# Improved T Cell Activation Bioassays for Development of Bispecific Antibodies and Engineered T Cell Immunotherapies

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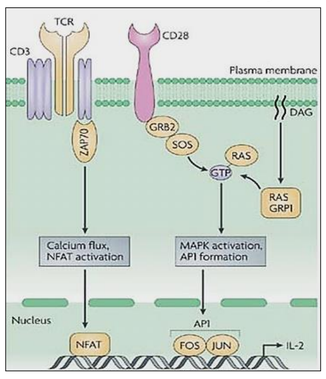
## 1. Introduction

Immunotherapy aims to boost a patient's own immune system to fight disease. In recent years, a variety of immunotherapy strategies aimed at inducing, strengthening or engineering T cell responses have emerged as promising approaches for the treatment of cancer and autoimmune disease.

Here we describe a platform of T cell activation bioassays for the development of CD3 bispecific antibodies and engineered T cell immunotherapies.

**T Cell Activation Pathways**

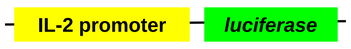



Lineberry-N et al. Nature Reviews Immunology 2007

Specifically, we developed two bioluminescent reporter-based bioassays to measure T cell activation via CD3 (NFAT-RE) or CD3 + CD28 (IL-2 promoter). These bioassays include the following:

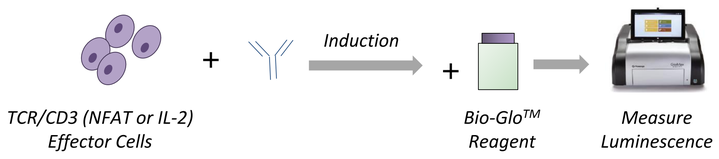
**TCR/CD3 (NFAT) effector cells:** Jurkat cells engineered with an NFAT-RE driving luciferase expression. Responds to CD3, but not CD28 stimulation.


**TCR/CD3 (IL-2) effector cells:** Jurkat cells engineered with an IL-2 promoter driving luciferase expression. Responds to CD3 and CD3+CD28 stimulation.




## 2. Both TCR/CD3 (NFAT) and TCR/CD3 (IL-2) Cells Respond to TCR/CD3 Stimulation

**T Cell Activation Protocol**

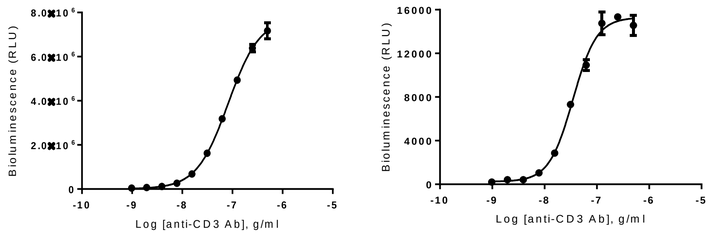



**TCR/CD3 (NFAT)**

EC50=82ng/ml
Fold Induction=165

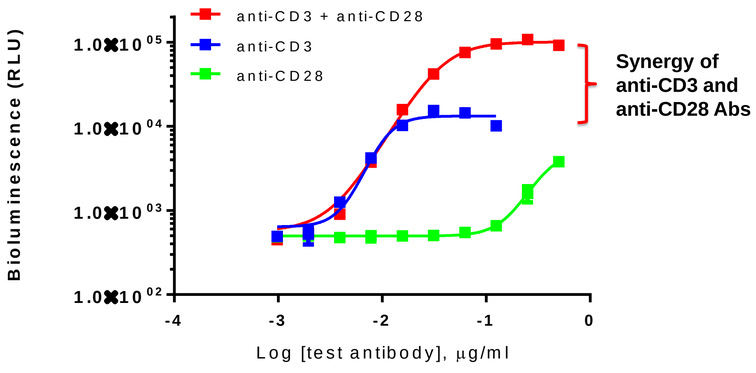
**TCR/CD3 (IL-2)**

EC50=35ng/ml
Fold Induction=76




TCR/CD3 (NFAT) (**Left**) and TCR/CD3 (IL-2) (**Right**) effector cells were stimulated with increasing concentrations of an anti-CD3 Ab.

## 3. T Cell CD3+CD28 Co-stimulation Measured using TCR/CD3 (IL-2) Effector Cells



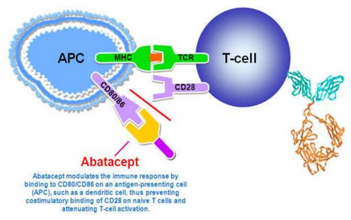

**anti-CD3 + anti-CD28 Abs**
- EC50=36ng/ml
- Fold Induction=230

**anti-CD3 Ab:**
- EC50=11ng/ml
- Fold Induction=31

**anti-CD28 Ab:**
- EC50=>368ng/ml
- Fold Induction=8

TCR/CD3 (IL-2) effector cells were stimulated with increasing concentrations of anti-CD28, anti-CD3 or a combination of anti-CD3+anti-CD28 Abs, as indicated.

## 4. Abatacept Modulates CD3+CD28 T Cell Activation using TCR/CD3 (IL-2) Cells



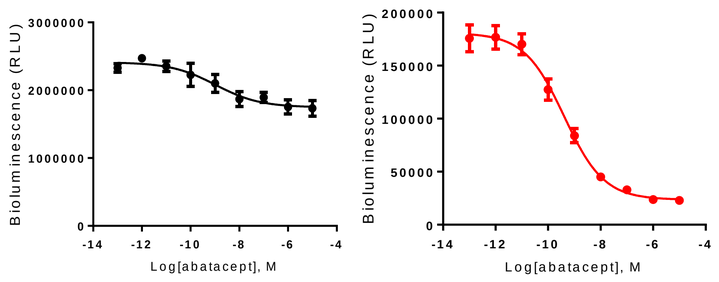

**Experimental Design**

(1) TCR/CD3 (NFAT or IL-2) effector cells are incubated with Raji (CD80/86$^+$) target cells

(2) T cell activation is induced via crosslinked anti-CD3 Ab and CD28 engagement by its ligand CD80/86 expressed on the Raji target cells

(3) Addition of a CTLA-4/IgG fusion protein (Abatacept) binds CD80/86 and inhibits CD28-mediated T cell activation.

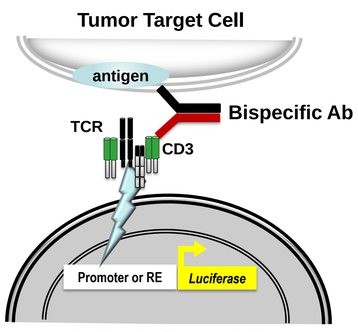
**TCR/CD3 (NFAT)** **TCR/CD3 (IL-2)**




Increasing concentrations of Abatacept were added to either TCR/CD3 (NFAT) or TCR/CD3 (IL-2) effector cells, as indicated. Abatacept induced a significant decrease in TCR-mediated luciferase activity in TCR/CD3 (IL-2) effector cells compared to TCR/CD3 (NFAT) effector cells. This is expected because CD28 functions independently of the NFAT response element (see Introduction).

## 5. Measurement of CD3 Bispecific Antibody Activity: Assay Design and Protocol

**Assay Design for Measuring CD3 Bispecific Antibody Activity**



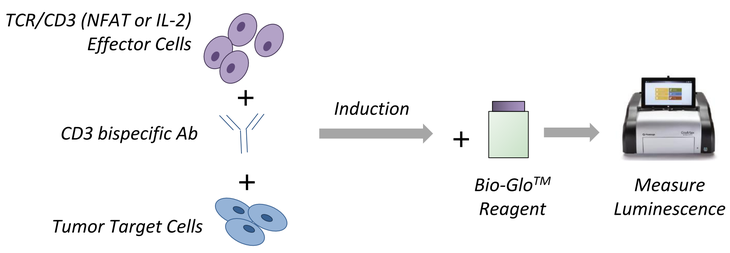

**Experimental Design**

(1) TCR/CD3 (NFAT or IL-2) effector cells are incubated with increasing concentrations of a CD3 bispecific Ab

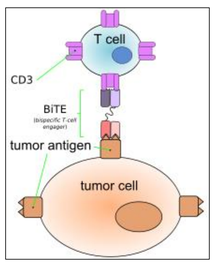
(2) The bispecific Ab simultaneously binds to TCR/CD3 on the effector cells and tumor antigen on the target cells

(3) Bispecific Ab binding stimulates IL-2 or NFAT luciferase activity

**Assay Protocol for Measuring CD3 Bispecific Antibody Activity**

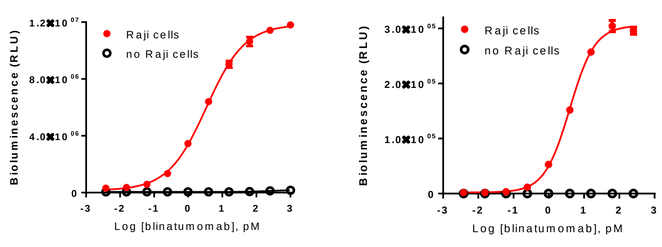



## 6. Analysis of Blinatumomab CD3 x CD19 Bispecific Antibody Activity




Blinatumomab belongs to a class of constructed monoclonal antibodies, bi-specific T-cell engagers (BiTEs), that exert action selectively and direct the human immune system to act against tumor cells. Blinatumomab specifically targets the CD19 antigen present on B cells.

**TCR/CD3 (NFAT)** **TCR/CD3 (IL-2)**




Increasing concentrations of Blinatumomab were added to either TCR/CD3 (IL-2) or TCR/CD3 (NFAT) effector cells, as indicated. Blinatumomab induced a dose-dependent increase in luciferase in both TCR/CD3 (IL-2) and TCR/CD3 (NFAT) effector cells in the presence of Raji (CD19+) target cells. No response was detected in the absence of Raji (CD19+) target cells.

## 7. Assay Qualification with Blinatumomab: Assay Precision, Accuracy and Linearity

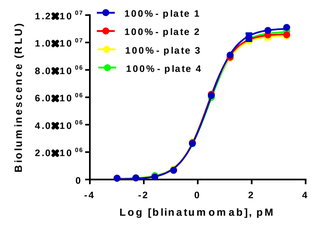
**Accuracy and Intermediate Precision (N = 6)**

**Assay Qualification Design:**
Two analysts
Three days
Four plates per day
- 100% vs 50%
- 100% vs 75%
- 100% vs 150%
- 100% vs 200%

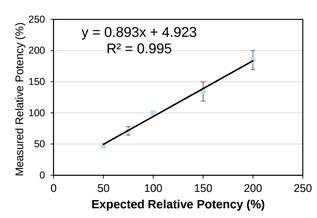
Data shown are generated using TCR/CD3 (NFAT) effector cells, binatumomab, and Raji (CD19+) target cells.

| Expected Relative Potency | Assay | Analyst | Measured Relative Potency % | Mean % | SD% | Accuracy Recovery % | Precision RSD % |
|---|---|---|---|---|---|---|---|
| 50% | 1 | 1 | 45.3 | 47.8 | 3.6 | 95.7 | 7.6 |
| | 2 | 1 | 45.0 | | | | |
| | 3 | 1 | 45.5 | | | | |
| | 4 | 2 | 46.2 | | | | |
| | 5 | 2 | 52.2 | | | | |
| | 6 | 2 | 52.8 | | | | |
| 75% | 1 | 1 | 62.2 | 71.2 | 6.8 | 95.0 | 9.5 |
| | 2 | 1 | 73.9 | | | | |
| | 3 | 1 | 63.3 | | | | |
| | 4 | 2 | 78.4 | | | | |
| | 5 | 2 | 75.9 | | | | |
| | 6 | 2 | 73.6 | | | | |
| 150% | 1 | 1 | 144.5 | 134.4 | 15.5 | 89.6 | 11.5 |
| | 2 | 1 | 143.5 | | | | |
| | 3 | 1 | 121.4 | | | | |
| | 4 | 2 | 108.8 | | | | |
| | 5 | 2 | 143.4 | | | | |
| | 6 | 2 | 144.7 | | | | |
| 200% | 1 | 1 | 174.9 | 184.8 | 15.0 | 92.4 | 8.1 |
| | 2 | 1 | 195.5 | | | | |
| | 3 | 1 | 179.8 | | | | |
| | 4 | 2 | 162.0 | | | | |
| | 5 | 2 | 198.9 | | | | |
| | 6 | 2 | 198.0 | | | | |
| | | | Overall | 93.2 | | | 9.2 |

**Repeatability (%CV) = 3.01%**

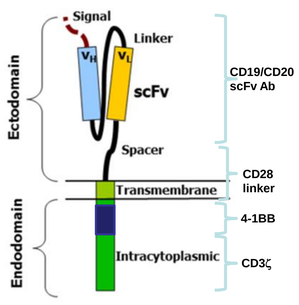



**Linearity and Range ($R^2$=0.995)**

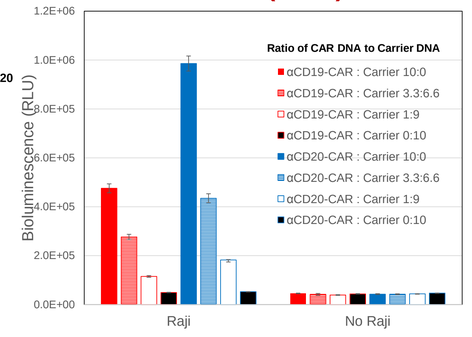



## 8. Measurement of Chimeric Antigen Receptor T (CAR-T) Cell Activity

**CAR-T Design**




**TCR/CD3 (NFAT)**

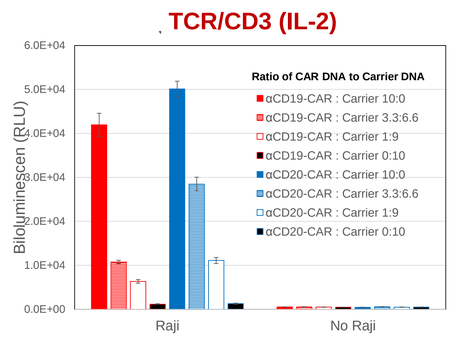



**Experimental Design**

(1) TCR/CD3 (NFAT or IL-2) effector cells were engineered to express anti-CD19/CD20 CAR-T receptors

(2) The effector cells were incubated in presence or absence or Raji (CD19/CD20+) target cells

**TCR/CD3 (IL-2)**




Serial dilutions of anti-CD19/CD20 CAR-T effector cells were incubated in the presence and absence of Raji (CD19/CD20+) target cells. Luciferase activity was detected in the presence of Raji cells, but not with the CAR-T effector cells alone.

## 9. Conclusions

We have developed a platform of T cell activation bioassays that incorporate a bioluminescent reporter-based readout of T cell activation via CD3 (NFAT-RE) or CD3 + CD28 (IL-2 promoter). These assays reflect the mechanisms of action of biologics designed to engage, recruit, and stimulate T cell activation to attack target disease cells. Specific applications include measurement of **anti-CD3 bispecific Ab** and **CAR-T cell** activity.

The bioassays provide the following:

**Mechanism of action (MOA)-based measure of biologics activity**

- Specific measure of CD3 or CD3 + CD28 T cell activation pathways
- Quantitative measure of anti-CD3 Ab and bispecific Ab potency

**Consistent and reliable measure of biologics activity**

- Demonstrated precision, accuracy, reproducibility, robustness
- All assays can be used as "Thaw-and-use" cell format, no cell culture required
- Functional performance suitable for development into potency, stability, and NAb assays (data not shown)

**Easy-to-implement**

- Rapid and convenient workflow
- Amenable to standard 96-well and 384-well plate formats

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