

30 Plex Human Luminex (Invitrogen Kit, Single Plate)

1. Defrost samples and bring to room temperature.
2. Bring Kit components to room temperature: Wash solution 20x.
Assay Diluent.
Incubation buffer.
3 Vial standard set:
(Human 25 plex, Death receptors, Growth factors)

Note: The number of vials of standards may change from Kit to Kit so check with Kit for preparation of the standards.

3. Mix samples by inversion (Do not vortex). Centrifuge at 14,000RPM for 10 minutes.

Dilutions of serum or plasma should be done with assay diluent. Tissue culture supernatants should be diluted using a 50:50 mixture of assay diluent and tissue culture medium.

4. Prepare Diluent for Supernatants and Standards:

10ml assay diluent + 10ml RPMI

5. Prepare Wash Solution:

For one plate use 15ml 20x concentrate + 285ml Deionised water in a glass beaker, mix well.

6. Prepare Standards: (Based on a 3 vial set)

Your 30 plex standard kit may contain 6 vials i.e. two sets of 3 vials (Human 25 plex, Death receptors, Growth factors). Reconstitute one of each of these vials with 330ul of your prepared diluent (Return the other set to 4°C to use on a second plate run at another time). Allow to stand for 10 minutes at room temperature. Gently swirl and invert the vial 2 or 3 times to ensure complete reconstitution, allow the vial to sit for a further 5 minutes. Combine 200ul from each vial mixing by pipetting up and down a few times.

This mixture is your top standard (7). From this serial 1:3 dilutions are prepared:

To 6 tubes add 300ul of diluent. Take 150ul standard (7) add it to standard tube (6) and mix.

..	(6)	(5)	..
..	(5)	(4)	..
..	(4)	(3)	..
..	(3)	(2)	..
..	(2)	(1)	..

i.e. 7 standards + blank

Blank = Diluent

After completing assay discard any remaining reconstituted and diluted standards. Assay diluent can be returned to 4°C.

Prepare Plate Plan (e.g.):

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S4	1	9	17	25	33	41	49	57	65	73
B	BL	S4	2	10	18	26	34	42	50	58	66	74
C	S1	S5	3	11	19	27	35	43	51	59	67	75
D	S1	S5	4	12	20	28	36	44	52	60	68	76
E	S2	S6	5	13	21	29	37	45	53	61	69	77
F	S2	S6	6	14	22	30	38	46	54	62	70	78
G	S3	S7	7	15	23	31	39	47	55	63	71	79
H	S3	S7	8	16	24	32	40	48	56	64	72	80

Assay Procedure:

1. Remove the lid from the plate and turn the top face down onto the bench. Always work with the plate standing across the lid so that none of the solution added to the wells wicks through the membrane. Pre-wet plate by adding 200ul wash solution to the wells, wait for 15 - 30 seconds then aspirate using the vacuum manifold. Blot the bottom of the plate on clean paper towels to remove residual liquid and return plate to sit across lid.

2. Prepare Beads:

Immediately prior to use in assay vortex beads for 30 seconds then sonicate for 30 seconds. As we are going to use these beads over two plates only use half the amount of beads from the vial i.e. 1.25 ml (Return the remainder to 4^oC for use on a second plate run at another time). Make the 1.25ml bead aliquot to 20 ml with wash solution and mix well.

3. Adding Beads to Wells:

Transfer the bead solution to a weigh boat. Add 200ul beads to each well of the 96 well plate and allow to soak for 15 – 30 seconds. Remove the wash solution by aspiration using the vacuum. Rinse out the container that the beads were prepared in with wash buffer and add to the weigh boat that contained the beads. Repeat the wash step. This should ensure that the maximum amounts of beads are added to the wells. Blot the bottom of the plate on paper towels to remove residual liquid and return plate to sit across lid.

4. Pipette 50ul of incubation buffer into each well.
5. Pipette 100ul standard or blank into the appropriate wells.
6. Pipette 50ul of your assay diluent to the remaining wells then 50ul of your samples. (Note this is a 1:2 dilution of your samples and should be accounted for when setting up the Protocol on the Bio-Plex).
7. Cover the plate with foil, making sure that the foil does not touch the plate's membrane, and incubate at room temperature for 2 hours with shaking on an orbital shaker. 10 to 15 minutes before the end of this incubation the Biotinylated Ab should be prepared.

8. Preparation of Biotinylated Ab:

As we are going to use this kit over two plates we only use half of the Biotinylated Ab (Return the remainder to 4°C for use on a second plate run at another time). To 480ul Biotinylated Ab add 10ml of biotin diluent, mix well.

9. After the 2 hour capture bead incubation remove the liquid by vacuum aspiration. Add 200ul wash solution to all the wells; allow to soak for 15 – 30 seconds then aspirate the fluid by vacuum. Repeat this washing step once more then blot the bottom of the plate on paper towels to remove residual liquid and return plate to sit across lid.
10. Add 95ul of the Biotinylated detection Ab to each well, cover the plate in foil as before and incubate at room temperature for 60 minutes with shaking. 10 to 15 minutes before the end of this incubation the Streptavidin-RPE should be prepared.

11. Preparation of Streptavidin-RPE:

To 10ml of Streptavidin-RPE Diluent add the 1ml vial of Streptavidin-RPE and mix well.

12. Repeat wash steps as step 9.
13. Add 95ul Streptavidin-RPE to each well, cover the plate with foil and incubate with shaking at room temperature for 30 minutes.
14. After incubation wash the beads by adding 200ul wash solution to each well, allow the beads to soak for 10 seconds then aspirate the fluid by vacuum. Repeat this wash step a further twice for a total of three washes. Blot the bottom of the plate on paper towels to remove residual liquid and return plate to sit across lid.
15. Add 100ul wash solution to each well, cover the plate with foil and shake at room temperature for 2 - 3 minutes to resuspend the beads. At this point the plate can either be uncovered, the lid removed and then put on the Bio-Plex to be read or it can be stored at 4°C wrapped in the foil for reading the next day.
16. If the plate was left overnight, bring the plate and the wash buffer to room temperature. Aspirate the liquid by vacuum then blot the bottom of the plate on paper towels to remove residual liquid and return plate to sit across lid. Add 100ul wash to each well, cover the plate with foil and shake at room temperature for 2 - 3 minutes to resuspend the beads then remove the foil and the lid and read on the Bio-Plex.

Setting up the Bio-Rad Bio-Plex to Read the Luminex Plate

1. Check that there is enough sheath fluid in the container (Box on the floor) and that the waste tube is draining to a sink.
2. Switch on at the wall the High-Throughput Fluidics (HTF) Pump, Microplate Platform, Array Reader, Computer and Monitor. **Note:** To ensure accurate and reproducible results the lasers in the Bio-Plex must be allowed to heat up for 30 minutes prior to calibration and reading assays. The optics begin to warm up when you first switch on the array reader.

3. Start up

Open up the Bio-Plex Manager 4.1 programme.

Click on the start up button and follow the on screen graphic instructing you to fill the appropriate wells of the Maintenance, Calibration and Validation (MCV) plate with distilled water and 70% isopropanol. Click the eject button and load the plate into the platform and then click OK to begin the start up. You do not have to wait until the optics have warmed up before performing the start-up procedure.

4. Calibration

Calibration of the Bio-Plex is essential for optimal performance and is done after the optics warm up has been completed and should be run after the start up every time the array reader is used.

Click on the calibrate button and a dialog box will open. Check that the radial button for CAL 1 & CAL 2 has been selected. Check the Control Numbers for CAL 1 & CAL 2 are the same as the numbers on the calibration microsphere bottles in the calibration kit you are using and the displayed target values are the same. If you have a new kit add the new control numbers to the Calibrate dialog by clicking on the add button and filling in the required fields in the dialog box that opens, do this for both CAL 1 & CAL 2. **Note:** CAL 2 has two RPI Targets a high one that is used for Bio-Plex phosphoprotein assays. Bio-Plex cytokine assays can use the high or lower setting but some Luminex assays require the lower one so it is better to set the CAL 2 RPI Target to the lower setting when using cytokine assays.

Calibration Set Up

When you have checked and entered your calibration parameters click OK in the Calibrate dialog and another dialog box will open. Follow the instructions in this box on how to load up the Bio-Plex MCV Plate and run the set up. Note. You can use 4 drops of beads instead of the 5. Click on the Eject/Retract Plate button to eject the microplate platform plate carrier, place the MCV Plate in the carrier with the arrow facing toward the platform, then retract the plate. Click on OK to start the calibration process. As calibration proceeds, the status bar at the bottom of the Bio-Plex Manager window will monitor the progress of calibration. The number of beads per second should be 100 or higher; fewer beads per second may indicate a problem with the fluidics system. An alert box will notify you whether calibration has succeeded or failed. Because calibration beads are highly concentrated, calibration is followed by three wash cycles using distilled water.

You are now ready to set up your protocol and run your plate.

Protocol Set Up

An existing saved protocol can be opened and used or modified by clicking on the open button on the main toolbar or file menu, then use the save as button to save it as a new protocol.

Note: If using a saved protocol you should check that the standard concentrations in the new kit are the same strength.

To create a new protocol from scratch click on the new button on the main toolbar or file menu. A new protocol window will open. The new protocol window has 7 different settings options, some of them are optional and some need to be completed for the protocol to run:

1. Describe Protocol
2. Select Analytes
3. Format Plate
4. Enter Standards Info
5. Enter Controls Info
6. Enter Samples Info
7. Run Protocol

1. Click on Describe Protocol button

Protocol description is optional

2. Click on Select Analytes button

Select Add Panel Button. Add Panel field will now open. Start by adding the name of your new panel then click on the Add button. An Add Analyte dialog box will open; enter the bead region and the analyte name. Click on the Add Continue button until you have entered your last bead region and analyte then click on the Add Button and close the Add Analyte dialog box. It is worth checking at this point that the bead region and the analyte in the available column match. The region number refers to a specific bead set that represents the analyte in a fluorescent colour map that the Bio-Plex reader will detect. Highlight all the analytes in the available list and click on the Add All button to select them and they will move to the selected area.

3. Click on Format Plate button

Clicking on the **Format Plate** button will open up a window displaying the plate template. Use the plate formatting tools to enter your blanks, standards, controls (If you have any) and your unknown samples (e.g.).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S4	1	9	17	25	33	41	49	57	65	73
B	BL	S4	2	10	18	26	34	42	50	58	66	74
C	S1	S5	3	11	19	27	35	43	51	59	67	75
D	S1	S5	4	12	20	28	36	44	52	60	68	76
E	S2	S6	5	13	21	29	37	45	53	61	69	77
F	S2	S6	6	14	22	30	38	46	54	62	70	78
G	S3	S7	7	15	23	31	39	47	55	63	71	79
H	S3	S7	8	16	24	32	40	48	56	64	72	80

S1 can be your highest or lowest concentration. It is important to remember what you have chosen as the position of your highest standard as it is used when calculating the other standard concentrations.

4. Click on Enter Standards Info button

Clicking on the enter standards info button will open up a window that will allow you to set up the standard curves that correspond to your plate plan and use the programme to calculate their concentrations automatically. If your analytes are differing concentration ranges make sure that the 'Same conc. values for all analytes' is not selected. Enter your concentration

units (pg/ml) and tick 'Same units for all analytes'. For the amount of non zero standards in the Invitrogen kits the 'Regression type' selected should be Logistic – 5PL. The 'Axis Transformation' should be Log(x) – Linear(y) and the 'Acceptable Recovery range' 70 – 130% with the 'Same recovery range for all analytes' ticked. To set up your first standard curve check the analyte showing in the window then select 'Enter Automatically' and then select the position of your highest standard concentration i.e. either position S1 or S7. In the Calculate Concentration box enter the value of your most concentrated standard and its dilution factor (This is 3 for the Invitrogen kits). Click on the calculate button and the standards concentrations will be calculated and automatically entered. Repeat this step for all your analytes.

5. Click on Enter Controls Info button

Controls info is optional. (There are no controls in the Invitrogen kit)

6. Click on Enter Samples Info button

Clicking on the enter samples info button will open up a window that will allow you to name your samples and enter any dilution factor of the sample you wish to add. Samples description is optional. Set your dilution factor, there is a 1:2 dilution of sample in the Invitrogen kit therefore the Dilution Factor should be entered as 2 and the 'Set All Dilution Factors' button clicked. Check that the factor 2 has been entered for all your samples. (Save your new protocol at this juncture before proceeding)

7. Click on Run Protocol button

You are now ready to run the protocol. Clicking on the run protocol button will open the run protocol window. Click on the 'Show/Hide Histogram/Bead Map' button to get your desired view, you will need to maximise the window to see the bead map and the raw data table in the same window. The bead count should be 100 per region. Clicking on the 'Advanced Settings' button will give you access to the sample size. The recommended volume is 50ul. The 'Auto save after run' button should be selected. The results will be automatically saved and you will be prompted to give the file a name when you click on the start button.

If your plate is prepared you are now ready to start to read the plate. Click on the start button and the Run Protocol dialog box will open. Enter your user name and plate ID if your plate has one. If you selected auto save results after run, you will be prompted to enter a file name for your saved results. Click on the Eject/Retract Plate button and insert your plate into the platform, remember to remove any lid or tape covering the plate. Click on the OK button to begin reading the plate.

When the plate is being read, if you are viewing the Histogram and the bead map, you will see the double discriminator histogram, the bead map with the bead regions and below this is the Raw Data table. (Refer to Bio-Plex Manager software 4.1 user guide for a more detailed explanation) The Raw Data table will be displaying Fluorescent intensity and if you click on Show/Hide Bead Statistics Columns button on the table toolbar the bead count will be displayed also. Other columns will show:

Region- The number of beads that fell within defined regions.

Gate- The number of beads that passed the Double Discriminator gate.

% Aggregated Beads- The percentage of aggregated beads in the well.

Error Codes- Sampling Error codes.

After the plate has read a Results file is generated and automatically opens. Remember if you selected Auto save after run, this file will have been saved under the file name you supplied.

Analysing Results

The Bio-Plex Manager software allows you to change a lot of parameters from the protocol file including plate format, standards info and sample dilution then recalculate a new results file, remember to 'save as' a new file. In the results window you will be able to access three sub-windows; **Raw Data**, **Standard Curve** and **Report Table**.

Raw Data

This Raw Data table is the same as the Raw Data table seen in the Protocol window the only difference is that in the Results Window you can export and print the file if you want.

Standard Curve

Clicking on the Standard Curve button will open a window that will display the curve for each analyte when selected. Here you can check your standard curve and or change the regression type. Note if you change this, the results in your Report Table will be automatically recalculated. If your regression type is Logistic – 5PL (This gives the best result for most assays) you need 6 non-zero standards to use this regression. In the Invitrogen kit you will have 7 non-zero standards this means that if on looking at the standard curve and one of your sets of standards is not correct you can remove it and still use this regression type.

Report Table

Clicking on the Report Table will open the Report Table window. The table opened will be for the analyte showing in the Analyte box above the table, other analytes can be selected from the pull-down list.

Results from Replicates and Individual Wells:

If you formatted your plate with replicate samples these results will be shown as an average of the replicates. If you want to see the data from each well click on the Show Replicates button, you should now see the average of the replicates plus the results from each well.

Removing Outliers:

In some cases you might now want to remove an Outlier result, to do this click on the Show/Hide Outliers Column button. Each row will now have a checkbox beside each replicate average and single well result. To exclude any of these results select the appropriate checkbox, these results will be excluded and the table automatically recalculated.

Exporting the Report Table

To export the Report table to an excel spreadsheet click on the Export Report Table button in the toolbar. In the table options window select Table per column; this will display one column of data for all the analytes in an individual spreadsheet. In the Advanced Options, if you selected table per column, you can select the data to export and the Export Destination to an Excel file. Clicking on the OK button will create your excel worksheet and you will then be prompted with a Save As dialog window to name and save your file.

30 Plex Human Luminex (Invitrogen Kit) Split Over Two Plates in One Run with One Set of Standards

This is essentially the same as running a single plate but you need an Extracellular Protein Buffer Reagent Kit to use with the 30 Plex Kit. The extracellular base kit contains a second plate plus Streptavidin-RPE and various buffers; it does NOT contain beads, standards or Biotinylated Ab therefore you have to use the beads and the Biotinylated Ab diluted 1:2 to have sufficient volume for 2 plates. The standards are only used on plate one and will not need to be diluted. The first plate will contain blanks, standards and unknown samples; the second plate will run with blanks and unknown samples only. The results from the standards from the first plate will be imported into the second plate and used to calculate the unknown results.

1. Defrost samples and bring to room temperature.
2. Bring both Kits components to room temperature: Wash solution 20x.
Assay Diluent.
Incubation buffer.
3 Vial standard set:
(Human 25 plex, Death receptors, Growth factors)

Note: The number of vials of standards may change from Kit to Kit so check with Kit for preparation of the standards.

3. Mix samples by inversion (Do not vortex). Centrifuge at 14,000RPM for 10 minutes.

Dilutions of serum or plasma should be done with assay diluent. Tissue culture supernatants should be diluted using a 50:50 mixture of assay diluent and tissue culture medium.

4. Prepare Diluent for Supernatants and Standards:

15ml assay diluent + 15ml RPMI

5. Prepare Wash Solution:

To 20ml 20x concentrate wash solution add 385ml deionised water in a glass beaker, mix well.

6. Prepare Standards: (Based on a 3 vial set)

Your 30 plex standard kit may contain 6 vials i.e. two sets of 3 vials (Human 25 plex, Death receptors, Growth factors). Reconstitute one of each of these vials with 330ul of your prepared diluent (Return the other set to 4°C). Allow to stand for 10 minutes at room temperature. Gently swirl and invert the vial 2 or 3 times to ensure complete reconstitution, allow the vial to sit for a further 5 minutes. Combine 200ul from each vial mixing by pipetting up and down a few times.

This mixture is your top standard (7). From this serial 1:3 dilutions are prepared:
To 6 tubes add 300ul of diluent. Take 150ul standard (7) add it to standard tube (6) and mix.

..	(6)	(5)	..
..	(5)	(4)	..
..	(4)	(3)	..
..	(3)	(2)	..
..	(2)	(1)	..

i.e. 7 standards + blank

Blank = Diluent

After completing assay discard any remaining reconstituted and diluted standards. Assay diluent can be returned to 4°C.

Prepare Plate Plans (e.g.):

Plate 1 (With Blanks, Standards and Unknowns)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S4	1	9	17	25	33	41	49	57	65	73
B	BL	S4	2	10	18	26	34	42	50	58	66	74
C	S1	S5	3	11	19	27	35	43	51	59	67	75
D	S1	S5	4	12	20	28	36	44	52	60	68	76
E	S2	S6	5	13	21	29	37	45	53	61	69	77
F	S2	S6	6	14	22	30	38	46	54	62	70	78
G	S3	S7	7	15	23	31	39	47	55	63	71	79
H	S3	S7	8	16	24	32	40	48	56	64	72	80

Plate 2 (With Blanks and Unknowns only)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	7	15	23	31	39	47	55	63	71	79	87
B	BL	8	16	24	32	40	48	56	64	72	80	88
C	1	9	17	25	33	41	49	57	65	73	81	89
D	2	10	18	26	34	42	50	58	66	74	82	90
E	3	11	19	27	35	43	51	59	67	75	83	91
F	4	12	20	28	36	44	52	60	68	76	84	92
G	5	13	21	29	37	45	53	61	69	77	85	93
H	6	14	22	30	38	46	54	62	70	78	86	94

Assay Procedure:

1. Remove the lids from the plates and turn the tops face down onto the bench. Always work with the plates standing across the lids so that none of the solution added to the wells wicks through the membrane. Pre-wet plates by adding 200ul wash solution to the wells in both plates, wait for 15 - 30 seconds then aspirate using the vacuum manifold. Blot the bottom of the plates on clean paper towels to remove residual liquid and return plates to sit across lids.

2. Prepare Beads:

Immediately prior to use in assay vortex beads for 30 seconds then sonicate for 30 seconds. Add the beads(2.5ml) from the vial to 20ml wash solution and mix well.

3. Adding Beads to Wells:

Transfer the bead solution to a weigh boat. Add 95ul beads to each well of the two 96 well plates and allow to soak for 15 – 30 seconds. Remove the wash solution by aspiration using the vacuum. Rinse out the container that the beads were prepared in with wash buffer and add to the weigh boat that contained the beads. Repeat the wash step. This should ensure that the maximum amounts of beads are added to the wells. Blot the bottom of the plates on paper towels to remove residual liquid and return plates to sit across lids.

4. Pipette 50ul of incubation buffer into each well of the two plates.

5. Pipette 100ul standards or blanks into the appropriate wells on the first plate and 100ul blanks on the second plate.

6. Pipette 50ul of your assay diluent to the remaining wells then 50ul of your samples. (Note this is a 1:2 dilution of your samples and should be accounted for when setting up the Protocol on the Bio-Plex).

7. Cover the plates with foil, making sure that the foil does not touch the plate's membrane, and incubate at room temperature for 2 hours with shaking on an orbital shaker. 10 to 15 minutes before the end of this incubation the Biotinylated Ab should be prepared.

8. Preparation of Biotinylated Ab:

To 20ml of Biotin Diluent add the 1ml vial of Biotinylated Ab, mix well.

9. After the 2 hour capture bead incubation remove the liquid by vacuum aspiration. Add 200ul wash solution to all the wells in both plates; allow to soak for 15 – 30 seconds then aspirate the fluid by vacuum. Repeat this washing step once more then blot the bottom of the plates on paper towels to remove residual liquid and return plates to sit across lids.

10. Add 95ul of the Biotinylated detection Ab to each well of both plates, cover the plate in foil as before and incubate at room temperature for 60 minutes with shaking. 10 to 15 minutes before the end of this incubation the Streptavidin-RPE should be prepared.

11. Preparation of Streptavidin-RPE:

To 20ml of Streptavidin-RPE Diluent add the 2x 1ml vials of Streptavidin-RPE and mix well.

12. Repeat wash steps as step 9.
13. Add 95ul Streptavidin-RPE to each well in both plates, cover the plates with foil and incubate with shaking at room temperature for 30 minutes.
14. After incubation wash the beads by adding 200ul wash solution to each well in both plates, allow the beads to soak for 10 seconds then aspirate the fluid by vacuum. Repeat this wash step a further twice for a total of three washes. Blot the bottom of the plates on paper towels to remove residual liquid and return plates to sit across lids.
15. Add 100ul wash solution to each well in both plates, cover the plates with foil and shake at room temperature for 2 - 3 minutes to resuspend the beads. At this point the first plate can either be uncovered; the lid removed and then put on the Bio-Plex to be read. Store the second plate at 4^oC wrapped in the foil for reading after the first plate, this plate should be taken out of the cold and brought to room temperature with shaking before the first plate has finished reading, or both plates can be stored at 4^oC wrapped in the foil for reading the next day.
16. If the plates were left overnight, take out the first plate (Containing your standards) and the wash buffer, bring to room temperature. Leave the second plate at 4^oC. Aspirate the liquid by vacuum then blot the bottom of the plate on paper towels to remove residual liquid and return plate to sit across lid. Add 100ul wash solution to each well, cover the plate with foil and shake at room temperature for 2 – 3 minutes to resuspend the beads then remove the foil and the lid from the plate and read on the Bio-Plex. Before the first plate has finished reading take the second plate from 4^oC and repeat the wash and re-suspension of the beads and read this plate on the Bio-Plex.

Note: You can, if you want, perform a wash between plates by clicking on the Wash Between Plates icon and follow the instructions in the new window that opens.

Setting up the Bio-Rad Bio-Plex to Read the Luminex Plate

1. Check that there is enough sheath fluid in the container (Box on the floor) and that the waste tube is draining to a sink.
2. Switch on at the wall the High-Throughput Fluidics (HTF) Pump, Microplate Platform, Array Reader, Computer and Monitor. **Note:** To ensure accurate and reproducible results the lasers in the Bio-Plex must be allowed to heat up for 30 minutes prior to calibration and reading assays. The optics begin to warm up when you first switch on the array reader.
3. **Start up**

Open up the Bio-Plex Manager 4.1 programme.

Click on the start up button and follow the on screen graphic instructing you to fill the appropriate wells of the Maintenance, Calibration and Validation (MCV) plate with distilled water and 70% isopropanol. Click the eject button and load the plate into the platform and then click OK to begin the start up. You do not have to wait until the optics have warmed up before performing the start-up procedure.

4. Calibration

Calibration of the Bio-Plex is essential for optimal performance and is done after the optics warm up has been completed and should be run after the start up every time the array reader is used.

Click on the calibrate button and a dialog box will open. Check that the radial button for CAL 1 & CAL 2 has been selected. Check the Control Numbers for CAL 1 & CAL 2 are the same as the numbers on the calibration microsphere bottles in the calibration kit you are using and the displayed target values are the same. If you have a new kit add the new control numbers to the Calibrate dialog by clicking on the add button and filling in the required fields in the dialog box that opens, do this for both CAL 1 & CAL 2. **Note:** CAL 2 has two RPI Targets a high one that is used for Bio-Plex phosphoprotein assays. Bio-Plex cytokine assays can use the high or lower setting but some Luminex assays require the lower one so it is better to set the CAL 2 RPI Target to the lower setting when using cytokine assays.

Calibration Set Up

When you have checked and entered your calibration parameters click OK in the Calibrate dialog and another dialog box will open. Follow the instructions in this box on how to load up the Bio-Plex MCV Plate and run the set up. Note. You can use 4 drops of beads instead of the 5 Click on the Eject/Retract Plate button to eject the microplate platform plate carrier, place the MCV Plate in the carrier with the arrow facing toward the platform, then retract the plate. Click on OK to start the calibration process. As calibration proceeds, the status bar at the bottom of the Bio-Plex Manager window will monitor the progress of calibration. The number of beads per second should be 100 or higher; fewer beads per second may indicate a problem with the fluidics system. An alert box will notify you whether calibration has succeeded or failed. Because calibration beads are highly concentrated, calibration is followed by three wash cycles using distilled water.

You are now ready to set up your protocol and to run your plate.

Protocol Set Up

An existing saved protocol can be opened and used or modified by clicking on the open button on the main toolbar or file menu, then use the save as button to save it as a new protocol.

Note: If using a saved protocol you should check that the standard concentrations in the new kit are the same strength.

To create a new protocol from scratch click on the new button on the main toolbar or file menu. A new protocol window will open. The new protocol window has 7 different settings options, some of them are optional and some need to be completed for the protocol to run:

1. Describe Protocol
2. Select Analytes
3. Format Plate
4. Enter Standards Info
5. Enter Controls Info
6. Enter Samples Info
7. Run Protocol

1. Click on Describe Protocol button

Protocol description is optional

2. Click on Select Analytes button

Select Add Panel Button. Add Panel field will now open. Start by adding the name of your new panel then click on the Add button. An Add Analyte dialog box will open; enter the bead region and the analyte name. Click on the Add Continue button until you have entered your last bead region and analyte then click on the Add Button and close the Add Analyte dialog box. It is worth checking at this point that the bead region and the analyte in the available column match. The region number refers to a specific bead set that represents the analyte in a fluorescent colour map that the Bio-Plex reader will detect. Highlight all the analytes in the available list and click on the Add All button to select them and they will move to the selected area.

3. Click on Format Plate button

Clicking on the **Format Plate** button will open up a window displaying the plate template. Use the plate formatting tools to enter your blanks, standards, controls (If you have any) and your unknown samples (e.g.).

Format for Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	S4	1	9	17	25	33	41	49	57	65	73
B	BL	S4	2	10	18	26	34	42	50	58	66	74
C	S1	S5	3	11	19	27	35	43	51	59	67	75
D	S1	S5	4	12	20	28	36	44	52	60	68	76
E	S2	S6	5	13	21	29	37	45	53	61	69	77
F	S2	S6	6	14	22	30	38	46	54	62	70	78
G	S3	S7	7	15	23	31	39	47	55	63	71	79
H	S3	S7	8	16	24	32	40	48	56	64	72	80

Use this Format when setting up the protocol for Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	7	15	23	31	39	47	55	63	71	79	87
B	BL	8	16	24	32	40	48	56	64	72	80	88
C	1	9	17	25	33	41	49	57	65	73	81	89
D	2	10	18	26	34	42	50	58	66	74	82	90
E	3	11	19	27	35	43	51	59	67	75	83	91
F	4	12	20	28	36	44	52	60	68	76	84	92
G	5	13	21	29	37	45	53	61	69	77	85	93
H	6	14	22	30	38	46	54	62	70	78	86	94

S1 can be your highest or lowest concentration. It is important to remember what you have chosen as the position of your highest standard as it is used when calculating the other standard concentrations.

4. Click on Enter Standards Info button

Clicking on the enter standards info button will open up a window that will allow you to set up the standard curves that correspond to your plate plan and use the programme to calculate their concentrations automatically. If your analytes are differing concentration ranges make sure that the 'Same conc. values for all analytes' is not selected. Enter your concentration units (pg/ml) and tick 'Same units for all analytes'. For the amount of non zero standards in the Invitrogen kits the 'Regression type' selected should be Logistic – 5PL. The 'Axis Transformation' should be Log(x) – Linear(y) and the 'Acceptable Recovery range' 70 – 130% with the 'Same recovery range for all analytes' ticked. To set up your first standard curve check the analyte showing in the window then select 'Enter Automatically' and then select the position of your highest standard concentration i.e. either position S1 or S7. In the Calculate Concentration box enter the value of your most concentrated standard and its dilution factor (This is 3 for the Invitrogen kits). Click on the calculate button and the standards concentrations will be calculated and automatically entered. Repeat this step for all your analytes.

Remember your second plate does not contain any standards.

5. Click on Enter Controls Info button

Controls info is optional. (There are no controls in the Invitrogen kit)

6. Click on Enter Samples Info button

Clicking on the enter samples info button will open up a window that will allow you to name your samples and enter any dilution factor of the sample you wish to add. Samples description is optional. Set your dilution factor, there is a 1:2 dilution of sample in the Invitrogen kit therefore the Dilution Factor should be entered as 2 and the 'Set All Dilution Factors' button clicked. Check that the factor 2 has been entered for all your samples. (Save your new protocol at this juncture before proceeding)

7. Click on Run Protocol button

You are now ready to run the protocol. Clicking on the run protocol button will open the run protocol window. Click on the 'Show/Hide Histogram/Bead Map' button to get your desired view, you will need to maximise the window to see the bead map and the raw data table in the same window. The bead count should be 100 per region. Clicking on the 'Advanced Settings' button will give you access to the sample size. The recommended volume is 50ul. The 'Auto save after run' button should be selected. The results will be automatically saved and you will be prompted to give the file a name when you click on the start button.

If your plates are prepared you are now ready to start to read the first plate. Click on the start button and the Run Protocol dialog box will open. Enter your user name and plate ID if your plate has one. If you selected auto save results after run, you will be prompted to enter a file name for your saved results. Click on the Eject/Retract Plate button and insert your plate into the platform, remember to remove any lid or tape covering the plate. Click on the OK button to begin reading the plate. Put your second plate wrapped in foil at 4°C.

When the plate is being read, if you are viewing the Histogram and the bead map, you will see the double discriminator histogram, the bead map with the bead regions and below this

is the Raw Data table. (Refer to Bio-Plex Manager software 4.0 user guide for a more detailed explanation) The Raw Data table will be displaying Fluorescent intensity and if you click on Show/Hide Bead Statistics Columns button on the table toolbar the bead count will be displayed also. Other columns will show:

Region- The number of beads that fell within defined regions.

Gate- The number of beads that passed the Double Discriminator gate.

% Aggregated Beads- The percentage of aggregated beads in the well.

Error Codes- Sampling Error codes.

After the plate has read a Results file is generated and automatically opens. Remember if you selected Auto save after run, this file will have been saved under the file name you supplied.

Analysing Results

The Bio-Plex Manager software allows you to change a lot of parameters from the protocol file including plate format, standards info and sample dilution then recalculate a new results file, remember to 'save as' a new file. In the results window you will be able to access three sub-windows; **Raw Data**, **Standard Curve** and **Report Table**.

Raw Data

This Raw Data table is the same as the Raw Data table seen in the Protocol window the only difference is that in the Results Window you can export and print the file if you want.

Standard Curve

Clicking on the Standard Curve button will open a window that will display the curve for each analyte when selected. Here you can check your standard curve and or change the regression type. Note if you change this, the results in your Report Table will be automatically recalculated. If your regression type is Logistic – 5PL (This gives the best result for most assays) you need 6 non-zero standards to use this regression. In the Invitrogen kit you will have 7 non-zero standards this means that if on looking at the standard curve and one of your sets of standards is not correct you can remove it and still use this regression type.

Report Table

Clicking on the Report Table will open the Report Table window. The table opened will be for the analyte showing in the Analyte box above the table, other analytes can be selected from the pull-down list.

Results from Replicates and Individual Wells:

If you formatted your plate with replicate samples these results will be shown as an average of the replicates. If you want to see the data from each well click on the Show Replicates button, you should now see the average of the replicates plus the results from each well.

Removing Outliers:

In some cases you might now want to remove an Outlier result, to do this click on the Show/Hide Outliers Column button. Each row will now have a checkbox beside each replicate average and single well result. To exclude any of these results select the appropriate checkbox, these results will be excluded and the table automatically recalculated.

Exporting the Report Table

To export the Report table to an excel spreadsheet click on the Export Report Table button in the toolbar. In the table options window select Table per column; this will display one column of data for all the analytes in an individual spreadsheet. In the Advanced Options, if you selected table per column, you can select the data to export and the Export Destination to an Excel file. Clicking on the OK button will create your excel worksheet and you will then be prompted with a Save As dialog window to name and save your file.

Calculating Results on the Second Plate.

After your second plate has been prepared and run the report table generated will not contain any standards and to calculate the unknown results you will need to import the standards from your first plate.

To Import Standards to Second Plate

When the report table without standards is open select the Enter Standards Info button. Click on Select External Standards tab and in the window that opens select Fill Available List. Select the results file that contains your standards and select open, this will load the standards from this plate, click add all. Click on the Report Table button and the results will be calculated using these standards and the standards added to the report table with a lower case e before the standard to show they came from an external source. Save the new calculated results report table and then export your results to an excel file.