

Genome Engineering Workflow

Reading, reaching and re-writing the code of life



Dr Kalpana Surendranath

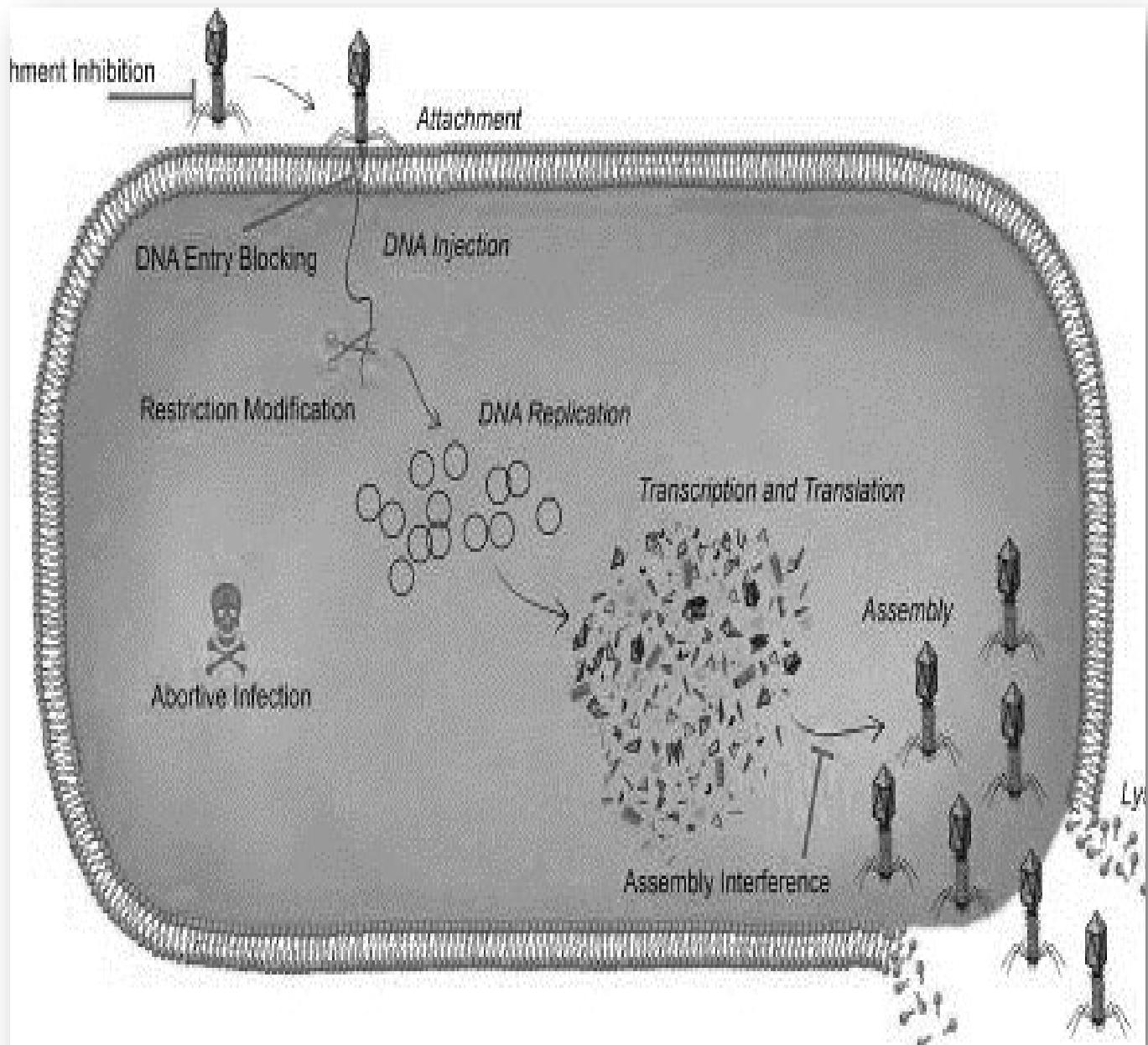
Associate professor in genome engineering

Director - Gene Editors of the Future

Recap of Day1 ...

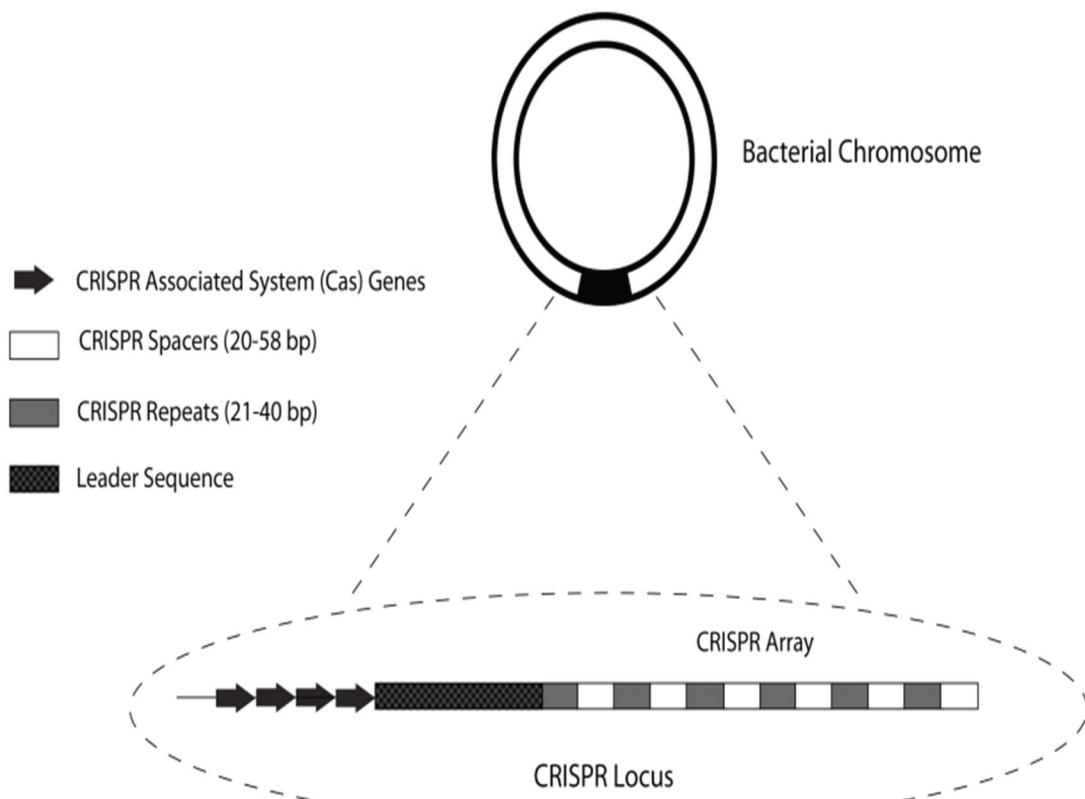
- 1) Genes and Genomes
- 2) Tools for Guide RNA design

Bacterial defense mechanisms against phages



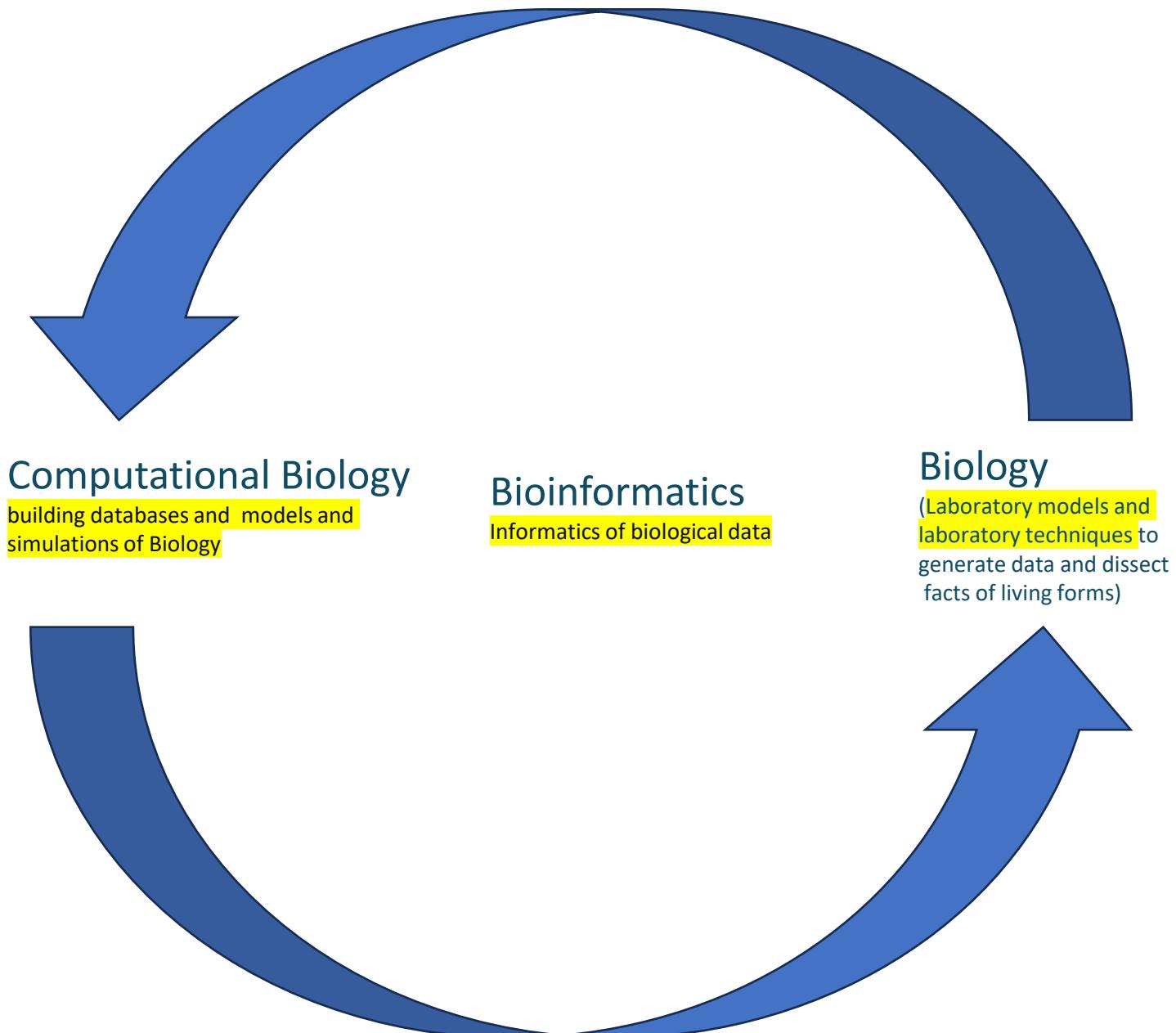
CRISPR LOCUS

CRISPR Locus on a Bacterial Chromosome

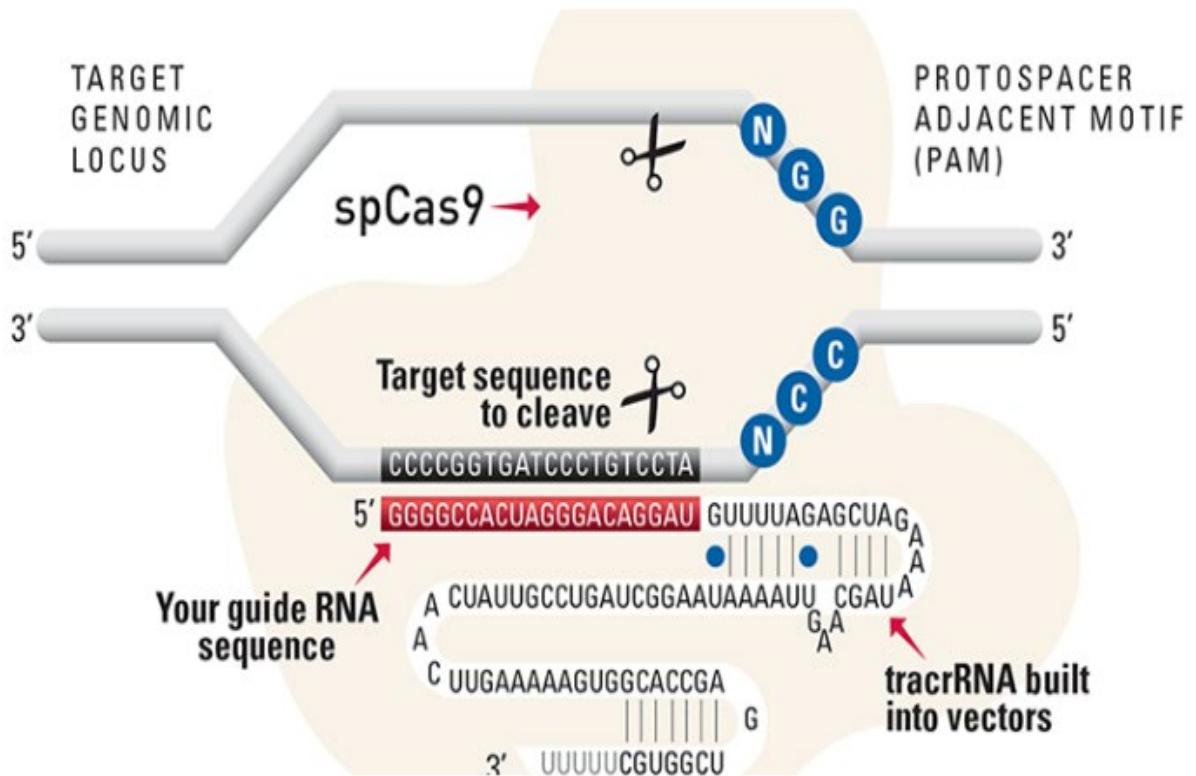


- Prokaryotes generally have a combination of different Cas genes, but Cas1 and Cas2 are conserved.
- The Cas genes are located several hundred basepairs upstream of the CRISPR array
- The number of CRISPR repeat/spacer groups varies by species, but each repeat is always followed by a unique spacer

THE CRISPR BUBBLE



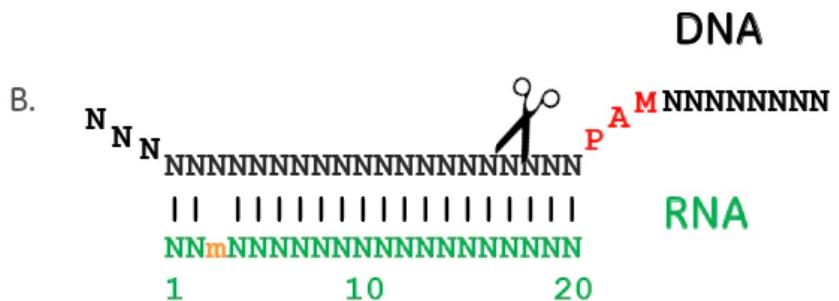
THE GUIDE RNA



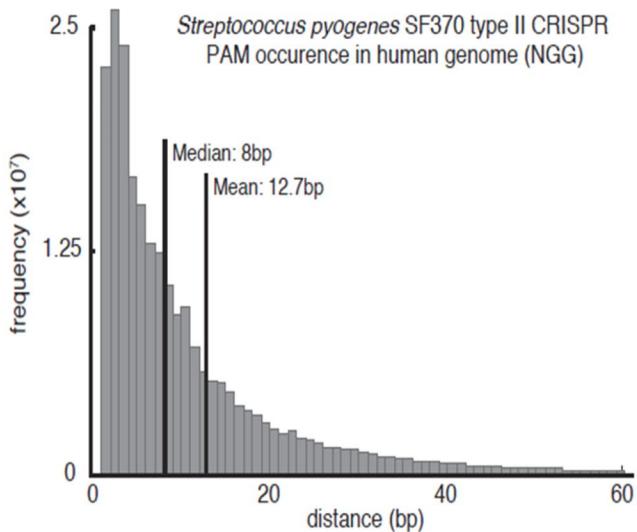
<https://www.stratech.co.uk/news/crispr/>

The Key Determiners

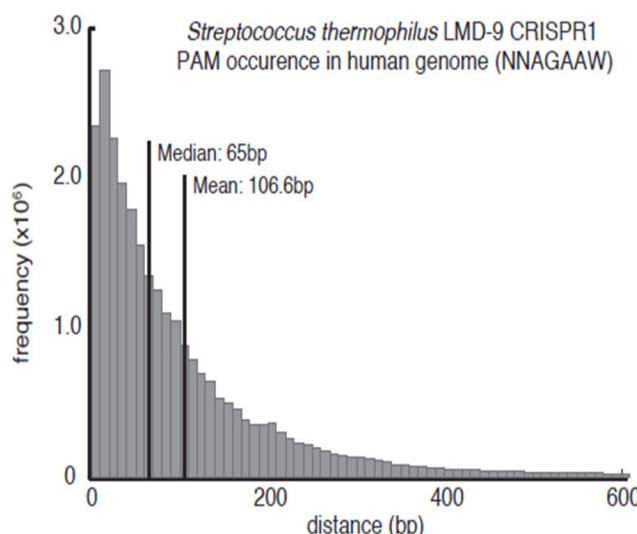
- Specificity
 - Efficiency
 - PAM



PAM occurrence on the human genome 2



B



Chr	NGG		NNAGAAW	
	median	mean	median	mean
1	7	12.8	67	115.8
2	8	12.7	64	100.8
3	8	13.0	63	98.5
4	9	14.0	61	94.5
5	8	13.1	63	97.9
6	8	13.1	63	98.5
7	8	12.4	64	102.9
8	8	12.8	64	100.9
9	7	13.9	65	120.5
10	7	12.1	66	107.0
11	7	12.0	65	105.8
12	8	12.4	65	103.5
13	8	13.6	62	94.6
14	8	12.0	65	101.5
15	7	11.5	68	107.7
16	7	11.7	74	136.8
17	6	10.3	76	127.9
18	8	13.4	63	101.8
19	6	9.4	82	145.4
20	7	11.1	72	121.8
21	7	13.4	64	111.4
22	6	9.2	85	140.3
X	8	13.2	63	99.0
Y	8	29.2	62	223.7

Species/Variant of Cas9	PAM Sequence
<i>Streptococcus pyogenes</i> (SP); Sp-Cas9	3' NGG
SpCas9 D1135E variant	3' NGG (reduced NAG binding)
SpCas9 VRER variant	3' NGCG
SpCas9 EQR variant	3' NGAG
SpCas9 VQR variant	3' NGAN or NGNG
xCas9	3' NG, GAA, or GAT
SpCas9-NG	3' NG
<i>Staphylococcus aureus</i> (SA); Sa-Cas9	3' NNGRRT or NNGRR(N)
<i>Acidaminococcus</i> sp. (AsCpf1) and <i>Lachnospiraceae</i> bacterium (Lb-Cpf1)	5' TTTV
AsCpf1 RR variant	5' TYCV
LbCpf1 RR variant	5' TYCV
AsCpf1 RVR variant	5' TATV
<i>Campylobacter jejuni</i> (CJ)	3' NNNNRYAC
<i>Neisseria meningitidis</i> (NM)	3' NNNNGATT
<i>Streptococcus thermophilus</i> (ST)	3' NNAGAAW
<i>Treponema denticola</i> (TD)	3' NAAAAC

Early days of CRISPR

2013...

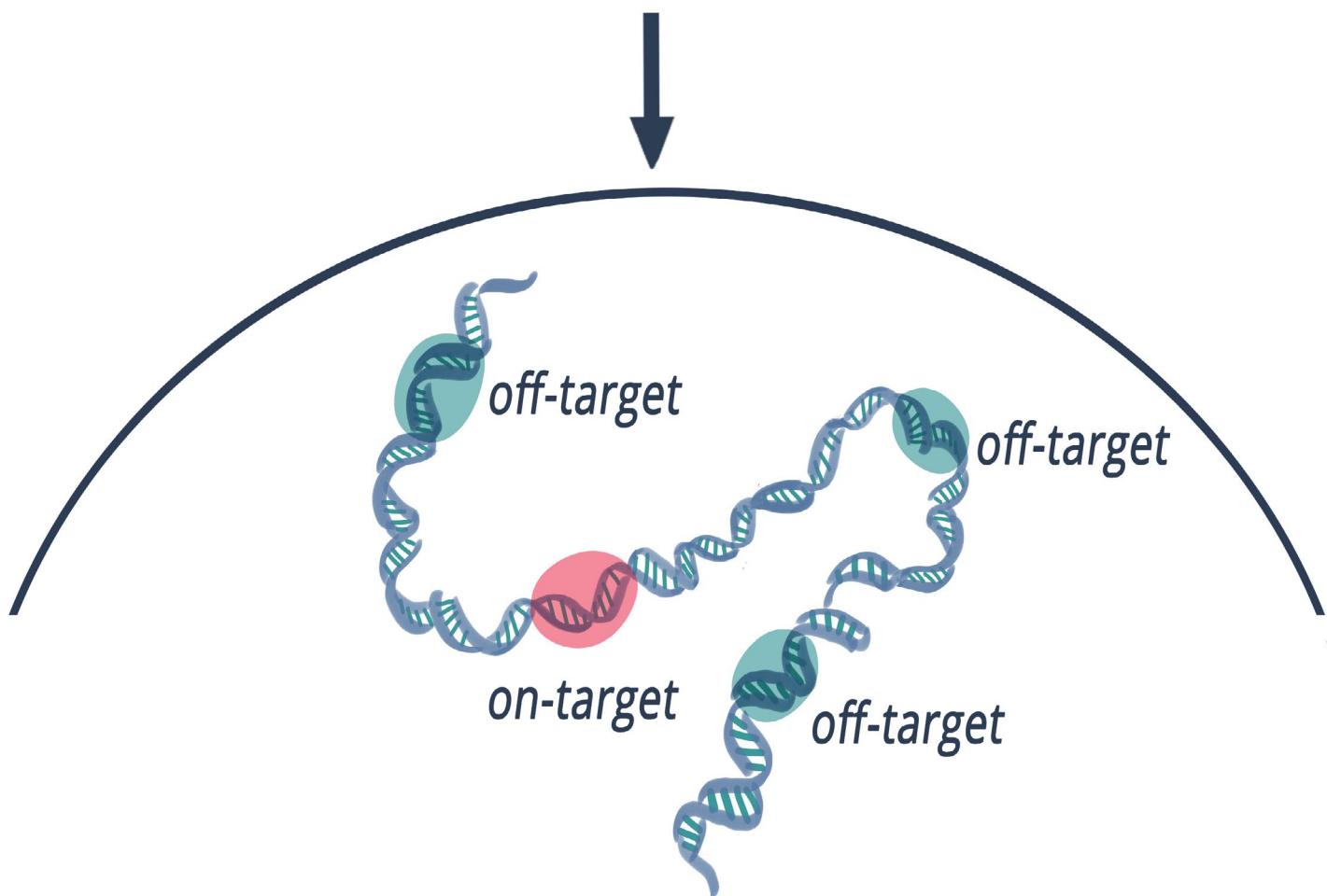
<http://crispr.mit.edu>

EMX1 guide, CRISPOR and CasOffFinder predict 1288 off-targets with up to four mismatches while the MIT site predicts only 334 and as a result does not find five out of 15 validated off-targets

Haeussler, M., Schönig, K., Eckert, H. *et al.* Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol* **17**, 148 (2016).

Downside of CRISPR

Cas9 + Guide RNA



Downside of CRISPR

No of mismatches	off-target sites
1	100
2	1, 000
3	100,000
4	1,000,000
5	10,000,000
6	100,000,000

For a single guide

Table 4.2 Web tools for precise genome engineering

Algorithm	Editing	URL
Cas-Designer	CRISPR-Cas mediated editing	http://www.rgenome.net/cas-designer/
Cas-OFFinder		http://www.rgenome.net/cas-offinder/
CHOPCHOP		https://chopchop.cbu.uib.no/
CRISPR-P 2.0		http://crispr.hzau.edu.cn/CRISPR2/
CRISPOR		http://crispor.tefor.net/
CRISPRdirect		https://crispr.dbcls.jp/
E-CRISP		http://www.e-crisp.org/
CCTop		http://crispr.cos.uni-heidelberg.de/
DeepCpf1		http://deepcrispr.info/
DeepSpCas9		http://deepcrispr.info/DeepSpCas9
DeepHF		http://www.DeepHF.com/
CINDEL		http://big.hanyang.ac.kr/cindel
SSC		http://cistrome.org/SSC/
Prediction web tool		https://crispr.ml
CRISPRscan		https://www.crisprscan.org
Microhomology-Predictor		http://www.rgenome.net/michcalculator
FORECasT		https://partslab.sanger.ac.uk/FORECasT
inDelphi		https://indelphi.giffordlab.mit.edu
TIDE		http://shinyapps.datacurators.nl
Cas-Analyzer		http://www.rgenome.net/cas-analyzer/
CRISPREssO2		https://crispresso.pinellolab.partners.org/
CRISPRAnalyzeR		http://crispr-analyzer.dkfz.de/
CRISPR-Sub		http://www.rgenome.net/crispr-sub
BE-Designer	Base editing	http://www.rgenome.net/be-designer/
DeepBaseEditor		http://deepcrispr.info/DeepBaseEditor
BE-Hive		https://www.crisprbehive.design/
BE-Analyzer		http://www.rgenome.net/be-analyzer/
PE-Designer	Prime editing	http://www.rgenome.net/pe-designer
PrimeDesign		https://drugthatgene.pinellolab.partners.org/
PE-Analyzer		http://www.rgenome.net/pe-analyzer/
DeepPE		http://deepcrispr.info/DeepPE/
pegFinder		http://pegfinder.sidichenlab.org

*Surendranath, K. and Akram, K., et al. (2024). **Progress and Prospects in CRISPR Genome Engineering Nucleases**. In Translational Research in Biomedical Sciences: Recent Progress and Future Prospects (Chapter 4). SpringerNature. ISBN 978-981-97-1776-7

Guide RNA design

[Benchling](#)

[Broad Institute GPP](#)

[CasOFFinder](#)

[CHOPCHOP](#)

[CRISPOR](#)

[Deskgen](#)

[E-CRISP](#)

[Horizon Discovery](#)

[IDT](#)

[Off-Spotter](#)

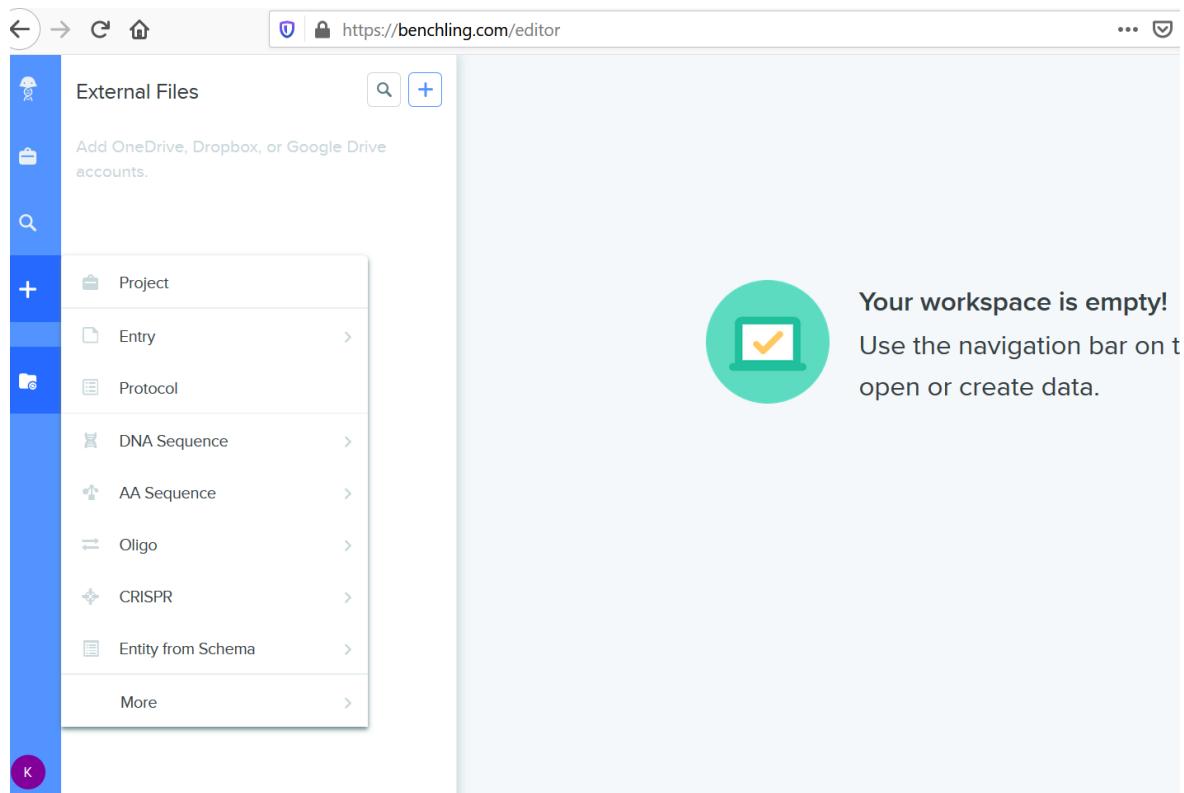
[Synthego](#)

**Choose a gRNA score
greater in both criteria**

<https://zlab.bio/guide-design-resources>

Benchling – Step1

Login using google account



<https://benchling.com/editor>

Benchling – Step2

Select CRISPR guides

The screenshot shows the Benchling software interface. At the top, there is a browser-style header with back, forward, and home buttons, a URL field containing <https://benchling.com/editor>, and a search bar. Below the header, the main workspace has a blue sidebar on the left with icons for External Files, Add OneDrive/Dropbox/Google Drive accounts, a search function, and a plus sign for creating new files. The main area displays a modal dialog titled "Design CRISPR Guides: Import sequence". The dialog has an "Import From" section with a dropdown menu set to "Gene ID or Name" and a search input field containing "Search by ENSEMBL Gene ID or Name (e.g. BRCA2)". There is also an "Example" link. A "Next" button is located at the bottom right of the dialog. The background of the workspace is mostly gray.

Benchling – Step3

Choose the gene of interest

The screenshot shows the 'Import From' section of the Benchling CRISPR Design Tool. The URL in the browser bar is https://benchling.com/kankalpu/f/lib_kWptgShR-gene-editors-of-the-future-university-of-westminster.

Import From

Gene ID or Name: Tp53

Genome: GRCh38 (hg38, Homo sapiens)

Gene: TP53 (ENSG00000141510)

Location: chr17 7,661,779—7,687,550 (-)

Import in sense orientation

Transcript: TP53-001 (ENST00000269305, CCDS11118)

Sequence Name: TP53 (ENSG00000141510)

Folder: EXAMPLE PROJECT

Show Advanced Settings

Next

Benchling – Step4

Choose the gene of interest

CRISPR Design Tool X TP53 (ENSG00000141510) · Ben X CHOPCHOP X +

https://benchling.com/kankalpu/f/lib_ZtqqxZal-example-project/seq_HAQzrqvU-tp53-ensg000001415 ...

Kalpana-Gene editors X TP53 (ENSG00000141510) X

Design CRISPR Guides: Guide parameters

Design Type Single guide
Wild-type Cas9, single gRNA (higher efficiency)
 Paired guides
Double Cas9 nickase, two gRNAs (lower off-target effects)
 Guides for "base editing" (Komor et al., 2016)
C > T (or G > A) substitution, no dsDNA breaks

Guide Length 20

Genome GRCh38 (hg38, Homo sapiens)
Don't see the genome you're looking for? We may be able to import it, just ask.

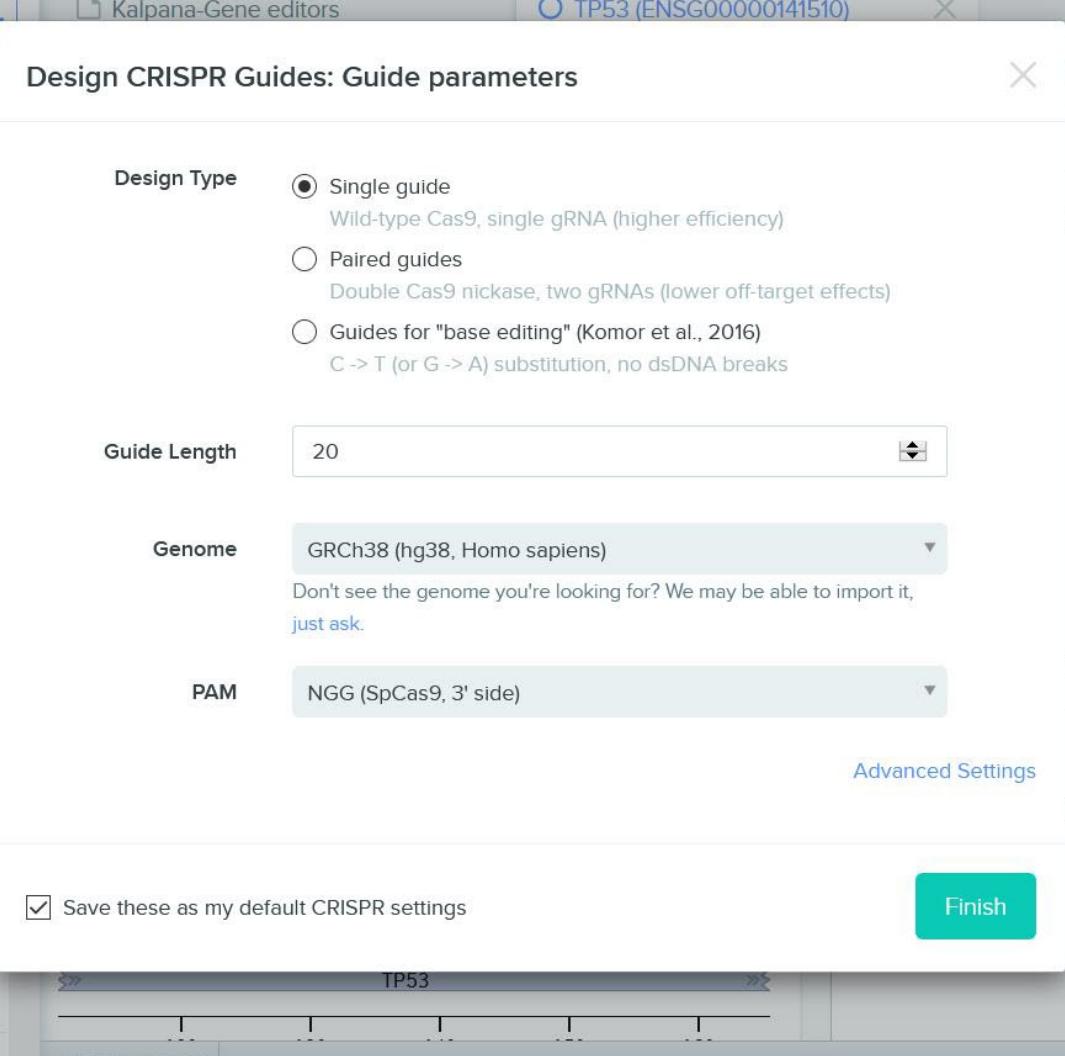
PAM NGG (SpCas9, 3' side)

[Advanced Settings](#)

Save these as my default CRISPR settings Finish

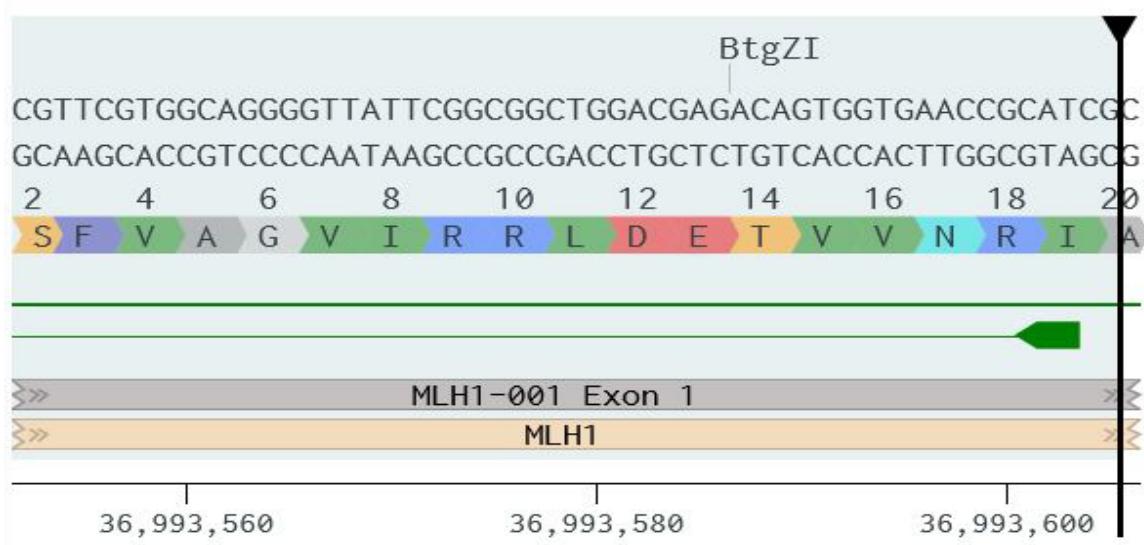
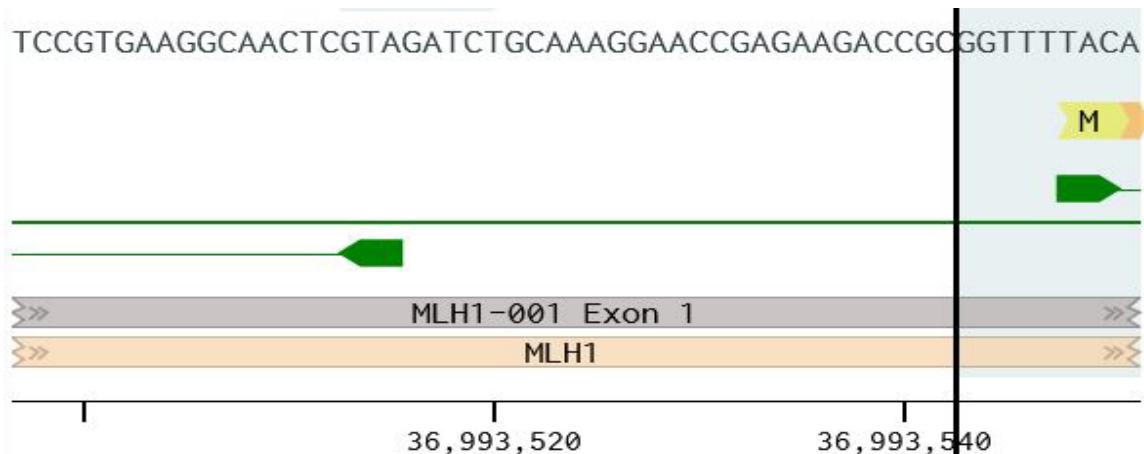
TP53

BASES 25772 AS



Benchling – Step5

Choose the gene of interest



Benchling – Step6

Choose the gene of interest

The screenshot shows the Benchling software interface. On the left, a "SEQUENCE MAP" panel displays a genomic sequence (TCCGTGAAGGCAACTCGTAGATCTGCAAAGGAACCGAGAAGACCGGGTTTACA) with a green arrow indicating the direction of transcription. Below the sequence are two exons: "MLH1-001 Exon 1" and "MLH1". A scale bar at the bottom indicates positions 36,993,520 and 36,993,540. A protein sequence for "BtgZI" is shown below the exons, with amino acid positions 2 to 20 labeled. Above the protein sequence, several SNPs are highlighted with colored boxes and numbers (e.g., 52 88, 49 90, 31 90, 43 91, 68 83). On the right, a "LINEAR MAP" panel titled "Untitled" shows a blue bar labeled "group". A "DESIGN CRISPR" panel lists 10 potential CRISPR guide sequences, each with its PAM site, On-Target Score (ranging from 27.4* to 69.0), and Off-Target Score (ranging from 83.5 to 95.4). The On-Target Score is described as an optimized score from Doench, Fusi et al. (2016) for 20bp guides with NGG PAMs.

Index	Strand	Sequence	PAM	On-Target Score	Off-Target Score
572	+	TCGTGGCAGGGTTATTCGG	CGG	69.0	89.9
588	+	TCGGCGGCTGGACGAGACAG	TGG	68.4	83.5
561	+	CAAAATGTCGTTCTGGCAG	GGG	52.7	88.0
560	+	CCAAAATGTCGTTCTGGCA	GGG	49.7	90.2
576	+	GGCAGGGTTATTCGGCGGC	TGG	43.9	91.3
569	+	CGTTCGTGGCAGGGTTATT	CGG	31.2	90.9
549	-	CCCTGCCACGAACGACATTT	TGG	27.4*	95.4

The Benchling-Ensembl shuttle

Gene: RAD51

Transcript: RAD51-211

Transcript: RAD51-211 ENST00000645673.2

Description

RAD51 recombinase [Source:HGNC Symbol;Acc:[HGNC:9817](#)]

Gene Synonyms

BRCC5, FANCR, HsRad51, HsT16930, RAD51A, RECA

Location

Chromosome 15: 40,695,174-40,732,340 forward strand.

About this transcript

This transcript has [10 exons](#), is annotated with [17 domains and features](#), is associated with [10614 variant alleles](#) and maps [probes](#).

Gene

This transcript is a product of gene [ENSG00000051180.17](#)

[Hide transcript table](#)

Show/hide columns (1 hidden)

Name	Transcript ID	bp	Protein	Biotype	CCDS	UniProt	RefSeq Match	Flag
RAD51-211	ENST00000645673.2	2439	340aa	Protein coding	CCDS53931	Q06609	-	GENCODE basic
RAD51-201	ENST00000267868.8	2436	339aa	Protein coding	CCDS10062	Q06609	NM_002875.5	TSL:1 GENCODE basic
RAD51-203	ENST00000423169.6	1611	280aa	Protein coding	CCDS53932	Q06609	-	TSL:1 GENCODE basic
RAD51-202	ENST00000382643.7	1588	340aa	Protein coding	CCDS53931	Q06609	-	TSL:2 GENCODE basic
RAD51-208	ENST00000532743.6	1539	339aa	Protein coding	CCDS10062	Q06609	-	TSL:2 GENCODE basic
RAD51-210	ENST00000557850.5	1397	242aa	Protein coding	-	Q06609	-	TSL:2 GENCODE basic
RAD51-205	ENST00000526763.6	665	75aa	Protein coding	-	Q9NZG9	-	CDS 3' incomplete
RAD51-206	ENST00000527860.5	566	172aa	Protein coding	-	E9PNT5	-	CDS 3' incomplete
RAD51-204	ENST00000525066.5	1545	151aa	Nonsense mediated decay	-	E9PJ30	-	TSL:2 GENCODE basic
RAD51-207	ENST00000531277.2	658	100aa	Nonsense mediated decay	-	E9PI54	-	TSL:2 GENCODE basic

https://www.ensembl.org/Homo_sapiens/Transcript/Exons?db=core;g=ENSG00000051180;r=15:40694774-40732340;t=ENST00000645673

Benchling gRNA design

Choose the exon to target

The screenshot shows the Benchling gRNA design interface. On the left, the "SEQUENCE MAP" tab is active, displaying the genomic sequence of the RAD51 gene (ENSG00000051180) with two exons. Exon 1 (RAD51-009) is located between 40,698,740 and 40,698,760, and Exon 2 is located between 40,698,760 and 40,698,780. The sequence map shows the amino acid translation for each exon. On the right, the "LINEAR MAP" tab is active, showing the protein sequence MAMQMLEEAN with positions 2 through 10 indicated above the letters. Below the sequence, the "RAD51" gene structure is shown with two exons. A red circle highlights the green button with a plus sign (+) next to the "Target" input field, which contains the value 40698758. The "Region" input field contains the value 40698842. The "RAD51" gene name is displayed prominently below the map.

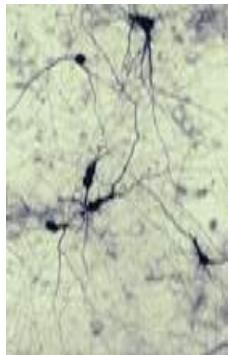
RAD51

1) Click on the + symbol to generate guides to the target site

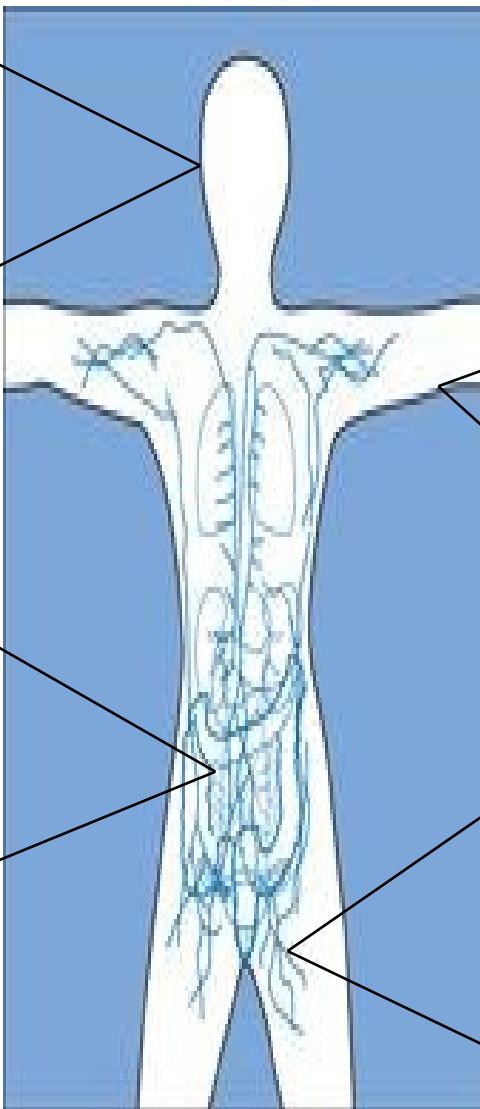
2) Enter in Benchling project book



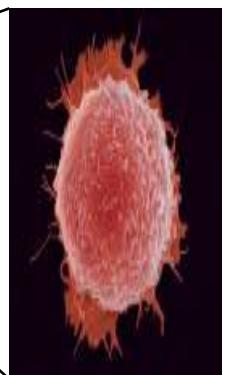
Cells...



Nerve cells



Skin cells



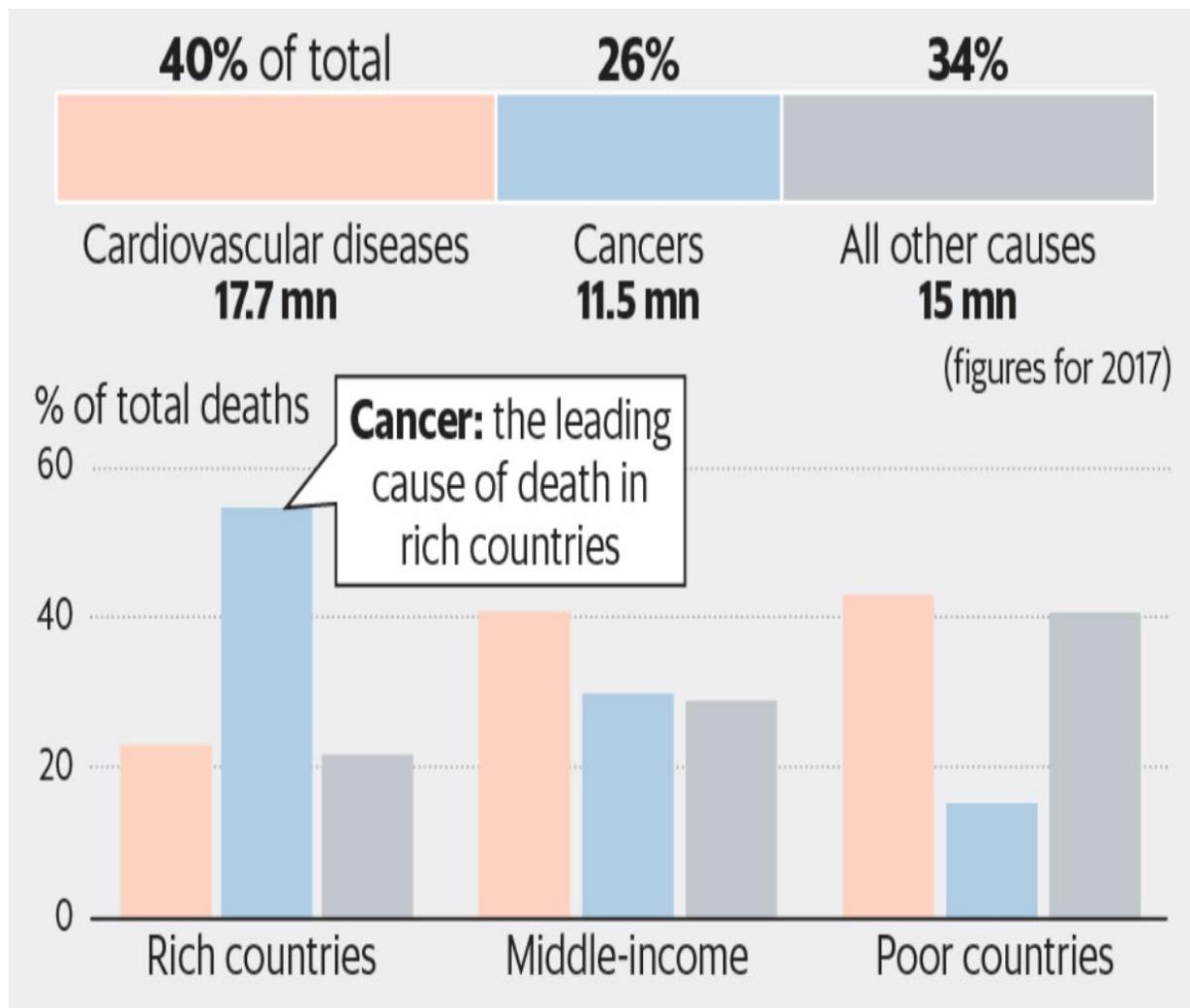
Red blood cells



Gut cells

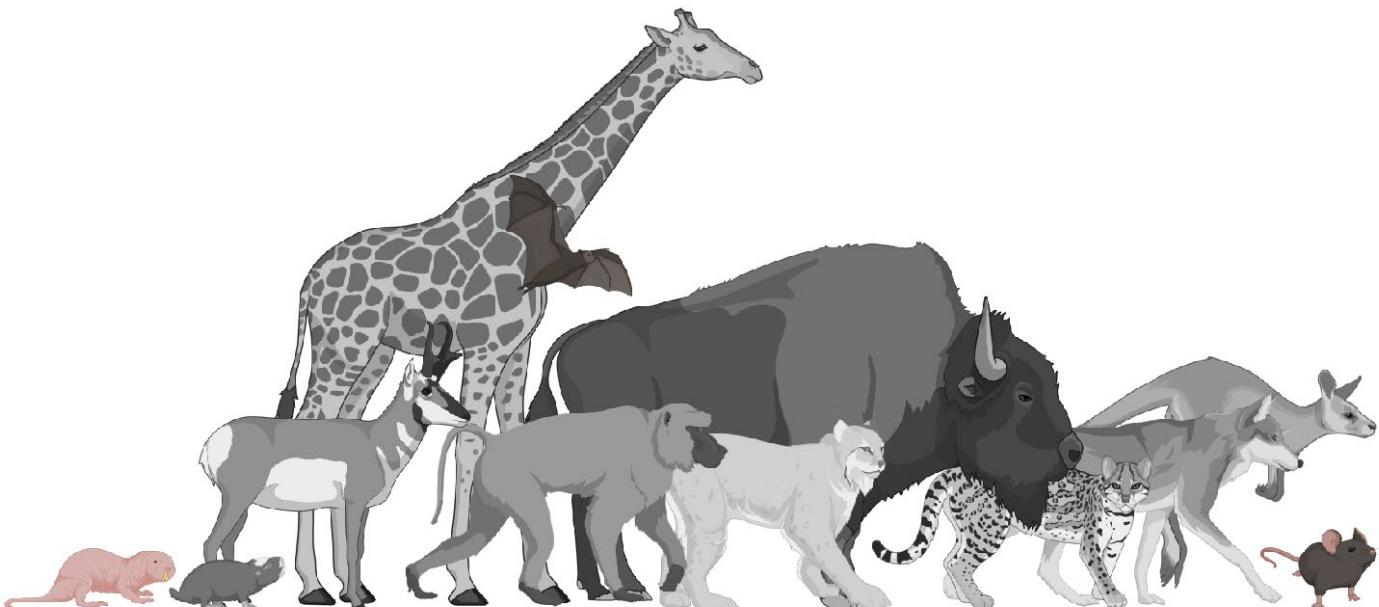


Causes of death in the world



The Lancet

Cancer risk across mammals

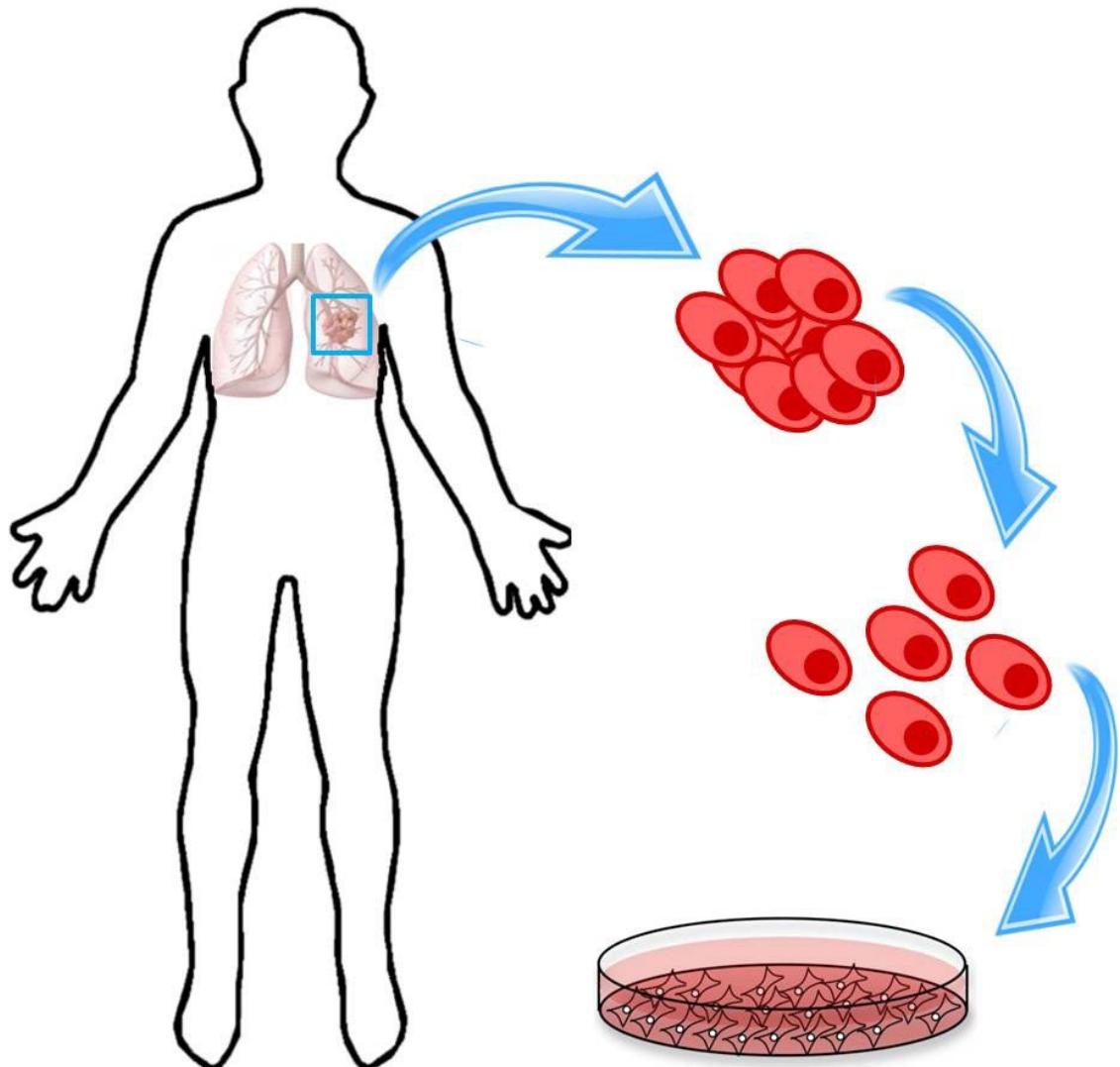


< 0.001% > 20%

<https://www.nature.com/articles/s41586-021-04224-5>

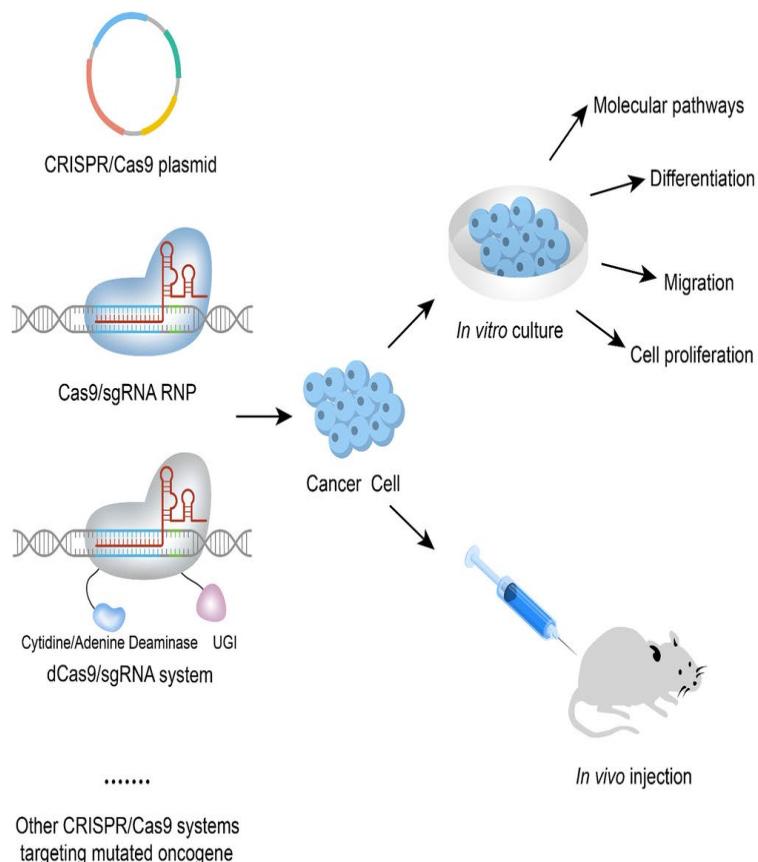
Immortalized cell lines

Supporting drug discovery for decades

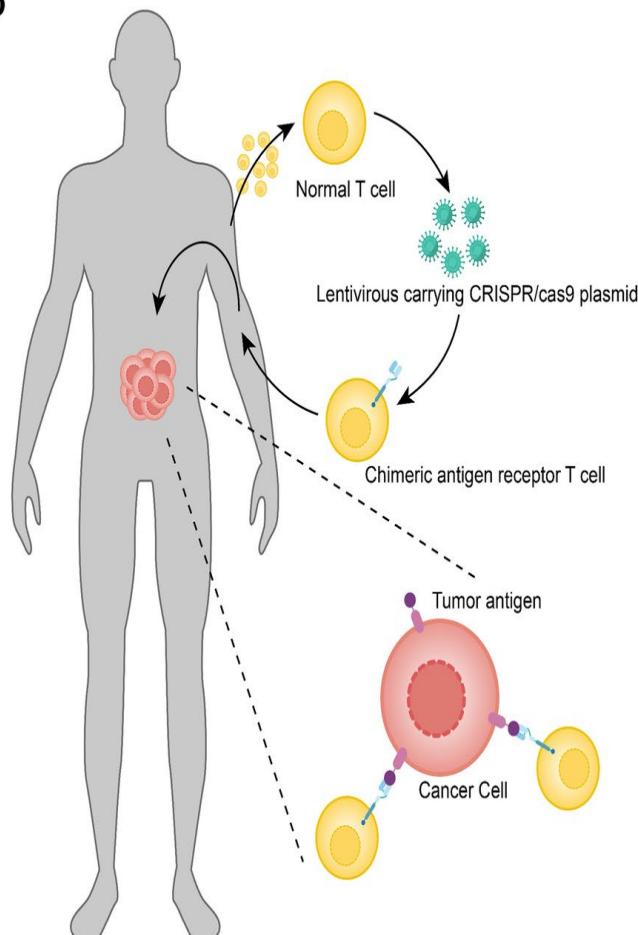


CRISPR in Cancer Research

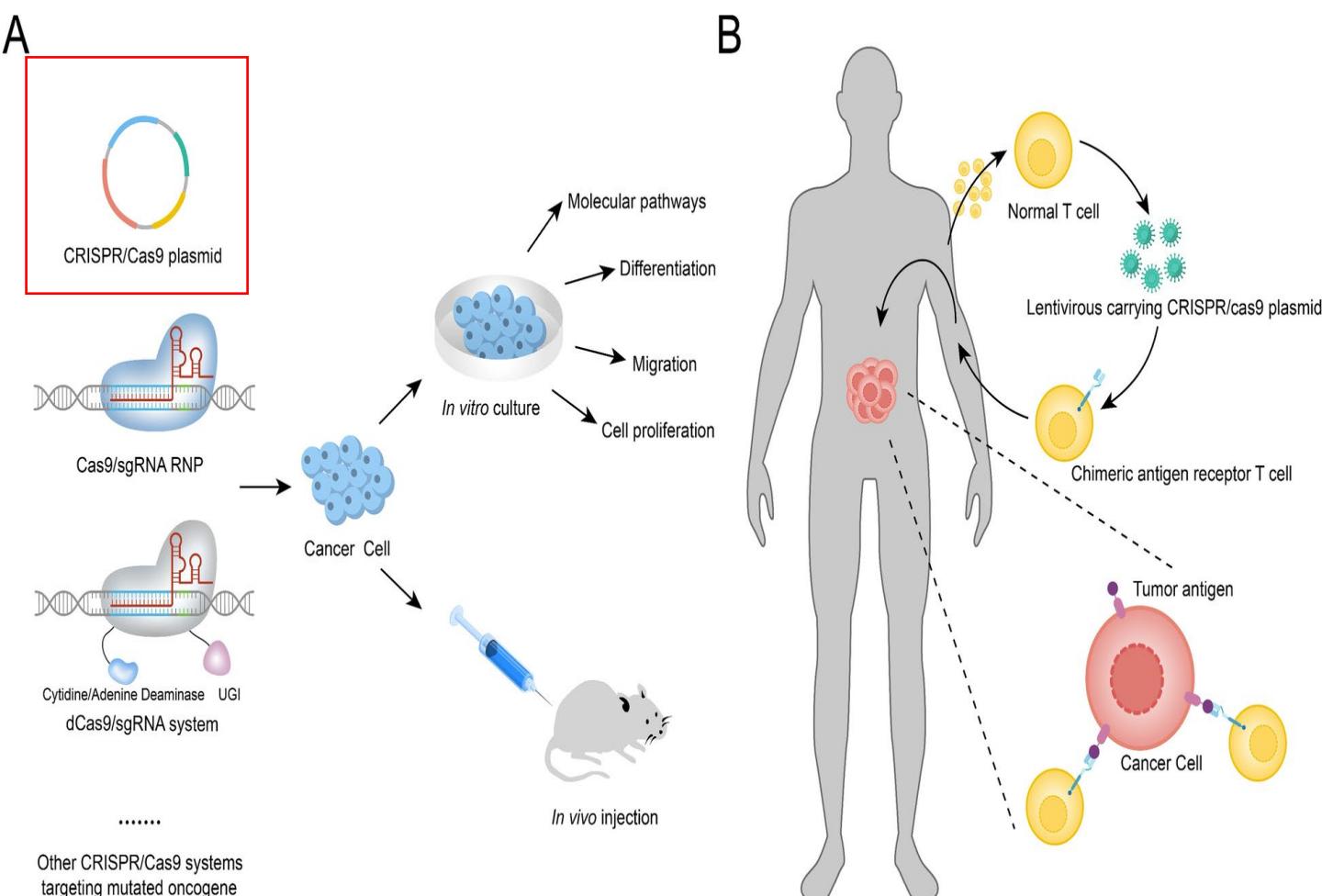
A



B



CRISPR in Cancer Research



Before you start

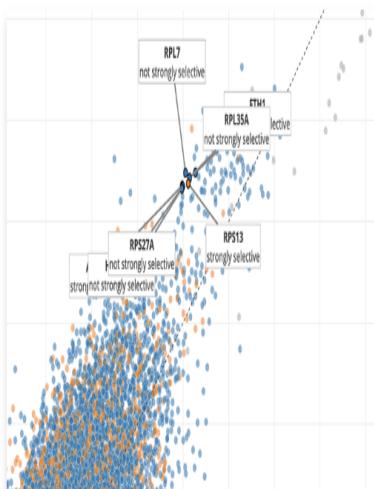


Project **Achilles**



Before you start

Data Explorer



Use the Data Explorer tool to dive deeper into DepMap datasets and explore relationships across cell lines.

Cell Line Selector



Today...

- 1)CRISPR/Cas9 Workflow
- 2)Case studies - Munuse
- 3)CRISPRESSO - Khalid

Today...

- 1)CRISPR/Cas9 Workflow
- 2)Case studies
- 3)CRISPRESSO

CRISPR workflow

Stage 1
2 weeks

1. Design and selection of **guide sequences** (online algorithm)
2. Synthesis of single stranded guide oligos
3. Clone into **CRISPR/Cas9 expression vector pX330 series**
4. Sanger Sequencing
5. Plasmids purification

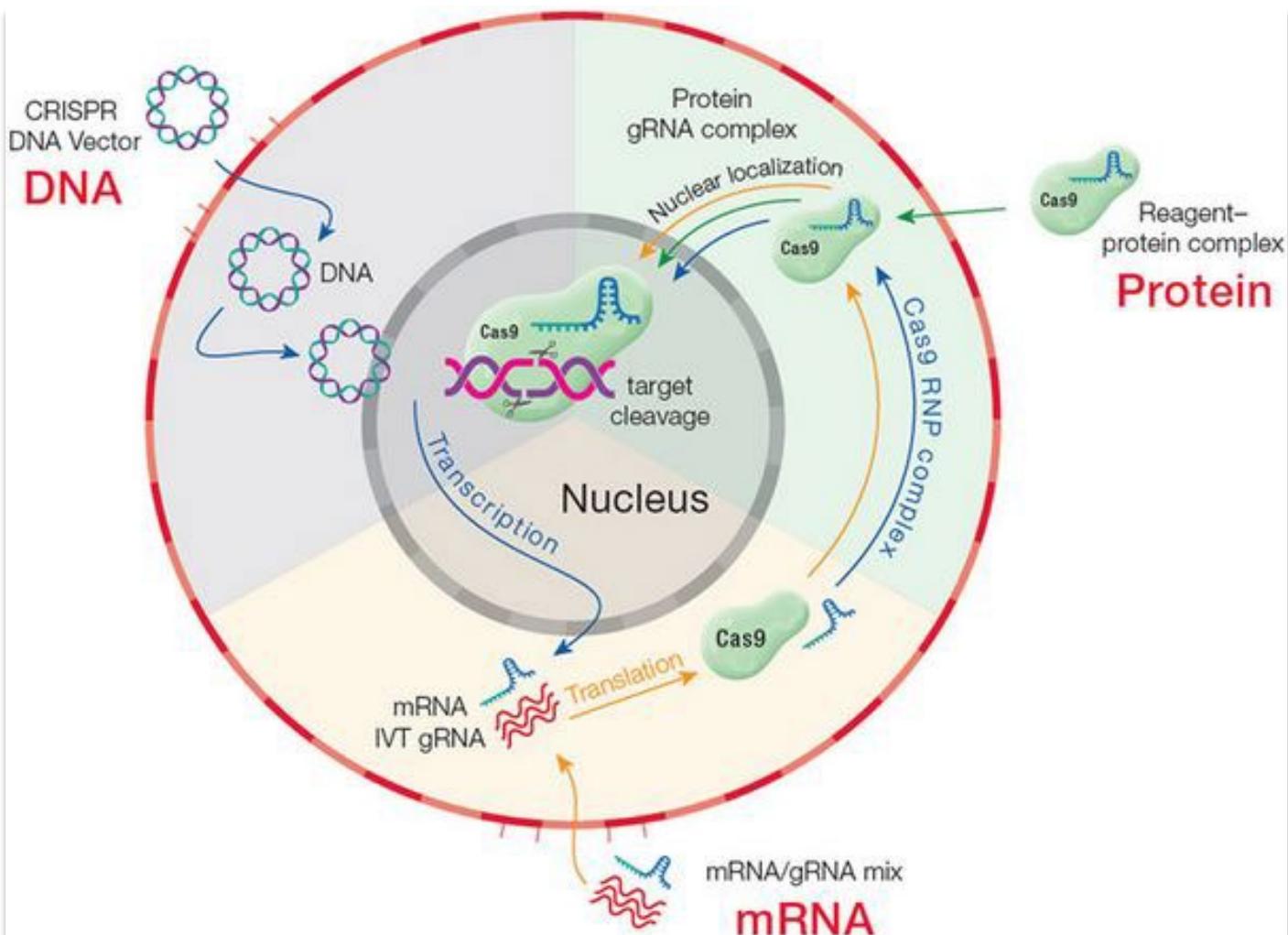
Stage 2
~ 2 months

6. Transfect cells
7. Selection e.g. **Puromycin**
8. Clonal Isolation and expansion

Stage 3

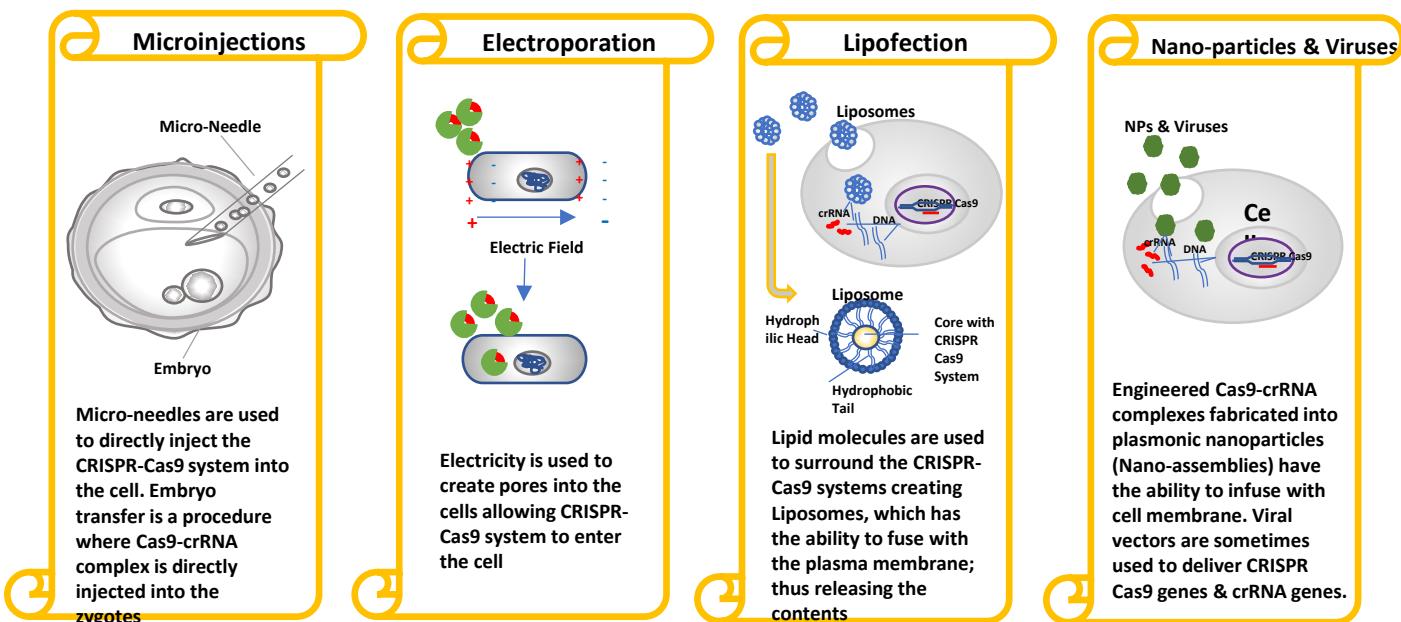
9. Functional characterization and phenotype and genotype analysis

Roadmap of CRISPR tools



Thermofisher

Delivery methods



Sirisha Yeramalli-PhD student UoW

Plasmid-mediated editing

 addgene

[Log In](#) | [Create Account](#)

[Catalog](#) ▾ [Deposit](#) ▾ [Education & Tools](#) ▾ [Help Center](#) ▾

CRISPR Search

We found 21,356 results for: CRISPR X

Select a Category to Narrow Your Search

Catalog

20,604 in Plasmids

18 in Viral Preps

255 in Pooled Libraries

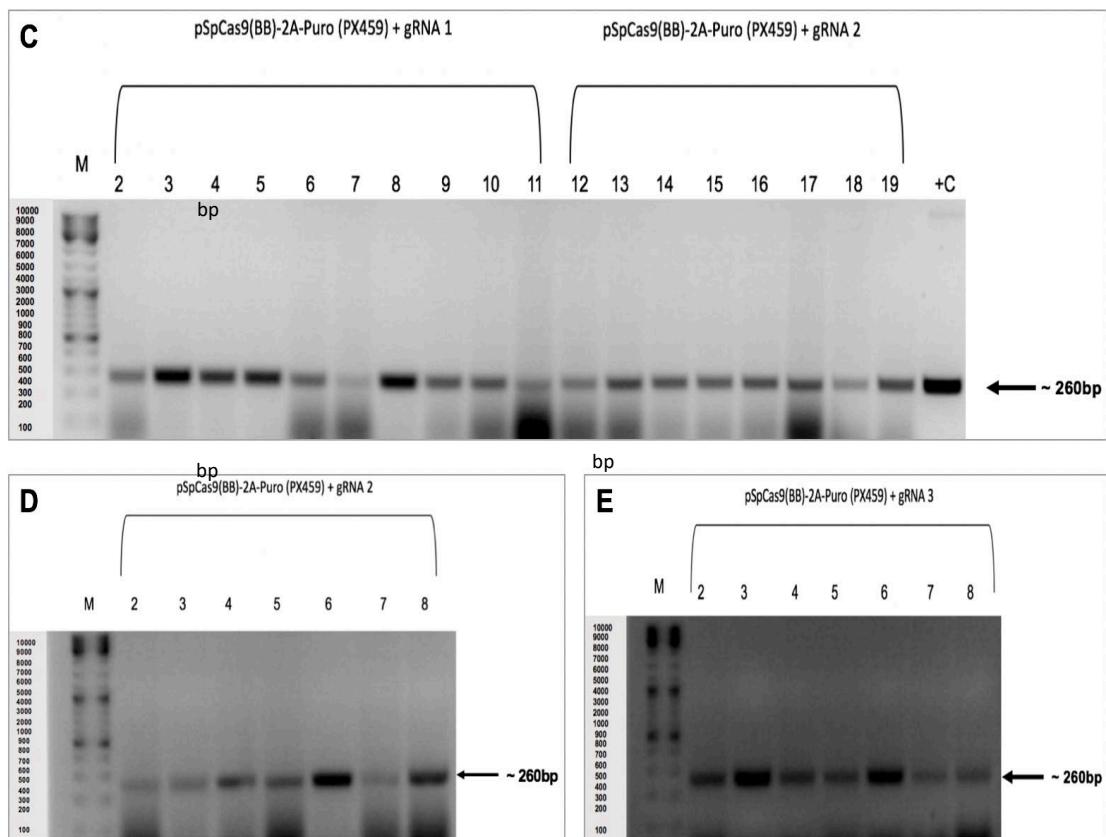
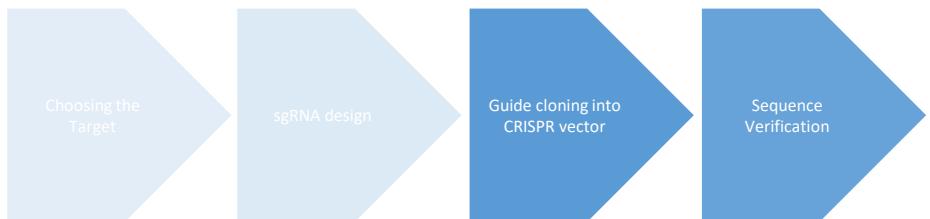
26 in Plasmid Kits

12 in Bacteri

www.addgene.org

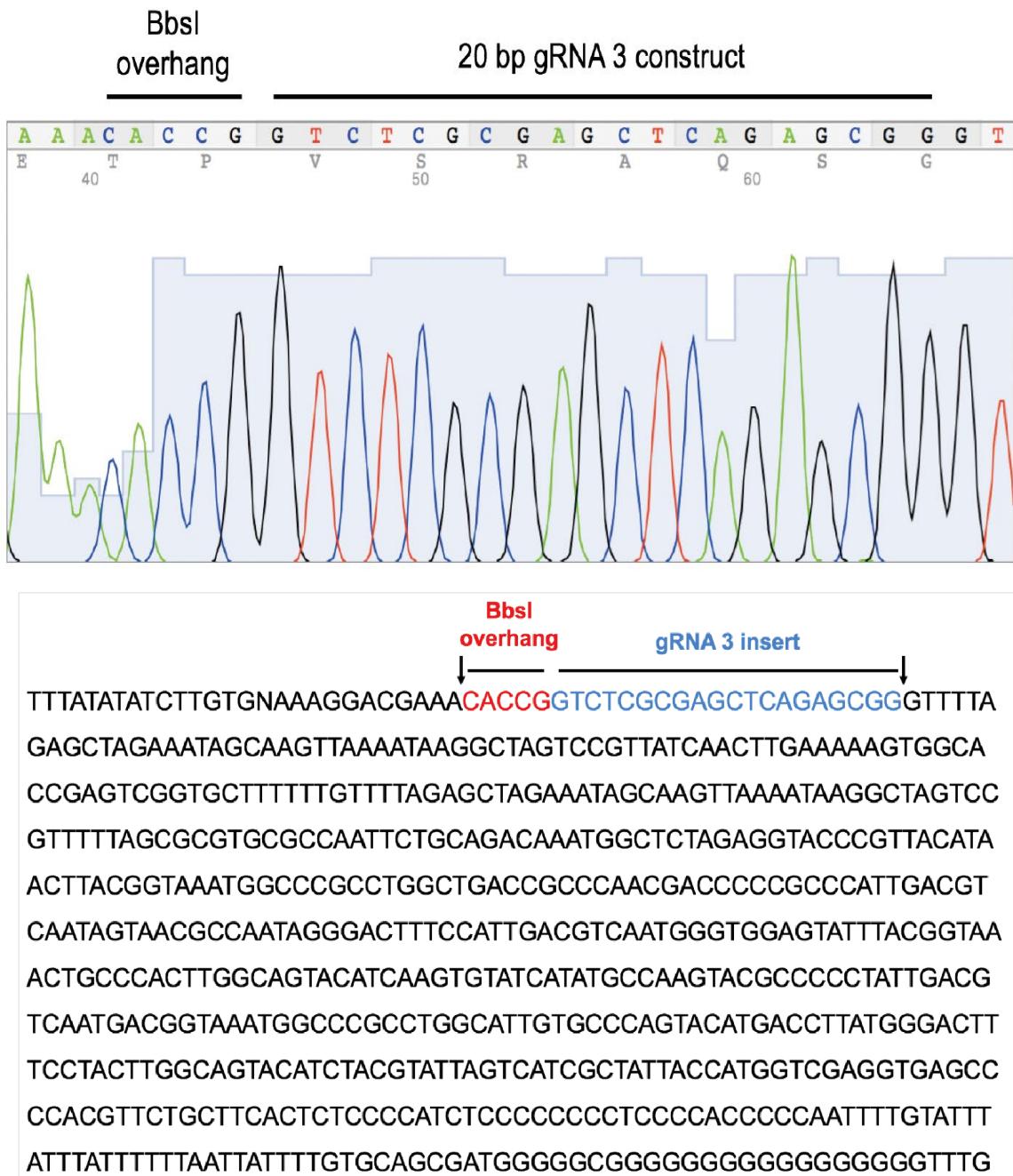
Construction of the guide expression plasmids

Stage 1



Sanger sequencing

U6- Forward primer

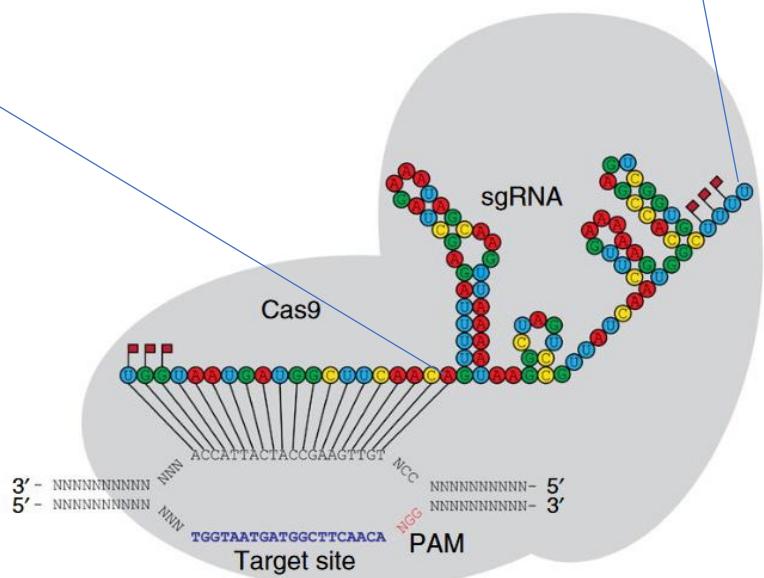


pSpCas9(BB)-2A-Puro (PX459) V2.0 Sequence

1-450bp

GAGGGCCTATTCCATGATTCTCATATTGCATATACGATACA
AGGCTGTTAGAGAGATAATTGGAATTAATTGACTGTAAACACA
AAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTCTT
GGGTAGTTGCAGTTAAAATTATGTTTAAAATGGACTATCAT
ATGCTTACCGTAACTTGAAAGTATTCGATTCTGGCTTATATA
TCTTGTGGAAAGGACGAAACACCGGGTCTTCGAGAAGACCTGT
TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTAT
CAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTGTTTAG
AGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTAGCG
CGTGCGCCAATTCTGCAGACAAATGGCTTAGAGGTACCCGTTA
CATAAC

- Guide sequence
- sgRNA scaffold



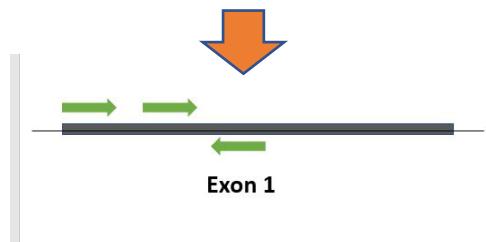
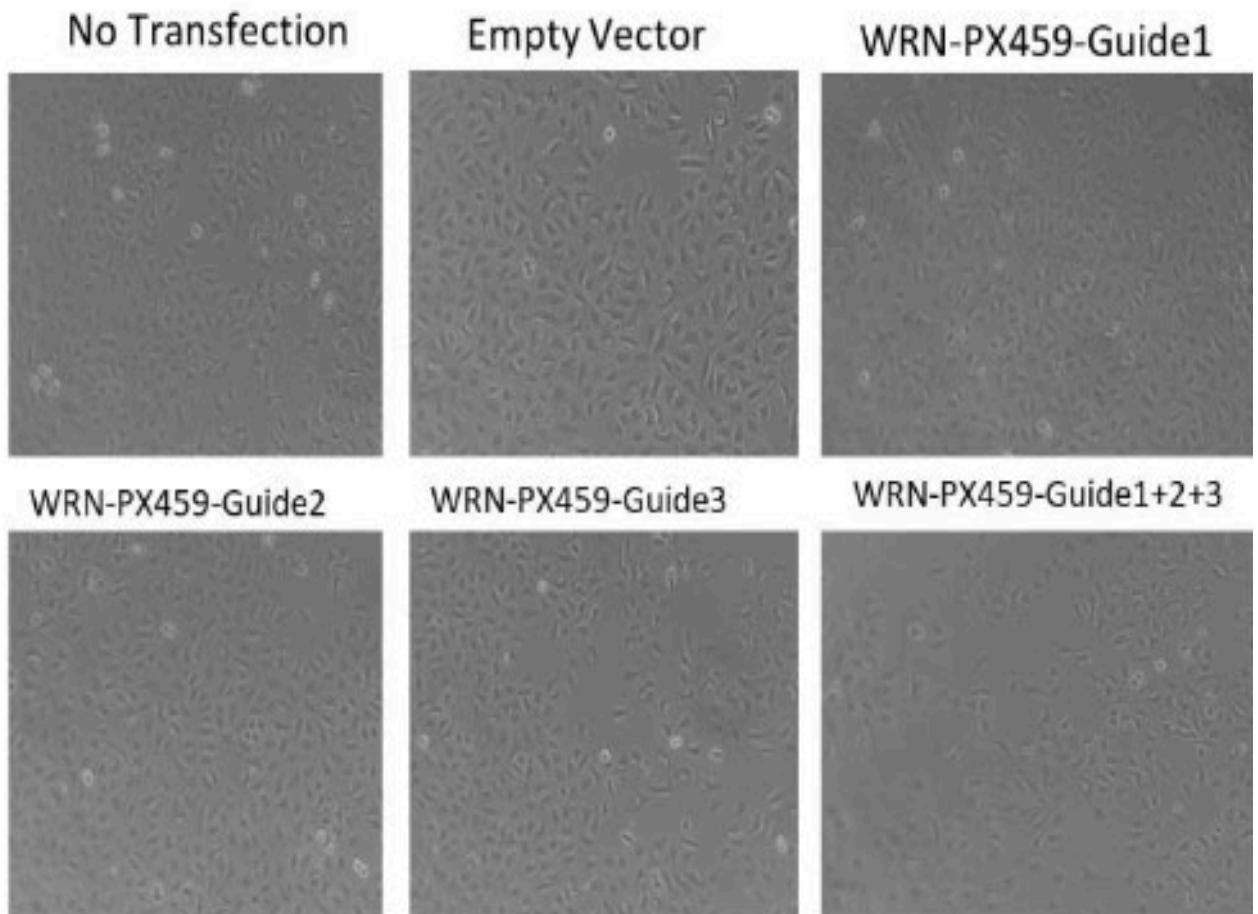
Ran A et al Nature protocols 2013

CRISPR workflow

1. Design and selection of **guide sequences (online algorithm)** ✓
2. Synthesis of single stranded oligos ✓
3. Clone into **CRISPR/Cas9 expression vector** (from addgene) ✓
4. Sanger Sequencing ✓
5. Plasmids purification
6. Transfect cells
7. Selection e.g. antibiotic puromycin
8. Clonal Isolation and expansion
9. Clonal characterization with further analysis and Phenotypic assay

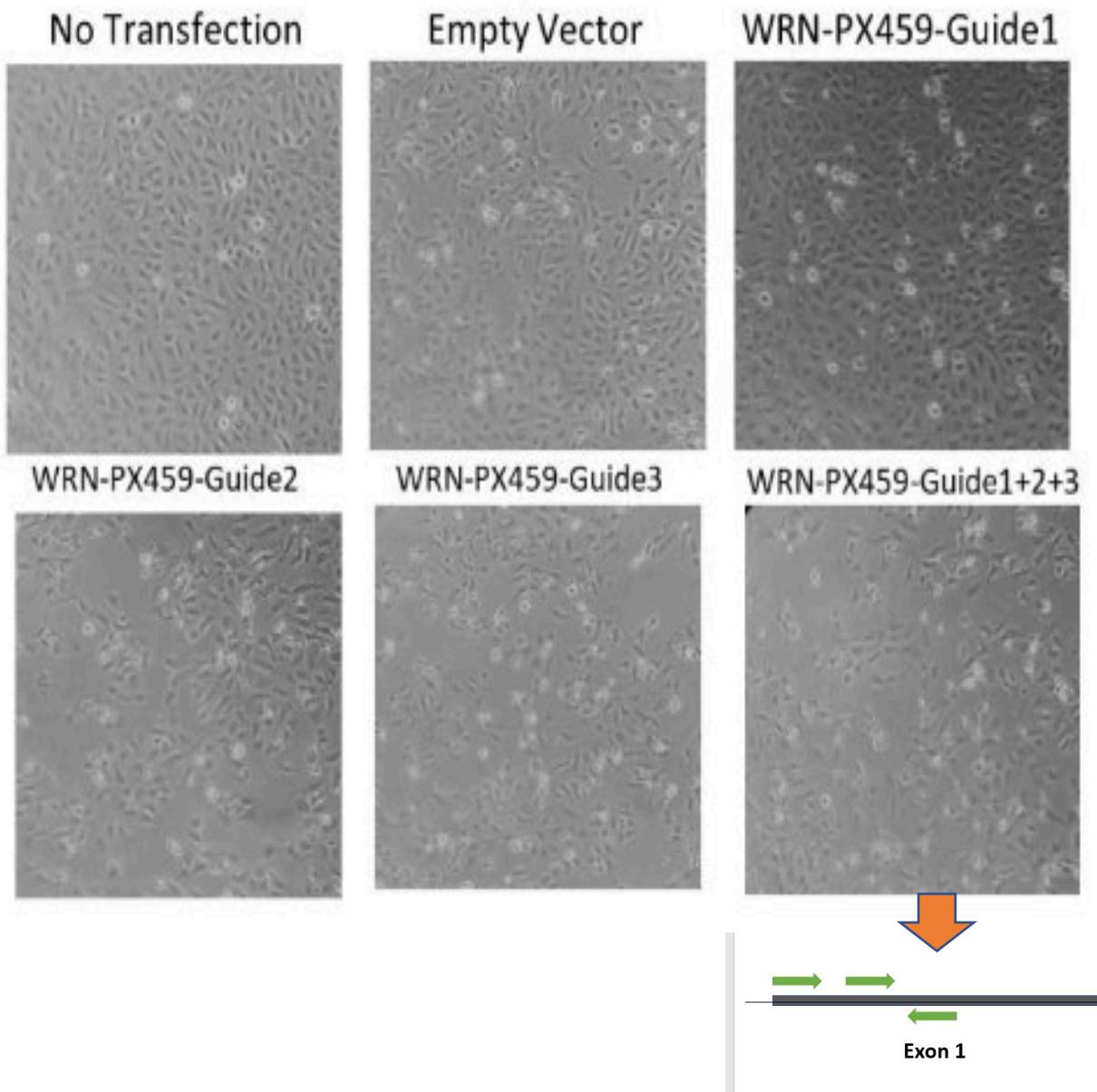
Morphology and Viability

(a) U2OS WT Day-1 Transfection



Morphology and Viability

(c) U2OS WT Day2 - Transfection



Morphology and Viability

(e) U2OS WT - Post antibiotic selection

No Transfection



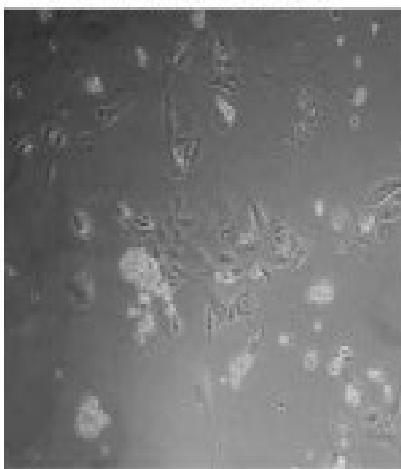
Empty Vector



WRN-PX459-Guide1



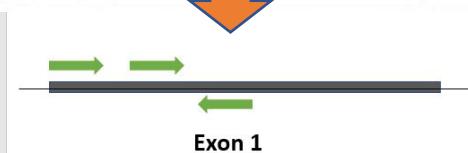
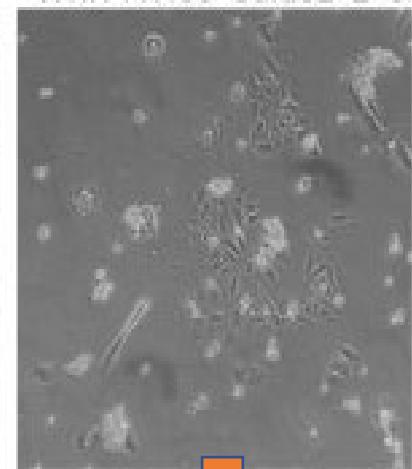
WRN-PX459-Guide2



WRN-PX459-Guide3

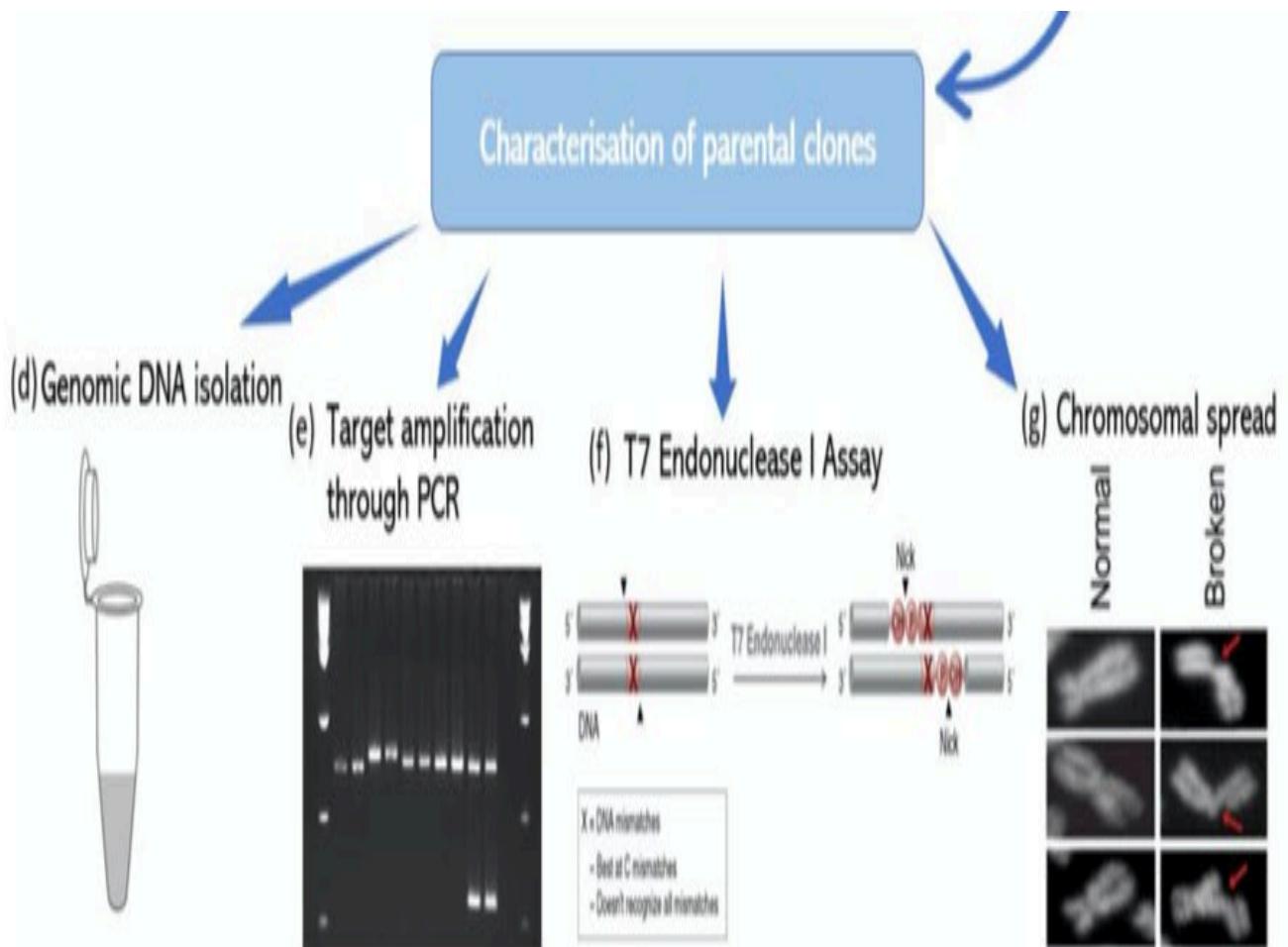


WRN-PX459-Guide1+2+3

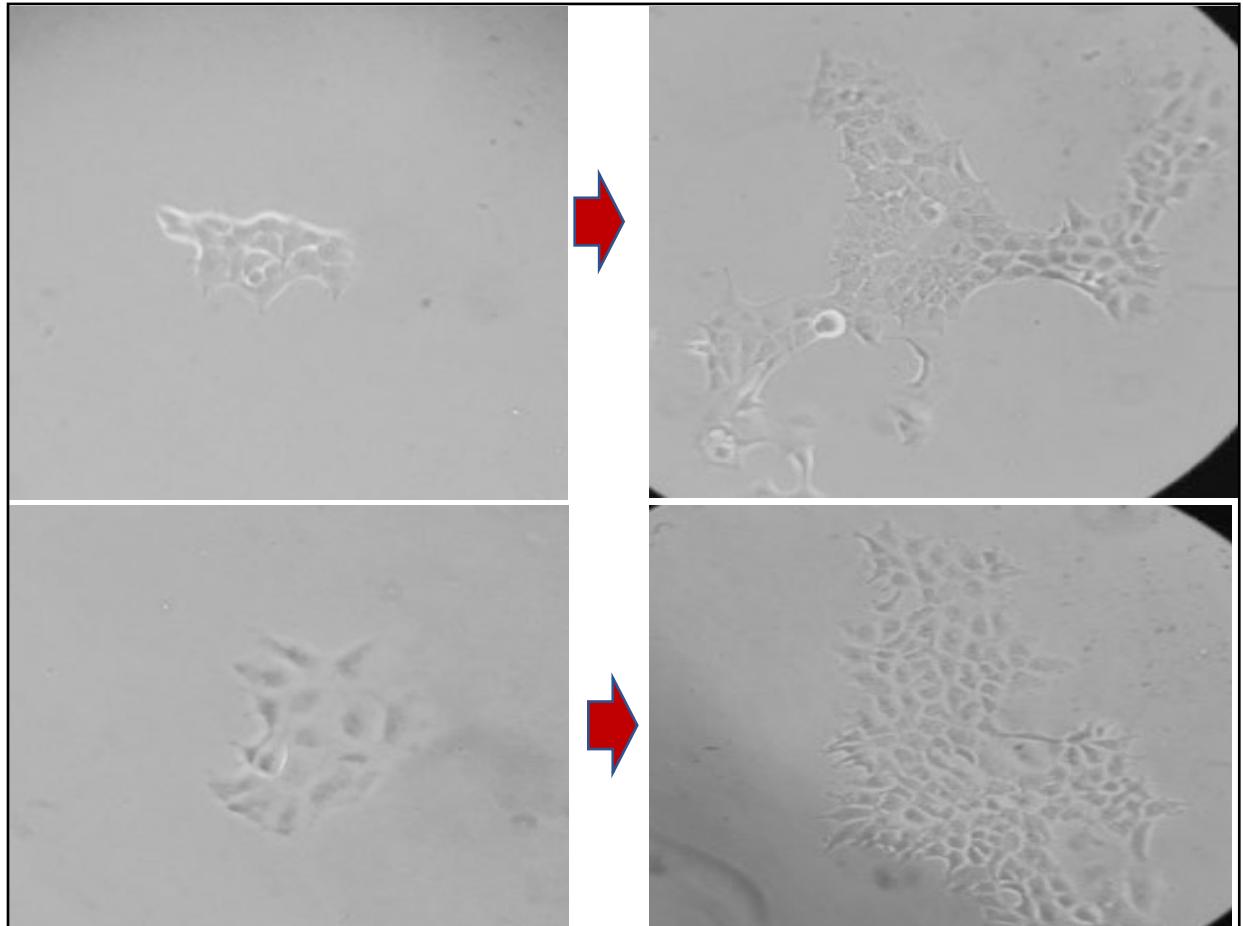


Analysis of parent population

- Important to determine potential editing in your parent Population before generating a monoclonal population
- Saves you time
- Can be used to troubleshoot
- Simple PCR amplification of your target site
- Followed by a simple mismatch detection assay (T7 endonuclease)



Expansion of single colonies

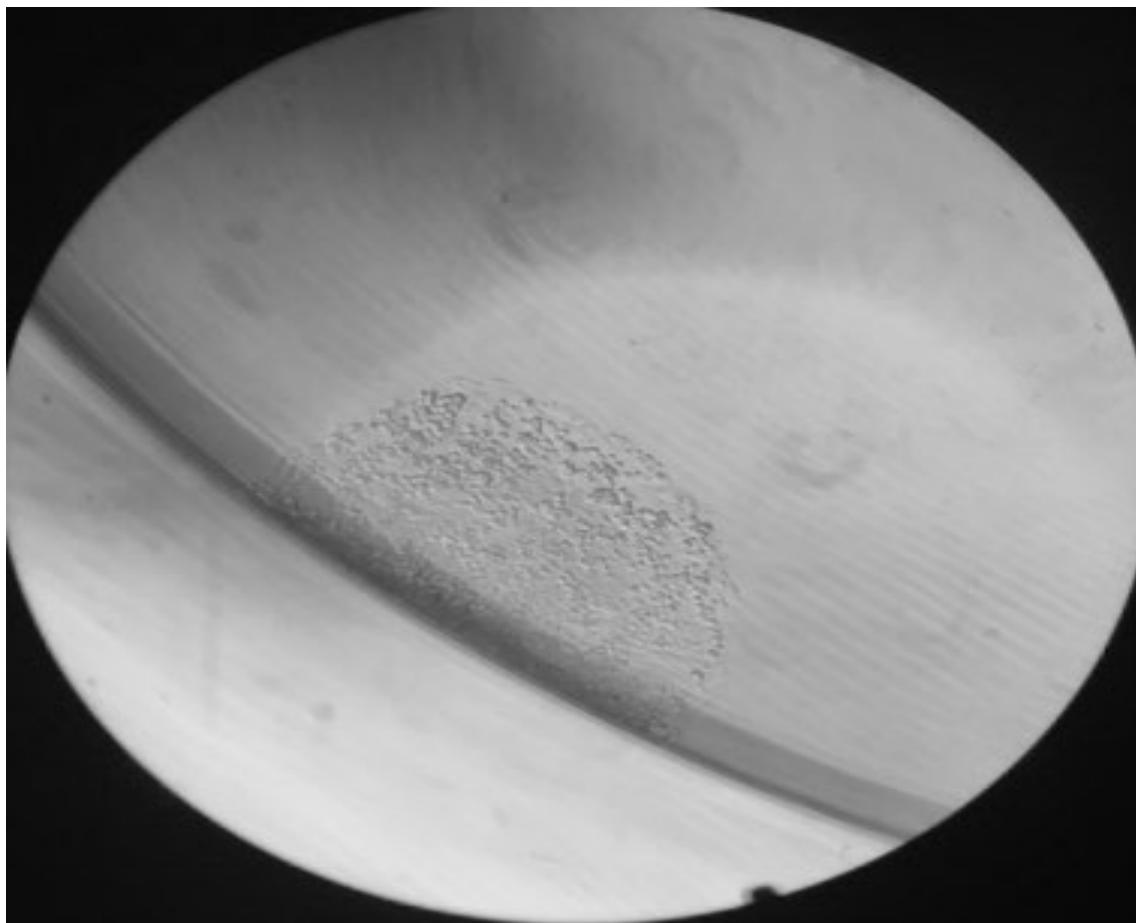


4 Days post-seeding

7 Days post-seeding

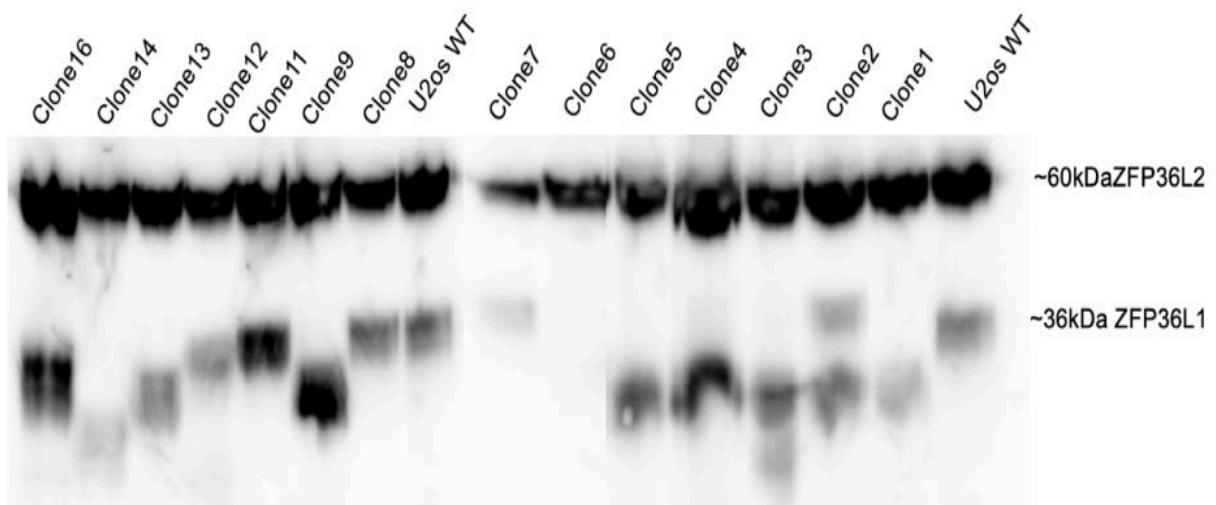
HEK 293 cells 150 cells /10cm dish

Representative image of a single colony of CRISPR Knock-out in a 96 well plate



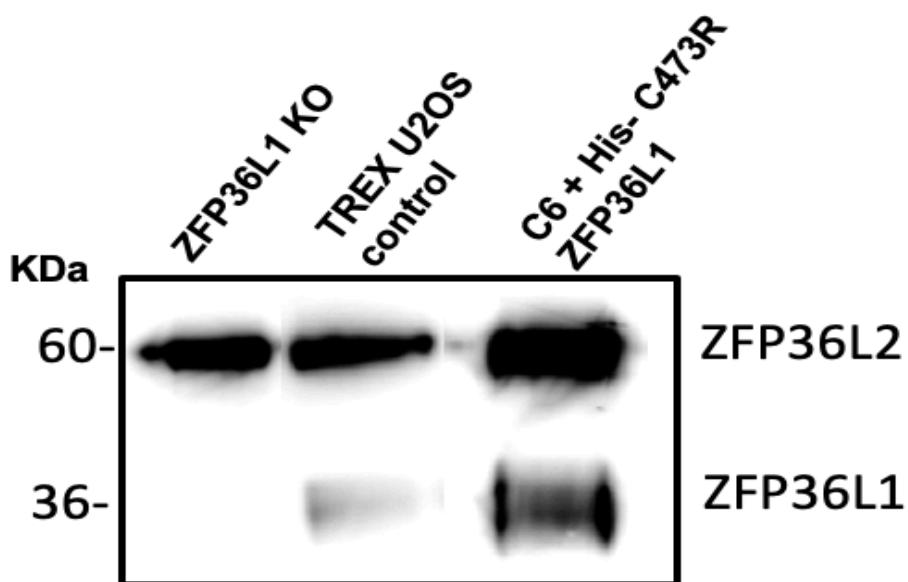
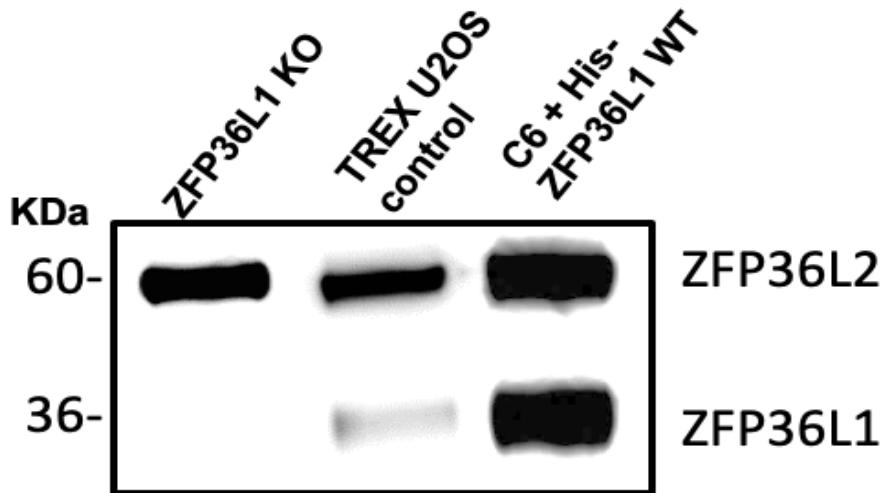
Screening monoclonal antibodies

Activity 2



Dr. Ahmed Sidali, unpublished

Complementation studies



Dr Ahmed Sidali, unpublished

**Verification of Gene Editing in
Parent and Monoclonal lines – in
CRISPRESSO session today**

CRISPR workflow

Stage 1
2 weeks

1. Design and selection of **guide sequences** (online algorithm)
2. Synthesis of single stranded guide oligos
3. Clone into **CRISPR/Cas9 expression vector pX330 series**
4. Sanger Sequencing
5. Plasmids purification

Stage 2
~ 2 months

6. Transfect cells
7. Selection e.g. **Puromycin**
8. Clonal Isolation and expansion

Stage 3

9. Functional characterization and phenotype and genotype analysis

All the best!



Suggested reading list

M. Jinek, K. Chylinski, et al. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*. 337(6096), 816-821 (2012).

P. Mali, L. Yang, et al. RNA-guided human genome engineering via Cas9. *Science*. 339(6121), 823-826 (2013).

T. R. Sampson, D. S. Weiss. Exploiting CRISPR/Cas systems for biotechnology. *Bioessays*. 36(1), 34-38 (2014).

K. Li, G. Wang, et al. Optimization of Genome Engineering Approaches with the CRISPR/Cas9 system. *PLoS ONE*. 9(8), e105779 (2014). doi:10.1371

A. Hendel, E.J. Kildeback, et al. Quantifying Genome-Editing Outcomes at Endogenous Loci with SMRT Sequencing. *Cell Reports*. 7, 293-305 (2014).