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Substance P EIA

Product Number **CS0180**
Storage Temperature –20 °C

ProductInformation

Technical Bulletin

Product Description

Substance P EIA is a 3-hour competitive enzyme immunoassay for the quantitative determination of Substance P (SP) concentrations in cell culture supernatants, saliva, urine, serum and plasma. Substance P present in the samples or standards competes with the fixed amount of Substance P conjugated to alkaline phosphatase for the limited number of binding sites on the rabbit anti-Substance P antibody. During the first incubation, the antigen-antibody complex binds to the anti-rabbit IgG antibody-coated multiwell plate. The excess conjugate and unbound sample are washed away and a substrate is added. During the second incubation the bound enzyme reacts with the substrate. The enzyme reaction is stopped and the absorbance read at 405 nm. The intensity of the yellow color is inversely proportional to the concentration of Substance P in the standards or the samples. The concentration is calculated based on optical reading of standard dilution.

Substance P belongs to the tachykinins, a family of amidated neuropeptides that share a carboxy terminal sequence Phe-X-Gly-Leu-Met-NH2 and are found in both vertebrates and invertebrates. One gene encodes a precursor containing both substance P and neurokinin A, while the other encodes a precursor containing only neurokinin B. Substance P is an 11 (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2) amino acid peptide that is synthesized in 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 neurotransmitter.

In the peripheral system, Substance P is localized in the primary sensory neurons and the neurons intrinsic to the gastrointestinal tract. Zimmer showed that mice that cannot produce these peptides display no significant pain responses following formalin injection and have an increased pain threshold in the hotplate test, while they react normally in some other tests. The results demonstrated that substance B and/or substance K have essential functions in specific responses to pain. The vast array of processes in which substance P is involved such as pain, anxiety, depression and inflammation have resulted in the development of substance P antagonists with a wide potential application to a number of medical conditions outside of the nausea and vomiting realm. Aprepitant (Emend(R)), Merck & Co., Inc.), the first of this class, was recently approved by the FDA for the prevention of both acute and delayed chemotherapy-induced nausea and vomiting (CINV).[1-5]

Reagents

- **Substance P Standard, 1 vial, Product No S 9943** – 0.5 mL (100,000 pg/mL) synthetic peptide in a buffer with preservative.
- **Anti-Rabbit IgG-coated 96 well plate, 1 plate, Product No. I 6283**
- **Assay Buffer, 30 mL, Product No. A 4228 –** contains sodium azide. Ready to use.
- **Anti-Substance P, 6 mL, Product No. S 9693–** from rabbit, contains sodium azide, yellow dye. Ready to use.
- **Substance P-Alkaline Phosphatase Conjugate, 6 mL, Product No. S 9818 -** contains sodium azide, blue dye. Ready to use.
- **p-Nitrophenylphosphate (pNpp) Substrate, 20 mL, Product No. N 6911 -** Ready to use.
- **Wash Buffer Concentrate, 10X, 30 mL, Product No. W 3139 –** TRIS buffered saline with detergents and sodium azide.
- **Stop Solution, 5 mL, Product No. S 9443 –** a solution of trisodium phosphate. Ready to use.
- **Plate sealer, 1 each, Product No. P 1496**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 405 nm, preferably with corrections between 570 and 590 nm.
- Horizontal orbital multiwell plates shaker capable of maintaining a speed of 500 +/- 50 rpm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- If the sample requires extraction, cell extraction materials are needed (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.
- Serine protease inhibitor Aprotinin (Prod. No. A 6279)

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples containing rabbit IgG may interfere with this assay.
- The assay is validated for cell culture samples, saliva, urine, plasma and serum.
- Approximately 500 Kalkrein Inhibition Units/mL (equals 10.6 Trypsin Inmhibition Units/mL) of Aprotinin should be added to all samples immediately after sample collection.
- Samples may be assayed directly or after extraction.
- Store samples in plastic tubes.
- Cell culture supernatants - centrifuge 10 min at 1000 x *g*, aliquot into plastic tubes and store at –70 °C.
- If the samples are assayed directly, dilute before run in cell culture media.
- Samples, which normally have low levels of Substance P, (below assay sensitivity) require extraction.
- After extraction dilute 1:2 in Assay Buffer
- Serum - collect with serum separator. Clot 30 min, Centrifuge 10 min at 1000 x *g*, store aliquotted in plastic tubes at –70 °C or assay diluted 1:2 in Assay Buffer.
- Urine **-** collect aseptically first urine, store aliquotted in plastic tubes at –70 °C or assay diluted 1:8 in Assay buffer.
- Saliva - aliquot into plastic tubes and store at –70 °C or assay diluted 1:20 in Assay Buffer**.**
- Plasma – collect in EDTA, immediately place on ice, centrifuge at 2-8 °C for 15 minutes, aliquot into plastic tubes and store at –70 °C. Assay diluted 1:2 in Assay buffer.
- To ensure accurate results, always dilute the standards and blanks in the same diluent as the samples.

Materials Requited for Extraction

- 1% Trifluoroacetic Acid (TFA)
- Acetonitrile (ACS Reagent Grade)
- 200 mg C_{18} column for sample extraction
- Centrifugal concentrator (Speed Vac) for sample extraction

Protocol for Sample Extraction

1. Add an equal volume of 1% trifluoroacetic acid (TFA) in water to the sample. Centrifuge at
2. 17,000 x g for 15 minutes at 4 °C to clarify and save the supernatant.
3. Equilibrate a 200 mg C_{18} Sep-Pak column with 1 mL of acetonitrile, followed by 10-25 mL of 1% TFA in water.
4. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 1% TFA in water. Discard wash.
5. Elute the sample slowly by applying 3 mL of acetonitrile: 1% TFA in water 60:40. Collect eluant in a plastic tube.
6. Evaporate to dryness using a centrifugal concentrator under vacuum. Store at -20°C.
7. Reconstitute with Assay Buffer and measure immediately.
8. 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.

Reagent Preparation

SUBSTANCE P Standard

1. Standards can be made either in glass or plastic tubes.
2. Pre-rinse pipette tips and change the tips before each dilution.
3. Equilibrate standard and all reagents to room temperature.
4. Prepare serial standard dilutions as follows:

Tube #	Assay Buffer or Cell Culture Media mL	Standard from tube #: mL	Final Standard Concentration pg/mL
0	Standard vial 100,000 pg/mL		
1	0.9 mL	0.1 mL (0)	10,000
2	0.75 mL	0.25 mL (1)	2,500
3	0.75 mL	0.25 mL (2)	625
4	0.75 mL	0.25 mL (3)	156.3
5	0.75 mL	0.25 mL (4)	39.1
6	0.75 mL	0.25 mL (5)	9.8

5. Diluted standards should be used within 60 minutes of preparation.

Substance P-Alkaline phosphatase conjugate

1. The activity of the Substance P conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions.
2. The activity of Substance P conjugate is affected by high concentrations of chelators, such as EDTA and EGTA. Samples that contain <10 mM EDTA or EGTA can be assayed without interference. Samples containing higher concentrations of chelators must be diluted prior to assay.
3. Equilibrate conjugate to room temperature before use.

Wash Buffer

1. Use only Wash Buffer provided in the kit.
2. Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; **avoid the use of PBS-based wash buffers** and other sources of inorganic phosphate contamination.
3. Warm buffer to room temperature.
4. Dilute 30 mL wash concentrate with 270 mL deionized or distilled water.
5. Label as **Working Wash buffer**

Diluted buffer may be stored at room temperature for 3 months.

Storage/Stability

- The kit is shipped on dry ice.
- Store unopened kit at <–20 °C until use.
- After opening store the components as follows:
  Standard - aliquot and store at –20 °C.
  Conjugate - aliquot and store at –20 °C.
  Unused Multiwell strips – return to pouch, seal with desiccant, store at 2-8 °C
  All other components – store at 2-8 °C

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www sigma-aldrich.com

Procedure

Precautions

- Allow all reagents to equilibrate to room temperature (15-30 ºC) for at least 30 minutes before opening the kit.
- Use only the pre-coated 96 multiwell capture plate provided with the kit.
- Multiwell strips should be equilibrated to room temperature in the sealed foil bag.
- Remove desired number of strips, reseal the bag and refrigerate unused wells desiccated at 2 – 8°C to maintain plate integrity.
- When not in use all kit components should be stored at 2 to 8 °C**.**
- Do not use reagents after the kit shelf life.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Minimize contamination by endogenous alkaline phosphatase, present especially in the substrate solution. Avoid touching pipette tips and other items with bare hands.
- Alkaline Phosphatase is a temperature sensitive enzyme. Optical Density (OD) units may vary with temperature changes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.

- Pipette standards and samples to the bottom of the wells.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Read absorbances within 2 hours of assay completion.

Washing directions

1. The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
2. Incomplete washing will adversely affect the assay and render false results.
3. Avoid the use of phosphate-based buffers to prevent contamination with endogenous phosphate.
4. Washing may be performed using automated washer, manifold pipette or squirt bottle.
5. Wash cycle three times, blotting as dry as possible after the 3rd wash.
6. When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
7. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Refer to the diagram at the end of this bulletin to view the assay layout.
The following wells should be included in each run as the quality control: Total Activity (TA), Non-Specific Binding (NSB), Maximum Binding (Bo), Substrate Blank (SB) and control blank (Blank).

1st Incubation

a. Remove the appropriate number of strips and return the unused strips to the pouch. Reseal pouch and refrigerate.
b. Add 100 µL of Assay Buffer to the NSB wells
c. Add 50 µL of Assay Buffer to the Maximum Binding (B_o) wells.
d. Add 50 µL of standards #1-6 to the appropriate wells.
e. Add 50 µL of samples to the appropriate wells
f. Add 50 µL of Substance P-alkaline phosphatase conjugate to all wells (except the TA and SB).
g. Add 50 µL of Anti-Substance P to all wells (except NSB, TA and SB wells).
h. Cover with plate cover and incubate 2 hours at room temperature on orbital shaker set at 500 rpm.
i. All wells should be green, except NSB wells, which are blue.
j. Wash wells for a total of 3 times following washing instructions.
k. After the final wash, blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate Incubation

a. Add 5 µL of Substance P conjugate to the TA wells.
b. Add 200 µL of pNpp substrate to all wells. Cover.
c. Incubate 1 hour at RT on a benchtop.

Stop Reaction

a. Add 50 µL of Stop Solution to each well.
b. Yellow color develops immediately and can be read in the multiwell plate reader at 405 nm with corrections at 570 or 590 nm.
c. Subtract the readings at 590 nm from the readings at 405 nm, to correct for optical imperfection of the plate.

Results

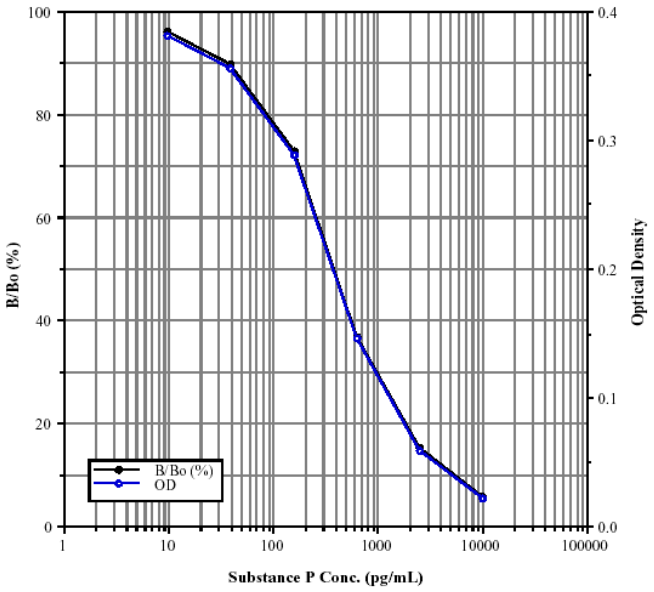
Average the duplicate readings for each standard and sample and subtract the average NSB optical density.

1. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit.
2. As an alternative, construct a standard curve by plotting the mean absorbance for each standard (nm) on a linear y-axis against the concentration (pg/mL) on a logarithmic x-axis and draw the best fit curve through the points on the graph.
3. % B/B_o can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.
4. Calculate the concentration of Substance P corresponding to the mean absorbance or % B/B_o from the standard curve.
5. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Product Profile

Typical Results

Wells pg/mL	Mean OD_{405} (-Blank)	Corrected Net OD_{405}	% Bound B/Bo
Blank OD	(0.086)		
TA	0.269	0.268	
NSB	0.001	0.000	
O (B_o) 0 pg/mL	0.398	0.397	100%
9.8 pg/mL	0.382	0.381	5.8%
39.1 pg/mL	0.357	0.356	15.2%
156 pg/mL	0.289	0.288	36.9%
625 pg/mL	0.147	0.146	72.8%
2,50 pg/mL	0.060	0.059	89.7%
10,000 pg/mL	0.023	0.022	96.1%



Typical Standard Curve for Substance P EIA

This standard curve is provided for demonstration only. A standard curve should be generated for each assay.

Quality Control

Each laboratory should establish a quality control program to monitor the performance of the Substance P immunoassay. As a part of this program, TA, NSB, B_0, and Substrate Blank wells should be run in each assay. The average readings are calculated over the time. Any time the assay readings exceed the average, the assay may need to be re-run.

Typical Quality Control Parameters

Substrate Blank (OD)	= 0.078
TA (TA x 10)	= 2.68
% NSB (NSB/TA x 100)	= 0.0%
% Bo (Bo/TA x 100)	= 14.8%
Quality of Fit	= 0.999

Performance Characteristics

Sensitivity

The sensitivity of the Substance P assay is typically less than 8.04 pg/mL. Sensitivity was determined by subtracting two standard deviations from the mean absorbance value of sixteen zero standard (Bo) replicates and calculating the corresponding concentration.

Linearity

To assess the linearity of the assay, serum samples spiked with high concentrations of Substance P were diluted with Assay Buffer to produce samples with values within the dynamic range of the assay.

Dilution	Mean % of Expected	Range (%)
1:2	90	83-96
1:4	92	82-101
1:8	92	87-96

Precision

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

	Substance P pg/mL	Intra-Assay %CV	Inter Assay %CV
Low	101.6	6.7	
Medium	1,116.1	4.5	
High	6,257.0	5.2	
Low	96.8		4.2
Medium	1,120.4		7.3
High	6,401.9		7.3

Recovery
The recovery of Substance P spiked into samples in various matrices was evaluated.

Sample	%Recovery	Recommended Dilution
Tissue Cultures	81.0	1:2
Human Saliva	108.3	1:20
Human Urine	105.8	1:8
Heparin Plasma (porcine)	109.3	1:2
Porcine Serum	97.7	1:2

Cross Reactivity
The cross reactivity for a number of related compounds were determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 10 to 100,000 pg/mL. These samples were measured in the Substance Passay and the 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.

Compound	Cross Reactivity
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%

References

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SUBSTANCE P Assay Flow Chart (CS0180)

Well ID	**Blank**	**TA**	**NSB**	**B_0**	**Standards**	**Samples**
	A1, B1	**C1, D1**	**E1, F1**	**G1, H1**	**A2-C3**	**D3-H12**
Assay Buffer			100 µL	50 µL		
Standard and/or Sample					50 µL	50 µL
Conjugate			50 µL	50 µL	50 µL	50 µL
Anti-Substance P Antibody				50 µL	50 µL	50 µL

Incubate 2 hours @RT with shaking
Wash 3X, blot dry

Conjugate		5 µL				
pNpp Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL

Incubate 1 hour @ RT without shaking

Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Read at 405 nm