

Basic method in molecular biology II

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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

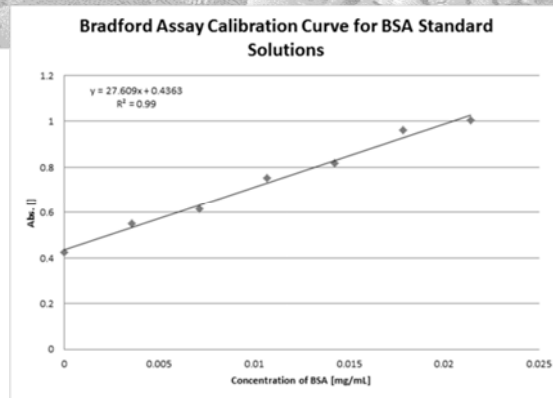
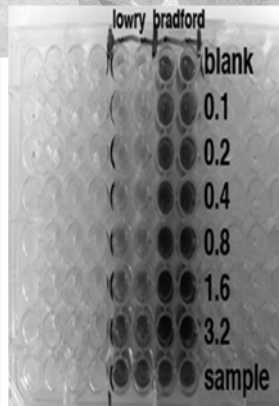


Spectrophotometers

- Transfer of protein to PVDF membrane

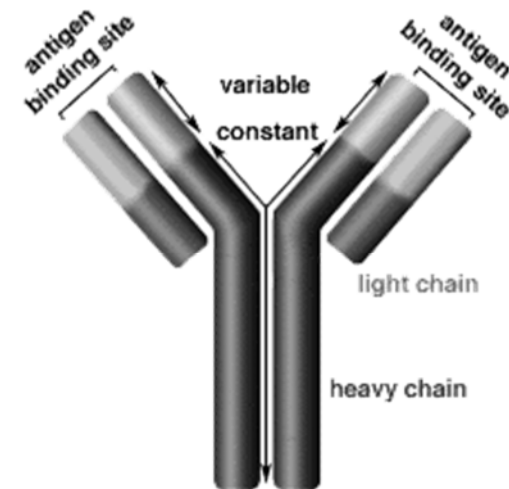
Protein measurement assays

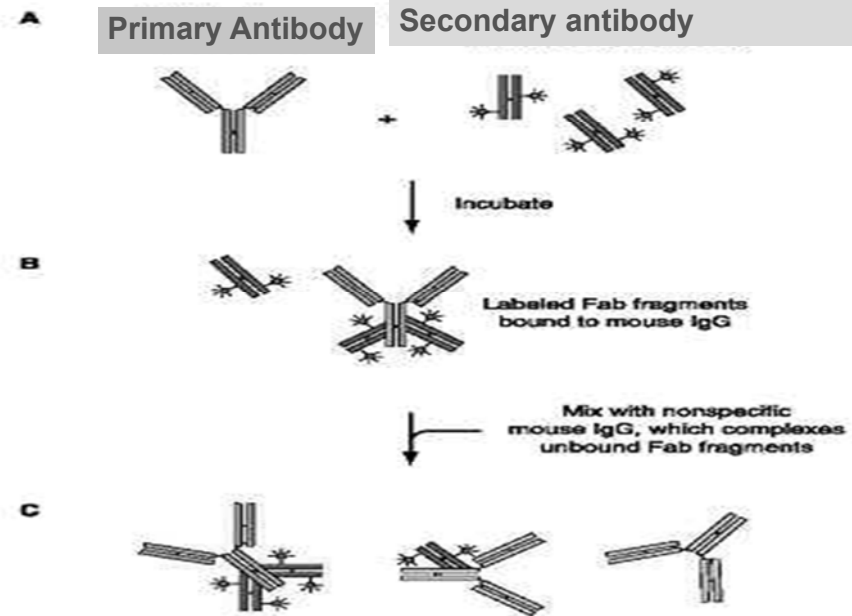
- Several methods are commonly used for determination of protein concentration. Measurement of the UV absorbance at 280 nm is most useful for pure protein solutions.
- Bradford (Coomassie Brilliant Blue), Lowry (Folin-Ciocalteu), Biüret, Pesce and Strande (Ponceau-S/TCA), and modified method of Schaffner-Weismann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



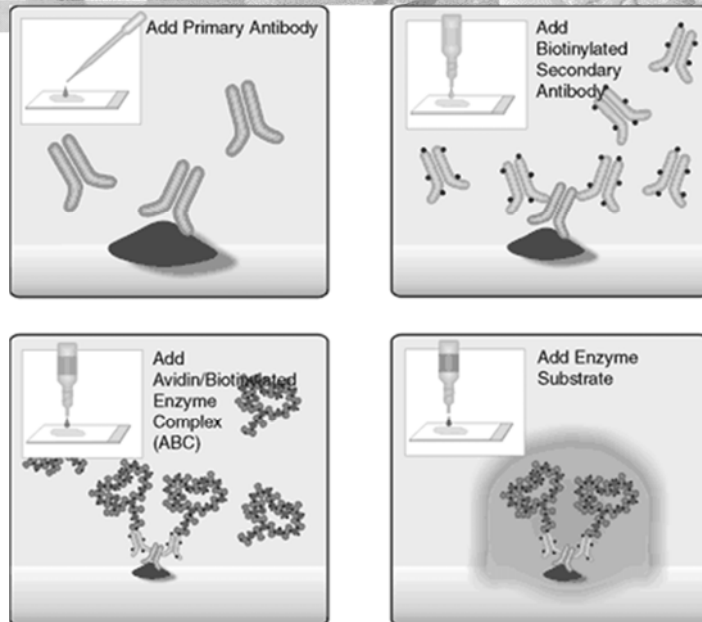
Wikipedia Bradford protein assay

Antibody

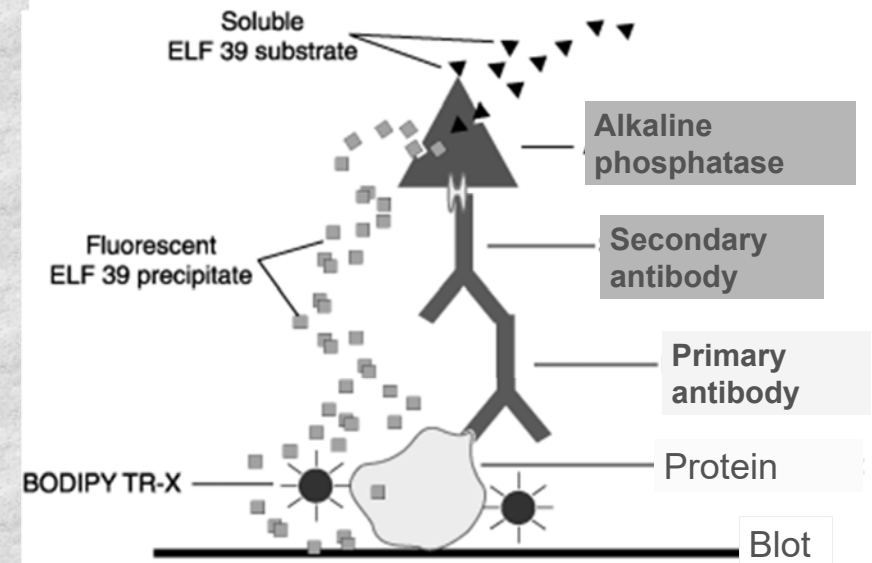


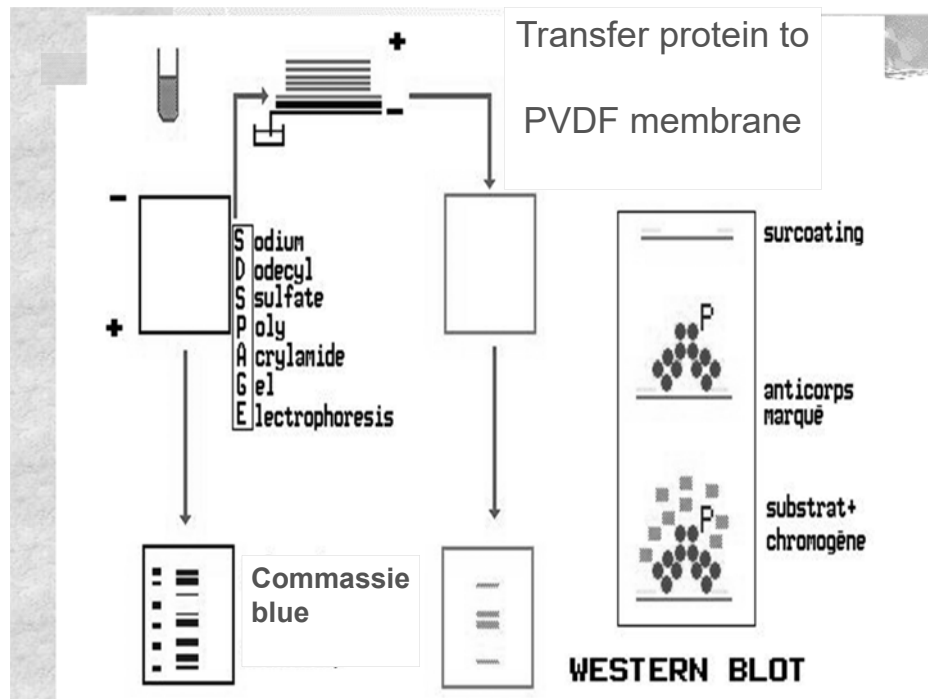


Animation



• Hybridized with antibody (primary & secondary)





Transgenic technology

Two photographs of fish are shown. The left image shows several normal, translucent fish. An arrow points to the right image, which shows a group of fish, some of which are opaque and white, indicating they are transgenic (GloFish).

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>

THE HUMAN GENOME - THE BLUEPRINT OF LIFE

The Human Genome project sequenced DNA, the molecules that make up chromosomes in cells. The information derived from this project presented scientists with a valuable opportunity to not only uncover the secrets of DNA but also the manner in which genes are associated with diseases. Scientists now are able to compare the genomes of people who have a certain condition with those who do not, in order to determine whether genetic variation plays a role in that condition. This information will help them to predict and possibly prevent disease in the future.

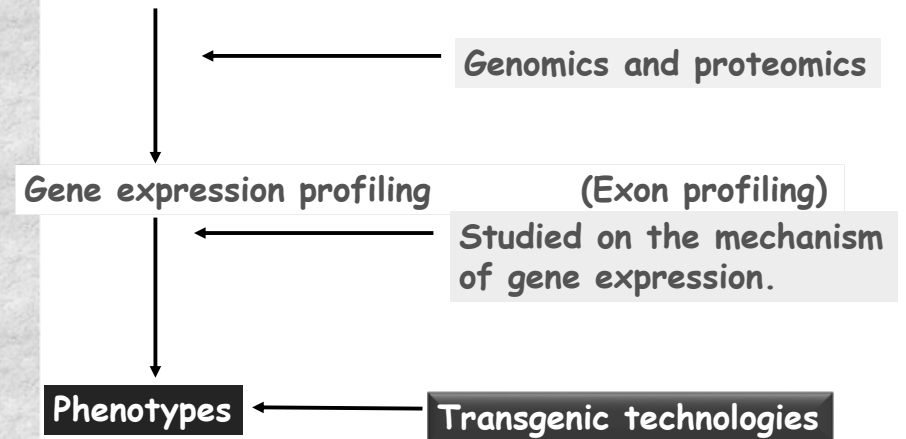
- 1. Cell**
Each of the trillions of cells in the human body contains 46 chromosomes packed tightly into the region called the nucleus.
- 2. Chromosomes**
Half of the chromosomes in the nucleus come from your mother, and half from your father. Each chromosome is a long, tightly coiled molecule called DNA, or deoxyribonucleic acid.
- 3. DNA**
If unwound, the DNA from all the chromosomes in a single cell placed end to end would stretch more than six feet.
- 4. Genome**
DNA is made up of chemical building blocks abbreviated A, C, T, and G. The entire length of a DNA strand consists of these four blocks in different combinations. Together, all the DNA in all the chromosomes - more than 3 billion letters - makes up the human genome. When scientists say they have "sequenced" the human genome, they mean that they have figured out the order of all those A's, C's, T's, and G's in sequence.
- 5. Genes: 30,000 DNA Segments**
Much of the DNA in the genome is organized into units called genes. There may be as many as 30,000 genes in the genome. These are the instructions for making all the proteins in the body. These proteins are the physical "hull" that makes up the hair, skin, teeth, and bones, and does other things. They also control chemical reactions, regulate blood sugar and heart rate, and control how food or medicine is metabolized in the body.
- 6. Misspellings in the Sequence**
The way the genes are "spelled" makes all the difference - one letter out of place in a gene can cause disease. Now that we know the correct sequence of the human genome, researchers can compare the DNA sequence from people who have a disease or condition to those who don't. If there are differences in the spelling of certain genes between the two groups, it's possible that the condition may be caused by or related to that misspelling in that gene.
- 7. Genes and Disease**
Scientists have identified about 6000 diseases, such as Huntington's disease and cystic fibrosis, that are directly caused by misspellings or physical problems in single genes. But the genetic contribution to many common conditions - such as diabetes and heart disease - is part of a larger puzzle that could include diet, lifestyle, environment, and even other genes. Forewarned of these common conditions, however, misspellings probably make only a small contribution to disease relative to other factors, or work in concert with them to cause illness.

<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)

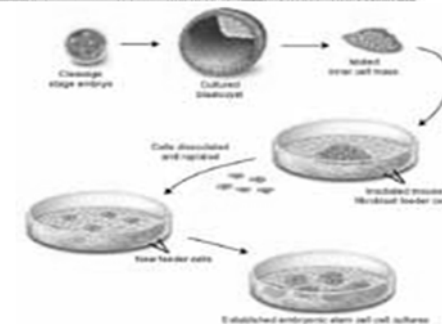


Transgenic technology

- 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

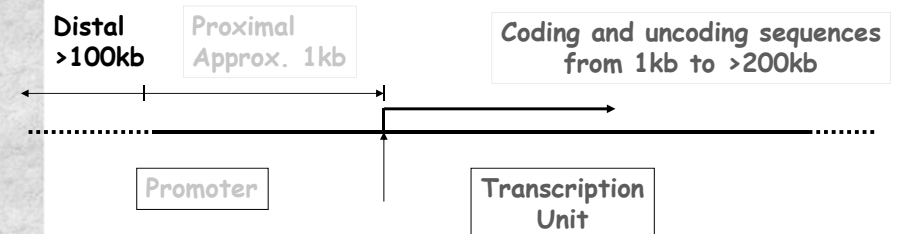
A method to express exogenous genes from specific endogenous regulatory elements *in vivo*



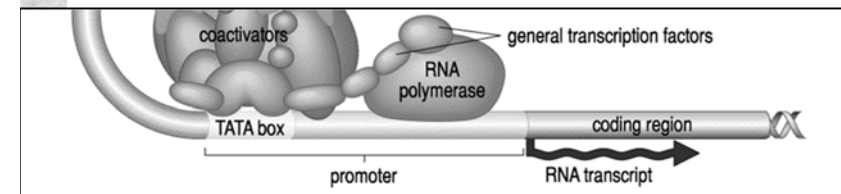
Targeting construct

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

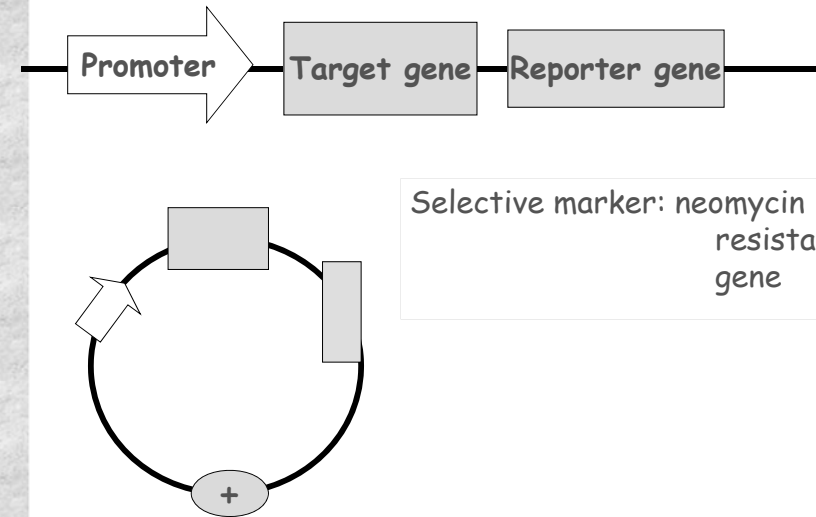
Targeting construct

exogenous specific gene: cDNA from other species

Human → mouse

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

Targeting construct: transgenic



Transgenic Technology

Promoter

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

cDNA

Gain of Function

- wild-type gene
- mutant

Loss of Function

- 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

Chloramphenicol 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.

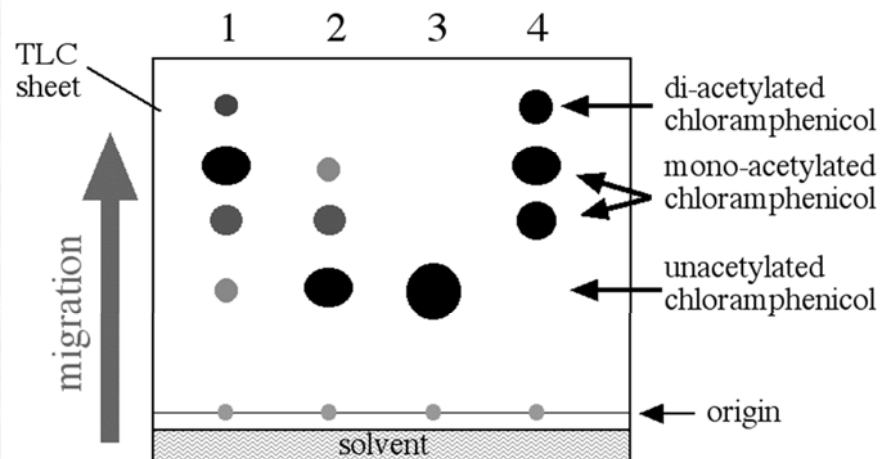
GFP

Fluorescence on irradiation with UV.

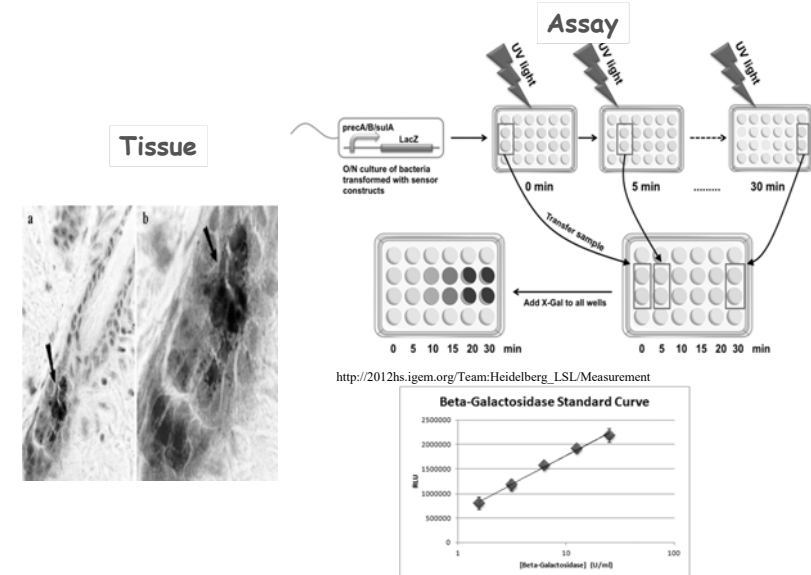
RFP

Fluorescence on irradiation with UV.

Chloramphenicol acetyltransferase (CAT)



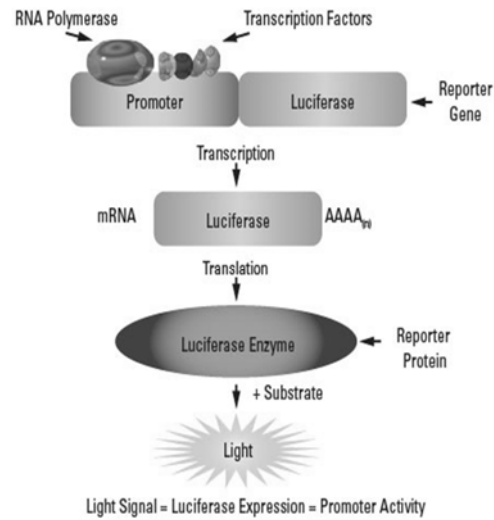
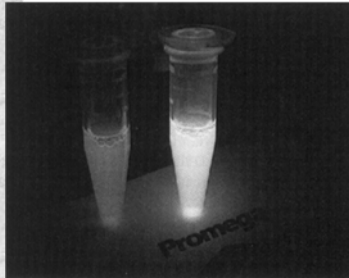
Beta galactosidase (GAL)



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

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

Luciferase (LUC)

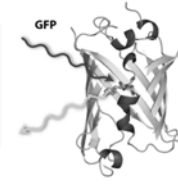


<http://www.piercenet.com/method/luciferase-reporters>

Green fluorescent protein (GFP)

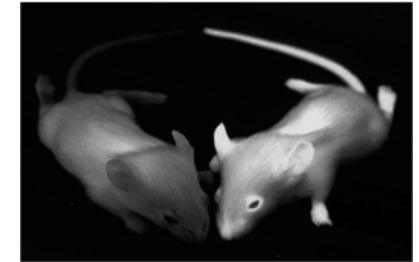


Aequorea victoria



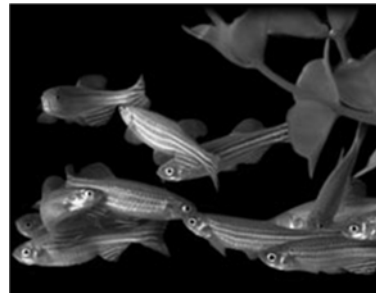
high energy

low energy



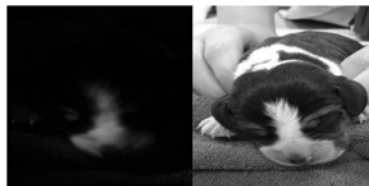
http://2008.igem.org/Team:Heidelberg/Human_Practice/Phips_the_Phage/Technical_Background

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>



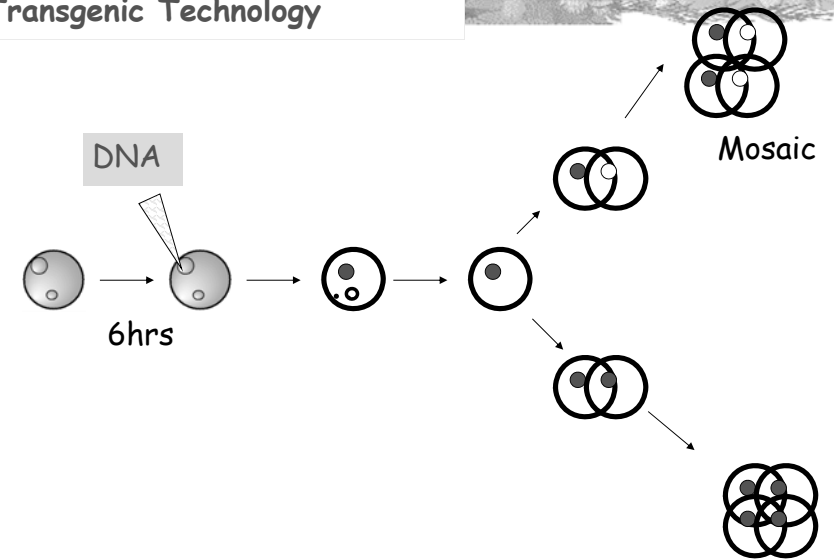
http://news.xinhuanet.com/english/photo/2013-10/20/c_132814711_13



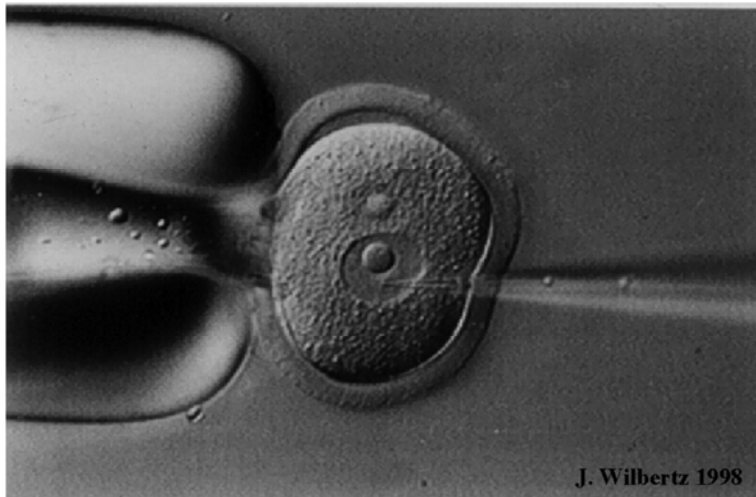


<http://www.dreamstime.com/royalty-free-stock-images-modern-system-making-transgenic-animals-image23617449>

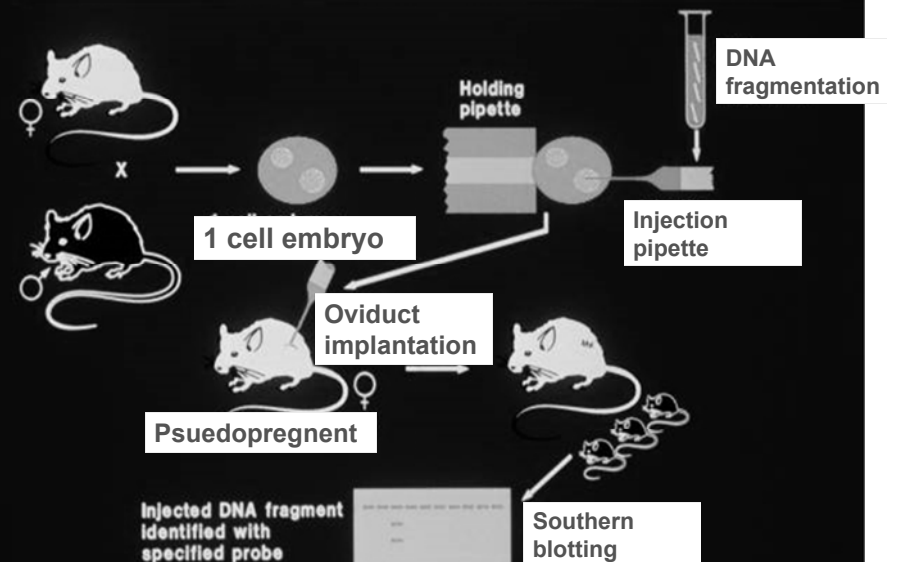
Transgenic Technology



Pronucleus injection



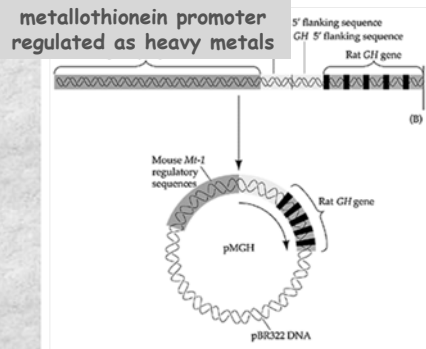
CONSTRUCTION OF TRANSGENIC MICE



Transgenic mice

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

The result: **BIG ANIMALS**



Mice fed heavy metals are 2-3 times larger



antifreeze gene promoter with GH transgene in atlantic salmon



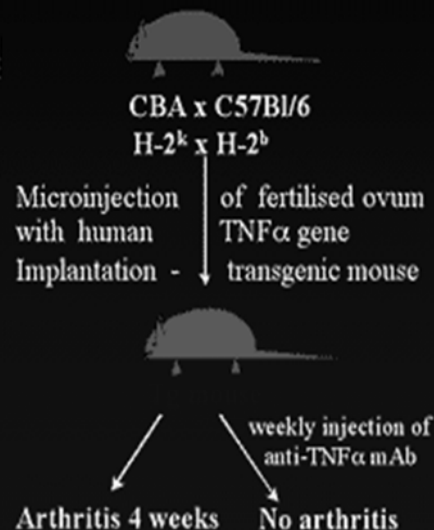
GH gene comes from larger chinook salmon

Human TNF α Transgenic Mice

promoter hu TNF α 3' β -globin

- 5 copies of human TNF α gene plus substitution of AU repeats with β -globin gene
- TNF α 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

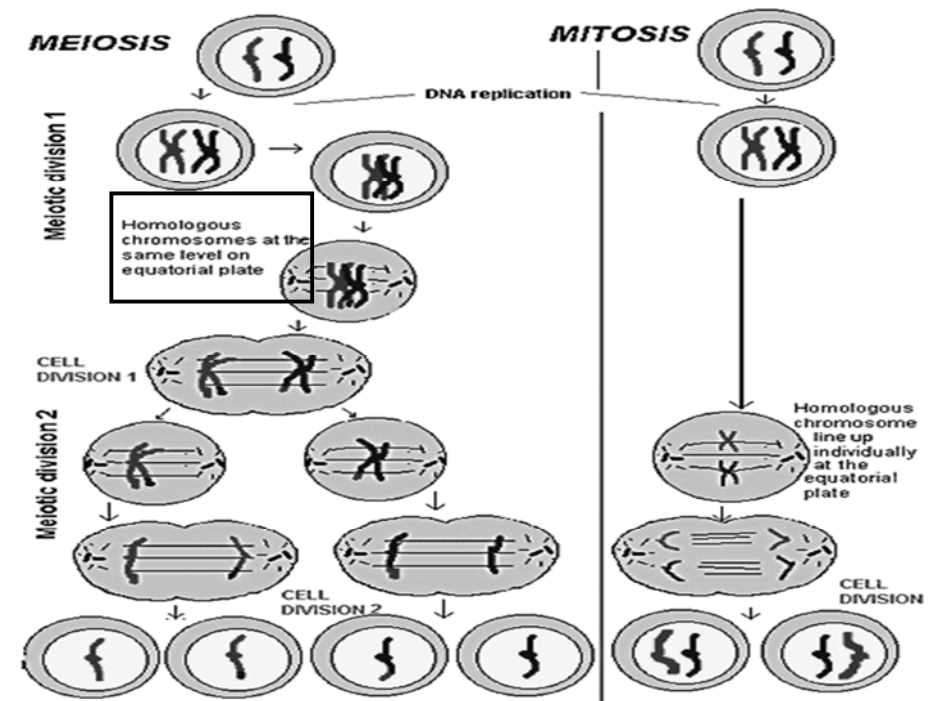
deletion

insertion

Selective markers

Positive selection: neomycin resistance gene

Negative selection: Thymidine kinase gene



Homologous recombination

Cloned DNA from target gene

Delete central region and insert neo

Add tk DNA

Neo gives resistance to neomycin

tk gives sensitive to acyclovir

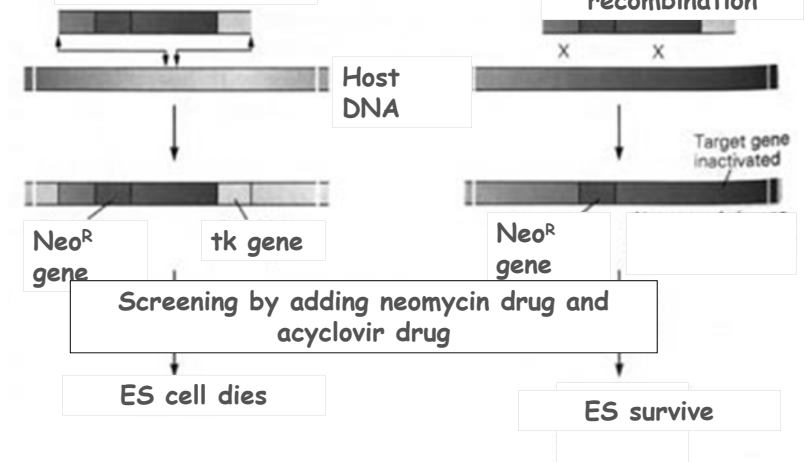
Homologous recombination

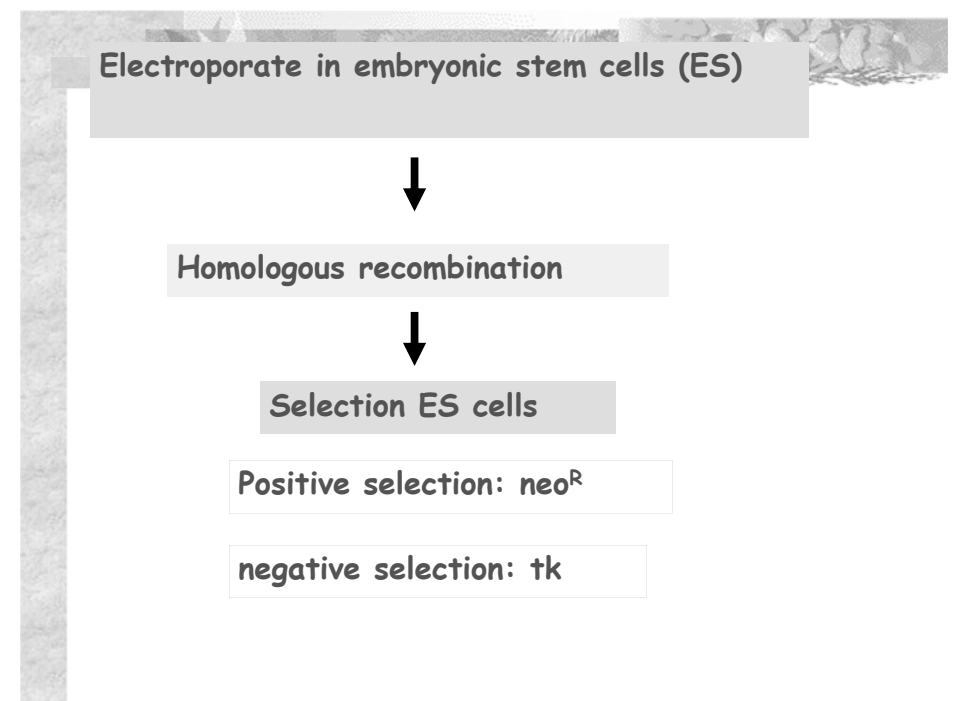
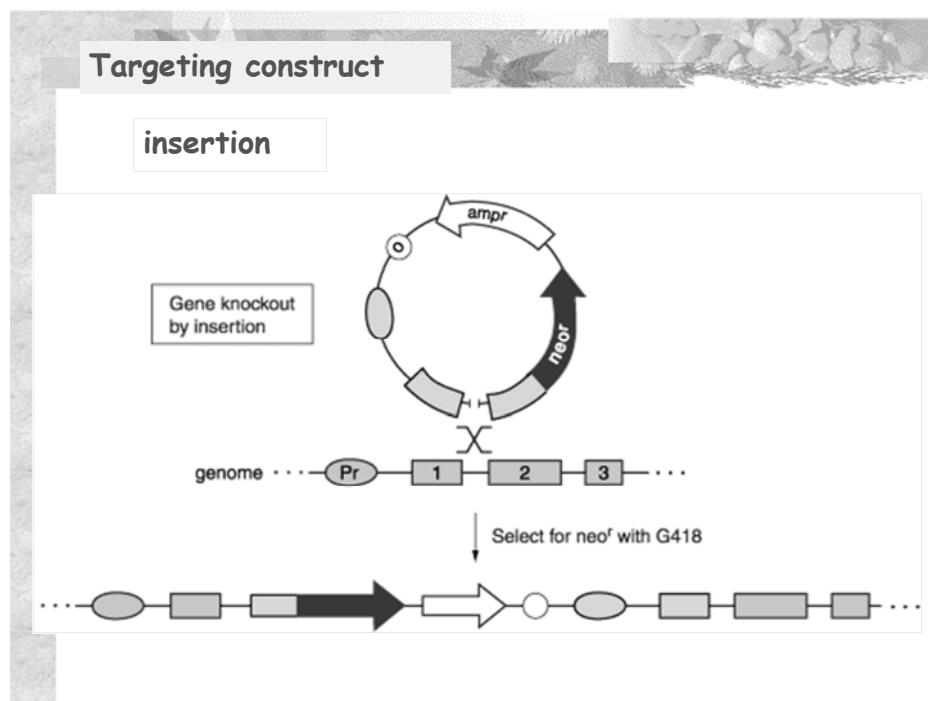
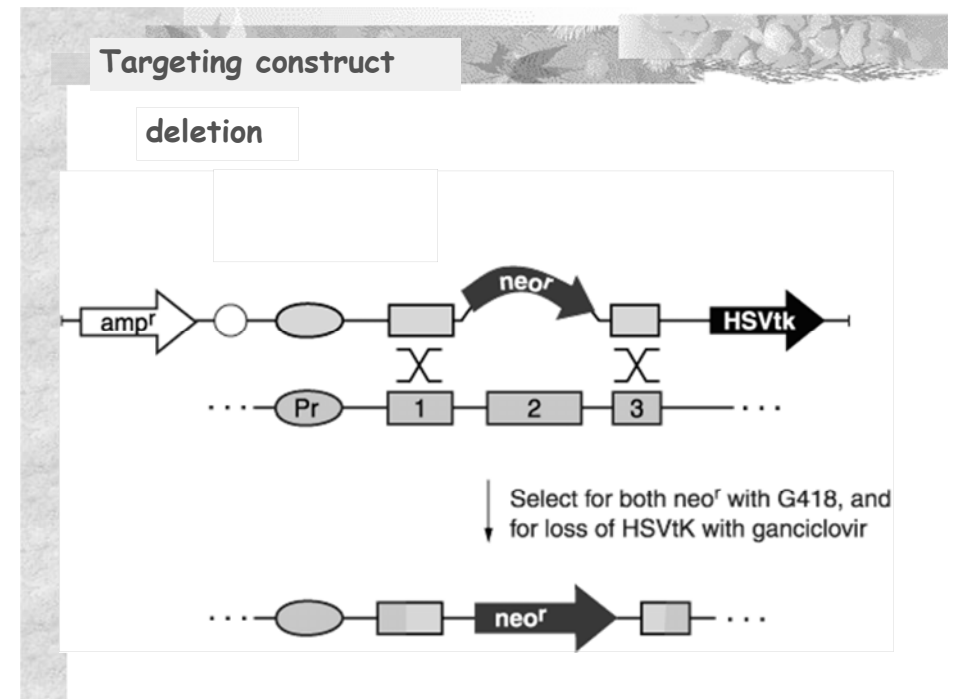
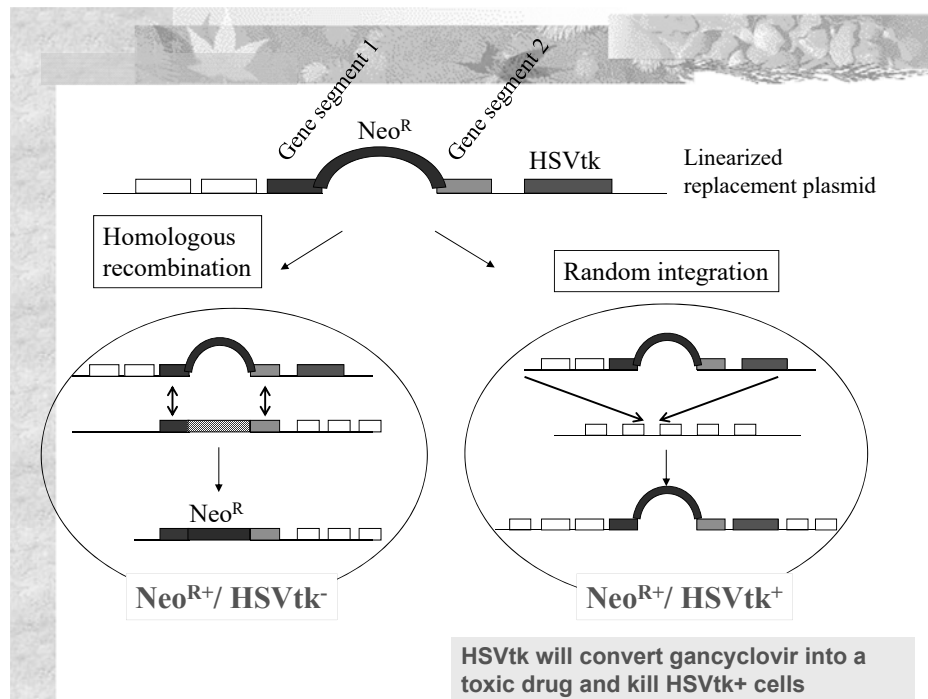
A

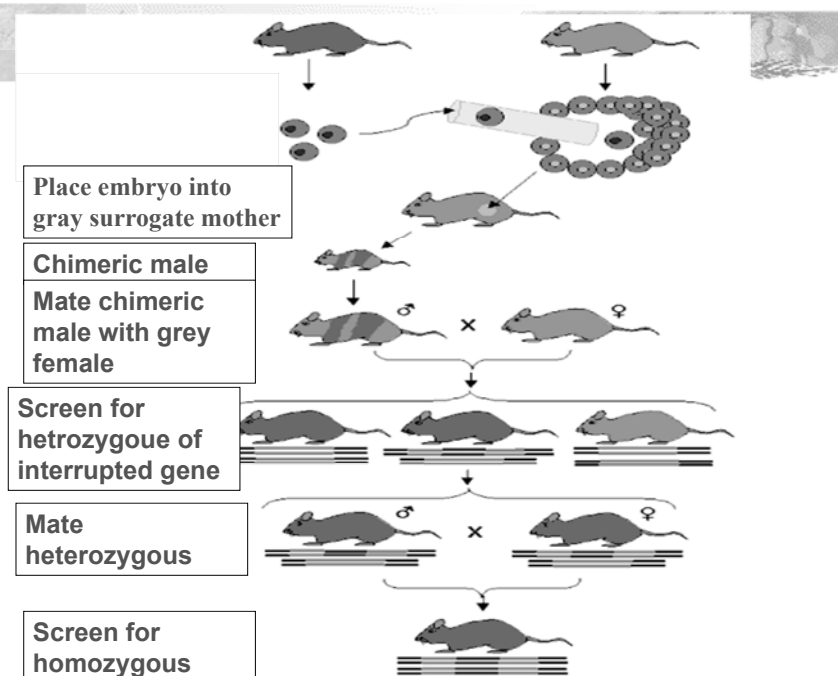
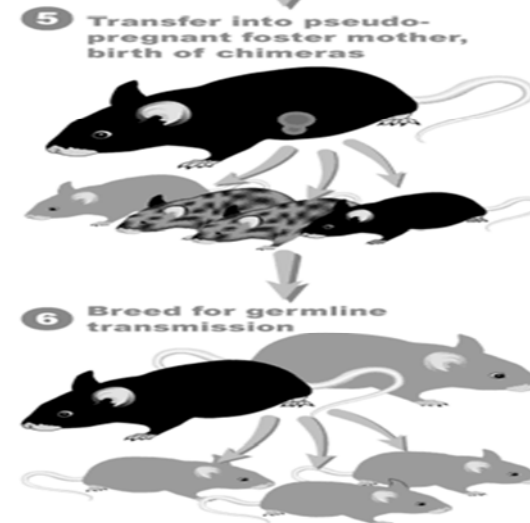
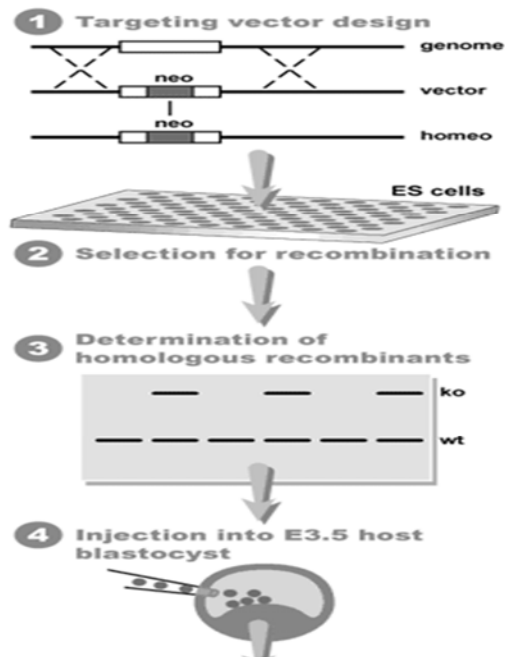
Nonhomologous recombination

B

Homologous recombination







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

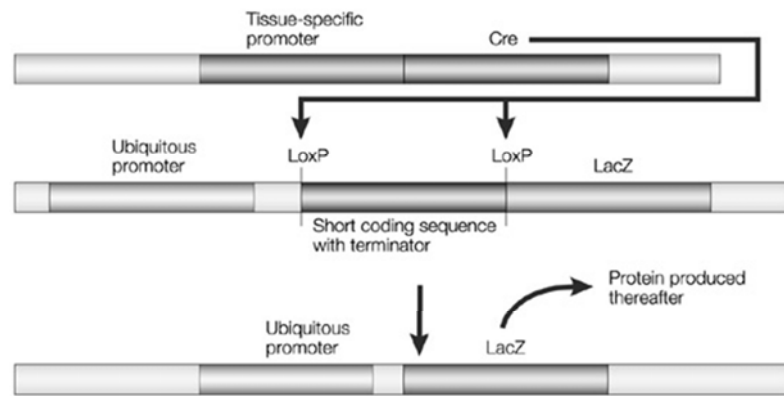
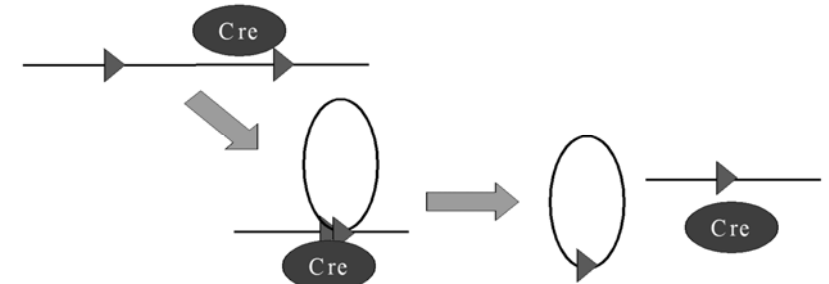
Saccharomyces cerevisiae

Cre-lox system

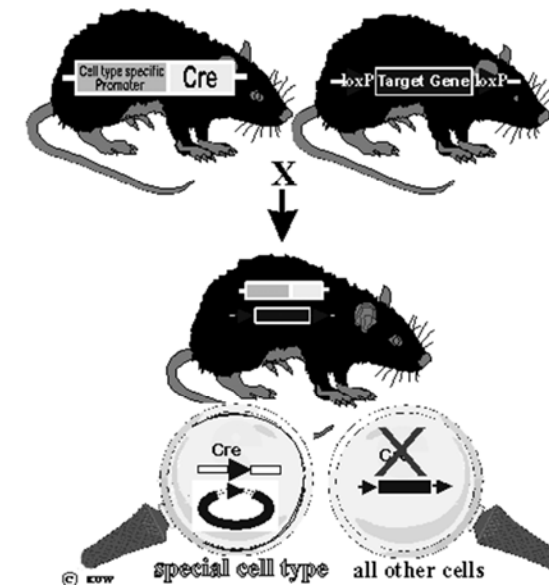
Cre-lox technology

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

Advanced transgenic technology

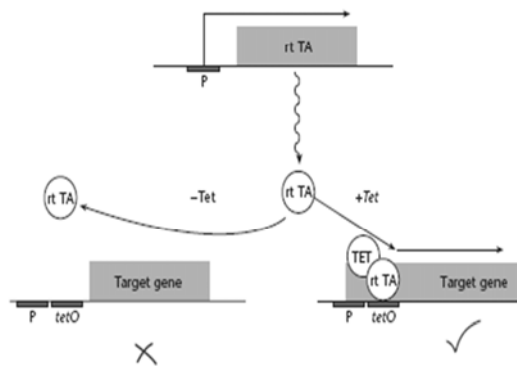


Fig. 15.3 The reverse tet transactivator (rtTA) system.

Tet-off

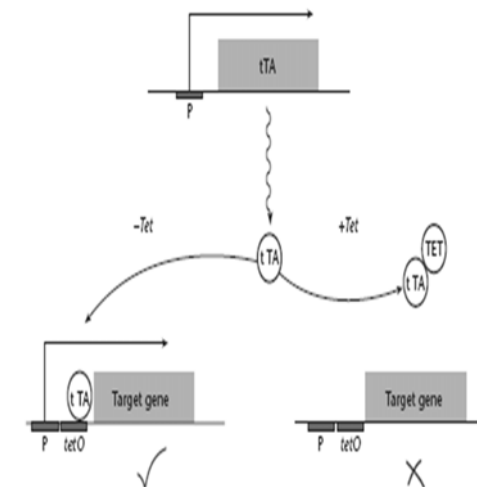


Fig. 15.2 The tet transactivator (tTA) system.

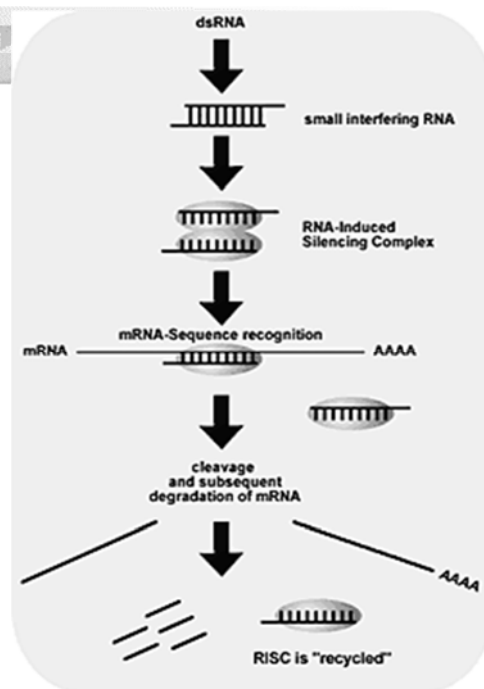
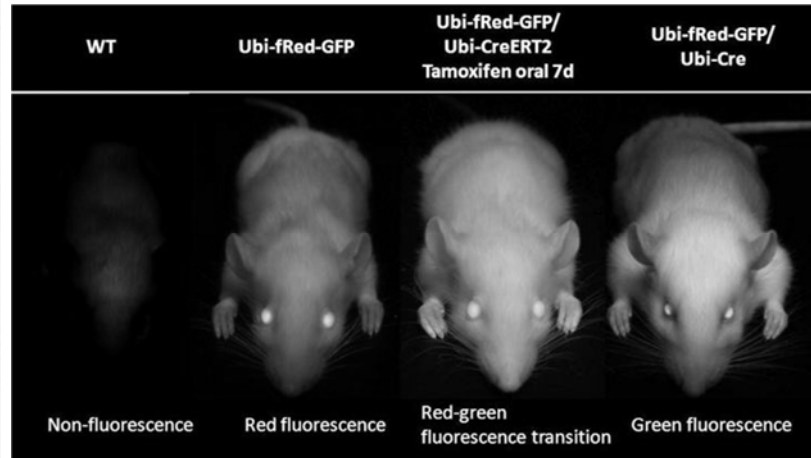
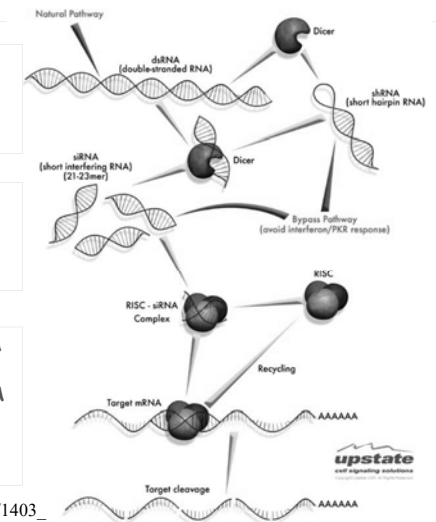
Knock-down: siRNA

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. Its catalytically active enzyme is called argonaute

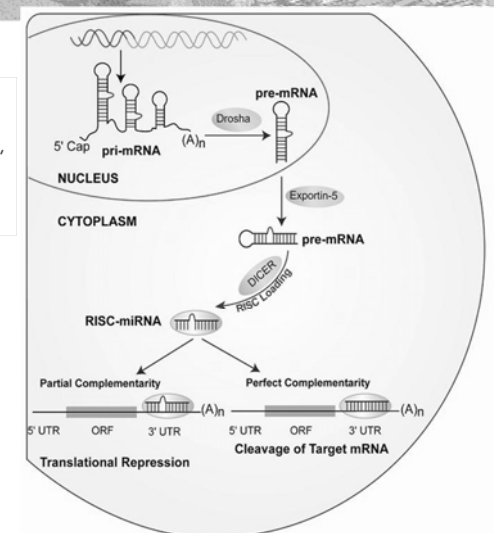
Dicer is an RNase that cleaves dsRNA into short double-stranded RNA fragments called small interfering RNA (siRNA) about 20-25 nucleotides long, usually with a two-base overhang on the 3' end.

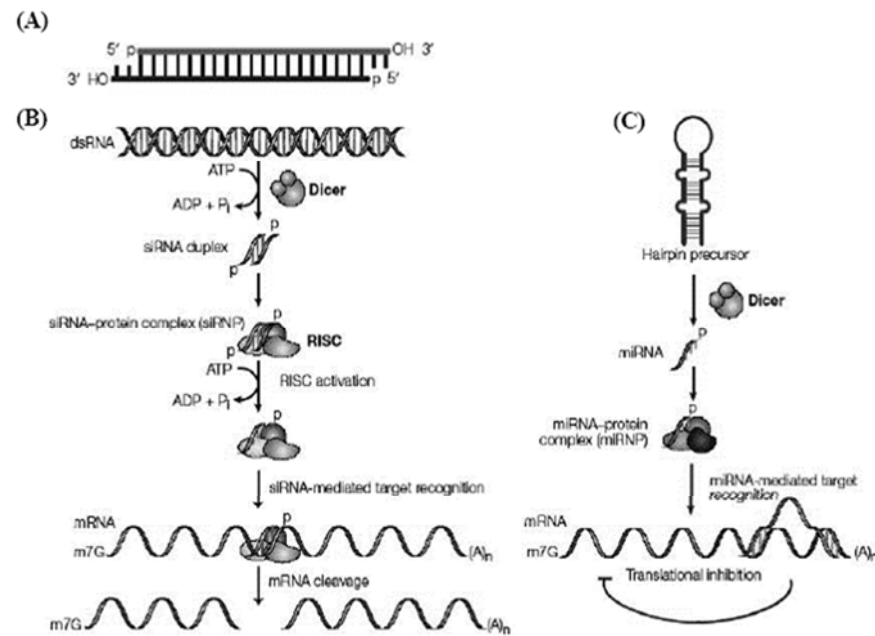
http://genue.unex.es/Genetica/tema12/12_Animations/1403_RNA_interference.swf



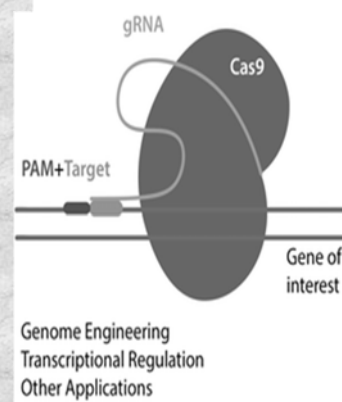
RITS is a complex with siRNAs complementary to the local genes and stably binds local methylated histones, acting co-transcriptionally to degrade any nascent pre-mRNA transcripts that are initiated by RNA polymerase

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





CRISPR Cas9



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).

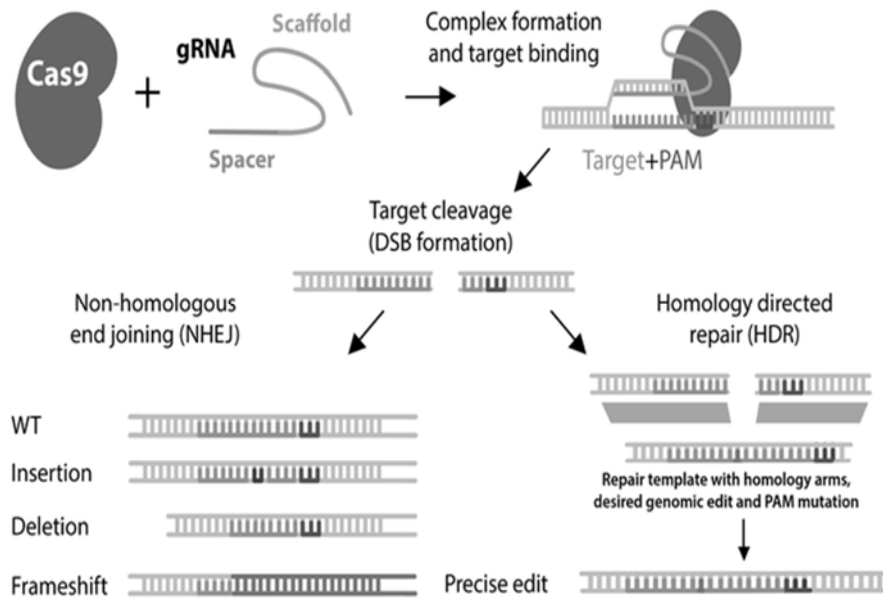
The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas-binding and a user-defined ~20 nucleotide spacer that defines the genomic target to be modified.

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 their PAM sequences.

CRISPR Nucleases	Organism Isolated From	PAM Sequence (5' to 3')
SpCas9	<i>Streptococcus pyogenes</i>	NGG
SaCas9	<i>Staphylococcus aureus</i>	NGRRT or NGRRN
NmeCas9	<i>Neisseria meningitidis</i>	NNNNGATT
CjCas9	<i>Campylobacter jejuni</i>	NNNNRYAC
StCas9	<i>Streptococcus thermophilus</i>	NNAGAAW
LbCpfI	<i>Lachnospiraceae</i> bacterium	TTTV
AsCpfI	<i>Acidaminococcus</i> 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

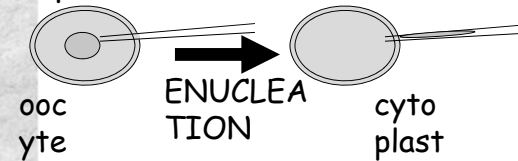
2. Nuclear transfer

A. electrofusion

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

From the a mature unfertilized oocyte (egg)

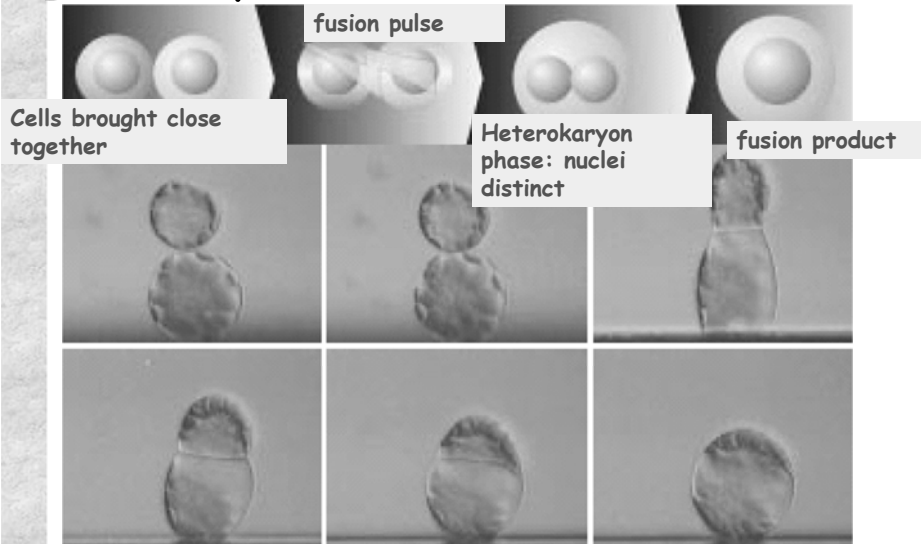
Or from the cell in quiescent state (inactive G0 phase of cell cycle) OR metaphase II



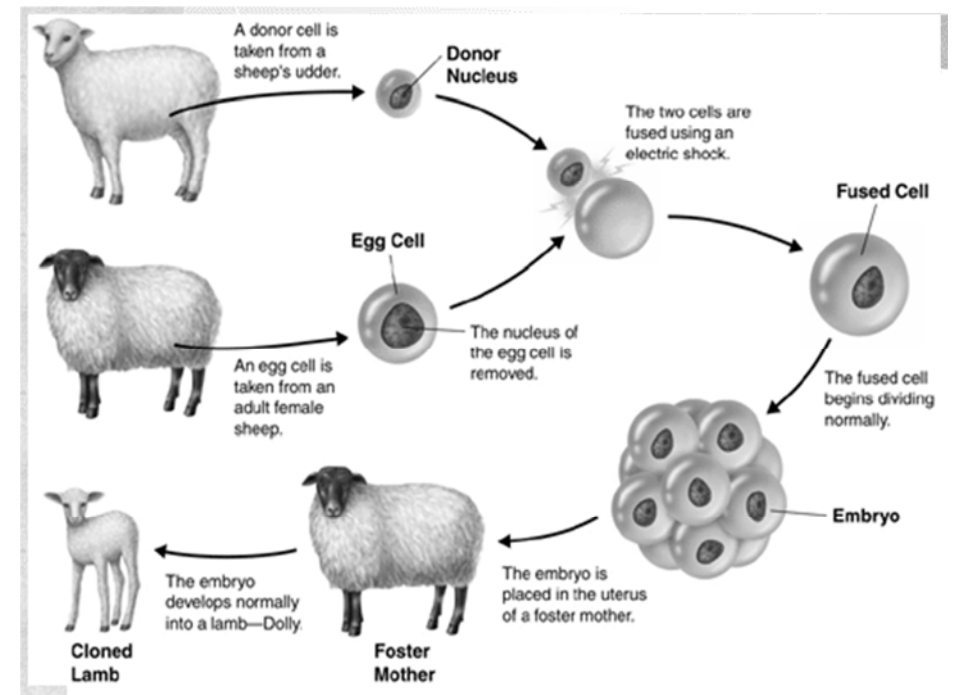
B. nuclear injection

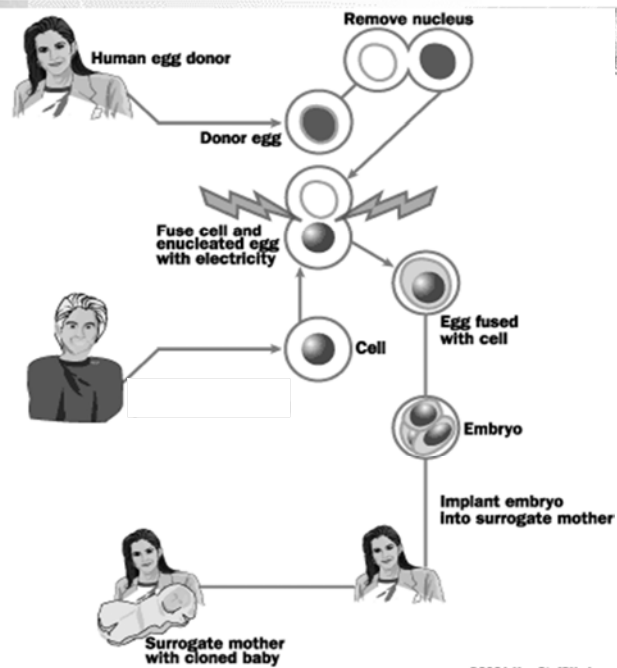
naked nucleus
microinjected into cytoplasm

Electrofusion



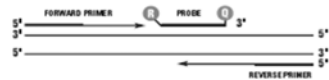
Fusion induced by electric pulse





TAQMAN® PROBE-BASED ASSAY CHEMISTRY

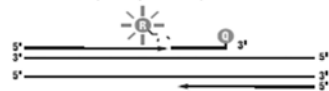
1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.



2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



SYBR® GREEN I DYE ASSAY CHEMISTRY

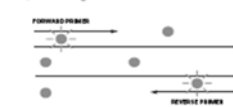
1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.



2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.



3. **Polymerization:** During extension, primers anneal and PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.

