Abstract Number 4072

Functional Screen to optimize logic gate potency and selectivity

Sara Martire, Tanveer Gill, Michele McElvain, Vasantika Suryawanshi, Mark Daris, Tim Riley, Alexander Kamb, Chawita Netirojjanakul

A2 Biotherapeutics, Inc., Agoura Hills, CA USA

ABSTRACT

Logic-gated cell therapy systems are considerably more complicated than conventional therapeutics and, therefore, more challenging to optimize. Here we describe the design and testing of a flow-cytometry-based screening system to rapidly select functional inhibitory receptors from a pooled library of candidate constructs. In proof-of-concept experiments, this approach identifies inhibitory receptors that can operate as NOT gates when paired with activating receptors. When further modules (e.g., potency boosters) are added to the system, the functional screen can be used to identify boosters from large libraries of variants.

INTRODUCTION

Logic-gated cell therapy systems are challenging to optimize, due to the complexity of the mechanism. One type of logic gate , the NOT gate, responds to two inputs: the presence of antigen A and absence of antigen B. The best studied NOT gate, the Tmod™, consists of an activator (e.g., a chimeric antigen receptor [CAR]) that is co-expressed with an inhibitory receptor, or blocker (1) (Figure 1). Progress has been made toward understanding the properties of Tmod (2,3). However, the complexity of the system limits the amount of structure-activity information that can readily be obtained.

Here, we developed a high-throughput screening to optimize the Tmod system. This approach utilizes FACS (fluorescence-activated cell sorter) combined with fluorescent reporters to select for cells that contain the gene of interest. As a proof of concept, we designed libraries of blockers and identified ones that function in the context of Tmod. Jurkat coculture assays were used to confirm top hits from the screen (4). In another set of experiments, a library of almost 30,000 variants of a signal 1 booster that activate signaling in the presence of a small molecule (rimiducid) was screened. Signal 1 boosters with specific domain structures were enriched and validated in subsequent experiments.

Figure 1: A schematic representation of the Tmod system

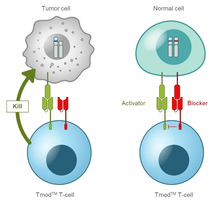


Figure 1: Tmod, which consists of an activator and a blocker, selectively kills tumor cells that express only the activator antigens, while protecting normal cells expressing both activator and blocker antigens.

JURKAT REPORTER CELL LINE

Figure 2: Jurkat reporter cell line

Figure 2: The Jurkat cell line used in this study was engineered to fit for the purpose of enriching blockers by FACS. Jurkat cells containing the fluorescent reporter (GFP), which is located downstream of NFAT-regulated promoter.

FLOW SCHEMA OF FUNCTIONAL SCREEN

Figure 3: Flow schema of the Functional Screen

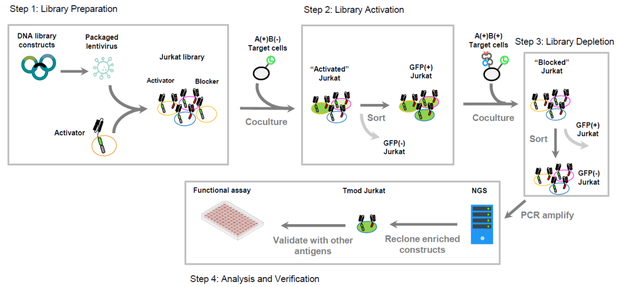
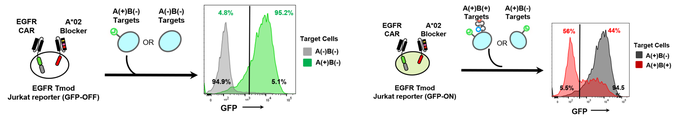


Figure 3: Step 1-Library Prep: A library of blockers is cloned into a lentiviral vector and transduced into an optimized GFP-reporter line of Jurkat cells, which express a CAR activator. **Step 2-Activation:** Jurkat cell library is exposed to target cells expressing only the activator antigen (A(+)B(-)) and sorted using FACS to isolate the activated GFP(+) population. **Step 3-Depletion:** The GFP(+) Jurkat cells are exposed to target cells expressing both activator and blocker antigens (A(+)B(+)) and sorted to isolate GFP(-) fraction. **Step 4-Analysis and Verification:** Cells are collected and analyzed by NGS to identify candidate blocker sequences enriched during the process.

VALIDATION OF THE JURKAT REPORTER CELL LINE

Figure 4: Validation of the Jurkat reporter cell line using Tmod system



$$\text{Activation} = \frac{\%\ \text{GFP (+) cocultured with A(+)B(-)}}{\%\ \text{GFP (+) cocultured with A(-)B(-)}}$$

$$\text{Blocking} = \frac{\%\ \text{GFP (-) cocultured with A(+)B(+)}}{\%\ \text{GFP (-) cocultured with A(+)B(-)}}$$

Activation: HeLa cells that express A antigen (EGFR) were presented to the Jurkat reporter cell lines (transduced with EGFR CAR), to activate the NFAT promoter and GFP signal.

Blocking: Jurkat cells that express both the EGFR CAR and an HLA-A*02 blocker were cocultured with HeLa cells that express both target antigens (A(+)B(+)).

NY-ESO-1 BLOCKER LIBRARY FUNCTIONAL SCREEN

Figure 5: NY-ESO blocker library

Figure 5: The blocker library comprised of 46 constructs with NY-ESO-1 scFv, LILRB1 hinge (H) and transmembrane domains (TM), and 46 different intracellular domains (ICDs) (2 null ICDs, 10 LILRB1 ICD variants, and 34 different ICDs).

Figure 6: Functional Screen coculture and sorting rounds

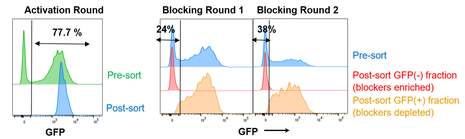


Figure 6: Jurkat reporter cells that express an EGFR CAR and blocker library were cocultured with A(+) HeLa cells that express EGFR antigen, sorted for the GFP(+) population to enrich cells able to activate. After recovery in media, activated cells were cocultured with A(+) B(+) HeLa cells that express EGFR antigen and NY-ESO-1 trimer antigen and sorted for GFP(+) and GFP(-) fractions. Flow plots show the gates for the activation step and the blocking rounds.

Figure 7: Top 10 enriched constructs identified by Functional Screen

Gene	Enrichment vs. Negative Control
EPOR	20.6
SCIMP	10.2
CD244	9.4
SLAF5	8.7
CD22	8.6
SIG10	8.6
LIRB2	8.4
CLIM1	8.3
LIRB1 positive control	7.7
KIR3DL2	7.5
LAIR1	7.5

Figure 7: Ranked list of library constructs from Functional Screen run (top 10 out of 46 are shown). Enrichment is calculated as the ratio of variant frequencies in the round 2 sample divided by the frequency in the activated sample prior to round 1. This enrichment is divided by the negative control (LILRB1 with truncated ICD) to give enrichment relative to the negative control.

FUNCTIONAL ASSAY TO CONFIRM BLOCKING

Figure 8: Validation of top enriched ICDs in Jurkat NFAT-luciferase

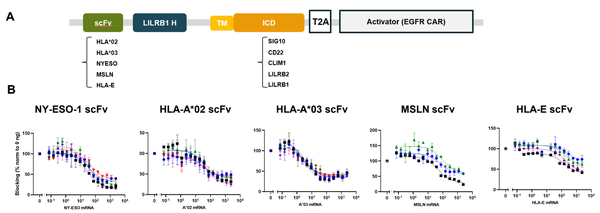


Figure 8: (A) Diagram of the 5 blocker constructs that were chosen and cloned into a single vector containing 5 different scFvs fused to the LILRB1 backbone plus the EGFR CAR. **(B)** Functional assays in Jurkat NFAT-luciferase reporter cells using HeLa target cells that express the different activator antigens, titrated with blocker antigen mRNA.

MACHINE LEARNING MODELS IDENTIFY PREDICTORS OF ENRICHMENT

Figure 9: Machine learning models using functional screen data

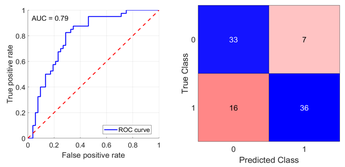


Figure 9: To further analyze the data from enrichment, a linear regression machine learning models were developed to predict enrichment. 8 features were selected to construct a model predicting successful or unsuccessful enrichment for each blocker and CAR pair. Most models predicted the blocker behavior with 70-80% accuracy.

POTENCY BOOSTER CAN BE IDENTIFIED IN COMPLEX LIBRARY

Figure 10: Flow schema of the Functional Screen for booster identification

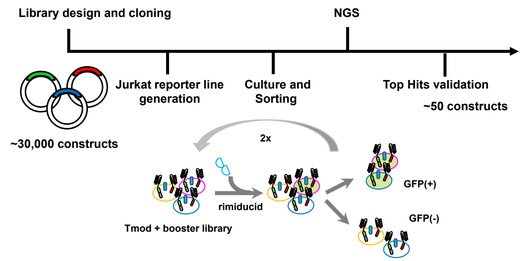


Figure 10: A library of booster is cloned into a lentiviral vector and transduced into an optimized GFP-reporter line of Jurkat cells, which express a CAR activator. Jurkat cell library is exposed to rimiducid and sorted using FACS to isolate the activated/boosted GFP(+) population. The step is repeated 2 more times. Cells are collected and analyzed by NGS to identify candidate booster sequences enriched during the process.

Figure 11: Booster identified after Functional Screen

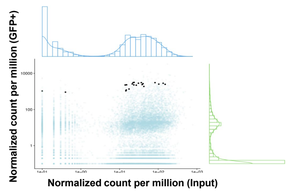


Figure 11: Scatterplot depicting the expression (normalized counts per million) of booster constructs in the Input sample versus the GFP+ sample after 3 rounds of rimiducid induction. Black dots represent the top 50 constructs with the highest enrichment score for the Signal 1 booster, while blue dots indicate all other constructs. Marginal histograms display the distribution of normalized counts in the Input sample (blue) and GFP+ sample (green) along the X- and Y-axes, respectively.

Figure 12: Validation of top hits identified by Functional Screen

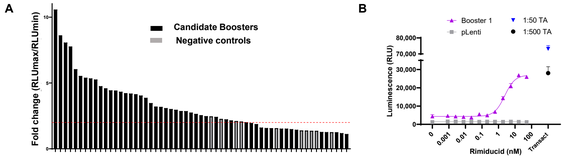


Figure 12: Functional assays in Jurkat NFAT-luciferase reporter cells that express boosters identified in the Functional Screen. rimiducid was titrated and cells were incubated for 5 hours. **(A)** Plot showing fold induction after incubation of top 50 candidate constructs with rimiducid. **(B)** Plot shows dose-dependent activation of one of the constructs tested.

REFERENCES

1. **DiAndreth B.,** et al., *The Tmod cellular logic gate as a solution for tumor-selective immunotherapy*. Clin Immunol, 2022.
2. **Manry, D.,** et al., *Robust In Vitro Pharmacology of Tmod, a Synthetic Dual-Signal Integrator for Cancer Cell Therapy*. Front Immunol, 2022
3. **Partin A.C.**, et al., *Geometric parameters that affect the behavior of logic-gated CAR T cells.* Front Immunol, 2024
4. **Martire S.,** et al. *High-throughput screen to identify and optimize NOT gate receptors for cell therapy*. Cytometry Part A, 2024

Presented at the 2025 AACR Annual Meeting, April 25-30, 2025, Chicago, Illinois

smartire@a2biotherapeutics.com