Basic method in molecular biology II

Assoc. Prof. Suwattanee Kooptiwut

Department of Physiology

Faculty of medicine Siriraj hospital

Aims

Protein

• Antibodies

• Western blot analysis: protein

• Other techniques: HPLC, 2D-gel

Transgenic technology

Western blot application

• Gene translation

- Where

- Amount

Western blot analysis

• protein preparation: lysis of cell

• Quantitation of protein

• Separation of protein

• Transfer of protein to PVDF membrane

• Hybridized with antibody (primary & secondary)

• Exposed to x-ray film

- protein preparation: lysis of cell
- Quantitation of protein: Protein assay

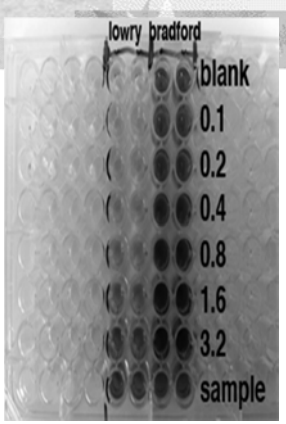
⇩

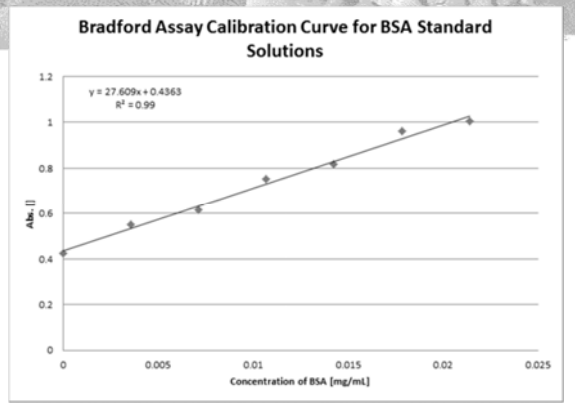
Spectophotometers

- Transfer of protein to PVDF membrane

Protein measurement assays

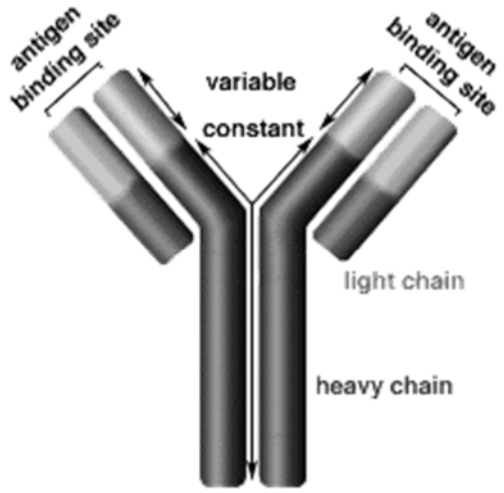
- Several methods are commonly used for determination of protein concentration. Measurement of the UV absorbance at 280 nm is most useful for pure protein solutions.
- Bradford (Coomassie Brilliant Blue), Lowry (Folin-Ciocalteau), Biüret, Pesce and Strande (Ponceau-S/TCA), and modified method of Schaffner-Weismann (Amido Black 10B), BCA were used during protein purification and screening.

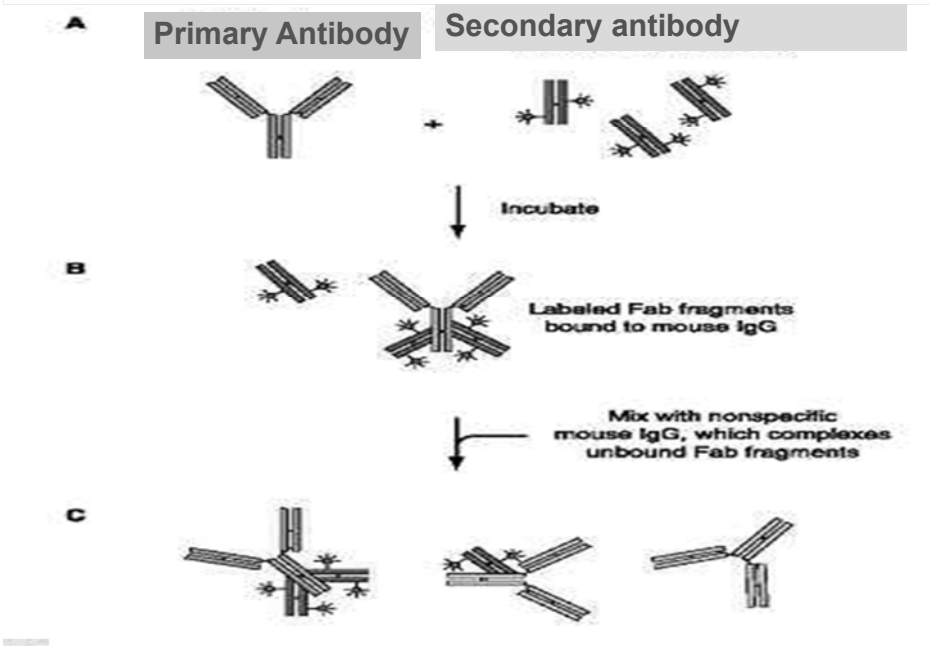




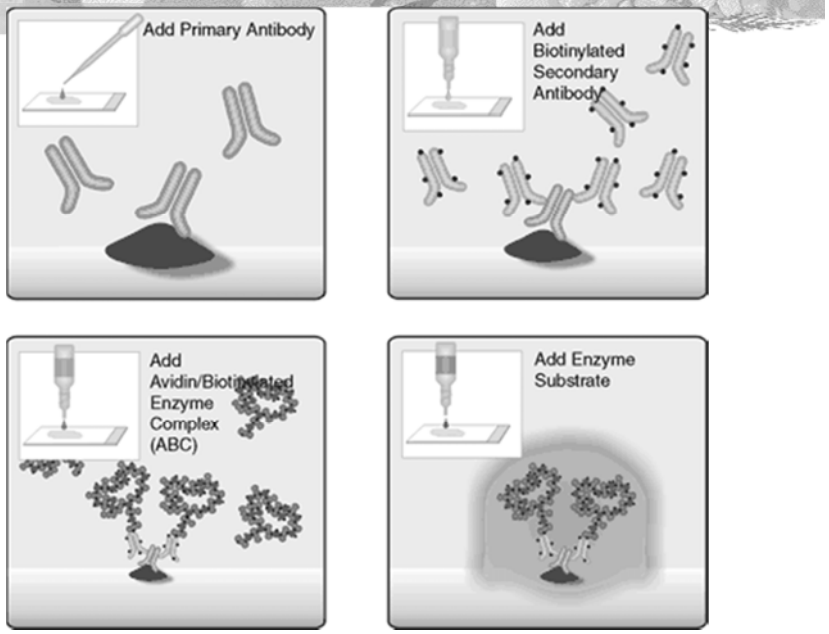
Wikipedia Bradford protein assay

Antibody

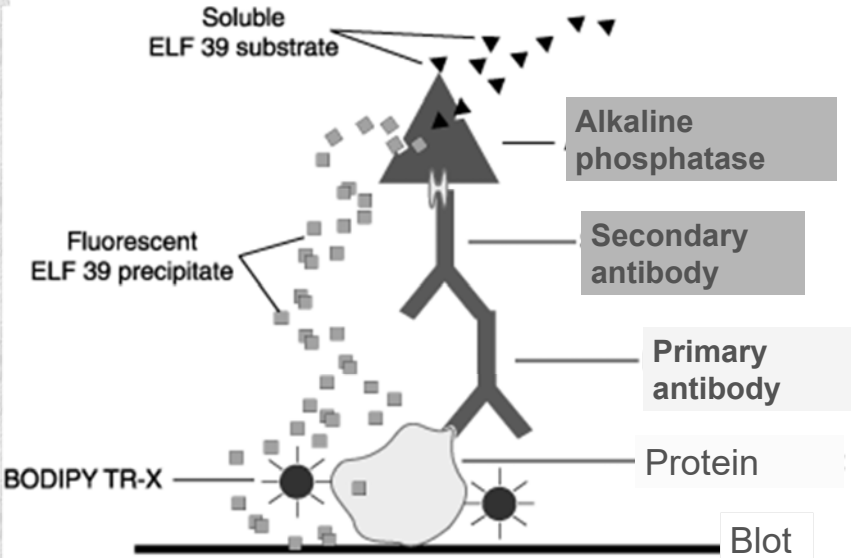


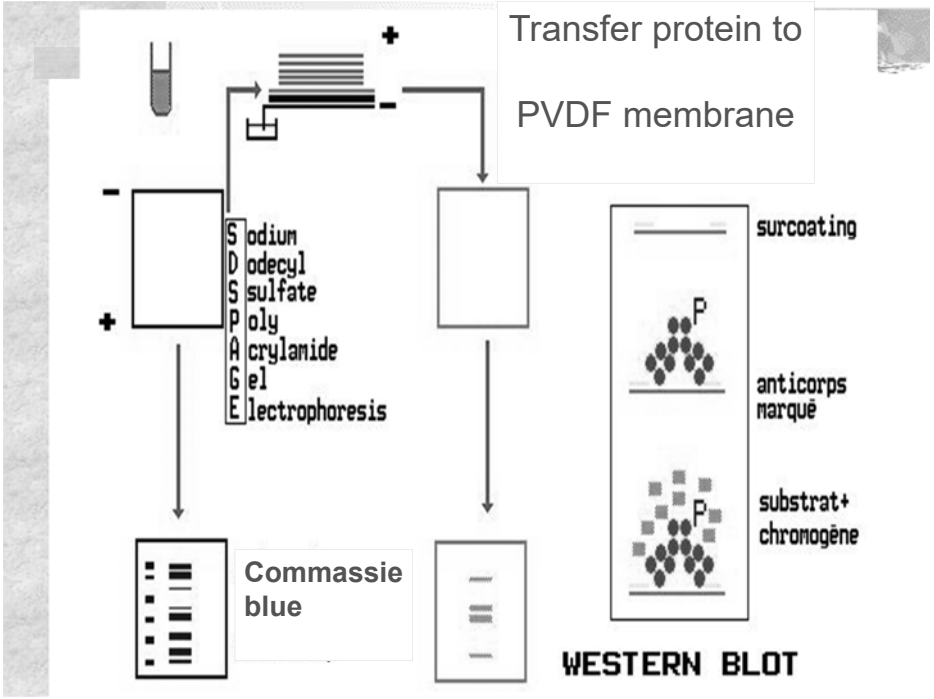


Animation

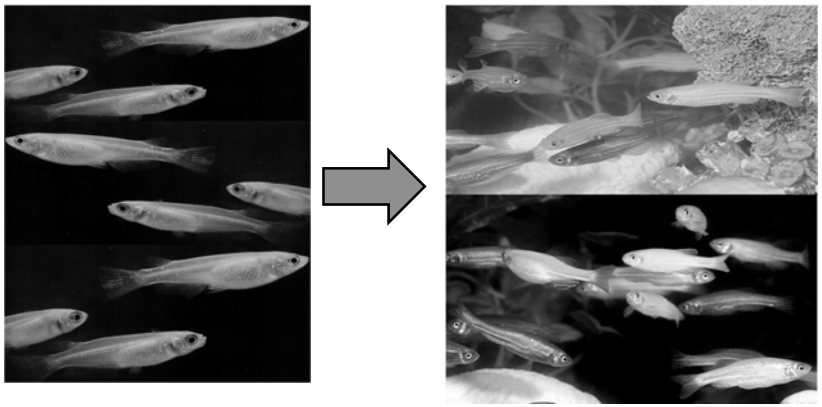


• Hybridized with antibody (primary & secondary)





Transgenic technology



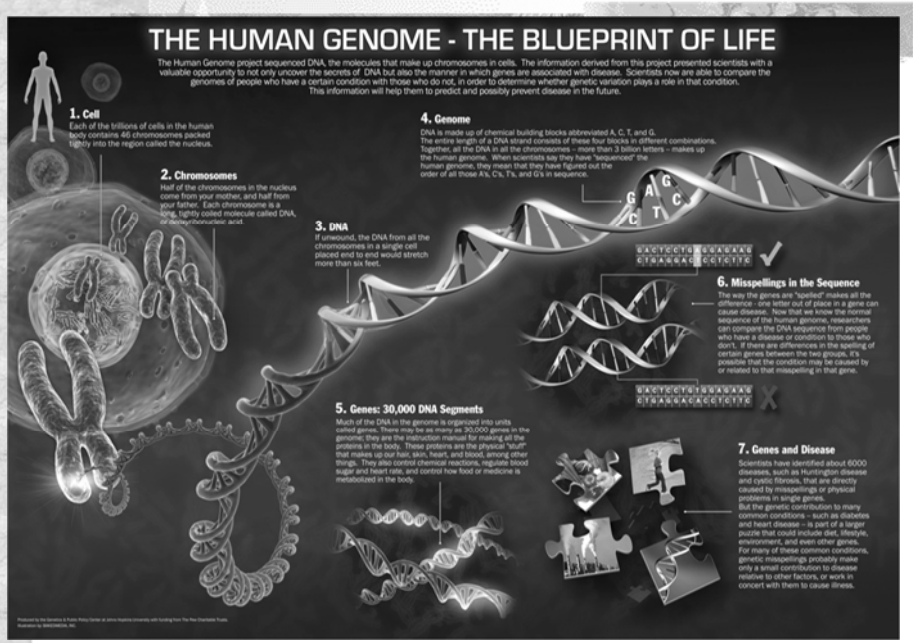
Assoc. Prof. Dr. Suwattanee Kooptiwut

Physiology Department

How many genes are there in organisms?

HIV	9700 b	9 genes
E. coli	4.6 Mb	3,200 genes
S. cerevisiae	12.1 Mb	6,000 genes
D. melanogaster	137 Mb	13,000 genes
C. elegans	97 Mb	19,000 genes
M. Musculus	2,600 Mb	30,000 genes
H. Sapiens	3,300 Mb	**30,000** genes

http://doegenomes.org



http://doegenomes.org
http://www.sanger.ac.uk/HGP/overview.shtml

Human genome project facts

·Almost all (99.9%) nucleotide bases are exactly the same in all people.

• Less than 2% of the genome codes for proteins.

• The total number of genes is estimated at around 30,000--much lower than previous estimates of 80,000 to 140,000.

• The functions are unknown for over 50% of discovered genes.

Genome project was completed in 2003 (completed)

Genomics and proteomics

Gene expression profiling (Exon profiling)

Studied on the mechanism of gene expression.

Phenotypes ← Transgenic technologies

Transgenic technology

·Identification of gene function

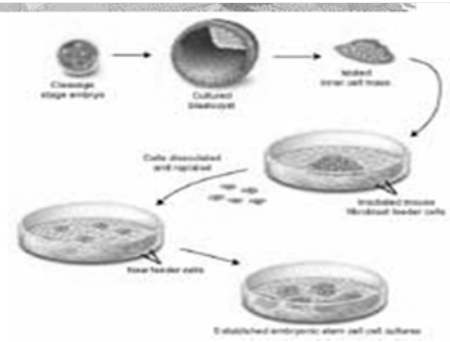
·Generation of animal models of diseases

·Drug validation

·Cell and organ research

Others…

MAMMALIAN EMBRYO MANIPULATIONS



Animal models?

In theory, all mammalian embryos can be used.

Mouse and rats	Research
Farm animals	Commercial uses:
Human	Improve health, Cure diseases , others?

Knock out

- Delete specific gene and observe function

- Gene function: Observe phenotypic (behavior or condition) differences between knock out and normal animals

Transgene

Increase specific gene and observe function

Transgenic: many copies of a specific gene

- Gene function: Observe phenotypic (behavior or condition) differences between transgenic and normal animals

Transgene

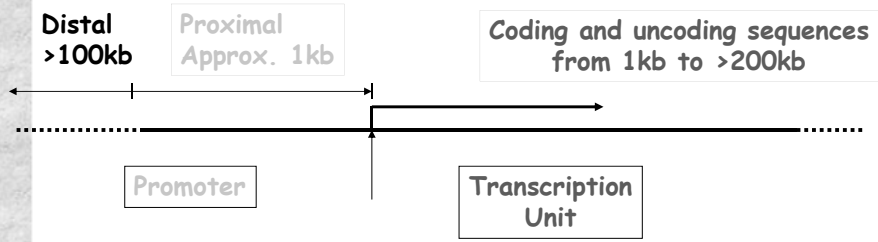
A method to express exogenous genes from specific endogenous regulatory elements *in vivo*

⇩

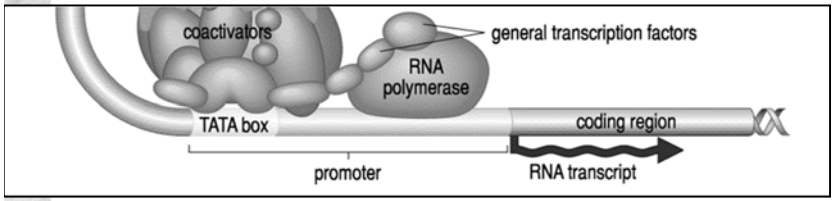
Targeting construct

Normal gene structure

Gene Locus: Includes both the promoter and the transcription unit!



The promoter is the site where *RNA polymerase* and the so-called *general transcription factors* bind.



Transgenic technology

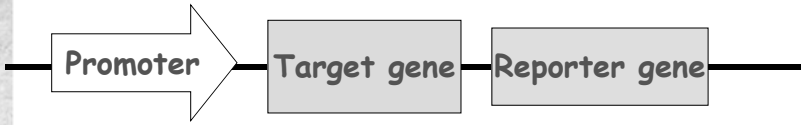
Targeting construct

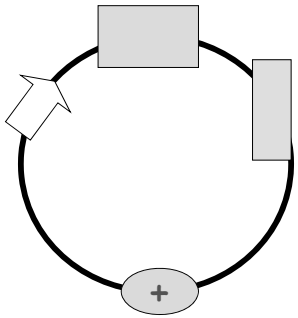
exogenous specific gene: cDNA from other species

Human → mouse

Reporter genes: are nucleic acid sequences encoding easily assays proteins.

Targeting construct: transgenic





Selective marker: neomycin resistance gene

Transgenic Technology

Promoter

1- Tissue specific (brain, liver, muscle …)
2- Ubiquitous
3- Inducible (tetracyclin, interferon…

cDNA

Gain of Function	***Loss of Function***
- wild-type gene - mutant	- dominant negative - antisense - ribozyme

Reporter genes

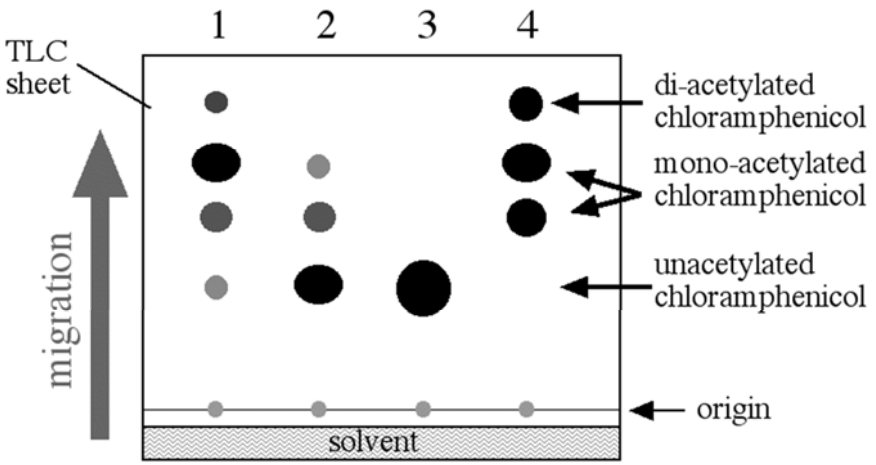
Reporter genes are nucleic acid sequences encoding easily assayed proteins. They are used to replace other coding regions whose protein products are difficult to assay.

Reporter genes

- Chloramphenical acetyltransferase (CAT)
- Beta galactosidase (GAL)
- Beta glucuronidase (GUS)
- Luciferase (LUC)
- Green fluorescent protein (GFP)
- Red fluorescent protein (RFP)

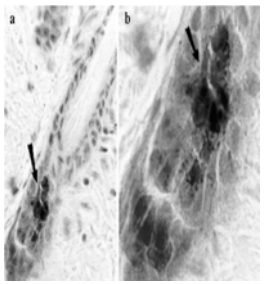
Protein	Activity & Measurement
CAT	Transfers radioactive acetyl groups to chloramphenicol; detection by thin layer chromatography and autoradiography
GAL	Hydrolyzes colorless galactosides to yield colored products
GUS	Hydrolyzes glucuronic acid to yield colored products (used in plant)
LUC	Oxidizes luciferin, emitting photons.
GFP	Fluorescence on irradiation with UV.
RFP	Fluorescence on irradiation with UV.

Chloramphenical acetyltransferase (CAT)

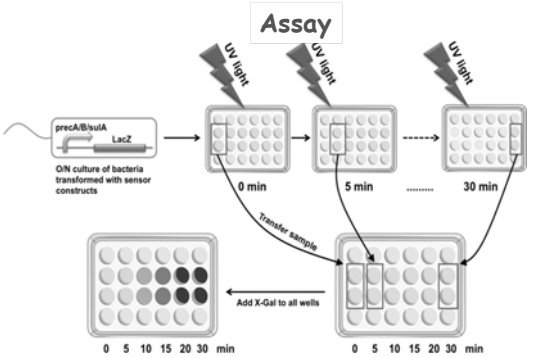


Beta galactosidase (GAL)

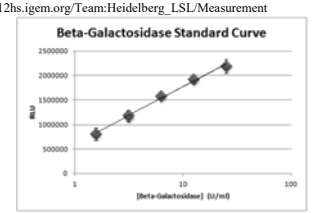
Tissue



Assay

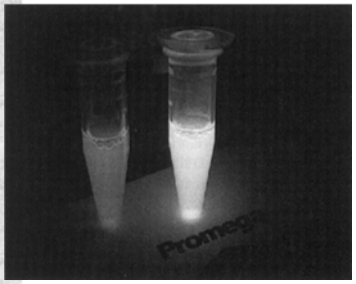


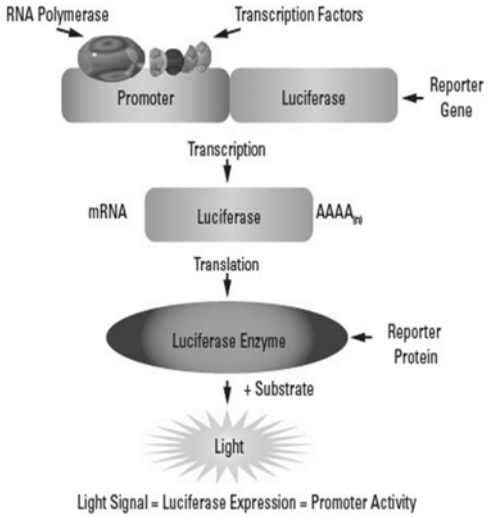
http://2012hs.igem.org/Team:Heidelberg_LSL/Measurement



http://www.markergene.com/ProductDetails.php/M1968

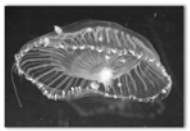
Luciferase (LUC)



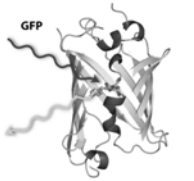


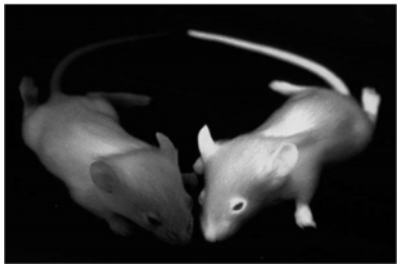
http://www.piercenet.com/method/luciferase-reporters

Green fluorescent protein (GFP)



Aequorea victoria

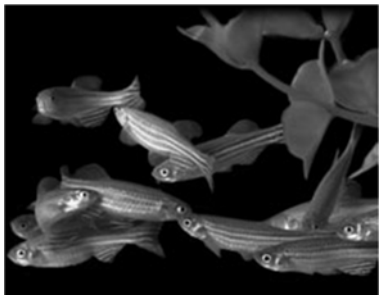




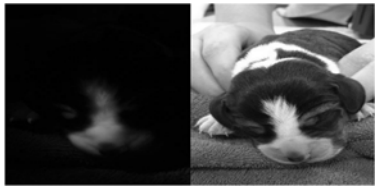
http://2008.igem.org/Team:Heidelberg/Human_Practice/Phips_the_Phage/Technical_Backround

Red fluorescent protein (GFP)

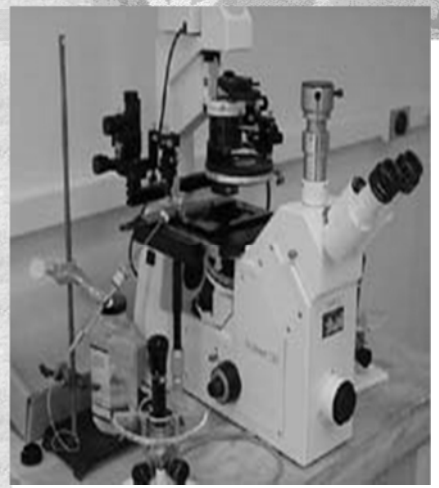
http://mol-biol4masters.masters.grkraj.org/html/Genetic_Engineering7B-Application-Animal_Biotechnology.htm

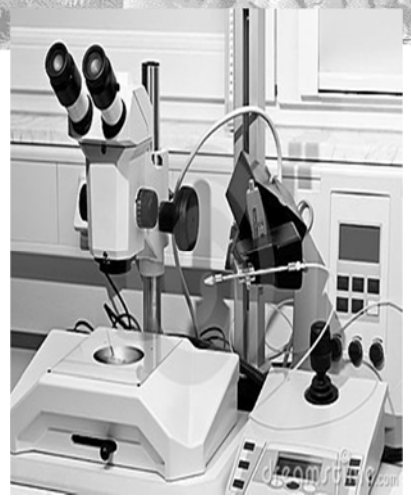


http://www.nature.com/nature/journal/v426/n6965/full/426372b.html



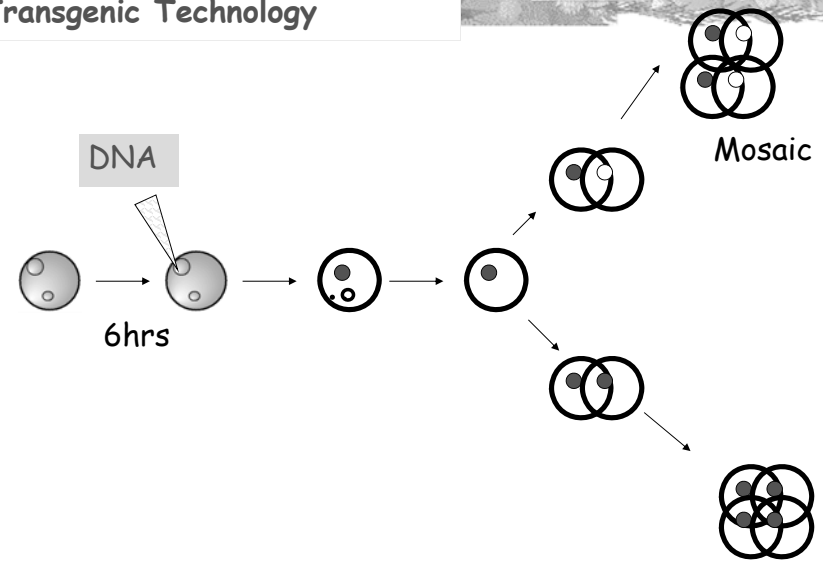
http://news.xinhuanet.com/english/photo/2013-10/20/c_132814711_13.



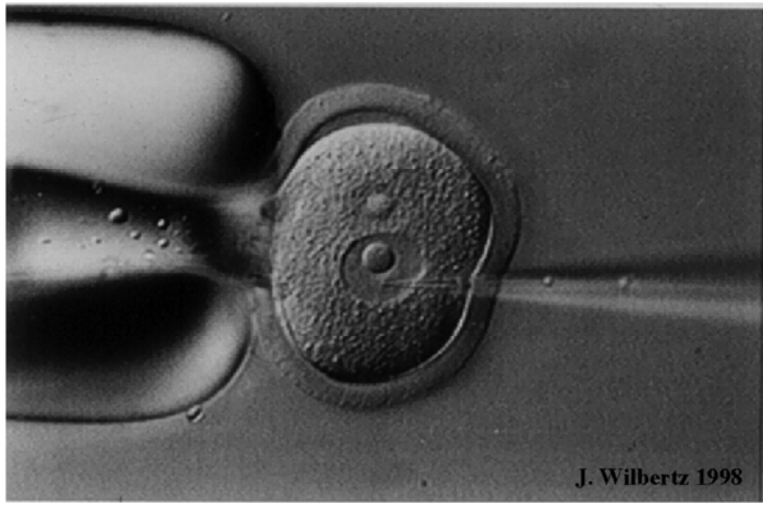


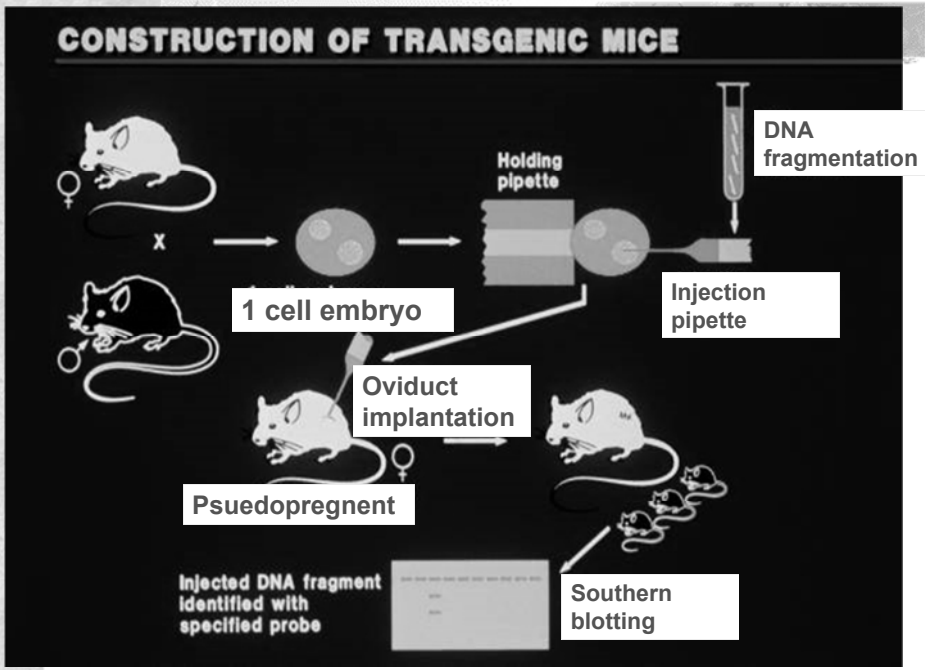
http://www.dreamstime.com/royalty-free-stock-images-modern-system-making-transgenic-animals-image23617449

Transgenic Technology



Pronucleus injection



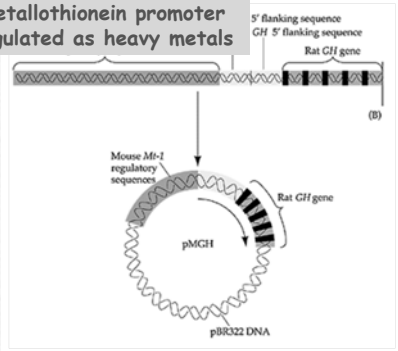


Transgenic mice

The growth hormone gene has been engineered to be expressed at high levels in animals.

The result: BIG ANIMALS

metallothionein promoter regulated as heavy metals



Mice fed heavy metals are 2-3 times larger

antifreeze gene promoter with GH transgene in atlantic salmon

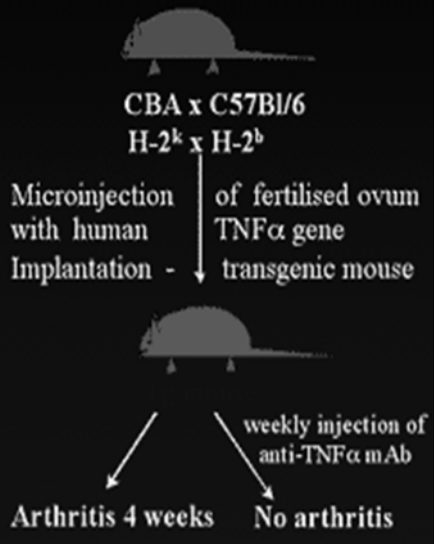
GH gene comes from larger chinook salmon

Human TNFα Transgenic Mice

promoter | hu TNFα | 3' β-globin

- 5 copies of human TNFα gene plus substitution of AU repeats with β-globin gene
- TNFα over-expressed in tissues
- Rheumatoid-like arthritis with joint destruction

Keffer et al 1991 EMBO J. 10: 4025.



Knock out

Knock out (total)

Knockout kill early embryo

Conditional knock out

Site specific recombination system

Inducible expression system

Knock out (total)

Targeting construct

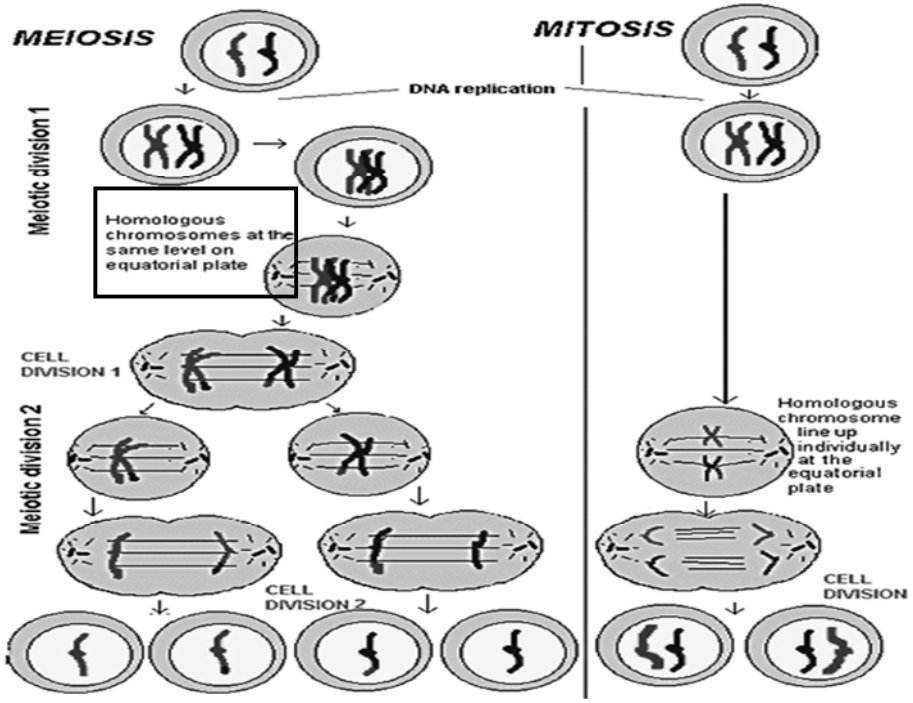
deletion

insertion

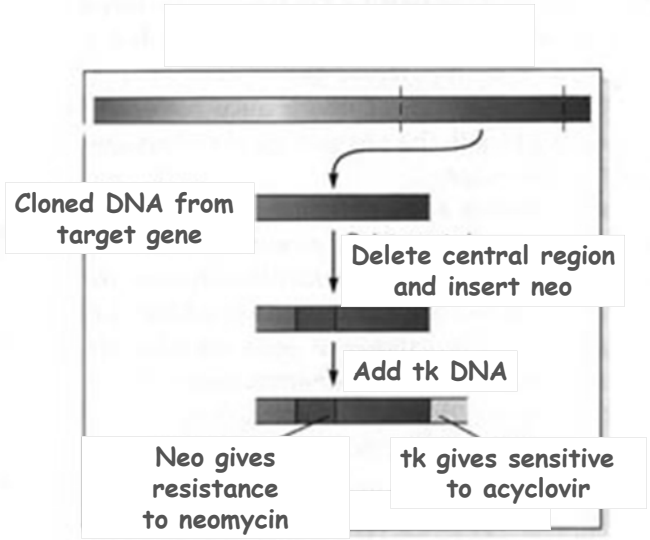
Selective markers

Positive selection: neomycin resistance gene

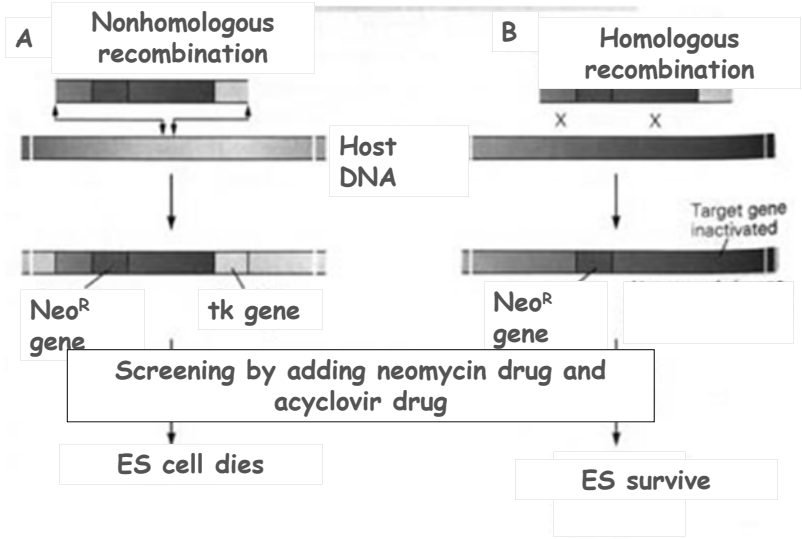
Negative selection: Thymidine kinase gene

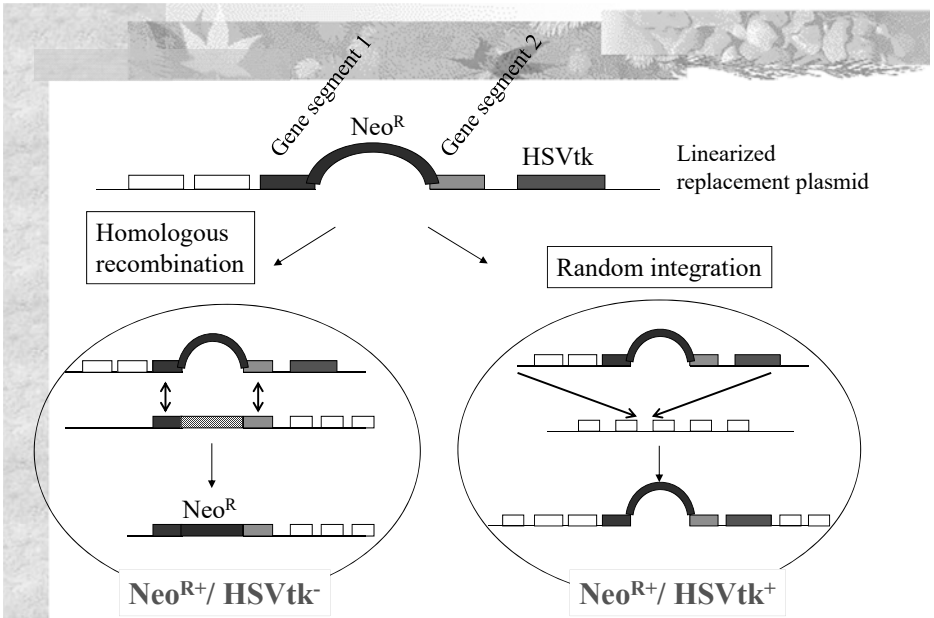


Homologous recombination



Homologous recombination

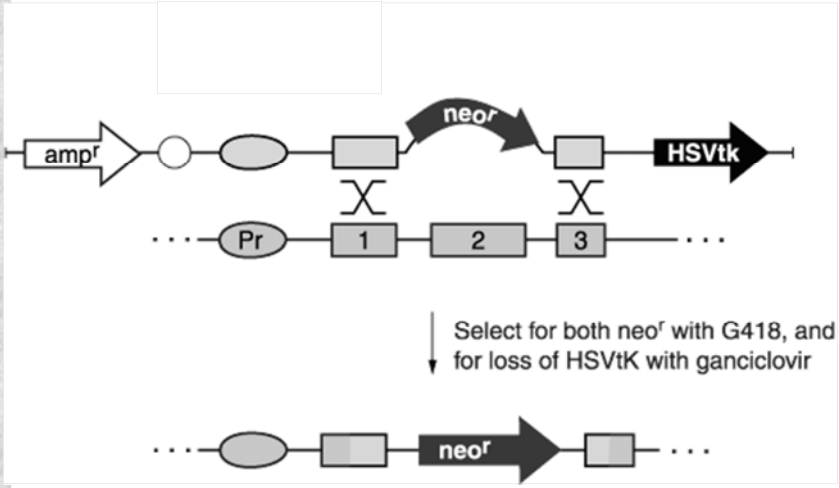




HSVtk will convert gancyclovir into a toxic drug and kill HSVtk+ cells

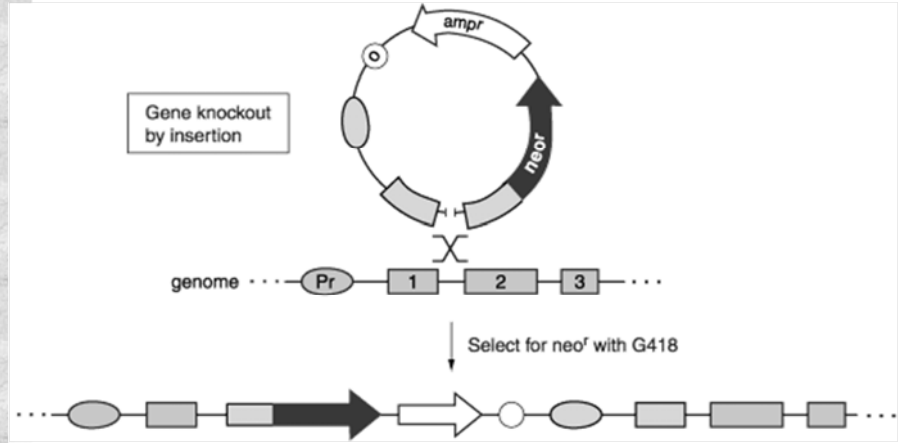
Targeting construct

deletion



Targeting construct

insertion



Electroporate in embryonic stem cells (ES)

↓

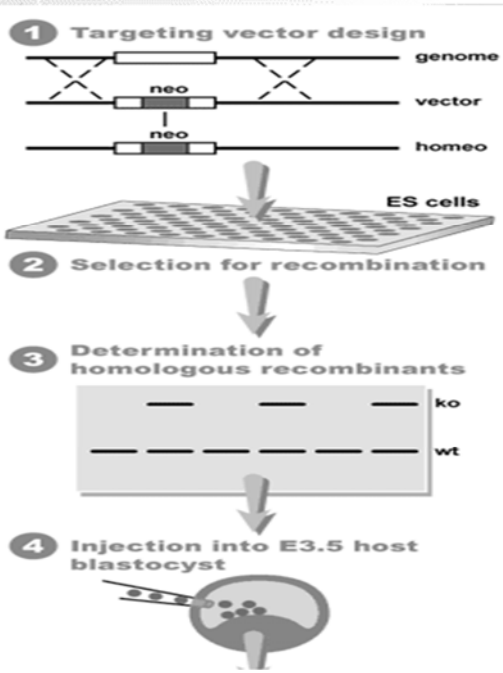
Homologous recombination

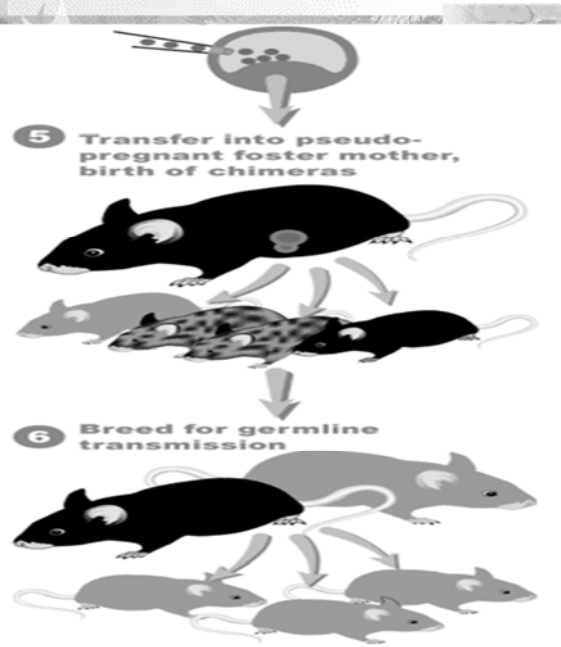
↓

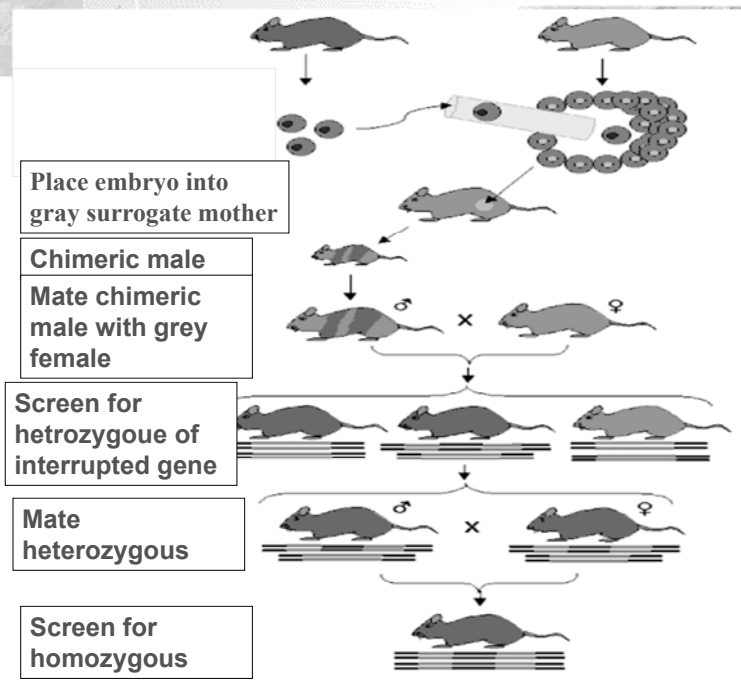
Selection ES cells

Positive selection: neo^R

negative selection: tk







Problems with interpretation of knock-out experiments

1) Knockout kills early embryo. How to estimate effect of adult?

2) No phenotype. Redundancy or just subtle change?

3) Variable phenotype

4) Combinatorial action of genes – the pinball model.

Knock-outs by themselves are not enough to tell you what your gene does to every organ

Conditional knock out

Site specific recombination system

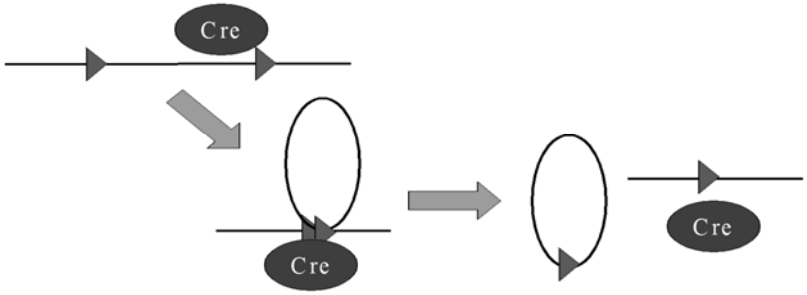
- Cre-lox system
  - Bacteriophage P1
- Flp-FRT system
  - Saccharomyces cerevisiae

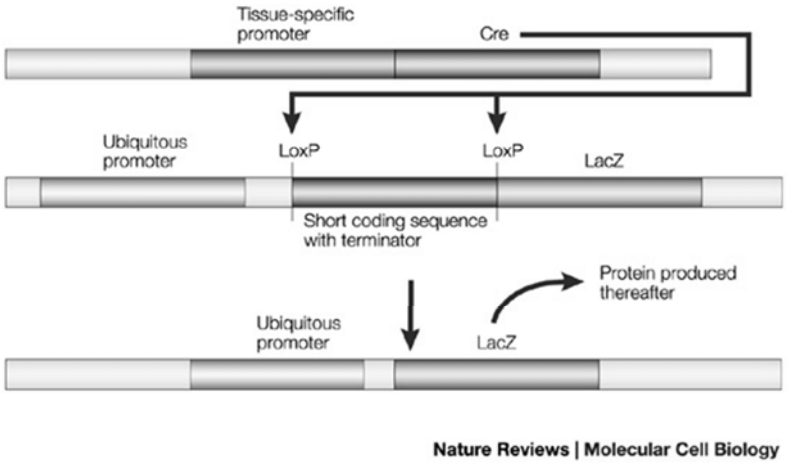
Cre-lox system

Cre-lox technology

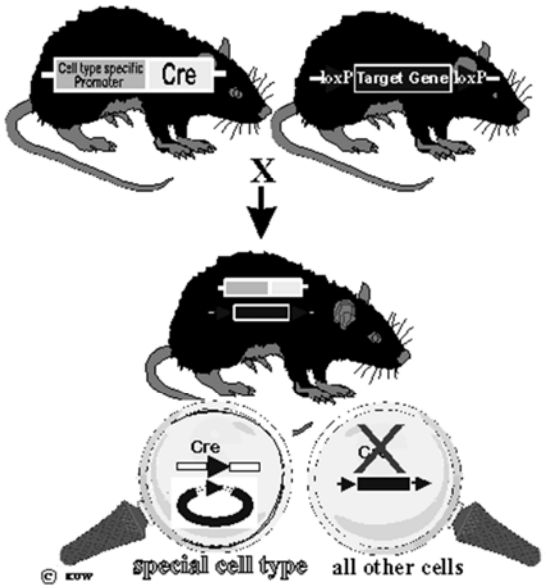
Cre – a site-specific recombinase enzyme from the P1 phage.

Recognises a 34bp DNA sequence *loxP* =





Nature Reviews | Molecular Cell Biology



Inducible expression system

Tet-off

Tet-on

Tet-on and Tet-off systems

Reminder!!!

Now we have 3 transgenes in the same mouse

1. Tetracycline Transactivator (tTA) with constitutive promoter

2. CRE recombinase with Tet-regulated promoter

3. Your gene with loxP sites for CRE

Tet-on

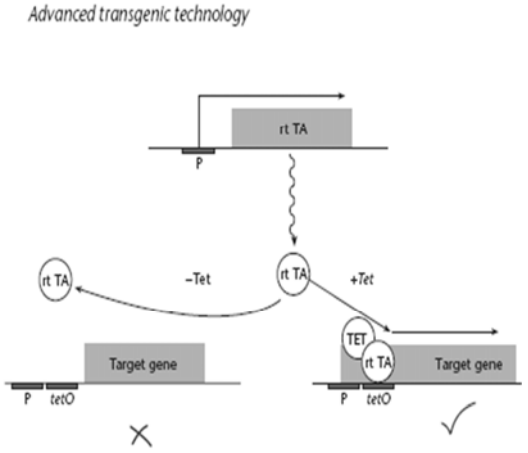


Fig. 15.3 The reverse *tet* transactivator (rtTA) system.

Tet-off

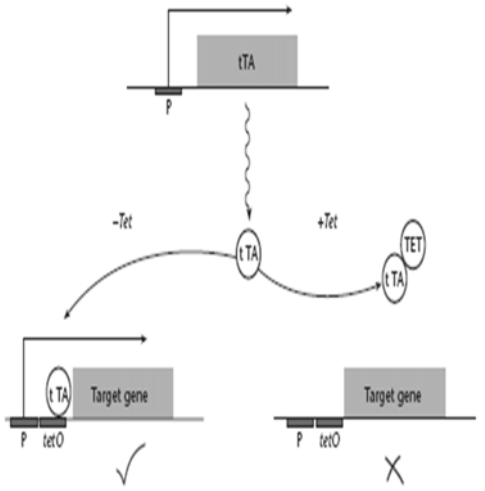
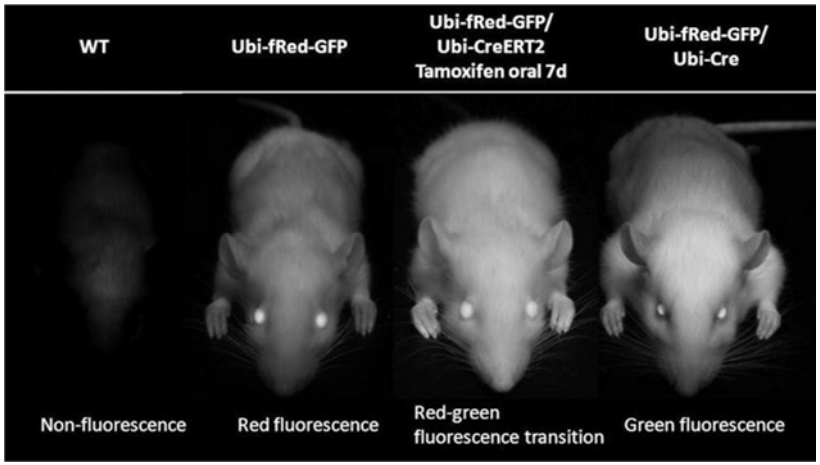


Fig. 15.2 The *tet* transactivator (tTA) system.

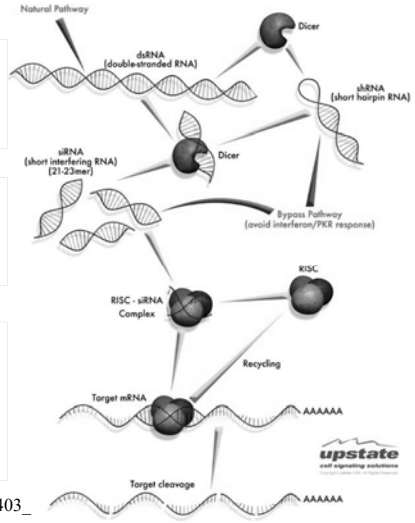


Knock-down: siRNA

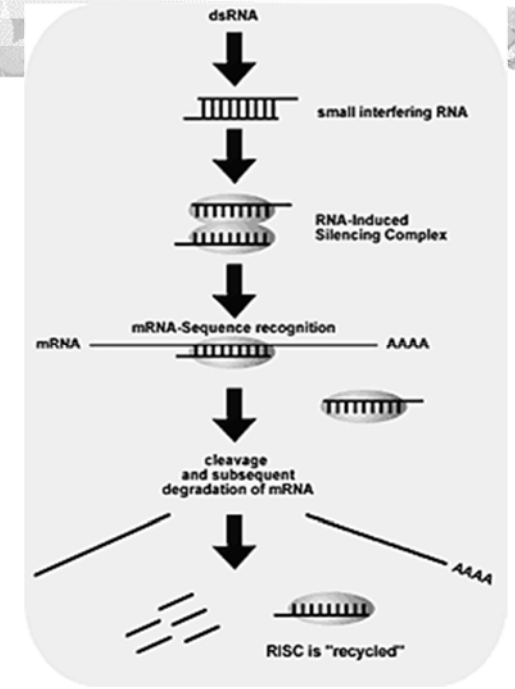
siRNA is 20-25 bp RNA hybridizing to target can trigger degradation of target

RISC is the complex which mediates RNA interference by degrading the target DNA. It's catalytically active enzyme is called argonaute

Dicer is an RNAse that cleaves dsRNA into short double-stranded RNA fragments called small interfering RNA (siRNA) about 20-25 nucleotides long, usually with a two-base overhang on the 3' end.

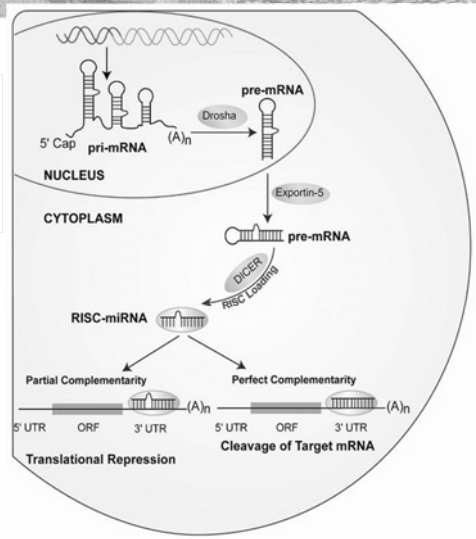


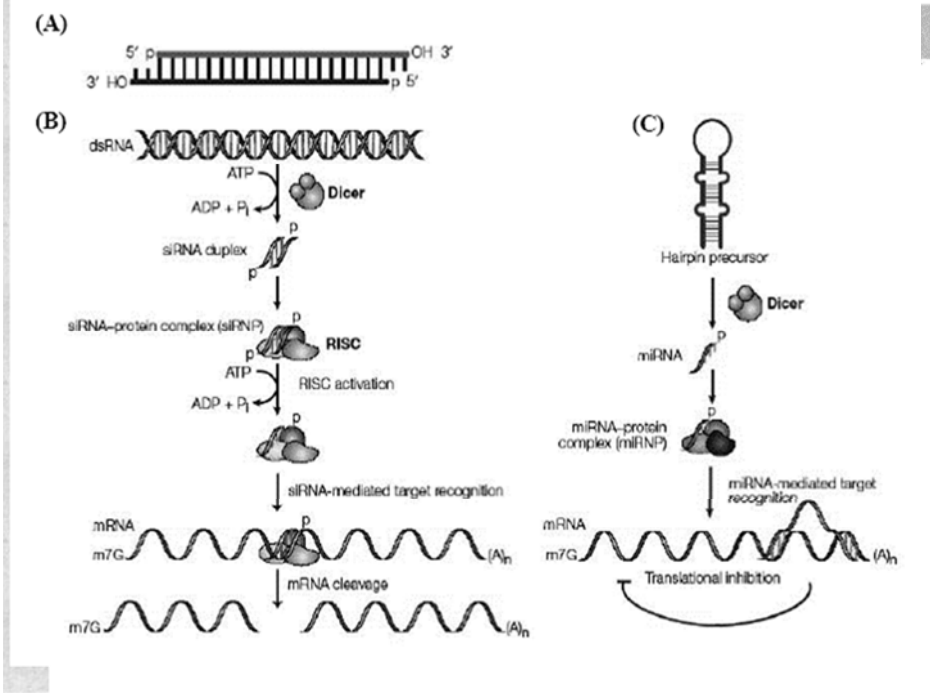
http://genuex.unex.es/Genetica/tema12/12.Animations/1403_RNA_interference.swf



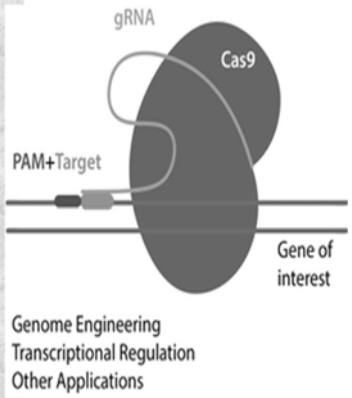
RITS is a complex with siRNAs complementary to the local genes and stably binds local methylated histones, acting co-transcriptionally to degrade any nascent pre-mRNA transcripts that are initiated by RNA polymerase

miRNAs is are genomically encoded non-coding RNAs that help regulate gene expression, particularly during development.





CRISPR Cas9



Genome Engineering
Transcriptional Regulation
Other Applications

CRISPR (clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found within the genomes of prokaryotic organisms such as bacteria.

Cas9 (or "CRISPR-associated protein 9") is an enzyme that uses CRISPR sequences as a guide to recognize and cleave specific strands of DNA that are complementary to the CRISPR sequence.

Engineered CRISPR systems contain two components: a guide RNA (gRNA or sgRNA) and a CRISPR-associated endonuclease (Cas protein).

CRISPR Cas9

Engineered CRISPR systems contain two components: a guide RNA (gRNA or sgRNA) and a CRISPR-associated endonuclease (Cas protein).

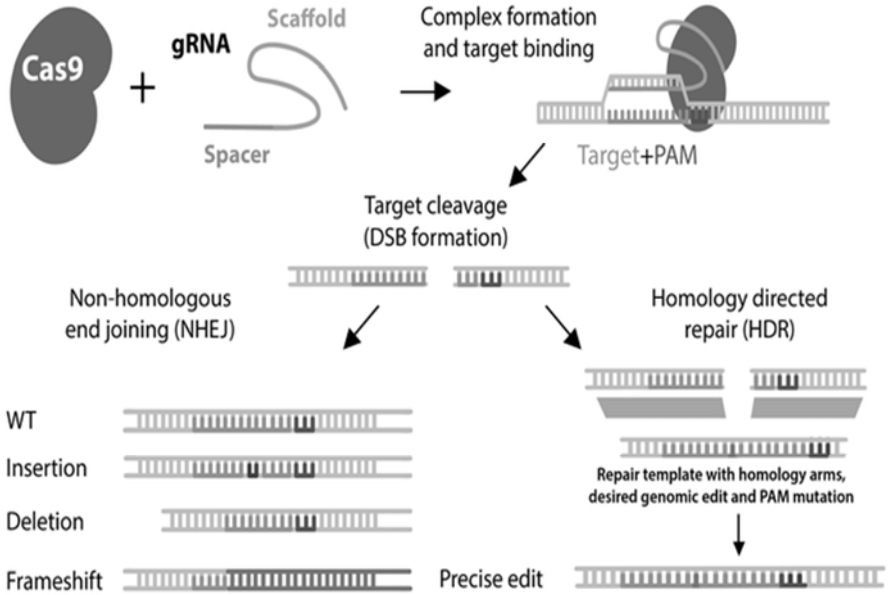
The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas-binding and a user-defined ~20 nucleotide spacer that defines the genomic target to be modified.

The PAM sequence serves as a binding signal for Cas, but the exact sequence depends on which Cas protein you use.

Table 1. *Summary of Cas and other nuclease variants used in CRISPR experiments and t heir PAM sequences.*

CRISPR Nucleases	Organism Isolated From	PAM Sequence (5' to 3')
SpCas9	*Streptococcus pyogenes*	NGG
SaCas9	*Staphylococcus aureus*	NGRRT or NGRRN
NmeCas9	*Neisseria meningitidis*	NNNNGATT
CjCas9	*Campylobacter jejuni*	NNNNRYAC
StCas9	*Streptococcus thermophilus*	NNAGAAW
LbCpf1	*Lachnospiraceae bacterium*	TTTV
AsCpf1	*Acidaminococcus* sp.	TTTV

CRISPR Cas9



Cloning technology

Mammal Cloning Timeline

1984 – A live lamb was cloned from sheep embryo cells
1986 – Early embryo cells were used to clone a cow
1993 – Calves were produced by transfer of nuclei from cultured embryonic cells
1995 – Two sheep, named Megan & Morag, were cloned using embryo cells
1996 – Birth of Dolly, the first organism to be cloned from a fully differentiated adult cell
1997 – Transgenic sheep named Polly was cloned containing a human gene

Megan and Morag

Dolly

1998 – 50 mice were cloned in three generations from a single mouse
1998 – 8 calves were cloned from a single adult cow, but only 4 survived to their first birthday
1999 – A female rhesus monkey named Tetra was cloned by splitting early embryo cells.
2000 – Pigs and goats reported cloned from adult cells
2002 – Rabbits and a kitten reported cloned from adult cells

Dolly with her surrogate mother

Dolly with her first newborn, Bonnie

- Born in July 1996 at the Roslin Institute in Scotland
- First mammal to be cloned from an adult mammal using the nuclear transfer technique
- 277 attempts were made before the experiment was successful
- Dolly died in February 14, 2003 of progressive lung disease at the age of 6; whereas normal sheep can live up to 12 years of age.

Nuclear Transplantation

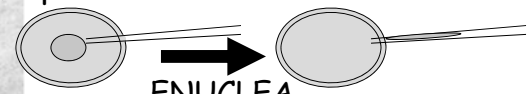
Nucleus comes from someone to be cloned

1. Enucleation of the cell

removal of the nucleus

From the a mature unfertilized oocyte (egg)

Or from the cell in quiescent state (inactive G0 phase of cell cycle) OR metaphase II



2. Nuclear transfer

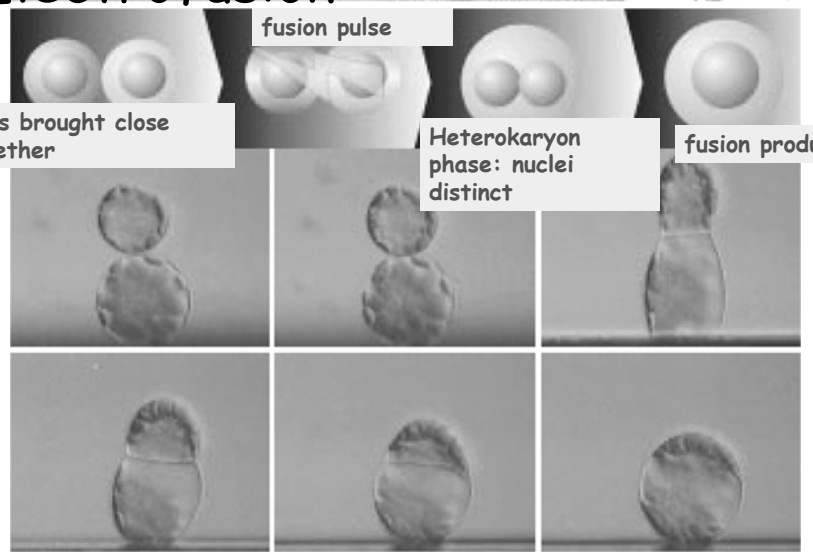
A. electrofusion

whole donor cell
injected beneath the zona pellucida (the outer membrane of the oocyte)
and fusion of cells
induced by electrical impulses

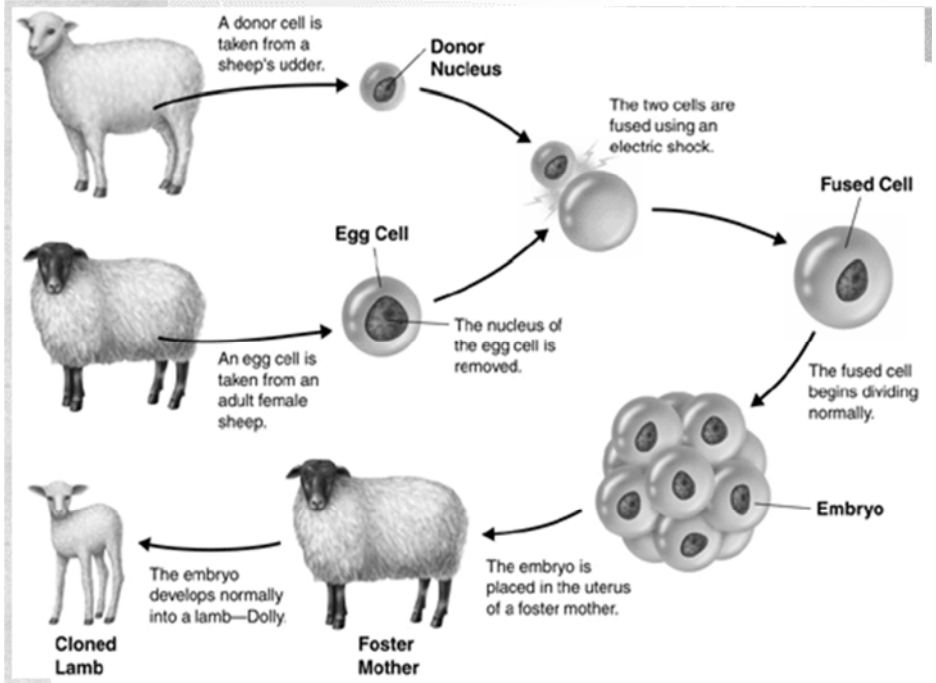
B. nuclear injection

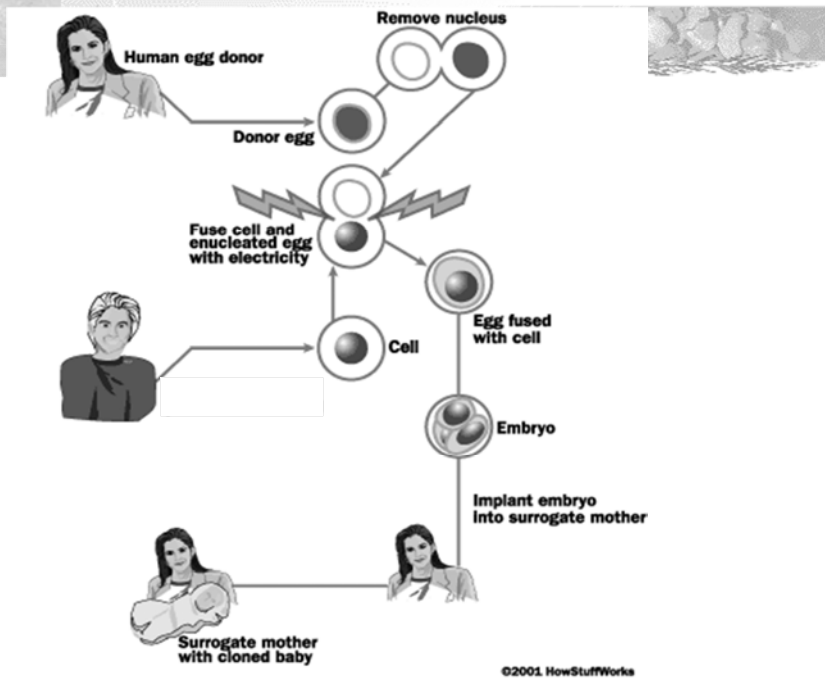
naked nucleus
microinjected into cytoplast

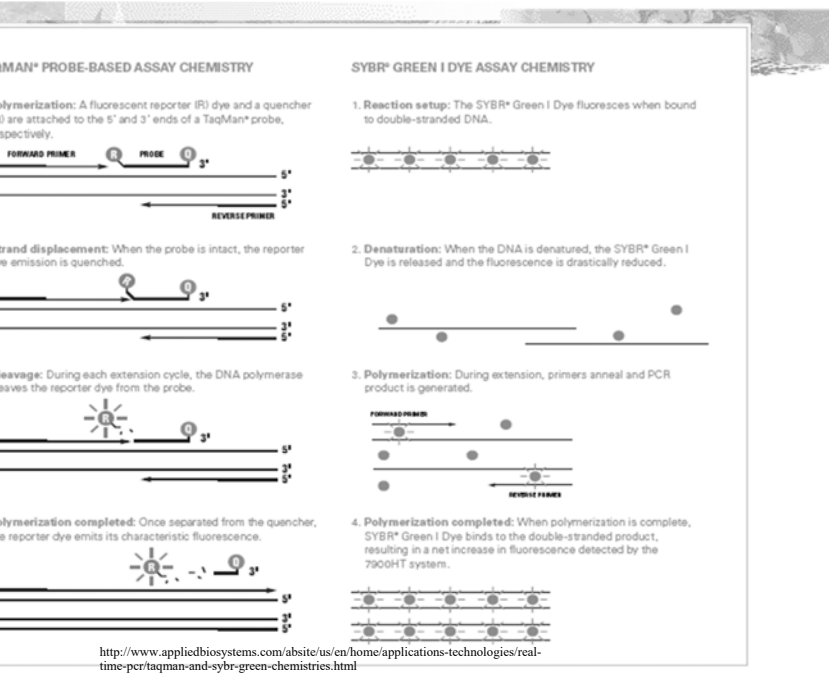
Electrofusion



Fusion induced by electric pulse







http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/taqman-and-sybr-green-chemistries.html