510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY
ASSAY ONLY TEMPLATE

A. 510(k) Number:

k111904

B. Purpose for Submission:

New assay, calibrator and control

C. Measurand:

Methotrexate

D. Type of Test:

Quantitative Homogeneous Enzyme Immunoassay

E. Applicant:

ARK Diagnostics, Inc.

F. Proprietary and Established Names:

ARK™ Methotrexate Assay, ARK™ Methotrexate Calibrator, ARK™ Methotrexate Control

G. Regulatory Information:

1. Regulation section:

Product Code	Classification	Regulation Section	Panel
LAO - Methotrexate enzyme immunoassay	II	Unclassified	Toxicology (91)
DLJ - Clinical toxicology calibrator	II	21 CFR §862.3200	Toxicology (91)
LAS - Clinical toxicology control material	I, reserved	21 CFR §862.3280	Toxicology (91)

H. Intended Use:

1. Intended use(s):

   See indications for use below.

2. Indication(s) for 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.

The ARK Methotrexate Calibrator is intended for the calibration of the ARK Methotrexate Assay.

The ARK Methotrexate Control is intended for the quality control of the ARK Methotrexate Assay.

3. Special conditions for use statement(s):

   For prescription use only.

   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.

4. Special instrument requirements:

   Performance characteristics were established on the Roche/Hitachi 917 automated clinical chemistry analyzer.

I. Device Description:

The ARK Methotrexate Assay consists of reagents R1 (anti-Methotrexate rabbit polyclonal antibody with substrate) and R2 (Methotrexate labeled with bacterial G6PDH enzyme.)

The ARK Methotrexate Calibrator consists of a six-level set to calibrate the assay. The calibrators consist of a synthetic protein matrix and the levels are 0.00 umol/L, 0.05 umol/L, 0.15 umol/L, 0.25 umol/L, 0.50 umol/L and 1.2 umol/L.

The ARK Methotrexate Control consists of a six-level set used for quality control of the assay (tri-level calibration range set and tri-level high range set). The controls consist of a synthetic protein matrix and the levels are 0.07 umol/L, 0.40 umol/L, 0.80umol/L, 5 umol/L, 50 umol/L and 500 umol/L.

The ARK Methotrexate Dilution Buffer is equivalent to zero calibrator (Calibrator A).

J. Substantial Equivalence Information:

1. Predicate device name(s):

   Abbott TDx®/TDxFLx® METHOTREXATE II

2. Predicate K number(s):

   k932615

3. Comparison with predicate:

Similarities		
Item	Proposed Device	Predicate Device (k932615)
Intended Use/Indications for use	For the quantitative determination of methotrexate. The measurements obtained are used in monitoring levels of methotrexate to ensure appropriate therapy.	Same
Specimen type	Serum and plasma	Same
Number of calibrators and controls	6 levels (each)	Same
Reagent matrix and storage condition	Liquid, 2-8° C	Same

Differences		
Item	Device	Predicate
Methodology	Homogenous enzyme immunoassay (EIA)	Fluorescence polarization immunoassay (FPIA)
Reagent Components	Two (2) reagent system: Anti-Methotrexate Antibody/Substrate Reagent (R1) (contains rabbit polyclonal antibodies to Methotrexate) Enzyme Reagent (R2) (contains Methotrexate labeled with bacterial G6PDH)	Reagent Pack: W (Wash solution) S (containing Methotrexate antibody (mouse monoclonal)) T (Methotrexate Fluorescein Tracer) P (Pretreatment Solution)
Matrix for calibrators and controls	Synthetic matrix	Human serum

K. Standard/Guidance Document Referenced (if applicable):

- CLSI document EP5-A2, *Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline*
- CLSI Guideline EP6-A: *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*
- CLSI Guideline EP9-A2*: Method Comparison and Bias Estimation Using Patient Samples*
- CLSI Protocol EP7-A2: *Interference Testing in Clinical Chemistry*
- CLSI Protocol EP17-A: *Protocols for Determination of Limits of Detection and Limits of Quantitation*

L. Test Principle:

The 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 coenyzme NAD functions only with the bacterial enzyme used in the assay.

M. Performance Characteristics (if/when applicable):

Performance was validated on the Hitachi 917 instrument.

1. <u>Analytical performance:</u>

   a. Precision/Reproducibility:

   Precision was determined as described in CLSI Guideline EP5-A2. Data for the precision evaluation studies (total and within-laboratory precision) were collected on a single Roche/Hitachi 917 automated clinical chemistry analyzer. Each level of control and patient sample was assayed in quadruplicate twice a day over twenty non-consecutive days. A total of 160 determinations were made for each sample. Four calibrations were performed during this interval. The ARK Methotrexate Controls (three levels) and three patient sample pools were tested in each run.

   Mean methotrexate concentration, standard deviation (SD) and coefficients of variation (% CVs) were calculated for within-run, between-day, and total precision.

   Patient specimens that contained methotrexate were pooled to create three levels with sufficient volume to complete the 20-day protocol.

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total	
			SD	%CV	SD	%CV	SD	%CV
Controls								
Low	160	0.06	0.005	8.2	0.005	7.3	0.007	10.7
Mid	160	0.37	0.011	3.0	0.008	2.1	0.014	3.8
High	160	0.76	0.032	4.3	0.030	4.0	0.045	5.9
Patient Pools								
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	0.029	7.2
High	160	0.82	0.037	4.5	0.043	5.2	0.057	7.0

*Samples were diluted in ARK Methotrexate Dilution Buffer. Mean result and SD were multiplied by the dilution factor.

The sponsor also performed a precision study to evaluate serum and plasma samples. The study was modified from the recommendations in CLSI Guideline EP5-A2; 5 days of precision studies were performed. The results showed that precision for serum and plasma was not significantly different.

b. Linearity/assay reportable range:

Linearity studies were performed as suggested in CLSI Guideline EP6-A: *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*.

Linearity studies were performed by testing concentrations of methotrexate across the claimed measuring range. Gravimetric addition of pure methotrexate (USP) to dimethylformamide and volumetric addition of this stock solution to human serum negative for methotrexate was made to achieve a concentration (HIGH Linearity Sample; 1.30 µmol/L) above the calibration range as suggested in CLSI Protocol EP-6A.

Dilutions of this 1.30 µmol/L HIGH Linearity Sample were made proportionally with pooled human serum negative for methotrexate. Two separately calibrated analytical runs of three replicates of each sample per run were assayed (N=6).

The results of the six replicates were averaged. Regression analyses were performed between the measured mean methotrexate and the theoretical values for each dilution, using first order and second order polynomial determinations. Predicted 1st and 2nd order regressed values were calculated from the regression equations and compared.

Theoretical (µmol/L)	Observed Results (µmol/L)	1st Order Predicted Results	2nd Order Predicted Results	Difference (µmol/L or %)
0.00	0.00	0.009	-0.003	na
0.02	0.02	0.026	0.016	-0.010 µmol/L
0.04	0.04	0.042	0.034	-0.008 µmol/L
0.05	0.06	0.059	0.053	-0.006 µmol/L
0.07	0.08	0.076	0.072	-0.004 µmol/L
0.11	0.11	0.110	0.109	-0.7 %
0.18	0.17	0.178	0.183	3.1 %
0.36	0.34	0.347	0.364	4.8 %
0.65	0.63	0.618	0.639	3.4 %
0.72	0.72	0.686	0.705	2.9 %
0.86	0.84	0.821	0.835	1.7 %
1.01	0.99	0.957	0.960	0.4 %
1.15	1.06	1.092	1.082	-1.0 %
1.30	1.19	1.228	1.199	-2.3 %

Linear regression analysis yields the following result:
$y = 0.9407x + 0.086$
$R^2 = 0.9978$

There are no concentrations at which the percent difference was more than 10% between the predicted 1st and 2nd order regressed values for concentrations >0.10 µmol/L or ±0.01 µmol/L at concentrations ≤ 0.10 µmol/L.

The claimed measuring range of this device is 0.04 - 1.20 µmol/L.

Evaluation of recovery:

An accuracy-by-recovery study was conducted to determine the trueness of quantitative measurements of methotrexate across the calibration range of the ARK™ Methotrexate Assay. Recovery of methotrexate was studied with two sources of methotrexate for comparison.

Samples were prepared by volumetric addition of methotrexate (Cerilliant Certified Stock solution 99.8% purity) to human serum negative for methotrexate. Drug concentrations across the assay range (0.06, 0.10, 0.30, 0.60, and 1.00 µmol/L) were tested. Each sample was assayed in triplicate in each of two separately calibrated runs for a total of six replicates. The results were averaged and compared to the theoretical target concentration and percentage recovery calculated.

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

Target concentration

For comparison, samples were prepared by gravimetric addition of methotrexate (USP > 99.9% purity) to dimethylformamide (DMF) and volumetric addition of this stock solution to human serum negative for methotrexate, then assayed as described above.

Recovery was acceptable if the mean methotrexate concentration measured was within ±10% of the target level for concentrations at >0.1 µmol/L and ±0.01 at ≤0.1 µmol/L.

Target Concentration (µmol/L)	**Cerilliant Methotrexate**		**USP Methotrexate**	
	Mean Recovered Concentration (µmol/L)	**Recovery (%)**	**Mean Recovered Concentration (µmol/L)**	**Recovery (%)**
0.06	0.067	+0.007 µmol/L (111.1%)	0.060	100.0
0.10	0.100	100.0	0.098	98.3
0.30	0.295	98.3	0.282	93.9
0.60	0.613	102.2	0.597	99.4
1.00	0.988	98.8	1.008	100.8

Evaluation of samples above the claimed measuring range:
Frequently, patient samples may have methotrexate levels higher than the claimed measuring range of the device. They require dilution into the measuring range for evaluation. Evaluation of linearity, precision and accuracy after dilution of samples was evaluated.

Evaluation of linearity for samples with elevated methotrexate levels:

A sample containing approximately 1200 µmol/L of methotrexate in human serum was prepared to assess the proportional measurement of the drug upon dilution into the calibration range with ARK Methotrexate Dilution Buffer. This HIGH sample was diluted proportionally in pooled human serum to obtain concentrations ranging from 2 to 1200 µmol/L. As a pre-analytical step, these serum samples were diluted in ARK Methotrexate Dilution Buffer. Ten-fold dilution factors were used. Two separately calibrated analytical runs of three replicates of each sample per run were assayed (N=6). Linear regression analysis was performed to demonstrate by visual analysis that measurement of methotrexate was adequately proportional for concentrations exceeding the calibration range.

Measurement of methotrexate is linear within the calibration range of the ARK Methotrexate Assay when the predicted 1st and 2nd order regressed values agree within 10% for concentrations >0.10 µmol/L or within ±0.01 µmol/L at concentrations ≤0.10 µmol/L.

Measurement of elevated methotrexate above the calibration range was linear. Regression analysis demonstrated proportionality along a straight line by visual inspection. Reported values (after calculating for dilution) were plotted versus the expected concentration of methotrexate (2.00 to 1200.00 µmol/L).

Expected (µM)	**2.00**	**8.00**	**20.00**	**80.00**	**200.00**	**800.00**	**1200.00**
Dilution	**1/10**	**1/10**	**1/100**	**1/100**	**1/1000**	**1/1000**	**1/10000**
1	0.21	0.75	0.20	0.83	0.20	0.79	0.12
2	0.21	0.76	0.20	0.78	0.20	0.76	0.11
3	0.20	0.82	0.21	0.85	0.20	0.75	0.12
4	0.21	0.75	0.19	0.82	0.20	0.74	0.11
5	0.20	0.78	0.20	0.80	0.20	0.76	0.11
6	0.20	0.79	0.19	0.82	0.19	0.75	0.12
Mean (µM)	**0.205**	**0.775**	**0.198**	**0.817**	**0.198**	**0.758**	**0.115**
SD	**0.005**	**0.027**	**0.008**	**0.024**	**0.004**	**0.017**	**0.005**
Reported (µM)	**2.05**	**7.75**	**19.83**	**81.67**	**198.33**	**758.33**	**1150.00**

Linear regression analysis of this study yielded the following:

$y = 0.9537x + 2.1285$
$R^2 = 0.9999$

Evaluation of precision in samples with elevated methotrexate levels:

Precision was evaluated as described above in 1a.

ARK Methotrexate High Range Controls are comprised of three levels (5, 50, and 500 µmol/L). These controls are comprised in a proteinaceous synthetic matrix equivalent to human serum/plasma.

High Range controls and patient sample pools with concentrations greater than the calibration range were diluted 10x, 100x or 1000x, respectively in ARK Methotrexate Dilution Buffer prior to testing. High Range controls and samples were diluted once for each run and these diluted samples were tested in quadruplicate

Human serum/plasma specimens that contained methotrexate were pooled to create six levels with sufficient volume to complete the 20-day protocol. High patient sample pools (approximately 5, 50 and 500 µmol/L) required that the sample pools were supplemented with methotrexate stock. The stock solution used was prepared by gravimetric addition of pure Methotrexate (USP >99.9 % purity) to dimethylformamide (DMF) and volumetric addition of this stock solution to the specimen pool.

Sample	N	Mean (µmol/L)	Within Run		Between Day		Total	
			SD	%CV	SD	%CV	SD	%CV
Controls								
Control 5	160	4.8	0.15	3.1	0.13	2.8	0.20	4.2
Control 50	160	49	1.36	2.8	2.32	4.8	2.72	5.6
Control 500	160	476	15.17	3.2	30.75	6.5	34.66	7.3
Patient Pools								
Patient Pool – 5	160	4.6	0.14	3.1	0.183	4.0	0.24	5.3
Patient Pool – 50	160	45	1.33	3.0	2.63	5.9	2.93	6.6
Patient Pool – 500	160	461	11.84	2.6	27.04	5.9	29.60	6.4

Evaluation of accuracy in samples with elevated methotrexate levels:

A method comparison study was performed to evaluate the agreement between the ARK Methotrexate Assay and predicate device Abbott TDx/TDxFLx Methotrexate II assay (k932615) using samples with elevated methotrexate levels (i.e., samples that require dilution into the assay's measuring range for evaluation). The study was performed using Clinical and Laboratory Standards Institute (CLSI) Protocol EP9-A2 *Method Comparison and Bias Using Patient Samples*.

Forty five patient specimens above the claimed measuring range were evaluated with the proposed and predicate assay; eight samples were spiked with methotrexate. All samples required dilution before testing on both devices (ARK Methotrexate Dilution Buffer was used for the proposed device. The samples ranged from 1.56 – 1440 µmol/L on the predicate. Results of the study are summarized in the table below:

Parameter	Range 1.56 to 1440 µmol/L
Number of Samples	45
Slope	0.96 (0.94 to 0.99)
y-intercept	0.11 (-0.52 to 0.33)
Correlation Coefficient (r^2)	0.997 (0.994 to 0.998)

c. *Traceability, Stability, Expected values (controls, calibrators, or methods):*

ARK Methotrexate Calibrators and controls are prepared by volumetric dilution of high purity, certified Methotrexate solution into a synthetic proteinaceous matrix free of Methotrexate.

ARK Methotrexate Calibrator

A master calibrator lot is prepared volumetrically using a certified solution of methotrexate. The ARK Methotrexate Calibrator is traceable to a certified methotrexate solution and the uncertainty in the methotrexate concentration is 5% relative to the concentration in the certified solution.

The concentration of methotrexate in the certified solution is traceable to HPLC; the purity of methotrexate in the certified solution is determined by HPLC and other spectral procedures as performed by the supplier of the certified solution.

Bulk solutions of the ARK Methotrexate Calibrator are prepared volumetrically using the certified solution of methotrexate. The concentration of methotrexate in the respective bulk solution must agree within 5% of its corresponding master calibrator.

Value Assignment: Testing is performed with the ARK Methotrexate Assay on the Roche/Hitachi 917 automated analyzer. Two calibrated runs are performed using the Master Calibrator. In each run, five replicates of Master Lot (reference) and Test Lot are tested as matched pairs for each calibrator level. Mean values for ten replicates are calculated. Test lot mean values are expected to match the Master lot mean values within 5% allowance. Target values for the six levels of calibrator are 0.00 umol/L, 0.05 umol/L, 0.15 umol/L, 0.25 umol/L, 0.50 umol/L and 1.2 umol/L.

ARK Methotrexate Control

ARK uses USP methotrexate powder to produce the controls. Six levels are provided: three levels (0.07, 0.40 and 0.80 umol/L) are within the calibration range and three levels above the calibration range (5, 50 and 500 μmol/L) are available for controlling the dilution step. ARK manufactures the controls gravimetrically to contain methotrexate within 10% of the target levels.

Value Assignment: Testing is performed with the ARK Methotrexate Assay on the Roche/Hitachi 917 automated analyzer, calibrated with the master calibrator lot. Three calibrated runs are performed using five replicates of each level per run. The 5, 50, 500 μmol/L controls are pre-diluted 1:10, 1:100, and 1:1000 respectively with the ARK Methotrexate Dilution Buffer and tested. Results for high range controls are multiplied by the applicable dilution factor. Mean values (15 replicates) for the test lots are expected to result within 10% of the nominal concentration. The control ranges are set to be +/- 25% from the mean values. Each laboratory should establish the mean value for each control level and its own ranges for each new lot of controls.

Calibrator and Control Stability:

The calibrators and controls are stable until the expiration date printed on the vial when stored unopened and opened at 2-8 °C.

Real time stability studies are ongoing for both unopened and opened calibrators and controls. Stability testing protocols and sponsor's acceptance criteria were reviewed and found to be acceptable.

d. Detection limit:

Limit of Detection (LoD) Limit of Quantitation (LoQ) studies were conducted using CLSI Guideline EP 17-A: *Protocols for Determination of Limits of Detection and Limits of Quantitation*. The study was performed on the Roche/Hitachi 917 automated clinical chemistry analyzer.

Samples were prepared by gravimetric addition of pure methotrexate (USP) to dimethylformamide and volumetric addition of this stock solution to human serum negative for methotrexate. Pooled human serum representative of the patient specimen matrix was supplemented with methotrexate to give concentrations of 0.02, 0.03, 0.04, and 0.05 µmol/L.

The LoB and LoD were evaluated by testing 60 replicates of pooled human serum (BLANK) and 60 replicates of the first positive level 0.02 µmol/L methotrexate. The analyzer was calibrated and three analytical runs were performed. In each run, 20 replicates of BLANK and 20 replicates of 0.02 µmol/L methotrexate were analyzed. The grand mean and root mean square standard deviation (RMS SD) were calculated. Statistical analyses were performed according to the CLSI Guideline. Since the analyzer did not report values below 0.00 µmol/L, the standard deviation for 0.02 µmol/L methotrexate was used to calculate the LoB based on the equation $LoB = \mu B + 1.645(\sigma B)$, the standard deviation being relatively constant in this area of measurement. The error of the BLANK (σB) was taken to equal the standard deviation of the sample (SDs) as a fair estimate.

The LoQ was determined after testing samples containing 0.03, 0.04, or 0.05 µmol/L. Eight replicates of each sample were tested in each of five runs (each run performed on a separate day) to yield 40 replicates of each LoQ sample tested. The grand mean and RMS SD were calculated for each sample.

The LoQ of the ARK Methotrexate Assay is defined as the lowest concentration for which acceptable inter-assay precision (≤0.01 SD) and recovery (± 0.01 µmol/L) is observed. Where this concentration's 95% confidence interval exceeds the LoD, the definition of LoQ according to the CLSI Guideline will be met. The criteria of LoQ were met at 0.04 µmol/L. (The same LoQ was established when a similar study was performed as above but also incorporating three lots of reagents, controls and calibrators.)

The LoB was determined as 0.01 µmol/L, and the LoD was determined as 0.02 µmol/L using SD = 0.005 µmol/L. LoQ was calculated to be 0.04 µmol/L.

e. Analytical specificity:

Potential interferences of endogenous materials were evaluated according to Clinical and Laboratory Standards Institute (CLSI) Guideline EP7-A2: *Interference Testing in Clinical Chemistry*.

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. Pooled human serum supplemented exogenously with endogenous interfering compounds was prepared prior to addition of methotrexate.

Gravimetric addition of methotrexate (USP > 99.9% purity) in dimethyformamide (DMF) and volumetric addition of this stock solution to samples was made to obtain the levels of methotrexate.

Six replicates of each sample and their respective serum controls were tested. Respective runs were co-calibrated with the ARK Methotrexate Calibrator. Tri-level calibration range quality controls of the ARK Methotrexate Control were used to qualify runs. The mean results of methotrexate were calculated and the percentage recoveries relative to the respective serum control mean results were determined.

Interference was defined as follows:

- At methotrexate concentrations near 0.05 µmol/L, the mean concentration of methotrexate in the presence of elevated endogenous substances should fall outside ± 0.02 µmol/L of the mean result for the respective serum control.
- At methotrexate concentrations near mid calibration range (0.50 µmol/L), the mean concentration of methotrexate in the presence of elevated endogenous substances should result in > 10% error in detecting methotrexate in comparison to the mean result for the respective serum control.

Interfering Substance	**Interferent Concentration**
Albumin	12 g/dL
Bilirubin - conjugated	70 mg/dL
Bilirubin - unconjugated	70 mg/dL
Cholesterol	400 mg/dL
Gamma-Globulin	12 g/dL
Hemoglobin	1000 mg/dL
Intralipid®	500 mg/dL
Rheumatoid Factor	1100 IU/mL
Triglycerides	749 mg/dL
Uric Acid	30 mg/dL

Interference with any of these substances was not detected at either level of methotrexate tested.

Methotrexate's metabolites, structurally similar compounds, folate derivatives, and potentially co-administered medications were tested to determine whether these compounds affect the quantitation of methotrexate concentrations using the ARK™ Methotrexate Assay.

7-Hydroxymethotrexate (7-OH-MTX) is the main metabolite in serum following high-dose methotrexate (HDMTX) treatment. The concentration of 7-OH-MTX may exceed that of the parent compound by up to 100-fold in plasma shortly after MTX infusion. Methotrexate is also metabolized by intestinal bacteria to the minor, inactive metabolite 2,4-diamino-N^{10}-methylpteroic acid (DAMPA).

Pooled human serum was supplemented with methotrexate prior to addition of potentially cross reacting metabolites (7-OH-MTX and DAMPA) or other compounds with structural similarity. Preparation of serum pools with 0.05 and 0.50 µmol/L methotrexate were identical to the interference study above. Then the potentially cross reactive compounds at their respectively solvated concentrations were added to serum either in the absence of methotrexate or to serum containing methotrexate. Solvent controls for each potentially cross-reacting interferent sample were also prepared.

The respective concentrations of cross reacting interferents were as recommended by CLSI EP7-A2, or in the case of trimethoprim and triamterene, above therapeutic levels as tested. Six (6) replicates of each sample and their respective solvent serum controls were tested. The mean results of methotrexate were calculated. The percentage cross reactivity was determined for each compound tested.

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

Compound	**Tested (µmol/L)**	**Compound**	**Tested (µmol/L)**
Adriamycin	1000	5-Methyltetra-hydrofolic acid	1000
Cyclophosphamide	1500	Prednisolone	1000
Cytosine	1000	Pyrimethamine	1000
Dihydrofolic Acid	1000	Sulfamethoxazole	1600
DL-6-Methyl-5,6,7,8-Tetrahydropterine	1000	Tetrahydrofolic Acid	1000
Folic Acid	1000	Vinblastine	1000
Folinic Acid (leucovorin)	1000	Vincristine	1000
5-Fluorouracil	3000		
6-Mercaptopurine	1000		

Cross reactivity to 7-Hydroxymethotrexate, the major metabolite

The ARK Methotrexate Assay did not cross react (≤ 0.07%) with the major metabolite 7-OH-MTX.

Cross reactivity to the minor, inactive metabolite 2,4-diamino-N^{10}-methylpteroic acid (DAMPA)

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 from 64.3 to 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.

Compound	Tested (µmol/L)	Methotrexate Absent		Methotrexate Present 0.05 µmol/L		Methotrexate Present 0.50 µmol/L	
		MTX (µmol/L)	Cross Reactivity (%)	MTX (µmol/L)	Cross Reactivity (%)	MTX (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

f. Assay cut-off:

Not applicable.

2. Comparison studies:

a. Method comparison with predicate device:

Method comparison studies were performed using Clinical and Laboratory Standards Institute (CLSI) Protocol EP9-A2 *Method Comparison and Bias Using Patient Samples*. Method comparisons were performed to evaluate the agreement between the ARK Methotrexate Assay and fluorescence polarization immunoassay (FPIA), predicate device Abbott TDx/TDxFLx Methotrexate II assay, k932615.

102 leftover specimens within the claimed measuring range were used for method comparison of the ARK Methotrexate Assay and the FPIA predicate device (TDx Assay). Both serum and plasma specimens were included in the study. Singlicate measurements of all specimens were used for method comparison.

Ten specimens were created by pooling or supplementation with methotrexate (USP) to contain methotrexate concentrations useful for the method comparison.

Summary of the method comparison data:

Parameter	Range 0.04 to 1.19 µmol/L
Number of Samples	102
Slope	1.00 (1.00 – 1.02)
y-intercept	0.01 (0.00 to 0.01)
Correlation Coefficient (r^2)	0.978 (0.968 to 0.985)

b. Matrix comparison:

Anticoagulated plasma and serum were evaluated to demonstrate equivalency of these matrices for measurement of methotrexate with the ARK™ Methotrexate Assay. Matched samples for serum and plasma from eight subjects were evaluated.

In this study, blood was separated into plasma or serum prior to supplementation with methotrexate. Each subject donated in three different anticoagulant tubes and a serum tube to produce a matched set. A methotrexate stock solution was prepared by gravimetric addition of methotrexate (USP > 99% purity) to dimethylformamide (DMF). For each matrix and subject, an aliquot was supplemented by volumetric addition of methotrexate stock solution.

For eight subjects, methotrexate was added to each matrix to give 1.00 µmol/L, and then serial two-fold dilutions were made with its own matrix/subject to give 0.50 and 0.25 µmol/L, respectively.

For four subjects, methotrexate was added to each matrix to give 0.05 µmol/L.

Since the calibration range spans 0.00 to 1.20 µmol/L, equivalency of serum and plasma matrices for measurement of methotrexate with the ARK Methotrexate Assay was evaluated from near the lower limit of quantitation (0.04 µmol/L) to the upper calibration range.

Compared to the mean methotrexate level in the individual's matched serum control, the mean methotrexate concentration in the individual's corresponding plasma was acceptable if the concentration measured was within ±10% of the serum control for concentrations at > 0.1 µmol/L and within ±0.02 µmol/L at methotrexate concentrations ≤ 0.1 µmol/L.

The overall percentage of serum levels of spiked methotrexate above 0.10 µmol/L in plasma ranged 93.7 to 107.4%. At 0.05 µmol/L methotrexate, the difference in measurement of methotrexate between the plasma samples and serum controls was ≤0.01 µmol/L.

3. Clinical studies:

   a. Clinical Sensitivity:

   Not applicable. Not typical for this type of assay.

   b. Clinical specificity:

   Not applicable. Not typical for this type of assay.

   c. Other clinical supportive data (when a. and b. are not applicable):

   The sponsor provided a discussion with balanced and representative literature discussing clinical use of methotrexate measurements.

4. Clinical cut-off:

   See expected values below.

5. Expected values/Reference range:

   The following is included in the package insert:

   Methotrexate serum levels depend on indication for use, dosage, mode of administration, treatment regimen, individual pharmacokinetics, metabolism and other clinical factors.[1,2] While the serum level may typically reach approximately 10 to 100 μmol/L in treatment of breast cancer (for example),[6] concentrations may exceed 1000 μmol/L[7] 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.[8] For treatment of osteosarcoma,[7] 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, 3] Some centers follow ≤ 0.10 μmol/L.[7, 9]

   From prescribing and other information: Laboratory Indicators of Toxicity Following Leucovorin Rescue Schedules with High Dose Methotrexate.[1, 3, 10]

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, [5, 10] for example vancomycin.[11] Other forms of toxicity can occur, including digestive disorders (e.g., nausea, vomiting, abdominal pain), cutaneous–mucous disorders (especially mucositis), hematological abnormalities (e.g., neutropenia and thrombocytopenia), liver function test disturbances, and neurotoxicity.[12-19]

Given the profile of the appearance of the 7-hydroxymethotrexate metabolite,[6, 18] its molar ratio to methotrexate of up to approximately 100-fold,[20] and relative insolubility versus the parent drug,[5, 10] possible nephrotoxicity due to precipitation of the metabolite in renal tubules[20] 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.[5] Elimination of DAMPA may take several days before it no longer interferes with the monitoring of methotrexate by immunoassay.[4]

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N. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

O. Conclusion:

The submitted information in this premarket notification is complete and supports a substantial equivalence decision.