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Version 2

# Luminex Magnetic Bead Assay for Assessment of the Immune Response to *Vibrio cholerae* V.2

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**We use this protocol and it's working**

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## Abstract

This protocol provides instructions for assessing antibody responses to *Vibrio cholerae* in human blood plasma samples using a multiplex bead assay (MBA).

Magnetic microspheres (beads) are conjugated with target antigens according to the manufacturer's instructions. Individual bead stocks are added to a buffered solution to create a pooled, master bead stock and dispensed into 384-well plates. Plasma samples are diluted and tested in duplicate. A serial dilution of a pooled plasma standard, positive and negative controls are included on each plate. After an incubation step to allow antibody-antigen binding, magnetic beads are washed, and incubated with fluorescently labeled isotype-specific secondary antibodies. Beads are washed once more and then resuspended and analyzed on a FlexMap3D instrument.

## Guidelines

Human blood collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee.

## Materials

### Materials

Antibody Coupling Kit, Luminex: 40-50016

EDC; Thermo Scientific 77149

Antigen panel (see Table 1, section 7.1.3)

Secondary antibodies, PE-conjugated

- IgG, Southern Biotech Cat # 9040-09
- IgA, Southern Biotech Cat # 2050-09
- IgM Southern Biotech Cat # 9020-09
- IgG1 Southern Biotech Cat # 9052-09
- IgG2 Southern Biotech Cat # 9070-09
- IgG3 Southern Biotech Cat # 9210-09
- IgG4 Southern Biotech Cat # 9200-09
- IgA1 Southern Biotech Cat # 9130-09
- IgA2 Southern Biotech Cat # 9140-09

Sample Dilution Buffer; 1% BSA with 0.05% Tween20

Bead Stock Buffer; 0.1% BSA in 1x PBS

Wash Buffer; 1% BSA with 0.05% Tween20 in 1X DPBS

10X sterile DPBS for making 1X wash buffer, Corning, 20-031-CV

Sheath Fluid Plus, Fisher Scientific NC0695082

Bovine Serum Albumin (BSA), for assay buffer and antigen, Sigma A7906-50G

15-mL and 50-mL Falcon tubes

Sterile pipet tips, all sizes

Sterile Reservoirs, VWR 89094-664

1.5 mL Microcentrifuge tubes

384-Well Polystyrene Non-Binding Flat Bottom Microplate, Fisher Scientific Greiner Bio-One™ 07000892

Aluminum foil

Foil Plate Seal, Bio-Rad, MSF 1001

Milli-Q water, or equivalent

U-bottom 96-well plates

### Equipment

Dynabead2Mag-Magnet, Thermofisher 12321D

Plate washer, e.g., Tecan Hydrospeed

Microplate Shaker

Sonicated water bath

Vortex mixer

Luminex FlexMap3D instrument

12-channel multichannel pipets

24-channel multichannel pipets

P2, P20, P200 and P1000 single channel pipets



## Safety warnings

### ! Biosafety/PPE

- This protocol requires working with human blood plasma samples that should be handled as potentially infectious.
- Lab staff are required to complete biosafety training prior to performing this protocol.
- Standard lab PPE is recommended for this protocol (e.g., lab coats, gloves, and goggles).
- All solid waste should be discarded in biohazardous waste containers. Liquid waste should be treated with a 10% solution of bleach for 20 minutes before discarding in the sink. Wear eye and/or face protection when disposing liquids in sink.
- Spills should be treated as infectious and cleaned using the lab standard protocol (i.e. cover spill with absorbent material, spray with 10% bleach to soak thoroughly, treat for at least 15 minutes, use tongs to discard material in biohazard waste, change gloves and clean area with 70% ethanol)

### Chemical Hazards

- EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) CAS # 25952-53-8
- Acute toxicity: Irritant. Oral, Dermal. Wear protective gloves, lab coat, eye protection/face protection when handling.
- MSDS: <https://www.sigmaaldrich.com/US/en/sds/mm/341006>

## Definitions/Abbreviations

- 1 **Beads:** Luminex® MagPlex® microspheres  
**BSA:** bovine serum albumin  
**MFI:** mean fluorescent intensity  
**PBS:** phosphate buffered saline  
**PPE:** personal protective equipment  
**rpm:** revolutions per minute  
**O1 Standard:** A high-titer pooled plasma sample prepared from convalescent plasma of patients who have recovered from V. cholerae O1 infection. Serial dilutions are used as a standard curve to normalize MFI data to minimize inter-run variation.  
**O139 Positive Control:** A high-titer pooled plasma sample prepared from convalescent plasma, reactive with the O139 antigen.  
**Sialidase/TcpA Positive Control:** A high-titer pooled plasma sample prepared from convalescent plasma, reactive with the sialidase and tcpA antigens.  
**Negative Control:** A pooled heat-inactivated plasma sample is prepared from North American volunteers without prior exposure to cholera.

## Procedure - Antigen Conjugation to MagPlex Beads

- 2 Antigens are conjugated to beads following the manufacturer's instructions in the Luminex xMAP® Antibody Coupling Kit User Manual, RevE, using a ratio of 5µg of antigen for every  $1 \times 10^6$  beads. Stocks are quantitated using a cell counter and stored at 4C, wrapped lids in parafilm to prevent evaporation and foil to protect from photobleaching.

The panel consists of the following antigens and bead regions:

| A                                    | B                     | C           | D              |
|--------------------------------------|-----------------------|-------------|----------------|
| Antigen                              | Antigen Source        | Bead Region | Bead Catalog # |
| V. cholerae O1 Inaba OSP-BSA         | Edward Ryan, MGH      | 44          | MC10042-01     |
| V. cholerae holotoxin (CTh)          | List Biological, 100B | 77          | MC10044-01     |
| V. cholerae toxin, B subunit (CtxB)  | Sigma, C9903-1MG      | 38          | MC10077-01     |
| V. cholerae sialidase, N16961 strain | Richelle Charles, MGH | 35          | MC10038-01     |



| A                                                       | B                                  | C  | D          |
|---------------------------------------------------------|------------------------------------|----|------------|
| V. cholerae toxin-regulated pilus (TcpA) N16961 strain  | Harris Lab, MGH                    | 49 | MC10035-01 |
| V. cholerae O1 Ogawa OSP-BSA                            | Edward Ryan, MGH                   | 42 | MC10049-01 |
| V. cholerae O139 OSP-BSA                                | Edward Ryan, MGH                   | 61 | MC10061-01 |
| V. cholerae cytolysin (VCC)                             | Richelle Charles, MGH              | 25 | MC10025-01 |
| Influenza A HA antigen HA1(A/California/07/2009) (H1N1) | Immune Technology, IT-003-SW12p    | 18 | MC10018-01 |
| E. coli heat-labile toxin, human isoform (LTh)          | Dennis Clements, Tulane University | 55 | MC10055-01 |
| E. coli heat-labile toxin, B subunit (LT-B)             | Dennis Clements, Tulane University | 20 | MC10020-01 |

**V. cholerae MagPlex Bead Panel**

## Preparation of Standards and Controls

- 3 Standard and controls should be prepared in 1.5-mL microcentrifuge tubes, to allow for thorough mixing using a vortex mixer.
- 4 **Standard Dilutions**

Using sample dilution buffer as a diluent, prepare an 8-step dilution series of the O1 Standard, beginning with a 1:10 dilution of the stock and then performing  $7 \times 4$ -fold additional dilutions. The resulting series will represent the following dilutions of standard: 1:10, 1:40, 1:160, 1:640, 1:2560, 1:10240, 1:40960 and 1:163840.

Adjust the volume for dilutions based on the number of plates and/or antibody isotypes to be tested. 5  $\mu$ L of each dilution will be used per replicate (384-well plate).
- 5 **Controls**

Prepare Positive and Negative Controls by diluting 1:100 in sample dilution buffer. Factor in any previous dilutions of the plasma stock. 5  $\mu$ L of each dilution will be used per replicate (384-well plate).

Prepare an additional aliquot of sample dilution buffer in a 1.5 mL centrifuge tube to use as a blank (plate background measurement).

## Sample Preparation

- 6 Sample dilutions are prepared in low-protein binding round-bottom 96-well plates to allow for use of multi-channel pipet when transferring samples to the 384 well plate.

Prepare plasma samples by diluting 1:100 in sample dilution buffer, factoring in any previous dilutions of the plasma sample.

Using a multichannel pipet, add 148.5  $\mu$ L of sample dilution buffer to 96-well plate.

Mix samples briefly by pulsing on a vortex mixer and then using a P2 pipet, transfer 1.5  $\mu$ L of plasma into buffer to make the 1:100 dilution.

Seal plate with a foil plate seal and place on a microplate shaker at room temperature to mix at 550 rpm while preparing bead stocks (approximately 30 minutes).

## Preparation of Pooled Bead Stock

- 7 A 10x concentrated bead stock is prepared and mixed well before diluting to the final 1x stock concentration dispensed into the 384 well plate. The final 1x stock contains 810 beads for each bead region (antigen type) in a 45  $\mu$ L volume. An overage of 20% is included to account for loss during pipetting.

The table below provides an example calculation.

| A                           | B       | C              |
|-----------------------------|---------|----------------|
| Step                        | Example | Formula        |
| number of samples           | 24      | 24             |
| number of controls          | 12      | 12             |
| replicate number            | 2       | 2              |
| number of secondary Abs     | 1       | 1              |
| total number of wells       | 72      | =(B2+B3)*B4*B5 |
| number of 384-well plates   | 0.2     | =B6/384        |
| number of beads/region/well | 810     | 810            |

| A                                        | B     | C                |
|------------------------------------------|-------|------------------|
| total number of beads + 20%              | 69984 | =B6*B8*1.2       |
| volume beads per well (μL)               | 45    | 45               |
| total volume of 1x bead stock + 20% (mL) | 3.888 | =B6*B10*1.2/1000 |
| total volume of 10x bead stock (mL)      | 0.389 | =B11/10          |

### Bead Stock Calculation Example

- 8 Prepare bead pool in a 50-mL centrifuge tube. Protect tube from light by wrapping in foil.

Vortex bead stocks for 1 minute to resuspend beads and then sonicate for 2 minutes in an ultrasonic water bath at a frequency of 46 KHz.

Add the predetermined volume of Bead Stock Buffer to the 50-mL tube for the 10x pooled bead stock, then add beads for each of the antigens in the panel. Vortex each bead stock for 5-10 seconds to mix immediately before making the transfer.

Vortex 10x pooled bead stock for 1 minute, then add the remaining Bead Stock buffer to dilute bead stock to final 1x concentration. Vortex 1x pooled bead stock again for 1 minute to mix.

## Incubation Steps

- 9 Transfer half of the 1x pooled bead stock to a reagent reservoir and use a multichannel pipet to transfer 45 μL to the wells of the 384-well plate. Ensure that the bead pool in the reservoir remains mixed by pipetting up and down 3-5 times before each transfer. Mix the 50-mL tube for 1-minute before transferring the remaining portion of 1x pooled bead stock to the reservoir.
- 10 Using a 12-channel pipettor, transfer 5 μL of diluted sample from the 96-well plates to the 384-well plate. Cover plate with an opaque foil seal and incubate for 2 hours at room temperature on a microplate shaker set to 850 rpm.
- 11 Prepare a 1:150 dilution of each secondary antibody in sample dilution buffer at the tail end of the incubation step and protect from light. Prepare sufficient volume to cover

addition of 40  $\mu$ L per well plus 15-20% overage for loss during pipetting and vortex gently for 10 seconds to mix.

- 12 After the 2-hour incubation, wash beads on a magnetic plate washer (e.g. Tecan HydroSpeed) using instrument dispense and aspiration rates suitable for magnetic beads. Use 6 cycles of 50  $\mu$ L of Wash Buffer.

An example wash protocol for a Tecan HydroSpeed Instrument is provided below:

Grenier 384-well plate

Aspiration Rate: 1

Stage 1

1. Cycles: 1
2. Soak
3. Intensity: Off Shake
4. Time: 90 seconds

Stage 2

1. Cycles: 6
2. Aspirate
3. Z-position: Bottom, Normal
4. Set: 8.0 [mm]
5. Time: 1 second
6. Head speed: 5 [mm/s]
7. Dispense
8. Z-position: Bottom
9. Set: 8.0 [mm]
10. Dispense rate: 50 [ $\mu$ L/s]
11. Channel: 2
12. Volume: 50 [ $\mu$ L]
13. Soak
14. Intensity: Medium Shake
15. Time: 15 seconds
16. Soak
17. Intensity: Off Shake
18. Time: 60 seconds
19. Cycles: 1
20. Aspirate
21. Z-position: Bottom, Normal
22. Set: 8.0 [mm]
23. Time: 1 second
24. Head speed: 5 [mm/s]



- 13 Using a reagent reservoir and a multichannel pipet, add 40  $\mu\text{L}$  of secondary antibody to each well. Cover plate with a foil seal and incubate at room temperature for 1 hour on a microplate shaker set at 850 rpm.
- 14 After the 1-hour incubation, wash beads on a magnetic plate washer (e.g. Tecan HydroSpeed) using instrument dispense and aspiration rates suitable for magnetic beads. Using 6 cycles of 50  $\mu\text{L}$  of Luminex Sheath Buffer and a final resuspension step with 60  $\mu\text{L}$  of Luminex Sheath Buffer.

An example wash protocol for a Tecan HydroSpeed Instrument is provided below:

Grenier 384-well plate

Aspiration Rate: 1

Stage 1

1. Cycles: 1
2. Soak
3. Intensity: Off Shake
4. Time: 90 seconds

Stage 2

1. Cycles: 6
2. Aspirate
3. Z-position: Bottom, Normal
4. Set: 8.0 [mm]
5. Time: 1 seconds
6. Head speed: 5 [mm/s]
7. Dispense
8. Z-position: Bottom
9. Set: 8.0 [mm]
10. Dispense rate: 50 [ $\mu\text{L}/\text{s}$ ]
11. Channel: 1
12. Volume: 50 [ $\mu\text{L}$ ]
13. Soak
14. Intensity: Medium Shake
15. Time: 15 seconds
16. Soak
17. Intensity: Off Shake
18. Time: 60 seconds
19. Cycles: 1
20. Aspirate
21. Z-position: Bottom, Normal
22. Set: 8.0 [mm]
23. Time: 1 second



24. Head speed: 5 [mm/s]

Stage 3

1. Dispense

2. Z-position: Overflow

3. Set: 8.0 [mm]

4. Dispense rate: 50 [ $\mu$ l/s]

5. Channel: 1

6. Volume: 60 [ $\mu$ l]

- 15 Cover plate with a foil seal and sonicate in an ultrasonic water bath for 1 minute to disperse beads well before removing foil seal and loading on the Luminex instrument.

## Luminex Run Protocol

16 **Luminex acquisition settings:**

Volume: 50 microliters

Timeout: 200 seconds

Bead Type: MagPlex

Plate Name: Greiner Luminex

DD Gating: 7500 to 15000

Reporter Gain: Enhanced PMT (High)

Assign the antigens to their corresponding beads regions (see table in step 2). Set the target bead count to a minimum of 50 for each antigen.

We also typically add 4-8 alcohol flush steps at regular intervals throughout the 384-well plate, depending on how many samples are being tested.

17 **Run QC:**

- Retest plates if over half of the dilutions of standards had 5 or more antigens with a coefficient of variation greater than 20% (calculated from triplicate MFI measurements)
- Exclude any measurements with a bead count less than 30

## Analysis

- 18 Follow the following steps to estimate relative antibody units.

*Note: Our initial publications reported values as 1/RAU, but we have subsequently simplified and report as RAU. The values are calculated in the same manner, are proportional and comparable.*

19 **Average Replicates**



For each standard dilution and sample, calculate the arithmetic mean NetMFI value from replicate wells.

## 20 **Log10 Transformation**

- Transform the averaged NetMFI values using log10.
- Transform the dilution factor for each standard (e.g., 1:100 → 100 →  $\log_{10} = 2$ ).

## 21 **4-Parameter Logistic (4PL) Curve Fit**

Fit a 4-parameter logistic (4PL) model using  $\log_{10}(\text{dilution factor})$  as the independent variable and  $\log_{10}(\text{NetMFI})$  as the dependent variable. Assess curve fit and ensure all interpolations fall within the standard range.

## 22 **Interpolation of Sample Values**

For each sample, use its  $\log_{10}(\text{NetMFI})$  value and the fitted 4PL curve to interpolate the corresponding  $\log_{10}$  dilution equivalent ( $x_{\text{interp}}$ ).

## 23 **Calculation of Relative Antibody Units (RAU)**

Convert the interpolated value into RAU using:  $\text{RAU} = 10^{(\log_{10}(D_{\text{sample}}) - x_{\text{interp}})}$ , where  $D_{\text{sample}}$  is the dilution factor at which the sample was assayed.

## 24 **Reporting**

Report RAU values along with dilution factors used, curve diagnostics, handling of values below the limit of detection (if applicable), and any offsets applied to zero or near-zero NetMFI values.