

FlexPlex™ System Assay

MSFX

Rev.5

DESCRIPTION

To perform this assay, the FlexPlex™ Starter Pack (MSFX-ST) and a minimum of one FlexPlex™ Module are required.

FlexPlex™ Starter Pack (Catalog # MSFX-ST)

Part Number	Item	Amount
8209800	20X Wash Buffer	12 mL
8209801	1X Sample Extraction Buffer	20 mL
8209802	10X Blocking Buffer	6 mL
8209803	1X Development Solution	12 mL

FlexPlex™ Module

Item	Amount
10X Detector Antibody	0.2 mL
10X HRP Label	0.2 mL
Microplate strips (4-pack)	1

Storage:

Store all components at 4°C. The kits are stable for at least 6 months.

Principle:

The microplate strips have a monoclonal antibody pre-bound to each well. After incubation with cell lysate or tissue homogenate a specific detector antibody is added. Then an HRP-conjugated label is added to bind to the detector antibody. Finally an HRP-substrate is added, and color change of this substrate is measured, which is proportional to the quantity of bound target protein.

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer plate reader (Molecular Devices SpectraMax recommended) capable of measuring absorbance at 600 nm (or 450 nm after addition of 1N HCl (not supplied)).
- Method for determining protein concentration
- Deionized water
- Multichannel pipette
- PBS (phosphate buffered saline – for recipe see www.mitosciences.com/PDF/western.pdf)
- Optional for 450 nm endpoint data measurement – 1N HCl
- Optional plate shaker for all incubation steps

ASSAY PROTOCOL

A. Preparation of buffers

1. It is highly recommended to first label each microplate strip with the corresponding FlexPlex™ Module number on the bottom tab of each strip using a waterproof pen such as a Sharpie®.
2. Prepare 1X Wash Buffer by adding 12 mL 20X Wash Buffer to 228 mL nanopure water.
3. Prepare 1X Incubation Buffer by adding 1 mL 10X Blocking Buffer to 9 mL 1X Wash Buffer.
4. Immediately before use (in Step C4) prepare 1X Detector Antibody. For 4 strips add 0.2 mL 10X Detector Antibody to 1.8 mL 1X Incubation Buffer.
5. Immediately before use (in Step C6) prepare 1X HRP Label. For 4 strips add 0.2 mL 10X HRP Label to 1.8 mL 1X Incubation Buffer.

B. Sample preparation

Note: *Samples must be detergent extracted before use. Suggestions for sample preparation, pelleting and detergent extraction can be found in Mitosciences Sample Preparation Guide - Method I: (<http://www.mitosciences.com/PDF/mitosciences-sample-preparation-guide.pdf>).*

1. A. Mitochondrial preparations are typically centrifuged at 12000 g for 20 min at 4°C to pellet the mitochondria. Cultured cells are typically pelleted at 500 g for 10 min at 4°C. Estimate the volume of the mitochondrial or cell pellet and then resuspend the sample pellet in 9 volumes of Sample Extraction Buffer.

B. Tissue homogenates are typically prepared by dounce homogenization of minced tissues in PBS to produce suspensions with a total concentration of approximately 25 mg/mL protein. Four volumes of Sample Extraction Buffer are added to extract.
2. Place the sample in Extraction Buffer on ice for 20 minutes. Centrifuge 12000 x g 4°C for 20 minutes. Save the supernatant and discard the pellet.

3. Determine the protein concentration of the supernatant extract (e.g. by BCA assay).
4. The sample should be diluted to within the working range of the assay in 1X Incubation Buffer. Undiluted extract can be frozen at -80°C. The working range of each FlexPlex assay is described in the specific Technical Data Sheet accompanying that assay.

C. Procedure

1. Add 50 µL of each diluted sample into individual wells. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a buffer control (50 µL Incubation Solution only) as a null or background reference. A procedure for setting up a dilution series of a control sample as a reference is shown on page 5 (“Setting up a reference dilution series”). It is recommended to use the plate map on page 9 to record the FlexPlex™ assay number of each strip and the location of samples.
2. Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
3. Wash the plate
 - a. Empty the wells by turning the plate over a receptacle and firmly shaking out the well contents in one rapid downward motion.
 - b. Rapidly add 300 µL 1X Wash Buffer to each well. The wells must not become dry during any step. Repeat this wash once more for a total of two washes. After the last wash, strike the microplate surface onto paper towels to remove excess liquid.
4. Add 50 µL of 1X Detector Antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature.
5. Repeat the wash procedure in step C3.
6. Add 50 µL of 1X HRP Label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. Meanwhile, prepare the microplate spectrophotometer using the parameters described below.
7. Repeat the wash procedure in step C3, however, performing a total of **three** washes.
8. Add 100 µL HRP Development Solution to each empty well and immediately record the blue color development in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Alternative– In place of a kinetic reading, at a **user defined**, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 50 µL stop solution (1N HCl) to each well and record the OD at 450 nm.

9. Analyze the data as shown on page 6 (“Data analysis”).

Setting up a reference dilution series

When comparing an unknown sample to a reference sample, make a dilution series of the reference in the incubation buffer. An example is described below:

- (1) Extract the reference cell line/tissue in extraction buffer as in Section B.
- (2) Measure the protein concentration of all extracted samples. Dilute the reference sample to the maximum specified concentration described in the FlexPlex™ Technical Data Sheet. Prepare a two-fold dilution series in incubation buffer by diluting at least 50 μL sample volume in an equal volume of incubation buffer in the serial fashion shown below. The dilution series should be prepared in clean microtubes, do not prepare samples by dilution directly in the microplate wells.

When using a pure protein standard simply prepare a dilution series in incubation buffer.

- (3) Load 50 μL of each diluted reference sample per well. Alongside, load 50 μL of the experimental samples diluted to a concentration which is within the reference dilution series. The unknown amount of FlexPlex™ analyte can thus be determined by extrapolation using graphing or microplate software (see the Data Analysis section below). It is recommended to load 2-4 replicates of each sample for improved accuracy.

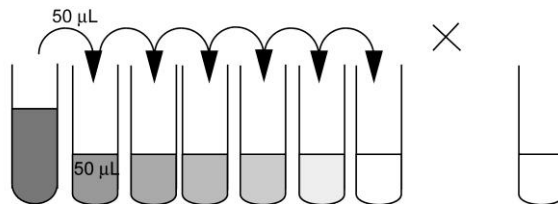
(1) Preparation of reference sample.

Reference sample extracted, protein assayed and diluted in incubation buffer .

In this example dilute the reference sample to the highest concentration in the series - 40 $\mu\text{g}/50 \mu\text{L}$ i.e. 0.8 mg/mL



(2) Serial dilution of reference sample in incubation buffer.



(3) Plate loading reference and unknowns.

Reference sample (50 μL)		1	2	3	4
40 μg	A	●	○	○	○
20 μg	B	●	○	○	○
10 μg	C	●	○	○	○
5 μg	D	●	○	○	○
2.5 μg	E	●	○	○	○
1.25 μg	F	●	○	○	○
0.6 μg	G	○	○	○	○
0 μg	H	○	○	○	○

Experimental samples loaded at 5 $\mu\text{g}/50 \mu\text{L}$ in columns 2,3 and 4

DATA ANALYSIS

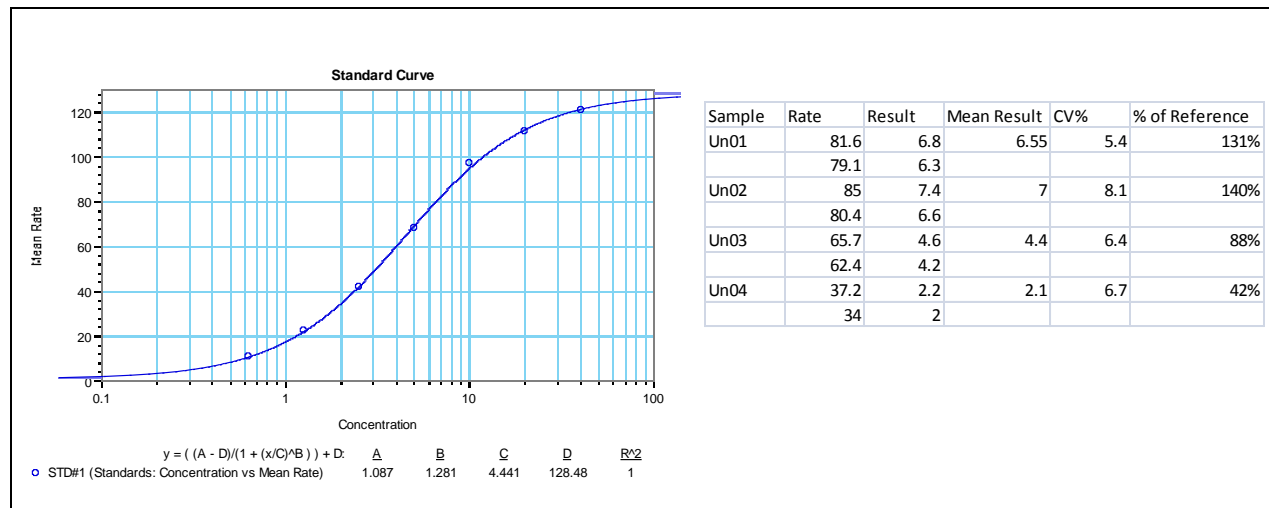
Creating a standard curve of reference sample and comparing unknown samples.

A standard curve was set up using a reference sample as described on page 5. Also unknown samples #1-#4 were measured at 5 µg total protein/50 µl (the midrange of the standard curve) and their resulting signals (mOD/min) were interpolated into this standard curve to determine the quantity of reference protein required to generate the same signal as the 5 µg of unknown.

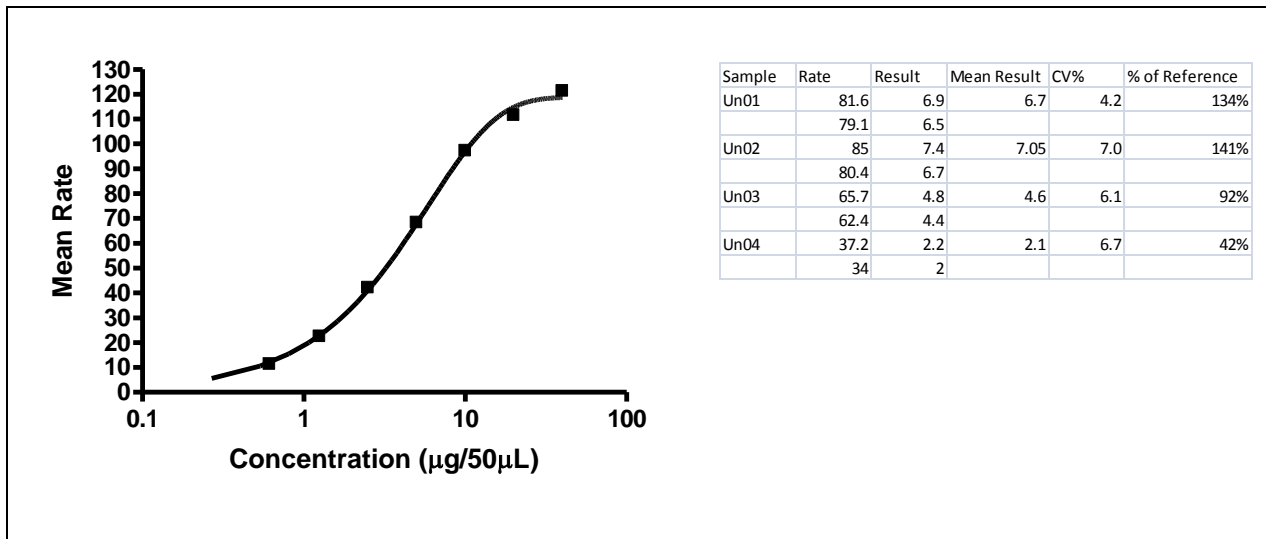
The concentration of reference sample required to generate the same signal in the unknown sample provides a measure of the relative concentration of target antigen in that unknown sample.

As an example using the curve and data below, the unknown sample #1 (Un01) loaded at 5 µg/well generates the same signal as 6.55 µg of reference sample after interpolation from the reference standard curve. Therefore Un01 contains $6.55/5=1.31$ of the concentration of target antigen in the reference sample. Furthermore the CV of the measurement is 5% therefore the sample Un01 is $131 \pm 5\%$ of the concentration of target antigen in the reference sample.

Example of curve fitting and sample interpolation using microplate software (e.g., SoftMaxPro, 4-PL fit):

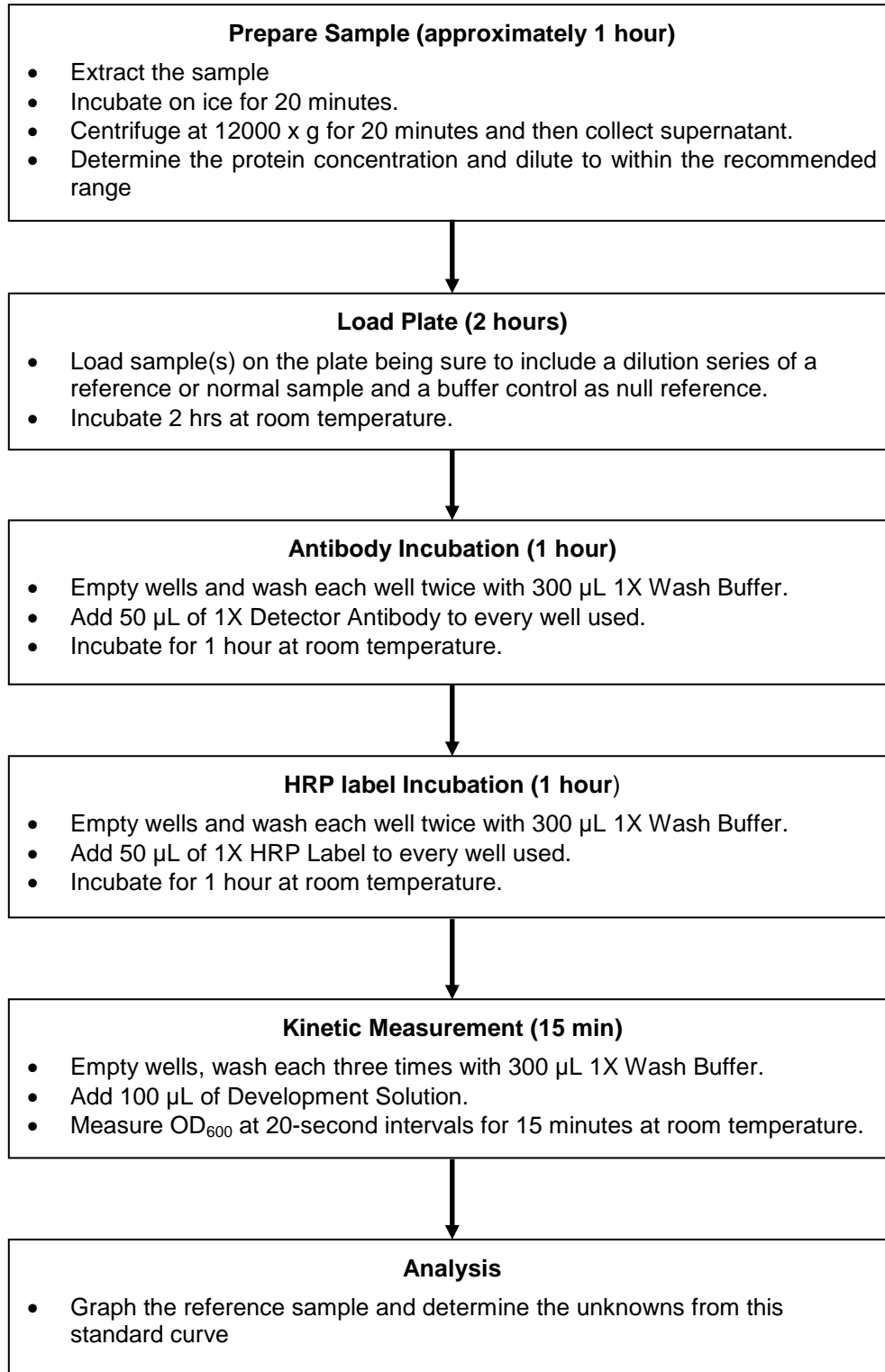


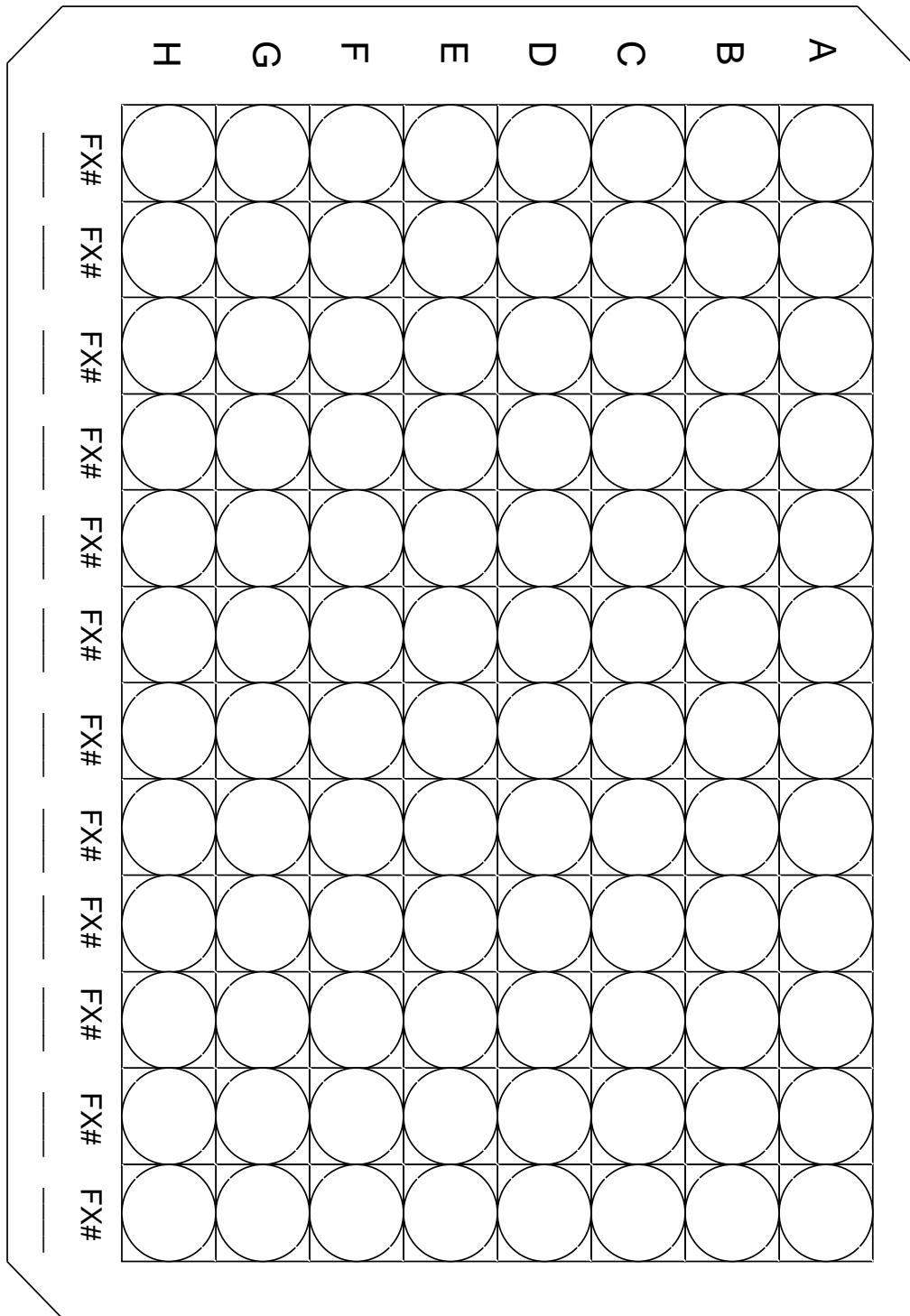
Another example of curve fitting and sample interpolation but instead using graphing software (e.g. GraphPad Prism4, 4-PL - 'Sigmoidal dose response variable fit'):



FLOW CHART

(For quick reference only. Be completely familiar with previous details of this document before performing the assay.)





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