ThermoFisher SCIENTIFIC

CRISPR-based Genome Editing Tools: New Applications & Streamlined Workflows

Jason Potter

SynBio R&D

What makes CRISPR work?

Design

- Cut on-target
- Minimize off-target

Delivery

- Get in cell
- Get in nucleus

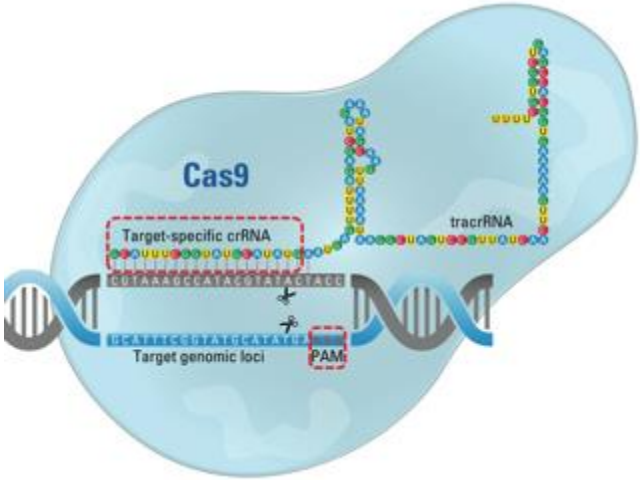
Find gRNA

Find target

Accessibility

- Chromatin
- Sequence variability

CRISPR-Cas9 and Targeted Genome Editing



- ✓ **Easy to design;**
- ✓ **simple workflow;**
- ✓ **fast implementation**

Available CRISPR-Cas9 delivery formats

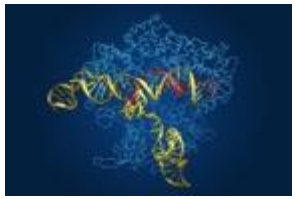
Plasmid

Market entry
Lowest efficiency
3 week workflow

mRNA

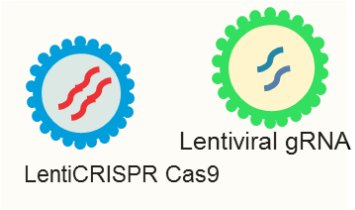
Greater versatility
Higher efficiency
1-2 week workflow

Cas9 RNP



Highest efficiency
Customer facing design tool
3-4 day workflow

Lentiviral particles

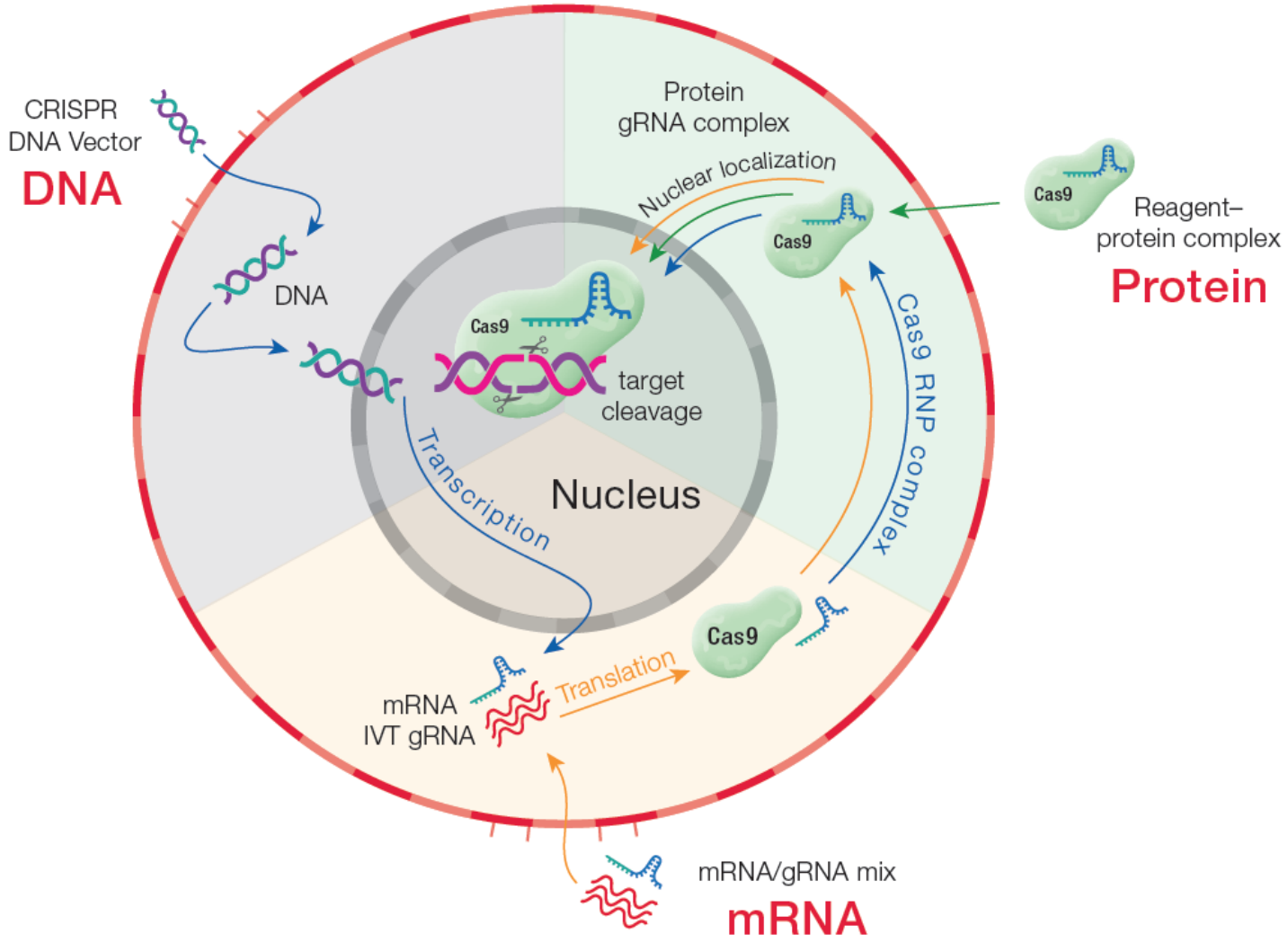


Enabling for
functional LOF
screening

ThermoFisher SCIENTIFIC

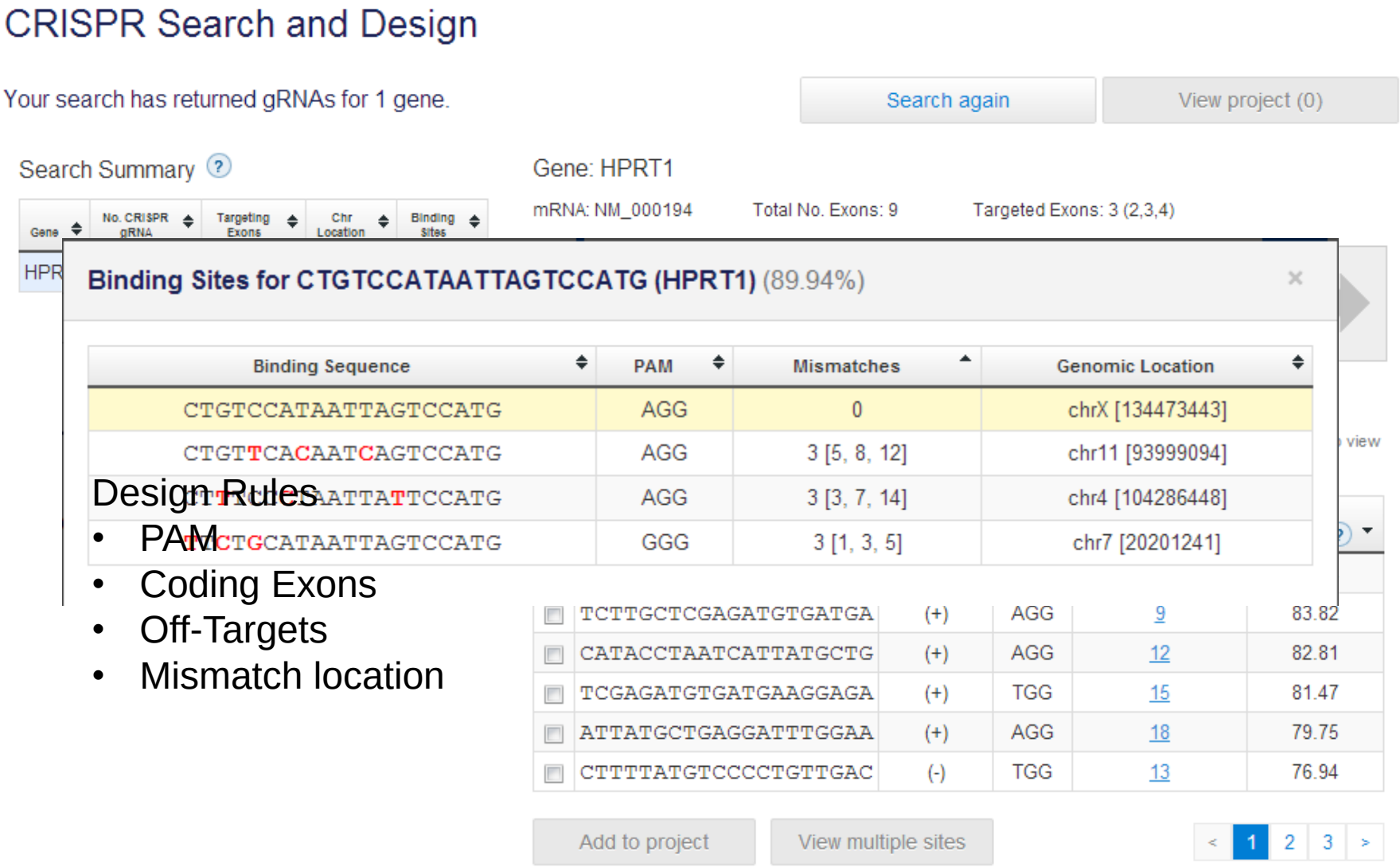
What has to happen for delivery?

Comparing the CRISPR-Cas9 Formats



ThermoFisher SCIENTIFIC

gRNA Design Tool

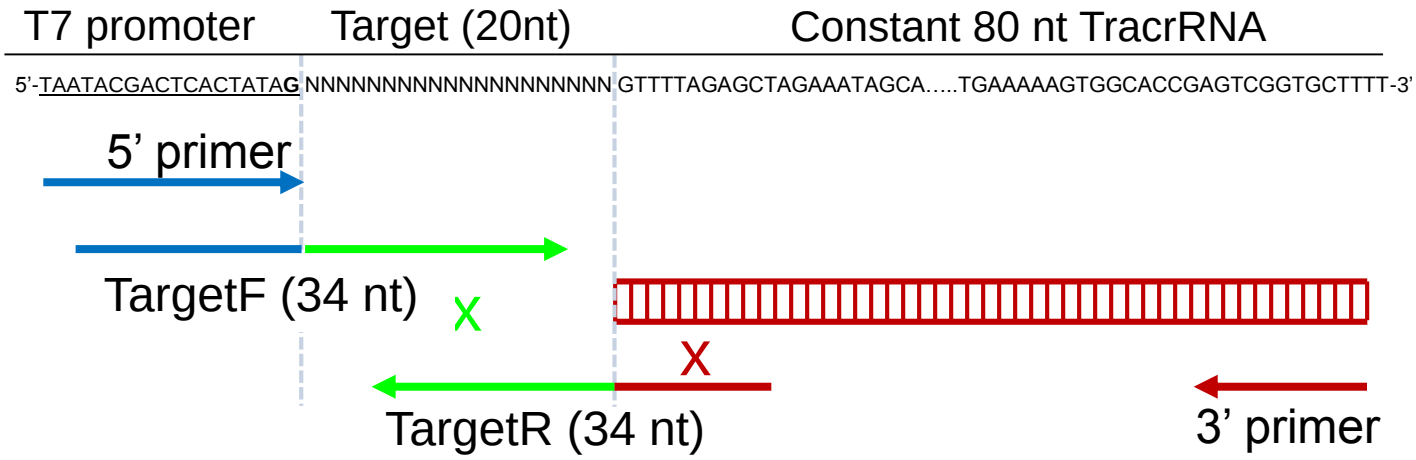


Design Rules

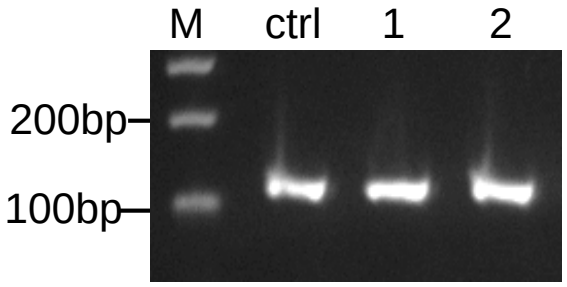
- PAM
- Coding Exons
- Off-Targets
- Mismatch location

Improved gRNA Synthesis Protocol

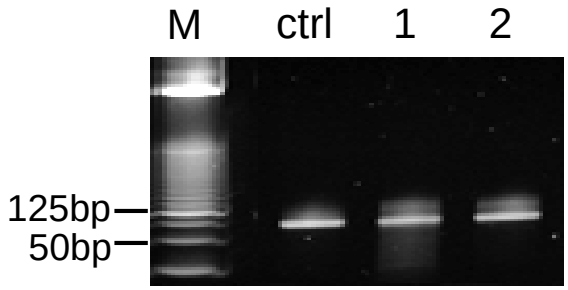
(1) Oligo design for gRNA template



(2) gRNA template by PCR



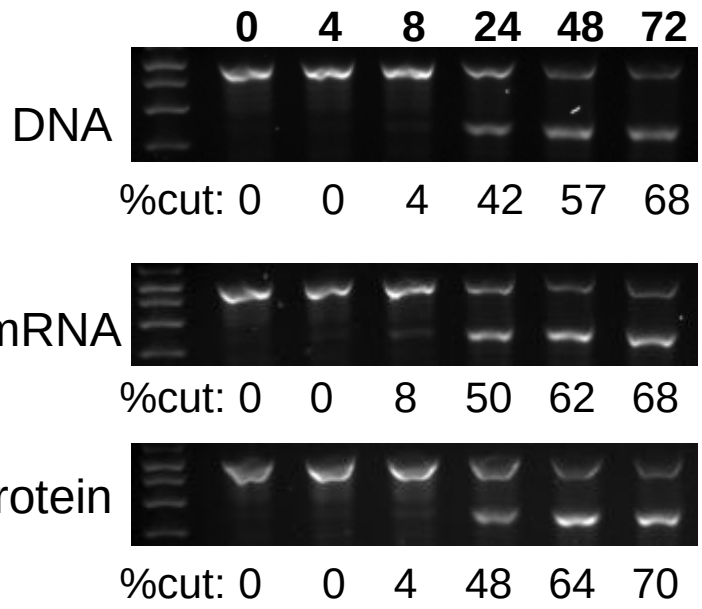
(3) synthetic gRNA by IVT



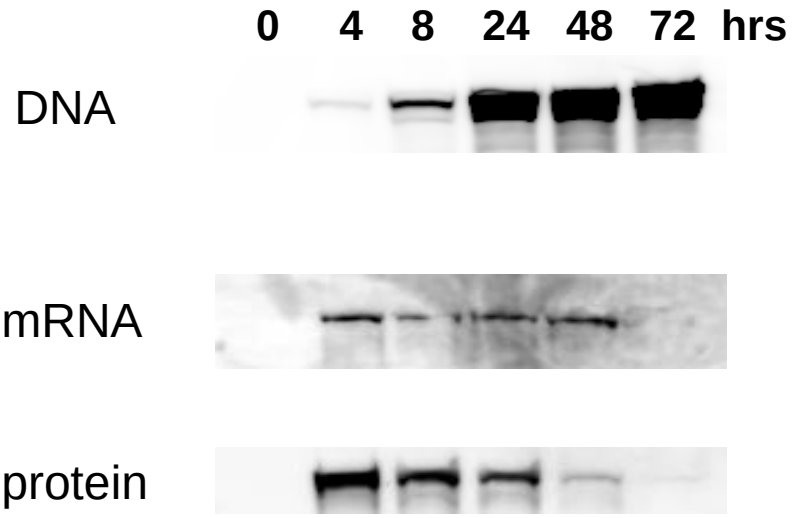
Time Course of Cas9 Activity

293FT

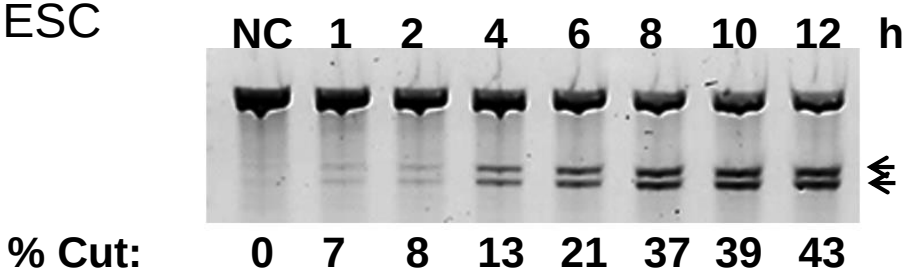
Time course of cleavage

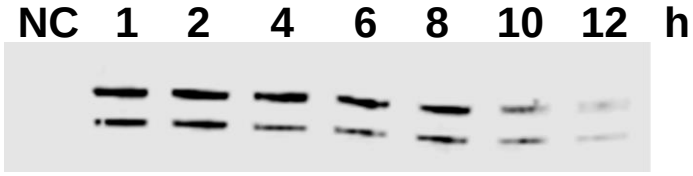


Western Blot

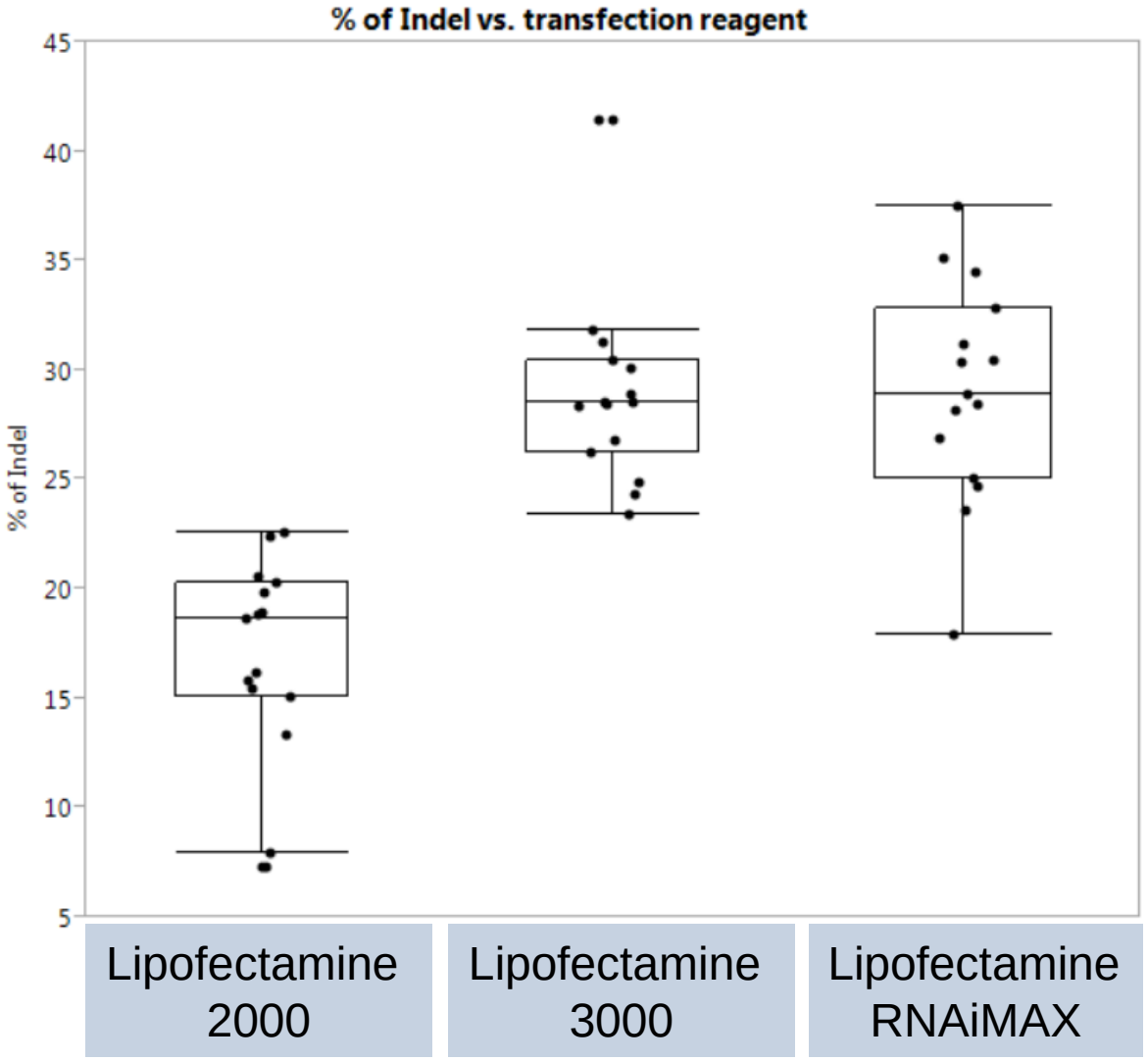


mESC





Lipid-mediated Transfection of Cas9 RNPs

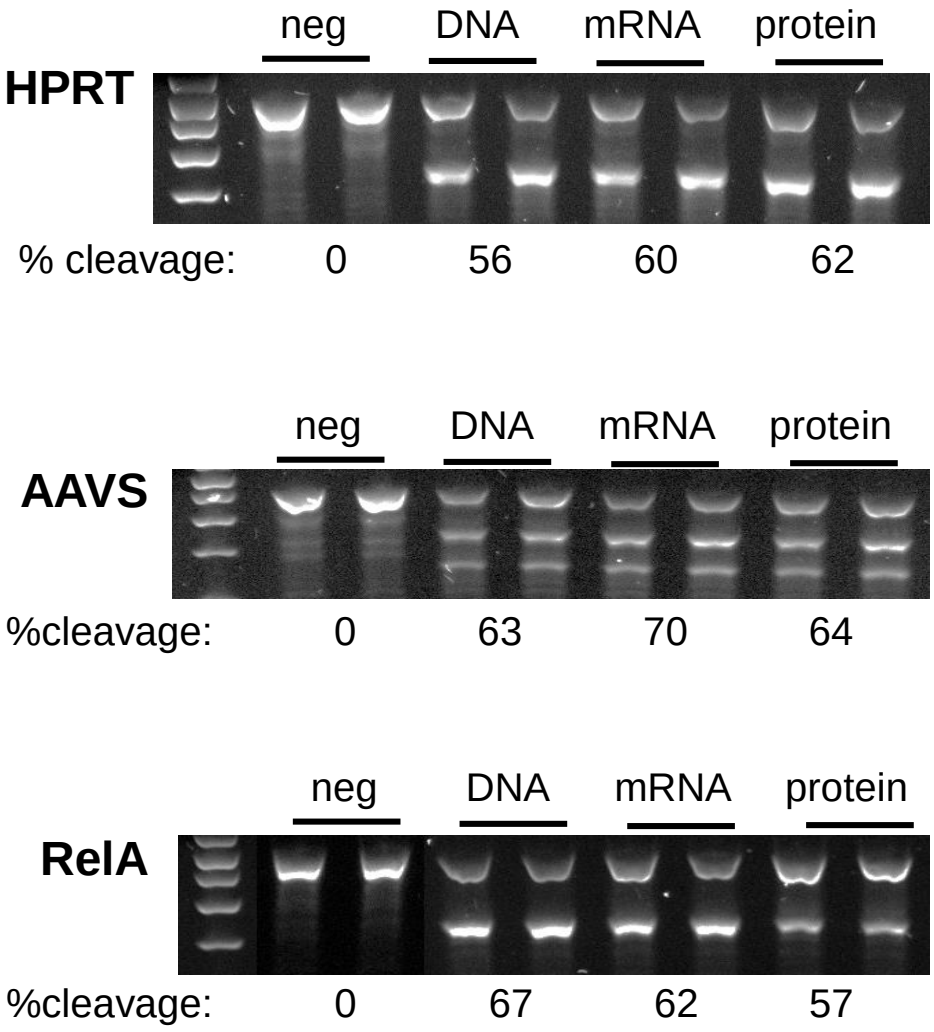


MessengerMAX for mRNA

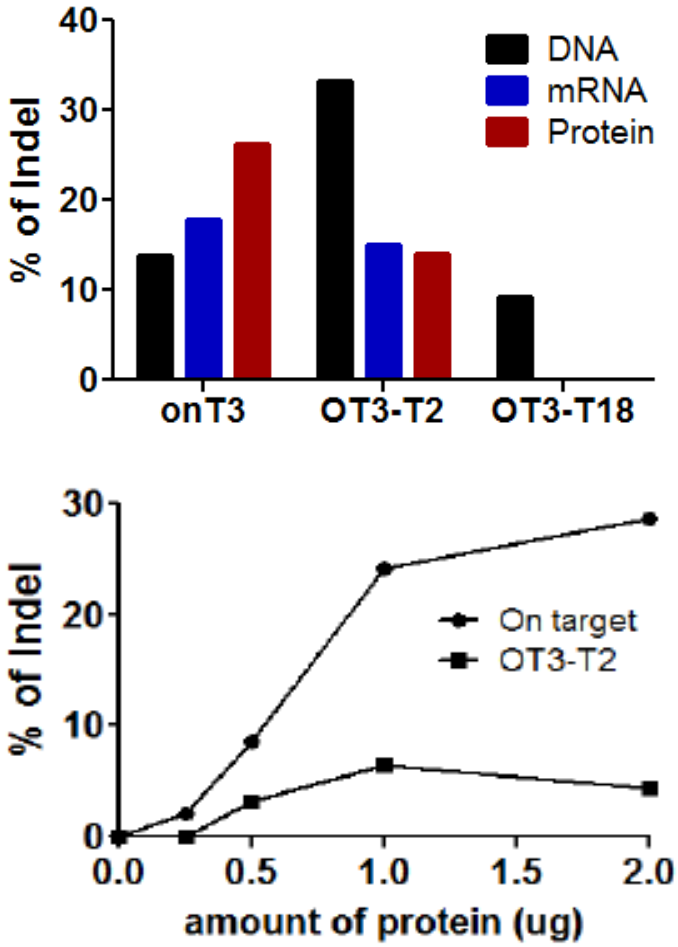
ThermoFisher SCIENTIFIC

Lipid-mediated Transfection in 293FT

DNA vs. mRNA vs. Protein



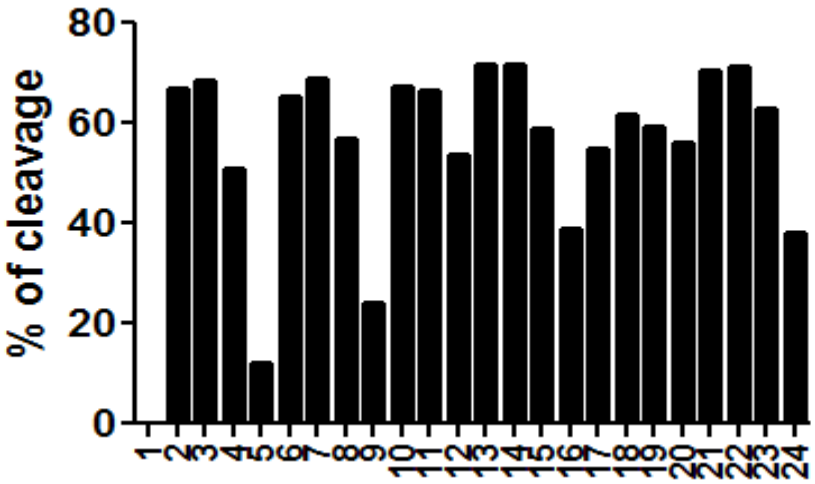
Off-target effect - VEGFA



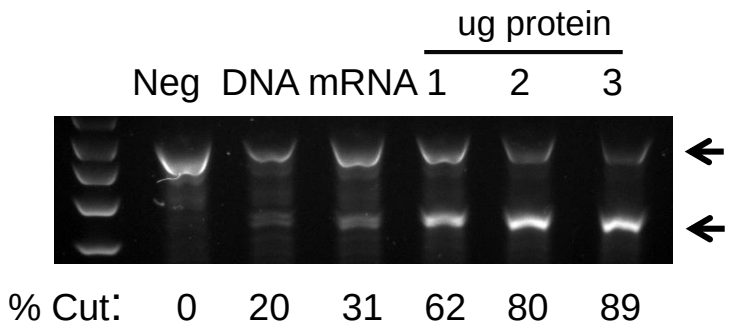
ThermoFisher SCIENTIFIC

Electroporation-mediated transfection of iPSC

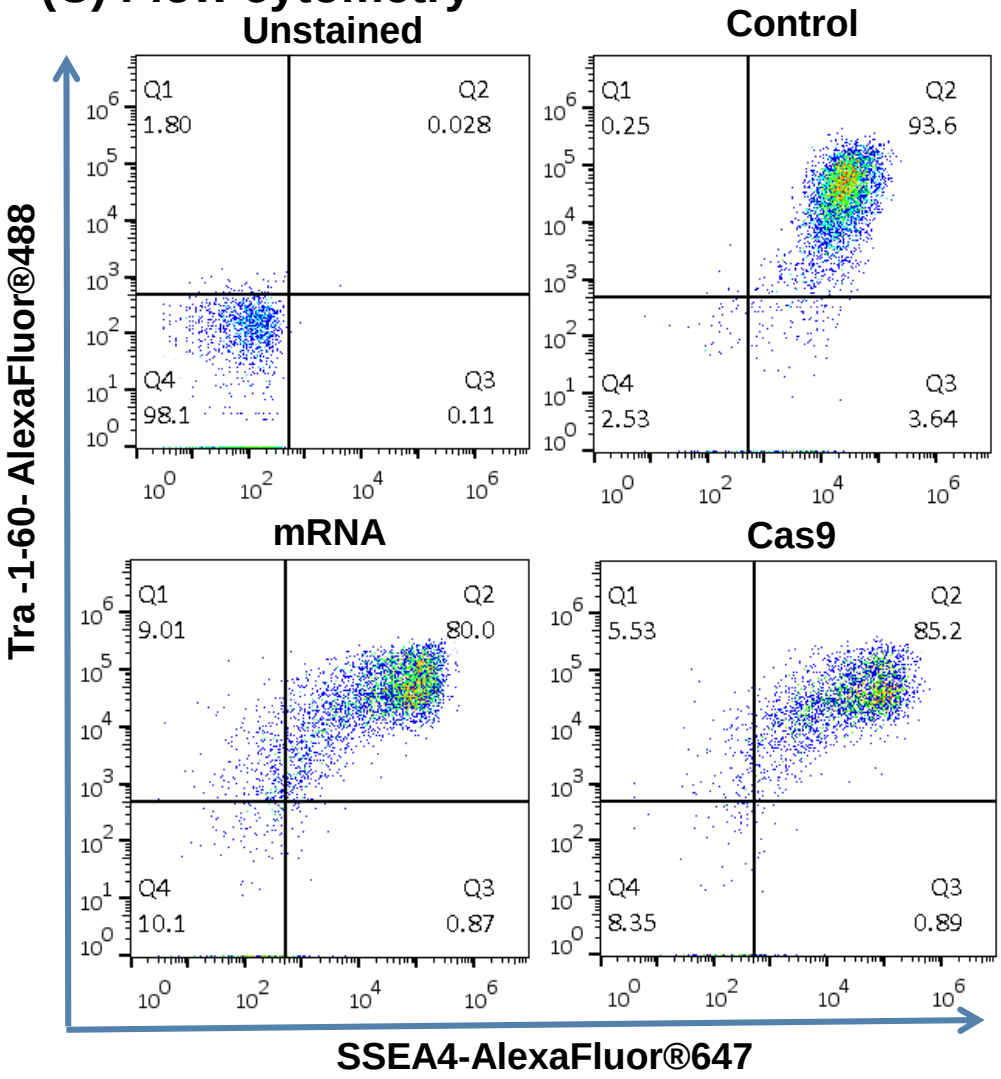
(A) 24 optimized protocol



(B) Transfection via Electroporation



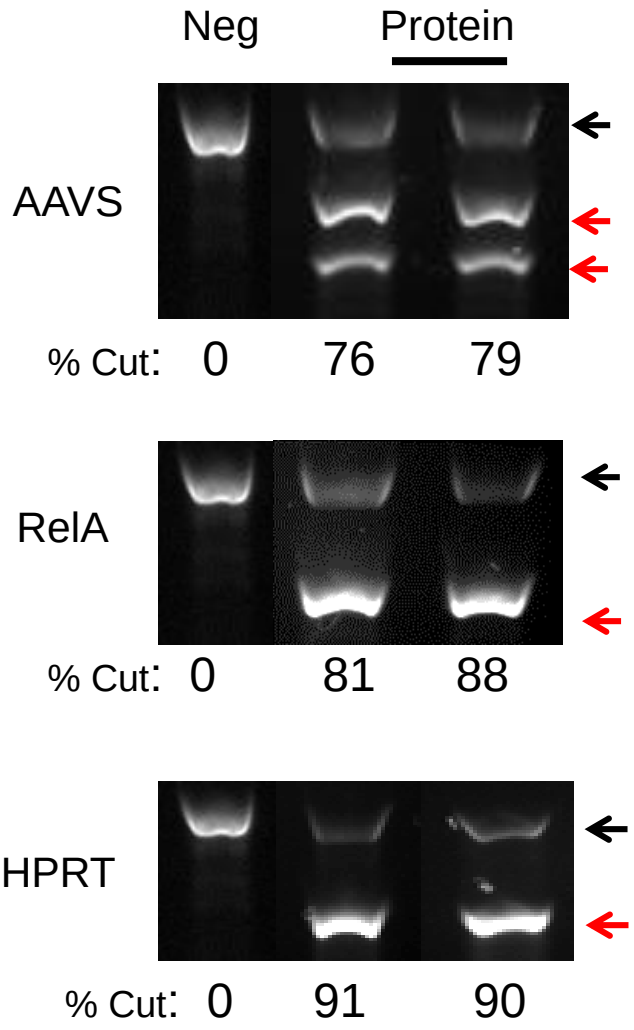
(C) Flow cytometry



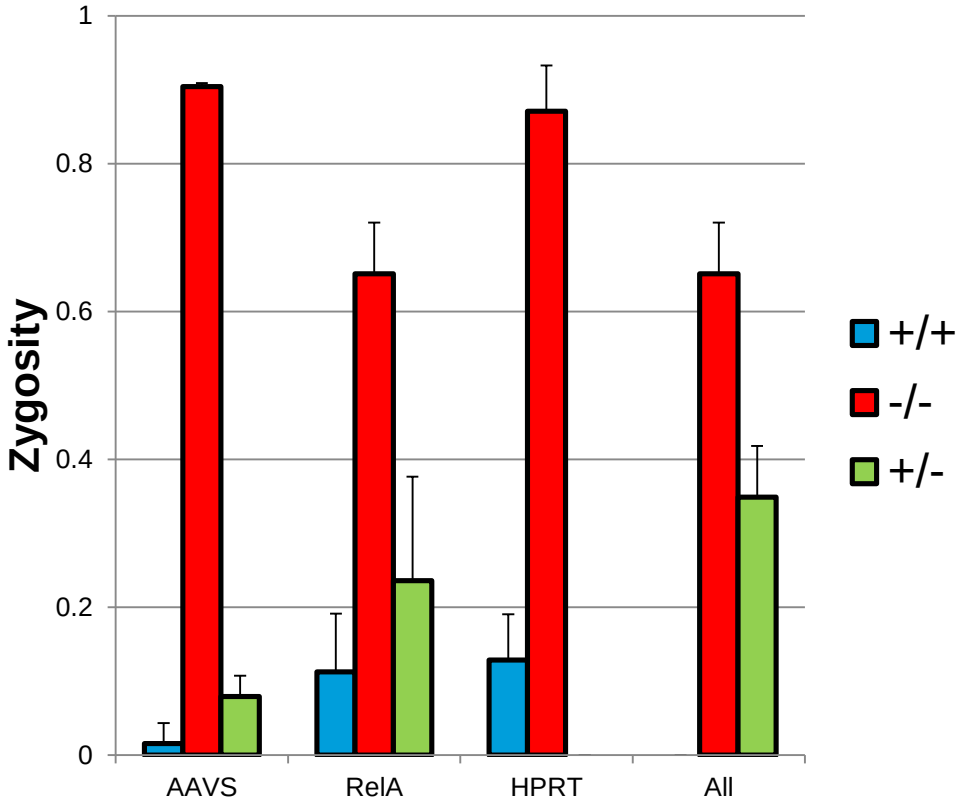
ThermoFisher SCIENTIFIC

Multiplex Knockout with Cas9 in Jurkat T (Male)

(A) AAVS, RelA, and HPRT targets



(B) Efficiency of triple knockout



ThermoFisher SCIENTIFIC

Transfection efficiency in variety of cell lines

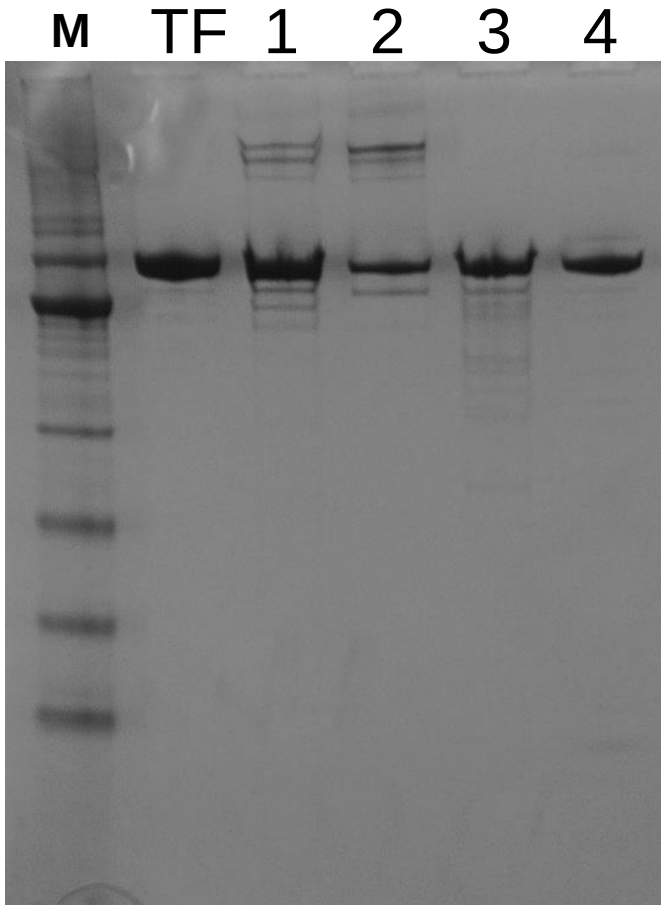
Cell lines	Plasmid		mRNA		Protein	
	Lipid	Electro	Lipid	Electro	Lipid	Electro
293FT	49	49	70	40	64	88
U2OS	15	50	21	24	18	70
Mouse ESCs	30	45	45	20	25	70
Human ESCs (H9)	0	8	20	50	0	64
Human iPSCs	0	20	66	32	0	87
N2A	66	76	66	80	66	82
Jurkat T	0	63	0	42	0	94
K562	0	45	0	27	0	72
A549	15	44	23	29	20	65
Human keratinocytes (NHEK)	0	0	0	n/a	n/a	35
Human Cord blood cells CD34+	n/a	n/a	n/a	0	n/a	24

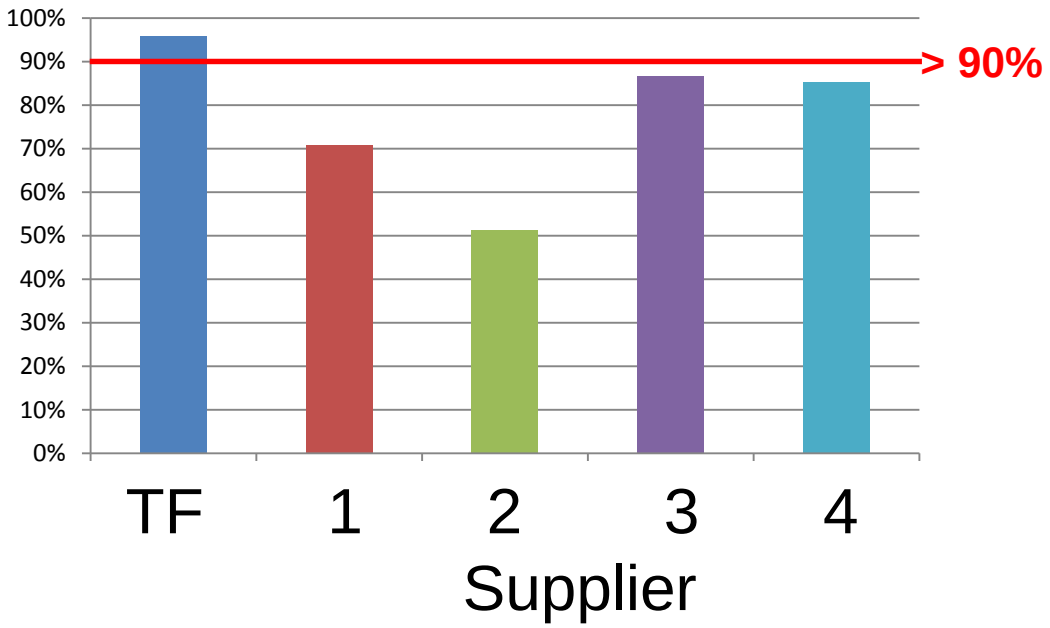
Observed higher toxicity with plasmids

New Lipofectamine Formulation

Cell line	GCD %
	CRISPRmax (24 wells)
293FT	95
HEK293	85
HCT116	80
N2A (mouse)	70
3T3 (mouse)	70
U2OS	70
HeLa	70
A-549	55
COS-7 (monkey)	55
HepG2	35
iPSC (stem cells)	30
MCF-7	20
Jurkat (suspension)	20
K562 (suspension)	20
THP-1 (suspension)	<10

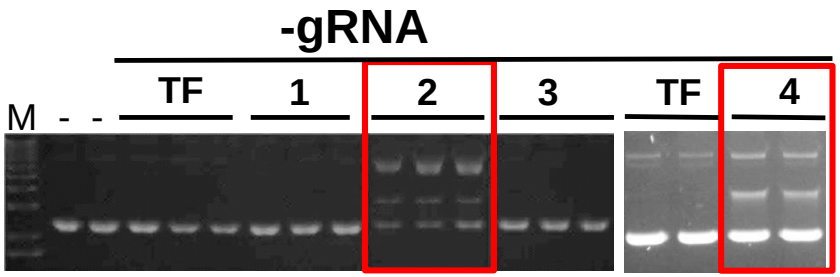
Comparison of Supplier Cas9 Purity

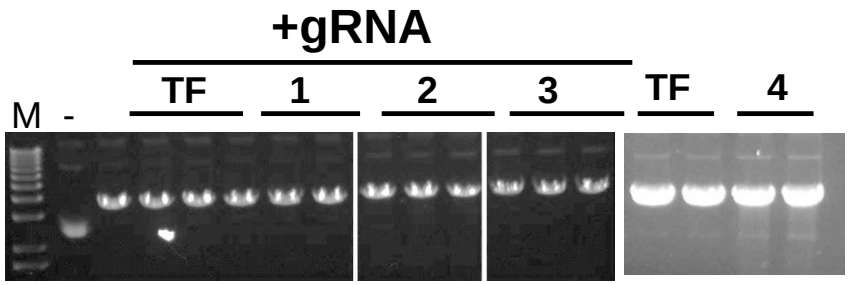


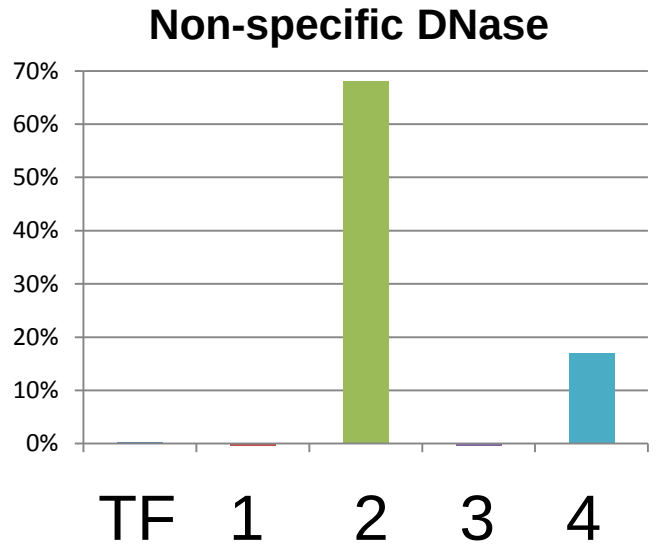


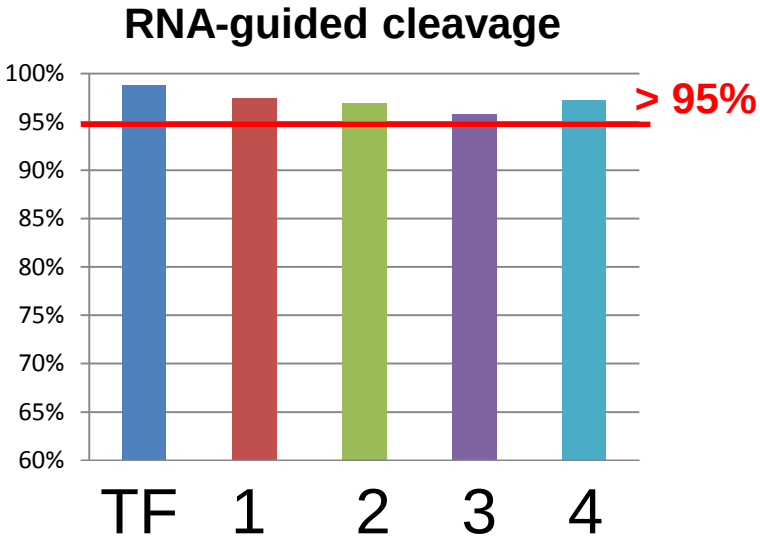
ThermoFisher SCIENTIFIC

DNA endonuclease cleavage comparison

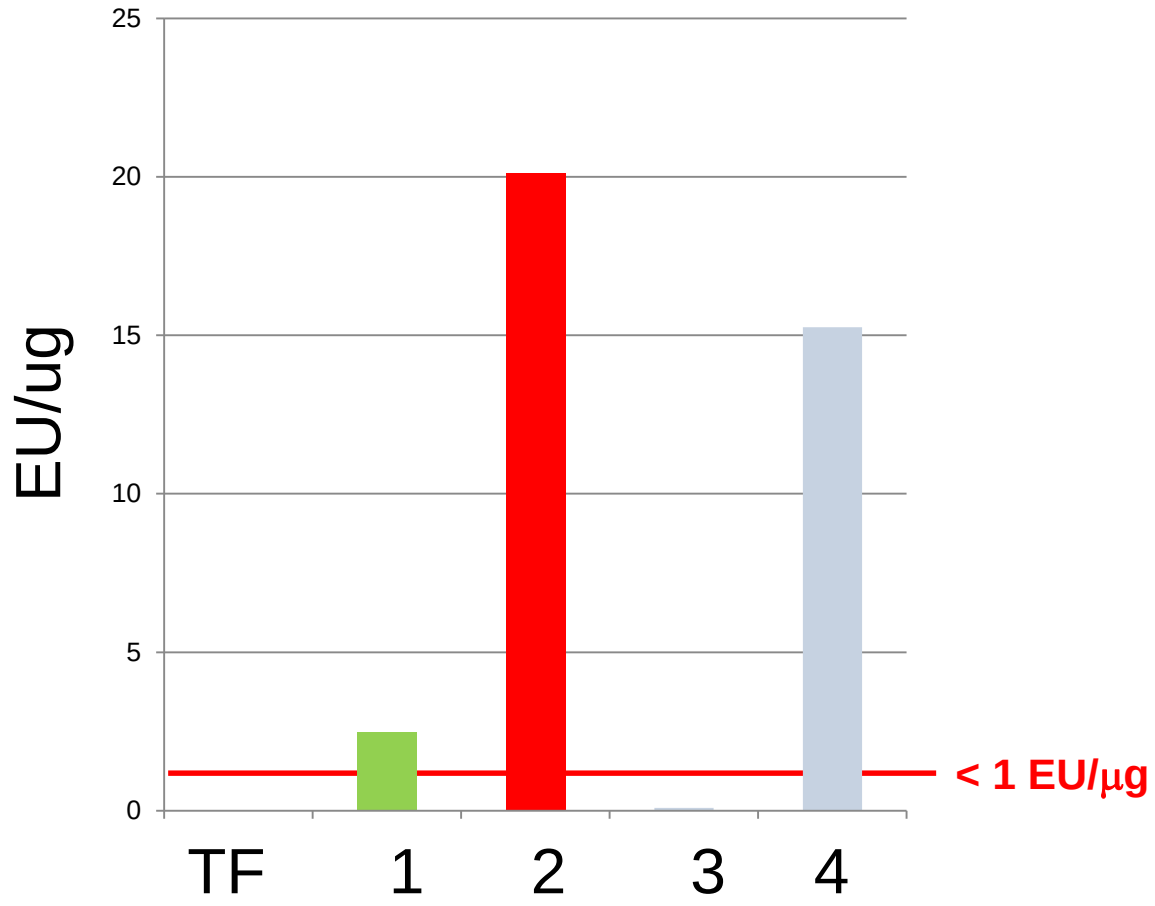






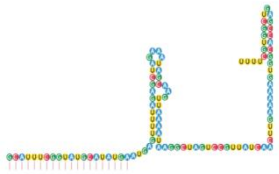


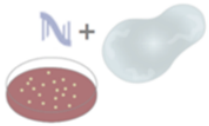
Endotoxin levels comparison



Supplier	EU/µg
TF	0.005
1	2.48
2	20.1
3	0.09
4	15.25

GeneArt® CRISPR-Cas9 Protein Workflow





1. Preparation

- Design CRISPR targets
- Order primers (next day)
- Cell seeding

2. Make gRNA

- Assemble gRNA template
- IVT reaction
- Purify gRNA

3. gRNA /Cas9 delivery

- Complex gRNA + Cas9 protein
- Lipid or Electroporation
- Incubate 48 hours

4. Analyze editing efficiency

Day 1 | Day 2 | Day 4

Advantages of Cas9 RNP mediated genome editing:

- Ready to act once delivered into the cell
- No DNA footprint
- Controlled dose
- Efficient for both single and multiplexing
- Fast turnover reduces off-target chance

Acknowledgements

Jon Chesnut

Xiquan Liang
Jason Carte
Wen Chen

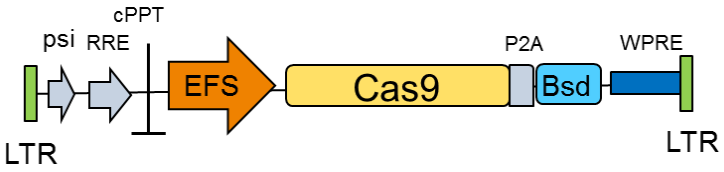
Namritha Ravinder
Yanfei Zou
Shantanu Kumar
JP Yang

Uma Lakshmipathy

Kevin Clancy
Sridhar Ranganathan

Lentiviral Particles for CRISPR Libraries

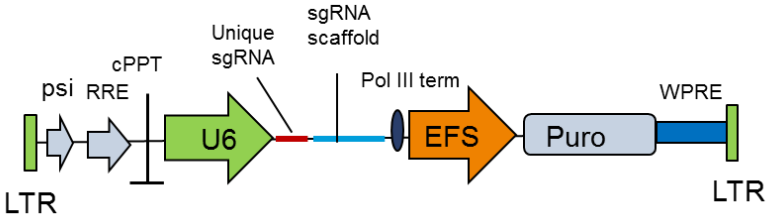
pLenti-Cas9-P2A-Bsd



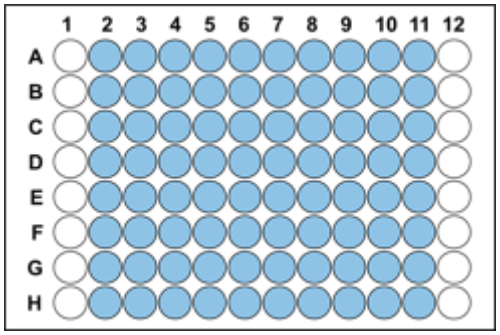
- Human codon-optimized S. pyogenes Cas9 gene
- Blasticidin resistance linked to Cas9 through a self cleavage 2A peptide

---

pLentiCRISPR-EFS-Puro



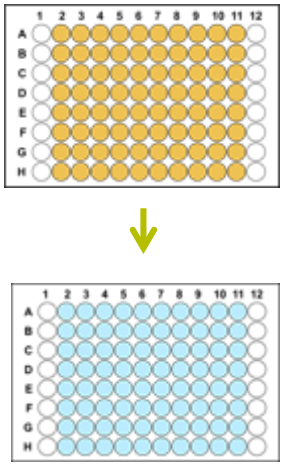
- Specific sgRNA expressed from U6 promoter
- Puromycin resistance from EF-1a promoter



- Four targets for each gene
- Targeting 5' exons
- Filtered to minimize off-target cleavage

Arrayed and Pooled Library Screening

Screening of Arrayed Library



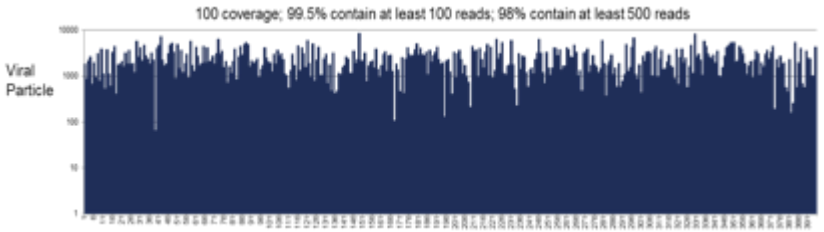
- 4 gRNAs per gene
- > 10^6 TU/mL

One gene per well

- Much more controlled delivery of gRNA per well
- Eliminate time-consuming deconvolution step
- Require some level of automation

Screening of Pooled Library

- 10 gRNAs per gene
- 10^8 TU/mL

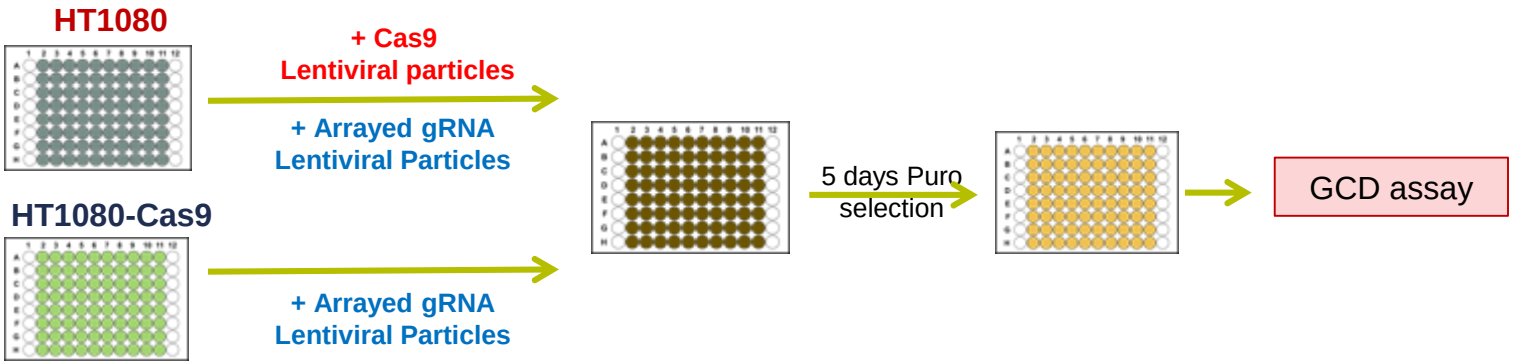


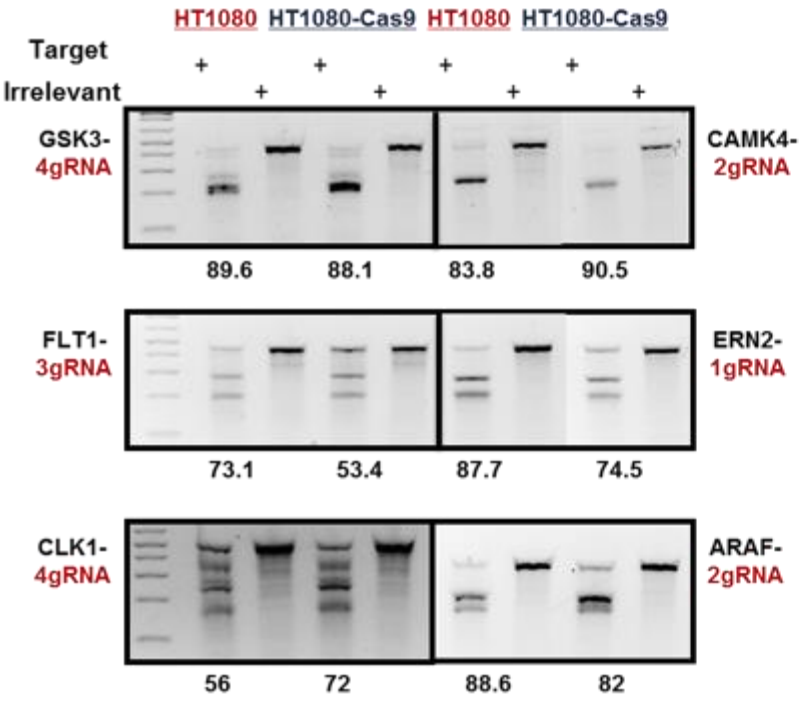
Multiple genes in parallel

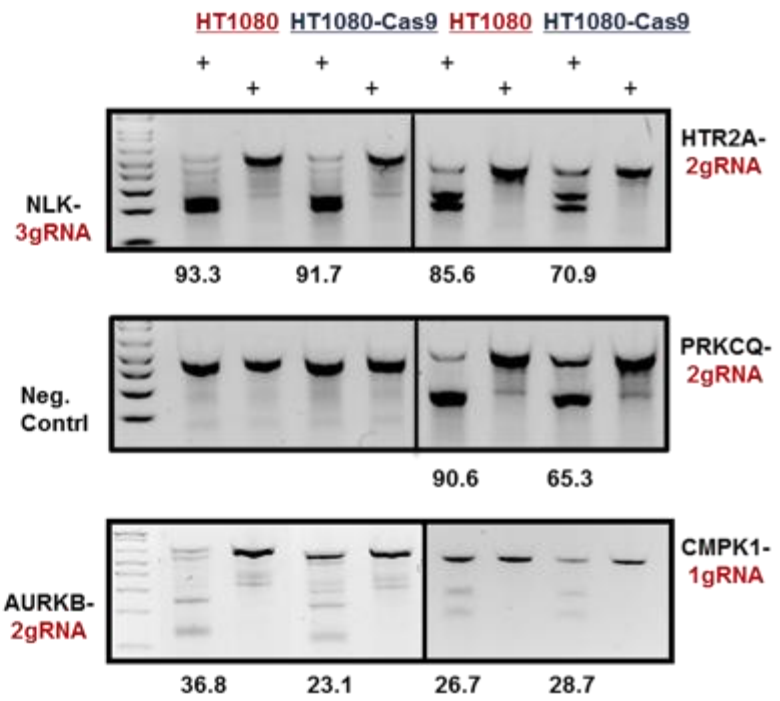
- Much less expensive than arrayed format
- Do not require special infrastructure

Focused gene Libraries: Kinases, Phosphatases, GPCR

Evaluation of gene targeting in HT1080-Cas9 and HT1080 cells

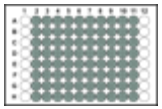






ThermoFisher SCIENTIFIC

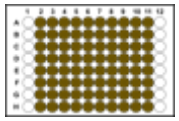
LentiCRISPR viral Particles for CellSensor® NFκB-bla ME-180 cell line



CellSensor NFκB ME180 cell

+ Cas9 Lentiviral particles

+ Arrayed gRNA Lentiviral Particles

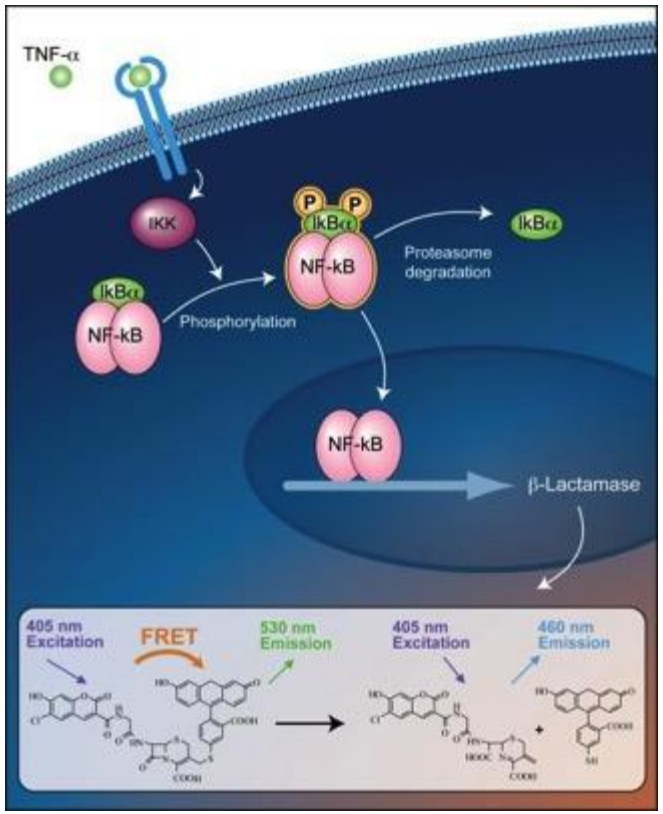


5-12 days Puro selection

384-well plate

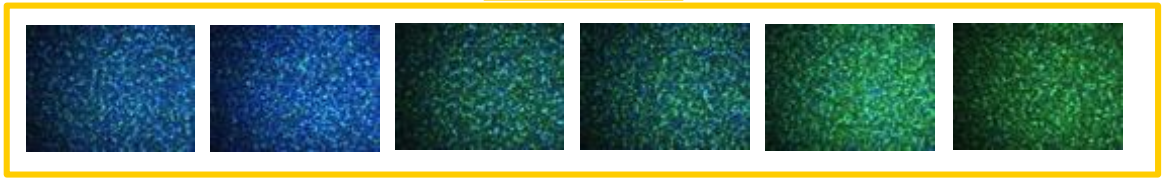
Add TNF

Measurement (Blue to Green)



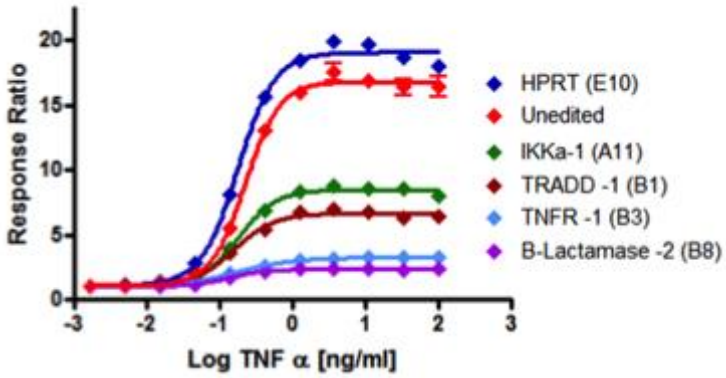
No TNF α

+ TNF α



Unedited HPRT IKKa TRADD TNFR BLA

Cells infected with lentiviral particles



ThermoFisher SCIENTIFIC

Figure 3s. Optimization of electroporation using Neon 24 optimized protocol

(A) 24 optimized protocol

Protocol	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Pulse Voltage	0	1400	1500	1600	1700	1100	1200	1300	1400	1000	1100	1200	1100	1200	1300	1400	850	950	1050	1150	1300	1400	1500	1600
Pulse Width	0	20	20	20	20	30	30	30	30	40	40	40	20	20	20	20	30	30	30	30	10	10	10	10
# of Pulse	0	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	3	3

(B) Transfection of Jurkat T cells via electroporation

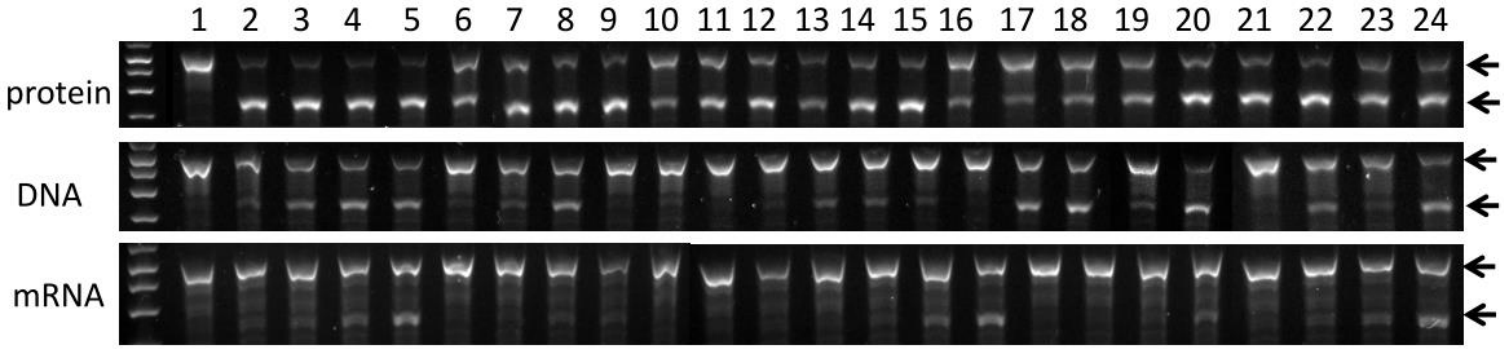
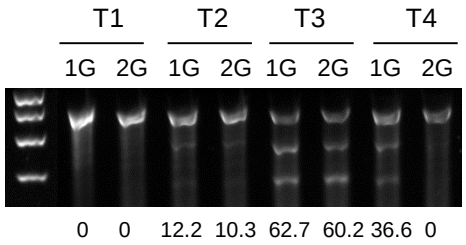


Figure 3s. A mastermix of plasmid DNA, mRNA or Cas9 protein/gRNA were prepared in 250 µl of Resuspension Buffer R and then mixed with 50 x 10^5 Jurkat T cells, which were previously washed with PBS. An aliquot of 10 µl sample was drawn with a Neon 10 µl pipette tip, followed by electroporation using Neon 24 optimized protocol, which varies in pulse voltage, pulse width and number of pulses. The percentage of genome cleavage was estimated 48-hour post transfection using genomic cleavage assay.

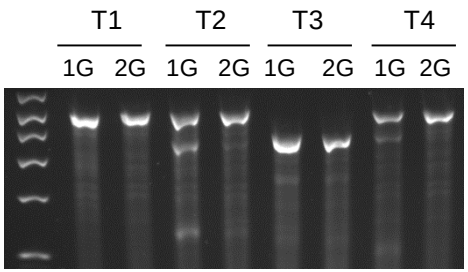
Effect of extra 5’ Gs on Genome Editing

Design: A “G” will be added to 5’end of a 20bp target unless a target starts with a “G” at 5’end.
Six genes with four gRNA each are investigated. Targets with 0% cleavage in both 1G and 2G samples are Excluded for statistical analysis

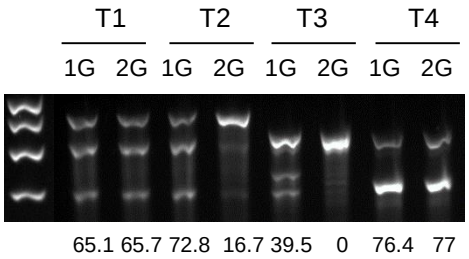
T1: AAGAGTCTGTGACATCTTTG
T2: GGAAGAATTAGTCTCATTGC
T3: GCATCTCCATGAGTTCCAGT
T4: AGAACACCCACTGGAACTCA

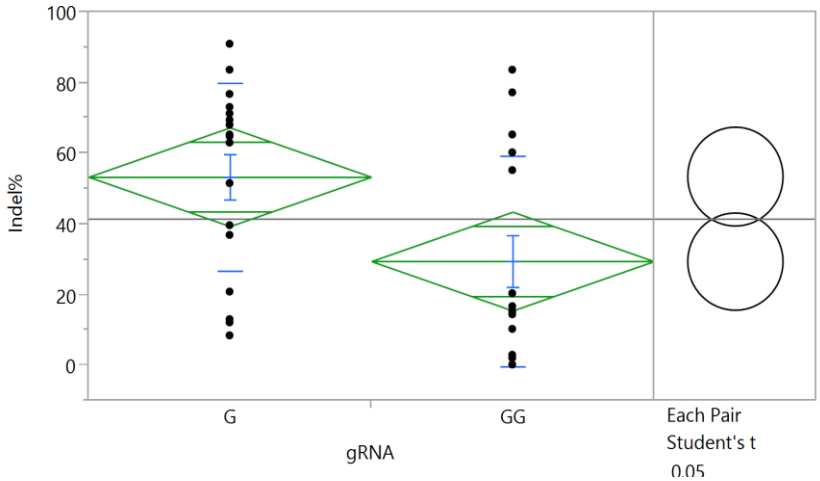


T1: CAGAGTAAGGTGCGATCTTC
T2: CAATACTGTTGAGTGACTTC
T3: GGATCAGTTGATGACCGGCC
T4: TACAAGCAAACCGAAATCTC



T1: GATCGCCATGAAAGGTGTAC
T2: ACCTTCCGGTACACCTTTCA
T3: ACAAGCCCTCAAATTTCATC
T4: GAACAAATCTCCTCAGGCAG





ThermoFisher SCIENTIFIC

You can purchase the Cas9 Nuclease protein product via our custom services team, see how to order below.

Product details are:

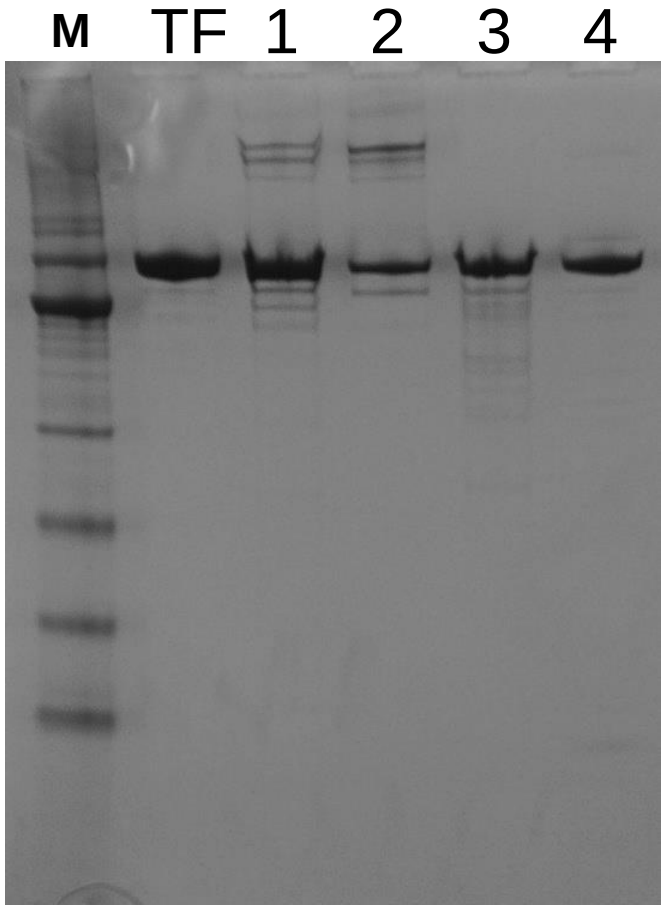
- Name: Cas9 Nuclease
- Sku #: A27865
- Concentration: 1ug/uL
- Volume: 50uL
- List price: $675
- The Cas9 nuclease has been tested for contaminating nucleases and endotoxins and shown to work in a large variety of cell lines.

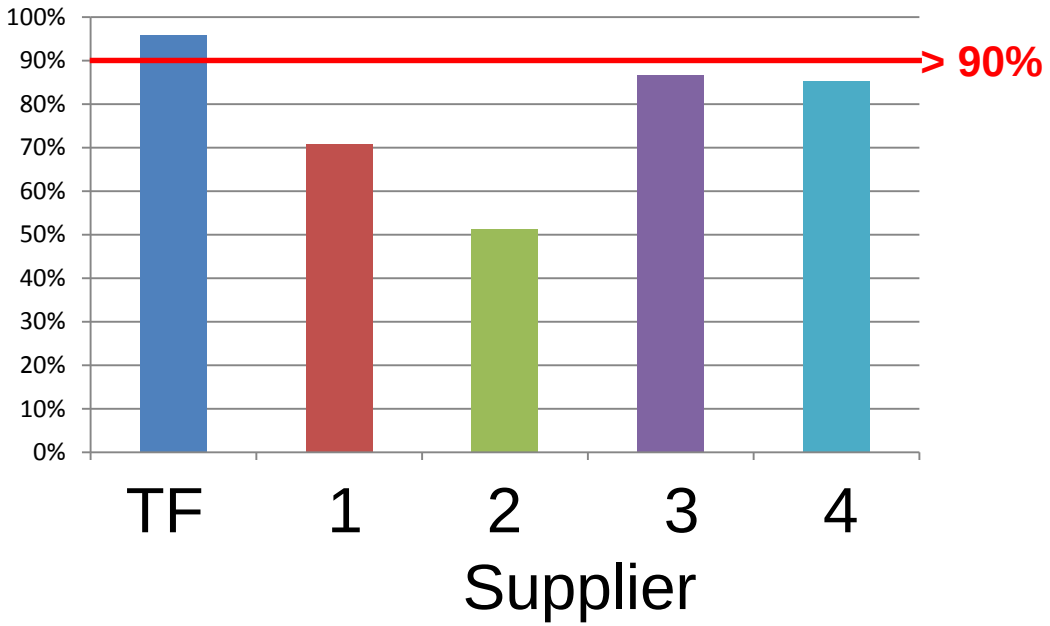
How to order:

- The Cas9 nuclease will not be in the catalog or website for a few more months. Until then we are making it available through our custom services. Please contact me or work with your local technical sales specialist if you have any questions.
- Send an email to: Custom.Services@lifetech.com
- Provide number of vials and customer name, shipping address, email, and phone number.
- Custom services will generate a quote
- Customer sets up a purchase order (PO)
- Customer submits PO to services team
- We will ship the material to the ‘bill to’ address specified on the PO (or an alternative address if provided)
- Protocol and COA will be sent electronically to the customers when the product ships

If you have any question please contact me at Jason.potter@lifetech.com

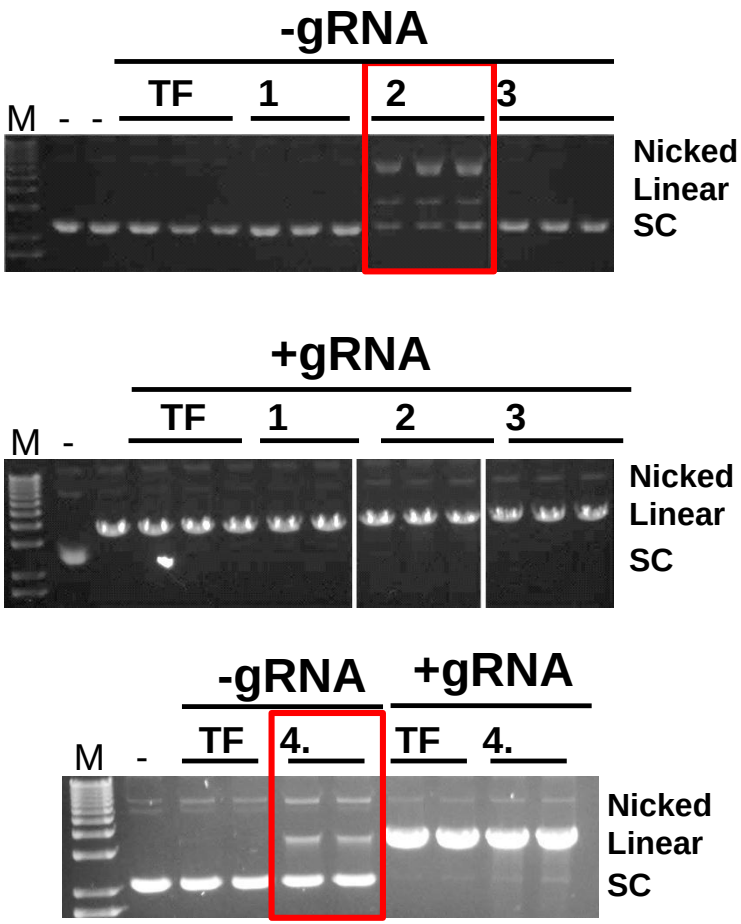
Comparison of Supplier Cas9 Purity

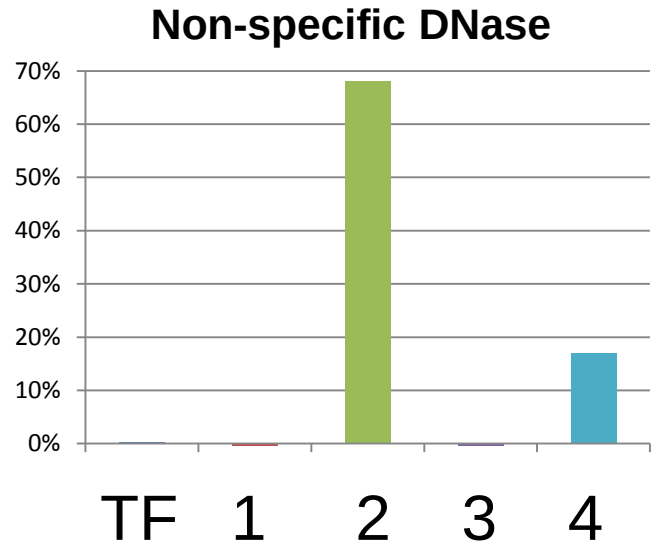


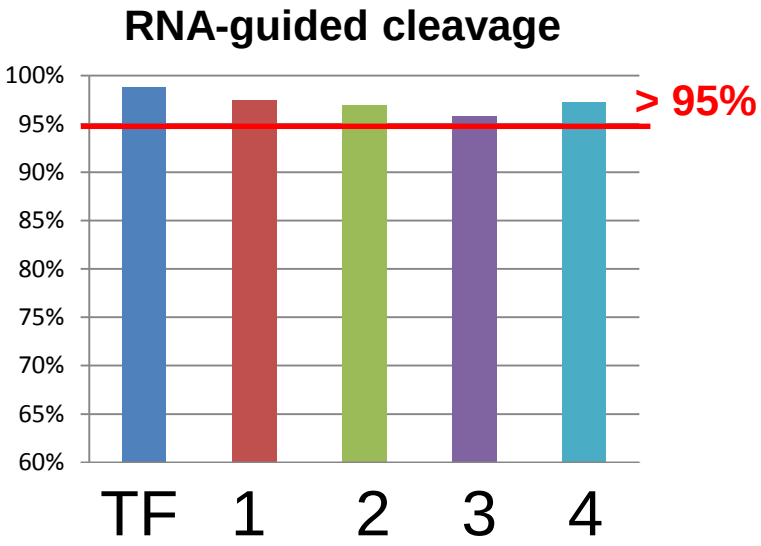


ThermoFisher SCIENTIFIC

DNA endonuclease cleavage comparison

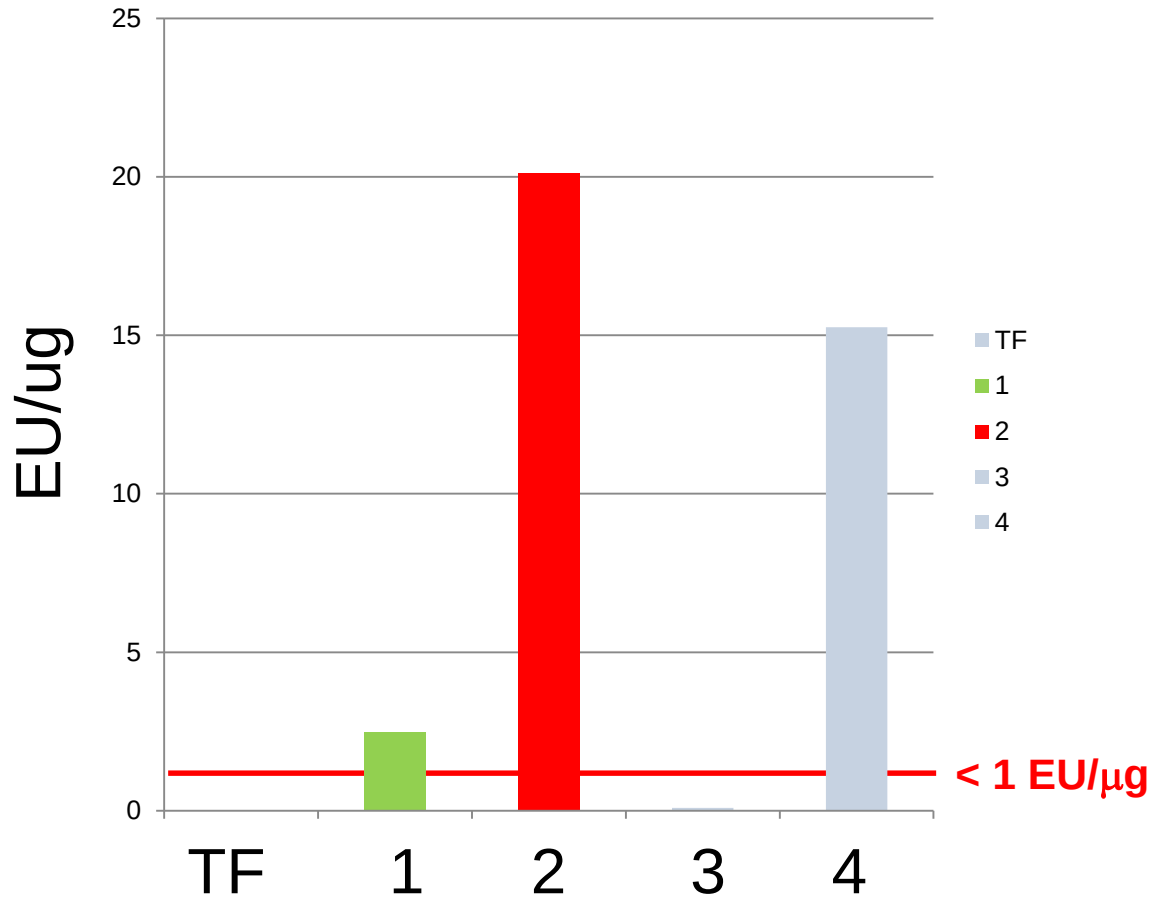






ThermoFisher SCIENTIFIC

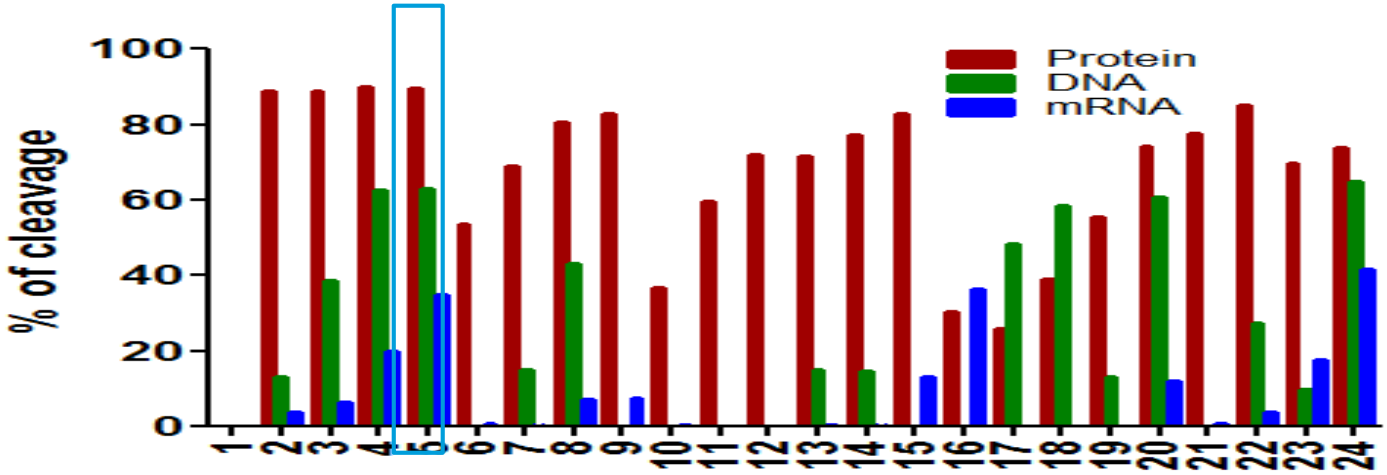
Endotoxin levels comparison



Supplier	EU/µg
TF	0.005
1	2.48
2	20.1
3	0.09
4	15.25

Electroporation of Jurkat T

(A) Neon Electroporation Optimizations / Quantitation of Genome Cleavage



(B) Protein Dose Response