APPLICATION GUIDE

AM100097 **96 Reactions**

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Ordering Information

Catalog Number	Description	Quantity
AM100097	TruePLEX™ Extracellular Core Reagent Kit	96 Reactions

Components and Component Sets

Catalog Number	Description	Quantity
AM100109	TruePLEX™ Detection Reagent	2 x 96 Rxns
AM100110	TruePLEX™ Wash Buffer Set: Wash Buffers I and II	2 x 10 ml 2 x 25 ml
AM100111	TruePLEX™ Assay Buffer Set Assay Diluent, Assay Buffer A, Assay Buffer B	2 x 10 ml 2 x 10 ml 2 x 50 ml
AM100112	Filter Plates with Sealers	Pk/5 plates, 15 Sealers

Important Information

- **Store the reagents at the temperature and condition specified on the labels.**
- **Read the entire protocol before use**
- **Research Use Only.** The product you have received is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic or therapeutic application. Uses other than the labeled intended use may be a violation of applicable law.
- **Hazards.** It is the end-user's responsibility to consult the applicable MSDS(s) before using this product. Disposal of waste materials must comply with all appropriate federal, state and local regulations. If you have any questions concerning the hazards associated with this product, please call OriGene Technologies Inc at 1-888-267-4436.
- **Terms and Conditions:** By opening the packaging containing this Assay Product (which contains fluorescently labeled microsphere beads authorized by Luminex Corporation) or using this Assay Product in any manner, you are consenting and agreeing to be bound by the following terms and conditions. You are also agreeing that the following terms and conditions constitute a legally valid and binding contract that is enforceable against you. If you do not agree to all of the terms and conditions set forth below, you must return the product unopened within

ten (10) day of receipt under the same shipping conditions as received to receive a full refund. You, the customer, acquire the right under Luminex Corporation's patent rights, if any, to use this Assay Product or any portion of this Assay Product, including without limitation the microsphere beads contained herein, only with Luminex Corporation's laser based fluorescent analytical test instrumentation marketed under the name Luminex® 100/200™.

- **Safety and Use:** All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of potentially infectious or hazardous agents. This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic application. Uses other than the labeled intended use may be a violation of applicable law.

Components supplied with this kit

Component	Vol for 96 Rxns
Assay Diluent	10 mL
Assay Buffer A	10 mL
Assay Buffer B	50 mL
Wash Buffer I (5X)	10 mL
Wash Buffer II (20X)	25 mL
Detection Reagent (Streptavidin-phycoerythrin) (10X)	960 µL
Filter Plate	1
Plate Sealers	3

Storage Instructions

- The kit is shipped on blue ice.
- The following components may be stored at room temperature.
  - Filter Plate
  - Plate Sealers
- All other components should be stored at 2-8ºC.

Materials required but not provided

- Single-plex assay reagents compatible with this kit. Single-plex assay reagents include bead mixes, biotinylated detection antibody and protein standards.
- Calibrated, adjustable micropipettors with disposable plastic tips.
- An 8-well multichannel pipettor is recommended but not required.
- De-ionized or molecular biology grade water
- Propylene tubes
- Absorbent paper towels (for example, Wypall X60 from Kimberly Clark)

Equipment and software required:

Description	***Recommended Supplier / Cat. No.***
Luminex 100™, 200™ or equivalent	Luminex, Millipore, Hitachi, Bio-Rad
Luminex Data Acquisition Software	xPONENT 3.1 Luminex IS 2.3 Luminex LDS1.7 BioPlex Manager
Vortex Mixer	VWR Analog Vortex Mixer 58816-121 (120V) 58816-123 (230V)
Mini centrifuge	VWR Minifuge 93000-196 (120V) 93000-198 (230V)
Orbital Shaker	Eppendorf Mix Mate 022674200 (120V/60Hz) 022674226 (230V/50Hz)
Water Bath Sonicator	Bransonic Model B3/B5 000-951-005 (Model B3, 115V) 000-951-103 (Model B5, 230V)
Microplate vacuum manifold with pressure gauge	Pall Multi-well plate vacuum manifold (PN 5017).
Data Analysis Software	MasterPlex QT from Hitachi Software or equivalent

Overview and Intended Use

OriGene's TruePlex™ immunoassay kits are designed to measure the concentration of one or more proteins in a complex biological sample. The kit reagents are intended to be used in conjunction with the Luminex 100™ or 200™ multianalyte flow analyzers manufactured by Luminex Corporation and sold by Invitrogen, Bio-Rad, Millipore, and others.

Advances in genomics, proteomics, and cell biology have allowed researchers to discover and characterize a wide range of complex extracellular and intracellular biological pathways. Researchers studying these pathways have also uncovered multiple deviations from these pathways are often found in diseases and particularly cancer. These deviations are often manifested by profound increases or decreases in levels of gene expression and concomitant changes in protein levels. Immunoassays are the traditional tools for measuring protein levels in biological systems. However, traditional microplate assays can be expensive and slow. Multiplexed bead-based systems based on Luminex technology can dramatically increase the efficiency of protein measurements at reasonable cost.

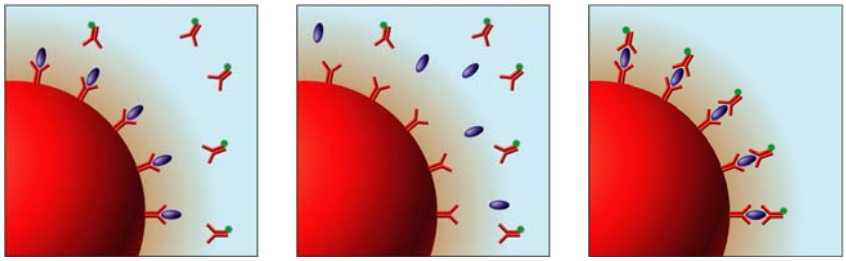
OriGene's TruePlex™ immunoassays replace the solid phase microplates used in traditional immunoassays with color-coded latex beads. Each bead color, or type, can be coated with a specific antibody or antigen to make a specific assay. Assays can be run as single-plexes or may be combined in multiplexes according to a user's needs. The assay is performed in a 96-well plate and the results are generated with the Luminex 100 or 200 instruments instead of a a traditional microplate reader. The sensitivity, linearity and dymanic range of OriGene's TruePlex™ immunoassays are comparable to traditional ELISA assays performed in a microplate.

OriGene's TruePlex™ Extracellular Core Reagent Kit is designed to be used in conjunction with one or more TruePlex Reagent Sets. Each TruePlex Reagent Set contains a single-plex bead mix, biotinylated detection antibody, and a protein standard. Up to ten reagent sets can be mixed to create a custom multiplex. The Core Reagent Kit contains all of the buffers and accessories necessary to run the assay with a single-plex reagent set or a custom multiplex.

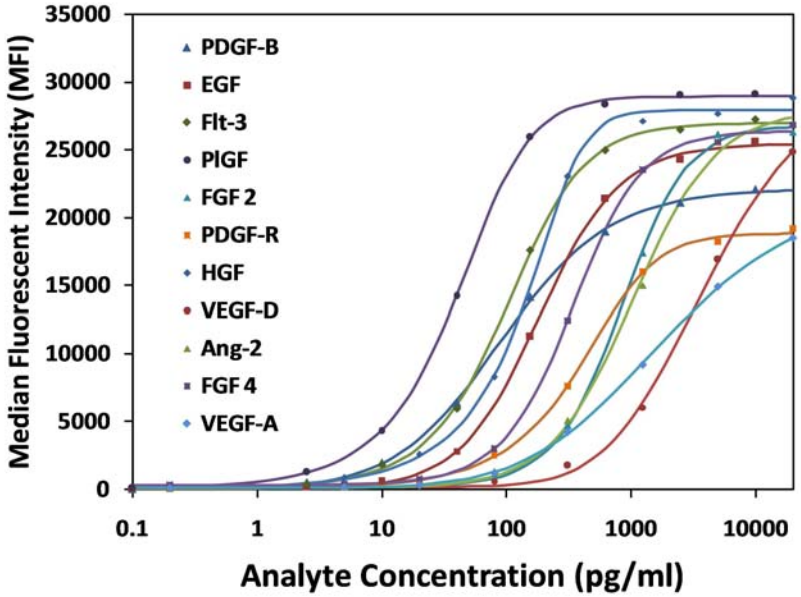
Assay Principles

OriGene TruePlex™ immunoassays use Luminex xMAP® technology to enable the efficient multiplexing of up to 100 different assays for simultaneous analysis. Luminex xMAP® technology utilizes color-coded 5.6 µm beads that have been internally dyed with two different fluors. By varying the concentration of fluors within each bead, the beads can be easily distinguished by the Luminex analyzer.

For immunoassays, capture antibodies for each target analyte are bound to a specific bead type or color. Different bead types can then be mixed to create defined multiplexes. Beads and samples are added to a 96-well filter plate and allowed to incubate. During the incubation, target analytes are captured onto the bead surfaces by the bound antibodies. Following a wash step, a mixture of biotinylated detector antibodies are added and allowed to incubate with the beads. The biotinylated detector antibodies subsequently bind to captured target analytes. After removal of excess biotinylated detector antibodies, streptavidin-phycoerythrin conjugate (Detection Reagent) is added to the beads. After incubation and another wash, the beads are read in the Luminex instrument.



The fluorescent intensity of the phycoerythrin bound to each bead is proportional to the amount of captured analyte. Recombinant protein standards are used to generate standard curves for each analyte and protein levels in each sample are determined by interpolation of the signals from for each analyte.



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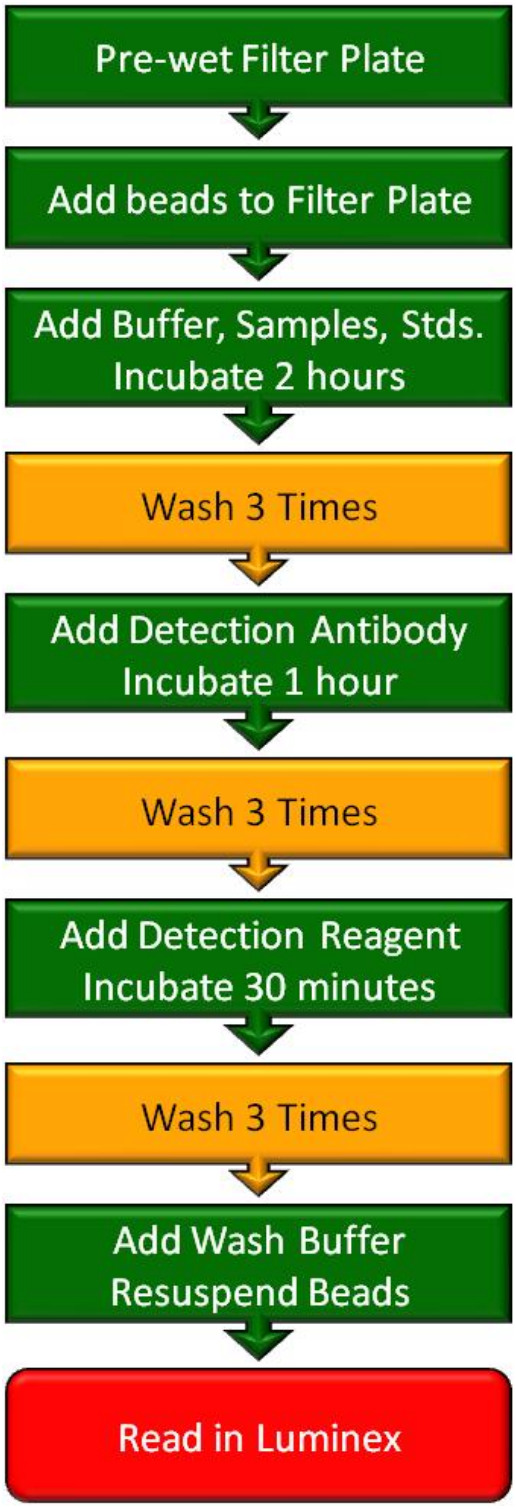
Procedure Outline



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Note : To achieve optimal results, this kit requires the use of the "High Gain" of "High PMT" setting on the Luminex 100 or 200. Some instruments may use this setting as the default. Check with your instrument supplier for more information.

Setting the Luminex 100 or 200 for "High Gain" of "High PMT" Reading

1. **Create a new lot number for Cal 2 and enter it into the Luminex software as a** new Cal 2 lot number (use the actual lot number with an HG at the end to designate High Gain).
2. Record the Cal 2 target value, which is usually around 3800.
3. Multiply the Cal 2 target value by 4.55 to get a new Target value of approximately 17,290.
4. Enter the new Target Value as the value for your "New" Cal 2 lot.
5. Run the Cal 2 Calibration.

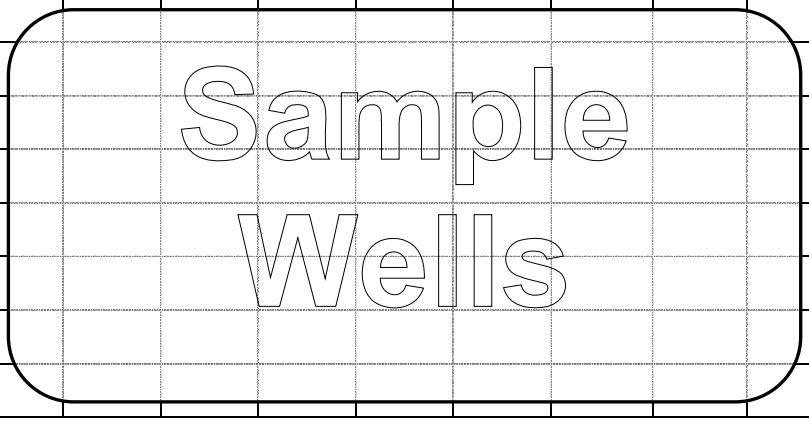
Procedure Notes

1. Before mixing beads to create a custom multiplex, ensure that each assay analyte is formatted onto a separate bead region.
2. Two different wash buffers (I and II) are used for the first wash step after analyte capture. The use of these two wash buffers minimizes potential clogging of the filter plate. Subsequent wash steps only use Wash Buffer II.
3. The vacuum pressure on the vacuum manifold should not exceed 5 mm Hg. Optimal filtration occurs between 2 and 4 mm Hg.
4. The fluorescent beads are light sensitive and are especially sensitive to direct sunlight and incandescent light. Use aluminum foil to cover plates and tubes. Store plates in a dark location during incubations.
5. After vacuum filtration, blot plates on clean absorbent material such as WYPALL X60 towels from Kimberly Clark.
6. During loading or incubations do not place the filter plate on absorbent material as liquid will be drawn through the plate by wicking.

Recommended Plate Layout

Before starting, it is recommended that a plate plan be designed. Such a plan will assist in assay workflow and data analysis. A suggested plate plan is shown below. A plate plan template is provided on page 18.

	1	2	3	5	6	7	8	9	10	11	12
A	Std 1	Std 1									
B	Std2	Std2									
C	Std 3	Std 3									
D	Std 4	Std 4									
E	Std 5	Std 5									
F	Std 6	Std 6									
G	Std 7	Std 7									
H	Neg	Neg									



Std 1 is the highest concentration and Std 7 is the lowest concentration.

Running all standards, samples, and controls in duplicate or triplicate is recommended.

Sample Collection and Preparation

1. Serum, plasma and tissue culture supernatants are suitable for use in this assay. Additional sample types may also be suitable, but have not been validated with this assay.
2. Avoid the use of lipemic of hemolyzed samples.
3. Collect samples according to standard protocols, immediately mix and separate, and then aliquot the samples into polypropylene tubes. If not tested immediately, store samples at -80ºC.
4. Analyze fresh samples as quickly as possible after collection. Allow frozen samples to thaw on ice, and then mix well. Avoid multiple freeze-thaw cycles of frozen samples.
5. Turbid samples should be clarified by centrifugation before use.
6. If measured analyte concentrations exceed the value of the upper range of the standard curve, dilute samples appropriately and reanalyze. Dilute serum and plasma samples in Assay Diluent. Dilute tissue culture supernatants in tissue culture medium.

Creating Custom Multiplexes

Custom multiplex assays can be prepared by purchasing one or more single-plex reagent sets and the Core Reagent Kit. Each of the single-plex reagent sets contains bead mix, biotinylated detection antibody and protein standard. Up to ten beads and detection antibodies can be mixed to create a custom multiplex. The protein standard is pre-mixed. The Core Reagent Kit contains all of the buffers and accessories necessary to run the assay with a single-plex reagent set or a custom multiplex.

Reagent Preparation

1X Wash Buffer I

Prepare 1X Wash Buffer I by diluting the entire contents of the 5X Wash Buffer I bottle with 40 ml of sterile, deionized water. If desired, add 20 mg sodium azide to bring the sodium azide concentration to 0.05% (7.5 mM). Store diluted Wash Buffer I at 2-8ºC. Alternatively, a portion of the 5X Wash Buffer I can be diluted. Mix 1 volume 5X Wash Buffer I with 4 volumes of deionized water.

1X Wash Buffer II

Prepare 1X Wash Buffer II by diluting the entire contents of the 20X Wash Buffer II bottle with 475 ml of sterile, deionized water. If desired, add 0.24 g sodium azide to bring the sodium azide concentration to 0.05% (7.5 mM). Store diluted Wash Buffer II at 2-8ºC. Alternatively, a portion of the 20X Wash Buffer II can be diluted. Mix 1 volume 20X Wash Buffer II with 19 volumes of deionized water.

Standards – for serum or plasma samples

Dilute one vial of Standard just prior to use. Do not store diluted standards. Additional standards may be purchased from OriGene.

Thaw the Standard vial at room temperature for 20 minutes. Mix well by inversion a minimum of 6 times. Use Standard 1 as supplied. Dilute Standard 1 as follows to prepare the remaining standards:

To Make	Add	To
Standard 2	150 µl of Standard 1	200 µl of Assay Diluent
Standard 3	100 µl of Standard 2	200 µl of Assay Diluent
Standard 4	100 µl of Standard 3	200 µl of Assay Diluent
Standard 5	100 µl of Standard 4	200 µl of Assay Diluent
Standard 6	100 µl of Standard 5	200 µl of Assay Diluent
Standard 7	100 µl of Standard 6	200 µl of Assay Diluent

Use Assay Diluent as the Negative Standard (blank).

Standards – for cell culture supernatants

Prepare as above, but use cell culture medium or other matrix in the place of Assay Diluent.

Use cell culture medium or other matrix as the Negative Standard (blank).

Capture Buffer – for serum or plasma samples

Prepare the following buffer just before use. Prepare enough for several extra wells to account for losses during pipetting.

Component	Volume per Reaction	Number of Wells	Final Volume
Assay Buffer A	65 µL		
Assay Diluent	35 µL		

Capture Buffer – for cell culture supernatants

Prepare as above, but use cell culture medium or other matrix in the place of Assay Diluent.

1X Bead Mix

Use the table below to prepare the 1X Bead Mix from one or more 10X Bead Mixes.

Note that all volumes are given on a per well basis. Multiply given volumes by the number of wells to be run. Add one or two additional wells to ensure a sufficient volume of the 1X mixes.

- Briefly vortex (5 seconds) the vial of Bead Mix Concentrate.
- Sonicate the Bead Mix Concentrate for 30 seconds.
- Prepare 1X Bead Mix according to the table below.

Plex Size	Volume of Assay Buffer B to add (µl)	Volume of each Bead to Add (µl)	Total Volume of Beads Added (µl)	Final Volume (µl)
1	90	10	10	100
2	80	10	20	100
3	70	10	30	100
4	60	10	40	100
5	50	10	50	100
6	40	10	60	100
7	30	10	70	100
8	20	10	80	100
9	10	10	90	100
10	0	10	100	100

- Mix thoroughly by inversion. Protect from light by covering tube in aluminum foil.

Filter Plate

Before starting the assay, cover the wells that will not be used in the assay with an aluminum plate sealer (supplied). Press sealer down so that all wells are tightly sealed. Trim edges with a razor blade.

Assay Protocol

Analyte Capture

1. Cover unused wells with an aluminum plate sealer.
2. Add 100 µl Wash Buffer II to each of the wells that will be used.
3. Remove liquid from the wells by gentle vacuum (do not exceed a vacuum pressure of 5 mm Hg). Blot plate thoroughly on absorbent paper.
4. Briefly vortex the 1X Bead Mix. Add 100 µl of 1X Bead Mix to each assay well. Remove liquid from the plate by gently vacuum. Blot plate thoroughly on absorbent paper.
5. Add 100 µl Capture Buffer into all standard, blank and sample wells.
6. Add 50 µl standards into designated standard wells (including negative).
7. Add 50 µl sample into designated sample wells.

8. Cover and incubate the plate for 2 hours at room temperature on an orbital plate shaker (600-800 rpm).
9. Ten to fifteen minutes before the end of this incubation, prepare 1X Detection Antibody.

Preparation of 1X Detection Antibody

Use the table to prepare the 1X Detection Antibody from one or more 10X Detection Antibodies.

Note that all volumes are given on a per well basis. Multiply given volumes by the number of wells to be run. Add one or two additional wells to ensure a sufficient volume of the 1X mixes.

- Briefly vortex (5 seconds) the vial of 10X Detection Antibody.
- Prepare 1X Detection Antibody according to the table below

Plex Size	Volume of Assay Buffer B to add (µl)	Volume of each Det. Ab. to Add (µl)	Total Volume of Det. Ab. Added (µl)	Final Volume (µl)
1	90	10	10	100
2	80	10	20	100
3	70	10	30	100
4	60	10	40	100
5	50	10	50	100
6	40	10	60	100
7	30	10	70	100
8	20	10	80	100
9	10	10	90	100
10	0	10	100	100

- Mix thoroughly by inversion. Protect from light by covering tube in aluminum foil.

Analyte Detection

1. Remove the liquid from the wells by gentle vacuum.
2. Wash beads two times by adding 100 µL Wash Buffer I to the wells and removing liquid with gentle vacuum.
3. Wash beads one times by adding 100 µL Wash Buffer II to the wells and removing liquid with gentle vacuum. After washing, blot plate thoroughly on absorbent material.
4. Add 100 µl Detection Antibody into each well.
5. Cover and incubate the plate for 1 hour at room temperature on an orbital plate shaker at 600-800 rpm.
6. During this incubation, prepare the Luminex instrument for reading (see below).
7. During the last 10 minutes of this incubation, prepare the Detection Reagent (Streptavidin-Phycoerythrin).

Prepare the Luminex instrument for reading

1. Set up the instrument as described in the user's manual. Instruments from different vendors may have different set-up procedures.
2. Warm up the instrument. This may take up to 30 minutes.
3. Parameters specific to this kit:
   - The XY platform heater should be off.
   - The total number of beads to count is 1100.
   - The minimum events setting should be 30.
   - The sample size should be set to 50 µL.
   - The flow rate should be set to Fast.
4. Enter the analyte names and bead numbers as indicated in the Table 1.
5. Check the probe height and adjust it, if necessary to accommodate the filter plate
6. Perform 1 prime with sheath fluid, 1 alcohol flush, and 2 sheath fluid washes.
7. Calibrate to "High Gain" setting if necessary (see page 9).

Preparation of Detection Reagent (Streptavidin-Phycoerythrin Conjugate)

- Prepare Detection Reagent according to the table below.

Component	Volume per Reaction	Number of Wells	Final Volume
Assay Buffer B	90 µL		
SAPE Conjugate	10 µL		

Detection with Streptavidin-Phycoerythrin

1. Remove the liquid in the wells by gentle vacuum.
2. Wash beads three times by adding 100 µl Wash Buffer II to all wells and removing liquid with gentle vacuum.
3. Blot plate thoroughly on absorbent material.
4. Add 100 µl Detection Reagent (Streptavidin-PE conjugate) into each well.
5. Cover and incubate the plate for 30 minutes at room temperature on an orbital plate shaker at 600-800 rpm.
6. Wash beads three times by adding 100 µl Wash Buffer II to all wells and and then removing liquid with gentle vacuum.
7. Blot plate thoroughly on absorbent material.
8. Add 100 µL 1X Wash Buffer II to each well
9. Shake plate for 2 minutes on an orbital plate shaker at 600-800 rpm.
10. Read plate in the Luminex instrument.

Data Analysis using MasterPlex QT

1. A free two-week trial of MasterPlex QT from Hitachi software is available for quantitative analysis of your data. If you don't already have a copy, download a copy of MasterPlex QT from www.miraibio.com. Follow the instructions for installing the software and licenses.
2. MasterPlex QT Tutorials in PDF format, PowerPoint presentations, FAQs and other training materials are also available on the MiraiBio website.
3. The standard curves generally give the best fit with a 5-parameter logistic regression with 1/Y weighting.

Protocol Summary

1.	Prepare 1X Wash Buffer I and II, Standards, 1X Bead Mix, and Capture Buffer.
2.	Add 100 µl Wash Buffer II to each well.
3.	Remove liquid from wells by gentle vacuum. Blot thoroughly.
4.	Add 100 ul 1X Bead Mix to each well.
5.	Remove liquid from wells by gentle vacuum. Blot thoroughly.
6.	Add 100 µl of Capture Buffer to all wells. Add 50 µl of standards or samples into designated wells
7.	Cover and incubate for 2 hours with shaking (600-800 rpm).
8.	Prepare 1X Detection Antibody.
9.	Remove liquid from plate by gentle vacuum.
10.	Wash two times with 100 µl 1X Wash Buffer I. Wash one times with 100 µl 1X Wash Buffer II. Blot thoroughly.
11.	Add 100 µl of 1X Detection Antibody to each well.
12.	Cover plate and incubate for 1 hour with shaking (600-800 rpm).
13.	Prepare Luminex instrument for reading.
14.	Prepare Detection Reagent.
15.	Remove liquid from wells by gentle vacuum.
16.	Wash three times with 1X Wash Buffer II. Blot thoroughly.
17.	Add 100 µl 1X Detection Reagent to each well.
18.	Cover and incubate for 30 minutes with shaking.
19.	Wash three times with 1X Wash Buffer II.
20.	Remove liquid from wells by gentle vacuum. Blot thoroughly.
21.	Add 100 µl of 1X Wash Buffer II to each well.
22.	Shake for 2 minutes (600-800 rpm).
23.	Read in Luminex instrument.

Plate Setup Template

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

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Limitations of the Procedure

1. Do not calculate the concentrations of analytes if the MFI value is higher than the highest standard or lower than the lowest standard.
2. If measured analyte concentrations exceed the value of the upper range of the standard curve, dilute samples appropriately and reanalyze. Dilute serum and plasma samples in Assay Diluent. Dilute tissue culture supernatants in tissue culture medium.
3. To measure samples with very low analyte levels, it may be possible to extend the range at the low end of the curve by additional dilution of Standard 7.
4. The influence of drugs or other substances not usually found in serum or plasma have not been investigated.
5. The influence of abnormal (jaundiced, hemolyzed, lipemic) sera or plasma samples has not been investigated.
6. The rate of degradation of analytes in serum, plasma, or tissue culture supernatants has not been investigated.
7. The affect of heterophilic antibodies on the assay has not been investigated.

Troubleshooting

To troubleshoot problems with the Luminex instrument, consult the appropriate Luminex manuals, contact Luminex technical support (http://www.luminexcorp.com/support), or contact your instrument supplier.

To troubleshoot problems with the using MasterPlex QT for data analysis, contact Hitachi Software (http://www.miraibio.com/support).

Problem	Cause	Solution
Insufficient bead count	Bead mix not prepared correctly	Prepare new bead mix and re-run samples. Sonicate and vortex vial containing 10X Bead Mix.
	Vacuum pressure too high	Adjust vacuum pressure to 2-5 mm Hg during filtration
	Plate leaked in the Luminex instrument	See below.
	Clogged sample probe	Clean sample probe. Remove and sonicate, if necessary.
Wells in filter plate will not vacuum	Clogged wells	Spin samples @ 14,000 x g for 2 minutes before removing aliquot for testing.
	Plate has not made a tight seal with the vacuum manifold	Replace gasket on vacuum manifold.
Leaking plate	Probe Height not adjusted correctly	Adjust probe height with two alignment disks using the filter plate in the kit.
	Insufficient blotting of filter plate	Blot filter plate thoroughly onto paper towels after each washing cycle
	Vacuum pressure too high	Ensure that the vacuum pressure is less than 5 mm Hg.
High Background	Cross-contamination of wells	Pipette carefully to ensure that no material from the standard or sample wells reaches the background (Neg) wells.
	Ineffective or omitted wash steps	Follow wash instructions carefully. Increase number of washes if necessary.

Problem	Cause	Solution
Low signal across the plate	Luminex instrument gain setting is incorrect	Make sure that the reporter channel is calibrated to the “High Gain” or “High PMT” setting.
	1X Detection Antibody prepared incorrectly	Prepare new 1X Detection Antibody
	Incubations were too short, or shaking was insufficient	Check assay conditions and remedy as necessary
Beads not in region	Instrument is out of calibration	Recalibrate instrument.
	Beads are photo-bleached	Protect beads from light at all times. Use amber colored tubes.
	Incorrect bead regions entered.	Check protocol template. Correct if necessary.
Sample readings are out of range	Analyte level is below the detection limit of the assay.	Prepare an additional low standard by diluting Standard 7 1:3 and re-run the sample and standard curve.
	Analyte level is above of the quantifiable range of the assay	Dilute samples in Assay Diluent and re-run.