The Benzodiazepines Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical test method must be used in order to obtain a confirmed analytical result. Gas chromatography/mass spectrometry (GC-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.

**SUMMARY AND EXPLANATION**

The Benzodiazepines Direct ELISA Kit is a sensitive in-vitro test to detect the presence of Benzodiazepines in samples such as whole blood, serum, plasma and urine. 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 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)

**PRINCIPLES OF THE TEST**

The Benzodiazepines Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled Benzodiazepine derivativ e 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 2 ng/ml. The Benzodiazepines Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It incorporates autoradiography and a more sensitive monoclonal antibody complex, reducing matrix effects and interference with binding proteins(s) or other Macromolecules.

**REFERENCES**

1. This kit is designed for Research Use Only. There should be no eating or drinking within work area. Always wear gloves and a protective lab coat.

2. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.

3. Do not add sodium azide to samples as preservative. Don’t use external controls containing sodium azide.

4. Use disposable pipette tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.

5. Do not pour chromogenic substrate back into container after use.

6. Keep reagents out of direct sunlight.

7. Handle reagents with care, since it is corrosive. Bring all reagents to room temperature.

8. Viscous forensic specimens should always be diluted in phosphate buffered saline or distilled water prior to pipetting.

9. Ensure the bag containing the micro-plate strips and desiccant is well sealed if only a partial plate is used.

10. 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 C.

ASSAY 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 Buffer Saline pH 7.0. (Urine samples are normally diluted 1:10 for a Oxazepam cutoff of 200 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory’s cutoff.

2. Add 10 µl of appropriately diluted 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 (20-25 C) preferably in the dark, after addition of enzyme conjugate to the last well.

6. 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 mF Phosphate buffered saline pH 7.0-7.4). This will lower potential non-specific binding of hemoglobin to the well, thus lowering background color.

7. Invert plates and vigorously tap 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 30 minutes at room temperature, preferably in the dark.

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.

12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

<table>
<thead>
<tr>
<th>Oxazepam (ng/ml)</th>
<th>Mean Abs</th>
<th>S.D.</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.86</td>
<td>0.163</td>
<td>5.3</td>
</tr>
<tr>
<td>25</td>
<td>0.957</td>
<td>0.091</td>
<td>9.1</td>
</tr>
<tr>
<td>50</td>
<td>0.665</td>
<td>0.047</td>
<td>6.6</td>
</tr>
<tr>
<td>100</td>
<td>0.458</td>
<td>0.051</td>
<td>11.2</td>
</tr>
</tbody>
</table>

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

ASSAY 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.

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<tbody>
<tr>
<td>0</td>
<td>3.043</td>
<td>0.750</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>0.548</td>
<td>0.388</td>
<td>100</td>
</tr>
</tbody>
</table>

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

SPECIMEN HANDLING

Urine samples should be stored at 2 - 4 C 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.