



# BENZODIAZEPINES DIRECT RIA KIT [I-125]

Ver: 06/2001

**Immunoanalysis Corporation:**

**Catalog Number 114-0100 100 tubes**

**Catalog Number 114-0500 500 tubes**

## INTRODUCTION

A radioimmunoassay (RIA) for Benzodiazepines in urine is described. A 25 ul aliquot of sample is incubated with a 100 ul dilution of sheep anti-benzodiazepine antibody and 200 ul of I-125-Benzodiazepines reagent. Separation of the bound I-125-Benzodiazepine is by a second antibody complex. The technique is sensitive to 5 ng/ml without dilution or other manipulation. The assay features a cutoff equivalent to 100 ng/ml of oxazepam.

## INTENDED USE

The Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] is intended for detection and semi-quantitation of Benzodiazepines in urine at 100ng/ml or higher.

**The Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] 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). Clinical consideration and professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.**

## EXPLANATION OF THE TEST

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)

The Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] provides a positive reference standard of 100 ng/ml of oxazepam and a normal reference control, I-125 Benzodiazepine radioligand, Benzodiazepine antibody and a second antibody complex to precipitate antibody-bound Benzodiazepines.

## PRINCIPLES OF THE PROCEDURE

The Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] is based upon the competitive binding to antibody of I-125 radio active labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. An unknown specimen is mixed in a test tube with fixed amounts of Benzodiazepine antibody and radiolabeled antigen. Antigen present in a patient sample competes with labeled antigen for the limited antibody present. After precipitation of the antigen-antibody complex with a second antibody reagent and centrifugation, the tubes are aspirated or decanted and the pellets containing bound antigen are counted in a gamma scintillation counter. Sample CPM values equal to or less than the CPM value of the reference standard are indicative of the presence of Benzodiazepines in the urine specimen. A reference standard containing 100 ng/ml Oxazepam is supplied for use as a cut-off

The Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] requires only one 60 minute incubation, and avoids extraction of urine sample for measurement. There is little or no interference with binding proteins(s) or other macromolecules.

## MATERIALS AND METHODS

Materials and equipment required but not supplied with the Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] are itemized below:

### Materials

- 12x75 mm Disposable Glass or Plastic Culture Tubes.
- Test Tube Racks.
- Manual micropipets or automated pipetting stations

### Equipment

- Refrigerator (for kit storage).
- Vortex Mixer.
- Interval Timer.
- Centrifuge.
- Gamma Counter calibrated for <sup>125</sup>I.
- Calculator.

## REAGENTS

### Immunoanalysis Benzodiazepines Direct RIA Kit I-125 Contents.

Each Immunoanalysis Benzodiazepines Direct RIA Kit [I-125] contains:

Contents	100 Test Kit
1 bottle Benzodiazepine Antibody	11 ml
1 bottle I-125-Benzodiazepine (not more than 10 uCi)	21 ml
1 bottle containing 100 ng/ml Oxazepam in synthetic urine	4 ml
1 bottle Normal Control (synthetic urine)	4 ml
1 Bottle Second Antibody-PEG Complex	22 ml

Benzodiazepine Antibody. The antibody solution is sheep anti-Benzodiazepine serum diluted in 0.1 M phosphate buffer, pH 7.0, with BSA 0.1%, sodium chloride 0.9%, and sodium azide 0.1% (colored blue).

I-125-Benzodiazepine. The tracer solution contains not more than 10 uCi of I-125-Benzodiazepine in 0.1 M phosphate buffer, pH 7.0, with BSA (0.1%), sodium chloride (0.9%) and sodium azide (0.1%) (colored green).

Positive Reference Standards. This contains 100 ng/ml of Oxazepam dissolved in synthetic urine with 0.1% sodium azide.

Normal Control. This bottle contains drug free synthetic urine.

Second Antibody-PEG Complex. The separation reagent contains a second antibody-PEG complex in 0.1 M phosphate buffered saline (colored pink).

#### Precautions

For In Vitro Diagnostic Use.

Not for Internal or External Use in Humans or Animals.

Radioactive Warning. This radioactive material may be received, acquired, possessed and used by physicians, clinical laboratories and hospital laboratories possessing a specific license and only for in-vitro clinical or laboratory tests not involving internal or external administration of the material or the radiation therefrom to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission or of the State with which the Commission has entered into an agreement for the exercise of regulatory authority.

This kit contains radioactive material which should be handled according to the following guidelines:

- (1) All radioactive materials should be stored and used in specially designated areas.
- (2) No pipetting should be done by mouth.
- (3) There should be no smoking or eating within the work area.
- (4) Hands should be washed after using radioactive materials.
- (5) Work should be carried out on a surface covered with absorbant materials.
- (6) Any spills of radioactive material should be cleaned immediately and all contaminated materials disposed of as radioactive waste. Contaminated surfaces should be washed with a detergent.

Sodium Azide Hazard. Sodium azide can violently react with the copper, lead, brass or solder in plumbing systems. Since sodium azide has been added in a concentration of 0.1% to the buffer, standards and antibody, these reagents should be disposed into the drain system together with copious amounts of water. Copper- free and lead-free plumbing is recommended.

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.

Treatment Required. There is no sample treatment or reagent purification required for the use of the Immunalysis Benzodiazepines Direct RIA Kit [I-125].

Indications of Deterioration. A drop of greater than 25% in the zero-dose (maximum bound %) for a constant incubation time indicates deterioration of the antibody or tracer. A significant shift of the standard curve to the right would result from deterioration of the standards. Non-specific binding above 15% indicates deterioration of the tracer.

#### SPECIMEN COLLECTION

##### Precautions.

The Immunalysis Benzodiazepines Direct RIA Kit [I-125] is to be used with human urine samples.

##### Additives.

Urine specimens to which sodium fluoride has been added or preservatives necessary to prevent bacterial growth do not affect the assay.

##### Interfering Substances.

There are no commonly abused drugs which alter the values obtained with the Immunalysis Benzodiazepines Direct RIA Kit [I-125]. Samples containing radioactive contamination from previous in vivo diagnostic procedures should be rejected.

##### 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 before use. The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added simultaneously using an automated pipettor. After simultaneous addition of all reagents proceed directly to step 7 below.

1. Set up and label as many duplicate tubes as are required for the Positive Reference Standards, the Normal Control and the urine specimens to be assayed.
2. Add 25 ul of Positive Reference Standard and Normal Control to the appropriate tubes.
3. Add 25 ul of each urine specimen to the appropriate tubes.
4. Add 200 ul of the green I-125 Benzodiazepine to each tube.
5. Add 100 ul of blue Anti-Benzodiazepine Serum Reagent to each tube
6. Add 200 ul of the red Second Antibody Reagent (**shake well before use**) to each tube.
7. Gently vortex mix all tubes and incubate for 60 minutes or any

interval up to 3 hours at room temperature (25°C). Standards, samples and controls must be incubated together for the same time period. The assay rack may be covered with parafilm.

8. Centrifuge the tubes for 15 minutes, at approximately 1200-2500 x g in a swinging bucket rotor, or at least 3500-4000g in a fixed angle head rotor. Centrifugation time may be extended, if necessary, to optimize formation of suitable pellets.
9. Decant supernatant, drain (optional) and blot each tube.
10. Count each tube in a gamma scintillation counter to obtain counts per minute (CPM).
11. Compare average counts per minute obtained from each unknown specimen with the average CPM obtained from the 100 ng/ml Oxazepam Positive Reference Standard

Should you desire to determine NSB, use 100ul of Normal control in place of antibody. It is recommended that at least one in house positive quality control sample be included with each assay run.

**RESULTS**

If the average sample CPM is equal to or less than the average CPM of the Positive Reference Standard the sample is **POSITIVE** for Benzodiazepines (has a Benzodiazepines concentration equal to or greater than 100 ng/ml of oxazepam equivalent). If the average sample CPM is greater than the average CPM of the Positive Reference Standard the sample is called **NEGATIVE** for Benzodiazepines (less than 100 ng/ml oxazepam equivalent). Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding CPM (ordinate). Values for unknown samples are obtained by interpolation from the curve.

THE FOLLOWING DATA REPRESENT A TYPICAL DOSE/RESPONSE CURVE.

NOTE THAT THE ENTIRE CLINICAL RANGE IS PERFECTLY LINEAR WITHOUT MANIPULATION.

Benzodiazepine ng/ml	Mean CPM
0	96696
50	54085
100	26841
200	15083

The dose/response curve shown above should not be used in assay calculations. One generated at time of assay is done easily and is suitable for calculation of drug concentration in sample. The dose/response plot is sharp and linear from the low point to the high point and through the 100 ng/ml cut-off.

**Interpretation.**

Benzodiazepines and their metabolites appear in urine within hours after drug use and may persist for days. Thus a positive result documents Benzodiazepines use. GC/MS is recommended for confirmation.

**SPECIFIC PERFORMANCE CHARACTERISTICS**

Accuracy

Forty urine samples collected from presumed non-users were tested in the Immunalysis Urine Benzodiazepines RIA Kit [I-125]. One hundred percent of these normal urines measured negative at 100 ng/ml. No individual urine exceeded 10 ng/ml. Fifty five samples which were previously confirmed positive for Benzodiazepines by GC-MS, were tested using the Immunalysis Benzodiazepines Direct RIA Kit [I-125] employing a cut-off of 100 ng/ml. Fifty three samples were found to be positive i.e. above the cut-off of 100 ng/ml.

Precision

The precision of the Immunalysis Benzodiazepines Direct RIA Kit [I-125] 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 50, 100, and 200 ng/ml standards were assayed five times in the same assay. The results are tabulated in Table 1.

Table 1  
Intra-assay Precision (Reference Controls)

Benzodiazepine (ng/ml)	Mean CPM	S.D.	C.V.%
50	53662	1489.35	2.77
100	25174	1104.82	4.39
200	16085	799.26	4.97

Inter-assay Precision

Inter-assay precision was performed on reference controls. A 50, 100 and 200 ng/ml reference controls were assayed in ten separate runs over a 24 hour period and a SD and CV determined. Results are tabulated in Table 2.

Table 2  
Inter-assay Precision (Reference controls)

Benzodiazepine (ng/ml)	No of determinations	Mean CPM	S.D.	C.V.%
50	10	52974	1371.56	2.59
100	10	24881	1194.36	4.80
200	10	14997	769.34	5.13

Sensitivity

Assay sensitivity based on the minimum Benzodiazepines concentration required to produce a four standard deviation from assay Bo is 5 ng/ml. A conservative 100 ng/ml cut off of oxazepam is recommended.

### Specificity

The specificity of the Immunalysis Radioimmunoassay for Benzodiazepines [I-125] for various Benzodiazepines was determined by generating inhibition curves for each of the compounds and then determining by extrapolation the percentage cross-reactivity at assay cut-off (approximately 50 percent B/Bo). The antisera cross-reactivities are listed in Table 3.

Table 3

### Cross Reactivities with Related Drugs

Compound	Approx. ng/ml equivalent to 100 ng Oxazepam	Cross-reactivities at 50% Inhibition
Alprazolam	70	145
Alpha-OH Alprazolam		78 128
Chlordiazepoxide	435	23
Clorazepate	385	26
Demoxepam	238	42
Diazepam	130	77
Flurazepam	208	48
Flunitrazepam	154	65
Halazepam	345	29
Lorazepam	263	38
Medazepam	222	45
Nitrazepam	119	84
Prazepam	333	30
Temazepam	128	78
Triazolam	74	135

### 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, Benzoylcegonine, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro-carbamazepine, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimid 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

### Recovery

Normal urines were spiked with Oxazepam to give a final

theoretical concentration of 50, 100 and 200 ng/ml. Each of these controls were assayed in replicates of 10 within a test run, and the subsequent experimental concentration and recovery calculated. The results are tabulated in Table 4.

Table 4  
Recovery

Spiked urine concentration Benzodiazepine	Observed Benzodiazepine concentration (ng/ml)	% Recovery
50	49.4	98.8
100	104.3	104.3
200	193.6	96.8

### Interpretation

Benzodiazepines appear in the urine within hours after drug use and may persist for days(3). Thus this Immunalysis highly sensitive, single step, single incubation assay, characterized by a sharp linear plot through the 100 ng/ml cut-off from the low point throughout the high concentration points, reliably documents recent Benzodiazepines use.

### Limitations of the Procedure

Samples containing radioactive contamination from previous in-vivo diagnostic procedures should be rejected. It is possible that other substances and/or factors may interfere with the test and cause false results e.g. technical or procedural errors. Clinical consideration and professional judgement should be applied to any drug abuse test, particularly when preliminary positive results are used.

### 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<sup>th</sup> 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.

This package conforms to the conditions and limitations specified in 49 CFR 173.421 for radioactive material, excepted package-limited quantity of material, UN2910.

**IMMUNALYSIS CORPORATION**  
**Pomona, CA 91767**  
**(909) 394 2203**