Version 26-04/24

User's Manual

Methotrexate ELISA kit

REF **DEIA-US209**

Σ **96T**

RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Methotrexate ELISA kit 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.

General Description

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.

Principles of Testing

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.

Reagents And Materials Provided

1. Methotrexate Microtiter Plate, One Plate of 96 Wells (Catalog No. 80-2678): A plate using break-apart strips coated with a methotrexate-BSA conjugate.
2. Methotrexate Antibody, Lyophilized (Catalog No. 80-2677): Lyophilized methotrexate rabbit polyclonal

antibody.

3. Antibody Diluent, 6ml (Catalog No. 80-2680): Buffer for reconstitution of the methotrexate antibody.
4. Methotrexate Conjugate, 10ml (Catalog No. 80-2679): A blue solution of goat anti-rabbit IgG conjugated to HRP.
5. Methotrexate Standard, 2 vials, 1,000ng (Catalog No. 80-2676): Two vials containing 1,000ng each of lyophilized methotrexate.
6. Wash Buffer Concentrate, 100ml (Catalog No. 80-1287): Tris buffered saline containing detergents.
7. Assay Buffer 13, 1×, 50ml (Catalog No. 80-1500): Tris buffered saline containing proteins and detergents.
8. TMB Substrate, 10ml (Catalog No. 80-0350): A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Protect from prolonged exposure to light.
9. Stop Solution 2, 10ml (Catalog No. 80-0377): A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: Caustic.
10. Methotrexate Assay Layout Sheet, 1 each (Catalog No. 30-0330)
11. Plate sealer, 3 each (Catalog No. 30-0012)

Materials Required But Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 50µl and 1,000µl.
3. Repeater pipet for dispensing volumes between 50 and 100µl.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 450nm, preferably with correction between 570nm and 590nm.

Storage

All components of this kit, are stable at 4°C until the kit's expiration date. Any unused reconstituted Antibody or Standard should be stored at -20°C as soon as possible after reconstitution. Repeated freeze thaws should be avoided.

Specimen Collection And 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 13 (see Reagent Preparation) can be read directly from a standard curve. Please refer to the Sample Matrix Properties section below for minimum recommended dilutions for validated matrices.

Only standard curves generated in Assay Buffer 13 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.

Sample Matrix Properties

Serum, plasma and urine from human, mouse and rat samples were diluted in Assay Buffer 13 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%	1:16
	0.385	71%	1:16
Human Plasma	500	77%	1:16
	13.85	107%	1:16
	0.385	91%	1:16
Mouse Serum	83.35	135%	1:32
	2.315	140%	1:32
	0.385	141%	1:32
Mouse Plasma	83.35	56%	1:32
	2.315	168%	1:32
	0.385	90%	1:32
Rat Serum	83.35	93%	1:32
	2.315	127%	1:32
	0.385	78%	1:32
Rat Plasma	166	86%	1:32
	6.17	75%	1:32
	0.23	160%	1:32
Human Urine	83.35	107%	1:128
	13.9	161%	1:128
	2.31	100%	1:128

Other Sample Types: 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 13 to eliminate matrix interference.

Reagent Preparation

1. Wash Buffer

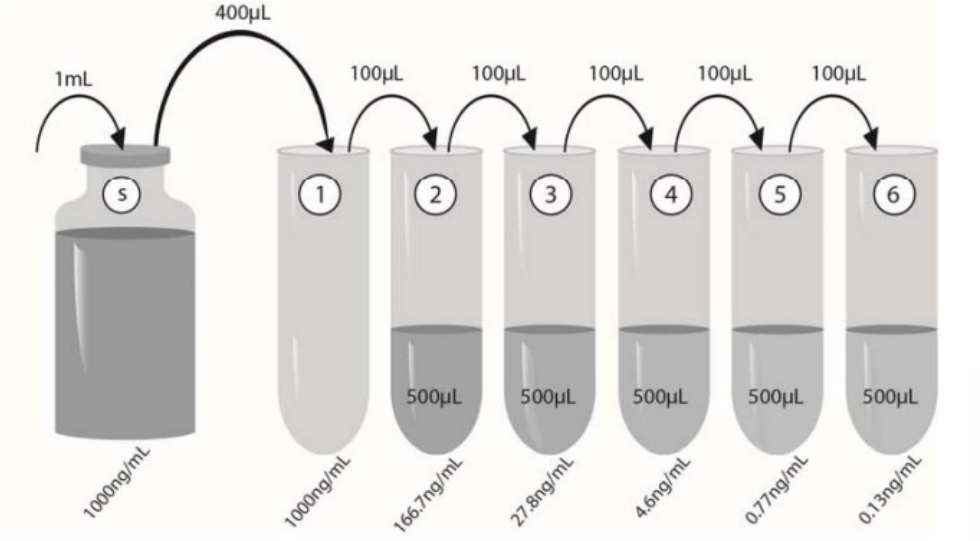
Prepare the Wash Buffer by diluting 50ml of the supplied concentrate with 950ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Methotrexate Standard Curve

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

   Reconstitute the Methotrexate Standard with 1ml of Assay Buffer 13. Lyophilized methotrexate may have dislodged from the bottom of the vial during shipping. Lightly vortex the vial to assure complete reconstitution. Label six 12×75 mm polypropylene tubes #1 through #6. Pipet 400µl of Methotrexate Standard into tube #1. Pipet 500µl of Assay Buffer 13 into tube #2 through tube #6. Transfer 100µl from tube #1 into tube #2 and vortex. Discard pipet tip. Transfer 100µl from tube #2 into tube #3 and vortex. Discard pipet tip. Continue this for tubes# 4 through #6.

   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.



3. Methotrexate Antibody

   Reconstitute the lyophilized Methotrexate Antibody in 6ml 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.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4°C.
2. Add 150µl of Assay Buffer 13 into the NSB wells.
3. Add 100µl of Assay Buffer 13 into the S0 (0ng/ml standard) wells.

4. Add 100µl of Standards #1 through #6 into the appropriate wells.
5. Add 100µl of the Samples into the appropriate wells.
6. Add 50µl of the yellow methotrexate antibody in all wells except for the NSB and blank.
7. Seal the plate and incubate at room temperature on a plate shaker for 30 min at ~500rpm*.
8. Empty the contents of the wells and wash by adding full well volume, ~400µl, 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.
9. Add 100µl of blue methotrexate conjugate solution into each well except the Blank.
10. Seal the plate and incubate at room temperature on a plate shaker for 30 min at ~500rpm.
11. Wash as above (Step 8).
12. Add 100µl of Substrate Solution into each well.
13. Seal the plate and incubate for 30 minutes at room temperature on a plate shaker at ~500rpm.
14. Add 100µl Stop Solution to each well.
15. Blank the plate reader against the Blank wells, read the optical density (OD) at 450nm, preferably with correction between 570 and 590nm. 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.

* The plate shaker speed was based on a BellCo Mini Orbital Shaker (mod no. 7744-08096). 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.

Note:

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in polypropylene tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
8. Prior to addition of conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

Calculation

The concentration of methotrexate can be calculated as follows:

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

2. Using data analysis software, plot the Average Net OD for each standard versus the methotrexate

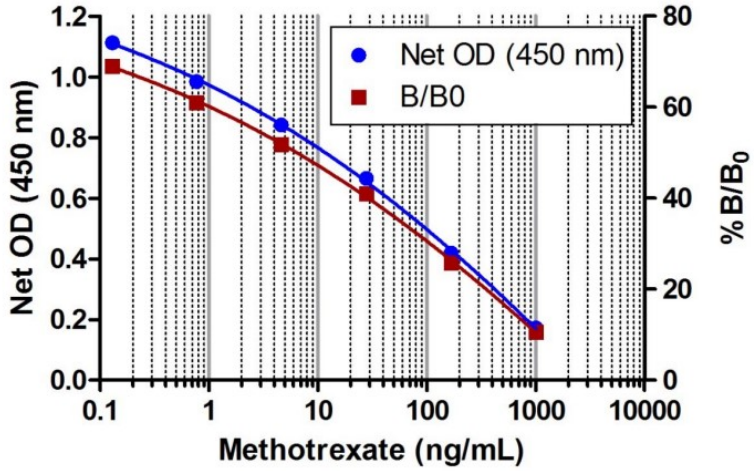
concentration in each standard. We recommend that the data be handled by a software package utilizing a 4 parameter logistic (4PL) or semilog curve fitting program.

Typical Standard Curve

The results shown below are for illustration only and should not be used to calculate results.

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	---	---

Typical standard curves fit to 4 parameter logistic equations are shown below. These curves must not be used to calculate methotrexate concentrations; each user must run a standard curve for each assay.



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).

Precision

Intra-assay precision was determined by assaying 20 replicates of methotrexate controls at two concentrations in a single assay.

Intra-assay precision	
ng/ml	%CV
265.2	9.8
4.5	20.5

Inter-assay precision was determined by measuring methotrexate controls at two concentrations in multiple

assays (n=10) over several days.

Inter-assay precision	
ng/ml	%CV
298.9	17.1
5.2	22.4

Note: %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.

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 0.087ng/ml.

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 ED50 was determined for each cross reactant. Each ED50 was divided by the ED50 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%**

Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test the assay performance with a variety of buffers, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The methotrexate standard provided, Catalog No. 80-2676, should be handled with care because of the known and unknown effects of methotrexate.
5. Once the methotrexate standard and methotrexate antibody are reconstituted, they should be used within 60 min. Any remaining reconstituted standard or antibody may be stored at -20°C for future use. Avoid repeated freeze-thaw cycles.
6. Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.

7. Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

References

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3. Rajagopalan, P. T. Ravi, et al., Proceedings of the National Academy of Sciences (2002) 99 (21): 13481–6.
4. Goodsell DS., The Oncologist, (1999) 4 (4): 340–341.
5. Wessels, JA, et al. Rheumatology, (2008) 47(3): 249–55.
6. Braun, J. Therapeutic Advances in Musculoskeletal Diseases, (2011); 3: 151-8.