Immunotag™ Transforming growth factor beta receptor type 3 ELISA Kit

A Complete ELISA kit for the detection of Transforming growth factor beta receptor type 3

(Cat. #IT13996)

INTRODUCTION ... 3
ITEMS SUPPLIED ... 3
STORAGE CONDITIONS ... 3
SPECIFICATIONS ... 3
ADDITIONAL ITEMS NEEDED ... 3
PRECAUTIONS ... 4
SAMPLE COLLECTION AND STORAGE ... 4
- SERUM ... 4
- PLASMA ... 4
- URINE ... 4
- TISSUE HOMOGENATES ... 4
- CELL CULTURE SUPERNATANTS & OTHER BIOLOGICAL FLUIDS ... 4

PREPARATION BEFORE USE ... 5
- WASH BUFFER ... 5
- ELISA STANDARD ... 5
- PREPARATION OF DETECTION REAGENT A ... 5
- PREPARATION OF DETECTION REAGENT B ... 5

PROTOCOL ... 6
- FOR MANUAL WASHING ... 6
- FOR AUTOMATED WASHING ... 6
- SAMPLE DILUTION GUIDELINE ... 6
- ASSAY PROCEDURE ... 6

PROTOCOL SUMMARY ... 7
TYPICAL DATA & STANDARD CURVE ... 7
SPECIFICITY ... 7
PRECISION ... 7
ELISA TROUBLESHOOTING GUIDE ... 8
RELATED PRODUCTS ... 10

INTRODUCTION

This ELISA kit allows for the in vitro quantitative determination of mouse Transforming growth factor beta receptor type 3 concentrations in serum, plasma, urine, tissue homogenates and cell culture supernatants and other biological fluids.

The microtiter plate provided has been pre-coated with an antibody specific to mouse Transforming growth factor beta receptor type 3. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for mouse Transforming growth factor beta receptor type 3 and avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. A TMB substrate solution is added to detect the biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of mouse Transforming growth factor beta receptor type 3 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

ITEMS SUPPLIED

Description	Size
Coated Microtiter Plate	1
Standard	2 vials
Sample Diluent	20ml
Assay Diluent A	10ml
Assay Diluent B	10ml
Detection Reagent A	120µl
Detection Reagent B	120µl
Wash Buffer [25X]	30ml
Substrate	10ml
Stop Solution	10ml

STORAGE CONDITIONS

The kit is shipped on blue ice. Upon arrival, store assay plate, standard, Detection Reagent A and Detection Reagent B at -20°C. Store other reagents at 4˚C for up to 6 months. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to moisture.

SPECIFICATIONS

- **Reactivity:** Mouse
- **Range:** 0.156-10ng/ml
- **Sensitivity:** <0.085ng/ml

ADDITIONAL ITEMS NEEDED

- Microplate reader (wavelength: 450nm)
- 37°C incubator
- Automated plate washer (Optional)
- Precision single and multi-channel pipette and disposable tips
- Clean tubes and Eppendorf tubes
- Deionized or distilled water

PRECAUTIONS

- We recommend performing pilot experiments using standards and a small number of samples.
- After opening and before using, keep plate dry.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Detection Substrates must be protected from light.
- False positives may arise if washing steps are not completed.
- The use of duplicate well assays are recommended for both standard and sample testing.
- Do not let the plate dry out during the assay as this may inactivate active components.
- Do not reuse tips and tubes to avoid cross contamination.

SAMPLE COLLECTION AND STORAGE

Serum

Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 × g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C.

Plasma

Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g at 2-8°C within 30 minutes of collection. Store samples at -20 or -80°C. Avoid repeated freeze-thaw cycles.

Urine

Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Tissue homogenates

The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissue was rinsed with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at ≤ -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Remove the supernatant and assay immediately or aliquot and store at ≤ -20°C.

Cell culture supernatants & other biological fluids

Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20 or -80°C. Avoid repeated freeze-thaw cycles.

NOTE:

- Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (1 month) or -80°C (2 months) to avoid loss of bioactivity and contamination.
- Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
- Influenced by the factors including cell viability, cell number and sampling time, samples from cell culture supernatant may not be detected by the kit
- Sample hemolysis will influence the result, so hemolytic specimen cannot be detected.
- When performing the assay slowly bring samples to room temperature.
- Do not use heat-treated specimens.

PREPARATION BEFORE USE

Bring all reagents to room temperature before use.

Wash Buffer

Dilute 30mL Wash Buffer [25X] into 750 mL of Wash Buffer with deionized or distilled water. If crystals have formed in the concentrate, you can warm in a 40°C water bath (Heating temperature should not exceed 50°C) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use. Store diluted wash buffer at 4°C.

ELISA Standard

1. ***10ng/ml of ELISA Standard:*** Add 1 ml of Sample Diluent into a Standard tube, keep the tube at room temperature for 15 min and mix thoroughly.
2. ***Dilute ELISA Standard:*** Label 6 Eppendorf tubes (1-6) and aliquot 0.5ml of the Sample Diluent into each tube. Add 0.5 ml of the above 20ng/ml standard solution into 1st tube and mix thoroughly. Transfer 0.5 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.5 ml from 2nd tube to 3rd tube and mix thoroughly, and so on. The resulting standard will be repeating 1:1 dilutions of the starting standard
   NOTE: *The standard solutions are best used within 2 hours. The standard solution should be at 4°C for up to 12 hours. Or store at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

Preparation of Detection Reagent A

Prepare within 1 hour before starting the experiment.

1. Calculate the total volume of the Detection Reagent A working solutions: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
2. Dilute the Detection Reagent A with the Assay Diluent A at 1:100 and mix thoroughly. (i.e. Add 1µl of Detection Reagent A into 99µl of Assay Diluent A.)

Preparation of Detection Reagent B

Prepare within 1 hour before starting the experiment.

3. Calculate the total volume of the Detection Reagent B working solutions: 0.1 ml / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume)
4. Dilute the Detection Reagent B with the Assay Diluent B at 1:100 and mix thoroughly. (i.e. Add 1µl of Detection Reagent B into 99µl of Assay Diluent B.)

NOTE: *Carefully reconstitute Standards or working Detection Reagent A and B according to the instructions, avoid foaming and mix gently until the crystals have completely dissolved. The reconstituted Standards, Detection Reagent A and B* ***can only be used once****.*

PROTOCOL

For Manual Washing

1. Discard the solution in the plate without touching the side walls.
2. Clap the plate on absorbent filter papers or other absorbent material.
3. Fill each well completely with 350µl wash buffer and soak for 1 to 2 minutes
4. Aspirate contents from the plate, and clap the plate on absorbent filter papers or other absorbent material.
5. Repeat this procedure two more times for a total of THREE washes.

For Automated Washing

Aspirate all wells, and then wash plate THREE times with 350µl wash buffer. After the final wash, invert plate, and clap the plate on absorbent filter papers or other absorbent material. It is recommended that the washer be set for a soaking time of 1 minute.

Sample Dilution Guideline

End user should estimate the concentration of the target protein in the test sample first, and select a proper dilution factor to ensure the diluted target protein concentration falls in the optimal detection range of the kit. Dilute the sample with the ELISA Standard Diluent. The test sample must be well mixed with the ELISA Standard Diluent.

- High target protein concentration (200-2000ng/ml): Dilution: 1:100. (i.e. Add 1µl of sample into 99µl of ELISA Standard Diluent)
- Medium target protein concentration (20-200ng/ml): Dilution: 1:10.(i.e. Add 10µl of sample into 90µl of ELISA Standard Diluent)
- Low target protein concentration (0.313-20ng/ml): Dilution: 1:2.(i.e. Add 50µl of sample into 50µl of ELISA Standard Diluent)
- Very low target protein concentration (outside the lower range of the assay), it is unnecessary to dilute, or dilute at 1:2.

Assay Procedure

Before adding to wells, equilibrate the Detection Reagent working solutions and substrate for at least 30 min at room temperature. When diluting samples and reagents, they must be mixed completely and evenly. It is recommended to plot a standard curve for each test.

1. Wash plate 2 times before adding standard, sample and control (zero) well as directed above.
2. Aliquot 0.1ml standard solutions into the standard wells. Perform in duplicate
3. Add 0.1 ml of ELISA Standard Diluent into the control (zero) wells.
4. Add 0.1 ml of properly diluted sample into test sample wells.
   NOTE: *See Sample Dilution Guideline above*
5. Seal the plate with a cover and incubate at 37°C for 120 min.
6. Remove the cover and discard the plate content, clap the plate on the absorbent filter papers or other absorbent material. Do NOT let the wells completely dry at any time.
   NOTE**:** *Do not wash the plate at this time***.**
7. Add 0.1 ml of Detection Reagent A working solution into the above wells (standard, test sample & zero wells). Add the solution at the bottom of each well without touching the side wall.
   NOTE: *Detection Reagent A may appear cloudy, if so warm to room temperature and mix gently until solution appears uniform.*
8. Seal the plate with a cover and incubate at 37°C for 60 min.
9. Remove the cover, and wash plate 3 times with Wash buffer, as directed above.
10. Add 0.1 ml of Detection Reagent B working solution into each well, cover the plate and incubate at 37°C for 60 min.

11. Remove the cover and wash plate 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min.
12. Add 90µl of Substrate Solution into each well, cover the plate and incubate at 37°C in the dark for 10-20 min. ***NOTE**: This incubation time is for reference use only; the optimal time should be determined by end user. A blue color should be seen in the first 3-4 wells (with most concentrated standard solutions); the other wells show no obvious color change.*
13. Add 50µl of Stop solution into each well and mix thoroughly. The color changes from blue to yellow immediately.
14. Read the O.D. absorbance at 450 nm in a microplate reader immediately after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be interpolated from the standard curve.

***NOTE:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.*

PROTOCOL SUMMARY

1. Add 100µL standard or sample to each well for 90 minutes at 37°C
2. add 100µL Detection Reagent A working solution to each well for 60 minutes at 37°C
3. Aspirate and wash 3 times
4. Add 100µL Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C
5. Aspirate and wash 5 times
6. Add 90µL Substrate Solution. Incubate 15 -30 minutes at 37°C
7. Add 50µL Stop Solution. Read at 450nm immediately
8. Calculation of results

TYPICAL DATA & STANDARD CURVE

Results of a typical standard run are shown below. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment. (N/A=not applicable)

X	ng/ml	0	0.156	0.312	0.625	1.25	2.5	5	10
Y	OD450	0	0.051	0.099	0.222	0.482	1.036	1.947	2.995

SPECIFICITY

This assay has high sensitivity and excellent specificity.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between the protein of interest and all the analogues, therefore, cross reaction may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level of protein were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level of protein were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/meanX100

Intra-Assay: CV<4.8%

Inter-Assay: CV<7.5%

ELISA TROUBLESHOOTING GUIDE

Problem	Possible Source	Solution
Poor Standard Curve	Improper standard solution	Confirm dilutions are made correctly
	Standard improperly reconstituted	Briefly spin vial before opening, inspect for undissolved material after reconstituting
	Standard degraded	Store and handle standard as recommended
	Curve does not fit scale	Try plotting using different scales e.g. log-log, 5 parameter logistic curve fit
	Pipetting error	Use calibrated pipettes and proper pipetting technique
	Standard was incompletely reconstituted or was inappropriately stored	Reconstitute standard according to protocol. Store reconstituted standard in appropriate vials. Store reconstituted standard at -70 °C
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and correct reagent volume.
	Incubations done at inappropriate temperature, timing or agitation	Assay conditions need to be checked
No signal	Incubation time too short	Incubate samples overnight at 4°C or follow the manufacturer guidelines.
	Target present below detection limit of assay	Decrease dilution factor or concentrate samples.
	Incompatible sample type	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect a positive control.
	Recognition of epitope impeded by absorption to plate	To enhance detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto the microtiter plate.
	Assay buffer compatibility	Ensure assay buffer is compatible with target of interest (e.g. enzymatic activity retained, protein interactions retained)
	Incorrect or no Detection Reagent A was added	Add appropriate Detection Reagent A and continue.
	Detection Reagent B was not added	Add Detection Reagent B according to protocol and continue.
	Substrate solution was not added	Add substrate solution and continue.
	Wash buffer contains sodium azide	Avoid sodium azide in the wash buffer.
	Multichannel pipette errors	Calibrate the pipettes.
	Plate washing was not adequate or uniform	Make sure pipette tips are tightly secured. Confirm all reagents are removed completely in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before pipetting
	Samples may have high particular matter	Remove the particular matter by centrifugation.
	Cross-well contamination	When reusing plate sealers check that no reagent has touched the sealer. Care should be taken when using the same pipette tips used for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.
	Not enough detection reagent	Increase concentration or amount of detection reagent following manufacturer guidelines.
	Sample prepared incorrectly	Ensure proper sample preparation/dilution. Samples may be incompatible with microtiter plate assay format.
	Insufficient antibody	Try different concentrations/dilutions of antibody
	Incubation temperature too low	Ensure the incubations are carried out at the correct

Problem	Possible Source	Solution
		temperature. All reagents including plate should be at room temperature or as recommended by the manufacturer before proceeding.
	Incorrect wavelength	Verify the wavelength and read plate again
	Plate washings too vigorous	Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
	Wells dried out	Do not allow wells to become dry once the assay has started. Cover the plate using sealing film or tape for all incubations
	Slow color developments of enzymatic reaction	Prepare substrate solution immediately before use. Ensure the stock solution has not expired and is not contaminated. Allow longer incubation.
High variation in samples and/or standards	Bubbles in wells	Ensure no bubbles are present prior to reading plate
	Wells not washed equally/thoroughly	Check that all ports of the plate washer are not obstructed. Wash wells as recommended.
	Incomplete reagent mixing	Ensure all reagents are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and proper technique to ensure accurate pipetting
	Edge effects	Ensure the plate and all reagents are at room temperature.
	Inconsistent sample preparation	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaw cycles).
High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Prepare fresh water buffer
	Too much detection reagent	Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent.
	Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)	Try different blocking reagent and/or blocking reagent to wash buffer.
	Salt concentration of incubation/wash buffers	Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.
	Waiting too long to read plate after adding stop solution.	Read plate immediately after adding stop solution.
	Non-specific binding of antibody	Use suitable blocking buffers e.g. BSA or 5010% normal serum-species same as primary antibody if using a directly conjugated Detection Reagent A or same as secondary if using conjugated.
	Background wells were contaminated	Avoid cross-well contamination by using the sealer appropriately. Use multichannel pipettes without touching the reagents on the plate.
	Matrix used has endogenous analyte or interference	Check the matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
	High antibody concentration	Try different dilutions for optimal results
	Substrate incubation carried out in light	Substrate incubations should be carried out in the dark or as recommended by manufacturer.
	Precipitate formed in wells upon substrate addition.	Increase dilution factor of sample or decrease concentration of substrate
	Dirty plate	Clean the plate bottom.
Low Sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note that all reagents may not have identical storage requirements.
	Not enough target	Concentrate sample or reduce sample dilution

Problem	Possible Source	Solution
	Inactive detection reagent	Ensure reporter enzyme has the expected activity.
	Plate reader settings incorrect	Ensure plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
	Assay format not sensitive enough	Switch to a more sensitive detection system (e.g. colorimetric to chemiluminescence / fluorescence) Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
	Target poorly absorbs to microtiter plate	Covalently link target to microtiter plate.
	Not enough substrate	Add more substrate
	Incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
	Interfering buffers or sample ingredients	Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibit HRP enzyme and EDTA used as anticoagulant for plasma collection inhibits enzymatic reactions.
	Mixing or substituting reagents from different kits	Avoid mixing components from different kits.

RELATED PRODUCTS

Download our Protein Assay Development or Bioassay Handbook

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Last saved: 10/22/2019 CMH

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