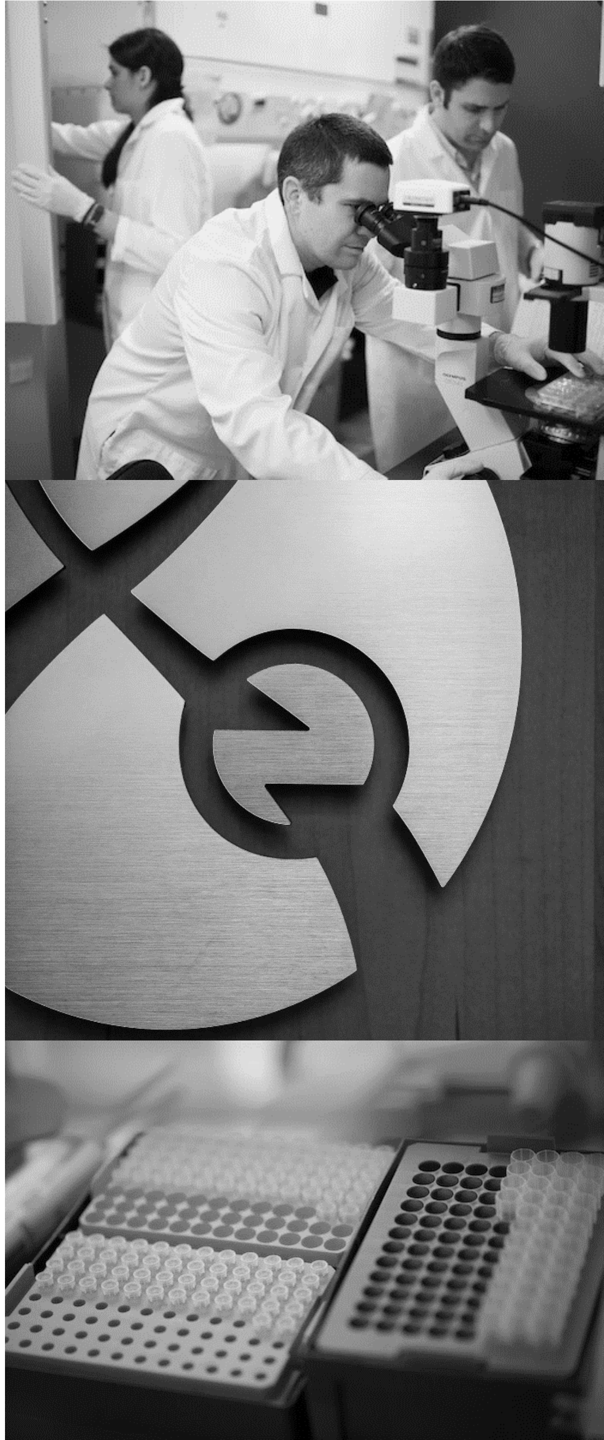
Developing Meganucleases for Clinical Applications

Derek Jantz PhD, Chief Scientific Officer

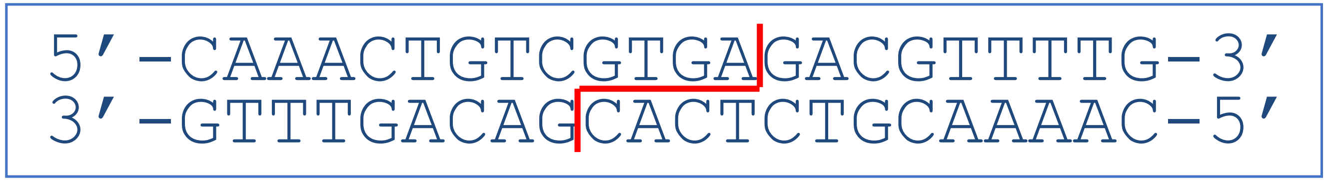


PRECISION
BIOSCIENCES

Meganucleases

Meganucleases are engineered variants of the I-CreI homing endonuclease from *Chlamydomonas reinhardtii*

- I-CreI targets the insertion of a Group I intron into the 23S rRNA gene
- The I-CreI target site is 22bp; cleavage produces a pair of 4bp 3′ overhangs

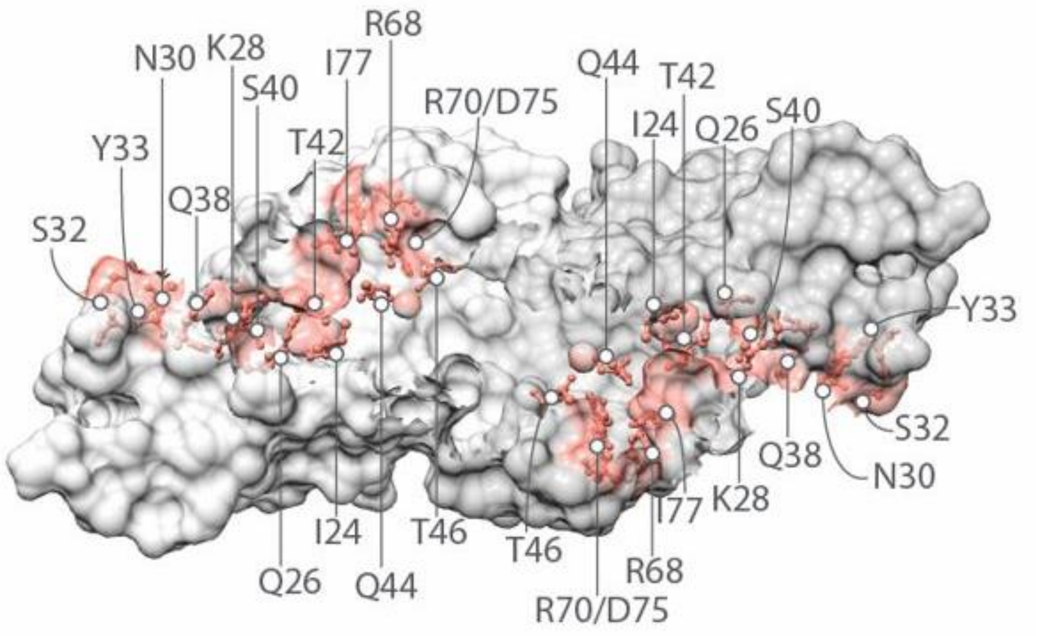


5′-CAAACTGTCGTGAGACGTTTTG-3′
3′-GTTTGACAGCACTCTGCAAAAC-5′

- Meganucleases are small (310 aa) and monomeric
- Meganucleases recognizes DNA through direct amino acid side-chain-to-base contacts that can be changed to confer novel binding specificities.

TRC 1-2x.87EE: a meganuclease for T cell editing

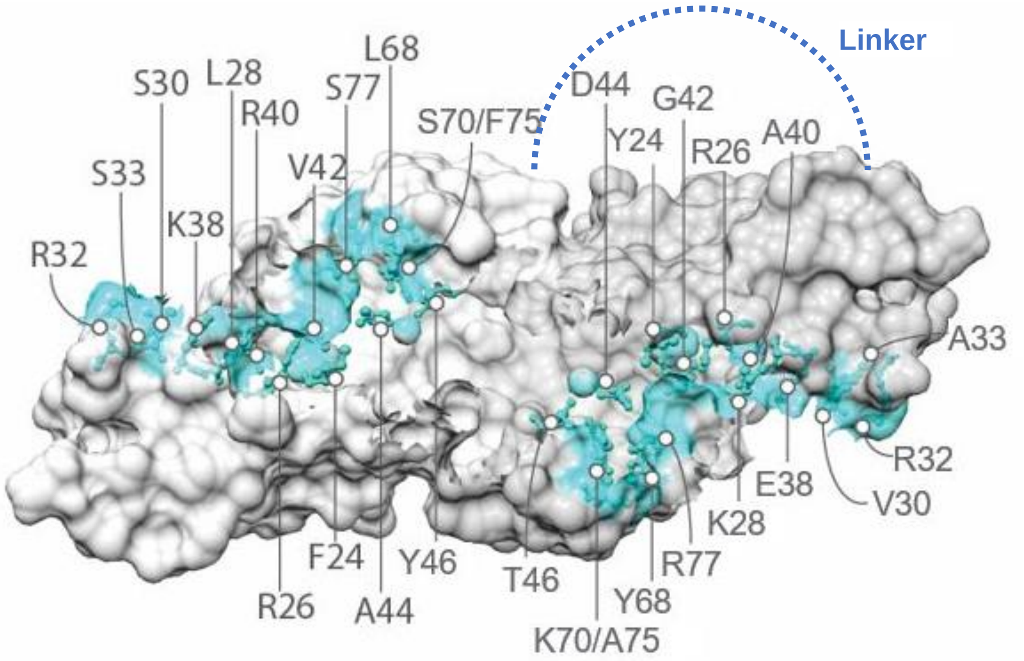
I-CreI



GCAAACGTCGTGAGACAGTTTCG

wild-type I-CreI target site in *Chlamydomonas*

TRC 1-2x.87EE

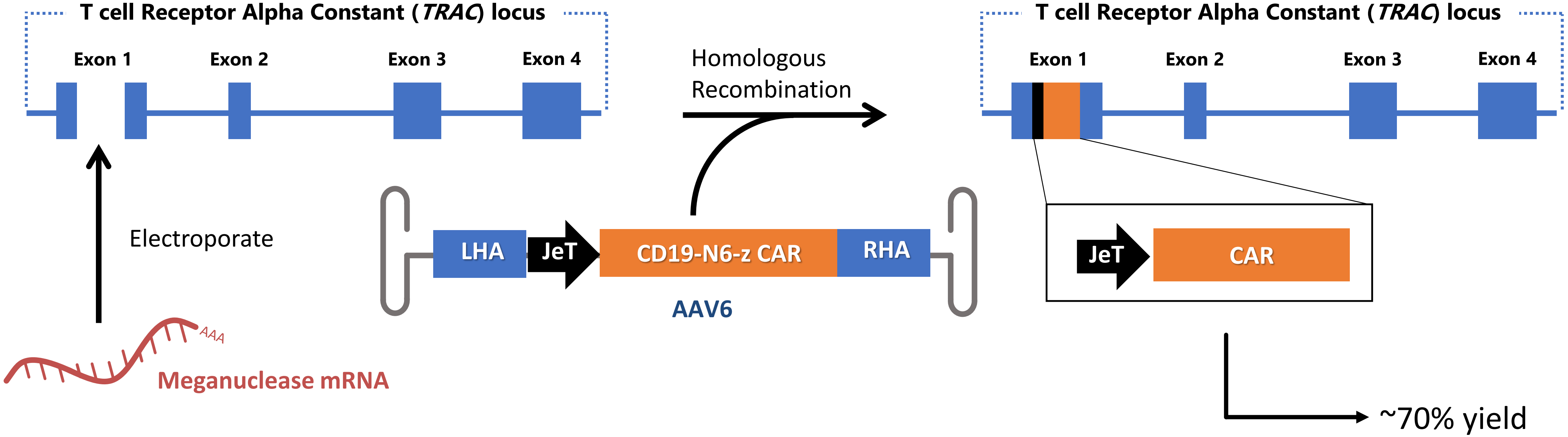


TGG**CC**TGGAGCAACAAA**T**CTGA

target site in human *TRAC* gene

- TRC 1-2x.87EE is a single-chain meganuclease that recognizes Exon 1 of the human *TRAC* gene

“One step” allogeneic CAR-T cells

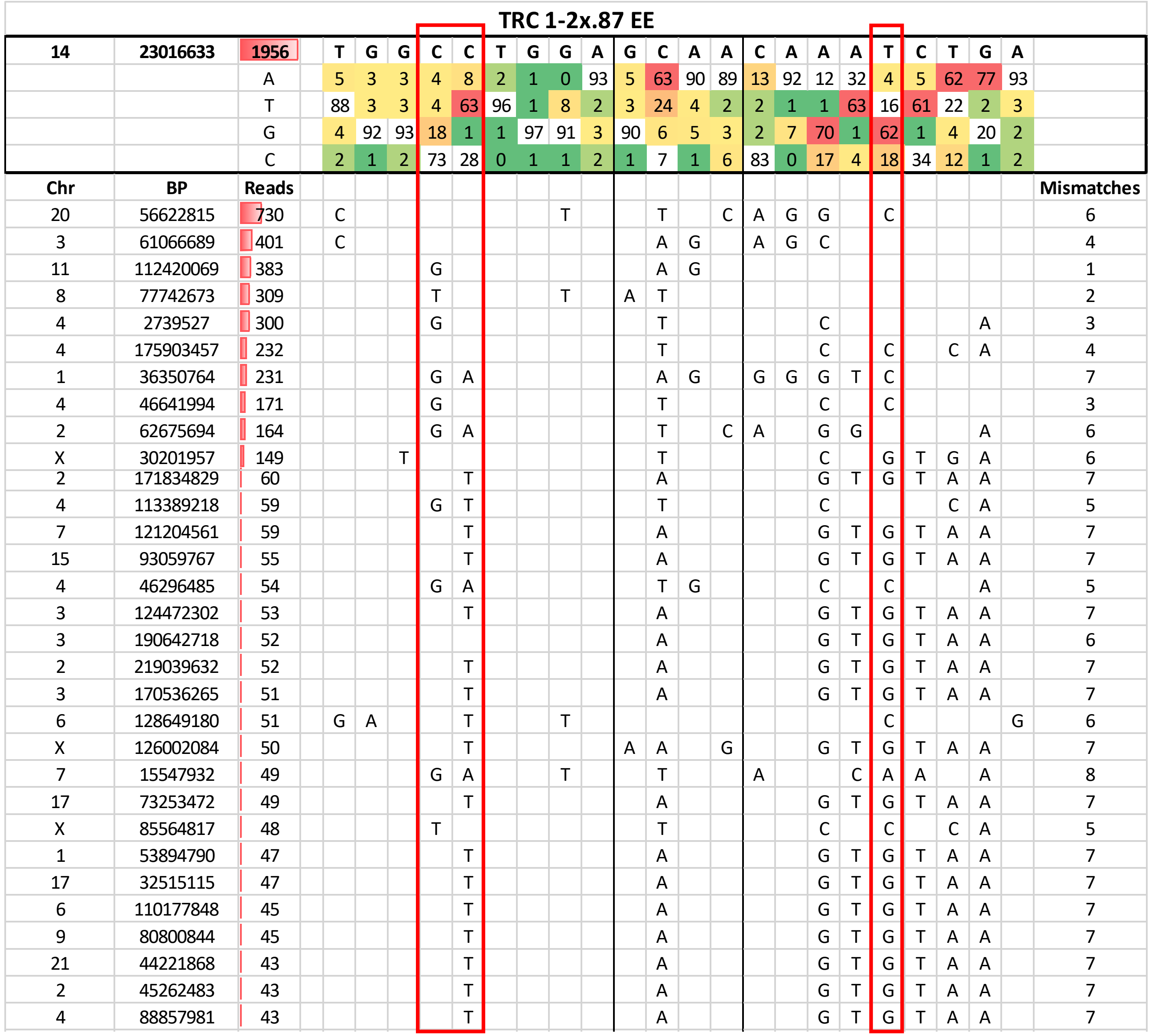


TRC 1-2x.87EE cuts off-target frequently

44 confirmed off-target sites

Off-target sites cut with high frequency

Repeated sequence

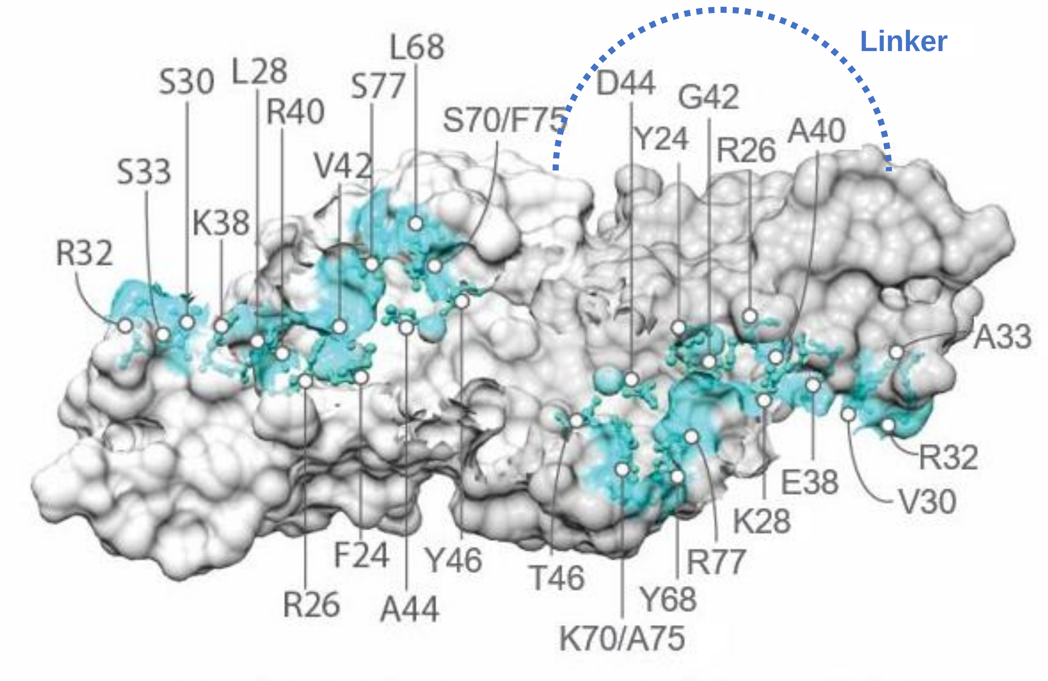


TRC 1-2x.87 EE

14	23016633	1956	T	G	G	C	C	T	G	G	A	G	C	A	A	C	A	A	A	T	C	T	G	A		
		A	5	3	3	4	8	2	1	0	93	5	63	90	89	13	92	12	32	4	5	62	77	93		
		T	88	3	3	4	63	96	1	8	2	3	24	4	2	2	1	1	63	16	61	22	2	3		
		G	4	92	93	18	1	1	97	91	3	90	6	5	3	2	7	70	1	62	1	4	20	2		
		C	2	1	2	73	28	0	1	1	2	1	7	1	6	83	0	17	4	18	34	12	1	2		
Chr	BP	Reads																							Mismatches	
20	56622815	730	C							T			T		C	A	G	G		C					6	Off-2
3	61066689	401	C										A	G		A	G	C							4	
11	112420069	383				G							A	G											1	
8	77742673	309				T				T		A	T												2	
4	2739527	300				G							T					C					A		3	
4	175903457	232											T					C		C		C	A		4	
1	36350764	231				G	A						A	G		G	G	G	T	C					7	
4	46641994	171				G							T					C		C					3	
2	62675694	164				G	A						T		C	A		G	G				A		6	
X	30201957	149			T								T					C		G	T	G	A		6	
2	171834829	60					T						A					G	T	G	T	A	A		7	Off-1
4	113389218	59				G	T						T					C				C	A		5	
7	121204561	59					T						A					G	T	G	T	A	A		7	
15	93059767	55					T						A					G	T	G	T	A	A		7	
4	46296485	54				G	A						T	G				C		C			A		5	
3	124472302	53					T						A					G	T	G	T	A	A		7	
3	190642718	52											A					G	T	G	T	A	A		6	
2	219039632	52					T						A					G	T	G	T	A	A		7	
3	170536265	51					T						A					G	T	G	T	A	A		7	
6	128649180	51	G	A			T			T										C				G	6	
X	126002084	50					T					A	A		G			G	T	G	T	A	A		7	
7	15547932	49				G	A			T			T			A			C	A	A		A		8	
17	73253472	49					T						A					G	T	G	T	A	A		7	
X	85564817	48				T							T					C		C		C	A		5	
1	53894790	47					T						A					G	T	G	T	A	A		7	
17	32515115	47					T						A					G	T	G	T	A	A		7	
6	110177848	45					T						A					G	T	G	T	A	A		7	
9	80800844	45					T						A					G	T	G	T	A	A		7	
21	44221868	43					T						A					G	T	G	T	A	A		7	
2	45262483	43					T						A					G	T	G	T	A	A		7	
4	88857981	43					T						A					G	T	G	T	A	A		7	

A Second-Generation Nuclease Was Produced

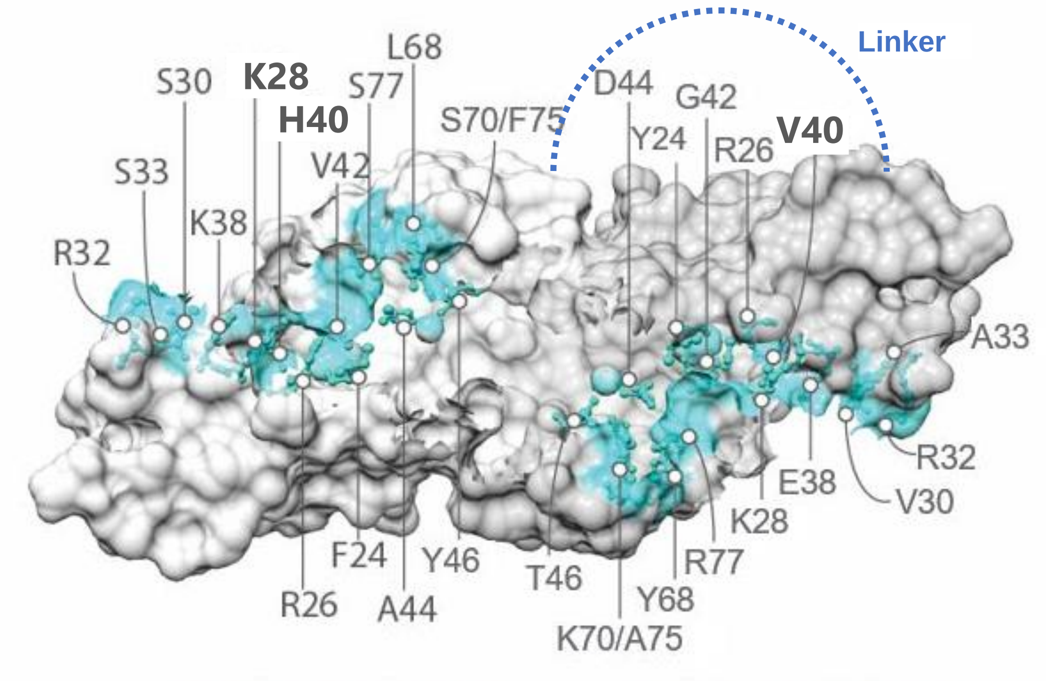
TRC 1-2x.87EE
(Generation 1)



TGGCCTGGAGCAACAAATCTGA

target site in human *TRAC* gene

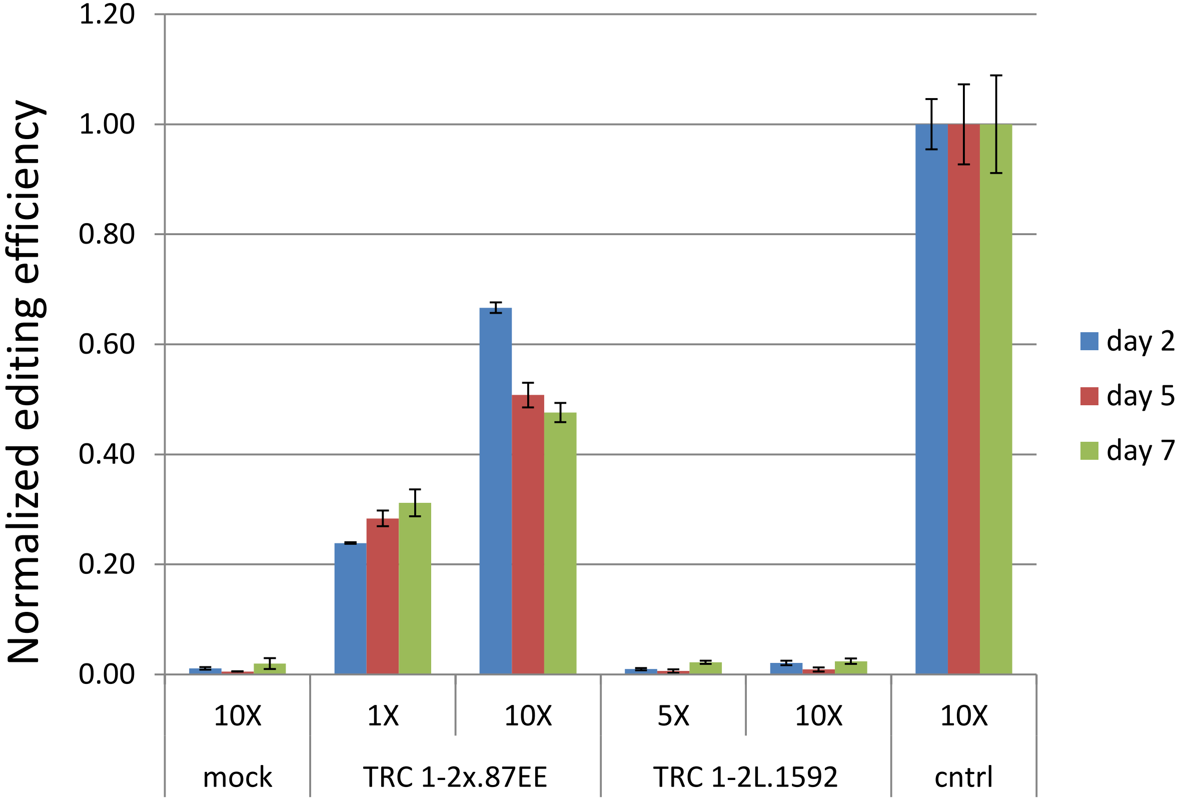
TRC 1-2L.1592
(Generation 2)



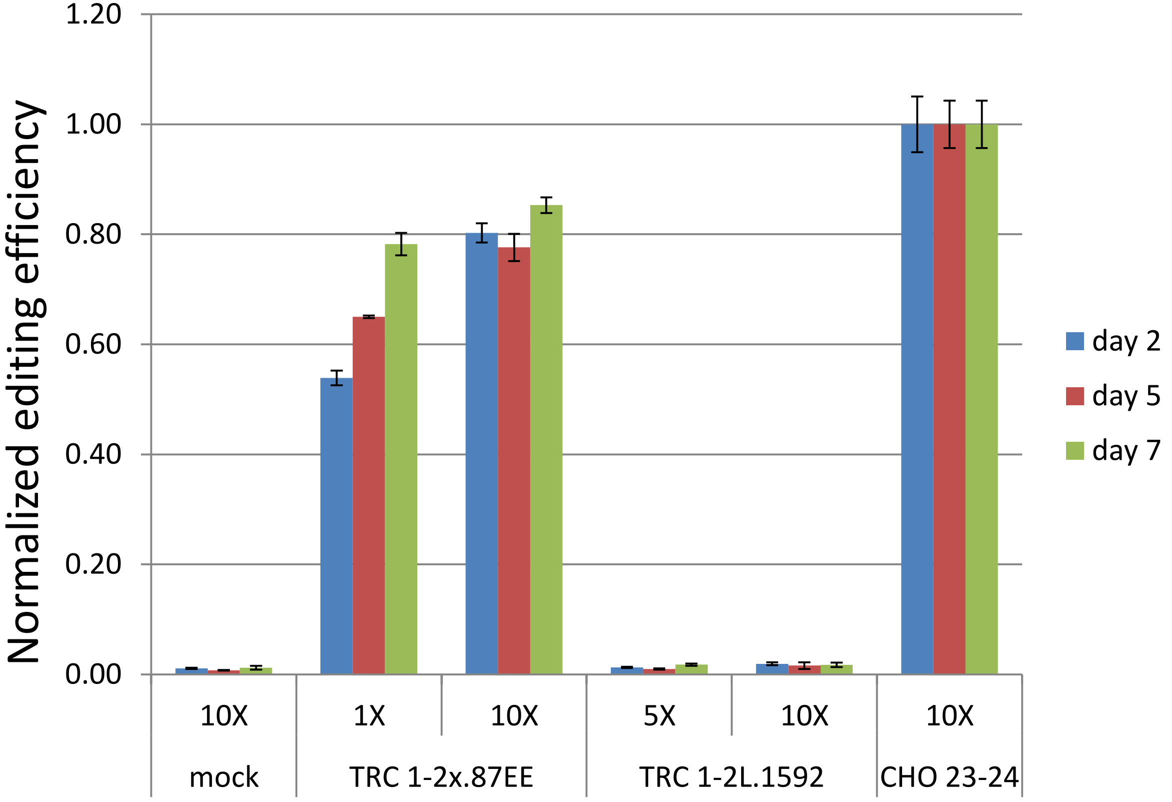
TGG**CC**TGGAGCAACAAA**T**CTGA

target site in human *TRAC* gene

TRC 1-2L.1592 does not cut Off-1 or Off-2 in a reporter assay

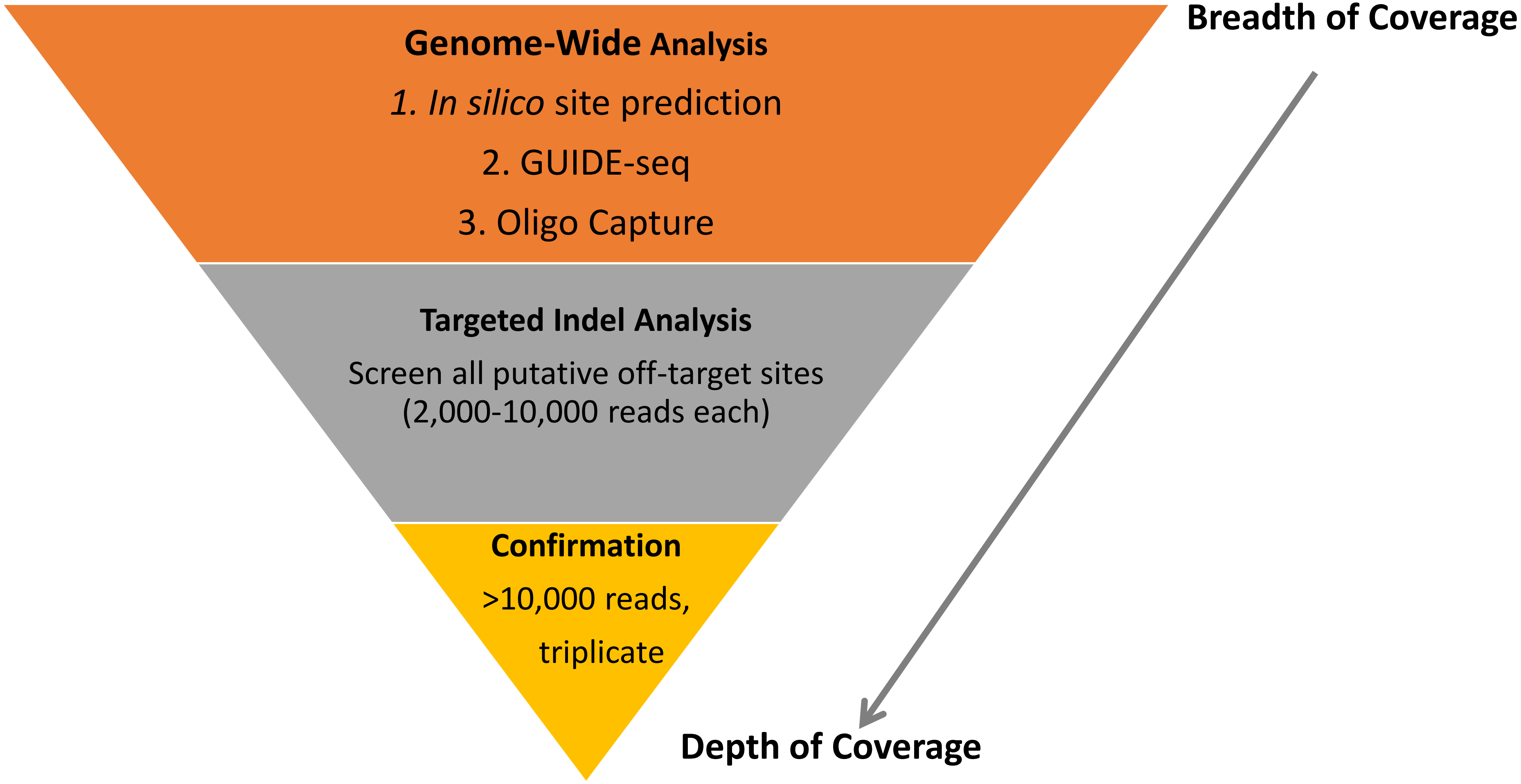


Off-1 target: Repeat



Off-2 target: High Frequency

TRC 1-2L.1592 off-target analysis



Genome-Wide Analysis

1. In silico site prediction

2. GUIDE-seq

3. Oligo Capture

Targeted Indel Analysis

Screen all putative off-target sites
(2,000-10,000 reads each)

Confirmation

>10,000 reads,
triplicate

Breadth of Coverage

Depth of Coverage

Genome-Wide Analysis 1: *in silico* site prediction

All sites in the genome <4bp different from the intended target site (COSMID)

site #	Chr	Pos	Sequence																						Triplicate?	Indel Frequency (%)
site 0	14	23016633	T	G	G	C	C	T	G	G	A	G	C	A	A	C	A	A	A	T	C	T	G	A	Y	61.35
site 1	19	50167592	A											C	C										N	N.S
site 2	2	121248255	C									A								C					N	N.S
site 3	2	168041009	G			A														A					N	N.S
site 4	7	124214889	G			T																A			N	N.S
site 5	11	579363			C								A					T							N	N.S
site 6	8	1220286			T													G	C						N	N.S
site 7	X	90307430				A														A	T				N	N.S
site 8	11	112420069				G							A	G											Y	N.S
site 9	6	146944540				T			T												A				N	N.S
site 10	X	130371540						A	A	T															N	N.S
site 11	2	192882479											A			T					T				N	N.S
site 12	18	52058914												C		G		C							N	N.S
site 13	11	65311098													C			G		G					N	N.S
site 14	17	70963906													T		C		C						Y	0.23
site 15	1	30736654																		A		A			N	N.S

N.S.: not significant (<1 STDEV over mock)

Genome-Wide Analysis 2: GUIDE-seq

Standard protocol and software modified to remove requirement for PAM sequence

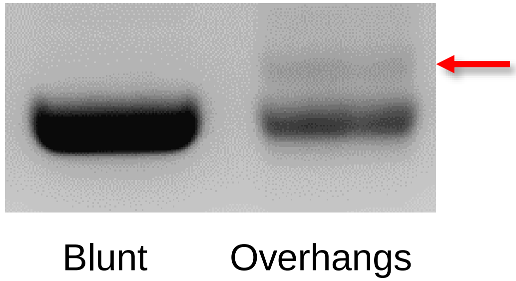
site #	Chr	Pos	Sequence																						Triplicate?	Indel Frequency (%)
site 0	14	23016633	T	G	G	C	C	T	G	G	A	G	C	A	A	C	A	A	A	T	C	T	G	A	Y	61.35
site 200	3	45249461	G							A	G	A					T	C		C					N	N.S
site 201	4	149232383				T	T						T	T		T	T				T				N	N.S

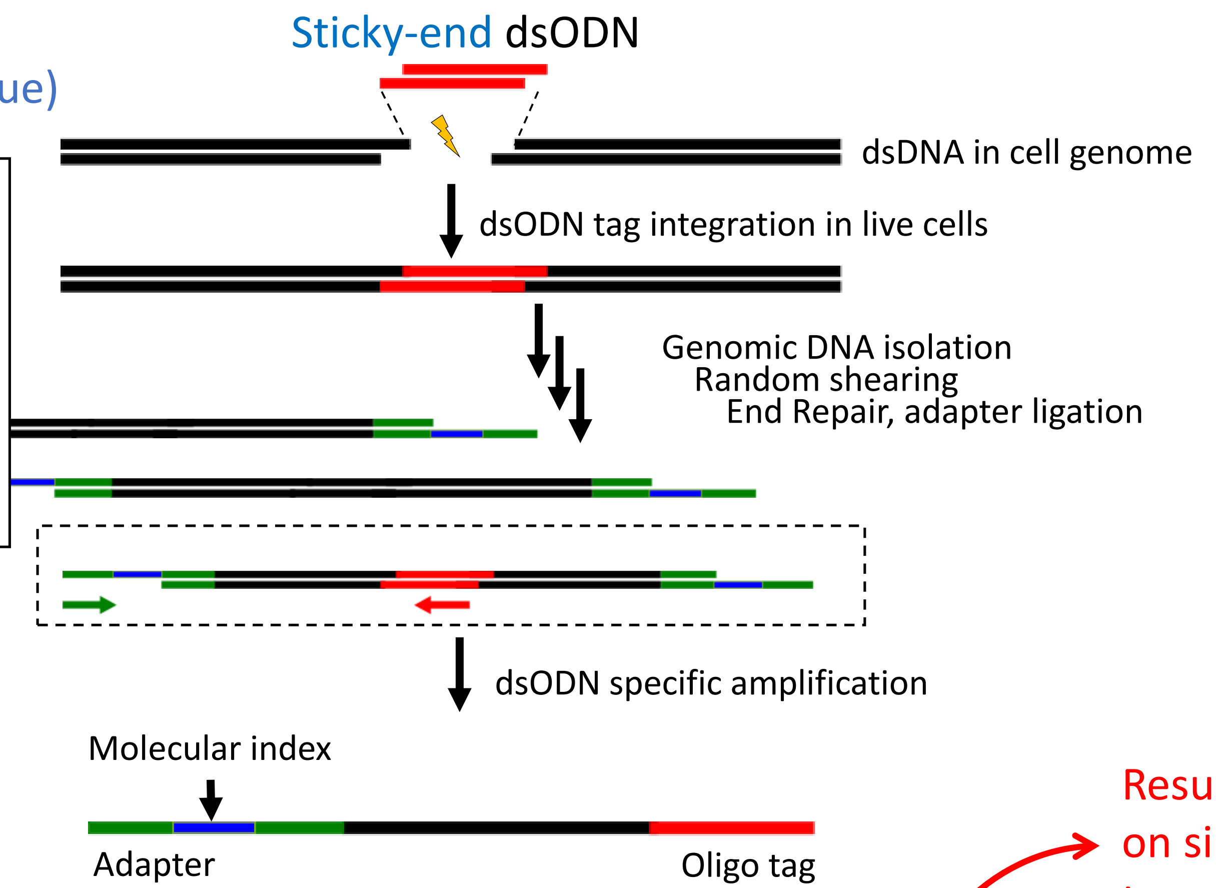
N.S.: not significant (<1 STDEV over mock)

Oligo Capture

(GUIDE-seq differences highlighted in blue)

cassette captured in *TRAC* gene





Sticky-end dsODN

dsDNA in cell genome

dsODN tag integration in live cells

Genomic DNA isolation
Random shearing
End Repair, adapter ligation

dsODN specific amplification

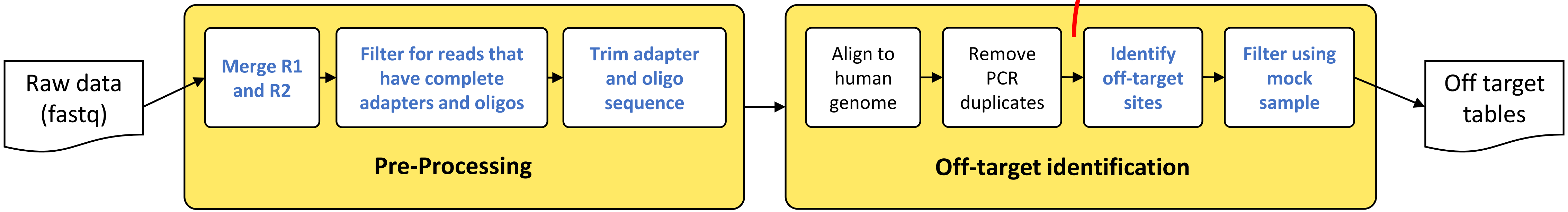
Molecular index

Adapter

Oligo tag

NGS library prep and sequencing

Results not filtered based on similarity to intended target site



Raw data (fastq)

Pre-Processing

- **Merge R1 and R2**
- **Filter for reads that have complete adapters and oligos**
- **Trim adapter and oligo sequence**

Off-target identification

- Align to human genome
- Remove PCR duplicates
- **Identify off-target sites**
- **Filter using mock sample**

Off target tables

Adapted from Tsai, et al. Nature Biotech 2015, 33(2), 187-197

Genome-Wide Analysis 3: Oligo Capture

site #	Chr	Pos	Sequence																						Triplicate?	Indel Frequency (%)
site 0	14	23016633	T	G	G	C	C	T	G	G	A	G	C	A	A	C	A	A	A	T	C	T	G	A	Y	61.35
site 8	11	112420069				G							A	G											Y	N.S
site 14	17	70963906													T		C		C						Y	0.23
site 43	7	45886383	G				G						A		T	A		C							Y	0.44
site 46	11	117707291											T			A		C				C	T		Y	0.85
site 79	8	70953326	G										T			A	C		C						Y	2.83
site 80	15	91730805					T			T			G		T	A		C						T	Y	0.45
site 81	6	134362399				G						T	A	G	G				G	A		A		T	N	N.S
site 83	20	51802795			T				A	A		T	T	G		A		C			T				N	N.S
site 84	2	85864762						A		A		C	A	C	T	T				C		A		C	N	N.S
site 85	5	75148730	A	A			T					T		T					T	A	A				N	N.S
site 86	14	96013249	A			T	G		T		T		G	T	G			C							N	N.S
site 89	8	68898594						A	A						G	G			C			C	A	T	N	N.S
site 92	22	48584419				A	A			A		A	G		T	A			C				C	T	N	N.S
site 93	2	9176023			A	A	G	A				T	T	T	C	T				C	T				N	N.S
site 94	4	183627898		T		T			T			A		T	C		T	G		C			A		Y	N.S
site 95	6	86300063			A					T	T	T	T	G	G	A				A	A			T	N	N.S
site 97	11	67957795			A	A	G			T		T	G	T	C			G	C					T	N	N.S
site 98	10	94357492					T			A		C		C		G	G		G	G		G			Y	N.S
site 100	2	200426071		T		T		A			T	T	T	G	T					G	A		C		N	N.S
site 101	7	102266445		A						T	G		A	T	G	A		C	G					T	N	N.S
site 102	12	133336771				A		A				A	T	C	C		C		C			A	A	T	N	N.S
site 103	1	91577468	A		T				A		C	T	A			G	C					A			N	N.S
site 105	2	207017087		A		T	A		T	T	T	T	A			A						A			Y	N.S

(47 total candidate sites identified)

Off-target site locations

- No sites in exons
- Three sites in non-coding regions of genes
- No known oncogenes
- No known association with relevant pathology

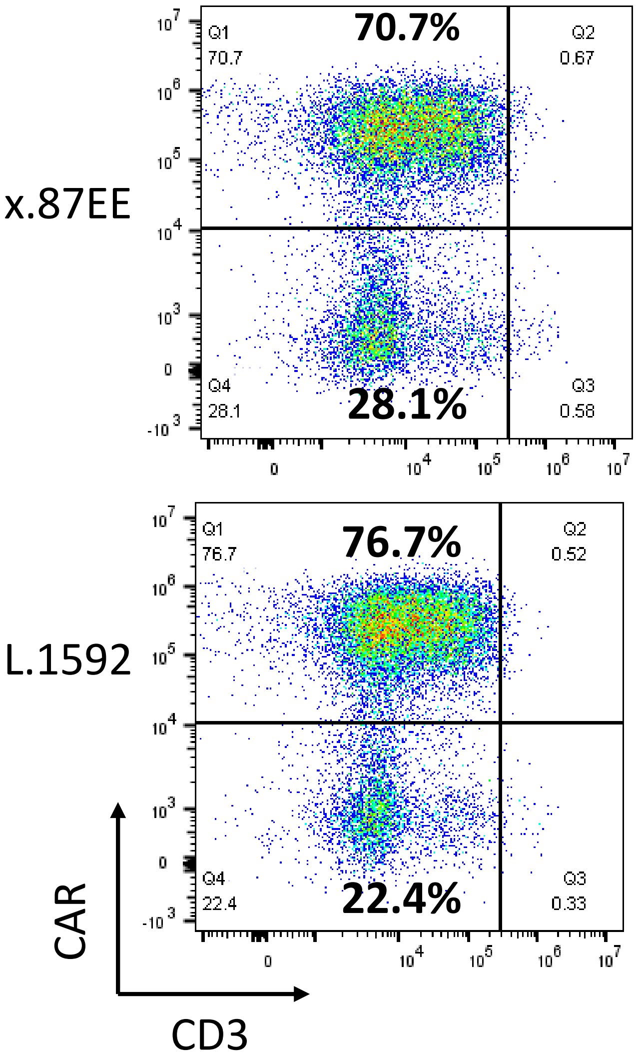
Site #	**% indel**	**Location**	
site 14	0.23	Intron 4 of ***SLC39A11*** , >10 kb from the nearest exon	Zinc transporter
site 43	0.44	>10 kb from closest known gene transcript	
site 46	0.85	3' UTR of ***FXYD6,*** 3225 bp from the nearest exon	Na,K-ATPase regulation
site 79	2.83	>10 kb from closest known gene transcript	
site 80	0.45	Intron 1 of ***SV2B*** *,* >10kb from the nearest exon	Uncharacterized secretory role

Editing safety studies

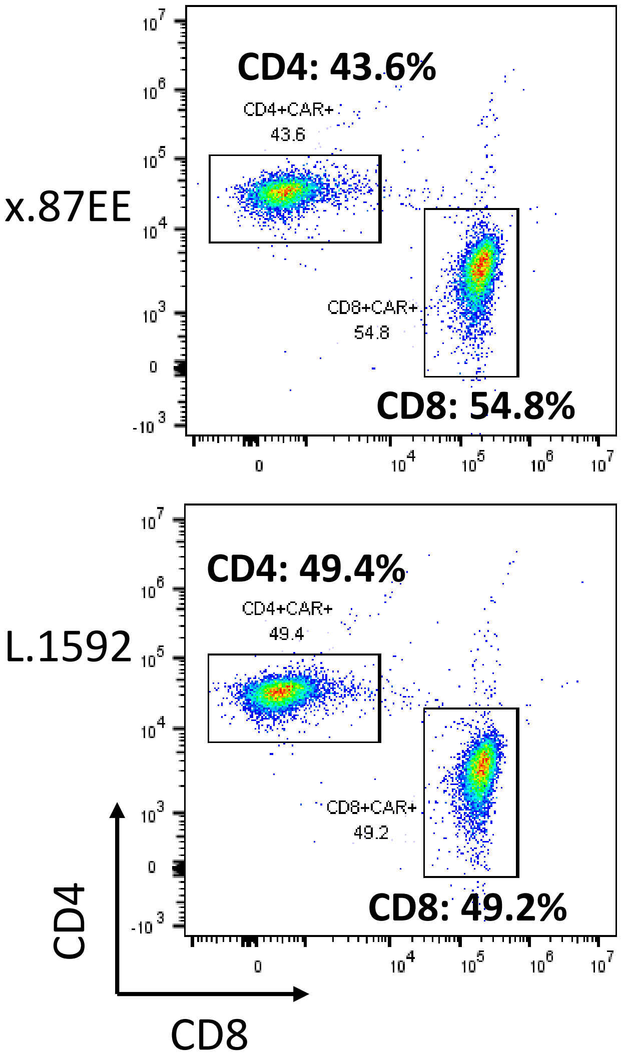
Study	TRC 1-2x.87EE (gen 1)	TRC 1-2L.1592 (gen 2)
Karyotype	N.D.	Negative
IL-2 Independent Growth	Negative	Negative
NSG Mouse Tumorigenesis	Negative	Negative

x.87EE vs. L.1592

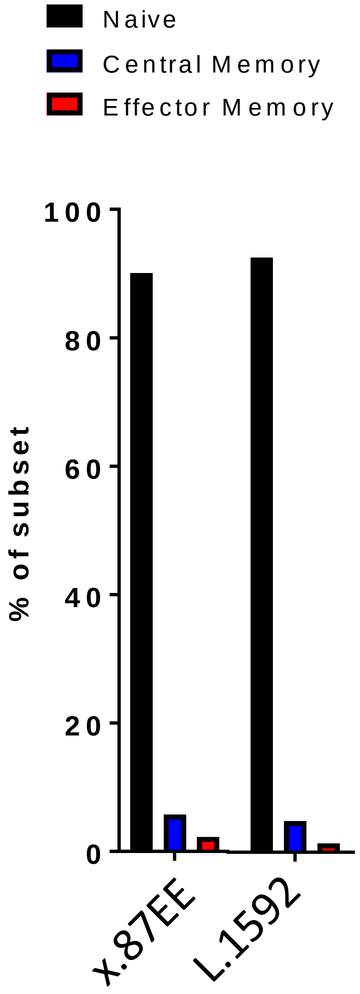
Knock-In Efficiency



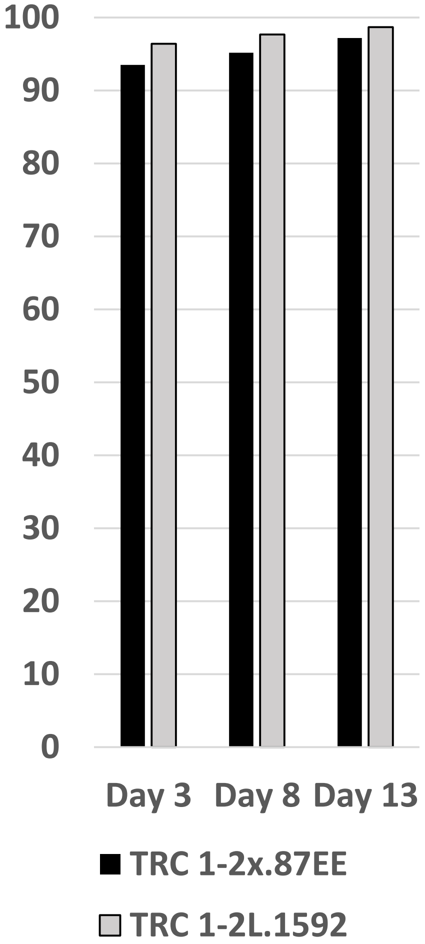
CD4:CD8 Ratio



Phenotype



Viability



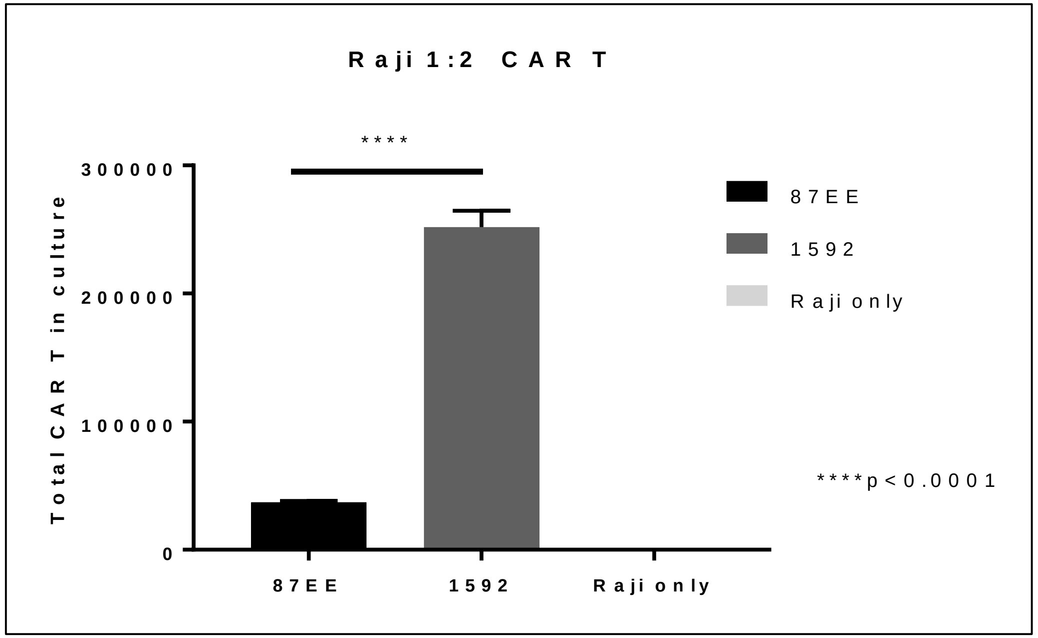
x.87EE vs. L.1592

Co-culture CAR T cells
And targets at
1 target: 2 CAR T cells

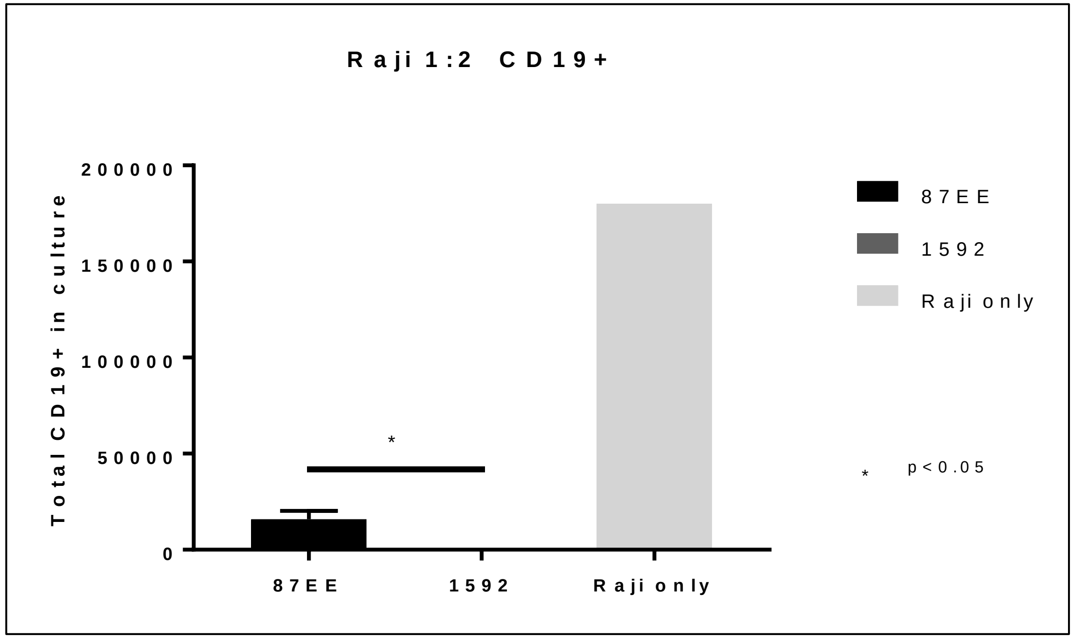
5 days →

Enumerate CAR T cell and
target number by flow
cytometry

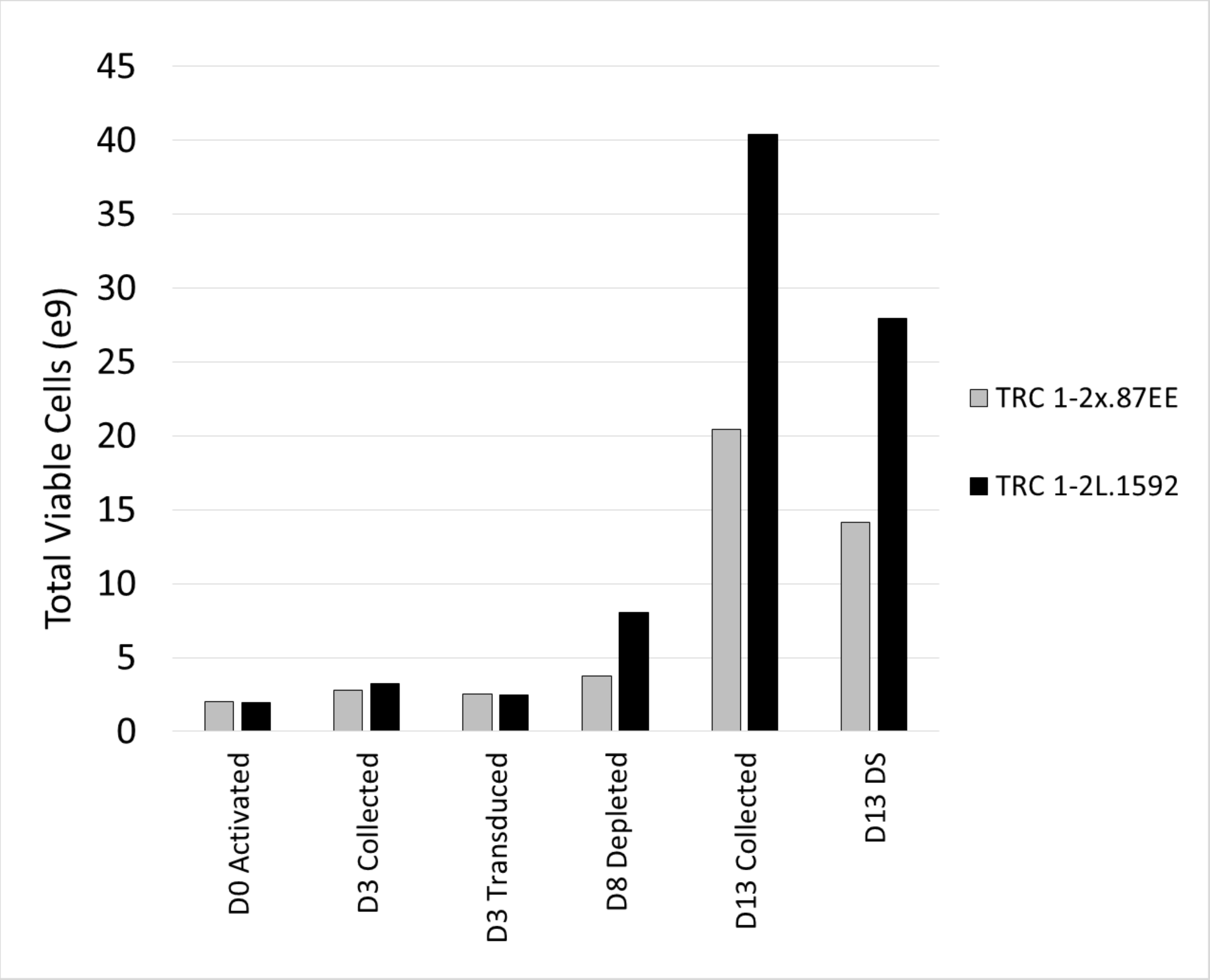
Cell expansion



Target cell killing expansion



x.87EE vs. L.1592



Concluding remarks

- Standardization across editing platforms will be challenging.
- Off-targeting can manifest itself in subtle ways that are probably more likely to affect efficacy/performance than safety.

Regarding off-targeting:

> “Try the best we can…”
>
> -Ed Rebar (yesterday, at least 3 times)

Thank You!