

Measurement of Active TGF- β Generated by Cultured Cells

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1. Introduction

1.1. TGF- β Latency and Activation

The transforming growth factors- β (TGF- β s) constitute a family of potent regulators of cellular differentiation, proliferation, migration, and protein expression (1,2). Three isoforms of TGF- β have been described in mammals: TGF- β 1, 2, and 3 (3–5). Most cell lines and tissues secrete TGF- β as a large latent complex formed by three components: TGF- β , LAP (latency-associated protein), and LTBP (latent TGF- β binding proteins). TGF- β is noncovalently associated to its prodomain LAP (6–8), and LAP is disulfide-bonded to LTBP (9). Four LTBPs (LTBP-1, 2, 3, and 4) have been described (10–14). Mature TGF- β must be released from the complex to bind to its high-affinity receptor and elicit its biological functions (15). This process, called TGF- β activation, appears to be a critical step in the control of TGF- β activity (16). An additional regulatory step involved in the activation process is the LTBP-mediated incorporation of latent TGF- β into the extracellular matrix (2). Activation of latent TGF- β has been described in various cell systems (17–19). However, the molecular mechanisms involved in extracellular TGF- β activation are not fully understood. It also remains to be elucidated whether latent TGF- β incorporation into the extracellular matrix regulates TGF- β activation in a positive or negative manner (2).

1.2. Detection of Active TGF- β

The availability of sensitive, specific, and quantitative assays for the detection of mature TGF- β is of fundamental importance in studying TGF- β

activation. The purpose of this chapter is to describe some of the assays used in our laboratory to measure active TGF- β in cell systems. These assays are as follows:

1. Wound assay for bovine aortic endothelial cell migration.
2. Cellular plasminogen activator assay for TGF- β .
3. Mink lung-cell growth-inhibition assay.
4. Mink lung epithelial cells luciferase assay.

In all these assays, the active TGF- β generated by the test cells induces a known and measurable biological response in the reporter cells such as inhibition of endothelial cell migration (20,21), inhibition of epithelial cell proliferation (22), decreased plasminogen activator activity (23), and increased production of plasminogen activator inhibitor-1 (24). All of these assays can be used to measure active TGF- β released by the test cells into their medium.

Only a few primary cells and established cell lines secrete significant amounts of active TGF- β into their culture medium when properly treated. Some examples are treatment of keratinocytes with retinoids (25) or vitamin D analogs (26), treatment of cancer cells or normal fibroblasts with antiestrogens (27,28), and treatment of MG-63 osteosarcoma cells with corticosteroids (29,30). Otherwise, little, if any, soluble active TGF- β is generated by most cultured cells. The absence of detectable levels of active TGF- β in the medium of TGF- β -producing cells is a common situation. However, the lack of active TGF- β in a cell culture supernatant does not necessarily mean lack of TGF- β activation. This may be because of two reasons. First, in some cases, TGF- β activation occurs at the cell surface (17,31,32), generating a high local concentration of active TGF- β . Second, active TGF- β is cleared from solution by binding to cell-surface receptor and/or to the extracellular matrix. As a result, only a small fraction may be released into the medium and therefore diluted to undetectable levels. High local concentration of active TGF- β can be detected by reporter cells cocultured with the activating cells (17,33). A useful TGF- β assay must be both sensitive and specific. Neutralizing antibodies to TGF- β should be included to verify that there are no other factors present that may affect the assay. Addition of isoform-specific neutralizing antibodies and use of the appropriate standard curves will allow quantification of specific TGF- β isoforms. When analyzing the effect of a treatment on TGF- β activation, one must determine if increased active TGF- β is the result of increased activation of latent TGF- β or increased production of total (active plus latent) TGF- β without any change in the latent versus active TGF- β ratio. In most TGF- β assays, the amount of total TGF- β released into the culture medium can be measured upon activation of the latent fraction by either acidification (31) or heat treatment (34).

2. Materials

2.1. General

1. Minimum essential medium (α MEM), store at 4°C.
2. Dulbecco's modified Eagle medium (DMEM), store at 4°C.
3. Fetal calf serum (FCS), store at -20°C, keep at 4°C after thawing.
4. Bovine serum albumin (BSA), store at 4°C.
5. Penicillin-streptomycin-L-glutamine (PSG) stock (100 \times): 20 g/L strepto mycin, 50 \times 10⁶ U/L penicillin G, 29.2 g/L L-glutamine. Filter sterilize and store aliquots at -20°C. Keep at 4°C after thawing.
6. Phosphate-buffered saline (PBS) pH~7.3: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄. Filter sterilize and store at 4°C.
7. Trypsin solution pH 7.2: 0.25% trypsin, 1 mM EDTA. Filter-sterilize and store aliquots at -20°C. Keep at 4°C after thawing.
8. Recombinant TGF- β (rTGF- β) stock solution: 5 mM HCl, 0.1% BSA, 2 μ g/mL TGF- β . Store at 4°C.
9. Neutralizing anti-TGF- β antibodies and nonimmune IgG. Store aliquots at -30°C. Keep at 4°C after thawing.
10. Control medium (serum-free medium): To avoid effects of serum factors, most experiments are conducted in the absence of serum. Serum-free medium contains α MEM or DMEM, depending on the cell type used in each assay, 0.1% BSA and 1 \times PSG. Filter sterilize and store at 4°C.
11. Test cells conditioned medium (*see Notes 1–4*): (a) Plate the cells at sub-confluence in regular growth medium and let them attach at 37°C for 2–4 h; (b) wash twice with PBS, (c) add serum-free medium and incubate at 37°C for 24 h; (d) collect the medium and centrifuge to remove cell debris. The conditioned medium is ready to be tested for the presence and levels of total and active TGF- β .
12. Acid-or heat-activated conditioned medium (*see Notes 5 and 6*). Acidification: (a) Acidify the conditioned medium to pH 2 with 1 M HCl; (b) incubate 1 h at room temperature; (c) neutralize with 1N NaOH. Use immediately. Heat treatment: (a) incubate the conditioned medium for 10' at 80°C; (b) let the medium cool down to 37°C. Use immediately.

2.2. Wound Assay for BAE Cell Migration

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH₂O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Rigid razor blade (*see Note 7*).
4. Absolute methanol.
5. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetyl LDL (DiI-acetyl-LDL).

6. 3% Formaldehyde in PBS: Formaldehyde is usually obtained as a 37% solution in H₂O. Dilute the stock solution 1:12.3 in PBS. Formaldehyde vapors are toxic; prepare the solution in a chemical hood.
7. Light microscope with ocular grid.

2.3. Mink Lung-Cell Growth-Inhibition Assay

1. CCL-64 cells (American Type Culture Collection, Rockville, MD).
2. ³H-Thymidine (³H-TdR), 40-60 Ci/mmol.
3. ¹²⁵I-Deoxyuridine (¹²⁵I-UdR), 5 Ci/mg.
4. 3:1 (v/v) Methanol-acetic acid.
5. 80% methanol.
6. 0.5% trypsin.
7. 1% sodium dodecyl sulfate (SDS).
8. Liquid scintillation counter.
9. 1N NaOH.
10. Gamma counter.

2.4. Cellular Plasminogen Activator Assay

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH₂O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Lysis buffer: 0.1 M Tris-HCl, pH 8.1, 0.5% Triton X-100.
4. Bovine fibrinogen.
5. 0.1X PBS: 13.7 mM NaCl, 0.27 mM KCl, 0.43 mM Na₂HPO₄, 0.14 mM KH₂PO₄.
6. ¹²⁵I-Fibrinogen, prepared by the iodine chloride method (35).
7. 2.5% FCS in αMEM, prepare freshly.
8. Assay buffer: 0.1 M Tris-HCl, pH 8.1, 250 μg/mL BSA, 8 μg/mL plasminogen. Prepare freshly.
9. Urokinase stock: 0.1 M Tris-HCl, pH 8.1, 0.1% BSA, 1000 U/mL urokinase. Store 10-μL aliquots at -20°C. Just before use, diluted with 5 mL of Tris-HCl, 0.1 M, pH 8.1, 0.1% BSA. Keep the dilutions on ice. Urokinase activity is not stable to repeated freezing and thawing.
10. Plasminogen. Purification of plasminogen is carried out at 4°C using a 100-mL lysine-Sepharose column per 500 mL of serum:
 - a. Equilibrate the column with PBS.
 - b. Load the serum.
 - c. Wash with at least three column volumes of 0.3 M potassium phosphate, pH 7.4, 2 mM EDTA; wash the column until the optical density (OD)_{λ280} returns to the basal value.
 - d. Elute the plasminogen with 0.2 M ε-aminocaproic acid in 0.1 M potassium phosphate, pH 7.4; collect 5-mL fraction and read the OD_{λ280} to follow the elution profile (see Note 8).

- e. Pool the eluted proteins and dialyze against PBS to remove the ϵ -amino-caproic acid.
 - f. Measure the OD λ_{280} (OD of 1.7 units = 1.0 mg/mL of plasminogen), aliquot, and store at -20°C .
11. Gamma counter.

2.5. MLEC Luciferase Assay

1. Mink lung epithelial cells (MLEC) permanently transfected with the expression construct p800neoLUC (36).
2. Geneticin stock solution (Invitrogen, Carlsbad, CA): 80 mg/mL in PBS. Filter-sterilize and store at -20°C .
3. Lysis buffer (Analytical Luminescence, San Diego, CA). Dilute 1:3 with dH $_2$ O the 3X stock solution. Prepare freshly.
4. Assay buffer. Prepare freshly from the following stock solutions: 5X luciferin buffer [1 M tricine, 5.35 mM (MgCO $_3$) $_4$ Mg(OH) $_2$, 13.35 mM MgSO $_4$, 0.5 mM EDTA, 166.5 mM DTT]; 50X ATP (37.5 mM); 20X luciferin (16 mM). Keep luciferin in the dark. (Luciferin is rapidly oxidized by exposure to light.) Store stock aliquots at -30°C .
5. Luminometer.

3. Methods

3.1. Wound Assay for BAE Cell Migration

This assay is based on the ability of TGF- β to inhibit cell migration in “wounded” monolayer cultures of BAE cells (20,21). The number of cells that migrate across the original edge of the wound is inversely proportional to the concentration of TGF- β present in the conditioned medium.

3.1.1. Cell Culture

1. Grow BAE cells on gelatin-coated dishes in α MEM containing 10% FCS and 1X PSG.
2. Use cells at early passages (not after passages 15–20).

3.1.2. Wound Assay

Portion of a confluent culture of BAE cells is removed by mechanical abrasion using a rigid razor blade (37).

1. Sterilize the razor blade in the pilot light of a Bunsen burner and let it cool down.
2. Use a surgical hemostat to manipulate the razor blade. Press the razor blade down onto the plate to cut the cell monolayer and to lightly mark the original edge of the wound by scoring the plastic surface (see Notes 7 and 9–11).
3. Gently move the blade to one side to remove part of the cell monolayer.
4. Wash twice with PBS to remove loose cells.
5. According to the experimental design, add the following:
 - a. Control medium to determine the basal level of cell migration.

- b. Control medium containing increasing amounts of rTGF- β to generate a standard curve; this assay can be used to detect concentrations of TGF- β as low as 0.4 pM (32).
 - c. Conditioned medium from the experimental cultures to measure active TGF- β .
 - d. Acid-or heat-activated conditioned medium to measure total (active plus latent) TGF- β .
 - e. Conditioned medium with neutralizing anti-TGF- β antibodies or nonimmune IgG to test the specificity of the migration inhibition.
6. Incubate at 37°C for 16–20 h.
 7. Remove the medium and wash once with PBS.
 8. Fix the cells with absolute methanol for 10–15' at room temperature.
 9. Count the number of cells that have migrated more than 125 μ m from the wound edge in seven successive 125- μ m increments. The cells present in the first 125 μ m segment are not included in the calculation in order to exclude those cells which moved across the origin before TGF- β had an effect (38,39). Cells are counted at 40X magnification using a light microscope with an ocular grid.
 10. Data are presented as percent of migration observed in the control wound. For each experimental condition, the number of migrating cells is counted in four to six random fields from each of two replicate dishes and the mean value is used to calculate the percent of migration inhibition.

3.1.3. Coculture Assay

1. Immediately after wounding, the second cell type is suspended in serum-free medium and inoculated into the culture dish (*see Note 12*).
2. Incubate at 37°C for 16–20 h.
3. Count the migrating BAE cells as in the standard wound assay (*see Note 13*).

3.2. Mink Lung-Cell Growth-Inhibition Assay

CCL-64 mink lung epithelial cells have been shown to be extremely sensitive to growth inhibition by TGF- β (40). A very sensitive and specific assay for TGF- β has been described by Danielpour and colleagues (22). They have shown that CCL-64 cells plated in DMEM containing 0.2% FCS are half-maximally growth inhibited by about 0.5 pM of TGF- β after 22 h of treatment. Because of this sensitivity, conditioned media can be assayed without concentration. Because other growth factors such as insulin, EGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been shown not to stimulate or inhibit CCL-64 cell proliferation, this assay is relatively specific for TGF- β (22).

3.2.1. Cell Culture

1. Grow CCL-64 cells in the high-glucose formulation of DMEM supplemented with 10% FCS and 1X PSG.
2. Pass the cells at a seed density of 5×10^5 cells/75 cm² T-flask at 3 d intervals.

3.2.2. Growth-Inhibition Assay

CCL-64 cells in logarithmic growth phase are used to initiate the growth inhibition assay.

1. Trypsinize and suspend the cells in 10 mL of DMEM 10% FCS
2. Centrifuge the cells at 500g for 5'.
3. Wash the pellet once with 10 mL of DMEM containing: 0.2% FCS and 1X PSG.
4. Resuspend the cells in the same medium.
5. Count and dilute the cells to a final concentration of 10^6 cells/mL.
6. Seed 0.5 mL/well of cell suspension in 24-well plates.
7. Let the cells attach at 37°C for 2 h.
8. Remove the medium and add the following according to the experimental design:
 - a. Control medium to determine the basal level of proliferation.
 - b. Control medium containing various concentrations of rTGF- β to generate a standard curve; this assay can be used to measure TGF- β in the range 0.08–2.4 pM (41).
 - c. Conditioned medium from the experimental culture to measure active TGF- β .
 - d. Acid- or heat-activated conditioned medium to measure total (latent plus active) TGF- β .
 - e. Conditioned medium with neutralizing anti-TGF- β antibodies or nonimmune IgG to test the specificity of the growth inhibitory response.
9. Incubate at 37°C for 22 h.
10. Remove the medium and pulse the cells with 0.25 mCi (40–60 Ci/mmol) of ^3H -TdR or 0.25 mCi (5 Ci/mg) of ^{125}I -UdR diluted in DMEM, 0.2% FCS, 1X PSG for 2 h at 37°C.
11. Remove the radioactive medium.
12. Fix the cells with 1 mL of methanol–acetic acid (3:1 v/v) for at least 1 h at room temperature.
13. Wash the wells twice with 1 mL of 80% methanol.
14. If ^3H -TdR is used, incubate with 250 μL of 0.5% trypsin for 30' at 37°C; solubilize the radioactivity with 250 μL of 1% SDS; measure the radioactivity by liquid scintillation counting.
15. If ^{125}I -UdR is used: lyse the cells with 1 mL of 1N NaOH for 30' at room temperature; ^{125}I -UdR is counted in a γ -counter.

3.3. Cellular PA Assay for TGF- β

This assay is based on the observation that TGF- β suppresses plasminogen activator (PA) activity of endothelial cells (23). The inhibitory effect of TGF- β is predominantly the result of the increased synthesis of plasminogen activator inhibitor-1 (PAI-1) (42).

Plasminogen activator activity in cell extracts or conditioned media can be measured using the ^{125}I -fibrin assay (43). Samples are tested in the presence of a known amount of plasminogen in ^{125}I -fibrin-coated plates. The PA present in

the test samples converts plasminogen into plasmin, and plasmin degrades fibrin. The amount of ^{125}I -fibrin degradation products released into the supernatant correlates with the levels of PA activity present in the sample. PA activity can be quantitated using a standard curve generated with purified urokinase plasminogen activator (uPA).

3.3.1. Cell Culture

Bovine aortic endothelial cells are grown as described in **Subheading 3.1.1.**

3.3.2. PA Assay

1. Grow BAE cells to confluence in 96-well plates in complete growth medium.
2. Remove the medium and wash the cells with PBS.
3. Add the following in duplicate:
 - a. Control medium to determine the basal level of PA activity
 - b. Control medium containing increasing concentration of rTGF- β to generate a standard curve; this assay can be used to measure TGF- β in the 0.08–2.4-pM range (**41**).
 - c. Conditioned medium from the experimental medium to measure active TGF- β .
 - d. Acid-or heat-activated conditioned medium to measure total (latent plus active) TGF- β .
 - e. Conditioned medium with neutralizing anti-TGF- β antibodies or nonimmune IgG to test the specificity of the PA inhibitory response.
4. Incubate the cells at 37°C for 12 h.
5. Remove the medium and wash twice with ice-cold PBS.
6. Lyse with 50 μL /well of lysis buffer.
7. Determine protein concentration.
8. Measure PA levels in the cell extracts using the ^{125}I -fibrin plate assay (*see* **Note 14**).

3.3.3. ^{125}I -Fibrin Plate Assay

3.3.3.1. PREPARATION OF ^{125}I -FIBRIN PLATES (**43**)

1. Dilute bovine fibrinogen in warm (37°C) 0.1X PBS. Do not mix or vortex. Fibrinogen is diluted such that a volume can be spread over the bottom of the well to give a concentration of 10 $\mu\text{g}/\text{cm}^2$. If using 24-well plates, add 250 μL /well of 120 $\mu\text{g}/\text{mL}$ fibrinogen solution.
2. Add ^{125}I -fibrinogen to bring the solution to approximately 160,000 counts per minute (cpm)/mL (40,000 cpm/well).
3. Aliquot 250 μL to each well, making sure that the entire bottom surface is covered.
4. Dry the open plates overnight under the hood.
5. Add 250 μL /well of medium containing 2.5% FCS. Fibrinogen is cleaved to fibrin by the action of thrombin present in serum.
6. Incubate at 37°C for 3 h.
7. Remove medium, wash twice with dH_2O , and store dry plates at 4°C.

3.3.3.2. ^{125}I -FIBRIN ASSAY (44)

Each sample is assayed in duplicate after dilution in assay buffer. Leave two wells with assay buffer only to give the background counts released by buffer alone. Add 500 μL of trypsin to each of the two wells. Trypsin will remove all the counts from the bottom of the wells, giving the total counts releasable.

1. Prepare 1 mL aliquots of assay buffer.
2. Add 1–5 μg of total cell extract protein to the assay buffer aliquots.
3. Add increasing amounts of urokinase (2–20 mU) to a separate set of assay buffer aliquotes.
4. Add 500 μL /well of each aliquot to the ^{125}I -fibrin-coated wells.
5. Incubate at 37°C for 1–2 h.
6. After 1 and 2 h, take 100 μL from each well and count the amount of ^{125}I -fibrin degradation products with a γ -counter (*see Note 15*).
7. Use the urokinase standard curve to quantitate the PA activity in the BAE cell extracts (mU/ μg). The data can also be presented as percentage of control, where 100% represents the PA activity of BAE cells incubated in serum-free medium.
8. Use the TGF- β standard curve to determine the TGF- β levels in the original experimental samples (pg/mL).

3.4. MLEC Luciferase Assay

This quantitative bioassay is based on the ability of TGF- β to upregulate PAI-1 (24). TGF- β activity is determined using MLEC permanently transfected with the expression construct p800neoLUC containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene (36). The specificity and sensitivity of the assay are the result of using a truncated PAI-1 promoter which retains the two regions responsible for maximal response to TGF- β (45).

3.4.1. Cell Culture

Mink lung epithelial cells are grown in DMEM containing 10% FCS, 1X PSG, and 250 $\mu\text{g}/\text{mL}$ Geneticin (Invitrogen).

3.4.2. Standard Luciferase Assay (36)

1. Detach MLEC with trypsin and suspend them at 5×10^5 cells/mL in complete growth medium.
2. Plate 50 μL /well (2.5×10^4 cells) in a 96-well plate (*see Note 16*).
3. Let the cells attach for 3–4 h.
4. According to the experimental design, replace the medium with 50 μL of each of the following:
 - a. Control medium to determine the basal levels of TGF- β produced by the transfected MLEC.
 - b. Control medium containing increasing concentrations of rTGF- β to generate a standard curve; this assay can be used to measure TGF- β in the 0.2–30 pM range (36).

- c. Conditioned medium from the experimental culture to measure active TGF- β .
 - d. Acid- or heat-activated conditioned medium to measure total (latent plus active) TGF- β .
 - e. Conditioned medium with neutralizing anti-TGF- β antibodies or nonimmune IgG to test the specificity of the PAI-1–luciferase induction.
5. Incubate the MLEC for 16–20 h at 37°C (*see Note 17*).
 6. Wash the cells two times with PBS and aspirate all the PBS after the second wash.
 7. Luciferase activity can be measured by various assays (**46**). In all protocols, cells transfected with luciferase expression plasmids are lysed to release the reporter protein luciferase. ATP and luciferin are added to the lysate in a luminometer. The enzyme catalyzes the ATP-dependent oxidation of the substrate, which emits light. Below, we describe the protocol used in our laboratory using a ML3000 Microtiter Plate Luminometer (Dynatech Labs. Inc., Chantilly, VA). Lyse the cells with 35 μ L of 1X cell lysis buffer (Analytical Luminescence, San Diego, CA) for 20' at room temperature.
 8. Transfer 30 μ L of the cell-extract to a Microlight1 96-well plate (Dynatech Labs. Inc., Chantilly, VA).
 9. 2" after injection of 110 μ L of freshly prepared luciferin buffer containing 800 μ M luciferin and 750 μ M ATP, emitted light is measured for 3" (*see Note 18*).
 10. The luciferase activity is recorded as relative light units (RLU). RLU values are converted to TGF- β activity (pg/mL) using the TGF- β standard curve.

3.4.3. Coculture Assay (**17,33**)

1. Detach MLEC and test cells with trypsin and suspend them at 5×10^5 cells/mL in complete growth medium.
2. Plate 50 μ L/well (2.5×10^4 cells) of MLEC in a 96-well plate.
3. Add 50 μ L/well (2.5×10^4 cells) of test cells (*see Notes 19–21*).
4. Add neutralizing anti-TGF- β antibodies to one set of wells to test the specificity of the PAI-1–luciferase induction (*see Note 22*).
5. Incubate the coculture for 16–20 h at 37°C.
6. Wash the cells two times with PBS and aspirate all the PBS after the second wash.
7. Lyse the cells and measure TGF- β activity as described in the standard luciferase assay.
8. The luciferase activity is recorded as relative light units (RLU). RLU values are converted to TGF- β activity (pg/mL) using the TGF- β standard curve obtained with the MLEC. The TGF- β activity in the coculture is compared with the TGF- β activity of the MLEC alone. TGF- β activity induced by different test cells can also be compared.

4. Notes

1. The amount of cells and the incubation time used to produce the conditioned medium may vary according to the experimental necessities and may need to be optimized.
2. Depending on the amount of active TGF- β produced by cells, the conditioned medium may need to be concentrated or diluted with fresh control medium in

order to fall within the optimal range of the assay. Note that concentration of the samples can result in losses of mature TGF- β (32) or activation of latent TGF- β .

3. When comparing several samples, one must normalize the conditioned medium to either the cell number or cell-extract protein concentration in the culture used to prepare the media.
4. The medium should be used immediately or kept at 4°C for short-term storage. Repeated freezing and thawing may result in activation of latent TGF- β .
5. The amount of TGF- β present in the conditioned medium after acidification or heating may be high. Serial dilutions of samples in fresh serum-free medium should be used in order for TGF- β concentrations to fall within the optimal range of the assay.
6. Although easier to perform, heat treatment may result in protein precipitation.
7. The razor blade must be a rigid one because the flexible type bends when pressure is applied and produces uneven wounds.
8. If using FCS, pass the eluate a second time through the column.
9. A blade should not be used for more than 20 wounds because a much-used blade has gaps in the cutting edge and leaves lines of cells attached to the plate.
10. Take care not to make the initial score in the plastic too deep, because cells will not be able to migrate across.
11. Multiple wounds can be made in the same plate.
12. The appropriate ratio between BAE cells and test cells has to be determined experimentally.
13. Bovine aortic epithelial cells can often be distinguished from other cell types by shape, size, and nuclear morphology. Otherwise, the BAE monolayer can be labeled before wounding with DiI-acetyl-LDL:
 - a. Incubate the cells with 10 mg/mL of DiI-acetyl-LDL in regular growth medium for 4 h at 37°C.
 - b. Wound the monolayer and wash three times with PBS.
 - c. Add the second cell type suspended in serum-free medium.
 - d. Incubate at 37°C for 16–20 h.
 - e. Wash three times with PBS.
 - f. Fix with 3% formaldehyde in PBS for 15' at room temperature.
 - g. Visualize labeled BAE cells by fluorescence microscopy using standard rhodamine excitation.
14. If not analyzed immediately, the sample can be stored at –20°C. Repeated freezing and thawing may result in loss of PA activity.
15. If after 2 h the counts are still low, the incubation can be continued. The reaction is usually stopped when <20% of the total radioactivity is released from the plates.
16. Test triplicates of each sample for accurate statistical manipulation.
17. Keep assay times less than 20 h in order to avoid complications as a result of the effect of TGF- β on the MLEC proliferation.
18. The delay time and measuring time of light detection may need to be optimized (46).
19. Test triplicates of each sample for accurate statistical manipulation.
20. The optimal ratio between MLEC and test cells has to be determined experimentally.

21. Test cells may need to be plated first (24 h, 48 h in advance).
22. Various test cells may nonspecifically suppress or induce basal luciferase expression by the MLEC.

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