Best Practices for Achieving Optimal *Ex Vivo* Genome Editing in Your Research

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Building a Genomic Medicine Leader

CRISPR Gene Editing to Develop Differentiated, Transformational Medicines for High Unmet Need

In Vivo CRISPR Medicines	Engineered Cell Medicines
Leverage AAV-mediated editing with SaCas9 into additional therapeutic areas	Develop best-in-class medicines for hemoglobinopathies and cancers using Cas12a engineered cells

Maintain Best-in-class Platform & Intellectual Property, and Advance Organizational Excellence

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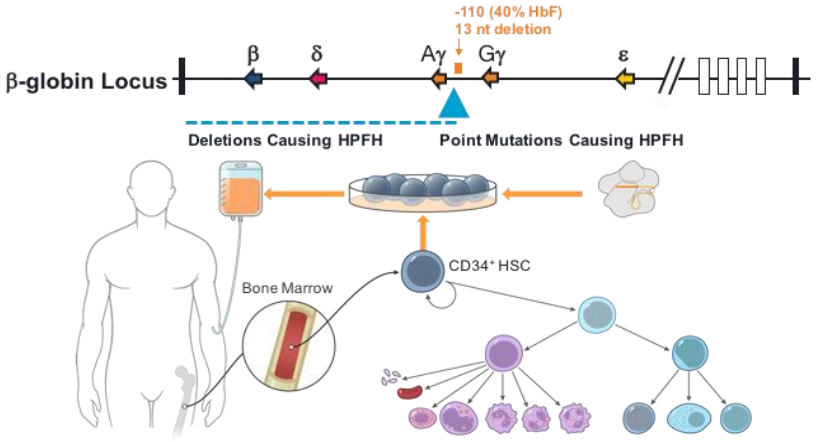
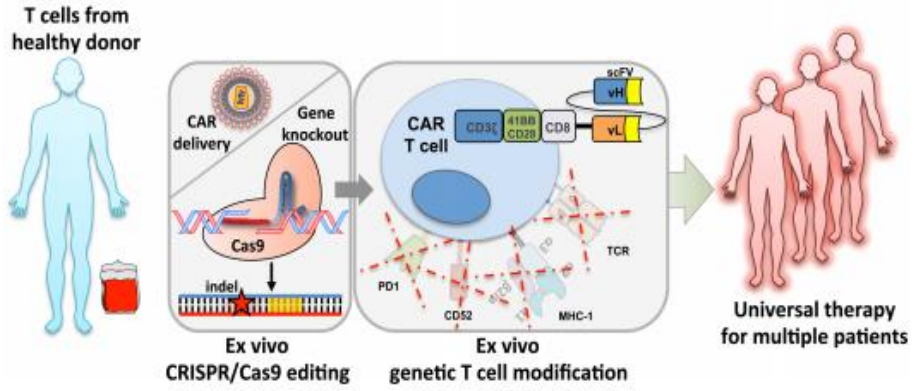
Examples of potential gene edited engineered cell therapies

Autologous HSC cell medicines

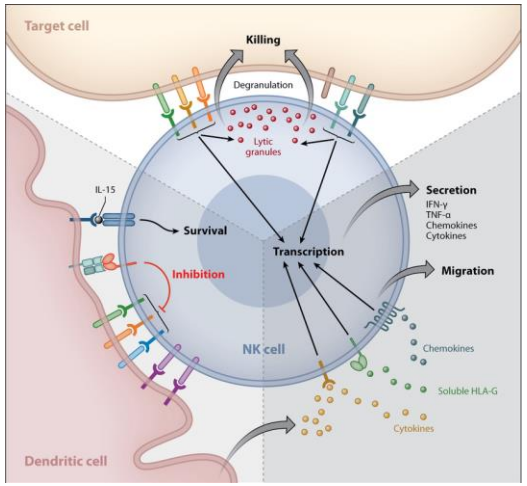


Allogeneic T cell medicines



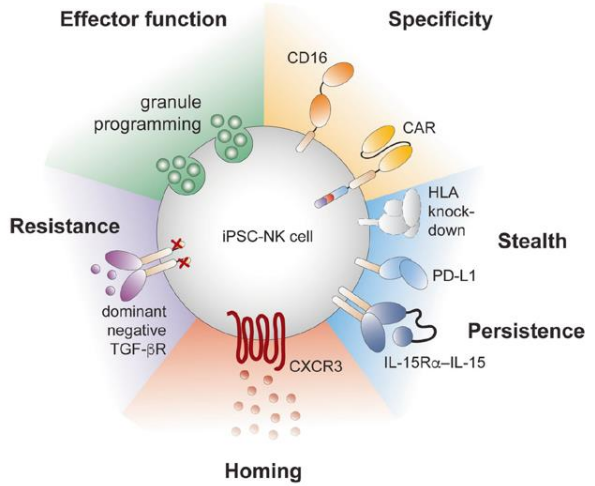
Preece et al. *Emerging Topics in Life Sciences* 2019

Healthy donor-derived NK cell medicines



Long et al. *Annual review of immunology* 2013

iPSC-derived NK cell medicines



Saetersmoen et al. *Seminars Immunopathology* 2019

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CRISPR-based gene editing enables highly efficient disruption of endogenous genes as well as the ability to insert new genes into cells

GENE DISRUPTION (KNOCK-OUT)

- For ex vivo gene editing a common strategy is to deliver CRISPR proteins directly complexed with their guide RNAs, conventionally referred to as ribonucleoproteins (RNPs)

- Why use RNP?
  - Better specificity due to transient nature
  - Nucleases and guides are commercially available
  - Widely used electroporation devices and protocols have been vastly improved over last few years
  - You can even have a company make your edited cell, which is easier to do than you might realize

GENE INSERTION (KNOCK-IN)

- Adeno-associated virus serotype 6 (AAV6)
  - AAV6 has become the gold standard for transgene delivery to primary cell types like HSCs, T cells, and NK cells
  - Making AAV yourself can be challenging, but several vendors offer very competitive prices (~$2000 can cover several experiments)
  - The price can be justified by the quality of the data that can be obtained and potential speed to publication
  - Many groups have already published optimized guides and AAV6 constructs for knock-in into your favorite targets like *TRAC*

- Short and long (>1 kb) ssDNA oligos:
  - More appropriate for small corrections, not large knock-ins
  - Making multi-kb long ssODNs is not trivial but companies offer this

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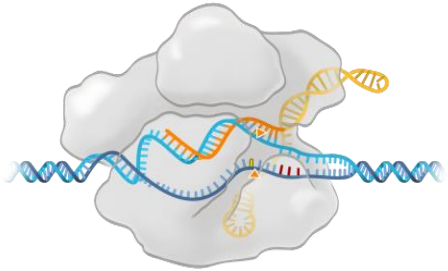
There are many nucleases to choose from but for *ex vivo* gene editing using AsCas12a offers several potential advantages for your experiments

Editas general suite of nucleases

Variant	PAM	Frequency (bp)
SpCas9	NGG	1 in 8
SaCas9	NNGRRT	1 in 32
SaCas9 KKH	NNNRRT	1 in 8
AsCas12a	TTTV	1 in 43
AsCas12a RR	TYCV/CCCC	1 in 18
AsCas12a RVR	TATV	1 in 43
LbCas12a	TTTV	1 in 43

If you do not need to make an edit at an exact bp location the PAMs should generally not be restricting to your KO/KI experiments

SpCas9

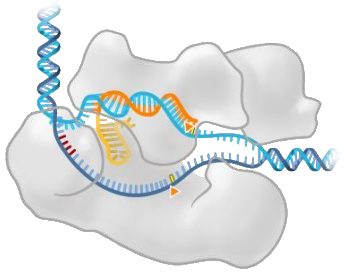


Targets G-rich PAMs

Separate crRNA and trRNA that can be linked (~100 nt)

Predominantly blunt DNA cut or 1 nt overhang

AsCas12a



Targets T- and C-rich PAMs

Naturally occurring ~40 nt single guide RNA

5’ staggered DNA cut with 4 nt overhangs

AsCas12a is highly specific primarily due to intrinsic DNA target engagement mechanism that is distinct from SpCas9

References on AsCas12a target specificity:

Strohkendl et al. Mol Cell 2018, vol 71, 816-824
Swarts et al. Biochem Soc Trans 2019, vol 47, 1499-1510

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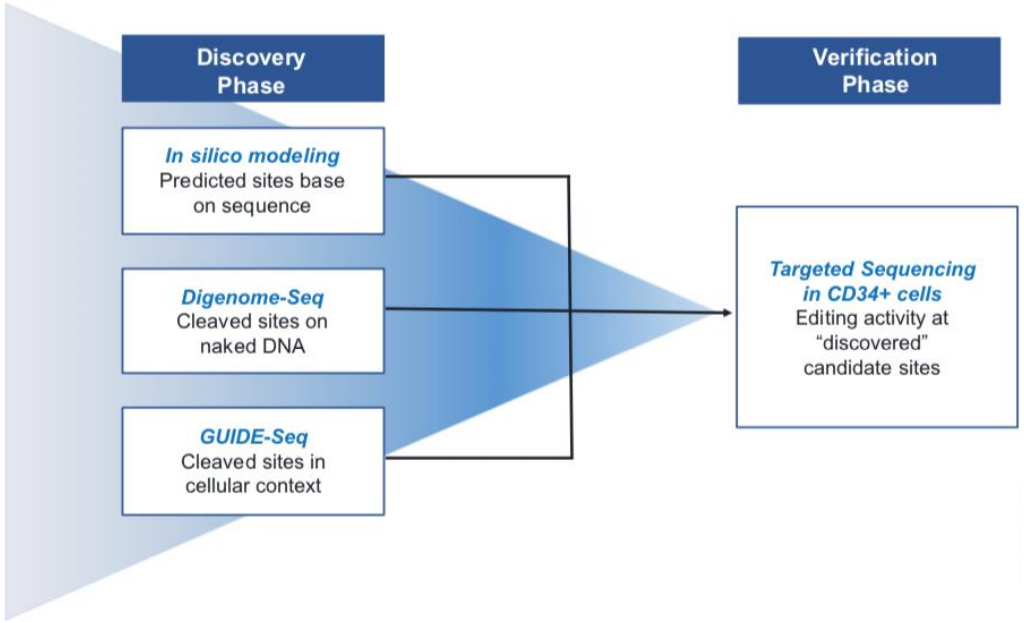
The case for AsCas12a to maximize specificity for your *ex vivo* applications

Multiple orthogonal specificity methods are needed to appropriately capture potential off-target sites that might occur in your cell type

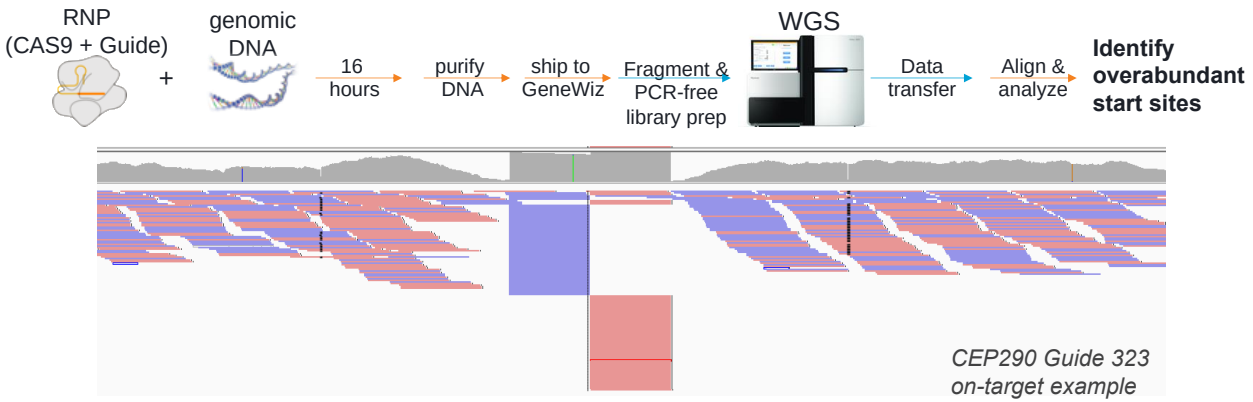


De Dreuzy et al. Annual Meeting of the American Society of Hematology, Orlando. FL, December 9, 2019

Do SpCas9, SaCas9, and AsCas12a (AsCpf1) cut genomic DNA at significantly different efficiencies from one another?

Optimized versions of GUIDE-seq based on Tsai et al. Nature Biotech 2015
Optimized versions of Digenome based on Kim et al. Nature Biotech 2015

Digenome-seq can be expensive to run, but it casts a wide-net



Selection of matched target sites to test this hypothesis

Step 1

Matchted Target Site (20-Ns):
TTTVNNNNNNNNNNNNNNNNNNNNNGGRRT

Concept from Kleinstiver et al. *Nature Biotech* 2016

Step 2

Applied filters:
1) Removed sites on chromosomes labeled as: alt, random, X, Y, M
2) GC% >35% and <70% in target sequence
3) No more than 3 identical bases in a row in target sequence
4) Removed sites with more than 1 guide site in the genome
5) No repetitive element within 500bp of either side of guide

Step 3

List randomized, working from top down
1) No common variants >0.1% in guide or PAM
2) Target sites are at least 10kb apart
3) Inspection of target region from previous WGS runs to ensure >15x coverage and no SNPs / indels / other variants
4) Successfully design PCR primers to amplify target region

Step 4

25 sites chosen

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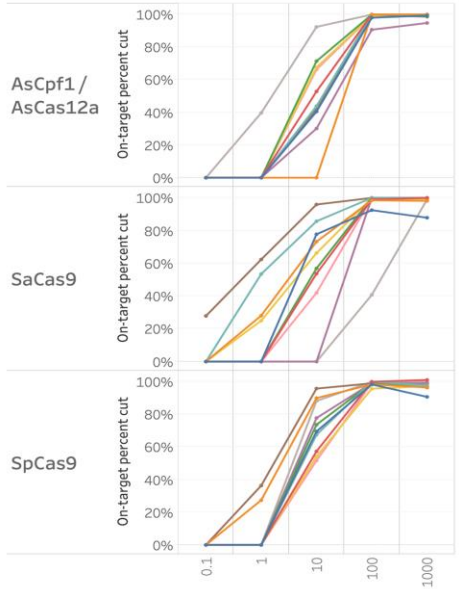
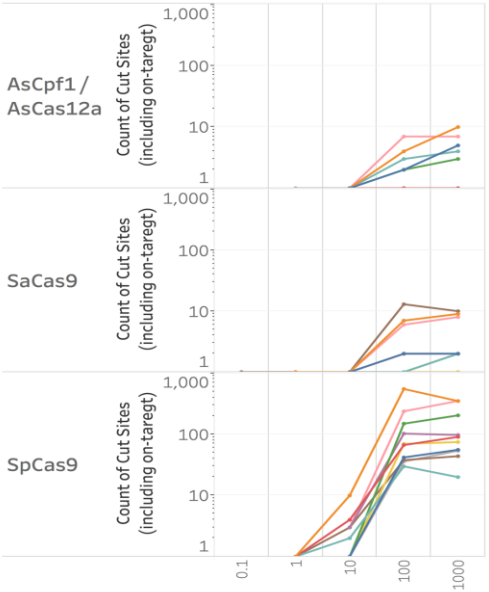
Digenome-seq biochemical assay shows that SpCas9 has similar on-target activity to AsCas12a and SaCas9 but far worse target specificity

SpCas9 has 10x–100x more off-target cut sites than AsCas12a and SaCas9 at 1 µM RNP

On-target potencies are similar between the three enzymes but off-targets for SpCas9 are much higher





Much smaller dose window between on-target and off-target when using SpCas9

Gotta et al. Genome Engineering: Frontiers of CRISPR/Cas oral presentation 2019

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Findings from Digenome-seq and Cellular Assays

- SpCas9 had 10x-100x more cut sites than SaCas9 and AsCas12a assayed with Digenome-Seq at saturating RNP concentrations (1000 nM)
  - The average SpCas9 had 78 off-target cut sites, while the SaCas9 had 0.9 and AsCas12a had 1.2

- On-target cutting was found to be concentration-dependent
  - Half-maximal biochemical potency was similar for all 3 enzymes at ~3 to 30 nM
  - Half-maximal cellular potency was ~300 nM to 3,000 nM in T cells

- Off-target cutting was also concentration-dependent with the “off-to-on” target ratio often reaching 100-1000x for SaCas9 and AsCas12a but not exceeding 10 for SpCas9

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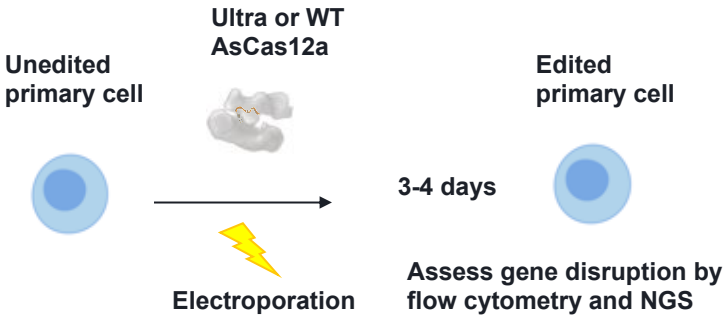
The case for an engineered AsCas12a for maximizing efficacy in challenging cell types

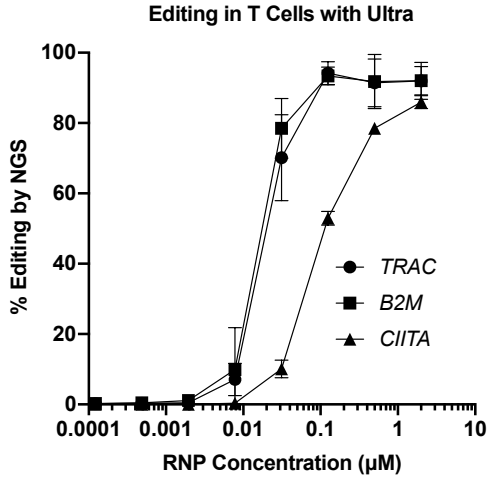
Engineered AsCas12a from IDT (Ultra) was shown to be efficient for all guides tested, potentially minimizing the number of guides to screen

RNP composed of AsCas12a Ultra + IDT CRISPR-Cas12a Alt-R guides

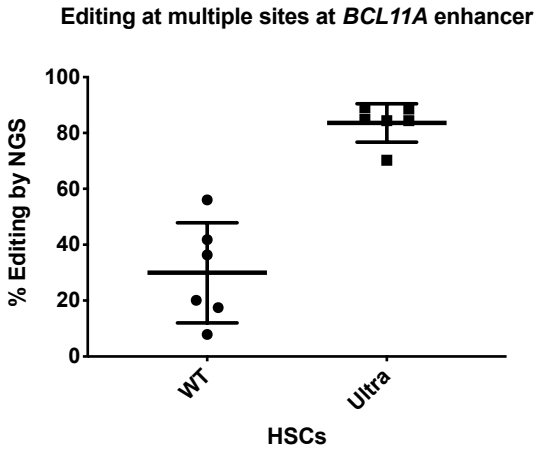
IDT™ INTEGRATED DNA TECHNOLOGIES

Ex vivo editing of primary cells





EC_{50} in 10-100 nM range with max editing at 1 μM or less mean far less consumption of RNP



Reduces need to extensively screen targets for a potent guide

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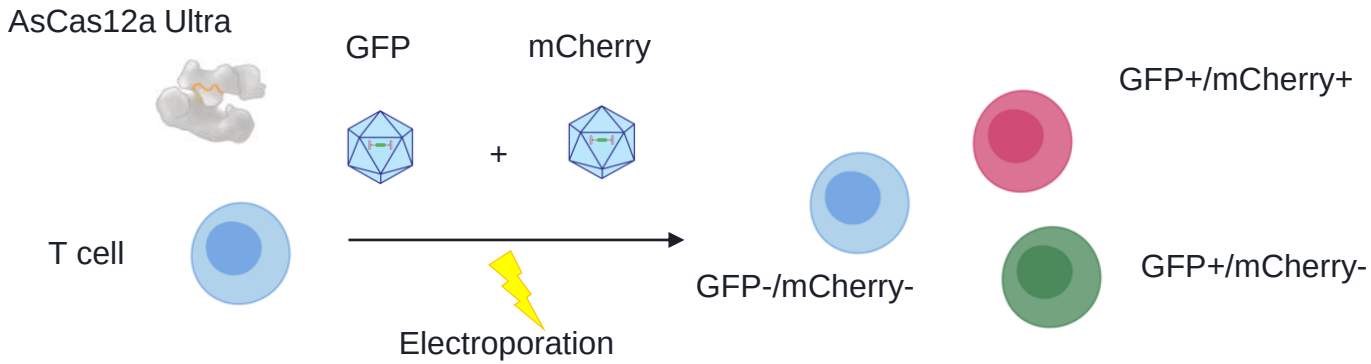
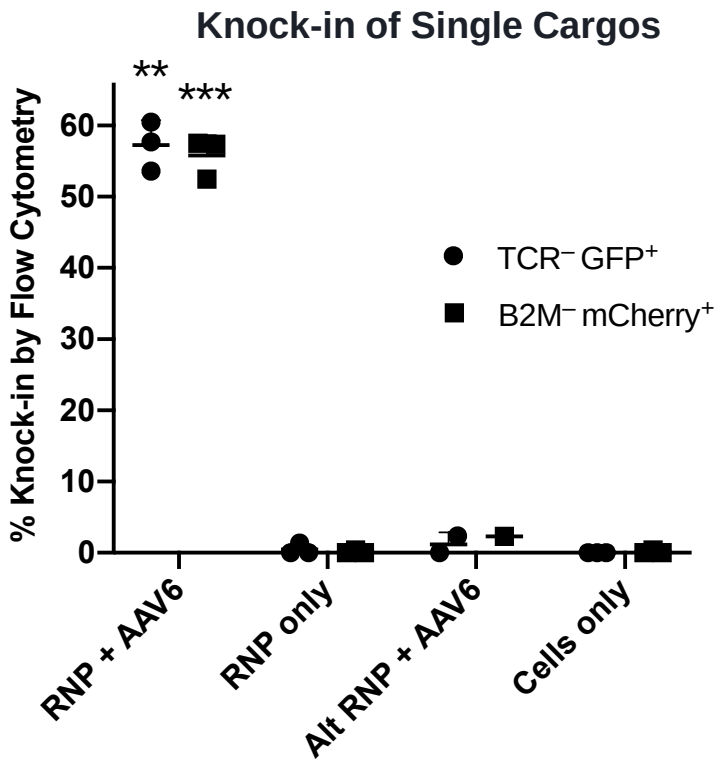
Engineered AsCas12a mediated highly efficient single and double knock-in using AAV6 donor templates

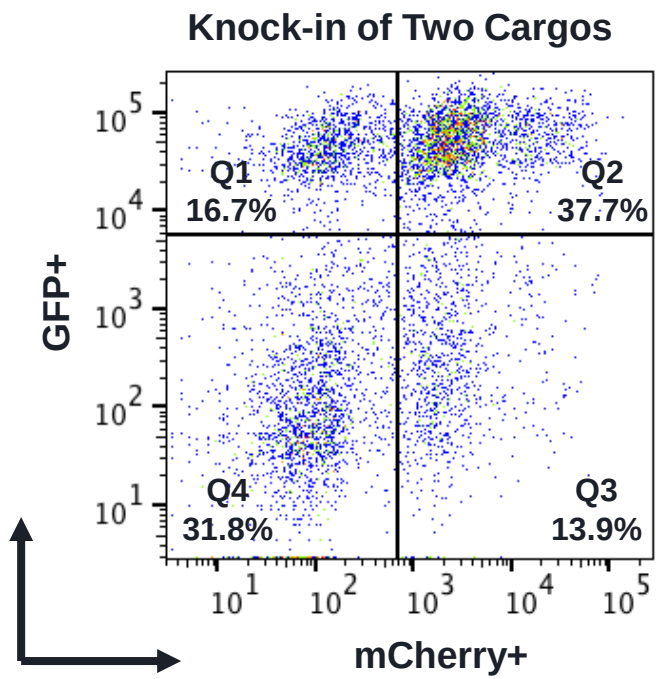
RNP composed of AsCas12a Ultra + IDT CRISPR-Cas12a Alt-R guides

AAV6

Lonza 4D nucleofector device







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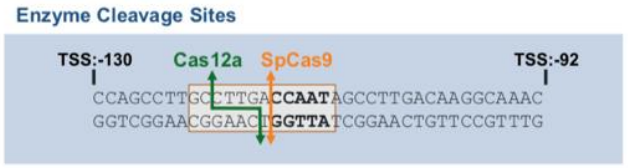
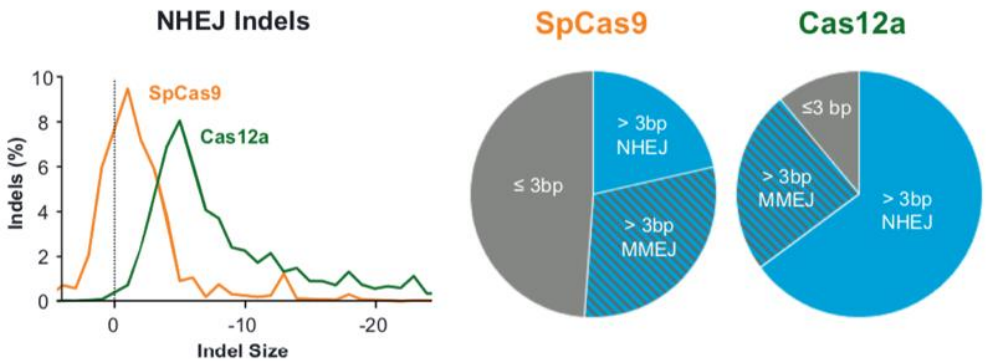
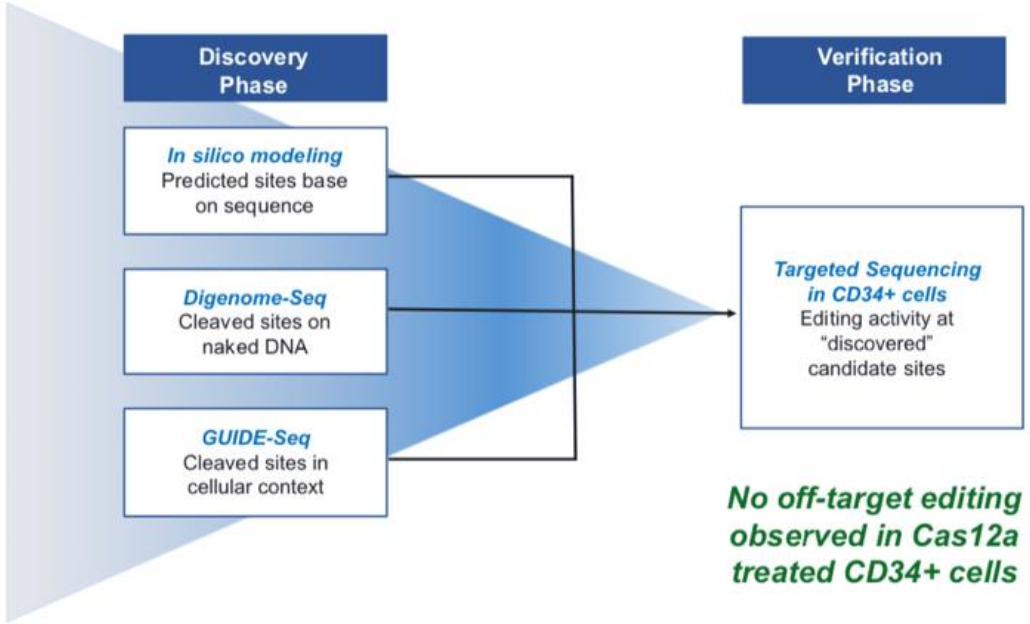
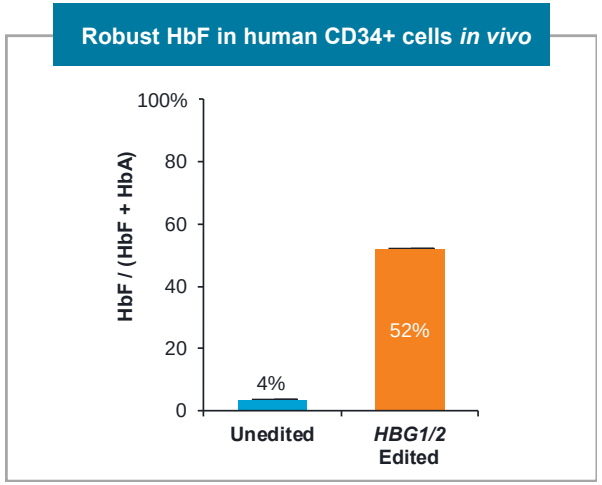
Engineered AsCas12a retains its high specificity, and its deletion profile may offer unique benefits









>90% editing in HSCs

High specificity for engineered AsCas12a verified across multiple cell types

De Dreuzy et al. Annual Meeting of the American Society of Hematology, Orlando. FL, December 9, 2019

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Troubleshooting and best practices regardless of your choice of CRISPR nuclease

Common reasons your ex vivo gene editing is not working

PROTEIN REAGENT	▪ Did you purify you're his-tagged nuclease using a single-step nickel affinity column and not remove excess endotoxin or solube aggregates use in primary? ▪ Perform SEC and endotoxin removal steps or purchase protein commercially
GUIDE REAGENT	▪ Did you extract your guide via in vitro transcription (IVT) and use it directly in electroporation step, potentially carrying over salts and residual ethanol? ▪ Use synthetic guides, they work, and the costs are less of a burden than you think
ELECTROPORATION	▪ Are you using a "cell-friendly" pulse code and buffer meant for plasmid DNA or unmodified mRNA/siRNA for your high-quality commercially purchased RNP? ▪ These new reagents are more friendly to cells, so dial up the pulse code intensity
CELL HANDLING	▪ Are you culturing a new cell type that you have limited experience with? ▪ Primary cells aren't HEK293Ts; 1) they don't like plasmid, and 2) find an expert on how to properly handle these cells and make sure you use optimal culture media
PROTOCOLS	▪ There are a lot of detailed protocols available, sometimes follow-up reports contain new details on editing optimization, and this is often critical for achieving efficient editing and/or viability, stay up-to-date with the literature!

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How to up your gene editing game

REACH OUT TO EXPERTS	▪ Protocols in papers are generally not detailed enough for even an expert to reproduce them with the same efficiency on the first try ▪ Consult gene editing product vendors that have a track record of success as there are product experts at these companies that are very knowledgeable
CONSIDER ENGINEERED CAS12A (ULTRA) FOR KO/KI	▪ Our experience with the IDT Ultra AsCas12a is that it is a superior product – to date, all targets edited with high efficiency in multiple cell types ▪ The smaller guide means lower cost and the high specificity and potency increasing the potential for optimal editing
CONSIDER AAV6 FOR EX VIVO GENE KNOCK-IN	▪ AAV6 seems expensive at first but it is robust and the quality of data you get justify the higher up-front cost ▪ Commercial vendors are abundant now and many universities also have their own vector cores which can help you

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Acknowledgements, Key references, and helpful links

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Component	Vendor	Website
Nucleases, guide RNAs	IDT	www.idtdna.com
Sequencing	GeneWiz	www.genewiz.com
”ICE” Editing Analysis	Synthego	www.synthego.com
AAV Production	Sirion Biotech	www.sirion-biotech.com
Electroporation	Lonza, Maxcyte	bioscience.lonza.com, www.maxcyte.com

IDT INTEGRATED DNA TECHNOLOGIES

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