Manipulating DNA

What can we do with DNA ?

What can we do with DNA ?

Extract, purify

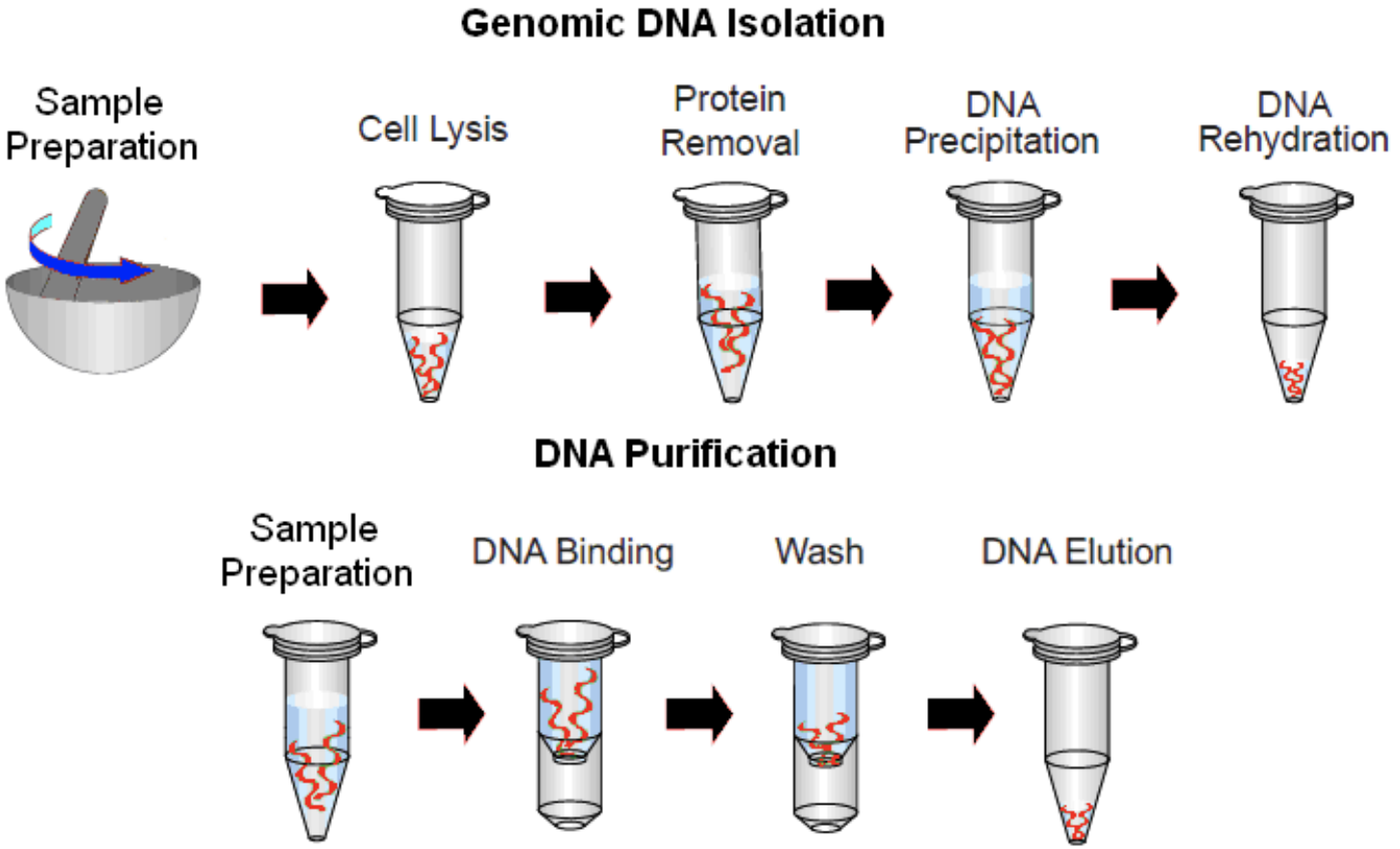
Make more
Amplify
Clone
Synthesize

Examine
Quantify
Examine length
Stain, probe
Sequence
Examine 3D structure
Measure physical properties of DNA molecules

Modify
Cut
Ligate
Recombine fragments
Introduce foreign DNA
Mutate

Extract DNA

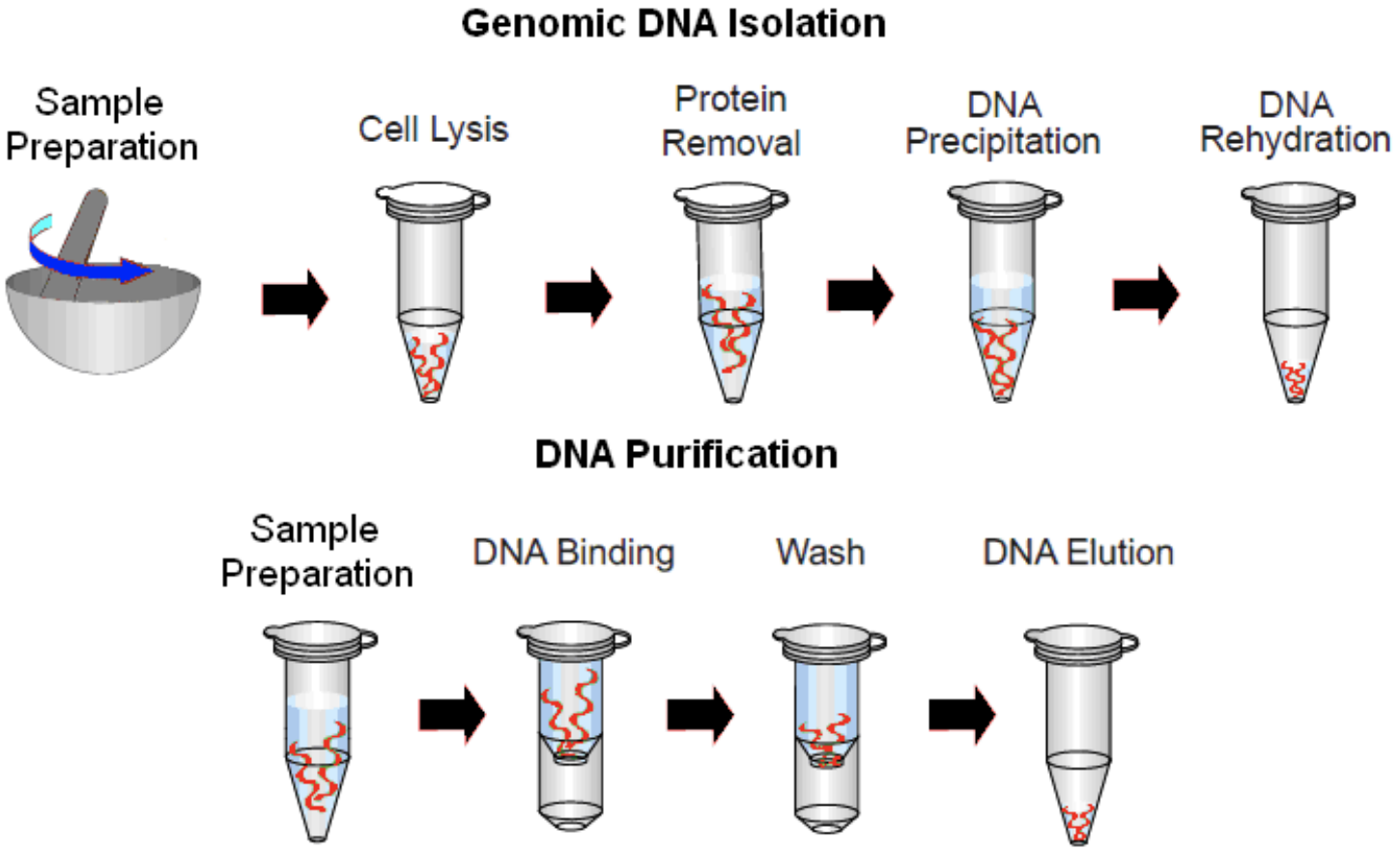
Break cells, remove lipids and proteins,
precipitate DNA, remove liquid, resuspend in aquaeous solution



Be aware of contaminants!

Extract DNA

Break cells, remove lipids and proteins,
precipitate DNA, remove liquid, resuspend in aquaeous solution



Be aware of contaminants!

(DNA from mitochondria, viruses, bacteria, researcher, symbionts...)

Amplify DNA

Mix:
Genomic DNA
Probes (oligonucleotides)
Nucleotides
Taq polymerase
Ions ($MgCl_2$)

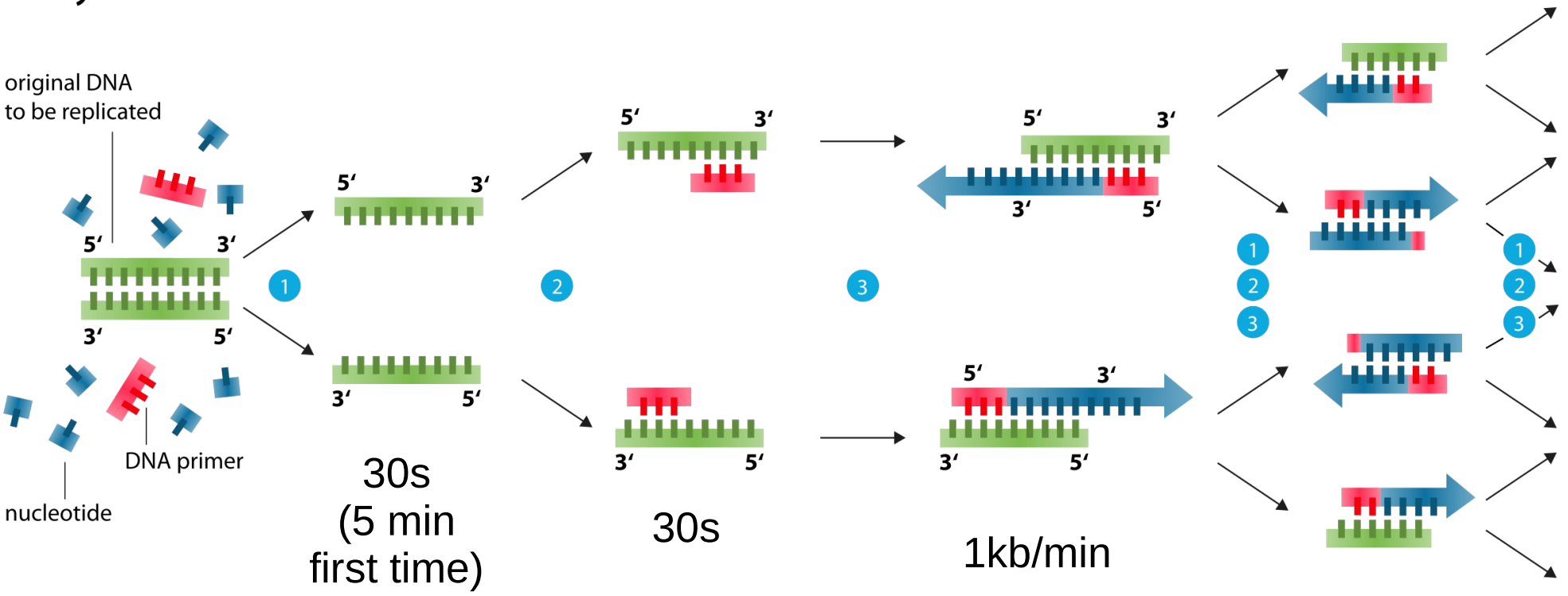
Cycles of Denaturation, Annealing, Elongation

PCR: Polymerase Chain Reaction

Amplifies DNA fragments of between 0.1 and 10 kb (up to 40 kb)

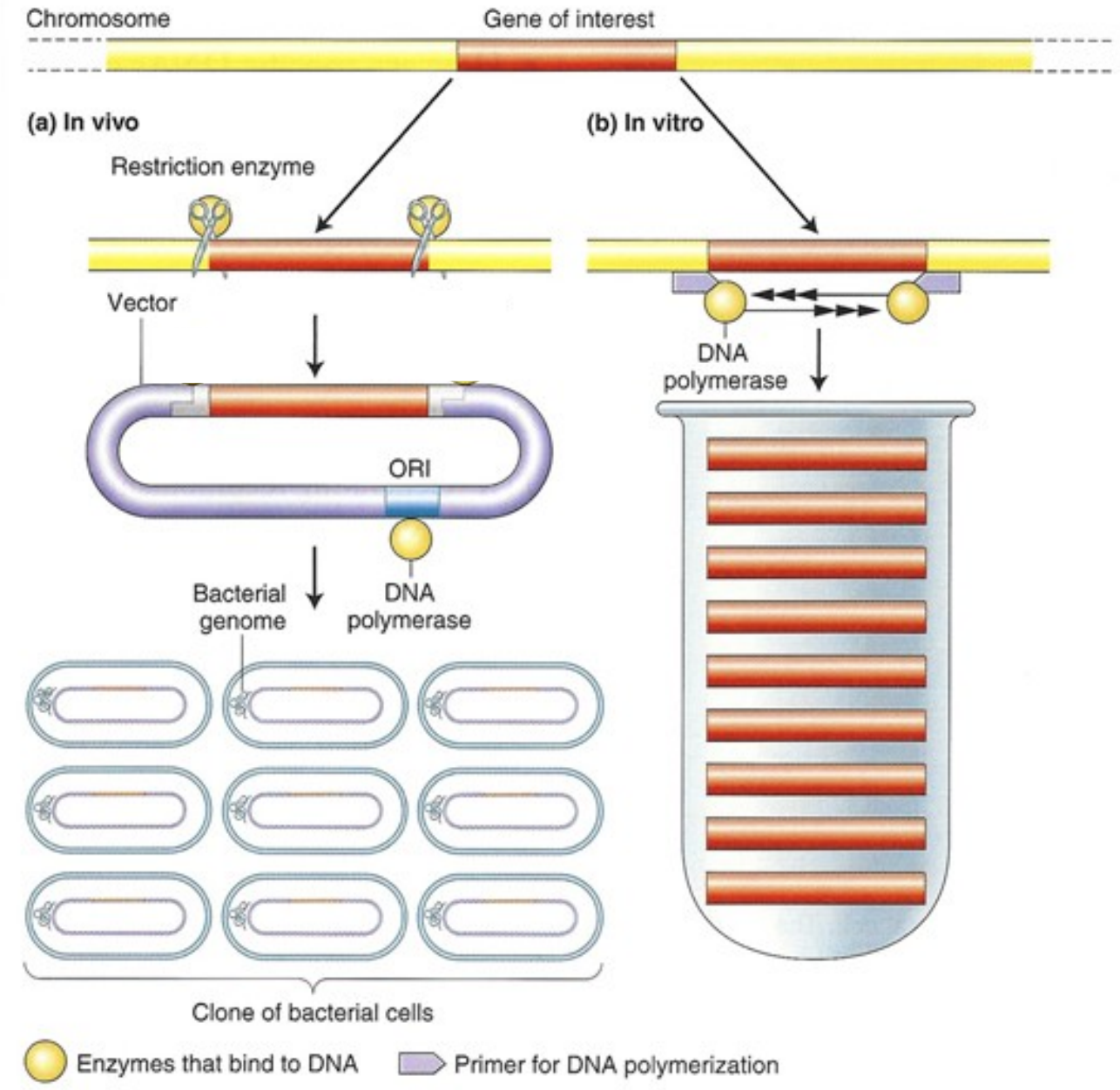
Amplify DNA

Polymerase chain reaction - PCR



1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C

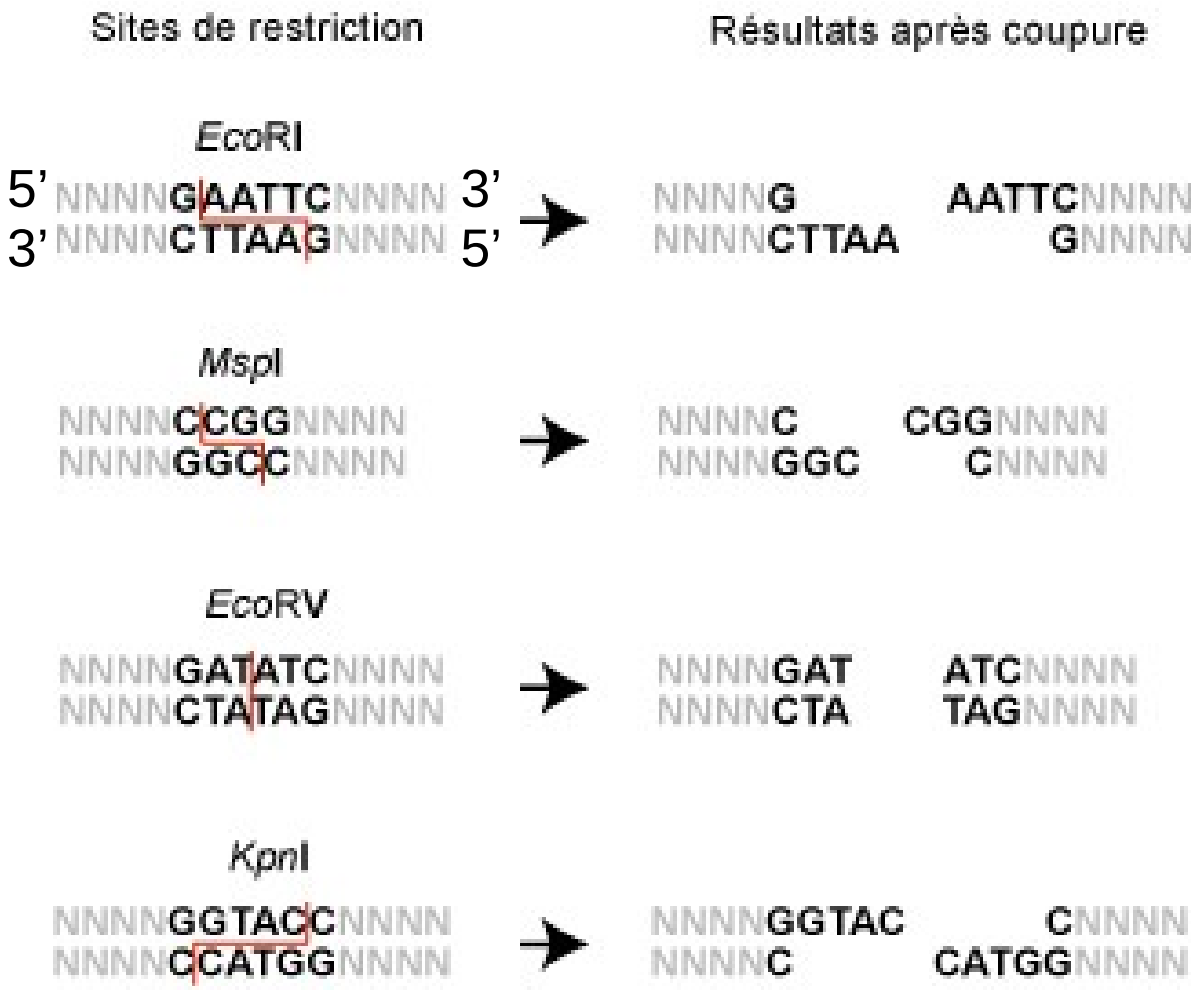
Cloning vs. PCR



Amplify DNA

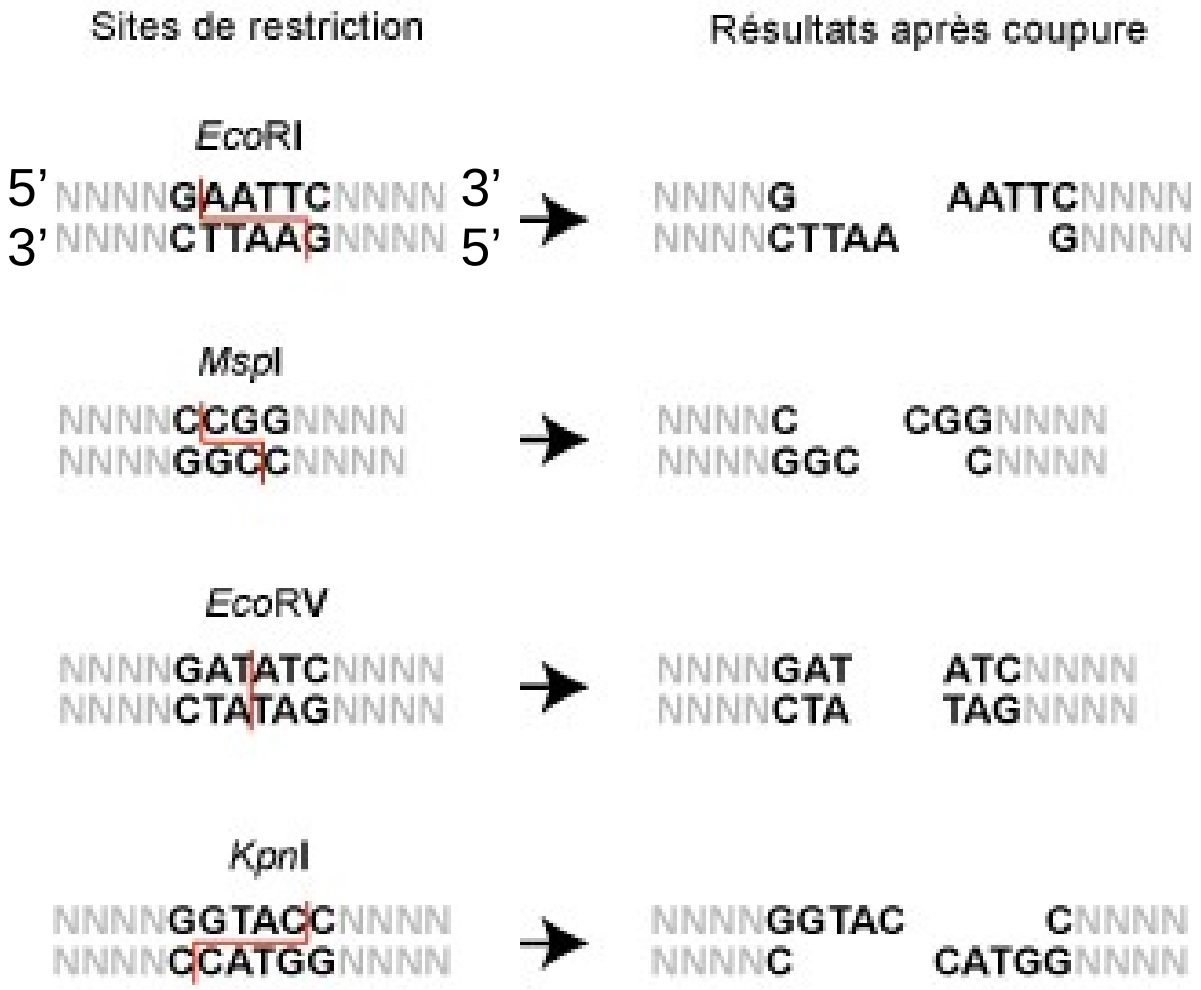
DNA fragments
5 kb-15 kb: plasmids in bacteria
~10 kb: lambda phage-based vectors
Up to 40 kb: fosmids in bacteria
~100-300 kb: bacterial artificial chromosomes (BAC)

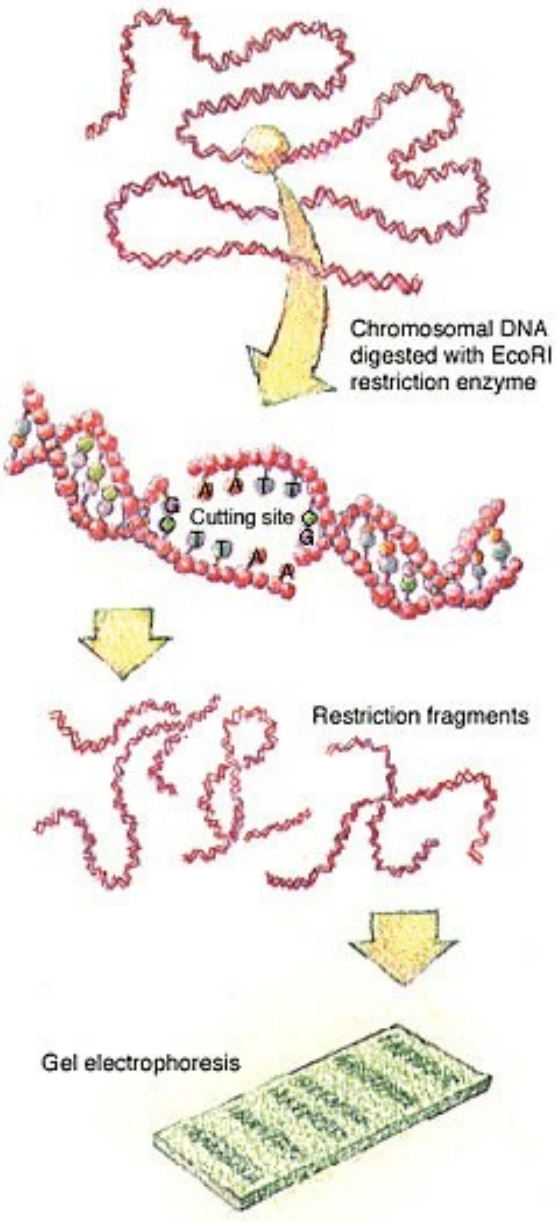
Cut DNA with restriction enzymes



Blunt ends, 3' protruding ends, 5' protruding ends

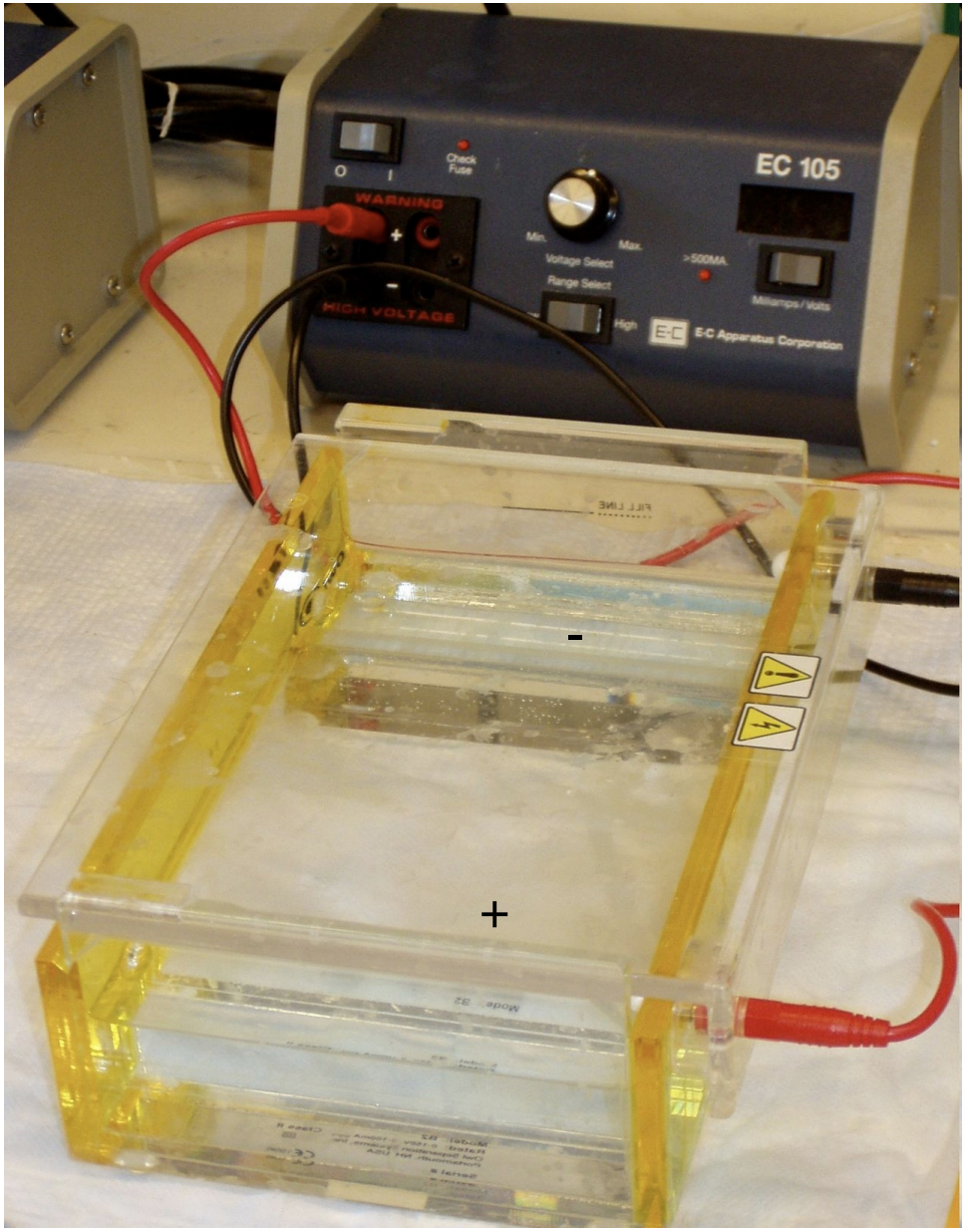
Cut DNA with restriction enzymes



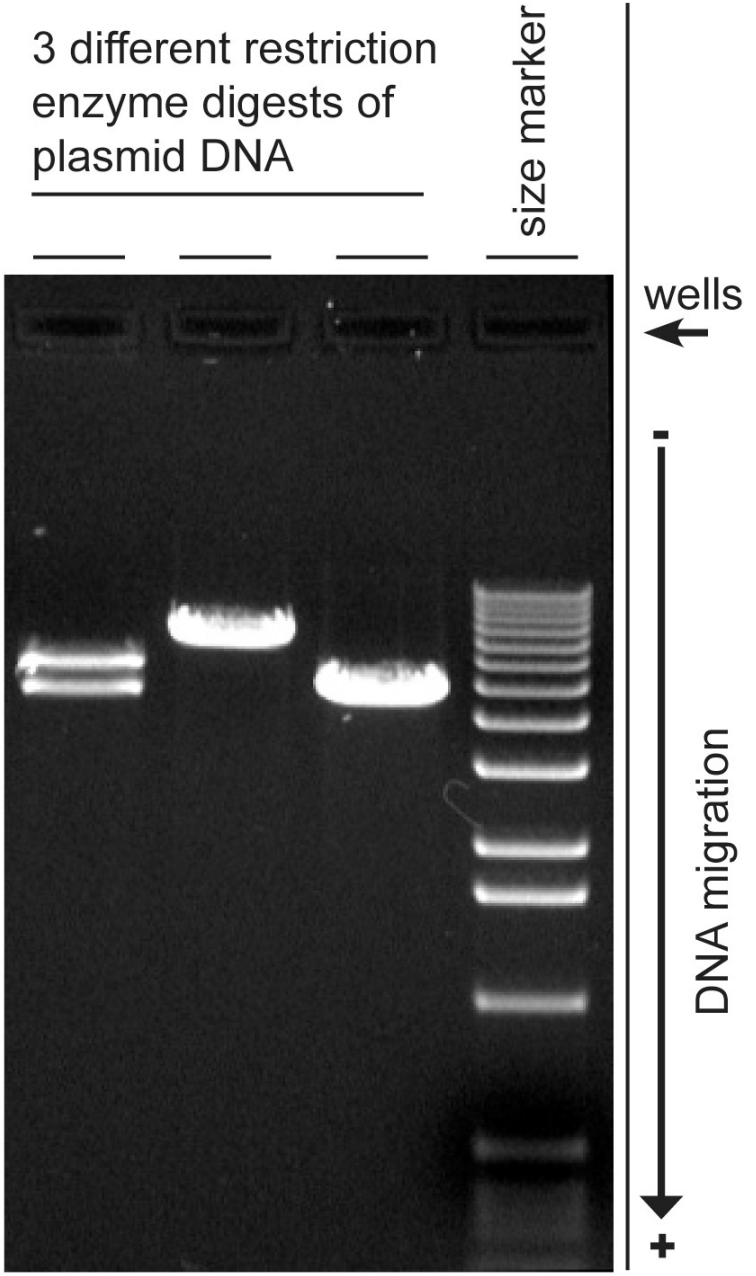


Blunt ends, 3' protruding ends, 5' protruding ends

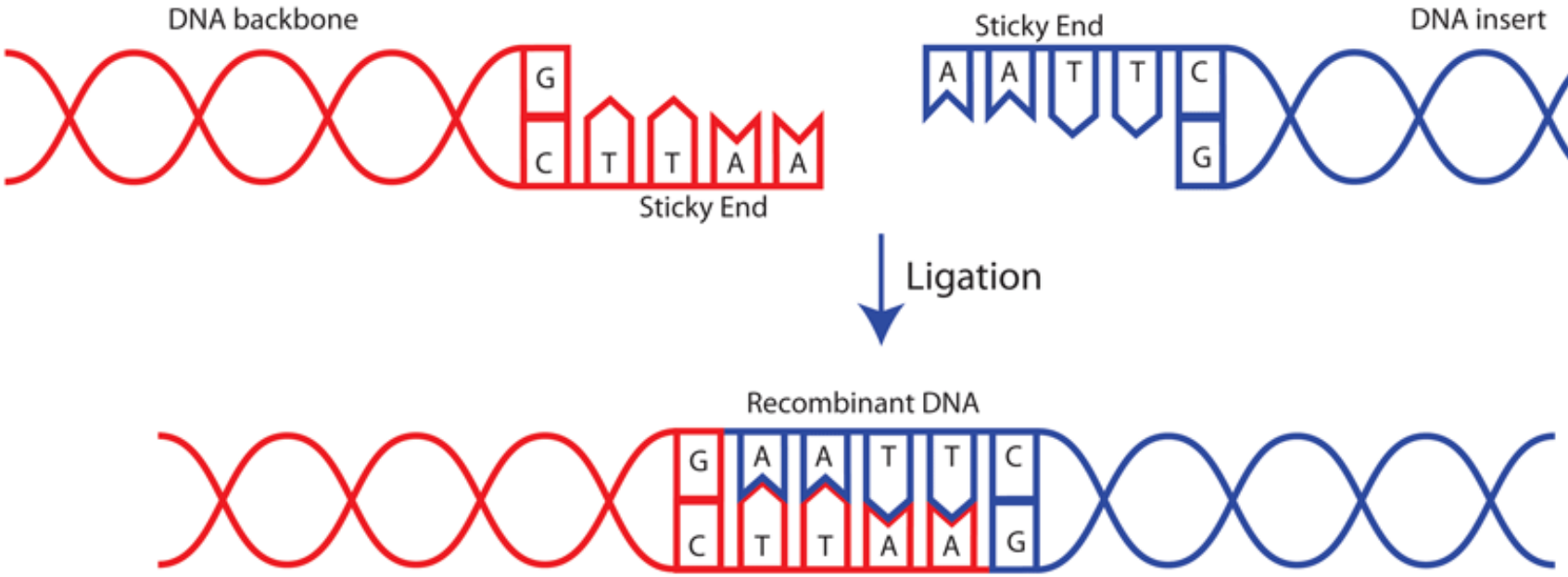
Examine length of DNA

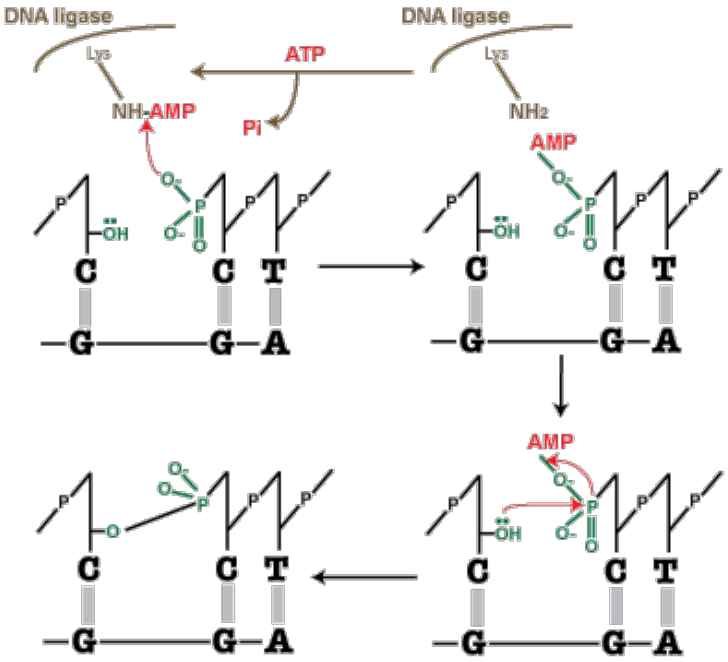


TAE (Tris-acetate-EDTA) buffer



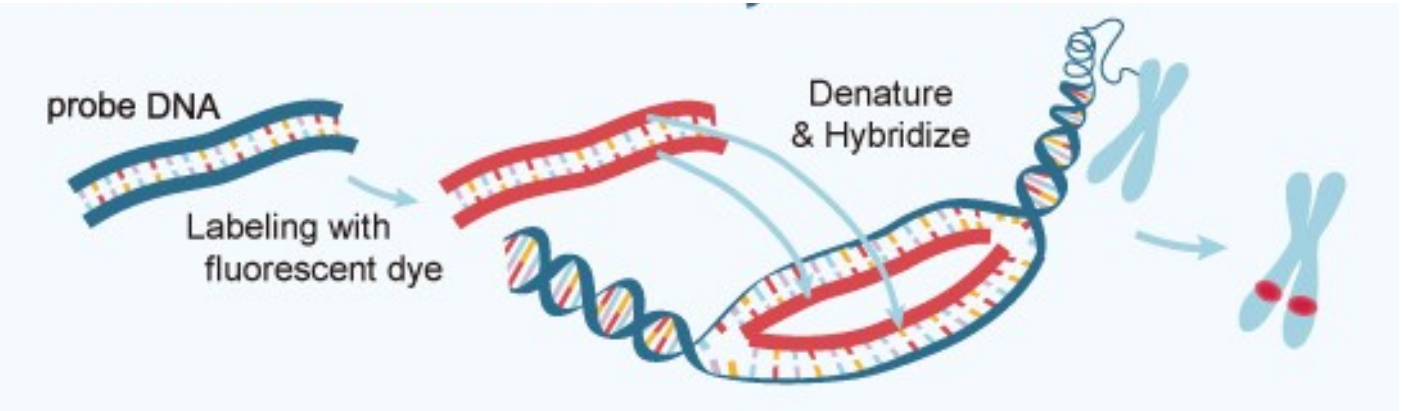
Ligate DNA

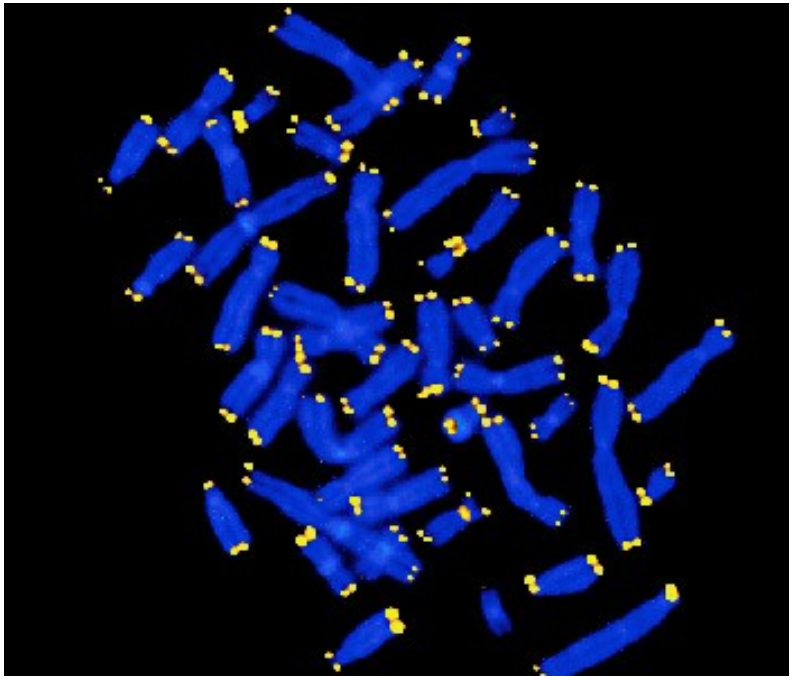




Fragments have to be phosphorylated but only on one strand
Dephosphorylate the vector to inhibit self-circularization

Probe DNA:
Fluorescent In Situ Hybridization



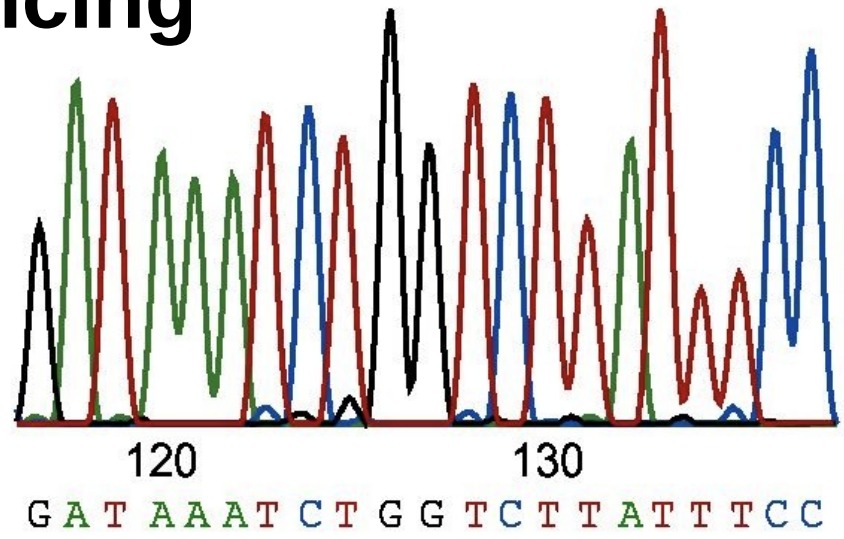


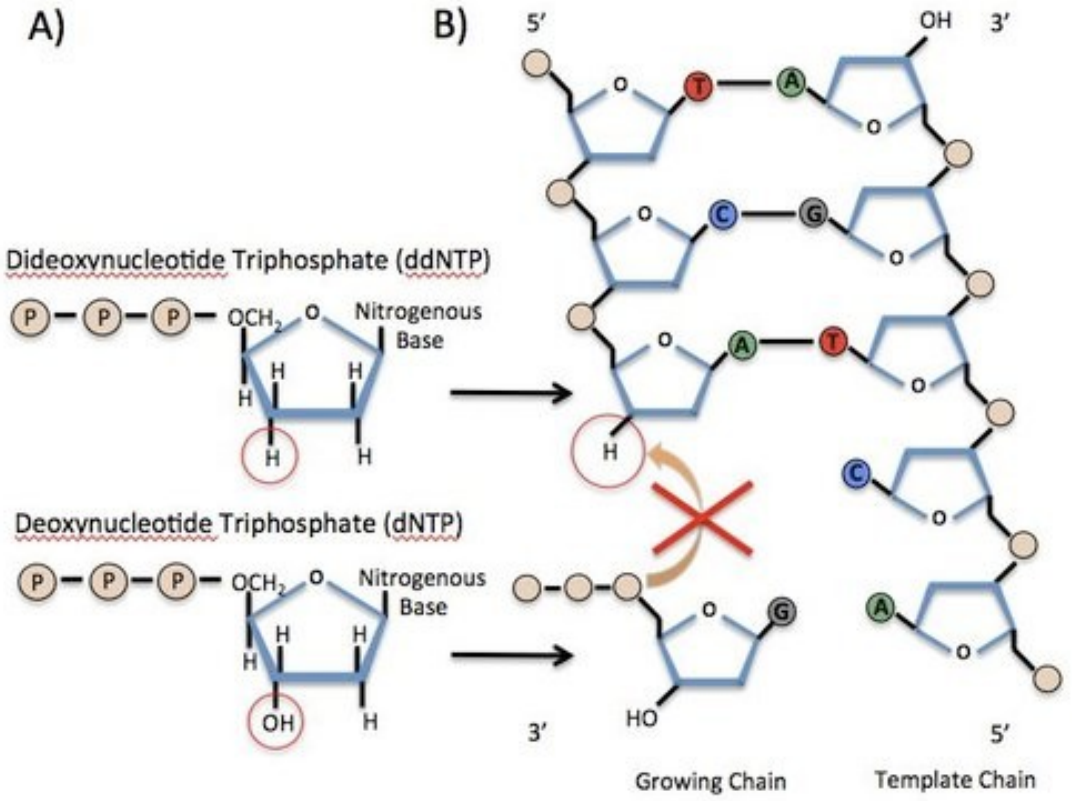
Probes for telomere sequences

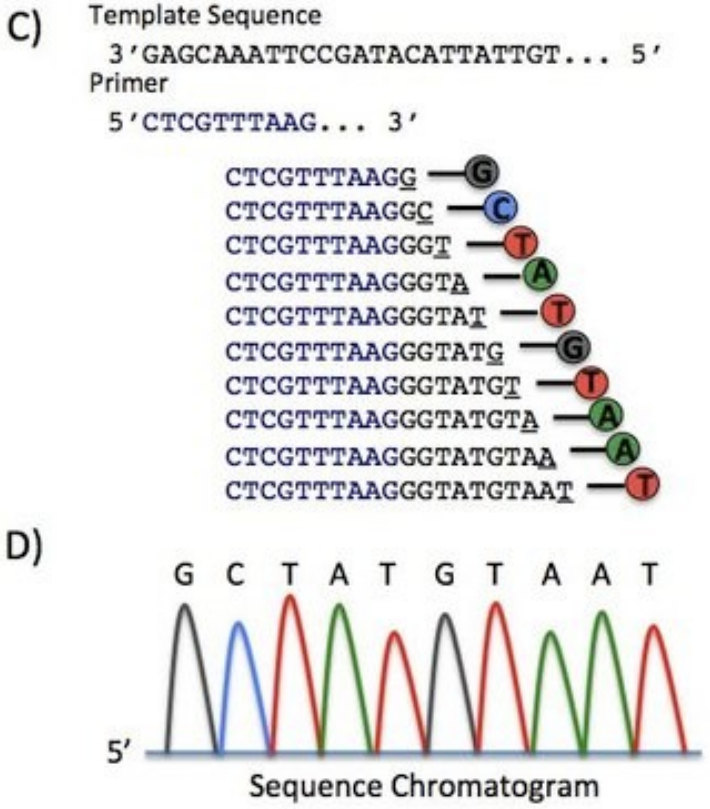
Sanger sequencing

800 bp long
Starts based on oligonucleotide (primer)
~4 euros per reaction

Dye terminator sequencing

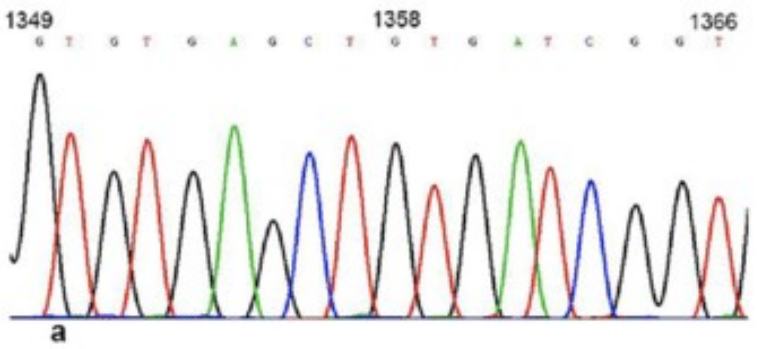


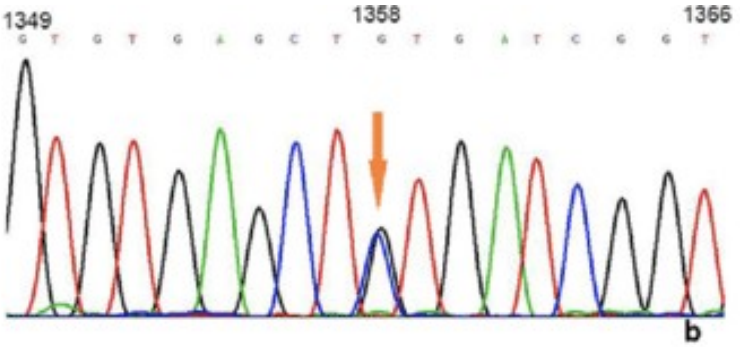


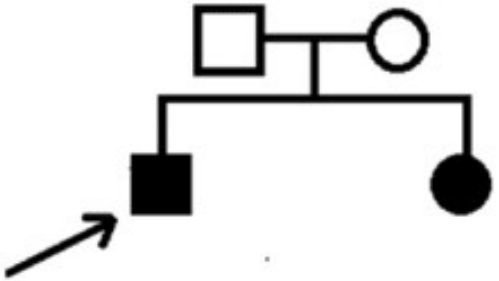


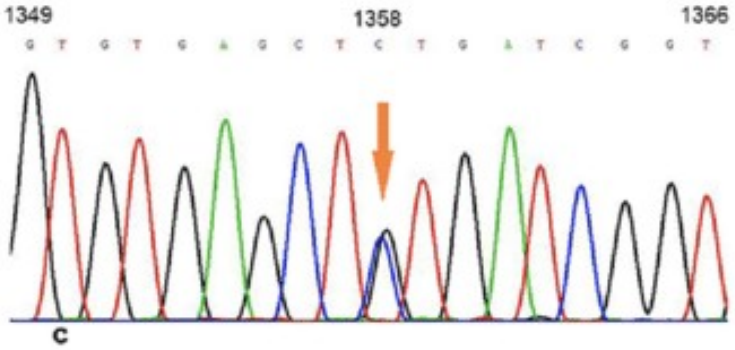
McGovern 2015

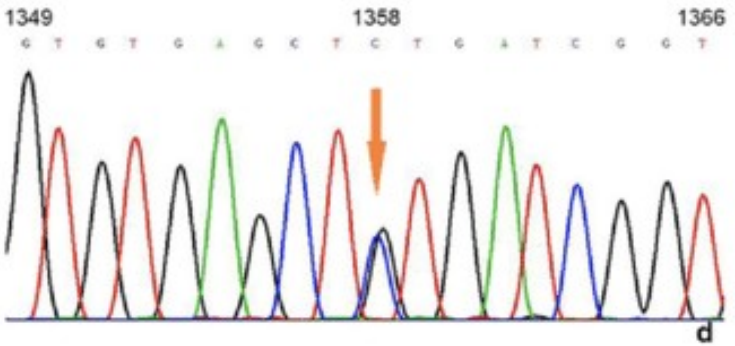
GTGTGAGCTGTGATCGGT



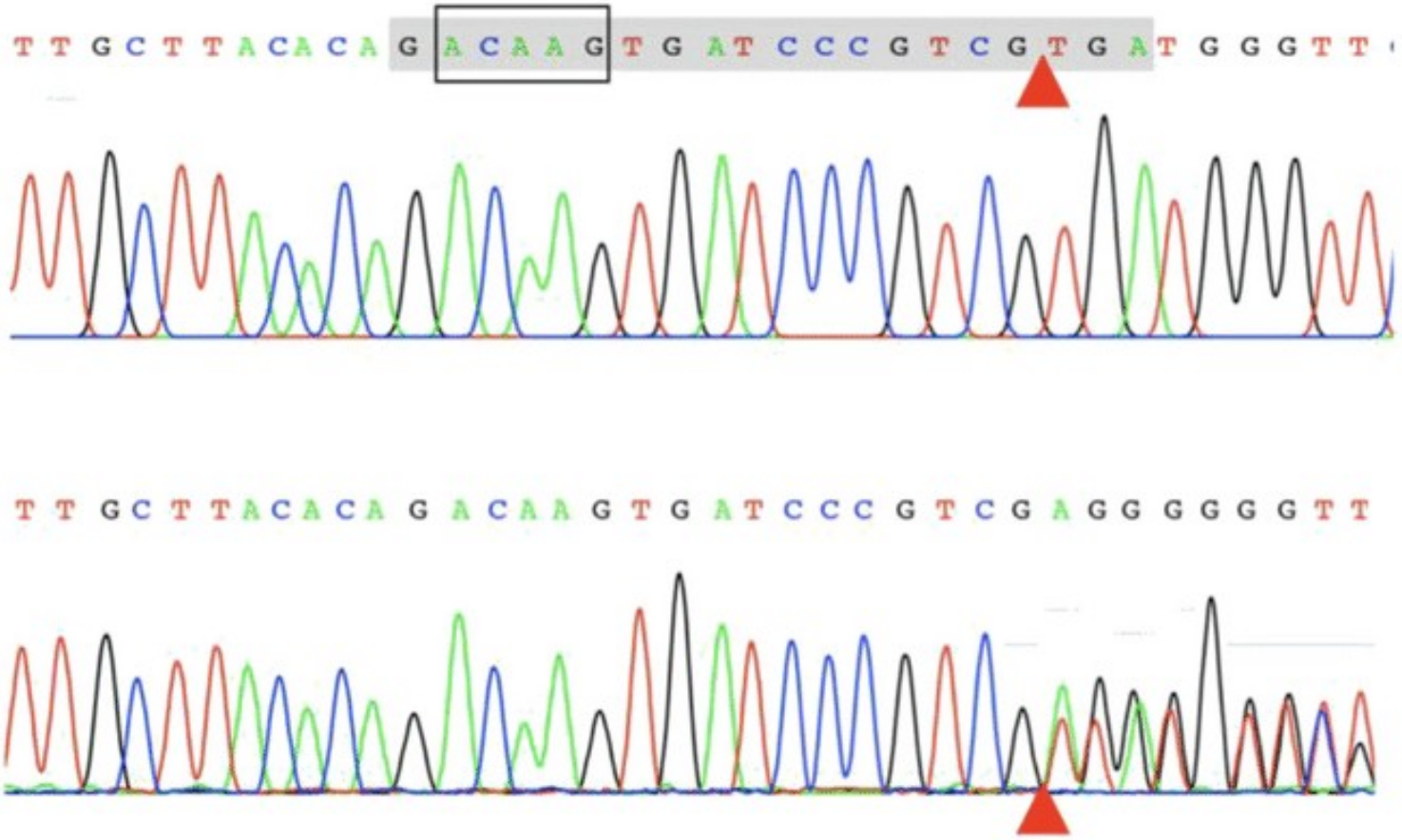






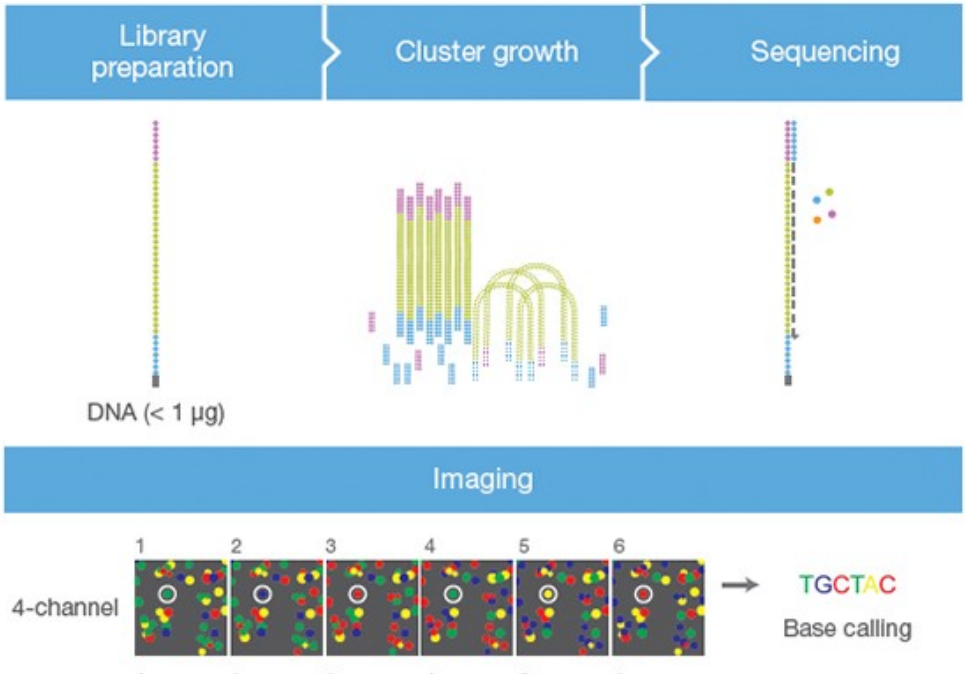


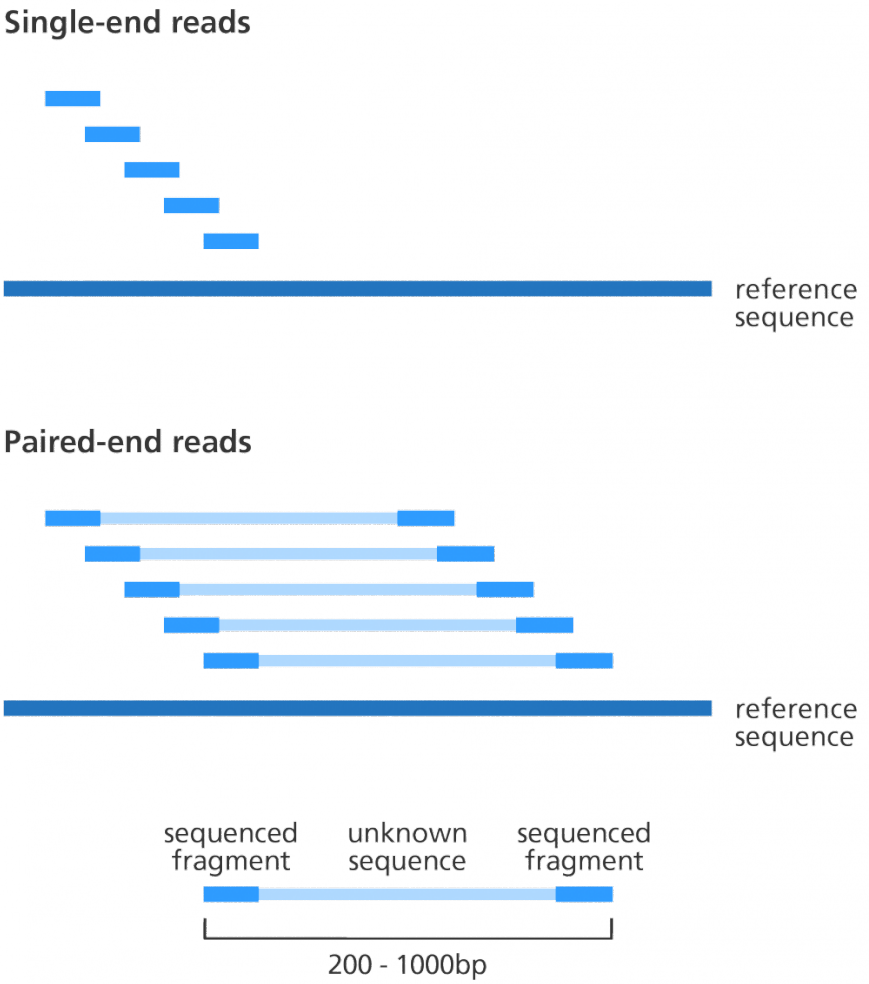
Ding et al 2015


T T G C T T A C A C A G A C A A G T G A T C C C G T C G T G A T G G G T T
T T G C T T A C A C A G A C A A G T G A T C C C G T C G A G G G G G G T T

Illumina sequencing

Millions of reads, each ~100 bp long
Starts at all possible positions
~500 euros per run



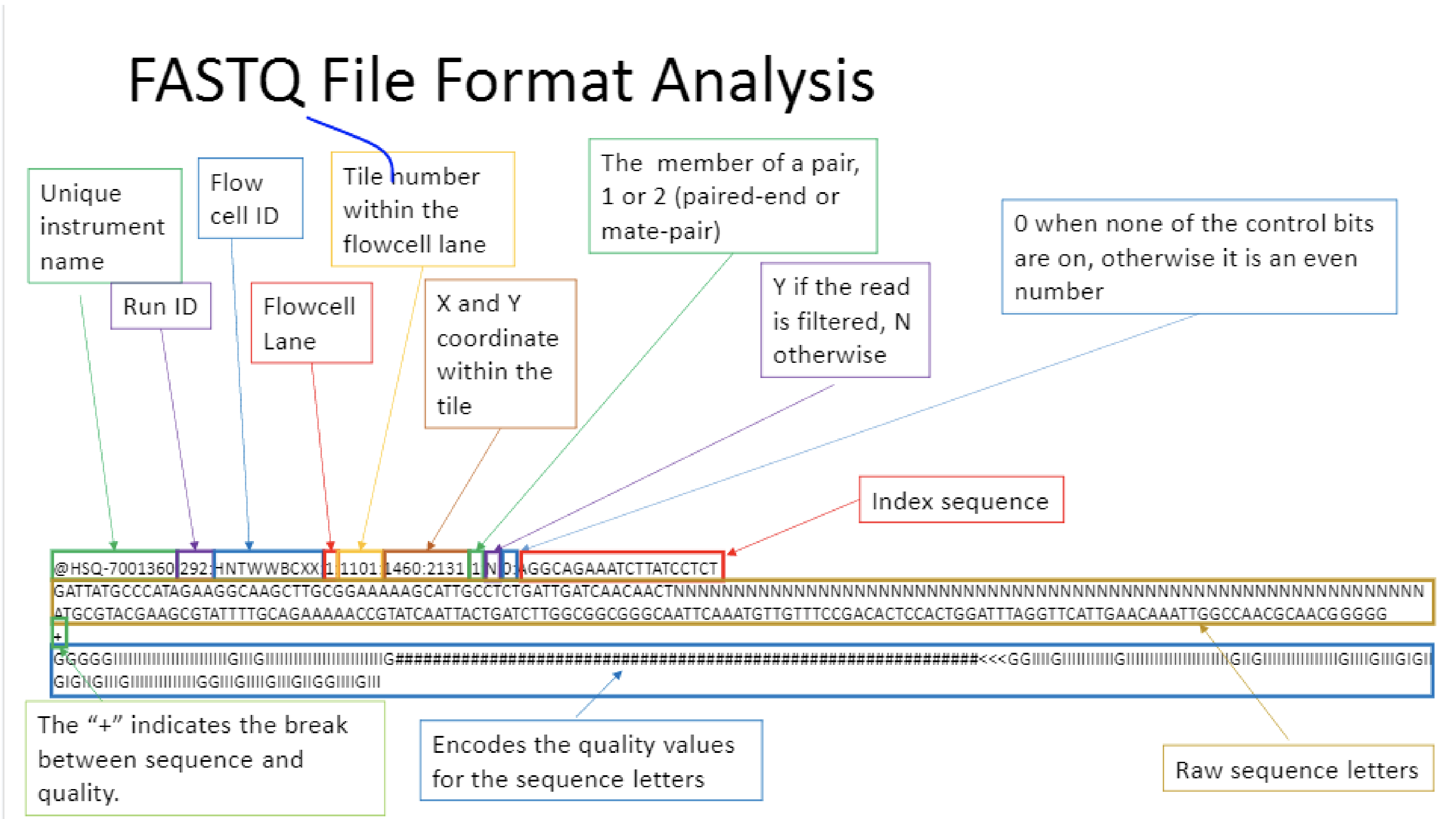


For transcriptome: 2x 75 bp
For whole genome: 2x 150 bp

https://www.youtube.com/watch?v=fCd6B5HRaZ8&t

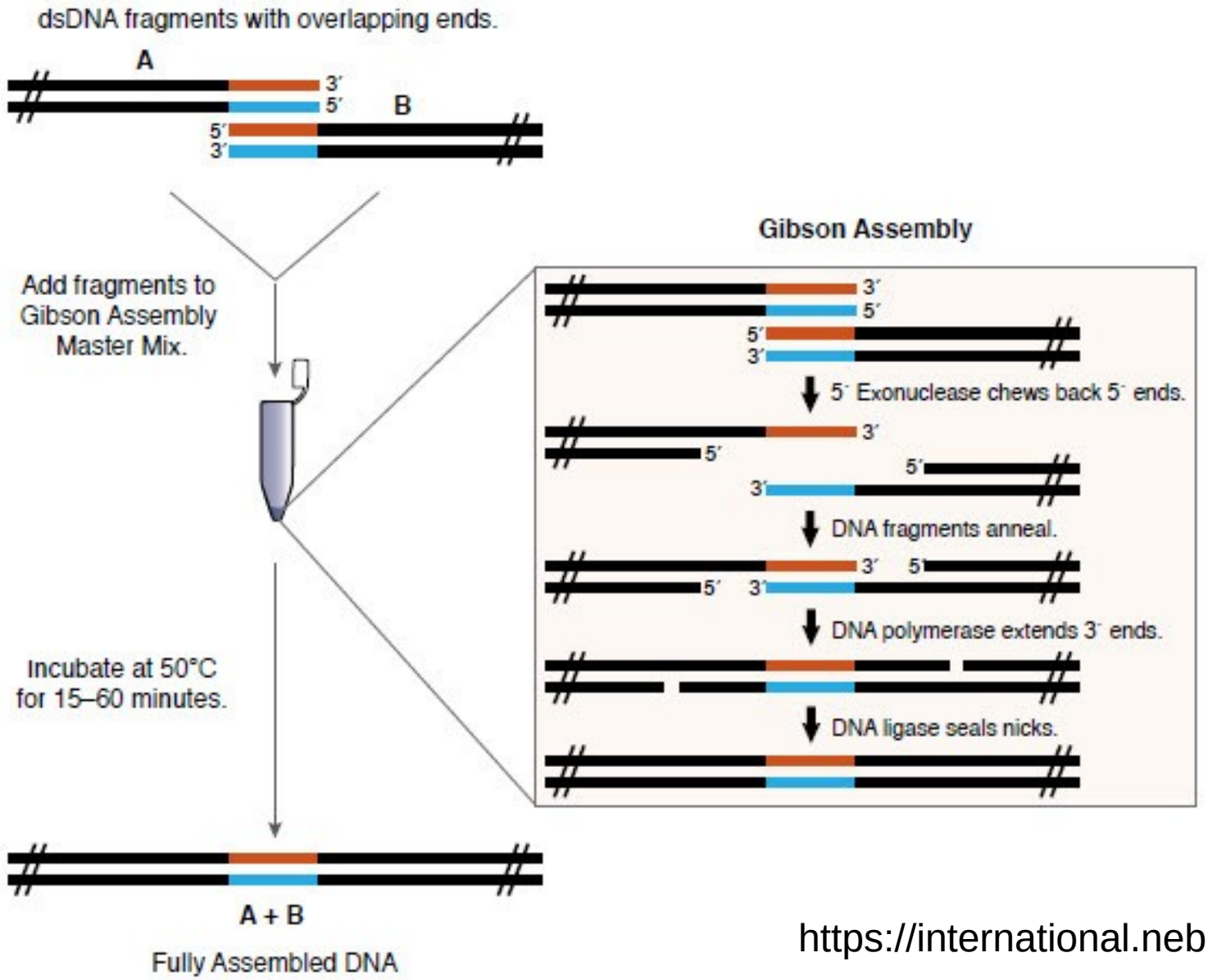
https://www.illumina.com/science/technology/next-generation-sequencing/sequencing-technology/2-channel-sbs.html

Output of Illumina sequencing



Recombine DNA: Gibson cloning

Prepare fragments using PCR and special primers



https://international.neb.com/products

Synthetize DNA

GenScript
Make Research Easy

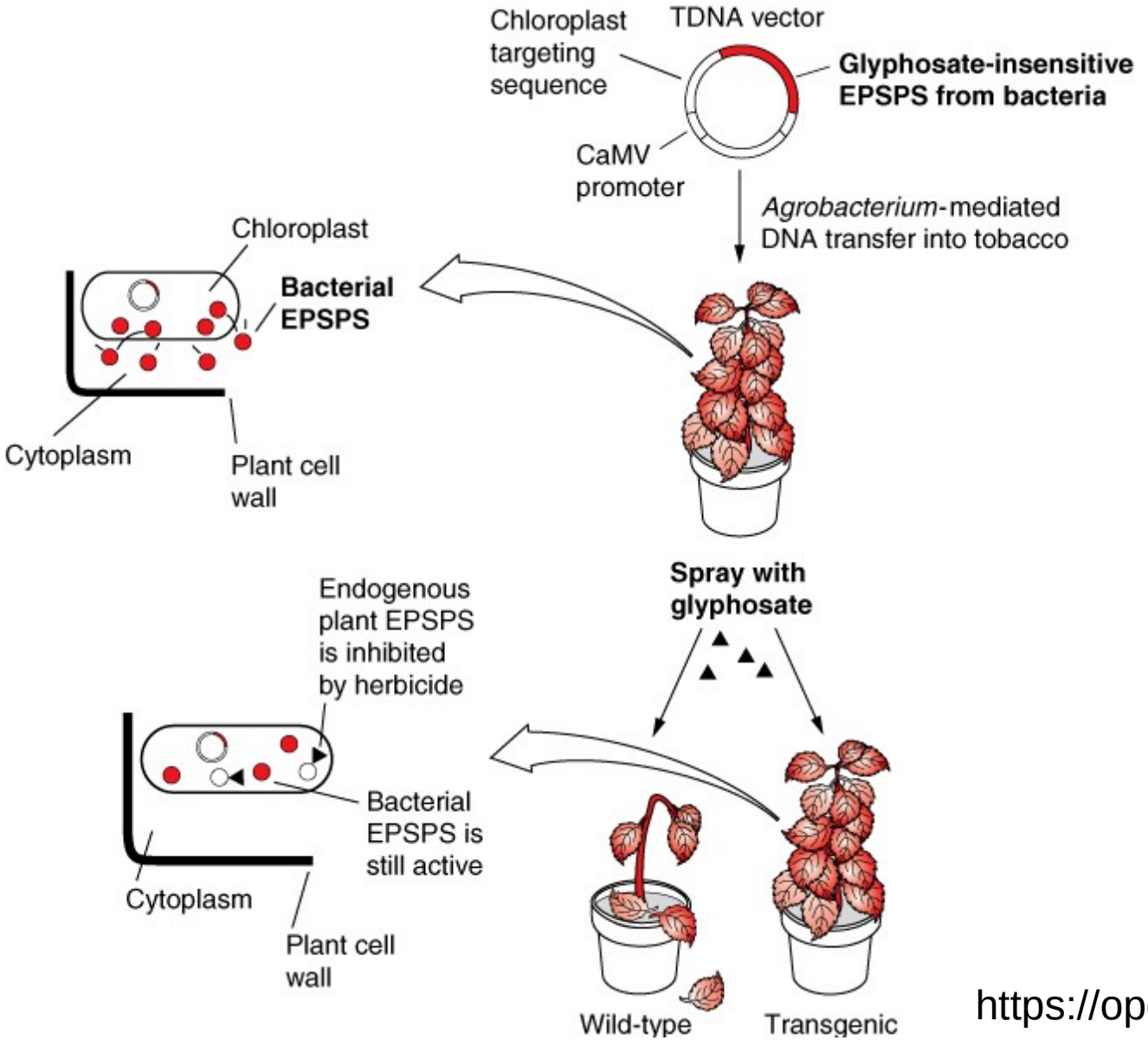
Gene Synthesis Service Options

Types	Gene Length	Price (No hidden charge promise) †	Starting Turnaround Time (Business Days) *	Starting Turnaround Time with Plasmid Prep Service (Business Days)
Standard Gene Synthesis *Guaranteed*	≤ 8 kb	View your discounted price online in as short as 1 minute	8	10
Fast Gene Synthesis *Guaranteed*	≤ 5 kb		7	9
Rush Gene Synthesis *Guaranteed*	≤ 4 kb		4 *US Manufacture*	6 *US Manufacture*
GenPlus HT Gene Synthesis	≤ 3 kb		18	20
GenPlus Economy Gene Synthesis	≤ 8 kb		15	17
GenBrick® Gene Synthesis	> 8 kb		23	25

Introduce foreign DNA

most widespread transgenic crop in 2005-2015 = soybean resistant to glyphosate

In tobacco:



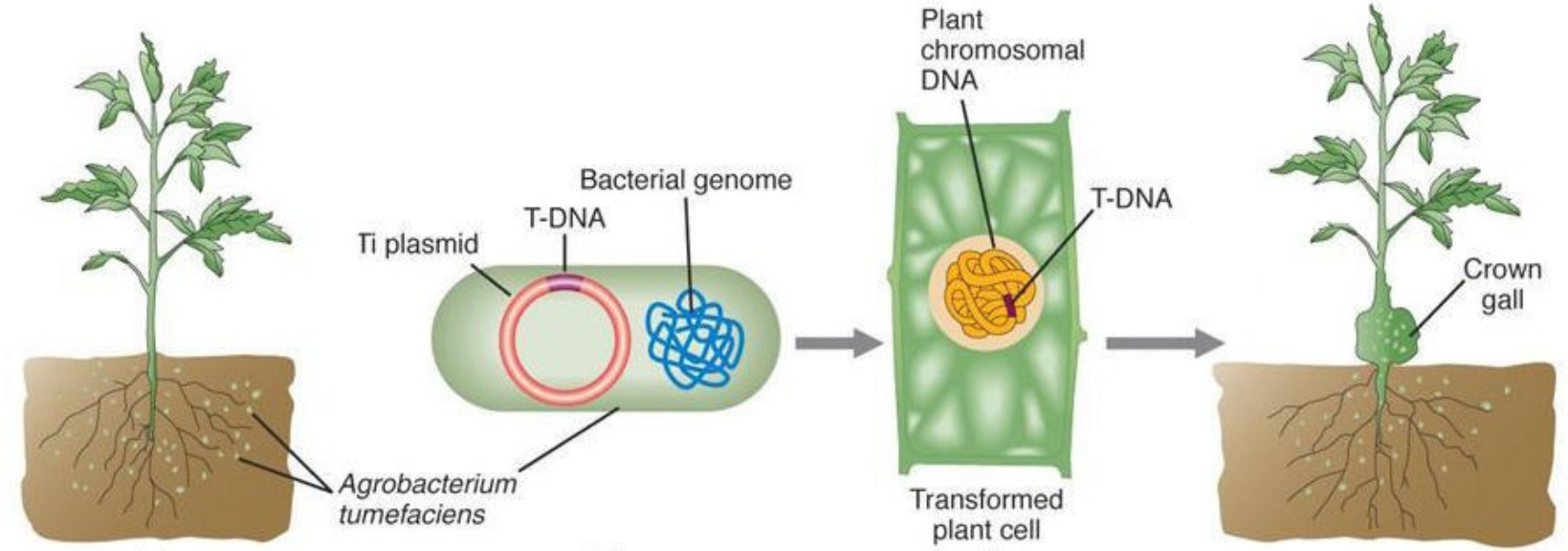
https://openwetware.org

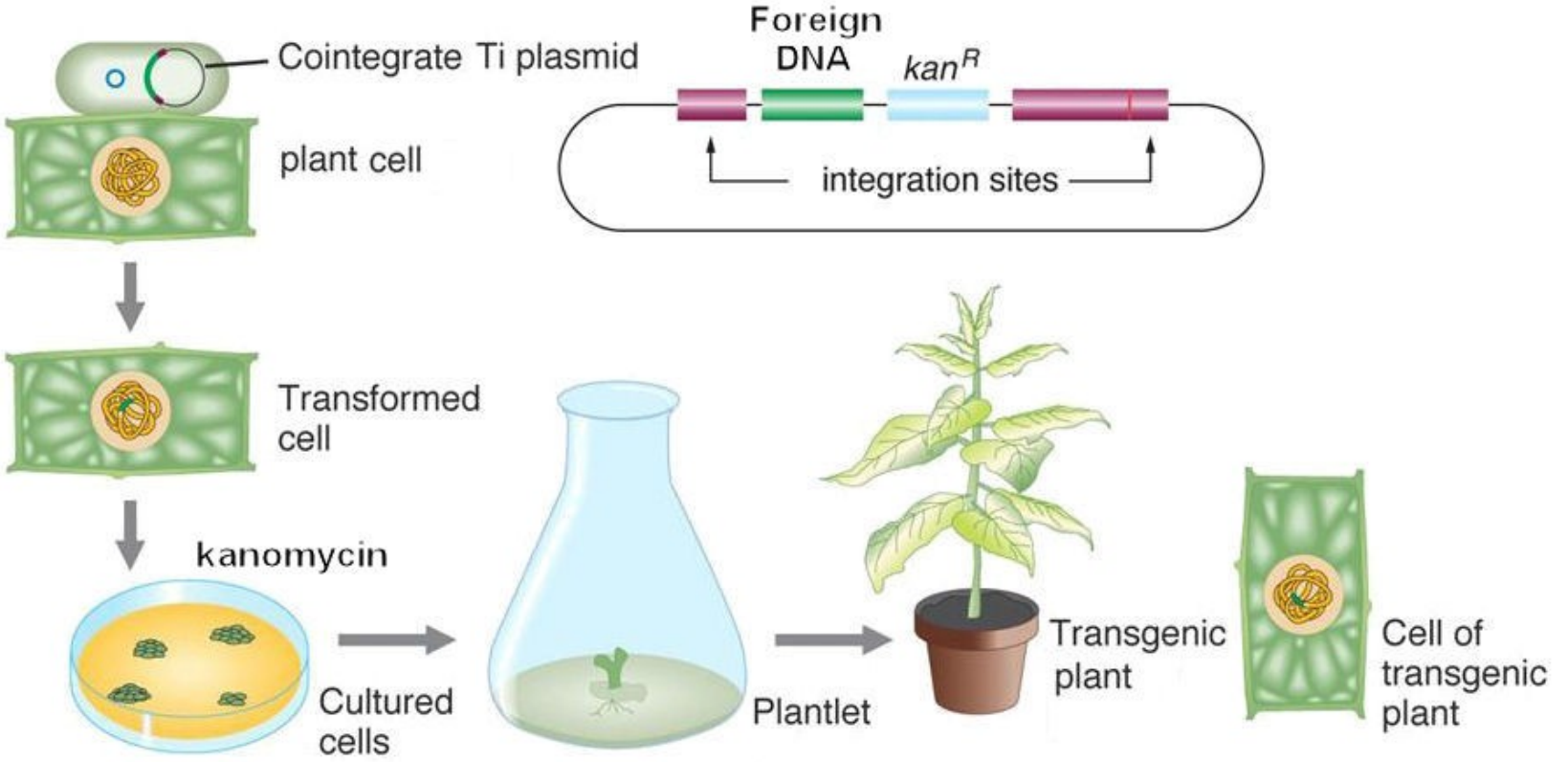
Methods to introduce DNA in plants

Physical
Microinjection
Biolistics: gene gun method
Electroporation
Laser-mediated

Chemical
Polyethyleneglycol (PEG)
Calcium phosphate
Diéthylaminoethyl-dextran (DEAE-dextran)
Artificial lipids

Biological
Agrobacterium tumefaciens
Agrobacterium rhizogenes
Virus





https://www.mun.ca/biology/scarr/Transgenic_Plants.html

Manipulating RNA

What can we do with RNA ?

What can we do with RNA ?

Extract, purify

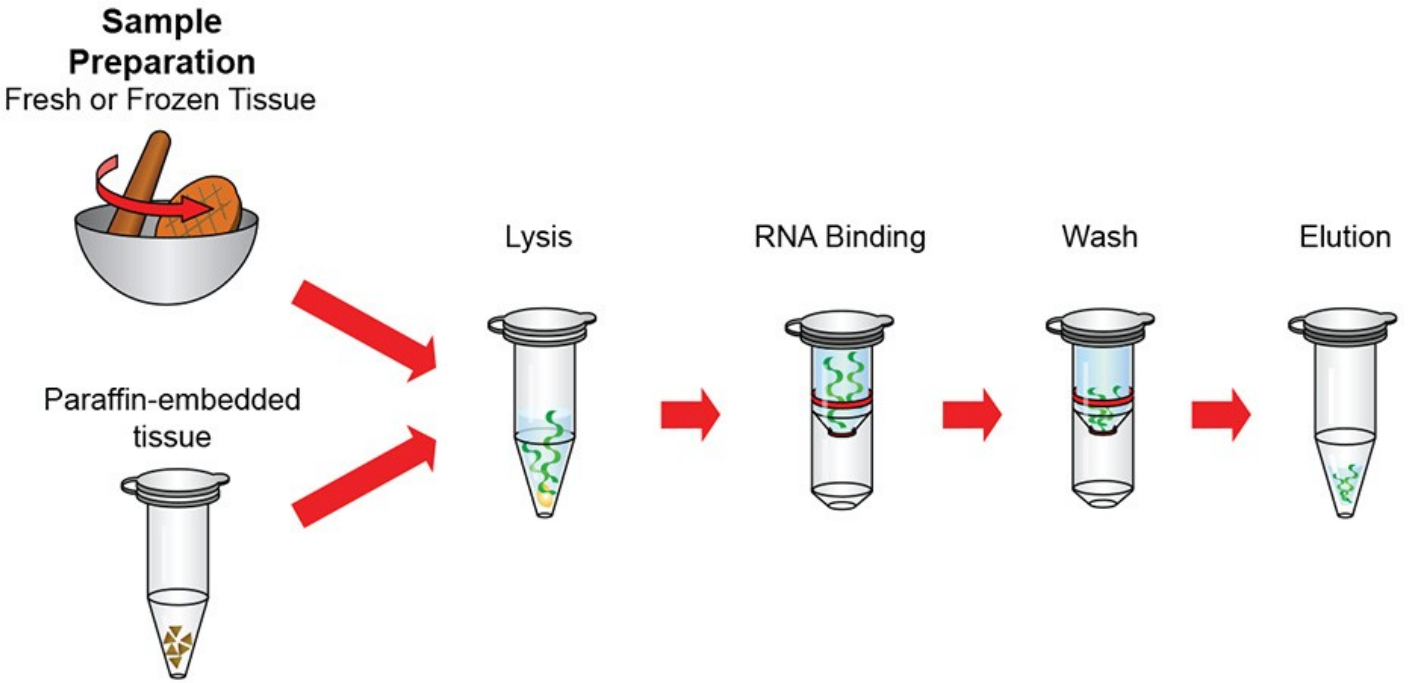
Make more
RNA → DNA → RNA (reverse transcription, transcription)

Examine
Quantify
Examine length
Stain, probe
Sequence
Examine 3D structure
Measure physical properties of RNA molecules

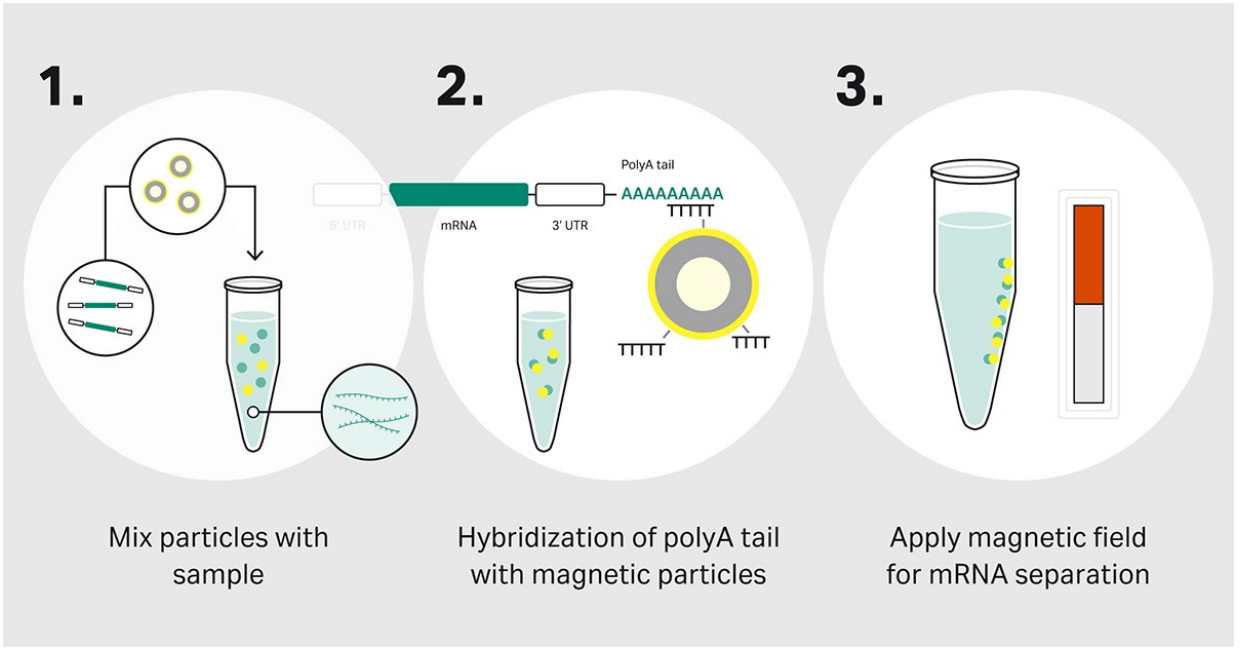
Modify
Mostly via DNA

Extract RNA

Total RNA

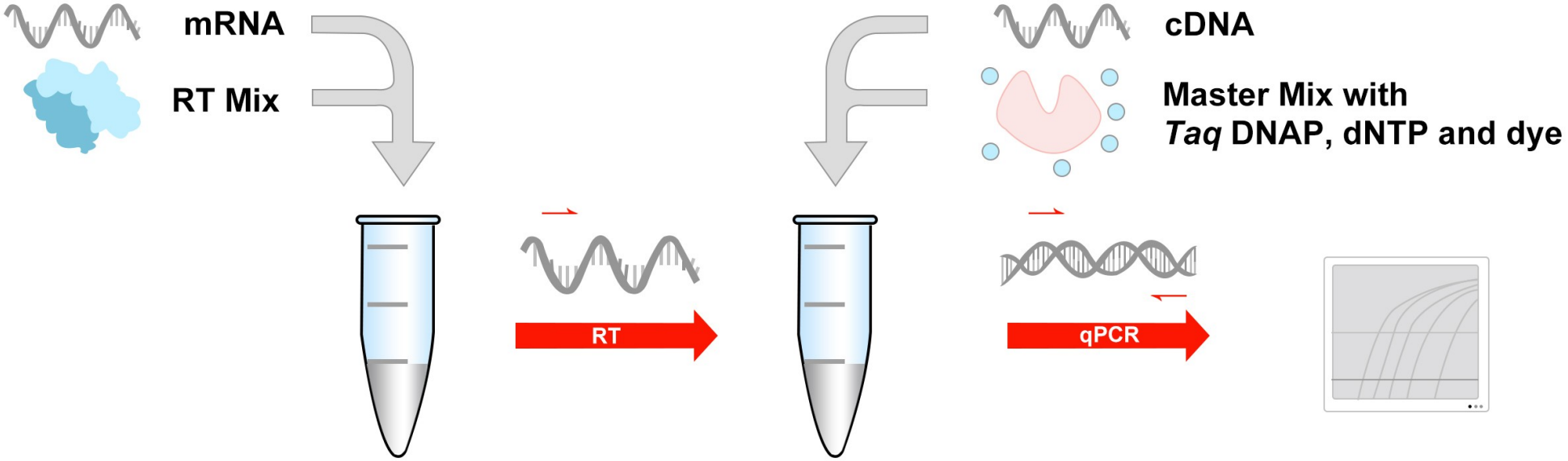


mRNA



RT-qPCR

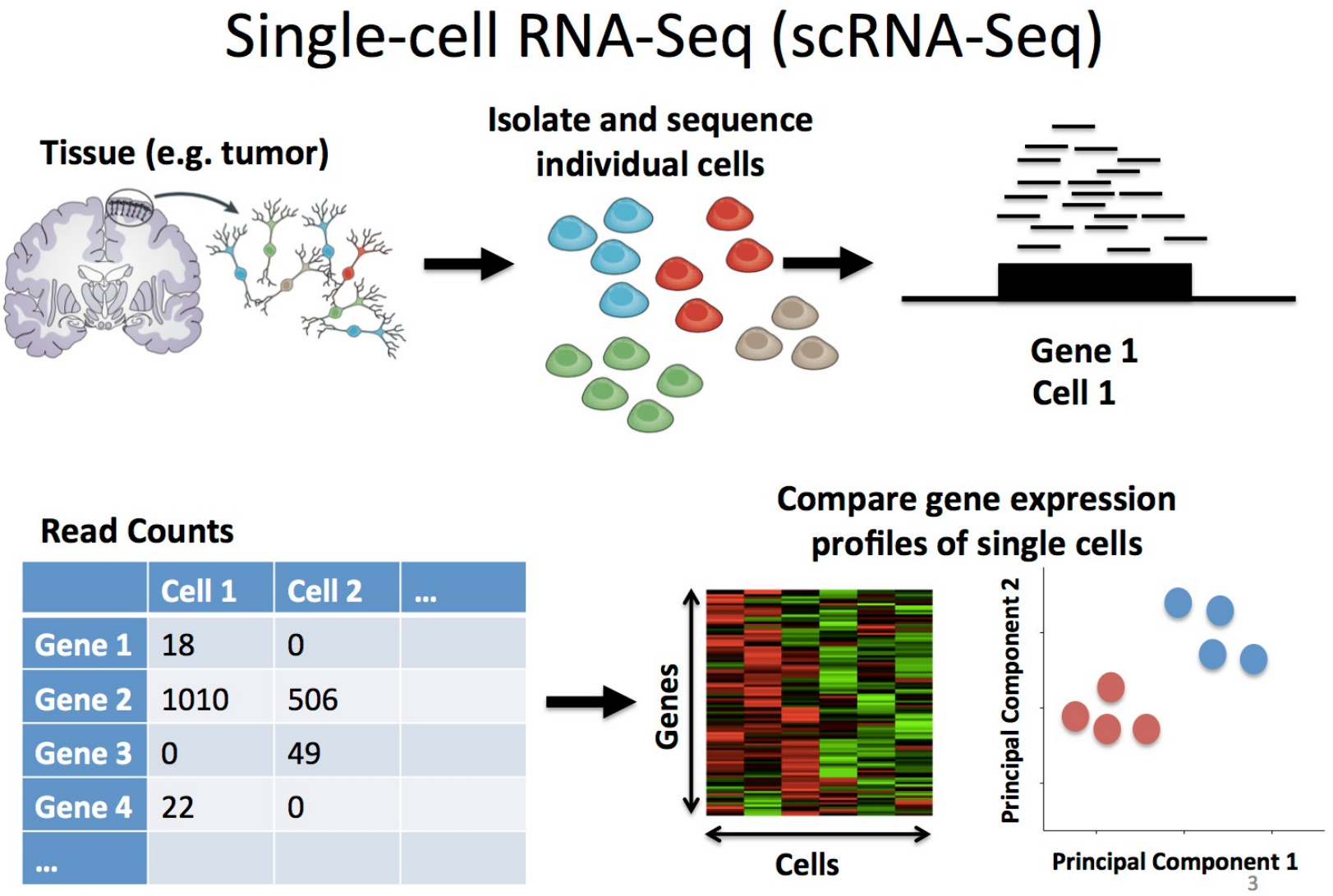
BlazeTaq™ Two-Step RT-qPCR Kit



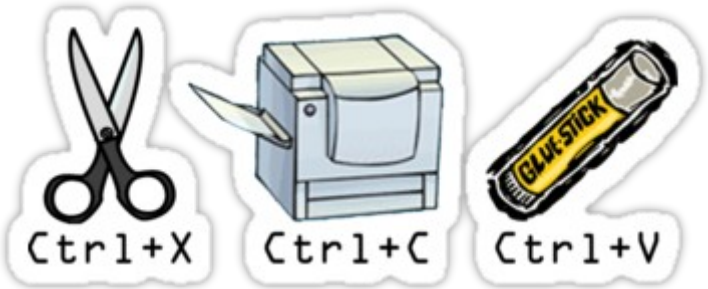
https://www.youtube.com/watch?v=iu4s3Hbc_bw

RNAseq

Starting with tissues/organs/single cells



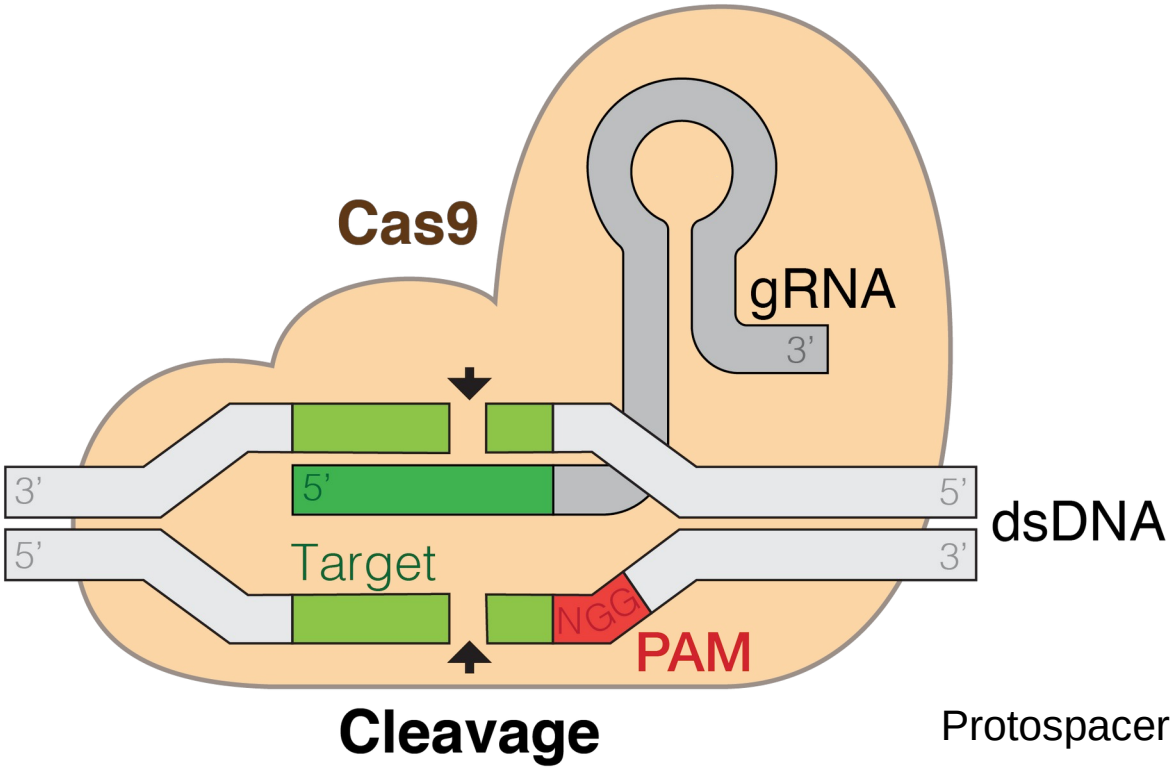
	Cell 1	Cell 2	...
Gene 1	18	0	
Gene 2	1010	506	
Gene 3	0	49	
Gene 4	22	0	
...			



CRISPR

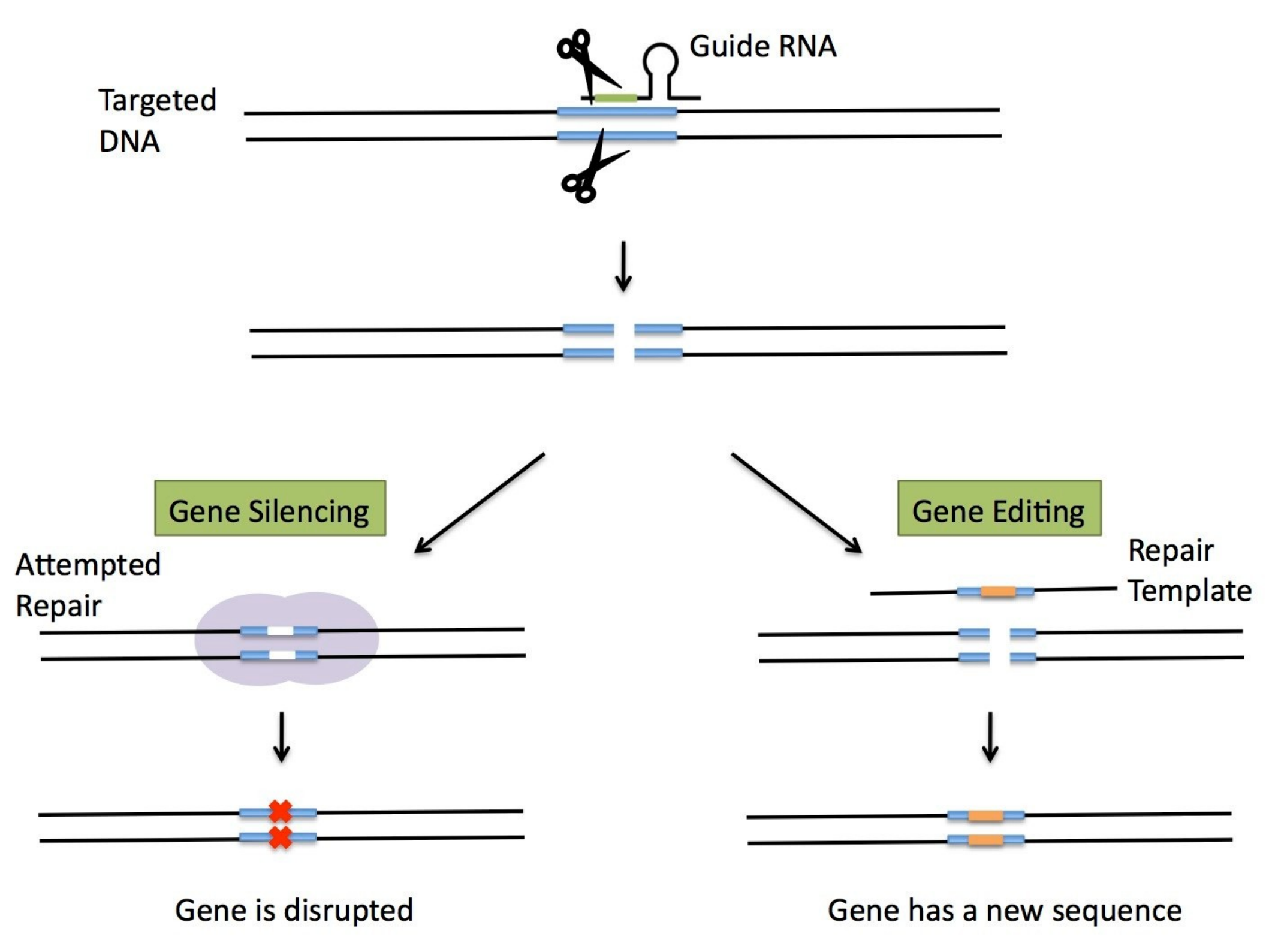
Marion Montagne

CRISPR = clustered regularly interspaced short palindromic repeats
= family of DNA sequences present in bacteria and used to detect and destroy virus DNA



Protospacer adjacent motif (PAM)

Can recognize and cut a specific DNA sequence (recognized by guide RNA)
More versatile than restriction enzymes, Zn finger nucleases and transcription activator-like effector nucleases (TALENs).


Guide RNA
Targeted
DNA
Gene Silencing
Gene Editing
Attempted
Repair
Repair
Template
Gene is disrupted
Gene has a new sequence

Creating mutants with CRISPR/Cas9

Agraulis vanillae

dorsal *ventral*

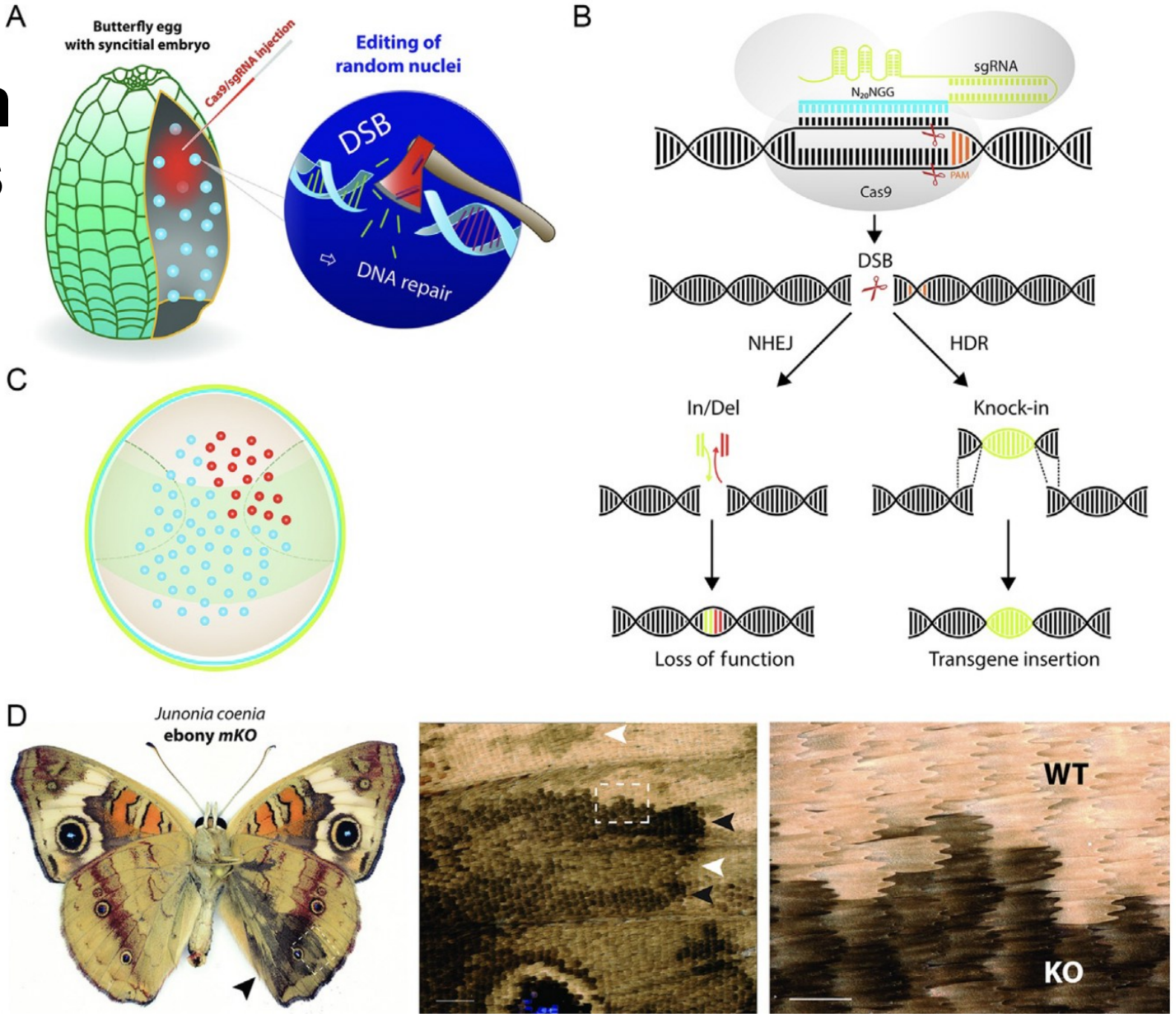
Wild-type **mutant *optics* CRISPR** **Wild-type** **mutant *optics* CRISPR**

Reed et al. 2017

normal

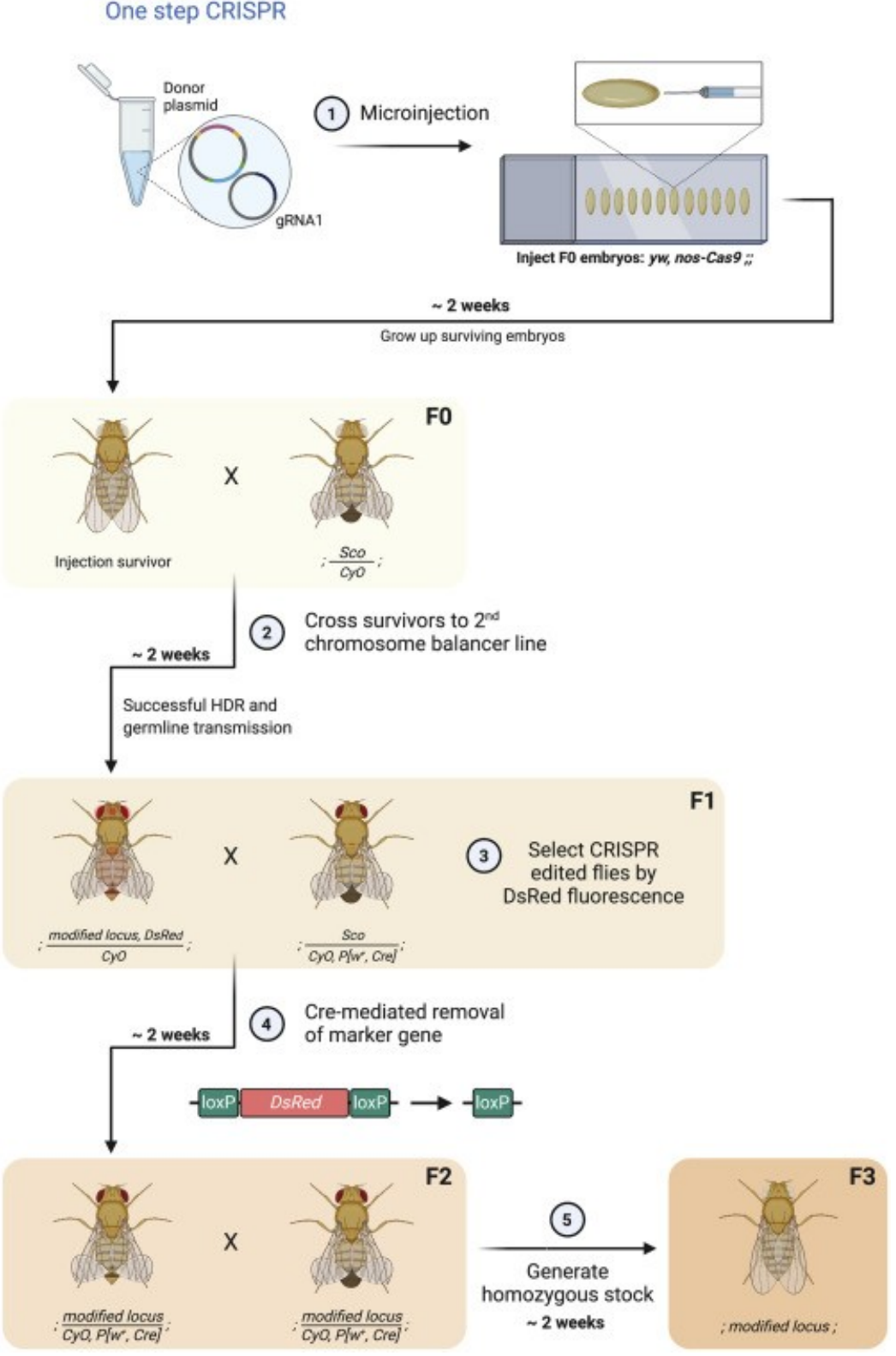
mutant
***WntA* CRISPR**

CRISPR in butterflies



Livraghi et al. 2018

CRISPR in Drosophila flies



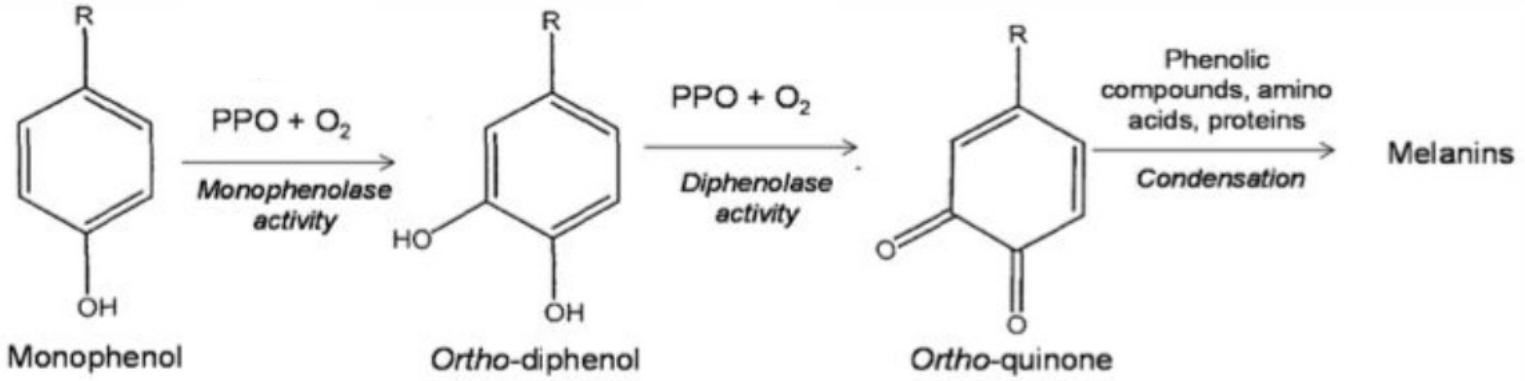
Hoppe and Ashe 2021

The first CRISPR food

Deletion in 1 of the 6 polyphenol oxidase genes
Reduction of 30% polyphenol oxidase activity

"Animal and Plant Health Inspection Service (APHIS) has concluded that your CRISPR/Cas9-edited white button mushrooms as described in your letter do not contain any introduced genetic material. APHIS has no reason to believe that CRISPR/Cas9-edited white button mushrooms are plant pests"

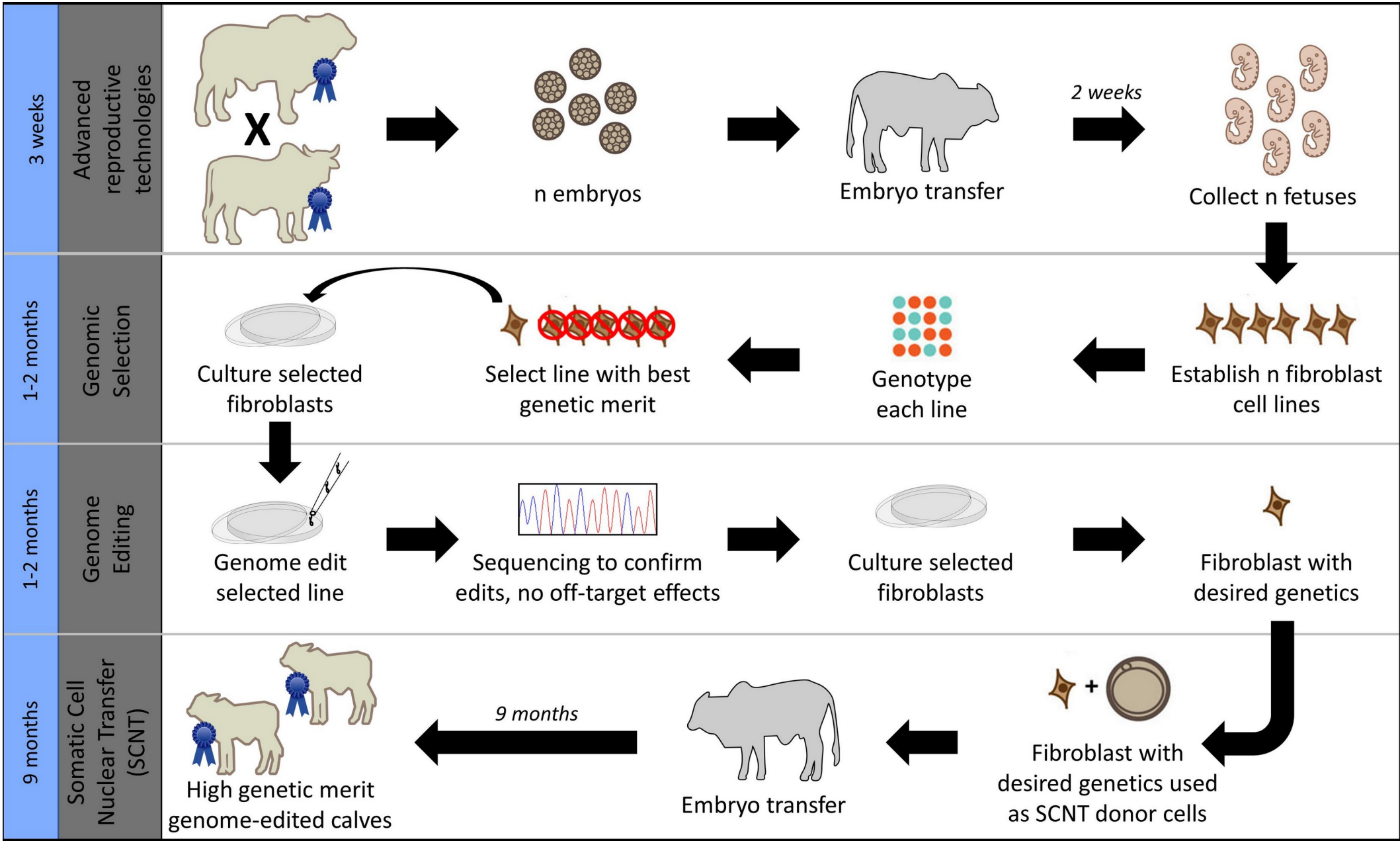
April 2016



FDA does not consider CRISPR-edited food as GMO

Advanced reproductive technologies

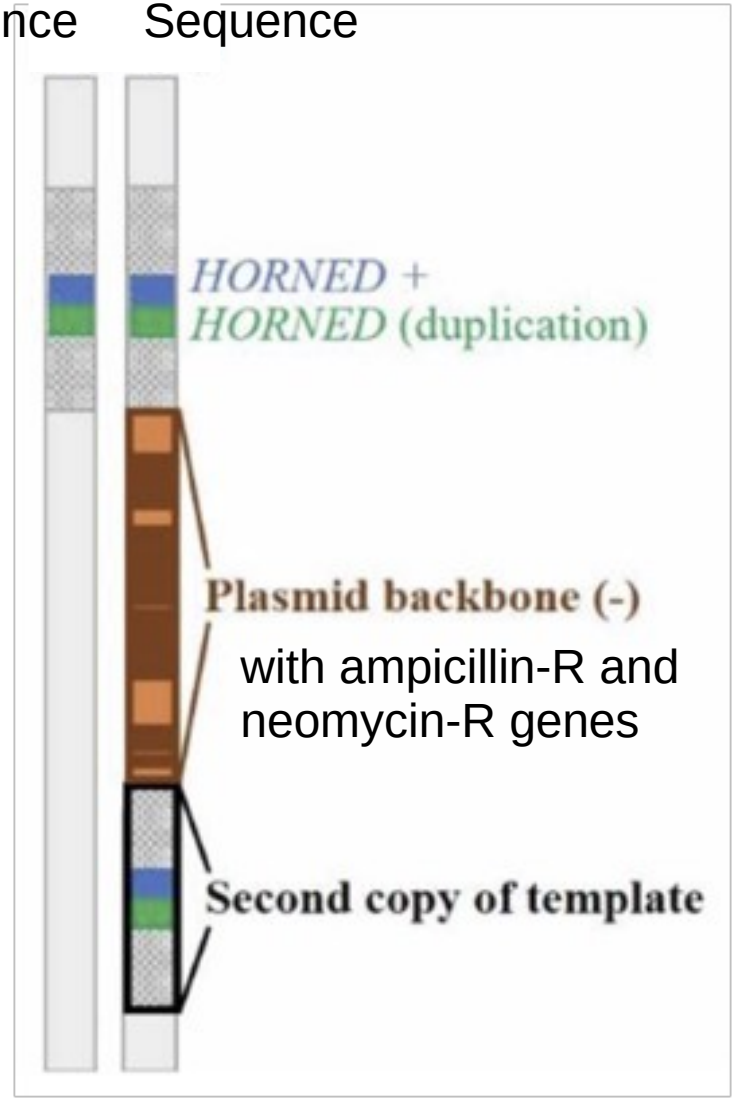
Cattle advanced reproductive technologies: somatic cell nuclear transfer cloning (SCNT), embryo transfer (ET)



https://www.frontiersin.org/files/Articles/593154/fgene-12-593154-HTML/image_m/fgene-12-593154-g001.jpg

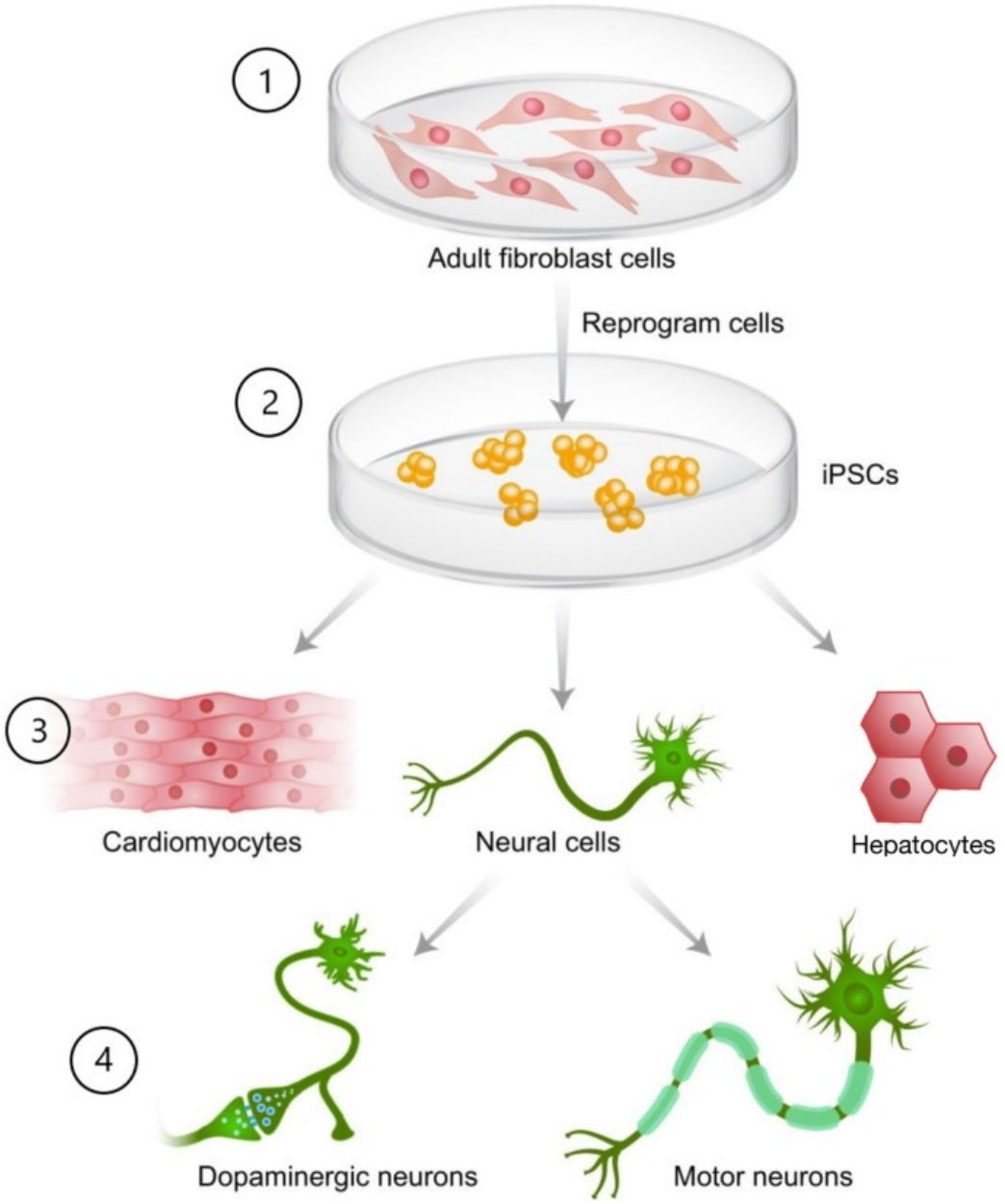
CRISPR-edited hornless cows were supposed to be exempt of transgenes

Expected Sequence | Obtained Sequence



Young 2019 Nature Biotechnolgy

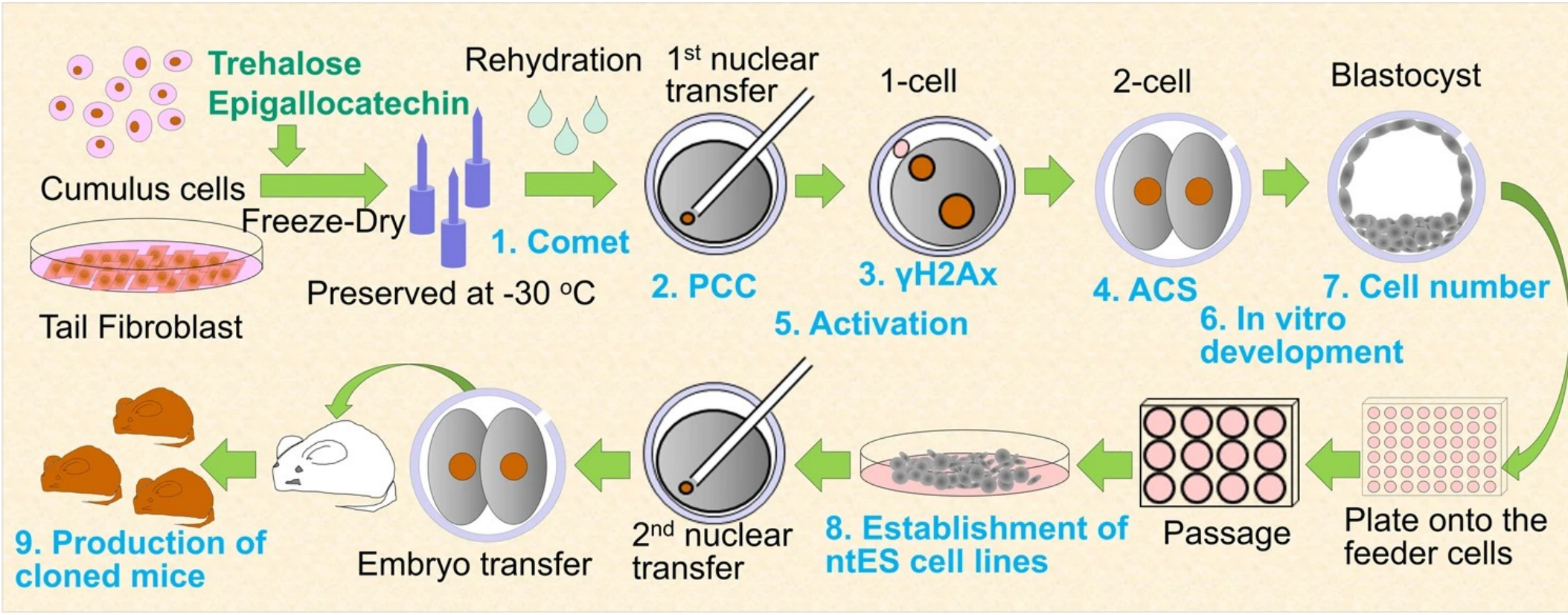
Induced Pluripotent Stem Cells (iPS)



Started in 2006
Nobel Price in 2012

Scarfone et al. 2020

Healthy mice
derived from freeze-dried somatic cells



nuclear transfer embryonic stem (ntES) cell lines established from cloned blastocysts

One of the ntES cell lines lost its Y chromosome and became an XO cell line. All the cloned mice produced from that cell line became female.

Wakayama et al. 2022

CRISPR: regulations and applications

Regulation about CRISPR-edited organisms in Europe and the US

Augustin Martin

In the US

- For Crops : No legal framework : **co-regulation** by the USDA, FDA and EPA
- What matters is the **final characteristics** of the genetically edited organism
- 2020 USDA-APHIS' "SECURE" initiative : **disregulation** of gene-edited organisms with mutations that could have naturally occurred (CRISPR-induced SNP or addition of an endemic gene)
- Considers this type of gene editing to be an acceleration of what is **naturally occurring**

USDA

« The newest of these methods, such as genome editing, **expand traditional plant breeding tools** (...) potentially saving years or even decades in bringing needed new varieties to farmers. »

Secretary of State Perdue for the USDA

- CRISPR-edited animals are evaluated as **animal drugs** by the FDA : strict safety evaluation

In Europe

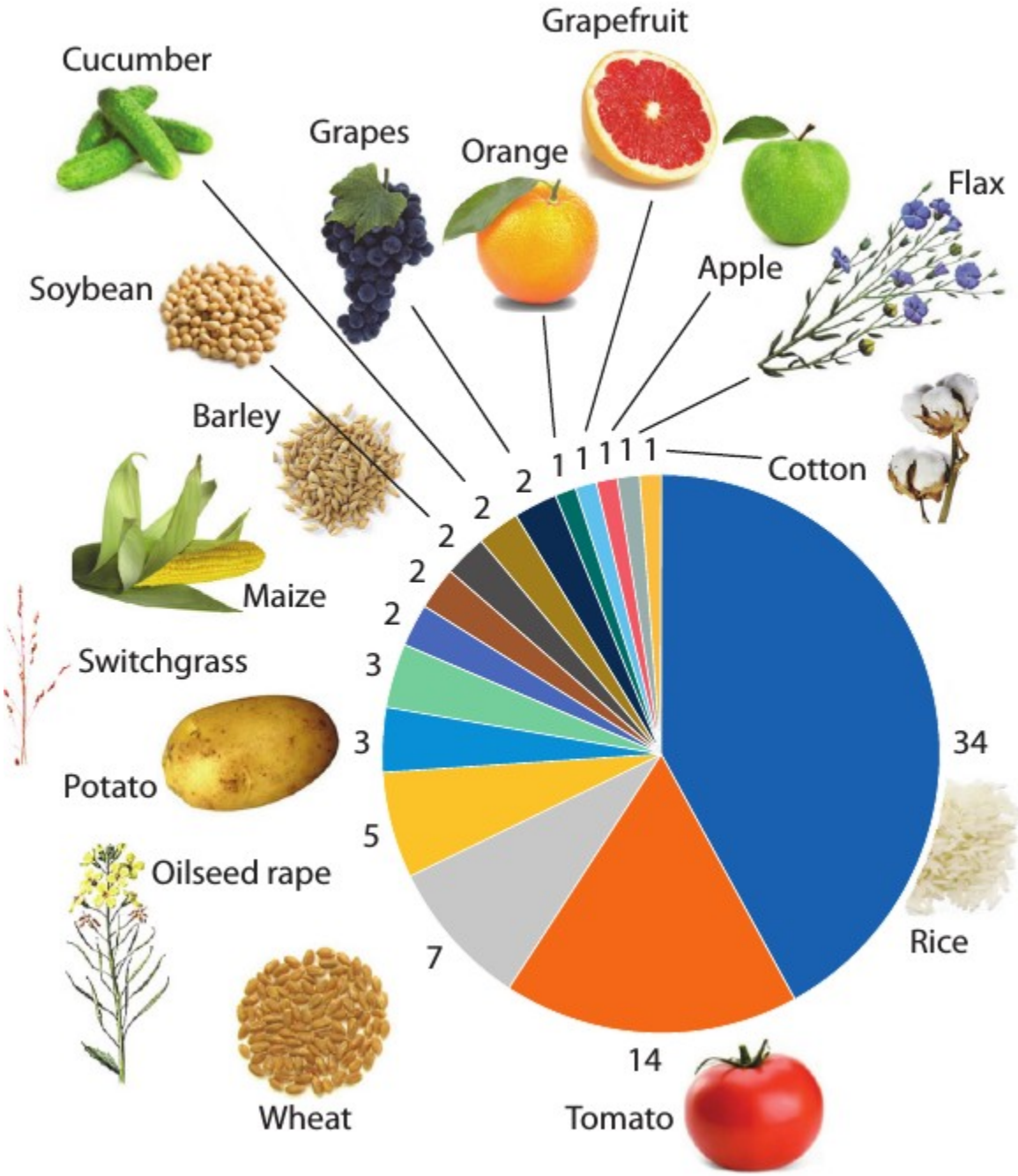
- Major judgment : ECJ "Confédération paysanne" 2018 ruling

CVRIA

« The Court considers that the **risks linked to the use of these new mutagenesis techniques might prove to be similar to** those that result from the production and release of a **GMO through transgenesis** »

- What matters is the **process** through which crops are obtained
- Concluded that **mutagenesis and transgenesis** are similar according to
  - Their potential danger
  - The rate of production
  - Their action of "denaturing" the genome
- CRISPR-edited crops and animals: regulated as **GMOs**
- Considers gene editing to be an **unnatural** modification of the genome

Numerous plants modified using CRISPR



Korotkova et al. 2019

The first CRISPR-edited foods are not “to save the world”

Herbicide-tolerant oilseed rape

(in 2015, the German authorities authorized the release of oilseed rape developed by the US company CIBUS without being regulated as genetically engineered.)

Soybean with modified fatty acid composition
Potato with improved storage capacity at cool temperatures
“Waxy” maize with a modified starch composition
Herbicide-tolerant flax
Sweeter-tasting strawberries
Seedless tomatoes

Ueta et al. 2017

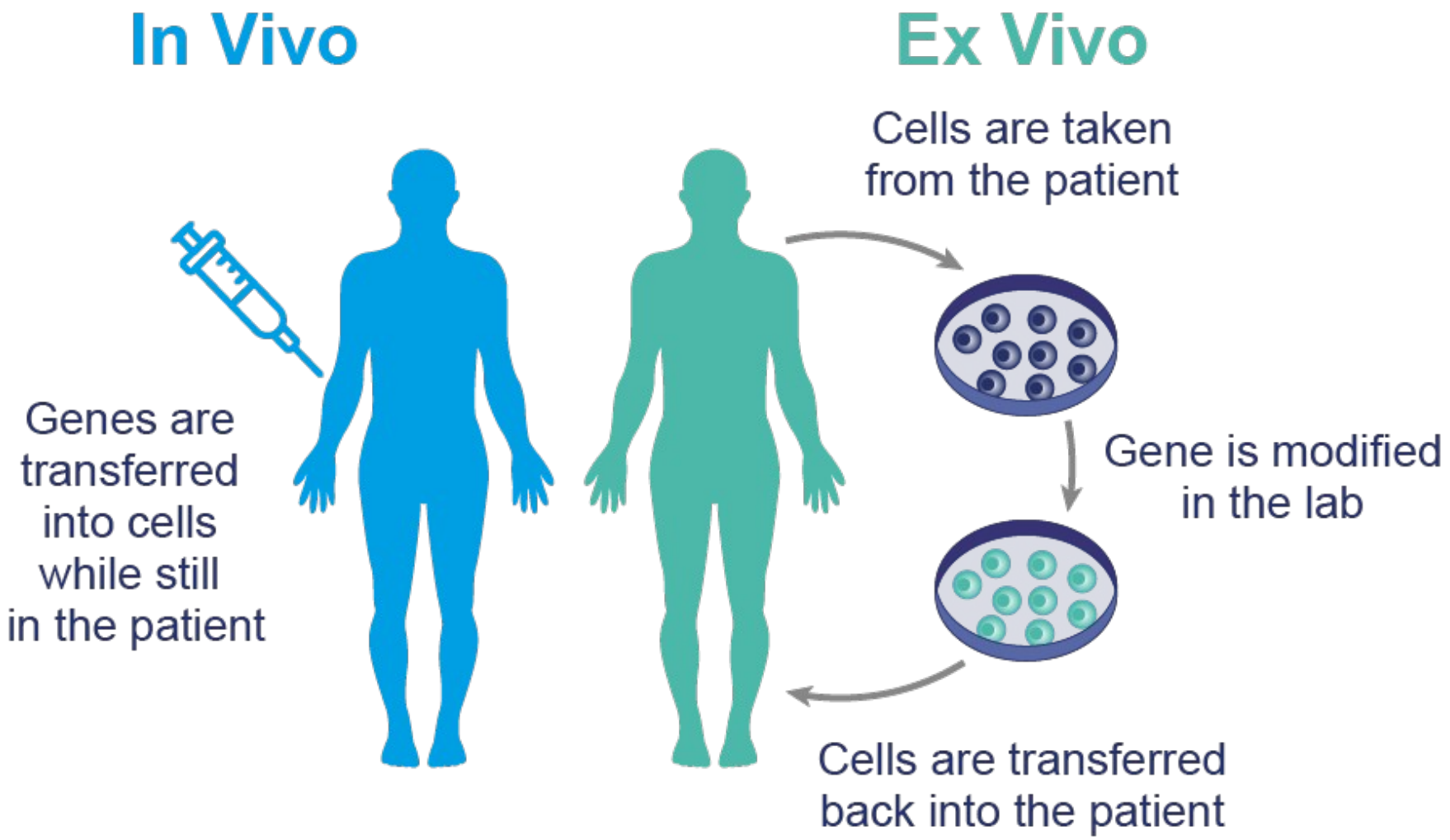
Making plants that are resistant to drought or salt requires exogenous genes

The coding loci of evolution and domestication: current knowledge and implications for bio-inspired genome editing

Virginie Courtier-Orgogozo[1,*] and Arnaud Martin[2]

J. Exp. Biol.

Ongoing CRISPR clinical trials



Retina disease
First CRISPR trial in March 2020
BRILLANCE trial, Editas

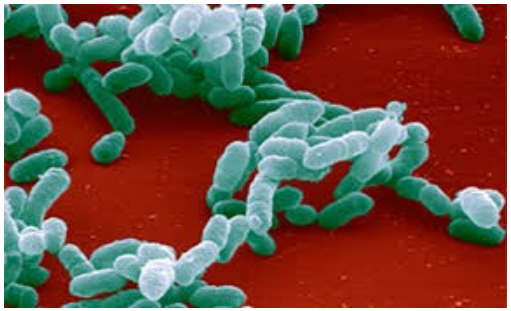
Sickle Cell Disease
Beta-Thalassemia

Fundamental research is important

bacteria *Thermus aquaticus*

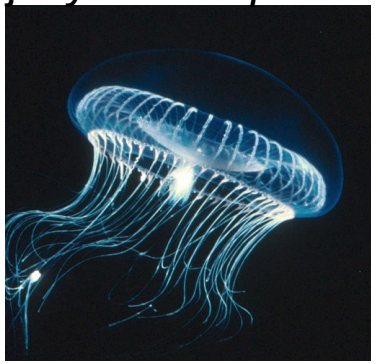
1969 → Taq-polymerase to amplify DNA

bacteria *Haemophilus influenzae*



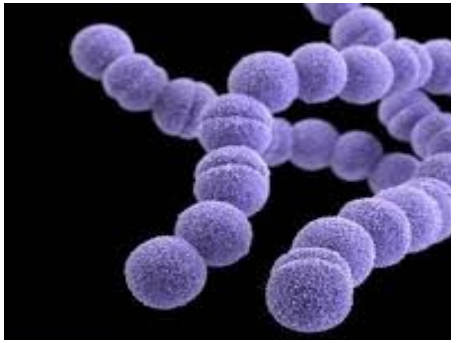
1970 → Restriction enzymes To cut DNA

jellyfish *Aequorea*



1992 → Fluorescent proteins

bacteria *Streptococcus pyogenes*



2012 → CRISPR

CRISPR-based gene drive

What is gene drive?

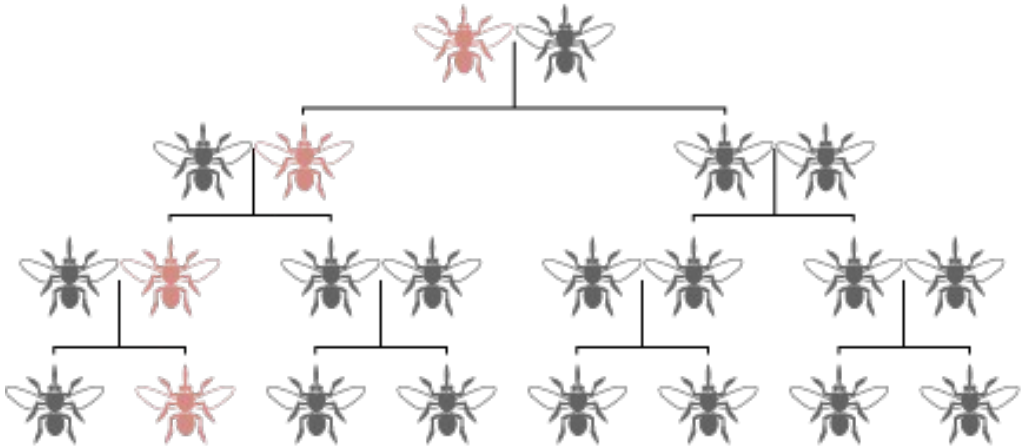
A novel biotechnology under development which aims to bias inheritance and control disease vectors, invasive species and other pests.

Public health, agriculture, conservation biology

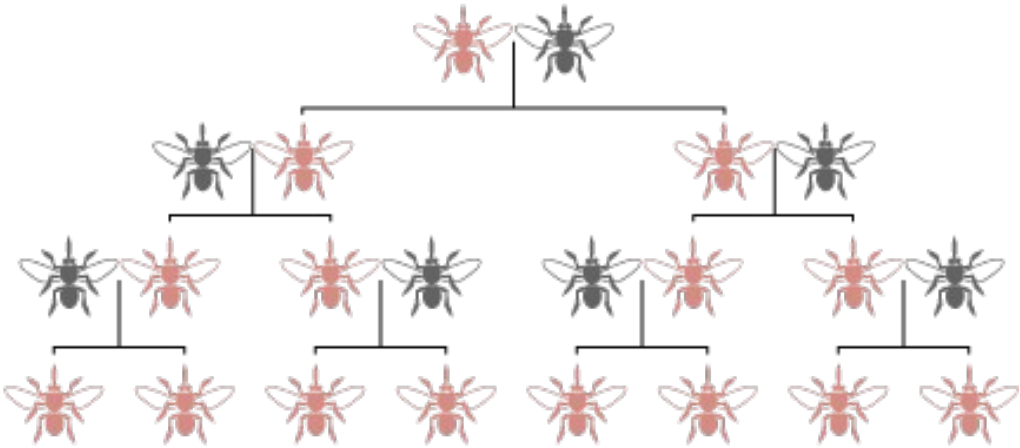
"*A natural phenomenon in several species*"

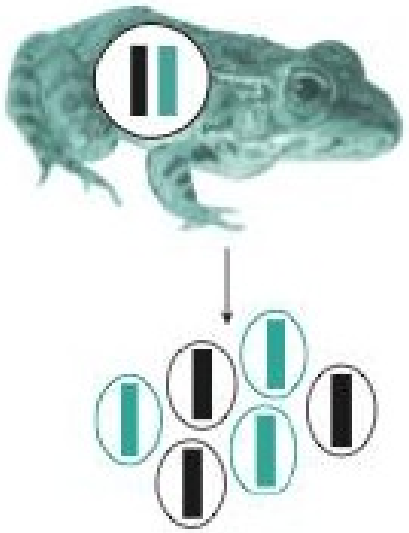
(Austin Burt, 2020)

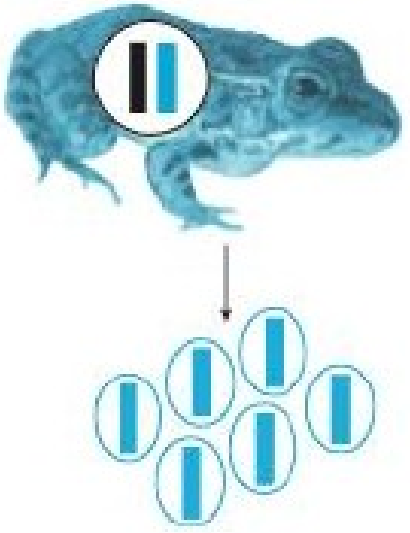
Normal reproduction



Reproduction with gene drive

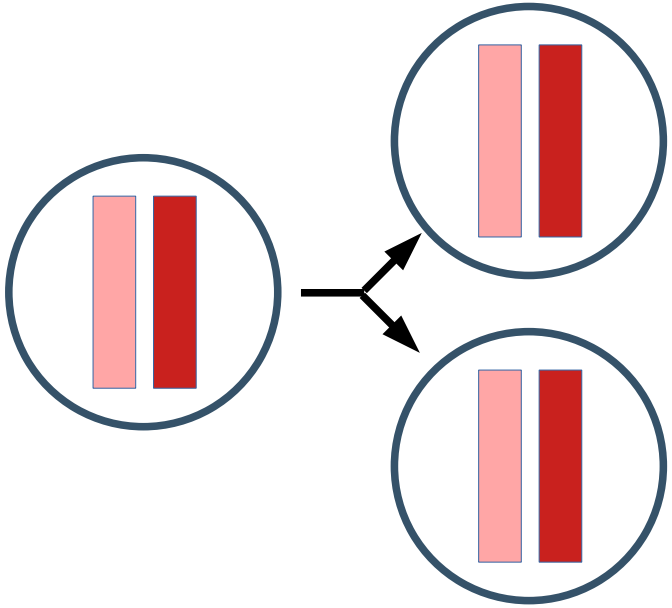




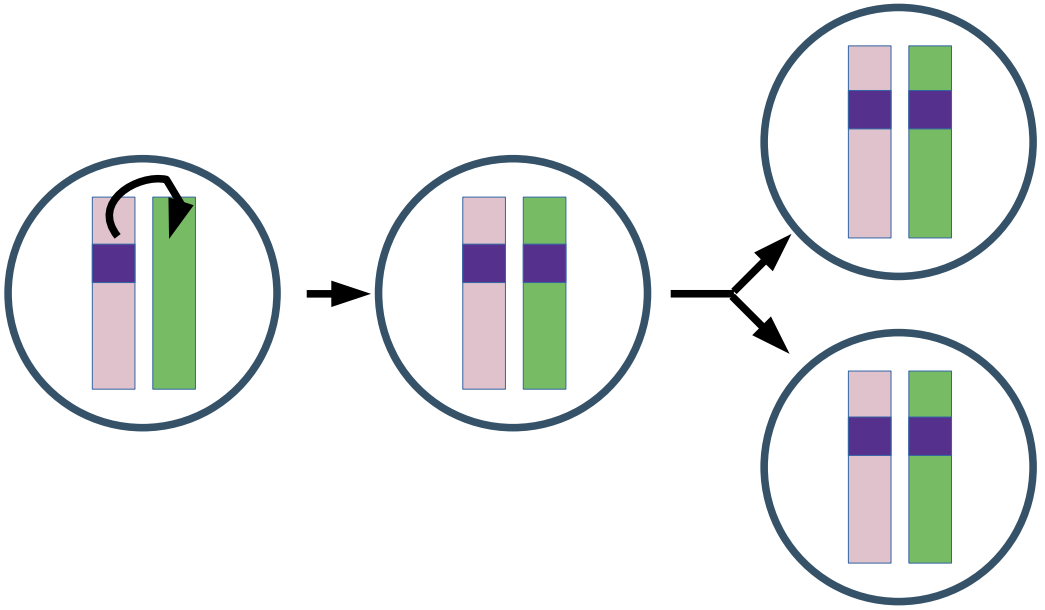


Rode et al. 2019

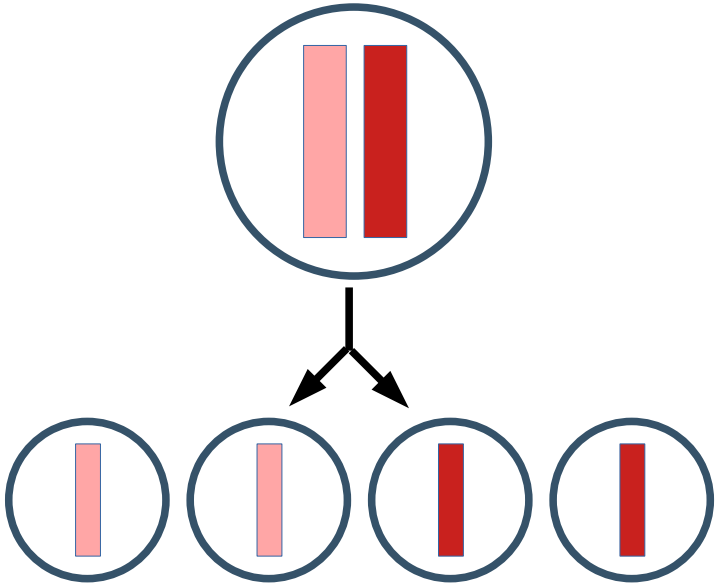
Cell division (mitosis)



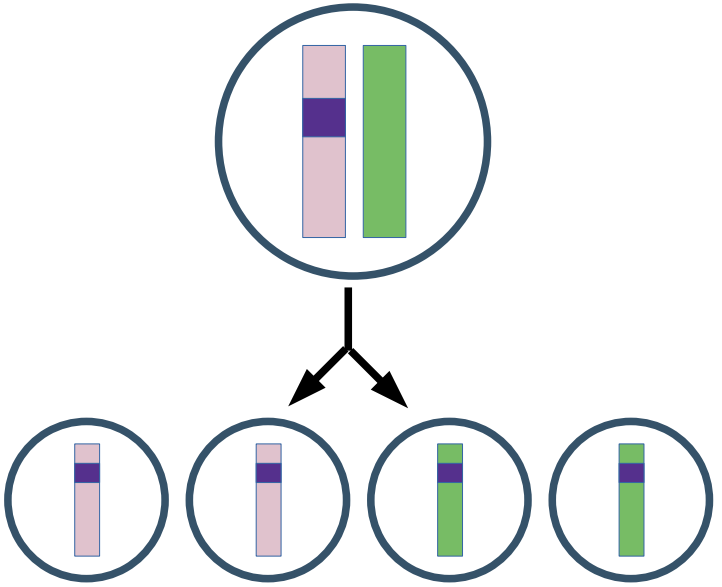
Gene drive

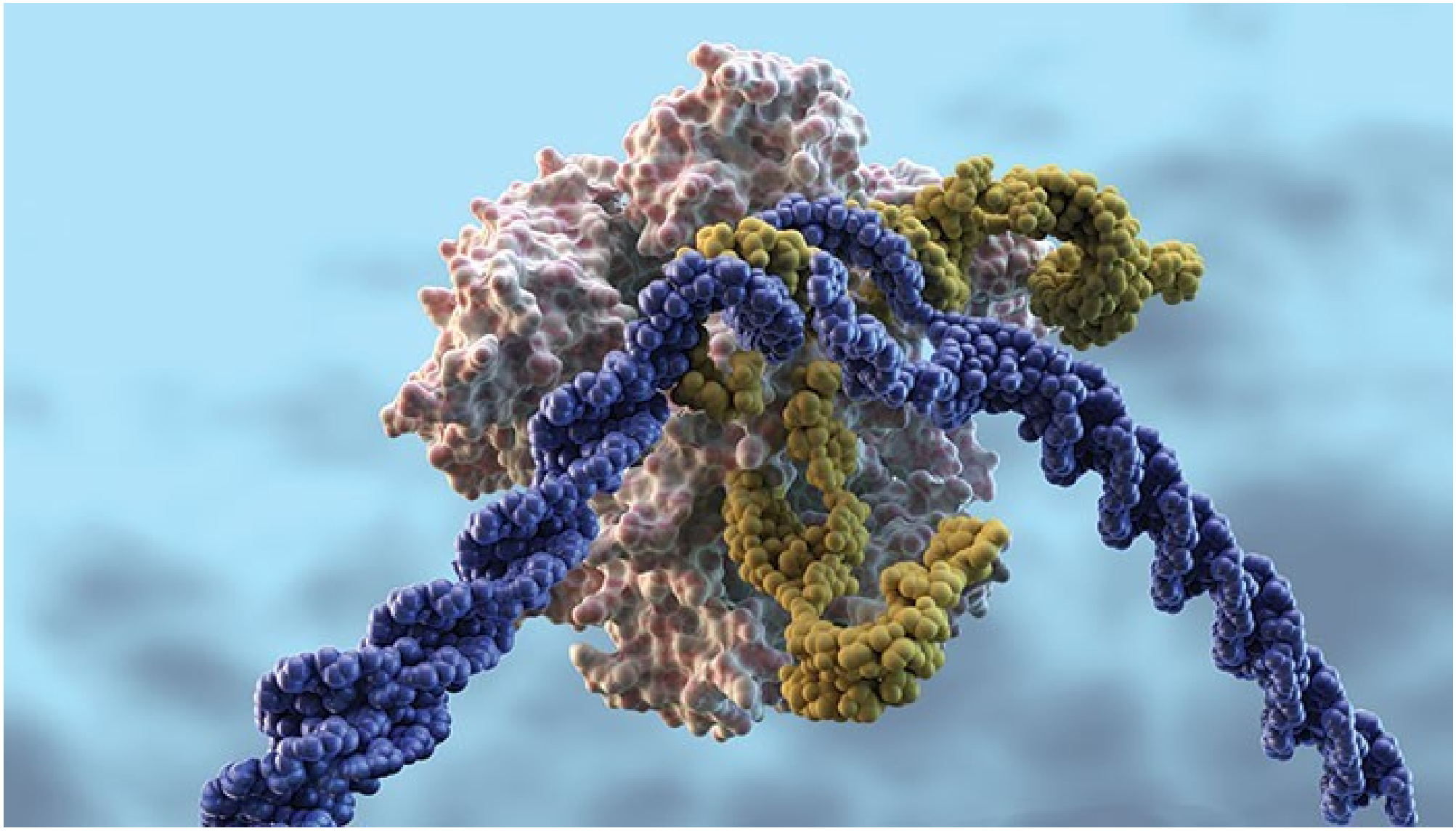


Normal reproduction

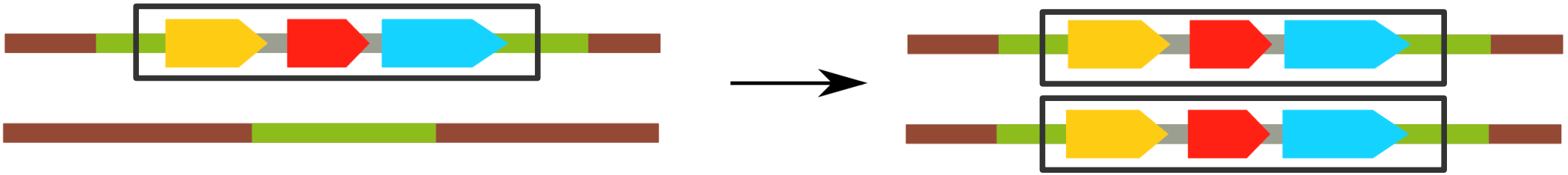


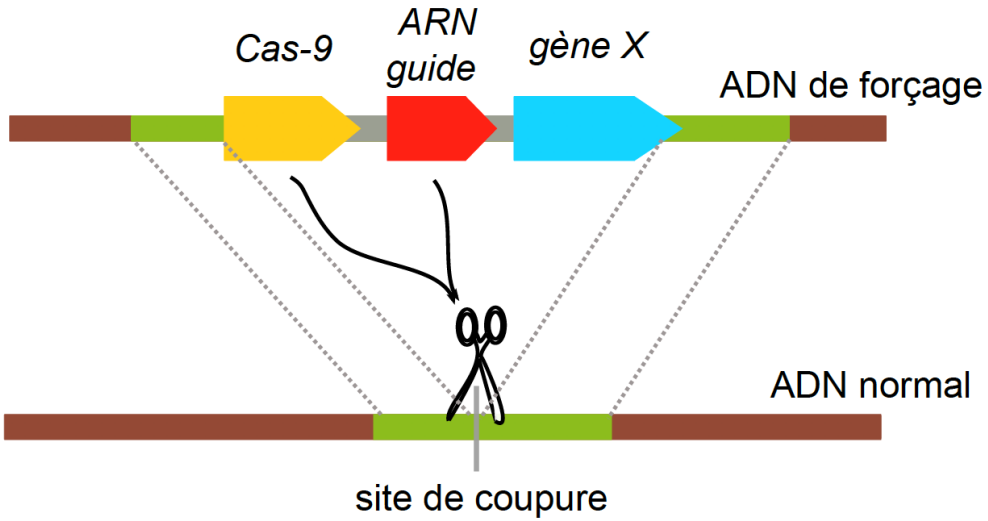
Gene drive





How a gene drive construct copies itself





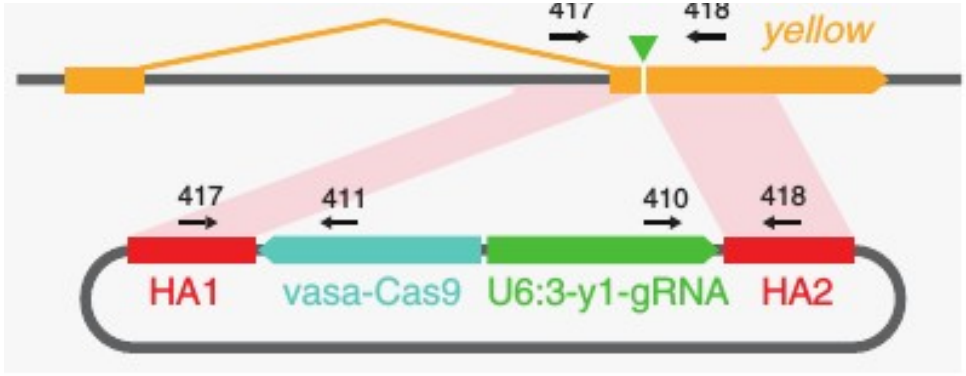
1rst gene drive organisms

GENOME EDITING

Science, April 2015

The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations

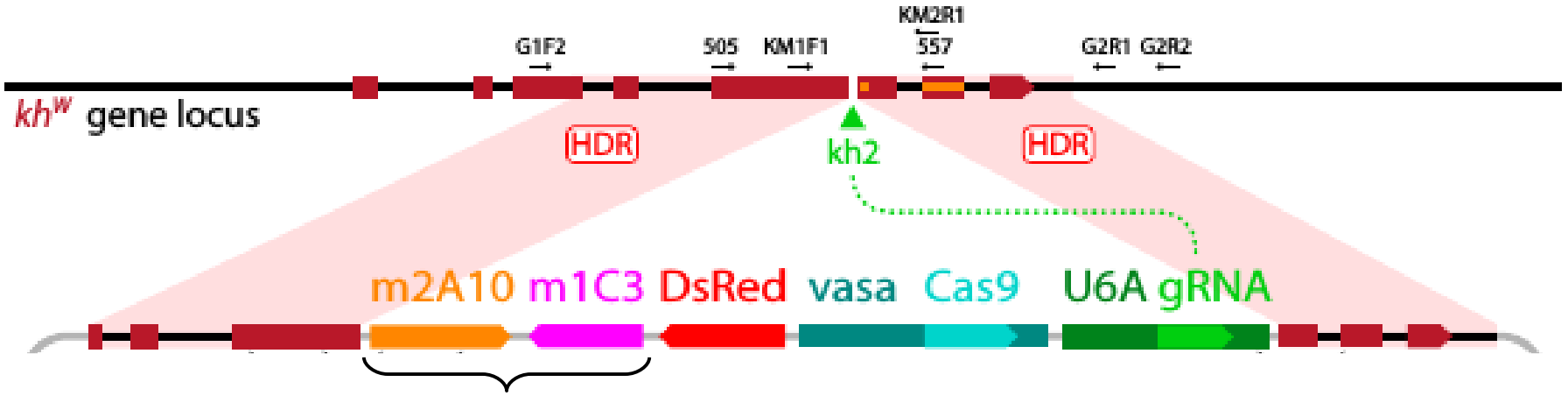
Valentino M. Gantz* and Ethan Bier*



E

	y^- ♂	y^- ♀	mosaic ♀	y^+ ♂	y^+ ♀	total
y^{MCR} ♂ x y^+ ♀	0	40	0	50	1	91
y^{MCR} ♀ x y^+ ♂	214	203	11	2	6	436

Mosquitoes without parasites



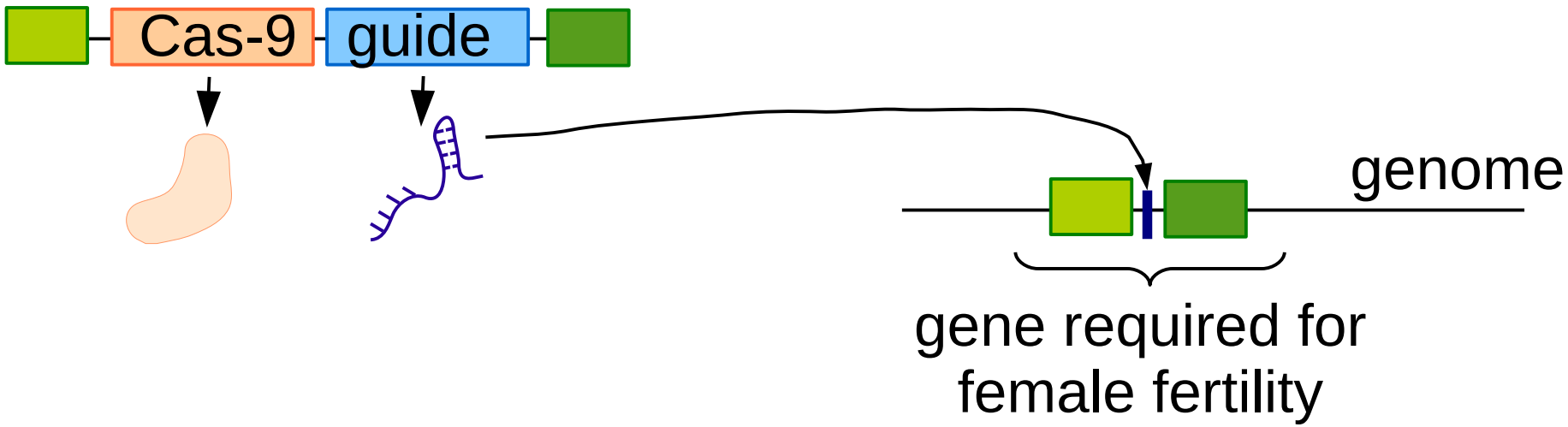
antibodies
anti-parasites

PNAS, November 2015

Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*

Valentino M. Gantz[a,1], Nijole Jasinskiene[b,1], Olga Tatarenkova[b], Aniko Fazekas[b], Vanessa M. Macias[b], Ethan Bier[a,2], and Anthony A. James[b,c,2]

Sterile mosquitoes

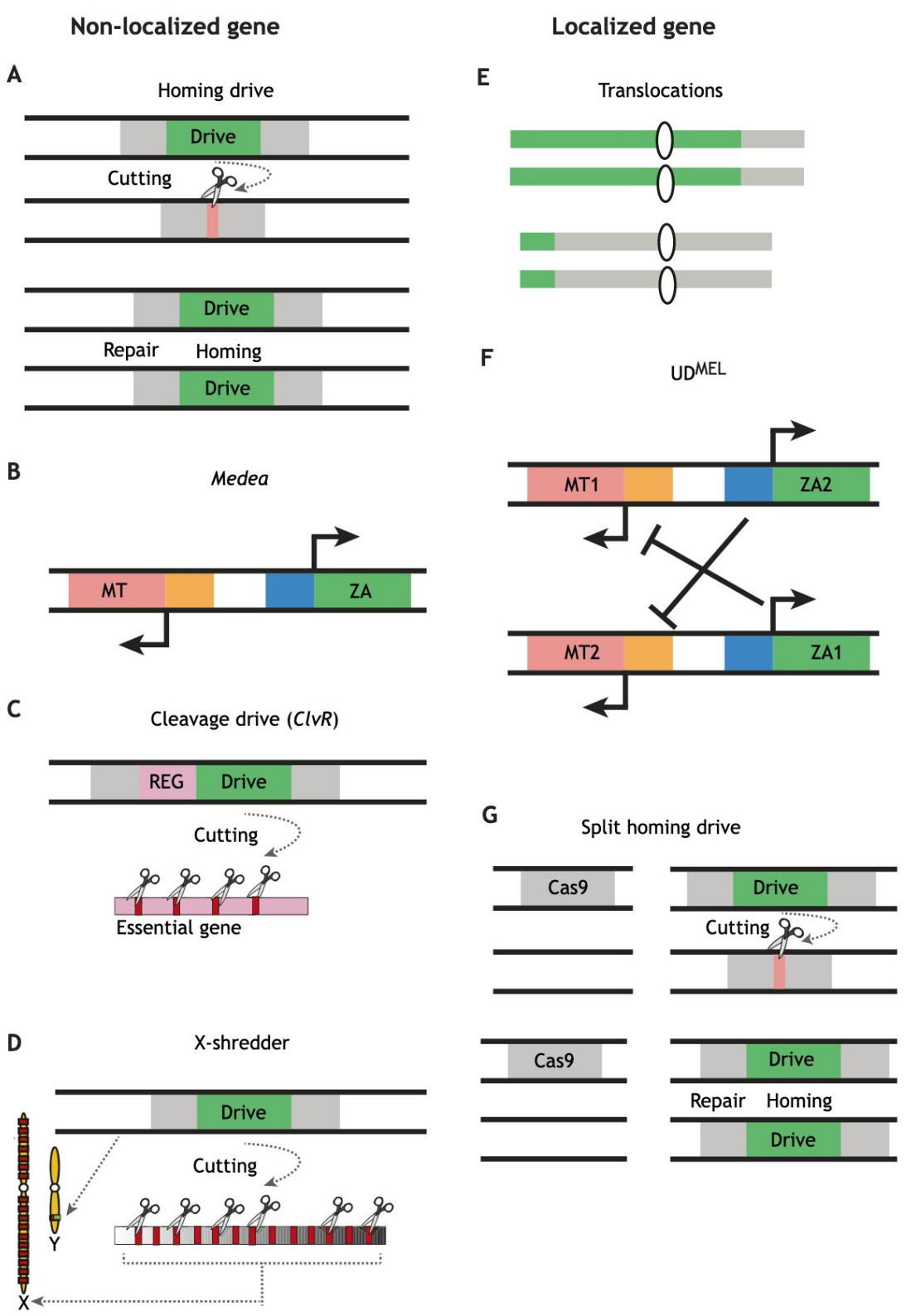


Nature Biotechnology, décembre 2015

A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*

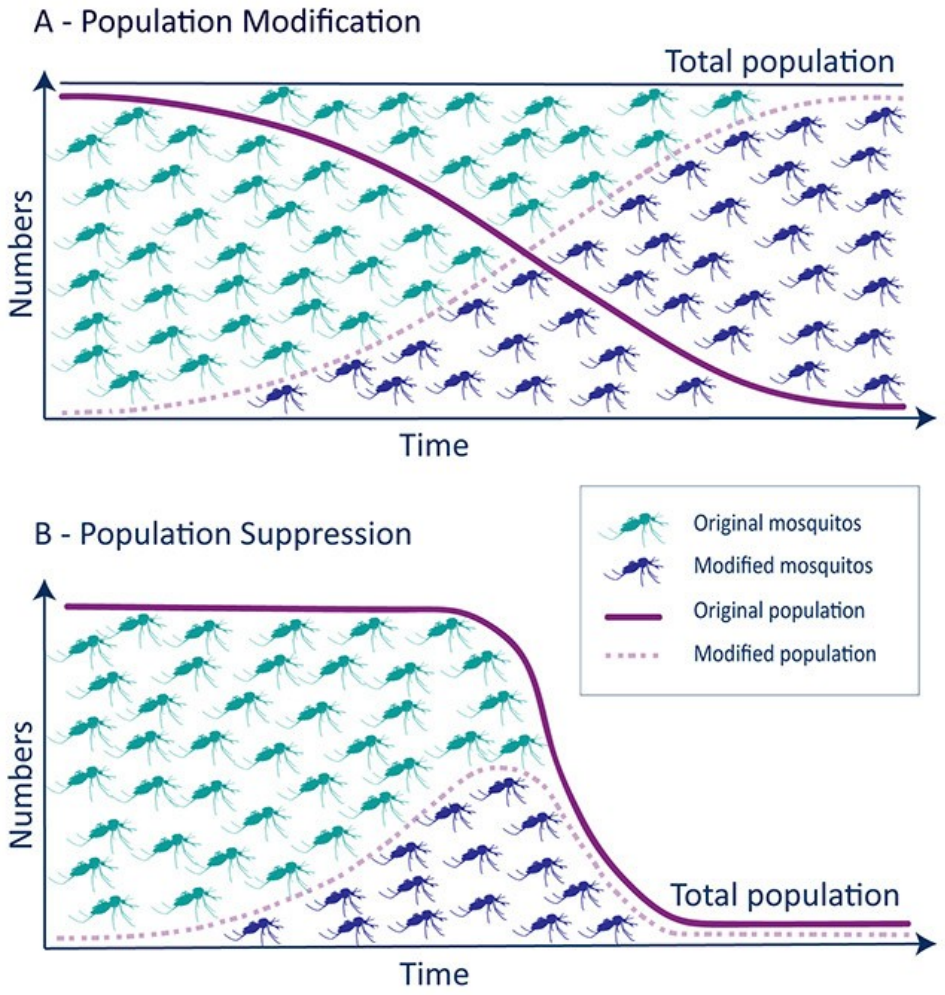
Andrew Hammond[1], Roberto Galizi[1], Kyros Kyrou[1], Alekos Simoni[1], Carla Siniscalchi[2], Dimitris Katsanos[1], Matthew Gribble[1], Dean Baker[3], Eric Marois[4], Steven Russell[3], Austin Burt[1], Nikolai Windbichler[1], Andrea Crisanti[1] & Tony Nolan[1]

Various gene drives



Low threshold drives
High threshold drives
Integral drives
Tethered drives
Split drives
Daisy drives
Under-dominant drives
Sex-limited drives
...

Raban et al, JEB 2020



"*Outcome conceptually similar to "vaccinating" pest populations*"
(Luke Alphey, 2020)

"*Agronomic science has been modifying **crops** to increase productivity or resistance to pests or pathogens.*

*Gene drive now allows manipulating **pests**.*"

Agricultural pest control with CRISPR-based gene drive: time for public debate

Should we use gene drive for pest control?

Virginie Courtier-Orgogozo[1], Baptiste Morizot[2] & Christophe Boëte[3]

EMBO J. 2017

Various applications of Gene Drive

(A) ERADICATION DRIVES

spreading strongly deleterious mutations in invasive populations

Eradicating invasive pest species

Eradicating invasive black rats that threaten the kereru (New Zealand pigeon) and other endemic species in New Zealand (NASEM 2016)
Image: David Mudge; Ngā Manu Nature Images

Eradicating invasive disease vectors

Eradicating invasive mosquitos, vector of avian malaria in Hawaiian honeycreeper birds (NASEM 2016)
Image: Sean McCann; Flickr

(B) SUPPRESSION DRIVES

spreading mildly deleterious mutations in invasive populations

Reducing the height of invasive common ragweed to decrease its competitive pressure on native plants (Neve 2018)
Image: Ashley Bradford; inaturalist.org

(C) RESCUE DRIVES

spreading beneficial mutations in endangered populations

Protecting lowland leopard frogs from highly pathogenic fungus (Esvelt et al 2014)
Image: Brian Gratwicke; Flickr

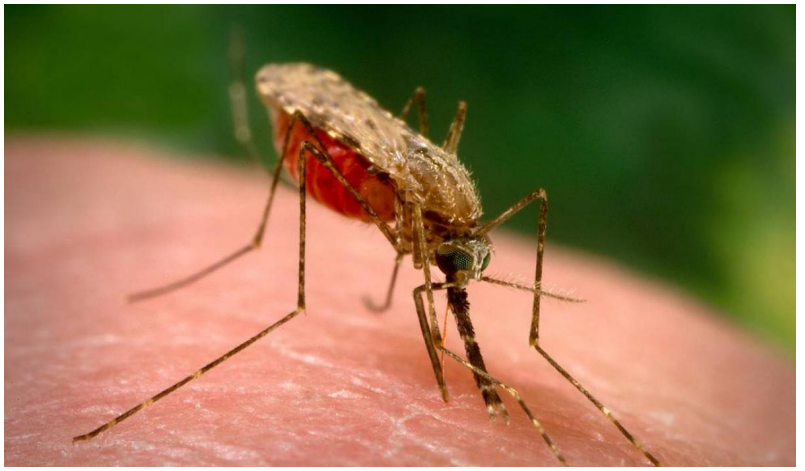
Rode et al. 2019

Two advanced gene drives

Drosophila suzukii
Invasive pest species

Scott et al. 2018

***Anopheles* mosquitoes**
Vector of malaria



TARGET MALARIA

https://targetmalaria.org

Risks and ethical issues associated with gene drives

What is novel about gene drive?

Several DNA pieces assembled together
Eukaryote cis-regulatory regions with bacteria coding regions

Manipulates the 2 pillars of evolution
- mutation
- transmission
-> can bypass selection and spread deleterious alleles

Potentially more effective than other biotechnologies
- ease of use
- speed of change
- unprepared regulatory environment

Classical Darwinian Evolution

1

Variation

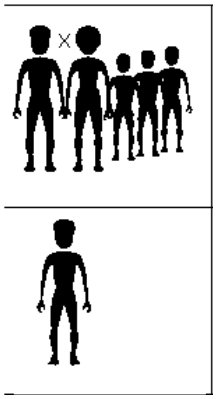
Mutations in DNA

2

Transmission to the next generation

3

Reproduction
Variability between individuals



Selection

Genetic Drift

http://evolution.berkeley.edu

What are the risks?

Molecular off-targets

Propagation to non-target populations

Propagation to non-target species

Consequences for ecosystems

Failure of counter-measures

What are the risks?

Molecular off-targets

Propagation to non-target populations

Propagation to non-target species

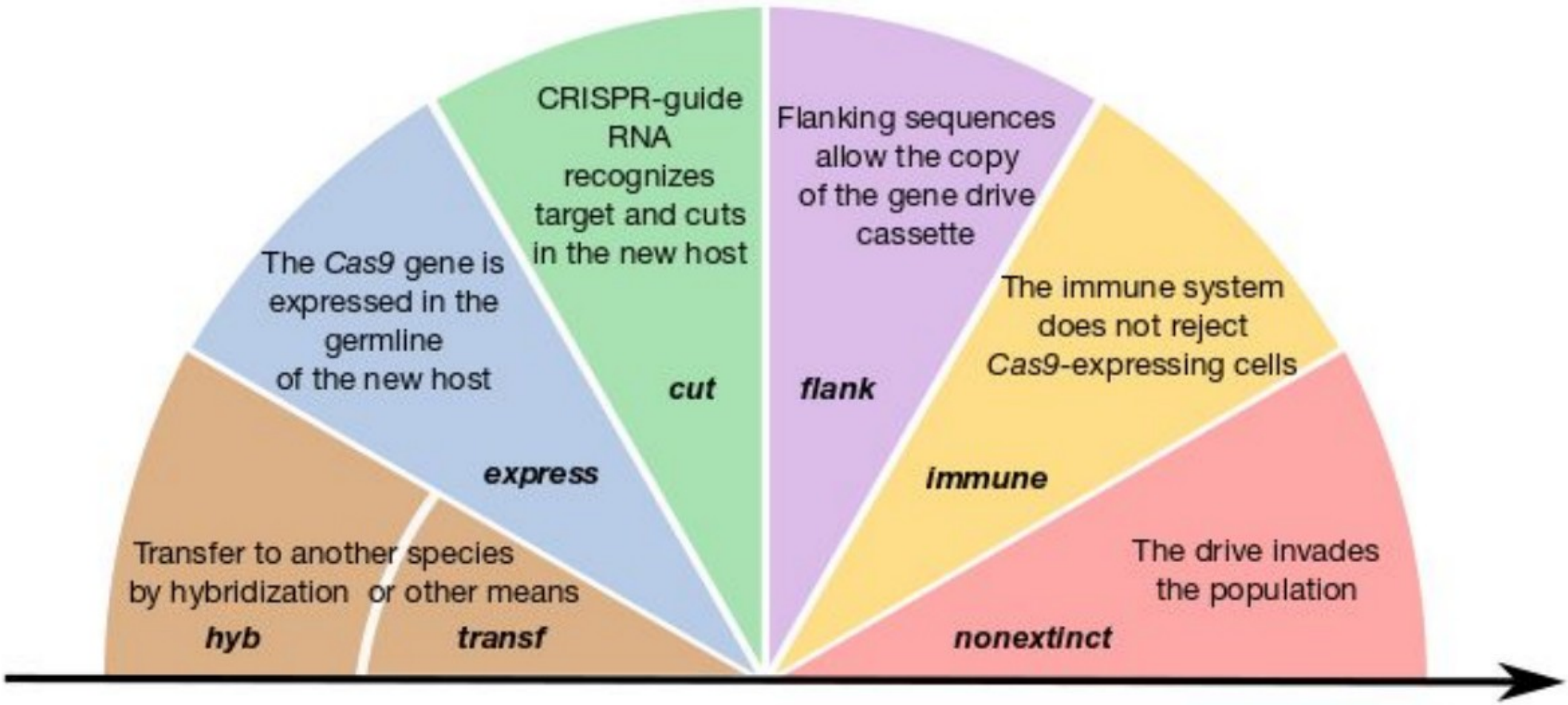
Consequences for ecosystems

Failure of counter-measures

Evaluating the probability of CRISPR-based gene drive contaminating another species

2019

Virginie Courtier-Orgogozo[1] | Antoine Danchin[2] | Pierre-Henri Gouyon[3] | Christophe Boëte[4]



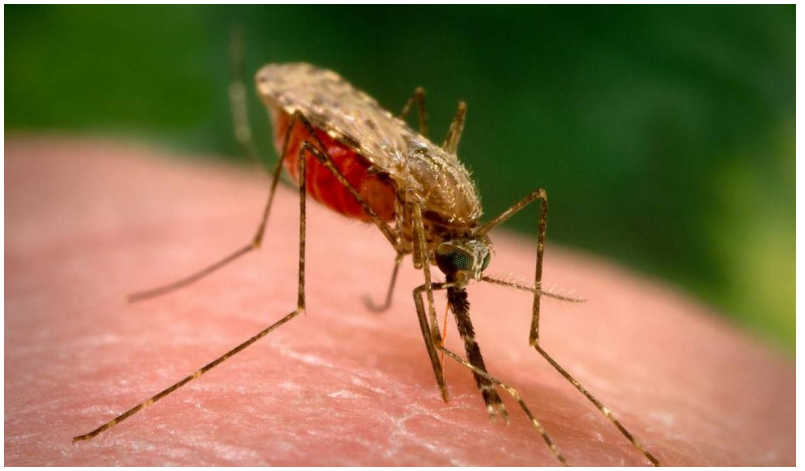
Risk of hybridization

Drosophila suzukii
Invasive pest species

D. subpulchrella India, South East Asia, China, Japan
D. pulchrella India, South East Asia, southern China

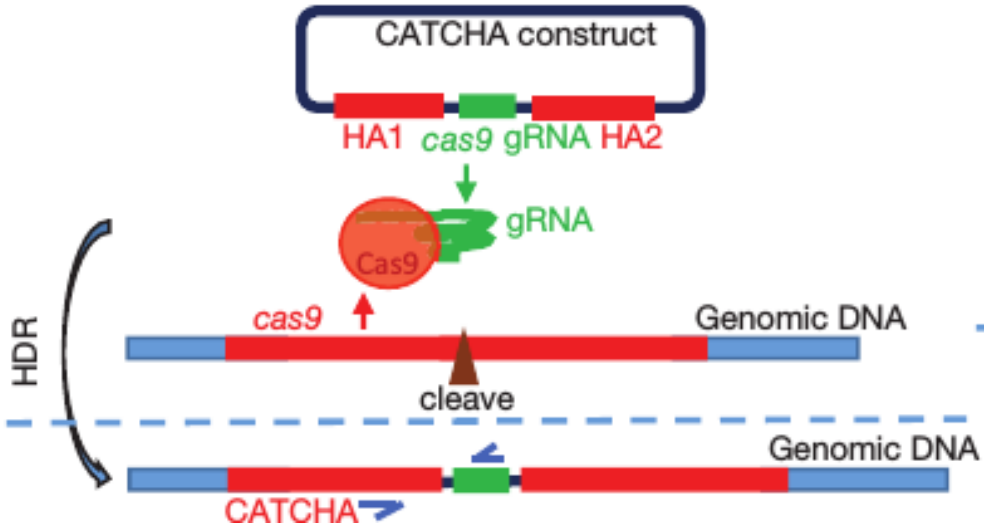
temperate
tropical

Anopheles mosquitoes
Vector of malaria



An. gambiae s.s.
An. arabiensis
An. coluzzii
An. amharicus
An. melas
An. merus
An. bwambae
An. quadriannulatus

Need to stop a drive? Use another one!

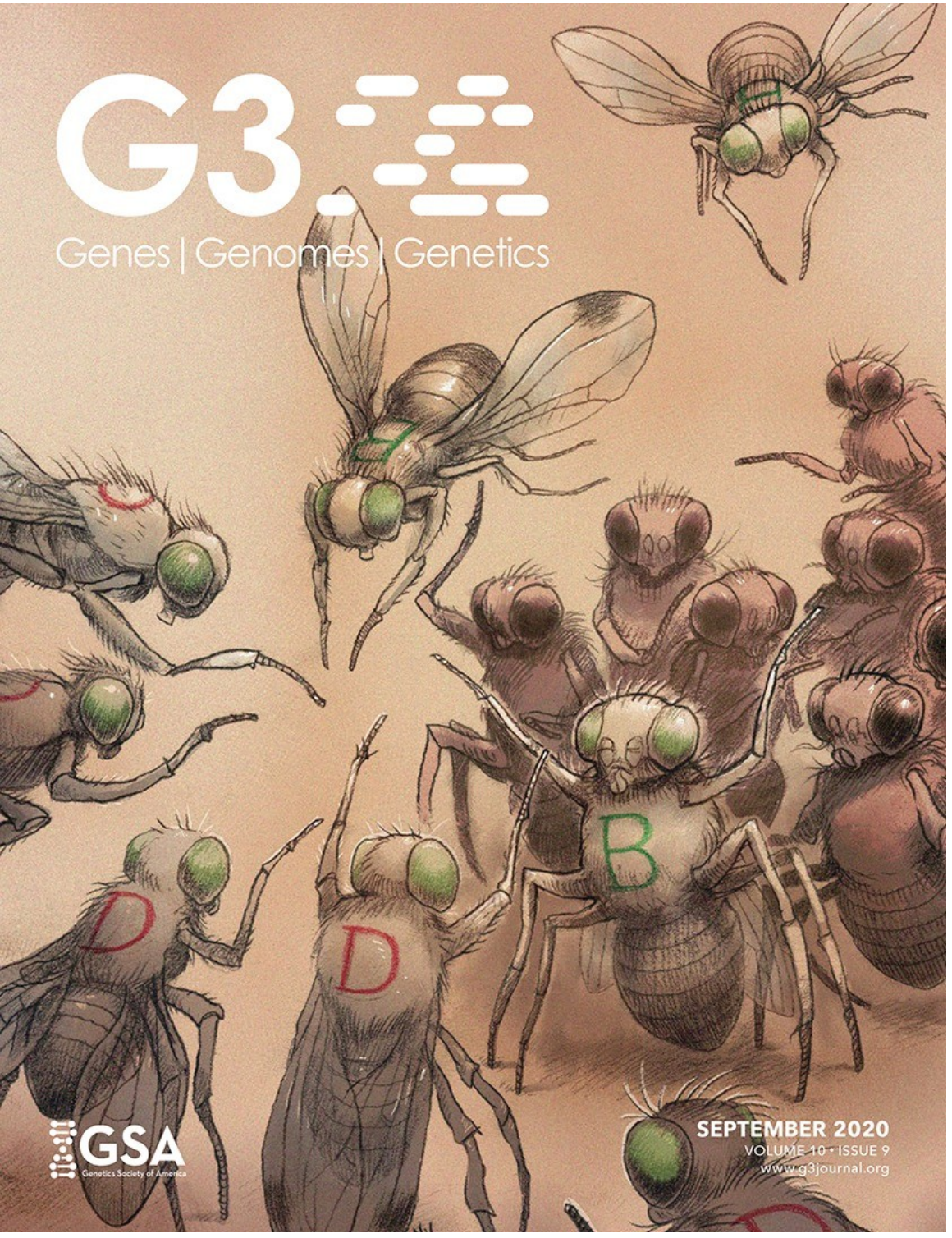


CORRESPONDENCE

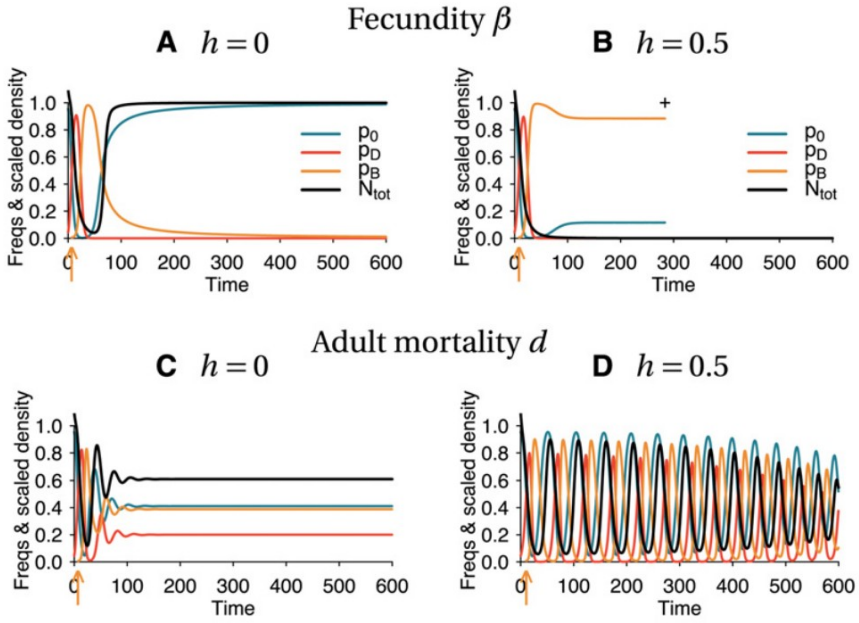
Nature Biotechnologies, Feb 2016

Bing Wu[1,2], Liqun Luo[1] & Xiaojing J Gao[1–3]

Cas9-triggered chain ablation of *cas9* as a gene drive brake



A brake is not guaranteed to stop an eradication drive



Rode et al., G3 2020

Gene drives: good or bad?

May eradicate diseases and pest species

Less expensive than other methods

Potentially faster than other methods

Potentially more powerful than other methods

Potentially less efficient than expected (resistance via mutations in the target site, cryptic species)

An uncontrolled system released in the wild

Impact on other species and ecosystems not quantified

Gene drives

Biases:

Living
in malaria area

Developing
gene drives

etc.

Regulation

Falls under the GMO regulation

Cartagena Protocol: international agreement, established as a supplement to the Convention on Biological Diversity (CBD), which aims to protect biological diversity from the potential risks imposed by LMOs (*Living Modified Organisms=any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology*)

Researchers added extra safety rules in their laboratories

What to do if it goes wrong?

International regulation?

DOI : 10.4267/2042/70673

LE FORÇAGE GÉNÉTIQUE (GENE DRIVE) ET SES APPLICATIONS

@Biol4Ever

GENE DRIVE AND ITS APPLICATIONS

Par Virginie COURTIER-ORGOGOZO[1]

http://documents.irevues.inist.fr/handle/2042/70673

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