Design high specificity CRISPR-Cas9 gRNAs: principles and tools

Heidi Huang, PhD

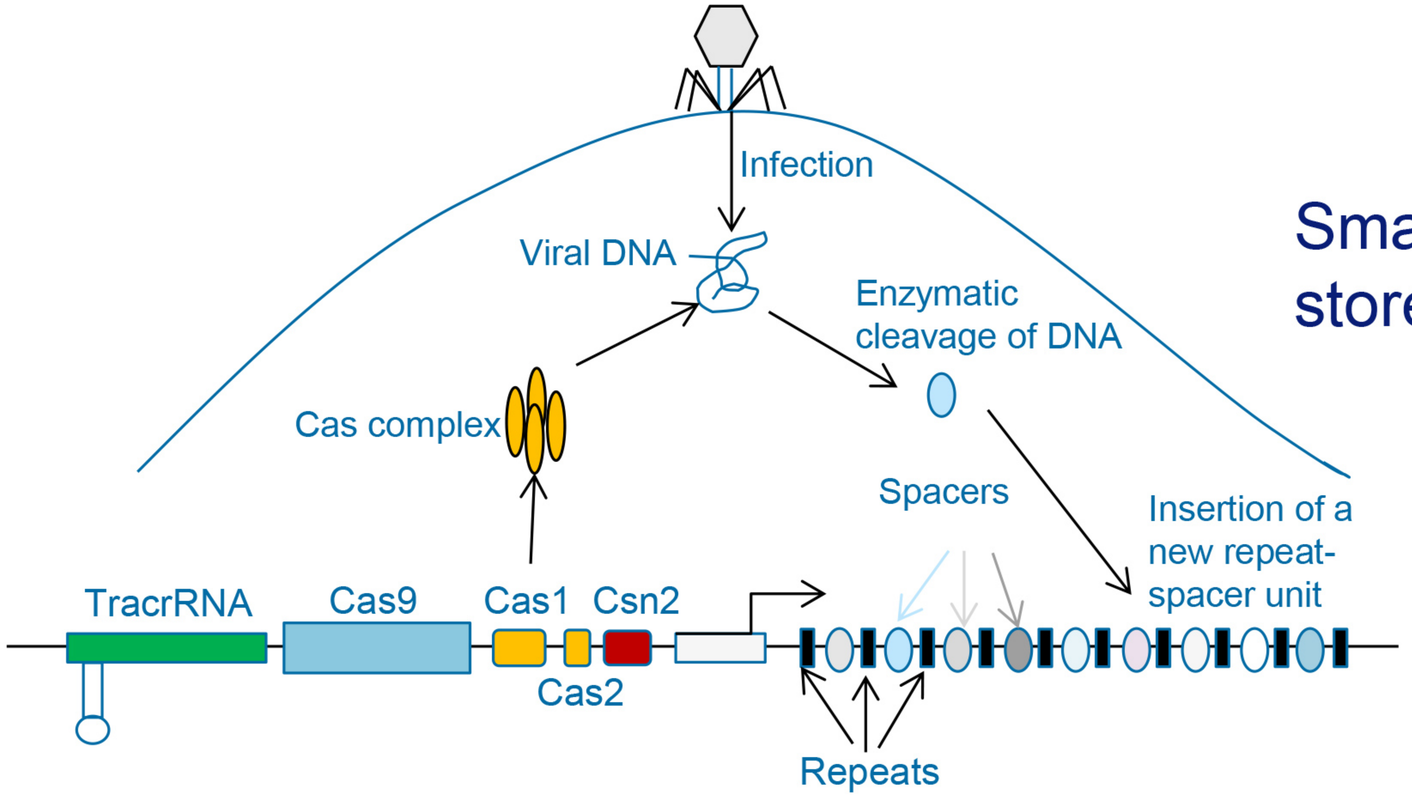
GenScript
Scripting Possibilities

Webinar Agenda

1. Introduction of CRISPR-Cas9
2. gRNA Design
3. Resources and Services
4. Q&A

What is CRISPR?

- **CRISPR** – **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats
- **Cas9** – **C**RISPR **a**ssociated **s**ystem. RNA-guided dsDNA-binding protein that has nuclease activity
- Type II CRISPR/Cas systems
  CRISPR locus: TracrRNA + Cas complex proteins + spacers/repeats



Small fragments of viral DNA are stored in CRISPR locus as **spacers**

Adapted from: Mali P. et al. **Cas9 as a versatile tool for engineering biology**. *Nat. Methods* (2013), 10(10):957-963

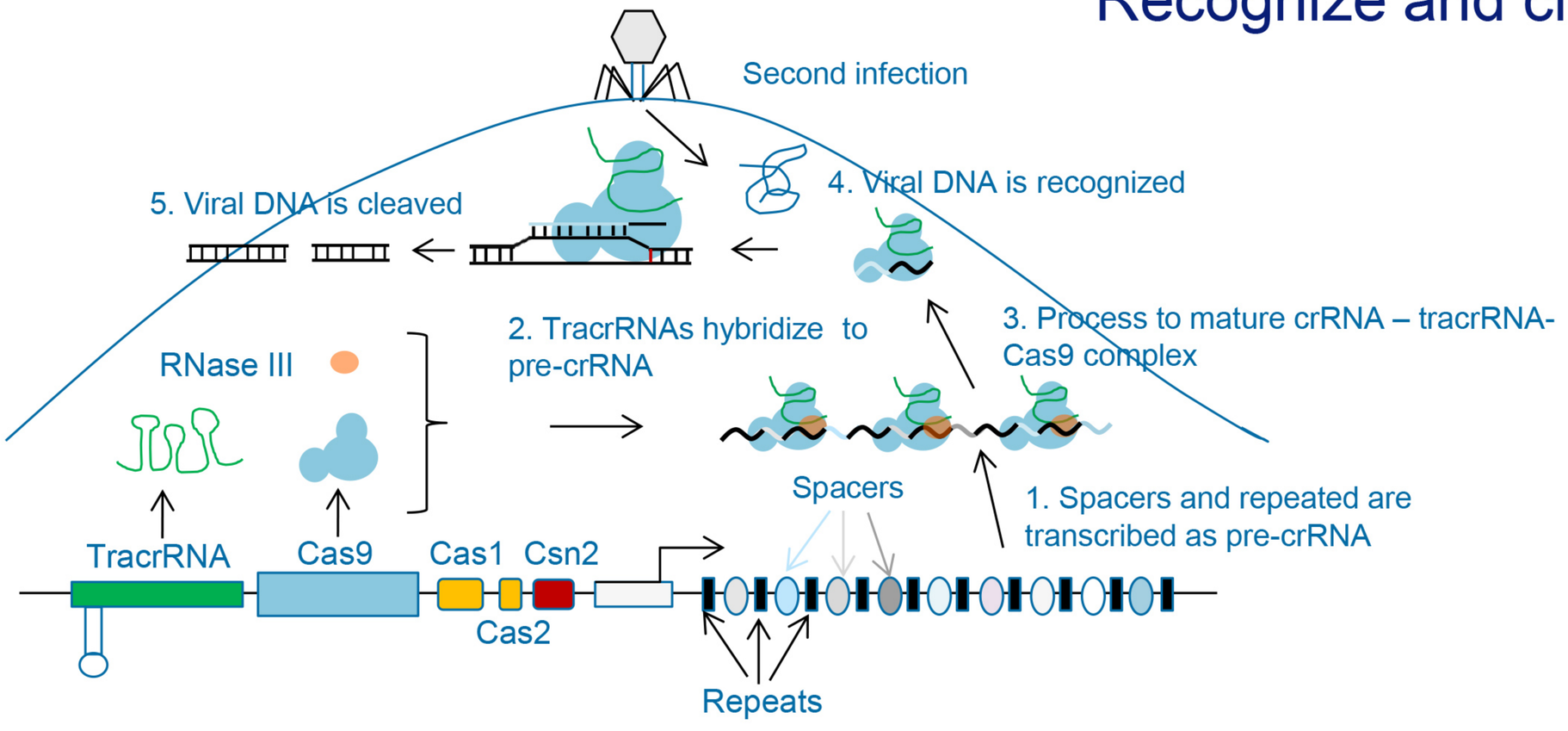
What is CRISPR?

spacers + repeats → pre-crRNA

tracrRNA, Cas9

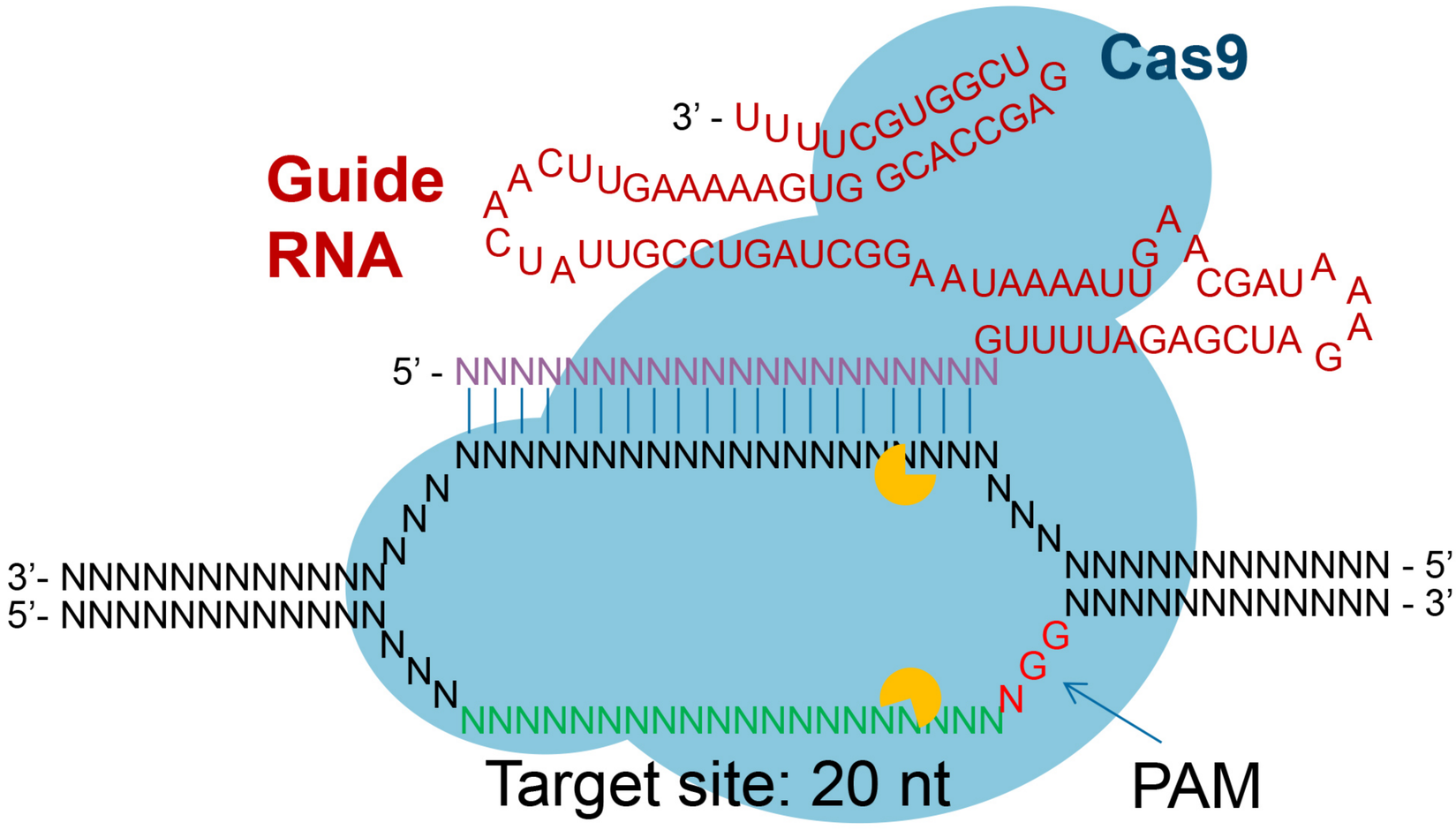
crRNA-tracrRNA-Cas9 Complex

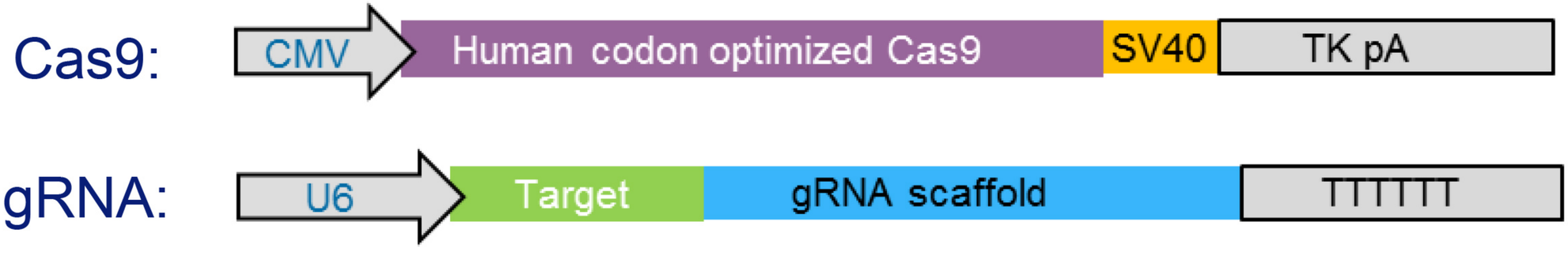
Recognize and cleave viral DNA



Adapted from: Mali P. et al. **Cas9 as a versatile tool for engineering biology**. *Nat. Methods* (2013), 10(10):957-963

Engineer CRISPR-Cas9 for Genome Editing





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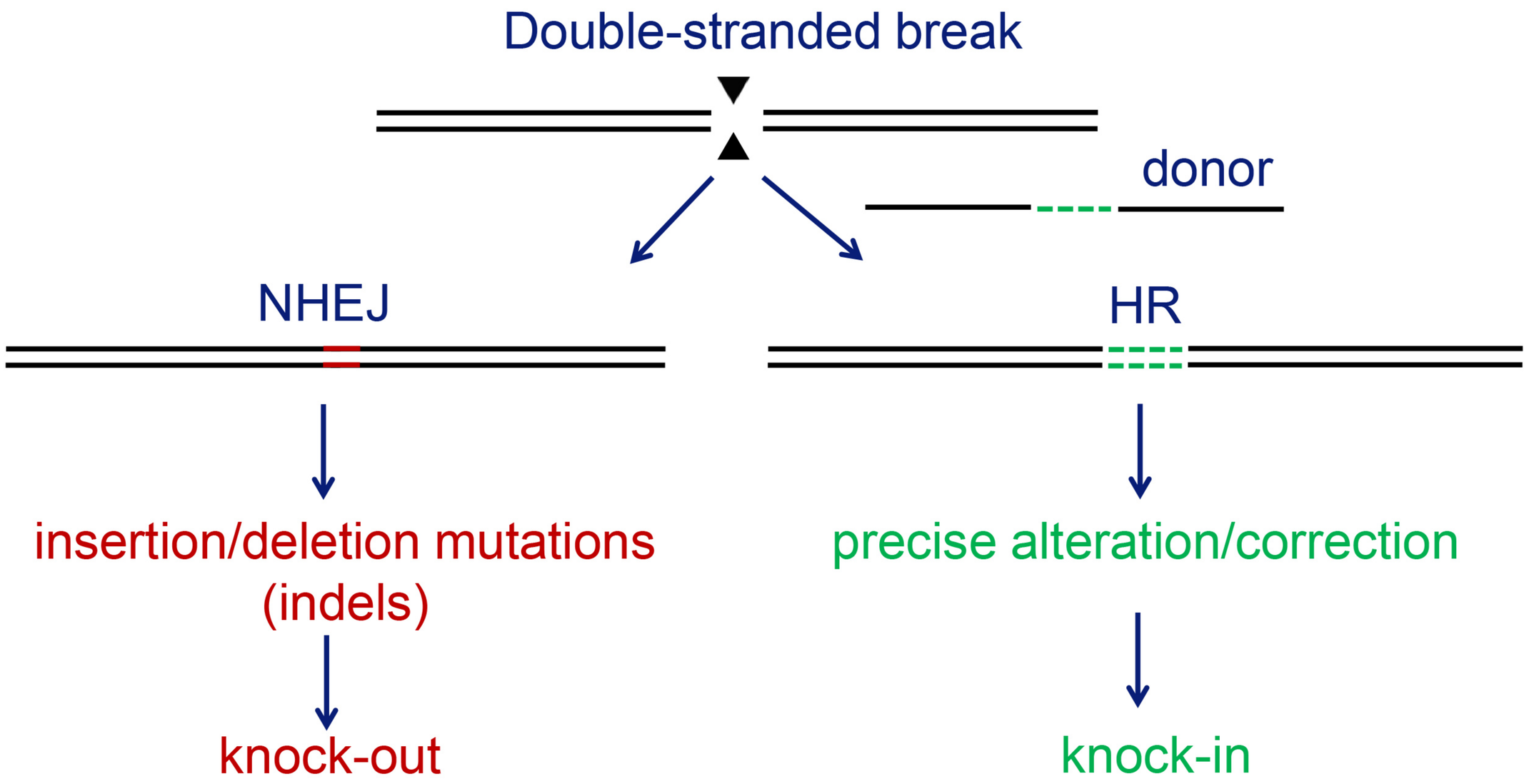
DNA Repair Enables Targeted Genome Editing

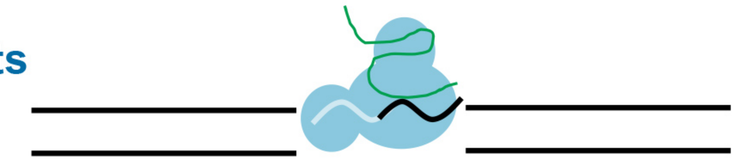


Potential Applications for CRISPR-Cas9

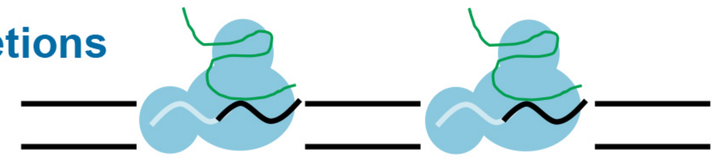
Genome editing

Wilde-type Cas9

Cuts

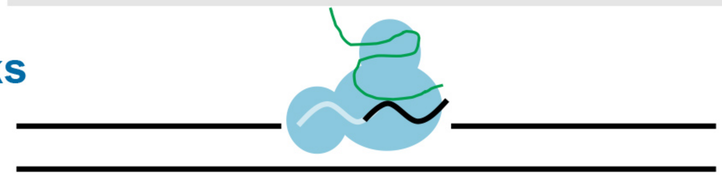


Deletions

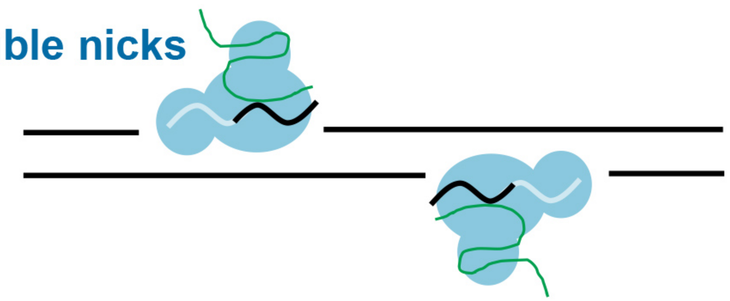


Cas9 Nickase

Nicks



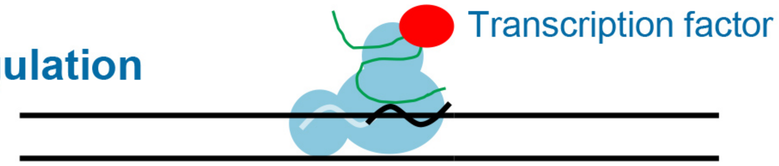
Double nicks



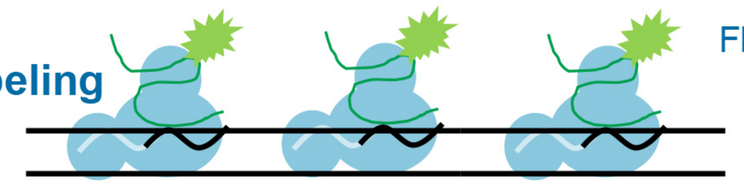
Genome regulation, reorganization and visualization

$Cas9_{nuclease\text{-}null}$ Protein Fusions

Regulation

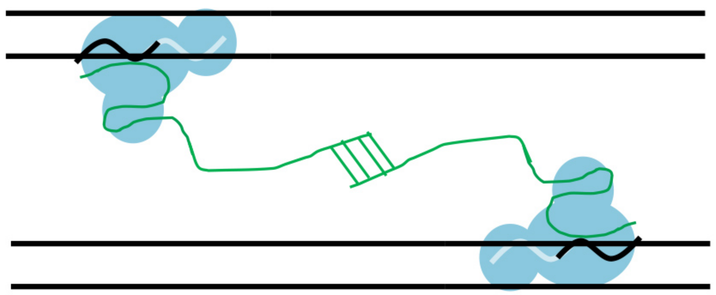


Labeling



$Cas9_{nuclease\text{-}null}$ Nucleic Acid

Structural aggregation



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Off-target of CRISPR-Cas9

Target site: **5’- NNNNNNNNNNNNNNNNNNNNNGG**

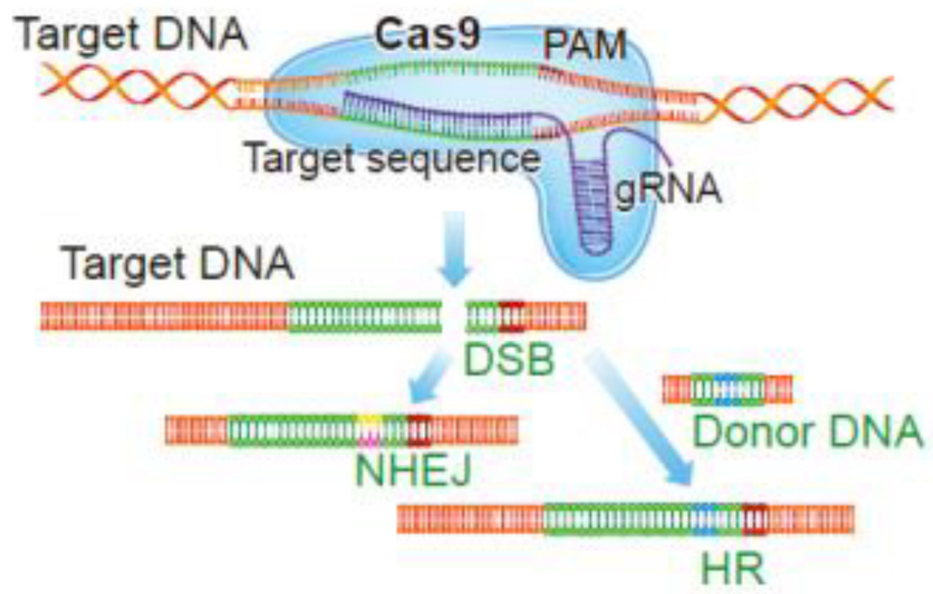
20 nt PAM

- CRISPR/Cas9 can tolerate 1-3 mismatches between gRNA and target sequence, which can lead to off target nuclease activity.

- Cas9 induces double stranded breaks, any off target nuclease activity can cause mutations in those genes, leading to possible oncogenesis.

gRNA Design - The Goal

- Accurate gRNA target sites
- Lowest off-target potential
- Optimum target location



gRNA Design – Step by Step

Step 1: Target gene analysis

Step 2: Find gRNA canonical sequences

Step 3: Off-target analysis

Step 4: Location analysis

Step 5: gRNA delivery

Step 1: Target Gene Analysis

- Gene sequence analysis
  - NCBI database
  - Genome sequencing
  - Our recommendation: Always sequence the target gene in the cells/strains you work with before designing gRNA

- Gene structure analysis
  - Exons/CDS
  - Introns

Step 2: Find gRNA Canonical Sequences

- gRNA canonical sequences

5’- NNNNNNNNNNNNNNNNNNNNNGG

20 nt PAM

- GN(20)GG

5’- GNNNNNNNNNNNNNNNNNNNNGG

20 nt PAM

- PAM sequences
  - NGG
  - NAG

Step 3: Off-target Analysis

- Genome wide analysis to find similar sequences to GN(20)GG
  - Higher similarity, higher off-target risk

Target site: **5’- GTGTAAACGGATAATGGACANGG**

Distal Seed PAM

- 3 criteria to gauge potential off-target sites
  - Existing of PAM sequence
    - NGG
    - NAG
  - Sequence similarity
    - higher similarity, higher risk
  - Sequence similarity at 3’ increase off-target risk

Step 4: Location Analysis

- Knock-out: NHEJ to induce indels
  - early exons
  - function domain

- Knock-in: HR with donor template
  - Both exon and intron can be targeted
  - CDS (Coding DNA sequence) on exon can be better than intron for donor design
  - Avoid mis-targeting of donor template by gRNAs

Recommendation: 2 or more gRNAs for each target gene

Step 5: gRNA Delivery

- gRNA expression plasmid
  - U6 promoter
  - gRNA targeting region
  - gRNA scaffold
  - Termination signal

- RNA
- Transgenic

Enhancing Specificity By Truncating gRNA Length

- gRNA sequences can be 17-20 nt in length to achieve similar levels of on-target gene editing

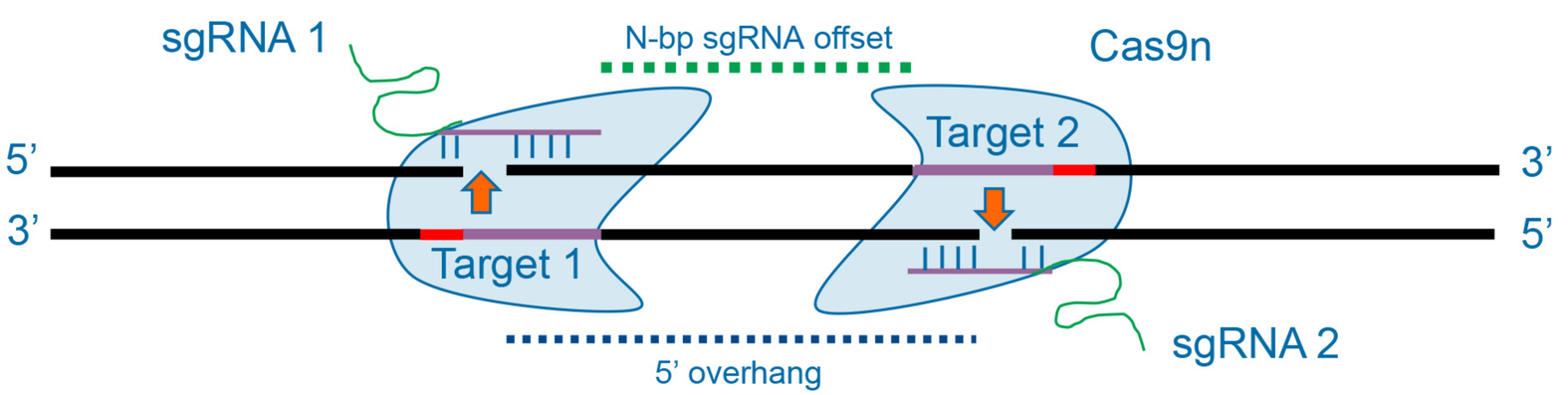
- Up to 10,000 fold improvement in target specificity when truncated (17 or 18 base pair) gRNA is used

- Using a shorter gRNA (17 or 18 nt) can greatly improve off-target specificity

Fu Y. et al. **Improving CRISPR-Cas nuclease specificity using truncated guide RNAs**. *Nat. Biotech*. (2014). 32:279-284

Enhancing Specificity with Modified Nucleases

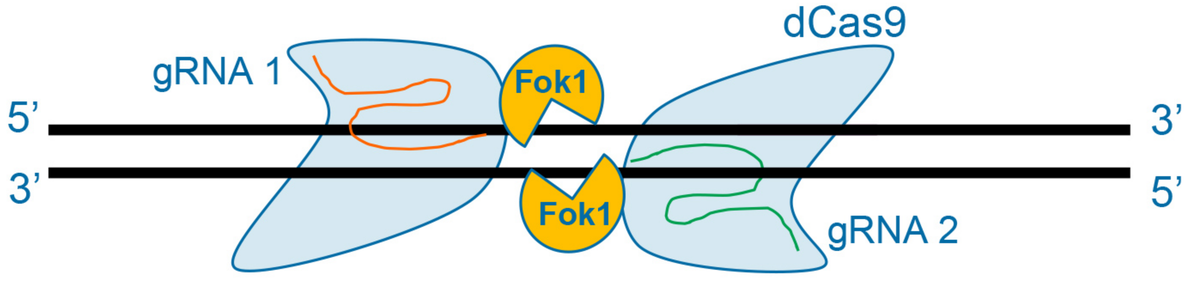
- Paired Cas9 nickases (Cas9n)
  - D10A Cas9 mutant allows for single strand nicking



Up to 1500-fold increase in specificity

Adapted from Ran AF. et al. **Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity**. *Cell* (2013). 154:1380-1389

- Dimeric CRISPR RNA-guided FokI nuclease (Fok1-dCas9)
  - FokI was fused to a catalytically inactive Cas9 (dCas9) mutant



Up to 10,000-fold less mutagenic activity

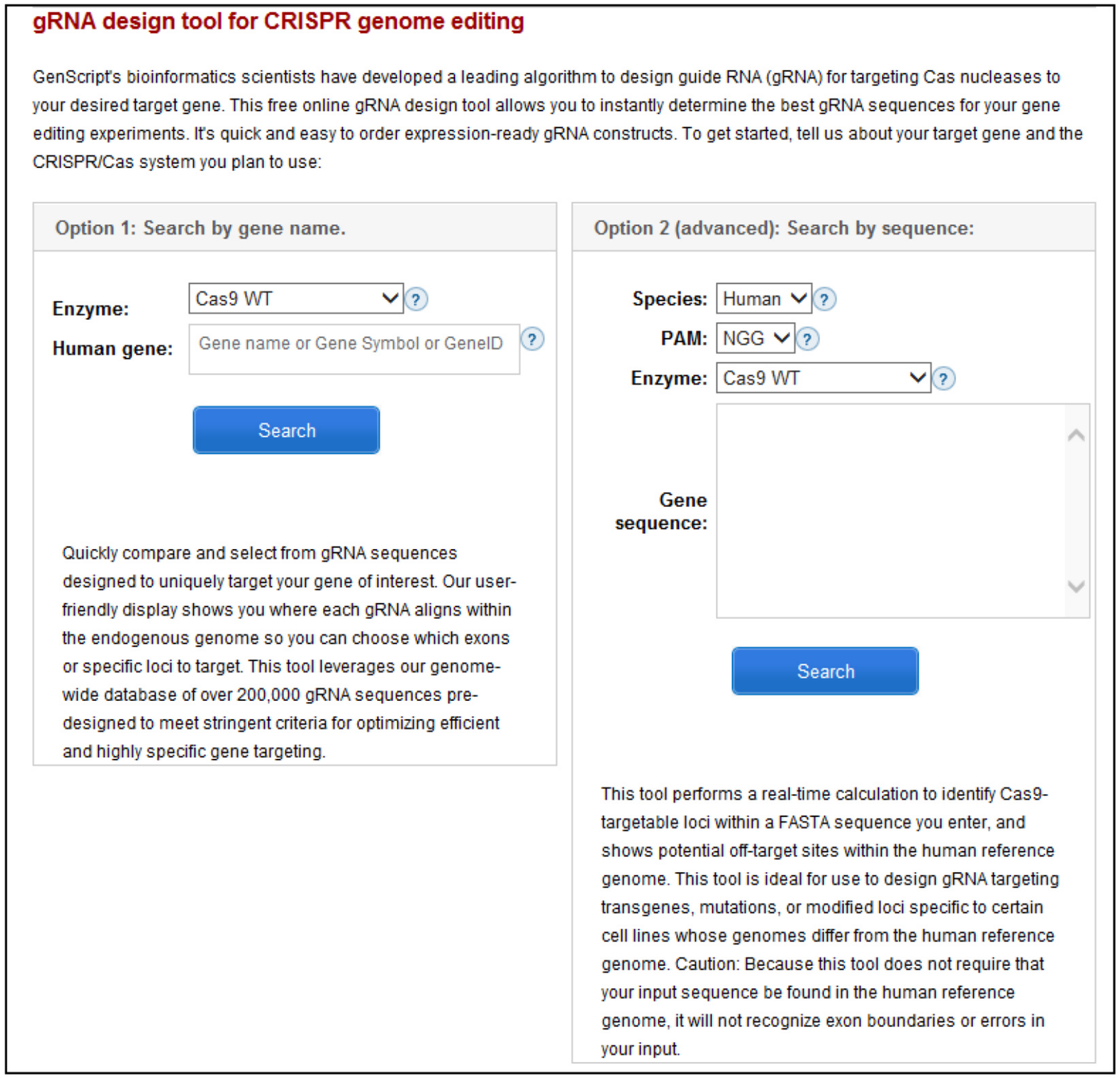
Adapted from: Tsai SQ et al**. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing**. *Nat. Biotech.* (2014). 32:569-575

Paired gRNA Design for Cas9n and FokI-dCas9

- Similar principle as gRNA design for wild-type Cas9
- Two gRNAs are needed to target each strand of the target gene respectively
- Off-set, the distance between two gRNAs, should be considered
  - Cas9n: -4 to 20bp, wider range -8 to 100bp
  - FokI-dCas9: 14-17bp

Ran AF. et al. **Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity**. *Cell* (2013). 154:1380-1389
Tsai SQ et al**. Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing**. *Nat. Biotech.* (2014). 32:569-575

gRNA Online Design Tool



- WT-Cas9 and Cas9n
- Multiple input options
- Human, mouse, CHO

http://www.genscript.com/gRNA-design-tool.html

gRNA Online Design Tool

TP53 CRISPR guide RNA, tumor protein p53 CRISPR guide RNA[human]

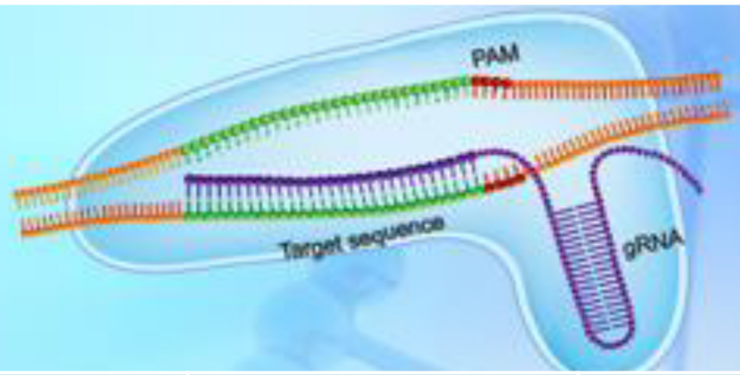
gRNA (name)	Exon	gRNA sequence	On-target sequence	Highest Risk Score	Potential off-target site with highest risk score	Price (per gRNA sequence)	Select
TP53 CRISPR guide RNA 1	1	CCACCGTC CAGGGAGC AGGTAGC	NC_000017.11 7687475..7687453 GCTACCTGCTCCCTGGACGGTGG ||||||||||||||||||||||| CCACCGTCCAGGGAGCAGGTAGC	0	not found	$199.00	☐
TP53 CRISPR guide RNA 4	1	GGACACTTT GCGTTCGG GCTGGG	NC_000017.11 7687439..7687417 GGACACTTTGCGTTCGGGCTGGG ||||||||||||||||||||||| GGACACTTTGCGTTCGGGCTGGG	0	not found	$199.00	☐
TP53 CRISPR guide RNA 5	10	GCATGGGC GGCATGAAC CGGAGG	NC_000017.11 7674238..7674216 GCATGGGCGGCATGAACCGGAGG ||||||||||||||||||||||| GCATGGGCGGCATGAACCGGAGG	0	NC_000003.11 168368424..168368446 GCATGGGCGGCATGAACCGGAGG |||||| |||||||||| | TGATGGGCAGCATGAACCGGATG	$199.00	☐
TP53 CRISPR guide RNA 6	6	GGGCAGCT ACGGTTTCC GTCTGG	NC_000017.11 7676057..7676035 GGGCAGCTACGGTTTCCGTCTGG ||||||||||||||||||||||| GGGCAGCTACGGTTTCCGTCTGG	47	NC_000005.9 179022511..179022533 GGGCAGCTACGGTTTCCGTCTGG |||||||||||||||| | ||| GGGCAGCTACGGTTTCTTTTTGG	$199.00	☐
TP53 CRISPR guide RNA 7	6	GGCAGCTA CGGTTTCC GTCTGGG	NC_000017.11 7676056..7676034 GGCAGCTACGGTTTCCGTCTGGG ||||||||||||||||||||||| GGCAGCTACGGTTTCCGTCTGGG	53	NC_000002.11 203053552..203053574 GGCAGCTACGGTTTCCGTCTGGG |||||||||||| |||| || CCAAGCTACGGTTTCTGTCTTGG	$199.00	☐

- Off-target risk scores (0- 58) by analyzing genome-wide sequence similarity
- Risk score <49 is recommended.

GenCRISPR™ gRNA Construct Service

GenCRISPR™ gRNA constructs

- free gRNA design
- ready-to-use plasmids for **$199**



	Service Steps	Deliverables	Timeline	Price
GenCRISPR gRNA construct service	• gRNA design • gRNA synthesis • Cloning into vector • Validation by PCR and enzyme digestion • Validation by sequencing	• 4ug plasmid for each gRNA construct • Final report with QC data	10 business days	$199/construct

- Full service covers both design and construction
- $199/construct, lowest price on the market
- Free standard gRNA vector and complementary human codon-optimized Cas9 expression vector
- All-in-one vectors are available now!

GenCRISPR™ Cell Line Service

Service details

GenCRISPR™ Cell line Service	Deliverables	Timeline	Pricing
Knock-out cell line, single clone (SC1652) Customer specifies gene/locus region and cell line of interest*	• Single clone, target sequence validated • Detailed report with gRNA targeting sequence design and sequencing data of target region	**9-10 weeks**, depending on complexity of target and cell line growth	**$8000**
Knock-in cell line, single clone (SC1663) Customer specifies target gene/locus and provides insert sequence or mutation on target Customer also specifies cell line of interest*	• Single clone, target sequence and knock-in gene sequence validated • Detailed report with gRNA targeting sequence design, and knock-in homology arm sequence	**12 weeks**, depending on complexity of target and insertion sequence, and cell line growth	**$9000**

Summary

- CRISPR-Cas9 is an efficient and easy to implement system for genome editing.

- Cas9 can tolerate 1-3 mismatches and generate off-target mutations.

- gRNA design steps: Target gene analysis, find canonical gRNA sequences, off-target analysis, location analysis, and gRNA delivery.

Summary

- Modified Cas9 nucleases, including Cas9n and FokI-dCas9, improve targeting specificity. Paired gRNAs are needed.

- GenScript offers free gRNA online design tool, featuring off-target risk ranking.

- GenScript offers GenCRISPR™: a complete genome editing solution including gRNA design and construction, and custom cell line development.

Q&A

Thank you for your participation

We wish you all success in your research

Email me: Heidi.Huang@GenScript.com

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Identify the optimal protein purification strategy for your recombinant protein production

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Anti-idiotypic antibodies – A powerful tool for antibody drug studies