

PRODUCT
INFORMATION



Substance P
Enzyme Immunoassay Kit

Catalog No. IB09650

96 Well Kit

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Description

The IBL-America Substance P kit is a competitive immunoassay for the determination of Substance P in biological fluids. Please read the complete kit insert before performing this assay. The kit for the measurement of Substance P uses a polyclonal antibody to Substance P to bind, in a competitive manner, the Substance P in the standard or sample or an alkaline phosphatase molecule which has Substance P covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of Substance P in either standards or samples. The measured optical density is used to calculate the concentration of Substance P. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Substance P is a undecapeptide that displays a number of biological activities. The peptide was first discovered in 1931 by von Euler and Gaddum³. They reported that extracts of equine brain and intestine contained a hypotensive and spasmogenic factor. The preparation, termed preparation P, was later found to be proteinaceous⁴. The isolation and characterization of Substance P was carried out by Leeman's group in 1970^{5,6}. The structure is shown below.

Substance P H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

Substance P is synthesized in the ribosomes as a larger protein and then enzymatically converted into the active peptide. The peptide is widely distributed in the peripheral and central nervous systems of vertebrates, where it is thought to act as a neurotransmitter⁷⁻⁹. In the peripheral system, Substance P is localized in the primary sensory neurons and neurons intrinsic to the gastrointestinal tract. Excellent reviews on the distribution and action of Substance P and other tachykinins are available¹⁰⁻¹³.

Precautions

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1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The Substance P Standard provided, Catalog No. 80-0206, is supplied in ethanolic buffer at a pH optimized to maintain Substance P integrity. Care should be taken in handling this material because of the known and unknown effects of Substance P.

Materials Supplied

- 1. Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 wells**
A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
- 2. Substance P EIA Conjugate, 6 mL**
A blue solution of alkaline phosphatase conjugated with Substance P.
- 3. Substance P EIA Antibody, 6 mL**
A yellow solution of a rabbit polyclonal antibody to Substance P.
- 4. Assay Buffer, 30 mL**
Tris buffered saline containing proteins and sodium azide as preservative.
- 5. Wash Buffer Concentrate, 30 mL**
Tris buffered saline containing detergents.
- 6. Substance P Standard, 0.5 mL**
A solution of 100,000 pg/mL Substance P.
- 7. p-Npp Substrate, 20 mL**
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
- 8. Stop Solution, 5 mL**
A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic**.
- 9. Substance P Assay Layout Sheet, 1 each**
- 10. Plate Sealer, 1 each**

Storage

All components of this kit, **except the Conjugate and Standard**, are stable at 4 °C until the kit's expiration date. The Conjugate and Standard **must** be stored frozen at -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

The IBL-America Substance P kit is compatible with samples in a wide range of matrices. Sample diluted sufficiently in Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing rabbit IgG may interfere with the assay.**

Plasma samples should be drawn into chilled EDTA tubes (1mg/mL blood) containing Aprotinin (500 KIU/mL or 10.6 TIU/mL of blood). Centrifuge the blood at 1,600 x g for 15 minutes at 0 °C. Transfer the plasma to a plastic tube and store at -70 °C or lower for long term storage.

Samples in the majority of Tissue Culture Media can also be read in the assay, provided the standards have been diluted into the Tissue Culture Media instead of Assay Buffer. There will be a small change in the binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of Substance P in the appropriate matrix. Because of the labile nature of Substance P we recommend the addition of protease inhibitors during collection and storage of samples. We recommend storage of all samples at -70 °C or lower, and the addition of protease inhibitors prior to freezing. Some samples normally have very low levels of Substance P present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Extraction of the sample should be carried out using a similar protocol to the one described below.

1. Add an equal volume of 1% trifluoroacetic acid (TFA) in water to the sample. Centrifuge at 17,000 x g for 15 minutes at 4 °C to clarify and save the supernatant.
2. Equilibrate a 200 mg C₁₈ Sep-Pak column with 1 mL of acetonitrile, followed by 10-25 mL of 1% TFA in water.
3. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 1% TFA in water. Discard wash.
4. Elute the sample slowly by applying 3 mL of acetonitrile: 1% TFA in water 60:40. Collect the eluant in a plastic tube.
5. Evaporate to dryness using a centrifugal concentrator under vacuum. Store at -20 °C.
6. Reconstitute with Assay Buffer and measure immediately.

Please note that recovery of peptides from extraction processes can be variable. It is important to optimize any process to obtain optimum recoveries. Extraction efficiencies can be determined by spiking a known amount of Substance P into paired samples and determining the recovery of this known amount of added Substance P.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Substance P Standard

Allow the 100,000 pg/mL Substance P standard solution to warm to room temperature. Label six 12 x 75 mm glass tubes # 1 through # 6. Pipet 1 mL of standard diluent (Assay Buffer or Tissue Culture Media) into tube # 1. Pipet 750 µL of diluent into tubes # 2 through # 6. Remove 100 µL of diluent from tube # 1. Add 100 µL of the 100,000 pg/mL standard to tube # 1. Vortex thoroughly. Add 250 µL of tube # 1 to tube # 2 and vortex thoroughly. Add 250 µL of tube # 2 to tube # 3 and vortex. Continue this for tubes # 4 through # 6.

The concentration of Substance P in tubes # 1 through # 6 will be 10,000, 2,500, 625, 156.25, 39.06 and 9.76 pg/mL respectively. See Substance P Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

2. Substance P Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C.

3. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 50 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the B₀ (0 pg/mL Standard) wells.
3. Pipet 50 µL of Standards # 1 through # 6 into the appropriate wells.
4. Pipet 50 µL of the Samples into the appropriate wells
5. Pipet 50 µL of Assay Buffer into the NSB wells.
6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~ 500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes**.
10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 5 µL of the blue Conjugate to the TA wells.
12. Add 200 µL of the p-Npp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.
13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of Substance P in the samples. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program. If this type of data reduction software is not readily available, the concentration of Substance P can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using Logit-Log paper plot Percent Bound (B/Bo) versus Concentration of Substance P for the standards. Approximate a straight line through the points. The concentration of Substance P in the unknowns can be determined by interpolation.

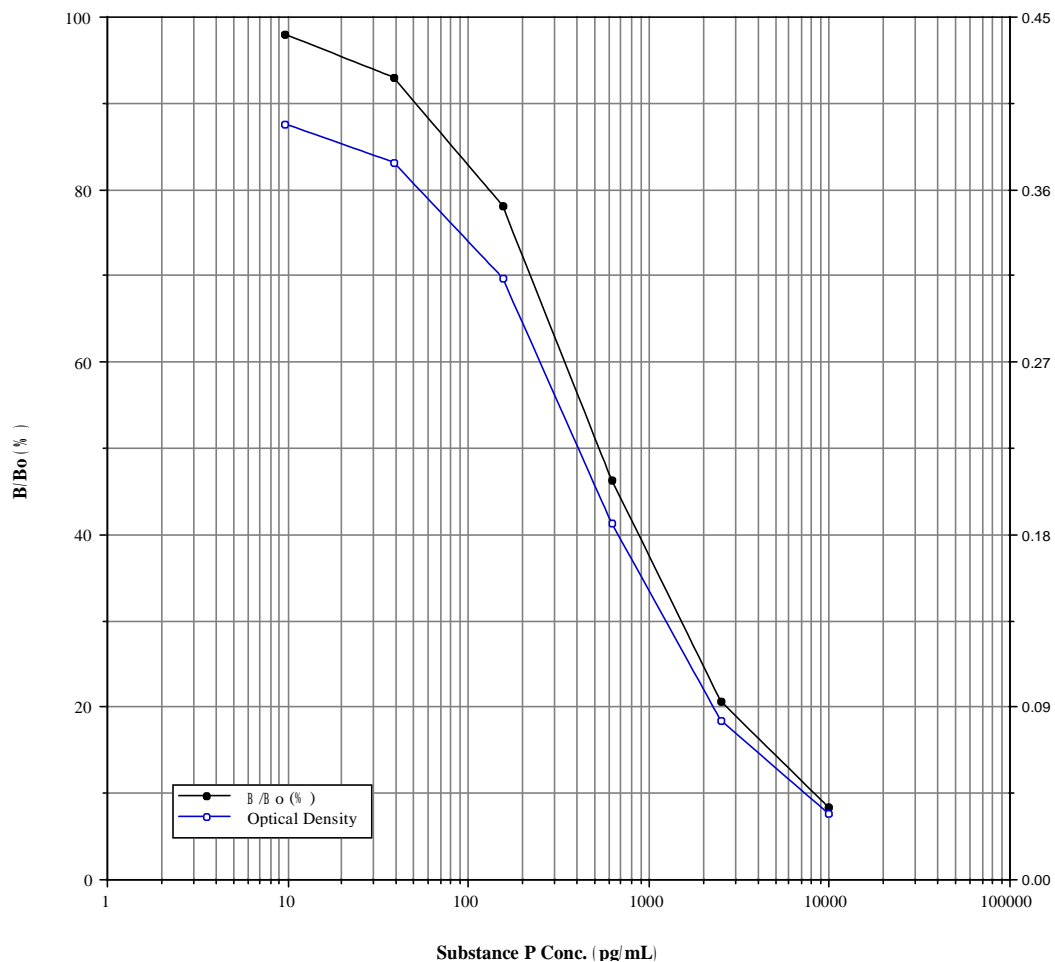
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

| <u>Sample</u> | <u>Mean OD (- Blank)</u> | <u>Average Net OD</u> | <u>Percent Bound</u> | <u>Substance P (pg/mL)</u> |
|---------------|--------------------------|-----------------------|----------------------|----------------------------|
| Blank OD | (0.091) | | | |
| TA | 0.103 | | | |
| NSB | 0.001 | 0.000 | | |
| Bo | 0.403 | 0.402 | 100% | 0 |
| S1 | 0.035 | 0.034 | 8.4% | 10,000 |
| S2 | 0.084 | 0.083 | 20.6% | 2,500 |
| S3 | 0.187 | 0.186 | 46.3% | 625 |
| S4 | 0.315 | 0.314 | 78.1% | 156.25 |
| S5 | 0.375 | 0.374 | 93.0% | 39.06 |
| S6 | 0.395 | 0.394 | 98.0% | 9.76 |
| Unknown 1 | 0.097 | 0.096 | 23.9% | 1,874 |
| Unknown 2 | 0.202 | 0.201 | 50.0% | 543 |

Typical Standard Curves

Typical standard curves are shown below. These curves **must not** be used to calculate Substance P concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added = 0.103 x 10 = 1.03
 % NSB = 0.0%
 % Bo/TA = 39.0%
 Quality of Fit = 1.00

20% Intercept = 2,486 pg/mL
 50% Intercept = 547 pg/mL
 80% Intercept = 134 pg/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹⁴.

Sensitivity

Sensitivity was calculated in Assay Buffer by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #6. The detection limit was determined as the concentration of Substance P measured

Average Optical Density for the Bo = 0.446 ± 0.007 (1.53%)
Average Optical Density for Standard # 6 : 0.429 ± 0.012 (2.8%)

Delta Optical Density (0-9.76 pg/mL) = 0.446 - 0.429 = 0.017

2 SD's of the Zero Standard = 2 x 0.007 = 0.014

Sensitivity = $\frac{0.014}{0.017} \times 9.76 \text{ pg/mL} = \mathbf{8.04 \text{ pg/mL}}$

Linearity

A sample containing 1,668 pg/mL Substance P was serially diluted 7 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Substance P concentration versus measured Substance P concentration.

The line obtained had a slope of 1.006 with a correlation coefficient of 0.998.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Substance P and running these samples multiple times (n= 24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Substance P in multiple assays (n= 8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of Substance P determined in these assays as calculated by a 4 parameter logistic curve fitting program.

| | <u>Substance P</u> <u>(pg/mL)</u> | <u>Intra-assay</u> <u>% CV</u> | <u>Inter-assay</u> <u>% CV</u> |
|--------|--------------------------------------|-----------------------------------|-----------------------------------|
| Low | 101 | 6.7 | |
| Medium | 1,116 | 4.5 | |
| High | 6,257 | 5.2 | |
| Low | 97 | | 4.2 |
| Medium | 1,120 | | 7.3 |
| High | 6,402 | | 7.3 |

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 1,000,000 to 10 pg/mL. These samples were then measured in the Substance P assay, and the measured Substance P concentration at 50 B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

| <u>Compound</u> | <u>Cross Reactivity</u> |
|----------------------|-------------------------|
| Substance P | 100% |
| Substance P (3-11) | 85.9% |
| Physalaemin | 75.3% |
| Substance P (4-11) | 11.7% |
| Substance P (7-11) | 5.9% |
| α -Neurokinin | 0.8% |
| β -Neurokinin | 0.2% |
| Somatostatin | < 0.001% |
| Substance P (1-4) | < 0.001% |

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Substance P concentrations were measured in a variety of different samples including tissue culture media, porcine serum and plasma, and human saliva and urine. Substance P was spiked into the undiluted samples which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

| <u>Sample</u> | <u>% Recovery</u> | <u>Recommended Dilution*</u> |
|----------------------------|-------------------|------------------------------|
| Tissue Culture Media | 81.0 | 1:2 |
| Porcine Serum | 97.7 | 1:2 |
| Porcine heparinized Plasma | 109.3 | 1:2 |
| Human Saliva | 108.3 | 1:20 |
| Human Urine | 105.8 | 1:8 |

* See Sample Handling instructions on page 4 for details.

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LIMITED WARRANTY

IBL-America warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

IBL-America must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if IBL-America is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

For more details concerning the information within this kit insert, or to order any IBL-America products, please call (763) 780-2955 between 8:00 a.m. and 5:00 p.m. CST.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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