

# **ab133029 – Substance P ELISA Kit**

Instructions for Use

For quantitative detection of Substance P in biological fluids.

This product is for research use only and is not intended for diagnostic use.

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## 1. BACKGROUND

Abcam's Substance P *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Substance P in biological fluids.

A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-Substance P antigen and a polyclonal rabbit antibody specific to Substance P. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of Substance P captured in the plate.

Substance P is an undecapeptide that displays a number of biological activities. The peptide was first discovered in 1931 by von Euler and Gaddum. 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 proteinaceous. The structure is shown below:

**Substance P:** H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH<sub>2</sub>

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 neurotransmitter. In the peripheral system, Substance P is localized in the primary sensory neurons and neurons intrinsic to the gastrointestinal tract.

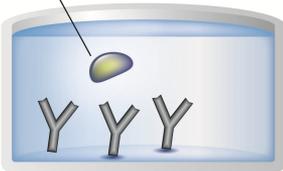
## 2. ASSAY SUMMARY

**Capture Antibody**



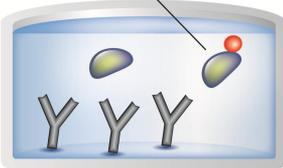
Prepare all reagents and samples as instructed.

**Sample**



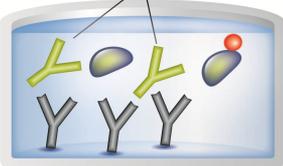
Add standards and samples to appropriate wells.

**Labeled AP-Conjugate**



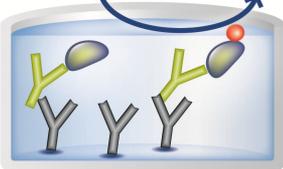
Add prepared labeled AP-conjugate to appropriate wells.

**Target Specific Antibody**



Add Substance P antibody to appropriate wells. Incubate at room temperature.

**Substrate**      **Colored Product**



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

### 3. PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

- 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.
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
- The activity of the alkaline phosphatase conjugate is dependent on the presence of  $Mg^{2+}$  and  $Zn^{2+}$  ions. The activity of the conjugate is affected by concentrations of chelators ( $>10$  mM) such as EDTA and EGTA.
- We test the performance of this kit with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
- The Substance P Standard provided, is supplied in ethanolic buffer at a pH optimized to maintain Substance P integrity. Care should be taken handling this material because of the known and unknown effects of Substance P.

#### 4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the Substance P Alkaline Phosphatase Conjugate and Substance P Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

#### 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-Rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4°C
Substance P Alkaline Phosphatase Conjugate	5 mL	-20°C
Substance P Antibody	5 mL	+4°C
Substance P Standard	500 µL	-20°C
Assay Buffer	27 mL	+4°C
20X Wash Buffer Concentrate	27 mL	+4°C
pNpp Substrate	20 mL	+4°C
Stop Solution	5 mL	+4°C

### **6. 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  $\mu\text{L}$  and 1,000  $\mu\text{L}$
- Repeater pipets for dispensing 50  $\mu\text{L}$  and 200  $\mu\text{L}$
- 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

### **7. LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

### 8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- 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
- 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
- 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
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

## 9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

### 9.1 **Substance P Conjugate**

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and store at -20°C.

### 9.2 **1X 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.

## 10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

- 10.1 Allow the 100,000 pg/mL Substance P **Stock Standard** solution to warm to room temperature. Avoid repeated freeze-thaw samples.
- 10.2 Label six tubes with numbers #1 – #6.
- 10.3 Add 900  $\mu$ L of standard diluent (Assay Buffer or Tissue Culture Media) to tube # 1.
- 10.4 Add 750  $\mu$ L of the standard diluent (Assay Buffer or Tissue Culture Media) to tubes #2 – #6.
- 10.5 Add Prepare a 10,000 pg/mL **Standard 1** by adding 100  $\mu$ L of the 100,000 pg/mL Stock Standard to tube #1. Mix thoroughly and gently
- 10.6 Prepare **Standard 2** by transferring 250  $\mu$ L from tube #1 to tube #2. Mix thoroughly and gently.
- 10.7 Prepare **Standard 3** by transferring 250  $\mu$ L from tube #2 to tube #3. Mix thoroughly and gently.
- 10.8 Repeat for tubes #4 through to #6.

# ASSAY PREPARATION

Standard	Sample to Dilute	Volume to Dilute ( $\mu\text{L}$ )	Volume of Diluent ( $\mu\text{L}$ )	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Standard	100	900	100,000	10,000
2	Standard 1	250	750	10,000	2,500
3	Standard 2	250	750	2,500	625
4	Standard 3	250	750	625	156.25
5	Standard 4	250	750	15.25	39.06
6	Standard 5	250	750	39.06	9.76



## 11. SAMPLE COLLECTION AND STORAGE

- The Substance P EIA kit is compatible with Substance P 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 in Section 16 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.
- 11.1 Some samples normally have very low levels of Substance P present and extraction may be necessary for accurate measurement. A suggested extraction procedure is outlined below:
- 11.1.1. 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.
  - 11.1.2. Equilibrate a 200 mg C18 Sep-Pak column with 1 mL of acetonitrile, followed by 10-25 mL of 1% TFA in water.

- 11.1.3. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 1% TFA in water. Discard wash.
- 11.1.4. Elute the sample slowly by applying 3 mL of acetonitrile: 1% TFA in water 60:40. Collect the eluant in a plastic tube.
- 11.1.5. Evaporate to dryness using a centrifugal concentrator under vacuum. Store at -20°C.
- 11.1.6. Reconstitute with Assay Buffer and measure immediately.

The 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.

## 12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at +4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

### Recommended plate layout

	1	2	3	4
A	B <sub>s</sub>	Std 1	Std 5	Sample 3
B	B <sub>s</sub>	Std 1	Std 5	Sample 3
C	TA	Std 2	Std 6	etc
D	TA	Std 2	Std 6	etc
E	NSB	Std 3	Sample 1	
F	NSB	Std 3	Sample 1	
G	B <sub>0</sub>	Std 4	Sample 2	
H	B <sub>0</sub>	Std 4	Sample 2	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

### Key:

**B<sub>s</sub>** = Blank; contains substrate only.

**TA** = Total Activity; contains conjugate (5 µL) and substrate.

**NSB** = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

**B<sub>0</sub>** = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate.



## **13. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
  - **It is recommended to assay all standards, controls and samples in duplicate**
  - **Refer to the recommended plate layout in Section 12 before proceeding with the assay**
- 13.1 Add 50  $\mu$ L appropriate diluent\* into the NSB (non-specific binding) wells. (\*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).
  - 13.2 Add 50  $\mu$ L appropriate diluent (Assay Buffer or Tissue Culture Media) into the B<sub>0</sub> (0 pg/mL standard) wells.
  - 13.3 Add 50  $\mu$ L of prepared standards #1 to #6, and 50  $\mu$ L diluted samples to appropriate wells.
  - 13.4 Add 50  $\mu$ L of Substance P Alkaline Phosphatase Conjugate (blue) to NSB, B<sub>0</sub>, standard and sample wells, i.e. not Total Activity (TA) and B<sub>s</sub> wells.
  - 13.5 Add 50  $\mu$ L of Substance P antibody (yellow) to B<sub>0</sub>, standard and sample wells, i.e. not TA, B<sub>s</sub> and NSB wells.  
*Note:* Every well used should be green except the NSB wells which should be blue. B<sub>s</sub> and TA wells are empty at this point and have no color.
  - 13.6 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.
  - 13.7 Empty the contents of the wells and wash by adding 400  $\mu$ L of 1X Wash Buffer to each well. 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.
  - 13.8 Add 5  $\mu$ L of the Substance P Alkaline Phosphatase Conjugate to the TA wells.

## ASSAY PROCEDURE

- 13.9 Add 200  $\mu\text{L}$  of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.
- 13.10 Add 50  $\mu\text{L}$  Stop Solution into each well. The plate should be read immediately.
- 13.11 After blanking the plate reader against the  $B_s$  wells, read optical density at 405nm. If the plate reader is not capable of adjusting for the  $B_s$ , manually subtract the mean OD of the  $B_s$  from all readings.

\*The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.

## 14. CALCULATIONS

- 14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

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

- 14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells ( $B_0$ ), using the following formula:

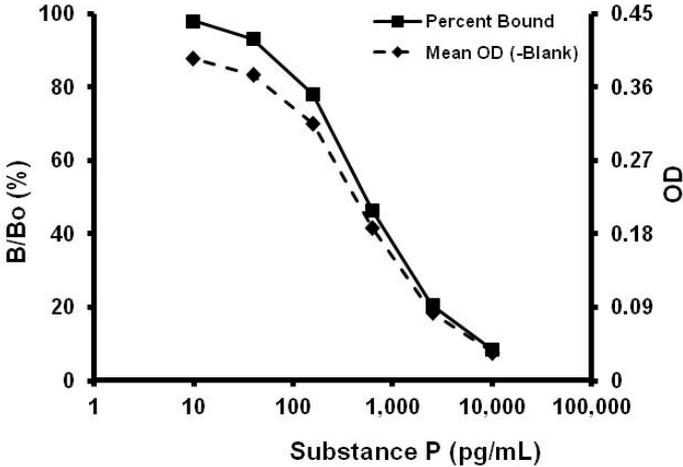
$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net } B_0 \text{ OD}} \times 100$$

- 14.3 Plot the Percent Bound ( $B/B_0$ ) versus concentration of Substance P for the standards. Fit a line through the data. The concentration of Substance P in the unknowns, can be determined by interpolation.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

## 15. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Mean OD (-B <sub>s</sub> )	Percent Bound	Substance P (pg/mL)
B <sub>s</sub>	(0.091)		
TA	0.103		
NSB	0.001	0	
Standard 1	0.035	8.4	10,000
Standard 2	0.084	20.6	2,500
Standard 3	0.187	46.3	625
Standard 4	0.315	78.1	156.25
Standard 5	0.375	93.0	39.06
Standard 6	0.395	98.0	9.76
B <sub>0</sub>	0.403	100	0
Unknown 1	0.097	23.9	1,874
Unknown 2	0.202	50.0	543

## TYPICAL QUALITY CONTROL PARAMETERS

Total Activity Added =  $0.103 \times 10 = 1.03$

%B<sub>0</sub>/TA = 39.0%

Quality of Fit = 1.00

20% Intercept = 2,486 pg/mL

50% Intercept = 547 pg/mL

80% Intercept = 134 pg/mL

## 16. TYPICAL SAMPLE VALUES

### SENSITIVITY –

Sensitivity was calculated in Assay Buffer by determining the average optical density bound for sixteen wells run as Bo, and comparing to the average optical density for sixteen wells run with Standard 6. The detection limit was determined as the concentration of Substance P measured at two standard deviations from the zero along the standard curve. The detection limit was found to be 8.04 pg/mL.

### SAMPLE RECOVERY –

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:

Sample Type	Average % Recovery	Recommended Dilution
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

### LINEARITY OF DILUTION –

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

	<b>Substance P (pg/mL)</b>	<b>Intra-Assay %CV</b>
Low	101	6.7
Medium	1,116	4.5
High	6,257	5.2

### Inter-Assay

	<b>Substance P (pg/mL)</b>	<b>Inter-Assay %CV</b>
Low	97	4.2
Medium	1,120	7.3
High	6,402	7.3

**17. ASSAY SPECIFICITY****CROSS REACTIVITY –**

The cross reaction of the antibody calculated at 50% is:

<b>Compound</b>	<b>Cross Reactivity (%)</b>
Substance P	100
Substance P (3-11)	85.9
Physalaemin	75.3
Substance P (4-11)	11.7
Substance P (7-11)	5.9
$\alpha$ -neurokinin	0.8
$\beta$ -neurokinin	0.2
Somatostatin	<0.001
Substance P (1-4)	<0.001

## 18. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES







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