Seramun
Diagnostica GmbH

SeramunGelb® fast

the ready-to-use pNPP/Substrate Solution for ELISA

Evaluation Report

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TABLE OF CONTENTS

1. INTRODUCTION

p-Nitrophenylphosphate (pNPP) is the most commonly used substrate for alkaline phosphatase (AP) in enzyme immunoassay techniques. The phosphate residue is ester interchanged by the AP to an acceptor, preferably amino alcohols. The colourless solution turns into the intensive yellow colour of *p*-nitrophenol.

The results reported here are mainly based on experimental data obtained from Seramun Diagnostica GmbH. Customers have also confirmed the properties of SeramunGelb® fast demonstrated in this report.

At present, a growing number of ready-to-use pNPP solutions are available. The ready-to-use substrate SeramunGelb® offers the following advantages:

- long shelf life,
- high absorbance yield,
- low background.

An optimized buffer system enhances the activity of the alkaline phosphatase and reduces spontaneous hydrolysis of pNPP. The non-hazardous, non-aggressive and non-flammable solution guarantees the safety of transport and of use in the laboratory. SeramunGelb® fast is produced according to a standardized operating procedure that guarantees a high lot-to-lot consistency.

In this report, we compare SeramunGelb® fast with pNPP substrate tablets from Sigma and with ready-to-use substrate solutions from Moss, SurModics Inc. (formerly BioFX Laboratories) and KemEnTec.

2. SENSITIVITY OF SERAMUNGELB® FAST

A highly active substrate is essential for ELISA tests demanding high sensitivity and steep standard curves. Furthermore, short substrate reaction times contribute to reduce the overall assay time as well as the conjugate concentration. The latter point is of importance to prevent background problems and may help to reduce reagent costs.

Kinetic measurement of substrate reaction

Substrate conversion was monitored by the following procedure: microtitration plates (Nunc, Maxisorp) coated with 10 µg/ml human IgG were incubated for 60 minutes at room temperature with 100 µl of anti-human IgG-AP conjugate (Seramun) adjusted to a concentration of 0.10 µg/ml and 0.05 µg/ml respectively. After washing the wells five times, the substrate reaction was started by addition of 100 µl SeramunGelb® fast. Absorbances were recorded at 405/620 nm wavelength every 5 minutes over a period of 45 minutes.

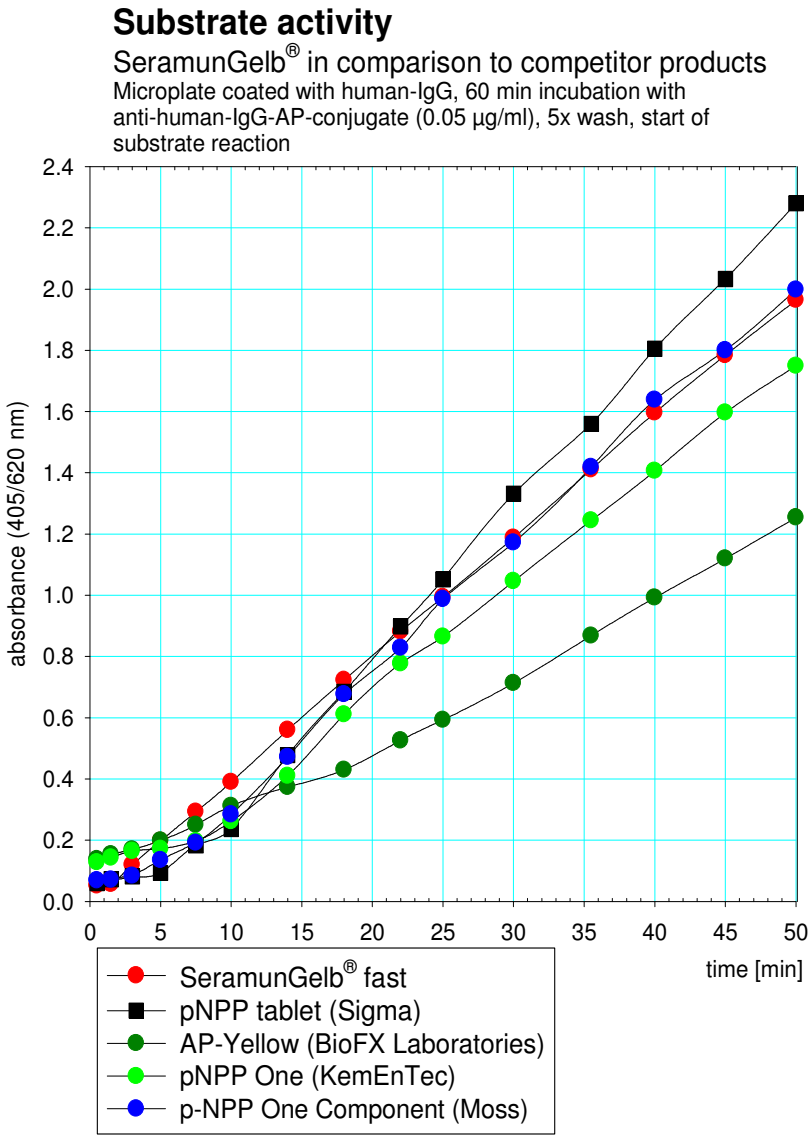


TABLE 1: Slope [AU/min] and linear coefficient of correlation

	conjugate concentration 0.10 µg/ml		conjugate concentration 0.05 µg/ml	
	coeff. of corr.	slope	coeff. of corr.	slope
SeramunGelb® fast	0.9991	0.0683	0.9996	0.0389
pNPP tablet (Sigma)	0.9898	0.0807	0.9919	0.0470
AP-Yellow (BioFX Laboratories)	0.9933	0.0402	0.9907	0.0237
pNPP One (KemEnTec)	0.9887	0.0609	0.9907	0.0345
p-NPP One Component (Moss)	0.9932	0.0701	0.9953	0.0410

Results

During the first 10 min, the reaction time and colour development was delayed in all substrate solutions. This effect was lowest with SeramunGelb® fast and highest with the solution prepared from the pNPP tablet.

Highest absorbance yield was achieved with freshly prepared substrate solution from pNPP tablets (Sigma) followed by SeramunGelb® fast and the substrate from Moss, whereas the substrates from KemEnTec and especially from BioFX Laboratories resulted in lower absorbances.

3. BACKGROUND OF SERAMUNGELB® FAST

Very low background absorbances are decisive for high S/N ratios in ELISA systems. This is especially important when the ELISA results are read visually e.g. in screening procedures under field conditions.

pNPP in aqueous solutions undergoes a spontaneous and irreversible hydrolysis. To minimize this effect a correct storage at 2-6 °C, protected from light, is recommended. Any refilling of the solution to the original vessel should be avoided.

Freshly produced SeramunGelb® fast shows a background between 0.030 AU and 0.040 AU and may increase about 0.010 AU per month. Freshly prepared solution of a pNPP tablet (Sigma) shows a background between 0.040 AU and 0.070 AU and ready-to-use solutions from different competitors show a background between 0.050 AU and 0.150 AU when the respective solution is measured immediately after removing from a freshly opened bottle.

TABLE 2: Background signals of different production lots of SeramunGelb® fast

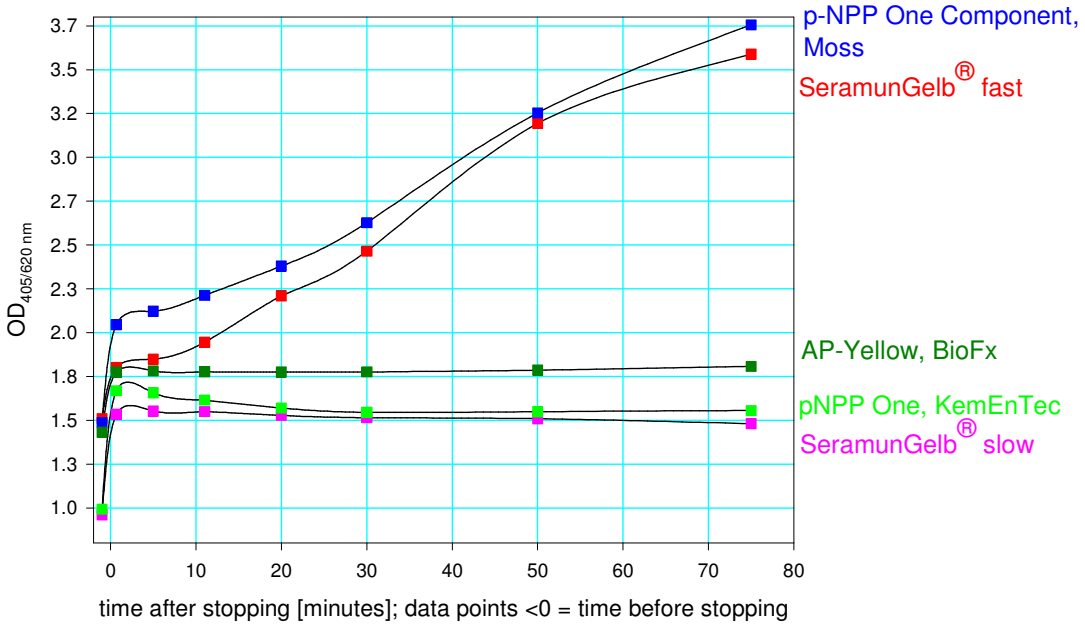
Lot	0010802	0030902	0051102	0061202	0071202
Mean absorbance 405/620 nm	0.034	0.031	0.033	0.033	0.031

4. INFLUENCE OF STOP SOLUTIONS

The use of stop solution should terminate the enzymatic reaction and result in a time independent stable absorption signal. If possible, the reaction stop should enhance the absorption yield in comparison to the unstopped solution.

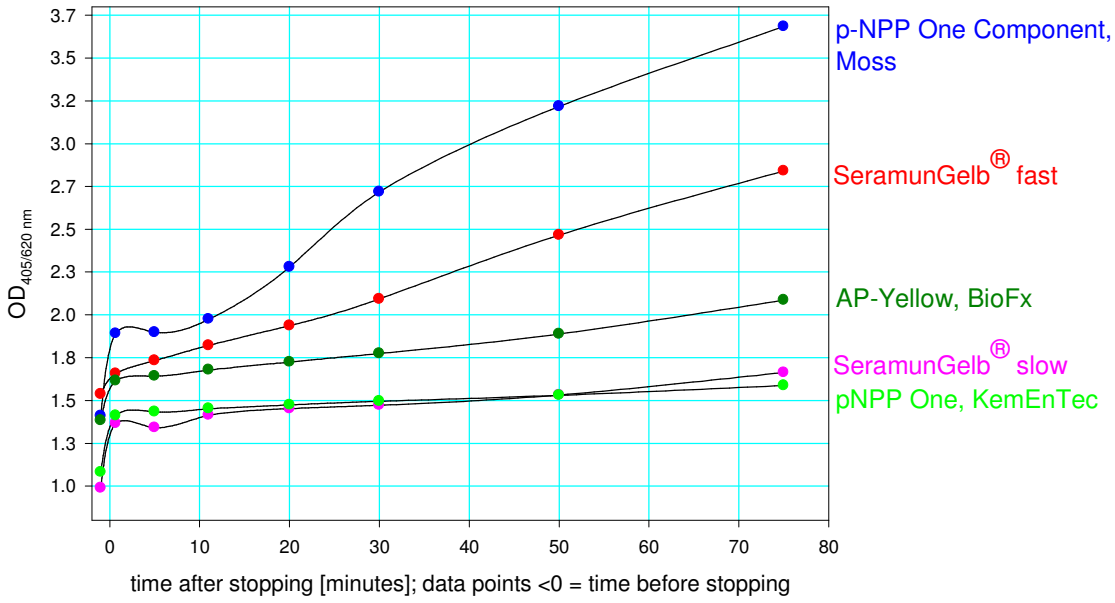
Various stop solutions are described and commercially available. However, the different substrate solutions react differently independent of the utilized stop solution.

Use of Sodium Hydroxide



The substrates from BioFX, KemEnTec and SeramunGelb® slow form a stable product with sodium hydroxide as a stop solution, whereas the reaction of the substrates from Moss and SeramunGelb® fast cannot be stopped.

Use of Sodium Carbonate



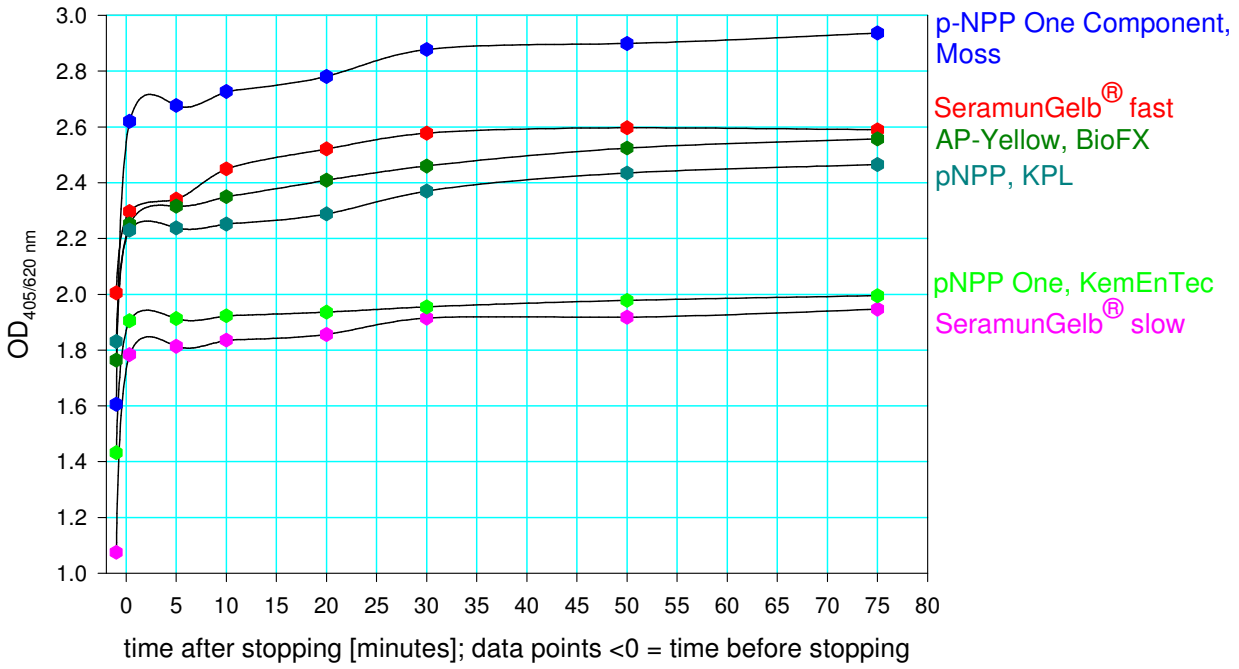
The use of sodium carbonate effectively stops the reaction of SeramunGelb® slow and pNPP One (KemEnTec), whereas the signal of SeramunGelb® fast is still growing slowly. The increase of absorbance is reduced by a factor of 2 when compared to the reaction stop with sodium hydroxide. The substrate from Moss cannot be stopped at all and the absorbance increase of the BioFX substrate is about 10-15 % over the investigated time course.

Use of Sodium Phosphate

The use of sodium phosphate is not recommended to stop SeramunGelb® fast due to the formation of precipitates. This result was consistent across all of the tested substrate solutions.

Use of SeramunGelb® stop

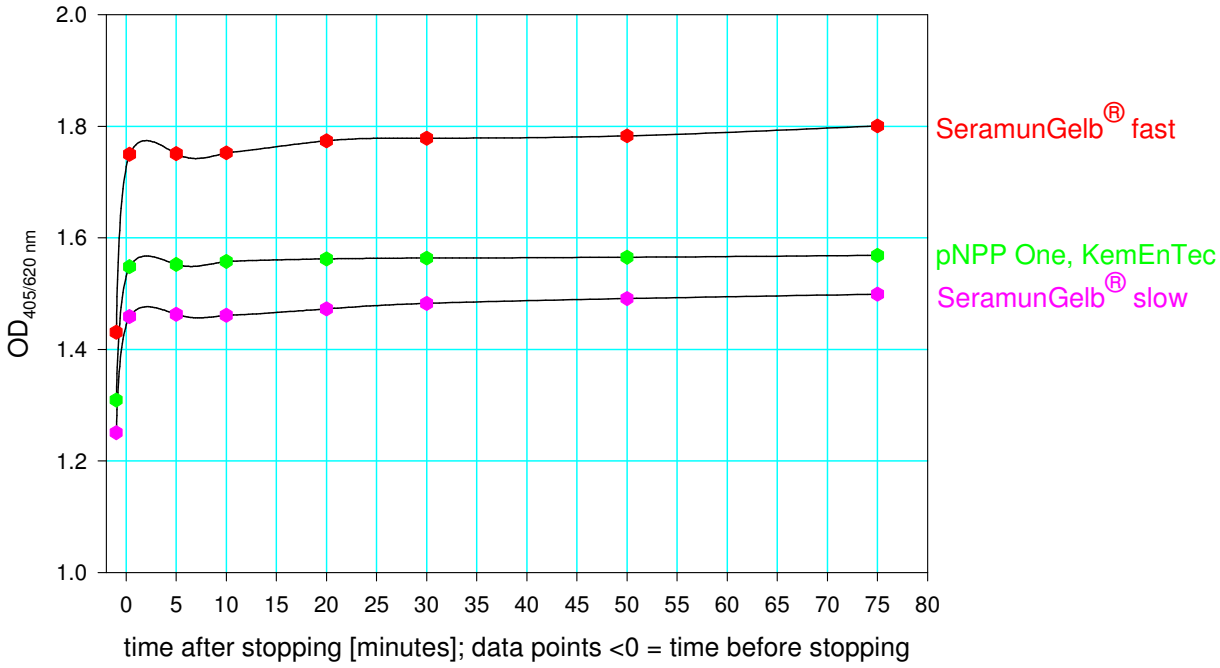
To overcome the insufficiency of the various stop solutions, the multi-component solution SeramunGelb® stop was developed.



SeramunGelb® stop shows for all pNPP substrate solutions satisfying results in colour stabilization if one accepts the absorbance increase of about 10 % in all the active substrate solutions within the first 30 minutes.

Use of SeramunGelb® stop2

Recently SeramunGelb® stop2 containing a well working inhibitor of alkaline phosphatase was developed.



The signal stability after stopping with SeramunGelb® stop2 is improved for all pNPP substrate solutions tested.

Additional advantages of SeramunGelb® stop2 are the lower content of chemicals, reduction of precipitate formation during storage at 2-8 °C as well as the reduction of costs and environmental pollution in case of disposal.

Results

The substrate solution, SeramunGelb® fast, shows the best results without stopping the reaction. If a stop solution is used, further increase of the absorbance will occur by using conventional stop solutions.

If the design of a test requires stopping, SeramunGelb® stop or SeramunGelb® stop2 are recommended.

5. LONG TERM STABILITY OF SERAMUNGELB® FAST

Stability experiments

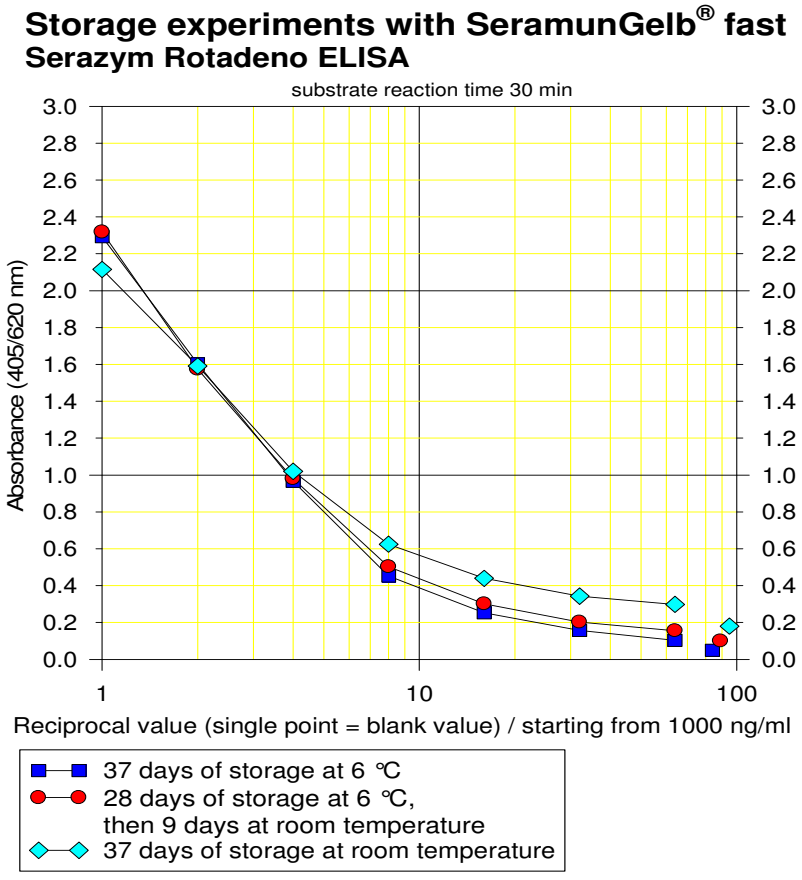
Long term stability is not only of great importance for the manufacturers and distributors of ELISA kits with respect to their expiry. Long term stability is also important for end-users who investigate small numbers of samples, thereby exposing the kit reagents, including the pNPP substrate solution, to air and different temperatures for several times.

The storage of SeramunGelb® fast between 4-25 °C over a period of 37 days neither affects the dynamic of kinetically measured substrate conversion nor the absorbance yield in the ELISA. The consistency of activity and background is proven by the kinetic measurements of stored lots.

To simulate laboratory conditions, the substrate bottles were stored overnight in the refrigerator, warmed up to room temperature (22 °C) during the day and were opened for a time period of five hours every day.

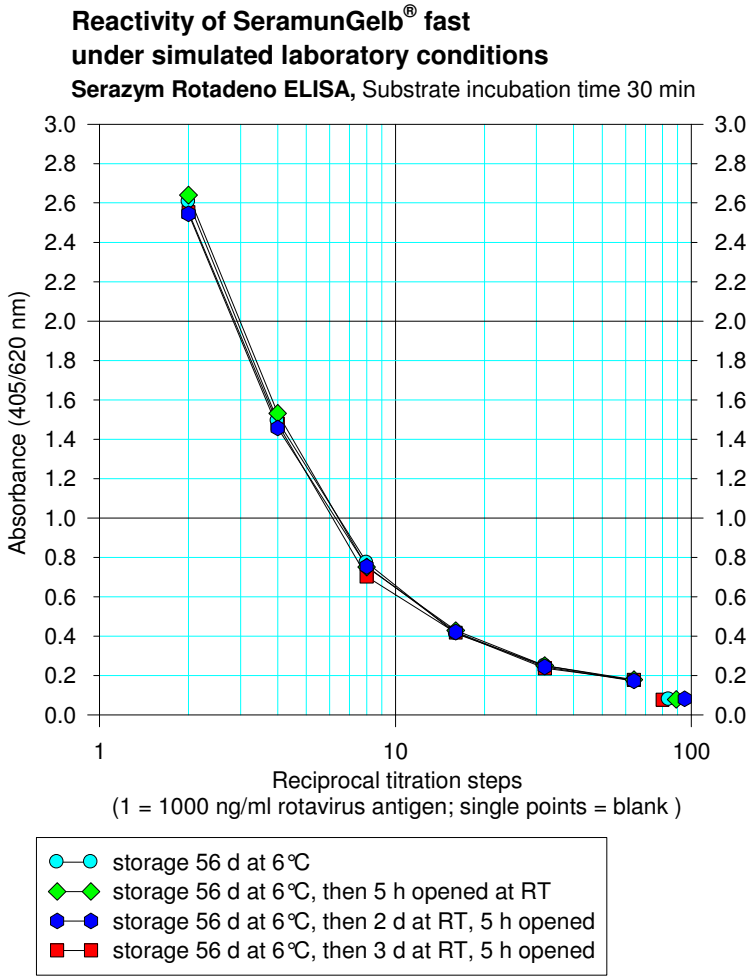
ELISA measurement

Long term stability of SeramunGelb® fast was determined by using a Rotavirus antigen-ELISA (Serazym® Rotadeno).



This experiment shows the different aging of the solution dependent on the storage conditions. It is possible to store the solutions three to five days at room temperature but long term storage requires refrigeration.

The following figure shows the simulation of laboratory conditions after 56 days of storage in the refrigerator. The solutions were warmed up to room temperature, opened and allowed to stay for five hours. The bottles were closed again and refrigerated. This procedure was repeated up to three times.



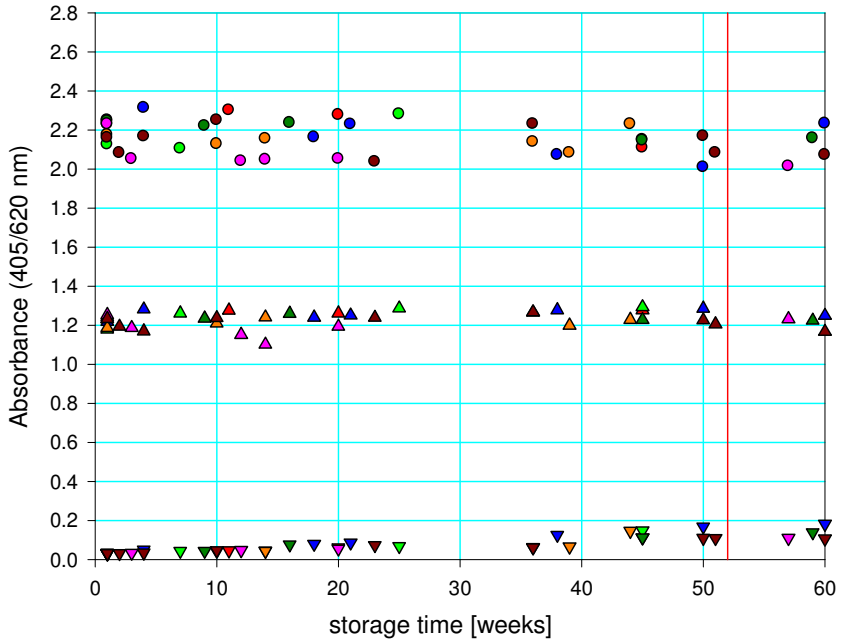
Results

Under normal laboratory handling conditions, neither loss of activity nor blank value increases are observed.

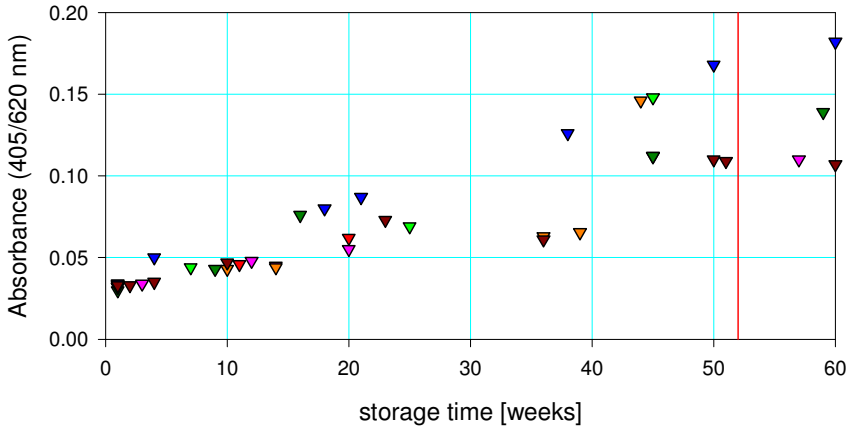
Real time stability

Retention samples of all production lots of SeramunGelb® fast were stored refrigerated until expiry and tested again according to the protocol described in chapter 2.

Background and activity of different lots of SeramunGelb® fast in dependence of the storage time



Detailed view of Background development in dependence of the storage time



Results

There was no significant loss of activity. The variation of absorbances over the investigated period is less than 10 % and reflects the within run coefficient of variation of the assay.

The background of the freshly prepared solution is < 0.04 AU and shows a shift of about 0.01 AU per month and will not exceed 0.20 AU during the shelf-life.

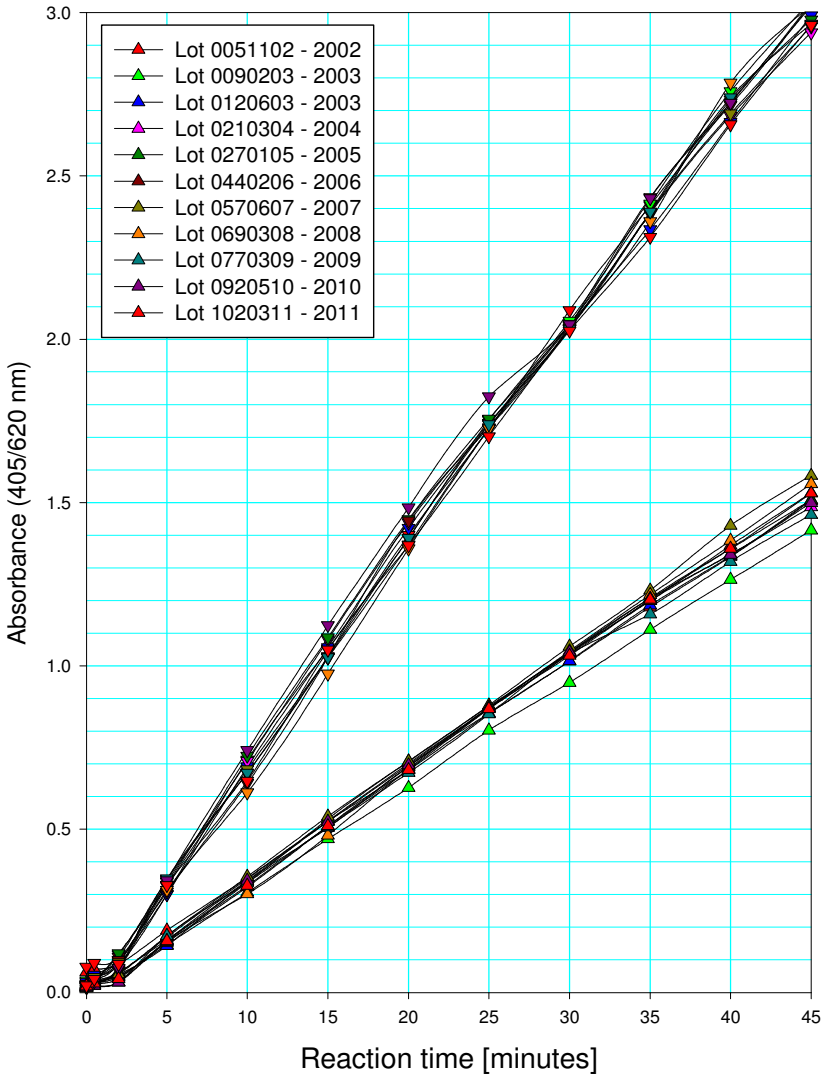
6. LOT-TO-LOT CONSISTENCY OF SERAMUNGELB® FAST

A low lot-to-lot variation of the substrate solution activity is important for ELISA manufacturers and for users of ELISA kits as well. For the latter, this is especially important when follow up studies exceed the shelf life of the ELISA kits of the respective lot, because a high lot-to-lot consistency of the substrate solution is one condition to yield comparable results.

Kinetic substrate comparison

The absorbances of every manufactured lot of SeramunGelb® fast were checked. For every year from 2002 to 2011, one example of the kinetic measurement using two different quantities of alkaline phosphatase (0.10 µg/well and 0.05 µg/well) is shown below.

Comparison of activity from lots SeramunGelb® fast produced from 2002 to 2011, one example per year



Results

The coefficient of variation of kinetically measured absorbances was less than 5 % when the different lots of SeramunGelb® fast during the last 9 years were compared.

7. SUMMARY

SeramunGelb® fast, a substrate for alkaline phosphatase, meets all the requirements of ELISA users:

- ready-to-use
- high absorbance yield
- low background signals
- long term stability at 2-8 °C storage and during usual laboratory practice
- excellent lot-to-lot consistency
- substrate reaction times between 20 and 60 minutes
- high product stability after reaction stop with SeramunGelb® stop2