

Recombinant DNA Technology

Cloning: *Production of multiple, identical copies of a particular DNA sequence*

Vector: *Self-replicating DNA molecule
Copies foreign DNA to be cloned*

*Bacterial plasmids (e.g. pBR322)
Phage (e.g. lambda)
Cosmids (packaged as phage; replicate as plasmids)
Artificial chromosomes
Bacterial (BACs); Yeast (YACs)*

Host Cell: *Site of vector replication (E. coli, yeast, etc)*

Restriction Endonucleases:

Enzymes that cleave dsDNA at specific recognition sites

*Function in bacteria to degrade phage DNA
Names reflect bacterial origin
EcoRI = E. coli Restriction Enzyme #1
> 200 different types identified from various bacteria*

Restriction enzymes differ in:

*Recognition sequence
Physical nature of cut
Average lengths of fragments produced
Ability to cut methylated DNA
Cost and availability*

Recognition sites are randomly dispersed in genome

I----------*-----*-----I Cloned DNA*

** = Recognition site for restriction enzyme*

*Add Enzyme: dsDNA fragments, variable lengths
Identical sequences at ends*

*Blunt ends: Both strands cut at same bp
Sticky ends: Uneven cut; short overhang*

*Fragments with sticky ends hybridize spontaneously
Add DNA ligase --> Recombinant DNA molecule*

Most recognition sites are palindromic

*EcoRI: ---G//A-A-T-T-C---
 ---C-T-T-A-A//G---*

Plasmid Vector: pBR322

*Small circular bacterial plasmid
Contains origin of replication
Single sites for several endonucleases
Antibiotic resistance genes (tet; amp)*

Generating a Recombinant DNA with pBR322 and PstI

*Isolate: Plasmid from bacterial culture
 Foreign DNA from organism of choice*

Cut plasmid and foreign DNA with PstI

*Plasmid: Linearized
Foreign: Multiple fragments*

*Mix tubes – Allow spontaneous hybridization
Add DNA ligase --> Recombinant DNAs
Transform DNAs into E. coli to propagate
Select for transformants with chimeric plasmids*

*Host E. coli: tet(s)amp(s)
Transformants: tet(r)amp(s)*

*Grow large number of desired bacteria
Isolate chimeric plasmids; cleave with PstI
Separate foreign DNA using gel electrophoresis*

Cloning with a Phage Vector (e.g. lambda)

*Central portion of lambda genome is dispensable
This region can be replaced with foreign DNA*

Procedure:

*Cut phage DNA with endonuclease
Mix with cleaved foreign DNA
Seal hybridized fragments with ligase
Mix recombinant DNAs with phage proteins*

*Phage particles form spontaneously
Amplify phage by infecting bacteria*

Bacterial Artificial Chromosomes:

*Advanced vector based on F factor
Major advantage: Accepts large inserts
Starting material for many genome projects*

Plasmid: < 10 kb inserts

Phage: < 20 kb

Cosmids: < 40 kb

BACs: 100-500 kb

YACs: 100-1,000 kb

Polylinker regions with multiple, unique restriction sites

Identifying Bacteria with Recombinant Plasmids

1. *Use antibiotic resistance (pBR322)*

Resistance lost when foreign DNA inserted into resistance gene

2. *Use visual color screen (pUC18)*

Polylinker in vector lacZ gene

Functional enzyme + substrate (X-gal): Blue colonies

Insertion into polylinker site: white colonies

Library:

*Collection of recombinant phage or bacteria containing many different DNA fragments
from same organism*

Extremely valuable starting material for research in molecular genetics

Common Types of Libraries:

Genomic: Start with total genomic DNA

Entire genome preserved in fragments

Challenge = Identifying desired gene

cDNA: Start with mRNAs from desired tissue

Produce ds DNA copies of mRNAs

Clone these complementary DNAs
Library preserves transcribed sequences
Entire genome not represented
Introns, non-coding regions missing
Sequences in library depends on tissue used

Expression: Allows production of protein from cloned cDNA in bacteria

Promoter drives transcription of foreign DNA in host cell

YAC/BAC: Large genomic fragments incorporated into yeast/bacterial artificial chromosomes

How to Identify Specific Gene in Library?

1. If you have an antibody to protein product

Antibody: Protein that selectively binds to antigen
Produced in mice or rabbits

Labeled antibody used to identify desired protein

Screen expression library with labeled antibody

Spread out library on agar plates
Transfer cells/phage to filter paper
Incubate filter paper with labeled antibody
Identify colony/plaque with antigen protein

Labeled cell/phage carries foreign gene that codes for desired antigen protein

2. If you have purified the desired protein

Common technique: 2-D gel electrophoresis
Separates protein mixtures ---> spots on gel
Determine partial AA sequence of polypeptide

Synthesize short DNA sequences (oligonucleotides)
that might code for sequenced part of polypeptide
Label these oligonucleotides (e.g. 32-P)

Probe: Labeled ssDNA that hybridizes to complementary target sequence

*Screen genomic/cDNA library with labeled probe
Identify recombinant cell with complementary DNA*

3. *If gene already cloned from another organism*

*Use that gene as “heterologous” probe
Identify recombinant cell with equivalent gene*

*Problem: Sequence divergence during evolution
Hybridization may not occur*

*Solution: Focus on most highly conserved regions
Sequences here often very similar
Use this region as hybridization probe*

How are conserved regions of genes identified?

Use computer to compare sequences of same gene from many different organisms

4. *If mutant phenotype and map location known*

True for many human genes cloned in recent years

*Cystic fibrosis gene; Muscular dystrophy gene
Genes that increase susceptibility to colon and breast cancers
Genes responsible for many heritable disorders*

Chromosome walking used to isolate gene involved

Map-based cloning:

1. *Identify DNA sequence closely linked to mutant gene*

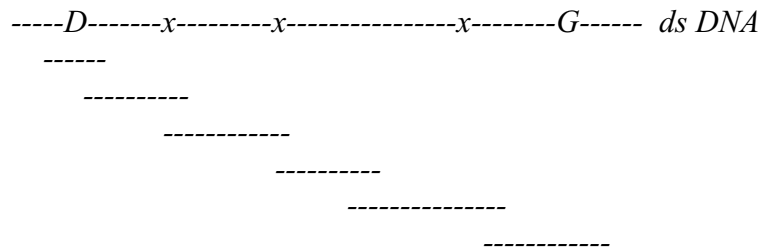
Involves analyzing DNA samples from many large families segregating for trait

Use DNA sequence differences (polymorphisms) in same way as mutations to look for genetic linkage

2. *Use linked DNA marker as probe to screen genomic library for overlapping (adjacent) clones*
3. *Find clones more tightly linked to mutant gene*
4. *Repeat process many times*

Summary of Process:

D = DNA marker; x = crossovers; G = target gene



Overlapping YAC/BAC/cosmid/plasmid/phage clones

How do you know when desired gene has been reached?

1. *No recombinants between gene and cloned DNA*
2. *Cloned region should be transcribed in expected cells*
Cystic fibrosis: Transcribed in epithelium
Included in cDNA library
3. *Affected individuals should have altered DNA sequence (mutation) in this region*
4. *Protein product predicted from mRNA sequence should make sense in cellular context*
Cystic fibrosis: Transmembrane protein
Regulates ion transport
Consistent with physiology
5. *Introduction of wild-type DNA into recessive mutant individual should correct mutant phenotype*
Not feasible with human disorders
Common approach with plant genes