Oxycodone ELISA Kit

Item No. 501590

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401592	Oxycodone ELISA Antiserum	1 vial/100 dtn
401590	Oxycodone AP Tracer	1 vial/100 dtn
401594	Oxycodone ELISA Standard	1 vial
400080	Tris Buffer Concentrate (10X)	2 vials/10 ml
411007	AP Wash Buffer Concentrate (150X)	1 vial/5 ml
400004/400006	Mouse Anti-Rabbit IgG Coated Plate	1 plate
400012	96-Well Cover Sheet	1 cover
400089	pNPP Substrate Solution	2 vials/12 ml
400040	ELISA Tracer Dye	1 vial
400042	ELISA Antiserum Dye	1 vial

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman's Oxycodone ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC-MS or GC-MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC-MS or GC-MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance at 405 nm.
2. Adjustable pipettes and a repeating pipettor.
3. Materials used for **Sample Preparation** (see page 11).
4. A source of pure water; glass distilled water or deionized water is acceptable.

INTRODUCTION

About This Assay

Cayman's Oxycodone ELISA Kit is a competitive assay that can be used for quantification of oxycodone. This assay has been tested in plasma, serum, and urine. The ELISA typically displays a 50% B/B_0 value of approximately 44 pg/ml and sensitivity (80% B/B_0) of approximately 5.2 pg/ml.

Principle of the Assay

This assay is based on the competition between free o xycodone and an Oxycodone Tracer (Oxycodone linked to alkaline phosphatase (AP)) for a limited number of oxycodone-specific rabbit antiserum binding sites. The concentration of the Oxycodone Tracer is held constant while the concentration of free oxycodone (standard or sample) varies. Thus, the amount of oxycodone Tracer that is bound to the rabbit antiserum will be inversely proportional to the concentration of free oxycodone in the well. This rabbit antiserum-oxycodone (either free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. After incubation the plate is washed to remove any unbound reagents and pNPP substrate solution is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Oxycodone Tracer bound to the well, which is inversely proportional to the amount of free oxycodone present in the well during the incubation; or

$$\text{Absorbance} \propto \text{[Bound Oxycodone]} \propto 1/\text{[Oxycodone]}$$

A schematic of this process is shown in Figure 1, below.

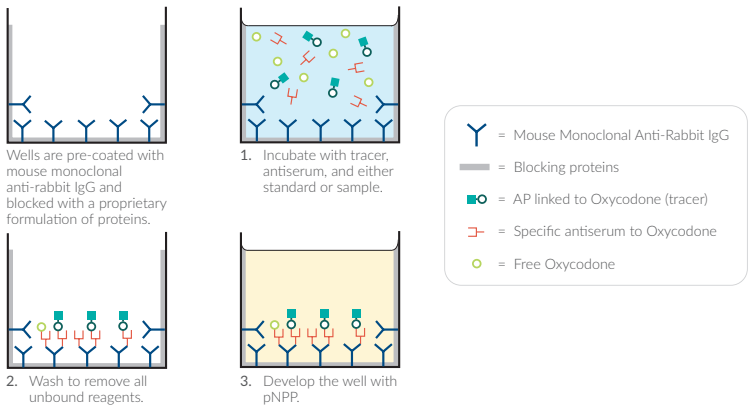


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blank: background absorbance caused by pNPP substrate solution. The blank absorbance should be substracted from the absorbance readings of all the other wells, including NSB wells.

Total Activity (TA): total enzymatic activity of the AP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Subtract the Blank absorbance values from raw NSB values.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Binding): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\text{\% Cross Reactivity} = \left[\frac{\text{50\% } B/B_0 \text{ value for the primary analyte}}{\text{50\% } B/B_0 \text{ value for the potential cross reactant}} \right] \text{x } 100\%$$

Lower Limit of Detection (LLOD): The smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a point, two standard deviations away from the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they should be stable for up to two months.

1. **Tris Buffer Preparation**

   Dilute the contents of one vial of Tris Buffer Concentrate (10X) (Item No. 400080) with 90 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **AP Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (150X) (Item No. 411007): Dilute to a total volume of 750 ml with pure water. Smaller volumes of 1X AP Wash Buffer can be prepared by diluting the AP Wash Buffer Concentrate 1:150 in pure water.

Sample Preparation

Oxycodone and oxymorphone can be measured in plasma, serum, and urine without extraction, however the major urine metabolite is oxymorphone3-glucuronide. Urine samples may be treated with β-glucuronidase prior to assay, as decribed by Sharke, C. *et. al.*[1] Treatment of urine with β-glucuronidase will remove a sugar reside from oxycodone or oxymorphone, improving the recognition of urinary oxycodone or oxymorphone by the oxycodone antiserum.

If you wish to treat urine with β-glucuronidase prior to immunoassay, the following protocol is recommended.

1. Dilute 1 ml of urine with 1 ml of 1 M sodium acetate, pH 5.0.
2. Add 100 µl of β-glucuronidase from *H. pomatia.*
3. Incubate overnight at 37°C.
4. Neutralize by the addition of 1 ml of 1 M potassium phosphate, pH 7.4. These neutralized samples can be used directly in the ELISA.

Testing for Interference

This assay has been tested using human plasma, serum, and urine. Other sample types will need to be assessed for interference by the end user. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 15 and 150 pg/ml (i.e., between 20-80% B/B_0, which is the linear portion of the standard curve). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated Oxycodone concentration, extraction is not required. If you do not see good correlation of the different dilutions, extraction is advised. Extraction protocols are described in references [1-3] and must be tested for use in the assay by the end user.

Sample Matrix Properties

Dilutional Linearity

A human plasma, serum, and urine sample with biologically relevant level of oxycodone was checked at multiple dilutions using the Oxycodone ELISA. The results are shown in the table on page 13. The minimum required dilution (MRD) to avoid sample matrix intererference was found to be 1:4 in urine, plasma and serum. In dilutional linearity experiments a minimum dilution of 1:40 was needed as samples contained high amounts of oxycodone and a 1:40 dilution was required to read the sample on the linear portion of the curve.

Dilution	Concentration (ng/ml)	Dilution Linearity (%)
Urine		
1:40	4615	92.3
1:80	5025	100.5
1:160	5359	107.2
1:320	5705	114.1
Plasma		
1:40	4912	98.2
1:80	5285	105.7
1:160	5550	111.0
1:320	6049	121.0
Serum		
1:40	5072	101.4
1:80	5223	104.5
1:160	5388	107.8
1:320	5407	108.1

Table 1. Dilutional Linearity of human, urine, plasma, and serum in Oxycodone ELISA

Spike and Recovery

Human plasma, serum, and urine was spiked with Oxycodone, diluted as described in the **Sample Preparation** section and analyzed using the Oxycodone ELISA Kit. The results are shown below. The y-intercept corresponds to the amount of endogenous Oxycodone in the urine sample. Error bars represent standard deviations obtained from multiple dilutions of each sample.

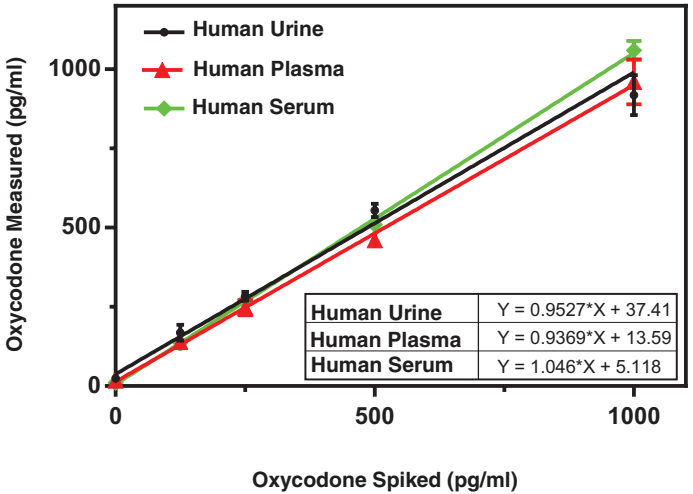


Figure 2. Spike and recovery in the Oxycodone ELISA

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Oxycodone ELISA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the Oxycodone ELISA Standard (Item No. 401594) into a clean test tube, then dilute with 900 µl of pure water. The concentration of this solution (the bulk standard) will be 5 ng/ml.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl Tris Buffer to tube #1 and 500 µl Tris Buffer to tubes #2-8. Transfer 100 µl of the bulk standard 5 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. The diluted standards may be stored at 4°C for no more than 24 hours.

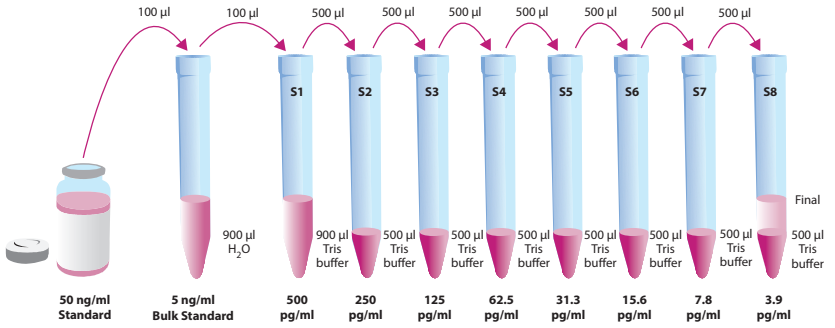


Figure 4. Preparation of the Oxycodone standards

Oxycodone AP Tracer

Reconstitute the Oxycodone AP Tracer as follows:

100 dtn Oxycodone AP Tracer (Item No. 401590): Reconstitute with 6 ml 1X Tris Buffer.

Store the reconstituted Oxycodone AP Tracer at 4°C (*do not freeze*) and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer).

Oxycodone ELISA Antiserum

Reconstitute the Oxycodone ELISA Antiserum as follows:

100 dtn Oxycodone ELISA Antiserum (Item No. 401592): Reconstitute with 6 ml 1X Tris Buffer.

Store the reconstituted Oxycodone ELISA Antiserum at 4°C. It should be stable for at least four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody).

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all of the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B0), two total activity (TA) wells, and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown below in Figure 5. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 28, for more details). We suggest you record the contents of each well on the template sheet provided (see page 28).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well(s).

Addition of the Reagents

1. **Tris Buffer**

   Add 100 µl Tris Buffer to NSB wells and 50 µl to B_0 wells.

2. **Oxycodone ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Oxycodone AP Tracer**

   Add 50 µl to each well except the TA and the Blk wells.

5. **Oxycodone ELISA Antiserum**

   Add 50 µl to each well except the TA, the NSB, and the Blk wells.

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate for 18-24 hours at 4°C.

Development of the Plate

1. Empty the wells and rinse five times with Wash Buffer
2. Add 200 µl of pNPP substrate solution to each well
3. Add 5 µl of tracer to the TA wells
4. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker for 90 minutes.

See page 27 for Procedure Summary

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep pNPP substrate solution from splashing on the cover. *NOTE: Any loss of pNPP substrate solution will affect the absorbance readings. If pNPP substrate solution is present on the cover, use a pipette to transfer the pNPP substrate solution into the well.*
3. Read the plate at a wavelength of 405 nm.

ANALYSIS

Many plate readers come with data reduction software that plots data automatically. Alternatively, a spreadsheet program can be used. The data should be plotted as either %B/B_0 versus log concentration using a four-parameter logistic fit or as logit B/B_0 versus log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B0 or corrected maximum binding.
4. Calculate the B/B_0 (sample or standard bound/maximum bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool during any troubleshooting exercise to help eliminate the tracer as a source of variability.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 versus Oxycodone concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation*:

$$\text{logit } B/B_0 = \ln [B/B_0/(1-B/B_0)]$$

Plot the data as logit (B/B_0) versus log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample by identifying the %B/B_0 on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well. Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.*

Performance Characteristics

Representative Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the value of your samples.

Absorbance at 405 nm at 90 min.

Oxycodone Standards (pg/ml)	Blank Corrected Absorbance	NSB Corrected Absorbance	%B/B_0	%CV Intra-assay variation	%CV Inter-assay variation
NSB	0.002	--	--	--	--
TA	0.477	0.475	--	--	--
B_0	1.10	1.08	--	--	--
500	0.073	0.071	6.6	6.4	12.0
250	0.132	0.130	12.0	4.6	9.0
125	0.257	0.255	21.9	4.0	8.8
62.5	0.416	0.414	37.0	5.0	3.6
31.3	0.651	0.649	60.1	5.6	5.2
15.6	0.859	0.857	79.4	14.9	4.0
7.8	0.968	0.966	89.5	13.6	20.3
3.9	1.046	1.044	96.7	25.0*	30.4*

Table 2. Typical results *Data in this range of the standard curve should be evaluated cautiously.

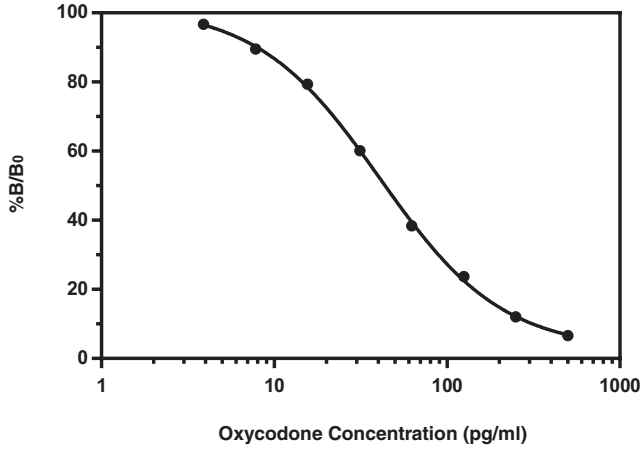


Figure 7. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (Spiked human urine samples) in a single assay.

Matrix Control (ng/ml)	%CV
151.1	6.2
57.2	5.2
15.3	15.6

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (Spiked human urine samples) in separate assays spanning across several days.

Matrix Control (ng/ml)	%CV
167.4	9.6
58.6	8.5
19.1	26.7

Table 4. Inter-assay precision

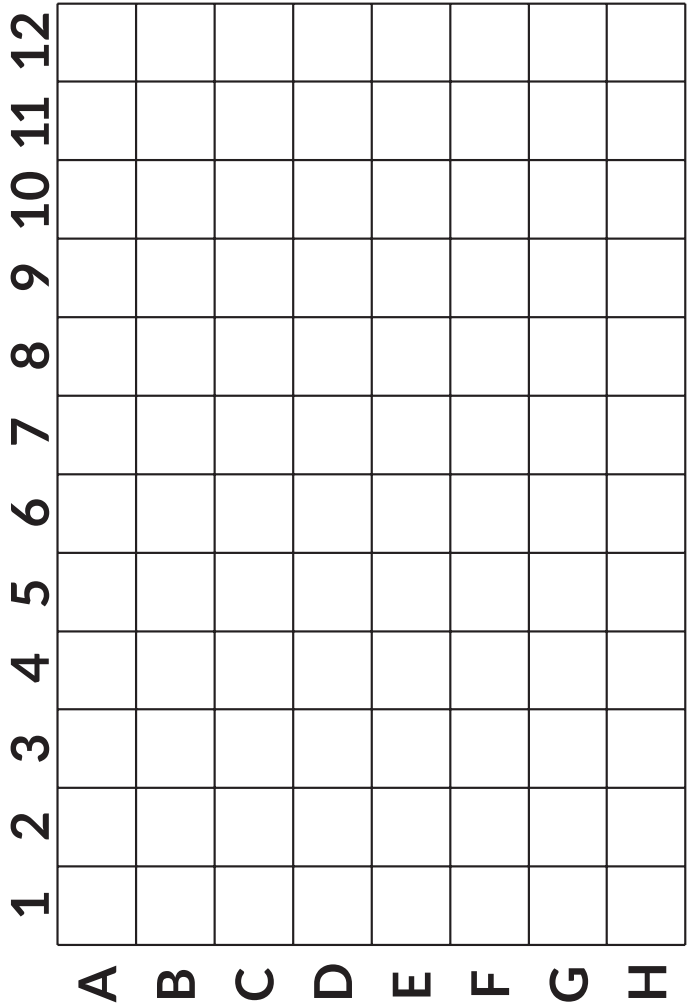
Cross Reactivity:

Compound	Cross Reactivity
Oxymorphone	42%
Oxymorphone 3-Glucuronide	32%
Hydrocodone	4.0%
Dihydrocodeine	1.8%
Hydromorphone	0.9%
Noroxycodone	0.77%
Codeine	0.59%
Heroin	0.26%
6-Acetylmorphine	0.11%
Morphine	0.10%
Levorphanol tartrate	0.09%
Morphine 6β glucuronide	0.04%
Morphine 3β glucuronide	0.03%
Noroxymorphone	0.01%
Norcodeine	0.001%
Norbuprenorphine	<0.001%
Buprenorphine	<0.001%

Table 5. Cross Reactivity of the Oxycodone ELISA

RESOURCES

Oxycodone Assay Summary					
Procedure	**Blk**	**TA**	**NSB**	**B_0**	**Standards/ Samples**
Reconstitute and Mix	Mix all reagents gently				
Tris Buffer (1X)	-	-	100 µl	50 µl	-
Standards/Samples	-	-	-	-	50 µl
Oxycodone AP-Tracer	-	-	50 µl	50 µl	50 µl
Oxycodone Antiserum	-	-	-	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate overnight a 4°C				
Aspirate	Aspirate wells and wash 5 x ~300 µl with 1X AP Wash Buffer				
Apply pNPP Substrate	200 µl	200 µl	200 µl	200 µl	200 µl
Total Activity TA - Apply Tracer	-	5 µl	-	-	-
Seal	Seal plate and incubate for 60-90 min. at room temperature on orbital shaker, protect from light				
Read	Read O.D. at 405 nm				



Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (> 0.100 O.D.)	A. Poor washing, ensure proper washing is used B. Exposure of NSB wells to specific antibody
Very Low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose response curve)	Standard is degraded or contaminated
Analysis of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present - Consider sample purification prior to analysis.[4]

References

1. Cone, E.J., Heltsley, R., Black, D.L., *et al.* Prescription opioids. II. Metabolism and excretion patterns of hydrocodone in urine following controlled single-dose administration. *J. Anal. Toxicol.* **37(8)**, 486-494 (2013).
2. Rudd, R.A., Seth, P., David, F., *et al.* (2016). Increases in drug and opioid-involved overdose deaths - United States, 2010-2015. *MMWR Morb. Mortal Wkly. Rep.* **65(5051)**, 1445-1452 (2016)..
3. Kenan, K., Mack, K., and Paulozzi, L. Trends in prescriptions for oxycodone and other commonly used opioids in the United States, 2000–2010. *Open Med.* **6(2)**, e41-e47 (2012).
4. Maxey, K.M., Maddipati, K.R., and Birkmeier, J.Interference in enzyme immunoassays. *Journal of Clinical Immunoassay* **15**, 116-120 (1992).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.