WideScreen®

WideScreen® Human Metabolism Panel 2

Table of Contents

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About the Kit

WideScreen ® Human Metabolism Panel 2 72290-3

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 of bead-based assays 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 Metabolism Panel 2 is a pre-mixed multiplex bead kit of quantitative antibody-based assays for simultaneous detection of seven human peptide hormones involved in regulating metabolism: AgRP (Agouti-related protein), ASP (acylation-stimulating protein), CNTF (ciliary neurotrophic factor), C-peptide (connecting peptide), GLP-1 active (glucagon-like peptide-1, active), PYY (peptide YY), and secretin. 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.

WideScreen Human Metabolism Panel 2 contains two types of immunoassays: conventional and competitive. AgRP, CNTF, C-peptide, GLP-1 (active), PYY, and secretin assays are conventional (non-competitive) sandwich-based immunoassays. The ASP assay is a competitive assay in which purified ASP antigen is immobilized on the bead and binds biotinylated detection antibody in the blocking buffer; upon sample addition, analyte can compete away bound detection antibody, resulting in a decrease in fluorescent signal.

Analyte	Full name
AgRP	Agouti-related protein
ASP	Acylation-stimulating protein, also known as C3a desArg
CNTF	Ciliary neurotrophic factor
C-peptide	Connecting peptide
GLP-1 (active)	Glucagon-like peptide-1, active (7-36 amide)
PYY	Peptide YY
Secretin	Secretin

Regulation of metabolism is one of the central functions of the body. A host of hormones and enzymes secreted into the bloodstream act in concert to control processes such as blood glucose levels, hunger, cellular energy usage and storage, and blood pressure, among many others. Understanding the functions and complex interplay between these factors is instrumental in controlling cardiovascular diseases and conditions resulting from aberrant energy homeostasis, such as diabetes mellitus and obesity. Measurement of these biomarkers in biological samples is used in the clinical setting for detecting disease, monitoring treatment, and studying normal metabolic processes. The WideScreen Human Metabolism Panel 2 is a non-diagnostic kit for use in the pre-clinical and primary research settings.

- Agouti-related protein (AgRP) is a neuropeptide produced by the hypothalamus. It acts as a potent stimulator of appetite, mediated by antagonism of melanocortin receptors. AgRP secretion is inhibited by the adipose-derived hormone leptin.
- Acylation-stimulating protein (ASP) is an adipocyte-derived hormone that is formed by complement factor C3 (C3) cleavage and desargination to generate C3a desarg (also known as ASP). ASP stimulates fatty acid incorporation into adipose triglyceride (FIAT) and also brings about increased glucose uptake. ASP acts on energy homeostasis independently of insulin.
- Ciliary neurotrophic factor (CNTF) is a polypeptide hormone and growth factor that is secreted from glial cells. CNTF acts as a survival factor for neuronal cells and also may reduce food intake without hunger or stress via a non-leptin, but “leptin-like” pathway.

- Connecting peptide (C-peptide) is a 31-amino acid peptide that is a product of the cleavage of proinsulin to form both insulin and C-peptide. In newly diagnosed patients, C-peptide levels are frequently measured as a means of distinguishing type 1 and type 2 diabetes mellitus. C-peptide levels are representative of the insulin produced endogenously by the pancreas.
- Glucagon-like peptide-1, active (GLP-1, active) is an incretin hormone derived from the proglucagon transcript. It is secreted primarily by L cells in the small intestines in response to a meal, and is a potent stimulator of insulin secretion and inhibitor of glucagon secretion. The GLP-1 (active) assay is specific for the active form of GLP-1 (7-36 amide), and has < 2% cross-recognition of inactive GLP-1
(9-36 amide, 9-37).
- Peptide YY (PYY) is a 36-amino acid peptide that is a member of the pancreatic polypeptide family. Following a meal, PYY is secreted by L cells of the gastrointestinal tract and released into the circulation where it is believed to inhibit subsequent food intake.
- Secretin is a 27-amino acid linear peptide hormone that is involved in the digestion process. It is secreted in response to acidification of the duodenum where it regulates pH by inducing the pancreas and bile duct to release a bicarbonate-rich neutralizing fluid.

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® Metabolism Panel 2		**72290-3**
1.1 ml	Human Metabolism Panel 2 Capture Beads PBS with BSA, Tween 20 and 0.009% ProClin® 300	Store all components at 4°C*
1 vial	Human Metabolism Panel 2 Detection Antibodies Lyophilized, biotinylated detection antibody premix	
1 vial	Human Metabolism Panel 2 Standards Mix Lyophilized recombinant, purified native and synthesized protein standards (AgRP, ASP, CNTF, C-peptide, GLP-1 active, PYY, and secretin)	
1 vial	Human Metabolism Panel 2 Control 1 Lyophilized, low levels of AgRP, ASP, CNTF, C-peptide, GLP-1 active, PYY, and secretin in human serum	
1 vial	Human Metabolism Panel 2 Control 2 Lyophilized, high levels of AgRP, ASP, CNTF, C-peptide, GLP-1 active, PYY, and secretin 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	Human Metabolism Panel 2 Blocking Buffer Lyophilized, proprietary mix of antibodies, biotinylated antibody (for competitive assay), and buffered domestic animal proteins to minimize non-specific interactions	
2 x 3.6 ml	Sample Dilution Buffer Type 4 1X, proprietary mix of buffered domestic animal proteins in PBS with 0.025% ProClin 300	
1 vial	Standard Curve Diluent Type 6 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 5).

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

Caution: Human Metabolism Panel 2 Standards Mix, 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 HBsAg. 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)

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 Metabolism Panel 2 Standards Mix	150 µl
Human Metabolism Panel 2 Control 1	100 µl
Human Metabolism Panel 2 Control 2	100 µl
Human Metabolism Panel 2 Blocking Buffer	1.5 ml
Standard Curve Diluent Type 6	1.0 ml

Note: Do not resuspend the Human Metabolism Panel 2 Detection Antibodies at this time. This reagent should be prepared after the overnight incubation step (see Step 11 *of the* Immunoassay Protocol *on p 7).*

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.

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: Human Metabolism Panel 2 analyte levels can be measured in plasma or serum blood samples. However, due to the labile nature of some of the analytes, the ideal sample type is EDTA plasma collected into pre-chilled tubes. It is recommended that GLP-1 be stabilized by adding a DPP4 inhibitor to EDTA plasma samples immediately after collection. Due to loss during clotting, AgRP levels may be lower in serum samples compared to plasma. Due to C3 conversion during clotting, ASP levels are significantly higher in serum samples compared to plasma.

1. Thaw and dilute samples within 1 h of use. Remove any particulates by centrifugation or filtration. Avoid multiple freeze/thaw cycles.
2. Dilute serum or plasma samples 1:5 in Sample Dilution Buffer Type 4 (Duplicate Samples: 15 µl sample + 60 µl Sample Dilution Buffer Type 4). 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 4 to ensure reading within the range of the assay standards.
3. Dilute tissue culture supernatants 1:5 in Sample Dilution Buffer Type 4 (Duplicate Samples: 15 µl supernatant + 60 µl Sample Dilution Buffer Type 4). Assaying duplicate samples is recommended. Mix well and store on ice. If desired, further dilutions can also be performed in Sample Dilution Buffer Type 4 to ensure reading within the range of the assay standards.

   Note: Some cell culture media supplements contain hormones such as insulin, ASP, and cortisol analogs that are detected by the corresponding immunoassays. Therefore, it is recommended to perform a media alone control and to check the media composition prior to performing the assay.

Standard Dilution Series Preparation

This preparation provides sufficient volume to run 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 6 into labeled tubes as described below. Transfer the reconstituted Human Metabolism 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 6	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: 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 (included) 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 an unused 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 Human Metabolism Panel 2 Blocking Buffer 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 Metabolism 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 Metabolism Panel 2 Capture 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. Apply plate sealer to all used wells to prevent evaporation during overnight incubation.
9. Incubate overnight at 4°C on a plate shaker (750 rpm). Cover plate with aluminum foil to protect from light.
10. The next day, warm samples to room temperature on a plate shaker (750 rpm) for 30 minutes. Cover plate with aluminum foil to protect from light.
11. During the 30 minute room temperature incubation, prepare the Detection antibody reagent. Add 4.4 ml deionized water to the lyophilized tube of Human Metabolism Panel 2 Detection Antibodies. Vortex at medium speed for 15 sec. Incubate at room temperature for a minimum of 5 minutes (not to exceed 30 min). Vortex again at medium speed for 15 sec.

    Note: Human Metabolism Panel 2 Detection antibody reagent will be stable at room temperature for upto 2 hours.
12. Remove liquid from filter plate by vacuum filtration (5 in. Hg or 127 mm Hg maximum).
13. 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 the second wash and vacuum, tap the 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.
14. Add 40 µl Human Metabolism Panel 2 Detection Antibodies to each well. Vortex or shake the plate as described in Step 5.
15. 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.
16. 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.
17. Add 20 µl 1X Streptavidin-PE to each well.
18. Cover plate with aluminum foil to protect from light and incubate 30 min at room temperature on a plate shaker (750 rpm).

19. Remove liquid from filter plate by vacuum filtration.
20. 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.
21. Add 100 µl Assay Buffer Type 2 to each well.
22. Cover plate to protect from light. Incubate 3–5 min at room temperature on a plate shaker (750 rpm).
23. 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
AgRP	03	GLP-1 (active)	66
ASP	19	PYY	55
CNTF	34	Secretin	49
C-peptide	44		

3. If desired, enter the standard concentrations into the assay template. WideScreen ® Human Metabolism Panel 2 standard concentrations are lot-specific. Refer to Certificate of Analysis of appropriate lot for specific standard concentrations.
4. 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™	default (50 µl)	50–100*	60 sec	default (4335-10000)	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 and peptide standards are supplied in the Human Metabolism 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 concentration standards are plotted using Median Fluorescence 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.
	DD gate setting not optimal	Use the DD gate setting recommended in the Data Acquisition Section. If necessary, raw data results can be reanalyzed with different DD gate settings; see software user manual.
	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 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 experiment, making a slightly more dilute working stock (e.g., 20-fold instead of 15-fold) will not adversely affect results.
Sample measurements not within 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 4 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 Metabolism Panel 2 Immunoassay