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## FLUNITRAZEPAM

ELISA KIT INSTRUCTIONS PRODUCT #109519 & 109516  
FORENSIC USE ONLY

**INTENDED USE:** For the determination of trace quantities of Flunitrazepam and/or other metabolites in human urine, blood, oral fluid.

### DESCRIPTION

Neogen Corporation's Flunitrazepam ELISA (Enzyme-Linked ImmunoSorbent Assay) test kit is a qualitative one-step kit designed for use as a screening device for the detection of drugs and/or their metabolites. The kit was designed for screening purposes and is intended for forensic use only. It is recommended that all suspect samples be confirmed by a quantitative method such as gas chromatography/mass spectrometry (GC/MS).

### ASSAY PRINCIPLES

Neogen Corporation's test kit operates on the basis of competition between the drug or its metabolite in the sample and the drug-enzyme conjugate for a limited number of antibody binding sites. First, the sample or control is added to the microplate. Next, the diluted drug-enzyme conjugate is added and the mixture is incubated at room temperature. During this incubation, the drug in the sample or the drug-enzyme conjugate binds to antibody immobilized in the microplate wells. After incubation, the plate is washed 3 times to remove any unbound sample or drug-enzyme conjugate. The presence of bound drug-enzyme conjugate is recognized by the addition of K-Blue<sup>®</sup> Substrate (TMB). After a 30 minute substrate incubation, the reaction is halted with the addition of Red Stop Solution. The test can be read visually or with a microplate reader equipped with a 650 nm filter. The extent of color development is inversely proportional to the amount of drug in the sample or control. In other words, the absence of the drug in the sample will result in a dark blue/purple color, whereas the presence of the drug will result in light pink to no color development.

### STORAGE AND STABILITY

This kit can be used until the expiration date on the label when stored refrigerated at 2-8°C. Store controls frozen if not used within 10 days. Note: Some kits require controls to be stored frozen immediately upon receipt. Reference kit label for details.

### MATERIALS PROVIDED – SINGLE KIT (96 WELL)

1. **EIA Buffer:** 40 mL (ready-to-use). Phosphate buffered saline solution with bovine serum and a preservative. Provided for dilution of drug-enzyme conjugate concentrate and samples.
2. **Wash Buffer Concentrate (10X):** 20 mL. Phosphate buffered saline solution with a surfactant. Dilute 10 fold with deionized or ultrapure water before use. Diluted wash buffer is used to wash all unbound conjugate and samples from the plate after the conjugate incubation.
3. **K-Blue Substrate:** 20 mL (ready-to-use). Stabilized 3, 3', 5, 5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) in a single bottle. It is used to develop the color in the wells after washing. Light sensitive.
4. **Drug-Enzyme Conjugate:** 200 µL. Drug-horseradish peroxidase concentrate. Dilute 180X before use.
5. **Antibody Coated Plate:** A 96 well Costar plate, in strips of 8 break-away wells, coated with anti-drug antiserum. The plate is ready for use as is. Do not wash.
6. **Red Stop Solution:** 20 mL (ready-to-use). Non-acidic reagent used to stop the enzyme reaction.
7. **Qualitative QC Positive Control:** 750 µL provided (synthetic human urine). Do not dilute.
8. **Qualitative QC Negative Control:** 750 µL provided (synthetic human urine). Do not dilute.

## MATERIALS PROVIDED – BULK KIT (480 WELL)

1. **EIA Buffer:** 200 mL (ready-to-use). Phosphate buffered saline solution with bovine serum and a preservative. Provided for dilution of samples.
2. **Wash Buffer Concentrate (10X):** 100 mL. Phosphate buffered saline solution with a surfactant. Dilute 10 fold with deionized or ultrapure water before use. Diluted wash buffer is used to wash all unbound conjugate and samples from the plate after the conjugate incubation.
3. **K-Blue Substrate:** 100 mL (ready-to-use). Stabilized 3, 3', 5, 5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide ( $H_2O_2$ ) in a single bottle. It is used to develop the color in the wells after washing. Light sensitive.
4. **Drug-Enzyme Conjugate:** 1 mL. Drug-horseradish peroxidase conjugate. Dilute 180X before use.
5. **Antibody Coated Plate:** 5 x 96 well Costar plate, in strips of 8 break-away wells, coated with anti-drug antiserum. The plate is ready for use as is. Do not wash.

## MATERIALS NEEDED BUT NOT PROVIDED

1. Deionized water.
2. Precision pipettes that range from 10  $\mu$ L - 1000  $\mu$ L and disposable tips.
3. Graduated cylinder to dilute and mix wash buffer.
4. Plate cover or plastic film to cover plate during incubation.
5. Clean glassware (i.e. test tubes) to dilute drug-enzyme conjugate.
6. Microplate reader with 650 nm filter if Red Stop is used, or a 450 nm filter if 1N HCl is used to stop the reaction. Note: It is not necessary to stop the reaction if reading immediately. Unstopped reactions should be read with a 650 nm filter.
7. Cut-off calibrator.

## OPTIONAL TEST MATERIALS

1. **Qualitative QC Positive Control:** 5 x 750  $\mu$ L (synthetic human urine). 1-5 vials available upon request. Do not dilute.
2. **Qualitative QC Negative Control:** 5 x 750  $\mu$ L (synthetic human urine). 1-5 vials available upon request. Do not dilute.
3. **Red Stop Solution:** 100 mL (ready-to-use). Non acidic reagent used to stop the enzyme reaction. Available upon request. Product No. 301473.

## OPTIONAL MATERIALS

1. 1N HCl if Red Stop Solution is not used.
2. Microplate shaker.

## PRECAUTIONS AND NOTES

1. **DO NOT** use kits or components beyond expiration date.
2. **DO NOT** mix conjugates and plates from different kit lots.
3. **DO NOT** pipette reagents by mouth.
4. Pour K-Blue Substrate out of the bottle into a clean reservoir. To prevent contamination of the substrate, **DO NOT** pipette out of the bottle.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. Keep plate covered except when adding reagents, washing or reading.
7. Kit components should be refrigerated at all times when not in use.
8. Keep the controls frozen if storing longer than 10 days. Avoid repeated freeze-thaw cycles. Note: Some kits require controls to be stored frozen immediately upon receipt. Reference kit label for details.
9. Use aseptic technique when opening and removing reagents from vials and bottles.
10. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
11. Do not substitute DI water for the wash step of this protocol. Use only Neogen's wash buffer.
12. Do not use Sodium Azide with samples, standards and/or calibrators.
13. Do not reuse wells, they are for one use only.

## PROCEDURAL NOTES

1. Desiccant bag must remain in foil pouch with unused strips. Keep zip lock pouch sealed when not in use to maintain a dry environment.
2. Use clean pipette tips for the buffer, drug-enzyme conjugate, controls and samples.
3. Before pipetting a reagent, rinse the pipette tip three times with that reagent.
4. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well or any of the reagent already inside the well. This may result in cross contamination.
5. Controls and samples should be assayed in duplicate.
6. The drug-enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
7. Before opening the drug-enzyme conjugate vial, tap the vial in an upright position to remove any liquid in the cap.
8. Before substrate addition, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
9. Gently mix specimens and reagents before use. Avoid vigorous agitation.

## SAMPLE TREATMENT

Recommended minimum sample dilutions are listed below. These dilutions may change based on your laboratory's determination. All sample dilutions should be made in Neogen's EIA Buffer.

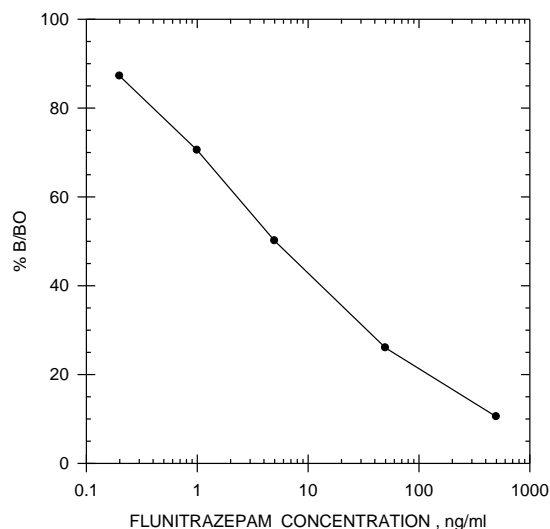
- a. **Urine:** A dilution of 1:4 (i.e. 1 part sample to 3 parts EIA Buffer provided) may be necessary to reduce natural background as well as bring desired cutoff concentration into assay range. Please contact your Neogen Representative for assistance.
- b. **Whole blood:** A dilution of 1:5 (i.e. 1 part sample to 4 parts provided EIA Buffer) is recommended. Please contact your Neogen Representative for assistance.
- c. **Other Forensic sample types:** Please contact your Neogen Representative for assistance.

## TEST PROCEDURES

The following test procedures can be run manually or on an automated instrument. Please contact your Neogen representative for assistance with protocols for automated instruments.

1. Determine the number of wells to be used.
2. Prepare the enzyme conjugate solution by diluting the 180X enzyme conjugate stock 1 to 180 in the EIA Buffer provided. Mix the solution by inversion. Do not vortex. Example: for two eight well strips, add 25  $\mu\text{L}$  of the 180X enzyme conjugate stock to 4475  $\mu\text{L}$  of EIA Buffer.
3. Add 20  $\mu\text{L}$  of sample, laboratory calibrators and Neogen controls to the appropriate wells in duplicate. Do not dilute Neogen's positive and negative controls.
4. Add 180  $\mu\text{L}$  of diluted drug-enzyme conjugate to each well. Use 8-channel pipetter or 12-channel pipetter for rapid addition.
5. For manual runs, mix by gently shaking plate. A microplate shaker may be used.
6. Cover plate with plastic film or plate cover and incubate at room temperature for 45 minutes.
7. During the conjugate incubation, dilute concentrated wash buffer 10 fold with deionized water (i.e. 20 mL of concentrated wash buffer plus 180 mL of deionized water). Mix thoroughly. Diluted wash buffer is stable for 5 days at room temperature or 7 days at 2-8°C.
8. Once the incubation is complete, dump or aspirate the liquid from the wells. Tap the plate on a clean lint-free towel to remove any remaining liquid in the wells.
9. Wash each well with 300  $\mu\text{L}$  of diluted wash buffer. Manual Wash: For manual wash procedures repeat for a total of 3 washings, invert and tap dry the plate following each step. After completing the last wash step wipe the bottom of the wells with a lint-free towel to remove any liquid on the outside of the wells. Automated Wash: If an automated plate washer is used wash the plate for a total of 5 washings with 300  $\mu\text{L}$  of diluted wash buffer. It is important for the automated washer to conduct a final aspirate cycle to eliminate residual amounts of wash buffer. Residual amounts of buffer in the wells will affect assay performance. Note: DI water should never be used for the plate wash.
10. Add 150  $\mu\text{L}$  of the K-Blue Substrate to each well. For manual runs, use a multi-channel pipetter for best results.
11. Incubate at room temperature for 30 minutes. Gently shake immediately before measuring the absorbance.
12. Add 50  $\mu\text{L}$  of Neogen's Red Stop Solution to each well to stop enzyme reaction. Mix gently before measuring the absorbance. For automated systems a 10 second shake is sufficient. Measure the absorbance at a wavelength of 650 nm. Wells should be read within 2 hours of stopping the reaction. Note: When Neogen's Red Stop is used, it will result in a color ranging from a dark blue/purple to light pink based on the concentration of drug present. If 1 N HCl is preferred, use 50  $\mu\text{L}$  per well and read plate with a 450 nm filter. All QC data is generated without using a stopping reagent. Note: When acid stop is used, OD values will approximately double as compared to the OD values obtained with Red Stop.

## STANDARD CURVE IN EIA BUFFER



## SENSITIVITY

Compound	I-50 in EIA Buffer	I-50 in Human Urine (1:4 dilution : 1 part sample to 3 parts EIA Buffer)
	7-aminoflunitrazepam	3.2 ng/mL
Flunitrazepam	4.1 ng/mL	41 ng/mL
Diazepam	9.6 ng/mL	N/A

The term I-50 is used to define the sensitivity of the test. This number is derived from a standard curve generated with the drug in EIA Buffer. The drug concentration that shows 50% less color activity than the zero standard is considered to be the I-50.

## SPECIFICITY

Compound	Flunitrazepam		
	Concentration (ng/mL)	Equivalents (ng/mL)	% Cross- Reactivity
Flunitrazepam	7.2	7.2	100%
7-aminoflunitrazepam	3.41	7.2	212%
Diazepam	10.43	7.2	69%
Clobazam	218.2	7.2	3.3%
Clonazepam	342.91	7.2	2.1%
Midazolam Maleate	514.31	7.2	1.4%
7-aminoclonazepam	1,200	7.2	0.6%
N-desmethyflunitrazepam	1,440	7.2	0.5%
Halazepam	1,800	7.2	0.4%
Triazolam	2,400	7.2	0.3%
Nitrazepam	2,400	7.2	0.3%
Nordiazepam	3,600	7.2	0.2%
Methylene Blue	36,000	7.2	0.02%
Trimipramine	72,000	7.2	0.01%

Note: Flunitrazepam equivalents represents 50% B/B<sub>0</sub> assay displacement in EIA Buffer.

*The compounds having cross-reactivity below 0.01% did not show any significant reaction up to 10µg/mL.*

**ALL THE FOLLOWING HAVE A CROSS-REACTIVITY <0.01%.**

Acepromazine, Acetaminophen, Acetylsalicylic Acid, E-amino-n-caproic Acid, Amitriptyline, Ascorbic Acid, Benzoic Acid, Caffeine, Chlordiazepoxide, Chlorpromazine, Clenbuterol, Clozapine, Codeine, Cotinine, Dexamethasone, Dextromethorphan, Diclofenac, Dimethyl Sulfoxide, Dipyrone, Doxepin, Ephedrine, Erythromycin, Ethyl p-amino-benzoate, Fenopropfen, Flunixin, Flurazepam, Folic Acid, Folinic Acid, Furosemide, Gemfibrozil, Gentisic Acid, L-Glutamic Acid, Glutethimide, Glycopyrrolate, Heparin, Hippuric Acid, Hordenine, Hydrocortisone, Ibuprofen, Imipramine, Isoxsuprine, Lidocaine, Meperidine, Metaproterenol, Methadone, Methaqualone, Methocarbamol, Methyprednisolone, Nalorphine, Naproxen, Niacinamide, Nicotine, Nortriptyline, Orphenadrine, Oxyphenbutazone, PCP, Pencillin G-Potassium, Penicillin G-Procaïne, Pentoxifylline, Phenothiazine, Phenylbutazone, Polyethylene Glycol, Prednisolone, Primadone, Procaine, Procainamide, Promazine, Pseudoephedrine, Pyrantel, Pylamine, Pyrimethamine, Quinidine, Quinine, Salbutamol, Salicylamide, Salicylic Acid, Theophylline, Thiamine, Trimethoprim, Uric Acid.

## RESULTS INTERPRETATION

Each laboratory should determine the cutoff level for their individual application. When possible, cutoff calibrators and/or standards should be prepared in the same matrix being tested.

**Positive Result:** Samples with an absorbance less than or equal to the laboratory's designated cutoff calibrator should be considered positive. All positive samples should be confirmed by a quantitative method such as GC/MS.

**Negative Result:** Samples with an absorbance greater than the laboratory's designated cutoff calibrator should be considered negative.

**Qualitative QC Controls:** The Neogen positive and negative controls provided in the kit are for QC purposes only. The sole purpose of these controls is to verify that the test kit is performing properly. The controls are not intended for use as cutoff calibrators. The positive control is spiked at a high concentration and its approximate level can be found on the label.

Note: The kit was designed for screening purposes only. It is recommended that all suspect samples be confirmed by a quantitative method such as GC/MS or HPLC.

## TECHNICAL SUPPORT

For technical assistance, please contact our Technical Services Department at (859) 254-1221 or email at [techservice-toxicology@neogen.com](mailto:techservice-toxicology@neogen.com). Representatives are available Monday – Friday from 8:00 am – 6:00 pm EST.

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