Morphine ELISA Kit

Item No. 501940

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

3 Materials Supplied
4 Safety Data
4 Precautions
5 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION

6 Background
7 About This Assay
7 Principle Of This Assay
9 Definition of Key Terms

PRE-ASSAY PREPARATION

11 Buffer Preparation
12 Sample Preparation
14 Sample Matrix Properties

ASSAY PROTOCOL

17 Preparation of Assay-Specific Reagents
19 Plate Set Up
20 Performing the Assay

ANALYSIS

22 Calculations
26 Performance Characteristics

RESOURCES

31 Troubleshooting
32 Assay Summary
33 Plate Template
34 References
35 Notes
35 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	Storage Temperature
401940	Morphine-HRP Tracer	1 vial/100 dtn	-20°C
401942	Morphine ELISA Monoclonal Antibody	1 vial/100 dtn	-20°C
401944	Morphine ELISA Standard	1 vial	-20°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400008/400009	Goat Anti-Mouse IgG-Coated Plate	1 plate	4°C
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400040	ELISA Tracer Dye	1 ea	RT
400042	ELISA Antiserum Dye	1 ea	RT
400012	96-Well Cover Sheet	1 ea	RT

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.

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 in the Materials Supplied section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. An orbital microplate shaker
4. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Materials used for **Sample Preparation** (see page 12)

INTRODUCTION

Background

Morphine is an opioid receptor agonist that has been used in the management of severe pain not relieved by non-narcotic analgesics.[1] It is also formed *in vivo* by the metabolism of codeine and heroin.[2] Common adverse effects of morphine include tolerance development, addiction, and respiratory depression, and it has been associated with misuse and abuse.[3,4] It has also frequently been implicated in cases of overdose.[5,6] Morphine is glucuronidated by the UDP-glucuronosyltransferase (UGT) isoforms UGT2B7, UGT1A1, and UGT1A8, and also metabolized by sulfation and N-demethylation, to form the active metabolites morphine-6-glucuronide and normorphine, the toxic metabolite morphine-3-glucuronide, and the inactive metabolites 7,8-dihydromorphine and morphine-3-sulfate.[2,9,10] It has an elimination half-life of approximately 1.9 hours following intravenous administration and is excreted primarily in the urine in the form of its metabolites.[7,8]

About This Assay

Cayman's Morphine ELISA Kit is a competitive assay that can be used for quantification of morphine in human plasma, serum, and urine. The assay has a range of 16-10,000 pg/ml with a midpoint of approximately 395 pg/ml (50% B/B_0) and a sensitivity (80% B/B_0) of approximately 86 pg/ml.

Principle Of This Assay

This assay is based on the competition between free morphine and a morphine-horseradish peroxidase (HRP) conjugate (Morphine-HRP Tracer) for a limited amount of Morphine Monoclonal Antibody. Because the concentration of the Morphine-HRP Tracer is held constant while the concentration of free morphine varies, the amount of Morphine-HRP Tracer that is able to bind to the Morphine Monoclonal Antibody will be inversely proportional to the concentration of free morphine in the well. This antibody-morphine complex binds to goat anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and TMB Substrate Solution (which contains the substrate to HRP) is added to the well, followed by the HRP Stop Solution. The product of this enzymatic reaction has a distinct yellow color that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Morphine-HRP Tracer bound to the well, which is inversely proportional to the amount of free morphine present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound Morphine-HRP Tracer}] \propto 1/[\text{morphine}]$$

A schematic of this process is shown in Figure 1, on page 8.

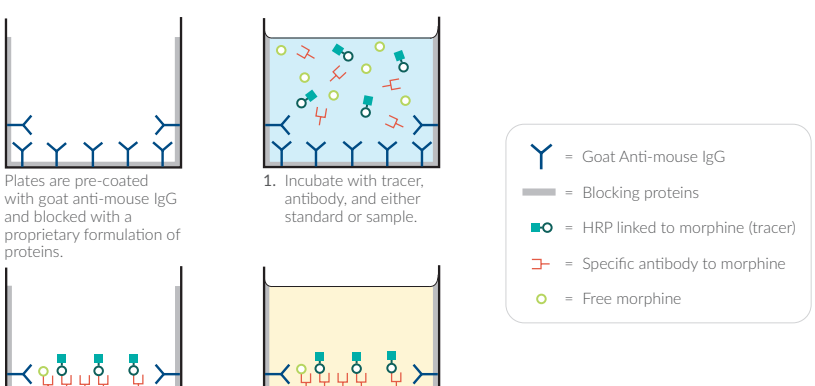


Figure 1. Schematic of the Morphine ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and the HRP Stop Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

TA (Total Activity): total enzymatic activity of the morphine HRP-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.

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 Bound): ratio of the absorbance of a sample or standard well to the average absorbance of the maximum binding (B_0) wells.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

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:

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

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 concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months.

NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.

1. **Immunoassay Buffer C (1X) Preparation**

   Dilute the contents of one vial of Immunoassay Buffer C Concentrate (10X) (Item No. 401703) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding Polysorbate 20 to an end concentration of 0.5 ml/L. *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.*

Sample Preparation

Testing for Interference

This assay has been validated using human plasma, serum, and urine. Other sample types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 1,100 and 180 pg/ml (*i.e.*, between 20-70% B/B_0, which is the linear portion of the standard curve). If two different dilutions of the same sample show good correlation (differ by 20% or less) in the final calculated morphine concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatability in the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.
- Samples of mouse origin may contain antibodies that interfere with the assay by binding to the goat anti-mouse plate. We recommend that all mouse samples be purified prior to use in the assay.

Plasma and Serum

Plasma and serum samples may require purification prior to quantification in the assay. Sample purification should be performed using a method similar to the following protocol.

1. To a 0.4 ml sample, add 1.2 ml of methanol. Mix and incubate at room temperature for 5 minutes.
2. Centrifuge at 500 x g for 5 minutes.
3. Carefully transfer the supernatant to a clean tube and set aside.
4. Repeat methanol purification two more times on the sample pellet, combining all supernatants.
5. Evaporate the combined supernatants to dryness under a stream of nitrogen.
6. Reconstitute with 0.4 ml (original volume of sample) of Immunoassay Buffer C (1X).
7. Dilute further in Immunoassay Buffer C (1X) for the assay, if needed. *NOTE: If samples cannot be assayed immediately, store at -80°C.*

Urine

It is recommended that urine samples be diluted into Immunoassay Buffer C (1X) prior to testing in the assay.

Sample Matrix Properties

Linearity

To assess dilutional linearity, human plasma, serum, and urine were spiked with the Morphine ELISA Standard, prepared as described in the Sample Preparation section, serially diluted and evaluated for linearity using the Morphine ELISA Kit. The results are shown in the table below.

Dilution Factor	Concentration (ng/ml)	Dilutional Linearity (%)
Human Plasma		
200	164.7	100
400	165.0	100
800	167.3	102
Human Serum		
200	87.5	100
400	80.7	92
800	76.8	88
Human Urine		
200	119.0	100
400	128.6	108
800	132.6	111

Table 1. Dilutional linearity of human plasma, serum, and urine samples

Spike and Recovery

Human plasma, serum, and urine were spiked with different amounts of Morphine ELISA Standard, prepared as described in the Sample Preparation section, serially diluted and analyzed using the Morphine ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

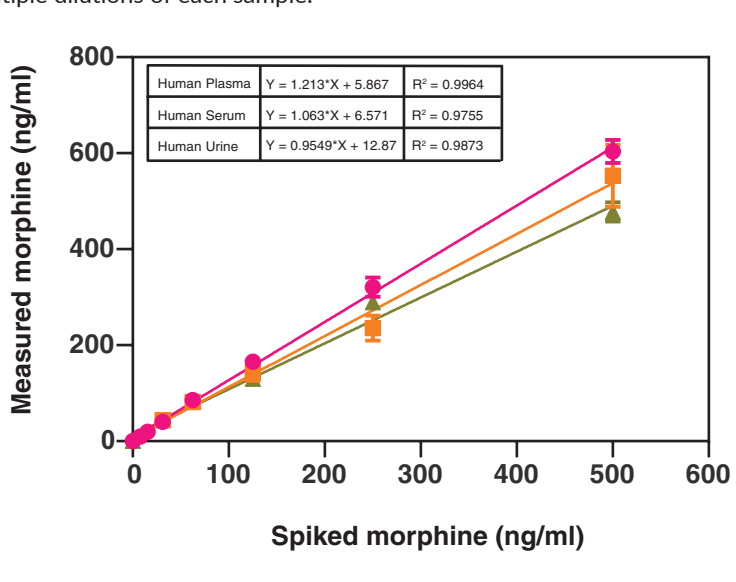


Figure 2. Spike and recovery in human plasma, serum, and urine

Parallelism

To assess parallelism, human plasma, serum, and urine samples were spiked with the Morphine ELISA Standard, prepared as described in the Sample Preparation section, serially diluted and evaluated using the Morphine ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below.

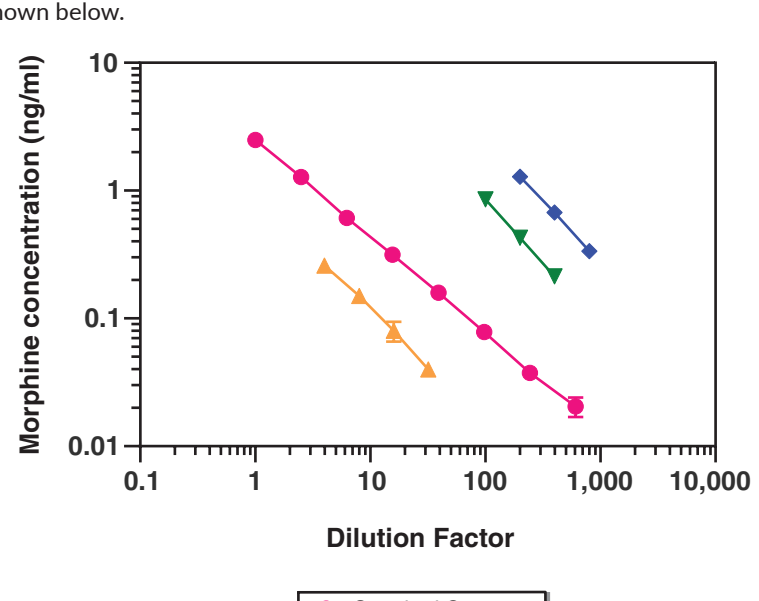


Figure 3. Parallelism of human plasma, serum, and urine in the Morphine ELISA Kit

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Morphine ELISA Standard

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 960 µl Immunoassay Buffer C (1X) to tube #1 and 600 µl Immunoassay Buffer C (1X) to tubes #2-8.

Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Morphine ELISA Standard (Item No. 401944) several times. Using the equilibrated pipette tip, transfer 40 µl of the standard into tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 30 minutes.

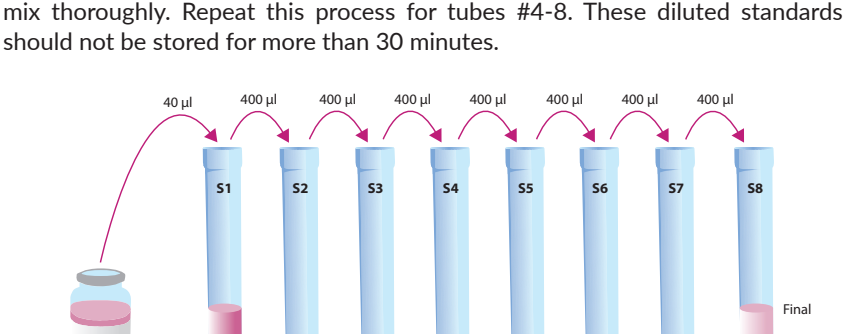


Figure 4. Preparation of the morphine standards

Morphine-HRP Tracer

Dilute the Morphine-HRP Tracer (Item No. 401940) with 5 ml of Immunoassay Buffer C (1X). Store the diluted Morphine-HRP Tracer at 4°C (*do not freeze!*) and use within three 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 diluted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer) *NOTE: Do not store tracer with dye for more than three weeks at 4°C.*

Morphine ELISA Monoclonal Antibody

Reconstitute the Morphine ELISA Monoclonal Antibody (Item No. 401942) with 6 ml of Immunoassay Buffer C (1X). Store the reconstituted Morphine ELISA Monoclonal Antibody at 4°C (*do not freeze!*) and use within three weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antibody 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). *NOTE: Do not store antibody with dye for more than three weeks at 4°C.*

Plate Set Up

The 96-well plate(s) included with this kit must be pre-washed five times with Wash Buffer (1X) (~300 µl/well) prior to use in the ELISA. *NOTE: If you do not need to use all the strips at once, place the unused unwashed 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 Blk, two NSB, and three B_0 wells, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at a minimum of 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 in Figure 5, below. 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 22 for more details). We suggest recording the contents of each well on the template sheet provided (see page 33).

	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.

Pre-Wash the Plate

Rinse the plate (or strips to be used) five times with ~300 µl Wash Buffer (1X).

Addition of the Reagents

1. **Immunoassay Buffer C (1X)**

   Add 100 µl Immunoassay Buffer C (1X) to NSB wells. Add 50 µl Immunoassay Buffer C (1X) to B_0 wells.

2. **Morphine 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 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 sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Morphine-HRP Tracer**

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

5. **Morphine ELISA Monoclonal Antibody**

   Add 50 µl to each well, except the TA, NSB, and Blk wells, within 15 minutes of addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-well Cover Sheet (Item No. 400012) and incubate two hours at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Add 5 µl of the diluted tracer to the TA wells.
4. Cover the plate with the 96-well Cover Sheet. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes.
5. Remove the plate cover being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
6. DO NOT WASH THE PLATE. Add 75 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Read the plate at a wavelength of 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plot 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 B_0 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. Erratic absorbance values could indicate the presence of organic solvents in the buffer or other technical problems (see page 31 for Troubleshooting). Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* morphine concentration using linear (y) and log (x) axes and perform a four-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.*

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

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 dilution of the original sample concentration prior to its addition to the well.*

Samples with %B/B_0 values greater than 70% 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.

NOTE: If there is an error in the B_0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

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 on page 27 to determine the values of your samples.

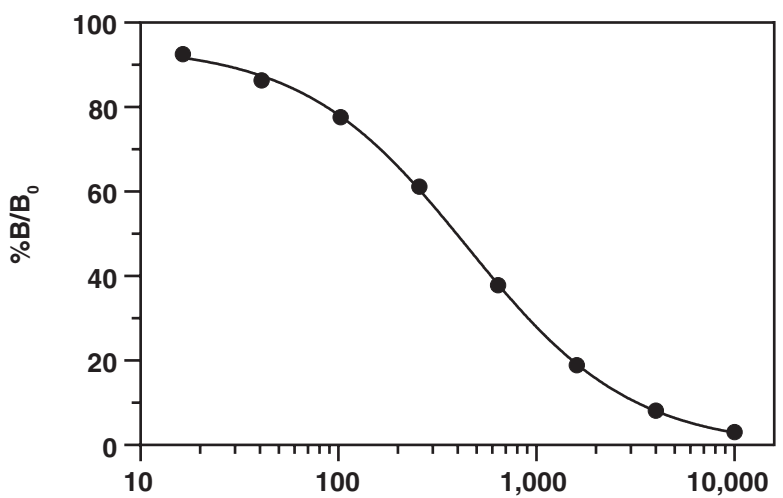
Absorbance at 450 nm (30 minutes)

Analyte Standards (pg/ml)	Blank-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV* Intra-assay Precision	%CV* Inter-assay Precision
NSB	0.002				
B_0	1.306	1.304			
10,000	0.041	0.039	3.0	9.7	8.2
4,000	0.107	0.105	7.7	4.0	1.6
1,600	0.249	0.247	18.6	3.1	4.9
640	0.496	0.494	37.3	4.3	1.4
256	0.799	0.797	60.3	4.2	6.0
102	1.015	1.013	76.7	7.5	4.3
41	1.131	1.129	87.3	27.3**	26.1**
16	1.203	1.201	89.3	36.3**	55.7**
TA	1.109				

Table 2. Typical results

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve

**Evaluate data in this range with caution



Assay Range = 16-10,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 86.4 pg/ml
Mid-point (defined as 50% B/B_0) = 394.6 pg/ml
Lower Limit of Detection (LLOD) = 3.5 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted in Immunoassay Buffer C (1X).

Figure 6. Typical standard curve

Precision:

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

Matrix Control (pg/ml)	%CV
679.2	4.3
14.7	4.3
1.3	4.1

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing four replicates of three matrix controls (urine) in 10 separate assays on different days.

Matrix Control (pg/ml)	%CV
640.7	19.5
14.7	16.1
1.4	17.0

Table 4. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity
Morphine	100
Codeine	433.3
Ethyl morphine	205.7
Morphine-3-β-D-glucuronide	188.7
Hydrocodone	138.7
3-Acetylmorphine	117.1
Dihydrocodeine	101.8
Heroin	101.6
6-Acetylmorphine	90.5
Hydromorphone	79.7
Dihydromorphine	51.2
Desomorphone	49.1
Thebaine	13.2
Oxycodone	1.4
6α-Oxymorphol	0.4
6β-Oxymorphol	0.1
Meperidine	0.1
Noroxycodone	<0.01

Table 5. Cross reactivity of the Morphine ELISA Kit

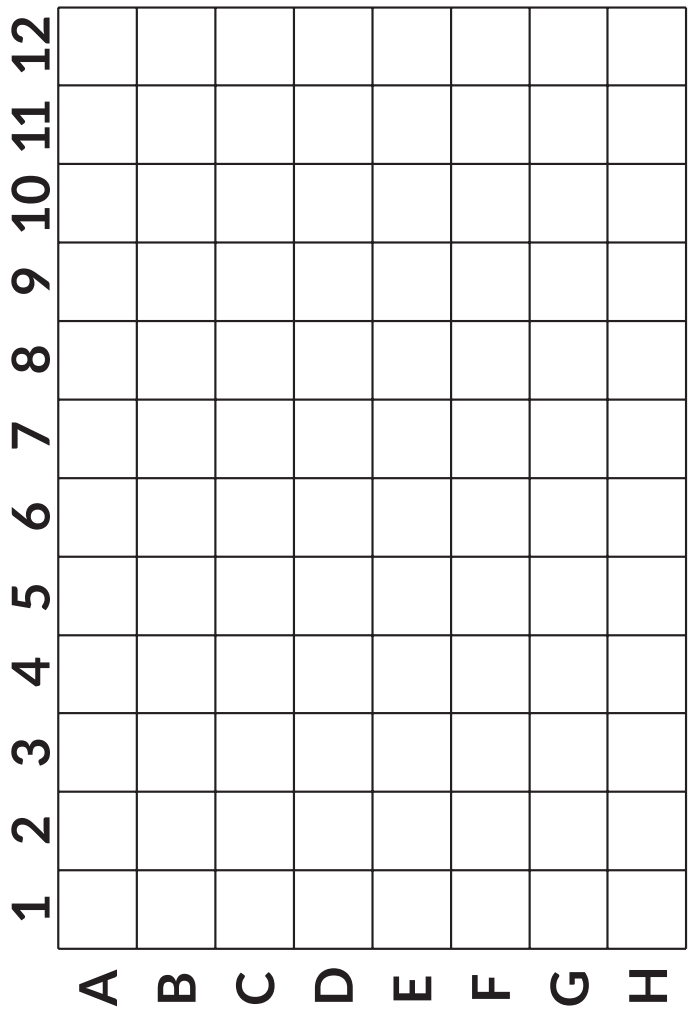
RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	Standard is degraded
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present
Only TA wells develop	A. Trace organic contaminants in the water source B. The tracer was not added to the well(s)

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Pre-Wash the Plate	Rinse the plate five times with ~300 µl Wash Buffer (1X)				
Reconstitute and mix	Mix all reagents gently				
Immunoassay Buffer C (1X)	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--	--	50 µl
Morphine-HRP Tracer	--	--	50 µl	50 µl	50 µl
Morphine ELISA Monoclonal Antibody	--	--	--	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate 2 hours at room temperature on an orbital shaker				
Aspirate and Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Apply TMB Substrate	175 µl	175 µl	175 µl	175 µl	175 µl
TA - Apply Tracer	--	5 µl	--	--	--
Development	Seal plate and incubate for 30 minutes at room temperature on an orbital shaker				
Apply HRP Stop Solution	Apply 75 µl HRP Stop Solution				
Read	Read optical density at 450 nm				

Table 6. Assay Summary



References

1. Dhawan, B.N., Cesselin, F., Raghubir, R., *et al.* International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol. Rev.* **48(4)**, 567-592 (1996).
2. Holmquist, G.L. Opioid metabolism and effects of cytochrome P450. *Pain Med.* **10(S1)**, S20-S29 (2009).
3. Atluri, S.A., Sudarshan, G., and Manchikanti, L. Assessment of the trends in medical use and misuse of opioid analgesics from 2004 to 2011. *Pain Physician* **17(2)**, E119-E128 (2014).
4. Pattinson, K.T.S. Opioids and the control of respiration. *Br. J. Anaesth.* **100(6)**, 747-758 (2008).
5. Warner, M., Trinidad, J.P., Bastian, B.A., *et al.* Drugs most frequently involved in drug overdose deaths: United States, 2010-2014. *Natl. Vital Stat. Rep.* **65(10)**, 1-15 (2016).
6. White, J.M. and Irvine, R.J. Mechanisms of fatal opioid overdose. *Addiction* **94(7)**, 961-972 (1999).
7. Hoskin, P.J., Hanks, G.W., Aherne, G.W., *et al.* The bioavailability and pharmacokinetics of morphine after intravenous, oral and buccal administration in healthy volunteers. *Br. J. Clin. Pharmacol.* **27(4)**, 499-505 (1989).
8. Brunk, S.F. and Delle, M. Morphine metabolism in man. *Clin. Pharmacol. Ther.* **16(1)**, 51-57 (1974).
9. Sullivan, A.F., McQuay, H.J., Bailey, D., *et al.* The spinal antinociceptive actions of morphine metabolites morphine-6-glucuronide and normorphine in the rat. *Brain Res.* **482(2)**, 219-224 (1989).
10. Andersson, M., Björkhem-Bergman, L., Ekström, L., *et al.* Detection of morphine-3-sulfate and morphine-6-sulfate in human urine and plasma, and formation in liver cytosol. *Pharmacol. Res. Perspect.* **2(6)**, e00071 (2014).

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.

This document is copyrighted. All rights are reserved. This document may not, in whole or part, be copied, photocopied, reproduced, translated, or reduced to any electronic medium or machine-readable form without prior consent, in writing, from Cayman Chemical Company.

©03/27/2026, Cayman Chemical Company, Ann Arbor, MI, All rights reserved. Printed in U.S.A.