Stabilization of ELISA plates – technology comparison

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The stability of the reagents in diagnostic kits is essential for any commercial test to be used worldwide in a high volume dur-ing a pandemic. Modern stabilizers not only allow for long shelf lives, but also increase the reliability of test kits. With the aid of SARS-CoV-2 antigens as examples, we explain how commercial stabilizers can improve assay quality while also optimizing workflows.

Even the most technically sophisticated and well-designed diagnostic test normally never sees its commercial launch if the stability of its components is not adequately considered during development. According to the EU regulation on in vitro diagnostics (IVDR), which will be in effect from May 2022, the analytical performance of a diagnostic kit must not change during its shelf life. This makes component stability a prerequisite for market introduction. The stability of the calibrators and the conjugated detector antibody can be greatly enhanced with commercially available protein- and conjugate-stabiliz-ers, such as CANDOR's Antibody Stabilizer, HRP-Protector™ and AP-Protector®[1]. This article focuses on the mechanisms and available solutions for the equally important solid-phase stabilization, the activity-preservation of surface-coated capture molecules, using ELISAs as an example. However, the outlined principles and solutions can also be transferred to other coated surfaces, like polystyrene- or glass-surfaces, in applications such as lateral flow assays, affinity columns, bead-based assays, Immuno-PCR or protein arrays.

The coated capture molecules like antibodies, or isolated viral proteins in the case of antigen-down assays, are a central component of a reliable immunoassay. Their misfolding, i.e. the loss of their native structure, is a huge concern for any assay, as it causes alterations of the paratope, the antigen binding region of antibodies, or the epitopes of antigens, both of which are essential for analyte binding. As a result, the capture molecules are unable to bind the analyte and might potentially even show nonspecific binding events due to the exposure of naturally not exposed amino acid sequences and structures, causing the diagnostic test to show incorrect results. Misfolding of antibodies occurs during coating due to changes in the steric orientation and conformation caused by surface effects. In the case of ELISA plates, only 3 - 10 % of coated antibodies are in good orientation and functionally active in binding the analyte[2]. Similar effects are to be expected for the coating of antigens. In most cases, the small active fraction is sufficient to establish an assay, as the capture molecule is normally used in large excess.

Even after successful coating, misfolding also takes place in a time- and temperature-dependent manner. Over time, the coated molecules lose their ability to bind the analyte. If the plate is part of a diagnostic kit with an envisioned shelf life of several years, however, state-of-the-art stabilization is essential for continued functionality. Otherwise, the performance of the kit will deteriorate with the misfolding of the capture molecule due to a decreased signal-to-noise ratio and potential nonspecific interactions, leading to false negatives and false positives. Especially monoclonal antibodies often show low stability on surfaces, but also some capture molecules in antigen-down-assays lose their ability to capture the analyte after two weeks at 4 °C if stored without stabilization[3].

Hence, stabilization should be considered early during development of a commercial assay. Simple approaches, like the use of BSA as a stabilizing agent, have no significant effect on the stability of the coated capture molecules and are unable to conserve plate functionality (Fig. 1a). More effective were the sugar or carbohydrate solutions that were employed in di-agnostic manufacturing for several decades since the 1980s. With the ability to achieve sufficient stabilization if continuous refrigeration is ensured, these solutions constituted the first real solid-phase stabilizers. Achievable shelf lives for plates with this sugar-based stabilization are normally in the range of one year, but a slight decrease in quality during this period

was common. In the early days of immunodiagnostics, this was not considered a major issue, since regulatory requirements were still much lower. However, these solutions have very little in common with the stabilizers commercially available today, like Liquid Plate Sealer® (Fig. 1b), which feature more advantages over carbohydrate solutions than simply a further increase in plate stability.

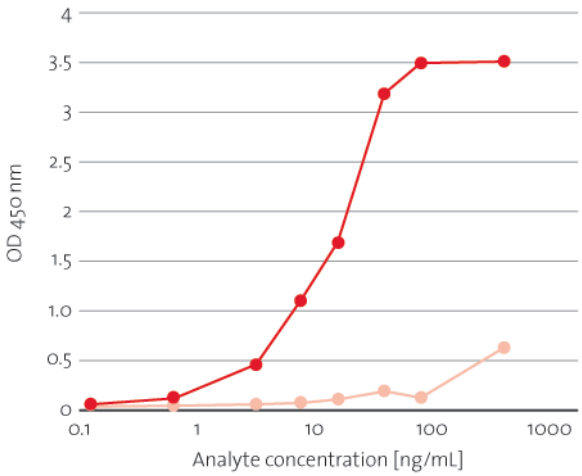


Fig. 1a

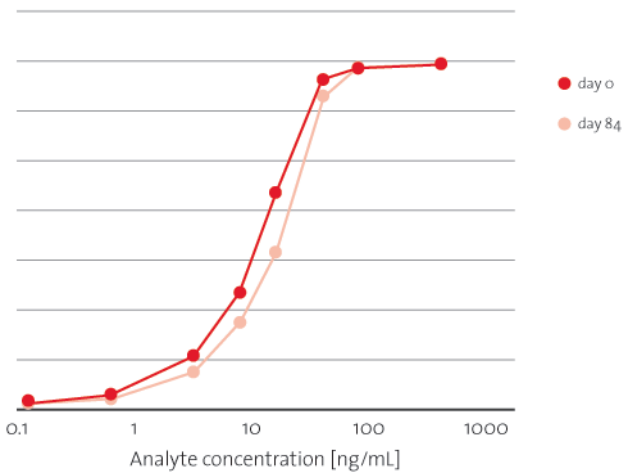


Fig. 1b

Figure 1: Calibration curves of an ELISA that was either blocked only with BSA (left panel) or blocked with BSA and additionally stabilized with Liquid Plate Sealer® (right panel). Calibration curves at the starting day are identical. After 84 days at 37 °C, no remaining binding activity of the capture antibodies is detectable in the unstabilized plate. In contrast, the stabilized plate does not show a significant decrease in sensitivity even after 84 days of incubation at 37°C.

Due to the evaporation of the hydration shell and direct air contact during drying, proteins lose their secondary and tertiary structure and hence their activity. To prevent this misfolding, it is necessary to seal the coated capture molecules with a closed and uniform protective layer. This is the primary mode of action of modern coating stabilizers, such as Liquid Plate Sealer®. In addition to this standard function, Liquid Plate Sealer® - due to components that weaken the negative influence of the surface - can additionally reactivate some antibodies or proteins that were misfolded during coating, leading to an increase in functional antibodies in many cases. The protective layer formed by Liquid Plate Sealer® maintains the conformation of the capture molecule even under temperature stress. This is especially advantageous if tests are to be distributed in countries with insecure cold chains. Despite its excellent protective properties, the formed layer is easily soluble and hence samples can be added to the stabilized ELISA plate without the need of an additional washing step after storage.

Apart from the conservation of assay performance, modern solid-phase stabilizers can also contribute to optimizations of the production process. A longer shelf-life allows for greater production batches, as the kits can be kept in stock for longer. This not only leads to cost reductions, as it saves on batch releases and machine set-up time, but also reduces inter-assay variations, as customers can keep using the same batch for an extended period. This is further supplemented by the high batch-to-batch consistency of modern stabilizers like Liquid Plate Sealer®. Longer shelf-lives can also be advantageous for hand-made laboratory assays, as they are frequently used for the detection of antibodies against SARS-CoV-2, since a larger batch sizes saves preparation time and improves inter-assay variation also in these settings, leading to more comparable and reliable results.

Moreover, state-of-the-art stabilizers can also be used as blocking reagents, allowing for surface blocking and stabilization of classical sandwich ELISAs in a convenient one-step process (Fig. 2). This reduction in manufacturing steps allows for optimizations and cost reductions in the large-scale manufacturing of diagnostic kits. However, some assays have a need for more efficient surface blocking. In such cases a superior surface blocker like PlateBlock™ in the case of serologic assays for SARS-CoV-2 antibodies[4] or The Blocking Solution for sandwich ELISA are a good choice. Both surface blockers were developed by CANDOR for high-quality immunodiagnostics. Liquid Plate Sealer® is compatible with all surface blocking reagents tested so far.

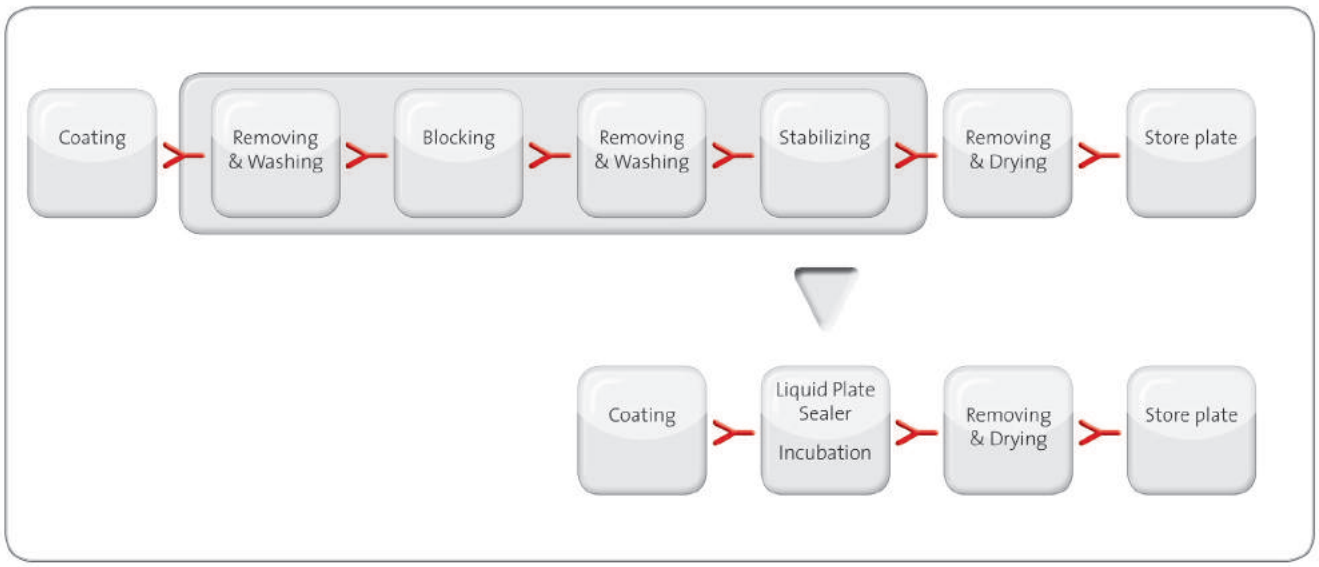


Figure 2: High-speed ELISA plate production with Liquid Plate Sealer® as blocking reagent and coating stabilizer in a single process step is suitable for sandwich ELISA and competitive tests. In serological assay formats however, it is sometimes better to use a more classical process as shown in the first row. In any case, washing after blocking can be commonly skipped when using Liquid Plate Sealer®.

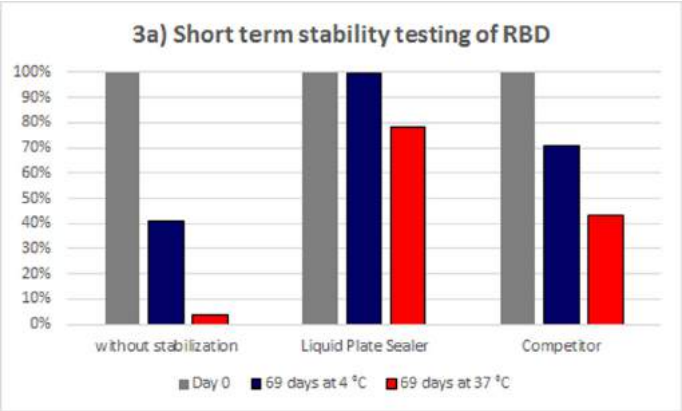
Independent of the exact assay setup, after removal of the commercial stabilizer, plates need to be dried before storage. This is best achieved at ambient temperature either in an environmental chamber at controlled humidity or in an incubator with forced convection followed by heat-sealing in an aluminum bag. An alternative often used in diagnostic manufacturing is to wrap the plate together with desiccant in aluminum bags directly after removal of the stabilizer. If enough desiccant is used, it absorbs all remaining moisture, allowing to skip the external drying process. In both cases, plates can then be stored at 2 - 8 °C for several years, depending on the capture molecule.

In the case of lab-made research ELISA, the aforementioned methods are often not available, leaving another option: plates can be air-dried on the lab bench, sealed with adhesive film and stored at 2 - 8 °C in a fridge. Under these conditions, the negative effect of moisture on the coated proteins cannot be prevented completely. Thus, some plates only show reduced shelf life of several months instead of several years, which is sufficient for many inhouse ELISA applications.

Stabilization of SARS-CoV-2 antigens

A crucial step in the development of an immunoassay is the selection of a suitable capture antigen. The detection of antibodies against SARS-CoV-2 is performed using serologic assays mainly based on three viral capture antigens: the nucleocapsid, the whole spike protein, or its subdomain, the receptor binding domain (RBD). The antibody tests will be required not only for the monitoring of the course of the pandemic and seroprevalence studies but are especially important for assessing the performance of vaccines in development and hopefully soon for surveying the effectiveness of vaccination campaigns. Hence, a large number of kits must be manufactured in a short period of time, to be stored and to be transported to remote locations around the world. In order to maintain the structural integrity of the coated viral antigens - which is essential for the accurate determination of a person's antibody status - under these circumstances, optimal stabilization is essential. Even more so, as experiments have shown that SARS-CoV-2 antigens are exceptionally unstable on the solid phase (Fig. 3). This is nicely illustrated by antigen-down-assays detecting CoViD-19-related antibodies employing two widely used antigens of SARS-CoV-2, the RBD (Fig. 3a) and the nucleocapsid (Fig. 3b). The loss of signal shows that, if not stabilized, both proteins lose more than 60 % of their ability to capture antibodies already after a few weeks, even at 4 °C. If stored at 37 °C for the same period, no more specific signal is detectable, indicating that both proteins are completely misfolded, hence making the assay completely useless. Despite these short half-lives, Liquid Plate Sealer® is able to stabilize the RBD and the nucleocapsid and hence conserve 80 % of the initial signal intensity even after continued heat-stress storage for several weeks at 37 °C. This short shelf-life of the RBD, the relevant antigen for detecting neutralizing antibodies[3], was not expected. In fact, it is a real surprise that RBD shows such a low stability, if not preserved optimally. Although most coated antigens need stabilization, they usually show a higher stability. So RBD is not only the most important antigen for SARS-CoV-2 antibody testing, but also a very good indicator for the technical potential of a coating stabilizer. The quick deterioration of RBD coated on plates is completely prevented by treating the plate with Liquid Plate Sealer®. After stabilization, even continued temperature stress did not significantly impact the activity of the coated capture antigens, negating any effect of a poten-

tially interrupted cooling chain during transport. Therefore, antibody tests employing Liquid Plate Sealer® are already in use in countries with challenges in maintaining cold chains[5].



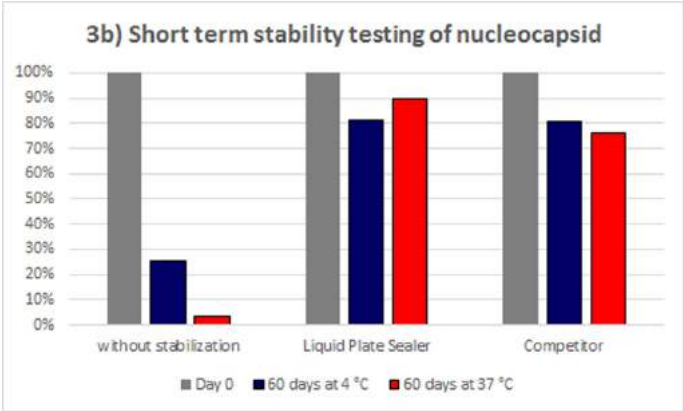


Figure 3: Stabilization of the Receptor-binding domain (RBD, Trenzyme) (a) or the nucleocapsid (Acro Biosystems) (b) of SARS-CoV-2: a Nunc MaxiSorp plate was coated with 100 ng RBD and saturated with PlateBlock™. A subset of wells was stabilized with Liquid Plate Sealer® or the stabilizer of a leading competitor brand for 60 min. Plates were stored for 69 days (RBD) or 60 days (nucleocapsid) at 4 °C or 37 °C and then incubated with human serum samples. After washing, captured antibodies from the serum are detected with a peroxidase-labeled anti-human-IgG-antibody. Values are normalized to the maximum value at day zero.

Due to the high reliability achievable with Liquid Plate Sealer®, it is on the other hand used for confirmatory assays in high-end research settings[6]. Of note, the incubation time with Liquid Plate Sealer® can be reduced to as little as 2 minutes (Fig. 4). The short incubation has no impact on the stabilizing activity of Liquid Plate Sealer® but is only possible if a separate surface blocking step is performed prior to stabilizing (cf. Fig. 2).

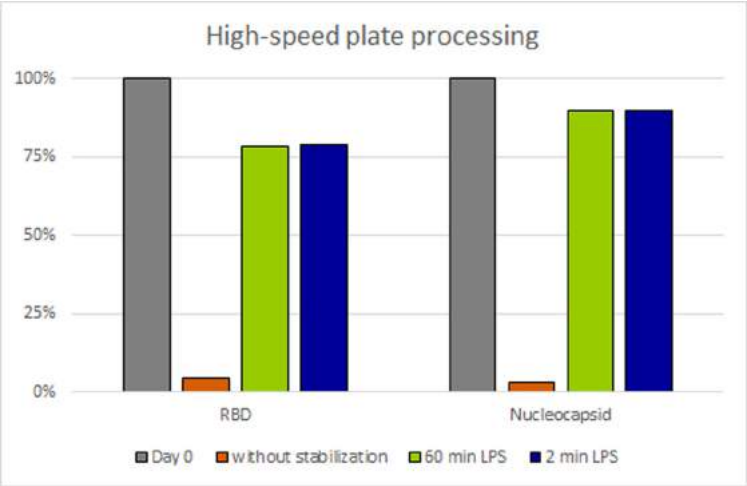


Figure 4: Two minutes of incubation with Liquid Plate Sealer® are sufficient for stabilizing the RBD or the nucleocapsid of SARS-CoV-2. Experiments were performed as described in Fig. 3, except that a subset of wells was incubated with Liquid Plate Sealer® for only 2 min instead of 60 min. Plates were stored for 69 days (RBD) or 60 days (nucleocapsid) at 37 °C.

The Liquid Plate Sealer® product group was introduced by CANDOR Bioscience about a decade ago and has since been successfully used with millions of samples. Apart from the classic Liquid Plate Sealer®, CANDOR also offers variants for specific applications: Liquid Plate Sealer® animal-free is free of animal derived components and hence especially useful for veterinary diagnostics. Liquid Plate Sealer® Plus is suitable for stabilizing especially challenging structural epitopes. Like the classic Liquid Plate Sealer®, both variants feature surface blocking activity and can hence be used in the one-step manufacturing process without separate blocking described above (Fig. 2).

Modern commercial stabilizers not only allow for shelf-lives of several years by preserving the native structure of capture molecules, but also improve assay performance and reliability, can function as surface blockers and thus help optimize workflows. Since all of these added benefits are available at the cost of only a few cents per sample, there is no rational reason for using inferior self-made stabilizers.

CANDOR Bioscience pursues the goal of progressively making immunodiagnostics and immunoassays for research use more reliable and supports all interested parties, also with personal advice. Like all products of CANDOR, the Liquid Plate Sealer® product group is available from 50 mL to bulk quantities and "Made in Germany" in a DIN EN ISO 9001:2015- and DIN EN ISO 13485:2016-certified production facility.

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The ELISA Experts

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