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AMFEPRAMONE (DIETHYLPROPION)

ELISA KIT INSTRUCTIONS PRODUCT #182119, 182116 & 182113
FORENSIC USE ONLY

INTENDED USE: For the determination of trace quantities of Amfepramone (Diethylpropion) and/or other metabolites in human samples.

DESCRIPTION

Neogen Corporation's Amfepramone (Diethylpropion) 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 PRINCIPLE

The Neogen forensic drug detection ELISA is a solid phase immunoassay designed to detect drugs of abuse for forensic application. The test is performed in microwells coated with a high affinity capture antibody. A control or sample is added to the wells followed by an enzyme conjugate. During the following incubation period, the enzyme conjugate competes with the drug in the sample for binding sites on the antibody coated well. After a wash step to remove any unbound material, substrate is added for the color development process. Acid stop solution is added to discontinue the enzyme-substrate reaction. The color intensity is inversely proportional to the amount of drug present in the sample. Therefore, those samples which contain the drug will inhibit binding of the enzyme conjugate to the capture antibody resulting in less color than the negative control. Negative and positive controls should be run along with the samples. Results should be obtained by reading the absorbance of the wells with a microplate reader.

STORAGE AND STABILITY

This kit can be used until the expiration date on the label and Certificate of Analysis when stored refrigerated between 2 - 8°C. Always check each kit for specific expiration dates and storage requirements.

MATERIALS PROVIDED – SINGLE KIT (96 WELL)

1. **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.
2. **K-Blue® Substrate:** 20 mL (ready-to-use). Stabilized 3,3',5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H₂O₂) in a single bottle. It is used to develop the color in the wells after washing. Light-sensitive.
3. **Drug-Enzyme Conjugate:** 200 µL. Drug-horseradish peroxidase conjugate. Dilute 100X before use.
4. **Drug-Enzyme Diluent:** 15 mL.
5. **Acid Stop Solution:** 18 mL of 1N H₂SO₄.
6. **Antibody-coated Plate:** 96 well plate coated with anti-drug antiserum. The plate is ready for use. Do not wash.

MATERIALS PROVIDED – BULK KIT (480 WELL)

1. **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.
2. **K-Blue Substrate:** 100 mL (ready-to-use). Stabilized 3,3',5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H₂O₂) in a single bottle. It is used to develop the color in the wells after washing. Light-sensitive.
3. **Drug-Enzyme Conjugate:** 1 mL. Drug-horseradish peroxidase conjugate. Dilute 100X before use.

4. **Drug-Enzyme Diluent:** 85 mL.
5. **Acid Stop Solution:** 90 mL of 1N H₂SO₄.
6. **Antibody-coated Plate:** 5 X 96 well plate coated with anti-drug antiserum. The plate is ready for use. Do not wash.

MATERIALS PROVIDED – 50 PACK (4800 WELL)

1. **Wash Buffer Concentrate (10X):** 1 L. 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.
2. **K-Blue Substrate:** 500 mL (ready-to-use). Stabilized 3,3',5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H₂O₂) in a single bottle. It is used to develop the color in the wells after washing. Light-sensitive.
3. **Drug-Enzyme Conjugate:** 9.5 mL. Drug-horseradish peroxidase conjugate. Dilute 100X before use.
4. **Drug-Enzyme Diluent:** 850 mL.
5. **Acid Stop Solution:** 500 mL of 1N H₂SO₄.
6. **Antibody-coated Plate:** 50 X 96 well plate coated with anti-drug antiserum. The plate is ready for use. Do not wash.

MATERIALS NEEDED BUT NOT PROVIDED

1. Precision pipettes that range from 10 uL - 1000 uL and disposable tips.
2. Graduated cylinder to dilute and mix wash buffer.
3. Clean glassware (i.e. test tubes).
4. Microplate reader capable of measuring absorbance at 450 nm (650 nm, 630 nm, or 620 nm wavelength reference filter).
5. Deionized water.

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. Use aseptic technique when opening and removing reagents from vials and bottles.
9. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
10. Do not substitute DI water for the wash step of this protocol. Use only Neogen's wash buffer.
11. Do not use Sodium Azide with samples, standards and/or calibrators.
12. Do not reuse wells, they are for one-time use only.

PROCEDURAL NOTES

1. Desiccant bag must remain in the zip-lock with unused strips. Keep zipped bag 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. Before opening the drug-enzyme conjugate vial, tap the vial in an upright position to remove any liquid in the cap.
7. Before substrate addition, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.

ENZYME PREPARATION

1. Prior to use, perform a 100X dilution of the drug-enzyme conjugate using the provided drug-enzyme diluent. The drug-enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used. For example:

# of plates	Volume of Conjugate	Volume of Drug-Enzyme Diluent
1	140 μ L	13.86 mL
5	700 μ L	69.3 mL
25	3.5 mL	346.5 mL

2. Gently mix the diluted drug-enzyme conjugate solution by inverting 10-15 times. **Do not vortex.** Store unused conjugate at 4°C.

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 and amount of reagents that will be required for immediate testing.
2. All ELISA components, controls, and samples must be at room temperature prior to use. Mix reagents by gentle inversion of bottles.
3. Pipette 10 μ L of standards, controls, and samples into wells.
4. Add 100 μ L of the diluted enzyme (refer to Enzyme Preparation) to each well.
5. Allow the reaction to incubate at room temperature for 45 minutes.
6. 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.
7. Wash each well with 300 μ 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 μ 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.
8. Proceed immediately to add 100 μ L of the K-Blue Substrate to each well. Ensure that the outside bottoms of the wells are clean and dry.
9. Allow the substrate to incubate for 30 minutes. Neogen recommends gentle agitation during this time, preferably on a plate shaker.
10. Stop the reaction by adding 100 μ L of Acid Stop Solution to each well.
11. Measure the absorbance at a wavelength of 450 nm.

SENSITIVITY/SPECIFICITY

Compound	Compound Concentration (ng/mL)	Amfepramone (Diethylpropion) Equivalents (ng/mL)	% Cross-Reactivity
Amfepramone (Diethylpropion)	1.02	1.02	100%
(+)-N,N-Diethylnorephedrine	140	1.02	0.73%
PCP	730	1.02	0.14%
4-Methylethcathinone	800	1.02	0.13%
Dextromethorphan	800	1.02	0.13%
Penicillin-G-Procaïne	900	1.02	0.11%
(+)-Norpseudoephedrine	1000	1.02	0.10%
Trimipramine	1300	1.02	0.08%
Ethcathinone	1400	1.02	0.07%
Procaïne	2000	1.02	0.05%
Chlorpromazine	2100	1.02	0.05%

Promazine	2400	1.02	0.04%
Acetopromazine	3100	1.02	0.03%
Hordenine	3100	1.02	0.03%
Methylene Blue	3100	1.02	0.03%
(±)-MDEA	3200	1.02	0.03%
Imipramine	3800	1.02	0.03%
Bupropion	4000	1.02	0.03%
Procainamide	4600	1.02	0.02%

Note: Amfepramone (Diethylpropion) equivalents represent 50% B/B₀ assay displacement in BPS Buffer

The compounds having cross-reactivity below 0.02% did not show any significant reaction up to 10µg/mL.

ALL THE FOLLOWING HAVE A CROSS-REACTIVITY <0.02%.

Acetaminophen; Acetylsalicylic Acid; Amitriptyline; R(-)-Amphetamine; S(+)-Amphetamine; Ascorbic Acid; Benzoic Acid; Caffeine; R(+)-Cathinone; Chlordiazepoxide; Clenbuterol; Codeine; Cotinine; Dexamethasone; Diclofenac; Dimethyl Sulfoxide; Doxepin; ε-amino-n-caproic Acid; 1S,2R(+) Ephedrine; 1R,2S(-) Ephedrine; Erythromycin; Ethyl p-amino benzoate; Fenoprofen; Flunixin; 4-Fluoromethcathinone; Folic acid; Folinic Acid; Furosemide; Gemfibrozil; Gentistic Acid; Glipizide; L-Glutamic Acid; Glutethamide; Glycopyrrolate; Heparin; Hippuric Acid; Hydrocortisone; Ibuprofen; Isoxuprine; Lidocaine; (±)-MDA; (±)-MDMA ; Meperidine; Mephedrone; Mephentermine; Metaproterenol; (±)-Methadone; R(-)-Methamphetamine; S(+)-Methamphetamine; Methaqualone; Methocarbamol; Methylone; Methylprednisolone; Nalorphine; Naproxen; Niacinamide; Nicotine; (±)N,N-Ethylcathinone Ephedrine metabolite; Nortriptyline; Orphenadrine; Oxyphenbutazone; Penicillin G-Potassium; Pentoxifylline; Phenothiazine; Phenylbutazone; (±)-Phenylpropanolamine; Polyethylene glycol; Prednisolone; Primadone; Promazine; R,R(-) Pseudoephedrine; S,S(+) Pseudoephedrine; Pyrantel; Pyrimethamine; Quinidine; Quinine; Salbutamol; Salicylamide; Salicylic Acid; Sodium Azide; 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.

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. Representatives are available Monday – Friday from 8:00 am – 6:00 pm EST.

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