ROCKLAND

Chemiluminescent Western Blot Kit

KCAE75

The AKT3 Chemiluminescent Western Blot Kit combines all the necessary reagents with a rapid proven protocol and the extremely high signal detection of our luminol chemiluminescent substrate for the detection of isoform-specific AKT3 protein, both phosphorylated and non-phosphorylated. The kit includes straightforward procedures and color-coded vials for ease-of-use with sufficient substrate for up to 5 mini blots at 7.5 x 8 cm^2 (1,800 cm^2). Use the AKT3 Chemiluminescent Western Blotting Kit for detection of isoform specific AKT3 proteins by Western blot. This kit is useful for both Western blotting and dot blotting methods.

This Chemiluminescent Western Blot Kit allows for the detection of endogenous AKT3 isoform protein present in cell lysates. After protein separation by SDS-PAGE and transfer, the membrane is probed with monoclonal Anti-AKT3. Detection of the membrane-bound antibody-antigen complex is achieved by the addition of a secondary antibody conjugated to the enzyme horseradish peroxidase. The enzyme reacts with a specialized formulation of luminol—an extremely sensitive, non-radioactive substrate that emits light and allows visualization using Xray film or other imaging methods, including highly sensitive CCD cameras and imaging systems.

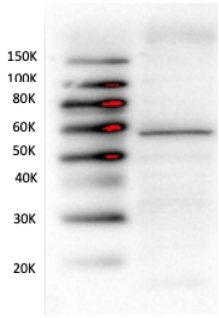


Fig 1. Western Blot. 50 µg of MDA-MB-468 Cell Lysate (#W09-001-GG9) was separated by SDS-PAGE using a 4-20% gradient gel. Proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with Blocking Buffer for Fluorescent Western Blots (#MB-070) for 1 hour at ambient temperature prior to probing the blot with the anti-AKT3 monoclonal antibody (#200-501-E75S) diluted 1:1,000 overnight at 4°C. Detection of the primary antibody by the HRP-conjugated anti-Rat IgG (#612-103-120) was performed at a dilution of 1:20,000 for 1h at ambient temperature. FemtoMax™ Super Sensitive Chemiluminescent Luminol Substrate was used for signal detection (see below).

I. Reagents Required

Reagent	Size	Cap Color	Notes
Anti-AKT3 (MOUSE) Monoclonal Antibody (#200-301-E75S)	25 µL	Red	Store at -20°C
Anti-Mouse IgG (RABBIT) Antibody Peroxidase Conjugated (#613-103-120)	100 µg	Red	Store at 4v
MDA-MB-468 Whole Cell Lysate (#W09-001-GG9)	500 µg	White	Store at -70°C. Use 50-100 µg lysate to detect AKT3.
Blocking Buffer for Fluorescent Western Blotting (#MB-070-0050)	50 mL	White	Store at 4°C
FemtoMax™ Super Sensitive Chemiluminescent HRP Substrate Luminol (#FEMTOMAX-020A)	10 mL	Brown	Store at 4°C
FemtoMax™ Super Sensitive Chemiluminescent HRP Substrate Reaction Buffer (#FEMTOMAX-020B)	10 mL	Brown	Store at 4°C
Western Blot Incubation Box	1 Unit	N/A	N/A

II. Materials Required but Not Supplied

1. SDS-PAGE electrophoresis equipment and related materials
2. Nitrocellulose, PVDF (polyvinylidene difluoride), or other membranes for protein transfer and transfer materials
3. Microfuge tubes
4. Rocker platform for gentle mixing during incubations
5. X-ray film, cassettes, and related materials or camera-based imaging system
6. Deionized water
7. Molecular-biology grade Tris base
8. Sodium chloride

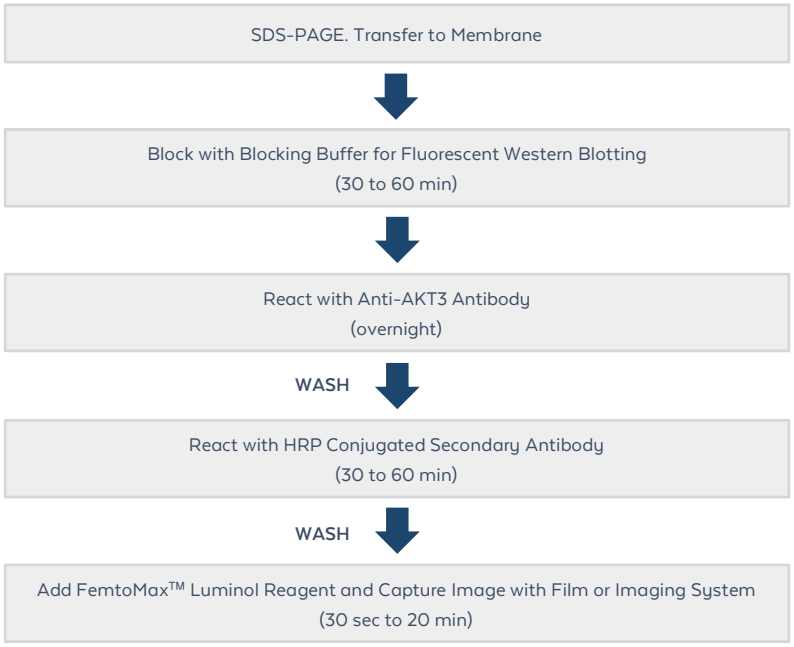
III. Storage and Stability

This kit is stable for at least one year when stored as indicated upon receipt. Individual components are stable for 3-4 weeks after dilution when stored at 4°C. The FemtoMax™ luminol chemiluminescent reagent is stable for up to 8 h at room temperature after mixing with buffer.

IV. Number of Assays

Components in this kit are sufficient to run approximately 5 mini blots at 7.5 x 8 cm^2 (1,800 cm^2). The amount of peroxidase conjugated secondary antibody supplied when diluted, as recommended in our protocol, will yield 200 mL of working solution. Adjustments in volumes for larger or smaller blots will affect the number of blots detected.

V. Flow Diagram for Chemiluminescent Western Blot Procedure



VI. Buffer Preparation

The user is to prepare the following buffers for this procedure. The exact volume of buffers required depends on the size of the membranes to be processed. We suggest preparation of 1.0 L of Tris Buffered Saline with Tween (TTBS) and 100 mL of TTBS with BSA. Prepare all solutions using ultrapure reagents and deionized (or equivalent) water. Filter the solutions and store at 4°C. Warm solutions to room temperature prior to use. Do not store solutions for more than one (1) month.

Note: Wash buffers MUST NOT contain SODIUM AZIDE or other inhibitors of peroxidase activity.

Buffer I: Tris Buffered Saline with Tween-20 (TTBS)

1. Add 800 mL of deionized water.
2. Dissolve 12.1 g of Tris base.
3. Dissolve 8.8 g Sodium Chloride (NaCl).
4. Adjust pH to 7.5 with HCl.
5. Add 1.0 mL of Tween-20 (provided).
6. Adjust volume to 1.0 L with deionized water.

VII. Preparation of Working Solutions

The Chemiluminescent Western Blot Kit for AKT3 detection comes with a concentrated stock of anti-AKT3 monoclonal antibody (primary) and peroxidase conjugated anti-Rat IgG antibody (secondary) and a 2X Stock of the Blocking Buffer for Fluorescent Western Blotting. Prior to use prepare a 1X Blocking Buffer for Fluorescent Western Blotting by diluting it 1:1 with deionized water. Prior to use, dilute the primary antibody to a final concentration of 1.0 µg/mL. Reconstitute and dilute the secondary antibody in a two-step process to a working solution. The working solution is ready for use. Reconstitute the peroxidase conjugated anti-Rat IgG antibody by adding 100 µL of deionized water. Mix thoroughly and maintain this stock at 4°C. Prepare an intermediate dilution (1:100) by pipetting 5 µL of the stock solution to 0.5 mL of 1X Blocking Buffer for Fluorescent Western Blotting in a clean microfuge tube. Prepare a second dilution (1:200) by pipetting 50 µL of the intermediate solution into 10 mL of 1X Blocking Buffer for Fluorescent Western Blotting. Mix thoroughly. This final dilution will contain 50 ng/mL peroxidase conjugated goat anti-mouse IgG antibody and will represent a 1:20,000 dilution of the stock solution.

FemtoMax™ Luminol Substrate Reagent. Just prior to use, prepare FemtoMax™ Super Sensitive Chemiluminescent Substrate by mixing 1 mL of the Luminol chemiluminescent reagent (Reagent A) with 1 mL of the Reaction Buffer (Reagent B). Mix well. Protect from intense light. Keep working solution in an amber bottle. Normal laboratory light will not harm the working solution. Larger or smaller volumes of the substrate can be prepared by mixing components at the same 1:1 ratio.

VIII. Western Blot Method

The following method is suggested as a guideline for the use of this kit. Each researcher must optimize Western blotting conditions for their protein of interest. Membranes composed of nitrocellulose or PVDF can be used. Nylon membranes may also be used (see "Additional Notes"). After your antigen has been immobilized onto the membrane by transfer, dotting or filtration, follow the numbered steps below to process the blot. Unless specified differently, all reactions occur at room temperature. Use a rocking platform set at low speed for gentle agitation. Always add enough solution to cover the membrane. Never let a membrane air dry during this process. Add the suggested volumes or just enough volume to cover the membrane to keep it wet. Do not touch the membrane with your skin!

Note: Some antigens in combination with the anti-AKT3 monoclonal antibody may require specific conditions other than those stated below. If so, use these recommendations as a starting point for further optimization.

1. After transfer is complete, block the membrane by immersing in 1X Blocking Buffer for Fluorescent Western Blotting and incubate at room temperature for 1 hour with gentle agitation.
   Note: See "Additional Notes" for other blocking agents that may be used.
2. Aspirate or decant the blocking solution.
   Note: Aspirate using a glass pipette attached to a vacuum. Alternatively, the solution may be poured off away from the blot.
3. Immediately add 5 mL of diluted anti-AKT3 monoclonal antibody (primary) solution to the membrane. The appropriate dilution should be determined by the end user. We would recommend a starting dilution of 1:1,000. Greater dilutions often result in lower backgrounds but may require longer incubation times.
4. Incubate for 1 hour at room temperature with gentle agitation. If desired, the membrane can be incubated with anti-AKT3 monoclonal antibody (primary) overnight at 4°C.
5. Aspirate or decant the anti-AKT3 monoclonal antibody (primary) solution.
6. Wash the blot 3x with Buffer I for 10 minutes each with gentle agitation.
   Note: Increasing the wash buffer volume or the number of washes may decrease background.
7. Aspirate or decant the wash solution and add 5 mL of the "Diluted Secondary Antibody" solution (see above for preparation).
8. Incubate for 1 hour at room temperature with gentle agitation.
9. Aspirate or decant secondary antibody solution.
10. Wash the blot as in Step 6.
11. Prepare FemtoMax™ Chemiluminescent reagent as described above just prior to use.
12. Transfer blot to incubation box then add 0.75 to 1.0 mL of freshly prepared FemtoMax™ reagent to the membrane (7.5 x 8.0 cm).
13. Immediately visualize the membrane by exposing X-ray film or by other imaging methods, including CCD camera-based imaging systems. For film, expose and process the film according to the manufacturer's instructions. Cover the blot with clear plastic wrap or equivalent and remove any excess liquid and any air bubbles to reduce imaging artifacts. Start with a 60 sec exposure. Exposure times may be varied for best results. For imaging systems, follow the manufacturer's instructions and vary exposure times and/or binning for best results. The length of time required to achieve optimum signal varies greatly depending on several factors. Incubation times can range from 30 seconds to 20 minutes or more.

IX. Additional Notes

- The methods given in these instructions are to be used as a guideline. Experienced users can make deviations from the stated method. Solutions have been optimized for the stated method and any change in reagent concentration, volume, reaction time or temperature will affect the overall performance of the kit. Generally, if a variable is to be modified, only alter one condition at a time.
- Nylon membrane is more difficult to block and may result in higher levels of non-specific staining. Using 10% BLOTTO (non-fat dry milk) (#B501-0500) is suggested to block nylon membranes. Allow the blocking step to proceed for several hours to overnight at 4°C. Do not use Tween-20 when using nylon membranes.
- The blocking of membranes for Western blotting can be accomplished with TTBS only (#MB-013). If using TTBS, we suggest adding BSA (#BSA-50) to lower non-specific staining. Users may omit BSA from the blocking step or use some other blocking agent, such as 5% normal goat serum, 3% fish gelatin, or other commercially available blocking agent, depending on previous experience.
- Always use enough solution to cover the membrane. Never let the membrane dry during the process.
- Protect the FemtoMax™ chemiluminescent reagent from light. Precise optimization is required to achieve maximum signal detection including optimizing the membrane, blocking conditions, antigen, and antibodies. Detection by FemtoMax™ chemiluminescent reagent requires much less sample and antibody than most commercially available ECL substrates.
- Western blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of detection reagents and reprobed.
- Use the same blotting conditions for FemtoMax™ chemiluminescent reagent as you would for Amersham ECL Plus™ Substrate or Pierce SuperSignal® West Femto Substrate.
- Use care not to touch the membrane with your skin! Wear gloves. Make certain that all equipment used in the process is free of foreign material.
- Unless specified differently, all reactions occur at room temperature.
- Use a rocking platform set at low speed for gentle agitation for all incubation steps.
- Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used to dilute the secondary antibody, substrate or any other FemtoMax™ chemiluminescent reagent.

X. Troubleshooting Guide

Table 3. Common Troubleshooting Techniques

Issue	Resolution	Solution
Little or no signal	**1.** Incomplete transfer of proteins	**1.** Follow all protocols included with your transfer apparatus. Check for the presence of transferred proteins using India ink stain as described in Reference 1.
	2. Poor binding of anti-AKT3 monoclonal antibody (primary)	**2.** Use provided control lysate in recommended concentrations. Decrease the dilution (increase the concentration) of anti-AKT3 monoclonal antibody. Increase the incubation time of anti-AKT3 monoclonal antibody from 30 minutes to several hours or overnight. Increase the incubation temperature to 37°C.
	3. Poor binding of peroxidase conjugated anti-IgG	**3.** Include Rat IgG as a control in your western blot or dot blot to ensure that the FemtoMax™ kit components are performing as described.
	4. Inactive Peroxidase Conjugate	**4.** Be certain that all buffers are free of sodium azide, which is a strong inhibitor of peroxidase activity.
Multiple Signals	**1.** Too much protein on the blot	**1.** Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 50-100 g of total protein (lysate) per lane.
	2. Too high concentration of anti-AKT3 monoclonal antibody	**2.** Increase the dilution of anti-AKT3 monoclonal antibody solution.
	3. Overexposure of signal	**3.** Decrease exposure time of film or decrease settings on camera system to decrease the signal from minor bands
High background / Poor signal-to-noise ratio	**1.** Insufficient blocking	**1.** Be certain blocking buffer has been properly prepared. Use other blocking agents. In most cases, the addition of 1.0% BSA will decrease background over the use of TTBS alone. In some cases, increased concentrations of BSA (up to 5%) are necessary.
	2. Insufficient Washing	**2.** Increase the number of wash steps and the volume of TTBS used for each wash.

XI. References

1. *Antibodies, A Laboratory Manuel.* Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988. Chapter 12 gives an excellent overview of Western Blotting techniques, including India Ink staining.
2. *Current protocols in Molecular Biology.* J. Ausebel, et al, eds. John Wiley and Sons, New York. Gives a complete protocol of Western Blotting and Dot Blotting.
3. *Molecular Cloning: A Laboratory Manuel.* 2nd Edition. J. Sambrook, E.F. Fritsch and T. Maniatis, eds. Cold Spring Harbor Press, 1989. Chapter 18 gives detailed protocols for both the production of cell lysates and electrophoresis and blotting of proteins.
4. *Antibodies, A Practical Approach.* 2nd Edition. Catty, D., ed. IRL Press, Oxford, England. 1990. Volumes I and II represent a detailed and complete reference for most current antibody technique