Modulating the Activity of CRISPR-Cas with Chemical Modifications in Single-guide RNAs

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CSHL Genome Engineering 2020: CRISPR Frontiers

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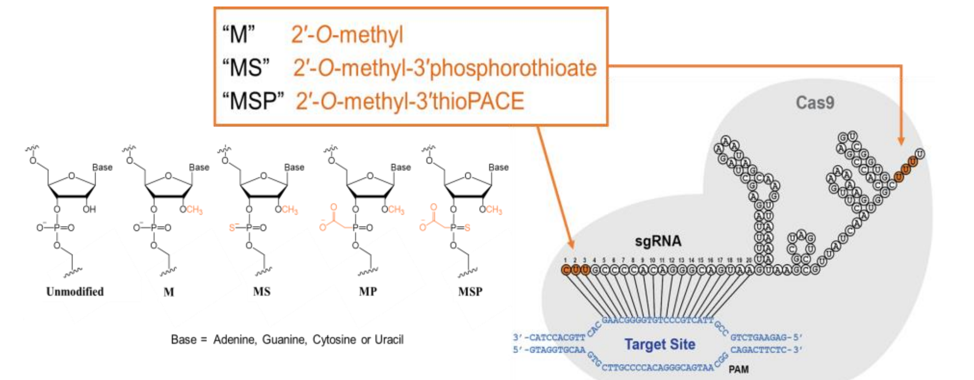
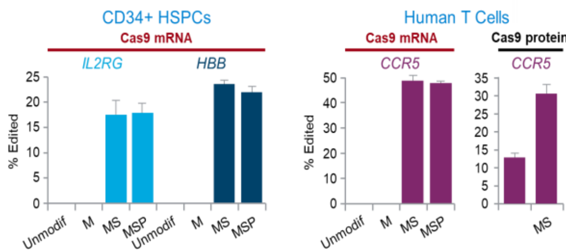
CRISPR is revolutionizing life science and drug development research. Through the targeted application of gene editing, gene knockins, knockouts, or gene expression control, new models and therapeutics are enabled and accelerated. An important consideration in the development of these capabilities is the activity of the CRISPR-Cas system.

With the novel RNA synthesis chemistry outlined in Dellinger et. al. (*JACS*, 2011) we robustly and routinely synthesize RNA oligos at higher yields per scale than other chemistries. Also, synthesis allows us to site-specifically modify nucleotides in sgRNAs to adjust their longevity and enhance their specificity.

Studies of the stability and activity of multiple guide RNAs demonstrate that different numbers and types of modifications incorporated at their 5' and 3' ends can enhance stability of the RNA in cells while maintaining the desired guide RNA functionality.

Chemical modifications enhance gRNA stability and activity





Human T Cells

Cas9 mRNA — CCR5 (% Edited; Unmodif, M, MS, MSP)

Cas9 protein — CCR5 (MS)

NATURE BIOTECHNOLOGY published online June 29, 2015; 33, 985–989

Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells

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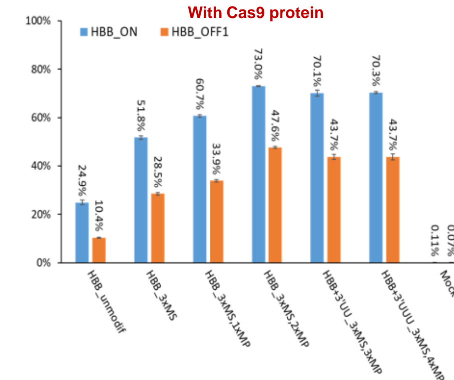
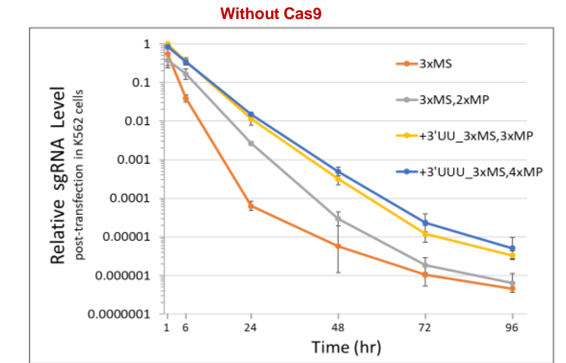
Agilent Patent
GUIDE RNA WITH CHEMICAL MODIFICATIONS
Ryan et al., priority Dec. 2014, issued July 2018

100nt guide RNAs targeting IL2RG, HBB and CCR5 genes were synthesized containing either no modification, M, MS, or MSP modifications at their 5' and 3' ends and were nucleofected into human primary cells with Cas9 protein or Cas9 mRNA. Indel frequencies were measured by TIDE analysis of PCR amplicons spanning the edited genomic target sites.

Alternative end-protection extends half-life of sgRNAs in cells

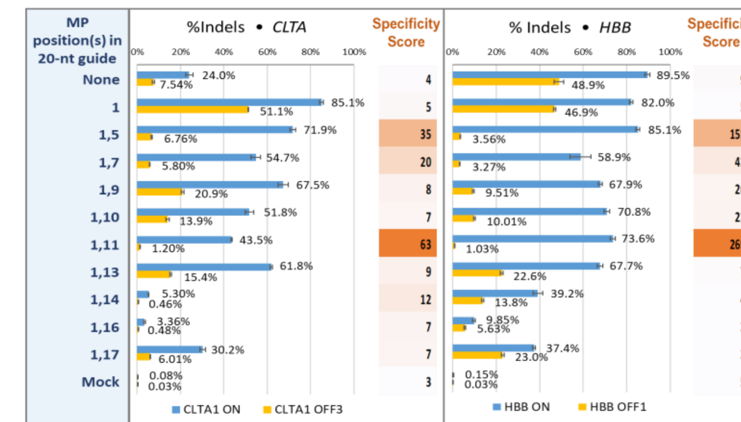




A 100-nt single guide RNA targeting the HBB gene was synthesized containing different numbers of MS or MP modifications at its 5' and 3' ends, and each sgRNA was nucleofected into human K562 cells with or without Cas9 protein.

qRT-PCR was used to directly measure the relative amounts of guide RNA persisting in the absence of Cas protein over timepoints sampled post-nucleofection. In a companion study using Cas9 protein, indel frequencies at the on-target site and a highly active off-target site were measured by amplicon deep sequencing of genomic DNA from HepG2 cells co-transfected with modified 100mer sgRNA and Cas protein.

MP modifications enhance targeting specificity of sgRNAs



MP position(s) in 20-nt guide	CLTA1 ON %Indels	CLTA1 OFF3 %Indels	CLTA Specificity Score	HBB ON %Indels	HBB OFF1 %Indels	HBB Specificity Score
None	24.0%	7.54%	4	89.5%	48.9%	9
1	85.1%	51.1%	5	82.0%	46.9%	5
1,5	71.9%	6.76%	35	85.1%	3.56%	155
1,7	54.7%	5.80%	20	58.9%	3.27%	42
1,9	67.5%	20.9%	8	67.9%	9.51%	20
1,10	51.8%	13.9%	7	70.8%	10.01%	22
1,11	43.5%	1.20%	63	73.6%	1.03%	269
1,13	61.8%	15.4%	9	67.7%	22.6%	7
1,14	5.30%	0.46%	12	39.2%	13.8%	4
1,16	3.36%	0.48%	7	9.85%	5.63%	2
1,17	30.2%	6.01%	7	37.4%	23.0%	2
Mock	0.08%	0.03%	3	0.15%	0.03%	5

Nucleic Acids Research published online Dec 4, 2017; 46:2, 792–803 (2018)

Improving CRISPR–Cas specificity with chemical modifications in single-guide RNAs

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Agilent Patent Application
HIGH SPECIFICITY GENOME EDITING USING CHEMICALLY-MODIFIED GUIDE RNAs
Dellinger, Ryan, Roy & Sampson, priority June 2016

MP modification of select positions in sgRNAs targeting HBB and CLTA enhance specificity of indel formation, as demonstrated by targeted deep sequencing of amplicons spanning the genomic DNA on- & off-target sites. This enhanced specificity is likely due to local weakening of RNA-Cas9 protein interactions in the gRNP:DNA complex by the guide-sequence modifications, thereby affecting the thermodynamic barriers that modulate specificity (Ryan et. al. *Nucleic Acids Research*, 2018).

Conclusions

Chemical modifications in chemically synthesized guide RNAs can provide significant advantages in affecting the duration of sgRNA activity in transfected cells (or in vivo). By introducing M, MS, MP or MSP modifications to the ends of the sgRNA, the half-life can be extended. In addition, incorporation of MP modifications at select positions in the sgRNA sequence can improve the specificity of on-target cleavage while maintaining, or improving, overall efficiency. Each of these factors can be combined to modulate the activity of the CRISPR-Cas system.

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