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Optimisation of assays: Interference in immunoassays recognize and avoid

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Interference in immunoassays

Recognize and avoid

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Immunoassays are analytical laboratory methods for the detection of different substances using antibodies. They are used commonly in bioanalytical and biochemical laboratories of the Life Science industry. The methods are Enzyme-linked immunosorbent assays (ELISA) and Enzyme immunoassays (EIA), Western blotting, Radio immunoassays (RIA), Protein-Arrays, Immunohistochemistry or also the immuno-polymerase chain reaction (Immuno-PCR). In all kinds of immunoassays the detection of the analytes can be influenced by interference. Very frequently cross reactivities, unspecific binding and matrix effects occur. Interfering substances are present in more or less significant concentrations in real specimen and interact with the analytes or with the capture respectively the detector antibodies directly. By application of a new buffer (LowCross-Buffer®) instead of the usually used buffers most interference effects can be avoided. Through this exchange the quality of the assays and the efficiency of the assay development is improved.

Key Words: LowCross-Buffer®, antibodies, interference factors in immunoassays, ELISA, Western blotting, protein arrays, immunohistochemistry

Niels Kaj Jerne, who received in 1984 the medicine Nobel Prize for his work on the specific construction and the control of the immune system, mentioned in his award lecture that every antibody is multi-specific. He related this statement to the antibodies building during the early phase of an immune response^{1,2}. Antibodies apparently characterized well with a high affinity to the target analytes show occasionally surprising results: During immunological detection on western blotting membranes unwanted bands are stained, on protein arrays one gets fluorescence signals for spots at the wrong positions from immobilized capture antibodies as well as a high background signal for blank samples. For ELISAs one gets a high background for the negative control or false negative signals in measurements. Misstatements due to the interference effects can lead to subsequent costs and also to false diagnosis³.

All immunoassays are characterized by a binding reaction between the target analyte and antibody. The problem of these methods, which is solved only insufficiently till now, is the regularly occurring interferences which lead to faulty measurements. Typical interferences are unspecific binding which lead to a poor signal to noise ratio and a high background, cross reactivities and matrix

effects. Expressed in simplified terms, most of these effects are based on a direct interaction of the analytes, the capture antibody or the detector antibody with external substances or surfaces. A simplified scheme of typical interference effects is represented in illustration 1. Known interfering factors, which are regularly described in the technical literature, are for example heterophilic antibodies as well as HAMAs (humane anti mouse antibody)² or rheumatism factors, albumins, complement factors, lysozyme⁴ and others.

Interference by immunoassay label

It is common for immunoassays to mark the detector antibody - in the case of competitive assays: the standard analyte - with a label. Frequently used labels are enzymes (very often alkaline phosphatase or (horseradish)-peroxidase), fluorescence dyes, radioactive isotopes or also DNA (for immuno-PCR.) The unwanted effects appear here too. The danger, using fluorescence dyes as a label, is often that hydrophobic dyes change the binding qualities of the detector antibody causing undesirable binding by the dye itself and reducing the solubility of the labelled protein. In addition the antigen to antibody binding can become weaker⁵. For example these

unspecific effects can cause increasing binding of the antibody to the surfaces (fig. 1 A and B), to extrinsic proteins in the sample (fig. 1 C) or to the capture antibody (fig. 1 D). In these cases false-positive measurement in absence of the analyte will occur or the entire assay suffers under a high background. For protein arrays increased background fluorescence for single spots is observed or altogether the signal-to-noise ratio deteriorates. Likewise proteins or antibodies from serum samples can bind to fluorescence dyes and reduce or even switch off the fluorescence of the dye. Therefore some researchers already recommend to abandon fluorescence dyes as a label for protein arrays or to choose other labels respectively⁶. The reactions on protein arrays are very complex since a variety of different capture and labelled detector antibodies are used simultaneously in one reaction volume. Therefore the probability of unspecific binding of proteins from the sample or labelled antibodies with single spots as well as the danger of interference effects of components from the sample with the antibodies is increased⁷.

Interference by cross reactions

Cross reactions are the ability of the antibody to bind also to other structures than the target analytes (fig. 1, I-K). Frequently these structures show a great similarity to the analytes. Examples therefore are metabolites or chemical substances with a similar molecular structure. Also proteins with a coincidental similarity or with homologies of the amino acid sequence can cross react. Especially in competitive assays cross reactions play a greater role since only one antibody is applied^{3,4}. Very often possible cross reacting substances and the cross reactivity need to be quantified for validation of these kinds of assays⁸.

Cross reactivities can play also a significant role at the detection of proteins on western blotting or at immunohistochemical applications. This becomes apparent in the staining of additional bands or cell structures, although one doesn't know the exact molecular causes for these unwanted bindings in every case. In Western blotting one assumes in many cases simply degradation products of the right protein, which result naturally or from the methodical approach. But in some cases that's not the truth and one

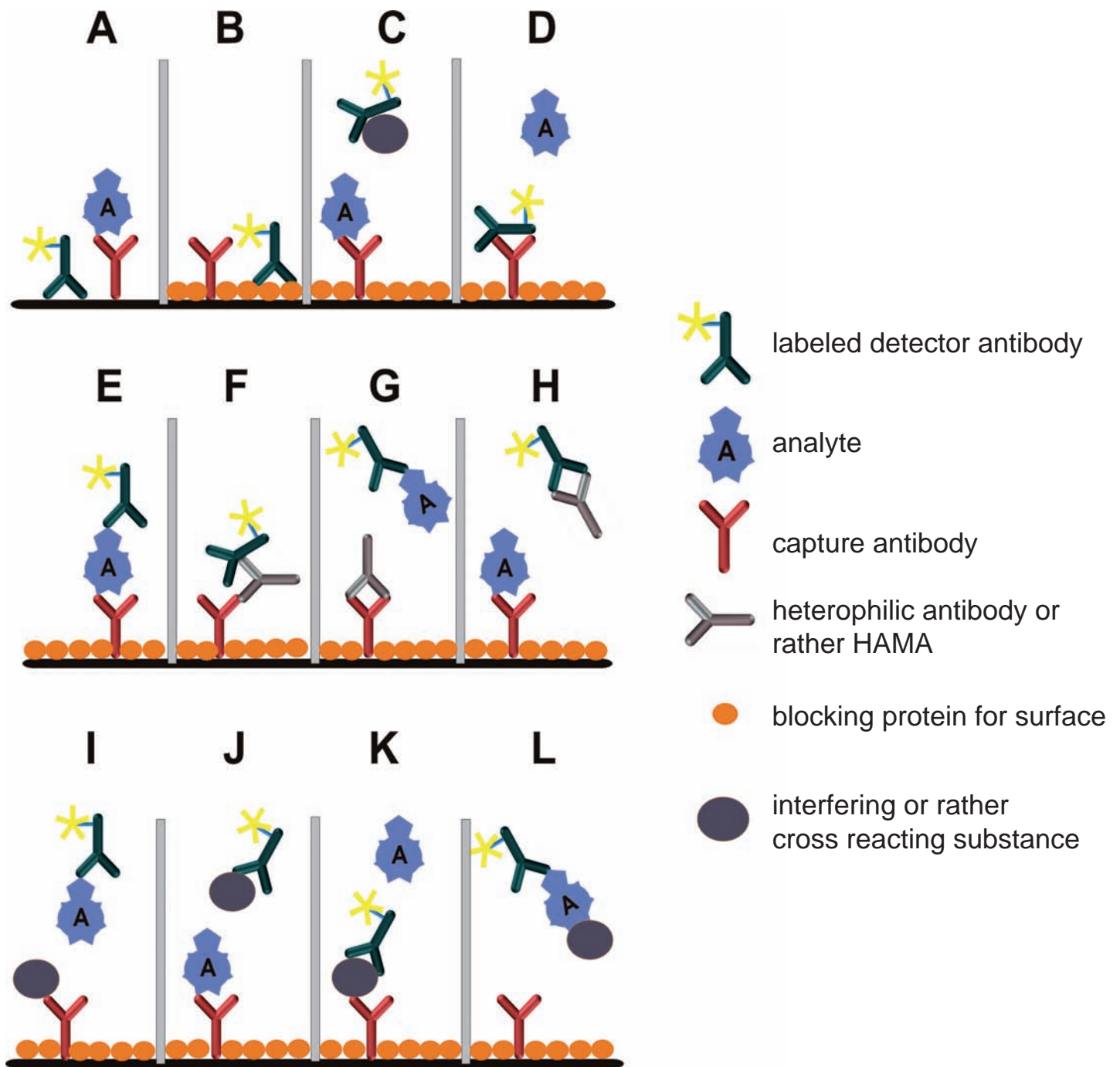


Fig. 1: Schematic picture of a choice of different interference effects which may appear in immunoassays. **A:** unspecific binding of a labeled detector antibody to a not blocked surface. The results are false positive signals. **B:** unspecific binding of a labeled detector antibody to a blocked surface. Despite blocking of the surface the antibody binds to the blocking proteins itself. The results are false positive signals. **C:** An interfering protein binds to the Fc segment of the detector antibody and hinders sterically the binding of the analyte. The results are false negative signals. **D:** The capture antibody binds to the Fc segment of the detector antibody. The results are false positive signals. The analyte cannot be bound by the capture antibody any more. **E:** Uninfluenced assay without any interference. The ideal state of affairs. **F:** A „bridge binding“ by heterophilic antibodies or by HAMAs. Through this the capture antibody is connected with the detector antibody so that false positive signals arise. **G:** A HAMA with anti-idiotypic binding qualities to the capture antibody. The interfering antibody binds in the area of the highly variable region of the Fab segment and thus prevents the binding of the analyte. False negative signals appear as a result of it. **H:** A HAMA with anti-idiotypic binding qualities to the detector antibody. The interfering antibody binds in the area of the highly variable region of the Fab segment and prevents the binding of the analyte. As a result of it false negative signals show up. **I:** Cross reactivity of an interfering substance with the capture antibody. The results are false negative signals. **J:** Cross reactivity of an interfering substance with the detector antibody. The results are false negative signals. **K:** Cross reactivity both with the capture and with the detector antibody. Such a phenomenon is rather seldom in practice, but definitely possible with antibodies having a lower specificity. Such an interference picture can happen also with antibodies directed to a target with a conserved amino acid sequence of a protein whose sequence motive also occurs at other proteins. **L:** Masking of the analyte by a protein of the specimen whereby the epitop is blocked for the capture antibody so that the binding to the analyte is not possible or in the case of a sterical hindrance is very bad. The results are false negative signals.

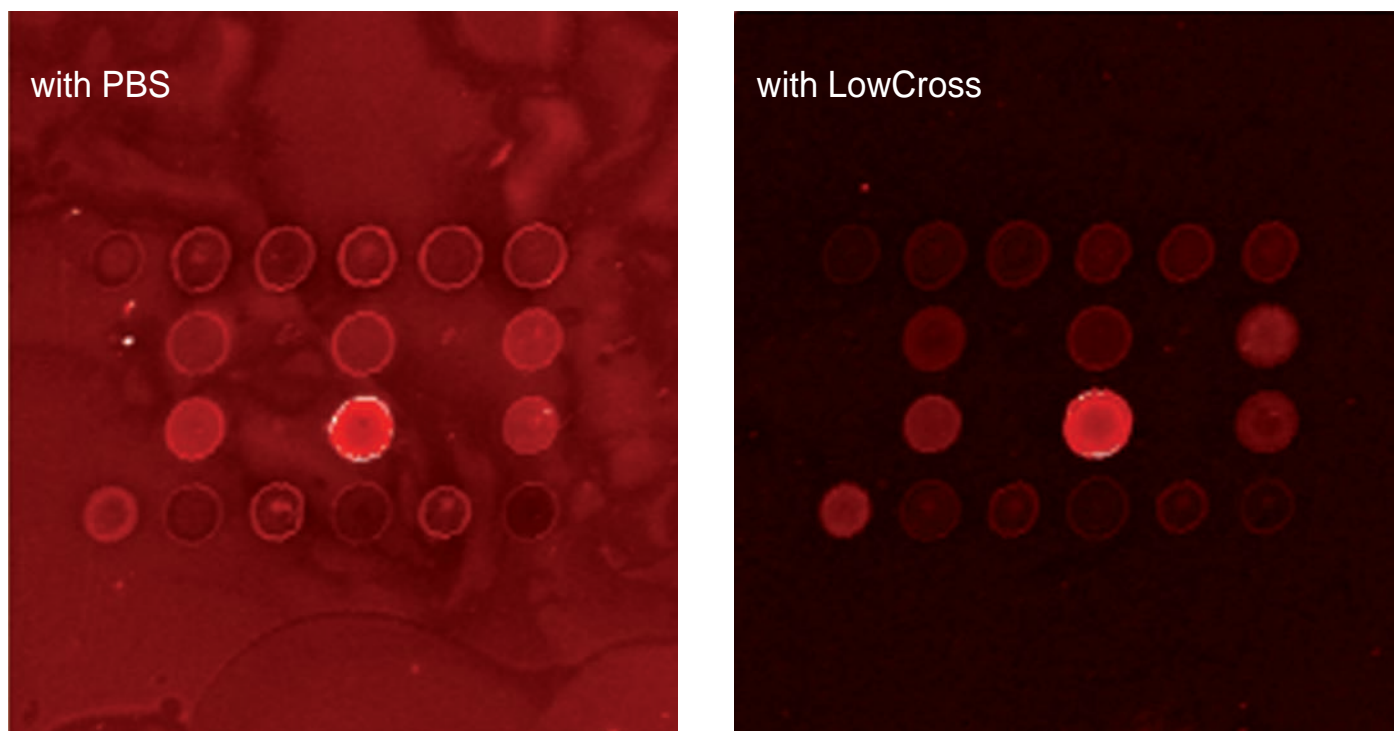


Fig. 2: Reduction of unspecific binding of the detector antibody to the surface of an protein array. Improvement of signal-to-noise ratio from 3,4 to 17,3 by the use of LowCross-Buffer® (data from N. Dankbar, university of Münster).

has to consider cross reactivities of the primary or secondary antibodies.

Interference by unspecific binding

Closely related to the cross reactivities are unspecific bindings. However the causes on a molecular level differ. In daily lab routine these differences are rarely noticeable. One deals with a cross reactivity, if the cross reactand is known and its cross reactivity can be measured for example with a competing concentration of the cross reactand⁸. In the case of unspecific binding the binding deals with substances, which are in far excess of the target analyte (i.e. unspecific binding to albumines or immuno globulines) or with surfaces (i.e. surfaces of ELISA-wells or of Western blotting membranes) or with spots of immobilised antibodies in protein arrays⁷.

Matrix effects

Matrix effects are the sum of all interference effects of all components, which appear in a specimen and influence the measurement of a target analyte⁹. If the true molecular cause for an influence is not determined, but one knows that it comes from the specimen, then you call it in general a “matrix effect”. The borders from one kind of effect to the other kind

are floating and sometimes unclear. Some matrix effects are derived from “anti-animal-antibodies” others from heterophilic antibodies, from endogenous interferers or just from viscosity, pH-value or simply the salt concentration.

Interference by „anti-animal-antibodies“

Human anti-animal-antibodies (HAAA) can be of the IgG-, IgA, IgM- or IgE-type. They are part of the immune systems answer to contacts with immune globulines from animal origin. HAAAs are well known interferers in many diagnostic immunoassays and can be part of sometimes 80% of all clinical specimen – depending on the study cited. The concentrations of HAAAs achieve ranges up to milligrams per milliliter¹⁰.

Human-anti-mouse-antibodies (HAMA) are the most popular under the interfering antibodies in immunoassays. HAMAs are human antibodies, binding with significant specificity and sometimes noticeable affinity mouse-antibodies. Reason for the build-up of these antibodies in patients are often therapeutic antibodies, which are given as drugs for cancer therapies. After medication the immune system of patient reacts to these extrinsic antibodies by forming own antibodies against the therapeutic mouse-antibodies. HAMAs therefore interfere with immunological

assays, which make use of mouse-antibodies as assay-reagents. In sandwich assays with mouse monoclonals this can result in a direct bridge binding between capture and detector without any analyte (fig. 1 F). This leads to false-positive signals. There are similarities in the sequences between antibodies derived from different species. That means, that HAMA-containing sera can also make problems in assays which make use of antibodies derived from other species than mouse with the same results of false signals.

HAMAs are not only derived from antibody therapies. Long exposure to domestic animals and pets can also end up in the formation of antibodies against these animals. One has found in patients sera antibodies against rabbits, mouse, dogs, hamsters just to name a few. These anti-animal-antibodies interfere with some assay-antibodies with different affinities and different kinds of problems. Some interfering antibodies are not only directed against the Fc-fragment, but also against the Fab-fragments of assay-antibodies. This can result in a reduction or total hindrance of correct binding, resulting in false negative signals (fig. 1 G and H). If the HAAAs bind to the Fc-fragment they are called anti-isotypical interferers. If they bind to the highly variable Fab-fragment they are called anti-idiotypal

interferers¹⁰.

Interference by heterophilic antibodies

Taber's Medical Dictionary defines heterophilic antibodies as „antibodies which bind other antigens than the specific antigen“¹¹. Heterophilic antibodies can be of the IgG, IgA, IgM or IgE type. Particularly the IgM type plays a particular role in sera of rheumatic patients. These sera contain so-called rheumatism factors in high concentration. Rheumatism factors are IgM antibodies that bind to the Fc sections of humane antibodies and who can bind therefore species independent also to Fc sections of the antibodies used in the assay. Therefore rheumatic sera connect capture with detector antibodies with the consequence of false positive signals. This is at the same time the general interfering mechanism of the heterophilic antibodies. The effect of the rheumatic sera resembles the effect of the HAAAs. The difference compared with the HAAAs is the origin of the heterophilic antibodies: These are not build on contact with animal immune globulins, but they are multi-specific antibodies of the early immune response or interfering antibodies with unknown immunological history of origins²,

Interference by HAAAs or by heterophilic antibodies are known already for more than 30 years. The interfering antibodies are general weakly binding antibodies² from animal origin and disturb predominantly assays that - due to the low concentration of the analytes - need to get along with a low dilution of serum or plasma samples¹². Additions of blocking substances to the sample buffer – commonly non-specific sera, antibody fragments or high concentrations of animal immune globulins – are able to reduce the interference effects of the HAAAs or heterophilic antibodies by competition but don't circumvent at all times¹⁰.

Interference by endogenous components of the specimen

Even naturally occurring proteins of specimen can interfere with immunoassays. Well known interfering substances in human sera are for example albumins, complement factors, lysozymes and fibrinogen⁴. Analytes of low molecular weight can bind to albumin. This makes the accessibility of an antibody to the analyte difficult. Numerous hormones are bound

to transportation proteins, what can lead to difficulties. Moreover, many proteins have the ability to bind other substances and proteins of course. This binding ability is often a substantial part of the biological function of the respective protein. Albumin, complement and C-reactive protein (CRP) are natural receptors for many substances. Unspecific binding or even cross reactivity – like with antibodies – are therefore possible, which complicates the recognition of certain analytes in an assay. Endogenous proteins can bind as an interfering factor to antibodies (fig. 1 C, I-K) or mask the target analyte (fig. 1 L). For example lysozymes bind non-specifically to proteins with a low isoelectric point. Therefore antibodies which have an isoelectric point of about 5 can be bound and build a bridge formation between capture and detector antibody⁴. An important aspect, which should be mentioned, is the interference by strongly lipid containing specimen, because some analytes are fat-soluble and as the case may be the binding between antibody and analyte can be affected by lipids.

The Hook effect

The Hook effect leads to false negative determinations which, however unlike the other interference effects, do not arise through interactions with interfering factors.

A Hook effect can appear in assays, where the specimen is mixed directly with the assay antibodies and the analyte appears in very high concentrations. In this case the high concentration of the analyte, when it exceeds the concentration of the assay antibodies, capture and detector antibodies can be saturated. Thus high concentrations simulate far lower concentration in the assay and lead to a significant underestimation of the true concentration in the assay⁴. The Hook effect can be avoided in the practice by using higher concentrations of the assay antibodies or by dilution of the specimen. Alternatively a systematic dilution of the test has to assure, that the measured value is not subject to a Hook effect. Known clinical parameters, which can be subject to a Hook effect, are CRP, AFP, CA 125, PSA, Ferritin, Prolactin and TSH for example⁴.

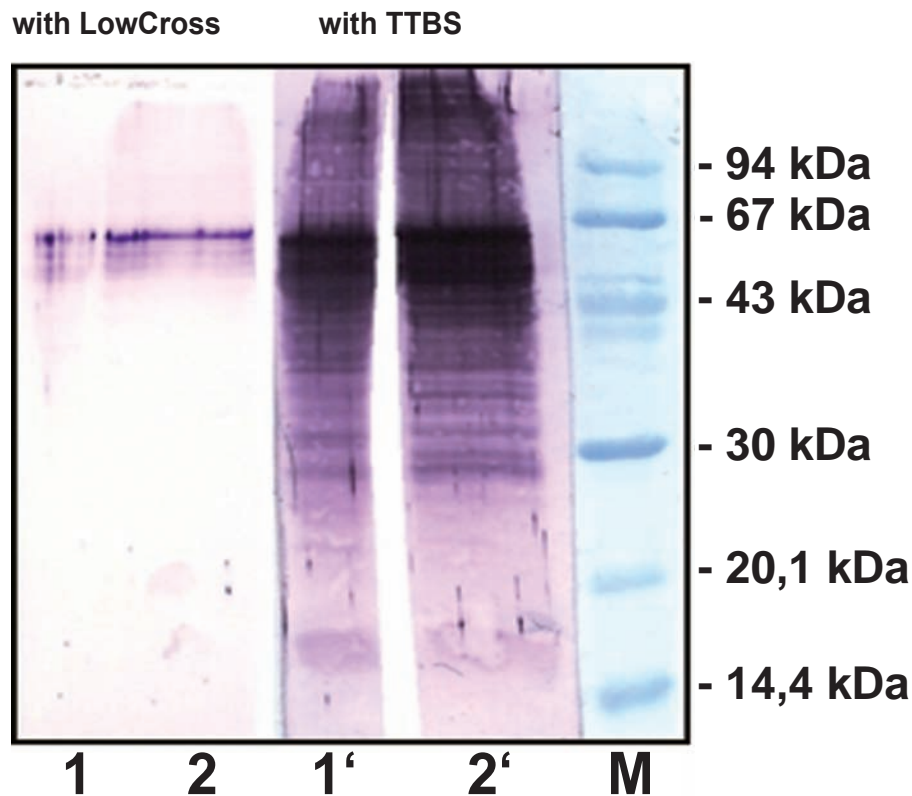


Fig. 3: Immunological detection of the cytokeratin 4, 5 and 6 after Western blotting (done by Dr. D. Sperling, MACHEREY-NAGEL, Düren). The figure shows the comparison between detection with LowCross-Buffer® and TTBS. LowCross-Buffer® could prevent unwanted binding totally. Cytokeratins 4, 5 and 6 in between 56 and 60 kDa are clearly detected with LowCross-Buffer®. Lanes 1 and 1' show detection from liver cells and lanes 2 and 2' show detection from HeLa-cells. M is the molecular weight marker, stained with Amido black. Blotting membrane is a nitrocellulose-membrane parablott NCP (MACHEREY-NAGEL).

Prevention of interference by LowCross-Buffer®

The reasons for the described interference effects are similar. There are unwanted low up to middle affine interactions of the interfering factors with the antibodies or the analytes. And there are low or middle affine binding of labeled antibodies to other proteins or surfaces as well as low to middle affine cross reactivities of the antibodies to structure related substances. These interference effects have something in common which the newly developed LowCross-Buffer® makes use of: The interference reaction is weaker than the specific binding of the real analytes. Of course there are rarely exceptions since very high affine cross reactivities can occur that achieve the same qualities as the real specific binding. In such cases one then must speak about a specific binding and one has in principle an antibody which is aimed at two different substances. Therefore such an antibody is not usable at all for specific assays. The LowCross-Buffer® was developed specifically to eliminate generally weak and middle affine binding, but not negatively affecting high affinity binding with high specificity in any way.

Figures 2 to 5 show different examples of typical interference effects in immu-

noassays that are prevented by the use of LowCross-Buffer®. Figure 2 shows a protein array application at which LowCross-Buffer® reduces a high background and improves the signal to noise ratio from 3.4 to 17.3. In this experiment different polyclonal anti EPIL antibodies (EPIL = early placenta insulin like growth factor) were tested for their suitability. The cleaned antibodies were immobilized on aminosilane-functionalised micro-array slides by means of a spotter (GMS 417) with a concentration of 500 µg/ml and a volume of 1.8 nl/spot. Afterwards 2 ml of medium of an EPIL-overexpressing cell line (SKBR3) were mixed with the dye Oyster650P (Denovo Biolabels GmbH) and all proteins in this set were labeled. The incubation on the slide was carried out at a dilution of the medium in a relationship of 1:20 with LowCross-Buffer® in comparison with PBS (medium:buffer). After washing of the slides these were read with a fluorescence-scanner (GMS 418) and the data were evaluated with ImaGene (Biodiscovery Inc.). By the use of LowCross-Buffer® a clear reduction of the background signal could be reached which made the distinction of single antibodies regarding their suitability for the EPIL possible.

Figure 3 shows the immunological detection of a western blotting of the cyto-

keratines 4, 5 and 6 from liver cells and from HeLa cells, where further bands than the proper bands were detected by cross reactivities in combination with unspecific binding. The protein samples were separated electrophoretically on a 12.5% SDS-poly acrylamide gel and then blotted on a nitro cellulose membrane. The immunoblot detection for cytokeratins in the protein samples was carried out with a modified standard protocol¹³ as well as with LowCross-Buffer®. Bound anti-cytokeratin antibodies (polyclonal rabbit anti cytokeratin, Biomedica, Foster City, USA), diluted 1:2500 in LowCross-Buffer® or in TTBS (Tween-Tris buffered saline), were detected with an alkaline phosphatase coupled to a secondary antibody (Goat anti rabbit IgG, abcam, Cambridge, UK) (diluted 1:1250 in LowCross-Buffer® or in TTBS), detected and visualized by means of BCIP/NBT.

It is very difficult to uncover the exact molecular reason for the unwanted binding reaction, which made detection without LowCross-Buffer® impossible. Possibly cross reactivities are visible due to other cytokeratins and their cleavage products or unspecific binding with different proteins from the cell disruption of the liver or the HeLa cells takes place. By simple exchange from the TTBS buffer, where the primary and secondary antibo-

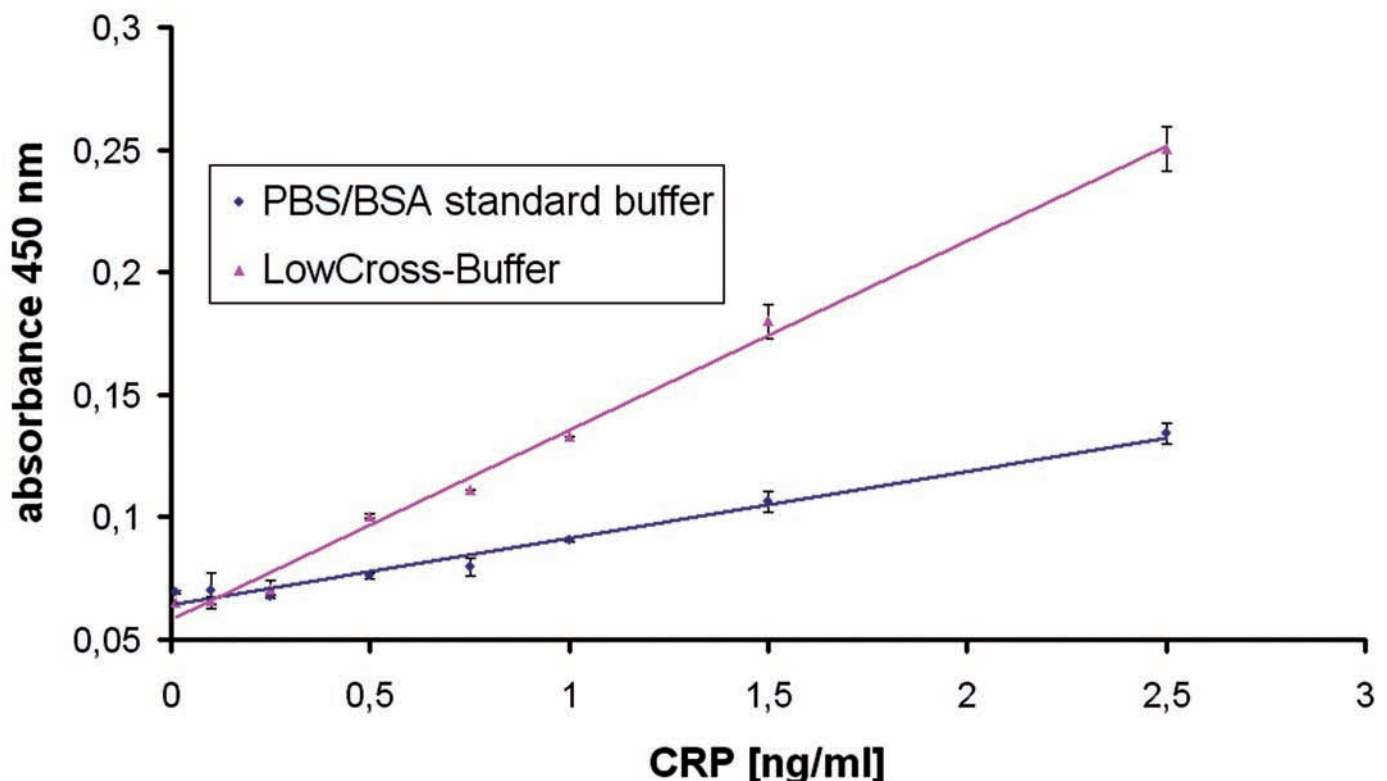


Fig. 4: ELISA of CRP in rabbit serum (carried out by A. Zellmer, CANDOR Bioscience). LowCross-Buffer® improves the sensitivity by removing a matrix effect.

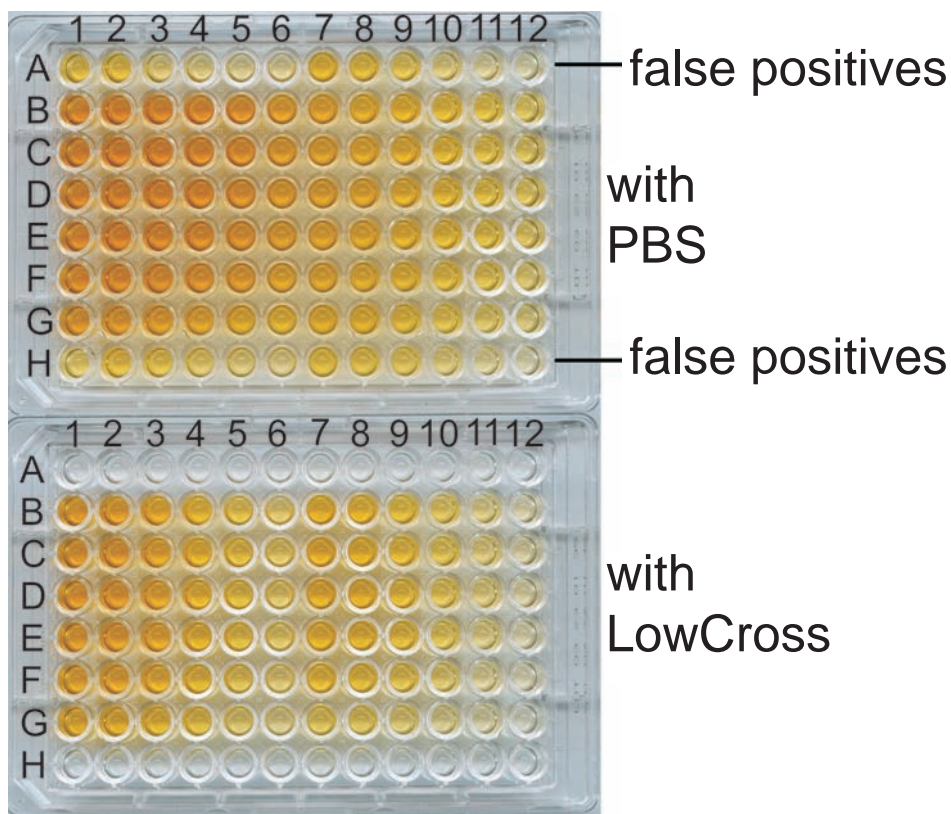


Fig. 5: Prevention of false-positive binding (control of specificity in row A1-12 and blank row H1-12) by the use of LowCross-Buffer® in an ELISA against guinea-pig IgG (done by Dr. C. Specht, PARA Bioscience, Gronau).

dies were diluted, to LowCross-Buffer®, the unwanted binding could be reduced to the extent that cytokeratins are stained exclusively in the correct molecular weight area of 56 to 60 kDa.

An example how a matrix effect can impact an ELISA is shown in illustration 4. With this model assay (developed by CANDOR Bioscience) a matrix effect was induced systematically. Rabbit serum was used as a matrix and spiked in defined concentrations with a humane C reactive protein (CRP, Biotrend). As capture antibody Clone C2 (Biotrend, 1 µg/ml Coating concentration in PBS), as a detector biotinylated Clone C6 (Biotrend, concentration 2 µg/ml) was used. The spiked serum samples were diluted either with a PBS-BSA buffer or with LowCross-Buffer® 1:2 and measured by ELISA. Detection was carried out via NeutrAvidin™-Horseradish peroxidase conjugated (Pierce, concentration 0.05 µg/ml in PBS-BSA buffer) with ImmunoPure® TMB-substrate (Pierce).

A matrix effect, whose exact molecular reason is not known, leads to a calibration curve with a bad sensitivity. Due to its physiological function CRP is able to bind many proteins and substances (scavenger function of the CRP) which probably

reduces significantly the accessibility of the epitope. Presumably an interference effect takes place as shown schematically in figure 1 (L) although interference effects as shown in figure 1 (I-K) cannot be excluded. LowCross-Buffer® prevents the binding of the CRP to endogenous substances of the rabbit serum and thus improves the sensitivity of the calibration curve by the factor of 3.

Figure 5 is an example of an ELISA against immune globuline from guinea pig (developed by PARA Bioscience, Gronau) which is used for immune toxicological studies in guinea pigs. In this assay false positive bindings in the specificity control (row of A1-A12) as well as at the blank value (H1-H12) spoiled the interpretation and evaluation. The use of LowCross-Buffer® prevented the false positive signals and moreover made the detection of the concentrations possible in the rows of B to G 1-6 or B to G 7-12. Goat-anti-guinea-pig IgG F(ab')₂ as a capture antibody and goat-anti-guinea-pig IgG (Fcγ) biotinylated as a detector (both Jackson ImmunoResearch Laboratories Inc., concentration range of 0.31 to 10 µg each/ml in PBS) were used. The guinea pig IgG was either diluted in LowCross-Buffer® or PBS (range 1-6 50

ng/ml, range 7-12 10 ng/ml). PBS-BSA buffer was used as a blocking buffer. The detection was carried out with Streptavidin-peroxidase (Sigma) and ortho-Phenylenediamine (Sigma).

Result

The phenomenon of interference in immunoassays is as old as the methodical use of antibodies for bioanalytical and diagnostic purposes. Numerous molecular causes were found in the course of the last 30 years and their interference mechanisms were evaluated which led to the development of prevention strategies. At today's level of the technological development many interference effects can be minimized and LowCross-Buffer® makes an essential contribution to it. It's new, that different interference effects with different molecular principles can be minimized with the same strategy. LowCross-Buffer® is applicable for different immunoassays. The results shown here are a clipping from different interference effects in different methods, which could be minimized or avoided with LowCross-Buffer®. Furthermore LowCross-Buffer® could prevent interference effects by HAMAs and rheumatism factors, unspecific bindings in immunohistochemical applications as well as false positive bands in immuno-PCR.

Altogether, the time consuming and costly effort for optimization strategies can be reduced and simplified significantly whereas improvements in reliability are reached simultaneously.

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Development of assays

Safety for ELISA development and validation

Dr. Tobias Polifke, CANDOR Bioscience GmbH, Weissensberg

Immunoassays for pharmaceutical research and diagnostics have to prove their reliability in validations. This point is critical for every assay, regardless of the assay format like ELISA, western blotting or RIA. In this stage you can improve the safety for developer and user. In conclusion you speed up the development, you get better results and you save money.

Key Words: ELISA development, pharmaceutical research, diagnostics

CANDOR Bioscience is specialised in optimising immunoassays. Due to optimisation, assays get safer and the reliability and the quality of results can be improved. You can obtain this by using services or new products of CANDOR. The company distributes own products like the innovative LowCross-Buffer® to customers in Life Sciences and in pharmaceutical research. CANDOR is also supplier for producers of diagnostics. For pharmaceutical research and diagnostics custom-made ELISAs are developed and validated according to the relevant guidances. Because of increasing demand of the diagnostics industry, the company had to upgrade its production capacities. This was realised by a relocation of the company from Münster to Weissensberg. In the border triangle of Germany, Switzerland and Austria CANDOR can also better support many customers in assay development.

CANDOR Bioscience is certified for DIN EN ISO 9001:2000 for assay

development and products for immunoassays. Therefore all products can be used directly in regulated areas like GLP laboratories. Also the diagnostics companies, which are supplied i.e. with LowCross-Buffer®, benefit from this certification.

Products – CANDOR Bufferline

CANDOR Bioscience offers the CANDOR Bufferline which gives the optimal solution for every immunoassay application. The buffers help to increase the analytical reliability and the economical efficiency of bioanalytics. The buffers are in use in research, pharmaceutical development and routine of diagnostic laboratories. For the diagnostics industry, all products are available as OEM supplies in bulk.

The innovative LowCross-Buffer® reduces cross reactivities, interferences and matrix effects which are characteristic for blood, serum and tissue specimens. With LowCross-Buffer® you can forget about false-positive results and interference. Validations - e.g. for FDA-



Contact persons: Dr. Peter Rauch, Dr. Sabine Glöggler, Dr. Tobias Polifke and Angela Zellmer

can be passed without loops. Therefore the duration of assay development is significantly reduced. This cost saving convinces an increasing number of customers especially from pharmaceutical research. CANDOR's Blocking Solution offers highest efficiency, too. The composition of Blocking Solution is based on highly purified casein. Due to a specially developed manufacturing procedure for this product, you are able to achieve a clearly better blocking. This can be very important in assays with small or critical analytes and in assays with difficult matrices. The use of Blocking Solution is as easy as with any other blocker. All buffers of the CANDOR bufferline are ready-to-use. The work gets easier, safer and mainly more efficient - thus more economic.

Services

CANDOR's team has many years of experience in development, optimisation and validation of ELISAs for pharmaceutical research and diagnostic industry. Customers get a systematically optimised ELISA as well as the standard operating procedures. For immunohistochemistry we offer sections, histological expertise and 3D reconstructions of histological and cytochemical stainings as well as multi stainings of one section to detect different analytes in one picture.

Technical Assistance

CANDOR attaches great importance on technical assistance for customers. Therefore you can call for any questions about immunoassays as well as about how to use the CANDOR Bufferline. The company's telephonic assistance service benefits from a long lasting experience in development of immunoassays for pharmaceutical research and diagnostics.

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