

FLOWPRA™ SCREENING TEST*

Flow Cytometric Reagents for Detection of Panel-Reactive Antibodies (PRA) Against HLA Class I and II Antigens

REF Catalog # Refer to Table 1.
IVD For In Vitro Diagnostic Use.

Table 1. FlowPRA Products

Catalog #	Description	Product Components
FL1-30*	FlowPRA HLA Class I Screening Test (50 tests) Uses a pooled panel of 30 different microparticles coated with different purified HLA Class I antigens to detect HLA Class I IgG antibodies by flow cytometry.	1. FlowPRA Class I Screening Beads (FL1-30-BD) – 300µl 2. FlowPRA Wash Buffer (10X) (FL-BF) – 26ml 3. FlowPRA Anti-Human IgG - FITC (100X) (FL-AB) – 50µl
FL2-30*	FlowPRA HLA Class II Screening Test (50 tests) Uses a pooled panel of 30 different microparticles coated with different purified HLA Class II antigens to detect HLA Class II IgG antibodies by flow cytometry.	1. FlowPRA Class II Screening Beads (FL2-30-BD) – 300µl 2. FlowPRA Wash Buffer (10X) (FL-BF) – 26ml 3. FlowPRA Anti-Human IgG - FITC (100X) (FL-AB) – 50µl
FL12-60*	FlowPRA HLA Class I & II Screening Test (50 tests) Uses two pooled panels of 30 different microparticles--one panel coated with different purified HLA Class I antigens and the other coated with different purified HLA Class II antigens to detect HLA Class I and II IgG antibodies simultaneously by flow cytometry.	1. FlowPRA Class I Screening Beads (FL1-30-BD) – 300µl 2. FlowPRA Class II Screening Beads (FL2-30-BD) – 300µl 3. FlowPRA Wash Buffer (10X) (FL-BF) – 26ml 4. FlowPRA Anti-Human IgG - FITC (100X) (FL-AB) – 50µl
FL1-PC	FlowPRA Class I Positive Control (10 tests)	240 µl
FL2-PC	FlowPRA Class II Positive Control (10 tests)	240 µl
FL-NC	FlowPRA Negative Control (10 tests)	240 µl

INTENDED USE



The FlowPRA Screening Test is intended for use in flow cytometric detection of HLA-specific antibodies in serum of pre- and post-transplant organ recipients.

SUMMARY AND EXPLANATION

The FlowPRA Screening Test is designed for flow cytometric screening of panel reactive antibody (PRA) against HLA using a panel of FlowPRA beads, which are microparticles (2-4 µm in diameter) coated with purified HLA antigens.

Individuals who have been sensitized to HLA during pregnancy, by blood transfusions, or by previous organ grafts, can develop antibodies against HLA. Studies have shown that HLA IgG antibodies against the donor organ are a contraindication to transplantation (1-4). In contrast, anti-HLA IgM and non-HLA antibodies are not a contraindication to transplantation (5). Although conventional PRA screening is done by lymphocyte cytotoxicity (6), it has now been demonstrated that flow cytometric methodology can detect antibodies at a more sensitive level (7). In addition, flow cytometry can easily distinguish between IgG and IgM antibodies by using anti-IgG secondary antibody (7) and can also detect non-complement-fixing antibodies.



Although pooled spleen cells have been used for flow cytometric PRA tests (10), creating a panel of frozen cells representing all the specificities is difficult. Furthermore, non-HLA antigens are also present on spleen cells, making it difficult to distinguish HLA from non-HLA antibodies.

Previously, Class II antibodies were often defined indirectly by B lymphocyte-positive and T lymphocyte-negative reactions. Therefore, Class II antibodies could not be clearly defined if the serum contained both Class I and Class II antibodies. Furthermore, many spleen cells have both T and B markers, making it difficult to determine whether the antibody is reacting to the Class I or Class II antigens.

The FlowPRA Screening Tests described here circumvent all of these problems and provide the following advantages:

1. Since FlowPRA beads are coated with purified HLA antigen, the tests exclude reactions to non-HLA antigens, which have been a troublesome source of false positive reactions with the standard cytotoxicity test and previous flow cytometric tests.
2. FlowPRA I beads consist of a pool of 30 Class I beads and FlowPRA II beads consist of a pool of 30 Class II beads. Each of these FlowPRA beads is coated with different purified HLA Class I or Class II antigens. (See the FlowPRA beads data sheet for HLA frequency in the panel.) All common HLA antigens, as well as many rare HLA antigens, are represented in the pool. Percent PRA is represented by the percentage of beads that react positively with the serum. Therefore, a screening test for not only a positive or negative PRA, but also for the percentage of PRA, can be performed using FlowPRA beads.
3. The FlowPRA tests can detect HLA Class I and Class II antibodies simultaneously and enable separation of Class I from Class II antibodies. FlowPRA I beads react specifically to HLA Class I antibodies and do not cross react with HLA Class II antibodies. FlowPRA II beads react specifically to HLA Class II antibodies and do not cross react with HLA Class I antibodies. The testing procedures for FlowPRA I and II are the same. In addition, the Class I and Class II beads can be distinguished by their different fluorescent properties when analyzed on a flow cytometer. Therefore, FlowPRA I and II beads can be mixed together to allow a single tube test for both Class I and Class II screening.
4. FlowPRA is a highly sensitive screening test. Studies show that FlowPRA tests are more sensitive than CDC/AHG tests (11, 12).
5. Results of the FlowPRA test are highly reproducible (11,12).

PRINCIPLE(S)

The FlowPRA Screening Test provides pre-calibrated reagents for rapid flow cytometric detection of PRA in human serum. Reactivity of each of these FlowPRA beads has been confirmed by flow cytometry with human allosera and specific HLA monoclonal antibodies. HLA antibodies in the serum react specifically to the FlowPRA beads. After incubation of serum with FlowPRA beads, followed by a staining with a fluorescent-labeled anti-human IgG antibody, the anti-HLA IgG positive serum shows a fluorescent channel shift as compared to the negative serum. Percent PRA is represented by the percentage of beads that react positively with the serum.

FlowPRA I beads are non-fluorescent particles, whereas, FlowPRA II beads are fluorescent particles. The latter can be excited at 488 nm, generating a maximum emission of approximately 580 nm, which is similar to phycoerythrin (PE) and can be detected by the FL2 channel. This allows FlowPRA II beads to be separated from FlowPRA I beads when they are run together.

Positive and negative control sera for the FlowPRA Screening Test should be tested daily as standards for sample analysis. The percentage of reaction of the positive serum should be consistent if the same lot of the FlowPRA beads and control sera is used.

REAGENTS

A. Identification

See Table 1.



B. Warning or Caution

1. For In Vitro Diagnostic Use.
2. **Warning:** The FlowPRA test reagents contain 0.1% sodium azide (NaN₃) as a preservative. Under acidic conditions, sodium azide yields hydrazonic acid, an extremely toxic compound. Reagents containing sodium azide should be diluted in running water prior to being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.
3. **Biohazard Warning:** The test and control sera should be treated as potentially infectious. The serum controls supplied for this product were tested and found negative in tests currently required by the FDA. However, no known test methods can offer complete assurance that products derived from human blood will not transmit infectious agents.
4. Refer to the Material Safety Data Sheet for detailed information.



C. Preparing Reagents for Use

If buffer salts have precipitated out of solution during shipment or storage, re-dissolve by gentle warming before preparing working dilution.



D. Storage Instructions

1. Upon receipt it is recommended that the package be kept intact and stored frozen within the temperature range of -80° to -65° C until use. This will keep the product stable for the duration of the shelf life of the product (see expiration date on package).
2. After first use and/or upon thawing of the product, store reagents at 2° to 5° C.
Important: Once thawed, do not refreeze the FlowPRA Beads or the FITC conjugated F(ab')₂ anti-human IgG.
3. Thawed beads may be stored at 2° to 5° C for three months or until the expiration date, whichever comes first. Wash Buffer can be stored at 2° to 8° C for 3 months or until the expiration date, whichever comes first. The FITC conjugated F(ab')₂ anti-human IgG can be stored at 2° to 5° C for 12 months.
4. The FITC conjugated F(ab')₂ anti-human IgG is sensitive to light and must be stored in the dark.
5. The Positive and Negative Control Sera may be stored at 2° to 8°C (after thawing) if used within 5 days. However, the Positive and Negative Control Sera may become unstable after repeated thawing and refreezing. Therefore, freeze aliquots, or re-freeze the original reagent vial (1X only) and store at -80° to -20° C if reagents are not used within 5 days.



E. Purification or Treatment Required for Use

See "Directions for Use."

F. Instability Indications

None.

INSTRUMENT REQUIREMENTS

- A. Align and quality control the flow cytometer daily according to the manufacturer's recommended start-up procedure.
- B. Run a control test using 5 µl FlowPRA I or II beads in 0.5 ml PBS to set up the FSC gains in order to locate the bead population on your flow cytometer (Figures 1, 2 and 4).
 - Note: Because the beads are smaller than regular lymphocytes, higher FSC gains may be expected in order to visualize the beads.
- C. Run positive and negative serum controls daily before the sample analysis.

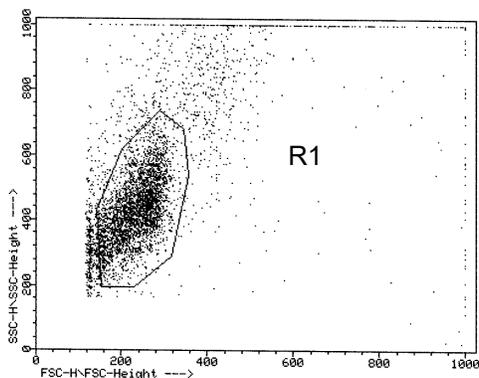


Figure 1. An example of FSC vs. SSC dot plot of FlowPRA I beads

SPECIMEN COLLECTION AND PREPARATION

1. Testing serum may be fresh or thawed. However, aggregates should be removed from the testing serum by centrifugation or filtration prior to testing. Any aggregates in the serum or contamination of the serum may generate invalid results.
2. Testing serum should not be heat inactivated, as it may give a high background in the test.

PROCEDURE

A. Materials Provided

See Table 1 for product components.

B. Materials Required, But Not Provided

Fixing solution: PBS with 0.5% formaldehyde (add 1.35 ml 37% formaldehyde to 100 ml PBS).

C. Directions for Use

Note: To screen for Class I or Class II antibody separately, use 5 μ l FlowPRA Class I or Class II beads per test. To screen for Class I and Class II antibody simultaneously, combine equal volumes of FlowPRA Class I and Class II beads. Mix well and use 10 μ l per test.

1. Vortex FlowPRA beads prior to use.
2. Incubate 20 μ l test serum with FlowPRA Class I and/or Class II beads in a 1.5 ml Eppendorf tube or a 96-well plate for 30 minutes in the dark at 20 - 25°C with gentle shaking.
3. Dilute 10X wash buffer in distilled water to make a 1X solution.
4. Add 1 ml 1X wash buffer to each tube or 150 μ l wash buffer to each well of a 96-well plate. Vortex. Centrifuge at 9,000 g for 2 minutes or at 1500 g for 10 minutes. Aspirate and discard the supernatant.
5. If tubes are used for the test, repeat step 4. If a 96-well plate is used, wash twice with 200 μ l wash buffer.
6. Dilute 1 μ l per test of 100X FITC-conjugated Goat anti-human IgG ($Fc\gamma$) with wash buffer to make a 1X solution.
7. Add 100 μ l of 1X FITC-conjugated Goat anti-human IgG ($Fc\gamma$) to beads and vortex. Incubate for 30 minutes in the dark at 20 - 25°C with gentle shaking.
8. Repeat step 4 twice if tubes are used for the test. If a 96-well plate is used, wash twice—first with 100 μ l wash buffer and then with 200 μ l wash buffer.
9. Add 0.5 ml 1X fixing solution to the tube or 200 μ l to each well of the 96-well plate. The sample is ready for flow analysis, or it can be stored at 2 - 5°C up to 24 hours before flow analysis.

RESULTS

Positive results obtained on FlowPRA tests are an indication of the sensitization to HLA antigens. Patients who have had multiple blood or platelet transfusions can be expected to have positive PRA. Sera from multiparous females may also have positive PRA.

Sera from healthy, untransfused males should give negative results.

FlowPRA tests are designed for HLA-specific IgG antibody detection; in contrast, conventional cytotoxicity tests measure all the antibodies that are cytotoxic to the cell through complement activation. Therefore, discrepancies between FlowPRA and cytotoxicity tests may occur due to the sera containing non-HLA antibodies, non-complement activating HLA antibodies, or IgM and IgA HLA antibodies. In these cases, FlowPRA tests give more specific results.

A. Data Acquisition

1. If only FlowPRA I beads are used:
 - a. Measure green fluorescence for 5,000-10,000 events for each sample.
 - b. Gate the major population of FlowPRA I beads on the FSC vs. SSC dot plot (R₁, Figure 1), and obtain FL1 histograms for each sample.
2. If only FlowPRA II beads are used:
 - a. Adjust the fluorescence compensation by using either commercially available compensation beads or using the FlowPRA I and II beads and Class I positive and negative control sera, as follows:
 - (1). Run a FlowPRA I test with Class I positive and negative control sera. FlowPRA I beads that have reacted with negative serum are used as blank beads.
 - (2). Dilute 5 μ l FlowPRA II beads into 0.5 ml PBS.
 - (3). Adjust (FL2 -%FL1) using the FlowPRA I beads that have reacted with the positive control serum so that high FL1 shifted beads are aligned along the FL2-axis with the blank beads.
 - (4). Adjust (FL1-%FL2) for the FlowPRA II beads so that they are aligned along the FL1-axis with the blank beads.
 - b. Measure fluorescence and gate populations:
 - (1). Measure green and yellow fluorescence for 5,000-10,000 events for each sample.
 - (2). Gate the major population of FlowPRA II beads on the FSC vs. SSC dot plot (R₁, Figure 2), and obtain an FL2 histogram (Figure 3).

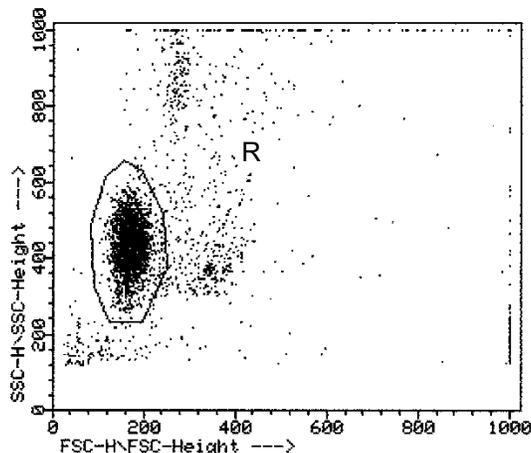


Figure 2. An example of FSC vs. SSC dot plot of FlowPRA II beads

- (3). Set up the gate on the high fluorescent population on the FL2 histogram (R₂, Figure 3), and obtain FL1 histograms of the gated R₁ (Figure 2) and R₂ regions for each sample.

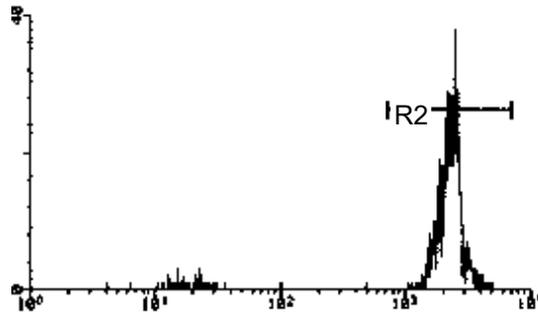


Figure 3. An example of FL2 histogram of FlowPRA II beads gated on region R₁

3. If FlowPRA I, FlowPRA II and control beads are used:
 - a. Adjust the fluorescence compensation by using either commercially available compensation beads or using the FlowPRA I and II beads and Class I positive and negative control sera, as follows:
 - (1). Run a FlowPRA I test with Class I positive and negative control sera. FlowPRA I beads that have reacted with negative serum are used as blank beads.
 - (2). Dilute 5 µl FlowPRA II beads into 0.5 ml PBS.
 - (3). Adjust (FL2 -%FL1) using the FlowPRA I beads that have reacted with the positive control serum so that high FL1 shifted beads are aligned along the FL2-axis with the blank beads.
 - (4). Adjust (FL1-%FL2) for the FlowPRA II beads so that they are aligned along the FL1-axis with the blank beads.
 - b. Measure fluorescence and gate populations:
 - (1). Measure green and yellow fluorescence for 5,000-10,000 events for each sample.
 - (2). Gate the major population of FlowPRA I and FlowPRA II beads on the FSC vs. SSC dot plot (R1, Figure 4).

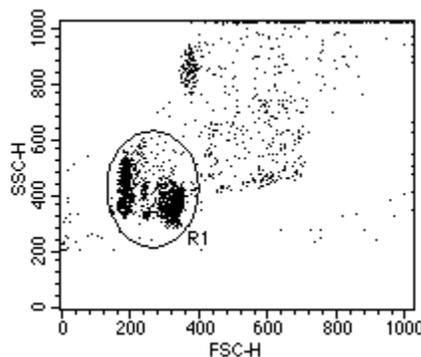


Figure 4. An example of dot plot of mixture of FlowPRA I and II Beads

- a. Get the FL2 vs. FSC dot plot of the R1 gated region (Figure 5). R2 is the gate for Class I beads; R3 is the gate for Class II beads; and R4 is the gate for control beads. Obtain FL1 histograms of each sample on the gated R1 and R2 regions for Class I analysis, on the gated R1 and R3 regions for Class II analysis, and on R1 and R4 regions for control bead analysis.

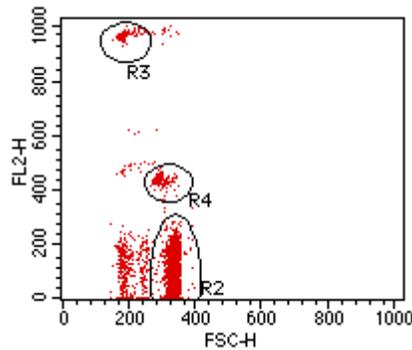


Figure 5. FL2 vs. FSC dot plot of the Class I, Class II, and Control Beads

B. Data Analysis

1. Use the positive and negative control sera to set the cut-off points on the FL1 histogram. The negative control serum contains no HLA antibody activity. The FL1 histogram of the negative control serum can be used as a reference for the negative samples. The positive control serum contains a certain percentage of reactivity and should show a fluorescent channel shift (Figures 6, 7)*. The positive and negative cut-off point should be set at the end of the peak of the FL1 histogram for the negative control serum. Set up a marker at the same cut-off point on the histogram for the positive control (Figures 6,7). The percentage of positive reactions is represented by the percentage of events shifted to the right of the cut-off point. Check the data sheet for the positive controls so that the expected value of percent PRA for the positive control can be obtained. If the percent PRA of the positive control is not consistent with the expected value, check the assay procedure or adjust machine settings. The negative control may generate a 1 to 7% positive signal due to background noise from the flow cytometer. However, if a second peak appears, the assay or the instrument has not been set up correctly. Please check the protocol.

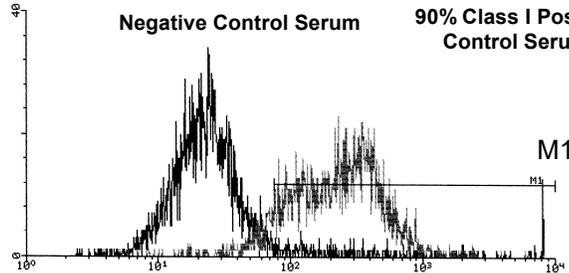


Figure 6. An example of overlaid FL1 Histograms of FlowPRA Beads Reacting with Class I Positive Control Serum on a Becton Dickinson FACStarPLUS®. Note: Please refer to the product datasheet for the current lot antigen panel information.

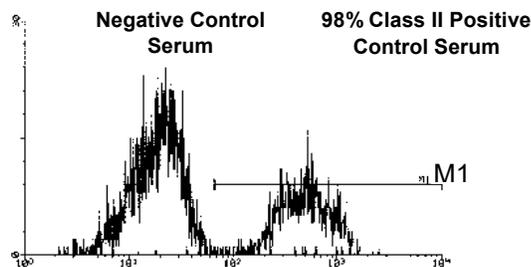


Figure 7. An example of overlaid FL1 histograms of FlowPRA II beads, gated on regions R1 and R2, reacting with Class II positive control serum and negative control serum Note: Please refer to the product datasheet for the current lot antigen panel information.

2. Use the same positive/negative cut-off point to set a marker on the histogram for each of the testing sera on the FL1 histogram. The percentage of positive reactions is measured by the percentage of events shifted to the right of the cut-off point.
3. The positive sera may generate a single shifted peak or multiple peaks on the FL1 histogram. If the percent PRA is less than 10%, carefully examine the histogram. Some sera may show a distinct second peak or a shoulder at the positive region. These sera are considered positive. Negative sera usually generate a single peak.

LIMITATIONS OF THE PROCEDURE

- FlowPRA tests detect only the IgG class of antibodies for diagnostic use. Detection of IgA or IgM HLA antibodies for research use could be conducted by using secondary antibodies (not provided) specific for IgA or IgM.
- Some of the rare HLA antigens may not be represented in the panel. Please reference lot specific data sheets for antigens present in the panel.
- Determination of % PRA is considered a first screen diagnostic test, but not the sole basis for a clinical decision affecting the patient's treatment. A final crossmatch test is routinely required prior to transplant.

EXPECTED VALUES

See RESULTS.

SPECIFIC PERFORMANCE CHARACTERISTICS

- A. Different negative sera may give different mean fluorescent channel shift on the FL1 histogram. The negative control serum provided by One Lambda, generates a slightly higher mean channel shift compared to all of the negative sera tested here. Therefore, it should be used as a reference for setting the positive and negative cut-off point. If different negative sera are to be used as the negative control, a group of known negative and positive samples has to be studied in order to decide the cut-off point.
- B. Different lots of positive serum controls may generate different profiles on the histogram. Carefully examine the data sheet for each lot of the controls.
- C. Different lots of FlowPRA beads may generate different histogram profiles for the positive control due to a slightly different distribution of the HLA antigens on the beads. Carefully examine the data sheets for each lot of the FlowPRA beads.

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EXPLANATION OF SYMBOLS

Symbol	Description
 <small>ISO 7000 Reg No. 2493</small>	Catalog number
	In vitro diagnostic medical device
 <small>ISO 7000 Reg No. 1641</small>	Consult instructions for use
 <small>ISO 7000 Reg No. 0434A</small>	Caution, consult accompanying documents
 <small>ISO 7000 Reg No. 0659</small>	Biological risks
 <small>ISO 7000 Reg No. 0632</small>	Temperature limitation
	CE mark

	CE mark of medical quality
 ISO 7000 Reg No. 3082	Manufacturer
	Authorized representative in the European Community

REVISION HISTORY

Revision	Date	Revision Description
17	12/17/2018	Changed volume of FL-AB from 60µl to 50µl in Table 1.
01	04/08/2019	Upgraded Internal Document Control System. No changes to the document content.
02	Current	Updated contact information and address to reflect change in legal manufacture site.



*0197 Applies to Annex II List B product only



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