CRISPR Design Considerations

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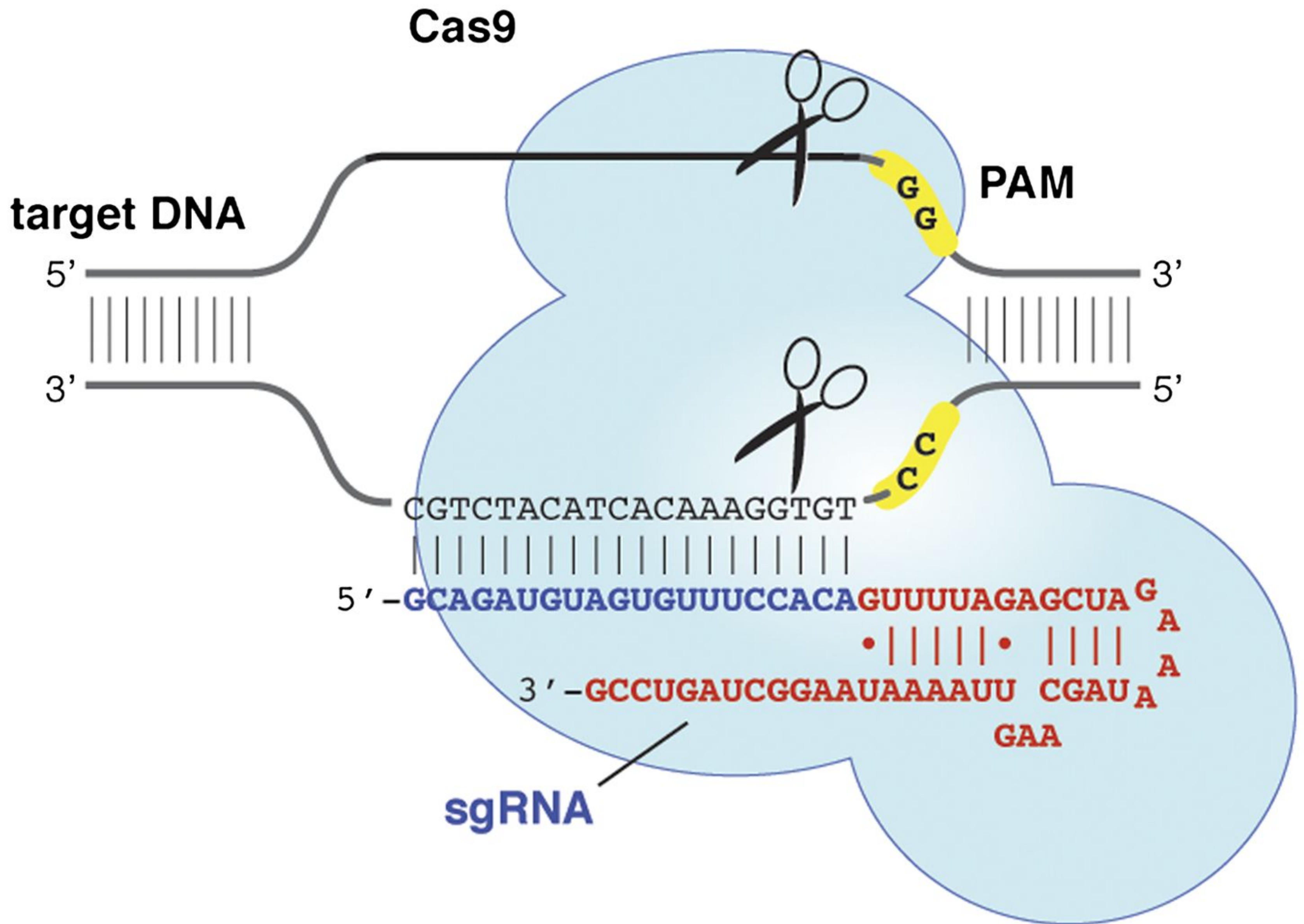
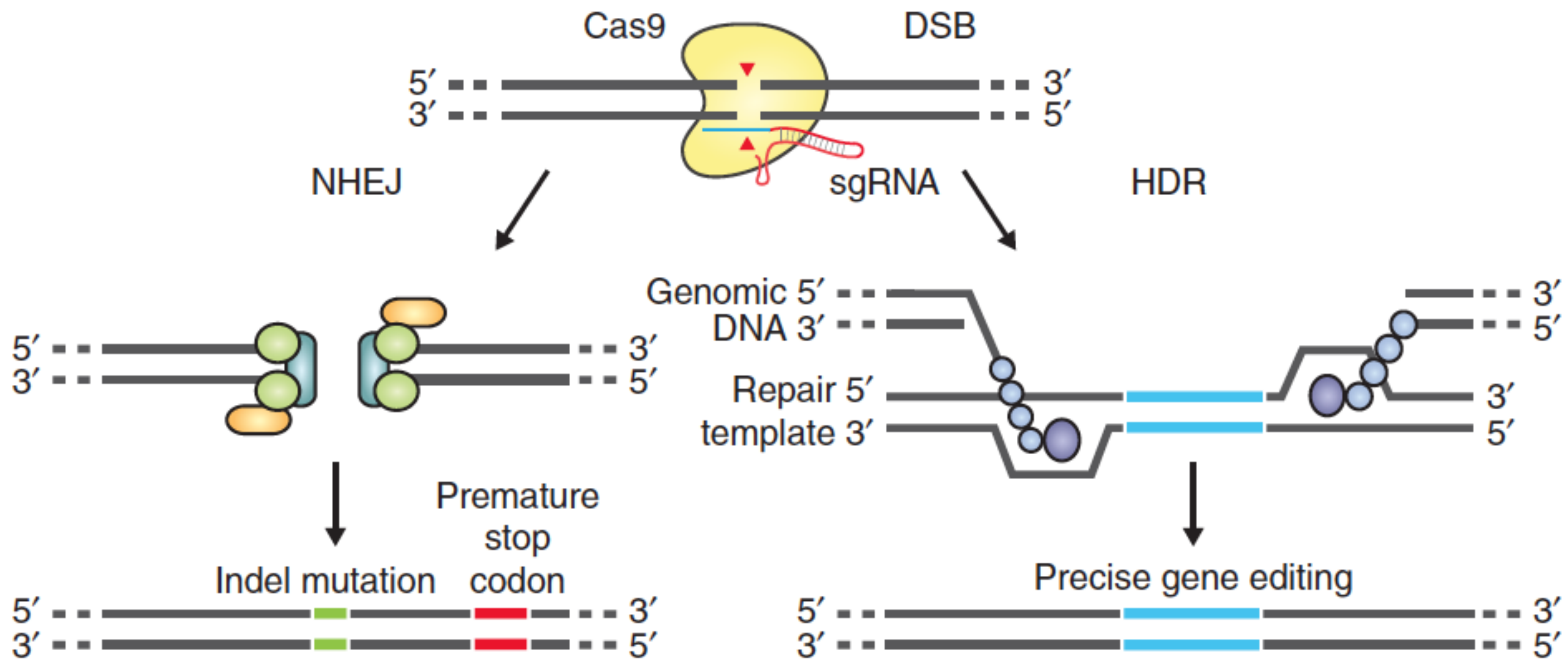
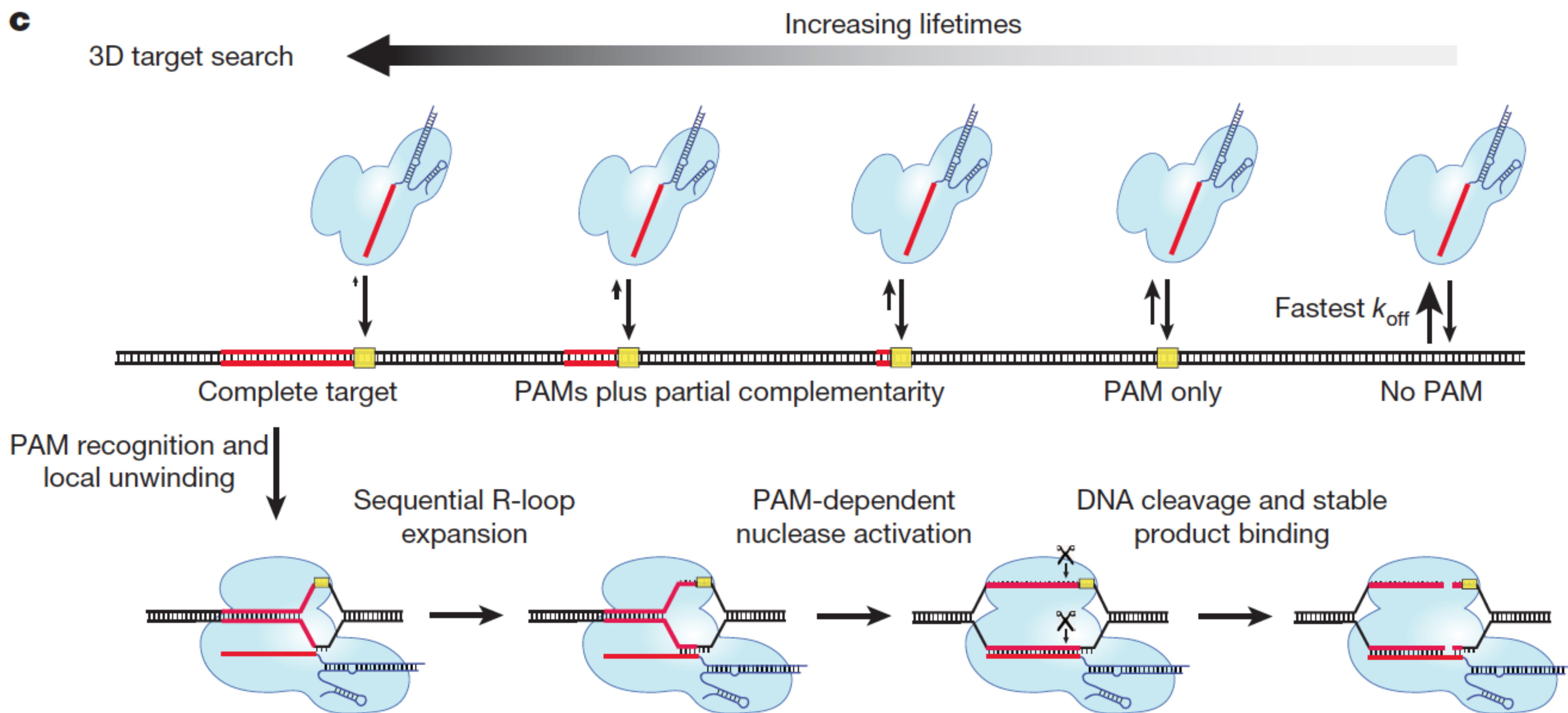


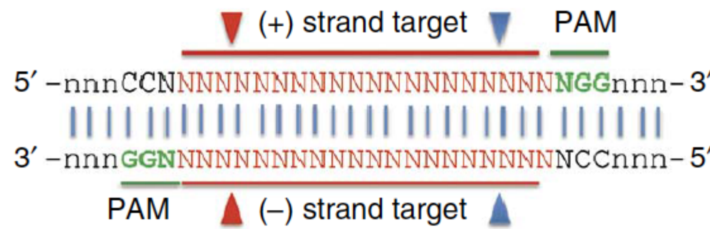
Jinek et al, eLife 2013;2:e00471


Cas9
DSB
5′
3′
3′
5′
NHEJ
sgRNA
HDR
5′
3′
3′
5′
Genomic 5′
DNA 3′
3′
5′
Repair 5′
template 3′
3′
5′
Premature
stop
codon
Indel mutation
5′
3′
3′
5′
Precise gene editing
5′
3′
3′
5′


c
Increasing lifetimes
3D target search
Fastest k_{off}
Complete target
PAMs plus partial complementarity
PAM only
No PAM
PAM recognition and
local unwinding
Sequential R-loop
expansion
PAM-dependent
nuclease activation
DNA cleavage and stable
product binding

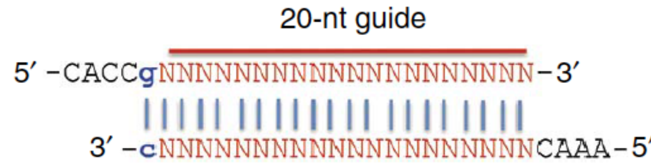
a

Targeted DNA



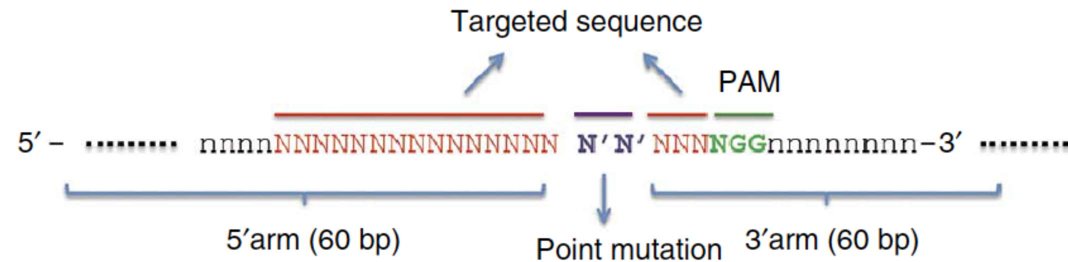
b

Guide oligo
for sgRNA



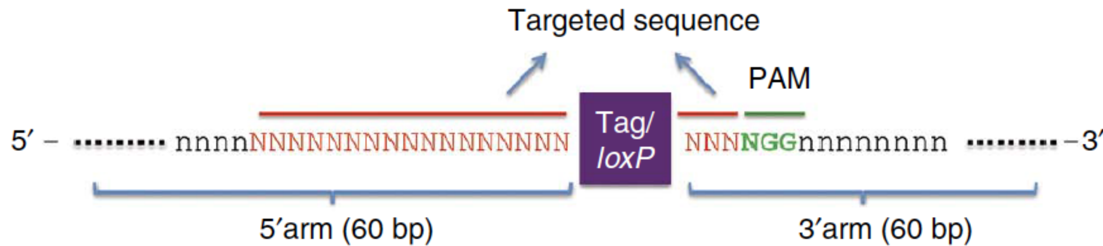
c

ssDNA for
point mutation



d

ssDNA for
tag/*loxP* insertion



e

Circular donor vector
for large fragment insertion

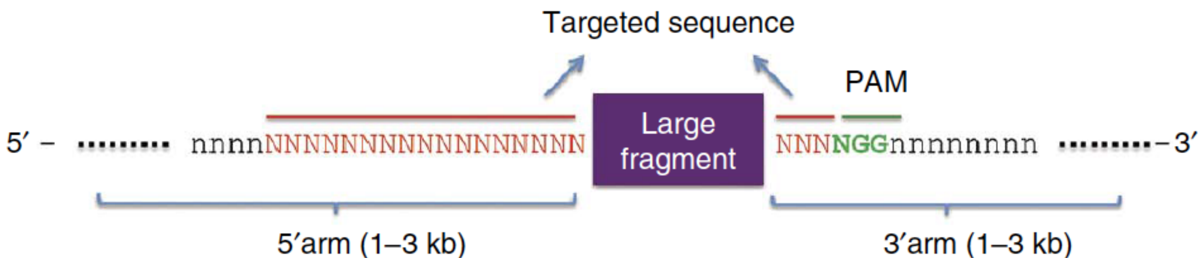


TABLE 1 | Genetically modified mice generated by CRISPR/Cas-mediated genome engineering[13,14].

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

[a]The 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  ACCACCAACTTCATCCACGTTCACCTTGC:::::::::::::::CGGCAGACTTCTCCTCAGGAG
           -9  ACCACCAACTTCATCCACGTTCACCTTGCCCC:::::::::TAACGGCAGACTTCTCCTCAGGAG
           -3  ACCACCAACTTCATCCACGTTCACCTTGC:::ACAGGGCAGTAACGGCGGACTTCTCCTCAGGAG
2x         -2  ACCACCAACTTCATCCACGTTCACCTTGCCC::CAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
3x         -1  ACCACCAACTTCATCCACGTTCACCTTGCCC:ACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
           -1  ACCACCAACT:CATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
          HBB  ACCACCAACTTCATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
13x        WT  ACCACCAACTTCATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAG
         R-03                 GACGTTCACCTTGCCCCACANGG
19x        +1  ACCACCAACTTCATCCACGTTCACCTTGCCCCCACAGGGCAGTAACGGCAGACTTCTCCTTAGGA
           +9  ACCACCAACTTCATCCACGTTCACCTTGCCTTGTTCACCGTTACAGGGCAGTAACGGCAGACTTC
          +10  ACCACCAACTTCATCCACGTTCTCATCCACGTTCACCTTGCCCACAGGGCAGTAACGGCAGACTT
```

Cradick et al *Nucleic Acids Research, 2013, Vol. 41, No. 20*

Outline

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

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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

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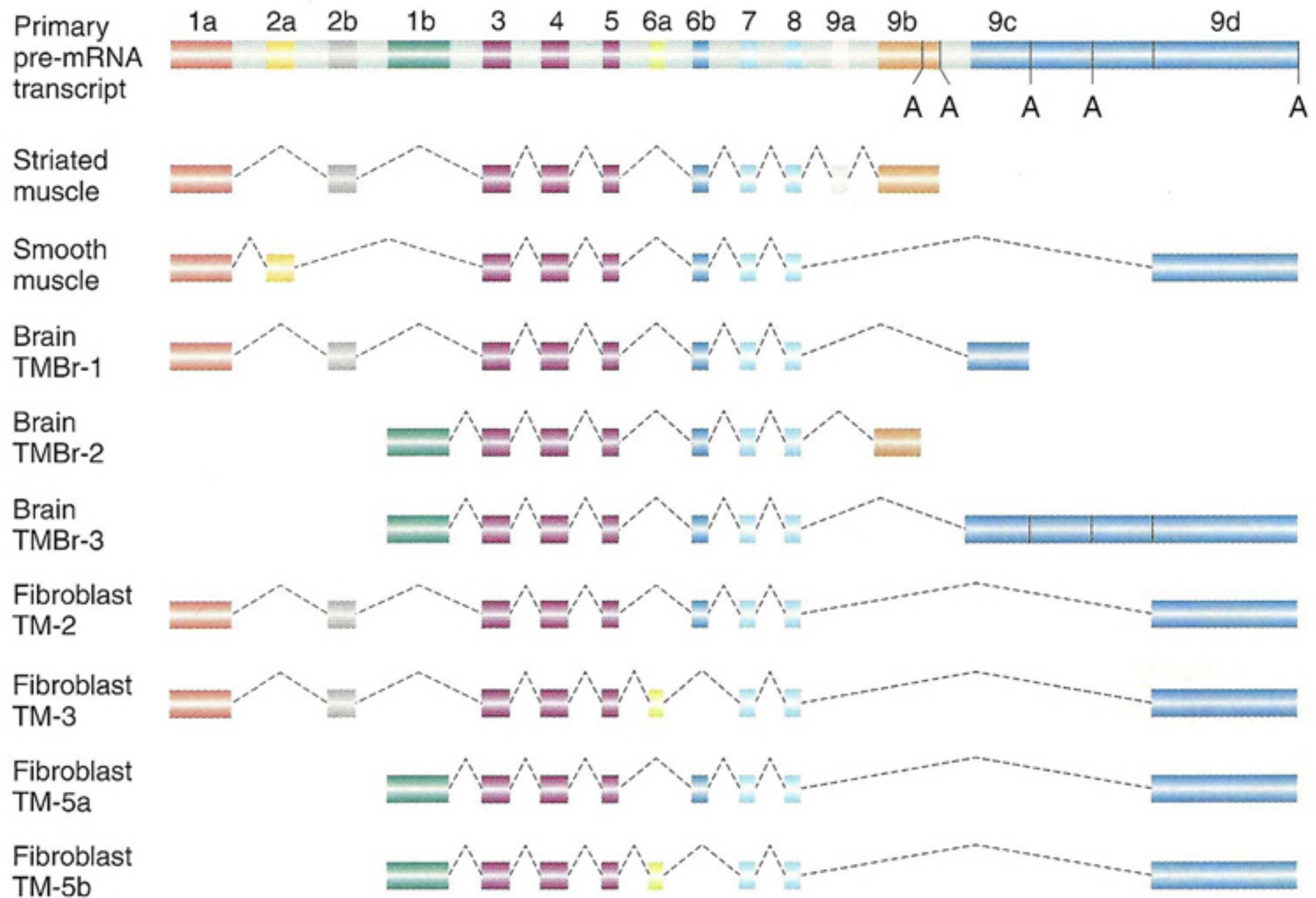
- Screening

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



Taken from: BIOL 202 Genetics taught by Dr. Paul Szauter at the University of New Mexico

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?

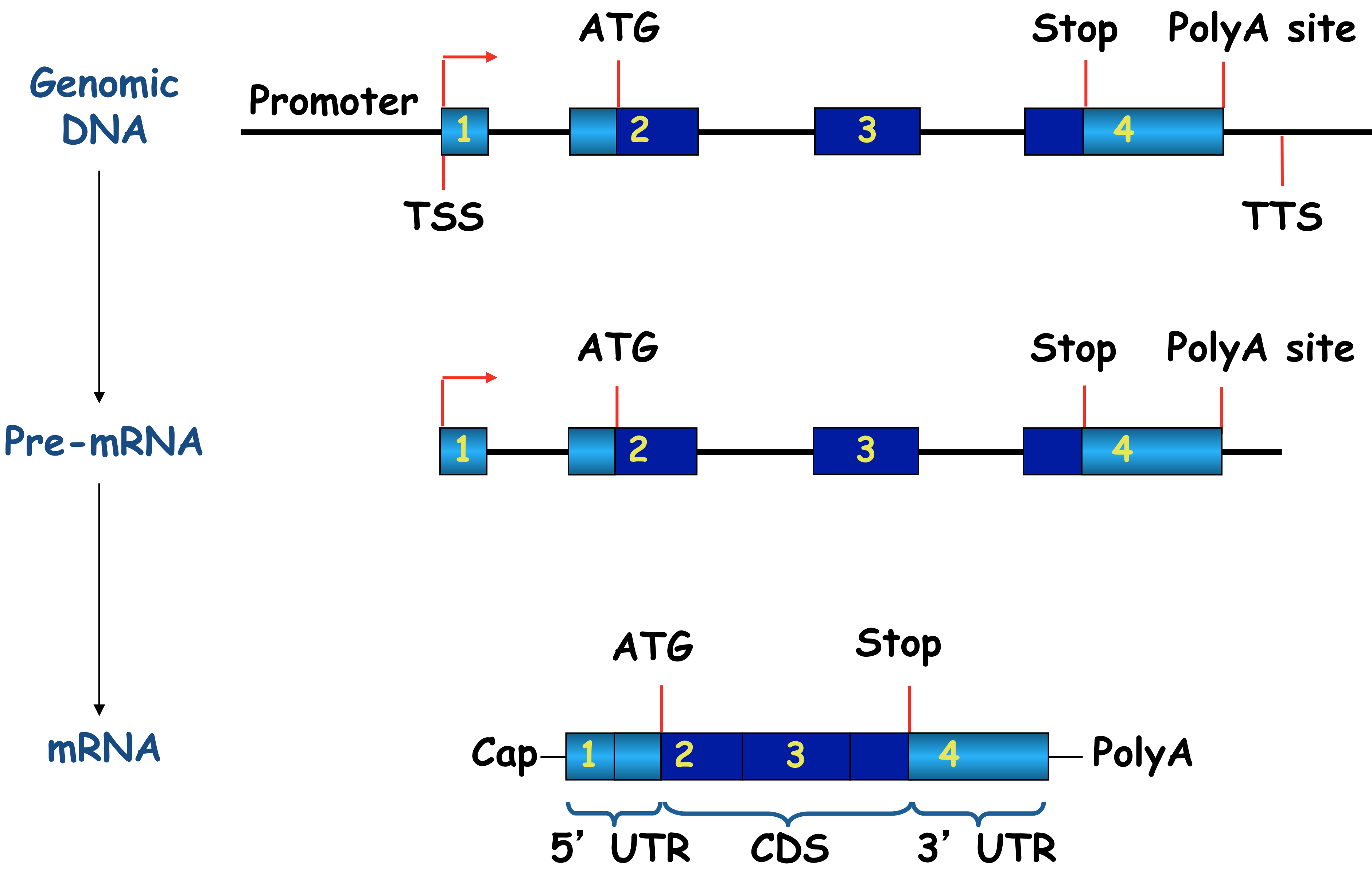
Other considerations

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

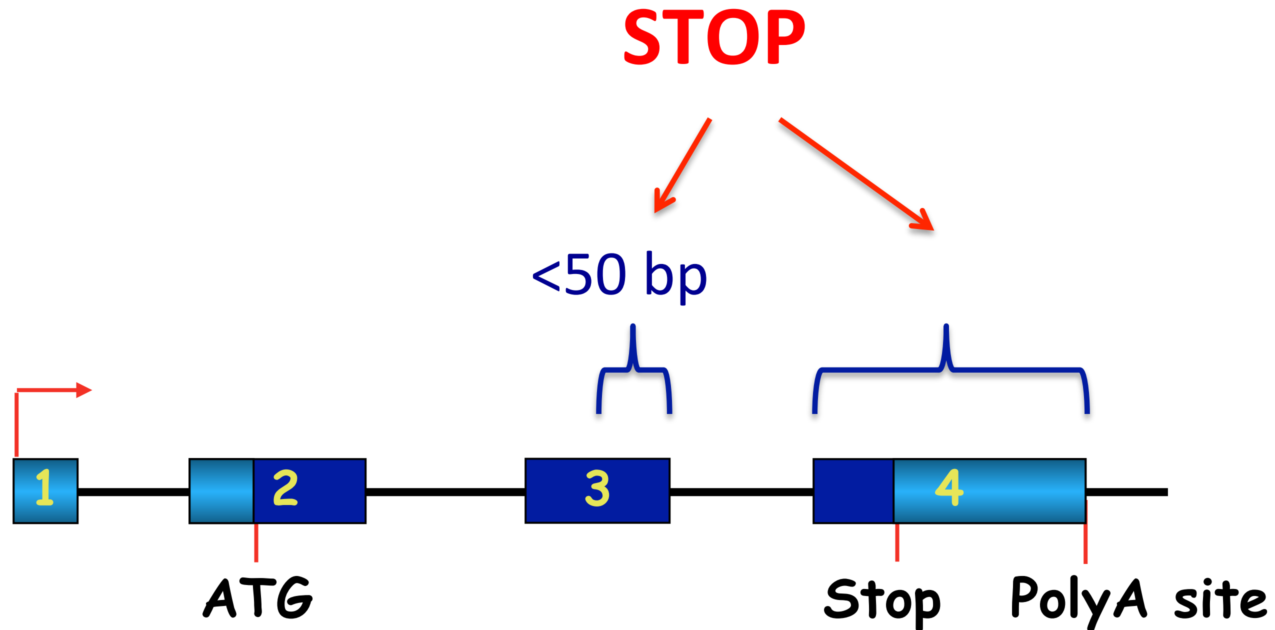
NMD

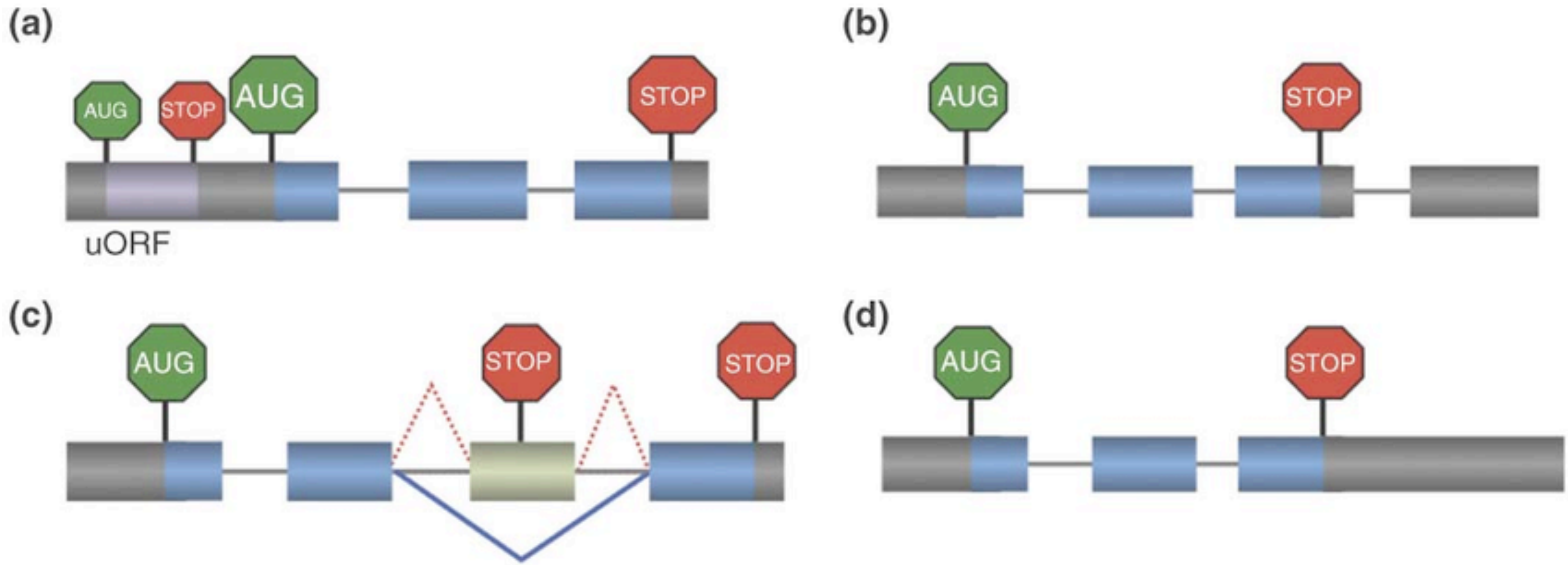
- Nonsense mediated decay



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





Regulation of nonsense-mediated mRNA decay. Huang L, Wilkinson MF. (2012)

Genomic Considerations

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

Guide Design

Starting Point

Sequence Guide

PAM

Cleavage Site

N N N N N N N N N N N N N N N N N N N N NGG

G

"Seed" Region

Promoter Required

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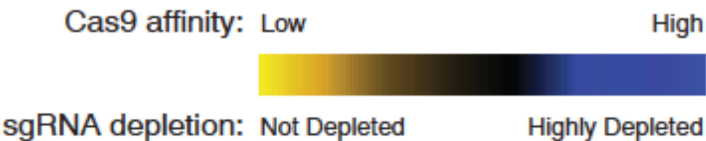
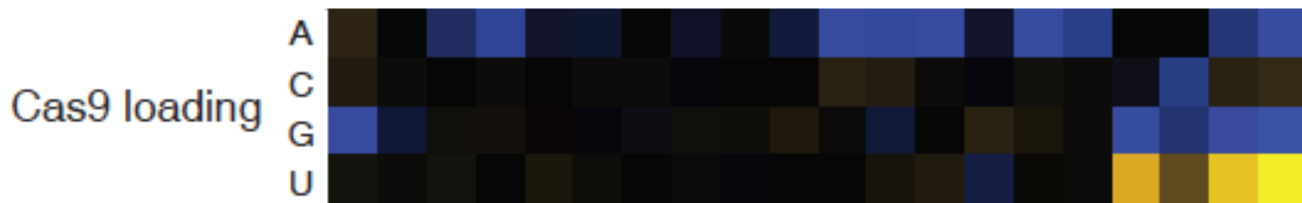
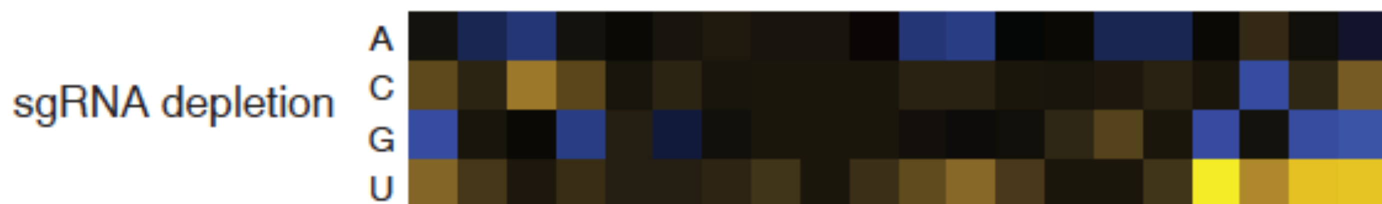
Positive Selection

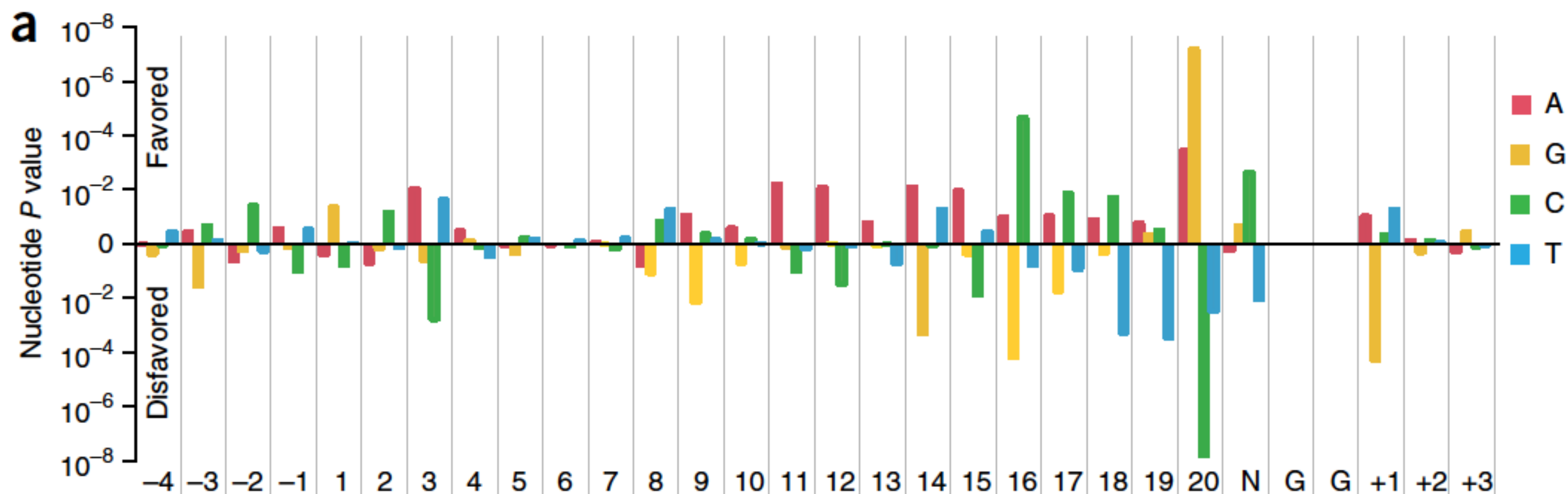
- G/C content
- Base Preferences

Wang, Science 2014









Doench, Hartenian, Nature Biotech. 2014

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.

Browse... No file selected.

Rule Set: 1 **Submit »**

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“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.

Outline

- Define your question
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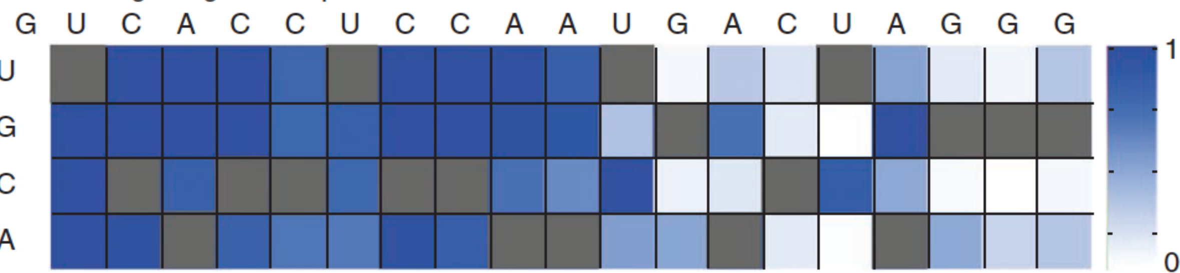
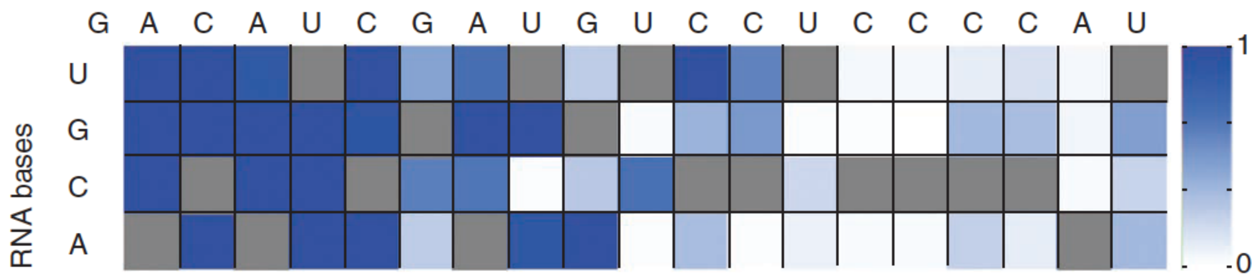
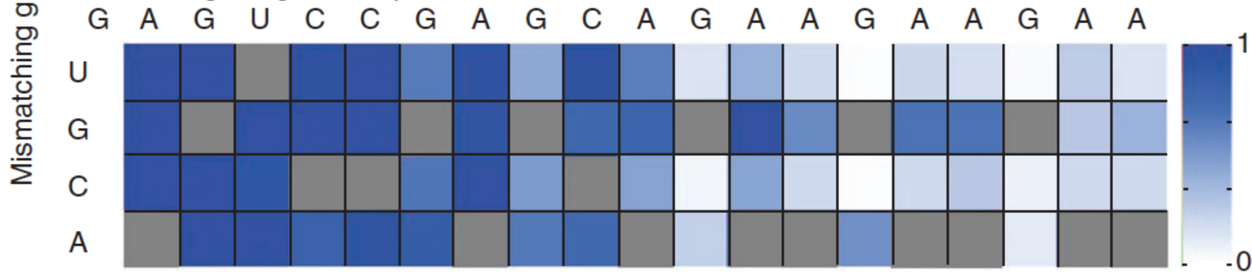
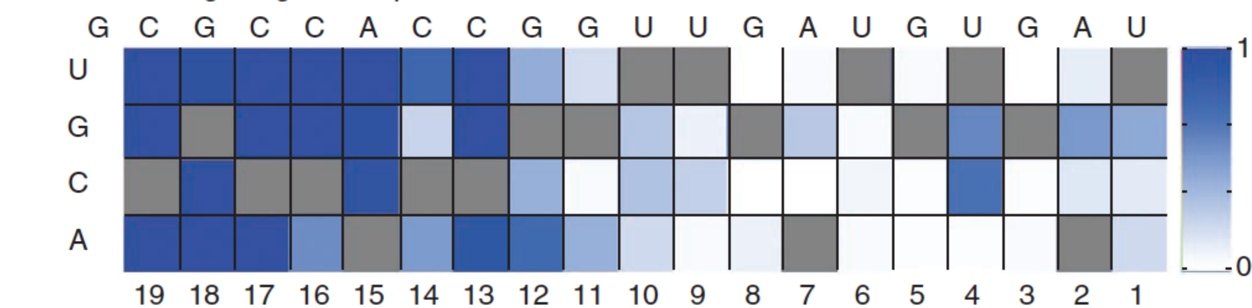
OFF TARGET HAPPENS

b









a

EMX1 target 1 guide sequence — GUCACCUCCAAUGACUAGGG — Indel (%) 0 5 10 15 20

EMX1 target 2 guide sequence — GACAUCGAUGUCCUCCCCAU — Indel (%) 0 2 4 6 8 10

EMX1 target 3 guide sequence — GAGUCCGAGCAGAAGAAGAA — Indel (%) 0 10 20 30 40

EMX1 target 6 guide sequence — GCGCCACCGGUUGAUGUGAU — Indel (%) 0 10 20 30 40

Concatenated mismatches

b

2 mismatches

c

3 mismatches

4 mismatches

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/
sgRNAcas9	http://www.biootools.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

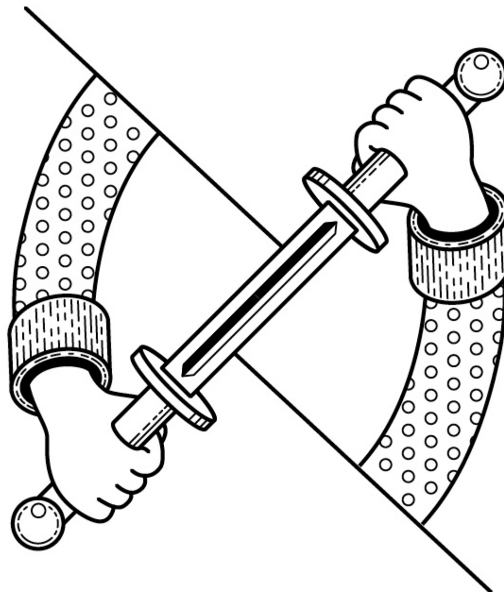
- NGG is the 'classic' but others may be acceptable
  - NAG
  - Possibly others

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

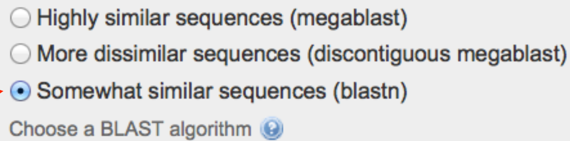
- Mismatches
- PAM
- G/C content
- Is an initial G required?
- What version of the genome is used?
- Are the criteria updated?
- Does it come with primer design?

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



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

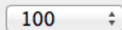
Show results in a new window

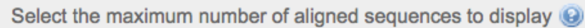
Algorithm parameters

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

General Parameters

Max target sequences

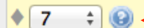




Short queries

Expect threshold

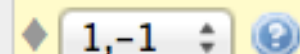
Word size



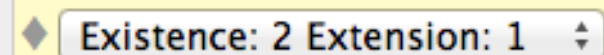
Max matches in a query range

Scoring Parameters

Match/Mismatch Scores

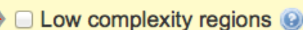


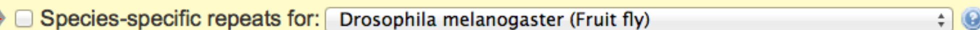
Gap Costs



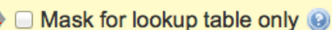
Filters and Masking

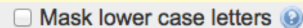
Filter





Mask





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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 to 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

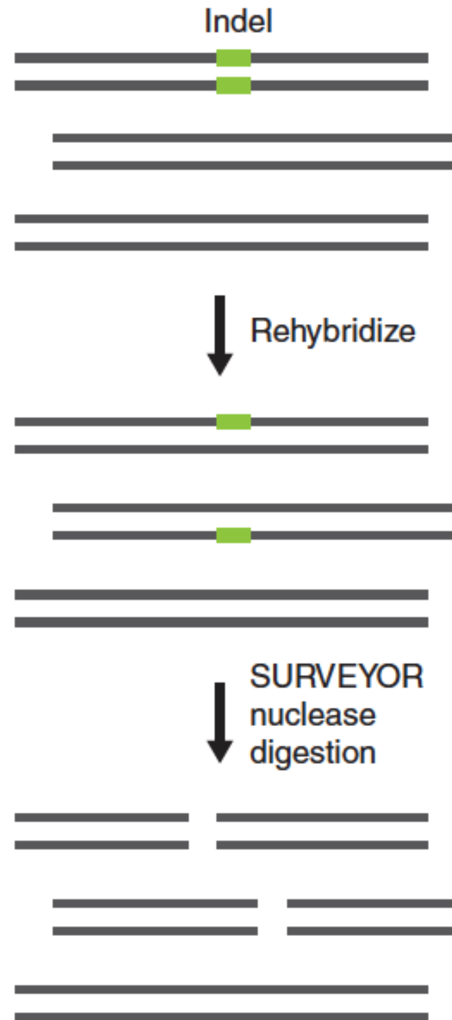
Activity vs Mutation

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

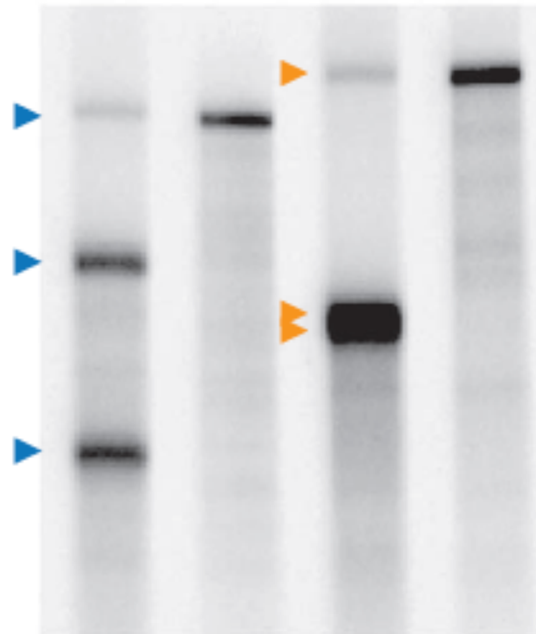
EMC



Ran FA et al, Genome engineering using the CRISPR-Cas9 system, Nature Protocols (2013)

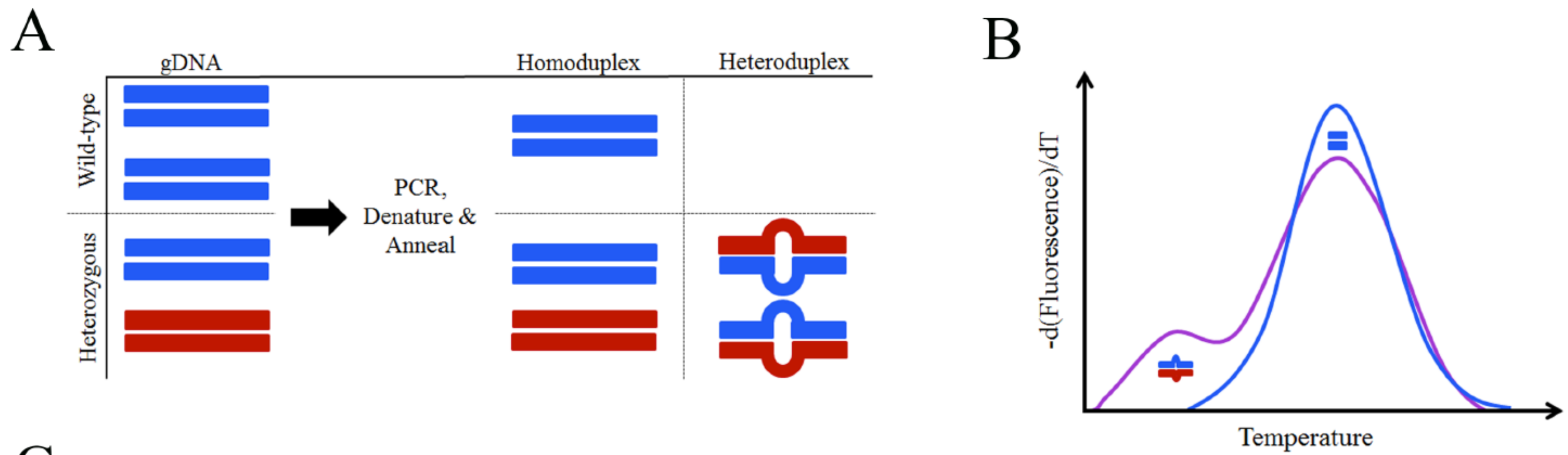
SURVEYOR: *DYRK1A* *GRIN2B*

sgRNA: 1 + 2 – 1 + 2 –

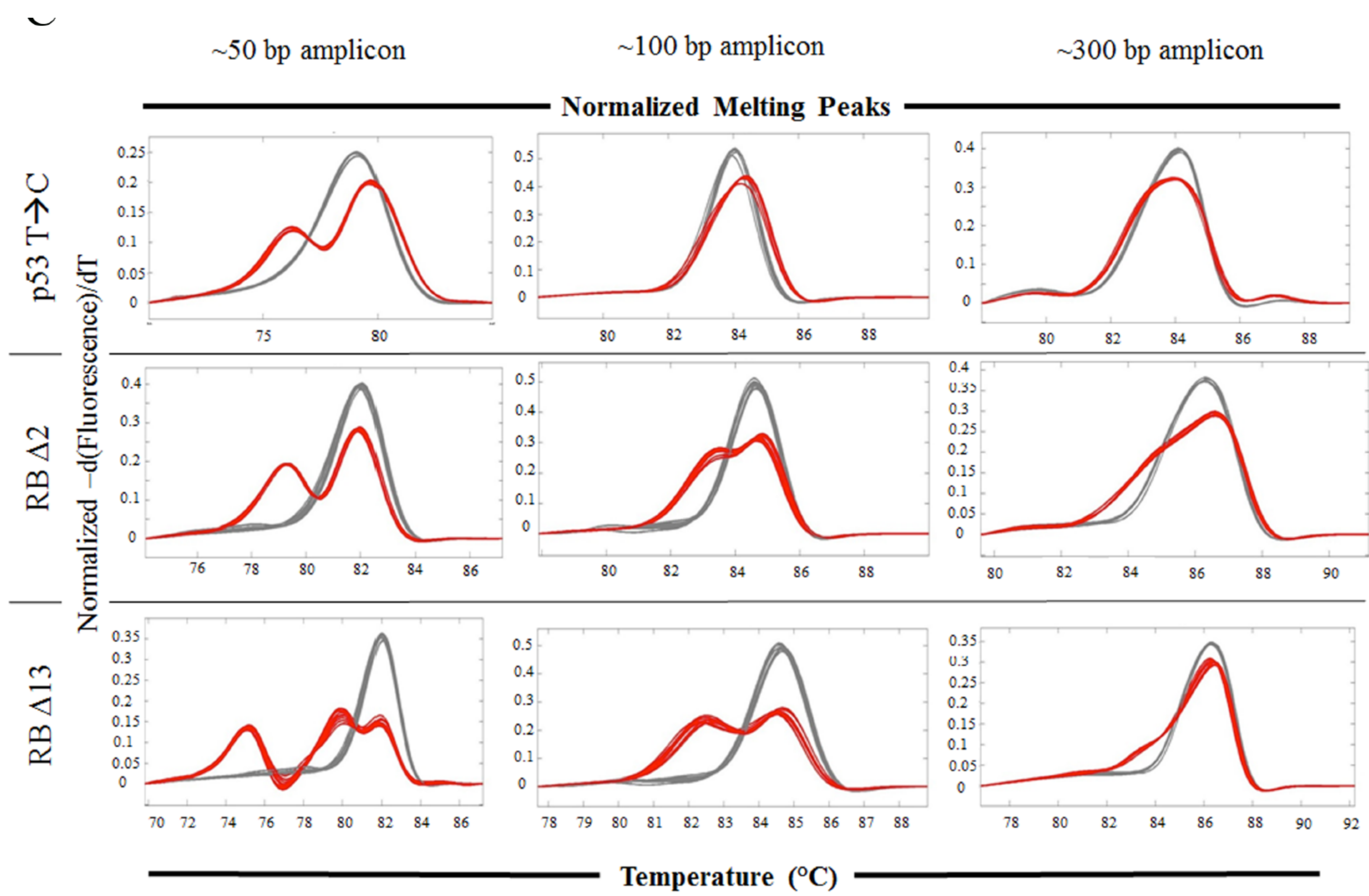


indel (%): 68 65

HRM

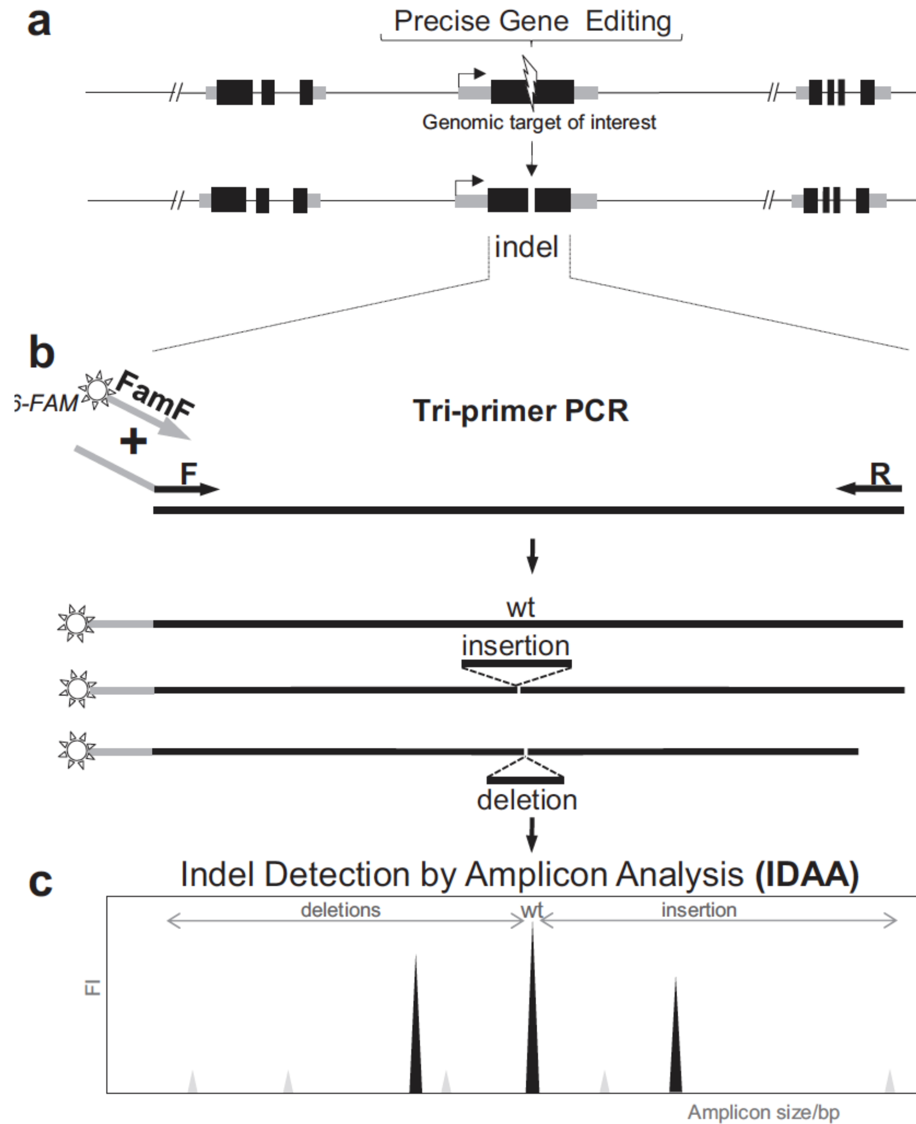


Thomas, HR et al. High-Throughput Genome Editing and Phenotyping Facilitated by High Resolution Melting Curve Analysis. PlosOne 2014

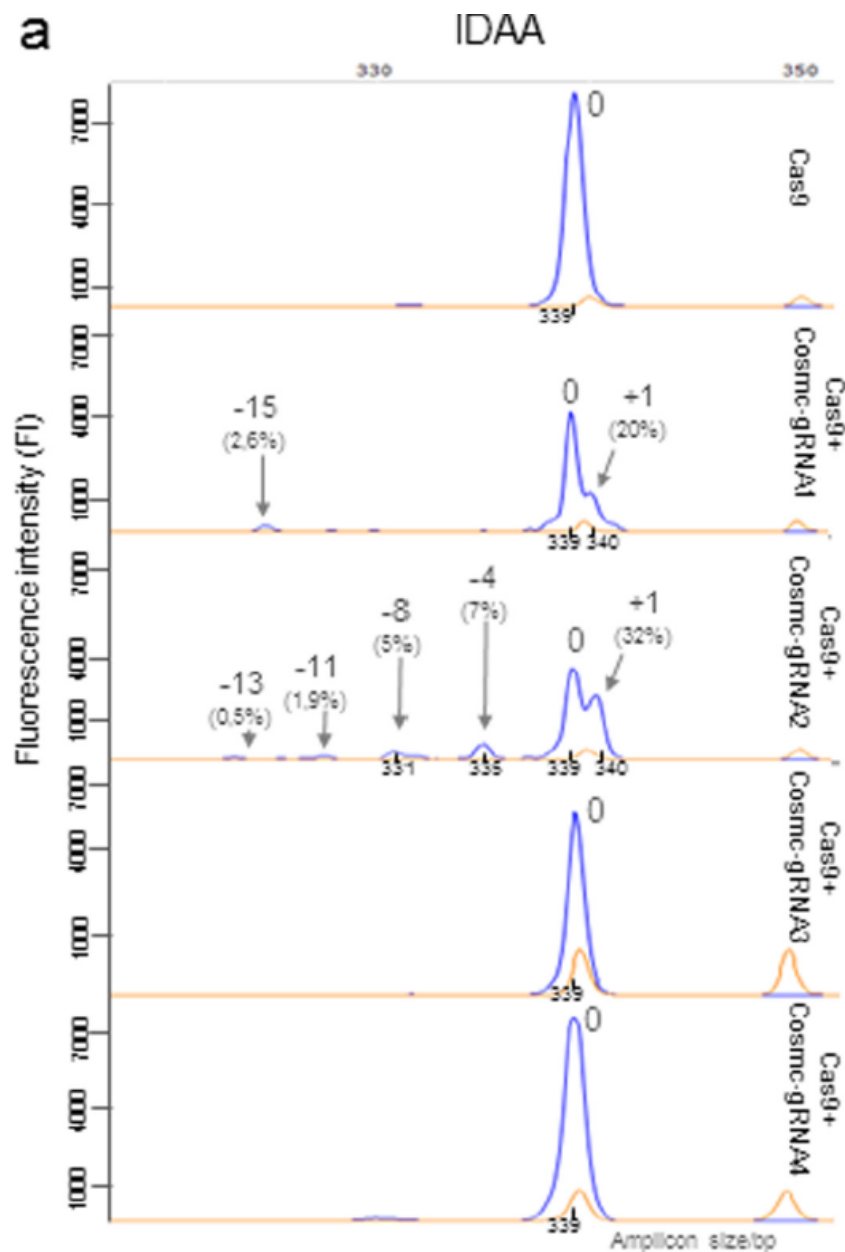


~50 bp amplicon
~100 bp amplicon
~300 bp amplicon
Normalized Melting Peaks
p53 T→C
RB Δ2
RB Δ13
Normalized −d(Fluorescence)/dT
Temperature (°C)

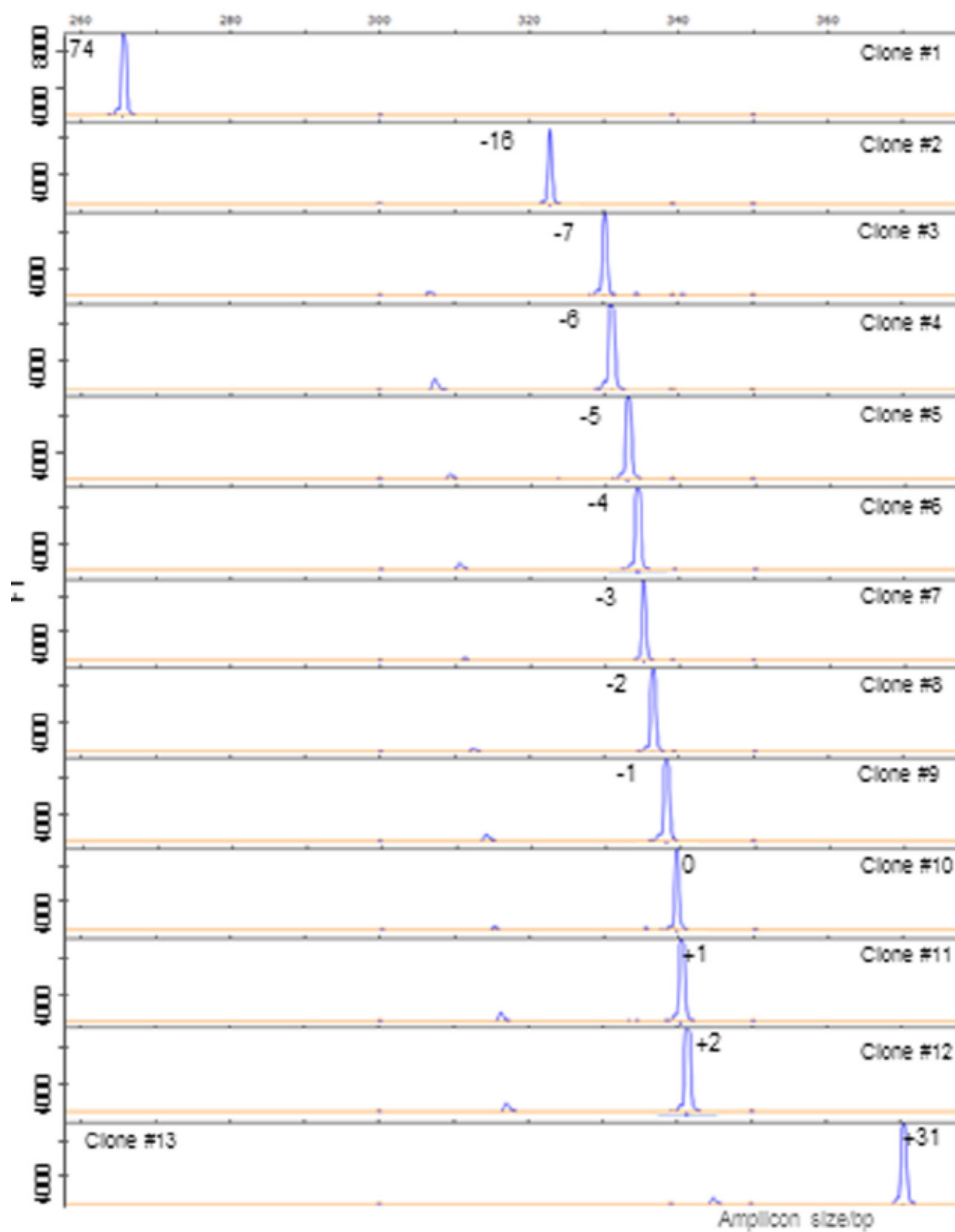
IDAA



Yang Z et al, Fast and sensitive detection of indels induced by precise gene targeting. NAR 2015


a
IDAA
330
350
0
Cas9
339
-15
(2,6%)
0
+1
(20%)
339
340
Cas9+
Cosmc-gRNA1
-13
(0,5%)
-11
(1,9%)
-8
(5%)
-4
(7%)
0
+1
(32%)
331
335
339
340
Cas9+
Cosmc-gRNA2
0
339
Cas9+
Cosmc-gRNA3
0
339
Cas9+
Cosmc-gRNA4
Fluorescence intensity (FI)
1000
4000
7000
Amplicon size/bp

e 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

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http://bip.weizmann.ac.il/toolbox/target/dna/cripsr.html