



# TruCytes Potency Cell Mimics Induce Robust and Reproducible Specific Activation of CAR-T Cells

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### ABSTRACT

**Background**  
 Reproducible assessment of target-specific activation is necessary for a robust validation strategy for CAR-T products. Antigen-expressing cell lines are commonly used to assess target-specific activation, but are prone to phenotypic drift after multiple passages or selection events introducing or knocking out a target or reporter and can be time-consuming and expensive to maintain. TruCytes™ Potency cell mimics are a renewable, stable activation alternative for potency assay development and standardization.

**Methods**  
 Cell mimics were loaded with either CD19 or BCMA and an enhancer protein and lyophilized. Commercially purchased CD19- or BCMA-specific CAR-T and donor-matched non-transduced T cells were co-cultured at defined E:T ratios and time points with mimics, cell lines, or target-coated microbeads. After indicated time points, supernatant was collected and assessed for interferon gamma (IFN $\gamma$ ) or panel of pro-inflammatory cytokines by CBA to assess CAR-T activation.

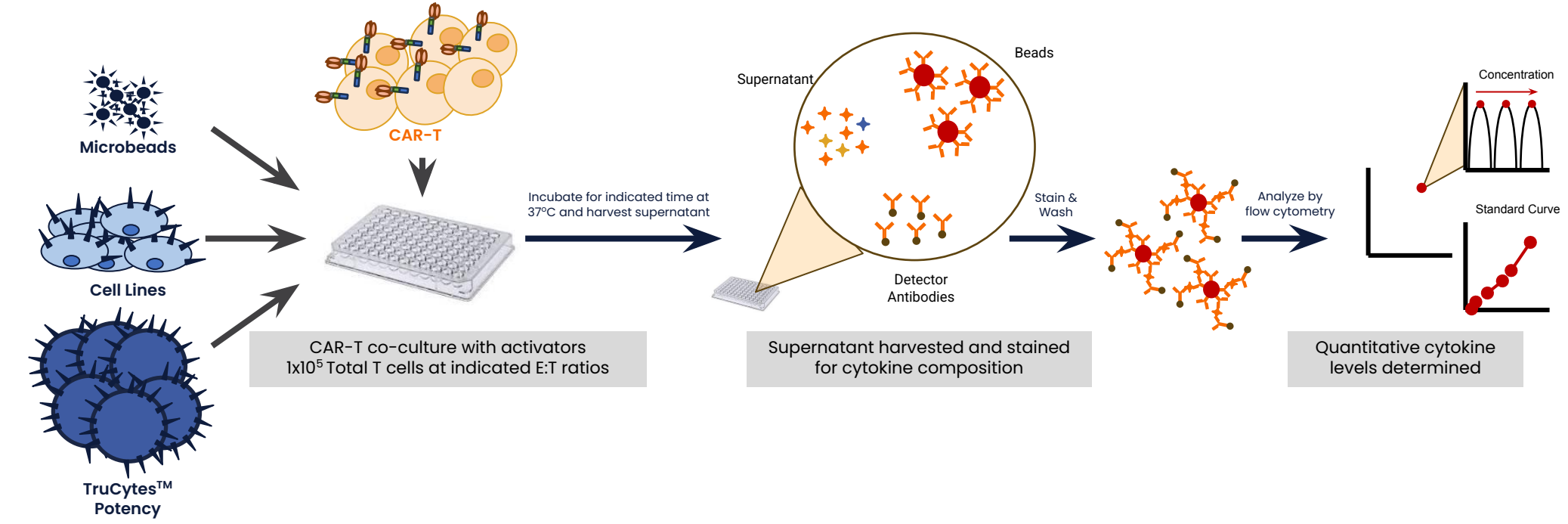
**Results**  
 TruCytes™ Potency cell mimics loaded with CD19, CD20, or BCMA activate their respective CAR-T, inducing IFN $\gamma$  release as early as 4 hours and up to 48 hours after co-culture. Mimics induced activation superior to target-coated microbeads, and enhancer protein increased specific activation on par with commonly used cell lines, including Raji and Daudi for CD19 and H929 and MML.S for BCMA. Unloaded mimics and enhancer-only mimics did not induce CAR-T activation above background. IFN $\gamma$  production was induced with E:T ratios between 5:1 to 1:5 for cognate CAR-T but not donor-matched non-transduced T cells, confirming specific activation. IFN $\gamma$  quantities correlated to CD19-specific CAR-T+ frequencies between 20-60% CAR-T+ and to the level of CD19 loading. To demonstrate reproducibility, cryopreserved BCMA-specific CAR-T from one lot were cultured with BCMA-loaded mimics and H929 cell line in 5 independent experiments, generating inter-day %CVs for BCMA-loaded mimics of 5.0% (6 hours) and 11.2% (24 hours), compared to 19.5% (6 hours) and 16.5% (24 hours) for H929 cells.

**Conclusions**  
 TruCytes™ Potency cell mimics provide a robust, chemically-defined alternative to cell lines for potency assay development and implementation. They deliver target-specific activation across varied E:T ratios, CAR-T frequencies, and time points, with superior lot-to-lot and inter-day reproducibility. Their defined composition and renewable format make them a reliable tool for assessing specific activation of CAR-T cells for potency assay development and implementation. CAR-T potency evaluation frequently utilizes cell lines for target-specific assessment activation. TruCytes™ Potency cell mimics are chemically-defined, stable, activation alternatives, inducing target-specific IFN $\gamma$  production by BCMA- and CD19-targeting CAR-T cells. TruCytes™ Potency delivered target-specific IFN $\gamma$  production across E:T ratios, CAR-T frequencies, and time points. TruCytes™ Potency's superior reproducibility and defined composition enable reliable specific activation of CAR-T cells for potency assay development and implementation.

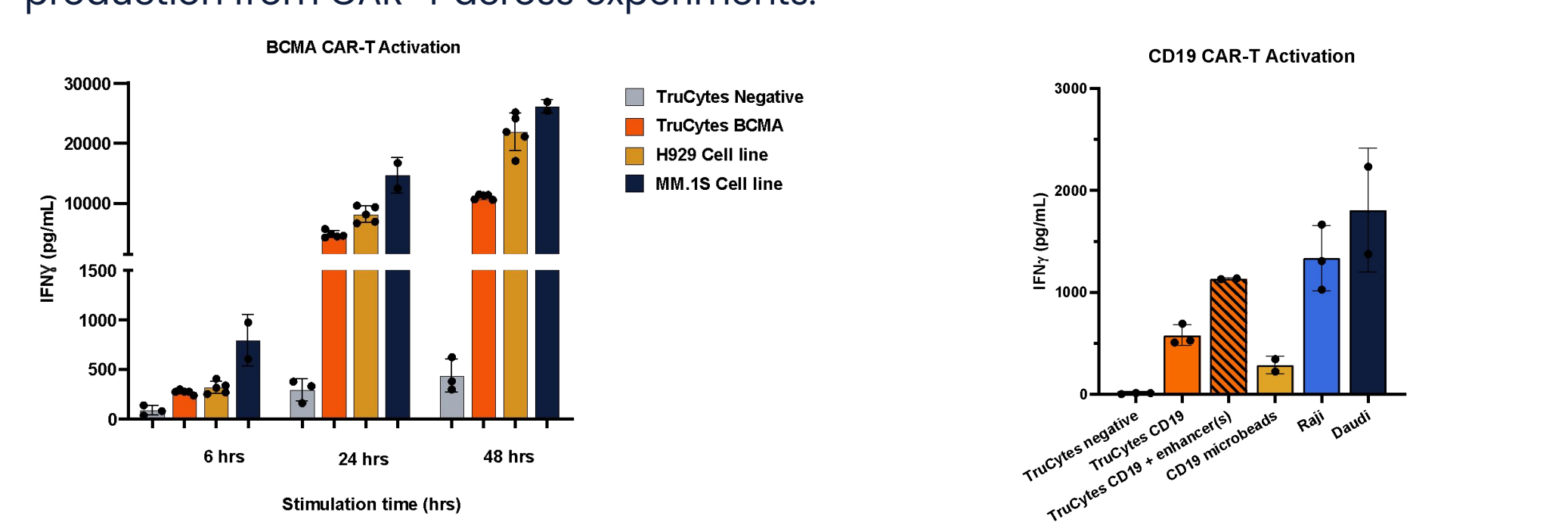
### TruCytes™ Potency: Highly reproducible cell mimics to demonstrate target-specific CAR-T activation and evaluate CAR-T function

#### EVALUATION METHODOLOGY

- TruCytes™ Potency are loaded with target antigen of choice, and enhancer protein(s) if indicated, and lyophilized.
- Target specific CAR-T are purchased commercially, thawed, and co-cultured at 1 x 10<sup>5</sup> total T cells per well with TruCytes™ Potency cell mimics or target positive cell lines at indicated E:T ratio.
- Supernatants are collected at indicated time point and stored at -20°C until cytokine assessment.
- IFN $\gamma$  in supernatant is quantified by CBA and plotted as mean +/- SD of technical duplicates unless otherwise indicated.



### Reproducibility: TruCytes™ Potency induce highly reproducible activation and IFN $\gamma$ production from CAR-T across experiments.



% CV for IFN $\gamma$ release in CAR-T Cell Potency Assays		
Culture Duration	TruCytes™ potency BCMA (n=5)	H929 (n=5)
6h	7.99%	19.2%
24h	11.6%	16.5%
48h	3.95%	14.4%

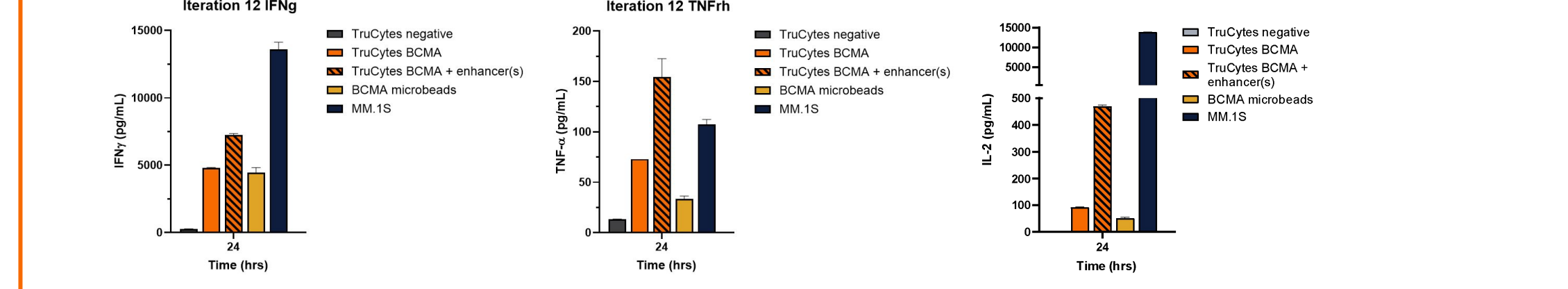
  

% CV for IFN $\gamma$ release in CAR-T Cell Potency Assays		
Activator	n	6h culture
TruCytes™ Potency CD19	3	17.6%
TruCytes™ Potency CD19 with Enhancer(s)	2	0.5%
CD19 microbeads	2	30.3%
Raji	3	24.0%
Daudi	2	33.6%

Figure 3. CAR-T specific for BCMA or CD19 from the same lot were thawed and cultured for indicated duration on different days with indicated synthetic activators or cell lines at 1:5 E:T. Symbols represent average IFN $\gamma$  detected in technical duplicate wells for each experiment, with bars representing mean +/- SD across experiments. %CV was calculated in Graphpad Prism.

### Customization: TruCytes™ Potency can be modified to meet optimal assay endpoints

#### A TruCytes™ Potency BCMA can induce multiple pro-inflammatory cytokines and production increases with enhancer(s)



#### B IFN $\gamma$ production level can be titrated by modifying CD19 protein loading

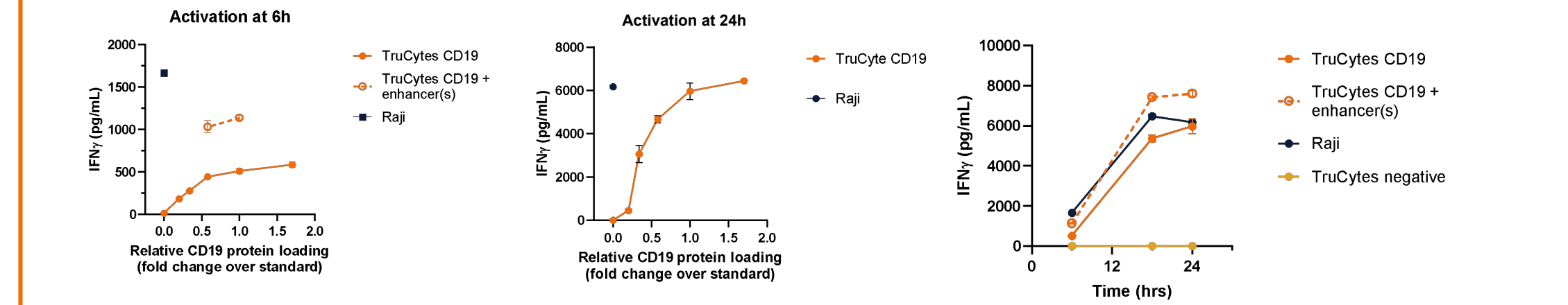
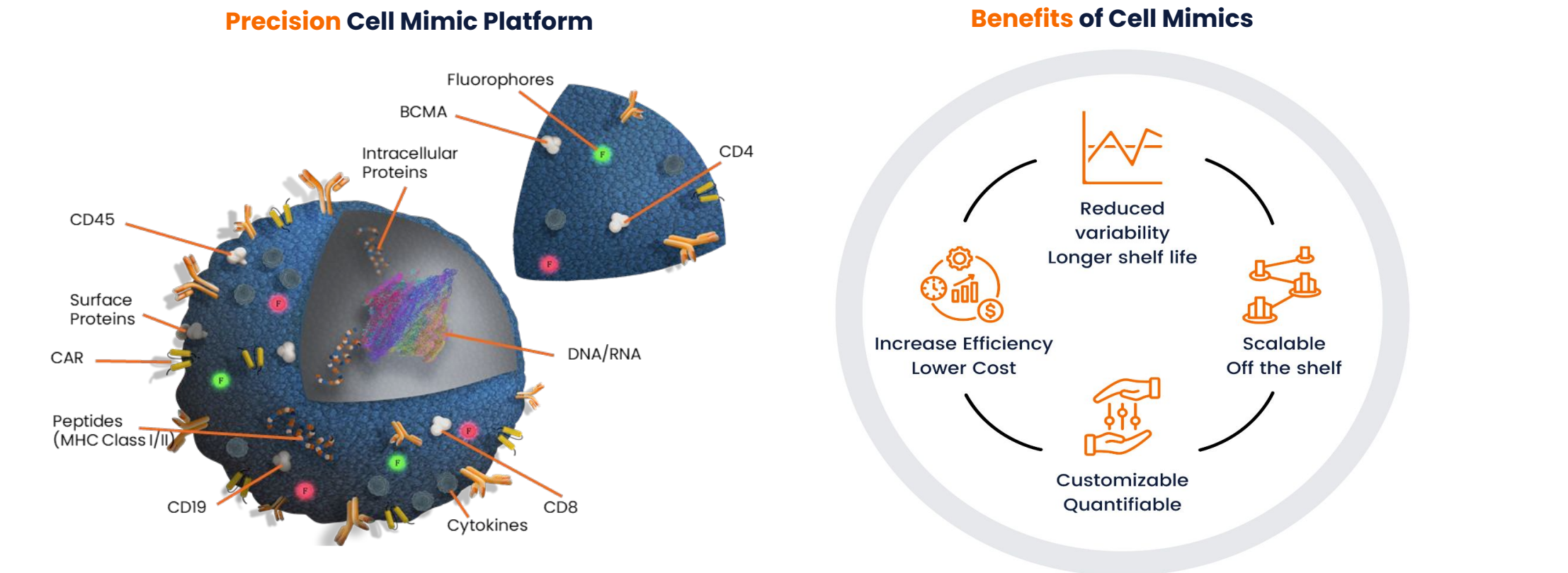


Figure 5. TruCytes™ Potency mimics can be modified to suit assay endpoint (A) BCMA-specific CAR-T were cultured with indicated activators at 1:5 E:T for 24h and supernatant was evaluated with BD Th1/2 CBA kit, with IFN $\gamma$ , TNF $\alpha$ , and IL-2 results shown. (B) CD19 CAR-T stimulated with TruCytes™ Potency loaded with titration of proprietary CD19 construct, at 1:5 E:T ratio and 1x10<sup>5</sup> T cells per well. Supernatant was collected at 6h, 18h, or 24h and stored at -20 °C and evaluated for IFN $\gamma$ .

### INTRODUCTION

Slingshot Biosciences leverages the principles of biochemistry, high-precision manufacturing, and polymer chemistry to engineer TruCytes™ cell mimics that match the features of biological cells, including optical, fluorescence and biochemical properties. Using this technology, TruCytes™ are customizable and reproducibly manufactured to express relevant surface antigens with controlled antigen density, making them ideal surrogates for biological controls that are prone to variability. TruCytes™ Lymphocyte Subsets is an example of this capability, a lyophilized cocktail composed of lymphocyte cell mimics, including B cells, NK cells, and CD4+ and CD8+ T cells, larger CD14+ monocyte cell mimics, and high scatter granulocyte cell mimics (Fig 1). When compared to commercial sources, TruCytes™ Lymphocyte Subsets demonstrate higher relevant precision (%CV) of cell frequencies across lots when stained with identical protocols and analyzed by flow cytometry (Table 1). They were also highly reproducible with %CV below 9% for all populations across 9 lots (data not shown), demonstrating Slingshot Biosciences' product reproducibility.



### TruCytes™ Lymphocyte Subsets are precision engineered to mimic white blood cell subsets in size, scatter, and antigen density.

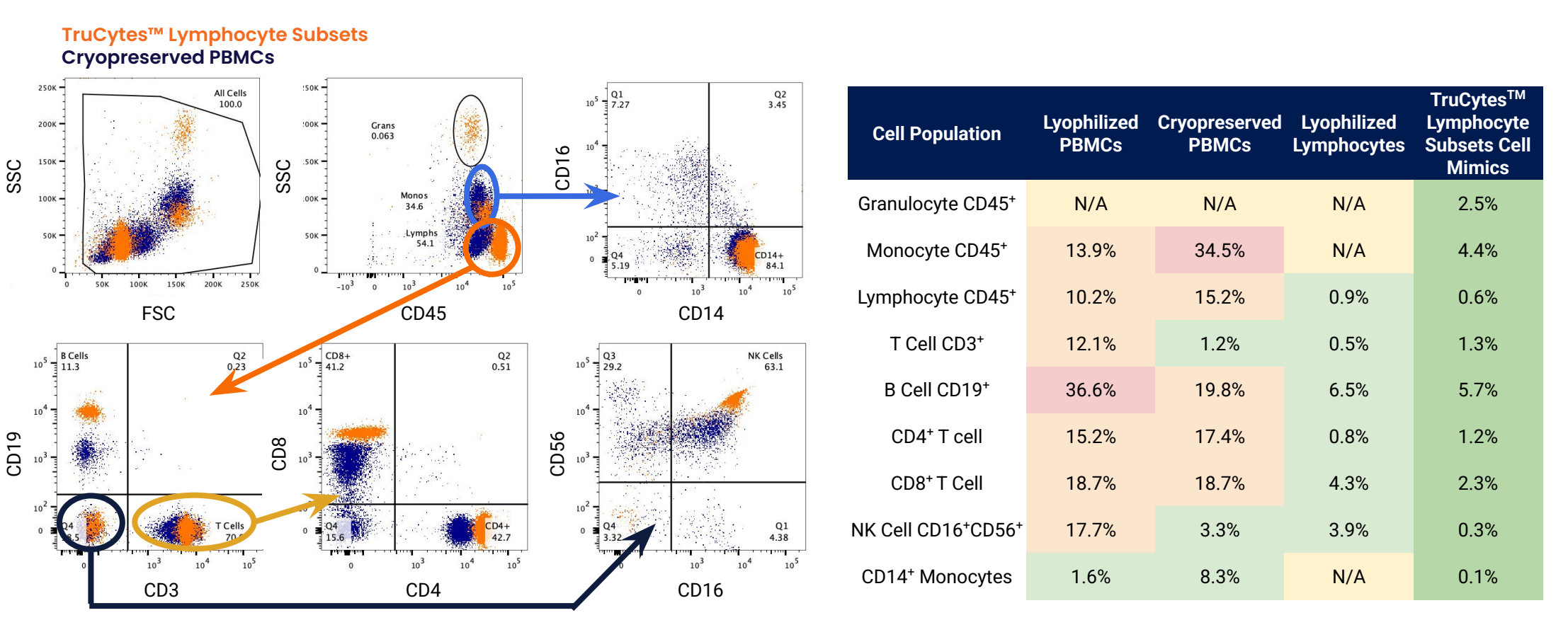
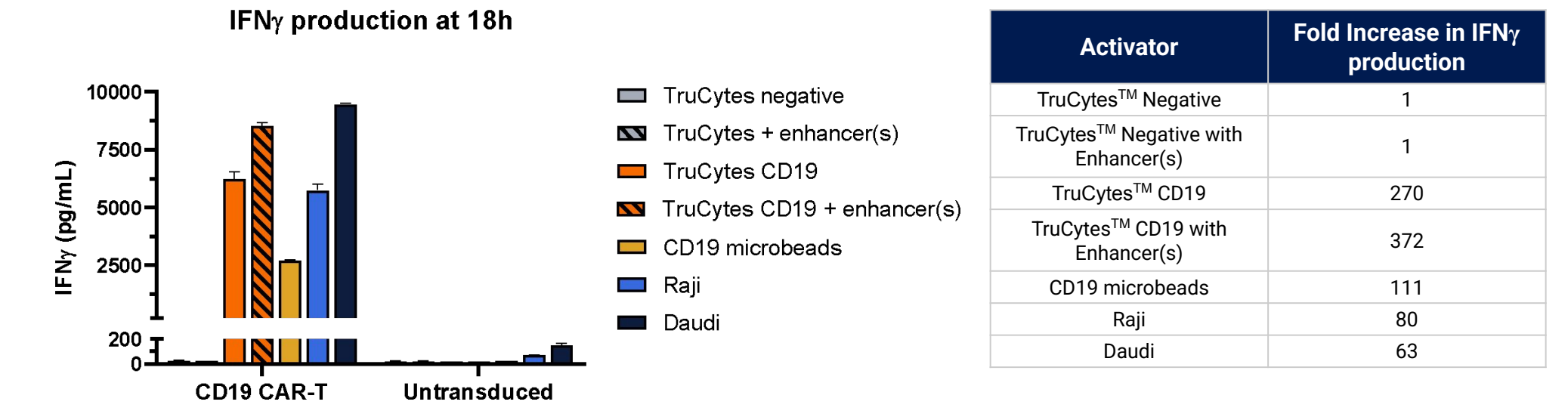


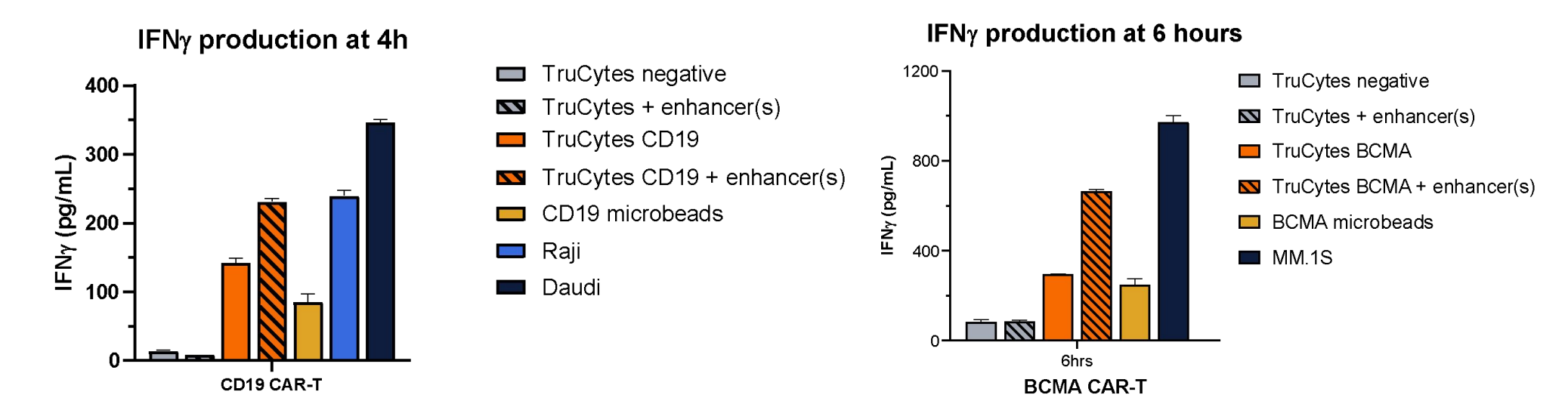
Figure 1. Representative flow plots for flow cytometry data for TruCytes™ Lymphocyte Subsets cell mimics compared to cryopreserved PBMCs. All samples underwent identical staining protocols using labeled monoclonal antibodies to CD45, CD3, CD19, CD4, CD8, CD14, CD16, and CD56, were collected on a Cytex Aurora spectral flow cytometer and analyzed via FlowJo. TruCytes™ Lymphocyte Subsets is overlaid in orange and PBMC sample in blue.

### Specificity: TruCytes™ Potency induce specific and reproducible activation of CAR-T that can be increased with additional enhancer protein

#### A IFN $\gamma$ production by $\alpha$ -CD19 CAR-T is robust and can be increased with addition of enhancer(s), increasing specific activation without increasing background.



#### B Addition of enhancer protein(s) augments IFN $\gamma$ production at early time points for both $\alpha$ -CD19 and $\alpha$ -BCMA CAR-T



#### C TruCytes™ Potency CD20 with Enhancer(s) reproducibly and specifically induce IFN $\gamma$ production by $\alpha$ -CD20 CAR-T

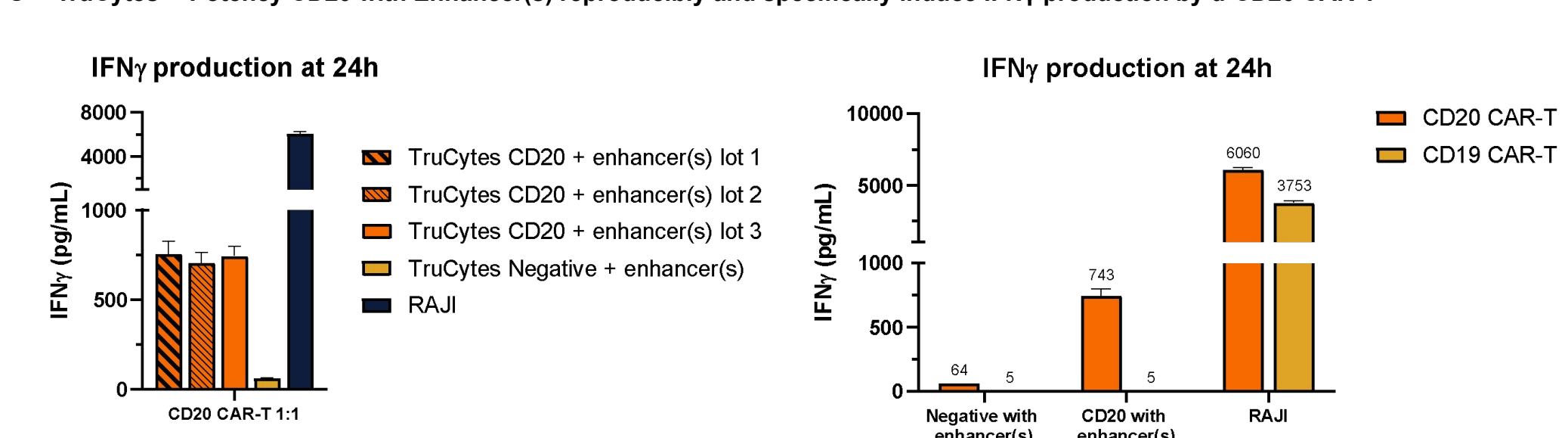
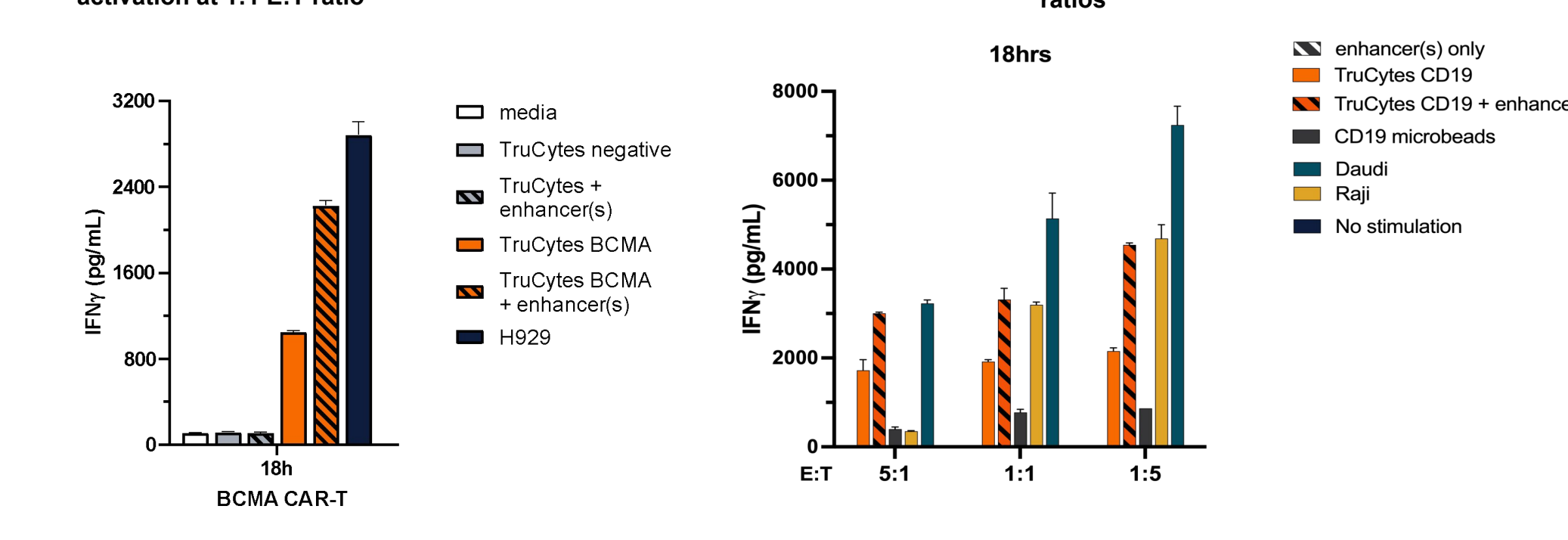


Figure 2. TruCytes™ Potency cell mimics provide robust and specific CAR-T activation (A) CD19-specific CAR-T or untransduced cells from same donor were plated at 1 x 10<sup>5</sup> cells per well at 1:5 E:T with indicated targets for 18 hours. Fold change in IFN $\gamma$  induction with CD19-specific CAR-T over untransduced cells for individual activators is displayed in table. (B) CD19- or BCMA-specific CAR-T were plated at 1 x 10<sup>5</sup> T cells per well at 1:5 E:T with indicated targets in technical duplicates for 4 or 6 hours, respectively. (C) CD20- or CD19-specific CAR-T were co-cultured with indicated mimics or cell lines at 1 x 10<sup>5</sup> T cells per well at 1:1 E:T in U-bottom plates for 24 hours. All supernatants were stored at -20°C and assayed for IFN $\gamma$  production by BD CBA on a Cytex Aurora. Mean +/- SEM is plotted for technical duplicates.

### Assay Compatibility: TruCytes™ Potency effectively stimulate CAR-T across E:T ratios and CAR+ frequencies

#### A Specific $\alpha$ -BCMA CAR-T activation at 1:1 E:T ratio



#### C $\alpha$ -CD19 CAR-T IFN $\gamma$ production correlates with CAR+ frequency

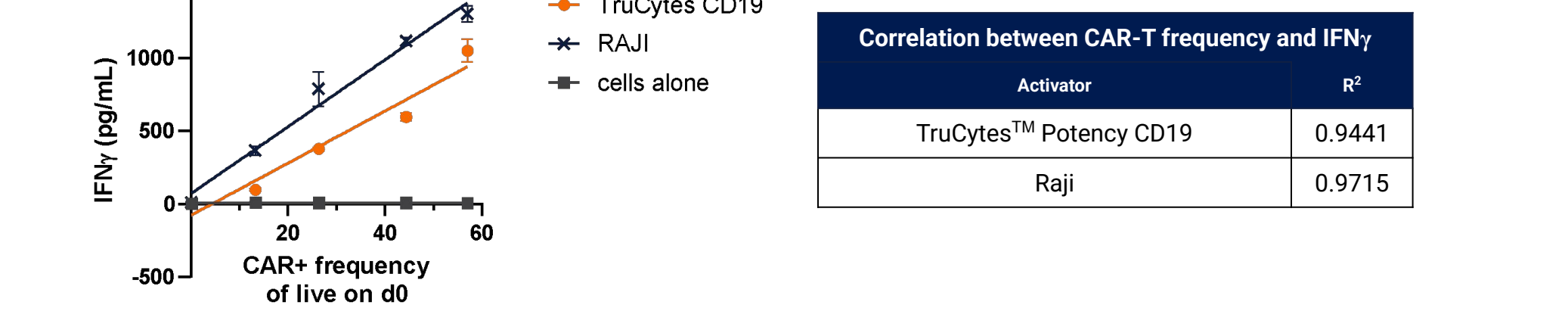
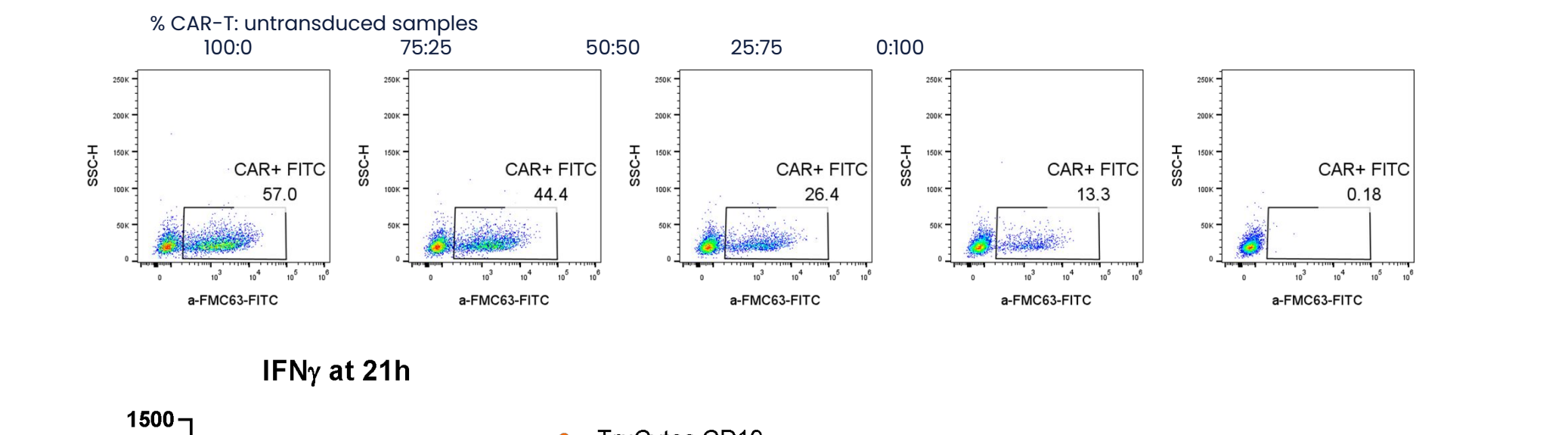


Figure 4. TruCytes™ Potency cell mimics activate CAR-T across E:T ratios and CAR+ frequencies (A) BCMA-specific CAR-T were cultured for 18h with indicated TruCytes™, CD19 microbeads, Daudi and Raji cell lines for 18h at indicated E:T ratios and assayed for IFN $\gamma$  in supernatant. (B) CD19-specific CAR-T were cultured with indicated TruCytes™, CD19 microbeads, Daudi and Raji cell lines for 18h at indicated E:T ratios and assayed for IFN $\gamma$  in supernatant. (C) CD19-specific CAR-T and untransduced T cells from the same donor were combined at various ratios to achieve a range of CAR+ frequencies, which were confirmed by flow cytometry using FM3C3-specific antibody. CAR-untransduced T cell mixtures were co-cultured with TruCytes™ CD19 or Raji cells at 1:1 E:T for 21h, and assayed for IFN $\gamma$  in supernatant. IFN $\gamma$  concentration was plotted against CAR+ frequency and analyzed for linear regression in Graphpad Prism, with calculated R<sup>2</sup> value shown in table.

### Stability: TruCytes™ Potency BCMA with Enhancer(s) demonstrate robust stability out to 24 months

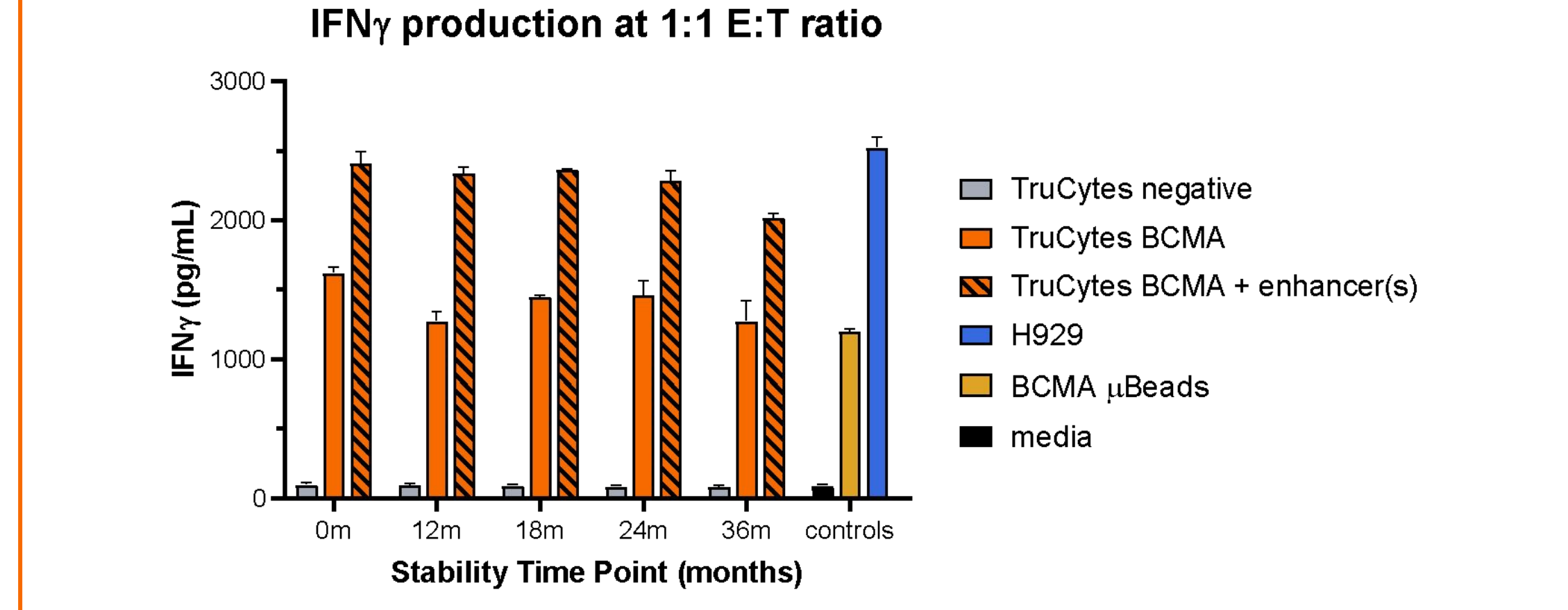


Figure 6. Stability of TruCytes™ Potency BCMA with Enhancer(s) demonstrate robust stability out to 24 months. TruCytes™ Potency were loaded with BCMA, BCMA and enhancer(s), or without protein and lyophilized. A reverse accelerated stability study was conducted to mimic storage at -20°C, and activation of BCMA-specific CAR-T at 20h of culture at 1:1 E:T ratio was conducted. Supernatant was evaluated for IFN $\gamma$ .

### CONCLUSIONS

Slingshot Biosciences has developed TruCytes™ Potency, a first-of-its-kind potency assessment solution using precision-engineered cell mimics that are chemically defined, highly consistent standards for potency QC of CAR-T products.

◆ **Biologically relevant specific activation:** CAR-T activation was observed only in response to cognate antigen, with response correlating to frequency of CAR+ cells in culture and low background activation observed in CAR-T cultured with mimics without antigen or untransduced T cells cultured with antigen-loaded mimics.

◆ **Enhanced precision:** In IFN $\gamma$  release assays, TruCytes™ Potency cell mimics delivered lower %CVs than cell lines when used as stimulators.

### ACKNOWLEDGEMENTS

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