arigoPLEX® Rat Fibrotic Marker Multiplex ELISA Kit (TGF beta 1, PDGF BB, IL6, TNF alpha)

arigoPLEX® Rat Fibrotic Marker Multiplex ELISA Kit (TGF beta 1, PDGF BB, IL6, TNF alpha) is an Enzyme Immunoassay kit for the quantification of TGF beta 1, PDGF BB, IL6, TNF alpha in serum, plasma and cell culture supernatant.

Catalog number: ARG83356

Package: 96 wells

For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION **Page**

INTRODUCTION 3
PRINCIPLE OF THE ASSAY 5
MATERIALS PROVIDED & STORAGE INFORMATION 6
MATERIALS REQUIRED BUT NOT PROVIDED 7
TECHNICAL NOTES AND PRECAUTIONS 7
SAMPLE COLLECTION & STORAGE INFORMATION...................... 8
REAGENT PREPARATION.. 10
ASSAY PROCEDURE... 12
EXAMPLE OF TYPICAL STANDARD VALUES 14
CALCULATION OF RESULTS 16

MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: Address: 9F.-7, No. 12, Taiyuan 2nd St., Zhubei City,

Hsinchu County 302082 , Taiwan (R.O.C.)

Phone: +886 (3) 621 8100

Fax: +886 (3) 553 0266

Email: info@arigobio.com

INTRODUCTION

Fibrosis is an important cause of global morbidity and mortality. Fibrosis, which is defined as the excessive accumulation of fibrous connective tissue, is a common pathological condition resulting from a dysregulated tissue repair response, most notably during chronic inflammatory disorders. Common diseases associated with fibrosis include hepatitis virus, nonalcoholic fatty liver disease (NAFLD), chronic kidney diseases, idiopathic pulmonary fibrosis (IPF), pneumonoconiosis, and cystic fibrosis.

Transforming growth factor-β1 (TGF-β1) is the key pro-fibrotic cytokine in most fibrosis pathways. TGF-β is secreted from cells in a latent tripartite complex consisting of its dimeric inactive form (named latency-associated peptide (LAP)) plus a latent TGF-β/LAP/LTBP inactive complex binds to the extracellular matrix (ECM) components. After cleavage by various proteases, the active form of TGF-β is released and is able to bind to TGFβR1 and TGFβR2 receptors. Two signaling pathways, known as Smad-mediated canonical and non-canonical, are activated by TGF-βs to regulate fibrotic evolution. Although fibroblasts are major sources of targets of TGF-β, some fibrogenic transformations reflect the activation of other cell types such as macrophages and epithelial cells.

Platelet-derived growth factor (PDGF) is identified as a growth factor in serum that stimulated proliferation of fibroblasts and vascular smooth muscle cells. Increased PDGF signaling has been associated with major human diseases including cancer, atherosclerosis, and fibrosis. PDGF-BB, a potent mitogen factor for dermal fibroblasts, which is majorly produced by lesional macrophages in fibrotic skin. PDGF-BB was previously found to cause increase inflammation, dermal thickening and collagen deposition in bleomycin mouse

model for skin fibrosis. Additionally, PDGF-BB, in mouse models of liver fibrosis, showed an increase in the liver as more platelets were recruited to the sinusoidal endothelium. In fibroblast-like synoviocytes, PDGF-BB acts in concert with TGF-β to potentiate TNF-α-induced release of pro-inflammatory cytokines.

Interleukin-6 (IL-6) is a multifunctional cytokine that is involved a variety of biological processes, including inflammation, immunological response, and hematopoiesis. IL-6 has been implicated in numerous fibrotic diseases, including lung fibrosis, renal fibrosis, dermal fibrosis and ocular fibrosis. IL-6 was shown to be upregulated in IPF patients and animal models of pulmonary fibrosis. Furthermore, epithelial-mesenchymal transition (EMT) plays a crucial role in embryonic development and wound healing and contributes pathologically to fibrosis and cancer progression. EMT can be induced by IL-6 in fibrotic diseases, in which epithelial cells lose their epithelial phenotypes and gain mesenchymal phenotypes with increased migration and invasion capacities. In peritoneal fibrosis, IL-6 stimulates EMT.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that is released in response to injury and inflammation and can stimulate ECM component production as well as promote cell proliferation and cell migration. Besides its modulation effect on ECM production, TNF-α has been considered a mediator of cell injuries in liver caused by alcoholism, reperfusion, graft rejection and endotoxic insult. TNF-α has been implicated in the pathogenesis of chronic liver inflammation that leads to liver fibrosis. TNF-α enhances hepatic stellate cell (HSC) survival, hepatocyte death, and immune cell activation, which are associated with enhanced liver fibrosis. Activated Kupffer cells, T-cells and

damaged hepatocytes also release the inflammatory cytokines (TNF-α and IL-6) which are closely related to fibrogenesis and could be used as biomarkers for liver fibrosis.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to Rat TGF beta 1, PDGF BB, IL6, TNF alpha has been pre-coated onto a microtiter plate. Standards Mixture and samples are pipetted into the wells and any target cytokines present is bound by the immobilized specific antibodies. After incubation, added mixed Antibody-Conjugate to each well and incubate. After washing away any unbound substances, the HRP-Streptavidin Solution is added to the wells and incubated. The wells are thoroughly washed to remove all unbound HRP-Streptavidin Solution. A TMB substrate is added to the wells and color develops in proportion to the amount of target cytokines in the initial step. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of O.D. 450 nm. The concentration of Rat Fibrotic Marker in the samples is then determined by comparing the O.D. 450 nm of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Upon received, store 10X Antibody Conjugate Mixture at ≤-20°C.

Store other components at 2-8°C at all times. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody Coated Microplate [1]	8 X 12 strips	4°C
Standards Mixture [2]	3 vials (lyophilized)	4°C
10X Antibody Conjugate Mixture	1.2 mL	≤ -20°C
40X HRP-Streptavidin Solution	300 µL	4°C
Standard / Sample Diluent Buffer	35 mL (ready to use)	4°C
Antibody Diluent Buffer	30 mL (ready to use)	4°C
10X Wash Buffer	60 mL	4°C
TMB substrate	12 mL (ready to use)	4°C (protect from light)
STOP solution	12 mL (ready to use)	4°C
Plate sealer	4 adhesive strips	RT

Note:

1. The Antibody Coated microplate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different antibody specific to one of the 4 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1
B	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB
C	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
D	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α
E	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1	TGF-β1
F	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB	PDGF-BB
G	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
H	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α	TNF-α

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: TGF beta 1: 2000 pg; PDGF BB: 2000 pg; IL6: 4000 pg; TNF alpha: 2000 pg.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm
- Deionized or distilled water
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Microtiter plate washer

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon received, store 10X Antibody Conjugate at ≤ -20°C. Store other component at 2-8°C at all times.
- Prior to beginning the assay procedure, bring all reagents and required number of strips to room temperature (20-25°C).
- Unused wells must be stored at 2-8 °C in the sealed foil pouch and used in the frame provided.
- All reagents must be mixed without foaming and briefly spin down the all vials before use.
- If crystals are observed in the 10X Wash Buffer warm to 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.

- Ensure complete reconstitution and dilution of reagents prior to use.
- Take care not to contaminate the mixed TMB Substrate. Do not expose the mixed TMB solution to glass, foil or metal. If the solution is blue before use, DO NOT USE IT.
- All reagents must be mixed without foaming before use.
- Change pipette tips between the addition of different reagent or samples.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.

SAMPLE COLLECTION & STORAGE INFORMATION

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at 4°C and aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at -20°C up to 1 month or -80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Note:

1. Do not use haemolytic, icteric or lipaemic specimens.
2. Samples containing sodium azide should not be used in the assay.
3. Avoid disturbing the white buffy layer when collection serum / plasma sample.
4. To obtain the data of each cytokine, **≥ 0.2 mL** of the sample is needed to complete one run of the assay. It is recommended to have a larger volume available in case that the experiments have to be repeated.

TGF beta 1 Sample activation:

Cell Culture Supernatants

a. Add 20 µl 1N HCl with 100 µl Cell Culture Supernatant samples.
b. Mix well and incubate for 10 min at room temperature.
c. Neutralize by adding 20 µl 1.2N NaOH / 0.5M HEPES, mix well.
d. Final predilution: 1:1.4.

Serum & Plasma

a. Add 10 µl 1N HCl with 40 µl Serum/Plasma samples
b. Mix well and incubate for 10 min at room temperature.
c. Neutralize by adding 10 µl 1.2N NaOH / 0.5M HEPES , mix well.
d. Dilute the activated sample 60-fold with Standard/Sample Diluent buffer.
e. Final predilution: 1:90.

REAGENT PREPARATION

- 1X Wash Buffer: Dilute **10X** Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 50 mL of 10X Wash Buffer into 450 mL of distilled water to a final volume of 500 mL) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.
- 1X Antibody Conjugate Mixture: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 10X Antibody Conjugate Mixture concentrate into **Antibody Diluent Buffer** to yield 1X detection antibody solution (e.g. 12 µl of 10X Antibody Conjugate Mixture concentrate + 108 µl of Antibody Diluent Buffer).
- 1X HRP-Streptavidin Solution: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 40X HRP-Streptavidin Solution into **Antibody Diluent Buffer** to yield 1X HRP-Streptavidin Solution buffer (e.g. 10 µl of 40X HRP-Streptavidin Solution +390 µl of Antibody Diluent Buffer).
- Sample: Diluent serum and plasma samples with equal volume of **Standard / Sample Diluent Buffer** before assay (1:1, dilution factor=2). If the initial assay found samples contain proteins higher than the highest standard, the samples can be diluted with Standard / Sample Diluent Buffer and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account. Cell culture supernatants could be assayed directly **(It is recommended to do pre-test to determine the suitable dilution factor)**.

- Standards Mixture:

A. Add **1 mL** of **Standard / Sample Diluent Buffer** to reconstitute the lyophilized Standard Mixture to obtain the high concentration stock of 4 cytokines at different concentrations (see table below). Brief vortex and allow the stock standard to sit for at least 15 minutes with gentle agitation to make sure the standard is dissolved completely before making serial dilutions.

B. Use the above high concentration Standards Mixture and a 32-fold diluted low concentration Standards Mixture to test together with up to 22 test samples. If more accurate results are required, a two-fold serial dilution with Dilution Buffer can generate a more accurate standard curve. However, the number of test samples will be reduced.

The concentrations of the 4 cytokines in different dilutions of the Standards Mixture are listed as below:

Cytokines (pg / mL)	High Conc. Standard Stock	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
TGF beta 1	2000	1000	500	250	125	62.5	31.3
PDGF BB	2000	1000	500	250	125	62.5	31.3
IL6	4000	2000	1000	500	250	125	62.5
TNF alpha	2000	1000	500	250	125	62.5	31.3

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use.

1. Add **100 µL** of the **Standards Mixture** and **diluted samples** in the wells of the Antibody Coated microplate.

   Note: To obtain the approximate concentrations of 4 cytokines on 22 test samples, the low concentration standard mixture (**S1**, 1:32 from high concentration mixture), the high concentration Standards Mixture (**S2**) and test samples (T1 to T22) can be added as the scheme below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
H	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22

2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 4 times for a **total 5 washes**. Wash by filling each well with **1X Wash Buffer (300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Keep the wash buffer in the wells for 30-60 sec before remove. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
4. Add **100 µL** of **1X Antibody Conjugate Mixture** to each well.
5. Cover the plate and incubate for **1 hour** at **room temperature**.

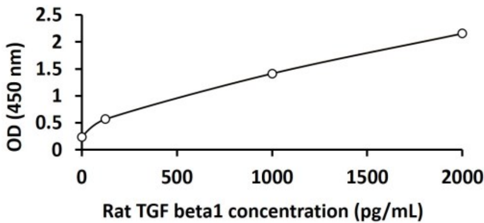
6. Aspirate each well and **wash as step 3**.
7. Add **100 µL** of **1X HRP-Streptavidin Solution** to each well. Cover the plate and incubate for **1 hour** at **room temperature**.
8. Aspirate each well and **wash as step 3**.
9. Add **100 µL** of **TMB Substrate** to each well. Cover and incubate for **10-20 minutes** at **room temperature** in the dark.

   (Note: The incubation time is for reference only, the optimal incubation time should be determined by end user. And the shades of blue color can be seen in the wells with the four highest concentrated standards)
10. Add **100 µL** of **Stop Solution** to each well, gently tap the plate to mix well. The color of the solution should change from blue to yellow. Gently tap the plate to ensure thorough mixing.
11. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance **within 5 minutes** after adding the stop solution.

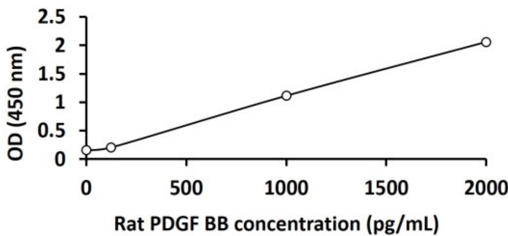
EXAMPLE OF TYPICAL STANDARD VALUES

The following table shows the OD readings of a run of this multiplex ELISA with two fold-serial diluted standards. It is for demonstration purpose only and cannot be used to replace the standard curve for testing. For each investigation, standards have to be assayed along with test samples and only the curve generated from the same test can be used.

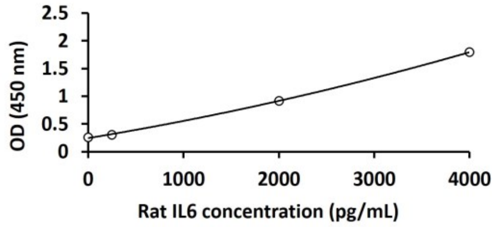
Example of Rat TGF beta1 standard curve



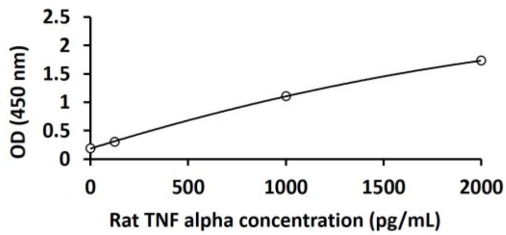
Example of Rat PDGF-BB standard curve



Example of Rat IL6 standard curve



Example of Rat TNF alpha standard curve



CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. The 4 curves for 4 targets can be generated from OD readings of high concentration standard and low concentration standard mixture. The standard curves might not be perfectly straight. To obtain more accurate results, more dilution points can be used when generating standard curves.
3. 4 Parameter Logistics is the preferred method for the result calculation. Other data reduction functions may give slightly different results.
4. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
5. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure.