

TGFβ₂ E_{max}[®] ImmunoAssay System



Technical Bulletin No. 224

INSTRUCTIONS FOR USE OF PRODUCT G7600. PLEASE DISCARD PREVIOUS VERSIONS.

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I. Description

The TGFβ₂ E_{max}[®] ImmunoAssay System is designed for the sensitive and specific detection of biologically active Transforming Growth Factor β₂ (TGFβ₂) in an antibody sandwich format (1) (Figure 1). In this format, flat-bottom 96 well plates are coated with TGFβ Coat mAb, which binds soluble TGFβ₂. A second antibody, Anti-TGFβ₂ pAb, is added to complete the sandwich. After washing, antibody conjugate (horseradish peroxidase, TGFβ HRP) is added and binds the sandwich complex. Finally, the chromogenic substrate 3,3',5,5'-tetramethyl benzidine (TMB) is added. The amount of specifically bound TGFβ₂ in the sample is proportional to the color generated in the coupled oxidation-reduction reaction and is quantitated against a standard curve generated with known amounts of TGFβ₂. Using this system, biologically active TGFβ₂ in media, plasma, serum or urine can be quantitated in the range of 32–1,000pg/ml.



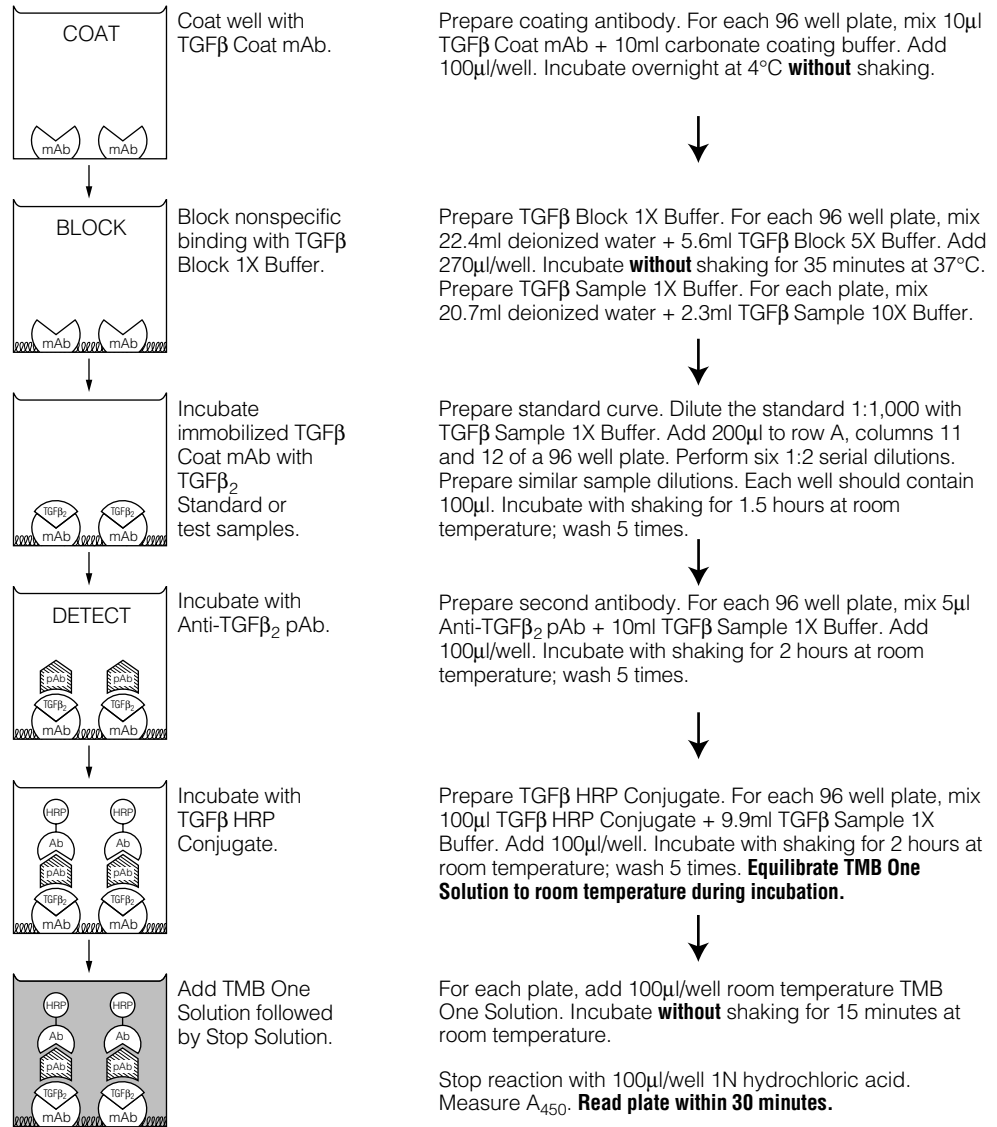


Figure 1. Schematic representation of the TGFβ₂ E_{max}[®] ImmunoAssay System. For a detailed protocol, or when using this system for the first time, please read Sections III through VI carefully.

The TGFβ₂ E_{max}[®] ImmunoAssay System offers several benefits:

- **Specificity:** Specific detection of TGFβ₂; ≤ 3% cross-reactivity with TGFβ₁ and TGFβ₃ at 10ng/ml.
- **Sensitivity:** Detects a minimum of 32pg/ml of TGFβ₂.
- **Flexibility:** ELISA plates can be set up in any desired configuration.
- **High Value:** Optimized reagents and protocol.

II. Product Components

Product	Size	Cat.#
TGFβ ₂ E _{max} [®] ImmunoAssay System	5 x 96 wells	G7600

Each system contains sufficient reagents for 400 determinations (plates not included) plus standard curves. Includes:

- 50μl TGFβ Coat mAb
- 28ml TGFβ Block 5X Buffer
- 20ml TGFβ Sample 10X Buffer
- 45μl TGFβ₂ Standard, 1μg/ml
- 25μl Anti-TGFβ₂ pAb
- 500μl TGFβ HRP Conjugate
- 2 x 25ml TMB One Solution
- 1 Protocol

Storage Conditions: Store the entire system in its original package protected from light at -20°C, where all components are stable for six months from the date of purchase. Once thawed, store at 4°C, where the system is stable for three months. Return each component to 4°C immediately after use. Avoid refreezing reagents. After dilution, use reagents the same day.

Note: The TGFβ₂ Standard supplied with this system is Natural Porcine TGFβ₂, which is 99% homologous to human TGFβ₂ and 97% homologous to mouse TGFβ₂.

III. General Considerations

The TGFβ₂ E_{max}[®] ImmunoAssay System has been tested using the following protocols. Plate coating requires an overnight incubation. The subsequent steps require approximately 7 hours to complete and should be performed on the following day. When transferring the TGFβ₂ Standard and experimental samples to the plate, take care not to disturb or scratch the surface of the wells, as this may dislodge coated antibodies. If unfamiliar with the technique, practice the pipetting procedures on a trial run.

A. Sample Preparation

The TGFβ₂ E_{max}[®] ImmunoAssay System may be used to quantitate TGFβ₂ in tissue culture supernatants, plasma, serum or urine. Collect plasma samples in EDTA rather than citrate, since citrate may interfere with the assay. Store experimental samples frozen at -20°C before use. Avoid multiple freeze-thaw cycles. Remove particulates from samples by centrifugation before use in the assay.


Due to endogenous TGFβ₂ and binding proteins in serum and plasma (see Table 1), we recommend a starting dilution of:

1:16 (in TGFβ Sample 1X Buffer) for samples containing 10% fetal bovine serum (e.g., conditioned medium), or


1:150 (in TGFβ Sample 1X Buffer) for human serum and plasma samples.

Table 1. Endogenous Levels of TGFβ₂ in Sera from Various Species.

Source	ng/ml	Source	ng/ml
bovine, adult	2.0	horse	60.0
bovine, fetal	1.5	mouse	5.0
chicken	1.2	pig	66.0
goat	4.0	sheep	5.0



DO NOT
attempt to acid treat the
TGF β ₂ Standard.



CAUTION:
HCl and NaOH are caus-
tic. Avoid contact with skin
or eyes.

B. Color Development

Allow the color development reaction to proceed for 15 minutes at ambient temperature. We recommend stopping the assay in the same sequence in which the TMB One Solution was added.

C. Limitations of the Assay

- For research use only. Not for use in diagnostic procedures.
- Absorbance values beyond the range of the standard curve are not valid.
- For consistent results, dilute samples using the prepared TGF β Sample 1X Buffer (TGF β Sample 10X Buffer is provided).
- Plasma samples containing citrate may interfere with the assay.

IV. Sample Preparation

TGF β ₂, in vivo, is processed from a latent form to the bioactive form of the protein. Only the bioactive form is immunoreactive and detectable by Anti-TGF β ₂ mAb and pAb. This processing can be performed in vitro by acid treatment.

Note: This assay is designed to measure biologically active TGF β ₂. **To measure the amount of naturally processed TGF β ₂ in your samples, proceed directly to the ELISA protocol in Section V.A without acid treatment.** To assay for total TGF β ₂, perform the acid treatment procedure and then neutralize the samples as described before proceeding with the ELISA protocol.

The TGF β ₂ Standard supplied with the system has been acid treated and will be neutralized upon dilution in TGF β Sample 1X Buffer.

Acid Treatment Procedure

This procedure acidifies samples diluted 1:5 in Dulbecco's PBS (DBPS) to approximately pH 2.6 (Step 2) and then neutralizes them to approximately pH 7.6 (Step 4). Depending on how much carrier protein the samples contain, additional dilutions may or may not require the use of the TGF β Sample 1X Buffer (which contains a proprietary carrier protein) to minimize loss of TGF β ₂.

For urine and other low protein matrices, we recommend direct acid treatment to a pH of 2.0–3.0 for 15–20 minutes. Following neutralization with NaOH, subsequent dilutions, if necessary, should be done with TGF β Sample 1X Buffer before adding samples to your assay plate.

For all matrices, verify that the pH is 3.0 or lower. In animal sera, the amount of 1N HCl to lower the pH will vary depending upon the species. We suggest adding 110–125 μ l of 1N HCl per milliliter of serum or plasma and checking the pH before adding additional amounts of acid. Samples can be acid treated in advance and stored at –20°C or –70°C.

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- DPBS
- 1N HCl, reagent grade
- 1N NaOH, reagent grade

Acid Treatment Procedure (continued)

1. Dilute the sample by adding 4 volumes of DPBS.
2. Add 1 μ l of 1N HCl for each 50 μ l of diluted sample. Verify that the pH is 3.0 or lower.
3. Mix and incubate for 15 minutes at room temperature.
4. Neutralize by adding 1 μ l of 1N NaOH per 50 μ l of sample. Check the pH to ensure that it is approximately 7.6.

V. Protocol for TGF β ₂ Quantitation

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- 96 well (flat bottom) ELISA plate
- carbonate coating buffer
- plate sealer
- TBST wash buffer
- 1N hydrochloric acid
- microplate reader capable of monitoring absorbance at 450nm
- pipettors capable of accurately delivering volumes of 1 μ l–1ml
- multichannel pipettor
- wash bottle or automated plate washer (DYNEX UltraWash Plus or equivalent)
- plate shaker (DYNEX Micro-Shaker[®] II or equivalent)
- 50ml (for better mixing) or 15ml polypropylene tubes for dilutions
- 37 $^{\circ}$ C incubator

A. Plate Preparation

1. Per 96 well plate, prepare the coating antibody solution by adding 10 μ l of the TGF β Coat mAb to 10ml of carbonate coating buffer in a 15ml or 50ml polypropylene tube. **Mix thoroughly**, but avoid creating excess bubbles. Use a multichannel pipettor to add 100 μ l of the coating antibody solution to each well of a polystyrene ELISA plate.
2. Seal the wells with a plate sealer and incubate overnight at 4 $^{\circ}$ C.

B. Preparing TGF β Blocking Buffer

Each 96 well plate requires approximately 28ml of TGF β Block 1X Buffer to be used on the second day. Per 96 well plate, combine 22.4ml of deionized water and 5.6ml of the TGF β Block 5X Buffer in a 50ml polypropylene tube. Use a sterile pipette when aspirating from the stock solutions. Mix gently and completely by inversion prior to use.

C. Blocking the Plate

1. Remove the coated plate from the refrigerator and allow it to warm to room temperature (approximately 15 minutes). Over a sink, flick out the contents of the wells and slap the plate upside down 3 times on a paper towel to help clear the wells. Add 270 μ l of TGF β Block 1X Buffer to each well using a multichannel pipettor. Do not touch or scratch the surface of the wells where antibody has bound to the plate.
2. Seal the wells with a 96 well plate sealer and incubate at 37 $^{\circ}$ C for 35 minutes without shaking.

Note: This assay has been tested using Corning Costar[®] (Cat.# 3590), Nunc MaxiSorp[™] (Cat.# 439454) and DYNEX Immulon[®]-4 (Cat.# 011-010-3855) plates. There are no observable differences in the performances of plates from these manufacturers. For best well-to-well accuracy, we recommend a high-quality, name brand polystyrene ELISA plate.

Note: Plate preparation has been optimized using the carbonate coating buffer prepared as described in Section VIII.B; other buffers may give poor results.

Note: Keep the undiluted TGF β Coat mAb on ice when removed from 4 $^{\circ}$ C storage.



DO NOT

allow wells to dry out completely between steps.



DO NOT

stack plates when incubating at 37 $^{\circ}$ C.

D. Preparing the Sample Buffer

Each 96 well plate requires approximately 23ml of TGFβ Sample 1X Buffer. This volume includes 3ml to determine the analyte (TGFβ₂) standard curve.

Per 96 well plate, combine 20.7ml of deionized water and 2.3ml of TGFβ Sample 10X Buffer in a 50ml polypropylene tube. Use a sterile pipette when aspirating from the stock solutions. Mix gently and completely by inversion prior to use.

E. Preparing the TGFβ₂ Standard Curve

The TGFβ₂ standard curve is linear between 32 and 1,000pg/ml. The TGFβ₂ Standard is supplied at a concentration of 1μg/ml and is already acid treated. Accurately dilute the supplied TGFβ₂ Standard 1:1,000 in TGFβ Sample 1X Buffer to achieve a concentration of 1,000pg/ml. For example, pipet 5μl of the TGFβ₂ Standard into 95μl of TGFβ Sample 1X Buffer (1:20 dilution), then pipet 10μl of this solution into 490μl of TGFβ Sample 1X Buffer (1:1,000 final dilution).

Hint: Keep the undiluted TGFβ₂ Standard on ice when removed from 4°C storage.

1. Following plate blocking, flick out the contents of the wells over a sink. Slap the plate 3 times upside down on a paper towel to remove residual liquid. Designate two columns of wells (16 wells) for the standard curve. To prepare the TGFβ₂ standard curve within the assay plate, add 100μl/well of the TGFβ Sample 1X Buffer to wells B through H in the two columns designated for the standard curve (Figure 2).
2. Add 200μl of the diluted TGFβ₂ Standard (1,000pg/ml) to the first well (row A) in each column designated for the standard curve.
3. Immediately perform serial 1:2 dilutions (100μl/well) in the two columns designated for the standard curve. In the last set of wells for the standard curve, do not add any TGFβ₂. The final concentrations (in duplicate) in the TGFβ₂ control columns will be 0–1,000pg/ml (Figure 2).

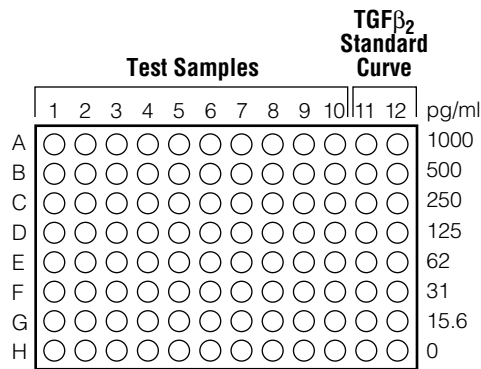


Figure 2. Recommended ELISA plate format for standard curve and test samples.

F. Addition of Sample

We recommend starting with a 1:4 dilution of each test sample and preparing 1:2 serial dilutions per column in the ELISA plate. Alternatively, screen samples at a single concentration (in triplicate) and subsequently reassay all positive samples to determine the exact TGF β ₂ concentration.

We also recommend performing a series of negative control reactions containing the carrier solution alone to determine background levels of TGF β ₂ (such as from serum in culture medium).

1. After preparing the TGF β ₂ standard curve, add 100 μ l of the acid treated (or naturally processed) samples to each of the remaining wells. (See Section IV for acid treatment of samples.)
2. Seal the wells with a 96 well plate sealer and incubate the plate for 1.5 hours at room temperature with shaking (500 \pm 100rpm).

Note: Best results are obtained using a plate shaker. Alternatively, plates may be incubated at 37°C without shaking, although a slight loss in sensitivity of the assay may occur.

3. Vigorously wash all wells with TBST wash buffer (Section VIII.B) using an automated plate washer, wash bottle or multichannel pipettor. If using a wash bottle or multichannel pipettor, wash plates by filling each well with TBST wash buffer, flicking out the contents over a sink and slapping the plate 3 times on a paper towel. Repeat this wash procedure an additional 4 times. Immediately proceed to the next step.

G. Addition of Anti-TGF β ₂ pAb

1. In a 15ml or 50ml polypropylene tube, add 5 μ l of the Anti-TGF β ₂ pAb to 10ml of TGF β Sample 1X Buffer (1:2,000 dilution) to prepare enough reagent for a full 96 well plate. **Mix thoroughly**, but avoid creating excess bubbles. Use a multichannel pipettor to add 100 μ l of the diluted Anti-TGF β ₂ pAb to each well, being careful not to touch or scratch the bottom or sides of the wells.
2. Seal the wells with a plate sealer and incubate for 2 hours at room temperature with shaking (500 \pm 100rpm).
3. Wash as described in Section V.F, Step 3.

H. Addition of TGF β HRP Conjugate

1. In a 15ml or 50ml polypropylene tube, add 100 μ l of the stock TGF β HRP Conjugate to 9.9ml of TGF β Sample 1X Buffer (1:100 dilution) to prepare enough reagent for a full 96 well plate. **Mix thoroughly**, but avoid creating excess bubbles. Add 100 μ l of the diluted TGF β HRP Conjugate to each well using a multichannel pipettor, being careful not to touch or scratch the bottom or sides of the wells.
2. Seal the wells with a plate sealer and incubate for 2 hours at room temperature with shaking (500 \pm 100rpm).

Note: During this incubation, equilibrate the TMB One Solution to room temperature.

3. Wash as described in Section V.F, Step 3.



Add samples
as quickly as possible to
minimize evaporation.

Note: We strongly recommend the use of an automated plate washer for consistent results.

Hint: Keep the Anti-TGF β ₂ pAb and TGF β HRP Conjugate on ice when removed from 4°C storage.

Note: During the incubation, equilibrate the TMB One Solution to room temperature.



CAUTION:

Take care to avoid contact of the TMB One Solution and 1N hydrochloric acid with skin and eyes.

Note: The exterior bottom of the plate must be optically clean for accurate measurement. Wipe the **exterior bottom** with 70% ethanol if necessary.



A standard curve must be prepared for each plate.

I. Color Development

1. Add 100µl of room temperature TMB One Solution to each well using a multichannel pipettor.
2. Incubate at room temperature for 15 minutes **without** shaking.

Note: Ambient (room) temperature variations may affect assay performance. If the assay is performed at less than 18°C, develop for at least 15 minutes to ensure maximum sensitivity. At temperatures greater than 23°C, color development will occur faster.

3. Stop the reaction by adding 100µl of 1N hydrochloric acid to the wells in the same order in which substrate was added in Step 2. The blue color will change to yellow as the pH decreases. Take care to avoid creating bubbles.
4. Record the absorbance at 450nm on a plate reader within 30 minutes of stopping the reaction. See Figure 3 for a representative TGFβ₂ standard curve.

J. Representative Standard Curve

Note: Best results are obtained using the average value of replicate samples.

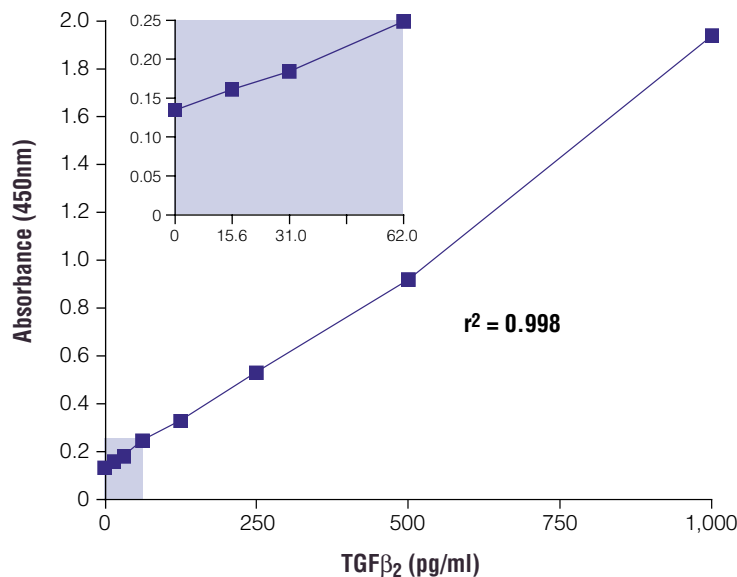


Figure 3. Representative TGFβ₂ standard curve obtained using the TGFβ₂ E_{max}[®] ImmunoAssay System. The inset is an enlargement of the 0–62.5pg/ml portion of the graph.

VI. Troubleshooting

Symptoms	Possible Causes	Comments
Sample absorbance is above range of standard curve	Sample too concentrated	Further dilute the sample. Assay multiple dilutions of each sample to ensure that at least one point will fall in the useful range of the standard curve.
Sample absorbance is below range of standard curve	Sample too dilute	Reassay at a higher sample concentration.
High absorbance in all samples	TGF β_2 present in buffer or media	Perform negative control reactions containing carrier solution alone whenever TGF β_2 may be present in buffer or media.
Low absorbance in all samples	Incorrectly diluted component	Recheck the dilutions for each component of the assay.
Low activity in TGF β_2 Standard	Improper storage	The standard is stable, if stored undiluted, for six months at -20°C and three months at 4°C .
Variability in replicate samples	Technique problems in performing assay	Ensure that all wells are washed completely. Allow plate to warm to room temperature for 10–15 minutes before starting blocking procedure. Add stop solution to wells in the same order as TMB substrate. Change pipette tips before adding each reagent. Perform additional replicates. Check calibration of the pipettor.

For questions not addressed here, please contact your local Promega branch office or distributor. Contact information available at: www.promega.com.

E-mail: techserv@promega.com

VII. Reference

- Hornbeck, P. (1994) Enzyme-linked immunosorbent assays. In: *Current Protocols in Immunology*, Vol. 1, Coico, R., ed., John Wiley & Sons, Inc., Unit 2.1, NY.

VIII. Appendix

A. Performance Characteristics of the TGF β_2 E $_{\text{max}}$ [®] ImmunoAssay System

Cross-Reactivity of the TGF β_2 E $_{\text{max}}$ [®] ImmunoAssay System

The TGF β_2 E $_{\text{max}}$ [®] ImmunoAssay System demonstrates very low cross-reactivity with other TGF β isoforms.

TGF β Isoform	Apparent TGF β_2 Concentration	% Cross-Reactivity
TGF β_1	0.027ng/ml	0.27%
TGF $\beta_{1,2}$	0.66ng/ml	6.6%
TGF β_3	0.068ng/ml	0.68%

10ng/ml of these TGF β isoforms were diluted in TGF β Sample 1X Buffer and assayed with this system. **Note:** TGF $\beta_{1,2}$ is a heterodimer composed of TGF β_1 and TGF β_2 chains.

Effect of Endogenous TGFβ₂ on Amount of Sample Measured

Due to endogenous TGFβ₂ in serum and plasma, we recommend acid treating human serum and EDTA plasma samples, then diluting the samples 1:150 in TGFβ Sample 1X Buffer before assaying.

Sample	Endogenous TGFβ ₂ (pg/ml)	TGFβ ₂ Spiked Amount (pg/ml)	Expected TGFβ ₂ (pg/ml)	Apparent TGFβ ₂ (pg/ml)
RPMI 1640				
+ 10% FBS	35	500	535	427
Serum	291	62	353	321
Plasma	302	62	364	364
Urine	0	500	500	540

Samples containing endogenous TGFβ₂ were acid treated, neutralized, diluted and spiked with activated Porcine TGFβ₂.

Intra-Assay Comparison

Three concentrations of Natural Porcine TGFβ₂ (acid treated) were diluted in TGFβ Sample 1X Buffer and assayed by one operator for a total of 8 determinations each.

	TGFβ ₂		
	Low	Medium	High
N	8	8	8
Mean (pg/ml)	229	402	767
SD (pg/ml)	21	35	57
CV (%)	9.2	8.7	7.4

N=sample size, SD=standard deviation, CV=coefficient of variance

Inter-Assay Comparison

Three concentrations of Natural Porcine TGFβ₂ (acid treated) diluted in TGFβ Sample 1X Buffer were assayed independently by 4 different operators for a total of 20 determinations.

	TGFβ ₂		
	Low	Medium	High
N	20	20	20
Mean (pg/ml)	94	399	756
SD (pg/ml)	25	29	46
CV (%)	27	7.3	6.1

N=sample size, SD=standard deviation, CV=coefficient of variance

B. Composition of Buffers and Solutions

1N hydrochloric acid

Add 82.7ml of concentrated hydrochloric acid to 917.3ml deionized water.

carbonate coating buffer

0.025M sodium bicarbonate

0.025M sodium carbonate

Leave out 5% of the water volume to accommodate the acid when preparing. Adjust pH to 9.2 using 2N HCl. **Filter-sterilize and treat aseptically or add 0.02% sodium azide.**

Dulbecco's PBS (per liter)

0.2g KCl
 8.0g NaCl
 0.2g KH₂PO₄
 1.15g Na₂HPO₄
 100mg 1M MgCl₂ • 6H₂O
 133mg 1M CaCl₂ • 2H₂O

Add room temperature deionized water to a final volume of 1 liter to the KCl, NaCl, KH₂PO₄ and Na₂HPO₄. Adjust pH to 7.35 using 1N HCl or 1N NaOH, if necessary. Add the MgCl₂ • 6H₂O, mix thoroughly; then add the CaCl₂ • 2H₂O, and mix thoroughly.

TBST wash buffer

20mM Tris-HCl (pH 7.6)
 150mM NaCl
 0.05% (v/v) Tween® 20

C. Related Products

E_{max}® ImmunoAssay Systems

Product	Size	Cat.#
TGFβ ₁ E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7590
	5 × 96 wells	G7591
BDNF E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7610
	5 × 96 wells	G7611
GDNF E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7620
	5 × 96 wells	G7621
NGF E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7630
	5 × 96 wells	G7631
NT-3 E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7640
	5 × 96 wells	G7641
NT-4 E _{max} ® ImmunoAssay Systems	2 × 96 wells	G7650
	5 × 96 wells	G7651

Items Available Separately

Product	Size	Cat.#
TGFβ Sample 10X Buffer	20ml	G1291
TGFβ ₁ , Human, Natural	2µg	G1241
Anti-TGFβ ₁ pAb	100µg	G1221
TMB One Solution*	100ml	G7431

*For Laboratory Use.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.



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