ELISA

Long-term stabilization of assay components

Dr. Tobias Polifke, Dr. Peter Rauch, CANDOR Bioscience GmbH, Germany;

Reliable long-term stabilization of immunoassay components is beneficial in many applications. Stabilization and storage of components are important for effective workflows not only for performing large numbers of ELISA but also for medium and small numbers of ELISA. This article deals with the state of the art - particularly with regard to food, veterinary or clinical diagnostics. Clinical immuno diagnostics require adequate stabilization of assay components according to EU guidance (IVDD 98/79/EC) or corresponding international regulations before product launch.

Key Words: Liquid Plate Sealer®, antibodies, long-term stabilization, ELISA, coating stabilizer

If you do only few ELISA tests in basic research it is better not to prepare for each measurement again new and freshly prepared components like conjugates and plates. It is easier and saves time to prepare the demand of half a year or one year in one run and to consume this stock from time to time. Results are more comparable (also in basic research) due to measurements with identical materials. Within the framework of larger studies e.g. in food diagnostics, medical research or pharmaceutical research, numbers of 10 - 100 ELISA plates have to be measured sometimes in labs of co-operating working groups. In this case not only the central preparation of ELISA in constant quality is important but also storage and shipment of the ELISA without degradation of components. In the field of immuno diagnostics – e.g. for clinical applications – it is general procedure that specialized companies manufacture ELISA kits and send them to customers which store the kits and use them. The requirements for immuno diagnostic products in terms of product quality and long-term stability are certainly very strict and challenging. Diagnostics manufacturers meet those requirements by using modern and industrial tools for stabilization. But also many smaller labs in other fields of application can make use of these industrial solutions.

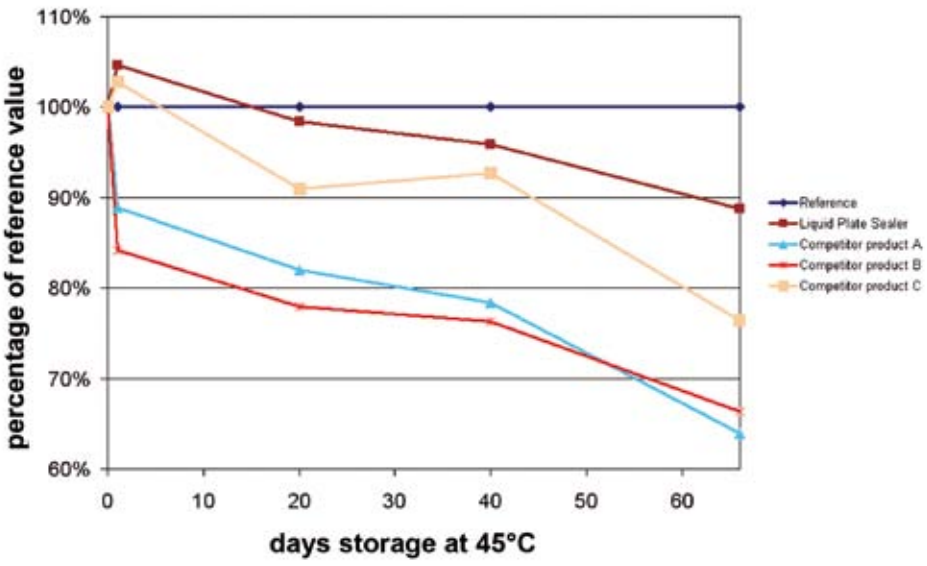

Fig. 2: Stress testing at 45°C with a monoclonal antibody. This antibody showed even after stabilization with carbohydrate containing solutions of the first generation no binding functionality after 24 hours (data not shown). The stabilizers of the second generation A, B and C show acceptable stabilization. After stabilization with Liquid Plate Sealer®, a modern stabilizer of the third generation, this unstable antibody shows even after 66 days of incubation at 45°C more than 85% of its activity and therefore an impressively enhanced stability.

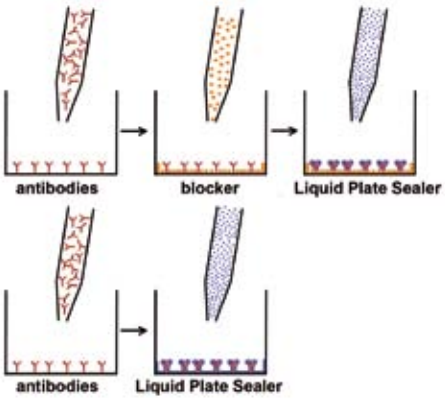

Fig. 1: Coating, blocking and stabilization of ELISA plates in a 3 step and in a 2 step process. The solutions are added, incubated and removed again. Then the plates are dried and sealed in plastic or aluminium foil for dry long-term storage.

State of the art

Integral part of each ELISA is an ELISA plate coated with antibodies or antigens. In the case of a sandwich ELISA the capture antibodies are immobilized with a coating buffer by adsorption. The choice of the best coating buffer depends on the amount of functional immobilized antibodies. Normally only 2 – 8% of the coated antibodies are in good orientation and functionally active (can bind the analyte). The reasons for this low percentage are changes in the sterical orientation and conformation due to surface effects during immobilization. The next step is to block and to saturate free binding capacities on the ELISA plate. This prevents false and non-specific binding of the specimen or detection antibody to the plastics surface. Quality of the blocking depends on the analyte, specimen and on the required reliability. Many different blockers are available on the market, for example well established protein-based blockers like BSA or casein or more or less effective synthetical blockers as well as highly effective chemical modified casein blockers. These chemical modified casein blockers are new products on the market. A new manufacturing process, developed by CANDOR Bioscience, made them available as industrial products with extremely low lot-to-lot variations. Now

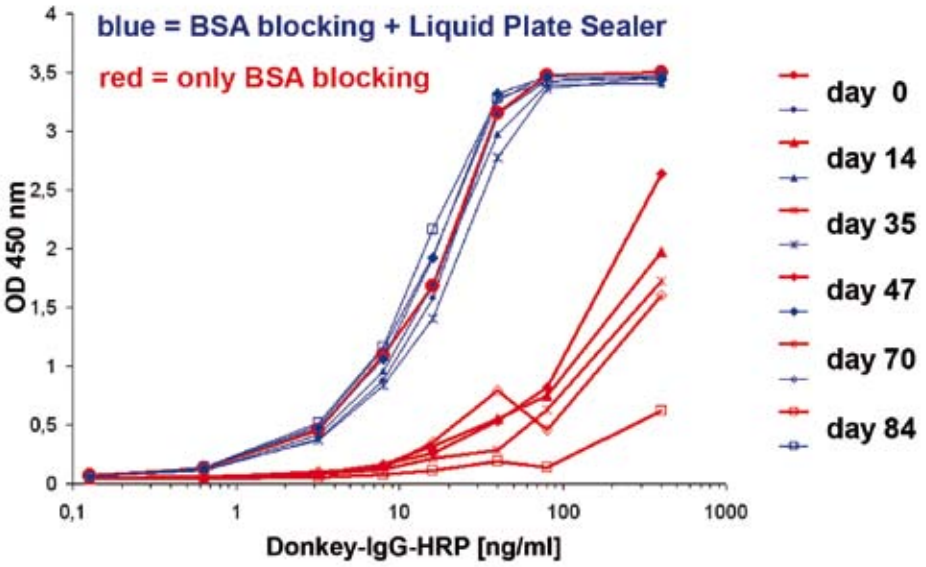

Fig. 3: Calibration curves of an ELISA are shown, which was either blocked only with BSA (red) or blocked with BSA and additionally stabilized with Liquid Plate Sealer® (blue). Calibration curves at the starting day are identical. The sensitivity of the assay without stabilization decreases rapidly during storage at 37°C. During this stress test the ELISA after stabilization does not show a decrease in sensitivity even after 84 days of incubation.

they are available for any lab and in bulk for diagnostics manufacturers as well. Third step is stabilization of the coated antibodies. After removing the blocker, the plate is incubated with the stabilizer solution. After removing this solution the plates can be dried and stored until use. Fig. 1 shows schematically the production process of coated ELISA plates in a 3-step and in a 2-step process. Basically the steps mentioned are identical independent of immobilized capture antibodies or antigens. In diagnostics manufacturing it was well established over decades to stabilize with sugar or carbohydrate containing solutions. These "homemade" formulations were the first generation of stabilizer solutions. These solutions show in many assays sufficient storage stability if continued refrigeration was provided. In addition to these homemade stabilizer solutions there are also some coating stabilizers on the market. These commercially available solutions of the second generation have two advantages: First you can save one step by doing blocking and stabilizing in one step without loosing too much of reliability. Second you can get better and longer stability. This is important if you have unstable antibodies.

In particular monoclonal antibodies often show low stability on surfaces. But also polyclonal sera can be affected by short shelf-life. The decision for an adequate blocker or stabilizer solution is made during development and validation of a diagnostics product. The EU guidance for in-vitro diagnostics regulates that criteria and activities of the product must not change during lifetime, because of potential risks for the patient's safety. That means that product characteristics must not change at all during transportation and storage, because every false diagnosis represents a potential risk for patients. Without sufficient stability it is not allowed to sell a diagnostical product on the European market. For important international markets corresponding standards apply.

Trends in diagnostics and new challenges

Diverse trends influence the development of modern immuno diagnostics. Some of them are exemplified and their effects on the stabilization standards of immuno diagnostics will be described.

Trend 1: There is an increasing cost pressure for diagnostics manufacturers in the industry nations. Main reason is political desired cost reduction in health care and more competition due to less discrimination of the diagnostics providers. This leads to a pressure on pricing which can only be met with adaptations in manufacturing. This trend has also an effect on established ELISA kits, because some product and manufacturing changes are necessary to lower the costs of production. During product changes the quality of diagnostics on the market has to be unchanged.

Trend 2: The markets of diagnostics in threshold countries grow continuously due to a rising economic status. Concurrently there are lacks of infrastructure in these countries. That means for diagnostics: sometimes broken cold chains – an especial technical challenge!

Trend 3: The medical research always discovers new and suitable molecules for diagnostics. Reasons for the discovery of new biomarkers are better analytics and sample preparation. Modern biomarkers are not easy to discover due to their availability in low concentrations or their short shelf-lives. Most of them even show a combination of both, low concentration and short shelf-life.

Requirements on the basis of the trends mentioned above

Trend 1: Lower costs of kit components are desirable. This also applies for commercially available stabilizer solutions. New technologies for stabilization must be more efficient.

Trend 2: If you also want to serve the strong rising markets of the threshold countries the security stock of the product stability has to be enlarged.

Trend 3: Low concentrations of analyte demand so far unknown good precision of immuno diagnostics. Loss in quality during storage like a decreasing amount of functional coated antibodies leads to a bad precision of the ELISA. The detection limit has to be exhausted to the technical maximum to meet the challenges of modern biomarkers. For product developments the stability of the standards becomes more and more important.

The requirements for the third generation of stabilizers are the following: Better stability and simultaneously low pricing.

The third generation: Liquid Plate Sealer®

CANDOR Bioscience is supplier of the diagnostics industry and developer of innovative tools for immunoassays. CANDOR can meet the demands mentioned above with the new product Liquid Plate Sealer®. Liquid Plate Sealer® shows an outstanding stabilization of antibodies and antigens after immobilization. A measurable reactivation can also be observed as well as a very good and

reproducible blocking. Additionally Liquid Plate Sealer® is more economic compared to the well-established market-dominating stabilizers of the second generation. Liquid Plate Sealer® can be used in production of immuno diagnostics like other stabilizers. After coating, the plates are incubated with Liquid Plate Sealer®. After removing the stabilizer the plates can be dried and stored for long-term periods. Shelf-lives up to several years are possible even with instable antibodies. Stress tests show this by conservative correlation according to Arrhenius. In addition Liquid Plate Sealer® makes it possible for any life science lab to store plates under moist conditions in the fridge. Such stored plates keep stable for several weeks up to several months (dependent on used antibodies). This is a good assistance for research applications where an industrial ELISA production is not profitable. Often the equipment like an oven with forced convection or heat-sealer also does not exist in these labs. The increased stability compared to three stabilizers of the second generation from the market leader has been quantified. Although the second generation of stabilizers shows very good results, CANDOR could enhance these results. The costs for Liquid Plate Sealer® are not higher than for the competitive products. Liquid Plate Sealer® is „made in Germany" and available from 50 ml for any lab to bulk quantities to manufacturers of test kits.

Mode of action

Liquid Plate Sealer® seals the coated biomolecules with an uniform and stabilizing but easy soluble layer. During incubation the coated antibodies are surrounded by a protective layer. Even some inactive and non-binding antibodies recover their binding ability and can give a measurement signal. In many cases the amount of functional capture antibodies increases because of reactivation of antibodies. Once activated most antibodies stay stable below the protective layer over long periods even under temperature stress. This allows ELISA which can better tolerate broken cold chains. Activation takes place by components which have an impact on the protein conformation. The negative influence of the plastics surface towards the proteins can be weakened. The generated protection layer prevents folding to an inactive conformation even under temperature stress. Altogether Liquid Plate Sealer® provides an active (reactivation) as well as a passive (stabilization of conformation) protection system for coated molecules. If you use the plate in an ELISA you can add the samples without further washing steps. The established ELISA procedure does not have to be changed. The user of an ELISA only recognizes the longer stability of the plates treated with Liquid Plate Sealer®. Exchanging the commercial stabilizers of the second generation with stabilizers of the third generation is mostly practicable without changes in manufacturing processes or products. Comparative stress tests – mandatory for clinical diagnostics – show stability data achieved with Liquid Plate Sealer®.

Stress testing

Fig. 2 shows the results of a stress test at 45°C with an unstable antibody. This antibody shows after 1 day of storage with a sugar containing stabilizer solution an extremely reduced signal in the ELISA. This antibody is well suited to show the qualitative differences between the 3 generations of stabilizers. Results after stabilization are shown in comparison to 3 competitor products of the second generation and Liquid Plate Sealer®. Applications and procedures of coating and measuring have been identical. If coating stabilizers of the second generation were used in production, switching to Liquid Plate Sealer® can be done very easily. Liquid Plate Sealer® is also suitable for applications which need an efficient blocking. It can be used not only in a two step production but also in the above shown 3 step production. It is compatible with all blockers tested so far. Stabilization in combination with the widely-used BSA blocking is shown exemplarily in fig. 3. You can see the drift of the calibration curve during storage when the plate is not stabilized. Calibration curves show that the ELISA is only useful over the whole storage period, when the plates are stabilized. This stabilized ELISA would also achieve reproducible results after 84 days at 37°C. Fig 4 shows the combination of different blockers with Liquid Plate Sealer® (3 step processing) and blocking and stabilization only with Liquid Plate Sealer® (2 step processing). One can see that the combination with many blockers is possible if needed.

Applications

Liquid Plate Sealer® is used for industrial production of coated ELISA plates, immunochromatographic strips (lateral flow devices), affinity columns and other commercial products.

Outlook

Liquid Plate Sealer® was the first product of the immunoassay stabilizer solution series from CANDOR on the market. In the meantime a number of further

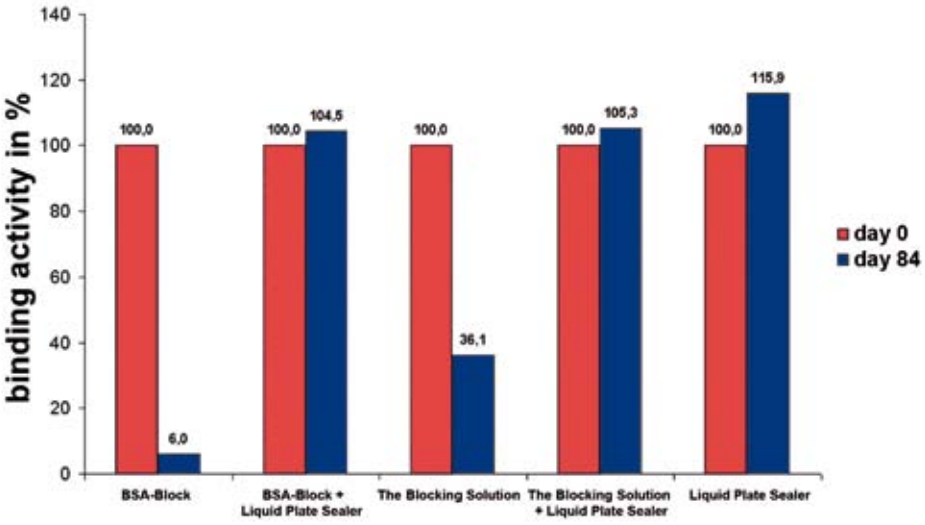

Fig. 4: Binding activity of an ELISA at the starting day and after storage for 84 days at 37°C is shown. In fig. 3 the complete calibration curves are shown (only for BSA blocking). Here we show the measurements at 40 ng/ml. Activation of binding capacity by Liquid Plate Sealer® can be seen quite clearly. It is also shown, that Liquid Plate Sealer® can be combined with different blockers without any problems.

stabilizer solutions like HRP-Protector™ and LowCross-HRP® are available. Both are stabilizers for peroxidase which show an outstanding stabilization of peroxidase conjugates. LowCross-HRP® combines peroxidase stabilization with the LowCross effect. Thus interferences from HAMAs (human anti mouse antibodies), other heterophilic antibodies and cross reactivities can be avoided. These stabilizers enable long-term storage of detection antibodies coupled to peroxidase when stored in very low end-user concentrations. Further dilution steps by the end-users are not necessary. The stabilizer series of CANDOR will be supplemented by stabilizers of alkaline phosphatase and protein (antigen) standards in solution. All products of CANDOR are available in bulk quantities and are manufactured according to the DIN EN ISO 9001:2000 certification of the production facility.

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Correspondence address

Dr. Peter Rauch
CANDOR Bioscience GmbH
Eggenwatt 12
88138 Weissensberg
Tel.: +49 (0)8389 92 93 992
Fax: +49 (0)8389 92 93 999
eMail: p.rauch@candor-bioscience.de
www.candor-bioscience.de

Stabilization of conjugates: LowCross-HRP®

One of the challenges by producing top-quality immunoassays is stabilization of antibody or neutravidin peroxidase conjugates for long-term storage over several years. LowCross-HRP® is a new stabilizer for this task. Shelf-lives of several years can be reached with most HRP conjugates (horse radish peroxidase). The acitivity of the detection enzyme as well as the binding capacity of the antibodies is protected well for long time periods. This was shown very impressively in stress testing with HRP conjugates. Conservative correlation to Arrhenius shows protection for many years. Another key advantage of this modern stabilization technology is that antibody conjugates can be stored in very low concentrations. This saves for conjugate during production, because lower concentrations can be used without loosing safety and quality of immuno diagnostics products. Conjugates can be used without any pre-dilution steps before using the kit. The LowCross-Buffer® effect of LowCross-HRP® helps to avoid interference and false-positive or false-negative results even when blood, serum or tissue specimen are measured. Using additional HAMA blocker is no longer needed and matrix effects as well as cross reactivities are minimized. Bulk quantities are available for kit manufacturers. LowCross-HRP® and HRP-Protector™ are used for industrial kit production.

Stress test at 45°C:

Shelf life of antibody conjugate bound to the analyte is measured as peroxidase signal in an ELISA and given as % value of freshly diluted HRP-antibody conjugate.