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ab213976
Human Methotrexate ELISA kit

For the quantitative determination of Human Methotrexate in serum, plasma and urine samples.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

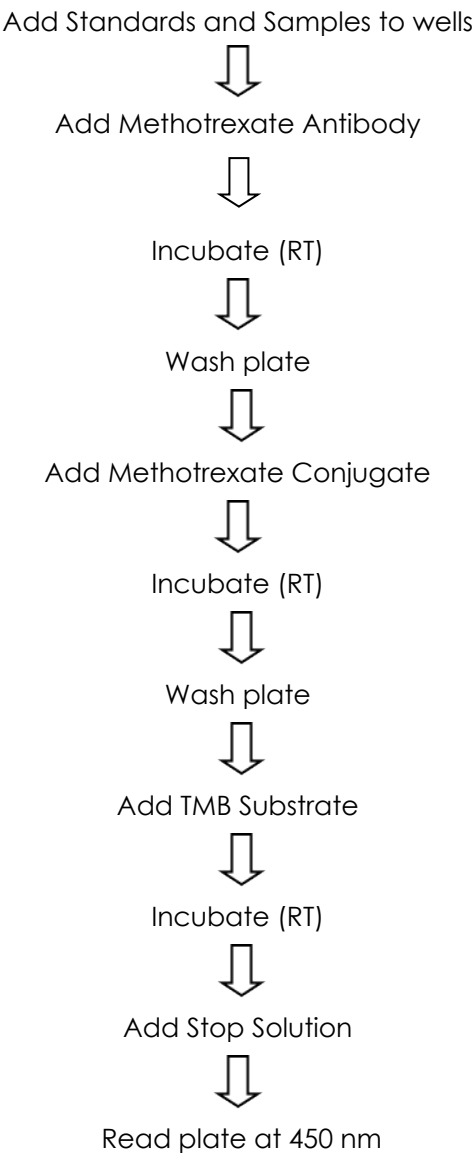
The Human Methotrexate ELISA Kit (ab213976) is a complete kit for the quantitative determination of methotrexate in serum, plasma and urine samples. Please read the complete kit insert before performing this assay. The methotrexate ELISA uses a methotrexate polyclonal antibody to bind methotrexate in the sample or standard competitively to that pre-bound to the wells as a bovine serum albumin (BSA) conjugate. Anti-methotrexate antibody bound to methotrexate in the sample or standard are washed away while those captured by the immobilized methotrexate are detected with a secondary antibody horseradish peroxidase (HRP) conjugate. The assay is developed with tetramethylbenzidine (TMB) substrate and the resulting absorbance is measured with a microplate reader at 450nm. The intensity of the yellow color is inversely proportional to the concentration of methotrexate.

Methotrexate is a drug used in the treatment of cancer and autoimmune disease. It is designed as an anti-folate to inhibit the metabolism of folic acid. Two distinct mechanisms of action have been described for methotrexate. In cancer treatments, methotrexate competitively inhibits the dihydrofolate reductase (DHFR) by blocking folate binding. DHFR converts dihydrofolate to active tetrahydrofolate. Inhibition of DHFR results in inhibition of the synthesis of purine and pyrimidine bases effectively limiting DNA and RNA synthesis and cancer cell growth. In autoimmune disease and specifically in the treatment of rheumatoid arthritis, methotrexate appears to impact several pathways resulting in inhibition of T cell activation. The effects include suppression of T cell expression of intercellular adhesion molecules, inhibition of methyl transferase activity and increased CD95 sensitivity leading to apoptosis in active T cells.

Monitoring methotrexate levels is important to assure appropriate levels are maintained during therapy or treatment. High levels of methotrexate can lead to toxicity and potential renal failure as well as immunosuppression. Additionally, methotrexate is known to interact with a wide variety of drugs leading to additional complications. Determining the presence of methotrexate in samples from subjects in blinded research studies can assist in the interpretation of study results.

Methotrexate is established as one of the most effective and safe therapeutics for rheumatoid arthritis. The safety profile assures that methotrexate will continue to be used in new studies in combination with other new or established drugs. The same is true in its use as a cancer therapeutic. The Methotrexate ELISA enables monitoring levels of methotrexate in both preclinical and clinical research. The methotrexate assay is also appropriate for the detection of methotrexate contamination after its use as a selective agent for recombinant protein production in mammalian cell lines.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All ELISA kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store ELISA kit at +2-8°C immediately upon receipt

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- ELISA kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition (Before prep)	Storage Condition (After prep)
Methotrexate coated microplate (12x 8 well strips)	96 wells	+2-8°C	+2-8°C
Methotrexate Antibody (Lyophilized)	1 Vial	+2-8°C	-20°C
Antibody Diluent	6 mL	+2-8°C	+2-8°C
Methotrexate Conjugate	10 mL	+2-8°C	+2-8°C
Methotrexate Standard (1,000 ng)	2 Vials	+2-8°C	+2-8°C
Wash Buffer Concentrate	100 mL	+2-8°C	RT
Assay Buffer	50 mL	+2-8°C	+2-8°C
TMB Substrate	10 mL	+2-8°C	+2-8°C
Stop Solution	10 mL	+2-8°C	+2-8°C
Plate Sealer	3 units	+2-8°C	+2-8°C

7. Materials Required, Not Supplied

These materials are not included in the ELISA kit, but will be required to successfully perform this assay:

- Deionized or distilled water
- Precision pipettes for volumes between 50 µL and 1,000 µL
- Repeater pipette for dispensing volumes between 50 µL and 100 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Absorbent paper for blotting
- Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- This ELISA kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 Methotrexate coated microplate (12x 8 well strips):

One plate (break-apart strips) of 96 wells coated with a methotrexate-BSA conjugate. Ready to use. Store at +2-8°C.

9.2 Methotrexate Antibody (Lyophilized):

Rabbit polyclonal antibody. Reconstitute the lyophilized Methotrexate Antibody in 6 mL of Antibody Diluent. Lyophilized antibody may have dislodged from the bottom of the vial during shipping. Lightly vortex the vial to assure complete reconstitution. Store any unused reconstituted antibody at -20°C. Allow no more than three freeze-thaw cycles.

9.3 Antibody Diluent:

6 mL. Ready to use. Store at +2-8°C.

9.4 Methotrexate Conjugate:

10 mL. A blue solution of goat anti-rabbit IgG conjugated to HRP. Ready to use. Store at +2-8°C.

9.5 Methotrexate Standard (1,000 ng):

2 Vials. Store at +2-8°C.

9.6 Wash Buffer Concentrate:

100 mL. Store conc. at +2-8°C. Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

9.7 Assay Buffer:

50 mL. Ready to use. Store at +2-8°C.

9.8 TMB Substrate:

10 mL. Ready to use. Store at +2-8°C.

9.9 Stop Solution:

10 mL. Ready to use. Store at +2-8°C.

9.10 Plate Sealer

3 units. Ready to use. Store at +2-8°C.

10. Standard Preparation

The Methotrexate standard stock as well as diluted standards and samples should be kept on ice and used within 60 minutes of preparation for optimal performance. Allow the Methotrexate standard to warm to room temperature.

10.1 Reconstitute the Methotrexate Standard with 1 mL of Assay Buffer. Lyophilized methotrexate may have dislodged from the bottom of the vial during shipping. Lightly vortex the vial to assure complete reconstitution.

10.2 Label six 12x 75 mm polypropylene tubes #1 through #6.

10.3 Pipette 400 µL of Methotrexate Standard into tube #1.

10.4 Pipette 500 µL of Assay Buffer into tube #2 through tube #6.

10.5 Transfer 100 µL from tube #1 into tube #2 and vortex. Discard pipette tip.

10.6 Transfer 100 µL from tube #2 into tube #3 and vortex. Discard pipette tip.

10.7 Continue this for tubes# 4 through #6.

Δ Note: Diluted standards should not be stored for re-use. Freshly reconstituted non-diluted standard can undergo 1 freeze-thaw cycle. Two standard vials are provided enabling preparation of at least 4 standard curves.

Standard #	Standard (µL)	Sample Diluent (µL)	Final volume (µL)	End Conc. (ng/mL)
1	400 Stock	N/A	300	1000
2	100 Std. 1	500	500	166.7
3	100 Std. 2	500	500	27.8
4	100 Std. 3	500	500	4.6
5	100 Std. 4	500	500	0.77
6	100 Std. 5	500	600	0.13

11. Sample Preparation

- The Methotrexate ELISA is compatible with serum and plasma samples from human, mouse and rat. The ELISA is also compatible with human urine. Samples diluted sufficiently into Assay Buffer can be read directly from a standard curve. Please refer to the Recovery section for minimum recommended dilutions for validated matrices.
- Only standard curves generated in Assay Buffer should be used to calculate the concentration of methotrexate. Samples must be stored frozen at or below -20°C. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4°C slowly and gently mixed. Samples may be clarified by centrifugation to reduce risk of matrix interference.
- The methotrexate ELISA kit may be appropriate for testing biological matrices from other species that have not been validated and may be compatible with other buffer matrix formulations. It is recommended that any matrix of interest undergo testing to determine the minimum dilution in Assay Buffer to eliminate matrix interference.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at +2-8°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1 Add 150 µL of Assay Buffer into the NSB wells.
13.2 Add 100 µL of Assay Buffer into the S0 (0ng/mL standard) wells.
13.3 Add 100 µL of Standards #1 through #6 into the appropriate wells.
13.4 Add 100 µL of the Samples into the appropriate wells.
13.5 Add 50 µL of the yellow methotrexate antibody in all wells except for the NSB and blank.
13.6 Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13.7 Empty the contents of the wells and wash by adding full well volume, ~400 µL, of wash buffer to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
13.8 Add 100 µL of blue methotrexate conjugate solution into each well except the Blank.
13.9 Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13.10 Wash as above (Step 13.7).
13.11 Add 100 µL of Substrate Solution into each well.
13.12 Seal the plate and incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
13.13 Add 100 µL Stop Solution to each well.
13.14 Blank the plate reader against the Blank wells, read the optical density (OD) at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean OD density of the Blank wells from all the readings.

ΔNote: The optimal speed for each shaker will vary. The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.

14. Calculations

The concentration of Methotrexate can be calculated as follows:

14.1 Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

Average Net OD = Average OD - Average NSB OD

14.2 Using data analysis software, plot the Average Net OD for each standard versus Methotrexate concentration in each standard. We recommend that the data be handled by a software package utilizing a 4 parameter logistic (4PL) curve fitting program.

15. Typical data

Typical standard curve – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Sample	Methotrexate (ng/mL)	Optical Density	Net Optical Density	%B/Bo
Bo	0	1.655	1.611	---
S1	1000.00	0.217	0.173	10.6
S2	166.70	0.464	0.420	25.8
S3	27.80	0.710	0.666	41.0
S4	4.60	0.886	0.842	51.8
S5	0.77	1.029	0.985	61.0
S6	0.13	1.157	1.113	69.0
NSB	NA	0.044	---	---

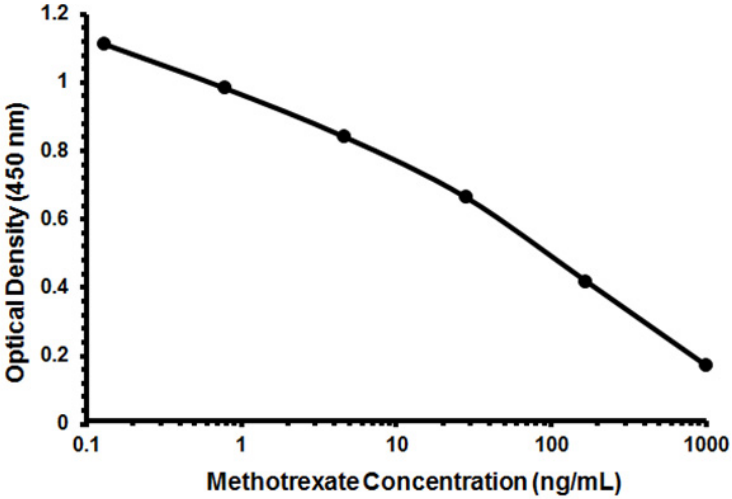


Figure 1. Human Methotrexate ELISA Kit (ab213976) Standard Curve

16. Typical sample values

SENSITIVITY –

This sensitivity was determined by interpolation from the average of 9 separate standard curves run with replicate data points at each concentration. The sensitivity was determined at 2 standard deviations below the average net OD of 18 zero standard replicates (2 per standard curve). The sensitivity or limit of detection of the assay is 0.087 ng/mL.

Units of Measure –

Samples measured in the methotrexate ELISA can be expressed in terms of concentration by mass or molarity. Nanograms/mL units are converted to µM by dividing by the molecular weight (454.5 Da).

RECOVERY –

Serum, plasma and urine from human, mouse and rat samples were diluted in Assay Buffer to levels that eliminated matrix interference and then spiked with methotrexate at three levels. The percent recoveries and the minimum required dilutions for each matrix are provided in the table below.

Sample	Spike Concentration (ng/mL)	% Recovery	Minimum Recommended Dilution
Human Serum	500	86	1:16
	13.85	89	
	0.385	71	
Human Plasma	500	77	1:16
	13.85	107	
	0.385	91	
Mouse Serum	83.35	135	1:32
	2.315	140	
	0.385	141	
Mouse Plasma	83.35	56	1:32
	2.315	168	
	0.385	90	
Rat Serum	83.35	93	1:32
	2.315	127	
	0.385	78	
Rat Plasma	166	86	1:32
	6.17	75	
	0.23	160	
Human Urine	83.35	107	1:128
	13.9	161	
	2.31	100	

PRECISION –

Intra-assay precision:

Determined by assaying 20 replicates of methotrexate controls at two concentrations in a single assay.

Mean (ng/mL)	CV%
265.2	9.8
4.5	20.5

Inter-assay precision:

Determined by measuring methotrexate controls at two concentrations in multiple assays (n=10) over several days.

Mean (ng/mL)	CV%
298.9	17.1
5.2	22.4

ΔNote: %CV values reflect the inherent variability imparted by the wide dynamic range of the assay. Increasing the number of replicates will reduce the variability of assigned values.

Specificity

The specificity of the assay was determined by serially diluting potential cross reactants in the kit assay buffer and running them in the assay. The results were fit to 4 parameter logistic equations and the ED_{50} was determined for each cross reactant. Each ED_{50} was divided by the ED_{50} of methotrexate and multiplied by 100 to provide the percent cross reactivity.

Cross Reactant	Cross Reactivity
Dihydrofolic Acid	0.61%
7-hydroxymethotrexate	0.19%
4-[N-(2,4-Diamino-6-pteridinylmethyl)-N-methylamino] benzoic acid hemihydrochloride hydrate (DAMPA)	20.5%

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check Pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check Pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	All components 4°C. Keep TMB substrate solution protected from light.

17. Troubleshooting

18. Notes

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