ab288318 – Substance P ELISA Kit

For the detection of Substance P in plasma, serum, saliva, urine, and supernatants.
For research use only - not intended for diagnostic use.

Storage and Stability

All components of this kit can be stored at -20°C and are stable at that temperature. Upon receipt or upon first thaw, the kit components should be stored as indicated on their individual labels. The Antibody, Conjugate and Standard can be stored frozen at -20°C, while the remaining components can be stored at 4°C and are stable at that temperature. Freeze thaws should be limited for the most optimal kit performance.

Materials Supplied

Item	Quantity	Storage Condition
20X Wash Buffer Concentrate	27 ml	4°C
Assay Buffer	27 ml	4°C
Goat anti-rabbit IgG Microplate (12 x 8 wells)	1 unit	4°C
Plate Sealer	1 unit	4°C
pNpp Substrate	20 ml	4°C
Stop Solution	5 ml	4°C
Substance P Alkaline Phosphatase Conjugate	5 ml	-20°C
Substance P Antibody	5 ml	-20°C
Substance P Standard	500 µl	-20°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

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

Reagent Preparation

- Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
- Pipet standards and samples to the bottom of the wells.
- Add the reagents to the side of the well to avoid contamination.
- 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.
- 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.

Substance P Standard: Allow the 100,000 pg/mL Substance P standard solution to warm to room temperature. Label six 12 x 75 mm polypropylene tubes #1 through #6. Pipet 900 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of diluent into tubes #2 through #6. 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.

Substance P Antibody: Allow the antibody to thaw and warm to room temperature. Any unused antibody should be aliquoted and stored at or below -20°C. Avoid repeated freeze-thaw cycles.

Substance P Conjugate: Allow the conjugate to thaw and warm to room temperature. Any unused conjugate should be aliquoted and stored at or below -20°C. Avoid repeated freeze-thaw cycles.

Wash Buffer: Prepare the Wash Buffer by diluting 10 mL of the supplied concentrate with 190 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Sample Preparation

- The Substance P ELISA kit is compatible with Substance P samples in a wide range of matrices.
- Biological fluids should be diluted in Assay Buffer and run directly in the assay. A minimum 1:64 dilution is required for EDTA plasma, 1:8 dilution for Heparin plasma, 1:64 dilution for serum, 1:128 dilution for saliva and 1:4 dilution for urine. Neat tissue culture media has been validated in the assay, provided the same tissue culture media is used to dilute the standards instead of assay buffer.
- 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 (1 mg/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.**

Assay Protocol

- Determine the number of wells to be used. Remove unneeded wells and return them with the desiccant to the plate bag and seal. Store unused wells at 4ºC.
- Let all reagents warm to room temperature before using them in the assay.

1. Add 50 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the Non-Specific Binding (NSB) and the Bo (0 pg/mL Standard) wells. Leave the TA (total activity) and Blank wells empty.
2. Add 50 µL of Standards #1 through #6 into the appropriate wells.
3. Add 50 µL of the Samples into the appropriate wells.
4. Add 50 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB wells.
5. Add 50 µL of blue Conjugate into each well, except the TA and Blank wells.
6. Add 50 µL of yellow Antibody into each well, except the TA, Blank and NSB wells.
7. Seal the plate. Incubate at room temperature (RT) on a plate shaker for 2 hours at ~500 rpm.
8. Empty the contents of the wells and wash by adding a full well volume (~400 µL) of 1x Wash Buffer to each well. Empty or aspirate the wells and repeat the wash 2 more times for a total of 3 Washes. 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.
9. Add 5 µL of the blue Conjugate to the TA wells.
10. Add 200 µL of the pNpp Substrate solution to every well. Incubate at RT for 1 hour while shaking.
11. Add 50 µL of Stop Solution to every well. This stops the reaction, and the plate should be read immediately.
12. 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.

∆ ***Please note:*** *TA control (total activity) is a measurement of the maximum enzymatic activity expected for an immunoassay. The TA value is not used for data analysis and is a control specification. NSB (non-specific binding) is the residual background control. The values of NSB and blank should be similar. The NSB well will detect anything in the diluent that might be non-specifically binding.*

Well I.D	Blank A1, B1	TA C1, D1	NSB E1, F1	Bo G1, H1	Stds. A2 - D3	Samples E3 - H12
Standard Diluent	---	---	50µL	50µL	---	---
Assay Buffer	---	---	50µL	---	---	---
Std. and/or Sample	---	---	---	---	50µL	50µL
Conjugate	---	---	50µL	50µL	50µL	50µL
Antibody	---	---	---	50µL	50µL	50µL
Incub. 2 hours @ RT, shaking	→→→	→→→	→→→	→→→	→→→	→→→
Asp. & Wash 3 x 400µL	→→→	→→→	→→→	→→→	→→→	→→→
Conjugate	---	5µL	---	---	---	---
Substrate	→→→	→→→	→→→	→→→	→→→	→→→
Incub. 1 hour @ RT, shaking	200µL	200µL	200µL	200µL	200µL	200µL
Stop Solution	50µL	50µL	50µL	50µL	50µL	50µL

Calculation

- 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 4-parameter logistic (4PL) curve fitting program.
- The concentration of Substance P can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

Average Net OD = Average OD - Average NSB OD

2. Using data analysis software, plot the Average Net OD for each standard versus Substance P concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalysed using alternative dilution(s).

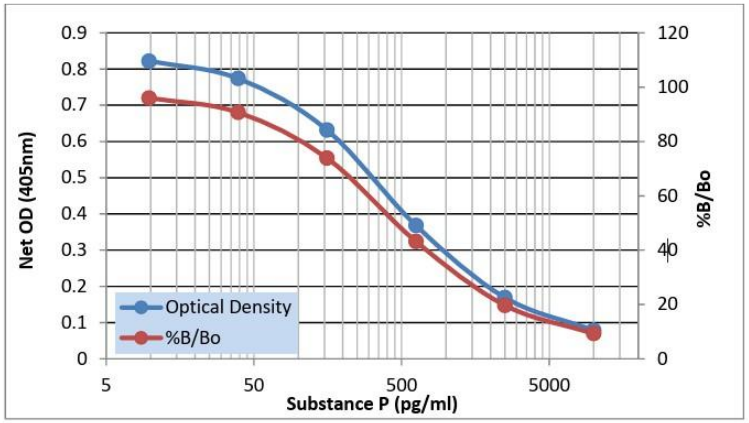
Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.
∆ ***Please note:*** *While blank OD is not used in data calculation, blank OD values should be very similar to NSB values.*

Sample	Mean OD	Net OD	%B/Bo	Substance P (pg/mL)
NSB	0.017			
TA	0.714			
Bo	0.87	0.853	100	0
S1	0.096	0.079	9.3	10,000
S2	0.186	0.169	19.7	2,500
S3	0.385	0.368	43.2	625
S4	0.649	0.632	73.9	156.25
S5	0.791	0.774	90.7	39.06
S6	0.839	0.822	96	9.76

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.



Specificity

The specificity of the assay was determined by running serial dilutions of the analytes, including the cross-reactants, in the assay, fitting the resulting dose response curves to a 4PL curve-fit and determining the ED50. The ED50 of each cross-reactant was then divided by the determined ED50 of the Substance P standard curve and multiplied by 100.

Analyte	Cross Reactivity
Substance P	100%
Substance P (4-11)	>100%
Substance P (7-11)	>100%
Substance P (3-11)	66%
Physalaemin	50%
Eledoisin	<0.001
α-Neurokinin	<0.001
β-Neurokinin	<0.001
Somatostatin	<0.001
Subs P (1-4)	<0.001

Sensitivity

The sensitivity or limit of detection of the assay is 5.3 pg/mL. This was determined by interpolation at 2 standard deviations below the background (0pg/mL) of 16 zero standard replicates. Data was collected from 23 standard curves.

Assay Precision

	Intra-Assay Precision	Inter-Assay Precision
CV (%)	5.3	10

Technical Support

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