Product Manual

Oxytocin ELISA kit

Catalog #: ADI-901-153A

5x96 Well Kit

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Product Manual

Please read entire booklet before proceeding with the assay.

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

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

The Oxytocin ELISA 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 standard or 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 is used to calculate the concentration of oxytocin. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

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 disorders[3], social/sexual behavior[4] and is important in male reproductive physiology[5,6]. Oxytocin and the related neurohypophysial peptide, Arg[8]-Vasopressin, maintain renal water and sodium balance[7].

Oxytocin: H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

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 isoleucine[8]. 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 pathway[8].

PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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 with its 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 (>10mM) such as EDTA and EGTA.
4. We tested this kit's performance with a variety of samples however it is possible that high levels of interfering substances may cause variations in assay results.
5. The Oxytocin Standard provided (Catalog No. 80-0251) is supplied in an ethanol containing 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, Component number 80-0060**

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

2. **Oxytocin Conjugate, 25 mL, Component number 80-0252**

   A blue solution of alkaline phosphatase conjugated with oxytocin.

3. **Oxytocin Antibody, 25 mL, Component number 80-2555**

   A yellow solution of a rabbit polyclonal antibody to oxytocin.

4. **Assay Buffer Concentrate, 27 mL, Component number 80-1545**

   Buffer containing proteins and sodium azide as preservative.

5. **Wash Buffer Concentrate, 100 mL, Component number 80-1287**

   Tris buffered saline containing detergents.

6. **Oxytocin Standard, 3 x 0.5 mL, Component number 80-0251**

   A solution of 10,000 pg/mL oxytocin.

7. **pNpp Substrate, 100 mL, Component number 80-0076**

   A solution of p-nitrophenylphosphate in buffer. Ready to use.

8. **Stop Solution, 30 mL, Component number 80-0248**

   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**

9. **Plate Sealer, 5 each, Component number 30-0012**

10. **Oxytocin Assay Layout Sheet, 1 each**
    Component number 30-0312

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 upon receipt. Please note that shipping conditions may not reflect long-term storage requirements.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1000 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.
8. Acetonitrile, anhydrous (>99%)
9. Trifluoroacetic acid (>99%)
10. C-18 resin cartridge/column (such as Waters Sep-Pak® 200 mg, product number WAT054945)

SAMPLE HANDLING

The Oxytocin ELISA is compatible with samples from 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 a small change in binding associated with running the standards and samples in media. Please refer to the Sample Recovery recommendations on page 17 for details of suggested dilutions. The user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing rabbit IgG may interfere with the assay.**

The extraction protocol outlined on page 8 is strongly recommended for all sample matrices that cannot be sufficiently diluted to avoid matrix interference without being too dilute to measure. Additionally, extraction of samples can serve to concentrate the analyte for aid in measurement.

Extraction efficiencies for a variety of sample matrices are listed below. For each matrix listed, 200pg/mL of oxytocin was spiked into the matrix, then extracted as per the sample extraction protocol and read in the assay. The efficiency of extraction was calculated as the amount returned off the standard curve divided by the theoretical amount (200pg/mL) x 100.

Sample	Extracted (pg/mL)	200 pg/mL Spiked, Extracted (pg/mL)	Extraction Efficiency
Human Serum	71.8	313.1	121%
Human Plasma	49.8	285.0	118%
Human Saliva	7.3	212.3	102%
Human Breast Milk	8.1	155.1	74%
Human Urine	21.3	218.7	99%
Human Cerebrospinal Fluid	42.5	269.5	113%
Conditioned Media	72.8	233.2	80%

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 (1 mg/mL blood) tubes containing Aprotinin (500 KIU/mL of blood). Centrifuge the samples at 1600 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.

OXYTOCIN EXTRACTION PROTOCOL

For a 200 mg C18 column we suggest a sample volume no greater than 3 mL.

1. Add an equal volume of 0.1% trifluoroacetic acid (TFA) in water (TFA-H_2O) to the sample. Centrifuge at 17000 g for 15 minutes at 4°C to clarify and save the supernatant.
2. Equilibrate a 200 mg C18 Sep-Pak column with 1ml of acetonitrile, followed by 10-25mL of 0.1% TFA-H_2O.
3. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 0.1% TFA-H_2O. Discard wash.
4. Elute the sample slowly (gravity-fed) by applying 3 mL of a solution comprised of 95% acetonitrile/5% of 0.1% TFA-H_2O. Collect the eluate in a plastic tube.
5. Evaporate to dryness under argon or nitrogen gas or with the aid of a centrifugal concentrator under vacuum. Evaporation under cold temperature is recommended. Store at -20°C.
6. Reconstitute with Assay Buffer and measure immediately. **You will need to have at least 250 µL volume (upon reconstitution) per sample in order to have enough material to run duplicates (n=2 per sample).** Please note that upon reconstitution insoluble material may be observed in some samples. Care should be taken to avoid this material when adding sample to plate wells.

   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.

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 variations 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 tubes #1 through #7. Transfer 1 mL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 500 µL of standard diluent into tubes #2 through #7. Remove 100 µL of buffer from tube #1. Add 100 µL of the 10,000 pg/mL standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL 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 divided into discrete aliquots 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 Media) into the NSB and the B_0 (0pg/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 out 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 four parameter logistic curve fitting program.

The concentration of Oxytocin 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. 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. Plot the average Net OD versus concentration of Oxytocin for the standards in order to generate a standard curve. Fit a line through the data points (4PL curve fit is suggested). The concentration of Oxytocin in the samples can be determined by interpolation off of the standard curve.

Samples that read outside of the standard curve range will need to be re-analyzed using a different dilution, or will require further concentration of the sample during the extraction protocol.

Be sure to multiple sample concentrations by the dilution factor used during sample preparation, or divide by the amount they were concentrated during extraction.

TYPICAL RESULTS

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

Sample	Mean OD (-Blank)	Net OD	Percent Bound	Oxytocin (pg/mL)
Blank OD	(0.073)			
TA	0.803			
NSB	0.002	0.000	0%	
B_0	0.609	0.607	100%	**0**
S1	0.160	0.158	26.0%	**1000**
S2	0.222	0.220	36.2%	**500**
S3	0.294	0.292	48.1%	**250**
S4	0.377	0.375	61.8%	**125**
S5	0.449	0.447	73.6%	**62.5**
S6	0.517	0.515	84.8%	**31.2**
S7	0.557	0.555	91.4%	**15.6**
Unknown 1	0.244	0.242	39.9%	**397**
Unknown 2	0.359	0.357	58.8%	**145**

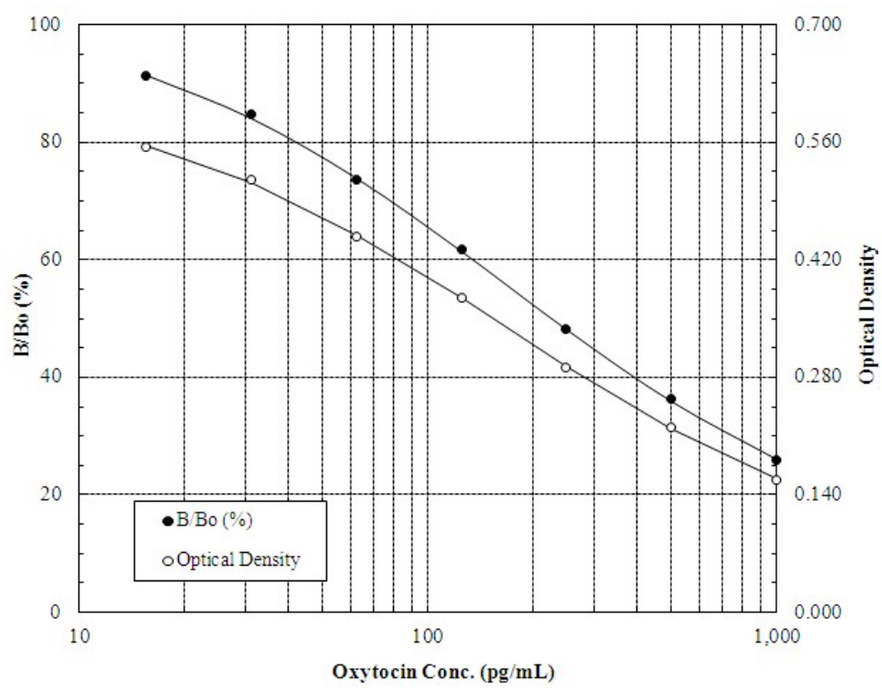
CALIBRATION

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

NIBSC/WHO 76/575 value (pg/mL) = Obtained oxytocin value (pg/mL) x 0.90.

TYPICAL STANDARD CURVES

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



TYPICAL QUALITY CONTROL PARAMETERS

Quality of Fit	=	1.0000*
20% Intercept	=	1791 pg/mL
50% Intercept	=	228 pg/mL
80% Intercept	=	43 pg/mL

(*Calculated from 4 parameter logistics curve fit)

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[11].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for forty-eight (48) wells run as B_0, and comparing to the average optical density for forty-eight (48) 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.

Average Optical Density for the B_0 = 0.663 ± 0.026

Average Optical Density for Standard #7 = 0.609 ± 0.024

Delta Optical Density (B_0-15.62 pg/mL) = 0.663-0.609 =0.054

2 SD's of the Zero Standard = 2 x 0.026 = 0.052

$$\text{Sensitivity} = \frac{0.052}{0.054} \times 15.62 \text{ pg/mL} = \mathbf{15.0\ pg/mL}$$

Linearity

A serum sample spiked with 200 pg/mL oxytocin was extracted then reconstituted at the same volume with assay buffer. Next the sample was serially diluted 1:2 with Assay Buffer and measured in the assay. The recovered spiked concentration was determined from a standard curve and the sample determined to be linear within a range of 100% ± 15% relative to a designated dilution.

Dilution Factor	Recovered Spike Concentration (pg/mL)	Dilutional Linearity
1	233.1	91
2	275.0	104
4	262.8	100
8	283.9	106

Precision

Intra-assay precision was determined by assaying 20 replicates of three controls containing oxytocin in a single assay.

Oxytocin (pg/mL)	%CV
39.9	12.6
121.4	10.2
363.7	13.3

Inter-assay precision was determined by measuring controls of varying oxytocin concentrations in multiple assays (n=17) over several days.

Oxytocin (pg/mL)	%CV
47.0	20.9
145.1	16.5
397.2	11.8

Cross Reactivity

A number of related compounds to oxytocin were dissolved in Assay Buffer and serially diluted to concentrations of 10,000 to 0.6 pg/mL (compound purity having been checked by NMR and other analytical methods). These samples were then measured in the oxytocin assay. Percent cross reactivity was calculated by comparing the EC_{50} of the compound to that of oxytocin.

Compound	Cross Reactivity	Compound	Cross Reactivity
Mesotocin	7.0%	Somatostatin	<0.02%
Arg^8-Vasotocin	7.5%	Met-Enkephalin	<0.02%
Ser^4,Ile^8-Oxytocin	<0.02%	VIP	<0.02%
TRH	<0.02%	Lys^8-Vasopressin	<0.02%
Growth Hormone	<0.02%	Arg^8-Vasopressin	<0.02%
Tocinoic acid	<0.02%	α−ANP	<0.02%
Melanostatin	<0.02%		

Sample Recoveries

Please refer to page 6 for Sample Handling recommendations.

Oxytocin concentrations were measured in a variety of different samples matrices. Oxytocin was spiked into undiluted samples which were subsequently serially diluted with Assay Buffer then assayed in the kit. The following results were obtained:

Sample	%Recovery	Recommended Dilution*
Human Serum	104%	1:8
Human Saliva	90%	1:32
Conditioned Media	92%	Neat
Human Cerebrospinal Fluid	106%	Neat
Human Urine	111%	1:16

* See Sample Handling instructions on page 6 for details.

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