Substance P ELISA Kit (Express)

Item No. 502892

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	Storage Temperature
401192	Substance P Express ELISA Antiserum	1 vial/100 dtn	-20°C
401191	Substance P Express HRP Tracer	1 vial/100 dtn	-20°C
401193	Substance P Express ELISA Standard	1 vial	-20°C
400108	Immunoassay Buffer D Concentrate (5X)	2 vials/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate	4°C
400012	96-Well Cover Sheet	1 ea	RT
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Substance P ELISA Kit (Express). This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

The stop solution provided with this kit is an acid solution. Please wear appropriate personal protective equipment (*e.g.*, safety glasses, gloves, and lab coat) when using this material.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000)*
5. Materials used for **Sample Preparation** (see page 13)

INTRODUCTION

Background

Substance P is a tachykinin neuropeptide and the primary ligand for neurokinin-1 (NK_1) receptors with roles in various physiological processes, including pain modulation, smooth muscle contraction, blood pressure regulation, water homeostasis, and renal function.[1-4] It is expressed in the central and peripheral nervous systems, including the enteric nervous system, and immune cells.[4] Substance P is released in the dorsal horn of the spinal cord in response to noxious stimuli at a magnitude proportional to the frequency and intensity of the stimulation.[5] Following noxious peripheral stimulation, it induces pain through NK_1 receptors located in the spinal cord. Substance P is also involved in depression, anxiety, seizures, and emesis among other functions.[4]

About This Assay

Cayman's Substance P ELISA Kit (Express) is a competitive assay that can be used for the quantification of Substance P in plasma, serum, and other sample matrices. The assay has a range of 15.6-2,000 pg/ml, an average sensitivity (80% B/B_0) of 33 pg/ml, and a lower limit of detection (LLOD) of 5.7 pg/ml.

Principle of this Assay

This assay is based on the competition between free Substance P and a Substance P HRP conjugate (Substance P Express HRP Tracer) for a limited number of Substance P polyclonal antiserum binding sites. Because the concentration of the Substance P Express HRP Tracer is held constant while the concentration of free Substance P varies, the amount of Substance P Express HRP Tracer that is able to bind to the Substance P Express ELISA Antiserum will be inversely proportional to the concentration of free Substance P in the well. This antibody-Substance P complex binds to the mouse anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and TMB Substrate Solution (which contains the substrate to HRP) is added to the well, followed by the HRP Stop Solution. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Substance P Express HRP Tracer bound to the well, which is inversely proportional to the amount of free Substance P present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound Substance P HRP tracer}] \propto 1/[\text{Substance P}]$$

A schematic of this process is shown in Figure 1, on page 9.

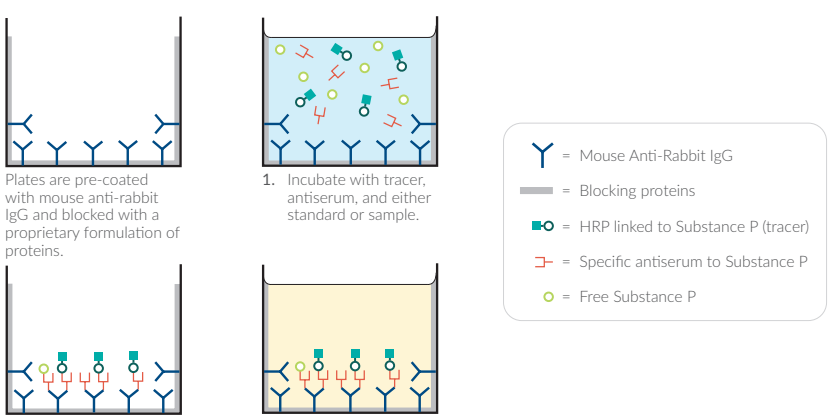


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and HRP Stop Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

TA (Total Activity): total enzymatic activity of the HRP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a sample or standard well to the average absorbance of the maximum binding (B_0) wells.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn (Determination): one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

LLOD (Lower Limit of Detection): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Store all diluted buffers at 4°C; they will be stable for at least two months. NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.

Buffer Preparation

1. **Assay Buffer Preparation**

   Dilute the contents of one vial of Immunoassay Buffer D Concentrate (5X) (Item No. 400108) with 40 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

Testing for Interference

This assay has been validated in plasma and serum. Other sample types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements.

To test for interference, test one or two samples at a range of dilutions, then select at least two different dilutions of each sample within the linear portion of the standard curve. If two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated Substance P concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. The **SPE (C-18) Purification Protocol** on page 14 and **Precipitation Protocol** on page 15 are two recommended methods of sample purification. Alternative protocols may be used based on the experimental requirements, sample type, and the user's expertise.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.
- Samples of rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit IgG plate. We recommend that all rabbit samples be purified prior to use in this assay.

Plasma and serum were processed using the following protocols.

Plasma

Collect blood in vacutainers containing heparin or EDTA. To obtain plasma, centrifuge blood at 1,000 x g for 15 minutes. Transfer the top plasma layer into a clean test tube without disturbing the white buffy layer. Samples should be assayed immediately after collection or stored at -80°C. Plasma samples can be tested after dilution with assay buffer at least 1:2. For samples with low Substance P levels or observed interference, sample purification by C18 SPE or precipitation is recommended (see protocols below).

Serum

Collect blood in vacutainers without an anticoagulant. Allow samples to clot undisturbed for 30-60 minutes at room temperature. To obtain serum, centrifuge at 1,000-2,000 x g for 15-30 minutes. Transfer the serum layer into a clean test tube. Samples should be assayed immediately after collection or stored at -80°C. Serum samples can be tested after dilution with assay buffer at least 1:2. For samples with low Substance P levels or observed interference, sample purification by C18 SPE or precipitation is recommended.

NOTE: It is recommended that non-human plasma and serum be purified using the SPE (C-18) protocol.

SPE (C-18) Purification Protocol[6]

1. Aliquot a known amount of each sample into separate tubes (600 µl is recommended). If samples need to be concentrated, a larger volume should be used.
2. Add 4 volumes of 4% acetic acid to the sample.
3. Activate a 6 ml SPE Cartridge (C-18) (Item No. 400020) by rinsing with 5 ml methanol, then with 5 ml ultrapure water. Do not allow the SPE cartridge (C-18) to dry.
4. Apply the sample slowly to the SPE cartridge (C18) and allow it to completely enter the packing material.
5. Wash the SPE cartridge with 10 ml of 4% acetic acid.
6. Prepare a solution of acetonitrile:1% trifluoroacetic acid (TFA) in ultrapure water (60:40 v/v). Elute the Substance P by passing 3 ml of the acetonitrile:TFA solution through the SPE cartridge one milliliter at a time. Do not allow the SPE cartridge (C-18) to dry. Be certain to pause between each milliliter of solution as reproducibility of the recovery is increased by the care taken during this step. This elution contains the sample. Save this for step 7.
7. Dry the purified sample by vacuum centrifugation or under a gentle stream of nitrogen with or without heating the sample at 37°C. It is imperative that all the organic solvent be removed as even trace quantities will adversely affect the ELISA.
8. Resuspend the samples in Assay Buffer to their original volumes and use for ELISA analysis. Samples can be concentrated in this step by using smaller volumes of buffer compared to the original sample volumes.

Precipitation Protocol[6]

1. Aliquot a known amount of each sample into separate tubes (600 µl is recommended). If samples need to be concentrated, a larger volume can be used.
2. Add 2 volumes of 0.1% trifluoroacetic acid in acetonitrile (ice-cold) to the sample and vortex thoroughly. Centrifuge the samples at 5,000 x g for 5 minutes at 4°C. Collect the supernatant to a clean tube. Repeat this step one more time, then combine the supernatants.
3. Dry the supernatant by vacuum centrifugation or under a gentle stream of nitrogen with or without heating the sample at 37°C. It is imperative that all the organic solvent be removed as even trace quantities will adversely affect the ELISA.
4. Resuspend the samples in Assay Buffer to their original volumes and use for ELISA analysis. Samples can be concentrated in this step by using smaller volumes of buffer compared to the original sample volumes.

Preparation of Assay-Specific Reagents

Substance P Express Standard

Reconstitute the lyophilized Substance P Express Standard (Item No. 401193) with 2 ml of Assay Buffer and mix gently. The concentration of this solution (the bulk standard) will be 10 ng/ml. Store this solution at 4°C; it will be stable for two weeks.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 800 µl Assay Buffer to tube #1 and 500 µl Assay Buffer to tubes #2-8. Transfer 200 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should be used within two hours.

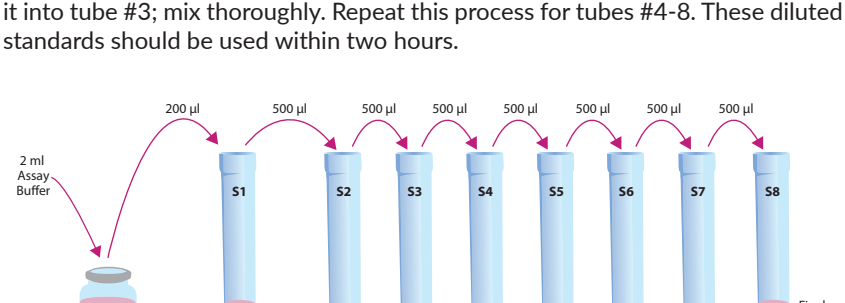


Figure 2. Preparation of the Substance P standards

Substance P Express HRP Tracer

Dilute the Substance P Express HRP Tracer (Item No. 401191) with 2.5 ml of Assay Buffer. Store the diluted Substance P Express HRP Tracer at 4°C (*do not freeze!*). It will be stable for at least three weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Substance P Express ELISA Antiserum

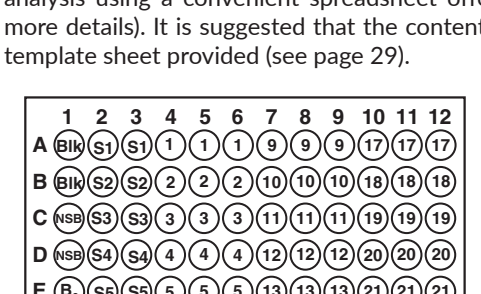
Reconstitute the Substance P Express ELISA Antiserum (Item No. 401192) with 3 ml of Assay Buffer. Store the reconstituted Substance P Express ELISA Antiserum at 4°C (*do not freeze!*). It will be stable for at least three weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Plate Set Up

The 96-well plate(s) included with this kit MUST be pre-washed five times with Wash Buffer (1X) (300 µl/well) prior to use in the ELISA. *NOTE: If you do not need to use all the strips at once, place the unwashed strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two Blk, two NSB, and three B_0 wells, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of two dilutions, and each dilution should be assayed in at least duplicate. For statistical purposes, assaying the samples in triplicate is recommended.

A suggested plate format is shown in Figure 3, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 22 for more details). It is suggested that the contents of each well be recorded on the template sheet provided (see page 29).



Blk = Blank Wells
TA = Total Activity Well
NSB = Non-Specific Binding Wells
B_0 = Maximum Binding Wells
S1-S8 = Standard Wells
1-24 = Samples Wells

Figure 3. Sample plate format

Performing the Assay

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Pre-Wash the Plate

Rinse the plate (or strips to be used) five times with ~300 µl Wash Buffer (1X).

Addition of the Reagents

1. **Assay Buffer**

   Add 125 µl Assay Buffer to NSB wells. Add 100 µl Assay Buffer to B_0 wells.

2. **Substance P Express Standard**

   Add 100 µl from tube #8 to both of the lowest standard wells (S8). Add 100 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 100 µl of sample per well.

4. **Substance P Express HRP Tracer**

   Add 25 µl to each well *except* the TA and Blk wells.

5. **Substance P Express ELISA Antiserum**

   Add 25 µl to each well *except* the TA, NSB, and Blk wells, within 15 minutes of addition of the tracer.

Well	Assay Buffer	Standard/ Sample	Tracer	Antiserum
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	125 µl	-	25 µl	-
B_0	100 µl	-	25 µl	25 µl
Std/Sample	-	100 µl	25 µl	25 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with a 96-Well Cover Sheet (Item No. 400012) and incubate for two hours at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Add 5 µl of the diluted tracer to the TA wells.
4. Cover the plate with the 96-Well Cover Sheet and protect from light. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes.
5. Remove the cover sheet being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
6. DO NOT WASH THE PLATE. Add 75 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman Chemical has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the Blk wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* Substance P concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the %B/B_0 (or B/B_0) value for each sample. Determine the concentration of each sample by identifying the %B/B_0 on standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any dilution of the original sample prior to its addition to the well.* Samples with %B/B_0 values outside the linear portion of the standard curve should be re-assayed. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference, which could be eliminated by purification.

NOTE: If there is an error in the B_0 wells, plot the absorbance values instead of %B/B_0 to calculate sample concentrations.

Performance Characteristics

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially.

Substance P Standard (pg/ml)	Blk-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV Intra-Assay*	%CV Inter-Assay*
NSB	0.000	--	--	--	--
B_0	1.322	1.322	--	--	--
TA	2.799	--	--	--	--
2,000	0.105	0.105	8.0	3.7	2.6
1,000	0.177	0.177	13.4	3.2	2.5
500	0.293	0.293	22.2	3.4	3.3
250	0.472	0.472	35.8	3.1	2.9
125	0.688	0.688	52.1	3.9	4.0
62.5	0.918	0.918	69.5	5.9	5.3
31.3	1.085	1.085	82.2	9.1	8.1
15.6	1.192	1.192	90.3	12.7	11.4

Table 2. Typical results
**%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.*

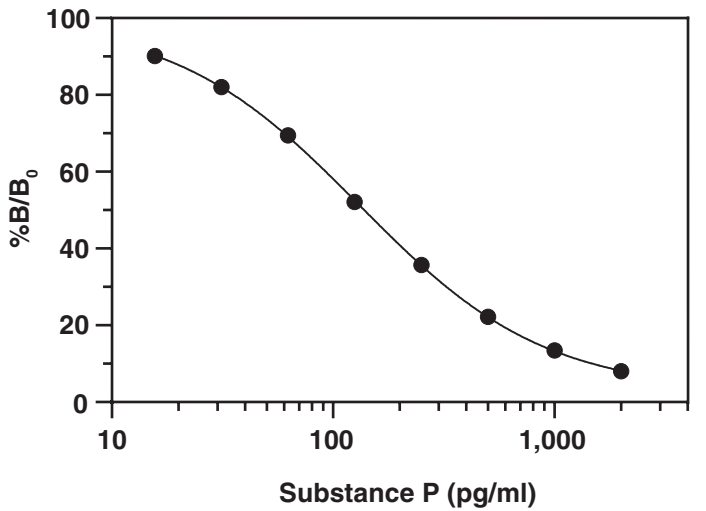


Figure 4. Typical standard curve

Cross Reactivity:

Compound	Cross Reactivity
Substance P	100%
Hemokinin-1	105%
Substance P (7-11)	29%
Neurokinin B (neuromedin K)	0.01%
Neurokinin A (substance K)	<0.01%
Substance P (1-7)	<0.01%

Table 3. Cross reactivity of the Substance P ELISA (Express)

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of replicates	A. Trace organic contaminants in the water B. Poor pipetting/technique C. The plate was not pre-washed
Low signal	A. HRP inhibitors are present; ensure that samples and buffers are free of HRP inhibitors, such as azide B. Sample requires further dilution
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	A. Dilution error in preparing the standard B. Standard or tracer is degraded
Analyses of two dilution of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present; purification is needed
Only TA wells develop	A. Trace organic contaminants in the water B. Tracer or antiserum was not added to the wells

References

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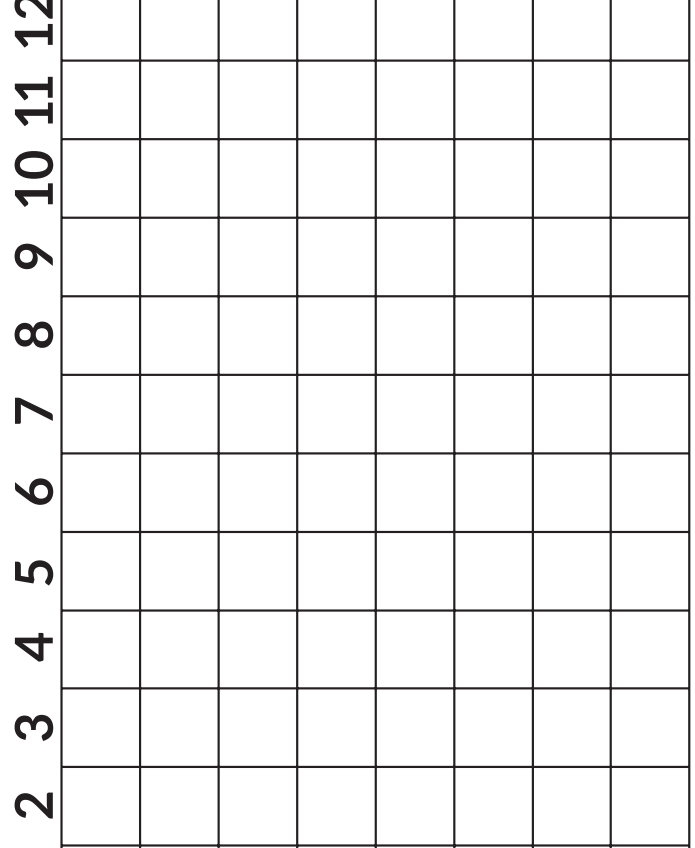
4. Muñoz, M. and Coveñas, R. Involvement of substance P and the NK-1 receptor in human pathology. *Amino Acids* **46(7)**, 1727-1750 (2014).
5. Mantyh, P.H. Neurobiology of substance P and the NK1 receptor. *J. Clin. Psychiatry* **63(Suppl 11)**, 6-10 (2002).
6. Fehder, W.P., Ho, W.-Z., Campbell, D.E., *et al.* Development and evaluation of a chromatographic procedure for partial purification of substance P with quantification by an enzyme immunoassay. *Clinical and Diagnostic Laboratory Immunology* **5**, 303-307 (1998).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.