Title: Quantitation of Methotrexate Using the Neogen Corporation ELISA **Kit**

SOP Number : 23107 Revision Number: 02

Supersedes: Revision 01 Effective Date: **AUG 15 2017**

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

Table of Contents

1.0 Purpose

This SOP describes the use of the Neogen Corporation Methotrexate ELISA kit for residual Methotrexate quantitation.

2.0 Scope

This SOP applies to Process Analytics/Quality Control (PNQC) personnelwho will perform the Methotrexate ELISA.

3.0 Authority and Responsibility

3.1 The Director, PN QC has the authority to define this procedure.

3.2 PN QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BOA).

3.3 PA/QC personnel are responsible for performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this procedure.

4.0 Supplies and Reagents

4.1 Methotrexate ELISA kit, BDP PN 30654, Neogen Corporation, or BDP approved equivalent.

4.2 PBS Buffer, BDP PN 30007, or BDP approved equivalent.

4.3 N,N-Dimethylformamide (DMF), HPLC Grade (≥ 99.9%), BDP PN 30694, or BDP approved equivalent.

4.4 Methotrexate, USP Grade, BDP PN 30658, or BDP approved equivalent.

4.5 Calibrated multi-channel pipettor (30-300 µL).

4.6 Calibrated pipettors (10-100 µL, 20-200 µL, and 100-1000 µL).

4.7 Aerosol Barrier Pipet tips, 10-100 µL, BDP PN 21484, 2-200 µL, BDP PN 20673 and 1-1000 µL, BDP PN 20769, VWR, or BDP approved equivalent.

4.8 Microtiter plate shaker, VWR Catalog Number 57019-600, or BDP approved equivalent.

4.9 Ziploc™ Bag, BDP PN 20339, or BDP approved equivalent.

4.10 Reagent reservoirs, BDP PN 20481, or BDP approved equivalent.

4.11 Microcentrifuge tubes 0.5 mL, BDP PN 20674, or BDP approved equivalent.

4.12 Ultrapure water, or BDP approved equivalent.

4.13 250 mL Graduated Cylinder for wash buffer and 10 mL Graduated Cylinder for stop solution.

4.14 Squirt/wash bottle with the tip cut off.

4.15 Kaydry EX-L Low-lint absorbent paper, BDP PN 21493, or BDP approved equivalent.

4.16 Hydrochloric Acid, N.F., BDP PN 10055, or BDP approved equivalent.

4.17 15mL Conical tubes, BDP PN 20006, or BDP approved equivalent.

4.18 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160 or BDP approved equivalent.

5.0 General Comments

5.1 Pipetting accuracy and reproducibility are critical for the success of this assay.

5.2 Use a new pipette tip for each pipetting procedure (between dilutions).

5.3 Avoid contamination of the workspace when handling Standards, ejecting pipette tips, etc. Pipette tips possessing an aerosol barrier must be used.

5.4 Good organization and attention to detail are essential to avoid confusion of sample identities and data.

5.5 Before pipetting a reagent, rinse the tip three times with that reagent. Once the tip is properly rinsed, it is ready to dispense the reagent into the well or tubes.

5.6 When pipetting into the wells, DO NOT allow the pipette tip to touch the inside of the well or any reagent already inside the well. This may result in cross contamination.

6.0 Preparation

NOTE: The reagents are stored at 2-8°C. Bring all reagents to ambient temperature (up to one hour). All standards, controls, and samples will be assayed in duplicate. The total volume of the samples must take this into account.

6.1 Sample Preparation: Linear Range of Assay = 0 – 5 ng/mL

6.1.1 Prepare the test article immediately before use, undiluted and at 1:5 and 1:50 dilutions using PBS Buffer as a diluent (see 4.2). If the absorbance results indicate that the value for the 1:50 dilution is greater than 5 ng/mL, then repeat the ELISA assay with appropriately diluted samples. (The original data is attached to the QC test request.) An example for making dilutions is the following.

6.1.1.1 For duplicate measurements of spiked and unspiked diluted test article (a total of four 20 µL aliquots), add 50 µL test article to 200 µL diluent in a microcentrifuge tube to give a 1:5 dilution.

6.1.1.2 A 1:50 dilution can be made up in microcentrifuge tubes by adding 50 µL of the 1:5 diluted test article to 450 µL diluent.

6.1.2 Prepare a spiked sample of the test article. Spiked samples can be prepared by the following.

6.1.2.1 A spiked sample can be prepared in microcentrifuge tubes prior to loading on the plate. For a spiked concentration of 1 ng/mL methotrexate (MTX), perform a 5x dilution using the 5 ng/mL prepared standard. For example, add 20 µL of the 5 ng/mL standard to 80 µL of the test article prepared in step 6.1.1. Vortex well.

6.2 Standard Preparation

6.2.1 Prepare standards at 0, 0.1, 0.2, 0.8 and 5.0 ng/mL in PBS.

6.2.2 Use sample buffer as the 0 ng/mL standard.

6.2.3 To prepare the other standards, perform the following.

6.2.3.1 Prepare at least 1 mL of a 1.0 mg/mL stock solution of MTX in DMF. Store the stock solution in a light-proof or amber container chemically compatible with DMF (i.e., glass with non-corrosive cap) at -10 to -30°C. Stock solution is stable for 1 month. Refer to ***SOP 22702 - Solutions Used in Process Analytics***.

6.2.4 Dilute the stock solution 1:100 with PBS to make a 10 µg/mL solution. For example, add 100 µL stock solution to 9.90 mL PBS. Fifteen (15) milliliter polypropylene tubes can be used for making standards.

6.2.5 Dilute the 10 µg/mL solution 1:100 to make a 100 ng/mL solution.

6.2.6 Dilute the 100 ng/mL solution 1:20 to make a **5 ng/ml standard**. For example, add 500 µL of the solution to 9.50 mL PBS.

6.2.7 Dilute the 100 ng/mL solution 1:100 to make a **1 ng/mL standard**.

6.2.8 Dilute the 1 ng/mL standard 1:10 to make a **0.1 ng/mL standard**.

6.2.9 Dilute the 1 ng/mL standard 1:1.25 to make a **0.8 ng/mL standard**. For example, add 1.0 mL of PBS to 4.0 mL of the standard.

6.2.10 Dilute the 1 ng/mL standard 1:5 to make a **0.2 ng/mL standard**. For example, add 4.0 mL of PBS to 1.0 mL of the standard.

NOTE: **Standards should be stored at 2-8°C and protected from light. Prepared standards are stable for 1 month after preparation**. Log and label the prepared standards per ***SOP 22702 - Solutions Used in Process Analytics***.

6.3 Positive and Negative Control Preparation

6.3.1 The Positive control is provided in the kit by Neogen.

6.3.2 The Negative control is provided in the kit by Neogen.

6.4 Wash Buffer Preparation

6.4.1 Dilute the concentrated wash buffer 10-fold with Ultrapure water. Empty the entire contents of the wash buffer bottle (20 mL) into a 250 mL graduated cylinder. Add 180 mL of Ultrapure water and mix thoroughly. Log and label the wash buffer in accordance with ***SOP 22702 - Solutions Used in Process Analytics***.

6.5 Enzyme Conjugate Solution Preparation

6.5.1 Prepare the enzyme conjugate solution by diluting 180X enzyme conjugate stock 1 to 180 in the EIA Buffer provided in the kit. For example, for four eight well strips; add 50 µL of the 180X enzyme conjugate stock to 8950 µL of EIA Buffer. Mix the solution by inversion. Do not vortex.

6.6 Stop Solution Preparation

6.6.1 The Neogen MTX ELISA kit includes a Red Stop Solution; however, a 1N HCL solution may be used as an alternative to stop the color reaction when absorbance at 450 nm is to be measured. To prepare a 1N HCL solution, pour 9 mL of Ultrapure water into a 10 mL graduated cylinder. Add 1 mL/10 N Hydrochloric Acid, N.F (4.16) to the graduated cylinder to make a total volume of 10 mL. Prepare the 1N HCL stop solution fresh immediately before use.

7.0 Procedure

The procedure is taken from the Neogen Corporation Methotrexate ELISA Kit Insert (Attachment 6).

7.1 Prepare an ELISA Worklist (Attachment 1) by labeling the wells where the samples and standards will be placed. Count the microtiter strips needed. Remove the required number of microtiter strips from the kit and place them in the provided frame.

7.2 Pipette 20 µL of standards, controls and samples into wells indicated on the ELISA Worklist (Attachment 1). All standards, controls and samples must be assayed in at least duplicate.

NOTE: Do not dilute the provided positive and negative controls.

7.3 Pipette 180 µL of the diluted **drug-enzyme conjugate** from step 6.5 into each well using a multichannel pipettor (4.5).

7.3.1 Pour the 15 mL conical tube of diluted drug enzyme conjugate into a new reagent reservoir (4.10).

7.3.2 Use a multi-pipettor to fill rows of wells. Use fresh pipette tips with each row that is filled.

7.4 Carefully place the plate into a Ziploc™ bag (4.9).

7.5 Transfer to the microtiter plate shaker (4.8) and gently shake the plate for 30-45 seconds at a setting of "2" (approximately 180 rpm). Incubate for 45 minutes ± 2 minutes at room temperature.

7.6 Using a manual microtiter plate procedure, wash the plate with at least 300 µL per well of diluted wash solution from step 6.4 and remove. Wash a total of 3 times.

7.6.1 Remove the liquid from the plate as follows: Grab the plate from the bottom with the thumb in the middle of one side and the fingers on the other side. With the thumb and fingers slightly overlapping the tabs of the strips, hold the plate over the sink, turn the plate upside down while accelerating the arm and wrist downward. Abruptly stop the arm movement, allowing the generated inertia to carry the liquid from the strips into the sink. Repeat the removal motion a second time.

7.6.2 Blot and strike the plate as follows: Immediately blot the plate upside-down onto the low-lint absorbent paper (4.15). Move the plate to an unused section of the blotting paper and allow it to drain upside down for up to 30 seconds. Strike the plate forcefully 4 times over the unused areas of the paper. Do not be afraid to strike vigorously. Anything short of breaking the strip holder or strips is not too hard. Wash the plate. Use the squirt bottle (4.14) with the narrow portion of the tip cut off to give the largest possible orifice so that the flow will be generous and gentle. Fill all wells with the diluted wash solution provided with the kit (See 6.4). Remove the wash solution and blot and strike the plate as described in steps 7.6.1 and 7.6.2. Repeat the washing, blotting, and striking procedure 2 more times for a total of 3 washes. After the last wash, let the plate rest upside down for at least 60 seconds to drain. Strike the plate again 4-6 times rotating the plate 180° between each strike. This rotation ensures that the ends of the plate receive on average the same energy and impact.

7.6.3 Wipe the bottom outside of all wells with clean absorbent paper to remove any liquid from the washing. If the washing technique has been performed correctly, the center of each well should have a small film of liquid (< 1 microliter). If the film is not uniform in terms of the area between the wells, or a significant amount of liquid remains in the circular edge of the wells, then strike the plate more forcefully and repeat step 7.6.3. Wells are now ready to have the K-Blue Substrate added to them.

7.7 Pipette 150 µL of the **K-Blue Substrate** (that is provided in the Neogen Kit) as in steps 7.3.1 - 7.3.2.

7.8 Place the microtiter plate in a Ziploc™ bag (4.9) and transfer to the microtiter plate shaker (4.8). Gently shake plate for 30-45 seconds at a setting of "2" (180 rpm).

7.9 Incubate at room temperature for 30 minutes ± 2 minutes. Gently shake the plate periodically to ensure uniform color development.

7.10 Remove the microtiter plate from the Ziploc™ bag and stop the reaction by one of two ways.

7.11 The reaction can be stopped by adding 50 µL of Neogen's red stop solution to each well as in step 7.3.1-7.3.2. Read the plate at an absorbance of 650nm. Refer to ***SOP 22100 - Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser***. Print out the Ascent Methotrexate results (Attachment 2).

7.12 As an alternative to Neogen's red stop solution, 1N HCL can be used to stop the reaction. Add 50 µL of the 1N HCL stop solution prepared in step 6.6 to each well as in steps 7.3.1-7.3.2. Read the plate at an absorbance of 450nm. Refer to ***SOP 22100 - Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser***. Print out the Ascent Methotrexate results (Attachment 2).

8.0 Data Analysis and Acceptance Criteria

8.1 **Methotrexate ELISA Analysis Worksheet** (Attachment 6). After the Ascent Methotrexate Results (Attachment 2) have been printed from the ELISA run, the results will need to be manually entered into the Methotrexate ELISA Analysis Worksheet (Attachment 4).

NOTE: That the Ascent software is not used for curve-fitting. It is used for the collection of raw absorbance data.

8.1.1 Go to windows "Start." Select programs and click on "Microsoft Excel."

8.1.2 Click on "File" and "Open". Select "[redacted]"

8.1.3 Select the Methotrexate ELISA Analysis Worksheet.

8.1.4 Enter each sample name in the left-most columns below the "POS" entry, replacing "sample" with the appropriate sample name.

8.1.5 For each standard, control, and sample, enter the observed OD readings from the Ascent methotrexate ELISA Results (Attachment 2). The worksheet calculates relevant parameters.

8.1.6 The column "Backfit" is the MTX concentration based on the standard curve.

8.1.7 The R value should be ≥ 0.99 for an acceptable assay.

8.2 **Excel Summary Worksheet (Attachment 3)**. The Summary Worksheet is also in Microsoft Excel format, and the requested information is transcribed from the Ascent Methotrexate Results (Attachment 2) and calculated by the Methotrexate ELISA Analysis Worksheet (Attachment 4). The acceptable spike recoveries and %CV's are calculated in this Excel Summary Worksheet.

8.2.1 Open Microsoft Excel.

8.2.2 Select "S[redacted]"

8.2.3 Select the Methotrexate Template.

8.2.4 Fill in the top portion of the summary sheet with all appropriate information, including QC number, Analyst, Date, Sample ID, Lot Number, Kit Lot Number, and Expiration Date.

8.2.5 Fill in the "Methotrexate Concentration" section with appropriate measured concentration for replicates 1 and 2 at each dilution from the corresponding Backfit column of the Methotrexate ELISA Analysis Worksheet (Attachment 4). The "Corrected Concentration," "Average," and "% CV" will automatically be calculated by the Summary Worksheet. The "%CV" should be less than 50%.

8.2.6 Fill in the "Expected" and "Found" Positive Control and the "% CV" from the Ascent ELISA Results. The positive control will be the positive control provided in the kit. The "%CV" should be less than 50%. The "Found" positive control should be 100 ± 50% of what is "Expected."

8.2.7 Fill in the “Found” Negative Control from the Ascent ELISA Results. The negative control is the negative control provided in the kit. The number should be close to 0 or less than the minimum (<min).

8.2.8 Fill in the “Spiked Test Article Concentration“ section with appropriate measured concentration for replicates 1 and 2 at each dilution from the corresponding Backfit column of the Methotrexate ELISA Analysis Worksheet (Attachment 4). These numbers are not corrected for dilutions. The “Average” and “% CV” will automatically be calculated by the Summary Worksheet. The % CV should be less than 50%.

8.2.9 The “Percent Spike Recovery of Test Article” will be automatically calculated. The %CV should be less than 50% and the average recovery should be 100 ± 50%.

8.2.10 If %CV and recoveries for the assay controls do not meet criteria, then the assay is invalid.

9.0 Documentation

9.1 Print out the Excel Summary Worksheet (Attachment 3). Sign and date the worksheet and attach it to the QC Test Request Form with a copy of the Methotrexate ELISA Analysis Worksheet (Attachment 4) and a copy of Ascent Methotrexate Results (Attachment 2).

9.2 The lowest dilution with the acceptable criteria listed above can be recorded on the QC Test Request Form.

NOTE: If the results are less than the lowest standard, it is recorded as < 0.1 ng/mL.

9.3 Record all solution preparation on Form 23107-01, Methotrexate ELISA Preparation (Attachment 5) and include with the QC Test Request Form.

10.0 References and Related Documents

10.1 **SOP 22100** *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser.*

10.2 **SOP 22702** *Solutions Used in Process Analytics*

10.3 Neogen Corporation Methotrexate ELISA Kit Instructions (Attachment 6).

11.0 Attachments

11.1 **Attachment 1** Sample ELISA Worklist

11.2 **Attachment 2** Sample Ascent Methotrexate ELISA Result Name

11.3 **Attachment 3** Sample Excel Summary Worksheet

11.4 **Attachment 4** Sample Methotrexate ELISA Analysis Worksheet

11.5 **Attachment 5** Form 23107-01, Methotrexate ELISA Preparation

11.6 **Attachment 6** Neogen Corporation Methotrexate ELISA Kit Insert

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 1

Sample ELISA Worklist

H	G	F	E	D	C	B	A	↑
Positive Control	Negative Control	5 ng/ml Standard	0.8 ng/ml Standard	0.2 ng/ml Standard	0.1 ng/ml Standard	0 ng/mL Standard		1
Positive Control	Negative Control	5 ng/ml Standard	0.8 ng/ml Standard	0.2 ng/ml Standard	0.1 ng/ml Standard	0 ng/mL Standard		2
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL Sample diluent	NEGATIVE CONTROL Sample diluent	3
SPIKED SAMPLE 1:50 dilution	SAMPLE 1:50 dilution	SPIKED SAMPLE 1:5 dilution	SAMPLE 1:5 dilution	SPIKED SAMPLE Undiluted	SAMPLE Undiluted	SPIKED NEGATIVE CONTROL Sample diluent	NEGATIVE CONTROL Sample diluent	4
								5
								6
								7
								8
								9
								10
								11
								12

Assay:

Sample ID:

Date:

Analyst:

Attachment 2

Sample Ascent Methotrexate **ELISA** Result

AScen)Software Curve Fill 7124/ffi 5:22 PIA

Session: [redacted]
Instrument: EMS Reader MF V2.S-OO
User name: K.Leib
Started at: 6112/065:25:36PM
Actual temperature: Amb.temp.

Layout map for oolibrotors Sheet; Measure1, A"3ay: A"3ay1 and for samples Sheet: Measure1Assay Assay1

	1	2	3	4	5	6	7	8	9	10	11
A	Blank	Blank		na	na		_009	_009		_016	_016
B							8009	s009		.016	3016
C	Cal1	Cal1		sna	sna		_009	_009		_016	_016
D	ca12	ca12					s009	$009		s016	s016
E	Cal3	Cal3		_009	_009		_016	_016			
F	Cal4	Cal4		s009	s009		s016	$()16			
G	nc	nc		_009	_009		_016	_016			
H	PC	pc		s009	s009		s016	s016			

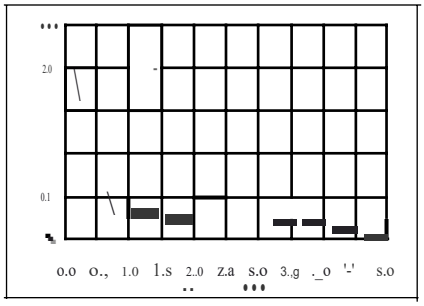
Source data lor calibrators Sheet: Measure1, Assay: Assay1 and for samples Sheet Measure1, Assay: Assay1

	1	2	3	4	6	6	I	8	9	10	11
A	2396	2.155		2.375.	2.312		0.150	0.145		0.155	0.528
B							0.176	0.184		0.156	0.160
C	2.150	2.177		1.070	1.052		0.262	0.268		0.254	0.271
0	1.796	1.817					0.215	0.225		0.231	0.244
E	0.758	0.701		0.078	0.072		0.064	0.065			
F	0.165	0.164		0.063	0.065		0.062	0.063			
G	3.081	2.949		0.077	0.072		0.069	0.070			
H	0.164	0.142		0.066	0.066		0.066	0.066			

Sheet: Measure1 AS$ay: AS$ay1

t:!illlil Mm		a,
Cal1	2.150	
	2.177	
	2.164	0.10
cal2	1.7£6	
	1.817	
	1.807	0.20
Cal3	0.758	
	0.701	
	0.730	0.80
Cal4	0.165	
	0.164	
	0.165	5.00

	Cone.	Meas.	CalcConc.	Residual
Cal!	0.1	2.164 0.1		-5.5511e-17
Cal2	0.2	1.807 02		-5.5511t-17
Cal3	08	0.730 0.8		-1.1102t-16
CaM	5	0.165 5		0



Status :
Fit type: Point to point
Meas. transfonnation: Linear
Cone. Iransfonnation Linear

Calculated ccnoontrations Sheet Measure1, Assay: Assay1

	1	2	3	4	6	6	7	8	9	10	11
A	0.035	0.102		0.041	0.058		255.389	257.248		253.531	114.894

This procedure is made available throughfederal funds from the National Cancer Institute,NIH, under contract [redacted]

Attachment 3

Sample EXCEL Summary Worksheet

Summary of Methotrexate ELISA Results

QC Number:	QC-00000	**Analyst:**	K.Leib	**Date:**	3/19/2006
Sample ID:		Project Name		**Lot #:**	LXX0000
Kit Lot#:	28082	**Expiration Date:**	7/31/2003		

I. Methotrexate Concentration (ng/ml)
Acceptable Criteria: %CV <50%

Dilution Factor	Measured Concentration		Corrected Concentration		Average	%CV
	Replicate 1	Replicate 2	Replicate 1	Replicate 2		
1.00	14.62	14.48	14.62	14.48	**14.55**	0.68
5.00	4.63	5.43	23.15	27.15	**25.15**	11.25
50.00	2.31	1.42	115.50	71.00	**93.25**	33.74

Final Result: 25.15ng/ml
Corrected Concentration = Measured Concentration x dilution factor

*n/c = not calculated

Calculated Concentration of " Methotrexate Spiking Solution" (ng/mL): 5.0

Correlation Coefficient of Standard Curve: point to point

Acceptable Criteria: %Recovery (100% ± 50) and %CV <50%

	Expected	Found	%Recovery	%CV
Avg. Positive Control (ng/ml):	5.00	5.24	104.80%	6.23
Avg. Negative Control (ng/ml):	0.00	<min		
Avg. Spike Control (ng/ml): (spiked dilution buffer)	1.00 [0.004ml x (conc. of "Methotrexate Spiking Sol'n." (in ng/ml))] / 0.02 ml	1.22	122.00%	6.53

Spiked Test Article Concentrations (ng/ml; not corrected for dilution):
Acceptable Criteria: %CV <50%

Dilution	Replicate 1	Replicate 2	Average	%CV
Neat	12.63	14.20	13.42	8.28
1:5	5.30	6.31	5.81	12.30
1:50	3.45	3.64	3.55	3.79

Attachment 3 (Continued)

Percent Spike Recovery of Test Article:
Acceptable Criteria: Average Recovery (100% ± 50) and %CV <50%

Dilution	Replicate 1	Replicate 2	Average	%CV
Neat	99.48	112.84	106.16	8.90
1:5	112.67	118.08	115.37	3.31
1:50	121.14	170.41	145.77	23.90

Calculated by: Spiked test article concentration, ng/ml/[[(0.016 ml) * (measured concentration, ng/ml) + (0.004ml)* (Concentration of "Methotrexate Spiking Solution", ng/ml)] / 0.02 ml]

Analyst/Date: _____

Reviewed By/Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 4

Methotrexate ELISA Analysis Worksheet

KIT: DATE: TECH:

STD (ng/ml)	OD1	OD2	OD AVG	STDEV	RSD	%B/Bo	Backfit	%ERR	LogCONC.	LOGIT
0	0.846	0.730	0.788	0.082	10.4	100.0				
0.1	0.732	0.572	0.652	0.113	17.4	82.7	0.09	14.70	-1.00	1.57
0.2	0.583	0.448	0.516	0.095	18.5	65.4	0.27	35.06	-0.70	0.64
0.8	0.356	0.261	0.309	0.067	21.8	39.1	1.03	28.56	-0.10	-0.44
5	0.153	0.125	0.139	0.020	14.2	17.6	4.02	19.57	0.70	-1.54
NEG	0.855	0.855	0.855	0.000	0.0	108.5	#NUM!	#NUM!	#VALUE!	#NUM!
POS	0.022	0.050	0.036	0.020	55.0	4.6	25.76	#VALUE!	#VALUE!	-3.04
sample	0.617	0.602	0.610	0.011	1.7	77.3	0.13	#VALUE!	#VALUE!	1.23
sample	0.711	0.736	0.724	0.018	2.4	91.8	0.03	#VALUE!	#VALUE!	2.42
sample	0.350	0.366	0.358	0.011	3.2	45.4	0.75	#VALUE!	#VALUE!	-0.18
sample	0.692	0.723	0.708	0.022	3.1	89.8	0.04	#VALUE!	#VALUE!	2.17
sample	0.655	0.638	0.647	0.012	1.9	82.0	0.09	#VALUE!	#VALUE!	1.52
sample	0.100	0.100	0.100	0.000	0.0	12.7	6.50	#VALUE!	#VALUE!	-1.93
sample	0.700	0.700	0.700	0.000	0.0	88.8	0.05	#VALUE!	#VALUE!	2.07
sample	0.550	0.550	0.550	0.000	0.0	69.8	0.21	#VALUE!	#VALUE!	0.84
sample	0.440	0.440	0.440	0.000	0.0	55.8	0.45	#VALUE!	#VALUE!	0.23
sample	0.321	0.321	0.321	0.000	0.0	40.7	0.95	#VALUE!	#VALUE!	-0.37
sample	0.120	0.120	0.120	0.000	0.0	15.2	5.00	#VALUE!	#VALUE!	-1.72
sample	0.110	0.110	0.110	0.000	0.0	14.0	5.67	#VALUE!	#VALUE!	-1.82
sample	0.550	0.550	0.550	0.000	0.0	69.8	0.21	#VALUE!	#VALUE!	0.84
sample	0.480	0.480	0.480	0.000	0.0	60.9	0.34	#VALUE!	#VALUE!	0.44
	I-50 =	0.60	ng/ml			Slope:	-1.8574			
	R =	0.9906				Inter:	-0.4184			

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 5
Form 23107-01, Methotrexate ELISA Preparation

FNLCR, BDP
Form No.: 23107-01
SOP No.: 23107
Revision 02: AUG 15 2017

METHOTREXATE ELISA PREPARATION

QC Number: _____ **Operator**:_____ **Date**:_____

Plate Reader MEF Number:_____ Calibration Due Date:_____ Wavelength Used: ___ nm

Diluent: Name: _____ Lot#_____ Exp. Date: _____

Test sample Preparation:

Test Sample #1
Name:_____ Appearance:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution :_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution :_____
Volume of diluent:_____µL
Volume of Initial Diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted test sample _____µL

Test sample #2
Name:_____ Appearance:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution :_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution :_____
Volume of diluent:_____µL
Volume of Initial Diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted test sample _____µL

Test sample #3
Name:_____ Appearance:_____
Lot #_____
Protein Concentration: _____mg/mL

Initial dilution :_____
Volume of diluent:_____ µL
Volume of test sample:_____ µL

Second dilution :_____
Volume of diluent:_____µL
Volume of Initial Diluted test sample_____µL

Third Dilution _____
Volume of Diluent: _____µL
Volume of Second Diluted test sample _____µL

Standard Curve Preparation

Methotrexate:	BDP Lot Number _____	Expiration _____
DMF:	BDP Lot Number _____	Expiration _____
PBS:	BDP Lot Number _____	Expiration _____
Balance:	MEF Number _____	Calibration Due Date _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 5 (Continued)

FNLCR, BDP
Form No.: 23107-01
SOP No.: 23107
Revision 02: AUG 15 2017

Preparation of MTX Stock Solution

Weigh 1-10 mg MTX. Record weight to the nearest 0.01 mg. Add a volume of DMF (in mL) equal to the weight of MTX (in mg). Store in a container chemically compatible with MTX protected from light at -10 to -30^{O}C.
Weight (mg) of MTX: _____ mg
Volume (mL) of DMF added: _____ mg MTX = _____ mL DMF added
Final Concentration of MTX Stock Solution: _____ mg/mL BDP Lot Number:_____
Expiration Date: _____

Preparation of MTX Standards

After preparation, standards should be stored at 2 to 8^{O}C protected from light. They expire in 1 month.

1. Make a 10 µg/mL MTX solution
   a. Add 100 µl of MTX Stock Solution to 9.90 mL of PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Stock Solution: _____ Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
2. Make a 100 ng/mL solution
   a. Add 100 µl of 10 µg/mL MTX solution (#1, above) to 9.90 mL of PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
3. Make a **5 ng/mL standard**
   a. Add 500 µL of the 100 ng/mL solution (#2, above) to 9.50 mL PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
4. Make a **1 ng/mL standard**
   a. Add 100 µL of the 100 ng/mL solution (#2, above) to 9.90 mL PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
5. Make an **0.1 ng/mL standard**
   a. Add 100 µL of the 1 ng/mL solution (#4, above) to 900 µL PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
6. Make an **0.8 ng/mL standard**
   a. Add 4.0 mL of the 1 ng/mL solution (#4, above) to 1.0mL PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____
7. Make an **0.2 ng/mL standard**
   a. Add 1.0 mL of the 1 ng/mL solution (#4, above) to 4.0mL PBS in a 15 mL polypropylene centrifuge tube. Vortex briefly.
      i. Volume of MTX Solution: _____Volume of PBS _____
      ii. Final Concentration of solution: _____ Expiration Date: _____

Template Procedure

Enzyme Conjugate Solution

Volume of EIA Buffer:_____
Volume of 180X Enzyme Conjugate Stock:_____ µL
Volume of Enzyme Conjugate Solution added to wells:_____ µL
Incubation Time:_____Washing times:_____

K-Blue Substrate

Lot# _____Exp. Date:_____
Volume of K-Blue Substrate added to wells:_____ µL
Incubation Time:_____

Stop Solution

Type of Stop Solution used:_____
Expiration Date:_____
Volume of stop added to wells:_____

Data Storage

File Name:_____
Performed by/Date: _____
Reviewed by/Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 6

Neogen Corporation Methotrexate ELISA Kit Insert

Neogen Corporation
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800/477-8201 USA/Canada | 859/254-1221
Fax: 859/255-5532 | E-mail: inform@neogen.com | Web: www.neogen.com/Toxicology

METHOTREXATE

ELISA KIT INSTRUCTIONS PRODUCT #107519 & 107516
FORENSIC USE ONLY

INTENDED USE: For the determination of trace quantities of Methotrexate and/or other metabolites in human urine, blood, oral fluid.

DESCRIPTION

Neogen Corporation's Methotrexate ELISA (Enzyme-Linked ImmunoSorbent Assay) test kit is a qualitative one-step kit designed for use as a screening device for the detection of drugs and/or their metabolites. The kit was designed for screening purposes and is intended for forensic use only. It is recommended that all suspect samples be confirmed by a quantitative method such as gas chromatography/mass spectrometry (GC/MS).

ASSAY PRINCIPLES

Neogen Corporation's test kit operates on the basis of competition between the drug or its metabolite in the sample and the drug-enzyme conjugate for a limited number of antibody binding sites. First, the sample or control is added to the microplate. Next, the diluted drug-enzyme conjugate is added and the mixture is incubated at room temperature. During this incubation, the drug in the sample or the drug-enzyme conjugate binds to antibody immobilized in the microplate wells. After incubation, the plate is washed 3 times to remove any unbound sample or drug-enzyme conjugate. The presence of bound drug-enzyme conjugate is recognized by the addition of K-Blue® Substrate (TMB). After a 30 minute substrate incubation, the reaction is halted with the addition of Red Stop Solution. The test can be read visually or with a microplate reader equipped with a 650 nm filter. The extent of color development is inversely proportional to the amount of drug in the sample or control. In other words, the absence of the drug in the sample will result in a dark blue/purple color, whereas the presence of the drug will result in light pink to no color development.

STORAGE AND STABILITY

This kit can be used until the expiration date on the label when stored refrigerated at 4°C. Store controls frozen if not used within 10 days. Note: Some kits require controls to be stored frozen immediately upon receipt. Reference kit label for details.

MATERIALS PROVIDED – SINGLE KIT (96 WELL)

1. **EIA Buffer:** 40 mL (ready-to-use). Phosphate buffered saline solution with bovine serum and a preservative. Provided for dilution of drug-enzyme conjugate concentrate and samples.
2. **Wash Buffer Concentrate (10X)**: 20 mL. Phosphate buffered saline solution with a surfactant. Dilute 10 fold with deionized or ultrapure water before use. Diluted wash buffer is used to wash all unbound conjugate and samples from the plate after the conjugate incubation.
3. **K-Blue Substrate**: 20 mL (ready-to-use). Stabilized 3, 3′, 5, 5′ Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after washing. Light sensitive.
4. **Drug-Enzyme Conjugate**: 200 µL. Drug-horseradish peroxidase concentrate. Dilute 180X before use.
5. **Antibody Coated Plate:** A 96 well Costar plate, in strips of 8 break-away wells, coated with anti-drug antiserum. The plate is ready for use as is. Do not wash.
6. **Red Stop Solution**: 20 mL (ready-to-use). Non-acidic reagent used to stop the enzyme reaction.
7. **Qualitative QC Positive Control**: 750 µL provided (synthetic human urine). Do not dilute.
8. **Qualitative QC Negative Control**: 750 µL provided (synthetic human urine). Do not dilute.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 6 (Continued)

Neogen Corporation Methotrexate ELISA Kit Insert

MATERIALS PROVIDED – BULK KIT (480 WELL)

1. **EIA Buffer:** 200 mL (ready-to-use). Phosphate buffered saline solution with bovine serum and a preservative. Provided for dilution of samples.
2. **Wash Buffer Concentrate (10X):** 100 mL. Phosphate buffered saline solution with a surfactant. Dilute 10 fold with deionized or ultrapure water before use. Diluted wash buffer is used to wash all unbound conjugate and samples from the plate after the conjugate incubation.
3. **K-Blue Substrate:** 100 mL (ready-to-use). Stabilized 3, 3′, 5, 5′ Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after washing. Light sensitive.
4. **Drug-Enzyme Conjugate:** 1 mL. Drug-horseradish peroxidase concentrate. Dilute 180X before use.
5. **Antibody Coated Plate:** 5 X 96 well Costar plate, in strips of 8 break-away wells, coated with anti-drug antiserum. The plate is ready for use as is. Do not wash.

OPTIONAL TEST MATERIALS

1. **Qualitative QC Positive Control:** 5 X 750 µL (synthetic human urine). 1-5 vials available upon request. Do not dilute.
2. **Qualitative QC Negative Control:** 5 X 750 µL (synthetic human urine). 1-5 vials available upon request. Do not dilute.
3. **Red Stop Solution:** 100 mL (ready-to-use). Non acidic reagent used to stop the enzyme reaction. Available upon request. Product No. 301473.

MATERIALS NEEDED BUT NOT PROVIDED

1. Deionized water.
2. Precision pipettes that range from 10 µL - 1000 µL and disposable tips.
3. Graduated cylinder to dilute and mix wash buffer.
4. Plate cover or plastic film to cover plate during incubation.
5. Clean glassware (i.e. test tubes) to dilute drug-enzyme conjugate.
6. Microplate reader with 650 nm filter if Red Stop is used, or a 450 nm filter if 1N HCl is used to stop the reaction. Note: It is not necessary to stop the reaction if reading immediately. Unstopped reactions should be read with a 650 nm filter.

OPTIONAL MATERIALS

1. 1N HCl if Red Stop Solution is not used.
2. Microplate shaker.

PRECAUTIONS AND NOTES

1. **DO NOT** use kits or components beyond expiration date.
2. **DO NOT** mix conjugates and plates from different kit lots.
3. **DO NOT** pipette reagents by mouth.
4. Pour K-Blue Substrate out of the bottle into a clean reservoir. To prevent contamination of the substrate, **DO NOT** pipette out of the bottle.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. Keep plate covered except when adding reagents, washing or reading.
7. Kit components should be refrigerated at all times when not in use.
8. Keep the controls frozen if storing longer than 10 days. Avoid repeated freeze-thaw cycles. Note: Some kits require controls to be stored frozen immediately upon receipt. Reference kit label for details.
9. Use aseptic technique when opening and removing reagents from vials and bottles.
10. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
11. Do not substitute DI water for the wash step of this protocol. Use only Neogen's wash buffer.
12. Do not use Sodium Azide with samples, standards and/or calibrators.
13. Do not reuse wells, they are for one use only.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 6

Neogen Corporation Methotrexate ELISA Kit Insert

PROCEDURAL NOTES

1. Desiccant bag must remain in foil pouch with unused strips. Keep ziplock pouch sealed when not in use to maintain a dry environment.
2. Use clean pipette tips for the buffer, drug-enzyme conjugate, controls and samples.
3. Before pipetting a reagent, rinse the pipette tip three times with that reagent.
4. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well or any of the reagent already inside the well. This may result in cross contamination.
5. Controls and samples should be assayed in duplicate.
6. The drug-enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
7. Before opening the drug-enzyme conjugate vial, tap the vial in an upright position to remove any liquid in the cap.
8. Before substrate addition, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
9. Gently mix specimens and reagents before use. Avoid vigorous agitation.

SAMPLE TREATMENT

Recommended minimum sample dilutions are listed below. These dilutions may change based on your laboratory's determination. All sample dilutions should be made in Neogen's EIA Buffer.

a. **Urine:** A dilution with EIA Buffer may be necessary to reduce natural background as well as bring desired cutoff concentration within the assay range. Please contact your Neogen Representative for assistance.
b. **Whole blood:** A dilution of 1:5 (i.e. 1 part sample to 4 parts provided EIA Buffer) is recommended. Please contact your Neogen Representative for assistance.
c. **Other Forensic sample types:** Please contact your Neogen Representative for assistance.

TEST PROCEDURES

The following test procedures can be run manually or on an automated instrument. Please contact your Neogen representative for assistance with protocols for automated instruments.

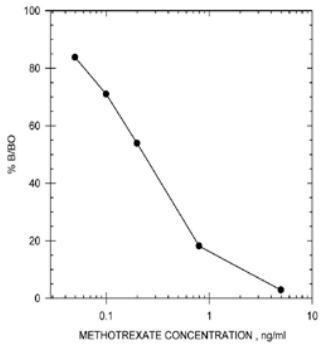
1. Determine the number of wells to be used.
2. Prepare the enzyme conjugate solution by diluting the 180X enzyme conjugate stock 1 to 180 in the EIA Buffer provided. Mix the solution by inversion. Do not vortex. Example: for two eight well strips, add 25 µL of the 180X enzyme conjugate stock to 4475 µL of EIA Buffer.
3. Add 20 µL of sample, laboratory calibrators and Neogen controls to the appropriate wells in duplicate. DO NOT dilute Neogen's positive and negative controls.
4. Add 180 µL of diluted drug-enzyme conjugate to each well. Use 8-channel pipetter or 12-channel pipetter for rapid addition.
5. For manual runs, mix by gently shaking plate. A microplate shaker may be used.
6. Cover plate with plastic film or plate cover and incubate at room temperature for 45 minutes.
7. During the conjugate incubation, dilute concentrated wash buffer 10 fold with deionized water (i.e. 20 mL of concentrated wash buffer plus 180 mL of deionized water). Mix thoroughly. Diluted wash buffer is stable for 5 days at room temperature or 7 days at 4°C.
8. Once the incubation is complete, dump or aspirate the liquid from the wells. Tap the plate on a clean lint-free towel to remove any remaining liquid in the wells.
9. Wash each well with 300 µL of diluted wash buffer. Manual Wash: For manual wash procedures repeat for a total of 3 washings, invert and tap dry the plate following each step. After completing the last wash step wipe the bottom of the wells with a lint-free towel to remove any liquid on the outside of the wells. Automated Wash: If an automated plate washer is used wash the plate for a total of 5 washings with 300 µL of diluted wash buffer. It is important for the automated washer to conduct a final aspirate cycle to eliminate residual amounts of wash buffer. Residual amounts of buffer in the wells will affect assay performance. Note: DI water should never be used for the plate wash.
10. Add 150 µL of the K-Blue Substrate to each well. For manual runs, use a multi-channel pipetter for best results.
11. Incubate at room temperature for 30 minutes. Gently shake immediately before measuring the absorbance.
12. Add 50 µL of Neogen's Red Stop Solution to each well to stop enzyme reaction. Mix gently before measuring the absorbance. For automated systems a 10 second shake is sufficient. Measure the absorbance at a wavelength of 650 nm. Wells should be read within 2 hours of stopping the reaction. Note: When Neogen's Red Stop is used, it will result in a color ranging from a dark blue/purple to light pink based on the concentration of drug present. If 1 N HCl is preferred, use 50 µL per well and read plate with a 450 nm filter. All QC data is generated without using a stopping reagent. Note: When acid stop is used, OD values will approximately double as compared to the OD values obtained with Red Stop.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 6 (Continued)

Neogen Corporation Methotrexate ELISA Kit Insert

STANDARD CURVE IN EIA BUFFER



SENSITIVITY

Compound	I-50 in EIA Buffer
Methotrexate	0.22 ng/mL
Aminopterin	0.66 ng/mL
Triamterene	29.7 ng/mL

The term I-50 is used to define the sensitivity of the test. This number is derived from a standard curve generated with the drug in EIA Buffer. The drug concentration that shows 50% less color activity than the zero standard is considered to be the I-50.

SPECIFICITY

Compound	Compound Concentration (ng/mL)	Methotrexate Equivalents (ng/mL)	% Cross-Reactivity
Methotrexate	0.22	0.22	100%
Aminopterin	0.66	0.22	33.33%
Triamterene	29.7	0.22	0.74%
Folic Acid	11000	0.22	0.002%
Trimethoprim	11000	0.22	0.002%

Note: Methotrexate equivalents represent 50% B/B_0 assay displacement in EIA Buffer.

The compounds having cross-reactivity below 0.01% did not show any significant reaction up to 10µg/mL.

ALL THE FOLLOWING HAVE A CROSS-REACTIVITY <0.01%.

Acepromazine ε-amino-n-caproic Acid; Ascorbic Acid; Clenbuterol; Dexamethasone; Diclofenac; Dimethyl Sulfoxide; Dipyrone; Ethyl p-amino-benzoate; Flunixin; Folinic Acid; Furosemide; L-Glutamic Acid; Glycopyrrolate; Hordenine; Hydrocortisone; Ibuprofen; Isoxsuprine; Lidocaine; Metaproterenol; Methocarbamol; Methylene Blue; 6α-Methylprednisolone; Naproxen; Niacinamide; Orphenadrine; Oxyphenbutazone; Pentoxifylline; Phenothiazine; Phenylbutazone; Polyethylene Glycol; Prednisolone; Procaine; Promazine; Pyrantel; Pyrilamine; Pyrimethamine; Salbutamol; Salicylamide; Salicylic Acid; Thiamine; Uric Acid.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 6 (Continued)

Neogen Corporation Methotrexate ELISA Kit Insert

RESULTS INTERPRETATION

Each laboratory should determine the cutoff level for their individual application. When possible, cutoff calibrators and/or standards should be prepared in the same matrix being tested.

Positive Result: Samples with an absorbance less than or equal to the laboratory's designated cutoff calibrator should be considered positive. All positive samples should be confirmed by a quantitative method such as GC/MS.

Negative Result: Samples with an absorbance greater than the laboratory's designated cutoff calibrator should be considered negative.

Qualitative QC Controls: The Neogen positive and negative controls provided in the kit are for QC purposes only. The sole purpose of these controls is to verify that the test kit is performing properly. The controls are not intended for use as cutoff calibrators. The positive control is spiked at a high concentration and its approximate level can be found on the label.

Note: The kit was designed for screening purposes only. It is recommended that all suspect samples be confirmed by a quantitative method such as GC/MS or HPLC.

TECHNICAL SUPPORT

For technical assistance, please contact our Technical Services Department at (859) 254-1221 or email at techservice-toxicology@neogen.com. Representatives are available Monday – Friday from 8:00 am – 6:00 pm EST.

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 D107519-2/8/2016

5