



Oxytocin

Enzyme Immunoassay Kit

Catalog No. 901-153

480 Well (5 by 96 Well) Kit

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Description

The Assay Designs' Oxytocin Enzyme Immunoassay (EIA) kit is a competitive immunoassay for the quantitative determination of Oxytocin in samples. The kit uses a polyclonal antibody to Oxytocin to bind, in a competitive manner, the Oxytocin in the sample or an alkaline phosphatase molecule which has Oxytocin 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 Oxytocin in either standards or samples. The measured optical density of the standards is used to calculate the concentration of Oxytocin in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Oxytocin is a neurohypophysial peptide which is produced in the paraventricular nuclei of the hypothalamus and stored in the posterior pituitary. The molecule consists of nine amino acids linked with a [1-6] disulfide bond and a semi-flexible carboxyamidated tail. A hormone once thought to be limited to female smooth muscle reproductive physiology, more current findings have determined that oxytocin also functions as a neurotransmitter^{1,2}, may be involved in neuropsychiatric disorders³, social/sexual behavior⁴ and is important in male reproductive physiology^{5,6}. Oxytocin and the related neurohypophysial peptide, Arg⁸-Vasopressin, maintain renal water and sodium balance⁷.

Oxytocin H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂

Highly conserved across species boundaries, oxytocin-like neurohypophysial peptides are substituted primarily at residues 4 and/or 8. In the oxytocin-like peptide, mesotocin, a common peptide found in some fishes, reptiles, amphibians, marsupials and nonmammalian tetrapods, the leucine at residue 8 is substituted for isoleucine⁸. Acting in classical endocrine fashion, Oxytocin elicits regulatory effects by binding specific cell surface receptors which in turn initiate a secondary intracellular response cascade via a phosphoinositide signaling pathway⁸.

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. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ 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 Oxytocin Standard provided, Catalog No. 80-0251, is supplied in ethanolic buffer at a pH optimized to maintain Oxytocin integrity. Care should be taken in handling this material because of the known and unknown effects of Oxytocin.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, Five 96 Well Plates, Catalog No. 80-0060**
Plates using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **Oxytocin Conjugate, 25 mL, Catalog No. 80-0252**
A blue solution of alkaline phosphatase conjugated with Oxytocin.
3. **Oxytocin Antibody, 25 mL, Catalog No. 80-1495**
A yellow solution of a rabbit polyclonal antibody to Oxytocin.
4. **Assay Buffer Concentrate, 27 mL, Catalog No. 80-1590**
Buffer containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
Tris buffered saline containing detergents.
6. **Oxytocin Standard, 3 x 0.5 mL, Catalog No. 80-0251**
A solution of 10,000 pg/mL Oxytocin.
7. **pNpp Substrate, 100 mL, Catalog No. 80-0076**
A solution of p-nitrophenylphosphate 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. **Plate Sealer, 5 each, Catalog No. 30-0012**
10. **Oxytocin Assay Layout Sheet, 1 each, Catalog No. 30-0233**

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 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 between 570 and 590 nm.

Sample Handling

Assay Designs' EIA is compatible with Oxytocin samples in a number of matrices. Oxytocin samples diluted sufficiently into the kit Assay Buffer can be read directly from the standard curve. Samples in the majority of tissue culture media, including those containing fetal bovine serum, 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 small change in binding associated with running the standards and samples in media. Please refer to the Sample Recovery recommendations on page 13 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.**

The Extraction protocol below is strongly recommended for all serum and plasma samples in addition to all other samples that cannot be sufficiently diluted to avoid matrix interference without being too dilute to measure. Data illustrating the benefits of extraction are outlined below.

<u>Sample</u>	<u>Neat, Unspiked (pg/mL)</u>	<u>Extracted, Unspiked (pg/mL)</u>	<u>Extracted, Spiked (200 pg/mL)</u>	<u>Extraction Efficiency</u>
Human EDTA Plasma	2,761	3.4	169	83
Human Serum	106	30	236	103

Because of the labile nature of Oxytocin we recommend several precautions in collecting and analyzing samples. Blood samples should be drawn into chilled serum or EDTA (1mg/mL blood) tubes containing Aprotinin (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.

Extraction of the sample should be carried out using a similar protocol to the one described below. For a 200 mg C₁₈ Sep-Pak column, we suggest a sample volume no greater than 3 mL.

1. Add an equal volume of 0.1% 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 0.1% TFA in water.
3. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 0.1% TFA in water. Discard wash.
4. Elute the sample slowly by applying 3 mL of acetonitrile: 0.1% 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. Upon reconstitution, an insoluble material may be observed in some samples. Care should be taken to avoid this material when adding sample to plate.

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 Oxytocin.

Procedure for clarification of milk

1. Centrifuge the sample at 10,000 rcf for 15 minutes.
2. Using a syringe, carefully pierce through the top layer and aspirate the lower layer of supernatant.
3. Centrifuge supernatant at 10,000 rcf for 15 minutes.
4. Repeat #2 and centrifuge resulting supernatant at 10,000 rcf for 15 minutes. Sample should now be ready to assay, following dilution in Assay Buffer.read immediately.

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

Reagent Preparation

1. Assay Buffer

Just before use, prepare the assay buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. **Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.**

2. Oxytocin Standard

Allow the 10,000 pg/mL Oxytocin standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 1 mL of standard diluent (Assay Buffer or Tissue Culture Medium) into tube #1. Pipet 500 μ L of standard diluent into tubes #2 through #7. Remove 100 μ L of diluent from tube #1. Add 100 μ L of the 10,000 pg/mL standard to tube #1. Vortex thoroughly. Add 500 μ L of tube #1 to tube #2 and vortex thoroughly. Add 500 μ L of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #7.

The concentration of Oxytocin in tubes #1 through #7 will be 1,000, 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL respectively. See Oxytocin Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.

3. Oxytocin Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C .

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 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 wells and wash by adding 400 μL of wash solution to each well. Repeat the wash 2 more times for a total of **3 Washes**.
10. After the final wash, empty or aspirate the wells, and firmly tap the plate dry on a lint free paper towel to remove any remaining wash buffer.
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 room temperature 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 Oxytocin 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 Oxytocin 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. 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 Oxytocin for the standards. Approximate a straight line through the points. The concentration of Oxytocin 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	Oxytocin (pg/mL)
Blank OD	(0.083)			
TA	0.929			
NSB	-0.004	0.000	0%	
Bo	0.484	0.488	100%	0
S1	0.056	0.060	12.3%	1,000
S2	0.098	0.102	20.9%	500
S3	0.159	0.163	33.4%	250
S4	0.235	0.239	48.9%	125
S5	0.326	0.330	67.6%	62.5
S6	0.392	0.396	81.1%	31.2
S7	0.443	0.447	91.6%	15.6
Unknown 1	0.142	0.146	29.9%	300
Unknown 2	0.430	0.434	88.9%	20

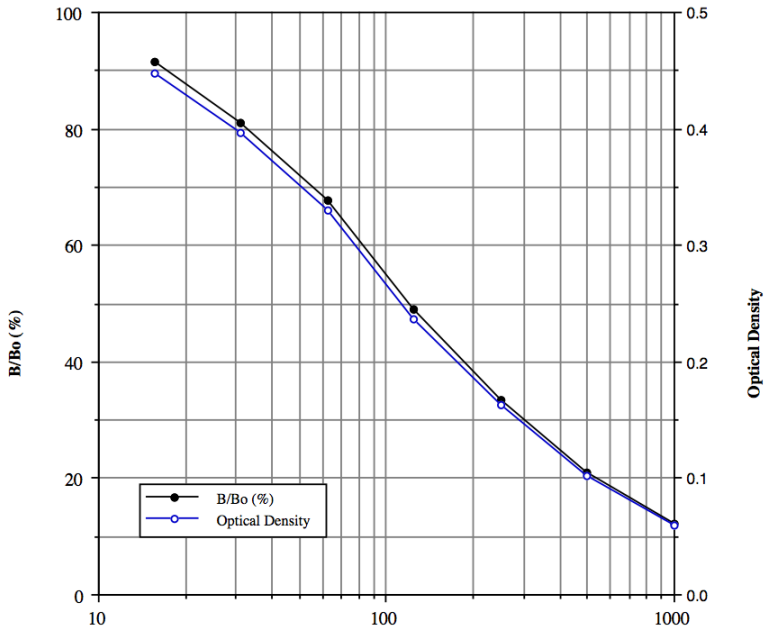
Calibration

Calibration to the NIBSC/WHO Oxytocin 4th International Standard 76/575 has been determined. To convert sample values obtained in the Oxytocin EIA Kit to this NIBSC/WHO Oxytocin Standard, use the equation below.

$$\text{NIBSC/WHO 76/575 value (pg/mL)} = \text{Obtained Oxytocin value (pg/mL)} \times 0.84.$$

Typical Standard Curves

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



Typical Quality Control Parameters

Total Activity Added	=	$0.929 \times 10 = 9.29$
%NSB	=	0.0%
%Bo/TA	=	5.25%
Quality of Fit	=	1.0000 (Calculated from 4 parameter logistics curve fit)
20% Intercept	=	519 pg/mL
50% Intercept	=	123 pg/mL
80% Intercept	=	34 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 Protocols¹¹.

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 Oxytocin measured at two (2) standard deviations from the zero along the standard curve.

$$\text{Average Optical Density for the Bo} = 0.490 \pm 0.025$$

$$\text{Average Optical Density for Standard \#7} = 0.423 \pm 0.027$$

$$\text{Delta Optical Density (0-15.62 pg/mL)} = 0.490 - 0.423 = 0.067$$

$$2 \text{ SD's of the Zero Standard} = 2 \times 0.025 = 0.050$$

$$\text{Sensitivity} = \frac{0.050}{0.067} \times 15.62 \text{ pg/mL} = \mathbf{11.7 \text{ pg/mL}}$$

Linearity

A sample containing 800 pg/mL Oxytocin was serially diluted five (5) times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Oxytocin concentration versus measured Oxytocin concentration.

The line obtained had a slope of 0.99959 with a correlation coefficient of 1.0626.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Oxytocin and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Oxytocin in multiple assays (n=8).

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

	Oxytocin (pg/mL)	Intra Assay <u>%CV</u>	Inter Assay <u>%CV</u>
Low	21.4	9.1	
Medium	81.2	8.7	
High	300.6	12.4	
Low	19.3		14.5
Medium	84.7		8.7
High	300.3		5.2

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 Oxytocin assay, and the measured Oxytocin 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.

<u>Compound</u>	<u>Cross Reactivity</u>	<u>Compound</u>	<u>Cross Reactivity</u>
Mesotocin	131%	Somatostatin	< 0.2%
Oxytocin	100%	Met-Enkephalin	< 0.2%
Arg ⁸ -Vasotocin	42.2%	VIP	< 0.2%
Oxytocin-SH	15.4%	Lys ⁸ -Vasopressin	< 0.2%
Arg ⁸ -Vasopressin-SH	0.6%	Arg ⁸ -Vasopressin	< 0.2%
Lys ⁸ -Vasopressin -SH	0.6%	α-ANP	< 0.2%
Ser ⁴ , Ile ⁸ -Oxytocin	< 0.2%	Growth Hormone	< 0.2%
TRH	< 0.2%	Tocinoic acid	< 0.2%
		Melanostatin	< 0.2%

Sample Recoveries

Please refer to page 4 for Sample Handling recommendations.

Oxytocin concentrations were measured in a variety of different samples including tissue culture media and human breast milk. Oxytocin was spiked into the undiluted samples which were diluted with the appropriate diluent and then assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	102	Neat
Human Breast Milk	95	1:2

* See Sample Handling instructions on page 4 for details.

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Notes

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LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

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

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