

Product Information & ELISA Manual

Human Antidiuretic Hormone ELISA Kit
(Colorimetric)
NBP2-62140

Enzyme-linked Immunosorbent Assay
for quantitative detection.

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Novus kits are
guaranteed for 6 months
from date of receipt.

**For research use only.
Not for diagnostic or
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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



Please contact Technical Support if necessary.

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For proper performance, use the insert provided with each individual kit received.

DESCRIPTION

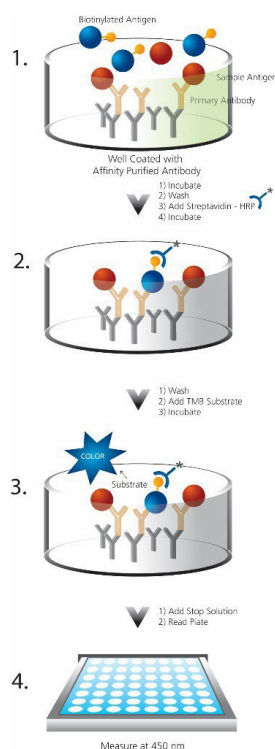
The Antidiuretic Hormone ELISA kit is a competitive immunoassay for the quantitative determination of Antidiuretic Hormone in plasma, serum, and tissue culture media. Please read the entire kit manual before performing the assay. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

INTRODUCTION

Arginine Vasopressin (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 temperature⁶⁻⁹. 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 psychogenic water intoxication¹⁰⁻¹⁴.

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PRINCIPLE



1. Standards and samples are added to wells coated with a goat-anti-rabbit IgG antibody. A blue solution of Antidiuretic Hormone conjugated to biotin is added, followed by a yellow solution of a rabbit polyclonal antibody to Antidiuretic Hormone.
2. The plate is incubated overnight at 4°C. During this incubation the specific antibody binds, in a competitive manner, the Antidiuretic Hormone in the sample or conjugate. The plate is then washed, leaving only bound Antidiuretic Hormone or the biotinylated Vasopressin conjugate.
3. A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind the biotinylated Antidiuretic Hormone. The plate is incubated shaking at room temperature.
4. The plate is washed to remove unbound HRP conjugate. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at **450nm**. The amount of signal is inversely proportional to the concentration of Antidiuretic Hormone in the sample.

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The standard should be handled with care due to the known and unknown effects of the antigen.



Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.



Protect TMB substrate from prolonged exposure to light.



Stop Solution 2 is caustic. Keep tightly capped.

MATERIALS SUPPLIED

1. **Goat anti-Rabbit IgG Clear Microtiter Plate, Five Plates of 96 Wells**

Plates using break-apart strips coated with goat antibody specific to rabbit IgG.

2. **Assay Buffer 28, 100ml**

Phosphate buffered saline containing BSA and detergent.

3. **Antidiuretic Hormone Standard, 3 x 0.5ml**

A solution of 10,000pg/ml Antidiuretic Hormone.

4. **Antidiuretic Hormone Conjugate, 25ml**

A blue solution of biotin conjugated with Antidiuretic Hormone.

5. **Antidiuretic Hormone Antibody, 25ml**

A yellow solution of a rabbit polyclonal antibody to Antidiuretic Hormone.

6. **Wash Buffer Concentrate, 100ml**

Tris buffered saline containing detergents.

7. **SA-HRP, 100ml**

A solution of streptavidin conjugated to horseradish peroxidase.

8. **TMB Substrate, 100ml**

A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide.

9. **Stop Solution 2, 50ml**

A 1N solution of hydrochloric acid in water.

10. **Antidiuretic Hormone Assay Layout Sheet, 1 each**

11. **Plate Sealer, 5 each**

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Reagents
require
separate
storage
conditions.

STORAGE

All components of this kit, **except the standard**, are stable at 4°C until the kit's expiration date. Upon receipt, the standard must be stored frozen at -20°C. Storage conditions do not necessarily reflect shipping conditions.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 5µl and 1,000µl.
3. Repeater pipets for dispensing 50µl and 200µl.
4. Disposable beaker for diluting buffer concentrates.
5. Graduated cylinders.
6. Lint-free paper for blotting.
7. Microplate reader capable of reading at 450nm.
8. A microplate shaker.
9. Nitrogen gas (required for the suggested extraction protocol)
10. Acetone (required for the suggested extraction protocol)
11. Petroleum Ether (required for the suggested extraction protocol)
12. Butanol (optional for the suggested extraction protocol)
13. Diisopropyl ether (optional for the suggested extraction protocol)

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Sample handling procedures should be completed prior to reagent preparation.



Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/thaw cycles.



If the end user chooses to vary from the extraction procedure noted, it is up to the end user to determine the appropriate dilution of samples and assay validation.

SAMPLE HANDLING

The Antidiuretic Hormone ELISA kit is compatible with Antidiuretic Hormone samples in a number of matrices. Antidiuretic Hormone samples should be reconstituted in kit Assay Buffer 28 for extracted serum and plasma samples and diluted into Assay Buffer 28 for tissue culture media samples. Please refer to the Sample Recovery recommendations on page 15 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.

Due to the low endogenous levels of native Antidiuretic Hormone we recommend extraction of samples, thereby concentrating them and allowing for accurate determinations of Antidiuretic Hormone. 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 Aprotinin (500 KIU/ml of blood). Centrifuge the samples at $1,600 \times g$ 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 $8,000 \times g$ 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 $3,000 \times g$ for 20 minutes.
3. Transfer supernatant to new tube.
4. Add 5x volume of ice cold petroleum ether. Vortex.
5. Centrifuge at $3,000 \times g$ 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.

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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, such as spiking into paired samples and determining the recovery of this known amount of added Antidiuretic Hormone.

Extraction efficiencies for matrices tested are listed below. For each matrix listed, high, middle, and low concentrations of Antidiuretic Hormone were spiked into the matrix, then extracted as per the sample extraction protocol and read in the assay. The same concentrations of Antidiuretic Hormone were spiked into the kit Assay Buffer 28, without performing the extraction protocol, for comparison purposes. The efficiency of extraction was calculated as the amount returned off the standard curve divided by the amount returned for the Assay Buffer 28 samples x 100 (after normalizing for endogenous levels).

Assay Buffer 28 Extraction Efficiency			
Antidiuretic Hormone Spike Level	Spiked Extracted (pg/mL)	Spiked Non-Extracted (pg/mL)	Percent Extraction Recovery
High	214	305	70.2%
Medium	100	140	71.7%
Low	46	61	75.3%

Human Plasma EDTA Extraction Efficiency			
Antidiuretic Hormone Spike Level	Spiked Extracted (pg/mL)	Percent Extraction Recovery	Plasma vs. AB 28 Extraction Efficiency
High	125	40.90%	58.30%
Medium	93	66.50%	92.70%
Low	36	59.20%	78.60%
Human Serum Extraction Efficiency			
Antidiuretic Hormone Spike Level	Spiked Extracted (pg/mL)	Percent Extraction Recovery	Plasma vs. AB 28 Extraction Efficiency
High	197	64.40%	91.80%
Medium	93	66.70%	93.00%
Low	28	46.10%	61.20%

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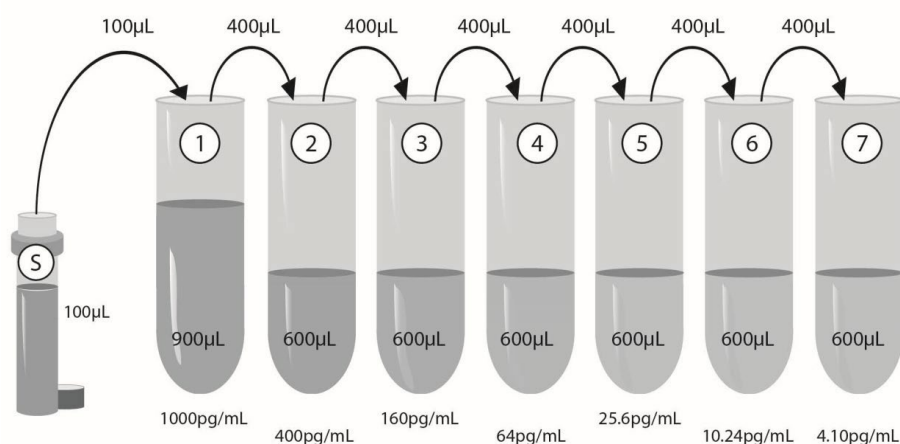
REAGENT PREPARATION



Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

1. Antidiuretic Hormone Standard

Allow the 10,000 pg/ml Antidiuretic Hormone standard solution to warm to room temperature. Label seven 12 x 75mm tubes #1 through #7. Pipet 900µl of Assay Buffer 28 into tube #1 and 600µl into tubes #2 through #7. Add 100µl of the 10,000 pg/ml standard to tube #1. Vortex thoroughly. Add 400µl of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 -#7.



Diluted standards should be used within 60 minutes of preparation. The concentrations of Antidiuretic Hormone in the tubes are labeled above.

2. Wash Buffer

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

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Bring all reagents to room temperature for at least 30 minutes prior to opening.



Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.



Pipet standards & samples to the bottom of the wells.



All standards and samples should be run in duplicate.



Prior to the addition of the SA-HRP and substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

ASSAY PROCEDURE

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.

1. Add 100µl of Assay Buffer 28 into the NSB and the Bo (0 pg/ml Standard) wells.
2. Add 100µl of Standards #1 through #7 into the appropriate wells.
3. Add 100µl of the Samples into the appropriate wells.
4. Add an additional 50µl of Assay Buffer 28 into the NSB wells.
5. Add 50µl of the blue Conjugate into each well, except the Blank wells.
6. Add 50µl of the yellow Antibody into each well, except the Blank and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank wells are empty at this point and have no color.

7. Tap the plate gently to mix. Seal the plate and incubate at 4°C for 18-24 hours.
8. Empty the contents of the plate and wash by adding full well volume (~400 µl) of wash solution to every 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.
9. Add 200µl of the SA-HRP to every well, except the Blank wells.
10. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm*. Wash as above (step 8).
11. Add 200µl of the TMB Substrate to every well.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm*.
13. Add 100µl of Stop Solution 2 to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, and read the optical density at **450nm**. 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.

* The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). The actual speed of the plate shaker should be such

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that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.



Samples with concentrations reading outside of the standard curve range will need to be re-analyzed using a different dilution or more concentrated extract.



Make sure to multiply sample concentrations by the dilution factor used during sample analysis.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Antidiuretic Hormone 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 Antidiuretic Hormone 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:

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

2. Plot the Net OD versus the Concentration of Antidiuretic Hormone for the standards. Sample concentrations of Antidiuretic Hormone may be calculated by interpolation off the standard curve using Net OD values.
3. Alternatively, calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = (\text{Net OD} / \text{Net Bo OD}) \times 100$$

4. Plot Percent Bound versus Concentration of Antidiuretic Hormone for the standards. The concentration of Antidiuretic Hormone in the unknowns may then also be determined by interpolation off of the binding curve.

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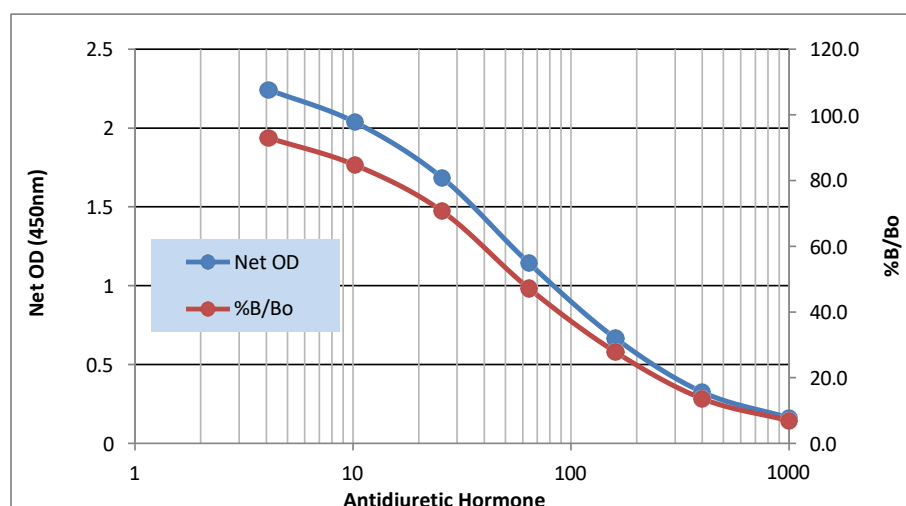
TYPICAL RESULTS

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

Sample	Antidiuretic Hormone (pg/mL)	Net Optical Density (450 nm)	%B/Bo
Bo	0	2.419	---
S1	1000	0.162	6.9
S2	400	0.325	13.6
S3	160	0.666	27.8
S4	64	1.144	47.2
S5	25.6	1.685	70.7
S6	10.24	2.032	84.7
S7	4.096	2.243	93.0
NSB	---	0.031	---

TYPICAL STANDARD CURVES

Typical standard curves are shown below. These curves must not be used to calculate Antidiuretic Hormone concentrations; each user must run a standard curve for each assay.



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PERFORMANCE CHARACTERISTICS

Sensitivity

The sensitivity of the assay was determined by interpolation from the average of 9 separate standard curves run with replicate data points at each concentration. The sensitivity was determined at 2 standard deviations below the average net OD of 54 zero standard replicates (6 per standard curve). The sensitivity (limit of detection) of the assay is 2.84 pg/ml.

Linearity

A buffer sample containing Antidiuretic Hormone was serially diluted 5 times 1:2 in the kit Assay Buffer and measured in the assay. The results follow:

Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
Neat	---	390.8	---
1:2	195.4	194.6	100%
1:4	97.7	105.1	108%
1:8	48.8	67.0	137%
1:16	24.4	27.9	114%
1:32	12.2	12.1	99%

The data was plotted graphically as expected Antidiuretic Hormone concentration versus observed Antidiuretic Hormone concentration. The line obtained had a slope of 1.0162 with a correlation coefficient of 0.9895.

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Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Antidiuretic Hormone and running these samples multiple times (n=22) in the same assay.

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

Intra-assay precision	
pg/mL	%CV
143.7	6.0%
70.7	6.7%
32.1	14.3%

Inter-assay precision was determined by measuring three samples with low, medium and high concentrations in multiple assays (n=13) over several days.

Inter-assay precision	
pg/mL	%CV
136.2	8.6%
66.4	6.4%
33.0	9.5%

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Cross Reactivities

The % cross reactivity for each related compound was determined by running serial dilutions of each compound (10,000 pg/mL – 10 pg/mL) in the assay, fitting the resulting dose response curve to 4PL curve and determining the ED50. The ED50 of the standard curve was then divided by the determined ED50 of the cross-reactant and multiplied by 100.

Analyte	Cross Reactivity
Antidiuretic Hormone	100%
Lys ⁸ -Vasopressin	9.8%
Oxytocin	<0.001%
TRH	<0.001%
VIP	<0.001%
Leu-Enkephalin	<0.001%
Met-Enkephalin	<0.001%
Mesotocin	<0.001%
Cyclo-Somatostatin	<0.001%
Vasotocin	4.8%
Desmopressin	3.1%
Ser ⁴ , Ile ⁸ -Oxytocin	<0.001%

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SAMPLE RECOVERIES

Please refer to pages 6-8 for Sample Handling recommendations and Standard preparation. Sample recovery dilutions should only be used to remove matrix interference in media such as Tissue Culture Media.

Plasma and serum samples will need to be extracted and reconstituted in Assay Buffer 28. Upon reconstitution, no further dilution is necessary.

Antidiuretic Hormone concentrations were measured in tissue culture media. Antidiuretic Hormone was spiked into the undiluted media and measured neat or following dilution with Assay Buffer 28. Control spikes into Assay Buffer 28 were also measured. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	116.2%	no dilution needed
Tissue Culture Media with 10% bovine serum	100.0%	1:2
Assay Buffer 28	111.4%	no dilution needed

*See Sample Handling instructions on page 6-7 for details.

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