

BARBITURATE ELISA KIT	DIRECT
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Corporation:

Catalog Number 210-0192 2 x 96 well plates

Catalog Number 210-0480 5 x 96 well plates

THE BARBITURATE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Barbiturate 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 Barbiturate Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of barbiturates in forensic samples like whole blood, serum, plasma and urine. Barbiturates - derivatives of Barbituric acid - are sedative drugs which at low doses induces relaxation and at high doses induce coma and even death(2). Barbiturates are usually administered orally but may also be taken intravenously or intramuscularly and are absorbed rapidly. The metabolism of Barbiturates is mainly in the liver, a number of metabolic pathways have been described which include oxidation, desulfuration and ring cleavage. Because the number and the proportion of the various Barbiturate metabolites varies with each individual the results are expressed in terms of the equivalents of the standard, secobarbital/ml.

PRINCIPLES OF THE PROCEDURE

The Barbiturate 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 barbiturate 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 1

ng/ml. The Barbiturate Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs a polyclonal high affinity, purified Barbiturate antibody. 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 Barbiturate 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

Barbiturate Direct ELISA Kit Contents.

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

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

Barbiturate-Enzyme Conjugate The conjugate solution contains a Barbiturate 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 25 ng/ml of secobarbital dissolved in a synthetic urine containing azide free preservatives. This can be diluted to the laboratory cutoff.

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

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 Barbiturate 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 Barbiturate 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 . (Whole blood and urine samples are diluted 1:4 for a cutoff level of 100 ng/ml of secobarbital equivalents.) The dilution factor can be adjusted based on the laboratory 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.

Secobarbital Absorbance ng/ml
0
1.955
5
0.758
10
0.652
25
0.514

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 Barbiturates. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Barbiturate.

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

45 whole blood samples and 50 urine samples collected from presumed non-users were tested in the Barbiturate Direct ELISA Kit . One hundred percent of these normal samples measured negative at 25 ng/ml of secobarbital equivalents for whole blood and 100 ng/ml of secobarbital equivalents for urine. Thirty five whole blood samples which were previously confirmed positive for barbiturates by GC-MS employing a cut-off of 25 ng/ml equivalents, were tested in the Barbiturate Direct ELISA Kit . All the samples were found to be positive i.e. above the cut-off of 25 ng/ml.

Precision

The precision of the Barbiturate 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 0,5,10 and 25 ng/ml standard was assayed eight times in the same assay. The results are tabulated in Table 1.

TABLE 1

Secobarbital C.V.% (ng/ml)	Mean Abs.	S.D.
0	1.944	0.149
7.67		
5	0.822	0.073
8.9		
10	0.695	0.057
8.2		
25	0.562	0.066
11.7		

Sensitivity

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

Specificity

The specificity of the Barbiturate 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	Approx.	ng/ml
Cross-reactivities	equivalent to	
	25 ng secobarbital	
Aprobarbital	45	
55		
Butabarbital	60	
42		
Barbital	125	
20		
Amobarbital	48	
52		
Butalbital	55	
46		
p-Hydroxyphen- barbital	60	
42		
Pentobarbital	52	
48		
Diallylbarbituric acid	60	
42		
Phenobarbital	114	
23		
Barbituric Acid	>1250	
<1		
Hexobarbital	>1250	
<1		
Mephobarbital	>1250	
<1		

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 2000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (1 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- hydrocarbamazepine,
Diazepam, Ethosuximide, Estriol, Estrone,
Estradiol, Ethotoin, Glutethimide, Ibuprofen,
Imipramine, Lidocaine, LSD, Methadone,
Methadone-primary metabolite, Methaqualone,
Methamphetamine, Mephenytoin, "-Methyl-"
propylsuccinimide, Methyl PEMA, Methsuximide,
4- Methylprimidone, Morphine, Meperidine,
Niacinamide, Norethindrone, N-Normethsuximide,
Phensuximide
PEMA, Primidone, Phencyclidine,
Phenothiazine, Phenylpropanolamine, Procaine,
Quinine, THC-COOH

REFERENCES

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