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New Substrate Incubation Time
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arg^{8}-Vasopressin

Enzyme Immunoassay Kit

Catalog No. 901-017

480 Well (5 by 96) Kit

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Description

Assay Designs' Vasopressin kit Enzyme Immunoassay (EIA) is a competitive immunoassay for the quantitative determination of Vasopressin in samples. The kit uses a polyclonal antibody to Vasopressin to bind, in a competitive manner, the Vasopressin in the standards or sample or an alkaline phosphatase molecule which has Vasopressin covalently attached to it. After a simultaneous incubation at 4°C 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 read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of Vasopressin in either standards or samples. The measured optical density is used to calculate the concentration of Vasopressin. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

ArginineVasopressin (AVP) is a 9 amino acid peptide with a 6-member disulfide ring. It is structurally related to oxytocin differing in 2 amino acids. It is synthesized in the hypothalamus supraoptic and paraventricular nuclei. It is stored in the posterior pituitary for release. AVP has powerful antidiuretic action and is also known as antidiuretic hormone (ADH)[2]. It acts upon the collecting tubule of the kidney increasing permeability to water and urea. It also has neurotransmitter and peripheral humoral functions.

AVP has been shown to be released upon both osmotic and non-osmotic stimuli[4,5], and its release into peripheral blood causes effects upon a number of factors, including emotional stress, posture, blood volume, and temperature[6-9]. Alcohol appears to inhibit AVP secretion. Serum AVP measurement is used clinically for studies involving diabetes insipidus, syndrome of inappropriate ADH secretion (SIADH), ectopic AVP production and pyschogenic water intoxication[10-14].

arg^8-Vasopressin H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

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. CAUSTIC; care should be taken in use.
3. 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.
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 Vasopressin Standard provided, Catalog No. 80-0181, is supplied in buffer at a pH optimized to maintain Vasopressin integrity. Care should be taken in handling this material because of the known and unknown effects of Vasopressin.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plates, 5 Plates of 96 Wells, Catalog No. 80-0060**
   Plates using break-apart strips coated with goat antibody to rabbit IgG.
2. **Vasopressin EIA Conjugate, 25 mL, Catalog No. 80-0262**
   A blue solution of alkaline phosphatase conjugated with Vasopressin.
3. **Vasopressin EIA Antibody, 25 mL, Catalog No. 80-0263**
   A yellow solution of a rabbit polyclonal antibody to Vasopressin.
4. **Assay Buffer Concentrate, 27 mL, Catalog No. 80-0011**
   Tris buffered saline, containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **Vasopressin Standard, 3 x 0.5 mL, Catalog No. 80-0181**
   A solution of 10,000 pg/mL Vasopressin.
7. **p-Npp Substrate, 100 mL, Catalog No. 80-0076**
   A solution of p-nitrophenyl phosphate in buffer. Ready to use.
8. **Stop Solution, 30 mL, Catalog No. 80-0248**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **Vasopressin Assay Layout Sheet, 1 each, Catalog No. 30-0041**
10. **Plate Sealers, 5 each, Catalog No. 30-0012**

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 beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. Adsorbent paper for blotting.
7. Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm.
8. 37 °C incubator.

Sample Handling

Assay Designs' EIA kit is compatible with Vasopressin samples in a number of matrices. Vasopressin samples should be in a matrix similar to the kit Assay Buffer. 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.**

We recommend extraction of samples for accurate determinations of Vasopressin. An extraction protocol is outlined below. Because of the labile nature of Vasopressin we recommend several precautions in collecting and analyzing samples.

Blood samples should be drawn into chilled EDTA (1mg/mL blood) or serum tubes containing Aprotonin (500 KIU/mL of blood). Centrifuge the samples at 1,600 x g for 15 minutes at 4°C. Transfer the plasma or serum to a plastic tube and store at -70°C or lower for long term storage. Avoid repeated freeze/thaw cycles. The stability of some peptides is improved by the addition of a protease inhibitor cocktail to the sample before freezing.

If samples are thought to be lipemic, the following procedure can be used to delipidate prior to extraction.

1. Prepare mixture of 40:60 butanol:diisopropyl ether. Vortex.
2. Add equal volume of butanol:diisopropyl ether to sample. Vortex.
3. Centrifuge at 8,000 x g for 5 minutes.
4. Remove top organic layer and discard. Measure aqueous layer and transfer to new tube.

Extraction Procedure:

1. Add 2x volume of ice cold acetone to sample. Vortex.
2. Centrifuge at 12,000 x g for 20 minutes.
3. Transfer supernatant to new tube.
4. Add 5x volume of ice cold petroleum ether. Vortex.
5. Centrifuge at 10,000 x g for 10 minutes.
6. Discard top ether layer. Carefully transfer remaining aqueous layer to glass tube and dry down under gas.
7. Reconstitute sample with Assay Buffer.

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 a number of methods, including the use of radioactive peptide, or by spiking into paired samples and determining the recovery of this known amount of added Vasopressin.

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 the 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 the addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in the assay results.**

Reagent Preparation

1. Assay Buffer

Prepare the Assay Buffer by diluting 10 mL of the supplied concentrate with 90 mL of deionized water. This can be stored at room temperature for 3 months.

2. Vasopressin Standard

Allow the 10,000 pg/mL Vasopressin standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 900 µL of standard diluent (Assay Buffer or Tissue Culture Medium) into tube #1 and 600 µL into tubes #2 through #7. Add 100 µL of the 10,000 pg/mL standard to tube #1. Vortex thoroughly. Add 400 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3-#7.

The concentration of Vasopressin in tubes #1 through #7 will be 1,000, 400, 160, 64, 25.6, 10.24, and 4.10 pg/mL respectively. See the Vasopressin Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

3. Vasopressin Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C. Avoid repeated freeze/thaws of the aliquots.

4. 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 100 µL of standard diluent (Assay Buffer or Tissue Culture Medium) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells

5. Pipet 50 µL of Assay Buffer into the NSB wells.

6. Pipet 50 µL of the blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 µL of the 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. Tap the plate gently to mix. Seal the plate and incubate at 4°C for 18-24 hours.

9. Empty the contents of the plate and wash the wells 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 the wells and tap the plate dry on a lint free paper towel.

11. Add 5 µL of the blue Conjugate to the TA wells.

12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37°C 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 Vasopressin in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Vasopressin 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:
   Average Net OD = Average Bound OD - 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 versus Concentration of Vasopressin for the standards. Approximate a straight line through the points. The concentration of Vasopressin in the unknowns can be determined by interpolation.

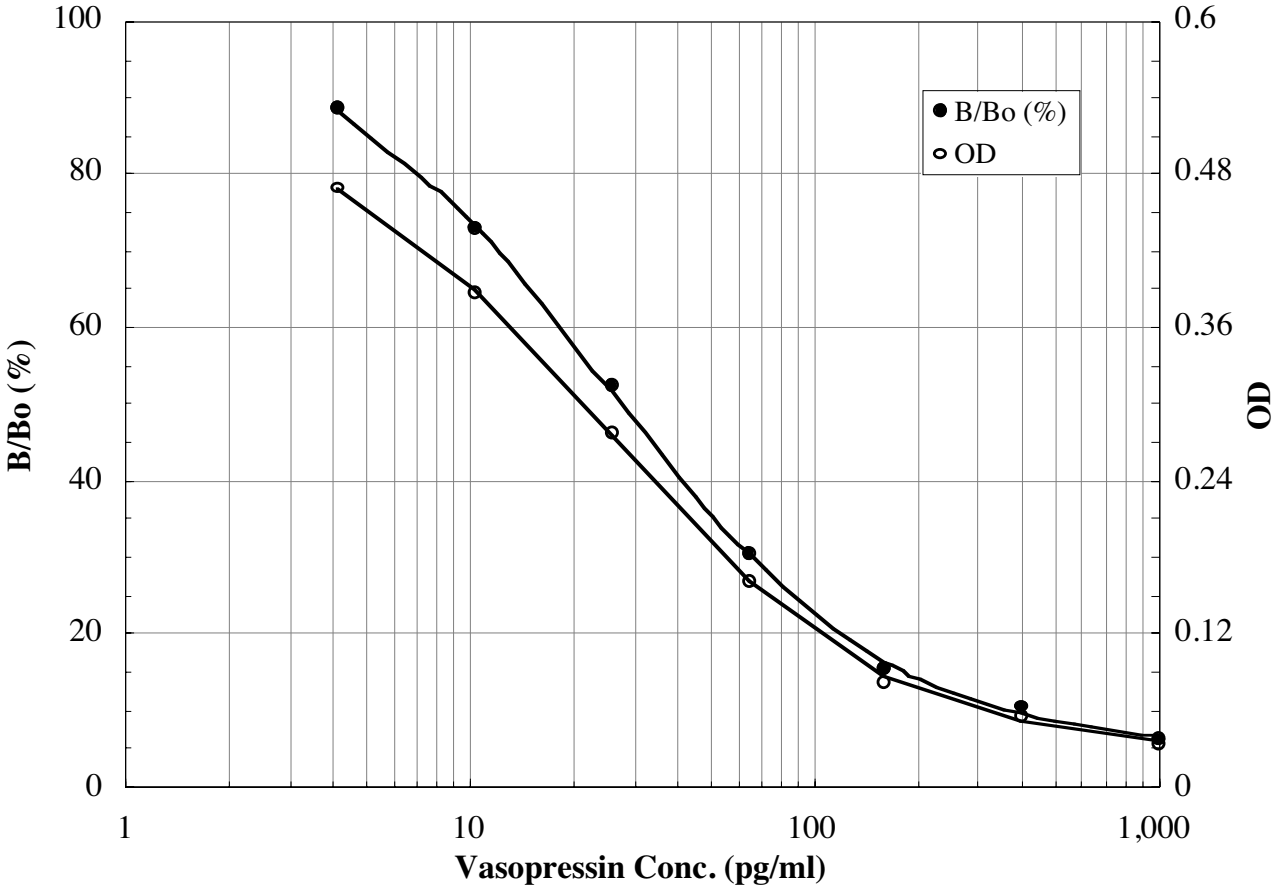
Typical Results

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

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	Vasopressin (pg/mL)
Blank OD	(0.109)			
TA	1.113	1.004		
NSB	0.123	0.014	0.00%	
Bo	0.652	0.529	100%	**0**
S1	0.156	0.033	6.2%	**1,000**
S2	0.179	0.056	10.5%	**400**
S3	0.205	0.082	15.50%	**160**
S4	0.285	0.162	30.6%	**64**
S5	0.401	0.278	52.6%	**25.6**
S6	0.510	0.387	73.2%	**10.24**
S7	0.593	0.470	88.8%	**4.10**
Unknown 1	0.292	0.169	31.9%	**59.7**
Unknown 2	0.513	0.390	73.7%	**10.3**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Vasopressin concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	1.004 x 10 = 10.04
%NSB	=	0.1%
%Bo/TA	=	5.3%
Quality of Fit	=	1.000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	119.2 pg/mL
50% Intercept	=	27.4 pg/mL
80% Intercept	=	7.3 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 Protocols[15].

Sensitivity

Sensitivity was calculated 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 #7. The detection limit was determined as the concentration of Vasopressin measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo =	0.319 ± 0.012 (3.78%)
Average Optical Density for Standard #7 =	0.290 ± 0.007 (2.45%)
Delta Optical Density (0-4.1 pg/mL) = 0.319 - 0.290 =	0.029
2 SD's of the Zero Standard = 2 x 0.012 =	0.024
Sensitivity = $\frac{0.024}{0.029}$ x 4.1 pg/mL =	**3.39 pg/mL**

Linearity

A sample containing 1,000 pg/mL Vasopressin was serially diluted 4 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Vasopressin concentration versus measured Vasopressin concentration.

The line obtained had a slope of 1.133 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Vasopressin and running these samples multiple times (n=20) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Vasopressin in multiple assays (n=6).

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

	AVP (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	6.31	10.2	
Medium	56.4	5.9	
High	260.6	10.6	
Low	5.54		8.5
Medium	46.0		6.4
High	194.5		6.0

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 10,000 to 1 pg/mL. These samples were then measured in the Vasopressin assay, and the measured Vasopressin 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
Arg^{8}-Vasopressin	100%
Lys^{8}-Vasopressin	3.6%
Oxytocin	<0.001%
TRH	<0.001%
VIP	<0.001%
Leu-Enkephalin	<0.001%
Met-Enkephalin	<0.001%
Mesotocine	<0.001%
Somatostatin	<0.001%
Vasotocin	<0.001%
Desmopressin	<0.001%
Arg^{8}-Vasotocin	<0.001%
Ser^{4}, Ile^{8}-Oxytocin	<0.001%

Sample Recoveries

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

Vasopressin concentrations were measured in tissue culture media. Vasopressin was spiked into the undiluted media which was diluted with the kit Assay Buffer and then assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	113.3	1:2

* See Sample Handling instructions on page 4 for details.

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

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