Genome editing

Cas9 (Blunt ends)

Cpf1 (Cas12a) (5' overhang)

Double-stranded break (DSB)

Homology-directed repair (HDR)

Non-homologous end joining (NHEJ)

DNA with homology to DSB sequence (double- or single-stranded)

Mutation/indel
Alt-R® CRISPR System—complete workflow

Design | Cut | Repair | Analyze

Cas9/Cas12a + Guide RNA (gRNA)
CRISPR-Cas9 genome editing

- RNA-guided endonuclease
- 20 nt protospacer
- PAM site (NGG)
- Native two-part crRNA and tracrRNA, or one-piece sgRNA
CRISPR-Cas9 genome editing

Alt-R crRNA:tracrRNA

- 67 nt universal tracrRNA
- 36 nt site specific crRNA
- Chemically modified to prevent immune stimulation, nuclease degradation

Alt-R sgRNA

- 100 nt site specific sgRNA
- Chemically modified to prevent immune stimulation, nuclease degradation
Optimizing 2-part Cas9 gRNAs

- Shorten crRNA and tracrRNA to minimal length that has full activity
  - Surprise that shortened species were more active

<table>
<thead>
<tr>
<th></th>
<th>crRNA</th>
<th>tracrRNA</th>
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<tbody>
<tr>
<td>Native</td>
<td>42mer</td>
<td>89mer</td>
</tr>
<tr>
<td>Optimized</td>
<td>36mer</td>
<td>67mer</td>
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</tbody>
</table>

- Introduce chemical modifications to:
  - Improve nuclease stability
  - Reduce risk of triggering innate immune response

- Manufacturing
  - crRNAs are target specific—need thousands in small scale
  - tracrRNA is universal—can synthesize in large scale and aliquot to smaller tubes at large cost savings compared to making this sequence for every gRNA
2′OMe modification walk in the Cas9 tracrRNA

Activity (T7EI assay)

Black = RNA       Red = 2′OMe RNA       * = Phosphorothioate (PS) bond
2′OMe modification walk in the crRNA

Activity (T7EI assay)

<table>
<thead>
<tr>
<th>Protospacer</th>
<th>tracrRNA-binding</th>
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Black = RNA
Red = 2′OMe RNA
Green = 2′F RNA
* = PS bond
What we learned from studying over 400 modified RNAs

tracrRNA

cGGAAuAAuUGAAGCUAC\*G\*A 5’

guCGuAUCAACUUG

AGCCACGGUGAA

G U C G G U G C U *U*U 3’

5’ C*U*U*AUAUCCAACACuuCGGGuuUAGAGCUAU*G*C*U 3’

20 base protospacer guide domain

16 base tracrRNA binding domain

AGCU = 2’OMe RNA
agcu = RNA
* = PS bond

= Major loss of function with 2’-mod
= Minor loss of function with 2’-mod
\(\blacktriangledown\) = Loss of function varies with sequence
Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells

Ayal Hendel¹,⁵, Rasmus O Bak¹,⁵, Joseph T Clark¹, Andrew B Kennedy², Daniel E Ryan², Subhadeep Roy³, Israel Steinfeld⁴, Benjamin D Lunstad³, Robert J Kaiser², Alec B Wilkens¹, Rosa Bacchetta¹, Anya Tsalenko², Douglas Dellinger³, Laurakay Bruhn² & Matthew H Porteus¹

For chemically synthesized sgRNA, this simple modification pattern gives very large benefits
Chemically modified Alt-R 2-part and sgRNAs have similar editing efficiency when used as RNP in most cells.

Modified sgRNAs cost ~4X more than modified 2-part gRNAs, but there is a role for both types of reagents.

- In some applications, sgRNAs perform better. For example, when using:
  - Cas9 mRNA instead of RNP
  - RNP in CD34+ cells
- In most cases, the 2 classes of reagents are similar, but the sgRNAs cost 3–5x more.
genome editing

Alt-R™ CRISPR-Cas9 System delivery—zebrafish embryo microinjection
Contributed by Jeffrey Essner, PhD., Associate Professor in the Department of Genetics, Development, and Cell Biology at Iowa State University

Mouse zygote microinjection
Alt-R™ CRISPR-Cas9 System ribonucleoprotein delivery
Contributed by Rolen Quadros, Donald Harms, and CB Gurumurthy
Mouse Genome Engineering Core Facility, University of Nebraska Medical Center, Omaha, NE, USA

Mouse zygote electroporation
Ribonucleoprotein delivery using the Alt-R™ CRISPR-Cas9 System
Contributed by Masakazu Hashimoto, Osaka University, and Tatsuya Takemoto, Tokushima University

C. elegans injection
Alt-R™ CRISPR-Cas9 System ribonucleoprotein delivery
Contributed by Dr Simone Köhler, postdoctoral fellow, and Dr Abby Dernburg, HHMI Investigator and Professor of Cell and Developmental Biology, at the University of California, Berkeley, CA, USA

http://www.idtdna.com/pages/products/genome-editing/crispr-cas9
Gene editing in plants using CRISPR/Cas9

• T-DNA (*Agrobacterium tumefaciens* infection) or Plasmid DNA encoding the gRNA + Cas9 and selectable marker
  – Delivered DNA frequently integrates into the genome
  – Foreign DNA integration is a concern for many regulatory authorities (‘GMO’)
  – Overexpression of Cas9 can lead to unwanted off-target cleavage
  – Can lead to plant chimerism, requires segregation

• Cas9 stably integrated into plant of interest
  – Requires time and resources for development and characterization of integrant lines

• Direct RNP delivery
  – DNA free editing alleviates regulatory concerns (no foreign DNA) and reduces the potential for off-target cleavage
DNA-free RNP delivery

- Lipid transfection
- Electroporation
- Microinjection

- Similar to mammalian tissue culture cells
- However, it is difficult or impossible to regenerate plants for many species

- Particle bombardment
RNP mediated genome editing in protoplasts

- PEG-mediated RNP delivery in multiple plant types
- Lettuce protoplasts edited using Cas9 RNP are able to regenerate into whole plants
  - Targeted deep sequencing of mutant calli show 40% (14/35) contained biallelic mutations at the target site

Biolistic delivery of Cas9 RNP in maize embryo cells

- High frequency of mutant plants with no selection
- Similar efficiencies between plasmid and RNP delivery
- RNP delivery reduced off target cleavage

S.p. Cas9 is likely to produce off-target cleavage
(particularly with plasmid expressed sgRNA and Cas9)

On-target site

Empirically determined off-target sites

RNP delivery of wild-type Cas9 reduces off-target editing

![Graph showing Indel by NGS (%) for different concentrations of Cas9 RNP in HEK293-Cas9 Cells and WT Alt-R S.p. Cas9 RNP, with on-target and off-target sites identified by NGS.]

**On target site:**

GAGTCCGAGCAGAAGAAGAA

**EMX1 on-target site:**

GAGTGAGCAGAAGAAGAAG

**Off-target site 1:**

GAGTCTAAGCAGAAGAAGAAG

**Off-target site 2:**

GAGTCTAAGCAGAAGAAGAAGA

HEK293-Cas9 Cells

WT Alt-R S.p. Cas9 RNP

Low-level constant expression

4 µM

2 µM

1 µM

0.5 µM

0

10

20

30

40

50

60

70

80

90

100

Indel by NGS (%)
Mitigating Cas9 off-target effects

• Delivery of Cas9 RNP complex reduces off-target editing, but it is not a total solution

• Other solutions to reduce OTE have significant drawbacks
  – crRNA length reduction (18–19 nt)
  – Chemical modification

• What about high-fidelity Cas9 proteins?
Published, rationally-designed, high-fidelity Cas9 mutants

- **eSpCas9 - Zhang/MIT mutant**

- **SpCas9-HF1 - Joung/MGH mutant**
eSpCas9 and SpCas9-HF1: On-target performance is significantly compromised

Alt-R® CRISPR-Cas9 System
10 nM RNP, Lipofection
HPRT locus
HEK-293 cells

Chemically modified sgRNAs
Amaxa® Nucleofector® (Lonza)
CD34+ HSPCs

eSpCas9 = Zhang/MIT mutant
SpCas9-HF1 = Joung/MGH mutant

Danny Dever
Matt Porteus
Stanford University
Bacterial screen to identify novel high-fidelity Cas9 mutants

- Double selection for avoidance of off-target cleavage and maintenance of on-target cleavage

250,000 mutants from low-fidelity PCR library

On-target site
Toxin
Must cleave

Off-target site
Essential gene
Must not cleave

100 hits from primary screen, then change on/off target sites & repeat
9 hits from secondary screen → purify protein, test in human cells
Alt-R® S.p. HiFi Cas9 Nuclease 3NLS: On-target performance is significantly improved

Alt-R® CRISPR-Cas9 System
10 nM RNP, Lipofection
HPRT locus
HEK-293 cells

Chemically modified sgRNAs
Amaxa® Nucleofector® (Lonza)
CD34+ HSPCs
Alt-R® S.p. HiFi Cas9 Nuclease 3NLS reduces off-target editing while maintaining on-target potency

Published, known off target sites

![Graph showing off-target and on-target cleavage for different Cas9 variants.]

- EMX1
  - On-target: GAGTCCGAGCAGAAGAAGAAAGG
  - Off-target: GAGTTAGCAGAAGAAGAAAAG

- HEKSite4
  - On-target: GGCACTGCCAGGTGGG
  - Off-target: GGCACGGACGGAGTTGGG

- VEGFA3
  - On-target: GGTGAGTGAGTGAGTGAGTGAGTG
  - Off-target: AGTGAGTGAGTGAGTGAGTGAGTG

WT Cas9
- eSpCas9
- SpCas9-HF1
- Alt-R HiFi Cas9
RNP dose response: WT Cas9 vs. Alt-R® HiFi Cas9

![Graph showing Indel by NGS (%; log10) for different concentrations of WT Alt-R S.p. Cas9 and Alt-R S.p HiFi Cas9. The graph includes data points for 4 µM, 2 µM, 1 µM, and 0.5 µM.](image)

- **WT Alt-R S.p. Cas9**
  - On target
  - Off target 1
  - Off target 2

**Alt-R S.p HiFi Cas9**
- On target

**NGS Sequence**
- GAGTCCGAGCAGAAGAAGAAAGG  EMX1 On Target Site
- GAGT**T**AGAGCAGAAGAAGAAAGG  Off Target Site 1
- GAGTCT**A**AGCAGAAGAAGAAAG**G**  Off Target Site 2

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Analyzing off-target editing globally

- Analyzing known off-target sites is an imprecise method for investigating off-target editing.
- We developed a proprietary method to analyze global off-target editing in a cell-free Cas9 cleavage system.
- The off-target editing percentage should be an overestimation:
  - Cas9 RNP should be stable in the absence of cellular proteases and nuclease.
  - Purified genomic DNA is absent factors that would encumber Cas9 binding.
Global and unbiased analysis of OTE

AR-S-1893
GTTGGAGCATCTGAGTCCAGGG

*On-target Cleavage Site

Cas9 cleavage (counts)

Genomic loci (Rank order: Most to least frequently cleaved by WT Cas9)

% Off-target cleavage

% On-target cleavage

On-target Cleavage Site
In silico predictions don’t capture all off target sites

AR-S-1893
GTTGGAGCATCTGAGTCCAGGG

% off-target read counts at in silico predicted sites

Predicted Not predicted
25%
9%

On target Off target CCTop predicted sites MIT predicted sites
Global and unbiased analysis of OTE

AR-S-1893
GTTGGAGCATCTGAGTCCAGGG

*On-target Cleavage Site
Alt-R® S.p. HiFi Cas9 Nuclease 3NLS reduces OTE

AR-S-1893
GTTGGAGCATCTGAGTCCAGGG

WT Cas9
Alt-R® HiFi Cas9

*On-target Cleavage Site
Global and unbiased analysis of OTE: WT vs. HiFi

WT Alt-R® S.p. Cas9

Alt-R® S.p. HiFi Cas9

On-target editing

Off-target editing
Guide-Seq screen: 7 gRNAs with WT and IDT HiFi Cas9

Wild Type Sp. Cas9
Avg Reads ON-target: 66.9%

- VEGFA3: 62% On-Target, 38% Off-Target
- EMX1: 66% On-Target, 34% Off-Target
- GRHPR: 98% On-Target, 2% Off-Target
- HPRT-38285: 100% On-Target
- HPRT-38087: 79% On-Target, 21% Off-Target
- AR: 64% On-Target, 36% Off-Target
- CTNNB1: 55% On-Target, 45% Off-Target

IDT HiFi Cas9
Avg Reads ON-target: 90.4%

- VEGFA3: 81% On-Target, 19% Off-Target
- EMX1: 80% On-Target, 20% Off-Target
- GRHPR: 99% On-Target
- HPRT-38285: 100% On-Target
- HPRT-38087: 96% On-Target, 4% Off-Target
- AR: 94% On-Target, 6% Off-Target
- CTNNB1: 82% On-Target, 18% Off-Target

GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nuclease

CRISPR editing by rhAmpSeq™ detection

• Many validated and predicted off-target sites – how do we capture them?
  – In vitro assays to define possible OTEs
  – GuideSeq identification of OTEs
  – Compilation of in silico sites

• rhAmpSeq – multiplexed amplification for targeted enrichment
Underlying RNaseH2 Technology

RNAse H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers

Joseph R Dobosy, Scott D Rose, Kristin R Beltz, Susan M Rupp, Kristy M Powers, Mark A Behlke* and Joseph A Walder

Dobosy et al. BMC Biotechnology (2011) 11:80
https://doi.org/10.1186/1472-6750-11-80
Global off-target editing experiments

• First determined the identity of each DSB using the GUIDE-seq procedure
• Second used rhAmpSeq™ multiplex amplicon sequencing coupled to NGS to better quantify editing efficiency at on/off-target loci
• Examined literature high OT sites, internal high OT sites, and clinically-relevant loci
• Examined in the context of stable cell line expression for WT and HiFi, and also delivered as RNP complexes
rhAmpSeq™ for CRISPR: off-target editing

**AR-S-1893**

GTTGGAGCATCTGAGTCCAGGG

![](chart.png)

*On-Target Editing efficiency = 83%*

rhAmpSeq™ panel of known off-target sites identifies editing at on and off-target genomic loci.
Alt-R CRISPR System—complete workflow

- Predesigned guides
- Custom designs
- Design checking
CRISPR gRNA design tools with OT analysis capability for plant species

- CGAT (http://cbc.gdcb.iastate.edu/cgat/)
- CHOPCHOP (http://chopchop.cbu.uib.no/)
- Crispr-P (http://crispr.hzau.edu.cn/CRISPR2/)
- CCTop (https://crispr.cos.uni-heidelberg.de/)
- CRISPR-PLANT (https://www.genome.arizona.edu/crispr/index.html)
Alt-R CRISPR System—complete workflow

**Guide RNA**
- crRNA:tracrRNA
- sgRNA
- Custom formats

**Cas9 protein**
- Cas9 Nuclease
- HiFi Cas9 Nuclease
- D10A and H840A Nickases
- Cas12a (Cpf1) Nuclease

**Supporting reagents**
- Electroporation enhancers
- ATTO™ 550 tracrRNA
- Control kits

ATTO is a trademark of ATTO-TEC.
Alt-R CRISPR System—complete workflow

Design  Cut  Repair  Analyze

Ultramer® Oligonucleotides
- Up to 200 bases

Megamer® ssDNA Fragments
- Up to 2000 bases
- From clonally purified DNA
- Sequence-verified via NGS
Alt-R CRISPR System—complete workflow

Design  Cut  Repair  Analyze

Genome Editing Detection Kit
• Simple, fast, T7EI-based assay

Custom oligos for Sanger, NGS
New multiplex amplicon NGS soon
Alt-R CRISPR System—complete workflow

Design → Cut → Repair → Analyze

Combine Reporter Mix and Master Mix
rhAmp® SNP Assay
Purified DNA sample

Reaction setup → Thermal cycling and detection → Data analysis

rhAmp® SNP Genotyping System
Conclusions

• Many paths are available to perform CRISPR genome editing
  – Use of RNP with chemically modified gRNAs gives high activity and reduced off-targets effects
  – 2-part crRNA:tracrRNA is less costly, but sometimes the more expensive sgRNA has benefits

• Off-target effects (OTEs) can be a problem, even when using RNP methods
  – Cleavage at the wrong site(s) is a problem which exists but is difficult to study except by NGS
  – Toxicity via stimulation of the innate immune system occurs if you do not use modified RNAs

• Cas9 mutant enzymes can improve off-target profiles but can also significantly hurt on-target cleavage, especially when using preferred RNP methods

• New IDT HiFi Cas9 mutant retains on-target cleavage and has reduced off-target risk
Thanks to the scientists who contributed to these studies …

Integrated DNA Technologies
- Ashley Jacobi
- Garrett Rettig
- Rolf Turk
- Chris Vakulskas
- Nicole Bode
- Bernice Thommandru
- Michael Christodoulou
- Mirna Jarosz
- Patty Mudivarti
- Matt McNeill
- Mark Behlke

Questions?
www.idtdna.com/CRISPR