Reducing Immunoassay Interferences through Innovative Blocking Technology

For immunodiagnostic assays, a false positive or false negative result can be detrimental to a patient's life. Clinicians and patients depend on the in vitro diagnostics (IVD) industry to develop assays that are quality products which deliver accurate results every time. With the increased use of antibody therapy, and animal derived products being used to treat patients, the prevalence of heterophilic antibodies, human anti-mouse antibodies (HAMA), human anti-animal antibodies (HAAA), and rheumatoid factors, is becoming an increasing problem for assay developers [1-5]. With immunoassays pursuing lower detection limits, multiplexing analytes, and higher sample through-put; the negative effects of heterophilic antibodies, HAMA, along with matrix effects needs to be addressed. A new fully synthetic sample diluent developed by Surmodics IVD allows the dramatic reduction of false positives in both in-house and commercially available kits, without having to sacrifice assay sensitivity.

ASSAY INTERFERENCE

Interference in an immunoassay, whether enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, bead-based assays, or immuno-PCR assays, has been a problem that continues to grow throughout the industry [1, 3, 4, 6-8]. Problems with HAMA, heterophilic antibodies, and rheumatoid factors have clinical significance as demonstrated by studies that show the vast number of patient samples that contain such interfering proteins [3, 4, 7, 9, 10]. The prevalence may be as high as 80% of all patients [4]. These issues have continued to grow in prevalence due to use of therapeutic antibodies, vaccinations, blood transfusions, certain drugs derived from animals, and other sources of exposure [3, 4].

Anti-animal Antibody Binding in Two-Site Assays

Two-site sandwich assays appear to be more susceptible to heterophilic antibody interference; however, it has also been shown in a letter to the editor by Zhu et al. (2008) that an ultra-sensitive three-site sandwich troponin I immunoassay also has the potential to show false positives. The important characteristics to understand about HAAA interference in immunoassays are the two types of binding that may be occurring. This happens when animal immunoglobulins elicit a human immune response to produce anti-animal antibodies specific to an antigen. In the first type, anti-idiotype antibodies recognize the Fab region of the animal immunoglobulin. In the second type, more prevalent anti-isotype antibodies recognize the Fc region of the immunoglobulin. Both types of binding can occur, but anti-isotype antibody binding is more common due to better sequence homology between species of the immunoglobulin molecule, specifically the Fc region [4].

Heterophilic Antibodies and Polyspecificity

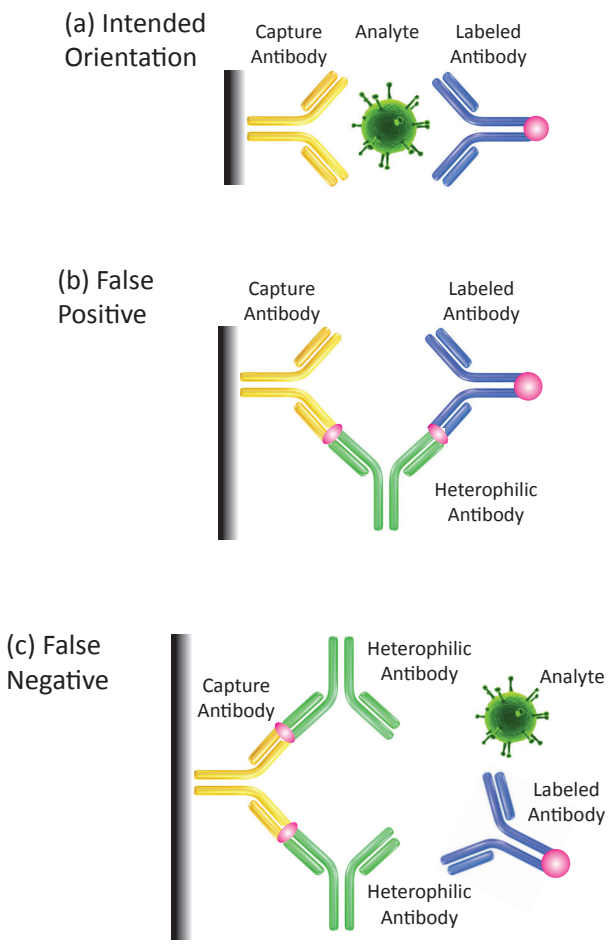
True heterophilic antibodies are dramatically different than HAAA, as explained by Levinson and Miller (2002). These antibodies are natural polyspecific antibodies that are highly variable and part of every person's immune system [7]. These antibodies are generally the IgG class, and due to their ability to have multiple variable Fab regions, they must find an antigen with the complimentary binding site to its Fab region. Most heterophilic antibodies are polyspecific and demonstrate anti-idiotypic binding. The variability allows for antigen-antibody interactions that are weak in nature due to the lack of a "lock and key" fit associated with specific antibodies that are produced as a result of antigen exposure [7]. Even though these heterophilic antibodies are present in every person, the prevalence of interference in two-site immunoassays, that employ blocking agents, is low. The increase in interference occurs when these natural idiotypic antibodies are presented with an antigen allowing the body to develop specific antibodies, which then can be classified as HAAA. Because of the more specific nature of these HAAA, it is more difficult to change the assay format. For example, if heterophilic antibody interference is suspected, changing either the capture or detection monoclonal antibody may effectively reduce the interference, due to the highly variable nature of heterophilic antibodies Fab binding. However, if this interference is due to HAMA, this is a more specific binding, and it is expected that this intense interference will be observed in most monoclonal formats, regardless of epitope [4, 7].

FALSE POSITIVIES AND FALSE NEGATIVES

Two-site sandwich ELISAs have the susceptibility of interference from heterophilic antibodies and HAAA by forming a bridge between the capture and detection antibody. A classic model for explaining this is the bridging of two mouse monoclonal antibodies by HAMA in the patient sample as explained by Kricka (1999) (Figure 1b).

Mechanisms of False Positives and False Negatives in Two-Site Sandwich ELISA

Figure 1: **(a)** The diagram depicts the intended antibody/analyte interaction. **(b)** Demonstrates a false-positive reading due to HAMA antibody bridging the capture and detection antibodies to mimic analyte binding. **(c)** The diagram depicts a false-negative reading. In this case, the analyte molecule is present, but is prevented from binding to the capture antibody because HAMA is blocking the binding site.



Alternatively, a false negative result occurs when HAMA interacts with an assay component, such as the capture antibody, and blocks the antigen binding site. The blocking of the binding site prevents the analyte from binding, even if it were present in the sample (Figure 1c). Other forms of false negative readings can occur if HAMA interacts with the detection antibody, which also prevents recognition of the analyte in the sample.

Interference in competitive binding assays is also an issue, although not as prevalent. In competitive assays, the affinity of heterophilic antibody or HAMA may be close to the affinity of the analyte for the capture antibody. In this case, a false negative result could occur as the heterophile or HAMA will compete for binding of the intended analyte, and could result in a lower signal than expected.

COMMON TECHNIQUES FOR INTERFERENCE REDUCTION

Many techniques have been developed for the reduction of interference in immunoassays. Most attempt to block the specific binding interactions of IgM and IgG antibodies by incorporating immunoglobulins with high affinity for the anti-animal antibody [1, 3, 4, 6, 7]. Some of these incorporate nonimmune serum, nonimmune mouse monoclonals, or fragments of IgG or IgM of various species to bind to the interfering antibodies in solution, or before dilution of the sample [1, 3, 6].

Other techniques have been developed, such as polyethylene glycol 6000 (PEG 6000) precipitation and chromatography [4]. The problem with the more elaborate purification processes is the invested time and materials it takes to run certain purification processes. These processes are impractical for industrial use and greatly add to the cost of goods for the product.

Inherent problems also exist with the specific and non-specific blocking products which utilize immunoglobulins and other nonimmune components. The majorities of the active reagents in products reduce the interference of heterophilic antibodies, but are directed against idiotypes of IgG and IgM, which fail to encompass all of the interference. There are complications with incorporating the mixtures of both non-specific and specific blockers into assays. The complications arise in the external validity of the product as it was designed. Simply stated, what works for one assay set up may not work for another. For multiple assays, the combination of immunoglobulins and nonimmune components have performed very well; however, from an industry point of view, there needs to be a solution for all heterophilic antibodies and HAAA. This is the driving force behind the Surmodics® Assay Diluent (Protein-Free).

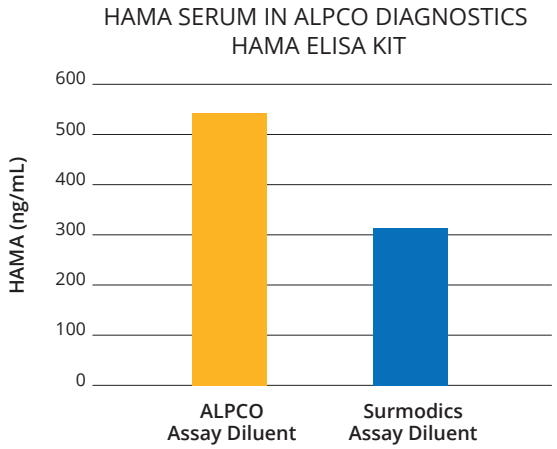
REDUCING INTERFERENCE THROUGH BLOCKING TECHNOLOGY

Surmodics IVD has recently developed a new protein-free assay diluent that utilizes innovative technology to block the non-specific binding of heterophilic antibodies, HAAA, and matrix interferences without compromising assay integrity. The proprietary formulation is fully synthetic to eliminate protein cross-reactivity issues, making it the ideal diluent for most assays. The novel chemistry allows for the reduction of interferences without loss of signal, thereby increasing assay signal to noise in most systems.

In multiple assay formats, the Surmodics assay diluent has proven to be an effective blocker of assay interferences. In a commercially available HAMA kit (Alpco Diagnostics, Salem, NH), human HAMA positive serum and plasma (BioReclamation, Inc., Westbury, NY) were analyzed per kit protocol, incorporating Surmodics assay diluent for sample dilution. The protein-free assay diluent was successful at blocking 42.5% of the HAMA serum signal (Figure 2).

HAMA Serum in Alpco Diagnostics HAMA ELISA Kit

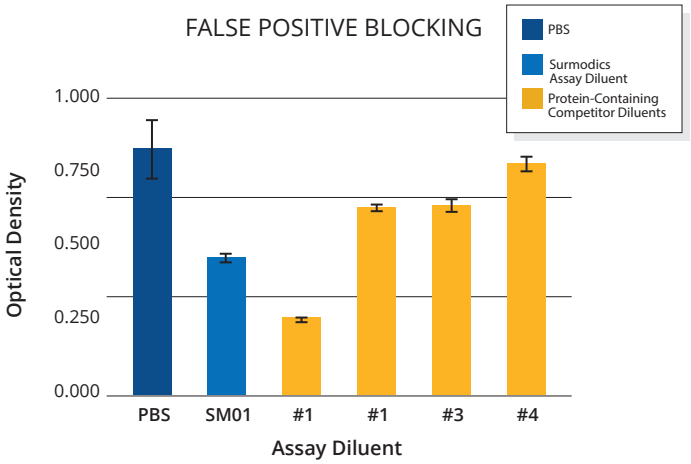
Figure 2: Signal of HAMA serum diluted in Surmodics assay diluent compared to Alpco kit diluent. Results show that the Surmodics assay diluent blocks non-specific HAMA interaction with 42.5% effectiveness.



The signal reduction demonstrated by Surmodics protein-free assay diluent shows how the proprietary formulation of the product effectively reduces the number of non-specific interactions of HAMA. The Surmodics assay diluent does not actively block HAMA activity, but instead reduces assay interferences synthetically, making the product applicable in all assay types, and remaining protein free.

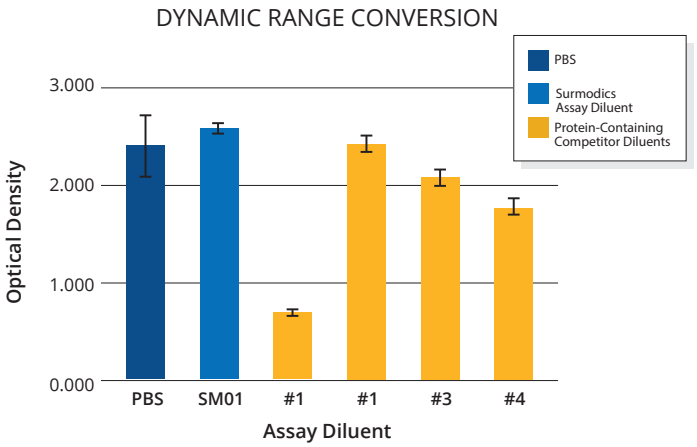
In a troponin I ELISA developed in-house, the blocking effectiveness and conserved assay integrity is demonstrated further. The data in Figure 3a demonstrate the blocking effectiveness of Surmodics assay diluent compared to PBS, and four competitors.

(a) False Positive Blocking



(b) Dynamic Range Conservation

Figure 3: In-house developed ELISA for human cardiac troponin I protein. Basic assay set up includes mouse monoclonal capture antibody to cardiac troponin I, native cardiac troponin I protein, and mouse monoclonal detection antibody to cardiac troponin I – HRP conjugate. False Positive Blocking (a): Surmodics assay diluent blocks 45-50% of the false positive signal. Competitor #1 blocks more of the signal than Surmodics assay diluent. Other competitors block less than 30% of the false positive signal. Dynamic Range (b): The troponin I sample, without HAMA interference, was run in each diluent. The Surmodics assay diluent does not block the specific troponin I analyte. Competitor #1, which had higher blocking than Surmodics protein-free assay diluent, also has extensive blocking of true troponin I signal.



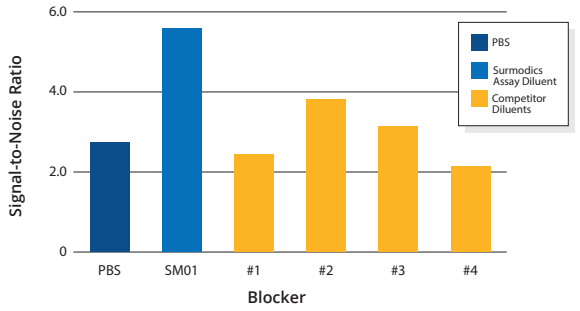
False positive reduction is apparent with the use of Surmodics assay diluent. The assay diluent outperforms most competitors in blocking false positive readings caused by HAMA. The ability to block the false positives seen in the assay is not completely impressive by itself. What makes Surmodics protein-free assay diluent better than protein containing competitors are the data shown in Figure 3b.

These data demonstrate that the dynamic range of the assay is conserved, and although 45-50% of the false positive signal is blocked, the signal-to-noise ratio of the assay is significantly improved (Figure 4). Competitor #1 (with protein), which demonstrated better blocking than the Surmodics assay diluent, also blocks specific binding of troponin to the antibody. This loss of sensitivity seen from use of other diluents is detrimental to diagnostic assays, which need to be sensitive, and provide the user with confidence.

The increased signal-to-noise ratio, which compares true signal to false positive signal, is superior with Surmodics assay diluent compared to PBS and other protein-containing competitors. The non-specific binding of heterophilic antibodies, HAAA, and rheumatoid factors is diminished with the use of *Surmodics* assay diluent, without having to sacrifice sensitivity and dynamic range.

Signal-to-Noise Ratio

Figure 4: Signal-to-Noise Ratio: Compares the true signal INCREASE SIGNAL-TO-NOISE RATIOS



of the troponin I analyte to the false positive signal from HAMA interference. A larger signal-to-noise ratio indicates increased sensitivity and greater confidence in signal.

CONCLUSIONS

Assay interference can be costly to researchers, clinicians, kit manufacturers, and most of all, patients. The continued increase in use of animal products and antibodies in medicine will continue to impact the diagnostic industry in many ways. As researchers and kit manufacturers continue to develop assays for detection of analytes in patient samples, assay interference due to heterophilic antibodies and HAAAs will need to be prevented. Current protein-containing products that utilize immunoglobulins and nonimmune components directed against HAAA and heterophilic antibodies do not provide an end-all solution that can be utilized across a variety of assays.

The new Surmodics assay diluent has shown selectivity with blocking non-specific interactions in both commercial and in-house ELISAs. It has demonstrated superiority over competitors in conservation of dynamic range. The assay diluent increases the signal-to-noise ratio of the assay, providing more sensitivity and greater confidence in the results. The Surmodics assay diluent's innovative technology allows for quicker optimization and greater external validity by not working preferentially towards one class or species of immunoglobulin over another. This ultimately increases a test's sensitivity, consistency, and confidence, which provides clinicians with a quality product to provide the patient with a reliable test result.

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