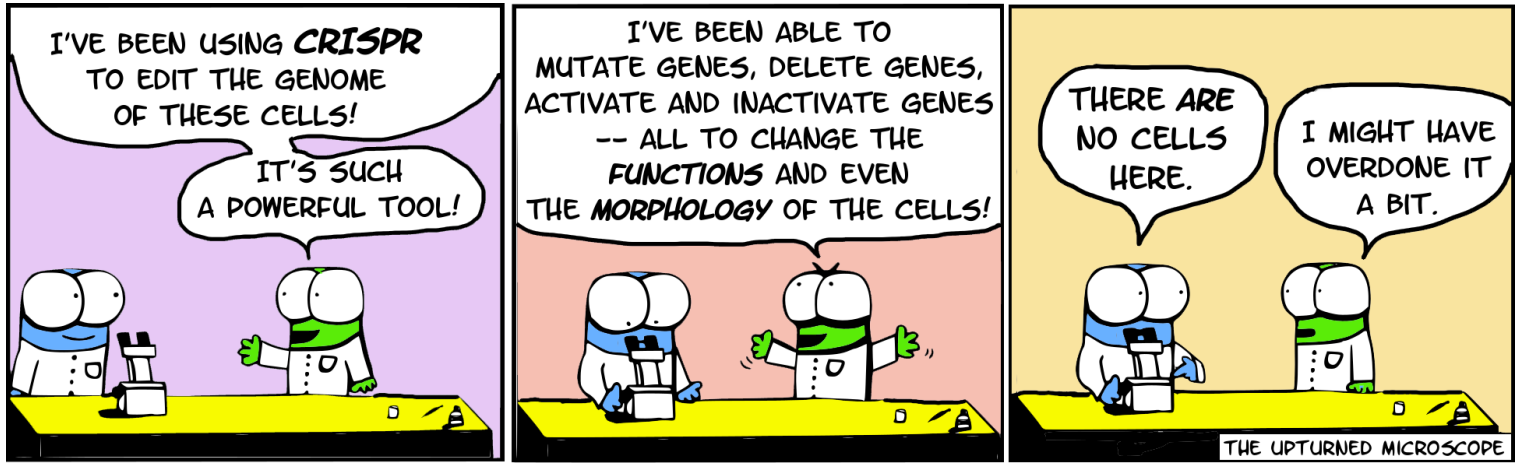
M2D1: Complete *in silico* cloning of dCas9 expression plasmid



1. Prelab
    1. M1 Recap
    2. *In silico cloning*
    3. Assignment reminders / Data Summary Questions

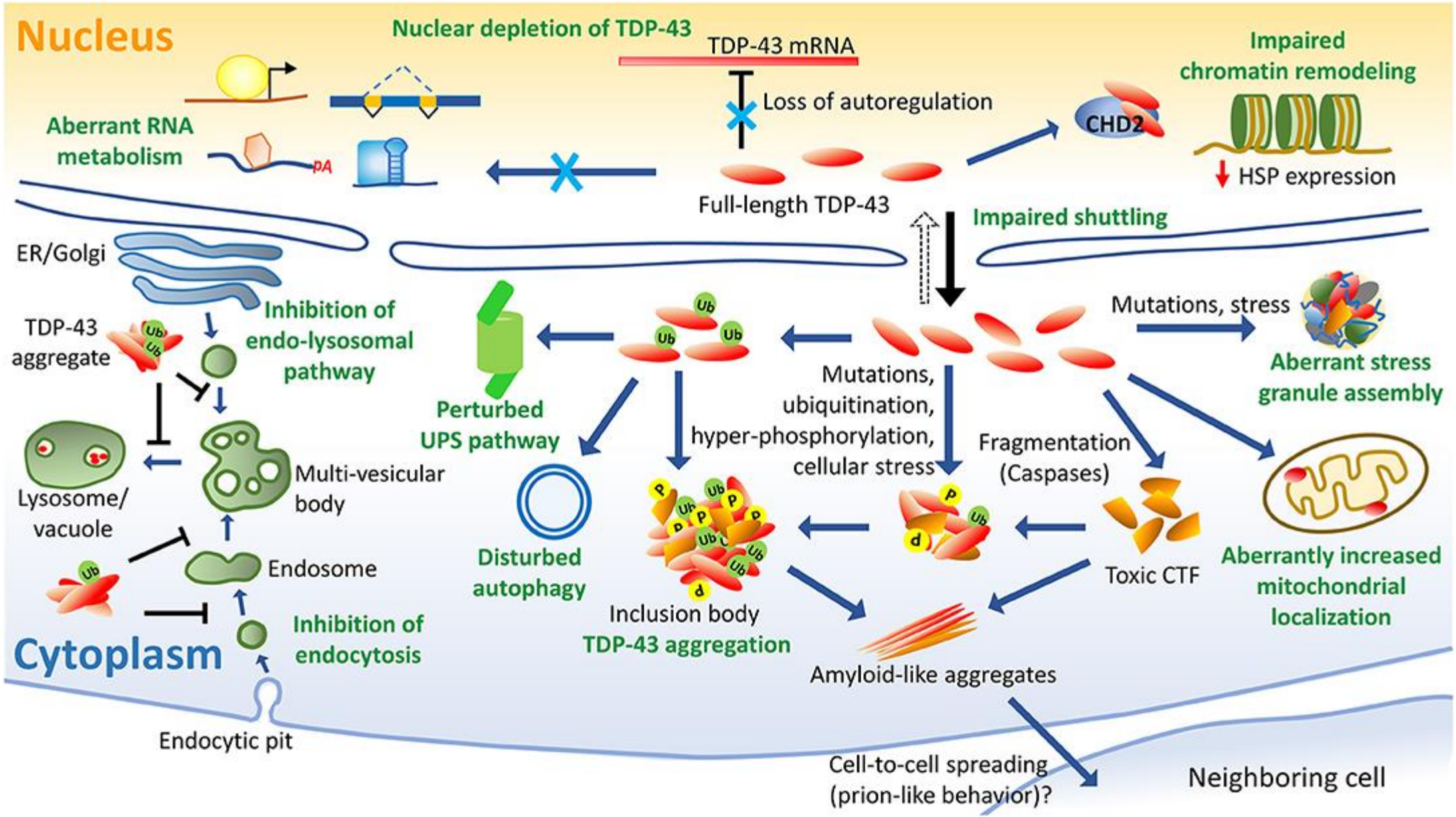
2. *In silico* cloning
3. Set up confirmation digest reaction by 4:30pm

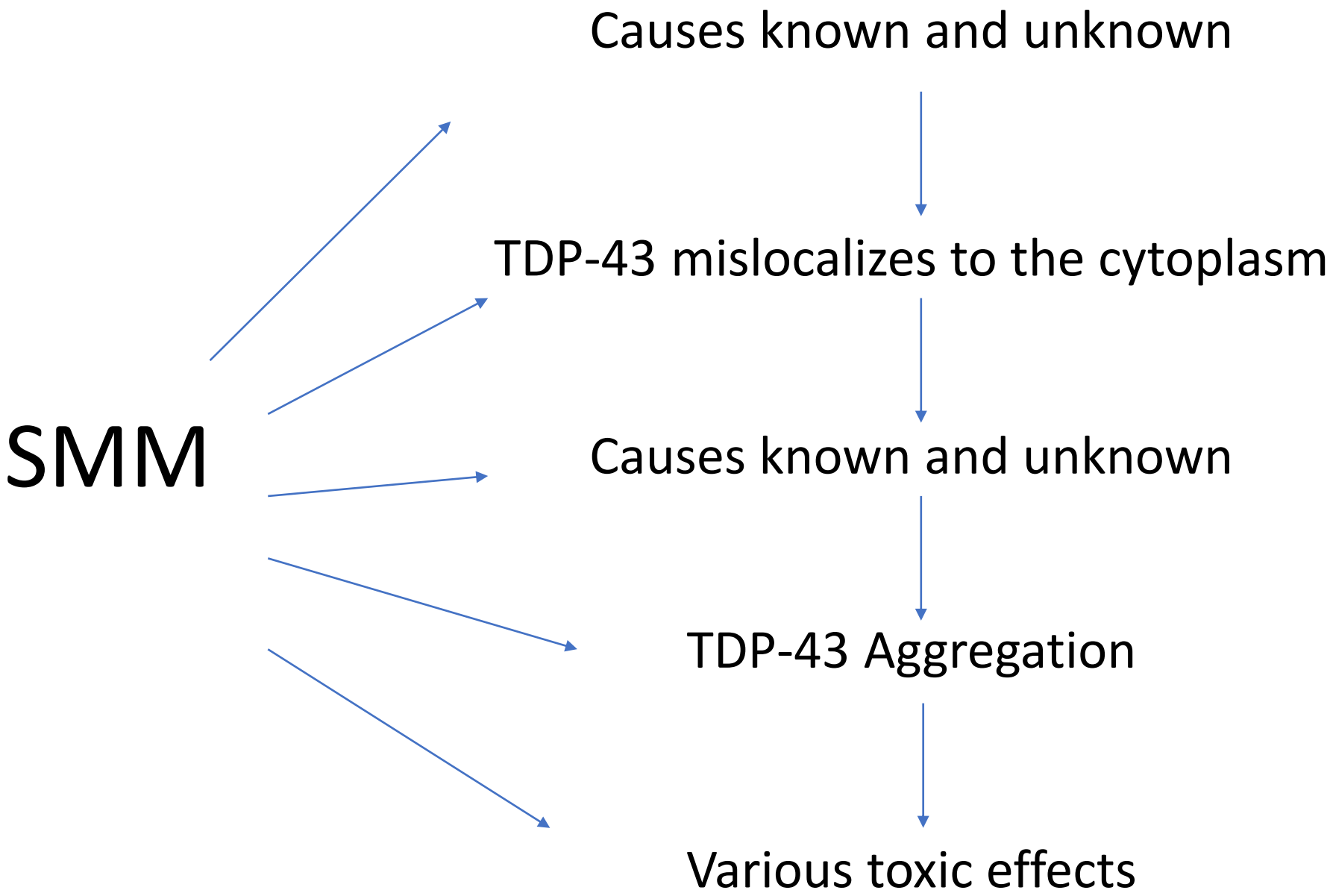
Why test localization & aggregation of TDP-43?

Observation: Aggregates of TDP-43 are present in many neurodegenerative diseases

Hypothesis: Stopping TDP-43 aggregates from forming may prevent neurodegenerative diseases

Why test whether your SMM affects TDP-43 localization at all?

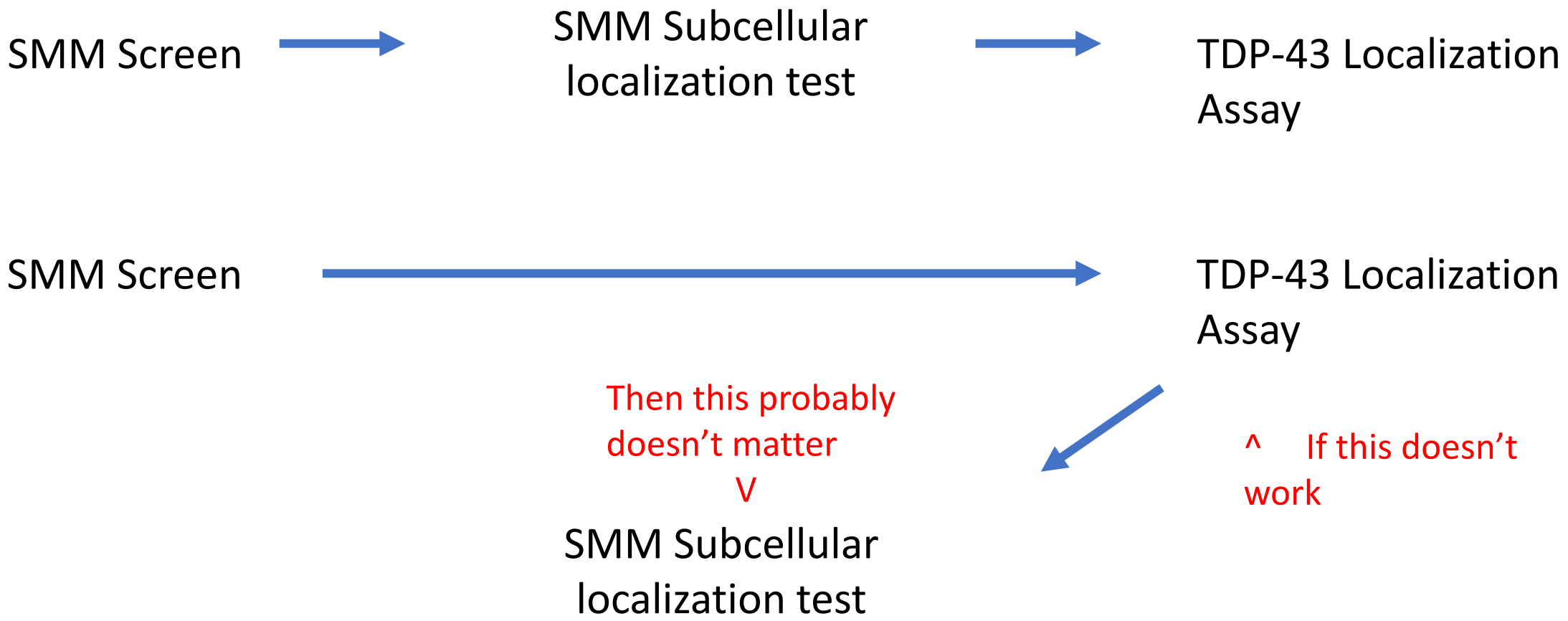

Nucleus
Nuclear depletion of TDP-43
TDP-43 mRNA
Loss of autoregulation
Aberrant RNA metabolism
pA
CHD2
Impaired chromatin remodeling
HSP expression
Full-length TDP-43
Impaired shuttling
ER/Golgi
Inhibition of endo-lysosomal pathway
TDP-43 aggregate
Ub
Perturbed UPS pathway
Mutations, stress
Aberrant stress granule assembly
Mutations, ubiquitination, hyper-phosphorylation, cellular stress
Fragmentation (Caspases)
Lysosome/ vacuole
Multi-vesicular body
Disturbed autophagy
P
d
Inclusion body
TDP-43 aggregation
Toxic CTF
Aberrantly increased mitochondrial localization
Endosome
Inhibition of endocytosis
Cytoplasm
Amyloid-like aggregates
Endocytic pit
Cell-to-cell spreading (prion-like behavior)?
Neighboring cell


Causes known and unknown
TDP-43 mislocalizes to the cytoplasm
SMM
Causes known and unknown
TDP-43 Aggregation
Various toxic effects

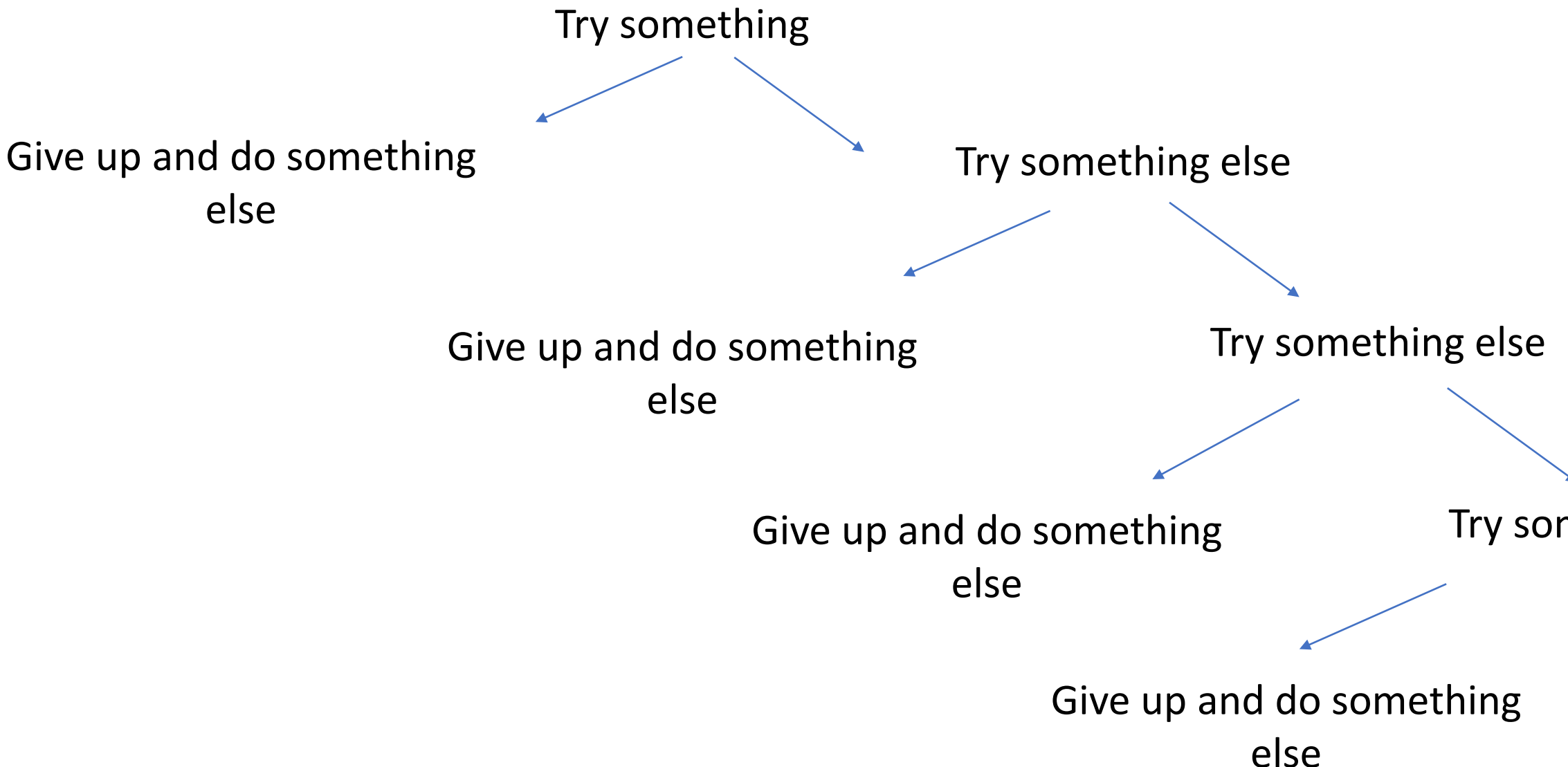
How do we know if our SMM will even make it to the nucleus?

We don’t. Our bad?

How do we know if our SMM will even make it to the nucleus?



A lot of science is based in failure modes



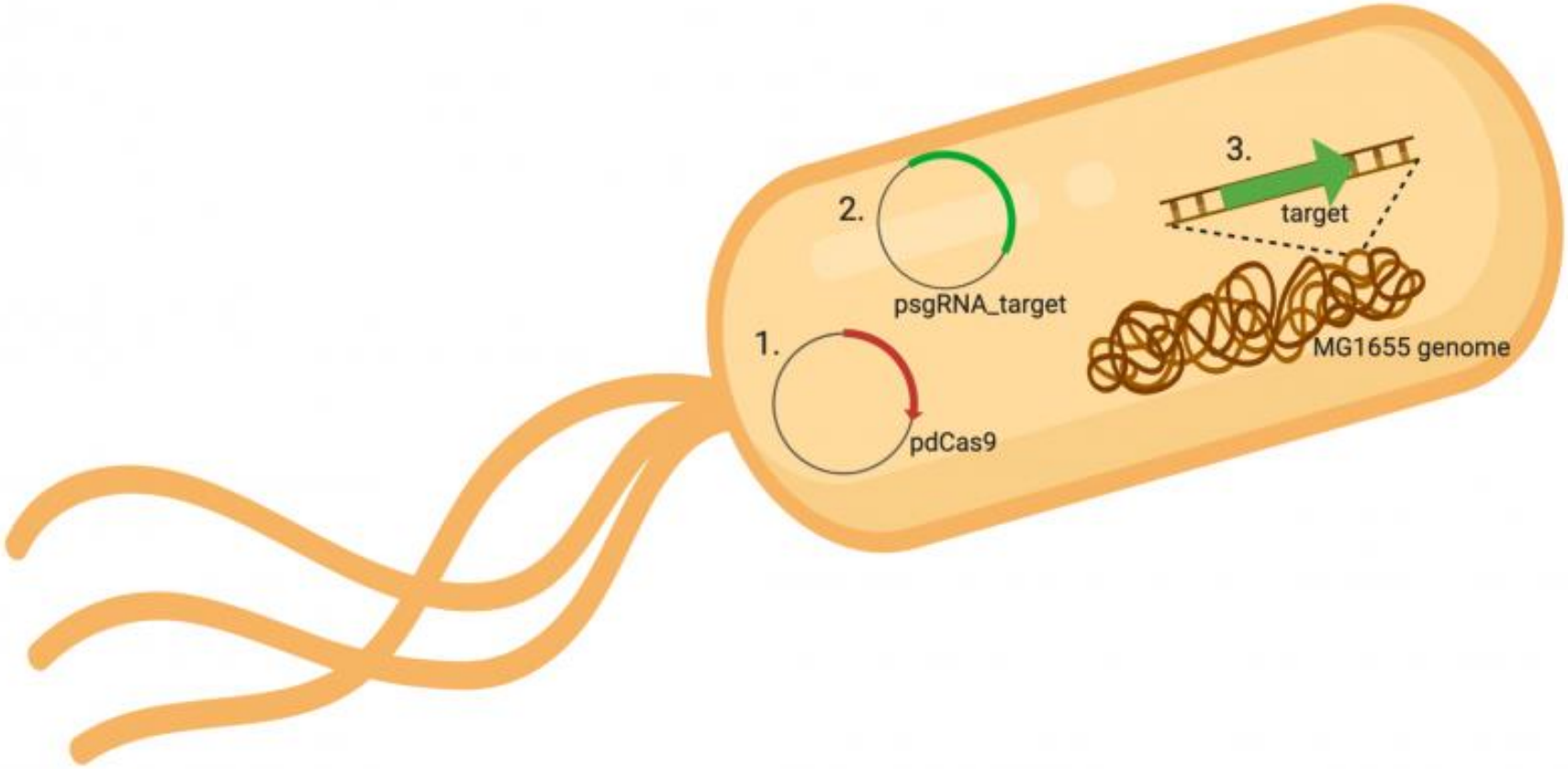
Mod 2 Research goal

Increase the yield of commercially valuable byproducts in *E.coli* using CRIPSRi technology to target genes involved in mixed-acid fermentation pathway.

Mod 2 Schematic

Create this *in silico*

Double check if this is real



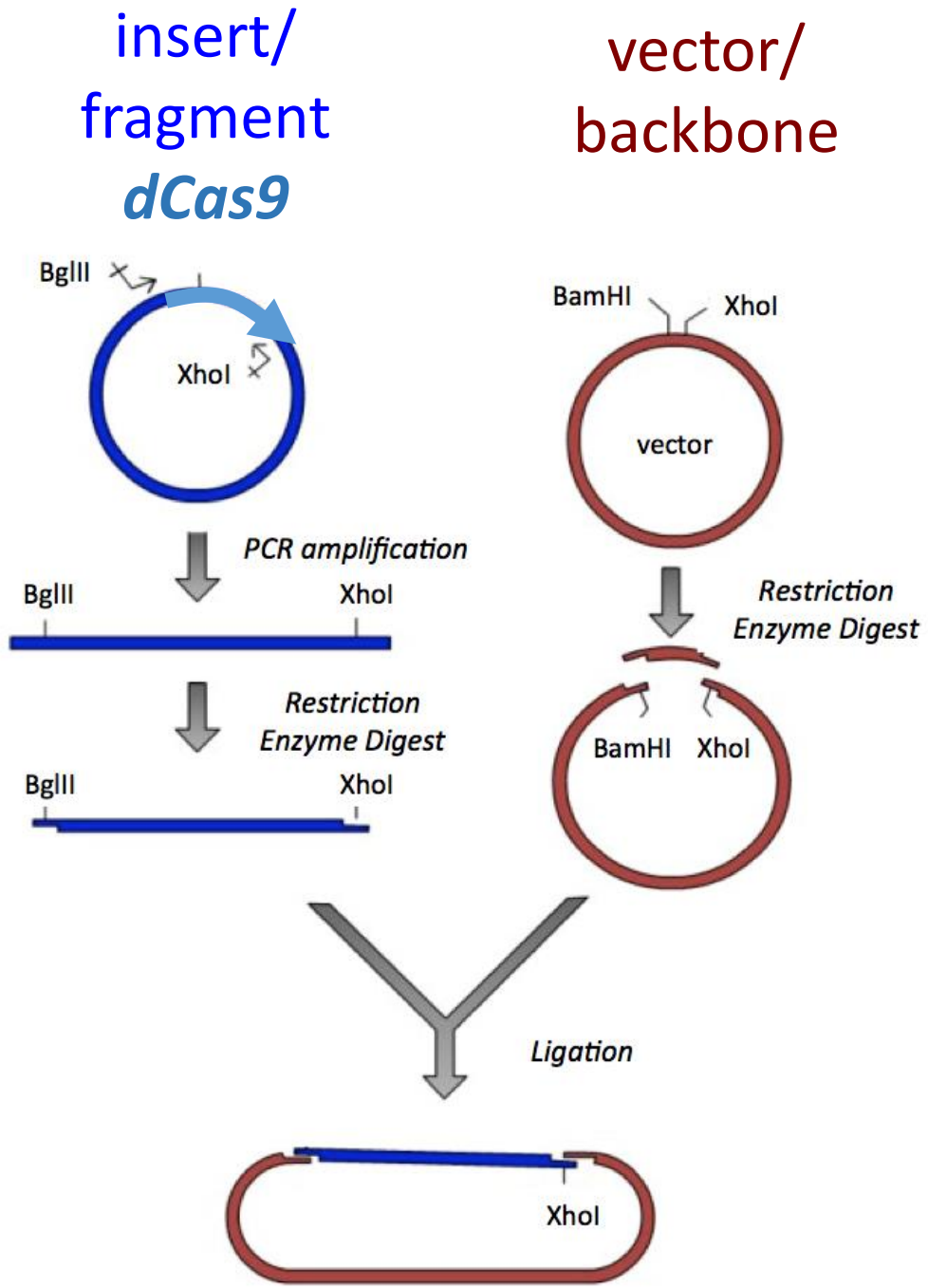
1) Prepare CRISPRi components
   1) pdCas9
   2) psgRNA_target of your design
2) Get these components into E.coli
3) See if your target increases ethanol or acetate

Cloning of pdCas9:

1. PCR amplification of DNA & *adding of convenient REnzyme sites:*

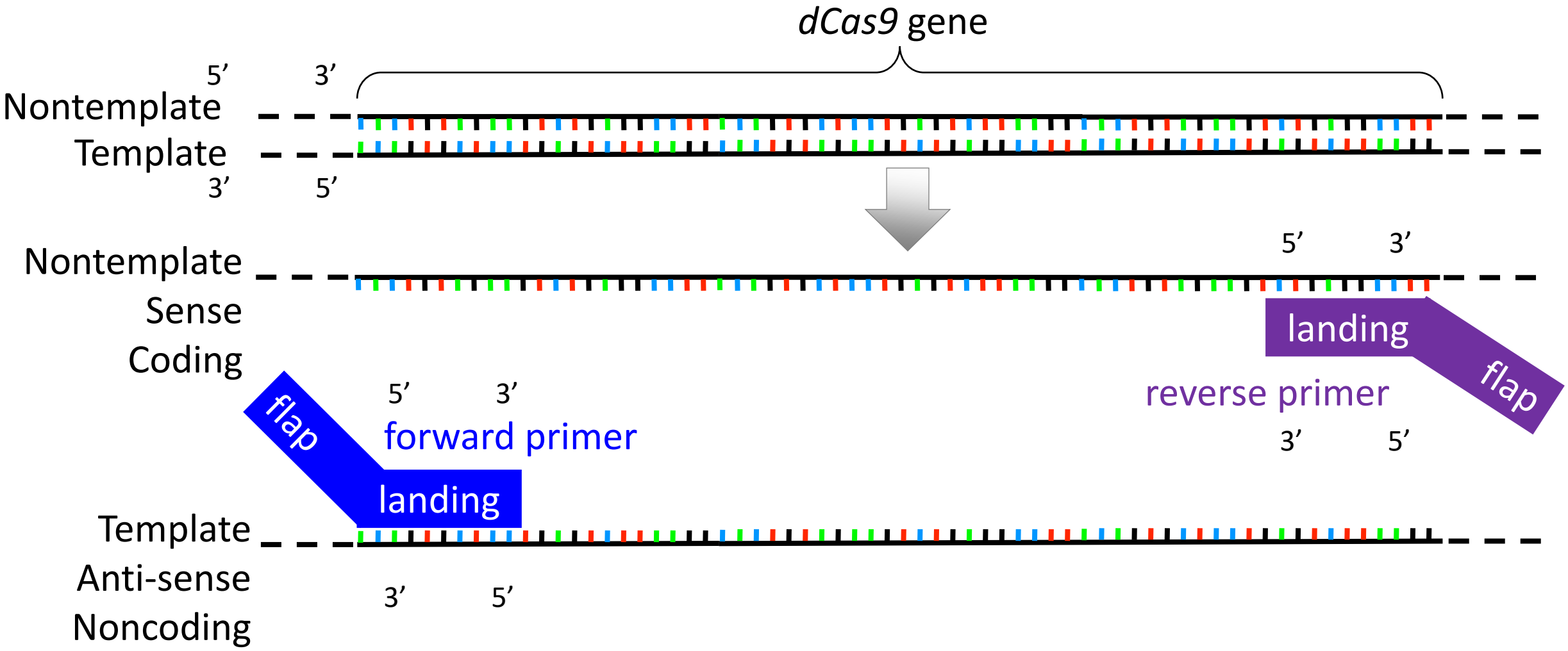
2. Digestion:

3. Ligation:

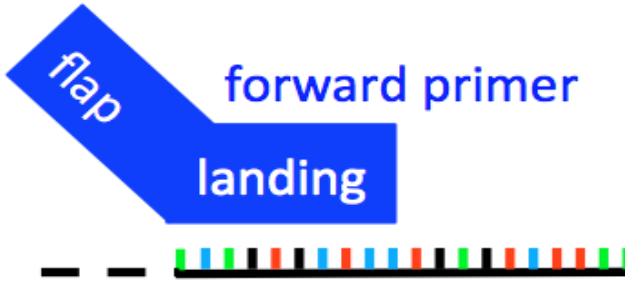


PCR amplification of DNA: Primers

- **Landing sequence**: match to dCas9
- **Flap sequence**: contains endonuclease recognition sequence and junk DNA



Primer design guidelines

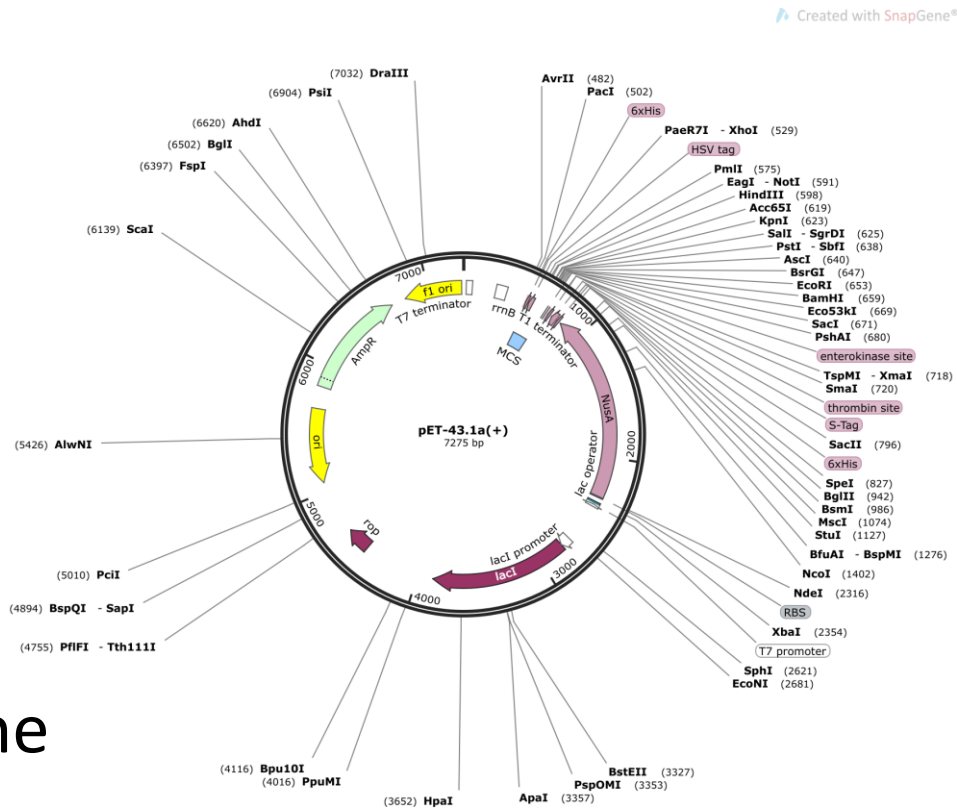


- Length: 17-28 base pairs
- GC content: 40-60%
  - GC has _____ hydrogen bonds; AT has _____ hydrogen bonds
  - GC clamp at ends
- T_m(primer) < 65 °C

- Avoid secondary structures
  - Hairpins [IDT FAQ has a guide here]
  - complementation w/in primer sequence [Try for < 1/3 complementation]
- Avoid repetitive sequences
  - Max of 4 di-nucleotide repeats (ex. ATATAT)
  - Max of 4 bp in a run (ex. GATGGGG)

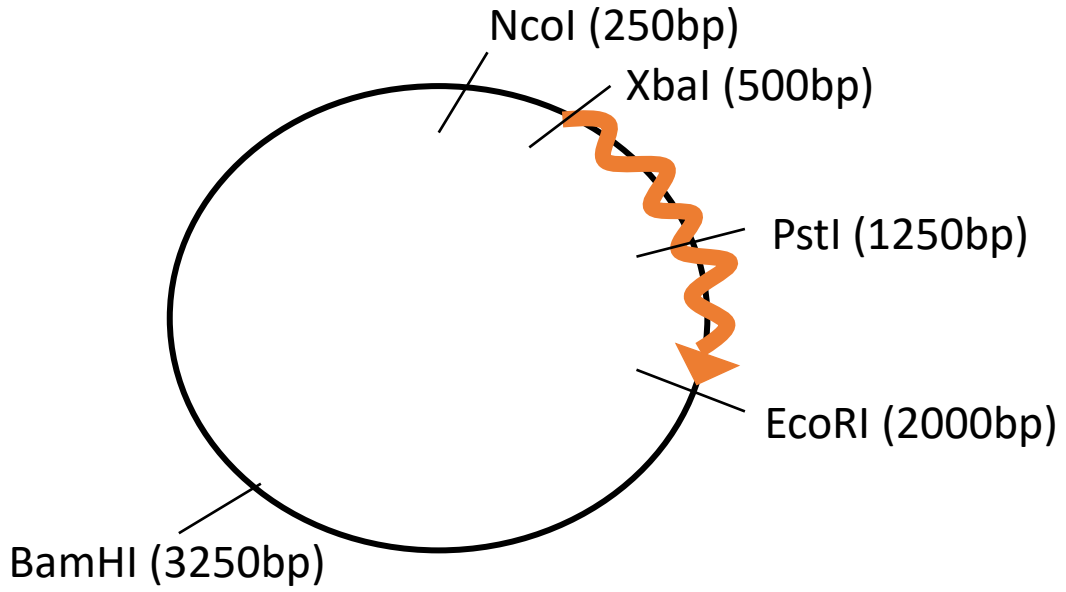
In silico cloning in SnapGene

- Work through the steps of cloning without the hours of incubations
  - Primer design and PCR amplification of insert
  - Restriction enzyme digest of insert and backbone
  - Ligation of insert into new backbone
- Generate plasmid map of dCas9 in new vector
- The plasmid you generate *in silico* is the same one we generated in the lab
  - Want to test that we generated the correct plasmid



Use a confirmation digest to test correct plasmid construction

- Use 2 restriction enzymes to cut the plasmid
  - Different from REs used to cut insert and backbone
  - One RE cuts only insert
  - One RE cuts only vector
- Can infer correct insertion of insert into backbone
- Considerations when choosing restriction enzymes:
  - Do you have access to the enzymes? [Wiki]
  - Are the two enzymes compatible? [NEB]
  - Are the resulting fragments *easily distinguished* on an agarose gel?



pNLL-PCR2 (6000bp)

For today

- Complete cloning and generate plasmid map of pdCas9
- Set up confirmation digest of pdCas9 based on plasmid map
  - Begin by 4:30pm
  - Printed maps available at front bench if needed

For M2D2

- Select article for Journal Club
- Sign up on wiki using instructions on the sign up page
  - Only one person per section can sign up for an article
  - First come first serve
- Write summary of why article is interesting, based on abstract and introduction

Mod 2 Due Dates
(because we haven't given you enough to think about…)

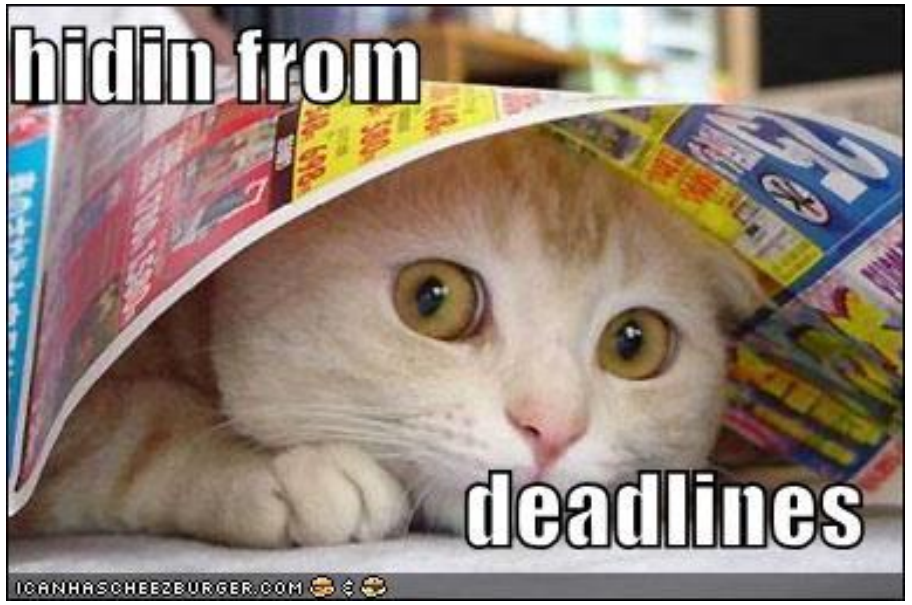
- **Journal Club presentation** (15%)
  - Individual
  - Presentations on 3/29 & 3/31
- **Research article** (20%)
  - Individual
  - due 4/23
- Laboratory quizzes (collectively 5%)
  - M2D4 and M2D7
- Notebook (collectively 5%)
  - one entry will be graded in detail by Christine
- Blog (part of 5% Participation)
  - due 4/1 & 4/25 via Slack channel

(Almost) done with Mod 1!

- Data summary due: **Saturday March 12 at 10pm**

- Thursday: Becky 5-7p on Zoom
- Friday: Noreen 5-7p on Zoom
- Saturday: Becky 10-12p and Noreen 12-2p, both on Zoom

- Revision (if you want) due: **Sunday March 20 at 10pm**



Data Summary Questions?