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Newly Developed Immunohistochemical Detection Polymer Permits Rapid Multiplex IHC™ Stains on Canine, Feline, Bovine, Equine, Ovine & Porcine Tissues

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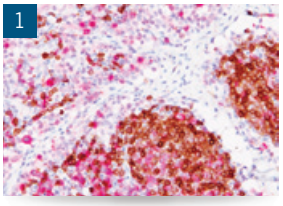
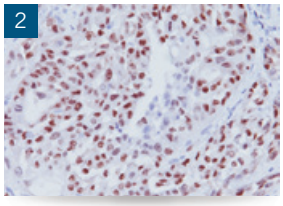
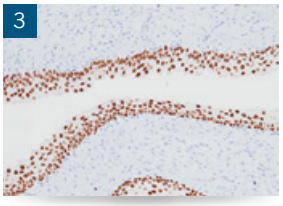
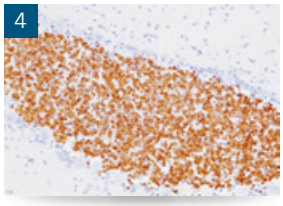
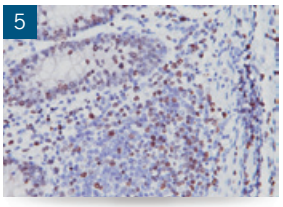
As Presented at ACVP/ASVCP Abstract #P-47

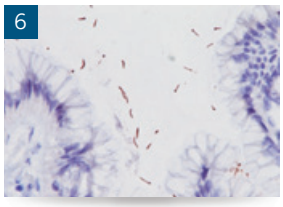


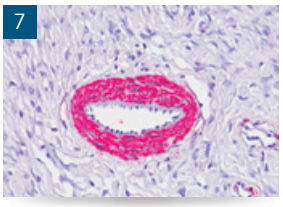


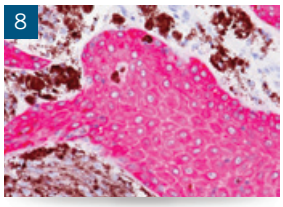












Introduction

A routine and simple method for immunohistochemistry (IHC) staining on canine, feline, bovine, equine and porcine tissues was developed using pre-adsorbed enzyme-conjugated polymers. The polymer detections are exceedingly sensitive, eliminate the need to biotinylate an antibody and require no avidin-biotin blocking. The pre-adsorbed polymers demonstrated minimal cross-reactivity to endogenous IgGs in all tissues including normal spleen. A wide variety of clinical primary antibodies were tested on various normal and neoplastic tissues, resulting in excellent staining. Double and triple staining techniques on animal tissues were also developed using a cocktail of primary mouse and rabbit antibodies and then sequentially detected with a cocktail of pre-absorbed polymer enzyme-conjugates of horseradish peroxidase (HRP) and alkaline phosphatase (AP). Visualization was achieved using DAB (brown) and Warp Red (fuchsia red) chromogens. A third antibody was detected and visualized with either a purple, green or blue chromogen to achieve the triple stain. The procedures were performed both manually and on an automated IHC stainer. These biotin-free pre-adsorbed polymer detection assays may provide a valuable diagnostic or research tool for the veterinary pathologist.

Background

IHC on animal tissues has traditionally been performed using avidin-biotin detection systems. Single stain procedures usually require a serum protein block, and in many cases, an avidin-biotin block. A primary antibody is applied, followed by a two-step detection method (14 steps including wash steps). For a double stain, a sequential method was necessary, which additionally adds another 14 steps, plus the denaturing step between antibodies. For a triple stain, over 40+ steps and 9 hours were required. Most automated stainers are not designed for double and triple stain procedures and a 6 to 9 hour process for a multiple staining protocol requires the full attention of the end-user. Recently, micro-polymer detections have been developed, which require fewer steps and provide added sensitivity; however most polymers are only human adsorbed, expensive, and targeted towards the clinical laboratory. New goat anti-mouse and anti-rabbit polymer detection systems (HRP and AP), has been developed that address these issues. By adsorbing against horse, cow and pig IgGs, the potential for single, double and triple-stain micro-polymer IHC procedures can now be tested and developed.

Methods & Materials

Goat anti-mouse and goat anti-rabbit serums were first purified and then placed in a column and adsorbed against canine, feline, equine, bovine and porcine IgGs. The micro-polymer HRP and AP enzymes were individually conjugated to the absorbed serum (Table 1: Detection Systems). Formalin-fixed paraffin-embedded (FFPE) tissues from dog, cat, horse, cow, pig, and sheep were collected and 4µm sections were cut and prepared for IHC staining. Single, double and triple stain procedures were developed using the HRP and AP detection polymers (Table 2: Protocols). HRP and AP polymers were also combined, thus allowing for antibody cocktails of mouse and rabbit primary antibodies. A selection of chromogens including brown, red, green, blue and purple were evaluated (Table 3: Chromogens). Stains were tested manually and on an open automated IHC staining platform (intelliPATH FLX®, Biocare Medical). Background reducing agents were also tested at different steps in the protocol for efficacy.

Image Legends

1 CD79a (DAB) & CD3 (WR) on Bovine Spleen 2 p63 on Canine Bladder Carcinoma 3 GATA-3 on Normal Canine Bladder 4 NeuN on Canine Brain 5 Ki-67 on Canine Colon 6 *Helicobacter pylori* on Canine Stomach 7 Muscle Specific Actin on Equine Artery 8 Cytokeratin 5 on Equine Melanoma 9 CD20 (DAB) & Muscle Specific Actin (WR) & CK8 (VG) on Feline Colon 10 CK8 (DAB) & Muscle Specific Actin (WR) on Feline Small Intestine 11 Microglia on Ovine Colon (macrophages) 12 Ki-67 on Ovine Spleen 13 Ki-67 (DAB) & Chromogranin (WR) & Smooth Muscle Actin (FB) on Porcine Colon 14 Ki-67 on Porcine Colon 15 Protein Block applied prior primary antibody 16 Protein Block applied after primary antibody

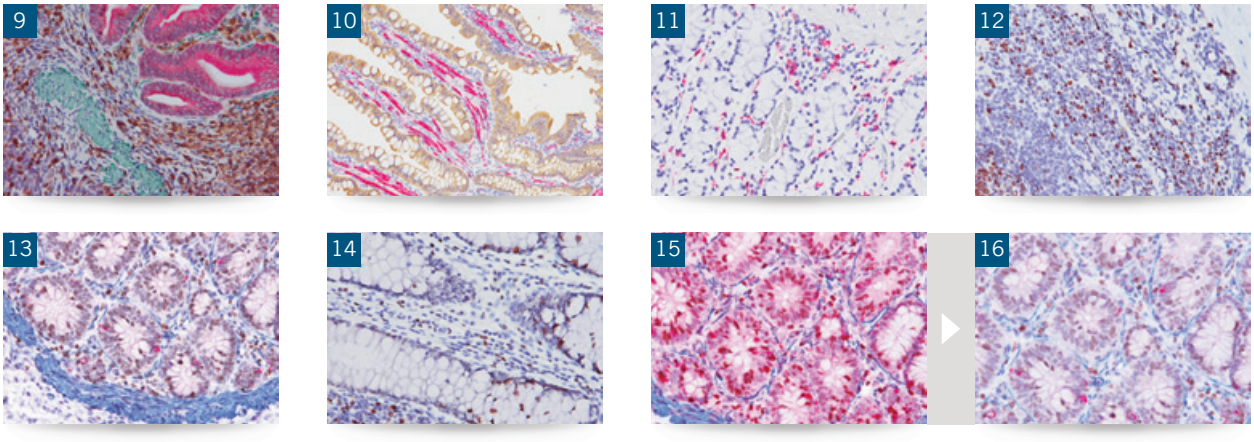


Table 1: Detection Systems

Product	Primary Antibody	Tissue Types	Adsorbed Against
Mouse-on-Farma HRP-Polymer	Mouse	Cow, Horse, Pig, Sheep	Bovine, Equine & Porcine IgG
Mouse-on-Farma AP-Polymer	Mouse	Cow, Horse, Pig, Sheep	Bovine, Equine & Porcine IgG
Rabbit-on-Farma HRP-Polymer	Rabbit	Cow, Horse, Pig, Sheep	Bovine, Equine & Porcine IgG
Rabbit-on-Farma AP-Polymer	Rabbit	Cow, Horse, Pig, Sheep	Bovine, Equine & Porcine IgG
Mouse-on-Canine HRP-Polymer	Mouse	Dog, Cat	Canine IgG
Mouse-on-Canine AP-Polymer	Mouse	Dog, Cat	Canine IgG
Rabbit-on-Canine HRP-Polymer	Rabbit	Dog, Cat	Canine IgG
Rabbit-on-Canine AP-Polymer	Rabbit	Dog, Cat	Canine IgG

The double stain polymer detection was achieved by cocktailing equal parts (1:1) of a mouse (HRP or AP) polymer and a rabbit (AP or HRP) polymer into a single detection mixture.

Table 2: Protocols

Table 2a: Single Stain

Step	Time
Antibody (mouse or rabbit)	30 – 60 minutes
Detection (HRP or AP polymer)	30 minutes
Chromogen	5 – 10 minutes

Table 2b: Double Stain

Step	Time
Antibody 1 & 2 cocktail (mouse & rabbit)	30 – 60 minutes
Protein block (optional)	10 minutes
Detection cocktail (HRP & AP polymer)	30 – 60 minutes
Chromogen 1	5 minutes
Chromogen 2	7 – 10 minutes

Table 2c: Triple Stain

Step	Time
Antibody 1 & 2 cocktail (mouse & rabbit)	30 – 60 minutes
Protein block (optional)	10 minutes
Detection cocktail (HRP & AP polymer)	30 - 60 minutes
Chromogen 1	5 minutes
Chromogen 2	7 – 10 minutes
Denaturing/Elution	5 minutes
Antibody 3 (mouse or rabbit)	30 minutes
Protein block (optional)	10 minutes
Detection (HRP or AP polymer)	30 minutes
Chromogen 3	5 – 10 minutes

Table 3: Chromogens

Enzyme	Color	Product	Abbreviation
Horseradish Peroxidase (HRP)	Brown	Betazoid DAB	DAB
HRP	Purple	Bajoran Purple	BP
HRP	Green	Vina Green	VG
Alkaline Phosphatase (AP)	Red	Warp Red	WR
AP	Blue	Ferangi Blue	FB

Results

Dog, cat, horse, cow, pig, and sheep tissue sections were stained manually and on an automated IHC system. Single, double and triple stains using HRP and AP-coupled polymers and multiple chromogens were achieved (Photos 1-14). Single and double stain procedures can now be accomplished in 2-3 hours and triple stains accomplished in 3-4 hours (Table 2: Protocols). Single stains did not require any protein-blocking or avidin-biotin blocking steps. Negative controls tested on a multitude of tissues including spleen and colon produced negative results.

Superior results for reducing non-specific background staining can be achieved by applying the protein block after the primary antibody rather than the conventional method of applying a protein block before the primary antibody (Photos 15-16).

Conclusion

Cost-effective, micro-polymer detection systems have been developed for use with dog, cat, horse, cow, pig, and sheep tissues. These detection systems also enable double and triple stains to be achieved both manually and on automated IHC staining platforms in less time.

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