Unlocking Agonism: A Comprehensive Strategy to Characterize Agonistic Antibodies for Immunotherapy & Autoimmunity

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Abstract

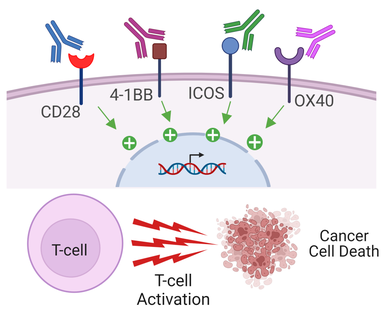
Checkpoint blockade antibodies, or checkpoint inhibitors, are established as effective cancer therapeutics. A contrasting class of therapeutics, agonistic checkpoint antibodies, has recently emerged for attenuating inflammation in autoimmune diseases. Despite showing clinical promise, their development remains challenging due to limitations in conventional checkpoint assay designs that often fail to replicate physiologically relevant conditions in vitro and are sometimes confined to assessing only inhibitory responses.

Here, we introduce a novel checkpoint assay design in a co-culture format involving Fcγ receptor-expressing cells, directly measuring the stimulatory activity of agonistic antibodies. Through testing various classes of Fcγ receptors and diverse cell backgrounds, we have established a specific and robust assay design for several key checkpoint receptors such as CD40, CD137, PD-1, and BTLA. Notably, we have identified previously unrecognized agonistic activity in a clinically approved blockade antibody, underscoring the importance of exploring similar activity in other therapeutics of this nature. The assay is optimized as a thaw-and-use format, exhibiting high reproducibility with ease of execution, and is qualified for potency and stability studies as needed for commercial release testing in a quality-controlled environment.

Active Areas of Clinical Development for Agonistic Antibodies

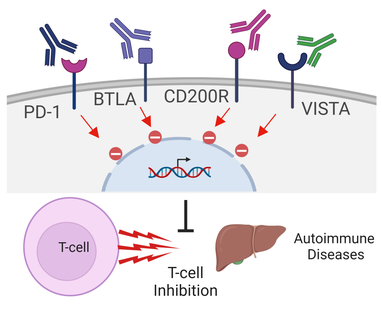
Cancer

Agonistic antibodies stimulating co-stimulatory receptors to activate T-cell to target cancer cells



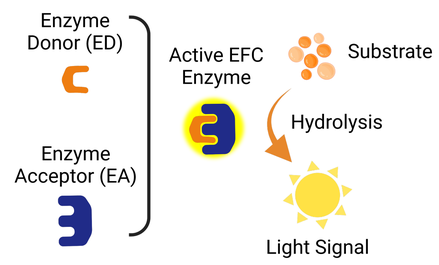
Autoimmune Diseases

Agonistic antibodies stimulating co-inhibitory receptors that inhibit T-cell activation and reduce inflammation in autoimmune diseases



Functional Cell Based Assays for Checkpoint Receptor Targets Based on EFC Technology

A **Enzyme Fragment Complementation (EFC) Technology**



B **PathHunter® NIK Signaling Assay Principle**

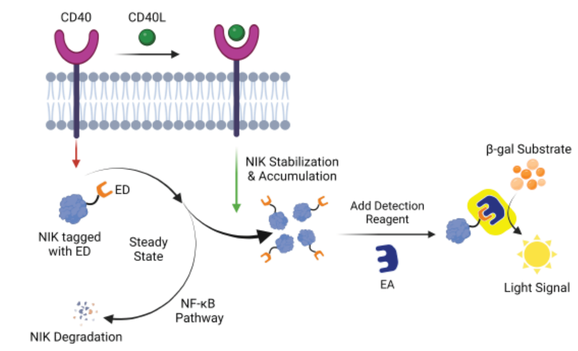
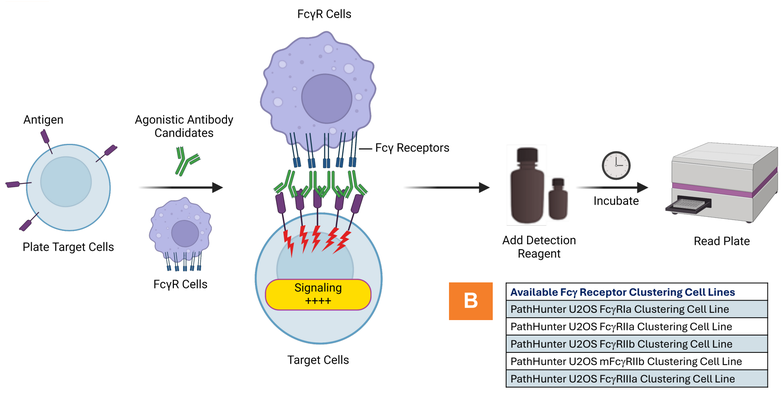


Figure 1. Eurofins DiscoverX's EFC assay design for the CD40 checkpoint receptor. A. The EFC technology uses a split β-galactosidase (β-gal) enzyme system. Individually, the enzyme donor peptide (ED) and enzyme acceptor (EA) are inactive fragments. When brought in proximity, ED complements with EA forming an active β-gal enzyme that hydrolyzes a substrate resulting in a highly sensitive chemiluminescent signal. B. The U2OS NIK signaling cell line is engineered to express ED-tagged NIK protein. Addition of CD40L (ligand) leads to activation of the NF-κB pathway resulting in stabilization and accumulation of the NIK protein with increasing concentrations of CD40L. Addition of the EFC substrate, EA, and buffer, lyses the cells and forces complementation of the ED and EA enzyme fragments. This results in the formation of a functional enzyme that hydrolyzes the substrate to generate a chemiluminescent signal that is proportional to the amount of NIK stabilized and accumulated in the cells.

FcγR Mediated Clustering Enhances Agonistic Effect of Antibodies Targeting Co-stimulatory Checkpoint Receptors

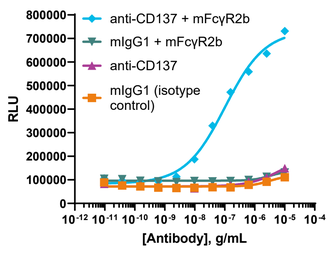
A **Assay Design with FcγR Clustering Cells for Studying Enhanced Agonistic Effect of Anti Co-stimulatory Checkpoint Antibodies**



B

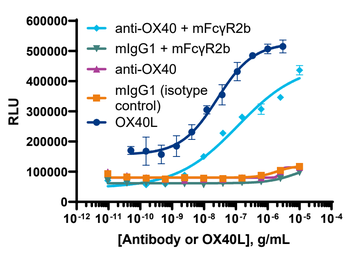
Available Fcγ Receptor Clustering Cell Lines
PathHunter U2OS FcγRIa Clustering Cell Line
PathHunter U2OS FcγRIIa Clustering Cell Line
PathHunter U2OS FcγRIIb Clustering Cell Line
PathHunter U2OS mFcγRIIb Clustering Cell Line
PathHunter U2OS FcγRIIIa Clustering Cell Line

C **PathHunter U2OS CD137 Signaling Assay**



	anti-CD137 + mFcγR2b	mIgG1 + mFcγR2b	anti-CD137	mIgG1 (isotype control)
S/B	8.8	1.4	6.6	1.7
EC_{50}	1.00E-07	3.00E-06	6.50E-06	3.10E-06

D **PathHunter U2OS OX40 Signaling Assay**

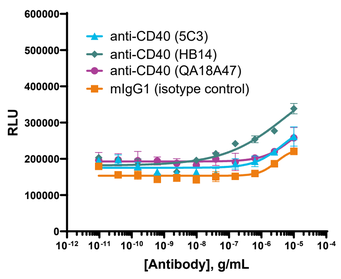


	anti-OX40 + mFcγR2b	mIgG1 + mFcγR2b	anti-OX40	mIgG1 (isotype control)	OX40L
S/B	9.9	2.0	1.4	1.5	3.4
EC_{50}	1.10E-07	7.60E-06	2.40E-06	1.90E-06	2.60E-08

Figure 2. FcγR mediated clustering enhances agonistic effect of antibodies. A. Including FcγR clustering cells in the assay design aids in clustering the antibodies and presenting them to the target cells resulting in enhanced signaling compared to currently used assay designs without FcγR mediated clustering. B. Available FcγR Clustering Cell Lines. C. and D. Enhanced agonism is observed with U2OS mouse FcγR2b (mFcγR2b) clustering cells in the PathHunter U2OS CD137 Signaling Assay and OX40 Signaling Assay, compared to the anti-CD137 or anti-OX40 antibodies alone, respectively E. Low agonistic activity was observed for three different clones of anti-CD40 antibodies (5C3, HB14, QA18A47) in the absence of FcγR clustering cells. F. Enhanced agonism was observed with clustering mediated by PathHunter U2OS mFcγR2b cells in all three clones of anti-CD40 antibodies exhibiting a rank order potency with HB14 being most potent and the 5C3 clone being the least potent.

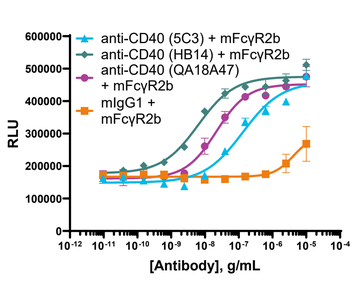
Rank Ordering Potencies of Candidate Antibodies

E **PathHunter U2OS CD40 Signaling Assay (Without FcγR Clustering Cells)**



	anti-CD40 (5C3)	anti-CD40 (HB14)	anti-CD40 (QA18A47)	mIgG1 (isotype control)
S/B	1.7	2.5	1.6	1.5
EC_{50}	4.30E-06	5.90E-06	7.70E-06	2.90E-06

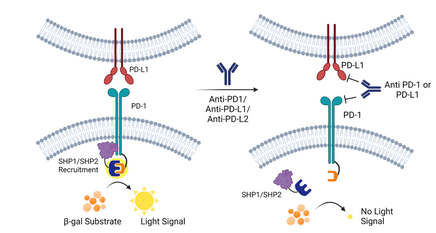
F **PathHunter U2OS CD40 Signaling Assay (With FcγR Clustering Cells)**



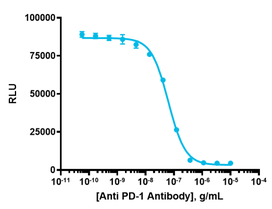
	anti-CD40 (5C3) + mFcγR2b	anti-CD40 (HB14) + mFcγR2b	anti-CD40 (QA18A47) + mFcγR2b	mIgG1 + mFcγR2b
S/B	1.8	3.1	2.7	1.5
EC_{50}	1.30E-07	6.30E-09	2.20E-08	4.20E-06

Introducing FcγR Clustering in the Assay Design Helps Identify a Strong Agonistic Effect in Antibodies Previously Established as Antagonists

A **PathHunter Jurkat PD-1 Signaling Assay Principle** (Antagonist Mode)

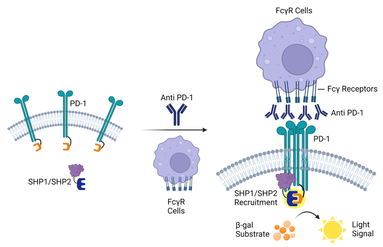


B **PathHunter Jurkat PD-1 Signaling Assay** (Tested in Antagonist Mode)

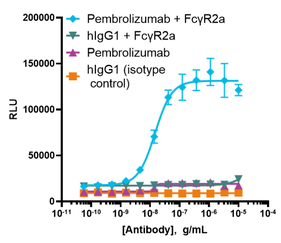


	Anti-PD1 Antibody
S/B	20.8
EC_{50}	1.02E-07

C **PathHunter Jurkat PD-1 Signaling Assay Principle** (Agonist Mode)

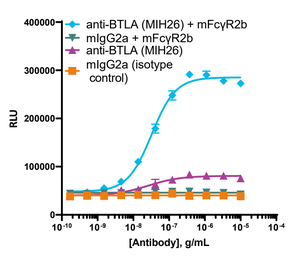


D **PathHunter Jurkat PD-1 Signaling Assay** (Tested in Agonist Mode)



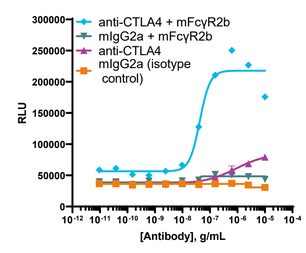
	Pembrolizumab + FcγR2a	hIgG1 + FcγR2a	Pembro-lizumab	hIgG1 (isotype control)
S/B	7.5	1.0	1.7	1.0
EC_{50}	14.7E-08	N/A	1.80E-08	N/A

E **PathHunter Jurkat BTLA Signaling Assay** (Tested in Agonist Mode)



	anti-BTLA + mFcγR2b	mIgG2a + mFcγR2b	anti-BTLA	mIgG2a (isotype control)
S/B	6.6	1.0	2.0	1.0
EC_{50}	3.20E-08	N/A	1.30E-07	N/A

F **PathHunter Jurkat CTLA4 Signaling Assay** (Tested in Agonist Mode)

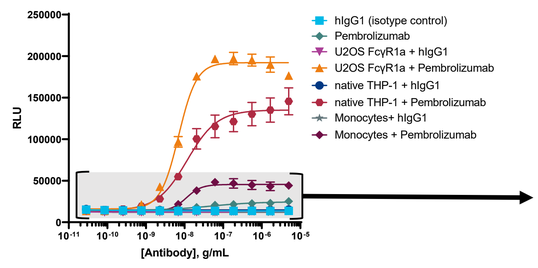


	anti-CTLA4 + mFcγR2b	mIgG2a + mFcγR2b	anti-CTLA4	mIgG2a (isotype control)
S/B	3.9	1.2	1.2	2.3
EC_{50}	4.30E-08	4.00E-08	2.60E-06	8.80E-07

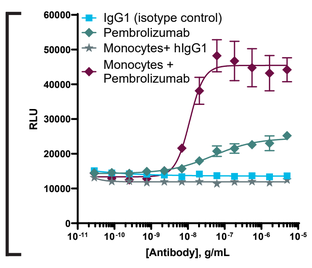
Figure 3. Including FcγR clustering cells in the assay design captures enhanced agonistic activity of antibodies previously defined as antagonists. A. The PathHunter Jurkat PD-1 Signaling Assay measures SHP1 recruitment to PD-1 ITIM motifs. When U2OS PD-L1 Ligand Cells are co-cultured with Jurkat PD-1 Signaling Cells, PD-L1 activates the PD-1 receptor and the SHP1 or SHP2 SH2-EA enzyme fragment fusion protein is recruited to PD-1 tagged with the complementary ED tag. Anti-PD-1 antibody disrupts the interaction between PD-1 and PD-L1, inhibiting PD-1 signaling, resulting in a loss of EFC-derived chemiluminescent signal. B. In antagonist mode, the PathHunter Jurkat PD-1 (SHP1) Signaling Assay exhibits dose-dependent blocking of PD-1 activation by anti-PD-1 antibody (NAT105). C. Including FcγR clustering cells in the assay design elicits enhanced agonistic activity driven by anti-PD1 antibodies like pembrolizumab in the Jurkat PD-1 signaling assay. D. FcγR mediated clustering significantly enhanced pembrolizumab's (a well-known anti-PD-1 therapeutic) agonistic activity in the Jurkat PD-1 Signaling Assay. E. and F. Results from the PathHunter Jurkat BTLA and CTLA4 Signaling Assays. These assays measure SHP recruitment as an activation mechanism. U2OS mFcγR2b cells enhance the agonistic effect of anti-BTLA and anti-CTLA4 antibodies as observed by the increased efficacy and potency in the presence of clustering cells compared to the antibody alone.

Primary and Immortalized Immune Cells with Endogenous FcγR Expression also Augment Agonistic Activity of Pembrolizumab

A **PathHunter Jurkat PD-1 Signaling Assay** (Tested in Agonist Mode)

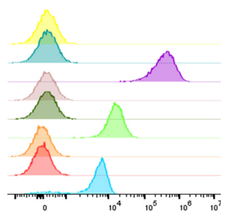


B **PathHunter Jurkat PD-1 Signaling Assay** (Tested in Agonist Mode) – Magnified from Figure 4. A.



	hIgG1 (isotype control)	Pembrolizumab	U2OS FcγR1a + hIgG1	U2OS FcγR1a + Pembrolizumab	native THP-1 + hIgG1	native THP-1 + Pembrolizumab	Monocytes + hIgG1	Monocytes + Pembrolizumab
S/B	1.0	1.8	1.0	14.6	1.0	9.5	1.0	3.5
EC_{50}	N/A	4.83E-08	N/A	7.00E-09	N/A	1.17E-08	N/A	1.11E-08

C **Clustering Mediated Agonistic Response to Pembrolizumab is Correlated to FcγR1a Cell Surface Expression**

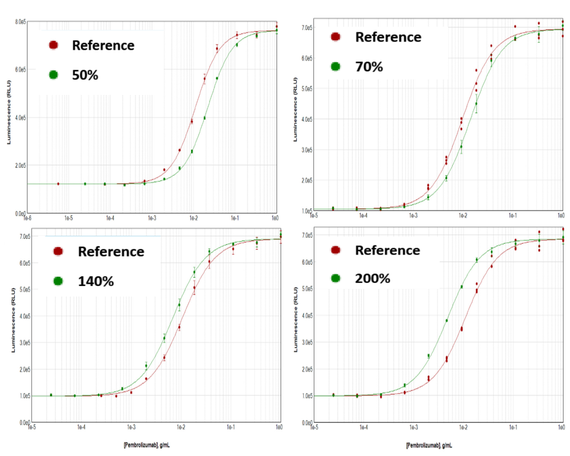


Sample Name	Antibody	Geometric Mean
FcγR1a Cell Line	Unstained	209
	Isotype Control	219
	Anti-FcγR1a	335524
Native THP-1 Cells	Unstained	191
	Isotype Control	198
	Anti-FcγR1a	14601
Primary Monocytes	Unstained	36.8
	Isotype Control	47.3
	Anti-FcγR1a	4158

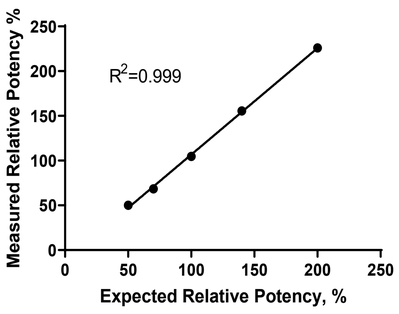
Figure 4. Agonistic activity of pembrolizumab is proportional to level of FcγR expression in multiple cell types. A. FcγR mediated clustering of pembrolizumab driven by U2OS FcγR1a, native THP-1, or monocytes demonstrates enhanced agonism in the Jurkat PD-1 Signaling Assay. B. Inset (magnified from A.) demonstrating increased agonistic activity of pembrolizumab with monocytes compared to pembrolizumab alone. C. Receptor density of FcγR1a in PathHunter U2OS FcγR1a Cell Line, native-THP1 cells, and primary monocytes using PE-labeled FcγR1a antibody.

PD-1 Bioassay Qualification with Pembrolizumab in Agonist Mode Using FcγR Clustering Cells

A



B **PathHunter Jurkat PD-1 (SHP1) Signaling Bioassay** Dilutional Linearity (50%-200%)



Parameter	Results	
	% Expected Relative Potency	% Recovery
Accuracy	50	100.4
	70	97.9
	100	104.7
	140	111.1
	200	113.0
Repeatability (%CV)	100% (Reference)	2.8%
Intermed. Pecision (%CV)		7.7%
Linearity (r^2)		0.999

Figure 5. PD-1 bioassay qualification with pembrolizumab in agonist mode. A. The assay was qualified using pembrolizumab with one analyst over 2 days for a range of concentrations relative to a 100% reference standard. The average measured relative potency was plotted against the expected relative potency (graph) in SoftMax Pro 7.1 using a 4PL fit. B. Dilutional linearity that was plotted using data from the 5 NCs tested (50%, 75%, 100%, 125%, and 150%), yielding in an R^2 value of 0.999.

Conclusions

- FcγR mediated clustering significantly augments agonistic activity of anti-checkpoint antibodies, offering a new avenue to design therapeutics against auto-immune diseases.
- Recording direct activation of checkpoint receptors through SHP recruitment provides a unique avenue for identifying previously unregistered agonistic activity in co-inhibitory checkpoint antibodies.
- Eurofins DiscoverX offers PD-1 signaling cell lines and ready-to-use bioassays for characterization through QC lot release. These cell-based, MOA-reflective functional assays are robust and sensitive.
- Eurofins DiscoverX cell-based assays for checkpoints with FcγR clustering cells enable screening and characterization for clinical development applications as well as potency lot-release testing for commercial release of agonistic antibodies.

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