PLNT2530
2024

Unit 6c

Finding a Gene in a Library

Molecular Biotechnology (Ch 4)

Analysis of Genes and Genomes (Ch 6)

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Two scenarios

A. You know the gene you are trying to isolate

B. You know the trait but don't know what the gene responsible is.

A. When you know the gene you are seeking

Screening a library

- Direct screening screen the library for the DNA sequence using a nucleotide probe

- Indirect screening of an expression library for the presence of the gene product

How do we get the probes?

a) heterologous probe: When a gene has been isolated from a closely related species, that gene sequence will likely share sufficient sequence homology to the gene from your plant of interest to be able to form a stable hybrid.

eg. Most cereal genes share considerable homology

wheat, barley, oat, rice, maize, rye

eg. Finding additional members of multigene family using one gene

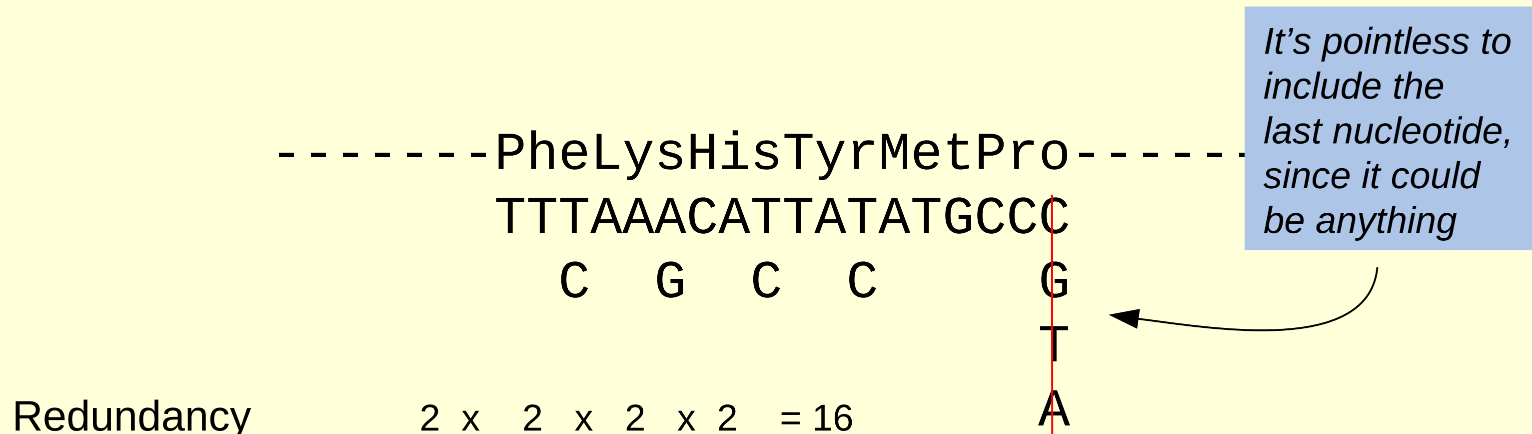
Highly conserved genes are suitable for even distantly related sources

- eg. histones, rRNA, ubiquitin, actin

How do we get the probes?

b) Use a synthetic oligonucleotide probe based on the amino acid sequence of the protein gene product (if this information is known). The oligonucleotide probe sequences are based on the codon sequences for an amino acid sequence in the protein

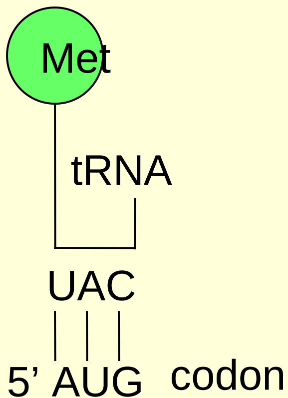
The synthetic oligonucleotide probes are normally redundant 17-26 nts long



There are 16 potential sequence combinations – only one correct for each gene!

Genetic code

		Second base U		Second base C		Second base A		Second base G	
First base	U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
		UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
		UUA	Leu	UCA	Ser	UAA	STOP	UGA	STOP
		UUG	Leu	UCG	Ser	UAG	STOP	UGG	Trp
	C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
		CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
		CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
		CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
	A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
		AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
		AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
		AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg
	G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
		GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
		GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
		GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly



mRNA

Oligonucleotide probes are normally redundant mixtures

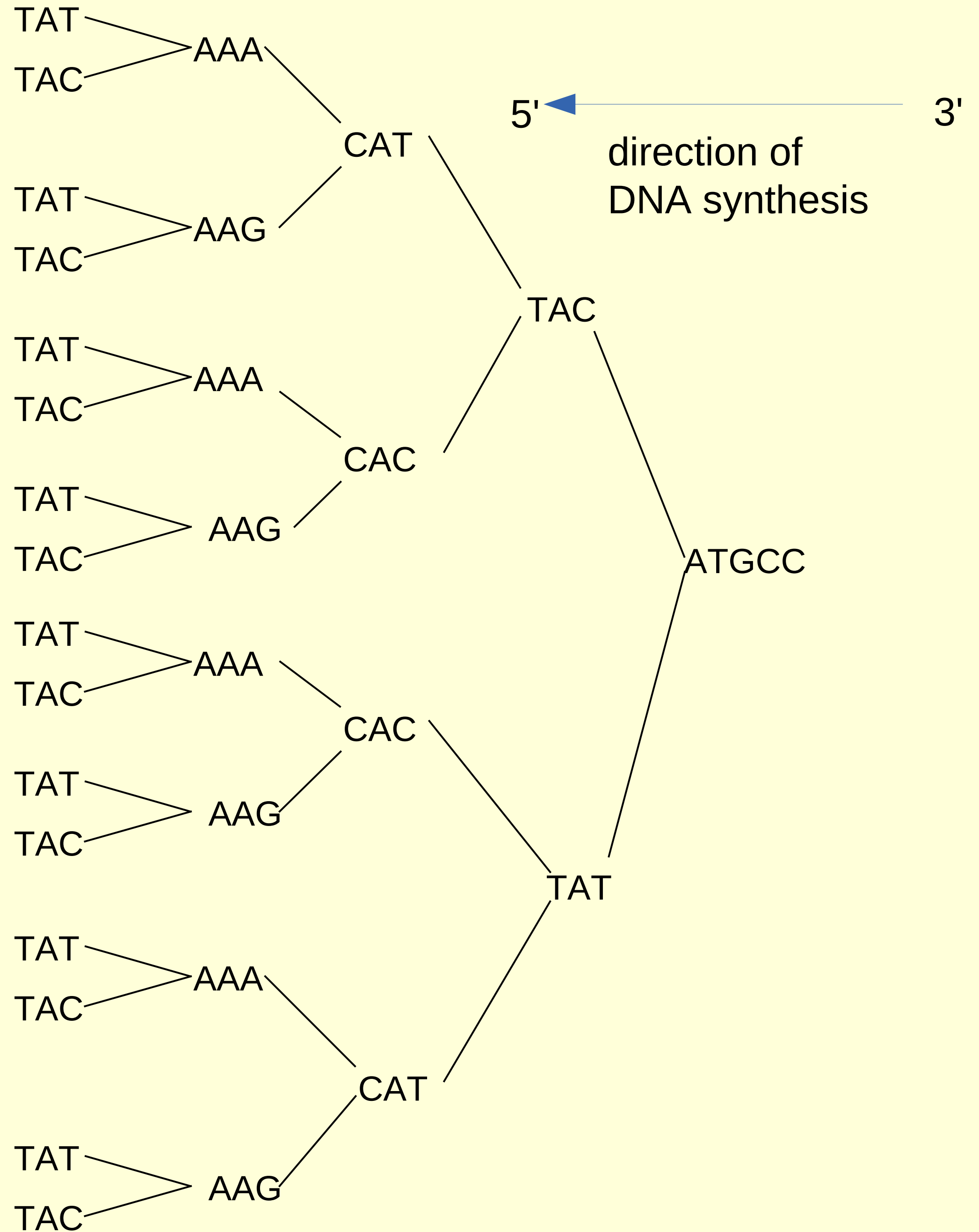
```
-------Phe   Lys  His   Tyr   Met  Pro-------
       TTT  AAA  CAT TAT  ATG CC
          C      G    C    C
```

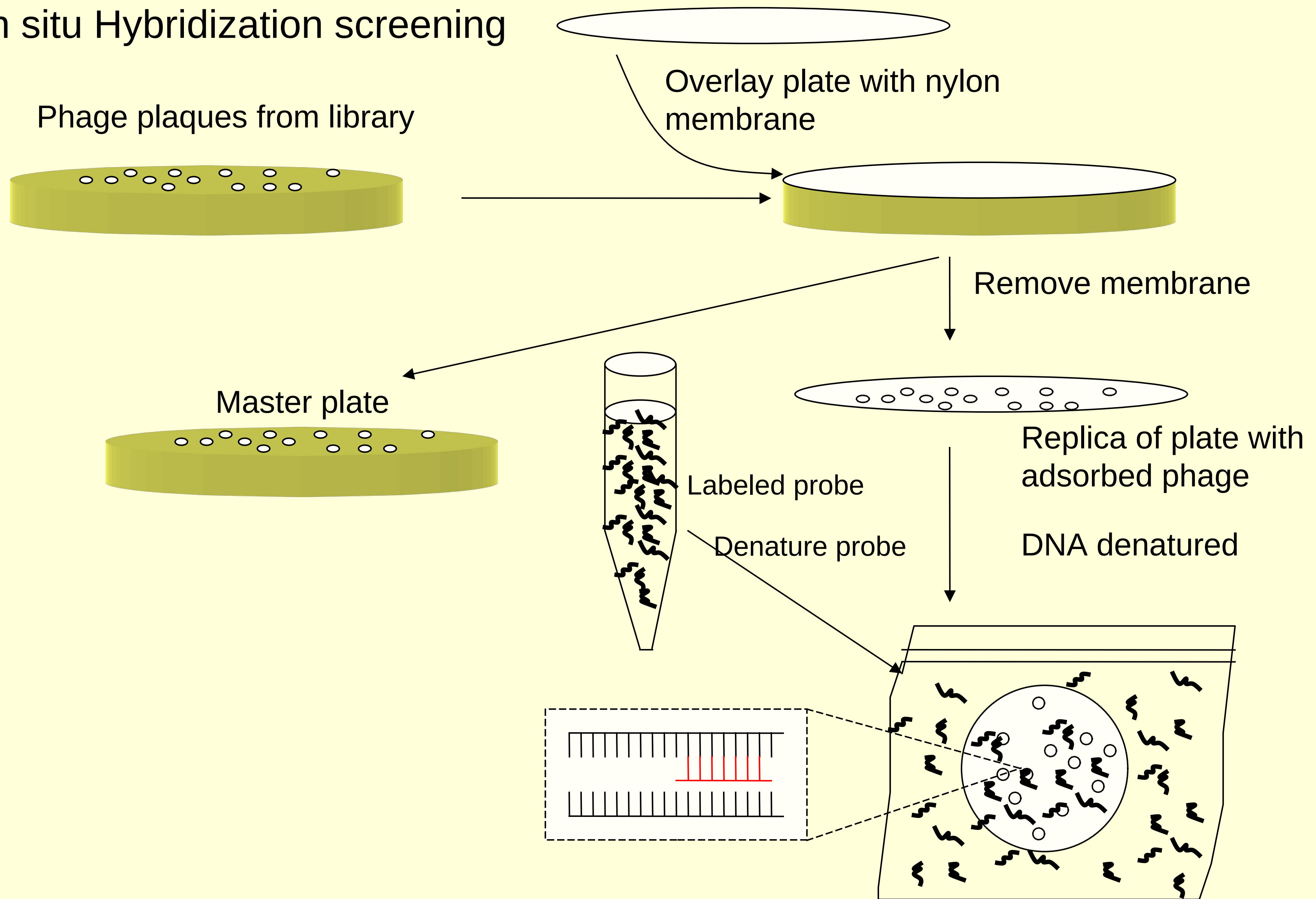
Oligonucleotides are normally labeled using polynucleotide kinase (PNK), which adds a phosphate group to the 5’ end.

Chemiluminescent or flourescent nucleotides can be used for end labeling.

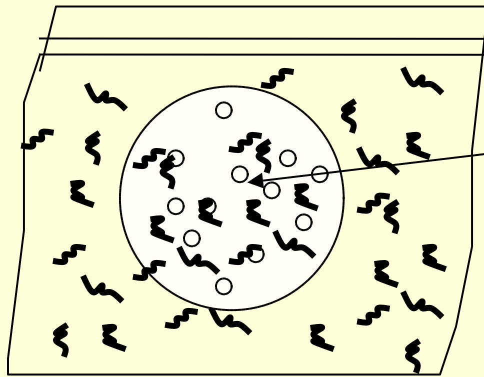
```
TTtAAgCAtTAcATGCC
TTcAAgCAtTAcATGCC
TTtAAaCAtTAcATGCC
TTcAAaCAtTAcATGCC
TTtAAgCAcTAcATGCC
TTcAAgCAcTAcATGCC
TTtAAaCAcTAcATGCC
TTcAAaCAcTAcATGCC
TTtAAaCAtTAtATGCC
TTcAAaCAtTAtATGCC
TTtAAgCAtTAtATGCC
TTcAAgCAtTAtATGCC
TTtAAaCAcTAtATGCC
TTcAAaCAcTAtATGCC
TTtAAgCAcTAtATGCC
TTcAAgCAcTAtATGCC
```

Synthesis pattern of oligonucleotide from the 3’ end with the 16 fold redundancy shown



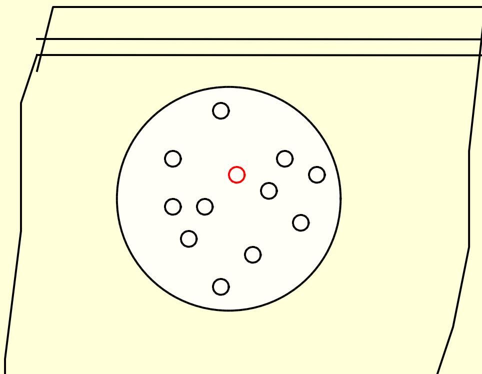

In situ Hybridization screening
Overlay plate with nylon membrane
Phage plaques from library
Remove membrane
Master plate
Replica of plate with adsorbed phage
Labeled probe
Denature probe
DNA denatured
Hybridize probe to filter membrane under salt and temperature to find near perfect match

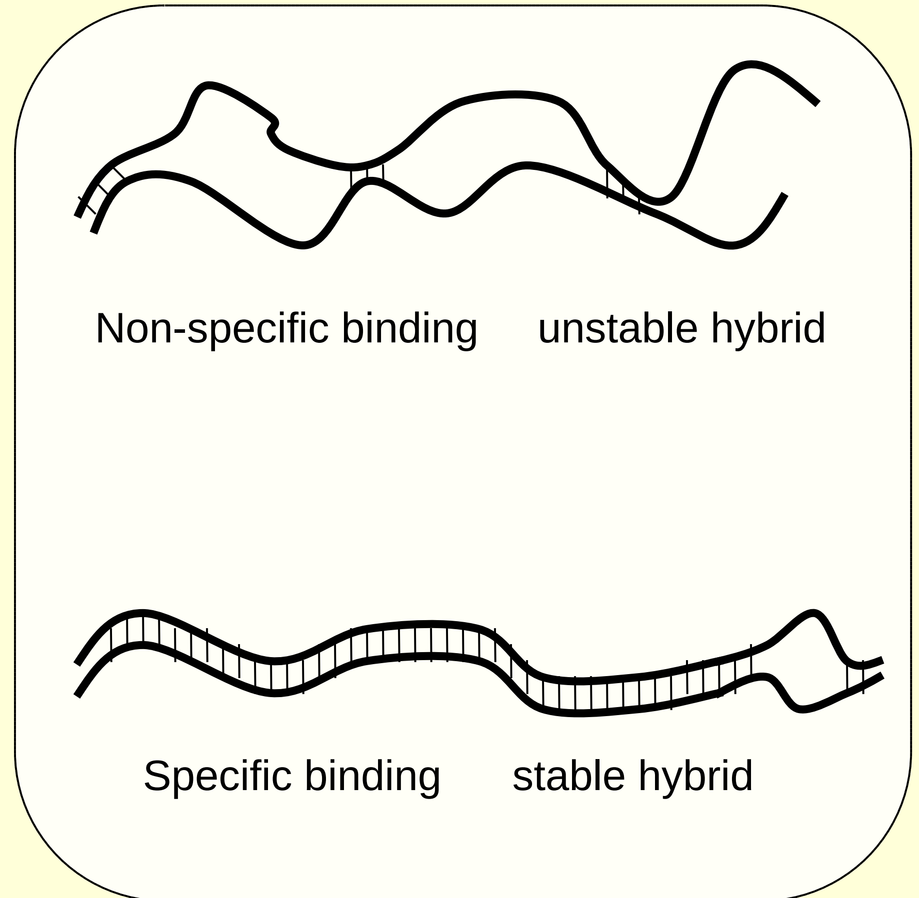
Hybridization screening



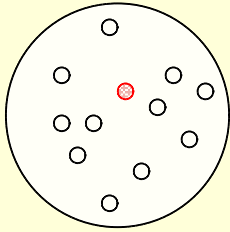
Probe hybridizes specifically to phage insert

Remove excess probe and wash membrane
to remove non-specific binding

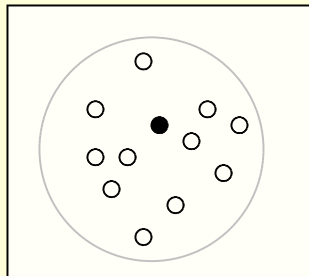




Hybridization screening

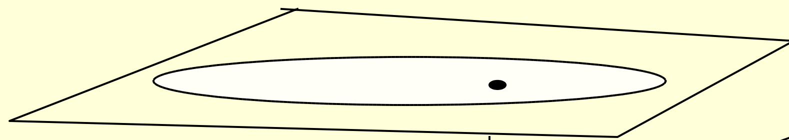


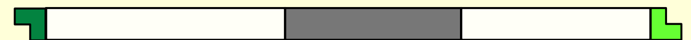
Expose to X-ray film

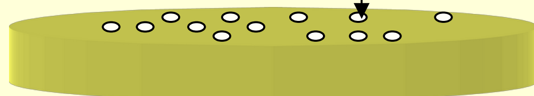


Exposed film blackened by region with bound chemiluminescent probe

Align film to master plate to find correct plaque







Master plate

Pick phage plaque and isolate phage DNA, cut out insert and transfer insert to a plasmid for further work

Generalizations -- hybridization screening

- If the vector used for the cDNA library was a plasmid rather than a lambda vector, the same process would be used except you would be dealing with colonies on the plate rather than plaques (colonies would have to be lysed on the membrane to release the DNA).

- Oligonucleotide probes are labeled with polynucleotide kinase at the 5’ end only (1 label per probe molecule)

- Heterologous probe (much larger) would be labeled by random hexanucleotide labeling using Klenow enzyme (many labeled sites along probe) Stronger signal, more sensitive. Probe can be labeled with fluorescent signals as an alternative to radioactivity.

Screening of cDNA libraries

Screening is about detecting a specific gene by its sequence or its expression

- Direct screening (in situ hybridization) involves use of an oligonucleotide probe to detect a specific sequence

- Indirect screening involves demonstrating the presence of the expressed protein product of the gene rather than the gene sequence itself.

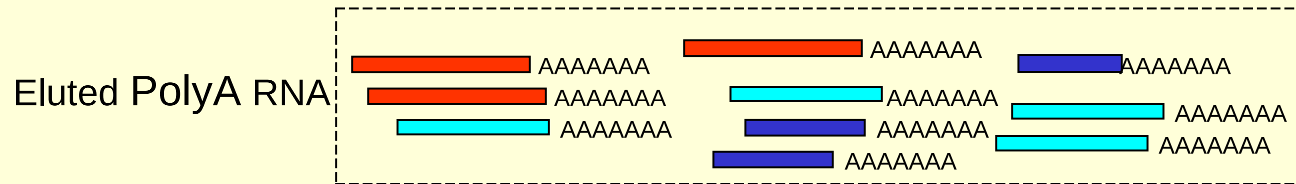
  - **Requires:** Expression of the genes in the cDNA library in their bacterial hosts (to protein products)

  - **Requires:** An efficient and specific means of detecting the expressed gene product.

    - If target gene encodes an enzyme and there was a way to demonstrate the presence of the enzyme in a bacterial colony—
      eg. something like the Xgal substrate for LacZ

    - Immunoscreening involves using antibodies that specifically recognize the target protein and bind to it.

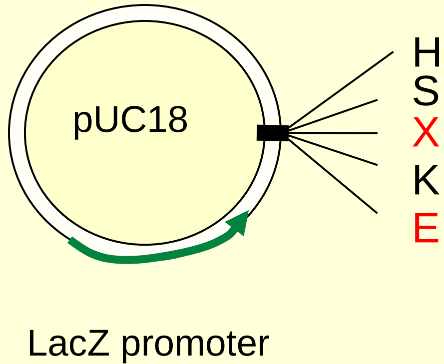
Directional cloning



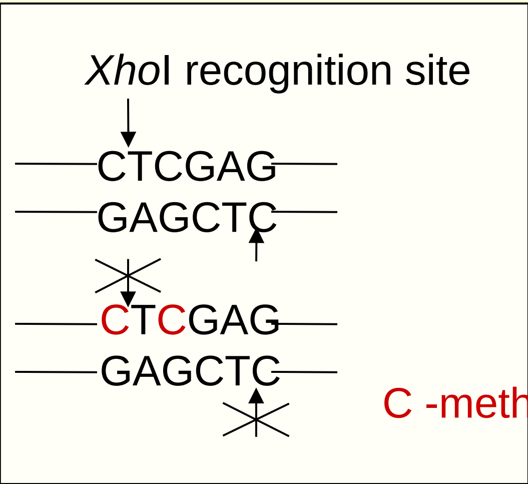
Using Reverse Transcriptase (RNA template-dependent DNA polymerase) and oligo-dT (15-18mer) primer, mRNA copied into complementary DNA (cDNA)

In non-directional cloning

Stratagene Directional Cloning strategy



LacZ promoter



XhoI linker-primer

5CTCGAGTTTTTTTTTTTT
3'AAAAAAAAAA RNA

Reverse transcriptase - dATP dGTP, dTTP 5-methyl dCTP

CH_3 CH_3 CH_3 CH_3

5'CTCGAGTTTTTTTTTTTT DNA
3'AAAAAAAAAA RNA

RNase H
DNA polymerase I
dATP, dGTP, dTTP, dCTP

CH_3 CH_3 CH_3 CH_3

5'CTCGAGTTTTTTTTTTTT DNA
3'GAGCTCAAAAAAAAAA DNA

EcoRI adapters
Ligase

CH_3 CH_3 CH_3 CH_3

5'AATTC—CTCGAGTTTTTTTTTTTT DNA — G
3'G—GAGCTCAAAAAAAAAA DNA — CTTAA5'

XhoI restriction enzyme

CH_3 CH_3 CH_3 CH_3

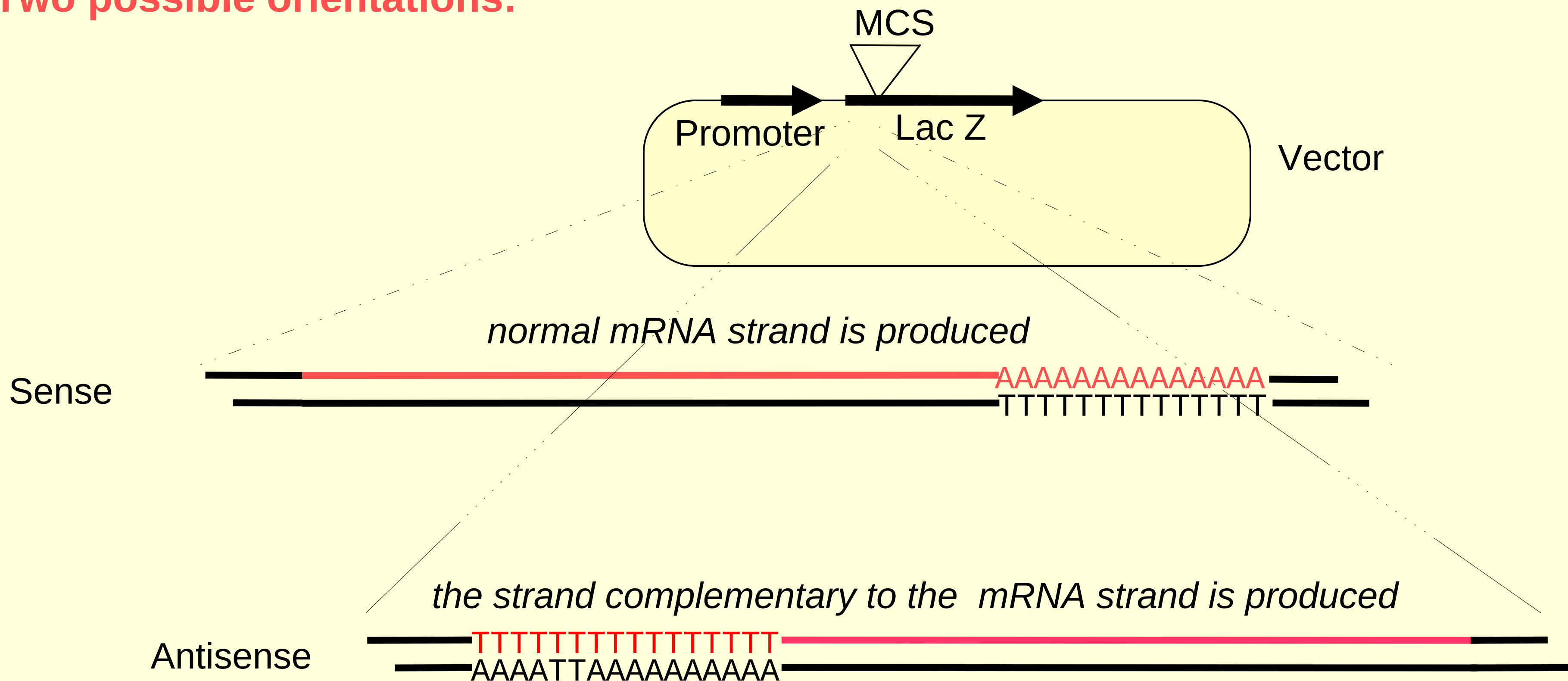
5'TCGAGTTTTTTTTTTTT DNA — G
3'CAAAAAAAAAA DNA — CTTAA5'

Complete directional cDNA

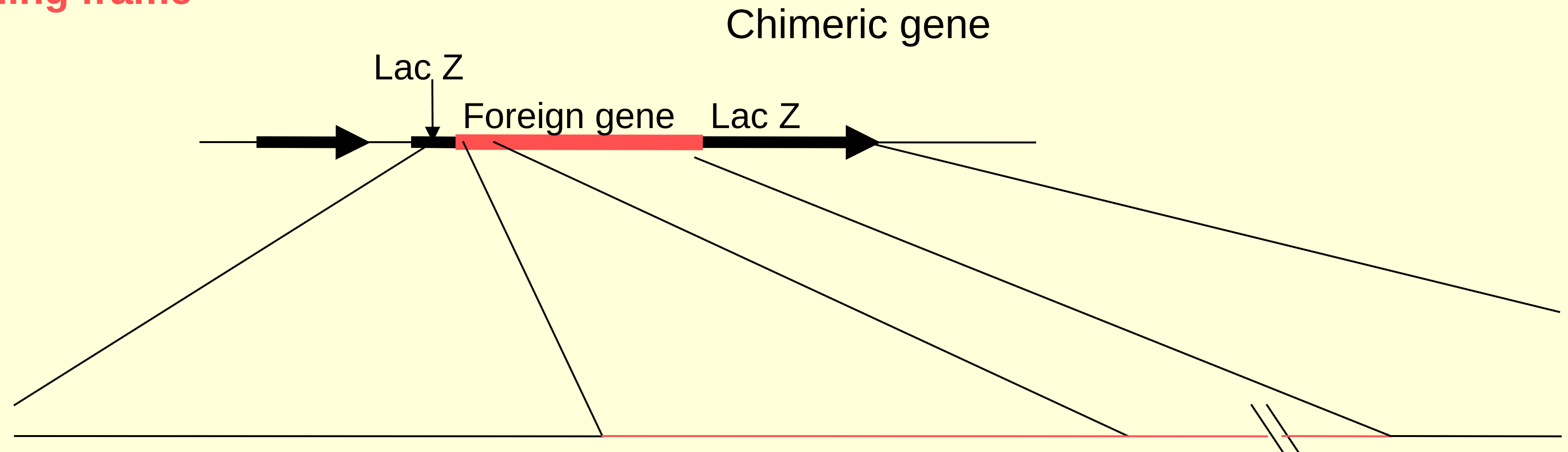
Expression of the genes in the cDNA library

- Expression requires use of the selectable marker gene promoter
  1. Correct (sense) orientation of gene sequence in the vector.
  2. Correct reading frame with the Lac Z gene

Two possible orientations:



Reading frame

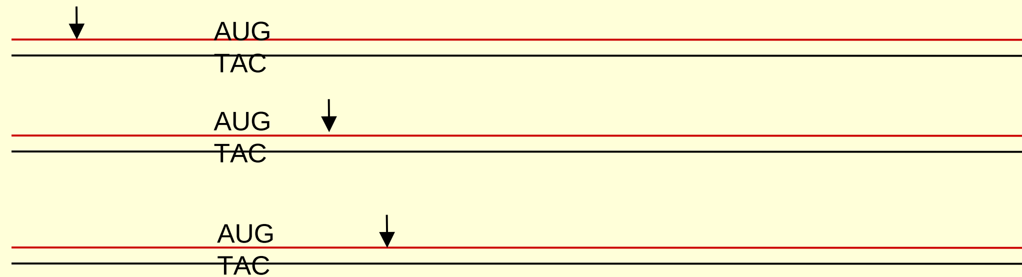


ATG ACC ATG ATT xxx xxx xxx xxx xxx xxx ATG GGA AGT AAG TAT ---

These codons must be in same reading frame

Met Thr Met Ile yyy yyy yyy yyy uuu uuu Met Gly Ser Tyr

What is the likelihood of this being the case?



3 mRNA from same gene in a heteroduplex

Cleavage by RNase H is random so there is an equal chance that subsequent insertion into the Lac Z gene will be in any of its 3 reading frames. Cleavage could either be in the 5'UTR, or, more commonly, in the coding region itslef.

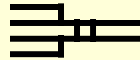
True for all mRNAs - hence at best only 1/3 of the clones in a library will be able to correctly express the encoded protein if insert is in the correct orientation.

If the cDNA gene has the same adapters on each end and can be inserted in either orientation then the potential for correct expression drops to 1/6

Thus it is important with a <u>cDNA expression library</u> to have the best selection of tissue and time for mRNA isolation for your gene of interest so that there are many copies present.

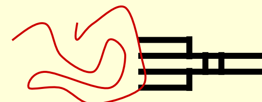
Can double chances by directional cloning

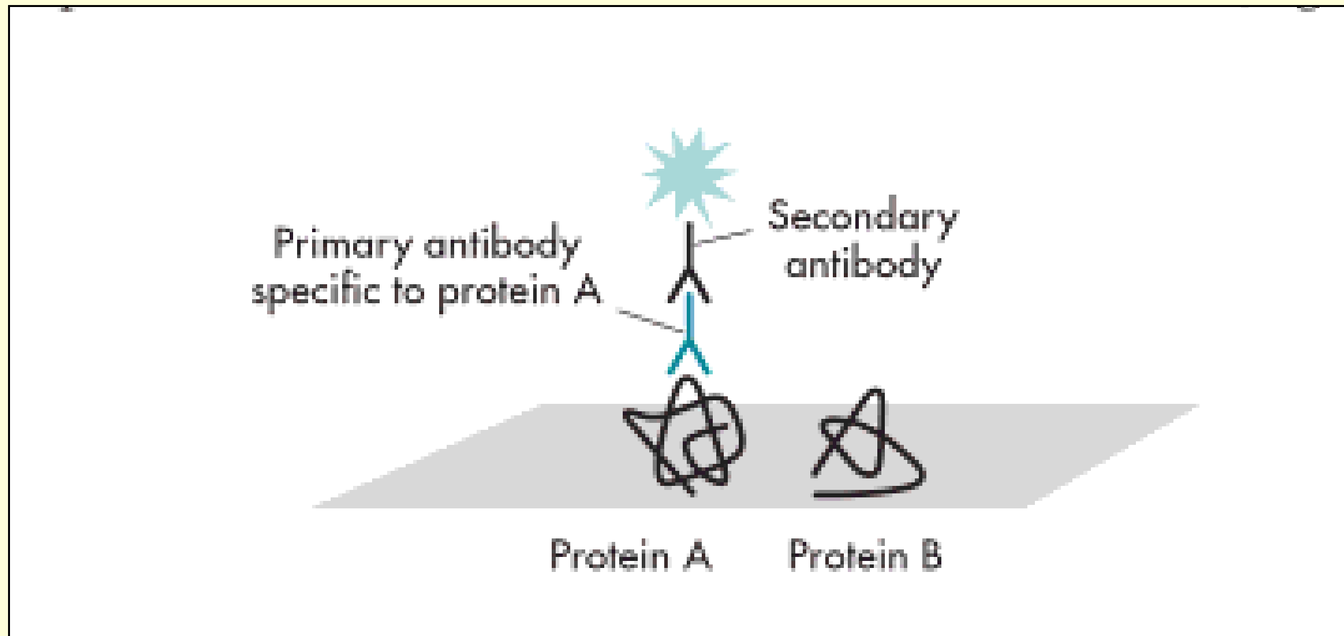
Antibodies -as specific probes

- Are part of the defensive immune system of animals and birds
  - Injection of foreign proteins into any animal of bird results in the generation by the animal of a mixture of antibodies which will recognize and bind to the foreign protein and trigger its removal from the system 

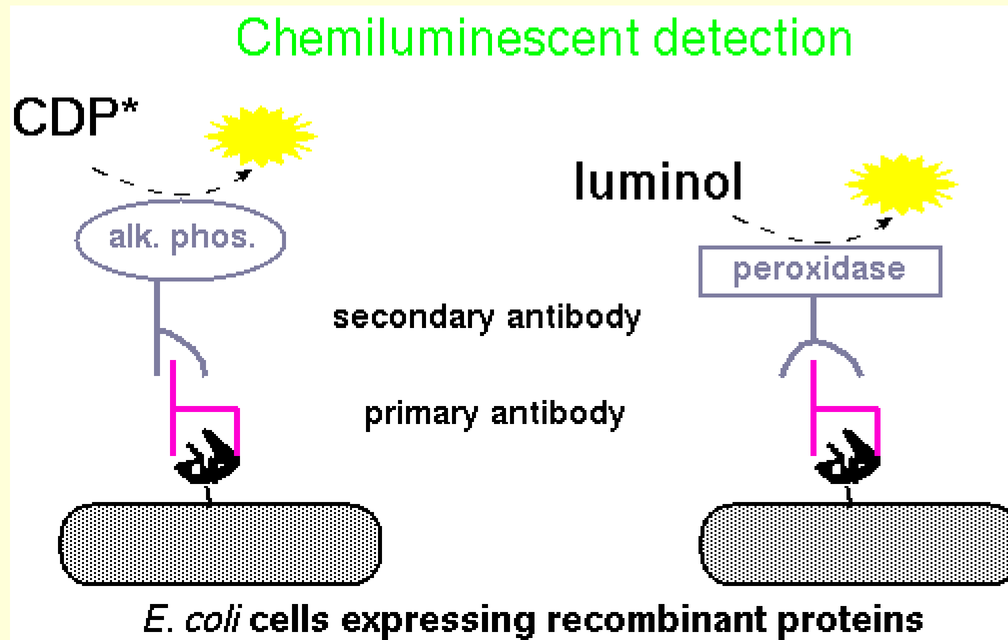
- To prepare specific antibodies for screening, you must isolate the pure protein for the target gene, inject it into a rabbit, rat, or mouse (normally) where it serves as the antigen. The animal produces antibodies in their blood which can be recovered by collecting the blood serum – antiserum.

- When antiserum is exposed to the antigen in a test-tube under proper conditions it will bind to it tightly.





Gene cloning: principles and applications: Julia Lodge, Peter A. Lund and Steve Minchin



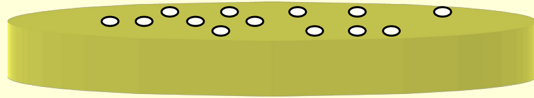
Chemiluminescent detection is typically done using substrates that emit photons of light when broken down by enzyme-conjugated antibodies. The antibody binds to a specific protein on the E. coli cell surface, carrying with it the enzyme.

For alkaline phosphatase, the substrate is CDP*

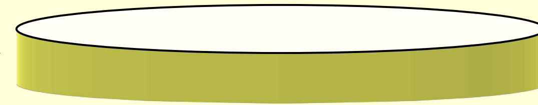
For horseradish peroxidase the substrate luminol peroxide from Clontech Inc.

Indirect Screening using Antibodies (Immunoscreening)

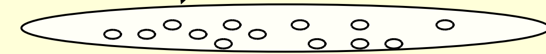
Colonies/plaques from cDNA library



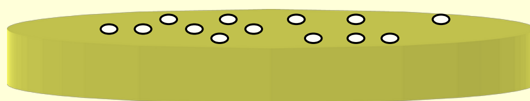
Overlay plate with nylon membrane



Remove membrane



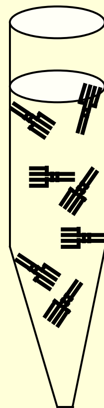
Master plate

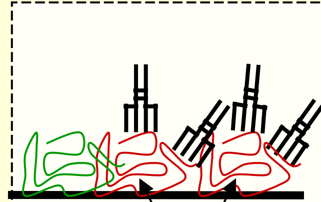


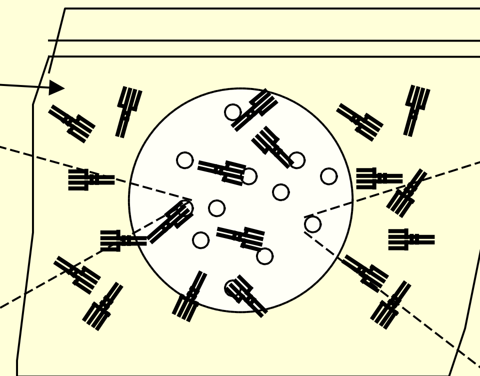
Replica of plate with adsorbed protein and DNA from each plaque

(If plasmid vector, colonies must be lysed before protein is bound)

Primary antiserum to target protein

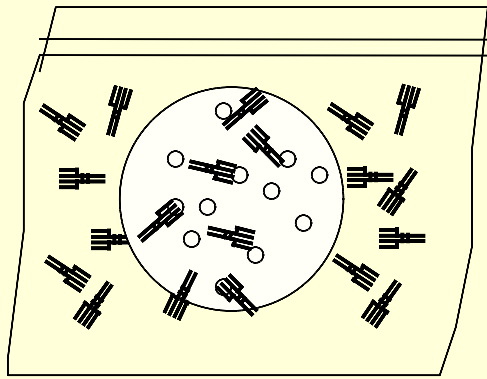




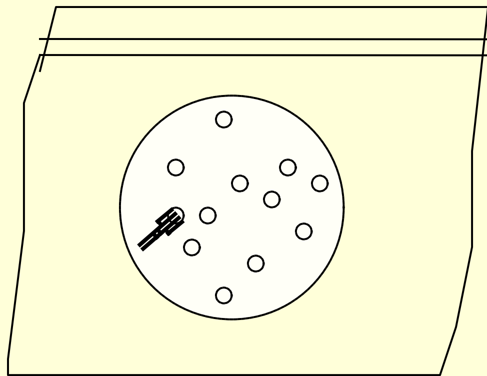


Proteins bound from colony/plaque

Antibodies bind to specific protein if it is present

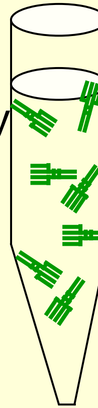


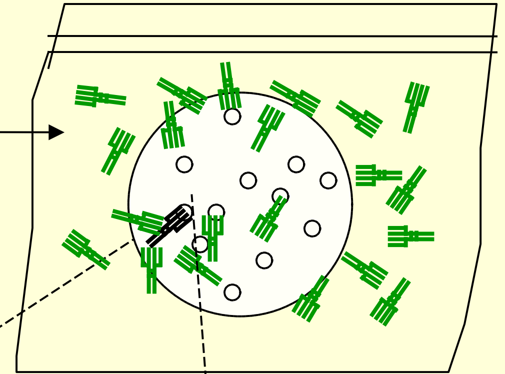
After allowing binding wash unbound primary antibody away

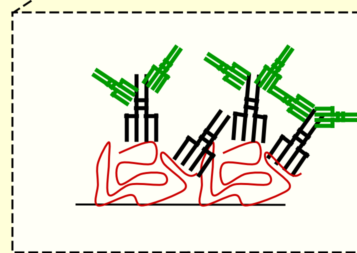


Add labeled secondary antibody that binds to the primary antibody

Secondary antibody

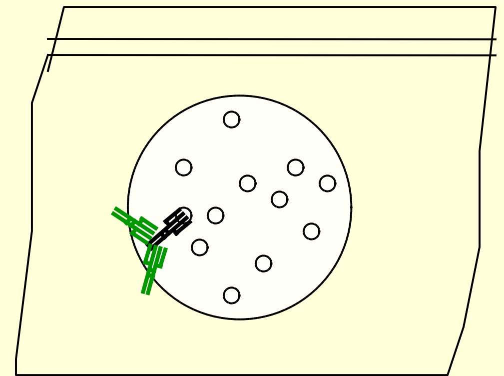






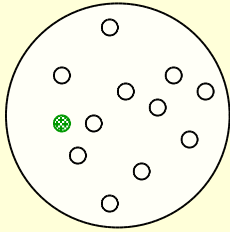
Wash away any unbound antibody

Add substrate

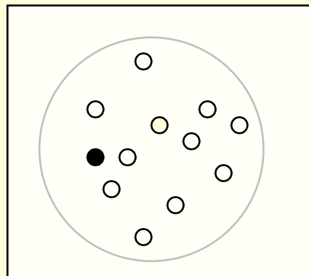


Expose to film to find where specific antibody was bound

Immunoscreening (cont'd)

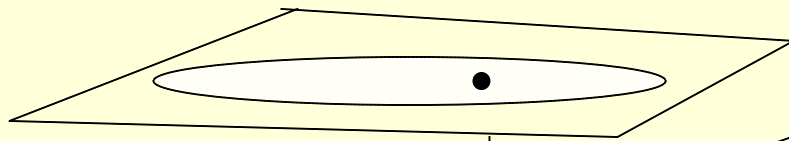


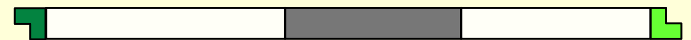
Expose to X-ray film



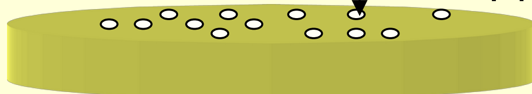
Exposed film blackened by region with bound labeled probe

Align film to master plate to find correct plaque





Pick plaque/colony and isolate recombinant vector and insert DNA



Master plate

Summary of gene isolation from a cDNA library

- Make cDNA library

- Screen library
  - Nucleotide probe (direct screening for gene)
  - Antibody probe (indirect screening for protein gene product)

- Select positive clone
  - If phage library –amplify selected clone and isolate insert and transfer to a plasmid vector for future work
  - If plasmid library – amplify cells from positive colony and isolate plasmid and retain cell line with selected clone

- Characterize the insert by sequencing it

- To verify you have the correct gene,
  - compare the sequence to other sequences in the available databases to see if there is similarity (sequence or motifs). This can be done at http://blast.ncbi.nlm.nih.gov

    See BLASTX search on next 2 slides

  - If you originally had protein sequence information you could verify if the nucleotide sequence would encode the protein by translating all reading frames

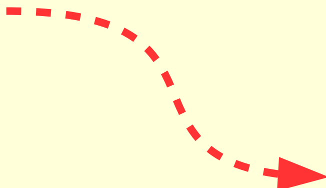
- Advantage of cDNA library is its simplicity but if you want the promoter and total unprocessed gene you would need to go to a genomic library.

  However, if you have the correct cDNA clone this is now easy!!

- This is how a gene is isolated when you know what the gene is --- i.e its sequence or identity based on the gene product

NCBI BLAST search

Set parameters, and paste your query sequence into the box



Note that we are searching the UniProt database, which is probably the most carefully annotated database for proteins.

Since the query is a plant sequence, we limit the search to the Magnoliophyta (flowering plants).

blastx: search protein databases using a translated nucleotide query - Mozilla Firefox

File Edit View History Bookmarks Tools Help

blastx: search protei...

blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE

Most Visited Information BIRCH Bit Lab cc.umanitoba.ca UM Webmail

BLAST® Basic Local Alignment Search Tool

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NCBI/ BLAST/ blastx

Translated BLAST: blastx

blastn | blastp | blastx | tblastn | tblastx

BLASTX search protein databases using a translated nucleotide query. more...

Reset page
Bookmark

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) Clear

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tttccagacacagttgagaagatctcatttgaggctaaactgtctgcaggaccaaatggaggatcca

Query subrange
From
To

Or, upload file Browse... No file selected.

Genetic code Standard (1)

Job Title
Enter a descriptive title for your BLAST search

Align two or more sequences

Choose Search Set

Database UniProtKB/Swiss-Prot(swissprot)

Organism Optional Magnoliophyta (taxid:3398) Exclude
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude Optional Models (XM/XP) Uncultured/environmental sample sequences

Entrez Query Optional YouTube Create custom database
Enter an Entrez query to limit search

BLAST

Search database UniProtKB/Swiss-Prot(swissprot) using Blastx (search protein databases using a translated nucleotide query)

RESULTS

NCBI Blast:Nucleotide Sequence (728 letters) - Mozilla Firefox

File Edit View History Bookmarks Tools Help

NCBI Blast:Nucleotid...

blast.ncbi.nlm.nih.gov/Blast.cgi

st Visited Information BIRCH Bit Lab cc.umanitoba.ca UM Webmail

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NCBI/ BLAST/ blastx/ Formatting Results - AXGZMSUD01R

Your search is limited to records matching entrez query: txid3398 [ORGN].

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Nucleotide Sequence (728 letters)

RID AXGZMSUD01R (Expires on 01-10 00:52 am)

Query ID lcl|149549
Description None
Molecule type nucleic acid
Query Length 728

Database Name swissprot
Description Non-redundant UniProtKB/SwissProt sequences
Program BLASTX 2.2.30+ Citation

Other reports: Search Summary [Taxonomy reports]

Graphic Summary

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	RecName: Full=Disease resistance response protein Pi176 [Pisum sativum]	317	317	65%	4e-110	100%	P13239.1
☐	RecName: Full=Disease resistance response protein Pi49; AltName: Full=PR10 [Pisum sativum]	301	301	65%	6e-104	96%	P14710.1
☐	RecName: Full=Disease resistance response protein DRRG49-C [Pisum sativum]	263	263	65%	9e-89	84%	P27047.1
☐	RecName: Full=Class-10 pathogenesis-related protein 1; Short=MtPR10-1; AltName: Full=Pathogen	249	249	65%	1e-83	80%	P93333.1
☐	RecName: Full=Class-10 pathogenesis-related protein 1; AltName: Full=MSPR10-1 [Medicago sativ	248	248	65%	8e-83	80%	Q43560.1