WESTERN BLOT PROCEDURE SUMMARY
Total Assay Time - 136 min. (60 + 15 + 60 + 1 min.)

	Protein is immobilized on membrane (use standard Dot blot or SDS blotting procedures)
Step 1	Block membrane with diluted (1:20) blocking buffer for **1 h** at room temperature. Drain excess liquid
Step 2	Dilute primary antibody/serum samples (use optimized concentrations) in blocking buffer (diluted 1:20) and incubate with the membrane for **1h** at room temperature. Gentle rocking may be used.
Step 3	Wash the membrane **three** times with 1:20 diluted wash buffer with a **five min**. soak time for each wash. Improper washing will cause high background.
Step 4	Add Ab-enzyme conjugate (diluted 1:3K-5K) or more in blocking buffer/Ab diluent). Incubate at room temperature for **1 h.**
Step 5	Wash the membrane **Four times** as above to remove unbound enzyme labeled antibodies..
Step 6	Add freshly prepared substrate solution (1:1:2 v/v of solns. A :B:H2O; **Example**: 5 ml Soln A+5 ml Soln. B+10 ml H2O). Incubate at room temperature for **one** minute.
Step 7	Remove substrate solution and completely drain excess liquid. Lay membrane between sheets of clear plastic or wrap with Saran Wrap and expose the membrane to X-ray film (few seconds to 30 min.) depending on the intensity of the signal. Intensity of the bands can be adjusted by varying exposure time.

KIT PROFILE

Date received:_____**Cat #** **Lot #**__ **Exp.** _____

Date kit opened _____ **Technician:** _____

Date used: _____

Remarks: _____

_____

_____

Instruction Manual # M-80200-80209

Enhanced NuGlo Western Blot Kit

For rabbit (#80200), goat (#80201), mouse (#80202), rat (#80203), chicken (#80204), monkey (#80205), guinea pig (#80206), human (#80207), sheep (#80208), and pig (#80209) primary antibodies

A Non-Radioactive Chemiluminescent Assay for Recording Immunoblotting (Western blot) Results

ALPHA DIAGNOSTIC INTERNATIONAL

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DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

Enhanced NuGlo Chemiluminescent Western Blot Kit for various primary Antibodies Cat. #. 80200-80209

Kit Contents: (Sufficient reagents for processing 3000 cm^2 or 200-400 membrane strips)

Components	Cat.#
The kit will contain one of the following secondary antibody-HRP conjugate stock, 0.5 ml (dilute 1:3K-1:5K before use) # 80200-Rb (conjugate #20321); # 80201-Gt (conjugate #30321) # 80202-Mo (conjugate #40321); # 80203-Rt (conjugate #50321) # 80204-Ch (conjugate #60321); # 80205-Mk (conjugate #60325) # 80206-Gp (conjugate #50325); # 80207-Hu (conjugate #10321) # 80208-Sh (conjugate #30326); # 80209-Pg (conjugate #90321)	
Blocking Buffer/Antibody Diluent Concn. (20X) 50 ml.	80062
Wash buffer concn.(20X), 100 ml	80081
Enhanced NuGlo Chemiluminescent substrate (2X); Solution A, 100 ml	80218
Enhanced NuGlo Chemiluminescent Substrate; Solution B (2X), 100 ml	80218
Complete Instruction Manuals # M - 80200 - 80209	

This kit is provided with appropriate antibody-HRP conjugates and supplied as concentrated solution in a stabilized buffer. It must be diluted before use. A suggested starting dilution is 1:3K-1:5K with the working solution of antibody diluent (in some cases it may be necessary to increase or decrease conjugate dilution to control intensity of the signal).

Ordering Information for various species Western blot kits

Cat #	Kit Description
80200-Rb	Western blot Kit for **Rabbit** Primary Antibodies,
80201-Gt	Western blot Kit for **Goat** Primary Antibodies,
80202-Mo	Western blot Kit for **Mouse** Primary Antibodies,
80203-Rt	Western blot Kit for **Rat** Primary Antibodies,
80204-Ch	Western blot Kit for **Chicken** Primary Antibodies,
80205-Mk	Western blot Kit for **Monkey** Primary Antibodies,
80206-Gp	Western blot Kit for **G. pig** Primary Antibodies,
80207-Hu	Western blot Kit for **Human** Primary Antibodies,
80208-Sh	Western blot Kit for **Sheep** Primary Antibodies,
80209-Pg	Western blot Kit for **Pig** Primary Antibodies,

STORAGE AND STABILITY

The reagents are stable at 2-8^{o}C until the expiration date printed on the label. Do not store diluted solutions of any reagents. Any unused mixture of substrate solutions A & B must be discarded.

Causes of No Signal

1. Procedure was not followed properly; a reagent may have been omitted or prepared improperly.
2. Protein was not transferred correctly from gel to the membrane.
3. Specificity of the peroxidase labeled antibody was not appropriate for the primary antibody.
4. Correct orientation of the membrane was not maintained throughout the procedure.
5. Presence of azide in any buffer has suppressed peroxidase activity.

Causes of Weak Signal

1. Antibody concentration was too low or antibody incubation time was too short. Conjugate dilution may be inappropriate.
2. Not enough protein was loaded onto the gel.
3. The primary antibody has low affinity for the target protein. (Affinity of the antibody may change after the denaturation of the sample protein in SDS buffer).
4. Exposure time of the membrane to film was too short.
5. Reagents were not warmed to room temperature before use.

Causes of Poorly Defined Signal

1. Protein transfer was performed incorrectly; check recommendation for use of blotting apparatus.
2. Excess substrate solution was not removed from membrane prior to signal development.
3. Film or membrane shifted position during signal development, causing `ghost' images (this can occur within a few seconds).
4. Certain types of membranes require special handling; check for manufacturer's recommendations.

Specificity of Antibodies or the ELISA Kit

The antibody conjugates provided in this kit are directed against the IgG (H+L) and designed to detect most common antibodies of IgG, IgA, or IgM isotypes.. It is possible to use a different antibody conjugates, such as anti-IgG (heavy chain) or IgM or IgA specific conjugates to detect IgG or IgM or IgA subtypes only. Please contact ADI for these specific conjugates.

Note: This kit is only designed to provide Western blot reagents to detect species specific primary antibodies when used in dot blot or western blot. It is not meant to measure the antibody concentration in the serum or samples. ADI has other ELISA kits to measure IgG, IgA, and IgM in various animal sera.

the Ab-enzyme conjugate. It can also be used to dilute primary antibody. Dilute only as needed. A preservative (merthiolate 0.02% can be added; DO NOT ADD SODIUM AZIDE**.** It is possible to substitute this milk-based buffer if it interferes with the assay or if BSA or gelatin based buffer are known to provide a better signal with a given antibody.

3. Wash Buffer. Dilute the wash buffer concentrate 1:20 with distilled water. Dilute only as needed. Diluted buffer can be stored at 4^{o}C for one week. A preservative (merthiolate 0.02% can be added). This is PBS-Tween (0.1%) based buffer. In house wash buffer can be substituted if more buffer is required.

4. Enzyme Conjugate. A suggested starting dilution is 1:3K-1:5K with the working solution of antibody diluent. The dilution factor may be adjusted if desired to optimize the reaction. Dilute only as needed. A preservative (merthiolate 0.02% can be added; DO NOT ADD AZIDE).

5. Enhanced NuGlo Substrate Solution. Mix equal volumes of substrate solution A:B:H2O (1:1:2 v/v; Example 10 ml of soln A+10 ml soln B+20 ml of H2O). The mixture is stable for up to 4 h at room temperature and need not be protected from light. Avoid storing soln. for periods longer than necessary and prepare only as needed.

OTHER PROCEDURAL AND MATERIAL REQUIREMENTS

Prior to application of the kit reagents, the protein must be immobilized onto the test membrane. Nitrocellulose, nylon, and polyvinylidene (PVDF) membranes are among the most common types of membranes determined to be suitable for use with this kit. For the Western blot, proteins are separated by SDS polyacrylamide gel electrophoresis (or a comparable method) and transferred to the membrane through either electrophoretic or passive transfer. It may be desirable to stain a test section of the unblocked membrane with a protein stain such as ADI's (Cat # StainALL) to verify transfer of proteins before proceeding with the immunoassay.

Incubation tubes or trays should be selected to minimize the volume of reagents required to immerse the membrane or strips in solution. If the entire membrane is reacted with a single primary antibody or serum, 15-20 ml of each reagent is required to immerse a 15 x 15 cm membrane in a reaction tray. If the membrane is cut into strips for reaction with serial dilutions or several different primary antibodies, the strips may be reacted in small tubes or a divided tray, and only 1-5 ml of solution is required to immerse each strip.

A platform shaker or rocker should be used during incubation to insure complete and uniform distribution of assay reagents over the membrane.

The emission of light is detected using a camera luminometer or any X-ray film suitable for use with radioisotopes.

IMMUNOBLOTTING PROCEDURE USING NuGLO SUBSTRATE

Step 1. Before starting the immunoassay, use a pencil to mark the orientation of the protein samples on the transfer membrane. The membrane may be cut into strips at this time if desired, although it may be more convenient to cut strips after the entire membrane has been blocked. Strips that have been reacted with identical reagents may be incubated in the same reaction vessel, but once strips have been incubated with different antibodies or several dilutions, they should be kept separate for the remainder of the procedure to avoid contamination of reagents.

Step 2. Block the membrane by immersing in the **diluted blocking buffer** working solution for **1 h** at room temperature. The membrane may be stored overnight at 2-8 ^{o}C while immersed in the blocking buffer. The membrane may be air dried and stored in a moist bag at 4oC.

Step 3. React whole membrane or strips with primary antibody or serum samples (diluted with the antibody diluent working solution). It may be desirable to perform serial dilutions to determine the optimal working dilution of the antibody. **Incubation with the primary antibody for 1h** at room temp. is usually sufficient.

Step 4. **Wash** the membrane or strips **three times** with diluted wash buffer by gently shaking at 180 rpm for 5 min. or longer per wash. Sufficient solution must be used to completely immerse the membranes.

Step 5. Incubate with the diluted Ab-enzyme conjugate (e.g., diluted 1:5K for example). **The optimal dilution may vary for different assay systems and it may be desirable to test serial dilutions to determine the optimum working dilution of the enzyme conjugate**. Incubate at room temperature for **1 h**. A gentle shaking may be used to assure proper distribution.

Step 6. Perform five-minute **washes** (4 times) as described in step 4. After the last wash, completely drain excess solution.

Step 7. **Incubate** the membrane with premixed chemiluminescent substrate solns. (equal volumes of solutions A & B) for **one minute**. The volume of substrate solution should be enough to allow free movement of membrane/strips and they should be completely immersed in soln. It is not necessary to protect the membrane or solution from light.

Step 8. Remove the membrane or strips from the substrate solution and drain the excess liquid. Wrap the membrane with Saran Wrap.

Step 9. Immediately measure light emission using a camera luminometer or a film. If using film, lay membrane or strips flat

between sheets of clear plastic and expose to the X-ray films (exposure time may vary between few seconds to 30 minutes). Be sure that the surface of the membrane to which the protein was applied is facing the film. A typical exposure time on X-ray film is 1-15 minutes. The optimal exposure should be determined for each application or assay system. Do not allow the X-ray film to get wet and be careful that the membrane and film are not moved in relation to one another during the exposure.

Step 10. Develop film according to the manufacturer's instructions.

TROUBLE SHOOTING AND GENERAL NOTES

1. Always incorporate a positive control, negative control, and reagent control (blank).
2. Use of a protein stain such as Amido black or StainALL, to confirm transfer of protein from gel to membrane, is recommended.
3. The high sensitivity of chemiluminescent substrate may allow reduction in antibody concentration or incubation times. Optimal dilutions and incubation times must be determined for each application.
4. Remove as much buffer as possible after washes, but do not allow the membrane to dry out.
5. Use reagent quality water for the preparation of all reagents.

Causes of Excess Signal or Background

1. Insufficient dilution of the peroxidase labeled antibody is the most common cause of high background. Try more diluted enzyme conjugate solution.
2. Excessive antibody incubation times. Try short incubation at RT.
3. Inadequate washing or blocking procedures. Increase number of washings.
4. Excessive amounts of protein loaded onto the gel. Load reduced concn.
5. Excessive exposure time of the film (delay of exposure of the membrane to film for several minutes after substrate incubation may enhance the signal-to-noise ratio).
6. Exposure of the film to light during development will cause fogging of the film.

INTRODUCTION

Immunoassays using enzyme labeled antibodies are highly specific for the analyses of a particular protein. Use of an enzyme labeled antibody together with a highly sensitive chemiluminescent substrate provides an excellent method for the detection and characterization of sample proteins bound to membranes through Western blot or Dot ELISA techniques. Following attachment of protein to a membrane, a primary antibody is used to selectively bind the protein of interest. Alternatively, a known protein is bound to the membrane for the screening of specific monoclonal antibodies or serum samples. An enzyme labeled second antibody directed against the primary antibody is then applied. The chemiluminescent substrate, provided as a stable two-component solution, is a luminol-based solution. In the presence of hydrogen peroxide, HRP converts luminol to an exited state dianion that emits light on return to its ground state. The resulting signal can be measured by using a camera luminometer or X-ray films to provide a permanent record. All necessary reagents required for blocking and washing of the membrane and for diluting the antibodies are included in the kit. Sufficient reagents are provided in a kit to process 3000 cm^2 of membrane (200-400 strips).

PRECAUTIONS

The ADI Western blot kit is intended for *in vitro research* use only. The reagents contain thimerosal as preservative; necessary care should be taken when disposing solutions. Gloves should always be worn while handling the membrane and all immunoassay reagents to avoid contamination with skin oils or proteins. All components of this kit are considered to be non-hazardous.

For proper analysis of results, be sure to include positive and negative controls, blanks, and/or protein standards as appropriate.

PREPARATION OF <u>WORKING</u> SOLUTIONS

1. **Blocking Buffer**. Dilute the blocking buffer/antibody diluents concentrate (1:20) with water or PBS. This solution is used to block membranes after the protein transfer. Dilute only in required amounts. Do not store diluted solutions. A preservative (merthiolate or thimerosal (0.02%) can be added. Do not add sodium azide that is an inhibitor of HRP. It is possible to substitute this milk-based buffer if it interferes with the assay or if BSA or gelatin based buffer are known to provide a better signal with a given antibody.

2. **Antibody Diluent.** Dilute the blocking buffer/antibody diluent 1:20 with preferably with PBS or water. This solution is used to dilute