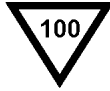


FIDIS™ HUMAN CYTOKINE Th1/Th2 FIVE-PLEX

English

REF

MX HC0502



For Research Use Only

INTRODUCTION

Advances in the field of cell biology have defined a complex and interdependent set of extracellular and intracellular signaling molecules that control normal cell function. Perturbations in signaling pathways may be important indicators, and possibly the root cause, of many diseases. Therefore, there is growing interest among clinicians as well as drug discovery groups in monitoring multiple components of signaling pathways simultaneously. Solid phase multiplex protein assays have become the primary tools of choice in these studies as they maximize efficiency by permitting several analytes to be profiled within individual samples.

BMD's Multiplex Bead Immunoassays are solid phase protein assays which use spectrally encoded antibody-conjugated beads as the solid support. The spectral beads are suitable for use in singleplex assays or may be mixed for multiplex assays according to the researcher's needs. Each assay is carefully designed and tested to assure that sensitivity, range and correlation are maximized. The assay is performed in a 96 well plate format and analyzed with a FIDIS™ instrument which monitors the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. Standard curves generated with this assay system extend over several orders of magnitudes of concentrations, while the sensitivity and quantitation of the assays are comparable to ELISAs (Enzyme Linked-Immuno-Sorbent Assays). Assay standards are calibrated to NIBSC reference preparations, when available, to assure accurate and reliable results.

This kit provides a series of combined reagents for the simultaneous measurement of five different human cytokines, including IFN- γ , IL-2, IL-4, IL-5 and IL-10.

The **FIDIS™ Human Cytokine Th1/Th2 5-Plex** assay is designed for the *in vitro* quantitative determination of the designated five analytes.

The **FIDIS™ Human Cytokine Th1/Th2 5-Plex** may also be combined with other extracellular bead reagents, allowing higher multiplexing of the assay. Samples may include serum, plasma, or tissue culture supernatant.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.

PRINCIPLE OF THE METHOD

BMD's Multiplex Bead Immunoassays are solid phase sandwich immunoassays, which are designed to be analyzed with a FIDIS™ instrument. The spectral properties of 100 distinct bead regions can be monitored with the FIDIS™ instrument, a capability that affords this assay system the potential for measuring up to 100 different analytes in a single sample.

- Beads of defined spectral properties conjugated to analyte specific capture antibodies and samples (including standards of known analyte concentration, control specimens, and unknowns) are pipetted into the wells of a filter bottom microplate and incubated for 2 hours. During this first incubation, analytes bind to the capture antibodies on the beads.
- After washing the beads, analyte-specific biotinylated detector antibodies are added and incubated with the beads for 1 hour. During this second incubation, the analyte-specific biotinylated detector antibodies recognize their epitopes and bind to the appropriate immobilized analytes.
- After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (Streptavidin-RPE), is added and incubated for 30 minutes. During this final incubation, the Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich.
- After washing to remove unbound Streptavidin-RPE, the beads are analyzed with the FIDIS™ instrument. By monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin (RPE) fluorescence, the concentration of one or more analytes can be determined.

MATERIAL REQUIRED BUT NOT PROVIDED

- FIDIS™ system. Please contact BMD for instrument and software placement services.
- BMD filtration manifold for bead washing.
- BMD data analysis and graphing software program.
- Sonicating water bath (e.g., Cole Parmer, Cat. # 08849-00).
- Orbital shaker (e.g., Fisher, Cat. # 14-271-9).
- Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable.)
- Distilled or deionized water.
- Glass or plastic tubes.
- Beakers and graduated cylinders in various sizes.
- Aluminum foil.
- Extra filter plate covers.

REAGENTS PROVIDED

Note: Store all reagents at +2°C / +8°C.

	MX HC0502
<i>Filter plate, 96 wells per plate</i> (Extra plates can be purchased from Millipore/Fisher Cat. # MSBVN-1250 or Pall Cat. # S5039)	1 plate
<i>Hu Cytokine Th1/Th2 Five-Plex Bead Concentrate (10x).</i> Contains 7.5 mM sodium azide (0,25mL per vial)	1 vial
<i>Hu Cytokine Th1/Th2 Five-Plex Biotinylated Antibody Concentrate (10x).</i> Contains 15 mM sodium azide (1 mL per vial)	1 vial
<i>Hu Cytokine Th1/Th2 Standard.</i>	2 vials
<i>Wash Solution Concentrate (20x)</i> (15 mL per bottle)	1 bottle
<i>Incubation Buffer.</i> Contains 15 mM sodium azide (12 mL per bottle)	1 bottle
<i>Biotin Diluent.</i> Contains 3.3 mM thymol (12 mL per bottle)	1 bottle
<i>Streptavidin-RPE Diluent.</i> Contains 3.3 mM thymol (12 mL per bottle)	1 bottle

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

PROCEDURAL NOTES / LAB QUALITY CONTROL

1. The **FIDIS™ Human Cytokine Th1/Th2 5-Plex** assay may be combined with other Extracellular Antibody Bead Kits for higher level multiplexing. See the **INFORMATION SHEET and APPENDIX I** for further information. To ensure that several Antibody Bead Kits are compatible in a multiplexed assay, verify that the bead region for each analyte (stated on the **INFORMATION SHEETS** in the Antibody Bead Kits used) is unique.
2. Do not freeze any component of this kit. When not in use, kit components should be stored at +2°C to +8°C. All reagents should be brought to room temperature before use.
3. **The fluorescent beads are light-sensitive.** Protect the beads from light to avoid photobleaching of the embedded dye. Aluminum foil should be used to cover test tubes used in the assay. Filter plates containing beads should be shielded with an aluminium foil-wrapped plate cover. The amber vial does not provide full protection, and should therefore be kept covered in the box when not in use.
4. Do not expose beads to organic solvents.

5. Do not place filter plates on absorbent paper towels during loading or incubations, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.
6. Do not invert the filter plates during the assay. The filter plates are designed to be used in conjunction with a vacuum manifold (DO NOT EXCEED 5 inches Hg) and emptied from the bottom.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This method ensures equal incubation times for all wells.
8. Avoid touching the filter plate membrane with pipette tips to prevent tearing.
9. Do not use reagents after kit expiration date.
10. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.

PREPARATION OF WASH SOLUTION

The *Wash Solution Concentrate* is provided as a 20x concentrate. Upon storage at +2°C to +8°C, a precipitate may form. If this occurs, warm the 20x *Wash Solution Concentrate* to +37°C and vortex until the precipitate is dissolved.

The *Wash Solution Concentrate* is provided as a 20x concentrate. To prepare the Working Wash Solution for use with a 96 well plate, transfer the entire contents of bottle to a 500 mL container and add 285 mL of deionized water. If not using an entire 96 well plate, smaller volumes of 1x Working Wash Solution can be made by mixing 1 part of 20x concentrate with 19 parts deionized water.

The Working Wash Solution is stable for up to two weeks when stored at +2°C to +8°C.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect assay outcome. All washing must be performed with the Wash Solution provided. All phases of the assay, including incubation steps, washing steps, and loading the beads into the FIDIS™ instrument, are performed in the filter bottom plate provided. Unused wells may be left dry during the assay and used at a later time.

1. To wash the beads, place the filter plate on the vacuum manifold and aspirate the wash solution with gentle vacuum. The vacuum setting should be adjusted so 3 seconds are required to empty 0.2 mL solution from the wells. (DO NOT EXCEED 5 inches Hg) Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface.
2. If solution remains in the wells during vacuum aspiration **DO NOT DETACH THE BOTTOM OF THE 96 WELL FILTER PLATE.** In some cases, minor clogs in the filter plate may be dislodged by carefully pressing the bottom of the plate under the clogged well with the pointed end of a 15 mL plastic conical tube.
3. Following the final aspiration step, blot the bottom of the filter plate on clean paper towels to remove residual liquid.

PREPARATION OF SAMPLE

1. Serum, plasma, and tissue culture medium samples have been evaluated with BMD's Multiplex Bead Assays. When possible, avoid the use of hemolyzed or lipemic sera. Suitable sample types are defined on the **INFORMATION SHEET** included in this kit.
2. Samples should be collected in pyrogen/endotoxin-free polypropylene tubes.
3. Samples should be analyzed shortly after collection or frozen. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well (DO NOT VORTEX) prior to analysis.
4. All samples need to be clarified by centrifugation (14,000 rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates.
5. Samples with concentrations that exceed the standard curve should be diluted and reanalyzed. Serum or plasma samples should be diluted in *Assay Diluent*. Tissue culture supernatants should be diluted in the corresponding tissue culture medium.

REAGENT PREPARATION

The **FIDIS™ Human Cytokine Th1/Th2 5-Plex** kit may also be used in conjunction with other bead reagents allowing for a higher level of multiplexing (please see **APPENDIX I** for preparation).

Prior to starting the assay, prepare standard and antibody conjugated beads in accordance with instructions below, then proceed to **Assay Procedure, Step 1**.

• Preparation of Standard

Reconstitute the protein standard within one hour of performing the assay. All standards are calibrated to NIBSC preparations, when available.

The standard included in this kit is provided as a premixed set of related markers. The concentrations of the protein components of the standard are indicated on the **INFORMATION SHEET** included in this kit. Standard dilutions may be performed in glass or plastic tubes.

When using serum or plasma samples, reconstitute the standard with *Assay Diluent* provided.

If using other sample types (e.g., tissue culture supernatant), reconstitute the standard with a mixture composed of 50% *Assay Diluent* and 50% of the matrix which most closely matches the sample type (50%/50% mixture).

For example: When the sample type is RPMI medium containing 5% FBS, the standards should be reconstituted in a mixture composed of 50% *Assay Diluent* and 50% RPMI containing 5% FBS.

• Reconstitution of Lyophilized Standards

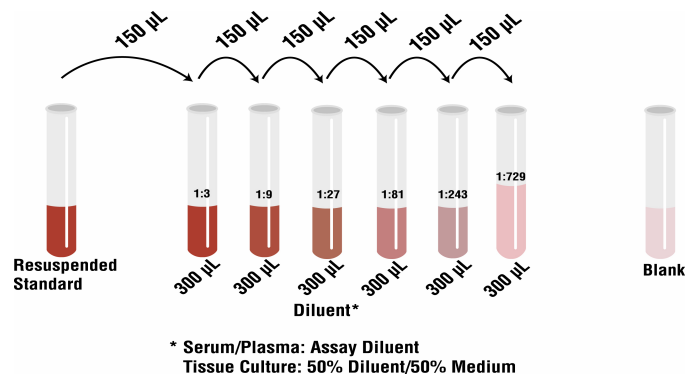
To the standard vial(s) add the suggested reconstitution volume listed below of the appropriate diluent (see above). Do not mix. Replace the vial stopper and allow the vial to stand undisturbed for 10 minutes.

Gently swirl and invert the vial 2-3 times to ensure complete reconstitution and allow the vial to sit for an additional 5 minutes. Protein standards may be analyzed alone, or may be combined with other protein standards for higher levels of multiplexing. **DO NOT COMBINE MORE THAN 4 VIALS.**

One vial of standard	Reconstitute the standard vial in the suggested reconstitution volume, usually 1 mL, of appropriate diluent (see above).
Two vials of standards	Reconstitute each vial with 0.5 mL of appropriate diluent (see above). Combine 300 µL from each vial and mix by pipetting up and down 5-10 times.
Three vials of standards	Reconstitute each vial with 0.333 mL of appropriate diluent (see above). Combine 200 µL from each vial and mix by pipetting up and down 5-10 times.
Four vials of standards	Reconstitute each vial with 0.250 mL of appropriate diluent (see above). Combine 150 µL from each vial and mix by pipetting up and down 5-10 times.

• Prepare the Standard Curve

The standard curve is made by serially diluting the reconstituted standard in *Assay Diluent* (serum and plasma samples) or 50% *Assay Diluent*/50% culture medium. See diagram below. DO NOT VORTEX. Mix by pipetting up and down 5-10 times.



Discard all remaining reconstituted and diluted standards after completing assay. Return the *Assay Diluent* to the refrigerator.

• Preparation of antibody conjugated beads

Determine the number of wells required for the assay. Standard curves and samples may be run singly or in replicates, as desired.

The beads are provided as a 10x concentrate and must be diluted prior to use. Immediately before dispensing, vortex the 10x bead concentrate for 30 seconds followed by sonication in a sonicating water bath for 30 seconds. To make a 1x stock, dilute 2.5 µL 10x beads in 25 µL Working Wash Solution per assay well. Each well requires 25 µL of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Beads	Vol. Working Wash Solution
24	0.06 mL	0.60 mL
32	0.08 mL	0.80 mL
40	0.10 mL	1.00 mL
48	0.12 mL	1.20 mL
56	0.14 mL	1.40 mL
64	0.16 mL	1.60 mL
72	0.18 mL	1.80 mL
80	0.20 mL	2.00 mL
88	0.22 mL	2.20 mL
96	0.24 mL	2.40 mL

ASSAY PROCEDURE

1. Pre-wet the wells designated for the assay. Pipette 0.2 mL of Working Wash Solution into designated wells. Wait 15 to 30 seconds then aspirate the Working Wash Solution from the wells using the vacuum manifold. Wells not used during the assay should be kept dry for future use. An adhesive plate cover may be used to seal the unused wells.
2. Vortex the diluted bead solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.
3. Pipette 25 µL of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then remove the Working Wash Solution from the wells by aspiration with the vacuum manifold. Repeat this washing step. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.

- Pipette 50 μ L *Incubation Buffer* into each well.
- To the wells designated for the standard curve, pipette 100 μ L of appropriate standard dilution.
- To the wells designated for the sample, pipette 50 μ L Assay Diluent followed by 50 μ L sample.
- Incubate the plate for 2 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500-600 rpm).
- Ten to fifteen minutes prior to the end of this incubation, prepare the biotinylated detector antibody in accordance with instructions below, then proceed to ASSAY PROCEDURE, Step 10.

Preparation of Detector Antibody

The Biotinylated Detector Antibody is provided as a 10x concentrate and must be diluted prior to use. To make a 1x stock, dilute 10 μ L 10x Biotinylated Detector Antibody Concentrate in 100 μ L Biotin Diluent per assay well. Each well requires 100 μ L of the diluted Biotinylated Detector Antibody. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Biotinylated Detector Antibody	Vol. Biotin Diluent
24	0.240 mL	2.4 mL
32	0.320 mL	3.2 mL
40	0.400 mL	4.0 mL
48	0.480 mL	4.8 mL
56	0.560 mL	5.6 mL
64	0.640 mL	6.4 mL
72	0.720 mL	7.2 mL
80	0.800 mL	8.0 mL
88	0.880 mL	8.8 mL
96	0.960 mL	9.6 mL

- After the 2 hour capture bead incubation, remove the liquid from the wells by aspiration with the vacuum manifold. Add 0.2 mL *Working Wash Solution* to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
- Add 100 μ L of prepared 1x Biotinylated Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).
- Ten to fifteen minutes prior to the end of the detector incubation step, prepare the *Streptavidin-RPE* in accordance with instructions below, then proceed to Assay Procedure, Step 13.

Preparation of Streptavidin-RPE

The *Streptavidin-RPE* is provided as a 10x concentrate and must be diluted prior to use. To make a 1x stock, dilute 10 μ L 10x *Streptavidin-RPE Concentrate* in 100 μ L *Streptavidin-RPE Diluent* per assay well. Each well requires 100 μ L of the diluted *Streptavidin-RPE*. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Streptavidin-RPE	Vol. Streptavidin-RPE Diluent
24	0.240 mL	2.4 mL
32	0.320 mL	3.2 mL
40	0.400 mL	4.0 mL
48	0.480 mL	4.8 mL
56	0.560 mL	5.6 mL
64	0.640 mL	6.4 mL
72	0.720 mL	7.2 mL
80	0.800 mL	8.0 mL
88	0.880 mL	8.8 mL
96	0.960 mL	9.6 mL

- Remove the liquid from the wells by aspiration with the vacuum manifold. Add 0.2 mL *Working Wash Solution* to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
- Add 100 μ L of prepared 1x *Streptavidin-RPE* to each well and incubate the plate for 30 minutes at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).
- Prepare the FIDIS 100™ instrument during this incubation step. Please see APPENDIX II and software user manual.

- Remove the liquid from the wells by aspiration with the vacuum manifold (DO NOT EXCEED 5 inches Hg). Wash the beads by adding 0.2 mL *Working Wash Solution* to the wells; allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step two additional times for a total of 3 washes. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
- Add 100 μ L of *Working Wash Solution* to each well. Shake the plates on an orbital shaker (500-600 rpm) for 2-3 minutes to resuspend the beads. If the plates cannot be read on the day of the assay, they may be covered and stored in a dark location overnight at +2°C to +8°C for reading the following day without significant loss of fluorescent intensity. Aspirate *Working Wash Solution* from stored plates and add 100 μ L fresh *Working Wash Solution*. Place the plate on an orbital shaker 2-3 minutes prior to analysis.
- Uncover the plate; insert the plate into the XY platform of the FIDIS™ instrument, and analyze the samples.
- Determine the concentration of samples from the standard curve using curve fitting software. The four parameter algorithm usually provides the best fit. **IMPORTANT:** In addition to dilutions performed on the sample prior to running the assay, the sample concentration calculated from the standard curve must be multiplied by an additional factor of 2 to correct for the 1:2 dilution in Step 7.

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the highest standard point; the dose-response is non-linear in this region and inaccurate. Dilute samples that are greater than the highest standard with *Assay Diluent* or appropriate matrix diluent; reanalyze these samples and multiply results by the appropriate dilution factor. The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum/plasma and tissue culture medium samples have not been thoroughly investigated. The rate of degradation of analytes in various matrices may not have been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies.

**This kit is for research use only.
Not for human therapeutic or diagnostic use.**

PERFORMANCE CHARACTERISTICS

Please refer to analyte specific **INFORMATION SHEET** for performance claims.

APPENDIX I: MULTIPLEXING ASSAYS

Before mixing plexes it is important to check that each analyte is represented by a unique bead region. Up to 10 bead concentrates (singleplexes or pre-mixed multiplexes) can be combined to increase the number of analytes being monitored.

CAPTURE BEAD PREPARATION

Volume from each vial of 10x Bead Concentrate to combine:

$$\frac{0.025 \text{ mL} \times \text{Assay Wells}}{10} = \text{mL per vial}$$

Volume Working Wash Solution:

$$(0.0275 \text{ mL} \times \text{Assay Wells}) - (\text{mL } 10\text{x Capture Bead per vial} \times \text{vials}) = \text{mL}$$

Final Volume of diluted multiplexed Capture Beads:

$$(\text{mL Working Wash Solution}) + (\text{mL } 10\text{x Capture Bead per vial} \times \text{vials}) = \text{mL}$$

Example Calculation: Combining 5 vials of 10x Capture Bead Concentrate for 48 assay wells.

Volume from each vial of each 10x Capture Bead Concentrate to combine:

$$\frac{0.025 \text{ mL} \times 48 \text{ Assay Wells}}{10} = 0.120 \text{ mL per vial}$$

Volume Working Wash Solution:
 (0.0275 mL x 48 Assay Wells) – (0.120 mL 10x Capture Beads per vial x 5 vials) = 0.720 mL

Final Volume of diluted multiplex Capture Beads:
 (0.720 mL Working Wash Solution) + (0.120 mL 10x Capture Beads per vial x 5 vials) = 1.32 mL

If desired, premixed beads can be stored at +2°C/+8°C until the expiration date printed on the kit box.

BIOTINYLATED ANTIBODY PREPARATION

Volume from each vial of each 10x Biotinylated Antibody to combine:

$$\frac{0.100 \text{ mL} \times \text{ Assay Wells}}{10} = \text{ mL per vial}$$

Volume Biotin Diluent:

$$(0.110 \text{ mL} \times \text{ Assay Wells}) - (\text{ mL } 10x \text{ Biotinylated Ab. per vial} \times \text{ vials}) = \text{ mL}$$

Final Volume diluted Biotinylated Antibody:

$$(\text{ mL Biotin Diluent }) + (\text{ mL } 10x \text{ Biotinylated Ab. per vial} \times \text{ 10x vials}) = \text{ mL}$$

Example Calculation: Combining 5 vials of 10x Biotinylated Antibody Concentrate for 48 assay wells.

Volume of each 10x Biotinylated Antibody to combine:

$$\frac{0.100 \text{ mL} \times 48 \text{ Assay Wells}}{10} = 0.480 \text{ mL per vial}$$

Volume Biotin Diluent:
 (0.110 mL x 48 Assay Wells) – (0.480 mL 10x Biotinylated Ab. per vial x 5 vials) = 2.88 mL

Final Volume diluted multiplexed Biotinylated Antibody:
 (2.88 mL Biotin Diluent) + (0.480 mL 10x Biotinylated Ab. per vial x 5 vials) = 5.28 mL

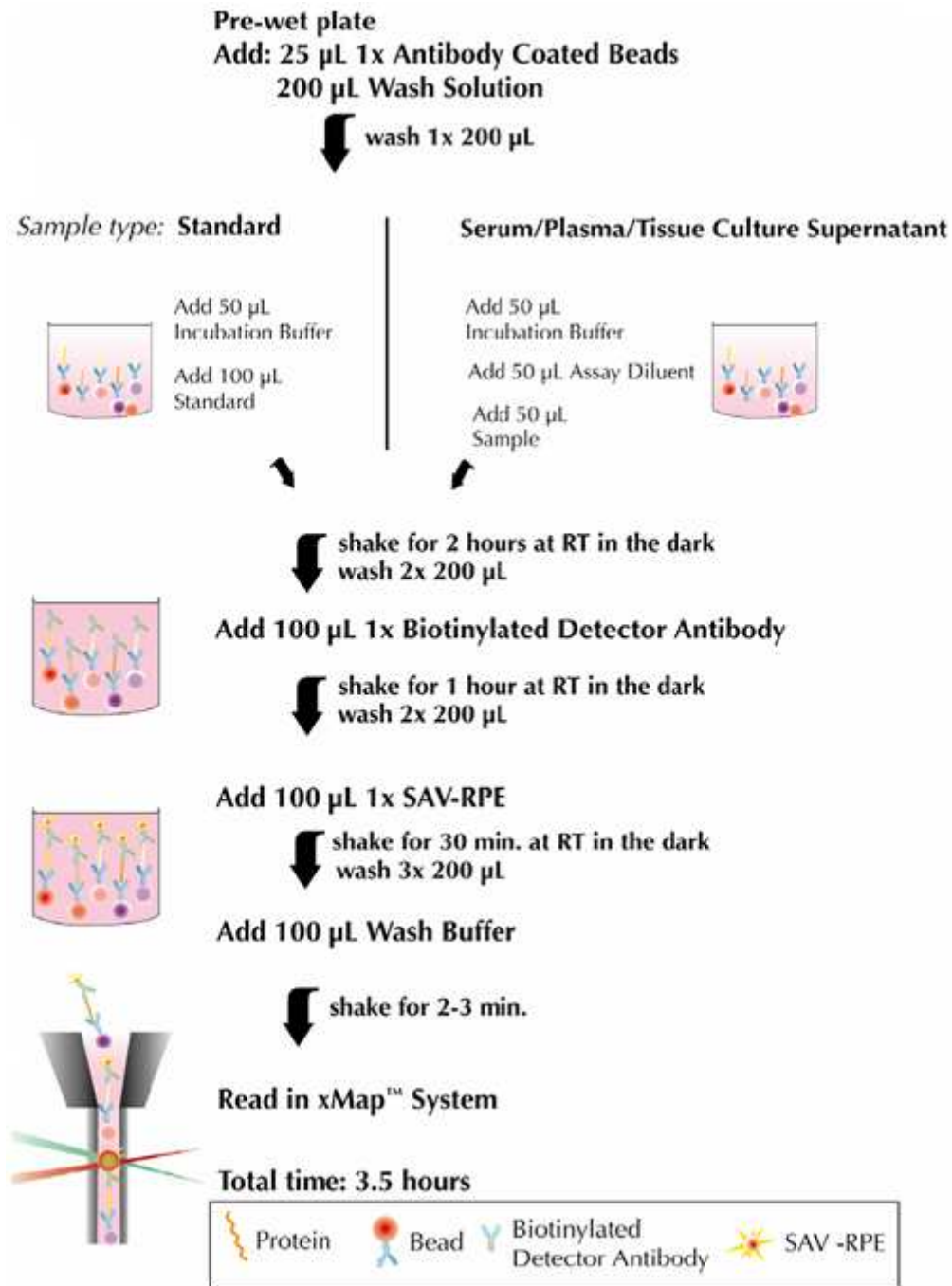
If desired, premixed Biotinylated Detector Antibody can be stored at +2°C - +8°C until the expiration date printed on the kit box.

Helpful Bmd guides for FIDIS™ cytokine users

- Creating a Cytokine method, April 2006
- How to run Cytokine, April 2006

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