Seramun
Diagnostica GmbH

SeramunBlau® fast2

and

SeramunBlau® slow2

ready-to-use

TMB-substrate-solution for ELISA

Performance data

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Table of Contents

1 Introduction

Rising regulatory requirements lead to labelling and trade bans of raw materials of the long-term established products SeramunBlau® fast (SBf) and SeramunBlau® slow (SBs). New formulations where developed which are free from clp- (classification labelling and packaging of substances and mixtures) labelling requirements. This report shows a summary of the SeramunBlau® fast2 (SBf2) validation and quality control data. Furthermore, the data transferability to the derived substrate formulations SeramunBlau® slow2 (SBs2) (50%, 70% and 85% activity) is presented.

Kinetic measurements of the blue intermediate of the HRP-substrate-reactions are carried out at 650/492 nm. The resulting reaction product is measured at 450/650 nm after stopping the reaction with 0.25 M sulphuric acid.

2 Activity

Highly active substrates enable high sensitivities and short reaction times of only a few minutes in ELISAs (Enzyme-linked Immunosorbent Assay). Our new formulation SBf2 has got the same sensitivity and activity as SBf. Figure 1 shows the reaction kinetics of SBf and SBf2 at two different horseradish peroxidase (HRP) concentrations. There is no significant difference in absorbance during time between SBf and SBf2 (Table 1). The kinetic measurements show a parallel graph progression of both substrate solutions.

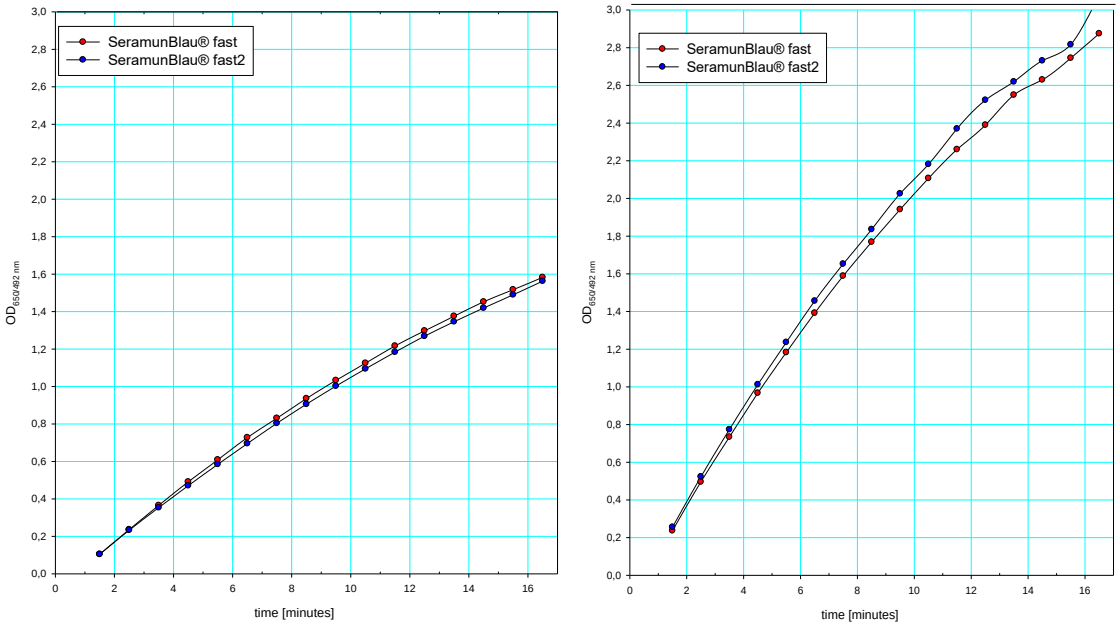

Figure 1: 16-min reaction kinetics of SBf (red) and SBf2 (blue) at 25 °C (left: 100 pg HRP/well; right: 200 pg HRP/well).

Table 1: Difference in activity of SBf2 in relation to SBf (100%).

HRP-concentration per well	Difference in activity of SBf2
100 pg	-2.7%
200 pg	3.5%

The results obtained in the 16-min reaction kinetic could be reproduced in a genuine, commercially available ELISA test (Fig. 2).

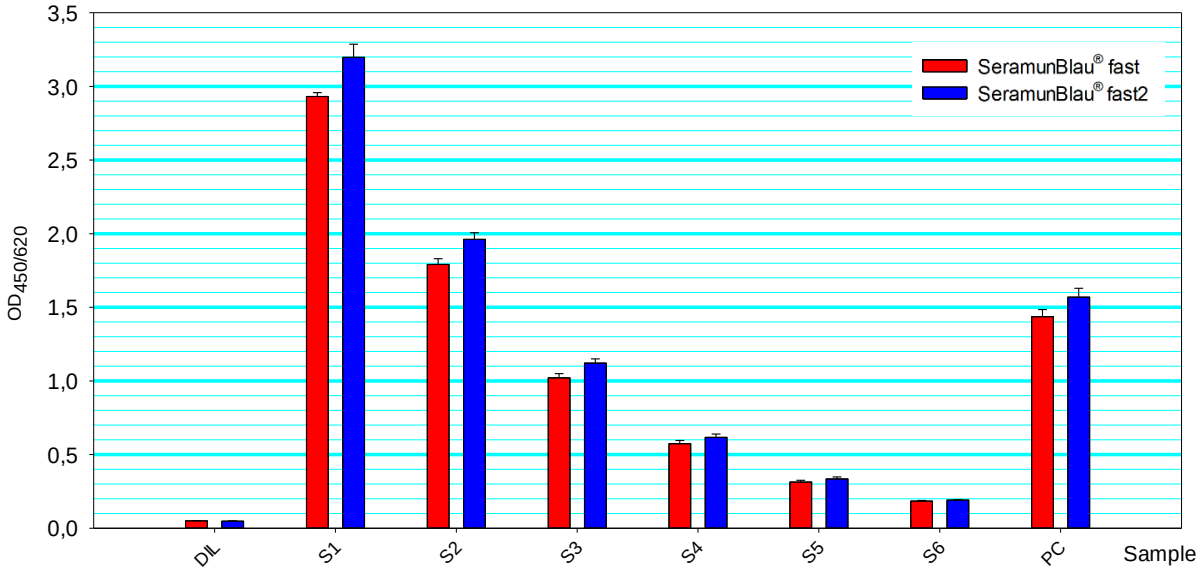

Figure 2: Ovalbumin-ELISA: Comparison of SBf2 (blue) and SBf (red), standard curves (simplified as bar chart with mean values & whiskers; S1: highest concentration, S6: lowest concentration).

At high signal levels SBf2 is generating slightly higher absorbance yields than SBf. No increase of the blank signal could be observed.

Lower activities can be adjusted by decreasing the concentration of TMB (3,3',5,5'-tetramethylbenzidine). Consequently, various activities of the established SeramunBlau® slow (SBs) solutions can be adjusted by using SBf2 and less amounts of TMB resulting in SeramunBlau® slow2 (SBs2) substrates.

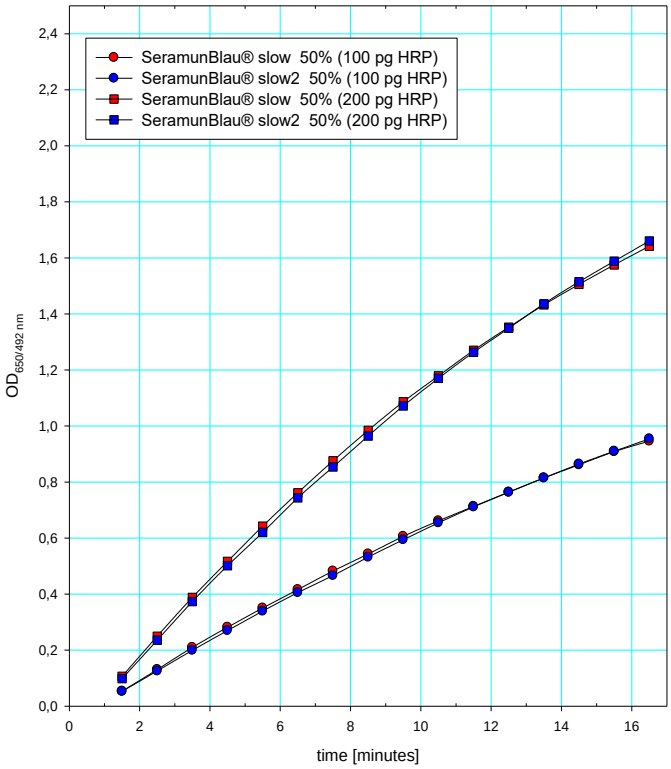

Figure 3: 16-min reaction kinetic of SBs 50% activity (red) and SBs2 50% activity (blue) at 25 °C (circles: 100 pg HRP/well; rectangles: 200 pg HRP/well).

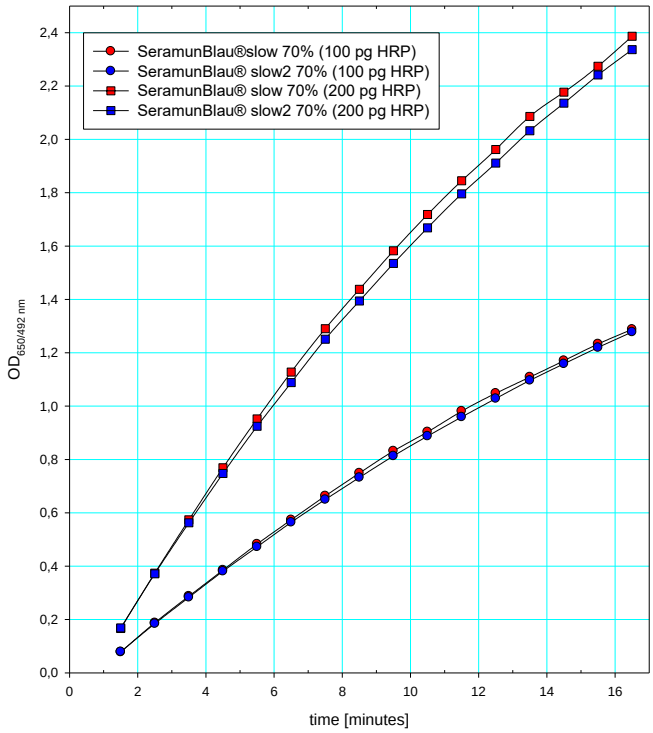

Figure 4: 16-min reaction kinetic of SBs 70% activity (red) and SBs2 70% activity (blue) at 25 °C (circles: 100 pg HRP/well; rectangles: 200 pg HRP/well).

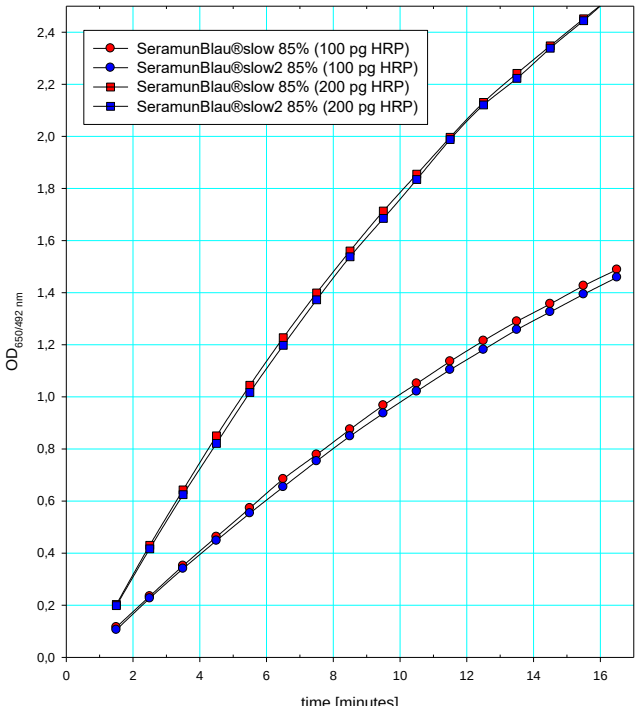

Figure 5: 16-min reaction kinetic of SBs 85% activity (red) and SBs2 85% activity (blue) at 25 °C (circles: 100 pg HRP/well; rectangles: 200 pg HRP/well).

3 Signal Stability after Reaction Stop

Sometimes an immediate photometric measurement after stopping the substrate reaction is not possible. Therefore, a constant signal over time after stopping the reaction is desirable. Signal stability of the new formulation SBf2 was compared with the signal stability of SBf. The measurements were taken out at three different HRP-concentrations and oxidation of TMB was stopped after 10 min reaction time with SeramunBlau® stop (0.25 M sulphuric acid). Afterwards, the absorbance was measured over a time period of 80 min (fig. 6).

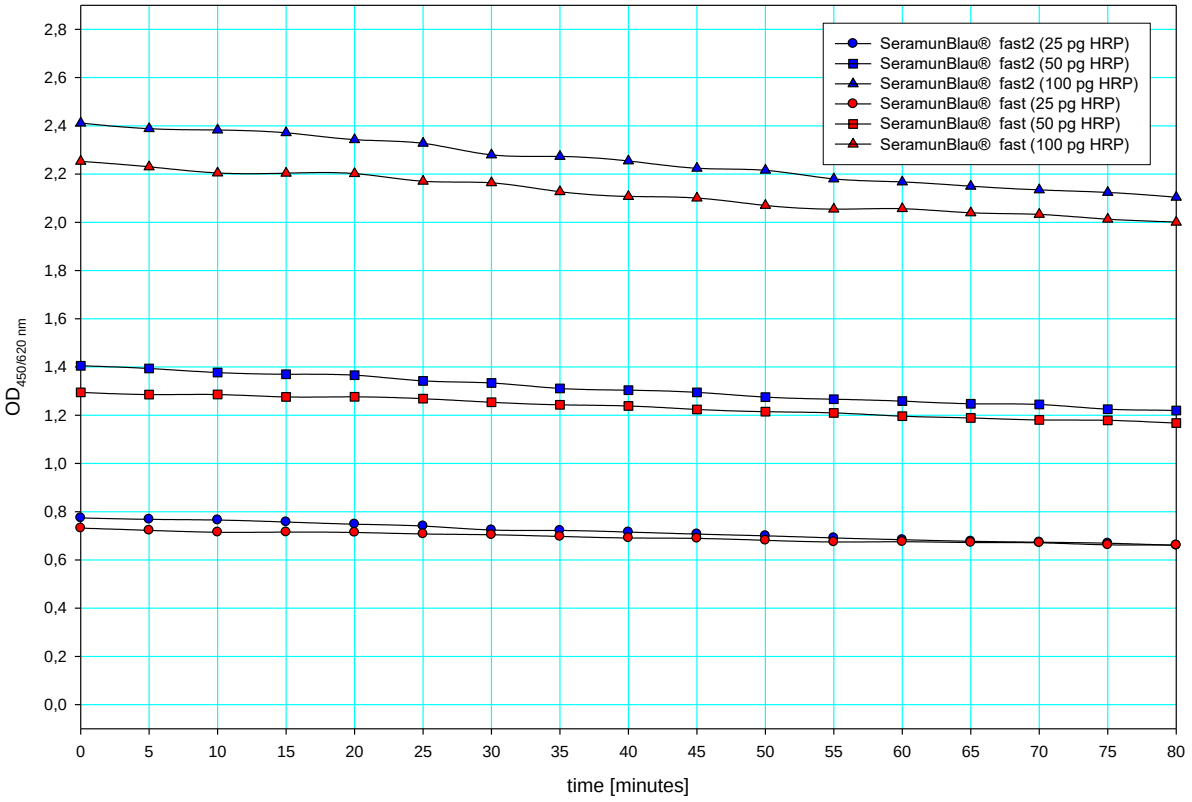

Figure 6: Signal stability after reaction stop. Comparison of SBf2 (blue) with SBf (red). Circles: 25 pg HRP/well; rectangles: 50 pg HRP/well; triangles: 100 pg HRP/well.

Table 2: Signal decrease after reaction stop.

HRP-concentration/ time after reaction stop	Signal decrease of SBf	Signal decrease of SBf2
25 pg HRP/well, 5 minutes	-1.2%	-0.8%
25 pg HRP/well, 30 minutes	-3.8%	-6.5%
100 pg HRP/well, 5 minutes	-1.0%	-1.0%
100 pg HRP/well, 30 minutes	-3.9%	-5.5%

Signal stability after reaction stop of SBf2 is slightly worse than the signal stability of SBf.

4 Influence of Washing Buffer Residues

Residues of washing buffer in the wells can influence the substrate reaction. This influence on SBf2 and SBf was investigated in the next experiment.

Before adding the substrate solution, 10 µl of water or 10 µl of washing buffer were filled into the wells. Subsequently, a 16-min reaction kinetic was measured (fig. 7). The volume of buffer and water is representative for the residual liquid which can be left over due to automatic job processing and in routine procedures.

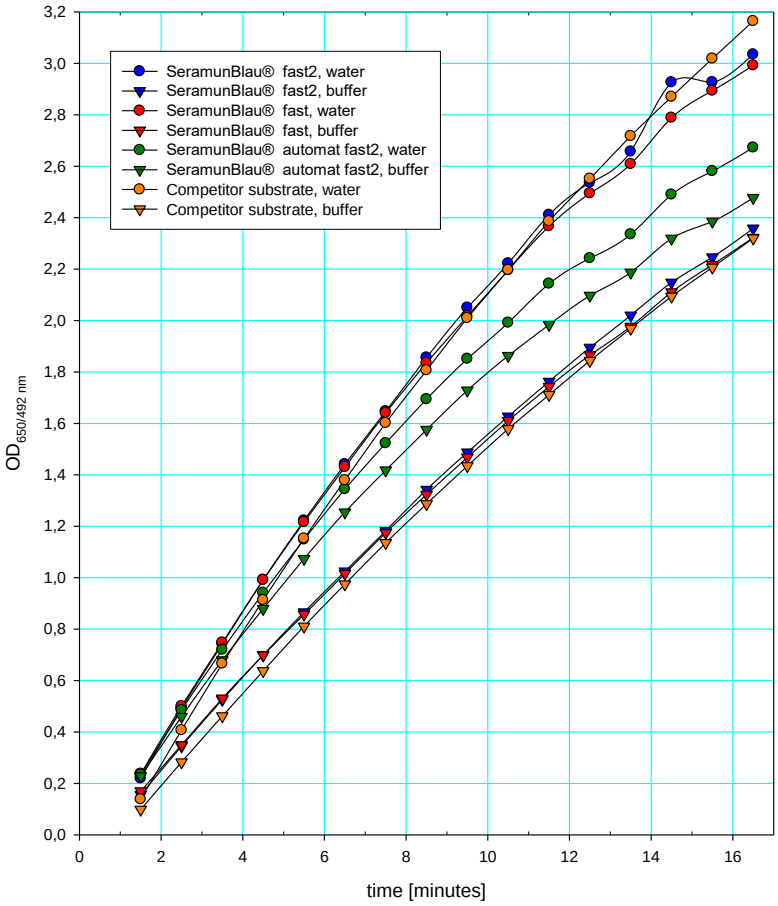

Figure 7: 16-minutes reaction kinetic at 25°C. Influence of washing buffer on substrate activity. Blue: SBf2; red: SBf; green: SeramunBlau® Automat fast; orange: competitor; circles: 10 µL water/well; triangles: 10 µL washing buffer/well; 200 pg HRP/well.

Table 3: Decrease of activity at presence of washing buffer residues.

Substrate	SBf2	SBf	SeramunBlau® automat fast	Competitor substrate
Decrease of activity	-26.8	-26.7	-6.5	-25.9

The activity of SBf2 is influenced by washing buffer residues. The impact on the absorbance values is comparable to that of SBf and to that of the competitor substrate (table 3). Our product SeramunBlau® automat fast can be offered as an alternative substrate if better washing buffer resistances are needed.

5 Accelerated Stability

Accelerated stability experiments were done to assess a preliminary shelf life of the newly developed product. For this purpose, samples of the substrate solution were stored at 37 °C and 4 °C. Reaction kinetics were measured after storage periods of 7, 14, 28 and 56 days (fig. 8).

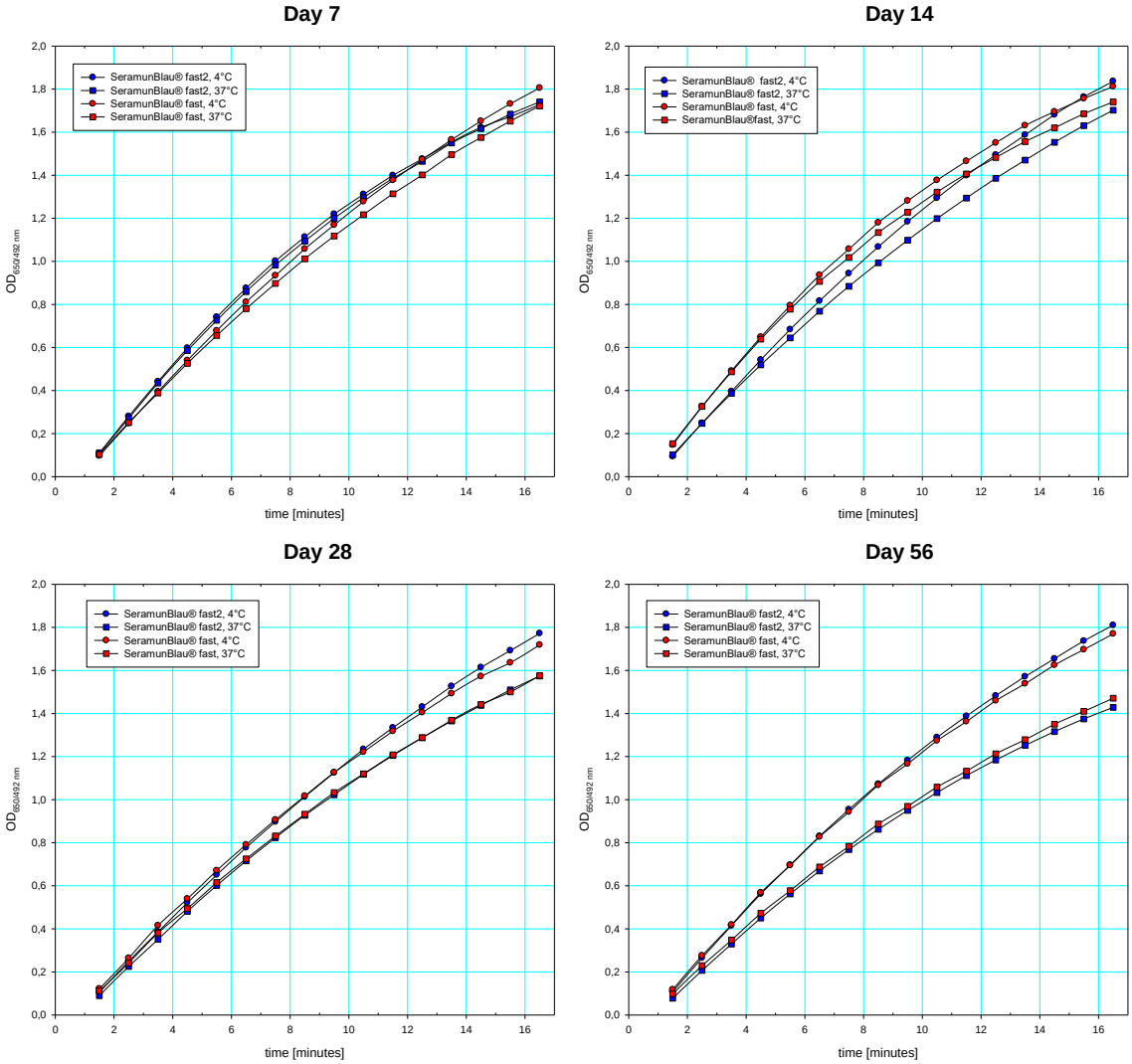

Figure 8: 16-min reaction kinetics at 25 °C of SBf2 (blue) and SBf (red) after storage at 37 °C (rectangles) and 4 °C (circles); 100 pg HRP/well.

Table 4: Activity decrease of SBf and SBf2 after storage at 37 °C.

Time	7 days		14 days		28 days		56 days	
Substrate	**SBf2**	SBf	**SBf2**	SBf	**SBf2**	SBf	**SBf2**	SBf
Decrease 100 pg HRP	**-4.8**	-1.1	**-7.3**	-4.0	**-9.4**	-5.5	**-19.9**	-16.9
Decrease 200 pg HRP	**-3.7**	-0.3	**-5.4**	-3.3	**-9.9**	-8.3	**-18.7**	-20.8

The activity decrease of TMB-substrate solutions under temperature stress is not linear to time. Activity increases might be observed after short storages at 37 °C. The signal decrease of SBf2 is not parallel to the one of SBf. After 7 and 14 days the signal decrease of SBf2 is larger than the signal decrease of SBf. After 28 and 56 days the values of SBf2 adopt to the values of SBf. Consequently, a comparable shelf life of three years can be expected.

Comparable data were obtained for SBs2. The substrates SBs and SBs2 were stored at 4 °C and 37 °C over a period of 28 days and reaction kinetics were measured (fig. 9). The results indicate a SBs2 shelf life of three years.

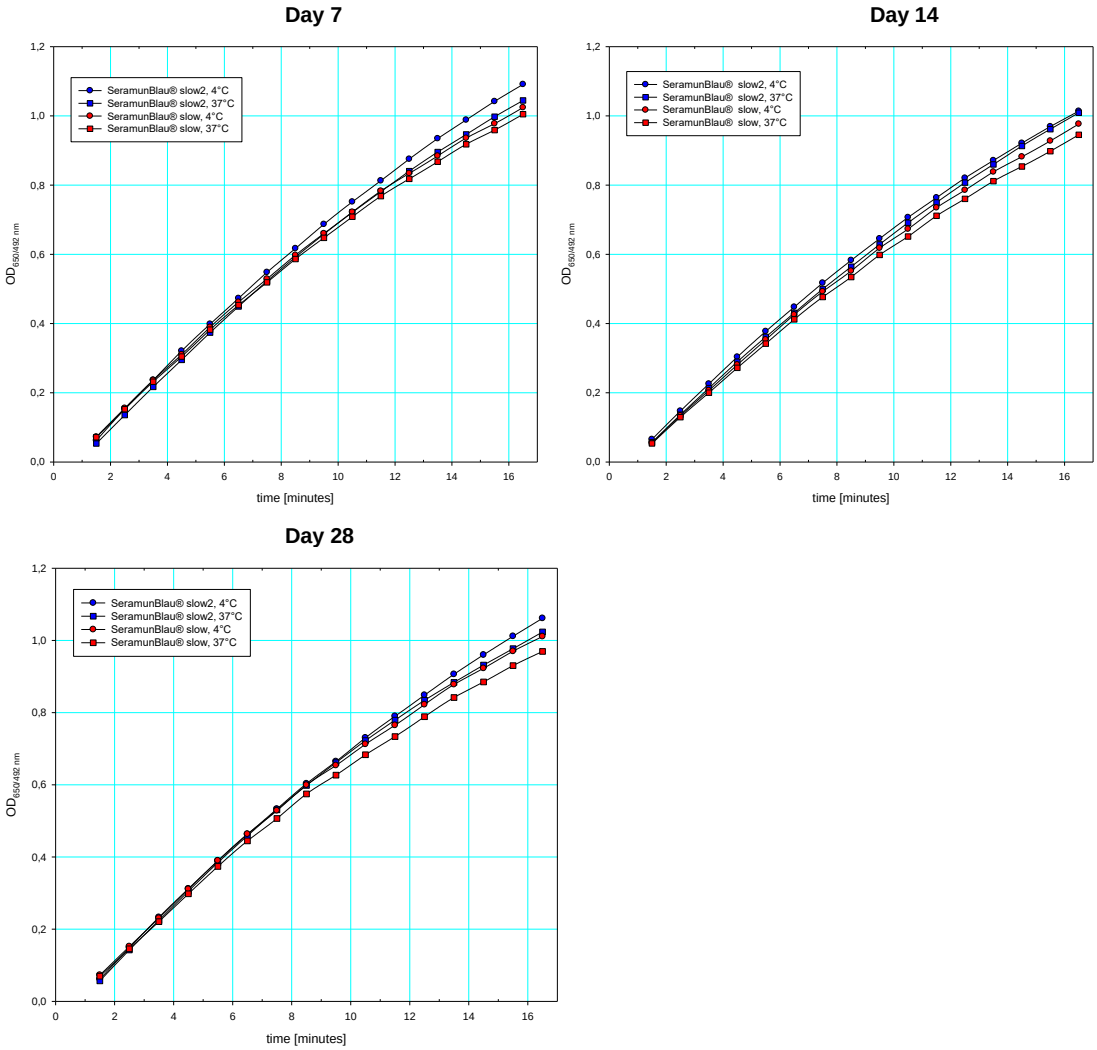

Figure 9: 16-min reaction kinetics at 25 °C of SBs2 (blue) and SBs (red) after storage at 37 °C (rectangles) and 4 °C (circles); 100 pg HRP/well.

Table 5: Activity decrease of SBs and SBs2 after storage at 37 °C.

Time	**7 days**		**14 days**		**28 days**	
Substrate	**SBs2**	SBs	**SBs2**	SBs	**SBs2**	SBs
Decrease 100 pg HRP	**+3.3**	-1.9	**+2.2**	-3.2	**-1.1**	-4.1
Decrease 200 pg HRP	**+3.3**	+0.8	**+2.2**	-1.8	**-5.3**	-4.6

The results of day 7 and day 14 show a signal increase of SBs2 (activity 50%) after storage at 37 °C. After 28 days the activity decrease is similar to that of SBs. The results indicate a SBs2 shelf life of three years.

6 Freeze-Thaw Cycles

During transportation or storage processes freezing of the substrate solution is possible. This can lead to a loss of activity. Impacts of freezing were investigated with the following experiment: A sample of SBf2 was stored at -20 °C overnight and thawed again. This procedure was repeated four times and a reaction kinetic was performed (fig. 10). The experiment was also performed with SBs2 (data not shown). No loss of activity compared to samples stored at 4 °C could be observed. Consequently, freezing and thawing has no influence on the substrate activity of SBf2 and SBs2.

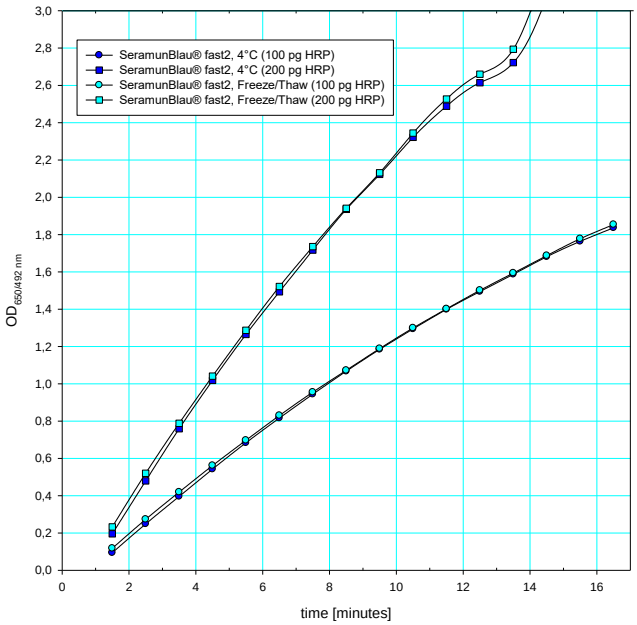

Figure 10: 16-min reaction kinetic at 25 °C of SBf2. Blue: storage at 4 °C; turquoise: 5 freeze-thaw cycles; circles: 100 pg HRP/well; rectangles: 200 pg HRP/well.

7 Open-vial-test

Often the substrate solution is not used at once. Thus, a substrate vessel can be opened several times and the temperature is changing from 4 °C storage temperature to room temperature. To investigate the influence of bottle opening and temperature change on the substrate activity an open-vial test of three different lots of SBf2 was performed. Therefore, the substrate bottles of SBf2 were opened for 6 h at room temperature. Afterwards, the bottles were closed and stored at 2-8 °C for three months. To investigate the influence on the substrate activity a reaction kinetic was performed (fig. 11).

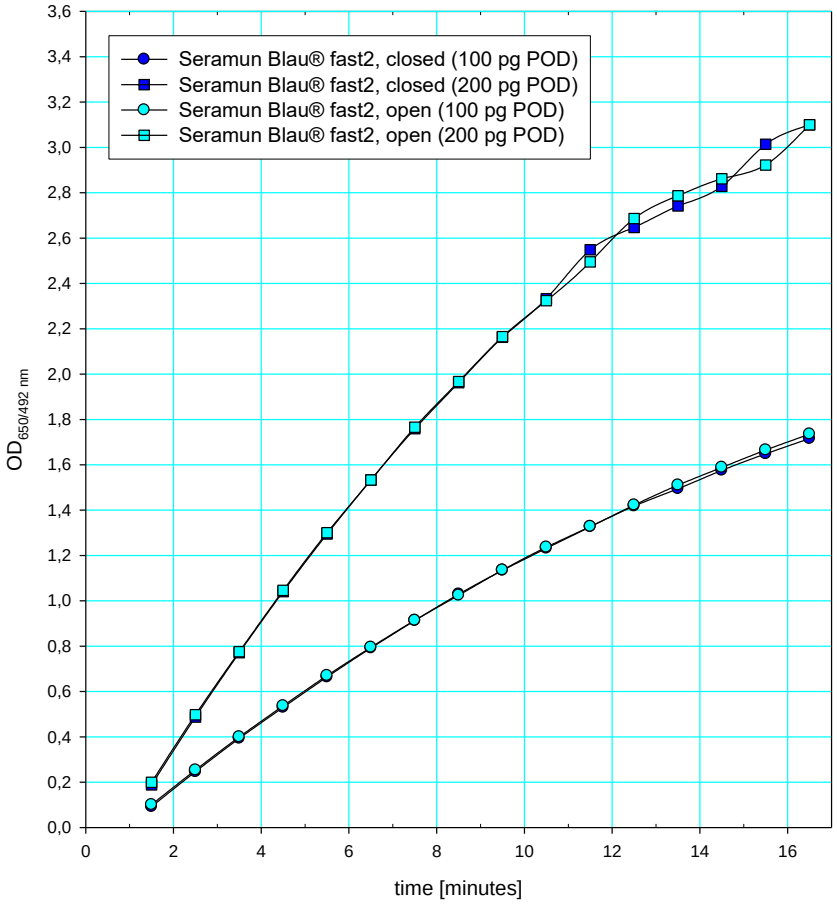

Fig. 11: 16-min reaction kinetic at 25 °C. Comparison of open-vial tested SBf2 (turquoise) and at 2-8 °C stored SBf2 (blue). Circles: 100 pg HRP/well, rectangles: 200 pg HRP/well.

No loss of activity could be observed for the open-vial tested SBf2. Furthermore, no difference in background signals of open-vial tested SBf2 and at 2-8 °C stored SBf2 could be observed.

8 Real time Stability

Real time stability data is generated by measuring a reaction kinetic of retained and freshly produced substrate batches. The activity of the freshly produced batch was set to 100%.

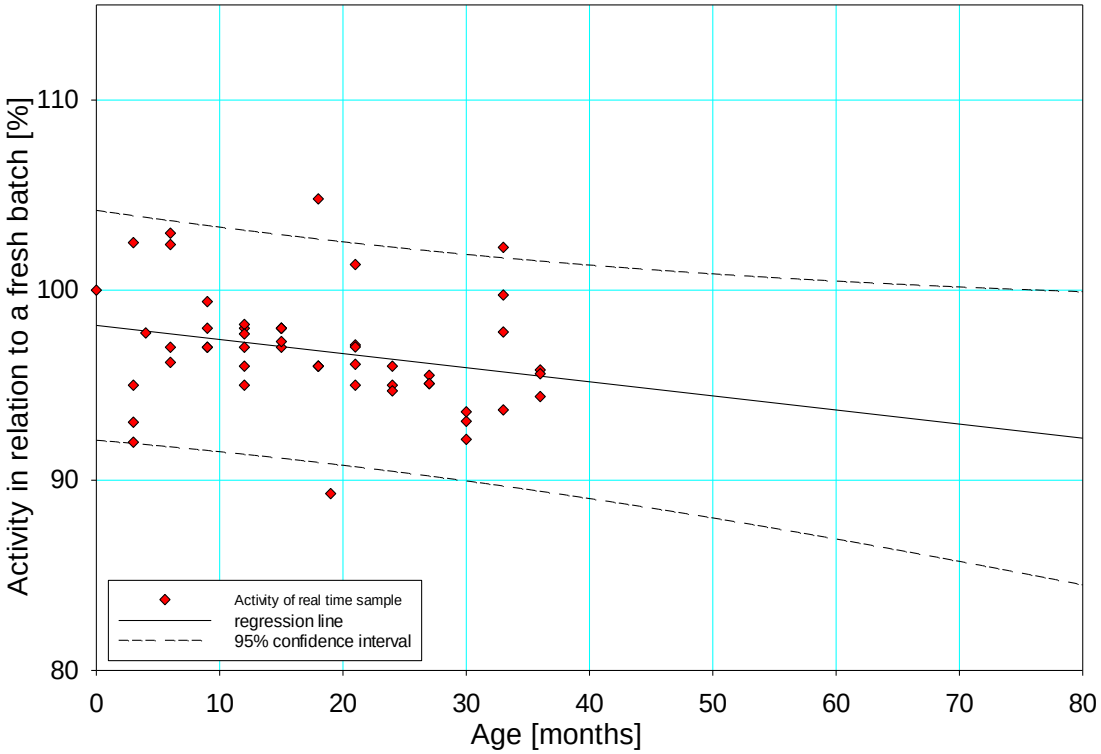

Figure 12: Activities of SBf2 real-time samples in relation to a freshly produced batch (100%).

Within the first months an activity increase of SBf can be observed (fig. 12). SBf2 shows analogous activity behaviour. The results indicate a SBf2 shelf life of three years.

Additional to the reaction kinetic the SBf2 background signal of the retained and the freshly produced batched was measured (fig. 13). The current data shows no significant increase of the blank signals. All recorded background signals lie within specification (OD_{Blank} < 0.050).

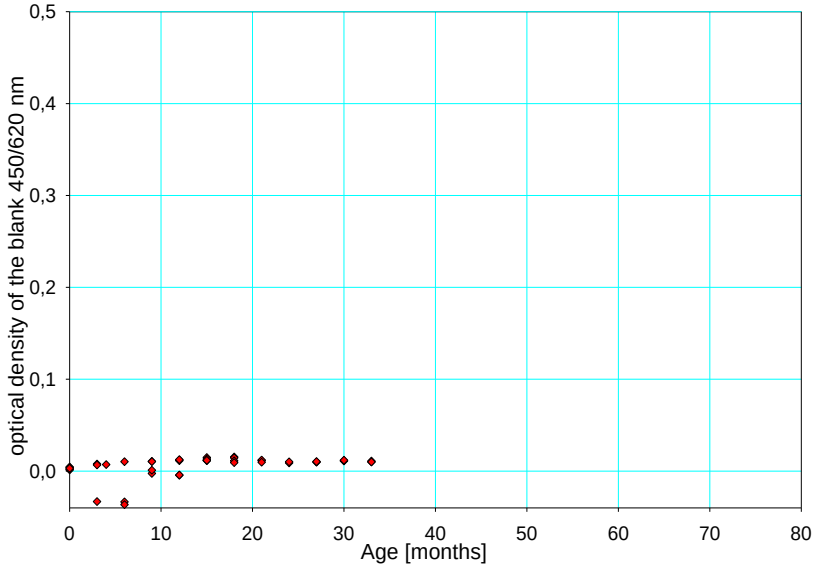

Figure 13: Background signal of SBf2 real time samples.

For SBs2 (50% activity) real-time data is generated as well. Reaction kinetics of retained and freshly produced substrate batches were measured. The activity of the freshly produced batch was set to 100%.

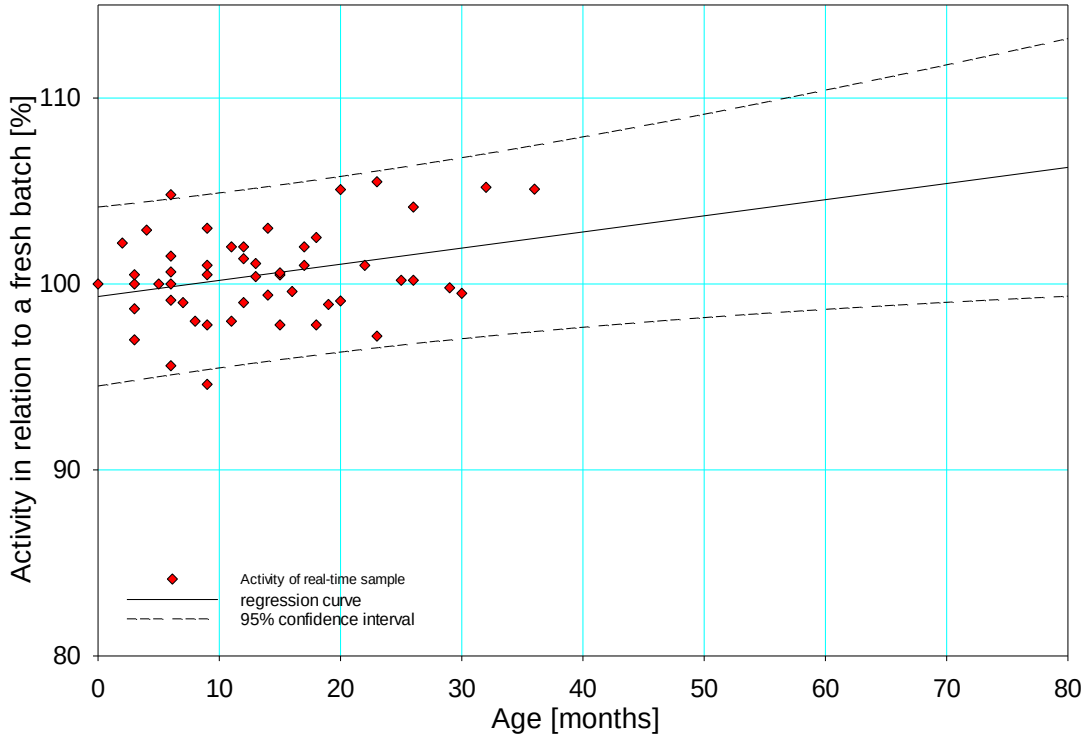

Figure 14: Activities of SBs2 real-time samples in relation to a freshly produced batch (100%).

Contrary to SBf2, SBs2 (50% activity) is gaining in activity during the first 36 months after production (fig. 14). The area of activity decline has not yet been reached by the batches with an age of up to 36 months. Therefore, the results indicate a SBs2 (50% activity) shelf life of three years.

Additional to the reaction kinetic the SBs2 background signal of the retained and the freshly produced batches was measured (fig. 15). The current data shows no significant increase of the blank signals. All recorded background signals lie within specification (OD_{Blank} < 0.050).

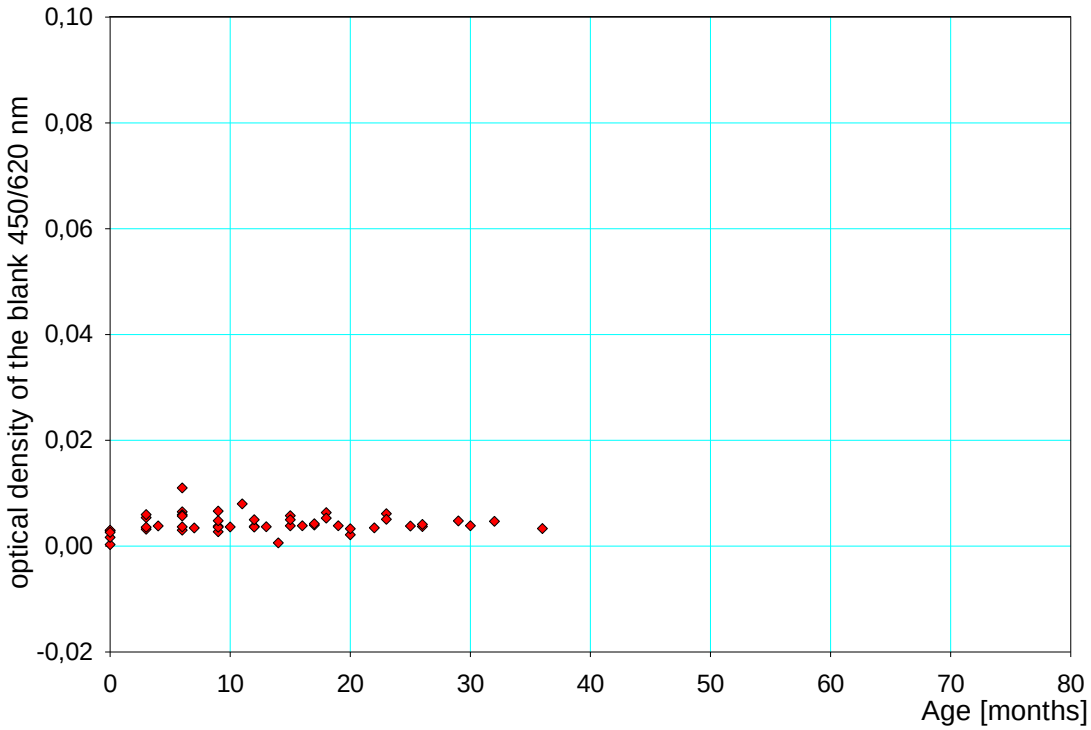

Figure 15: Background signal of SBs2 real time samples.

9 Reproducibility

The reproducibility of SBf2 was tested by producing three different lots of SBf2 out of different raw material batches. Afterwards, all three SBf2 lots were tested in a reaction kinetic (fig. 16).

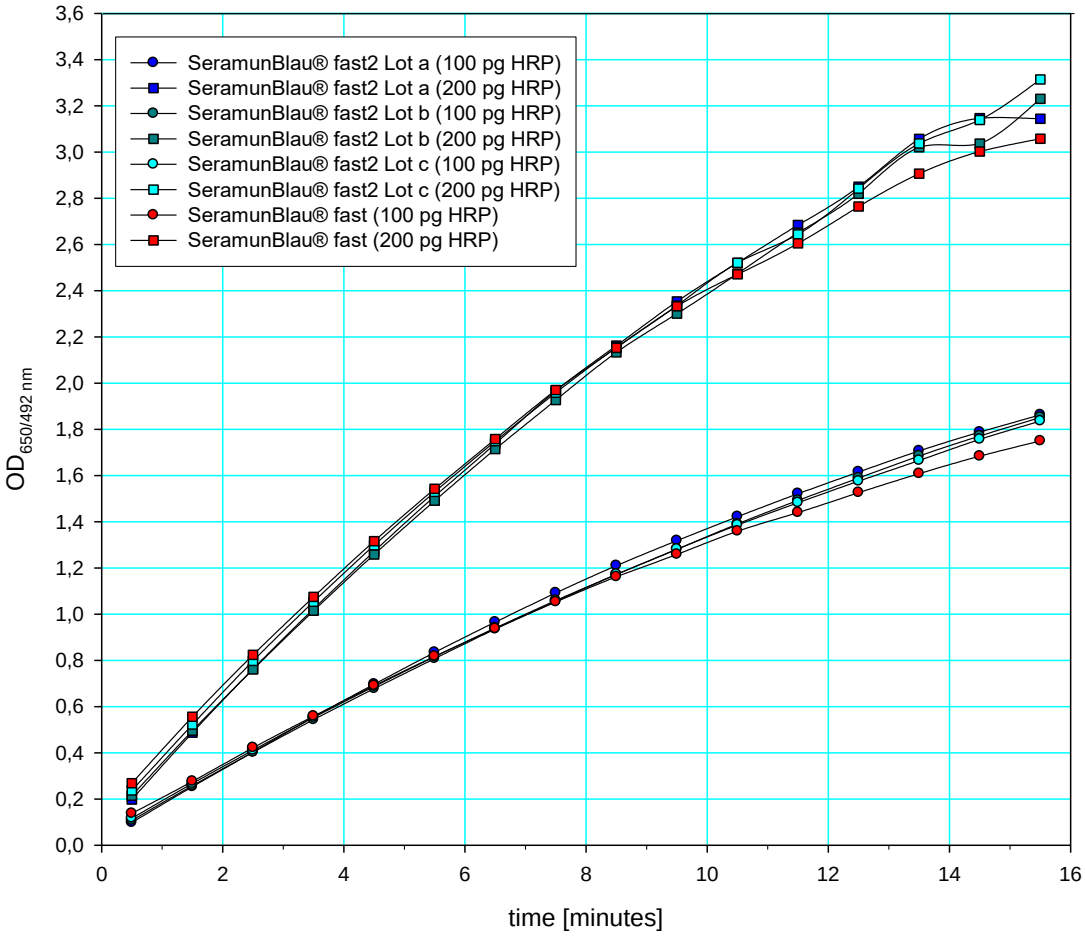

Figure 16: 16-min reaction kinetic at 35 °C of three lots SBf2 and one lot SBf.

All lots of SBf2 show the same activity and are comparable to that of SBf. The coefficient of variation (COV) of activity values between these batches lies below 2%. Consequently, a reproducible production can be implemented.

10 Additional Characteristics

10.1 Elongated Incubation Period

SBs2 is designed for incubation periods of 10 to 15 minutes at room temperature. The next experiment shows how elongated incubation times and increased temperatures affect the SBf2 activity. Therefore, a 46-min reaction kinetic at 37 °C was performed.

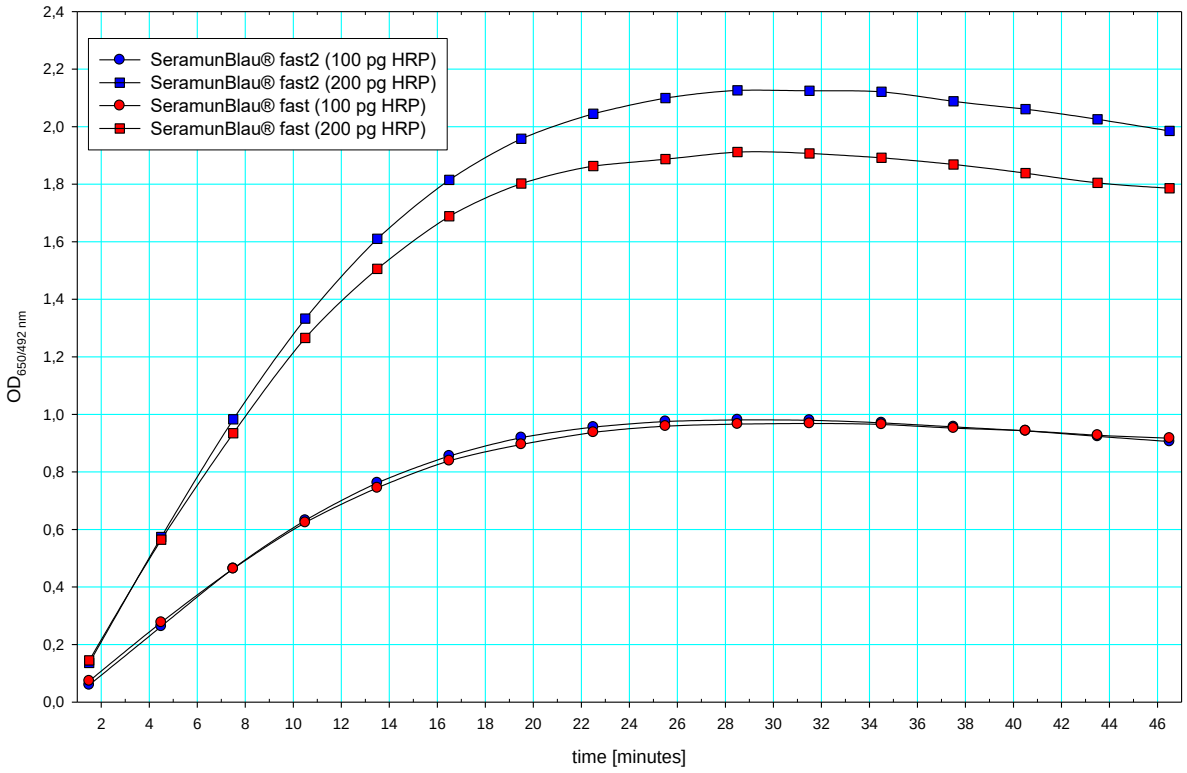

Figure 17: 45-min reaction kinetic of SBf2 (blue) and SBf (red) at 37 °C. Circles: 100 pg HRP/well, rectangles: 200 pg HRP/well.

After 20 minutes reaction time the absorption slope decreases (fig. 17). The inflection point is reached after 30 minutes incubation. Especially at HRP-concentrations of 200 pg per well SBF2 shows better curve progression than SBf.

Incubation periods of up to 30 minutes and higher incubation temperatures of up to 37 °C are tolerated by SBf2. False negative signals at high HRP–concentrations are highly unlikely with SBf2.

10.2 Light Exposure

Oxidation of TMB to the blue iminium cation can be initiated by light exposure. This effect induces high blank values. All SeramunBlau® substrate solutions contain a mechanism which leads to a reduction of TMB. Consequently, blue staining of the substrate solutions can be avoided and blank values stay low.

The following experiment shows the effects of light exposure on SBf2 and the decoloration kinetic of the substrate. The samples were exposed either to laboratory light (fluorescent lamp) or sunlight (bright sunshine, window ledge) for 30 min. The comparative solution was kept in a black HDPE–bottle.

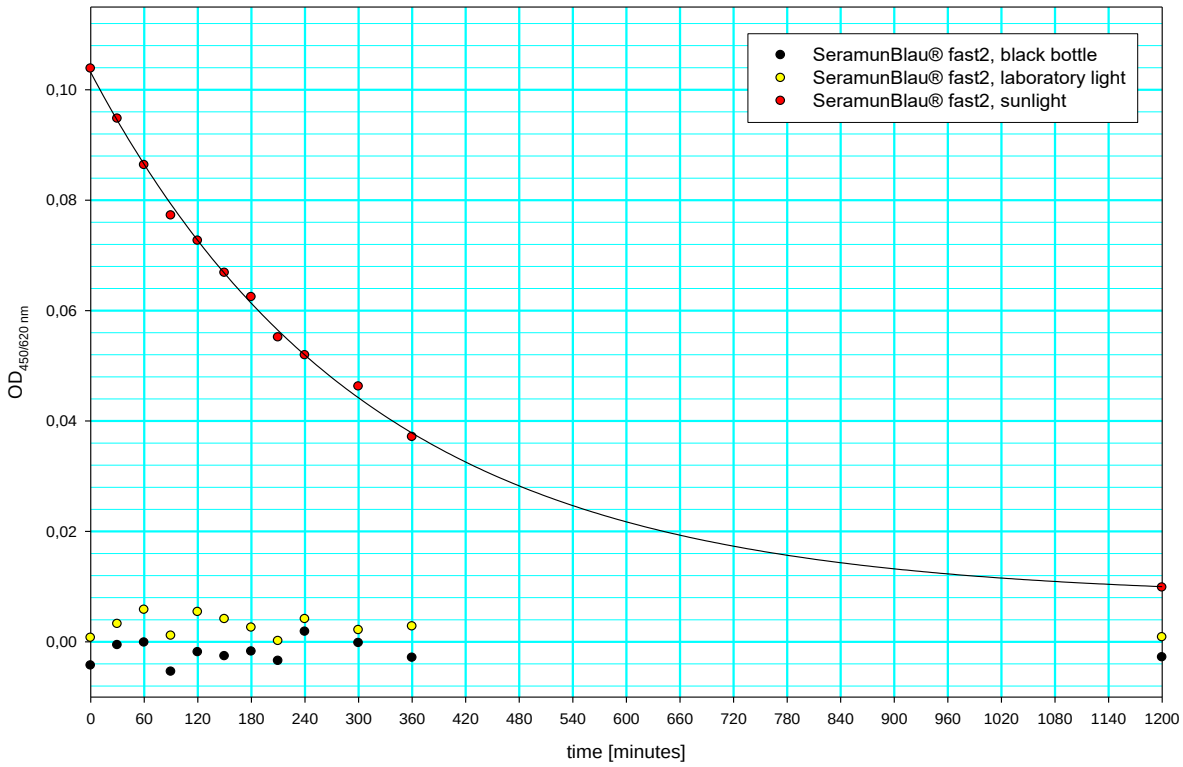

Figure 18: Blank values von SBf2. Black: SBf2 kept in a black HDPE-bottle; yellow: SBf2 exposed to laboratory light; red: SBf2 exposed to sunlight.

Sunlight exposure results in an increase of blank signals (fig. 18). Storing the solution in a dark bottle leads to a decoloration and thus, to a decrease of the blank signals. After around 24 the substrate solution should be regenerated. Laboratory light did not lead to a significant increase of blank signals.

Sunlight exposure of the solution should be avoided. Temporary exposure to light, however, can be tolerated.

11 Transport Stability

During transportation of substrate solutions temperature changes are likely. In this experiment the influence of temperature changes on the substrate solutions where investigated. Therefore SBf2 and SBs2 (50% activity) were stored for 4 days at 30 °C, for 3 days at 4 °C, for 1 day at 30 °C, for 1 day at 4 °C and finally for 2 weeks at 37 °C. Afterwards, a 16-min reaction kinetic of the temperature-stressed probes and probes stored at 4…8 °C were measured.

Table 6: Difference in activity of temperature stressed probes compared to probes stored at 4…8 °C.

Substrate	SBf2, #012	SBf2, #015	SBf2, #019	SBs2, #001	SBs2, #004	SBs2, #006
Signal Decrease	-5%	-6%	-5%	-7%	-3%	-1%

The maximal activity decrease observed in this experiment is 7% (table 6). After such temperature changes an activity decrease of 15% would be still tolerable. Consequently, the substrate formulations are stable against multiple temperature changes.

12 Miscibility with Original Formulations

The performance of mixtures can be of interest, if customers are using remains of original formulations (SBf and SBs) together with new formulations (SBf2 and SBs2). To ensure equal activities by mixing both formulations, different mixtures were prepared and a reaction kinetic was measured (fig. 19).

The results show no significant change of activity of the mixtures compared to SBf2 (pure) or SBs2 (pure, 50% activity). Consequently, the performance is not hindered, if remains of the original formulations are mixed with the new formulations in a 1:4, 1:1 or 4:1 ratio.

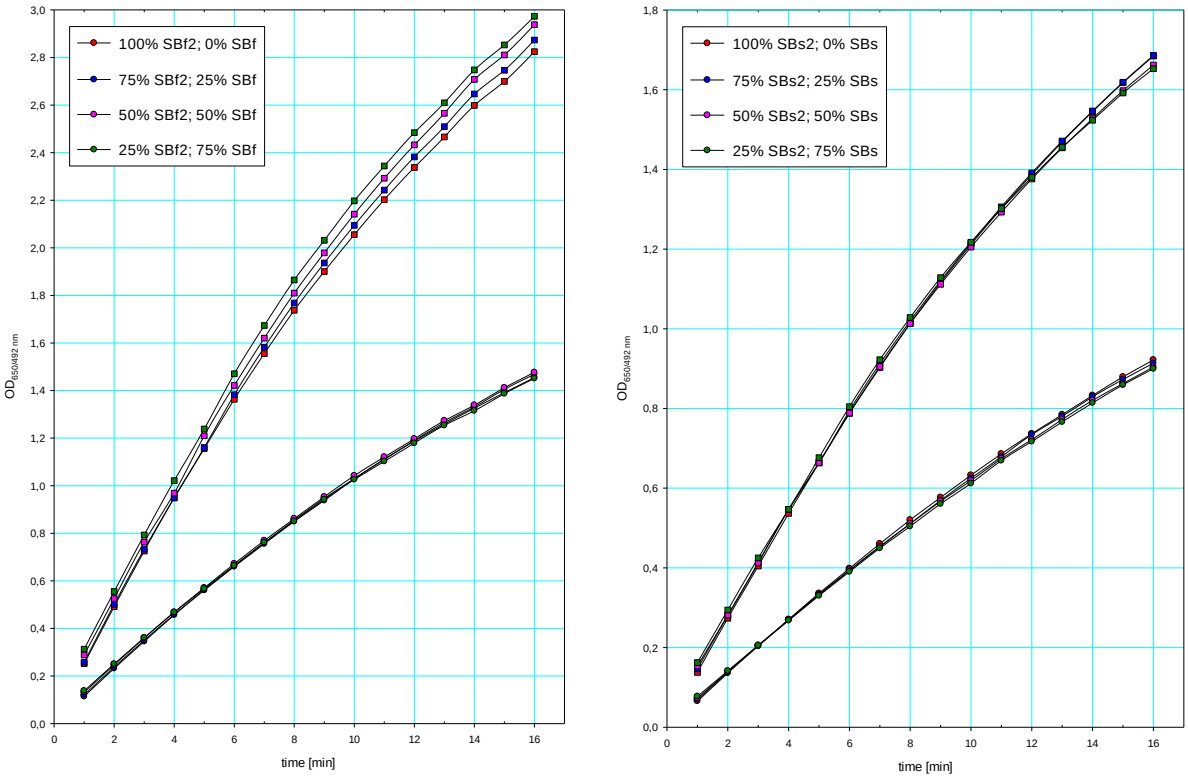

Figure 19: 16-min reaction kinetic of formulation mixtures. Right: Mixture of SBf and Sbf2; left: Mixture of SBs2 and SBs; circles: 100 pg POD/well; rectangles: 200 pg POD/well.

13 Summary

SBf2 is a ready-to-use, labelling-free TMB-substrate solution. The substrate shows similar performance and validation data as SBf. Furthermore, activity reduced formulations based on SBf2 leading to SBs2 can be prepared.

Based on accelerated stability data (Section 5) a preliminary shelf life of 36 months was expected. The results of the real time stability (Section 8) support this, as none of the batches with an age of up to 36 months has reached the area of activity decline.

Accelerated stability, freezing and thawing during transport (Sections 5 and 6) and light exposure (Section 10.2) during usage in the laboratory do **not** have a negative effect on the performance.