MSD® 96-Well MULTI-ARRAY® Bioprocess Assay

Methotrexate Assay

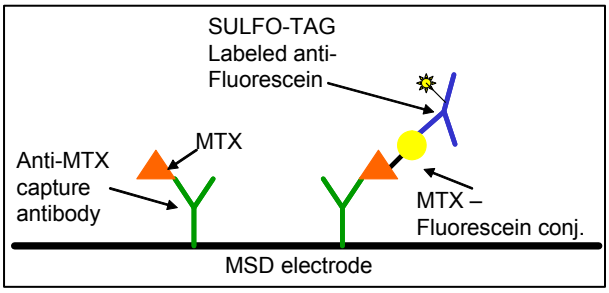
Summary

MSD's bioprocess assays are designed for single and multiplex measurements of common bioprocess contaminants. Assays for several common contaminants including Insulin, Methotrexate (MTX), host cell proteins, and protein A are currently available. This insert describes how to run the MTX assay independently. MSD also offers a multiplex kit that includes CHO host cell protein, Insulin and MTX.

Comments on the assay

The Methotrexate (MTX) assay is formulated as a competitive assay (Figure 1). MTX from the sample competes with an MTX-Fluorescein conjugate for binding sites on an electrode coated with anti-MTX antibody. An Anti-Fluorescein Detection Antibody labeled with MSD SULFO-TAG™ acts as a reporter. To run this assay, MTX-Fluorescein conjugate is first added to each well. After sample addition, the plate is incubated in order to establish a competitive equilibrium at the electrode surface. Then, Anti-Fluorescein Detection Antibody is added and the plate is read after incubation, wash, and addition of Read Buffer.

Figure 1: Diagram of competitive assay for Methotrexate (MTX).



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Reagents Provided

Storage

Read Buffer T	4X Read Buffer T with surfactant	RT
MULTI-SPOT® Plate	96-well MSD plate spotted with specific capture antibodies	2-8 °C
Anti-Fluorescein Antibody	Detection antibodies labeled with SULFO-TAG reagent. These antibodies are supplied at 50X concentration.	2-8 °C
MTX-Fluorescein Conjugate	MTX conjugated with Fluorescein. Stock concentration is 500 ng/mL in DMSO.	≤ -70 °C
Diluent 21	Buffered solution containing blocking and stabilizing agents in addition to detergents	≤ -10 °C
Methotrexate Calibrator	Stock concentration is 100 µg/mL.	≤ -70 °C

Required Materials and Equipment – not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of accurately dispensing 20 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

Reagent Preparation

Bring all reagents to room temperature. The MTX-Fluorescein conjugate will not thaw on ice because it is supplied in DMSO.

Preparation of MTX-Fluorescein Conjugate Solution

Samples and MTX standard solutions are premixed in a 1:1 ratio with MTX-Fluorescein conjugate solution. This solution is prepared in Diluent 21 with the conjugate at 2 ng/mL. For one plate, mix 3.0 mL of Diluent 21 with 12 µL of MTX-Fluorescein Conjugate stock solution at 0.5 µg/mL.

(Warning: Methotrexate is a known reproductive toxin and should be handled carefully with appropriate personal protective equipment including gloves and safety precautions.)

Preparation of Calibrator dilutions for standard curve

The table below shows recommended values for the highest and lowest Calibrators on the standard curve for the MSD Bioprocess assays. An 8-point standard curve is recommended which includes the high and low Calibrators below, as well as a 0 pg/mL standard.

Assay	High Calibrator level	Low Calibrator level
MTX	1000 ng/mL	244 pg/mL

To make the high standard Calibrator solution, dilute the Calibrator stock solution into Diluent 21. Then, make 4-fold serial dilutions into Diluent 21. To dilute the standards, be sure to use Diluent 21 that **does not** include the MTX-Fluorescein conjugate. The example below demonstrates how to quickly prepare mixed Calibrator standards:

Step 1: Make 400 µL of High Calibrator according to the recipe below:

4 µL of 100 µg/mL MTX Calibrator
396 µL of Diluent 21
400 µL total volume

Step 2: Make a series of 4-fold serial dilutions of the High Calibrator. For each serial dilution step, mix 100 µL of Calibrator with 300 µL of Diluent 21.

Prepare dilutions of samples

Samples should be diluted into Diluent 21 that **does not** include the MTX-Fluorescein conjugate. Several dilution ratios (e.g. 4-fold, 16-fold, 64-fold) may need to be tested in order to bring some samples into the detection range.

Prepare premixes of samples and standards

For each sample and standard, prepare a 1:1 premix of MTX-Fluorescein Conjugate Solution with the diluted sample or standard. For each well, 40 µL of premix is required. For duplicate measurements, mix 50 µL of sample or standard with 50 µL of MTX-Fluorescein Conjugate solution.

Prepare Detection Antibody Solution

The Detection Antibody should be diluted 1:50 to a final concentration of 1X in a common volume of Diluent 21 just prior to addition to the plate. Combine 60 µL of Detection Antibody stock with 2.94 mL of Diluent 21. After use, the stock Detection Antibody should be stored at 4 ^{o}C, shielded from light.

Prepare Read Buffer

The Read Buffer should be diluted 4-fold in deionized water to make a final concentration of 1X Read Buffer T. For each plate, add 5 mL of stock Read Buffer T (4X) to 15 mL of deionized water to make 20 mL of 1X Read Buffer T.

Protocol

1. ***Addition of Sample or Standards:***
   Dispense 40 µL of Sample or Standard premixed with MTX-Fluorescein conjugate into each well. Pipette to the bottom of the plate so as to allow the fluid to cover the entire bottom of the well. A slight tap may be necessary to allow the fluid to settle to the bottom. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (approx. 500 rpm) at room temperature.

2. ***Wash:*** Wash the plate 3X with PBS + 0.05% Tween-20.

3. ***Addition of Detection Antibody Solution:*** Dispense 25 µL of the 1X Detection Antibody solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (approx. 500 rpm) at room temperature.

4. ***Wash and Read:*** Wash the plate 3X with PBS + 0.05% Tween-20. Add 150 µL of 1X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR® Imager. Plates may be read immediately after addition of Read Buffer. Note: Bubbles in the fluid will interfere with reliable reading of the MULTI-SPOT plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

Notes

1. ***Sample compatibility:*** Bioproduction samples vary in their pH, ionic strength and protein concentration, and these factors can influence assay results. Users are encouraged to substitute an application-specific diluent for the Diluent 21 supplied in the kit. The most accurate quantitation will be achieved when the composition of the Calibrator diluent closely matches that of the samples.

2. ***Alternate protocol:*** The assay can be run without premixing the samples with MTX-Fluorescein conjugate. To do this, add 20 µL of sample to the well, and then quickly add 20 µL of MTX-Fluorescein Conjugate Solution. Mix the solution in wells on a plate shaker as soon as possible. When these steps cannot be completed in 5 minutes or less, a premix of sample and conjugate is recommended.

Topics of Interest

1. ***Background signal and negative signals:*** The output signal produced by electrochemiluminescence assays is in units of counts of light measured by a charge-coupled device (CCD) camera or photodiode. As with any measurement technique, there is a certain amount of normal variation in this signal (instrument noise) which sets the threshold for the lowest levels of signal that can be measured (noise floor). This variation is different depending upon the size of the working electrode with typical values of about 10 counts for 96-well small spot and 96-well 4 Spot plates, 15 counts for 96-well 7 Spot plates, and 30 counts for 96-well 10 Spot plates. When the background signal of an assay approaches the noise floor (i.e. the mean signal of negative controls or sample blanks is close to zero), it is possible to observe negative counts for some wells.

2. ***Signal Levels:*** The camera system is linear over nearly a 6 log-dynamic range. The highest achievable signals on the SECTOR Imager 6000 and 2400 are between 1.0 and 2.0 million counts. If the signals from the highest point on the calibration curve are not approaching 1.0 million counts, the high end of the calibration curve may be extended. The lowest observed signals using the Read Buffer T (1X) are between 10 and 50. Negative signal values may occur due to instrument noise, omission or usage of the incorrect Read Buffer, or incorrect amount of Detection Antibody.

3. ***Fitting methods:*** To utilize the quantitative value of electrochemiluminescent detection, a titration curve is produced using a known standard. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. MSD's cytokine assays have a wide dynamic range (typically 3-5 logs) which allows accurate quantitation in many samples without the need to dilute prior to running the assay. MSD recommends using software to fit the data that utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting functionality is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve. An alternative analysis approach is to subtract the background signal from all data points, and then use a linear model to fit the data. The disadvantage of this approach is that a skewed calibration curve may be created if the background signal used for subtraction is not an appropriate indicator of background signal over the complete curve. Also, negative numbers may be produced if background-corrected signal values are less than the instrument noise observed in signals at the low end of the curve.

4. ***Antibody pairs and cross-reactivity:*** The Capture and Detection Antibody pairs used in MSD cytokine assays have been selected by an optimization process that is designed to minimize cross-reactivity with other bioprocess assays.

5. ***Reverse pipetting:*** Most manual hand pipettes have two plunger positions for pipetting liquids. The first position is calibrated to allow aspiration and dispensing of user-specified amounts of liquid and the second (blow-out) position enables the user to expel any residual liquid after the pipette has been pushed to the first position. When a pipette is used to dispense liquid by moving the plunger to the first position followed by the second (blow-out) position, bubbles may be created in the dispensed liquid. The reverse pipetting technique is designed to allow precise pipetting while avoiding the creation of bubbles. The technique is to push the pipette plunger past the first position to the second position prior to aspirating liquid into the tip, thereby aspirating slightly more liquid than the desired volume (overdraw). In order to dispense the liquid from the tip, the pipette plunger is pushed to the first position only. This allows precise dispensing without the introduction of bubbles. When using the reverse pipetting technique, it is important not to overdraw excess liquid into the pipette mechanism.

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Methotrexate Assay

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the MSD Bioprocess Assays

STEP 1: Sample and Reagent Preparation

Bring appropriate diluents, Calibrators, and plates to room temperature
Store Detection Antibody mix at 4 °C; shield from light.
Prepare MTX-Fluorescein Conjugate solution by diluting MTX-Fluorescein to 2 ng/mL concentration in Diluent 21.
Samples should be diluted 1:1 in MTX-Fluorescein Conjugate to give a final concentration of 1 ng/mL of MTX-Fluorescein Conjugate. Samples with high MTX concentrations should be prediluted in Diluent 21 in order to get the analyte level in range.
Prepare Calibrator solutions and calibration curve.

- Use the provided Calibrator stocks to prepare an 8-point calibration curve of MTX: 1000, 250, 62.5, 15.6, 3.9, 0.98, 0.24, and 0 ng/mL.
  Note: The calibration curve can be modified as necessary to meet specific assay requirements.
- Use Diluent 21 as the Calibrator diluent.

(if necessary) Dilute samples in Diluent 21 prior to premixing with the Conjugate solution
Just prior to addition to the plate in Step 4, prepare Detection Antibody solution by diluting Detection Antibody 1:50 to a final 1X concentration in 3.0 mL of Diluent 21 (per plate)
Prepare 20 mL of 1X Read Buffer T by diluting 4X Read Buffer T 4-fold with deionized water.

STEP 2: Addition of Standard/Samples

Dispense 40 µL of Sample or Standard premixed with MTX-Fluorescein conjugate into each well. Incubate at room temperature with vigorous shaking (approx. 500 rpm) for 2 hours.

STEP 3: Wash

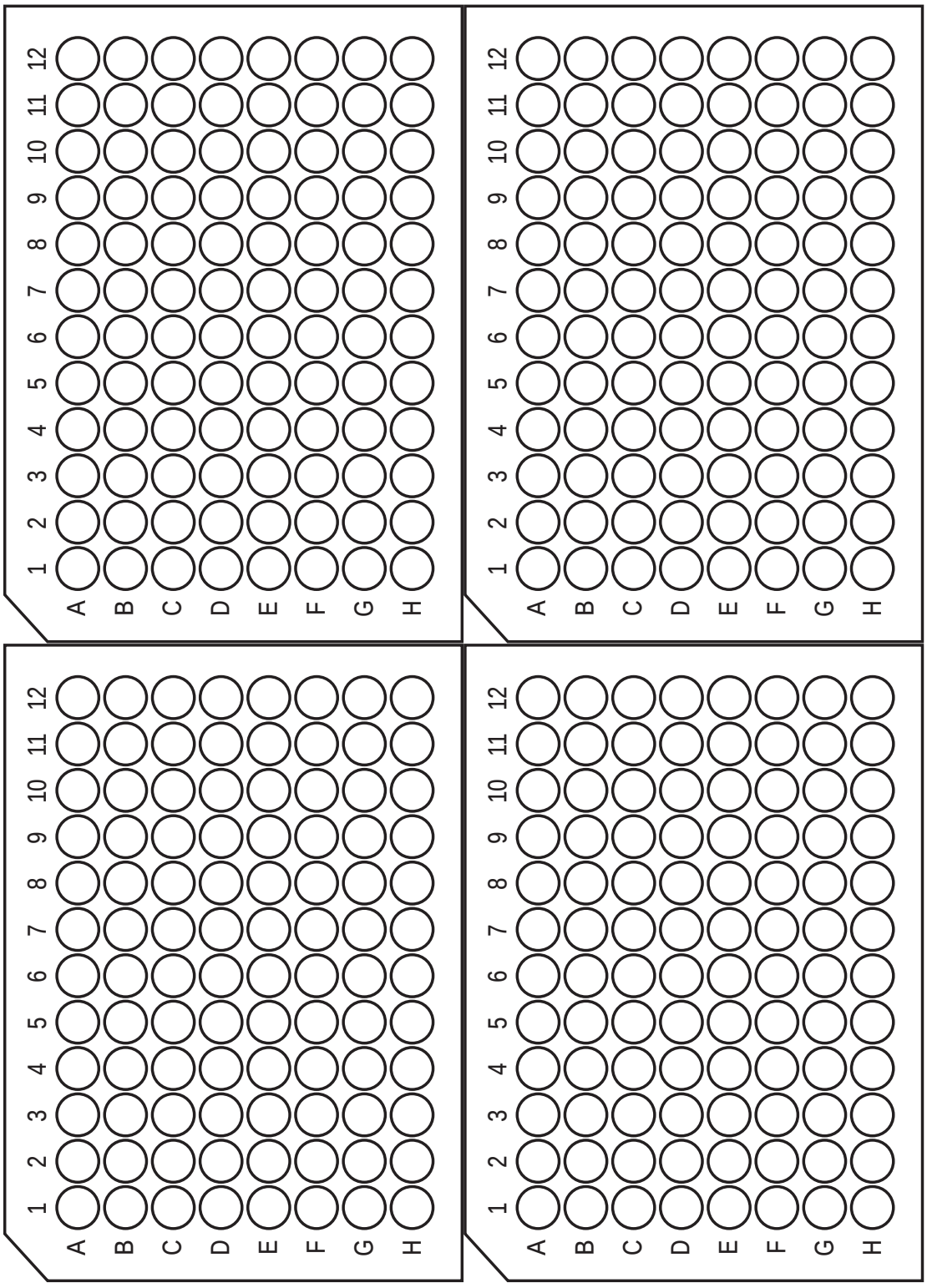
Wash plate 3X with PBS-0.05% Tween-20

STEP 4: Add Detection Antibody

Dispense 25 µL/well 1X Detection Antibody solution.
Incubate at room temperature with vigorous shaking (approx. 500 rpm) for 2 hours.

STEP 5: Wash and Read Plate

Wash plate 3X with PBS-0.05% Tween-20.
Dispense 150 µL/well 1X Read Buffer T.
Analyze plate on SECTOR Imager.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