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The development of an immunohistochemical digoxigenin-labeled mouse on mouse single and double stain methodology

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Introduction

When a mouse monoclonal antibody is desired for immunohistochemical detection on mouse tissues, the anti-mouse secondary antibody will also bind to endogenous mouse IgG in the tissue and may produce unwanted background staining. In the past, mouse-on-mouse (MM) detection utilized a biotinylated primary antibody or biotinylation kit. Tissues rich in endogenous biotin such as kidney or liver made it difficult to use, as streptavidin-horseradish peroxidase or anti-biotin is required for detection. Attempts to completely negate endogenous biotin are ineffective as avidin-biotin blocking steps are unlikely to work acceptably in problematic tissues. Therefore, the design of a digoxigenin-labeled detection system is advantageous for MM detection as it would eliminate these issues. This technology has broad application as it allows the use of a variety of monoclonal antibodies reactive on mouse tissues.

Materials and Methods

Mouse tissues were formalin-fixed for 24 hours and embedded in paraffin (FFPE). Sections were cut between 5-7 microns and dried on slides before deparaffinizing and peroxidase blocking in the usual manner. Slides were then heat retrieved in a citrate-based buffer prior to application of antibody. Mouse antibody concentrates were selected (Table 1) and mixed (0.1 - 1.0 µg / ml) with an anti-mouse linker labeled with digoxigenin and incubated at room temperature (RT) for 1 hour. An absorption reagent is then added to the mixture, and further incubated for 30 minutes. The mixture is then diluted in antibody diluent (optimized per antibody) to the desired concentration and applied to mouse tissue for 60 minutes at RT, followed by a rabbit anti-digoxigenin secondary antibody for 15 minutes. Detection of rabbit anti-digoxigenin is performed with an incubation in either a goat anti-rabbit Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) polymer for 30 minutes. Visualization is accomplished by using a DAB or Warp Red (WR) chromogen. For double stains, a sequential method using two monoclonal antibodies was employed using a heating step in retrieval solution after the first chromogenic reaction followed by a second dig-labeled monoclonal antibody and detection with rabbit anti-digoxigenin followed by an anti-rabbit AP polymer. Visualization of the second antibody target is accomplished by using Warp Red or a Ferangi Blue (FB) chromogen (Figure 3). Slides were lightly counterstained in hematoxylin or light green and coverslipped with a permanent mounting medium prior to visualization using brightfield microscopy. All reagents, antibodies, detections and chromogens were provided by Biocare Medical.

Table 1

Primary mouse monoclonal antibodies used in single and double stain method.

Antibody	Clone	Mouse Tissue	Single Stain	Double Stain
Neurofilament (NF)	2F11	Brain	DAB	NF (DAB) + GFAP (FB)
Glial Fibrillary Acidic Protein (GFAP)	GA-5	Brain		
Hepatocyte Specific Antigen (HSA)	OCH1E5	Liver	DAB	CD10 (DAB) + HSA (FB)
PAX8	BC12	Kidney	DAB	
CD10	56C6	Kidney/Liver	DAB	PAX8 (DAB) + CD10 (FB)
Proliferating Cell Nuclear Antigen (PCNA)	PC10	Colon	DAB	
Beta Catenin	14	Colon	DAB	Beta Catenin (DAB) + SMA (WR)
Smooth Muscle Actin (SMA)	1A4	Kidney/Colon	DAB	

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Table 2

Single Stain MM digoxigenin and MM biotinylation methods used in testing. Sections were washed between steps in TBS.

MM Digoxigenin Protocol	Time	MM Biotinylation Protocol	Time
Mouse Antibody + Mouse Linker (Digoxigenin)	60 min.	Mouse Antibody + Mouse Linker (Biotin)	30 min.
Absorption Reagent	30 min.	Absorption Reagent	15 min.
Deparaffinize & perform heat retrieval optimal for antibody	30 min.	Deparaffinize & perform heat retrieval optimal for antibody	30 min.
Apply digoxigenin-labeled antibody to tissue sections	60 min.	Block tissues in Avidin / Biotin (30 minutes each)	60 min.
Apply Anti-Digoxigenin (Rabbit)	15 min.	Apply biotin-labeled antibody to tissue sections	60 min.
Apply anti-rabbit HRP or AP polymer	30 min.	Apply Streptavidin-HRP	15 min.
Apply Chromogen	5-10 min.	Apply Chromogen	5-10 min.
If single-stain counterstain in hematoxylin or light green. If dual (see Table 3)	30 sec.	Counterstain in hematoxylin	30 sec.

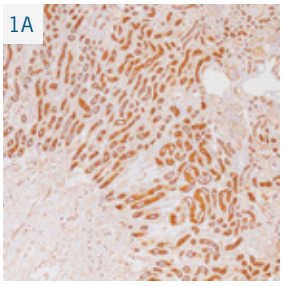
Table 3

Dual Stain (multiplex) MM digoxigenin method. Sections were washed between steps in TBS.

Follow MM Digoxigenin Single Stain Protocol Excluding Counterstain	Time
Perform second heat retrieval	10 min.
Apply second digoxigenin-labeled monoclonal antibody (previously prepared)	60 min.
Apply Anti-Digoxigenin (Rabbit)	15 min.
Apply anti-rabbit AP polymer	30 min.
Apply contrasting chromogen: WR or FB	10 min.
Counterstain in hematoxylin or light green	30 sec.

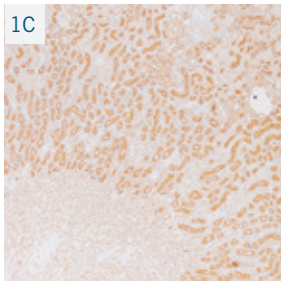
Figure 1

Comparison of MM digoxigenin system versus a MM biotinylation system using monoclonal CD10 [56C6] on mouse kidney. No protein blocking was performed for MM digoxigenin system. Thirty minutes of blocking in avidin followed by thirty minutes in biotin was required to dampen staining of endogenous biotin in MM biotinylation system.

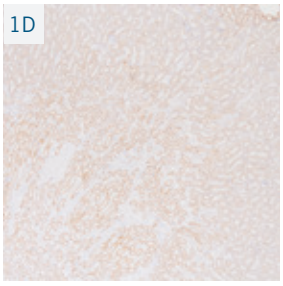


CD10 detected with MM digoxigenin system

Isotype matched concentration control



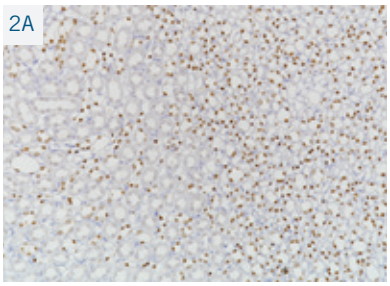
CD10 detected with MM biotinylation system



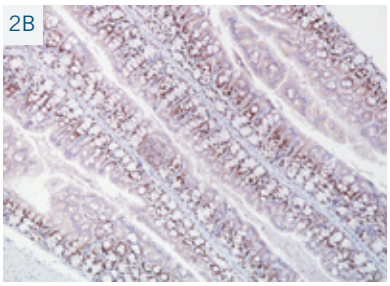
Isotype matched concentration control

Figure 2

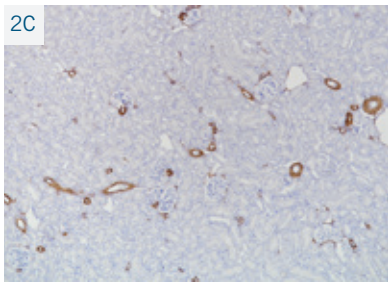
Images of MM digoxigenin system following single staining methodology. All primary antibodies utilized are mouse monoclonal antibodies listed in Table 1.



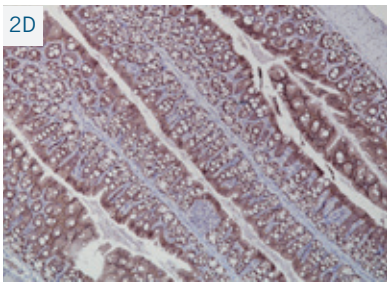
Mouse kidney stained with PAX8 [BC12]



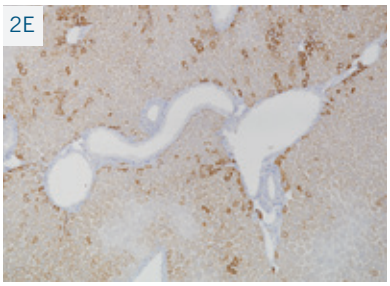
Mouse colon stained with PCNA [PC10]



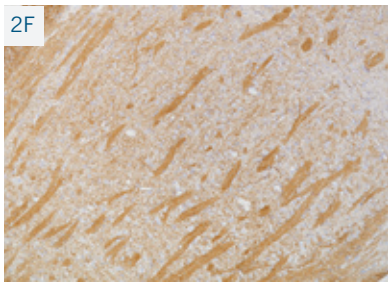
Mouse kidney stained with SMA [1A4]



Mouse colon stained with Beta Catenin [14]



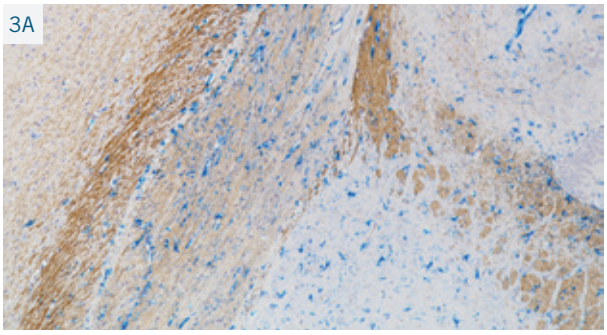
Mouse liver stained with HSA [OCH1E5]



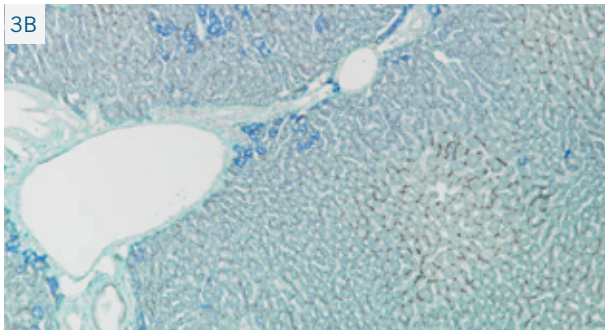
Mouse brain stained with Neurofilament [2F11]

Figure 3

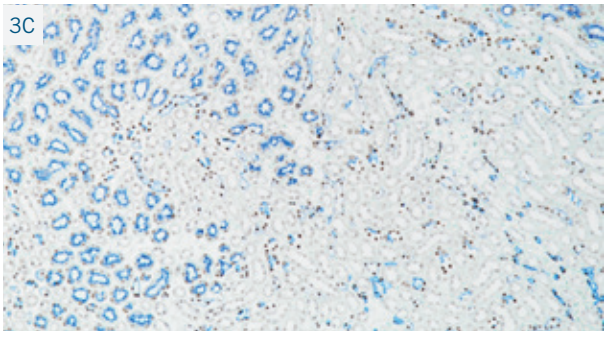
Images of dual MM digoxigenin sequential staining procedure (Table 2, 3).



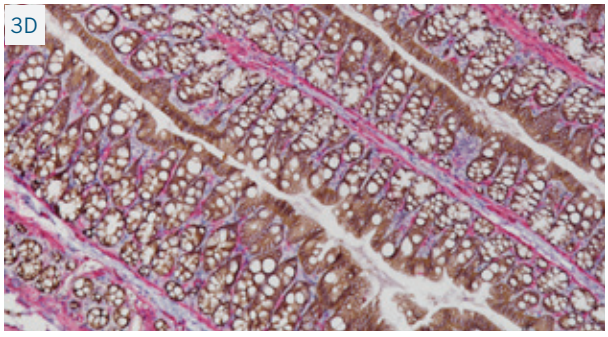
Mouse brain stained with Neurofilament [2F11] (DAB) & GFAP [GA-5] (FB)



Mouse liver stained with CD10 [56C6] (DAB) & HSA [OCH1E5] (FB)



Mouse kidney stained with PAX8 [BC12] (DAB) & CD10 [56C6] (FB)



Mouse colon stained with Beta Catenin [14] (DAB) & SMA [1A4] (WR)

Results

MM single stains with a variety of antibodies were easily achieved with specific, clean and biotin-free staining (Figure 1, 2). Both MM detection systems can be performed with a similar number of steps; however, the sensitivity and the signal-to-noise of the MM biotin system cannot compare to the newly developed MM digoxigenin system (Figure 1). Incorporating significant blocking with avidin and biotin for 30 minutes cannot completely eliminate recognition of endogenous biotin in kidney (Figure 1D). The sequential double stain techniques using monoclonal antibodies were also achieved and visualized with DAB, WR or FB chromogens (Figure 3). Single stains were accomplished in as little as 4 hours while sequential dual stain methods require an additional two hours. Single and dual protocols were developed manually and then transitioned to an automated platform (single assays).

Discussion

Methods used to stain monoclonal mouse antibodies on same target specific tissues has been problematic in the past.[1-4] By employing techniques that enable an intermediary antibody step (post primary antibody labeling with digoxigenin) enables one to bypass this situation entirely. Utilizing an anti-hapten intermediary step and following with a polymer methodology (anti-rabbit HRP or AP) allows an indirect detection without recognition of endogenous mouse immunoglobulins. The MM digoxigenin method provides a sensitive and specific identification of monoclonal mouse antibodies reactive on same species tissues. A critically important requirement when using mouse tissues and/or xenografts in a research or preclinical setting.[2]

Alternate approaches (other than biotin / anti-biotin or streptavidin methods) to mouse-on-mouse require the use of highly cross-adsorbed polymers, typically anti-mouse or anti-rabbit HRP or AP polymers followed by contrasting chromogens. These methods are successful when probed on tissues that require no blocking of endogenous proteins or IgG in problematic tissues. This is a significant drawback that potentially compromises signal-to-noise when these systems are attempted in lymphoid or other FFPE mouse tissues containing significant amounts of immunoglobulins. Therefore, blocking endogenous IgG or polymer modifications may be necessary to generate acceptable results when performing immunohistochemistry with monoclonal antibodies on mouse tissues. Although these methods have been determined to work acceptably, avoiding the use of biotin or anti-same species detections has merit in certain circumstances.

This methodology has potential to incorporate the use of other haptens such as dinitrophenyl (DNP) to label antibodies that can be detected by anti-hapten antibodies followed by detection polymers. These systems may also be used in multiplexing strategies to identify separate target antigens in mouse tissues.[2] This primary labeling technique and detection strategy can potentially be translated to rat-on-rat and human-on-human systems without worry of background noise.

Conclusion

The replacement of biotin-labeled MM technology with a digoxigenin MM technology is a superior method that not only eliminates endogenous biotin blocking, but allows utilization of HRP and AP polymers for double stain technology. This methodology has the potential to be adapted to other same species antibodies as the target tissue with high sensitivity and specificity. This approach can readily be transitioned to automated platforms allowing one to screen large number of slides with excellent signal-to-noise.

References

1. Goodpaster T, Randolph-Habecker J. A Flexible Mouse-On-Mouse Immunohistochemical Staining Technique Adaptable to Biotin-Free Reagents, Immunofluorescence, and Multiple Antibody Staining. J Histochem Cytochem. 2014; 62 (3): 197 - 204 2. Brown JK, *et al.* Primary antibody-Fab fragment complexes: a flexible alternative to traditional direct and indirect immunolabeling techniques. J Histochem Cytochem. 2004; 52:1219-1230 3. Negoescu A, *et al.* F(ab) secondary antibodies: a general method for double immunolabeling with primary antisera from the same species. Efficiency control by chemiluminescence. J Histochem Cytochem. 1994; 42:433-437 4. Wood GS, Warnke R. Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem Cytochem. 1981; 29:1196-1204.

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