ELISpot

Human Latent TGF-β1

Catalog Number EL246

For the quantitative determination of the frequency of cells releasing human latent TGF-β1.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Transforming growth factor beta (TGF-β) proteins (including the three closely related mammalian isoforms TGF-β1, 2 and 3) are pleiotropic cytokines that regulate extracellular matrix production, wound healing, immune functions, cell proliferation and differentiation. They belong to the large TGF-β superfamily, which also includes the activins and inhibins, bone morphogenetic proteins (BMPs), and growth/differentiation factors (GDFs) (1, 2).

The TGF-β1 cDNA encodes a 391 amino acid residue (aa) pre-proprotein with a 23 aa signal peptide and a 368 aa proprotein that is proteolytically processed via a furin-like proprotein convertase to generate an N-terminal 256 aa latency-associated peptide (LAP) and the C-terminal 112 aa mature TGF-β1 (3 - 7). Both LAP and mature TGF-β1 exist as disulfide-linked homodimers. After proteolytic cleavage and secretion, the two homodimers remain non-covalently associated as the small latent TGF-β1 complex. In most cell types, the small latent complex is also covalently linked via LAP to a latent TGF-β binding protein (LTBP) to form a secreted, large latent complex. The TGF-β1 present in the small or the large latent complex is not available for TGF-β receptor binding and activation, and is therefore latent. Whereas LAP is both necessary and sufficient to confer latency to TGF-β1, LTBP facilitates the proper folding and secretion of the small latent complex. Since LTBP is a structural component of the extracellular matrix, it also directs the localization of the latent complex to the extracellular matrix (8 - 13). To date, four LTBPs that share multiple EGF-like domains and four LTBP- and fibrillin-specific eight-cysteine domains have been cloned. Three of the four LTBPs [1, 3, or 4] have been shown to bind the small latent complexes of all TGF-β isoforms (14). The activation of the large and/or small latent TGF-β complexes is an important step that regulates TGF-β functions *in vivo.* Multiple activation mechanisms, involving protease-dependent (plasmin and matrix-metalloprotease) and protease-independent (binding of LAP to thrombospondin 1 or a subset of integrins) pathways, have been identified (4, 13, 15 - 19).

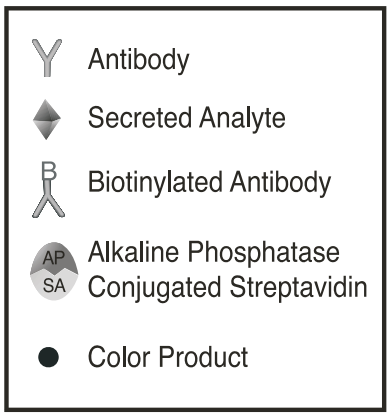
Although mature TGF-β1 is highly conserved (>99% aa sequence identity) across mammalian species, the sequences of LAP are more divergent. Human LAP (TGF-β1) shares approximately 85% aa sequence identity with that from mouse, rat and guinea pig. Human LAP (TGF-β1) also shares 98%, 92% and 91% aa sequence identity with monkey, dog and pig LAP (TGF-β1), respectively. Most cells, including hippocampal neurons (20), hepatocytes (21), vascular endothelial cells (22), $CD34^+$ stem cells (23), fetal osteoblasts (24), naive $CD4^+$ T cells (25), breast epithelium (26), macrophages (27, 28), thymic epithelium (29), fibroblasts (30), and platelets (31) can be induced to secrete latent TGF-β1. It is also suggested that B cells produce active TGF-β1 complexed to IgG (32, 33)

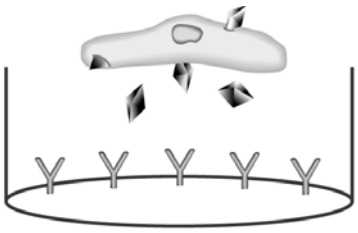
The Human Latent TGF-β1 ELISpot assay is designed for the detection of latent TGF-β1 secreting cells at the single cell level, and it can be used to quantitate the frequency of latent human TGF-β1 secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and they have been used for the quantitation of antigen-specific CD4 and/or CD8 T cell responses. Other methods for the assessment of antigen-specific T cell responses, such as the chromium release assay with quantitation by limiting dilution, are tedious, and require previous *in vitro* expansion of T cells for several days. These assays typically are not suitable for measuring infrequent T cell responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive, and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of T cells, and they are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in areas as diverse as antigen recognition, vaccine development, cytokine secretion and the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

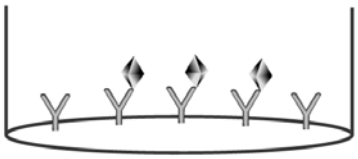
The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (34, 35). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (36, 37). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody specific for human LAP (TGF-β1) has been pre-coated onto a PVDF (polyvinylidene difluoride)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37° C CO_2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted latent TGF-β1. After washing away any cells and unbound substances, a biotinylated monoclonal antibody specific for human LAP (TGF-β1) is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual latent TGF-β1 secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

ELISpot SCHEMATIC

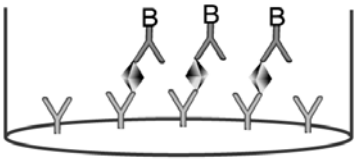




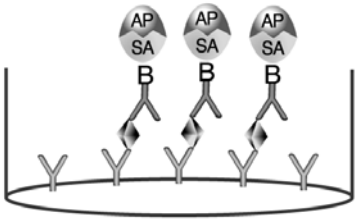
Incubate latent TGF-β1 secreting cells in an antibody-coated well.



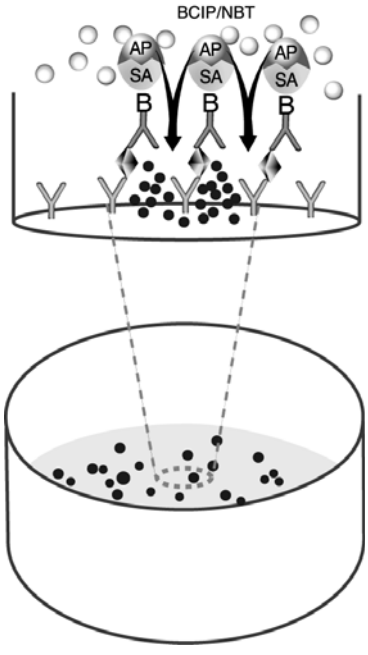
Remove cells by washing. Secreted latent TGF-β1 is captured by the immobilized antibody.



Incubate with biotinylated anti-latent TGF-β1 antibody.



Incubate with alkaline phosphatase conjugated streptavidin.



Add substrate and monitor the formation of colored spots.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in density of spots, intensity of specific staining and background levels.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Do not use reagents from this kit with components from other R&D Systems' ELISpot or ELISA kits and/or components manufactured by other vendors.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with BCIP/NBT chromogen.

Although the toxicity of the chromogenic substrate BCIP/NBT is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used BCIP/NBT.

MATERIALS PROVIDED

Human Latent TGF-β1 Microplate (Part 892205) - One 96-well PVDF-backed microplate coated with monoclonal antibody specific for human LAP (TGF-β1).

Detection Antibody Concentrate (Part 892206) - 150 µL of a 120-fold concentrated solution of biotinylated monoclonal antibody specific for human LAP (TGF-β1) with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 µL of a 120-fold concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives.

Dilution Buffer 2 (Part 895354) - 12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.

Wash Buffer Concentrate (Part 895308) - 50 mL of a 10-fold concentrated solution of a buffered surfactant with preservative.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3′-Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Human Latent TGF-β1 Positive Control (Part 892207) - 1 vial (8 ng) of recombinant human LAP (TGF-β1); lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO_2 incubator
- Sterile culture media
- Dissection microscope or an automated ELISpot reader

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membranes.
- After completion of experiment, do not dry the microplate at a temperature higher than 37° C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment.

  Positive Control - Use recombinant human LAP (TGF-β1).

  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

  Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

REAGENT PREPARATION

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Human Latent TGF-β1 Positive Control - Reconstitute lyophilized LAP (TGF-β1) with 250 µL of culture medium that is used to incubate cells.

Detection Antibody - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare Detection Antibody immediately before use.**

Streptavidin-AP - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare Streptavidin-AP immediately before use.**

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator. R&D Systems' cell selection products are suitable for the purification of effector and responder cells. For a complete product listing of human, mouse, and rat cell selection products, see the R&D Systems catalog or visit our website at www.RnDSystems.com.

ASSAY PROCEDURE

Bring all reagents as needed to room temperature. The Detection Antibody Concentrate and Dilution Buffer 1 should remain at 2 - 8° C. All samples and controls should be assayed at least in duplicate. An Assay Record Template is provided at the back of this insert to record controls and samples assayed.

1. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells and controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37° C CO_2 incubator. Optimal incubation time for each stimuli should be determined by each investigator. **Do not disturb the cells during the incubation period.**
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250 - 300 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
   Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.
5. Add 100 µL of diluted Detection Antibody into each well and incubate at 2 - 8° C overnight.
6. Repeat step 4.
7. Add 100 µL of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat step 4.
9. Add 100 µL of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light.**
10. Discard the chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels and dry completely either at room temperature (60 - 90 minutes) or 37° C (15 - 30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots either manually using a dissection microscope or by using a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

Transfected CHO cells (5 x 10^3 cells/mL) were incubated overnight at 37° C in a 5% CO_2 incubator. The sample was assayed in seven wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	78
2	80
3	76
4	75
5	78
6	80
7	77

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with distilled water, the dark-blue background color of filter membrane attenuates visualization and quantitation of spots	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry. Usually it takes from 15 to 30 minutes at ~37° C or about 60 - 90 minutes at room temperature
The number of spots in the wells that contained the cells is high but their contrast as well as intensity of staining in the Positive Control wells is low	Underdevelopment perhaps as a result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted to room temperature	Adjust the temperature of the reagents to room temperature before adding to the wells
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue	Cell stimulation problem	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells added to the wells	Increase the number of cells added per well
Following incubation with BCIP/NBT and drying the microplate, the density of the spots makes it difficult to quantify them	Too many cells were added to the wells	Make dilutions of cells, *i.e.,* 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4 cells per well to determine the optimal number of cells that will result in formation of distinct spots

REFERENCES

1. Kingsley, D.M. (1994) Genes Dev. **8**:133.
2. Burt, D.W. and A.S. Law (1994) Prog. Growth Factor Res **5**:99.
3. Derynck, R. *et al.* (1985) Nature **316**:701.
4. Clark, D.A. and R. Coker (1998) Int. J. Biochem. Cell Biol. **30**:293.
5. Lawrence, D.A. (2001) Mol. Cell. Biochem. **219**:163.
6. Gentry, L.E. *et al.* (1988) Mol. Cell. Biol. **8**:4162.
7. Dubois, C.M. *et al.* (1995) J. Biol. Chem. **270**:10618.
8. Brunner, A.M. *et al.* (1989) J. Biol. Chem. **264**:13660.
9. Mittl, P.R.E. *et al.* (1996) Protein Sci. **5**:1261.
10. Gray, A.M. and A.J. Mason (1990) Science **247:**1328.
11. Oklu, R. and R. Hesketh (2000) Biochem. J. **352**:601.
12. Saharinen, J. *et al.* (1996) EMBO J. **15**:245.
13. Mangasser-Stephan, K. and A.M. Gressner (1999) Cell Tissue Res. **297**:363.
14. Koli, K. *et al.* (2001) J. Cell Sci. **114**:2869.
15. Yang, L. *et al.* (2000) Wound Rep. Reg. **8**:538.
16. Pedrozo, H.A. *et al.* (1999) Endocrinology **140**:5806.
17. Yu, Q. and I. Stamenkovic (2000) Genes Dev. **14**:163.
18. Maeda, S. *et al.* (2001) J. Bone Miner. Res. **16**:1281.
19. Munger, J.S. *et al.* (1999) Cell **96**:319.
20. Zhu, Y. *et al.* (2000) Brain Res. **866**:286.
21. Roth-Eichhorn, S. *et al.* (1998) Hepatology **28**:1588.
22. Schultz-Cherry, S. and J.E. Murphy-Ullrich (1993) J. Cell. Biol. **122**:923.
23. Majka, M. *et al.* (2001) Blood **97**:3075.
24. Wu, Y. and R. Kumar (2000) J. Bone Miner. Res. **15**:879.
25. Seder, R.A. *et al.* (1998) J. Immunol. **160**:5719.
26. Chakravarthy, D. *et al.* (1999) Int. J. Oncol. **15**:187.
27. Singhal, P.C. *et al.* (1999) J. Immunol. **162**:3031.
28. Marriott, I. and K.L. Bost (1998) Cell. Immunol. **183**:113.
29. Schluns, K.S. *et al.* (1995) Int. Immunol. **7**:1681.
30. Ghahary, A. *et al.* (2000) Growth Factors **17**:167.
31. Miyazono, K. *et al.* (1988) J. Biol. Chem. **263**:6407.
32. Rowley, D.A. and R.M. Stach (1998) Int. Immunol. **10**:355.
33. Carver, T.E. *et al.* (1996) J. Clin. Invest. **98**:2496.
34. Czerkinsky, C.C. *et al*. (1983) J. Immunol. Methods **65**:109.
35. Sedgwick, J.D. and P.G. Holt (1983) J. Immunol. Methods **57**:301.
36. Czerkinsky, C.C. *et al*. (1984) J. Immunol. Methods **72**:489.
37. Helms, T. *et al.* (2000) J. Immunol. **164**:3723.

ASSAY RECORD TEMPLATE

This template may be used as a record of samples and controls run in an assay.