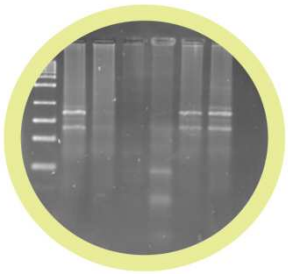
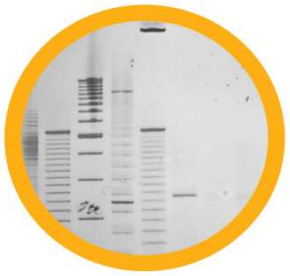
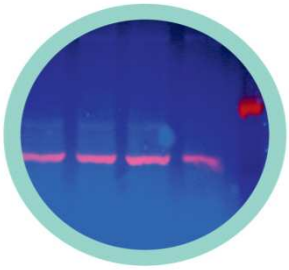


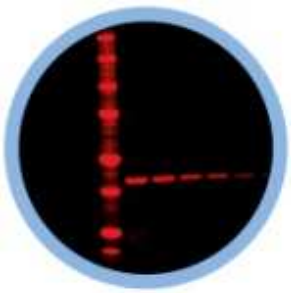


SYNGENE

APPLICATION

TRAINING GUIDE





SYNGENE

A DIVISION OF THE SYNOPTICS GROUP

Contents Page

Lighting and Filter combinations…… 3
Examples…… 6
Chemiluminescence versus Fluorescence Western blot detection…… 8
Film versus digital imaging Chemiluminescence Western blot…… 9
Dot Blot Protocol…… 10
Fluorescent Western blot general protocol…… 11
General Tips and advice for Fluorescence Western blots…… 13
General Tips and advice for IR Western blots…… 15

Lighting and Filter combinations

Fluorescence

Excitation

When a photon of energy supplied by an external source such as a LED or a laser is absorbed by a fluorophore it creates an excited unstable electronic singlet state (when an electron in a molecule with a singlet ground state is excited to a higher energy level).

Emission

When a fluorochrome molecule falls from the excited state to the ground state light is often emitted at a characteristic wavelength.

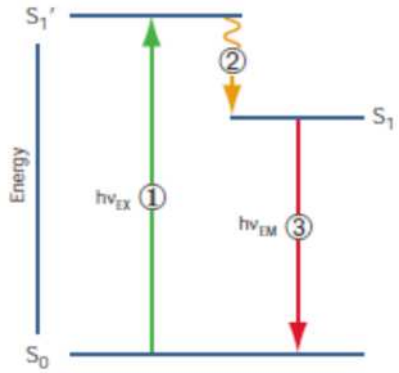
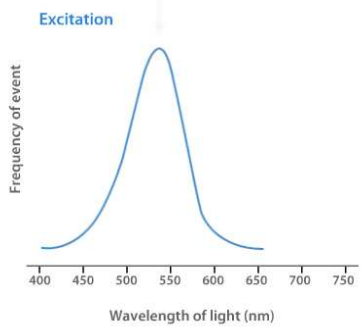


Figure 1- Jablonski diagram illustrating the processes involved in creating an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. 1) Excitation; 2) vibrational relaxation; 3) Emission

Excitation and Emission spectra

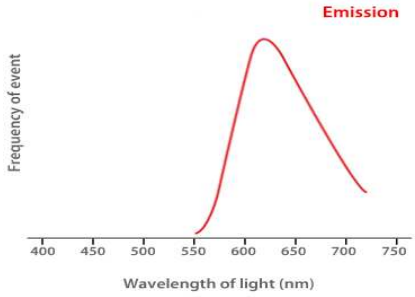
Excitation Spectra

This spectrum is a plot of emitted fluorescence versus excitation wavelength.



Emission spectra

A plot of relative intensity of emitted light as a function of the emission wavelength.



Excitation sources

Light sources fall into two broad categories—wide-area, broad-wavelength sources, such as UV and xenon arc lamps, and line sources with discrete wavelengths, such as lasers. Broad wavelength excitation sources are used in fluorescence camera imaging systems. The spectral output of a lamp is broad, but it can be tuned to a narrow band of excitation light with the use of filters.

Excitation light is delivered to the sample by direct illumination of the imaging field, with the excitation source positioned either above or below the sample.

Filter Selection

Matching a fluorochrome label with a suitable excitation source and emission filter is the key to optimal detection efficiency.
There are three main types of filters;

Long-pass (LP) filters pass light that is longer than a specified wavelength and reject all shorter wavelengths. A good quality long-pass filter is characterized by a steep transition between rejected and transmitted wavelengths. Long-pass filters are named for the wavelength at the midpoint of the transition between the rejected and transmitted light (cutoff point).

Short-pass (SP) filters reject wavelengths that are longer than a specified value and pass shorter wavelengths. Like long-pass filters, short-pass filters are named according to their cutoff point.

Band-pass (BP) filters allow a band of selected wavelengths to pass through, while rejecting all shorter and longer wavelengths. Band-pass filters provide very sharp cutoffs with very little transmission of the rejected wavelengths.
The full-width at half-maximum transmission (FWHM). Band-pass filters with an FWHM of 20–30 nm are optimal for most fluorescence applications, including multi-label experiments.

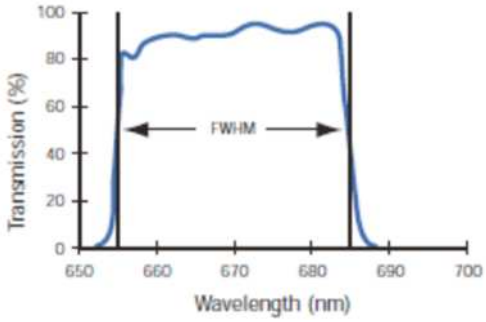
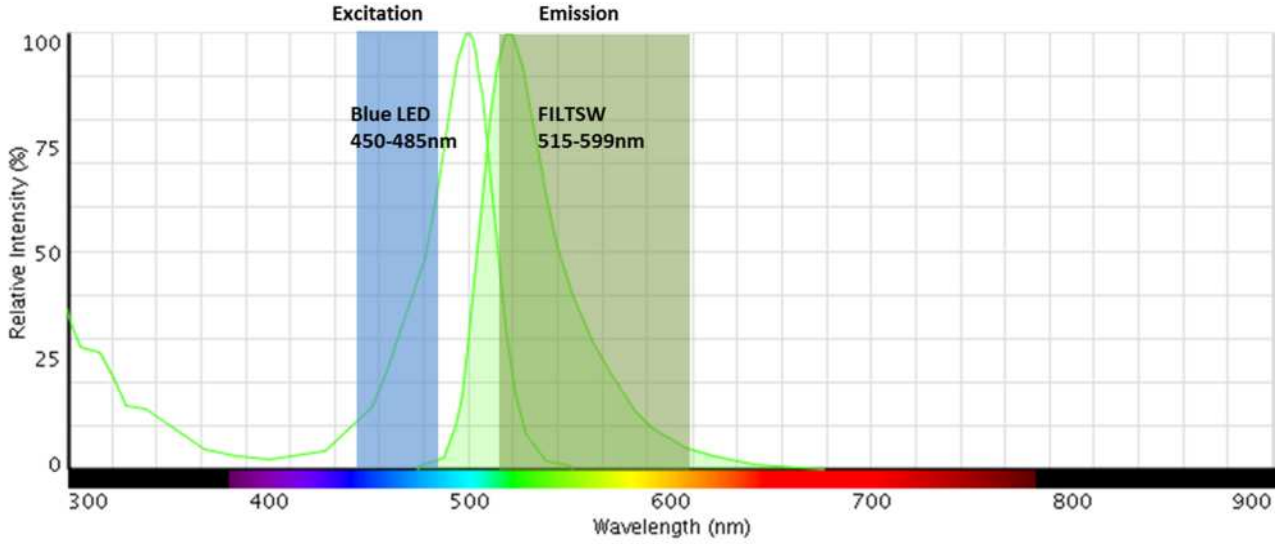


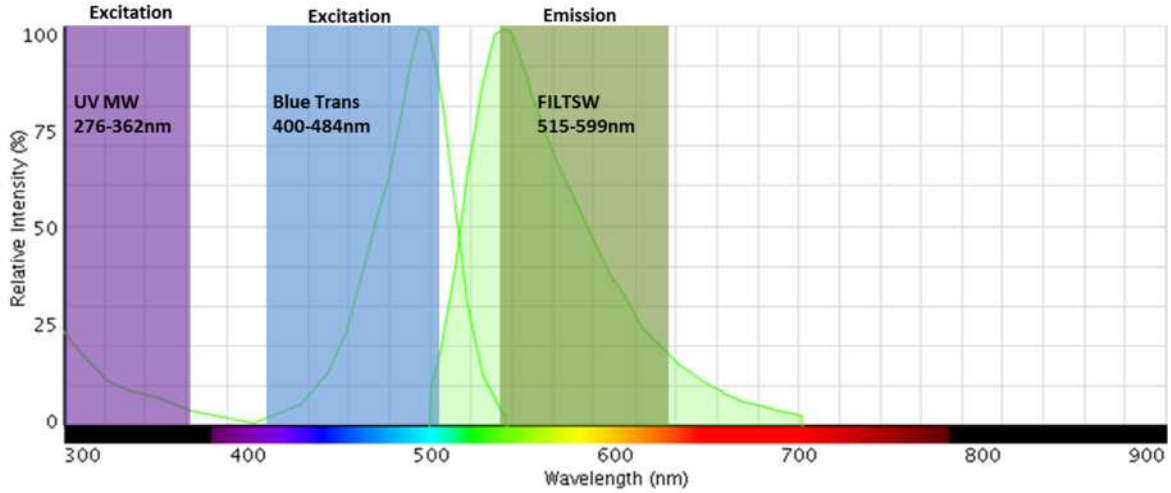
Figure 2- Transmission profile for a band-pass (670 BP30) filter. The full-width at half maximum (FWHM) transmission of 30nm is indicated by the arrows.

Examples

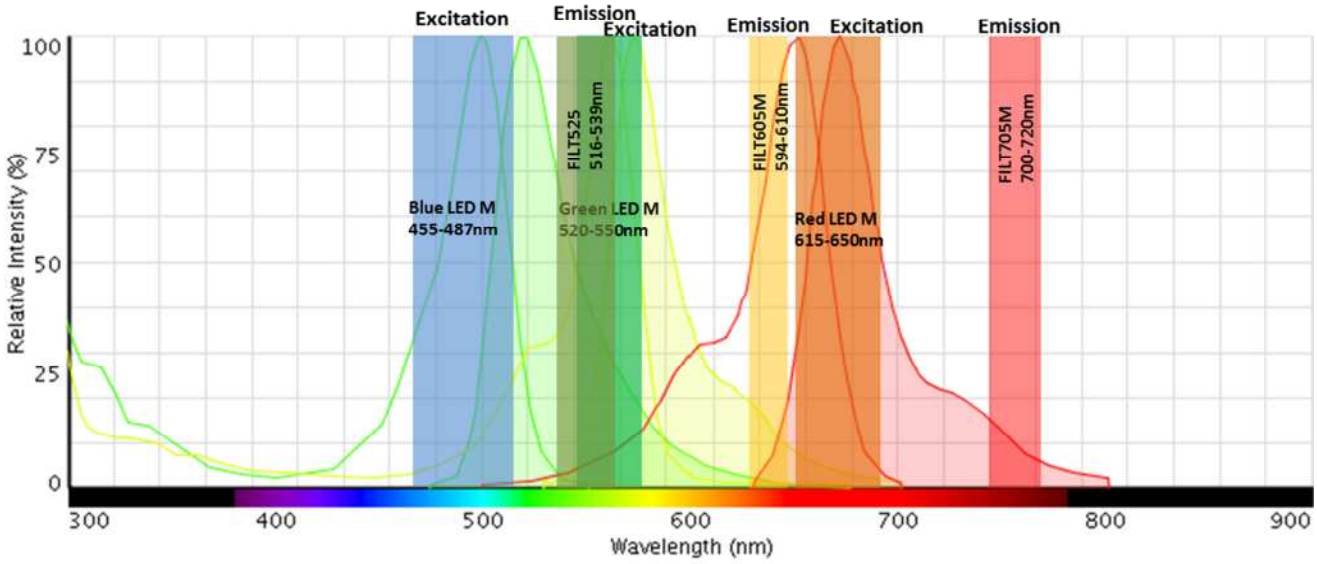
Alexa Fluor 488



SYBR GOLD



Alexa Fluor 488,546 and 647nm multiplex



Chemiluminescence versus Fluorescence Western blot detection

The current trend with many researchers is the move towards changing their Western blot detection method from chemiluminescence to fluorescence and fluorescence multiplexing.

There are several reasons for this trend the main reason is that fluorescent detection allows the user to multiplex their Western blots which allows simultaneous detection of several target proteins at once reducing or eliminating the need to strip and re-probe the membrane which can lead to a loss of sensitivity and is time-consuming.

The following table outlines the advantages of using fluorescence over chemiluminescence.

Feature	Chemiluminescence	Fluorescence
Detection	Single-plex reaction cannot distinguish light from light	Multiplex detection can detect more than 2 fluorophores simultaneously
Quantification	Cannot quantify target protein compared to internal control (housekeeping gene) at the same time. Film has a relatively restricted linear dynamic range.	Easy quantification by comparing one protein's abundance to an internal control (housekeeping gene). Relatively wide linear dynamic range up to 3 orders of magnitude.
Reproducibility	Small differences during set-up e.g. timing, freshness of reagents can change the total light signal output. Enzymatic reaction.	Fluorescence is fairly constant. Given fluorophore will produce a specific amount of light no more no less. Stable.
Sensitivity	Good sensitivity depending on which chemi kit is used. Maybe better for very low abundant protein detection.	Sensitive. Better sensitivity in red and IR wavelengths.
Stability	Depends on chemiluminescent kit used but typically stable for no more than 24 hours. Can image only once for optimum results.	Stable for several months or longer. Can be re-imaged several times without significant loss of signal.

Film versus digital imaging Chemiluminescence Western blot

The question of whether digital imaging is more sensitive than film has been greatly debated over the years. With the advancement of CCD imaging systems more researchers are moving away from exposing their blots to film.

When assessing the performance of both methods the most important criteria to look at are dynamic range and the limit of detection (LOD).

A digital imager typically records from 4,096 to 65,536 different levels of intensity, thus covering a greater dynamic range of grey scale. Thus, the dynamic range digital imagers are on average 2-4 orders of magnitude higher than film. This however is theoretical and in practice the dynamic range is determined from running a dilution series of protein (e.g 10, 5, 2.5ng) to determine the LOD. The following equation is then used

$$\frac{\text{Max (concentration protein)}}{\text{Min (concentration protein)}} = X \quad \text{then } LOG_{10}(X) = \text{dynamic range}$$

The majority of chemiluminescent kits provide a dynamic range of about 2 orders of magnitude with an imaging system.

One of film's major limitations is its limited linear dynamic range for light detection; film is easily saturated by chemiluminescent signals from the blot. It's this ease of signal saturation that gives scientists the idea that film is more sensitive than digital imaging.

Film has a higher LOD compared to that of digital imaging systems and therefore, weak signals need extensive exposure time to be detected and quantified on film. Digital imaging systems will capture less intense signals that are missed by film and do so without compromising stronger signals to saturation.

The following table highlights the advantages of using an imaging system over film.

Feature	Film	Digital Imaging system
Dynamic Range	Limited linear dynamic range difficult to see faint and bright bands without over saturation	Much broader dynamic range can detect bright and faint bands without causing saturation
Linearity	Non-linear	Large linear response
Cost	Expensive maintaining a darkroom, chemicals and film	Cheaper running costs only need to invest in an imaging system and lower operational costs

Dot Blot Protocol

A technique for detecting, analyzing, and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane or paper substrate.

Concentration of proteins in crude preparations (such as culture supernatant) can be estimated semiquantitatively by using “Dot Blot” method if you have both purified protein and specific antibody against it.

Reagents

TBS: or PBS

20 mM Tris-HCl

150 mM NaCl

pH 7.5

TBS-T: (PBST)

0.05% Tween20 in TBS

BSA/TBS-T:

3-5% BSA in TBS-T (PSB-T)

PVDF membrane

Procedure

1. Have PVDF membrane ready, draw grid by pencil to indicate the region you are going to blot (seebelow).

Decide what concentrations of primary and secondary antibody you wish to test and the concentration range of protein.

2. Pipet 2µl of protein sample onto the PVDF membrane at the center of the grid. Minimize the area that the solution penetrates (usually 3-4 mm diam.) by applying it slowly.

3. Block non-specific sites by soaking in 5% BSA in TBS-T (0.5-1 hr, RT). Use 10cm Petri Dish for reaction chamber. Cut up the grid.

4. Incubate with primary antibody (each square being incubated with a different concentration of primary antibody) dissolved in BSA/TBS-T for 30 min at RT.

5. Wash three times with TBS-T (3 x 5 min).

6. Incubate with secondary antibody (again different concs of the secondary antibody) for optimum dilution, follow the manufacturer’s recommendation) for 30 min-1hr at RT.

7. Wash three times with TBS-T (15 min x 1, 5 min x 2), then once with TBS (5 min).

8. Image using the gel imager. Try several different lengths of exposure.

9. See which concentration combination works best. Gives you the most signal.

Fluorescent Western blot general protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transfer the protein to the membrane (electroblotting) either by wet transfer or semi-dry transfer.

2. Wash the membrane twice with distilled water. If desired, stain the membrane with Ponceau Red solution for 5 minutes to visualize protein bands. (Stock solution: 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid; dilute 1:10 for use.) Rinse the membrane in water until protein bands are distinct then once the bands have been detected wash the membrane in TBST to remove the Ponceau red stain. **Note:** Do not let the blot dry out at any step through development, as this will cause an increase in background staining.

3. Block the blotted membrane in freshly prepared TBS and/or PBS containing BSA (3–5%) (Other blocking reagents are available) for 30–60 minutes at room temperature with constant agitation. A maximum blocking time of 2 hours at room temperature should not be exceeded since staining artifacts will appear. **Note:** If longer blocking times are required, the membrane should be kept at 4°C.

4. Dilute the primary antibody to the recommended concentration/dilution in fresh blocking solution (TBS and/or PBS /3%or 5% BSA). Incubate the membrane in the primary antibody solution for 1 to 2 hours at room temperature or overnight at 4°C with agitation.

5. Wash the membrane three times for 3 to 5 minutes each with TBS and/or PBS containing 0.05% Tween-20. Incubate the membrane in the secondary antibody reagent of choice for 30 minutes to 1 hour at room temperature. For example, if multiplexing two or more fluorophores requires careful selection of primary and secondary antibodies. If two primary antibodies are derived from different host species (e.g. primary antibodies from mouse and chicken), secondary antibodies derived from the same host and labelled with different colour fluorophores must be used e.g. IRDye 800CW Donkey anti-mouse and IR dye 680LT donkey anti-chicken.

   Note: It is highly recommended that before combining primary antibodies in a two or more colour experiment you should always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible background bands (non-specific).

   Slight cross-reactivity may occur and can complicate the interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the

amount of antibody. Always use highly cross-absorbed secondary antibodies for two colour detection. Failure to use highly crossed absorbed antibodies may result in cross-reactivity.

6. Wash the membrane five times for 3 to 5 minutes each time with TBS and/or PBS containing 0.05%Tween-20.

   Note: Tween-20 detergent has the potential to strip low affinity primary antibodies, and therefore the membrane is briefly washed to improve the background.

7. Imaging the membrane either completely the dry the membrane or keep it wet whilst imaging. If the membrane starts to dry out then patches can appear on the membrane which can possibly obscure bands. Make sure that the membrane is placed on the black plastic anti-reflective screen whilst imaging.

General Tips and advice for Fluorescence Western blots

General Tips

1. It is important to block membranes for at least 1hr or overnight at 4^0C. Be sure to use enough blocking buffer to cover the membrane (minimum recommended is 0.4mL/cm^2). If using anti-goat antibodies a milk based blocker can interfere with detection. They can also deteriorate rapidly at 4^0C. BSA blocking buffers may cause high membrane background.
2. Diluting antibodies- depends on the antibody and should be determined empirically. Usually you can find a suggested starting range on the manufacturer's data sheet.
3. Protect the membrane from light during secondary antibody incubations and washes
4. To concentrate the target protein use the narrowest well size possible for the loading volume
5. For proteins <100KDa, try blotting in standard Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane.
6. It is recommended to soak the gel in transfer buffer for 10-20 minutes before setting up transfer. Soaking the gel allows it to equilibrate the gel and removes buffer salts that will be carried over into the transfer tank.
7. To maximise retention of transferred proteins on the membrane, allow the membrane to completely air-dry after transfer (approx. 1-2 hours).
8. To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at 4^0C. Try and avoid extended incubations in secondary antibodies.

Optimisation Tips

1. No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. It is difficult to detect target protein, changing the blocking solution may dramatically improve performance.
2. Let the membrane dry out completely before imaging.

Two or more colour detection

Two or more colour detection requires careful selection of primary and secondary antibodies. If two primary antibodies are derived from different host species (e.g. primary antibodies from mouse and chicken), secondary antibodies derived from the same host and

labelled with different colour fluorophores must be used e.g. IRDye 800CW Donkey anti-mouse and IR dye 680LT donkey anti-chicken.

It is highly recommended that before combining primary antibodies in a two or more colour experiment you should always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible background bands (non-specific).

Slight cross-reactivity may occur and can complicate the interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody. Always use highly cross-absorbed secondary antibodies for two colour detection. Failure to use highly crossed absorbed antibodies may result in cross-reactivity.

General Tips and advice for IR Western blots

General Tips

1. It is important to block membranes for at least 1hr or overnight at 4^0C. Be sure to use enough blocking buffer to cover the membrane (minimum recommended is 0.4mL/cm^2). If using anti-goat antibodies a milk based blocker can interfere with detection. They can also deteriorate rapidly at 4^0C. BSA blocking buffers may cause high membrane background.
2. Diluting antibodies- depends on the antibody and should be determined empirically. Usually you can find a suggested starting range on the manufacturer's data sheet.
3. Protect the membrane from light during secondary antibody incubations and washes
4. To concentrate the target protein use the narrowest well size possible for the loading volume
5. For proteins <100KDa, try blotting in standard Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane.
6. It is recommended to soak the gel in transfer buffer for 10-20 minutes before setting up transfer. Soaking the gel allows it to equilibrate the gel and removes buffer salts that will be carried over into the transfer tank.
7. To maximise retention of transferred proteins on the membrane, allow the membrane to completely air-dry after transfer (approx. 1-2 hours).
8. To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at 4^0C. Try and avoid extended incubations in secondary antibodies.

Optimisation Tips

1. No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. It is difficult to detect target protein, changing the blocking solution may dramatically improve performance.
2. Let the membrane dry out completely before imaging.

Two colour detection

Two colour detection requires careful selection of primary and secondary antibodies. If two primary antibodies are derived from different host species (e.g. primary antibodies from mouse and chicken), IRdye secondary antibodies derived from the same host and labelled

with different IRDye fluorophores must be used e.g. IRDye 800CW Donkey anti-mouse and IR dye 680LT donkey anti-chicken.

It is highly recommended that before combining primary antibodies in a two colour experiment you should always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible background bands (non-specific).

Slight cross-reactivity may occur and can complicate the interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody. Always use highly cross-absorbed secondary antibodies for two colour detection. Failure to use highly crossed absorbed antibodies may result in cross-reactivity.

Imaging Tips

1. It is recommended to either image the blot wet or to let the blot completely dry out. If the membrane starts to dry out this can create blotches on the membrane which can prevent bands being detected easily.
2. For more even illumination place one of each colour LED module opposite each other inside the darkroom.
3. Make sure that you zoom in on the sample to completely fill the screen.
4. If you are imaging IRDye800 you may not see any bands in the preview screen of the image capture software which can make it difficult to make sure your sample is in focus. Please use a business card or a piece of paper with writing on it to make sure you have the sample in focus.
5. Always handle the membrane with gloves and tweezers.
6. Do not write in pencil or pen on the membrane as they will fluoresce and can interfere with autocapture mode in GeneSys software.