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Chemiluminescent Western Blot Kit - *Research Use Only*

Catalog No. KCAE71

I. Overview

Rockland Immunochemicals' Chemiluminescent Western Blot Kit for AKT2 (KCAE71) combines all of 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 AKT2 protein both phosphorylated and non-phosphorylated. The Chemiluminescent Western Blot Kit design includes straightforward procedures and color-coding to allow for ease of use. This kit contains sufficient substrate for up to 5 mini blots at 7.5 x 8 cm^2 (1,800 cm^2) and is stable for at least 1 year when stored as indicated.

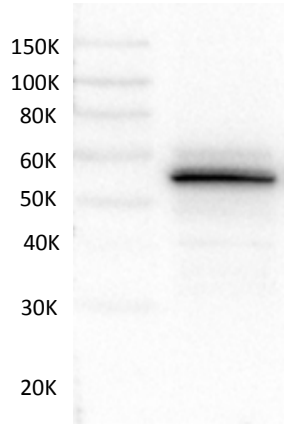
Please read the entire product insert prior to use.

II. Kit Principle

This Chemiluminescent Western Blot Kit allows for the detection of endogenous AKT2 isoform protein present in cell lysates. After protein separation by SDS-PAGE and transfer, the membrane is probed with monoclonal **Anti-AKT2**. 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 X-ray film or other imaging methods, including highly sensitive CCD cameras and imaging systems.

III. Intended Use

Use Rockland Immunochemicals' **Anti-AKT2 Chemiluminescent Kit for Western Blotting** for detection of isoform specific AKT2 proteins by western blot. This kit is useful for both "western blotting" and "dot blotting" methods. If you require additional assistance please call or e-mail technical service at 800-656-7625 or tech@rockland-inc.com.



Western Blot: 50 µg of MDA-MB-468 Cell Lysate (p/n 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 (p/n MB-070) for 1 hour at ambient temperature prior to probing the blot with the anti-AKT2 monoclonal antibody (p/n 200-501-E71) diluted 1:1,000 overnight at 4°C. Detection of the primary antibody by the HRP-conjugated anti-Rat IgG (p/n 612-103-120) was performed at a dilution of 1:20,000 for 1h at ambient temperature. **Femto*Max***^{TM} Super Sensitive Chemiluminescent Luminol Substrate was used for signal detection (see below).

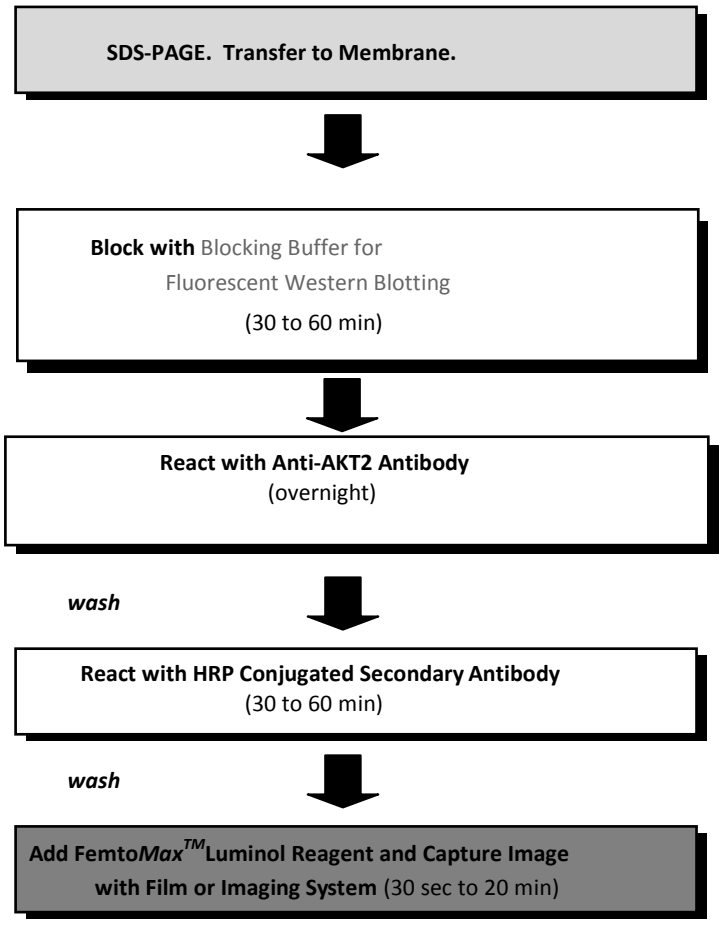
IV. Storage and Stability

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**^{TM} luminol chemiluminescent reagent is stable for up to 8 h at room temperature after mixing with buffer.

V. 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 in excess of 200 mL of working solution. Adjustments in volumes for larger or smaller blots will affect the number of blots detected.

VI. Flow Diagram for Chemiluminescent Western Blot Procedure



VII. Materials Required but Not Supplied

Nearly all components required for the detection of AKT2 by western blot are provided for your convenience in Rockland Immunochemicals' Chemiluminescent Western Blot Kit for AKT2. Some additional materials not included are required:

- SDS-PAGE electrophoresis equipment and related materials
- Nitrocellulose, PVDF (polyvinylidene difluoride) or other membranes for protein transfer and transfer materials
- Microfuge tubes
- Rocker platform for gentle mixing during incubations
- X-ray film, cassettes and related materials or camera based imaging system
- Deionized water and molecular biology grade Tris base and sodium chloride

VIII. Kit Components and Layout

1. (1) x 25 µL **anti-AKT2 monoclonal antibody** in clear plastic 0.5 mL vial with **RED** cap. Store at -20° C.
2. (1) x 100 µg Peroxidase anti-Rat IgG antibody in amber plastic 0.5 mL vial with **RED** cap. Store at +4° C.
3. (1) x 500 µg of positive control cell lysate in clear plastic 1.5 mL vial with **WHITE** cap. Store at -70° C. Use 50 - 100 ug lysate to detect AKT2.
4. (1) x 50 mL 2X Blocking Buffer for Fluorescent Western Blotting in plastic vial with **WHITE** cap. Store at +4° C.
5. (1) x 10 mL **Femto*Max***^{TM} Reagent A - Luminol - in an amber plastic bottle with **BROWN** cap. Store at +4° C.
6. (1) x 10 mL **Femto*Max***^{TM} Reagent B - Reaction Buffer - in an amber plastic bottle with **BROWN** cap. Store at +4° C.
7. Incubation box with lid.
8. Instruction Manual.

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IX. Solutions Required but Not Supplied

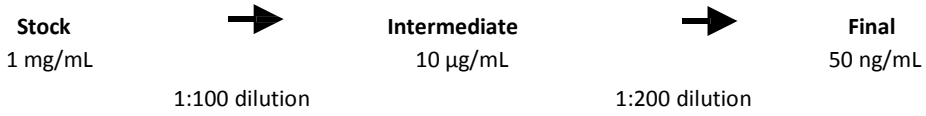
The user is to prepare the following buffers for this procedure. The exact volume of buffers required depends on the size of the membranes to be processed. We suggest preparation of 1.0 L of Tris Buffered Saline with Tween (TTBS) and 100 ml of TTBS with BSA. Prepare all solutions using ultra pure reagents 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)** Add 800 mL of deionized water. Dissolve 12.1 g of Tris base. Dissolve 8.8 g Sodium Chloride (NaCl). Adjust pH to 7.5 with HCl. Add 1.0 mL of Tween-20 (provided). Adjust volume to 1.0 L with deionized water.

X. Preparation of Working Solutions

The Chemiluminescent Western Blot Kit for AKT2 detection comes with a concentrated stock of **anti-AKT2 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-rat IgG antibody and will represent a 1:20,000 dilution of the stock solution.



Femto*Max*™ Luminol Substrate Reagent. Just prior to use, prepare **Femto*Max*™** 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.

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XI. Western Blot Method

The following method is suggested as a **guideline** for the use of Rockland Immunochemicals' Chemiluminescent Western Blot Kit for AKT2 detection. 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-AKT2 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 h with gentle agitation. Other blocking agents may be used (see "Additional Notes").

2. Aspirate or decant the blocking solution[1]. Immediately add 5 ml of diluted **anti-AKT2 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. Incubate for 1 h at room temperature with gentle agitation. If desired, the membrane can be incubated with **anti-AKT2 monoclonal antibody** (primary) overnight at 4° C.

3. Aspirate or decant the **anti-AKT2 monoclonal antibody** (primary) solution. Wash the blot with 3 changes of *Buffer I* for 10 min each with gentle agitation. Increasing the wash buffer volume or the number of washes may decrease background.

4. Aspirate or decant the wash solution and add 5 ml of the "Diluted Secondary Antibody" solution (see above for preparation). Incubate for 1 h at room temperature with gentle agitation.

5. Aspirate or decant secondary antibody solution. Wash the blot as in Step 3.

6. Prepare **Femto*Max*™** chemiluminescent reagent as described above just prior to use. Transfer blot to incubation box or film cassette then add 0.75 to 1.0 mL of freshly prepared **Femto*Max*™** reagent to the membrane (7.5 x 8.0 cm).

7. 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 sec to 20 min or more.

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[1] Aspirate using a glass pipette attached to a vacuum. Alternatively, the solution may be poured off away from the blot.

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XII. 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) (p/n 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 (p/n MB-013). If using TTBS, we suggest adding BSA (p/n 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 re-probed.

- 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.

- A positive control is provided in a WHITE capped vial. We recommend to use 50-100 ug of lysate/lane to detect AKT2.

- Store the components of this kit as specified.

- Individual components of this kit may be ordered separately (see "Replacement Parts List").

XIII. Troubleshooting Guide

Little or no signal

Incomplete transfer of proteins. Follow all protocols included with your transfer apparatus. Check for the presence of transferred proteins using India ink stain as described in Reference 1.

Poor binding of anti-AKT2 monoclonal antibody (primary). Use provided control lysate in recommended concentrations. Decrease the dilution (increase the concentration) of anti-AKT2 monoclonal antibody. Increase the incubation time of anti-AKT2 monoclonal antibody from 30 minutes to several hours or overnight. Increase the incubation temperature to 37°C.

Poor binding of peroxidase conjugated anti-IgG. Include Rat IgG as a control in your western blot or dot blot to ensure that the **Femto*Max*™** kit components are performing as described.

Inactive Peroxidase Conjugate. Be certain that all buffers are free of sodium azide, which is a strong inhibitor of peroxidase activity.

Multiple signals

Too much protein on the blot. Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 50-100 µg of total protein (lysate) per lane.

Too high concentration of anti-AKT2 monoclonal antibody. Increase the dilution of anti-AKT2 monoclonal antibody solution.

Overexposure of signal. 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

Insufficient blocking. 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.

Insufficient Washing. Increase the number of wash steps and the volume of TTBS used for each wash.

XIV. References

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.

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.

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.

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

XV. Trademarks

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XVI. Disclaimer

This product is for research use only and is not intended for therapeutic or diagnostic applications. Please contact a technical service representative for more information. All products of animal origin manufactured by Rockland Immunochemicals are derived from starting materials of North American origin. Collection was performed in United States Department of Agriculture (USDA) inspected facilities and all materials have been inspected and certified to be free of disease and suitable for exportation. All properties listed are typical characteristics and are not specifications. All suggestions and data are offered in good faith but without guarantee as conditions and methods of use of our products are beyond our control. All claims must be made within 30 days following the date of delivery. The prospective user must determine the suitability of our materials before adopting them on a commercial scale. Suggested uses of our products are not recommendations to use our products in violation of any patent or as a license under any patent of Rockland Immunochemicals, Inc. If you require a commercial license to use this material and do not have one, then return this material, unopened to: Rockland Inc., P.O. BOX 5199, Limerick, Pennsylvania, USA.

XVII. Additional Products and Services

Additional Chemiluminescent Western Blotting Kits

Product	Code	Size	Price
Femto*Max*™ Chemiluminescent Western Blot Kit for use with Human Primary Antibody	KCA001	1 each	inquire
Femto*Max*™ Chemiluminescent Western Blot Kit for use with Mouse Primary Antibody	KCA002	1 each	inquire
Femto*Max*™ Chemiluminescent Western Blot Kit for use with Rabbit Primary Antibody	KCA003	1 each	inquire
Femto*Max*™ Chemiluminescent Western Blot Kit for use with Goat Primary Antibody	KCA004	1 each	inquire
Femto*Max*™ Chemiluminescent Western Blot Kit for DYKDDDDK (FLAG®) proteins	KCA383	1 each	inquire
Femto*Max*™ Chemiluminescent Western Blot Kit for GFP recombinant proteins	KCA215	1 each	inquire

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Replacement Parts or additional products of interest for AKT2 Chemiluminescent Kit for Western Blotting

Anti-AKT2 monoclonal antibody	200-501-E71	100 µg	BSA, Protease and IgG Free	BSA-10	10 g
MDA-MB-468 Whole cell lysate	W09-001-GG9	0.5 mg	Ultra Pure Tween-20	TW0020	50 mL
Peroxidase Anti-Rat IgG	612-103-120	1.0 mg	10X TTBS pH 7.5	MB-013	1.0 L
AKT2 Control Protein	000-001-E71	100 µg	BLOTTO Immunoanalytical Grade (Non-Fat Dry Milk)	B501-0500	500 g
Femto*Max*™ Super Sensitive Chemiluminescent Substrate	FEMTOMAX-110	110 mL	Western Incubation Box SMALL	WIB-2875-010	10 PACK