VISUAL PROTEIN

Manual

Western Blot Tool Box

BOX12/BOX12-03/BOX12-05

V3.0

Store at 2-8 °C
For Research Use Only

Introduction

The Western Blot Tool Box is designed to conveniently provide reagents/buffers needed for Western blotting, from cell lysis, protein quantification, protein separation, protein transfer, antibody incubation, and chemiluminescent detection. All items on this all-in-one box are available individually.

Product Components

Prodcut Name	Cat. No.	BOX12	BOX12-03	BOX12-05	Content	Working Reactions
RIPA Cell Lysis Buffer (5X)[1]	RP05-10	●	●	●	10 mL	50 runs of cell lysis
Dual-Range™ BCA Protein Assay Kit	BC03-100	●	●	●	100 mL Reagent A + 12 mL Reagent B + 1 mL BSA (2 mg/mL)	50 tube assays or 500 microplate assays
SDS-PAGE Running Buffer[2]	RB500	●	●	●	10 Powder Packs	10 runs of mini-gels
Western Blot Transfer Buffer[3]	WTB500	●	●	●	10 Powder Packs	10 runs of mini-gels transfer
BlockPRO™ Protein-Free Blocking Buffer (20X)	BF20-50	●	●	●	10 Packs	10 blots
TBS Tween-20 Buffer (25X)[4]	TBST200	●	●	●	10 Packs	10 blots
LuminolPen™, HRP System	LH03-10	–	●	–	1 Pen	100 membrane use
LuminolPen™ EZ, HRP System	LH05-10	–	–	●	1 Pen	100 membrane use
LumiFlash™ Ultima Chemiluminescent Substarte , HRP System	LF08-100	●	●	●	50 mL Solution A + 50 mL Solution B	50-100 blots (0.1 mL/cm²)

1. RIPA Cell Lysis Buffer (1X): 25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS
2. SDS Running Buffer (1X): 25mM Tris, 192mM Glycine, 0.1% SDS, pH8.3
3. Western Blot Transfer Buffer (1X): 25mM Tris, 192mM Glycine, pH8.3
4. TBS Tween-20 Buffer (1X): 25mM Tris, 150mM NaCl, 0.05% Tween-20, pH7.4

Safety Information

Please wear gloves, lab coat and goggles while operating. Prevent contact product directly. In case of contacting, wash with large amount of water.

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Storage

Store **Western Blot Tool Box** at 2-8 °C or follow the recommended storage temperature on the label of each component. The Expiration date is noted on the component label.

Materials needed but not provided

1. Centrifuge
2. Microcentrifuge tubes
3. PBS (Phosphate Buffered Saline) wash buffer
4. Protease or Phosphatase Inhibitor Cocktails (if desired)
5. Cell scraper
6. 96 well plate
7. Water bath
8. Plate Reader capable of measuring absorbance in the region of 560 nm
9. SDS-PAGE gel
10. Electrophoresis Unit & Power Supply Unit
11. Either nitrocellulose or PVDF
12. Western blot transfer tank
13. Filter paper (e.g. Whatman #50)
14. Specific primary antibody for interested protein, diluted in blocking buffer
15. HRP-conjugated secondary antibody, specific for primary antibody, diluted in blocking buffer
16. X-ray film or chemiluminescence image acquisition systems

Instruction

A. Sample Lysis (based on a typical adherent cell culture condition)

> **NOTE:** Reconstitute one bottle of **RIPA Cell Lysis Buffer (RP05-10)** with 40 mL ddH_2O to make 1X RIPA Cell Lysis Buffer. If desired, dilute the Protease Inhibitor Cocktails in recommended ratio with 1X RIPA Cell Lysis Buffer immediately before applying to cells.

1. Remove culture media from the cells by decantation or aspiration.
2. Carefully wash cells twice with a volume of cold PBS equal to that of the culture media removed.

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A. Sample Lysis (based on a typical adherent cell culture condition) (~continued)

3. After removal of the final wash solution from the cells, add an appropriate volume of RIPA Buffer (1 mL for 0.5-5 x 10^7 cells). Incubate on ice or in a refrigerator (2-8 °C) for 5-15 minutes.
4. Use cell scraper to scrape off cells. Pass the cell lysate through pipette several times to form a homogeneous lysate and transfer the lysate to ice-cold 1.5 mL microcentrifuge tube in an ice bucket.
5. Centrifuge the lysate at 14,000 x g for 15 minutes at 4 °C to pellet the cell debris.
6. Transfer supernatant to a clean tube for further analysis.
7. If necessary, aliquot the protein samples for long-term storage at -20 °C. Repeated freeze and thaw cycles cause protein degradation and it should be avoided.

B. Protein Quantitation (based on the Standard Protocol of Microplate Procedure)

NOTE: Prepare Working Reagent of **Dual-Range™ BCA Protein Assay Kit (BC03-100)** by mixing 50 parts of Reagent A and 1 part of Reagent B. 200 µL of Working Reagent is required for each sample in the Microplate Procedure.

1. Preparation of diluted protein standards: prepare a set protein standards.

Table 1. Preparation of Diluted Albumin (BSA) Standards for Microplate Procedure (working range: 20-2,000 µg/mL)

Tube	Volume of Diluent (µL)	Volume and source of protein Standards (µL)	Final BSA Standard Concentration (µg/mL)
A	0	60 of Stock	2,000
B	40	80 of Stock	1,500
C	60	60 of Stock	1,000
D	60	60 of tube B dilution	750
E	60	60 of tube C dilution	500
F	60	60 of tube E dilution	250
G	60	60 of tube F dilution	125
H	240	60 of tube G dilution	25
I	60	0	0

2. Add 200 µL of the Working Reagent to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37 °C for 30 minutes.
4. Cool plate to room temperature.
5. Measure the absorbance at or near 562 nm on a plate reader.
6. Prepare a standard curve of BSA by plotting the average blank-corrected 562 nm measurement and determine the protein concentration of each unknown sample by using the standard curve.

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C. Protein Separation

> **NOTE:** Reconstitute one pack of **SDS-PAGE Running Buffer (RB500)** with 500 mL ddH_2O to make 1X running buffer.

1. Take 20-30 µg of each sample. Dilute 5 parts sample with 1 part of **6X Laemmli Sample Buffer (SBR06-15)**.
2. Boil each cell lysate in sample buffer at 95 °C for 5 minutes and centrifuge at 16,000 × g in a microcentrifuge tube for 1 minute.
3. Fill the upper and lower buffer chamber with 1X running buffer.
4. Load equal amounts of protein into the wells of a mini or midi format SDS-PAGE gel, along with 3-5 µL of **VisColor™ Pre-Stained Protein Marker (VC01-250)**
5. Run the gel for 5 minutes at 50 V.
6. Increase the voltage to 100–150 V to finish the run in about 1 hour.

Table 2. The gel percentage required is dependent on the size of your protein of interest

Protein Size	Gel Percentage
4-40 kDa	20%
10-45 kDa	15%
12-70 kDa	12.5%
15-100 kDa	10%
25-100 kDa	8%

D. Protein Transfer

> **NOTE:** Reconstitute one pack of **Western Blot Transfer Buffer (WTB500)** with 500 mL ddH_2O (or 400 mL ddH_2O + 100 mL MeOH for PVDF) to make 1X transfer buffer.

1. Move the electrophoretic gel into appropriate 1X transfer buffer and equilibrate for 10 minutes.
2. Wet the PVDF or nitrocellulose membrane in 1X transfer buffer (pre-wet the PVDF membrane in methanol prior to use).
3. Assemble the transferring sandwich as the order of two filter papers, gel, membrane and two filter papers. For wet transfer, the gel side of the cassette holder should face the cathode (-) while the membrane side should face the anode (+). For semi-dry transfer, the gel side should face the cathode plate (-), while the membrane side should face the anode plate (+).
4. Transfer proteins according to blotting apparatus manufacturer's instruction.

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E. Antibody incubation

NOTE: Reconstitute one pack of **BlockPRO™ Protein-Free Blocking buffer (BF20-50)** with 50 mL ddH_2O to make 1X blocking buffer and reconstitute one pack of **TBS Tween-20 Buffer (TBST200)** with 200 mL ddH_2O to make 1X wash buffer.

1. For taking notes on the membrane, please remove excess buffer from the membrane with filter paper. Keep the membrane wet and do not let the membrane over-dry.
2. Use **LuminolPen™, HRP System (LH03-10)** to mark the pre-stained ladder on membrane before blocking (one drawing should be enough for delivering strong signals.
3. Block the membrane at room temperature for 1 hour with 1X blocking buffer.
4. Incubate the membrane with appropriate dilutions of primary antibody in 1X blocking buffer at 4°C overnight.
5. Wash the membrane three times for 5 minutes with 1X wash buffer.
6. Incubate the membrane with the recommended dilutions of conjugated secondary antibody in 1X blocking buffer at room temperature for 1 hour.
7. Wash the membrane three times for 5 minutes with 1X wash buffer.

F. Chemiluminescent detection

NOTE: Prepare HRP working substrate of **LumiFlash™ Serires Chemiluminescent Substrate (LF01/LF08/LF16)** by mixing equal volume of Solution A and Solution B in a clean tube freshly. 0.1 mL of HRP working substrate is sufficient per 1 cm^2 membrane area.

1. Before performing the ECL development (adding ECL substrates), gently remove the residual solution from the PVDF or nitrocellulose membrane by using filter paper. Keep the membrane wet and don't let the membrane over-dry.
2. For taking notes on the membrane, use **LuminolPen™ EZ, HRP System (LH05-10)** to mark the pre-stained ladder on membrane before blocking (one drawing should be enough for delivering strong signals.
3. In the dark room or box, place the protein side up in a clean box or plastic wrap. Add HRP working substrate onto the membrane.
4. Incubate the membrane at room temperature for 10 seconds.
5. Overlay plastic wrap or a transparency sheet on the wet membrane.
6. Expose the membrane to appropriate X-ray film or by chemiluminescent image acquisition system. It is recommended to use 1 minute as the initial exposure time.

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Related Visual Protein Products

6X Laemmli SDS Sample Buffer, non-reducing	SBN06-15	15 mL
6X Laemmli SDS Sample Buffer, reducing	SBR06-15	15 mL
BlockPRO™ 1 Min Protein-Free Blocking Buffer	BM01-500	500 mL
BlockPRO™ Blocking Buffer	BP01-1L	1 L
Dual-Range™ Bradford Protein Assay Kit	BR05-500-K	1 kit
ExtractPRO™ Protein Extraction Reagent	EP05-30	30 mL
LumiFlash™ Prime Chemiluminescent Substrate	LF01-500	500 mL
LumiFlash™ Ultima Chemiluminescent Substrate	LF08-500	500 mL
LumiFlash™ Infinity Chemiluminescent Substrate	LF16-500	500 mL
StripPRO™ 1 Min Stripping Buffer	SP01-500	500 mL
PBS Buffer (10X)	PBS10-1L	1 L
PBS Buffer (25X), 30 packs	PBS200P	1 set
PBS Tween-20 Buffer (10X)	PBST10-1L	1 L
PBS Tween-20 Buffer (25X), 30 packs	PBST200P	1 set
TBS Buffer (10X)	TBST10-1L	1 L
TBS Buffer (25X), 30 packs	TBST200P	1 set
TBS Tween-20 Buffer (10X)	TBST10-1L	1 L
TBS Tween-20 Buffer (25X), 30 packs	TBST200P	1 set
VisColor™ Pre-Stained Protein Marker	VC01-500	500 µL
VisColor™ Full Range Pre-Stained Protein Marker	VC03-500	500 µL
VisPRO™ 5 Minutes Protein Stain Kit	VP01-500	1 L