GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Block Buffer to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

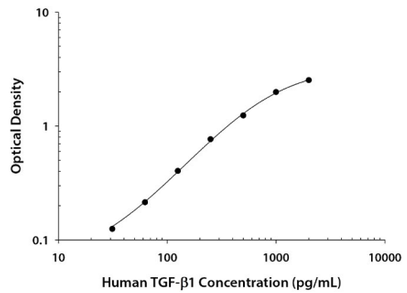
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human TGF-β1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.



SPECIFICITY

The factors below were assayed and exhibited the following cross-reactivity:

Factor	Concentration Tested (pg/mL)	Observed Value (pg/mL)	% Cross-reactivity
pTGF-β2	50,000	145	0.3%
rhTGF-β1.2	2500	1421	57%
rhTGF-β2	50,000	74	0.15%
rhTGF-β3	50,000	481	0.96%
raTGF-β5	50,000	904	1.8%

TECHNICAL HINTS & LIMITATIONS

- We recommend the use of R&D Systems' Reagent Diluent Concentrate 1 (Catalog # DY997) to prepare Reagent Diluent for use in this kit.
- It is important that the Reagent Diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured.
- Avoid microbial contamination of reagents and buffers.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is recommended that all standards and samples be assayed in duplicate.

TROUBLESHOOTING

Note: *For more detailed troubleshooting, please visit:*
www.RnDSystems.com/ELISADevelopment

Poor Standard Curve

- Improper reconstitution and/or storage of standard.
- Improper dilution of highest standard and standard curve.
- Incomplete washing and/or aspiration of wells.
- Unequal volumes added to wells/pipetting error.
- Incorrect incubation times or temperatures.

Poor Precision

- Unequal volumes added to wells/pipetting error.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

Low or No Color Development

- Inadequate volume of substrate added to wells.
- Incorrect incubation times or temperatures.

DuoSet® ELISA DEVELOPMENT SYSTEM

Human TGF-β1

Catalog Number: DY240-05 (5 plates)

INTENDED USE

For the development of sandwich ELISAs to measure natural and recombinant human Transforming Growth Factor beta 1 (TGF-β1). The Reagent Diluent recommended may be suitable for most cell culture supernate, serum, and plasma samples. The Reagent Diluent selected for use can alter the performance of an immunoassay. Reagent Diluent optimization for samples with complex matrices such as serum and plasma, may improve their performance in this assay.

This kit contains sufficient materials to run ELISAs on at least five 96 well plates, provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

This package insert must be read in its entirety before using this product.
Refer to insert provided in this kit for component concentrations as they may vary.
For research use only. Not for use in diagnostic procedures.

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MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human TGF-β1 Capture Antibody	840116	1 vial	Store at 2-8 °C for up to 8 weeks or aliquot and store at -20 °C to -70 °C in a manual defrost freezer for up to 12 weeks.*
Human TGF-β1 Detection Antibody	840117	1 vial	
Human TGF-β1 Standard	840118	1 vial	Store at 2-8 °C for up to 4 weeks or aliquot and store at -70 °C for up to 12 weeks.*
Streptavidin-HRP	893975	1 vial	Store at 2-8 °C for up to 12 weeks. **DO NOT FREEZE.**

* Provided this is within the expiration date of the kit.

OTHER MATERIALS & SOLUTIONS REQUIRED

DuoSet Ancillary Reagent Kit 1 (5 plates):
(R&D Systems, Catalog # DY007) containing 96 well microplates, plate sealers, substrate solution, stop solution, plate coating buffer (PBS), block buffer, wash buffer, and Reagent Diluent Concentrate 1.

The components listed above may be purchased separately:

96 well microplates: (R&D Systems, Catalog # DY990).

Plate sealers: (R&D Systems, Catalog # DY992).

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006).

Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer: 5% Tween 20 in PBS, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalog # DY004).

Reagent Diluent: Add 1.4 mL of Reagent Diluent Concentrate 1 (R&D Systems, Catalog # DY997) to 98.6 mL of 0.05% Tween 20 in PBS, pH 7.2-7.4, 0.2 µm filtered.

Substrate Solution: 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution: 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

CALIBRATION

This DuoSet is calibrated against a highly purified CHO cell-expressed recombinant human TGF-β1 produced at R&D Systems.

PRECAUTIONS

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

The Stop Solution suggested for use with this kit is an acid solution.

The Color Reagent B suggested for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

REAGENT PREPARATION

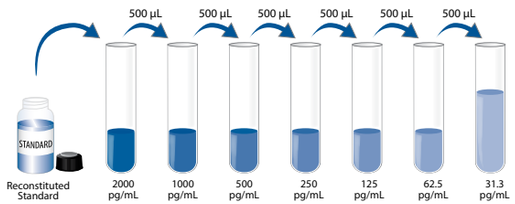
Bring all reagents to room temperature before use. Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately.

Streptavidin-HRP: 2.0 mL of streptavidin conjugated to horseradish-peroxidase. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Human TGF-β1 Capture Antibody: 240 µg/mL of mouse anti-TGF-β1 antibody when reconstituted with 0.5 mL of PBS. Dilute to a working concentration of 2.0 µg/mL in PBS, without carrier protein.

Human TGF-β1 Detection Antibody: 18 µg/mL of biotinylated chicken anti-human TGF-β1 antibody when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 300 ng/mL in Reagent Diluent.

Human TGF-β1 Standard: 220 ng/mL of recombinant human TGF-β1 when reconstituted with 0.5 mL of Reagent Diluent. A seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 2000 pg/mL is recommended. Prepare 1000 µL of high standard per plate assayed.



ACTIVATION REAGENT PREPARATION

To activate latent TGF-β1 to the immunoreactive form, prepare the following solutions for acid activation and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month.

Caution: *Wear protective clothing and safety glasses during preparation or use of these reagents.*

1 N HCl (100 mL) - To 91.67 mL of deionized water, slowly add 8.33 mL of 12 N HCl. Mix well.

1.2 N NaOH/0.5 M HEPES (100 mL) - To 75 mL of deionized water, slowly add 12 mL of 10 N NaOH. Mix well. Add 11.9 g of HEPES. Mix well. Bring final volume to 100 mL with deionized water.

TGF-β1 SAMPLE ACTIVATION

To activate latent TGF-β1 to immunoreactive TGF-β1, follow the activation procedure outlined below. Assay samples after neutralization (pH 7.2-7.6). **Use polypropylene test tubes.**

Note: *Do not activate the kit standards. The kit standards contain active recombinant TGF-β1.*

CELL CULTURE SUPERNATE ASSAY	SERUM/PLASMA ASSAY
To 100 µL of cell culture supernate, add 20 µL of 1 N HCl.	To 40 µL serum/plasma, add 20 µL of 1 N HCl.
Mix well.	Mix Well
Incubate 10 minutes at RT.	Incubate 10 minutes at RT.
Neutralize the acidified sample by adding 20 µL of 1.2 N NaOH/0.5 M HEPES.	Neutralize the acidified sample by adding 20 µL of 1.2 N NaOH/0.5 M HEPES.
Mix well.	Mix well.
Assay Immediately.	Prior to assay, dilute the activated sample 20-fold with Reagent Diluent.*
The concentration read off the standard curve must be multiplied by the dilution factor, 1.4.	The concentration read off the standard curve must be multiplied by the appropriate dilution factor.

*A suggested 20-fold dilution is 10 µL of activated sample + 190 µL of Reagent Diluent. (Final dilution factor is 40).

Note: *For each new lot of activation reagents, measure the pH of several representative samples after neutralization to ensure that it is within pH 7.2-7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.*

Cell Culture Supernates Note
Significant levels of latent TGF-β1 are found in bovine, porcine, equine, and caprine sera. The reported levels of TGF-β1 in bovine and fetal bovine sera can be as high as 16 ng/mL after activation (1, 2). Therefore, conditioned medium containing 10% fetal bovine serum can be expected to have a TGF-β1 concentration of about 1600 pg/mL. The background level of TGF-β1 in control medium can be determined and subtracted from samples of conditioned medium. As an alternative, the background level of TGF-β1 in medium can be lowered using the following procedure prior to assaying (reference 1 with modifications). After growth to confluence in medium containing 10% serum, the medium is changed to serum-free medium supplemented with 200 µg/mL crystalline BSA with four changes of medium over 12-24 hours. Cells are then switched to medium alone or medium containing 200 µg/mL crystalline BSA. Particular cell lines may require specific additions to the serum-free medium for maintenance. After 24 hours, the serum-free conditioned medium is clarified by centrifugation and samples are stored at ≤ -70 °C. Optionally, 2 µg/mL aprotinin, leupeptin, pepstatin A, and 120 µg/mL PMSF can be added before freezing. Thawed or fresh samples of serum-free or serum-containing conditioned medium are processed further as described above. If bovine serum added as a supplement to conditioned media exceeds 5%, further dilute the activated sample at least 2-fold using Reagent Diluent. The dilution as a result of the sample activation procedure (1.4-fold) should be taken into consideration in the final concentration of TGF-β1 in culture media samples.

Plasma Note
TGF-β1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of TGF-β1, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

REFERENCES

1. Danielpour, D. *et al.* (1989) Growth Factors **2**:61.
2. Danielpour, D. *et al.* (1993) J. Immunol. Meth. **158**:17.