AppliCations
No.4

Improving quality & stability of ELISA

Using ready-to-use ELISA kits from manufacturers is easy and convenient. Sometimes however, home-made ELISA are required, because there is no kit available with the right antibodies or characteristics, such as limits of detection are not appropriate. Ready-to-use ELISA kits from good suppliers may be stored for two years at 4°C without any problem. With home-made ELISA kits it is a completely different story. For any new measurement one has to coat a new plate, because after storage of some days the plates don't perform as well as before.

AppliChem

Keywords

Immunoassays

Antibody Stabilisation

ELISA Plates

Cross-reactivity

Interfering effects

Improved stability during storage

Why is there such a great difference in the suitability for storage between home-made ELISA and commercial ELISA kits?

The reason is that in commercial ELISA kit production the plates are not only blocked after coating, but also stabilised. This easy to perform process has been an industry standard for thirty years. For stabilisation of a plate one has to incubate with a coating stabiliser solution. It is just as simple as a "second blocking step". But there were no such high quality stabiliser solutions freely available in low volumes for use in research lab until now. AppliChem now offers a product for use in every research lab in volumes starting as small as 50 ml, which is called the AppliCoat Plate Stabiliser (Cat. No. A7708). This stabiliser solution is easy-to-use and has a great advantage compared to almost any stabiliser used in industry. It gives better storage stability for coated antibodies and antigens than most other products do. And there is a second benefit.

Two benefits with one solution

When antibodies are coated onto ELISA plates, most of the antibodies are not active. When the antibodies (or any proteins) come into close contact to the plastics surface of the ELISA plate, conformational changes can occur due to surface-protein interactions. The result is that most antibodies coated on a plate are unfolded or inactive. Only around 2–8 % of all coated antibodies remain active and can bind to analytes and this is greatly variable depending on the surface characteristics of the ELISA plate, which can really differ from batch to batch or even from well to well.

These differences from well to well can affect the variability of an assay, because the antibodies can be affected. If there was a way of refolding antibodies and of preserving antibodies from conformational changes during storage, this could help to decrease such variabilities in assay performance. This is a key benefit of AppliCoat Plate Stabiliser. It assists antibodies and coated proteins to refold and then to preserve active conformation over a long time. Thus it has two benefits: 1. Refolding of antibody conformation of some of the coated antibodies and 2. Preserving correct conformation during storage. These benefits are used for production of high-quality ELISA kits as well as in research applications now. Even with AppliCoat Plate Stabiliser the percentage of active antibodies will still be in the range of 2–8 %. But the great difference is that the variability from well to well and from plate to plate can be minimised in most assays by using AppliCoat Plate Stabiliser. Such effects depend on the used antibodies, but when ELISA are validated (e.g. according to "Guidance for Industry: Bioanalytical Method Validation", FDA, 2001) or according to other validation strategies, the difference can be measured in many assays.

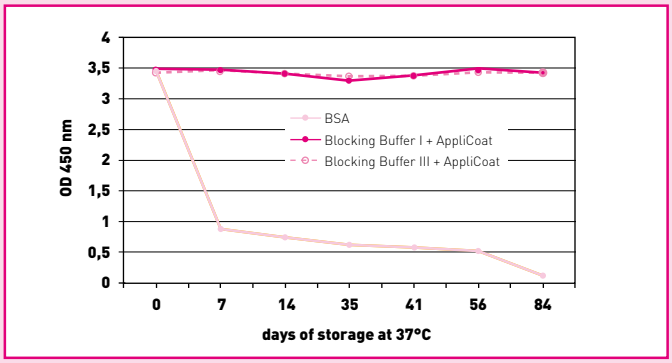


Figure 1. High temperature "stress test" at 37°C for 84 days after application of the plate stabiliser solution. The true OD signal is shown without any normalisation, which could potentially falsify interpretation of the results. One can clearly see the better binding activity when AppliCoat Plate Stabiliser is used. If stored not at 37°C but dry at 4°C the test would correlate to around 2 years of storage!

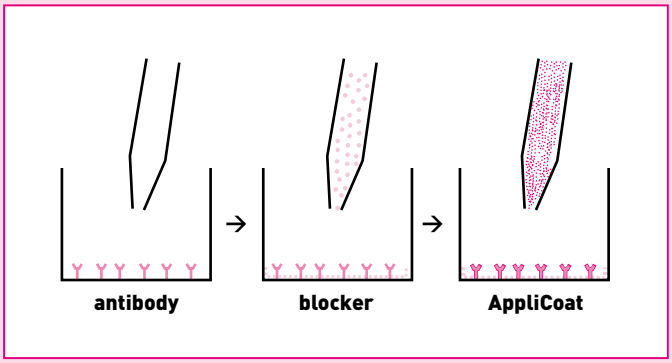


Figure 2. Procedure of the application of AppliCoat Plate Stabiliser. Stabilisation is similar to a "second blocking step". After this the ELISA plates are dried in an incubator and can be stored in a fridge for a long time.

The positive effects of AppliCoat Plate Stabiliser are shown in Fig. 1. A sandwich ELISA with a monoclonal antibody has been done. This monoclonal AB lost its binding activity when coated on a plate and stabilised only with BSA without applying AppliCoat Plate Stabiliser. Figure 1 shows a high temperature "stress test" at 37°C for 84 days after application of the stabiliser solution. The real OD signal is shown without any normalisation, which could potentially falsify interpretation of the results. One can clearly see the better binding activity when AppliCoat Plate Stabiliser is used. If this plate was stored dry at 4°C the test would correlate to around 2 years of storage.

An improved workflow for most labs

This advantage of a prolonged storage time can help to set up a completely new workflow in research labs. Whilst nowadays it is best practise to produce a new and fresh ELISA plate whenever some measurements with a home-made ELISA are done, this can change. One can easily coat, block and stabilise as much plates as needed for the next 3 to 6 months. Those plates are dried in an incubator at 20°C or 30°C for 60 to 120 minutes, the plates can be stored in the fridge for several months. Those plates can be used from time to time for measurements. All those plates are therefore produced in one batch in your lab minimising plate-to-plate variations coming from differences in plate production.

Thus the new workflow in labs has three great advantages:

1. Less work, because coating and blocking of many plates can be done in one run instead of doing this work for any new plate again and again.
2. Lower variations in measurements from plate to plate as described above.
3. Saving time and potentially improving the quality of the results of a lab.

Industrial technology now available even for small research labs

According to the needs of research labs AppliCoat Plate Stabiliser (A7708) is available in volumes of 50 ml, 125 ml and 500 ml. It can be used in combination with standard blocking solutions like casein (e.g. Blocking Buffer I, A7099) or BSA based solution (e.g. Blocking Buffer III BSA, A7252) and other blockers. For any researcher this industrial stabilisation technology is made available by AppliChem. Even small labs can make use of it. Test it for yourself!

How to use AppliCoat Plate Stabiliser?

Figure 2. shows the simple three step production process, which can be used for kit production as well as for homemade ELISA.

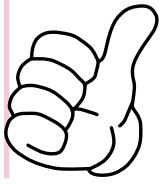
1. Coating as routinely.
2. Blocking as routinely.
3. Plates are washed 3 times with 200–300 µl PBS or a Washing Buffer without detergents (e.g. Washing Buffer TrisT- (10x) order number A7137)
4. Add 200 µl AppliCoat Plate Stabiliser and incubate for 30–90 minutes at approx. 20–30°C
5. Remove AppliCoat Plate Stabiliser by suction. Incubate the plates at 37°C until dry. This needs around 60 to 120 minutes depending on incubator type, number of plates in the incubator and speed of air circulation in the incubator.
6. Store the plate with a lid in the fridge for 3 to 6 months. Alternatively plates can be sealed with plastic or aluminium foil under dryness. When plates are stored dry, the shelf life will be around 1 to 3 years.

Achievable shelf life can differ from antibody to antibody. The used plates, coating concentration, procedures and buffers have additional impact on achievable stability. Thus if plates should be stored for more than 6 months, a stress test at 37°C or 45°C for storage should be done with the plates to easily measure the shelf life of the plate in a short time.

Related Products

Blocking Buffer I (A7099) saturates free binding capacities on plastic consumables and other surfaces like ELISA plates and blotting membranes. Thus a reduction of unspecific binding on surfaces can be achieved.

Efficiency of blocking is significantly improved in comparison to standard blocking procedures by a special production method, which leads to casein molecules with many different molecular sizes.
Can be used: in ELISA, EIA, RIA, Western blotting, immuno-PCR, protein arrays as well as immunohistochemistry.



Blocking Buffer III BSA (A7252) saturates free binding capacities on surfaces of plastic consumables and other surfaces like ELISA plates and blotting membranes. Thus, a reduction of unspecific binding on surfaces can be achieved. Blocking Buffer III BSA is the standard surface blocker for many applications. If a blocker on basis of BSA (bovine serum albumin) is efficient enough for an assay, Blocking Buffer III BSA is the well-priced alternative to universally applicable and more complex blockers. Blocking Buffer III BSA can be used for ELISA, EIA, Western blotting, Immuno-PCR as well as protein arrays and immunohistochemistry.

Recognise and avoid interference in Immunoassays

Antibodies are useful tools to specifically detect many substances. Methods like Enzyme-linked immunosorbent assays (ELISA), Enzyme immunoassays (EIA), Western blotting, Radio immunoassays (RIA), Protein-Arrays, Immunohistochemistry or the immuno-polymerase chain reaction (Immuno-PCR) belong to this category of the immuno detection technology. Even if they are highly specific, they all have in common to be disturbed by unwanted cross-reactivities. By applying a new buffer (CrossDown™ Buffer) instead of the usually used buffers most interfering effects can be avoided.

All immunoassays are characterised by a binding reaction between the target analyte and antibody. In theory, an antibody binds to a single, specific antigen only. In reality, cross-reactivities, non-specific binding and matrix effects negatively influence all assays. Interfering substances are present in more or less significant concentrations in specimen, interacting directly with the analytes, the capture or the detector antibodies, respectively. Apparently well characterised antibodies, with a high affinity to the target analytes, occasionally show surprising results. For example during immunological detection on Western blotting membranes, unwanted bands are stained; on protein arrays one observes fluorescence signals for spots at the wrong positions from immobilised capture antibodies as well as a high background signal for blank samples. In ELISAs one gets a high background for the negative control or false-negative signals during measurements. Known interfering factors, which are regularly described in the technical literature, are e.g. heterophilic antibodies as well as HAMAs (humane anti mouse antibody) or rheumatism factors, albumins, complement factors, lysozyme and others.

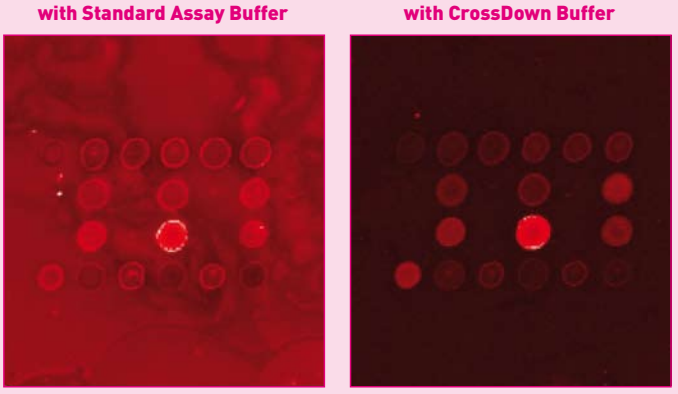


Figure 1. Reduction of unspecific binding of a detector antibody to the surface of a protein array. Improvement of signal-to-noise ratio from 3.4 (left) to 17.3 (right) by the use of CrossDown™ Buffer (data from N. Dankbar, University of Münster, Germany).

Figure 2. Western Blot without (left) and with (right) Blocking Solution I and CrossDown Buffer. Detection of Myostatin in mouse myoblasts with anti-GDF-8 primary antibody and rabbit anti-goat IgG-HRP secondary antibody (assay and image by Dipl.-Biol. Siewert, University of Ulm, Germany).

Interference by immunoassay label

Usually, detector antibodies or standard analytes are labelled with enzymes (e.g. alkaline phosphatase or (horseradish) peroxidase), fluorescence dyes, radioactive isotopes or DNA (immuno-PCR). Such labels may influence the affinity of antibody/antigen; reduce the solubility of labelled proteins. Likewise, proteins or antibodies from serum samples can bind to fluorescence dyes and reduce or even switch off the fluorescence of the dye.

Interference by cross-reactions

Cross-reactions are the ability of the antibody to bind to other structures than the target analytes. Frequently, these structures show a great similarity to the analytes. Examples thereof are metabolites, chemical substances with a similar molecular structure, and fragments of degraded target proteins or proteins with a similar amino acid sequence.

Interference by unspecific binding

Closely related to cross-reactivity is unspecific binding. However, the causes on the molecular level differ. While the wrong binding partners in the mechanism of cross-reactivity are known, unspecific binding is caused by substances, which are in far excess of the target analyte (i.e. unspecific binding to albumins or immuno globulins) or with surfaces (i.e. surfaces of ELISA plates or Western blotting membranes) or with spots of immobilised antibodies in protein arrays.

Matrix effects

Matrix effects are the sum of all interfering effects of all components from the specimen, and influencing the measurement of a target analyte. Some matrix effects are derived from "anti-animal-antibodies" others from heterophilic antibodies, from endogenous interfering substances 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 system's answer to contacts with immuno globulins from animal origin or contact of humans to pets and other animals. Human anti-mouse-antibodies (HAMA) are the most popular ones. The reason for the presence of these antibodies in patients are often therapeutic applications of such antibodies (e.g. cancer therapy). Sequence similarities between antibodies derived from different species may be detected by HAMA-containing sera too. 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. The binding of HAAAs to the Fc-fragment is called anti-isotypical, while binding to the highly variable Fab-fragment is called anti-idiotypical.

Interferences by heterophilic antibodies

Taber's Medical Dictionary defines heterophilic antibodies as "antibodies, which bind other antigens than the specific antigen". 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. Rheumatism factors are IgM antibodies that bind to the Fc sections of humane antibodies and therefore bind species-independent to Fc sections of antibodies used in the assay. Rheumatic sera join 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 to the HAAAs is the origin of the heterophilic antibodies: These are not build on contact with animal immunoglobulins, but they are multi-specific antibodies of the early immune response or interfering antibodies with unknown immunological history or origin.

Prevention of interference by CrossDown™ Buffer

The reasons for the described interference are similar. There are unwanted low to middle affine interactions of the interfering factors with the antibodies or the analytes. And there is 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 structurally related substances. These interferences have something in common that the newly developed CrossDown™ Buffer makes use of: The interference is weaker than the specific binding of the real analytes. Of course there are rare exceptions, since very high affine cross-reactivities can occur that achieve the same quality as the real specific binding. In such cases one must speak about a specific relationship and principally, one has an antibody that is aimed at two different substances. Therefore such an antibody is useless for specific assays. The CrossDown™ Buffer was developed specifically to eliminate weak and middle affine binding, without negatively affecting high affinity binding with high specificity in any way. It's new that different interferences with different molecular principles can be minimised with the same strategy. CrossDown™ Buffer is applicable for different immunoassays. CrossDown™ Buffer can prevent interfering effects by HAMAs and rheumatism factors, unspecific binding in immunohistochemical applications as well as false-positive bands in Immuno-PCR. Taken together, the time consuming and costly efforts for optimisation strategies can be reduced and simplified significantly whereas improvements in reliability are attained simultaneously.

with Standard Assay Buffer

Figure 3. Prevention of false-positive binding (control of specificity in row A1-12 and blank row H1-12) by the use of CrossDown™ Buffer in an ELISA against guinea pig IgG. For both plates, identical assay diluents were applied (by Dr. C. Specht, PARA Bioscience, Gronau, Germany).

Description	Product No.
Antibody Stabilizer-PBS	A7148
Antibody Stabilizer-Tris	A7135
AppliCoat Plate Stabilizer	A7708
Blocking Buffer I	A7099
Blocking Buffer II *EGrade*	A7516
Blocking Buffer III BSA	A7252
Coating Buffer C pH 9,6	A7150
Coating Buffer PBS pH 7,4	A7136

Description	Product No.
CrossDown Buffer	A6485
CrossDown Peroxidase-Stabilizer	A8625
Peroxidase-Stabilizer	A8647
Sample Buffer T+	A7134
Sample Buffer T-	A7101
Stripping Buffer I	A7140
Washing Buffer TrisT+ (10X)	A7158
Washing Buffer TrisT- (10X)	A7137

For more information about improving your immunoassays please see our comprehensive brochure.

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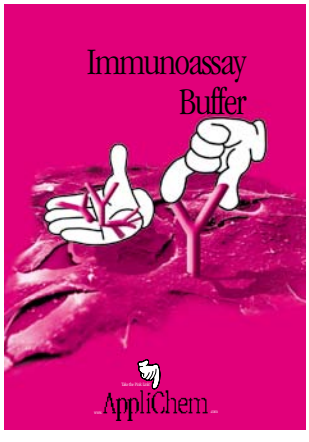

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