BioLegend®

LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 740449

Human Free Active/Total TGF-β1 Assay with Filter Plate

Cat. No. 740450

Human Free Active/Total TGF-β1 Assay with V-bottom Plate

Please read the entire manual before running the assay.

BioLegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Tel: 858-768-5800

Chapter 1: KIT DESCRIPTION

Introduction

Transforming growth factor beta 1 (TGF-β1) belongs to the transforming growth factor beta superfamily of cytokines. It has diverse biological functions in multiple cellular processes such as proliferation and differentiation of various cell types. TGF-β1 is also an important immunoregulatory cytokine which is involved in the maintenance of self-tolerance, Th17 differentiation and T-Cell homeostasis. It is nearly 100% conserved across mammalian species.

TGF-β1 exists mostly in inactive homodimeric form in circulation, complexed to LAP and latent TGF-β binding protein (LTBP). Non-covalent binding of LAP to TGF-β1 confers latency of TGF-β1, while covelent binding of LTBP to LAP is for efficient secretion of the complex to extracellular sites. Free active TGF-β1 can be released (activated) by many factors including enzymes and low or high pH. It is expected that normal serum, plasma and other biological fluids contain a lower concentration of free active TGF-β1 and a higher concentration of latent TGF-β1. It is the free active TGF-β1 that binds to the TGF-β receptor and exerts biological functions. In order to fully understand the biological functions of TGF-β1, it is necessary to measure both the free active and total TGF-β1 forms in biological samples. However, it has been difficult to quantify the level of free active TGF-β1 due to the insufficient sensitivities of most assay products currently available on the market.

The LEGENDplex™ Free Active/Total TGF-β1 is a single plex assay of a multiplex beads-based assay system using fluorescence–encoded beads suitable for use on various flow cytometers. This assay allows quantification of either free active TGF-β1 without sample treatment or total TGF-β1 after sample treatment. This assay provides higher detection sensitivity and broader dynamic range than traditional ELISA methods and it has been validated for use on serum, plasma, cell culture supernatant and urine samples.

Since TGF-β1 is nearly 100% conserved across mammalian species, this assay may be used for other species as well. For Mouse/Rat Free Actvie/Total TGF-β1 measurement, please use Cat# 740489 and 740490. The capability of this assay to measure free active TGF-β1 without sample treatment makes multiplexing TGF-β1 with other targets possible. Please visit http://www.biolegend.com/legendplex and email CST@biolegend.com or contact your local BioLegend representatives for ordering cutom panels.

This assay is for research use only.

Principle of the Assay

BioLegend's LEGENDplex™ assays are bead-based immunoassays using the same basic principle as sandwich immunoassays.

Beads are differentiated by size and internal fluorescence intensities. Each bead set is conjugated with a specific antibody on its surface and serves as the capture beads for that particular analyte. When a selected panel of capture beads is mixed and incubated with a sample containing target analytes specific to the capture antibodies, each analyte will bind to its specific capture beads. After washing, a biotinylated detection antibody cocktail is added, and each detection antibody in the cocktail will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Streptavidin-phycoerythrin (SA-PE) is subsequently added, which will bind to the biotinylated detection antibodies, providing fluorescent signal intensities in proportion to the amount of bound analytes.

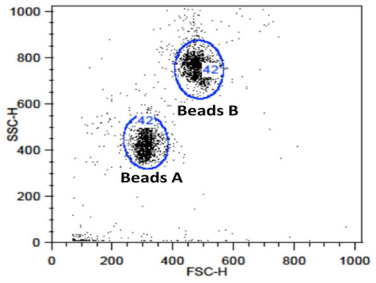
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and quantified by the PE fluorescent signal. The concentration of a particular analyte is determined by a standard curve generated in the same assay.

Beads Usage

The LEGENDplex™ beads-based assay usually includes two sets of beads. Each set has a unique size that can be identified on flow cytometer based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A and Beads B, Figure 1). Each bead set can be further resolved based on their internal fluorescence intensities. The internal dye can be detected using FL3, FL4, or APC channel, depending on the type of flow cytometer used. The smaller Beads A consists of 6 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

The Free Active /Total TGF-β1 Assay uses only one of the 13 bead populations (A6) distinguished by size and internal fluorescent dye.

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

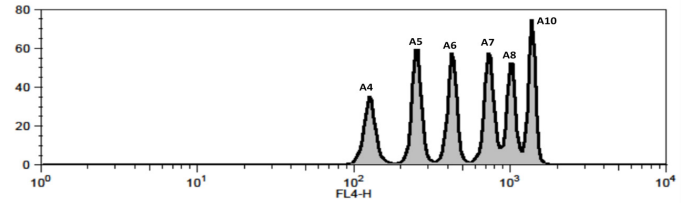


Figure 3. Beads B Classification by FL4

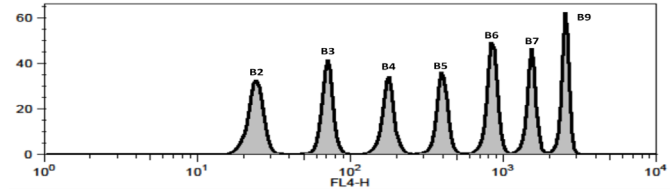


Table 1. Beads ID and Target Information

Target	Bead ID	Top Standard Concentration
TGF-β1	A6	The top standard concentration of TGF-β1 was set at 10 ng/mL, but may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).

*Bead ID is used to associate a bead population to a particular analyte when using the LEGENDplex™ data analysis software program. For further information regarding the use of the program please visit biolegend.com/en-us/legendplex

Storage Information

Recommended storage for all original kit components is between 2°C and 8°C. DO NOT FREEZE Beads, Detection Antibodies or SA-PE.

- Once the standards have been reconstituted, immediately transfer contents into polypropylene vials. DO NOT STORE RECONSTITUTED STANDARDS IN GLASS VIALS.
- Upon reconstitution, leftover standard and Matrix should be stored at ≤-70°C for use within one month. Avoid multiple (>2) freeze-thaw cycles. Discard any leftover diluted standards.

Materials Supplied

The LEGENDplex™ assay contains reagents for 100 tests listed in the table below. When assayed in duplicate, this is enough for an 8-point standard curve and 40 samples.

Kit Components	Quantity	Volume	Part #
Setup Beads: PE Beads	1 vial	1 mL	77842
Setup Beads: Raw Beads	1 vial	1.8 mL	77844
Free Active/Total TGF-β1 Capture Bead, A6	1 bottle	3.3 mL	77576
Free Active/Total TGF-β1 Detection Antibody	1 bottle	3.3 mL	77578
Free Active/Total TGF-β1 Standard	1 vial	lyophilized	77580
Acidification Solution	1 vial	1.5 mL	78260
Neutralization Solution	1 vial	1.5 mL	78250
Sample Diluent	1 bottle	25 mL	78248
LEGENDplex™ SA-PE	1 bottle	3.3 mL	77743
LEGENDplex™ Matrix B, Lyophilized	1 vial	lyophilized	77549
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Filter Plate* or V-bottom Plate**	1 plate		76187* or 76883**
Plate Sealers	4 sheets		78101

Either a filter plate* or a V-bottom plate will be provided in each kit.**

Materials to be Provided by the End-User

- A flow cytometer equipped with two lasers (e.g., a 488 nm blue laser or 532 nm green laser and a 633-635 nm red laser) capable of distinguishing 575-585 nm and 660 nm or a flow cytometer equipped with one laser (e.g., 488 nm blue laser) capable of distinguishing 575 nm and 670 nm.

Partial list of compatible flow cytometers:

Flow Cytometer	Reporter Channel	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD Accuri™ C6™	FL2	585 nm	FL4	675 nm	No*
BD FACSCanto, BD FACSCanto™II	PE	575 nm	APC	660 nm	No*
BD™ LSR, LSR II BD LSRFortessa™	PE	575 nm	APC	660 nm	No*
Gallios™	PE	575 nm	APC	660 nm	No*
CytoFLEX	PE	585 nm	APC	660 nm	No*
NovoCyte	PE	572 nm	APC	660 nm	No*
Attune™ NxT	PE	574 nm	APC	670 nm	No*

***Compensation is not required for the specified flow cytometers when set up properly.**

For setting up various flow cytometers, please visit: **www.biolegend.com/legendplex** and click on the **Instrument Setup** tab.

Multichannel pipettes capable of dispensing 5 µL to 200 µL

- Reagent reservoirs for multichannel pipette
- Polypropylene microfuge tubes (1.5 mL)
- Laboratory vortex mixer
- Sonicator bath (e.g., Branson Ultrasonic Cleaner model #B200, or equivalent)
- Aluminum foil
- Absorbent pads or paper towels
- Plate shaker (e.g., Lab-Line Instruments model #4625, or equivalent)
- Tabletop centrifuges (e.g., Eppendorf centrifuge 5415 C, or equivalent)
- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, catalog # TN0946-01R, or equivalent).

If the assay is performed in a filter plate,

- A vacuum filtration unit (Millipore MultiScreen ® HTS Vacuum Manifold, cat # MSVMHTS00 or equivalent). Instructions on how to use the vacuum manifold can be found at the supplier's website.
- A vacuum source (mini vacuum pump or line vacuum, e.g., Millipore Vacuum Pump, catalog # WP6111560, or equivalent)
- If needed, additional Filter plate can be ordered from BioLegend (Cat# 740377 or 740378)

If the assay is performed in a V-bottom plate,

- Centrifuge with a swinging bucket adaptor for microtiter plates (e.g., Beckman Coulter Allegra™ 6R Centrifuge with MICROPLUS CARRIER adaptor for GH3.8 and JS4.3 Rotors) .
- If needed, additional V-bottom plate can be ordered from BioLegend (Cat# 740379)

• Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and Beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes and centrifuge for 10 minutes at 1,000 x *g*.
- Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

- Collect blood samples in a tube containing an anticoagulant (heparin is preferred). Centrifuge for 10 minutes at 1,000 x g within 30 minutes of collection.

  Note: Platelet activation results in relasing of TGF-β1 from platelet granules. Avoid using anticoagulant that may activate platelet. Also, plalelet-poor plasma is recommended for measuing TGF- β1 in the circulation. To remove platelet from plasma preparation, an additional centrifugation step at 10,000 x g for 10 minutes at 2-8 °C is recommended.
- Remove plasma and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed well and centrifuged to remove particulates prior to use.

Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately. If not possible, aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

  Note: Animal serum used in cell culture media may contain latent TGF-β1. For best results, use serum-free medium for cell culture. If animal serum is used as a supplement in the medium, appropriate control should be run to determine the baseline concentration of TGF-β1 (see Sample Preparation on page 12 for details).

Reagent Preparation

Preparation of Antibody-Immobilized Beads

Sonicate the beads bottle for 1 minute in a sonicator bath and then vortex for 30 seconds prior to use. If no sonicator bath is available, increase the vortexing time to 1 minute to completely resuspend the beads.

Preparation of Wash Buffer

- Bring the 20X Wash Buffer to room temperature and mix to bring all salts into solution.
- Dilute 25 mL of 20X Wash Buffer with 475 mL deionized water. Store unused portions between 2°C and 8°C for up to one month.

Preparation of Matrix B

Note: Matrix B is only needed for measuring free active TGF-β1 in serum or plasma samples.

- Add 5.0 mL LEGENDplex™ Assay Buffer to the bottle containing lyophilized Matrix B. Allow at least 15 minutes for complete reconstitution. Vortex to mix well. Leftover reconstituted Matrix B should be stored at ≤-70°C for up to one month.

Standard Preparation

1. Prior to use, reconstitute the lyophilized TGF-β1 Standard with 250 µL Assay Buffer.
2. Mix and allow the vial to sit at room temperature for 10 minutes, and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the top standard C7.

   Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).
3. Label 6 polypropylene microfuge tubes as C6, C5, C4, C3, C2 and C1, respectively.
4. Add 75 µL of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 µL of the top standard C7 to the C6 tube and mix well. This will be the C6 standard.
5. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2

and C1 standards (see the table below, using the top standard at 10,000 pg/mL as an example). Assay Buffer will be used as the 0 pg/mL standard (C0).

Tube/ Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL)
C7	--	--	--	10,000
C6	1:4	75	25 µL of C7	2,500
C5	1:16	75	25 µL of C6	625
C4	1:64	75	25 µL of C5	156.3
C3	1:256	75	25 µL of C4	39.1
C2	1:1024	75	25 µL of C3	9.8
C1	1:4096	75	25 µL of C2	2.4
C0	--	75	--	0

Sample Treatment and Dilution

For Measuring Free Active TGF-β1

- Serum or plasma samples must be diluted 2-fold with Assay Buffer before testing (e.g. dilute 50 µL of sample with 50 µL of Assay Buffer). If further sample dilution is desired, dilution should be done with Matrix B to ensure accurate measurement.

  NOTE: Adding serum or plasma samples without dilution will result in low assay accuracy and possibly, clogging of the filter plate.

- For cell culture supernatant samples, while samples can be tested without dilutions, a preliminary experiment may be required to determine the appropriate dilution factor. If sample dilution is desired, dilution should be done with Assay Buffer to ensure accurate measurement.

For Measuring Total TGF-β1

In order to measure total TGF-β1, samples must be treated to release free TGF-β1 from complex.

For Serum/Plasma samples

- Add 20 µL of each serum/plasma sample to a polypropylene microplate

well or a polypropylene microfuge tube, then add 10 µL of Acidification Solution, mix well, and incubate for 10 minutes at room temperature. Add 10 µL of Neutralization Solution, mix well. Make sure each sample is treated the same.

Note: A polypropylene microplate is preferred for sample treatment and dilution as it allows using multichannel pipette for convenience and to ensure equal sample treatment.

- Dilute treated serum/plasma samples in Sample Diluent immediately after sample treatment. A recommended dilution factor is 50 (final dilution factor of the original sample is 100). To make a 50-fold dilution, add 5 µL treated samples in 245 µL of Sample Diluent. If further sample dilution is desired, dilution should be done with Sample Diluent to ensure accurate measurement.

For Cell Culture Supernatant Samples

- Add 40 µL of each cell culture supernatant sample to a polypropylene microplate well or a polypropylene microfuge tube, then add 10 µL of Acidification Solution, mix well, and incubate for 10 minutes at room temperature. Add 10 µL of Neutralization Solution, mix well.

- If cells have been cultured in serum-free medium, dilute treated cell culture supernatant in Sample Diluent according to estimated sample concentrations, which can be determined by a pilot study. For example, to make a 2-fold dilution of treated cell culture supernatant samples in Sample Diluent, dilute 50 µL treated serum free cell culture supernatant samples in 50 µL Sample Diluent (final dilution factor of the original sample is 3).

- If cells have been cultured in medium containing serum (e. g. 10% fetal bovine serum), the medium should be treated using the same protocol as a control because the significant level of latent TGF-β1 present in animal serum. To measure TGF-β1 in serum-containing cell culture supernatants, more dilution of treated samples may be required prior to the assay. For example, to make a 10 fold dilution of treated samples, add 10 µL treated samples in 90 µL Sample Diluent (final dilution factor of the original sample is 15). If further sample dilution is desired, dilution should be done with Sample Diluent to ensure accurate measurement.

Chapter 3: ASSAY PROCEDURE

The LEGENDplex™ assay can be performed in a filter plate, or in a V-bottom plate.

- The in-filter plate assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 7)**. If you have performed bead-based multiplex assays before, your lab may already have the vacuum filtration unit set up.
- If the in-filter plate assay procedure is not possible or if you prefer, the assay can be performed in a V-bottom plate.

Performing the Assay Using a Filter Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Set the filter plate on an inverted plate cover at all times during assay setup and incubation steps, so that the bottom of the plate does not touch any surface. Touching a surface may cause leakage.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 30). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

1. Pre-wet the plate by adding 100 µL of 1X Wash Buffer to each well and let it sit for 1 minute at room temperature. To remove the excess volume, place the plate on the vacuum manifold and apply vacuum. Do not exceed 10" Hg of vacuum. Vacuum until wells are drained (5-10 seconds). Blot excess Wash Buffer from the bottom of the plate by pressing the plate on a stack of clean paper towels. Place the plate on top of the inverted plate cover.

   For measuring free active TGF-β1 in serum/plasma, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix B	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution and Treatment**

For measuring free active or total TGF-β1 in cell culture supernatant, or total TGF-β1 in serum/plasma, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix B	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

2. Vortex mixed beads for 30 seconds. Add 25 µL of mixed beads to each well. The total volume should be 75 µL in each well after beads addition. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).

3. Seal the plate with a plate sealer. **To avoid plate leaking, do not apply positive pressure to the sealer when sealing the plate.** Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker, secure it and shake at approximate 500 rpm for 2 hours at room temperature.

4. **Do not invert the plate**! Place the plate on the vacuum manifold and apply vacuum as before in Step 1. Add 200 µL of 1X Wash Buffer to each well. Remove Wash Buffer by vacuum filtration. Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels. Repeat this washing step once more.

5. Add 25 µL of Detection Antibodies to each well.

6. Seal the plate with a new plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximately 500 rpm for 1 hour at room temperature.

7. **Do not vacuum!** Add 25 µL of SA-PE to each well directly.

8. Seal the plate with a new plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximate 500 rpm for 30 minutes at room temperature.

9. Repeat step 4 above.

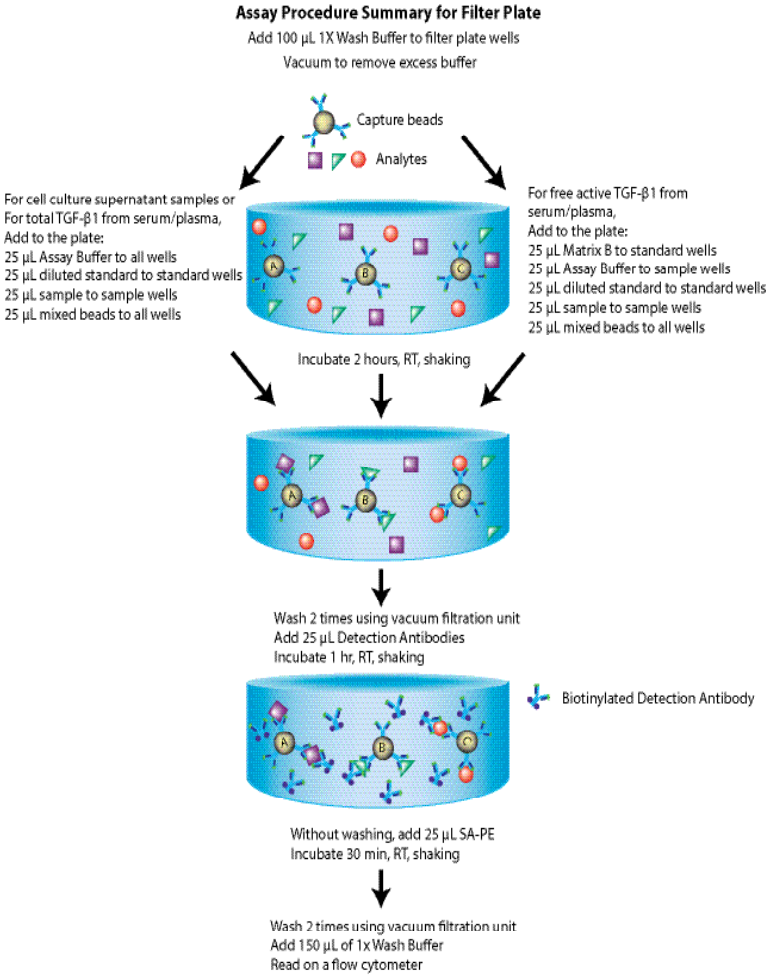
10. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads on a plate shaker for 1 minute.

11. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

    If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **Please be sure to program the autosampler**

to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler.

If an autosampler is not available, the samples can be transferred from the filter plate to micro FACS (or FACS) tubes and read manually.



Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 30). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

1. **For measuring free active TGF-β1 in serum/plasma,** load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix B	Standard	Sample*
Standard wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution and Treatment**

For measuring free active or total TGF-β1 in cell culture supernatant, or total TGF-β1 in serum/plasma, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix B	Standard	Sample*
Standard wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

2. Vortex mixed beads for 30 seconds. Add 25 µL of mixed beads to each well. The total volume should be 75 µL in each well after beads addition. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).

3. Seal the plate with a plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 2 hours at room temperature **(Depending on the shaker, the speed may need to be adjusted. The optimal speed is one that is high enough to keep beads in suspension during incubation, but not too high so it causes spill from the wells)**.

4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 7).** Do not use excessive centrifugation

speed as it can make it harder to resuspen beads in later steps. **Make sure the timer of the centrifuge works properly and standby to make sure the centrifuge reaches preset speed.**

5. Immediately after centrifugation, dump the supernatant into a sink by quickly inverting and flicking the plate **in one continuous and forcefull motion**. Do not worry about losing beads even if the pellet is not visible. The beads will stay in the tip of the well nicely. Blot the plate only once by pressing on a stack of clean paper towel and drain the remaining liquid from the well as much as possible. Be careful not to disturb the bead pellet.

   Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

6. Wash the plate once by dispensing 200 µL of washing buffer into each well. Shake the plate at 800 rpm for 1 minute and repeat step 4 and 5.

7. Add 25 µL Detection Antibodies to each well.

8. Seal the plate with a new plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 1 hour at room temperature.

9. **Do not wash the plate!** Add 25 µL of SA-PE to each well directly.

10. Seal the plate with a new plate sealer. Wrap the entire plate with aluminum foil and shake the plate on a plate shaker at approximate 800 rpm for 30 minutes at room temperature.

11. Repeat step 4 and 5.

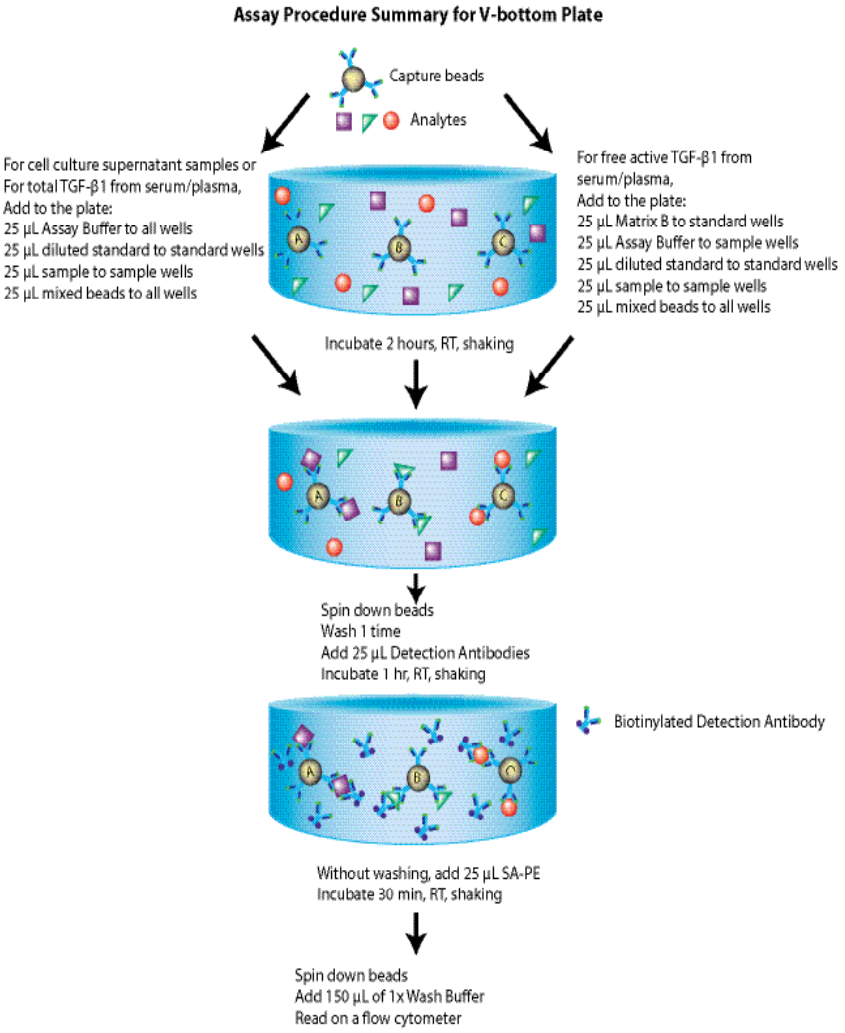
12. Wash the plate by dispensing 200 µL of 1X Wash Buffer into each well and incubate for one minute. Repeat step 4 and 5 above. **This washing step is optional but it helps to reduce the background.**

13. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

14. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

    If the flow cytometer is equipped with an autosampler, the samples can be read directly. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler**.

    If an autosampler is not available, the samples can be transferred from the plate to micro FACS (or FACS) tubes and read manually.


Assay Procedure Summary for V-bottom Plate
Capture beads
Analytes
For cell culture supernatant samples or
For total TGF-β1 from serum/plasma,
Add to the plate:
25 µL Assay Buffer to all wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells
For free active TGF-β1 from serum/plasma,
Add to the plate:
25 µL Matrix B to standard wells
25 µL Assay Buffer to sample wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells
Incubate 2 hours, RT, shaking
Spin down beads
Wash 1 time
Add 25 µL Detection Antibodies
Incubate 1 hr, RT, shaking
Biotinylated Detection Antibody
Without washing, add 25 µL SA-PE
Incubate 30 min, RT, shaking
Spin down beads
Add 150 µL of 1x Wash Buffer
Read on a flow cytometer

Chapter 4: FLOW CYTOMETER SETUP

In order to generate reliable data, the flow cytometer must be set up properly before data acquisition.

The setup instructions have been removed from this manual and uploaded onto our website to save paper.

To access the setup instructions, please visit: **www.biolegend.com/legendplex** and click on the **Instrument Setup** tab.

Chapter 5: DATA ACQUISITION AND ANALYSIS

Data Acquisition

1. Before reading samples, make sure that the flow cytometer is set up properly.

2. Create a new template or open an existing template (for details on how to create a cytometer-specific template, please refer to the Flow Cytometer Setup Guide).

3. Vortex each sample for 5 seconds before analysis.

4. Set the flow rate to low. Set the number of beads to be acquired to 300. Do not set to acquire total events as samples may contain large amounts of debris. Instead, acquire the number of events in gate A. This will exlude majority of the debris.

   Note: Do not acquire too few or too many beads. Too few beads acquired may result in high CVs and too many beads acquired may result in slow data analysis later.

5. Read samples.

   When reading samples, set the flow cytometer to setup mode first and wait until bead population is stabilized before recording or switching to acquisition mode.

   To simplify data analysis using the LEGENDplex™ Data Analysis Software, read samples in the same order as shown on the PLATE MAP attached at the end of the manual. For an in-plate assay, read column by column (A1, B1, C1...A2, B2, C2...).

   When naming data files, try to use simple names with a consecutive num-

bering for easy data analysis (e.g. for standards, C0.001, C0.002, C1.003, C1.004, C2.005, C2.006, C3.007, C3.008, ... C7.015, C7.016; for samples, S1.017, S1.018, S2.019, S2.020, S3.021, S3.022…)

Store all FCS files in the same folder for each assay. If running multiple assays, create a separate folder for each assay.

6. Proceed to data analysis using LEGENDplex™ Data Analysis Software when data acquisition is completed.

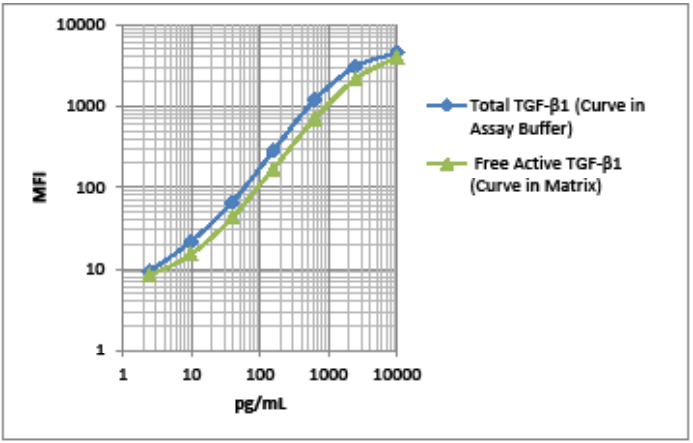
Data Analysis

- The assay FCS files should be analyzed using BioLegend's LEGENDplex™ data analysis software. The program is offered free of charge with the purchase of any LEGENDplex™ assay. For further information regarding acccess to, and use of the program please visit biolegend.com/en-us/legendplex.

Chapter 6: ASSAY CHARACTERIZATION

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Free Active/Total TGF-β1 Assay for demonstration purpose only. A standard curve must be run with each assay.



Assay Sensitivity

The assay sensitivity is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-paramater curve fitting algorithm. Assay Sensitivity presented here is ≤Mean LOD + 2x STDEV LOD.

Analyte	LOD in Assay Buffer (pg/mL)	LOD in Matrix (pg/mL)
TGF-β1	2.30	4.80

Cross-Reactivity

The following recombinant proteins were tested at 50 ng/mL except that Latent TGF-β1 was tested at 200 ng/mL using the LEGENDplex™ Free Active/Total TGF-β1 Assay. No or negligible cross-reactivity was found.

Human:

IL-1α	IL-11	IL-21	CCL5	CXCL9	PDGF-BB
IL-2	IL-12p40	IL-22	CCL11	CXCL10	TGF-α
IL-3	IL-13	IL-23	CCL17	CXCL11	TGF-β2
IL-4	IL-15	IL-27	CCL20	CXCL12	TGF-β3
IL-5	IL-17	IL-33	CCL21	sFASL	TSLP
IL-6	IL-17A/F	CCL2	CCL22	G-CSF	Latent TGF-β1
IL-7	IL-17F	CCL3	CXCL1	IFN-β	
IL-8	IL-18	CCL4	CXCL5	IFN-ω	

Mouse:

IL-1α	IL-10	IL-17E	CCL9	CXCL9	G-CSF
IL-1β	IL-11	IL-21	CCL11	CXCL10	GM-CSF
IL-2	IL-12p40	IL-22	CCL17	CXCL11	ICAM-1
IL-3	IL-12p70	IL-23	CCL20	CXCL12	IFN-β1
IL-4	IL-13	IL-27	CCL22	CXCL13	IFN-γ
IL-5	IL-15	IL-33	CXCL1	EGF	Isthmin-1
IL-6	IL-17A	IL-34	CXCL4	FGF-b	M-CSF
IL-7	IL-17F	CCL8	CXCL5	EPO	MMP-9

Rat:

IL-1α	IL-9	IL-17E	IL-23	IFN-γ	TNF-α
IL-1β	IL-12	IL-17F	IL-33	KC	CX3Cl1
IL-2	IL-13	IL-18	CCL20	MCP-1	EPO
IL-5	IL-17A	IL-22	GM-CSF	RANTES	

Accuracy (Spike Recovery)

For Free Active TGF-β1

For spike recovery in cell culture medium or urine, TGF-β1 protein with known concentrations (625, 156.3, 39.1 pg/mL) was spiked into RPMI and DMEM with 10% FBS or 2 urine samples. The spiked samples were then assayed and the measured concentrations were compared with the expected values.

For spike recovery in serum or plasma, TGF-β1 protein with known concentrations (625, 156.3, 39.1 pg/mL) was spiked into 8 human serum/plasma

samples or 8 pooled serum/plasma samples from 4 different mouse and rat species. The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

Species	Sample type	Cell Culture Medium	Serum	Plasma	Urine
Human	Spike recovery	87%	105%	99%	101%
Mouse		87%	90%	103%	N/A
Rat		87%	64%*	48%*	N/A

*For rat serum and plasma samples, the spike recovery for free active TGF-β1 is low.

For Total TGF-β1

For spike recovery in cell culture medium or urine, TGF-β1 protein with known concentrations (625, 156.3, 39.1 pg/mL) was spiked into 2 treated and diluted samples. The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

For spike recovery in serum or plasma, TGF-β1 protein with known concentrations (625, 156.3, 39.1 pg/mL) was spiked into 6 treated and diluted serum or plasma samples (Samples were treated and diluted to 100 fold with Sample Diluent before spike). The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

Species	Sample type	Cell Culture Medium	Serum	Plasma	Urine
Human	Spike recovery	98%	106%	102%	99%
Mouse		98%	105%	85%	N/A
Rat		98%	81%	78%	N/A

Linearity of Dilution

For Free Active TGF-β1

For linearity of dilution in cell culture medium or urine, samples with high concentrations of free active TGF-β1 were serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed. The measured concentrations of serially diluted samples were then compared with that of undiluted samples.

For linearity of dilution in serum or plasma, samples with high concentrations of free active TGF-β1 were serially diluted 1:2, 1:4, 1:8 with Matrix and assayed. The measured concentrations of serially diluted samples were then compared with the concentration of the original samples.

Species	Sample type	Cell Culture Medium	Serum	Plasma	Urine
Human	Linearity of Dilution	105%	97%	114%	84%
Mouse		105%	114%	156%*	N/A
Rat		105%	104%	165%*	N/A

***** For mouse and rat plasma samples, the linearity of dilution for free active TGF-β1 is not optimal. However, levels of free active TGF-β1 in samples are low and further sample dilution is usually not required.

For Total TGF-β1

For linearity of dilution in cell culture medium or urine, samples were first treated, then serially diluted 1:2, 1:4, 1:8 with Sample Diluent and assayed. The measured concentrations of serially diluted samples were then compared with that of the treated, undiluted samples.

For linearity of dilution in serum or plasma, samples were first treated to release free active TGF-β1, diluted 50-fold with Sample Diluent, then further serially diluted 1:2, 1:4, 1:8 with Sample Diluent and assayed. The measured concentrations of serially diluted samples were then compared with that of the treated, 50-fold diluted samples

Species	Sample type	Cell Culture Medium	Serum	Plasma	Urine
Human	Linearity of Dilution	80%	96%	87%	86%
Mouse		80%	94%	88%	N/A
Rat		80%	105%	92%	N/A

Intra-Assay Precision

Two samples with different concentrations of target proteins were analyzed

in one assay with 16 replicates for each sample. The intra-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
TGF-β1	Sample 1	35.82	1.55	4%
	Sample 2	578.02	27.00	5%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in three independent assays with duplicates for each sample. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
TGF-β1	Sample 1	36.71	1.96	5%
	Sample 2	595.07	38.17	6%

Biological Samples

Human serum, plasam and urine samples were tested for free active or Total TGF-β1. The concentrations measured are shown below (after mulitplying dilution factors).

Sample type	Serum		Plasma		Urine	
Analyte	Free Active TGF-β1 (pg/mL)	Total TGF-β1 (ng/mL)	Free Active TGF-β1 (pg/mL)	Total TGF-β1 (ng/mL)	Free Active TGF-β1 (pg/mL)	Total TGF-β1 (ng/mL)
# of samples	35	16	16	8	2	2
Mean	55.2	32.1	5.1	9.0	13.41	1.76
Minimum	3.1	10.6	ND	4.1	n/a	n/a
Maximum	127.0	93.0	18.6	16.2	n/a	n/a
% Detected	100%	100%	87.5%	100%	100%	100%

Mouse/rat serum and plasma samples from different species were tested for free active or total TGF-β1. The concentrations measured are shown below (after mulitplying dilution factors).

Sample Type	Serum		Plasma
Species	**Free Active TGF-β1 (pg/mL)**	**Total TGF-β1 (ng/mL)**	**Total TGF-β1 (ng/mL)**
Balb/C	243.2	41.1	41.1
C57/B6	285.2	36.8	36.8
Swiss Webster	260.1	42.3	
CD1	161.5		
Hannover	173.6	29.2	29.2
S.P	210.0	32.6	32.6
Fisher	90.2	22.1	
Long Evans	177.0		

TROUBLESHOOTING

Problem	Possible Cause	Solution
Bead population shifting upward or downward during acquisition	The strong PE signal from high concentration samples or standards may spill over to classification Channel (e.g., FL3/FL4/APC) and mess up the bead separation.	Optimize instrument settings using Kit Setup Beads, and make appropriate compensation between channels.
Filter plate will not vacuum or some wells clogged	Vacuum pressure is insufficient or vacuum manifold does not seal properly.	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Clean the vacuum manifold and make sure no debris on the manifold. Press down the plate on the manifold to make a good seal.
	Samples have insoluble particles or sample is too viscous (e.g., serum and plasma samples)	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous. If some wells are still clogged during washing, try the following: 1). Add buffer to all the wells, pipette up and down the clogged wells and vacuum again. 2). Use a piece of clean wipe, wipe the under side of the clogged wells and vacuum again. 3). Take a thin needle (e.g., insulin needle), while holding the plate upward, poke the little hole under each of the clogged wells and vacuum again. Do not poke too hard or too deep as it may damage the filter and cause leaking.
	Filter plate was used without pre-wet.	Pre-wet plate with wash buffer before running the assay.

Insufficient bead count or slow reading	Beads inappropriately prepared	Sonicate bead vials and vortex just prior to addition. Agitate mixed beads intermittently in reservoir while pipetting this into the plate.
	Samples cause beads aggregation due to particulate matter or viscosity	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
	Beads were lost during washing for in-tube assay	Make sure beads are spun down by visually check the pellet (beads are in light blue or blue color). Be very careful when removing supernatant during washing.
	Probe might be partially clogged	Sample probe may need to be cleaned, or if needed, probe should be removed and sonicated.
Plate leaked	Vacuum pressure set too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Do not exceed 10” Hg of vacuum.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Liquid present on the under side of the plate after vacuum	After washing, press down plate firmly on a stack of clean paper towels to dry the underside of the plate.
	Pipette touching and damaged plate filter during additions	Pipette to the side of wells.
High Background	Background wells were contaminated	Avoid cross-well contamination by changing tips between pipetting when performing the assay using a multichannel pipette.
	Insufficient washes	The background may be due to non-specific binding of SA-PE. Increase number of washes.
Debris (FSC/SSC) during sample acquisition	Debris or platelet may exist in sample solution	Centrifuge samples before analyzing samples. Remove platelet as much as possible.

Variation between duplicate samples	Beads aggregation	Sonicate and vortex the Beads prior to use.
	Multichannel pipette may not be calibrated or inconsistent pipetting	Calibrate Pipette. Ensure good pipetting practice. Prime pipette before use may help.
	Plate washing was not uniform	Make sure all reagents are vacuumed out completely in all wash steps.
	Samples may contain particulate matters.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
Low or poor standard curve signal	The standard was incorrectly reconstituted, stored or diluted	Follow the protocol to reconstitute, store and dilute standard. Double check your calculation.
	Wrong or short incubation time	Ensure the time of all incubations was appropriate.
Signals too high, standard curves saturated	PMT value for FL2/PE set too high	Make sure the PMT setting for the reporter channel is appropriate
	Plate incubation time was too long	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	Make sure the experiment to generate the samples worked. Use proper positive controls.
	Samples concentrations higher than highest standard point.	Dilute samples and analyze again.
	Standard curve was saturated at higher end of curve.	Make sure the PMT setting for the reporter channel is appropriate. Use shorter incubation time if incubation time was too long
Missed beads populations during reading, or distribution is unequal	Sample may cause some beads to aggregate.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
	Beads populations are not mixed properly	Make sure all bead populations are mixed. and in similar numbers.

PLATE MAP (for in-plate assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A	C0	C4	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
B	C0	C4	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
C	C1	C5	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
D	C1	C5	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
E	C2	C6	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
F	C2	C6	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
G	C3	C7	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40
H	C3	C7	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40

Tel: 858-768-5800

BioLegend®

LEGENDplex™ Kits are manufactured by **BioLegend**
8999 BioLegend Way
San Diego, CA 92121
Tel: 1.858.768.5800
Tel: US & Canada Toll-Free: 1.877.Bio-Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
biolegend.com

For a complete list of world-wide BioLegend offices and distributors,
please visit our website at: biolegend.com

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