Radiance Plus

Chemiluminescent substrate for the most sensitive Western blotting experiments

Long Protocol for Catalog Numbers

AC2102 Radiance Plus Sample, sufficient for 200 cm^2 membrane

512103 Radiance Plus, 150 ml, sufficient for 1500 cm^2 membrane

Important Information

The following instructions are for use with the Radiance Plus enhanced chemiluminescent substrate kit, catalog numbers AC2102 and AC2103. Please see the Kit Contents section for details.

Storage Information

The Radiance Plus reagents are stable at room temperature for at least one year. For more information, see the Shipping and Storage Conditions section on page 3.

Warnings and Precautions

- Radiance Plus is for research use only.
- Always wear gloves when handling membranes and reagents.
- Refer to MSDS for additional safety information.
- The product is guaranteed to be free of manufacturer defect, and to function as described when the enclosed protocol is followed by properly trained personnel. Please see the Warranty section for more information.

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1. Kit Contents

AC2102

Radiance Plus Chemiluminescent HRP Substrate, Sample size, sufficient for 200 cm^2 of membrane surface

• Radiance Plus Luminol/enhancer solution	7 ml
• Radiance Plus Peroxide Chemiluminescent Detection Reagent	7 ml

AC2103

Radiance Plus Chemiluminescent HRP Substrate, Sample size, sufficient for 1000 cm^2 of membrane surface

• Radiance Plus Luminol/enhancer solution	50 ml
• Radiance Plus Peroxide Chemiluminescent Detection Reagent	50 ml

2. Shipping and Storage Conditions

Product may be shipped at ambient temperature. No extra temperature control or insulation is required. All shipping methods (ground and express) are acceptable.

The Radiance Plus reagents are stable at room temperature (up to +25°C) for at least one year. Accidental freezing does not significantly affect the performance, but multiple freeze-thaw cycles are not recommended.

3. Additional Materials Required

- Electrophoresis apparatus and buffers for SDS-PAGE
- Tank and buffers for electrophoretic transfer of proteins from gel to membrane
- Nitrocellulose or PVDF membrane, cut to size of gel. All membrane products available from Azure (see the Related Products section) are compatible with Radiance Plus.
- Washing buffer (PBS-T or TBS-T). For best results, use Azure Fluorescent Blot Washing Buffer (see the Related Products section)
- Blocking buffer
- Primary antibody compatible with your application
- Secondary antibody, conjugated to Horseradish peroxidase (HRP) corresponding to your primary antibody (see the Related Products section)
- CCD-based detection system, or film (see the Related Products section)

4. Background

Radiance Plus is a horseradish peroxidase (HRP) substrate specially developed for Western blotting detection of very low abundance proteins. Radiance Plus produces a strong, long-lived signal, which, combined with very low background levels, allows for long exposure times enabling the detection of low-abundance proteins. Additionally, the signal from Radiance Plus is linear with respect to protein amount over a broad range of concentrations, allowing the user to accurately quantify protein bands. Radiance Plus is most suitable for detection of low-abundance proteins or in situations when amounts of available primary antibodies are very limited and high dilution factors are desired, or when primary antibodies have relatively low binding constants. Radiance Plus is also compatible with X-ray film detection, though the limited dynamic range of film will make resulting data less quantitative. Radiance Plus also produces a chemifluorescent signal that can be detected with a fluorescence imaging system using appropriate excitation and emission settings.

5. Western Blotting

Western blotting is a protein analysis tool that has become commonplace in a molecular biology and protein chemistry laboratory. The principle of chemiluminescent Western blotting is shown in Figure 1. The general protocol, including the role of Radiance Plus, can be seen on page 7. Proteins are separated by size via electrophoresis, and then transferred electrophoretically from the gel to a membrane support, usually nitrocellulose or PVDF. This membrane containing the transferred proteins is commonly referred to as a blot. The location of a protein of interest is detected on the blot by applying the primary antibody, which binds to the protein. The primary antibody bound to the blot is then visualized using a secondary antibody that binds to the primary antibody. The secondary antibody is labeled in some way to make it detectable, such as with a radioactive isotope or an enzyme that can be detected by its activity.

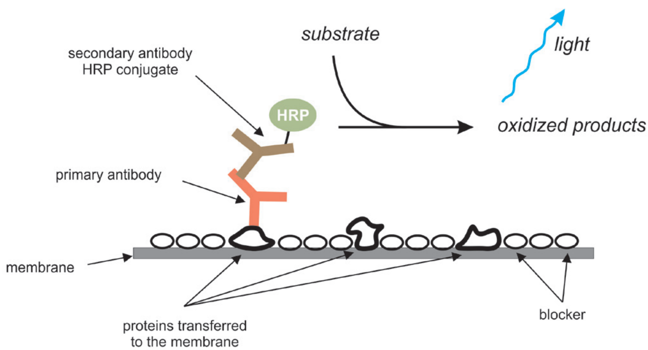


Figure 1. The principle of chemiluminescent Western blotting.

Since 1988, enhanced chemiluminescence or ECL (1) has become one of the most common detection methods in Western blotting (2). In this method, the secondary antibody is conjugated to the enzyme Horseradish peroxidase (1,2). Once bound to the membrane, the secondary antibody is detected by incubating the blot with a solution containing an HRP substrate that generates a light-emitting product after reaction with HRP (Figures 1, 2). The chemiluminescent signal can be detected by exposing the blot to X-ray film, or by imaging with a CCD camera.

Radiance Plus is an enhanced chemiluminescent substrate specially formulated to achieve high sensitivity. Radiance Plus produces a bright signal with very low background for femtomolar detection levels. Additionally, the Radiance Plus signal is long lasting, which combined with the low background, allows long-term exposures to detect low-abundance proteins.

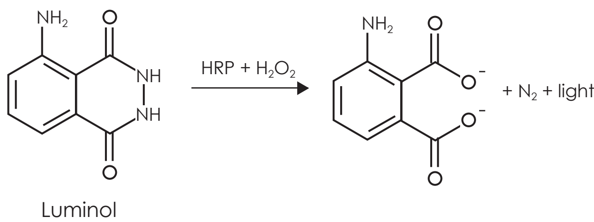
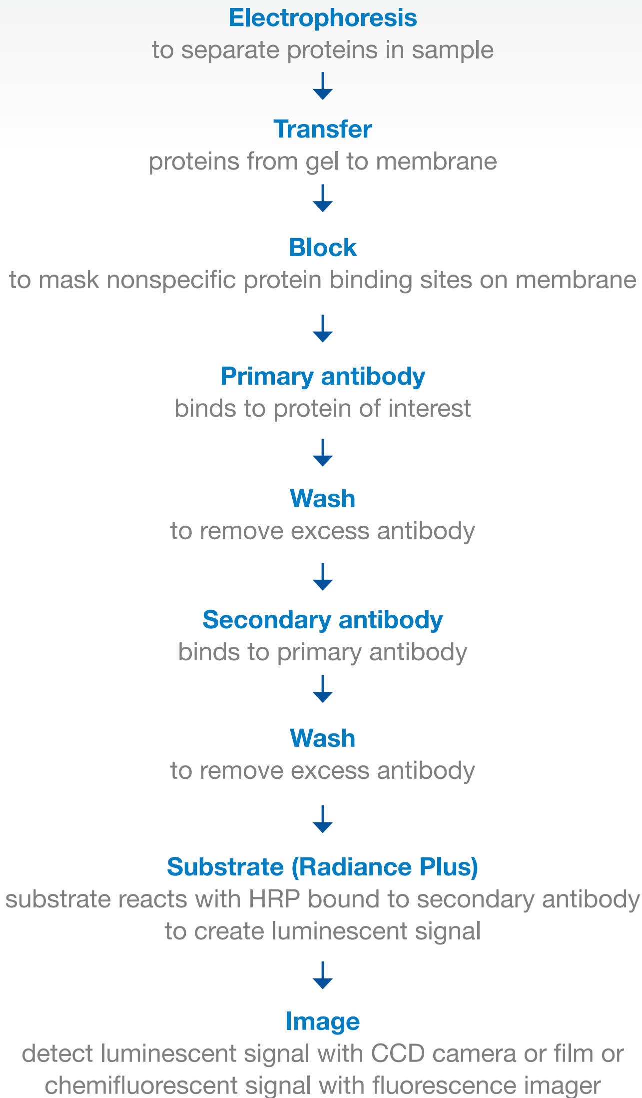


Figure 2. Chemiluminescence of luminol.

6. Overview of the Protocol for Chemiluminescent Western Blots



7. Quick Protocol

For additional information, see the detailed protocol which follows.

Step	User Notes
1. Prepare your protein blot	
2. Block membrane for 1 hour at room temperature (RT)	
3. Incubate blot with primary antibody for one hour at RT with gentle agitation	
4. Wash blot: • 1 x quickly • 1 x 15 min, with 0.7 ml/cm^2 membrane • 3 x 5 min, with at least 0.3 ml/cm^2 membrane each time	
5. Incubate blot with secondary antibody for one hour at RT with gentle agitation	
6. Wash blot: • 3 x 5 min, with at least 0.3 ml/cm^2 membrane each time	
7. Mix Radiance Plus components 1:1 to obtain 0.1 ml/cm2 and place on blot for 2 minutes	
8. Drain excess reagent	
9. Cover damp blot with plastic wrap and image with CCD camera or by exposure to X-ray film or with fluorescence imaging system	

8. Detailed Protocol

Step	Notes
1. Prepare a protein blot	
1.1. Separate the protein sample(s) via electrophoresis 1.2. Transfer proteins to membrane • Prewet membrane in transfer buffer, and assemble transfer sandwich according to tank manufacturer's instructions. • Dot-blots or slot blots can also be detected with Radiance Plus.	• Any electrophoresis system and buffer, such as Laemmli, is compatible with Radiance Plus. • A wet or tank transfer method is preferred, though semi-dry methods should also be compatible. We have found that the buffer system developed by Bolt et all (3) works well. • Both nitrocellulose and PVDF membranes are compatible with Radiance Plus. • If using PVDF, first wet membrane with a 1 min incubation in 100% MeOH followed by water for ~5 min and then transfer buffer for 5-10 min. • For slot blot applications, nitrocellulose is much more convenient than PVDF because it is more difficult to avoid bubbles with PVDF.
2. Block membrane	
• Incubate the blot in a blocking buffer with gentle agitation for 1 hour at room temperature (RT). Use 0.2 to 0.5 ml of blocking buffer per cm^2 of blot to provide adequate blocking.	• Blocking masks non-specific protein binding sites on the membrane, reducing background and increasing the specificity of binding of the primary antibody to the protein of interest. • The optimal blocking buffer will depend in part on the nature of the antigen of interest, and on the quality of the primary antibody. Common blocking agents including non-fat dry milk have been found to be compatible with Radiance Plus. • 10 to 20 ml is usually sufficient for a typical 7 x 9 cm mini-blot.
3. Incubate blot with primary antibody	
• Dilute primary antibody in blocking buffer. • Incubate blot with primary antibody solution for 1 hour at RT with gentle agitation.	• Optimal primary antibody dilutions must be determined empirically. • For CCD imaging, we recommend primary antibody dilutions from 1:1000 to 1:10,000. A good initial dilution is 1:5000.

8. Detailed Protocol, continued

Step	Notes
3. Incubate blot with primary antibody, continued	
	• If the blot will be imaged on film, use 2–5x less primary antibody than for CCD imaging. For example, if 1:1000 dilution of the primary antibody was optimal for CCD detection, 1:5000 is suitable for film detection. • Antibody can be added to a dish and placed on a shaker, or a smaller volume (5-10 ml) can be used by sealing the blot into a bag and placing it on a rotary or rocking platform.
4. Wash blot to remove excess primary antibody	
• 1 x quickly • 1 x 15 min, with 0.7 ml/cm membrane • 3 x 5 min, with at least 0.3 ml/cm^2 membrane each time.	• For best results, use Azure Fluorescent Blot Washing Buffer (AC2113) which is optimized for chemiluminescent as well as fluorescent blots. PBS-T or TBS-T are also compatible with Radiance Plus. • We recommend washing or rocking blots in a clean dish on a shaker to provide gentle agitation. • For example, a standard 7x9 membrane requires: ~50 ml of washing solution for the 15 min wash; and ~20 ml of washing solution for 5 min washes.
5. Incubate blot with secondary antibody	
• Dilute secondary antibody in blocking buffer. • Incubate blot with secondary antibody solution for 1 hour at RT with gentle agitation.	• Optimal secondary antibody dilutions must be determined empirically. • We recommend secondary antibody dilutions of 1:5,000 to 1:20,000. A good initial dilution is 1:10,000. • If the blot will be imaged on film, use 2–5x less secondary antibody than for CCD imaging. 1:50,000 dilution is a good starting point for film detection. • See also notes for step 3.
6. Wash blot to remove excess secondary antibody	
• 3 x 5 min, with at least 0.3 ml/cm^2 membrane each time.	• See notes for step 4.

Step	Notes
7. Incubate blot with Radiance Plus	
• Mix components 1 and 2 in a 1:1 ratio in sufficient amounts to obtain at least 0.1 ml/cm^2 of the blot and add to the blot. • It is better to prepare the working solution just before use. However, mixed reagent is stable for several hours at RT. • Allow substrate to react with blot for 2 minutes.	• Be careful not to touch or put pressure on the blot as this can result in non-specific background. • Use only plastic forceps, not metal; metal forceps damage the blocked surface, creating new adsorption sites. Also, traces of metal may act as a catalyst for non-enzymatic substrate oxidation, resulting in very high background. • The minimal amount of working reagent is 0.1 ml/cm^2. For example, for a 7 x 9 cm blot, this minimal volume is 7x9x0.1=6.3 ml. • If using the minimal amount of working reagent, incubation may be done without agitation. Make sure the membrane surface is level so adequate reagent is held by surface tension. • Incubation may also be done with gentle agitation in a tray just slightly larger than the membrane. Increase the reagent volume as necessary to ensure the membrane is adequately covered with reagent.
8. Drain excess reagent	
	• Remove excess substrate via capillary action by touching a KimWipe® or other absorbent material to the edge of the blot.
9. Image blot	
• While blot is damp, cover with transparent plastic wrap and either place blot in CCD imager, or expose blot to film. • If you are going to use fluorescent imaging, incubation time with the substrate may be increased to 5 to 10 minutes.	• We recommend trying three exposures; 30 sec, 2 min, and 5 min. • The blot can be imaged and re-imaged for several hours; for medium intensity bands about 60% of the initial signal will remain after 60 minutes, and substantial signal will remain after 8-10 hours. • Excitation range is from 430nm to 490nm and can be done with most blue light sources. Optimal emission range is from 500nm to 530nm.

8. Detailed Protocol, continued

Step	Notes
9. Image blot, continued	
• Remove excess reagent by blotting the membrane against clean filter paper or tissue.	• Products of the substrate oxidation during the reaction with HRP are insoluble in water and will remain adsorbed to the membrane. • Membrane may be imaged while still damp, or it can be dried completely. The best imaging conditions should be determined by the user depending on the imaging instrument.

9. Troubleshooting & FAQ

Western blotting can require substantial optimization due to the multiple steps involved. The correct amount of protein to load on the gel and the best dilutions of primary and secondary antibodies must be determined empirically. Some common questions are addressed below:

Problem	Possible Soutions
High background	• Reduce primary antibody concentration by increasing the dilution factor. • Try a different blocking buffer. • Try a shorter exposure time. • Increase washing time.
No or low signal	• Check that correct primary antibody used. • Check that secondary antibody recognizes primary (for example if the primary is a rabbit antibody, that the secondary is goat-anti-rabbit).
White spots within bands	• Improve transfer, making sure to remove any bubbles between the gel and the membrane.
Speckled background	• Filter secondary antibody. • Filter blocking and washing buffers. • Ensure that the laboratory environment is clean, to minimize dust, debris or any other particles that might come in contact with the blot. Cover the dish during incubation or washing steps. • Use non-powdered gloves, or switch to a different kind of gloves. We recommend powder-free nitrile gloves or polyethylene gloves.

10. References

1. Thorpe GH, Kricka LJ, Moseley SB, Whitehead TP, Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminolhydrogen peroxide reaction: application in luminescence-monitored enzyme immunoassays. *Clin Chem.* 1985 Aug; 31(8): 1335-41.
2. Leong MM, Fox GR., Enhancement of luminol-based immunodot and Western blotting assays by iodophenol. *Anal Biochem.* 1988 Jul; 172(1): 145-50.
3. Bolt M.W., Mahoney P.A, High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide gel electophoresis. Anal Biochem. 1997 May 1; 247(2): 185-192.

11. Related Products

Catalog Number	Product	Size
AC2113	Azure Fluorescent Blot Washing Buffer	500 ml
AC2114	Goat-anti-rabbit HRP-conjugated secondary antibody	500 µl
AC2115	Goat-anti-mouse HRP-conjugated secondary antibody	500 µl
AC2105	Low Fluorescence Western Membrane (PVDF) 7x9 cm	10 sheets
AC2106	Nitrocellulose Transfer Membrane 0.45 µm 7x9 cm	10 sheets
AC2107	Nitrocellulose Transfer Membrane 0.22 µm 7x9 cm	10 sheets

12. Warranty

This product is warranted to be free of defects of material or workmanship, and to perform as described in the published specifications when stored according to the documentation included with the product, and used according to the accompanying instruction manual by appropriately trained personnel. If the product is found to have a defect upon first use and within 30 days of shipment, the product may be replaced. This warranty extends only to the original purchaser of the product. There is no obligation to replace the product as a result of misuse, improper storage, or negligence of the buyer.

13. User Notes

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