revvity

MANUAL

ELAST™ ELISA Amplification System

Part Number: NEP116E001EA NEP116001EA

Storage: 2 - 8°C

Version: 1 Date: April 2024

For research only. Not for use in diagnostic procedures

TABLE of CONTENTS

INTRODUCTION	2
PRINCIPLE OF THE PROCEDURE	2
REAGENTS	3
STORAGE RECOMMENDATIONS	3
SAFETY	3
PROCEDURES	4
GENERAL INFORMATION	4
ELISA AMPLIFICATION STEPS	
I. REAGENT PREPARATION	5
II. AMPLIFICATION	5
TROUBLESHOOTING GUIDE	12
REAGENT FORMULATIONS	13
REFERENCES	15

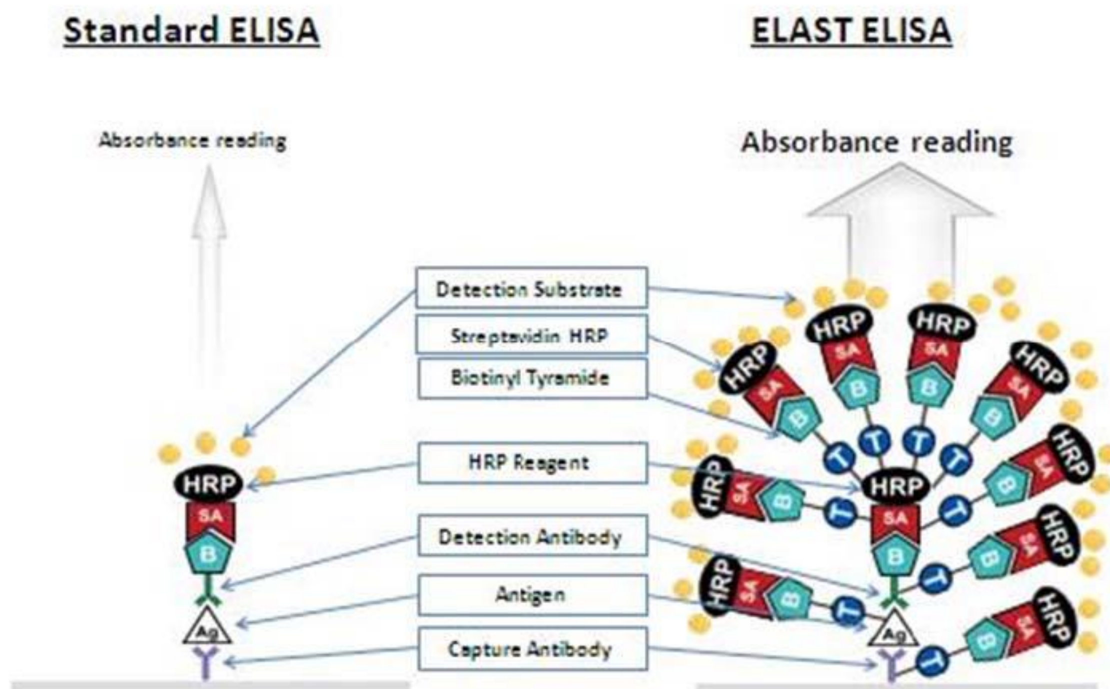
INTRODUCTION

The Revvity ELAST™ ELISA Amplification system is designed to amplify the signal generated by the enzyme horseradish peroxidase (HRP) when applied to solid phase analytical methods such as the ELISA. The final enhanced signal is primarily due to the catalytic activity of additional HRP deposited on the solid phase

PRINCIPLE OF THE PROCEDURE

Revvity ELAST is based on the catalyzed reporter deposition technology described by Bobrow et al. (1,2). When H_2O_2 and biotinyl-tyramide (a biotin-phenolic compound) are added to an assay system containing immobilized HRP, the enzyme catalyzes the activation of the phenolic group resulting in covalent binding to electron rich moieties on the solid phase. Subsequent reaction with streptavidin-HRP results in the binding of additional HRP to the now biotinylated solid phase. Restated, the ELISA reporter enzyme, HRP, catalyzes the covalent binding of biotinyl-tyramide to the solid phase of the assay vessel. Subsequent reaction with Streptavidin-HRP causes additional HRP to be bound to the solid phase resulting in signal amplification. In practice, the ELAST procedure is inserted into the user's protocol after the HRP reporter incubation (and washing) and before the addition of the chromogenic substrate.

Figure 1



Other than the optimization suggestions made in the Procedures section below, under many circumstances no changes to the ELISA assay format currently in use need be made as long as appropriate blocking agents are used for the solid phase. The researcher can use any number of methods for HRP reporter addition. For example, an anti-analyte-HRP conjugate or a secondary reporter such as an anti-rabbit or anti-mouse IgG-HRP conjugate can be used. The procedure will also work with biotinylated antibody/streptavidin-HRP systems or hapten/anti-hapten-HRP formats.

REAGENTS IN THE ELAST KIT (NEP116001EA)

BIOTINYL-TYRAMIDE SOLUTION (FP317) -One (1) bottle containing 2 ml of biotinyl-tyramide in ethanol.

AMPLIFICATION DILUENT CONCENTRATE (2X) (FP485) -One (1) bottle containing 70 ml of a borate buffer with hydrogen peroxide.

STREPTAVIDIN-HRP CONCENTRATE (FP105) -One (1) vial containing 0.22 ml of streptavidin-horseradish peroxidase in citrate buffer with added protein and preservative.

Note: The ELAST evaluation size kit (NEP116E001EA) contains the same reagents in smaller amounts to process 1 96-well microtiter plate.

STORAGE RECOMMENDATIONS

All kit components should be kept refrigerated at 2°- 8°C.

SAFETY

OSHA WARNING

ETHYL ALCOHOL

CAUTION

MAY CAUSE RESPIRATORY TRACT, SKIN, AND EYE IRRITATION.
CENTRAL NERVOUS SYSTEM DEPRESSANT.
HIGHLY FLAMMABLE

HAZARD INFORMATION: WARNING: This product contains a chemical known to the state of California to cause cancer.

For Best Results

Read the entire manual before attempting to use the kit.

Blocking: Proper blocking, of the solid phase is critical to the efficiency of amplification. Use only the recommended blocking reagent. The use of bovine serum albumin (BSA) as the protein blocker is preferred. A suggested formulation is listed in the Reagent Formulation section.

Prepare all working dilutions within 30 minutes of use. Prepare only enough for the assay being run. Discard any excess in appropriate containers.

Use only the reagent lots assigned to the kit. Do not interchange vials or bottle caps.

Do not use the kit beyond the expiration date on the kit label.

This kit has been formulated specifically for use in ELISA formats. Use for other applications may not produce the desired amplified signal.

PROCEDURES

GENERAL INFORMATION

Before proceeding to use the ELAST kit, it is important to optimize the unamplified assay to achieve the lowest background possible (preferably 0.01 OD units, or lower). This can be achieved by decreasing detector reagent concentrations or incubation times.

While the most important parameter affecting the extent of amplification with the ELAST kit is the streptavidin-HRP dilution, it is essential to understand the direct relationship of the streptavidin-HRP dilution to the biotinyl-tyramide concentration. For full assay optimization, both steps should be cross matched. To help users, a section of Examples demonstrating how the various components and/ or conditions affect the resultant assay sensitivity is included in this manual.

Modulation of the extent of amplification can also be achieved by changing the concentration of the HRP reporter or other members of the reporter system in the unamplified assay (e.g., unlabeled anti-analyte antibody). This may be desirable in order to save critical assay reagents.

The protocol below is written for use with 96 well microplates. If other vessels are utilized, adjust volumes as necessary .

ELISA AMPLIFICATION STEPS

I REAGENT PREPARATION

The ELISA is performed as usual through the HRP reporter incubation step and subsequent washing. DO NOT ADD SUBSTRATE. A suitable wash buffer and antibody diluent are described in the "Reagent Formulations" section.

A. Prepare the biotinyl-tyramide (B-T) Working Solution:

Dilute an appropriate amount of the Amplification Diluent Concentrate 1: 1 with deionized H20. (As100 µl per well will be needed, 10 m1 of final dilution will be adequate for an entire 96 well plate).

Add 10 µl of biotinyl-tyramide solution per ml of the diluted Amplification Diluent from step Al. (For one 96 well plate use 100 µl of B-T to 10 ml of diluent.)

Initially, amplify the assay by using this fixed dilution of the biotinyl-tyramide solution. This concentration will be adequate for most applications and is recommended as a starting point. More or less may be used to modulate the extent of amplification.

II AMPLIFICATION

A. Add 100 µl of the B-T Working Solution to each well of the microplate and incubate for 15 minutes at room temperature.

B. Wash the wells at least 4x with PBS-T[1]

C. Dilute the streptavidin-HRP 1:500 (2 µl /ml) in1% BSA-PBS-T[1] (For one 96 well plate, use 20 µl to 10 ml of diluent)

This 1:500 dilution is recommended as a starting point. The extent of amplification can be modulated by varying the Streptavidin-HRP concentration (see example 1)

D. Add 100 µl to each well of the microplate and incubate for 15 to 30 minutes at room temperature.

E. Wash the wells at least 4x with PBS-T.

F. Add the customary ELISA substrate and complete the assay "as usual".

[1]Described in the "Reagent Formulations" section

Examples

Example 1. Amplification of an HIV -1 p24 ELISA

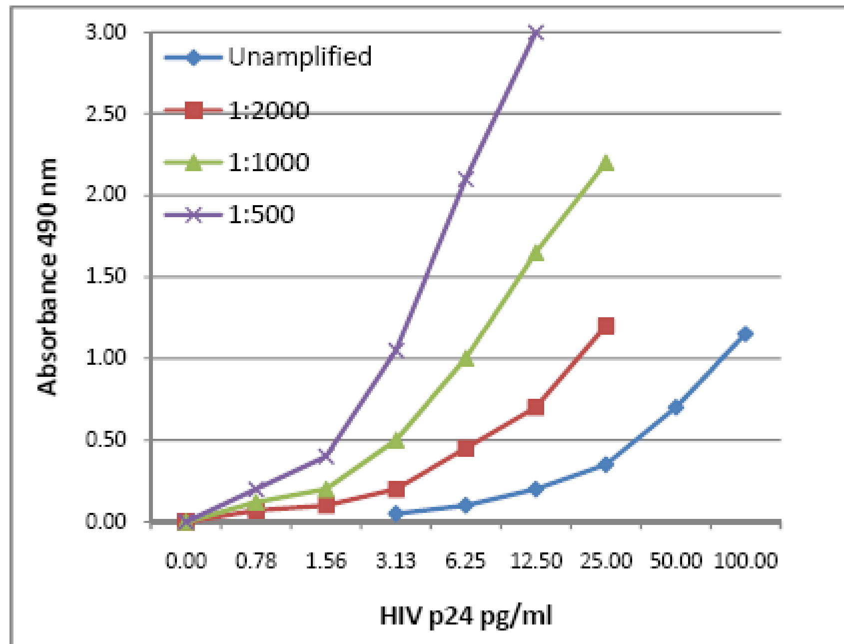
Determining optimal Streptavidin-HRP dilution

The Revvity HIV-1 p24 Core Profile ELISA (NEKO60) was run as described in the kit manual. (Note: the NEK060 kit has been replaced with NEK050001KT Alliance HIV-1 p24 Antigen ELISA Kit). This is a typical sandwich ELISA where the analyte (HIV-l p24) is captured by an immobilized monoclonal antibody and detected with a biotinylated polyclonal/ streptavidin- HRP reporter system.

The standard curve range (normally 0 to 100 pg/ml) was changed to 0 to 25 pg/ml for the amplified assay. Briefly, antigen was captured for 2 h at 37°C; biotinylated detector antibody was incubated for 1 h at 37°C; streptavidin-HRP (diluted as indicated in the kit manual) was incubated for 15 min at37°C. All washes were performed as recommended in the kit. Following the HIV-l p24 ELISA kit streptavidin-HRP incubation and washing, optimization using the ELAST amplification reagents was run as follows:

> Step 1. The Amplification Diluent Concentrate was diluted 1:1with deionized H20, and biotinyl-tyramide was added to10 µl /m1. 100 µl was added to all wells for amplification and incubated for 15 min. at room temperature. The wells were washed as recommended in the HIV-l p24ELISA manual (two 5-cycle washes of at least 300 µl per well per wash using automated microplate washer).
>
> Step 2. The ELAST streptavidin-HRP concentrate was diluted 1/500,1/1000, and 1/2000 in 1% BSA-PBS-T (see the Reagent Formulation section). One dilution was used for each standard curve.100 µl was added to the wells and incubated for 15 min. at room temperature. The wells were washed as recommended in theHIV-1 p24 ELISA manual.
>
> OPD substrate was added and incubated for 30 min. at room temperature, then stopped with the kit stop solution. The absorbance at 490 nm was determined. Results are shown in Fig. 2.

Figure 2



Conclusions: The 1/500 dilution yielded the highest absolute signal (over20 times the unamplified assay) as well as net signal (background subtracted)On the other hand, the *1/1000* dilution yields a greater than 10fold signal increase with the highest signal noise ratio due primarily to the lower background. Therefore, the best improvement in assay sensitivity is achieved with the 1/1000 dilution.

NOTE:

Commercial kits are formulated and optimized to give a fixed signal for each level of analyte concentration in an unamplified assay. Use of a different lot of the same commercial kit or of the ELAST amplification kit may require some reoptimization.

Example 2. Amplification of an Interleukin-2 ELISA

Varying Biotinyl-Tyramide dilution

An in house ELISA for Interleukin-2 (IL-2) ELISA was used to generate data for Figure 3. Streptavidin-HRP was used at a 1/100 dilution This IL-2 assay is a sandwich ELISA where the analyte is captured by an immobilized polyclonal antibody and detected with a biotinylated polyclonal/streptavidin-HRP reporter system. The IL-2 ELISA was optimized to give maximum linear range at the expense of assay sensitivity for illustrative purposes.

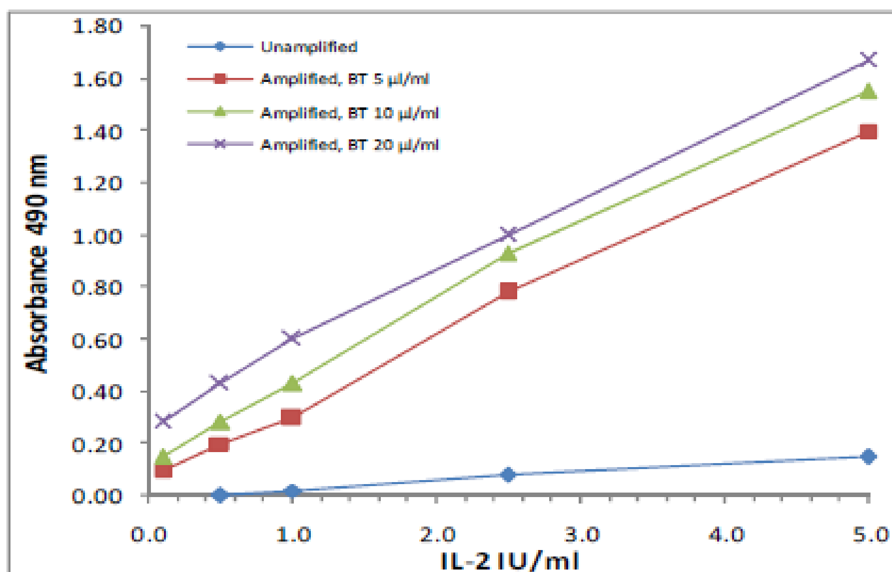
The standard curve range (normally 0 to 100 IU/ml) was changed to 0 to 5 IU/ml for the amplified assay. Briefly, antigen was captured overnight at 4°C; biotinylated detector was incubated for 2 h at 37°C; streptavidin-HRP diluted 1/100 was incubated for 2 h at 37°C. All washes were performed manually. Following the IL-2 ELISA kit streptavidin-HRP incubation and washing, optimization using the ELAST amplification reagents was run as follows.

Step 1. The Amplification Diluent Concentrate was diluted 1: 1with deionized H20, and biotinyl-tyramide was added to5 µl/m1, 10 µl /m1, and 20 µl /m1. 100 µl of the diluted material was added to all wells for amplification followed by incubation for 15 minutes at room temperature. The wells were washed four times.

Step 2. The ELAST Streptavidin-HRP concentrate was diluted1/1000 in 1% BSA-PBS-T (see the Reagent Formulations section). 100 µl was added to the wells and incubated for15 min. at room temperature. The wells were washed four times.

OPD substrate was added and incubated for 30 min. at room temperature and stopped with kit stop solution. The absorbance at 490 nm was determined. Results are shown in Fig. 3.

Figure 3



Conclusions: From Fig. 3, the optimal BT concentration would be 5 µl /ml. Using higher concentrations does not improve assay sensitivity based on net signal and signal/noise ratio. The amplified assay reflects the type of optimization used in this assay (i.e., range vs. sensitivity) by also giving a relatively shallow response (higher blanks and lower signals) which is shifted approximately 10-fold lower in concentration from the unamplified assay.

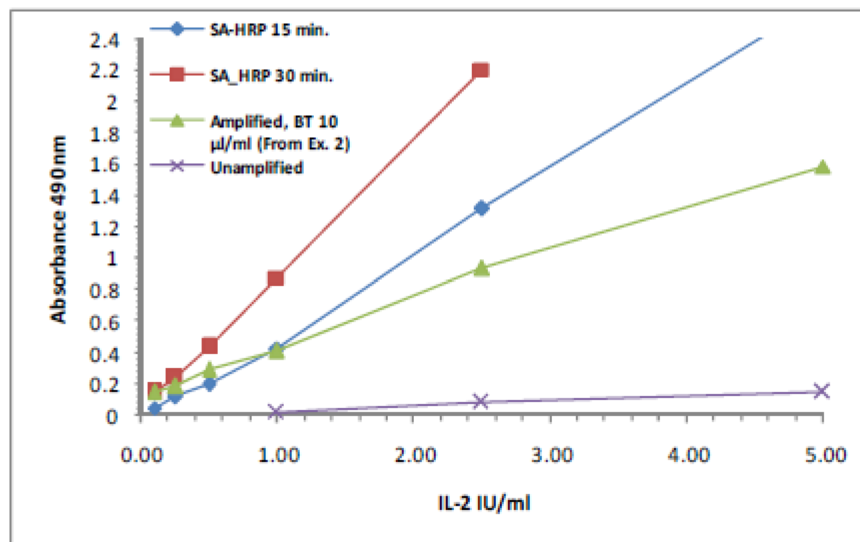
Example 3. Improvements to the Amplified IL-2 ELISA

Modifying ELISA Conditions for Optimal Amplification

The IL-2 ELISA described in Example 2 yielded a very shallow response due to the type of optimization of the original assay. It was found that changing three parameters (concentration, time, and diluent composition) of the IL-2 SA-HRP incubation step improved the amplified assay. The IL-2-SA-HRP and diluent were replaced with a more concentrated SA-HRP diluted in 1% BSA-PBS-T. The SA-HRP incubation was for 15 min. at 37°C (as compared to 2 hr. at 37°C in the IL-2 kit). All other IL-2 assay steps remained the same. The assay was amplified using biotinyl-tyramide at 10 µl/ml and (ELAST) SA-HRP at a 1/500 dilution for 15 and 30 min. at room temperature.

The results are shown in Fig. 4. Included, for comparison, is the amplified assay from Example 2 where biotinyl-tyramide was used at 10 µl/ml.

Figure 4



Conclusion: The increased slope of the response curve clearly shows improved sensitivity of the modified assay. If higher absorbances are desired, the ELAST SA-HRP can be incubated for 30 min. instead of 15 min.

Example 4. Amplification of a *Salmonella* ELISA

A sandwich ELISA for the detection of *Salmonella* antigen was developed utilizing a goat anti-*Salmonella* capture antibody and a peroxidase labeled goat anti-*Salmonella* detector antibody. Affinity purified unlabeled and peroxidase labeled anti-*Salmonella* antibodies, and *Salmonella typhimurium* positive control were purchased from Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.

Optimization of the unamplified assay to maximize signal-to-noise ratio resulted in the following assay parameters:

A. Microplates were coated with l00 µl unlabeled capture antibody at 2.5 mg/ml in 0.1M carbonate buffer overnight at 4°C, blocked with 200 µl 2% BSA-PBS, and stored at 4°C until used.

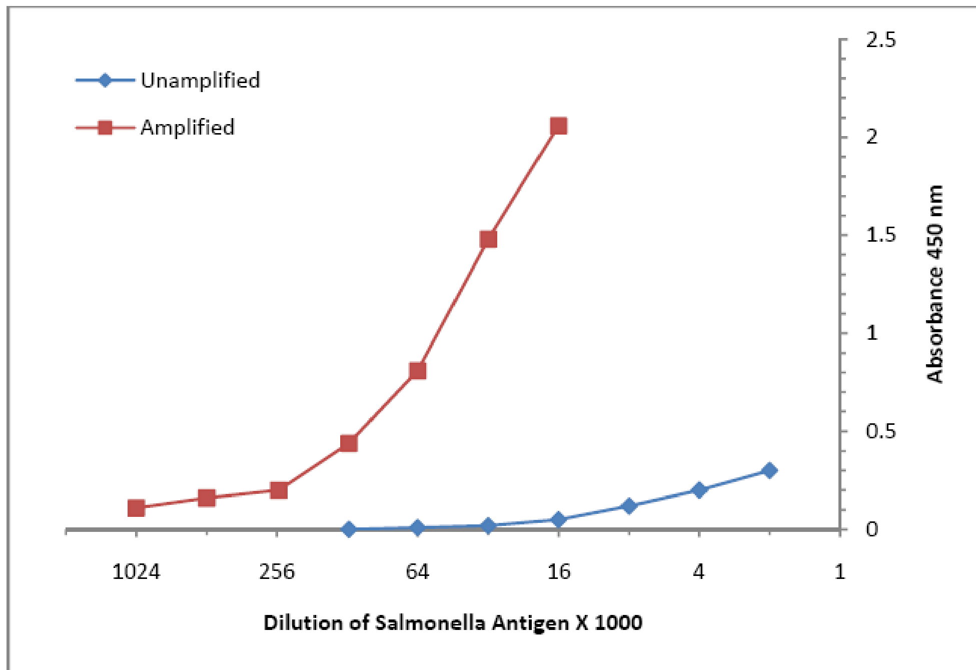
B. *Salmonella* positive control was diluted in 1% BSA-PBS-T and 100 µl was added to the wells for 2 hr. at room temperature.

C. Peroxidase labeled goat anti-*Salmonella* antibody was diluted 1/500 in PBS-T containing 40% goat serum and 100 µl was added to the wells for 1hr. at room temperature.

D. A tetramethylbenzidine (TMB) chromogen was incubated for 7 minutes at room temperature, stopped with 3N H_2S0_4/ 1N HCl, and the absorbance at 450nm determined.

For amplification, the ELAST reagent incubations were inserted between steps 3 and 4. Biotinyl-tyramide was diluted to 10 µl/mL and incubated for 15 min. at room temperature. Streptavidin-HRP was diluted 1/500 in 1% BSA-PBS-T and incubated for 30 min. at room temperature. The results are shown in Fig.5.

Figure 5



Troubleshooting Guide

While we feel that a careful reading of Examples 1-4 will give the ELAST user a good grounding in the optimization of assays to achieve high sensitivity , in Table 2 we have summarized the potential problems and listed possible solutions

Observation	Things to try
High Background	*Determine the optimal ELAST streptavidin-HRP/ biotinyl-tyramide concentrations to give lower backgrounds. *Rework the original assay to decrease non-specific binding by identifying specific reagents at fault. Revise diluent, concentration and/ or incubation conditions. *Insufficient washing
Inadequate Amplification	*Was the appropriate blocking reagent used? VERY CRITICAL! *Check the unamplified assay. Did it work right? * Review the relationship between concentrations of BT and SA-HRP (Examples 1 and 2) *Were the critical reagents added (BT; SA-HRP)?
Shallow Response Curve	*Increase concentration of SA-HRP (Examples 1 and 3) * Increase time of SA-HRP incubation (Example 3) *Rework the original assay detection system
Variable Results	*Check the precision of the original assay. As the amplification system requires extra steps * Increased signal in the area of the inherent precision in the original assay (ultra- low levels) ,some increase in CVs are to be expected but this should not be excessive (See Reference 1 in Manual for typical expectations) . *Check the incubation time for the BT and the streptavidin- HRP steps. We have found that a 30 minute incubation of streptavidin-HRP often gives better precision than a 15 minute incubation *May be due to pipetting errors

Reagent Formulations

Phosphate Buffered Saline Reagent Formulations

10X PBS serves as a "base" for many of the buffer formulations used. Two formulations have been used successfully.

Formulation A:
For 1 liter:
2.03 g NaH_2PO_4 H_20
11.49 g Na_2HPO_4
85 g NaCl
The pH of the10X solution is 6.7 to 6.9. The pH of the 1X solution should be 7.3 to 7.5 (if not, adjust the 1X). Storage: Room Temperature

Formulation B:
For 1 liter:
21.6 g Na_2HPO_4 $7H_20$
2 g KH_2PO_4
80 g NaCl
2 g KCl
The pH of the 10X solution is 6.7 to 6.9. The pH of the 1X solution should be 7.0 to 7.2 (if not, adjust the 1X). Storage: Room Temperature

Alternatively, Dulbecco's Phosphate Buffered Saline without calcium chloride or magnesium chloride (available from commercial sources) may be used.

PBS-Tween 20 10X (10X PBS-T)

For 1 liter:
995 ml 10X PBS
Tween 20 5 ml
A preservative (1 g/liter) may be added to prolong the life of the reagent.
Do not use sodium azide.
Storage: Room Temperature

Streptavidin-Enzyme/General Assay Wash Buffer (PBS-T)

For 1 liter:
10 ml 10X PBS-T
900 ml H_2O
Storage: Room Temperature

ELISA Blocking Reagent (2% BSA-PBS)

For 1 liter:
100 ml 10X PBS
800 ml H_2O
20 g BSA*
Adjust the pH to 7.4, add H20 to 1 liter, and filter through a 0.22 µm filter unit.
Storage: 4°C

ELISA Streptavidin-HRP Diluent. 1% BSA-PBS-T

For 1 liter:
100 ml 10X PBS-T
800 ml H_2O
10 g BSA*
Adjust the pH to 7.4, add H^20 to 1 liter, and filter through a 0.22 µm filter unit.
Storage: 4°C

* Bovine Serum Albumin -Sigma A7888 or equivalent.

References

1. Bobrow, M.N., Harris, T.D., Shaughnessy, K.J. and Litt, G.J. (1989) Catalyzed reporter deposition, a novel method of signal. amplification. Application to immunoassays. *J. Immunol. Methods* 125,279-285.

2. Bobrow, M.N., Shaughnessy, K.J. and Litt, G.J. (1991). Catalyzed reporter deposition, a novel method of signal amplification. II. Application to membrane immunoassays. *J. Immunol Methods* 137,103-112.

3. Helle, M., Boeije, L., de Groot, E., de Vos, A. and Aarden, L. (1991) Sensitive ELISA for interleukin 6. Detection of IL-6 in biological fluids: synovial fluids and sera. *J. lmmunol Methods* 138,47-56.

This product is covered by US patents 5,196,306, 5,583,001 and 5,731,158 owned by Revvity, Inc. It includes a license for research use only.

The information provided in this document is for reference purposes only and may not be all-inclusive. Revvity, Inc., its subsidiaries, and/or affiliates (collectively, "Revvity") do not assume liability for the accuracy or completeness of the information contained herein. Users should exercise caution when handling materials as they may present unknown hazards. Revvity shall not be liable for any damages or losses resulting from handling or contact with the product, as Revvity cannot control actual methods, volumes, or conditions of use. Users are responsible for ensuring the product's suitability for their specific application. REVVITY EXPRESSLY DISCLAIMS ALL WARRANTIES, INCLUDING WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, REGARDLESS OF WHETHER ORAL OR WRITTEN, EXPRESS OR IMPLIED, ALLEGEDLY ARISING FROM ANY USAGE OF ANY TRADE OR ANY COURSE OF DEALING, IN CONNECTION WITH THE USE OF INFORMATION CONTAINED HEREIN OR THE PRODUCT ITSELF

www.revvity.com

revvity

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com

Copyright ©2023, Revvity, Inc. All rights reserved.