
Electroblot to Nitrocellulose
Block
Diluted in ReliaBLOT™ Block
Wash
Substitute ReliaBLOT™ HRP Conjugate Diluted in ReliaBLOT™ Block
Wash
1
2
3
4
5
6
7
8
9
DETECT
SDS-PAGE
Substitute ReliaBLOT™ Block
Primary Ab
HRP Conjugated 2° Ab
Substrate

Reagents supplied:

- Five 50 ml conical tubes containing dry ReliaBLOT® Block
- ReliaBLOT® HRP Conjugate
- Packet of powder to make 1 liter of TBST (Tris-buffered Saline with Tween-20)

For In vitro laboratory use only. Not for any clinical, therapeutic, or diagnostic use in humans or animals. Not for animal or human consumption.

Overview:

Your standard protocol for Immunoprecipitation will work with **ReliaBLOT®**. For Western Blotting, use **ReliaBLOT®** Reagents as outlined below and detailed in the subsequent section titled Western Blotting.

Prepare cell lysate

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Perform immunoprecipitation reaction

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SDS PAGE and Transfer

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Reconstitute **ReliaBLOT® Block** - 50 ml TBST added 30 minutes prior to use

↓

Block 1 hour in 20 ml **ReliaBLOT® Block**

↓

Incubate membrane in rabbit primary antibody
diluted in 15 ml reconstituted **ReliaBLOT® Block**
2 hours to overnight

↓

Wash membrane 3 times in TBST

↓

Incubate membrane with **ReliaBLOT® HRP Conjugate**
diluted in 15 ml reconstituted **ReliaBLOT® Block**
1 hour

↓

Wash membrane 3 times with TBST

↓

Develop and Expose

Suggested Protocols:

(Formulations for suggested buffers are included in a subsequent section)

Preparation of Cell Lysate used for Immunoprecipitation:

1. Allow cells to grow to approximately 80% confluence on 100mm polystyrene tissue culture plates. Approximately $8*10^6$ cells (approximately one plate at 80% confluence) are needed per IP reaction.
2. Wash cells two times in **cold** PBS.
3. Lyse cells to release soluble cellular proteins using 500 mcl of cold lysis buffer containing 1X protease inhibitors (Calbiochem, Cat. No. 539131) per 100 mm plate. Scrape cells from the plates, transfer to 1.5 ml micro-centrifuge tubes, and place on ice for 30 minutes to assure efficient lysis.
4. Centrifuge (10,000 x g; 5 minutes) lysates.
5. Remove and pool the supernatant. Determine protein concentration.

Immunoprecipitation Reaction:

1. Place 500 mcl of the pooled cell lysate (1-3mg/ml) into a 1.5 ml micro-centrifuge tube.
2. To this tube add 2 to 10 mcg of the primary antibody (If using neat sera or an IgG fraction such as Protein-A purified antibody, larger amounts are likely to be required. For best results, optimal amounts of antibody should be empirically defined.)
3. To a negative control reaction, add an equivalent amount of normal IgG from the species of the animal in which the primary antibody was raised. For example, if your antibody was made in rabbit, your negative control should be normal rabbit IgG (Bethyl, Cat. No. P120-101).
4. Add 100 mcl of a 20% Protein A suspension. (Amersham Biosciences, Cat# 17-0780-01) to the mixture of antibody and cell lysate. Rotate the immunoprecipitation reactions (end-to-end) for 3 hours to overnight at 4^o C.
5. Centrifuge (200 x g; 5 minutes) to pellet the complex.
6. Remove the supernatant and add 500 mcl cold cell lysis buffer without protease inhibitors. Centrifuge (200 x g; 5 minutes).
7. Repeat wash step 6 twice more. After each centrifugation remove as much of the supernatant as possible.
8. After removing the supernatant from the third wash, add 40 mcl of freshly prepared 1X sample buffer to each tube and heat at 90^0 C for 5 minutes.
9. Load 8 to 16 mcl (20 to 40% of the IP reaction) to a polyacrylamide gel.

Note: For optimal results, complete reduction of the sample is required. We recommend the use of 0.1 M DTT in SDS-PAGE sample buffer and immediately heating samples, loading and running gels.

Electrophoresis in SDS-Polyacrylamide Gel and Transfer to Nitrocellulose:

Many of the reagents for these procedures are commercially available. Sources that are preferred by Bethyl Laboratories, Inc. are:
Polyacrylamide gels (4-12% Tricine), running buffer and transfer buffer from PAGEgel (San Diego, CA).
SeeBlue2 and HiMark molecular weight markers - Invitrogen (Carlsbad, CA).
Nitrocellulose membranes - Invitrogen (Carlsbad,CA).

1. Cut open the package that contains the gel cassette and drain away the buffer.
2. Rinse the wells with distilled water.
3. Rinse the wells with fresh running buffer.
4. Place the gels on the buffer core so that the shorter plates face inward. If only using one gel, use a buffer dam to seal the other side.
5. Fill the inner core with fresh running buffer. If there are no leaks, fill the outer core with running buffer. Load samples.
6. Run the gels at 150V until the dye front reaches the bottom of the gel (approximately 60 minutes).
7. Soak nitrocellulose membrane and blotting paper in transfer buffer for at least 5 minutes prior to opening gel cassette.
8. Open gel cassettes and place the gel on the nitrocellulose membrane sandwiched between two pieces of blotting paper.
9. Place in transfer apparatus and fill with fresh transfer buffer.
10. Run transfer apparatus for 60-75 minutes on 35V.

Western Blotting:

1. Prepare 1 L of TBST by adding the contents of the foil package of dry TBST powder to 1L Ultrapure H2O. Store unused portion at 4° C.
2. Reconstitute the **ReliaBLOT® Block** by adding 50 ml of TBST to one conical tube containing powdered **ReliaBLOT® Block**. This should be done approximately 30 minutes prior to use to ensure the powder dissolves completely. *The resulting solution should be sufficient for one Western Blot (20 ml for step 3, and 15 ml each for steps 4 and 7 below).*
3. Remove the membrane from the transfer apparatus and place in 20 ml of reconstituted **ReliaBLOT® Block** for one hour, with gentle shaking.
4. Dilute the primary antibody in 15 ml of reconstituted ReliaBLOT® Block. When using affinity purified antibodies, the recommended dilution for blotting immunoprecipitates is 1 mcg/ml. (If using neat sera or an IgG fraction such as Protein-A purified antibody, higher concentrations are likely to be required. For best results, the optimal concentration of antibody should be empirically defined.)
5. Incubate the membrane in diluted primary antibody for two hours to overnight with gentle shaking at room temperature.

6. Wash the membrane three times, 10 minutes each time in TBST. *(It is recommended that the user prepare the TBST used for washing the membrane. The amount of TBST supplied with ReliaBLOT® is an amount sufficient to reconstitute the ReliaBLOT® Block. There is not excess to be used for washing.)*
7. Dilute the **ReliaBLOT® HRP Conjugate** in 15 ml of reconstituted **ReliaBLOT® Block**. It is recommended that the ReliaBLOT® HRP Conjugate be used at a dilution of 1:10,000 to 1:50,000. For best results, the optimal concentration of ReliaBLOT® HRP Conjugate should be empirically defined.
8. Incubate the membrane in diluted **ReliaBLOT® HRP Conjugate** for 60 minutes.
9. Wash as directed in step 6.
10. Develop blots with substrate solution and place in plastic membrane protector.
11. Expose membrane to film or CCD camera.

Formulations for Buffers

PBS (Phosphate Buffered Saline, pH 7.2)

NaCl	8.0 g
KCl	0.2 g
NaH_2PO_4	0.23 g
$Na_2H\,PO_4$	0.12 g

Adjust the volume to 1 liter with distilled water.

Store at 4-25^{0}C.

Packets for 1 liter of PBS are available from Sigma (Cat# P3813)

TBST (Tris Buffered Saline with Tween 20, pH8.0)

Tris	6.1 g
NaCl	8.68 g
Tween-20	500 mcl

Adjust the volume to 1 liter with distilled water. Adjust pH to 8.0 with HCL.

Store at 4-25^{0}C.

Packets for 1 liter of TBST are available from Sigma (Cat# T-9039)

IP Lysis Buffer

NaCl	7.31 g
1 M Tris, pH 8	25 ml
0.5 M EDTA, pH 8	5 ml
10% NP-40	25 ml
Distilled Water	445 ml

Store at 4^{0}C.

Protein -A Agarose Beads

Protein A Sepharose Beads are available from Amersham (Cat# 17-0780-01). To prepare the beads, swell 400 mg of beads in 10 ml of distilled water for 30 minutes. Centrifuge at 200 x g for 60 seconds. Remove the supernatant and replace with 10 ml cell lysis buffer. Centrifuge as above, remove the buffer and replace with 10 ml cell lysis buffer. Repeat this step three times, for a total of 4 washes with cell lysis buffer. After the last wash add 8 ml of cell lysis buffer. The resulting slurry an approximately 20% slurry of beads. Store at 4°C for one month. **Discard after one month.**

4X Sample Buffer

Glycerol	4.0 g
Tris Base	0.68 g
Tris HCL	0.67 g
LDS	0.80 g
EDTA	6 mg
Brilliant Blue G250	2.5 mg
Phenol red	2.5 mg

Adjust volume to 10 ml with ultra pure water.

Store at 4^0C.

4X sample buffer is available from Invitrogen (Cat# NP0007)

DTT Reducing Agent

DTT (Sigma Cat# D-0632)	1.54 g
Ultra pure water	10 ml

Aliquot and **store** at -20°C. At time of use, thaw an aliquot. **Discard excess. Do not refreeze.**

1X Sample Buffer

4X sample buffer	150 mcl
1M DTT	60 mcl
Distilled water	390 mcl

Make **fresh** for each use.

10X Transfer Buffer

Tris (free base)	15.2 g
Glycine	72.1 g
SDS	5.0 g

Ultra pure water to 500 ml

10X Transfer Buffer is available from PAGEgels (Cat# CB82500)

Store at 4^0C.

1X Transfer Buffer

10X Transfer Buffer	50 ml
Methanol	100 ml
Distilled water	350 ml

Make fresh for each use.

20X Running Buffer

Tricine (free base)	71.7 g
Tris (free base)	72.6 g
SDS	10.0 g
Sodium Bisulfite	2.5 g

Adjust to 500 ml with ultra pure water.

Store at 4^0C. For 1X Running Buffer, add 10 ml of 20X Running Buffer to 190 ml of distilled water.

20X Running Buffer is available from PAGEgels (Cat#CB60500)

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Rev 160125

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