

## Introduction and Background

### A. Overview

Flunitrazepam (Rohypnol) is available in a number of western European countries and Mexico for use as a hypnotic and anesthetic induction agent. It is administered orally or by intravenous injection in doses of 2 mg. Flunitrazepam is metabolized via N-demethylation, 3-hydroxylation and glucuronidation and the reduction of the nitro group to an amine with subsequent acetylation. Over a seven day period an average of 84% of a labeled dose is eliminated in urine and 11% in feces. Major metabolites include 7-aminoflunitrazepam, 3-hydroxyflunitrazepam, 7-acetamidonorflunitrazepam and 3-hydroxy-7-acetamidoflunitrazepam.

### B. Test Principle

The Flunitrazepam 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 20 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled Flunitrazepam derivative in micro-plate wells, coated with fixed amounts of high affinity purified polyclonal anti-Flunitrazepam. 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 0.5 ng/ml. The Flunitrazepam Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs a Flunitrazepam 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 allows an extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macromolecules.

### C. Notice for Application of Kit

This kit has been configured for research use only and is not for diagnostic and clinical use.

### D. Application

The Flunitrazepam Direct ELISA Kit is a sensitive in-vitro test to detect the presence of Flunitrazepam and 7-amino flunitrazepam in forensic samples such as whole blood, serum, plasma and urine.

## Material and Method

### A. List of component

	96 Tests
Microwells coated with polyclonal anti- Flunitrazepam	12x8x1
Positive Control 1 vial (ready to use)	2 ml
Negative Control 1 vial (ready to use)	2 ml
Enzyme conjugate: 1 bottle (ready to use)	15 ml
TMB Substrate: 1 bottle (ready to use)	28 ml
Stop Solution: 1 bottle (ready to use)	25 ml

### B. Additional Required Materials But Not Provided

1. Distilled or deionized water
2. Precision pipettes
3. Disposable pipette tips
4. ELISA reader capable of reading absorbance at 450nm
5. Absorbance paper or paper towel
6. Graph paper

### C. Stability and Storage

1. Store the kit at 2-8 °C.
2. Keep microwells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun, or strong light.

### D. Warnings and Precautions for Users

1. Potential biohazardous materials:  
The calibrator and controls contain human source components which have been tested and found non-reactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents. However, as there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2, as recommended in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories." 1984.
2. This test kit is designed for Research Use Only. Not for use in diagnostic procedures.
3. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.
4. The components in this kit are intended for use as an integral unit. The components of different lots should not be mixed.

5. It is recommended that serum samples be run in duplicate.
6. Optimal results will be obtained by strict adherence to this protocol. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from this may yield invalid data.

#### **E. Specimen Collection and Handling**

1. Collect blood specimens and separate the serum immediately.
2. Specimens may be stored refrigerated at (2-8 °C) for 5 days. If storage time exceeds 5 days, store frozen at (-20 °C) for up to one month.
3. Avoid multiple freeze-thaw cycles.
4. Prior to assay, frozen sera should be completely thawed and mixed well.
5. Do not use grossly lipemic specimens.

#### **F. Protocol**

Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before use.

1. Secure the desired number of coated strips in the frame holder.
2. Add 20 µl of calibrators and standards to each well in duplicate.
3. Add 20 µl of the diluted specimens in duplicate (recommended) to each well.
4. For post mortem forensic samples add 100 µl of 100 mM Phosphate Buffer saline to each well. (Optional)
5. Add 100 µl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
6. Incubate for 60 minutes at room temperature (20-25 °C) preferably in the dark, after addition of enzyme conjugate to the last well.
7. Wash the wells 6 times with 350 µl distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
8. 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.
9. Add 100 µl of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
10. Incubate for 30 minutes at room temperature, preferably in the dark.
11. Add 100 µl of Stop Solution to each well, to change the blue color to yellow.
12. 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.
13. Wells should be read within 1 hour of yellow color development.

**Example of a standard curve**

The following data represent a typical dose/response curve.

7-Amino Flunitrazepam ng/ml	OD 450nm
0	1.755
5	0.902
10	0.372
25	0.137

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.

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

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.

**References**

1. R.C. Baselt, R.H. Cravey. Disposition of Toxic Drugs and Chemicals in Man. Chemical Toxicology Institute, Foster City 325-327.