Lecture 8

Research Technologies in Developmental Biology

Gene and protein expression analysis methods

Methods to study gene function in embryos *in vivo*

Chapter 5 in Larsen's *Human Embryology* (4th edition)
Chapter 4 in Scott Gilbert's *Developmental Biology* (8th edition)

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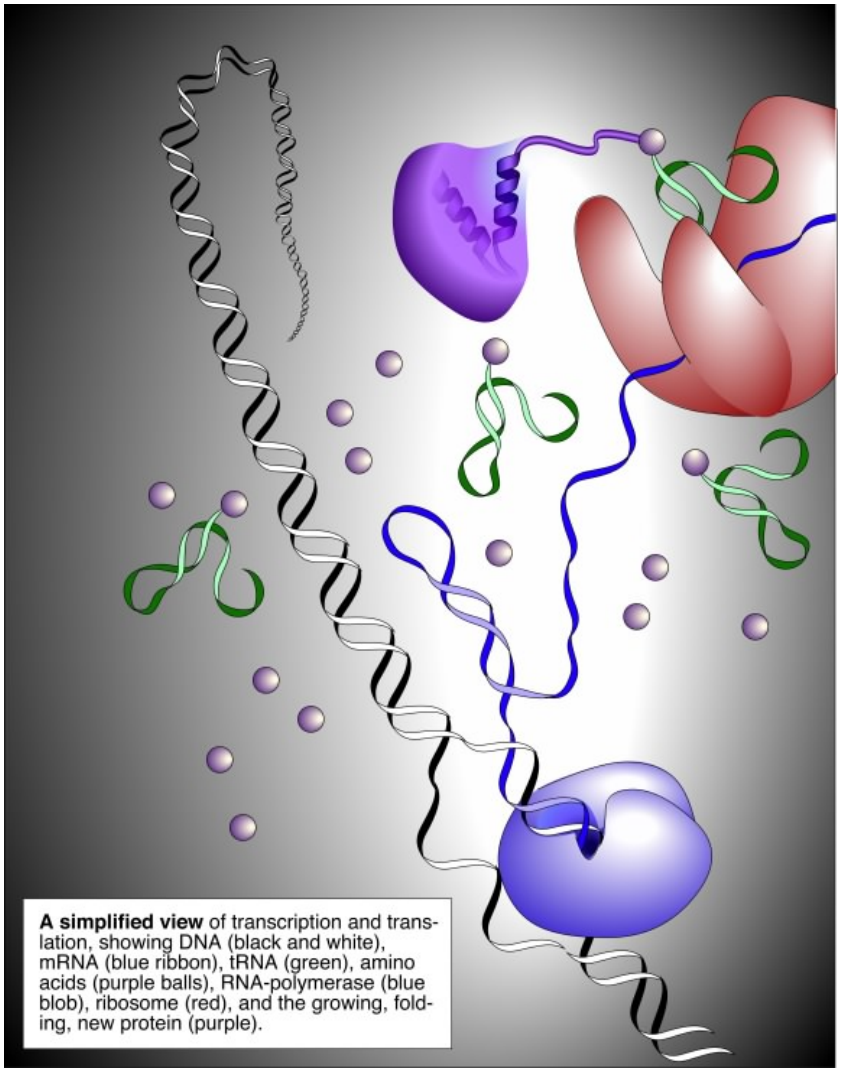
Developmental Genetics Research is driven by two main questions:

Where is the gene/protein expressed during development?

What function does the gene/protein have?

Gene and protein expression analysis methods



A simplified view of transcription and translation, showing DNA (black and white), mRNA (blue ribbon), tRNA (green), amino acids (purple balls), RNA-polymerase (blue blob), ribosome (red), and the growing, folding, new protein (purple).

Gene expression:

DNA

Transcription ↓

mRNA

Translation ↓

Proteins

↓

Function

Gene and protein expression analysis methods

Detection of **protein** expression:

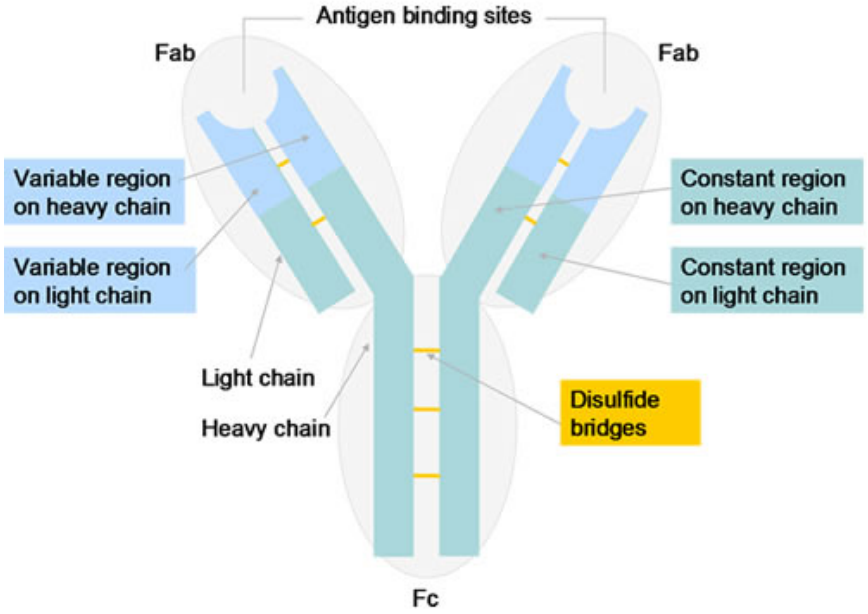
- Antibodies
- Immunodetection

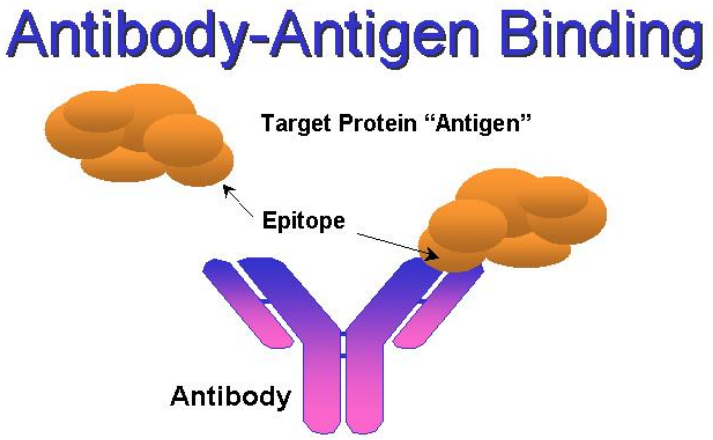
Detection of **RNA** expression:

- RTPCR
- Quantitative RT PCR
- In situ hybridization
- RNA sequencing

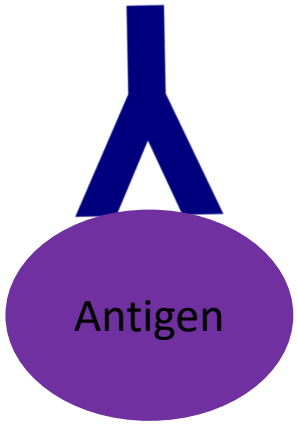
Antibodies – not only for immunity!

An **antibody**, also known as an **immunoglobulin**, is a large, Y-shaped protein produced mainly by the immune system to identify and neutralize pathogens such as bacteria and viruses

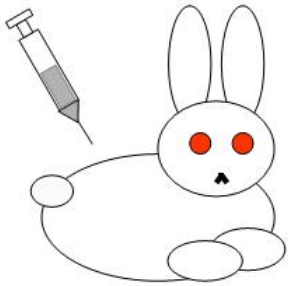




We can produce antibodies binding defined antigens at large scale

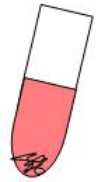


immunization



A solution containing a specific antigen is injected into a rabbit; the rabbit is immunized.

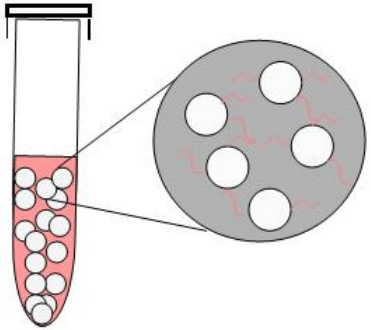
collect serum



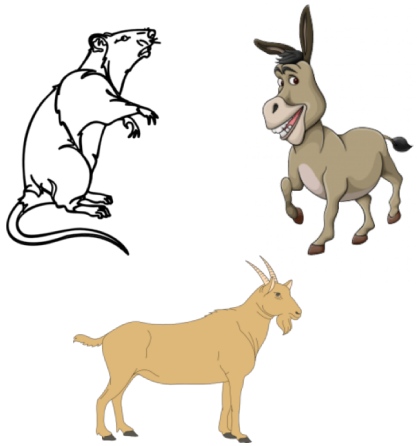
serum

Antiserum is taken from the rabbit; the supernatant contains the antibodies of interest.

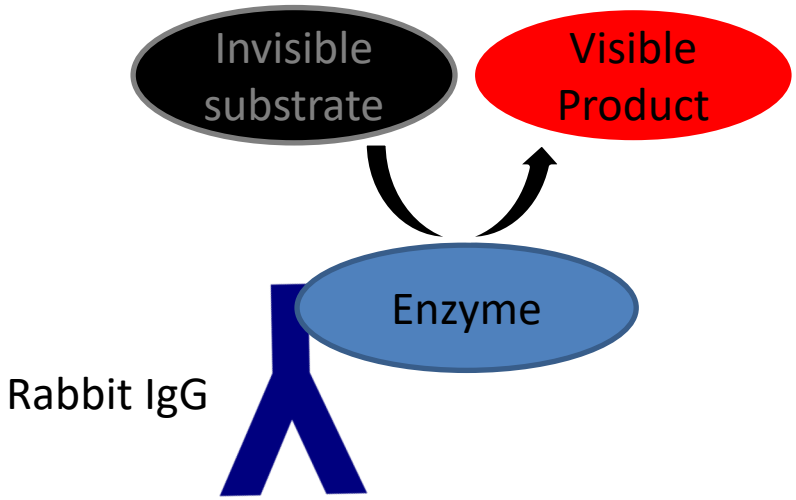
purification

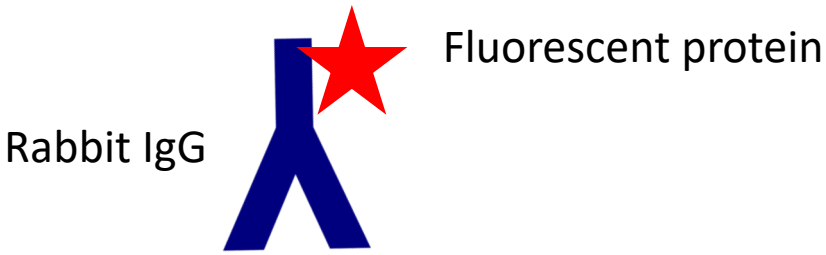


Antibodies are incubated with sepharose beads that is conjugated to the original antigen.



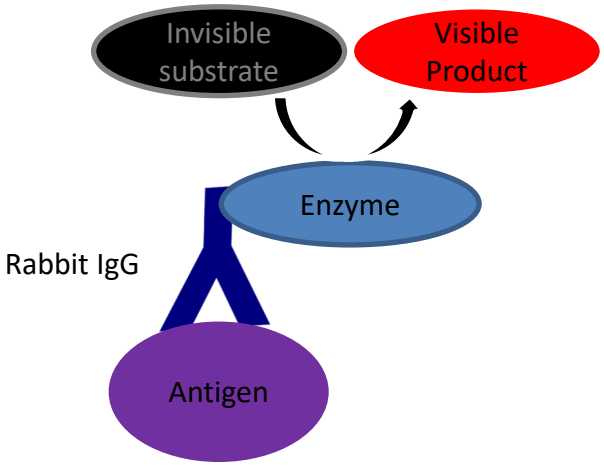
We can modify antibodies – add conjugates to Fc portion IgG

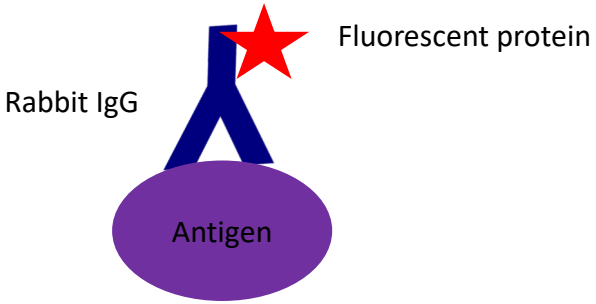




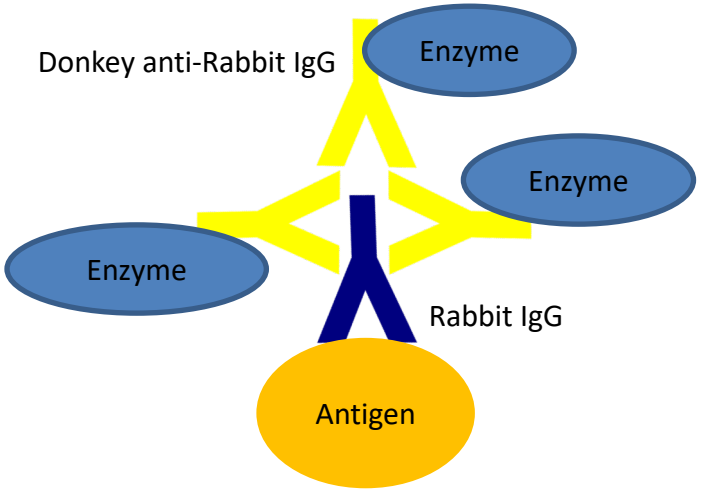
We use conjugated antibodies for direct and indirect immunodetection

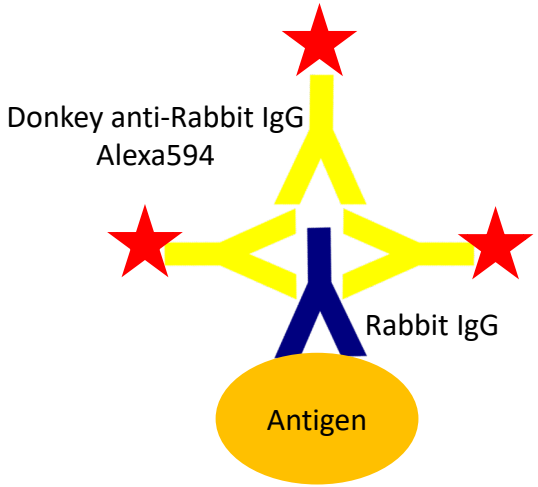
Direct





Indirect





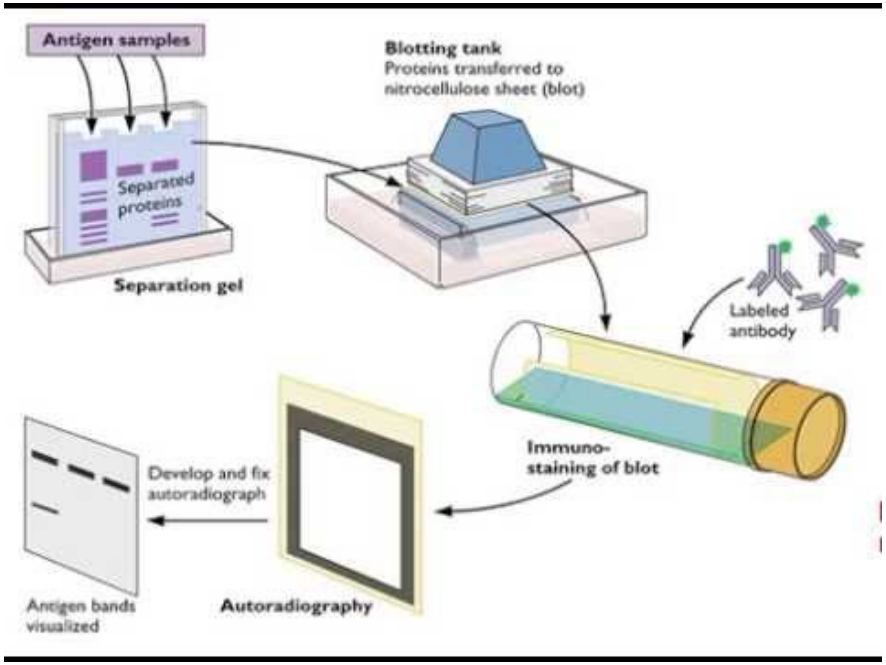
Advantage over direct immunodetection: signal amplification!

Protein expression analyses

Immunodetection in lysates

Cell tissue lysates

Polyacrylamide gel electrophoresis (PAGE) and Western Blotting



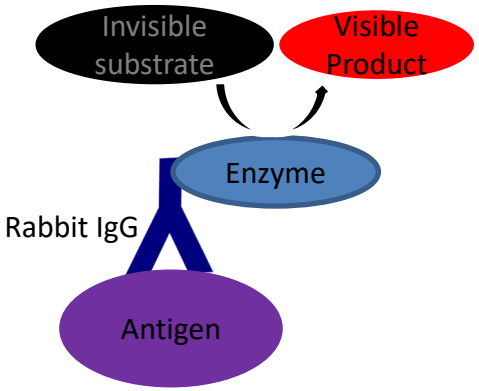
Quantitative

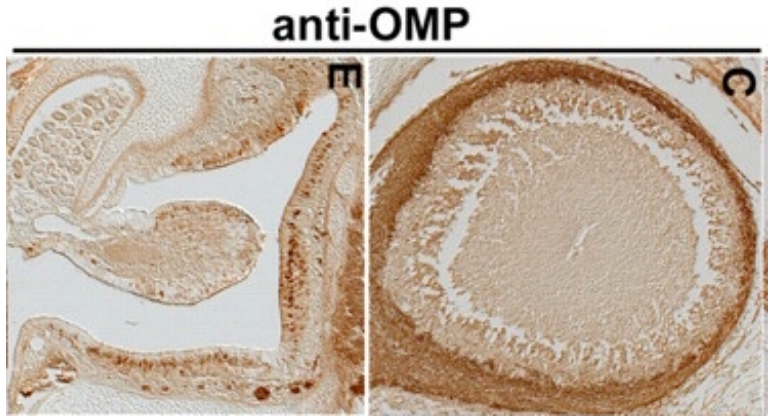
Rapid

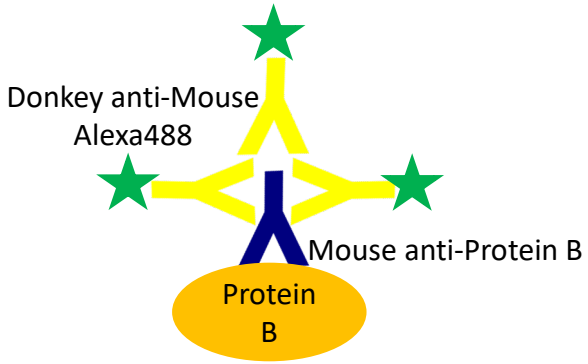
No spatial expression information

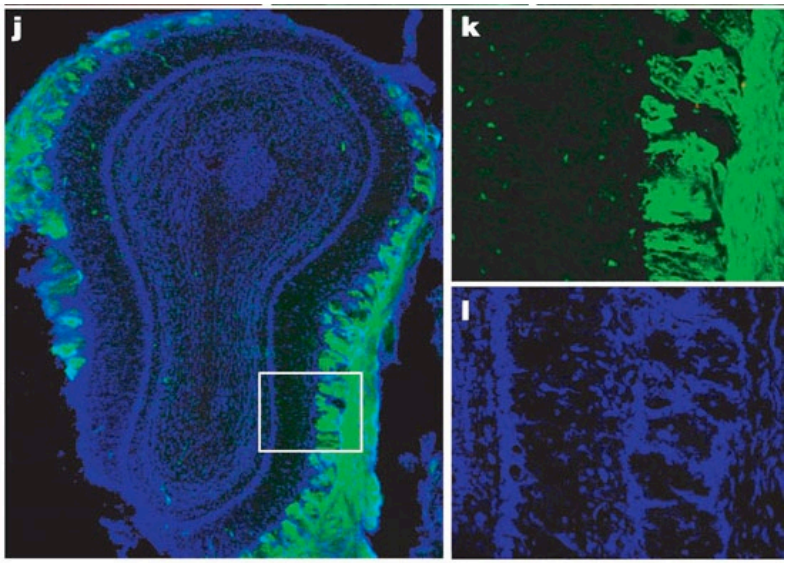
Protein expression analyses

Immunodetection *in situ*

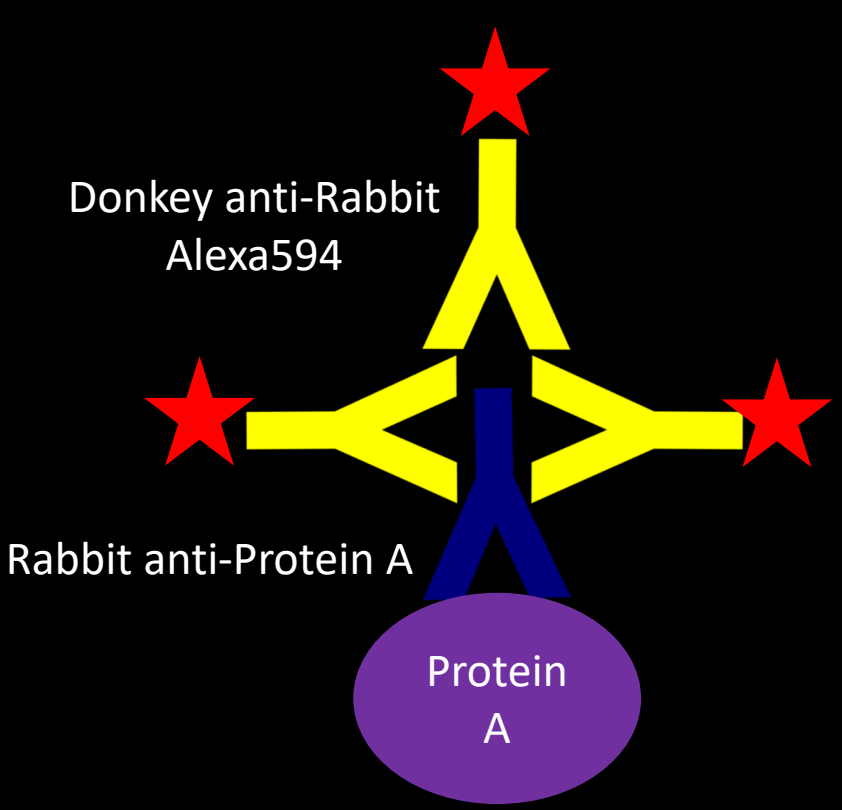


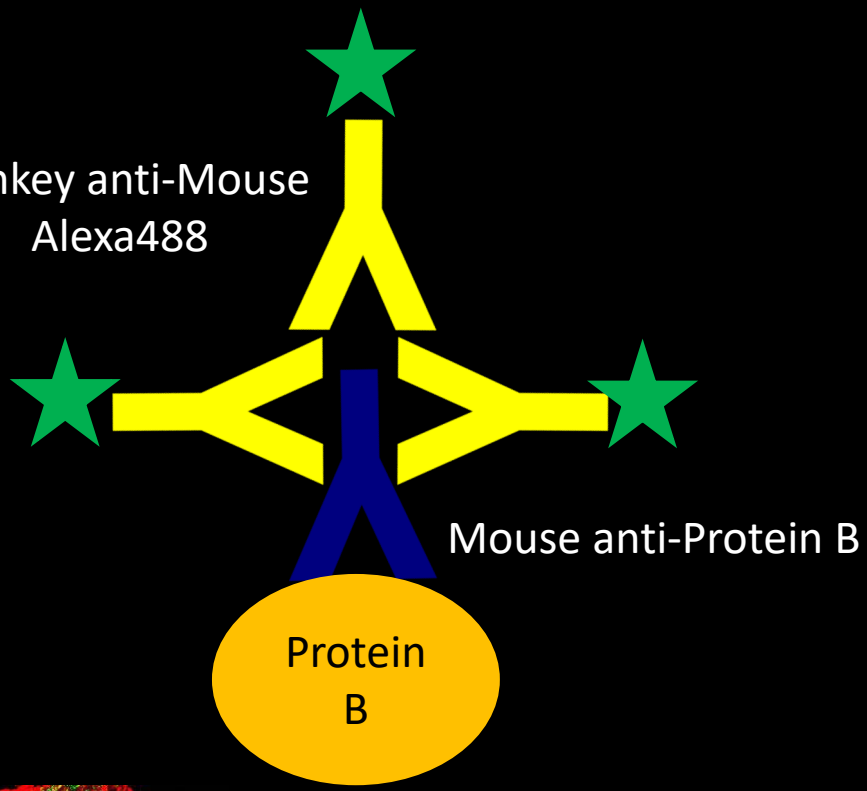


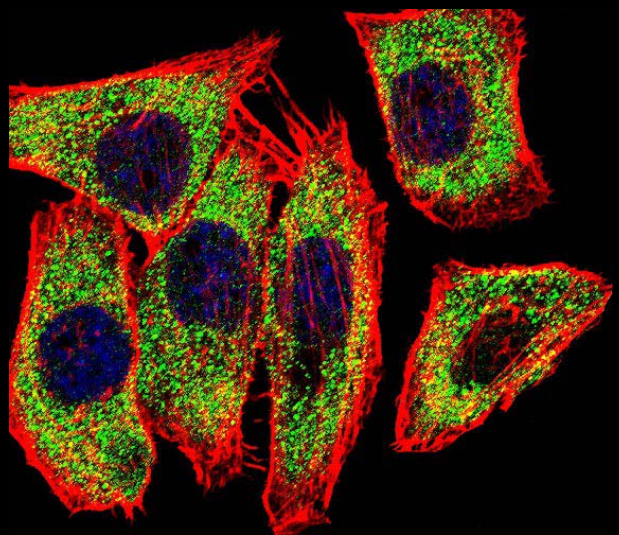




Immunofluorescence – Double Labelling

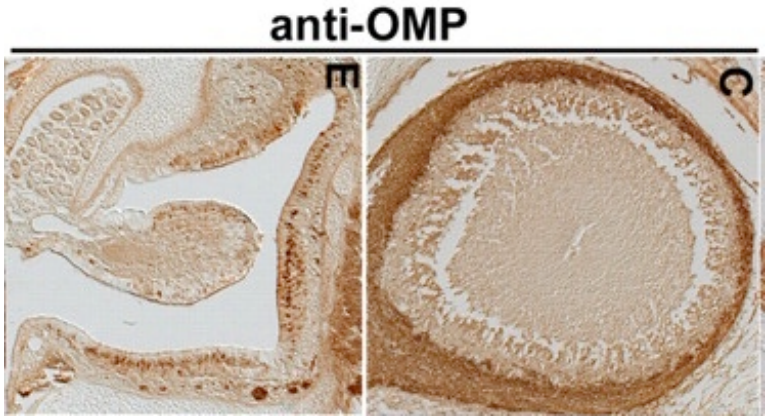




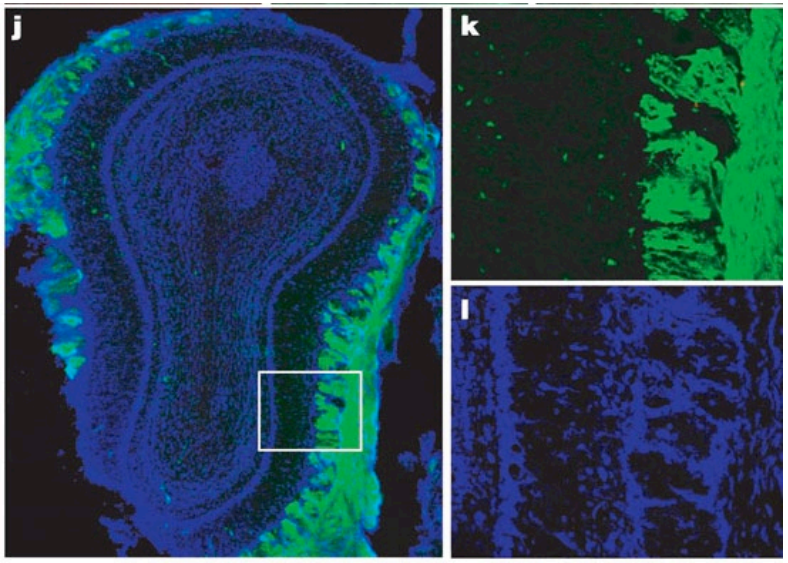


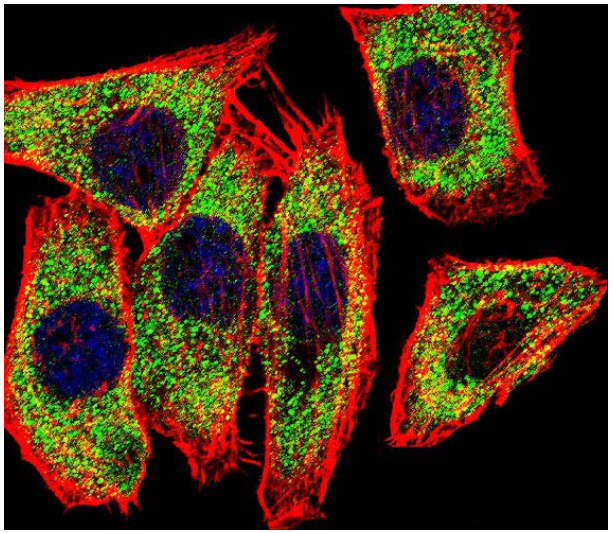
Protein expression analyses

Immunodetection *in situ*



OMP/DAPI



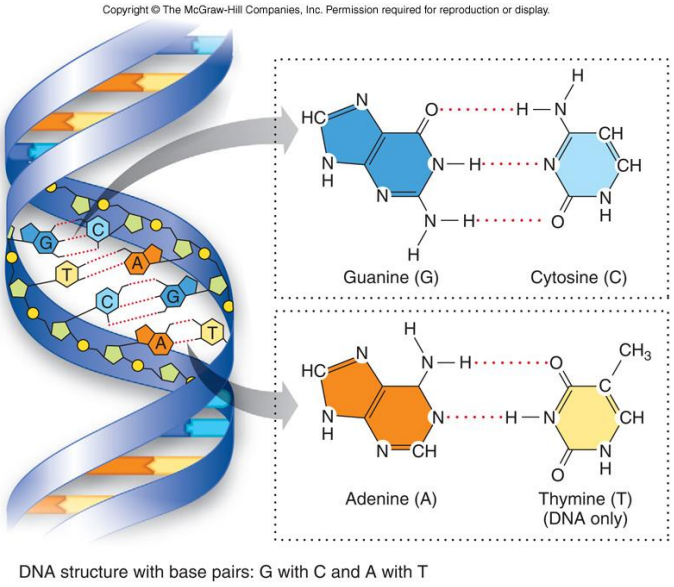


Limited quantitative information
Rapid
Excellent spatial expression information

Gene expression analyses

Detection by Nucleotide Base Pairing

Base Pairing in the genome
DNA:DNA



DNA structure with base pairs: G with C and A with T

Base Pairing in transcription
DNA:RNA

Complementary base pairing

DNA Base	Complementary RNA Base
G	C
C	G
A	U
T	A

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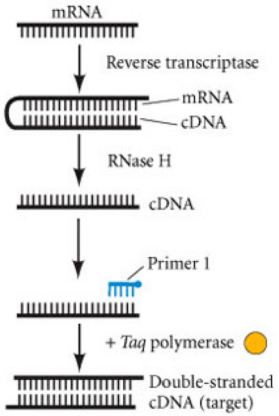
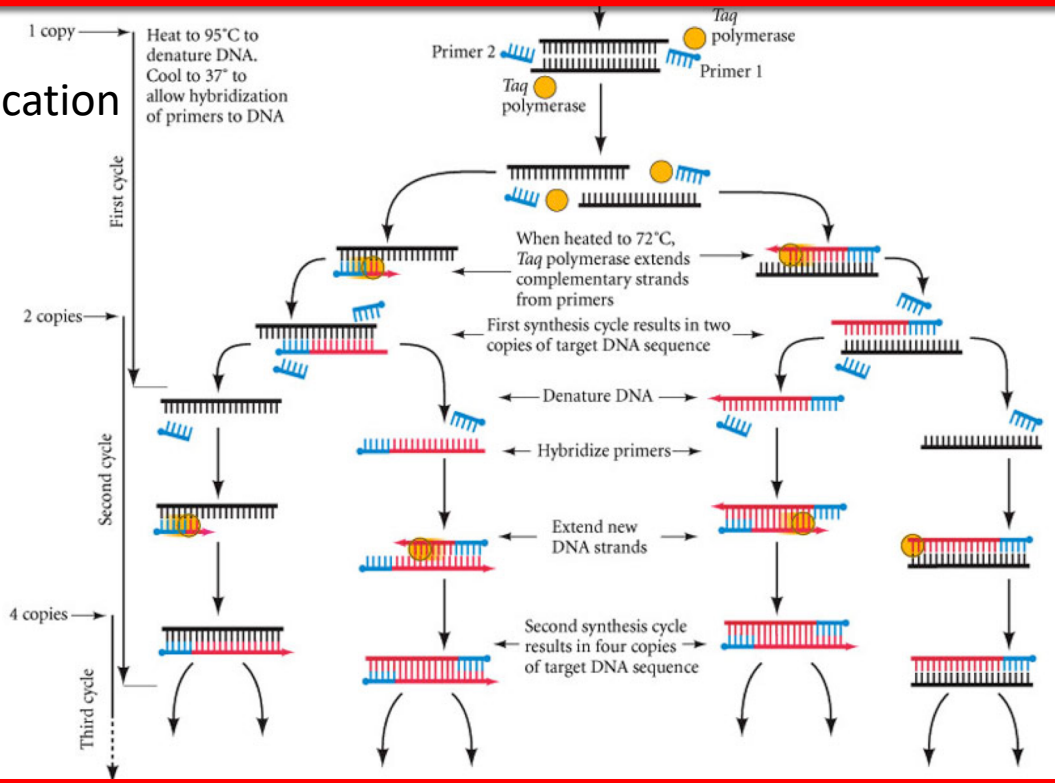
Gene expression analyses

RT PCR – RNA solution

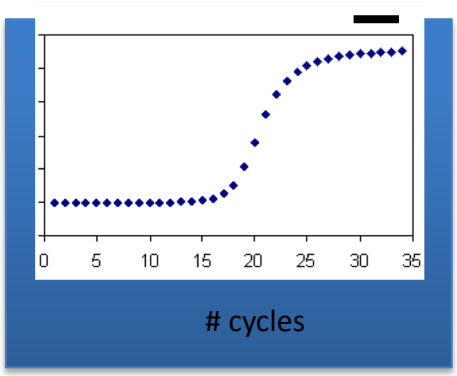
Step 1
Reverse transcription,
Generation of double stranded cDNA



Step 2:
PCR amplification



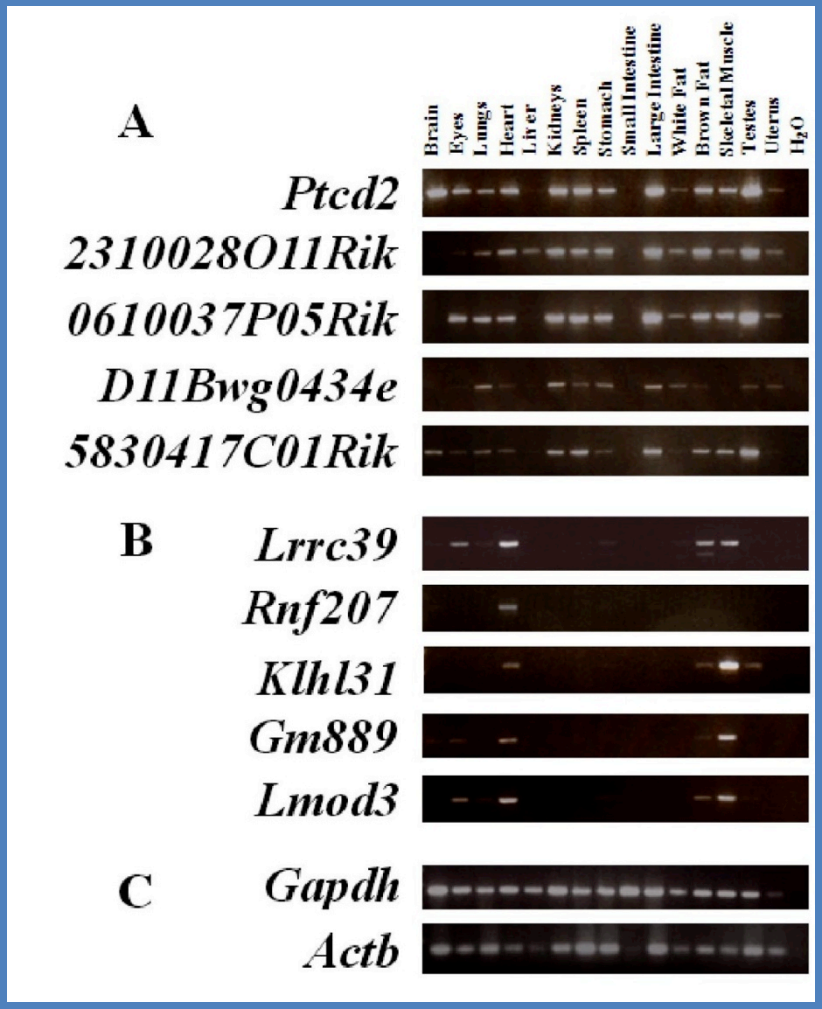
Transfer
Product to
DNA gel



Gene expression analyses

RT PCR – RNA solution

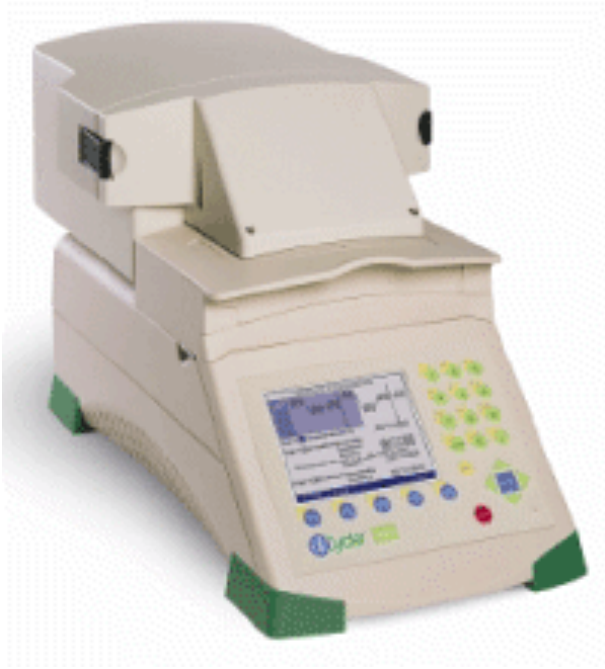
Example experimental results



Not quantitative (Y/N answer)
Rapid (2-3 hours)
No spatial expression information

Gene expression analyses
quantitative real time RT PCR

- Used for
  - Quantitative gene expression (both relative and absolute),
  - Genotyping,
  - miRNA analysis
  - SNP analysis,
  - Pathogen detection
- Measures PCR amplification as it occurs
- More sensitive than conventional RTPCR



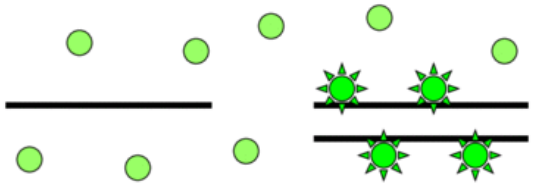
Gene expression analyses

quantitative real time RT PCR

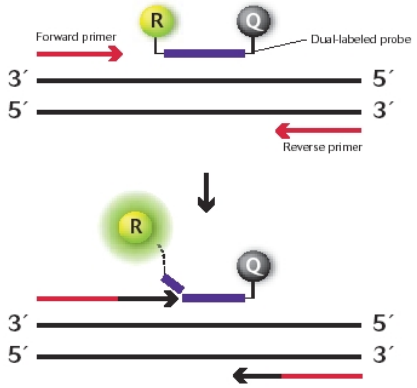
Method:

- Isolate RNA
- Make cDNA with reverse transcriptase
- Carry out PCR with primers to amplify genes of interest and intercalating fluorescent dye SYBR Green or Taqman probes
- Detect fluorescent signal during linear amplification phase as measure for amount of PCR product made

SYBR green intercalates double stranded cDNA



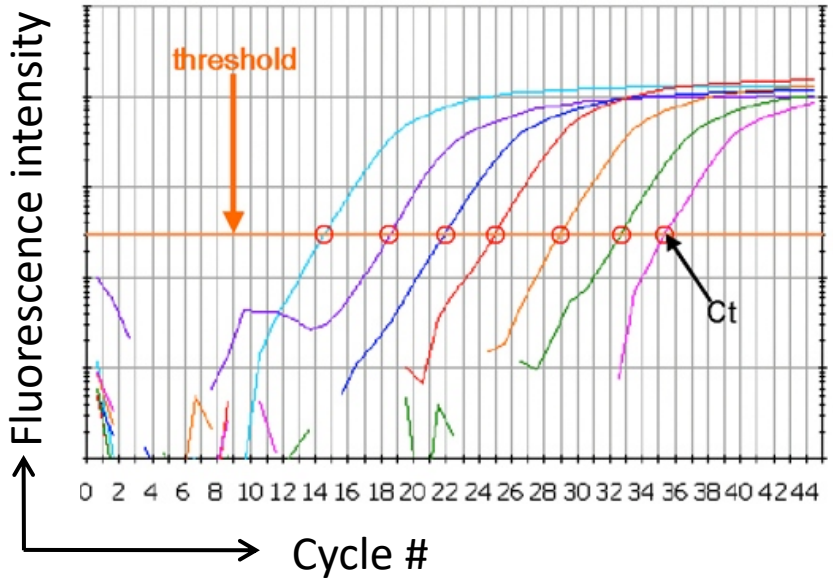
Taqman probes specific for target cDNAs
cDNA duplication results in signal emission

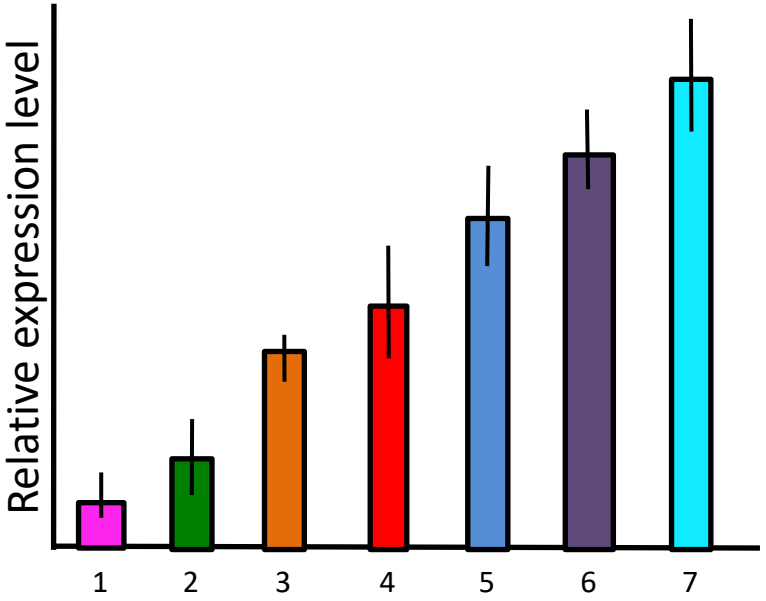


Gene expression analyses

quantitative real time RT PCR

Fluorescent signal intensity (Ct) is measure for amount of product



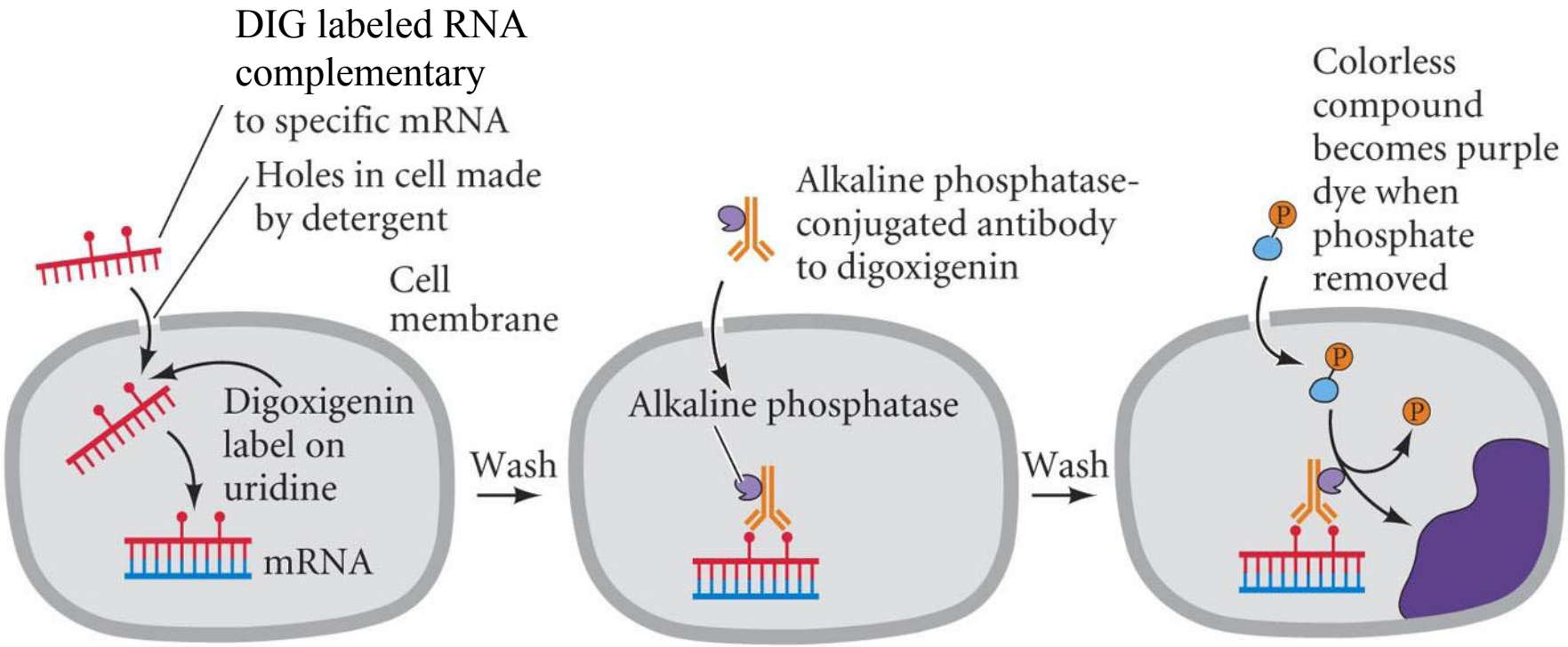


- Quantification of expression levels of:
  - different genes within one RNA sample
  - the same gene in different samples (against a reference ‘house hold gene’)
- Relative vs absolute quantification (against a known standard)

Gene expression analyses

in situ hybridization

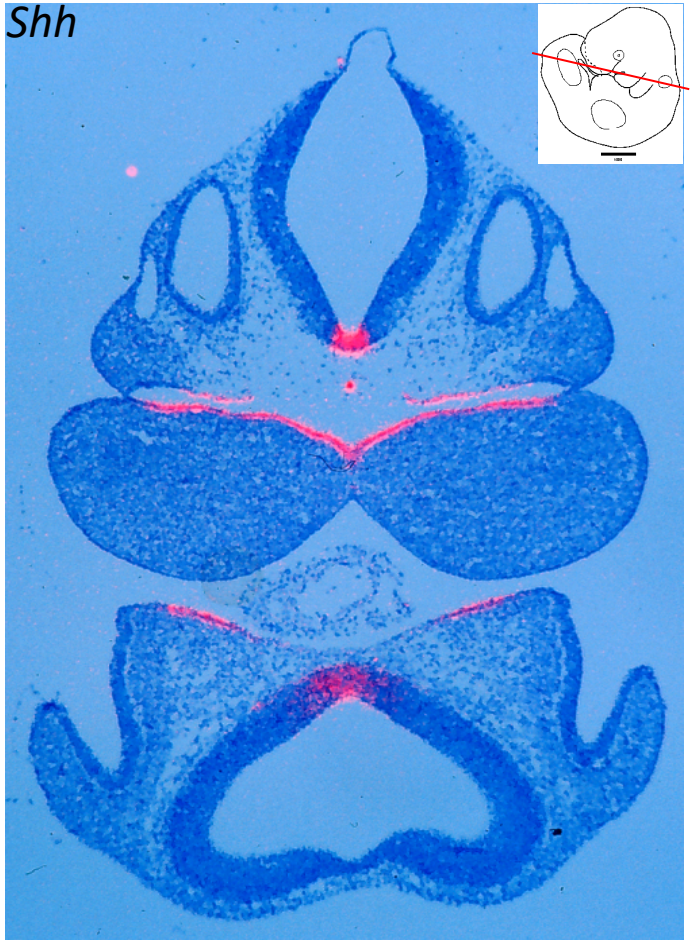
Prepare labeled antisense RNA probe:
- Digoxigenin (DIG)
- Radioactive label



Gene expression analyses

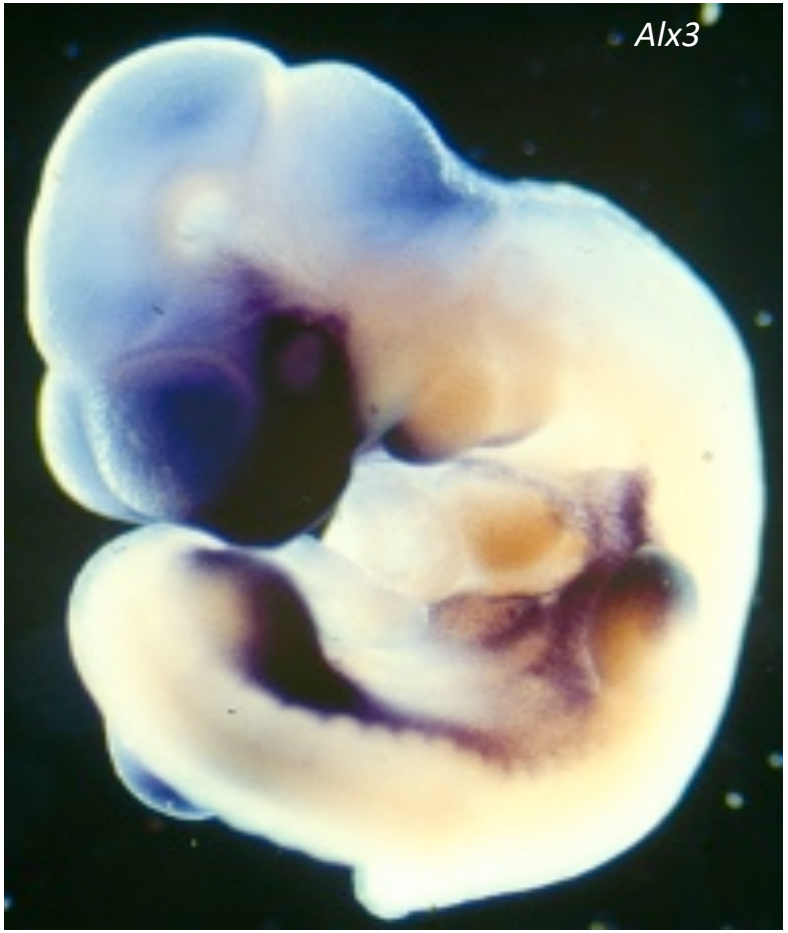
in situ hybridization

On sections:



Radioactively labelled probe

On whole embryos/whole tissues:

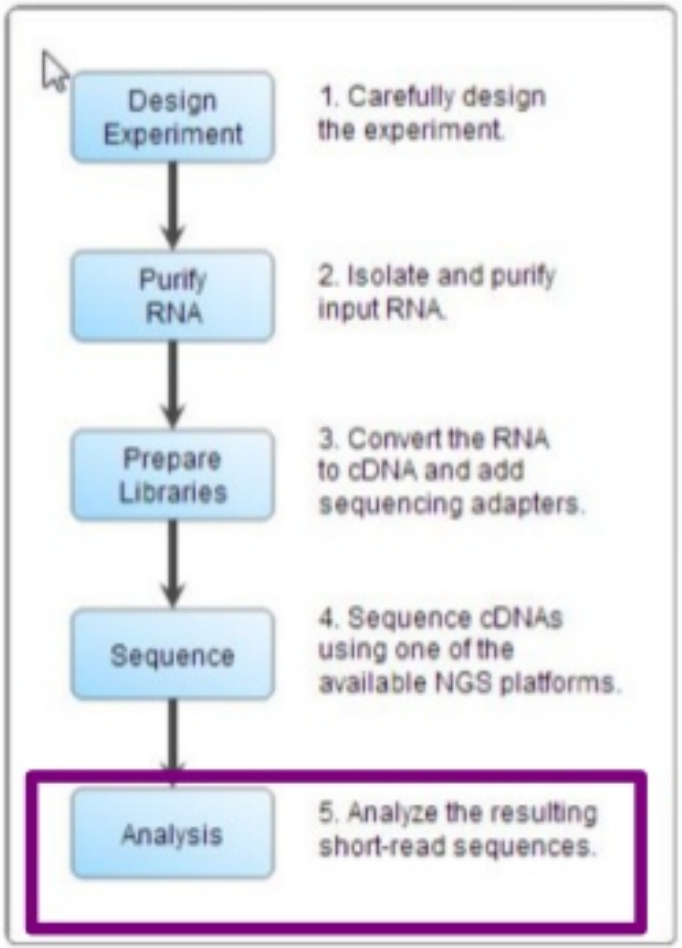


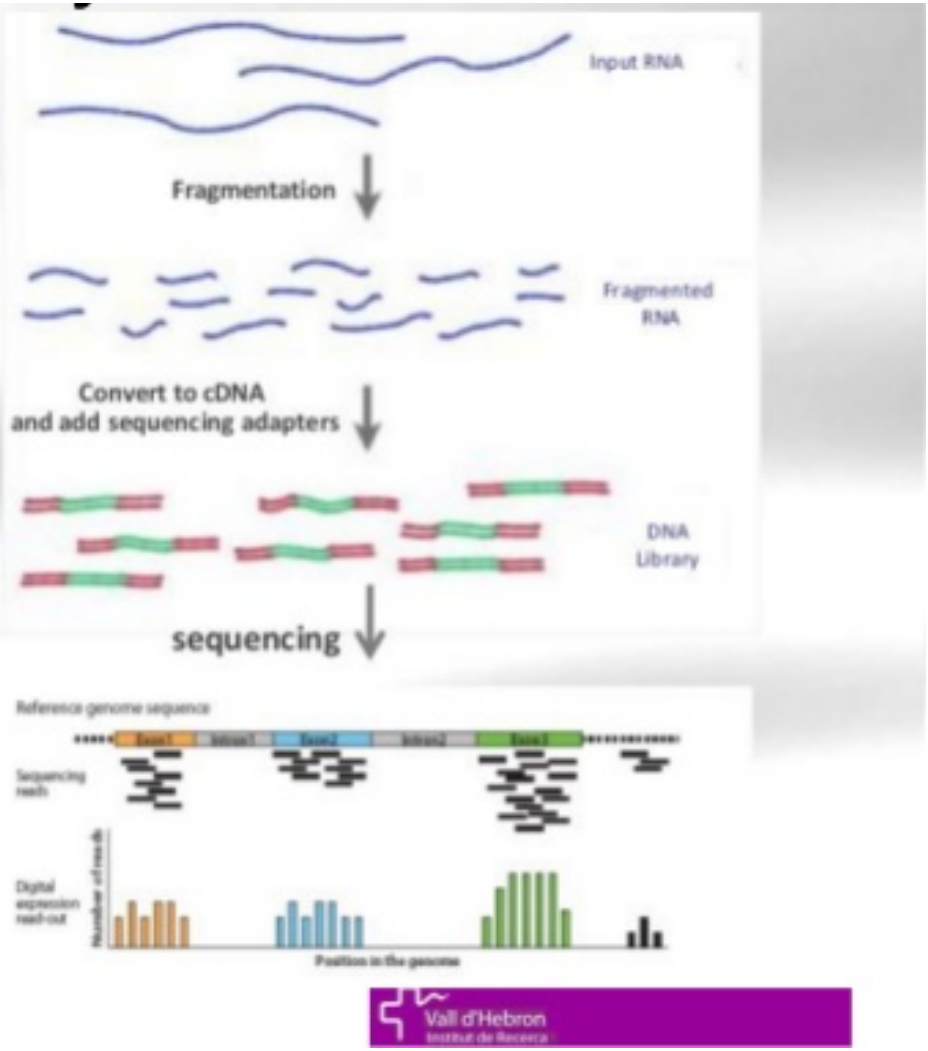
Alkaline phosphatase labelled probe

Gene expression analyses

RNA sequencing

Analyse and compare the transcriptome of thousands of genes in samples





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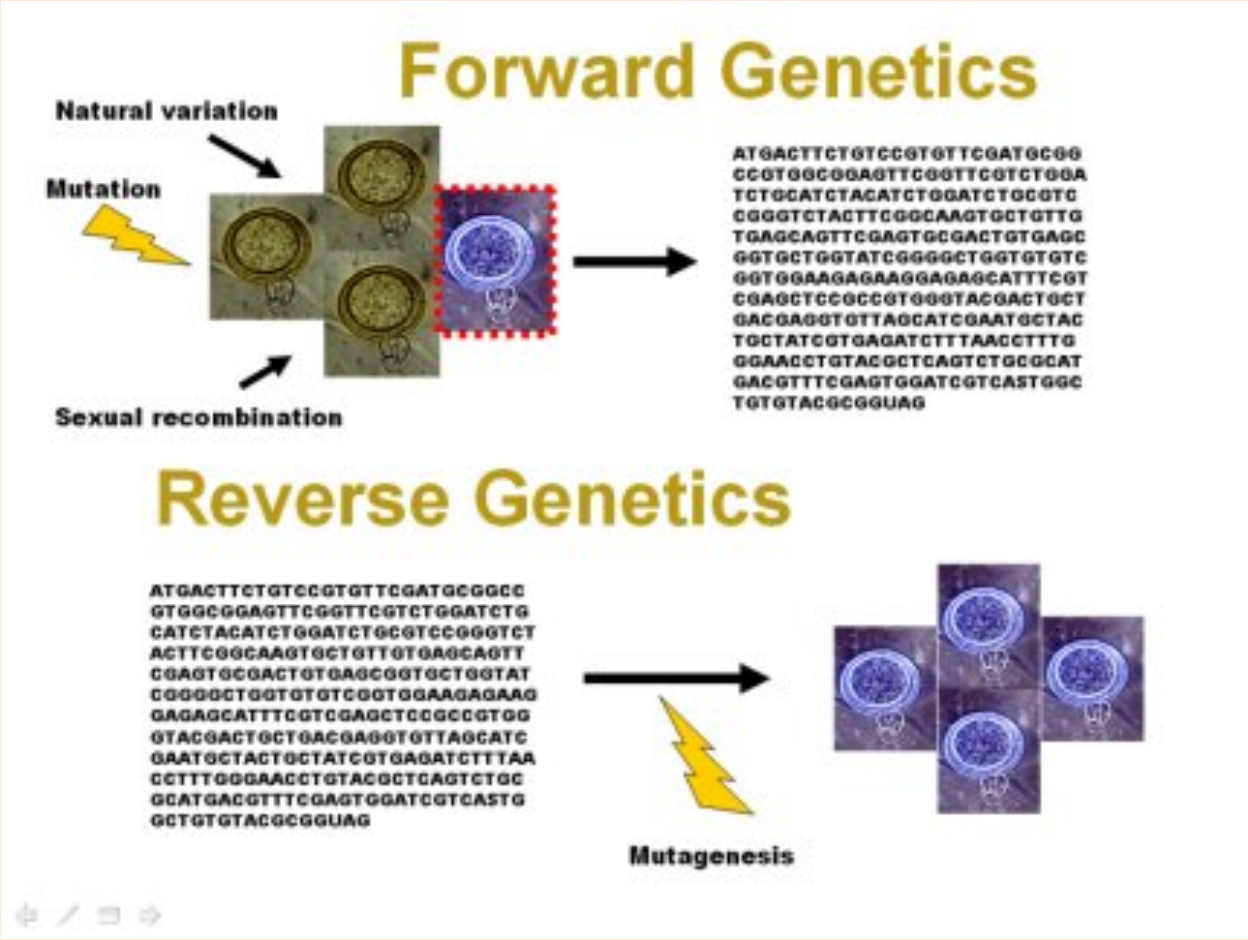
Expression analyses

overview

Method	Detection of	Quantitative	Spatial information	Results within
Protein gel Western blot	Protein	Yes	No/Little	2 Days
IHC/IF	Protein	Limited	Yes	2 Days
RT PCR	RNA	No	No/Little	1 Day
Real Time PCR	RNA	Yes	No/Little	1 Day
Section *in situ* hybridization	RNA	Limited	Yes	Few weeks
Whole mount *in situ* hybridization	RNA	Limited	Yes	1 Week
RNAseq	Transcriptome	Yes	No/Little	Weeks

Methods of studying gene function

Forward genetics: phenotype -> gene
Reverse genetics: gene -> phenotype



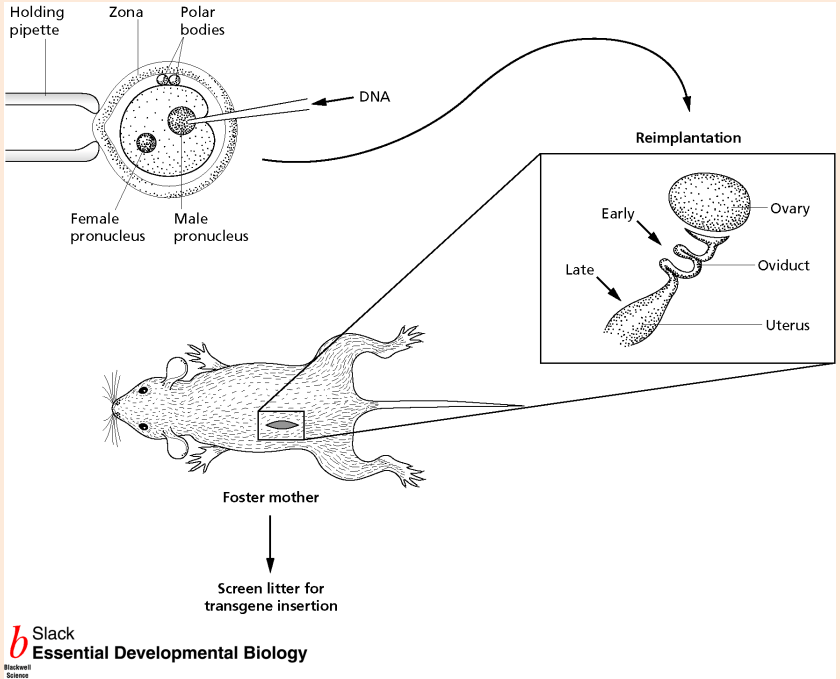
(Random) gene mutagenesis
Caenorhabditis elegans
Drosophila melanogaster
Zebrafish
(Mouse)

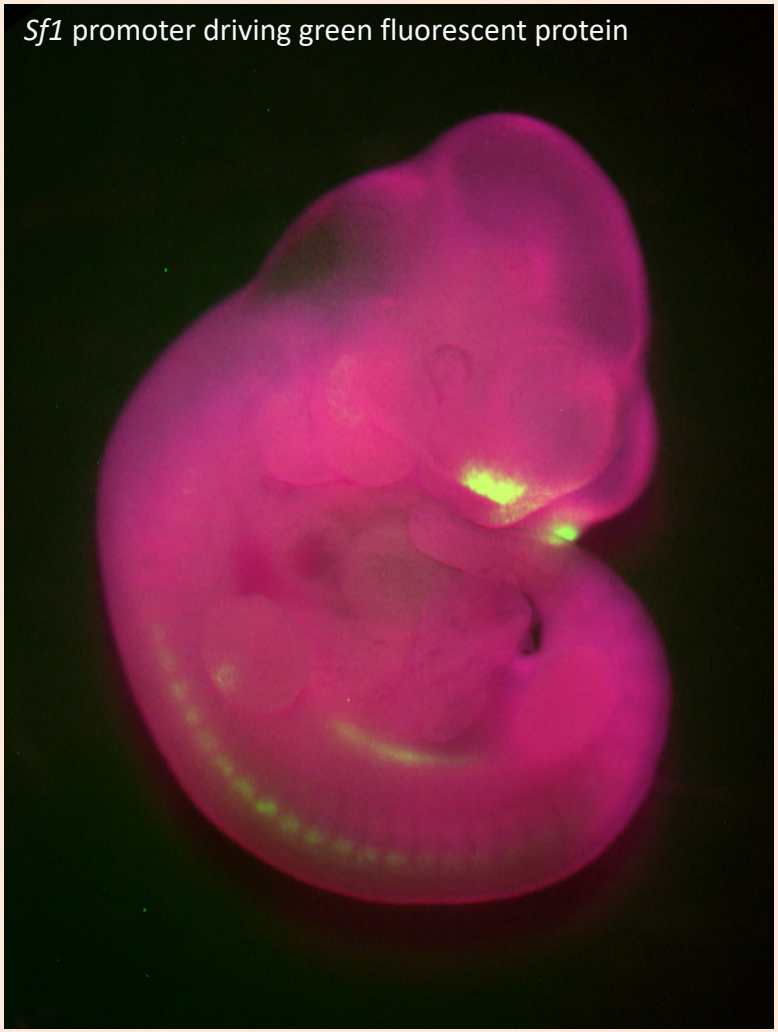
Gene gain- or loss-of-function
Caenorhabditis elegans
Drosophila melanogaster
Zebrafish
Mouse

Gain- or Loss-of-function transgenesis

Generation of Transgenic mice:

1. Generate transgenic construct
   promoter + cDNA/shRNA/Fluorescent marker
2. Inject transgene into zygotes
3. Transgene is integrated into genome
4. Transfer zygotes to pseudopregnant mouse
5. Transgene is expressed by mouse (embryo)

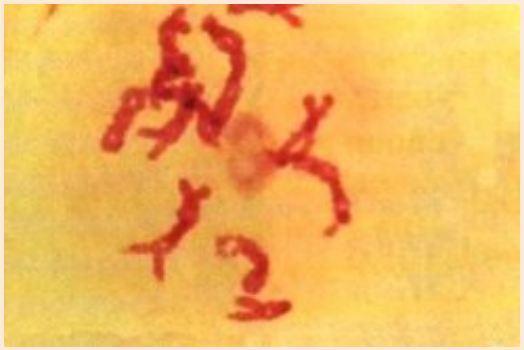


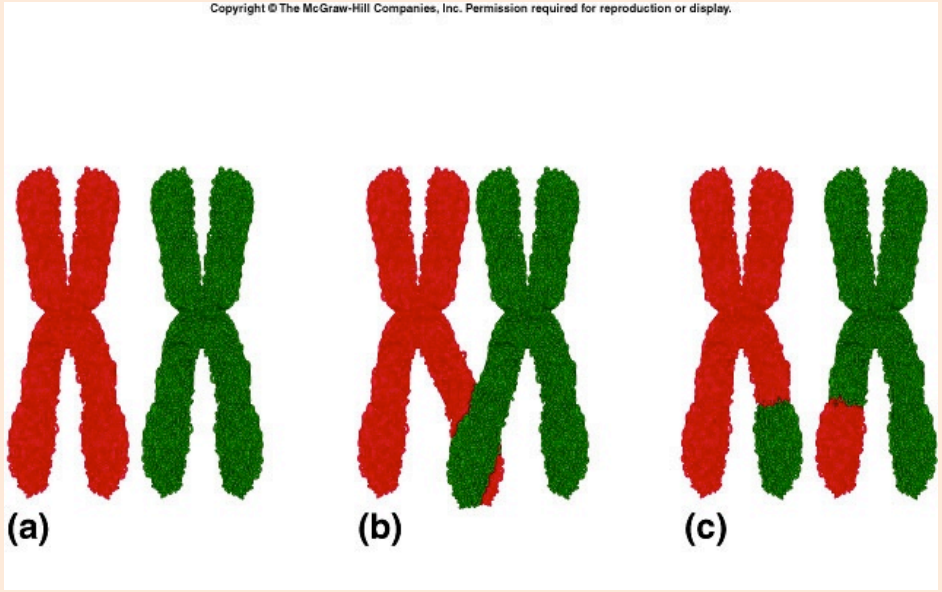


Knock out technology

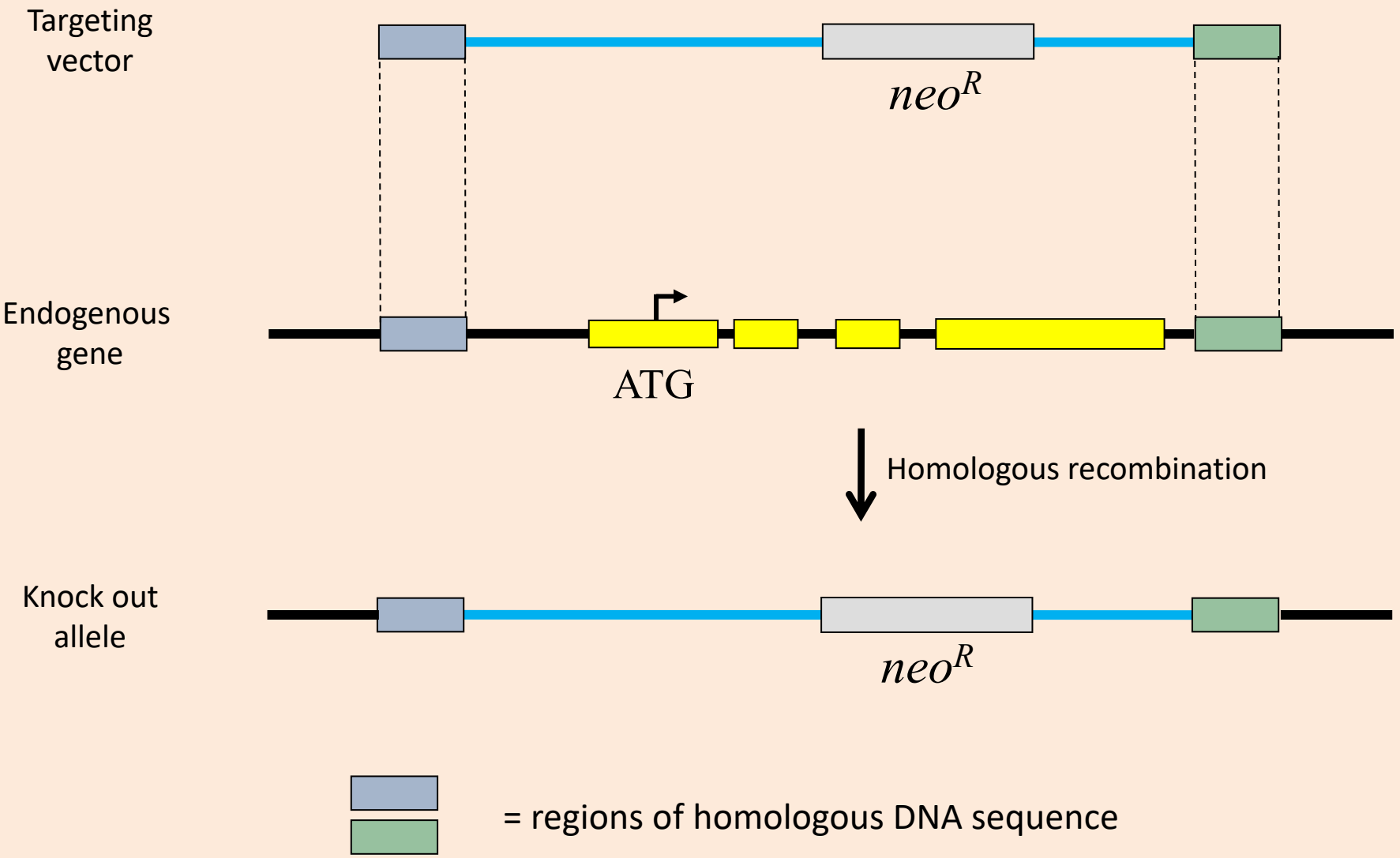
Crossing over is a natural process that happens during meiosis

Knock out technology =
directed homologous recombination in omnipotent ES cells

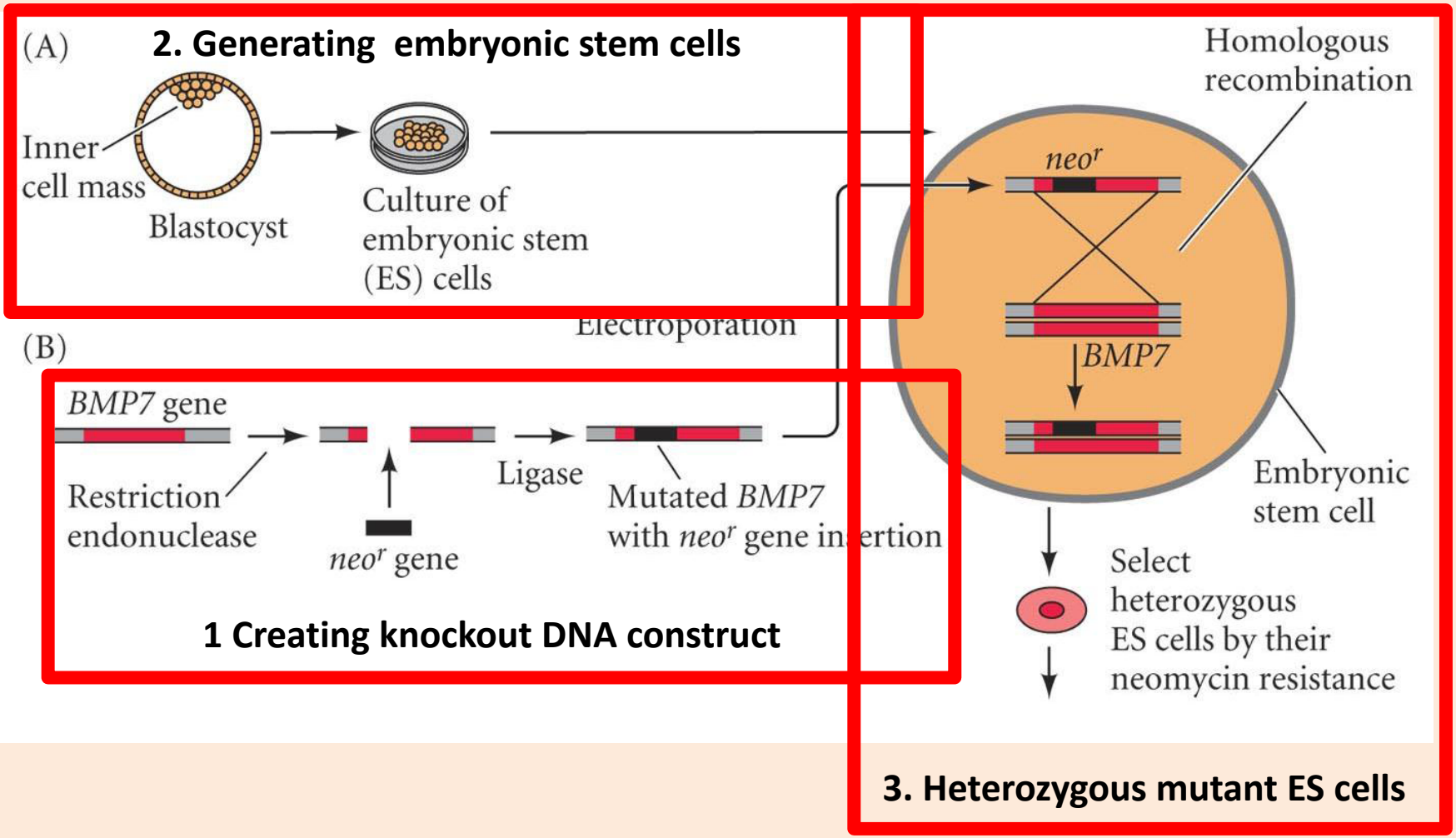




Knock out technology

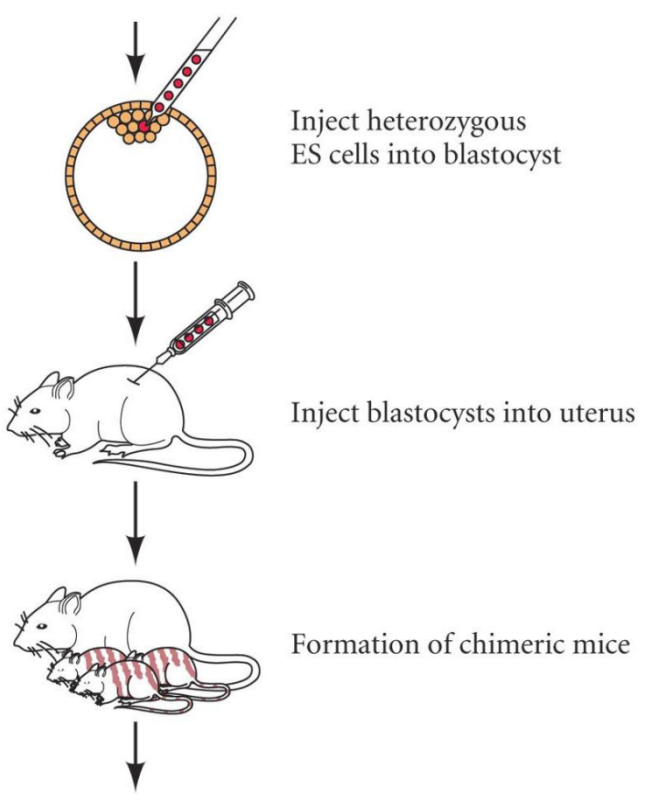


Knock out technology



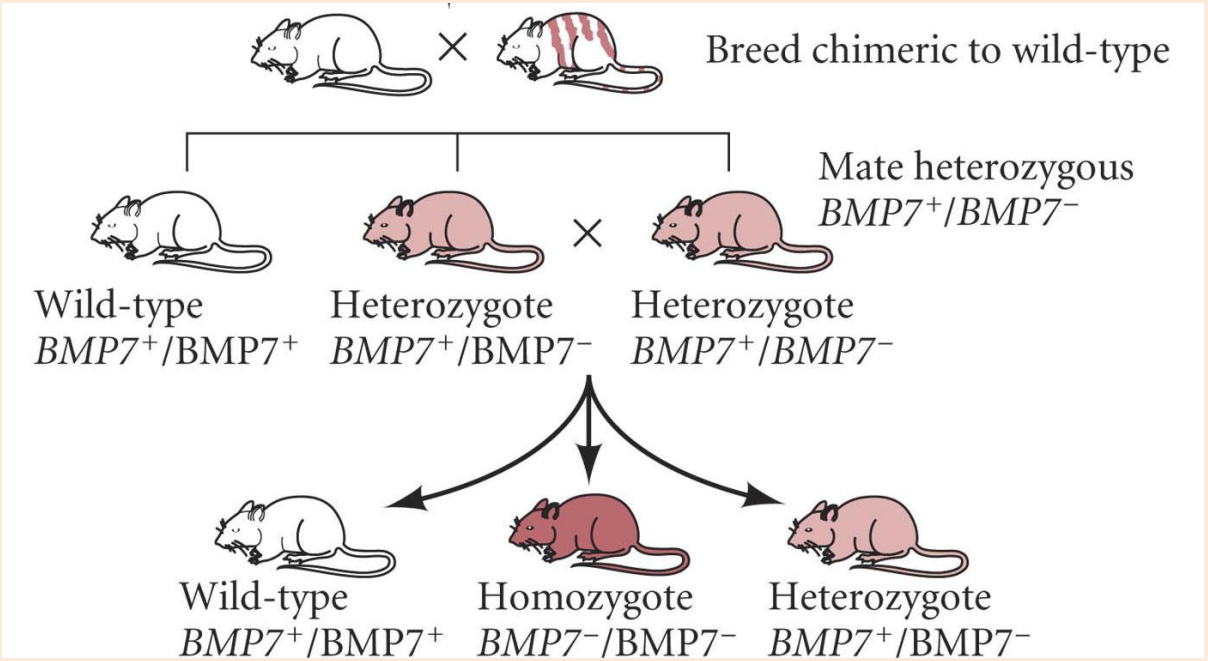
Knock out technology

Heterozygous mutant
Embryonic stem cells



Chimeric mice

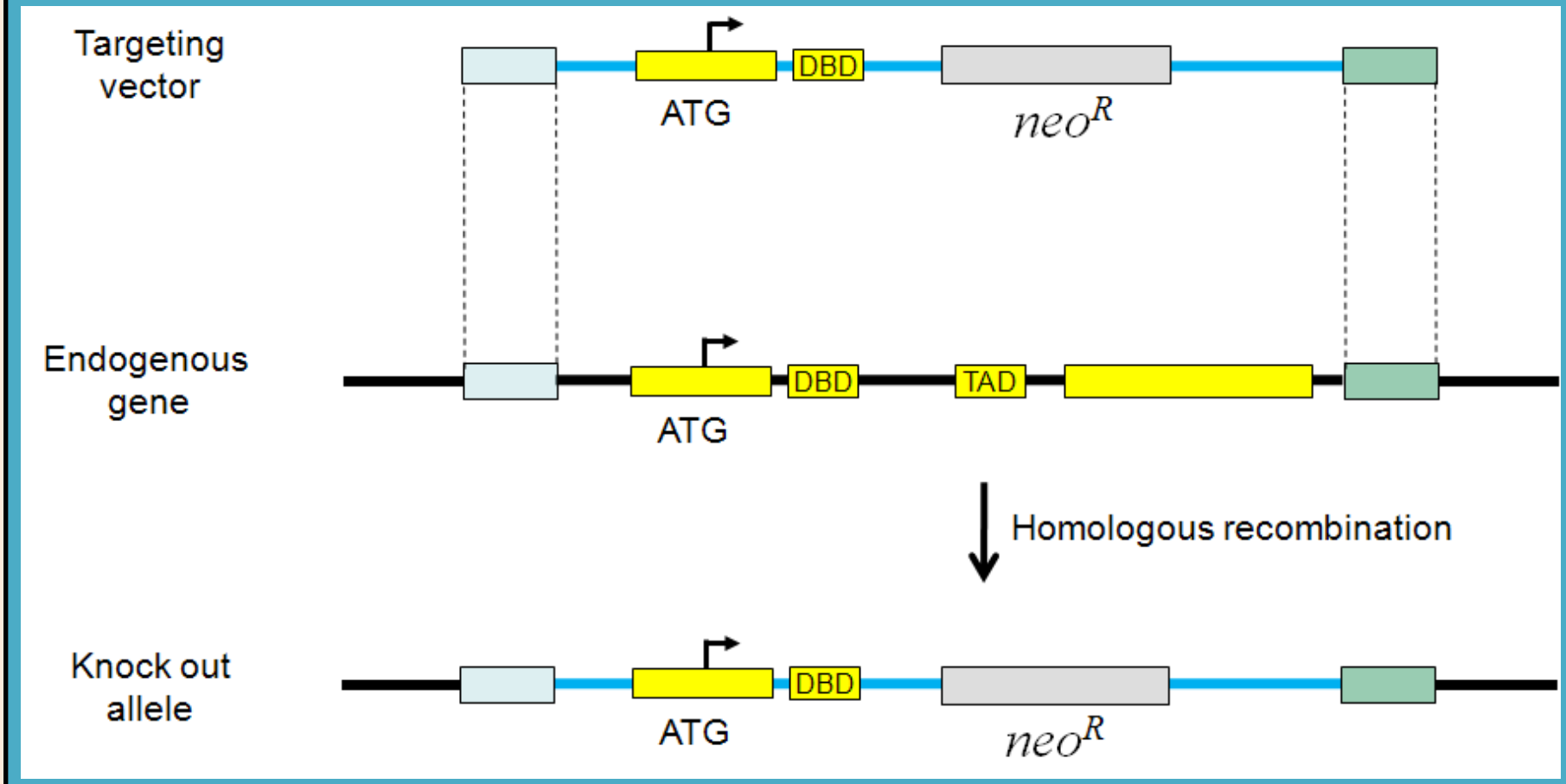
Genetic crosses to obtain
Homozygous mutant mice



Knock out technology

Engineering of targeting vectors

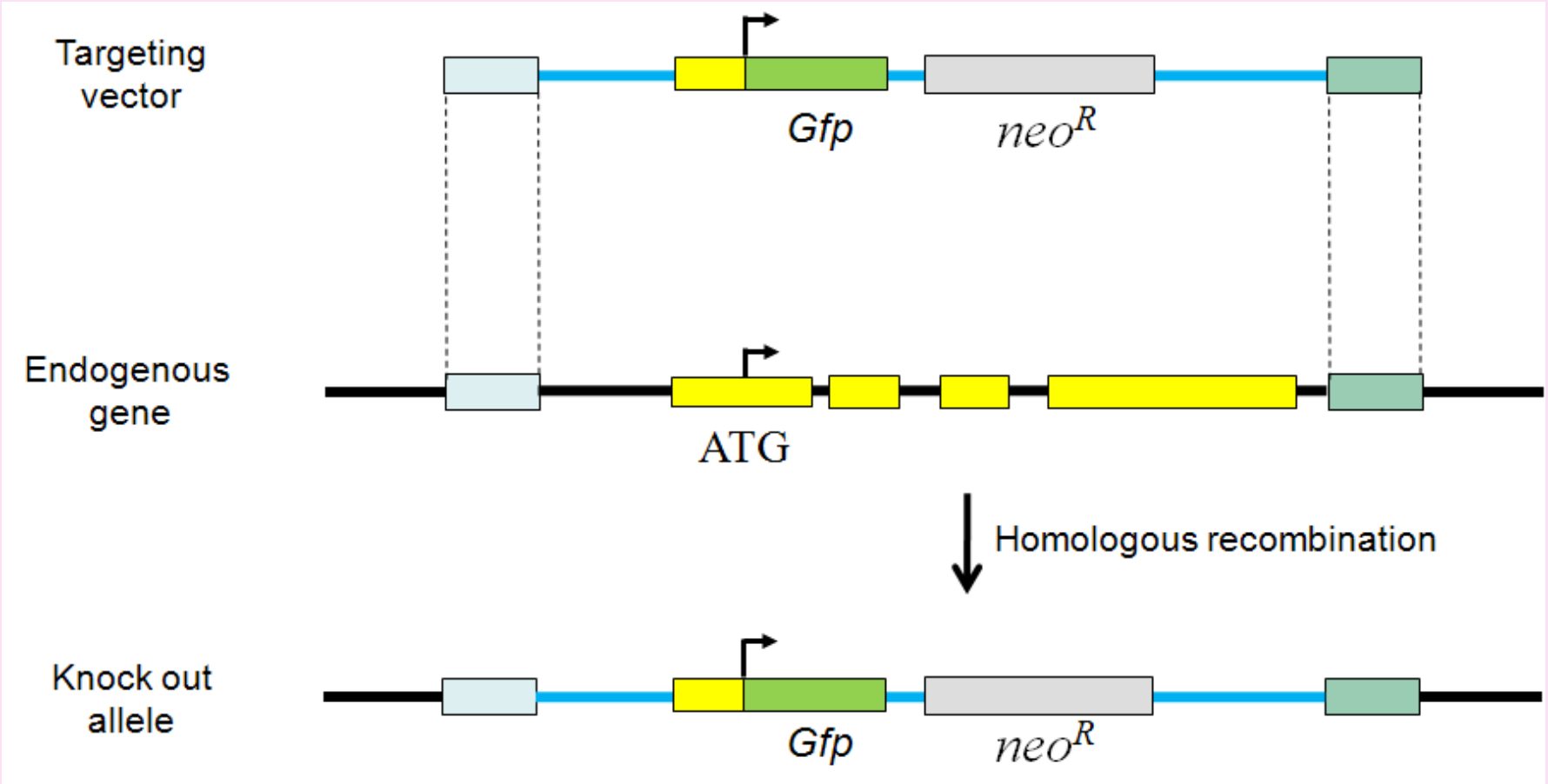
Generating dominant negative proteins



Knock out technology

Engineering of targeting vectors

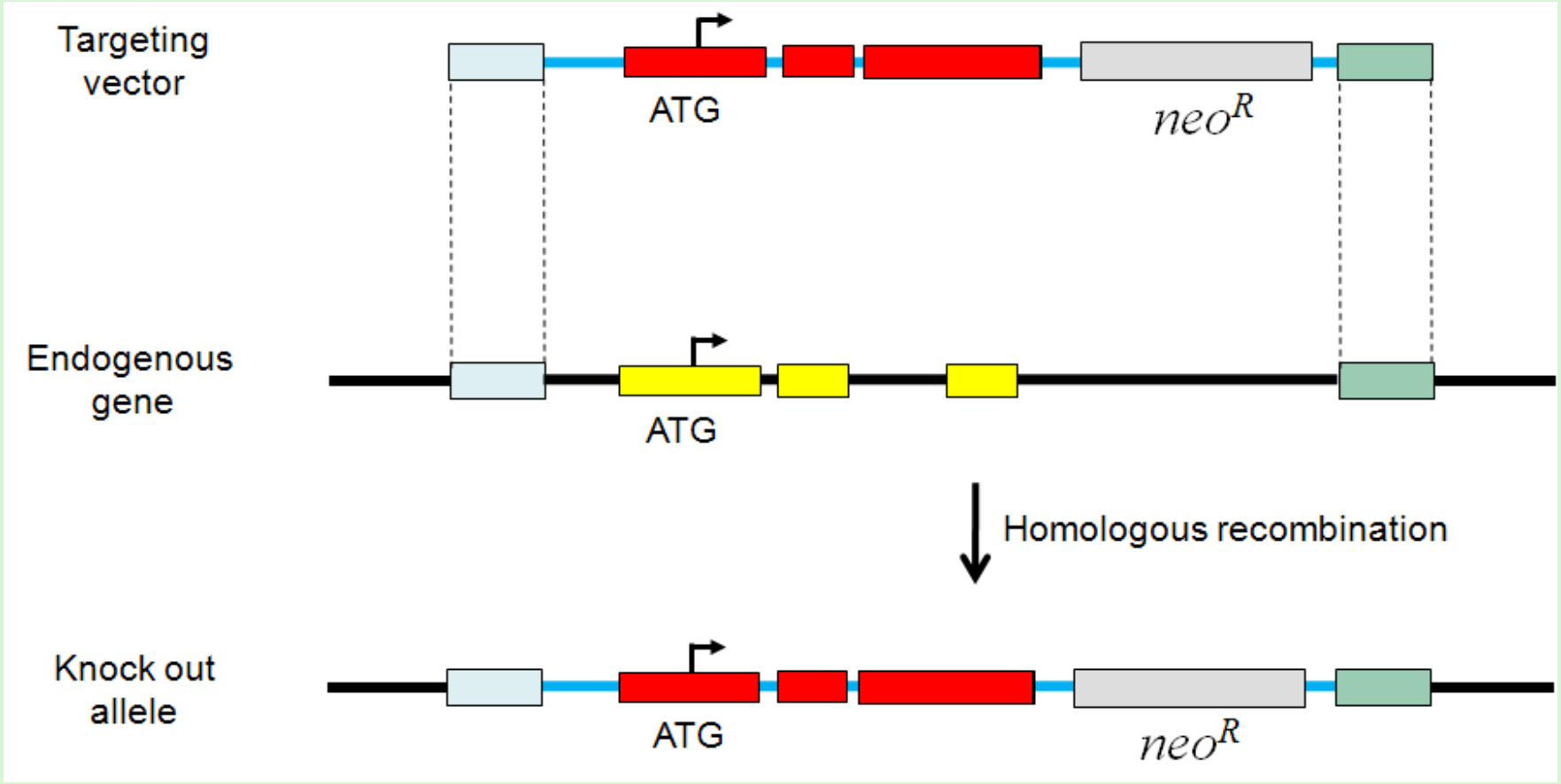
Expressing reporter genes from mutant locus



Knock out technology

Engineering of targeting vectors

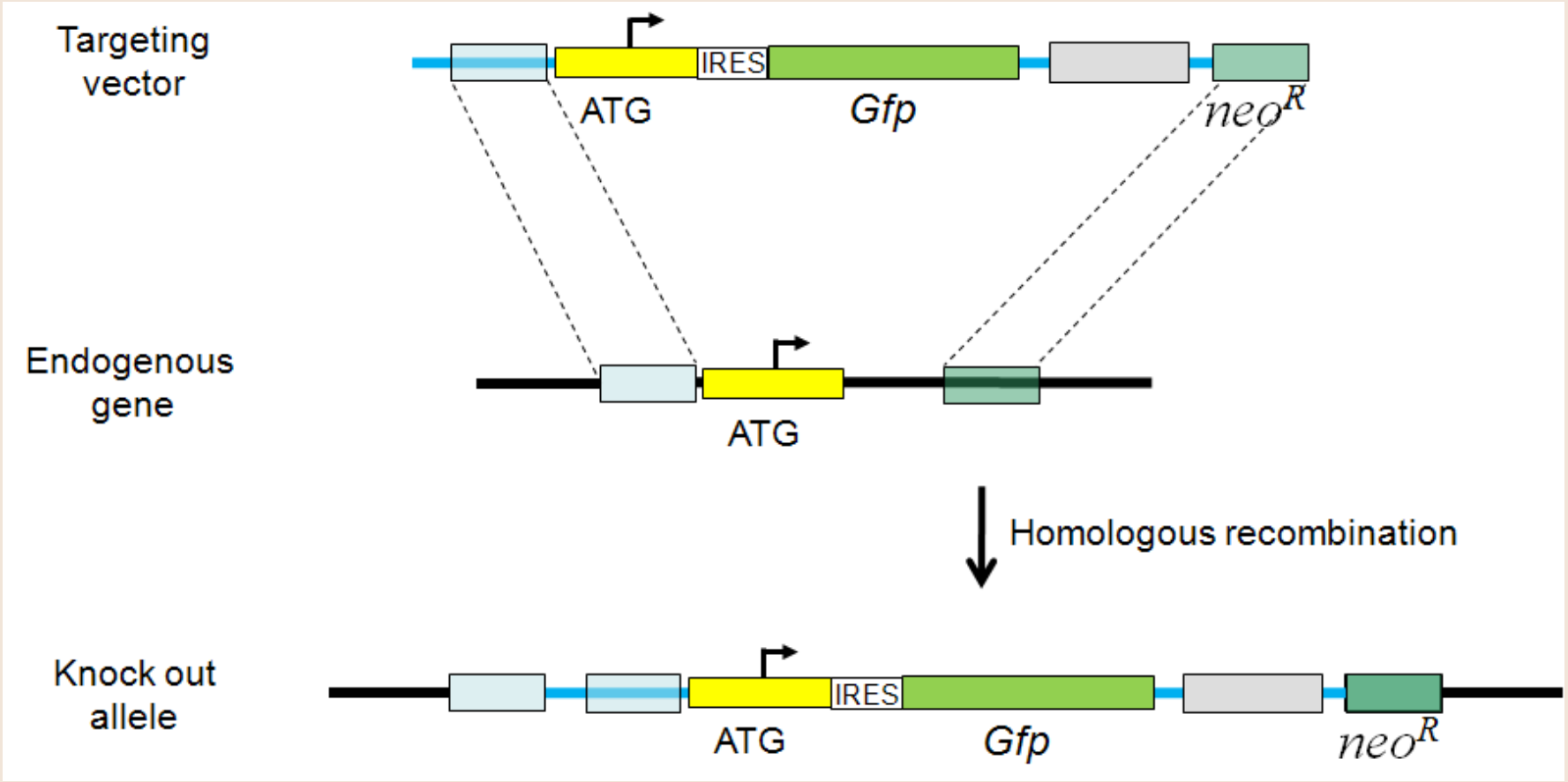
Gene swapping



Knock out technology

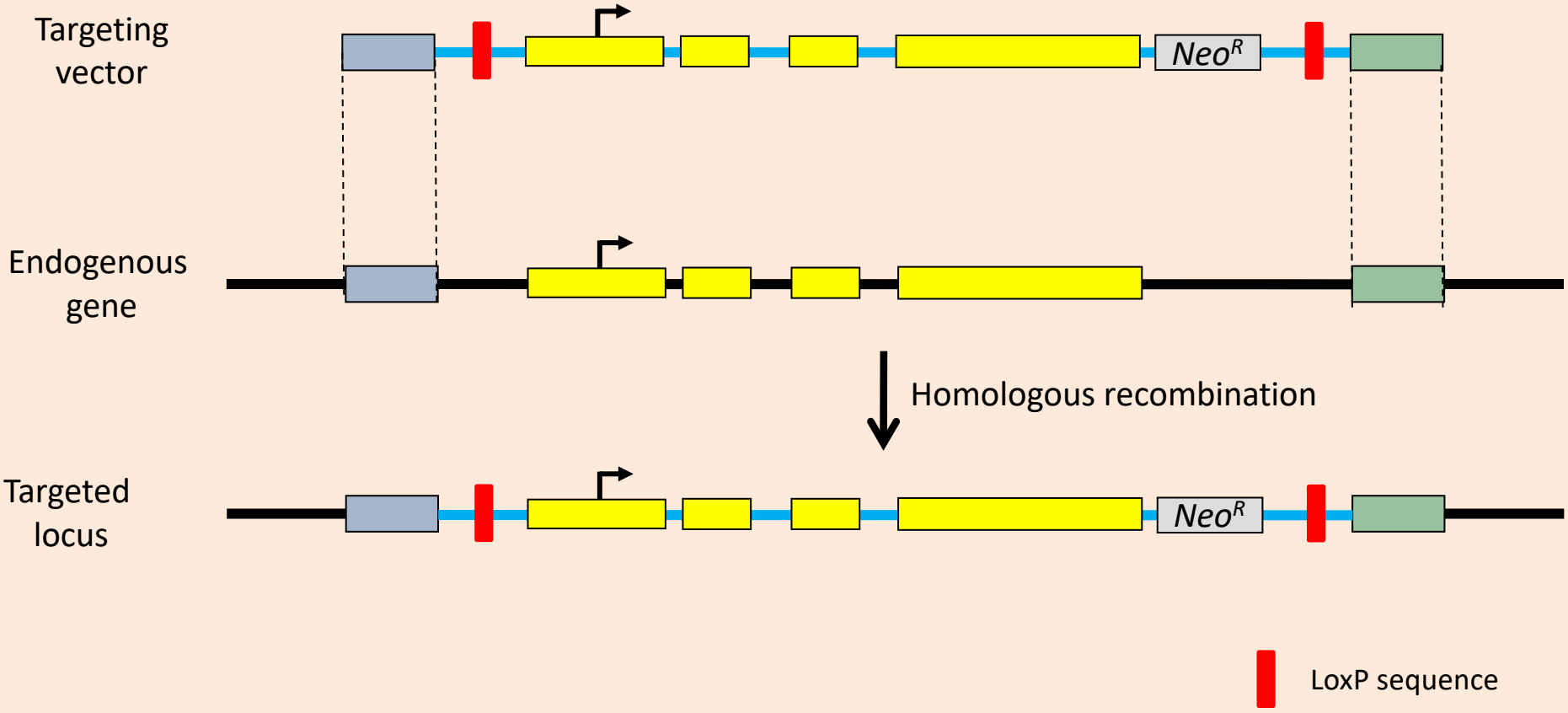
Engineering of targeting vectors

Bicistronic messengers

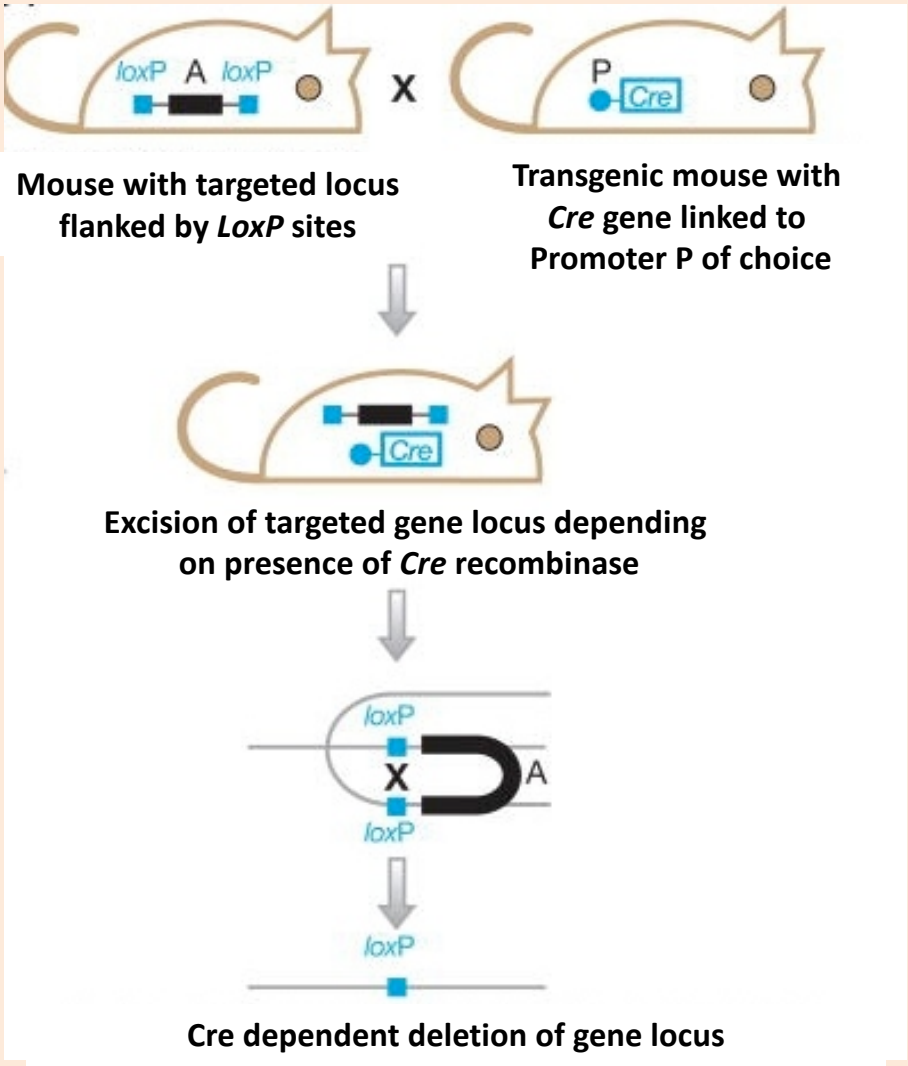


Conditional knock out technology

Conditional LOF mutants: excision of gene dependent on presence of
- *LoxP* sites in gene locus
- Cre recombinase



Conditional knock out technology

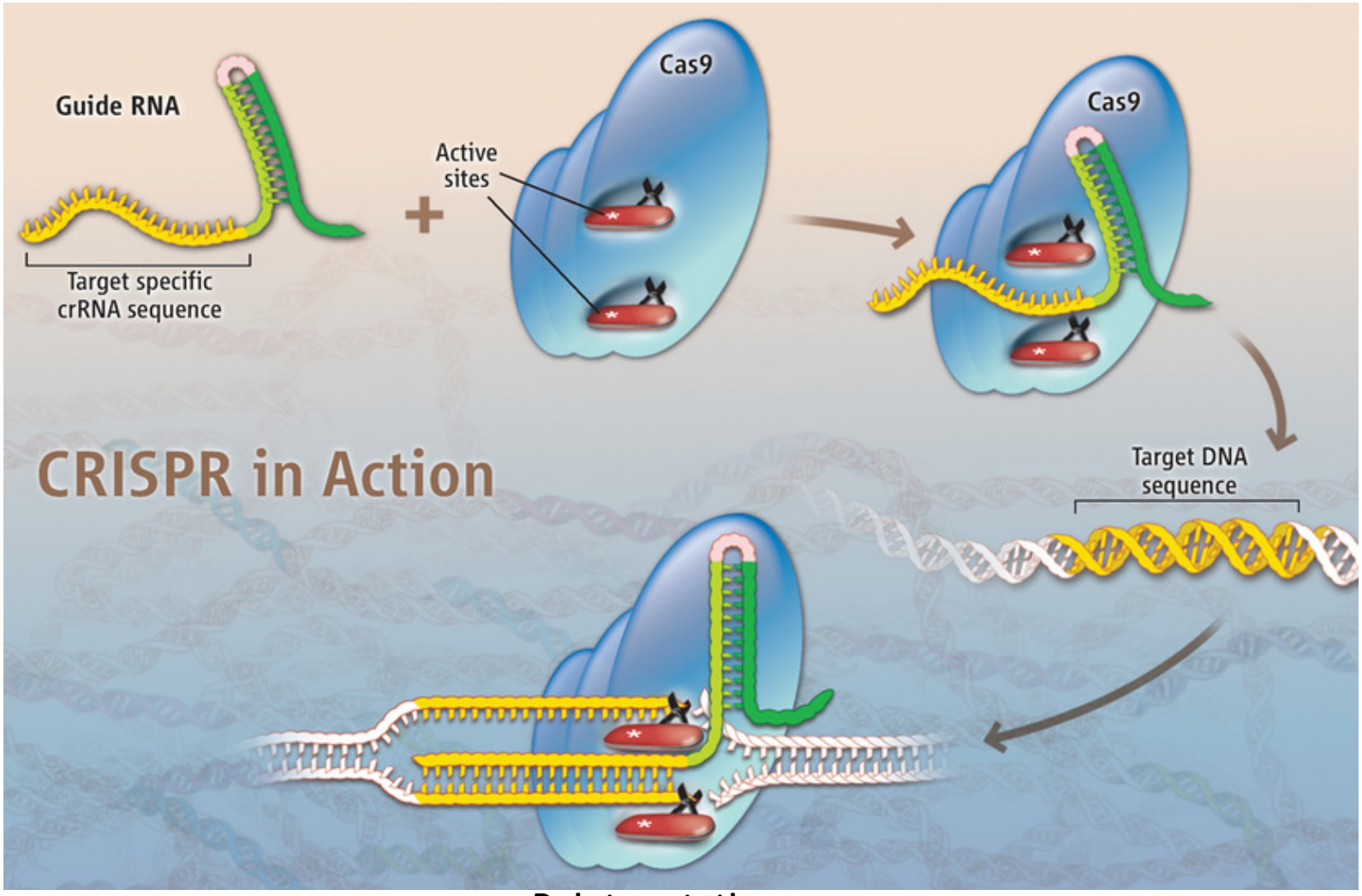


Advantages:

Cell/tissue type specific
Timing specific
Inducible

CRISPR/Cas9 Genome Engineering

Guide RNA and Cas9



Point mutations

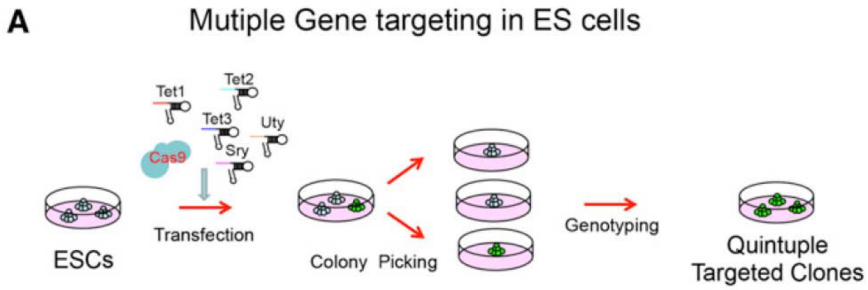
Homologous-driven repair

Mutation of single or multiple genes

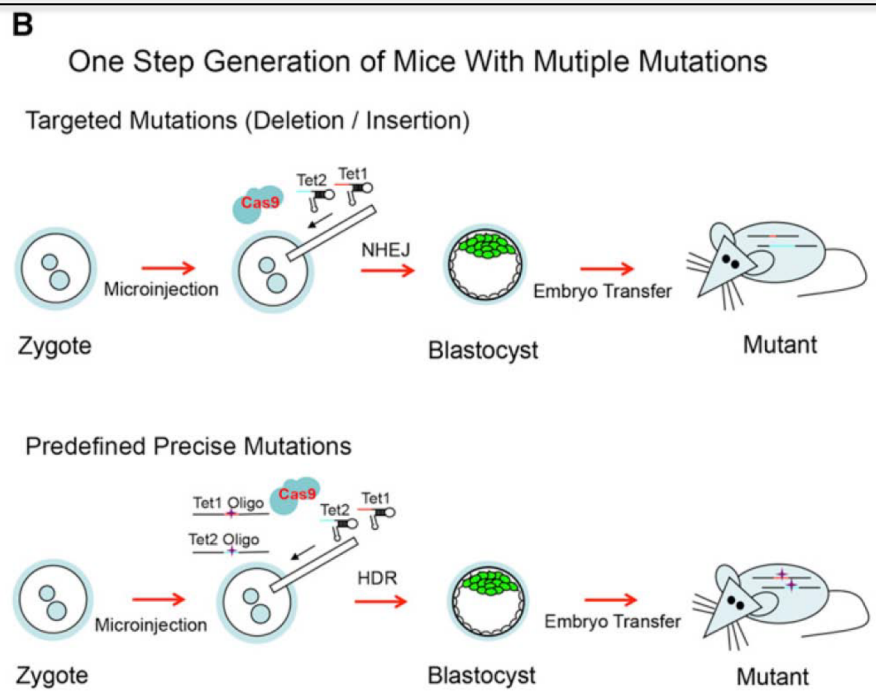
CRISPR/Cas9 Genome engineering

Applications in Stem Cells and Mice

ES cells



Zygotes



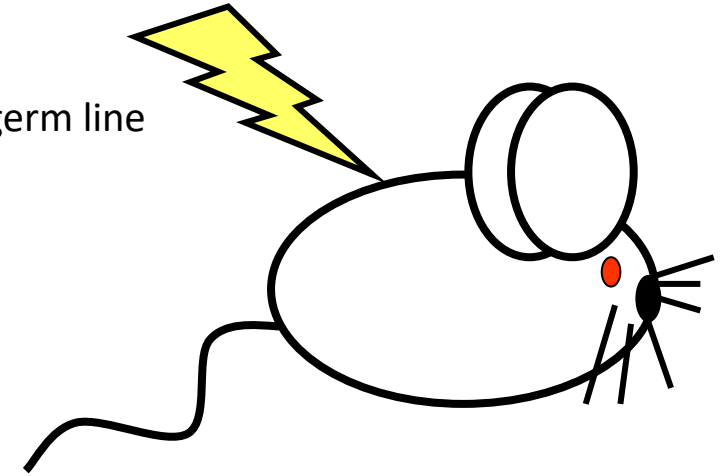
Random mutagenesis screens

Forward genetics

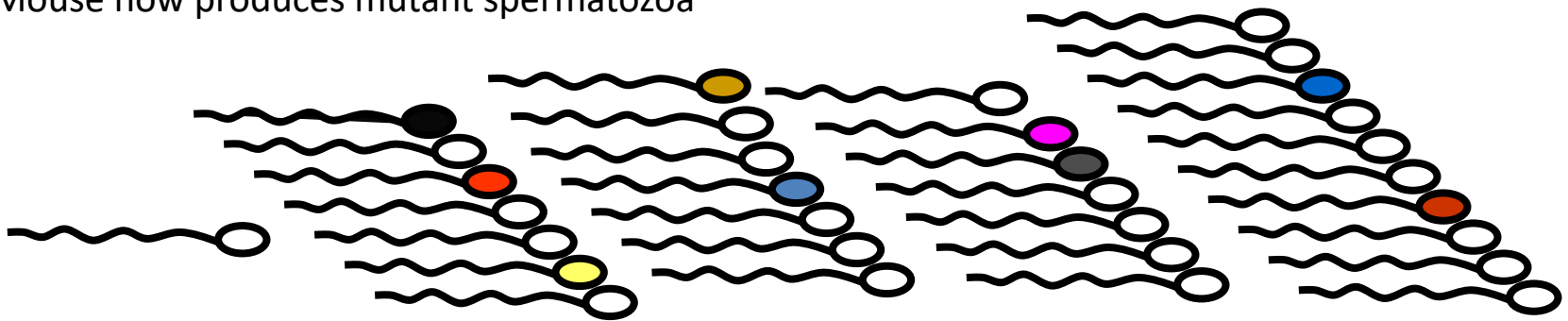
You want to identify new genes that are involved with a certain process

The male animal is subjected to a mutagen, e.g. radiation, or chemical mutagens such as ethylnitrosurea (ENU) or ethylmethyl sulphate (EMS).

Mutagen hits the germ line



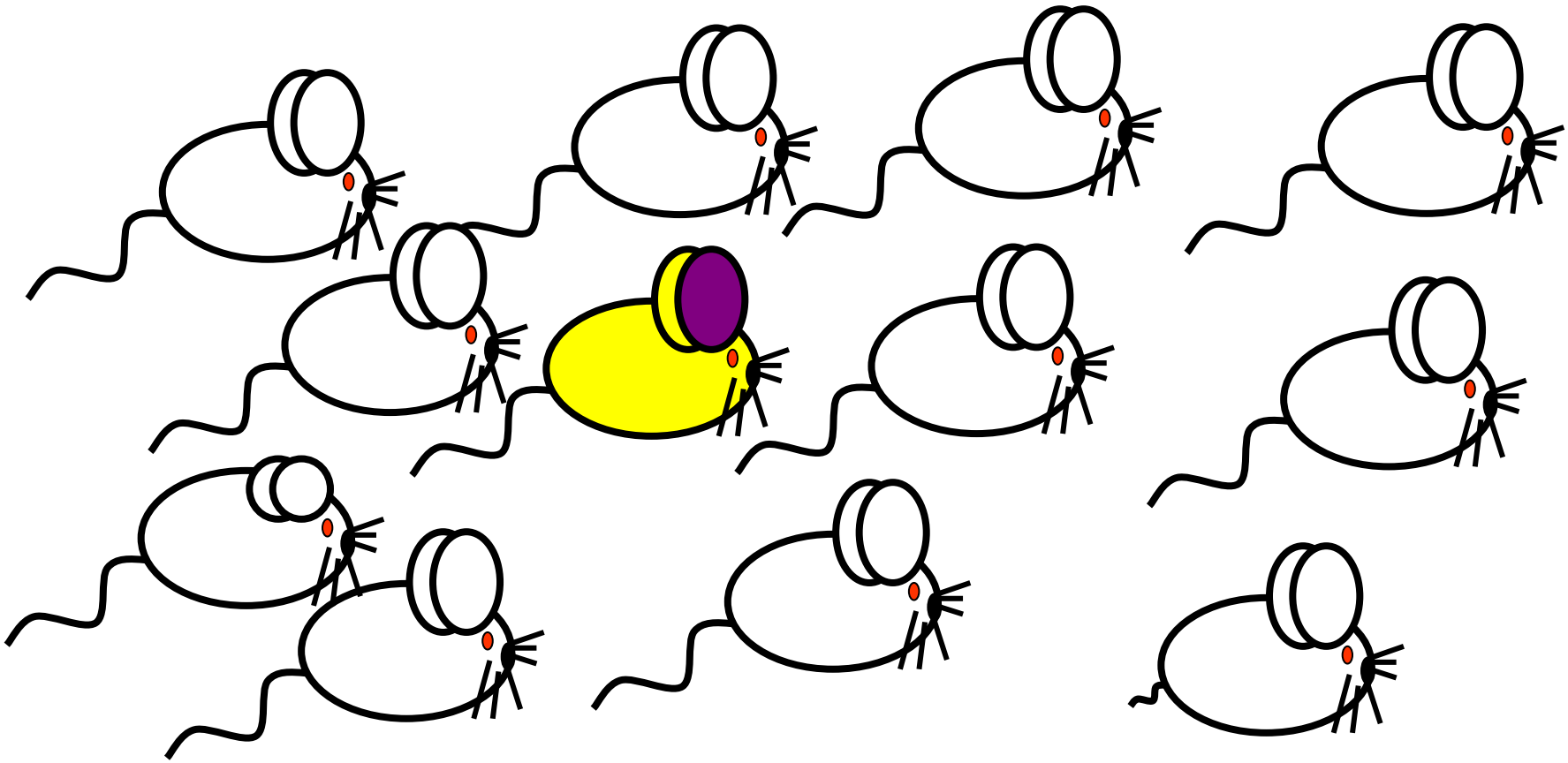
Mouse now produces mutant spermatozoa



Random mutagenesis screens

A screen for dominant mutations:

Mate mutated animals with wildtype females.
Screen babies for the desired phenotypes.
Those that are heterozygous mutant for a dominant gene will show the phenotype.

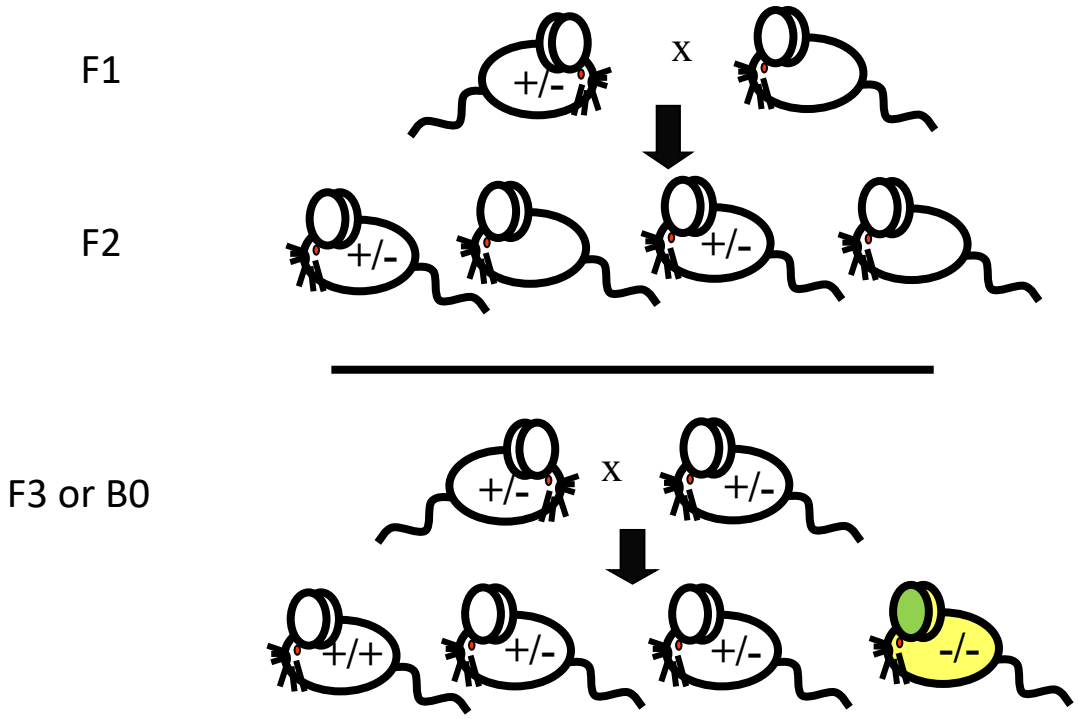


Random mutagenesis screens

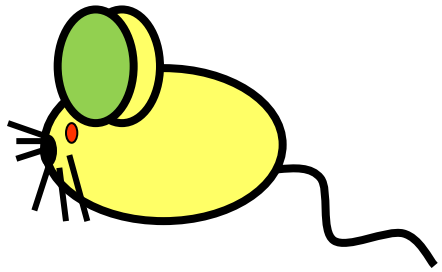
A screen for recessive mutations:

Some of the F1 progeny of the mutagenized animals may LOOK normal, but be heterozygous for a recessive mutation.

Have to breed a litter of progeny then do brother-sister matings to get homozygous mutant animals to reveal recessive phenotype.



Random mutagenesis screens



Select animals with desired phenotypes

Start identification of mutated genes:
Combination of genetic linkage mapping and sequencing

Manipulation of gene function in embryos

Transgenesis:
- gain of function transgenesis
- loss of function transgenesis
- reporter overexpression

Mutagenesis:
- Conventional knock out mice
- Conditional knock out mice
- CRSPR/CAS9 mice
- Random mutagenesis

Lecture 8

Research Technologies in Developmental Biology

Gene and protein expression analysis methods

Methods to study gene function in embryos *in vivo*

Chapter 5 in Larsen's *Human Embryology* (4th edition)
Chapter 4 in Scott Gilbert's *Developmental Biology* (8th edition)

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