WHITEPAPER

# Creating Sequential 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, as shown below.

| Single Antigen Immunostaining | Multi-Antigen Immunostaining |
|---|---|
| Interpret results from two or more individual slides | Improved correlation of the immuno-staining patterns of different antigens within adjacent cells and tissues on the same slide |
| Several slides need to be examined to evaluate clinical cases or research experiments | One slide needs to be examined, decreasing the time necessary to make a diagnosis or collect investigational data |
| Increased risk of cutting through small areas of interest, especially on core biopsies | Conservation of specimen material |
| At least two or more slides to be prepared, stained, and stored | Only one slide needed, resulting in decreased 'labor' and material costs |

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 paper focuses on sequential multiplexing procedures; simultaneous multiplexing is the subject of another paper later in this series.

In an ideal situation, the antibodies that will be employed in a multiplexing protocol:

1. Would be prepared in different species
2. Would identify one cytoplasmic and one nuclear antigen
3. Would be detected with reagents labeled with different enzymes (i.e. HRP and ALP).

However, sequential multiplexing procedures are particularly effective when the antigens of interest:

1. Are found in the same location of target cells (i.e. both are within the nucleus or cytoplasm)
2. When available antibodies to these antigens are produced in the same animal species
3. When the same enzyme 'label' will be employed to polymerize different 'colored' chromogens.

Creating a sequential multiplexing procedure starts with identifying primary antibodies that are appropriately reactive with the desired target antigens, followed by acquisition of detection reagents that are capable of binding to (i.e. 'species-specific' for) the selected primaries. As a result of there being so many different species used to prepare immuno-staining reagents, it is essential that individuals who wish to develop a sequential multiplexing procedure have a substantial understanding of the compatibilities/incompatibilities of species-specific secondary antibodies (incorporated into detection reagents), enzymes and chromogens. One should avoid use of so-called 'universal' detection reagents (i.e. that are capable of binding to primary antibodies raised in both mouse and rabbit) since doing so could result in avoidable background staining. The next step involves experimenting with the order in which primary antibodies, species specific detection reagents and chromogens are applied. Once the investigator has established an acceptable protocol, it will likely resemble, schematically, the procedure shown in Figure 2, which is outlined in greater detail in Figure 3. Alternatively, 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.

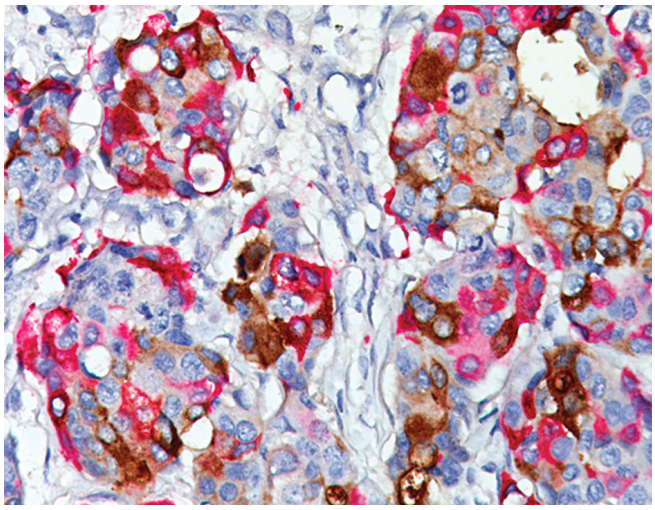
Another important consideration when performing sequential multiplexing procedures is the very common requirement to denature/elute the primary antibody (and detection reagent) that is applied in the first 'pass' before applying a second (or third, etc.) set of reagents. This is especially true when an investigator wishes to employ primary antibodies that were prepared in the same species because, if this step is not performed, the detection reagent applied in the second (or third, etc.) pass will likely bind to the 'first' (or second, etc.) primary antibody, potentially resulting in a small degree of specific but undesirable staining. Denaturing/elution typically consists of application of a weak acid-alcohol solution to slides after the 'first' (insoluble) chromogen is deposited, as shown in 'Step 4' of Figure 2.

In conclusion, multi-antigen immuno-staining procedures are histologic techniques that, although often challenging, represent opportunities to improve clinical diagnostics, conserve tissue specimens and reduce operational costs.

Biocare Medical currently offers over 300 antibodies, a variety of detection reagents and seven (7) different chromogens that can be incorporated into a sequential multiplexing procedure. 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 "Performing Simultaneous Chromogenic IHC Multiplexing Procedures".

## Figure 1

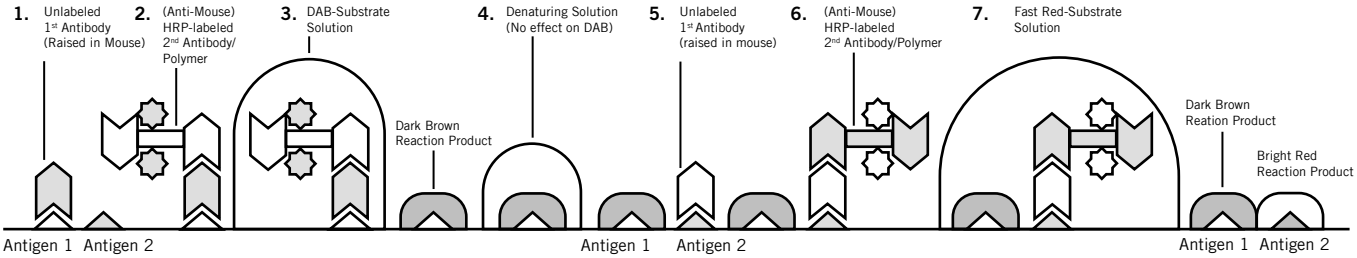
Photomicrograph of a multi-antigen immunostaining procedure



Breast Cancer Stained with GCDFP-15 + Mammaglobin

## Figure 2

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



## Figure 3

Sample Sequential Multiplexing Immunostaining 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 (first) **primary** antibody solution - incubate for 30 to 60 minutes at room temperature (RT);
6. Rinse slides with TBS;
7. Apply HorseRadish-Peroxidase (HRP)-labeled **secondary** antibody/polymer solution – incubate for 10 to 20 minutes at RT – with approximately 10 minutes of incubation period remaining, prepare appropriate HRP-compatible substrate-chromogen solution (e.g. hydrogen peroxide and DAB);
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 DI water;
11. Rinse slides thoroughly with TBS;
12. If 'second' **primary** antibody is prepared in the same species as the previously-applied ('first' primary antibody), apply an appropriate 'elution' reagent^ - incubate for 3 to 5 minutes at RT;
13. Rinse slides thoroughly with TBS;
14. Apply (second) primary antibody solution – incubate for 30 to 60 minutes at RT;
15. Rinse slides with TBS
16. Apply Alkaline-Phosphatase (ALP)-labeled **secondary** antibody/polymer solution – incubate for 10 to 20 minutes at RT – With approximately 10 minutes of incubation period remaining, prepare appropriate ALP-compatible substrate-chromogen solution (e.g. alpha-naphthol phosphate and Fast Red*);
17. Rinse slide thoroughly with TBS;
18. Apply second substrate-chromogen solution (e.g. alpha-naphthol phosphate and Fast Red*) – incubate for 5 to 10 minutes at RT;
19. Rinse slides thoroughly with DI water;
20. Counterstain slides as desired;
21. 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
22. 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 Denaturing Solution – Product code: DNS001

*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.