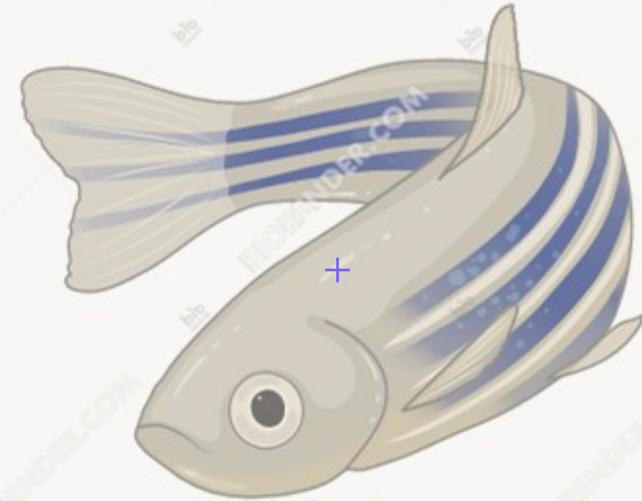


# Transgenic zebrafish for the study of physiology and pathology



# What is a transgenic animal?

**FELASA (*Federation of European Laboratory Animal Associations*) definition:**

Each animal whose genome has been deliberately modified.

**Common definition:**

Animals in which a foreign gene has been introduced into all cells, including the germline, allowing it to be passed on to the offspring.

# Generation of a transgenic reporter line: Overview of the Protocol

- **Cloning** of the regulatory region in a plasmid
- **Cloning** of the reporter gene in a plasmid
- **Assembling** of a **Tol2** destination vector (regulatory region:reporter)
- **Inject** the F0 eggs with the vector and the Tol2 transposase (or its mRNA)
- When possible, **screen** the fluorescent embryos for potential founders.
- **Grow** the F0
- **Outcross** the F0 founders to find F1 positive embryos-> larvae->adults
- **Outcross** F1 or F2 or F3..... to do the experiment.

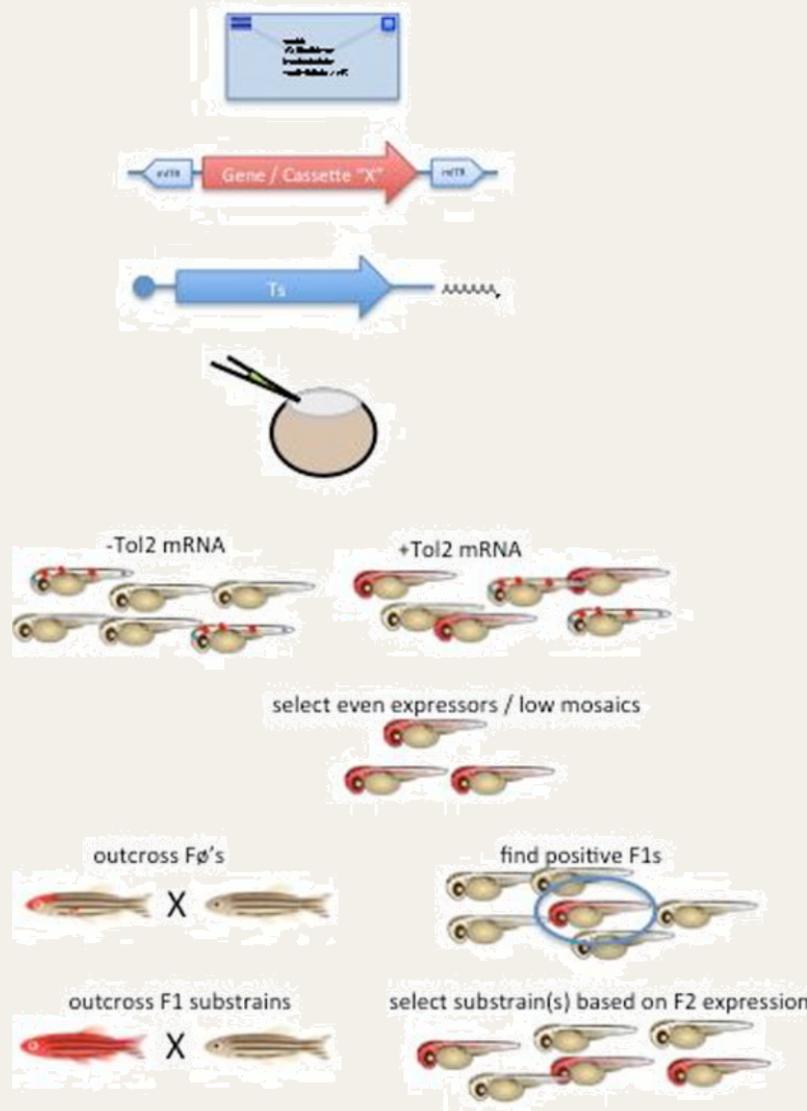
# Crucial Factors for Obtaining Reliable Transgenic Fish

- Choosing the most appropriate **transgenesis method**
- Selecting the correct **promoter/enhancer region**
- Adding a **strong minimal promoter**
- Choosing the most appropriate **reporter protein**

# Transgenesis method: Tol2 strategy

- Transposon-based system → **Tol2 transposon**
- Advantages:
  1. high **germline transmission** rate → about 70%
  2. **single-copy** (non-concatemered) integration of the transgene
  3. high activity and integration efficiency
- Originally isolated from the medaka fish genome
- Leads to the precise integration of a single-copy of the transgene at a random location.
- Transgenesis tool of choice for zebrafish
- Requires the delivery of:
  1. **Transposase mRNA**
  2. **Transgenesis vector** containing a desired transgene cargo **flanked by Tol2 transposon repeats**

# Transgenesis method: Tol2 strategy



II. Request *Tol2* system reagents.

IIA. Prepare your favorite *Tol2* transposon.

IIB. Prepare *Tol2* transposase mRNA.

III. Microinject embryos with mix of transposon DNA and transposase mRNA.

IV. Quality check for transposition.

V. Select F<sub>0</sub> embryos to raise.

VI. Out cross F<sub>0</sub> to non-transgenic partner, select positive F1 embryos.

VII. Raise F1's. Select best line(s) by observing F2 embryos.

# Transgenesis method: Multisite Gateway-based Tol2 strategy

## Lamba-based recombination

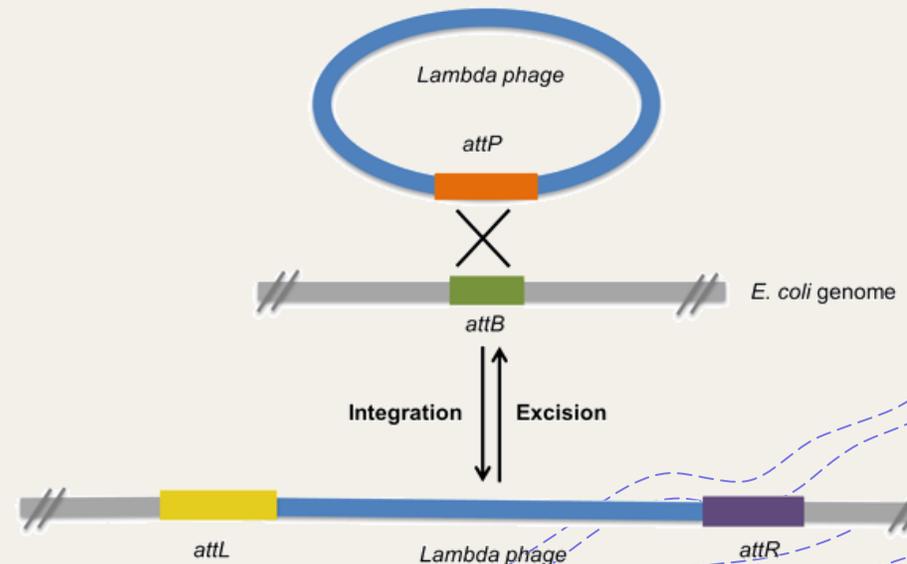
- Universal cloning method based on the site-specific recombination properties of **bacteriophage lambda** which ease the integration of lambda into **E. Coli**
- DNA sequences are cloned into **multiple vectors** → **3 vector-based technique**
- Two major components:
  1. The DNA recombination sequences (***att* sites**)
  2. The proteins that mediate the recombination reaction (*i.e.* **Clonase**)
- Recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules.
- **Conservative recombination** (no net loss or gain of DNA)

# Transgenesis method: Multisite Gateway-based Tol2 strategy

## Att sites and recombination

- Att-site: site-specific attachment sites:
  1. **attB** → originally present on *E. Coli* chromosome
  2. **attP** → originally present on lambda chromosome
- Binding sites for recombination proteins
- The DNA segments flanking the recombination sites (att) are switched
- After recombination, the **remaining att sites are hybrid sequences** comprised of sequences donated by each parental vector
- Strand exchange occurs within a **15 bp core region** that is **common to all att sites**

Give rise to **attL** and **attR** sites



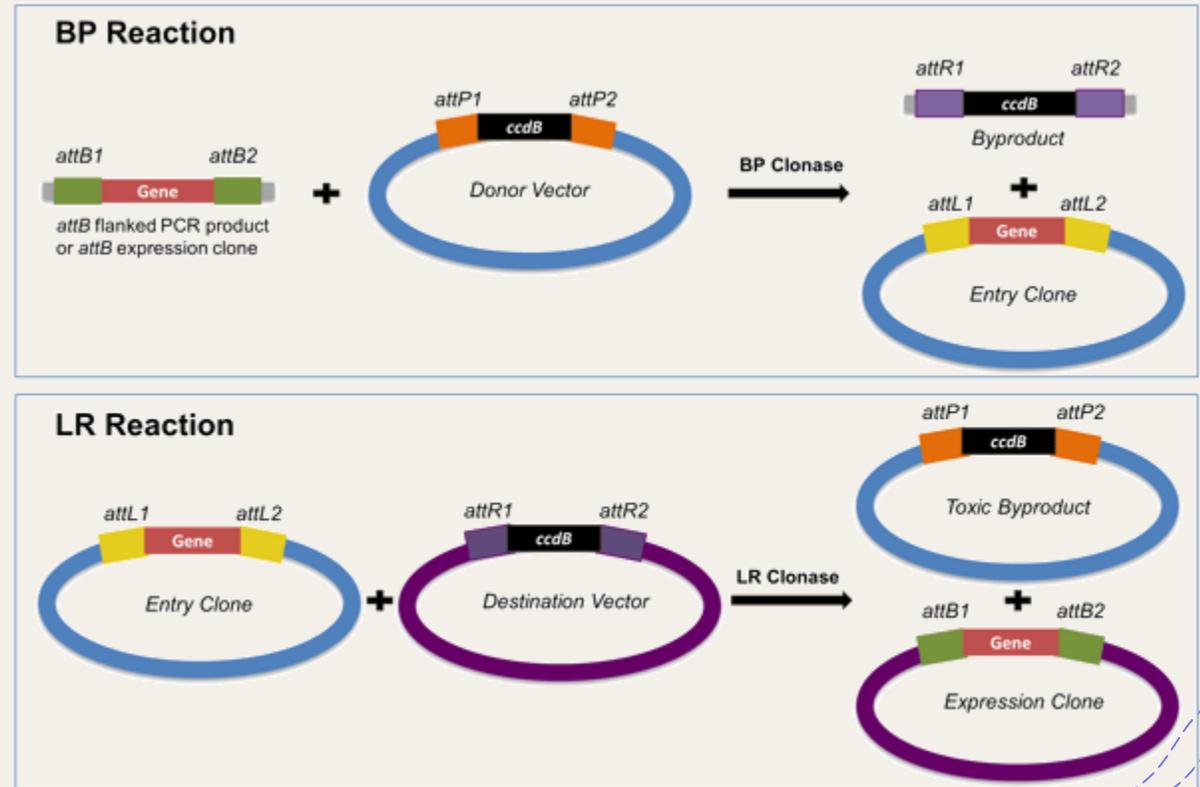
# Transgenesis method: Multisite Gateway-based Tol2 strategy

## Att sites and recombination

Two types of recombination reaction:

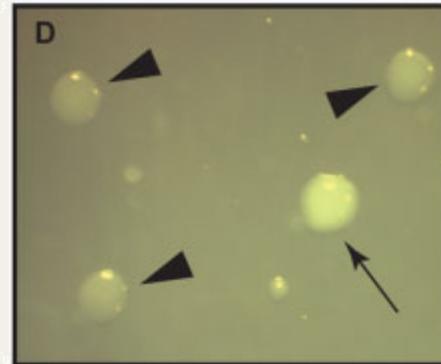
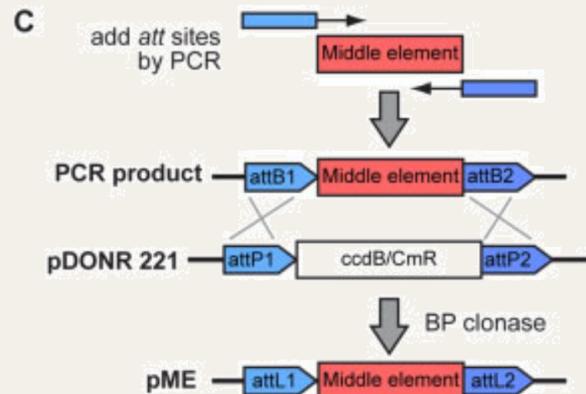
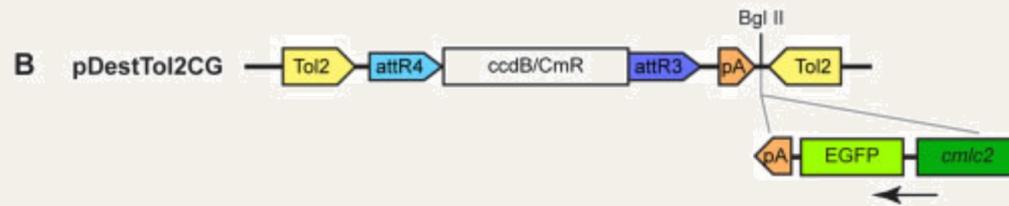
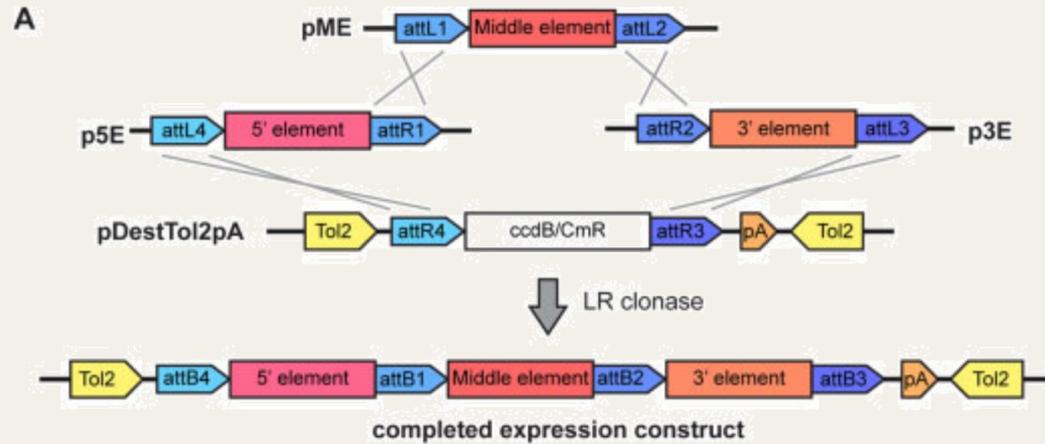
**BP Reaction:** Recombination of an *attB* substrate with an *attP* substrate to create an *attL*-containing entry clone → **Lysogenic Pathway**

**LR Reaction:** Recombination of an *attL* substrate with an *attR* substrate to create an *attB*-containing expression clone → **Lytic Pathway**



# Transgenesis method: Multisite Gateway-based Tol2 strategy

## Vectors



**Transformation of TOP10 cells.**  
Clear colonies yield the correct recombination product 99% of the time, whereas opaque colonies never do.

# Transgenesis method: Multisite Gateway-based Tol2 strategy

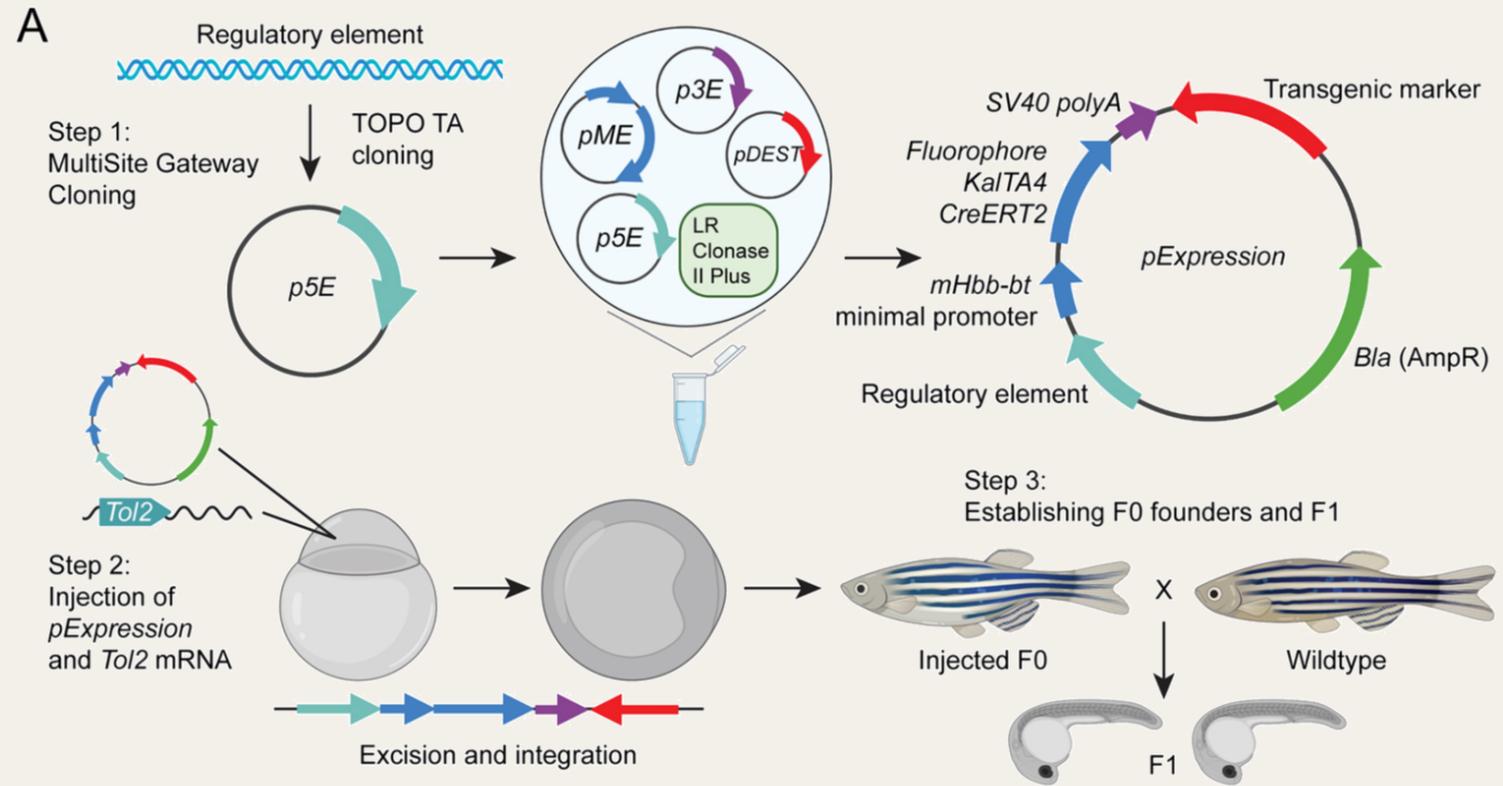
## Vectors

TABLE 1. Components of the Tol2kit<sup>a</sup>

Name	Description	Test	Figure
<b>5' entry clones</b>			
p5E- <i>bactin2</i>	5.3-kb beta-actin promoter (ubiquitous)	F0, F1	2
p5E- <i>h2afx</i>	1-kb H2A-X promoter (quasi-ubiquitous)	F0	2b
p5E-CMV/SP6	1-kb CMV/SP6 cassette from pCS2+	F0	
p5E- <i>hsp70</i>	1.5-kb <i>hsp70</i> promoter for heat-shock induction	F0, F1	4
p5E-UAS	10x UAS element and basal promoter for Gal4 response	F0	
p5E-MCS	Multicloning site from pBluescript	F0	
p5E-Fse-Asc	Restriction sites for 8-cutters <i>FseI</i> and <i>AscI</i>	F0	
<b>Middle entry clones</b>			
pME-EGFP	EGFP	F0	
pME-EGFPCAAX	Membrane-localized (prenylated) EGFP; fused to the last 21 amino acids of H-ras	F0, F1	3
pME-nlsEGFP	Nuclear-localized EGFP	F0	2f
pME-mCherry	Monomeric red fluorophore mCherry	F0, F1	2c
pME-mCherryCAAX	Membrane-localized (prenylated) mCherry	F0	2e, 4
pME-nlsmCherry	Nuclear-localized mCherry	F0	2abd
pME-H2AmCherry	mCherry fused to the zebrafish histone H2A.F/Z	F0, F1	
pME-Gal4VP16	Gal4 DNA binding domain fused to the VP16 transactivation domain	F0	
<b>3' entry clones</b>			
p3E-polyA	SV40 late poly A signal sequence from pCS2+	F0, F1	3, 4
p3E-MTpA	6x myc tag for protein fusions, plus SV40 late polyA		
p3E-EGFPpA	EGFP for protein fusions, plus SV40 late polyA	F0, F1	
p3E-mCherrypA	mCherry for protein fusions, plus SV40 late polyA	F0, F1	
p3E-IRES-EGFPpA	EMCV IRES driving EGFP plus SV40 late polyA	F0	2d
p3E-IRES-EGFPCAAXpA	EMCV IRES driving EGFPCAAX (prenylated EGFP) plus SV40 late polyA	F0, F1	2abc
p3E-IRES-nlsEGFPpA	EMCV IRES driving nlsEGFP (nuclear EGFP) plus SV40 late polyA	F0	2e
<b>Destination vectors</b>			
pDestTol2pA/pDestTol2pA2	attR4-R3 gate with SV40 polyA flanked by Tol2 inverted repeats	F0, F1	3
pDestTol2CG/pDestTol2CG2	pDestTol2pA/pDestTol2pA2 with <i>cmlc2</i> :EGFP transgenesis marker	F0, F1	4
<b>Other</b>			
pCS2FA-transposase	For in vitro transcription of capped Tol2 transposase RNA		3, 4

<sup>a</sup>EGFP, enhanced green fluorescent protein; F0, yields appropriate expression in transient transgenics; F1, yields appropriate expression in stable transgenics.

# Transgenesis method: Multisite Gateway-based Tol2 strategy Workflow Overview



# THE PROMOTER/ENHANCER

- **SIMPLE**

- **TISSUE SPECIFIC** (e.g. insulin promoter, CMLC promoter; crystallin promoter)

- Inserted (isolated in a plasmid, fused and injected together with the reporter gene) in a vector
- Trapped (enhancer trap screening)

- **TIME SPECIFIC** (e.g. HSP70 promoter)

- **UBIQUITOUS** (ubiquitin, alpha-actin)

- **COMBINATORIAL**

- **SIMPLE: SPACE** (e.g. *Ins:Gal4* and *UAS:GFP*) or **TIME** (*HSP70:Gal4* and *UAS:GFP*)

- **CONDITIONAL (SPACE AND TIME)** (*HuC:ERT2-Gal4* and *UAS:GFP*)



# THE PROMOTER/ENHANCER

- TISSUE/CELL SPECIFIC PROMOTER/ENHANCER
  - Span: 0.3-30 kb (to Mb)
  - **Characterized** *in vitro* (cell transfection)
  - **Characterized** *in silico*: comparative alignments of evolutionary conserved non coding regions of genes (evolutionary footprinting analysis)
  - **Characterized** targeted KI (insertion of the reporter in the regulatory region of a known gene by CRISPR/Cas or TALEN)
  - **Non characterized** (trial and error in transient injection experiments or enhancer trap)

# ENHANCER/GENE TRAP EXPERIMENTS (non characterized regulatory elements/random insertion)

The introduction of the yeast **Gal4 system** in Zebrafish

**TWO TRANSGENIC LINES:**

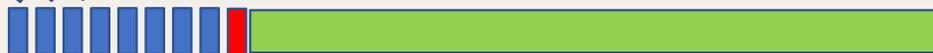
- THE **DRIVER** (ACTIVATOR) EXPRESSING Gal4 TF IN SPECIFIC FEATURES (CELLS TISSUE, TIME)
- THE **REPORTER** (ACTIVATED) BEARING UAS:REPORTER

are crossed to **produce animals with both alleles**, expressing the reporter

Gal4 transcription factor

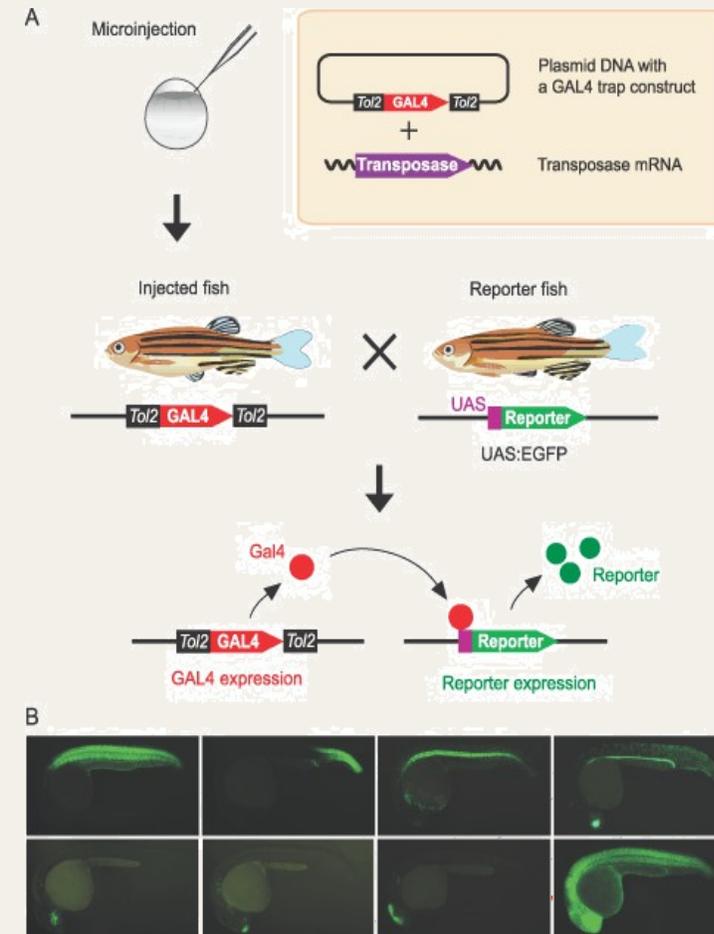


GFP



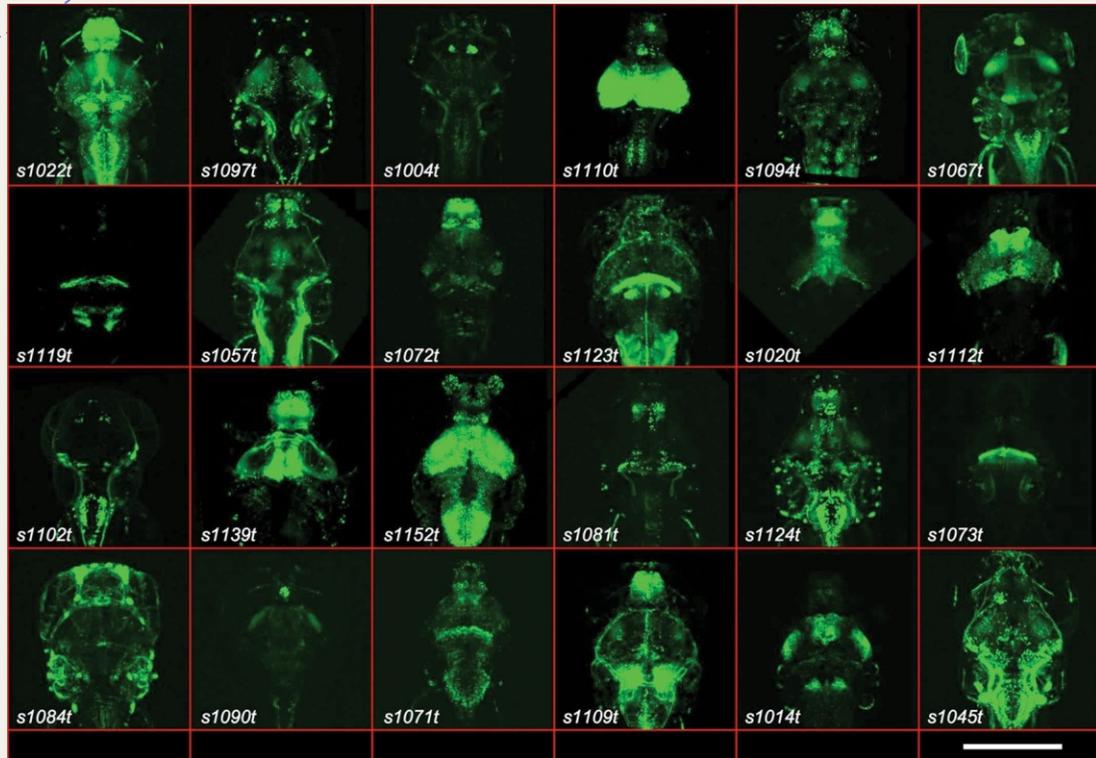
8xUAS (binding element)

The **DRIVER** (GAL4 expressing line) is produced by multiple **RANDOM INSERTION** in the zebrafish genome.

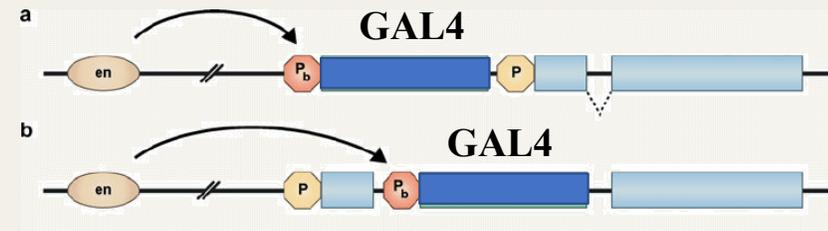


# ENHANCER/GENE TRAP EXPERIMENTS (non characterized regulatory elements/random insertion)

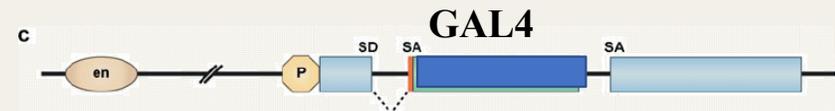
EVERY INDEPENDENT INSERTION OF THE GAL4 DRIVER NEAR OR IN THE CODING REGION OF A GENE WILL RESULT IN A SPECIFIC PATTERN WHEN CROSSED WITH THE REPORTER (GFP)



## Enhancer Trap



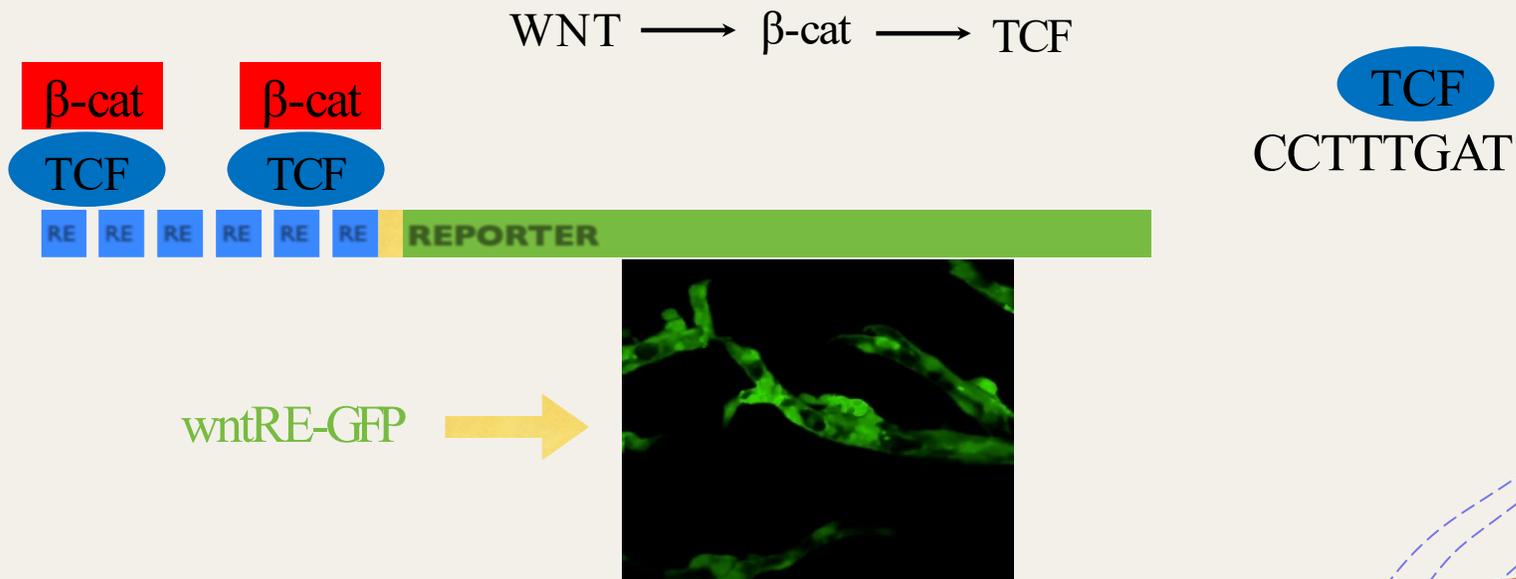
## Gene Trap



N.B.: (GFP is produced by the «reporter» allele)

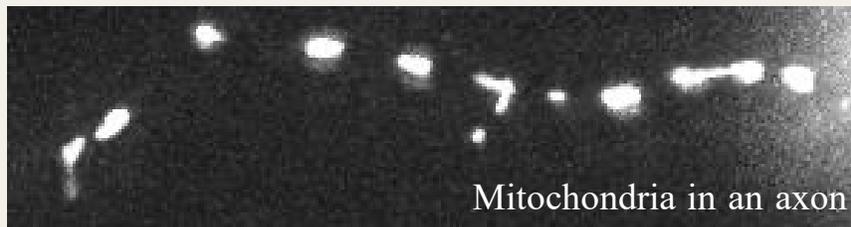
# REGULATORY ELEMENTS (REs)

- **Transcription factors** and **cis-elements** are **conserved** in vertebrates
- Polymerized cis elements increase **signal sensitivity**
- **Multimerized REs** recruit more signal-dependent TF
- Can be chosen from **literature** or **bioinformatic tools** (es. UCSC) → Search for conserved regions close to the target
- Useful for the creation of **transgenic lines** for the study of **specific signalling pathways**

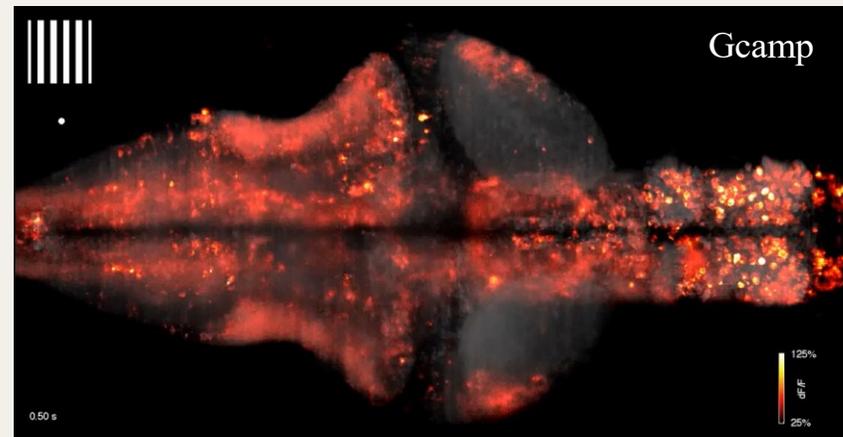


# THE REPORTER PROTEIN

- STRICTLY QUANTITATIVE REPORTERS (e.g. Luciferase)
- «**MORPHOLOGICAL**» AND QUANTITATIVE FLUOPRESCENCE REPORTER (e.g. GFP/RFP/KAEDE/others)
- **COMBINED DOMAINS** QUANTITATIVE FLUORESCENT REPORTER
  - SUBCELLULAR RESOLUTION (NUCLEAR, MITOCHONDRIAL, MEMBRANES ETC)
  - SPECIFIC INTRACELLULAR SIGNAL (Ca<sup>++</sup>, ROS, pH, Oxygen)



Barazzuol et al., 2023



Benjamin F. Fosque, 2015

# THE REPORTER PROTEIN

**STRICTLY QUANTITATIVE REPORTERS** (e.g. Luciferase) rarely used, mostly in physiology.

- **DISADVANTAGES: NO SPATIAL INFORMATIONS**
- **ADVANTAGES: VERY FAST SIGNAL DYNAMICS**

«**MORPHOLOGICAL**» AND QUANTITATIVE FLUOPRESCENCE REPORTER (e.g. GFP/RFP/KAEDE/others)

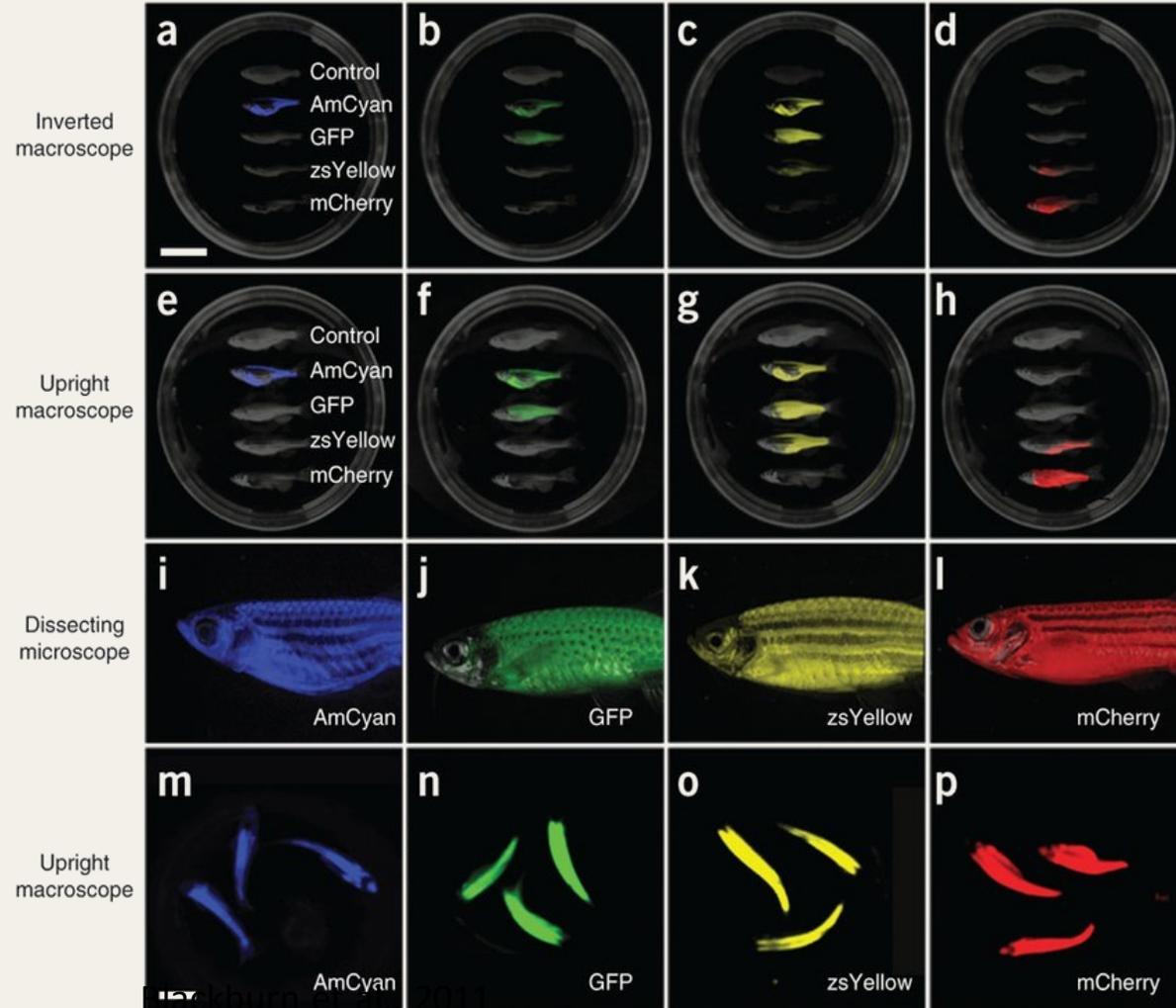
- **DISADVANTAGES: ACCUMULATED (SLOW DYNAMICS)**
- **ADVANTAGES: SPATIAL INFORMATIONS**

**PHYSIOLOGICAL FLUORESCENT REPORTER**

SUBCELLULAR RESOLUTION (NUCLEAR, MITOCHONDRIAL, MEMBRANES ETC) SPECIFIC INTRACELLULAR SIGNAL (Ca<sup>++</sup>, ROS, pH, Oxygen...)

# FLUORESCENT PROTEIN: PALETTE

GFP, RFP, VENUS, KAEDE CERULEAN...



# THE REPORTER PROTEIN: How to choose?

Factors determining the choice of the most suitable fluorescent protein:

- **Colour**
- **Brightness**
- **Toxicity**
- **Tissue Penetration**
- **Subcellular Localization**
- **Availability of Modified Version** (e.g. convertible, toxic, stable/destabilized)

## STABLE REPORTER

Which use?

- Cell Signalling Dynamics
- Cell Lineage Tracking

Drawbacks:

- Slow accumulation

## DESTABILIZED REPORTER

Which use?

- Studying rapid dynamics

Drawbacks:

- Low amount of detectable accumulating protein during a biological event

vs

# To Sum Up

- The regulatory element targets the cell/tissue
- The reporter collect the signal
- Different combinations of regulatory elements and reporters are possible,
- Reporters can be in multiple colors (filters or spectral microscopes are needed)
- Combinatorial control of gene expression uses Gal4 or CRE systems
- The reporter can be targeted in any tissue/cell and in any subcellular compartment

# Microinjection in Zebrafish

Best stages:

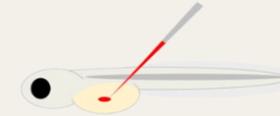
Transgenesis and genome editing:  
1-cell (zygote) stage



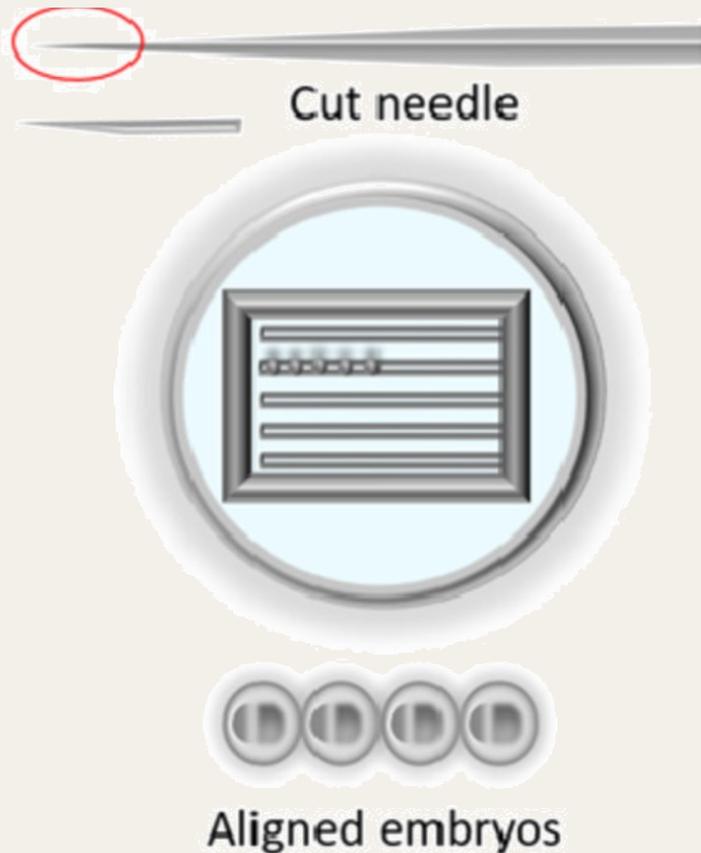
Antisense technology (eg: morpholino oligos): 1-4 cells stage



Cell transplantation: usually 1-2  
days post-fertilization



# Microinjection in Zebrafish

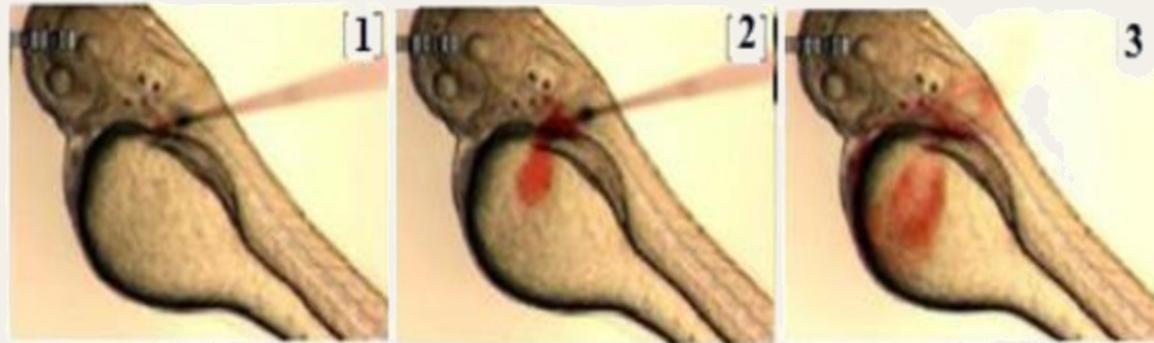
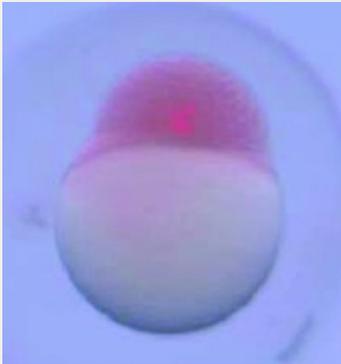


- Embryos or larvae obtained from pair or population crosses
- Egg collection after about 15 min from laying (to allow fertilization)
- Alignment along agarose-made lanes or microscope slide borders
- Glass needles with filament (for solutions) or without filament (for cells)

# Microinjection in Zebrafish

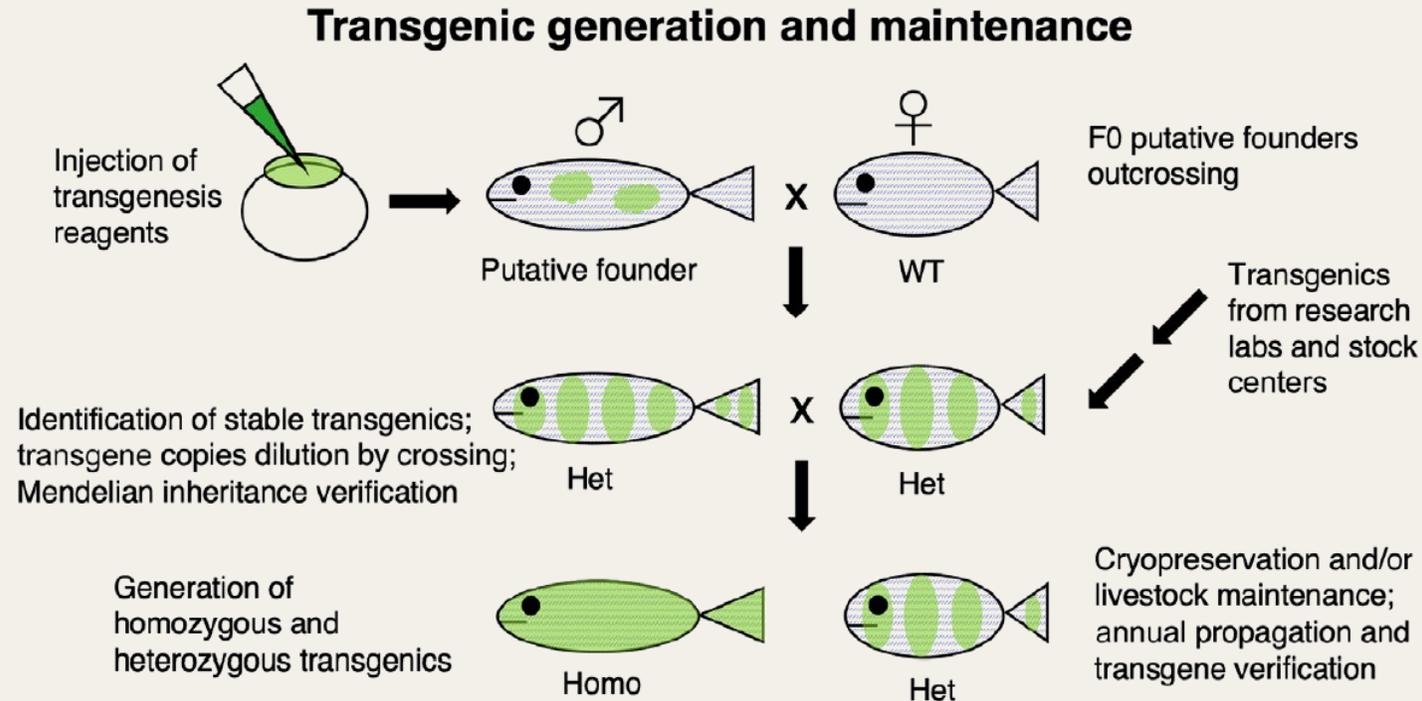
Tracking the microinjection:

- Typical dyes: phenol red (red under visible light; pH indicator), rhodamine, dextrans
- Stable membrane dyes: DiI (fluorescent red), DiO (fluorescent green)
- An mRNA for GFP or other reporters can track nucleic acid translation/quality/quantity/distribution/degradation (for 1-2 days)



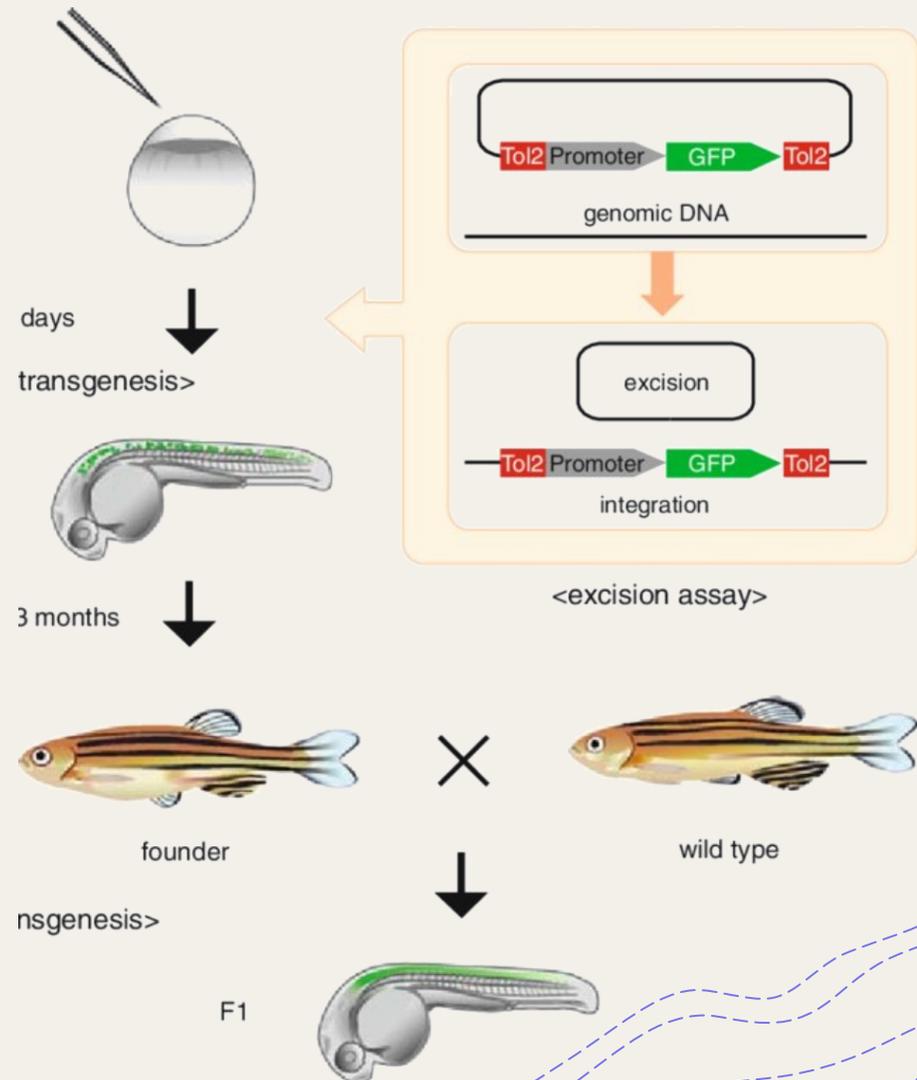
# Transgenic line stabilization, genotyping and maintenance

- How to manage a newly produced transgenic line?



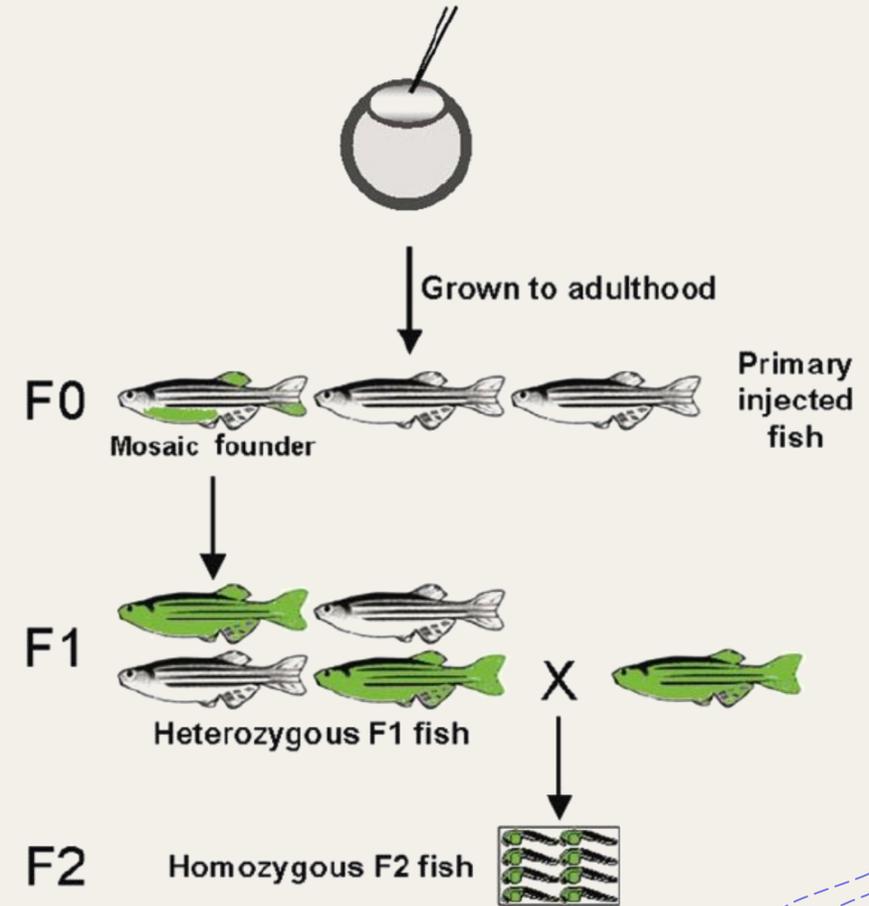
# Transgenic line stabilization

- After F0 (mosaic) production, founders are outcrossed to verify the **germline transmission**
- If transgene **multi-copies** are present (variable phenotypes, non-Mendelian inheritance), fishes are further out-crossed to dilute/select single copies
- Stabilized line: homogeneous phenotypes, **Mendelian inheritance** of the transgene



# Monitoring the transgene presence

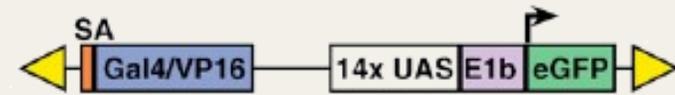
- **Fluorescence analysis**
- In many cases single/double copies correspond to different fluorescence intensities
- this can help to identify heterozygous and homozygous transgenic animals



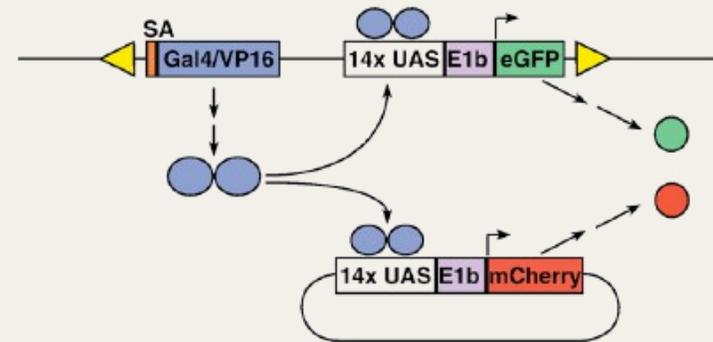
# Monitoring the transgene presence

- Analysis of transgene presence by **PCR (transgene-specific primers)**
- Useful in case of non-fluorescent transgenes (Eg: classical GAL4 is not fluorescent in itself (but it can be combined with a fluorescent reporter))

Green reporter



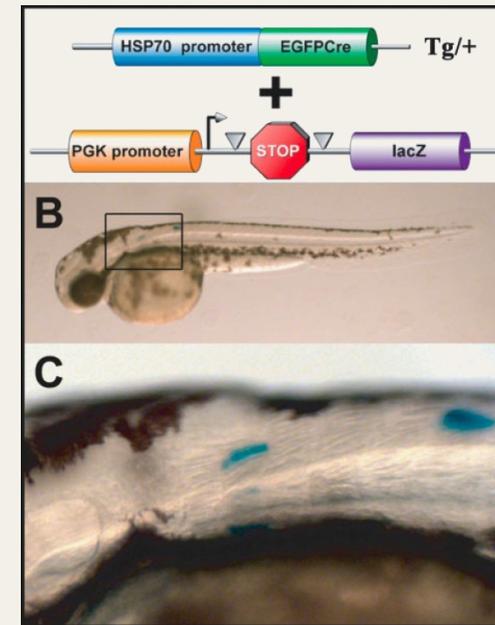
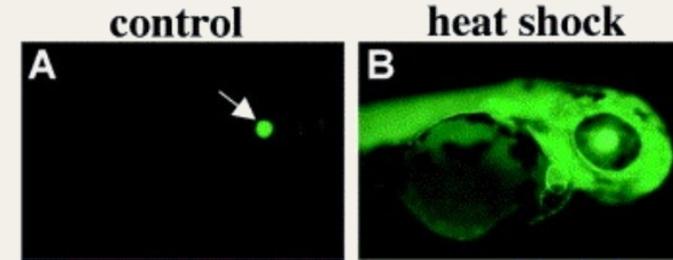
Green and Red reporter



# Monitoring the transgene presence

Various methods depend on the type of transgene e.g:

- **heat-shock** based transgenes (eg: hsp70 transgenes)
- Combination in **binary systems** (eg: GAL4/UAS, Cre/Lox, Flp/FRT)
- **Enzymatic activity** revealed by a substrate (eg: LacZ, NTR)
- Transcriptional activity depending on **compound administration** (eg: Tet-Off and Tet-On systems) and so on ...



Cre-dependent  
LacZ expression  
in zebrafish

(PGK:  
ubiquitous  
promoter)

# Methods for zebrafish genotyping

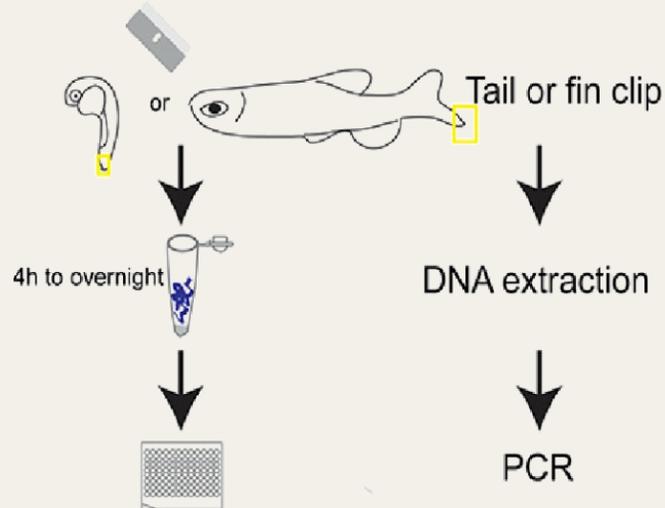
Genotyping can be performed on:

- Zebrafish embryos
- Zebrafish larvae
- Zebrafish juveniles
- Zebrafish adults

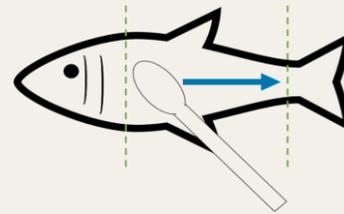
Genotyping can be:

- Destructive (eg: from whole embryos or larvae)
- Mildly invasive (eg: fin clipping)
- Non-invasive (cutaneous cells, skin swabs); this is a refinement in animal experimentation

## Fin clip



## Skin swab



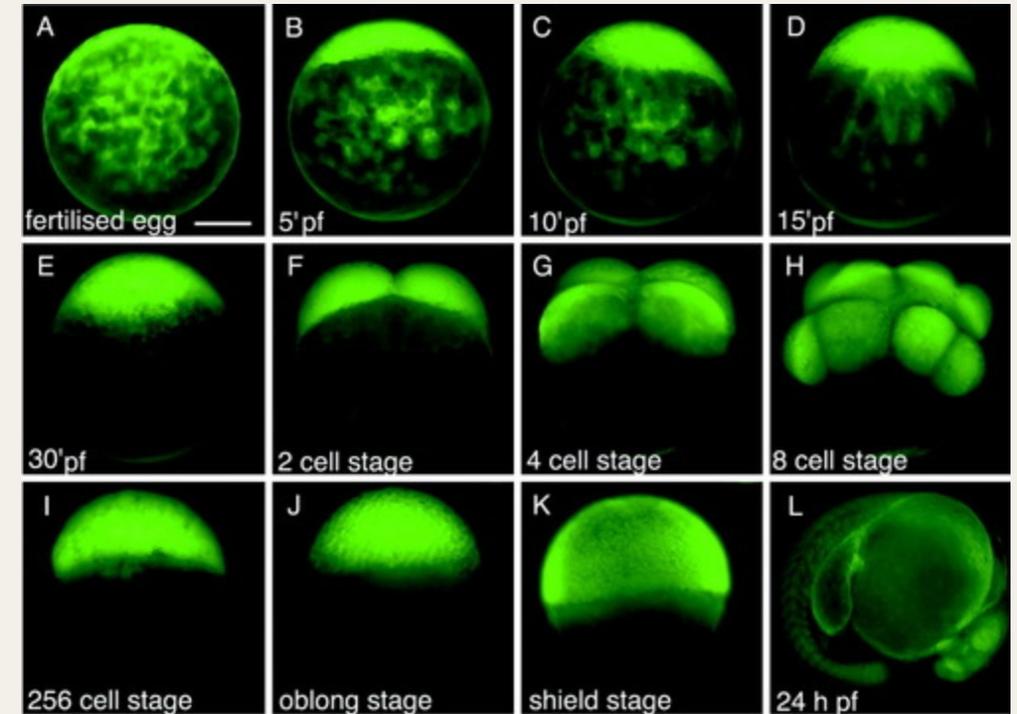
Genotyping can be based on PCR, directly inspected or followed by post-PCR assays (eg: digestion, HRMA).

# Transgenic line maintenance along the generations

- Lines can be maintained alive or frozen (a **frozen backup** is recommended)
- To avoid repeated inbreeding within the transgenic line (with deleterious effects due to homozygosity - **inbreeding depression**), outcrosses are recommended every
- Fluorescent transgenes should be monitored at every generation, discarding weaker ones (due to **gene silencing, epigenetic modifications**, other reasons), and keeping the selection on brighter ones.
- Should this strategy be insufficient, the generation of a new transgenic line, reinjecting the original transgene constructs/reagents, may be required.

# Transgenic line maintenance along the generations

- To check for possible cross-contaminations among adjacent tanks, fluorescent embryos/larvae are always checked under **different filter/illumination conditions**, keeping individuals with the expected pattern, and discarding those bearing wrong transgenes.
- Specific attention is paid to **maternally activated transgenes**. For instance, in case of a heterozygous mother, maternal fluorescence can mask negative genotypes in the offspring. In this case, we suggest monitoring and reevaluating the offspring after 2- 4 dpf, usually sufficient for the fading of the maternal signal.
- Criteria to propagate: internal/external requests, low numbers, old age (we schedule **new crosses every 6-12 months**)

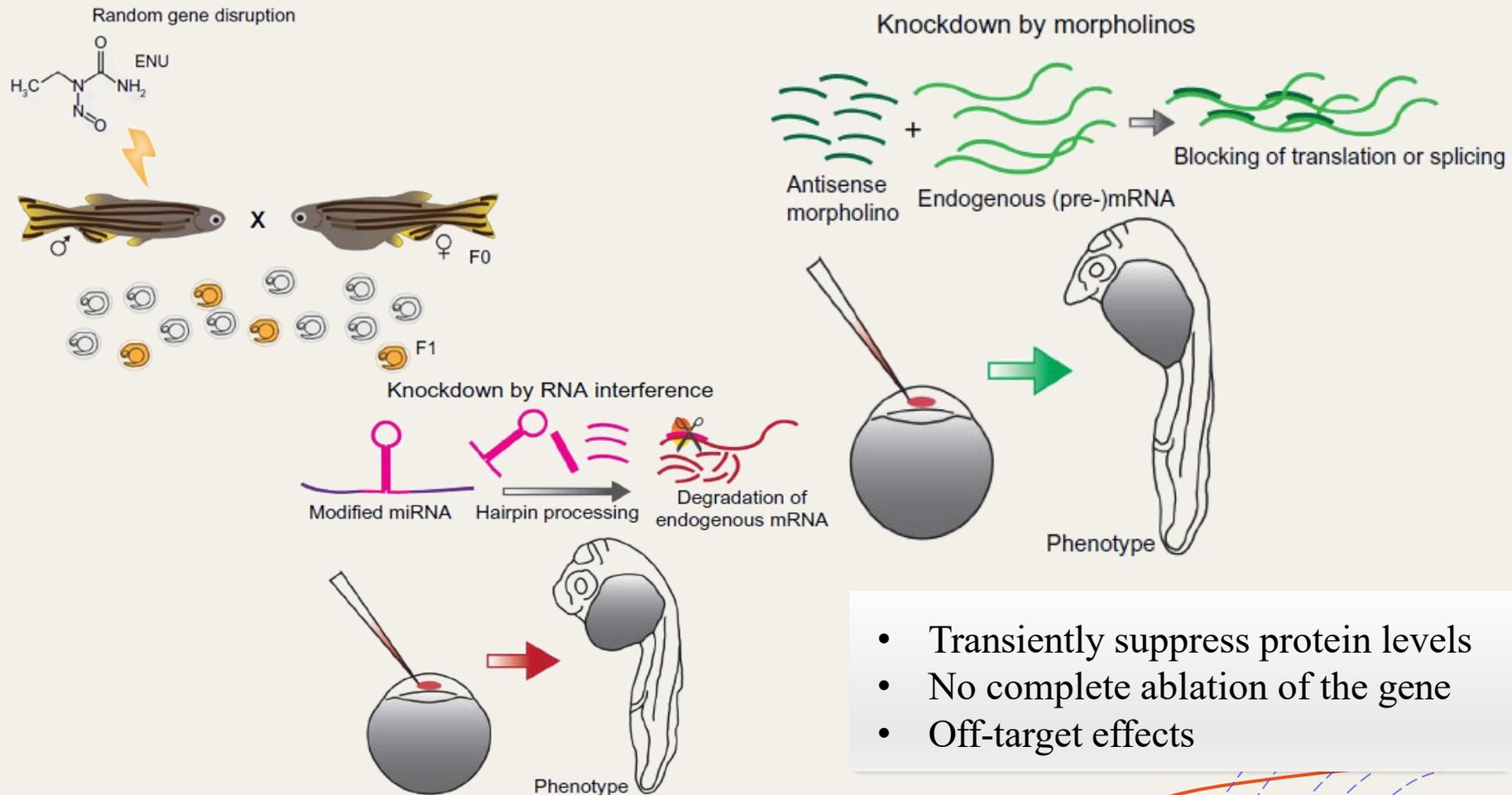


# Useful Videos

- <https://app.jove.com/v/21012/tol2-transposonmediated-zebrafish-transgenesis-a-procedureto-generate-transgenic-zebrafish-following-thecoinjection-of-tol2-system-into-fertilizedzebrafish-embryos>
- <https://app.jove.com/it/v/5130/zebrafish-microinjection-techniques>

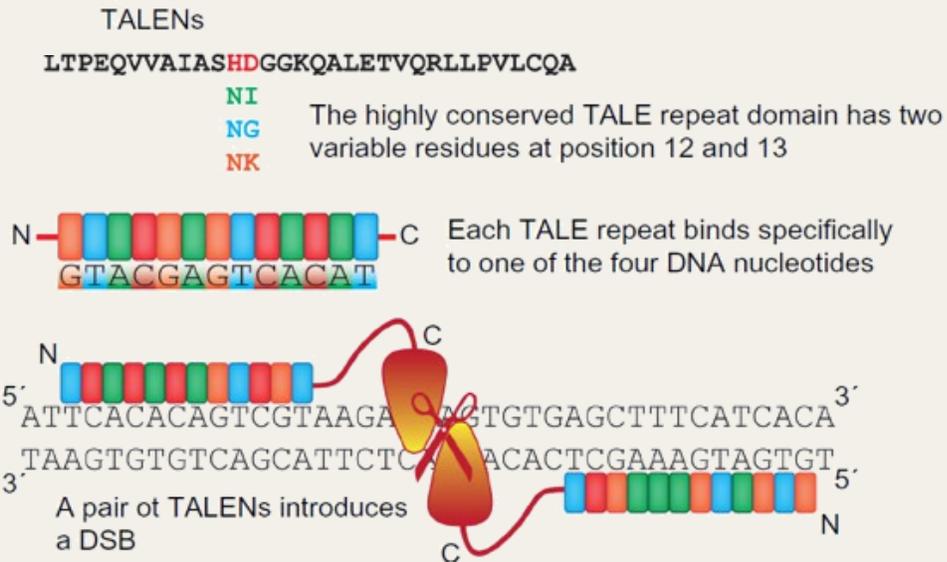
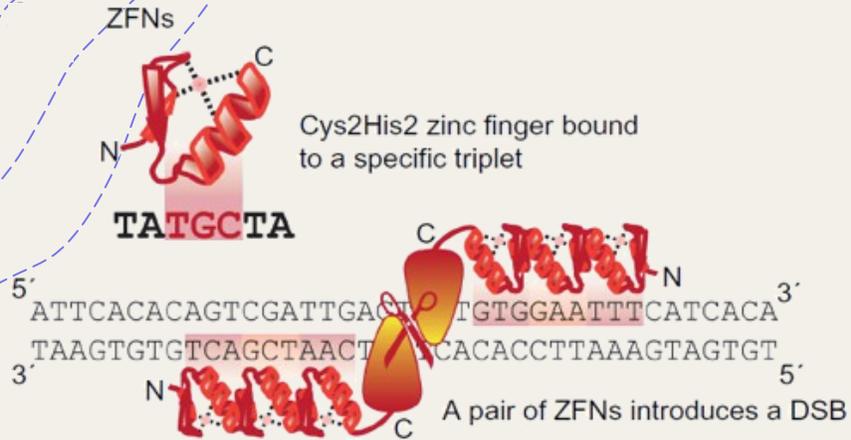
# CRISPR/Cas9 technology in zebrafish: an efficient approach for human genetic diseases modeling

How can we study gene function with zebrafish?



- Transiently suppress protein levels
- No complete ablation of the gene
- Off-target effects

# New experimental approaches with genome editing



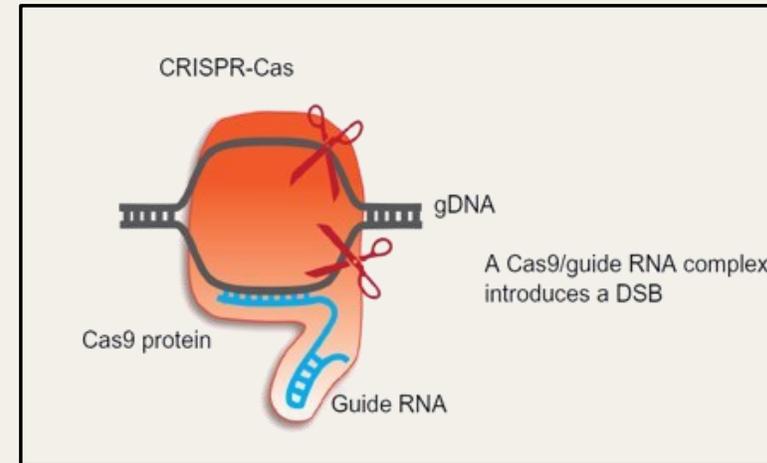
NATURE PROTOCOLS | VOL.8 NO.11 | 2013 | 2281

PROTOCOL

## Genome engineering using the CRISPR-Cas9 system

F Ann Ran<sup>1-5,8</sup>, Patrick D Hsu<sup>1-5,8</sup>, Jason Wright<sup>1</sup>, Vineeta Agarwala<sup>1,6,7</sup>, David A Scott<sup>1-4</sup> & Feng Zhang<sup>1-4</sup>

<sup>1</sup>Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard, Cambridge, Massachusetts, USA. <sup>2</sup>McGovern Institute for Brain Research, Cambridge, Massachusetts, USA. <sup>3</sup>Department of Brain and Cognitive Sciences, MIT, Cambridge, Massachusetts, USA. <sup>4</sup>Department of Biological Engineering, MIT, Cambridge, Massachusetts, USA. <sup>5</sup>Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, USA. <sup>6</sup>Program in Biophysics, Harvard University, MIT, Cambridge, Massachusetts, USA. <sup>7</sup>Harvard-MIT Division of Health Sciences and Technology, MIT, Cambridge, Massachusetts, USA. <sup>8</sup>These authors contributed equally to this work. Correspondence should be addressed to EZ. (zhang@broadinstitute.org).



Simple and efficient method (2013)

Sassen & Köster, 2015. Adv. Genomics Genetic

# CRISPR-Cas9

- Zebrafish was the **first vertebrate model** used to demonstrate that CRISPR/Cas9 can efficiently edit the genome in vivo (Hwang et al., 2013)

## Components:

- **guide RNA** (crRNA + tracrRNA): short synthetic RNA composed of a scaffold sequence necessary for Cas-binding and a user-defined **~20 nucleotide spacer** that defines the genomic target to be modified.
- **CRISPR-associated endonuclease (Cas protein)**

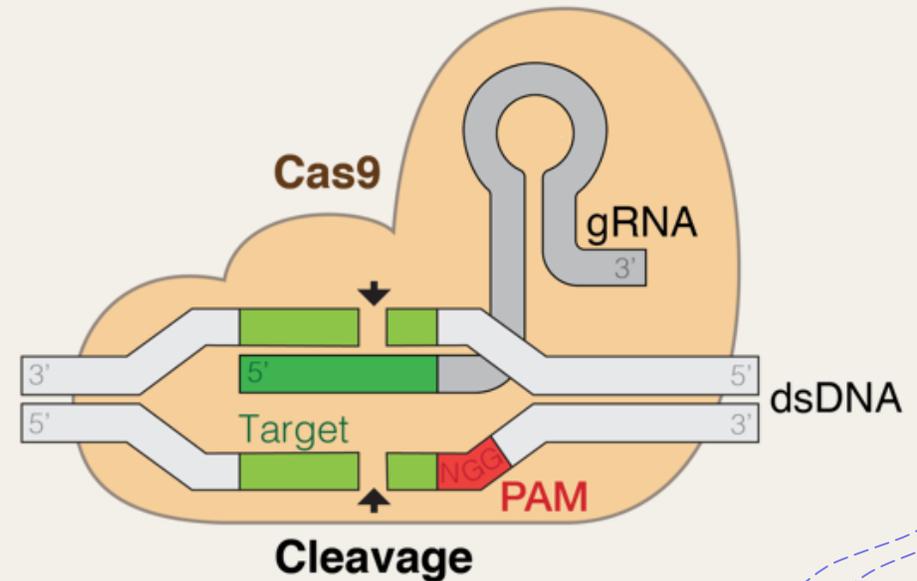
## **Target sequence: 20 bp**

Microinjecting an *in vitro* complex of guide RNA and Cas9 protein into one-cell stage embryos.

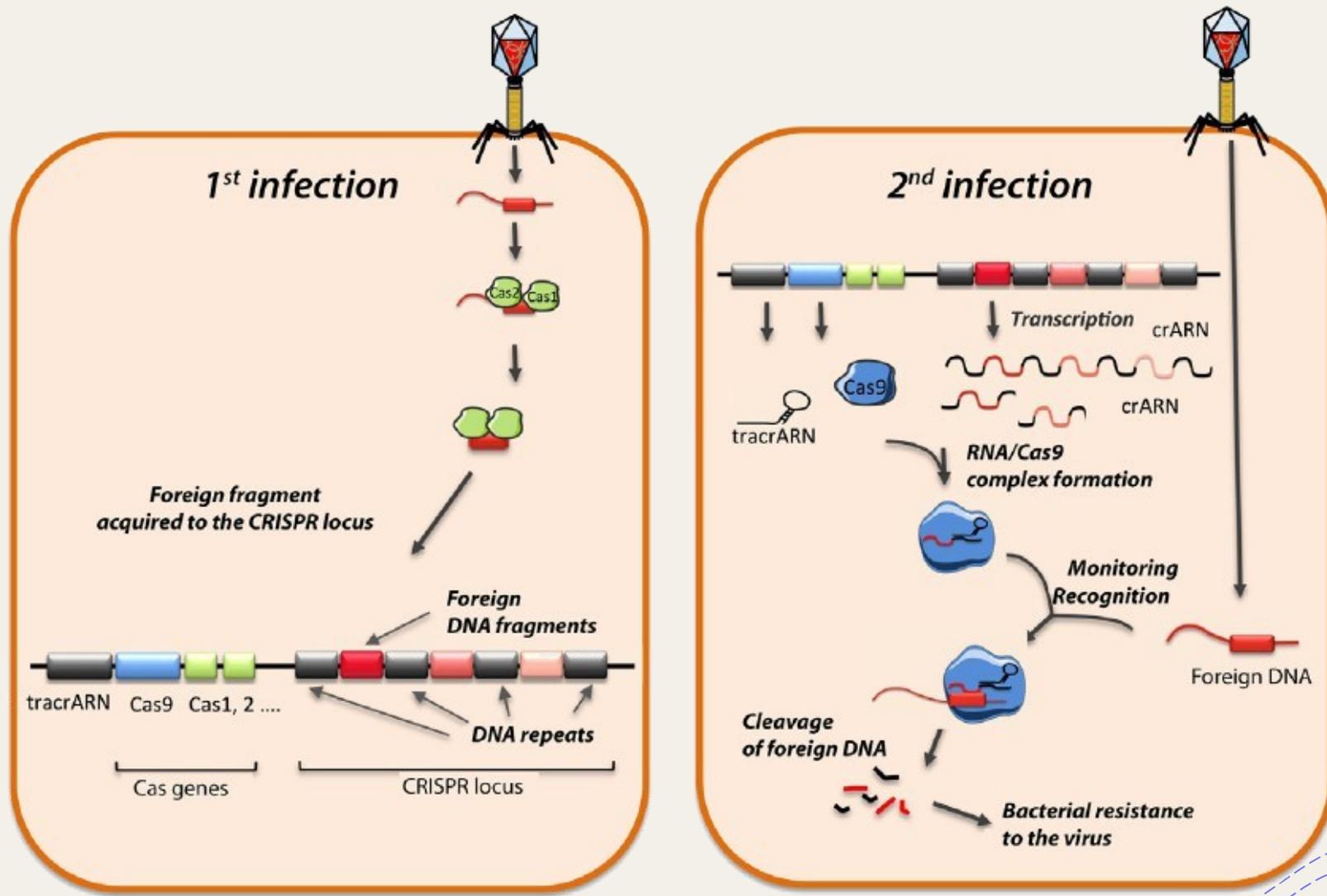
CRISPR-mediated knockout zebrafish models are extremely popular and have been used to **model several diseases**.



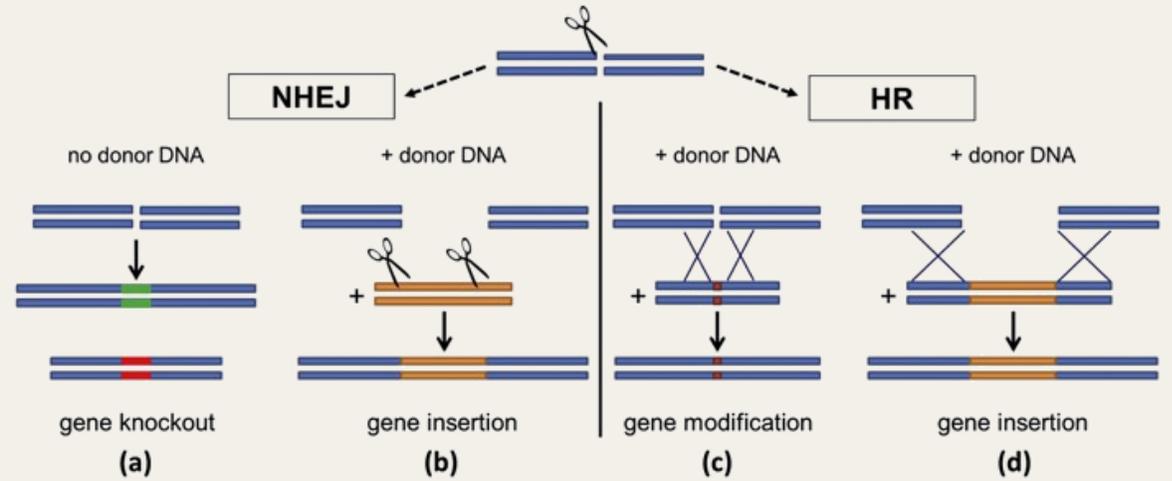
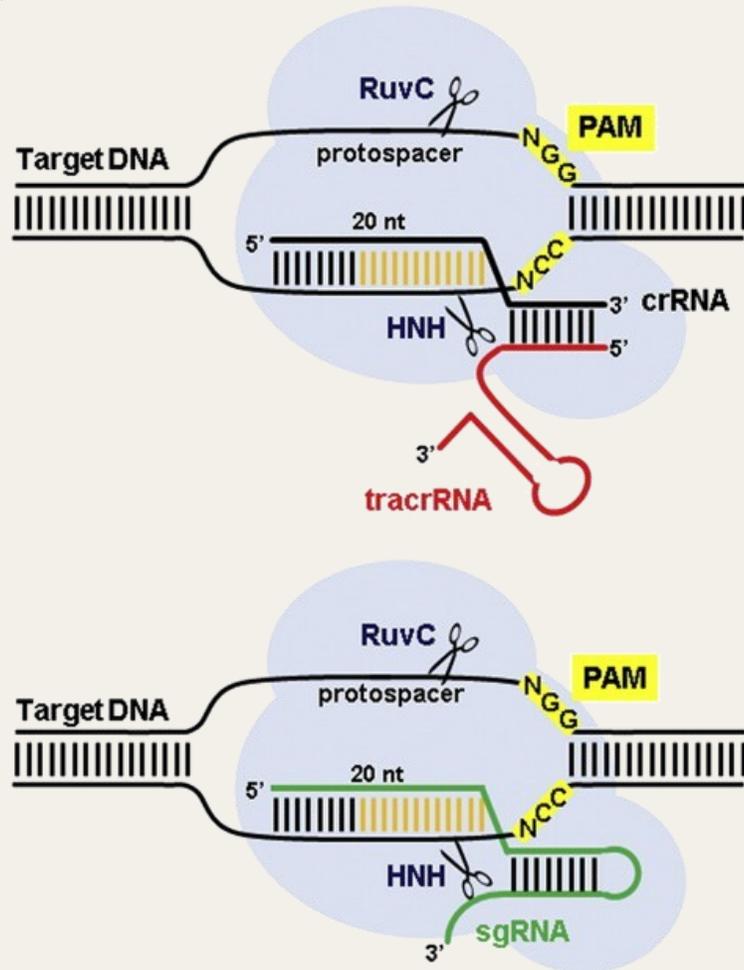
Emmanuelle Charpentier and Jennifer Doudna, Nobel Prize 2020



# CRISPR/Cas: the Immune System of Bacteria

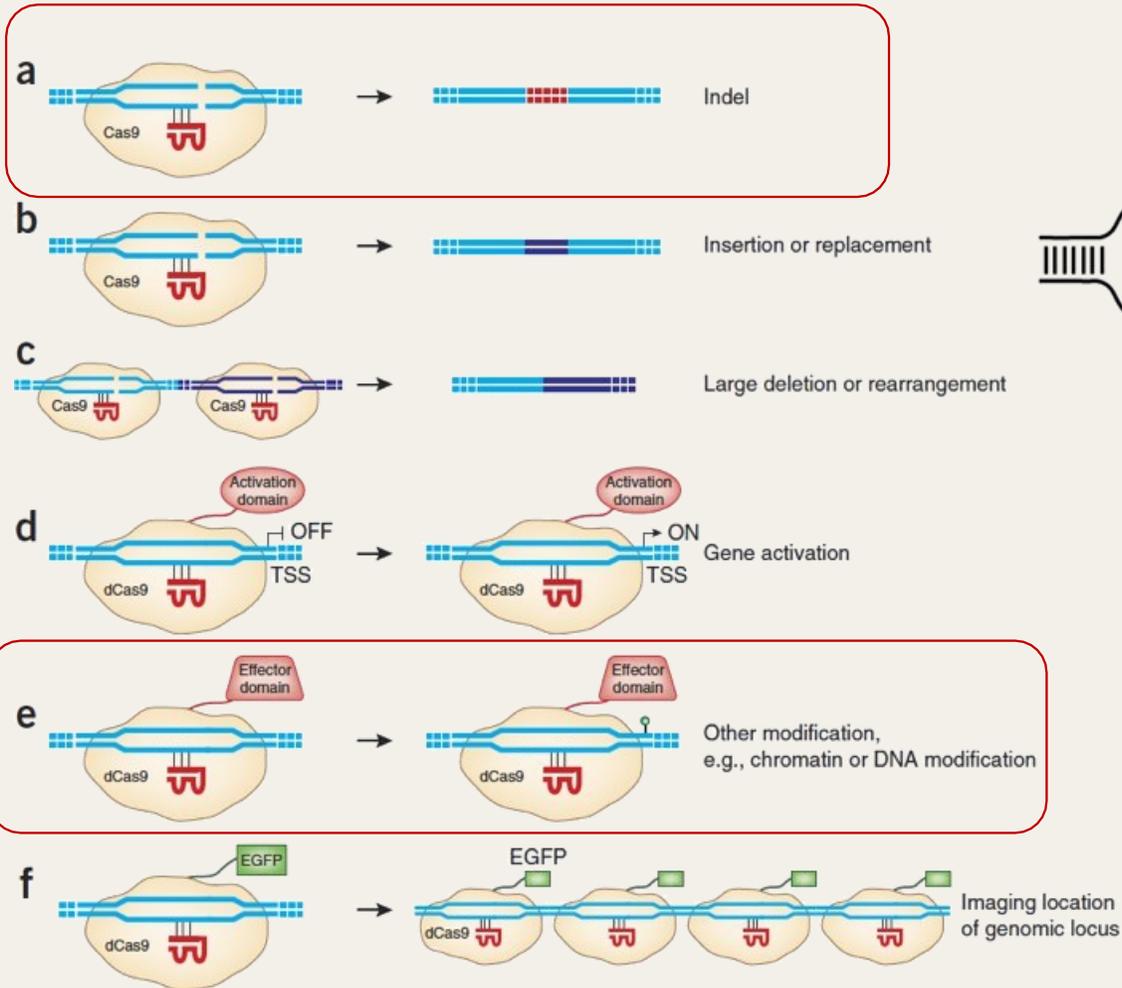


# CRISPR/Cas9 for targeted genome editing

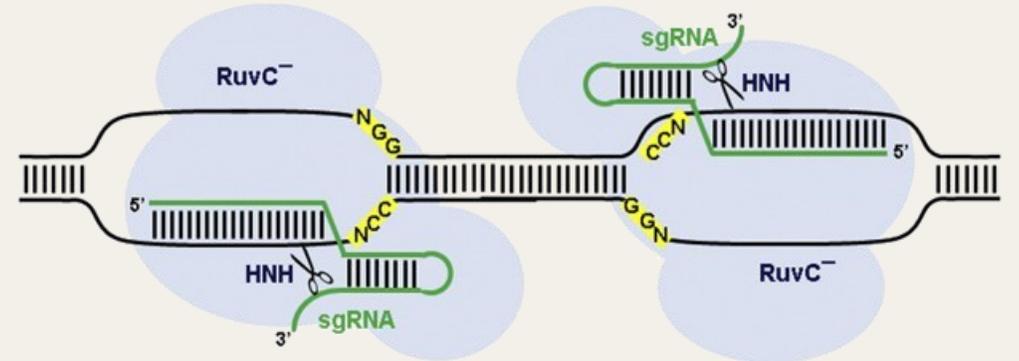


Bortesi & Fisher, 2015. Biotechnol. Adv.

# CRISPR/Cas9 for Gene Activation and Repression



Sander & Joung, 2014. Nat. Biotech



Bortesi & Fisher, 2015. Biotechnol. Adv.



# Steps to generate and characterize a mutant in zebrafish

## 1. Choosing a guide



## 2.1 sgRNA production

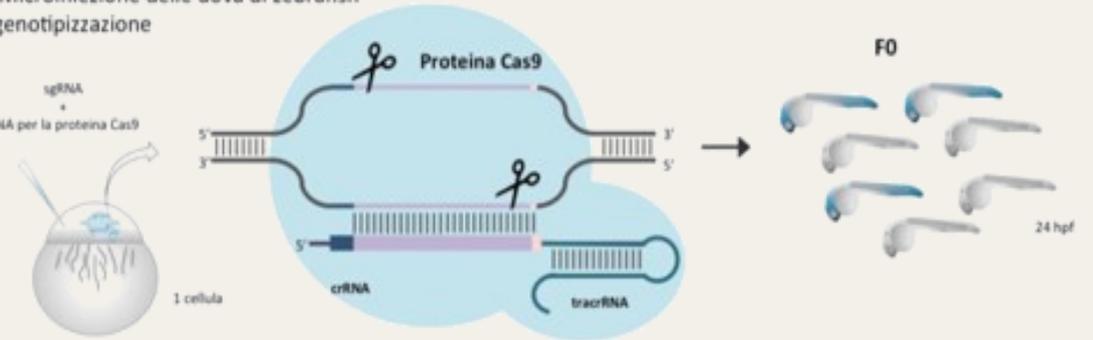


## 2.2 Cas9 RNA production or Cas9 protein



## 3. Injection and downstream analysis

### 4. Microiniezione delle uova di zebrafish e genotipizzazione



### ANALISI

#### • digestione enzimatica



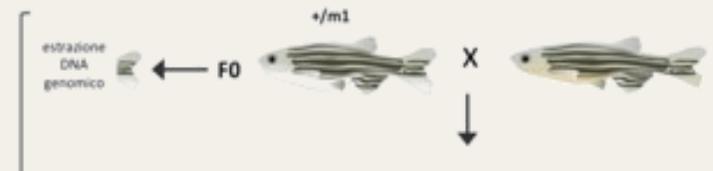
#### • sequenziamento



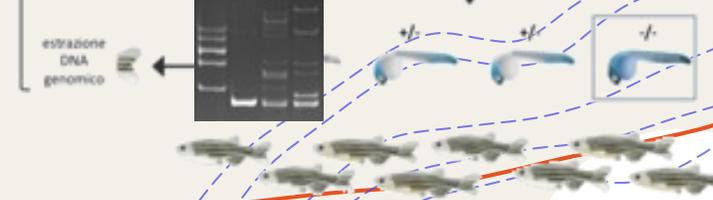
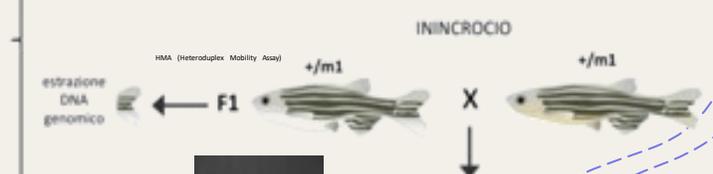
#### • HRMA



### ESOINCROCIO



### ININCROCIO

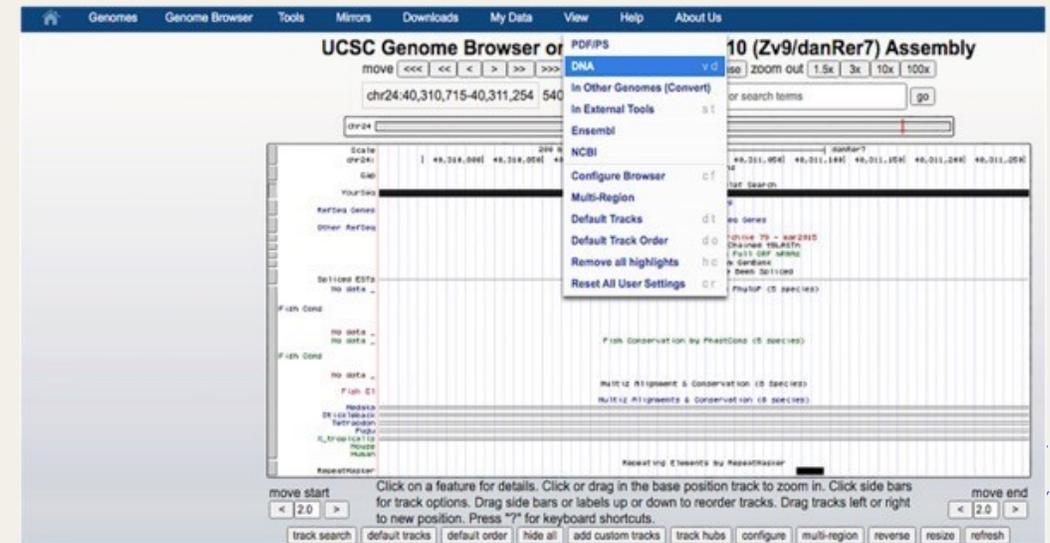


# Selection of Target Sequence - Gene Analysis in UCSC

Choose your target sequence in the zebrafish genome. You can search for a DNA sequence in a genome browser such as UCSC Genome Browser (<https://genome.ucsc.edu/>) (Kent et al., 2002) (Figure 1). For this, in the website:

## 1. Figure 1. Snapshot of the UCSC Genome Browser Website Showing How to Get a DNA Sequence of Interest, Using the Example Shown (Nog2E3)

1. Select: Genomes - zebrafish
2. Search for a specific gene or genomic coordinates (e.g., Nog2E3 genomic coordinates – chr24:40,310,715-40,311,254; danRer7)
3. Zoom on your sequence of interest
4. Select: View → DNA
5. Select: get DNA
6. Copy and save the obtained sequence



# Selection of Target Gene- Gene Analysis in Ensemble

<https://www.ensembl.org>

The screenshot shows the Ensembl genome browser homepage. At the top, there is a navigation bar with the Ensembl logo and links for BLAST/BLAT, VEP, Tools, BioMart, Downloads, Help & Docs, and Blog. A search bar is located in the top right corner. Below the navigation bar, there are four main tool categories: Tools, BioMart, BLAST/BLAT, and Variant Effect Predictor. The search section is prominently displayed, with a search box containing 'Zebrafish' and 'emilin1a'. Below the search box, there are examples of search results. The 'All genomes' section shows a dropdown menu for species selection, with 'Pig breeds' selected. The 'Favourite genomes' section lists Human, Mouse, and Zebrafish. On the right side, there is a section for 'Ensembl Release 108 (Oct 2022)' with a list of changes. Below that, there is a 'More release news' link. At the bottom right, there is a 'Rapid Release' section with a 'Go' button and a link to 'Rapid Release news'.

**Ensembl** BLAST/BLAT | VEP | Tools | BioMart | Downloads | Help & Docs | Blog Login/Register

**Tools** [All tools](#)

**BioMart >** Export custom datasets from Ensembl with this data-mining tool

**BLAST/BLAT >** Search our genomes for your DNA or protein sequence

**Variant Effect Predictor >** Analyse your own variants and predict the functional consequences of known and unknown variants

**Search**

Zebrafish  for

emilin1a

e.g. [BRCA2](#) or [rat 5:62797383-63627669](#) or [rs699](#) or [coronary heart disease](#)

**All genomes**

-- Select a species --

**Pig breeds**  
Pig reference genome and 12 additional breeds

[View full list of all species](#)

**Favourite genomes**

**Human**  
GRCh38.p13  
[Still using GRCh37?](#)

**Mouse**  
GRCm39

**Zebrafish**  
GRCz11

Ensembl is a genome browser for vertebrate genomes that supports research in comparative genomics, evolution, sequence variation and transcriptional regulation. Ensembl annotate genes, computes multiple alignments, predicts regulatory function and collects disease data. Ensembl tools include BLAST, BLAT, BioMart and the Variant Effect Predictor (VEP) for all supported species.

**Ensembl Release 108 (Oct 2022)**

- Changes in the default tracks in the Location view: cDNAs EST cluster (UniGene) CCDS to be removed when MANE Select is available
- RNASeq tracks including data from GeneSWITCH consortium for chicken
- Variation data for crab-eating macaque, pike-perch, prairie vole, Japanese quail and collared flycatcher
- Retirement of postGAP tool

[More release news](#) on our blog

**Ensembl Rapid Release**

**New assemblies with gene and protein annotation every two weeks.**

Note: species that already exist on this site will continue to be updated with the full range of annotations.

The Ensembl Rapid Release website provides annotation for recently produced, publicly available vertebrate and non-vertebrate genomes from biodiversity initiatives such as Darwin Tree of Life, the Vertebrate Genomes Project and the Earth BioGenome Project.

[Rapid Release news](#) on our blog

# Selection of Target Gene- Gene Analysis in Ensemble

The screenshot shows the Ensembl genome browser interface. At the top, the Ensembl logo is on the left, and navigation links for BLAST/BLAT, VEP, Tools, BioMart, Downloads, Help & Docs, and Blog are in the center. On the right, there is a search bar with the text "Search all species..." and a "Login/Register" link.

The main content area is titled "New Search". On the left side, there are several filter panels:

- Current selection:** A dropdown menu showing "< all Species" and "Only searching Zebrafish".
- Restrict category to:** A list with "Gene" (1), "Transcript" (2), and "GeneTree" (1).
- Per page:** Radio buttons for "10", "25", "50", and "100".
- Layout:** Radio buttons for "Standard" and "Table".
- Tip:** A message stating: "You can choose which results appear near the top of your search by updating your favourite species."

The search results are displayed in a light blue box. At the top of this box, it says "Only searching Zebrafish" and "emilin1a". Below this, it indicates "4 results match emilin1a when restricted to species: Zebrafish".

The results list includes:

- emilin1a (Zebrafish Gene):** ENSDARG00000024537 20:34845672-34865031:1. Description: Elastin microfibril interfacier 1a [Source:ZFIN;Acc:ZDB-GENE-041001-191].
- emilin1a-201 (Zebrafish Transcript):** ENSDART00000035612 20:34845997-34864519:1. Description: Elastin microfibril interfacier 1a [Source:ZFIN;Acc:ZDB-GENE-041001-191].
- emilin1a-202 (Zebrafish Transcript):** ENSDART00000128895 20:34845672-34865031:1. Description: Elastin microfibril interfacier 1a [Source:ZFIN;Acc:ZDB-GENE-041001-191].
- ENSGT01030000234633 (Zebrafish GeneTree):** ENSGT01030000234633. Description: Gene emilin1a (ENSDARG00000024537) is a member of GeneTree ENSGT01030000234633.

At the bottom of the results box, there are navigation arrows: "<< < 1 > >>".

# Selection of Target Gene- Gene Analysis in Ensembl

**Ensembl** BLAST/BLAT | VEP | Tools | BioMart | Downloads | Help & Docs | Blog Login/Register

**Zebrafish (GRCz11)** Search all species...

Location: 20:34,845,672-34,865,031 **Gene: emilin1a** **Transcript: emilin1a-202**

### Transcript-based displays

- Summary
- Sequence
  - Exons
  - cDNA
  - Protein
- Protein Information
  - Protein summary
  - Domains & features
  - Variants
  - PDB 3D protein model
  - AlphaFold predicted model
- Genetic Variation
  - Variant table
  - Variant image
  - Population comparison
  - Comparison image
- External References
  - General identifiers
  - Oligo probes
- Supporting evidence
- ID History
  - Transcript history
  - Protein history

**Transcript: ENSDART00000128895.4** emilin1a-202

**Description** elastin microfibril interfacier 1a [Source:ZFIN;Acc:[ZDB-GENE-041001-191](#)]

**Gene Synonyms** im:7159005, si:dkey-20n10.2

**Location** [Chromosome 20: 34,845,672-34,865,031](#) forward strand.

**About this transcript** This transcript has **8 exons**, is annotated with **24 domains and features**, is associated with **513 variant alleles** and maps to **169 oligo probes**.

**Gene** This transcript is a product of gene [ENSDARG00000024537.9](#) [Hide transcript table](#)

Show/hide columns (1 hidden) <span style="float: right;">Filter</span>						
Transcript ID	Name	bp	Protein	Biotype	UniProt Match	Flags
<a href="#">ENSDART00000128895.4</a>	emilin1a-202	3882	<a href="#">1014aa</a>	Protein coding	<a href="#">F1QC17</a>	Ensembl Canonical APPRIS P5
<a href="#">ENSDART00000035612.7</a>	emilin1a-201	3027	<a href="#">1008aa</a>	Protein coding	<a href="#">Q5TZ63</a>	APPRIS ALT2

### Summary

[Export Image](#)

emilin1a-202 - ENSDART00000128895.4 > protein coding

**Statistics** Exons: 8, Coding exons: 8, Transcript length: 3,882 bps, Translation length: 1,014 residues

**Version** ENSDART00000128895.4

**Type** Protein coding

**Annotation Method** Manual annotation (determined on a case-by-case basis) from the Havana project.

Ensembl release 108 - Oct 2022 © [EMBL-EBI](#) [Permanent link](#) - [View in archive site](#)



# Selection of Target Gene- Gene Analysis in Ensembl

**Ensembl** BLAST/BLAT | VEP | Tools | BioMart | Downloads | Help & Docs | Blog

**Zebrafish (GRCz11)**

Location: 20:34,845,672-34,865,031 Gene: emilin1a Transcript: emilin1a-202

**Transcript-based displays**

- Summary
- Sequence
  - Exons
  - cDNA
  - Protein
- Protein Information
  - Protein summary
  - Domains & features**
  - Variants
  - PDB 3D protein model
  - AlphaFold predicted model
- Genetic Variation
  - Variant table
  - Variant image
  - Population comparison
  - Comparison image
- External References
  - General identifiers
  - Oligo probes
  - Supporting evidence
- ID History
  - Transcript history
  - Protein history

**Transcript: ENSDART00000128895.4** emilin1a-202

**Description** elastin microfibril interfacer 1a [Source:ZFIN;Acc:ZDB-GENE-041001-191]

**Gene Synonyms** im:7159005, si:dkey-20n10.2

**Location** Chromosome 20: 34,845,672-34,865,031 forward strand.

**About this transcript** This transcript has **8 exons**, is annotated with **24 domains and features**, is associated with **513 variant alleles** and maps to **169 oligo probes**.

**Gene** This transcript is a product of gene [ENSDARG00000024537.9](#) [Hide transcript table](#)

Show/hide columns (1 hidden) Filter

Transcript ID	Name	bp	Protein	Biotype	UniProt Match	Flags
<a href="#">ENSDART00000128895.4</a>	emilin1a-202	3882	<a href="#">1014aa</a>	Protein coding	<a href="#">F1QC17</a>	Ensembl Canonical APPRIS P5
<a href="#">ENSDART00000035612.7</a>	emilin1a-201	3027	<a href="#">1008aa</a>	Protein coding	<a href="#">Q5T263</a>	APPRIS ALT2

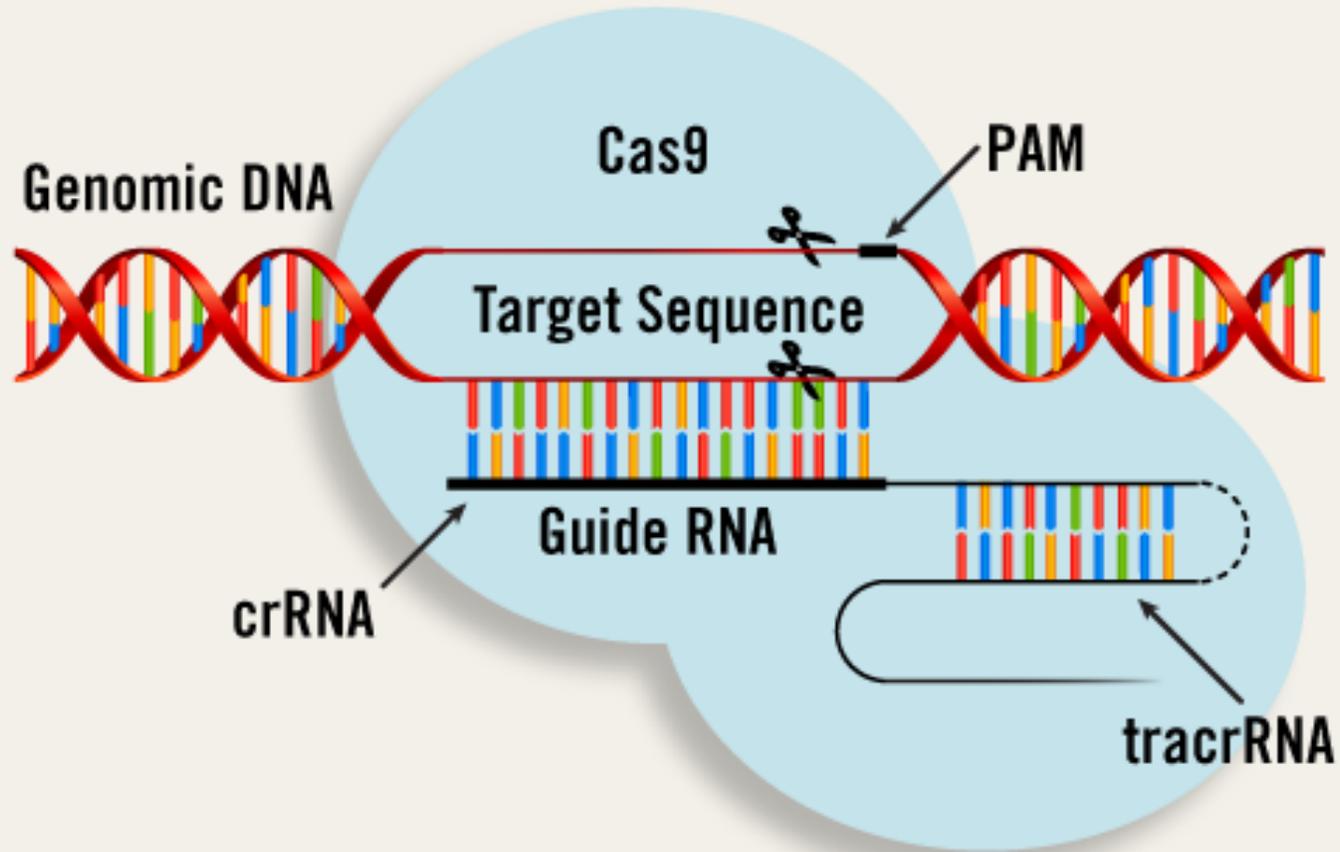
**Domains & features**

**Domains**

Show/hide columns Filter

Domain source	Start	End	Description	Accession	InterPro
PANTHER	2	1009	EMILIN ELASTIN MICROFIBRIL INTERFACE-LOCATED PROTEIN	PTHR15427	-
PANTHER	2	1009	EMILIN-1	PTHR15427.SF1	-
Smart	864	1009	C1Q_2	<a href="#">SM00110</a>	<a href="#">IPR001073</a> [Display all genes with this domain]
Prosite_profiles	866	1012	C1Q	<a href="#">PS50871</a>	<a href="#">IPR001073</a> [Display all genes with this domain]
Pfam	873	1006	C1q	<a href="#">PF00386</a>	<a href="#">IPR001073</a> [Display all genes with this domain]
Pfam	815	865	Collagen	<a href="#">PF01391</a>	<a href="#">IPR008160</a> [Display all genes with this domain]
Prosite_profiles	53	128	EMI	<a href="#">PS51041</a>	<a href="#">IPR011489</a> [Display all genes with this domain]

# Selection of crRNA



# Selection of crRNA

IDT's contact information has changed! Visit our [Contact Us](#) page for updated information.



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OLIGO DESIGN & HANDLING	CRISPR GENOME EDITING	qPCR ASSAY DESIGN	GENE REGULATION AND RNAI
<ul style="list-style-type: none"><li>OligoAnalyzer™ Tool</li><li>UNAFold Tool</li><li>Resuspension Calculator</li><li>Dilution Calculator</li></ul>	<ul style="list-style-type: none"><li>Alt-R Predesigned Cas9 crRNA Selection Tool</li><li>Alt-R Custom Cas9 crRNA Design Tool</li><li>CRISPR-Cas9 Design Checker</li><li>Alt-R HDR Design Tool</li><li>rhAmpSeq Design Tool</li><li>rhAmpSeq CRISPR Analysis Tool</li></ul>	<ul style="list-style-type: none"><li>PrimerQuest™ Tool</li><li>Realtime PCR Tool</li><li>Predesigned qPCR Assays</li><li>PrimeTime Multiplex Dye Selection</li></ul>	<ul style="list-style-type: none"><li>Predesigned DsiRNA Selection Tool</li><li>RNAi Design Tool</li></ul>
GENOTYPING	GENES & GENE FRAGMENTS	NGS TOOLS	
<ul style="list-style-type: none"><li>rhAmp Genotyping Design Tool</li></ul>	<ul style="list-style-type: none"><li>Codon Optimization Tool</li><li>gBlocks Gene Fragments Entry Tool</li><li>Genes &amp; Gene Fragments order status</li></ul>	<ul style="list-style-type: none"><li>Library Concentration Conversion Calculator</li><li>Target Capture Probe Design &amp; Ordering Tool</li><li>Custom Adapter Configurator Tool</li><li>NGS Solutions Builder Tool</li></ul>	

◀ Browse all tools ▶

Which factors do you consider when determining the best NGS approach for your cancer research?

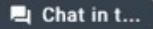
[FIND OUT](#)

○ ○ ●

 COVID-19 solutions

 DNA & RNA

 CRISPR genome edit

 Chat in t...

<https://eu.idtdna.com/>

# Selection of crRNA

## Custom Alt-R® CRISPR-Cas9 guide RNA

Generate CRISPR-Cas9 guide RNAs (gRNAs, such as crRNA and sgRNA) targeting any sequence from any species. Currently, analysis of off-target effects against human, mouse, rat, zebrafish, or *C. elegans* genes are available. For HDR experiment designs, please see the following [HDR design tool](#).

Search for predesigned gRNA

**Design custom gRNA**

CRISPR-Cas9 gRNA checker

Species

Danio rerio

Input format

FASTA Sequence



DESIGN

CLEAR AND RESET

Paste/Type input

Upload file

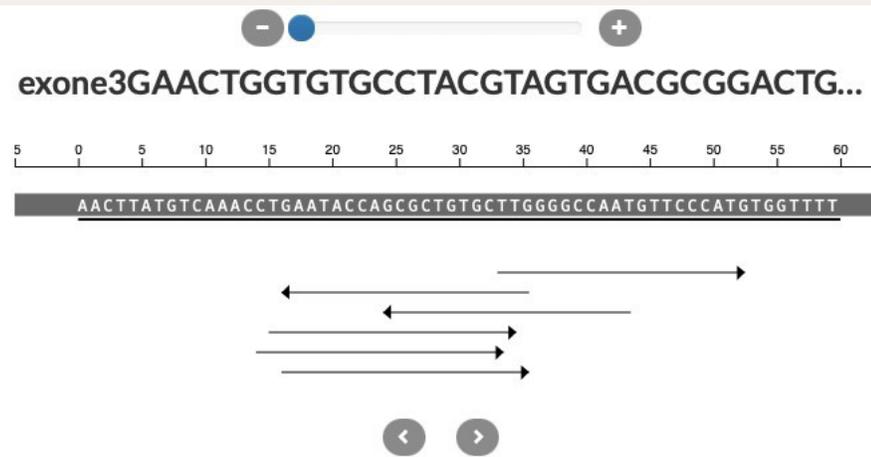
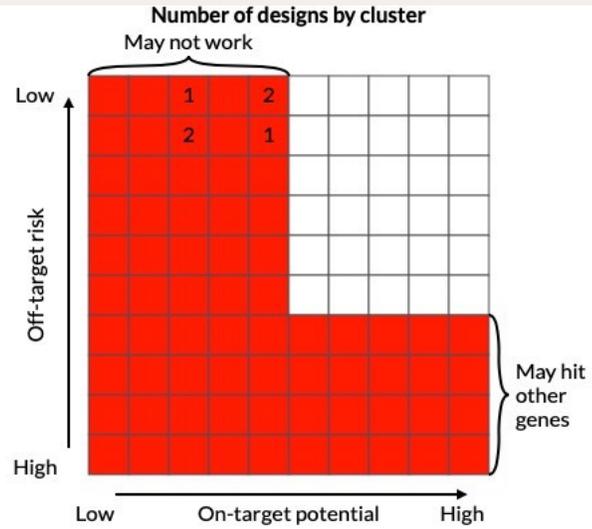
Enter up to 10 FASTA Sequences.

Please enter sequences in standard FASTA formatting.

No more than 1000 bases accepted.

```
> exon3GAACTGGTGTGCCTACGTAGTGACGCGGACTGTAAGCTGTGTTATGGAAGATGGAGTGGA  
AACTTATGTCAAACCTGAATACCAGCGCTGTGCTTGGGGCCAATGTTCCCATGTGGTTTT
```

# Selection of crRNA



Your search returned 6 results. [Clear results](#)

- Result sets**
- Search results >
  - My design set (0 items) >
  - Design tool help ⓘ

Select all ACTIONS SORT ⓘ QUICK ORDER ⓘ

Design ID: CD.Cas9.QSJH8104.AA exon3GAACTGGTGTGCCTACGTAGTGACGGGACTGTAAGCTGTG... Custom Alt-R CRISPR-Cas9 gRNA

Position	Strand	Sequence	PAM ⓘ	On-target score ⓘ	Off-target score ⓘ	crRNA, 2 nmol tube ⇅
33	+	TTGGGGCCAATGTTCCCATG	TGG 49		92	127,59 € EUR

Show off-target details + | Show related products

+ ADD TO DESIGN SET

# Selection of crRNA

CNB-CSIC | BioinfoGP | tools

**Breaking-Cas**

[object HTMLParagraphElement] Genomes: [object HTMLInputElement]

**Breaking-Cas**

**Genome Databases Available:**

Please select one of the available releases of ENSEMBL or ENSEMBLGENOMES. It is always recommended to use the most recent version of any database although we will keep active the previous one in order to support already started projects.

**SARS-CoV-2 genome available [HERE!](#)**

<b>Ensembl (108)</b> Ensembl collection of vertebrate genomes. Release 108 (October 2022) ( <b>316 genomes</b> )	<b>EnsemblGenomes (55)</b> Ensembl collection of protists, fungi, plants and invertebrate metazoa genomes. Release 55 (October 2022) ( <b>2071 genomes</b> )
<b>Ensembl (107)</b> Ensembl collection of vertebrate genomes. Release 107 (July 2022) ( <b>316 genomes</b> )	<b>EnsemblGenomes (54)</b> Ensembl collection of protists, fungi, plants and invertebrate metazoa genomes. Release 54 (July 2022) ( <b>2044 genomes</b> )

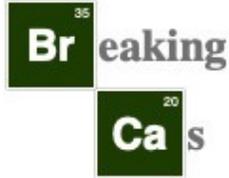
**Latest news** Contact: [bioinfo@cnb.csic.es](mailto:bioinfo@cnb.csic.es)

- 2020-02-04: Breaking-Cas server maintenance finished.
- 2019-01-23: Breaking-Cas server temporarily down for maintenance.
- 2019-06-21: Breaking-Cas server maintenance finished.

<https://bioinfo@cnb.csic.es/tools/breakingcas/>

# Selection of crRNA

CNB-CSIC | BioinfoGP | tools



## Breaking-Cas

Ensembl collection of vertebrate genomes. Release 108 (October 2022) Genomes: 316

Please cite:

"Juan C. Oliveros, Mònica Franch, Daniel Tabas-Madrid, David San-León, Lluís Montoliu, Pilar Cubas and Florencio Pazos (2016). Breaking-Cas—interactive design of guide RNAs for CRISPR-Cas experiments for ENSEMBL genomes. *Nucleic Acids Research* (2016) doi: 10.1093/nar/gkw407. <https://bioinfoGP.cnb.csic.es/tools/breakingcas>"

[Tutorial](#)

**1** Choose organism: ([alphabetic list](#)) Danio rerio, leopard danio, zebra danio, : *Write 3 letters or more and select it.*

**2** Paste one or several query DNA sequences in FASTA format (up to 20,000 nucleotides in total):

```
> esone3
GTATCGGACTTACAGAAAACCACGGTATAAGGTTGCCTATAAAGTGGTATCAGAGATGGA
ATGGAAGTGCTGTCATGGTACTCAGGTGATGACTGCAGTGACGGTTCCTCTGCCATACA
TGACAGCAGGGCAAGACCTACTGGTGAAGAAG
```

Or upload FASTA file (DNA):  nessun file selezionato

**3** Select nuclease settings:

Or set your own parameters:

PAM sequence:

PAM position:  5'  3'

Guide length:

Mismatches:

*Use predefined settings for Cas9 or Cpf1, or set custom parameters for other nucleases. If necessary, write a different PAM sequence (in IUPAC notation). For Cas9, positional weights based on Hsu et al. (2013) are used by default. See [tutorial pages](#) for details on off-targets score's calculation.*

Position-dependent weights

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	(PAM)	
5'-	0	0	0.014	0	0	0.395	0.317	0	0.389	0.079	0.445	0.508	0.519	0.651	0.732	0.828	0.615	0.804	0.685	0.581	NGG	
																						-3'

Confirmation email (optional):

*To receive a message as soon the job finishes. Write it carefully (it will not be checked).*

# Selection of crRNA

## esone 3

*Danio rerio* (leopard danio, zebra danio, zebra fish, zebrafish)

### Analysis Settings:

-Database='Ensembl\_108'  
 -Genome(FASTA)='Danio\_rerio.GRCz11.dna.primary\_assembly.fa'  
 -Genome(GTF)='Danio\_rerio.GRCz11.108.gtf'  
 -PAM='NGG'  
 -PAM Position=3'  
 -Oligo Size=20  
 -Max. mismatches=4  
 -Positional Weights (1 to 20)=0|0|0.014|0|0|0.395|0.317|0|0.389|0.079|0.445|0.508|0.613|0.851|0.732|0.828|0.615|0.804|0.685|0.583



Filter boxes [?](#) [Export Oligos](#) (text-tabulated format)

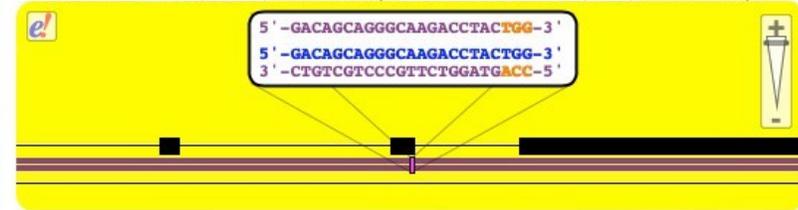
START#	END#	STRAND#	OLIGO#	ONTARGETS#	OFFTARGETS#	GENES#	SCORE↓
23	42	-	TTATAGGCAACCTTATACCGTGG	1	12	12	99.1
65	84	+	AAGTGCTGTCATGGTTACTCAGG	1	48	41	93.9
10	29	+	TTACAGAAAACCACGGTATAAGG	1	62	40	93.4
39	58	-	CATCTCTGATAACCACTTATAGG	1	43	32	92.5
111	130	-	CCTGCTGTCATGTATGGCAGAGG	1	47	38	92.4
3	22	+	ATCGGACTTACAGAAAACCACGG	1	17	14	91.1
109	128	+	CTCTGCCATACATGACAGCAGGG	1	45	36	90.6
25	44	+	GTATAAGGTTGCCTATAAAGTGG	1	53	44	89.5
108	127	+	CCTCTGCCATACATGACAGCAGG	1	47	34	89.4
42	61	+	AAGTGGTATCAGAGATGGAATGG	1	70	45	88.3
117	136	-	TCTTGCCCTGCTGTCATGTATGG	1	39	30	87
83	102	+	TCAGGTGATGACTGCAGTGACGG	1	57	47	86.8
122	141	+	GACAGCAGGGCAAGACCTACTGG	1	59	43	83.7
56	75	+	ATGGAATGGAAGTGCTGTCAATGG	1	48	35	82.8
37	56	+	CTATAAAGTGGTATCAGAGATGG	1	72	59	80.3

### GACAGCAGGGCAAGACCTAC(TGG)

[Export Targets](#) (text-tabulated format)

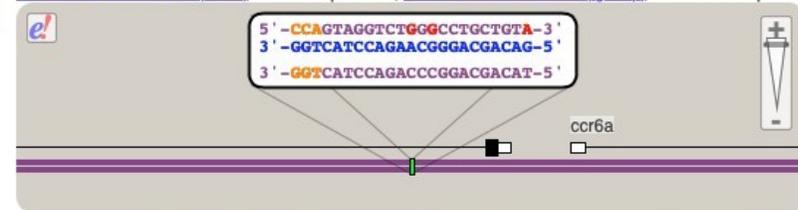
100 20:34858728-34858750(+)

ENSDARG00000024537 (emilin1a): 23 nts overlap; ENSDARG00000030106 (stmn4): 23 nts overlap



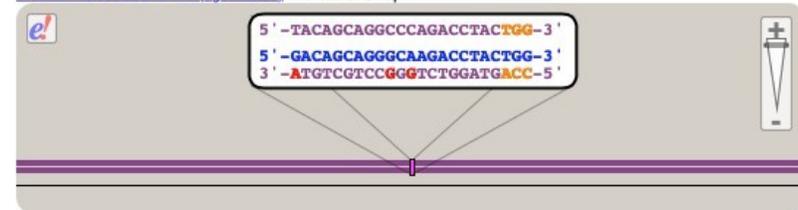
1.3 20:30609528-30609550(-)

ENSDARG00000087474 (ccr6a): 997 nts upstream; ENSDARG0000003058 (fgfr1top): 23 nts overlap



1.3 8:4659140-4659162(+)

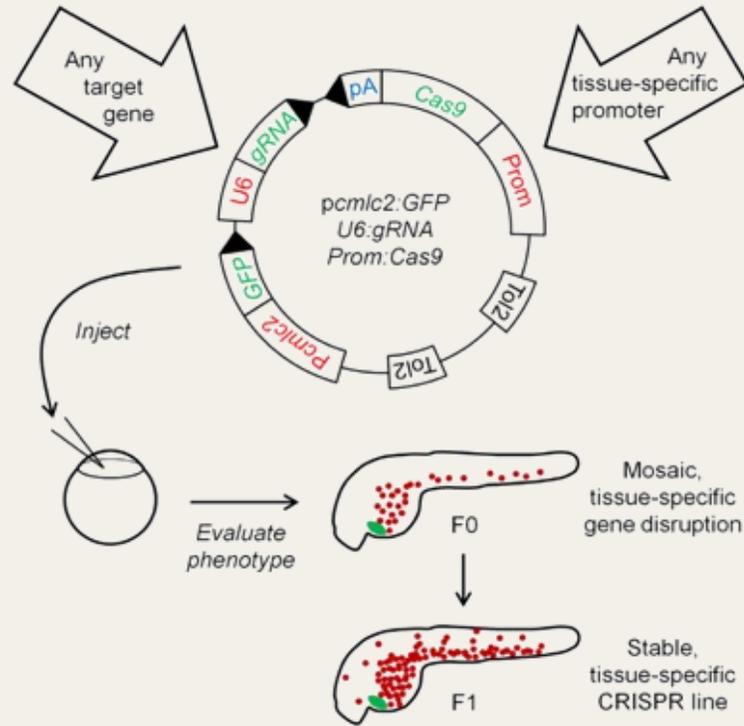
ENSDARG00000017140 (zgc:63587): 23 nts overlap



# Top tips for success with CRISPR-Cas9

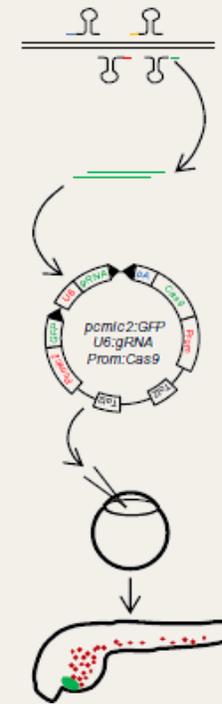
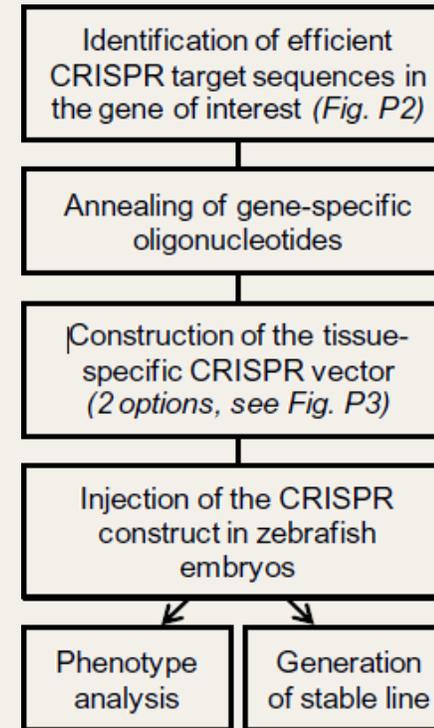
- *Identify a target region*
  1. The target region should be 5' to domains that you suspect may impart functionality.
  2. The target region should be in a constitutive exon. Avoid exons that are sometimes excluded from transcripts unless your project focuses on function of a particular splice form.
  3. Preferred exons have few or no SNPs shown in Ensembl's "Variation Features" track.
  4. Exons longer than 150 bp are likely to contain ideal CRISPR sites.
- *Finding Guides* **IDT, CHOPCHOP, CRISPRSCAN, Breaking Cas9 and Synthego**
- *Choosing a guide* How do you choose which one to use?
  1. Never choose a guide that has any significant **off-target** sites (perfect match for the 8-12 bases closest to the PAM) in a coding of the genome
  2. Eliminate candidate CRISPR target sites with an obvious stretches of self-complementary sequence that might cause hairpin formation
  3. **GC content** should be between ~ 30-80%, the higher the better (but not too high!).
  4. Consider the method to assay mutagenesis and genotype (restriction enzyme, HRMA, agarose gel 4%)

# Conditional KO in zebrafish



## Advantage

- Flexibility of the tissue-specific CRISPR vector system.
- Avoid the embryonic lethality associated with the global knockout of certain genes.
- Insertion marker in the vector (GFP) facilitates sorting of transgenic animals.
- Greater level of biallelic inactivation by the vector.
- Faster than the time required to generate KO or conditional knockout mice (CRE/Lox system).



## Disadvantage

The efficiency of gene inactivation is likely to be lower than in a straight knockout or a conditional knockout such as the Cre/Lox system in the mouse.

# Knock-in

> *Nat Biotechnol.* 2016 Mar;34(3):339–44. doi: 10.1038/nbt.3481. Epub 2016 Jan 20.

## Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA

Christopher D Richardson<sup>1 2</sup>, Graham J Ray<sup>1 2</sup>, Mark A DeWitt<sup>1 2</sup>, Gemma L Curie<sup>1 2</sup>, Jacob E Corn<sup>1 2</sup>

Affiliations + expand

PMID: 26789497 DOI: [10.1038/nbt.3481](https://doi.org/10.1038/nbt.3481)



# Indel detection following CRISPR/Cas9 mutagenesis by PCR: Oligo design

**Primer3web** version 4.1.0 - Pick primers from a DNA sequence. [disclaimer](#) [code](#)  
[cautions](#)

Select the **Task** for primer selection

**Template masking before primer design (available species)**

Select species Example: Mus musculus   
Primer failure rate cutoff

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINES, etc.) or use a [Mispriming Library \(repeat library\)](#)

```
CTGTGTTATGGAAAGATGGAGTggaacctatgtcaaacctgaatccagcgcgtgctggggccaatgittccatggtgtttgtaagttatcgttaagcactgccaaat  
tacatttcattaaactgttgaccacctgttattatgacagtGTCAAGCTGGAAAGGCTGG
```

Pick left primer, or use left primer below  Pick hybridization probe (internal oligo), or use oligo below  Pick right primer, or use right primer below (5' to 3' on opposite strand)

[Pick Primers](#) [Download Settings](#) [Reset Form](#)

**Sequence Id**  A string to identify your output.

**Targets**  E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the [source sequence](#) with [ and ]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

**Overlap Junction List**  E.g. 27 requires one primer to overlap the junction between positions 27 and 28. Or mark the [source sequence](#) with -: e.g. ...ATCTAC-TGTCAT.. means that primers must overlap the junction between the C and T.

**Excluded Regions**  E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the [source sequence](#) with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

**Pair OK Region List**  See manual for help.

**Included Region**  E.g. 20,400: only pick primers in the 400 base region starting at position 20. Or use { and } in the [source sequence](#) to mark the beginning and end of the included region: e.g. in ATC{TTC...TCT}AT the included region is TTC...TCT.

**Start Codon Position**

**Internal Oligo**

**Excluded Region**

**Force Left Primer Start**  **Force Right Primer Start**   
**Force Left Primer End**  **Force Right Primer End**

**Sequence Quality**

**Min Sequence Quality**  **Min End Sequence Quality**  **Sequence Quality Range Min**  **Sequence Quality Range Max**

[Pick Primers](#) [Download Settings](#) [Reset Form](#)



# Guidelines to design successful primers for PCR

Before designing a gRNA, ensure that you can design good primers flanking the CRISPR target site for HRMA and sequencing.

## **Primers for agarose gel 4%**

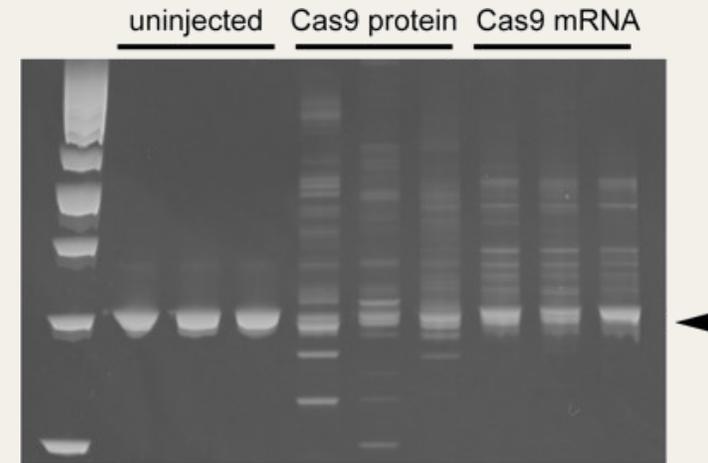
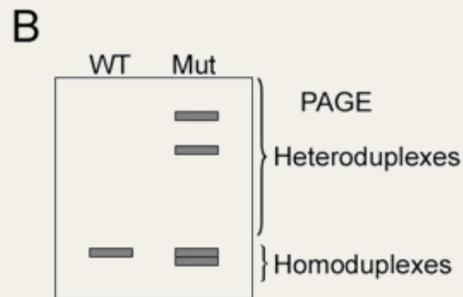
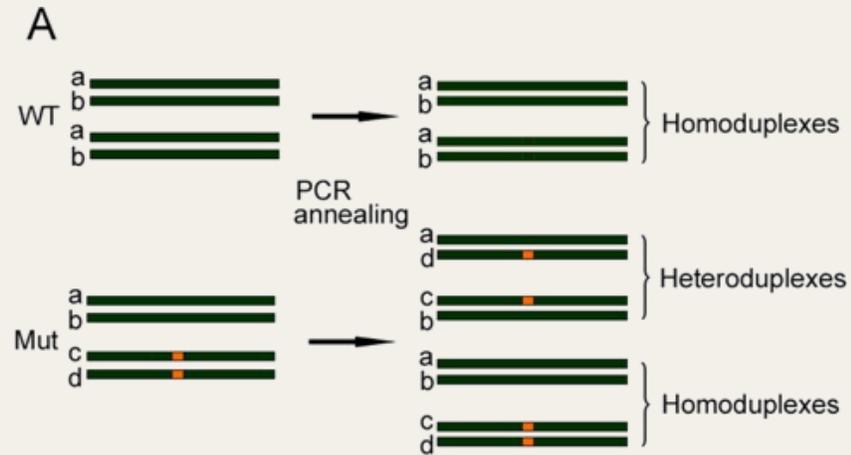
- Check for predicted SNPs in the target region using the “variation features” track on Ensembl
- The product size should be 100-160 bp; with these short PCR products, small sequence changes can produce large changes in melting temperature
- Ensure that at least 20 bp are present between each primer and the CRISPR target site, so longer insertions or deletions (indels) can be detected.

## **Primers for sequencing reactions:**

- Ensure that at least 50 bp are present between each sequencing primer and the CRISPR target site.
- Ensure that at least one of the primers for sequencing is found in the same exon as the CRISPR target site.
- Intronic sequence and untranslated regions (UTRs) sometimes contain silent indels, which can complicate lesion identification.

# PAGE-based genotyping protocol for identification of CRISPR/Cas9-mediated indel mutations

## F0 adults screening (mutation efficiency)



Cas9 protein-gRNA

spns2

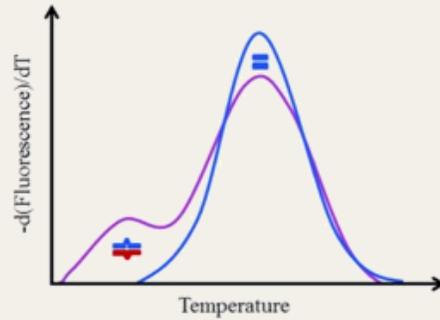
indel mutations rate : 15/15 (100%)

TCTCTCCGCAGCGGTCTGGGCTACATCCTGGGA	WT	x0
TCTCTCCGCAG---TCTGGGCTACATCCTGGGA	-3	x5
TCTCTCCGC-----TACATCCTGGGA	-12	x4
TCTCTCCGCAG-----GA	-20	x2
TCTCTCCG-----GTCTGGGCTACATCCTGGGA	-5	x1
TCTCT-----GGGCTACATCCTGGGA	-12	x1
TCTCTCCGCAGAGAGAGAG---CTACATCCTGGGA	-9/+7	x1
TCTCTCCGCAAAACATGTTATAGCTCCGGTCTG	-1/+16	x1

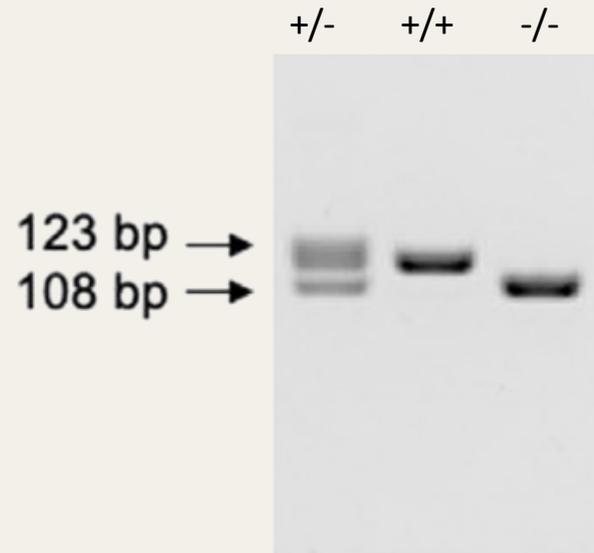
# Identification of founders

## F1 embryos screening (founders identification)

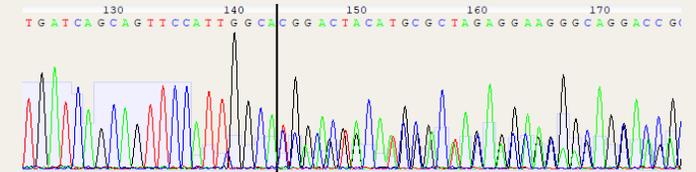
### HRMA (High Resolution Melting Analysis (HRMA))



### PCR on 4% agarose gel

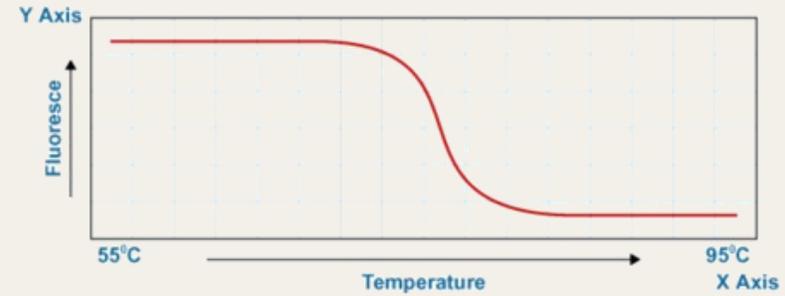
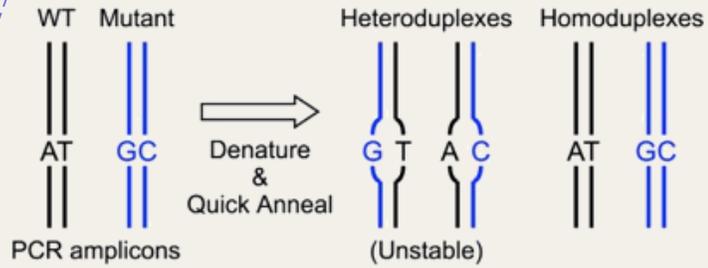


### Sequencing

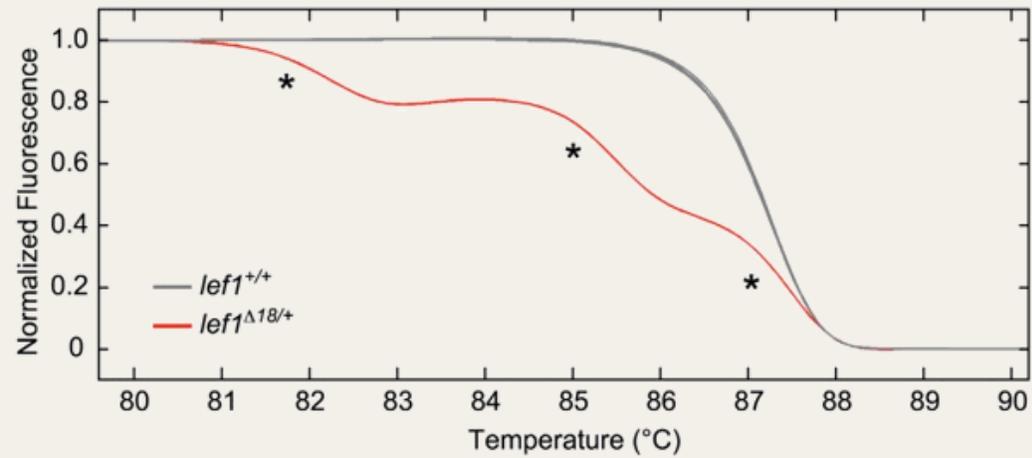


# Identification of founders: High Resolution Melt Analysis (HRMA)

**A**



**B**



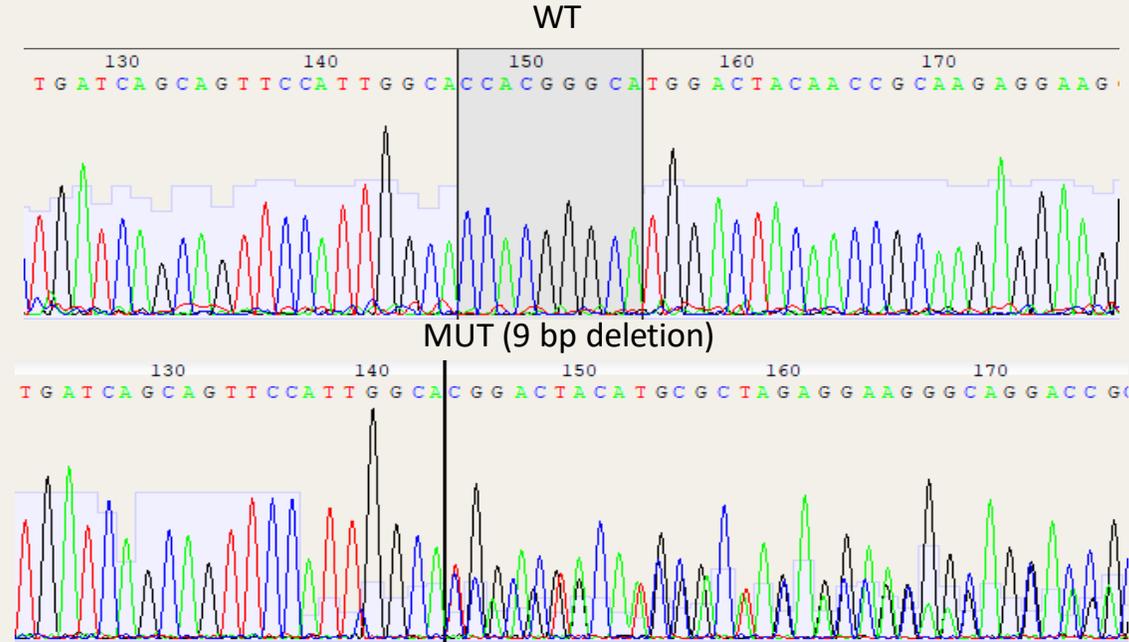
# Identification of mutations

## F1 embryos sequencing (mutations identification)

Sequencing:

BMR genomics → <https://www.bmr-genomics.it>

Eurofins → <https://eurofinsgenomics.eu>



Detect actual mutations by sequencing in the mutants identified by HMA/PCR

- Poly Peak Parser
- Ice Analysis (Synthego)
- TIDE (<https://tide.nki.nl>)

```
652 ATTGAATACGGGGACATGCAGCTGATCTGTGAGGCCTATCATCTGATGAAGGACGTGCTTTGTATGAAC 660
218 -I--E--Y--G--D--M--Q--L--I--C--E--A--Y--H--L--M--K--D--V--L--C--M--N--240
652 ATTGAATACGGGGACATGCAGC-----TGAGGCCTATCATCTGATGAAGGACGTGCTTTGTATGAAC 660
218 -I--E--Y--G--D--M--Q-----L--R--P--I--I--*--*--230
```

# To Sum Up

## *1. Identify the target region*



<https://genome.ucsc.edu/>

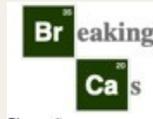


<https://www.ensembl.org/>

## *2. crRNA Design*



<https://eu.idtdna.com/>



<https://bioinfogp.cnb.csic.es/tools/breakingcas/>

CRISPRscan

<http://www.crisprscan.org>

## *3. Indel detection following CRISPR/Cas9 mutagenesis by PCR*

Primer3web

<https://primer3.ut.ee/>



<https://tide.nki.nl>