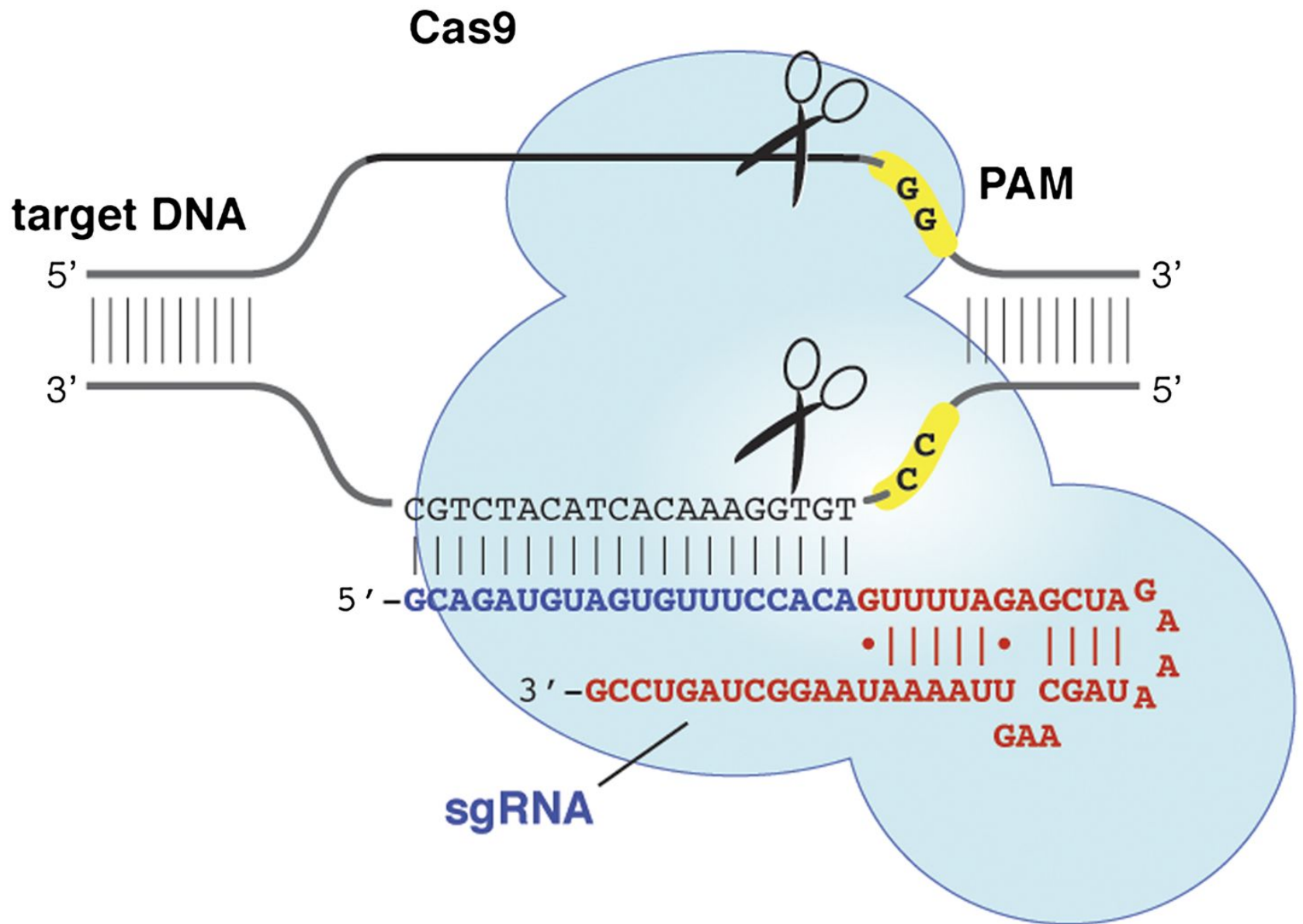


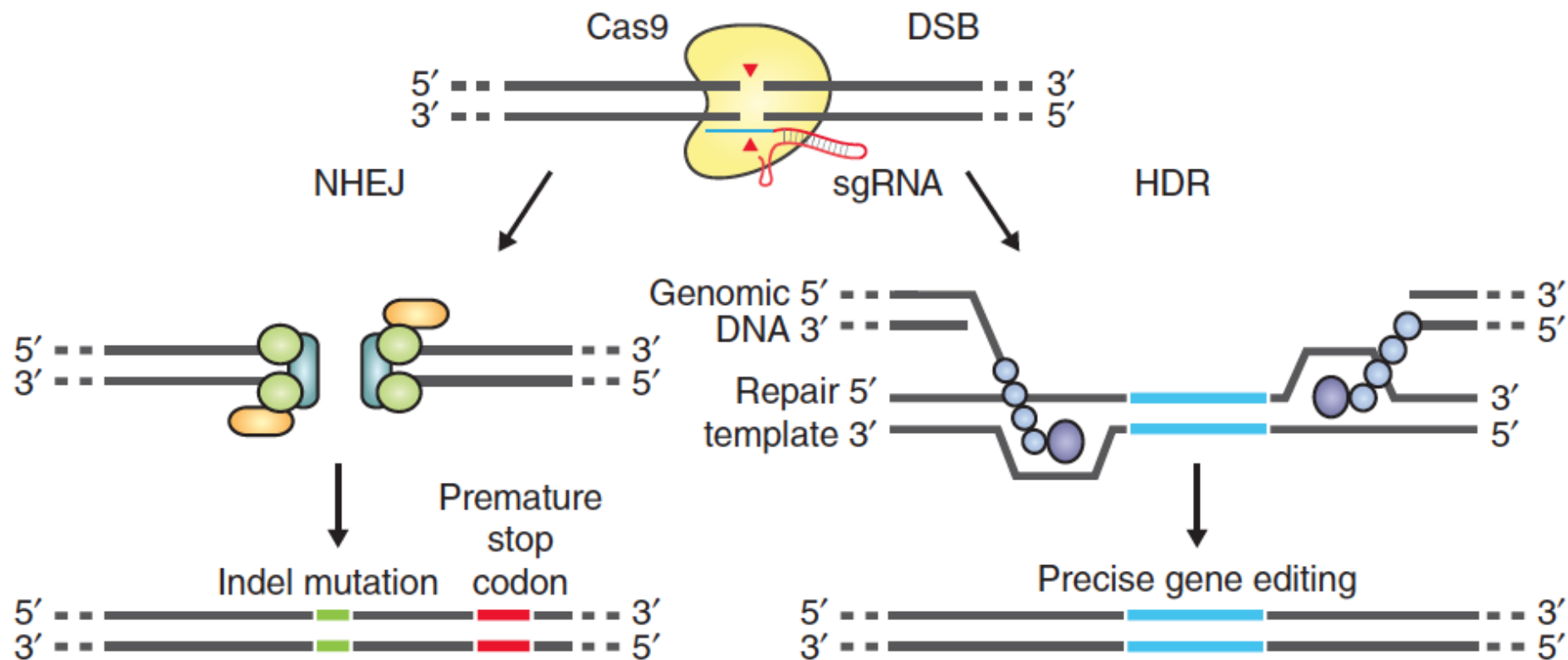
CRISPR Design Considerations

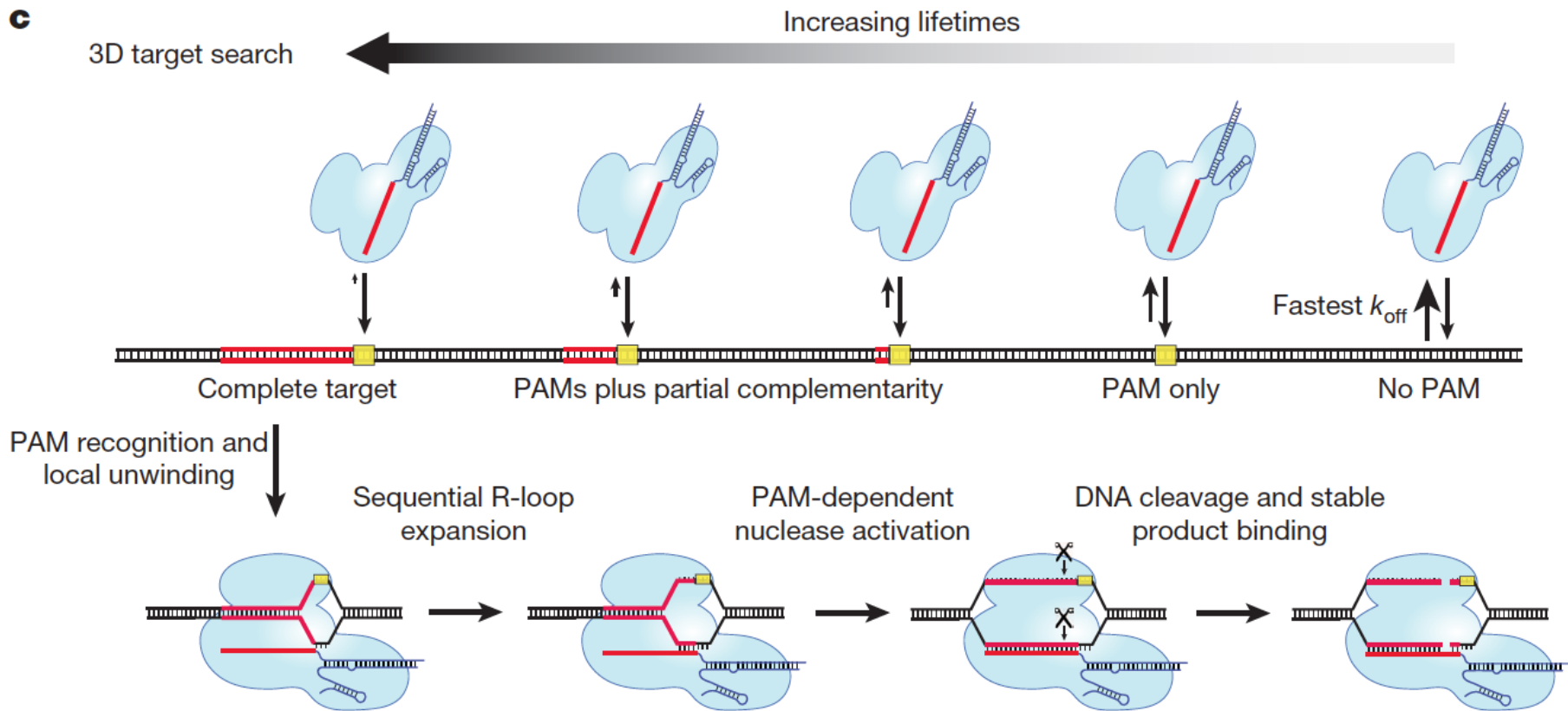
Shifra Ben-Dor

Bioinformatics Unit, Biological Services

April-May 2016

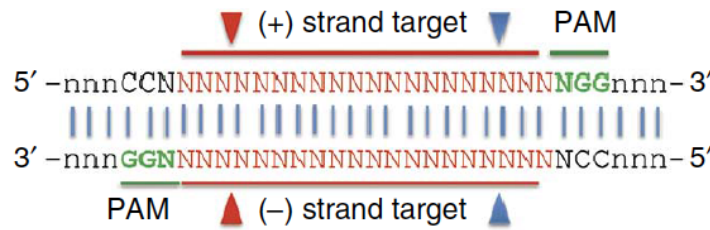




c

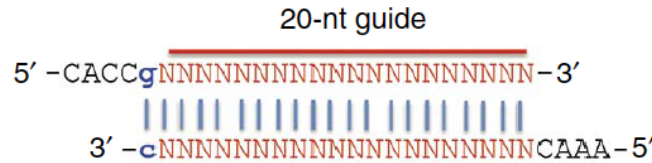
a

Targeted DNA



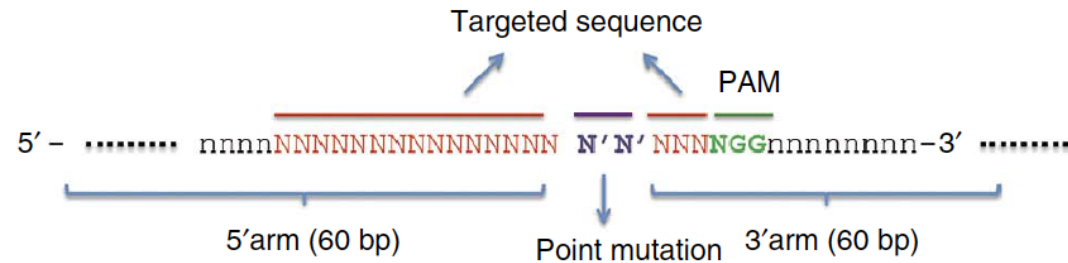
b

Guide oligo for sgRNA



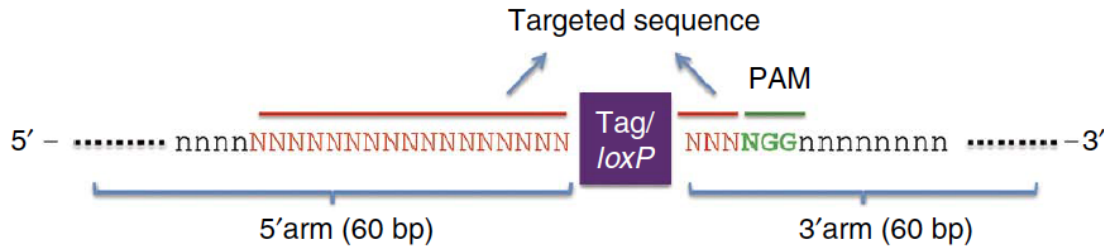
c

ssDNA for point mutation



d

ssDNA for tag/loxP insertion



e

Circular donor vector for large fragment insertion

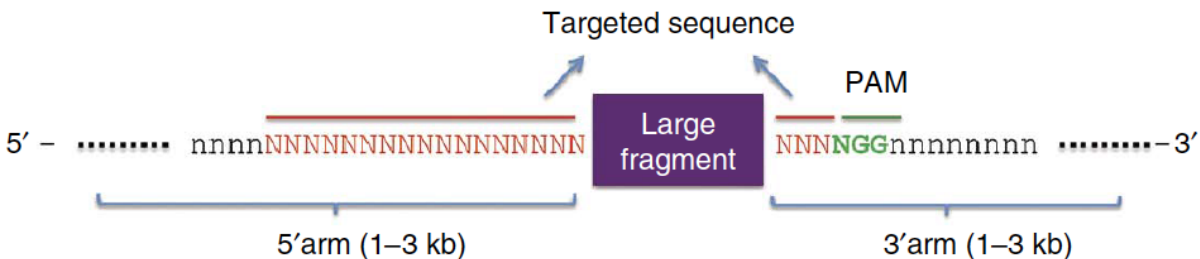


TABLE 1 | Genetically modified mice generated by CRISPR/Cas-mediated genome engineering^{13,14}.

Type of gene modification	Components	Repair	Targeting efficiency (%)	Application
Indels	Cas9 and sgRNA	NHEJ	80–90	Gene disruption by frame shift
Point mutation	Cas9, sgRNA and ssDNA	HDR	50–80	Precise gene modification
Small tag insertion	Cas9, sgRNA and ssDNA	HDR	30–50	Gene labeling
Conditional allele generation	Cas9, sgRNA and ssDNA	HDR	10–20	Conditional gene knockout
Large fragment deletion	Cas9, sgRNA	NHEJ	30 ^a	Gene disruption of specific domain
Large fragment insertion	Cas9, sgRNA and plasmid DNA	HR	10–20	Gene reporter or gene expression

^aThe efficiency of a 700-bp deletion is ~30%. The larger the fragment to be deleted, the lower the efficiency.

A R-03 HBB 31/ 44 = 70%

```

-22 ACCACCAACTTCA:GGGCAGTAACGGCAGACTTCTCCTCAGGAG
-15 ACCACCAACTTCATCCACGTTACCTTGC:CGGCAGACTTCTCCTCAGGAG
-9 ACCACCAACTTCATCCACGTTACCTTGCCCC:TAACGGCAGACTTCTCCTCAGGAG
-3 ACCACCAACTTCATCCACGTTACCTTGC:ACAGGGCAGTAACGGCGGACTTCTCCTCAGGAG
2x -2 ACCACCAACTTCATCCACGTTACCTTGCCCC:CAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
3x -1 ACCACCAACTTCATCCACGTTACCTTGCCCC:ACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
-1 ACCACCAACT:CATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
HBB ACCACCAACTTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
13x WT ACCACCAACTTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
R-03 GACGTTACCTTGCCCCACANGG
19x +1 ACCACCAACTTCATCCACGTTACCTTGCCCCCACAGGGCAGTAACGGCAGACTTCTCCTTAGGA
+9 ACCACCAACTTCATCCACGTTACCTTGCCTTGTTACCGTTACAGGGCAGTAACGGCAGACTTC
+10 ACCACCAACTTCATCCACGTTCTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTT

```

Outline

- Define your question
- Know your gene
- Positive selection (on target efficiency)
- Negative selection (off target probability)
- Repair Oligo Design
- Screening

Outline

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Define your question

- Why are you doing CRISPR?
 - Gene knock-out
 - Point mutation
 - Deletion
 - Insertion (tag, lox sites)
- What?
 - NHEJ or HDR?
 - Cleave or nick (One strand or two)?

Define your question

- When?
 - Conditional or constitutive?
- Where?
 - Cell line or whole animal?
 - Tissue specific?

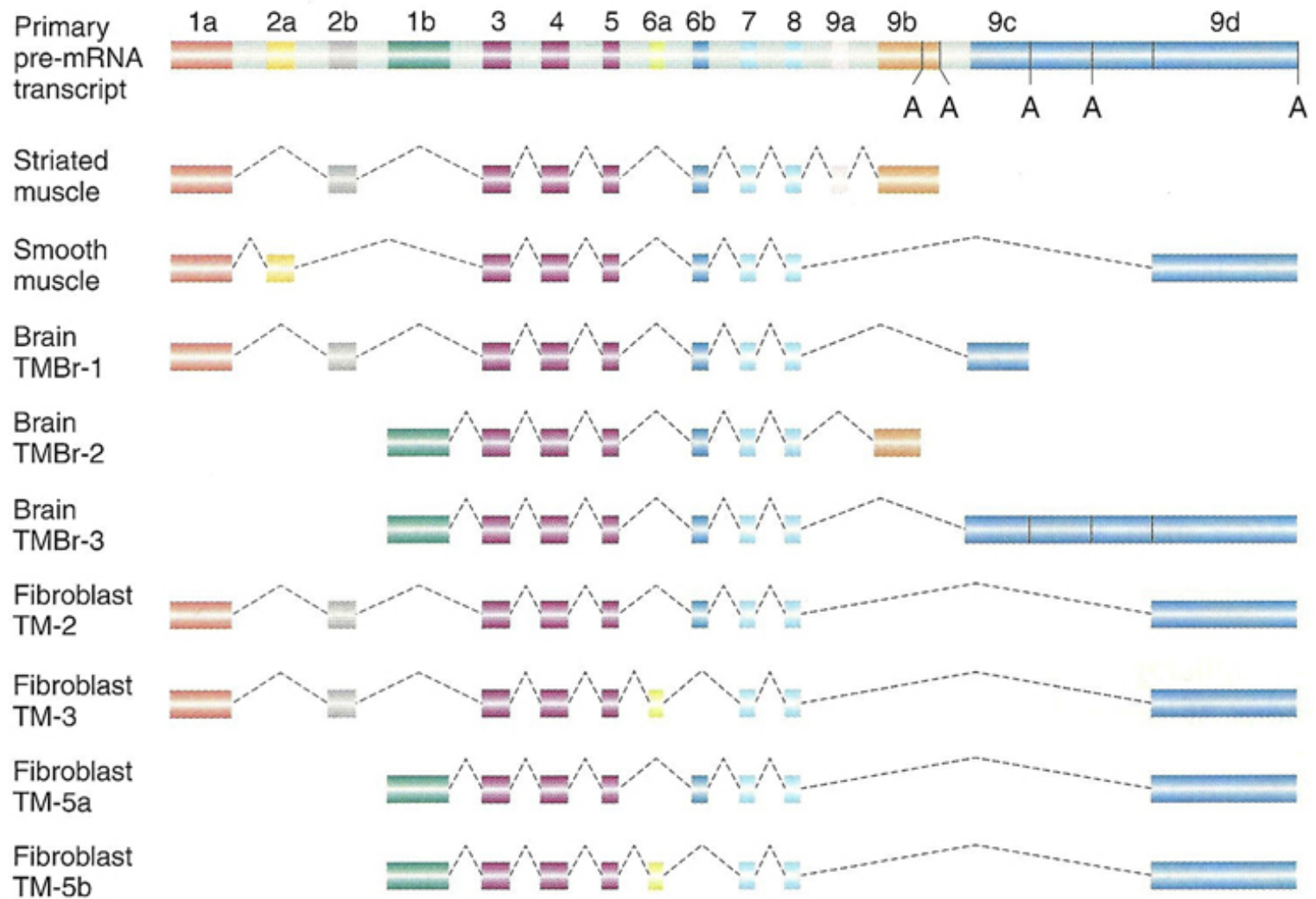
Outline

- Define your question
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Know your gene

- Splice Variants
 - Are there consistent exons?
 - Do they all have the same start?
 - Are the exons in frame?
- How far into the gene can you go?
- Does your gene overlap with other genes?

Alternative splicing in tropomyosin



Know your gene

- Splice Variants
 - Are there consistent exons?
 - Do they all have the same start?
 - Are the exons in frame?
- How far into the gene can you go?
- Does your gene overlap with other genes?

Your Favorite Protein



Active Site

Know your gene

- Splice Variants
 - Are there consistent exons?
 - Do they all have the same start?
 - Are the exons in frame?
- How far into the gene can you go?
- Does your gene overlap with other genes?

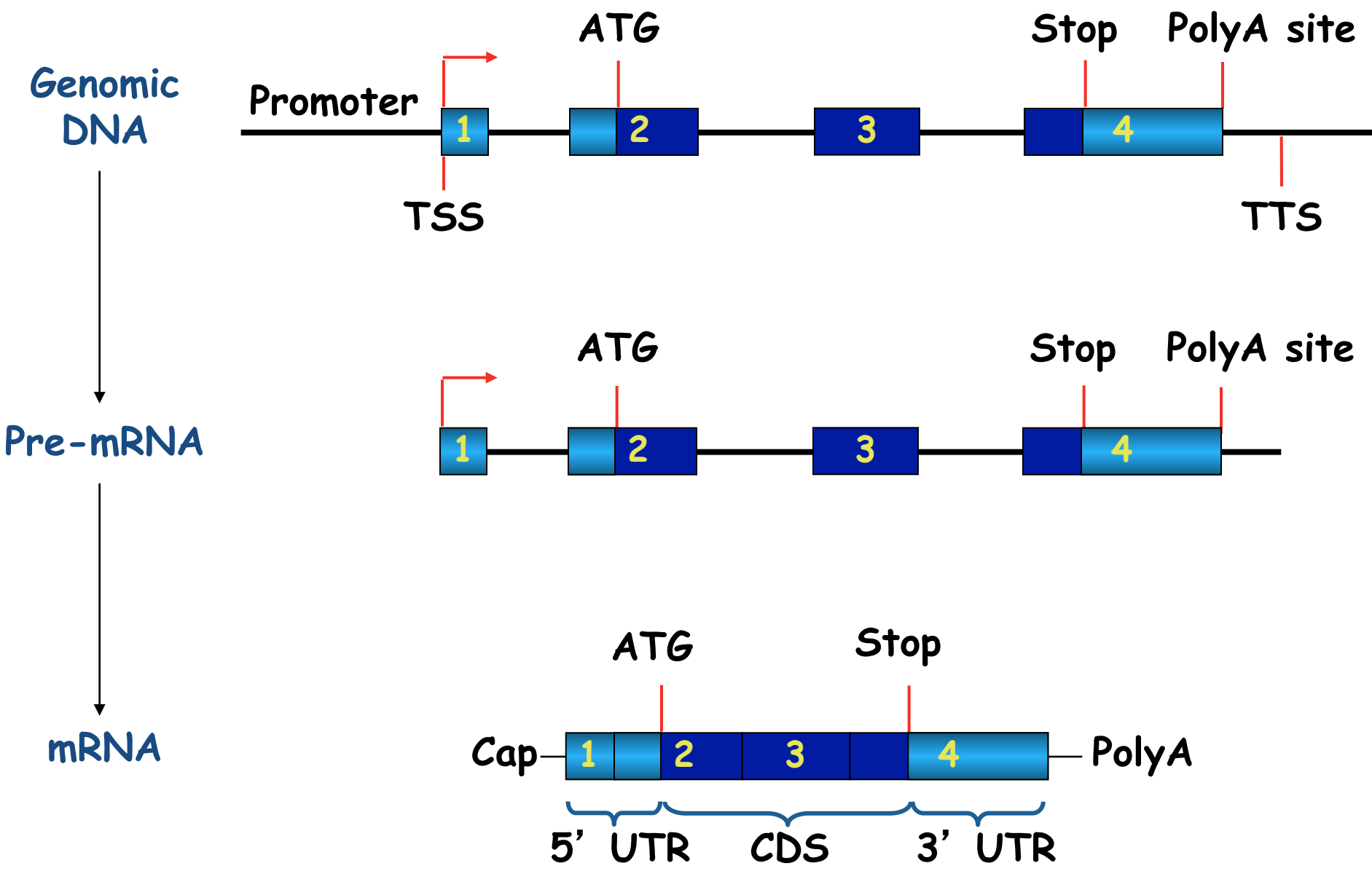
Other considerations

Residual protein

- If a whole exon was removed
- If an alternate ATG can be activated
- If the stop codon isn't introduced in the right place

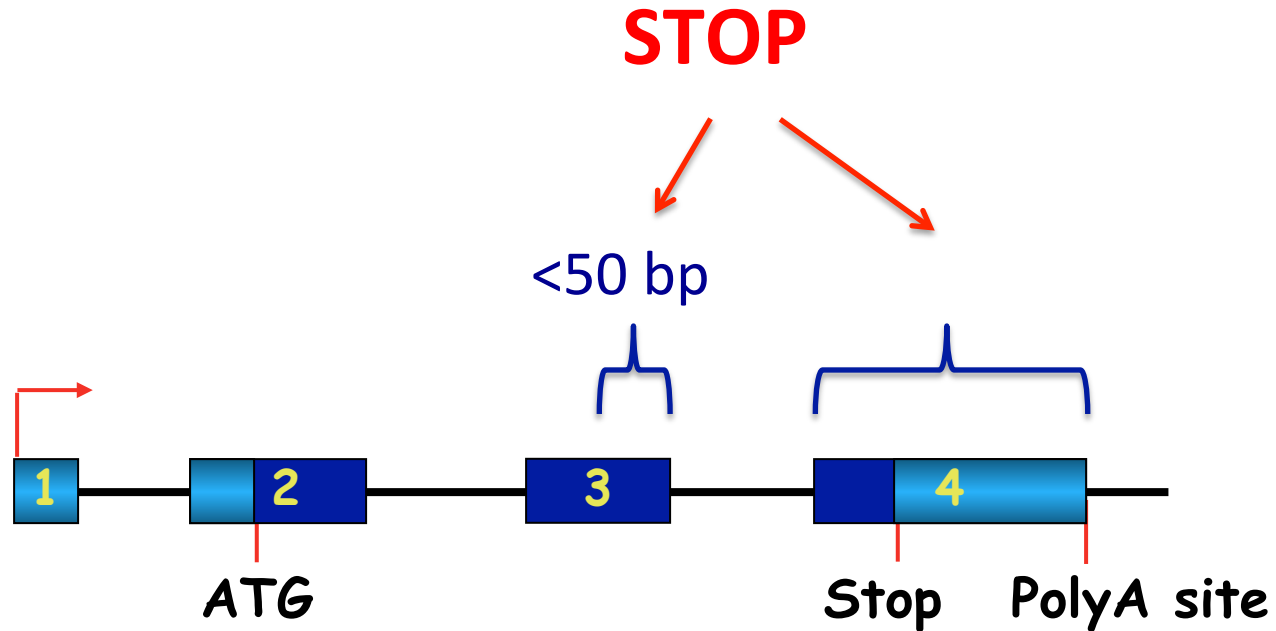
NMD

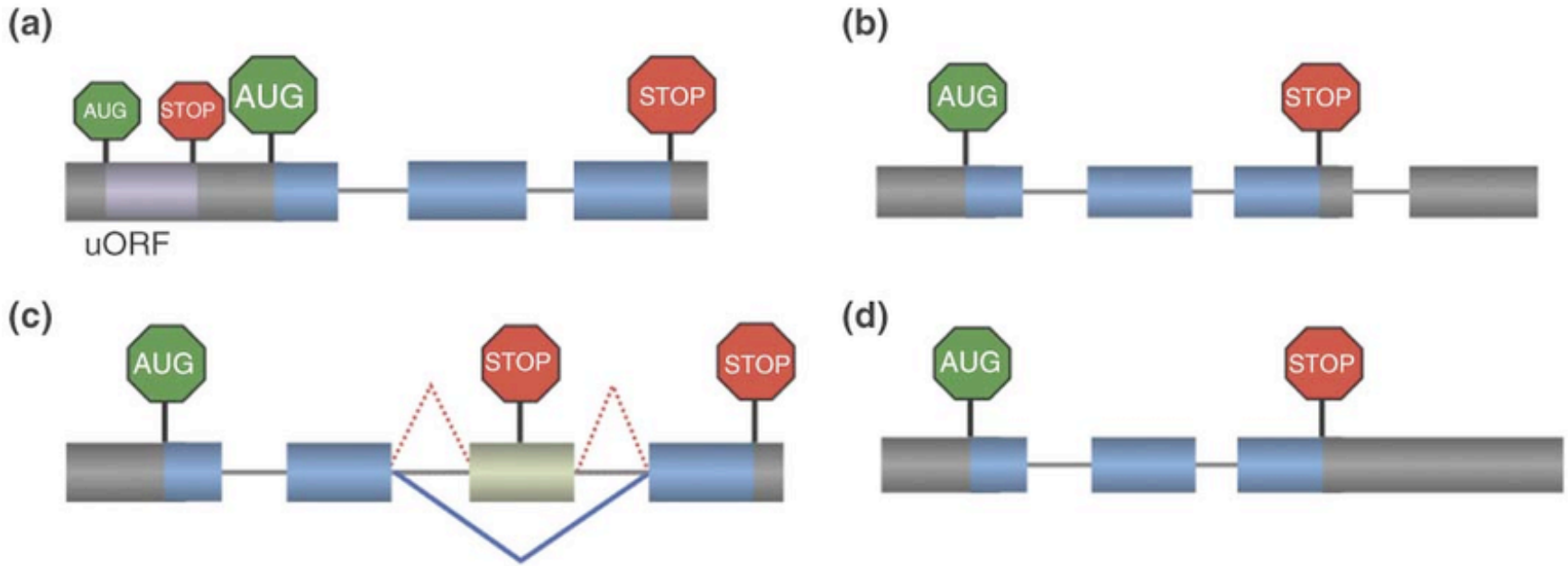
- Nonsense mediated decay



Modified from Zhang MQ Nat Rev Genet. 2002 Sep;3(9):698-709.

Stop codon must be in last exon, or within 50 bp of last junction





Genomic Considerations

- Family members
- Pseudogenes
- Conditional Knockouts
 - Control regions (Promoter, Enhancer)
 - Overlapping genes

Guide Design



Starting Point

Sequence Guide

PAM

Cleavage Site



NNNNNNNNNNNNNNNNNNNNNNNN NGG



G



Promoter
Required



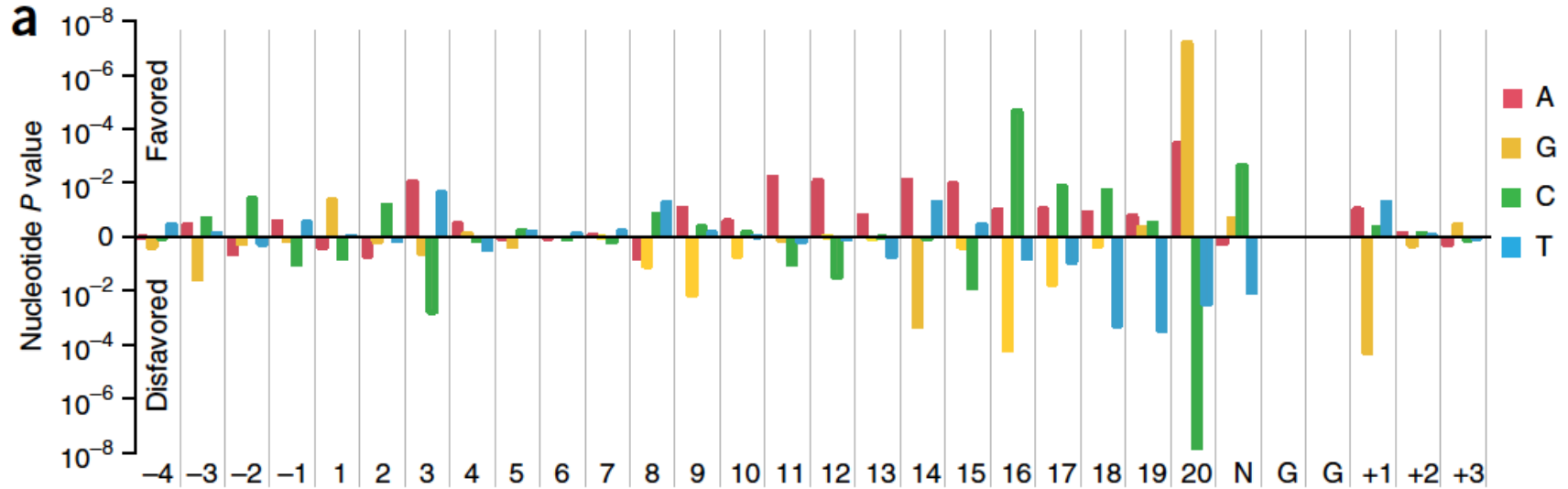
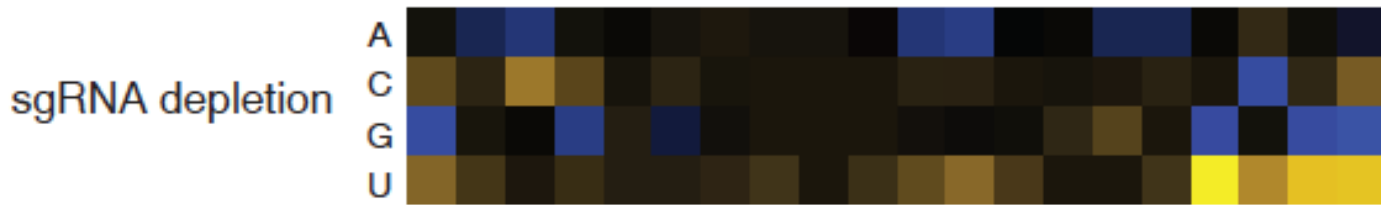
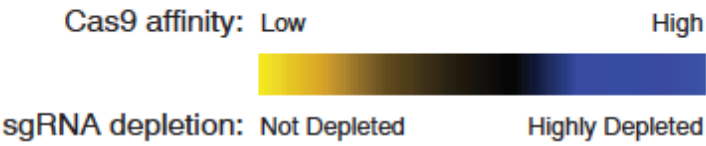
“Seed” Region

Outline

- Define your question
- Know your gene
- **Positive selection (on target efficiency)**
- Negative selection (off target probability)
- Repair Oligo Design
- Screening

Positive Selection

- G/C content
- Base Preferences



sgRNA Designer

<http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design>

Score is from 0 to 1, the higher the better.
Over 0.2 is supposed to work well



Design sgRNAs

This website implements the sgRNA scoring algorithm described in [Doench, Hartenian, et al., Nature Biotechnology, 2014](#).

For a stand-alone version to score sgRNAs, please see this short [python script](#).

For instructions detailing how to use this tool, please see our [sgRNA Designer Help Page](#). Please also visit [Addgene](#) for further discussion on sgRNA design.

Note: This form accepts up to 10 Human or Mouse ENSEMBL (e.g., 'ENST00000544455', 'ENSMUST00000044620', etc.) transcript IDs or a single nucleotide sequence.

File inputs must be smaller than 10kb in size, and any sequences submitted via file *must* be in FASTA format.

No file selected.

Rule Set:

“Facts are stubborn things,
but statistics are more pliable.”



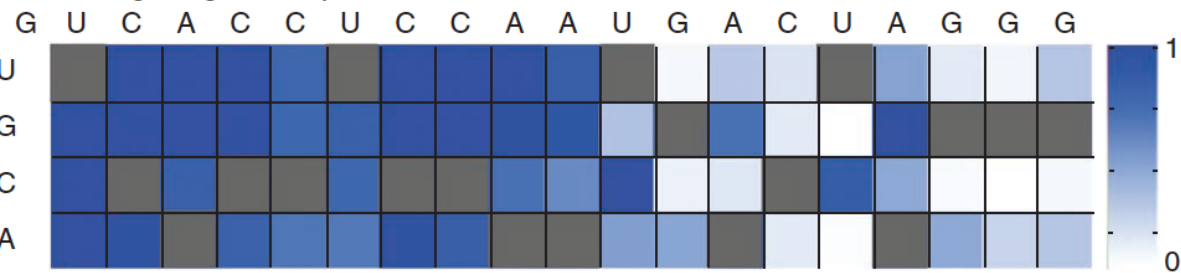
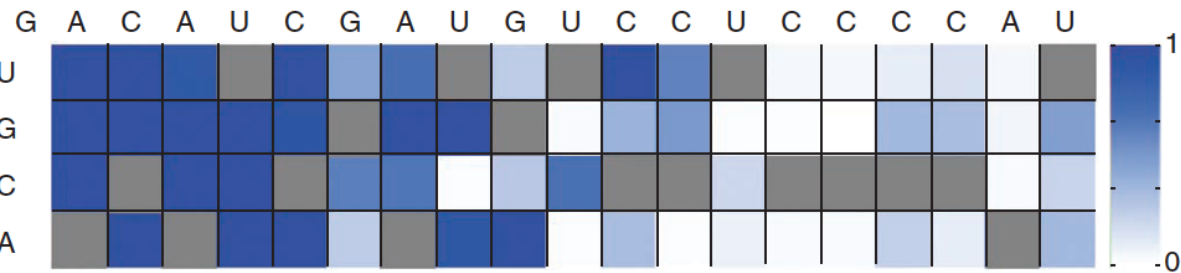
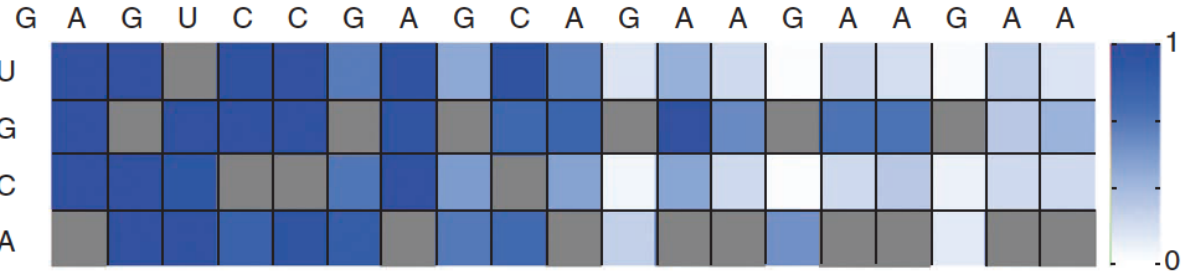
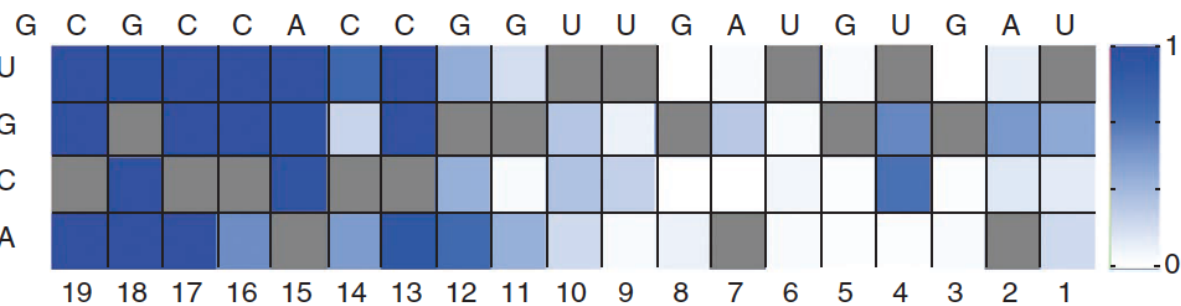
A group at WIS designed CRISPR based on off-target alone, and retroactively checked the positive scores: One scored 0.0629 and the other scored 0.04859. They both worked beautifully.

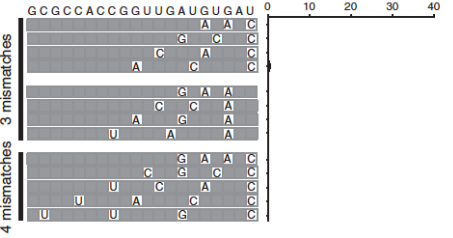
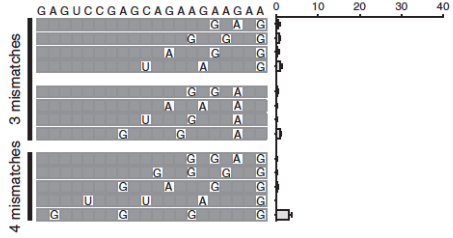
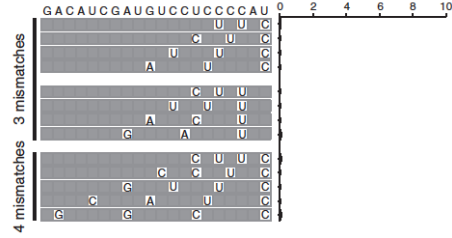
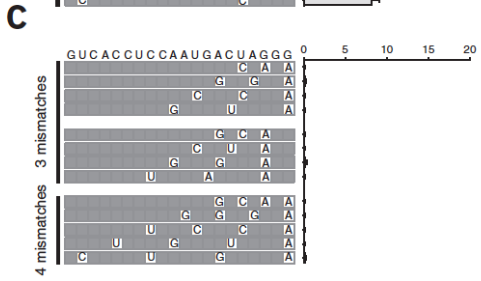
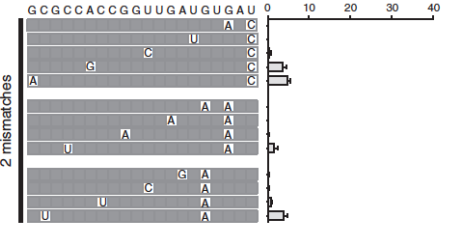
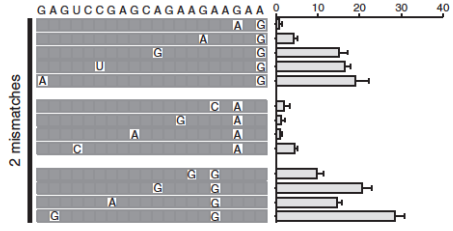
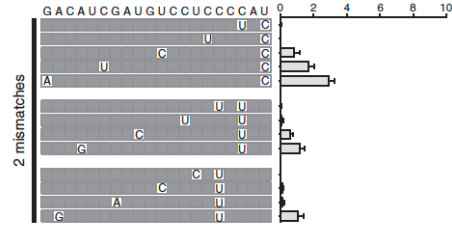
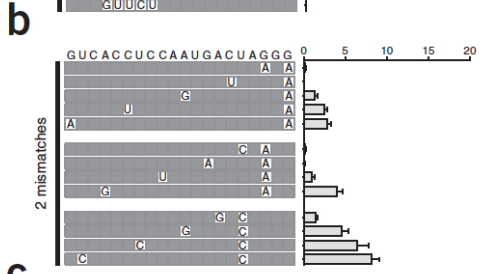
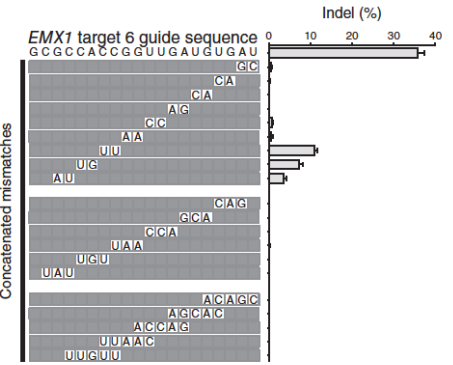
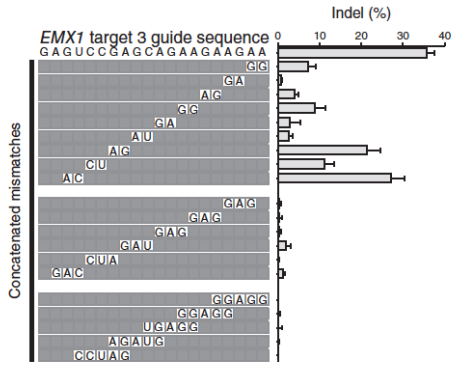
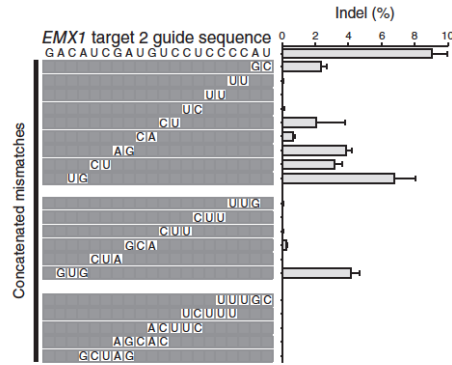
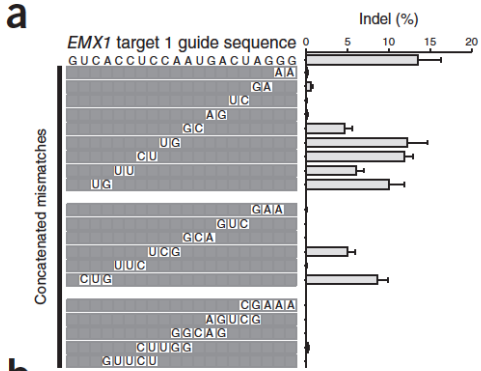
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OFF TARGET HAPPENS



b*EMX1* target 1 guide sequence*EMX1* target 2 guide sequence*EMX1* target 3 guide sequence*EMX1* target 6 guide sequence



Off Target programs

Zhang lab	http://crispr.mit.edu/
E-CRISP	http://www.e-crisp.org/E-CRISP/
CHOPCHOP	https://chopchop.rc.fas.harvard.edu/
ZIFIT	http://zifit.partners.org/ZiFiT/
GT-Scan	http://gt-scan.braembl.org.au/
COSMID	https://crispr.bme.gatech.edu/
sgRNAscas9	http://www.biotoools.com/
CasOT	http://eendb.zfgenetics.org/casot/
DNA 2.0	https://www.dna20.com/eCommerce/cas9/input

Considerations in off-target

- Mismatches
- PAM
- G/C content
- Is an initial G required?
- What version of the genome is used?
- Are the criteria updated?
- Does it come with primer design?

Mismatches

- How many mismatches are allowed?
 - 2, 3, 4 or more?
- Where are the mismatches allowed?
 - PAM proximal as compared to PAM distal
- What type of mismatches are allowed?
 - Insertions and deletions?

Considerations in off-target

- Mismatches
- PAM
- G/C content
- Is an initial G required?
- What version of the genome is used?
- Are the criteria updated?
- Does it come with primer design?

PAM

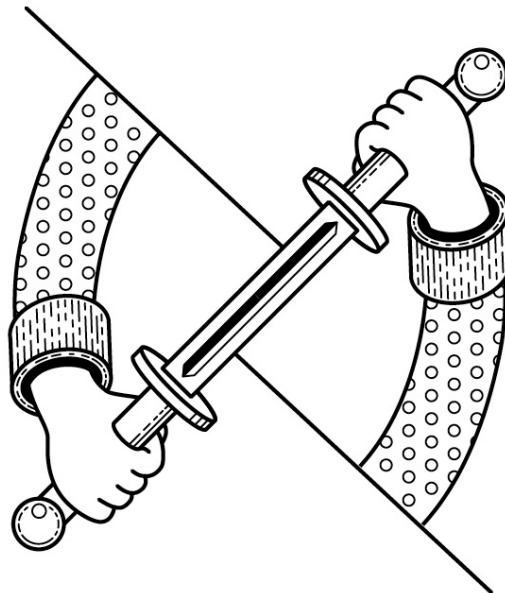
- NGG is the 'classic' but others may be acceptable
 - NAG
 - Possibly others

Considerations in off-target

- Mismatches
- PAM
- G/C content
- Is an initial G required?
- What version of the genome is used?
- Are the criteria updated?
- Does it come with primer design?

G/C content

- For activity, a higher G/C content is preferred
- For specificity, a lower G/C content is preferred



Considerations in off-target

- Mismatches
- PAM
- G/C content
- Is an initial G required?
- What version of the genome is used?
- Are the criteria updated?
- Does it come with primer design?

How can you double check?

- Run your own Blast (but change the parameters)
- Make sure you don't have a genomic repeat sequence
- Check if the genome sequence is masked

Program Selection

Optimize for

- Highly similar sequences (megablast)
- More dissimilar sequences (discontiguous megablast)
- Somewhat similar sequences (blastn)

Choose a BLAST algorithm 

Make sure you are doing genome blast, and run against only the reference version of the genome!

BLAST

Search **database Genome (reference only) - Drosophila melanogaster** using **Blastn (Optimize for somewhat similar sequences)**


Show results in a new window


Algorithm parameters

Note: Parameter values that differ from the default are highlighted in yellow and marked with **◆** sign

General Parameters

Max target sequences


100 

Select the maximum number of aligned sequences to display 


Short queries

Automatically adjust parameters for short input sequences 


Expect threshold

◆ 1000 

Word size


◆ 7 

Max matches in a query range


0 

Scoring Parameters

Match/Mismatch Scores


◆ 1,-1 


Gap Costs

◆ Existence: 2 Extension: 1 


Filters and Masking


Filter

◆ Low complexity regions 

◆ Species-specific repeats for: 

Mask

◆ Mask for lookup table only 

Mask lower case letters 

BLAST

Search **database Genome (reference only) - Drosophila melanogaster** using **Blastn (Optimize for somewhat similar sequences)**

Show results in a new window

Outline

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Repair Oligo Design

- Keep in mind:
 - We don't want the repair oligo to be cleaved, if possible mutate the PAM, if not, introduce mismatches
 - Add or delete cleavage site for screening
 - Don't add mutations too far from each other, need at least 60bp flanking, and a total length of no longer than 200bp

Other issues

Lox sites for conditional mutations may fold over on themselves (partly palindromic)

CRISPR Screening Considerations

So, you've done CRISPR...
now we have see if it worked

- Screening of CRISPR experiments is as variable as the types of use of CRISPR
- Different uses will have different methods of screening
- Some types have more options than others

Activity vs Mutation

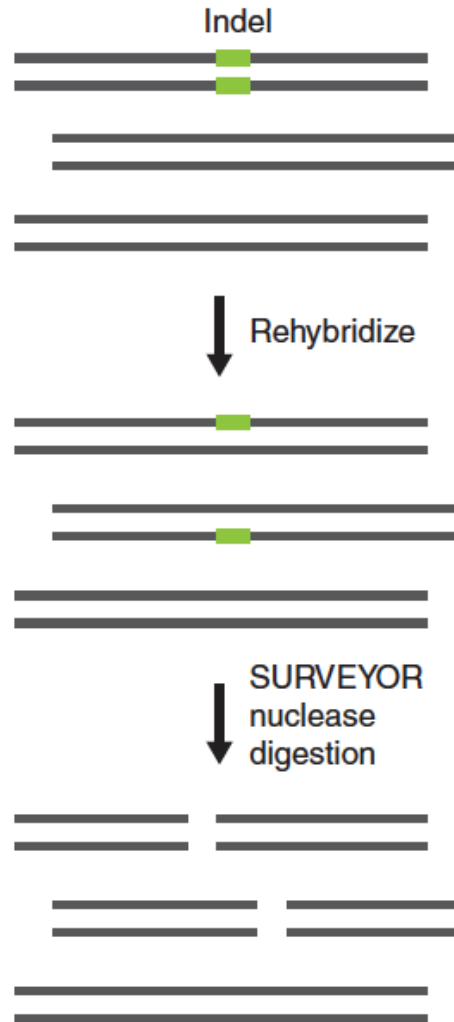
In cases where we are trying to knockout or mutate a protein, do we check the DNA for mutation, or do we 'go for the gold' and check protein presence or activity?

Types of indel screening

- EMC (Enzyme Mismatch Cleavage)
 - Surveyor, T7E1, etc
- HRM (High Resolution Melt)
- IDAA (Indel Detection by Amplicon Analysis)
- PCR
- Sequencing
 - NGS
 - Sanger

EMC

a



SURVEYOR:

DYRK1A

GRIN2B

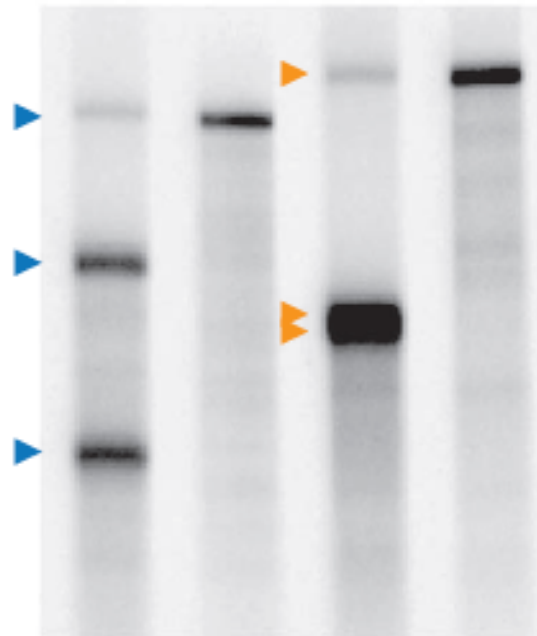
sgRNA:

1 + 2

-

1 + 2

-

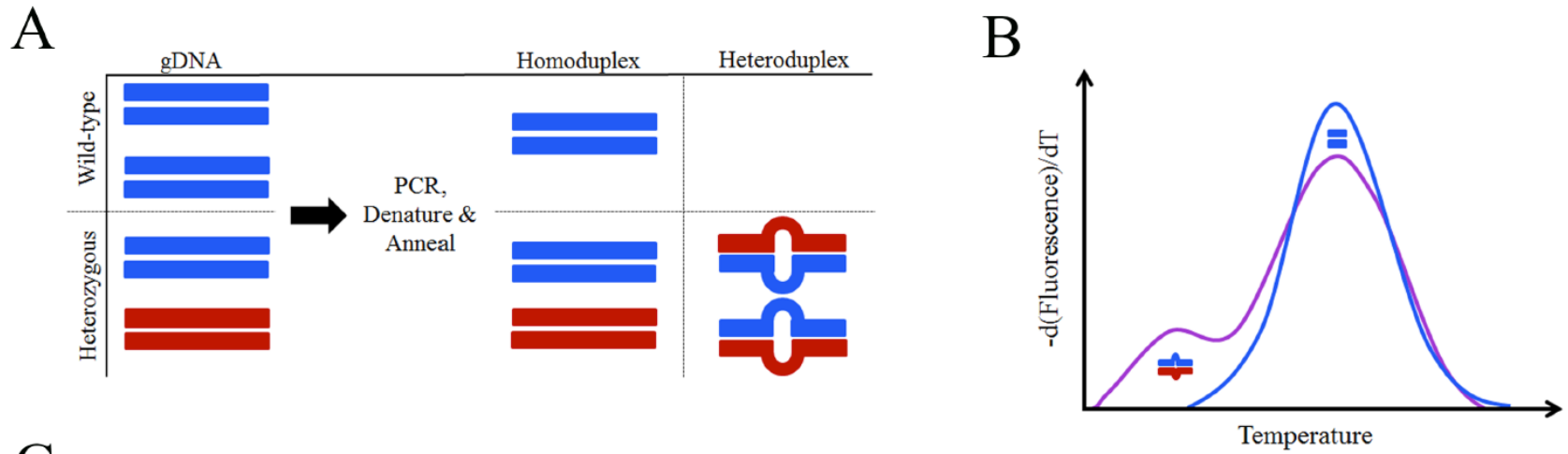


indel (%):

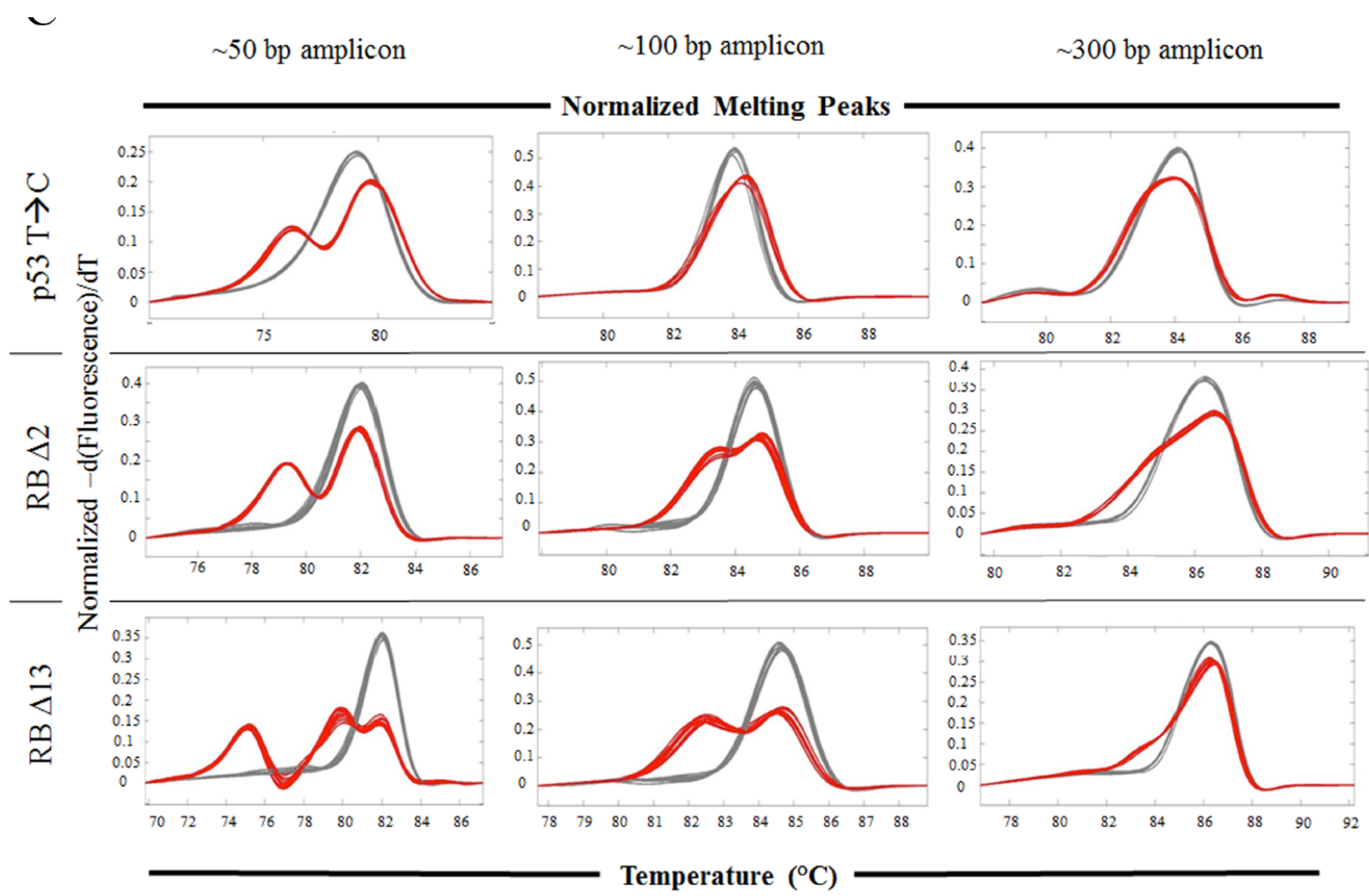
68

65

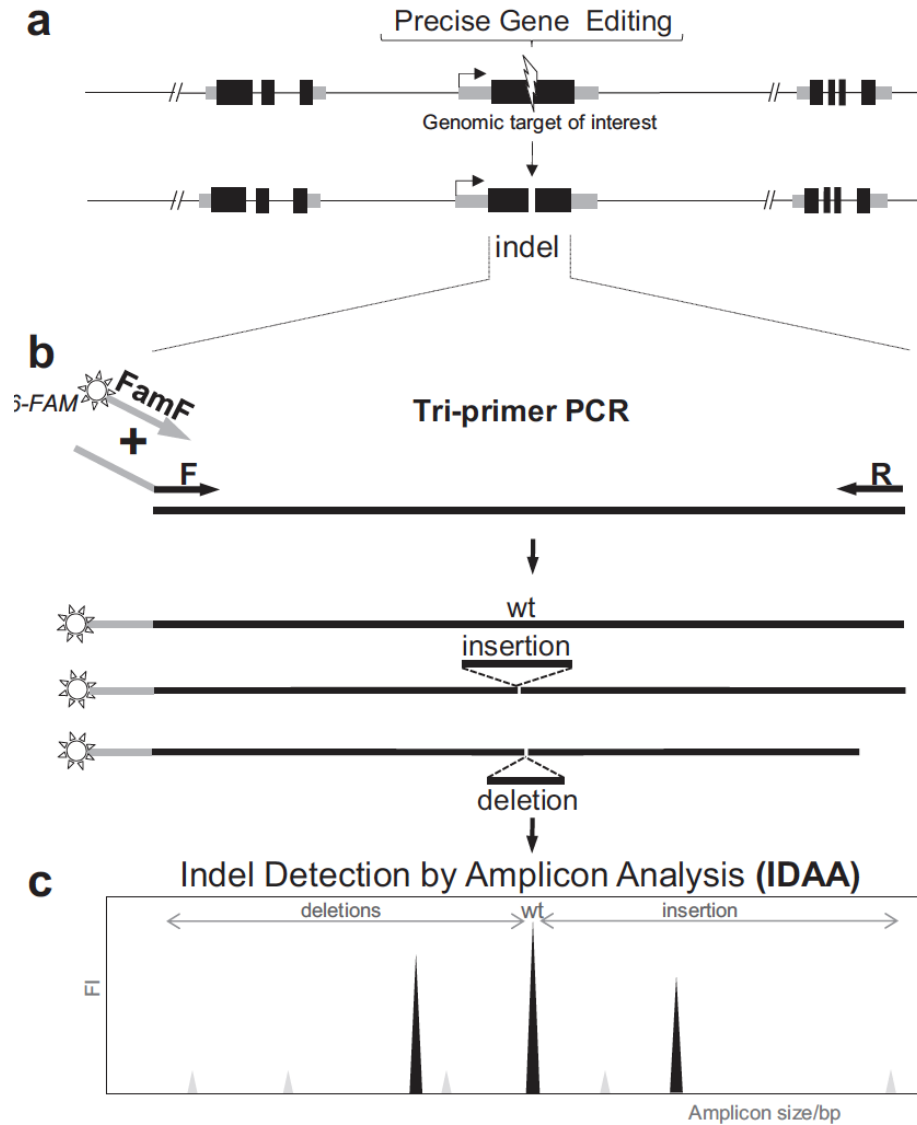
HRM

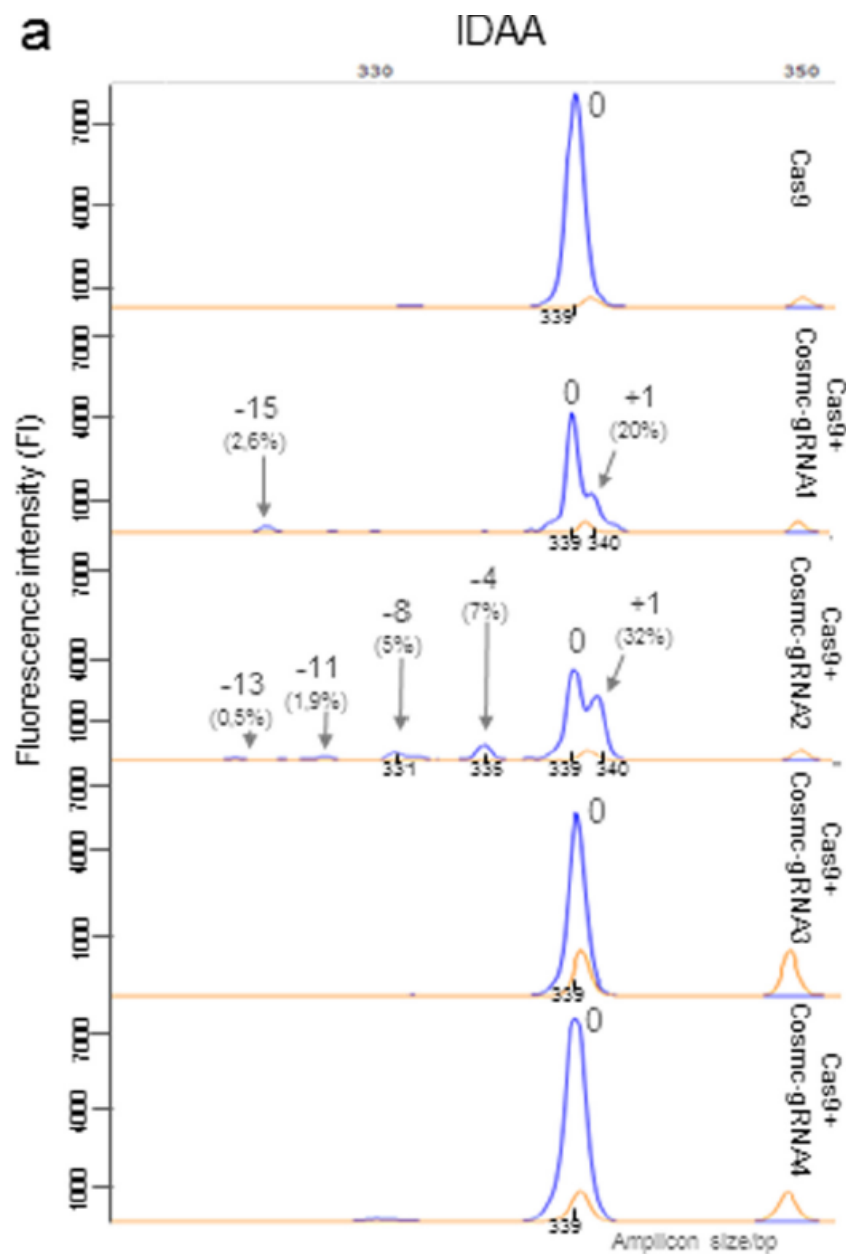


Thomas, HR et al. High-Throughput Genome Editing and Phenotyping Facilitated by High Resolution Melting Curve Analysis. PlosOne 2014



IDAA





Types of indel screening

- EMC (Enzyme Mismatch Cleavage)
 - Surveyor, T7E1, etc
- HRM (High Resolution Melt)
- IDAA (Indel Detection by Amplicon Analysis)
- PCR
- Sequencing
 - NGS
 - Sanger

Amplicon size

- EMC: Surveyor: 400-800
- HRM: 50-100 bp
- IDAA: 250-450
- NGS: Depends on kit

Which is better?

- EMC, HRM, IDAA only tell you of the presence of a change, and sometimes the length, but not the exact mutation
- Sequencing gives the exact mutation, but is more work and more expensive

Indel vs Deletion

- Current design recommendations are to use two guides where possible for easier screening, as if both work, you can easily see a size difference in a regular PCR
- But if only one works, there may still be an indel based mutation in one site, but it won't be detected by a standard PCR

Other issues

- Large deletions
 - Long Range PCR is very tricky
- Blastocysts give messy PCR/sequence
- G/C rich sequence

Contact Info

Phone: 2470

Email: shifra.ben-dor@weizmann.ac.il

<http://bip.weizmann.ac.il/toolbox/target/dna/crispr.html>

