Chemiluminescent Western Blot Kit: FemtoMax™ Chemiluminescent Substrate Kit for Western Blotting

for use with Rabbit Primary Antibody

Catalog # OKRA00004

I. Overview

Aviva Systems Biology's Chemiluminescent Western Blot Kit, namely our **Femto*Max***™ Chemiluminescent Substrate Kit for Western Blotting, combines all of the necessary reagents with a rapid proven protocol and the extremely high signal detection of our luminol substrate. 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 30 mini blots at 7.5 x 8 cm^2 (1,800 cm^2) and is stable for at least 1 year when stored at 4°C.

Please read the entire product insert prior to use.

II. Kit Principle

This Chemiluminescent Western Blot Kit allows for the detection of primary ***Rabbit*** polyclonal or monoclonal antibodies provided by the user. After protein separation and transfer, the membrane is probed with primary antibody. Detection of the membrane bound primary 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.

Because of the extremely high sensitivity of our **Femto*Max***™ Chemiluminescent Substrate Kit for Western Blotting, primary and secondary antibodies can be used at greater dilutions. If you currently dilute your primary antibody 1:1,000 using an ECL substrate equivalent, then dilute your antibody 1:5,000 using the **Femto*Max***™ substrate.

III. Intended Use

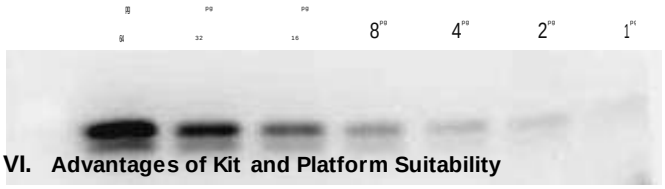
Use Aviva Systems Biology's Chemiluminescent Western Blot Kit with **Femto*Max***™ Chemiluminescent Substrate (included) for western blotting. This kit is for the detection of ***Rabbit*** polyclonal or monoclonal antibody complexed with antigen immobilized on a membrane (western blot). This kit is useful for both "Western blotting" and "dot blotting" methods. This kit *can* be used with the appropriate user supplied primary antibody for the detection of phosphoproteins and other post translational modifications. If you require additional assistance please call or e-mail our technical service representatives at 858-552-6979 or techsupport@avivasysbio.com.

IV. Storage and Stability

This kit is stable for at least one year when stored at 4°C. Individual components are stable for 3-4 weeks after dilution when stored at 4°C. The **Femto*Max***™ luminol substrate 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 30 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.



Western Blot. Chemiluminescent Western Blot Kit shows super sensitive signal. Known amounts of GST were spiked into a HeLa lysate followed by separation by SDS-PAGE using a 4-20 % gradient gel. Proteins were transferred to a Protran B85 membrane for 1 hour at 100 mV. The membrane was blocked with 5% BLOTTO in TTBS for 1 h at 4 °C. The blot was probed for 40 min with 1:1,000 dilution of Mab anti-GST followed by HRP anti-Mouse IgG at 1:10,000 dilution in blocking buffer for 1 h at 4°C.

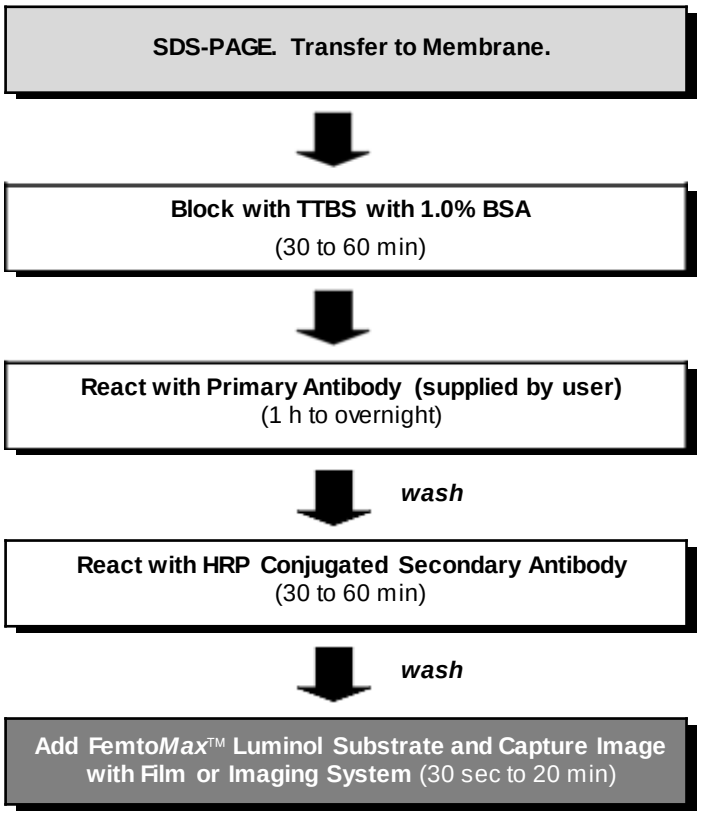
VI. Advantages of Kit and Platform Suitability

Aviva Systems Biology's Chemiluminescent Western Blot Kit with **Femto*Max***™ Chemiluminescent Substrate is more sensitive than most commercially available chemiluminescent detection products. This conserves precious amounts of limiting antigen and antibody and results in considerable savings.

- **Super Sensitive Signal.** Generally the signal obtained with **Femto*Max***™ substrate is twice as intense as competitor's luminol-based systems.
- **Conserves Costly Antibody.** Greater dilutions of antibody and less antigen are used for assay detection.
- **Femtogram Sensitivity.** Low femtogram sensitivity with moderate signal duration designed for both film and CCD/imaging system detection methods.
- **Excellent Stability.** The luminol reagent is stable for up to 8 hours as a working solution after mixing with reaction buffer.
- **Platform Flexibility.** This kit is compatible with the following imaging platforms. This kit will likely be suitable for other similarly designed instruments by manufacturers not listed below.

Supplier	Instrument
UVP	Chemi, BioChemi and OptChemi Systems
Bio-Rad	ChemiDoc and VersiDoc Imaging Systems
Kodak	Image Station 2000MM
Fujifilm	LAS-3000 Imaging System
Alpha Innotech	Alpha Imager
GE	Typhoon, Storm and Image Quant Systems

VII. Flow Diagram for Chemiluminescent Western Blot Procedure



VIII. Kit Components and Layout

1. (1) x 50 mL Ultra Pure Tween-20 in an amber glass bottle with **BLACK** cap
2. (1) x 100 µg Peroxidase Goat anti-Rabbit IgG antibody in amber plastic vial with **GREEN** cap
3. (1) empty dropper bottle with **GREEN** cap labeled "Diluted Secondary antibody"
4. (2) empty microfuge tubes for intermediate dilution of secondary antibody
5. (1) x 10 g Bovine Serum Albumin (BSA) in a plastic bottle with a **WHITE** cap
6. (1) x 0.5 mL Control Rabbit IgG in plastic vial with **YELLOW** cap
7. (1) x 10 mL **Femto*Max*** Reagent A - Luminol - in an amber plastic bottle with **BROWN** cap
8. (1) x 10 mL **Femto*Max*** Reagent B - Reaction Buffer - in an amber plastic bottle with **BROWN** cap
9. Instruction Manual

IX. Materials Required but Not Supplied

Nearly all components required for western blotting are provided for your convenience in Aviva Systems Biology's Chemiluminescent Western Blot Kit. 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
- Primary Antibody (***Rabbit*** derived monoclonal or polyclonal)
- Microfuge tube
- 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

X. 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.

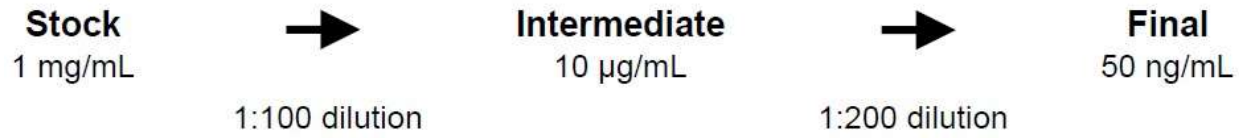
Buffer II **TTBS with 1.0% (w/v) BSA**

Add 100 mL of TTBS.
Dissolve 1.0 g of BSA (provided).
Use solution immediately.

XI. Preparation of Working Solutions

The **Femto*Max***™ Chemiluminescent Western Blot Kit comes with a concentrated stock of peroxidase conjugated goat anti-***Rabbit*** secondary antibody. Prior to use, the secondary antibody must be reconstituted and diluted in a two-step process to a working solution. The working solution is ready for use.

- **Secondary antibody.** Reconstitute the peroxidase conjugated goat anti-***Rabbit*** antibody by adding 100 µL of deionized water to the **GREEN** capped amber vial. Mix thoroughly and maintain this stock at 4 °C. Prepare an intermediate dilution (1:100) by accurately pipetting 5 µL of the stock solution to 0.5 mL of Buffer I (TTBS) in a clean microfuge tube. Prepare a second dilution (1:200) by accurately pipetting 50 µL of the intermediate solution to the **GREEN** capped dropper bottle labeled "Diluted Secondary Antibody." Add 10 mL of Buffer I (TTBS). Mix thoroughly. The final dilution will contain 50 ng/mL peroxidase conjugated goat anti-***Rabbit*** 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 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.

XII. Western Blot Method

The following method is suggested as a **guideline** for the use of Aviva Systems Biology's Chemiluminescent Western Blot 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 western blot. 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 primary antibodies and/or antigens 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 *Buffer II* (TTBS with BSA) and incubate at room temperature for 1 h with gentle agitation. The addition of 1.0% BSA (provided) as a blocking agent increases the signal-to-noise ratio when compared to using TTBS alone. Other blocking agents may be used (see "Additional Notes").

2. Aspirate or decant the blocking solution[1]. Immediately add 5 ml of primary antibody solution (not provided) diluted in *Buffer I* (TTBS) to the membrane. The appropriate dilution should be determined by the end user. If unknown, a starting dilution of 1:1,000 to 1:5,000 from a 1.0 mg/mL stock is suggested. 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 primary antibody overnight at 4° C.

3. Aspirate or decant the primary antibody solution. Wash the blot with 3 changes of *Buffer I* for 5 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 from the **GREEN** capped dropper bottle (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 luminol substrate reagent as described above just prior to use. Transfer blot to incubation box or film cassette then add approximately 1.25 ml of freshly prepared **Femto*Max***™ chemiluminescent luminol substrate 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 film according to manufacturer's instructions[2]. A trial and error approach is suggested to vary exposure time to obtain maximum signal. A good starting time is 60 sec. For imaging systems, follow the manufacturer's instructions and vary exposure time 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 a short as 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 western blot.

[2] Cover blot with clear plastic wrap or plastic sheet. Remove excess liquid and any air bubbles to reduce imaging artifacts.

XIII. 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, or 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) is suggested to block nylon membranes. Allow the blocking step to proceed for several hours to overnight at 37° C. Do not use Tween-20 when using nylon membranes.

- The blocking of membranes for western blotting can be accomplished with TTBS only. We suggest adding BSA 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.

- Some primary antibodies do not bind well in the presence of mild detergents like Tween-20. In these instances, replace the TTBS with TBS or PBS containing BSA or 1% to 10% normal goat serum (secondary antibody is goat host).

- Always use enough solution to cover the membrane. Never let the membrane dry during the process.

- Protect the **Femto*Max***™ luminol substrate reagent from light. This kit easily allows for the detection of femtogram (10^{-15}) amounts of antigen using photographic film or imaging methods, including highly sensitive CCD cameras. Precise optimization is required to achieve maximum signal detection including optimizing the membrane, blocking conditions, antigen, primary and secondary antibodies. Detection by **Femto*Max***™ luminol substrate reagent requires much less sample and antibodies 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 **Femto*Max***™ luminol substrate 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.

- 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 **Femto*Max***™ reagent.

- A positive control ***Rabbit*** IgG is provided in a YELLOW capped vial for use as a secondary antibody control. Spot 1 or 2 µl as a control on your Western or dot blot prior to the blocking step.

- Store the components of this kit at 4 °C.

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

XIV. 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 primary antibody. Decrease the dilution (increase the concentration) of your primary antibody. Increase the incubation time of the primary antibody solution from 30 minutes to several hours or overnight. Increase the incubation temperature to 37° C. If all of the above fails, contact the source of your primary antibody.

Poor binding of peroxidase conjugated anti-IgG. Be sure the source of the primary antibody is appropriate for this kit. Include 1 or 2 μL of ***Rabbit*** IgG in the YELLOW capped vial as a control in your western blot or dot blot to ensure that the **Femto*Max***™ Chemiluminescent Western Blot 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 western blot. Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 10 μg of total protein (lysate) per lane.

Too high concentration of primary antibody. Increase the dilution of primary 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. 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.

XV. 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 techniques.

XVI. Trademarks

XVII. Disclaimer