PRODUCT INFORMATION AND MANUAL

FlowCytomix Human Basic Kit PE

BMS8421FF

For research use only.

Not for diagnostic or therapeutic procedures.

96 Tests

Human Basic Kit PE
BMS8421FF

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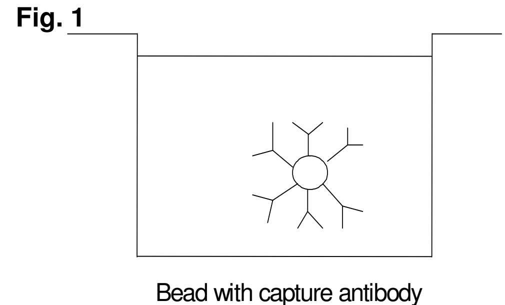
1 INTENDED USE

BMS8421FF is a Basic Kit to be used in combination with BMS Simplex Kits PE to perform the bead based Analyte Detection Assay for quantitative detection of soluble human analytes by Flow Cytometry. In case Simplex Kits PE are combined with other Simplex Kits (not PE Simplex Kits) Basic Kit BMS8420FF is required. **BMS8421FF is for research use only. Not for use in diagnostic or therapeutic procedures.**

2 PRINCIPLES OF THE TEST

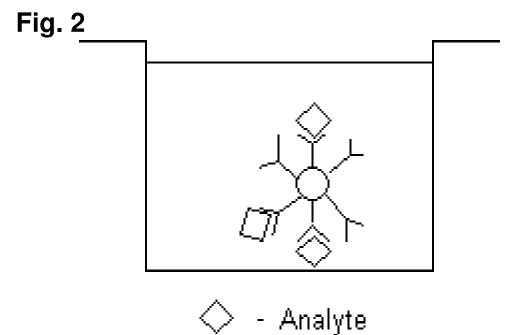
2.1 Principles of the Fluorescent Bead Immunoassay

Beads are coated with antibodies specifically reacting with each of the analytes to be detected in the multiplex system. The beads can be differentiated by their sizes and by their distinct spectral addresses.

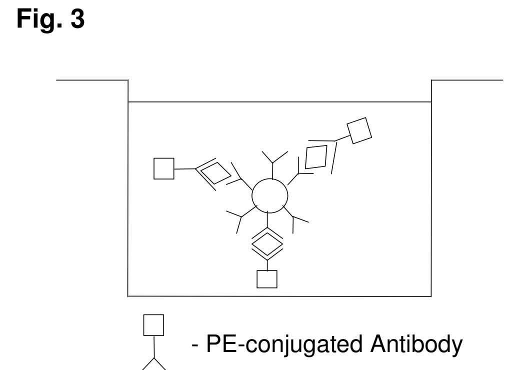


Bead with capture antibody

A mixture of coated beads for each analyte to be measured is incubated with the samples or standard mixture. The analytes present in the sample bind to the antibodies linked to the fluorescent beads.



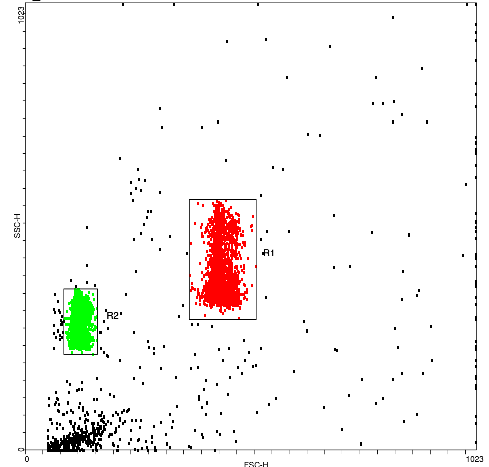
A Phycoerythrin (PE)-conjugated second antibody mixture is added, the specific antibodies bind to the analytes captured by the first antibodies and emit fluorescent signals.



2.2 Principles of the FlowCytomix

Two sets of microspheres of different size are used for the FlowCytomix:
Size A: 5 µm
Size B: 4 µm

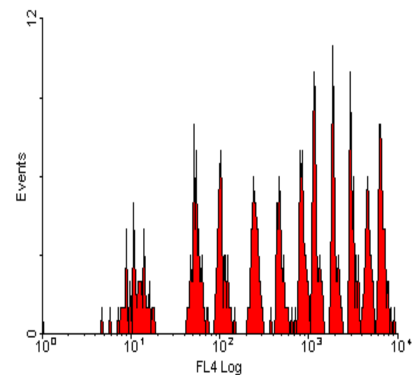
Fig. 4



Size A set consists of 11 bead populations, size B set consists of 9 bead populations internally dyed with different intensities of a fluorescent dye (see Fig. 6 and Fig. 7).

The dye excites with an Argon, He-Ne or even UV laser, and emits in the red (612 nm).

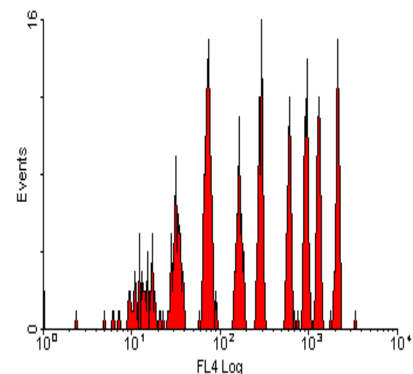
Fig. 5



The two different bead sizes make it possible to distinguish 20 bead sets in one fluorescence channel.

20 different bead sets distinguished by internal dye intensity and bead size allow the simultaneous quantification of 20 analytes in a single small volume sample using the same principle as an ELISA (refer to 2.1).

Fig. 6



3 REAGENTS PROVIDED

1 vial **Setup Beads** (SB)

1 bottle (50 ml**) Assay Buffer** (10x) (PBS with 10% BSA)

1 bottle (13 ml) **Reagent Dilution Buffer** (RDB), ready to use

1 96 - well **Filter Plate**

6 **Adhesive Films**

4 STORAGE INSTRUCTIONS

Store kit and components at 2 to 8°C. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

5 SPECIMEN COLLECTION

For detailed information regarding specimen collection refer to the Simplex Kit Manuals.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly lipemic specimens. Fat causes agglutination of the beads. Centrifugation of lipemic samples (about 16.000 x g for 5 min) before analysis is recommended.

Pay attention to a possible **“Hook Effect”** due to high sample concentrations (see also Simplex Kit Manuals).

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactivity. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

6 MATERIALS REQUIRED BUT NOT PROVIDED

- A flow cytometer equipped with one laser (488 nm or 532 nm) capable of detecting and distinguishing fluorescence emissions at 575 nm and red (612 nm)
- In the case of the test procedure using the filter plate being chosen, a **Filtration manifold** and a **vacuum pump** is required:

  We recommend using the "Multi-Well Plate Vacuum Manifold" (PALL, cat # 5017) for bead washing.

  The Filtration manifold can be ordered via Bender MedSystems in combination with the FlowCytomix Kit:

  Multi-Well Plate Vacuum Manifold, PALL (BMS497FF)
- Centrifuge
- Sample acquisition tubes for a flow cytometer
- Aluminium foil
- 5 ml and 10 ml graduated pipettes
- 10 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 20 µl to 300 µl adjustable multichannel micropipettes with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Glass-distilled or deionized water
- Vortex mixer

- **FlowCytomix Pro Software** (Cat. No. BMS8401FF):
  The FlowCytomix Pro Software is complimentary and can be ordered at customerserv@bendermedsystem.com or downloaded at www.bendermedsystems.com/software-download

7 PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice (www.bendermedsystems.com).
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to light during storage or incubation; the beads and PE-conjugates are photosensitive!
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugates.
- Exposure to acids will inactivate the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.

- Decontaminate and dispose of specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.

- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

8 PREPARATION OF REAGENTS AND SAMPLES

Bring all reagents to **room temperature and vortex well** before usage!

Calculation of reagent volumes using FlowCytomix Pro Software:
Before starting the hands-on work this function facilitates the calculation of reagent volumes specifically for the number of samples you choose. Open the calculator in the FlowCytomix Pro Software. Enter the number of parameters and the number of your samples. Choose single, dual or triple evaluation for both standards and samples. The program automatically provides you with the customized volumes. The volumes given by the program are rounded values. Pipetting losses are already considered.

8.1 Assay Buffer

If crystals have formed in the **Assay Buffer Concentrate (10x)**, warm it gently until they have completely dissolved.

Mix the contents of the bottle well. Add contents (50.0 ml) **Assay Buffer Concentrate (10x)** to 450 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C.

8.2 Preparation of Standard

The standard vials are components of the analyte specific BMS Simplex Kits.

Reconstitution of Standard Protein:

Determine the number of analytes.

It is recommended spinning down vials for a few seconds in a microcentrifuge before opening to collect lyophilized standard at the bottom. The lyophilized standard must be reconstituted by adding distilled water **according to the label on the standard vial**. Swirl vial thoroughly to ensure quantitative solubilization of contents. Wait 10-30 minutes before pipetting the standard.

It is recommended spinning down vials for a few seconds in a microcentrifuge before pipetting reconstituted standard.

Preparation of Standard Mixture:

Add **10 µl** of each reconstituted standard to a vial labeled **standard 1**.

Fill up to the **final volume of 200 µl** with Assay Buffer (1x). See Table 1 for pipetting scheme. (This is a 1:20 dilution of each reconstituted standard.)

Table 1
Examples for the preparation of Standard Mixture depending on the number of analytes (up to 20 analytes within one species can be tested simultaneously).

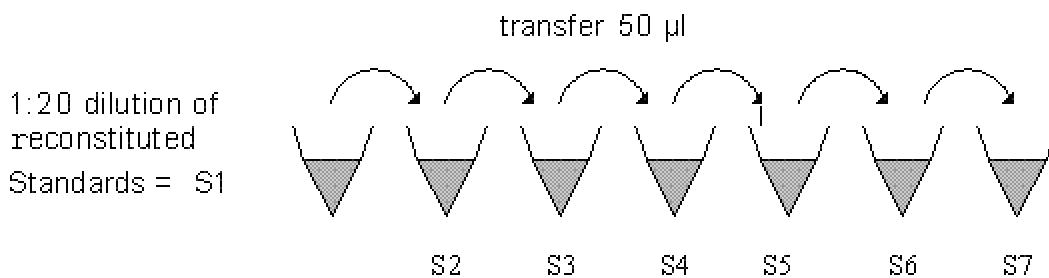
Number of analytes per assay	Volume of each reconstituted standard (µl)	Volume of Assay Buffer (1x) (µl)	Final volume of Standard Mixture (µl)
simplex	10	190	200
2-plex	10	180	200
3-plex	10	170	200
4-plex	10	160	200
5-plex	10	150	200
6-plex	10	140	200
7-plex	10	130	200
8-plex	10	120	200
9-plex	10	110	200
10-plex	10	100	200
11-plex	10	90	200
12-plex	10	80	200
13-plex	10	70	200
14-plex	10	60	200
15-plex	10	50	200
16-plex	10	40	200
17-plex	10	30	200
18-plex	10	20	200
19-plex	10	10	200
20-plex	10	-	200

<u>Serial Dilution of Standard Mixture:</u>

Add **100 µl Assay Buffer (1x)** to 6 tubes labelled **standard 2 to 7.** Transfer **50 µl of standard 1** to **tube 2**, mix the contents of tube 2 and transfer 50 µl to tube 3. Repeat the procedure creating a row of 7 standard dilutions.

Discard immediately any solubilized or prediluted standard left after usage.

Fig. 7



8.3 Preparation of Bead Mixture

The bead set vials are components of the analyte specific BMS Simplex Kits.

Prepare the Bead Mixture in a centrifuge tube according to the following calculation (alternatively refer to chapter 8: Calculation of reagent volumes using FlowCytomix Pro Software).

a. Per test (= well) **25 µl** of the Bead Mixture is required. Consider tests for standard curves, blanks and samples plus an additional test for standard 1 for setup.
   Calculate the final volume (V fin) of the Bead Mixture needed.
   V fin = number of tests x 25 µl
   Round up for pipetting reservoir.
   e.g.: 96 tests x 25 µl = 2400 µl, round up: V fin = 3000 µl

b. Vortex individual bead vial for 5 seconds and pipette 1/20 of final volume (V fin) of each bead set to a vial labelled “Bead Mix”.
   e.g.: V fin = 3000 µl; 1/20 of final volume = 150 µl

c. Fill up to the final volume (V fin) with Reagent Dilution Buffer (RDB).

d. Centrifuge at 3000 x g for 5 min.

e. Carefully remove excess liquid from the surface, leaving 50 µl in the vial (e.g.: total volume 3000 µl, take off 2950 µl). Avoid resuspension of beads.

f. Add the same volume of Reagent Dilution Buffer that has been removed (e.g.: 2950 µl) and vortex for 5 seconds.

For examples see Table 2.

Table 2
Examples for the preparation of Bead Mixture depending on the number of analytes and tests (up to 20 analytes within one species can be tested simultaneously).

	48 tests Final volume of Bead Mixture: 1500 µl		**96 tests Final volume of Bead Mixture: 3000 µl**	
Number of analytes per assay	**Volume of each Bead Set (µl)**	**Volume of RDB (µl)**	**Volume of each Bead Set (µl)**	**Volume of RDB (µl)**
simplex	75	1425	150	2850
2-plex	75	1350	150	2700
3-plex	75	1275	150	2550
4-plex	75	1200	150	2400
5-plex	75	1125	150	2250
6-plex	75	1050	150	2100
7-plex	75	975	150	1950
8-plex	75	900	150	1800
9-plex	75	825	150	1650
10-plex	75	750	150	1500
11-plex	75	675	150	1350
12-plex	75	600	150	1200
13-plex	75	525	150	1050
14-plex	75	450	150	900
15-plex	75	375	150	750
16-plex	75	300	150	600
17-plex	75	225	150	450
18-plex	75	150	150	300
19-plex	75	75	150	150
20-plex	75	0	150	0

8.4 Preparation of PE-Conjugate Mixture

The PE-Conjugate vials are components of the analyte specific BMS Simplex Kits.

Prepare the the PE-Conjugate Mixture according to the following calculation (alternatively refer to chapter 8: Calculation of reagent volumes using FlowCytomix Pro Software).

a. Per test (= well) **50 µl** of the the PE-Conjugate Mixture is required. Consider tests for standard curves, blanks and samples plus an additional test for standard 1 for setup.
   Calculate the final volume (V fin) of the PE-Conjugate Mixture needed.
   V fin = number of tests x 50 µl
   Round up for pipetting reservoir.
   e.g.: 96 tests x 50 µl = 4800 µl, round up: V fin = 6000 µl

b. Pipette 1/20 of final volume (V fin) of each PE-Conjugate to a vial labelled “PE-Conjugate Mix”.
   e.g.: V fin = 6000 µl, 1/20 of final volume = 300 µl

c. Fill up to the final volume (V fin) with Reagent Dilution Buffer (RDB).

For further examples see Table 3.

Table 3

Examples for the preparation of PE-Conjugate Mixture depending on the number of analytes and tests (up to 20 analytes within one species can be tested simultaneously).

	48 tests Final volume of PE-Conjugate Mixture: 3000 µl		**96 tests Final volume of PE-Conjugate Mixture: 6000 µl**	
Number of analytes per assay	**Volume of each PE-Conjugate (µl)**	**Volume of RDB (µl)**	**Volume of each PE-Conjugate (µl)**	**Volume of RDB (µl)**
simplex	150	2850	300	5700
2-plex	150	2700	300	5400
3-plex	150	2550	300	5100
4-plex	150	2400	300	4800
5-plex	150	2250	300	4500
6-plex	150	2100	300	4200
7-plex	150	1950	300	3900
8-plex	150	1800	300	3600
9-plex	150	1650	300	3300
10-plex	150	1500	300	3000
11-plex	150	1350	300	2700
12-plex	150	1200	300	2400
13-plex	150	1050	300	2100
14-plex	150	900	300	1800
15-plex	150	750	300	1500
16-plex	150	600	300	1200
17-plex	150	450	300	900
18-plex	150	300	300	600
19-plex	150	150	300	300
20-plex	150	0	300	0

9 TECHNICAL TIPPS

Please read carefully before you start:

9.1 Mixing

When preparing the Bead Mixture, pipette the bead solution all the way down **to the bottom of the tube**, so that you do not lose any material on the walls of the tube.
Inadequate mixing can lead to little or no beads being detected. **Vortex the beads** immediately before mixing with the standards or samples and again before analysis on the flow cytometer. Vortex each sample tube for 3-5 seconds before placing the tube on the flow cytometer as this will yield better discrimination of the bead populations in the red fluorescence channel.

9.2 To avoid double bead populations in the analysis plot

When applying the bead solution to the assay plate, put the beads **into the standard- / sample solution** in the well to make sure that the beads do not stick on the side of the tube/well.
(Beads which do not come in contact with the detection antibody during the incubation step will show a much lower PE signal. These beads will lead to a second, almost “PE-negative” population.)

9.3 To optimize sensitivity

It is critical that all steps after the addition of the detector reagents are **protected from light** to obtain maximum fluorescence of the PE reporter system.
When establishing the instrument settings with Standard one (highest concentration), it is essential to **place the bead populations at the very right margin of the acquisition plot**. Thereby the distribution of all standard concentrations across all decades in the PE channel will be optimized.

9.4 Blocked filter plate

Serum or plasma samples can sometimes block the filter plate during washing. If this occurs very carefully push a needle through the hole underneath the respective well of the plate. Do not stick the needle all the way through the filter as this will damage the membrane!

10 TEST PROTOCOL

10.1 Test Procedure Using the Filter Plate

In this case a filtration manifold (see chapter 6) is required for test performance.

Prepare Assay Buffer referring to 8.1. Prepare PE-Conjugate, Bead Mixtures and Standards referring to 8.4, 8.3 and 8.2.

a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards and calculate amount of reagents needed respectively (see chapter 8). Place adhesive film over unused wells so that vacuum filtration works efficiently.

   We highly recommend preparing 2 standard curves and an additional standard 1 (see Table 4 for pipetting scheme) in order to have enough solution of standard 1 also for cytometer setup (section 11 of this manual).

b. Add 50 µl **Assay Buffer (1x)** to the filter plate to pre-wet the wells. Aspirate using the vacuum filtration manifold. Blot the bottom of the plate after filtration.

c. Add 25 µl of **Standard Mixture dilutions 1 to 7** to designated wells of the plate (refer to Preparation of Standard 8.2).

Table 4

An example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3
A	Standard 1	Standard 1	Standard 1 for Setup
B	Standard 2	Standard 2	Sample 1
C	Standard 3	Standard 3	Sample 2
D	Standard 4	Standard 4	Sample 3
E	Standard 5	Standard 5	………..
F	Standard 6	Standard 6	………..
G	Standard 7	Standard 7	………..
H	Blank	Blank	………..

d. Add 25 µl of **Assay Buffer (1x)** to the blank wells.

e. Add 25 µl of **Standard Mixture dilution 1** to well A3 (see Table 4) which is designated for cytometer setup (highly recommended).

f. Add 25 µl of **sample** to the designated wells.

g. Add 25 µl of **Bead Mixture** (refer to Preparation of Bead Mixture 8.3) to all wells, including the blank wells.

h. Add 50 µl of **PE-Conjugate Mixture** (refer to Preparation of PE-Conjugate Mixture 8.4) to all wells, including the blank wells.

i. Cover wells with **adhesive film**, avoid putting an excessive pressure on the top of the plate. Protect from light with an aluminium foil and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker at 500 rpm.

j. Remove **adhesive film** and empty wells using the vacuum filtration manifold. Add 100 µl **Assay Buffer (1x)** to microwell strips and empty wells again using the vacuum filtration manifold. Repeat this

step once. Remove any liquid on the bottom of the filter plate with an absorbent towel. Remove towel before next step.

k. Add 200 µl **Assay Buffer (1x)** to each well.

l. Mix the contents of each well by repeated aspiration and ejection, and transfer these 200 µl of each well into a separate sample acquisition tube for a flow cytometer and fill up to 500 µl final volume by adding 300 µl of **Assay Buffer (1x)**.

m. Protect tubes from light.

n. Before analysing samples on a flow cytometer make **cytometer setup** (refer to chapter 11).

10.2 Test Procedure Using Tubes

In this case incubations are performed in tubes. Centrifugation steps are necessary for test performance.

Prepare Assay Buffer referring to 8.1. Prepare PE-Conjugate, Bead Mixtures and Standards referring to 8.4, 8.3 and 8.2.

a. Determine the number of cytometer tubes required to test the desired number of samples plus appropriate number of tubes needed for running blanks and standards standards and calculate amount of reagents needed respectively (see chapter 8).

   We highly recommend preparing 2 standard curves and an additional standard 1 (see Table 4 for pipetting scheme) in order to have enough solution of standard 1 also for cytometer setup (section 11 of this manual).

b. Add 25 µl of **Standard Mixture dilutions 1 to 7** in designated tubes (refer to Preparation of Standard 8.2).

c. Add 25 µl of **Assay Buffer (1x)** to the blank tubes.

d. Add 25 µl of **Standard Mixture dilution 1** to the tube designated for cytometer setup (highly recommended).

e. Add 25 µl of **sample** to the designated sample tubes.

f. Add 25 µl of **Bead Mixture** (refer to Preparation of Bead Mixture 8.3) to all tubes, including the blank tubes.

g. Add 50 µl of **PE-Conjugate Mixture** (refer to Preparation of PE-Conjugate Mixture 8.4) to all tubes, including the blank tubes.

h. Mix the contents of each tube well and incubate at room temperature (18° to 25°C) for 2 hours. Protect from light with an aluminium foil.

i. Add 1 ml of **Assay Buffer (1x)** to all tubes and spin down at 200 x g for 5 minutes.

j. Carefully discard the supernatant from each tube leaving 100 µl of liquid in each tube.

k. Repeat steps i and j.

l. Add 500 µl **Assay Buffer (1x)** to each tube.

m. Protect tubes from light.

n. Before analysing samples on a flow cytometer make **cytometer setup** (refer to chapter 11).

11 CYTOMETER SETUP

Flow cytometer requirements:

For the most commonly used flow cytometers (e.g. Becton Dickinson or DakoCytomation) you can immediately start cytometer setup as below. If you run a FlowCytomix assay on a **FC500 Instrument from Beckman Coulter** ensure that the Forward Scatter (FS) measurements are collected at 1-8°. To accomplish this insert the FS 1-8° Field Stop into place: Remove the front cover to locate the FS 1-8° Field Stop. Slide the knob from the right (= default 1-19° position) to the left and push to lock in place.
On the **Beckman Coulter XL Series** systems the EPICS XL/XL-MCL FS Low Angle Collection Kit is recommended accomplishing this.

General information:

- PE emission is measured at 595 nm (X-axis), red emission is measured at 612 nm (Y-axis).
- Start the setup using the setup beads, use standard 1 (prepared three times for this purpose) for final instrument settings. Save the setup protocol.
- For following experiments before starting sample acquisition adjust voltage (PE/red emission) as well as compensation with standard 1 in Setup Mode.
- Do not change Voltage and Compensation, nor Flow Through (small – medium – high) during measurement.

The **setup beads** provided in the assay are needed

- to adjust FS/SSC parameters.
- to create regions for the bead populations.
- to adjust voltage of PE/red emission so that the bead populations are positioned in the left part of the dot blot. This ensures that the bead populations of standard 1 will be visible on the screen.

Standard 1 for setup is needed

- to adjust voltage of PE emission so that the bead populations are positioned in the right part of the dot blot.
- to adjust compensation.

Please contact us if you require instrument specific setup guides:
techserv@bendermedsystems.com

11.1 Instrument setup

Instrument setup before running the setup beads:

a. Perform instrument start up following the manufacturer’s recommendations.

b. Perform flow check to verify alignment and fluidic stability of the system.

c. Open a new protocol.

d. Create a dot plot window with FS (Forward Scatter) for X- and SSC (Side Scatter) for Y-axis.

e. Set FS and SSC to linear mode.

f. Create a second and a third dot plot window with PE emission, measured at 595nm, for X-axis and red emission, measured at 612 nm for Y-axis. The FL channel number for the specific nm range depends on the instrument. Table 5 shows recommended fluorescence channels for different flow cytometers.

Table 5
Recommended fluorescence channels for different flow cytometers

flow cytometer	PE emission X-axis	red emission Y-axis
BC FC500, BC XL	FL-2	FL-4
BD Calibur, BD Scan	FL-2	FL-3
BD Canto, BD LSRII, BD Aria	PE	PerCP-Cy5.5 or APC
BD Array	Yellow-A	Far Red-A
Dako Cyan	FL-2	FL-8

g. Set the parameters of the fluorescent dot plot to Log mode.

h. Set all Compensation to zero.

i. Save your protocol frequently during setup.

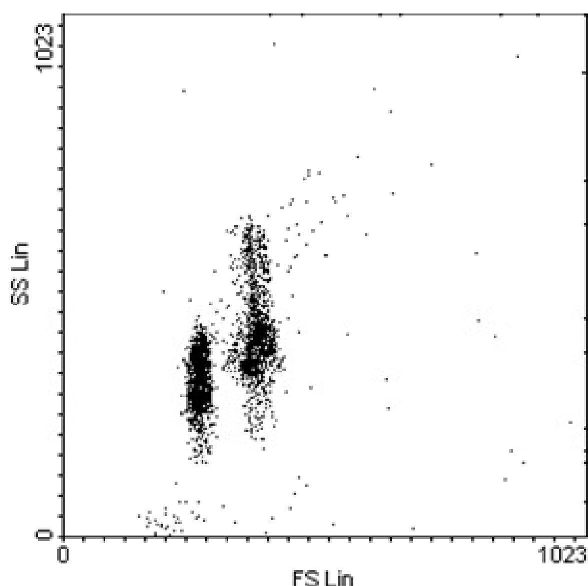
<u>Running the setup beads:</u>

j. Prepare setup beads: Vortex the vial with Setup Beads for several seconds. Pipette 500 µl of Setup Beads into a tube labelled SB.

k. In Setup Mode run tube SB (with Setup Beads).

l. Adjust the parameters of FS and SSC so that both bead populations in the setup beads are visible in the opened window (see Fig. 8).

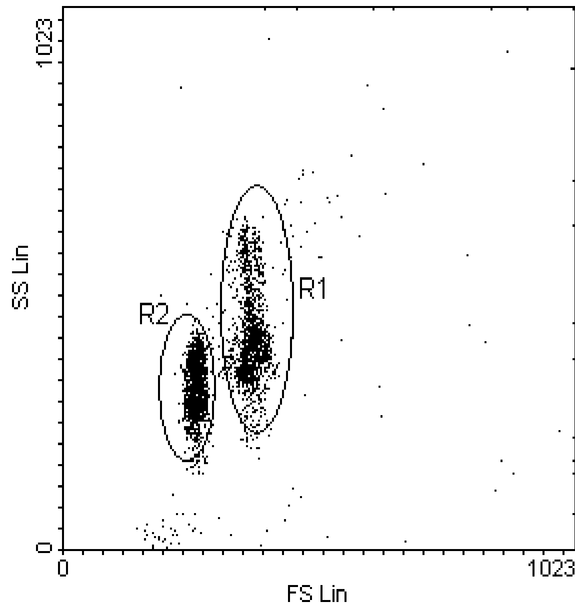
Fig. 8



Pause and restart acquisition frequently during the cytometer setup in order to reset detected values after setting adjustment.

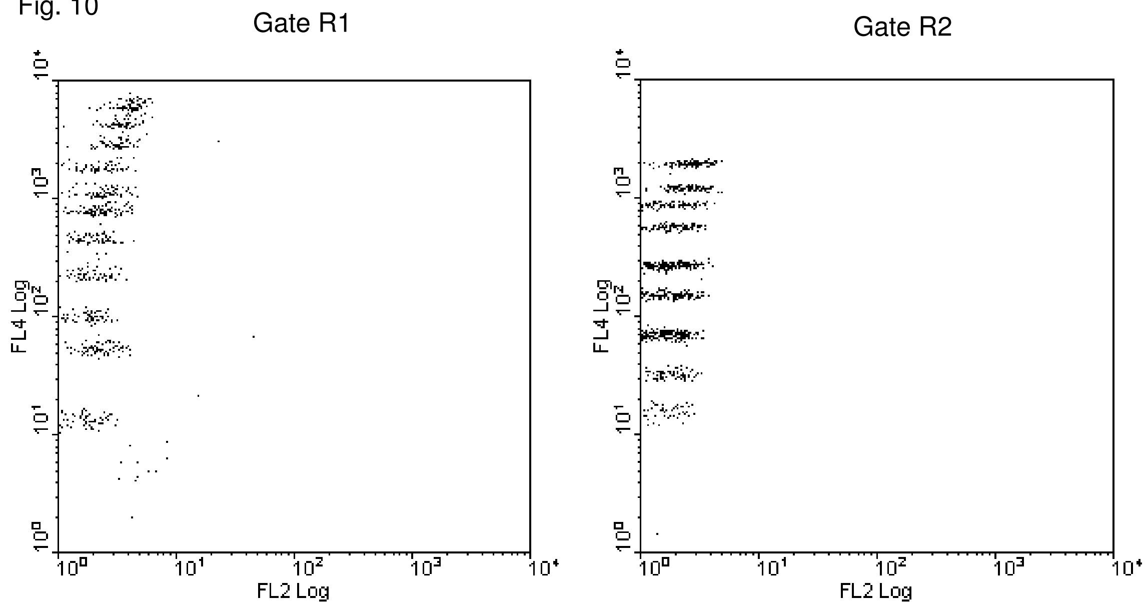
m. Create regions “R1” for the large beads and “R2” for the small beads (see Fig. 9).

Fig. 9



n. Select gate R1 in the first fluorescent Dot Plot (see Fig. 10, Gate R1). Select gate R2 in the second fluorescent Dot Plot (see Fig. 10, Gate R2).

Fig. 10



o. Adjust voltage of the PE emission (X-axis) so that the bead populations are positioned in the left part of the dot blot. This ensures that the bead populations of standard 1 will be visible on the screen (see Fig. 10).

p. Adjust voltage of red emission (Y-axis) so that the bead populations are clearly separated (see Fig. 10).

q. Save your protocol frequently during setup.

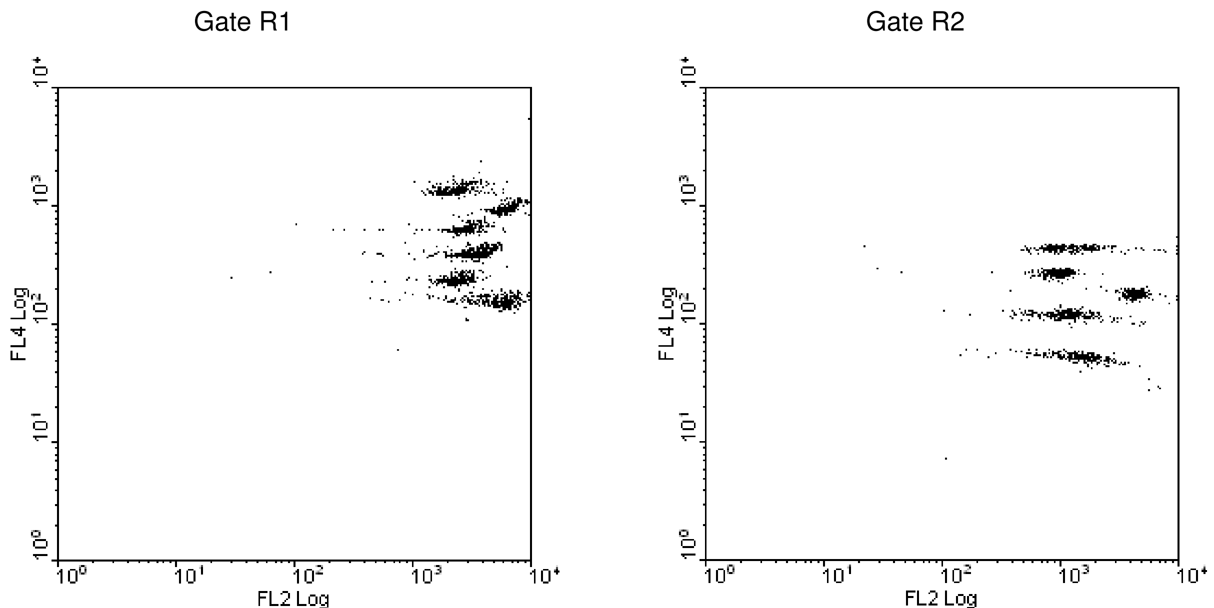
Running standard 1 for setup:

Before starting the acquisition of standards and samples stay in Setup Mode and adjust the settings using standard 1, which gives the highest PE signal.

r. In Setup Mode run standard 1 for setup.

s. Adjust the parameters of PE emission so that the bead population with the highest PE signal **touches the right margin of the** dot plot (to make sure that bead populations with low PE signal are detectable) while the whole population is visible (see Fig. 11, example of a 11-plex).

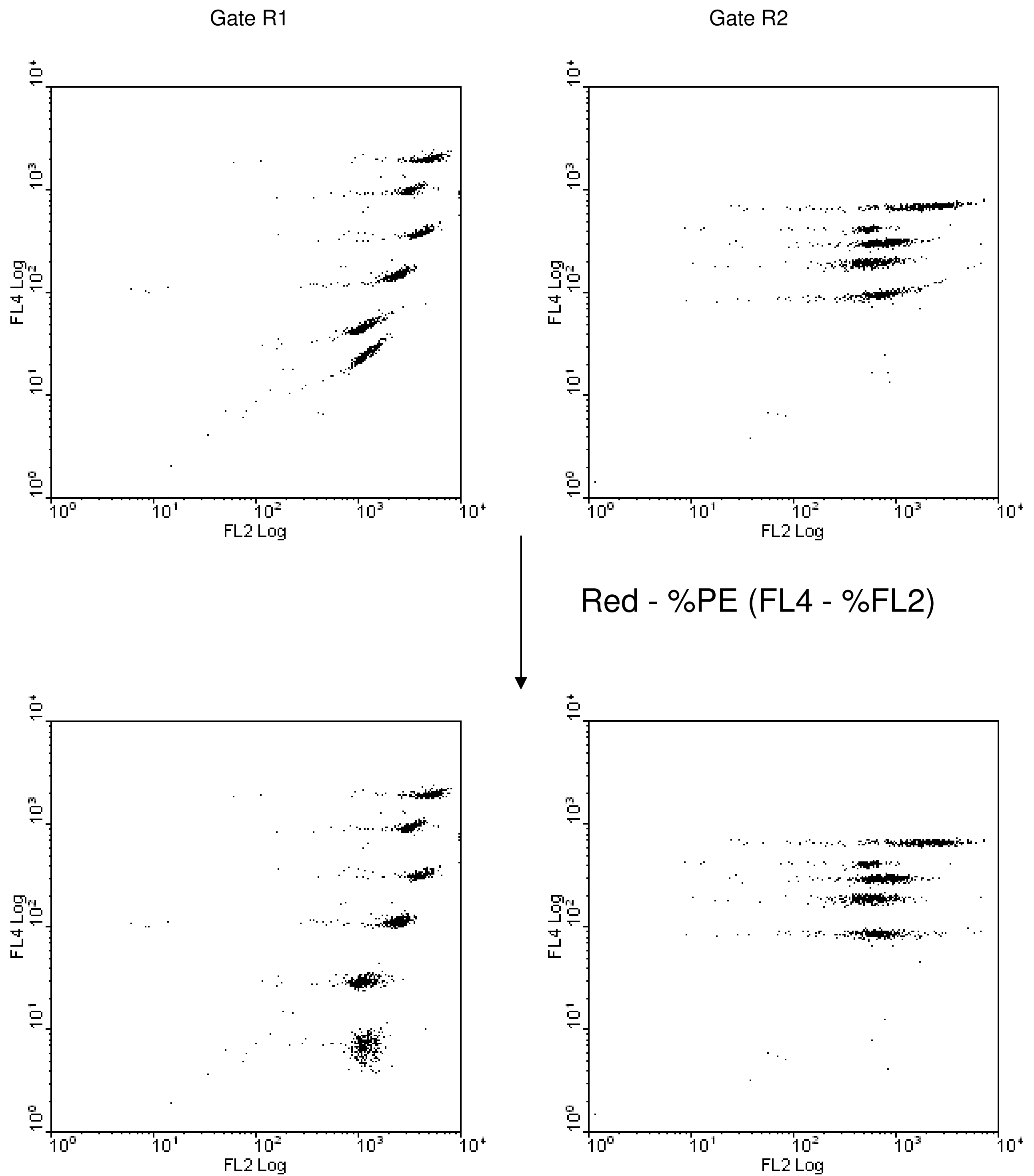
Fig. 11



t. Restart acquisition of standard 1 for setup.
(Pause and restart acquisition frequently during the cytometer setup in order to reset detected values after setting adjustment.)

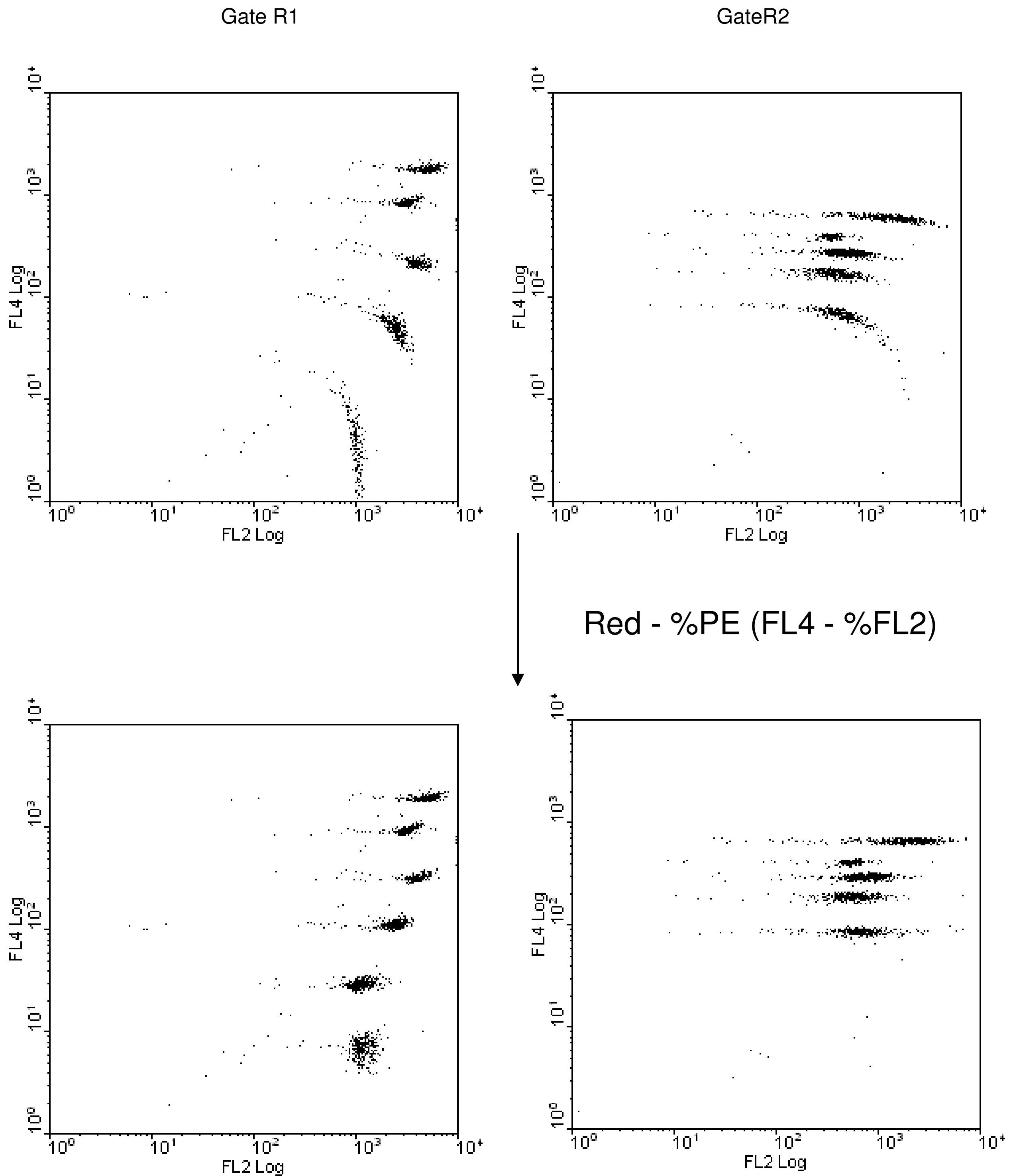
u. If the bead populations are not in a horizontal position (as shown in Fig. 12) **increase** compensation setting for Red - %PE.

Fig. 12



v. If the bead populations are not in a horizontal position (see Fig. 13) **decrease** compensation setting for Red - %PE.

Fig. 13



Please note:

There is no compensation PE – %Red (PE - %Red = 0)!

w. Define number of events so that 300 events per analyte are measured within gate R2 (small beads) e.g. 5 small bead populations; 300 events per analyte –>> count 1500 events of gated bead population in R2. In case only larger beads are measured define number of events so that 300 events per analyte are measured within gate R1 (large beads).

Please note:

Too many beads acquired can lead to huge data file sizes, which can not be processed by the FlowCytomix Pro software!

x. Save your protocol.

A final setup **may** look like shown here:

A final setup (e.g.: for **BD FACSCalibur**) **may** look like that shown in Table 6.
Please note: individual setup is required

Table 6

Detector	Voltage	Amp	Mode
FS	E00	5.4	Lin
SSC	350	2	Lin
FL1	600	1	Lin
FL2	650	-	Log
FL3	610	-	Log

Compensation		
FL1	0.0	FL2
FL2	0.0	FL1
FL2	0.0	FL3
FL3	7.5	FL2

A final setup (e.g.: for **BD FACScan**) **may** look like that shown in Table 7.
Please note: individual setup is required

Table 7

Detector	Voltage	Amp	Mode
FS	E01	1.9	Lin
SSC	336	1	Lin
FL1	163	-	Log
FL2	316	-	Log
FL3	501	-	Log

Compensation		
FL1	0.0	FL2
FL2	0.0	FL1
FL2	0.0	FL3
FL3	**10.0**	FL2

A final setup (e.g.: for **BC FC500**) **may** look like that shown in Table 8.
Please note: individual setup is required

Table 8

	Detectors	Gain
FS	200-400	20
SSC	200-400	5 to 10
FL1	200-400	1
FL2	500	1
FL3	250	1
FL4	580	1
FL5	250	1

Compensation		
	FL1	FL2
FL1		
FL2		
FL3		
FL4		10
FL5		

11.2 Acquisition of standards and samples

a. Switch from Setup Mode to Acquisition Mode.

b. To simplify auto file loading in the analysis software save all measured data with the same file name and consecutive numbering (e.g.: Data.001, Data.002, Data.003, …).

c. Begin analysing samples starting with the standard curve (S1-S7 and blank), followed by the samples.

12 CALCULATION OF RESULTS

For calculation of results refer to the BMS FlowCytomix Software manual.

The FlowCytomix Pro (Cat. No. BMS8401FF) Software is complimentary and can be ordered at customerserv@bendermedsystem.com or downloaded at www.bendermedsystems.com/software-download

- To determine the concentration of circulating analytes for each sample, first find the fluorescent intensity value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding concentration.
- It is suggested that each testing facility establishes a control sample of known concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

13 LIMITATIONS

- Since exact conditions may vary from assay to assay, standard curves must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

14 FAQ FOR FLOWCYTOMIX KITS

Is there a particular flow cytometer required for the use of FlowCytomix Kits?

The FlowCytomix beads are detectable in the most commonly used flow cytometers. The flow cytometer needs to be equipped with one laser (488 nm or 532 nm) capable of detecting and distinguishing fluorescence emissions at 575 nm and red (612 nm).

FlowCytomix suitability has been tested for:

Beckman Coulter:
EPICS® XL™ / XL-MCL ™ (see section 11 of this booklet)

Cytomics ™ FC500 (to resolve the two bead populations, the Forward Scatter collection angles have to be at the correct position. Move the Field Stop mechanism to the left position in order to change the Forward Scatter collection angles from 1-19 degrees to 1-8 degrees.)

BD FACScan ™
BD FACSCalibur ™
BD FACSCanto ™
BD ™ LSR I
BD ™ LSR II

Is there a special software required?

Yes, the FACS raw data is analysed with the specifically designed FlowCytomixPro Software. The FlowCytomix Pro (Cat. No. BMS8401FF) Software is complimentary and can be ordered at customerserv@bendermedsystem.com or downloaded at www.bendermedsystems.com/software-download

Does the software interfere with the flow cytometer operating system?

Software has been virus checked with latest version of McAfee VirusScan. FlowCytomixPro Software has been successfully operated in conjunction with all commonly used flow cytometer models. It does not interfere with the flow cytometer operating system.

In addition the FlowCytomixPro Software does not need to be installed on the flow cytometer working station; data files can be transferred to any other computer.

In which case is a Basic Kit required?
A Basic Kit is required for any combination of Simplex Kits. In order to run a FlowCytomix assay some reagents are needed only once, even in the case of combining several Simplex Kits. These reagents are provided in a Basic Kit.

Are the assays performed in plates or in tubes?
The FlowCytomix assays can be either performed like an ELISA in a 96-well filter plate, in this case a filtration manifold is required, or alternatively in tubes, which requires centrifugation of the tubes during the washing procedure.

Why is a cytometer setup required before measuring standards and samples?
The cytometer setup is required by the mode of operation of the flow cytometer. Setup Beads are included in the Basic Kits and in the Multiplex Kits.
With every new experiment, before starting the acquisition of standards and samples stay in SET UP MODE and adjust the settings using the highest standard 1.

What wavelength is chosen for measurement?
The maximum emission of PE is at 578 nm and the emission of the beads is in the red region (612 nm).

Is it possible to measure with high "flow through" settings?
There are three flow through settings: low-medium-high.
Increasing the "flow through" may result in a dispersed bead population. Therefore it is recommended starting with the low "flow through"; it can only be increased if the bead population does not start to disperse.
Once the final instrument settings are saved, do not change the "flow through" **during** measurement.

Does a FlowCytomix Kit work on a Luminex™ instrument?
No, the FlowCytomix Kit can only be measured on a flow cytometer.

Is it correct to use the non linear part of the standard curve?
Yes, it is possible to use the non linear part of the standard curve for calculation of results. Dilutions of samples behave in the same way as the standard curve.

FAQ´s regarding Combination:

How many analytes can be combined in one assay?
Up to 20 analytes are possible.

Which analytes can be combined?
There are 20 different bead sets that can be distinguished in the FlowCytomix assay. Analytes containing different bead sets can be analysed simultaneously. The analytes and the corresponding bead sets are indicated in the Simplex Kit manuals.

Which Simplex Kits can be combined with each other?
All Simplex Kits can be combined with each other, as long as they contain different bead sets.
For analysis of Simplex Kits a Basic Kit is required.

Can Simplex Kits be combined with a Multiplex Kit?
It is possible in case all analytes contain different bead sets. Pay attention to the kind of Conjugate (PE or Biotin). For detailed information about possible combinations refer to “Combination Table“ on www.bendermedsystems.com.

Can components from different lots be mixed?
The bead sets, standards and conjugates are lot-specific and must be used in combination with each other. Do not mix these components from different kit lots.
Assay Buffer, Reagent Dilution Buffer and Streptavidin-PE are not lot-specific and can therefore be exchanged between different kit lots.

Is it possible to combine Biotin Conjugates with PE-conjugates in one Conjugate Mixture?
In case you are using at least one Biotin-Conjugate in your combination a further incubation step with Streptavidin-PE is necessary. This does not interfere with the PE-conjugates.

15 ORDERING INFORMATION

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16 REAGENT PREPARATION SUMMARY

16.1 Assay Buffer

Table 9

Assay Buffer Concentrate (10x) (ml)	Distilled Water (ml)
50	450

16.2 PE-Conjugate Mixture

Make a 1:20 dilution of each PE- Conjugate creating a Mixture of conjugates.

16.3 Bead Mixture

Make a 1:20 dilution of each bead set creating a mixture of beads. Wash Bead Mixture once with Reagent Dilution Buffer.

16.4 Standard

a. Centrifuge vials for a few seconds to collect lyophilized standard at the bottom. Reconstitute the standard by adding distilled water according to the label on the standard vial.

b. Make a 1:20 dilution of the reconstituted standards all diluted in the same vial to get standard 1. Make further serial dilutions.

17 TEST PROTOCOL SUMMARY

17.1 USING THE FILTER PLATE: vacuum manifold required

- Prepare **Assay Buffer**
- Prepare **PE-Conjugate Mixture, Bead Mixture and Standard Mixture**
- Pre-wet microwell strips of filter plate with **Assay Buffer (1x)**
- Pipette 25 µl diluted **Standard Mixture dilution 1-7** into designated wells
- Add 25 µl **Assay Buffer (1x)** to the blank wells
- Add 25 µl of **Standard Mixture dilution 1** to well A3 which is designated for cytometer setup (highly recommended)
- Add 25 µl **sample** to designated wells
- Add 25 µl **Bead Mixture** to all wells
- Add 50 µl of **PE-Conjugate Mixture** to all wells
- Cover microwell strips, protect from light and incubate 2 hours at room temperature (18° to 25°C) on microplate shaker (500 rpm)
- Wash microwell strips twice with **Assay Buffer (1x)** using the vacuum filtration manifold
- Add 200 µl of **Assay Buffer (1x)**
- Mix the contents of each well by repeated aspiration and ejection, and transfer these 200 µl of each well into a separate sample acquisition tube for a flow cytometer. Fill up to 500 µl final volume with **Assay Buffer (1x)**
- Analyse samples on a flow cytometer

17.2 USING TUBES: centrifugation steps required

- Prepare **Assay Buffer**
- Prepare **PE-Conjugate Mixture**, **Bead Mixture** and **Standard Mixture**
- Pipette 25 µl diluted **Standard Mixture dilution 1-7** into designated tubes
- Add 25 µl **Assay Buffer (1x)** to the blank tubes
- Add 25 µl of **Standard Mixture dilution 1** to the tube which is designated for cytometer setup (highly recommended)
- Add 25 µl **sample** to designated tubes
- Add 25 µl **Bead Mixture** to all tubes
- Add 50 µl of **PE-Conjugate Mixture** to all tubes
- Incubate 2 hours at room temperature (18° to 25°C) protect from light
- Add 1 ml **Assay Buffer (1x)** to all tubes
- Wash tubes twice by centrifugation (5 min at 200 x g) and discard supernatant carefully leaving 100 µl of liquid in each tube
- Add 500 µl **Assay Buffer (1x)** to all tubes
- Analyse samples on a flow cytometer