ARK Diagnostics, Inc.

ARK™ *Methotrexate Assay*

This ARK Diagnostics, Inc. package insert for the ARK Methotrexate Assay must be read carefully prior to use. Package insert instructions must be followed accordingly. Reliability of the assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

CUSTOMER SERVICE

ARK Diagnostics, Inc.
48089 Fremont Blvd
Fremont, CA 94538 USA
Tel: 1-877-869-2320
Fax: 1-510-270-6298
customersupport@ark-tdm.com
www.ark-tdm.com

CE EC REP
Emergo Europe
Westervoortsedijk 60
6827 AT Arnhem
The Netherlands

CH REP
MedEnvoy Switzerland
Gotthardstrasse 28
6302 Zug
Switzerland

KEY TO SYMBOLS USED

LOT	Batch Code	YYYY-MM-DD	Use by/Expiration Date
REF	Catalog Number		Manufacturer
EC REP	Authorized Representative	CE	CE Mark
IVD	In Vitro Diagnostic Medical Device		Temperature Limitation
	Consult Instructions for Use	R1 R2	Reagent 1/Reagent 2
Rx Only	For Prescription Use Only		

Reagent Kit REF 5026-0001-00
Reagent Kit REF 5026-0001-02
Reagent Kit REF 5026-0001-03

1 NAME

ARK™ *Methotrexate Assay*

2 INTENDED USE

The 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.

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.

3 SUMMARY AND EXPLANATION OF THE TEST

Methotrexate [N-[4[[(2,4-diamino-6-pteridinyl) methyl] methylamino]benzoyl]-L-glutamic acid], formerly Amethopterin, is an antimetabolite used in the treatment of certain neoplastic diseases, severe psoriasis, and adult rheumatoid arthritis.[1-3] 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 cancer.[4-8]

4 PRINCIPLES OF THE PROCEDURE

ARK Methotrexate Assay is a homogeneous 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 latter 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 converts 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.

5 REAGENTS

REF	Product Description	Quantity/Volume
5026-0001-00 5026-0001-02 5026-0001-03	**ARK Methotrexate Assay** **Reagent R1 – Antibody/Substrate** rabbit polyclonal antibodies to methotrexate, glucose-6-phosphate, nicotinamide adenine dinucleotide, bovine serum albumin, preservative, and stabilizers	1 X 16 mL
	Reagent R2 – Enzyme methotrexate labeled with bacterial G6PDH, buffer, bovine serum albumin, preservative, and stabilizers	1 X 8 mL

Reagent Handling and Storage

ARK Methotrexate Assay reagents are provided liquid, ready to use and may be used directly from the refrigerator. When not in use, reagents must be stored at 2–8°C (36–46°F), upright and with screw caps tightly closed. If stored as directed, reagents are stable until the expiration date printed on the label. Do not freeze reagents. Avoid prolonged exposure to temperatures above 32°C (90°F). **Improper storage of reagents can affect assay performance.**

6 WARNINGS AND PRECAUTIONS

- For **In Vitro Diagnostic** Use. For prescription use only.
- Reagents R1 and R2 are provided as a matched set and should not be interchanged with reagents from different lot numbers.

7 SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

- Serum or plasma is required. For consistency, using the same specimen matrix for individual patients is a good practice.
- The sampling time of methotrexate will be dependent on dose, duration of infusion, and clinical status of the patient. Consult specific treatment protocols for sampling time.
- Whole blood cannot be used. The following anticoagulants may be used with this assay.
  - Sodium heparin
  - Lithium heparin
  - Potassium EDTA
- Blood collection must be performed with collection tubes compatible for use with therapeutic drug monitoring (TDM).
- Do not induce foaming and avoid repeated freezing and thawing to preserve the integrity of the specimen from the time it is collected until the time it is assayed.

- Fibrin, red blood cells, and other particulate matter may cause an erroneous result. Ensure adequate centrifugation.
- Clarified specimens may be stored up to two weeks at 2 to 8°C. If testing will be delayed, specimens may be stored frozen (≤ -10°C) prior to being tested. Care should be taken to limit the number of freeze-thaw cycles. Specimens were shown to withstand 3 freeze-thaw cycles when stored at -20°C.
- **Handle all patient specimens as if they were potentially infectious.**

8 PROCEDURE

Materials Provided

ARK Methotrexate Assay – REF 5026-0001-00

ARK Methotrexate Assay, Roche® cobas c pack – REF 5026-0001-02

ARK Methotrexate Assay, Roche® cobas c pack green – REF 5026-0001-03

Materials Required – Provided Separately

ARK Methotrexate Calibrator – REF 5026-0002-00
Quality Controls – ARK Methotrexate Control – REF 5026-0003-00
ARK Methotrexate Dilution Buffer – REF 5026-0004-00

Instruments

Reagents R1 and R2 may need to be transferred to analyzer-specific reagent containers prior to use. Avoid cross-contamination of R1 and R2 .

Assay Sequence

To run or calibrate the assay, see the instrument-specific operator's manual.

Calibration

Perform a full calibration (6-point) procedure using the ARK Methotrexate Calibrators A, B, C, D, E, and F; test calibrators in duplicate. Calibration is required with each new reagent kit lot number. Verify the calibration curve with at least two levels of quality controls according to the established laboratory quality assurance plan.

When to Re-Calibrate

- Whenever a new lot number of reagents is used
- Whenever indicated by quality control results
- Whenever required by standard laboratory protocols

Quality Control (QC)

Laboratories should establish QC procedures for the ARK Methotrexate Assay. All quality control requirements and testing should be performed in conformance with local, state and/or federal regulations or accreditation requirements.

Good laboratory practice suggests that at least two levels (low and high medical decision points) of quality control be tested each day patient samples are assayed and each time a calibration is performed. Monitor the control values for any trends or shifts. If any trends or shifts are detected, or if the control does not recover within the specified range, review all operating parameters according to your clinical laboratory quality procedures. Contact Customer Service for further assistance.

Manual Dilution Protocol

The measurement range of the ARK Methotrexate Assay is 0.04 - 1.20 µmol/L. Specimens and controls containing methotrexate in higher concentrations (>1.20 µmol/L), are assayed by dilution of the specimen and controls into the measurement range.

Manually dilute the high specimen or control with ARK Methotrexate Dilution Buffer by preparing the appropriate ten-fold serial dilution as shown below.

	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
50 µL	1:1000 sample	450 µL	1:10000	10000

$$\text{Manual Dilution Factor} = \frac{(\text{Volume of Specimen} + \text{Volume of Dilution Buffer})}{\text{Specimen Volume}}$$

Multiply the assayed result by the dilution factor.

9 RESULTS

To convert µmol/L to µg/mL, divide the value obtained by the conversion factor of 2.2005.

10 LIMITATIONS OF PROCEDURE

This assay is designed for use with serum or plasma only; refer to the sections Specimen Collection and Preparation for Analysis. It is generally good practice to use the same method (as well as matrix) consistently for individual patient care due to the potential for method-to-method variability. See the section Expected Values below.

As with all analyte determinations, the methotrexate value should be used in conjunction with information available from clinical evaluation and other diagnostic procedures.

IMPORTANT: 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)[10-12] that result from metabolism of methotrexate by glucarpidase. DAMPA crossreacts 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 methotrexate may return.[13] 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]

11 EXPECTED VALUES

Methotrexate serum levels depend on indication for use, dosage, mode of administration, treatment regimen, individual pharmacokinetics, metabolism and other clinical factors.[1,3] While the serum level may typically reach approximately 10 to 100 µmol/L in treatment of breast cancer (for example),[15] concentrations may exceed 1000 µmol/L[16] with high dose therapy for osteosarcoma, and up to 3100 µmol/L methotrexate was reached following a 4-hour infusion in pediatric patients with osteosarcoma.[17] For treatment of osteosarcoma,[16] the methotrexate decay curve has wide variability: 24 hours, 30 to 300 µmol/L; 48 hours, 3 to 30 µmol/L; and 72 hours, less than 0.3 µmol/L. A dose of 10 mg of leucovorin is usually administered intravenously 24 hours after initiation of the MTX infusion. Subsequent doses are adjusted and administered according to the MTX levels obtained at 24, 48, and 72 hours. Methotrexate levels in excess of 50 µmol/L at 24 hours, 10 µmol/L at 48 hours, and 0.5 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 MTX level is <0.1 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] Some centers follow ≤ 0.10 µmol/L.[16, 18]

From prescribing and other information: Laboratory Indicators of Toxicity Following Leucovorin Rescue Schedules with High Dose Methotrexate.[1, 9, 19]

Clinical Situation	Laboratory Findings	
	Methotrexate Level (µmol/L)	Hours after administration
Normal Methotrexate Elimination	~10	24
	~1	48
	<0.2	72
Delayed Late Methotrexate Elimination	>0.2	72
	>0.05	96
Delayed Early Methotrexate Elimination and/or Evidence of Acute Renal Injury	≥50	24
	≥5	48
	OR	
	≥100% increase in serum creatinine	24

Renal toxicity is a significant risk and may be exacerbated by coadministration of other drugs,[14, 19] for example vancomycin.[20] Other forms of toxicity can occur, including digestive disorders (e.g., nausea, vomiting, abdominal pain), cutaneous–mucous disorders (especially mucositis), haematological abnormalities (e.g., neutropenia and thrombocytopenia), liver function test disturbances, and neurotoxicity.[21-28]

Given the profile of the appearance of the 7-hydroxymethotrexate metabolite,[15, 27] its molar ratio to methotrexate of up to approximately 100-fold,[29] and relative insolubility versus the parent drug,[14, 19] possible nephrotoxicity due to precipitation of the metabolite in renal tubules[29] may delay elimination of methotrexate itself.

Glucarpidase therapy (available for compassionate use) reduces the circulating level of methotrexate rapidly, not intracellular drug. A rebound effect in the serum level of methotrexate following glucarpidase therapy has been observed.[14] Elimination of DAMPA may take several days before it no longer interferes with the monitoring of methotrexate by immunoassay.[13]

12 SPECIFIC PERFORMANCE CHARACTERISTICS

Each laboratory is responsible for verification of performance using instrument parameters established for their analyzer. The following performance characteristics were obtained on the Beckman Coulter AU680 System.

Limit of Quantitation (LoQ)

The following characteristics were determined according to CLSI EP17-A2 for the ARK Methotrexate Assay. Analyzer-specific performance may vary.

Criterion	MTX Concentration (µmol/L)
Limit of Blank (LoB); N = 60 µB + 1.645 SD , where SD = 0.002	0.00
Limit of Detection (LoD); N = 60 LoB + 1.652 SD, where SD = 0.012	0.02
Limit of Quantitation (LoQ); N = 40 LoQ – 2 SD > LoD	0.04

Each laboratory is responsible for determining reporting criteria for methotrexate concentrations. The following suggestion from CLSI EP17-A2 may be appropriate:

Result ≤ LoB report "not detected; concentration < LoD"

LoB < Result < LoQ report "analyte detected; concentration < LoQ"

Result ≥ LoQ report the result as measured

Measurement Range

The measurement range of the ARK Methotrexate Assay is 0.04 - 1.20 µmol/L. Specimens containing methotrexate in higher concentrations are assayed by dilution of the specimen. Report assayed values exceeding the LoD according to the information provided for LoQ. Multiply the assayed result by the dilution factor for specimens containing methotrexate above the measurement range.

Recovery

Accuracy (analytical recovery) was performed by adding concentrated methotrexate drug into human serum negative for methotrexate. A certified stock concentrate of highly pure methotrexate was added volumetrically to human serum negative for methotrexate, representing drug concentrations across the assay calibration range. Six replicates of each sample were assayed on an automated clinical chemistry analyzer. The results were averaged and compared to the target concentration and percent recovery calculated. Results are shown below.

$$\% \text{ Recovery} = 100 \times \frac{\text{Mean recovered concentration}}{\text{Theoretical concentration}}$$

Theoretical Concentration (µmol/L)	Mean Recovered Concentration (µmol/L)	Percentage Recovery (%)
0.06	0.06	102.8
0.10	0.11	108.3
0.30	0.30	101.1
0.60	0.62	103.3
1.00	1.06	105.7

Mean percentagerecovery:104.2

Linearity

Linearity studies were performed as suggested in CLSI EP6-A. A 1.40 µmol/L serum sample was prepared and dilutions were made proportionally with human serum negative for methotrexate. The ARK Methotrexate Assay was linear between 0.03 to 1.20 µmol/L. Results are shown below.

Theoretical (µmol/L)	Observed Results (µmol/L)	1st Order Predicted Results	2nd Order Predicted Results	Difference (µmol/L or %)
0.00	0.01	-0.002	0.010	NA
0.03	0.04	0.029	0.038	0.009 µmol/L
0.05	0.06	0.050	0.057	0.007 µmol/L
0.12	0.13	0.124	0.125	1.1 %
0.24	0.24	0.250	0.243	-2.7 %
0.36	0.36	0.375	0.363	-3.2 %
0.48	0.49	0.501	0.486	-3.0 %
0.72	0.72	0.753	0.740	-1.8 %
0.96	1.02	1.005	1.003	-0.2 %
1.20	1.27	1.257	1.277	1.6 %

Samples containing methotrexate between 2 and 1200 µmol/L were prepared proportionally in pooled human serum and then diluted into the calibration range with ARK Methotrexate Dilution Buffer. Regression of assayed methotrexate concentrations was linear throughout the range.

Method Comparison

Correlation studies were performed using CLSI EP9-A3. Results from the ARK Methotrexate Assay on the Beckman Coulter AU680 analyzer were compared with results on the Roche/Hitachi 917 analyzer.

Methotrexate concentrations by Roche/Hitachi 917 ranged 0.04 to 1050 µmol/L (µM). ARK Methotrexate values on the Beckman Coulter AU680 ranged 0.04 to 1070 µmol/L. Results of the Passing-Bablok[30] regression analysis for the study are shown below (with 95% confidence limits) for 112 specimens within the measurement range as well as for all 142 specimens including those above the measurement range requiring dilution.

Parameter	Range 0.04 to 1.11 µM		Range 0.04 to 1050 µM	
Slope	0.99	(0.96 to 1.00)	1.00	(1.00 to 1.02)
y-intercept	0.00	(0.00 to 0.01)	0.00	(0.00 to 0.00)
Correlation Coefficient (r^2)	0.98	(0.97 to 0.98)	1.00	(1.00 to 1.00)
Number of Samples	112	NA	142	NA

Precision

Precision was determined as described in CLSI EP5-A3. The six-level ARK Methotrexate Control and pooled human serum 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. Acceptance criteria: ≤10% total CV at >0.1 µmol/L, SD ≤0.01 at ≤0.1 µmol/L.

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total	
			SD	CV (%)	SD	CV (%)	SD	CV (%)
ARK Methotrexate Control								
LOW	160	0.08	0.006	7.8	0.004	5.5	0.008	9.6
MID	160	0.39	0.009	2.2	0.007	1.7	0.012	3.1
HIGH	160	0.78	0.026	3.3	0.027	3.5	0.038	4.9
5	160	5.2	0.186	3.6	0.247	4.8	0.309	6.0
50	160	48.7	3.674	7.6	2.264	4.6	4.439	9.2
500	160	516.8	13.284	2.6	35.641	6.9	38.813	7.5
Human Serum								
LOW	160	0.08	0.007	8.9	0.006	7.2	0.009	11.2
MID	160	0.41	0.011	2.6	0.008	2.1	0.015	3.7
HIGH	160	0.82	0.038	4.6	0.031	3.8	0.050	6.1
5	160	5.2	0.278	5.3	0.381	7.3	0.464	8.9
50	160	53.0	1.624	3.1	3.319	6.3	3.705	7.0
500	160	507.9	12.222	2.4	22.957	4.5	26.177	5.2

Interfering Substances

Interference studies were conducted using CLSI EP7-A2 as a guideline. Clinically high concentrations of the following potentially interfering endogenous substances in serum with known levels of methotrexate (approximately 0.05 and 0.50 µmol/L) were evaluated. 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 tested.

		Methotrexate (~ 0.05 µmol/L)		Methotrexate (~ 0.50 µmol/L)	
Interfering Substance	Interferent Concentration	Serum Control	Test	Serum Control	Test (% Control)
Albumin	12 g/dL	0.05	0.05	0.48	0.50 (103.5)
Bilirubin - conjugated	70 mg/dL	0.05	0.06	0.48	0.49 (101.4)
Bilirubin - unconjugated	70 mg/dL	0.05	0.05	0.48	0.48 (101.4)
Cholesterol	620 mg/dL	0.05	0.04	0.47	0.48 (103.2)
Gamma-Globulin	12 g/dL	0.05	0.06	0.48	0.49 (100.7)
Hemoglobin	1000 mg/dL	0.06	0.06	0.48	0.49 (101.4)
Rheumatoid Factor	1080 IU/mL	0.06	0.07	0.46	0.45 (96.7)
Triglycerides	835 mg/dL	0.05	0.04	0.48	0.48 (98.6)
Uric Acid	30 mg/dL	0.05	0.06	0.48	0.49 (100.7)

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.

Crossreactivity to 7-Hydroxymethotrexate, the major metabolite

After administration of high-dose methotrexate (HDMTX), the serum/plasma concentration of 7-hydroxymethotrexate typically exceeds that of methotrexate at later time points. It has been reported that 7-hydroxymethotrexate levels exceed those of methotrexate by up to 100-fold 12 to 48 hours after HDMTX administration.[15, 27, 29, 31, 33-34]

Crossreactivity 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 crossreact (≤ 0.1%) with the major metabolite 7-hydroxymethotrexate.

Crossreactivity 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 measurement of methotrexate.[32] However, following glucarpidase rescue therapy, the serum concentration of DAMPA can be substantial.[13, 14] The ARK Methotrexate Assay crossreacts substantially with the minor metabolite DAMPA. Tests were performed in the absence of the parent drug methotrexate. Crossreactivity to DAMPA ranged 76.3% to 100% based on data observed. The assay should not be used during possible compassionate therapy with glucarpidase (carboxypeptidase G2) that rapidly converts circulating methotrexate to DAMPA.

Drugs that crossreact

The ARK Methotrexate Assay crossreacts 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. In the absence of methotrexate, crossreactivity to triamterene (1.15%) and trimethoprim (0.01%) was observed. In the presence of methotrexate, crossreactivity to triamterene (≤3.3%) and trimethoprim (≤0.5%) resulted based on data observed.

Crossreactivity to folate analogs and other compounds

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

Compound	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-Methyltetrahydrofolic acid	1000
Prednisolone	1000
Pyrimethamine	1000
Sulfamethoxazole	1600
Tetrahydrofolic Acid	1000
Vinblastine	1000
Vincristine	1000

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14 TRADEMARKS

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