WideScreen®

WideScreen® Human CVD Panel 2

(Inflammation)

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About the Kit

WideScreen® Human CVD Panel 2 72014-3

Overview

Bead-based flow cytometric assays enable sensitive, precise quantitation of analytes within a sample. When directed toward proteins or peptides, such assays are essentially ELISAs on a bead. Samples are combined with fluorescently labeled microparticles (beads) covalently conjugated to a capture antibody. Analytes captured on the beads are identified with detection antibodies and a fluorescent label. A major advantage over traditional protein quantitation methods (such as ELISA) is the capacity for multiplexing, as bead-based assays allow simultaneous quantitation of multiple analytes in a small sample volume.

WideScreen® Human CVD (Cardiovascular Disease) Panel 2 is a pre-mixed multiplex bead kit of antibody-based assays for simultaneous quantitation of six cardiovascular disease-associated cytokines found in serum, plasma, and tissue culture supernatants.

Analyte	Full name
IL-6	Interleukin 6
IL-8	Interleukin 8
MCP-1	Monocyte chemotactic protein 1
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
TNF-α	Tumor necrosis factor alpha

Conditions that affect the heart or vasculature are termed cardiovascular diseases (CVD) and include hypertension, congenital heart disease, and coronary heart disease. As a group, CVDs are a heterogeneous set of conditions and there are numerous causative and accessory factors. Atherosclerosis (narrowing and hardening of the arteries), internal bleeding and blood clots, inflammation, infection, genetic and environmental factors underlie many CVDs and are the subject of large and growing research and therapeutic efforts.

As low density lipoprotein cholesterol levels increase in plasma, it can accumulate as an atheroma, or plaque, in the subendothelial space of arteries where it is oxidized and stimulates the expression of MCP-1 and IL-8, which recruit T-lymphocytes and monocytes to the area. The presence of IL-8 in the nascent atheroma also facilitates strong adhesion of monocytes to nearby epithelial cells. Following adhesion, monocytes secrete MIP-1α and MIP-1β. TNF-α expression by the T-lymphocytes stimulates the expression of factors that induce monocytes to differentiate into lipid-rich foam cells and macrophages. Growth factors, chemokines, cytokines and proteases (including IL-6, IL-8, MCP-1 and TNF-α) expressed by foam cells further augment the inflammatory cascade within the growing atheroma.

The WideScreen® Human CVD Panel 2 (Inflammation) is a pre-mixed multiplex bead kit of quantitative antibody-based assays for simultaneous detection of six human inflammatory cytokines associated with cardiovascular diseases: IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α. The kit includes all the reagents and buffers needed to analyze the above proteins in serum, plasma, and tissue culture supernatants using the Luminex® xMAP® System.

Components and Storage

The kit includes all the reagents and buffers needed to assay the above proteins in serum, plasma, and tissue culture supernatants using the Luminex® xMAP® System. Whole blood or grossly hemolyzed samples cannot be used with this kit. The kit contains sufficient components to assay one 96-well plate.

WideScreen® Human CVD Panel 2		**72014-3**
1.1 ml	Human CVD Panel 2 Capture Beads PBS with BSA, Tween 20 and 0.025% ProClin® 300	Store all components at 4°C*
1 vial	Human CVD Panel 2 Detection Antibodies Lyophilized, biotinylated detection antibody premix	
1 vial	Human CVD Panel 2 Standards Mix Lyophilized recombinant and purified protein standards for IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α	
1 vial	Human CVD Panel 2 Control 1 Lyophilized, low levels of IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α in human serum	
1 vial	Human CVD Panel 2 Control 2 Lyophilized, high levels of IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α in human serum	
60 ml	Assay Buffer Type 2 1X, proprietary mix of domestic animal proteins in PBS with 0.025% ProClin 300	
1 vial	Blocking Buffer Type 2 Lyophilized, proprietary mix of domestic animal proteins to minimize non-specific interactions	
3.6 ml	Sample Dilution Buffer Type 1 1X, proprietary mix of domestic animal proteins in PBS with 0.025% ProClin 300	
1 vial	Standard Curve Diluent Type 1 Lyophilized, proprietary mix of domestic animal proteins	
150 µl	15X Streptavidin-Phycoerythrin PBS with 2 mM NaN_3	
1	96-well Filter Plate and Sealer	

*****Following reconstitution of lyophilized reagents, store any unused reagent at –70°C. See Reagent Preparation section (p 4).

Note: WideScreen® Human CVD Panel 2 is not compatible with other bead kits and buffers sold by EMD or other vendors.

Caution: Human CVD Panel 2 Control 1 and Control 2 contain components derived from human sources. All human source materials have been tested negative for HIV-1, HIV-2, HCV antibodies, HIV Ag and HBs Ag. However, all materials derived from human fluids or tissues should be considered biohazardous and handled accordingly. Refer to MSDS for additional information.

Additional Materials Required But Not Supplied

- Luminex® xMAP® System (or equivalent)
- Vacuum manifold for filter plates (Pall 5017 or Millipore MSVMHTS00)
- 96-well plate platform shaker, such as IKA MTS4
- Polypropylene microcentrifuge tubes
- 15 ml polypropylene centrifuge tubes
- Vortex mixer
- Ultrasonic bath, such as Cole Parmer EW-08849 (optional)
- Multichannel pipet (optional)

Bead-Based Human CVD Panel 2 Protocol

Considerations Before You Begin

- Guidelines when using filter plates and vacuum manifold:
  - Excessive vacuum will cause the filter plate membrane to perforate. Adjust the pressure using a non-filter (ELISA or tissue culture) plate, ensuring that vacuum does not exceed 5 in. (127 mm) Hg.
  - After adjusting the vacuum with a non-filter plate, place filter plate on the manifold. Use fingertips to apply pressure evenly across the plate. The liquid should drain in 2–5 sec.
  - To avoid drying out the beads, vacuum only long enough to drain all wells. Do not allow drained beads to sit for >5 min before rehydrating with buffer.
  - It is critical to remove excess buffer from the underside of the filter plate by tapping it on a paper towel several times before adding samples or reagents. This prevents samples from wicking out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations.
  - To avoid perforating the filter plate membrane, make certain that the probe height on the xMAP® system is adjusted correctly. Do not touch the membrane with pipet tips. For accurate pipetting, touch tips to the sides of the filter plate wells. Change tips as necessary to prevent cross-contamination.
- Capture Beads and Streptavidin-PE are light sensitive. To avoid photobleaching, keep beads and Streptavidin-PE in microcentrifuge tubes covered. Cover filter plates with aluminum foil during incubation steps.
- To prevent fluorescent dye loss, do not use organic solvents with capture beads. Beads are incompatible with DMSO concentrations >1%.
- Many of the washing and reagent dispensing steps may be done with an 8-channel or 12-channel pipet (manual or automatic). For accurate results, use calibrated single-channel pipets for manipulation of standards and test samples.
- Test samples (serum, plasma, tissue culture supernatant) should be stored at –70°C prior to use.

Reagent Preparation

1. Resuspend each of the following lyophilized reagents in deionized water, immediately prior to performing the assay:

Reagent	dH_2O Volume
Human CVD Panel 2 Standards Mix	150 µl
Human CVD Panel 2 Control 1	100 µl
Human CVD Panel 2 Control 2	100 µl
Blocking Buffer Type 2	1.5 ml
Standard Curve Diluent Type 1	1.0 ml
Human CVD Panel 2 Detection Antibodies	4.4 ml

2. Mix each vial by vortexing at medium speed for 15 sec. Incubate at room temperature for a minimum of 5 min (not to exceed 30 min) and repeat vortexing step. Human CVD Panel 2 Detection Antibodies can remain at room temperature for up to 2 hours.

   Note: Following reconstitution, store any unused reagents at –70°C. Unused reagents can be stored at –70°C for up to one month. Avoid multiple freeze-thaw cycles.

Test Sample Preparation

Note: Thaw and dilute samples within 1 h of use. Remove any particulates by centrifugation or filtration. Avoid multiple freeze/thaw cycles.

1. Dilute serum or plasma samples 5-fold in Sample Dilution Buffer Type 1. For pipetting accuracy, we recommend 15 µl sample + 60 µl Sample Diluion Buffer Type 1. Assaying duplicate samples is recommended. Mix well and store on ice. If desired, further dilutions of serum or plasma samples can also be performed in Sample Dilution Buffer Type 1 to ensure reading within the range of the assay standards.
2. Dilute tissue culture supernatants in PBS. Cytokine secretion by cell lines varies considerably depending on cell type and stimulation conditions. As a starting point, we recommend diluting unstimulated cell culture supernatants 5-fold in PBS (for pipetting accuracy, we recommend 15 µl sample + 60 µl PBS). Stimulated cell culture supernatants may require dilution of 500-fold or greater. For pipetting accuracy, we recommend using two serial 1:22.5 dilutions (5 µl sample + 220 µl PBS) to reach the final dilution of 1:500. Assaying duplicate samples is recommended. Mix well and store on ice. If desired, further dilutions of tissue culture supernatants can also be performed to ensure reading within the range of the assay standards.

Standard Dilution Series Preparation

This preparation provides sufficient volume to run two duplicate standard dilution curves. Label 8 polypropylene tubes S8 through S1. Alternatively, prepare standard dilutions in a 96-well plate. Pipet Standard Curve Diluent Type 1 into labeled tubes as described below. Transfer the reconstituted Human CVD Panel 2 Standards Mix to the S8-labeled tube. Prepare 3-fold serial dilutions of S8 following the table below. Ensure that each new standard is mixed well by vortexing before proceeding to the next dilution. Change tips between each dilution.

Standard	Volume of Standard Curve Diluent Type 1	Volume of Standards Mix
S8	0 µl	150 µl from vial
S7	80 µl	40 µl of S8
S6	80 µl	40 µl of S7
S5	80 µl	40 µl of S6
S4	80 µl	40 µl of S5
S3	80 µl	40 µl of S4
S2	80 µl	40 µl of S3
S1	80 µl	40 µl of S2

Note: Sufficient standards are provided in this preparation for two standard dilution curves. Standard concentrations are lot-specific. Refer to Certificate of Analysis of appropriate lot for specific standard concentrations.

Immunoassay Protocol

1. Seal any unused wells of the 96-well filter plate with plate sealer or lab tape for future use.
2. Pre-wet 96-well filter plate wells with 50 µl Assay Buffer Type 2 and incubate for a minimum of 5 min. Immediately prior to Step 3, remove liquid from filter plate by vacuum filtration. Do not exceed 5 in. Hg or 127 mm Hg vacuum; liquid should drain in 2–5 sec. Tap filter plate on a paper towel to remove any buffer remaining on the underside.

   Note: It is critical to remove excess buffer from the underside of the filter plate before adding samples or reagents. Otherwise, samples may wick out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations. If a well does not drain, use the non-tip end of a 200 µl pipet tip to flick the center of the plastic support on the underside of the well, then reapply vacuum.
3. Add 10 µl of Blocking Buffer Type 2 to each filter plate well that will be used.
4. Add 30 µl of each standard, sample or control to appropriate well of the 96-well filter plate.

   Note: Human CVD Panel 2 Control 1 and Control 2 do not need to be diluted.
5. Vortex the plate by gently gliding the plate over the vortex mixer.

   Note: Gradually increase the vortex speed from low to medium. Hold the plate with a loose grip. Mix thoroughly for 10 sec. Avoid splashing. Alternatively, mix using a plate shaker for 10 sec on high speed (1200 rpm).
6. Sonicate 10 sec (optional) and vortex the tube of Human CVD Panel 2 Beads for 10 sec. Add 10 µl to each well.
7. Vortex or shake the plate 10 sec as described above in Step 5.
8. Cover plate with aluminum foil to protect from light and incubate 1 hr at room temperature on a plate shaker (750 rpm).
9. Remove liquid from filter plate by vacuum filtration (5 in. Hg or 127 mm Hg maximum).
10. Wash beads by adding 100 µl Assay Buffer Type 2 to each well and applying vacuum to remove buffer. Repeat wash step for total of two washes in Assay Buffer Type 2. After second wash and vacuum, tap filter plate on paper towels to remove any buffer remaining on the underside.

    Note: Do not resuspend beads in Assay Buffer Type 2 after second wash.
11. Add 40 µl Human CVD Panel 2 Detection Antibodies to each well. Vortex or shake the plate as described in Step 5.
12. Cover plate with aluminum foil to protect from light and incubate 1 h at room temperature on a plate shaker (750 rpm)

    Note: Do not wash beads after Detection Antibody incubation.
13. Microcentrifuge 15X Streptavidin-PE briefly (5 sec) to ensure all material is in the bottom of the tube. If using all 96 wells, dilute 15X Streptavidin-PE to 1X by adding 144 µl concentrated Streptavidin-PE to 2016 µl Assay Buffer Type 2.

    Note: Do not dilute the whole vial of Streptavidin-PE if the entire kit will not be used. Dilute only what is needed based on the number of wells. Allow 10% extra for pipetting error. If there is an insufficient volume of 15X Streptavidin-PE for your final experiment, making a slightly more dilute working stock will not adversely affect results.
14. Add 20 µl 1X Streptavidin-PE to each well.
15. Cover plate with aluminum foil to protect from light and incubate 30 min at room temperature on a plate shaker (750 rpm).
16. Remove liquid from filter plate by vacuum filtration.
17. Wash beads by adding 100 µl Assay Buffer Type 2 to each well and applying vacuum to remove buffer. Repeat wash step for total of two washes in Assay Buffer Type 2. After second wash and vacuum, tap filter plate on paper towels to remove any buffer remaining on the underside.
18. Add 100 µl Assay Buffer Type 2 to each well.
19. Cover plate to protect from light. Incubate 3–5 min at room temperature on a plate shaker (750 rpm).
20. Analyze using a Luminex® instrument.

Collecting Data and Data Analysis

Data Acquisition

For detailed instructions on the operation of Luminex® systems, refer to the user guide for your specific instrument and software. General recommendations are given below.

1. Using your Luminex system software, prepare a protocol for the assay you will run. Enter information for each bead target, and for the standards, samples, and controls.
2. Select the following bead regions:

Analyte	Bead Region	Analyte	Bead Region
IL-6	49	MIP-1α	17
IL-8	39	MIP-1β	51
MCP-1	64	TNF-α	33

3. Acquire data using the system settings shown below:

Software	Sample Size	Events per Bead Region	Timeout	Doublet Discriminator	CAL2 Gain Setting
Luminex® IS™ or equivalent	50 µl	50–100*	60 sec	7500–15500	default
Bio-Plex® Manager™	50 µl	50–100*	default	default	RP1 low

*If the time spent acquiring samples needs to be reduced, collect as few as 50 events per bead region.

Generation of Standard Curves and Quantitation of Experimental Samples

- Protein standards are supplied in the Human CVD Panel 2 kit, allowing for accurate quantitation using a titrated standard curve. Representative standard curves and assay performance information can be found in the Certificate of Analysis for the specific lot.
- Refer to the Certificate of Analysis for expected control ranges.
- The eight data points obtained with the standard dilution series are plotted using Median Fluorescent Intensity (MFI) as the signal readout (Y-axis), against concentration of standard dilutions (X-axis).
- Five-parameter logistic (5PL) curve fitting is recommended for modeling data obtained from bead-based immunoassays. Most ranges of standard concentrations are too wide for accurate linear regression analysis. Four-parameter logistic (4PL) equations will often give a good fit, but are not ideal because they assume the standard curve is symmetrical.
- If the signal from an unknown sample exceeds the highest point of the standard curve, the concentration of the unknown should *not* be calculated by extrapolation, because the non-linear shape of the standard curve cannot be accurately modeled past the last measured point. In this case, dilute the samples and test again.
- When concentrations of unknowns have been determined by reading off of the standard curve, remember to multiply this value by the dilution factor to obtain the concentration of the target in the original sample.

Troubleshooting

Problem	Probable Cause	Solution
Leaking wells in filter plate	Wicking due to adherent drops	Tap filter plate several times on paper towel before adding samples or reagents. Do not place filter plate on an absorbent surface during incubations. If wells leaked during data acquisition, it is possible to reacquire these wells. Blot underside of wells and add 100 µl/well Assay Buffer Type 2.
	Perforation of filter plate membranes	Adjust the vacuum setting to <5 in. (127 mm) Hg. Do not touch membranes with pipet tips.
Filter plate wells not draining under vacuum	Vacuum is too low	Adjust vacuum setting to 3–5 in. (76–127 mm) Hg. Clean rubber seals. Apply fingertip pressure to filter plate to ensure formation of a good seal. Use the plate sealer to cover wells not in use.
	Clogged membranes	Clarify samples by centrifugation or filtration. If samples are viscous, dilute further before assaying. Use the non-tip end of a 200 µl pipette to flick the center support on the underside of the well, then reapply vacuum.
Low bead counts during data acquisition	No beads in the wells	See "Leaking wells in filter plate" solutions above. Verify that beads were added at the correct concentration, and that correct bead regions and wells were selected during acquisition setup.
	xMAP® fluidics system is clogged	Clear system of clogs or air using maintenance steps described in the instrument user manual (sanitize, alcohol flush, probe sonication, etc.). Make sure that the probe height is set correctly. Make sure that beads are in suspension by incubating plate for 3–5 min on plate shaker (750 rpm) immediately before analysis. Microbial growth in buffers can cause beads to stick to the filter plate membrane. Do not use contaminated reagents.
	Timeout limit is set too low	50–100 events per bead region should be acquired within the 60 sec timeout limit. If necessary, the timeout limit can be set higher, e.g. 75 sec.
Beads are not falling into the gates properly	Beads were not resuspended in Assay Buffer Type 2 before analysis	The setting of the Doublet Discriminator (DD) gate is buffer-specific. This gate can be adjusted, but Assay Buffer Type 2 is the recommended buffer for running samples. Other buffers may also cause bead aggregation.
	Beads were exposed to organic solvents	Do not use organic solvents in the immunoassay, as they will damage beads irreversibly.
	Beads are falling outside the bead region gates due to photobleaching	Do not use expired beads. Do not expose the beads to ambient light for >10 min. Avoid intense light.
	Fluidics system is not running properly	Confirm that the waste container is not full, the sheath fluid is not empty, and the SD fluidics module is turned on. Check system calibration using approved calibration beads. Verify correct system pressure. Confirm that the system is free of air or particulate buildup. Follow maintenance steps described in the instrument user manual.
Insufficient volume of an immunoassay reagent	Solutions were not prepared or used as described in protocol	Confirm correct buffer dilutions and use. If additional Assay Buffer Type 2 is needed, PBS can be used for the final bead resuspension step. If additional 96-well filter plates are required, we recommend Millipore Cat. No. MSBVN1210. If there is insufficient volume of 15X Streptavidin-PE for your final experiement, making a slightly more dilute working stock (e.g., 20-fold instead of 15-fold) will not adversely affect results).
Sample measurements not falling on the standard curve	Dilution of sample is too low or too high	If values are higher than the standard curve, dilute samples further in Sample Dilution Buffer Type 1 and repeat assay.
	Target concentration is below detection	Verify that curve fitting at the lower end of the standard curve is accurate. Not all serum/plasma samples contain detectable levels of all analytes.

Appendix A: Flowchart for Human CVD Panel 2 Immunoassay