ARK Diagnostics, Inc.

ARK *Methotrexate II Assay*

This ARK Diagnostics, Inc. package insert for the ARK Methotrexate II 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.

Report any serious incident that has occurred in relation to the device to the manufacturer and the appropriate competent authority as applicable. A Summary of Safety and Performance is available through Eudamed (European database on medical devices), SRN: US-MF-000023925.

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KEY TO SYMBOLS USED

LOT	Batch Code	YYYY-MM-DD	Use by/Expiration Date
EC REP	Authorized Representative	CE 2797	CE Mark with notified body number
REF	Catalog Number		Manufacturer
IVD	In Vitro Diagnostic Medical Device		Temperature Limitation
	Consult Instructions for Use	R1 R2	Reagent 1/Reagent 2
Rx Only	For Prescription Use Only		

1 NAME

ARK *Methotrexate II Assay*

2 INTENDED USE

The ARK Methotrexate II 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 obtained from patients who have received glucarpidase (carboxypeptidase G2) as a high dose methotrexate rescue therapy should not be tested with the ARK Methotrexate II Assay.

Reagent Kit REF 5071-0001-00

Reagent Kit REF 5071-0001-01

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.

Methotrexate monitoring is commonly used for patients undergoing high-dose methotrexate treatment for cancer; the results are used to guide supportive therapy while methotrexate is being eliminated by the kidneys. Guidelines for methotrexate therapy with leucovorin rescue should be consulted.[1,4] Intermediate to high doses of methotrexate (approximately 35 mg/m2 - 12 g/m2) 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.[5-9]

4 PRINCIPLES OF THE PROCEDURE

The ARK Methotrexate Assay is a homogeneous enzyme immunoassay. The assay uses specific antibodies that can bind to methotrexate. The assay is based on competition between a drug labeled with recombinant glucose-6-phosphate dehydrogenase (rG6PDH) and free drug from the sample, for a fixed amount of specific antibody binding sites. In the absence of free drug from the sample, rabbit monoclonal anti-methotrexate antibody binds to the drug labeled with rG6PDH and causes a decrease in enzyme activity. In the presence of drug from the specimen, enzyme activity increases and is directly related 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
5071-0001-00	**ARK Methotrexate II Assay** **Reagent R1 – Antibody/Substrate** Rabbit monoclonal antibody to methotrexate, glucose-6-phosphate, nicotinamide adenine dinucleotide, bovine serum albumin, sodium azide, and stabilizers	1 X 16 mL
	Reagent R2 – Enzyme Methotrexate labeled with bacterial rG6PDH, buffer, bovine serum albumin, sodium azide, and stabilizers	1 X 8 mL

REF	Product Description	Quantity/Volume
5071-0001-01	**ARK Methotrexate II Assay** **Reagent R1 – Antibody/Substrate** Rabbit monoclonal antibody to methotrexate, glucose-6-phosphate, nicotinamide adenine dinucleotide, bovine serum albumin, sodium azide, and stabilizers	1 X 28 mL
	Reagent R2 – Enzyme Methotrexate labeled with bacterial rG6PDH, buffer, bovine serum albumin, sodium azide, and stabilizers	1 X 14 mL

Reagent Handling and Storage

ARK Methotrexate II 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.**

ARK Methotrexate II products contain ≤0.09% sodium azide. As a precaution, affected plumbing including instrumentation should be flushed adequately with water to mitigate the potential accumulation of explosive metal azides. No special handling is required regarding other assay components.

6 WARNINGS AND PRECAUTIONS

- For ***In Vitro* Diagnostic**, laboratory professional use.
- For prescription use only. *Caution: U.S. Federal Law restricts this device to sale by or on the order of a licensed practitioner.*
- Reagents R1 and R2 are provided as a matched set and should not be interchanged with reagents from different lot numbers.
- Reagents contain ≤0.09% sodium azide.
- The assay should only be used in conjunction with information available from clinical evaluations and other diagnostic procedures.

7 SPECIMEN COLLECTION AND PREPARATION FOR ANALYSIS

- Each laboratory is responsible for supplying a valid specimen for analysis according to their quality procedures.
- 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 times.
- 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).
- Follow the collection tube manufacturer's recommendations for collection, processing and centrifugation.
- CLSI document GP44-A4 outlines procedures for minimizing artifacts due to specimen collection and handling for common laboratory tests.[10]
- 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.
- The presence of bubbles or foam on specimens can lead to short sample delivery and erroneous results.
- Each laboratory should consult available literature and internal data regarding specimen stability.
- Based on studies performed by ARK Diagnostics, clarified specimens may be stored up to one week at 2 to 8°C based on supporting data. If testing will be delayed more than one week, specimens should be stored frozen (≤ -10°C) up to four weeks prior to being tested. Care should be taken to limit the number of freeze-thaw cycles; supporting data showed no adverse impact from three freeze-thaw cycles.
- **Handle all patient specimens as if they were potentially infectious.**

8 PROCEDURE

Materials Provided

ARK Methotrexate II Assay – REF 5071-0001-00, 5071-0001-01

Materials Required – Provided Separately

ARK Methotrexate II Calibrator – REF 5071-0002-00
Quality Controls – ARK Methotrexate II Control – REF 5071-0003-00
Quality Controls – ARK Methotrexate II Control – REF 5071-0003-01
Quality Controls – ARK Methotrexate II Control – REF 5071-0003-02
ARK Methotrexate II Dilution Buffer – REF 5071-0004-00

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 .

Many automated clinical chemistry analyzers with photometric rate determination at 340 nm are suitable. Consult the analyzer-specific application sheet for programming the ARK Methotrexate II Assay, available from your distributor or ARK Customer Service. Application Protocol Sheets which have been CLIA categorized or bear the CE Mark have been verified by the manufacturer. It is the responsibility of the laboratory to perform all appropriate validation for use of the assay with other settings or analyzers.

Refer to the instrument-specific operator's manual for daily maintenance.

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 II Calibrators A, B, C, D, E, and F; run 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 II 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

To estimate drug levels in specimens exceeding the upper limit of quantitation, manually dilute the specimen with ARK Methotrexate II Dilution Buffer. Multiply the assayed result by the dilution factor.

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

An expanded assay range has been validated up to 1200 µmol/L through serial 1:10 sample dilutions of 1:10, 1:100, or 1:1000 as needed.

9 RESULTS

Report result units as µmol/L or µg/mL. To convert µmol/L to µg/mL, divide the value obtained by the conversion factor of 2.2005. The methotrexate value from this assay should be used in conjunction with other clinical information. Refer to the instrument specific operator's manual for any result error codes.

10 LIMITATIONS OF PROCEDURE

This assay is designed for use with serum or plasma only; refer to the section **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 variabilities. See the section **Expected Values** below.

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 II Assay. These specimens have increased serum levels of 4-[[2,4-diamino-6-(pteridinyl) methyl]-methylamino]-benzoic acid (DAMPA)[11-13] 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.[14] 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.14 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.[15]

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),[16] concentrations may exceed 1000 µmol/L[17] 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.[18] For treatment of osteosarcoma,[17] 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, 4] Some centers follow ≤0.10 µmol/L.[17, 19]

From prescribing and other information: Laboratory Indicators of Toxicity Following Leucovorin Rescue Schedules with High Dose Methotrexate.[1, 4, 20]

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

Renal toxicity is a significant risk and may be exacerbated by coadministration of other drugs,[15, 20] for example vancomycin.[21] 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.[22-29]

Given the profile of the appearance of the 7-hydroxymethotrexate metabolite,[16, 28] its molar ratio to methotrexate of up to approximately 100-fold,[30] and relative insolubility versus the parent drug,[15, 20] possible nephrotoxicity due to precipitation of the metabolite in renal tubules[30] may delay elimination of methotrexate itself.

Glucarpidase therapy 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.[15] Elimination of DAMPA may take several days before it no longer interferes with the monitoring of methotrexate by immunoassay.[14]

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® automated clinical chemistry analyzer System.

Sensitivity

Limit of Quantitation (LoQ)

The LoQ of the ARK Methotrexate II Assay was determined according to CLSI EP17-A2 and is defined as the lowest concentration for which acceptable inter-assay precision (≤0.010 SD) and recovery (±0.010 µmol/L of nominal) is observed. The LoQ was determined to be 0.030 µmol/L, and may depend on analyzer-specific performance.

Nominal Concentration (µmol/L)	Grand Mean (µmol/L)	SD	CV (%)
0.030	0.034	0.002	4.87
0.040	0.043	0.002	4.01
0.050	0.052	0.002	4.00

Measurement Range

The analytical measurement range of the ARK Methotrexate II Assay is 0.030 – 1.300 µmol/L. Specimens containing methotrexate in higher concentrations (>1.300 µmol/L) may be assayed by dilution of the specimen into the measurement range for a quantitative result or otherwise reported as detected above the measurement range. Multiply the assay result by the dilution factor to obtain the concentration of methotrexate in the undiluted specimen. Report results below this range as <0.030 µmol/L or below a higher analyzer-specific LoQ established in your laboratory. Report results above this range as >1.300 µmol/L or above the analyzer-specific upper LoQ established in your laboratory.

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 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.

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

Theoretical Concentration (µmol/L)	Mean Recovered Concentration (µmol/L)	Percent Recovery
0.060	0.063	104.4
0.100	0.105	105.2
0.300	0.322	107.2
0.600	0.628	104.7
1.000	1.079	107.9
1.200	1.293	107.8

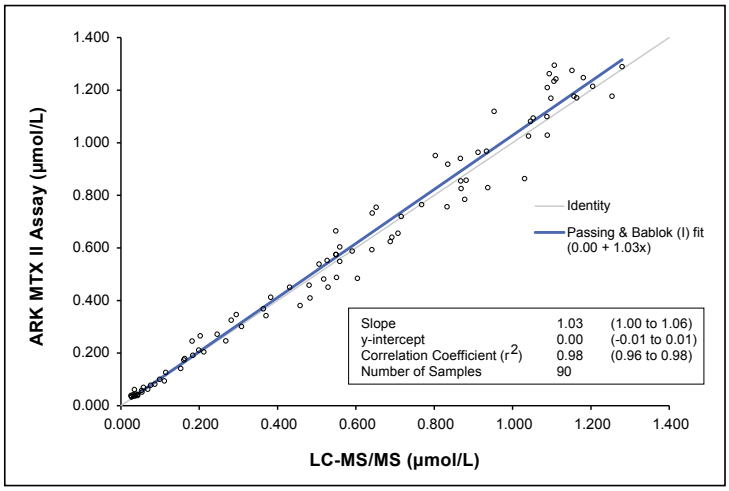
Linearity

Linearity studies were performed as suggested in CLSI Protocol EP06-Ed2. A 1.600 µmol/L serum sample was prepared and dilutions were made proportionally with human serum negative for methotrexate. Methotrexate concentrations ranged from 0.030 to 1.300 µmol/L. A linear relationship was demonstrated between 0.030 and 1.300 µmol/L. Results are shown below.

Nominal Concentration (µmol/L)	Observed Results (µmol/L)	Predicted Results (µmol/L)	% Difference
0.000	0.000	NA	NA
0.030	0.035	0.033	5.78
0.060	0.062	0.065	-4.96
0.130	0.129	0.141	-8.73
0.260	0.296	0.283	4.66
0.390	0.399	0.424	-5.98
0.520	0.549	0.565	-2.89
0.650	0.721	0.707	2.07
0.780	0.877	0.848	3.36
0.910	1.012	0.989	2.32
1.040	1.157	1.131	2.34
1.170	1.261	1.272	-0.87
1.300	1.380	1.413	-2.40

Method Comparison

Correlation studies were performed using CLSI Protocol EP9-A3. Measurements of methotrexate in human serum (from patients treated with high-dose methotrexate therapy) by the ARK Methotrexate II Assay were compared to those obtained by liquid chromatography tandem mass spectrometry (LC-MS/MS). The methotrexate concentrations ranged from 0.026 to 1.280 µmol/L by LC-MS/MS. Results of the Passing-Bablok[31] regression analysis for the study are shown below (with 95% confidence limits).



Method comparison was also performed against the original ARK Methotrexate Assay for 123 patient samples with methotrexate values ranging from 0.054 to 1.168. Statistics with confidence intervals from the Passing-Bablok comparison are slope = 0.98 (0.95 to 1.01); y-intercept = -0.02 (-0.03 to -0.01); and correlation coefficient (r^2) = 0.97 (0.96 to 0.98).

Precision

Precision was determined as described in CLSI Protocol EP5-A3. All six control levels of the ARK Methotrexate II Control (Low, Mid, High, 5, 50, and 500) and the six corresponding pooled human serum sample counterparts 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.

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total	
			SD	CV (%)	SD	CV (%)	SD	CV (%)
ARK Methotrexate II Control								
LOW	160	0.069	0.002	2.84	0.001	1.23	0.002	3.00
MID	160	0.411	0.006	1.40	0.002	0.43	0.006	1.40
HIGH	160	0.811	0.014	1.79	0.008	0.97	0.017	2.05
5	160	4.868	0.070	1.44	0.036	0.74	0.077	1.58
50	160	49.660	1.108	2.23	0.397	0.80	1.141	2.30
500	160	493.769	8.012	1.62	2.483	0.50	8.012	1.62
Human Serum								
LOW	160	0.070	0.002	2.50	0.001	1.49	0.002	2.88
MID	160	0.404	0.008	1.86	0.003	0.65	0.008	1.92
HIGH	160	0.846	0.016	1.93	0.008	0.95	0.017	2.06
5	160	5.247	0.076	1.45	0.028	0.54	0.078	1.49
50	160	51.614	0.723	1.40	0.285	0.55	0.777	1.51
500	160	507.988	7.632	1.50	4.240	0.83	8.538	1.68

Interfering Substances

Interference studies were conducted using CLSI Protocol EP07-A3 as a guideline. Clinically high concentrations of the following potentially interfering substances in serum with known levels of methotrexate (approximately 0.050 and 0.500 µmol/L) were evaluated. Each sample was assayed using the ARK Methotrexate II Assay, along with a serum control of methotrexate. Measurements of methotrexate were within ±10% interference or ±0.010 µmol/L of serum control when methotrexate concentrations were ≤0.100 µmol/L.

Interfering Substance	Interferent Concentration	Results	
		± µmol/L from Control (0.050 µmol/L Methotrexate)	% Interference (0.500 µmol/L Methotrexate)
Human Albumin	12 g/dL	0.002	-1.04
Bilirubin - conjugated	72 mg/dL	0.001	1.96
Bilirubin - unconjugated	72 mg/dL	0.003	0.23
Cholesterol	500 mg/dL	0.005	3.49
Human Gamma-Globulin	12 g/dL	0.003	2.42
Hemoglobin	1000 mg/dL	-0.006	-2.72
Rheumatoid Factor	1080 IU/mL	0.001	3.52
Triglycerides	1000 mg/dL	-0.007	7.48
Uric Acid	30 mg/dL	0.000	1.60

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 II Assay. High levels of these compounds were spiked into serum pools containing no methotrexate, 0.050 µmol/L or 0.500 µmol/L of methotrexate. The samples were analyzed and the methotrexate concentrations of samples containing interferent were compared to a serum control.

Interference with 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.[16, 28, 30, 32, 34-35]

Cross-reactivity with 7-hydroxymethotrexate methotrexate was determined for the ARK Methotrexate II Assay by testing both 0.050 and 0.500 µmol/L methotrexate in human serum supplemented with 50 µmol/L 7-hydroxymethotrexate (1000- and 100-fold excess). Cross-reactivity to this metabolite was negligible at less than 0.01%. An extreme 1000-fold excess of the 7-hydroxymethotrexate metabolite causes less than 10% interference in the measurement of methotrexate in the ARK Methotrexate II Assay.

Crossreactivity to 2,4-Diamino-N10-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 II Assay crossreacts substantially with the minor metabolite DAMPA. Tests were performed in the absence of the parent drug methotrexate. Cross-reactivity to DAMPA ranged from 19% to 58% based on data observed. The assay should not be used during therapy with glucarpidase (carboxypeptidase G2) that rapidly converts circulating methotrexate to DAMPA.

Interference with folate analogs and other compounds

The compounds listed below did not interfere with the ARK Methotrexate II Assay when tested in the presence of methotrexate (±10% interference at 0.500 µmol/L methotrexate concentration and ±0.010 µmol/L from control at 0.050 µmol/L methotrexate concentration). Compound concentrations were tested according to CLSI EP37 guidelines.

Compound	Conc. Tested (µmol/L)
Adriamycin	1000
Cyclophosphamide	2200
Cytosine	1000
Dihydrofolic Acid	1000
Tetrahydrofolic Acid	1000
DL-6-Methyl-5,6,7,8-Tetrahydropterine	1000
Folic Acid	1000
Folinic Acid	1000
5-Fluorouracil	3000
6-Mercaptopurine	1000
5-Methyltetrahydrofolic Acid	1000
Prednisolone	1000
Pyrimethamine	1000
Sulfamethoxazole	1600
Vinblastine	1000
Vincristine	1000
Trimethoprim	150
Triamterene	25

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14 TRADEMARKS

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