Description:

The design of this kit simplifies the process of Western blotting. The kit contents provide a rapid and sensitive system for the detection of proteins that have been transferred to a membrane and stained with immuno-specific primary and AP-conjugated secondary antibodies. The basis of detection relies on the use of AMRESCO’s VisiGlo™ reagents for alkaline phosphatase (AP) detection. This dioxetane-based, chemiluminescent detection system offers superior advantage in sensitivity compared to chromagenic substrates.

Number of Assays:

The supplied materials are sufficient for performing 20 – 30 Western blots equal to 10 cm x 10 cm (100cm² each). The antibody supplied will yield approximately 200 mL of working solution when diluted to the recommended concentration.

Storage / Stability:

This kit is stable for at least one (1) year when stored at +4°C.
Buffer Preparation:

The WesternMAX™ AP Detection Kit contains all the necessary components for preparing a Dilution Buffer, Wash Buffer and Blocking Buffer. The volume of buffers required will vary depending on the number and size of membrane blots that are to be treated. Although this kit suggests the use of the supplied Dilution Buffer Powder for preparation of working reagents, Phosphate Buffered Saline (PBS) may be substituted if desired (not supplied). All solutions should be prepared using deionized water. If solutions are to be stored for subsequent use, it is recommended they be sterile-filtered and stored at 4°C. Do not store solutions for greater than one (1) month. The use of preservatives or anti-microbial agents (i.e., Sodium Azide) may have functional consequences on the performance of the product.

Dilution Buffer
- Add 1.2g WesternMAX™ Dilution Buffer Powder to 100 mL deionized water.

Wash Buffer
- Add 100µL of Tween® 20 to 100 mL of Dilution Buffer to make a 0.1% (v/v) Tween® 20 solution.

Blocking Buffer (optional)
- Add 100 mg Bovine Serum Albumin (BSA) per 10 mL of Wash Buffer to make a 0.1% (w/v) BSA solution.

Secondary Antibody:

Approximately 10 mL of solution is used to cover a 10 cm x 10 cm membrane. To prepare 10 mL of diluted secondary antibody solution (sufficient for 1 – 2 100cm² membranes), add 5 µL of concentrated AP-conjugated secondary (Rabbit or Mouse) antibody to 10 mL of Wash Buffer. This is a recommended starting concentration. Improved results may require some degree of optimization.

Immunoblot Method:

The WesternMAX™ AP Chemiluminescent Detection Kit can be used for detection of protein antigens on a variety of membranes including PVDF and nitrocellulose. The following method provides a guideline for use. However, it may be necessary, depending upon the antigen, antibody, and specific conditions being used, for further optimization. All steps should be performed at room temperature. The use of a rocking or orbital platform to provide gentle agitation is suggested. In order to reduce background, membranes should not be allowed to dry at any time during the process. Use care when handling membranes. **Always use gloved hands and never allow for skin contact.**
**Protocol Overview:**

- Prepare sample for assay (Western blot or dot blot).
- Block membrane with Wash Buffer or Blocking Buffer (30 minutes).
- React with Primary antibody.
- React with Alkaline Phosphatase conjugated secondary antibody.
- React with VisiGlo™ AP reagent.
- Visualize proteins by autoradiography.

1. Transfer proteins from an SDS-PAGE gel via electroblotting or other equivalent methods. It is recommended the provided IgG matched control be loaded to one lane of the gel for use as a positive control.
2. If the membrane has been allowed to dry following transfer, re-hydrate by submersion in Deionized water for 1 – 2 minutes. If PVDF is being used, re-hydrate membrane in a 10% Methanol solution.
3. Block membrane with Wash Buffer or Blocking Buffer for 30 – 60 minutes at room temperature with gentle agitation.
4. Discard blocking solution.
5. Dilute the primary antibody in Wash Buffer or Blocking Buffer. The proper dilution must be determined through trial and error or per the supplier’s recommended starting concentration. Add approximately 10 mL or a volume of antibody solution sufficient to cover the surface of the membrane. Incubate membrane at room temperature for 30 – 120 minutes with gentle agitation.
6. Discard primary antibody solution. (Primary antibody solutions may be re-used if stored at 2°C – 8°C for up to one week).
7. Wash membrane with an ample amount of Wash Buffer for 5 – 10 minutes with gentle agitation and discard. Repeat wash step twice more.
8. Add approximately 10 mL or a volume of secondary antibody solution sufficient to cover the surface of the membrane. Incubate membrane at room temperature for 30 – 60 minutes with gentle agitation.
10. Wash membrane with an ample amount of Wash Buffer for 5 – 10 minutes with gentle agitation and discard. Repeat wash step twice more.
11. For detection, cover membrane with an ample amount of VisiGlo™ AP Substrate. The volume used should be sufficient to just cover surface of membrane. Excess amount of reagent or submersion of membrane may contribute to background.
12. Incubate membrane in VisiGlo™ AP Substrate for 1 – 10 minutes.
13. Gently blot membrane dry with a Kimwipe®, or alternatively, allow excess reagent to run off.
14. Proteins may be visualized via autoradiography following exposure to film.
Additional Notes:

- The above protocol is intended to be used as a general guideline. Deviations from this may be made by experienced users. The buffers and reagents provided have been optimized for use with the given protocol. If conditions, volumes, reaction times, or temperatures are altered, the overall performance of the kit and its reagents may be affected. As a general rule, modifications should be made sequentially one at a time.
- There are several manufacturers of electrophoresis equipment used for Western blotting and gel-running applications. Refer to your owner’s manual for an optimized blotting / transfer protocol.
- In most cases, sufficient blocking of the membrane can be achieved with Wash Buffer only. It may be necessary, however, to use Blocking Buffer for improved signal-to-noise ratio.
- It is possible that some primary antibodies may not bind well in the presence of non-ionic detergents such as Tween® 20. For best results, dilute the primary antibody in Blocking Buffer when dealing with an unknown antigen-antibody pair. Alternatively, Dilution Buffer or PBS containing 1% – 10% goat serum may be used.

Troubleshooting:

Problem 1 – No Signal
- Inactive Alkaline Phosphatase
  - Verify enzyme activity by mixing 10µL of diluted conjugate with 1mL of substrate. (The substrate should glow in the dark.)
- No binding of conjugate to the primary antibody
  - Confirm correct specificity of the conjugate for the primary antibody (i.e., no alkaline phosphatase conjugated anti-rabbit with a mouse primary antibody).
- No transfer of target to membrane
  - Use a protein stain on unblocked membrane to verify attachment of target protein or use a pre-stained protein marker to monitor transfer.
- Detection of non-blotted side of membrane
  - Ensure correct orientation of the membrane during the assay and film exposure.
- Missed step in procedure
  - Review procedure to ensure all steps were followed.

Problem 2 – Weak Signal
- Insufficient amount of antibody
  - Optimize antibody concentrations. Affinity of the primary antibody may change after proteins are denatured through SDS-PAGE.
- Insufficient protein loaded or transferred
  - Increase the amount of protein loaded onto the gel.
- Insufficient incubation of primary antibody
  - Increase the incubation times for weak primary antibodies.
- Insufficient exposure time
  - Increase the time of exposure to film.
- Excessive washing beyond recommended procedure
  - Follow the procedure as written.
Problem 3 – Excessive Signal, Nonspecific Bands or General Background

- Overexposure of film to signal
  - Expose the membrane to film for a shorter period of time.
- Insufficient blocking or washing
  - Increase blocking and washing time or increase number of washes. Vary type of block used.
- Excessive antibody used for detection
  - Optimize conjugate concentration. Reduce antibody concentrations. Optimal conjugate dilution should be 1/10,000 – 1/100,000 of a 0.1mg/mL stock – OR – decrease the amount of primary antibody.
- Excessive protein loaded on the gel
  - Decrease the amount of protein loaded onto the gel.

Problem 4 – Poorly Defined or “Fuzzy” Bands or Dots

- Poor transfer of protein to membrane
  - Follow manufacturer’s recommended procedure or contact the manufacturer for additional support regarding the blotting apparatus.
- Excessive substrate
  - Remove excess substrate before exposure of the membrane to film.
- Ghost images from shifted position of film during development
  - Avoid movement of film over membrane during exposure period.
- Inadequate handling of membranes
  - Certain membranes require special handling. Check with the membrane vendor for correct procedures.

References: