Solutions and buffers used in Luminex assay

**AB-A (1 x Assay Buffer –A)**
Final concentration:
- PBS (1:10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
- 1% BSA (optimized. A7906, A7030, A3059 etc)
- 0.05% Tween-20 (Sigma # P7949)
- 0.08% Azide (Sigma # S8032)
- 5mM EDTA-Na2 (Sigma # E7889, 0.5M)
- 0.22µ filtrated

Prepare 2 litters
1. Prepare 200mL 10% BSA in dd H2O and filtrate vial 0.2µ syringe filter.
2. Dilute 10 x PBS into 1.5 litters of milli-Q water with a magnetic stirring rod.
Add 10ml 10% tween-20, 1.6g NaN₃ and 20ml 0.5M EDTA-Na₂. Stir to complete dissolve of all components. Adjust pH to 7.4. Adjust total volume to 1.8 litters with dd H2O. Filtrate the buffer through 0.22µ membrane into 2 x 1000 ml bottle.
3. Add 100mL filtered 10% BSA to each bottle.

Store at 4°C for 2 years.

**AB-B (1 x)**
- 1 x PBS (Sigma P7059)
- 1% BSA (optimized. A7906, A7030, A3059 etc)
- 0.08% Azide
- 0.22µ filtrated

**AB-C (1 x)**
- AB-A +
Protease inhibitors (Sigma S8820), 1 tablet/100ml

**AB-D (1 x)**
- AB-A +
Protease inhibitors (Sigma S8820), 1 tablet/100ml
DPP4 inhibitor (1:100)

**AB-E**
- 1 x PBS (Sigma# P7059)
- 0.2% BSA
- 0.05% Tween-20 (Sigma # P7949)
- 0.08% Azide (Sigma # S8032)
- 5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
- 0.22µ filtrated
AB-F (1 x Assay Buffer –F)
Final concentration:
PBS (1:10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
1% Chicken albumin (Sigma A5503 or A2512)
0.05% Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
0.22μ filtrated

AB-G (1 x Assay Buffer –G)
Final concentration:
PBS (1:10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
0.2% Chicken albumin (Sigma A5503 or A2512)
0.05% Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
0.22μ filtrated

AB-H (1 x Assay Buffer –H)
Final concentration:
PBS (1:10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
0.05% Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
0.22μ filtrated

Beads Diluent A (BD-A)
1 x PBS (Sigma# P7059)
1% BSA (optimized. A7906, A7030, A3059 etc)
0.05% Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
IIR (Bioreclamation, # 6LD1074), 150µg/ml IIR)
0.22μ filtrated

Beads Diluent B (BD-B)
1 x PBS (Sigma# P7059)
0.1 % BSA (optimized. A7906, A7030, A3059 etc)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
IIR (Bioreclamation, # 6LD1074), 100µg/ml IIR)
0.22μ filtrated
**Beads Diluent C (BD-C)**
1 x PBS (Sigma# P7059)
0.1 % Ovalbumin(Sigma A5503 or A2512)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
IIR (Bioreclamation, # 6LD1074), 100µg/ml IIR)
0.22µ filtrated

**Beads Diluent D (BD-D)**
1 x PBS (Sigma# P7059)
0.1 % BSA (optimized. A7906, A7030, A3059 etc)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na₂ (Sigma # 7889, 0.5M)
IIR (Bioreclamation, # 6LD1074), 10µg/ml IIR)
0.22µ filtrated

**10 X Wash Buffer (WB), 1000ml**
Dissolve 10 bag of PBS Sigma P3563) and 5 g of NaN₃ in a 1000ml bottle. Stir to dissolve completely and filtrate through 0.22µ membrane. Store at RT

1 x WB was diluted from 10 x stock (see below) with water
Final concentration: 10mM PB, pH7.4, 138mM NaCl, 2.7mM KCl, 0.05% Tween-20
0.05% NaN₃

**Blocking Buffer (BB-A)**
Same as AB-B

**Blocking Buffer C (BB-C)**
1 x PBS (Sigma P7059)
1% Ovalbumin(Sigma A5503 or A2512)
0.08% Azide
0.22µ filtrated

**Activation Buffer (100mM NaH₂PO₄, pH 6.0), 1000ml**
Deionized water 800 ml
Sodium phosphate monobasic (Sigma S3139, NaH₂PO₄), 12g
adjust pH to 6.0 with 5 N NaOH (about 70 drops)
Bring final volume to 1000ml

**Coupling Buffer-A (CB-A) (50mM MES, pH5.0), 1000ml**
Deionized water 800 ml
MES (Sigma M2933), 9.76g
adjust pH to 5.0 with 5 N NaOH (about 15 drops)
Bring final volume to 1000ml

**Coupling Buffer-B(CB-B) (100mM acetic acid, 300mM NaCl, pH 4.0), 250ml**
Deionized water 233ml
5M NaCl (Sigma 5150), 15ml
Acetic acid (Sigma #A6283, MW=60.05, d=1.049), 1.5ml
Adjust pH to 4.0 with 5N NaOH (about 30 drops)

**PBS, pH 7.4 (1 x)**
Purified water 900 ml
PBS (Sigma P7059, 10 X), 100 ml
Final concentration (10mM PB, 155 mM NaCl, pH 7.4)

**1 x TBS**
Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl

**SAPE**

**Cat#-xx**
Assay Buffer + xx µg/ml SAPE
e.g. S866-15 (Invitrogen SAPE Cat#S866, 15µg/mL)

**30 x BSA-NaN3**
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
3% BSA (optimized. A7906, A7030, A3059 etc)
2.4% Azide (Sigma # S8032)
0.22µ filtrated

**30 x OA-NaN3**
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
3% Ovalbumin (Sigma A5503 or A2512)
2.4% Azide (Sigma # S8032)
0.22µ filtrated

**Antibody biotinylation (production scale)**

**Document History**
Amicon Ultra-filter tube has been changed from 20mL to 4mL, (MWCO=30 kD), to reduce antibody loss during buffer exchange. Antibody buffer with azide requires buffer exchange
Use
This protocol is to biotinylate antibodies for Luminex assay

Safety
NaN₃ is used in some solutions for this protocol. *EZ-Link Sulfo-NHS-LC-LC-Biotin* may cause eye irritation. Please always wear goggles, gloves and lab coat during experiment. Follow all necessary safety procedures in the lab.

Materials needed
1 x PBS pH 7.4 (diluted from Sigma P7059)
1 M Tris-HCl, pH 7.0 (for small scale biotinylation of Ab <100µg only)
1 x TBS (Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl)
30 x OA-NaN₃ (3% Ovalbumin-2.4%NaN₃) (see [protocol for buffer preparation.doc](#))
30 x BSA-NaN₃ (3% BSA-2.4%NaN₃) (see [protocol for buffer preparation.doc](#))
Assay Buffer-A or -F (AB-A or AB-F) (see [protocol for buffer preparation.doc](#))
Antibody to be biotinylated
EZ-Link Sulfo-NHS-LC-LC-Biotin (PIERCE: #21338), stored at –20ºC in desiccator (each opened vial should be used within three months under proper storage)
Filter tube (Amicon Ultra-4, MWCO=30 kD), need 2 tubes/antibody
1.5 ml screw cap microtube and 15ml tube
Aluminum foil
Plate shaker
Centrifuge (with swing bucket rotor for 50ml Conical tubes)
A print copy of work sheet (Click “..\Assay Panels\1st year panels\Master work sheet for antibody biotinylation for 1st year targets.xls”)

Protocol
A. Preparation

1. Bring buffers, *EZ-Link Sulfo-NHS-LC-LC-Biotin* and *antibodies* to RT for at least 30 min.
2. Double check data sheet and vial label to make sure the antibody contains no BSA glycine, or Tris or NaN₃. Azide, Glycine and Tris can be removed by filtration through *Amicon Ultra-4 tube (30 kD)* for three times with 3 x 15 ml PBS at 4,000 RPM centrifugation for 10-15 min (at 25C). BSA can be removed by *NAb Protein A/G Spin Kit* (Pierce # 89950). After buffer exchange or purification, the final concentration of antibody must be measured again.
3. Calculate amount of reagents based on ratio at:
   **1mg Antibody/150 µg Biotin (Molecular ration≈1:30)**
4. Prepare one filter tube (*Amicon Ultra-15 tube, MWCO 30 kD*) for each antibody to be biotinylated. Add 15 ml TBS to each tube and stay at RT until use at step C.

B. Biotinylation

5. Adjust antibody solution to about 0.1-1mg/ml with PBS
6. Calculate the total volume needed for biotinylation of all antibodies at same time. Prepare appropriate amount of Biotin (1mg/ml, freshly prepared in PBS) at the ratio above. Biotin is light sensitive, please use amber vials or wrap the tube with aluminum foil. The prepared solution should be used as soon as possible.

7. Pipette required volume of Biotin to each antibody tube on the table. (Note: please change tips for each tube) Wrap each tube with aluminum foil before wrapping multiple tubes together. Discard the leftover of Biotin solution.

8. Fix the wrap with rubber belts on the shaker stage. Shake the tubes for 1 hour at RT with maximum intensity of shaker setting.

9. Go next step to stop biotinylation.

C. Stop biotinylation
   (light protection is no longer needed from now)

10. Check the filter tubes for leaking. There should not be any TBS flow through to the bottom of collection tubes without centrifugation. Replace with a new filter tube if leaking is observed. Empty all filter tubes.

11. Briefly spin the biotinylation tubes and transfer each labeling solution to the bottom of upper part of filter tubes. Rinse each biotinylation tube with 500µL TBS and pool into filtrate tube. (Change tip for each antibody to avoid inter-tube contamination). Balance all tubes for centrifugation at 4,000 RPM at RT for about 5-10 min until the retention volume reach to 100-200 µl. Don’t over drain the filter tubes. Collect the filtrate into each microtube and store at 4°C (in case of antibody loss due to membrane leakage).

12. Add 13-15 ml 1 x TBS to each filter tube and centrifuge for 15 min at 4,000 g at 25°C (until the final retention volume is about 0.2ml). Discard the filtrate in collection part.

13. Repeat step 12 for two additional times (total three times)

14. Carefully transfer each labeled antibody to a new microtube (Change tip for each antibody). Rinse the filter tube with additional TBS. Keep the total volume smaller than starting volume. Record the volume of antibody.

15. Measure OD$_{280}$ of each biotinylated antibody with NanoDrop spectrophotometer. Record the OD$_{280}$, dilution factor and concentration to the worksheet (For IgG: OD$_{280}$/1.37 = mg/ml).

16. Add 1/30 of BSA-NaN$_3$ (30X) or OA-NaN$_3$ (30 x) for each biotinylated antibody. The final concentration for BSA or OA and NaN$_3$ is 0.1% and 0.08%, respectively.

17. The stock concentration of biotinylated antibody can be adjusted (500µg/mL for production scale are suggested) with Assay Buffer (AB-A or AB-F)

18. Label each biotinylated antibody with date and antibody name. Store at 4°C. (e.g. Bt-MPO Mab, Sigma# M3156, clone# 12345, 500µg/mL, 9/23/2008)

*Description of the reagent (From product insert)*
*EZ-Link® Sulfo-NHS-LC-LC-Biotin, 50 mg*

**Molecular Weight:** 669.75

**Spacer Arm:** 30.5 Å

**Storage:** Upon receipt store desiccated at -20°C. Product is shipped at ambient temperature.

*N*-Hydroxysuccinimide (NHS) esters of biotin are the most popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amino groups (-NH2) in pH 7-9 buffers to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for labeling with NHS-activated biotin reagents. Several different NHS esters of biotin are available, with varying properties and spacer arm lengths. Sulfo-NHS ester reagents are water soluble, enabling reactions to be performed in the absence of organic solvents such as DMSO or DMF.

![Reaction of NHS-LC-Biotin with a primary amine.](image)

**Conjugation of antibodies to Magnetic microsphere (Production scale)**

**Use**
This protocol is to make antibody conjugated beads for Luminex assay in large scale format (1 x 10⁹)

**Safety**
*NaN₃* is used in some solutions for this protocol. *Sulfo-NHS* and *EDC* may cause eye irritation. Please always wear safety goggles, gloves and lab coat during experiment. Follow all necessary safety procedures in the lab.
**Materials needed**
1 x PBS (diluted from Sigma P7059, 10mM PB, 0.9% NaCl, pH7.4)  
Activation buffer (100mM PB, pH 6.0), (see **Buffer preparation protocol**)
Coupling buffer (50mM MES, pH5.0 or 100mM acetic acid, 300mM NaCl, pH 4.0), (see **Buffer preparation protocol**)
1 x TBS (Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl)
Blocking buffer-A (BB-A) (see **Buffer preparation protocol**)
Blocking buffer-C (BB-C) (see **Buffer preparation protocol**)
Antibody to couple
Luminex microspheres
Sulfo-NHS (N-hydroxysulfosuccinimide) (Sigma #56485 or PIERCE: #24510, stored at 4°C in desiccator.
EDC (N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride) Sigma E7750, stored at –20°C in desiccator
15mL amber bottle (for conjugation)
50mL amber bottles (for storage)
Aluminum foil
Ultrasonic cleaner
Plate shaker
Magnetic separator or centrifuge (swing rotor with 15mL tube adaptor)
Microscope and hemacytometer

**Protocol**

D. Preparation

19. Bring buffers, Sulfo-NHS, EDC and beads and antibodies to RT for at least 30 min.
20. Double check data sheet and vial label to make sure the antibody contains no BSA
glycine, Tris or NaN3. Glycine, Tris and azide can be removed by filtration
through Amicon Ultra-15 tube (30 kD) for three times with three buffer exchanges
(3 x 15 ml PBS at 4,000 RPM centrifugation for 15-20 min at 25C). BSA can be
removed by Nab Protein A/G Spin Kit (Pierce # 89950). After buffer exchange or
purification, the final concentration of antibody must be measured again.
21. Calculate total amount of antibody and beads needed: Without indication, use
200µg antibody for1x10⁸ beads
22. Calculate volume of Sulfo-NHS (50mg/ml) and EDC (10mg/ml) needed for all
coupling work at this time.
23. Record details of antibodies and beads to be used in production document

E. Beads activation

24. Sonicate beads for 20 seconds and then vortex for 15 seconds. Beads are light
sensitive and should not be exposed to strong light. Use aluminum foil for light
protection.
25. Mark 15mL amber bottle with item # and beads # on cap. Pipette appropriate amount of each bead (1 x 10^8/8mL) into the bottle. Collect beads by magnetic separator or centrifugation.

26. Discard supernatant carefully by pipette. Don’t touch bead pellet (Change tips for different beads #). Add 5 ml PBS (pH7.4), vortex for 10 seconds and then separate beads as in step 7.

27. Repeat step 8 for two more times (total three times).

28. Resuspend beads (1 x 10^8) in 3 mL activation buffer (pH6.0), vortex for 20 seconds.

29. Freshly prepare enough volume of Sulfo-NHS (50mg/ml in activation buffer) and EDC (10mg/ml in activation buffer) in aluminum foil wrapped tubes. Vortex for 20 sec to dissolve all powder.

   **Note**: Sulfo-NHS and EDC have a short active life. Freshly prepare these stocks before use. If there are more than 5 antibodies for coupling, reduce time during transfer.

30. Add 1mL Sulfo-NHS into each tube, vortex for 10 seconds. Then add 1mL EDC to each tube. Vortex for 30sec. Wrap each tube first with aluminum foil before wrapping together. Shake for 20 min at RT.

31. Separate beads and discard supernatant.

32. Wash beads twice with 5 ml coupling buffer as above.

**C. Conjugation**

33. Resuspend the beads in 4mL coupling buffer, sonicate for 30 seconds, then vortex for 30 seconds.

34. Add 200µg antibody for 1 x 10^8 beads. Bring the final volume to 5mL with coupling buffer.

35. Wrap and shake tubes in dark for 2 hr at RT at maximum intensity.

   **a. Blocking**

36. Separate beads and discard supernatant.

37. Wash beads once with 5 mL 1 x TBS.

38. Resuspend beads in each amber bottle in 5 mL **Blocking buffer (BB-A or BB-C, same for next indications)**. Shake bottles in dark for overnight at 4°C with moderate intensity on plate shaker (scale 6).

39. Wash beads twice with 5 mL **Blocking Buffer** as above.

40. Resuspend beads in 10 mL **Blocking Buffer**. Vortex for 10 seconds and move to 50 mL amber bottles. Rinse the original bottle with 3 x 5 mL **Blocking Buffer** and then pool into the same amber bottle. Sonicate for 10 seconds and vortex for 10 seconds.

**E. Beads counts**

41. In a 0.5 ml microtube, make dilution of each conjugated bead at 1:5 in PBS pH7.4 (20 µl beads + 80µl PBS). Vortex briefly and Charge both sides of a
hemacytometer with 10 µl of the sample. Count the number of beads in the center square on both sides of the hemacytometer.

\[
\text{# of beads/ml} = \left( \frac{\text{total # of beads counted}}{2} \right) \times 10^4 \times 5(\text{dilution})
\]

42. Adjust the beads density with **Blocking Buffer** to 2.4 \times 10^6/ml (30 X Beads stock). Label vial for each conjugated beads and store at 4ºC. **Don’t freeze beads**.

43. Record all information into production document.

**Principle of Crosslinking mediated by EDC**

EDC reacts with a carboxyl group on molecule #1, forming an amine-reactive O-acylisourea intermediate. This intermediate may react with an amine on molecule #2, yielding a conjugate of the two molecules joined by a stable amide bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The addition of Sulfo-NHS (5 mM) stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions\(^1,2\). The amine-reactive Sulfo-NHS ester intermediate has sufficient stability to permit two-step crosslinking procedures, which allows the carboxyl groups on protein #2 to remain unaltered.
# REAGENTS

| Product                                                                 | Source                                      | Catalog Number |
|------------------------------------------------------------------------|---------------------------------------------|----------------|
| EDC (1-ethyl-3-[3dimethylaminopropyl]carbodiimide hydrochloride)       | Pierce (Thermo Scientific)                  | 77149          |
| Sulfo-NHS (N-hydroxsulfosuccinimide)                                   | Pierce (Thermo Scientific)                  | 24510          |
| Streptavidin-Alexa Fluor® 532                                          | Molecular Probes (Invitrogen)               | S-11224        |
| Streptavidin-R-phycoerythrin (1 mg/ml)                                 | Molecular Probes (Invitrogen)               | S-866          |
| PhycoLink® Streptavidin-R-phycoerythrin                                 | Prozyme                                     | PJ31S          |
| PhycoLink® Streptavidin-R-phycoerythrin                                 | Prozyme                                     | PJRS34 (ROU)   |
| Streptavidin-PE conjugate with BSA                                      | MOSS Inc.                                   | SAPE-001       |
| Streptavidin-PE conjugate without BSA                                   | MOSS Inc.                                   | SAPE001NB      |
| Flogen® Streptavidin-phycoerythrin                                      | Far East Bio-Tec                            | 1SA            |
| Streptavidin R-Phycoerythrin LumiGrade                                  | Roche Applied Science                       | 05065925103    |
| Streptavidin R-Phycoerythrin LumiGrade Ultrasensitive                   | Roche Applied Science                       | 05351693103    |
| 2-Propanol                                                             | VWR                                         | MK304306       |
| MES (2-[N-Morpholino]ethanesulfonic acid hydrate)                      | Sigma                                       | M2933          |
| TWEEN® 20 (Polyoxyethylene sorbitan monolaurate)                        | Sigma                                       | P9416          |
| SDS (Sodium lauryl sulfate), 10% solution                               | Sigma                                       | L4522          |
| Sarkosyl (N-Lauroylsarcosine sodium salt), 20% solution                 | Sigma                                       | L7414          |
| TE (Tris-EDTA) Buffer, pH 8.0, 100X                                    | Sigma                                       | T9285          |
| 5M TMAC (Tetramethylammonium chloride solution)                         | Sigma                                       | T3411          |
| 1M Tris-HCL, pH 8.0                                                    | Sigma                                       | T3038          |
| 0.5 M EDTA, pH 8.0                                                     | Invitrogen                                  | 15575-020      |
| Sodium Chloride                                                        | Sigma                                       | S6191          |
| Triton® X-100                                                          | Sigma                                       | T8787          |
Quick Protocol for P3-CVD Multiplex Assay

P3-CVD is a multiplex panel of Luminex assay to quantify the concentration of five high abundant protein targets (Clusterin, Apo-A1, Apo-B100, LP(a) and Hs-RP) from human plasma in a 96-well microplate format. Samples are diluted at 1/10,000 and incubated with capture antibody coated beads for 18-20 hours at 4°C. After washing to remove unbound proteins, beads are mixed with a cocktail of five Biotin-labeled detection antibodies. The bead mounted antibody-protein complex is then detected with Streptavidin-Phycoerythrin by BioPlex-200 reader. Bio-Plex Manager V 6.0 software is applied to analyze raw data with standard curves and calculate the concentration of five proteins from unknown samples.

Special notice
1. All FHS samples are classified to be biohazard level 2 materials. Please follow all BSL-2 lab guidelines and wear proper Personal Protection Equipments (PPE) during work.
2. Always keep tools and hands free of possible contaminates from samples or waste during assay. Change new tips and gloves whenever is suspicious.

Kit Components and Storage

| Cat# P3-CVD | Lot# P3-0611 |
|-------------|-------------|
| **P3-CVD Reagent Bag** | **Cat#** | **Lot#** | **Quantity** | **Storage** |
| Assay Buffer-A | AB-A | AB-A-0311 | 150mL | 2nd FL cold room (#2321 in Lab2317) |
| Bead Mix | P3-CAP | P3-0511 | 9mL |
| Detection Mix | P3-DET | P3-0511 | 9mL |
| SAPE | SAPE-15 | P3-0511 | 10mL |
| **P3-CVD Accessories Bag** | | | | Lab2305 (RT, storage box for P3-CVD) |
| Assay plate (Bio-Rad) | 171025001 | N/A | 3 |
| Deep well block (0.5mL) | Costar 3957 | N/A | 1 |
| Dilution plate (360µL) | Costar 3363 | N/A | 1 |
| Sample Diluent Tube (15mL) | Corning 430052 | N/A | 1 |
| Strip tubes | N/A | 8/strip | 2 strips |
| Plate sealer (Sealplate®) | 100SEALPLT | 6 sheets |
| Premade labels | N/A | N/A | 6 |
| **Frozen reagents** | | | Lab2305 (-80°C freezer, 13FR-0166, shelf 2, rack A) |
| Standard Mix | P3-STD | P3-0511 | 0.8mL |
| Quality Control 1 | P3-QC1 | P3-0511 | 0.8mL |
| Quality Control 2 | P3-QC2 | P3-0511 | 0.8mL |
| Sample Diluent (10X) | P3-SMD | P3-0511 | 1mL |

Daily pre-assay check list:
1. Pipettes and tips:
   - Adjustable multichannel pipettes: 10µL, 20µL, 50µL, 300µL, and 1mL
   - Adjustable single channel pipettes: 100µL, 200µL, 1mL, 5mL
2. 50mL Reagent Reservoir (Corning, Can be get from basement supply room)
3. Aluminum foil
4. Ice tray
5. Rack for 1mL strip tubes (for preparation of standard and QCs)
6. Plate centrifuge. Rotina 38R, change rotor for plate (#1760)
7. Sonicator (Brookstone)
Day 1

1. Preparations

1). Take one **P3-CVD Reagent Bag** from cold room #2321 to Lab2305 fridge and one **P3-CVD Accessories Bag** and 3 **Assay Plates** (**Bio-Rad Cat#171025001**) from stock box onto bench. Take Assay Buffer bottle out onto the bench only. Mark the initial, date and mother plate ID on reagent bottles and bags.

2). Label 3 assay plates with premade labels (Plate # A, B and C) on the front side of plate. Mark A, B or C to 3 circular labels for labeling wraps later on. Label the 15mL Corning tube with “Sample Diluent Tube”. Put the STD and QCs labels to the front side of each strip tubes.

3). Dispense 200µL 1 x Wash Buffer to each well of assay plate by Microplate dispenser (Statmatic I) or Bio-Rad wash station (program “DISP”). **Put plates on a safe place to avoid contamination during sample dilution.**

4). Transfer 9mL AB-A into the Sample Diluent Tube. Then transfer 1mL (10X) P3-SMD into the tube. Rinse the stock tube once. Vortex the 1X sample diluent tube briefly.

5). Take one vial of each: P3-STD (0.8mL), P3-QC(0.8mL), P3-QC2(0.8mL) and P3-SMD (10X) from Lab2305 -80C freezer (13FR-0166) to bench. Once standard and QCs are completely thawed up, move them into ice tray or 4° C until use.

2. Prepare samples

Please wear gloves and use BSL-2 hood for dilution of FHS samples. Use ice tray during sample dilution.

**For 10µL aliquot samples:**

| Tools               | µL of STD | µL of AB-A | Dilution fold |
|---------------------|-----------|------------|---------------|
| Source plate        | 10        | 190        | 1/20          |
| deep well plate     | 10        | 490        | 1/50          |
| 360µL plate         | 20        | 180        | 1/10          |
| **Final dilution**  |           |            | 1/10,000      |

**For 20µL aliquot samples:**

| Tools               | µL of STD | µL of AB-A | Dilution fold |
|---------------------|-----------|------------|---------------|
| Source plate        | 20        | 180        | 1/10          |
| deep well plate     | 10        | 490        | 1/50          |
| 360µL plate         | 10        | 190        | 1/20          |
| **Final dilution**  |           |            | 1/10,000      |

1). Take one daughter plate of FHS samples (10 or 20µL aliquot) to the hood. Use the computer for Luminex#04 to open the file "A copy to FHS ID for production.xls" containing FHS sample ID information from C:\Mysessions\P03-CVD-prod. Scan plate ID into the Plate ID sheet and input user information.

2). When samples are completely thawed up, pellet samples by centrifugation with plate rotor (Rotina 38R) at 1,000 RPM for 30 sec. Move the plate into ice tray or 4C until use.

3). Carefully remove the plate sealer to avoid sample spillover cross wells. Pipette indicated volume of diluent (AB-A) to the 1st row of wells and then mix them by pipetting up and down for 5 times. Change tips for next row of samples and finish the 1st dilution.

4). Transfer 10µL 1st dilutions to the deep well plate with 490µL AB-A. Mix them row by row with multichannel pipette (200µL volume) for 10 times. This is the 2nd dilution.

5). Dispense indicated volume of AB-A to each well of the 96-well dilution plate and make the final dilution (1/10,000) with indicated volume of 2nd dilutions. Move the plate to ice tray until use. The final diluted samples can be mixed now with plate shaker (covered with a new plate sealer) for 1-3 min or mixed by pipette before loading to assay plates (see step 4.3). **Store the daughter plate to -80° C freezer(13FR-0166) with initial, date and dilution information on the aluminum plate sealer.** Store all other diluted sample plates at 4°C until all three plates are set up at step 4.5.
3. Prepare Standards and QCs

Change a new pair of gloves before making standard dilutions.

Dilution table (5 fold serial dilution)

| Standard | µL of STD | µL of AB-A | Dilution fold |
|----------|-----------|------------|---------------|
| STD1     | 800       | 0          | Starting tube |
| STD2     | 100       | 400        | 5             |
| STD3     | 100       | 400        | 5             |
| STD4     | 100       | 400        | 5             |
| STD5     | 100       | 400        | 5             |
| STD6     | 100       | 400        | 5             |
| STD7     | 100       | 400        | 5             |
| Background | 0    | 400        | Background    |

1). Make sure frozen standard and QCs are completely thawed up at RT (about 15-30min). Mix each vial by brief vortex and move to ice tray until use. Put the STD and QC strip tubes onto an empty rack with label side towards front. For the convenience of using multiple plates and reducing variations, an 8-channel pipette will be used to transfer standard and QCs from strip tubes to 3 assay plates.

2). Pipette 400µL of AB-A to the bottom 7 wells of Standard strip. Transfer all standard solutions from the original tube into the 1st well of Standard strip. Transfer 100µL standard from well 1 to well 2, mix by pipetting up and down for 15 times. Change tips and make remaining serial dilutions until well 7. The well 8 has buffer only.

3). QC1 and QC2 are ready to use. No further dilution is required. Directly dispense 200µL of QC1 to each top 4 wells of QC strip and 200µL QC2 to each bottom 4 wells.

4. Set up assay plate

Dump and blot plates that were prewet with Wash Buffer. The following dispense steps should go column by column.

Platemap

1). Use multichannel pipette (with 8 channels) to dispense 25µL AB-A to standard wells and 25µL

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|----|----|----|
| A | STD1 | STD1 | STD1 | QC1 | S1 | S1 | S9 | S9 | S17 | S17 | S25 | S25 |
| B | STD2 | STD2 | STD2 | QC1 | S2 | S2 | S10 | S10 | S18 | S18 | S26 | S26 |
| C | STD3 | STD3 | STD3 | QC1 | S3 | S3 | S11 | S11 | S19 | S19 | S27 | S27 |
| D | STD4 | STD4 | STD4 | QC1 | S4 | S4 | S12 | S12 | S20 | S20 | S28 | S28 |
| E | STD5 | STD5 | STD5 | QC2 | S5 | S5 | S13 | S13 | S21 | S21 | S29 | S29 |
| F | STD6 | STD6 | STD6 | QC2 | S6 | S6 | S14 | S14 | S22 | S22 | S30 | S30 |
| G | STD7 | STD7 | STD7 | QC2 | S7 | S7 | S15 | S15 | S23 | S23 | S31 | S31 |
| H | BKG | BKG | BKG | QC2 | S8 | S8 | S16 | S16 | S24 | S24 | S32 | S32 |

1 X sample diluent (P3-SMD) to all other wells of each plate (see platemap above).

2). Pipette 25µL of standard dilutions, QCs into each plate.

3). Before pipetting samples, mix them by pipetting up and down for 10 times and then transfer 25µL to sample wells of each plate. Change tips for each column of samples

4). Sonicate Bead Mix for 30 sec and vortex for 15 sec. Pipette 25µL of Beads Mix into each well.

5). Seal the plate with plate sealer and wrap the plate with aluminum foil. Mark the plate# and starting time of shaking and projected end time (18-20hr). Move plates to shaker at 4C and set shaking speed at 6. Shake plate for 18-20 hours. Put remaining reagents back to the bag.
Day 2

5. Preparation

1. Take P3-CVD reagents (Detection Mix, SAPE) and instrument calibration kits to bench
2. Turn on the plate shaker on bench, set temperature to 25°C, speed to 600 RPM
3. Turn on 3 Bio-Plex 200 readers, check sheath fluid and waste bottles. Refill as necessary.
4. Warm up instrument (require 30min). Calibrate each instrument at the beginning of each day (1st user only). If validation recommendation is prompt (every month), run validation.
5. Turn on Bio-Rad wash station. Prime washers (channel=1, volume=30mL) with wash buffer.

6. Run assay

1. Take assay plates out from shaker and wash each plate twice with Bio-Rad washer (Program “MAG 2X”).
2. Pipette 25µL Detection Mix to each well of plates. Use a new plate sealer for each plate.
3. During this period, run start up, calibration or validation for three Bioplex-200 instruments (1st user only) after warm-up is finished (30min). Then:
   a. From the file menu, open the assay protocol “P03-CVD prod” from C:\Mysessions\P03-CVD-prod folder. Do not change any default settings.
   b. Identify and double check the sample ID related to the mother plate from the “FHS ID for production.xls” which is located in G:\R&D\PEP\Luminex. Copy the 1st 32 sample IDs (yellow highlight) to plate# A profile, 2nd 32 sample IDs (orange) to plate# B and the 3rd 32 sample IDs (green) to plate# C.
   c. Click “Run protocol” and Set file destination to C:\Mysessions\P03-CVD-prod folder as below.
   d. Name the raw data file as “P03-plate ID-A, B or C-YYYYMMDD”).
   e). For wells with no samples: Copy the well positions and sample IDs from sample ID sheet to the description page to notify data analysis.
4. Wash plates twice. Pipette 25µL SAPE-15 into each well of plates. Seal, wrap and shake plates or 30min at 25°C. Put all remaining reagents back to the bags.
5). Wash plates twice. Pipette 100µL sheath fluid into each well of plates. Seal, wrap and shake plate for 5min at 25°C.

6). Carefully remove plate sealer to avoid spillover cross wells. Move the plate to BioPlex-200 reader. **Input user name and plate IDs plus A, B or C for corresponding instrument.**

7). Monitor the first 2-5 well readings to make sure beads regions are correct. After reading plate, Bioplex reader will automatically save the raw data (.rbx) to previously indicated folder at C:\Mysessions\P03-CVD-prod. Then:
   a). Within the pop-up window of “Report table”, click the icon “Standard Curve” on left side of window, check the options as show below. Click “Optimize” then close the pop-up window “Optimization Report”.

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![Standard Curve Optimization Interface](image)

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For Help, press F1
b). Switch to “Report Table” on the left side of window. Select the “Export Table” from “File” menu and choose “Multiple Analyte Layout” (see figure below). Click “OK” to generate a report file (Excel format).

For wells with no samples:
Overwrite the correspondent wells in the spreadsheet “Concentration in range” of report table with letter “E” before.
Save the report file into C:\Mysessions\P03-CVD-prod folder.
c). Click “Save” from File menu to save the updated raw data.
d). Copy both the raw data file (.rbx) and report file (.xls) from local PC to G: drive at G:\R&D\PEP\Luminex\CVD Biomarkers. NIH projects\Screening Data for Framingham Sample\P03-CVD
8). Remove plates from readers and save them at 4°C until verification of the raw data is done.
9). Clean the instrument as instructions. Turn off the instruments at the end of day (last user only)

7. Data verification (for reviewer use only)
1). Verify the agreement of plate ID appeared in raw data file name(.rbx) and report file (Excel format) with FHS ID for production.xlsx.
2). Input the QC concentrations (mean) from Report file of each assay plate to the file “P03-CVD QC track.xls” which is located in G:\R&D\PEP\Luminex\CVD Biomarkers. NIH projects\Screening Data for Framingham Sample\P03-CVD. Sign the sheet.
3). Check the mark of wells with no sample
Attachment 1: Luminex Assay Flow Chart

**Day 1 preparations:**
1. Take Standard, QCs, sample diluent from -80°C freezer to bench. Take one daughter plate of FHS samples (20µL aliquot) to the hood after scanning the barcode.
2. Take one bag of P3-CVD reagents one bag of P3-CVD accessories.
3. Label 3 plates, 15mL tube and strip wells
4. Make 1 x sample diluent

**Day 2 preparations:**
1. Turn on the plate shaker, set temperature to 25°C, speed to 600 RPM.
2. Take P3-CVD reagents (Detection Mix, SAPE) and instrument calibration kits to bench
3. Turn on 3 Bio-Plex 200 readers, check sheath fluid and waste bottles. Do warm up and calibration (1st user only).
4. Turn on Bioplex wash station. Prime instrument and refill buffer if needed.
5. Edit assay protocol

Prewet plate by on plate washer with 200µL/well of Wash Buffer

\[\text{RT >5 minutes}\]

Dump and blot plates

\[\text{Add 25µL AB-A to STD wells and 25µL 1 x Sample Diluent to QC and sample wells}\]

Add 25µL/well standard, QCs or samples

Add 25µL/well Beads

Seal, wrap plates and shake for 18-20 hr at 4°C

Wash plate twice

Add 25µL/well detection antibodies

Seal, wrap plates and shake for 1 hr at 25°C

Wash plates twice

Add 25µL/well SAPE-15

Seal, wrap plates and shake for 30 min at 25°C

Wash plate twice

Add 100µL/well sheath fluid

Seal, wrap plates and shake for 5 min at 25°C

Read plate on Bioplex-200 Reader

(Naming raw data: P3-Plate ID-A/B/C-YYYYMMDD)

Export file destination: C:\Mysecssions\P03-CVD
# Attachment 2: Settings for Standard and QCs

(Assay Protocol: P03-CVD-prod)

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**Cat# P3-STD  Lot# P3-0511  Exp. 06/01/2012**

| Standard | Clusterin (008) | Apo-Al (012) | Apo-B100 (013) | Lp(a) (021) | Hs-CRP (026) |
|----------|----------------|--------------|----------------|-------------|-------------|
| STD1     | 26693.00       | 328084.00    | 3889609.00     | 5414013.00  | 3476.00     |
| STD2     | 5338.60        | 65616.80     | 777921.80      | 1082802.60  | 695.20      |
| STD3     | 1067.72        | 13123.36     | 155584.36      | 216560.52   | 139.04      |
| STD4     | 213.54         | 2624.67      | 31116.87       | 43312.10    | 27.81       |
| STD5     | 42.71          | 524.93       | 6223.37        | 8662.42     | 5.56        |
| STD6     | 8.54           | 104.99       | 1244.67        | 1732.48     | 1.11        |
| STD7     | 1.71           | 21.00        | 248.93         | 346.50      | 0.22        |

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**Panel: P03-CVD, Lot P3-0611**

| QC1       | Mean | Clusterin (08) | Apo-Al (12) | Apo-B (13) | Lp(a) (21) | CRP (26) |
|-----------|------|---------------|-------------|------------|------------|---------|
|           |      |               |             |            |            |         |
| QC2       | Mean | 1380.39       | 72690.23    | 114961.15  | 226307.82  | 542.01  |
|           |      |               |             |            |            |         |

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