AvivaBlot™ ECL Reagents

AvivaBlot™ Pico One (OKCF00001)
AvivaBlot™ Femto (OKCF00002)
AvivaBlot™ Ultra Femto (OKCF00003)

Instructions for Use

Enhanced Chemiluminscent (ECL) Substrates for Western Blotting

This product is intended for research use only and
shall not be used in any clinical procedure or for diagnostic purposes.

Table of Contents

1. Brief Protocol

Product Name	Catalog, Size	Features
AvivaBlot™ Pico One ECL Reagent	**OKCF00001, 1 X 10 mL**	One component, premixed RT Storage, ready to use Mid-picogram detection Ideal for everyday detection needs
	OKCF00001, 1 X 250 mL	
AvivaBlot™ Femto ECL Reagent	**OKCF00002, 2 X 5 mL**	Two component, RT storage, working solution stable for 3 days High range flexibility, mid-femtogram detection Extended signal duration
	OKCF00002, 2 X 125 mL	
AvivaBlot™ Ultra Femto ECL Reagent	**OKCF00003, 2 X 5 mL**	Two component, RT storage, working solution stable for 3 days Low femtogram detection Market leading sensitivity for rare and precious samples
	OKCF00003, 2 X 50 mL	

1. Perform electrophoresis, membrane transfer and antibody incubation and washes.

2. For AvivaBlot Pico One: Apply Pico One room temperature substrate directly to semi-wet blot using 1 mL per 10 cm^3 of membrane. Standard sized mini blots are 8 X 8 cm, 64 cm^2 requiring ~7 mLs of solution and midi blots are 8.5 X 14 cm, 119 cm^2, requiring 12 mLs of solution.

3. For AvivaBlot Femto or Ultra Femto: Femto Part A and Femto Part B 1:1 in a conical tube thoroughly. ~7 mLs of solution and midi blots are 8.5 X 14 cm, 119 cm^2, requiring 12 mLs of solution. Working solutions can be stored at 4° C for up to 3 days but should be warmed to room temperature before use.

4. Apply Femto or Ultra Femto working solution at room temperature directly to semi-wet blot using 1 mL per 10 cm^3 of membrane.

5. Incubate 2 minutes at RT.

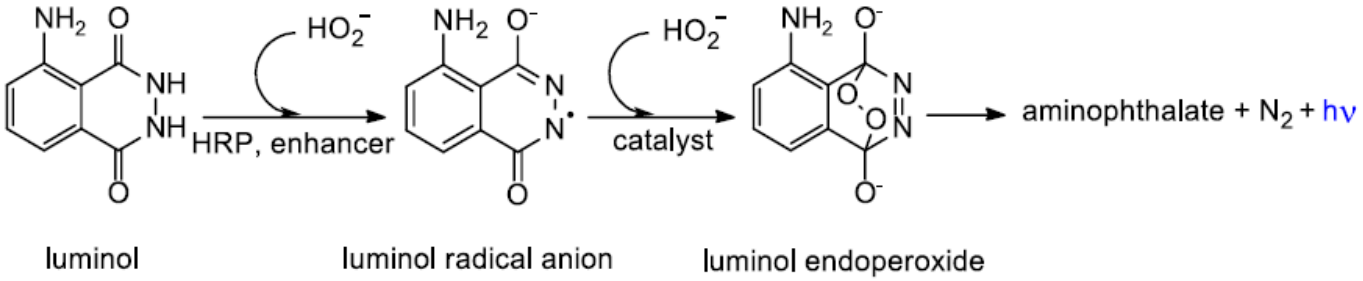
6. Expose membrane using chemiluminescent imager or x-ray film. Use plastic wrap to avoid wetting film where appropriate.

    - Exposure time is empirically determined, typical exposures require 60 to 180 seconds.

2. Background

Principle

The peroxidase-catalyzed oxidation of luminol and its derivatives produces a weak flash of light at 425 nm. The incorporation of an electron transfer mediator into the buffer forces the flash signal into a glow and greatly improves the analytical characteristics of the reaction in terms of increased signal intensity and duration (1,2). Recent works (3÷6) have shown that, by addition of a suitable acylation catalyst, a further large increase in light output is observed.



The Aviva Systems Biology **AvivaBlot™** ECL Reagents are non-isotopic, chemiluminescence substrates, designed for the chemiluminescent detection of immobilized proteins and immobilized nucleic acids conjugated with horseradish peroxidase (HRP**). AvivaBlot Pico One, Femto** and **Ultra Femto** are intended for research use only, and shall not be used in any clinical procedures, or for diagnostic purposes.

1. Kricka, L.J. (2000) Methods Enzymol. 305, 370-390. 2. Heindl, D. and Josel, H.P. (1997) Non-radioactive Analysis of Biomolecules, 258-261. Springer, Berlin. 3. Marzocchi, E., Grilli. S., Della Ciana, L., Prodi, L., Roda, A. and Mirasoli, M., (2008) Anal. Biochem., 377, 189-194. 4. Vdovenko, M. M., Della Ciana, L., Sakharov, I. Yu., (2009) Anal. Biochemistry, 392, 54-58. 5. Vdovenko, M. M., Della Ciana, L., Sakharov, I. Yu., (2010) Biotechnology Journal, 5(8),886-90. 6. Vdovenko, M.M., Zubkov, A.V., Kuznetsova, G.I., Della Ciana, L., Kuzmina, N.S., Sakharov, I. Yu., (2010) J Immunol Methods, 362 (1-2), 127-130.

The AvivaBlot Product line includes 3 separate product options based on the customer's western blotting needs. **AvivaBlot Pico One** is a pre-mixed and stabilized single solution, whereas **AvivaBlot Femto** and **Ultra Femto** are two component solutions. **All products are stable at Room Temperature (18-25°C) for One Year from Date of Receipt.**

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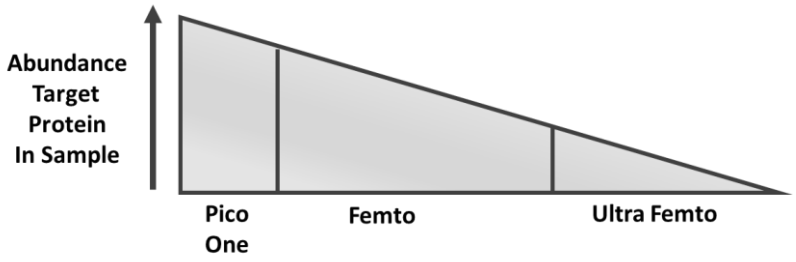


Product selection and use is based on the end user's western blotting needs.

- **AvivaBlot Pico One ECL Reagent** is formulated for general use in detecting abundant proteins such as loading controls and ubiquitous proteins. As a one component solution, Pico One can address basic western blotting needs, for up to 35 mini blots (2500 cm2 of membrane, 1 mL per 10 cm2, 7 mL per application on mini blots typically 64 cm2 each) or up to 21 midi blots (12 mL per application, typically 119 cm2 each).
- **AvivaBlot Femto ECL Reagent** is formulated for high intensity and extended signal duration with coverage over a broad range of protein abundance, from picogram to mid femtogram. Most applications can be accommodated by Femto, and as a two-component system, the blended final solution is capable of processing 35 mini 35 mini blots (2500 cm2 of membrane, 1 mL per 10 cm2, 7 mL per application on mini blots typically 64 cm2 each) or up to 21 midi blots (12 mL per application, typically 119 cm2 each).
- **AvivaBlot Ultra Femto ECL Reagent** is formulated for high performance low femtogram detection when detecting low abundance proteins or otherwise using precious sample types. The extreme sensitivity allows a user to reduce the amount of antibody required per application. Ultra Femto is sufficient for 1000 cm2 of membrane, covering 15 mini gels or 8 midi gels. It is also used at 7 mL per application on mini blots and 12 mL on midi blots.

All three products are available in single application sample size to validate performance in digital imaging or x-ray film chemiluminescent detection methods.

3. Kit Components

• The following reagents are the provided contents of the kit.

Part Number	Description	Quantity	Storage Conditions
OKCF00001	AvivaBlot Pico One ECL Reagent	10 mL (Sample) 250 mL (Standard) Amber Bottle	Room Temperature 1 year from date of receipt
OKCF00002	AvivaBlot Femto ECL Reagent	2 X 5 mL Part A (Amber) & B (White) 2 X 125 mL Part A & B	
OKCF00003	AvivaBlot Ultra Femto ECL Reagent	2 X 5 mL Part A (Amber) & B (White) 2 X 50 mL Part A & B	

For **OKCF00001**, the solution contains a luminol derivative, enhancer and peroxide solution stabilized and ready to use in an amber bottle.

For **OKCF00002 and 3**, Part A contains the luminol derivative and enhancer in an amber bottle, while Part B contains a peroxide solution in a white bottle. Take care to use sterile pipettes when transferring solutions to mix Part A & Part B in a clean conical tube or equivalent.

4. Precautions

• Read instructions fully prior to beginning use of the product.

• Successful western blotting requires adherence to standard protocols involving Sample Preparation, SDS-PAGE, Protein Transfer, and appropriate dilutions of antibodies following blocking of a PVDF or nitrocellulose membrane validated for efficient transfer. For details on Western/Immunoblotting see “ebook”.

• Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.

• For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).

• Product cannot be used beyond 12 months following receipt.

5. Western Blot Detection Procedure

Standard sample preparation, SDS-PAGE, Electro-transfer to membrane, and membrane blocking are all required prior to performing the stand western blot/immunoblotting method. For details regarding these methods, see Aviva Systems Biology website Protocol & Procedure area (**https://www.avivasysbio.com/technical-resources/protocols-procedures**).

Ensure AvivaBlot reagents are at ambient room temperature prior to use in the procedure.

5.1 Following transfer to membrane, ensure consistent transfer in the protocol under use with ponceau S or other staining method. Transfer can also be validated using a "loading control" antibody immunoblot.

5.2 Antibody incubation. Optimal primary and secondary antibody dilutions may vary between different applications and depend on the quality and affinity for the target protein, as well as the abundance of the target protein in the sample. A useful resource for assessing the relative abundance of your target protein can be found by querying various databases such as https://pax-db.org/. While RNA expression profiles may suggest the presence of a given protein in a sample, such correlations are rarely one to one. Aviva Systems Biology recommends considering the relative abundance by leveraging peptide occurrences in mass spectrometry datasets which is performed and query able at Pax-db.org. Consult the table below for rough estimates of both primary and secondary antibody dilution ranges when using AvivaBlot products.

Product	Primary Antibody Dilutions	Secondary Antibody Dilutions
AvivaBlot Pico One ECL Reagent (OKCF00001)	1:100 to 1:5,000	1:1000 to 1:15,000
AvivaBlot Femto ECL Reagent (OKCF00002)	1:1,000 to 1:15,000	1:25,000 to 1:150,000
AvivaBlot Ultra Femto ECL Reagent (OKCF00003)	1:5,000 to 1:100,000	1:100,000 to 1:300,000

5.3 Following western blot processing and incubation, prepare **AvivaBlot Femto** or **Ultra Femto** by mixing 1:1 for the volume required based on the surface area of the membrane. Do not contaminate Part A or Part B bottles with used pipettes. Conical tubes of the appropriate size may be used for preparation. Typically, **7 mL** of working solution is sufficient for mini blots and **12 mL** of working solution is sufficient for a midi blot. **AvivaBlot Pico One** can be used immediately without dilution or mixing. While the working solution is stable at room temperature for up to 3 days post mixing, optimal conditions require fresh reagent for each use.

5.4 Remove the membrane from tray containing TBS-T Buffer, rinse the membrane twice with TBS-T Buffer, and equilibrate in TBS until ready to apply AvivaBlot working solution.

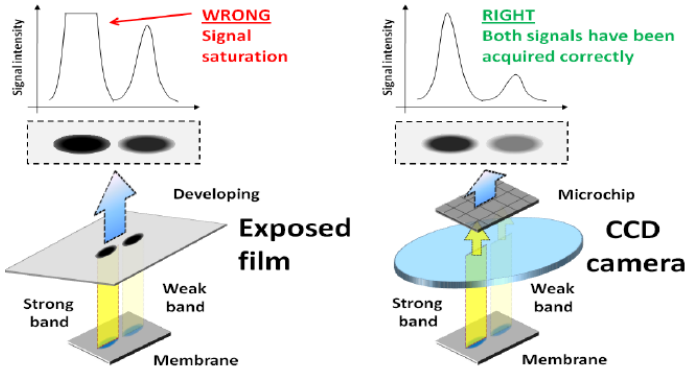
5.5 Use 0.1 mL of **Pico One** or **Working Solution (Femto or Ultra Femto)** per cm2 of membrane. Allow excess buffer to run off from a corner. Ensure the membrane does not dry out but stays moist after removal of excess reagent. Pipette the entire volume onto the membrane, protein side up and incubate for 1.5 minutes (90 seconds) ensuring that the entire surface is covered.

5.6 Acquire the chemiluminescent signal with autoradiography film or imaging device. For an unknown target, expose for 15s, 30s, 1 min, and 5 min to evaluate signal.

6. Interpretation

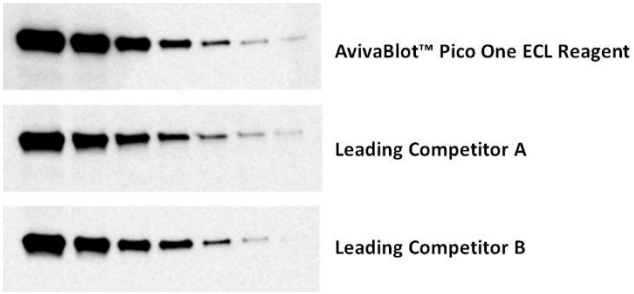
Autoradiography film vs. imaging devices

Currently, western blotting is used either for absolute quantification (in combination with a calibration curve of the recombinant protein of known concentration) or for quantification of samples relative to a control sample. Through the development of new technologies most imagers offer a wide dynamic range (3 to 5 orders of magnitude) generating a high-quality image compared with the limited linear dynamic range of film (1.5 orders of magnitude). This means that is possible to quantify both strong and weak signals on the same blot with reliable results. Instead, on film strong signals get saturated resulting in a wrong quantitation. See the figure below for an example of issues of saturation with autoradiography film versus a more dynamic CCD camera.



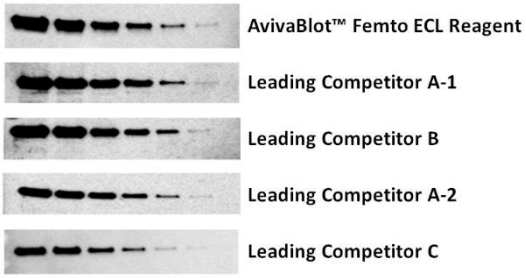
7. Competitive Performance

7.1 AvivaBlot Pico One ECL Reagent (OKCF00001)



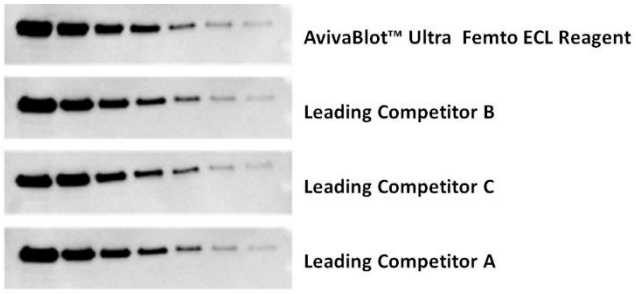
AvivaBlot™ Pico One Competitor Analysis. Hela whole cell lysates were 2 fold serially diluted from 5 ug/lane to 0.008 ug/lane Total Protein. An Anti-HDAC1 antibody was used at 1:2000, with a Goat anti-Rabbit IgG HRP used at 1:20,000. Exposure time of 120 seconds on an GE Imagequant LAS 4000 was used for image acquisition.

7.2 AvivaBlot Femto ECL Reagent (OKCF00002)



AvivaBlot™ Femto Competitor Analysis. Hela whole cell lysates were 2 fold serially diluted from 5 ug/lane to 0.016 ug/lane Total Protein. An Anti-HDAC1 antibody was used at 1:5000, with a Goat anti-Rabbit IgG HRP used at 1:75,000. Exposure time of 180 seconds on an GE Imagequant LAS 4000 was used for image acquisition.

7.3 AvivaBlot Ultra Femto ECL Reagent (OKCF00003)



AvivaBlot Ultra Femto Competitor Analysis. Hela whole cell lysates were 2 fold serially diluted from 2.5 ug/lane to 0.004 ug/lane Total Protein. An Anti-HDAC1 antibody was used at 1:10,000, with a Goat anti-Rabbit IgG HRP used at 1:300,000. Exposure time of 120 seconds on an GE Imagequant LAS 4000 was used for image acquisition.

8. Troubleshooting

Issue	Cause	Solution
High membrane background	High concentration of Primary or Secondary Antibody	Dilute primary and/or secondary antibody according to section 5.2.
	Inefficient Blocking	Increase Tween-20 in TBS-T buffer (0.1% to 0.5% v/v). Use non-fat dried milk as blocking buffer if possible.
	Insufficient Washing	Increase both the volume, length and number of wash steps. Always use sufficient volumes to submerse the membrane.
	Primary antibody is not specific or the protein is not detectable.	Use monospecific or antigen affinity purified antibodies. Always incubate your primary antibody at 4°C overnight and not at room temperature. Reduce NaCl in TBS-T Buffer (100 mM to 350 mM). Use monospecific or antigen affinity purified Ab. Consult resources to determine if you sample has been shown to contain the protein of interest.
	Non-specific binding of the secondary antibody	Confirm the secondary is specific by omitting the primary and running a secondary only blot. If bands develop, choose an alternative secondary antibody. If necessary, use species adsorbed secondaries where necessary.
	Incompatible blocking agent	Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin systems. Substitute with 5% BSA.
	Antibody quality	Quality and age of primary and secondary antibody may lead to background problems. Follow manufacturer's recommended storage and dating criteria.
	Membrane handling	Be sure to handle the membrane only with clean plastic tweezers and non-powdered gloves.
	Contaminated buffer solutions	Check buffers for particulate or bacterial contaminate. Replace old buffers.
Irregular black spots	Air bubble trapped in membrane	Remove air bubbles by gently rolling a clean pipette or a test-tube during sandwich assembling.
	Unevenly hydrated membrane	Make sure that the membrane is fully immersed during washes and antibody incubations.
	Contaminated equipment	Protein or pieces of gel remaining on the unit may stick to the membrane. Antibody can get trapped in the gel, and then are washed out poorly, resulting in intense localized signal.
	Aggregation of blocking agent	When blocking agent is powder stir it over night at 4°C to make sure it is completely dissolved.
	Interaction of membrane with sample tray	Always use clean plastic trays to avoid any type of cross-reaction

	Formation of aggregates in HRP-conjugate	Filter secondary antibody solution through a 0.2 µm filter. Use fresh antibody.
No bands or weak bands	Excessive signal generated	The enzyme in the system depleted the substrate and caused the signal to fade quickly. Further dilute secondary antibody.
	Inefficient transfer	Ensure that there is good contact between membrane and gel during sandwich assembling. High MW protein may require more time for transfer. Reduce voltage or time of transfer for low molecular weight proteins (< 10 kDa).
	Antibodies may have lost activity or degraded	Perform a Dot Blot. Follow manufacturer's recommended storage and avoid freeze/thaw cycles.
	Incorrect secondary antibody used	Confirm host species/Ig type of primary antibody.
	Low protein:antibody binding	Reduce the number of washes to minimum. Reduce NaCl in TBS-T Buffer (100mM minimum). Decrease blocking time.
	Non-fat dry milk may mask some antigens	Decrease milk percentage in Blocking Buffer or substitute with 5% BSA Blocking Buffer.
	Sodium azide contamination	Make sure buffers do not contain sodium azide as this will quench HRP signal.
	Contaminated stock solutions	Do not contaminate the chemiluminescent substrate stock solutions using the same pipette tip. Use new reagents.
Non-specific bands	Aggregation of analyte	Increase amount of reducing agent to ensure complete reducing of disulfide bonds.
	SDS interference	The presence of SDS may result in the development of unspecific bands caused by antibodies binding to the charged SDS molecules associated with the proteins. Wash thoroughly the membrane after transfer with water.
	High protein concentration	A commonly seen effect is the diffusion of protein bands. Reduce the amount of protein initially loaded (~5 ug to 50 ug max).
	Primary antibody is not specific for the protein of interest	Use monospecific or antigen affinity purified antibodies. Always incubate your primary antibody at 4°C overnight and not at room temperature. Reduce NaCl in TBS-T Buffer (range of 100mM to 350mM). Use monospecific or antigen affinity purified antibodies.
	Non-specific binding of the secondary antibody	Confirm the secondary is specific by omitting the primary and running a secondary only blot. If bands develop, choose an alternative secondary antibody.
White/Ghost bands	Excessive signal generated	Excessive antibodies or loaded protein can cause high levels of localized signal. This results in rapid consumption of substrate at this point. Since there is no light production after the completion of this

		reaction, white bands are the result. Try first to further dilute secondary antibody.
Uneven or jagged bands	Uneven gel run	Load all available wells. Empty wells can be loaded with sample buffer.
	Voltage or current too high during electrophoresis	Reduce voltage or current during electrophoresis.
	Effects of high salt in samples	Reduce NaCl concentration in TBS-T Buffer (acceptable range from 100mM to 350mM).

9. Technical Resources

For optimal service please be prepared to supply the lot number of the product used.

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