APPLICATION NOTE

Dual Color Staining Protocol Utilizing Anti-FluorTag Antibodies

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When moving from flow cytometry to microscopy, one is likely to be interested in visualizing the presence of more than one molecule or cell at the same time. In immunohistochemistry, this is accomplished by performing a dual color stain.

The anti-FluorTag antibodies are well suited for dual color stains because AP and HRP conjugates are available for each antibody, both conjugates can be added at the same time, and both conjugates are derived from the same species (goat).

What follows is a base protocol for performing a dual stain with the anti-FluorTag antibodies. It can be adapted as needed.

SECTIONING

Cryosections are preferred because the process that is necessary to prepare formalin-fixed paraffin-embedded (FFPE) tissues is known to damage epitopes. It is usually beneficial to freeze a tissue as rapidly as possible in order to minimize ice crystal formation that can damage tissue morphology. Thus, using liquid nitrogen cooled isopentane can be preferable to using dry ice or liquid nitrogen alone (isopentane is a liquid from -160 °C to 28 °C and helps extract heat rapidly without boiling). However, the freezing process should not be carried out for too long or the block of embedding media containing the tissue may crack. Cryosections are typically cut in thicknesses of 5-15 µm in order to have a single layer of cells. Using positively-charged slides can be helpful in retaining tissue sections during the entire staining process. After cryosections are applied to slides, they should be dried and stored frozen in the absence of moisture.

FIXATION (15 – 40 MINUTES)

The use of ice-cold acetone (i.e., acetone stored at -20 °C) is usually preferred over other methods and is more likely to preserve the epitopes that are recognized by most monoclonal antibodies. Slides are first warmed to room temperature in the absence of moisture. If not already done, it can be helpful to label or mark the top of each slide at this point in order to identify the side that contains the section(s). The slides are then immersed in ice-cold acetone for about 10 minutes. Afterwards, the slides are removed from the acetone and air dried for up to 30 minutes. An abbreviated drying procedure that preserves most epitopes involves removing the slide from acetone and placing it on a 65 °C block for a minute and following that with several minutes of air drying.

During air drying, slides can be divided into regions by using a PAP pen. This is especially helpful if slides contain multiple sections that will be stained differently. This can also reduce the volume of solutions that will be used later.

REHYDRATION (5 MINUTES)

At this point, the acetone-fixed and dried sections are still surrounded by embedding medium. Submerging the sections in Phosphate Buffered Saline (PBS) for 5 minutes will rehydrate them and dissolve the surrounding embedding medium. Once the slides are rehydrated, they must be kept moist and must not dry until the end of the staining procedure when they are mounted. Slides should be moved quickly from step to step.

ENDOGENOUS PEROXIDASE INACTIVATION (15 MINUTES)

Tissues may contain endogenous peroxidases and alkaline phosphatases. While this is usually not a concern in flow cytometry, it could lead to unwanted background signals in IHC. HistoMark® AP substrates contain Levamisole, which inhibits most endogenous alkaline phosphatases except for those found in epithelial cells of the intestine and certain other tissues.

When HRP substrates will be used, endogenous peroxidases should be inactivated and there are several possible methods that accomplish this. A method that preserves most epitopes and typically leads to minimal tissue damage involves first submerging sections in 0.1% Sodium Azide in water for 5 minutes. Subsequently, sections are submerged in 0.1% Sodium Azide with 0.3% Hydrogen Peroxide in water for 10 minutes. This two-step procedure minimizes the bubble formation that can occur if Hydrogen Peroxide is used alone while keeping the concentration of Hydrogen Peroxide low as well. It also does not require the use of organic solvents like methanol, ethanol and acetonitrile that may damage proteins or remove membrane proteins. However, Sodium Azide is toxic and solutions that contain it must be disposed of properly.

WASHING (5 MINUTES)

Submerge slides in Tris Buffered Saline (TBS) for 1 minute (in order to quickly dilute the inactivation mix) then in fresh TBS for another 4 minutes. PBS should be avoided in this and later

steps if AP conjugates will be used because free phosphate inhibits the removal of phosphate from AP substrates.

BLOCKING (10 – 30 MINUTES; 1 ML PER SLIDE)

Slides and tissues may adsorb proteins nonspecifically until blocked. Additionally, cells of the immune system may express Fc receptors that bind antibodies. Thus, sections should be incubated with a blocking mix that minimizes nonspecific binding of antibodies later. Several things need to be kept in mind and the strategy is similar to what would be used in flow cytometry.

First, the blocking mix should contain albumin or another protein or a suitable substitute that minimizes any nonspecific adsorption of antibodies to the slide or tissue later. Serum typically contains about 5% albumin, and 1% serum (0.05% albumin) is typically sufficient for this purpose. Adding 0.05% Tween 20 can help as well and will also reduce surface tension but can allow solutions to cross boundaries drawn by a PAP pen.

Second, the blocking mix should contain unlabeled antibodies that are bound by Fcγ receptors. Serum typically contains around 10 to 20 mg/mL of IgG. The IgG in 1% serum is usually over 100 times more concentrated than the primary antibodies that will be used later. Thus, if staining mouse sections, 1% Normal Mouse Serum (NMS) is helpful provided that one is not trying to detect a protein that is also found in mouse serum. Similarly, 1% NMS is also helpful when staining human sections if the monoclonal primary antibodies are mouse-derived (many are mouse IgG1κ). Purified unlabeled IgG can be added as well.

Third, the blocking mix should contain antibodies that compete with the goat-derived anti-FluorTag antibodies for any nonspecific binding. This can be accomplished by including 1% Normal Goat Serum (NGS).

Overall, for staining mouse tissues, a blocking mix of 1% NMS and 1% NGS in TBS with 0.05% Tween 20 (TBST) frequently works well (approximately 0.1% albumin; approximately 100 to 200 µg/mL IgG of each species). This can be prepared by mixing 1 part each of 10% NMS, 10% NGS and 10x TBST and diluting with 7 parts water. About 1 mL is needed to cover an entire slide at this point. Enough blocking mix should be prepared for this step and for primary antibody staining.

Sections should be covered completely with the blocking mix and blocked for 10 to 30 minutes. The blocking mix should be applied gently and it can be helpful to not pipette directly onto the tissue section but adjacent to it in this and subsequent steps. During the incubation, sections should be placed into a humidified chamber to minimize evaporation. A humidified chamber can be prepared from an empty pipette tip box by placing water and wet paper towels into the bottom compartment, placing the slides on the tip rack above and closing the top.

Finally, as in flow cytometry, it is possible to use unlabeled antibodies that are designed to block Fcγ receptors (e.g., anti-CD16/CD32) in order to minimize the background that can be caused by these receptors. However, because these antibodies may not bind to an Fcγ receptor that is currently binding IgG, anti-Fcγ antibodies should be applied before any other IgG is added (i.e., before serum).

PRIMARY ANTIBODY STAINING (10 – 30 MINUTES; 0.5 ML PER SLIDE)

For flow cytometry, fluorescently-labeled antibodies are usually diluted about 100-fold from their starting concentrations to about 1 to 10 µg/mL. For abundant proteins whose expression will be visualized with Alkaline Phosphatase (AP) conjugates and rare proteins whose expression will be visualized with Horseradish Peroxidase (HRP) conjugates, the use of the same concentration as for flow cytometry is a good starting point. For abundant proteins whose expression will be visualized with HRP conjugates later, 10- to 100-fold lower concentrations can often be used.

In order to prepare the staining mix, the fluorescently-labeled primary antibodies should be combined (if performing a dual stain) and diluted appropriately in the blocking mix that was prepared earlier. 0.2 to 0.5 mL is usually sufficient for an entire slide. Due to the presence of Tween 20 and the previous blocking step, the staining mix typically spreads over sections well and therefore less liquid is required to cover the sections.

After the blocking procedure is complete, the blocking mix is removed gently (e.g., by tipping the slide sideways onto a paper towel). The staining mix is then applied gently in order to cover the sections and the slides is incubated in a humidified chamber for 10 to 30 minutes. A longer time may be needed to detect some antigens. Keeping the slides shielded from light during this step can be helpful to minimize any photobleaching. If a slide contains several sections that were separated using a PAP pen and that will be stained differently, do not add too much staining mix to each section or it may spill to an adjacent section.

WASHING (10 MINUTES)

The staining mix(es) should be removed gently. Afterwards, submerge the slides in TBST for 1 minute (in order to quickly

dilute the staining mix), then in fresh TBST for another 4 minutes and then in more fresh TBST for 5 minutes.

VISUALIZING BY FLUORESCENCE MICROSCOPY

If desired, at this point, the sections can be covered in a layer of TBST (about 0.5 mL per slide) and visualized by fluorescence microscopy. Care should be taken to prevent the sections from drying and to minimize photobleaching, which can alter the chemical structure of the fluorescent tags. Using standard filter sets, FITC fluoresces green, PE fluoresces orange/red, APC fluoresces red and PerCP fluoresces red.

ANTI-FLUORTAG STAINING (10 – 30 MINUTES; 0.5 ML PER SLIDE)

A new blocking mix should be prepared that is similar to the initial blocking mix but does not contain Tween 20 (e.g., 1% NMS and 1% NGS in TBS). The appropriate anti-FluorTag antibody or antibodies should be added to the mix in order to create the anti-FluorTag mix. It is possible to combine an HRP conjugate with an AP conjugate in order to detect two tags at once. A good starting concentration for each anti-FluorTag antibody would be 0.5 µg/mL and about 0.5 mL is usually sufficient for an entire slide. If staining is low, try increasing the concentration or the concentration of the primary antibody used earlier. If unwanted background staining is high, try decreasing the concentration or the concentration of the primary antibody used earlier.

In this step, it is also possible to detect a biotinylated primary antibody with an HRP or AP conjugate of streptavidin. However, serum can contain free biotin, which is bound by and prevents the further binding of streptavidin to biotinylated antibodies. In this case, the staining mix should contain something like BSA (e.g., 1% BSA) instead of serum. It can be helpful to supplement this with purified IgG from the species used earlier.

After the slides have been washed, the anti-FluorTag mix is applied gently to cover the sections and the slides is incubated in a humidified chamber for 10 to 30 minutes.

WASHING (10 MINUTES)

The anti-FluorTag mix is removed gently. Afterwards, submerge the slides in TBS for 1 minute (in order to quickly dilute the anti-FluorTag mix), then in fresh TBS for another 4 minutes and then in more fresh TBS for 5 minutes. TBST should not be used at this stage in order to allow the substrates to deposit well in the subsequent steps.

VISUALIZING THE AP CONJUGATE (10 – 60 MINUTES)

[If an AP conjugate was not used, skip this step.]

The HistoMark RED substrate should prepared immediately before use from its component solutions, which can be done during the prior wash step and takes about 4 minutes. HistoMark BLUE substrate can also be used, but is only compatible with aqueous mounting media.

After the slides have been washed, residual wash solution should be removed gently. Then, the solution containing the prepared substrate should be applied gently to cover the sections.

The AP reaction should be carried out in the dark and is typically run for 10 to 60 minutes depending on the desired intensity. Unlike HRP reactions where the enzyme is slowly inactivated during the reaction, AP reactions can be carried out for extended periods of time, if needed. For long reactions, old substrate solution should be removed and replaced periodically with freshly-prepared substrate solution.

WASHING (5 MINUTES)

[If an AP conjugate was not used, skip this step.]

The solution containing the substrate should be removed gently. Afterwards, submerge the slides in TBS for 5 minutes.

VISUALIZING THE HRP CONJUGATE (5 – 20 MINUTES)

[If an HRP conjugate was not used, skip this step.]

The HistoMark BLACK and HistoMark ORANGE substrates should prepared before use from their component solutions (hydrogen peroxide, appropriate DAB solution and appropriate buffer solution), which can be done during the prior wash step and takes about 2 minutes.

TrueBlue substrate does not require any preparation and is used as is.

After the slides have been washed, residual wash solution is removed gently. Then, the solution containing the substrate should be applied gently to cover the sections.

The HRP reaction is typically run for 5 to 20 minutes depending on the desired intensity. If the reaction leads to visible coloration in under a minute, the amount of primary antibody or anti-FluorTag antibody should be reduced.

MOUNTING THE SLIDES

Submerge the slides in water for a minute. Counterstain the slides, if desired. Submerge the slides in water to rinse off the counterstain. If using Contrast BLUE, submerge the slides in dilute sodium bicarbonate in order to blue the slide, followed by water. Keep in mind that if a counterstain is used, the type of counterstain should be chosen carefully so as not to interfere with the colors of the substrate(s).

Dehydrate the slides by submerging them in 100% Ethanol for 10 seconds.

Tip the slides to allow the 100% Ethanol to flow off and air-dry the slides for at least 30 minutes.

Mount the section(s) under Xylene/Toluene-based permanent mounting media.

RELATED PRODUCTS

Antibodies – Unlabeled

01-40-01	Goat anti-FITC, Affinity Purified Antibody, 1.0 mg
01-40-02	Goat anti-PE, Affinity Purified Antibody, 1.0 mg
01-40-03	Goat anti-APC, Affinity Purified Antibody, 1.0 mg
01-40-04	Goat anti-PerCP, Affinity Purified Antibody, 1.0 mg

Antibodies – HRP-Labeled

04-40-01	Anti-FITC, HRP-Labeled Antibody, 0.1 mg
04-40-02	Anti-PE, HRP-Labeled Antibody, 0.1 mg
04-40-03	Anti-APC, HRP-Labeled Antibody, 0.1 mg
04-40-04	Anti-PerCP, HRP-Labeled Antibody, 0.1 mg

Antibodies – ReserveAP™-Labeled

051-40-01	Anti-FITC, ReserveAP-Labeled Antibody, 0.1 mg
051-40-02	Anti-PE, ReserveAP -Labeled Antibody, 0.1 mg
051-40-03	Anti-APC, ReserveAP -Labeled Antibody, 0.1 mg
051-40-04	Anti-PerCP, ReserveAP -Labeled Antibody, 0.1 mg

Substrates

71-00-64	TrueBlue Substrate, 50 ml
54-75-00	HistoMark® BLACK, 1000 slides
54-74-00	HistoMark Orange, 1000 slides
55-69-00	HistoMark RED, 1000 slides
54-11-00	StableDAB Peroxidase Substrate, 500 slides
54-10-00	DAB Reagent Set, 500 slides

Related Reagents

71-00-27	10% Normal Goat Serum, 50 ml
71-18-01	10% Normal Mouse Serum, 10 ml
71-00-28	10% Normal Rabbit Serum, 50 ml
51-18-02	10X Tris-buffered Saline with 0.5% Tween 20, 1 liter

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