# 20.109 MOD1 – DNA ENGINEERING
# Fall 2010

# DNA Engineering Reveals HR Function

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Engelward lab

## Going from Understanding to Engineered Solutions

-Exploiting Understanding of HR for genetic engineering

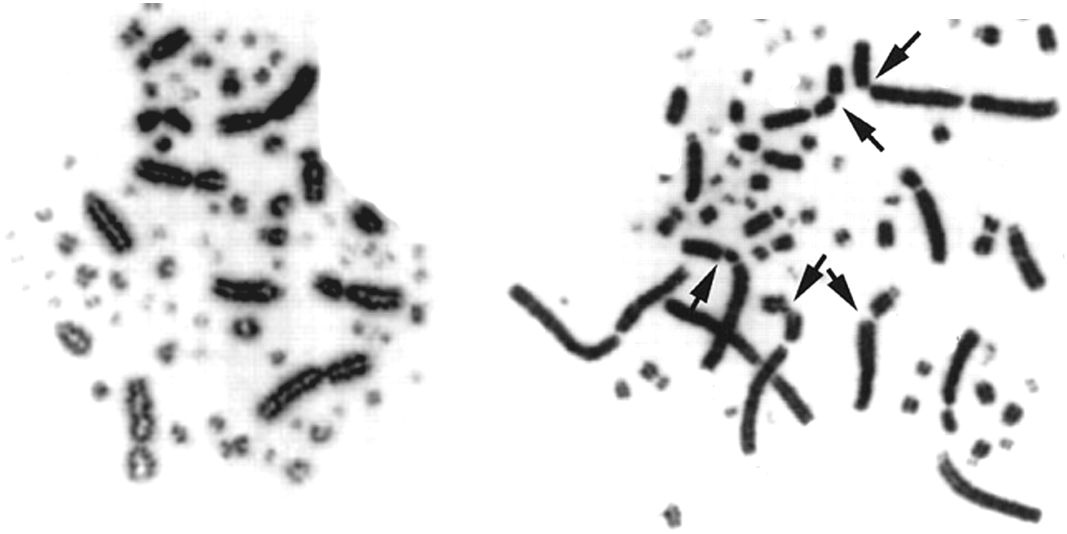
-Conditional Expression

## Mammalian Cell Culture: Methods and Logic

-Fundamentals & How To

# *Why you owe Your Life to Homologous Recombination…*

Normal



Rad51-/-

*But how do we that cells cannot survive without HR?*

Sonada *et al*., *EMBO J.* **17,** 598–608 (1998).

**To understand how the experiments were done to show that HR is essential, you need to understand:**

a) Gene Targeting
b) Conditional Expression
c) Cell Cycle Analysis by Flow Cytometry

The EMBO Journal Vol.17 No.2 pp.598–608, 1998

# Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death

Eiichiro Sonoda, Masao S.Sasaki[1], Jean-Marie Buerstedde[2], Olga Bezzubova[2], Akira Shinohara[3], Hideyuki Ogawa[3], Minoru Takata, Yuko Yamaguchi-Iwai and Shunichi Takeda[4]

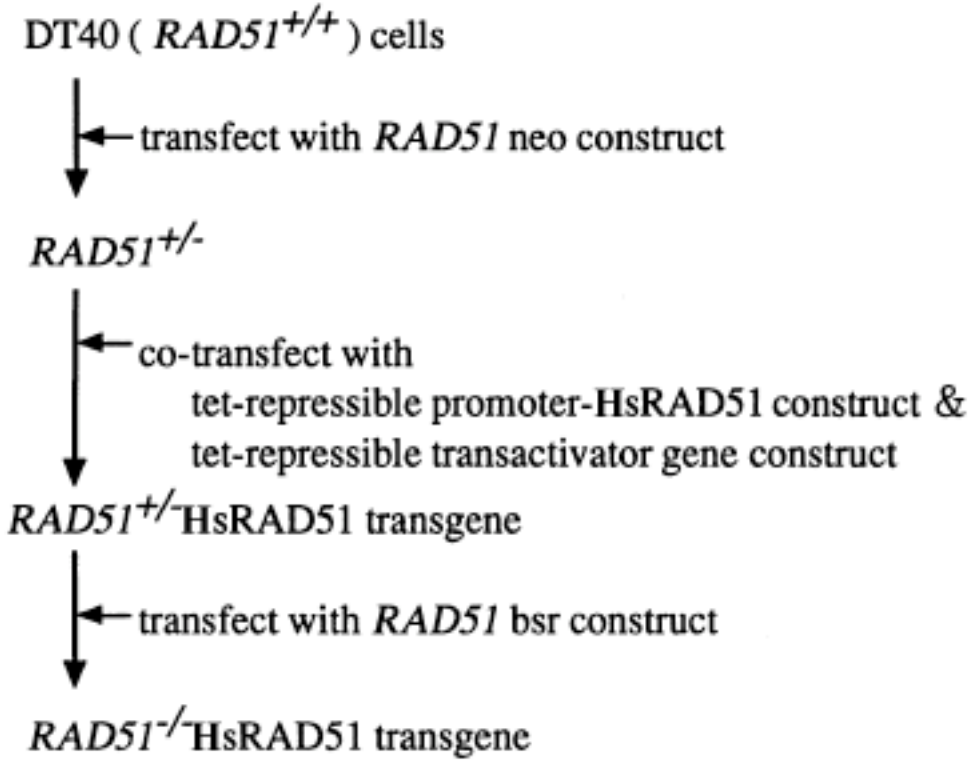
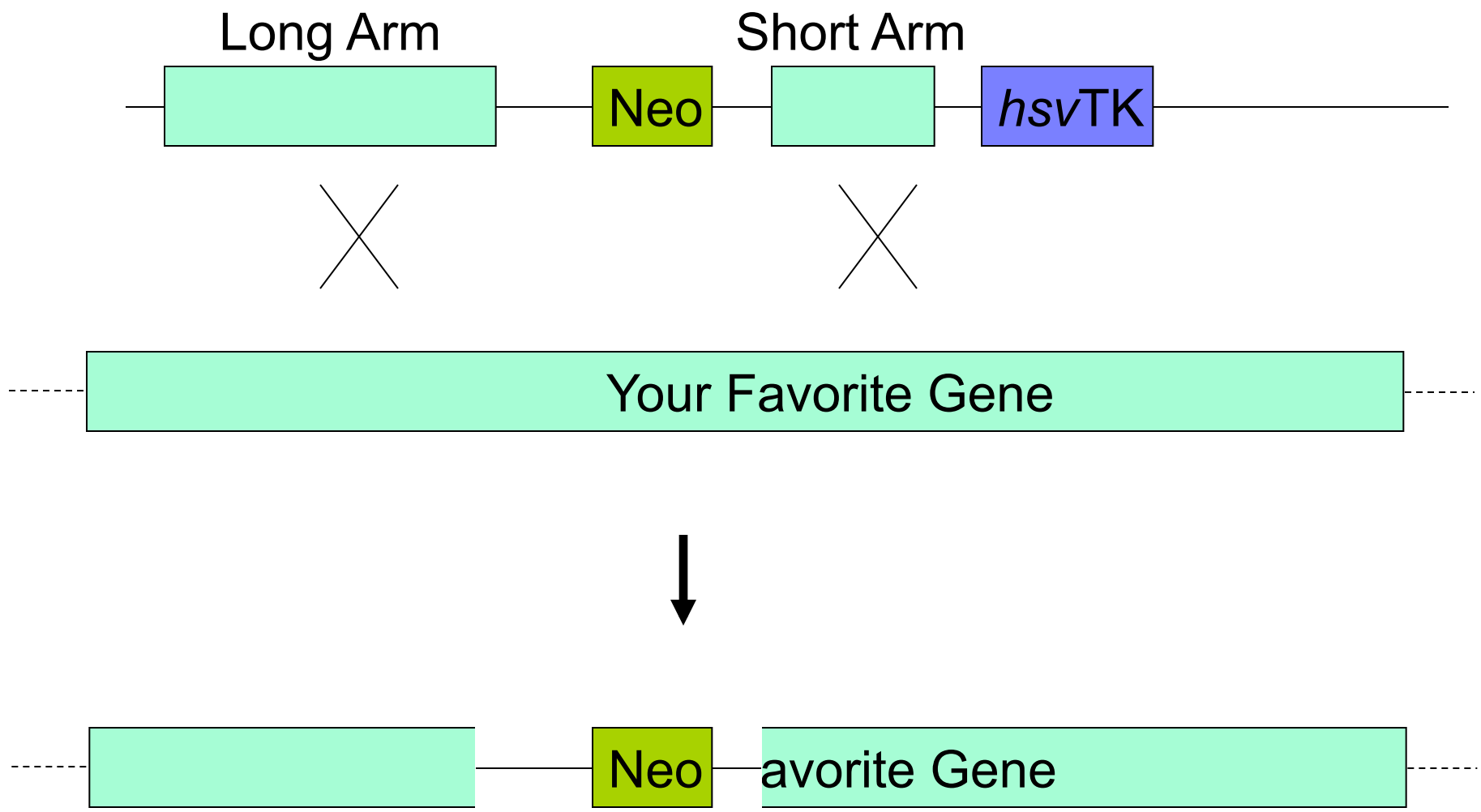
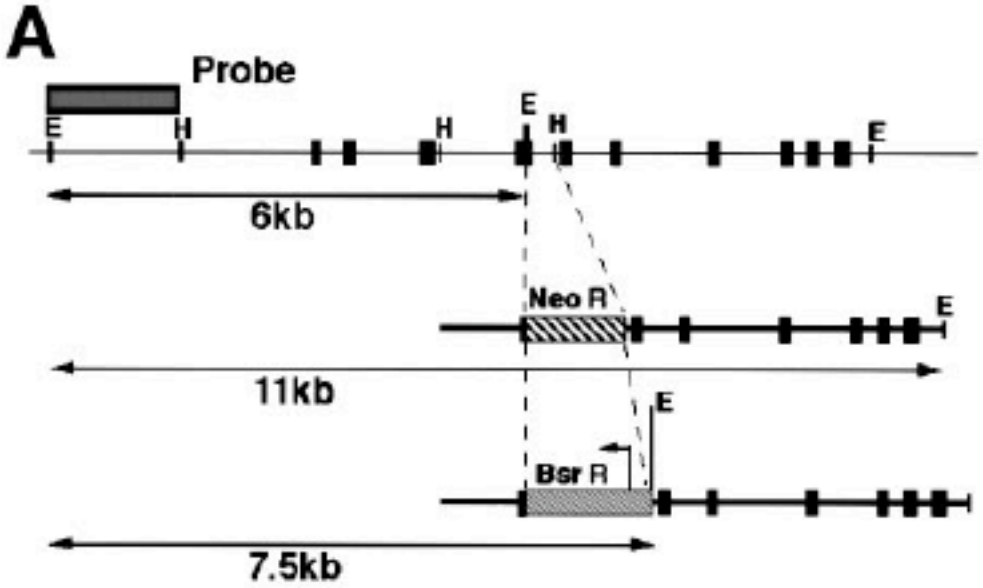


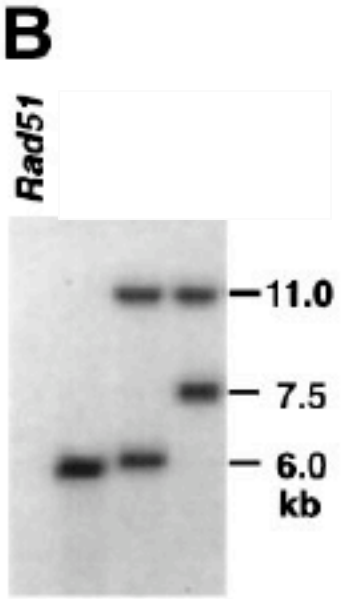

**Fig. 1.** Experimental strategy.

# Traditional Knock-Out Technology

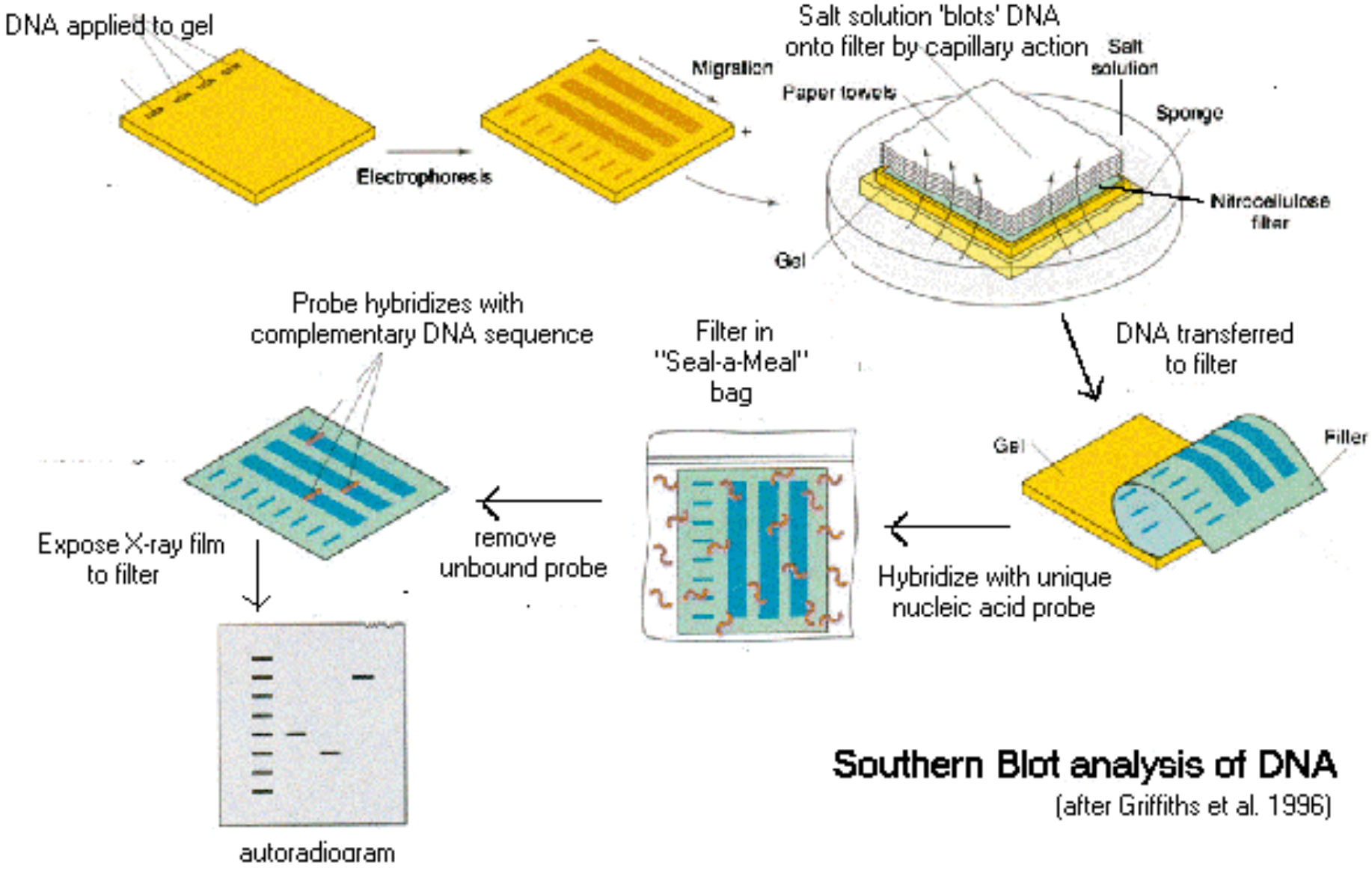
## Targeted Homologous Recombination




A
Probe
E
H
H
E
H
E
6kb
Neo R
E
11kb
E
Bsr R
7.5kb


B
Rad51
11.0
7.5
6.0
kb

# Southern Blot Analysis




**Southern Blot analysis of DNA**

(after Griffiths et al. 1996)

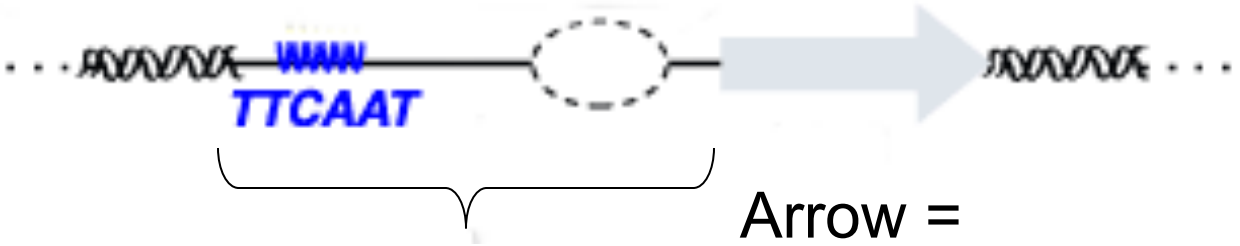
# Conditional Expression:

## Tet-Repressible Expression

# Regulation of Gene Expression

RNA Polymerase

Transcription Factor



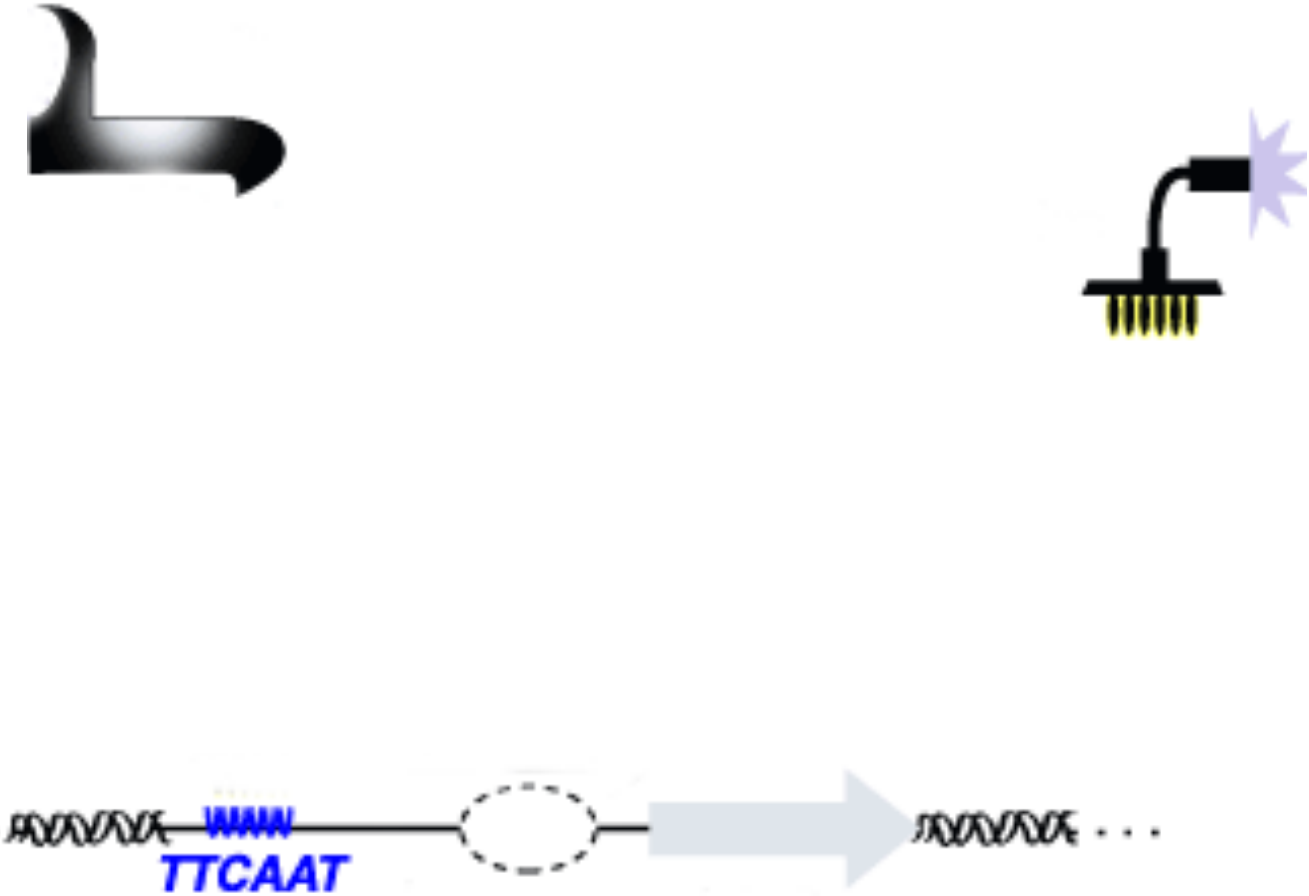

Promoter

Arrow =
Coding Sequence

# Regulation of Gene Expression

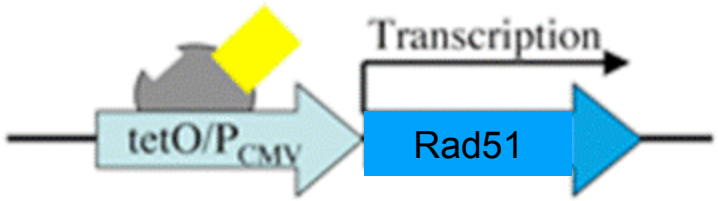

Expression is Off

# Regulation of Gene Expression




Expression is On

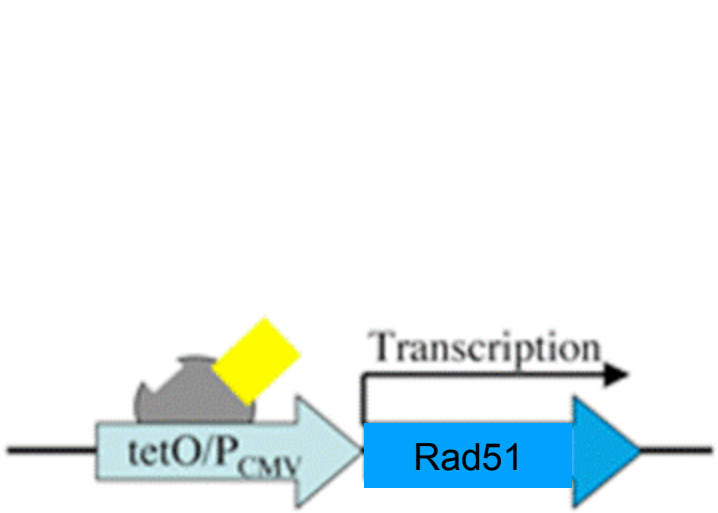
# Tet-Repressible Expression



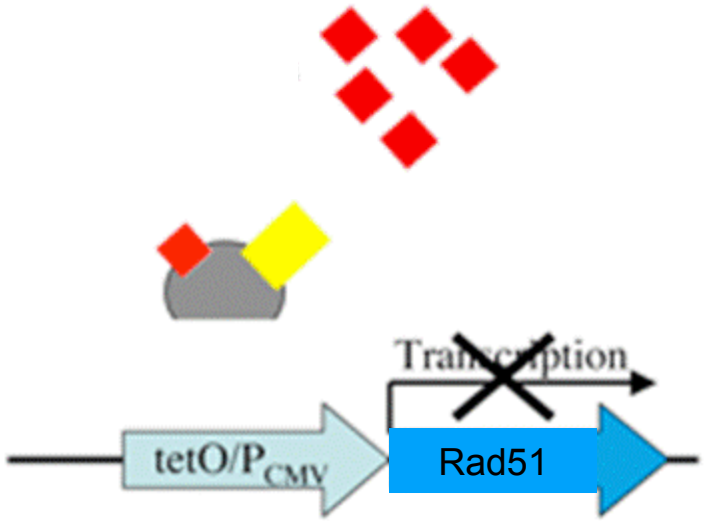

Expression On

# Tet-Repressible Expression

Add Doxycycline
(= a tetracycline analog)

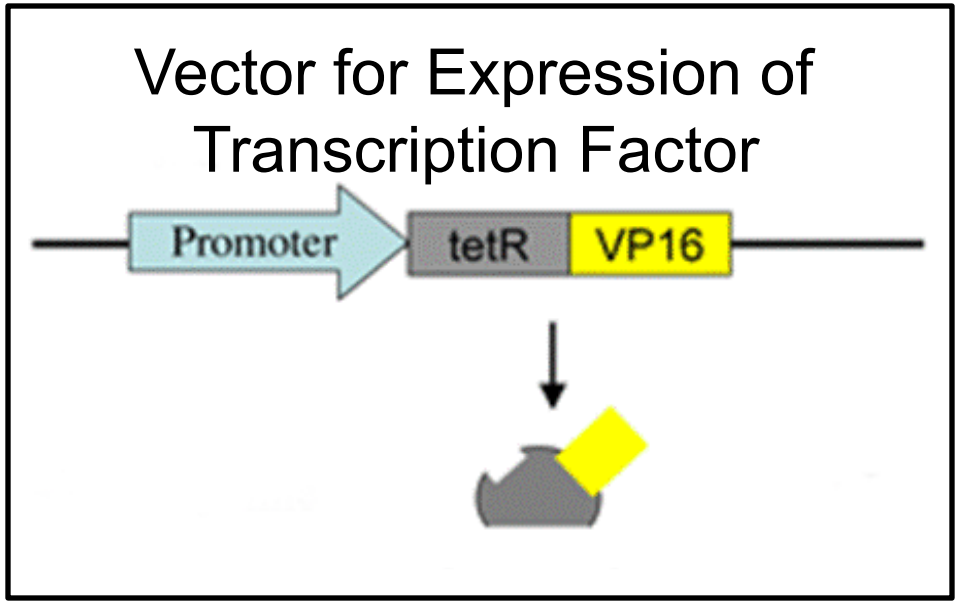



Expression On

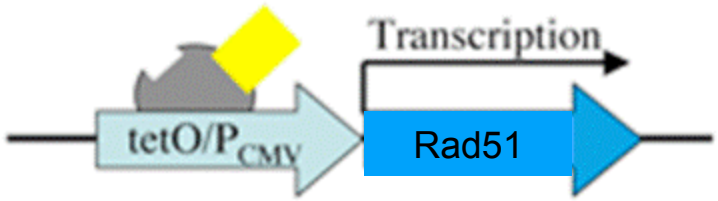



Expression Off

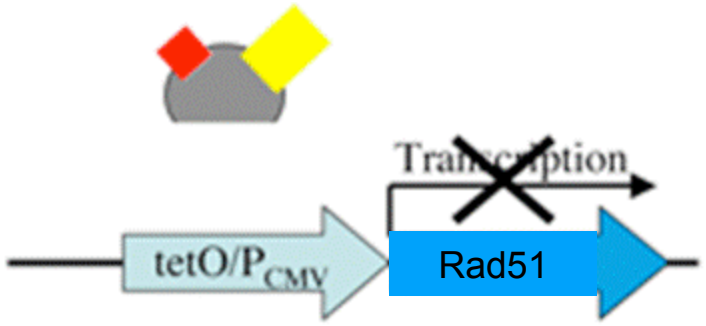
# Tet-Repressible Expression




Add Doxycycline
(= a tetracycline analog)




Expression On




Expression Off

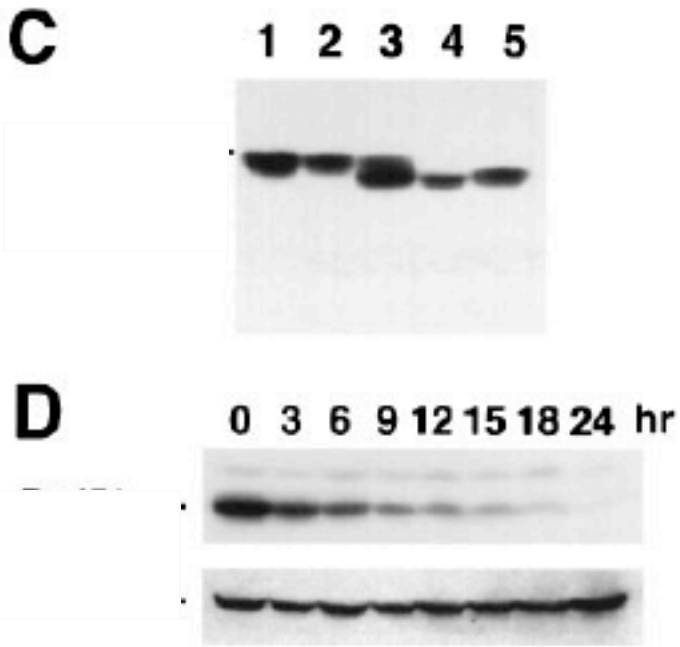
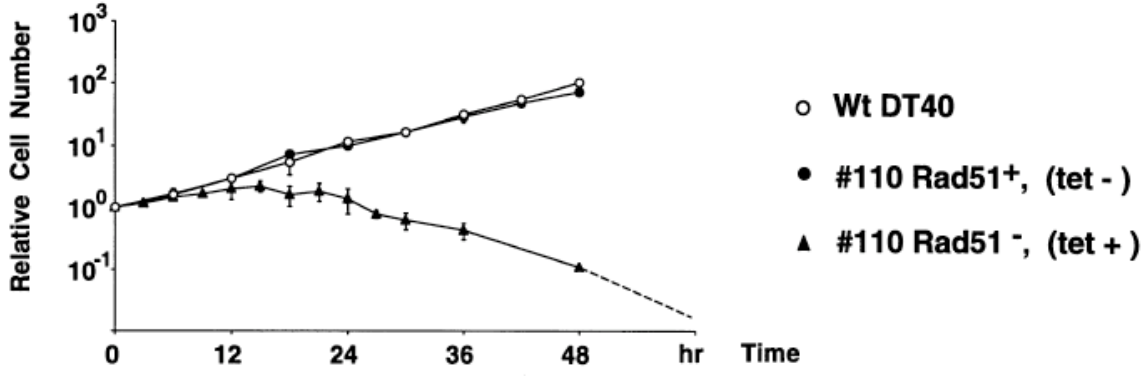
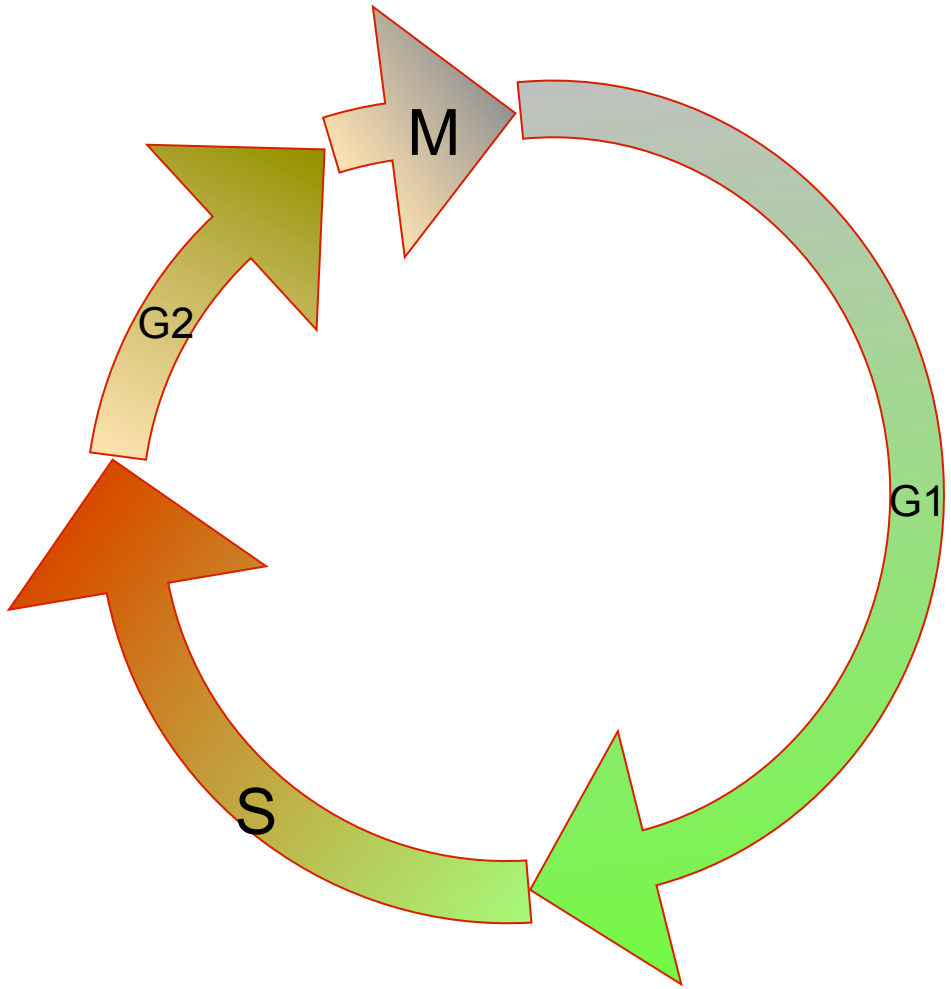



**Fig. 2.** Generation of a *RAD51*$^{-/-}$ clone carrying a human RAD51 transgene under the control of a tet repressible promoter.
(**A**) Schematic representation of part of the *RAD51* locus, the two gene disruption constructs and the configuration of the targeted loci. Closed boxes indicate the positions of exons deduced from the positions of primers in the cDNA sequence and from the sizes of PCR fragments. Relevant *Eco*RI and *Hin*dIII recognition sites and the position of the probe used in Southern blot analysis are indicated. New 11 kb and 7.5 kb *Eco*RI fragments are expected to hybridize with the probe following targeted integration of the indicated knockout constructs. (**B**) Southern blot analysis of *Eco*RI-digested DNA from wild-type DT40 (lane 1), a *RAD51*$^{+/-}$ (lane 2) and #110 *RAD51*$^{-/-}$ (lane 3) clone using the probe shown in (A). (**C**) Western blot analysis of the indicated cell extracts using anti-Rad51 antiserum. The following samples were loaded on each lane of a SDS–polyacrylamide gel: wild-type DT40 (lane 1), the *RAD51*$^{+/-}$ (lane 2), a *RAD51*$^{+/-}$ clone carrying the human Rad51 transgene (lane 3), #110 *RAD51*$^{-/-}$ clone (lane 4) and a human B lymphocyte line Ramos (lane 5). (**D**) Suppression of Rad51 expression from the transgene. Whole-cell lysates were prepared from *RAD51*$^{-/-}$ #110 cells at times indicated following the addition of tet. The filter was rehybridized with anti-Ku70 antibody to control for loading difference. Western blot analysis was performed in the same manner as in Figure 3C.

**A**

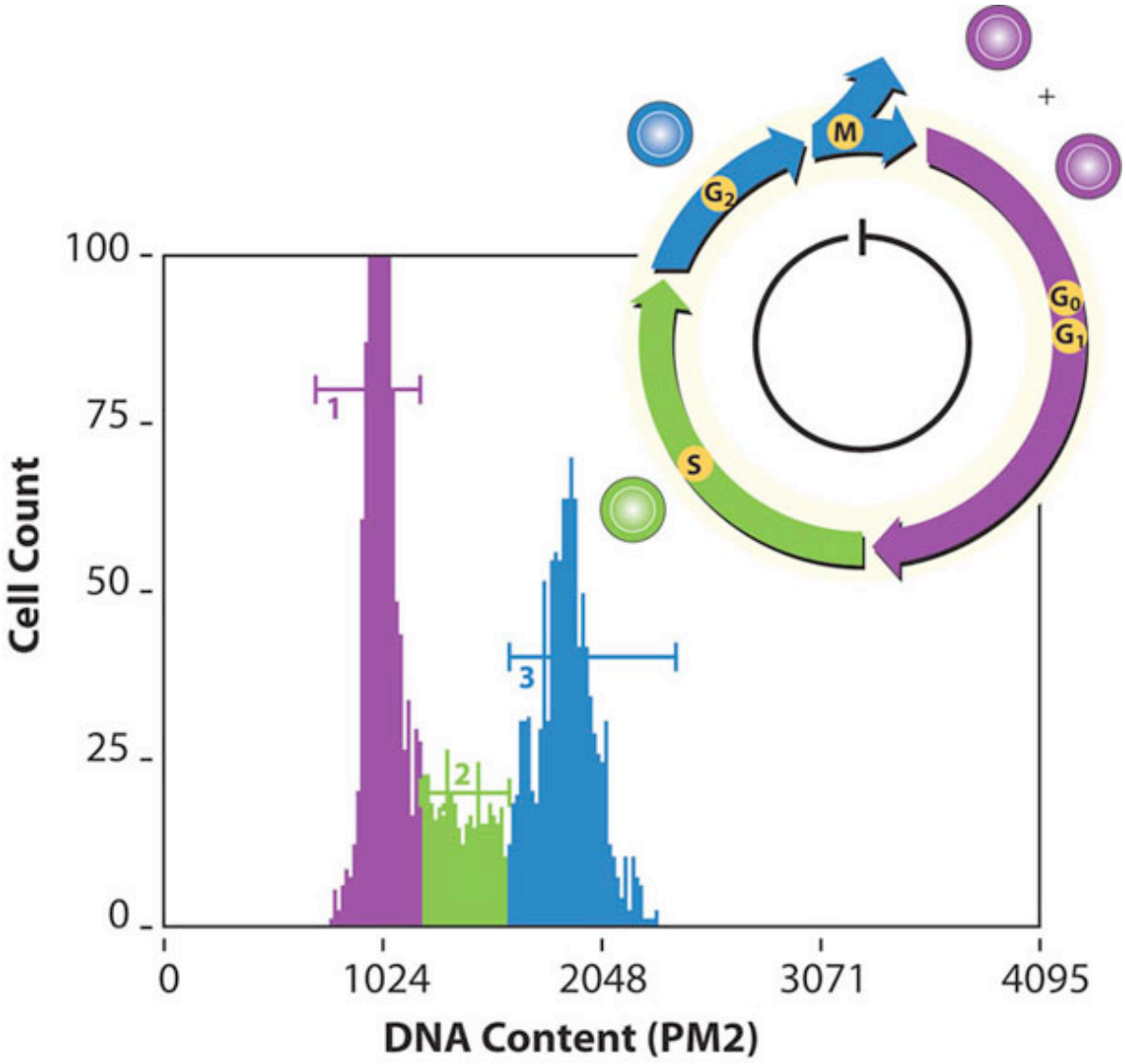

Relative Cell Number
$10^3$
$10^2$
$10^1$
$10^0$
$10^{-1}$
0
12
24
36
48
hr
Time
Wt DT40
#110 Rad51+, (tet - )
#110 Rad51 -, (tet + )

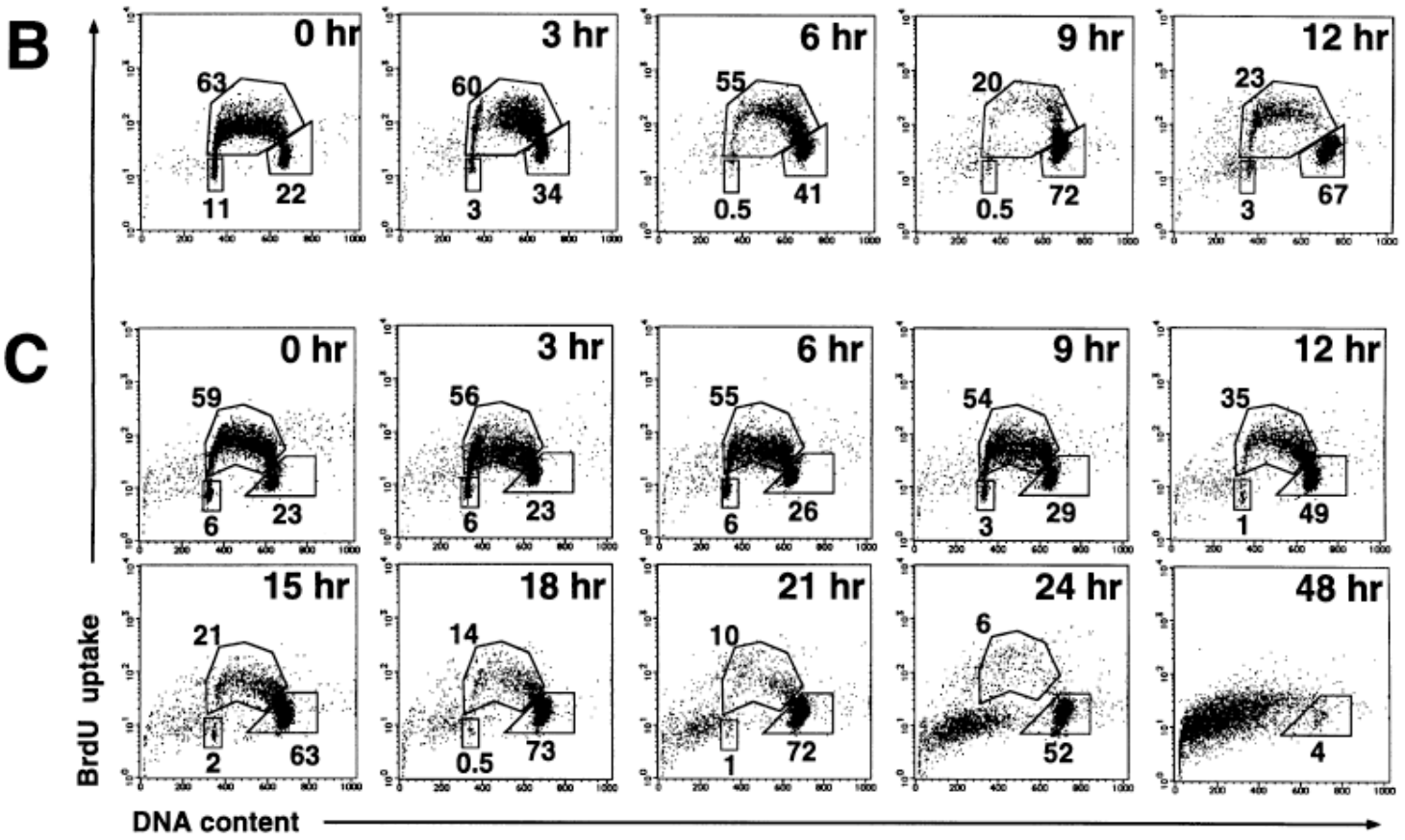
# Cell Cycle




How much DNA/ cell is there at different phases of the cell cycle?

# Cell Cycle Analysis by Flow Cytometry

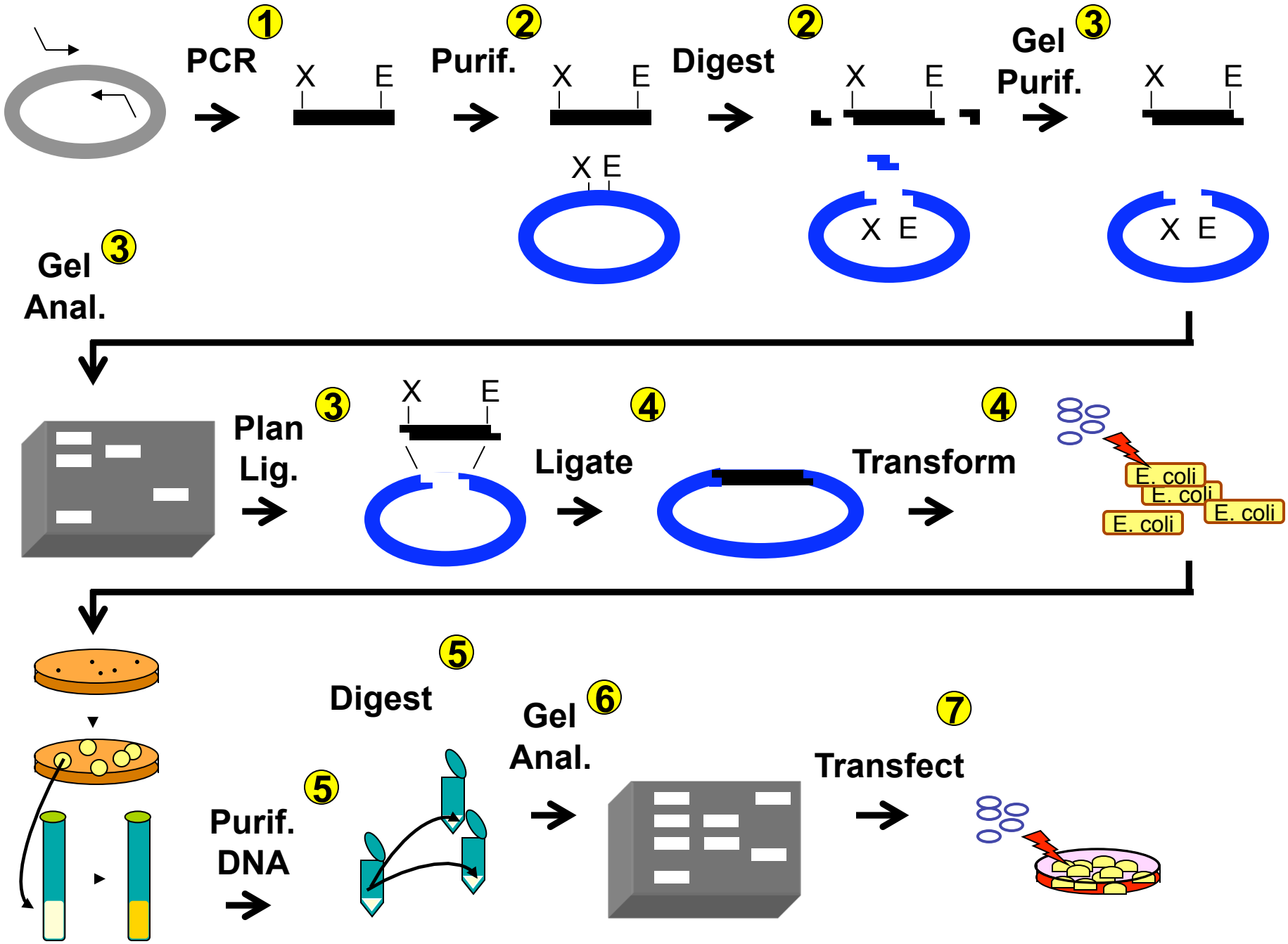



B
C
0 hr
63
11
22
3 hr
60
3
34
6 hr
55
0.5
41
9 hr
20
0.5
72
12 hr
23
3
67
0 hr
59
6
23
3 hr
56
6
23
6 hr
55
6
26
9 hr
54
3
29
12 hr
35
1
49
15 hr
21
2
63
18 hr
14
0.5
73
21 hr
10
1
72
24 hr
6
52
48 hr
4
BrdU uptake
DNA content

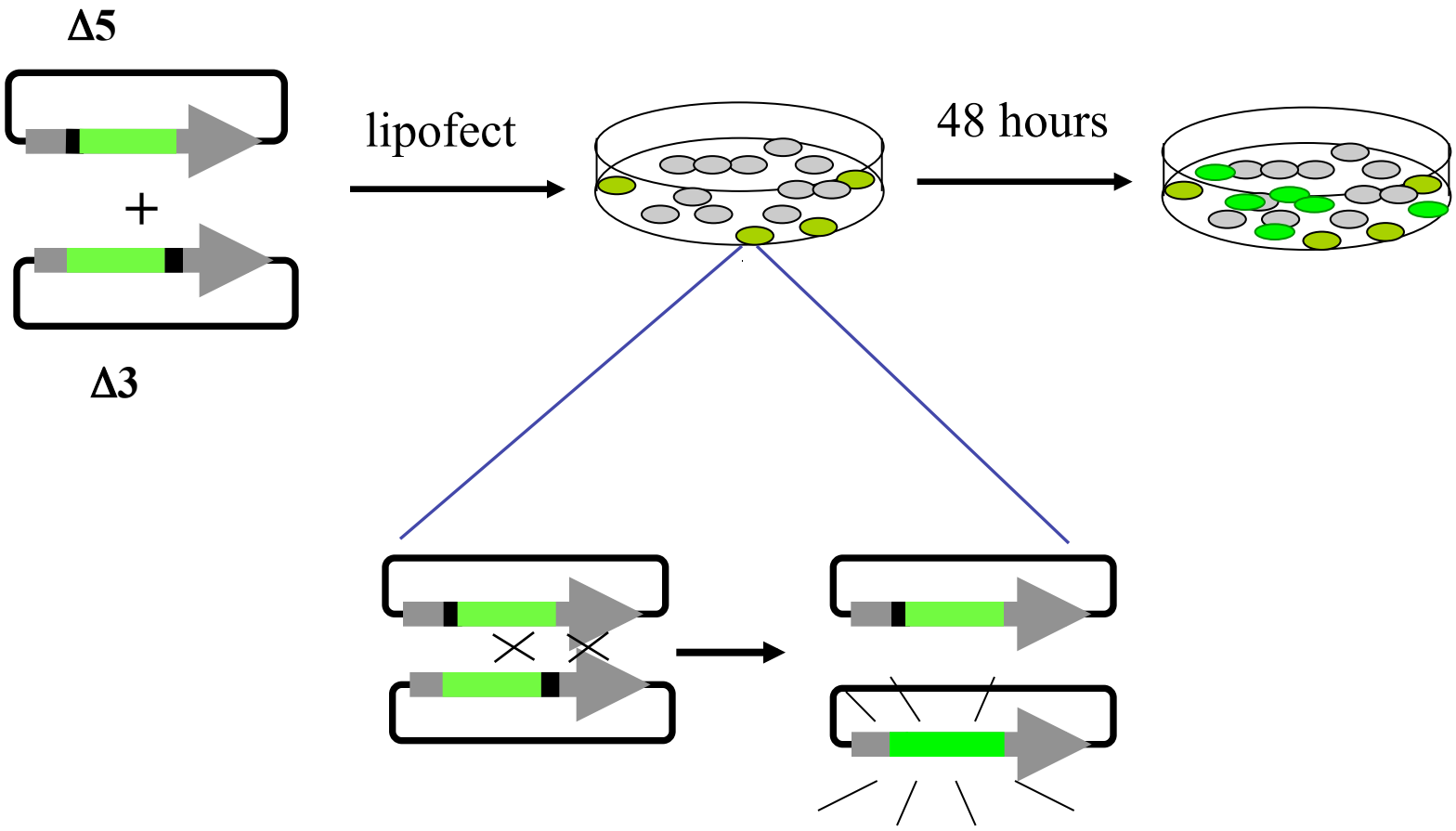
## Mod 1: DNA Engineering

# Engineering in vitro recombination assay

## Day 6

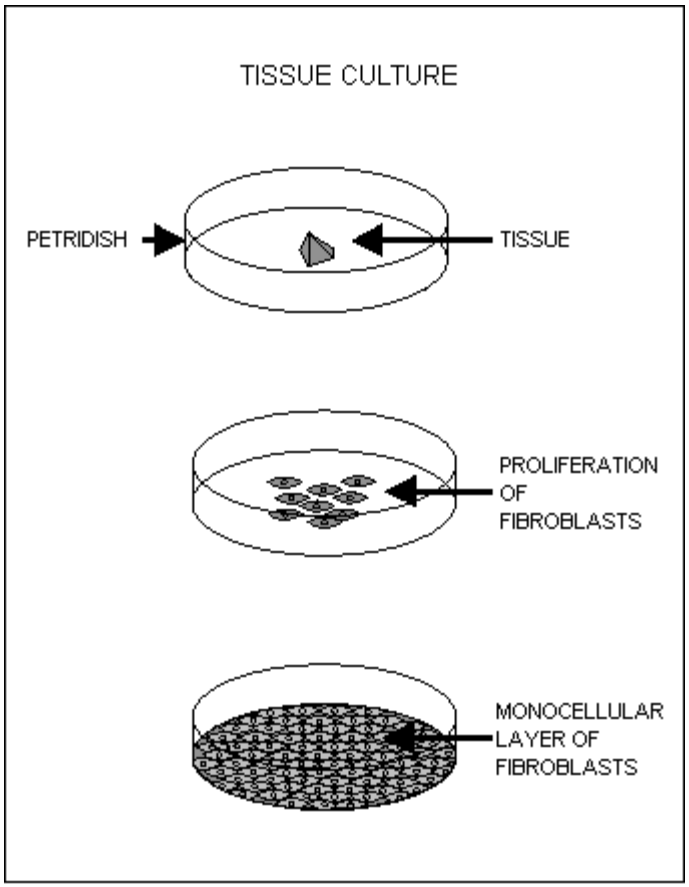

1
PCR
X
E
2
Purif.
X
E
2
Digest
X
E
3
Gel
Purif.
X
E
X E
X E
X E
3
Gel
Anal.
3
Plan
Lig.
X
E
4
Ligate
4
Transform
E. coli
E. coli
E. coli
E. coli
5
Digest
5
Purif.
DNA
6
Gel
Anal.
7
Transfect

# A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells



# Culturing Mammalian Cells

# Tissue Culture vs Cell Culture




# What do cells need to grow in culture?

Correct Temperature

Correct pH

Correct Osmolality

Amino Acids

Glutamine (used for energy)

Vitamins

Glucose

Salts

Growth Factors

(Antibiotics)

Lipids (usually in serum)

Minerals

Serum: Calf, Fetal, Horse, Bovine…

# What is in DMEM?

Salts

Amino Acids

Vitamins

| | D 0422 |
|---|---|
| | [1X] |
| **COMPONENT** | g/L |
| | |
| **INORGANIC SALTS** | |
| $CaCl_2 \cdot 2H_2O$ | 0.265 |
| $Fe(NO_3)_3 \cdot 9H_2O$ | 0.0001 |
| $MgSO_4$ | 0.09767 |
| KCl | 0.4 |
| $NaHCO_3$ | 3.7 |
| NaCl | 6.4 |
| $NaH_2PO_4$ | 0.109 |
| Succinic Acid | — |
| Sodium Succinate | — |
| **AMINO ACIDS** | |
| L-Arginine•HCl | 0.084 |
| L-Cystine•2HCl | — |
| L-Glutamine | — |
| Glycine | 0.03 |
| L-Histidine•HCl•$H_2O$ | 0.042 |
| L-Isoleucine | 0.105 |
| L-Leucine | 0.105 |
| L-Lysine•HCl | 0.146 |
| L-Methionine | — |
| L-Phenylalanine | 0.066 |
| L-Serine | 0.042 |
| L-Threonine | 0.095 |
| L-Tryptophan | 0.016 |
| L-Tyrosine (free base) | — |
| L-Tyrosine•2Na•$2H_2O$ | 0.10379 |
| L-Valine | 0.094 |

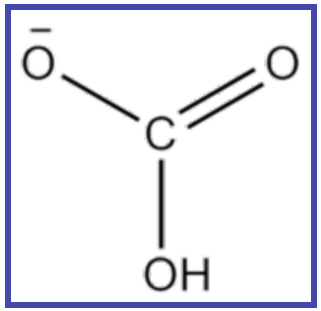
| **VITAMINS** | |
|---|---|
| Choline Bitartrate | — |
| Choline Chloride | 0.004 |
| Folic Acid | 0.004 |
| myo-Inositol | 0.0072 |
| Niacinamide | 0.004 |
| D-Pantothenic Acid•½Ca | 0.004 |
| Pyridoxal•HCl | — |
| Pyridoxine•HCl | 0.004 |
| Riboflavin | 0.0004 |
| Thiamine•HCl | 0.004 |
| **OTHER** | |
| D-Glucose | 4.5 |
| HEPES | — |
| Phenol Red•Na | 0.0159 |
| Pyruvic Acid•Na | 0.11 |

| **ADD** | |
|---|---|
| Glucose | — |
| L-Glutamine | 0.584 |
| L-Cystine•2HCl | — |
| L-Leucine | — |
| L-Lysine•HCl | — |
| L-Methionine | — |
| $NaHCO_3$ | — |
| $NaH_2PO_4$ | — |
| Phenol Red•Na | — |
| Pyruvic Acid•Na | — |
| | |
| Grams of powder required to prepare 1 L | N/A |

# How do you maintain a neutral pH?

Blood pH is 7.4

The most important buffer in extracellular fluids is a mixture of carbon dioxide ($CO_2$) and bicarbonate anion ($HCO_3^-$).




$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

The pH is determined by the concentration of $CO_2$ and bicarbonate.

# Typical media...

82 ml DMEM

15 ml Calf Serum

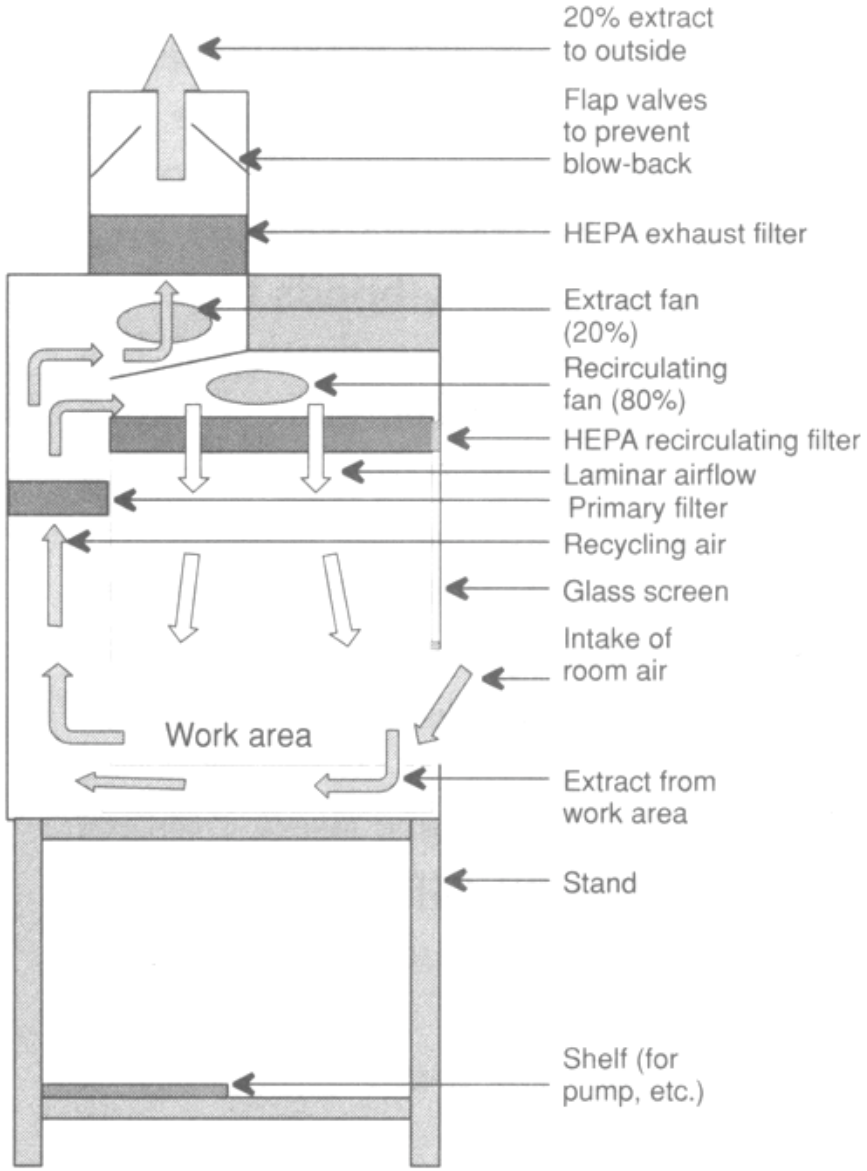
1.5 ml Glutamine

1.5 ml Pen/Strep

# Where do you grow your cells?

# Why is sterility important?

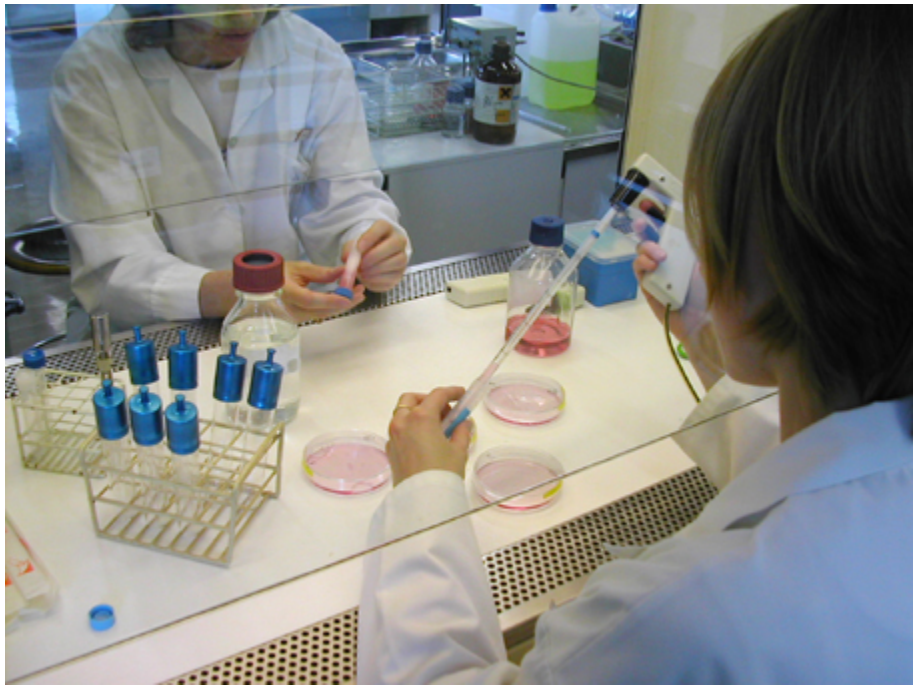
# How do you maintain sterility?




(b) VERTICAL LAMINAR FLOW

From Freshney's "Culture of Mammalian Cells"

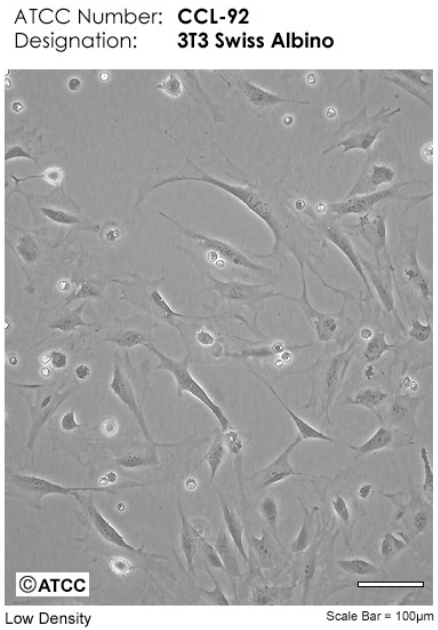
# Mammalian Cell Culture Hood

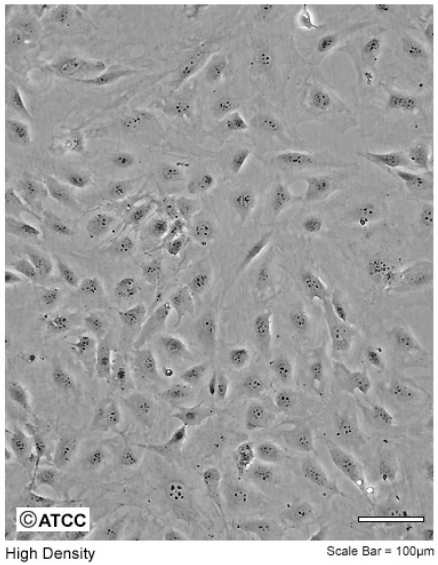


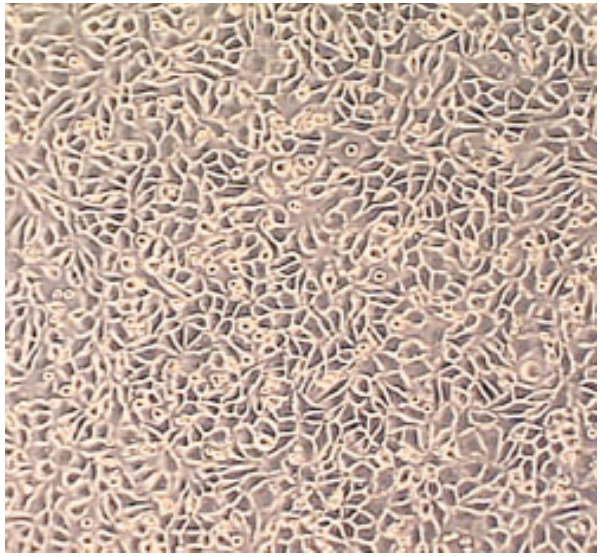
# Hands-On..

What does it mean to
“pass” your cultures?

# Cell Density (cells per $cm^2$)

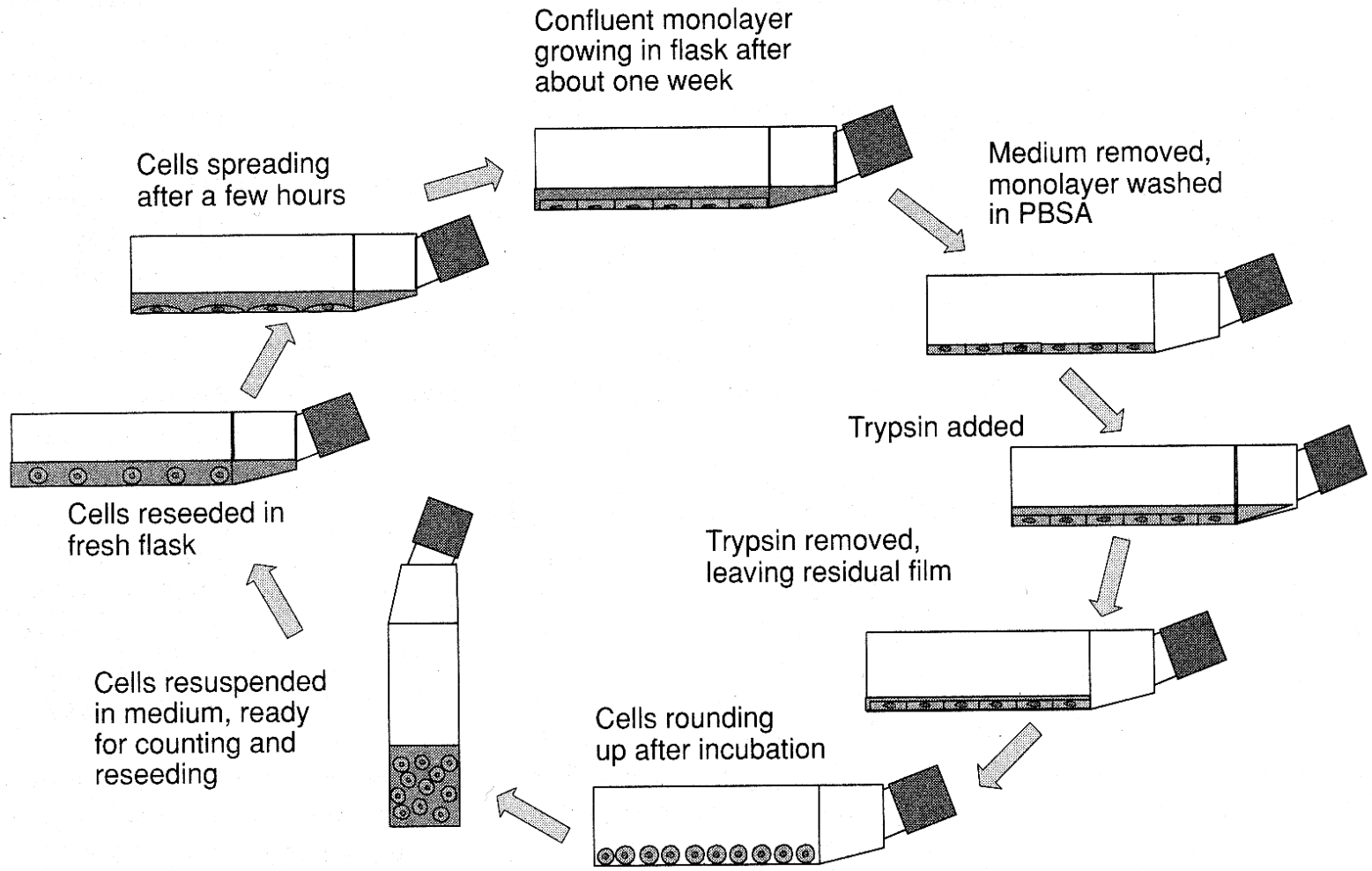


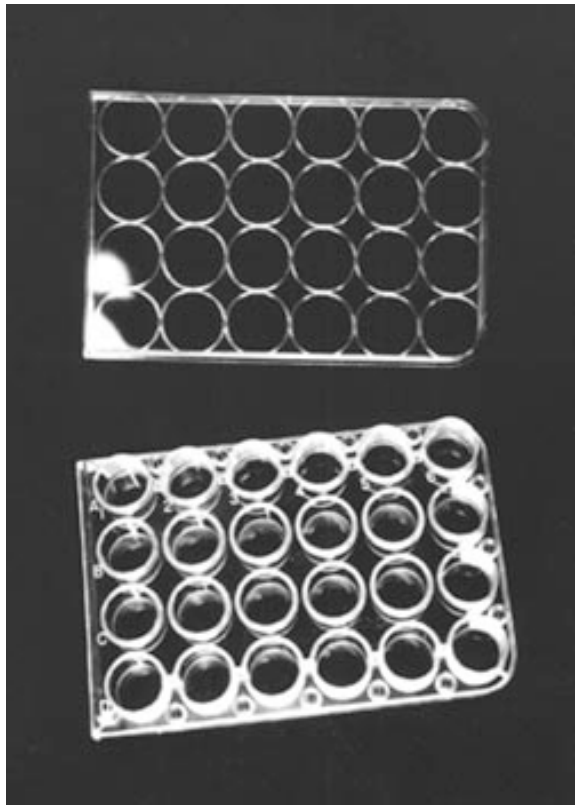



Low Density High Density Confluent

# Cell Density can be kept low through "Passaging"




Trypsin is used to release cells from the surface of the dish



# Cell Culture Growth Phases