Preparation and characterisation of Eukaryotic macromolecular complexes

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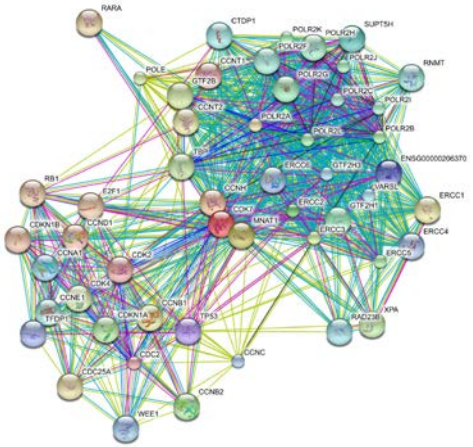
Preparation and characterisation of Eukaryotic macromolecular complexes

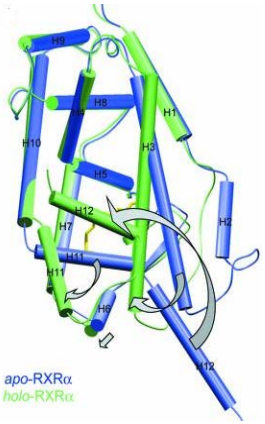
Contribution of the baculovirus expression system for reconstitution of multiprotein complexes and dissection of the protein-protein interaction network

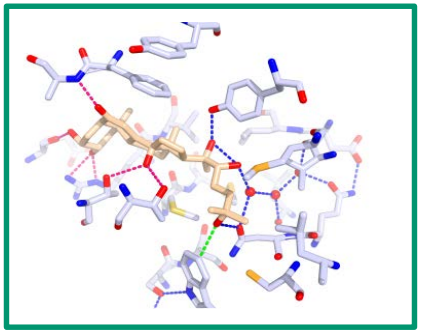
Potential inputs from genome engineering approaches for labelling mammalian proteins to facilitate isolation of endogenous complexes and their characterization in a cellular environment

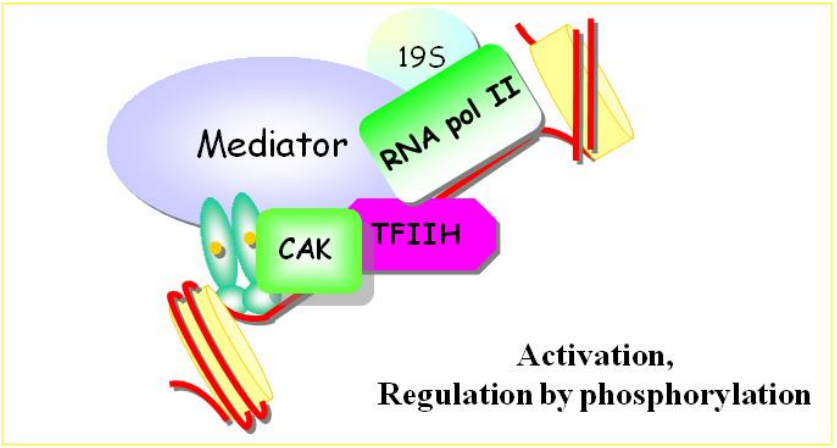
Most proteins do not function as isolated particules……

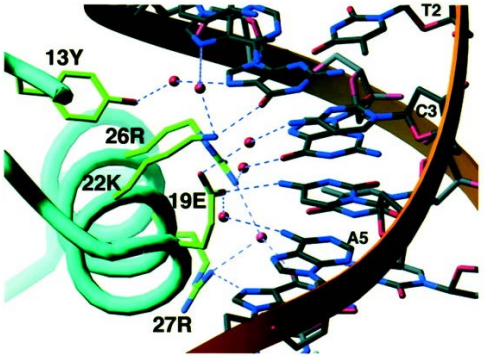
…. but interact with partners to fullfill their fonction.



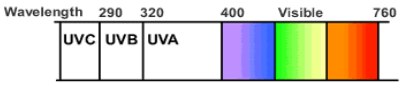


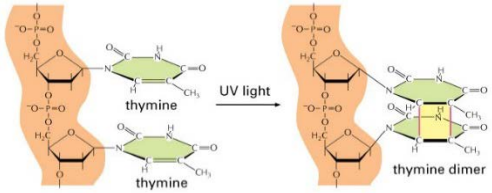


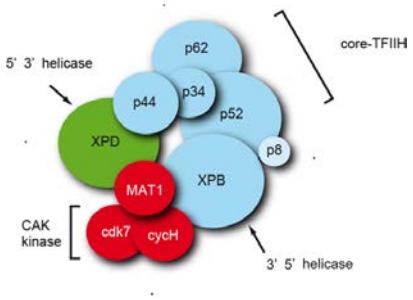


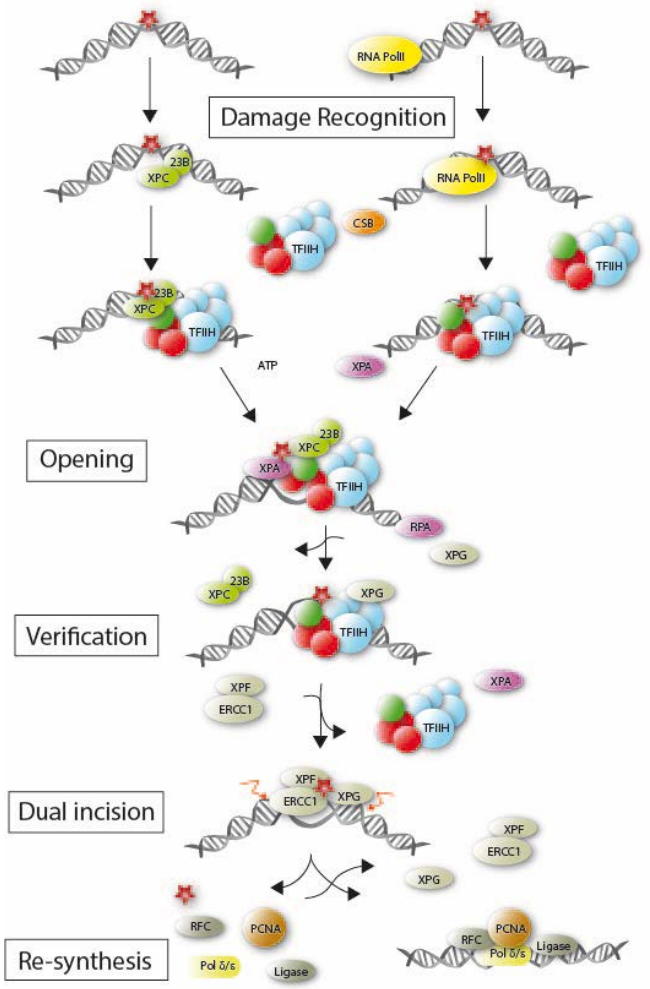


Components of the Nucleotide Excision Repair pathway









- XPA, encodes a protein that binds the damaged site and helps assemble the other proteins needed for NER.
- XPB and XPD, which are part of TFIIH, a 10 subunit complex
- XPF, with ERCC1 cuts the backbone on the 5' side of the damage
- XPG, which cuts the backbone on the 3' side.
- XPC interacts with HR23B in GGR and recognizes damage
- VPV is a by-pass polymerase

6 out 8 XP gene are part of obligate multi-protein complexes
Transiently assemble on damaged DNA to excise the lesion and repair

Types of complexes

Composition and structure

Protein-protein, protein-nucleic acid, protein-ligand

Homo- and hetero oligomeric complexes

Non obligate and obligate

Protomers are not found as stable structures in vivo

Subunits exist independantly

Lifetime of complexes

Permanant interactions: stable/only exist in complexed state

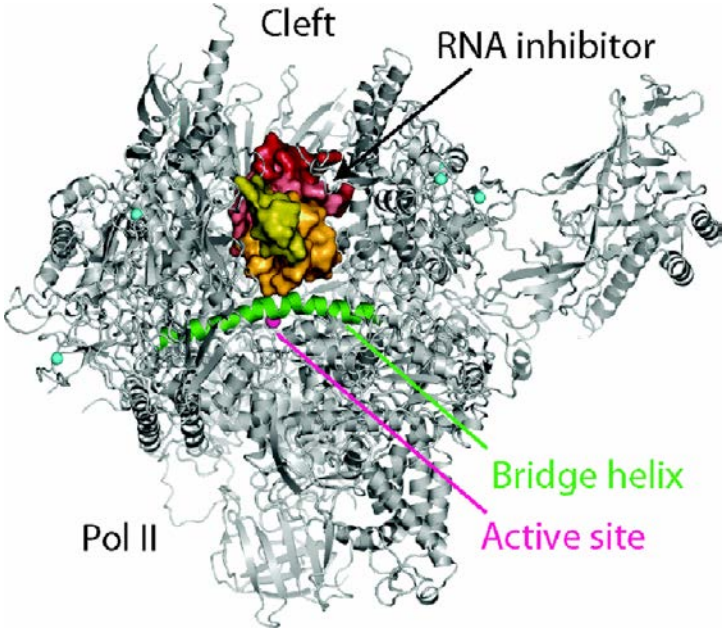
Transient interactions associate and dissociate in vivo
- weak: dynamic equilibrium in solution
- strong: molecular trigger to switch on and off

Subunits of RNA Pol II

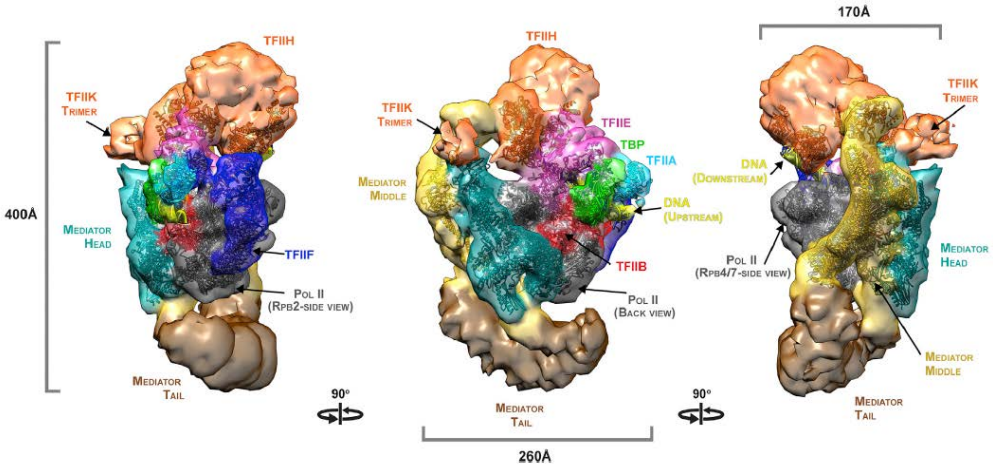
Obligate and non-obligate

Protomers are not found as stable structures in vivo

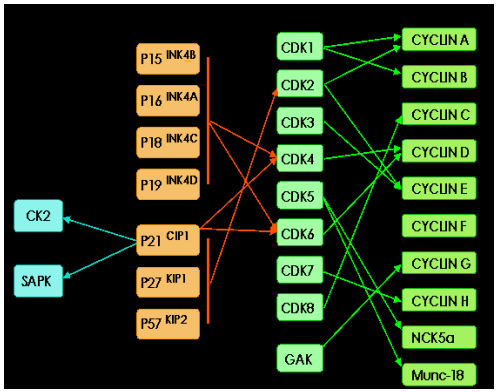
Protomers exist independantly



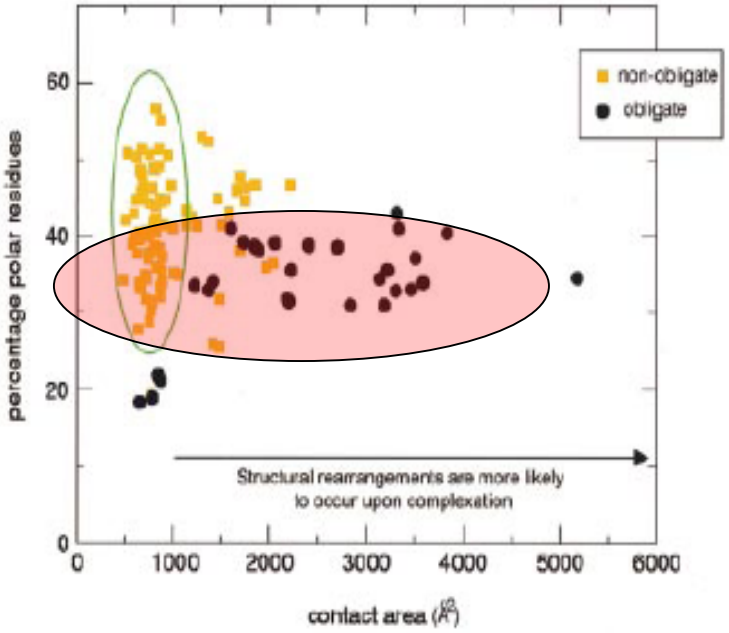
Transcription regulation complexes

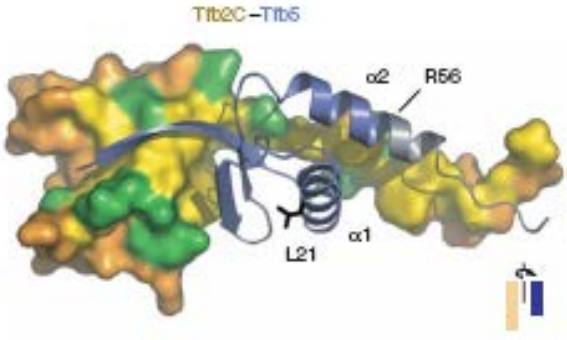


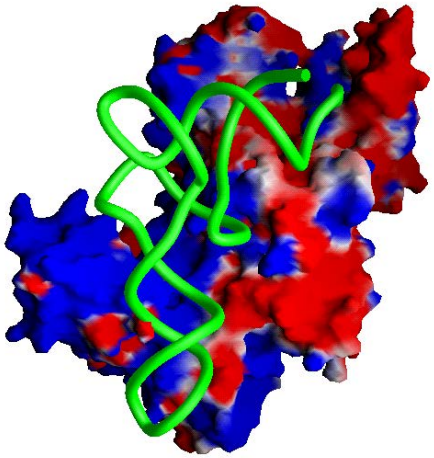
Cdk/Cyclin/Inhibitor



Structural characteristics of interfaces



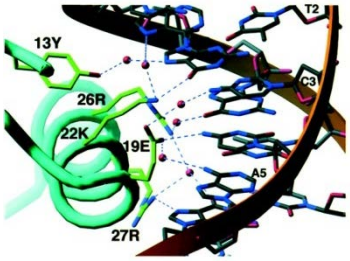


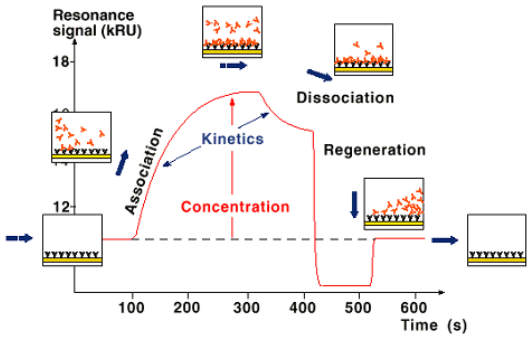


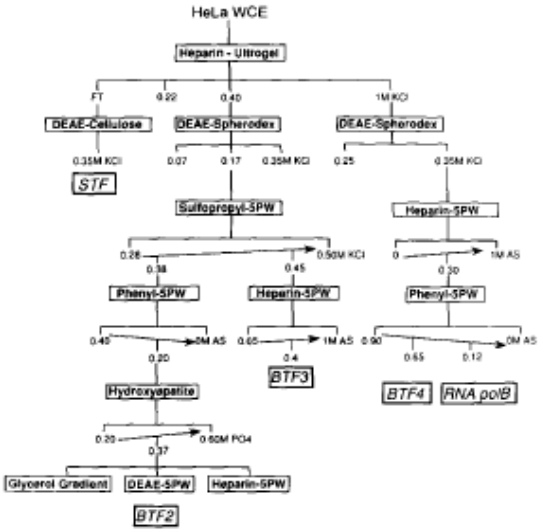
Contact area and polarity of various non obligate and obligate complexes. Thevertical ellipse denotes the aera-polarity space of weak transient interactions

The interfaces in obligate complexes) are generally larger and more hydrophobic than non-obligate associations.

Types of complexes







Lifetime of complexes

Permanant interactions: stable/only exist in complexed state: operational definition: that can be purified

Transient interactions associate and dissociate in vivo

- weak: dynamic equilibrium in solution
- strong: molecular trigger to switch on and off

Weak

(Tx, DNA repair electron transport complexes)

K_d mM-μM

Intermediate

(antibody-antigen, TCR-MHC-peptide, signal transduction PPI), K_d μM-nM

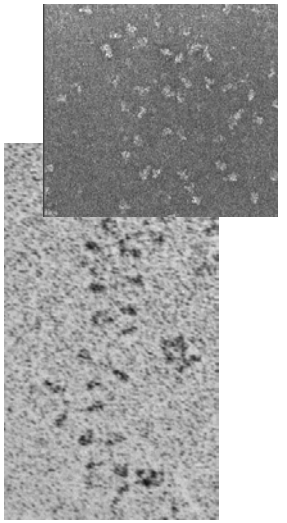
Strong

(require a molecular trigger to shift the oligomeric equilibrium)

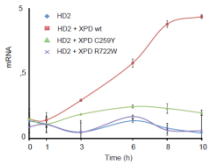
K_d nM-fM

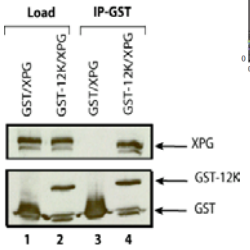
Functional characterization

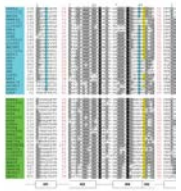
Cellular imaging



Bio computing

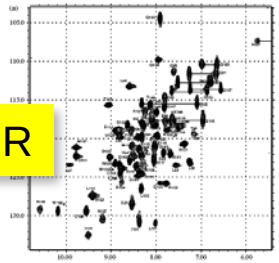






Partner identification

Cryo-EM



NMR

Domain/Orthologs indentification

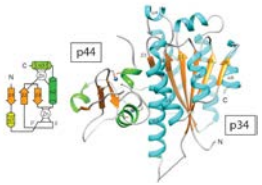
Targets

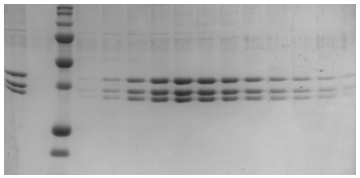
Structure

Production

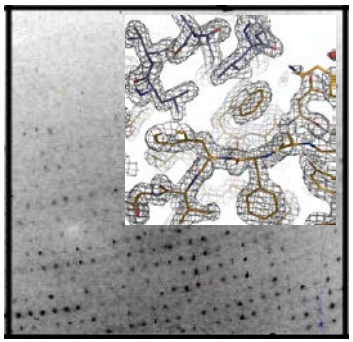
X ray crystallography

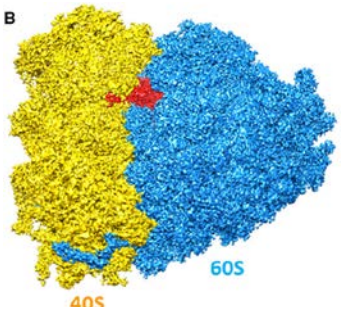
Modelling





Biophysical characterization





Recombinant or endogenous?

Isolate protein from native source

Advantages – Protein solubility, authenticity

Disadvantages – Expense/effort, yield, slaughter-houses
Waring blenders

Popular sources: E coli, yeast, HeLa cells

Bacterial expression system

Advantages – Easy, great over-expression, low protease activity,
no post-translational modifications

Disadvantages – Protein solubility, lack of post-translational modifications

Eukaryotic expression system

Advantages – Protein solubility, post-translational modifications

Disadvantages – Expensive, low yield, proteases, time consuming

Hierarchy: Bacteria, Yeast, SF9, Mammalian

Recombinant expression

Prokaryotic

E. coli,

....

Eukaryotic

Yeast

Insect cells

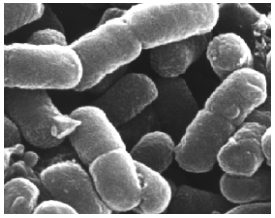
Mammalian

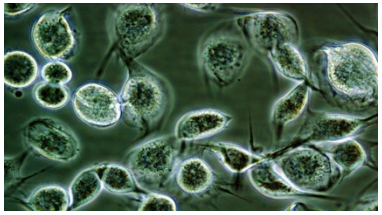
Cell free systems:

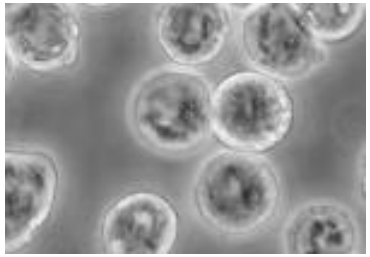
E coli

Wheat germ,

Insect



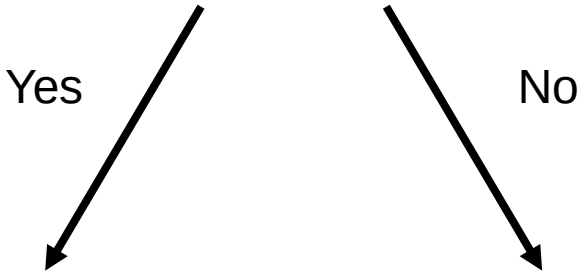




Implications for production

Non-obligatory / Obligatory

Transient / Stable



Produce components independently and reconstitute the complex *in vitro*

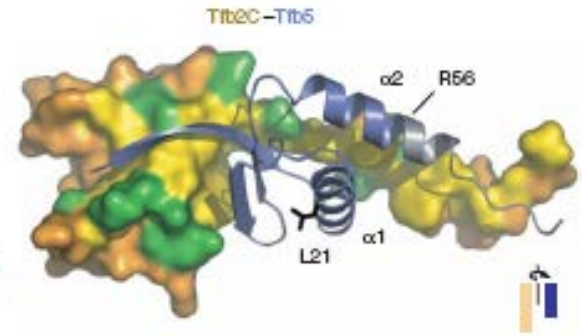
Single gene expression

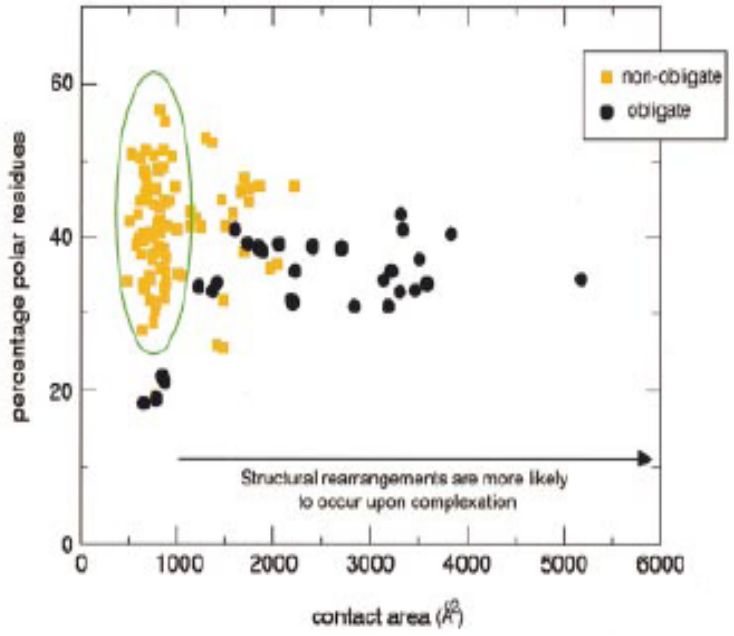
Produce components of the complex simultanously which are assembled *in vivo*

Co-expression

Strategies for production of multi-protein complexes

Separate expression of subunits purify and mix; mix and purify





No always possible. The interfaces in **obligate complexes** beeng generally large and hydrophobic.

Contact area and polarity of various **non obligate** and **obligate** complexes. The vertical ellipse denotes the aera-polarity space of weak transient interactions

Noreen & Thornthon, 2000

Strategies for production of multi-protein complexes

Separate expression of subunits purify and mix; mix and purify

E. coli

Co-transformation with several single promoter plasmids

Transformation with multigene expression plasmid

BVES

Co-infection of insect cells by several viruses

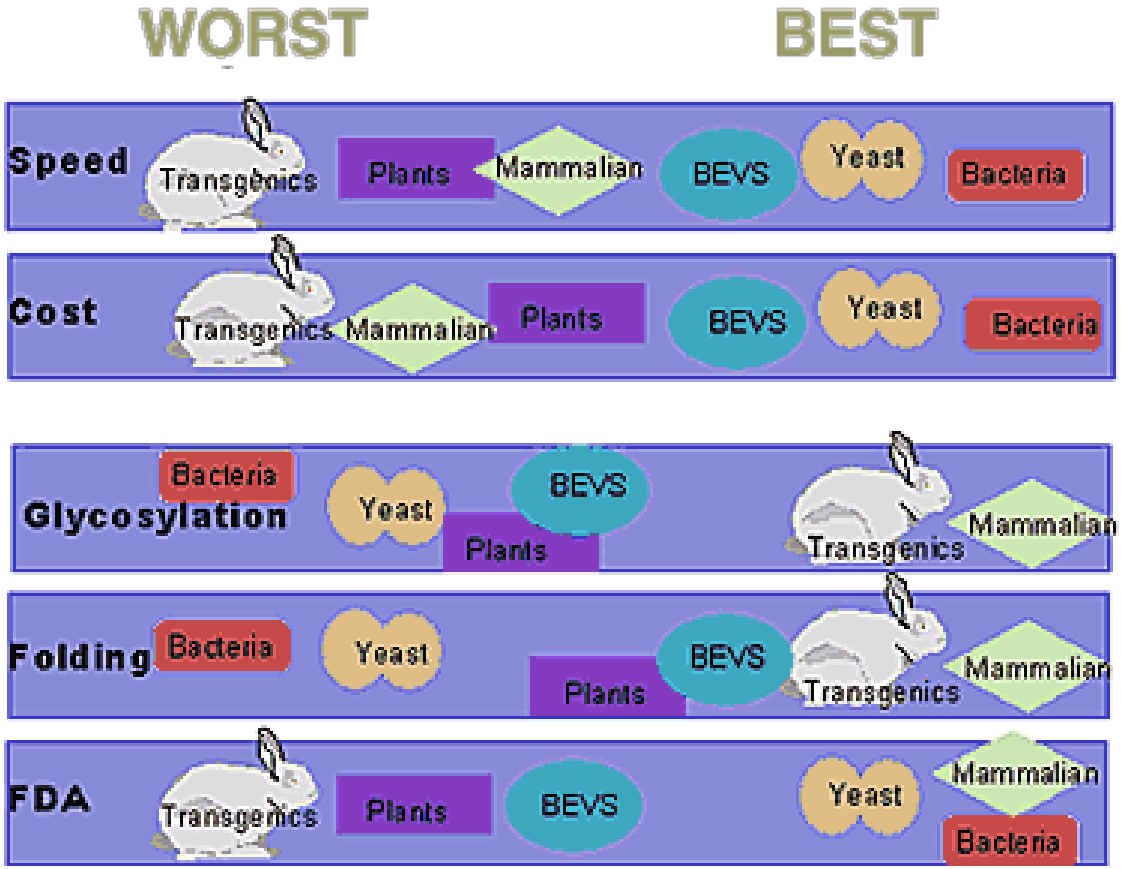
Infection with a multigene expression virus

Which expression system?

Suitable for co-expression

High levels of heterologous gene expression

BEVS



What are baculoviruses ?

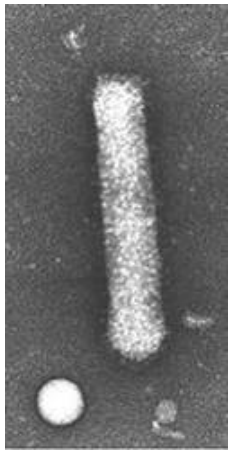
Baculoviruses are a group of viruses that infect specifically insects *

They are rod-shaped (latin baculum = stick), 40-50 nm in diameter and 200-400 nm in lengh

Double stranded , covalently closed and circular DNA (80 – 200 kbp)

Spodoptera frugiperda

Trichoplusia ni



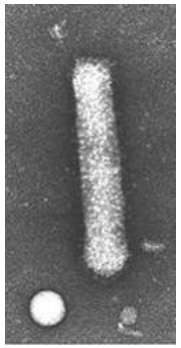
Autographa californica multiple nuclear polyhedrosis virus **(AcMNPV)**

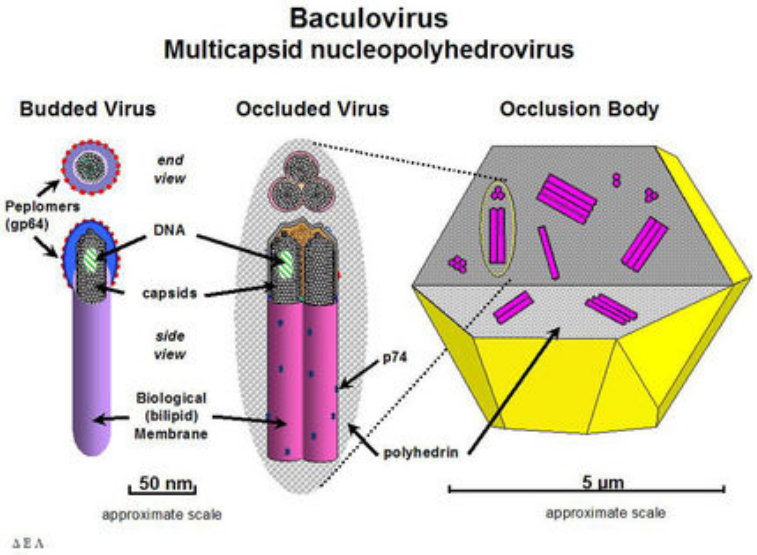
alfalfa looper = cabbage looper

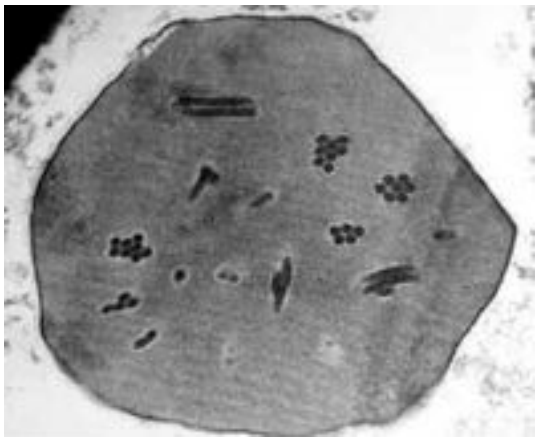
AcMNPV infects 30+ insects

In cell culture or when multiplying within an insect host, baculoviruses
Form so called virions, also referred to as non-occuded or budded virus (BV)

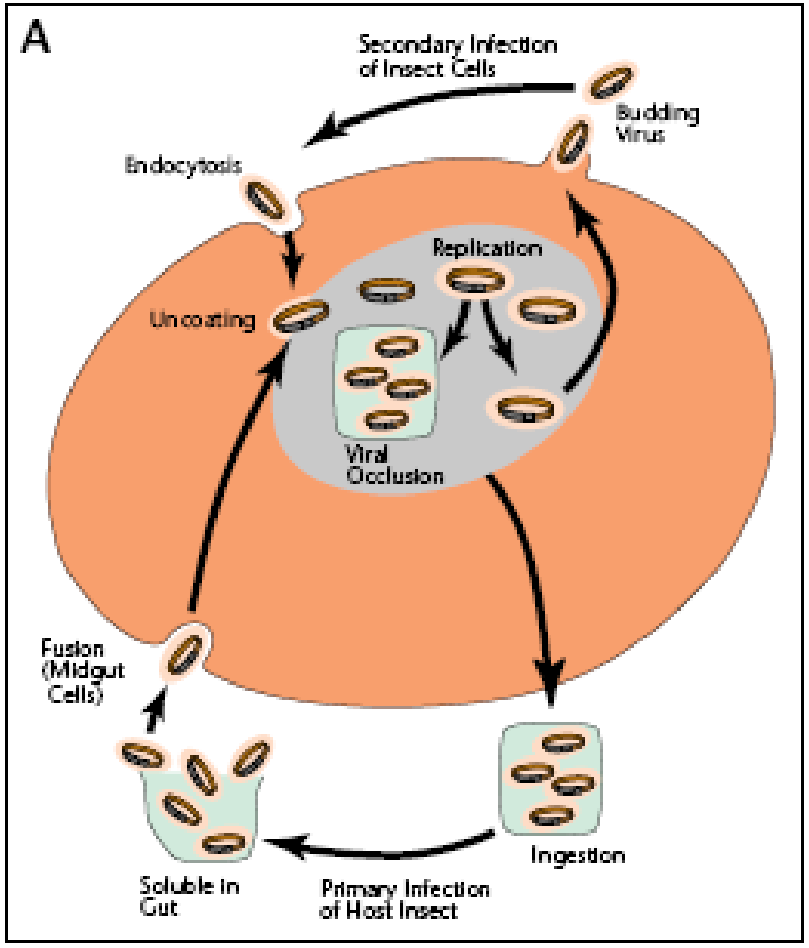
For long-term survival occlusion bodies (OB) or polyhedra are formed.
Para-crystalin matrix, composed of polyhedrin (50% of the total protein mass)

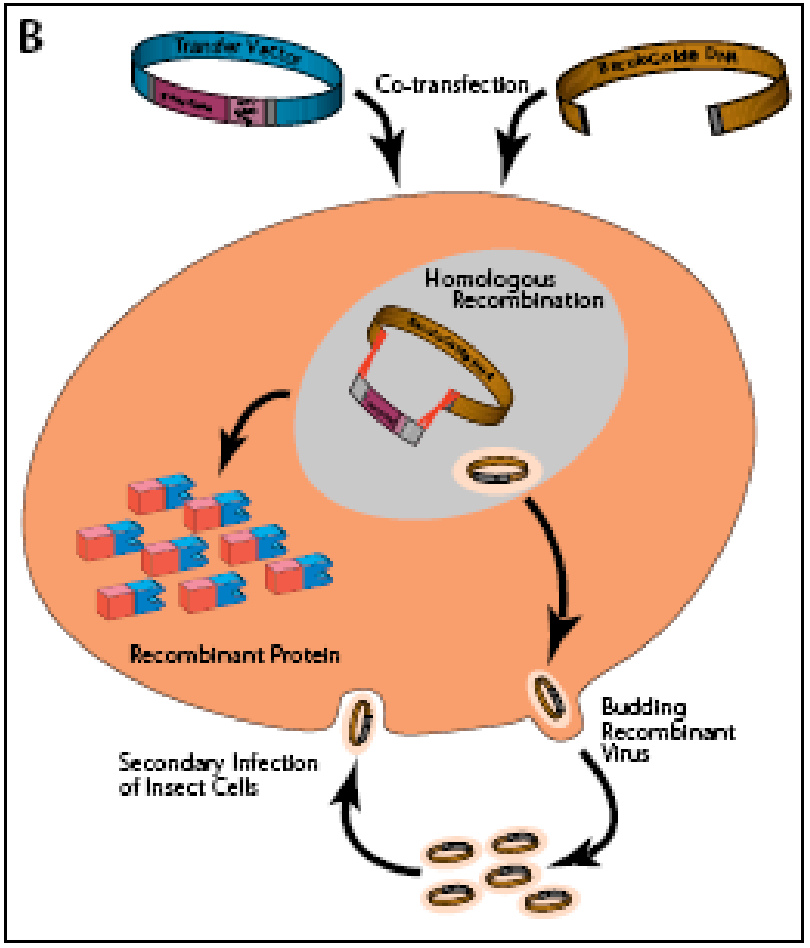






- Strong promoters (Polyhedrin, P10)
- Express lots of protein 36-48 h post-infection
- Don’t need polyhedron “package” in lab
- Replace polyhedrin coding seq. with GOI





What is needed to express a protein ?

The expression unit

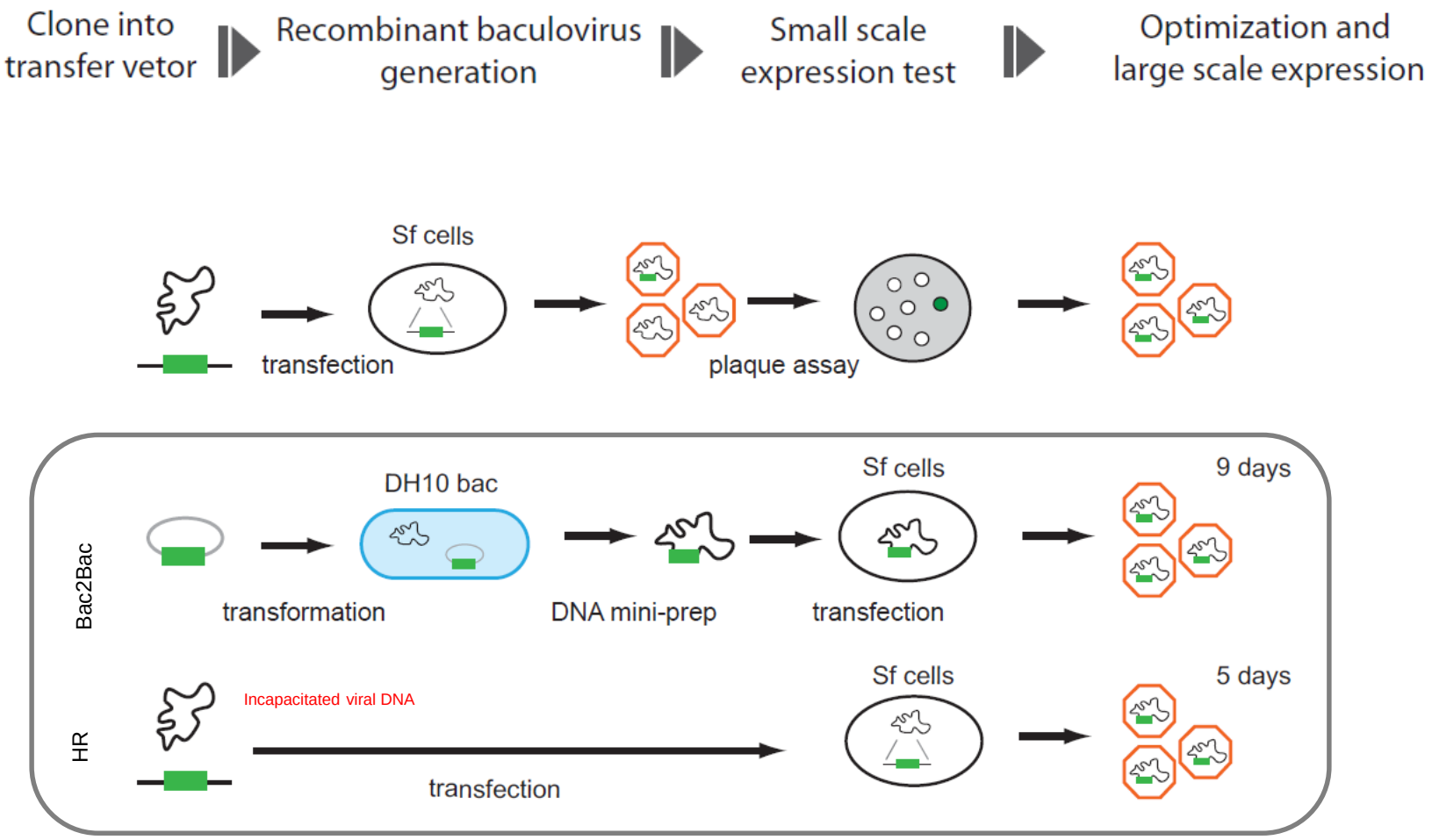
- Strong promoter: PH or p10
- Kozak sequence:
- Gene of interest
- Terminator

On both sides, elements that will allow the integration of the expression unit(s) into the viral genome:

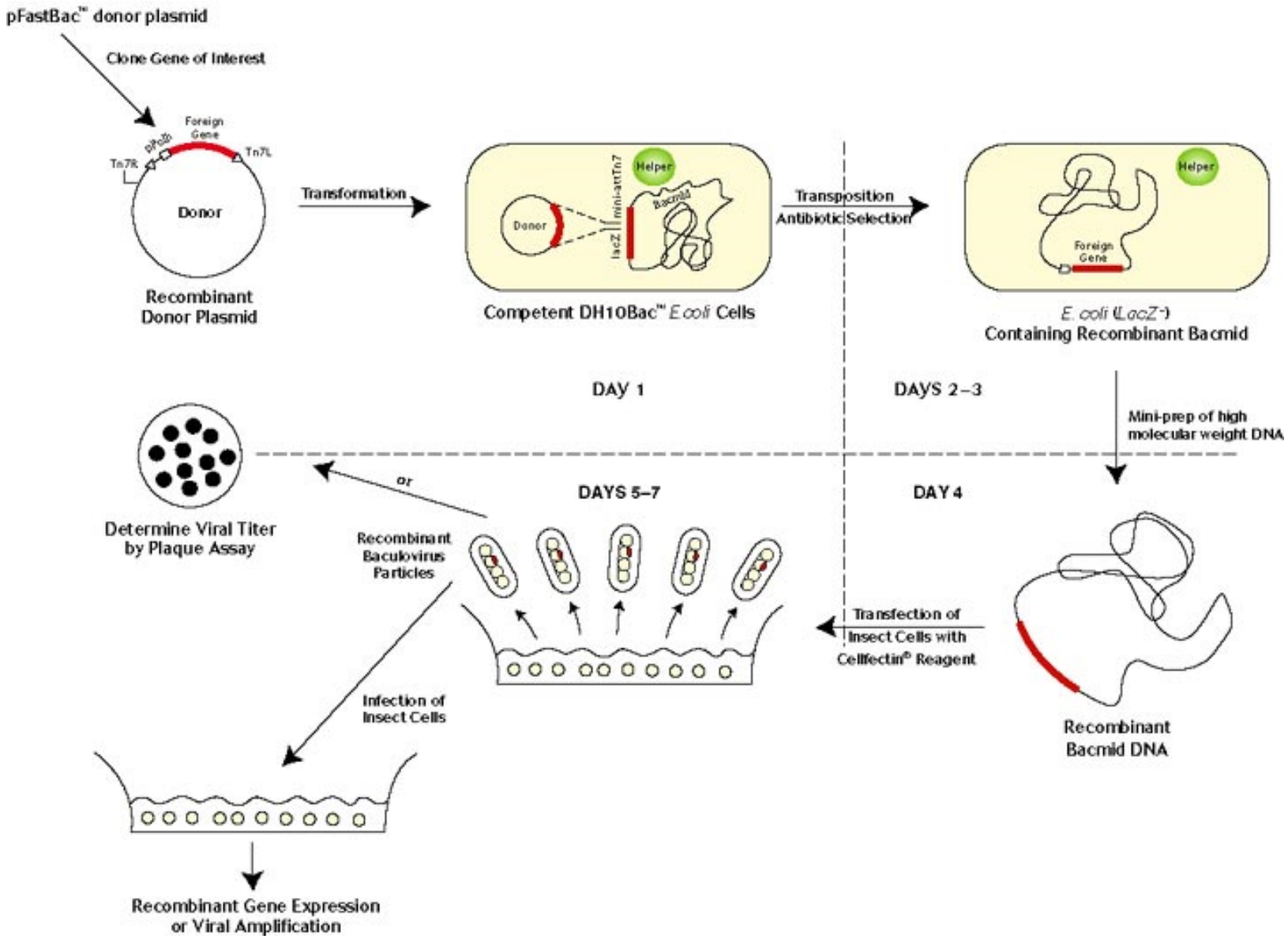
- Either segment from viral genome for homologous recombination in insect cells between the transfert vector and the viral DNA

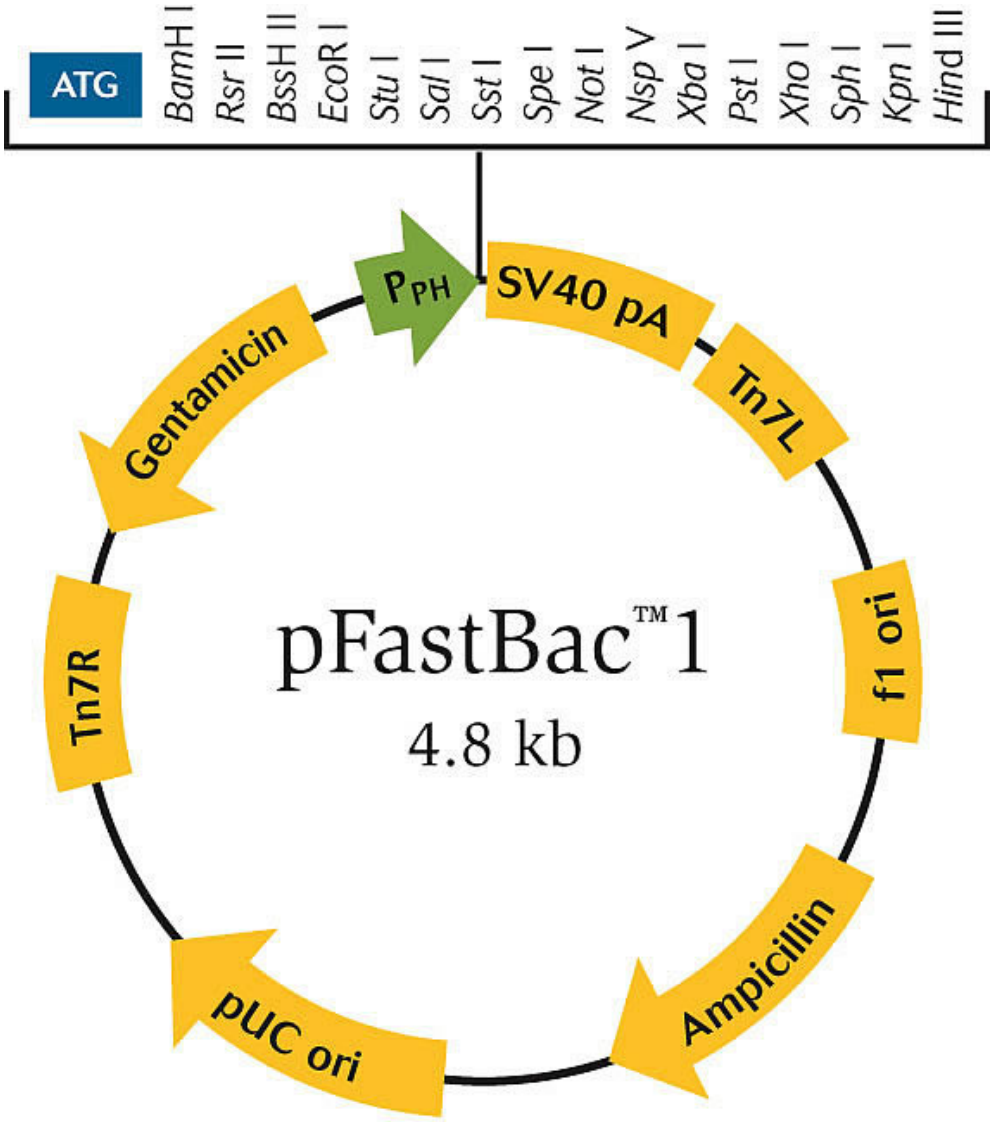
- Or transposons (Tn7L and Tn7R) recombination sites (LoxP) when a bacmid is to be used

An expression flowchart for BV expression

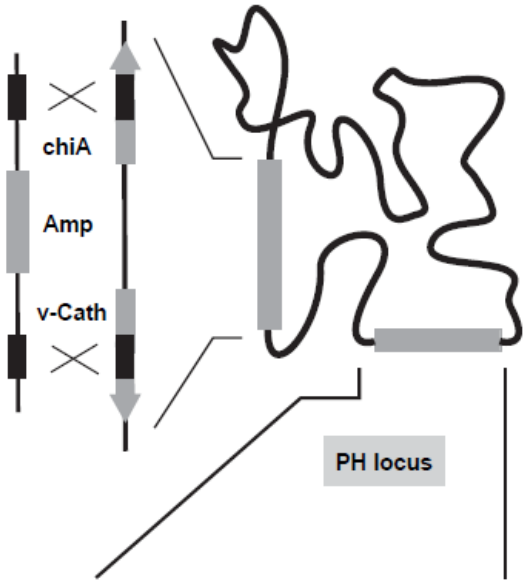


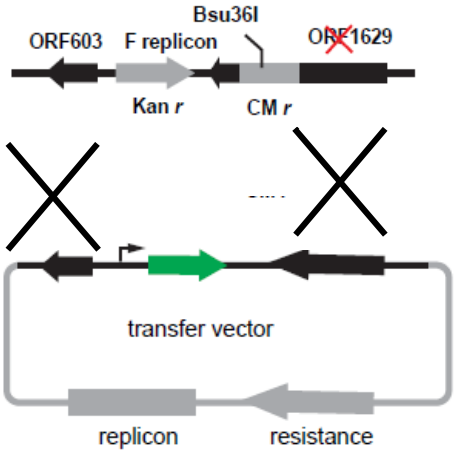
Transposition in E. Coli

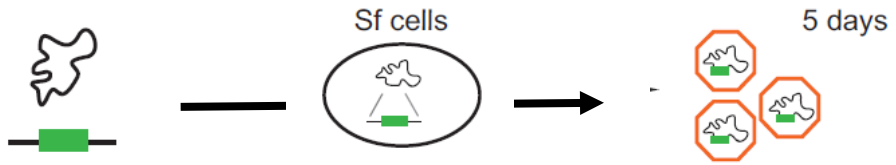



ATG
BamH I
Rsr II
BssH II
EcoR I
Stu I
Sal I
Sst I
Spe I
Not I
Nsp V
Xba I
Pst I
Xho I
Sph I
Kpn I
Hind III
P PH
SV40 pA
Tn7L
f1 ori
Ampicillin
pUC ori
Tn7R
Gentamicin
pFastBac™1
4.8 kb

Homologous recombination in insect cells with disabled viral genome







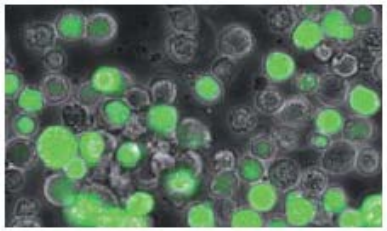
Disabled viral DNA

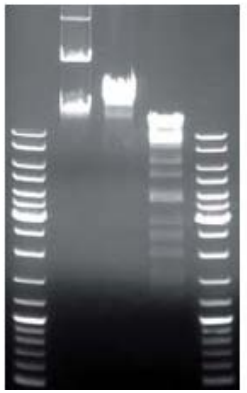
Co-transfection

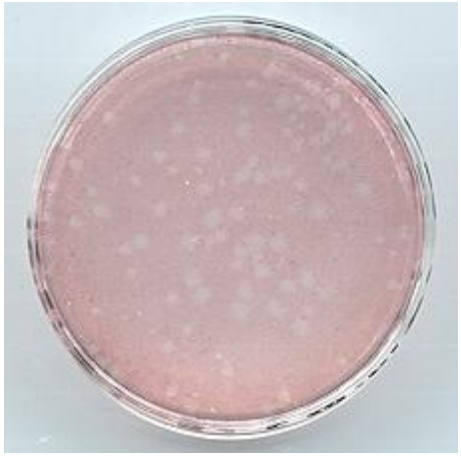
Viable genome can only be formed if the truncation is bridged and repaired by recombination with a suitable transfer vector.

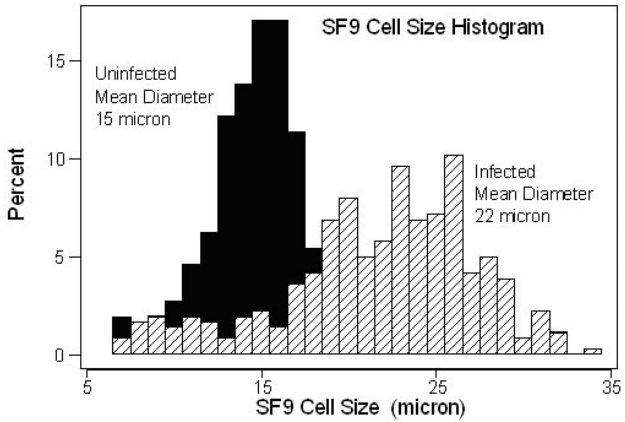
Recombination inserts the foreign gene (GFP) into the viral DNA, restores the deleted gene, allowing virus replication.

No need for plaque selection (screening) for medium size inserts



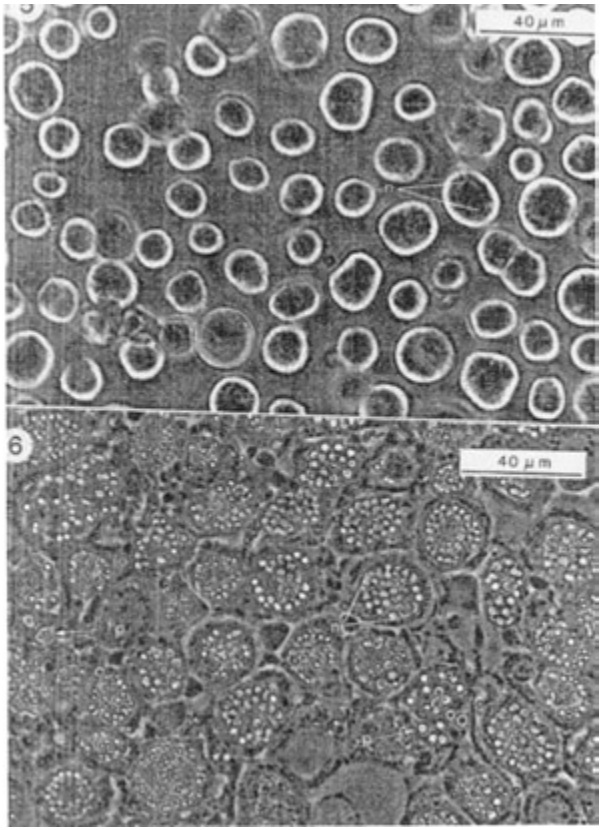






Sf9 non-infected

Sf9 infected



Upscaling and purification issues

For infections cells in exponential growth phase are required.

infect cells a 0.5 to 2.0 $1O^6$ cell/ml
T =27 °C, phosphate based buffer (no CO_2)
monolayers or suspension (Deep Well, Spinner, Bottles..)

Optimization fo the culture conditions

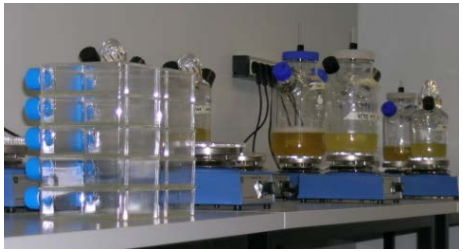
- harvest time post-infection: 48, 72 hrs
- multiplicities of infection: 0.1, 1, 5, 10

Very important for co-infections experiments

- cell line/media of choice:
  Sf9, Sf21, H5
  with or without serum

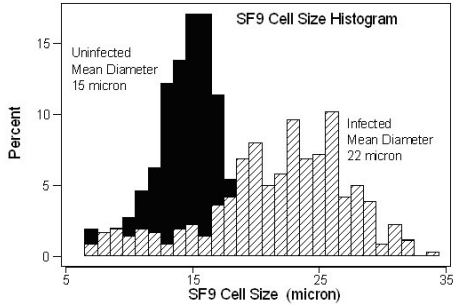
Small scale experiments: 4 ml cultures

Sf9, Sf21 and Hi5



Monolayers
6W, T25, T75, T175; 20 10 6 cells, # 25ml

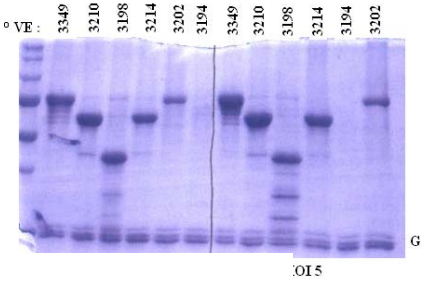
Suspension
Spinners (100-500 ml; 0.8 10 6 cells/ml)
Erlenmeyers (500 ml, 1L; 0.8 10 6 cells/ml)

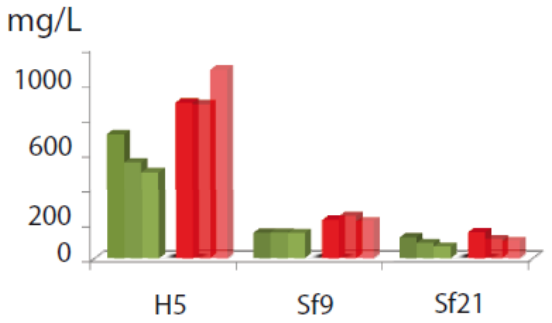


Automated cell counting system: counting and cell size distribution

Lysis
Centrifugation
Affinity Purification

96-wells
(batch purification)





Purification flowchart

Cell lysis: cell wall/plasma membrane,

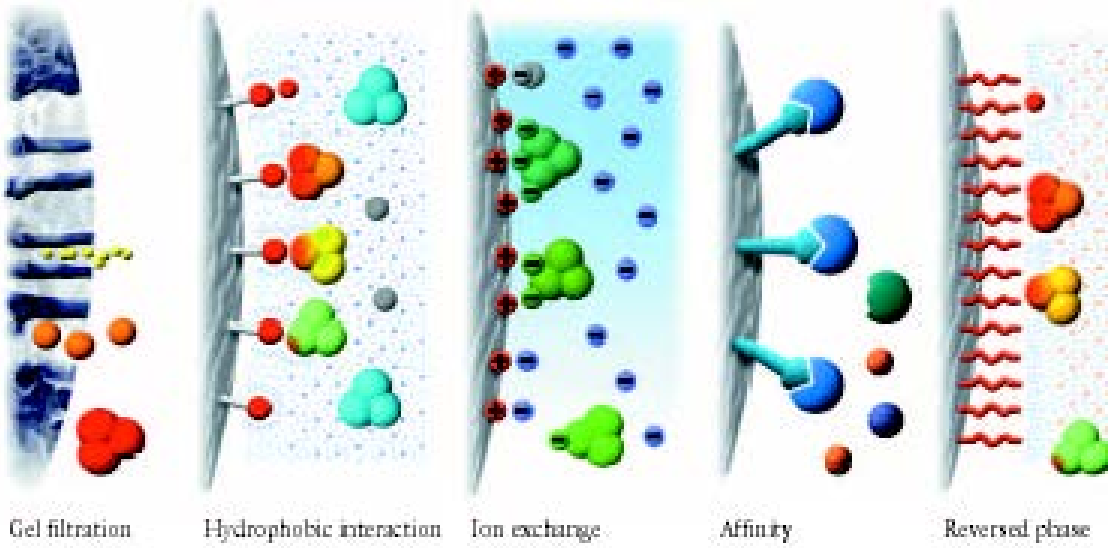
1. Physical means
1'. Sonication
1''. Osmotic shock

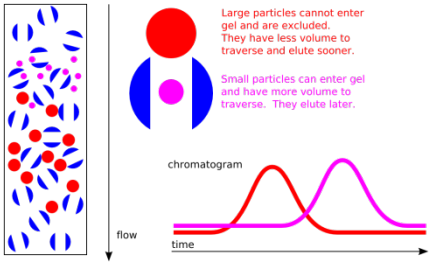
Centrifugation

Chromatographies

1. Affinity/ Specific binding
2. (Ion exchange)
3. Size exclusion

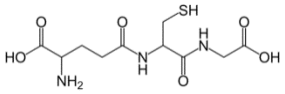
Concentration

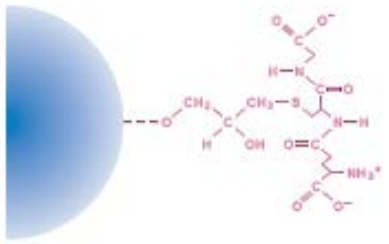


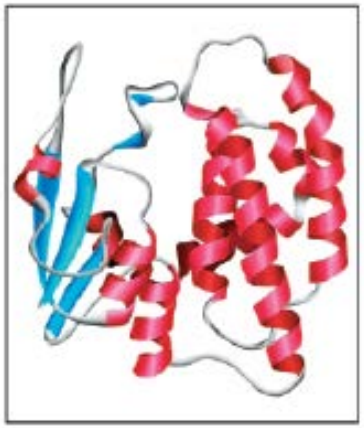


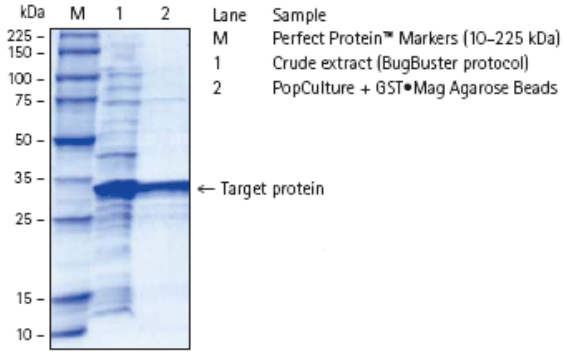
Most widely used tags: His, GST, strep, FLAG

GST-affinity

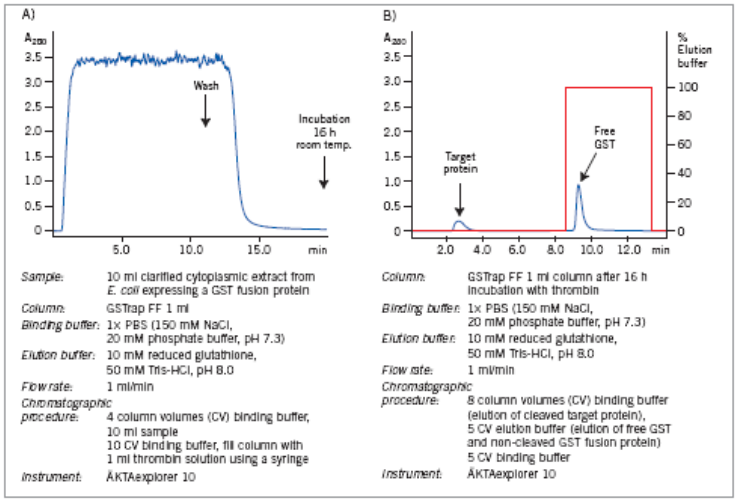








Lane	Sample
M	Perfect Protein™ Markers (10–225 kDa)
1	Crude extract (BugBuster protocol)
2	PopCulture + GST•Mag Agarose Beads

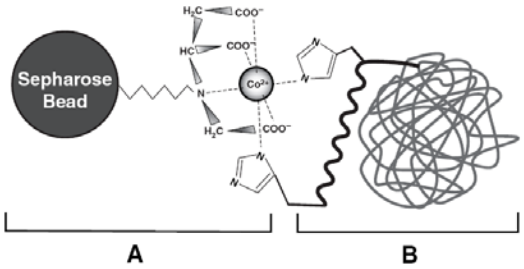


	A)	B)
Sample:	10 ml clarified cytoplasmic extract from *E. coli* expressing a GST fusion protein	
Column:	GSTrap FF 1 ml	GSTrap FF 1 ml column after 16 h incubation with thrombin
Binding buffer:	1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)	1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)
Elution buffer:	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0	10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0
Flow rate:	1 ml/min	1 ml/min
Chromatographic procedure:	4 column volumes (CV) binding buffer, 10 ml sample 10 CV binding buffer, fill column with 1 ml thrombin solution using a syringe	8 column volumes (CV) binding buffer (elution of cleaved target protein), 5 CV elution buffer (elution of free GST and non-cleaved GST fusion protein) 5 CV binding buffer
Instrument:	ÄKTAexplorer 10	ÄKTAexplorer 10

Fig 26. On-column thrombin cleavage of a GST fusion protein. A) Equilibration, sample application, and washing of a GST fusion protein on GSTrap FF 1 ml were performed using ÄKTAexplorer 10. After washing, the column was filled by syringe with 1 ml of thrombin (20 U/ml 1× PBS) and incubated for 16 h at room temperature. B) GST-free target protein was eluted using 1× PBS. GST was eluted using 10 mM reduced glutathione. The GST-free target protein fraction also contained a small amount of thrombin (not detectable by SDS-PAGE; see Fig 27, lane 6). The thrombin can be removed using a HiTrap Benzamidine FF (high sub) column.

Source: See Figure 27.

His-tag



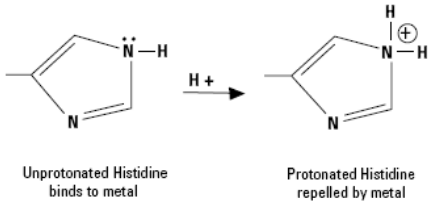
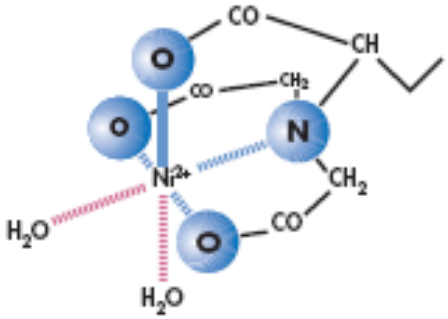
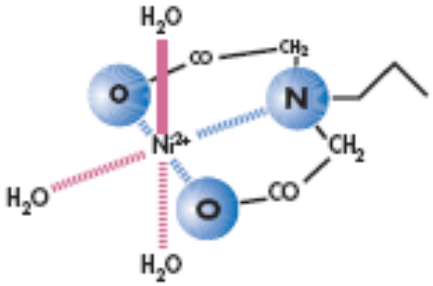


Table I: Histidine Tags

Tag	Amino acids
6xHis	His – His – His – His – His – His
6xHN	His – Asn – His– Asn – His – Asn– His – Asn – His – Asn – His – Asn
HAT	Lys – Asp – His – Leu – Ile – His – Asn – Val – His – Lys – Glu – His – Ala – His – Ala – His – Asn – Lys



Ni-NTA



Ni-IDA

10xHis

Ni, Co, Fe

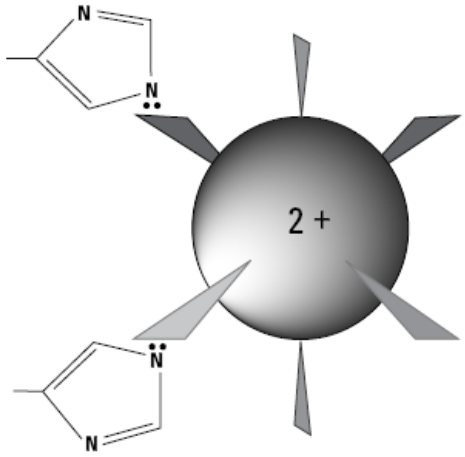
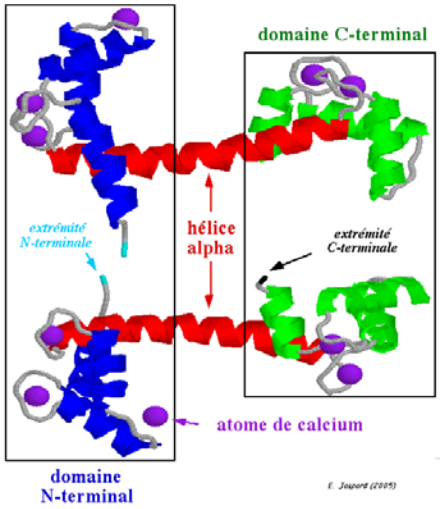
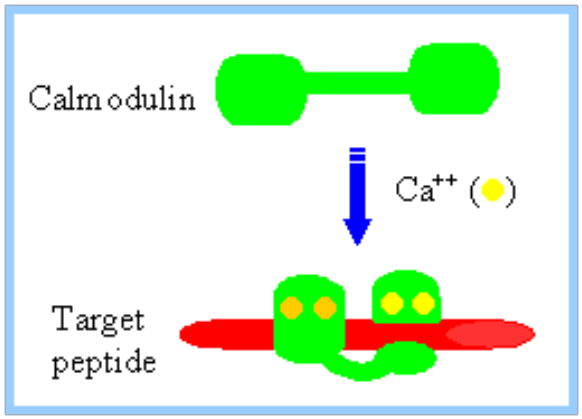


Figure 3. Binding of histidines to the TALON® Resin metal ion. Under conditions of physiological pH, histidine binds by sharing imidazole nitrogen electron density with the electron-deficient orbitals of the metal ion.

CBP affinity





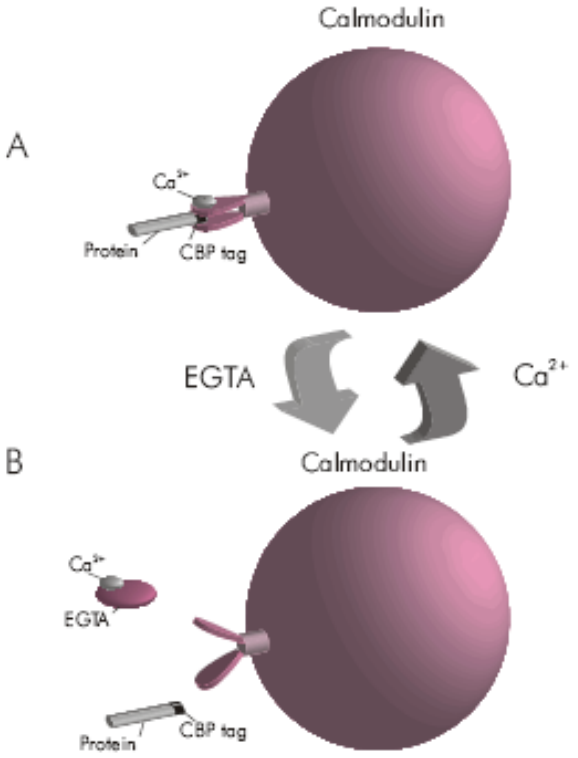
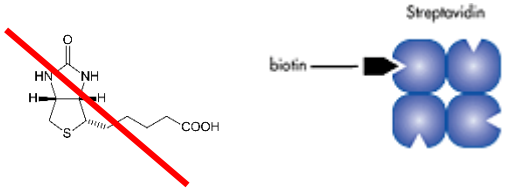
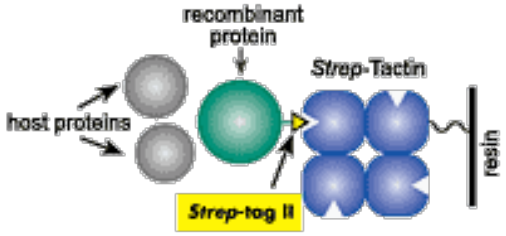


FIGURE 2 The Affinity protein expression and purification system. The highly conserved protein calmodulin binds to the CBP-tagged fusion protein in the presence of low concentrations of calcium at neutral pH (A). The fusion protein elutes from its ligand at neutral pH with 2 mM EGTA (B). The purified protein is now ready for storage, or if desired, proteolytic cleavage by thrombin or EK.

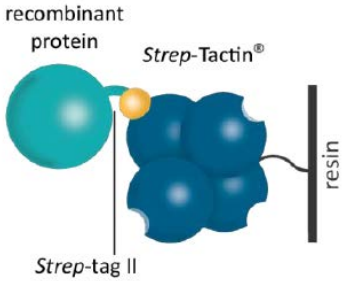
Strep tag-II ™ and twin-strep ™

Derived from strepavidin-Biotin

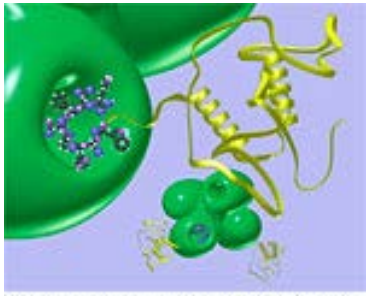


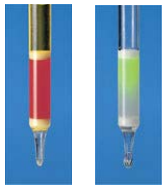


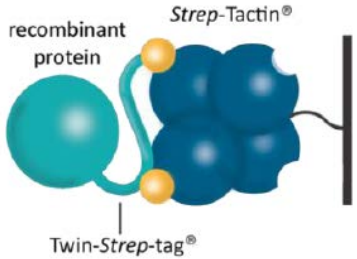
Strep tag-II: **WSHPQFEK**



Twin-strep: **WSHPQFEK**-GGGSGGGSGG-SA**WSHPQFEK**

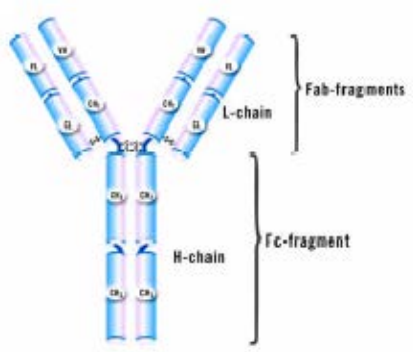

Strep-tag protein is binding to a **Strep-Tactin** tetramer.

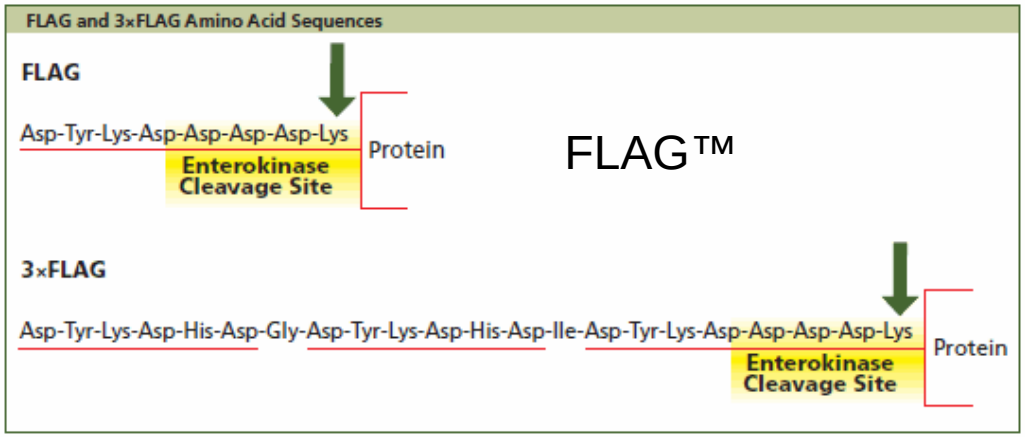


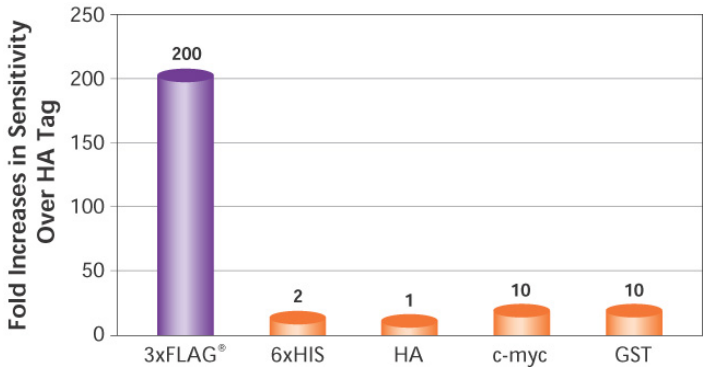


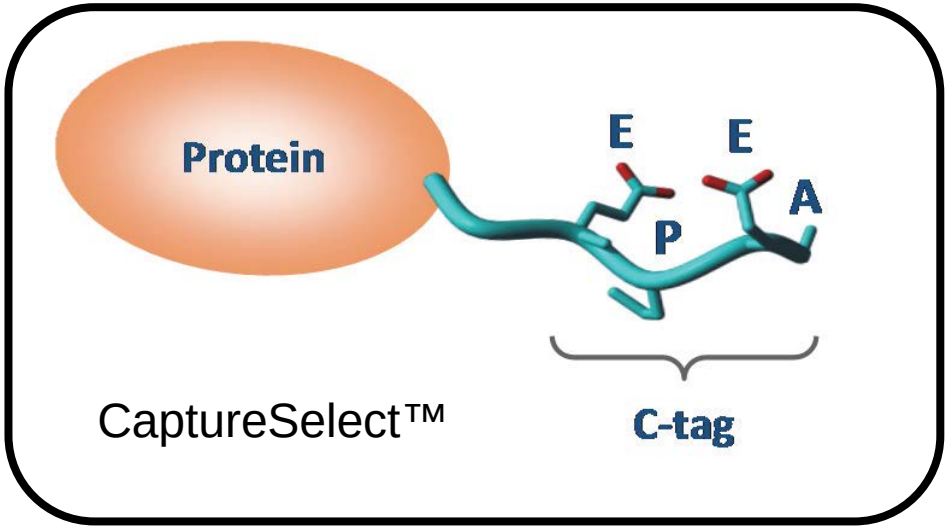
Elution with biotin analog: desthiobiotin

FLAG and Capture Systems



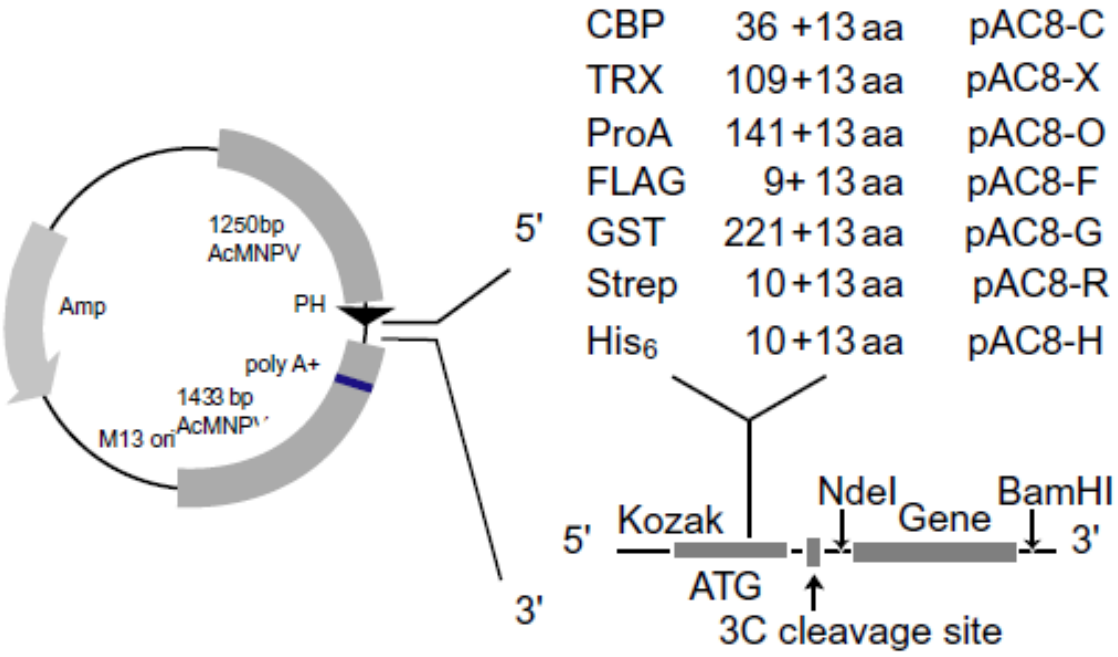






Transfer vectors for screening of affinity tags and parallel cloning of constructs

Standardize expression screening, enable constitent comparsons



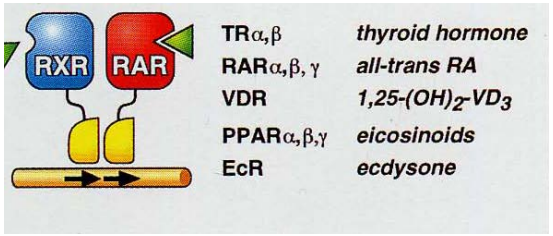
pBacPAK8 (Clontech) backbone
Restriction/Ligation/SLIC, or GW

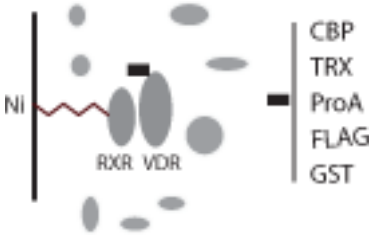
Abdulrahman, Anal Bioch, 2009

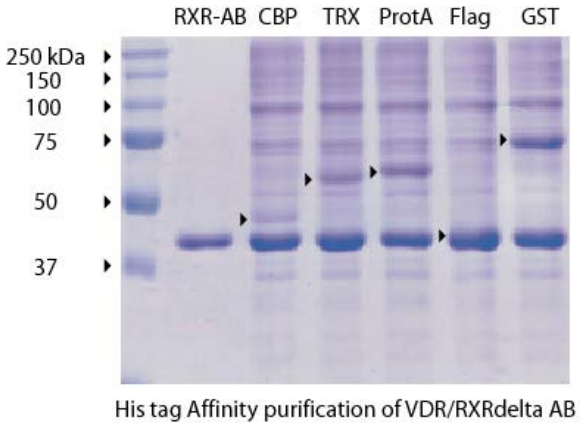
Protein A: Protein from Staphylococcus aureus that has affinity for immunoglobulins. Widely used for Ab pruifiction; Elution with pH shift or on collumn cleavage

The example of VDR

Expression screning to optimize expression of VDR (variant)

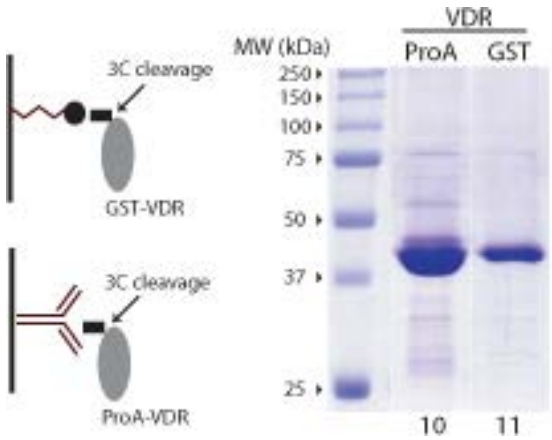






His tag Affinity purification of VDR/RXRdelta AB

RXR capture assay



On column tag cleavage

Abdulrahman, Anal Bioch, 2009

Tag	Resin	Elution	Cost/10mg
CBP	Calmodulin affinity	EGTA	181 €
TRX	Thiobond resin	ß-mercapto ethanol	n.a.
ProA	IgG Sepharose G	n.a.	275 €
FLAG	Anti flag M2 affinity gel	FLAG peptide	2343 €
GST	Glutathione sepharose 4B	Glutathione	41 €
Strep tag II	Streptactin sepharose	Desthiobiotin	67-134 €
His6	TALON Affinity	Imidazole	8-23 €
HA	Red Anti-HA affinity gel	HA peptide	4480 €

Flowchart for Baculovirus Expression

Clone the gene(s) of interest into a bacterial transfer vector

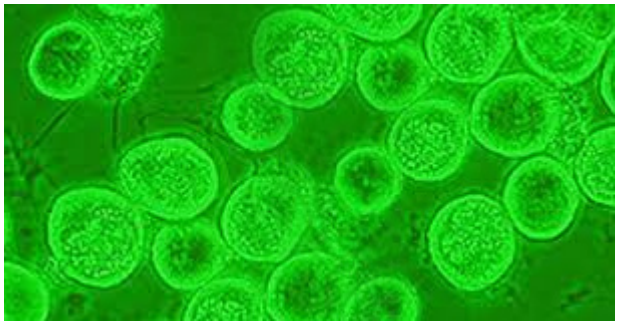
Generate the recombinant virus
Transfection/Co-transfection

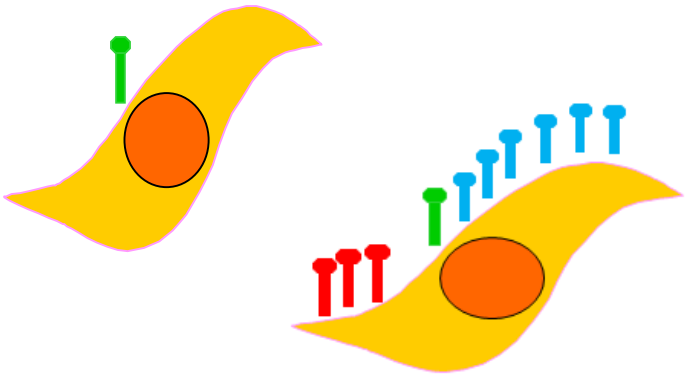
Small scale expression assay

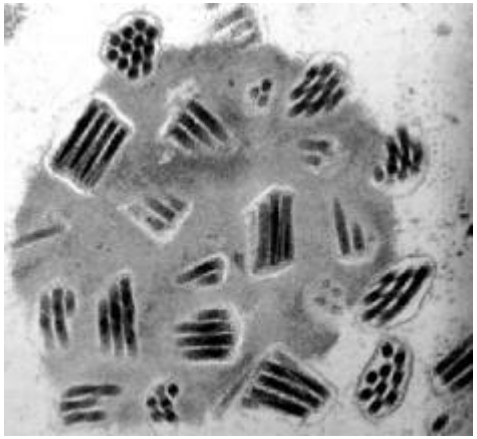
Prepare a high titer virus stock <108 pfu/ml

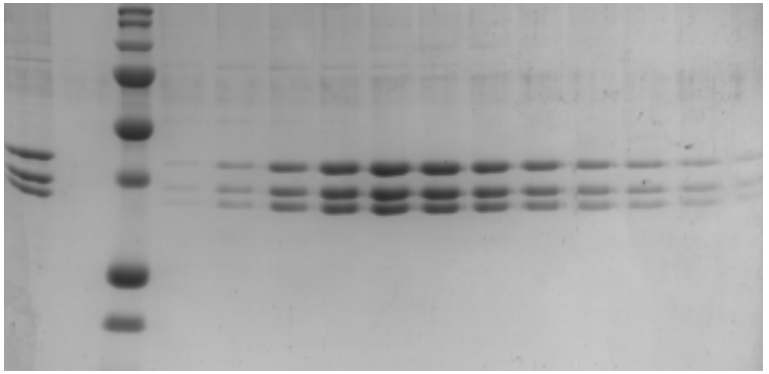
Optimization of the expression conditions and large scale production

Co-expression in insect cells using the BVES









Tandem affinity purification

Apply the cell lysate to the first affinity resin

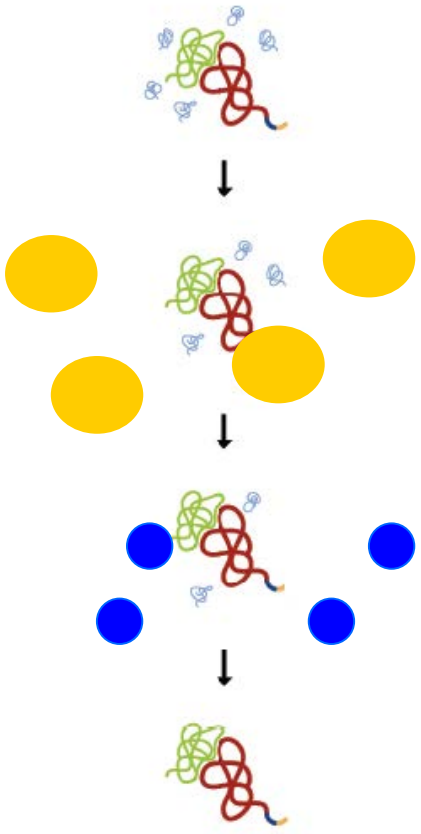
Wash unbound proteins and contaminants

Elute tagged protein I and interacting partner with elution buffer I

Apply the cell lysate to the second affinity resin

Wash unbound proteins and contaminants

Elute tagged protein II and interacting partner with elution buffer II



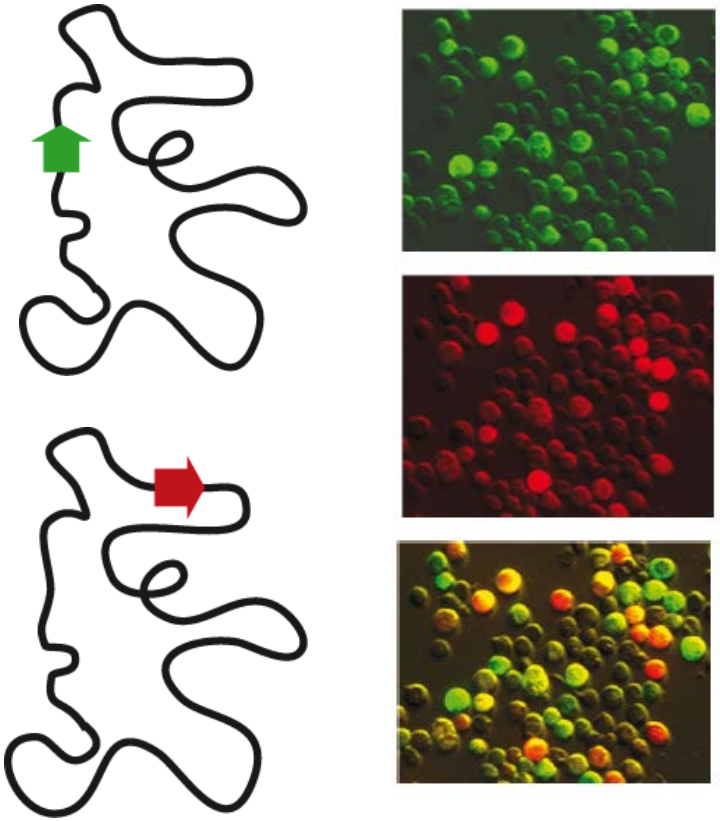
PROTEIN OF INTEREST WITH INTERACTING PARTNER

CONTAMINANTS

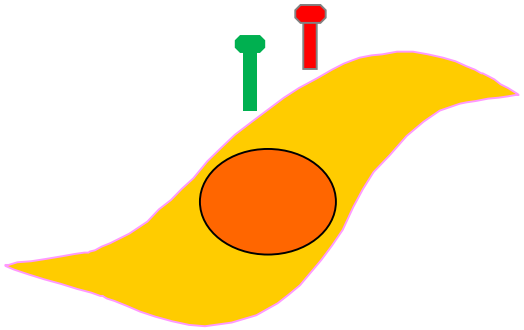
Affinity resin I

Affinity resin II

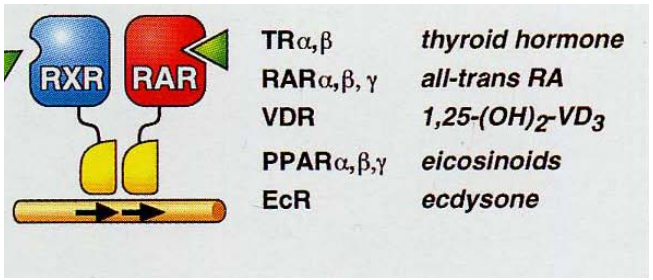
Co-infection: a simple way to co-express proteins

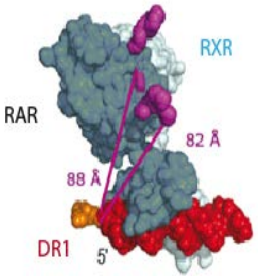


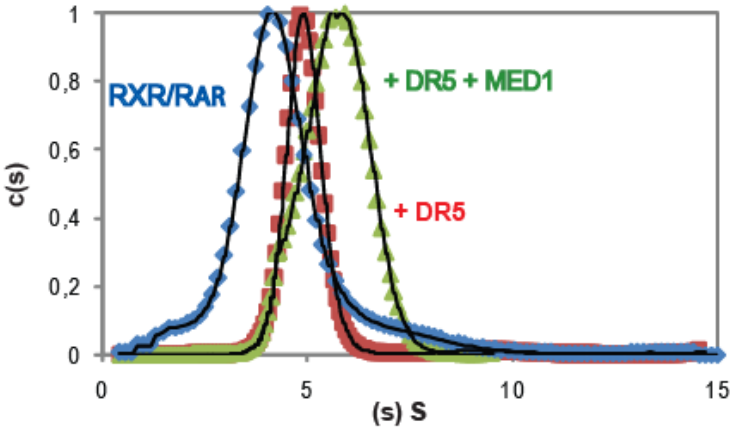
pH GFP+ p10 DsRed

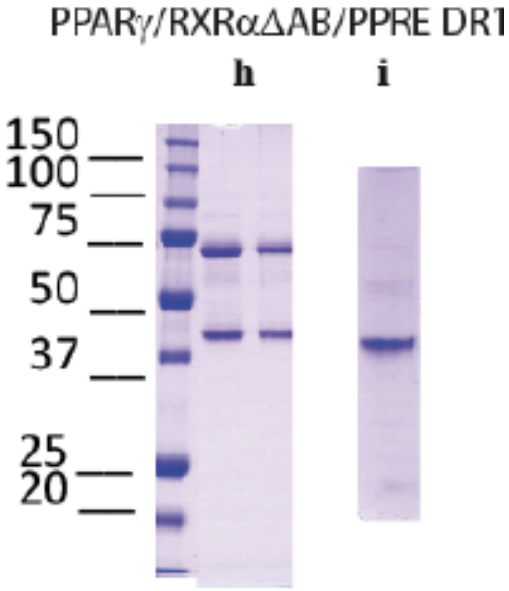


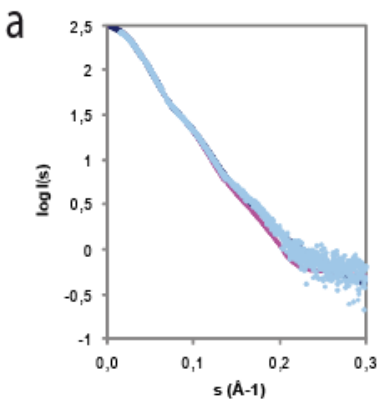
Production of nuclear hormone receptor complexes

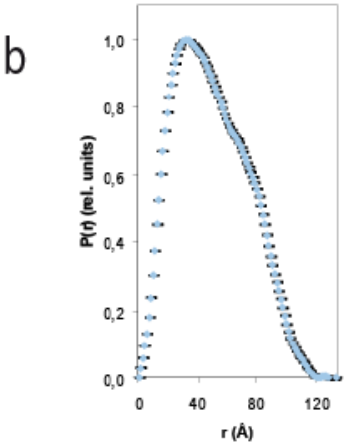






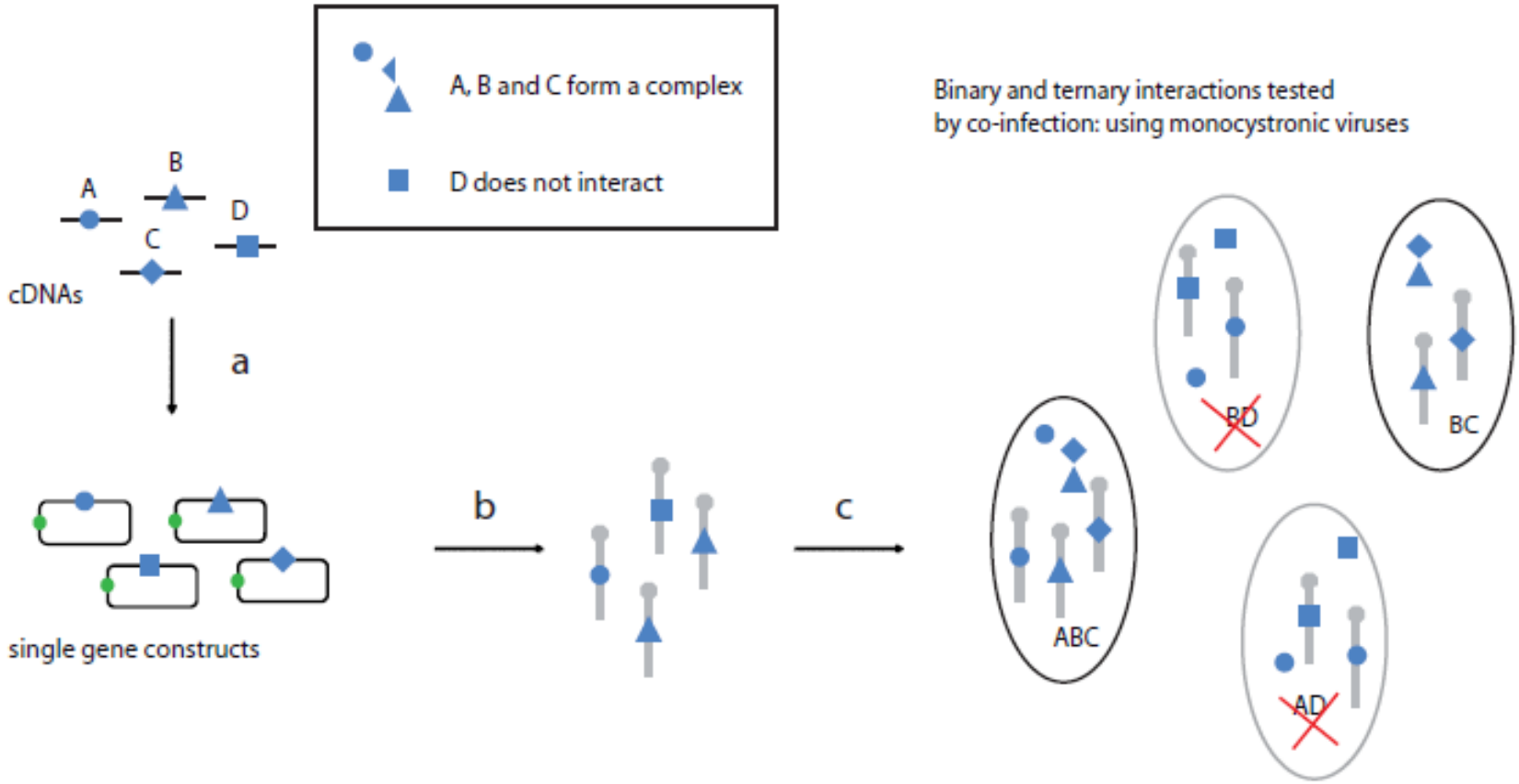




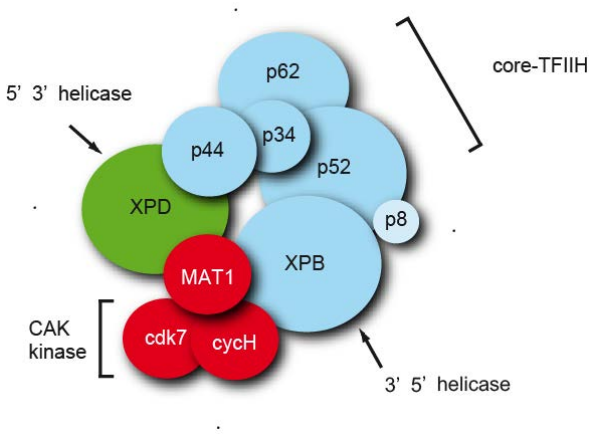


Rochel, et al. NSMB, 2011

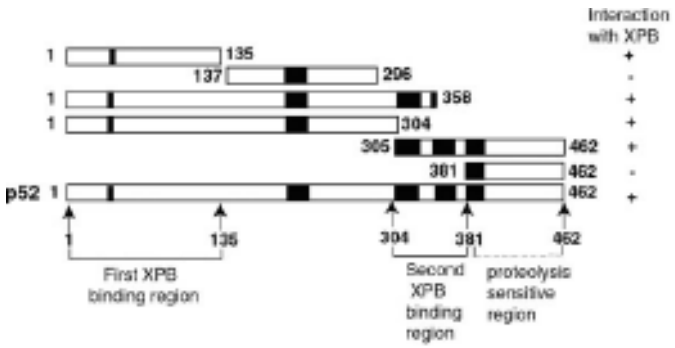
Analysis of protein-protein interaction networks

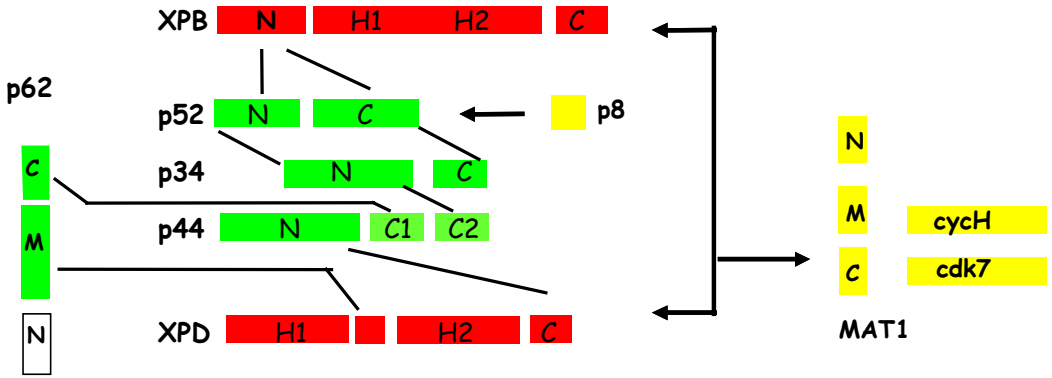


Systematic dissection of protein-protein interactions within TFIIH

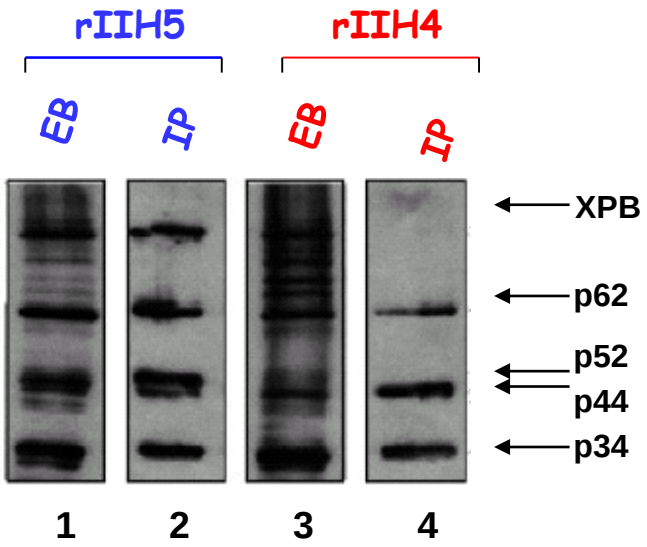


	p34	p44	p52	p62	XPB	XPD	MAT1
p34							
p44							
p52							
p62							
XPB							
XPD							

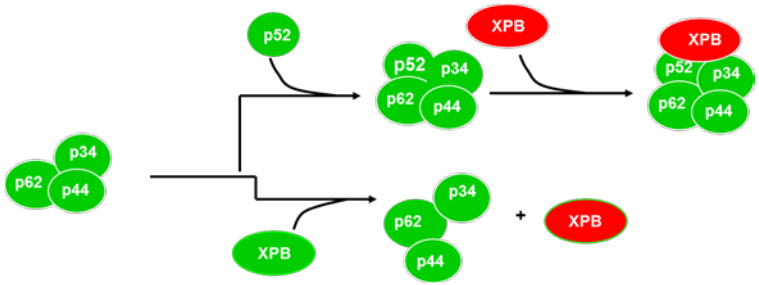


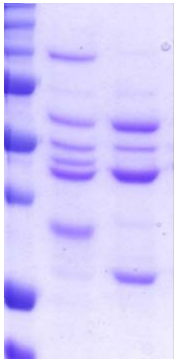


Systematic dissection of protein-protein interactions: deletion analysis



Analysis of the protein interaction network
Identification of key regulatory interactions



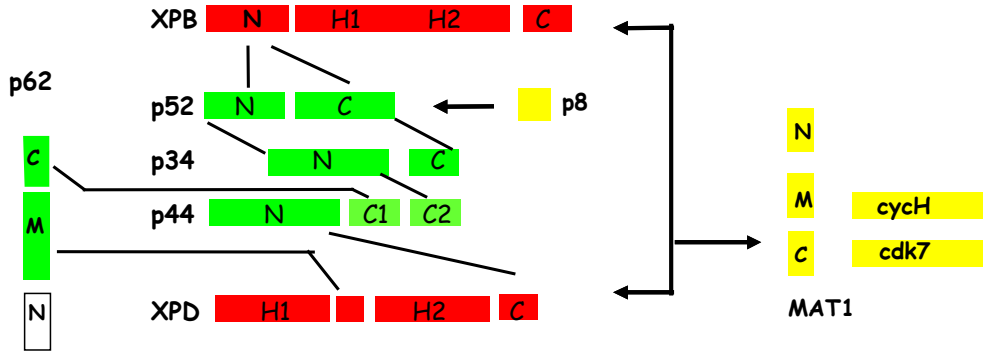


XPB

P62
*
P52,p44

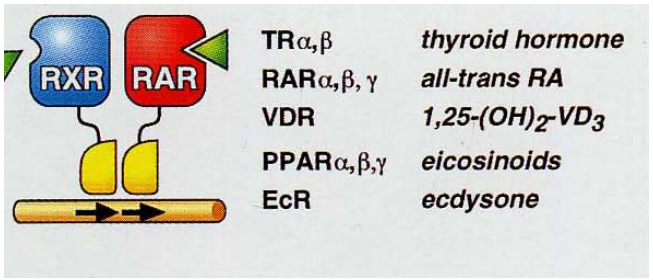
P34

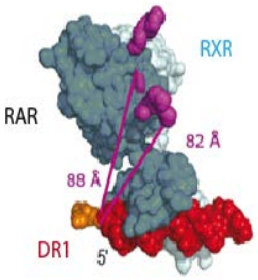
P34(1-233)

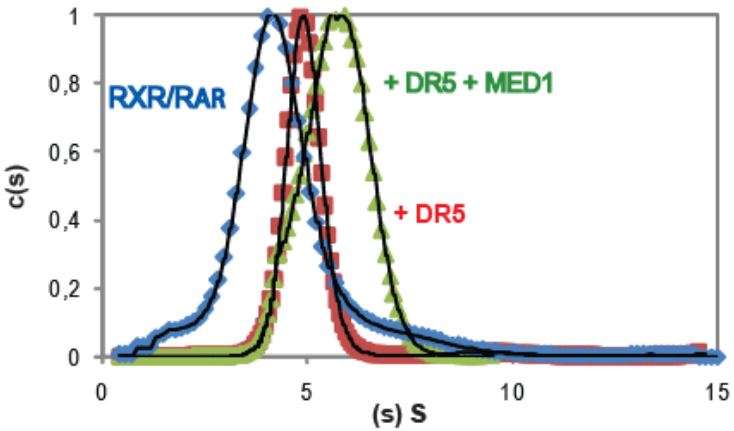


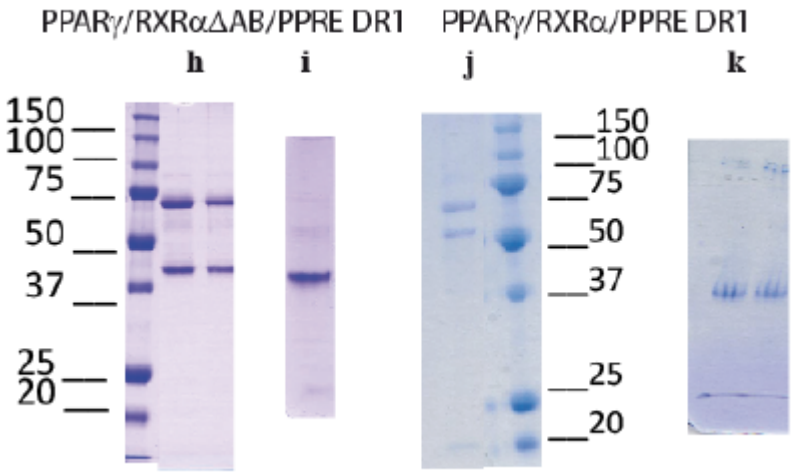
Jawahri at al. 2002, Radu et al, in prep

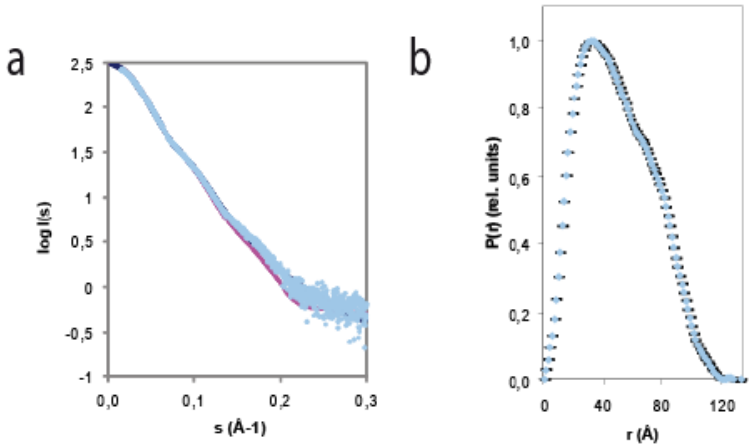
Production of nuclear hormone receptor complexes





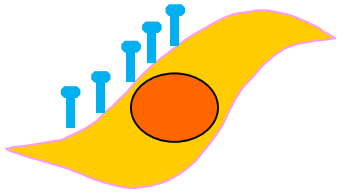


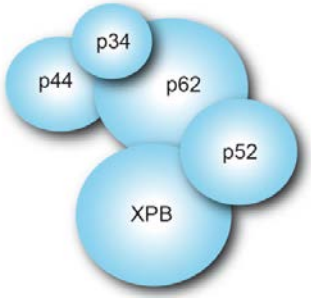




Rochel, et al. NSMB, 2011

Co-infection with multiple viruses for reconstitution of complexes



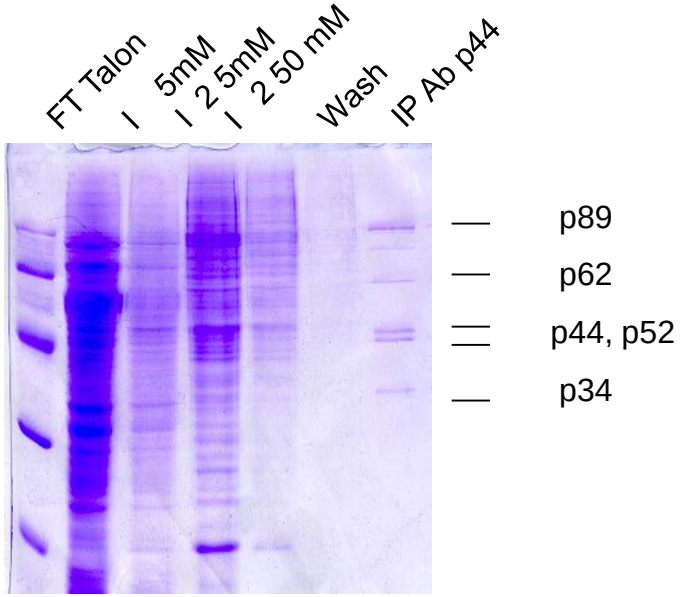


Cell extract

IMAC

Affinity purification

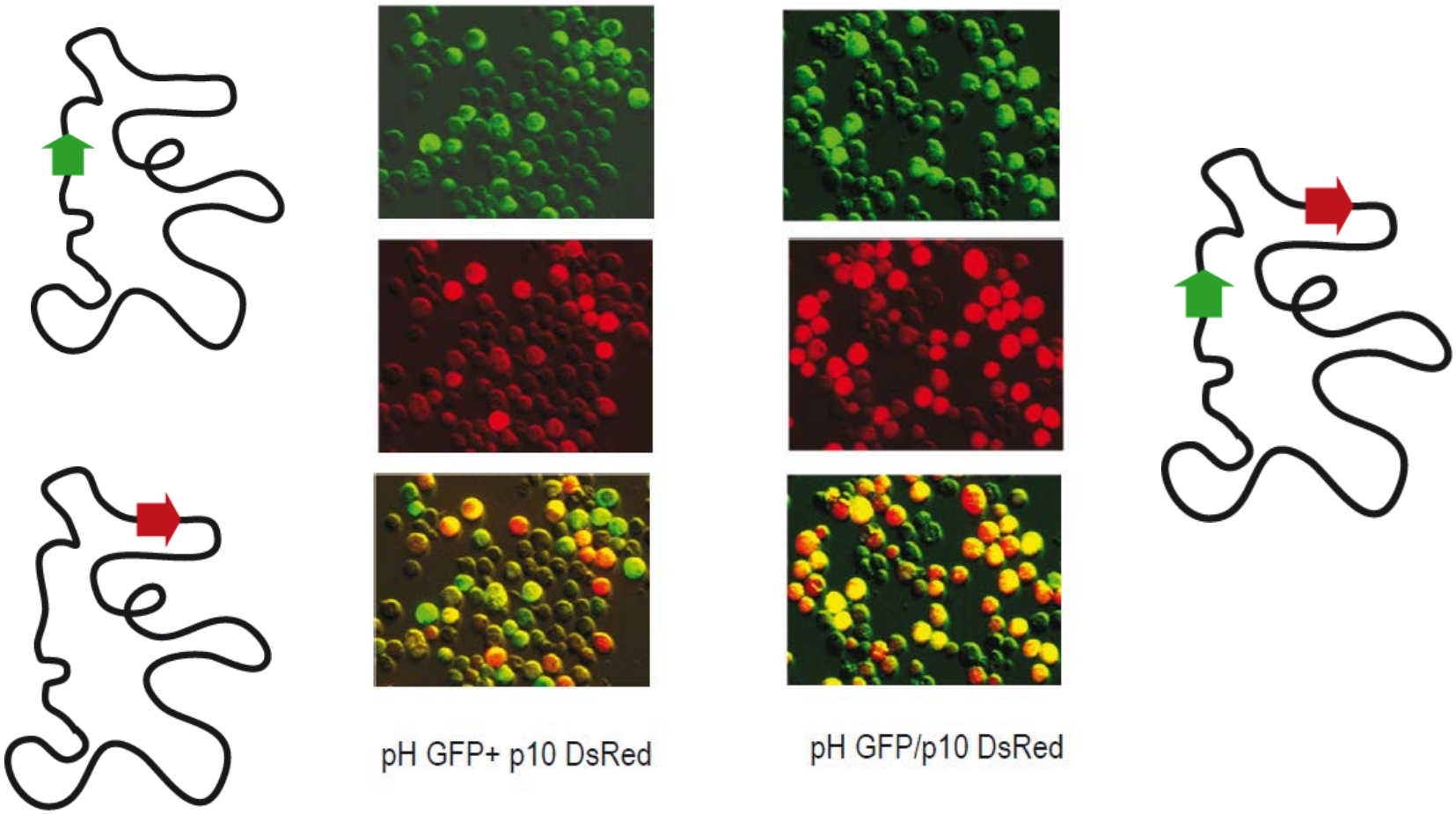
Size exclusion

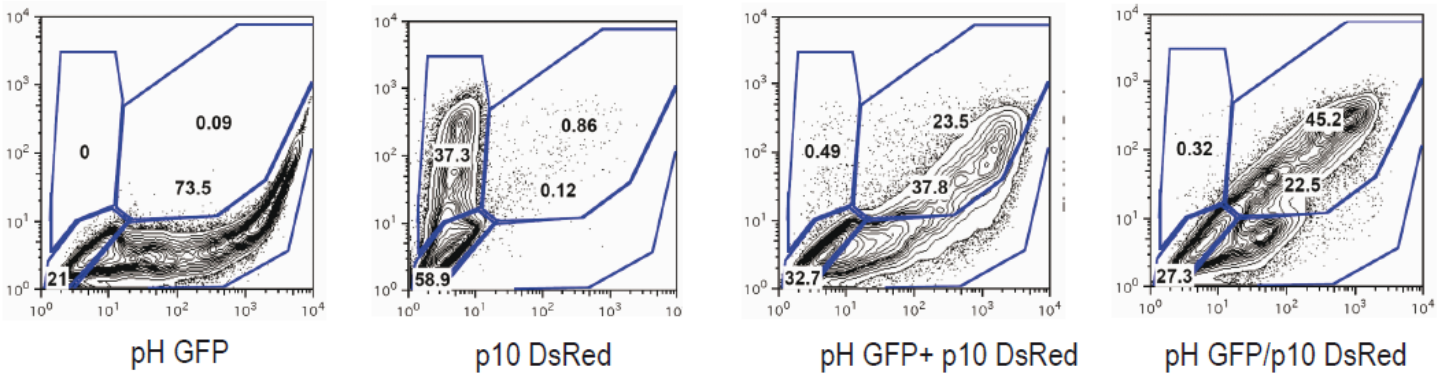


η # 50-100 μg/L

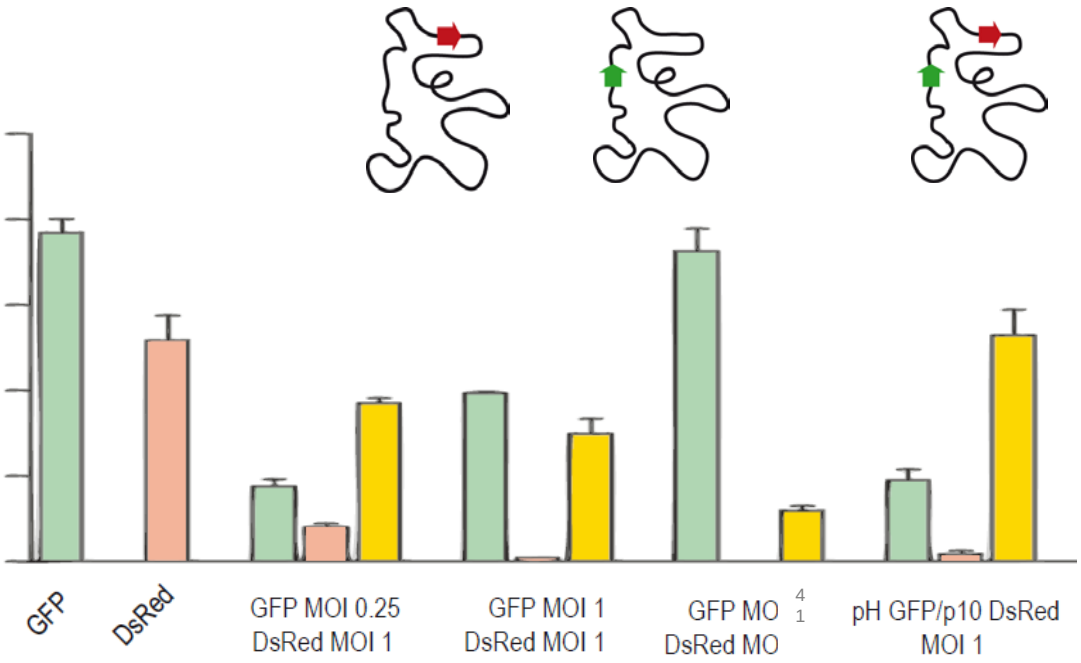
Low yields, labour intensive, poor reproducibility

Co-infection vs Multigene expression





Controls **Co-infection** **Multigene expression**

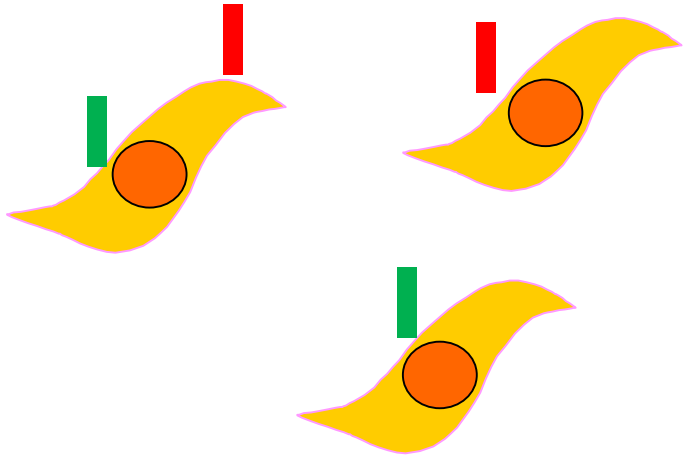


Idem for other MOIs

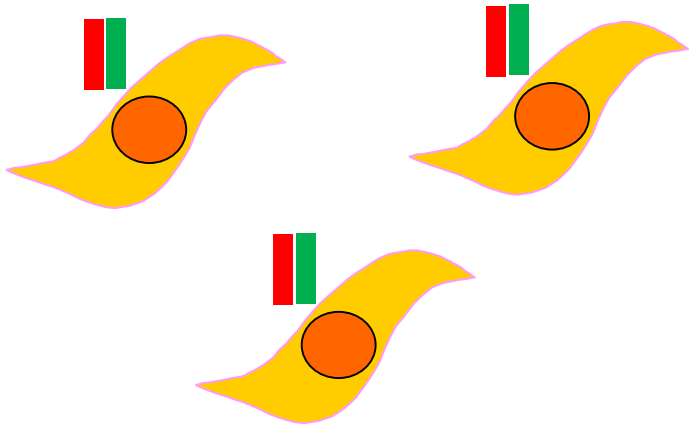
Co-infection vs Multigene expression

Two viruses encoding a single gene each

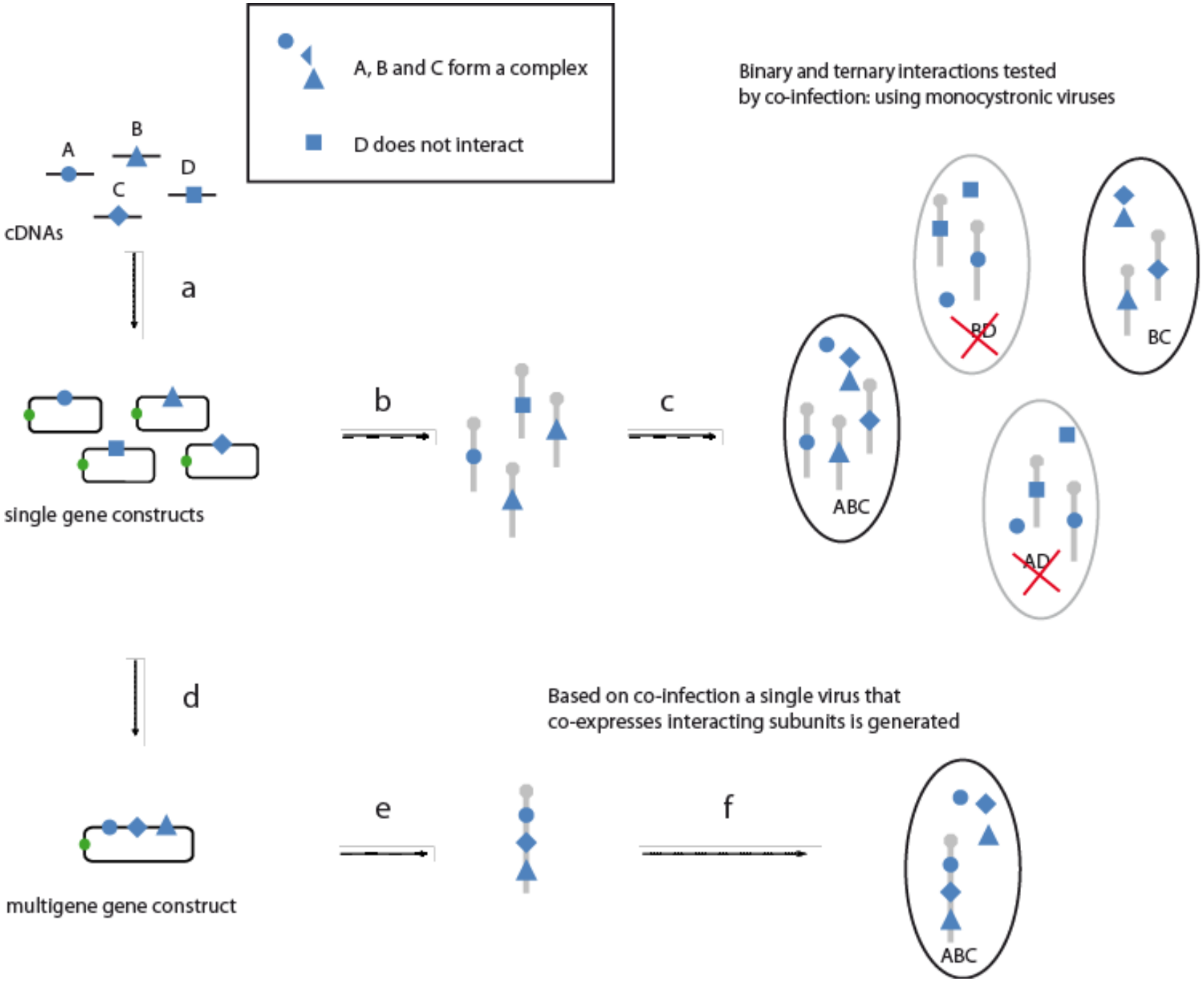
A single virus encoding the two genes



Co-infection



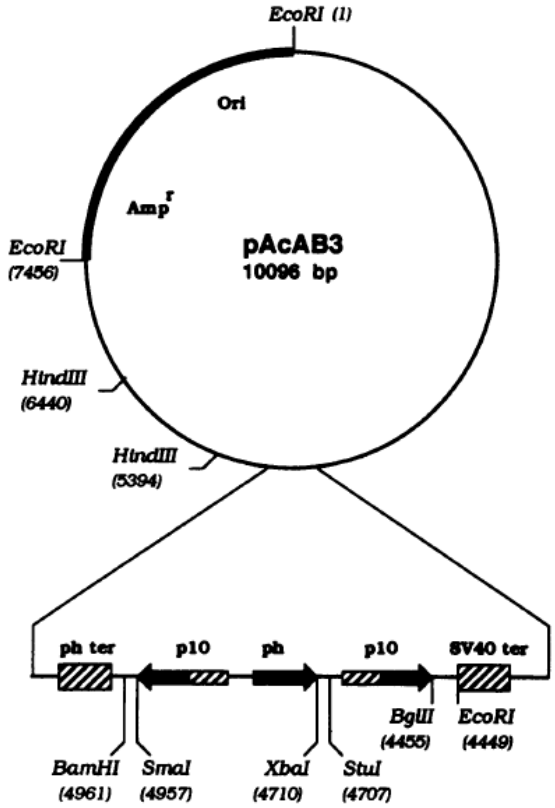
Infection by a single virus

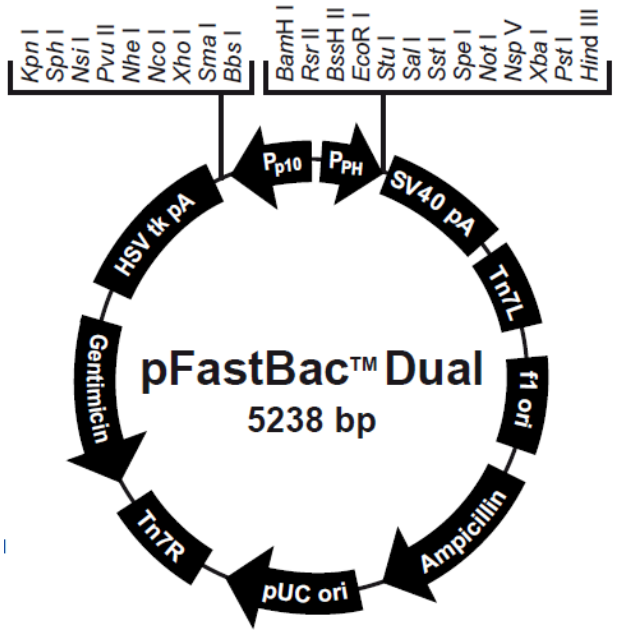

A, B and C form a complex
D does not interact
Binary and ternary interactions tested
by co-infection: using monocystronic viruses
A
B
C
D
cDNAs
a
single gene constructs
b
c
BD
BC
ABC
AD
d
multigene gene construct
Based on co-infection a single virus that
co-expresses interacting subunits is generated
e
f
ABC

Nucleic Acids Research, 1993, Vol. 21, No. 5 1219–1223

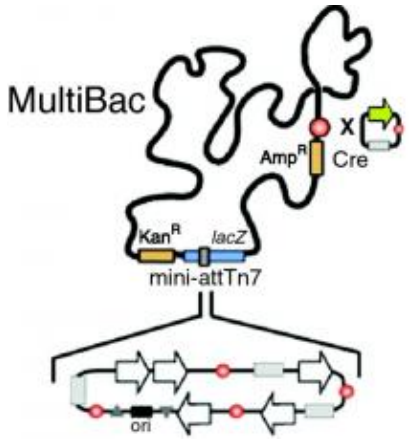
Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells

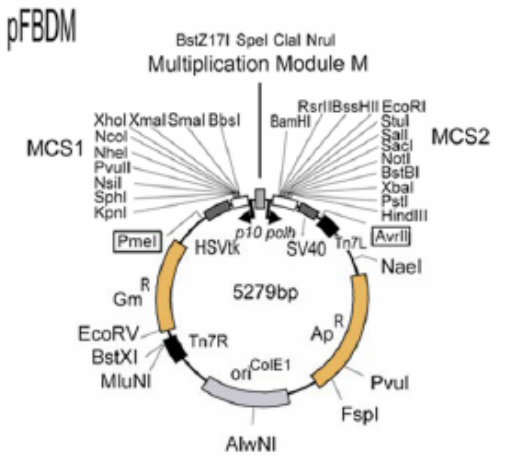
Alexander S.Belyaev[1] and Polly Roy[1,2,*]

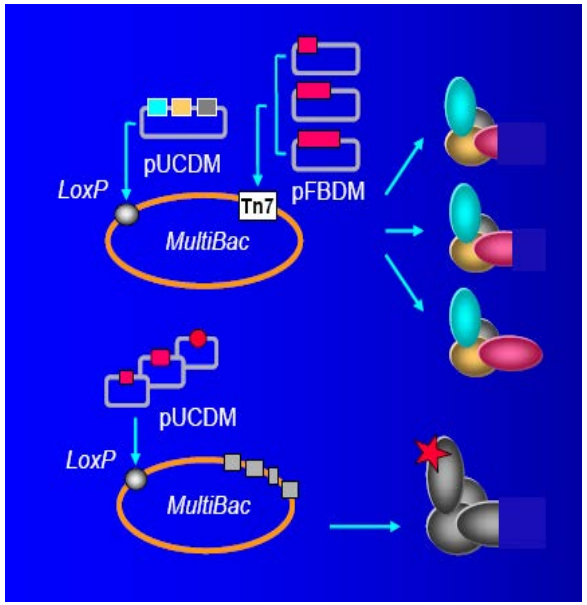




MultiBac technology: Combinatorial applications in protein expression



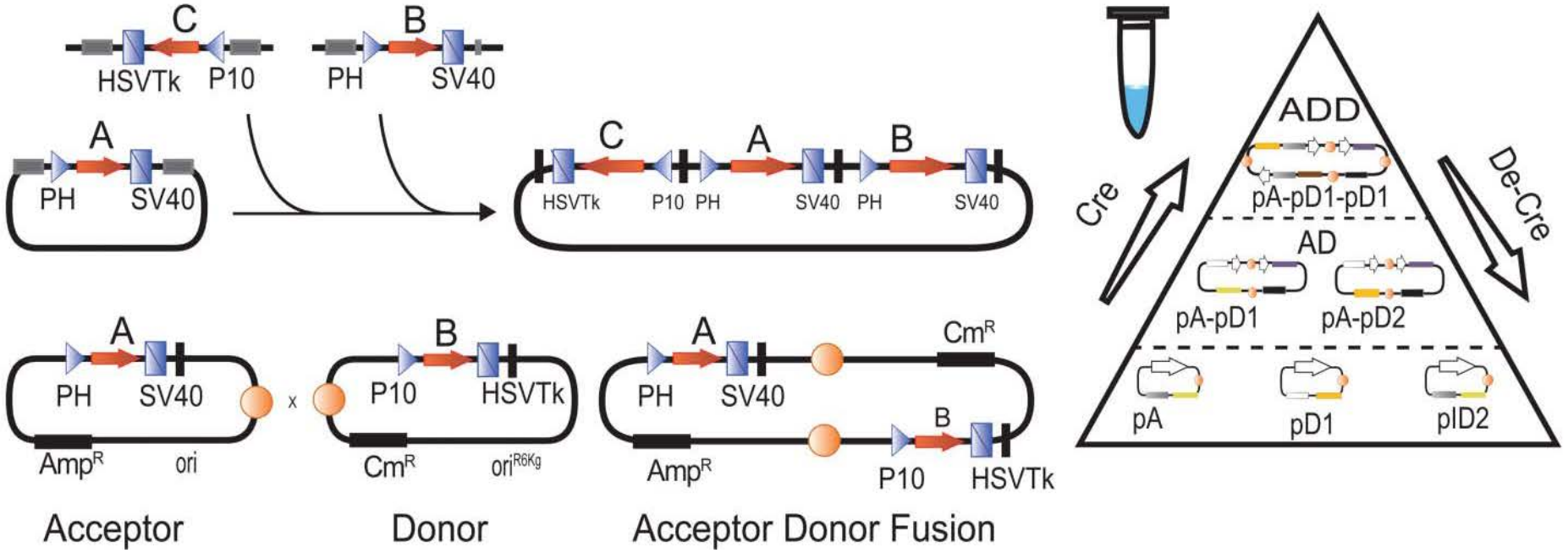




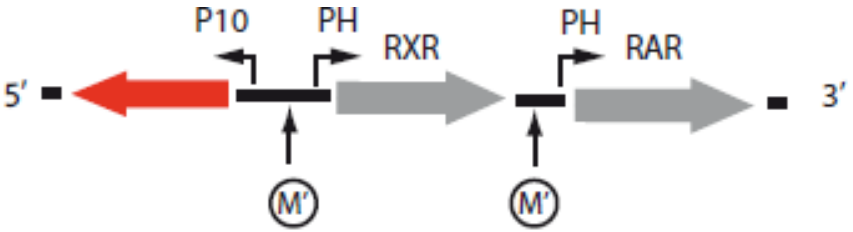
Tools to streamline design of multigene expression recombinnant baculoviruses

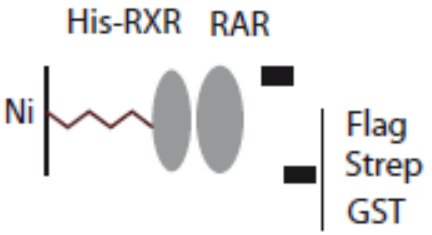
Imre BERGER, Bristol, UK

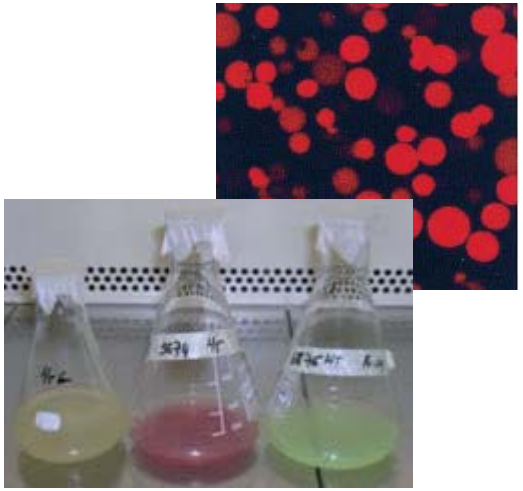
MultiBac technology: Combinatorial applications in protein expression

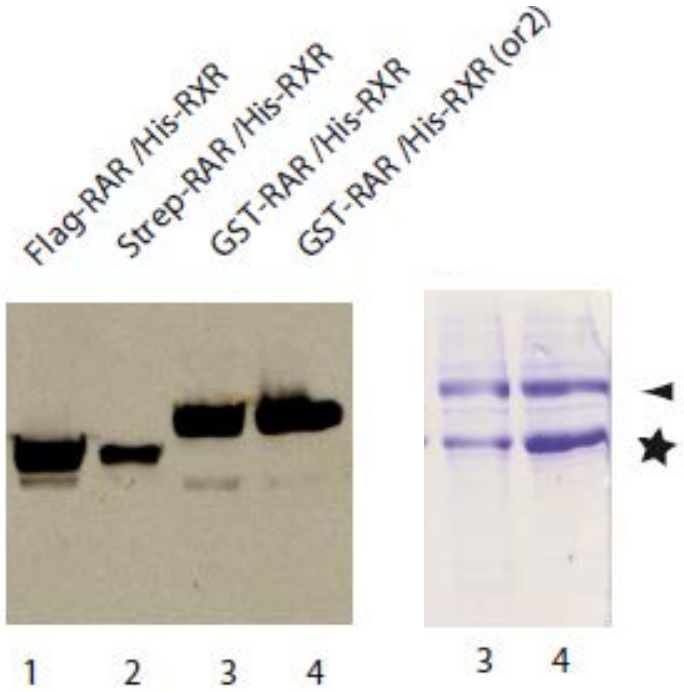


Insertion of an expression cassette into the multiplication module

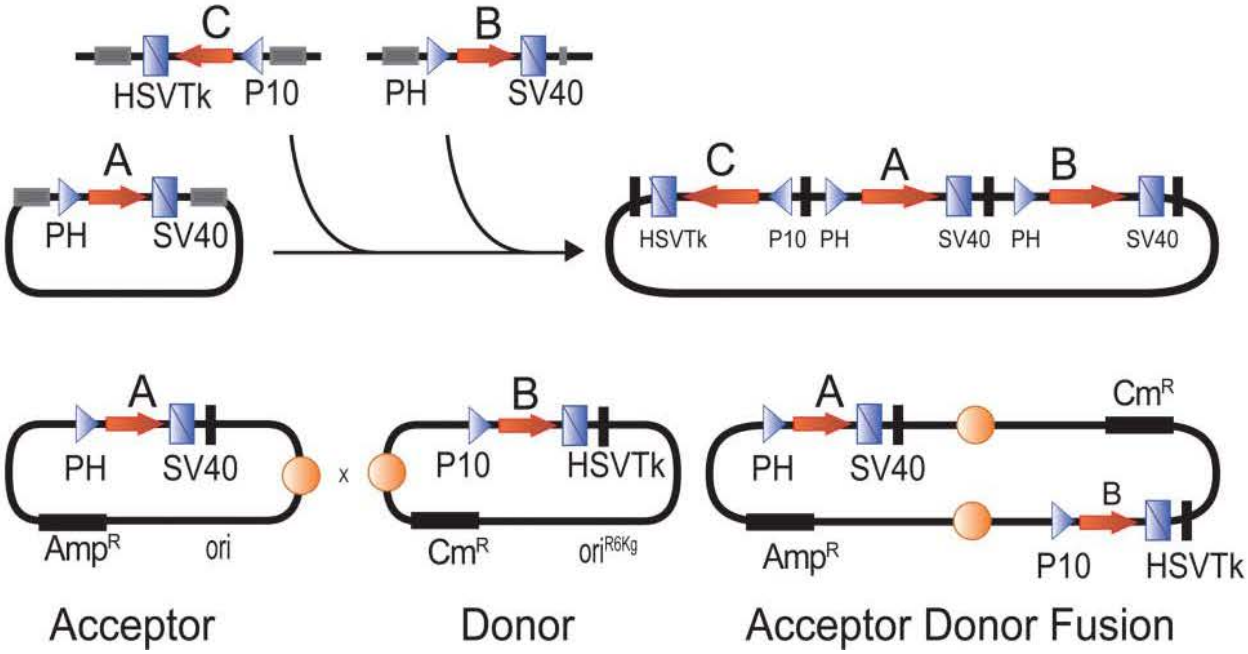


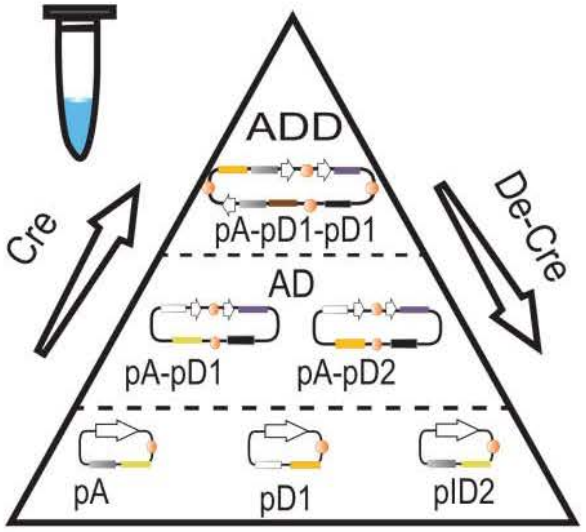






MultiBac technology: Combinatorial applications in protein expression





Cre-LoxP recombination *in vitro*

Cre recombinase binds to the loxP sites on both the donor vector and the acceptor vector, cleaves the DNA, and covalently attaches itself to the DNA which leads to strand exchange and concatenation.

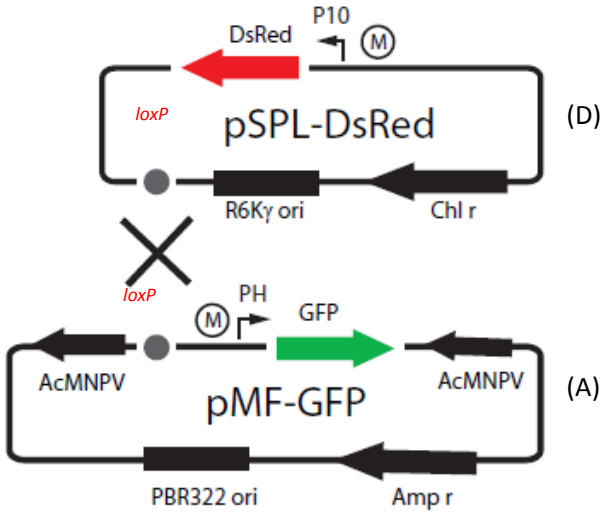
loxP sequence

8-bp overlap region

ATAACTTCGTATAGCATACATTATACGAAGTTAT

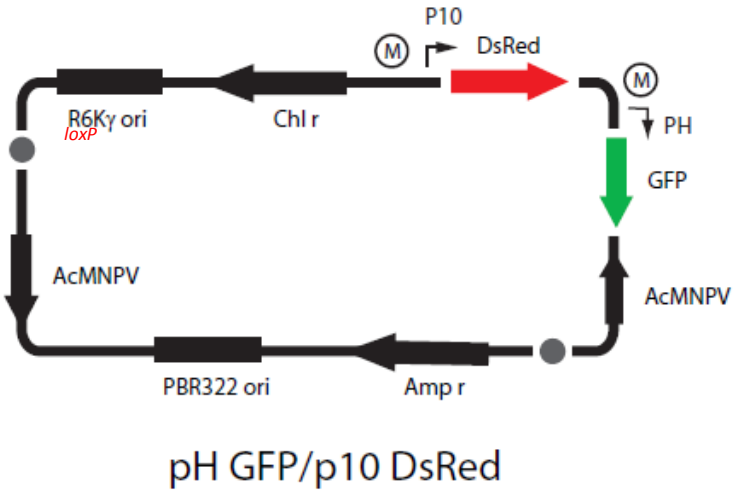
Inverted region Inverted region

- 34-bp recognition sequence
- 13-bp inverted repeats flanked by 8-bp spacer region

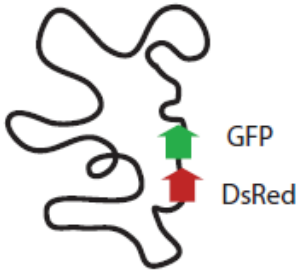


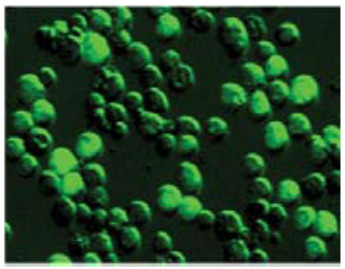
(D)

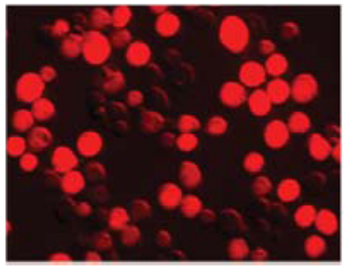
(A)

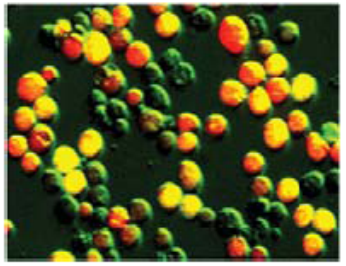


pH GFP/p10 DsRed









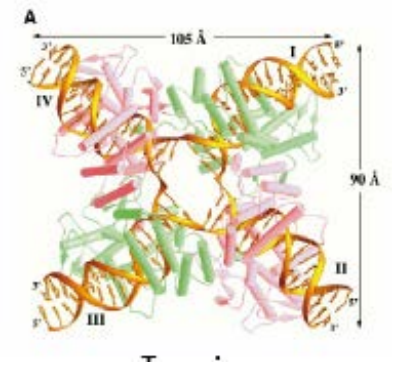
pSPL Multibac, (Fitzgerald, Nat Methods 2006)

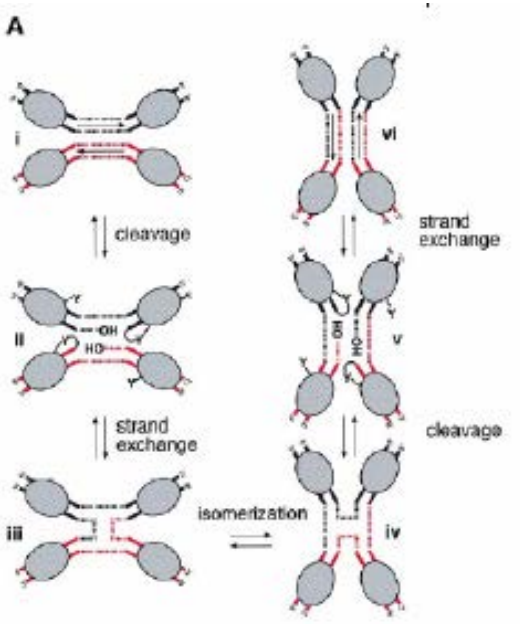
Site specific recombination cloning

- General
  - RecA
    - Promotes homologous strand recombination
- Site Specific
  - Cre/lox
    - P1 phage recombinase Cre
    - Lux sites 32 bp
  - Lambda integrase/att
    - L-phage integrase
    - attB
    - attP

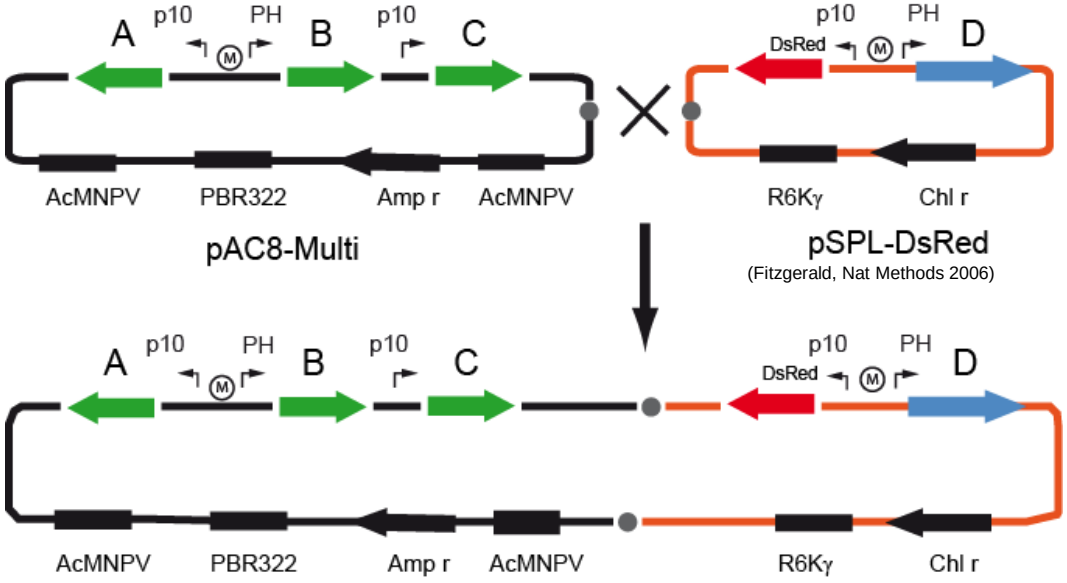
- MAIN APPLICATIONS
- *In VITRO*
  - Rapid restriction enzyme free subcloning
  - Plasmid excision
  - In vitro clone screening
- *In VIVO*
  - Genome rearrangements
  - Tightly regulated transcription

Cre-loxP recombination in bacteriophage P1





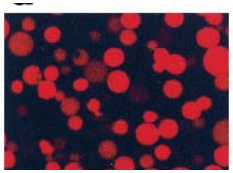
Expression of ternary and quaternary complexes with a single virus: a problem of DNA synthesis



pBac8 Multi-pSPL Fusion

A: strep-tag
D: Flag-tag

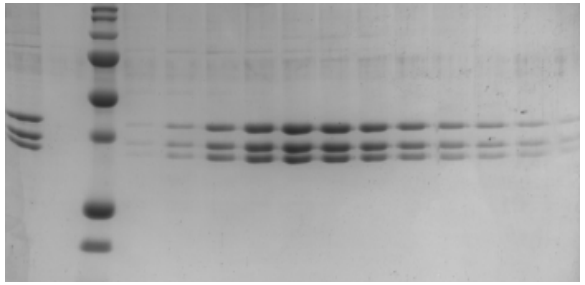
Flag-affinity



Elution with
Competitor-peptide

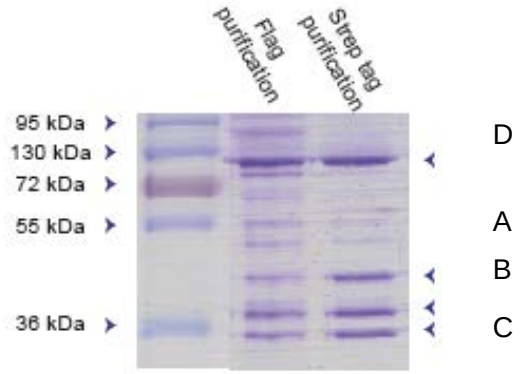
Strep-affinity

Elution with
Desthiobitin



A
B
C

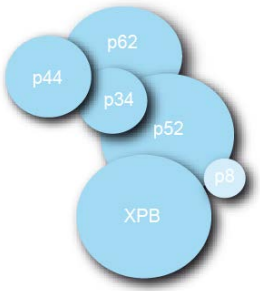
Ternary complex



Quaternary complex

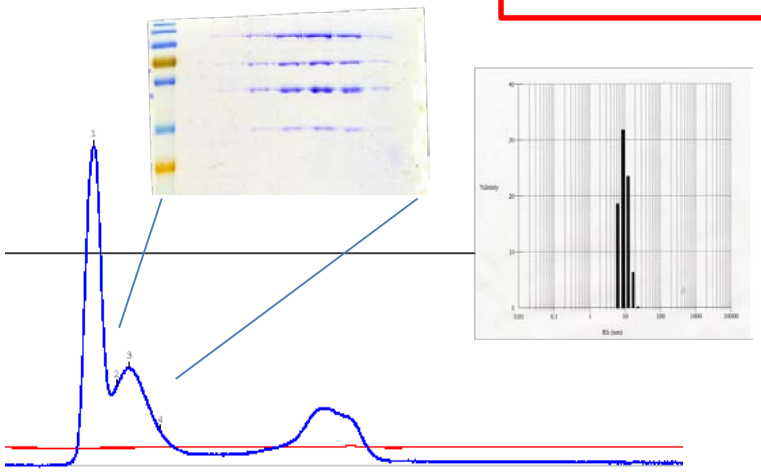
Production of core-TFIIH with a single virus

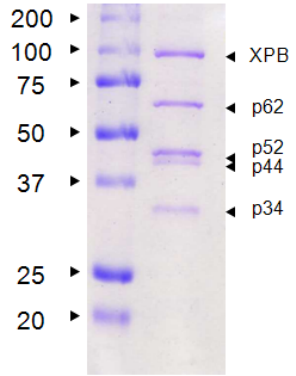
- 6 subunits: XPB, p62, p52, p44, p34, p8/TTDA (+ DsRed)
- Yield : 0.5 mg/L

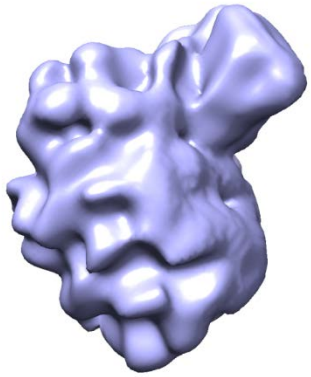


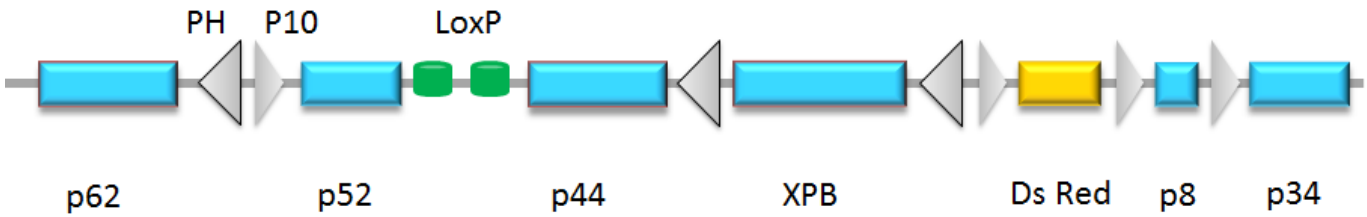
MS-cross linking, Cryo-EM and crystallization

Screening for XPB inhibitors



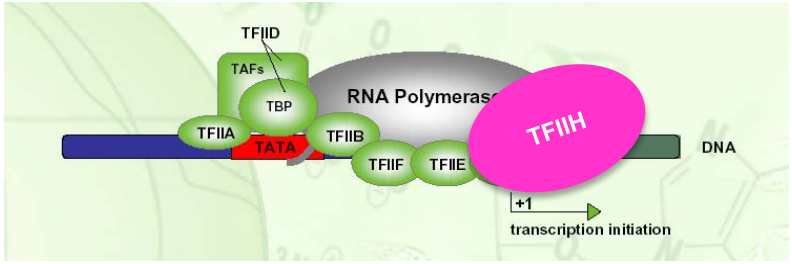


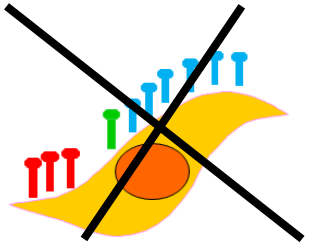


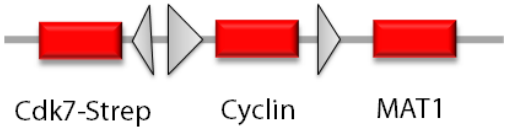


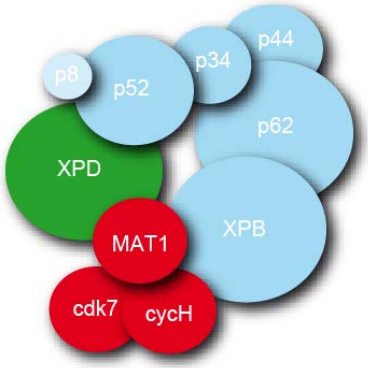
16 kbp

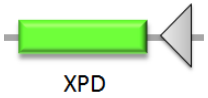
Reconstitution and in vitro assays

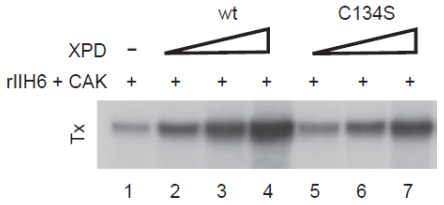


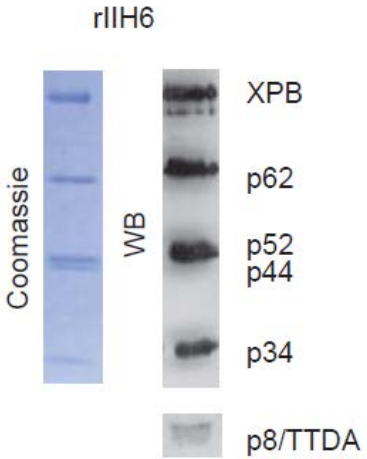


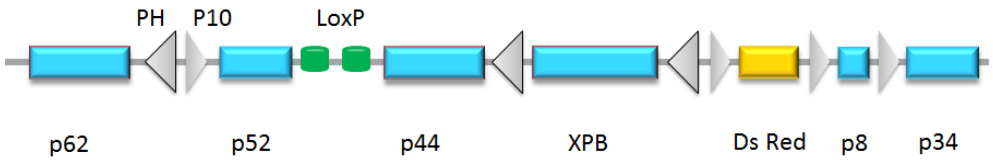






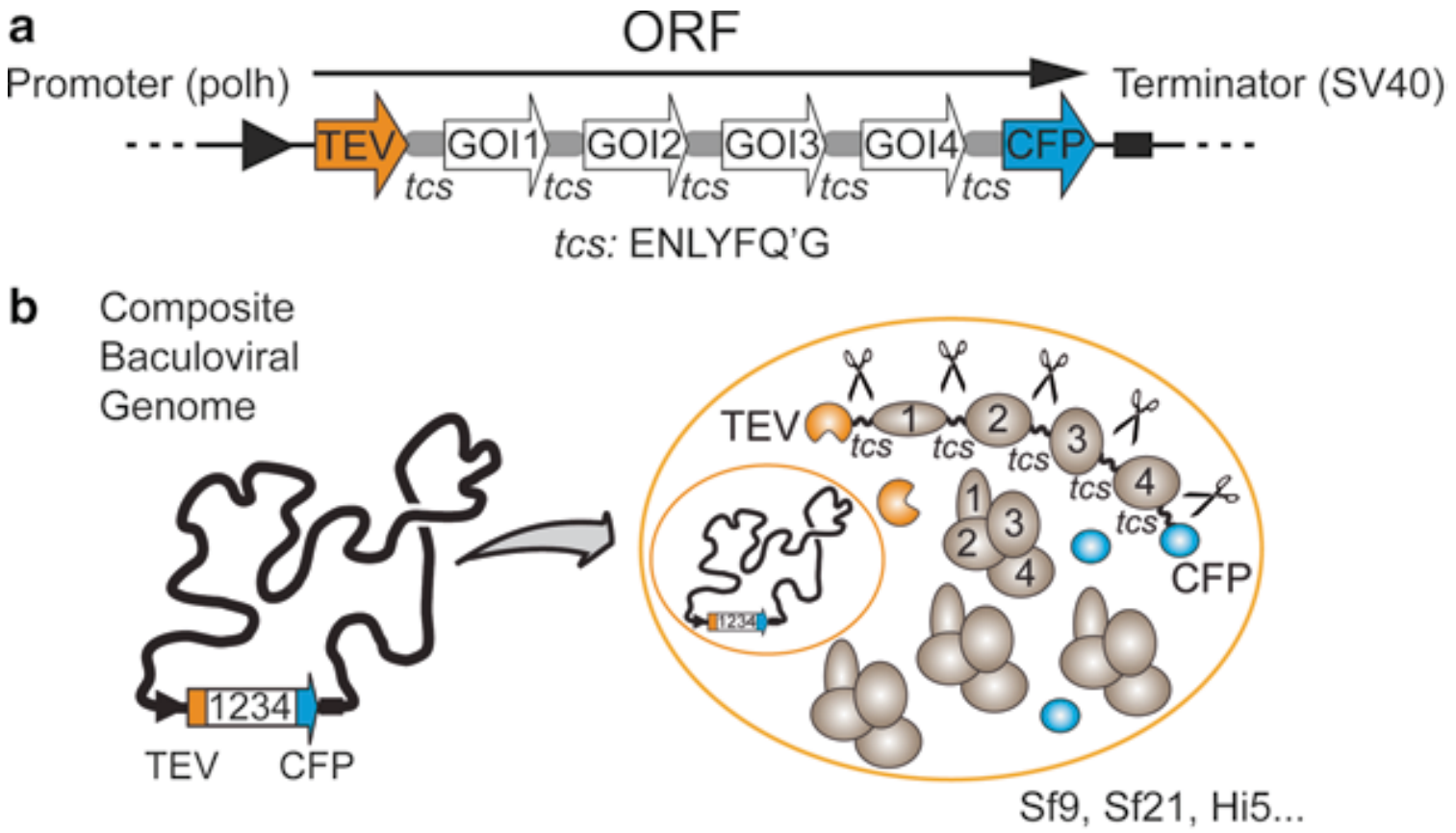




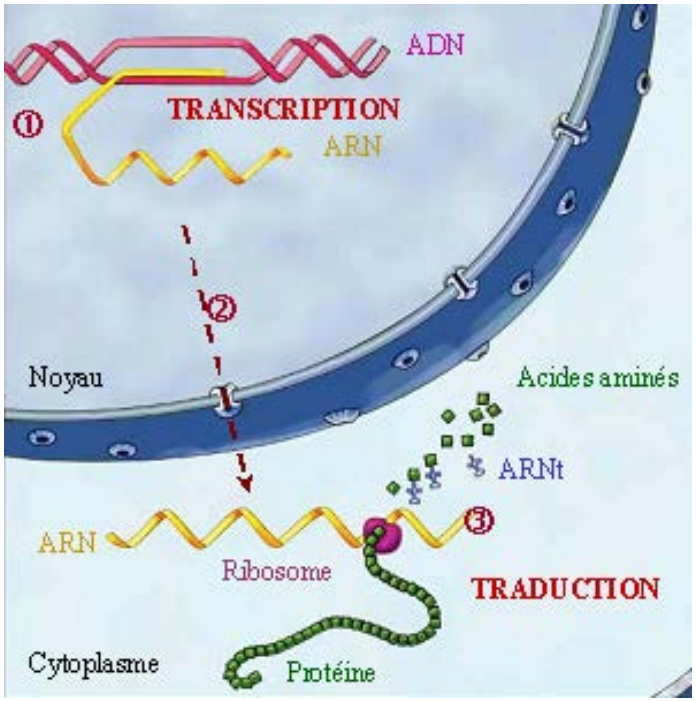


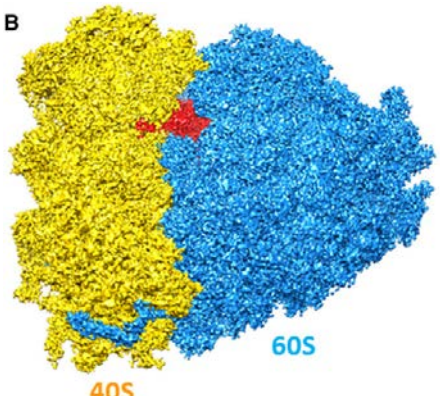
(Abdulrahman et al, PNAS 2013,
Kupper et al., Plos Biology 2014)

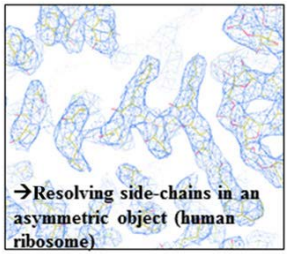
Polyproteins

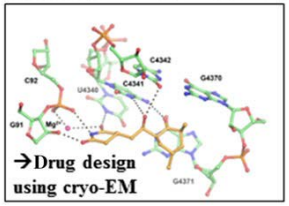


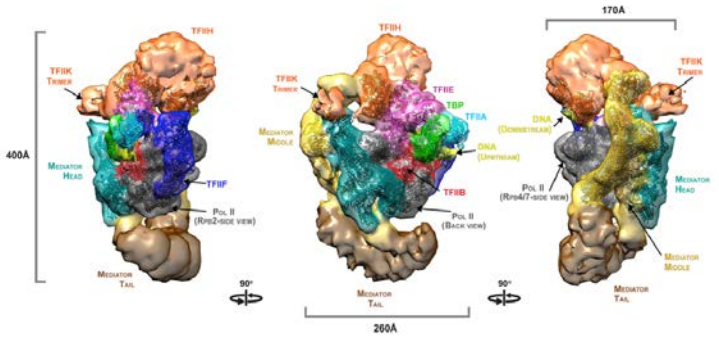
Preparation from endogenous sources











Tandem Affinity Purification

- Rapid purification of complexes without prior knowledge of the complex composition, activity, or function
- Ability to purify low abundant proteins/protein complexes
- Fusion of the TAP tag to the target protein
- Complex retrieval from tissue culture
- Large-scale studies

TAP-TAG in yeast

(Gavin, 2002)

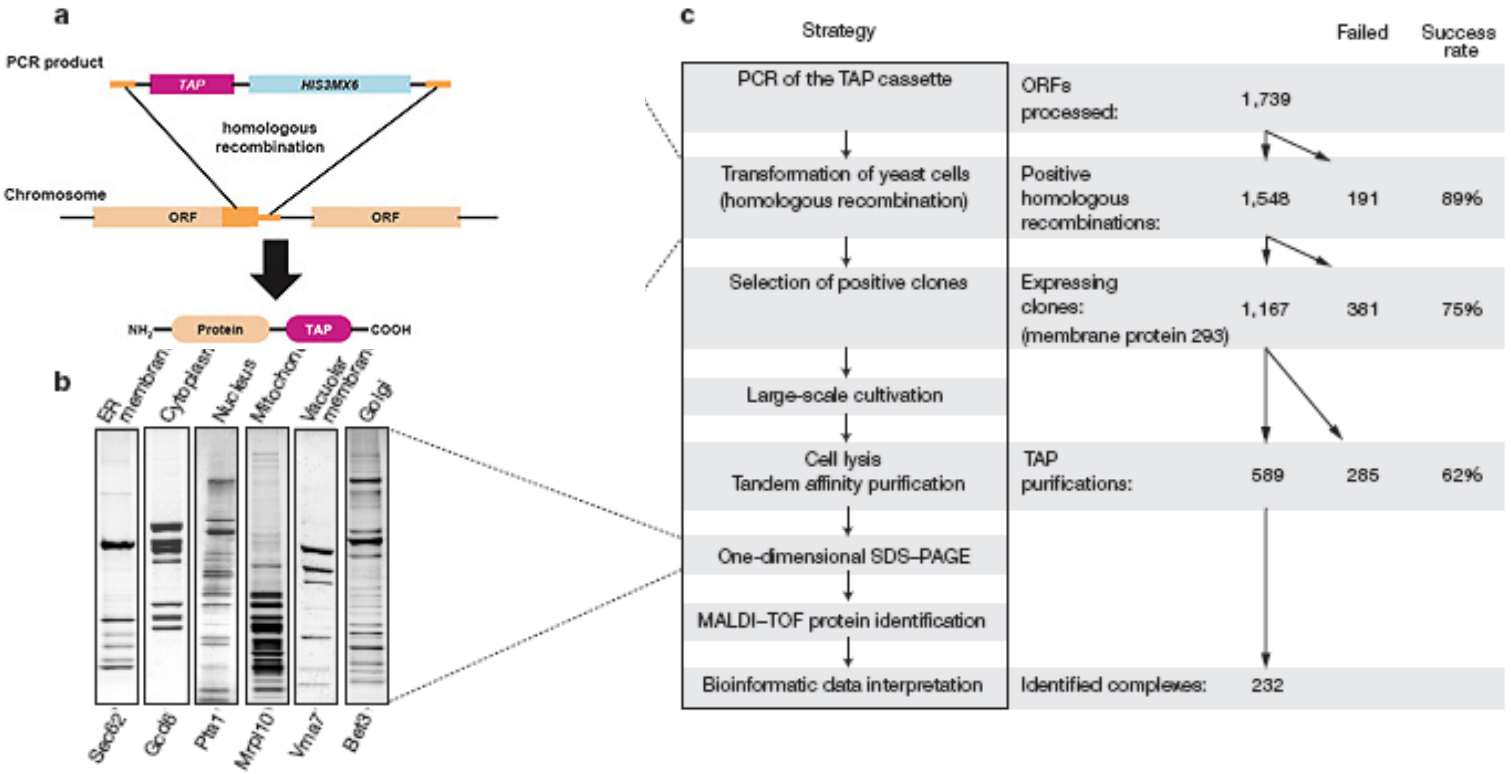
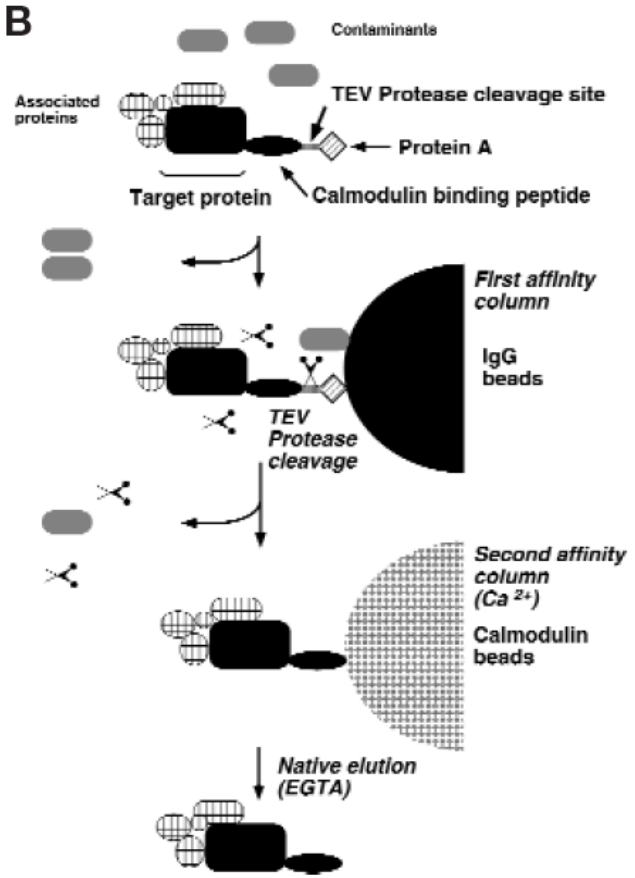
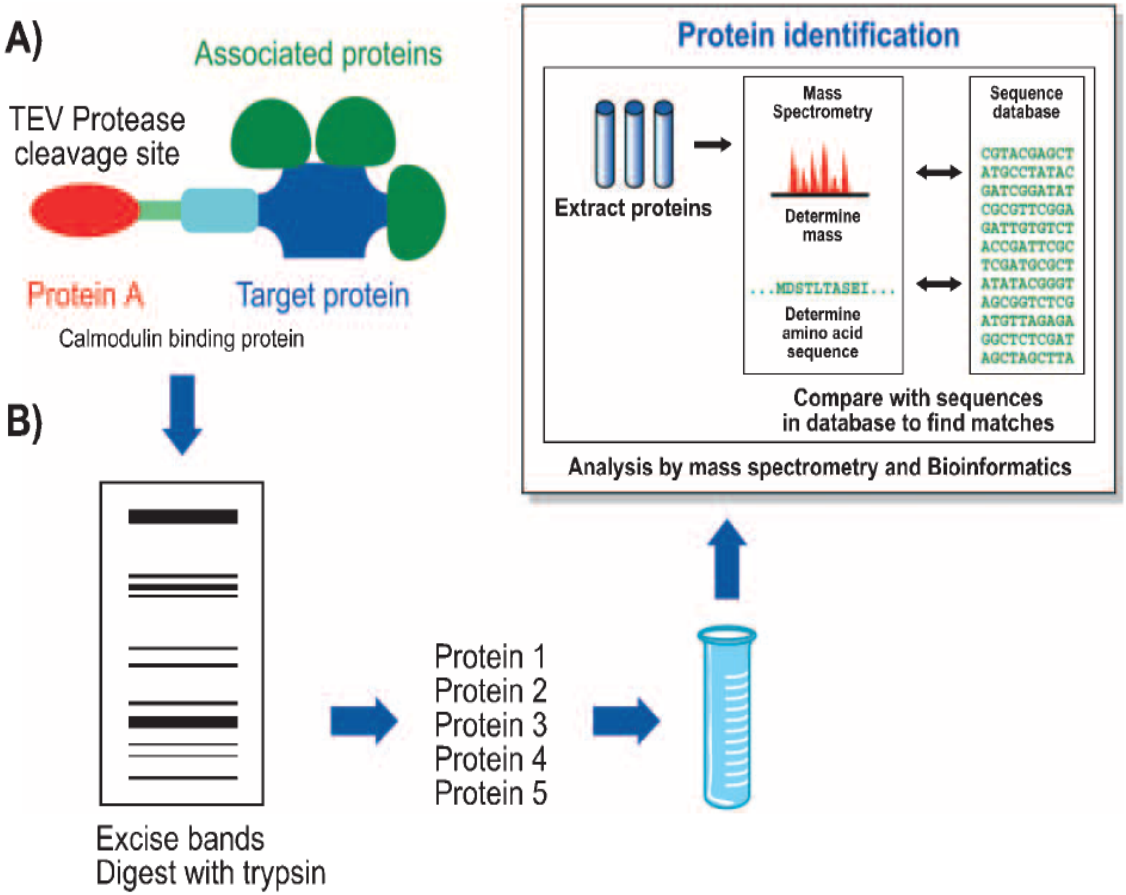


Figure 1 Synopsis of the screen. **a**, Schematic representation of the gene targeting procedure. The TAP cassette is inserted at the C terminus of a given yeast ORF by homologous recombination, generating the TAP-tagged fusion protein. **b**, Examples of TAP complexes purified from different subcellular compartments separated on denaturing protein gels and stained with Coomassie. Tagged proteins are indicated at the bottom. ER, endoplasmic reticulum. **c**, Schematic representation of the sequential steps used for the purification and identification of TAP complexes (left), and the number of experiments and success rate at each step of the procedure (right).

TAP-MS

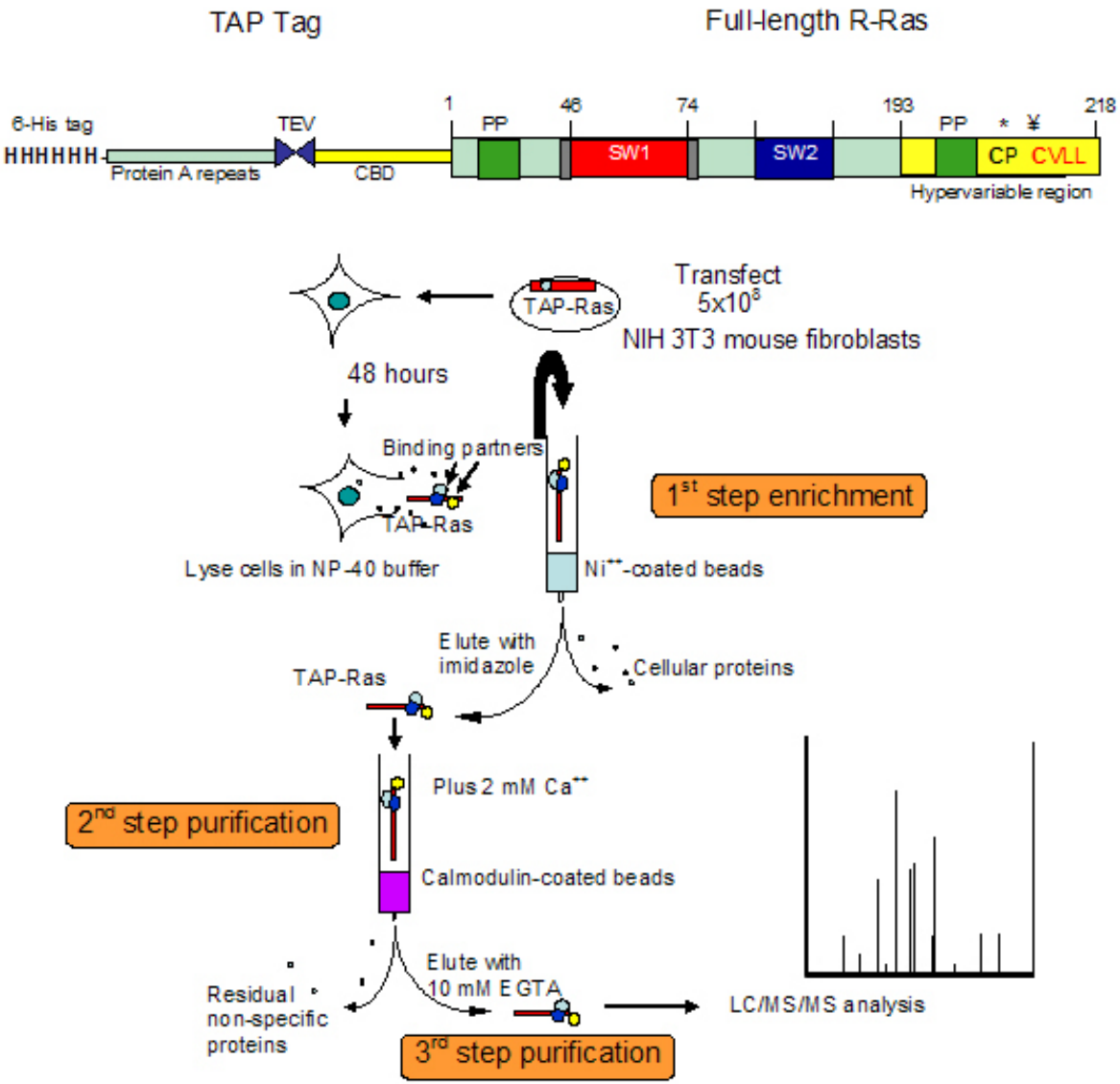


(Guillaume Rigaut, *et al.* 1999)



(Arnaud Droit, *et al.* 2005)

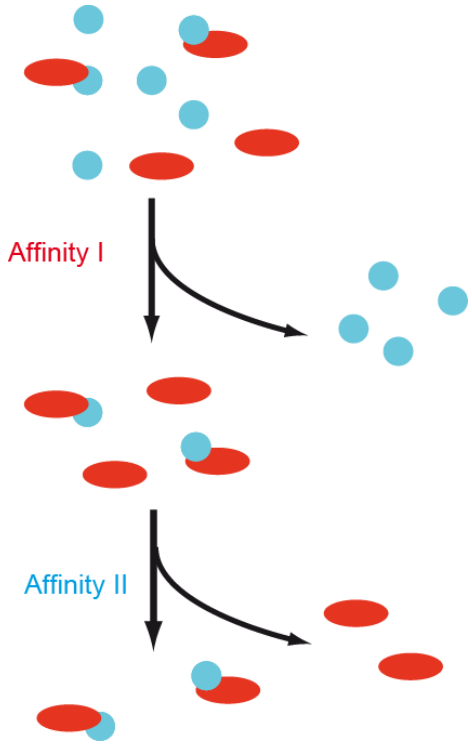
TAP-TAG in Mammalian cells



Tandem affinity purification protocols: Nature and position of the affinity tag

Right tag on the right place

Tandem affinity purification



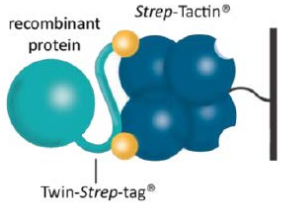
Which affinity tags?

10His, Twin-strep, 3Flag, (HA, GFP, CBP)

The position maters

Two different subunits

Location has tremendous impact



Abdulrahman, Anal Bioch, 2009
Koleschnikova et al, in prep

Engineering of Mammalian cell lines

Random integration (based on antibiotic selection)

Site specific integration by RMCE

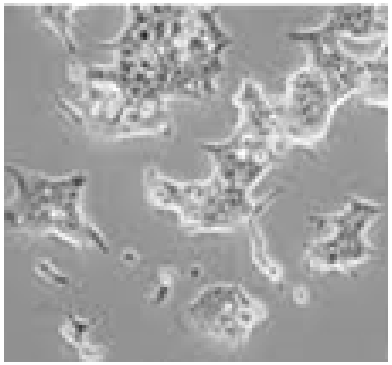
DNA Editing tools: Zinc-Fingers, Talen, CRISPR-Cas9

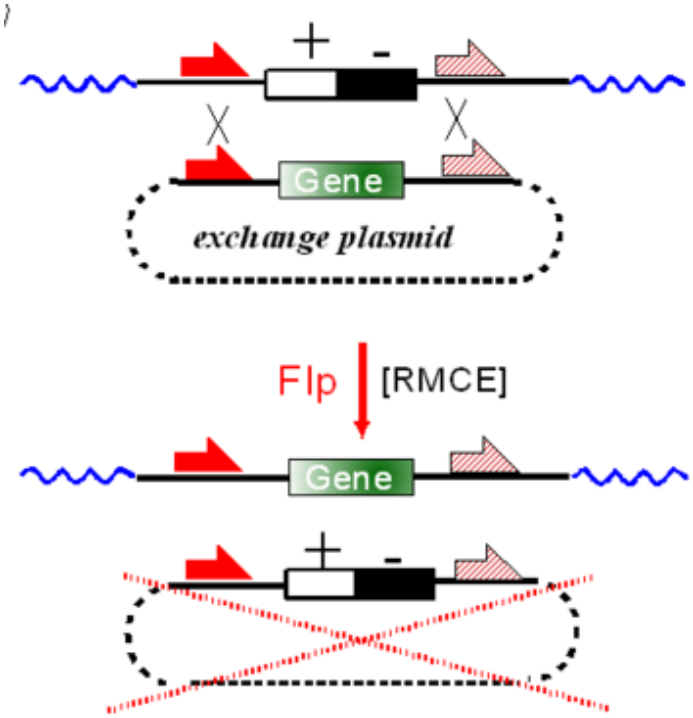
Recombinase Mediated Cassette Exchange (RMCE, Flp-IN)

Isogenic Expression Cell Lines

Targetted intergration

FRT sites (master cell line and transfer vector), recombinase





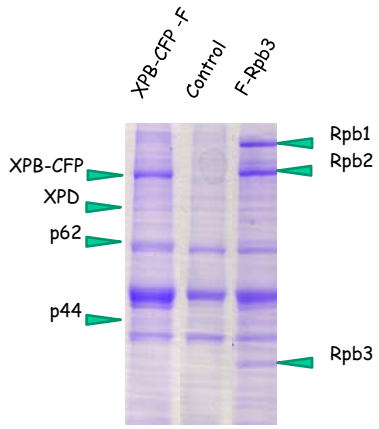
Clone the gene in pcDNA5FRT (1 week)

Transfection of HEK293 Flp-In cells and Hygromycin selection (3-4 weeks)
with pOG44 and pcDNA5FRT,

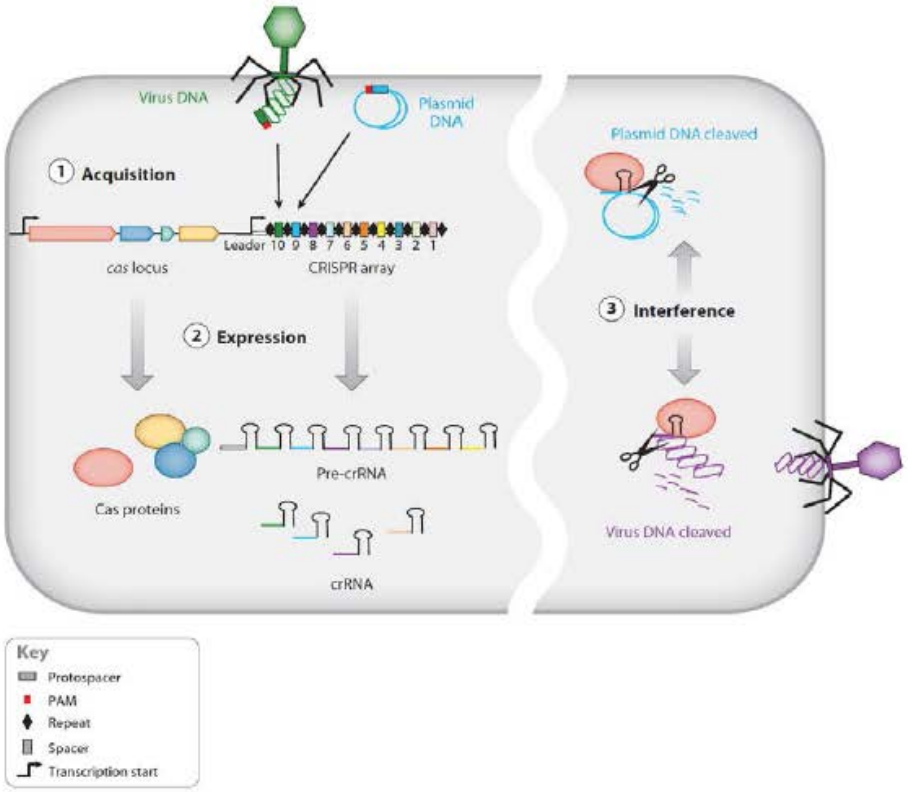
Establishment of a clonal population by limit dilution (3-4 weeks)

Clones screening by Western Blot (1-2 weeks)

Amplification of selected clones for first biochemical characterization (1-2 weeks)



What is a CRISPR-Cas system?



Bhaya et al., *Annu. Rev. Genet.* **45**, 273-97 (2011)

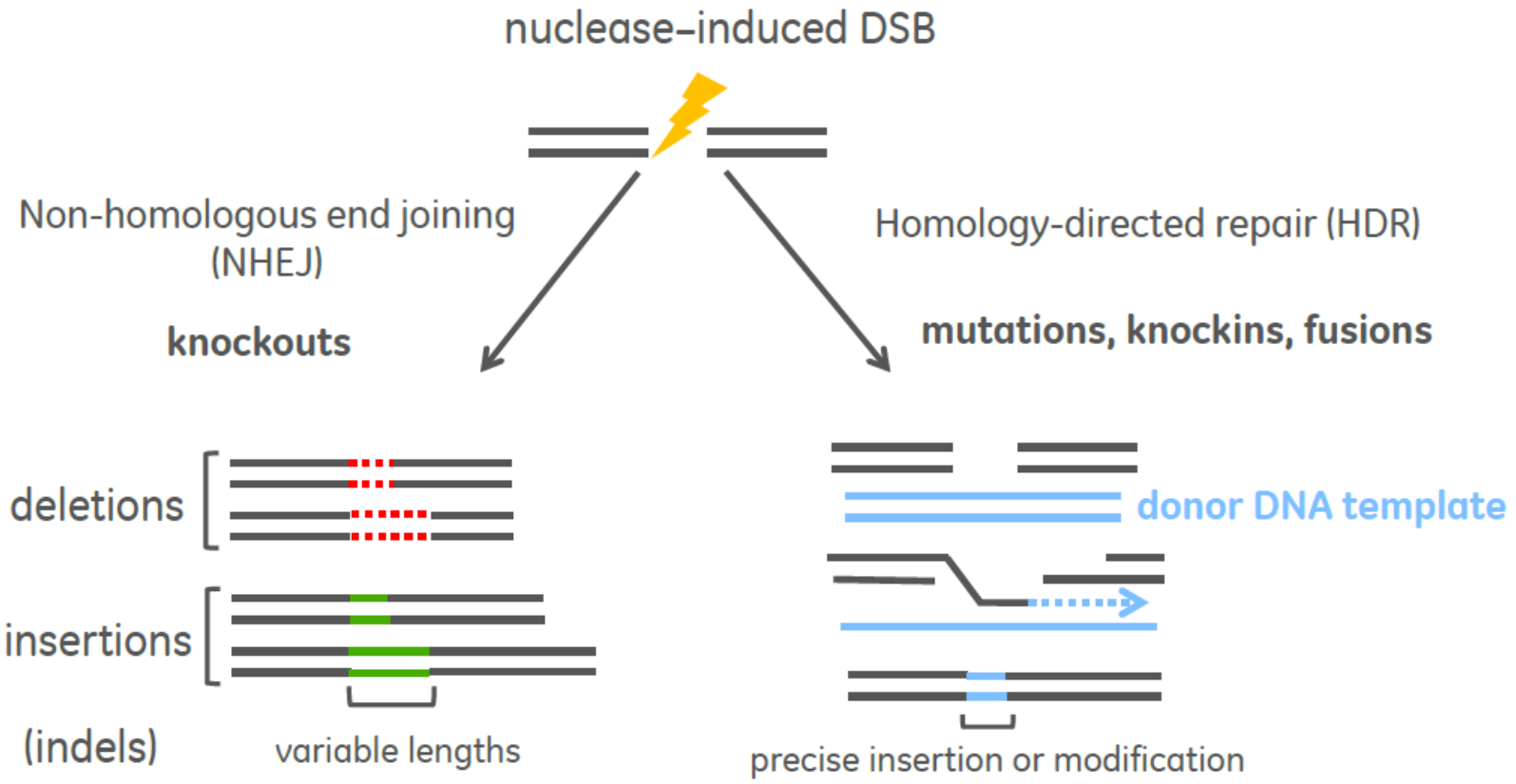
Mechanism of adaptive immunity in bacteria and archaea

Evolved to adapt and defend against foreign genetic material (i.e., phage, horizontal gene transfer, etc.)

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

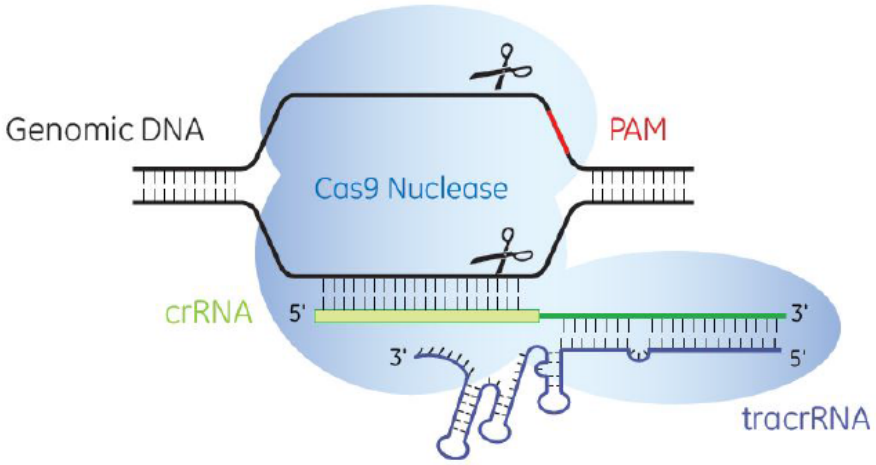
Cas: CRISPR-associated proteins

Editing by repair of double-strand breaks (DSB)



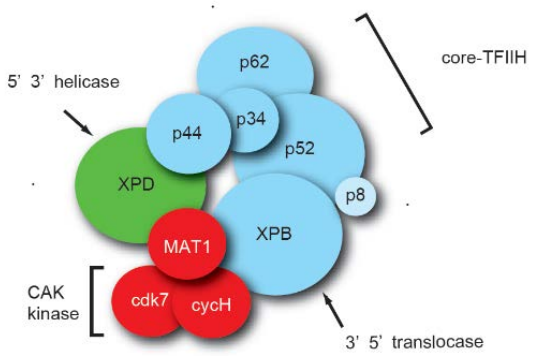
Required components for CRISPR-Cas9 gene knockout

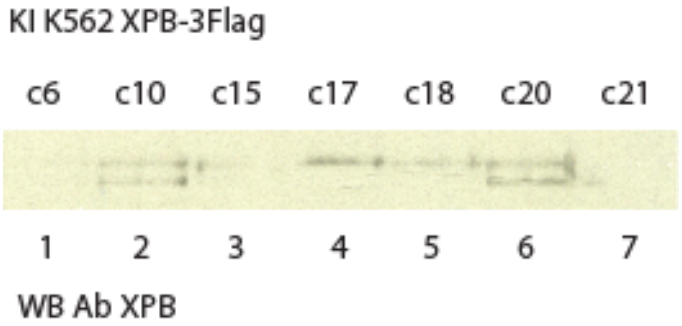
1. **Cas9 Nuclease** – creates double-strand break
2. **Guide RNA** – recruits Cas9 and directs target cleavage

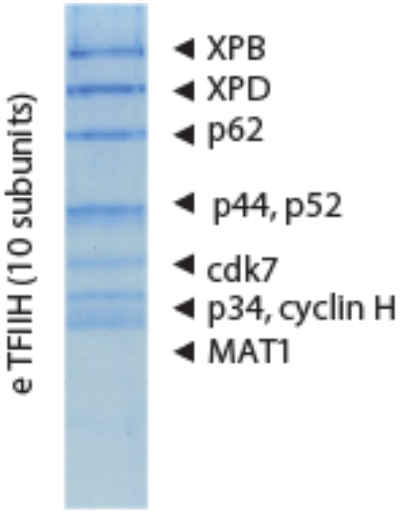


- **crRNA** – synthetic RNA comprising 20 nt target-specific sequence and fixed *S. pyogenes* repeat sequence
  - High-throughput synthesis to enable arrayed screening
- **tracrRNA** – Long synthetic RNA which hybridizes with crRNA

Two step purification from XPB-Flag KI cells







- Detailed analysis of the complex and its composition
- Endogenous sample as comparison
- Large scale production when the complex

- High resolution and live cell imaging

Coll JP Concordet/C Giovannangeli

Preparation and characterisation of Eukaryotic macromolecular complexes

Contribution of the baculovirus expression system for reconstitution of multiprotein complexes and dissection of the protein-protein interaction network

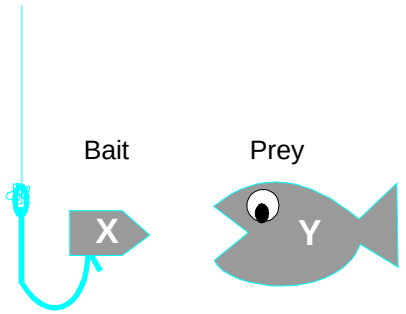
Potential inputs from genome engineering approaches for labelling mammalian proteins to facilitate isolation of endogenous complexes and their characterization in a cellular environment

Biochemical approaches for analysis of protein protein interactions

✓*In vitro*

- Co-immunoprecipitation
- GST, His, Strep-pull down assays
- ChIPs Protein arrays
- TAP-MS

Bait – Prey model



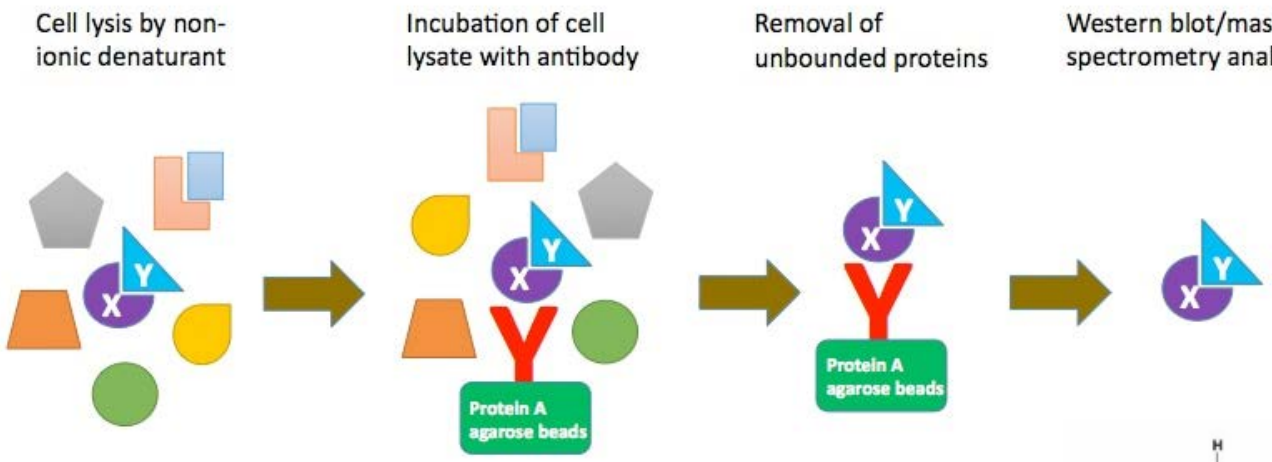
does X bind with a protein?

✓*In vivo*

- Yeast two-hybrid system
- Phage display

Physical interaction between protein binding domains

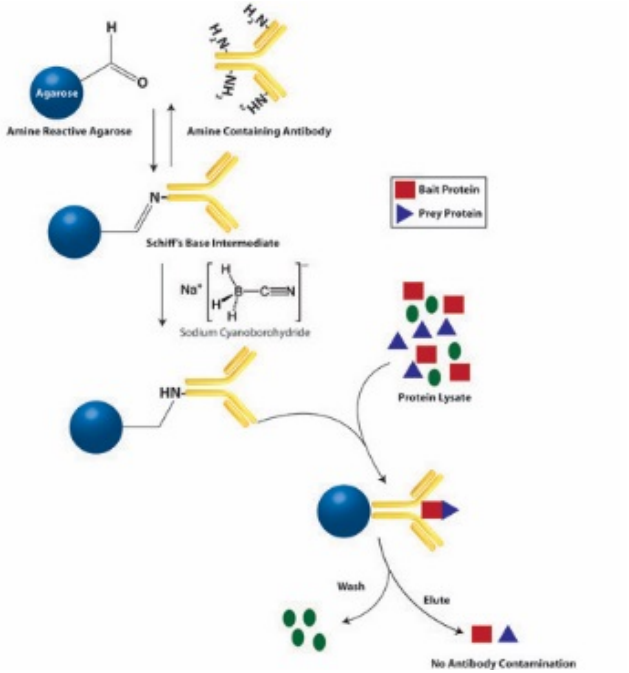
Pull down/Immuno-precipitations

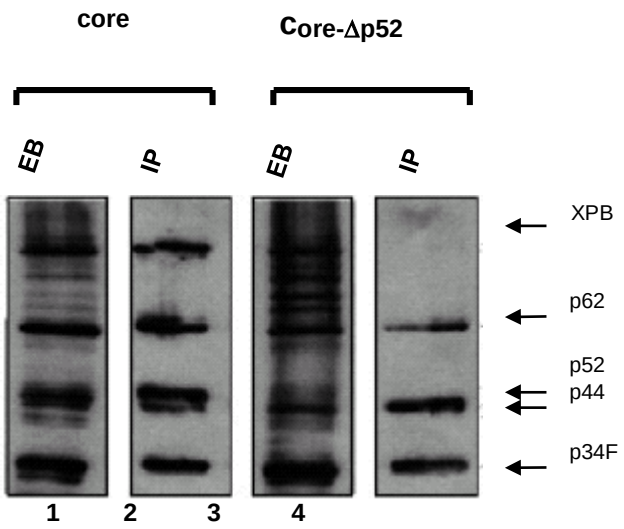


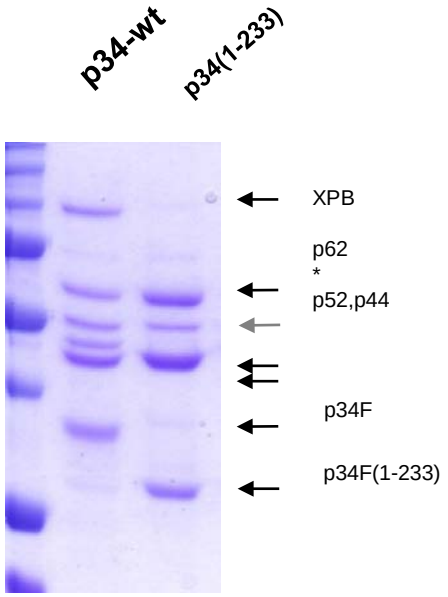
Popular (Flag, HA..) or specific epitopes

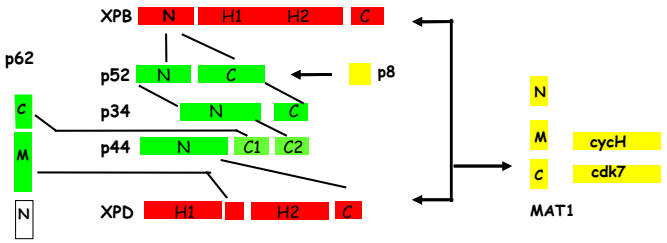
Non-Ab pull down: Affinity tags (His, Strep….)

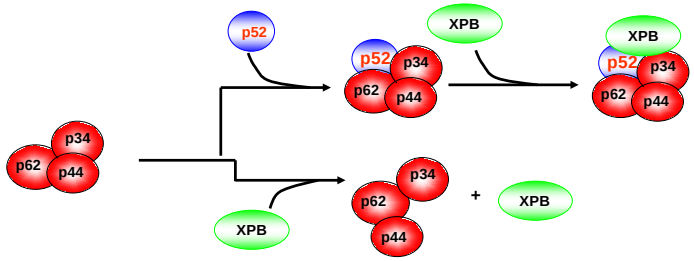
Conventional resin or magnetic beads









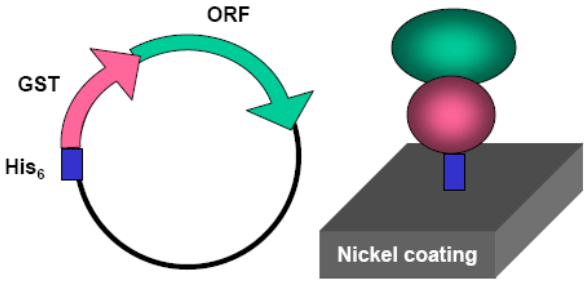


Do not forget controls

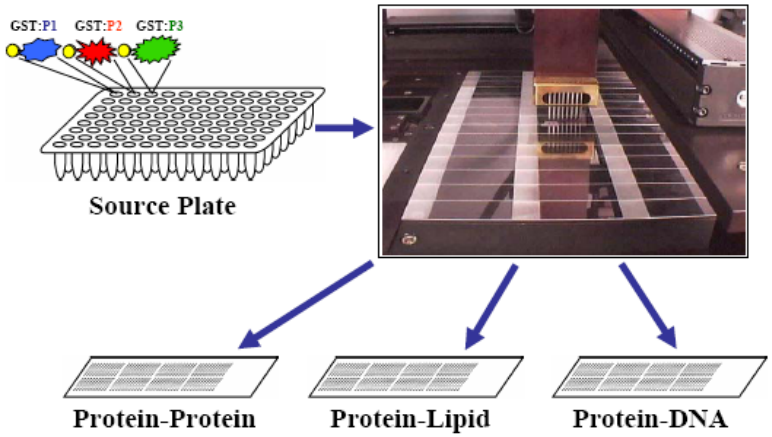
Protein arrays: the HTP version of co-IPs

Protein (Antigen) Chips

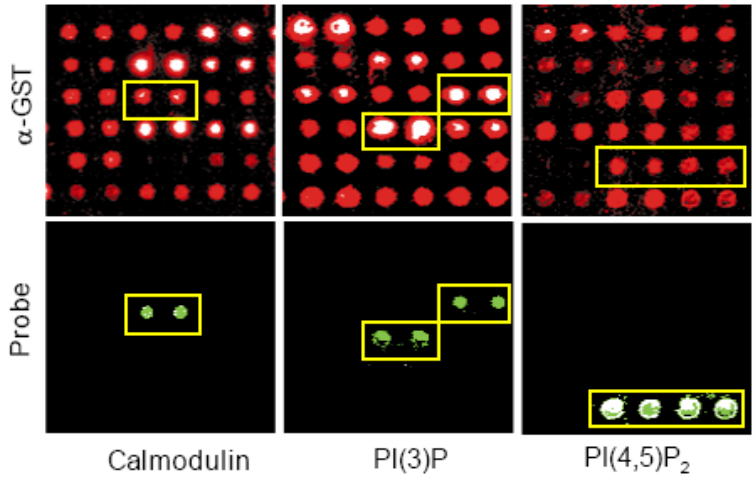
H Zhu, J Klemic, S Chang, P Bertone, A Casamayor, K Klemic, D Smith, M Gerstein, M Reed, & M Snyder (2000).**Analysis of yeast protein kinases using protein chips**. Nature Genetics 26: 283-289



Printing the Yeast Proteome

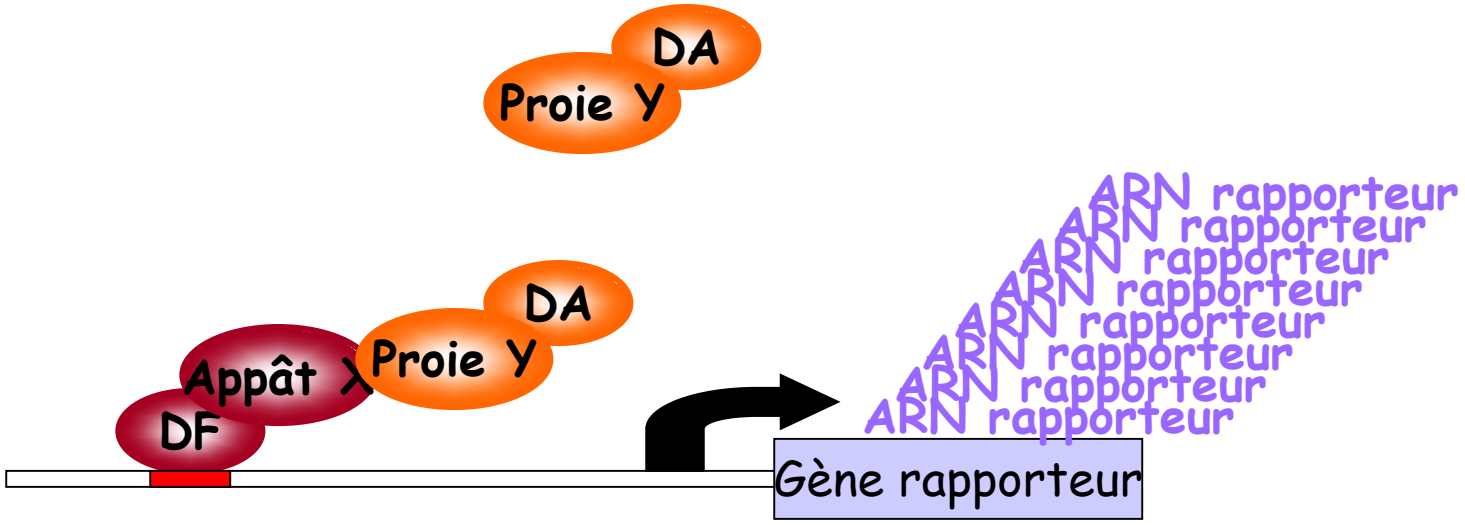


Cy3-labelled probes



Yeast two-hybrid system

- Detecting protein-protein interactions in yeast
- Transcriptional regulator system
- “prey”-”bait” model :fusion proteins with a transcriptional activating domain (AD, prey), a DNA-binding domain (DBD, bait)
- Term “two-hybrid” derives from these two chimeric proteins.
- Most commonly used method for large scale, high-throughput identification of potential protein-protein interactions

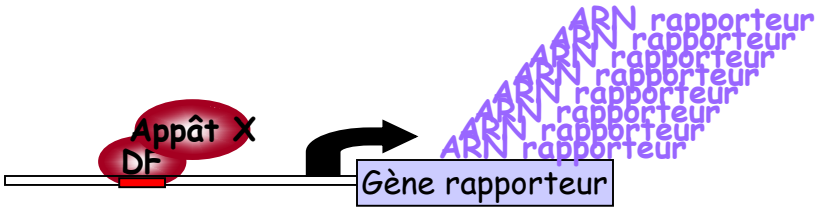


Quelles interactions sont détectées par un crible double hybride?

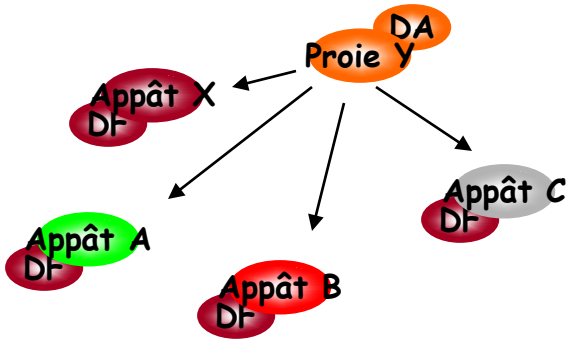
- Interactions permanentes

- Interactions transitoires dont les interactions enzyme-substrat (ex: 10 à 40 % des interactions kinase-substrat).

- Interactions qui n'existent pas physiologiquement
→ Faux-positifs



L'appât auto-activateur
(activation de la transcription en absence d'interacteur)



La proie collante
(interagit avec un très grand nombre d'appâts)

Quelles interactions ne sont pas détectées par un crible double hybride?

- Les interactions impliquant des protéines qui présentent des problèmes:

  - structuraux (repliement)
  - stabilité
  - toxicité
  - mauvaise localisation (protéines membranaires)
  - modification post-traductionnelle

- Estimation: 80 à 90 % des interactions sensées exister, n'auraient pas été détectées → Faux-négatifs

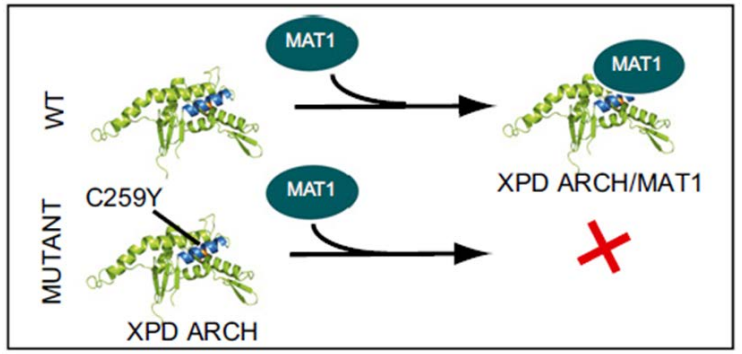
→ évolution des méthodes de double-hybrides pour palier à ces problèmes.

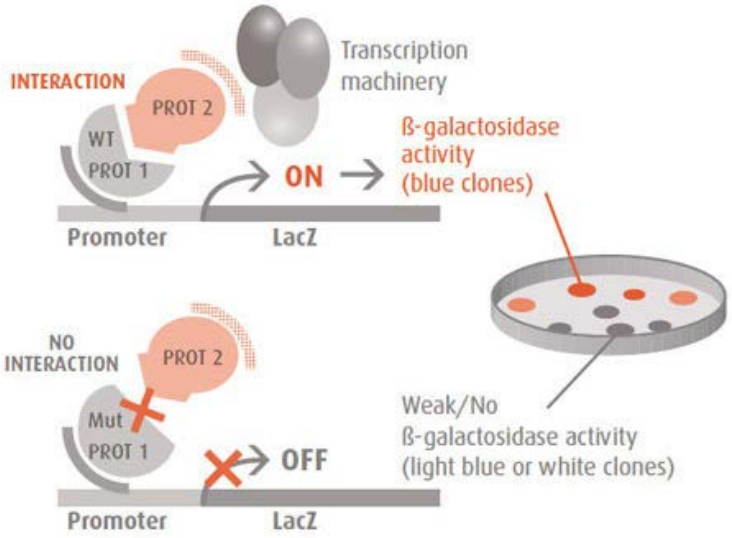
Loss of Affinity Mutant screening (LAM)

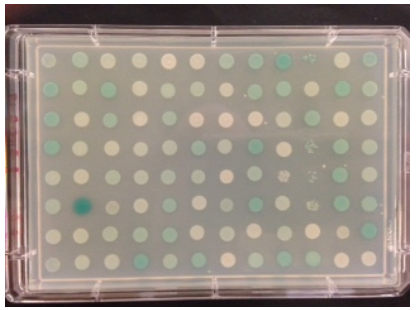
Y2H adapted to identify binders, inhibitors of PPI or select mutations

Indentification of XPD mutants that have lost the capaticity to interact with MAT1 but still interact with p44 using Y2H.

30 000 mutants have been tested, 150 sequenced
25 have been selected for further analysis







Coll JC Rain (Hybrigenics)

Method	Advantage	Disadvantage
Co-immunoprecipitation	Independent of cloning and ectopic gene expression Rapid procedures	Cross-reactivity of antibody Antibody bleeding from column
TAP-MS	Generically applicable approach Ability to purify low abundant proteins/protein complexes	Ectopic gene expression necessary Protein-tag might influence protein function
Far-western analysis	Detection of stable interaction	Fusion protein labeled protein and bait protein
GST-pull down assays	Applicable to very weak protein interactions	Complex formation in-vitro Competition with in-vivo pre-assembled complex
Protein arrays	High-throughput assay Disease diagnosis	Difficulty of protein chip production
Yeast two-hybrid system	Highly sensitive detection Applicable to a wide range of protein interactions No biochemical purification	Stability of folding and activity in yeast Not post-transcriptional modification
phage display	Random library screening of many cDNAs through panning cycle	Size of limitation of protein sequence Incorrect folding or modification

Preparation and characterisation of Eukaryotic macromolecular complexes

Contribution of the baculovirus expression system for reconstitution of multiprotein complexes and dissection of the protein-protein interaction network

Potential inputs from genome engineering approaches for labelling mammalian proteins to facilitate isolation of endogenous complexes and their characterization in a cellular environment

Overview of popular biochemical methods to analyse protein-protein interactions

Thank you