DATA SHEET

IDEL-F074
Enzyme Immunoassay for the Detection of Methotrexate in Urine or Serum

PRODUCT DESCRIPTION
The IDELISA™ Forensic Methotrexate ELISA Kit is a competitive enzyme immunoassay for the detection of Methotrexate in urine
or serum. Methotrexate belongs to a group of drugs known as anti-metabolites. It acts by inhibiting the metabolism of folic acid.
Methotrexate is used in treatment of cancer, autoimmune diseases and as an abortifacient in the induction of medical abortions. In
racing, human recombinant Erythropoietins have been used to drug horses. It is believed that Methotrexate is also given to horses
undergoing this drugging to prevent the horse from mounting an immune response to the foreign human recombinant protein.
The IDELISA™ Forensic Methotrexate ELISA Kit was designed for screening purposes and is intended for forensic or In Vitro
Research use only (NOT FOR THERAPEUTIC USE). All suspect samples should be confirmed by a quantitative method such as Gas
Chromatography-Mass Spectrometry (GC-MS).
This kit is a simple, rapid, sensitive and cost-effective screening method. The unique features of the kit are:

_ Consistent, reproducible results
_ High sensitivity 50% B/Bo (0.50 ng/ml)
_ Assay Range 0.1 ng/ml – 10.0 ng/ml
_ Fast, assay times can be less than 2 hours

Like most ELISA assays, this kit relies on a Horseradish Peroxidase (HRP) conjugated antibody and the TMB (3,3´,5,5´-
tetramethylbenzidine) substrate. TMB is a chromogen that yields a blue color when oxidized with hydrogen peroxide (catalyzed by
HRP) that has major absorbances at 370 nm and 652 nm. The color then changes to yellow with the addition of acid with maximum
absorbance at 450 nm. The relative amount of Methotrexate in the sample is directly proportional to the amount of signal that is
obtained at 450 nm.
This kit contains materials for the extraction and quantitative detection of Methotrexate in urine or serum.

PROCEDURE OVERVIEW
The method is based on a competitive colorimetric ELISA assay. The drug of interest has been coated in the plate wells. The sample
of interest is added along with a primary antibody specific for the target drug. If the target is present in the sample, it will compete for
the primary antibody, thereby preventing the antibody from binding to the drug attached to the well. After incubation, the sample is
removed, the wells are washed and a second antibody, which is directly conjugated to HRP, is added. Signal is generated by reaction
with the TMB substrate as described above. The intensity of the signal (measured at 450 nm) is directly proportional to the amount of
Methotrexate in the sample. Dilutions of the Methotrexate Standard are used to construct a standard curve, from which the
concentration of Methotrexate in the samples are determined by extrapolation. This is described in more detail in Section,
“Methotrexate Concentration Calculations.”

KIT REAGENTS SUPPLIED

The kit has the capacity for 96 determinations (96 wells) or testing of 42 samples in duplicate (assuming 12 wells for standards).
Return any unused microwells to the foil bag and reseal them with the desiccant provided in the original package. Store the kit at 2-
8°C*. The shelf life is noted on the kit label, when the kit and components are properly stored.

Kit Contents	Amount	Storage
Methotrexate-Coated Microtiter Plate	1 x 96-well plate (8 wells x 12 strips)	2-8°C
Methotrexate Antibody #1	15 ml	2-8°C
1000 ng/ml Standard	1.0 ml	2-8°C
100X HRP-Conjugated Antibody #2	300 µl	2-8°C
Antibody #2 Diluent **	20 ml	2-8°C
20X Wash Solution **	28 ml	2-8°C
Stop Buffer **	20 ml	2-8°C
TMB Substrate **	12 ml	2-8°C
10X PBS **	25 ml	2-8°C

If you are not planning to use the kit for over 3 months, store Methotrexate Antibody #1 and 100X HRP-Conjugated Antibody#2 at -20°C.

** These components are interchangeable between IDELISA™ Forensic Kits as long as they are used before the expiration dates on
the individual vials.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microtiter plate reader (450 nm)
- 10, 20, 100 and 1000 µl pipettes
- Multi-channel pipette: 50-300 µl (Optional)
- Distilled deionized water
- Vortexer
- Incubator

SENSITIVITY (Detection Limit)

Sample Type	Detection Limit (ng/ml or ppb)
Urine	5.0
Serum	5.0

SPECIFICITY (Cross Reactivity)

Drug	Sensitivity (ng/ml)	Cross reactivity
Methotrexate	~ 0.25	100%
N10-Methyl-4-amino-4-deoxypteroic acid	~ 0.25	100%
4-Amino-4-deoxypteroic acid	~ 0.25	100%

WARNINGS AND PRECAUTIONS

ID Labs™ strongly recommends that you read the following warnings and precautions to ensure your full awareness of ELISA
techniques and other details you should pay close attention to when running the assays. More information can also be found in the
Troubleshooting section. Periodically, optimizations and revisions are made to the kit and manual. Therefore, it is important to follow
the version of the protocol included with the kit.

Additional Technical Hints

• Do not use the kit past the expiration date.
• Do not intermix reagents from different kits or different lots. **Antibodies and plates are kit and lot specific.** Make sure that the
standards, detection antibody, avidin-HRP, and diluent are mixed in correct volumes.
• Make sure that the 100X HRP-Conjugated Antibody #2 and Antibody #2 Diluent are mixed in correct volumes.
• Try to maintain a laboratory temperature of (20 – 25°C / 68 – 77°F). Avoid running assays under or near air vents, as this may
cause excessive cooling, heating and/or evaporation. Also, do not run assays in direct sunlight, as this may cause excessive heat and
evaporation. Cold bench tops should be avoided by placing several layers of paper towel or some other insulation material under the
assay plates during incubation.
• Make sure you are using only distilled deionized water since water quality is very important.
• When pipetting samples or reagents into empty microtiter plate, place the pipette tips in the lower corner of the well, making
contact with the plastic.
• Incubations of assay plates should be timed as precisely as possible. Be consistent when adding standards to the assay plate. Add
your standards first and then your samples.

Add standards to plate only in the order from low concentration to high concentration as this will minimize the risk of
compromising the standard curve.
• Always refrigerate plates in sealed bags with a desiccant to maintain stability. Prevent condensation from forming on plates by
allowing them to equilibrate to room temperature (20 – 25°C / 68 – 77°F) before opening (plates provided in package containing
desiccant).

NOTE: ID Labs™ makes no warranty of any kind, either expressed or implied, except that the materials from which such products
are made are of standard quality. There is no warranty of merchantability of this product, or of the fitness of the product for any
purpose. ID Labs™ shall not be liable for any damages, including special or consequential damage, or expense arising directly or
indirectly from the use of this product.

SAMPLE PREPARATION
Be sure samples are properly stored. In general, samples should be refrigerated at 2-4°C for no more than 1-2 days. Freeze samples to
a minimum of -20°C if they need to be stored for a longer period. Frozen samples can be thawed at room temperature (20 – 25°C / 68
– 77°F) or in a refrigerator before use. Preparation protocols for other samples can be made available upon request.
Preparation of 1X PBS: Mix 1 volume of the 10X PBS with 9 volumes of distilled water.
Urine
1. Centrifuge 0.5 ml of the urine sample at 4,000x g for 5 minutes.
2. Take out 200 µl of the supernatant and add 800 µl of 1X PBS and mix well.
3. Use 100 µl of the diluted supernatant per well in the assay.
Note: Dilution factor: 5
If the Methotrexate concentration in the sample is too high, the sample can be further diluted in 1X PBS, and the assay re-run.
Serum
1. Blood should be collected without anticoagulant and left at room temperature for 3 hours or at 4°C overnight, to clot.
2. Centrifuge at 3,000 g for 10 minutes at 4°C.
3. Take 200 µl of the serum from the upper layer of the blood sample. Add 800 µl of 1X PBS and mix well.

4. Use 100 µl of the diluted serum per well in the assay.
Note: Dilution factor: 5
If the Methotrexate concentration in the sample is too high, the sample can be further diluted in 1X PBS and the assay re-run.

PREPARATION OF REAGENTS

IMPORTANT: All reagents should be brought up to room temperature before use (1 – 2 hours at 20 – 25°C / 68 – 77°F); Make sure
you read "Warnings and Precautions" section. Solutions should be prepared just prior to ELISA test. All reagents should be mixed by
gently inverting or swirling prior to use. Prepare volumes that are needed for the number of wells being run. Do not return the reagents
to the original stock tubes/bottles. Using disposable reservoirs when handling reagents can minimize the risk of contamination and is
recommended.

1. **Preparation of 1X HRP-Conjugated Antibody #2**
Mix 1 volume of 100X HRP-Conjugated Antibody #2 with 99 volumes of Antibody #2 Diluent.

2. **Prepare 1X PBS**
Mix 1 volume of the 10X PBS with 9 volumes of distilled water.

3. **Preparation of Standards**
Dilute the 1000 ng / ml Standard within 0.01-1 ng /ml range using 1X PBS before adding to the wells. Use 1X PBS alone as a
negative control standard.

4. **Preparation of 1X Wash Solution**
Mix 1 volume of the 20X Wash Solution with 19 volumes of distilled water.

ASSAY PROCEDURE
Label the individual strips that will be used and aliquot reagents as in the following example:

Component	Volume per Reaction	24 Reactions
Methotrexate Antibody #1	100 µl	2.4 ml
1X HRP-Conjugated Antibody #2	150 µl	3.6 ml
1X Wash Solution	2.0 ml	48 ml
Stop Buffer	100 µl	2.4 ml
TMB Substrate	100 µl	2.4 ml

1. Add 100 µl of each Methotrexate standards in duplicate into different wells **(Add standards to plate only in the order from low
concentration to high concentration).**
2. Add 100 µl of each sample in duplicate into different sample wells.
3. Add 100 µl of Antibody #1 and mix well by gently rocking the plate manually for 1 minute.
4. Incubate the plate for 30 minutes at room temperature (20 – 25°C / 68 – 77°F).
5. Wash the plate 3 times with 250 µl of 1X Wash Solution. After the last wash, invert the plate and gently tap the plate dry on paper
towels **(Perform the next step immediately after plate washings. Do not allow the plate to air dry between working steps).**
6. Add 150 µl of 1X HRP-Conjugated Antibody #2. Incubate the plate for 30 minutes at room temperature (20 – 25°C / 68 – 77°F)
**(Avoid direct sunlight and cold bench tops during the incubation. Covering the microtiter plate while incubating is
recommended).**
7. Wash the plate 3 times with 250 of µl 1X Wash Solution. After the last wash, invert the plate and gently tap the plate dry on paper

towels **(Perform the next step immediately after plate washings. Do not allow the plate to air dry between working steps).**
8. Add 100 µl of TMB substrate. Time the reaction immediately after adding the substrate. Mix the solution by gently rocking the
plate manually for 1 minute while incubating. **(Do not put any substrate back to the original container to avoid any potential**
contamination. Any substrate solution exhibiting coloration is indicative of deterioration and should be discarded. Covering
the microtiter plate while incubating is recommended).
9. After incubating for 10-30 minutes at room temperature (20 – 25°C / 68 – 77°F), add 100 µl of Stop Buffer to stop the enzyme
reaction.
10. Read the plate as soon as possible following the addition of Stop Buffer on a plate reader with 450 nm wavelength **(Before**
reading, use a lint-free wipe on the bottom of the plate to ensure no moisture or fingerprints interfere with the readings).

CALCULATION OF RESULTS – METHOTREXATE CONCENTRATION CALCULATION
A standard curve can be constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its
concentration in ng/ml on a logarithmic curve.
Relative absorbance (%) =
absorbance standard (or sample) x 100
absorbance zero standard
Use the mean relative absorbance values for each sample to determine the corresponding concentration of the tested drug in ng/ml
from the standard curve.
We would recommend a standard curve that is made up of at least 6 dilutions of standards, including zero (0), in duplicate.

TROUBLESHOOTING

No Color Development or No Signals with Standards

Possible Causes	Recommended Action
Reagents were used in the wrong order or a step was skipped.	Follow the protocol carefully and repeat the assay.
Wrong antibodies were used. Either Detection Antibody or Avidin-HRP was prepared incorrectly or has deteriorated.	Make sure that the antibodies used are the ones that came with the kit. All antibodies are kit- and lot-specific. Make sure that the Detection Antibody, Avidin-HRP and diluent are mixed in correct volumes.
TMB Substrate has deteriorated.	Use a new set of TMB substrate. Note, if TMB substrate shows any color before use, it should not be used for the assay.

Low Optical Density (OD) Readings

Possible Causes	Recommended Action
Reagents were expired or mixed with a different lot number.	Verify the expiration dates and lot numbers.
Wash solution was prepared incorrectly.	Verify that the solution was prepared as described in the protocol.
Too many wash cycles were used.	Make sure to use the number of washes per the protocol instruction.
Incubation times were too short.	Follow protocol and ensure accurate incubation time.
Lab temperature was too low.	Maintain the lab room temperature within 20°–25°C. Do not run assays under air conditioning vents or near cold windows.
Reagents and plates were too cold.	Make sure that the plates and reagents are brought up to room temperature.
Reader was at wrong wavelength, or reader was malfunctioning.	Make sure that the wavelength is set to 450 nm and read the plate again. Verify reader calibration and lamp alignment.
Excessive kit stress has occurred.	Check records to see how many times the kit has cycled from the refrigerator. Check to see if the kit was left at extreme temperatures for too long.
Assay plates were compromised.	Always refrigerate plates in sealed bags with a desiccant to maintain stability. Prevent condensation from forming on plates by allowing them to equilibrate to room temperature while in the packaging.

High Background or High Optical Density (OD) Readings

Possible Causes	Recommended Action
Poor quality water was used in wash solution.	If water quality is questionable, try substituting an alternate source of distilled deionized water to prepare the wash solution.
Substrate solution has deteriorated.	Make sure that the substrate is colorless prior to addition to the plate.
There was insufficient washing or poor liquid handling technique.	Use the number of washes per the protocol instruction. Make sure that 250 µL of wash solution is dispensed per well per wash. If you use a multichannel pipette or robotic liquid handling system, verify its performance; have the system repaired if any ports drip, dispense or aspirate poorly.
Reader was malfunctioning or not blanked properly. This is a high possibility if the OD readings were high and the color was light.	Verify the reader's performance using a calibration plate and check the lamp alignment. Verify the blanking procedure, if applicable, and re-blank.
Lab temperature was too high.	Maintain the room temperature within 20°–25°C .Avoid running assays near heat sources or in direct sunlight.
Reagents were intermixed, contaminated or prepared incorrectly.	Ensure that the correct reagents were used, that working solutions were prepared correctly and that contamination has not occurred.

High Intra-Plate or Inter-Plate Variance

Possible Causes	Recommended Action
Inconsistent time was taken when adding standards, reagents or samples within and/or between plates.	Make sure that all materials are set up and ready to use. Use a multichannel pipette to add reagents to multiple wells whenever possible. Do not interrupt procedure while adding standards, reagents and samples.
Multichannel pipette was not functioning properly.	Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.
There was inconsistent washing or poor liquid handling technique.	Use the number of washes per the protocol instruction. Make sure that 250 µl of wash solution is dispensed per well per wash. If you use a multichannel pipette or robotic liquid handling system, verify its performance; have the system repaired if any ports drip, dispense or aspirate poorly.
Inconsistent incubation times occurred from plate to plate.	Time each plate separately to ensure consistent incubation times.
Pipette was inaccurate.	Check the pipette calibration. Verify that pipette tips are on tight before use and that all channels draw and dispense equal volumes.
Kit plates, reagents, standards and samples were at different temperatures.	Make sure to allow sufficient time for kit plates, reagents, standards and samples to come to room temperature. Larger volumes will require longer equilibration time. If using a water bath to hasten equilibration, make sure that it is maintained at room temperature; do not use a warm water bath to warm reagents, samples and kit standards.
Reagents used were intermixed from different kit lots, or the kits were of different expiration dates.	Carefully label each user-prepared reagent to make sure that the reagents are not intermixed. Kits with different expiration dates might generate different range of OD readings, however, the relative absorbance values will typically be comparable. In general, a value of less than 1.0 reading for the highest standard may indicate deterioration of reagents.

One or More of the Standard Curve Points Are Out of Range

Possible Causes	Recommended Action
Standards were added in wrong order or recorded in wrong position.	Make sure that the standards are applied and recorded correctly.
Standards were contaminated or intermixed with other standards.	Prepare a new set of standards. Always add standards to plate in the order from low concentration to high concentration.
There was inconsistent washing or poor liquid handling technique.	Use the number of washes per the protocol instruction. Make sure that 250 µL of wash solution is dispensed per well per wash. If you use a multichannel pipette or robotic liquid handling system, verify its performance; have the system repaired if any ports drip, dispense or aspirate poorly.
Inconsistent time was taken to add standards and reagents to plate.	Make sure all materials are set up and ready to use. Add standards to plate only in the order from low concentration to high concentration at undisrupted constant pace. Use a multichannel pipette to add reagents to multiple wells simultaneously to increase consistency.
Multichannel pipette was not functioning properly.	Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.