

## BENZODIAZEPINES DIRECT ELISA KIT

**Catalog Number 214-0192 2 x 96 well plates**  
**Catalog Number 214-0480 5 x 96 well plates**

**THE BENZODIAZEPINES DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.**

**The Benzodiazepines Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.**

### EXPLANATION OF THE TEST

The Benzodiazepines Direct ELISA Kit is a sensitive in-vitro test to detect the presence of Benzodiazepines in forensic samples like whole blood, serum, plasma and urine.

Benzodiazepines - are a class of widely prescribed central nervous system depressant drugs with sedative, muscle relaxant and anti-convulsant activities. Chronic use does result in moderate dependence and tolerance to the drug. The use of alcohol in conjunction with the benzodiazepines has been shown to have a greater suppressive effect to the central nervous system than that attributable to either chemical alone. Benzodiazepines are usually administered orally and are absorbed rapidly. The metabolism of Benzodiazepines is mainly in the liver and excreted in the urine as a variety of structurally related metabolites. Metabolic similarities include removal of substituents from the B ring of the 1,4 benzodiazepines and alpha hydroxylation of the triazolobenzodiazepines, hydroxylation of the 3 position carbon of the B ring and conjugation of hydroxylated metabolites followed by urinary excretion as glucuronides.(6)

### PRINCIPLES OF THE PROCEDURE

The Benzodiazepines Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled Benzodiazepine derivative in micro-plate wells,

coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 5 ng/ml.

The Benzodiazepines Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs an Oxazepam directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This results in extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macromolecules.

### Materials and Methods

Materials and equipment required but not supplied with the BENZODIAZEPINES Direct ELISA Kit are itemized below:

#### Materials

12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).

Test Tube Racks.

Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.

#### Equipment

Refrigerator (for kit storage).

Interval Timer.

Wash bottle or Plate Washer.

Microplate reader capable of reading at 450 nm. And 650 nm.

### REAGENTS

#### Benzodiazepines Direct ELISA Kit Contents.

Component	192 Test Kit Cat#210-0192	480 Test Kit Cat#210-0480
96 well Micro-plate	2	5
Benzo-Conjugate	25ml	55 ml
Positive Ref. Std	2 ml	5ml
Neg Std	2 ml	5ml
TMB Substrate	28ml	55ml
Stop Reagent	25ml	55 ml

- Bring all reagents to room temperature.
- Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.

96 well micro-plate. The micro-plate is coated with polyclonal anti-Oxazepam via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Benzodiazepine-Enzyme Conjugate The conjugate solution contains a Benzodiazepine derivative labeled with horseradish peroxidase in a stabilized protein Tris buffer solution, pH 7.6 containing 0.02% thiomersal as a preservative. (Colored Red)

Positive Reference Standard. This contains 100 ng /ml of Oxazepam dissolved in a synthetic urine containing azide free preservatives. This should be diluted to the laboratory cutoff standard.

Normal Control. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent This contains 1 N hydrochloric acid.

#### Precautions

- Not for Internal or External Use in Humans or Animals.
- There should be no eating or drinking within work area.
- Always wear gloves and a protective lab coat.
- No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
- **Do not add sodium azide to samples as preservative.**
- **Do not use external controls containing sodium azide.**
- Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
- Do not pour chromogenic substrate back into container after use.
- Do not freeze reagents.
- Do not mix reagents from different kit lot numbers.
- Keep reagents out of direct sunlight.
- Handle stop reagent with care, since it is dilute acid.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation. Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 - 4 degrees centigrade.

Indications of Deterioration. A drop of greater than 50% in the A0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

#### SPECIMEN COLLECTION

##### Precautions.

The Benzodiazepines Direct ELISA Kit is to be used with human forensic samples, like whole blood, serum and plasma. has not tested all possible applications of this assay. **The Cutoff criteria is important in deciding the sample dilution.**

##### Additives.

Specimens to which sodium azide has been added affect the assay. Interfering Substances. There are no commonly abused drugs which alter the values obtained with the Benzodiazepines Direct ELISA Kit.

##### Storage and Handling Instructions.

Urine samples should be stored at 2 - 4 degrees centigrade until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

##### DETAILS OF THE PROCEDURE.

**All reagents must be brought to room temperature (20-25 ° C) before use.**

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor .

- 1) Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0 . No diluion is required for a cutoff of 100 ng/ml of oxazepam. The dilution factor can be adjusted based on the laboratories cutoff.
- 2) Add 10 µl. of calibrators and standards to each well in duplicate.
- 3) Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
- 4) Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
- 5) Incubate for 60 minutes at room temperature (20-25 ° C) preferably in the dark, after addition of enzyme conjugate to the last well.
- 6) Wash well 6 times with 350 µl distilled water using either a suitable plate washer or wash bottle **taking care not to cross contaminate wells.**
- 7) Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
- 8) Add 100 µl of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
- 9) Incubate for 20 minutes at room temperature, preferably in the dark.
- 10) Add 100 µl of Stop Solution to each well, to change the blue color to yellow.
- 11) Measure the absorbance at a dual wavelength of 450 nm. and 650 nm. Compare average absorbance readings obtained from each unknown specimen with the average absorbance obtained from the Positive Reference Standard.
- 12) Wells should be read within 2 hours of yellow color development.

The following data represent a typical dose/response curve.

Oxazepam ng/ml	Absorbance
0	2.105
25	1.122
50	0.758
100	0.564

The dose/response curve shown above should not be used in assay calculations. It is

recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

## RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Benzodiazepines. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Benzodiazepines .

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

## SPECIFIC PERFORMANCE CHARACTERISTICS

### Accuracy

50 whole blood samples and 35 urine samples collected from presumed non-users were tested in the Benzodiazepines Direct ELISA Kit . One hundred percent of these normal samples measured negative at 100 ng/ml of oxazepam . Thirty whole blood samples which were previously confirmed positive for Benzodiazepines by GC-MS, 28 of the samples were found to be positive i.e. above the cut-off of 100 ng/ml of oxazepam equivalents the remaining 2 samples would have screened positive at an oxazepam cutoff of 50 ng/ml.

### Precision

The precision of the BENZODIAZEPINES Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

### Intra-assay precision

Intra-assay precision was determined with reference controls. A 0,25,50 and 100 ng/ml Oxazepam standard was assayed five times in the same assay. The results are tabulated in Table 1.

TABLE 1

Oxazepam C.V.% (ng/ml)	Mean Abs.	S.D.	
0	2.061	0.153	7.4
25	1.197	0.099	8.3
50	0.845	0.056	6.6
100	0.558	0.063	11.2

Sensitivity

Assay sensitivity based on the minimum oxazepam concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

Specificity

The specificity of the Benzodiazepines ELISA for was determined by generating inhibition curves for each of the compounds listed below. The antisera cross-reactivities are listed in Table 2.

TABLE 2  
Cross-reactivities related drugs

Compound ng/ml	Cross-reactivities Oxazepam	Approx. equivalent to 100 ng
Alprazolam		118
	85	
Alpha-OH Alprazolam		133
	75	
Bromazepam		200
	50	
Chlordiazepoxide		760
	13	
Clorazepate		480
	21	
Demoxepam		285
	35	
Diazepam	155	65
Flurazepam		760
	13	
Flunitrazepam		110
	91	
Halazepam		398
	25	
Lorazepam		667
	15	
Medazepam		398
	25	
Nitrazepam		130
	77	
Nordiazepam		67
	150	
Prazepam		360
28		
Temazepam		77
	130	
Triazolam		300
	33	

**Cross-Reactivity with Unrelated Drugs**

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/ml. None of these compounds gave

values in the assay that were equal to or greater than the assay sensitivity level (5 ng/ml).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Ascorbic acid, Atropine, Benzoyllecgonine, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro-carbamazepine, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Methamphetamine, Mephentoin, "-Methyl"-propylsuccinimide, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phensuximide PEMA, Primidone, Phencyclidine, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, THC-COOH

## REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph, 73,1986.
2. S.C. Harvey, "Hypnotics and Sedatives" in The Pharmacological Basis of Therapeutics 7 th Ed.,1985 L.S. Goodman and A. Gilman, T.W. Rall and F. Murad, edd. (New York, Macmillan, ( pp339-51)
3. Greenblatt, D.J., Lacasse Y., and Shader, R.I.: "Acute Overdosage with Benzodiazepine Derivatives." Clin.Pharmacol. Ther. 21:4976, 1977.
4. Blum, K.: Handbook of Abusable Drugs, Gardner Press, p.371, 1984.
5. R.C. Kelley et al, "Association of Benzodiazepines with death in a major metropolitan area" Journal of Analytical Toxicology 6, 1982 p. 91-96.
6. Kaplan, S.A. and Jack, M.L.: "Metabolism of the Benzodiazepines: Pharmacokinetic and Pharmacodynamic Considerations" In: The Benzodiazepines: From Molecular Biology to Clinical Practice. E. Costa, Ed. Raven Press, New York p. 173, 1983.