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Data Sheet

GITRL:GITR[Biotinylated] Inhibitor Screening Assay Kit

Catalog # 72061
Size: 96 reactions

BACKGROUND: Glucocorticoid-induced TNFR-related protein (GITR) is a member of the TNFR superfamily, expressed in a number of cells including T cells, natural killer (NK) cells and antigen-presenting cells (APC). GITRL, GITR's natural ligand, is expressed mainly by APCs and GITR:GITRL interaction is important for activation of the immune system. Agonistic antibodies targeting GITR are actively being pursued as potential immuno oncology therapies.

DESCRIPTION: The *GITR:GITRL [Biotinylated] Inhibitor Screening Assay Kit* is designed for screening and profiling inhibitors of GITR:GITRL signaling. This kit comes in a convenient 96-well format, with biotin-labeled GITR (CD357), purified GITRL, streptavidin-labeled HRP, and Immunobuffer for 100 binding reactions. The key to this kit is the high sensitivity of detection of biotin-labeled GITR by streptavidin-HRP. Only a few simple steps on a microtiter plate are required for the assay. First, GITRL is coated on a 96-well plate. Next, GITR is incubated with GITRL 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	
71256	GITR, Biotin-labeled	2 µg	-80°C	***(Avoid freeze/ thaw cycles!)***
71190	GITRL-His	10 µg	-80°C	
79742	Streptavidin-HRP	15 µl	+4°C	
79311	3x Immunobuffer 1	50 ml	-20°C	
79728	Blocking Buffer 2	50 ml	+4°C	
79670	ELISA ECL substrate A (transparent bottle)	6 ml	RT	
	ELISA ECL substrate B (brown bottle)	6 ml	RT	
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 GITRL binding to GITR.

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

REFERENCES:

1. Clouthier, D.L., Watts, T.H. *Cytokine Growth Factor Rev.* 2014, **25(2):**91-106.
2. Pascutti, M.F., *et al. PLoS Pathog*. 2015, **11(3):**e1004675.

ASSAY PROTOCOL:
All samples and controls should be tested in duplicate.

Coating the plate with GITRL:

1) Thaw **GITRL** on ice. Upon first thaw, briefly spin tube containing **GITRL** to recover the full contents of the tube. Aliquot into single use aliquots. Immediately store remaining GITRL in aliquots at -80°C. *Note: GITRL is very sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles.*

2) Dilute **GITRL** to 2 µg/ml in PBS.

3) Add 50 µl of diluted **GITRL** 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 Immunobuffer 1** to **1x Immunobuffer 1** with water. Dilute only enough **3x Immunobuffer 1** required for washing the plate (below).

5) Decant to remove supernatant. Wash the plate 3 times with 100 µl **1x Immunobuffer 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 4.

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Step 1:

1) Prepare the master mixture: N wells × (10 µl **3x Immunobuffer 1** + 15 µl H_2O).
2) Add 25 µl of master mixture to each well. Use uncoated wells for the "Ligand Control".

3) Add 5 µl of inhibitor solution to each well designated "Test Inhibitor". For the "Positive Control", "Ligand Control" and "Blank", add 5 µl of the same solution without inhibitor (inhibitor buffer). Incubate at room temperature for one hour.

4) Thaw **GITR-biotin** on ice. Upon first thaw, briefly spin tube containing enzyme to recover full contents of the tube. Aliquot **GITR-biotin** into single use aliquots. Immediately store remaining undiluted enzyme in aliquots at -80°C. *Note: GITR-biotin is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots or diluted enzyme.*

	Blank	Ligand Control	Positive Control	Test Inhibitor
3x Immunobuffer 1	10 µl	10 µl	10 µl	10 µl
H_2O	15 µl	15 µl	15 µl	15 µl
Test Inhibitor/Activator	–	–	–	5 µl
Inhibitor buffer (no inhibitor)	5 µl	5 µl	5 µl	–
1x Immunobuffer 1	20 µl	–	–	–
GITR-biotin (0.25 µg/ml)	–	20 µl	20 µl	20 µl
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**

5) Dilute **GITR-biotin** to 0.25 µg/ml (5 ng/20 µl) in **1x Immunobuffer 1**. Keep diluted protein on ice until use. Discard any unused diluted protein after use.

6) Add 20 µl of **1x Immunobuffer 1** to the well designated "Blank".

7) Initiate reaction by adding 20 µl of diluted **GITR-biotin** (see Step 1-5) to wells labeled "Positive Control", "Ligand Control", and "Test Inhibitor". Incubate at room temperature for two hours.

8) Decant to remove supernatant. Wash the plate three times with 100 µl/well **1x Immunobuffer 1**. Tap plate onto clean paper towels to remove liquid.

9) Block wells by adding 100 µl of **Blocking Buffer 2** to each well. Incubate for 10 minutes at room temperature. Remove supernatant as in Step 1-8.

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Step 2:

1) Dilute **Streptavidin-HRP** 1000-fold with **Blocking Buffer 2**.

2) Add 100 µl to each well. Incubate for 1 hour at room temperature with slow shaking.

3) Wash plate three times with **1x Immunobuffer 1**. Tap plate onto clean paper towels to remove liquid.

4) Block wells by adding 100 µl 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 µl **ELISA ECL Substrate A** and 50 µl **ELISA ECLSubstrate B**, then add 100 µl 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.

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).

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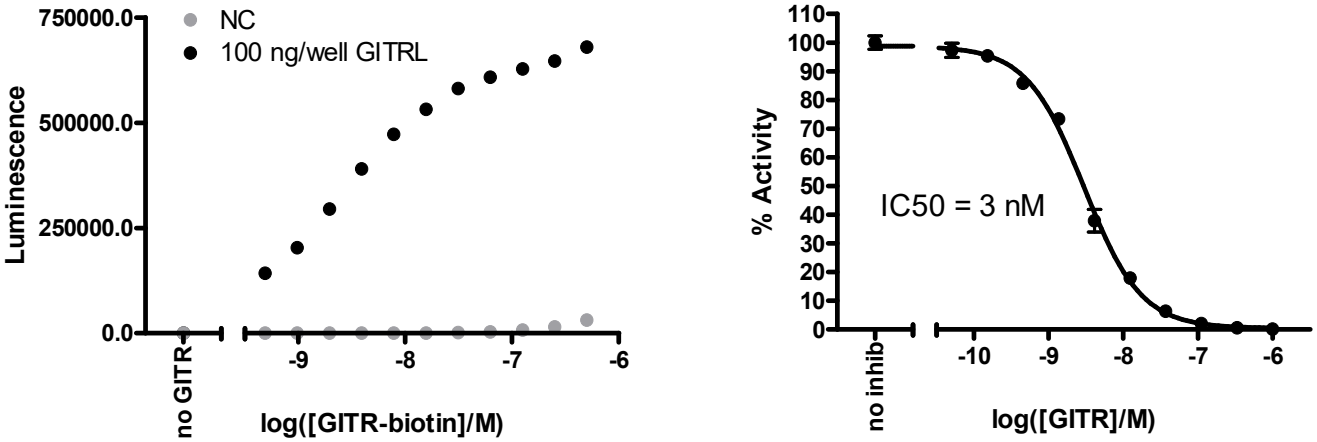
Example of Assay Results:



(Left) Interaction of GITRL with GITR-biotin using the *GITRL:GITR [Biotinylated] Inhibitor Screening Assay Kit*, BPS Bioscience, Catalog #72061 (non-coated wells, NC). (Right) Inhibition of GITRL:GITR interaction by unlabeled GITR. *Data shown is lot-specific. For lot-specific information, please contact BPS Bioscience, Inc. at info@bpsbioscience.com.*

RELATED PRODUCTS:

Product Name	Catalog #	Size
GITR (CD357), Fc fusion	71172	100 µg
GITRL, His-tag	71190	100 µg
Human CD137 (4-1BB), Fc fusion	71170	100 µg
Human CD137 (4-1BB), Fc Fusion, Biotin-labeled	71171	50 µg
Mouse CD137, Fc fusion, Avi-tag	71254	100 µg
Mouse CD137, Fc fusion, Biotin-labeled	71255	50 µg

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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
Luminescence signal of positive control reaction is weak	GITR or GITRL has lost activity	Enzyme loses activity upon repeated freeze/thaw cycles. Use fresh GITR-biotin, (BPS Bioscience #71256) and fresh GITRL (BPS Bioscience #71190). Store proteins in single-use aliquots. Increase time of enzyme incubation. Increase enzyme concentration.
	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 GITR-biotin (BPS Bioscience #71256) to create a standard curve.