WHITEPAPER

Performing Simultaneous Chromogenic IHC Multiplexing Procedures

BIOCARE MEDICAL

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Chromogenic multiplexing procedures represent a set of techniques that permit the identification of two or more target antigens that can be microscopically differentiated by cellular location and/or color. Multiplexing has become increasingly popular as pathologists and other scientists have gained an appreciation for the significant clinical and technical benefits that such procedures provide, including:

- Improved correlation of the immuno-staining patterns of different antigens within adjacent cells and tissues on the same slide, ratherthan having to interpret results from two or more individual slides
- Reduction in the number of slides that need to be examined in order to properly evaluate many clinical cases or validate research experiments – thereby decreasing the time necessary to make a diagnosis or collect investigational data
- Conservation of specimen material, which may be critical when only a small amount is originally collected or is available after slides for routine H&E staining are prepared (e.g. prostate and breast 'core' biopsies)
- Reduction in the number of slides that need to be prepared, stained and stored – resulting in decreased 'labor' and material costs

No discussion of multiplexing procedures would be complete without mentioning that they can also be performed with various fluorophoric/fluorescent reagents, resulting in preparations that must be examined with a darkfield-fluorescence microscope; to be clear, this document focuses on chromogenic multiplexing procedures. There are essentially two main types of chromogenic multiplexing methods: 1) a sequential protocol, where one complete staining procedure is performed immediately after the other; and 2) a simultaneous protocol, where two (or more) antigens are localized by the same staining procedure that employs 'cocktailed' antibodies and cocktailed detection reagents. This document focuses on simultaneous multiplexing procedures; sequential multiplexing is the subject of another document in this series.

Simultaneous multiplexing procedures were developed and made commercially practical much later than sequential multiplexing, and although simultaneous procedures take far less time and are more cost-effective, they have not been as widely adopted in the clinical/diagnostic arena as they should have been, primarily because: A) the manufacturers of so-called 'closed' automated slide-staining systems do not permit such instruments to perform simultaneous methods, on the basis software/programming, not hardware/available reagents); and B) reimbursement for multiplexing procedures (on the same slide) is less than performing immunostaining for the same target antigens on separate slides.

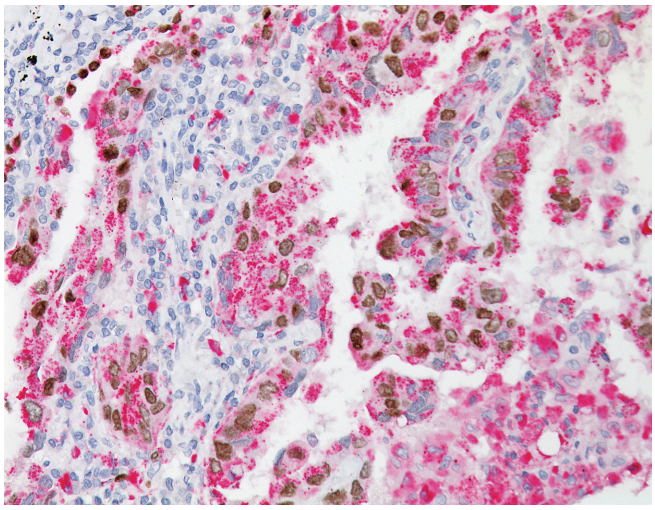
Performing a simultaneous multiplexing procedure starts with which set of pre-prepared primary antibodies your lab wishes to employ, followed by acquisition of the recommended detection reagent, shown in Figure 3. The next step involves implementing the recommended protocol parameters (shown on the applicable datasheet), which will resemble, schematically, the procedure shown in Figure 2 and outlined in greater detail in Figure 4. Another important consideration when performing simultaneous multiplexing procedures is that they do not require a denaturing/elution step, as is often necessary with sequential multiplexing procedures.

As a means of simplifying the implementation of simultaneous multiplexing, Biocare Medical offers 25 different antibody cocktails, where one of antibodies is prepared in mouse and the other in rabbit. Two detection-reagent cocktails were developed by Biocare and remain necessary as they permit the ability to identify (only) mouse and rabbit antibodies with different chromogens. Biocare's Mach 2 Double Stain 1 ("DS-1" in Figure 3) will label rabbit antibodies with HRP and mouse antibodies with ALP, while Mach 2 Double Stain 2 ("DS-2" in Figure 3) labels mouse antibodies with HRP and the rabbit antibodies with ALP. Through its internal optimization/validation efforts, Biocare has determined the ideal combination of antibody cocktail and detection cocktail and have pre-optimized protocol recommendations for several immunohistochemistry (IHC) instruments.

In conclusion, multi-antigen immuno-staining procedures are histologic techniques that, when performed in a sequential fashion are technologically challenging, but, when performed in a simultaneous manner – using Biocare Medical's ready-to-use (RTU) antibody and detection-reagent cocktails – are quite easy to implement. Such procedures improve clinical diagnostics, conserve tissue specimens, and reduce operational costs. Biocare Medical currently offers 25 different antibody cocktails for simultaneous multiplexing, two (2) unique detection reagents, and seven (7) different chromogens. If your laboratory is interested in learning more, please contact Biocare's Technical Support department (800-799-9499, option 3). You might also be interested in reviewing the companion document in this series, entitled "Creating Sequential Chromogenic IHC Multiplexing Procedures".

Figure 1

Photomicrograph of a multi-antigen immunostaining procedure



TTF-1 + Napsin A staining lung adenocarcinoma

Figure 2

Schematic representation of simultaneous multiplexing immunostaining procedure (for two target antigens)

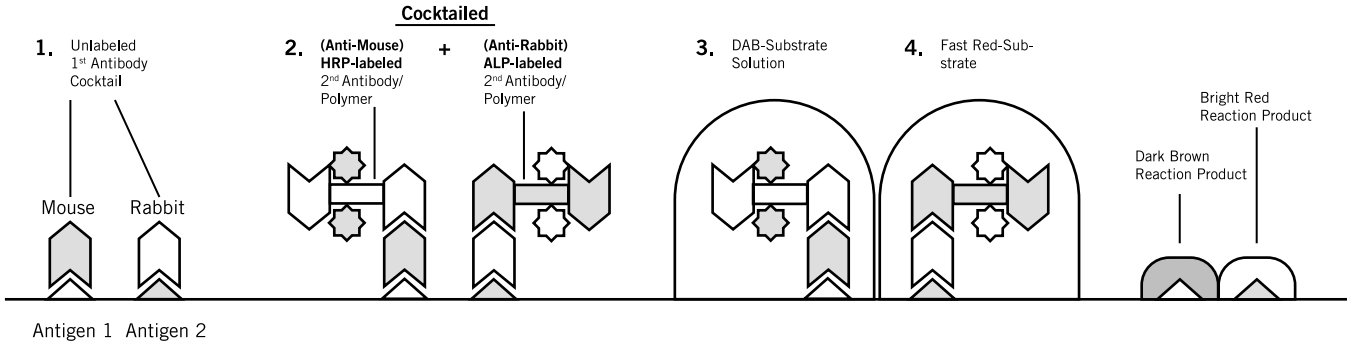


Figure 3

Biocare's primary antibody cocktails for multi-antigen immuno-staining

Item Code	Product Description	Recommended Detection	Volume (in mL)
API3157DSAA	CD4 + CD8	DS-2	6
PM237DSAA	CD20 (L26) + CD3	DS-1	6
API3169DSAA	CD138 + Ki-67	DS-1	6
PM367DSAA	CDX-2 + CK-7	DS-2	6
PM367DSH	Same as above, but larger volume vial	DS-2	25
API3135DSAA	CDX-2 + CDH17	DS-2	6
PM360DSAA	CK-5/14 + p63 + CK-7/18 (Breast Cancer Cocktail)	DS-2	6
PM360DSH	Same as above, but larger volume vial	DS-2	25
API3154DSAA	CK-HMW [34BE12] + p63 + AMACR (Prostate Cancer Cocktail 1)	DS-2	6
API3154DSH	Same as above, but larger volume vial	DS-2	25
PPM225DSAA	CK-5/14 + p63 + P504S (Prostate Cancer Cocktail 2)	DS-2	6
PPM225DSH	Same as above, but larger volume vial	DS-2	25
PPM428DSAA	Desmoglein-3 + Napsin-A	DS-2	6
API3132DSAA	Desmoglein-3 + p40 + Napsin-A	DS-2	6
API437DSAA	ERG-2 (i.e. ERG + CK-5)	DS-2	6
PM317DSAA	GCDFP-15 + Mammaglobin	DS-2	6
API3159DSAA	Kappa + Lambda	DS-2	6
PPM240DSAA	Ki-67 + Caspase-3	DS-2	6
API3198DSAA	Ki-67 + PHH3	DS-1	6
PM391DSAA	p63 + CK-5	DS-2	6
PPM427DSAA	p63 + TRIM29	DS-2	6
API3011DSAA	p120 + E-Cadherin	DS-1	6
PM362DSAA	Pan-Melanoma (MART-1+Tyrosinase) + Ki-67 [M2-7C10+ M2-9E3+T3-11 + SP6]	DS-1	6
PM362DSH	Same as above, but larger volume vial	DS-1	25
API3186DSAA	Pan-Melanoma (MART-1+Tyrosinase) + PHH3 [M2-7C10+ M2-9E3+T3-11 + BC37]	DS-1	6
PM425DSAA	TTF-1 + CK-5	DS-2	6
PPM394DSAA	TTF-1 + Napsin-A (Rabbit Polyconal)	DS-2	6
API3078DSAA	TTF-1 + Napsin-A (Rabbit Monoclonal)	DS-2	6
API3001DSAA	Uro-2™ (CK-20 + p53) - 'Double-Stain'	DS-2	6
PM370TSAA	Uro-3™ (CK-20 + CD44 + p53) - 'Triple-Stain' (i.e. three colors)	DS-1 and Mach 2-ALP	6

1. Nakane, *et. al.*, Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: A study on pituitary glands of the rat. J Histochem Cytochem, 1978.

2. Myers J: The technical, clinical and financial benefits of multi-antigen immuno-staining (MULTIPLEXING) procedures. HistoLogic 34(2): 25-29, 2006.

Figure 4

Sample simultaneous multi-antigen immuno-staining protocol

1. Deparaffinize slide-mounted tissue sections by established procedures;
2. Perform heat-induced or digestion-induced antigen retrieval procedures as needed;
3. Perform endogenous-enzyme 'quenching' and endogenous-protein 'blocking' procedures as needed;
4. Rinse slides thoroughly with Tris-Buffered Saline (TBS);
5. Apply primary antibody **cocktail** solution – incubate for 30 minutes at room temperature (RT);
6. Rinse slides with TBS;
7. Apply **cocktailed** HorseRadish-Peroxidase (HRP)-labeled AND AlkLine Phosphatase (ALP) secondary antibody/polymer solution (i.e. DoubleStain™ 2) – incubate for 30 minutes at RT – With approximately 10 minutes of incubation period remaining, prepare applicable substrate-chromogen solutions;
8. Rinse slides thoroughly with TBS;
9. Apply (first) substrate-chromogen solution (e.g. hydrogen peroxide and DAB) – incubate for 5 minutes at RT;
10. Rinse slides thoroughly with TBS;
11. Apply second substrate-chromogen solution (e.g. alpha-naphthol phosphate and Fast Red*) – incubate for 5 to 10 minutes at RT;
12. Rinse slides thoroughly with DI water;
13. Counterstain slides as desired;
14. Completed slides should be prepared for mounting by one of the following methods:
    a. Dehydrate slides in graded-ethanol^ solutions, and do not let slides 'stand in ethanol for extended periods of time; or
    b. Allow slides to dry, either by placement in a forced-air oven for 15 to 20 minutes, or at room temperature, overnight; or
15. Once slides are completely dry, they may be 'dipped' in xylene before cover slipping by established methods (using a permanent mounting media)

*Refers to Biocare Medical's Warp Red™ - Product code: WR806

^Must not be prepared from 'reagent-grade' products, since they often contain significant amounts of methanol and/or isopropanol, which can cause Fast Red chromogens to 'leach out' of the final preparation

1. Nakane, *et. al.,* Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: A study on pituitary glands of the rat. J Histochem Cytochem, 1978.

2. Myers J: The technical, clinical and financial benefits of multi-antigen immuno-staining (MULTIPLEXING) procedures. HistoLogic 34(2): 25-29, 2006.