sgRNA Design Workshop

Elizabeth Heyes

February 18, 2020

Assiut University

University of Veterinary Medicine, Vienna (Vetmeduni Vienna)

Overview

- Introduction to CRISPR/Cas9 system
- gRNA design
  - Important considerations
  - Available online tools
- Delivery options - Lentiviral vectors versus RNPs
- Determining CRISPR efficiency – genotyping cell pools (TIDE)
- Practical example

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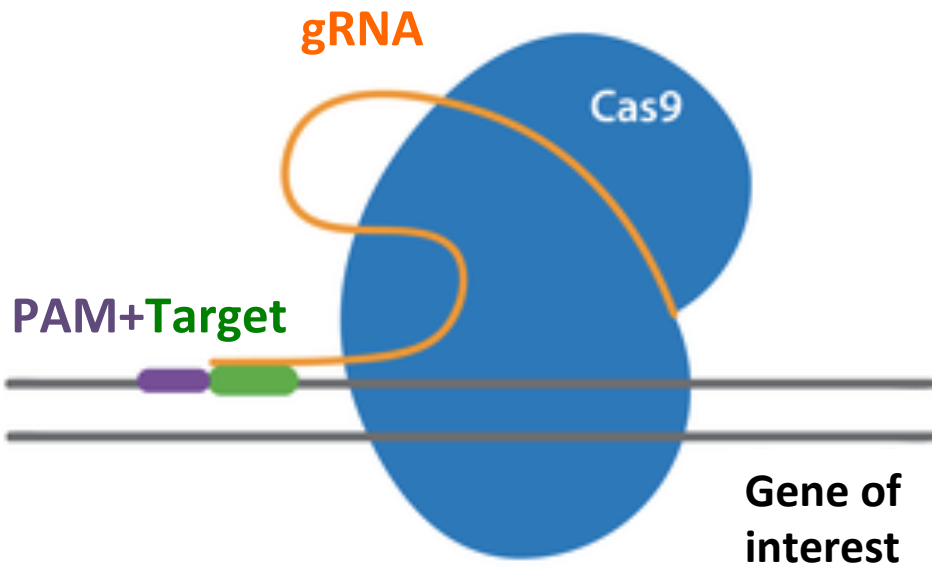
Introduction to CRISPR/Cas9



Components:

- CRISPR-associated endonuclease (Cas protein)
- gRNA

(= scaffold + 20 bp spacer)

https://www.addgene.org/guides/crispr/

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Generating a Knock-Out with CRISPR/Cas9

- Co-express Cas9 with specific gRNA

- gRNA must meet two requirements
    1. Unique sequence
    2. Target sequence is immediately adjacent to a Protospacer Adjacent Motif (PAM)

- PAM sequence
    - Binding site for Cas protein
    - Specific for different Cas proteins
    - 3'-NGG for S.pyogenes Cas9 (SpCas9)

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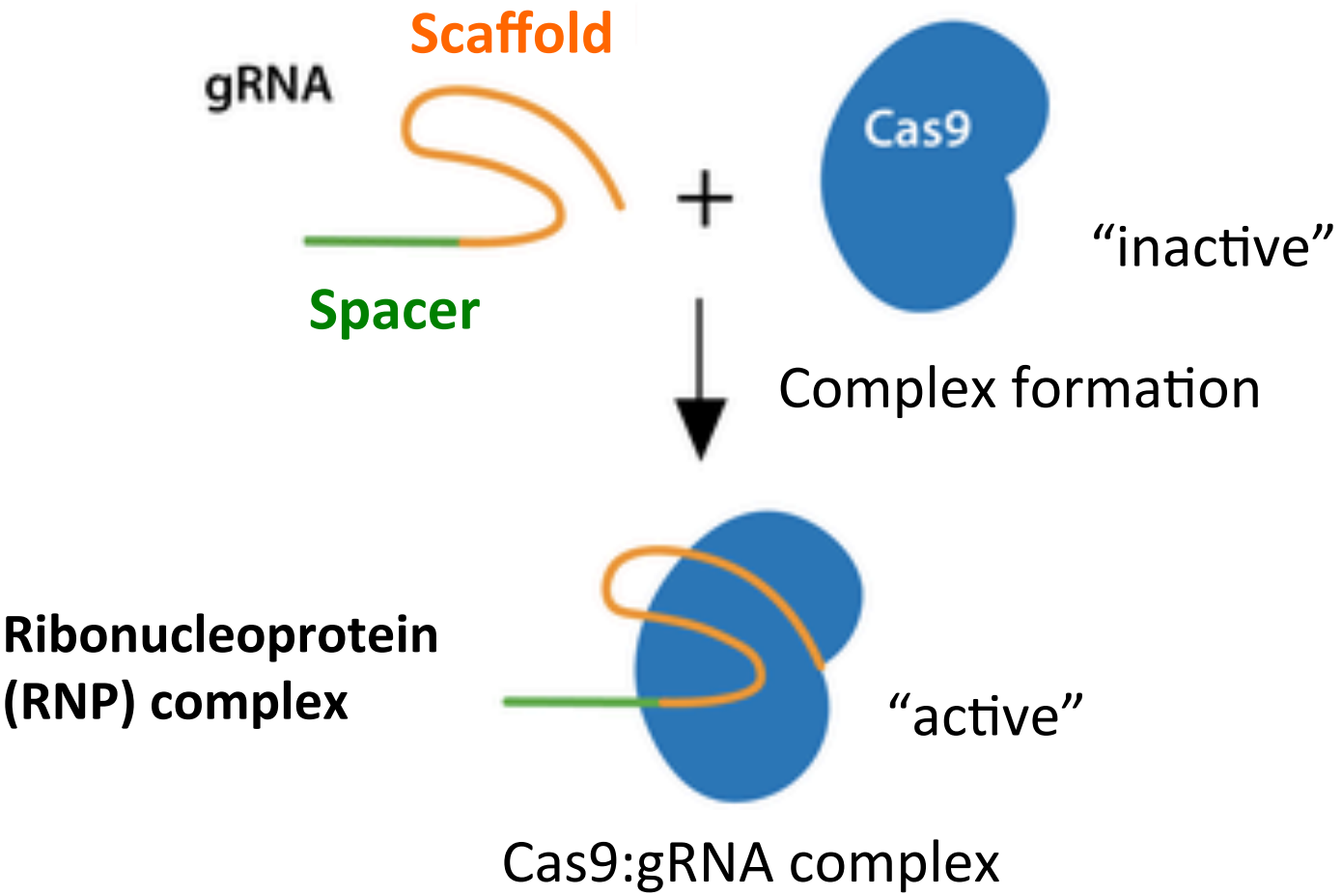
Generating a Knock-Out with CRISPR/Cas9



https://www.addgene.org/guides/crispr/

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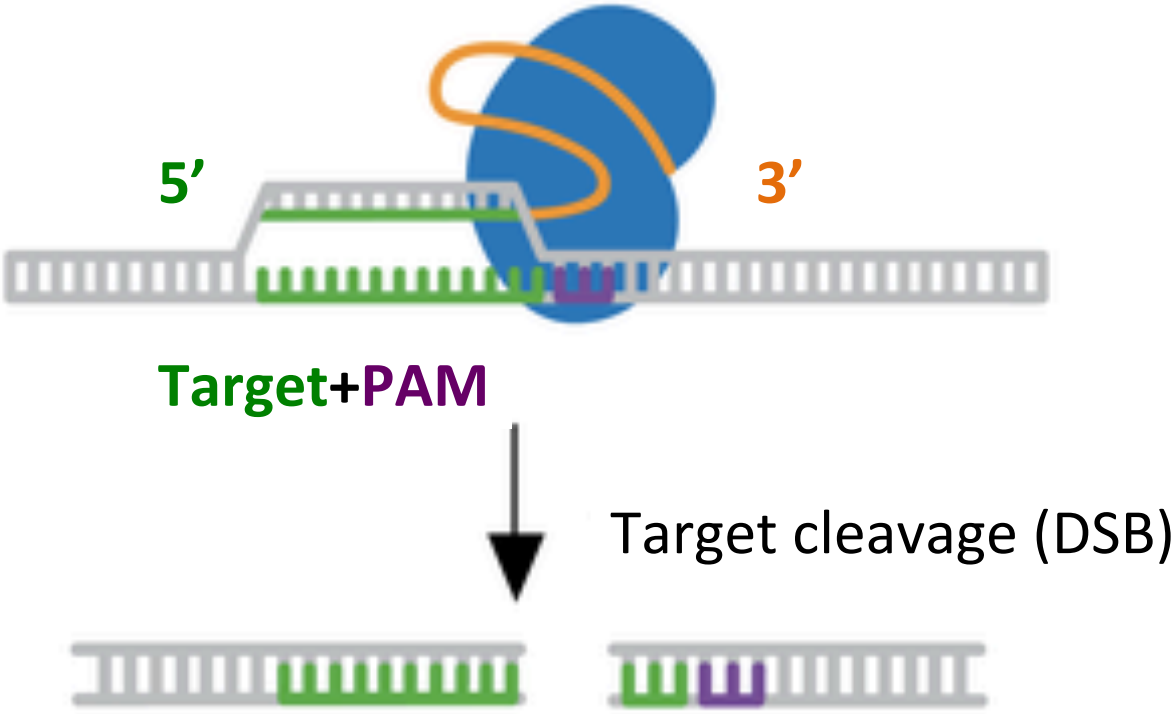
Generating a Knock-Out with CRISPR/Cas9



https://www.addgene.org/guides/crispr/

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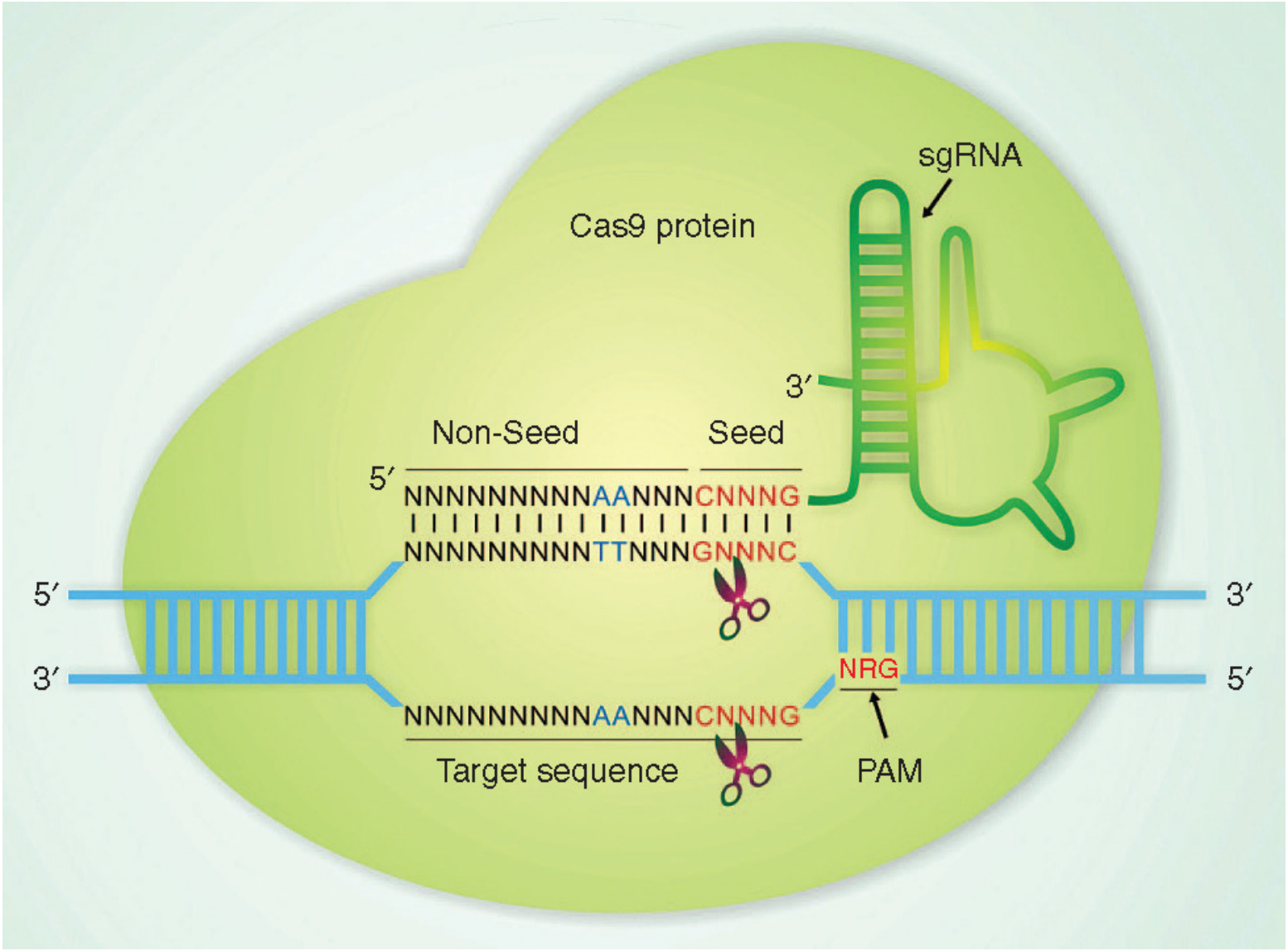
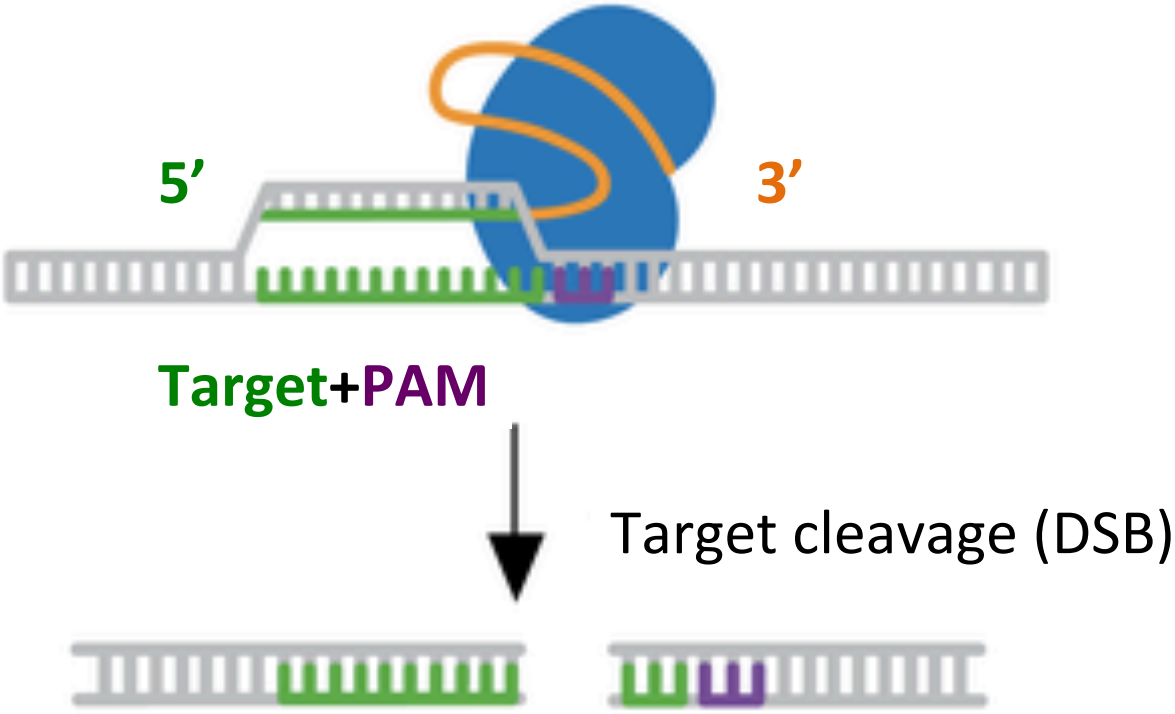


Zhang et al., 2015

Generating a Knock-Out with CRISPR/Cas9



https://www.addgene.org/guides/crispr/

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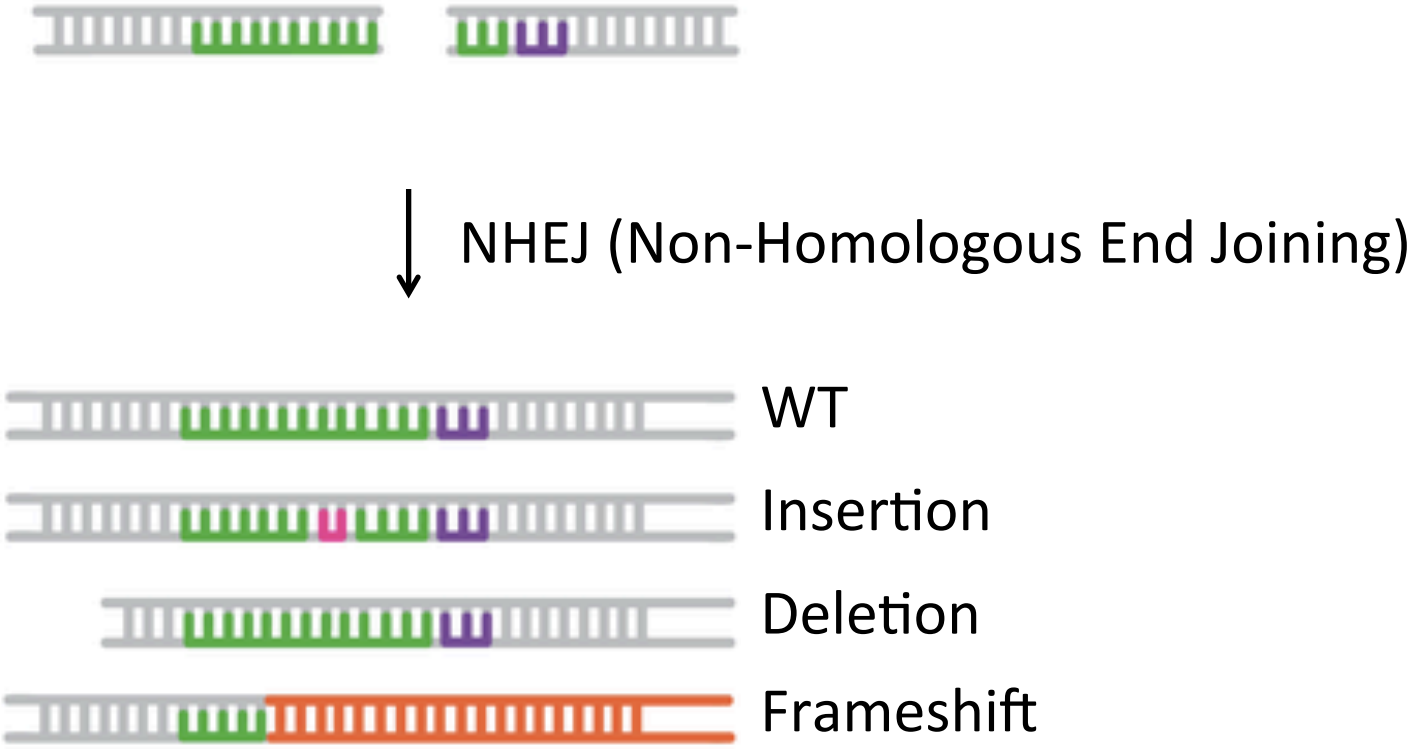
Generating a Knock-Out with CRISPR/Cas9



https://www.addgene.org/guides/crispr/

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gRNA design – Important considerations

- gRNA must target a specific, unique sequence.
- The number of off-target sites with mismatches must be kept to a minimum.

Bioinformatic tools for gRNA design
(with filters / 'scores' to predict effectiveness)

- The gRNA efficiencies for a single target vary dramatically (weak/strong secondary structures, chromatin accessibility, …)
- CRISPR-KO, CRISPRi or CRISPRa require different positions of gRNA.

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gRNA design – Available tools

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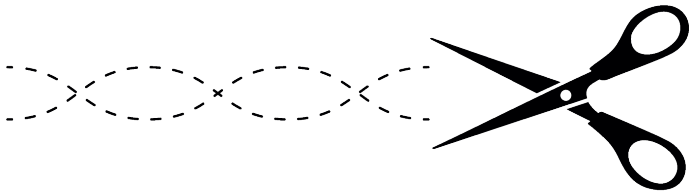

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UCSC Genome Browser

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- https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
- Distinguishes between CRISPR-KO, CRISPRi and CRISPRa
- Multiple genes as input
- Clear and detailed output, picks top gRNAs
- Fast and easy

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GPP Web Portal

Home | Search by Gene | Search by Clone

GPP sgRNA Designer

CRISPRko | CRISPRa | CRISPRi

CRISPR Enzyme: SpyoCas9 (NGG)

Target Genome: Human GRCh38 (NCBI RefSeq v.109)

Specify Target(s):

Input Transcript IDs, Gene IDs/Symbols, or raw DNA sequence:

GATA2

Enter up to 200 Transcript IDs (e.g., NM_014911.3, ENST00000456328, etc.), Gene IDs or Symbols (e.g., 988, CDC5L, ENSG00000223972, etc.), *or* a **single** DNA sequence.

File inputs must be smaller than 20kb in size, and any sequences submitted via file *must* be in FASTA format.

Please refer to our sgRNA Designer Help Page for details on how a transcript is chosen for a gene input.

-OR-

Upload a list of Transcript IDs, Gene IDs/Symbols, or a FASTA file of DNA sequences:

Choose File | No file chosen

Pick Quota: 5

Report Unpicked Sequences?

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https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

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GPP Web Portal

Home | Search by Gene | Search by Clone

GPP sgRNA Designer

CRISPRko | CRISPRa | CRISPRi

CRISPR Enzyme: SpyoCas9 (NGG)

Target Genome: Human GRCh38 (NCBI RefSeq v.109)

Specify Target(s):

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NM_032638

Enter up to 200 Transcript IDs (e.g., NM_014911.3, ENST00000456328, etc.), Gene IDs or Symbols (e.g., 988, CDC5L, ENSG00000223972, etc.), *or* a **single** DNA sequence.

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GPP Web Portal

Home | Search by Gene | Search by Clone

GPP sgRNA Designer

CRISPRko | CRISPRa | CRISPRi

CRISPR Enzyme: SpyoCas9 (NGG)

Target Genome: Human GRCh38 (NCBI RefSeq v.109)

Specify Target(s):

Input Transcript IDs, Gene IDs/Symbols, or raw DNA sequence:

TTGTGCAGCTTGTAGTAGAGGCCACAGGCGTTGCAGAC
AGGGTCCCCGTTGGCGTTTCGGCGCCATAAGGTGGTGG
TTGTCGTCTGACAATTTGCACAACAGGTGCCGGCTCTTC
TGGCGGCCGA

Enter up to 200 Transcript IDs (e.g., NM_014911.3, ENST00000456328, etc.), Gene IDs or Symbols (e.g., 988, CDC5L, ENSG00000223972, etc.), *or* a **single** DNA sequence.

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X I'm not a robot

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https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

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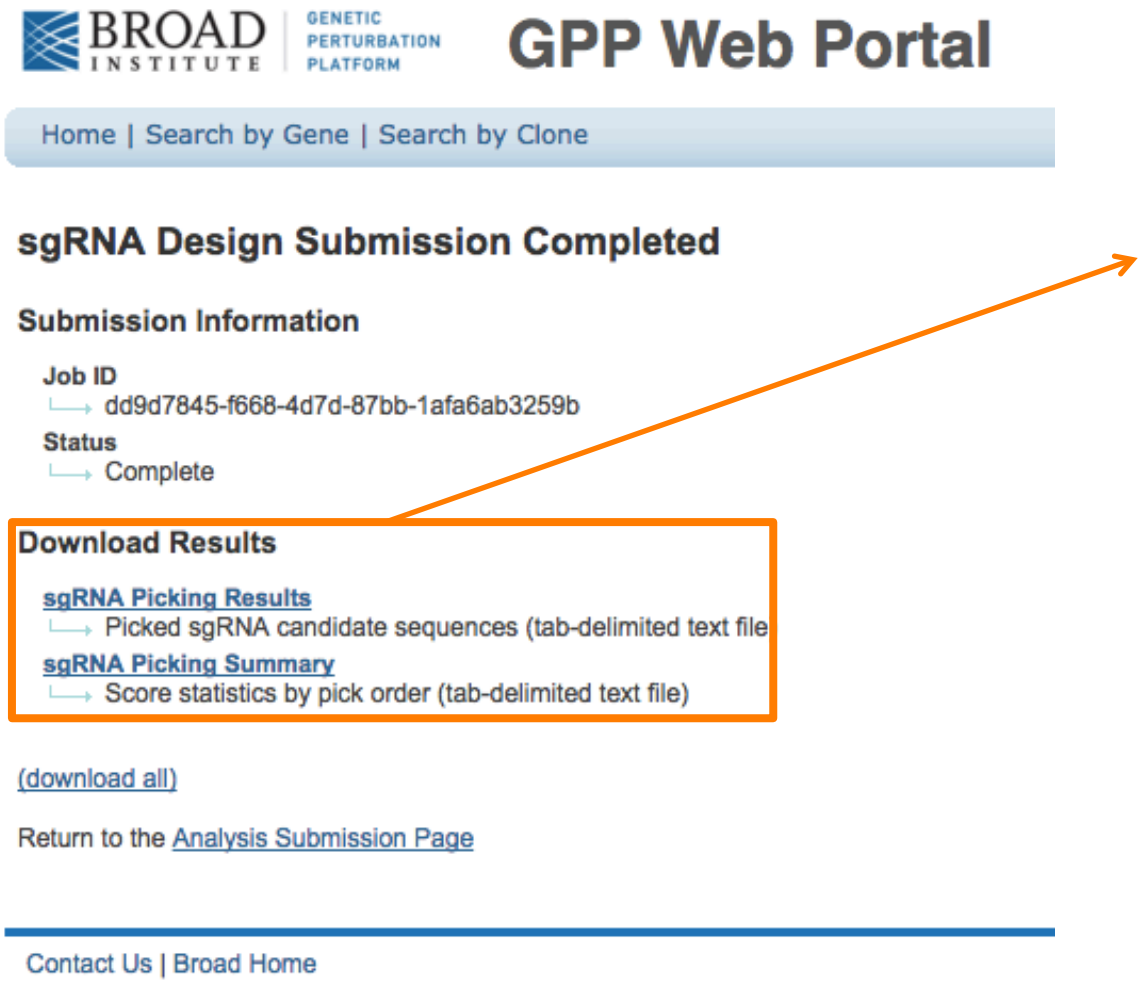
Broad Institute GPP - Output



txt-file:
sgRNA Sequence
Exon number
On- and off-Target ranks
Picking order
…

https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

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gRNA design – Available tools

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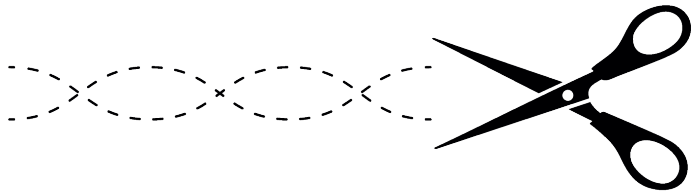
CHOPCHOP 

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UCSC Genome Browser

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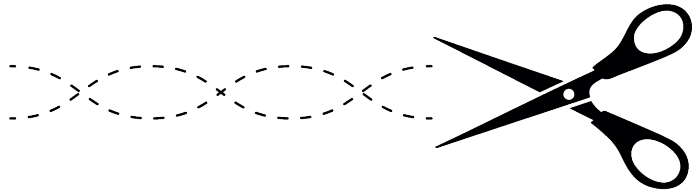

- chopchop.cbu.uib.no
- Clear overview of gRNA positions within the target gene
- Ranks gRNAs by off-targets and CRISPR efficiency
- Calculates primer sequences for genotyping of each provided gRNA
- Fast and easy
- Just one gene or genomic region as input

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Target

GATA2

RefSeq/ENSEMBL/gene name or genomic coordinates.

In

Homo sapiens (hg38/GRCh38)

Add new species.

Using

CRISPR/Cas9

- CRISPR/Cas9
- CRISPR/Cas9 nickase
- CRISPR/Cpf1 or CasX
- CRISPR/Cas13 (eg. C2C2)
- TALEN

For

knock-out

- knock-out
- knock-in
- activation
- repression
- nanopore enrichment

Paste Target Options

Find Target Sites!

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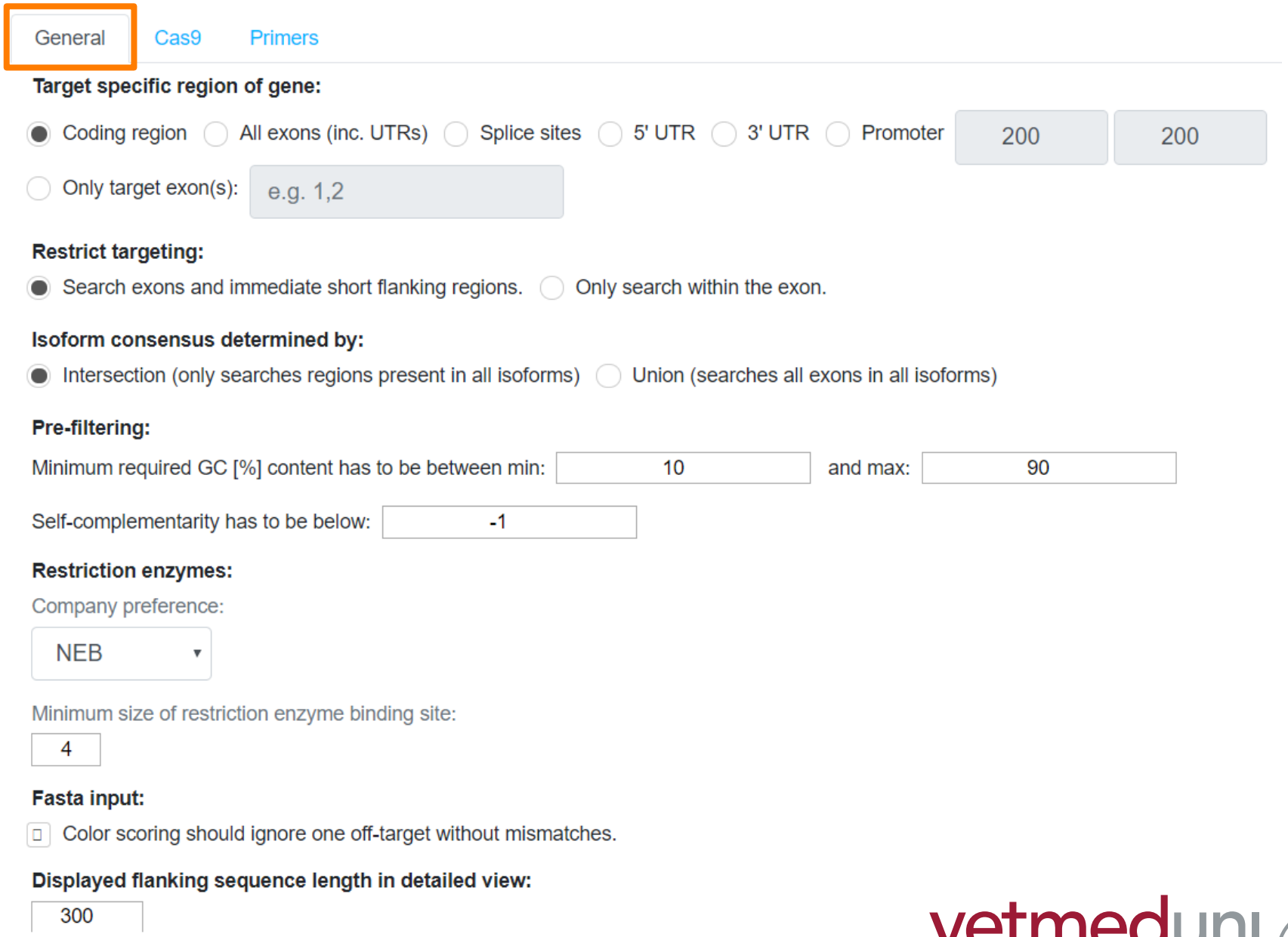
Options



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Options

General | Cas9 | Primers

sgRNA length without PAM:

20

PAM-3':

(•) NGG ( ) NAG ( ) NGA ( ) NRG (R = A or G) ( ) NNAGAAW (W = A or T) ( ) NNNNGMTT (M = A or C) ( ) NNGRRT (R = A or G)

( ) Custom PAM: e.g. NGAG

Method for determining off-targets in the genome:

(•) Off-targets with up to 3 mismatches in protospacer (Hsu et al., 2013)

( ) Off-targets may have no more than 0 mismatches in the protospacer seed region (Cong et al., 2013)

Efficiency score:

( ) Doench et al. 2014 - only for NGG PAM

(•) Doench et al. 2016 - only for NGG PAM

( ) Chari et al. 2015 - only NGG and NNAGAAW PAM's in hg19 and mm10

( ) Xu et al. 2015 - only for NGG PAM, but can be used with other PAMs

( ) Moreno-Mateos et al. 2015 - only for NGG PAM

( ) G20

...

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Options

General | Cas9 | Primers

Design primers

Product size:

From: 150 to: 290

Primer size:

From: 18 to: 25 Optimal: 22

Primer Tm:

From: 57 to: 63 Optimal: 60

Minimum distance from primer to target site:

20

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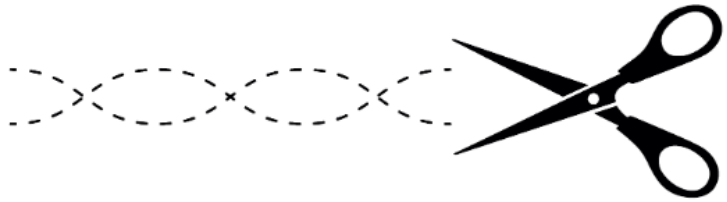

Target

GATA2

RefSeq/ENSEMBL/gene name or genomic coordinates.

In

Homo sapiens (hg38/GRCh38)

Add new species.

Using

CRISPR/Cas9

Change default PAM and guide length in Options.

For

knock-out

Presets can be adjusted in Options.

Paste Target | Options | Reset Options

Find Target Sites!

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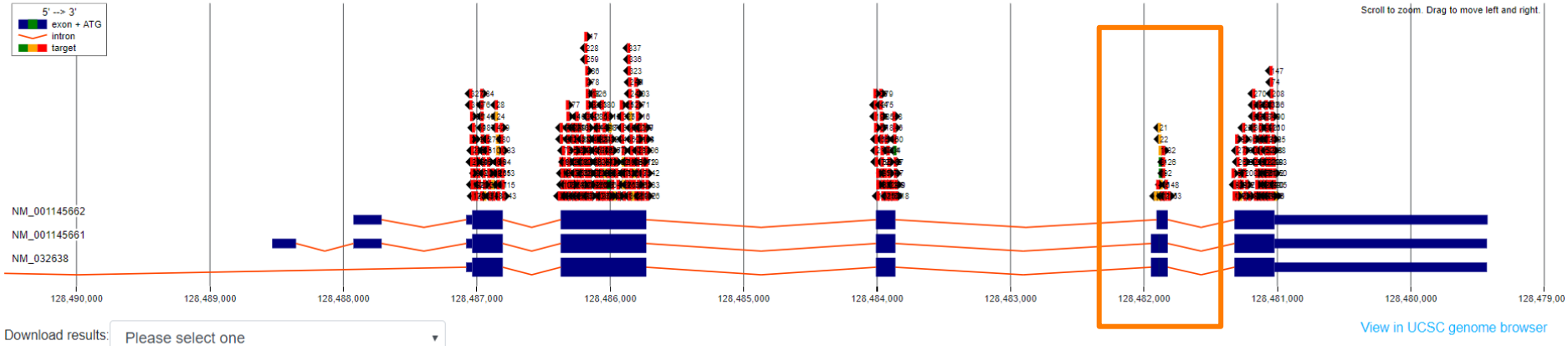
GATA2



Download results: Please select one

View in UCSC genome browser

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	ATGGCGCCGAAACGCCAACGGGG	chr3:128481863	-	65	0	0	0	0	0	67.35
2	ACTACCCCCCGCGGAAGATGAGG	chr3:128485995	+	65	1	0	0	0	0	55.51
3	TATGGCGCCGAAACGCCAACGGG	chr3:128481864	-	60	1	0	0	0	0	47.14
4	CGCTCCTACCAGTCTTCGCTTGG	chr3:128483851	+	60	0	0	0	0	0	31.05
5	CAAGGACGGCGTCAAGTACCAGG	chr3:128485955	-	60	1	0	0	0	1	55.33
6	GTTTCGGCGCCATAAGGTGGTGG	chr3:128481873	+	60	0	0	0	0	1	54.31
7	CGCCGGCACATAGGAGGGGTAGG	chr3:128485833	+	70	0	0	0	0	1	52.09
8	ATGCCAACCCCGCTCACGCGCGG	chr3:128486831	-	70	1	0	0	0	1	50.61
9	ACGCAGCCCCCGTGGTGCTAGGG	chr3:128486024	+	70	1	0	0	0	1	48.08

...

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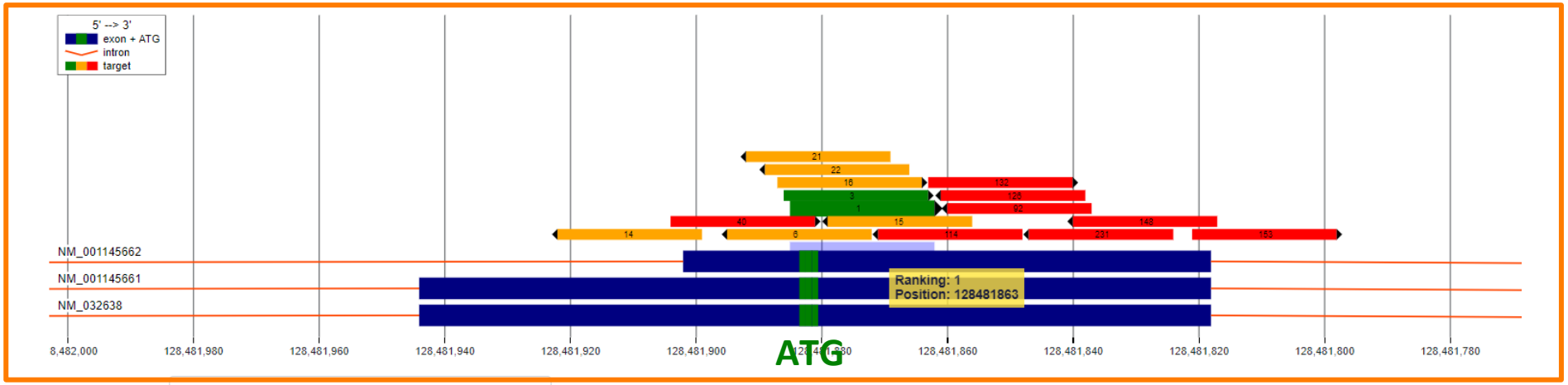
GATA2



Download results: Please select one

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2
1	ATGGCGCCGAAACGCCAACGGGG	chr3:128481863	-	65	0	0	0	0
2	ACTACCCCCCGCGGAAGATGAGG	chr3:128485995	+	65	1	0	0	0
3	TATGGCGCCGAAACGCCAACGGG	chr3:128481864	-	60	1	0	0	0
4	CGCTCCTACCAGTCTTCGCTTGG	chr3:128483851	+	60	0	0	0	0
5	CAAGGACGGCGTCAAGTACCAGG	chr3:128485955	-	60	1	0	0	0
6	GTTTCGGCGCCATAAGGTGGTGG	chr3:128481873	+	60	0	0	0	0
7	CGCCGGCACATAGGAGGGGTAGG	chr3:128485833	+	70	0	0	0	0
8	ATGCCAACCCCGCTCACGCGCGG	chr3:128486831	-	70	1	0	0	0
9	ACGCAGCCCCCGTGGTGCTAGGG	chr3:128486024	+	70	1	0	0	0
10	TGCCAACCCCGCTCACGCGCGGG	chr3:128486830	-	75	2	0	0	0

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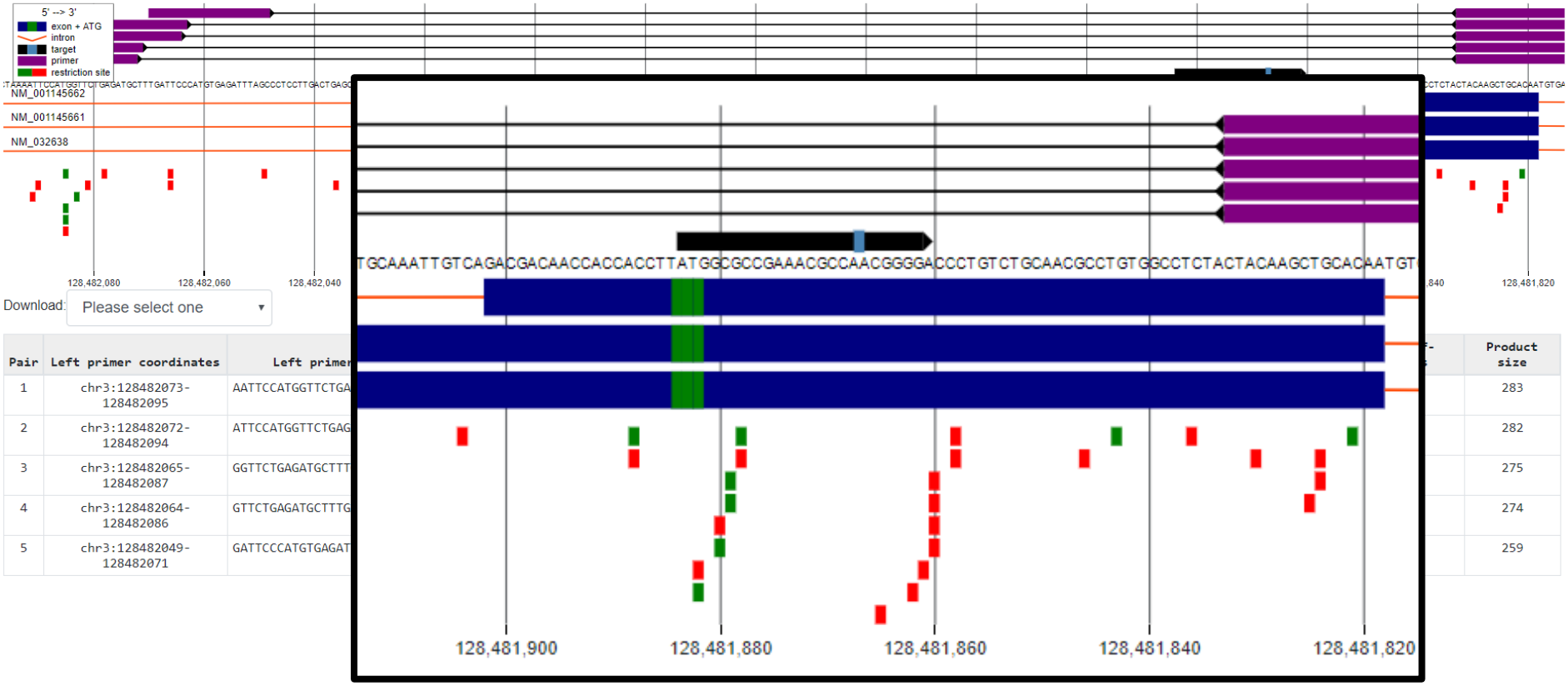
Target: **GATA2**
Rank: **1**
Target sequence: **ATGGCGCCGAAACGCCAACGGGG**



Download: Please select one

Pair	Left primer coordinates	Left primer	Product size
1	chr3:128482073-128482095	AATTCCATGGTTCTGA	283
2	chr3:128482072-128482094	ATTCCATGGTTCTGAG	282
3	chr3:128482065-128482087	GGTTCTGAGATGCTTT	275
4	chr3:128482064-128482086	GTTCTGAGATGCTTTG	274
5	chr3:128482049-128482071	GATTCCCATGTGAGAT	259

gRNA design – Available tools

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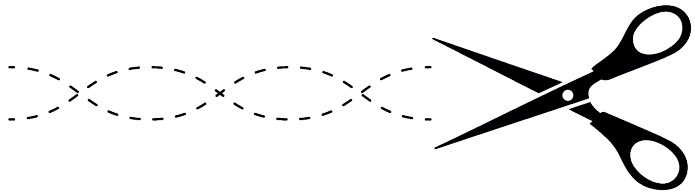
CHOPCHOP 

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Genome Browser

- https://genome.ucsc.edu/
- Broad collection of vertebrate and model organism assemblies and annotations.
- Shows DNA sequences targetable by CRISPR RNA guides using SpCas9 over the entire human genome.
- Shows the predicted specificity (off-target effects) and predicted efficiency (on-target cleavage).

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UCSC Genome Browser

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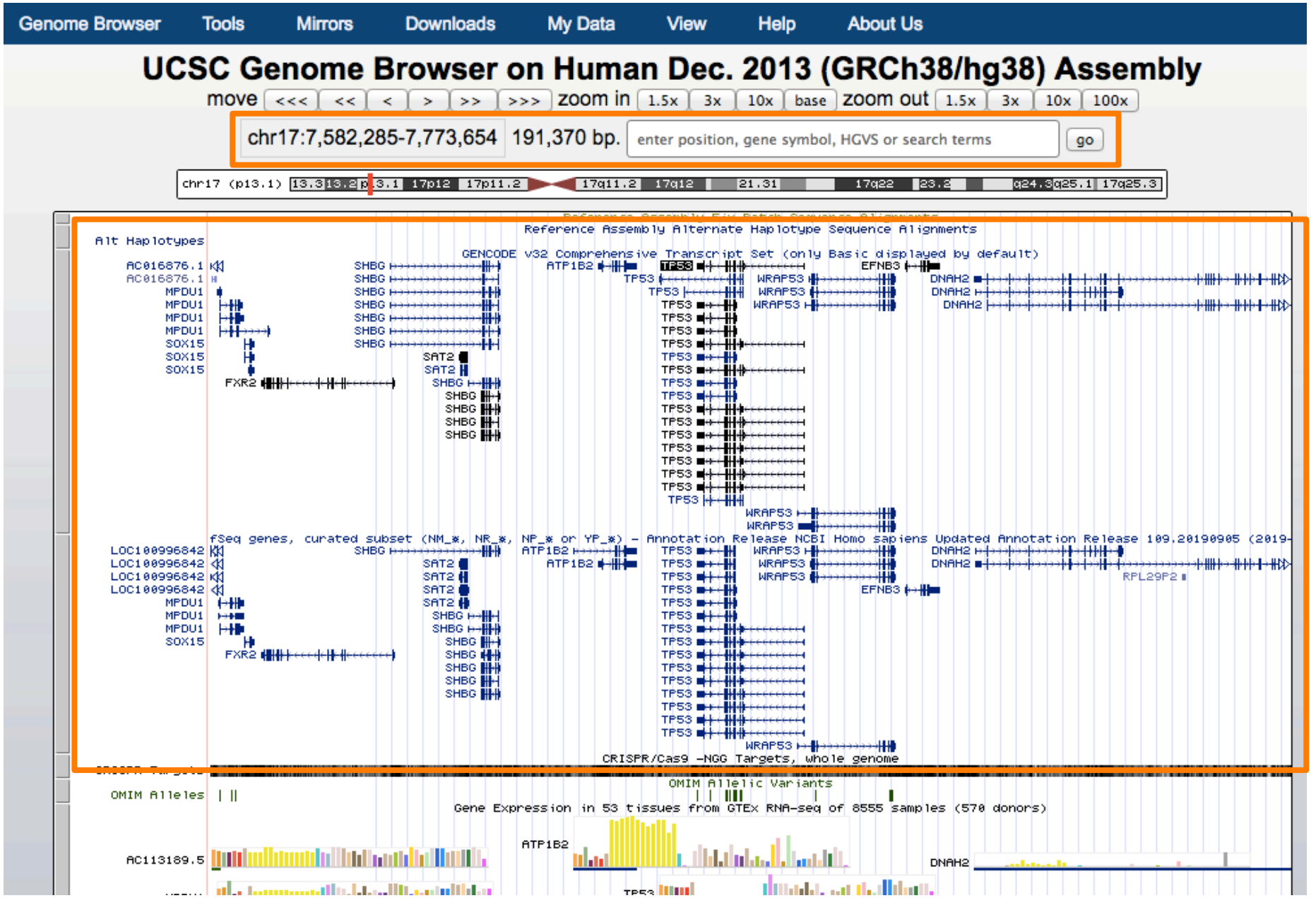
- Human GRCh38/hg38
- Human GRCh37/hg19
- Mouse GRCm38/mm10
- Mouse NCBI37/mm9
- Mouse: 16 strains
- Other

Our tools

- **Genome Browser**
  interactively visualize genomic data
- **BLAT**
  rapidly align sequences to the genome
- **Table Browser**
  download data from the Genome Browser database
- **Variant Annotation Integrator**
  get functional effect predictions for variant calls
- **Data Integrator**
  combine data sources from the Genome Browser database
- **Genome Browser in a Box (GBiB)**
  run the Genome Browser on your laptop or server
- **In-Silico PCR**
  rapidly align PCR primer pairs to the genome
- **LiftOver**
  convert genome coordinates between assemblies
- **Track Hubs**
  import and view external data tracks
- **REST API**
  returns data in JSON format

More tools...

https://genome.ucsc.edu/index.html



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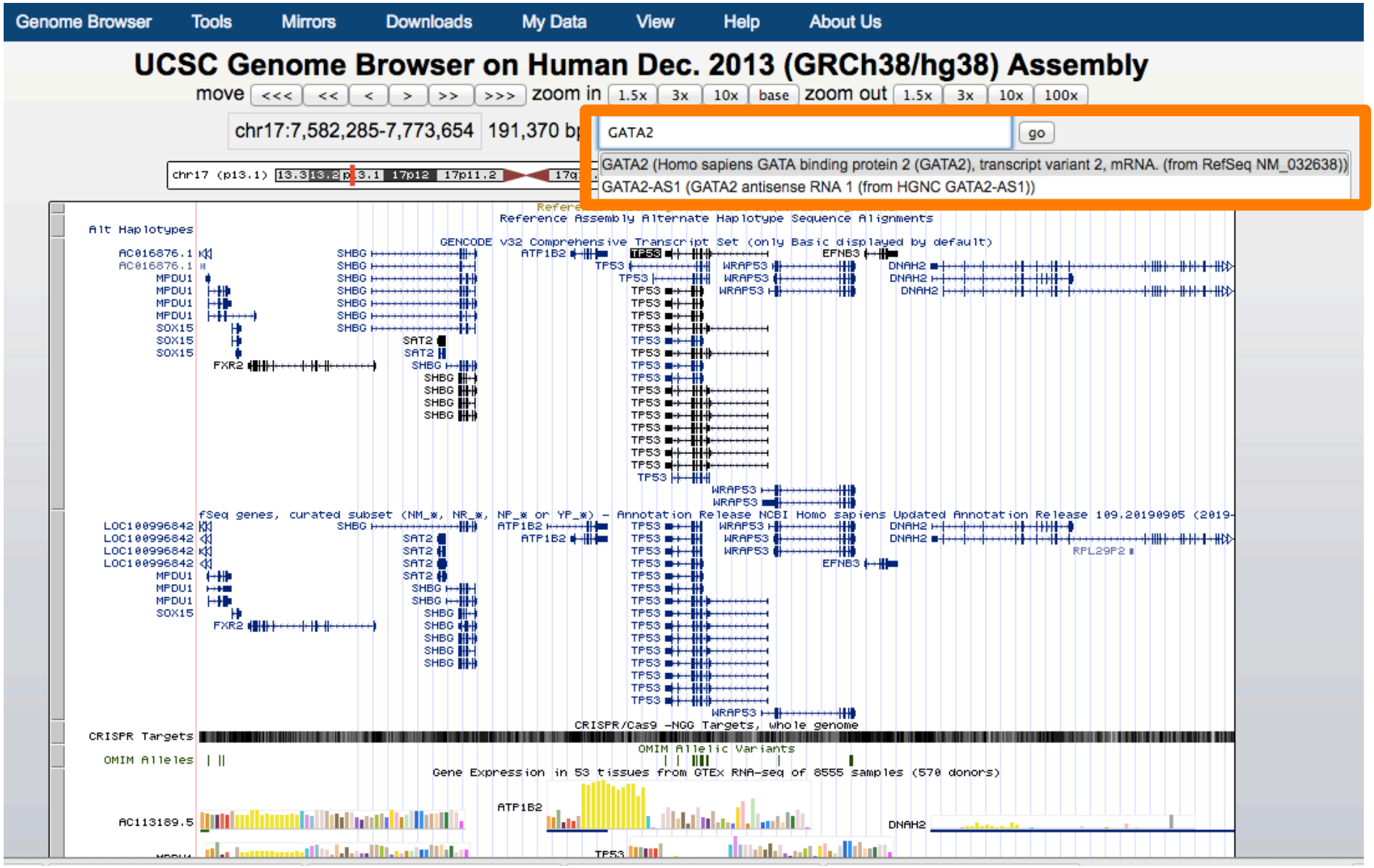

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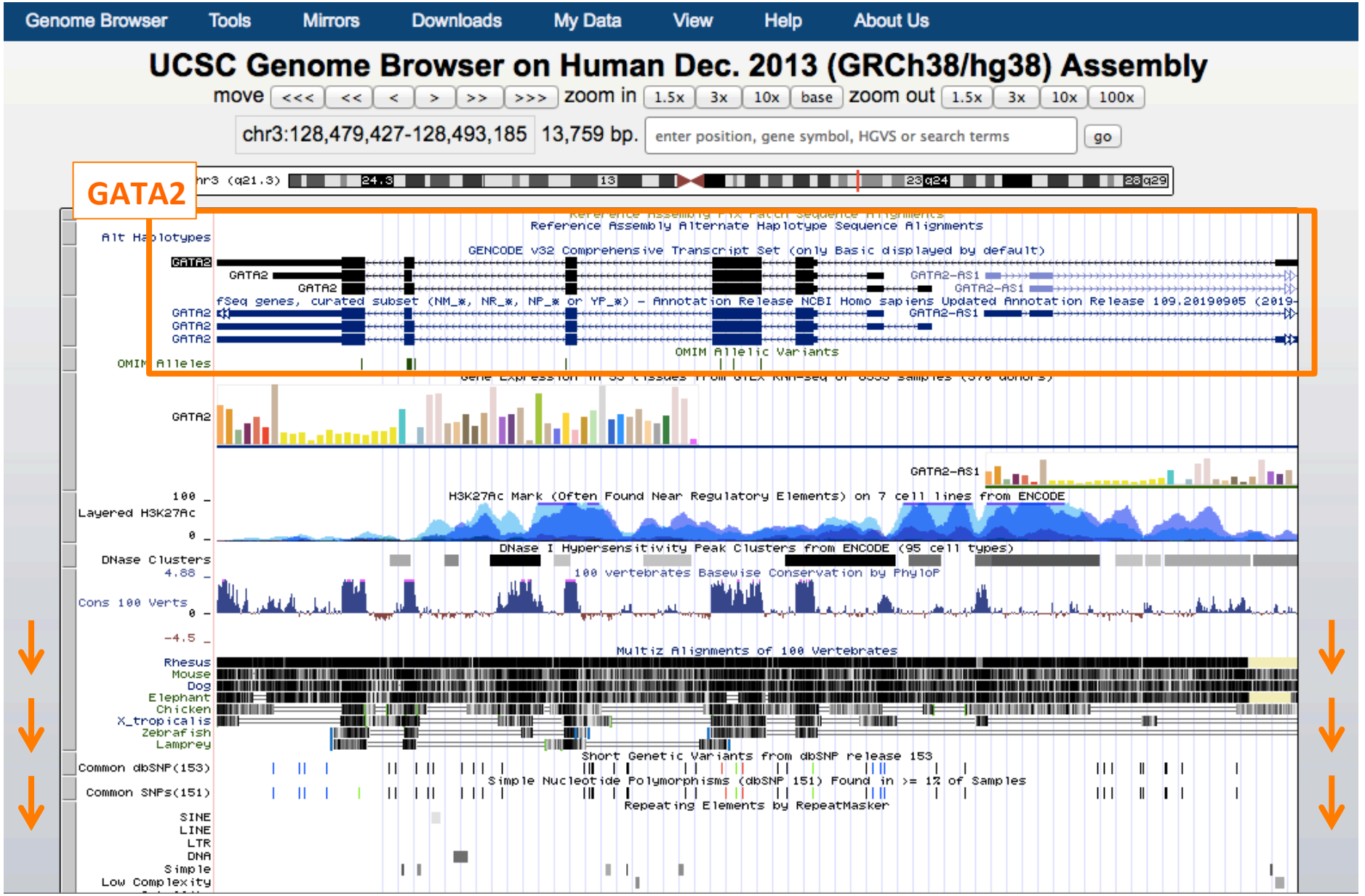

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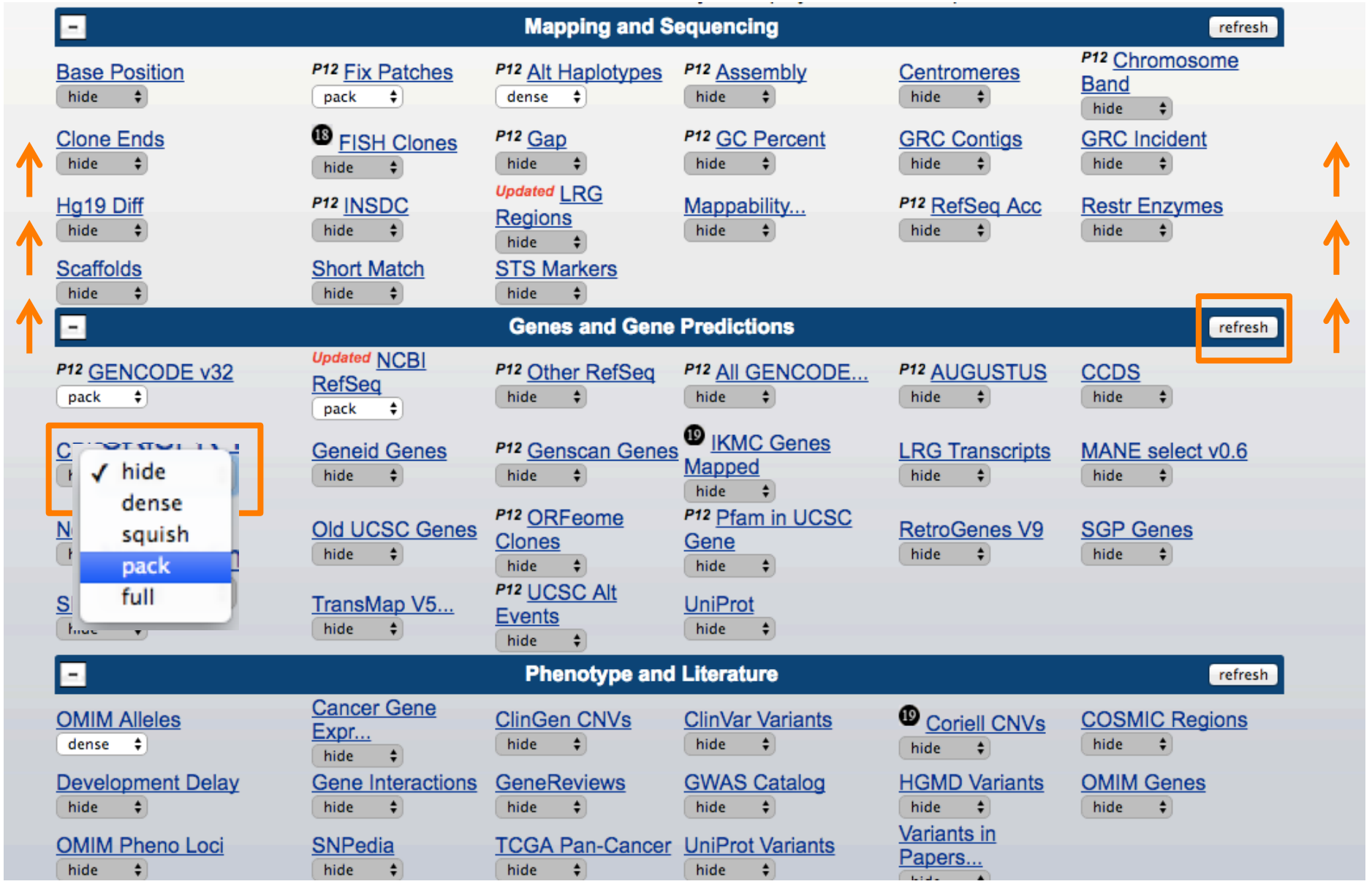

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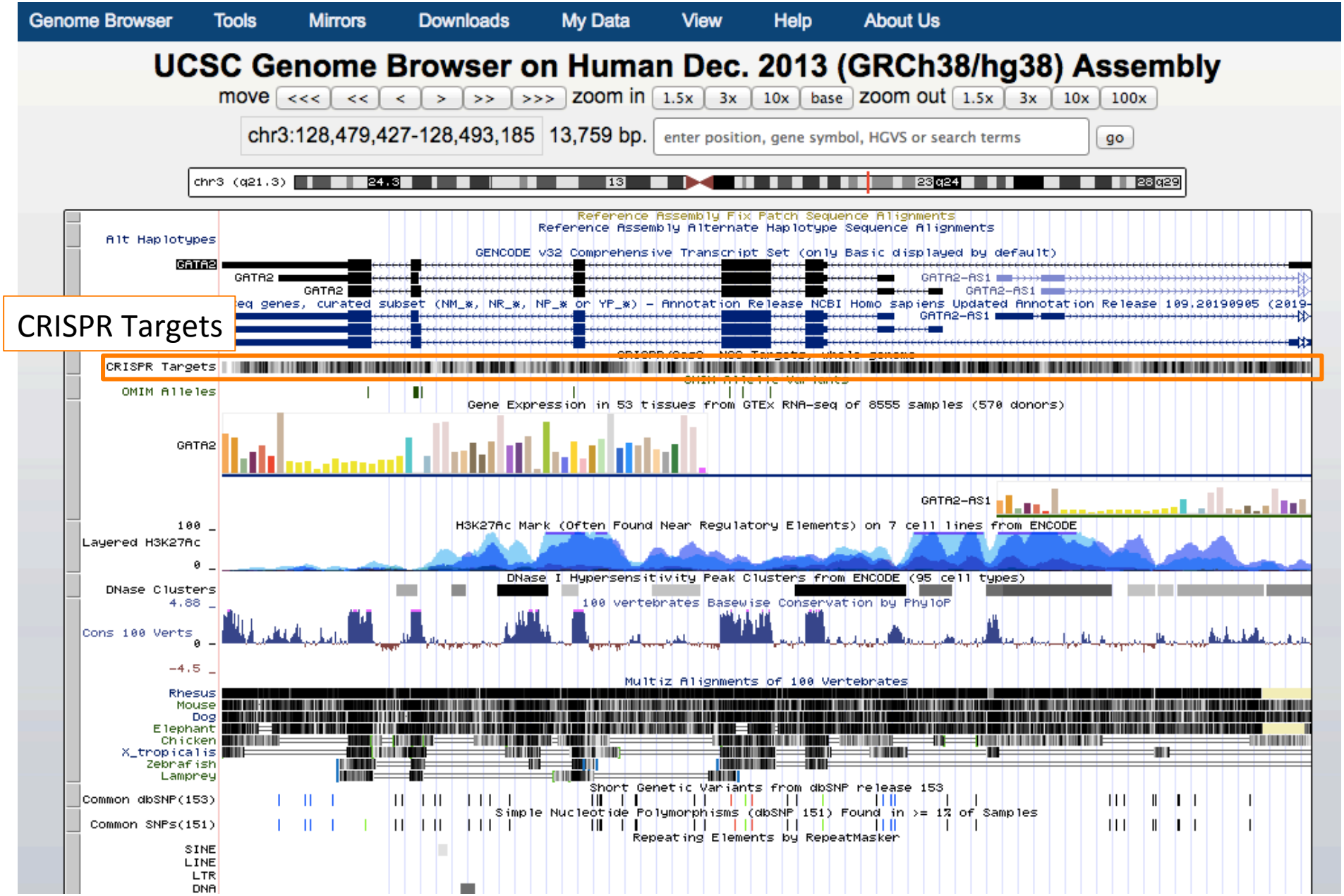
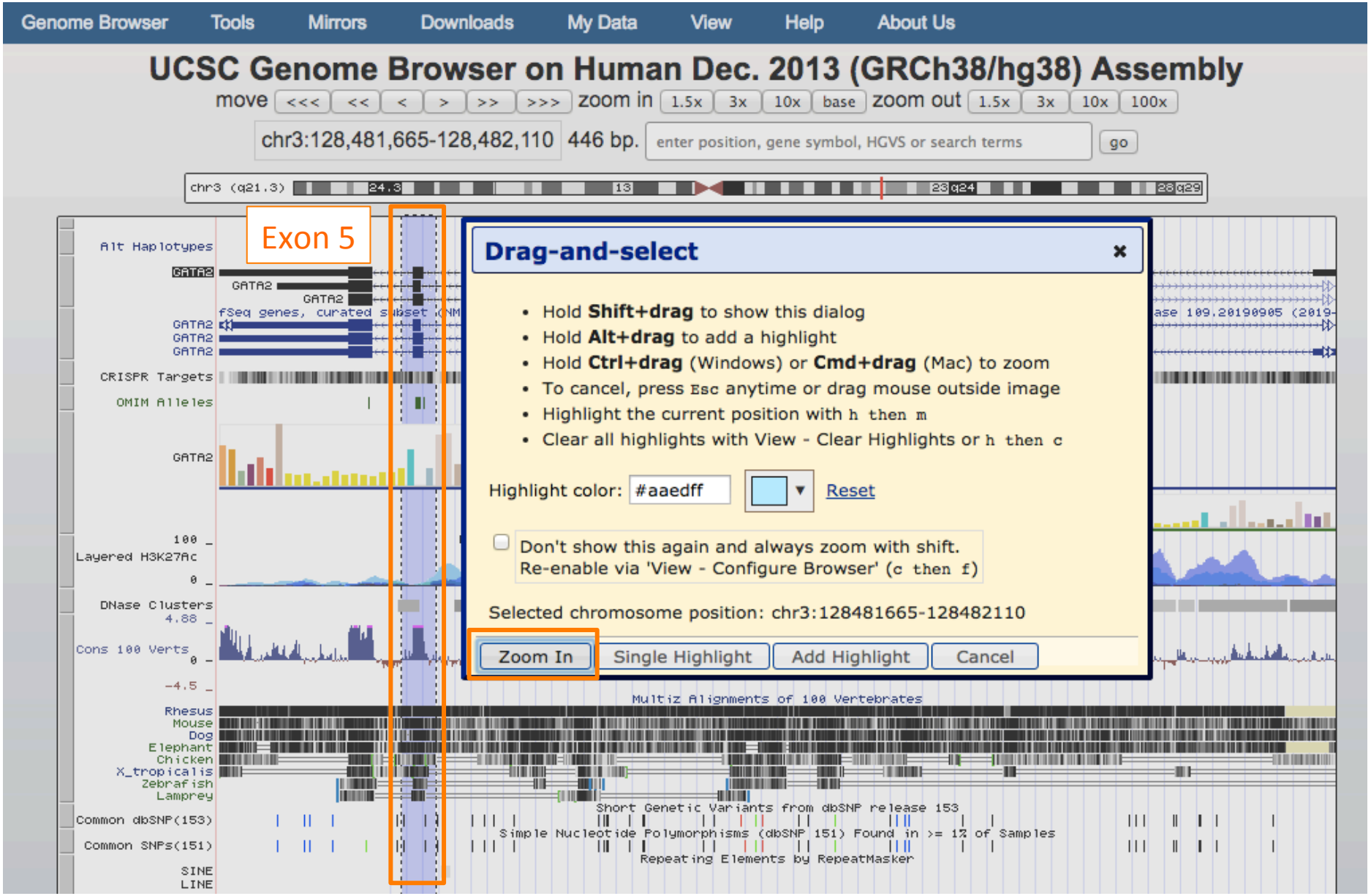


CRISPR Targets

https://genome.ucsc.edu/index.html



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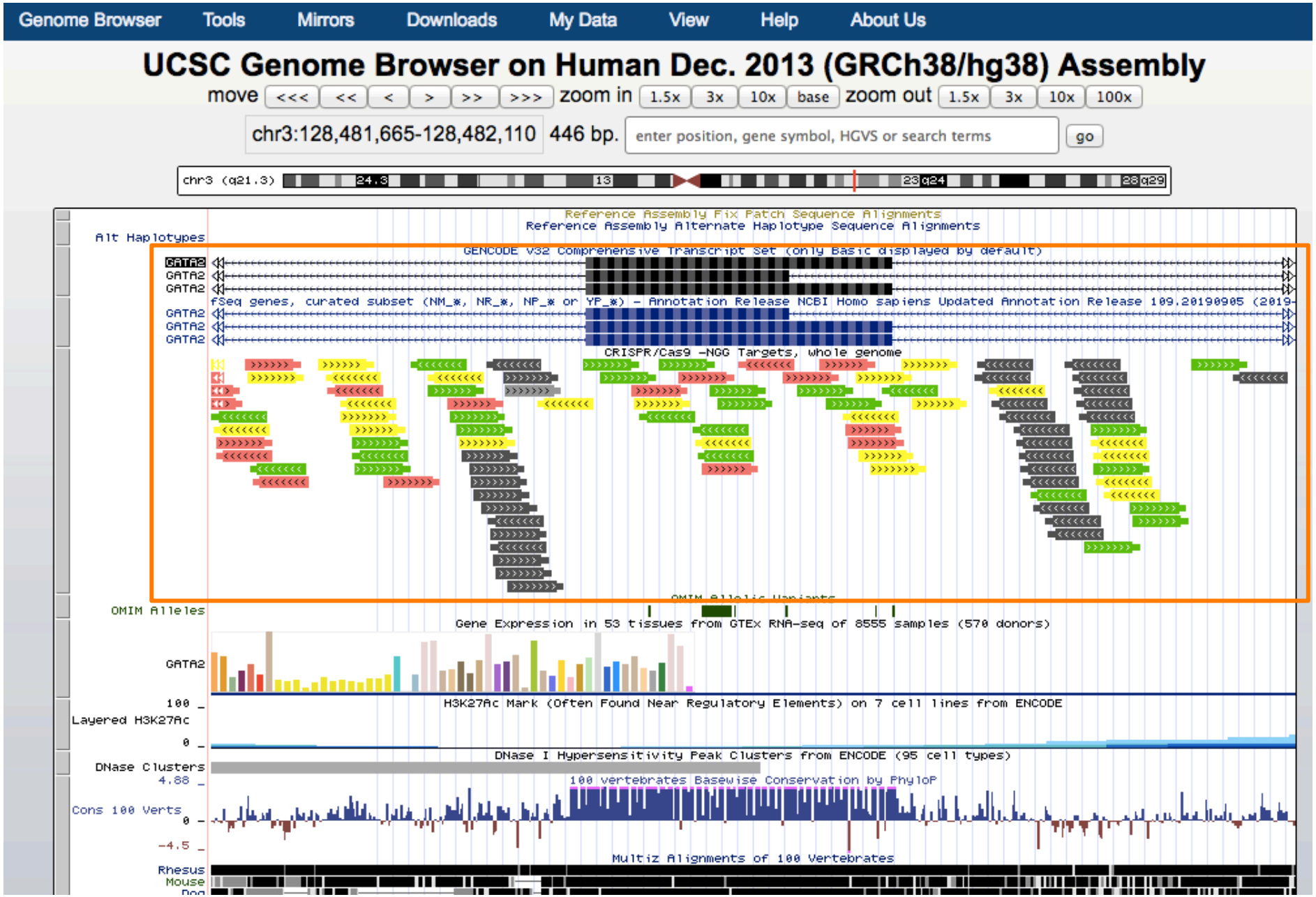

https://genome.ucsc.edu/index.html

CRISPR/Cas9 -NGG Targets, whole genome

Click here to show this guide on Crispor.org, with expression oligos, validation primers and more

MIT Guide Specificity Score: 81
Position: chr3:128481849-128481871
Band: 3q21.3
Genomic Size: 23
Strand: +
View DNA for this feature (hg38/Human)

Guide Sequence	GTTGCAGACAGGGTCCCCGT
Protospacer Adjacent Motif (PAM)	TGG
MIT Guide Specificity Score	81
Efficiency: Doench et al. 2016 Score	91% (64)
Efficiency: Moreno-Mateos (In-vitro) Score	91% (70)
Efficiency: Doench et al 2014 Score	22
Bae et al. Out-of-Frame Score	66

https://genome.ucsc.edu/index.html

Number of potential off-targets	0 mismatches: 0 off-targets	1 mismatches: 0 off-targets	2 mismatches: 1 off-targets	3 mismatches: 7 off-targets	4 mismatches: 59 off-targets

Potential Off-targets

Mismatched nucleotides	CFD Score	Locus	Position
A.....A. TGG	0.642	exon GATA3	chr10:8069546 (-)
....G.....A.A.... TGG	0.371	exon GAA	chr17:80118759 (-)
T.....G.....A.T.. GGG	0.357	exon LRP5	chr11:68403982 (-)
T..AT.....A.. GGG	0.308	exon UBP1	chr3:33391683 (-)
.C.....AC.....T. GGG	0.302	intron HMCN2	chr9:130428105 (-)
CAA.....T. GGG	0.287	intergenic RP11-401P9.7-RP11-401P9.6/NKD1	chr16:50591945 (-)
TG.....G.....T... GGG	0.287	intron DAP	chr5:10729891 (-)
CA.....G.....T.. GGG	0.284	intergenic TCERG1L-LINC01164	chr10:131708516 (-)
T....T.CA..... GGG	0.262	intergenic MIR552-SMIM12	chr1:34699977 (-)
.C..G.....A....A. TGG	0.227	intron PRELID2	chr5:145801962 (-)
C.....C..T.....A... AGG	0.202	intergenic RBMY2FP-TTTY25P	chrY:22327163 (-)

Show all 67 off-targets...

https://genome.ucsc.edu/index.html

gRNA-sequence designed – what now?

Constitutive expression of Cas9 and gRNA

Transient transfection of RNP-complex

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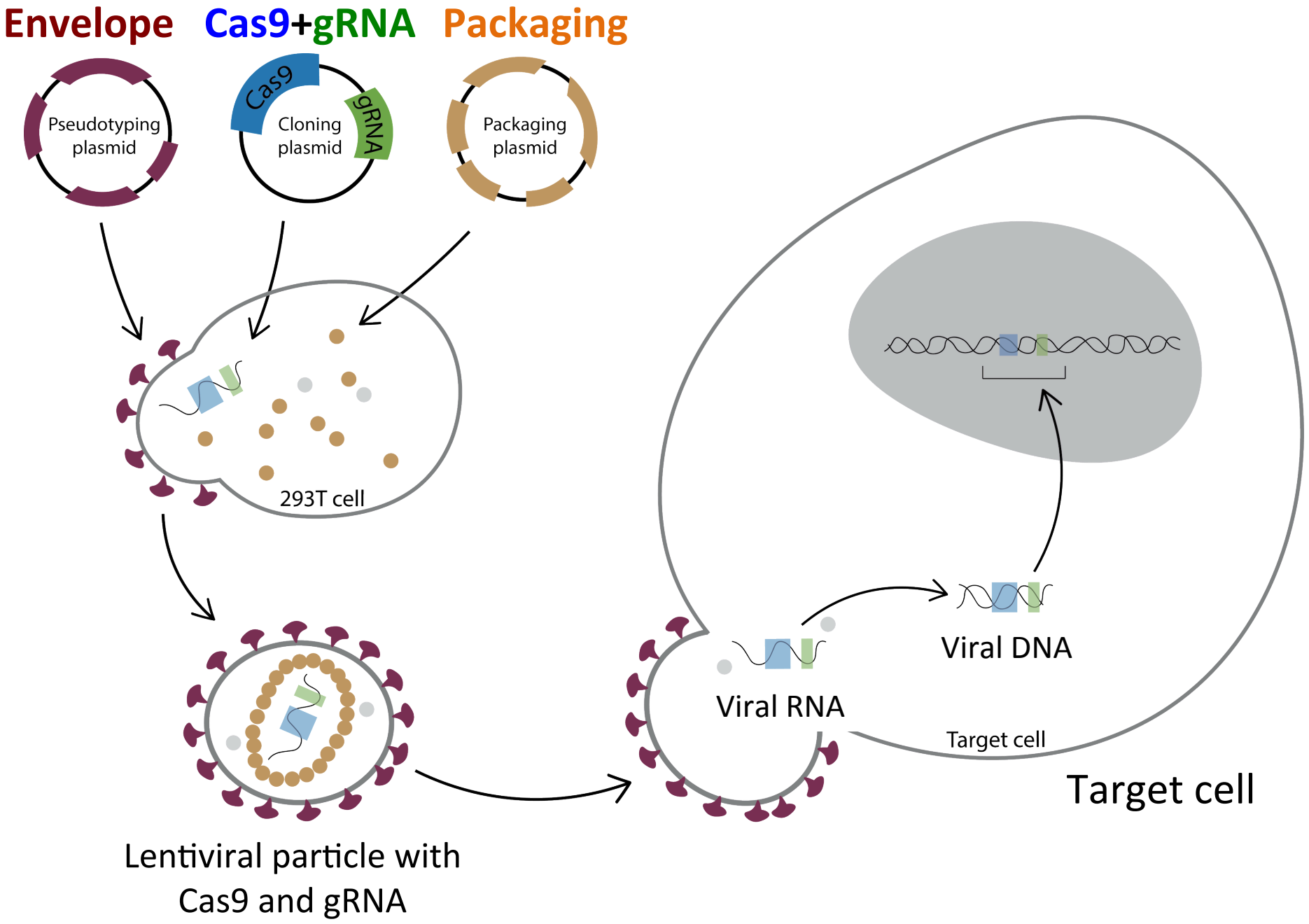
Constitutive expression of Cas9 and gRNA

- Lentiviral vectors (https://www.addgene.org)
- Cas9 and gRNA can be present in single or separate transfer vectors.
- May contain reporter gene (e.g. GFP) or selection marker (e.g. Puromycin) to identify and enrich for positive cells.
- Packaging and envelope plasmids provide the necessary components to make lentiviral particles.



https://www.benchling.com/2016/03/24/how-to-express-crispr-in-your-target-cells/

gRNA-sequence designed – what now?

Constitutive expression of Cas9 and gRNA

Transient transfection of RNP-complex

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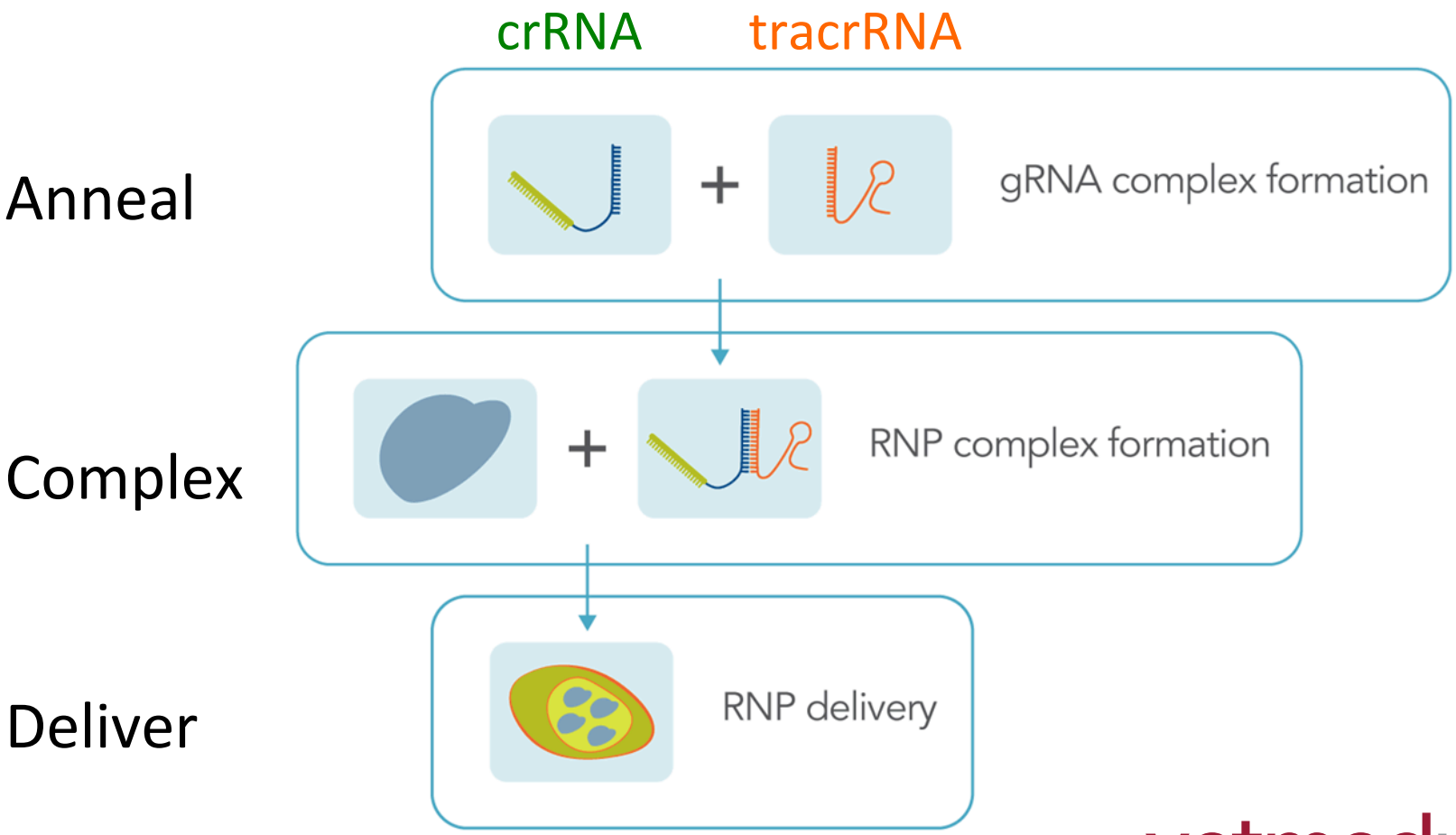
crRNA and tracrRNA complex to form gRNA



https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system

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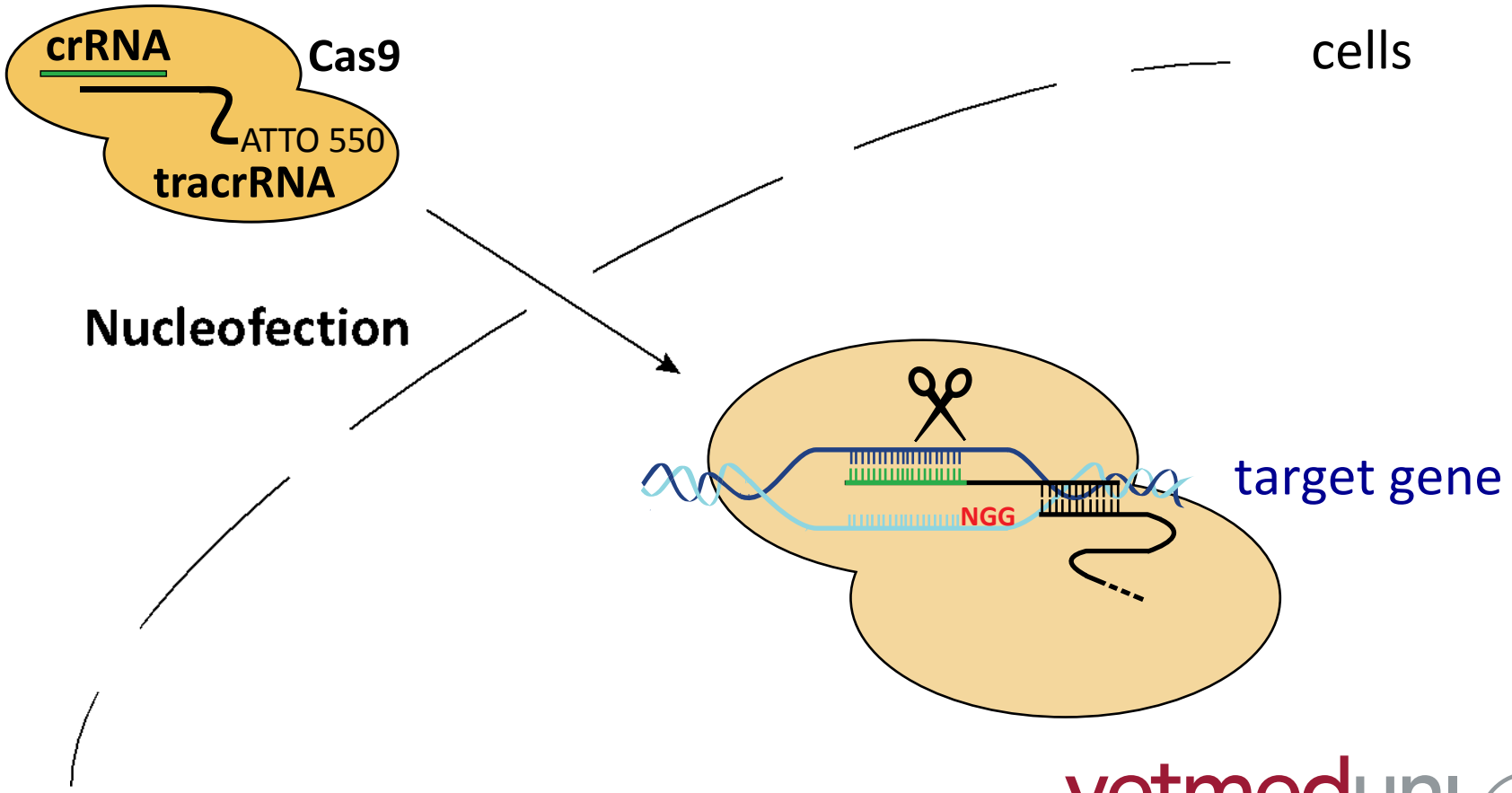
Transient transfection of RNPs

Ribonucleoprotein (RNP)



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Overview

- Introduction to CRISPR/Cas9 system
- gRNA design
  - Important considerations
  - Available online tools
- Delivery options - Lentiviral vectors versus RNPs
- **Determining CRISPR efficiency – genotyping cell pools (TIDE)**
- Practical example

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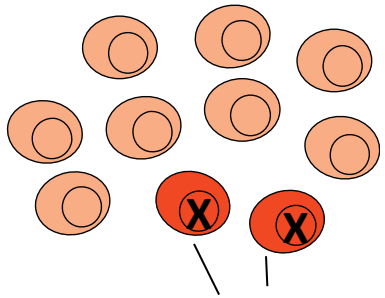
Genotyping of targeted cell pools

CRISPR-knockout experiment



cells with mutations
(insertions/deletions – indels)

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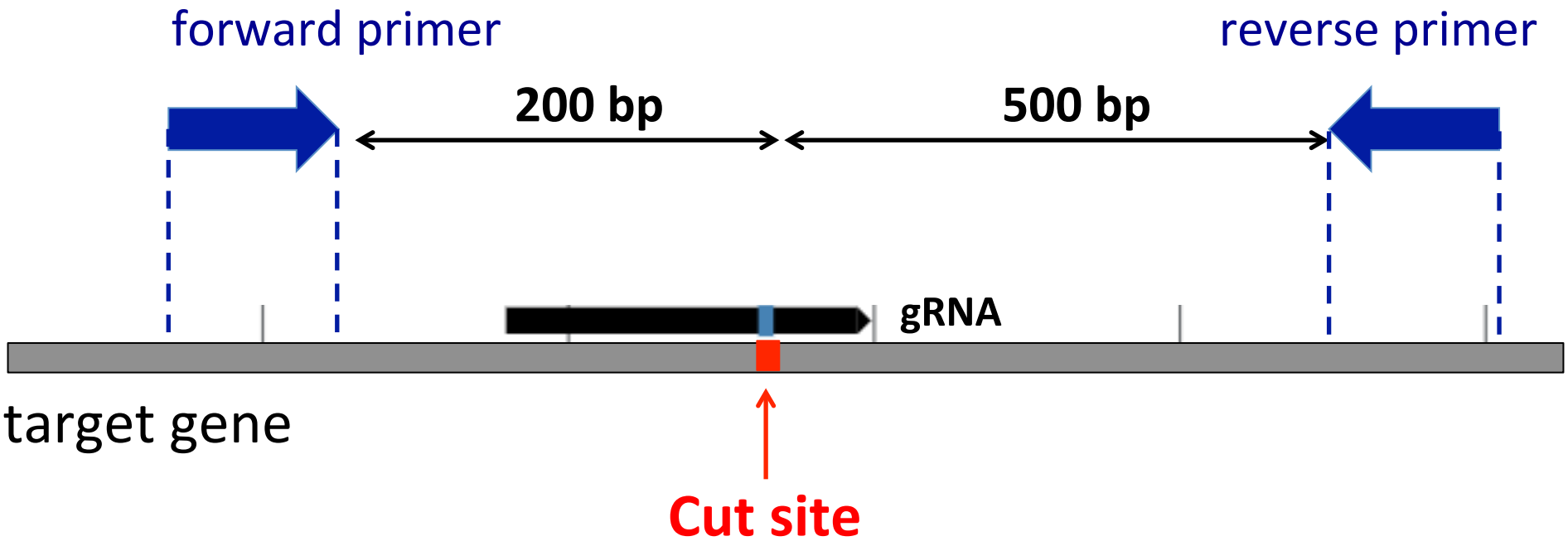
Genotyping of targeted cell pools



PCR → Sanger sequencing

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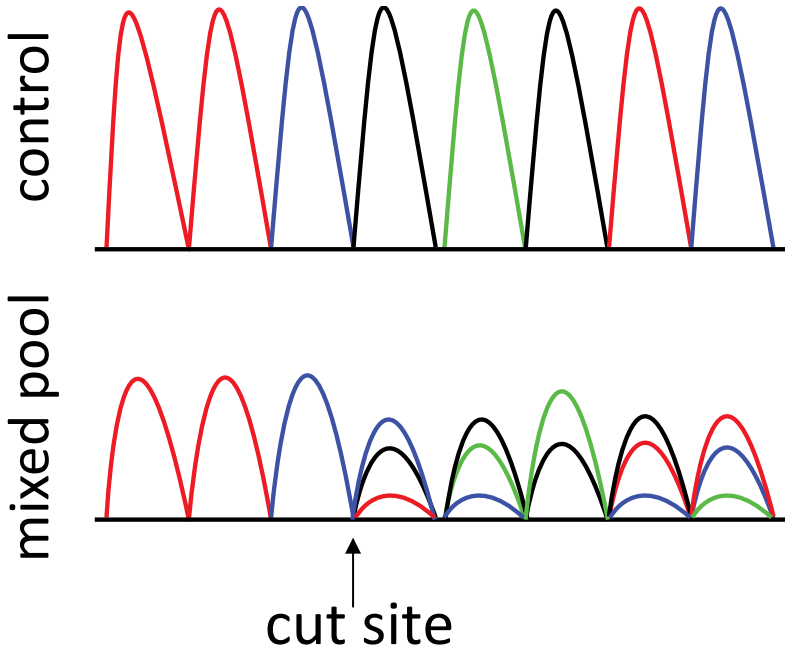
Quantification of genome editing efficiency



https://tide.deskgen.com/

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Practical example

- **gRNA design** – Broad Inst., chopchop, UCSC
- **gRNA delivery** - Lentiviral vs. RNPs
- **gRNA efficiency** – Genotyping using TIDE

Example:
Knock-out the gene ***Tet2*** in a **murine cell line** (gRNA should not be constitutively expressed) and determine knock-out efficiency.

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Step 1: gRNA design

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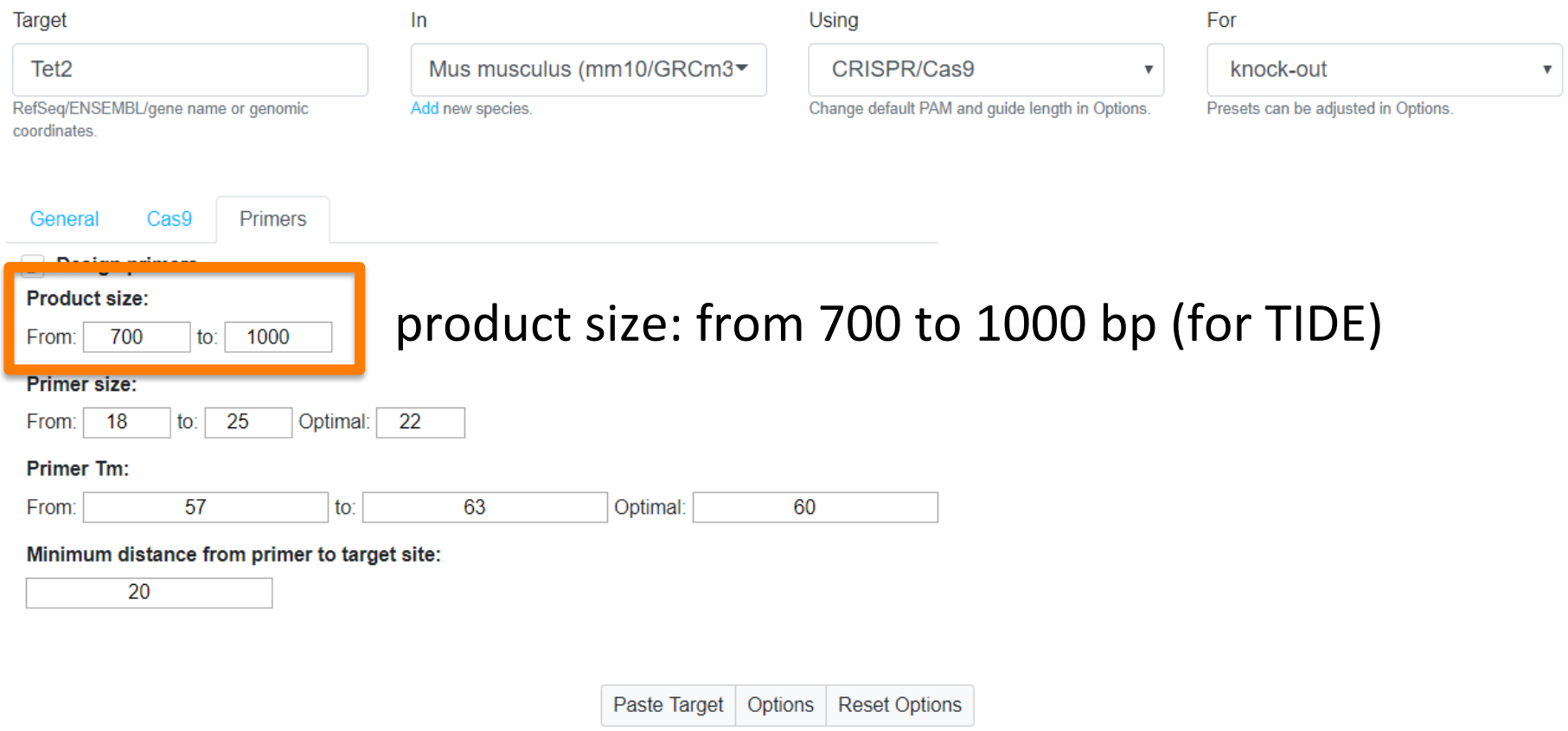
Step 1: gRNA design



product size: from 700 to 1000 bp (for TIDE)

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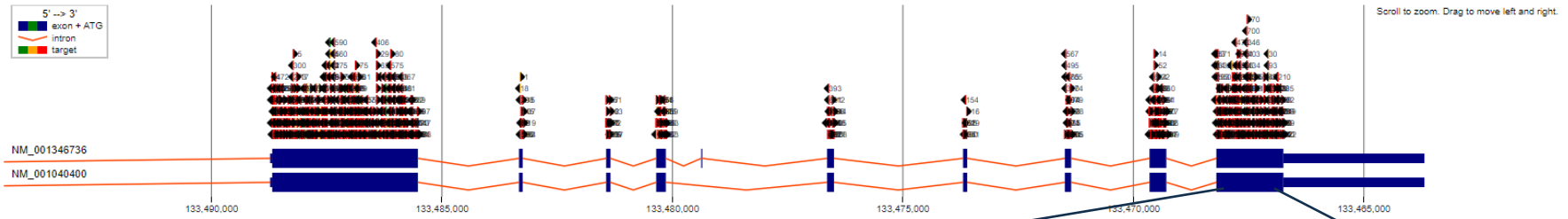
Step 1: gRNA design

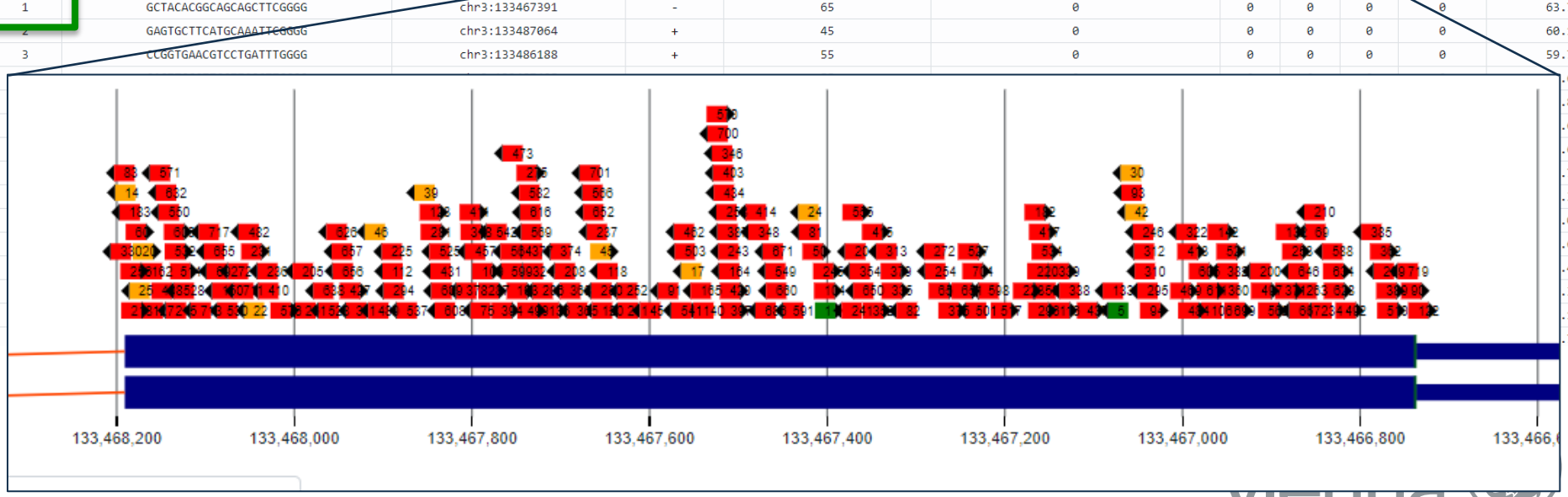
Tet2



Download results: Please select one

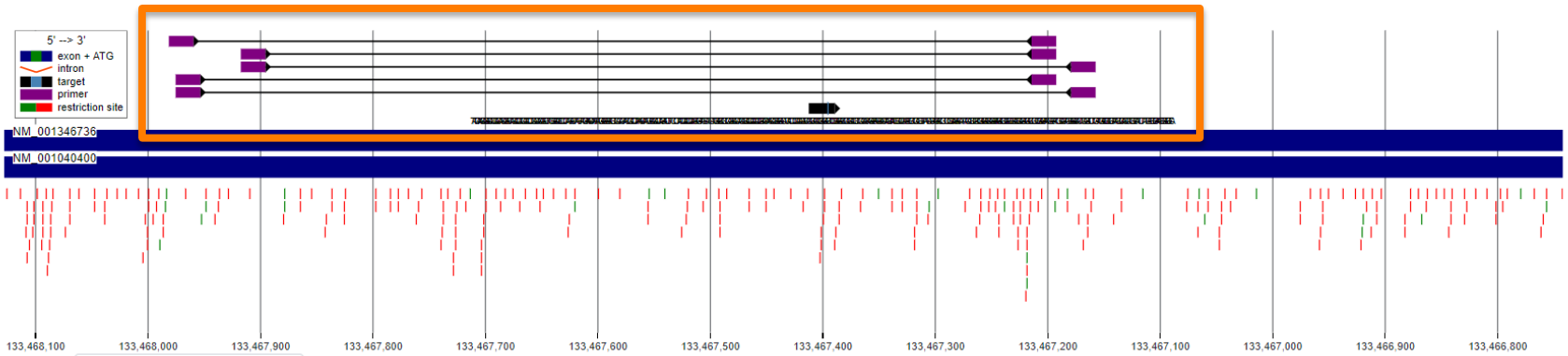
View in UCSC ge

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficie
1	GCTACACGGCAGCAGCTTCGGGG	chr3:133467391	-	65	0	0	0	0	0	63.7
2	GAGTGCTTCATGCAAATTCGGGG	chr3:133487064	+	45	0	0	0	0	0	60.1
3	CCGGTGAACGTCCTGATTTGGGG	chr3:133486188	+	55	0	0	0	0	0	59.7



Step 1: gRNA design

Target: **Tet2**
Rank: **1**
Target sequence: **GCTACACGGCAGCAGCTTCGGGG**



Download: Please select one

Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	chr3:133467954-133467976	ACTCCTCACAGTCCTTATCCCA	60.0	0	chr3:133467158-133467180	ATGCAAGGATCCTGAAAGTTGT	60.0	0	0	818
2	chr3:133467954-133467976	ACTCCTCACAGTCCTTATCCCA	60.0	0	chr3:133467193-133467215	CCAATACTCAATTTCCTGCACA	60.0	0	0	783
3	chr3:133467896-133467918	ACCATGTGAACTTTTACCCCAC	60.0	0	chr3:133467158-133467180	ATGCAAGGATCCTGAAAGTTGT	60.0	0	0	760
4	chr3:133467896-133467918	ACCATGTGAACTTTTACCCCAC	60.0	0	chr3:133467193-133467215	CCAATACTCAATTTCCTGCACA	60.0	0	0	725
5	chr3:133467960-133467982	CAGCCTACTCCTCACAGTCCTT	59.9	0	chr3:133467193-133467215	CCAATACTCAATTTCCTGCACA	60.0	0	0	789

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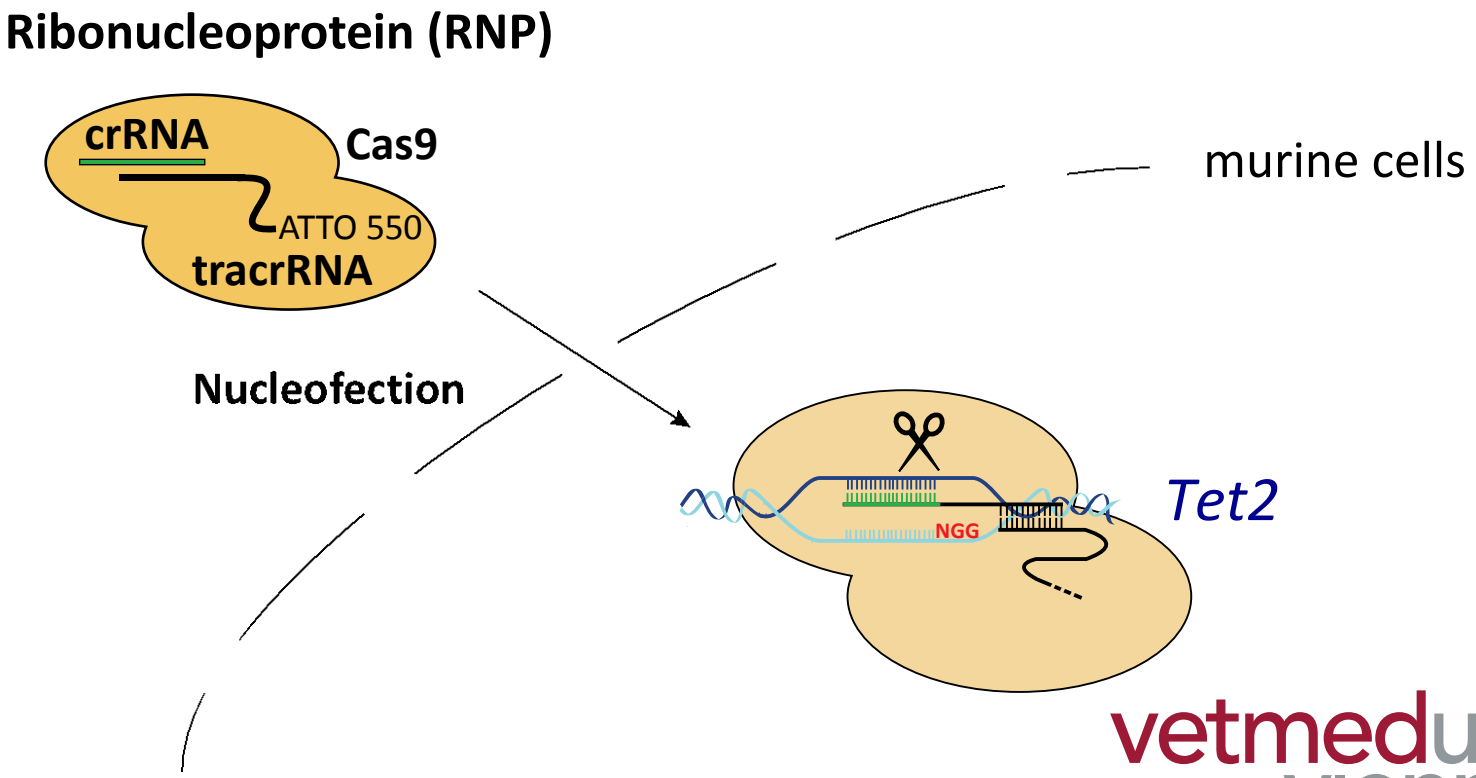
Step 2: gRNA delivery

- Order crRNA (Tet2-specific), tracrRNA and recombinant Cas9 at IDT (idt.dna.com)
- Nucleofect pre-assembled RNP (crRNA+tracrRNA+Cas9) into murine cells



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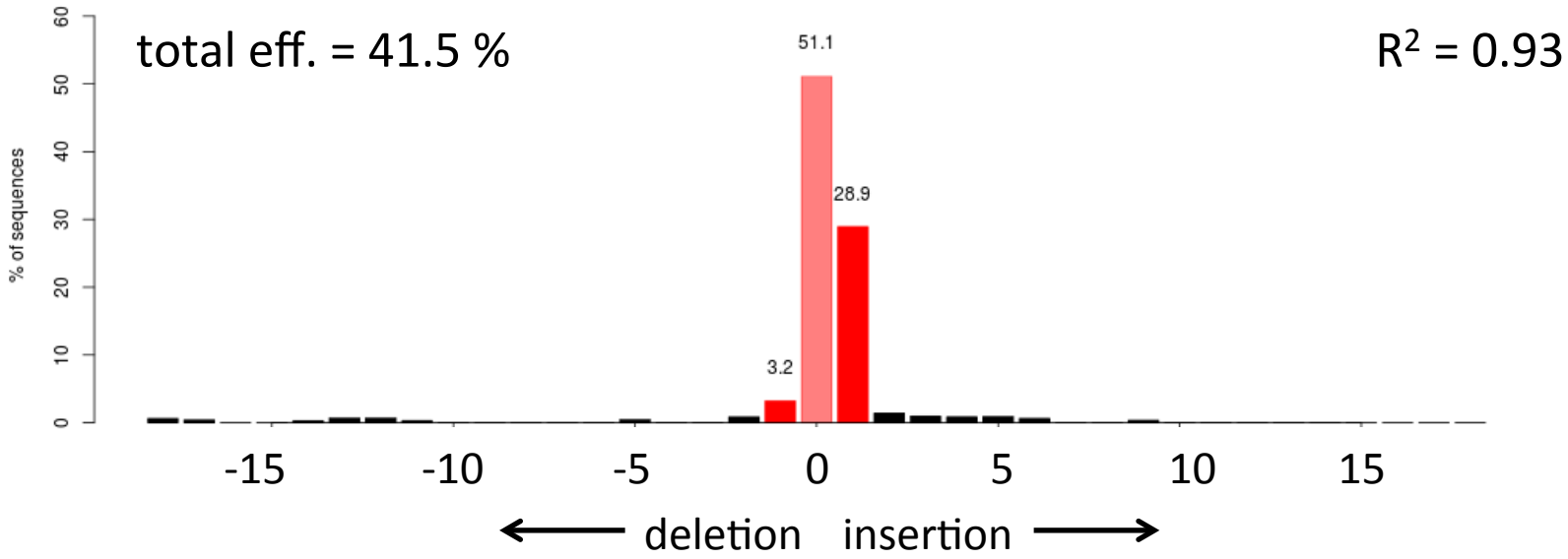
Step 3: gRNA efficiency

- PCR amplify wild-type (control) and *Tet2*-targeted murine cells
- Perform Sanger sequencing of PCR-amplified fragment
- Analyse with TIDE



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Additional suggestions

Use controls.

Check the literature.

Try out the online tools.

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Thanks for your attention!

grebienlab.com elizabeth.heyes@vetmeduni.ac.at @HeyesElizabeth