SCA124Mu 96 Tests
Chemiluminescent Immunoassay Kit
For Transforming Growth Factor Beta 1 (TGFb1)
Organism Species: Mus musculus (Mouse)
Instruction manual

FOR IN VITRO AND RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

6th Edition (Revised in July, 2013)

[INTENDED USE]

The Chemiluminescent Immunoassay kit is designed for the in vitro sensitive quantitative measurement of TGFb1 in mouse serum, platelet-poor plasma, tissue homogenates, cell culture supernates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
Substrate A	1×10mL	Substrate B	1×2mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Luminometer capable of reading 96-well microplates with the following parameters:
   lag time 30.0secs; read time 1.0 sec/well .
2. Precision single or multi-channel pipettes and pipette tips with disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

[STORAGE OF THE KITS]

1. **For unopened kit:** All the reagents should be kept according to the labels on vials. The **Standard**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20°C upon receipt while the others should be at 4 °C.
2. **For opened kit:** When the kit is opened, the remaining reagents still need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is within the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Assay (see activation procedure) freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Platelet-poor 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. It is recommended to centrifuge samples for 10 minutes at 10,000×g for complete platelet removal. Remove plasma and assay(see activation procedure) immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS(0.01mol/L,pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5-10mL of PBS with a glass homogenizer on ice(Micro Tissue Grinders woks, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000×g. Remove the supernate and assay immediately or aliquot and store at ≤-20°C.

Cell culture supernates and other biological fluids - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay (see activation procedure) immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be detected.
3. When performing the assay, bring samples to room temperature.
4. Do not use animal serum for growth of cell cultures. It may contain high levels of latent TGFb1 in the preparation of cell culture media and will affect the results.

[ACTIVATION REAGENT PREPARATION]

To activate latent TGFb1 to the immunoreactive form, prepare the following solutions for acid activation and neutralization.

1. **1 M HCl (100 mL) -** Slowly add 8.33 mL of 12 M HCl to 91.67 mL of deionized water. Mix well.
2. **1.2 M NaOH/0.5 M HEPES (100 mL) -** Slowly add 12 mL of 10 M NaOH to 75 mL of deionized water. Mix well. Add 11.9 g of HEPES. Mix well. Bring final volume to 100 mL with deionized water.

Note:

1. Ensure that samples after neutralization is within pH 7.2-7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.

2. Activated samples must be assayed immediately. Do not freeze activated samples.
3. The solutions may be stored in polypropylene bottles at room temperature for up to one month.
4. Wear protective clothing and safety glasses during preparation or use of these reagents.
5. **Do not activate the kit standards.**

[SAMPLE ACTIVATION PROCEDURE]

Serum/Plasma

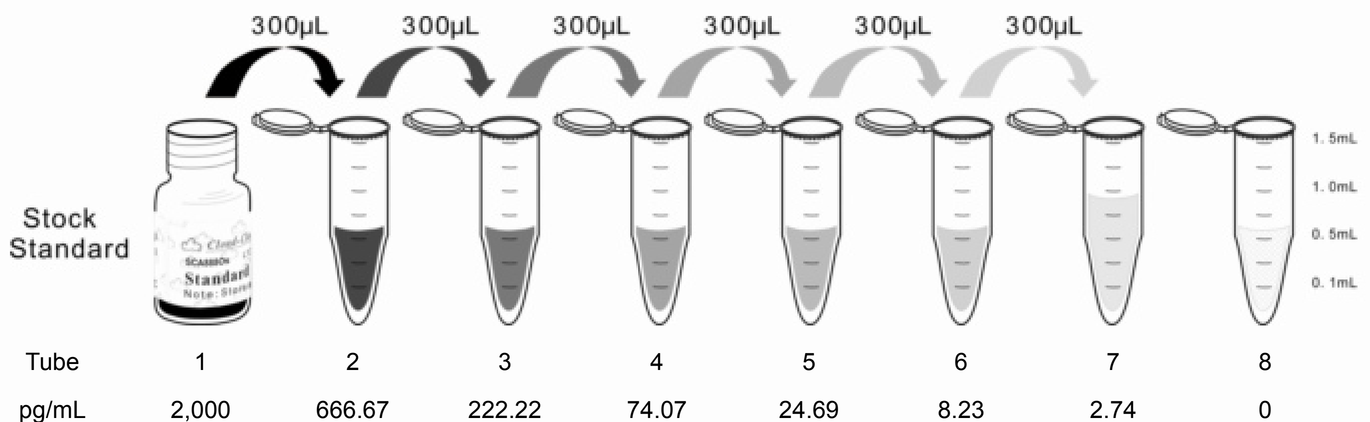
1. To 50μL of serum/plasma, add 10μL of 1 M HCl. Mix well.
2. Incubate 10 minutes at room temperature.
3. Neutralize the acidified sample by adding 10μL of 1.2 M NaOH/0.5 M HEPES. Mix well. Add 80μL Standard Diluent. Mix well. Assay immediately.
4. The concentration read off the standard curve must be multiplied by the appropriate dilution factor, 3.

Cell culture supernates

1. To 100μL of cell culture supernate, add 20μL of 1 M HCl. Mix well.
2. Incubate 10 minutes at room temperature.
3. Neutralize the acidified sample by adding 20μL of 1.2 M NaOH/0.5 M HEPES. Mix well. Assay immediately·
4. The concentration read off the standard curve must be multiplied by the dilution factor, 1.4.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25°C) before use.
2. **Standard -** Reconstitute the **Standard** with 1.0mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 2,000pg/mL. Please prepare 7 tubes containing 0.6mL Standard Diluent and produce a triple dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 2,000pg/mL, 666.67pg/mL, 222.22pg/mL, 74.07pg/mL, 24.69pg/mL, 8.23pg/mL, 2.74pg/mL, and the last EP tubes with **Standard Diluent** is the blank as 0pg/mL.



3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with **Assay Diluent A** and **B,** respectively (1:100).

4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **Substrate working Solution -** Mix the substrate A and B by the ratio of 99:1 to make the substrate working solution. Mix thoroughly. For example, prepare 1,000μL Substrate working Solution with 990μL Substrate A + 10μL Substrate B.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standard within 15 minutes before assay. Please do not dissolve the reagents at 37°C directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting.
4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
5. Prepare Substrate working Solution within 15 minutes before assay.
6. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
7. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments. Sample should be diluted by 0.01mol/L PBS(PH=7.0-7.2).
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected CLIA results due to the impacts of certain chemicals.
5. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
6. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don’t wash.

3. Add 100μL of **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100μL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for five times as conducted in step 4.
7. Add 100μL of **Substrate working Solution** to each well. Cover with a new Plate sealer. Incubate for 10 minutes at 37°C. Protect from light.
8. Measure the chemiluminescence signal in a microplate luminometer or as appropriate for the instrument used.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **For Substrate A** and **B**, please protect it from light.
6. Relative light units (RLUs) may differ from different luminometers.The Immunoassay was optimized using a Beijing Hamamatsu luminometer. Other instruments may require settings to be adjusted.

[TEST PRINCIPLE]

The microtiter plate provided in this kit has been pre-coated with an antibody specific to TGFb1. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to TGFb1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and

incubated. Then the mixture of substrate A and B is added to generate glow light emission kinetics. Upon plate development, the intensity of the emitted light is proportional to the TGFb1 level in the sample or standard.

[ASSAY PROCEDURE SUMMARY]

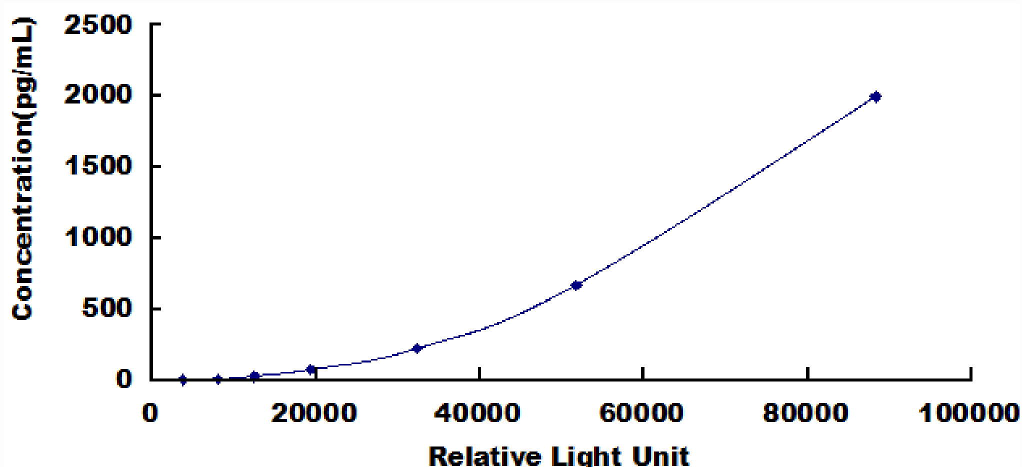
1. Prepare all reagents, samples and standards;
2. Add 100µL standard or sample to each well. Incubate 2 hours at 37°C;
3. Aspirate and add 100µL prepared Detection Reagent A. Incubate 1 hour at 37°C;
4. Aspirate and wash 3 times;
5. Add 100µL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
6. Aspirate and wash 5 times;
7. Add 100µL Substrate Solution. Incubate 10 minutes at 37°C;
8. Read RLU value immediately.

[CALCULATION OF RESULTS]

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard relative light unit (RLU). Create a standard curve on log-log graph paper, with TGFb1 concentration on the y-axis and the RLU value on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, such as curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the RLU value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is indeed the independent variable while RLU value is the dependent variable. Further, in this part, in order to help the customer perform the assay more visual, we provide the customer with the raw data (not the log of data). However, plotting log of the data to construct the curve will be recommended. The RLU values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). This curve is provided for demonstration only. The customers should establish their own standard curve for each test conducted.



Typical Standard Curve for TGFb1, Mouse CLIA.

[DETECTION RANGE]

2.74-2,000pg/mL. The standard curve concentrations used for the CLIA's were 2,000pg/mL, 666.67pg/mL, 222.22pg/mL, 74.07pg/mL, 24.69pg/mL, 8.23pg/mL, 2.74pg/mL.

[SENSITIVITY]

The minimum detectable dose of TGFb1 is typically less than 1.24pg/mL.

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean RLU value of 20 replicates of the zero standard added by their two standard deviations.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of TGFb1.

No significant cross-reactivity or interference between TGFb1 and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between TGFb1 and all the analogues, therefore, cross reaction may still exist.

[RECOVERY]

Matrices listed below were spiked with certain level of recombinant TGFb1 and the recovery rates were calculated by comparing the measured value to the expected amount of TGFb1 in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	82-99	92
EDTA plasma(n=5)	89-102	95
heparin plasma(n=5)	91-105	99

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of TGFb1 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	81-101%	79-95%	95-105%	85-97%
EDTA plasma(n=5)	86-99%	87-102%	83-103%	81-93%
heparin plasma(n=5)	80-92%	82-96%	78-90%	93-104%

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level TGFb1 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level TGFb1 were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

[STABILITY]

The stability of CLIA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

[IMPORTANT NOTE]

1. Limited by the current condition and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for information.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. We recommend using a single-mode luminometer or a multi-mode detection instrument set for luminescence measurement to measure light emission from 96-well microplates.
8. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled. Furthermore, a preliminary experiment before assay for each batch is recommended.
9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
10. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
11. The instruction manual also suits for the kit of 48T, but all reagents of 48T kit are reduced by half.