MOD1 – DNA ENGINEERING

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Spring 2008

Day 8
Module in Review
Experimental Approaches
PCR

MELT 94°C

ANNEAL 55°C

EXTEND 72°C
DNA Purification
Gel Electrophoresis
Data Analysis
Example of appropriate labeling.

Figure 1. Agarose gel analysis of digested backbone and insert. Bkb, pCX-NNX. Ins, Insert (PCR product of truncated EGFP coding sequence). Restriction enzymes used to digest DNA are indicated.
Ligation
Mammalian Cell Culture

-PETRISHE -> TISSUE

PROLIFERATION OF FIBROBLASTS

MONOCYTOCELLULAR LAYER OF FIBROBLASTS
Flow Cytometry: First Principles, Alice L. Givan, 1992
Key Concepts:

Nothing is 100%
Ask ‘What else might be happening’?
Avoid Assumptions (Controls!)
Double Check at Every Opportunity
Ask the same question in several ways
Biological Principles
DNA Damage & Repair via Homologous Recombination
Sunlight
Pollution & Food
Cigarette Smoke
Radiation
Oxidative Radicals
Nitric Oxide
Base Lesions
Single Strand Breaks
Double Strand Breaks
SDSA
Engineering an Assay for Homologous Recombination
A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells
Exploiting Homologous Recombination for Gene Targeting
Traditional ES Knock-Out Technology

Targeted Homologous Recombination

Long Arm  Neo  Short Arm  hsvTK

Your Favorite Gene

↓

Your Favorite Gene
Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2

Shyam K. Sharan†, Masami Morimatsu§, Urs Albrecht†, Dae-Sik Lim‡, Eva Regel†, Christopher Dinh††, Arthur Sands‡, Gregor Eichele†, Paul Hasty‡ & Allan Bradley††

Figure 1 Disruption of Brca2 gene in ES cells. a, Restriction map of the Brca2 genomic fragment containing exon 9-15 is shown. Restriction sites shown here are EcoRI (R) and BamHI (B). SalI (S) is from the cloning vector. Double-headed arrows correspond to the 5' and 3' homology regions and the dark shaded boxes show the probes used. b, Restriction map of the targeting vector pBrca2TV. c, Expected restriction map of the mutated Brca2 locus. A 2.8-kb genomic region is deleted and replaced by the 3.6-kb Hprt gene. d, Southern analysis to identify heterozygous ES cells by digesting genomic DNA with BamHI. The 3’ probe detects a 9.5-kb wild-type band and a 14.0-kb mutant band. e, Restriction map of
Engineering
Inducible
Gene Expression
Tet-Repressible Expression

Vector for Expression of Transcription Factor

Add Doxycycline
(= a tetracycline analog)

Expression On

Expression Off
Maintaining the Genome:

Using Homologous Recombination to Fix Broken Replication Forks
Double Strand Breaks

NHEJ

HR
Genomic Instability:

BRCA2: Without homologous recombination, cells suffer genomic instability
HR provides the only pathway to accurately repair broken replication forks.

Too Little HR: Mis-Repair of Broken Forks

Genomic Instability:

BRCA2: Without homologous recombination, cells become sensitive to chemo (xlinks)
Flow Cytometry
Principle of Fluorescence Activation
See Specific Colors By Restricting Ex and Em:

For Green, can capture emission when >510 nm

For Green, can excite using <510 nm

Your laser is 488 nm

Spectra from Clontech, Inc
Basic Optics

Laser(s) → Cell

Dichroic mirrors

1 2 3

Photo-multiplier tubes (PMTs)

PMTs turn light into analog signals

Slide Adapted From Terry Hoy, Department of Haematology, UWCM
Direct beam stop.

Laser

Light

High angle scatter (Side Scatter): Reflection & refraction; structure.

Low angle scatter (Forward Scatter): Diffraction. Cell size.

Direct beam stop.

Slide Adapted From Terry Hoy, Department of Haematology, UWCM
Sample Flow
FACS = Fluorescence Activated Cell Sorting

Vibrating the nozzle produces a stream that breaks into droplets.

Electronic delay until cell reaches break off point. Then the stream is charged.

Flow Cytometry: First Principles, Alice L. Givan, 199
Normal Cells

EGFP Expressing Cells

Rare Cells Expressing EGFP

515-545 nm (FL-1)

562-588 nm (FL-2)
515-545 nm (FL-1)

562-588 nm (FL-2)

Normal Cells

Emission

Wavelength (nm)

Emission

EBFP, ECFP, EGFP, EYFP, DsRed
Normal Cells

EGFP Expressing Cells

Rare Cells Expressing EGFP

515-545 nm (FL-1)

562-588 nm (FL-2)
515-545 nm (FL-1)

562-588 nm (FL-2)

File: 11-2-02.001
Sample ID: Y1 ears 0 MMC
Gated Events: 1261613
Log Data Units: Linear Values
Gate: No Gate
Total Events: 1261613

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<th>Y Geo Mean</th>
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File: 11-2-02.001
Sample ID: Y1 ears 0 MMC
Gated Events: 1088917
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Flow Cytometry

Flow cytometry analyzes cells one by one

Fluorescence, diffracted, and reflected light can be measured for each cell

Multiple lasers and multiple colors can be analyzed at millions of cells per minute

Resulting plots show the relative level of fluorescence of each cell for specific wave lengths (a dot is a single cell)

Flow cytometry is an analysis method, whereas FACS actually sorts cells
Module in Review:

Experimental Approaches & Biological Concepts

Flow Cytometry