Benzodiazepine ELISA Kit

Item No. 501800

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	Storage
401800	Benzodiazepine-HRP Tracer	1 vial/100 dtn	-20°C
401802	Benzodiazepine ELISA Monoclonal Antibody	1 vial/100 dtn	-20°C
401804	Benzodiazepine ELISA Standard	1 vial	-20°C
400108	Immunoassay Buffer D Concentrate (5X)	2 vials/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	Room Temperature
400035	Polysorbate 20	1 vial/3 ml	Room Temperature
400008/400009	Goat Anti-Mouse IgG-coated Plate	1 plate	4°C
400074	TMB Substrate Solution	2 vial/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	Room Temperature
400040	ELISA Tracer Dye	1 ea	Room Temperature
400042	ELISA Antiserum Dye	1 ea	Room Temperature
400012	96-Well Cover Sheet	1 cover	Room Temperature

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

Fax: 734-971-3640

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, on 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. An orbital microplate shaker
3. Adjustable pipettes and a repeating pipettor
4. A source of ultrapure water 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

Benzodiazepines are positive allosteric modulators of the $GABA_A$ receptor that have a core chemical structure containing a 1,4-benzodiazepine ring and a 5-aryl substitution.[1,2] Formulations containing benzodiazepines have been used as sedatives and in the treatment of anxiety, insomnia, seizure disorders, including status epilepticus, and alcohol withdrawal. Benzodiazepines are short-, intermediate-, or long-acting, based on elimination half-lives that range from one to 250 hours.[3] Metabolism occurs through the cytochrome P450 (CYP) and glucuronidation systems, with subsequent excretion in the urine. Toxicity can occur due to accumulation in the body, when lung, liver, or kidney damage is present, or when used in combination with other psychotropic agents.[3,4] Benzodiazepines have been associated with misuse and abuse and have been implicated in cases of overdose and attempted suicide.[4,5] In addition, derivatives of medically used benzodiazepines, called designer benzodiazepines, have been found in samples seized by law enforcement, screens for acute intoxication, and in cases of fatal polydrug overdose.[6]

About This Assay

Cayman's Benzodiazepine ELISA Kit is a competitive assay that can be used for quantification of benzodiazepines in plasma, serum, and urine. The assay has a range of 0.041-25 ng/ml with a midpoint of approximately 1.3 ng/ml (50% B/B_0) and a sensitivity (80% B/B_0) of approximately 0.3 ng/ml.

Principle Of This Assay

This assay is based on the competition between free benzodiazepines and a benzodiazepine-horseradish peroxidase (HRP) conjugate (Benzodiazepine-HRP Tracer) for a limited amount of Benzodiazepine Monoclonal Antibody. Because the concentration of the Benzodiazepine-HRP Tracer is held constant while the concentration of free benzodiazepines varies, the amount of Benzodiazepine-HRP Tracer that is able to bind to the Benzodiazepine Monoclonal Antibody will be inversely proportional to the concentration of free benzodiazepines in the well. This antibody-benzodiazepine 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 Benzodiazepine-HRP Tracer bound to the well, which is inversely proportional to the amount of free benzodiazepines present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound Benzodiazepine-HRP Tracer}] \propto 1/[\text{benzodiazepines}]$$

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

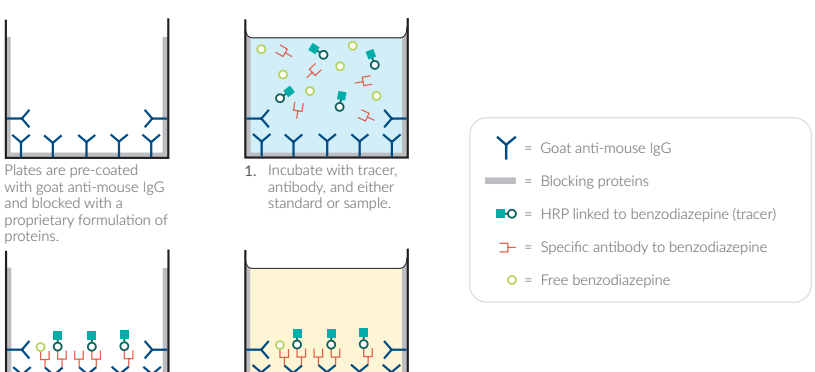


Figure 1. Schematic of the Benzodiazepine ELISA

Definition of Key Terms

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

Total Activity (TA): total enzymatic activity of the benzodiazepine-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 particular 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:

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

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 about two months.

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

   Dilute the contents of one vial of Immunoassay Buffer D Concentrate (5X) (Item No. 400108) with 40 ml of ultrapure 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 ultrapure water.*

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

This assay has been demonstrated to work with human plasma, serum, and urine. Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning 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.

Testing for Interference

To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 5.3 and 0.3 ng/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 same sample show good correlation (differ by 20% or less) in the final calculated benzodiazepine 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 compatibility in the assay.

Plasma and Serum

The following protocol is suggested prior to running plasma and serum samples in the ELISA.

1. To 0.4 ml of neat sample, add 1.2 ml of methanol. Mix and incubate at room temperature for 5 minutes.
2. Centrifuge at 5,000 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 supernatant to dryness under inert gas.
6. Reconstitute with 0.4 ml (original volume of sample) of ultrapure water and dilute further with ultrapure water, if needed.

Urine

Urine samples must be further diluted into ultrapure water for assay.

Sample Matrix Properties

Linearity

To assess dilutional linearity, human plasma, serum, and urine were spiked with the Benzodiazepine Standard, diluted or purified as described in the Sample Preparation section above, and evaluated for linearity using the Benzodiazepine ELISA Kit. The results are show in the table below.

Dilution Factor	Concentration (ng/ml)	Dilutional Linearity (%)
Plasma		
10	26.47	100
20	27.08	102
40	25.09	95
80	25.28	96
Serum		
10	25.78	100
20	26.04	101
40	22.92	89
80	25.22	98
Urine		
10	24.15	100
20	25.23	104
40	23.76	98
80	23.17	96

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

Spike and Recovery

Human samples were spiked with different concentrations of the Benzodiazepine Standard, diluted or purified as described in the Sample Preparation section (see page 12), and analyzed using the Benzodiazepine ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

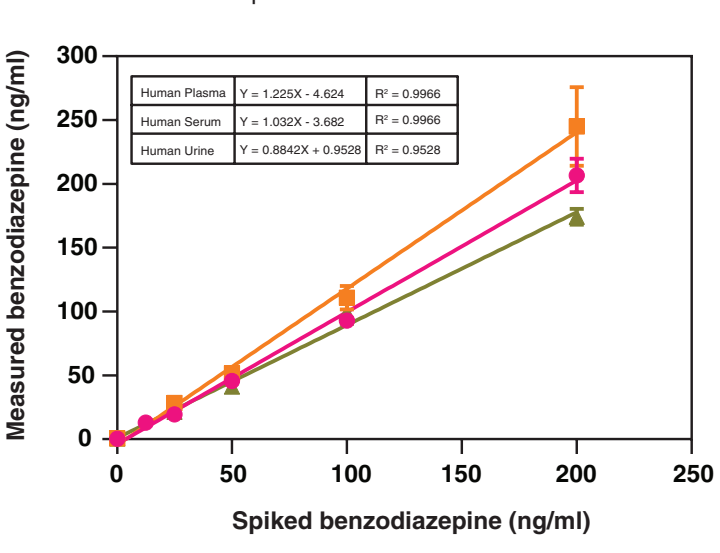


Figure 2. Spike and recovery in human plasma, serum, and urine

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Benzodiazepine ELISA Standard

Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Benzodiazepine ELISA Standard (Item No. 401804) several times. Using the equilibrated pipette tip, transfer 100 µl of the standard into a clean test tube, then dilute with 900 µl of ultrapure water. The concentration of this solution (the bulk standard) will be 250 ng/ml. The bulk standard should be stored at 4°C and used within two weeks.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl ultrapure water to tube #1 and 600 µl ultrapure water to tubes #2-8. Transfer 100 µl of the bulk standard (250 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing 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 two hours.

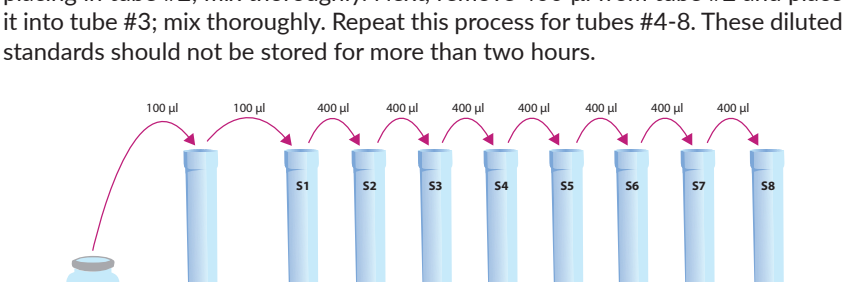


Figure 3. Preparation of the benzodiazepine standards

Benzodiazepine-HRP Tracer

Dilute the Benzodiazepine-HRP Tracer (Item No. 401800) with 5 ml of Immunoassay Buffer D (1X). Store the diluted Benzodiazepine-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). Store tracer with dye at 4°C and use within two weeks.

Benzodiazepine Monoclonal Antibody

Reconstitute the Benzodiazepine Monoclonal Antibody (Item No. 401802) with 6 ml of Immunoassay Buffer D (1X). Store the reconstituted Benzodiazepine 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)

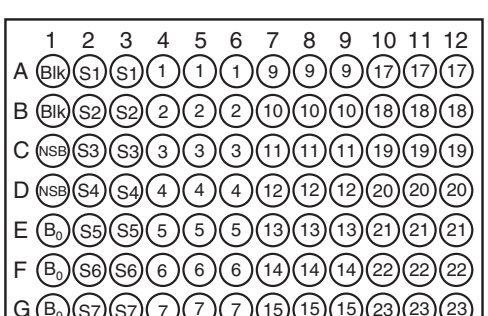
This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). Store antibody with dye at 4°C and use within two weeks.

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 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 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 4, on page 20. 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 23 for more details). We suggest recording the contents of each well on the template sheet provided (see page 33).



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

Figure 4. 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.

Addition of the Reagents

1. **Ultrapure Water**

   Add 50 µl ultrapure water to NSB wells. Add 50 µl ultrapure water to B_0 wells.

2. **Immunoassay Buffer D (1X)**

   Add 50 µl Immunoassay Buffer D (1X) to NSB wells.

3. **Benzodiazepine 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.

4. **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).

5. **Benzodiazepine-HRP Tracer**

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

6. **Benzodiazepine 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 Plate Cover Sheet (Item No. 400012) and incubate 2 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 plastic film. 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. *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 30 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* benzodiazepine 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.*

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

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 dilution of the original sample concentration prior to its 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.

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. Only the linear portion of this standard curve should be used in the calculations.

Performance Characteristics

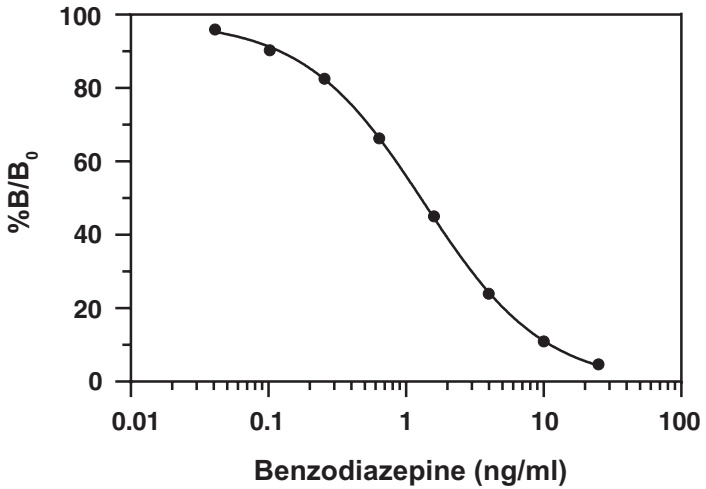
Representative Data

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 values of your samples.

Absorbance at 450 nm (30 minutes)

Analyte Standards (ng/ml)	Blank-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV* Intra-assay Precision	%CV* Inter-assay Precision
NSB	0.00				
B_0	1.191	1.191			
25	0.053	0.053	4.7	10.4	7.2
10	0.130	0.130	10.9	4.9	4.3
4	0.287	0.287	24.0	4.3	3.2
1.6	0.538	0.538	45.0	4.7	3.8
0.640	0.801	0.801	66.3	7.0	1.2
0.256	0.999	0.999	82.3	14.6	4.7
0.102	1.089	1.089	90.3	30.8**	9.9
0.041	1.156	1.156	96.0	77.5**	21.4**
TA	3.894				

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 = 0.041-25 ng/ml
Sensitivity (defined as 80% B/B_0) = 0.3 ng/ml
Mid-point (defined as 50% B/B_0) = 1.3 ng/ml
Lower Limit of Detection (LLOD) = 0.1 ng/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted in ultapure water.

Figure 5. Typical standard curve

Precision:

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

Matrix Control (ng/ml)	%CV
125.1	5.6
38.2	3.6
1.9	4.0

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three urine controls in eight separate assays on different days.

Matrix Control (ng/ml)	%CV
106.3	17.9
33.7	12.1
1.7	11.2

Table 4. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity	
Oxazepam	100	≥100%
Diazepam	1,216	
Estazolam	989	
Midazolam	461	
Temazepam	425	
Tetrazepam	411	
Nordiazepam	330	
Nimetazepam	289	
Prazepam	242	
Alprazolam	220	
Flurazepam	190	
Clobazam	149	
α-hydroxy Alprazolam	80	10-99%
Flubromazepam	62	
Flunitrazepam	61	
Bromazepam	25	
N-Desmethylflunitrazepam	16	
Chlordiazepoxide	8	1-9.99%
Diclazepam	7	
7-Aminoflunitrazepam	5	
Lormetazepam	2	
Triazolam	2	
Delorazepam	1	
Phenazepam	1	
Etizolam	1	
Clonazepam	0.5	<1%
Lorazepam	0.5	

Table 5. Cross reactivity of the Benzodiazepine ELISA Kit

RESOURCES

Troubleshooting

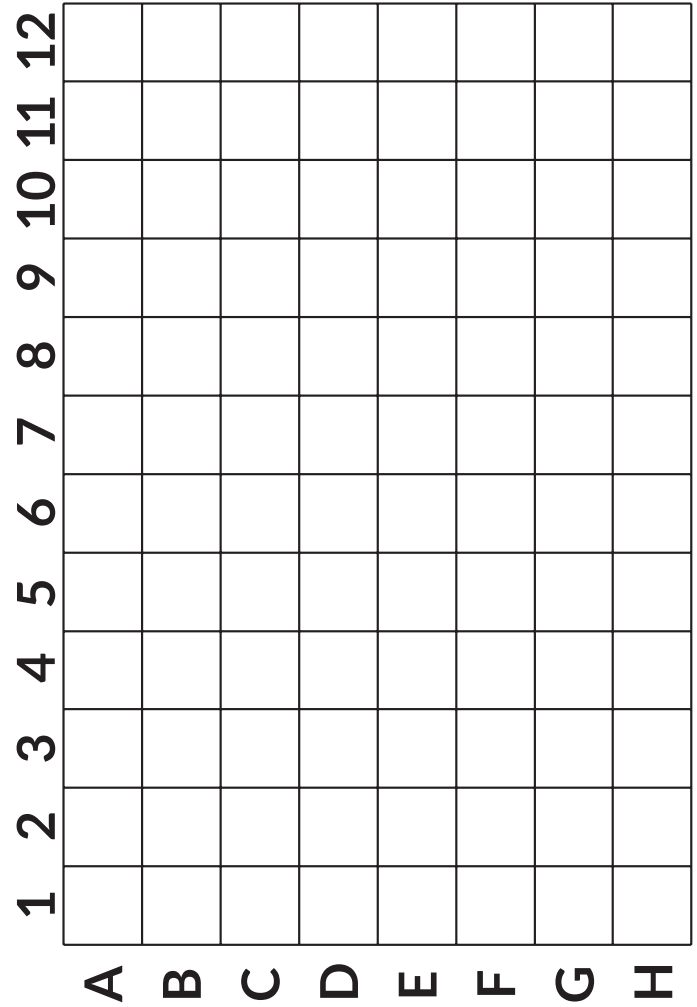
Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires 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)

References

1. Scott, S. and Aricescu, A.R. A structural perspective on $GABA_A$ receptor pharmacology. *Curr. Opin. Struct. Biol.* **54**, 189-197 (2019).
2. Sternbach, L.H. The benzodiazepine story. *J. Psychoactive Drugs* **15(1-2)**, 15-17 (1983).
3. Griffin, C.E., III, Kaye, A.M., Bueno, F.R., *et al.* Benzodiazepine pharmacology and central nervous system-mediated effects. *Ochsner J.* **13(2)**, 214-233 (2013).
4. Lader, M. Benzodiazepines revisted-will we ever learn? *Addiction* **106(12)**, 2086-2109 (2011).
5. Ait-Daoud, N., Hamby, A.S., Sharma, S., *et al.* A review of alprazolam use, misuse, and withdrawal. *J. Addict. Med.* **12(1)**, 4-10 (2018).
6. Zawilska, J.B. and Wojcieszak, J. An expanding world of new psychoactive substances-designer benzodiazepines. *Neurotoxicology* **73**, 8-16 (2019).

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and Mix	Mix all reagents gently				
Ultrapure Water	--	--	50 µl	50 µl	--
Immunoassay Buffer D (1X)	--	--	50 µl	--	--
Standards/Samples	--	--	--	--	50 µl
Benzodiazepine-HRP	--	--	50 µl	50 µl	50 µl
Benzodiazepine ELISA Antibody	--	--	--	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate for 2 hours at room temperature, on an orbital shaker				
Aspirate	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Apply TMB Substrate Solution	175 µl	175 µl	175 µl	175 µl	175 µl
TA - Apply Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 30 minutes at room temperature on orbital shaker, protected from light				
Stop Solution	75 µl	75 µl	75 µl	75 µl	75 µl
Read	Read optical density at 450 nm				

Table 6. Assay Summary



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.