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Supplemental Information

A High-Throughput Method for Characterizing Novel Chimeric Antigen Receptors in Jurkat Cells

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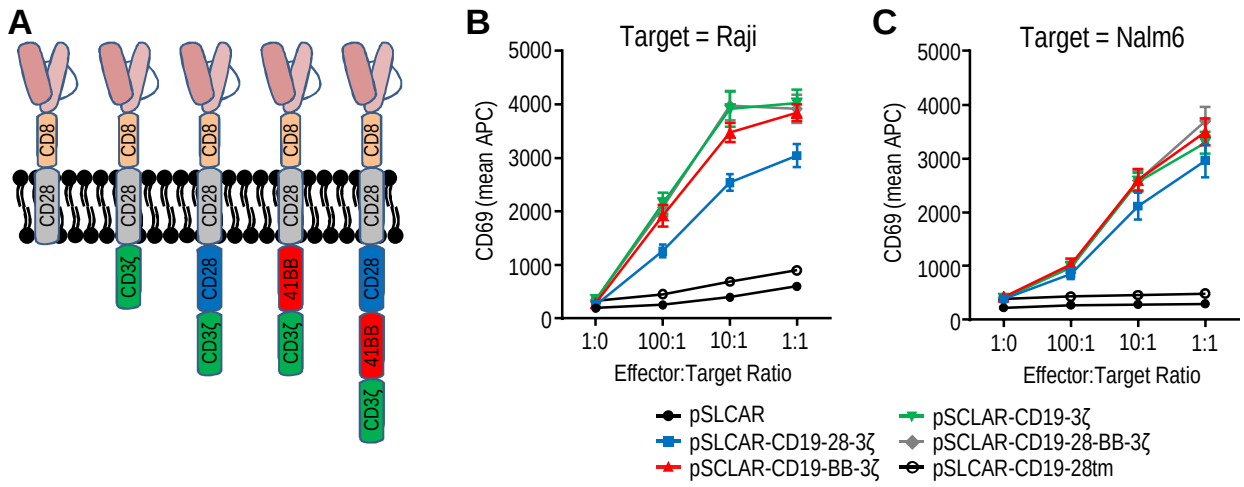


Figure S1: CAR-J does not robustly differentiate between signaling/co-stimulation domains for the anti-CD19 scFv derived from FMC63 (supplement to Figure 1).

(A) Depiction of CAR constructs containing different signaling/co-stimulation domains. (B) CAR-J dose-response when co-cultured with increasing numbers of CD19-positive Raji cells. (C) CAR-J dose-response when co-cultured with increasing numbers of CD19-positive Nalm6 cells. Results represent means +/- SEM of 3 independent experiments (n=3).

A

mAb clone	ELISA (OD at 405 nm)				Flow cytometry (MFI)	
	EGFRvIII protein		EGFR protein		U87-vIII	U87-MG
	Native	Denatured	Native	Denatured		
F260	1.736	1.743	0.001	0.000	4007	180
F263	1.527	0.997	1.698	0.001	3465[a]	175[a]
F265	0.874	0.623	0.002	0.000	2021	177
F269	1.125	0.517	0.011	0.001	2084	195
F271	0.935	1.054	0.004	0.000	1645	170
225	1.496	0.004	1.320	0.002	6574	11165
anti-GFP	-0.001	0.002	0.002	0.000	164	188

[a]performed with purified antibody

B

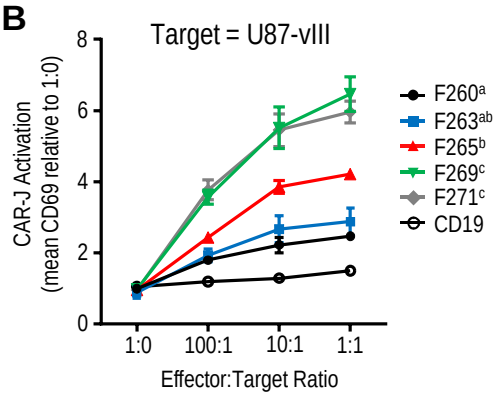


Figure S2: Characterization of novel anti-EGFRvIII mouse monoclonal antibodies (supplement to Figure 2). Monoclonal antibodies specific for the mutant form of EGFR (EGFRvIII) were generated using mouse immunization, hybridoma creation, and screening as described in the Methods section. (A) Monoclonal antibody characterization was then performed using indirect ELISA using purified mAbs (1 µg/ml). Antibodies were incubated with immobilized recombinant EGFRvIII or EGFR protein. Flow cytometry analyses using raw hybridoma supernatant was done on U87MG cell lines (wildtype U87 or EGFRvIII-expressing U87-vIII). Note that due to low IgG production from the hybridoma, flow cytometry analyses were performed using purified antibody for F263. Antibody 225 is a commercial anti-EGFR mAb that binds to a native epitope common to both EGFRvIII and EGFR which was used as a control antibody in these experiments. (B) Normalized data as presented in Figure 2C. Results in (B) represent means +/- SEM of 3 independent experiments (n=3). In (B), 1:1 ratio data was compared using 1-way ANOVA and significant differences are indicated with lower case letters, where groups with different letters are significantly different from each other.

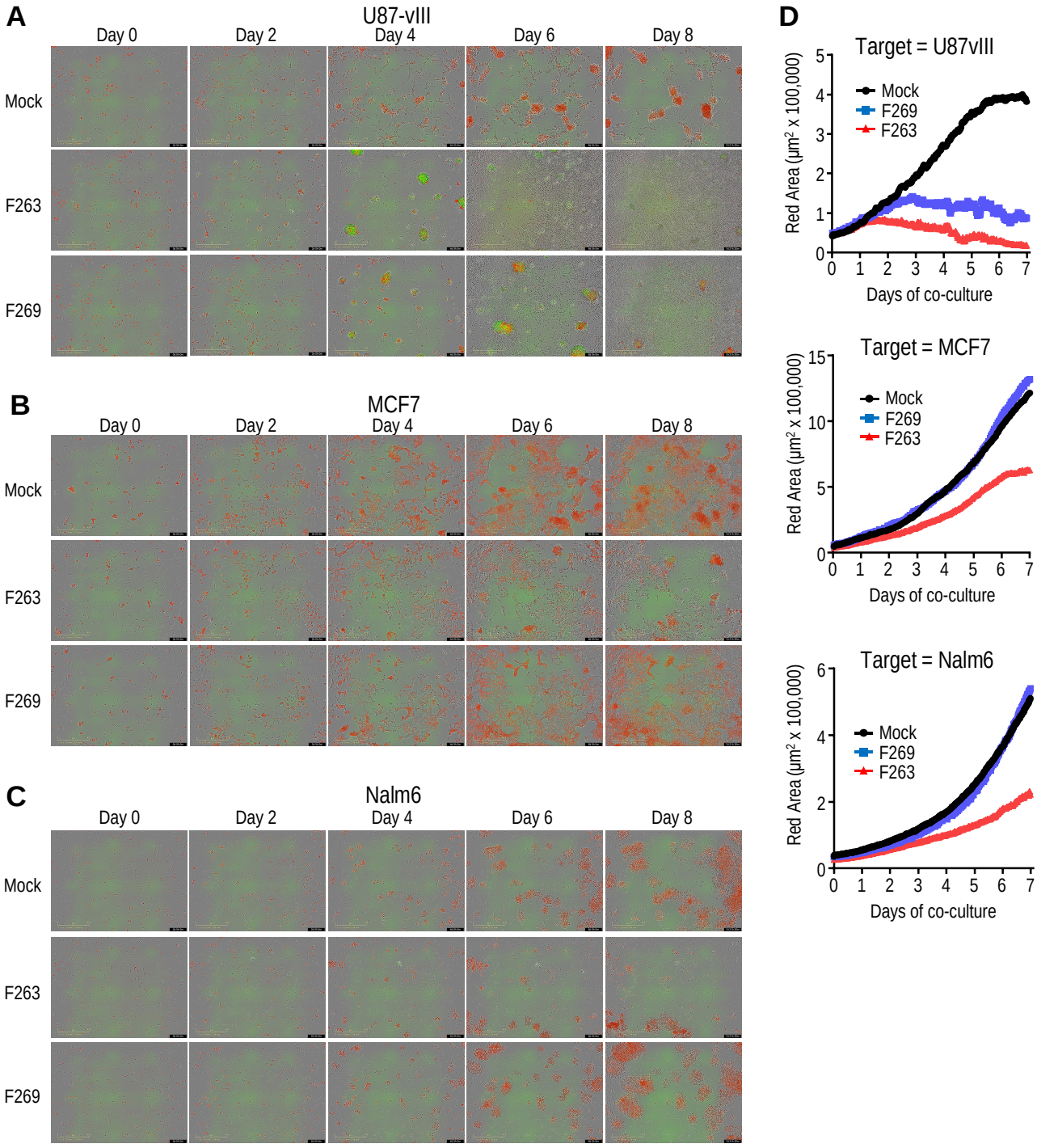


Figure S3: CAR-T effector function testing using continuous live-cell imaging: Donor 1 (supplement to Figure 4).

(A) Sample time-lapse images of mock (top) and F263 (middle) and F269 (bottom) EGFRvIII CAR-T cells (green) co-cultured at a 1:1 ratio alongside U87-vIII cells stably expressing nuclear-localized mKate2 (red). CAR-T activation and proliferation is apparent by formation of GFP-positive T cell blasts. (B) Similar sample images from MCF7 co-cultures. (C) Sample time-lapse images of mock (top) and F263 (middle) and F269 (bottom) EGFRvIII CAR-T cells (green) co-cultured at a 1:1 ratio alongside Nalm6 cells stably expressing nuclear-localized mKate2 (red). (D) Assessment of mKate2 (red) area measurements for co-cultures performed with CAR-T cells derived from Donor 1. These images correspond to Figure 5I & 5J.

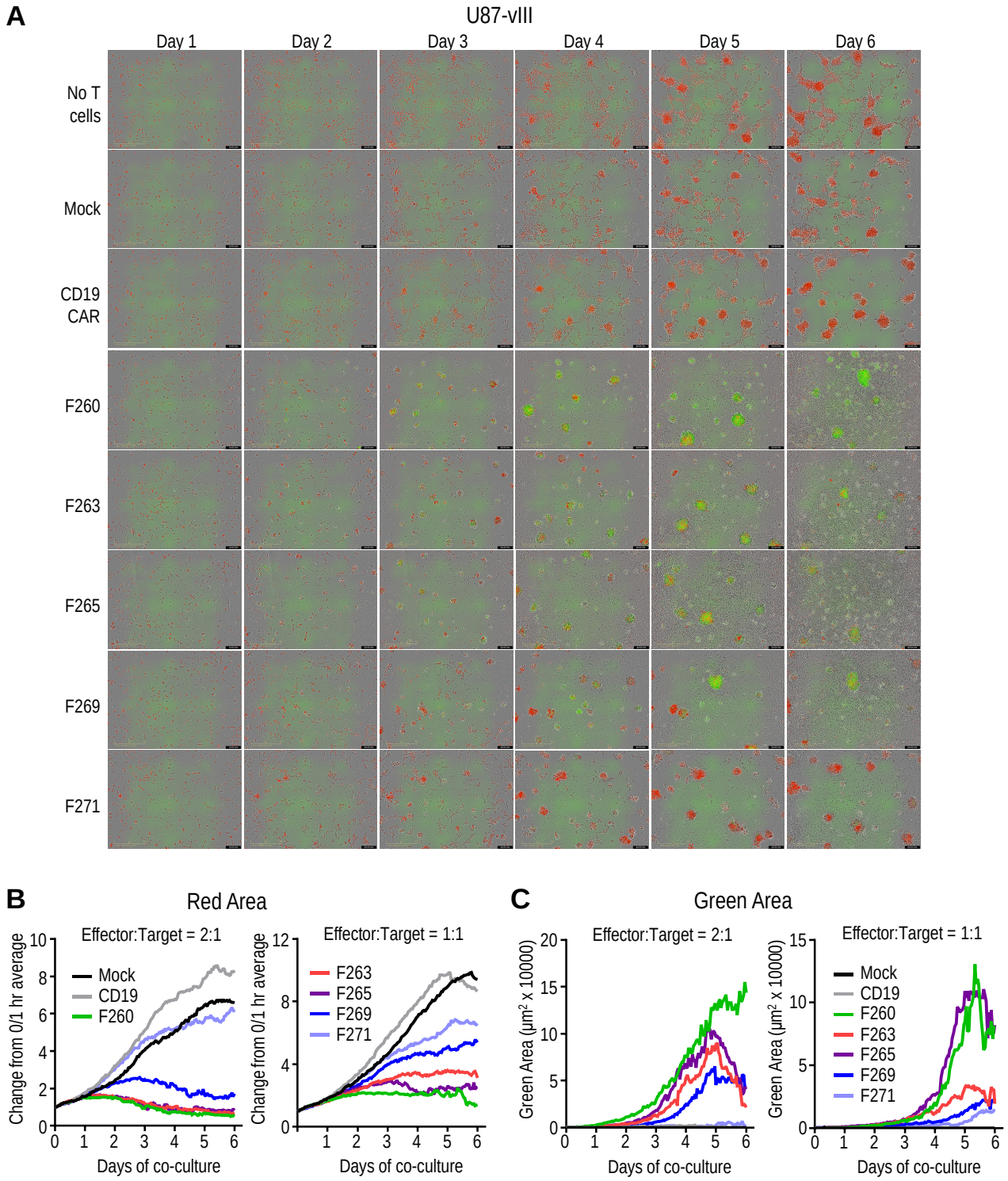


Figure S4: CAR-T effector function testing using continuous live-cell imaging: results from Donor 4 (supplement to Figure 4).

(A) Sample time-lapse images of EGFRvIII CAR-T cells (green) co-cultured at a 1:1 ratio alongside U87-vIII cells stably expressing nuclear-localized mKate2 (red). These images correspond to data reported in Figure 5I & 5J. Assessment of U87-vIII growth (mKate2 area) (B) and CAR-T proliferation (GFP area) (C) for Donor 4 co-cultures.

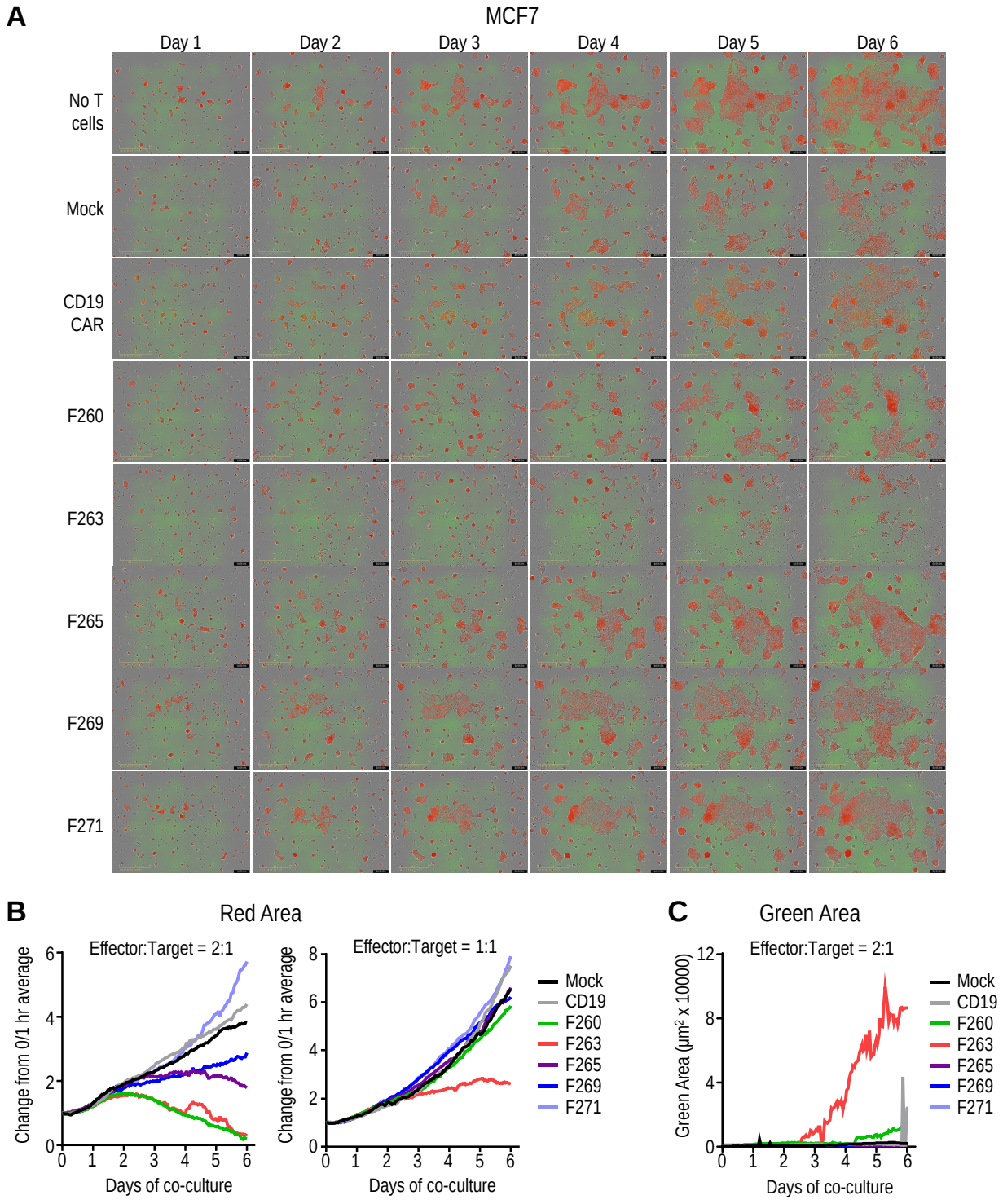


Figure S5. CAR-T effector function testing using continuous live-cell imaging: results from Donor 4 (supplement to Figure 4)

(A) Sample time-lapse images of EGFRvIII CAR-T cells (green) co-cultured at a 1:1 ratio alongside MCF7 cells stably expressing nuclear-localized mKate2 (red). These images correspond to Figure 5I & 5J. Assessment of MCF7 growth (mKate2 area) (B) and CAR-T proliferation (GFP area) (C) for Donor 4 co-cultures.

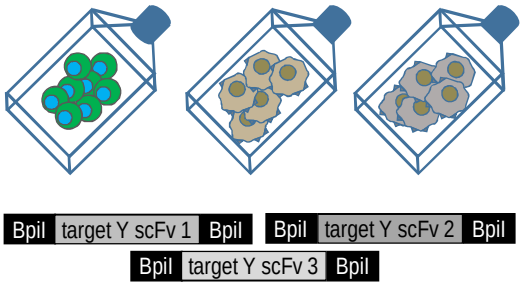
Day 0

Maintain healthy Jurkat and target cells.

Ensure adequate cell numbers by Day 2:
- 1 96-well plate = 4 x 10^6 Jurkat cells
- 1 96-well plate = 5 x 10^5 target cells

Ensure adequate pSLCAR-CD19 DNA:
- 16 wells = 600,000 Jurkat + 3 µg DNA per CAR construct being tested

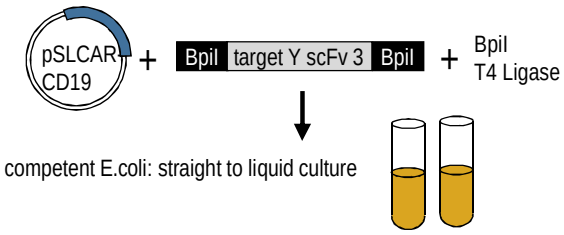
Design and acquire target-specific scFv/ABD DNA sequences.



Day 1

Clone scFv/ABD DNA into pSLCAR-CD19 and transform into competent cells.

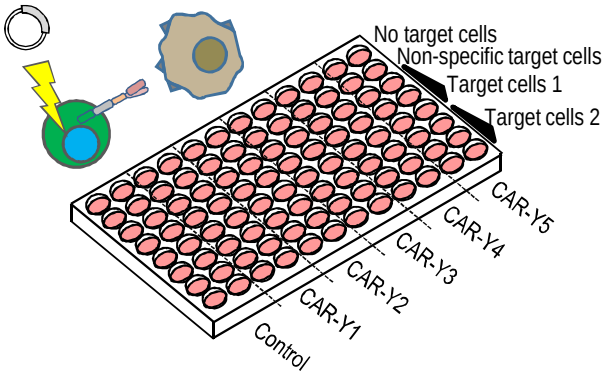
Alternate: use Gibson cloning.



Day 2

Mini-prep polyclonal culture.

Perform CAR-J assay:
- Morning: electroporate Jurkat cells
- 4 hours later, co-culture with appropriate numbers of target cells.



Day 3

Stain and analyze cells via flow cytometry.

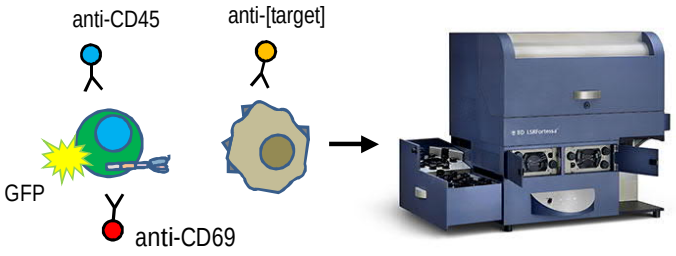


Figure S6. Overview of rapid CAR-J screening protocol. (supplement to Figure 6)

Supplemental Video Titles and Captions

Supplemental Video 1 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 non-transduced cells co-cultured with U87-vIII (red).

Supplemental Video 2 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 F263 CAR-T cells (green) co-cultured with U87-vIII (red).

Supplemental Video 3 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 F269 CAR-T cells (green) co-cultured with U87-vIII (red).

Supplemental Video 4 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 non-transduced cells co-cultured with MCF7 (red).

Supplemental Video 5 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 F263 CAR-T cells (green) co-cultured with MCF7 (red).

Supplemental Video 6 (supplement to Figures 3-5): Time-lapse video created using live-cell images of Donor 4 F269 CAR-T cells (green) co-cultured with MCF7 (red).