Protocol for MSD® 96-Well BioProcess Assays

Protein A Contamination Assay

Summary

MSD's bioprocess assays are designed for single- and multi-plex measurements of common bioprocess contaminants. Assays for several common contaminants including insulin, methotrexate (MTX), host cell proteins, and protein A are currently available. The MSD Protein A contamination assay employs a simple immunosandwich assay format using capture and detection antibodies specific to Protein A. This insert describes a recommended protocol for the Protein A contamination assay. A duplex assay measuring Protein A and CHO Host Cell Protein contaminants is also available in the product catalog. Upon request, MSD can supply custom multiplex kits that include assays for Protein A, CHO host cell protein and/or methotrexate.

Reagents Provided

		Storage
Protein A Detection Antibody	Detection Antibody labeled with SULFO-TAG™ reagent. The antibody is supplied as a 50X stock.	2-8 °C
Diluent 21	Buffered solution containing blocking and stabilizing agents in addition to detergents	≤ -10 °C
Protein A Calibrator	Stock concentration is 10 µg/mL.	≤ -70 °C
Diluent 100	Contains blocking and stabilizing agents	2-8 °C
Read Buffer T	4X Read Buffer T with surfactant	RT
MULTI-ARRAY® or MULTI-SPOT® Plate	96-well MSD plate spotted with specific capture antibody	2-8 °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.

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 Protein A Assay. 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
Protein A	250 ng/mL	61 pg/mL

To make Calibrator curve solutions for Protein A, first dilute the Protein A Calibrator stock into Diluent 21 to a concentration of 250 ng/mL. Then, make 4-fold serial dilutions into Diluent 21. The example below demonstrates how to prepare Calibrator standards:

Step 1: Make 400 µL of combined High Calibrator according to the recipe below:
10 µL of 10 µg/mL Protein A Calibrator.
390 µL of Diluent 21
400 µL total volume

Step 2: Make a series of 4-fold serial dilutions of the combined 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. 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 Detection Antibody solution

The Protein A Detection Antibody should be diluted 1:50 to a final concentration of 1X in Diluent 100 just prior to addition to the plate. After use, the Detection Antibodies 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 Diluent 21***
   Dispense 20 µL of MSD Diluent 21 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.

2. ***Addition of Sample or Calibrator:*** Dispense 20 µL of each Calibrator or Sample Solution into a separate well of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (approx. 500 rpm) at room temperature.

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

4. ***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.

5. ***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. ***Addition of Calibrators and samples to the Protein A assay plate:*** As an alternative protocol, the first and second steps of the protocol above can be combined into one addition. In this case, 40 ul per well of the Protein A Calibrators and samples are added after dilution of the samples by at least 2-fold in Diluent 21.
2. ***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.

3. ***Quantitation of Protein A in bioproduction samples:*** The Protein A assay is particularly sensitive to the IgG concentration in the well. This is because Protein A in complex with IgG is less detectable than free Protein A. For accurate quantitation of samples that contain IgG, it is important that the calibrator diluent has a similar IgG concentration to the tested samples. For

bioprocesses where the product is a monoclonal antibody, three approaches can be taken: 1) Pre-purification of samples to remove the product IgG, 2) Matching of product IgG levels between samples and calibrator diluent, and 3) Dilution of samples to normalize product IgG levels. Whichever approach is taken, it is particularly important to validate the Protein A assay for each individual application.

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 bio-process assays.

5. ***Reverse pipetting:*** Most manual hand pipets 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 pipet has been pushed to the first position. When a pipet 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 pipet 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 pipet 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 pipet mechanism.

Protocol for MSD® 96-Well Bio-Process Assays

Protein A Contamination Assay

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

STEP 1: Sample and Reagent Preparation

Samples may not require dilution prior to use in the assay.
Bring appropriate diluents, Calibrators, and plates to room temperature
Store Detection Antibody mix at 4 °C; shield from light.
Prepare Calibrator solutions and calibration curve.

- Use the provided Calibrator stock to prepare an 8-point calibration curve of 250, 62.5, 15.6, 3.9, 0.98, 0.24, 0.061, and 0 ng/mL. The calibration curve can be modified as necessary to meet specific assay requirements.
- Use Diluent 21 to dilute the Calibrator.
- If necessary, dilute samples in Diluent 21

Just prior to addition to the plate in Step 4, prepare Detection Antibody Solution by diluting the Protein A Detection Antibody 1:50 to a final 1X concentration in 3.0 mL of Diluent 100 (per plate)
Prepare 20 mL of 1X Read Buffer T by diluting 4X Read Buffer T 4-fold with deionized water.

STEP 2: Add Diluent 21

Dispense 20 µL/well Diluent 21.

STEP 3: Add Sample or Calibrator

Dispense 20 µL/well Calibrator or Sample.
Incubate at room temperature with vigorous shaking (approx. 500 rpm) for 2 hours.

STEP 4: Wash

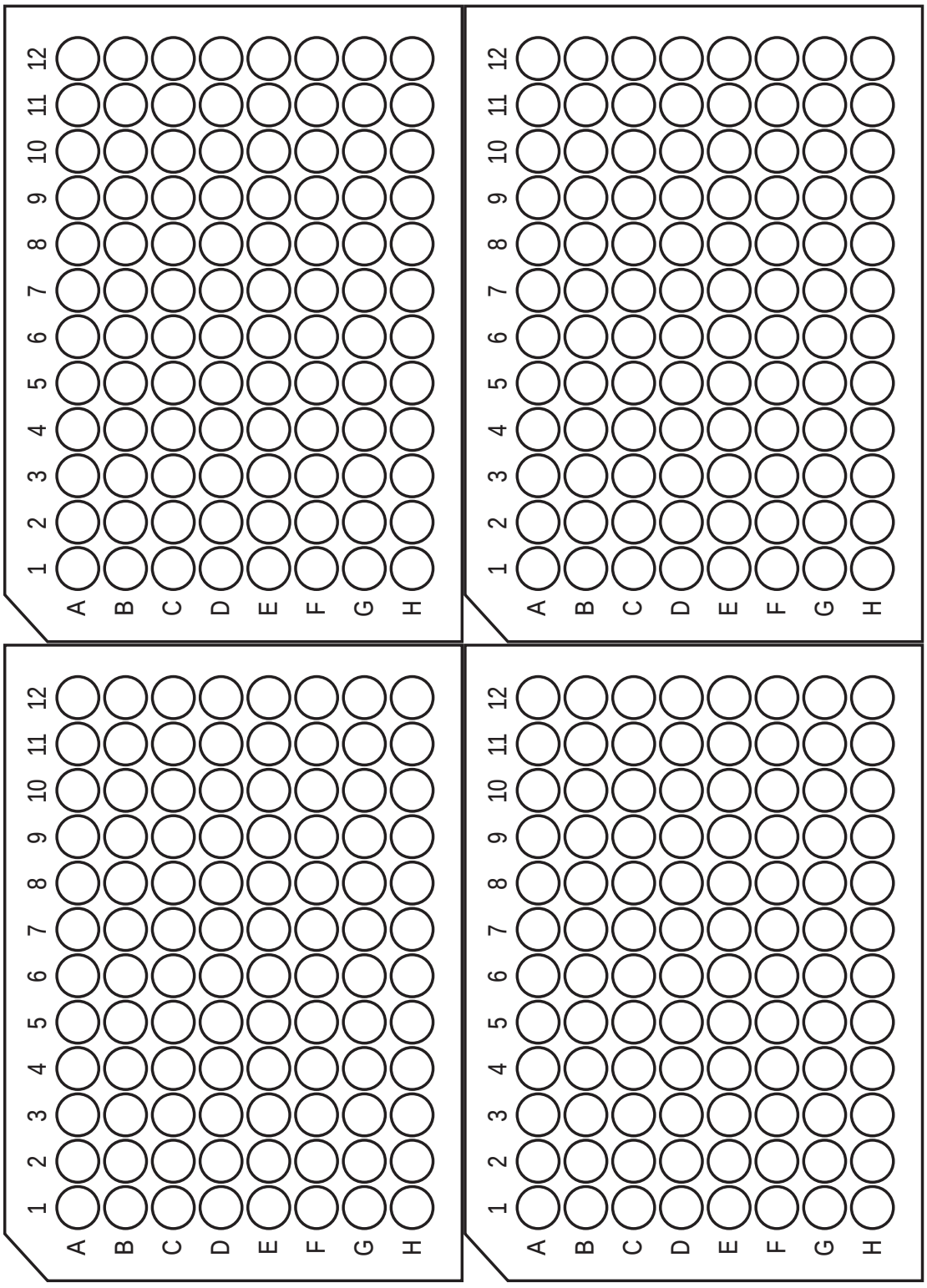
Wash plate 3X with PBS-0.05% Tween-20

STEP 5: 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 6: 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 instrument.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