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Data Sheet

CTLA4 [Biotinylated]:B7-1 Inhibitor Screening Assay Kit

Catalog #79973

Size: 96 reactions

DESCRIPTION: The activation of naïve T cells requires two signals, the specific T cell receptor recognition of MHC/Antigen on the surface of the antigen-presenting cell (APC), and the binding of B7-1 (CD80) ligand on the APC with the CD28 receptor on the T cell surface. Conversely, binding of CTLA4 to B7-1 on the T-cell surface results in an inhibitory signal and prevents T-cell activation. CTLA4B7-1 interaction is an important drug target for the regulation of the host's response to cancer. The *CTLA4 [Biotinylated]:B7-1 Inhibitor Screening Assay Kit* is designed for screening and profiling inhibitors of CTLA4:B7-1 signaling. This kit comes in a convenient 96-well format, with biotin-labeled CTLA4 (CTLA4[B]), purified B7-1, streptavidin-labeled HRP, and assay buffer for 100 binding reactions. The key to this kit is the high sensitivity of detection of biotin-labeled CTLA4 by streptavidin-HRP. Only a few simple steps on a microtiter plate are required for the assay. First, B7-1 is coated on a 96-well plate. Next, CTLA4[B] is incubated with B7-1 on the plate. Finally, the plate is treated with streptavidin-HRP followed by addition of an HRP substrate to produce chemiluminescence, which can be measured using a chemiluminescence reader.

COMPONENTS:

| Catalog # | Component | Amount | Storage | |
|-----------|--|--------|-----------|------------------------------------|
| 71125 | B7-1 | 10 µg | -80°C | (Avoid freeze/thaw cycles!) |
| 71152 | CTLA4, Biotin-labeled | >1 µg | -80°C | |
| 79742 | Streptavidin-HRP | 15 µl | +4°C | |
| 79311 | 3x Immuno Buffer 1 | 50 ml | -20°C | |
| 79728 | Blocking Buffer 2 | 50 ml | +4°C | |
| 79670 | ELISA ECL substrate A (transparent bottle) | 6 ml | Room temp | |
| | ELISA ECL substrate B (brown bottle) | 6 ml | Room temp | |
| 79699 | White 96-well microplate | 1 | +4°C | |

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MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED:

PBS (Phosphate buffered saline)
Luminometer or fluorescent microplate reader capable of reading chemiluminescence
Rotating or rocker platform

APPLICATIONS: This kit is useful for screening for inhibitors of B7-1 binding to CTLA4.

STABILITY: One year from date of receipt when stored as directed.

REFERENCES:

1. Linsley, P.S., *et al. J. Exp. Med.* 1991, **174(3)**: 561-569.
2. Hurwitz, A.A., *et al. Canc. Res.* 2000, **60**: 2444-2448.

ASSAY PROTOCOL:

All samples and controls should be tested in duplicate.

Coating the plate with B7-1:

- 1) Thaw **B7-1** on ice. Upon first thaw, briefly spin tube containing **B7-1** to recover the full contents of the tube. Aliquot into single use aliquots. Immediately store remaining **B7-1** in aliquots at -80°C. *Note: **B7-1** is very sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles.*
- 2) Dilute **B7-1** to 2 µg/ml in PBS.
- 3) Add 50 µl of diluted **B7-1** solution to each well and incubate overnight at 4°C. Leave a couple of wells empty (uncoated), for use with the "Ligand Control" (see below).
- 4) Dilute **3x Immuno Buffer 1** to **1x Immuno Buffer 1** with water. Reserve some undiluted **3x Immuno Buffer 1** for use in later steps of the assay (below).
- 5) Decant to remove supernatant. Wash the plate three times with 100 µl **1x Immuno Buffer 1**. Tap plate onto clean paper towels to remove liquid.
- 6) Block wells by adding 100 µl of **Blocking Buffer 2** to each well. Incubate for 1 hour at room temperature. Remove supernatant as described in step 5.

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Step 1:

- 1) Prepare the master mixture: N wells × (10 µl **3x Immuno Buffer 1** + 15 µl H₂O).
- 2) Add 25 µl of master mixture to each well. Use uncoated wells for the “Ligand Control.”
- 3) Prepare the test inhibitor solution.

The final concentration of DMSO in the assay should not exceed 1%. If the inhibitor compound is dissolved in DMSO, make a 100-fold higher concentration of the compound than the highest concentration you want to test in DMSO. Then make a 10-fold dilution in water (at this step the compound concentration is 10-fold higher than the final concentration).

If the inhibitor compound is soluble in water, make an aqueous solution of the compound 10-fold higher than the final concentration.

- 4) Add 5 µl of test inhibitor solution to each well designated “Test Inhibitor.” For the “Positive Control” and “Blank,” add 5 µl of inhibitor buffer (water or 10% DMSO in water, depending which inhibitor solution is used). Incubate at room temperature for one hour.
- 5) Thaw **CTLA4-biotin** on ice. Upon first thaw, briefly spin tube containing enzyme to recover full contents of the tube. Aliquot **CTLA4-biotin** into single use aliquots. Immediately store remaining undiluted enzyme in aliquots at -80°C. *Note: **CTLA4-biotin** is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots or diluted enzyme.*

| | Blank | Positive Control | Test Inhibitor |
|----------------------------|--------------|------------------|----------------|
| 3x Immuno Buffer 1 | 10 µl | 10 µl | 10 µl |
| H ₂ O | 15 µl | 15 µl | 15 µl |
| Test Inhibitor | – | – | 5 µl |
| Inhibitor buffer | 5 µl | 5 µl | – |
| 1x Immuno Buffer 1 | 20 µl | – | – |
| CTLA4-biotin (0.125 ng/µl) | – | 20 µl | 20 µl |
| Total | 50 µl | 50 µl | 50 µl |

- 6) Dilute **CTLA4-biotin** to 0.125 ng/µl in **1x Immuno Buffer 1**. Keep diluted protein on ice until use. Discard any unused diluted protein after use.
- 7) Add 20 µl of **1x Immuno Buffer 1** to the well designated “Blank.”

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- 8) Initiate reaction by adding 20 μ l of diluted **CTLA4-biotin** (see Step 1-5) to wells labeled "Positive Control" and "Test Inhibitor." Incubate at room temperature for two hours.
- 9) Decant to remove supernatant. Wash the plate 3 times with 100 μ l/well **1x Immuno Buffer 1**. Tap plate onto clean paper towels to remove liquid.
- 10) Block wells by adding 100 μ l of **Blocking Buffer 2** to each well. Incubate for 10 minutes at room temperature. Remove supernatant as in Step 1-9.

Step 2:

- 1) Dilute **Streptavidin-HRP** 1000-fold with **Blocking Buffer 2**.
- 2) Add 100 μ l to each well. Incubate for 1 hour at room temperature with slow shaking.
- 3) Wash plate three times with **1x Immuno Buffer 1**. Tap plate onto clean paper towel to remove liquid.
- 4) Block wells by adding 100 μ l of **Blocking Buffer 2** to each well. Incubate for 10 minutes at room temperature. Decant to remove supernatant. Tap plate onto clean paper towels to remove liquid.
- 5) Just before use, mix on ice 50 μ l **ELISA ECL Substrate A** and 50 μ l **ELISA ECL Substrate B**, then add 100 μ l to each well. Discard any unused chemiluminescent reagent after use.
- 6) Immediately read sample in a luminometer or microtiter-plate capable of reading chemiluminescence. "Blank" value is subtracted from all readings.

Reading Chemiluminescence:

Chemiluminescence is the emission of light (luminescence) which results from a chemical reaction. The detection of chemiluminescence requires no wavelength selection because the method used is emission photometry and is not emission spectrophotometry.

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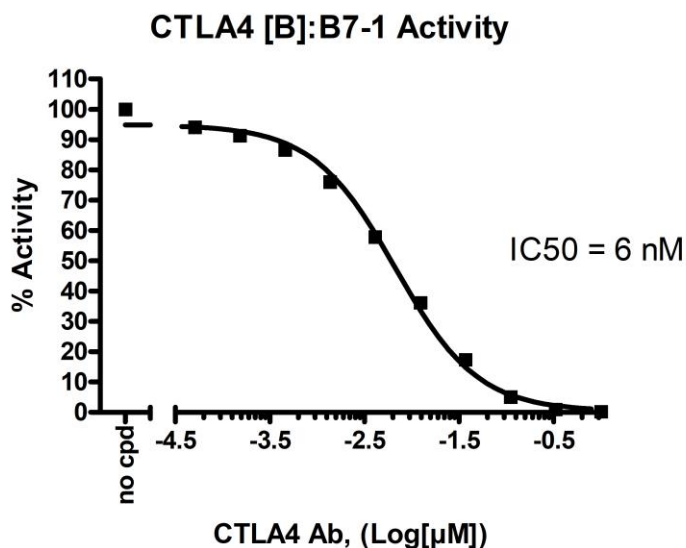
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To properly read chemiluminescence, make sure the plate reader is set for LUMINESCENCE mode. Typical integration time is 1 second; delay after plate movement is 100 msec. Do not use a filter when measuring light emission. Typical settings for the Synergy 2 BioTek plate reader are: use the "hole" position on the filter wheel; Optics position: Top; Read type: endpoint. Sensitivity may be adjusted based on the luminescence of a control assay without enzyme (typically we set this value as 100).

Example of Assay Results:



Inhibition of CTLA4 [B]:B7-1 binding using the *CTLA4 Neutralizing Antibody*, BPS Bioscience #71212 and the *CTLA4 [Biotinylated]:B7-1 Inhibitor Screening Assay Kit*. Luminescence was measured using a Bio-Tek fluorescent microplate reader. *Data shown is lot-specific. For lot-specific information, please contact BPS Bioscience, Inc. at info@bpsbioscience.com.*

RELATED PRODUCTS:

| <u>Product Name</u> | <u>Catalog #</u> | <u>Size</u> |
|---|------------------|-------------|
| CTLA4 (CD152), Fc fusion | 71149 | 100 µg |
| CTLA4 (CD152), Biotin labeled | 71152 | 50 µg |
| B7-1 | 71125 | 100 µg |
| B7-1, Biotin labeled | 71114 | 50 µg |
| B7-2 | 71150 | 100 µg |
| CD28 | 71113 | 200 µg |
| CTLA4 (CD152) Neutralizing Antibody | 71212 | 50 µg |
| CD28:B7-1[Biotinylated] Inhibitor Screening Assay Kit | 72007 | 96 rxns |

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TROUBLESHOOTING GUIDE

| Problem | Possible Cause | Solution |
|--|---|---|
| Luminescence signal of positive control reaction is weak | CTLA4 or B7-1 has lost activity | Enzyme loses activity upon repeated freeze/thaw cycles. Use fresh B7-1, (BPS Bioscience #71125) and fresh biotin-labeled CTLA4 (BPS Bioscience #71152). Store proteins in single-use aliquots. Increase time of enzyme incubation. Increase enzyme concentration. |
| | Antibody reaction is insufficient | Increase time for primary antibody incubation. Avoid freeze/thaw cycles of antibodies. |
| | Incorrect settings on instruments | Refer to instrument instructions for settings to increase sensitivity of light detection. |
| | Chemiluminescent reagents mixed too soon | Chemiluminescent solution should be used within 15 minutes of mixing. Ensure both reagents are properly mixed. |
| Luminescent signal is erratic or varies widely among wells | Inaccurate pipetting/technique | Run duplicates of all reactions. Use a multichannel pipettor. Use master mixes to minimize errors. |
| | Bubbles in wells | Pipette slowly to avoid bubble formation. Tap plate lightly to disperse bubbles; be careful not to splash between wells. |
| Background (signal to noise ratio) is high | Insufficient washes | Increase number of washes. Increase wash volume. Increase Tween-20 concentration to 0.1% in PBST. |
| | Sample solvent is inhibiting the enzyme | Run negative control assay including solvent. Maintain DMSO level at <1% Increase time of enzyme incubation. |
| | Results are outside the linear range of the assay | Use different concentrations of CTLA4-biotin (BPS Bioscience #71152) to create a standard curve. |

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