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Extending the lifespan of IVD kits

Assessing the stability of a range of different stabilizers to increase shelf-life, thereby maximizing usability and improving cost-efficiency. **BY LENA BRANDT LARSEN, TINA KRISTENSEN MARWOOD, AND THOMAS ANDERSEN**

The stability of all components in an IVD kit reflects its ability to maintain consistent, and therefore accurate, performance over time. Unlike commonly measured attributes, stability is problematic to assess prior to experimental use, and as such, there is a great onus on IVD manufacturers to ensure that any stability claims are substantiated. In today's culture of mass manufacturing, extending stability to ensure that shelf-life does not expire prior to shipping is becoming increasingly important.

Furthermore, guaranteed stability is perceived as a value added component, specifically in international markets or areas where storage can be problematic. Such guaranteed stability increases the product's usability, regardless of shipping time and any storage issues, including power failures or temperature deviations in a cold storage environment. In addition, once the IVD kits are shipped, they likely will undergo further storage before use, not necessarily at optimal temperatures. By extending the usable shelf-life of such products, concerns over stability are alleviated, and end users can continue to work with a significant level of assurance and confidence that resulting data are accurate.

Thermo Scientific Nunc Immobilizer Streptavidin and Nunc Immobilizer Amino surfaces.

Shelf-life is commonly assessed using two different types of stability testing: real-time and accelerated. In real-time stability testing, the IVD kit components are stored under recommended conditions and monitored until viability is lost. In accelerated stability testing, a product is stored in an environment in which conditions such as temperature and humidity are higher than recommended. Degradation at the recommended storage conditions can therefore be predicted using known relationships between the acceleration factor and the degradation rate. As the most commonly used acceleration factor, temperature has a strong relationship with the Arrhenius equation,

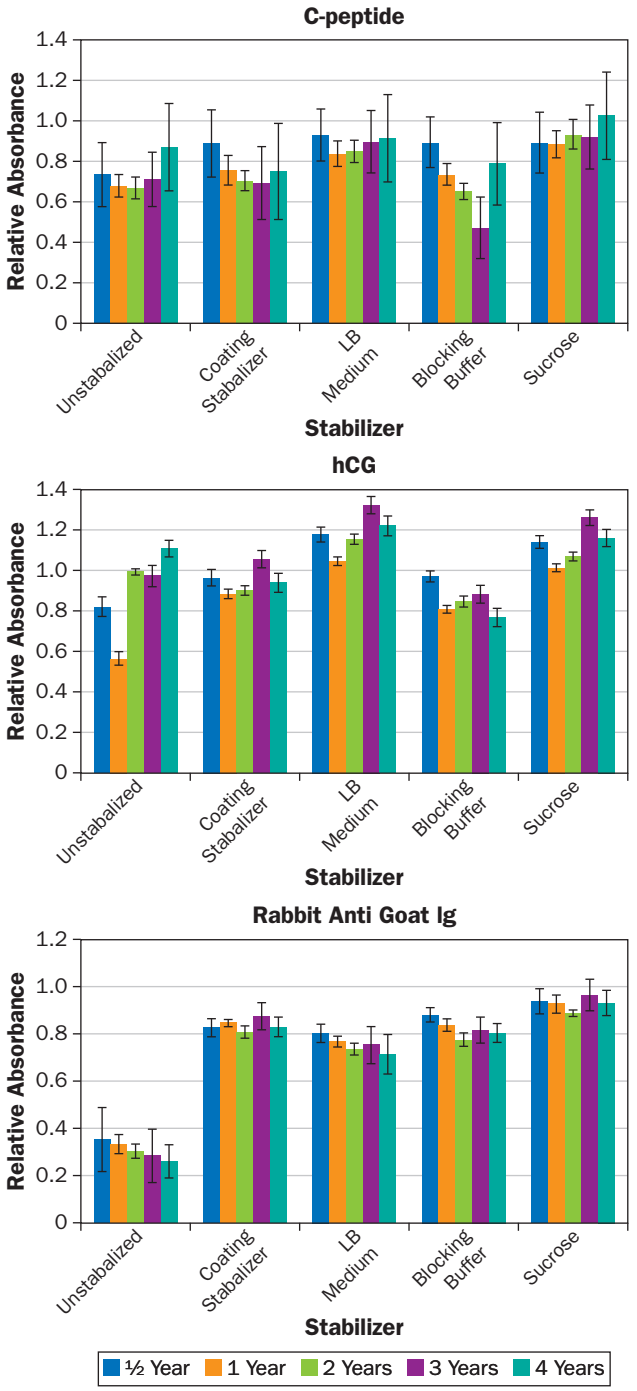

Figure 1. Determined activity for the immobilizer amino surface coated with C-peptide (A), hCG (B), and rabbit anti-goat Ig (C). The coated surfaces were stored as unstabilized or stabilized with either a coating stabilizer, LB medium, blocking buffer, or sucrose, and tested after storage at 50° C for 1, 2, 4, 6, and 8 months, corresponding to ½, 1, 2, 3, and 4 years' storage at room temperature (normalized data, n=7).

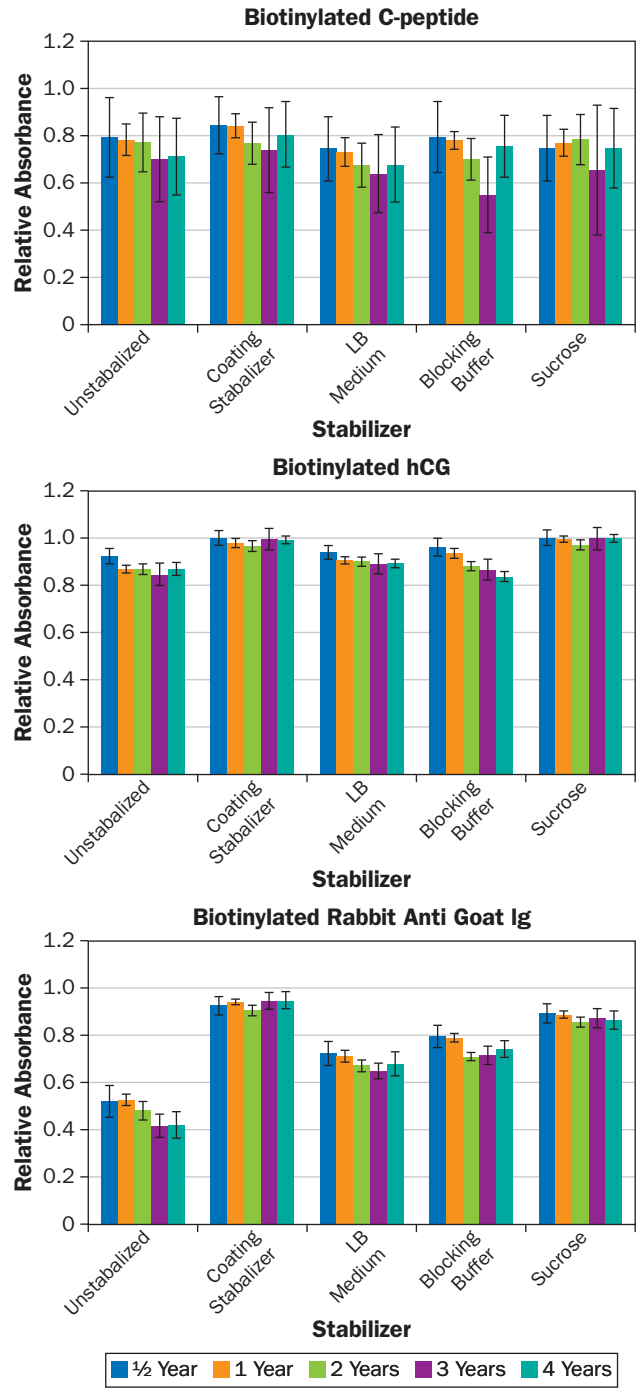

Figure 2. Determined activity for the immobilizer streptavidin surface coated with biotinylated C-peptide (A), hCG (B), and rabbit anti-goat Ig (C), unstabilized or stabilized with either a coating stabilizer, LB medium, blocking buffer, or sucrose, after storage at 50° C for 1, 2, 4, 6, and 8 months, corresponding to ½, 1, 2, 3, and 4 years' storage at room temperature (normalized data, n=7).

which is used to determine an estimation of the degradation rate.

Solid Capture Surfaces

As an integral component of any diagnostic kit, solid capture surfaces (e.g., microwell plates) are used to bind effectively the component of interest. In order to maximize usability and consequently obtain consistent and reliable results, these surfaces ideally need to remain effective over long periods of time. In order to increase the shelf life of diagnostic immunoassay kits and provide the required level of quality assurance, plate activity should remain constant with storage at room temperature. This eliminates the need for long-term storage in cooling facilities and makes the immunoassay techniques more accessible to laboratories where cooling facilities may not exist.

For diagnostic purposes, the solid surfaces of the plates are coated with a capture molecule before being used in a screening set-up. Capture proteins are fixed to the surface and used to detect specific biomarkers from serum or urine, or an antibody response to infection. The surface coating can consist of a range of different-sized molecules, from small peptides to large antibody molecules, the activity of which can be maintained during storage using a variety of stabilization methods. For example, previous methods of increasing reagent stability include freeze-drying, which preserves the protein-coated surfaces that are unstable in aqueous solutions.[1] Frozen samples are preserved through the removal of water, which blocks several degradation pathways.[2-5] However, the freeze-drying process itself can lead to protein degradation and subsequent loss.[6-8] These risks are significantly reduced by adding a stabilization agent prior to freeze-drying in order to provide a reliable method to simplify transport and storage, and extend shelf-life.

This article assesses the stability of a range of different stabilizers to increase shelf-life, thus maximizing usability and improving cost-efficiency. Such assessment is demonstrated via accelerated aging by which capture molecules of different sizes can maintain their full activity for at least four years, when attached to an amino binding or streptavidin immobilization surface.[9]

Accelerated Aging

In order to ensure experimental integrity is maintained, plates are often coated to modify surface properties and increase ligand binding affinity. With the ability to improve significantly enzyme immunoassay sensitivity, having a plate with low well-to-well variability and strong adsorption is crucial and minimizes the loss of any material during the repeated washing steps.[10] Such plate types are widely used for immobilizing captured antibodies, since they provide a generic surface that maintains molecular activity.[11] To demonstrate the suitability of plates with amino and streptavidin binding molecules as solid surfaces for diagnostic kits, different plate types (e.g., the Thermo Scientific Nunc Immobilizer Streptavidin and Nunc Immobilizer Amino surfaces) were coated with a typical capture molecule, and their activity were measured.

Three different capture molecules were adapted for covalent binding of NH_2- or SH-groups to the Immobilizer Amino surface, or were biotinylated for attachment to the Immobilizer Streptavidin surface: C-peptide from human insulin (3 kDa), human chorionic gonadotropin hormone (hCG) (37 kDa), or rabbit anti-goat immunoglobulin (Ig) (170 kDA).To assess the long-term stability of the coated surfaces, different commercially available stabilizers were compared to a coated, unstabilized surface. Since storage at 4° C is considered non-aging and room temperature storage is considered real-time aging, all surfaces were stored at 50° C. Doing so simulates accelerated aging, in which one day at 50° C equals 6.5 days at room temperature and enables stabilization experiments to be performed during a shorter timeframe.[9]

Materials and Methods

Amino binding strips were coated with either C-peptide (Bachman H2470), hCG (Sigma CG5), or polyclonal rabbit anti-goat Ig (Dako 2Z0228). C-peptide (1 mg/ml) was diluted in 0.1 M sodium phosphate buffer (pH 8.0) to a final concentration of 600 ng/ml, and 100 µl was added to the well. hCG (65 µg/ml) was diluted in 0.1 M sodium phosphate buffer (pH 8.0) to a final concentration of 400 ng/ml, and 100 µl was added to the well. Polyclonal rabbit anti-goat Ig (7.1 mg/ml) was diluted in 0.1 M sodium phosphate buffer (pH 8.0) to a final concentration of 10 µg/ml, and 100 µl was added to the well. The strips were incubated for two hours and agitated at 300 revolutions per minute (rpm), followed by three washes with 350 µl/well of 0.15 M phosphate buffered saline.

C-peptide and hCG were biotinylated using a commercially available biotinylation kit (EZ-Link Sulfo-NHS-LC-Biotinylation kit by Pierce), in accordance with the manufacturer's guidelines. Streptavidin strips were prewashed three times with 350 µl/well of 0.15 M phosphate buffered saline and PBS with 0.05% Tween 20 (pH 7.2). They were coated with either 300 ng/ml of biotinylated C-peptide, 200 ng/ml of biotinylated hCG, or 5 µg/ml of biotinylated polyclonal rabbit anti-goat Ig, all of which were diluted in PBS-Tween. Each well was coated with 100 µl of capture molecule for three hours and gently agitated at 100 rpm at room temperature. The strips were washed three times with 350 µl/well of 0.15 M phosphate buffered saline (pH 7.2).

The stabilization process was performed by adding 200 µl/well of either of the following: starting block blocking buffer by Thermo Fisher Scientific; 0.15 M (5% w/v) sucrose by Acros; coating stabilizer and blocking buffer diluted 1:1 with water by BioDesign International; or luria broth (LB) medium by Sigma for one hour with gentle agitation (100 rpm). The strips were aspirated and dried at 37° C for 30 minutes. After this stabilization, the strips were packed in alubags with desiccants and stored at 4° C, room temperature, or 50° C accordingly until the protocol began.

To assess the effectiveness of the stabilization protocol, the surfaces were tested and their conditions were recorded after two weeks, one month, two months, four months, six months, and eight months of storage at the specified temperatures. Uncoated strips were also freshly prepared as baseline reference values for normalization by using the coating process described above. The stabilized strips were washed three times with 350 µl/well of PBS-Tween prior adding the antibody solutions:

- The C-peptide-coated strips were incubated for three hours with a mixture of HRP-conjugated polyclonal mouse anti-human C-peptide Ig 1:64000 and monoclonal mouse anti-human C-peptide 1:400.
- The hCG-coated strips were initially incubated for ninety minutes with polyclonal rabbit anti-hCG Ig 1:400. They were subsequently incubated for an additional ninety minutes with a mixture of HRP-conjugated polyclonal swine anti-rabbit IgG 1:2000 and polyclonal swine anti-rabbit IgG 1:1000.
- The rabbit anti-goat Ig-coated strips were incubated for three hours with a mixture of HRP-conjugated polyclonal swine anti-rabbit IgG 1:2000 and a polyclonal swine anti-rabbit IgG 1:1000.

Immobilization surface	Capture molecule	Recommended stabilizer(s) for long-term storage	Effect of stabilization
Immobilizer Amino	C-peptide	LB Medium, Sucrose	++
	hCG	LB Medium, SucroseCoating stabilizer	+++
	Rabbit anti-goat Ig	Coating stabilizer, Sucrose, LB Medium, Blocking buffer	+++
Immobilizer Streptavidin	Biotinylated C-peptide	-	-
	BiotinylatedhCG	Coating stabilizer, Sucrose	++
	Biotinylated rabbit anti-goat Ig	Coating stabilizer, SucroseLB Medium, Blocking buffer	+++

Table 1: Recommended stabilizers and their degree of effect when used with Nunc Immobilizer Amino and Nunc Immobilizer Streptavidin surfaces. -: No effect, +: either long-term stabilizing effect or effect on activity level, ++: both effect on activity level and long-term stabilizing effect.

The substrate reactions were initiated by adding 100 µl/well of O-phenylenediamine dihydrochloride (OPD/H_2O_2). All incubation steps were performed using 100 µl/well of substrate at room temperature, gentle agitation at 100 rpm. Reactions were terminated after five minutes by adding 100 µl/well of 2N sulfuric acid. Absorbance was consequently measured at 492 nm.

Linear regression was used to evaluate the change of activity level due to aging. An α value greater than or equal to 0 is considered no change in aging, and an α value less than 0 is considered a decrease in activity level due to aging. A two-way analysis of variance was used to evaluate the effect of stabilization compared to the unstabilized assays, with a significance level of p less than 0.05 (n=9).

Results

In this study of accelerated aging, amino-binding and streptavidin surfaces were coated with a variety of different capture molecules and stored at 50° C. All six model assays demonstrated sustained activity at eight months after coating, which is equivalent to four years of storage at room temperature (see Figures 1 and 2).

In the majority of assays, activity levels were constant, and the relative absorbance was high compared to the unstabilized surfaces. The greatest decrease in measured activity was found in the biotinylated C-peptide assay stabilized with blocking buffer (see Figure 2a). The decrease was calculated to be 24.7% by linear regression. However, as an inherently variable structure, C-peptide is a problematic model to assess for stability, and there may be some inherent background noise which is not accounted for here.

Amino-Binding Surfaces

C-Peptide. As shown in Figure 1a, the C-peptide coated and unstabilized amino-binding surface maintained an activity level of approximately 70% for up to four years. Coated surfaces which are stabilized with either LB-medium or sucrose maintained an activity level of approximately 90% throughout the testing period, while surfaces stabilized using blocking- or coating-buffer showed a decrease in activity. LB medium or sucrose demonstrated a stabilization effect, as the activity of these surfaces was significantly higher than the unstabilized surface.

hCG. An activity level of greater than 100 % was achieved after four years of aging using the hCG-coated amino-binding surface in combination with LB medium or sucrose. This indicates that hCG is interacting with the stabilizing buffer to cause non-specific binding of the polyclonal antibody. This combined with minimal degradation of hCG shows that the effect of using LB medium or sucrose is significant compared to the unstabilized surface and the surfaces treated with a coating stabilizer, or blocking buffer, which demonstrated a significant decrease in stability between the ½- and four-year time points. However, the coating stabilizer resulted in an activity level of approximately 90% after four years of aging (see Figure 1b).

Polyclonal Rabbit Anti-Goat Ig. When stabilized with sucrose, Ig demonstrated a steady activity level of greater than 90% after four years of aging. Surfaces treated with coating stabilizer and blocking buffer both demonstrated a steady activity level of greater than 80 % after four years of aging. Unstabilized Ig lost activity immediately following coating, and after four years of aging, the activity levels were down to 25% (see Figure 1c). For this model assay, all stabilization methods resulted in a significant increase in activity compared to the unstabilized surface.

Discussion. Despite the size of the capture molecule, all stabilization methods had a positive effect on the molecules immobilized onto the amino-binding surface. Favorable results were obtained on the C-peptide- and hCG-coated plates with both sucrose and LB medium. Maintained stability and activity levels demonstrated that by using these methods, plates can be stored for up to four years. Data show that sucrose has the greatest stabilizing effect for the polyclonal rabbit anti-goat Ig-coated plate, while LB medium provided optimal stabilization for the hCG-coated plate.

Streptavidin Surfaces

C-Peptide. When C-peptide was immobilized to the streptavidin surface, no significant differences between an unstabilized surface and stabilized surfaces were observed (see Figure 2a).

hCG. As shown in Figure 2b, a high degree of stability is reached when hCG was immobilized onto the streptavidin surface. The activity level was maintained close to 1.0. Stabilization with sucrose or a coating stabilizer had a long-term effect. Unstabilized and LB medium set-ups have also shown activity levels between 80% and 90%, which is accurately maintained during four years of aging. Surfaces treated with sucrose and a coating stabilizer showed significant stabilization compared to the unstabilized surface and surfaces treated with a blocking buffer and LB medium.

Polyclonal Rabbit Anti-Goat Ig. When Ig immobilized on the streptavidin surface was treated with a coating stabilizer, activity levels are maintained at 95% for up to four years of real-time aging. Sucrose demonstrated a long-term stabilizing effect, with the ability to maintain an activity level of 90%. The activity level of unstabilized molecules decreased to 50%, and to a lesser extent, the coated surfaces stabilized with LB medium or a blocking buffer also decreased. As with the amino-binding surface, all stabilization methods resulted in a significant improvement in activity compared to the unstabilized surface.

Discussion. The stabilizers did not have any effect on the activity of the C-peptide. For hCG, the coating stabilizer and sucrose have improved the retention of activity over four years. However, the LB medium has shown no effect, while the blocking buffer appears to accelerate a loss of activity. Coating stabilizers provide the best stabilization for Ig, followed by sucrose, the blocking buffer, and finally the LB medium. Therefore, when using the most suitable stabilizers for each assay, data have shown that coated surfaces can be stored at room temperature for up to four years with a consistent and stable level of activity.

Conclusion

This accelerated shelf-life study has demonstrated that capture molecules can be immobilized onto amino-binding or streptavidin surfaces. They can subsequently be detected after accelerated storage at 50° C for eight months. By effectively stabilizing the interaction between the amino-binding or streptavidin surface and the capture molecule, degradation is not an issue when stored at room temperature.

For several capture molecules, introducing a stabilizer has a positive effect on long-term stability (see Table I). Where stabilization is necessary to maintain activity, stabilization can be achieved using the appropriate combination of capture molecule and immobilization surface. Data obtained in this study allows specific stabilizers to be recommended. For amino surfaces coated with C-peptide or hCG, LB medium or sucrose should be used. Amino plates coated with polyclonal rabbit anti-goat Ig should be stabilized with a coating stabilizer. For biotinylated surfaces coated with C-peptide, inconclusive results prevent specific recommendations to be made, and further investigation is required. With hCG and polyclonal rabbit anti-goat Ig, optimal stabilization can be achieved with coating stabilizer or sucrose.

In general terms, the data have shown that coated surfaces can be stored at room temperature for up to four years, while maintaining a stable level of activity. However, not all combinations of capture molecules and stabilization methods show a stable level of activity throughout the testing

period. Retention of activity can be enhanced through the use of a stabilization buffer to maintain the integrity of tertiary protein structures on the surface of the plate. As a result, after coating with the optimal stabilization buffer, immobilizing plates are suitable for inclusion in diagnostic kits, with a shelf-life of up to four years at room temperature.

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