Methotrexate (MTX) – Serum
Beckman UniCel DxC Systems

Technical Procedure 3146

Principle

Intended Use

ARK™ Methotrexate Assay, is a homogeneous enzyme immunoassay intended for the quantitative determination of methotrexate in human serum or plasma on automated clinical chemistry analyzers. The measurements obtained are used in monitoring levels of methotrexate to help ensure appropriate therapy.

Clinical Significance

Methotrexate [N-[4[[(2,4-diamino-6-pteridinyl) methyl] methyl(amino)benzoyl]-L-glutamic acid], formerly Amethopterin, is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis. (1,2,3)

Methotrexate has the potential for serious toxicity. Patients undergoing methotrexate therapy should be closely monitored so that toxic effects are detected promptly. Guidelines for methotrexate therapy with leucovorin rescue should be consulted. (1)

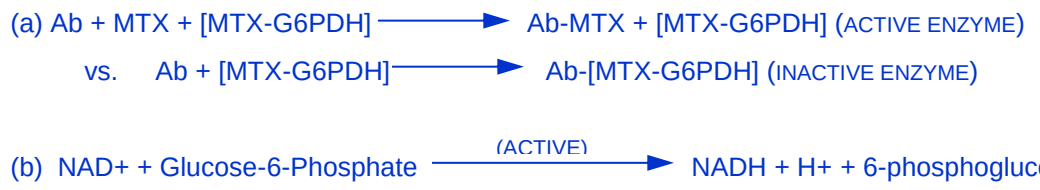
Intermediate to high doses of methotrexate (approximately 35 mg/m^2 –12 g/m^2) with leucovorin (citrovorum-factor) rescue have been used with favorable results in the treatment of osteogenic sarcoma, leukemia, non-Hodgkin's lymphoma, lung, and breast cancers. (4,5,6,7,8)

Methodology

ARK Methotrexate Assay is a homogenous immunoassay based on competition between drug in the specimen and methotrexate labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for binding to the antibody reagent. As the labeled methotrexate binds antibody, enzyme activity decreases. In the presence of drug from the specimen, enzyme activity increases and is directly proportional to the drug concentration. Active enzyme reduces the coenzyme nicotinamide adenine dinucleotide (NAD) to NADH that is measured spectrophotometrically as a rate of change in absorbance. Endogenous serum G6PDH does not interfere with the results because the coenzyme NAD functions only with the bacterial enzyme used in the assay.

The SYNCHRON® System(s) automatically proportions the appropriate sample and reagent volumes into a cuvette. The ratio for MTX is one part sample to 25 parts reagent. The system monitors the change in absorbance at 340 nanometers to calculate the MTX concentration.

Chemical Reaction Scheme



Specimen

Acceptable Sample Containers

13 x 75 Sodium Heparin BD tubes
Sodium Heparin microtainers

13 x 75 Red Top BD tubes
Red Top BD microtainers

Unacceptable Sample Containers

Serum tubes containing gel, whole blood, or urine are not recommended for use as a sample. Grossly lipemic samples should be ultra-centrifuged (90,000 x g for 10 minutes) prior to analysis. Grossly hemolyzed samples should be cancelled.

Type of Specimen

Biological fluid samples should be collected in the same manner routinely used for any laboratory test. Freshly drawn plasma is the preferred specimen.

Fibrin, red blood cells, and other particulate matter may cause an erroneous result. Ensure adequate centrifugation.

For consistency, use the same sample type while monitoring patients.

Specimen Storage and Stability

Tubes of blood are to be kept closed at all times and in a vertical position. It is recommended that the serum be physically separated from contact with cells within four hours from the time of collection.

Separated serum should not remain at room temperature longer than 8 hours. If assays are not completed within 8 hours, serum or plasma should be stored at +2°C to +8°C. for two weeks. If assays are not completed within 2 weeks, or the separated sample is to be stored beyond 2 weeks, samples should be frozen at -15°C to -20°C for one year. Avoid repeated freeze-thaw cycles.

Sample Volume

The optimum volume, when using a 0.5 mL sample cup, is 0.3 mL of sample. For optimum primary sample tube volumes and minimum volumes, refer to the *Primary Tube Sample Template* for UniCel DxC Systems.

Reagents

Contents

Each kit contains the following items Kit Reorder #: 5026-0001-00
Ready-to-use ARK Methotrexate Reagent 1 - 1 x 16 mL
Ready-to-use ARK Methotrexate Reagent 2 - 1 x 8 mL

Volumes per Test

Sample Volume	9 µL
Total Reagent Volume	225 µL

Reactive Ingredients

Reagent Constituents

REAGENT	CONTENTS
R1 – Antibody/Substrate	rabbit polyclonal antibodies to methotrexate glucose-6-phosphate nicotinamide adenine dinucleotide bovine serum albumin
R2 - Enzyme	methotrexate-labeled with bacterial G6PDH buffer bovine serum albumin

Also preservative and stabilizers necessary for optimal system performance

Reagents 1 and 2 are provided as a matched set and should not be interchanged with reagents from different lot numbers.

CAUTION

Avoid contact with skin and eyes. If this occurs wash immediately with water. Spills should be thoroughly washed with water. Reagent contains sodium azide which may react with copper or lead plumbing. Flush with plenty of water when disposing.

Materials Needed But Not Supplied With Reagent Kit

ARK Methotrexate Calibrator
ARK Methotrexate Dilution Buffer Kit Reorder #: 5026-0004-00
ARK Methotrexate Controls

Reagent Preparation

Reagent is provided liquid, ready-to-use, and may be used directly from the refrigerator. Transfer 8 mL of ARK Methotrexate R1 reagent into a User-Defined reagent cartridge compartment B. Transfer 4 mL of ARK Methotrexate R2 reagent into reagent cartridge C. Avoid cross-contamination of R1 and R2.

R1 and R2 are provided as a matched set and should not be interchanged with reagents from different lot numbers.

Document lot number in reagent log, date and initial and note expiration date of reagent on every cartridge before loading.

Acceptable Reagent Performance

The acceptability of this reagent is determined by successful calibration and by ensuring that quality control results are within acceptance criteria, as defined in the Clinical Chemistry Quality Control Procedure #3000.T.

Reagent Storage and Stability

Methotrexate reagent when stored upright, with screw caps tightly closed at 2°C to 8°C, will remain stable until the expiration date printed on the kit label. Once transferred to the SYNCHRON cartridge and loaded on the analyzer, the reagent is stable for 14 days unless the expiration date is exceeded.

DO NOT FREEZE.

Avoid prolonged exposure to temperatures above 32°C (90°F). Improper storage of reagents can affect assay performance.

Equipment

This test is performed on the Beckman UniCel DxC 800 Systems; Beckman-Coulter, Brea, California. For technical assistance, call the Beckman-Coulter hotline: 1-800-854-3633.

Refer to the Beckman *UniCel DxC 800 systems Reference Manual* for detailed instructions.

Calibration

Calibrator Required

ARK Methotrexate Calibrator Kit Reorder #: 5026-0002-00

Calibrator Preparation

Calibrators are provided ready to use. Mix each level by gentle inversion before dispensing. Use 2 drops per level. Return caps to their original bottles, and keep tightly closed.

Do not mix calibrators from different lots.

Calibrator Storage and Stability

ARK Methotrexate Calibrator is stable until the expiration date printed on the label if stored capped in the original container at 2°C to 8°C.

Opened calibrators when stored capped in the original container at 2°C to 8°C are stable for 12 months unless the expiration date is exceeded.

DO NOT FREEZE.

Calibration Information

The system must have a valid calibration curve in memory before control or patient samples can be run.

Under typical operating conditions the MTX reagent cartridge must be calibrated every 14 days or with each new lot, and also with certain parts replacements or maintenance procedures, as defined in the UniCel DxC800 System *Instructions For Use* (IFU) manual.

For detailed calibration instructions, refer to the UniCel DxC800 System *Instructions For Use* (IFU) manual.

The system will automatically perform checks on the calibration and produce data at the end of calibration. In the event of a failed calibration, the data will be printed with error codes and the system will alert the operator of the failure. For information on error codes, refer to the UniCel DxC 600/800 System *Instructions For Use* (IFU) manual.

Calibrator Summary

There is no internationally recognized standard for Methotrexate. ARK Methotrexate Calibrators are prepared by volumetric dilution of high purity, certified Methotrexate solution into a synthetic proteinaceous matrix free of Methotrexate with the following concentrations of Methotrexate:

CALIBRATOR LEVEL	STANDARD VALUE
A	0.00 µmol/L
B	0.05 µmol/L
C	0315 µmol/L
D	0.25 µmol/L
E	0.50 µmol/L
F	1.20 µmol/L

Quality Control

Three levels of control material will be analyzed each day of patient testing.

In addition, controls should be run under the following circumstances:

Upon loading a new reagent cartridge.
Following each new calibration.
Following specific maintenance or troubleshooting procedures as detailed in the UniCel DxC800 System *Instructions For Use* manual.

More frequent use of controls or the use of additional controls is left to the discretion of the user based on workload and workflow.

The following controls should be used in accordance with the package instructions for use inserts. Quality control results should be evaluated and handled with respect to the Clinical Chemistry Quality Control Procedure #3000.T. Controls are compiled statistically in the LIS and reagent lot changes are documented on DxC Reagent Log sheets.

Quality Control Material: ARK MTX Controls Kit Reorder #: 5026-0003-01

Control	Expected Range	Storage
LOW	0.05 – 0.09 µmol/L	2°C to 8°C
MID	0.30 – 0.50 µmol/L	2°C to 8°C
HIGH	0.60 – 1.00 µmol/L	2°C to 8°C
500*	375.0 -625.0 µmol/L	2°C to 8°C

Controls are stored at 2-8°C and are stable until their expiration date.
Opened calibrators when stored capped in the original container at 2°C to 8°C are stable for 12 months unless the expiration date is exceeded.
Do not mix controls from different lot numbers; use each lot as a set.
*Diluted per the Manual Dilution Protocol

Testing Procedure

1. If necessary, load the reagent onto the system.
2. After reagent load is completed, calibration may be required.
3. Program samples and controls for analysis.
4. After loading samples and controls onto the system, follow the protocols for system operation.

For detailed testing procedures, refer to the UniCel DxC800 System *Instructions For Use* (IFU) manual.

Calculations

UniCel DxC Systems perform all calculations internally to produce the final reported result. The system will calculate the final result for sample dilutions made by the operator when the dilution factor is entered into the system during sample programming.

Reporting Results

Equivalency between the SYNCHRON LX and UniCel DxC 800 Systems has been established. Chemistry results between these systems are in agreement and data from representative systems may be shown.

Reference Intervals

Methotrexate levels depend on indication for use, dosage, mode of administration, treatment regimen, individual pharmacokinetics, metabolism, and other clinical factors, and are determined by the individual service and/or study.(1,3)

All results will report with a comment: *"Methotrexate therapy is tailored to each patient. Refer to the specific treatment regimen for assessment of methotrexate levels."*

Methotrexate levels in excess of 50 µmol/L at 24 hours, 10 µmol/L at 48 hours, and 0.50 µmol/L at 72 hours portend potential toxicity and are usually treated with an increase in the dose of leucovorin in accordance with algorithms until the methotrexate level is < 0.10 µmol/L. Guidelines for methotrexate therapy with leucovorin rescue usually recommend continuance of leucovorin until the methotrexate level falls below 0.05 µmol/L.(1,9)

Procedural Notes

Anticoagulant Test Results

If plasma is the sample of choice, Sodium Heparin plasma was found to be compatible with this method.

Limitations

Specimens from patients who have received glucarpidase (carboxypeptidase G2) as a high dose methotrexate rescue therapy should not be tested with the ARK Methotrexate Assay. These specimens have increased serum levels of 4-[[2,4-diamino-6-(pteridinyl)methyl]-methylamino]-benzoic acid (DAMPA) that result from metabolism of methotrexate by glucarpidase.(10-12) DAMPA cross-reacts with the methotrexate antibody used in this assay, and may continue to circulate for at least five to seven days before accurate measurements of serum/plasma methotrexate may return. Oncologists on the clinical team should notify the laboratory when glucarpidase is administered to avoid the reporting of falsely elevated methotrexate concentrations due to interference by DAMPA that would confuse the efforts of glucarpidase therapy.(13) While glucarpidase is well tolerated and rapidly reduces circulating MTX, delayed renal elimination of MTX can still be a problem for adult and elderly patients.(14)

Interferences

Clinically high concentrations of potentially interfering endogenous substances in serum with known levels of Methotrexate (approximately 0.05 and 0.50 µmol/L) were evaluated using CLSI/NCCLS Protocol EP7-A2 as a guideline. Each sample was assayed using the ARK Methotrexate Assay along with a serum control of methotrexate. Measurement of methotrexate was not substantially affected at the levels of endogenous substances tests.

The following substances were tested:

Substance	Level tested	Methotrexate (~0.05 µmol/L)		Methotrexate (~0.50 µmol/L)	
		Serum Control	Test	Serum Control	Test (% Control)
Hemoglobin	1000 mg/dL	0.04	0.05	0.49	0.45 (92.8)
Intralipid®	500 mg/dL	0.05	0.05	0.43	0.45 (105.1)
Triglycerides	749 mg/dL	0.04	0.04	0.49	0.45 (91.4)
Albumin	12 g/dL	0.05	0.06	0.48	0.45 (92.8)
Bilirubin - conjugated	70 mg/dL	0.05	0.06	0.48	0.51 (105.5)
Bilirubin - unconjugated	70 mg/dL	0.05	0.06	0.48	0.52 (106.9)
Cholesterol	400 mg/dL	0.05	0.06	0.47	0.49 (105.4)
Gamma-Globulin	12 g/dL	0.05	0.06	0.48	0.51 (105.5)
Rheumatoid Factor	1100 IU/mL	0.05	0.06	0.43	0.41 (96.1)
Uric Acid	30 mg/dL	0.05	0.04	0.48	0.50 (102.8)

Specificity

Methotrexate's metabolites, folate analogs and other compounds having structural similarity were tested to determine whether these compounds affect the quantitation of methotrexate concentrations using the ARK Methotrexate Assay. High levels of these compounds were spiked into serum pools containing no methotrexate, 0.05 µmol/L or 0.50 µmol/L of methotrexate. The samples were analyzed and the methotrexate concentrations of samples containing interferent were compared to a serum control.

Cross-reactivity to 7-Hydroxymethotrexate, the major metabolite

After administration of high-dose methotrexate (HDMTX), the serum/plasma concentration of 7-hydroxy-methotrexate typically exceeds that of methotrexate at later time points. It has been reported that 7-hydroxy-methotrexate levels exceed those of methotrexate by up to 100-fold 12 to 48 hours after HDMTX administration.(15,27,29,32,34,35)

Cross-reactivity by 7-hydroxymethotrexate in the measurement of methotrexate was determined for the ARK

Methotrexate Assay by testing paired samples containing (1) both 0.05 µmol/L methotrexate and 5 µmol/L 7-hydroxymethotrexate and (2) both 0.50 µmol/L methotrexate and 50 µmol/L 7-hydroxymethotrexate in human serum.
The ARK Methotrexate Assay did not cross-react (≤ 0.07%) with the major metabolite 7-hydroxy-methotrexate.

Cross-reactivity to 2,4-Diamino-N^{10}-methylpteroic acid (DAMPA)

As a minor metabolite of methotrexate, DAMPA is not expected to circulate at concentrations that would interfere in a measurement of methotrexate.(33) However, following glucarpidase rescue therapy, the serum concentration of DAMPA can be substantial.(13,14) The ARK Methotrexate Assay cross-reacts substantially with the minor metabolite DAMPA. Tests were performed in the absence of the parent drug methotrexate. Cross-reactivity to DAMPA ranged 64.3 – 100%. The assay should not be used during possible compassionate therapy with glucarpidase (carboxypeptidase G2) that rapidly converts circulating methotrexate to DAMPA.

Drugs that cross-react

The ARK Methotrexate Assay cross-reacts slightly with triamterene and trimethoprim, however these drugs may be contraindicated for MTX cancer treatment due to additional adverse effects if co-administered. The structures of these compounds closely match the pteridine ring moiety of methotrexate.

		MTX ABSENT		MTX PRESENT 0.05 (µmol/L)		MTX PRESENT 0.50 (µmol/L)	
Compound	**Level Tested (µmol/L)**	**MTX (µmol/L)**	**Cross Reactivity %**	**(µmol/L)**	**Cross Reactivity %**	**(µmol/L)**	**Cross Reactivity %**
Triamterene	25	0.46	1.85	0.89	3.32	1.04	2.31
Trimethoprim	100	0.17	0.17	0.16	0.12	0.99	0.54

Cross-reactivity to folate analogs and other compounds

The ARK Methotrexate Assay did not cross-react (≤ 0.01%) with folate analogs or other compounds at ≥ 1000 µmol/L as tested.

Compound	**Level Tested (µmol/L)**
Adriamycin	1000
Cyclophosphamide	1500
Cytosine	1000
Dihydrofolic Acid	1000
DL-6-Methyl-5,6,7,8-Tetrahydropterine	1000
Folic Acid	1000
Folinic Acid (leucovorin)	1000
5-Fluorouracil	3000
6-Mercaptopurine	1000
5-Methytetrahydrofolic acid	1000
Predisolone	1000
Pyrimethamine	1000
Sulfamethoxazole	1600

Cross-reactivity to folate analogs and others, cont.

Tetrahydrofolic Acid	1000
Vinblastine	1000
Vincristine	1000

Performance Characteristics

Analytical Measurement Range

The ARK Methotrexate Assay for the determination of MTX provides the following analytical range:

Analytical Measurement Range (AMR)

Sample Type	Conventional Units
Serum or Plasma	0.04 – 1.20 µmol/L

Clinical Reportable Range:

Clinical Reportable Range (CRR) as determined at UCDMC

Sample Type	Conventional Units
Serum or Plasma	0.04 – 1000.00 µmol/L

Samples with concentrations below the AMR and CRR (0.04 µmol/L) will be reported as "**< 0.04 µmol/L**."

Samples with concentrations greater than the AMR (> 1.20 µmol/L) will be diluted following the Manual Dilution Protocol below.

Diluted samples with concentrations greater than the CRR (> 1000.00 µmol/L) will be reported as "**>1000.00 µmol/L**".

Manual Dilution Protocol for Methotrexate:

Manually dilute the specimen with ARK Methotrexate Dilution Buffer by preparing a series of 10-fold dilutions as shown below. Program the dilutions in Remisol using the dilution factors in the following chart, and run all three dilutions at the same time.

Sample Volume		Dilution Buffer Volume	Dilution	Dilution Factor
50 µL	Undiluted sample	450 µL	1:10	10
50 µL	1:10 sample	450 µL	1:100	100
50 µL	1:100 sample	450 µL	1:1000	1000

Analytical Sensitivity

Analytical sensitivity is defined as the lowest measurable concentration which can be distinguished from zero with 95% confidence. When run as recommended, the analytical sensitivity for the ARK Methotrexate Assay method is 0.04 µmol/L.

The following characteristics were determined according to CLSI EP17-A for the ARK Methotrexate Assay.

Limit of Blank (LoB); N=60	0.01 µmol/L
Limit of Detection (LoD); N=60	0.02 µmol/L
Limit of Quantitation (LoQ); N=40	0.04 µmol/L

Equivalency

Correlation studies were performed using CLSI/NCCLS Protocol EP9-A2. Results from the ARK Methotrexate Assay were compared with results from Fluorescence Polarization Immunoassay method (monoclonal FPIA).(30)

Methotrexate concentrations by FPIA ranged from 0.04 µmol/L to 1440 µmol/L. ARK Methotrexate values ranged from 0.04 µmol/L to 1500 µmol/L. Results of the Passing-Bablok regression analysis for the study are shown below (with 95% confidence limits) for 102 specimens within the measurement range as well as for all 147 specimens including those above the measurement range requiring dilution.

Serum or plasma (in the range of 0.04 – 1.19 µmol/L):

SLOPE	= 1.00
y-intercept	= 0.01
N	= 102
CORRELATION COEFFICIENT (r)	= 0.978

Serum or plasma (in the range of 0.04 - 1440 µmol/L):

SLOPE	= 0.99
y-intercept	= 0.01
N	= 147
CORRELATION COEFFICIENT (r)	= 0.998

Refer to References (6) for guidelines on performing equivalency testing.

Equivalency determined at UCDMC Specialty Testing Center Toxicology lab (STC) using Abbott TDxFLx Fluorescence Polarization Immunoassay (FPIA) technology:

Serum or Plasma (in the range of 0.05 – 989 µmol/L):

Y (UniCel DxC800-4449)	= 1.1X – 0.64
N	= 58
MEAN (SD) Bias	= -6.0 (24.6) µmol/L
P	= 0.403
CORRELATION COEFFICIENT (R^2)	= 0.99

Serum or Plasma (in the range of 0.05 – 989µmol/L):

Y (UniCel DxC800-4427)	= 1.1X – 0.37
N	= 58
MEAN (SD) Bias	= -8.1 (26.5) µmol/L
P	= 0.169
CORRELATION COEFFICIENT (R^2)	= 0.99

Serum or Plasma (in the range of 0.05 – 989 µmol/L):

Y (UniCel DxC800-4449)	= 1.0X – 0.52
N	= 58
MEAN (SD) Bias	= -2.1 (1.9) µmol/L
P	= 0.94
CORRELATION COEFFICIENT (R^2)	= 0.99

Precision

Precision was determined as described in CLSI/NCCLS Protocol EP5-A2. The six-level ARK Methotrexate Control and pooled human specimens containing methotrexate were used in the study. Each level was assayed in quadruplicate twice a day for 20 days. Each of the runs per day was separated by at least two hours. The within run, between day, total SD, and percent CVs were calculated. Results are shown below.

As determined by ARK

Precision Values: ARK Methotrexate Control

Level	n	MEAN (µmol/L)	WITHIN RUN		BETWEEN DAY		TOTAL	
			SD	CV (%)	SD	CV(%)	SD	CV (%)
LOW	160	0.06	0.005	8.1	0.005	7.1	0.007	10.6
MID	160	0.37	0.011	3.1	0.008	2.2	0.014	3.8
HIGH	160	0.76	0.039	5.1	0.029	3.8	0.048	6.4
5	160	4.8	0.13	2.8	0.013	2.8	0.19	4.1
50	160	48	1.40	2.9	2.13	4.4	2.71	5.6
500	160	470	15.63	3.3	27.64	5.8	33.35	7.0

Acceptable criteria: ≤10% total CV at >0.1 µmol/L, SD ≤ 0.01 at ≤ 0.10 µmol/L.

Precision Values: Patient Pool

Level	n	MEAN (µmol/L)	WITHIN RUN		BETWEEN DAY		TOTAL	
			SD	CV (%)	SD	CV(%)	SD	CV (%)
LOW	160	0.07	0.006	9.1	0.005	7.5	0.008	11.7
MID	160	0.41	0.013	3.3	0.026	6.4	0.030	7.2
HIGH	160	0.82	0.037	4.5	0.042	5.1	0.057	6.9
5	160	4.6	0.14	3.1	0.018	4.0	0.24	5.3
50	160	45	1.31	2.9	2.62	5.9	2.92	6.5
500	160	460	11.55	2.5	27.21	5.9	29.63	6.4

Acceptable criteria: ≤10% total CV at >0.1 µmol/L, SD ≤ 0.01 at ≤ 0.10 µmol/L.

Precision established at UCDMC

Type of Precision	Sample Type	n	Mean (µmol/L)	1 SD	%CV
DxC800-4427 Within-run	Low Control	20	0.07	0.01	14.38
	Mid Control	20	0.42	0.01	2.38
	High Control	20	0.87	0.07	8.05
	500 control	20	538.0	26.87	49.94
DxC800-4449 Within-run	Low Control	20	0.06	0.01	16.67
	Mid Control	20	0.47	0.02	4.26
	High Control	20	0.74	0.04	5.41
	500 Control	20	559.5	34.85	62.29

Type of Imprecision	Sample Type	n	Mean (mg/dL)	SD	%CV
DxC800-4427 Day to Day	Low Control	20	0.08	0.01	12.50
	Mid Control	20	0.43	0.03	6.98
	High Control	20	0.84	0.10	11.90
	500 Control	20	545.00	31.37	5.76
DxC800-4449 Day to Day	Low Control	20	0.08	0.01	12.50
	Mid Control	20	0.44	0.03	6.82
	High Control	20	0.85	0.09	10.59
	500 Control	20	525.00	35.01	6.67

Additional Information

For more detailed information on UniCel DxC Systems, refer to the *Instructions for Use* and *Reference* manual.

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