Assay Development

Examining diagnostic applications for porous adhesvies

Using affinity discrimination to avoid interference effects

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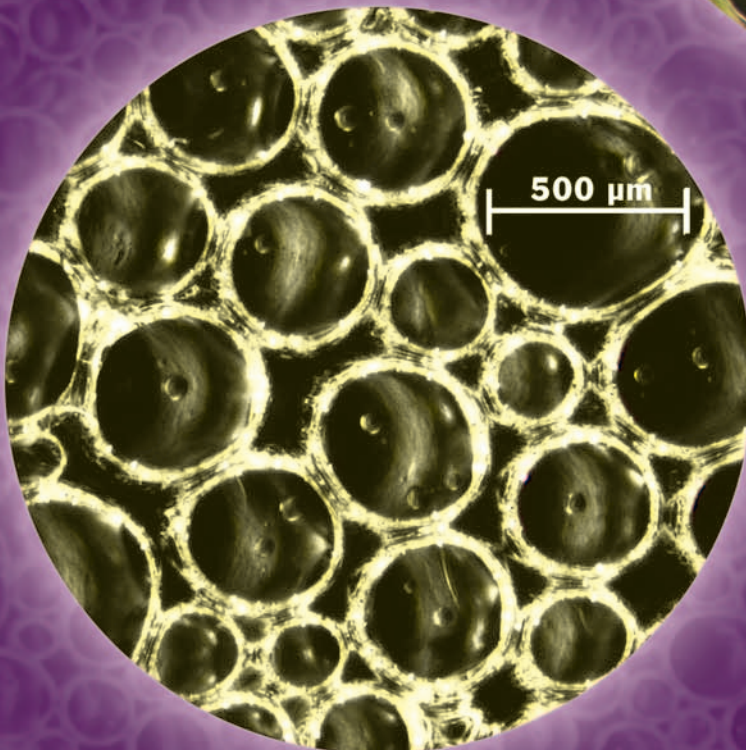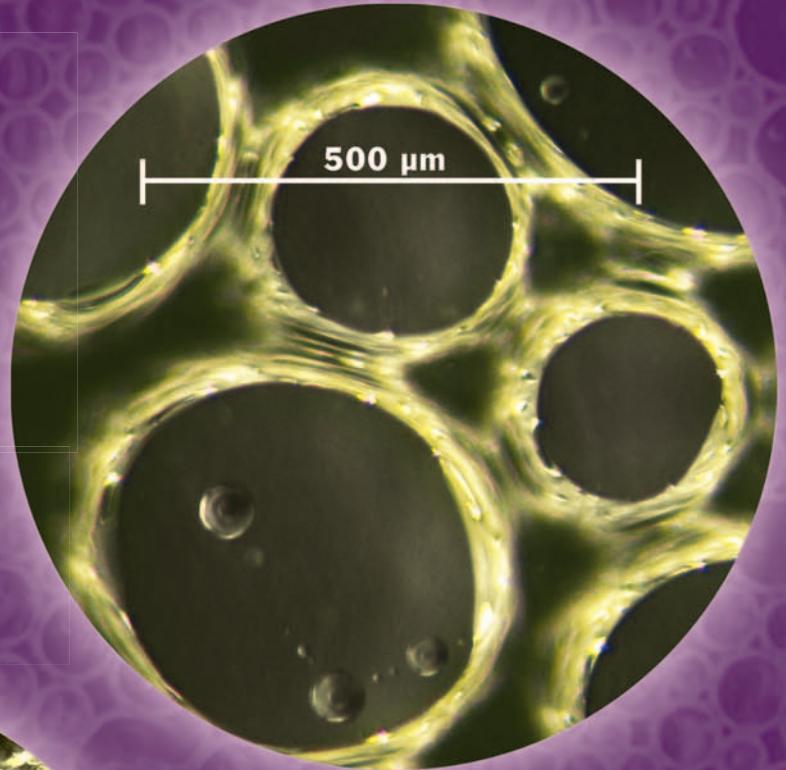
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# Affinity discrimination to avoid interference in assays

Heterophilic antibodies, HAMAs, rheumatoid factors, and matrix effects that cause problems in immunodiagnostics are all neutralized with one new approach. **BY TOBIAS POLIFKE AND PETER RAUCH**

Any immunodiagnostic assay can suffer from negative test effects, which fall under the rubric of interference. These effects lead to imprecision or can result in false-positive or false-negative outcomes. False negatives represent the worst case for most clinical diagnostic tests. However, it is hard to routinely detect them.

Now, though, a new buffer is available that, used instead of traditional assay buffers, enables most interference effects to be avoided. These include problems derived from human antimouse antibodies (HAMAs) and rheumatoid factors. Many test methods can benefit, including enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay, radioimmunoassay, lateral-flow assays, and bead-based assays (including automated assays), as well as modern formats such as immuno–polymerase chain reaction (immuno-PCR) and various multiplex assays.

The Problem of Interference

Most clinical users and all developers of immunodiagnostics are aware of the problems caused by HAMAs, heterophilic antibodies, and rheumatoid factors. Those, the most common known interference effects, are characterized by high clinical relevance and prevalence. Owing to the rising numbers of patients with autoimmune and allergic diseases, the HAMA and
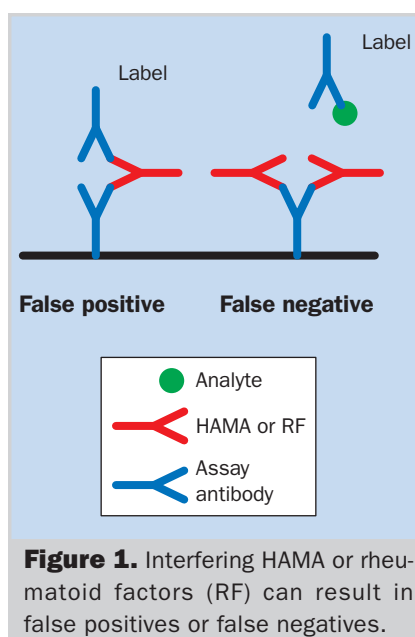
Figure 1. Interfering HAMA or rheumatoid factors (RF) can result in false positives or false negatives.

heterophilic antibody problems will become more and more critical. Another reason for their steadily growing clinical prevalence is the increasing number of patients treated with therapeutic monoclonal antibody drugs.

A recent biomarker study showed that more than 50% of all test subjects had heterophilic antibodies.[1] Other studies have been described that found as many as 80% of all patients thus affected.[2-4] Heterophilic antibodies produce a variety of interference effects.[2] HAMAs are the source of two kinds of potential interference (see Figure 1).

Heterophilic antibodies, HAMAs, and rheumatoid factors can cross-bridge capture and detector antibodies

or even agglutinate detector antibody conjugates in immunoassays. To avoid problems with heterophilic antibodies and HAMAs, HAMA-blockers have routinely been used. Until recently, this was the only available approach. Its disadvantage is that biologically active components—namely, more antibodies—are added to the assays. This can result in new kinds of interference derived from those active components. Additionally, for any new assay, it is necessary to test to discover which kind of HAMA-blockers, or which composition of them, can be used for that specific assay. The type and intensity of the interference effects of heterophilic antibodies depend on the assay layout, the assay antibodies, and the characteristics of the analyte.[4]

There are many different HAMA-blocker reagents on the market. Most producers offer several different HAMA-blockers, making it hard to choose the correct blocker. In fact, one producer offers some 10 different compositions of HAMA-blocking reagents. This shows that very extensive testing is necessary to find exactly the right HAMA-blocker composition for any new assay and any new kind of interfering sample. One composition of HAMA-blockers that has been found to be right for the interfering sample of one certain patient may be totally useless for other patients' interfering samples. HAMA-blockers have been the best approach available for a time; however, they lack general utility.

Analytic interference has been defined as the effect of a substance present in the sample that alters the correct value of the result.[5] Besides those derived from HAMAs and heterophilic antibodies, many other kinds of interference exist, though their effects are not discussed in detail much in the technical literature. There has been no generally applicable way to treat the range of negative effects. Interference can be caused by a lot of different endogenous biological substances.[4–7] Also, in routine diagnostics of patient samples, many other interference effects occur that may be caused by endogenous substances derived from samples, by analyte masking due to the binding of endogenous proteins, by cross-reactivities, or even by general matrix effects such as the viscosity, pH value, or high lipid or salt concentration of samples. The molecular origins of such interference, and their detection, which are discussed in this article, have been described in detail elsewhere.[4,5,8,9]

Interference effects are not limited to human serum samples. They can also appear in other matrices, such as plant specimens.[10]

Immunoassay Labels as Cause

Immunoassays commonly mark the detector antibody—or, in the case of competitive assay, the standard analyte—with a label. Frequently used as labels are enzymes—very often alkaline phosphatase or horseradish peroxidase (HRP)—fluorescent dyes, radioactive isotopes, and, for immuno-PCR, DNA. Unfortunately, even these labels can cause interference.

For example, most fluorescent dyes for labeling are hydrophobic and thus can change the binding characteristics of antibodies. Further, the dyes themselves can bind to other substances. Or, the solubility of the protein may be reduced dramatically when labeled with a fluorescent dye. The specific binding of the antigen to the antibody also can be affected negatively, which can lead to reduced affinity of a labeled
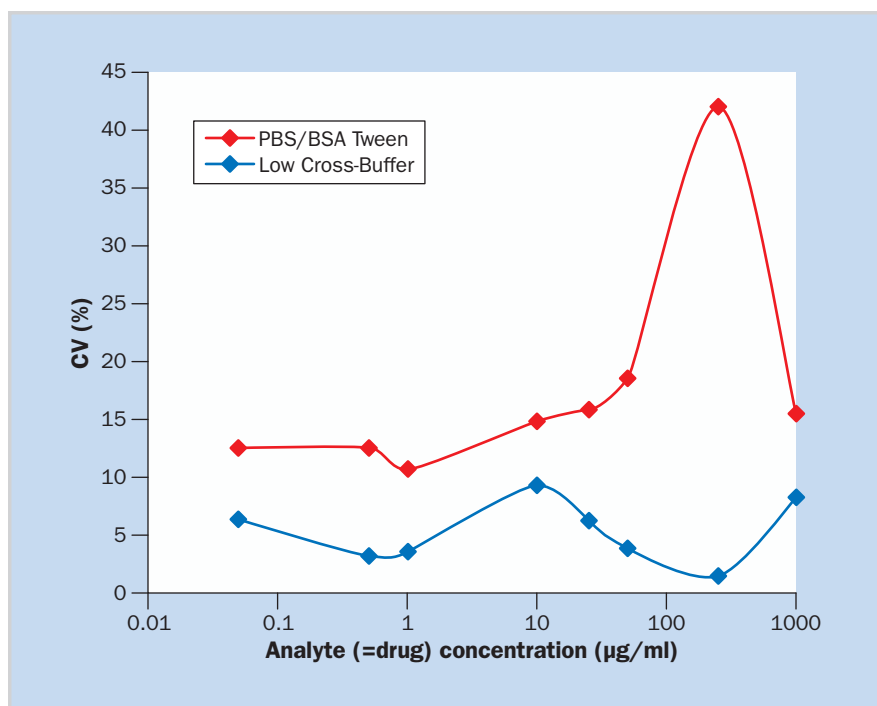
Figure 2. Precision profiles of an ELISA with a standard buffer (PBS/BSA Tween) and with the LowCross-Buffer (Candor Bioscience GmbH, Weissensberg, Germany). An experimental therapeutic protein drug was measured in a competitive format. The protein drug was spiked in human serum and then diluted at 1:750 in the two assay buffers for measurement. In an assay for drug safety studies, FDA specifies that the coefficient of variation (CV) must not exceed 15% over the whole measurement range.

antibody relative to that of the unlabeled antibody. These unspecific effects of the dyes can also cause increased binding of the antibody to test surfaces such as the plastic of ELISA plates, binding to endogenous proteins in the sample, or binding to the capture antibody. In these cases, false-positive measurement in the absence of the analyte will occur, or the entire assay will suffer from a high background. With protein arrays, increased background fluorescence of single spots is observed. The signal-to-noise ratio deteriorates.

Likewise, proteins or antibodies can bind to fluorescence dyes, with some of them being able to reduce, or even switch off, the fluorescence of the dye. All reactions taking place on protein arrays are very complex, because a variety of capture and labeled detector antibodies are used simultaneously in one reaction volume. Therefore, the probability of

nonspecific binding of proteins from the sample or of labeled antibodies with single spots is greater. Interference effects involving components from the sample affecting the antibodies are much more common in protein arrays than in traditional assays.[11]

Cross-Reactions as Cause

Cross-reactions, or cross-reactivities, are occasions when the antibody exercises its ability to bind structures other than the target analyte. Frequently, these structures show a great similarity to the analyte. Examples would be metabolites of the analyte, chemical substances with a similar molecular structure, and proteins with a random similarity or with homologies of the amino acid sequence.

Cross-reactions can affect competitive assays in a very dramatic manner, because only one assay antibody is
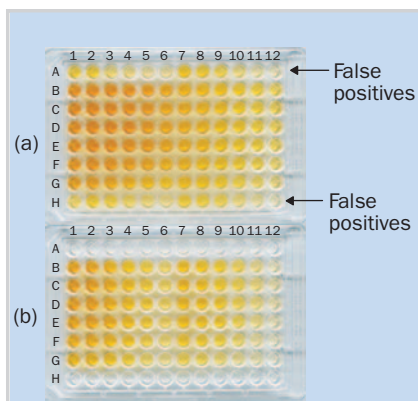
Figure 3. An ELISA against immunoglobulins from guinea pig that is used for immune toxicological studies is shown. The analyte was diluted in standard assay buffer (a) and in LowCross-Buffer (b) at concentrations of 50 ng/ml (range 1–6) and 10 ng/ml (range 7–12). Representing one step during the assay development, the plates show the titration of capture and detector antibody in rows B to G. With the standard assay buffer (a), false positives in the specificity control (row A) and in the blank values (row H) spoiled the interpretation and evaluation. The use of LowCross-Buffer (b), however, prevented false-positive signals.

SOURCE: PARA BIOSCIENCE GMBH (GRONAU, GERMANY).

applied.[4,6] Investigation of potential cross-reactivities is a recommended part of most assay validations, including when validations are done according to FDA guidelines.[12]

Cross-reactivities can play a significant role in the detection of proteins in Western blotting and in immunohistochemical applications. This becomes apparent in the staining of additional bands or cell structures. In many cases, the exact molecular reasons for these unwanted bindings are unknown. In Western blotting, the cause often will simply be naturally occurring degradation products of the analyte protein. Degradation can also result from the methodical approach. But, in some cases, this story of degradation is not the true one, and real cross-reactions of the primary or secondary antibodies have to be considered.

Nonspecific Binding and Matrix Effects

A phenomenon closely related to cross-reactivity is nonspecific binding. However, there are differences in the molecular origin of these effects. These differences are rarely noticeable in daily laboratory routine. If the cross-reactant is known and its cross-reaction can be measured with a competing concentration of the cross-reactant, then it is indeed a cross-reaction. In the case of nonspecific binding, substances with far higher concentrations than the target analyte are present. These can be albumins or immunoglobulins, or even surfaces, such as those of ELISA wells or of Western blotting membranes, or spots of immobilized antibodies in protein arrays.[11]

The term *matrix effects* is used to denote the sum of all interference effects of all components that appear in a specimen and influence the measurement of a target analyte.[13] If the actual molecular cause of an influence is undetermined but is known to come from the specimen, then it is called a matrix effect. The boundaries between one kind of effect and another are flexible and sometimes unclear. Some matrix effects derive from antianimal antibodies; others are the result of endogenous interferers or just caused by the viscosity, pH value, or the salt concentration of the specimen.

Endogenous Specimen Components as Cause

Even naturally occurring proteins in a specimen can interfere with immunoassays. Some well-known interfering substances in human sera are albumins, complement factors, lysozymes, alpha-1 antitrypsin, and fibrinogen.[4] Analytes of low molecular weight can bind to albumin. This makes access to the antibody difficult for the analyte. Also, numerous hormones are bound to transportation proteins, leading to sterical hindrance in assays. Therefore, hormone-determining immunodiagnostics are well known to have high coefficients of variation.

Moreover, many proteins are able to bind other substances; this is part of their biological function. Albumin, complement, and C-reactive protein are natural receptors for many substances. Nonspecific binding and cross-reactivity are expressions of their biological function but can interfere with assays. Endogenous proteins may bind the assay antibodies as an interfering factor, or may mask the target analyte. Lysozymes, for example, bind nonspecifically to most proteins with a low isoelectric point. Therefore, antibodies that have an isoelectric point of about five can be bound so as to build a bridge formation between the capture and detector antibodies.[4]

Another important endogenous-component effect is interference by a strongly lipid-containing specimen. Some analytes are fat soluble; thus, the bond between assay antibody and analyte can be affected by lipids.

A Universal Solution

Regardless of whether they derive from the matrix, from cross-reactivities, from endogenous substances, or from patient disorders, most interference effects have one thing in common: they relate to low- to medium-affinity binding reactions during the assay procedure.[6] Specific binding of the assay antibody to the analyte is high affinity binding. However, all kinds of binding—whether only low- to medium affinity or high affinity—can result in the same assay signals.

A general approach to preventing those interference effects would be to block all low- and medium-affinity binding instead of blocking only specifically heterophilic antibodies. This could be achieved by means of an assay buffer with high stringency. Such an approach could prevent all interference effects in one step, but it also would negatively affect the correct signals of the assay antibodies. Therefore, using high-stringency buffers is not an effective option. The high-affinity binding of the assay antibodies must not be compromised.

Discrimination between high-affinity binding on the one hand and low- to medium-affinity binding on the other is important for success in this area. That is the mode of action of an assay buffer called LowCross-Buffer, newly developed by Candor Bioscience GmbH (Weissensberg, Germany). By having no deleterious effect on high-affinity binding, this buffer does not reduce the true signal of high-affinity binders. But it does reduce dramatically low- to medium-affinity binding. This prevents the appearance of most interference effects, whether or not the molecular sources of the interference are known. The interference reducer can be used as an assay buffer or an antibody diluent. That is, either the specimen or the assay antibodies, or both, can be diluted in this buffer. What is important is that the binding reaction between the assay antibody and the analyte takes place in LowCross-Buffer.

The detector antibodies for immunodiagnostic kits have to be stored for a long term in the antibody diluent. Therefore, the new buffer is available also in a stabilizing formulation called LowCross-HRP. This stabilizer solution for HRP conjugates also exhibits the technology's basic minimization of interference effects, of course. With it, assay antibodies can be stored for long periods at 4°C in low ready-to-use concentrations. This saves predilution steps during the assay procedure.

Performance

The reliability of assay results can be shown by means of precision profiles. For preclinical studies, FDA requires an ELISA validation that includes precision profiles.

A comparative precision profile reveals the significant extent to which using LowCross-Buffer can enhance the reliability of assay results (see Figure 2). This ELISA, for use in pharmaceutical research, detected an experimental therapeutic protein (that is, a drug candidate) in human serum. It was carried out in a competitive format with the drug protein as a biotinylated tracer.
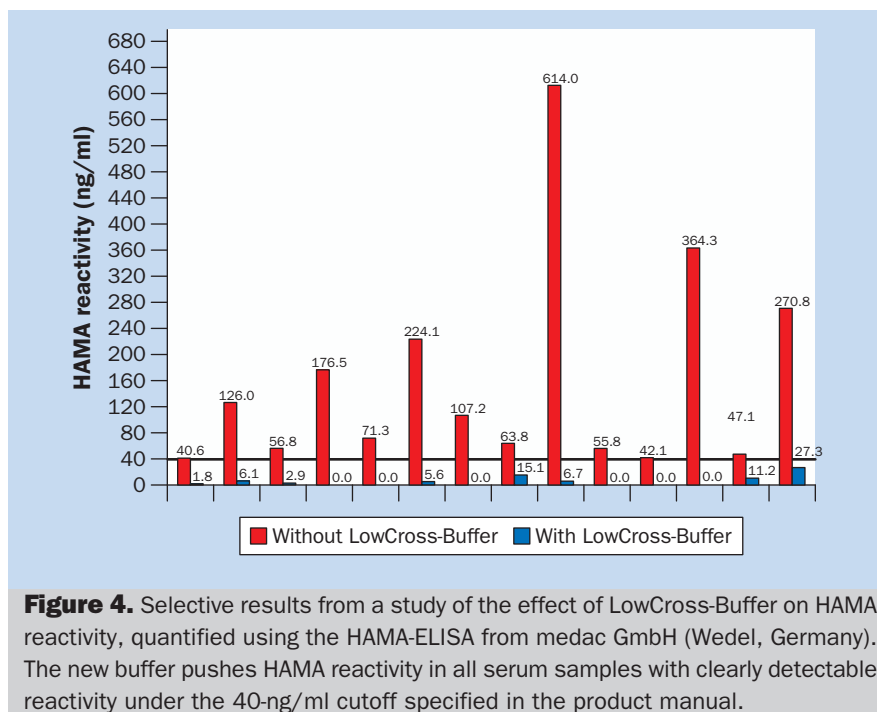
Figure 4. Selective results from a study of the effect of LowCross-Buffer on HAMA reactivity, quantified using the HAMA-ELISA from medac GmbH (Wedel, Germany). The new buffer pushes HAMA reactivity in all serum samples with clearly detectable reactivity under the 40-ng/ml cutoff specified in the product manual.

It was detected with NeutrAvidin HRP conjugate. The drug candidate was spiked in human serum to measure the calibration curve. Then the serum was diluted at 1:750 prior to measurement in the assay buffers. This ELISA was validated according to the FDA guidance.[12] Only with the use of the new interference-reducing buffer could the required precision be achieved over the entire measurement range.

Another ELISA, this an assay against immunoglobulins from guinea pig that is used for immune toxicological studies, shows how the new buffer can help avoid false-positive results (see Figure 3). Goat-anti–guinea pig IgG F(ab')2 as a capture antibody and goat-anti–guinea pig IgG (Fcγ) biotinylated as a detector were the antibodies used. The guinea pig IgG (the analyte) was diluted in standard assay buffer and in LowCross-Buffer. In the figure, ranges 1–6 represent a concentration of 50 ng/ml, while ranges 7–12 represent 10 ng/ml.

The plates in Figure 3 depict one step during the assay development. The titration of capture and detector antibody on the plate with standard assay buffer can be seen in rows B to G. False positives in the specificity control (row A) and in the blank values

(row H) spoiled the interpretation and evaluation. The use of LowCross-Buffer prevented the false-positive signals and, moreover, made possible the detection of serial dilutions in rows B to G 1–6 and B to G 7–12 (see Figure 3b).

The results from these two sets of comparative ELISAs show the effectiveness of the new buffer against matrix-derived and unknown interference effects that lead to assay imprecision or false positives. The general applicability of the buffer can be recognized as well in a record of its effectiveness with respect to heterophilic sera.

A CE-certified diagnostic assay, the HAMA-ELISA from medac GmbH (Wedel, Germany) was used to detect HAMA interference. This ELISA measures cross-bridging by heterophilic antibodies. The investigators used it to test commercially available HAMA serum panels obtained from Scantibodies Laboratory Inc. (Santee, CA) and from in.vent Diagnostica GmbH (Hennigsdorf, Germany) that are sold for the purpose of testing and showing the potential of a HAMA-blocker or HAMA-blocking reagent by preventing interference derived from such sera. Obviously, the providers of the panels include a wide range
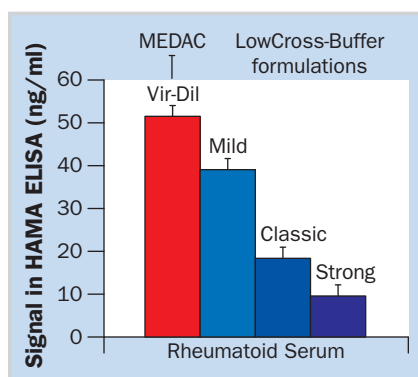
Figure 5. Effects of the various formulations of LowCross-Buffer on the cross-linking reactivity of a rheumatoid serum in comparison with that of the assay buffer (MEDAC Vir-Dil) of the HAMA-ELISA. Cross-linking would lead to false results in immunodiagnostics. All of the formulations of the new buffer can prevent unwanted binding between rheumatoid factor and assay antibodies according to the affinity of cross-linking. The binding between assay antibodies and analytes is of higher affinity.

of different and very critical heterophilic sera to reveal the potential of the HAMA-blocker and the problems that may arise from heterophilic patient sera.

When the complete panels were tested, every heterophilic interference effect was overcome by the interference-reducing buffer. A signal below 40 ng/ml indicates that there is no interference effect, according to the HAMA-ELISA manual. The short study showed that the interference could be effectively minimized simply by switching from the standard dilution buffer (called MEDAC Vir-Dil) to the new assay buffer (see Figure 4).

It is important to note that the positive controls of the HAMA-ELISA are still positive with LowCross-Buffer and not pushed under the cutoff. (These data are not shown in the figure.) The positive controls are not HAMAs but cross-linking secondary antibodies (goat antimouse IgG antibodies). This shows that signal from normal assay antibodies is not negatively affected by the new assay buffer.

Application Potential

The medical questions addressed by immunodiagnostics vary significantly in terms of the answers required.

Antigen-detecting assays for infectious-disease testing should detect even very low concentrations of the antigen—for example, virus particles. Neither false negatives nor false positives are acceptable. The assay antibodies can show extremely high affinities with K_D-values in the range of 10^{-11} or 10^{-12} M. Every kind of interference should be considerably reduced in order to get the most reliable diagnosis. Good reliability in initial testing can minimize the cost of second-line testing for critical infections. For infectious-disease assays, the difference between the affinity of the assay antibodies and the affinity of interference binding is very large.

On the other hand, autoimmune-disease testing and allergy testing address different medical questions. In these assays, patient autoantibodies or allergic antibodies that do not show very high affinities should be detectable. Patient antibodies with medium affinities can be part of the disease structure; therefore, even medium-affinity binding should be included in the signal.

The completely different medical requirements of infectious-disease testing versus allergy testing show that there is a need for different degrees of interference reduction. Obviously, a strong interference-reducing solution achieves a maximum of safety. However, in such a strong solution, the assay antibodies have to exhibit very high affinities. In other words, an assay performed with a very strong interference reducer has an extremely high resistance to any kind of interference. The milder the interference reduction, the more interference effects will occur even when the interference-reducing buffer is used. But, with such a mild interference reducer, even medium-affinity patient antibodies can be detected correctly.

To meet varying immunodiagnostic needs, Candor Bioscience offers LowCross-Buffer to immunodiagnostics manufacturers in formulations designated as Mild, Classic, and Strong. The Classic formulation provides the best compromise between the affinity required from the assay antibodies and the strength of most interference effects, and is suitable for most applications. The advanced formulations Mild and Strong were created for professional users, such as immunodiagnostics manufacturers, with special application needs. All three formulations can push an interference effect—cross-linking reactivity—derived from a rheumatoid factor serum under the 40-ng/ml HAMA-ELISA cutoff (see Figure 5), but Classic and Strong have a greater impact, leading to an assay with better reliability. These assay results show that this new buffer approach can be not only used universally but also adapted to specific assays and clinical requirements if necessary.

The mode of action of this interference reducer is to discriminate between low- and medium-affinity binding (of interference effects) on the one hand and high-affinity binding (of assay antibodies to analytes) on the other. Therefore, it is not a blocker of some substances, but rather an affinity discriminator. If too much HAMA is in a patient's sample, this cannot be blocked off with too-low concentrations of HAMA-blockers. However, this effect is not observed with the new buffer because of its different mode of action. While a HAMA-blocker might be administered in a concentration that is too low, the performance of the new affinity discriminator is not dependent on the patient's specific HAMA concentration. This further enhances the reliability and robustness of assays done with the new assay buffer.

Conclusion

The studies described in this article show that a new affinity-discriminating buffer has the potential to deal with a very wide spectrum of interference. Reducing interference increases assay reliability. FDA requires a very high level of precision in immunoassays conducted for preclinical or clinical

studies. Various assays have demonstrated the wide-ranging potential of the new assay diluent to ensure attainment of the required precision and reliability.

Until recently, only specific and very limited approaches to overcoming interference, such as HAMA-blockers, were available. But a new approach that can be used for nearly any kind of interference and is restricted neither to HAMAs or heterophiles nor to cross-reactivities or matrix effects has been developed. It is universal, eliminating all these problems with one technology. If necessary, it can be further adapted to accommodate specific needs. The assay buffer has displayed its potential in assays for preclinical and clinical studies and for food diagnostics as well as human diagnostics. It is being used in the production of lateral-flow assays, ELISAs, protein arrays, and Western blotting kits.

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CANDOR Bioscience GmbH

Corporate Description

CANDOR Bioscience is a supplier to ELISA kit manufacturers worldwide. CANDOR Bioscience GmbH is a privately owned company located at the border triangle Germany, Austria and Switzerland. CANDOR is a technology solution provider for optimising immunoassays. The buffer solutions are "Made in Germany" and are manufactured according to the DIN EN ISO 9001:2000 certification. CANDOR solutions are used in life sciences, food analytics, diagnostic assays and in GLP-laboratories.

CANDOR focuses on immunoassays.

Core competencies are

- improving of reliability and reproducibility of critical assays
- long-term stabilization of antibodies, proteins and enzymes
- assay development for pharmaceutical research, pre-clinial and clinical studies
- product development for immunodiagnostics companies

Products/Services

CANDOR offers a wide range of innovative buffer solutions applicable to immunoassays such as ELISA, Western blotting, lateral flow assays, protein arrays, immuno-PCR and immunohistochemistry. The product portfolio comprises special assay buffers, innovative blockers and outstanding stabilizers as well as complementary buffer solutions for all kinds of immunoassays. The innovative assay buffer LowCross-Buffer® was specially developed to minimize interference in immunoassays. It can substitute for HAMA blockers. Thus quality and reliability of the assay can be significantly improved. Non-specific binding of the antibodies, negative effects of interfering substances and low to medium cross reactivities of the antibodies are minimized. In addition matrix effects are reduced, which are derived from blood sera, plasma or other specimens.

The blockers prevent non-specific and unwanted binding to surfaces. CANDOR provides an appropriate blocking buffer for every application.

The most effective blocker is The Blocking Solution, a blocker based on chemically modified casein. The very high blocking effectiveness compared to alternative blocking agents like BSA can be easily quantified with the measurement of the coefficient of variation.

CANDOR also offers a wide range of excellent stabilizers.

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Liquid Plate Sealer® is a stabilizer for coated ELISA plates. After immobilization and blocking, Liquid Plate Sealer® seals the plates with a uniform stabilizing layer demonstrating good solubility and without affecting the assay afterwards.

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CANDOR offers their products and services to customers in Life Sciences and pharmaceutical research as well as to the diagnostics industry. For the diagnostics industry all products are offered as OEM supplies. Products are used in ELISA, Western blotting as well as in lateral flow assays. Due to DIN EN ISO 9001:2000 certification all products can be used directly in regulated areas like GLP laboratories.

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