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SEAP reporter assay for assessing NF-κB inhibition by TGF-β1/PD-L1 fusion protein

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We use this protocol and it's working

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Abstract

This protocol describes a secreted embryonic alkaline phosphatase (SEAP)–based reporter assay to evaluate NF-κB pathway inhibition mediated by a TGF-β1/PD-L1 fusion protein. Human TLR2 Reporter HEK 293 cells are stably modified to express the PD-1 receptor and used as a cellular model to assess downstream NF-κB activation following Toll-like receptor 2 stimulation. NF-κB signaling is induced using graded dilutions of Mycobacteria-containing Freund's Complete Adjuvant, and pathway activity is quantified via SEAP secretion into the culture medium. Treatment with the TGF-β1/PD-L1 fusion protein is compared against antibody-blocked and untreated controls to determine receptor-dependent inhibition of NF-κB activation. This assay provides a reproducible and modular framework for mechanistic validation of immunomodulatory proteins affecting inflammatory signaling pathways.

Protocol materials

Imject™ Freund's Complete Adjuvant (FCA) **Thermo Fisher Catalog #**77140

HEK Blue Detection Medium **InvivoGen**

Safety warnings

This assay is sensitive to the presence of endotoxins. Before performing the experiment, ensure first that your culture is not contaminated and your reagents are endotoxin-free.

Before start

This protocol uses Human TLR2 Reporter HEK 293 Cells from InvivoGen, lipofected stably with pCMV3-PD1 to express the PD-1 receptor. Please contact the author regarding inquiries on how to reconstitute the PD-1 protein in these cells or different eukaryotic cell models.

Confirmation of receptor expression in PD1^{+} TLR2^{+} HEK 293 cells

44m

1 Perform immunofluorescence (IF) analysis using anti-TGFBR1 and anti-PD1 antibodies. Note that you can use FITC-conjugated antibodies (direct) or premix the primary antibodies with FITC-conjugated secondary antibodies in 1:1 ratio (indirect)

1.1 Harvest 1 ×10^{6} cells and collect by centrifugation 500 x g, 25°C, 00:05:00 5m

1.2 Resuspend the cell pellet in 100 µL of PBS, repeat go to step #1.1 once

1.3 Add 100 µL of staining buffer and resuspend the cells well by pipetting

	A	B
	Reagent	**Amount needed**
	Bovine serum albumin (BSA)	0.05% (w/v)
	FITC-labelled antibody	1:100 (antibody:buffer ratio)
	PBS	Fill to 100 uL

Composition of staining buffer

1.4 Incubated at 4 °C for 00:30:00 in the dark with constant shaking. Note that over-exposure to light may photobleach FITC signal 30m

1.5 Wash the cells in 5 mL PBS-T, twice.

	A	B
	Reagent	**Amount needed**
	Tween-20	0.01% (w/v)
	PBS	5 mL

Composition of PBS-T buffer

1.6 Collect the cells by centrifugation 500 x g, 25°C, 00:03:00 and resuspend the cells in 25 µL of PBS with or without 0.5 µg/mL of Hoechst 33342. Incubate for 00:05:00 at room temperature 8m

1.7 Wash the cells once with 5 mL PBS and resuspend to a final volume 100 µL of PBS

1.8 Aliquot desired amount and dispense to poly-L-lysine coated slides, incubate in the dark for at least 00:01:00 prior to visualization under a fluorescence microscope 1m

SEAP reporter assay 2d

2 Seed 1×10^5 cells in a 96-well plate, each well should not exceed 200 µL

3 After 24:00:00, replace the medium with same volume of HEK Blue Detection Medium **InvivoGen** 1d

4 Challenge the cells with an increasing dilution of *Mycobacteria*-containing Imject™ Freund's Complete Adjuvant (FCA) **Thermo Fisher Catalog #**77140

	A	B
	Set-up	**Dilution**
	1	1:100
	2	1:1,000
	3	1:10,000
	4	1:50,000
	5	1:100,000
	Control	None (PBS only)

Dilution reactions for the experiment

Note: Consider the number of replicates in your assays

5 Divide the set-up into fusion protein-treated, antibody-blocked, and untreated reactions. For this protocol, the inhibition of NF-κB activation by the TGF-β1/PD-L1 (TP) fusion protein is determined. Note that you can use a different immunomodulating protein or molecule for this assay.

	A	B
	Reaction	**Purpose**
	TP-treated	Assess the direct effect of receptor binding of TP with receptors in NF-κB activation
	Antibody-blocked	Identify the consequences of blocking the receptors of TP in NF-κB activation
	Untreated	Serves as control

SEAP reporter assay set-up; TP = TGF-β1/PD-L1 fusion protein

5.1 For TP-treated cells, add 50 ng/mL of fusion protein to the wells

5.2 For antibody-blocked cell, add 1 ug/mL of either anti-TGFBR1 and anti-PD1 antibodies, incubate for 00:15:00 , then add 50 ng/mL of fusion protein to the wells

5.3 For untreated cells, add the same volume of PBS to the wells

5.4 Culture the cells as usual for 24:00:00 1d

6 Check the culture plate for color development, image the plate and then read the optical density (OD) at 620-655 nm on a plate reader