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p/n KFA011
Rb800/Ms700

*Max*Tag™ Kit for Duplex IRDYE™ Immunoblotting

For 1-color or 2-color immunoblots using Rabbit and Mouse Primary Antibodies

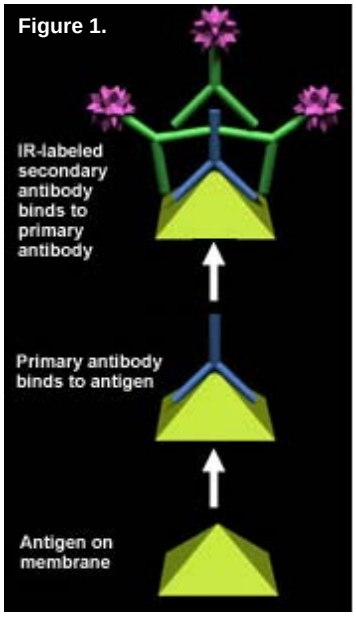
I. Overview

Rockland's ***Max*Tag**™ Duplex IRDYE immunoblotting kit provides for the near infrared fluorescent detection of up to two primary antibodies simultaneously (rabbit and mouse host) using the Odyssey® Infrared Imaging System developed by LI-COR (see Figure 1). IRDye™800 and IRDye™700DX conjugated secondary antibodies are included for the sensitive and economical detection of proteins present on immunoblots. Kit design simplifies the process of infrared fluorescent immunoblotting applications. Straightforward procedures and color-coding add to the ease of use. Controls and specially formulated blocking and washing buffers are provided for your convenience. Detection antibodies included in this kit can process blots up to 2500 cm^2. Maintain kit components at +4°C. Concentrated stocks of IRDye™ antibodies are stable for at least 6 months when properly stored at +4°C.

Please read the entire product insert prior to use.

II. Kit Principle

This kit allows for the individual (1-color) or simultaneous (2-color) detection of primary rabbit antibodies using an IRDye™800 conjugated secondary antibody (green) and primary mouse antibodies using an IRDye™700DX conjugated secondary antibody (red). The user is required to provide all primary antibodies. After protein separation and transfer, the membrane is blocked to prevent non-specific protein binding using a specially formulated blocking buffer for infrared fluorescent immunoblot detection. After washing the membrane, user-provided unlabeled primary antibodies are bound to the antigen. Additional washings are followed by the addition of IRDye™800 and IRDye™700DX conjugated secondary antibodies (see Figure 1). Final washing removes excess reagents and the membrane is further prepared for simultaneous scanning at two wavelengths to detect antibody complexes using a two channel infrared scanner such as LI-COR's Odyssey® Infrared Imaging System. The labeled antibodies are detected directly on the membrane, eliminating film, darkrooms, and messy substrates, while preserving high sensitivity (see Figure 2).



The ***Max*Tag™** Kit for Duplex IRDYE™ Immunoblotting relies on the use of two infrared dyes as labels. Infrared-labeled antibodies enable simultaneous two-color target analysis -- a technique not possible with chemiluminescence or radioactive methods. This kit allows for two-color analysis of proteins on one blot that results in faster and more precise measurements of proteins by eliminating the variability due to stripping or comparing separate blots.

III. Intended Use

Use Rockland's ***Max*Tag**™ Kit for Duplex IRDYE™ Immunoblotting for the detection of Rabbit and Mouse primary antibodies using infrared-labeled antibodies to form an antigen-antibody-antibody complex that is immobilized on a membrane (immunoblot) and detected using a two channel infrared scanner such as LI-COR's Odyssey® Infrared Imaging System. This Kit is useful for both “western blotting” and “dot blotting” methods and can be used for single (1-color) or duplex (2-color) antibody detection (see Figure 3). Nitrocellulose membrane formats are preferred

(see sections XI and XII for more details). If you require additional assistance please call or e-mail our technical service representatives at 800-656-7625 or tech@rockland-inc.com.

IV. Storage and Stability

Undiluted components within this kit are stable for at least six months when stored at +4°C. Individual components are stable for 3-4 weeks after dilution when stored at +4°C.

V. Number of Assays

Infrared-labeled antibodies and controls provided in this kit are sufficient to run approximately 25 immunoblots each measuring 10 cm x 10 cm. The amount of antibody supplied when diluted as recommended in our protocol will yield 1000 ml of working solution. Adjustments in volumes for larger or smaller blots will effect the number of blots detected. Blocking and washing buffers are provided in limited quantities so that the user can immediately begin to process immunoblots. These buffers can be re-ordered in larger quantities for economical use (see "Replacement Parts").

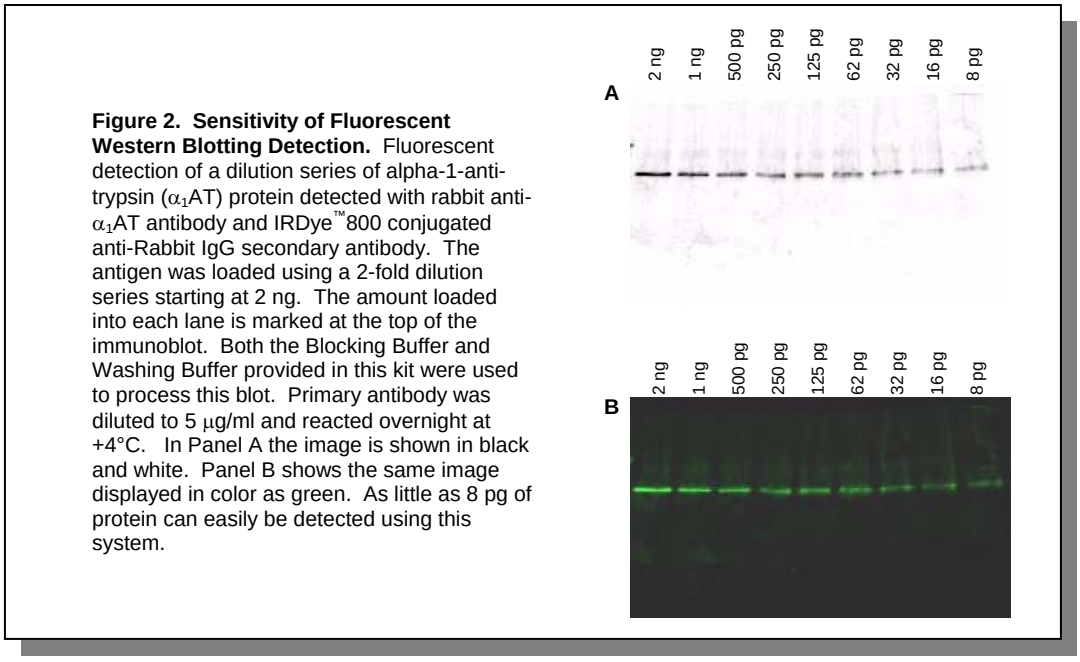


Figure 2. Sensitivity of Fluorescent Western Blotting Detection. Fluorescent detection of a dilution series of alpha-1-anti-trypsin (α_1AT) protein detected with rabbit anti-α_1AT antibody and IRDye™800 conjugated anti-Rabbit IgG secondary antibody. The antigen was loaded using a 2-fold dilution series starting at 2 ng. The amount loaded into each lane is marked at the top of the immunoblot. Both the Blocking Buffer and Washing Buffer provided in this kit were used to process this blot. Primary antibody was diluted to 5 μg/ml and reacted overnight at +4°C. In Panel A the image is shown in black and white. Panel B shows the same image displayed in color as green. As little as 8 pg of protein can easily be detected using this system.

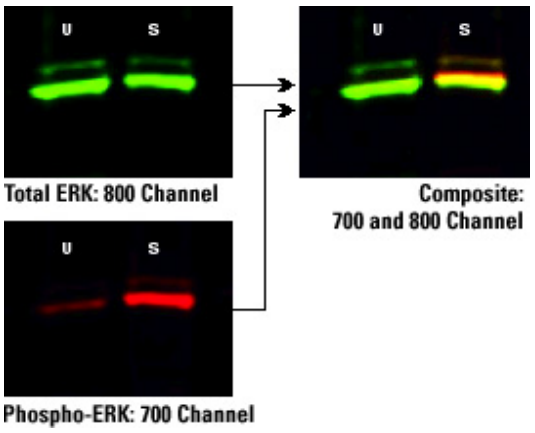


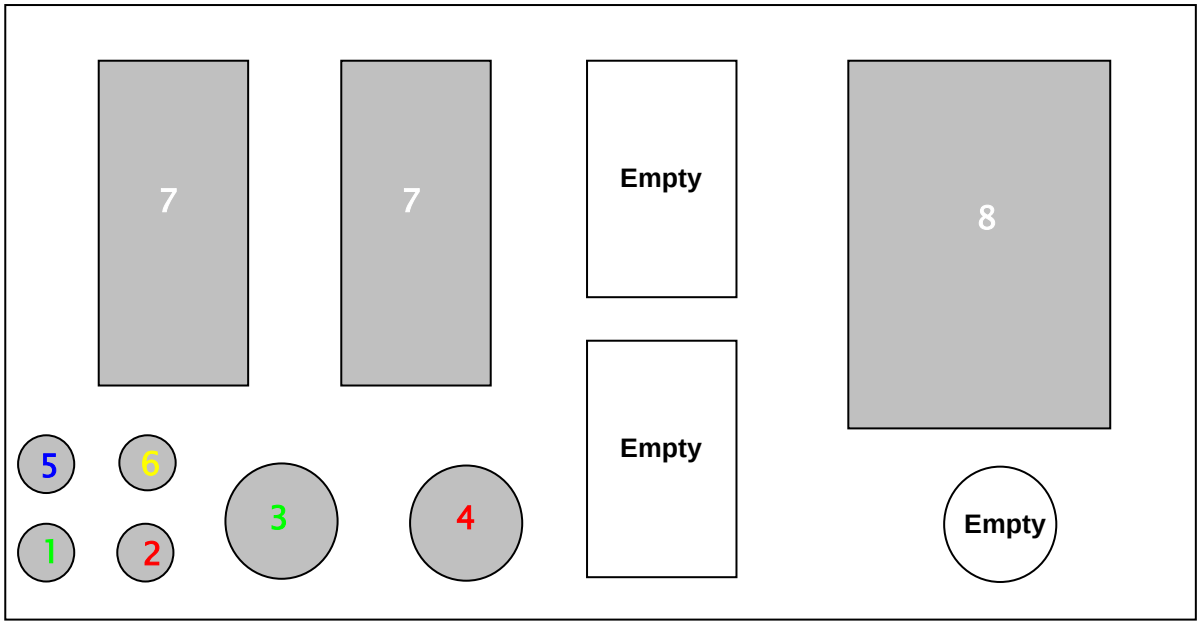
Figure 3. Western Blot Analysis of ERK Activation: ERK1/ERK2 and phospho-ERK were detected simultaneously in lysates of unstimulated (U) and EGF-stimulated (S) A431 cells. Total ERK protein was visualized with rabbit anti-ERK primary Ab and anti-rabbit-IRDye™ 800 infrared dye secondary Ab (green). Tyrosine-phosphorylated ERK was visualized with mouse anti-phospho-ERK primary Ab and anti-mouse secondary Ab (red). The single-color images can be overlaid (composite) to show both results (yellow color indicates overlap of red and green signals). The mobility shift caused by ERK phosphorylation can be seen in the EGF-stimulated sample.

VI. Kit Components and Component Layout

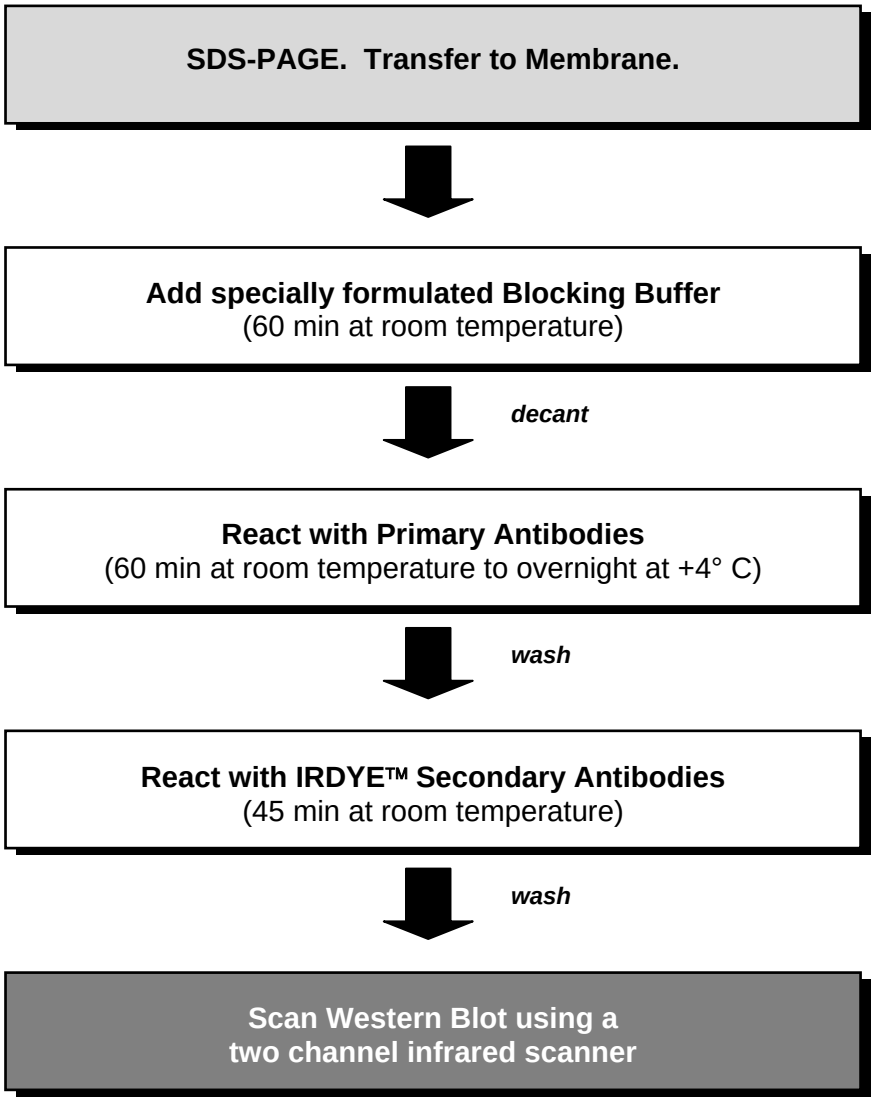
A. Kit Components

1. (1) x 100 µg IRDye™800 anti-Rabbit IgG antibody in amber vial with **GREEN** cap (*lyophilized*)
2. (1) x 100 µg IRDye™700DX anti-Mouse IgG antibody in amber vial with **RED** cap (*lyophilized*)
3. (1) empty 10 ml dropper bottle with **GREEN** cap labeled "Diluted IRDye™800 anti-Rabbit IgG antibody"
4. (1) empty 10 ml dropper bottle with **RED** cap labeled "Diluted IRDye™700DX anti-Mouse IgG antibody"
5. (1) x 0.5 ml Control Rabbit IgG in plastic vial with **BLUE** cap (*liquid*)
6. (1) x 0.5 ml Control Mouse IgG in plastic vial with **YELLOW** cap (*liquid*)
7. (2) x 50 ml 2X Blocking Buffer (specially formulated) in 60 ml Nalgene® plastic bottles with **WHITE** cap
8. (1) x 100 ml 10X Wash Buffer (PBST) in 125 ml Nalgene® plastic bottle with **WHITE** cap
9. Instruction Manual

B. Component Layout



VII. Flow Diagram of *Max*Tag™ Duplex IRDYE™ Kit Procedure



VIII. Preparation of Working Solutions

The ***Max*Tag**™ Kit for Duplex IRDYE™ Immunoblotting comes with a specially formulated 2X concentrate of Blocking Buffer for Infrared Fluorescent Detection (#7) and a 10X concentrate of Washing Buffer (#8) that must be diluted to 1X concentrations prior to use. In addition the kit comes with lyophilized stocks of IRDye™800 conjugated anti-Rabbit IgG antibody and IRDye™700DX conjugated anti-Mouse IgG antibody that must be reconstituted. The user must also prepare working dilutions of these solutions prior to use. Dropper bottles are provided for ease-of-use and are labeled and color coded to match the respective secondary antibody. Just add the appropriate volume of concentrated stock solution to Blocking Buffer (as described below). Replace the dropper-tip and cap, mix the solution, and the working solution is ready for use.

A. **Prepare Blocking Buffer.** Carefully transfer 25 ml of 2X specially formulated Blocking Buffer for Infrared Fluorescent Detection (#7) to a suitable container and then add 25 ml of deionized water (or equivalent). Thoroughly mix the solution. Store at +4° C prior to use. See "Replacement Parts" if you require additional buffer available in 500 ml bottles and also in convenient and economical packs of 3 and 10 bottle units (Rockland p/n MB-070, MB-070-003 or MB-070-010).

B. **Prepare Washing Buffer.** Carefully transfer the contents (100 ml) of 10X Washing Buffer (#8) to a suitable container and then add 900 ml of deionized water (or equivalent). This phosphate based buffer already contains 0.05% Tween-20. Thoroughly mix the solution. Store at +4° C prior to use. See "Replacement Parts" if you require additional buffer available in 1000 ml bottles (Rockland p/n MB-075-1000).

C. **Reconstitute IRDYE™ Conjugated Secondary antibodies.** Add 200 µl of deionized water (or equivalent) to one of the lyophilized (freeze-dried) IRDYE™ conjugated secondary antibodies to reconstitute the antibody solution. Replace the cap on the amber vial and mix thoroughly. Repeat the process for the other IRDYE™ conjugated secondary antibody. The resultant solutions contains 0.5 mg/ml of IRDYE™ conjugated antibody in a stabilized PBS buffer containing sodium azide as a preservative. Store the reconstituted antibody at +4° C for up to 6 months. Protect from direct light.

D. **Prepare Working Solutions of IRDYE™ Conjugated Secondary antibodies.** To prepare 5 ml of diluted secondary antibody solution first transfer 5 ml of Blocking Buffer to the **GREEN** capped dropper bottle labeled "Diluted IRDye™800 anti-Rabbit IgG antibody." Add 2 µl of the reconstituted stock of IRDye™800 conjugated anti-Rabbit IgG antibody from the **GREEN** capped amber vial. Mix thoroughly. Repeat this process for the IRDye™700 conjugate. Transfer 5 ml of Blocking Buffer to the **RED** capped dropper bottle labeled "Diluted IRDye™700 anti-Mouse IgG antibody." Add 2 µl of the reconstituted stock of IRDye™700 conjugated anti-Mouse IgG antibody from the **RED** capped amber vial. Mix thoroughly. For greater volumes of either antibody simply add 2 parts concentrate per 5000 parts Blocking Buffer. Diluted antibody may be stored at +4° C for up to 3 to 4 weeks. Protect from direct light.

IX. Materials Required but Not Supplied

Nearly all components required for immunoblotting are provided for your convenience in Rockland's ***Max*Tag**™ Kit for Duplex IRDYE™ Immunoblotting. Some addition materials are required:

A. SDS-PAGE electrophoresis materials

B. Nitrocellulose for protein transfer and transfer materials

C. Primary Antibodies (this kit is for the detection of Rabbit **AND** Mouse primary antibodies)

D. Rocker platform for gentle mixing during incubations

E. Incubation trays (we recommend black Perfect Western® containers available in many shapes and sizes from www.GenHunter.com for best results)

F. Deionized water

G. Two channel infrared scanner (LI-COR's Odyssey® Infrared Imaging System or other comparable systems)

H. Other blocking buffers (optional)

X. Immunoblot Method

The following method is suggested as a **guideline** for the use of Rockland's ***Max*Tag**™ Kit for Duplex IRDYE™ Immunoblotting using nitrocellulose membranes. The method is optimized for duplex (two-color) detection on the same blot. Two-color detection requires careful selection of primary antibodies so that two different antigens can be detected simultaneously and visualized in different fluorescence channels (700 and 800 nm). Users may modify these directions and use components to stain and process only one antigen (1-color). Nitrocellulose membranes are preferred, although PVDF membranes can be used (see Section XI. "Additional Notes") but are not recommended. After your antigen has been immobilized onto the membrane by transfer, dotting or filtration, carefully follow the numbered steps below to process your immunoblot. Some primary antibodies and/or antigens may require specific conditions other than those stated below. 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. Once processing your blot has started never let a membrane air dry. Add the suggested volumes or just enough volume to cover the membrane to keep it wet. Do not touch the membrane with your skin! Wear gloves.

A. Volumes specified in these directions are given for reactions using a 5 cm x 7 cm tray capable of holding a blot up to 4 cm x 6 cm in size. Users can adjust volumes accordingly depending on specific blot sizes.

B. Wet membranes in PBS for several minutes.

C. Immerse the membrane in specially formulated Blocking Buffer diluted as specified above. Incubate for 60 min with gentle agitation. Be sure to use sufficient blocking buffer to cover the membrane. See Section XI. "Additional Notes" for more information on blocking membranes.

D. Aspirate the Blocking Buffer [1]. Immediately add 5 ml of Rabbit host primary antibody **and** 5 ml of Mouse host antibody solutions (not provided) diluted in Blocking Buffer. The appropriate dilution should be determined by the end user. If unknown, a starting dilution of 1:500 to 1:1,000 is suggested. Greater dilutions often result in lower backgrounds but may require longer incubation times. Incubations for 60 min at room temperature are usually sufficient; however, some more weakly binding antibodies may require longer incubation times. If insufficient signal is detected, try incubating your primary antibody overnight at +4° C. Two-color detection requires that the primary antibodies are raised from different host species, specifically rabbit and mouse for this kit. Only by using different host primary antibodies can they be discriminated by the specific secondary antibodies included in this kit which have different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies, respectively). If primary antibodies compete for binding sites on a specific protein then consider adding the primary antibodies sequentially to modulate specific antibody binding. Add 10 ml of a single antibody if you desire to detect only one antibody on the blot (1-color).

E. Aspirate the primary antibody solution(s). Some solutions of primary antibodies may be saved for re-use. Wash the blot with 4 changes of Washing Buffer for 5 min each. Use at least twice the volume added for your primary antibody solutions.

F. Aspirate the Washing Buffer and add 5 ml of the “Diluted IRDye™800 anti-Rabbit IgG antibody" from the **GREEN** capped dropper bottle and 5 ml of the “Diluted IRDye™700DX anti-Mouse IgG antibody" from the **RED** capped dropper bottle. Incubate for 45 min. Using the recommended incubation trays (see above) can minimize exposure of the membrane and solutions to light. If you are processing only a single primary antibody on the blot (1-color), add only the secondary antibody that recognizes your primary antibody during this step and add an additional 5 ml of Blocking Buffer to obtain the proper final concentration.

G. Aspirate the IRDYE™ conjugated secondary antibody solution(s).

H. Wash the membrane with 4 changes of Washing Buffer for 5 min each. The membrane is now ready to scan. If desired, allow the membrane to dry and store in the dark for future analysis using the Odyssey® Infrared Imaging System. See "Additional Notes" for more information on storing membranes.

I. Scan in the membrane in the 800 nm and 700 nm channels using the appropriate intensity. Remember to protect the membrane from light until it has been scanned. If either signal on the membrane is too strong or too weak, re-scan the membrane at a lower or higher scan intensity. If a 1-color blot is processed, then scan the blot only in the appropriate channel matching the secondary antibody used for detection.

[1] Aspirate using a glass pipette attached to a vacuum. Alternatively, the solution may be poured off away from the immunoblot.

XI. Additional Notes

A. 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 effect the overall performance of the kit. Generally, if a variable is to be modified, only alter one condition at a time.

B. Nitrocellulose or PVDF membranes may be used for protein blotting, but nitrocellulose membrane is recommended for maximum performance. The best results using PVDF may be obtained using Millipore's Immobilon FL membrane (see www.millipore.com for additional details). If using PVDF membranes, pre-wet briefly in 100% methanol and rinse with double distilled water before incubating in PBS. Pure cast nitrocellulose is generally preferable to supported nitrocellulose. Protein should be transferred from gel to membrane by standard procedures. Membranes should be handled only by their edges, with clean forceps. Nylon membranes are not recommended for this application.

C. In some antibody/antigen/lysate systems extended blocking times may be required from several hours at room temperature to overnight at +4° C.

D. Ink from most pens and markers will fluoresce in the Odyssey® system. The ink may wash off and re-deposit elsewhere on the membrane, creating blotches and streaks. Mark the membrane with pencil to avoid this problem.

E. The use of the specially formulated Blocking Buffer for Infrared Fluorescent Detection supplied with this kit is recommended. In some instances, alternative blocking agents may be used. We suggest Users first block membranes with the supplied Blocking Buffer. If high backgrounds are detected, other agents, such as non-fat dry milk, normal goat serum, fish gelatin, or other commercially available blocking agents may be used depending on previous experiences.

F. Adding 0.01 - 0.02% SDS to the working solutions of IRDYE™ conjugated secondary antibodies can dramatically reduce overall membrane background and also reduce or eliminate non-specific banding. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process. The addition of SDS is particularly helpful for reducing the higher overall background seen when PVDF membranes are used. Never add SDS during the blocking step or to the diluted primary antibodies or to the Wash Buffer.

G. Some primary antibodies do not bind well in the presence of mild detergents like Tween-20. In instances where the diluted primary antibody binds antigen weakly, the antibody may be washed away by too much detergent in the Wash Buffer. To compensate for weak binding, replace the PBST with PBS or TBS containing 1% to 3% BSA or 1% to 10% normal goat serum (secondary antibody is goat host).

H. Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wetted for scanning. Keep the membrane wet if you plan to strip and re-use it. Once a membrane has dried, stripping is ineffective. The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry or in PBS buffer at +4°C. Membranes can be re-washed and re-scanned if background staining is too high.

I. Always use enough solution to cover the membrane. Never let the membrane dry during the process. Use care not to touch the membrane with your skin! Wear gloves. All reactions, unless otherwise stated, occur at room temperature. Use a rocking platform set at low speed for gentle agitation for all incubation steps.

J. IgG Controls are provided to ensure the ***Max*Tag™ Duplex IRDYE™** Immunoblotting kit components are performing as described. Spot 1 or 2 µl of each control on your western or dot blot prior to the blocking step. A control Rabbit IgG is provided in a BLUE capped vial (#5) and a control Mouse IgG is provided in a YELLOW capped vial (#6).

K. We recommend adding a prestained molecular weight marker to a lane of your gel prior to electrophoresis and transfer. Blue stained marker proteins will be strongly visible in the 700 nm channel image and also faintly visible in the 800 nm channel. For these reasons always load 1/3 to 1/5 of the amount you would normally use for conventional western transfer. Over loading marker can result in very strong marker bands that may interfere with visualization of sample lanes. Multicolored or "rainbow" markers may not always be visualized with two channel infrared scanner systems.

L. Store the components of this kit at +4° C.

M. Individual components of this kit may be ordered separately (see below).

XII. Suggestions for Superior Results

A. Follow the protocol carefully.

B. No single blocking buffer will be optimal for every antibody/antigen/lysate system. Some primary antibodies may exhibit greatly reduced signal or different nonspecific banding in different blocking solutions. If you have difficulty

detecting your target protein, changing the blocking solution may dramatically improve performance. If your primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for Odyssey® detection.

C. To avoid background speckles on blots, use high-quality deionized or double distilled water for all buffers. Use this water to also rinse plastic dishes before and after use. Never perform Western incubations or washes in dishes that have been used for Coomassie staining.

D. Membranes should be handled only by their edges using forceps. After you handle membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solutions, they can cause spots or streaks of background on the membrane that are difficult to wash away.

E. When scanning, always clean the scanning surface first to remove dust, residue, and smudges that may affect image quality or contaminate the membrane. If using a silicone mat over your membranes, make certain to clean the surface of the mat that will touch the membrane; a dirty mat can deposit dust and residue that will appear as speckles on your processed image. Do not wipe the mat with tissue. This will create lint that leads to speckling especially in the 700 nm channel.

F. Do not wrap the membrane in plastic when scanning.

G. It is strongly suggested that before combining primary antibodies in a duplex (two-color) experiment, the User always perform preliminary blots with each primary antibody alone to determine the optimal antibody dilution and to visualize the expected banding pattern and possible background bands. Slight reagent cross-reactivity may also occur and can complicate the interpretation of your blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody. Use the Control IgGs provided with kit.

H. Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of your primary antibody and the amount of target antigen you want to detect. Suggested dilutions are 1:500, 1:1500, 1:5000, and 1:10,000 (start with the dilution factor you would normally use for chemiluminescent detection). Optimize your primary dilution to achieve the maximum performance of the antibody while at the same time conserving precious reagents.

I. In instances where the amount of antigen to be detected is extremely low, the dilution of the working solutions of IRDYE™ conjugated secondary antibodies may need to be decreased. In these instances, add 4 to 8 μl per 5 ml of Blocking Buffer instead of the recommended 2 μl of concentrated secondary antibody.

J. Store the antibody vial at 4°C in the dark. Do not thaw and refreeze the vial, as this will affect antibody performance. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. Any particulates in the antibody solution can be removed by a brief spin in a micro centrifuge prior to use.

K. Protect membrane from light during secondary antibody incubations and washes.

L. Use the narrowest well size possible for your loading volume when you perform SDS-PAGE to concentrate the target protein.

M. The best transfer conditions, membrane, and blocking agent for your experiments will vary, depending on the antigen and antibody. If you have problems with high back ground or low signal level, a good first step is to try a different blocking solution.

N. For maximum sensitivity, use nitrocellulose membrane for transfer.

O. For proteins <20 kDa, try blotting using Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer greatly reduces binding of transferred proteins to the membrane (for both PVDF and nitrocellulose).

P. Soak the gel in transfer buffer for 5 min before setting up the transfer. Soaking the gel washes away excess SDS so that it will not be carried over into the transfer tank.

Q. To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours). To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at +4°C. Avoid extended incubations with the IRDYE™ conjugated secondary antibodies.

XIII. Troubleshooting Guide

A. Weak or no signal

Blocking buffer is not optimal. The primary antibody may perform substantially better using an alternative blocking buffer. See suggestions given above.

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 60 minutes at room temperature to overnight at +4° C. In some instances increased binding may occur when the incubation temperature is raised to +37° C. If all of the above fails then contact the source of your primary antibody.

Antibody inactivation. Primary or secondary antibody may have lost reactivity due to long or improper storage conditions. Obtain a new aliquot of antibody and confirm performance prior to repeating assay.

Incomplete transfer of proteins. Follow all protocols included with your transfer apparatus. Check transfer buffer choice and blotting procedure. Soak gel in transfer buffer to remove excess SDS prior to transfer. Load prestained molecular weight markers to monitor protein transfer. Especially note whether very large and/or very small proteins are effectively transferred.

Poor binding of IRDye Conjugated anti-IgG. Be sure the source of the primary antibody is appropriate for this kit. Include 1 or 2 μl of Control IgG on your western blot or dot blot to ensure that the ***Max*Tag**™ Kit components are performing as described.

Too much detergent present. Excess detergent will actually wash away primary or secondary antibodies in weakly binding systems. Decrease the amount of Tween-20 or SDS (if used) in diluted antibodies. Recommended SDS concentrations are from 0.01 to 0.02%, %, but some antibodies may require even lower concentrations. Try diluting antibody in 0.5X Blocking Buffer diluted in water.

Too little antigen loaded. Load more protein on the gel. Try using the narrowest possible well size to concentrate antigen.

Protein lost during detection. Extended blocking times or high concentrations of detergents in diluted antibodies may cause loss of protein from blotted membrane.

Protein not retained on membrane during transfer. Allow membrane to air dry for 1-2 h to make transfer irreversible.

B. Uneven blotchy or speckled background

More than one membrane was blocked in small volume. Ensure that adequate volume of blocking buffer is used when more than one membrane is blocked simultaneously. Membranes must all move freely and contact liquid.

Membrane not fully wetted or allowed to partially dry. Keep membrane wet at all times, especially if the membrane is to be stripped and re-probed. If using PVDF, remember to first pre-wet using 100% methanol.

Contaminated forceps or dishes. Always clean forceps after each use. Thoroughly rinse forceps with water and alcohol after each use. Use dedicated trays for membrane incubations. Never use a container that was used for Coomassie staining.

Dirty scanning surface or silicon mat. Clean surface and mat carefully before each use. Any dust, lint or residue will appear as speckles, especially using the 700 nm channel.

C. Multiple signals

Too much protein on the blot. Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 10 μg of total protein per lane.

Too high concentration of primary antibody. Increase the dilution of primary antibody solution. Reduce the incubation time for primary antibody reaction. Increase the concentration of Tween-20 in diluted antibodies.

Cross reactivity between antibodies in a two-color experiment. Double-check the source and specificity of primary antibodies. Confirm that primary antibodies from two different hosts are used. Decrease the amount of secondary antibody used.

Signal bleed through from one channel to the other. If the signal in one channel is very strong (near or at saturation) it may generate a small amount of bleed through signal in the other channel. Rescan the gel using a lower sensitivity setting in the problem channel.

D. High background / Poor signal-to-noise ratio

Membrane blocking. Try alternate blocking buffer without Tween-20 or BSA. Compare different blockers to determine the optimal blocking conditions for your specific system. Try blocking for longer times.

Nitrocellulose background. Make certain thatTween-20 is added to the diluted antibodies to reduce background. Try adding SDS to diluted secondary antibodies.

PVDF background. Reduce the amount of Tween-20 added to the diluted antibodies. Try adding SDS to diluted secondary antibodies.

Insufficient Washing. Increase the number of wash steps and the volume of wash buffer used for each wash. Try increasing the Tween-20 concentration in the wash buffer up to 0.2% for more stringent washing conditions.

Antibody concentration too high. Optimize the dilution of the primary antibody.

Inadequate antibody volume used. Increase the volume of antibody solutions so that no area of the membrane is allowed to dry.

Membrane contamination. Always handle membranes with clean forceps by the edges of the membrane only. Do not allow the membrane to dry. Always use clean trays for incubations.

XIV. References

Antibodies, A Laboratory Manuel. Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988.

Current protocols in Molecular Biology. J. Ausebel, et al, eds. John Wiley and Sons, New York.

Molecular Cloning: A Laboratory Manuel. 2nd Edition. J. Sambrook, E.F. Fritsch and T. Maniatis, eds. Cold Spring Harbor Press, 1989.

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.

Odyssey® Western Blot Analysis, October, 2004. LICOR Biosciences. Doc# 988-07737.

XV. Trademarks

XVI. USDA Certification

All products of animal origin manufactured by Rockland Immunochemicals are derived from starting materials of North American origin. Collection was performed in United States Department of Agriculture (USDA) inspected facilities and all materials have been inspected and certified to be free of disease and suitable for exportation.

XVII. Additional Products and Replacement Parts

Additional Products	Code	Size	Price
***Max*Tag™** Kit for Duplex IRDYE™ Immunoblotting Rb800/Ms700	KFA011	1 each	$225
***Max*Tag™** Kit for Duplex IRDYE™ Immunoblotting Ms800/Rb700	KFA012	1 each	$225

Replacement Parts	Code	Size	Price
IRDye™800 anti-Rabbit IgG antibody	611-132-122	500 µg	$185
IRDye™700DX anti-Rabbit IgG antibody	611-130-122	500 µg	$185
IRDye™800 anti-Mouse IgG antibody	610-132-121	500 µg	$185
IRDye™700DX anti-Mouse IgG antibody	610-130-121	500 µg	$185
Ultra Pure Tween-20	TW0020	50 ml	$20
BSA - IgG and Protease free	BSA10	10 g	$35
Normal Goat Serum (NGS)	B304	10 ml	$65

Replacement Parts	Code	Size	Price
Control Mouse IgG	KFC002	0.5 ml	$25
Control Rabbit IgG	KFC003	0.5 ml	$25
10X PBST pH 7.2	MB-075-1000	1.0 L	$35
10X PBS pH 7.2 w/ NaN_3	MB-011	1.0 L	$30
Blocking Buffer for Infrared FWB (1X)	MB-070	500 ml	$100
Blocking Buffer for Infrared FWB (1X)	MB-070-003	3 x 500 ml	$270
Blocking Buffer for Infrared FWB (1X)	MB-070-010	10 x 500 ml	$750

User Notes