SOP Title:	**Quantitation of Methotrexate Using the Methotrexate ELISA Kit from Creative Diagnostics**
SOP Number:	**23107**
Revision:	**04**

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1. PURPOSE

This SOP describes the use of the Methotrexate ELISA kit, from Creative Diagnostics (CD), for residual Methotrexate quantitation in product samples. It is based on methotrexate monoclonal antibody to bind methotrexate in the sample or standard competitively to that pre-bound to the wells as bovine serum albumin (BSA) conjugate.

2. SCOPE

This SOP applies to Process Analytics/Quality Control (PA/QC) personnel who will perform the Methotrexate ELISA.

3. RESPONSIBILITIES

3.1 Director / PA/QC

- Defines the procedure.

3.2 PC/QC Personnel

- Trains lab personnel.
- Performs this procedure.
- Reviews data.

3.3 Biopharmaceutical Quality Assurance (BQA).

- Provides quality oversight.

4. MATERIALS AND REAGENTS

Part Number	Description	BDP Approved Substitution Permitted?
20050	96-well cell culture plate with lid	☒ YES ☐ NO
20101	Aspiration pipettes, 1ml	☒ YES ☐ NO
20140	Centrifuge tubes, 50ml	☒ YES ☐ NO
20595	Microcentrifuge tubes, 1.5ml	☒ YES ☐ NO
21470	Pipette tips 200µl	☒ YES ☐ NO
21471	Pipette tips 1000µl	☒ YES ☐ NO
21472	Pipette tips 20µl	☒ YES ☐ NO
21493	Kaydry EX-L Low-lint absorbent paper	☒ YES ☐ NO
21602	Pipette tips 5000µl	☒ YES ☐ NO
21767	Pipette tips 250µl	☒ YES ☐ NO
22667	Reagent Reservoir 10ml	☐ YES ☒ NO
31398	Methotrexate ELISA kit; Creative Diagnostics, Catalog. # DEIA-US209 storage temperature of kit 2-8°C (do not freeze)	☐ YES ☒ NO
N/A	Beaker 500 ml	☒ YES ☐ NO
N/A	Graduated Cylinder 500ml volume	☒ YES ☐ NO
N/A	Formulation Buffer/Medium of Test Sample (to be submitted along with the Test Sample)	☐ YES ☒ NO

5. EQUIPMENT

- Stat-matic Plate-washer Model II (8-channel microplate dispenser), Catalog. #S1061-1EA, Sigma/Millipore or approved equivalent
- Calibrated SpectraMax M5e plate-reader, MEF #81410, Molecular Devices or approved equivalent
- Microtiter plate shaker, VWR Catalog. #57019-600, or BDP approved equivalent.
- Sartorius arium-Pro Type I Ultra-Pure Water system, MEF #3001-LWPS-009B or approved equivalent
- Calibrated refrigerator, 2-8°C, MEF #REFI-084-B or approved equivalent
- Calibrated freezer, ≤-70°C or approved equivalent
- Vortex mixer
- Vacuum System
- BDP issued calibrated timer
- Calibrated pipettes-2µl, 10µl, 20µl, 100µl, 200µl, 1000µl, 5000µl and 8 and 12-channel multichannel pipettes

6. PREPARATION

6.1 Precautions

6.1.1 The mean values of the absorbance obtained for standards and samples will be reduced if the reagents and samples have not been regulated to room temperature (20-25˚C)

6.1.2 Do not allow microwells to dry between steps and operate the next step immediately.

6.1.3 Ensure adequate mixing of plate contents during each step.

6.1.4 Observe caution when handling 2M H2SO4.

6.1.5 Do not use expired kits or interchange the reagents from kits of a different lot number.

6.1.6 Store the kit at 2-8°C and do not freeze. Avoid direct sunlight during all incubations. Covering the microtiter plate with a plate lid is recommended.

6.1.7 The substrate solution should be discarded if there is a color change (normally it is colorless). Reagents could have deteriorated if the absorbance value (A_{450}) of the 0-standard is <1.

6.1.8 The color development reaction requires approximately 12-15 minutes incubation after the addition of the substrate solution; therefore, adjust the time between 12-15 minutes to attain the optimal color intensity.

6.1.9 The best reaction temperature is RT (20-25˚C), too high/too low a temperature could lead to changes in sensitivity and absorbance values.

NOTE: 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.

6.2 Preparation of Wash Buffer (1X)

6.2.1 Measure 285ml of Type I DI water in a 500ml graduated cylinder.

6.2.2 Using a 5ml pipette, add 15ml of 20X Wash Buffer into the cylinder. Pour into a 500ml beaker, mix well and transfer into the polyethylene bottle of the 8-channel microplate dispenser (Section. 5). Press the plunger button several times in consistent steady strokes until all air/water is expelled from the tubing and the wash solution is dispensed uniformly through the 8-channel manifold and is ready for dispensing. Label the contents of the bottle as 1X Wash Buffer.

6.3 Preparation of Diluent Buffer (1X)

6.3.1 Measure 10ml of Type I (DI water) into a 50ml centrifuge tube.

6.3.2 Add 10ml of 2X Sample Diluent from the kit. Vortex and mix Well. Label as 1X Sample Diluent solution.

6.4 Preparation of Blank Medium-Diluent for standard curve and test sample dilutions.

Pipette 500µl of the Medium/Formulation buffer (to be provided by requesting Lab with the respective sample) into 4.5ml of 1X Diluent Buffer (Section 6.3) in a 15ml centrifuge tube. Gently vortex to mix contents and label as Blank Medium (or Formulation) - Diluent.

6.5 Preparation of Samples

NOTE: The dilutions shown below are only suggested sample dilutions and might vary with the media/formulation of the individual test sample that is submitted for the assay. In those instances, it is recommended that an appropriate higher or lower dilution (as deemed necessary) may be used.

6.5.1 Pipette 1800µl of 1X Diluent buffer into a 15ml centrifuge tube

Add 200µl of stock sample, gently vortex to mix contents. Label as Working Stock Sample.

6.5.2 Pipette 200µl of Blank Medium-Diluent (Sec. 6.4) into a 1.5ml microcentrifuge tube and label as A

6.5.3 Pipette 200µl of working stock sample (Sec. 6.5.1) into a microcentrifuge tube and label as B

6.5.4 Pipette 300ul of blank medium-diluent into another mc tube. Add 300ul of working stock sample (Sec. 6.5.1) to this, vortex to mix. Label as C. This will be a 2X dilution sample.

6.5.5 Pipette 200µl of blank medium-diluent into a microcentrifuge tube and add 200ul of the 2X dilution sample from tube C (Sec. 6.5.4), vortex to mix and label as D. This will be a 4X dilution sample.

6.6 Preparation of Methotrexate Spike Controls

6.6.1 Pipette 200µl of Blank Medium-Diluent into a microcentrifuge tube and label tube as A' for blank control

6.6.2 Pipette 195µl of Blank Medium-Diluent into a microcentrifuge tube. Add 5ul of 20ng/ml Methotrexate standard from the kit into it, mix by gentle vortex and label tubes as B'. This is the 0.5ng/ml spike control.

6.6.3 Pipette 190ul of Blank Medium-Diluent into an mc tube labeled C' and add 10µl of 20ng/ml Methotrexate standard to give a 1ng/ml spike control.

6.6.4 Similarly, take a microcentrifuge tube labeled D' and pipette 180µl of the blank medium-diluent and add 20µl of the 20ng/ml Methotrexate standard. This will be labeled as the 2ng/ml spike control.

6.7 Preparation of Standards for the Standard Curve

Concentrations for the standard curve are prepared directly in the Dilution Plate using the 100ng/ml (100ppb) standard from the kit. A concentration range of 100-0.024ng/ml is prepared directly in a single column of the 96-well cell culture plate (Figure 1. Dilution Plate-Template) using a 1:4 serial dilution in the Blank Medium- Diluent before transferring into the assay plate (Figure 2. Assay Plate-Template). A blank medium-diluent 'only' is included in the final well for blank subtraction.

7. PROCEDURE

7.1 Set all necessary reagents out at RT for >30 minutes, to equilibrate to Room Temperature (RT) before use.

7.2 Get the microwells needed from the kit along with its frame and return the rest to its pouch. Place pouch in a zip-lock bag inside the kit and return to 2-8°C immediately. Unused wells should be kept desiccated at 2 -8°C inside the sealed bag.

7.3 Addition of Standards, Test Samples and Spike Controls into Plate # 1 (Figure 1. Dilution Plate-Template). This step is included to enable standard and sample transfer into the assay plate quick and more efficient.

7.3.1 Pour 5-8ml of the Blank Medium-Diluent into the 10ml reagent reservoir. Using a single channel 200µl pipette, add 150µl/well into wells from B-H of column 1 of the 96-well plate. Pipette 200µl (neat) of 100ng/ml Standard from the kit to well A1 (Figure 1, Dilution Plate-Template) of the plate. Pipette 50µl of the Standard from well A1 and transfer to B1, pipette up and down a few times to mix and remove 50µl to C1, repeat the same process till G1, discarding the 50µl from G1 to waste and leaving 150µl of the Blank Medium-Diluent in H1 for blank reading.

7.3.2 Pipette 200µl each of the blank and test samples from microcentrifuge tubes A, B, C and D into wells 3A, 3B, 3C and 3D. Similarly pipette 200µl of the spike controls from tubes A', B', C' and D' into wells 3E, 3F, 3G and 3H.

7.3.3 Cover the Dilution Plate and set aside ready for transferring standards and samples into the Assay Plate.

NOTE: Ensure using new pipette tips when pipetting diluent only, standards, samples, and spike controls to prevent cross-contamination.

Figure 1. Dilution Plate – Template

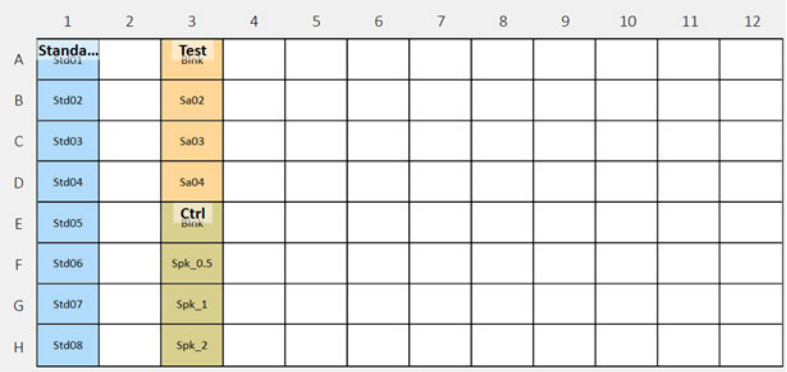
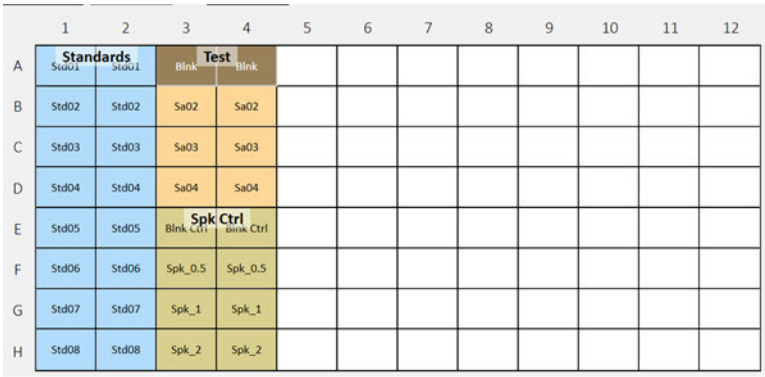


Figure 2. Assay Plate -Template



7.3.4 Transfer of Standards, Test Samples and Spike Controls into Assay Plate (Figure 2, Assay Plate-Template)

7.3.4.1 The required number of microtiter plate strips are fixed on to the frame firmly. The positions of the standards and samples are pre-designated before actual transfer of standards, samples and controls.

7.3.4.2 Once the standards, test samples and spike controls are pipetted into the Dilution Plate and the Assay Plate is ready, using an 8-channel pipette, remove 50µl/well each of the standards from column 1 (1A-1H) in the Dilution Plate into column 1 and 2 of the Assay plate (1A-1H and 2A-2H). Similarly, all the samples/controls from 3A-3H of the Dilution Plate are transferred to 3A-3H and 4A-4H of the Assay plate, i.e., standards, samples and spike controls are assayed in duplicate.

7.4 Addition of monoclonal Antibody solution

Pour 3ml of the antibody solution into a 10ml reagent reservoir. Using an 8-channel pipette add 50µl/well into columns 1, 2, 3 and 4 from A-H using new pipette tips for each column. Cover the plate with the lid of the cell-culture plate and place on the microplate shaker to gently shake (speed set at 500rpm, Section. 5), start timer and incubate for 30 minutes at RT. At this point take the Enzyme Conjugated Secondary Antibody, in the kit, from the refrigerator and let it equilibrate to RT.

7.5 Wash

At the end of incubation remove plate cover and gently invert plate, over the sink, to pour the liquid out of the wells. Using the manual 8-channel microplate dispenser (Section. 5) rinse the wells with 250-300µl of the wash buffer. Repeat the process 5 times. Each time gently invert the plate to empty the liquid in each well and tap any residual liquid over the absorbent sheet (Section. 4). Any remaining droplets in each well is removed by gentle aspiration (use fresh pipette tip for blank wells, standards, and samples to prevent any cross-contamination). Wash direction can be alternated/reversed between Right > Left and Left > Right during the wash.

7.6 Addition of Enzyme Conjugated Secondary Antibody Solution

7.6.1 Pour about 5ml of the secondary antibody solution into a 10ml reagent reservoir.

7.6.2 Using an 8-channel pipet add 100µl/well of the solution into the wells. Cover the plate, place it over the shaker, start the timer and let it incubate for 30 minutes at RT. Next take the TMB substrate and STOP solutions out of the refrigerator to equilibrate to RT.

7.6.3 Turn the plate-reader and computer ON. Create an assay template, if not already done, as in Figure1 Assay Template on the SoftMax Pro V7.

7.7 Wash

At the end of incubation, repeat the wash process as in Section 7.5

7.8 Addition of TMB Substate

Add 100µl of the substrate into each well, cover plate and let it mix gently on a shaker, start timer, and let it incubate for 12-15 minutes. Incubation time can be adjusted between 12-15 minutes and can be visually determined based on the intensity of color development.

7.9 STOP Reaction

7.9.1 At the end of incubation stop the reaction with 2M H2SO4. Pour 3ml of the solution into the reagent reservoir and pipette 50µl/well into the wells using an 8-channel pipette. Cover plate and let it mix on the shaker for a few seconds.

7.9.2 Remove the plate from shaker, remove cover and read plate at 450nm, preferably with correction at 630nm. Read plate within 5 minutes of STOP reaction.

7.9.3 Document the use and other relevant information in the dedicated logbook.

8. DATA COLLECTION

8.1 Use SpectraMax M5e installed with SoftMax Pro V7 or its upgrade for data acquisition.

8.2 Follow SOP 16144 Operation of SpectraMax Series Plate-Reader (Molecular Device) for instructions on system setup. The reading parameters selected on the plate-reader for the Quantitation of Methotrexate in this SOP are as follows:

- Read Type: End Point
- Read Mode: Absorbance (ODs)
- Number of wavelengths: 2 [450nm and 630nm]
- Enter the standard/sample names and concentration/dilutions following the
- Template layout in Figure 2
- Obtain the readout and save the raw data in the local hard drive and network.

8.3 Data Analysis and Acceptance Criteria

8.3.1 Data Analysis

The residual Methotrexate concentration in test samples and spike controls is estimated relative to the standard used. For the standard series, enter its protein concentration in the template (Figure 2 Assay-Plate Template). For the samples labeled as 'unknown' (dilution), enter the dilution factor in the template. Standard curve is plotted, and a 4-parameter curve fit analysis is performed with concentrations along the x-axis and mean ODs (OD_{450} – OD_{630}) along the y-axis with error Standard Deviation (Std. Dev.).

8.3.2 Acceptance Criteria

8.3.2.1 The R^2 value of the Standard Curve should be ≥0.985.

8.3.2.2 The coefficient of variation (%CV) for standards falling within the steep part of the standard curve should be ≤25%.

8.3.3 Reporting of Methotrexate concentration in the sample of interest

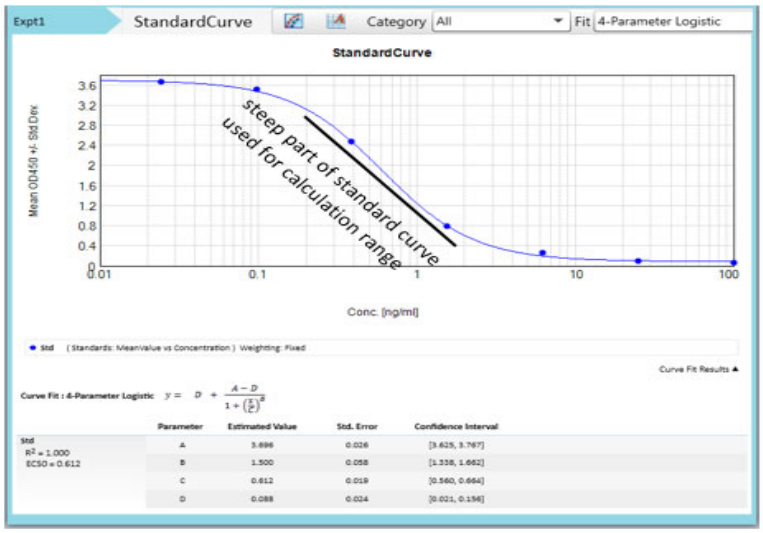
8.3.3.1 If the OD readouts are within the accepted range of the

8.3.3.2 standard curve, report the back-calculated value from the standard curve.

8.3.3.3 If the OD readouts are outside this range, i.e., in the upper plateau region of the standard curve, report result as below the limit of detection (LOD).

If readouts are in the lower plateau region of the standard curve, repeat the assay using higher sample dilutions.

Figure 3 Standard Curve



8.4 Data Storage

Save the raw data and the original template in the file under [redacted] folder using the respective QC # and date (e.g., QCXXXXXX_DD-MM-YY).

9. REFERENCES AND RELATED DOCUMENTS

Document Number	Title
16144	Operation of SpectraMax Series (190, 384 plus, M2, M5e, etc.)
21409	Good Documentation Practices
22702	Solutions Used in Process Analytics
23107-01	Buffer and Reagent Preparations
23107-02	Standard and Sample Preparation
23107-03	ELISA Procedure
23107-04	Raw Data

Document Number	Title
23107-05	Standard/Samples OD_{450} Table, B/B0 (%) Calculation Sheet
23107-06	Equipment/Material/Reagent Info

10. ATTACHMENTS

Attachment 1 Methotrexate ELISA Kit User Manual

Attachment 1 Methotrexate ELISA Kit User's Manual Provided in each Kit

CD Creative Diagnostics®

Version 07-03/23

User's Manual

Methotrexate ELISA kit

REF DEIA-US209

Σ 96T

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics
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Attachment 1 (Continued)

PRODUCT INFORMATION

Intended Use

The Methotrexate ELISA kit is a colorimetric competitive immunoassay kit with results in 1,5 hours.

Note: For research use only.

Unless otherwise specified expressly on the packaging, all products sold here under are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings.

General Description

Methotrexate is a drug used in the treatment of cancer and autoimmune disease. It is designed as an antifolate to inhibit the metabolism of folic acid. Two distinct mechanisms of action have been described for methotrexate. In cancer treatments, methotrexate competitively inhibits the dihydrofolate reductase (DHFR) by blocking folate binding. DHFR converts dihydrofolate to active tetrahydrofolate. Inhibition of DHFR results in inhibition of the synthesis of purine and pyrimidine bases effectively limiting DNA and RNA synthesis and cancer cell growth. In autoimmune disease and specifically in the treatment of rheumatoid arthritis, methotrexate appears to impact several pathways resulting in inhibition of T cell activation. The effects include suppression of T cell expression of intercellular adhesion molecules, inhibition of methyl transferase activity and increased CD95 sensitivity leading to apoptosis in active T cells.

Monitoring methotrexate levels is important to assure appropriate levels are maintained during therapy or treatment. High levels of methotrexate can lead to toxicity and potential renal failure as well as immunosuppression. Additionally, methotrexate is known to interact with a wide variety of drugs leading to additional complications. Determining the presence of methotrexate in samples from subjects in blinded research studies can assist in the interpretation of study results.

Methotrexate is established as one of the most effective and safe therapeutics for rheumatoid arthritis. The safety profile assures that methotrexate will continue to be used in new studies in combination with other new or established drugs. The same is true in its use as a cancer therapeutic. The ELISA enables monitoring levels of methotrexate in both preclinical and clinical research. The methotrexate assay is also appropriate for the detection of methotrexate contamination after its use as a selective agent for recombinant protein production in mammalian cell lines.

Principles of Testing

The Methotrexate ELISA kit is a complete kit for the quantitative determination of methotrexate in serum and plasma samples. Please read the complete kit insert before performing this assay. The methotrexate ELISA uses a methotrexate monoclonal antibody to bind methotrexate in the sample or standard competitively to that pre-bound to the wells as a bovine serum albumin (BSA) conjugate. Anti-methotrexate antibody bound to methotrexate in the sample or standard are washed away while those captured by the immobilized methotrexate are detected with a secondary antibody horseradish peroxidase (HRP) conjugate.

The assay is developed with tetramethylbenzidine (TMB) substrate and the resulting absorbance is measured with a microplate reader at 450nm. The intensity of the yellow color is inversely proportional to the concentration of methotrexate.

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Reagents And Materials Provided

1. Methotrexate Microtiter Plate, One Plate of 96 Wells
   A plate using break-apart strips coated with a methotrexate BSA conjugate
2. Methotrexate Antibody Solution, 7mL
   Methotrexate monoclonal antibody
3. Enzyme Conjugated Secondary Antibody Solution, 12 ml
   Rabbit anti-mouse IgG conjugated to HRP.
4. Methotrexate Standard Solution, 1ppm, 1mL
5. Methotrexate Standard Solution, 100ppb, 1mL
6. Methotrexate Standard Solution, 20ppb, 1mL
7. Methotrexate Standard Solution, 4ppb, 1mL
8. Methotrexate Standard Solution, 0.8ppb, 1mL
9. Methotrexate Standard Solution, 0.16ppb, 1mL
10. Methotrexate Standard Solution, 0 ppb, 1mL
11. 20× concentrated wash solution, 50 ml
    Phosphate buffered saline containing detergents.
12. 2× Sample Diluent, 50mL
    Phosphate buffered saline.
13. TMB Substrate, 6ml × 2
    A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. Protect from prolonged exposure to light.
14. Stop Solution, 7ml
    A 2M solution of sulphuric acid in water. Keep tightly capped. **Caution:** Caustic.

Materials Required But Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 50 µl and 1,000 µl.
3. Repeater pipet for dispensing volumes between 50 and 100 µl.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. Absorbent paper for blotting.
7. Microplate reader capable of reading at 450nm, preferably with correction between 620nm and 630nm.

Storage

1. The optimal storage temperature of the kit is 2-8 °C, do not freeze.

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Attachment 1 (Continued)

2. Unused ELISA strips must be sealed and stored at 2-8 °C.

Specimen Collection And Preparation

The Methotrexate ELISA is compatible with serum and plasma samples from human. Samples diluted sufficiently into Sample Diluent (see Reagent Preparation) can be read directly from a standard curve. the minimum recommended dilutions for validated matrices of sample is 1:20 (e.g. 50uL serum/plasma sample+950uL Sample Diluent).

Samples must be stored frozen at or below -20°C. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4°C slowly and gently mixed. Samples may be clarified by centrifugation to reduce risk of matrix interference.

Other Sample Types

The methotrexate ELISA kit may be appropriate for testing biological matrices from other species that have not been validated and may be compatible with other buffer matrix formulations. It is recommended that any matrix of interest undergo testing to determine the minimum dilution in Sample Diluent to eliminate matrix interference.

Reagent Preparation

Solution 1: 1× Sample Diluent

Dilute the 2× Sample Diluent with deionized water diluent in the volume ratio of 1:1, called Sample Diluent (10ml 2× Sample Diluent + 10ml deionized water).

Solution 2: 1× Wash solution

Dilute the 20× concentrated wash solution with deionized water in the volume ratio of 1:19, which will be used for washing the plates.

Assay Procedure

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
4. Pipet standards and samples to the bottom of the wells.
5. Add the reagents to the side of the well to avoid contamination.
6. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.
7. Prior to addition of conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

Procedure

1. Take all reagents out at room temperature (20-25°C) for more than 30min, homogenize before use.

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2. Get the microwells needed out and return the rest into the zip-lock bag at 2-8°C immediately.
3. The wash solution should be brought to room temperature (20-25°C) before use.
4. Number: Number every microwell position and all standards and samples should be run in duplicate. Record the standards and samples positions.
5. Add standard solution/sample and antibody solution: Add 50µl of standard solution or prepared sample to corresponding wells. Add 50µl of antibody solution to each well, mix gently by shaking the plate manually and incubate for 30min at room temperature (20-25°C) with cover.
6. Wash: Remove the cover gently and pour the liquid out of the wells and rinse the microwells with 300µl of diluted wash solution atinterval of 10s for 5 times. Absorb the residual water with absorbent paper (the rest air bubble can be eliminated with unused tip).
7. Add enzyme conjugate: Add 100µl of enzyme conjugate to each well, mix gently by shaking the plate manually and incubate for 30min at room temperature (20-25°C) with cover.
8. Wash: Remove the cover gently and pour the liquid out of the wells and rinse the microwells with 300µl of diluted wash solution at interval of 10s for 5 times. Absorb the residual water with absorbent paper.
9. Coloration: Add 100µl of substrate solution to each well. Mix gently by shaking the plate manually and incubate for 15 min at room temperature (20-25°C) with cover .
10. Measure: Add 50µl of the stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450nm, preferably with correction between 620 and 630 nm. (Read the result within 5min after addition of stop solution.)

Calculation

Percentage absorbance

The mean values of the absorbance values obtained from the standards and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100%.

Absorbance (%)=(B/B_0)*100%

B ——absorbance of standards or samples

B_0 ——absorbance of zero standard (0ng/ml)

Precision

Intra-plate coefficient of variation: <10%

Inter-plate variation coefficient: <10%

Sensitivity

0.16 ppb

Precautions

1. The mean values of the absorbance values obtained for the standards and the samples will be reduced if the reagents and samples have not been regulated to room temperature (20-25°C).

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Attachment 1 (Continued)

2. Do not allow microwells to be dry between steps to avoid unsuccessful repetitiveness and operate the next step immediately after tap the microwells holder.
3. Mix the homogenate and elute the plate adequately.
4. Avoid the stop solution touching skin for the 2M H_2SO_4.
5. Don't use the kits out of date. Don't exchange the reagents of different batches, or else it will drop the sensitivity.
6. Storage constitution: Keep the ELISA kits at 2-8°C without frozen. Avoid direct sunlight during all incubations. Covering the microtiter plates is recommended.
7. The reagents go bad: Substrate solution should be abandoned if its color has changed. The reagents may be turn bad if the absorbance value of the zero standard is less than 1 (A450nm<1).
8. The coloration reaction needs 15min after the addition of substrate solution; But you can prolong the incubation time ranges from 15min to 12min if the color is too light to be determined. On the contrary, shorten the incubation time properly.
9. The best reaction temperature is room temperature (20-25°C), temperature too high or too low both will lead to the changes of sensitivity and absorbance values.