Transgenic zebrafish for the study of physiology and pathology

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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

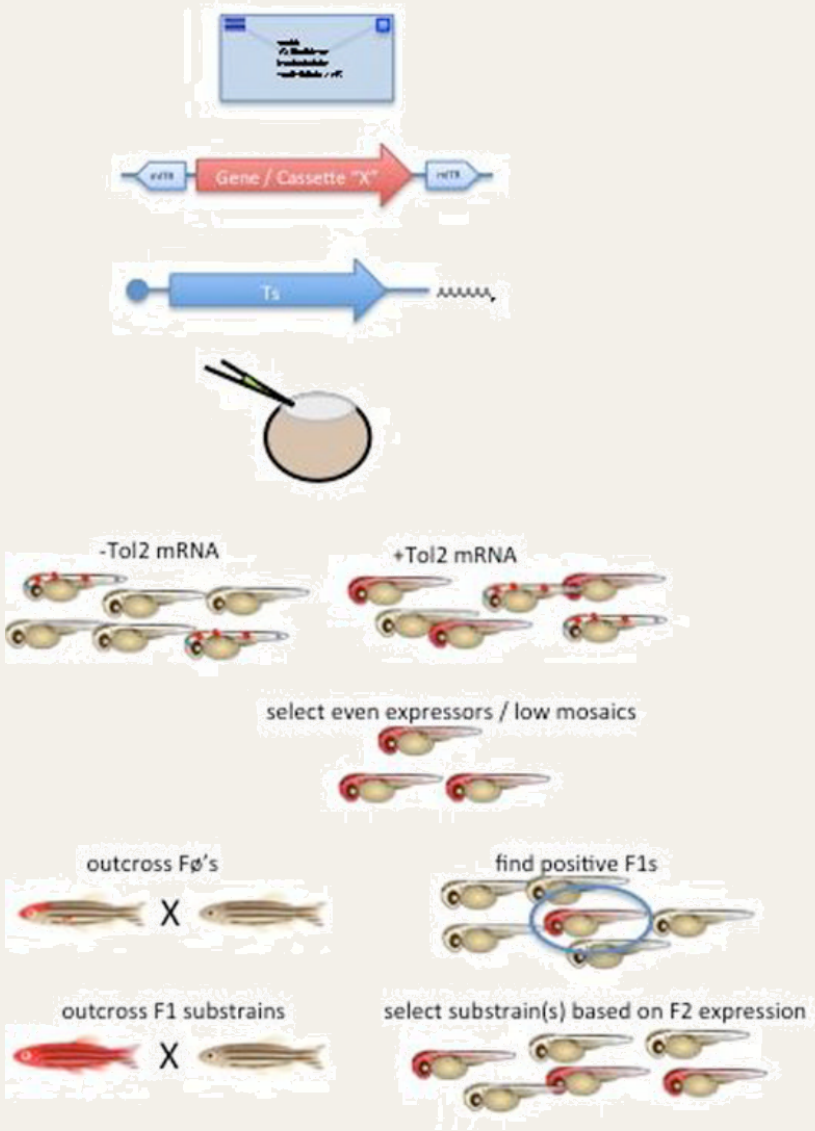
- Transposon-based system → **Tol2 transposon**
- Advantages:

1. high **germline transmission** rate → about 70%
2. **single-copy** (non-concatemerized) 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 Fø embryos to raise.

VI. Out cross Fø to non-transgenic partner, select positive F1 embryos.

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

Clark et al., 2011

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 lamba 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)

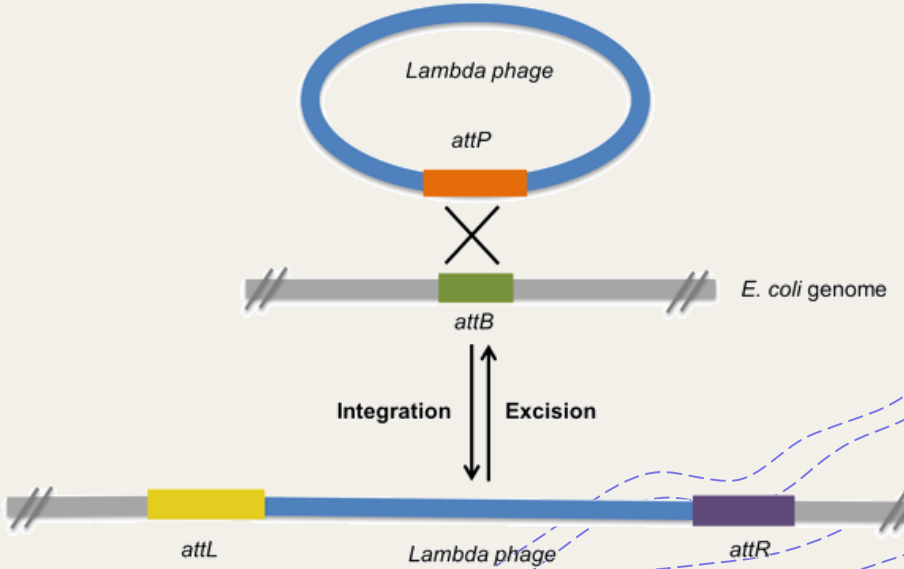
Transgenesis method: Multisite Gateway-based Tol2 strategy
Att sites and recombination

- Att-site: site-specific attachment sites:

1. **attB →** originally present on E. Coli chromosome
2. **attP→** originally present on lambda chromosome

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

- 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**

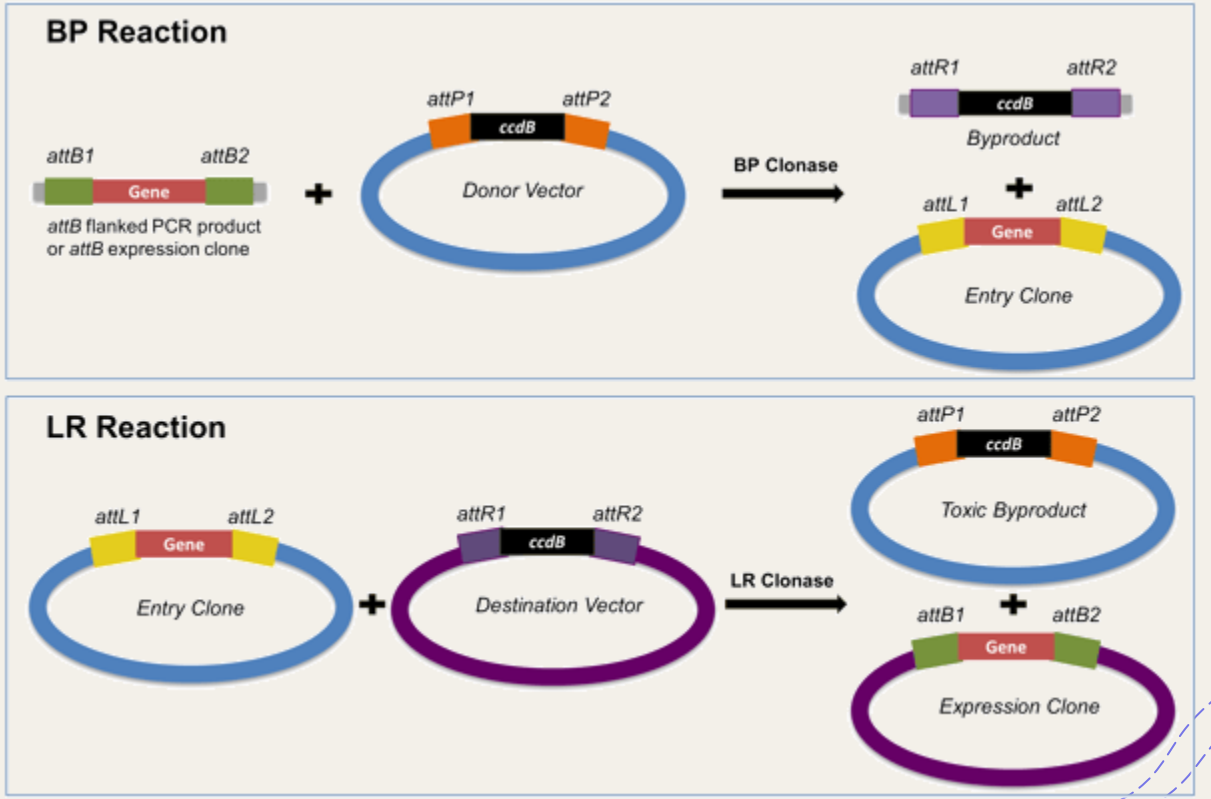


Transgenesis method: Multisite Gateway-based Tol2 strategy
Att sites and recombination

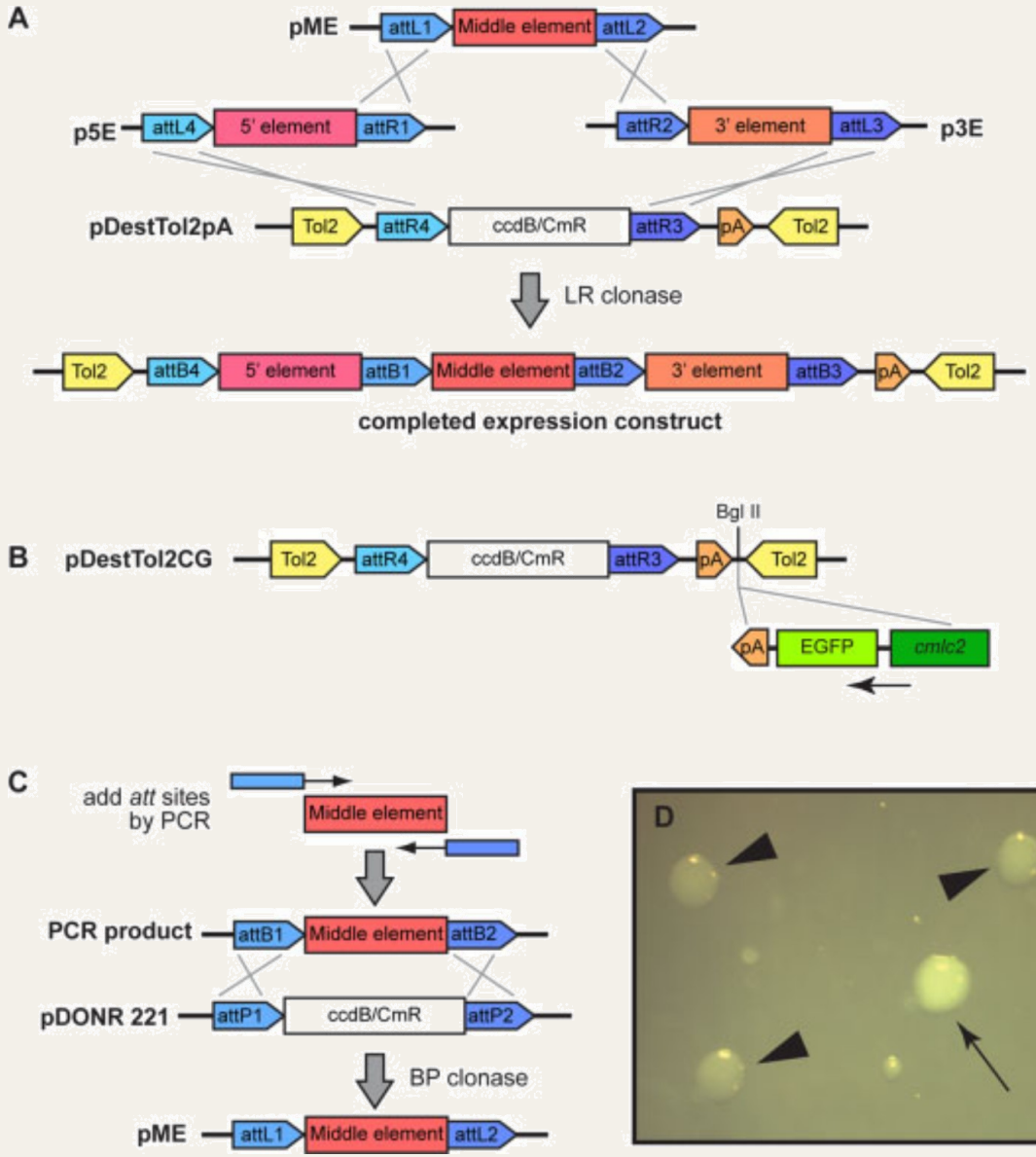
Two types of recombination reaction:

BP Reaction: Recombination of an *att*B substrate with an *att*P substrate to create an *att*L-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.

Kwan et al., 2007

Transgenesis method: Multisite Gateway-based Tol2 strategy Vectors

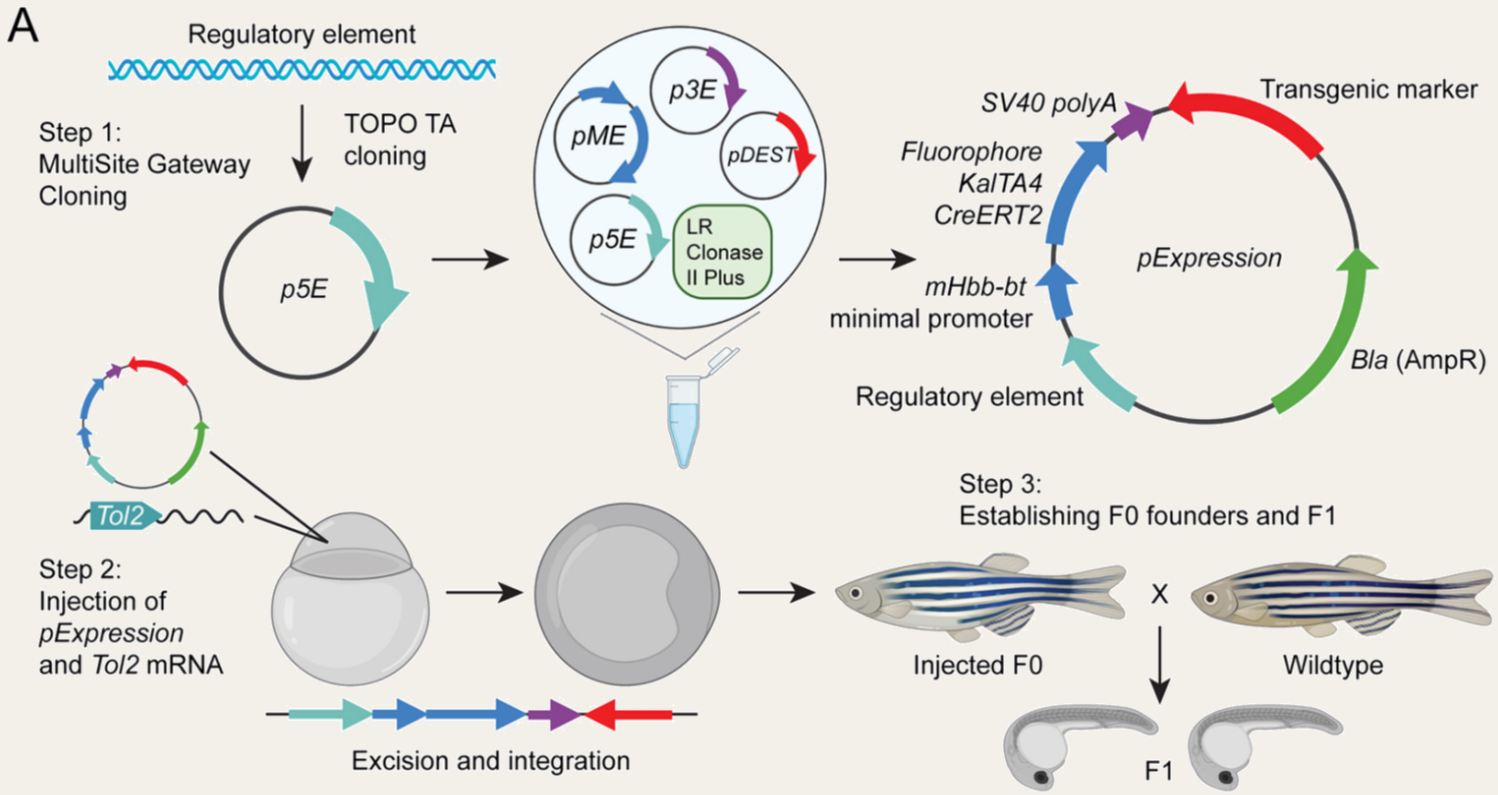
TABLE 1. Components of the Tol2kit[a]

Name	Description	Test	Figure
5′ entry clones			
p5E-*bactin2*	5.3-kb beta-actin promoter (ubiquitous)	F0, F1	2
p5E-*h2afx*	1-kb H2A-X promoter (quasi-ubiquitous)	F0	2b
p5E-CMV/SP6	1-kb CMV/SP6 cassette from pCS2+	F0	
p5E-*hsp70*	1.5-kb *hsp70* 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 *Fse*I and *Asc*I	F0	
Middle entry clones			
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	
3′ entry clones			
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	2abcf
p3E-IRES-nlsEGFPpA	EMCV IRES driving nlsEGFP (nuclear EGFP) plus SV40 late polyA	F0	2e
Destination vectors			
pDestTol2pA/pDestTol2pA2	attR4-R3 gate with SV40 polyA flanked by Tol2 inverted repeats	F0, F1	3
pDestTol2CG/pDestTol2CG2	pDestTol2pA/pDestTol2pA2 with *cmlc2*:EGFP transgenesis marker	F0, F1	4
Other			
pCS2FA-transposase	For in vitro transcription of capped Tol2 transposase RNA		3, 4

[a]EGFP, enhanced green fluorescent protein; F0, yields appropriate expression in transient transgenics; F1, yields appropriate expression in stable transgenics.

Kwan et al., 2007

Transgenesis method: Multisite Gateway-based Tol2 strategy Workflow Overview

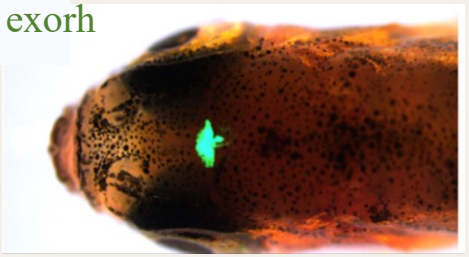


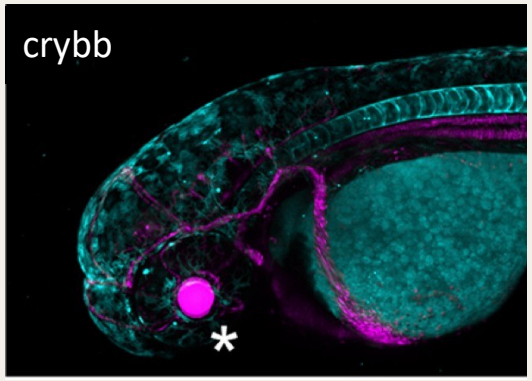
Kemmler et al., 2023

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)





Pictures from: Cassie L. Kemmler et al., 2023

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)

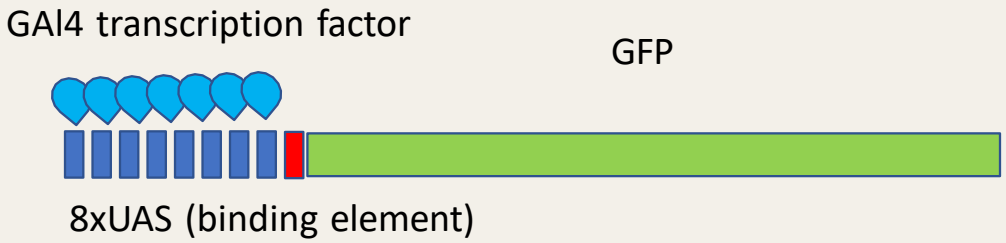
The introduction of the yeast **Gal4 system** in Zebrafish

TWO TRANSGENIC LINES:

a) THE **DRIVER** (ACTIVATOR) EXPRESSING Gal4 TF IN SPECIFIC FEATURES (CELLS TISSUE, TIME)

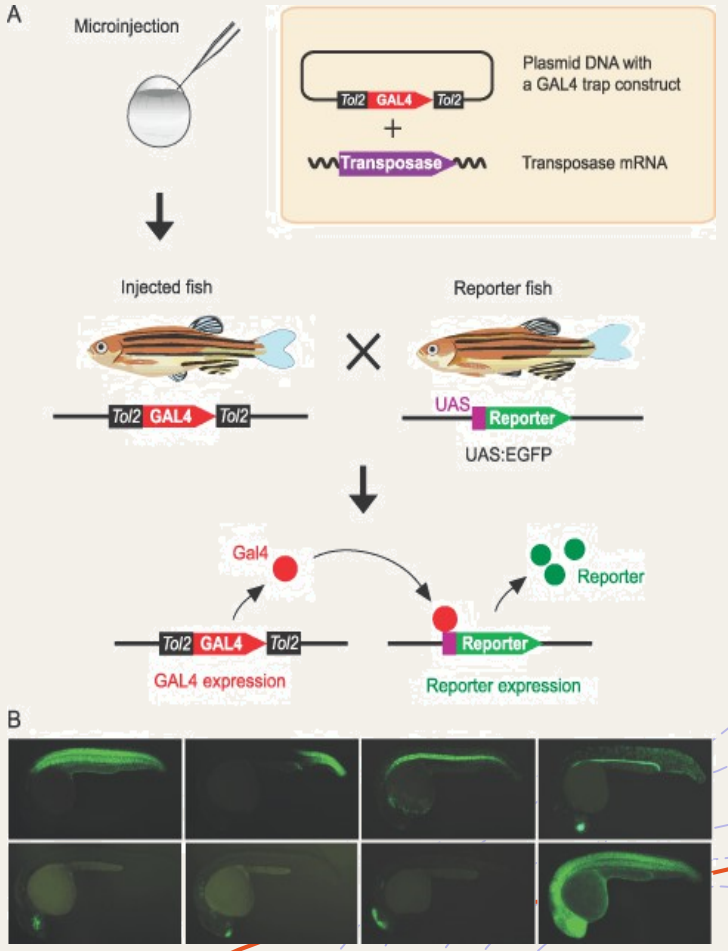
b) THE **REPORTER** (ACTIVATED) BEARING UAS:REPORTER

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



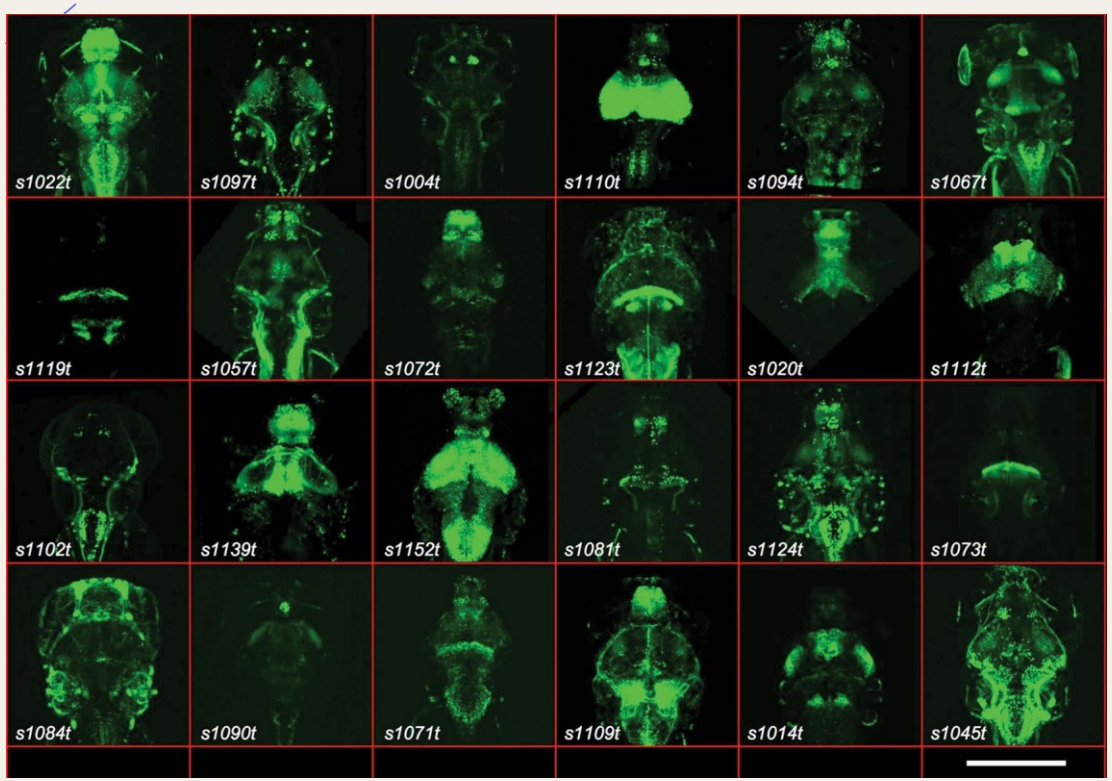
Asakawa, 2008

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

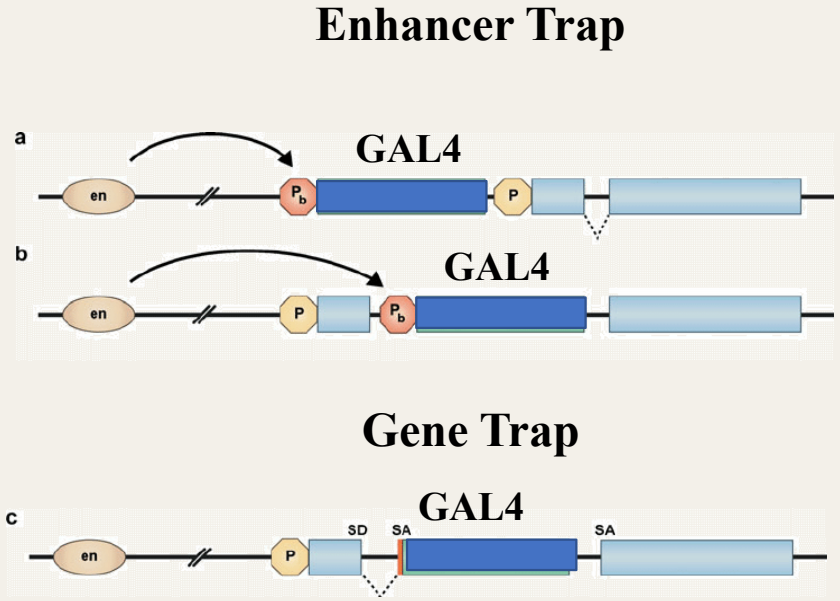


ENHANCER/GENE TRAP EXPERIMENTS
(non characterized regulatory elements/random insertion)

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)



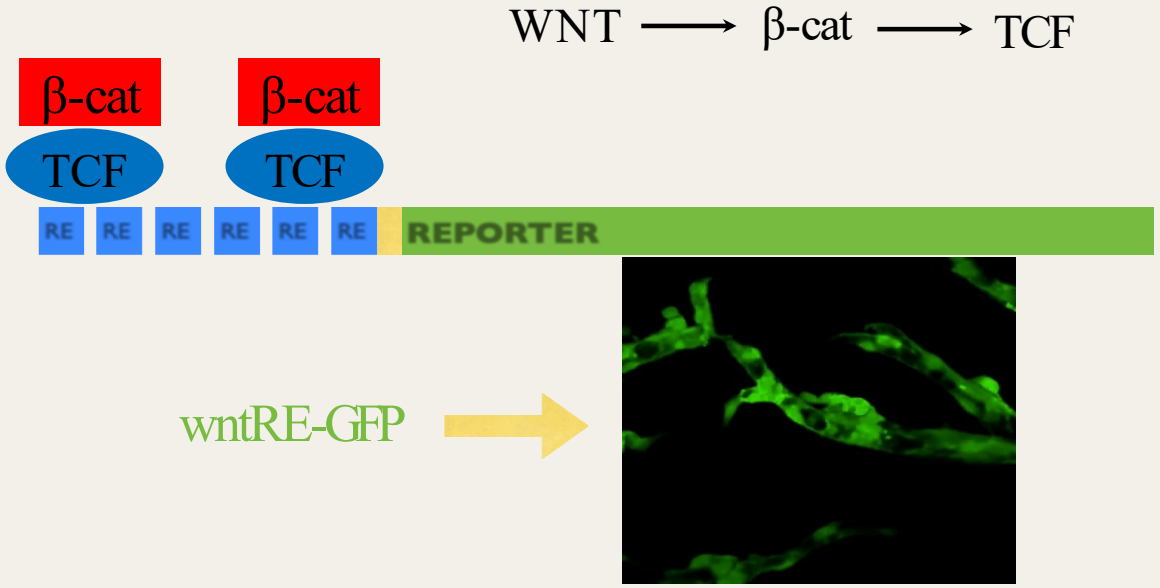
N.B.: (GFP is produced by the «reporter» allele)



Scott Baier, 2009

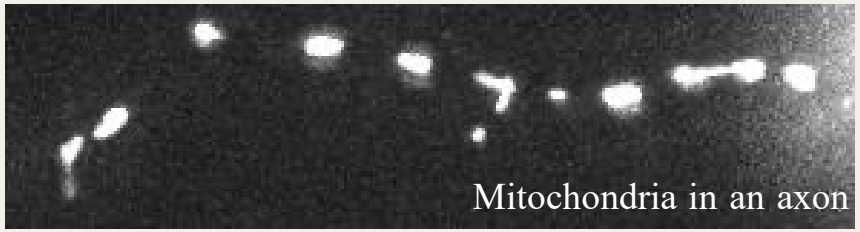
REGULATORY ELEMENTS (REs)

- **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**

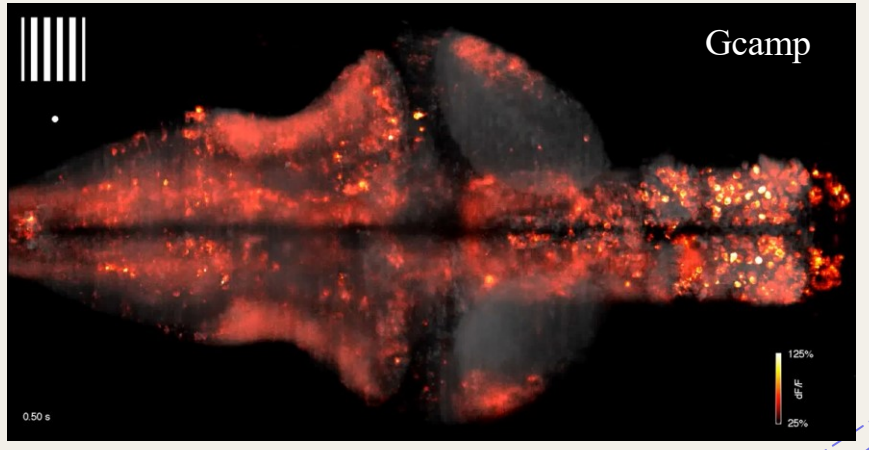


THE REPORTER PROTEIN

- 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++, 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)

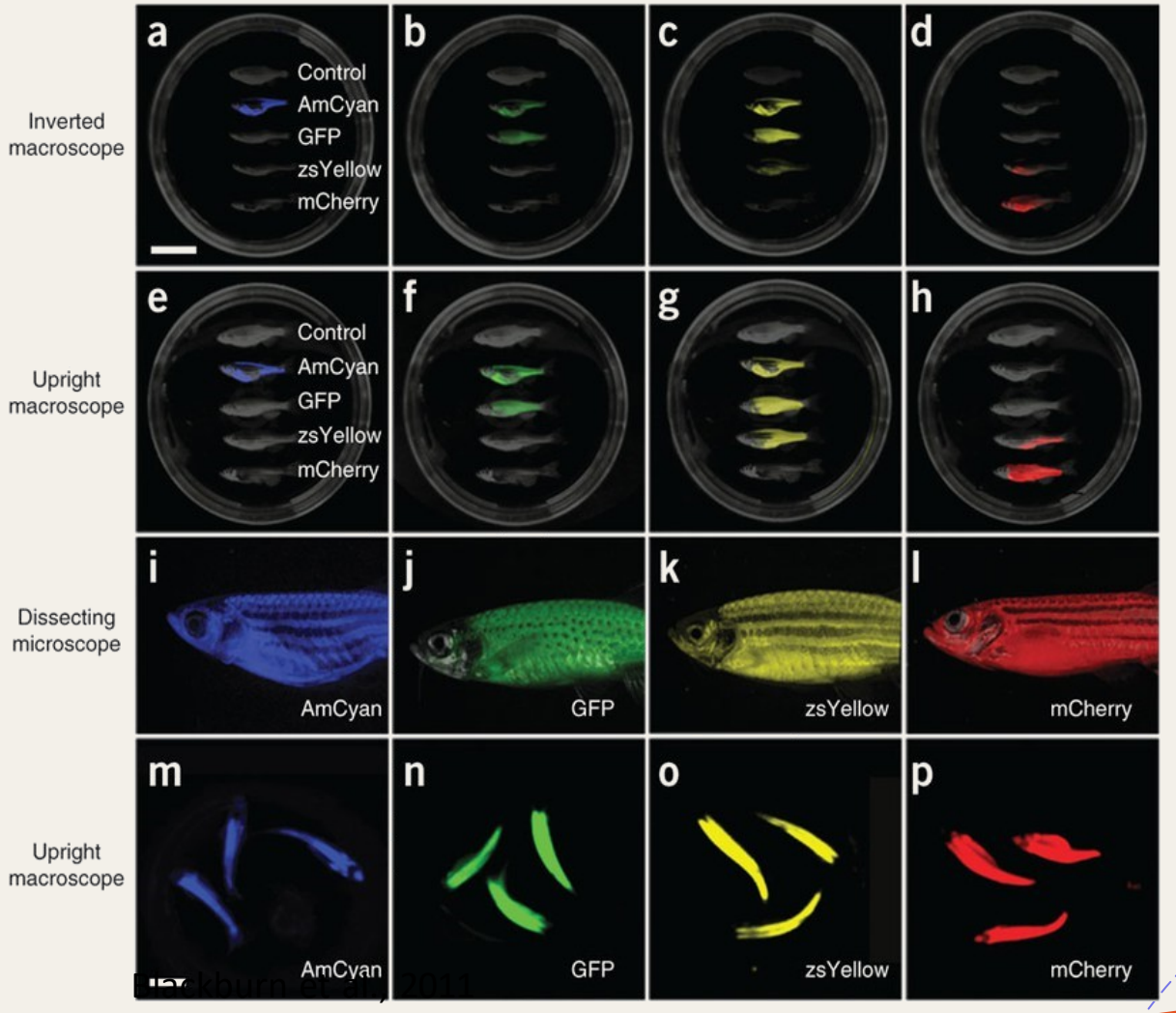
- DISADVANTAGES: ACCUMULATED (SLOW DYNAMICS)
- ADVANTAGES: **SPATIAL INFORMATIONS**

PHYSIOLOGICAL FLUORESCENT REPORTER

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

FLUORESCENT PROTEIN: PALETTE

GFP, RFP, VENUS, KAEDE CERULEAN…



Buckburn et al, 2011

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

vs

Drawbacks:
- Slow accumulation

DESTABILIZED REPORTER

Which use?
- Studying rapid dynamics

Drawbacks:
- Low amount of detectable accumulating protein during a biological event

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

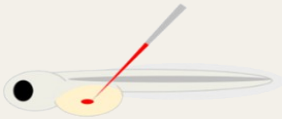
Microinjection in Zebrafish

Best stages:

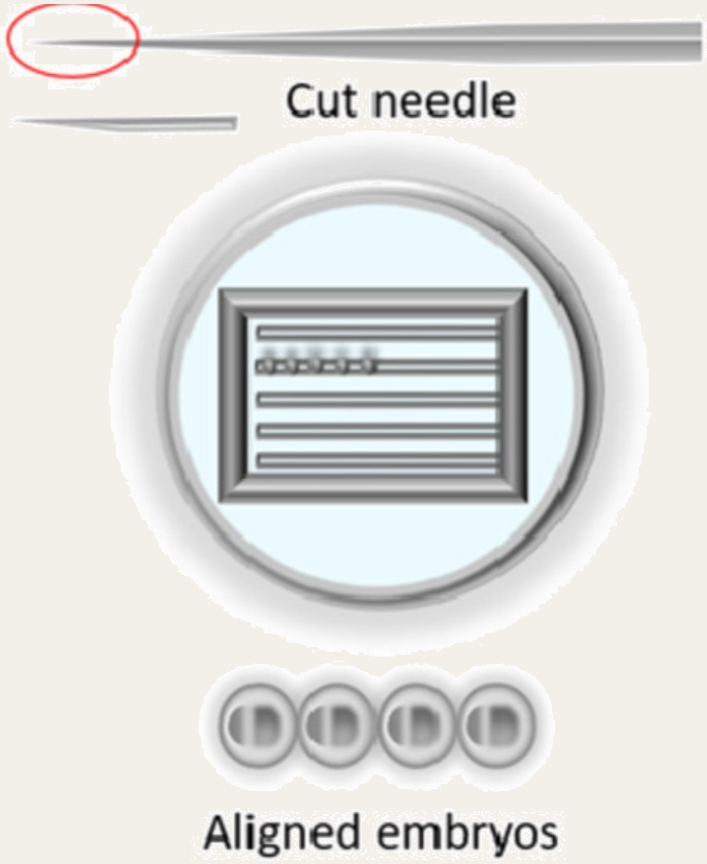
- Transgenesis and genome editing: 1-cell (zygote) stage

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

- Cell transplantation: usually 1-2 days post-fertilization



Microinjection in Zebrafish

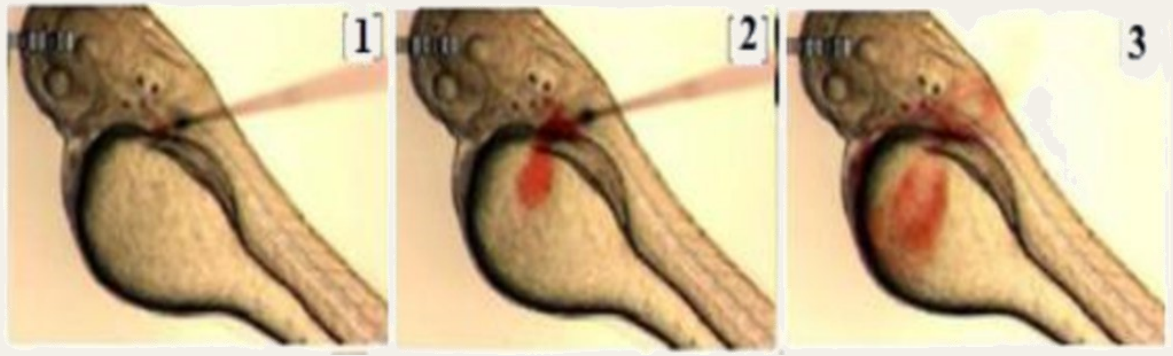


- Embryos or larvae obtained from pair or population crosses
- Egg collection after about 15 min from laying (to allow fertilization)
- Alignment along agarose-made lanes or microscope slide borders
- Glass needles with filament (for solutions) or without filament (for cells)

Microinjection in Zebrafish

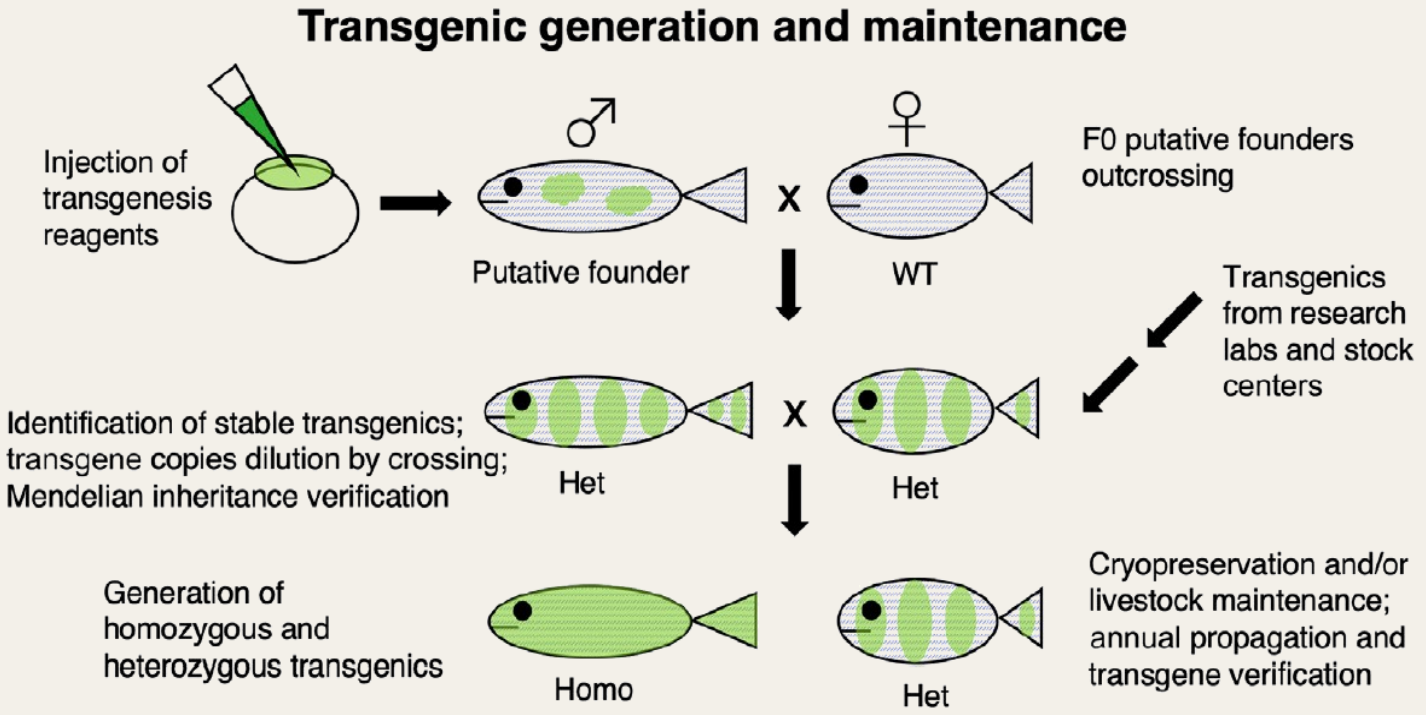
Tracking the microinjection:

- 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)



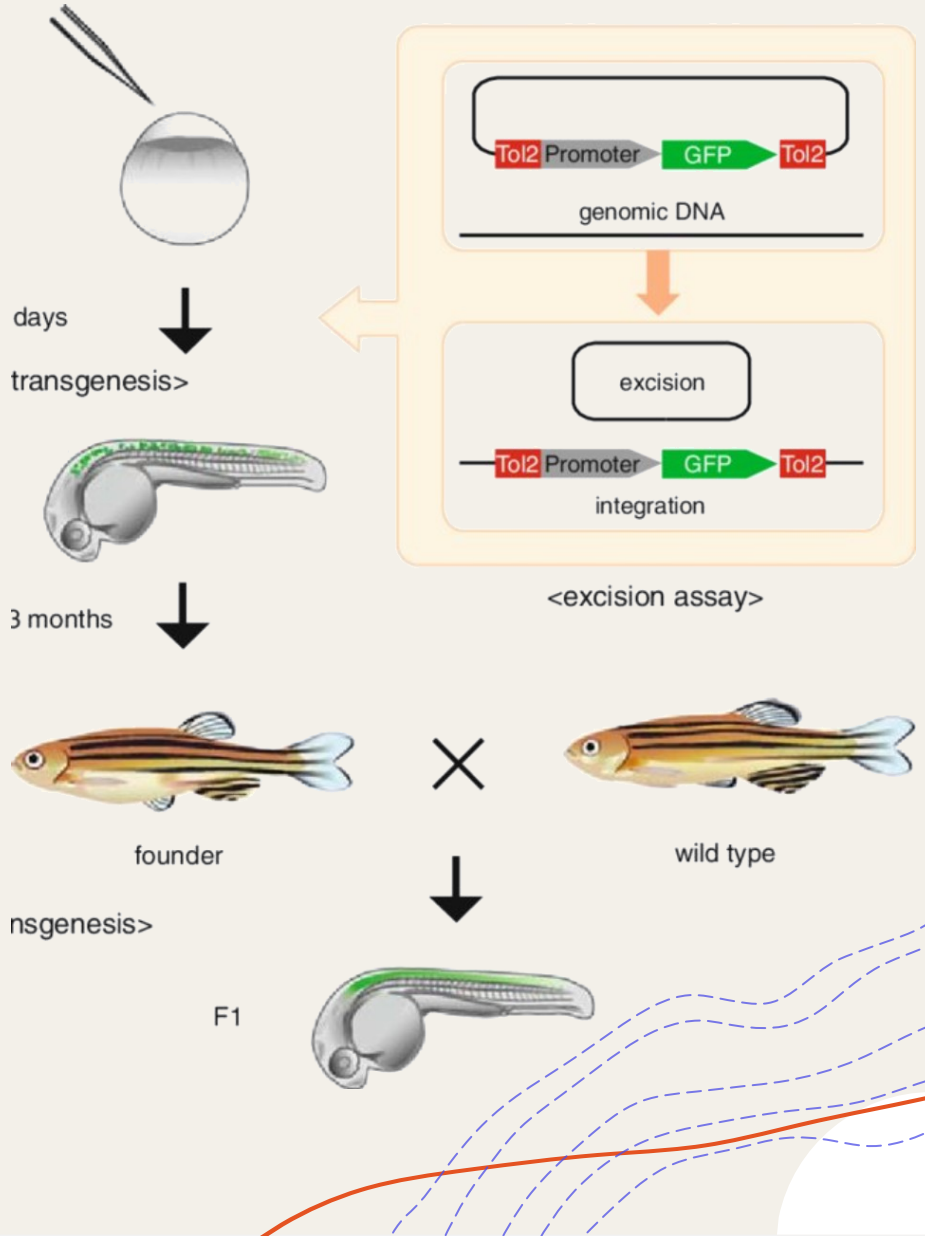
Transgenic line stabilization, genotyping and maintenance

- How to manage a newly produced transgenic line?



Transgenic line stabilization

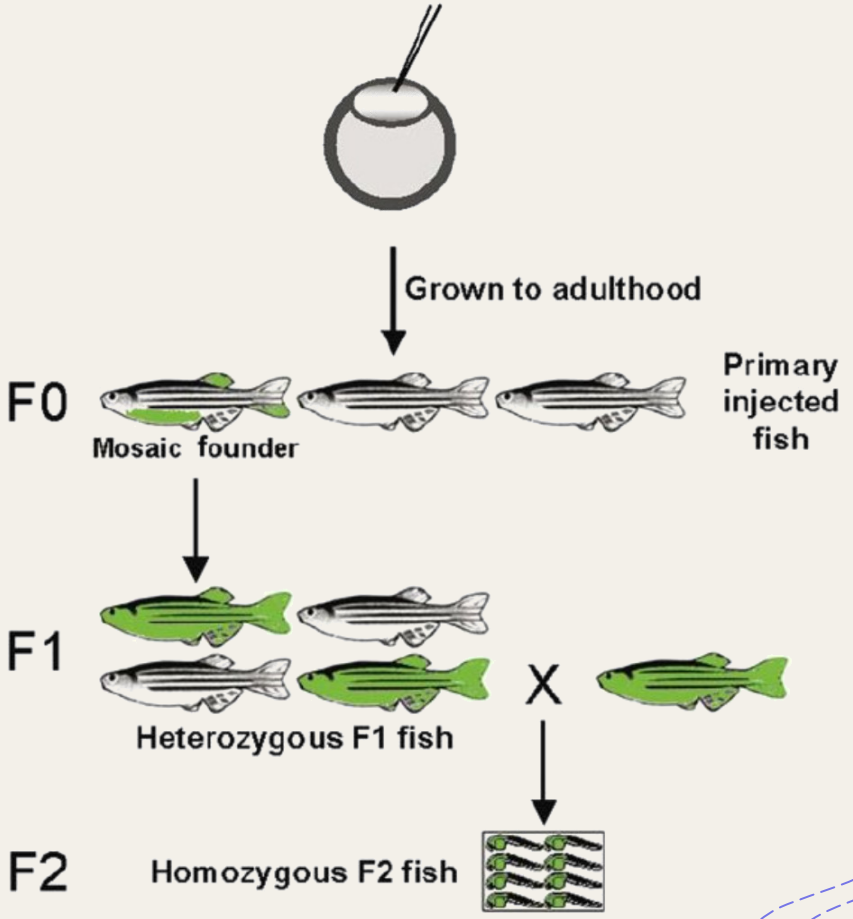
- After F0 (mosaic) production, founders are outcrossed to verify the **germline transmission**
- If transgene **multi-copies** are present (variable phenotypes, non-Mendelian inheritance), fishes are further out- crossed to dilute/select single copies
- Stabilized line: homogeneous phenotypes, **Mendelian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



Monitoring the transgene presence

- **Fluorescence** analysis
- In many cases single/double copies correspond to different fluorescence intensities

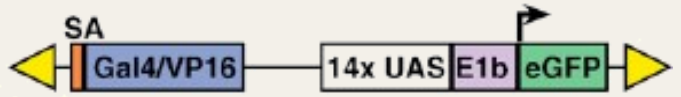
  → this can help to identify heterozygous and homozygous transgenic animals



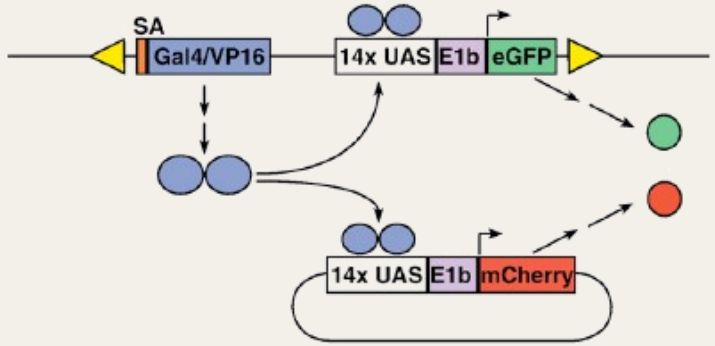
Monitoring the transgene presence

- 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



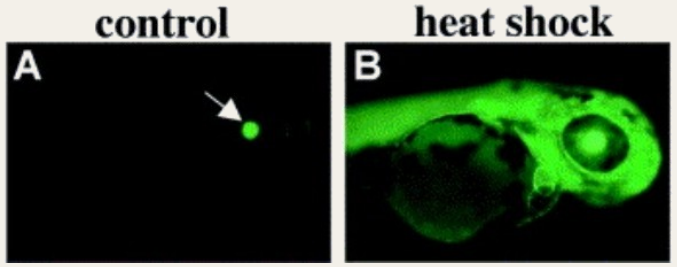
Green and Red reporter

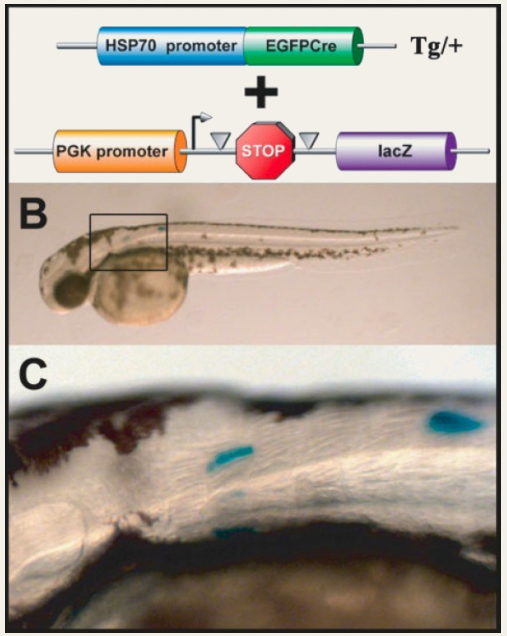


Monitoring the transgene presence

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

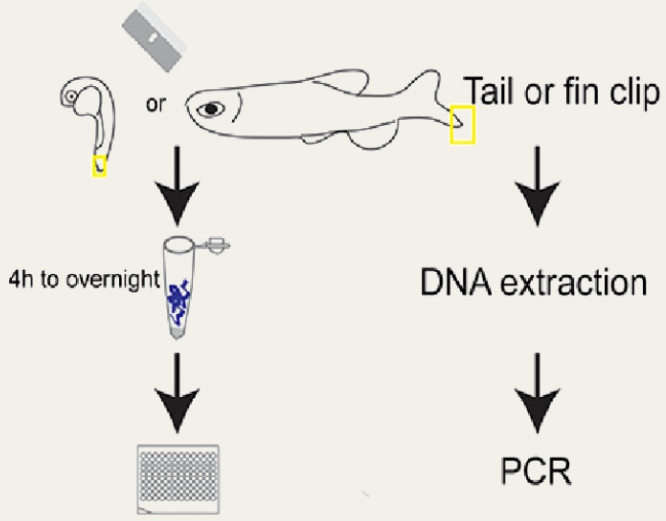
Genotyping can be performed on:

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

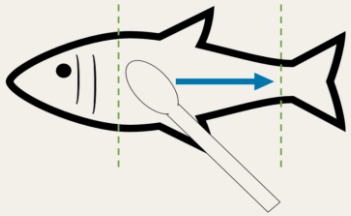
Genotyping can be:

- 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

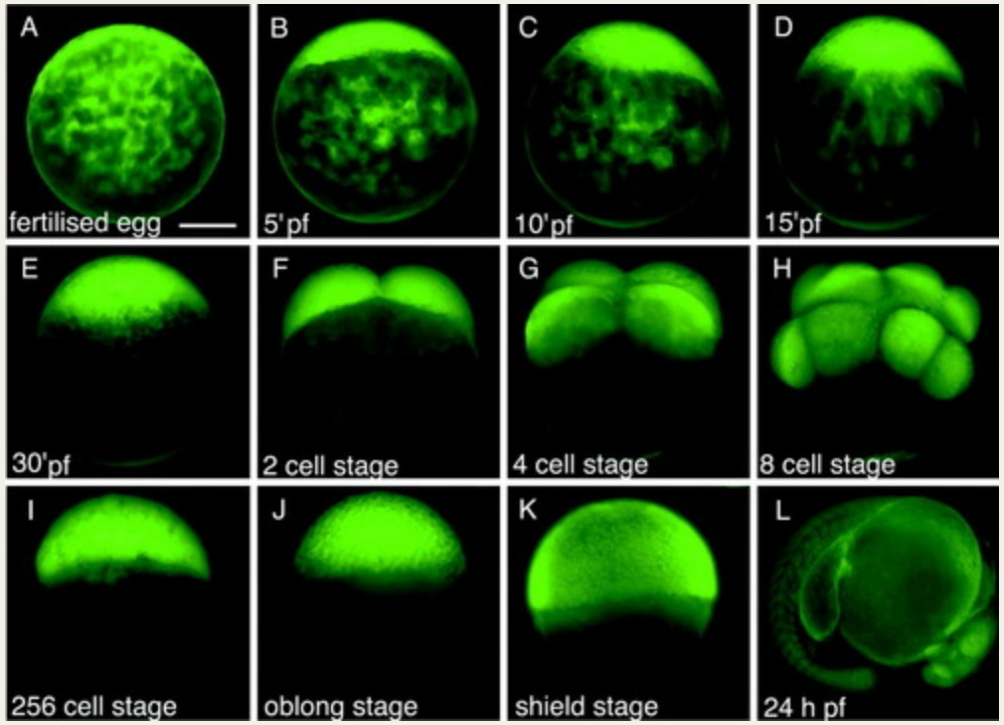
- Lines can be maintained alive or frozen (a **frozen backup** is recommended)
- To avoid repeated inbreeding within the transgenic line (with deleterious effects due to homozygosity - **inbreeding depression**), outcrosses are recommended every
- Fluorescent transgenes should be monitored at every generation, 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**)

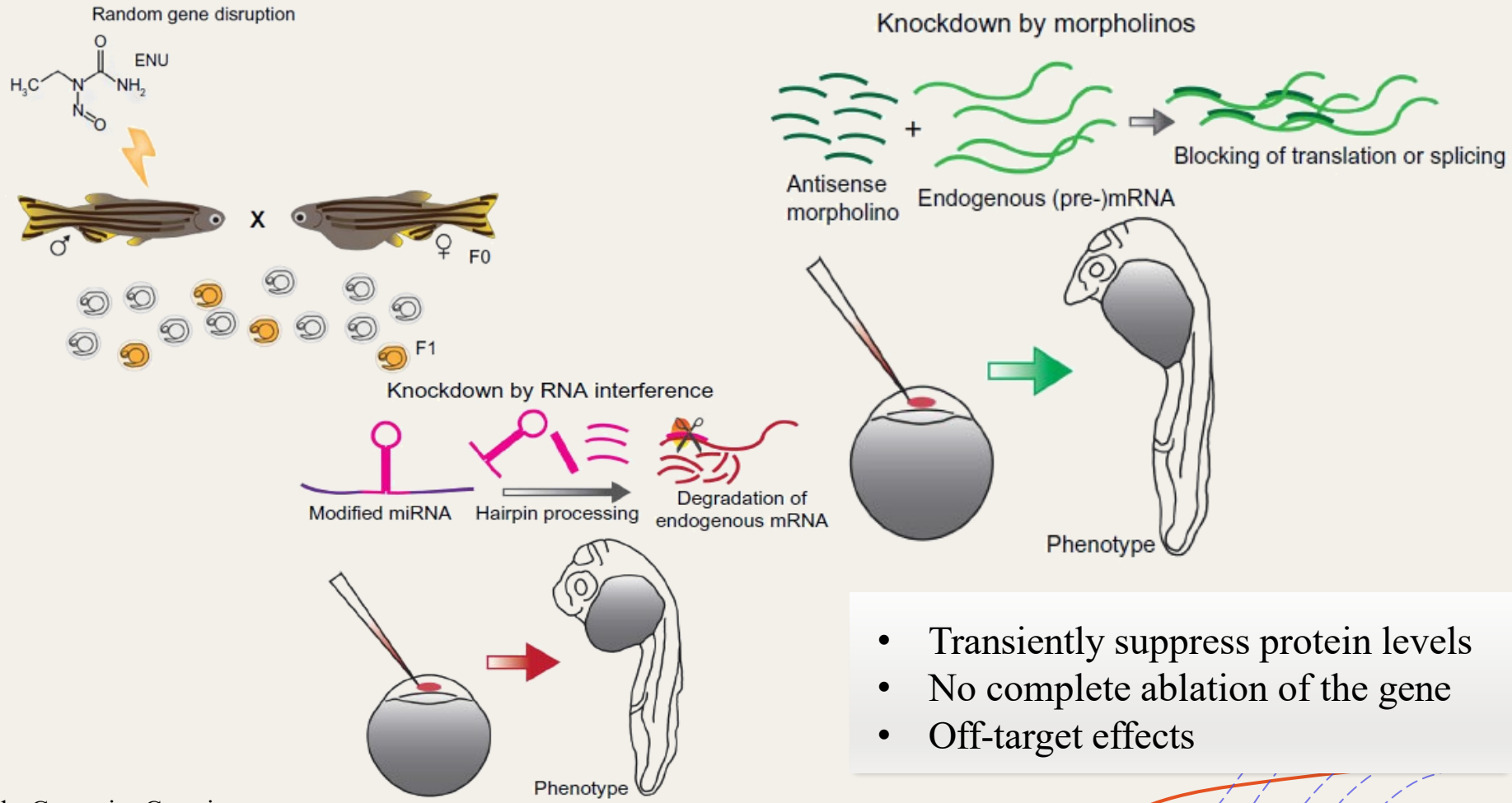


Useful Videos

- 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

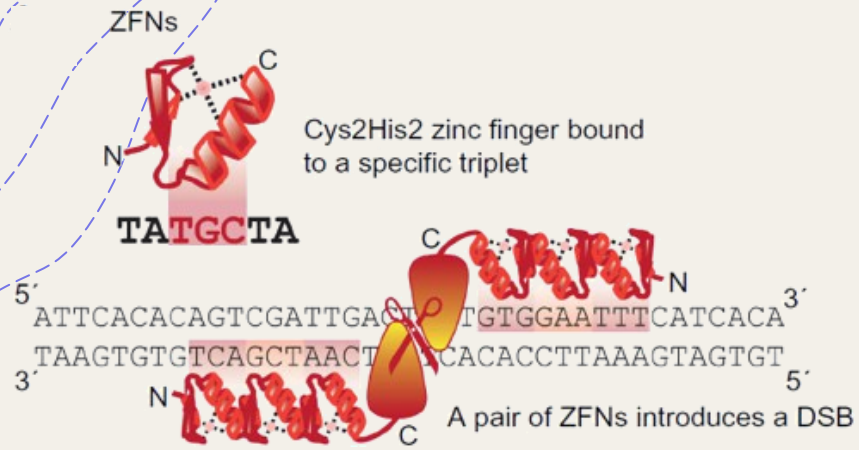
How can we study gene function with zebrafish?

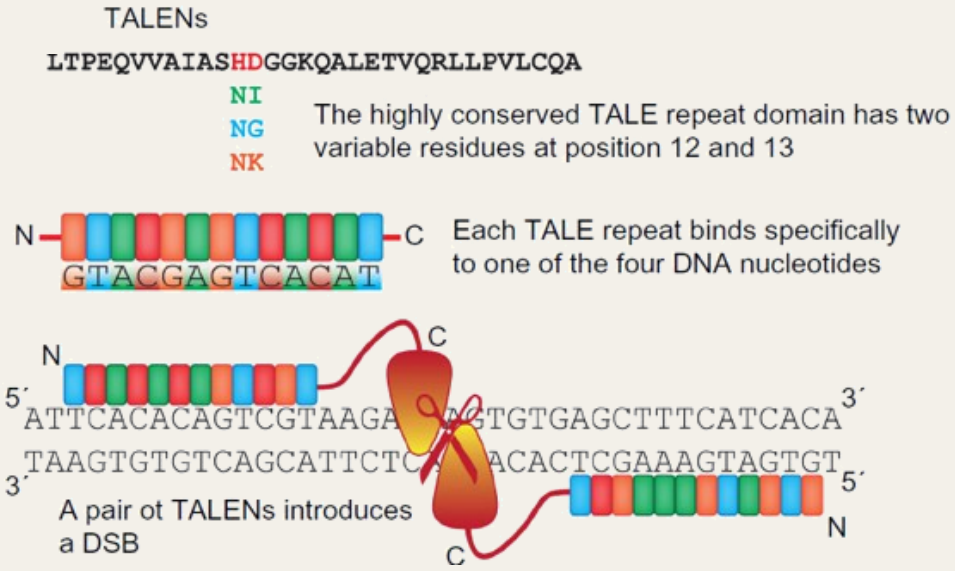


- Transiently suppress protein levels
- No complete ablation of the gene
- Off-target effects

Sassen & Köster, 2015. Adv. Genomics Genetic.

New experimental approaches with genome editing



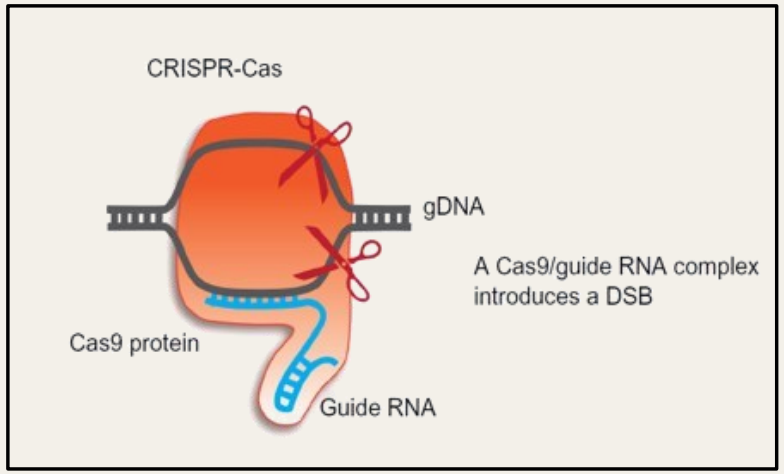


NATURE PROTOCOLS | VOL.8 NO.11 | 2013 | 2281 PROTOCOL

Genome engineering using the CRISPR-Cas9 system

F Ann Ran[1–5,8], Patrick D Hsu[1–5,8], Jason Wright[1], Vineeta Agarwala[1,6,7], David A Scott[1–4] & Feng Zhang[1–4]

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



Simple and efficient method (2013)

Sassen & Köster, 2015. Adv. Genomics Genetic

CRISPR-Cas9

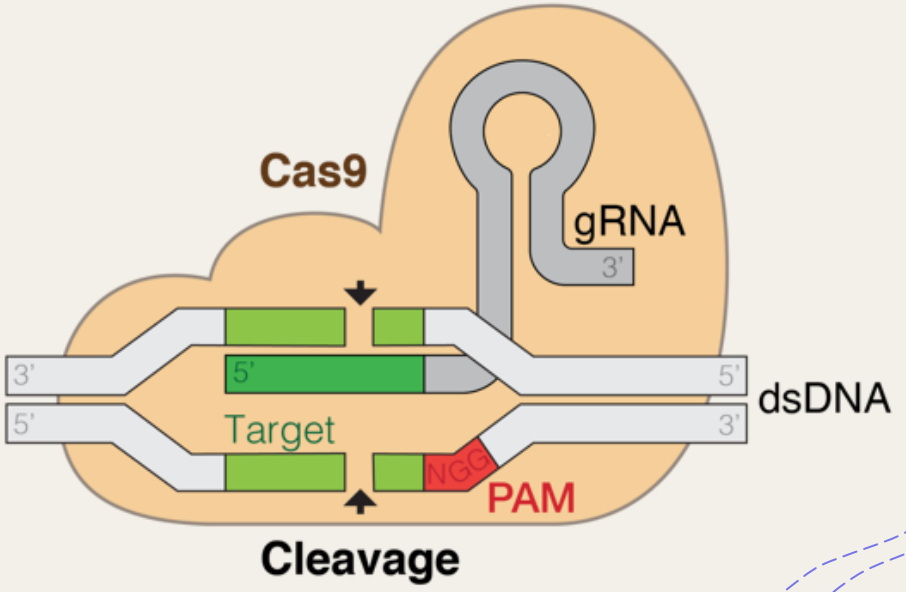
Emmanuelle Charpentier and **Jennifer Doudna, Nobel Prize 2020**

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

Components:

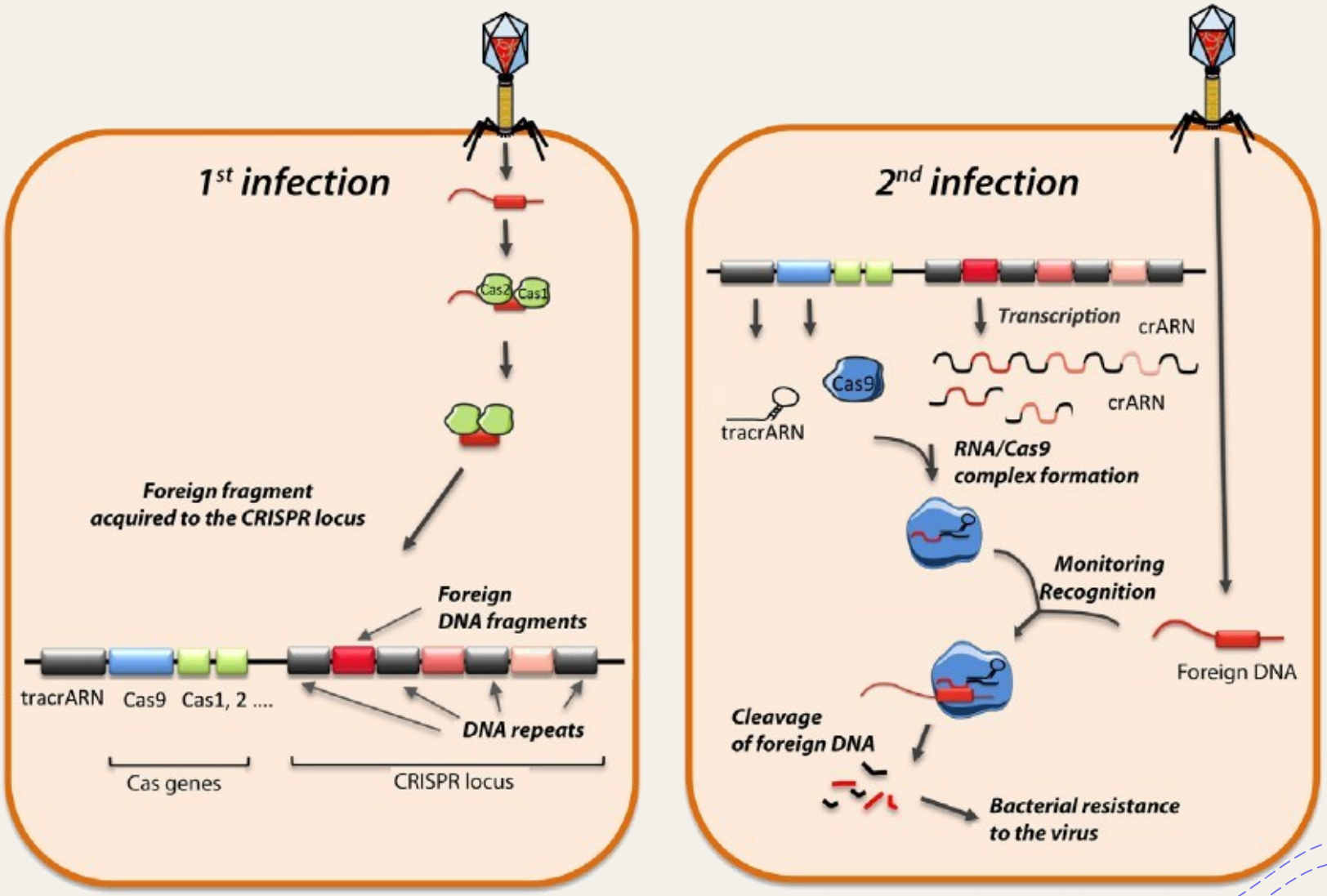
- **guide RNA** (crRNA + tracrRNA): short synthetic RNA composed of a scaffold sequence necessary for Cas-binding and a user-defined **~20 nucleotide spacer** that defines the genomic target to be modified.
- **CRISPR-associated endonuclease (Cas protein)**

Target sequence: 20 bp



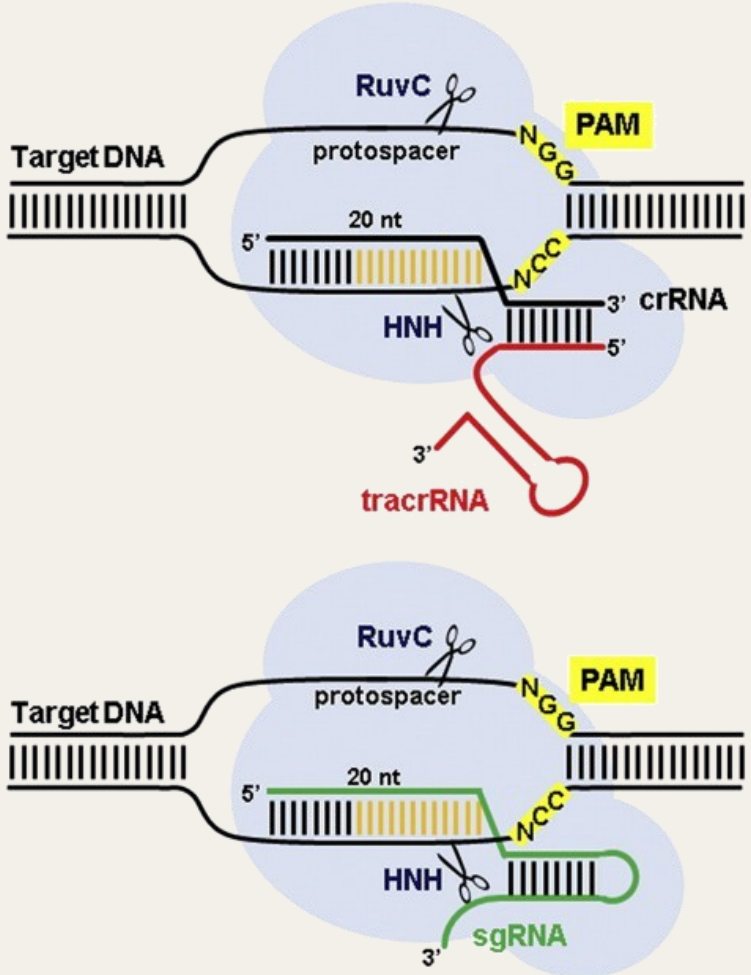
Microinjecting an *in vitro* complex of guide RNA and Cas9 protein into one-cell stage embryos.
CRISPR-mediated knockout zebrafish models are extremely popular and have been used to **model several diseases**.

CRISPR/Cas: the Immune System of Bacteria

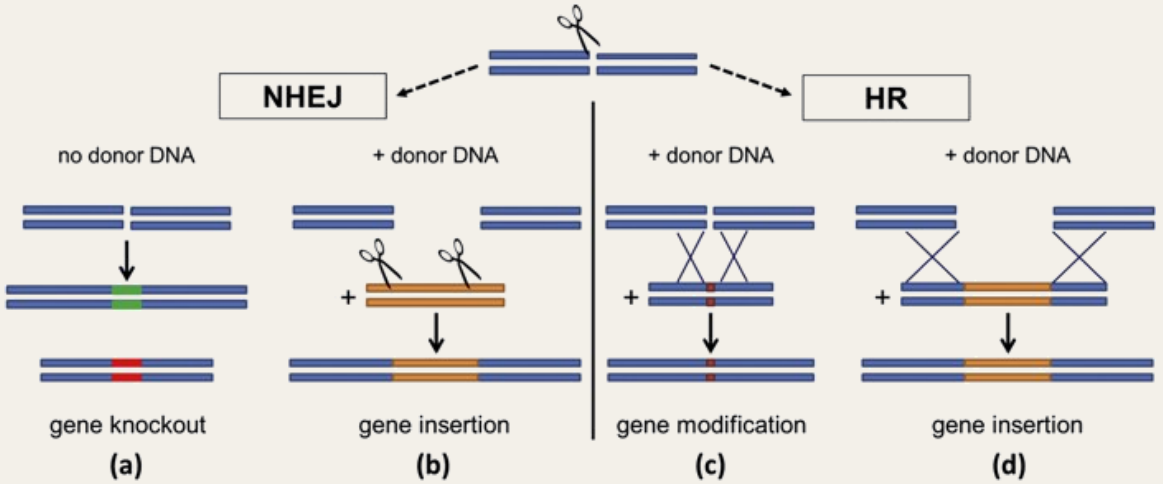


Duroux-Richard et al. 2017. Joint Bone Spine

CRISPR/Cas9 for targeted genome editing

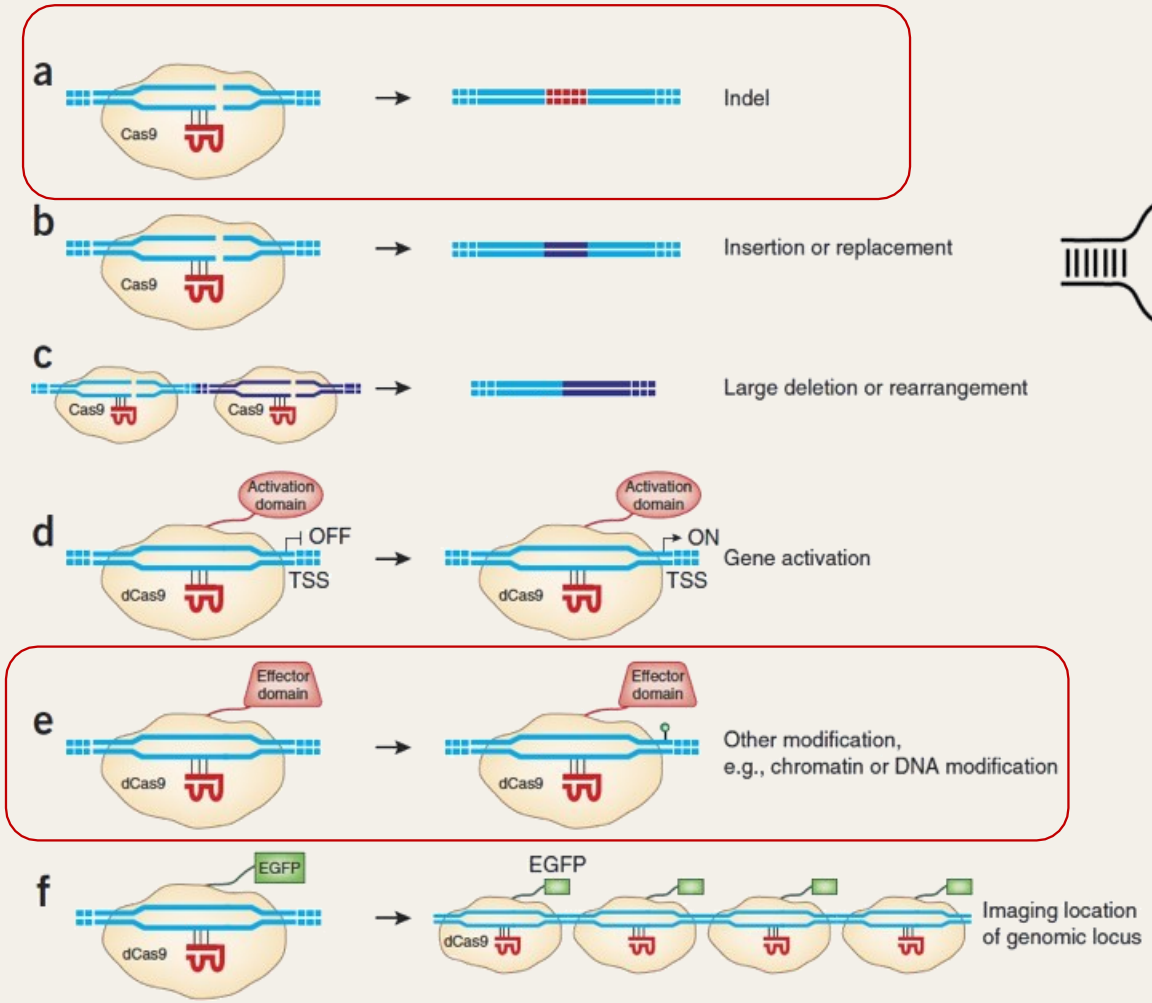


Bortesi & Fisher, 2015. Biotechnol. Adv.

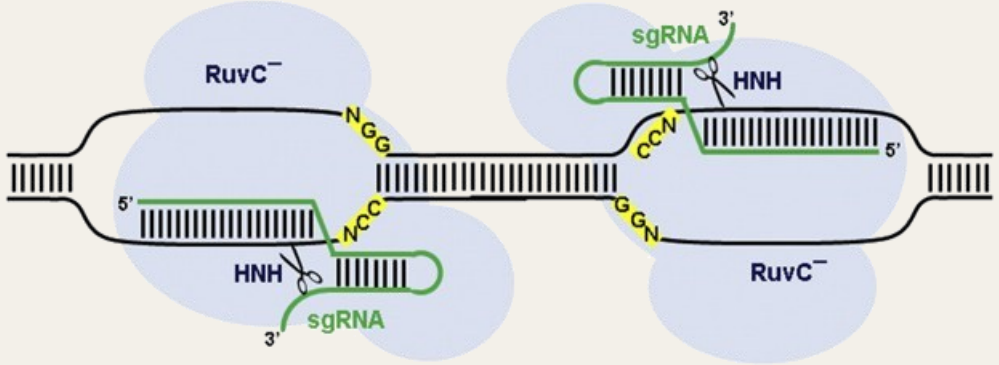


Bortesi & Fisher, 2015. Biotechnol. Adv.

CRISPR/Cas9 for Gene Activation and Repression

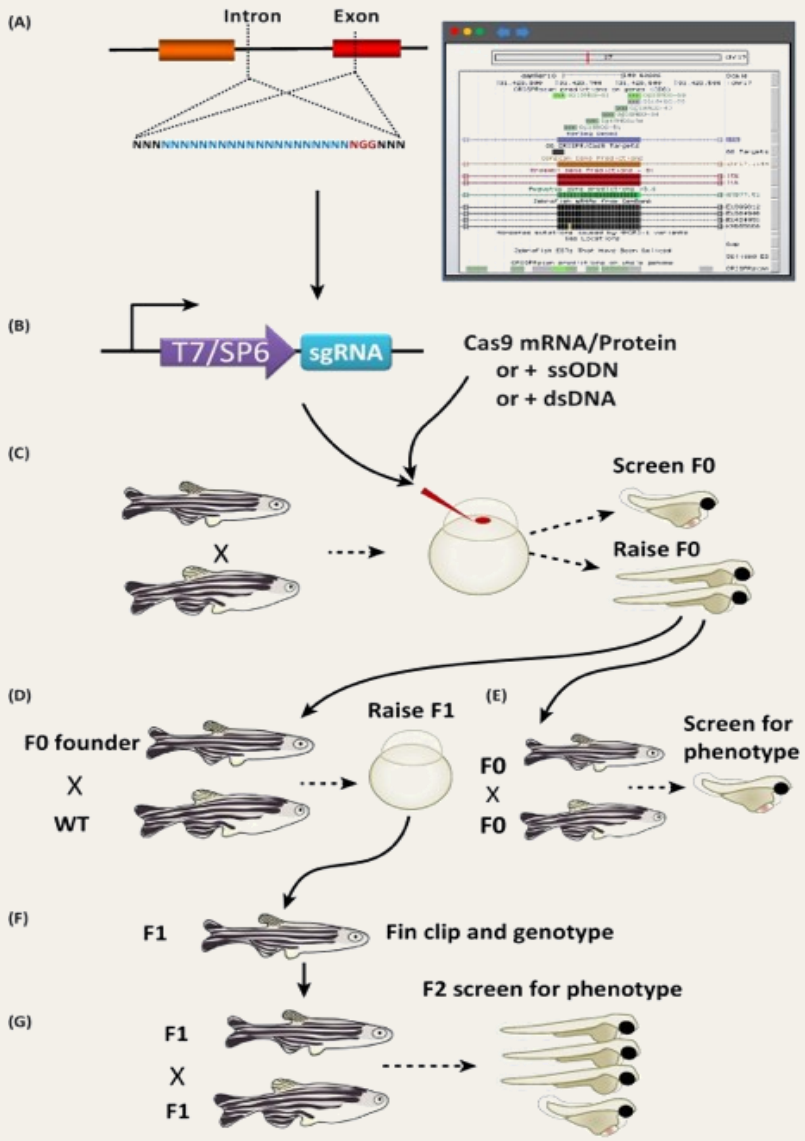


Sander & Joung, 2014. Nat. Biotech



Bortesi & Fisher, 2015. Biotechnol. Adv.

CRISPR/Cas9 and Zebrafish



From 2002, 9600 articles have been published on CRISPR/Cas and 300 in zebrafish

Li et al., 2016. Trends Genetics

Steps to generate and characterize a mutant in zebrafish

1. Choosing a guide

CHOPCHOP

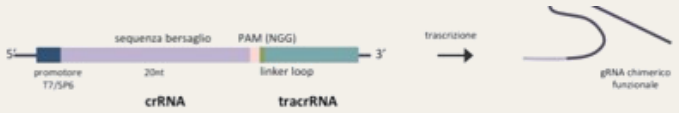
E-CRISP
Design of CRISPR constructs

dkfz. GERMAN CANCER RESEARCH CENTER

Breaking Cas

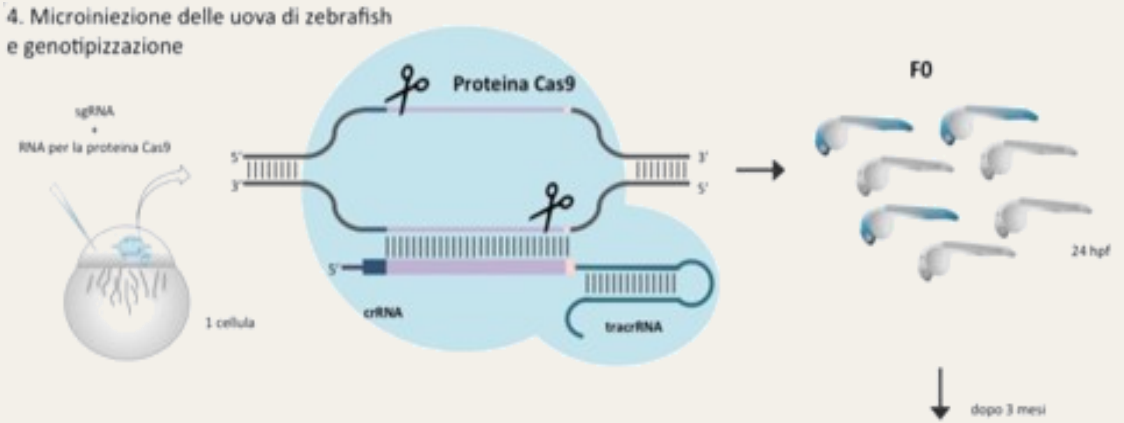
SYNTHEGO
Genome Engineering Solutions

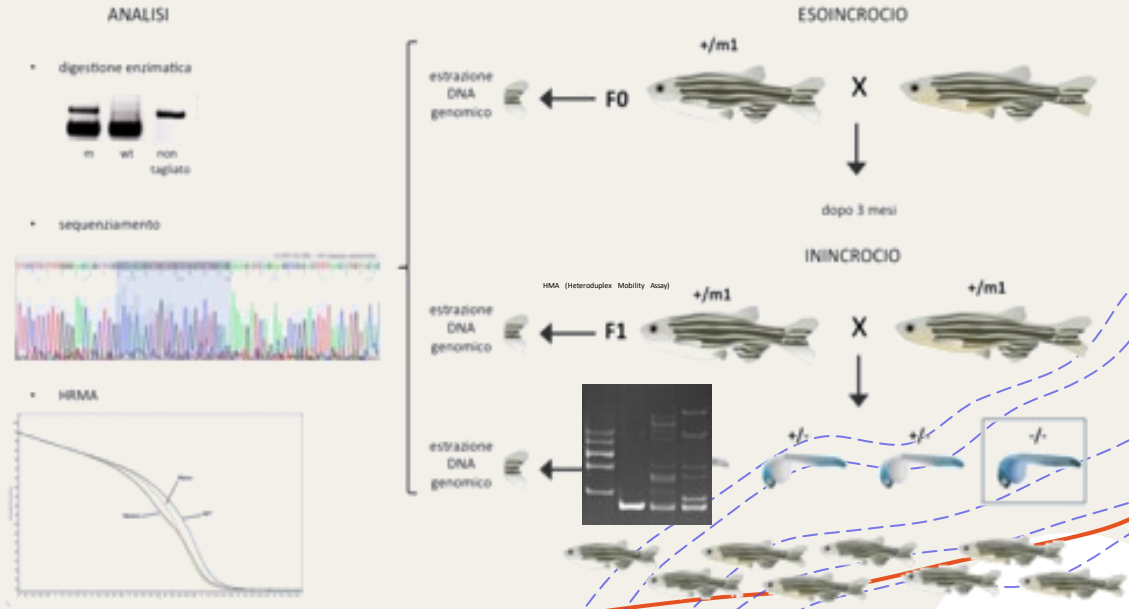
2.1 sgRNA production



2.2 Cas9 RNA production or Cas9 protein

3. Injection and downstream analysis



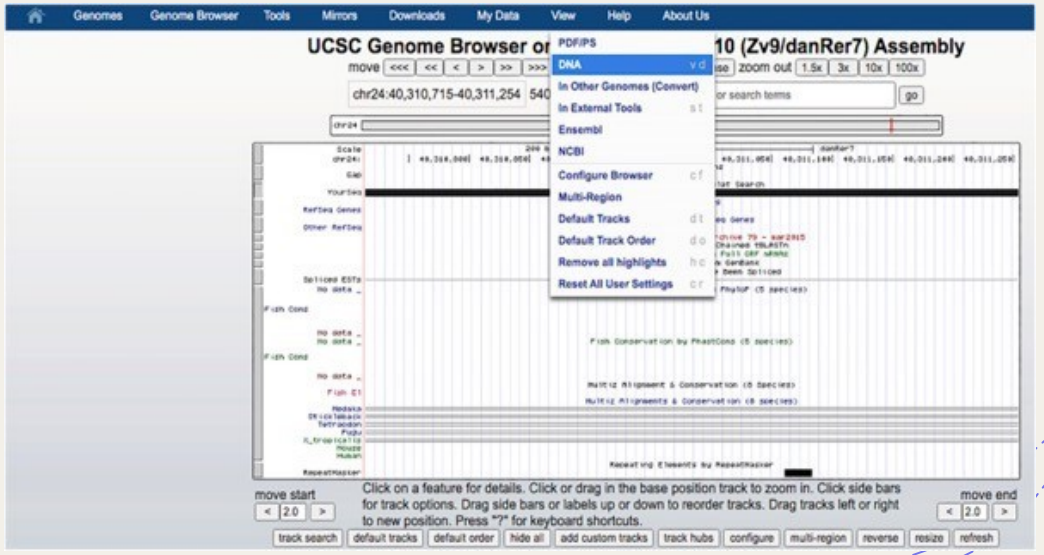


Selection of Target Sequence - Gene Analysis in UCSC

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:

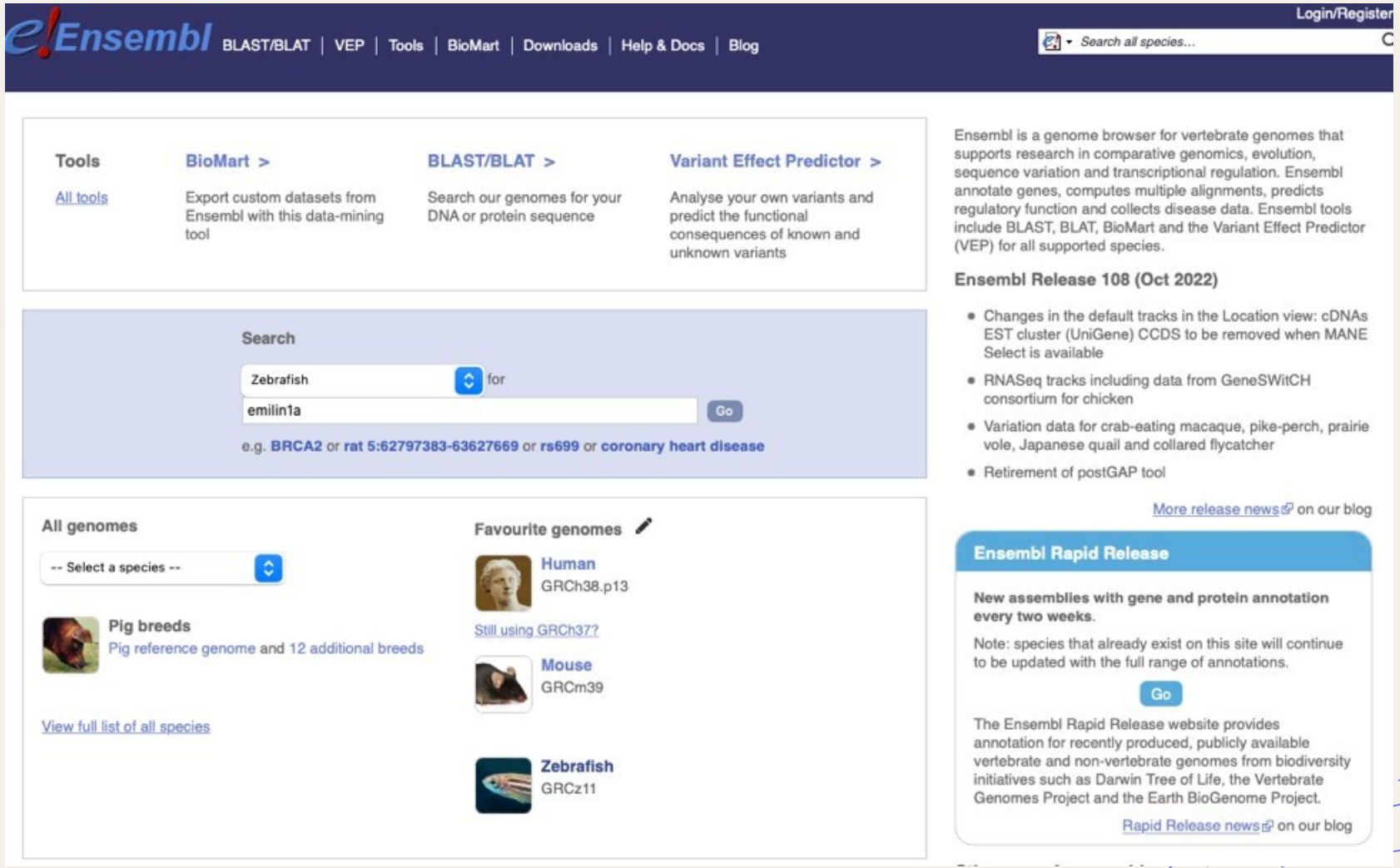
1.Figure 1. Snapshot of the UCSC Genome Browser Website Showing How to Get a DNA Sequence of Interest, Using the Example Shown (Nog2E3)

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

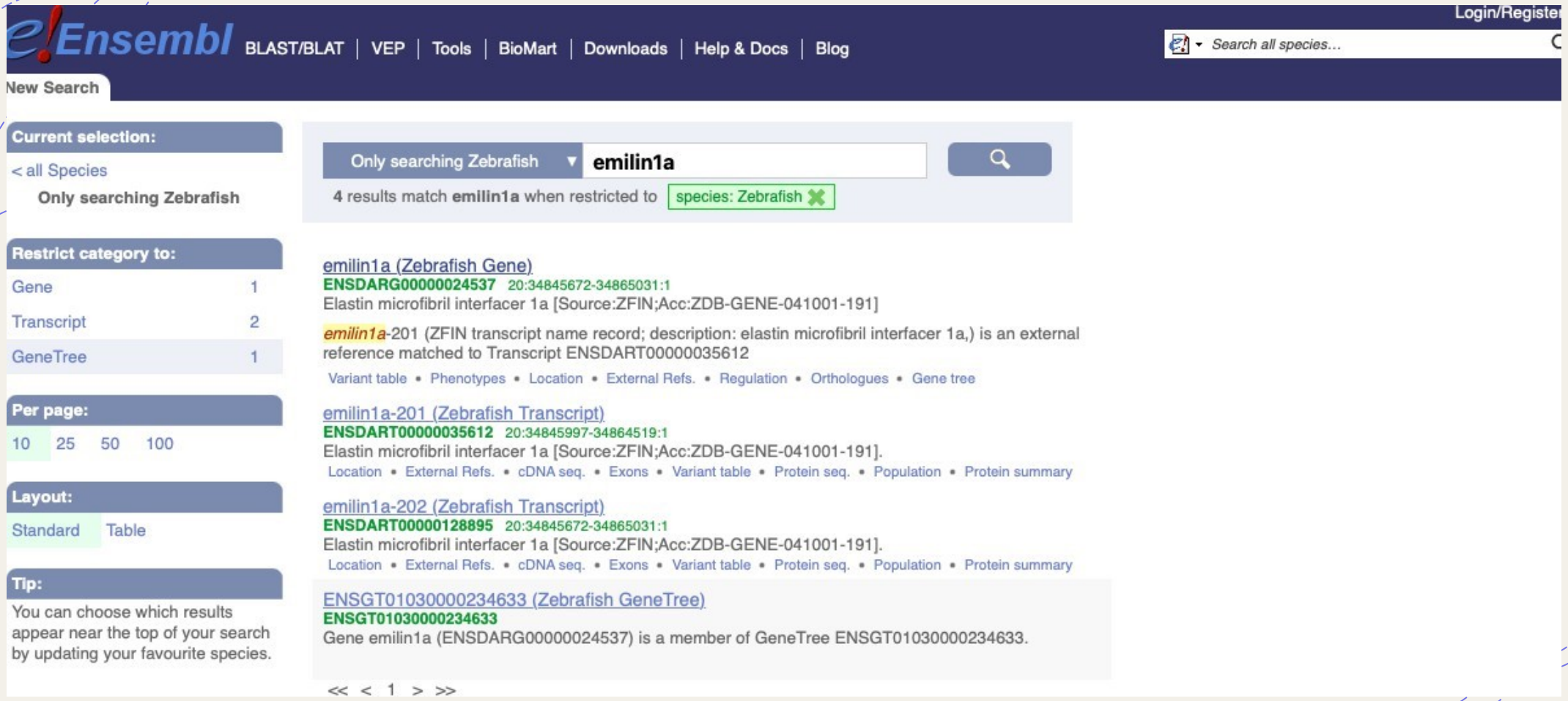


Selection of Target Gene- Gene Analysis in Ensemble

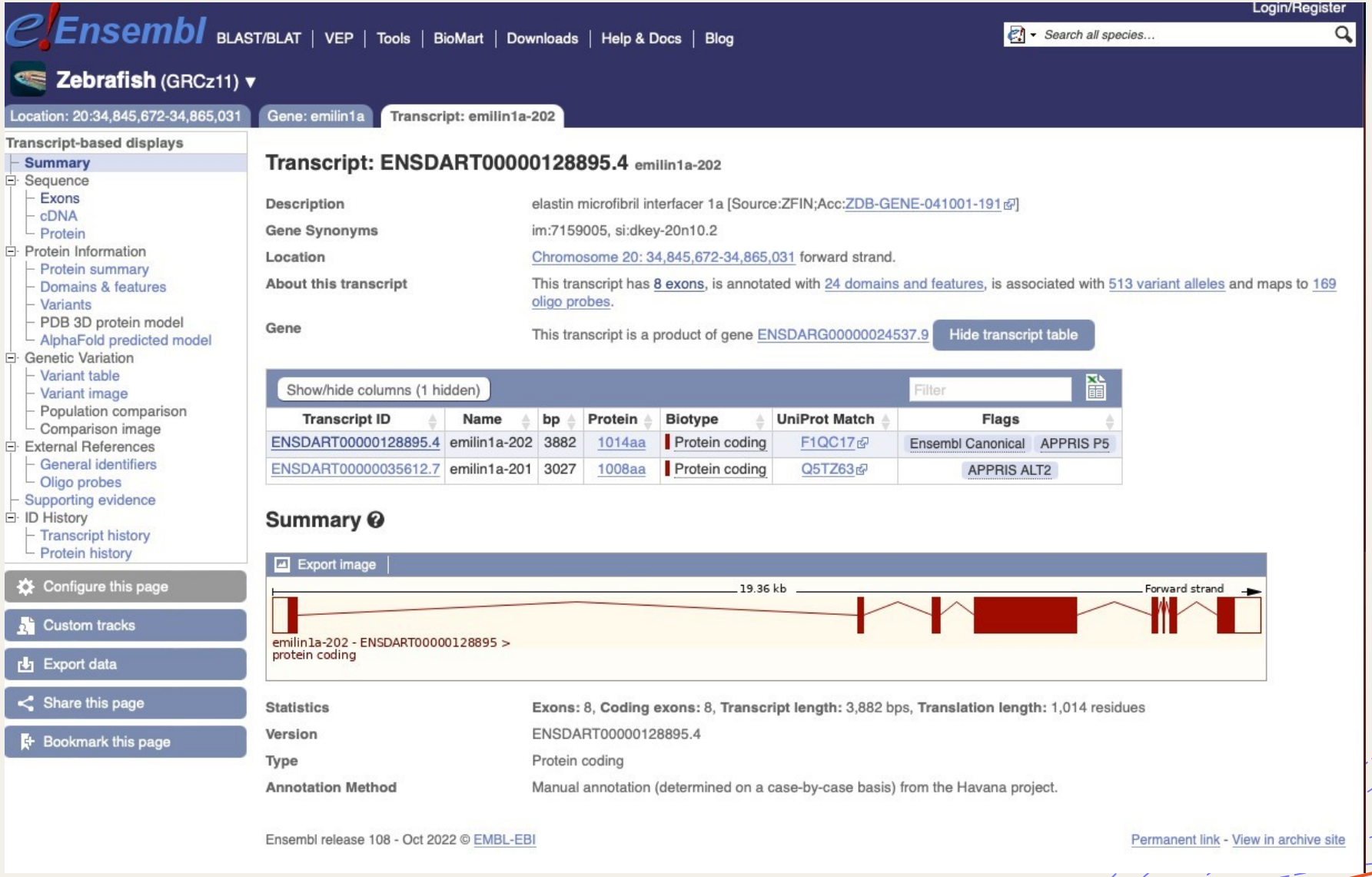
https://www.ensembl.org



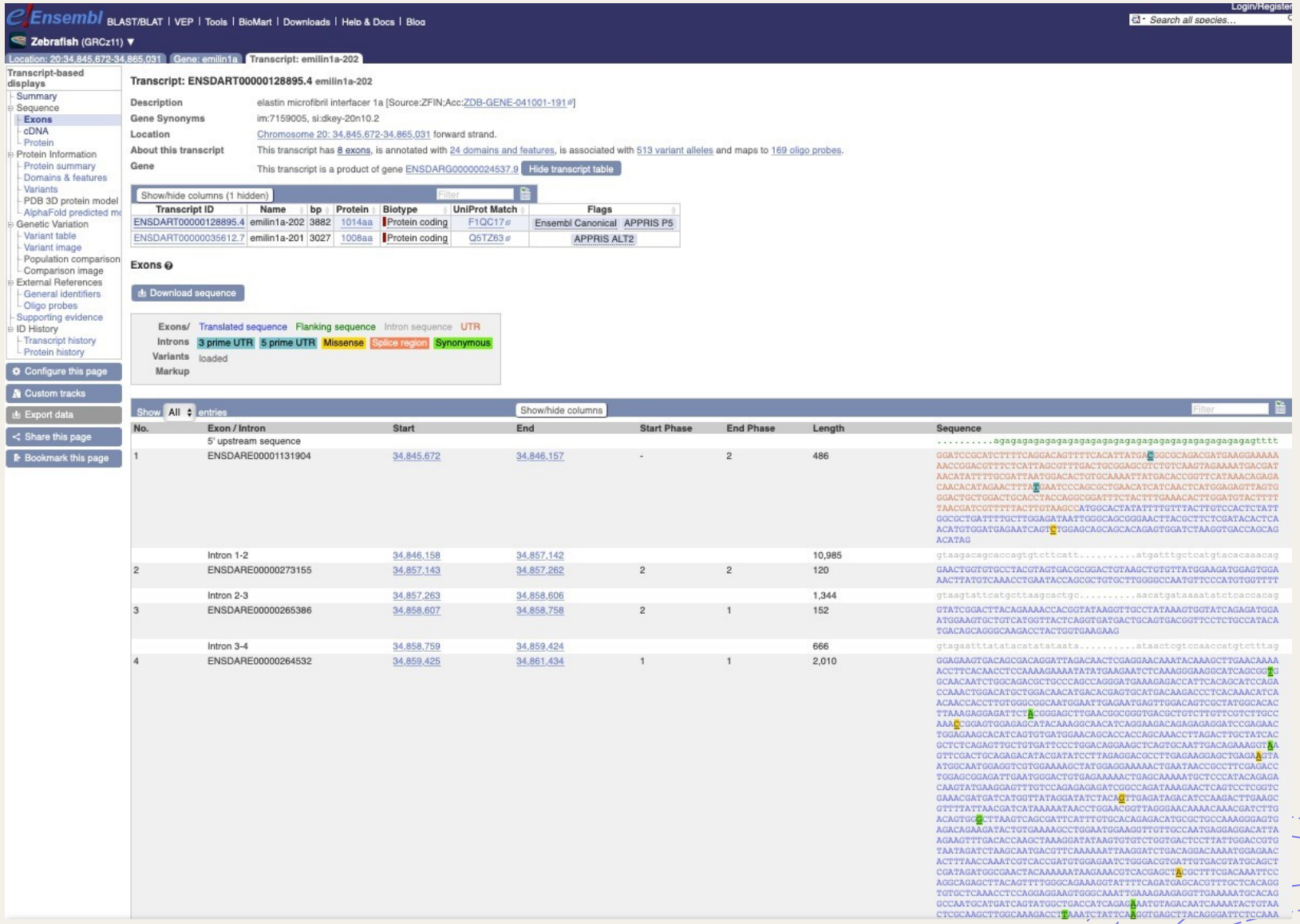
Selection of Target Gene- Gene Analysis in Ensemble



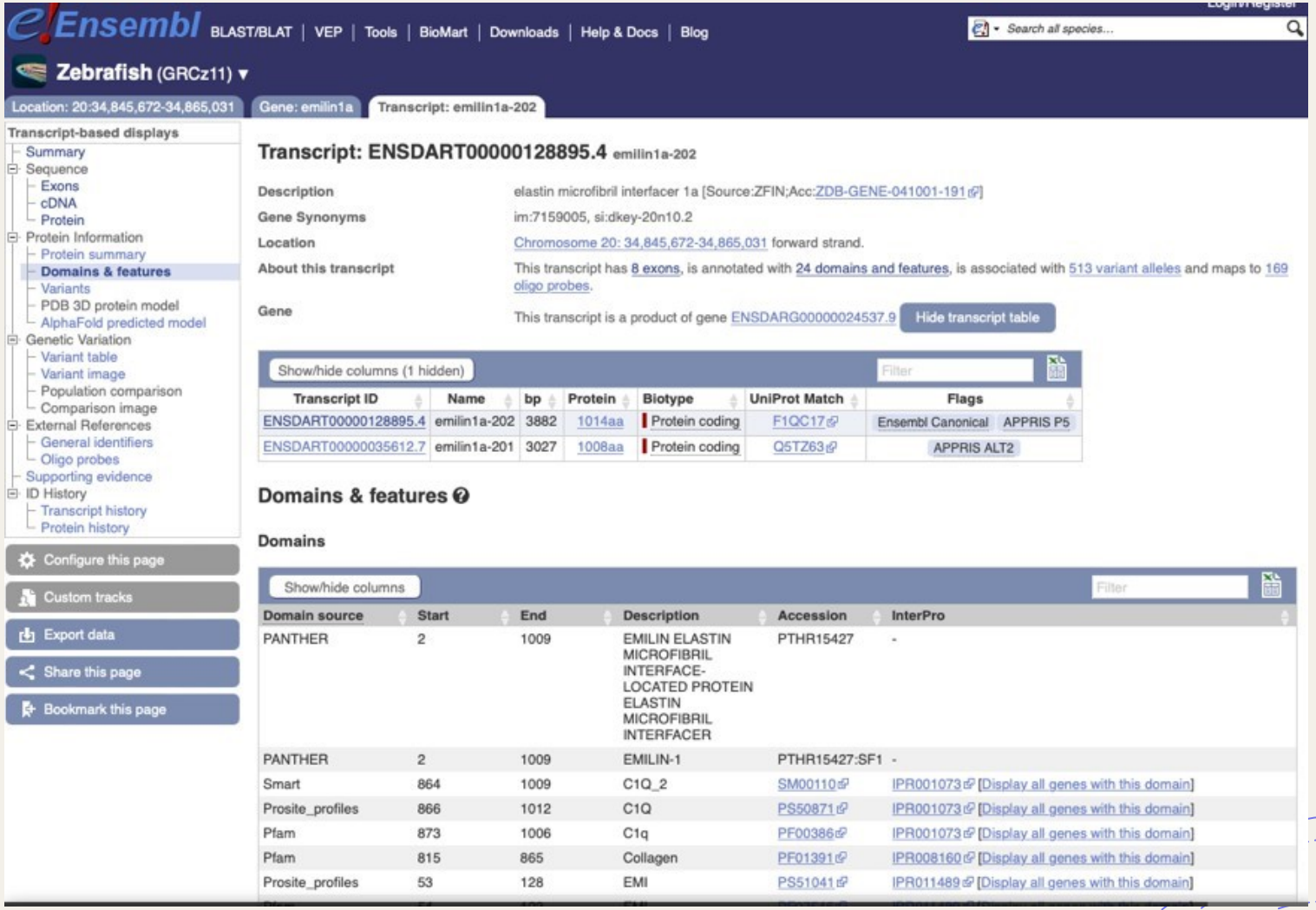
Selection of Target Gene- Gene Analysis in Ensemble



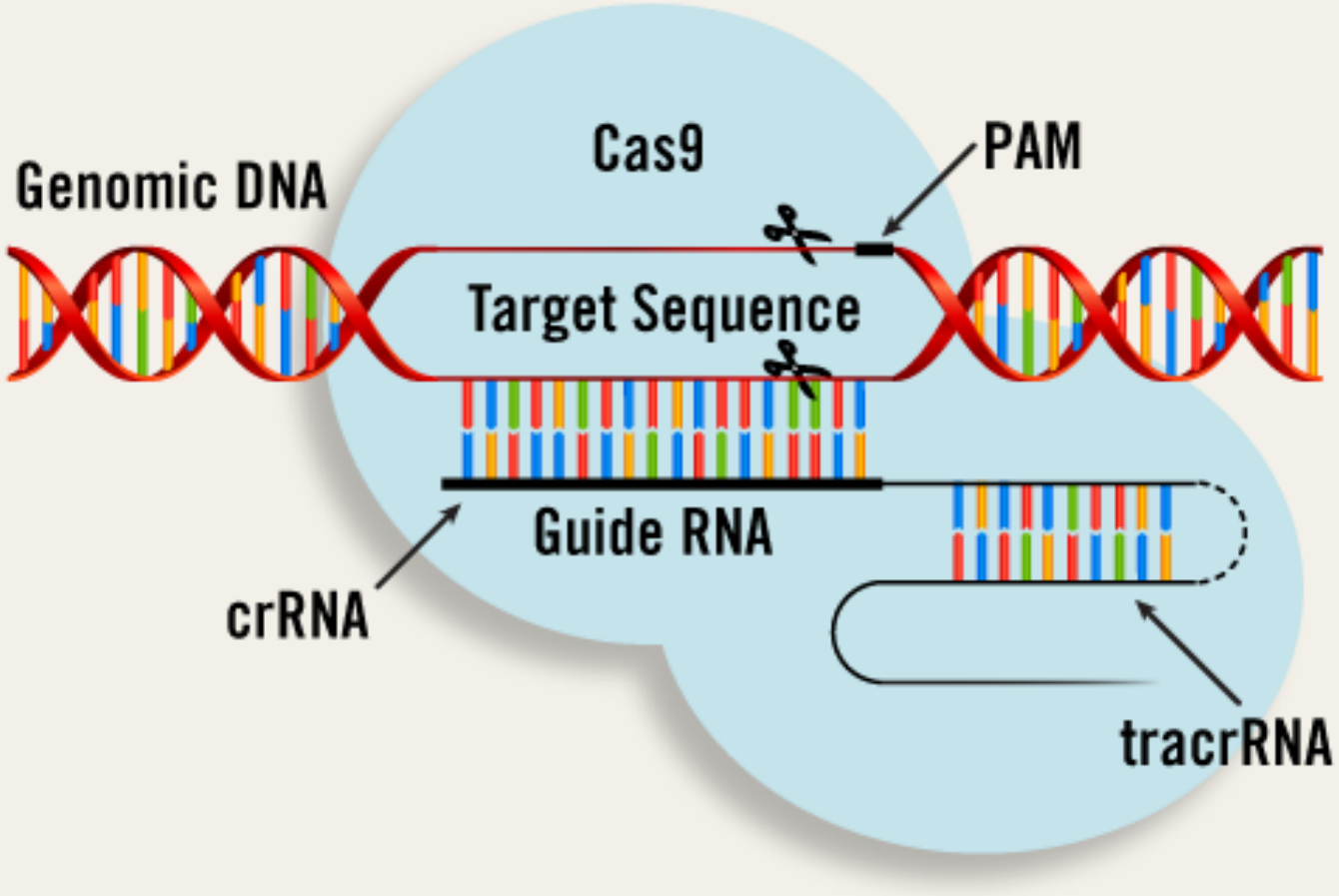
Selection of Target Gene- Gene Analysis in Ensemble



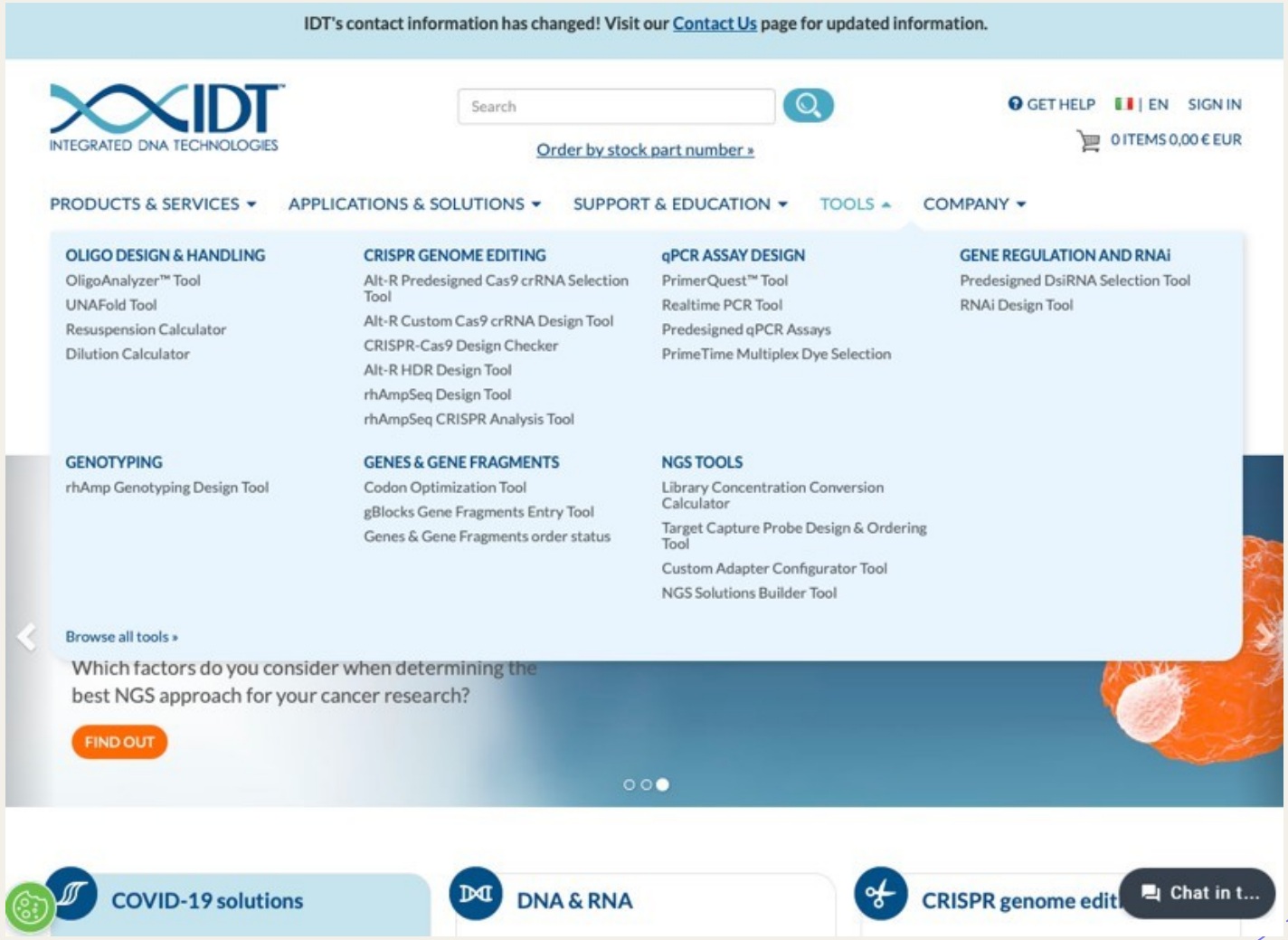
Selection of Target Gene- Gene Analysis in Ensemble



Selection of crRNA



Selection of crRNA



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

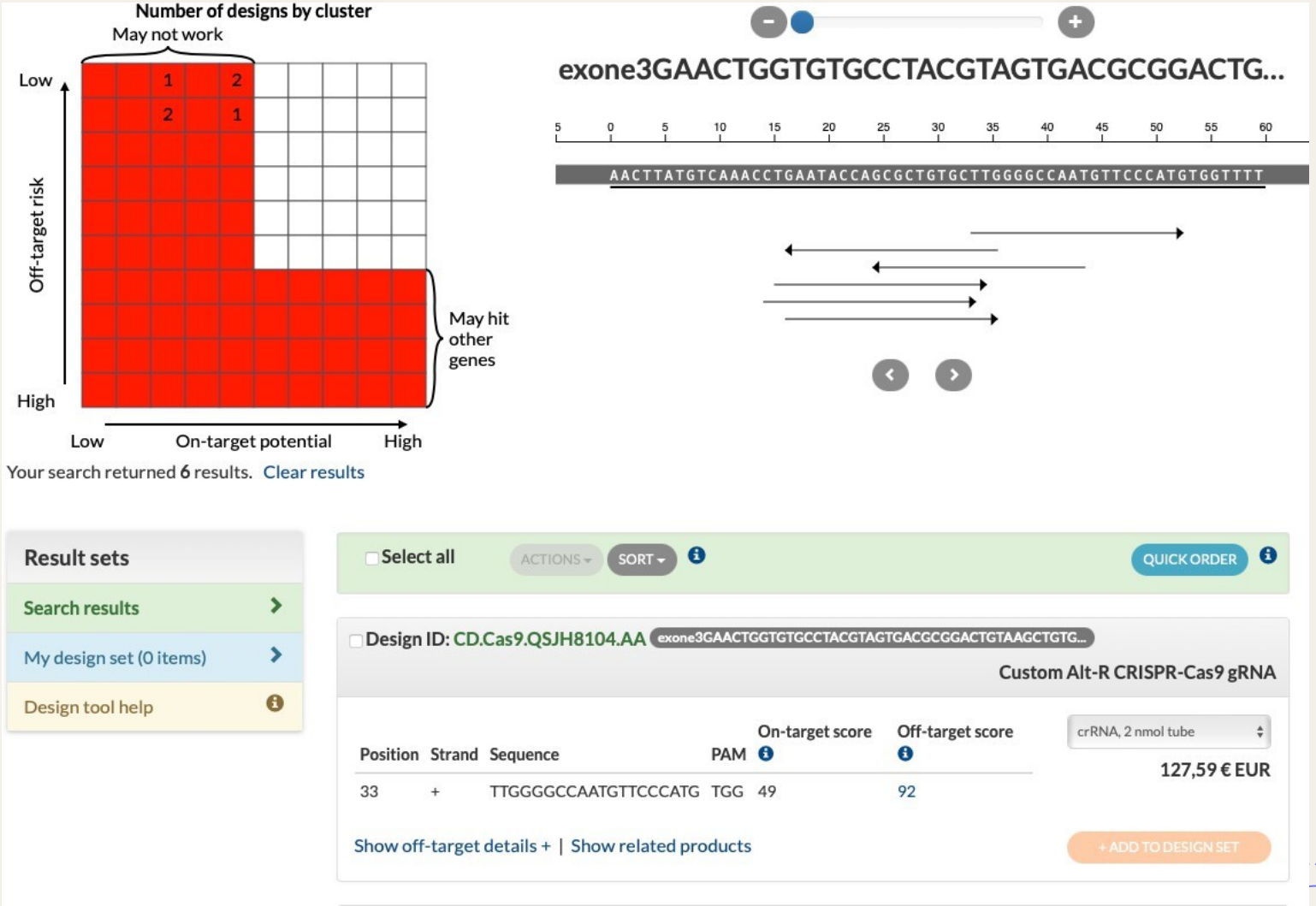
Enter up to 10 FASTA Sequences.

Please enter sequences in standard FASTA formatting.

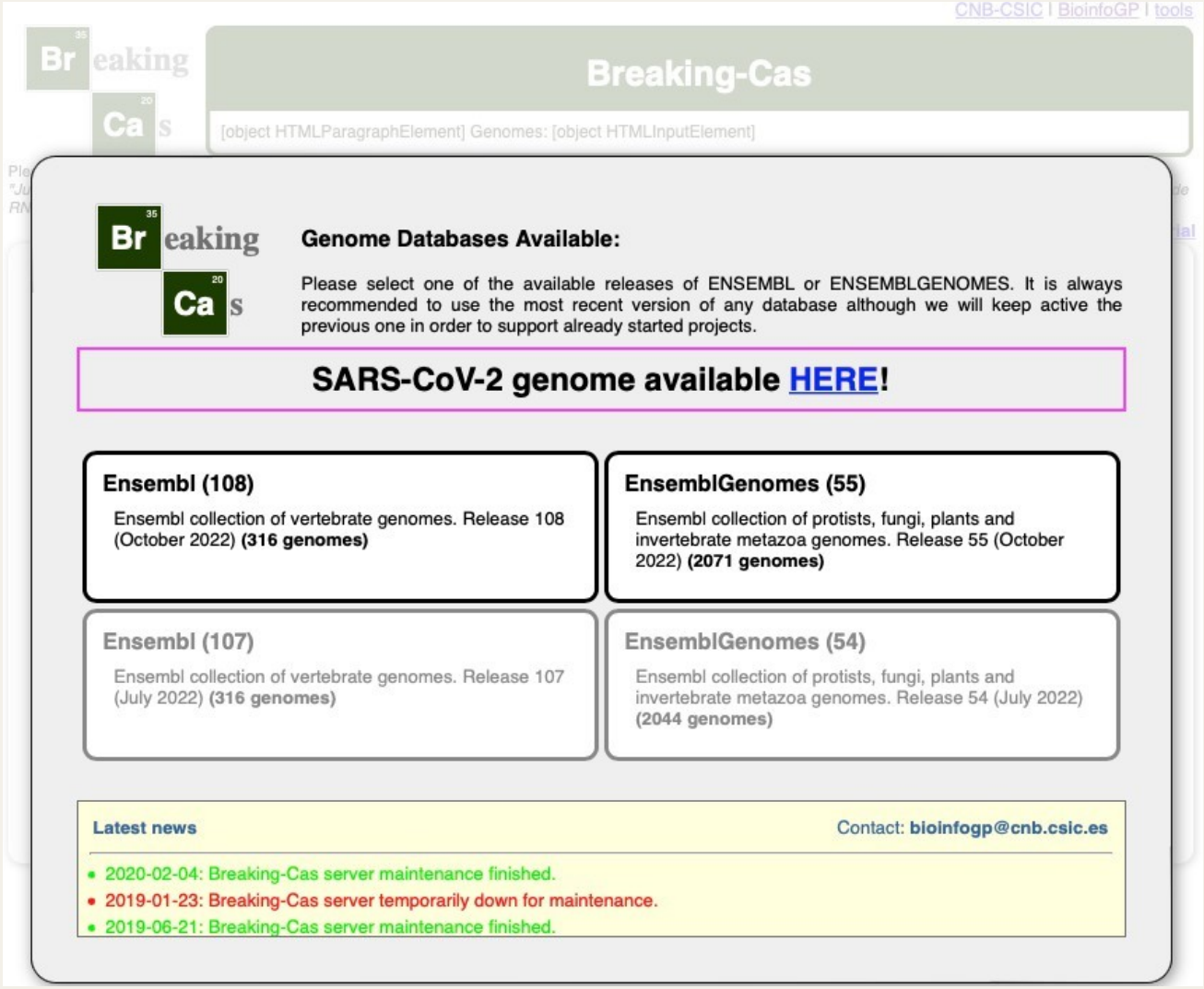
No more than 1000 bases accepted.

> exone3GAACTGGTGTGCCTACGTAGTGACGCGGACTGTAAGCTGTGTTATGGAAGATGGAGTGGA
AACTTATGTCAAACCTGAATACCAGCGCTGTGCTTGGGGCCAATGTTCCCATGTGGTTTT

Selection of crRNA

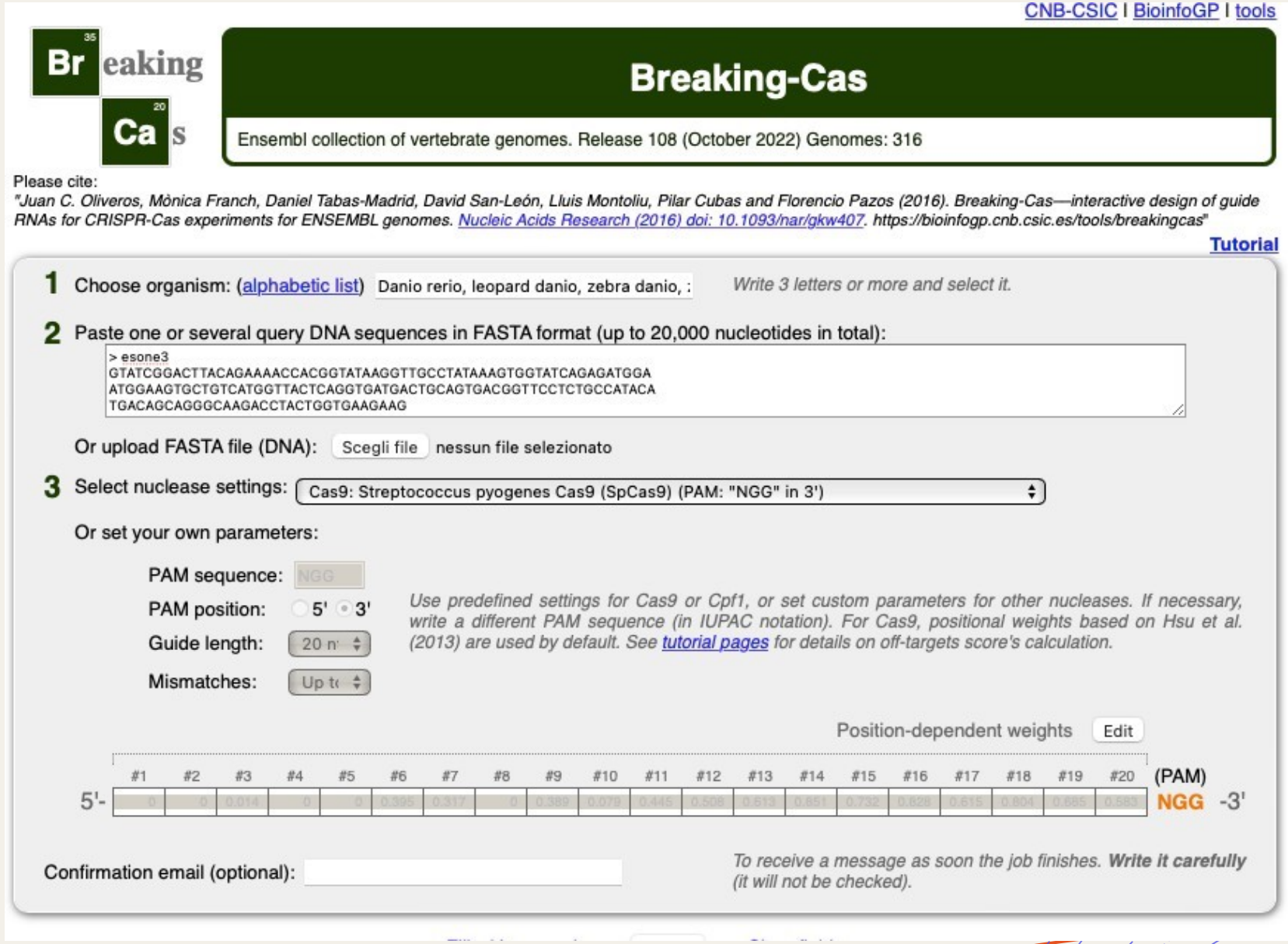


Selection of crRNA



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

Selection of crRNA



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

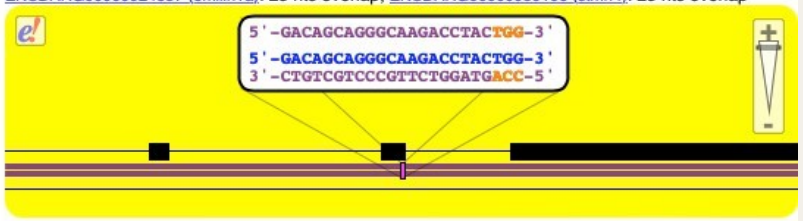
Filter boxes (?) Export Oligos (text-tabulated format)

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	-	CATCTCTGATACCACTTTATAGG	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	+	ATGGAATGGAAGTGCTGTCATGG	1	48	35	82.8
37	56	+	CTATAAAGTGGTATCAGAGATGG	1	72	59	80.3

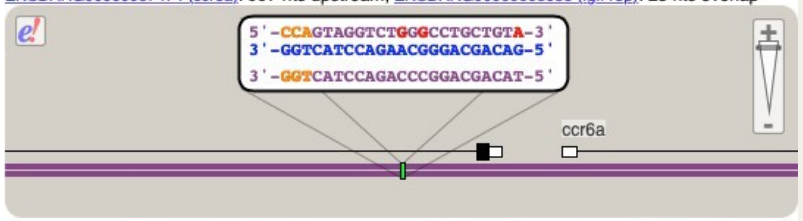
GACAGCAGGGCAAGACCTAC(TGG)

Export Targets (text-tabulated format)

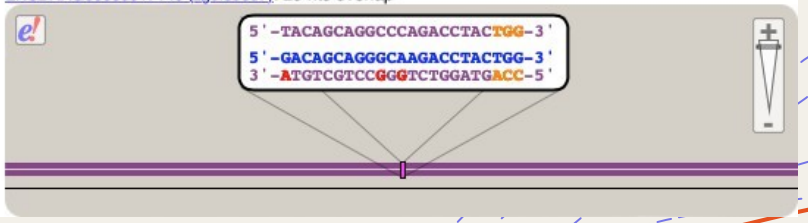
100 20:34858728-34858750(+)
ENSDARG00000024537 (emilin1a): 23 nts overlap; ENSDARG00000030106 (stmn4): 23 nts overlap



1.3 20:30609528-30609550(-)
ENSDARG00000087474 (ccr6a): 997 nts upstream; ENSDARG00000003058 (fgfr1op): 23 nts overlap



1.3 8:4659140-4659162(+)
ENSDARG00000017140 (zgc:63587): 23 nts overlap



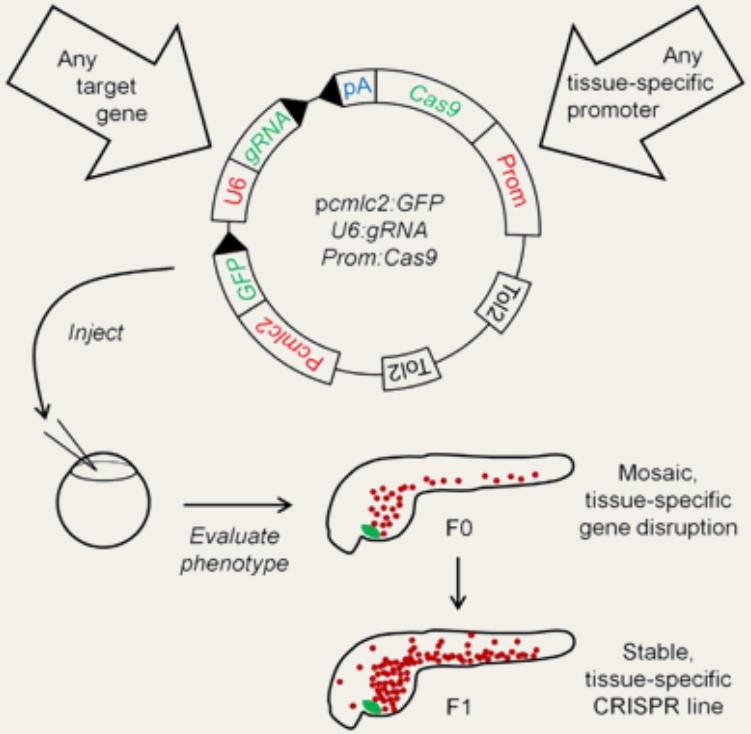
Top tips for success with CRISPR-Cas9

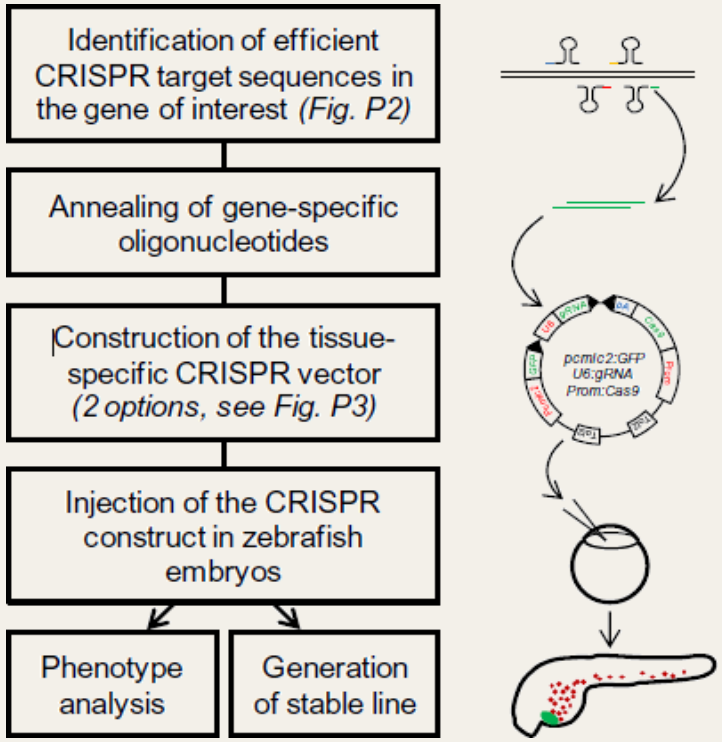
- *Identify a target region*
  1. The target region should be 5' to domains that you suspect may impart functionality.
  2. The target region should be in a constitutive exon. Avoid exons that are sometimes excluded from transcripts unless your project focuses on function of a particular splice form.
  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.

Ablain et al., 2015

Knock-in

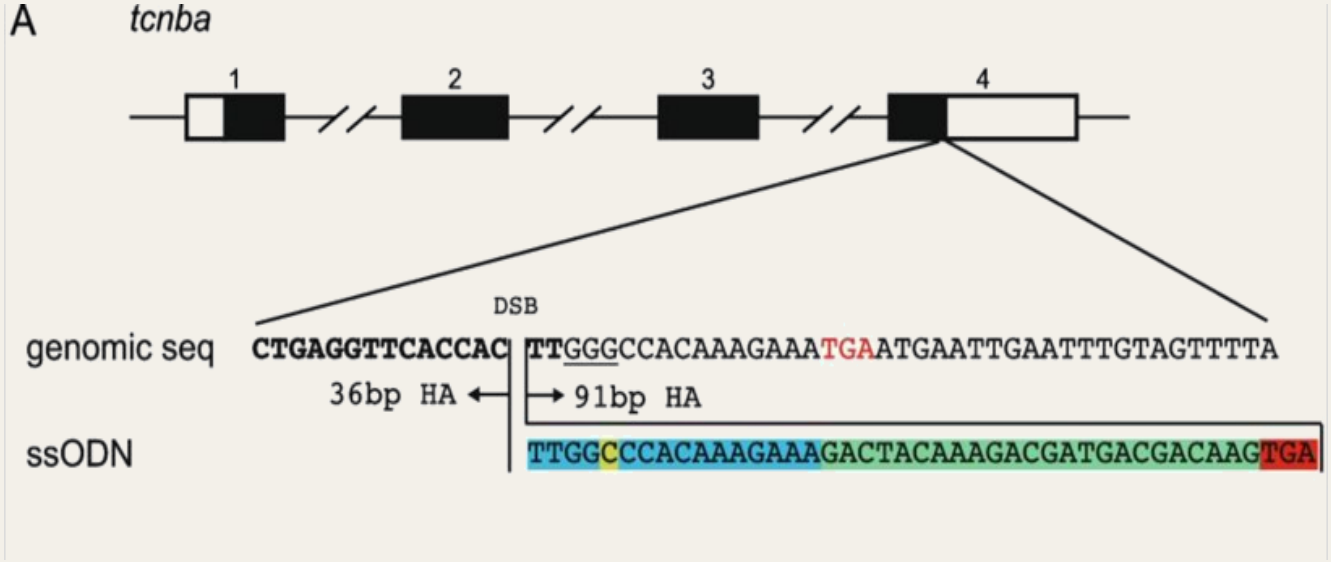
Nat Biotechnol. 2016 Mar;34(3):339-44. doi: 10.1038/nbt.3481. Epub 2016 Jan 20.

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

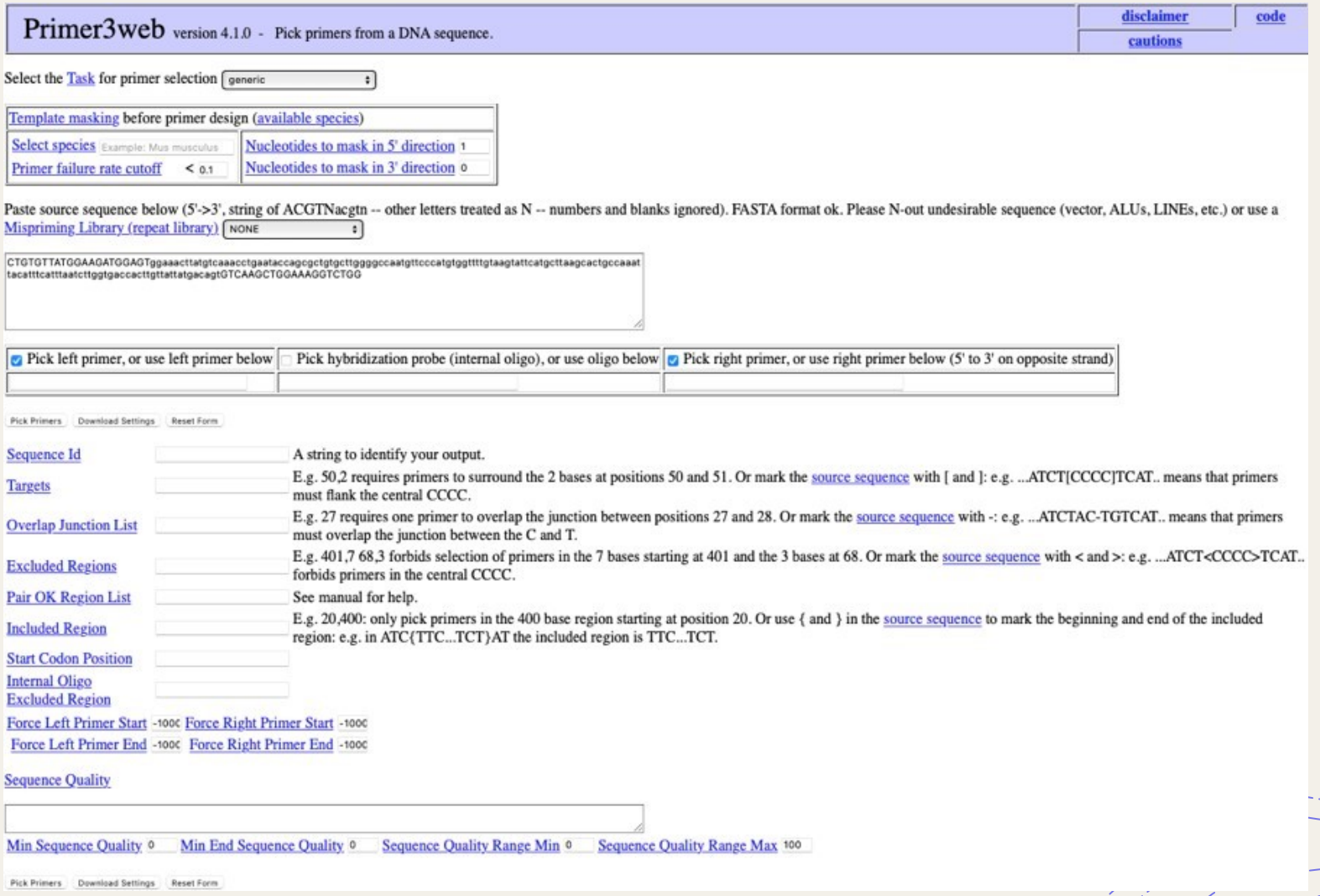
Christopher D Richardson [1 2], Graham J Ray [1 2], Mark A DeWitt [1 2], Gemma L Curie [1 2], Jacob E Corn [1 2]

Affiliations + expand

PMID: 26789497 DOI: 10.1038/nbt.3481



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



https://primer3.ut.ee/

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

Primer3 Output

```
PRIMER PICKING RESULTS FOR

Template masking not selected
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%  any_th  3'_th hairpin seq
LEFT PRIMER          2   23   59.16   43.48    0.00   0.00    0.00 TGTGTTATGGAAGATGGAGTgga
RIGHT PRIMER       177   20   58.47   55.00    0.00   0.00    0.00 CCAGACCTTTCCAGCTTGAC
SEQUENCE SIZE: 177
INCLUDED REGION SIZE: 177

PRODUCT SIZE: 176, PAIR ANY_TH COMPL: 15.07, PAIR 3'_TH COMPL: 8.30

    1 CTGTGTTATGGAAGATGGAGTggaaacttatgtcaaacctgaataccagcgctgtgcttg
       >>>>>>>>>>>>>>>>>>>>>>

   61 gggccaatgttcccatgtggttttgtaagtattcatgcttaagcactgccaaattacatt


  121 tcatttaatcttggtgaccacttgttattatgacagtGTCAAGCTGGAAAGGTCTGG
                                         <<<<<<<<<<<<<<<<<<<<

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm      gc%  any_th  3'_th hairpin seq

 1 LEFT PRIMER          8   22   57.34   40.91    0.00   0.00    0.00 ATGGAAGATGGAGTggaaactt
   RIGHT PRIMER       175   21   59.23   47.62    0.00   0.00    0.00 AGACCTTTCCAGCTTGACact
   PRODUCT SIZE: 168, PAIR ANY_TH COMPL: 15.07, PAIR 3'_TH COMPL: 0.00
```

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%

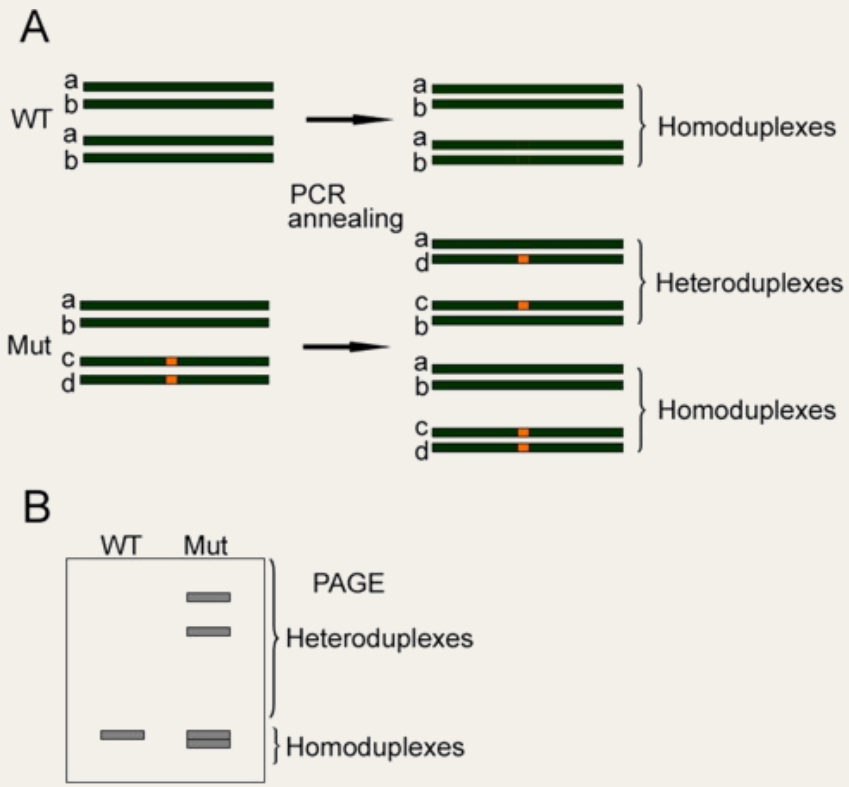
- Check for predicted SNPs in the target region using the “variation features” track on Ensembl
- The product size should be 100-160 bp; with these short PCR products, small sequence changes can produce large changes in melting temperature
- Ensure that at least 20 bp are present between each primer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

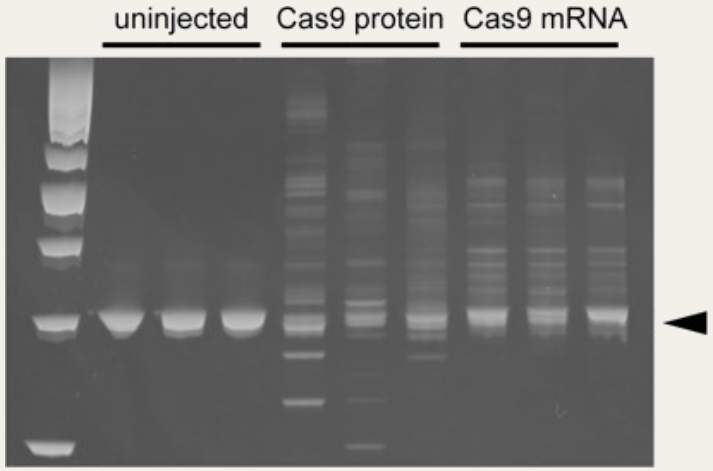
Primers for sequencing reactions:

- 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

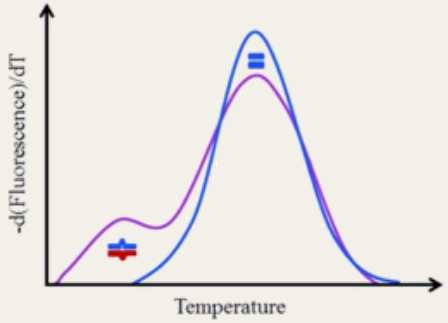
indel mutations rate : 15/15 (100%)

```
TCTCTCCGCAGCGGTCTGGGCTACATCCTGGGA  WT      ×0
TCTCTCCGCAG---TCTGGGCTACATCCTGGGA  -3      ×5
TCTCTCCGC----------TACATCCTGGGA    -12     ×4
TCTCTCCGCAG--------------------GA  -20     ×2
TCTCTCCG-----GTCTGGGCTACATCCTGGGA  -5      ×1
TCTCT----------GGGCTACATCCTGGGA    -12     ×1
TCTCTCCGCAGAGAGAGA--CTACATCCTGGGA  -9/+7   ×1
TCTCTCCGCAAAACATGTTATAGCTCCGGTCTG  -1/+16  ×1
```

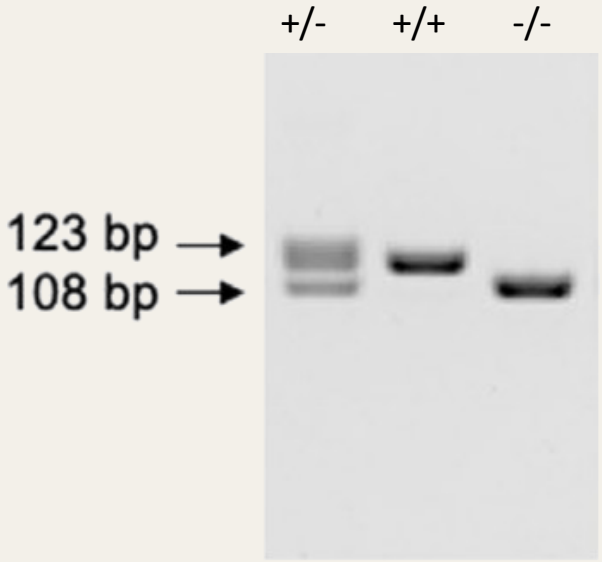
Identification of founders

F1 embryos screening (founders identification)

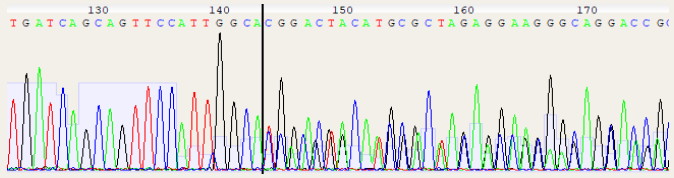
HRMA (High Resolution Melting Analysis (HRMA)



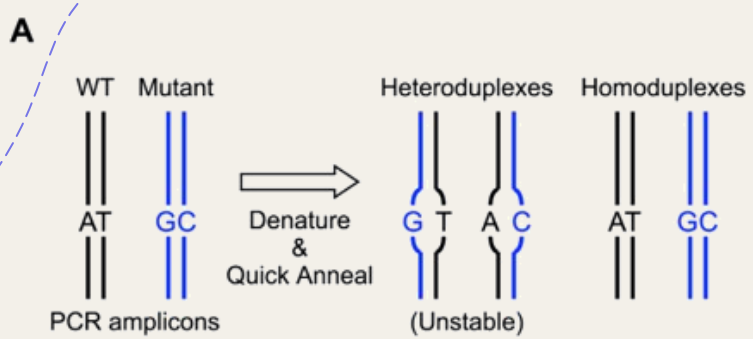
PCR on 4% agarose gel

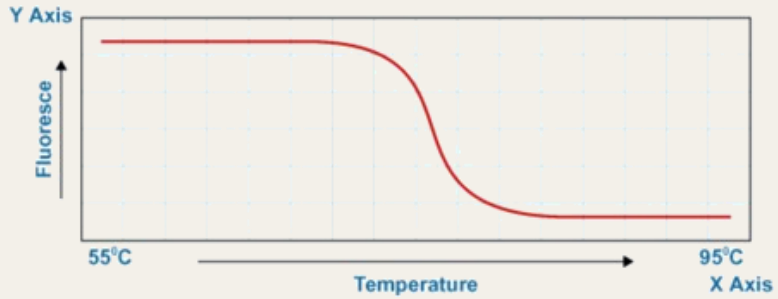


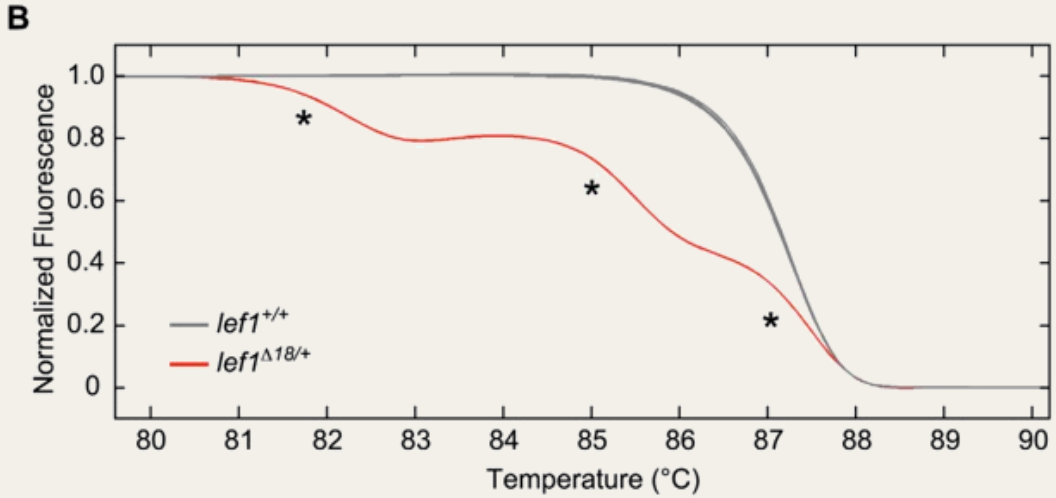
Sequencing



Identification of founders: High Resolution Melt Analysis (HRMA)







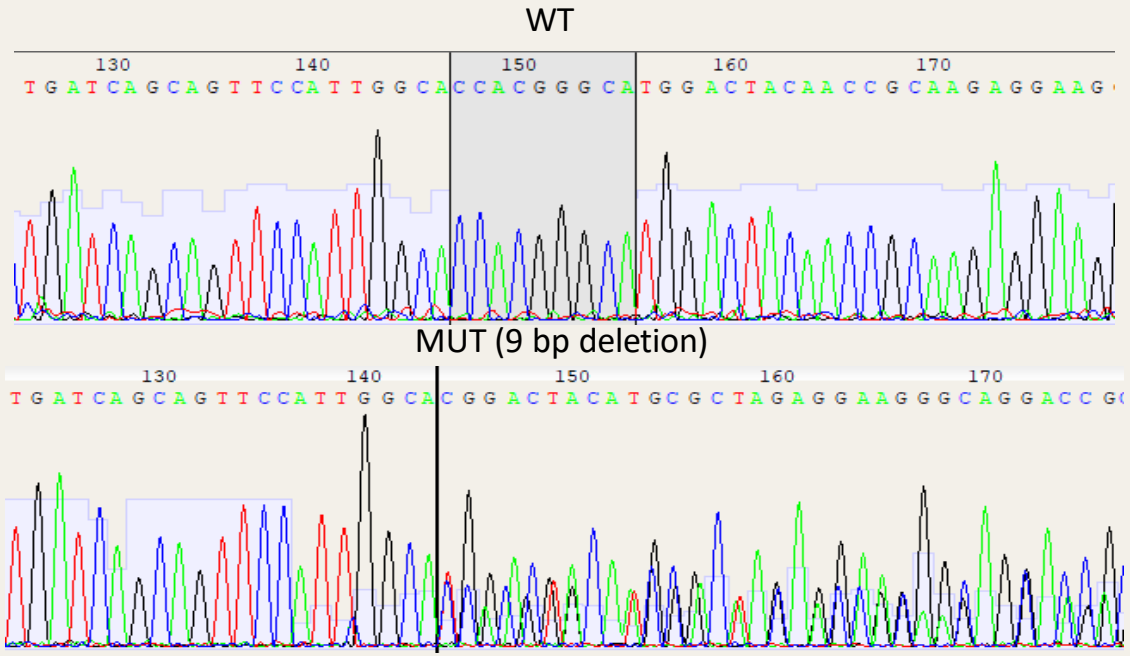
Identification of mutations

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/

http://www.crisprscan.org

3. Indel detection following CRISPR/Cas9 mutagenesis by PCR

https://primer3.ut.ee/

https://tide.nki.nl