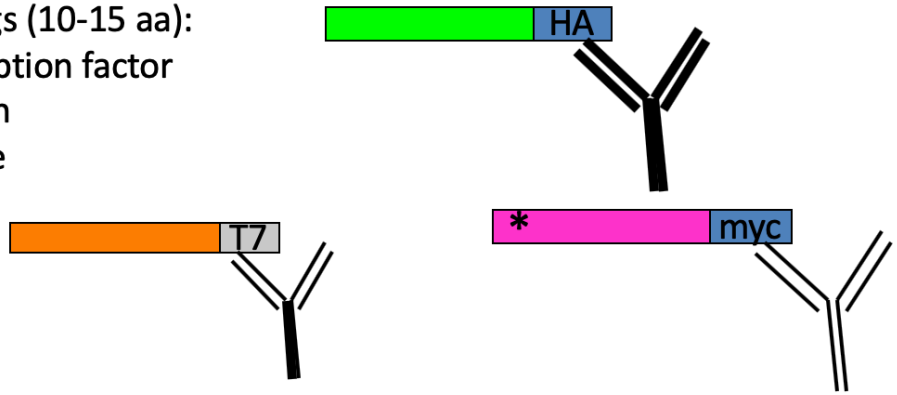
Tagged-proteins

Tagged-proteins

Definition:

Fusion of the target protein to either terminus (the C- or N- terminal) of a short peptide (epitope tag) which is recognized by an antibody (immunoprecipitation and Western blot analysis)

Exemple of epitope-Tags (10-15 aa):
myc: from myc transcription factor
HA: from hemagglutinin
T7: from T7 polymerase



Notice: The fusion protein must be continuous with the target protein - the same open reading frame must be maintained. Stop codons between the target protein and the fusion partner must be omitted.

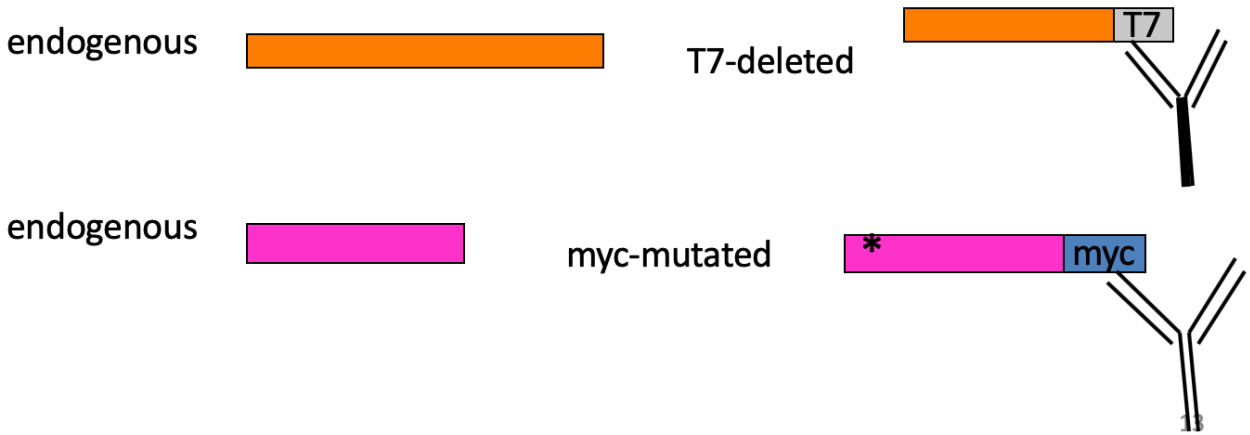
When are Tag-proteins used?

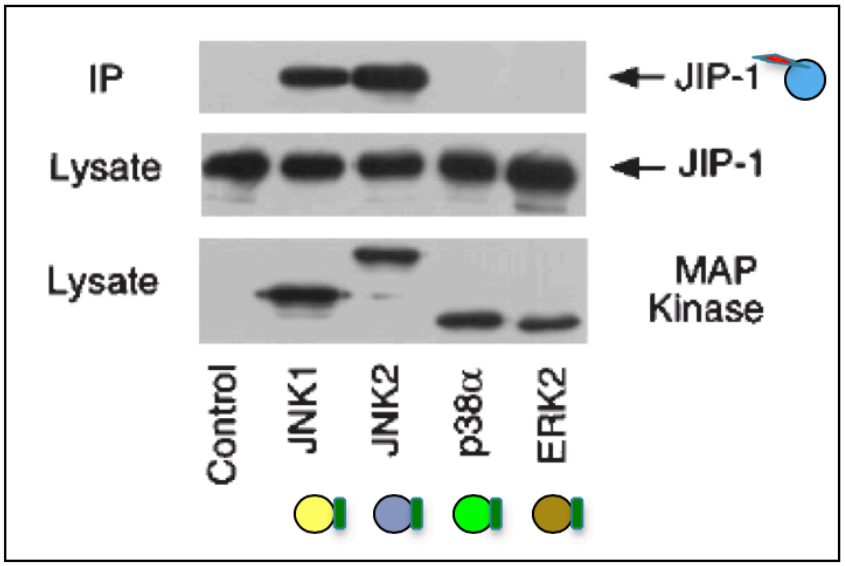
Ab against endogenous protein not available

If the Ab againt the protein of interest is not available

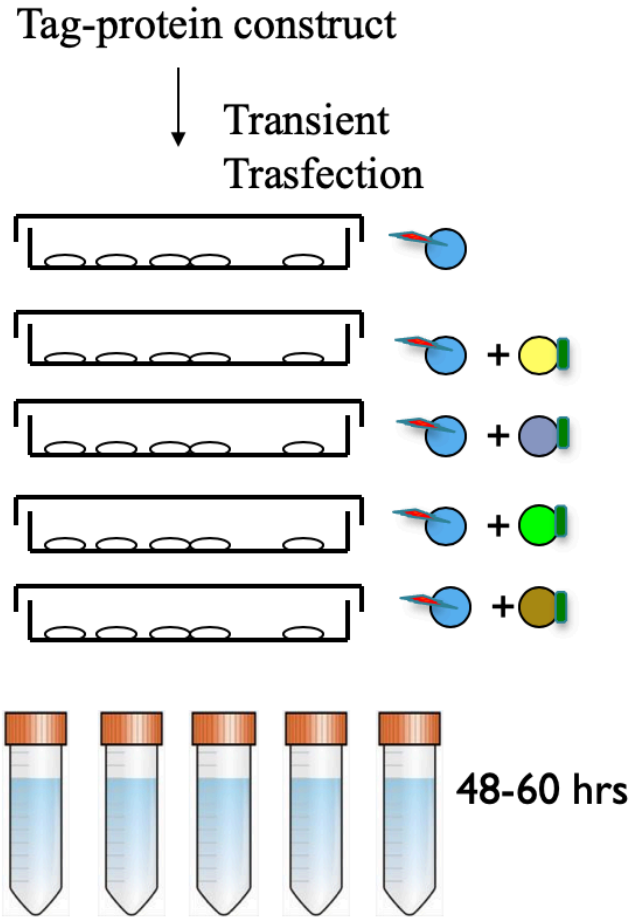
to distinguish recombinant constructs from endogenous proteins

To study recombinant construct of the protein of interest (deletions, site-directed mutations) even in the presence of expression of the endogenous WT protein.



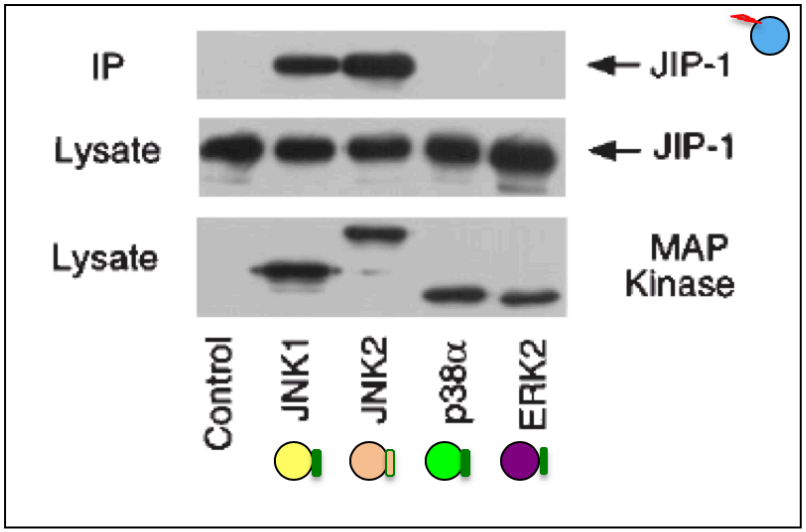


Epitope-tagged JIP-1 (T7-Tag) was ex-pressed in cells with the HA-tagged MAP kinases ERK2, p38α JNK1, and JNK2. The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag. The amount of JIP-1 and MAP kinases in the cell lysates was examined by protein immunoblot analysis.

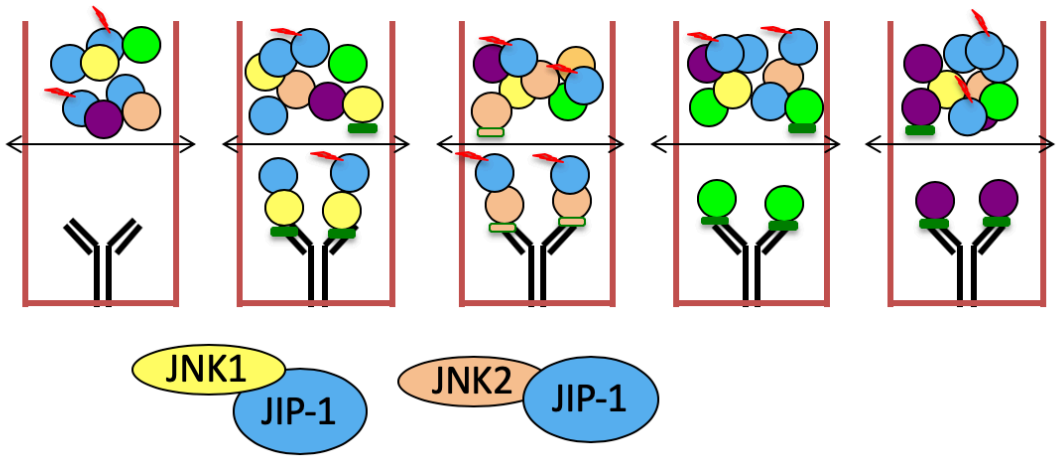


IP: Antibody against the Tag

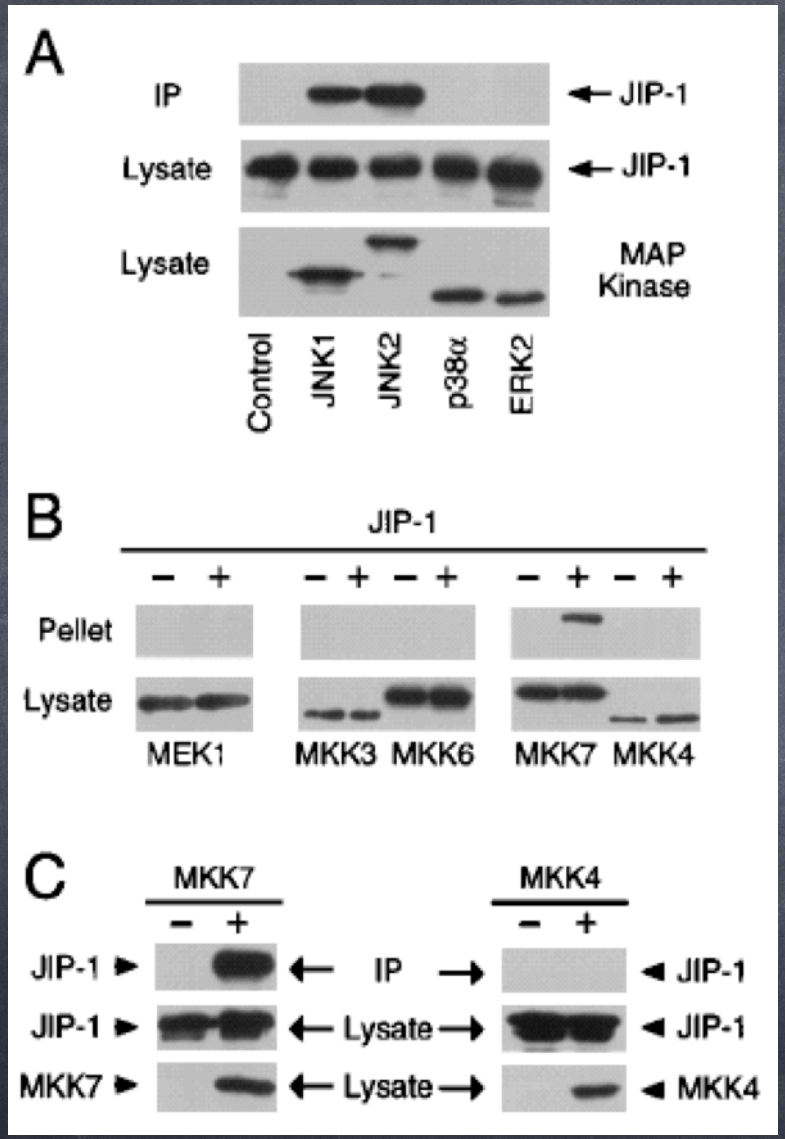
Selective binding of JIP-1 to the MAP kinase JNK



Epitope-tagged JIP-1 (T7-Tag) was expressed in cells with the HA-tagged MAP kinases ERK2, p38α JNK1, and JNK2. The MAP kinases were immunoprecipitated with an antibody to HA. The presence of JIP-1 in the immunoprecipitates (IP) was detected on immunoblots probed with an antibody to T7-Tag. The amount of JIP-1 and MAP kinases in the cell lysates was examined by protein immunoblot analysis.



IP


A
IP
JIP-1
Lysate
JIP-1
Lysate
MAP Kinase
Control
JNK1
JNK2
p38α
ERK2
B
JIP-1
– + – + – + – + – +
Pellet
Lysate
MEK1
MKK3
MKK6
MKK7
MKK4
C
MKK7
MKK4
– + – +
JIP-1
IP
JIP-1
JIP-1
Lysate
JIP-1
MKK7
Lysate
MKK4

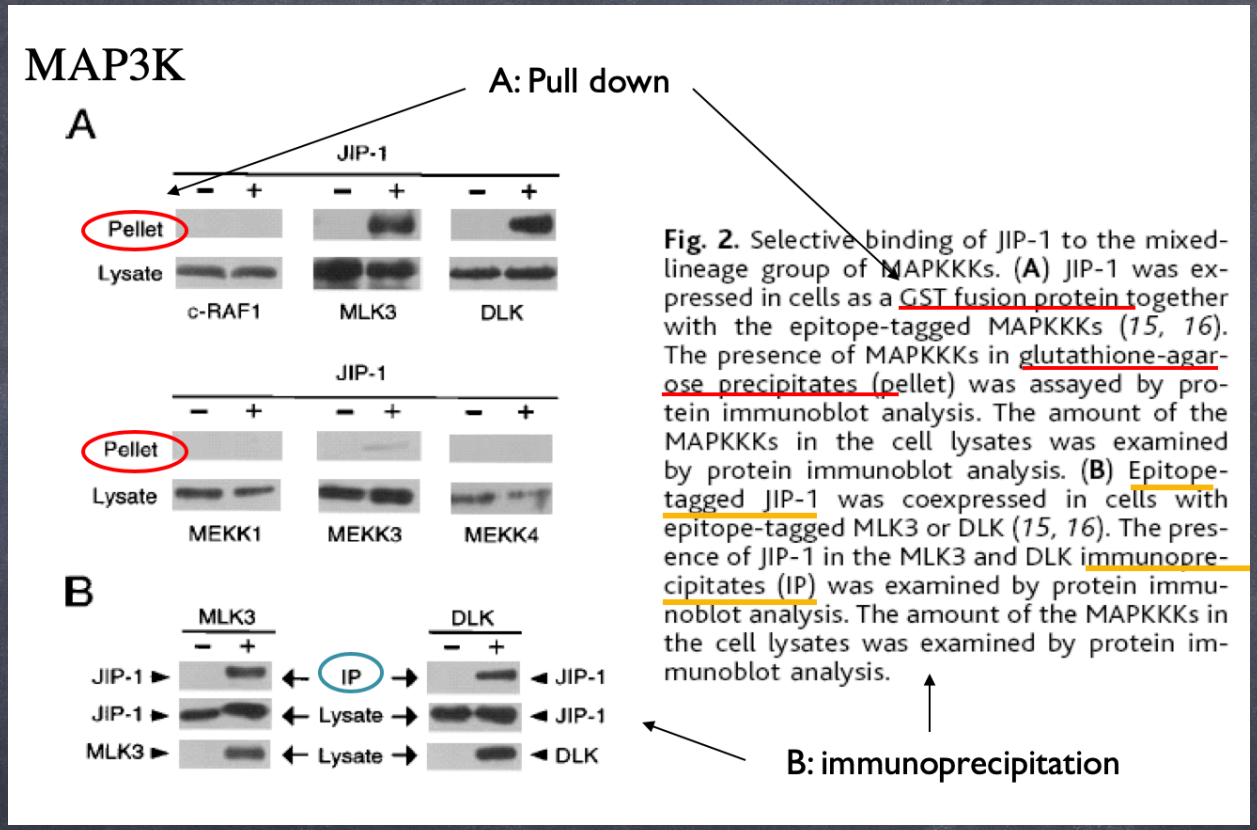
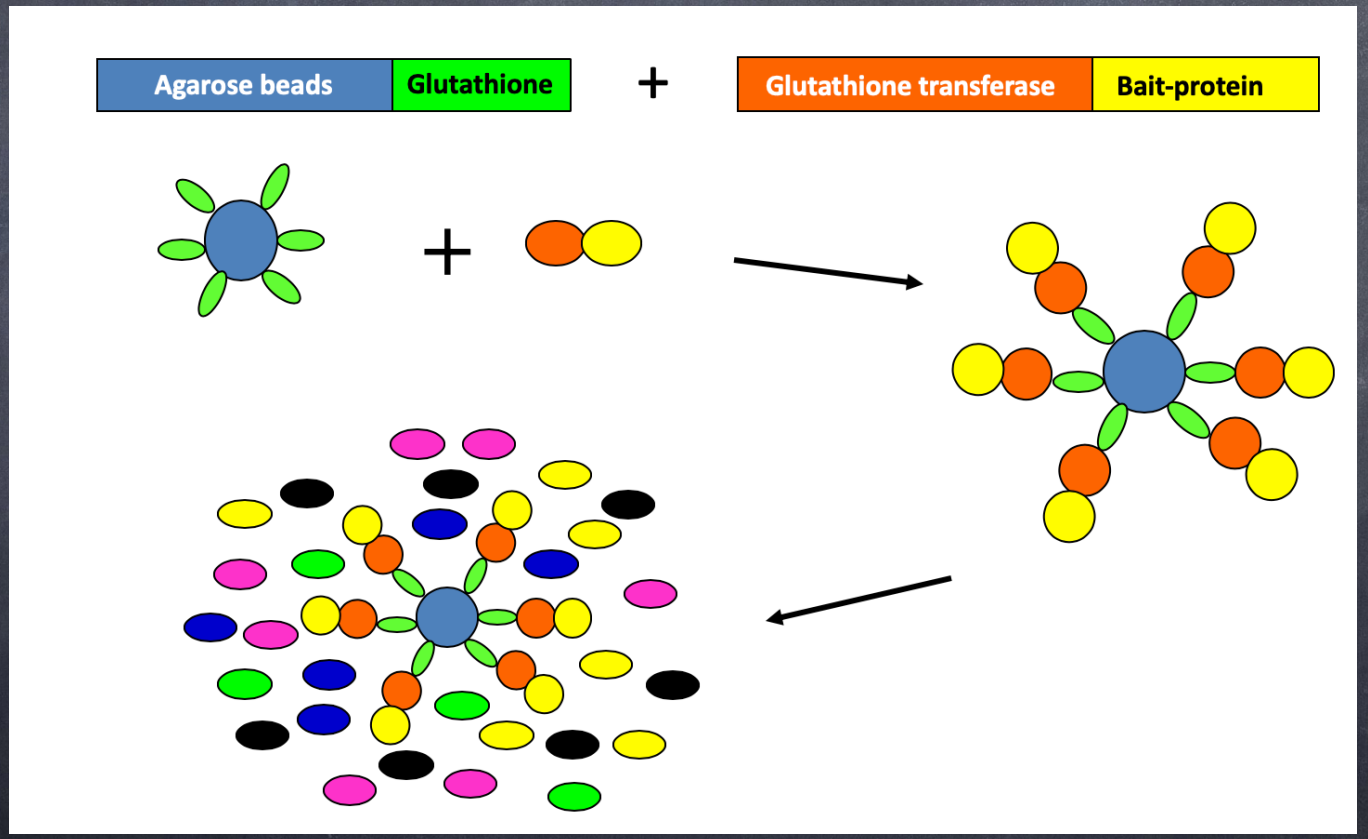
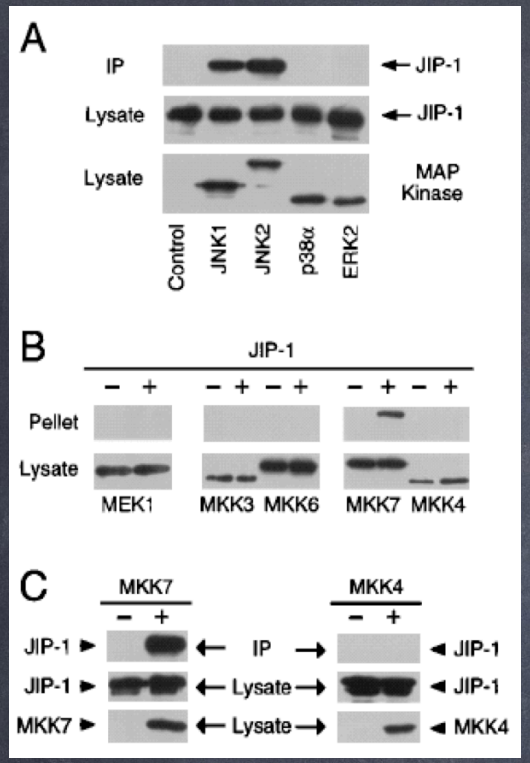


Fig. 2. Selective binding of JIP-1 to the mixed-lineage group of MAPKKKs. (**A**) JIP-1 was expressed in cells as a GST fusion protein together with the epitope-tagged MAPKKKs (*15*, *16*). The presence of MAPKKKs in glutathione-agarose precipitates (pellet) was assayed by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis. (**B**) Epitope-tagged JIP-1 was coexpressed in cells with epitope-tagged MLK3 or DLK (*15*, *16*). The presence of JIP-1 in the MLK3 and DLK immunoprecipitates (IP) was examined by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis.

Pull-down vs Co-IP

They are closely related methods to identify stable protein-protein interactions. The difference in pull-down assays is that affinity-tagged bait proteins replace antibodies.





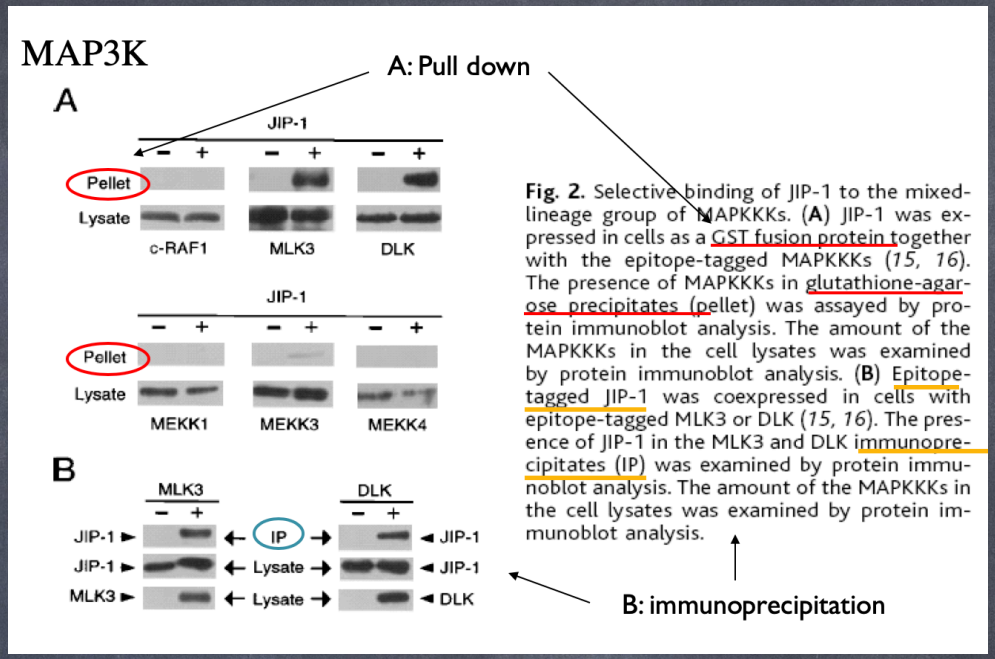
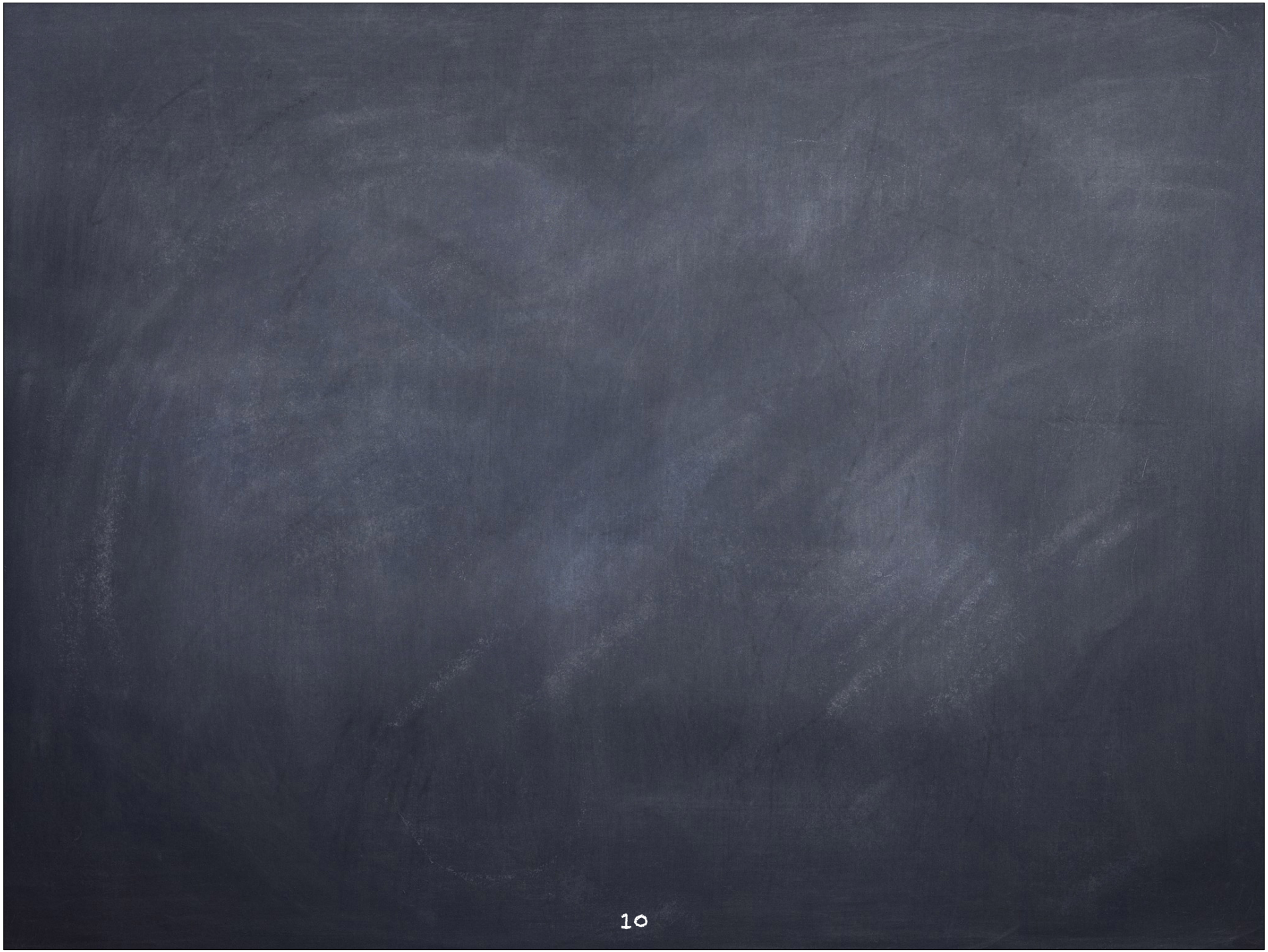


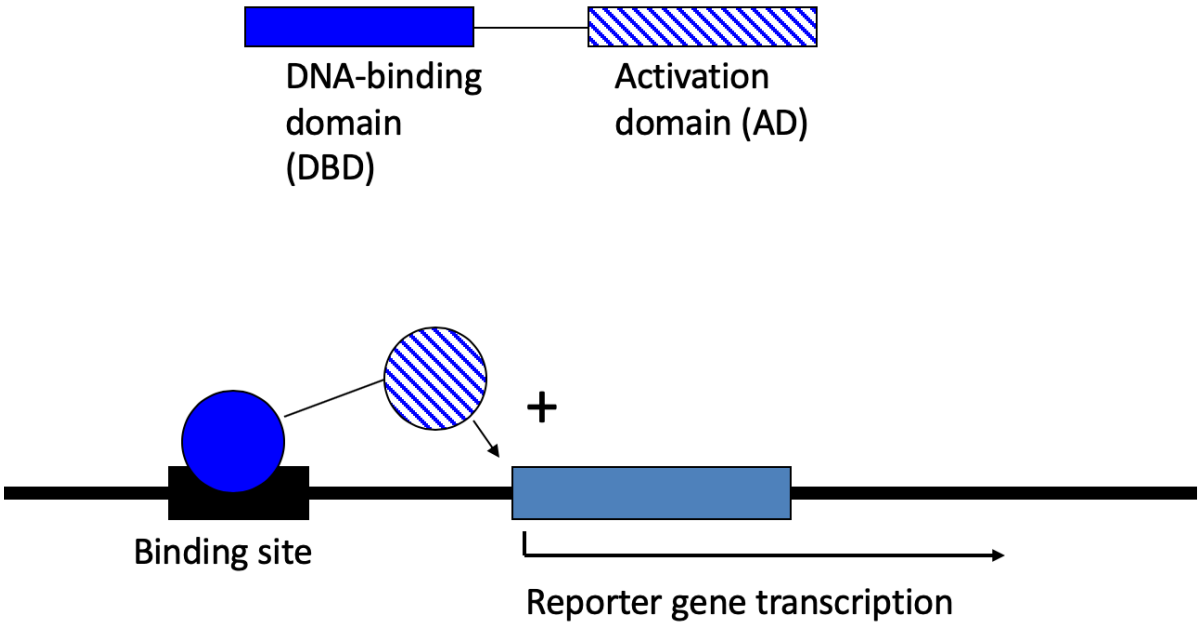
Fig. 2. Selective binding of JIP-1 to the mixed-lineage group of MAPKKKs. (**A**) JIP-1 was expressed in cells as a GST fusion protein together with the epitope-tagged MAPKKKs (*15*, *16*). The presence of MAPKKKs in glutathione-agarose precipitates (pellet) was assayed by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis. (**B**) Epitope-tagged JIP-1 was coexpressed in cells with epitope-tagged MLK3 or DLK (*15*, *16*). The presence of JIP-1 in the MLK3 and DLK immunoprecipitates (IP) was examined by protein immunoblot analysis. The amount of the MAPKKKs in the cell lysates was examined by protein immunoblot analysis.


10

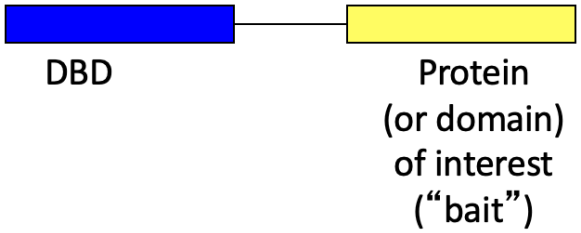
Yeast two-hybrid system

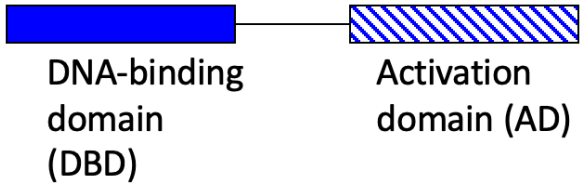
Y2H system → To formulate an hypothesis → co-immunoprecipitation Pull-down → To verify an hypothesis

Y2H systems exploit the modular nature of eukaryotic transcription factors, which consist of a sequence-specific DNA-binding domain (DBD) and an RNA Pol II-recruiting, transcription activation domain (AD).

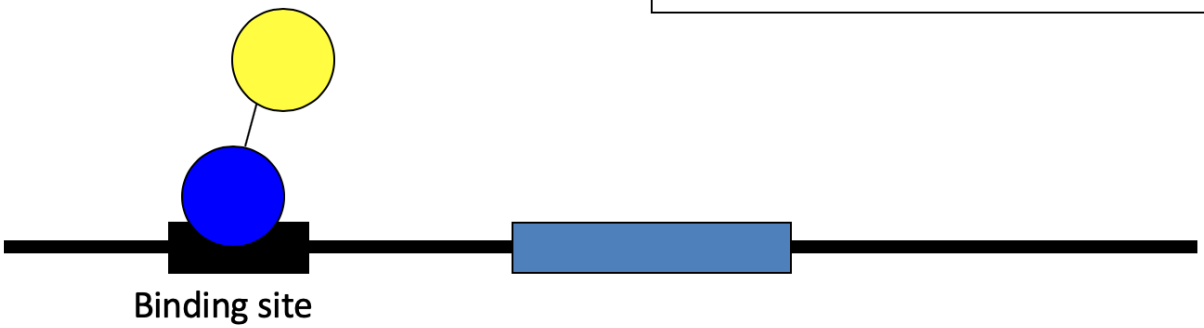


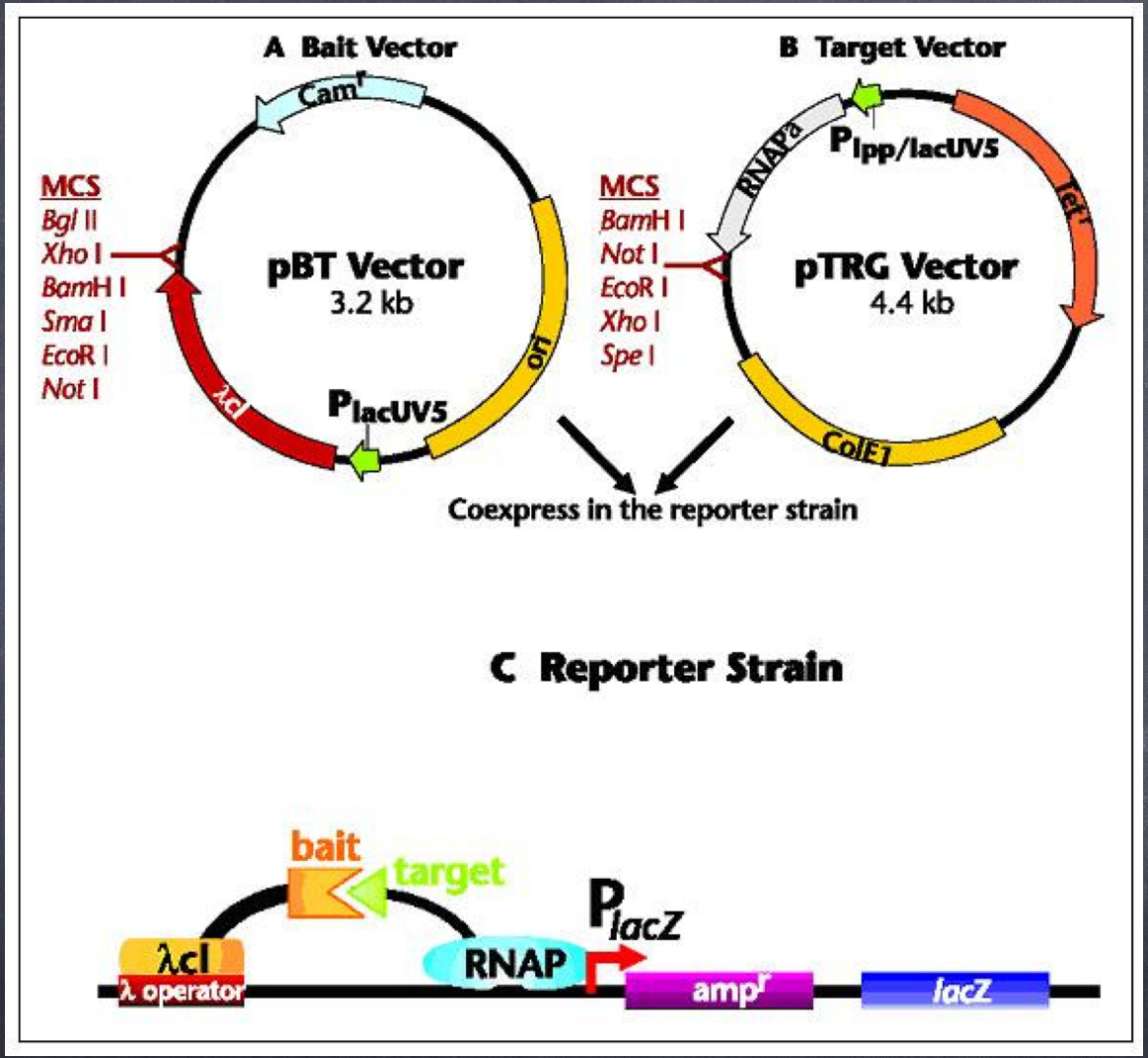
Two-hybrid system: two types of hybrids

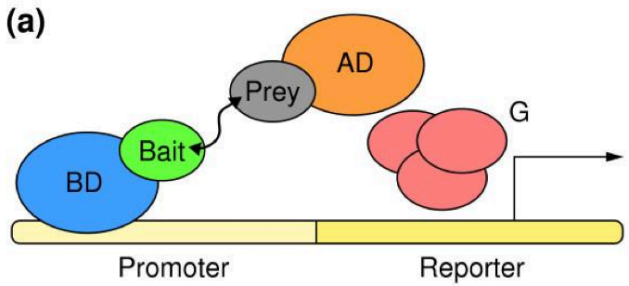


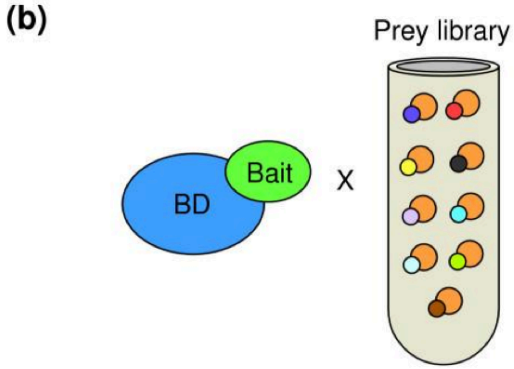


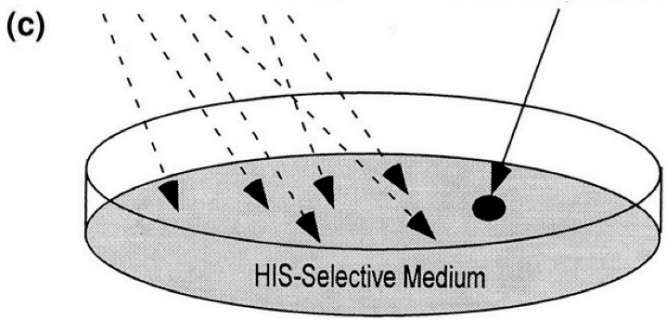
By itself, the DBD:bait fusion does not stimulate expression.



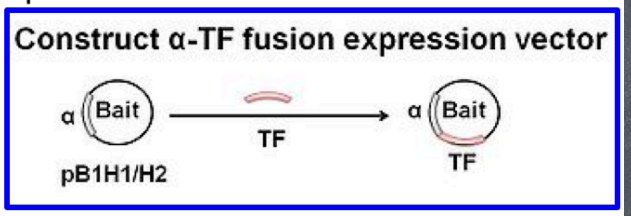

A Bait Vector
Cam^r
MCS
Bgl II
Xho I
BamH I
Sma I
EcoR I
Not I
pBT Vector
3.2 kb
λcI
P lacUV5
ori
B Target Vector
RNAPα
P lpp/lacUV5
MCS
BamH I
Not I
EcoR I
Xho I
Spe I
pTRG Vector
4.4 kb
ColE1
Coexpress in the reporter strain
C Reporter Strain
bait
target
P lacZ
λcI
λ operator
RNAP
amp^r
lacZ



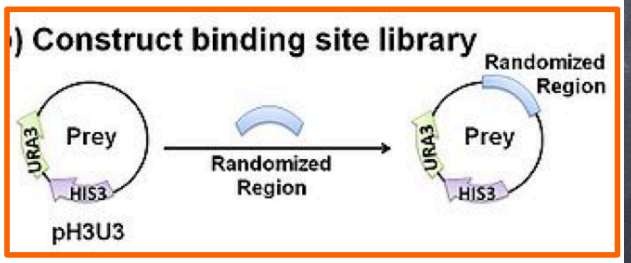




A known protein of interest is fused to the DNA-BD of the yeast GAL4 transcription factor to create a "bait" protein



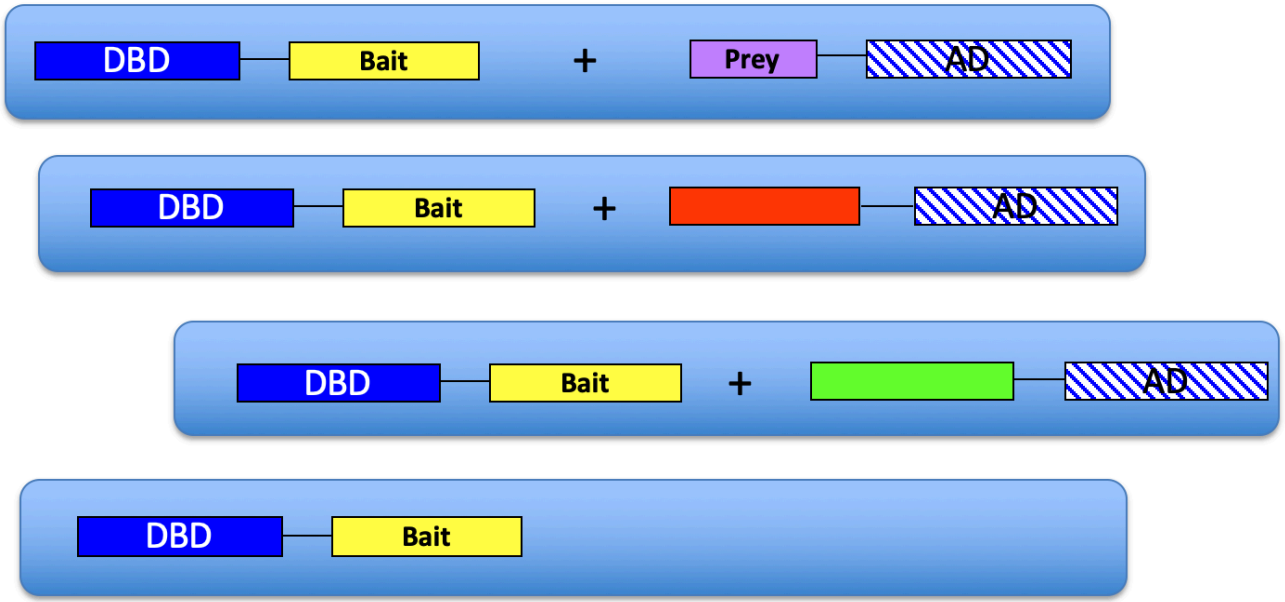
Interacting partner proteins, often derived from a library, are expressed as fusions to the AD of yeast GAL4, to create "prey" proteins



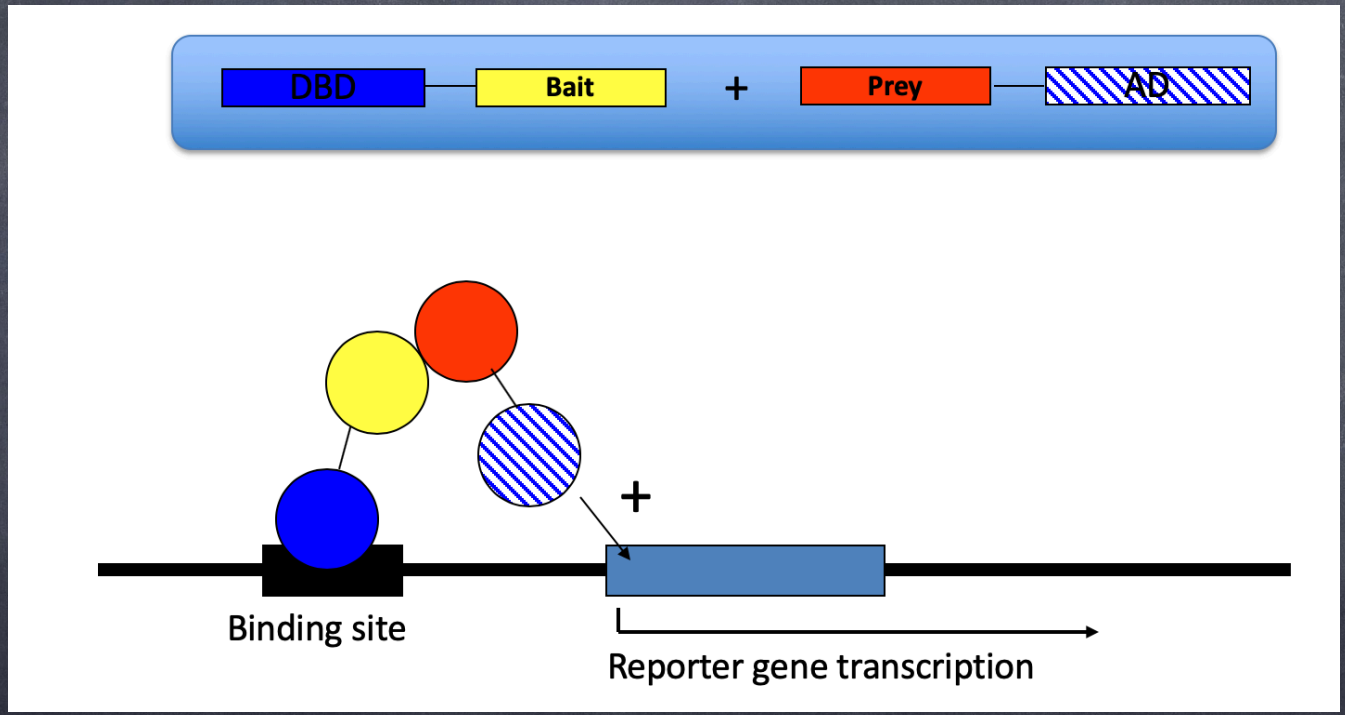
selective medium and reporter gene to identify positive colonies

Screening of the library

Pairs of bait- and prey-fusion proteins are coexpressed in yeast cells.



Transcription of the reporter gene: Prey and bait do interact



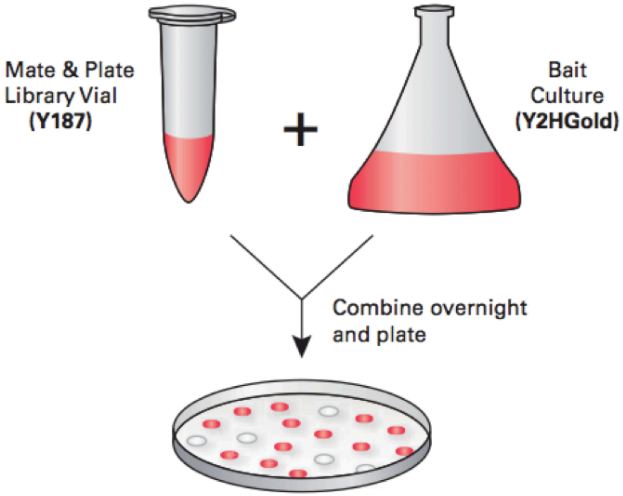
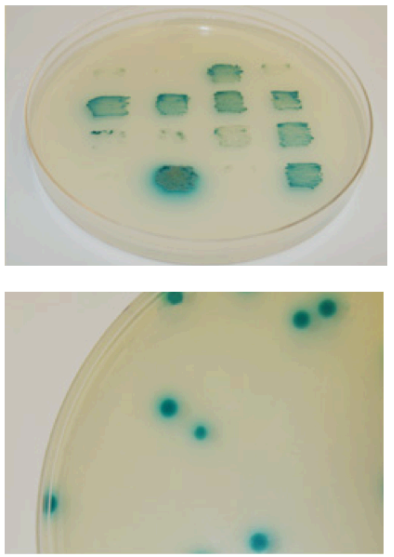


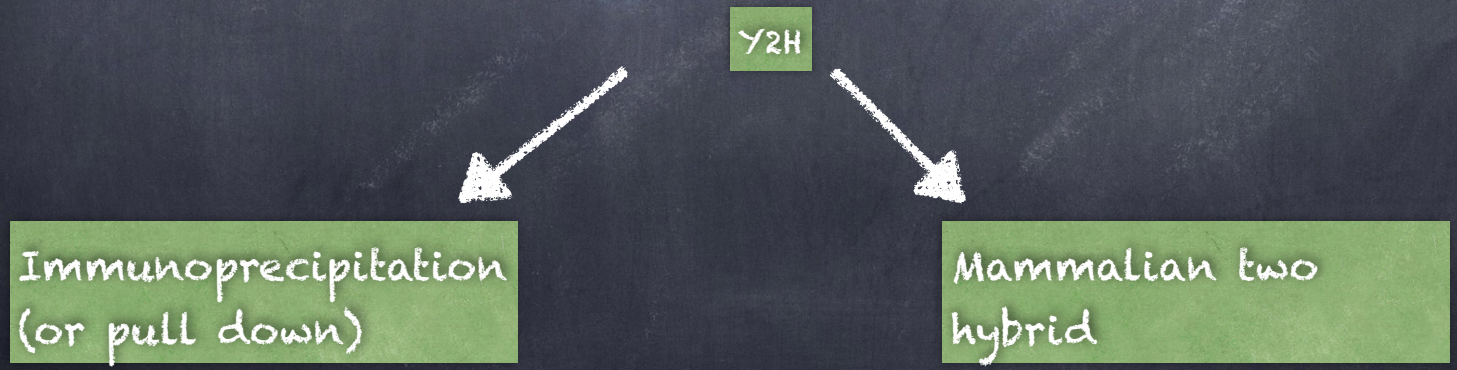
Figure 3. The Mate & Plate Protocol. To screen a Matchmaker Mate & Plate Library, an aliquot of the library in the Y187 strain (*MATα*) is simply mixed with a bait-expressing culture of the Y2HGold strain *(MATa)*. The mated strains are cultured overnight and plated on selective agar medium containing AbA.



X-α-Gal detects secreted α-galactosidase activity following a GAL4-based two-hybrid interactions in Y2HGold yeast patches and colonies.

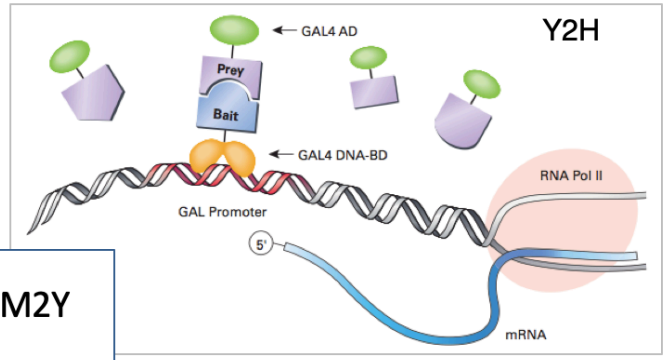
Limits of Y2H approach

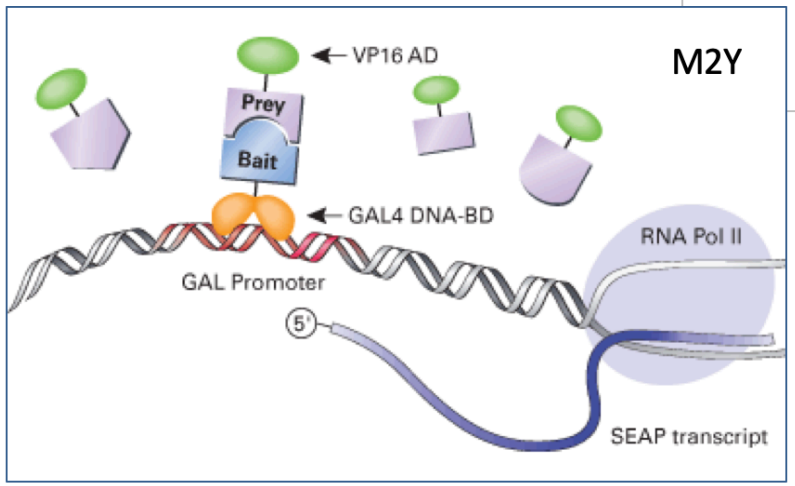
Performing two-hybrid screens in yeast is a powerful method of identifying novel protein binding relationships that involve a particular protein of interest. However, the conformation of a mammalian protein expressed in yeast may be quite different from its normal conformation in a mammalian cells. Thus, it is important to perform a two-hybrid assay in mammalian cells to confirm that the suspected interactions also take place when the proteins are folded and modified as they would be in their native environment. The mammalian assay often reflects interactions between mammalian proteins with greater authenticity than can be achieved in yeast.



Mammalian 2-H System

The bait protein is fused to the DNA binding domain of yeast GAL4 and the prey protein is fused to the transcriptional activation domain of HSV VP16. If the two proteins interact at the PGAL4-E1b promoter (GAL promoter), SEAP is secreted into the growth medium.





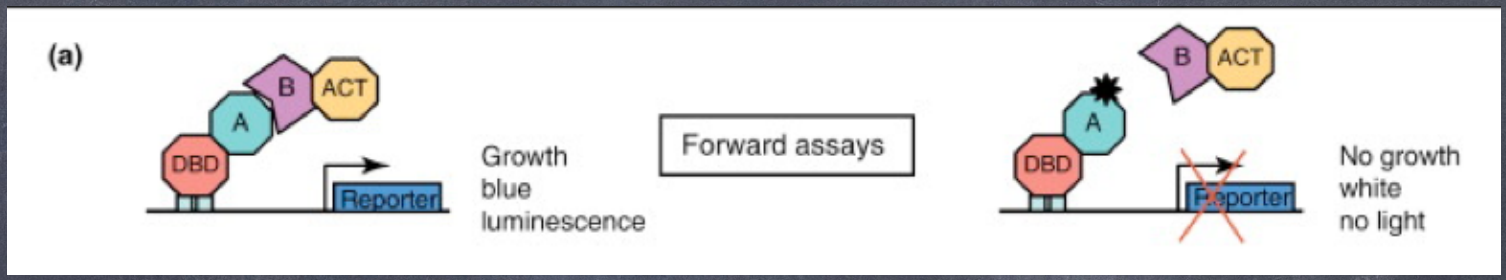
secreted alkaline phosphatase (SEAP), which is readily detected in the culture medium (chemiluminescence).

(from Clontech)

Y2H applications to drug discovery

detection of protein-protein interactions

detection of inhibition of protein interactions by small molecules



Phenotype

Phenotype

Y2H applications to drug discovery

detection of protein-protein interactions

detection of inhibition of protein interactions by small molecules

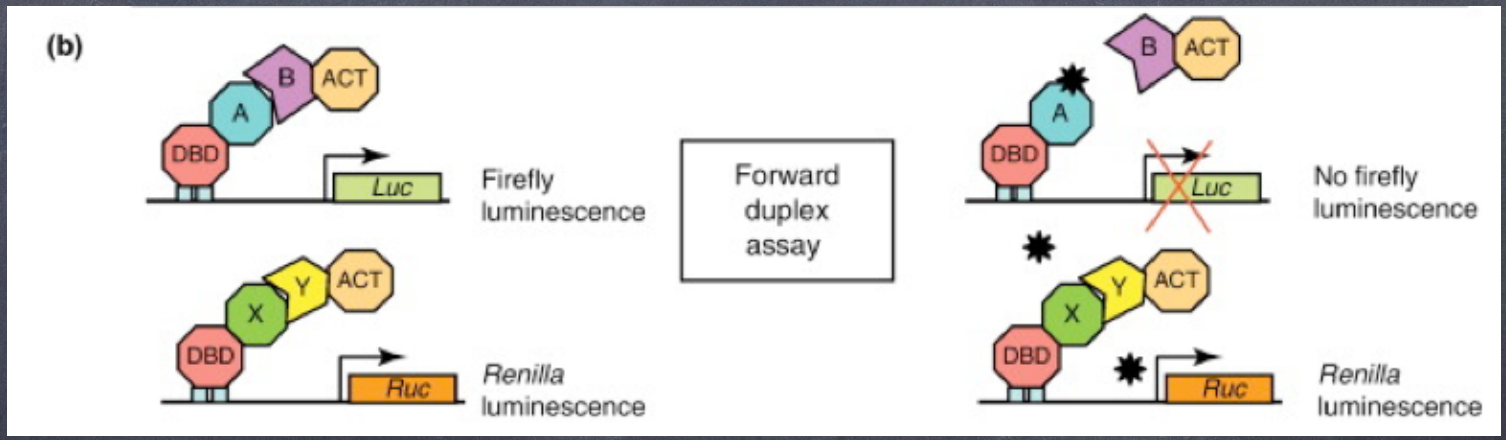
Phenotype

Phenotype

Y2H applications to drug discovery

detection of protein-protein interactions

detection of inhibition of protein interactions by small molecules



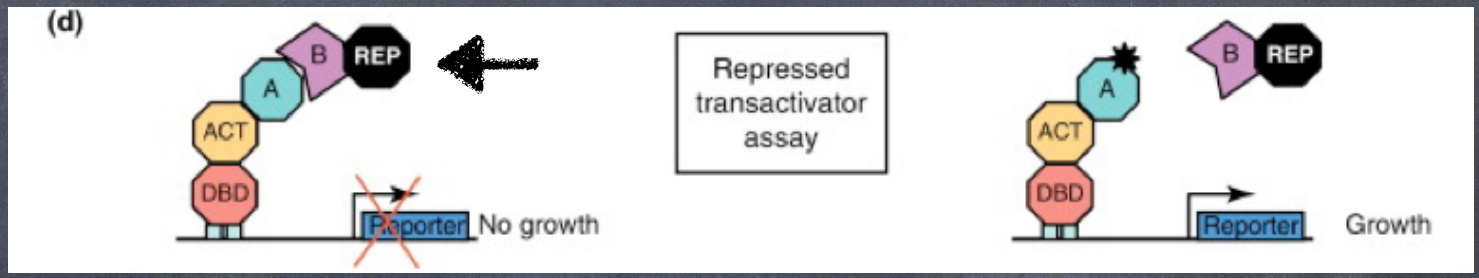
Phenotypes

Phenotypes

Y2H applications to drug discovery

detection of protein-protein interactions

detection of inhibition of protein interactions by small molecules



Phenotype

Phenotype

Opened-questions

- Scientific question that can be addressed using co-IP technique and flowchart of the technique
- Scientific questions that requires Tagged-proteins and flowchart of production and use
- Scientific question that can be addressed using Y-2H and Y-3H techniques and flowchart of the technique

In vitro site-directed mutagenesis

starting materials and aim

Site-directed mutagenesis* studies can be extremely useful for:

- elucidating the function of a gene or protein,
- creating variants of an enzyme with new and improved functions
- screening a variety of mutants to determine the optimal sequence for addressing the question at hand.

There are now many approaches available for generating site-directed mutants which are all based on PCR amplification.

(*) also called site-specific mutagenesis or oligonucleotide-directed mutagenesis

Common steps are...

- Design of synthetic oligo encoding desired mutation
- Annealing to target region of wt template DNA - serves as primer for initiation of DNA synthesis in vitro
- Extension of oligo by DNA polymerase generates dsDNA that carries desired mutation
- Selection of mutated DNA
- Subcloning in expression vector
- Transfection and expression of the mutated protein

Technique 1
Traditional PCR with modified primers for site-directed mutagenesis

Description:
This type of site-directed mutagenesis uses PCR primers designed to contain the desired change. The PCR primer sequence simply replaces the original sequence - as long as the changes are minimal enough to allow the primer to anneal to the intended target.

Use for:
Limited base identity changes at the end of the target sequence 5′ or 3′ terminal insertions <100 bases

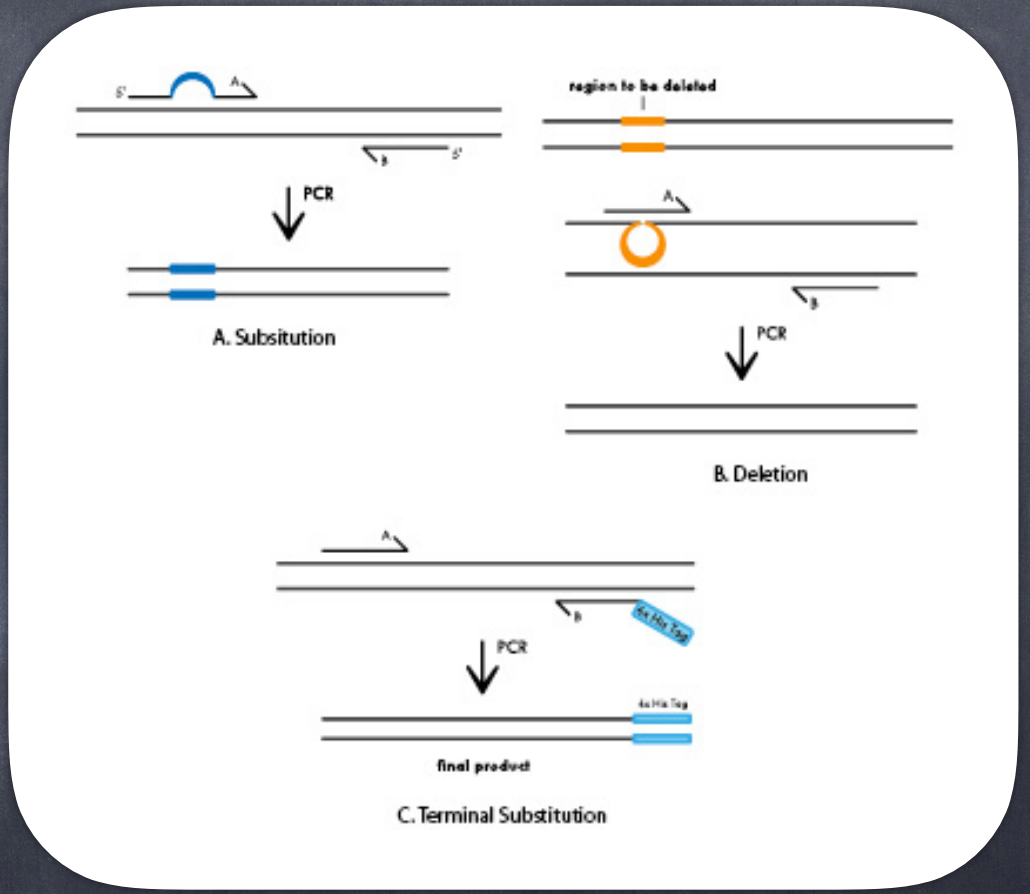
Technique 1
Traditional PCR with modified primers

Primers incorporating the desired base changes are used in PCR. As the primers are extended, the mutation is created in the resulting amplicon.

Primers are designed to include the desired change, which could be base substitution, addition, or deletion.

During PCR, the mutation is incorporated into the amplicon, replacing the original sequence. Mutations introduced by PCR can only be incorporated into regions of sequence complementary to the primers and not regions between the primers.

Technique 1:
Traditional PCR with modified primers



Technique 2: Primer extension PCR for site-directed mutagenesis

Primer extension uses nested primers to mutate a target region.

Use for:

Limited, non-random base changes internal to the target sequence

Insertions >100 bases

Deletions < 50 bases

Deletions > 50 bases**

Technique 2: Primer extension with nested primers

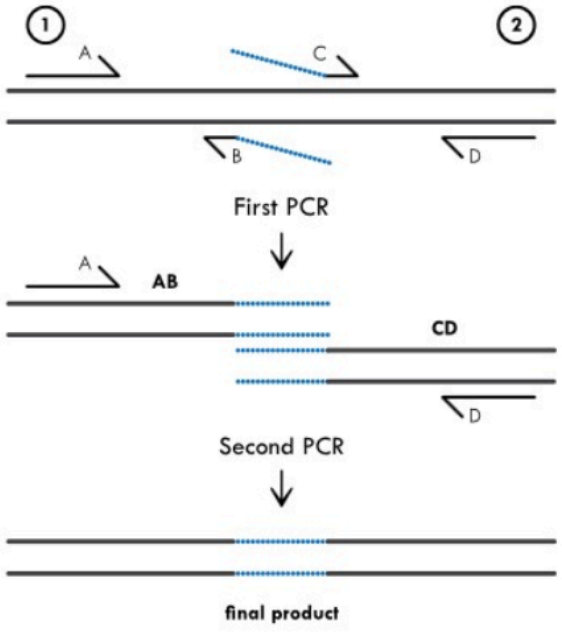
Description:

Primer extension uses nested primers to mutate a target region. In the diagram, primers B and C contain the mis-matched sequence to insert bases.

- The first round of PCR uses primers A-B and C-D to create two products with the mutated sequence. The second PCR round is where the smart stuff happens and the new sequence is created. Since primers B and C contain complementary sequences, the products from the first round will hybridize after they are denatured following the first PCR cycle.

- Primers A-D can then be used to amplify the full-length product that contains the desired mutation. Alterations to this method can also create deletions or longer additions.

Technique 2: Primer extension



Technique 3: Inverse PCR for site-directed mutagenesis

Inverse PCR enables amplification of a region of unknown sequence using primers oriented in the reverse direction. An adaptation of this method can be used to introduce mutations in previously cloned sequences. Using primers incorporating the desired change, an entire circular plasmid is amplified to delete, change, or insert the desired sequence.

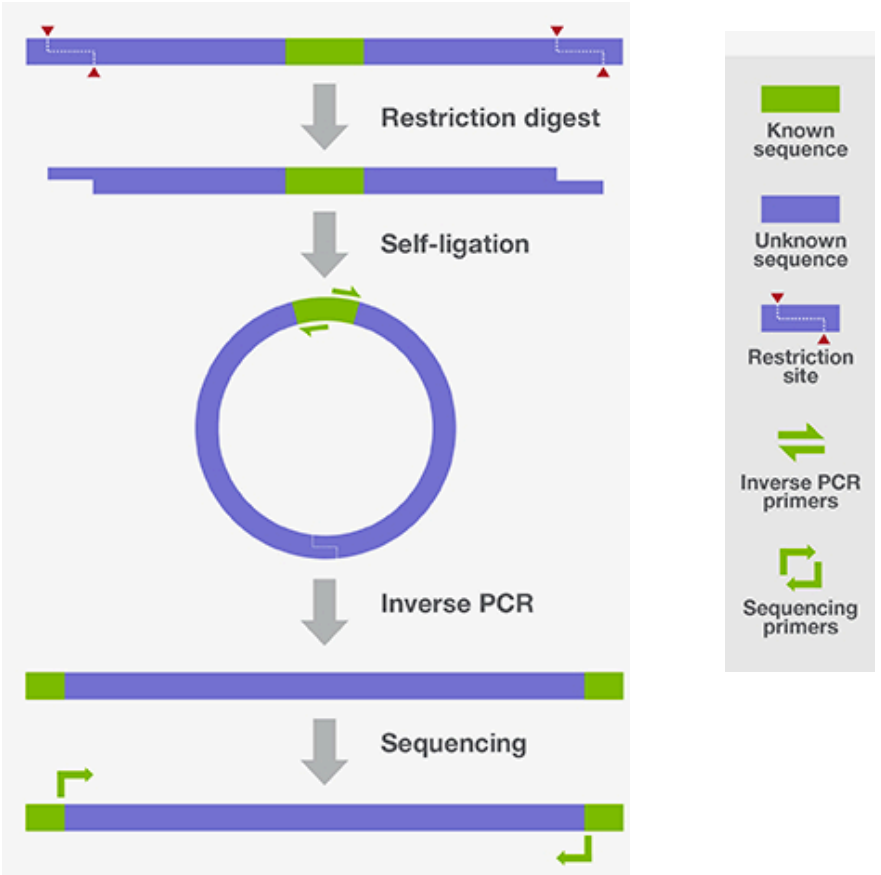
Principle of inverse PCR

Inverse PCR enables amplification of a region of unknown sequence using primers oriented in the reverse direction.

Inverse PCR (IPCR) was designed for amplifying anonymous flanking genomic DNA regions

Principle of inverse PCR

The technique involves the digestion of source DNA, circulation of restriction fragments, and amplification using oligonucleotides that prime the DNA synthesis directed away from the core region of a known sequence, i.e., opposite of the direction of primers used in normal or standard PCR



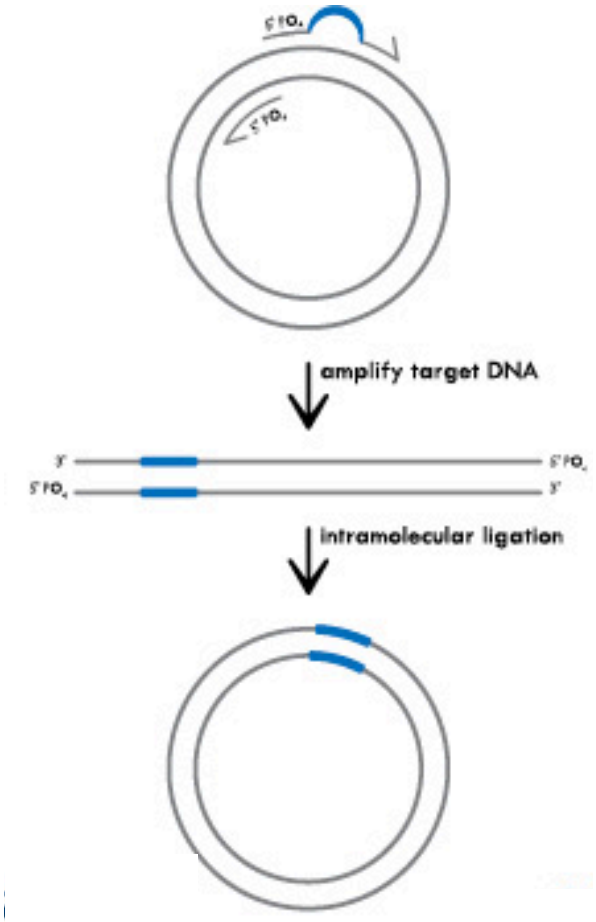
Technique 3:
Inverse PCR for site-directed mutagenesis

A high fidelity DNA polymerase that creates blunt-ended products is used for the PCR to produce a linearised fragment with the desired mutation, which is then recircularised by intramolecular ligation.

The primers used are 5'-phosphorylated to allow ligation of the amplicon ends after PCR.

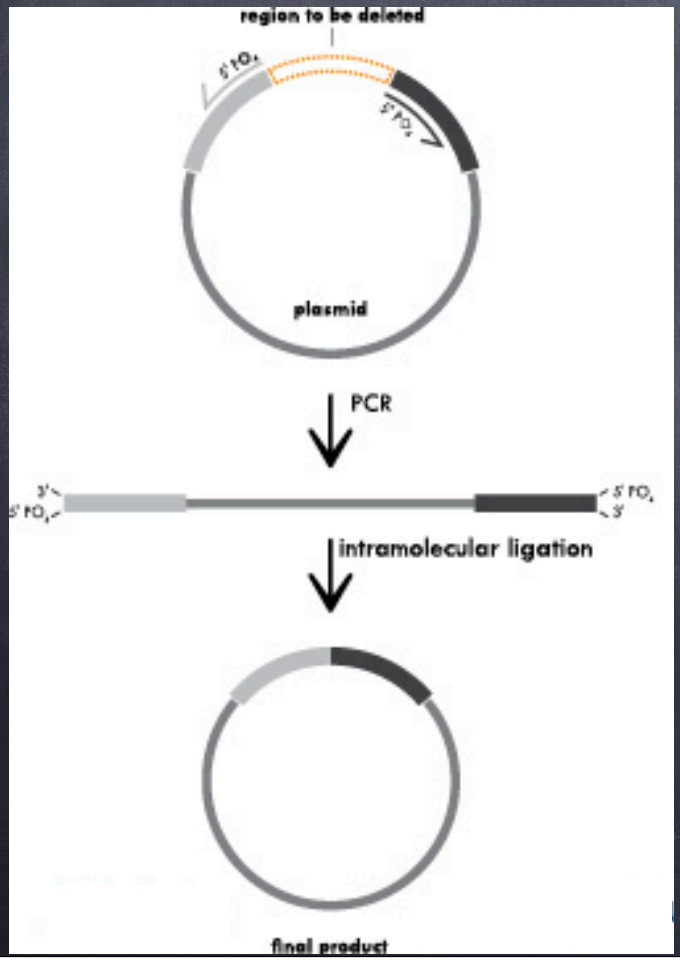
Technique 3:
Inverse PCR for site-directed mutagenesis

The primers used are 5'-phosphorylated to allow ligation of the amplicon ends after PCR. A high fidelity DNA polymerase that creates blunt-ended products is used for the PCR to produce a linearized fragment with the desired mutation, which is then recircularized by intramolecular ligation.

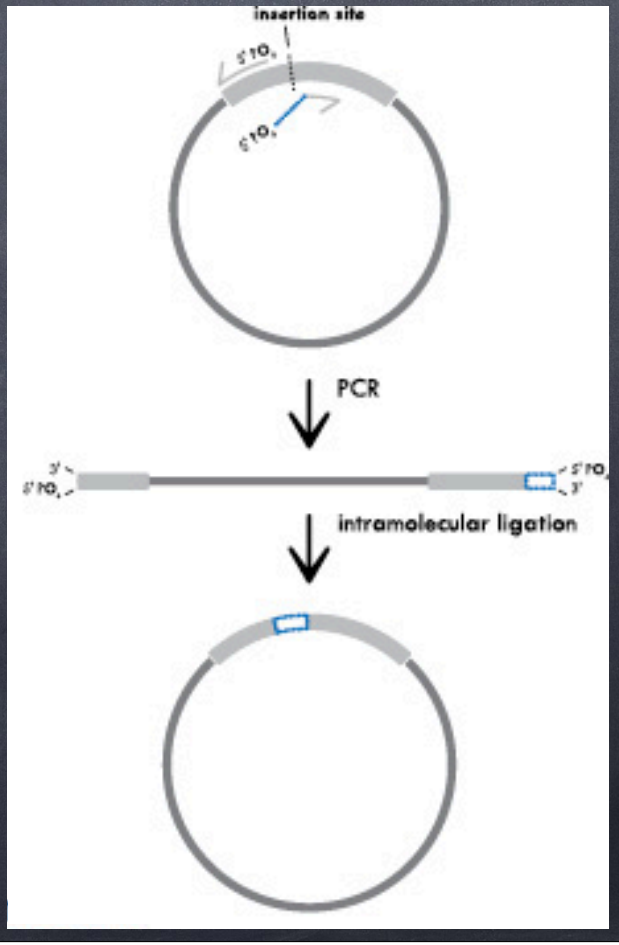


Technique 3:
Inverse PCR for site-directed mutagenesis

Primers that hybridise to regions on either side of the area to be deleted are used.



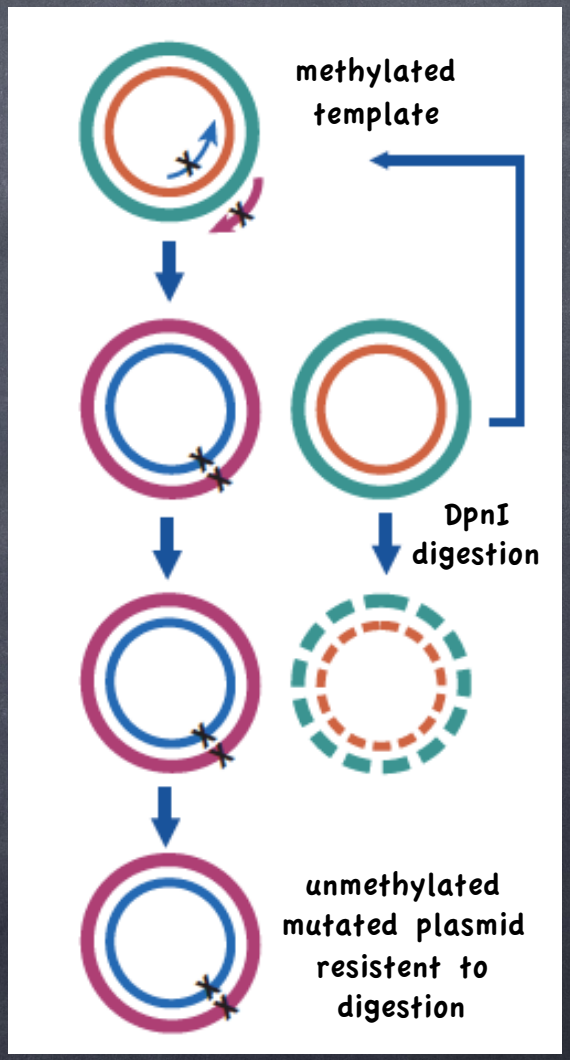
One primer contains the additional sequence that will be inserted (blue line).



Technique 3: Inverse PCR for site-directed mutagenesis

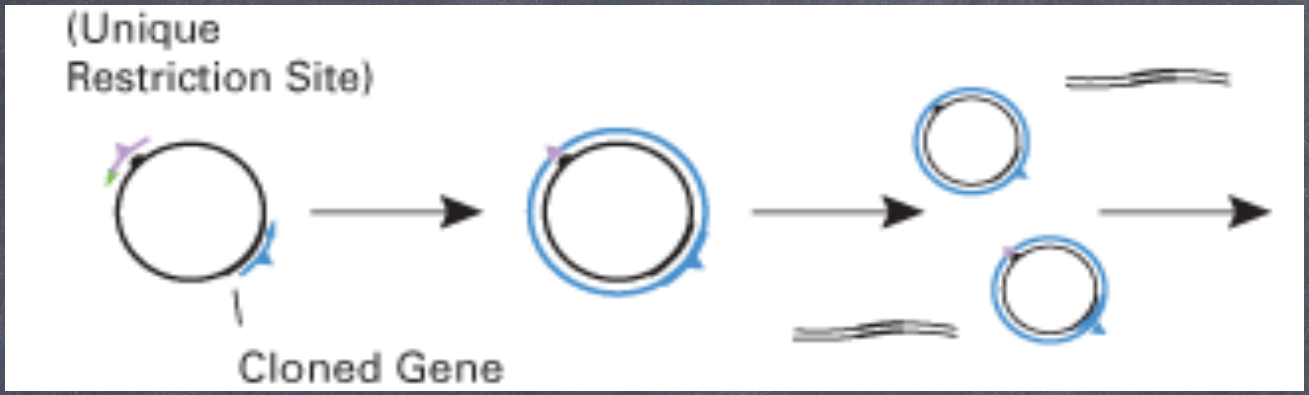
Mutated-plasmid selection

Digest parental methylated and hemimethylated DNA



From Stratagene

Use a selection primer to suppress a unique restriction site on the plasmid

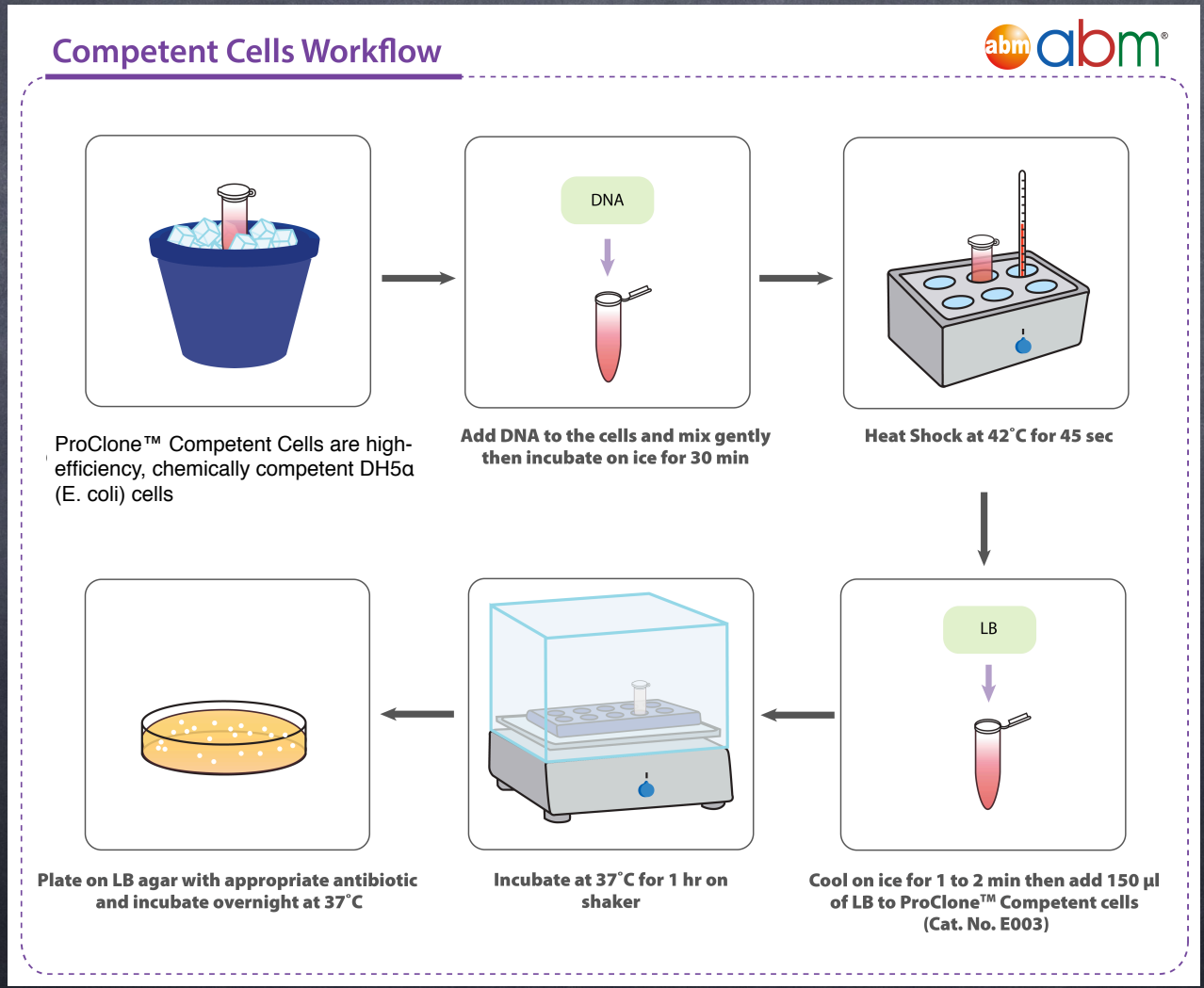


The Transformer Kit uses two oligonucleotide primers which are simultaneously annealed to one strand of a denatured double-stranded template. One primer introduces the desired mutation and the other mutates the unique restriction site in the plasmid, creating a new restriction site or eliminating the site completely. Elongation by T4 DNA polymerase, which lacks strand displacement activity, results in the incorporation of both mutations in the same newly synthesized strand. The DNA is then digested with a restriction enzyme that cuts at the original restriction site. The uncut, mutated DNA will transform E. coli more efficiently than the linear DNA with no mutations.

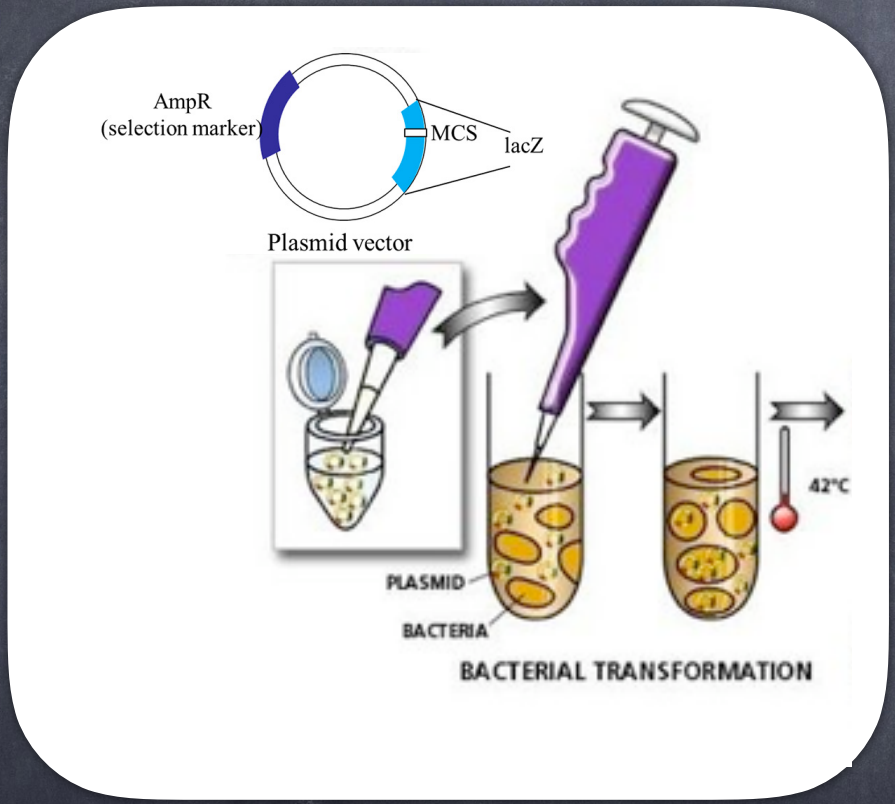
(from Clontech)

Next step following recombinant vector production?

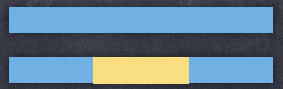
Bacterial transformation



Antibiotic selection



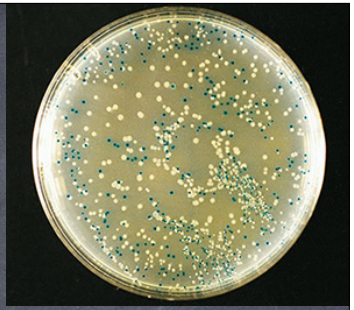
Ampicillin



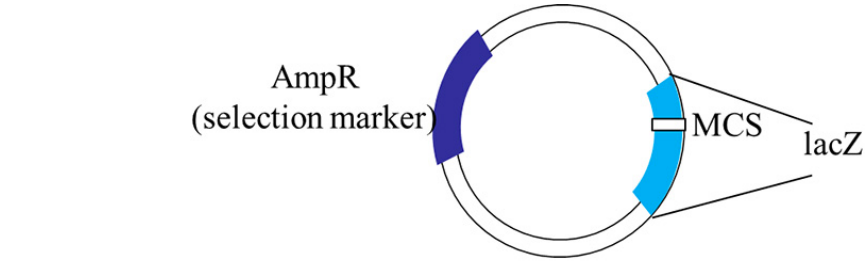
Growth of transformed colonies (with plasmid)

- plasmid w/o insert
- plasmid with insert

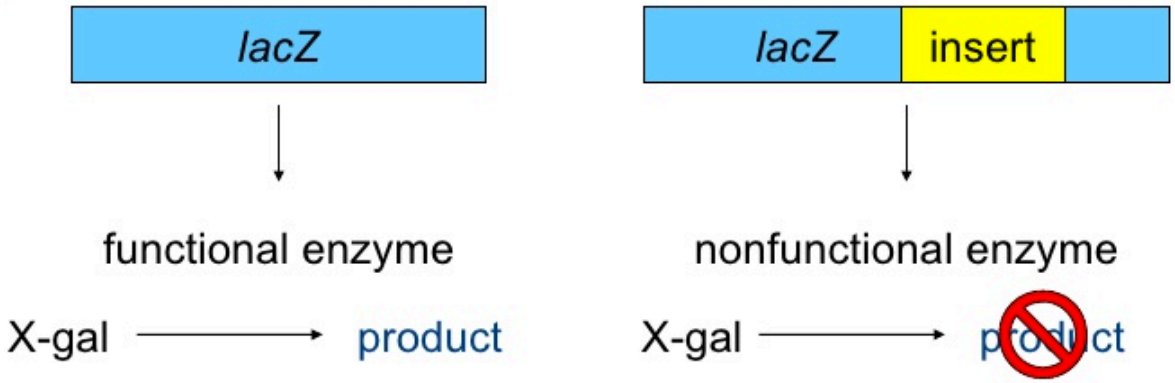
Blue-white color screening



select white colonies



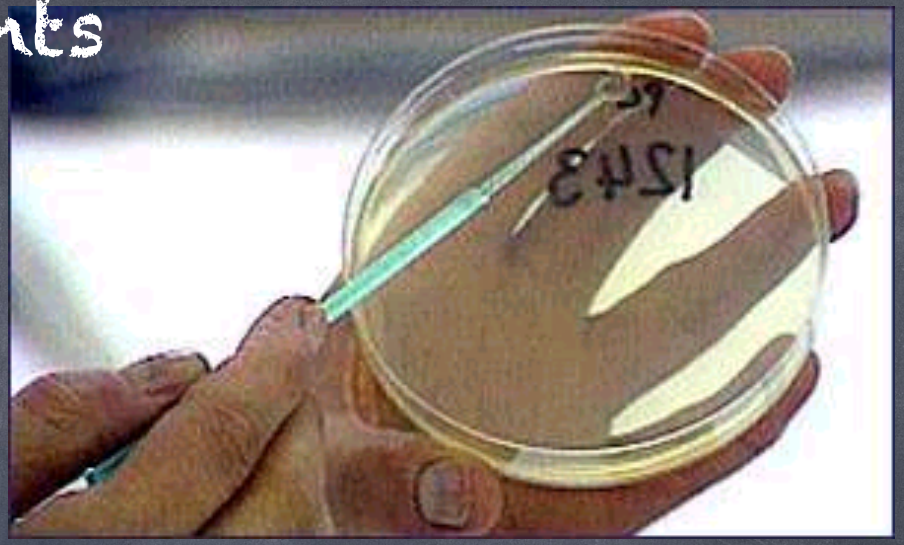
Plasmid vector



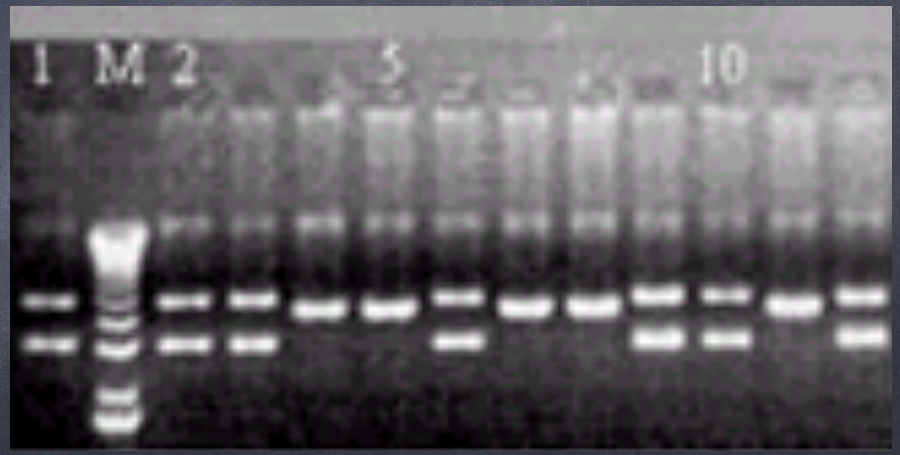
Transformed colonies w/o insert

Transformed colonies with insert

Analysis of Mutants



Miniprep and enzymatic digestion



Opened-questions

- Explain experimental results obtained using IP or co-IP techniques, with or W/O Tag-proteins, Y-2H and site-directed mutagenesis

- Choose among IP, co-IP, tagged-proteins, Y-2H and site-directed mutagenesis the appropriate approach to address a specific experimental question, defending your choice

Assignment for next Wednesday:

Regulators	Differences in ERK activity	Cellular responses
Temporal regulators PKC Rap1 Sprouty	Activity Sustained ERK activation Time Transient ERK activation	Differentiation PC12 cells Proliferation

Is the difference in the kinetics due to the rate of internalisation of the receptor?

How would you proceed to answer this question?