

AMPHETAMINE DIRECT ELISA KIT

Catalog Number 209-0192 2 x 96 well plates
Catalog Number 209-0480 5 x 96 well plates

THE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Amphetamine 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 Amphetamine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of d-amphetamine in forensic samples like whole blood, serum, plasma and urine. While the assay will detect amphetamine use, interference by l-amphetamine and pseudo-ephedrine is virtually nonexistent.

Amphetamine is a potent central nervous system stimulant. The (+)-isomer also referred to as d-amphetamine is three to four times more potent than the (-)-isomer, l-amphetamine (2). Amphetamine may be metabolized and excreted as the p-hydroxy isomer. Amphetamines act by inducing euphoria, irritability, anxiety and paranoia. Urinary excretion rates are influenced by the urinary pH with acidic urine favoring the excretion of unchanged drug(2). Up to 80% of a given dose may be excreted unchanged, especially in acid urine. Alkaline urine reduces the excretion of unchanged amphetamine to less than 5% of the dose.

PRINCIPLES OF THE PROCEDURE

The Amphetamine Direct ELISA Kit (for d-amphetamine measurement) 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 d-amphetamine 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 Amphetamine Direct ELISA Kit avoids extraction of urine sample for measurement. It employs a d-amphetamine 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 Amphetamine Direct ELISA Kit are itemized below:

Materials

12x75 mm Disposable Glass or Plastic Culture Tubes to pre-dilute samples (if required).
Test Tube Racks.
Manual or electronic micropipets (single channel or multi channel) or automated pipetting stations.

Equipment

Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Micro-plate reader capable of reading at 450 nm and 650 nm.

REAGENTS

Amphetamine Direct ELISA Kit Contents.

Component	192 Test Kit Cat#209- 0192	480 Test Kit Cat#209- 0480
96 well Micro-plate	2	5
d-Amph-Conjugate	25 ml	55 ml
Positive Ref. Std	2ml	5 ml
Neg Std	2 ml	5 ml
TMB Substrate	28 ml	55 ml
Stop Reagent	25 ml	55 ml

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

Enzyme Conjugate The conjugate solution contains d-amphetamine 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 50ng/ml of d-amphetamine dissolved in a synthetic urine containing azide free preservatives. This should be diluted to the laboratory cutoff level.

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 peroxide in buffer.

Stop solution This contains 1 N hydrochloric acid.

Precautions

1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is dilute acid.
13. Bring all reagents to room temperature.
14. 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 radio immunoassay. 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 value (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 Amphetamine 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 Amphetamine 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 Buffered Saline pH 7.0 . (Whole blood samples are normally diluted 1:2 for a cutoff level of 50 ng/ml of d-amphetamine and urine samples 1:20 for a cutoff level of 500 ng/ml.) 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 preferably in the dark (20-25 ° C), 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 (20-25 ° C).
- 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.

d-amphetamine ng/m	Absorbance
0	1.965
5	1.074
10	0.655
25	0.402

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 amphetamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference

standard the sample is called **NEGATIVE** for amphetamine. 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. Interpretation. D-Amphetamine and its metabolites appear in urine within hours after drug use and may persist for days. Thus a positive result documents amphetamine use. GC/MS is recommended for confirmation.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy

Forty whole blood samples and 40 urine samples collected from presumed non-users were tested in the Amphetamine Direct ELISA Kit . One hundred percent of these normal samples measured negative at 50 ng/ml for whole blood and 1000 ng/ml for urine. Thirty five whole blood samples which were previously confirmed positive for amphetamine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Amphetamine Direct ELISA Kit . All of the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

Precision

The precision of the Amphetamine 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 five times in the same assay. The results are tabulated in Table 1.

Table 1

S.D.	Amphetamine C.V.% (ng/ml)	Mean Abs.
	0	1.923
0.093	4.84	
	5	1.097
0.028	2.6	
	10	0.655
0.054	8.3	
	25	0.436
0.047	10.8	

Sensitivity

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

Specificity

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

Table 2

Compound	Cross-reactivities	Approx. ng/ml equivalent to 25ng amphetamine
I-Amphetamine		865
Hydroxyamphetamine HCl	2.9	57
I-Methamphetamine HCl	44	1250
d-Methamphetamine HCl	2	417
d-Ephedrine	6.5	>2500
I-Ephedrine	<1	>2500
d-Phenylpropanolamine	<1	>2500
I-Phenylpropanolamine	<1	>2500
d-Pseudoephedrine	<1	>2500
I-Pseudoephedrine	<1	>2500
MDMA.HCl	<1	>2500
Tyramine	<1	>2500
Methylphenidate	<1	>2500

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 5,000 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, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbitol, Benzoyllecgonine, Butabarbital, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chloropromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-

Diphenylhydantoin, 10-Diazepam, 11-Dihydrocarbamazepine, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Methbarbital, Mephenytoin, "-Methyl-"-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital, Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

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