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Rodent Multi-Species Multiplex Immunohistochemistry Using Digoxigenin and Polymer Detection Methods in Mouse Tissues

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Introduction

When mouse, rat, and rabbit antibodies are used, consideration of the target tissue is of critical importance. Endogenous IgGs present within tissues pose significant challenges to interpretation. When a species conflict exists between the antibody and the target tissue, it is likely to generate undesired background staining due to the detection system. In the past, mouse-on-mouse and rat-on-mouse utilized either a biotinylated primary antibody or a biotinylation approach to label the antibody.[1] Tissues rich in endogenous biotin, such as kidney or liver, were difficult to use in these systems as streptavidin-horseradish peroxidase (HRP) or anti-biotin techniques were used as the detection complement.[2] Attempts to negate endogenous biotin were minimally effective as avidin-biotin blocking steps were unlikely to work acceptably in tissues such as kidney and liver. Using anti-digoxigenin (dig) and polymer technology to generate multi-species multiplex stains may provide a solution.

Materials and Methods

Mouse tissues were fixed for 24 hours and embedded in paraffin. Sections were cut at 5 microns and dried on slides before deparaffinizing and peroxidase blocking. Slides were then heated at 110°C for 15 minutes (spleen, liver, kidney) or 80°C for 60 minutes (brain) in a citrate-based buffer (pH 6.2) prior to application of antibodies. Two mouse primary antibodies, either PAX8 (M) and CD10 (M) or Neurofilament (M) and GFAP (M), were dig labeled and detected individually using sequential application of a mouse-on-mouse (MM) digoxigenin system (Tables 1 and 5). Anti-rat alkaline phosphatase (AP) and anti-rat HRP polymer sequential applications were executed with rat monoclonals CD4 and CD8a (Tables 2 and 6). A multi-species sequential multiplex stain using an anti-rabbit polymer along with a MM anti-dig was achieved with rabbit CD3 and mouse hepatocyte specific antigen (HSA) and with rabbit Iba1 and mouse CD10 (Tables 3 and 7). A four-step cocktailed multi-species multiplex was developed using a rat monoclonal F-480 combined with a rabbit CD3 in conjunction with a cocktail of an anti-rat polymer and an anti-rabbit polymer (Tables 4 and 8). Visualization in each assay was achieved by the use of two chromogenic end-points including Betazoid DAB (HRP), Deep Space Black (DSB) (HRP), Warp Red (WR) (AP), and Ferangi Blue (FB) (AP). Slides were counterstained in either hematoxylin or light green prior to dehydration and mounting. Slides were scored based on IHC staining intensity ranging from 1 to 3+. Manual and/or automated staining methods using an intelliPATH FLX® automated platform (Biocare Medical, Pacheco CA) were used in all IHC protocols.

Results

Rodent multiplex stains were easily achieved with a sequential digoxigenin-based MM system. Stains achieved with dig detection were graded as a 3+ staining intensity when using antibody concentrations ranging from 0.1 µgs/ml to 1.25 µgs/ml respectively (Tables 1 and 5). In addition, the dig-based system generates no identifiable background and, thus, provides an exceptional signal-to-noise ratio in both mouse kidney and brain (Figures 1A and 1B).

A sequential approach using two rat CD markers, CD4 for T-helper cells and CD8a for cytotoxic T-cells, on mouse spleen and liver proved successful (Tables 2 and 6). Both antibodies were identified as high-affinity rat monoclonals that could be detected with anti-rat HRP and anti-rat AP polymers. Staining intensities achieved were 3+ for both antibodies with relatively little background noted for CD4 on spleen (Figure 2A).

Performing MM dig detection sequentially with a rabbit antibody application was equally robust (Tables 3 and 7). Clean and specific identification of each target yielded grade 3+ staining intensity with no background on mouse kidney and liver (Figures 3A and 3B). In this scenario, rabbit antibodies were initially applied and detected with a one-step polymer-based system. A second digoxigenin-labeled mouse antibody was immediately applied after an elution step and detected via an anti-digoxigenin detection. Reversing this approach with a mouse antibody and dig detection followed by a rabbit antibody demonstrated similar results indicating the flexibility of a sequential assay (picture not shown).

Clean, background-free 3+ staining on spleen and liver was achieved using a cocktail approach with rabbit CD3 and rat F-480 followed by a detection cocktail of anti-rabbit HRP and anti-rat AP (Figures 4A and 4B). The entire procedure was accomplished in as little as 2.5 hours whereas a sequential method may take twice as long (Tables 4 and 8).

Conclusion

All multiplexing methods that were developed demonstrated effectiveness for staining in mouse tissues generating strong and specific 3+ results. Using mouse-on-mouse anti-digoxigenin technology in multiplex staining is a superior approach to streptavidin or biotin strategies in rodent systems. Additional multiplexing techniques with rat or rabbit antibodies using polymer-based detections generates excellent staining with a significant reduction of background and an exceptional signal-to-noise ratio in mouse tissues.

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Table 1

Sequential MM method using primary antibodies labeled with digoxigenin*

MM Digoxigenin Protocol (Step 1)	Time	MM Digoxigenin Protocol (Step 2)	Time
Make 2 solutions: Combine first mouse antibody + Mouse Linker (Digoxigenin); Combine second mouse antibody + Mouse Linker (Digoxigenin)	60 min.	Perform elution step: Place slides in citrate-based buffer (6.0) at 95°C	10 min.
Add Absorption Reagent to both Antibody + Linker solutions	30 min.	Apply second digoxigenin-labeled antibody (previously prepared)	60 min.
Dilute using optimal antibody diluents – antibodies are now digoxigenin-labeled		Apply Anti-Digoxigenin (Rabbit)	15 min.
Apply first digoxigenin-labeled antibody to tissue sections	60 min.	Apply anti-rabbit AP polymer	30 min.
Apply Anti-Digoxigenin (Rabbit)	15 min.	Apply contrasting AP chromogen (FB)	10 min.
Apply anti-rabbit HRP polymer	30 min.	Counterstain in hematoxylin or light green	30 sec.
Apply HRP chromogen (DAB)	5 min.		

Table 2

Sequential rat monoclonal antibody detection using anti-rat polymers*

First Rat Antibody (Step 1)	Time	Second Rat Antibody (Step 2)	Time
Apply first rat antibody to tissue sections	60 min.	Apply second rat antibody to tissue sections	60 min.
Apply anti-rat HRP polymer	30 min.	Apply anti-rat AP polymer	30 min.
Apply HRP chromogen (DAB or DSB)	5 min.	Apply contrasting AP chromogen (WR)	10 min.
Perform elution step: Place slides in citrate-based buffer (6.0) at 95°C	10 min.	Counterstain in hematoxylin	30 sec.

Table 3

Sequential method using a rabbit primary antibody and a mouse primary antibody labeled with digoxigenin*

Rabbit Antibody (Step 1)	Time	MM Digoxigenin Protocol (Step 2)	Time
Apply rabbit primary antibody	60 min.	Combine mouse antibody + Mouse Linker (Digoxigenin)	60 min.
Apply anti-rabbit HRP polymer	30 min.	Add Absorption Reagent	30 min.
Apply HRP chromogen (DSB)	5 min.	Dilute using optimal antibody diluent – antibody is now digoxigenin-labeled	
Perform elution step: Place slides in citrate-based buffer (6.0) at 95°C	10 min.	Apply digoxigenin-labeled mouse antibody to tissue sections	60 min.
		Apply Anti-Digoxigenin (Rabbit)	15 min.
		Apply anti-rabbit AP polymer	30 min.
		Apply contrasting AP chromogen (WR or FB)	10 min.
		Counterstain in hematoxylin or light green	30 sec.

Table 4

4-step multiplex using an antibody cocktail of rabbit and rat antibodies and a cocktailed anti-rabbit and anti-rat polymer detection on mouse tissue*

4-Step Cocktailed Rabbit and Rat Multiplex	Time
Apply primary antibody cocktail (rabbit/rat)	60 min.
Apply cocktail of anti-rabbit HRP + anti-rat AP detection	30 min.
Apply first HRP chromogen (DAB or DSB)	5 min.
Apply second contrasting AP chromogen (WR)	10 min.
Counterstain in hematoxylin	30 sec.

Table 5

Mouse antibodies tested in sequential approach with MM digoxigenin system

Antibody	Species	Clone/Isotype	Vendor	Mouse Tissue Tested	Dilution / Concetration	HIER**
PAX8	Mouse	BC12 (IgG1)	Biocare Medical	Kidney	0.13 µg/ml	110°C for 15 min.
CD10	Mouse	56C6 (IgG)	Biocare Medical	Kidney	0.1 µg/ml	110°C for 15 min.
Neurofilament	Mouse	2F11 (IgG1/kappa)	Biocare Medical	Brain	0.17 µg/ml	80°C for 60 min.
GFAP	Mouse	GA-5 (IgG1)	Biocare Medical	Brain	1.25 µg/ml	80°C for 60 min.
Neurofilament	Mouse	2F11 (IgG1/kappa)	Biocare Medical	Brain	0.17 µg/ml	80°C for 60 min.
GFAP	Mouse	GA-5 (IgG1)	Biocare Medical	Brain	1.25 µg/ml	80°C for 60 min.

Table 6

Rat antibodies tested in sequential approach with anti-rat AP and anti-rat HRP polymers

Antibody	Species	Clone/Isotype	Vendor	Mouse Tissue Tested	Dilution / Concetration	HIER**
CD4	Rat	4SM95 (IgG1/kappa)	Thermofisher	Spleen	0.4 µg/ml	110°C for 15 min.
CD8a	Rat	4SM15 (IgG2a/lambda)	Thermofisher	Spleen	0.25 µg/ml	110°C for 15 min.

Table 7

Rabbit and mouse antibodies tested in a sequential approach with anti-rabbit HRP polymer and MM digoxigenin system

Antibody	Species	Clone/Isotype	Vendor	Mouse Tissue Tested	Dilution / Concetration	HIER**
HSA	Mouse	OCH1E5 (IgG1/kappa)	Biocare Medical	Liver	0.13 µg/ml	110°C for 15 min.
CD3	Rabbit	Polyclonal	Sigma Aldrich	Liver	19 µg/ml	110°C for 15 min.
CD10	Mouse	56C6 (IgG)	Biocare Medical	Kidney	0.1 µg/ml	110°C for 15 min.
Iba1	Rabbit	Polyclonal	Biocare Medical	Kidney	0.3 µg/ml	110°C for 15 min.

Table 8

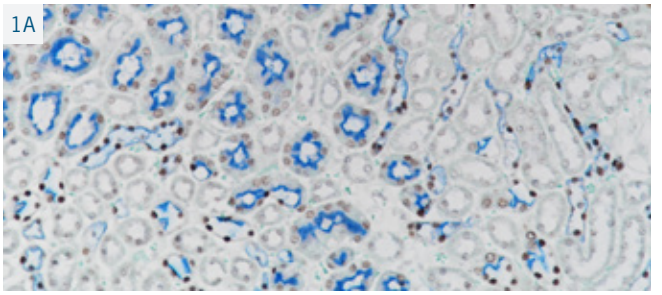
Rabbit and rat antibody cocktail tested in 4-step approach with cocktailed anti-rabbit HRP polymer and anti-rat AP polymer

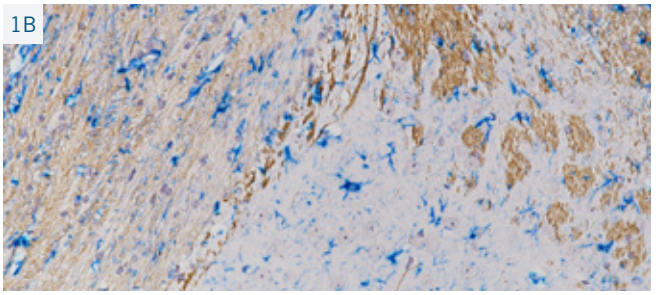
Antibody	Species	Clone/Isotype	Vendor	Mouse Tissue Tested	Dilution / Concetration	HIER**
CD3	Rabbit	Polyclonal	Sigma Aldrich	Spleen / Liver	19 µg/ml	110°C for 15 min.
F-480	Rat	A3-1 (IgG2b)	BioRad	Spleen / Liver	20 µg/ml	110°C for 15 min.

*Deparaffinization, peroxidase blocking and heat retrieval occurred prior to protocol. Sections were washed between steps in TBS.

**Heat Induced Epitope Retrieval

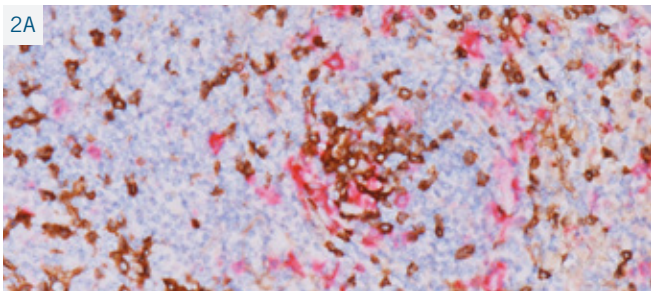
Figure 1: Sequential Mouse-on-Mouse Multiplex

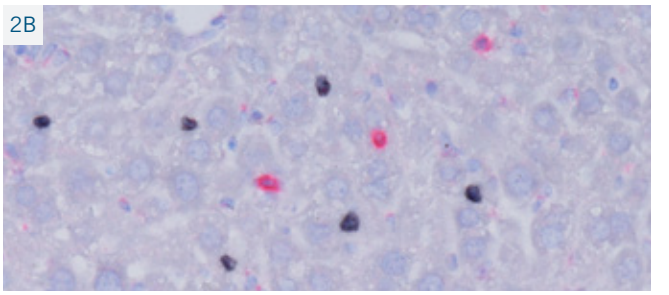




(1A) Mouse kidney stained with PAX8 (M) [BC12] (DAB) and CD10 (M) [56C6] (FB) using sequential approach with MM Digoxigenin System. (1B) Mouse brain stained with Neurofilament (M) [2F11] (DAB) and GFAP (M) [GA-5] (FB) using a sequential approach with MM Digoxigenin System. Original magnifications at 20X.

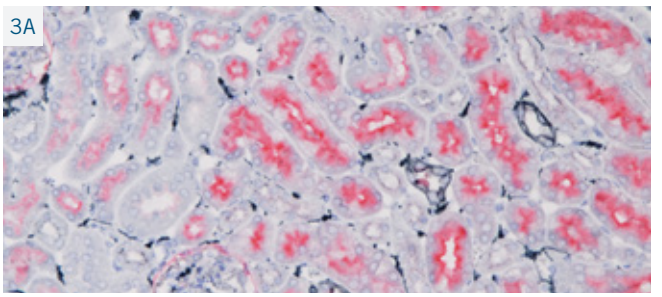
Figure 2: Sequential Rat-on-Rat Multiplex

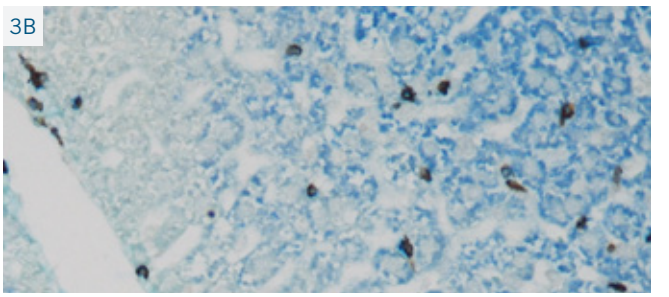




(2A) Mouse spleen stained with rat CD4 [4SM95] (DAB) and rat CD8a [4SM15] (WR) using a sequential anti-rat polymer approach. (2B) Mouse liver stained with rat CD4 [4SM95] (DSB) and rat CD8a [4SM15] (WR) using a sequential anti-rat polymer approach. Original magnifications at 20X.

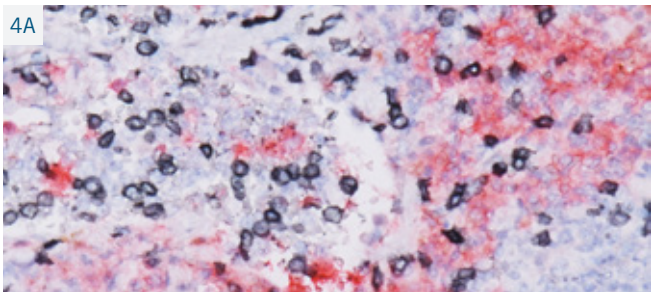
Figure 3: Sequential Multiplex using Rabbit & Mouse Antibodies

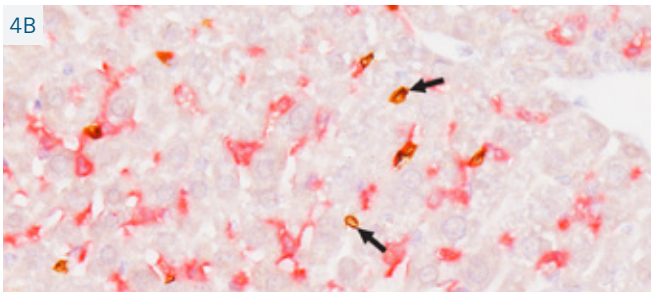




(3A) Mouse kidney stained with rabbit Iba1 (DSB) and CD10 (M) [56C6] (WR). Original magnification at 20X. (3B) Mouse liver stained with rabbit CD3 (DSB) and HSA (M) [OCH1E5] (FB). Original magnification at 10X.

Figure 4: 4-Step Cocktailed Rabbit & Rat Multiplex





(4A) Mouse spleen stained with rabbit CD3 (DSB) and rat F-480 [A3-1] (WR) cocktail. (4B) Mouse liver stained with rabbit CD3 (DAB) (arrows) and rat F-480 [A3-1] (WR) cocktail. Original magnifications at 20X.

References

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2. 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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