

Bio-Plex Pro™ Human Th17 Cytokine Assays

Instruction Manual

For technical support, call your local Bio-Rad office, or in the U.S., call 1-800-424-6723.
For research use only. Not for diagnostic procedures.



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Introduction

Bio-Plex Pro™ Human Th17 Cytokine Assays

Bio-Plex Pro human Th17 cytokine assays are designed to meet the needs of the most demanding preclinical and clinical research environments. These magnetic bead-based multiplex immunoassays include a comprehensive blend of soluble biomarkers involved in the T-helper cell type 17 immune response pathway, a key driver of autoimmune diseases, as well as the body's response to microbial infection. With diluents optimized for enhanced performance in clinical matrices, a fast protocol, and a flexible kit format, the Human Th17 cytokine assays promise to make multiplexing easier and more reliable than ever.

Multiplexing with Bio-Plex Pro Assays

Bio-Plex Pro assays enable researchers to quantify multiple protein biomarkers in a single well of a 96-well plate in just 3 to 4 hr. These robust immunoassays require as little as 12.5 µl of serum or plasma, or 50 µl of other biological fluid. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station. Magnetic separation offers greater convenience and reproducibility compared to vacuum filtration.

For more information please visit www.bio-rad.com/bio-plex

Principle

Technology

The Bio-Plex® suspension array system is built around the three core elements of xMAP technology:

- Fluorescently dyed microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of more than 100 different types of molecules in a single well of a 96-well microplate
- A dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads
- A high-speed digital signal processor that efficiently manages the fluorescence data

Assay Format

Bio-Plex Pro™ assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter.

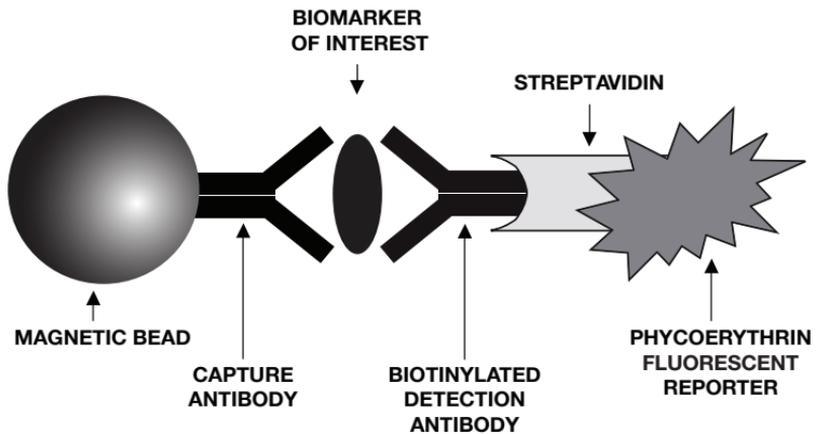


Figure 1. Bio-Plex sandwich immunoassay

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex® system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, for example, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites PE to generate a reporter signal, which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output, and Bio-Plex Manager™ software presents data as Median Fluorescence Intensity (MFI) as well as concentration (pg/mL). The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

Kit Contents and Storage

Reagents Supplied

Bio-Plex Pro™ human Th17 cytokine assays are available in a convenient all-in-one kit format that includes assay, reagent, and diluent components in a single box.

Table 1. Contents of 1 x 96 well premixed and custom x-Plex™ kits.

| Component | Quantity** |
|---|-------------------|
| Standard diluent HB | 10 ml |
| Sample diluent HB | 8 ml |
| Assay buffer | 50 ml |
| Wash buffer | 200 ml |
| Detection antibody diluent | 5 ml |
| Streptavidin-PE (100x) | 1 tube |
| Assay plate (96-well flat-bottom plate or filter plate) | 1 plate |
| Sealing tape | 1 pack of 4 |
| Assay Quick Guide | 1 booklet |
| Coupled magnetic beads (20x) | 1 tube |
| Detection antibodies (20x) | 1 tube |
| Standard | 1 vial |
| Quality Control High* | 1 vial |
| Quality Control Low* | 1 vial |

* Only premixed Th17 panels and custom Th17 x-Plex kits include quality controls.

** Volumes shown are approximate. Quantities in Express assays will vary.

Storage and Stability

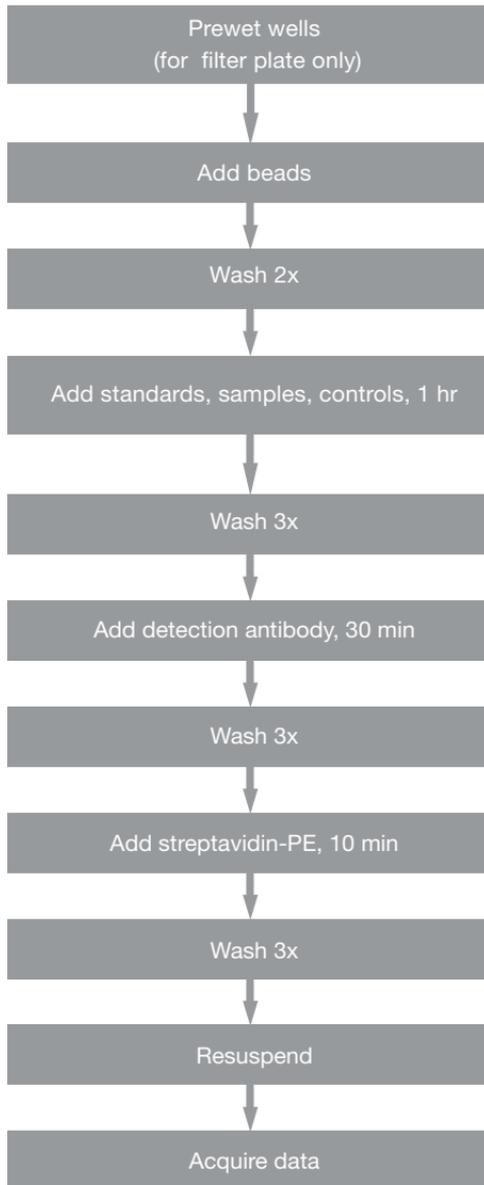
Kit contents should be stored at 4°C and never frozen. Coupled magnetic beads and streptavidin-PE should be stored in the dark. All components are guaranteed for a minimum of 6 months from the date of purchase when stored as specified.

Table 2. Recommended materials.

| Item | Ordering Information |
|--|--|
| Bio-Plex Pro Assays Quick Guide 1 | Item #10022581 (download at www.bio-rad.com/bio-plex) |
| Bio-Plex® 200 system or Luminex system with HTF | Bio-Rad catalog #171-000205 |
| Bio-Plex validation kit Note: the validation kit should be run monthly to ensure performance of fluidics and optics systems | Bio-Rad catalog #171-203001 |
| Bio-Plex calibration kit Note: the calibration kit should be run either daily or whenever the instrument is to be used to standardize fluorescence signal | Bio-Rad catalog #171-203060 |
| Bio-Plex Pro wash station For use with magnetic bead-based assays only | Bio-Rad catalog #300-34376 |
| Bio-Plex Pro II wash station For use with both polystyrene (nonmagnetic) and magnetic bead-based assays | Bio-Rad catalog #300-34377 |
| Bio-Plex handheld magnetic washer For use with magnetic bead-based assays only | Bio-Rad catalog #170-20100 |
| Bio-Plex Pro flat-bottom plates (40, 96-well plates) For magnetic separation on the Bio-Plex Pro wash station | Bio-Rad catalog #171-025001 |
| Microtiter plate shaker IKA MTS 2/4 shaker for 2 or 4 microplates or Barnstead/Lab-Line Model 4625 plate shaker (or equivalent capable of 300–1,100 rpm) | IKA catalog #320-8000 VWR catalog #57019-600 |
| Bio-Rad Aurum™ vacuum manifold For vacuum filtration | Bio-Rad catalog #732-6470 |
| BR-2000 vortexer | Bio-Rad catalog #166-0610 |
| Reagent reservoirs, 25 ml For capture beads and detection antibodies | VistaLab catalog #3054-1002 or VistaLab catalog #3054-1004 |
| Reagent reservoir, 50 ml (for reagents and buffers) | VistaLab catalog #3054-1006 |
| Pall Life Science Acrodisc: 25 mm PF syringe filter (0.8/0.2 µm Supor membrane) | Pall Life Sciences catalog #4187 |
| Filter plate, 1 x 96 with clear plastic lid and tray | Bio-Rad catalog #171-304502 |

Other: 15 ml polypropylene tubes for reagent dilutions. Calibrated pipets (multichannel pipets are optimal for this application) and pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, 1.5 or 2 ml microcentrifuge tubes, and standard flat-bottom microplate (for calibrating vacuum manifold).

Assay Workflow



Important Considerations

Instruments and Software

The Bio-Plex Pro™ assays described in this manual are compatible with all currently available Luminex-based life science research instruments. Assays can be read and analyzed with either Bio-Plex Manager™ software or Luminex xPonent software (Section 8).

Assay Procedures

Please pay close attention to vortexing, shaking, and incubation times, and Bio-Plex® reader PMT (RP1) setting, as these have been optimized specifically for each assay panel.

Assay Quick Guide

Each assay kit comes complete with a printed Bio-Plex Pro™ Assay Quick Guide (item #1002258), which can be used to prepare and run a full 1 x 96 well assay plate. Users can also download a copy at www.bio-rad.com/bio-plex.

Bead Regions and Multiplexing Compatibility

- Bead regions for all analytes are listed in the Read Plate section.
- For optimum performance of the human Th17 cytokine assays, mixing of Th17 analytes with other Bio-Plex assay panels or reagent kits is not recommended. For example:
 - a. Overlapping analytes in different antigen standards should not be mixed
 - b. Bio-Plex Pro reagent kit II contains diluents that have not been fully validated in other assays

In each case, standard curves and sample values may be inaccurate.

Detailed Instructions

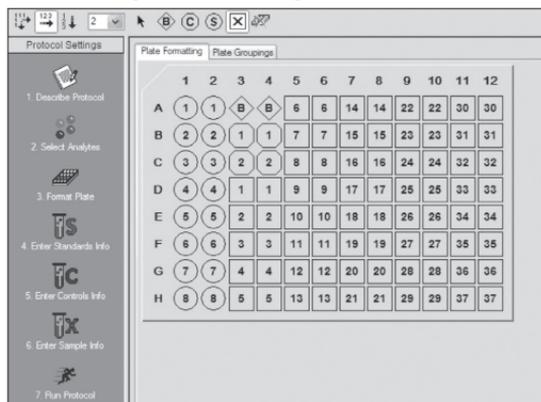
The following pages provide detailed instructions for each step of the assay procedure, including preparation, running the assay, and reading the plate with Bio-Plex Manager™ and Luminex xPonent software.

1. Plan Plate Layout

Prior to running the assay, determine the total number of wells in the experiment using the plate layout template on page 34, or the Plate Formatting tab in Bio-Plex Manager™. A suggested plate layout is shown in Figure 2, with all conditions run in duplicate.

1. Assign standards to columns 1 and 2, with the highest concentration in row A and the lowest concentration in row H.
2. Assign the blank to wells A3 and A4. The blank should consist of your chosen standard diluent. Note that Bio-Plex Manager™ automatically subtracts the Blank (B) FI value from all other assay wells.
3. User-specified controls, as well as the quality controls supplied in premixed Th17 cytokine kits, are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples.
5. Once the total number of wells is known, you can calculate the required volumes of beads, detection antibody, and streptavidin-PE. Use Tables 6, 9, and 11, respectively, or the Calculation Worksheet on pages 35–36.

Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager, see Section 8.



Legend

-  **Standards**
-  **Blank**
-  **Samples**
-  **Controls**

2. Prepare Instrument

Start up and calibrate the Bio-Plex® system with Bio-Plex Manager™ software prior to setting up the assay. The calibration kit should be run daily or before each use of the instrument to standardize the fluorescent signal. For instructions on using other xMAP system software packages, contact Bio-Rad Technical Support.

The validation kit should be run monthly to ensure performance of fluidics and optics systems. Refer to either the software manual or online Help for directions on how to conduct validation.

Start Up System (Bio-Plex 100, 200, or similar)

1. Empty the waste bottle and fill the sheath fluid bottle before starting if high throughput fluidics (HTF) are not present. This will prevent fluidic system backup and potential data loss.
2. Turn on the reader, XY platform, and HTF (if included). Allow the system to warm up for 30 min (if not already done).
3. Select Start up  and follow the instructions. If the system is idle for 4 hr without acquiring data, the lasers will automatically turn off. To reset the 4-hr countdown, select Warm up  and wait for the lasers/optics to reach operational temperature.

Calibrate System

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads. Use the Bio-Plex system low RP1 target value.
2. Select OK and follow the software prompts for step-by-step instructions for CAL1 and CAL2 calibration.

NOTE! In Bio-Plex Manager v6.1 and higher, startup, warm up and calibrate can be performed together by selecting the start up and calibrate icon. 

3. Prepare Wash Method

There are two methods of performing wash steps in the assay procedure: magnetic separation and vacuum filtration. Compatible wash stations and plate types are shown in the table below.

Table 3. Summary of compatible wash stations and plate types.

| Wash Method | Wash Station | Assay Plate |
|---------------------|--|--------------------|
| Magnetic separation | Bio-Plex Pro™ Bio-Plex Pro II (use MAG programs) Bio-Plex handheld magnetic washer | Flat-bottom plate |
| Vacuum filtration | Bio-Plex Pro II (use VAC programs) Vacuum manifold (manual) | Filter plate |

Magnetic Separation Using the Bio-Plex Pro or Bio-Plex Pro II Wash Station

The wash station does not require calibration, however it should be primed before use. For more information, refer to the Bio-Plex Pro and Pro II wash station quick guide (bulletin 5826).

1. Install the appropriate plate carrier on the wash station.
2. Use the Prime procedure to prime channel 1 with wash buffer.

Bio-Plex Handheld Magnetic Washer

Place an empty flat-bottom plate on the magnetic washer by sliding it under the retaining clips. Push the clips inward to secure the plate. Make sure the plate is held securely. If needed, the clips can be adjusted for height and tension. For detailed instructions, refer to the user guide (item #10023087).

Vacuum Filtration Using a Vacuum Manifold

Calibrate the vacuum manifold by placing a standard 96-well flat-bottom plate on the unit and adjusting the pressure to -1 to -3" Hg. In general, 100 ul liquid should take 3–4 sec to clear the well. For more detailed instructions, refer to item #10005042.

4. Prepare Standards and Controls

General Instructions

- It is essential to prepare standards and quality controls (if included) exactly as described in this section. Incorrect preparation may lead to low signal or variable measurements from plate to plate
- The product data sheet or peel-off sticker provided with the standards lists the most concentrated point on the standard curve (S1). Enter this information into Bio-Plex Manager™ software as instructed in Section 8

Using the Quality Controls (optional)

Two-level quality controls (high and low) are included with premixed Th17 cytokine panels and custom x-Plex kits. Their use is intended for monitoring the day-to-day quality of assay results.

Selecting a Diluent for Standards and Controls

Refer to Table 4 for recommended diluents based on different sample types.

In order to meet the lot-specific control ranges provided on the product data sheet, both the standards and controls should be reconstituted in Bio-Plex® standard diluent HB. If reconstituting in a different diluent, users will need to establish/validate their own control ranges or acceptance criteria.

Table 4. Summary of recommended diluents for standards and controls.

| Sample Type | Diluent for Standards and Controls* | Add BSA |
|------------------------------|--|-----------------|
| Serum and plasma | Standard diluent HB | None |
| Culture media, with serum | Culture medium | None |
| Culture media, serum-free | Culture medium | To 0.5% final** |
| Lavage, sputum, other fluids | Sample diluent HB | To 0.5% final** |
| Lysate | Sample diluent HB | To 0.5% final** |

* If using diluents other than standard diluent HB, users must establish their own control ranges.

** At least 0.5% final BSA is recommended to stabilize analytes and reduce absorption to labware.

Reconstitute Standards and Quality Controls

This procedure prepares enough standard to run each dilution in duplicate.

1. Gently tap the vial containing the lyophilized standards on a solid surface to ensure the pellet is at the bottom of the vial.
2. Reconstitute a single vial of standards with **781 μ l** of the appropriate diluent. Optional: at the same time, reconstitute the two control vials with **250 μ l** of the appropriate diluent as summarized in Table 4.
Controls do not require further dilution.
3. Gently **vortex** the reconstituted standards and controls for **5 sec**, then incubate **on ice for 30 min**. It is important that reconstitution of standards and controls is started and ended at the same time. Be consistent with this incubation time to ensure optimal assay performance and reproducibility.
4. During the incubation period, prepare the samples as instructed in the Prepare Samples section.

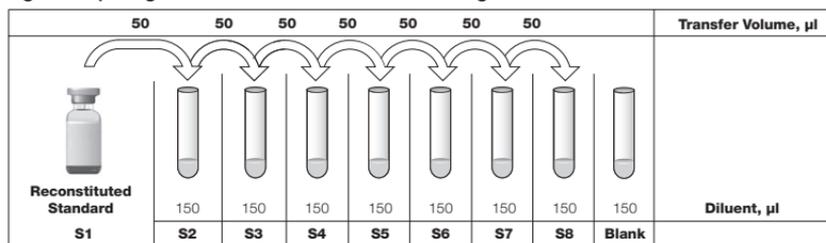
Prepare the Standard Dilution Series

The following procedure produces an eight-point standard curve with a four-fold dilution between each point. Pipet carefully using calibrated pipets and use new pipet tips for every volume transfer.

1. Label eight 1.5 ml polypropylene tubes S2 through S8 and Blank.
2. Add **150 μ l** of the appropriate diluent to tubes S2–S8 (Figure 3).
3. **Vortex** reconstituted standards gently for **5 sec** before removing any volume. Add **50 μ l** to the S2 tube containing the chosen standard diluent. **Vortex** for **5 sec**.

4. Use a new pipet tip to transfer **50 μ l** from the S2 tube to the S3 tube. **Vortex** for **5 sec**.
5. Continue with 1:4 (fourfold) serial dilutions as shown in Figure 3.
6. Use reconstituted and diluted standards and controls immediately. Do not freeze for future use.

Fig. 3. Preparing a fourfold dilution series with a single reconstituted standard.



5. Prepare Samples

General guidelines for preparing different sample types are provided here. For more information, consult publications listed in Bio-Rad bulletin 5297, available for download at www.bio-rad.com, or contact Bio-Rad Technical Support.

- Once thawed, keep samples on ice. Prepare dilutions just prior to the start of the assay and equilibrate to room temperature before use
- Do not freeze diluted samples.

Table 5. Summary of recommended sample diluents and dilution factors.

| Sample Type | Diluent | Add BSA | Sample Dilution* |
|------------------------------|-------------------|----------------|---|
| Serum and plasma | Sample diluent HB | None | Fourfold (1:4) |
| Culture media, with serum | Culture media | None | User optimized (neat to 1:10) |
| Culture media, serum-free | Culture media | To 0.5% final* | User optimized (neat to 1:10) |
| Lavage, lysate, other fluids | Sample diluent HB | To 0.5% final* | User optimized (neat to 1:10) |
| Lysate | Sample diluent HB | To 0.5% final* | User optimized (at least 1:2 for 50 to 500 ug/ml final protein) |

*At least 0.5% final BSA is recommended to stabilize analytes and reduce absorption to labware.

Sample Preparation

Serum and Plasma

EDTA or citrate is preferred as an anticoagulant. Heparin-treated plasma, while compatible with Bio-Plex Pro™ assays, may absorb certain soluble proteins of interest. Avoid using hemolyzed samples as this may lead to false positive results.

1. Draw whole blood into collection tubes containing anticoagulant. Invert tubes several times to mix.
2. For serum, allow blood to clot at room temperature for **30 to 45 min**. For plasma, proceed directly to the centrifugation steps.
3. Perform centrifugation at 1,000 x g for **15 min** at 4°C and transfer the serum or plasma to a clean polypropylene tube.
4. To completely remove platelets and precipitates, centrifuge again at 10,000 x g for **10 min** at 4°C.
5. Dilute samples fourfold (1:4) by adding 1 volume of sample to 3 volumes of Bio-Plex® sample diluent HB (for example, **40 µl** sample + **120 µl** sample diluent).
6. Assay samples immediately or aliquot into single-use tubes and store at -70°C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatant

1. Collect supernatants and centrifuge at 1,000 x g for **15 min** at 4°C. For cell lines cultured in serum-free culture media, collect samples and add BSA as a carrier protein to a final concentration of at least 0.5% to stabilize protein analytes and to prevent adsorption to labware.
2. Transfer to a clean polypropylene tube. If cellular debris or precipitates are present, centrifuge again at 10,000 x g for **10 min** at 4°C.
3. We recommend testing undiluted samples first. If levels are anticipated to be high, samples can be further diluted in culture medium. Rarely would samples need to be diluted greater than 1:10.
4. Assay immediately or store samples in single-use aliquots at -70°C. Avoid repeated freeze-thaw cycles.

Lavage, Sputum, and Other Biological Fluid Samples

Keep all samples on ice until ready for use. The appropriate sample dilution factor should be optimized by the user.

1. If required, dilute the sample in Bio-Plex sample diluent with BSA added to a final concentration of 0.5%.
2. Centrifugation at 10,000 x g for **10 min** at 4°C may be required to clarify the sample.

Lysates

The Bio-Plex cell lysis kit is required for lysate preparation (available separately, catalog #171-304011 and #171-304012). Refer to bulletin 5297 for a list of published articles on cytokine analysis in tissue samples.

1. Prepare the cell or tissue lysates according to the instructions provided with the Bio-Plex cell lysis kit. The protease inhibitors, Factor I and II, are included in the kit. PMSF needs to be added to lysis buffer at a final concentration of 2 mM. The lysates should be free of particulate matter.

2. Determine the total protein concentration of the lysate. It may be necessary to test lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.
3. Dilute at least 1:2 in sample diluent + 0.5% BSA, to a final protein concentration of 50–500 µg/ml.

For optimum antibody binding during sample incubation, it is important to dilute lysates as much as possible to reduce the detergent concentration.

4. If the lysate is not tested immediately, store at –20°C. Lysates are stable for up to five freeze-thaw cycles.

6. Prepare Coupled Beads

1. Use Tables 6–7 or the Calculation Worksheet on page 35 to calculate the volume of coupled beads and assay buffer needed. Tables 6–7 include ~20% excess to compensate for transfer loss.
2. Add the required volume of Bio-Plex® assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the 20x stock of coupled beads at mid speed for **30 sec**. Carefully open the cap and pipet any liquid trapped in the cap back into the tube. This is important to ensure maximum bead recovery. **Do not centrifuge the vial**; doing so will cause the beads to pellet.
4. Dilute coupled beads to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.

Each well of the assay requires 2.5 µl of the 20x stock adjusted to a final volume of 50 µl in assay buffer.

5. Protect the beads from light with aluminum foil. Equilibrate to room temperature prior to use.

Tables 6–7 summarize the volumes required to prepare 1x beads from a 20x stock. Also shown are volumes for preparing 1x beads when mixing two 20x stocks of single-plex assays.

Preparing 1x coupled beads from 20x stock

Table 6. Premixed panel or one singleplex assay.

| # of Wells | 20x Beads, μ l | Assay Buffer, μ l | Total Volume, μ l |
|------------|--------------------|-----------------------|-----------------------|
| 96 | 288 | 5,472 | 5,760 |
| 48 | 144 | 2,736 | 2,880 |

Table 7. Mixing singleplex assays.

| # of Wells | 20x Beads, μ l Singleplex #1 | 20x Beads, μ l Singleplex #2 | Assay Buffer, μ l | Total Volume, μ l |
|------------|-------------------------------------|-------------------------------------|-----------------------|-----------------------|
| 96 | 288 | 288 | 5,184 | 5,760 |
| 48 | 144 | 144 | 2,592 | 2,880 |

7. Run Assay

The following instructions apply to premixed multiplex, singleplex, x-Plex™, and Express assay formats.

NOTE! Bring all buffers, diluents, diluted standards, diluted coupled beads, and samples to room temperature prior to use. To ensure optimal performance, pipet carefully (avoiding bubbles) with a calibrated pipet, and use new pipet tips.

Assay Key — The terms in Table 8 are repeated throughout the assay procedure. Refer to the detailed instructions in the table when **wash** and **cover and incubate** are mentioned.

Table 8. Detailed wash and incubation instructions.

| WASH | | | |
|--|--|-------------------------------------|---|
| After each assay step, select the appropriate Bio-Plex Pro™ wash station program, or perform the appropriate manual wash step as summarized below. | | | |
| Assay Step | Bio-Plex Pro or Pro II Wash Station | Bio-Plex Pro II Wash Station | Handheld Magnet or Vacuum Manifold |
| | Magnetic Program | Vacuum Program | Manual Wash Steps |
| Add beads to plate | MAG x2 | VAC x2 | 2 x 100 µl |
| Sample incubation | | | |
| Detection Ab incubation | MAG x3 | VAC x3 | 3 x 100 µl |
| SA-PE incubation | | | |

Using the Bio-Plex Pro Series Wash Stations

For detailed instructions, please refer to the quick guide (bulletin 5826) or user manual (item #10013125). Both documents can be found at www.bio-rad.com/bio-plex.

Using the Bio-Plex® Handheld Magnetic Washer

For detailed instructions, please refer to the user guide (item #10023087), which can be found at www.bio-rad.com/bio-plex.

Using a Vacuum Manifold

Place the filter assay plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Add 100 µl wash buffer to each well and remove the liquid as before. Ensure that all wells are exposed to the vacuum. As a general guideline, 100 µl should take approximately 3–4 sec to completely clear the well. Repeat as specified. Thoroughly blot the bottom of the filter plate with a clean paper towel between each vacuum step to prevent cross contamination and plate leakage. Place the assay plate on the plastic plate holder.

COVER AND INCUBATE

Gently cover the plate with a new sheet of sealing tape. To prevent cross-well contamination or leaking from the bottom of filter plates, do not press down on the tape. Place the plate on a microplate shaker and cover with aluminum foil to prevent photobleaching. **Vigorous shaking is important.** Shake at room temperature at **850 ±50 rpm** for the specified incubation time. Slowly ramp up to the maximum speed to avoid splashing of samples.

Add Coupled Beads, Samples, Standards, Blank, and Controls

- Cover unused wells of the assay plate with sealing tape.
- Prewet the filter plate. Skip this step if using a flat-bottom plate.
 - Vacuum manifold: Prewet the wells with **100 µl** assay buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel.

3. **Vortex** the diluted (1x) beads for **30 sec** at medium speed. Pour into a reagent reservoir and transfer **50 µl** to each well of the assay plate.

***TIP:** A multichannel pipet is highly recommended for ease of use and efficiency.*

4. **Wash the wells twice** with **100 µl** Bio-Plex wash buffer according to your wash method of choice.
5. **Vortex** the diluted samples, standards, blank, and controls for **5 sec**. Transfer **50 µl** of each to the appropriate well of the assay plate, changing the pipet tip after every volume transfer.
6. **Cover and incubate** in the dark for **1 hour** at room temperature (RT) with shaking at **850 ±50 rpm**.

Prepare and Add Detection Antibodies

1. While the samples are incubating use Tables 9–10 or the Calculation Worksheet on page 35 to calculate the volume of detection antibodies and Bio-Plex detection antibody diluent needed. These calculations include ~20% excess to compensate for transfer loss. Detection antibodies should be prepared **10 min before use**.
2. Add the required volume of Bio-Plex detection antibody diluent to a 15 ml polypropylene tube.
3. **Vortex** the 20x stock of detection antibodies for **15–20 sec** at medium speed, then perform a **30 sec** spin to collect the entire volume at the bottom of the tube.
4. Dilute detection antibodies to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.

Each well of the assay requires 1.25 µl of the 20x stock adjusted to a final volume of 25 µl in detection antibody diluent.

Tables 9–10 summarize the volumes required to prepare 1x detection antibodies from a single 20x stock. Also shown are volumes to prepare 1x antibodies when mixing two 20x stocks from singleplex assays.

Preparing 1x detection antibodies from 20x stock

Table 9. Premixed panel or one singleplex assay.

| # of Wells | 20x Detection Antibodies, μl | Detection Antibody Diluent, μl | Total Volume, μl |
|------------|---|---|-----------------------------|
| 96 | 145 | 2,755 | 2,900 |
| 48 | 73 | 1,377 | 1,450 |

Table 10. Mixing singleplex assays.

| # of Wells | 20x Detection Antibodies, μl Singleplex #1 | 20x Detection Antibodies, μl Singleplex #2 | Detection Antibody Diluent, μl | Total Volume, μl |
|------------|---|---|---|-----------------------------|
| 96 | 145 | 145 | 2,610 | 2,900 |
| 48 | 73 | 73 | 1,304 | 1,450 |

- After incubating the beads, samples, standards, blank, and controls, slowly remove and discard the sealing tape.
- Wash the wells three times with 100 μl** wash buffer according to the wash method of choice.
- Vortex** the diluted (1x) detection antibodies gently for **5 sec**. Pour into a reagent reservoir and transfer **25 μl** to each well of the assay plate using a multichannel pipet.
- Cover and incubate** in the dark for **30 min** at RT with shaking at **850 \pm 50 rpm**.

Prepare and Add Streptavidin-PE (SA-PE)

1. While detection antibodies are incubating, use Table 11 or the Calculation Worksheet on page 35 to calculate the volume of SA-PE and assay buffer needed. These calculations include ~25% excess to account for transfer loss. SA-PE should be prepared **10 min before use**.
2. Add the required volume of assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the 100x stock of SA-PE for **5 sec** at medium speed. Perform a **30 sec** spin to collect the entire volume at the bottom of the vial.
4. Dilute SA-PE to 1x by pipetting the required volume into the 15 ml tube. **Vortex** and protect from light until ready to use.

Each well of the assay requires 0.5 μl of the 100x stock adjusted to a final volume of 50 μl in assay buffer.

Table 11. Preparing 1x SA-PE from 100x stock.

| # of Wells | 100x SA-PE, μl | Assay Buffer, μl | Total Volume, μl |
|------------|---------------------------|-----------------------------|-----------------------------|
| 96 | 60 | 5,940 | 6,000 |
| 48 | 30 | 2,970 | 3,000 |

5. After detection antibody incubation, slowly remove and discard the sealing tape.
6. **Wash the wells three times** with **100 μl** of wash buffer according to the wash method of choice.
7. **Vortex** the diluted (1x) SA-PE at medium speed for **5 sec**. Pour into a reagent reservoir and transfer **50 μl** to each well using a multichannel pipet.
8. **Cover and incubate** in the dark for **10 min** at RT with shaking at **850 \pm 50 rpm**

9. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.
10. **Wash the wells three times** with **100 µl** of wash buffer according to the wash method of choice.
11. To resuspend beads for plate reading, add **125 µl** assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake at RT at **850 ±50 rpm** for **30 sec** and slowly remove the sealing tape. Ensure that the plate cover has been removed before placing the plate on the reader.

8. Read Plate

Bio-Plex Manager™ software is recommended for all Bio-Plex Pro™ assay data acquisition and analysis. Instructions for Luminex xPONENT software are also included. For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your regional Bio-Rad field applications specialist.

Prepare Protocol in Bio-Plex Manager Software v 6.0 and Higher

The protocol should be prepared in advance so that the plate is read as soon as the experiment is complete.

A protocol file specifies the analytes in the assay, the plate wells to be read, sample information, the values of standards and controls, and instrument settings.

Bio-Plex Manager software versions 6.0 and higher contain protocols for most Bio-Plex® assays. Choose from available protocols or create a new protocol. To create a new protocol, select File, then New from the main menu. Locate and follow the steps under Protocol Settings.

1. Describe Protocol and enter information about the assay (optional).
2. Select Analytes and create a new panel. Visually confirm the selected analytes and proceed to step 3.
 - a. Click the Add Panel button  in the Select Analytes toolbar. Enter a new panel name. Select Bio-Plex Pro Assay Magnetic from the assay pull-down menu. If using Bio-Plex Manager version 5.0 or lower, select MagPlex from the assay pull-down menu.
 - b. Click the Add button. Enter the bead region number and name for the first analyte. Click Add Continue to repeat for each analyte in the assay. Refer to the bead regions in parentheses () listed on the peel-off label provided with the standards.

For reference, bead regions for human Th17 cytokine assays are shown in Table 12.
 - c. Click the Add button when the last analyte has been added and click OK to save the new panel.
 - d. Highlight analytes from the Available list (left) and move to the Selected list (right) using the Add button. To move all analytes at once, simply click the Add All button.

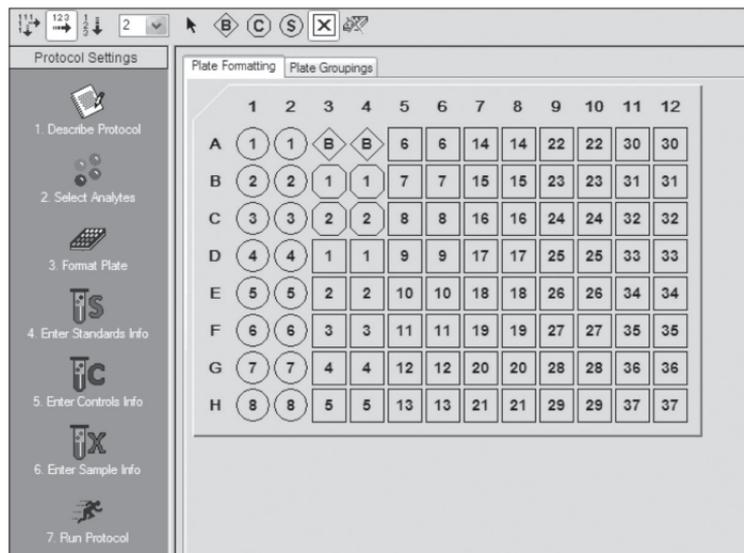
Table 12. Bead regions for human Th17 cytokine assays.

| Analyte | Bead Region | Analyte | Bead Region |
|--------------|-------------|---------------|-------------|
| IL-1 β | 39 | IL-23 | 43 |
| IL-4 | 52 | IL-25 | 55 |
| IL-6 | 19 | IL-31 | 62 |
| IL-10 | 56 | IL-33 | 46 |
| IL-17A | 76 | IFN- γ | 21 |
| IL-17F | 44 | sCD40L | 29 |
| IL-21 | 47 | TNF- α | 36 |
| IL-22 | 18 | IL-17A/F* | 66 |

* Available only as a singleplex and in Express assay format.

- e. If some of the analytes need to be removed from the Selected list, highlight them and select Remove. If desired, it is possible to rename the panel by clicking on Rename Panel and entering a new panel name.
3. Format the plate in Bio-Plex Manager™ according to the plate layout created in Section 1 (Plan Plate Layout). To modify the plate layout, follow the steps below (see Figure 4).
 - a. Select the Plate Formatting tab.
 - b. Select the standards icon (S) and drag the cursor over all the wells that contain standards. Repeat this process for Blanks (B), Controls (C), and Samples (X). Note that Bio-Plex Manager™ automatically subtracts the blank FI value from all other assay wells.

Fig. 4. Plate formatting



4. Enter Standards Info into Bio-Plex Manager.
 - a. Enter the highest concentration of each analyte in the top row (labeled S1) of the table. S1 concentration information is included with each vial of standards.
 - b. Enter a dilution factor of 4 and click Calculate. The concentrations for each standard point will be populated for all analytes in the table.
 - c. Optional: enter the lot number of the vial of standards into the Standard Lot box and click Save.
5. Enter Controls Info — for user specified controls, select an analyte from the pull-down menu, then enter a description and concentration. Repeat for each additional analyte in the assay.

For the quality controls supplied in premixed Th17 cytokine kits, format the appropriate wells as controls, enter descriptions, but leave the concentrations blank. Alternatively, the quality controls can be formatted as samples with clear descriptions such as “quality control high” and “quality control low”. In any case, **the expected control ranges provided on the product data sheet are not entered into Bio-Plex Manager software v6.1 and earlier.**

6. Enter Sample Info — enter sample information and the appropriate dilution factor.
7. Run Protocol — confirm that the assay settings are correct.
 - a. Human Th17 cytokine assays are run at the default setting in Bio-Plex Manager — **low RP1 target (low PMT)**.
 - b. Confirm that data acquisition is set to **50 beads per region**. In Advanced Settings, confirm that the bead map is set to **100 region**, the sample size is set to **50 µl**, and the DD gates are set to **5,000 (Low) and 25,000 (High)**. In Bio-Plex Manager software versions 4.0, 4.1, 4.1.1, and 5.0, check Override Gates and set the DD gate values as indicated.

Select Start, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click Eject/Retract to eject the plate carrier.

Acquire Data

1. Shake the assay plate at **850 ±50 rpm** for **30 sec**, and visually inspect the plate to ensure that the assay wells are filled with buffer. Slowly remove the sealing tape and any plate cover before placing the plate on the plate carrier.
2. Run Protocol – on the pop-up screen, select Load Plate and click OK to start acquiring data.
3. Use the Wash Between Plates  command after every plate run to reduce the possibility of clogging the instrument.
4. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF are not present). Select Wash Between Plates and follow the instructions. Then repeat the Prepare Protocol and Acquire Data instructions.
5. When data acquisition is complete, select Shut Down  and follow the instructions.

Reacquire Data

It is possible to acquire data from a well or plate a second time using the Rerun/Recovery mode located below Start in the Run Protocol step. Any previous data will be overwritten.

1. Check the wells from which data will be reacquired.
2. Aspirate the buffer with the wash method of choice, but do not perform wash step.
3. Add **100 µl** assay buffer to each well. Cover the plate with a new sheet of sealing tape.
4. Repeat the Acquire Data steps to reacquire data. The data acquired should be similar to those acquired initially; however, the acquisition time will be extended because the wells have fewer beads.

Data Analysis

Quality Controls

If the quality controls were run in the assay plate, open the results (.rbx) file, click on Report Table, and locate the control wells. Visually compare the observed concentrations of the high and low controls in the Report Table against the lot-specific control ranges shown in the product data sheet.

Expected control ranges are provided for reference and should be used as general guidelines. Actual results may vary for some operators. If the controls do not fall within the expected ranges, please refer to the troubleshooting section for possible causes and solutions.

Removing Outliers

Outliers are identified as standard data points that do not meet accuracy or precision requirements and should be considered invalid when performing curve fitting. As such, they should be removed to generate a more realistic and accurate standard curve. This may result in an extended assay working range and allow quantitation of samples that might otherwise be considered out of range (OOR).

In Bio-Plex Manager software version 6.0 and higher, outliers can be automatically removed by selecting the Optimize button in the Standard Curve window. In Bio-Plex Manager software 6.0 and earlier versions, outliers also can be manually selected in the Report Table. Visit online Help to learn more about the standard curve optimizer feature and how outliers are determined.

Previous Versions of Bio-Plex Manager Software

For instructions on using previous versions of Bio-Plex manager software, please contact Bio-Rad Technical Service.

Luminex xPONENT Software

Luminex xPONENT software may be used to analyze Bio-Plex assays. Although guidelines are provided here, consult the xPONENT software manual for more details. Perform a system initialization with Luminex's calibration and performance verification kit, as directed by Luminex. Select Batches to set up the protocol and follow the information under Settings.

1. Select MagPlex as the bead type for magnetic beads. This automatically sets the DD gates.
2. Volume = 50 μ l
3. Bio-Plex Pro Human Th17 cytokine assays are run at low PMT (Standard PMT).
4. Plate name: 96-well plate.
5. Analysis type: Quantitative, 5PL Curve Fit.
6. Number of standards: 8.

Select Analytes to set up the panel.

1. Enter "pg/ml" in the Units field.
2. Enter 50 in the Count field.
3. Select the bead region and enter the analyte name.
4. Click Apply all for Units and Count.

Select Stds and Ctrl.

1. Enter standard concentrations, lot number, dilution factor, and other information, as applicable.

After the assay is complete, select Results, then select Saved Batches.

Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ assays. If you experience any of the problems listed below, review the possible causes and solutions provided. Poor assay performance may also be due to the Bio-Plex® suspension array reader. To eliminate this possibility, use the validation kit to validate all the key functions of the array reader and to assist in determining whether or not the array reader is functioning properly.

Possible Causes

High Inter-Assay CV

Standards and controls were not reconstituted consistently between assays

Reconstituted standards, controls, and diluted samples were not stored properly

Bottom of filter plate not dry

Possible Solutions

Incubate the reconstituted standards for 30 min on ice. Always be consistent with the incubation time and temperature.

Reconstituted standards and diluted samples should be prepared on ice as instructed. Prior to plating, the reconstituted standards and diluted samples should be equilibrated to room temperature.

Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-well contamination.

Possible Causes

High Intra-Assay CV

Improper pipetting technique

Reagents and assay components not equilibrated to room temperature prior to pipetting

Contamination with wash buffer during wash steps

Slow pipetting of samples and reagents across the plate

Possible Solutions

Pipet carefully when adding standards, controls, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

All reagents and assay components should be equilibrated to room temperature prior to pipetting.

During the wash steps, be careful not to splash wash buffer from one well to another. Be sure that the wells are filtered completely and that no residual volume remains. Ensure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing

Sample pipetting across the entire plate should take less than 4 min. Reagent pipetting across the entire plate should take less than 1 min.

Possible Causes

Low Bead Count

Miscalculation of bead dilution

Beads clumped in multiplex bead stock tube

Vacuum on for too long when aspirating buffer from wells

Assay plate not shaken enough during incubation steps and prior to reading

Reader is clogged

Incorrect needle height of the reader

Low Signal or Poor Sensitivity

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE diluted incorrectly

Possible Solutions

Check your calculations and be careful to add the correct volumes

Vortex for 30 sec at medium speed before aliquoting beads.

Do not apply vacuum to the filter plate for longer than 10 sec after the buffer is completely drained from each well.

Shake the plate at 850 ± 50 rpm during incubation steps and for 30 sec immediately before reading the plate.

Refer to the troubleshooting guide in the Bio-Plex system hardware instruction manual (bulletin #10005042).

Adjust the needle height to coincide with the plate type provided in the kit.

Follow the cytokine standard instructions carefully.

Check your calculations and be careful to add the correct volumes.

Possible Causes

High Background Signal

Incorrect buffer was used (for example, assay buffer used to dilute standards)

Accidentally spiked blank wells

Detection antibodies or streptavidin-PE incubated too long

Poor Recovery

Expired Bio-Plex reagents were used

Incorrect amounts of components were added

Microplate shaker set to an incorrect speed

High end saturation of the standard curve

Quality controls do not fall within expected ranges.

Possible Solutions

Use sample matrix standard diluent to dilute standards.

Be careful when spiking standards. Do not add any antigens to the blank wells.

Follow the procedure incubation time precisely.

Check that reagents have not expired. Use new or nonexpired components.

Check your calculations and be careful to add the correct volumes.

Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker.

Make sure that correct shaker speed and incubation times are used. Remove S1 for data analysis if needed.

Make sure that the controls are reconstituted at the same time as standards, and in the same diluent (standard diluent HB). Incubate for precisely 30 min.

Possible Causes

Poor Recovery

Improper pipetting technique

Impact of Sample Matrix

Negative MFI values in samples or standards

Poor precision in serum and plasma sample measurements

Possible Solutions

Pipet carefully when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

If samples contain little or no analyte, negative values observed may be due to statistical variation. If assay drift is suspected, retest the samples by positioning them next to the standards. If contamination of standards is suspected, check the standard replicate value and be careful when adding samples to the wells. Matrix effects could also produce negative sample values.

Bio-Plex Manager™ software automatically subtracts the Blank (B) FI value from all other assay wells. While this has no impact on observed concentrations of samples within the assay working range, it may result in a negative FI value if the Blank's FI value is greater than either the standard or sample value. If this is undesirable, then reformat the blank wells as Sample (X) or Control (C), in the protocol or results file.

Check if any interfering components such as heparin-based anticoagulant, additives, or gel from separators were introduced into the samples. Avoid using hemolyzed and heavily lipemic samples. Remove visible particulate in samples by centrifugation. Avoid multiple freeze-thaw cycles of samples.

Plate Layout Template

| | | | | | | | | |
|-----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 12 | | | | | | | | |
| 11 | | | | | | | | |
| 10 | | | | | | | | |
| 9 | | | | | | | | |
| 8 | | | | | | | | |
| 7 | | | | | | | | |
| 6 | | | | | | | | |
| 5 | | | | | | | | |
| 4 | | | | | | | | |
| 3 | | | | | | | | |
| 2 | | | | | | | | |
| 1 | | | | | | | | |
| | A | B | C | D | E | F | G | H |

Calculation Worksheet

If using either a **premixed panel or one singleplex assay**, follow these directions.

Plan the plate layout and enter the number of wells to be used in the assay: _____
1

1. Determine the volume of 1x coupled beads needed.

- a. Each well requires 50 μl of coupled beads (1x): _____₁ x 50 μl = _____₂ μl
- b. Include a 20% excess to ensure enough volume: _____₂ μl x 0.20 = _____₃ μl
- c. Total volume of 1x coupled beads: _____₂ μl + _____₃ μl = _____₄ μl
- d. Volume of **20x coupled beads** stock: _____₄ μl / 20 = _____₅ μl
- e. Volume of **assay buffer** required: _____₄ μl - _____₅ μl = _____₆

2. Determine the volume of 1x detection antibody needed.

- a. Each well requires 25 μl detection antibodies (1x): _____₁ x 25 μl = _____₇ μl
- b. Include a 20% excess to ensure enough volume: _____₇ μl x 0.2 = _____₈ μl
- c. Total volume of 1x detection antibodies: _____₇ μl + _____₈ μl = _____₉ μl
- d. Volume of **20x detection antibodies** stock: _____₉ μl / 20 = _____₁₀ μl
- e. Volume of **detection antibody diluent** required: _____₉ μl - _____₁₀ μl = _____₁₁ μl

3. Determine the volume of 1x streptavidin-PE needed.

- a. Each well requires 50 μl streptavidin-PE (1x): _____₁ x 50 μl = _____₁₀ μl
- b. Include 25% excess to ensure enough volume: _____₁₀ μl x 0.25 = _____₁₁ μl
- c. Total volume of 100x streptavidin-PE: _____₁₀ μl + _____₁₁ μl = _____₁₂ μl
- d. Volume of **100x streptavidin-PE** required: _____₁₂ μl / 100 = _____₁₃ μl
- e. Volume of **assay buffer** required: _____₁₂ μl - _____₁₃ μl = _____₁₄ μl

If **mixing singleplex assays**, follow these directions.

Enter the number of wells to be used in the assay: _____
1

1. Determine the volume of 1x coupled beads needed.

- a. Each well requires 50 μl coupled beads (1x): _____ x 50 μl = _____ μl
1 2
- b. Include 20% excess to ensure enough volume: _____ μl x 0.20 = _____ μl
2 3
- c. Total volume of 1x coupled beads: _____ μl + _____ μl = _____ μl
2 3 4
- d. Enter the number of singleplex sets (or analytes) that will be multiplexed: _____
5
- e. Volume of **20x coupled beads** required from **each coupled beads tube**:
_____ μl / 20 = _____ μl
4 6
- f. Total volume of bead stock required: _____ x _____ μl = _____ μl
5 6 7
- g. Volume of **assay buffer** required: _____ μl - _____ μl = _____ μl
4 7 8

2. Determine the volume of 1x detection antibody needed.

- a. Each well requires 25 μl detection antibodies (1x): _____ x 25 μl = _____ μl
1 9
- b. Include a 20% excess to ensure enough volume: _____ μl x 0.2 = _____ μl
9 10
- c. Total volume of 1x detection antibodies: _____ μl + _____ μl = _____ μl
9 10 11
- d. Enter the number of singleplex sets (or analytes) that will be multiplexed: _____
5
- e. Volume of **20x detection antibodies** required from **each detection antibody tube**:
_____ μl / 20 = _____ μl
11 12
- f. Total volume of detection antibody stock: _____ μl x _____ = _____ μl
12 5 13
- g. Volume of **detection antibody diluent** required: _____ μl - _____ μl = _____ μl
11 13 14

3. Determine the volume of 1x streptavidin-PE needed.

- a. Each well requires 50 μl streptavidin-PE (1x): _____ x 50 μl = _____ μl
1 15
- b. Include 25% excess to ensure enough volume: _____ μl x 0.25 = _____ μl
15 16
- c. Total volume of 100x streptavidin-PE: _____ μl + _____ μl = _____ μl
15 16 17
- d. Volume of **100x streptavidin-PE** required: _____ μl / 100 = _____ μl
17 18
- e. Volume of **assay buffer** required: _____ μl _____ μl = _____ μl
17 18 19

Safety Considerations

Eye protection and gloves are recommended when using these products. Consult the MSDS for additional information. The Bio-Plex Pro™ assays contain components of animal origin. This material should be handled as if capable of transmitting infectious agents. Use universal precautions. These components should be handled at Biosafety Level 2 containment as defined by U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories* (Centers for Disease Control, 1999).

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Ordering Information

| Catalog # | Premixed Multiplex Assay Panel |
|--|---|
| 171-AA001M | Bio-Plex Pro Human Th17 Cytokine Panel 15-plex, 1 X 96-well |
| Human Th17 Cytokine Singleplex Sets, 1 x 96 well (requires reagent kit II and standards) | |
| 171-BA001M | IL-1 β |
| 171-BA002M | IL-4 |
| 171-BA003M | IL-6 |
| 171-BA004M | IL-10 |
| 171-BA005M | IL-17A |
| 171-BA006M | IL-17F |
| 171-BA007M | IL-21 |
| 171-BA008M | IL-22 |
| 171-BA009M | IL-23 |
| 171-BA010M | IL-25 |
| 171-BA011M | IL-31 |
| 171-BA012M | IL-33 |
| 171-BA013M | IFN- γ |
| 171-BA014M | sCD40L |
| 171-BA015M | TNF- α |
| 171-BA016M | IL-17A/F |
| Individual Components and Accessories | |
| 171-304055 | Bio-Plex Pro Reagent Kit II, 1 x 96-well Filter Plate |
| 171-304055M | Bio-Plex Pro Reagent Kit II, 1 x 96-well Flat-Bottom Plate |
| 171-DA0001 | Bio-Plex Pro Human Th17 Cytokine 16-plex Standards, pkg of 1 vial |
| 171-DA0501 | Bio-Plex Pro Human Th17 Cytokine 16-plex Standards, pkg of 50 vials |
| 171-304502 | Filter Plate, 1 x 96-well with clear plastic lid and tray |

Bio-Plex® x-Plex™ Assays (We Mix)

Premium, custom assay service using the Bio-Plex Assay Builder, www.bio-rad.com/bio-plex/assaybuilder, to select analytes and plate type of interest. Assays are supplied as premixed coupled beads and detection antibodies in the all-in-one kit format. Quality controls are included.

Bio-Plex Express Assays (You Mix)

Fast and economical custom assay service using the Bio-Plex Assay Builder, www.bio-rad.com/bio-plex/assaybuilder, to select analytes and plate type of interest. Assays are supplied as individual sets of coupled beads and detection antibodies in the all-in-one kit format. Quality controls not included.

Other Components and Accessories

Wash buffer, Bio-Plex Pro flat-bottom plates, and streptavidin-PE are also available individually. For more information, go to www.bio-rad.com/bio-plex.



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South Africa 27 861 246 723 **Spain** 34 91 590 5200
Sweden 08 555 12700 **Switzerland** 061 717 95 55
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