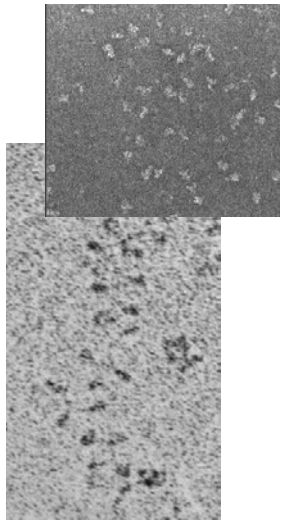
Preparation and characterisation of Eukaryotic macromolecular complexes

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

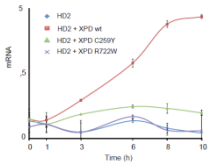
Potential inputs from genome engineering approaches for labelling mammalian proteins to facilitate isolation of endogenous complexes and their characterization in a cellular environment

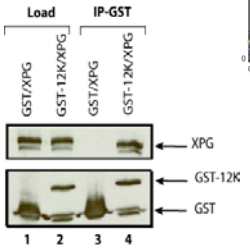
Functional characterization

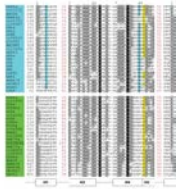
Cellular imaging



Bio computing

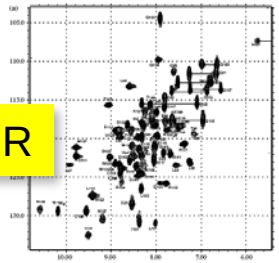






Partner identification

Cryo-EM



NMR

Domain/Orthologs indentification

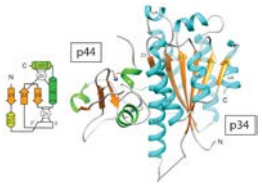
Targets

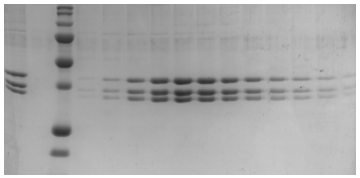
Structure

Production

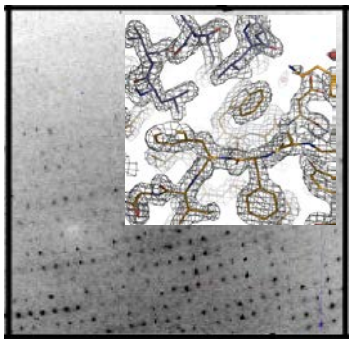
X ray crystallography

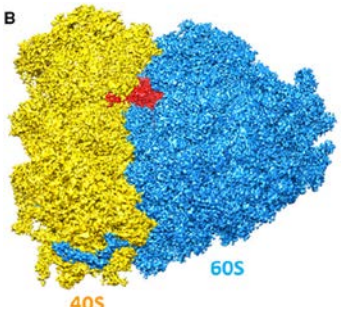
Modelling





Biophysical characterization





Recombinant or endogenous?

Isolate sample from native source

Advantages – Protein solubility, authenticity

Disadvantages – Expense/effort, yield, abundancy

Popular sources: E coli, yeast, HeLa cells
Model imposed by the biological question

Bacterial expression

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

Disadvantages – Protein solubility, lack of post-translational modifications

Eukaryotic expression

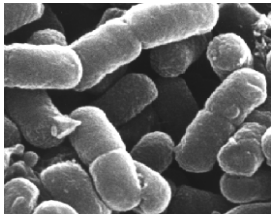
Advantages – Protein solubility, post-translational modifications

Disadvantages – Expensive, low yield, proteases, time consuming

Recombinant expression

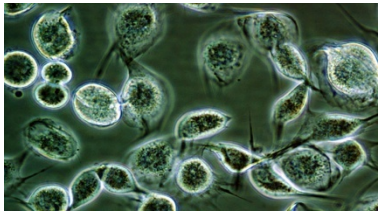
Prokaryotic

E. coli,
B. subtilis
....



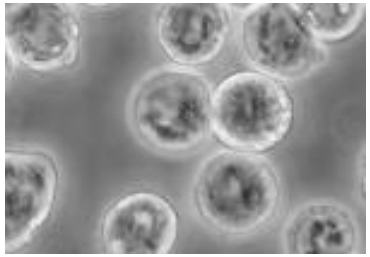
Eukaryotic

Yeast
Insect cells
Mammalian



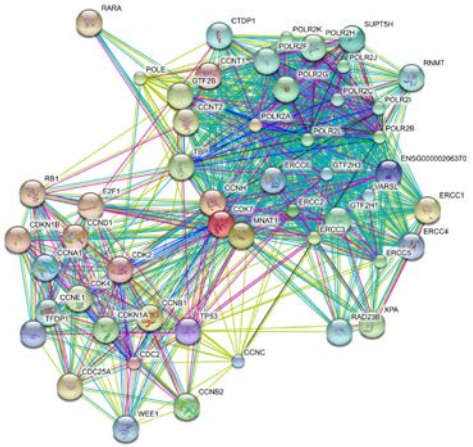
Cell free systems:

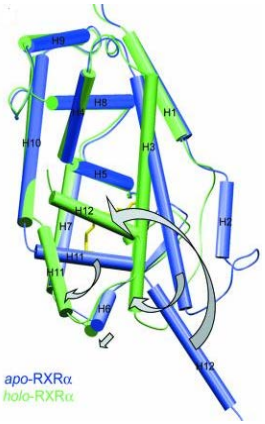
E coli
Wheat germ,
Insect

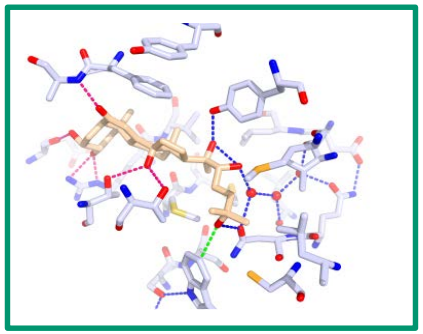


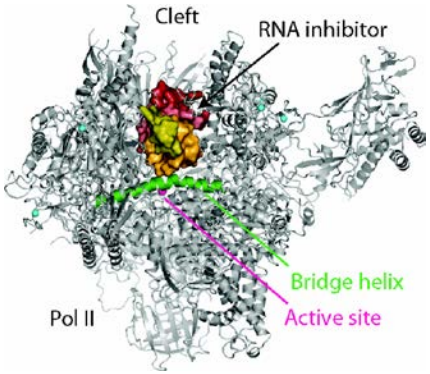
Most proteins do not function as isolated particules……

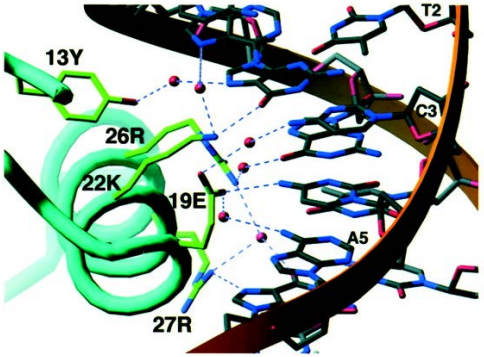
…. but interact with partners to fullfill their fonction.











Types of complexes

Composition and structure

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

Homo- and hetero oligomeric complexes

Non obligate and obligate

Protomers/Subunits are not found as stable structures in vivo

Protomers/Subunits exist independantly

Lifetime of complexes

Permanant interactions: stable/only exist in complexed state

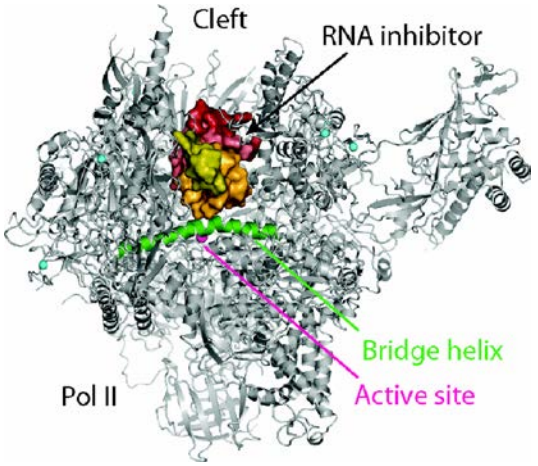
Transient interactions associate and dissociate in vivo

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

Obligate and non-obligate

Protomers/subunits are not found as stable structures in vivo

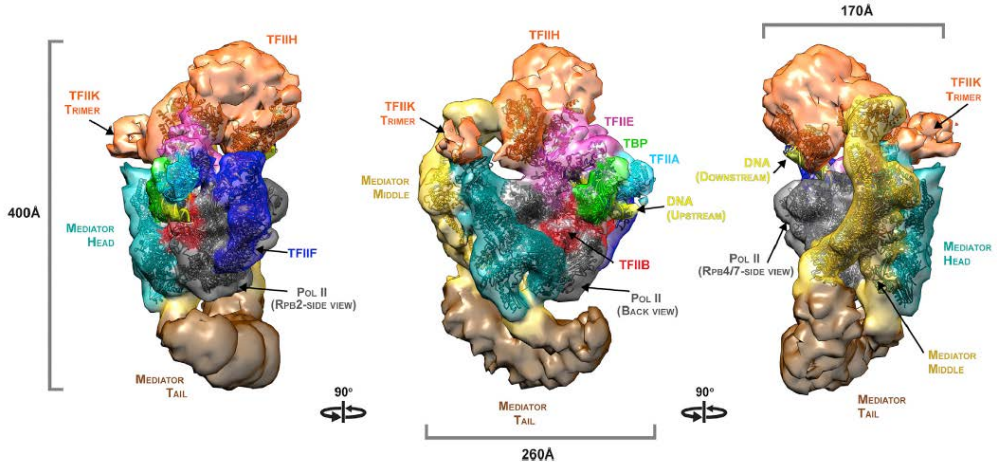
Protomers/subunits exist independantly



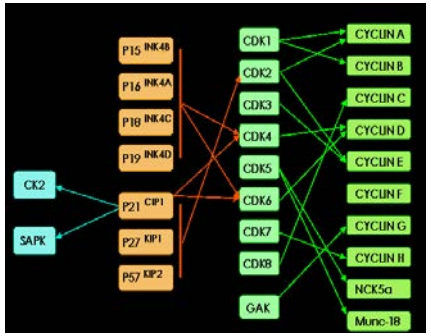
RNA Polymerase II

Regulation complexes

- transient interactions
- most protomers are obligate multi-protei,complexes



Eukaryotic pre-initiation complex



Cdk/Cyclin/Inhibitor

Transient and stable complexes

Lifetime

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

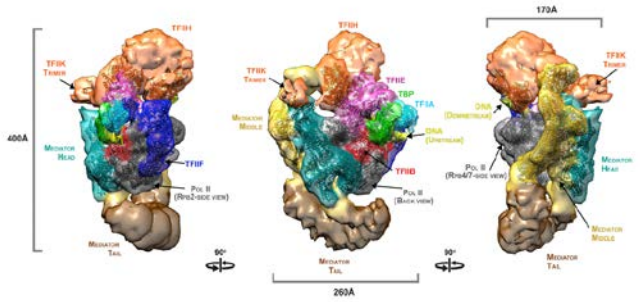
Intermediate

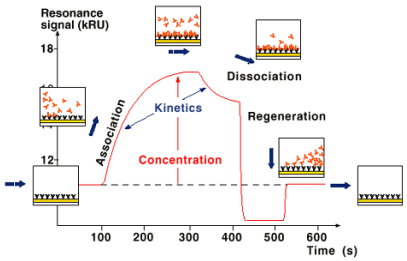
(antibody-antigen, TCR-MHC-peptide, signal transduction PPI), K_d μM-nM

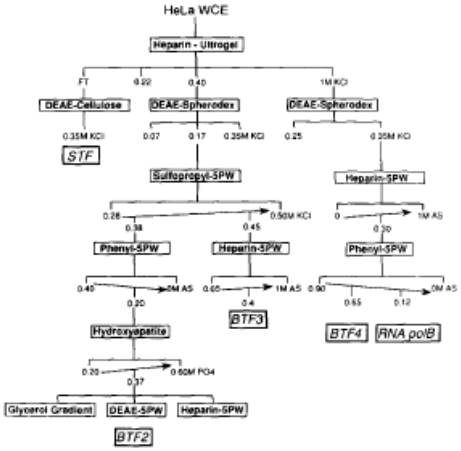
Strong

(require a molecular trigger to shift the oligomeric equilibrium)

K_d nM-fM



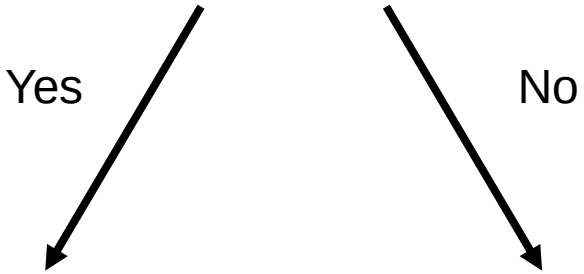




Implications for production

Non-obligatory / Obligatory

Transient / Stable



Produce components independently and reconstitute the complex *in vitro*

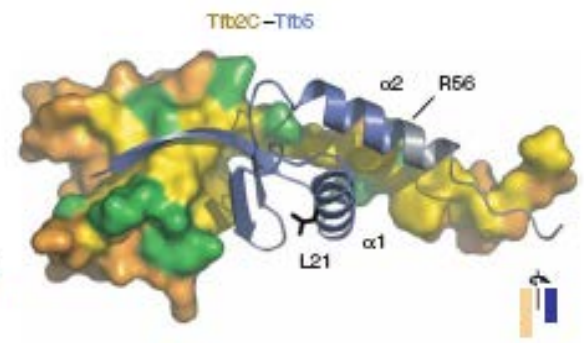
Single gene expression

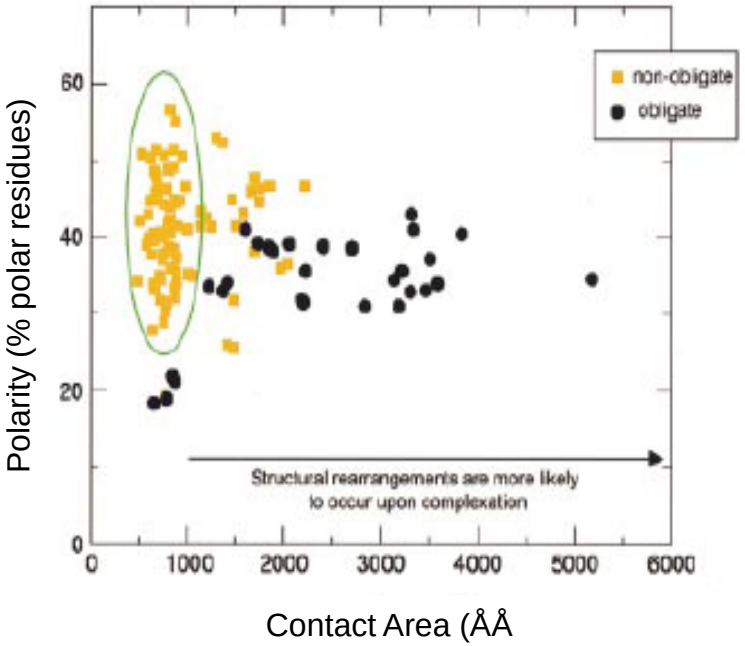
Produce components of the complex simultanously which are assembled *in vivo*

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** being 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

Bacteria (E. coli)

Co-transformation with several single promoter plasmids

Transformation with multigene expression plasmid

Viral system (baculorius, vaccinia)

Co-infection of insect cells by several viruses

Infection with a multigene expression virus

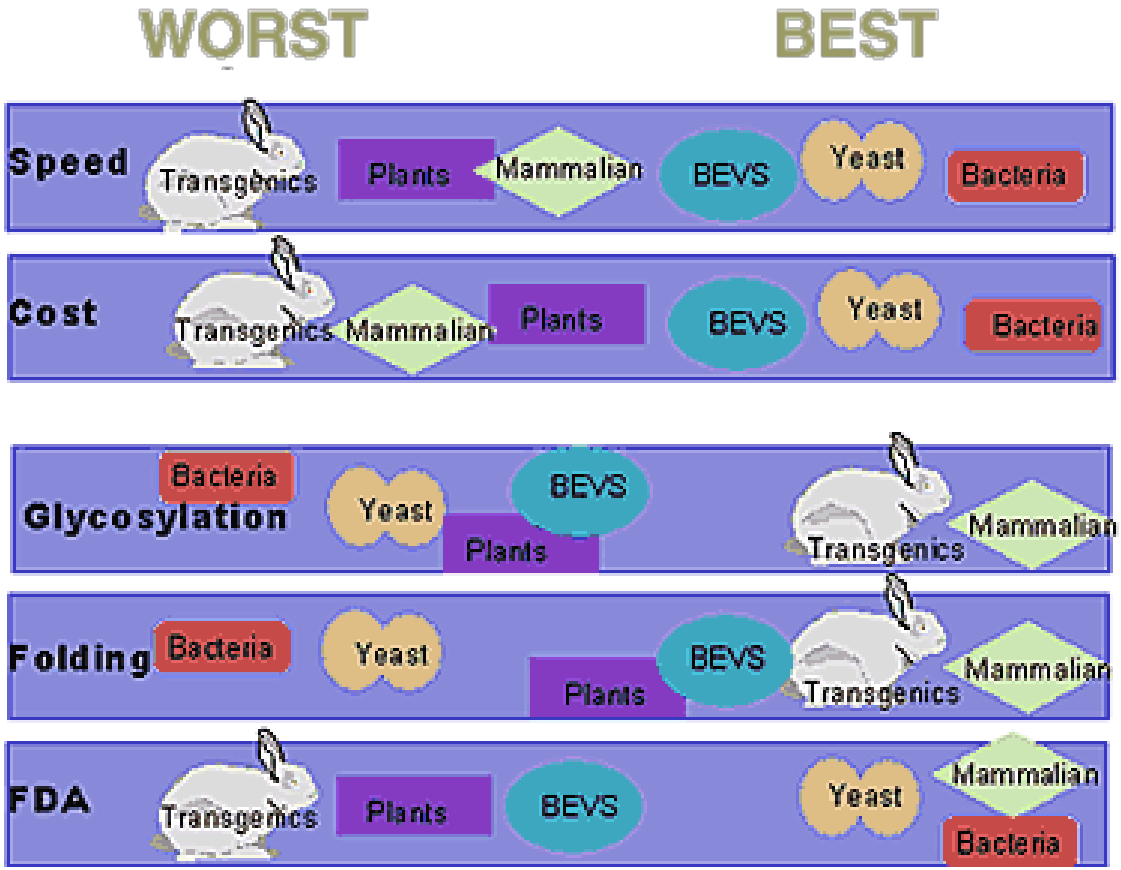
Which expression system?

Baculovirus Expression Vector System

Suitable for co-expression

High levels of heterologous gene expression

BEVS



What are baculoviruses ?

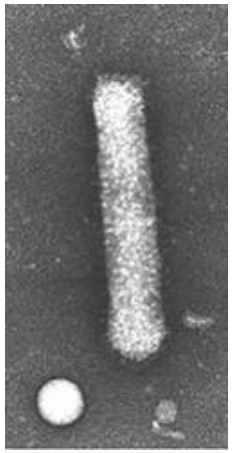
Baculoviruses form a group of viruses that infect specifically insects *

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



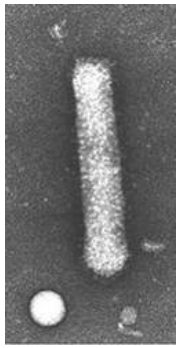
Autographa **c**alifornica **M**ultiple **N**uclear **P**olyhedrosis Virus **(AcMNPV)**

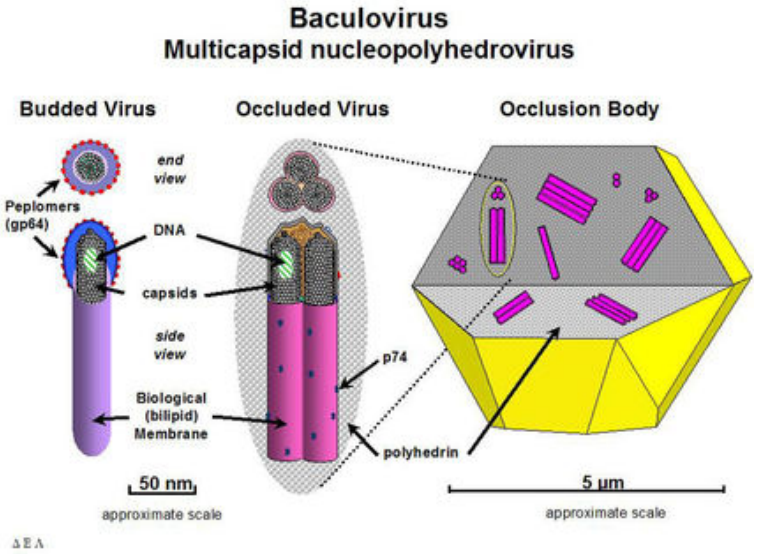
alfalfa looper = cabbage looper

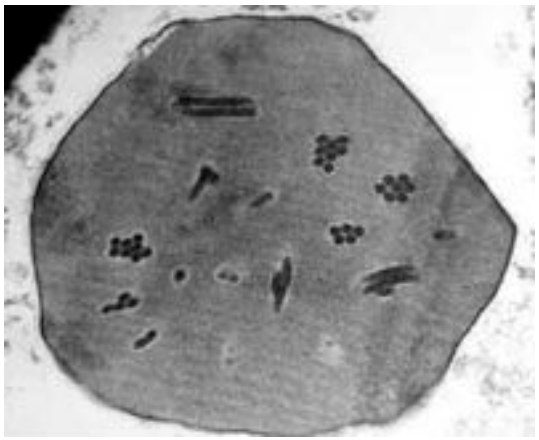
AcMNPV infects 30+ insects

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 (protection) virions are part of occlusion bodies (OB) or polyhedra. Para-crystalin matrix, composed of polyhedrin (50% of the total protein mass)

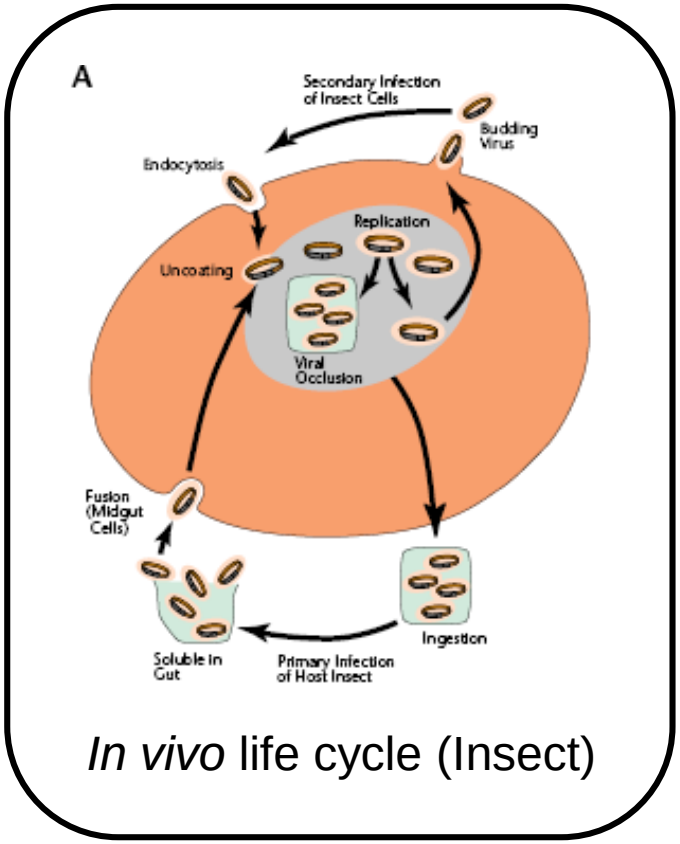




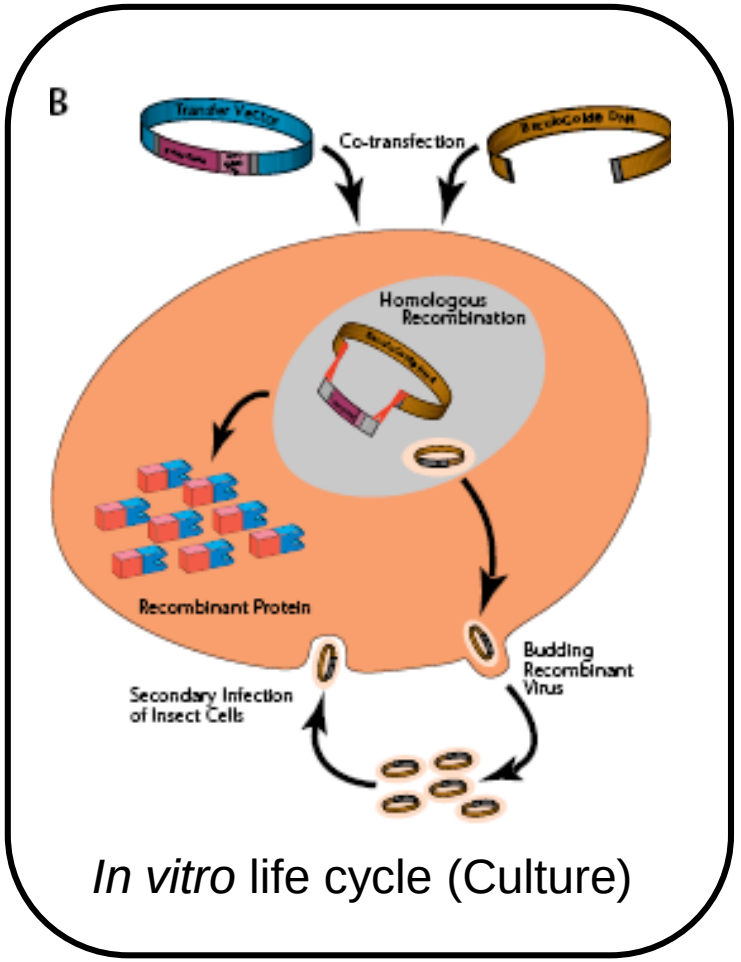


Strong promoters (Polyhedrin, p10)
Express lots of protein 36-48 h post-infection

Viral Occlusion (Polyhedron “package”) not required for virus replication in vitro (cellular system)



In vivo life cycle (Insect)



In vitro life cycle (Culture)

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

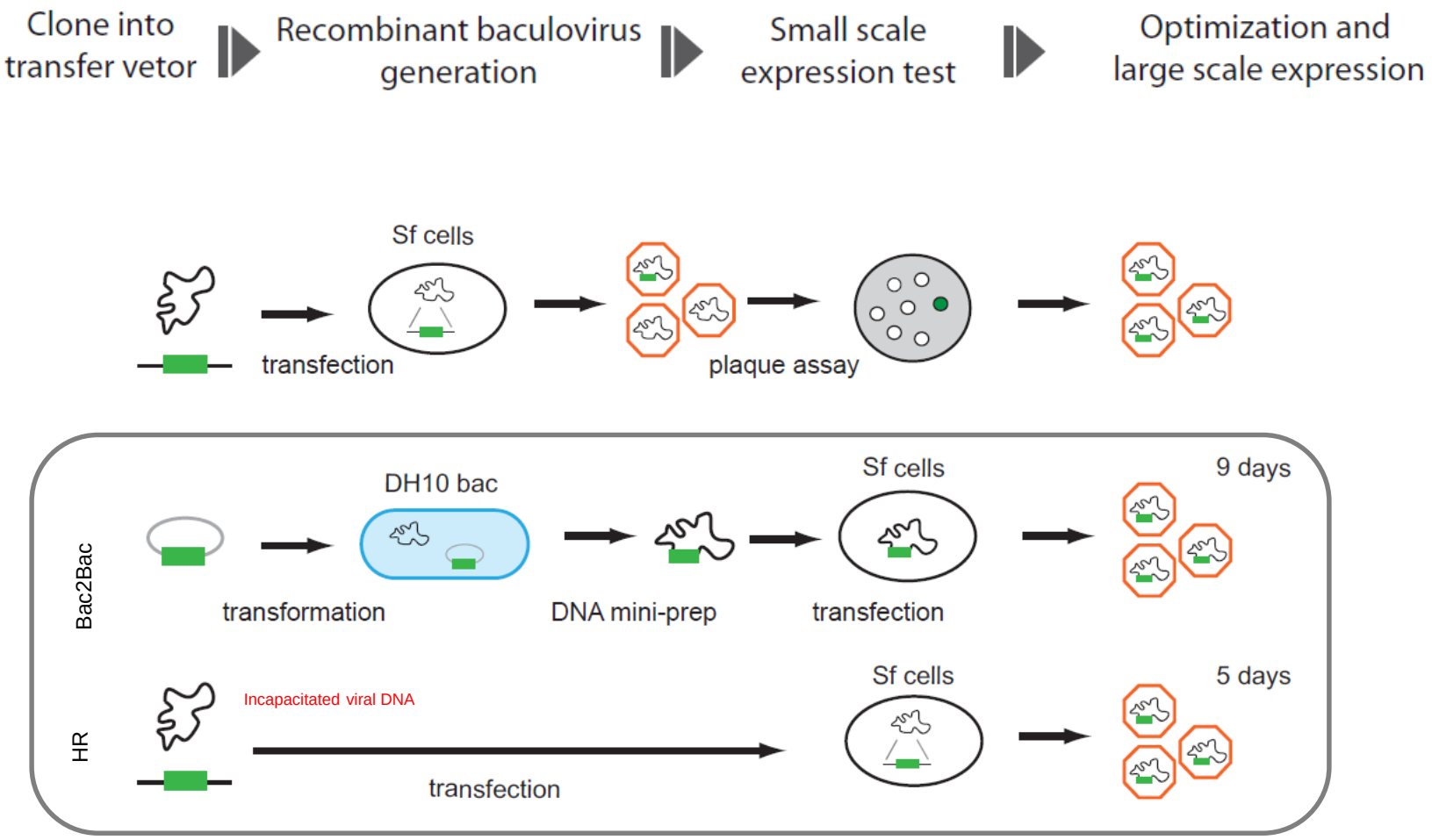
Promoter Gene (GOI) Terminator

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

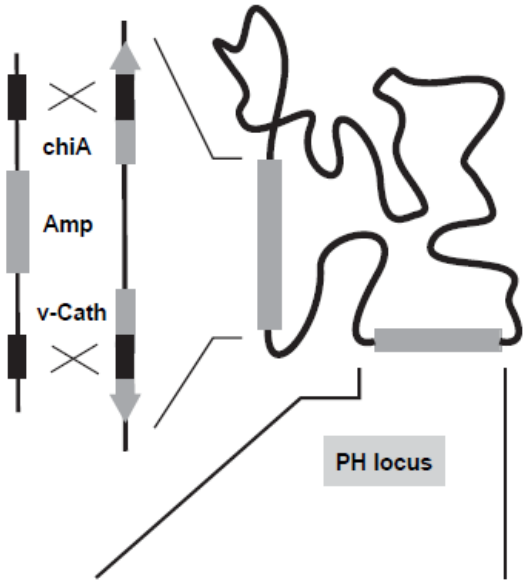
- Either segment from viral genome for homologous recombination in insect cells between the transfert vector and the viral DNA

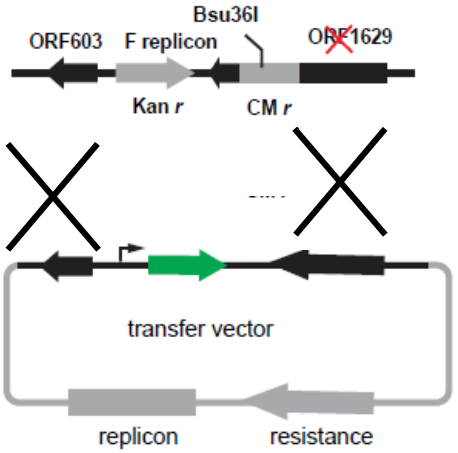
- Or transposons (Tn7L and Tn7R) recombination sites (LoxP) when a bacmid is to be used

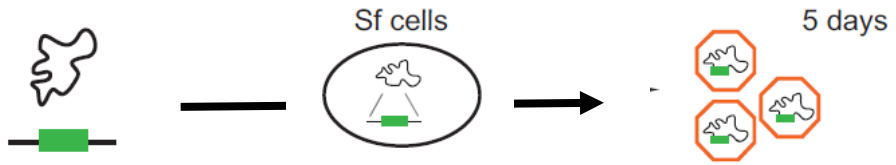
An expression flowchart for BV expression



Homologous recombination in insect cells with disabled viral genome







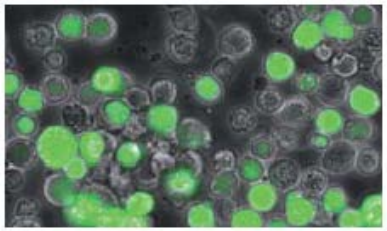
Disabled viral DNA

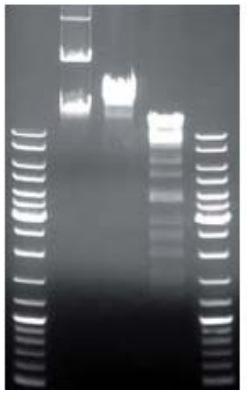
Co-transfection

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

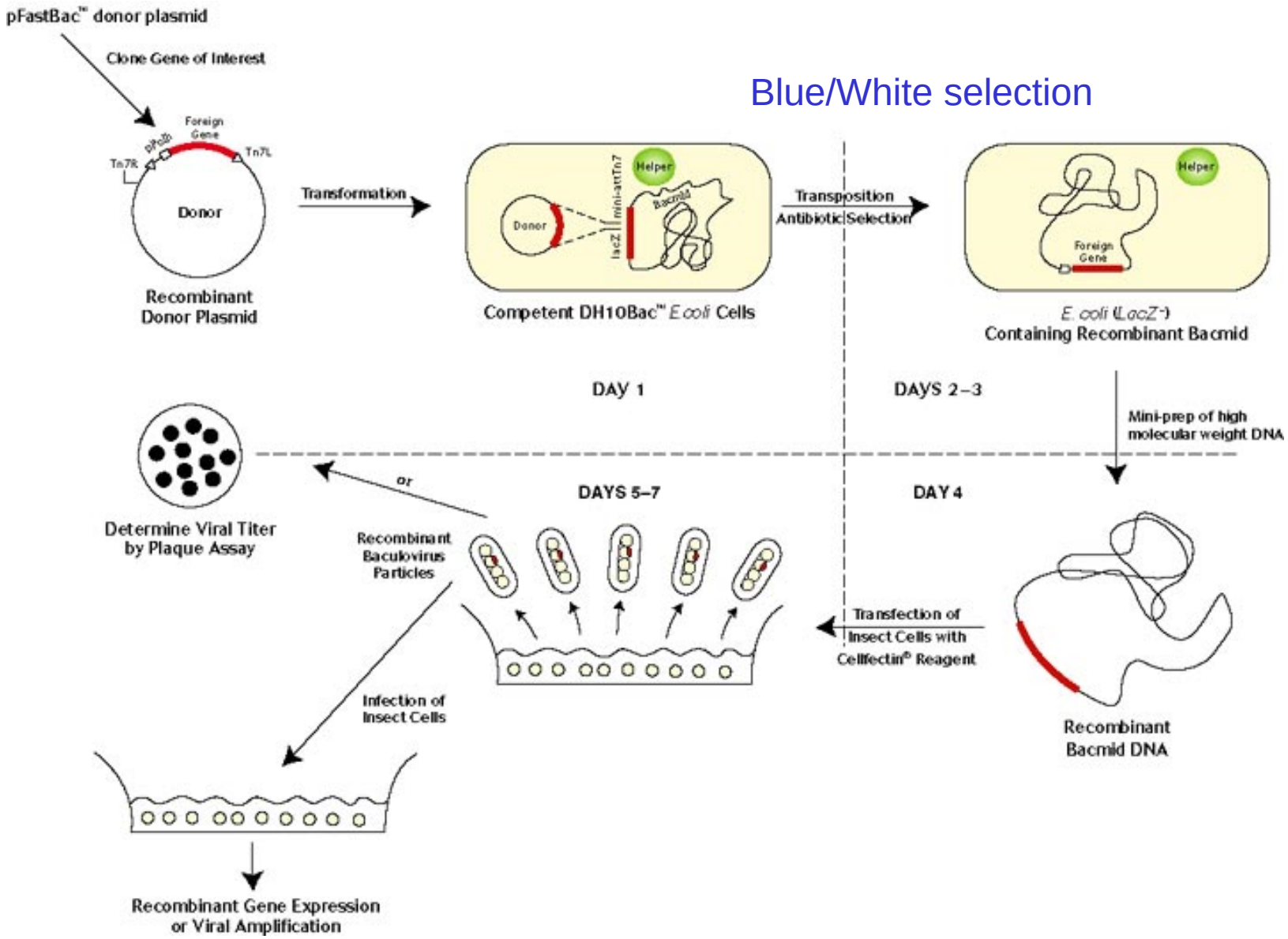
Recombination inserts the foreign gene (GFP) into the viral DNA, restores the deleted gene, allowing virus replication.

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

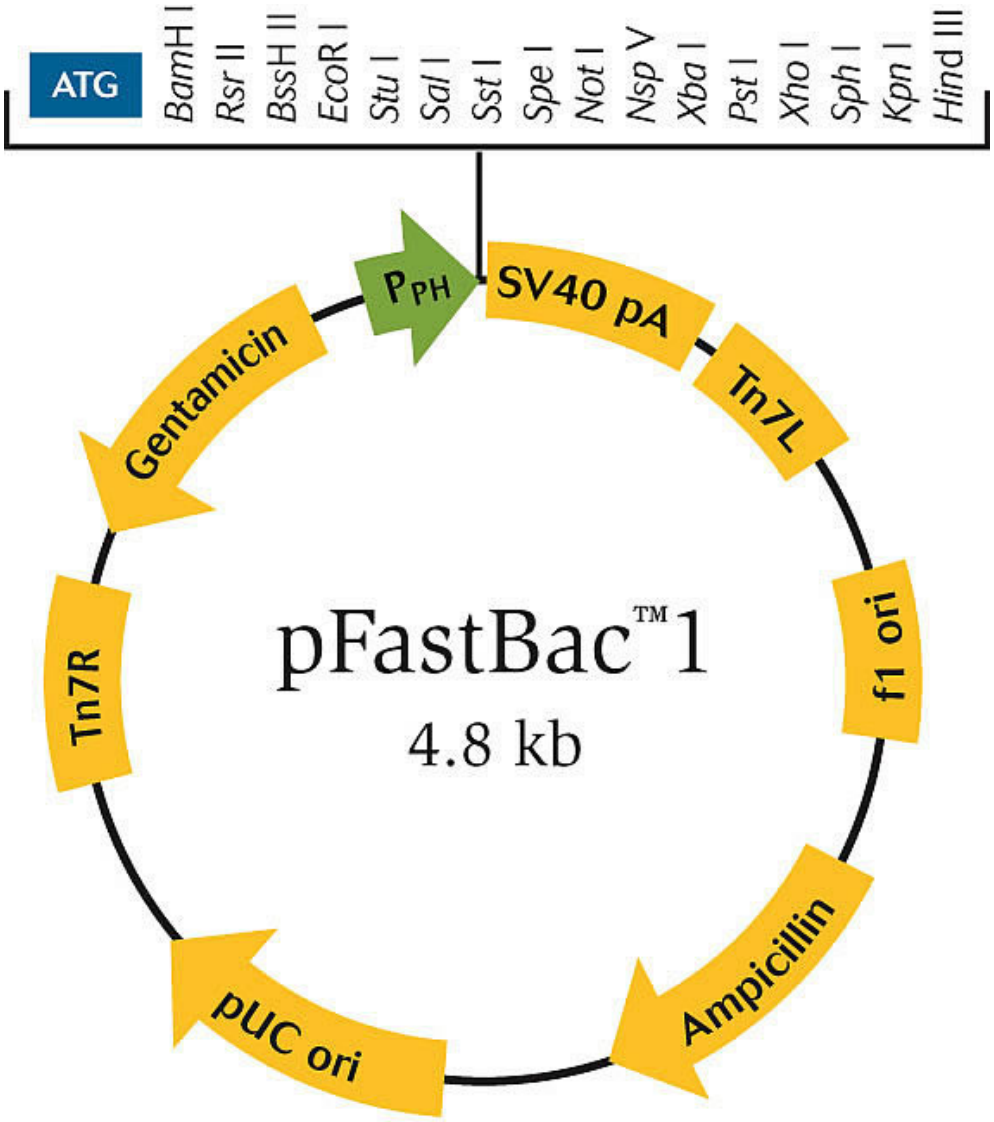




Transposition in E. Coli



Blue/White selection

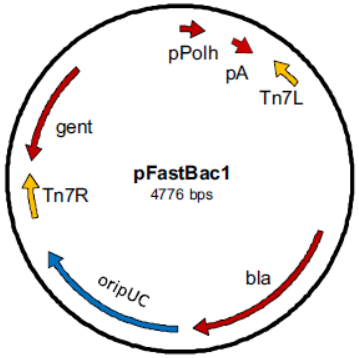

ATG
BamH I
Rsr II
BssH II
EcoR I
Stu I
Sal I
Sst I
Spe I
Not I
Nsp V
Xba I
Pst I
Xho I
Sph I
Kpn I
Hind III
P PH
SV40 pA
Tn7L
f1 ori
Ampicillin
pUC ori
Tn7R
Gentamicin
pFastBac™1
4.8 kb

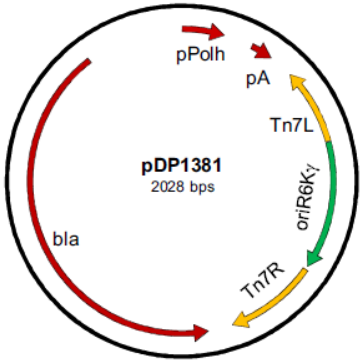
B2F vs B2B

1/ Low transposition efficiency: transposition into the E. coli genome and sub- optimal transposition system

2/ Bacmid preparation contanminated with transfer vector DNA

Bac to the Future

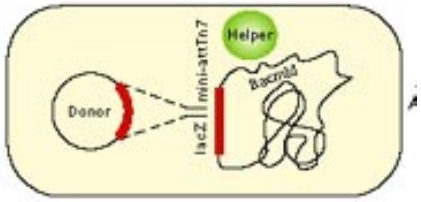




New baculovirus expression vectors: single antibiotic, conditional replication origin (oriR6Kγ)

New helper plasmid Tn7 transposase delivery vector

New E coli strain (chromosomal Tn7 site inactivated) that replaces the DH10 Bac: DE26

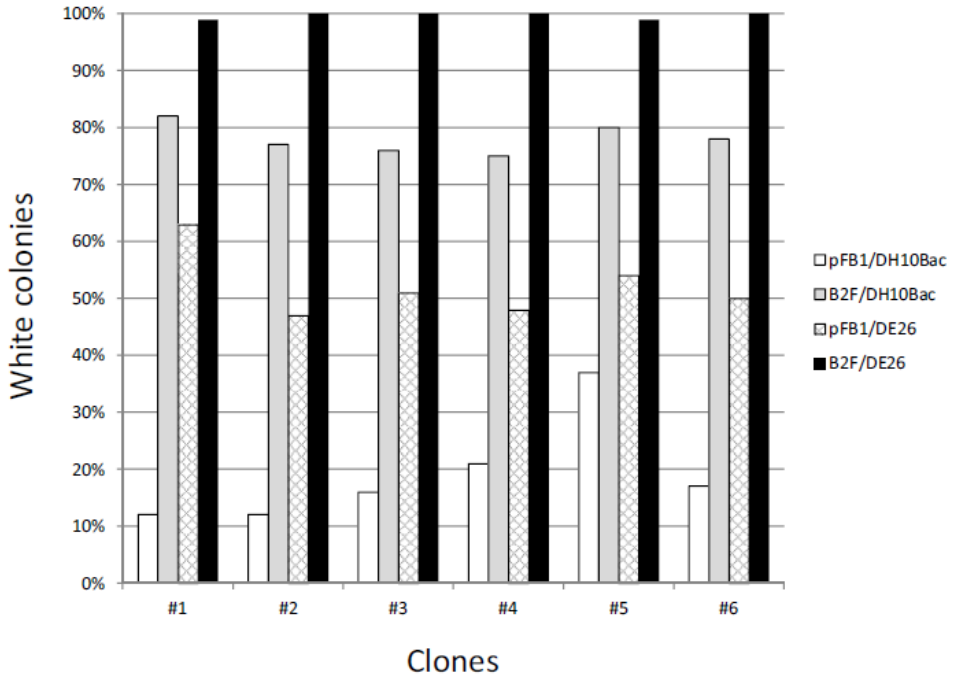


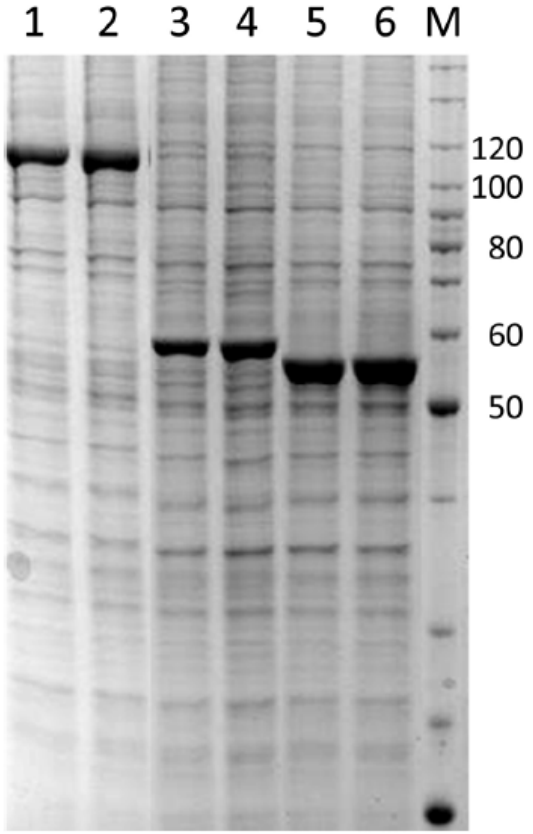
J Mehalko, D Esposito, J Biotech 2016

B2F vs B2B

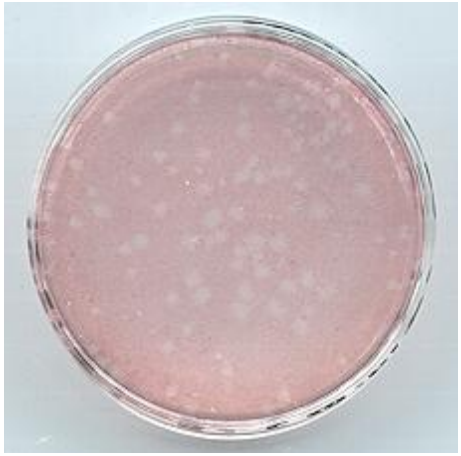
B2F transfer plasmid (no transfer plasmid replication, pir-)

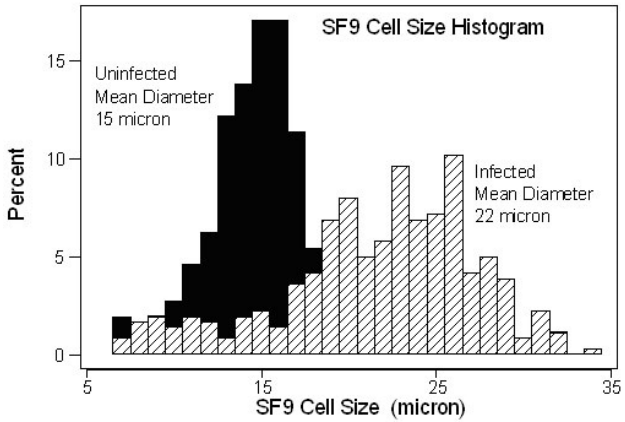
B2F transfer plasmid + new strain (no transfer plasmid replication and no transposition into the E. coli backbone)





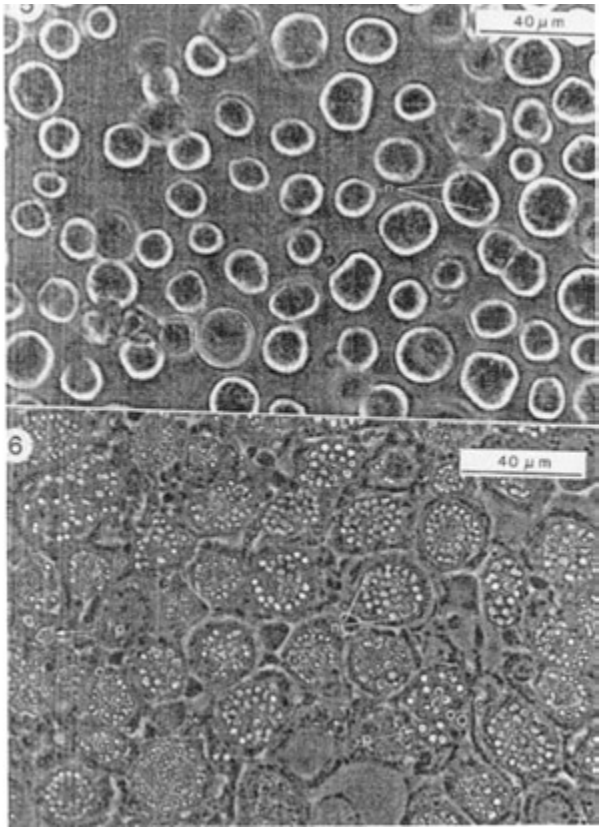
J Mehalko, D Esposito, J Biotech 2016





Sf9 non-infected

Sf9 infected



In practice: culture conditions

For infections cells in exponential growth phase are required.

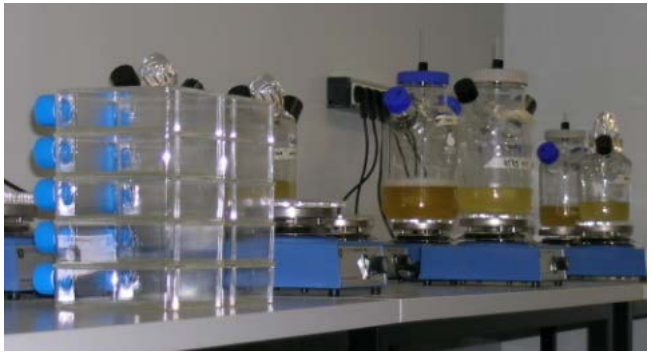
infect cells a 0.5 to 2.0 $1O^6$ cell/ml
T =27 °C, phosphate based buffer (no CO_2)
monolayers or suspension (Deep Well, Spinner, Bottles..)

Optimization of 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



Scale: from 3 ml to several L

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

Preparation and characterisation of Eukaryotic macromolecular complexes

1/ What is a macromolecular complex?

2/ Recombinant production

- The baculovirus expression system

- Purification strategies: tandem affinity

- Co-expression in insect cells

3/ Purification from endogenous sources

Tandem affinity purification

A single affinity step is usually not sufficent.

Sequential affinity steps that will select for the presence of two subunits

Apply the cell lysate to the first affinity resin

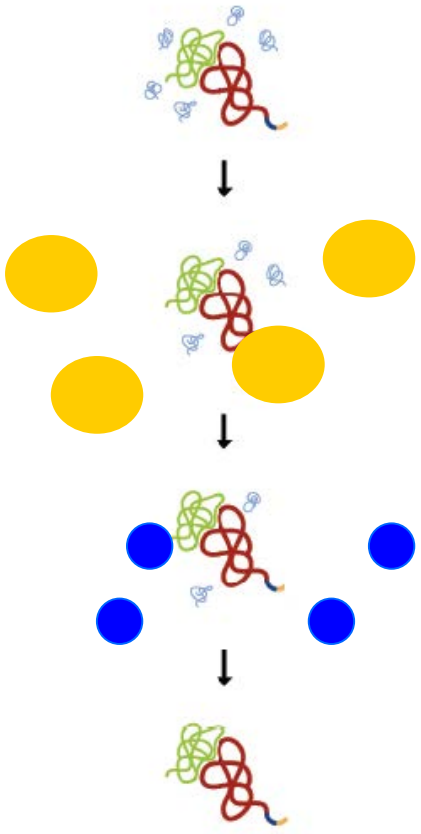
Wash unbound proteins and contaminants

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

Purification flowchart

Cell lysis: cell wall/plasma membrane,

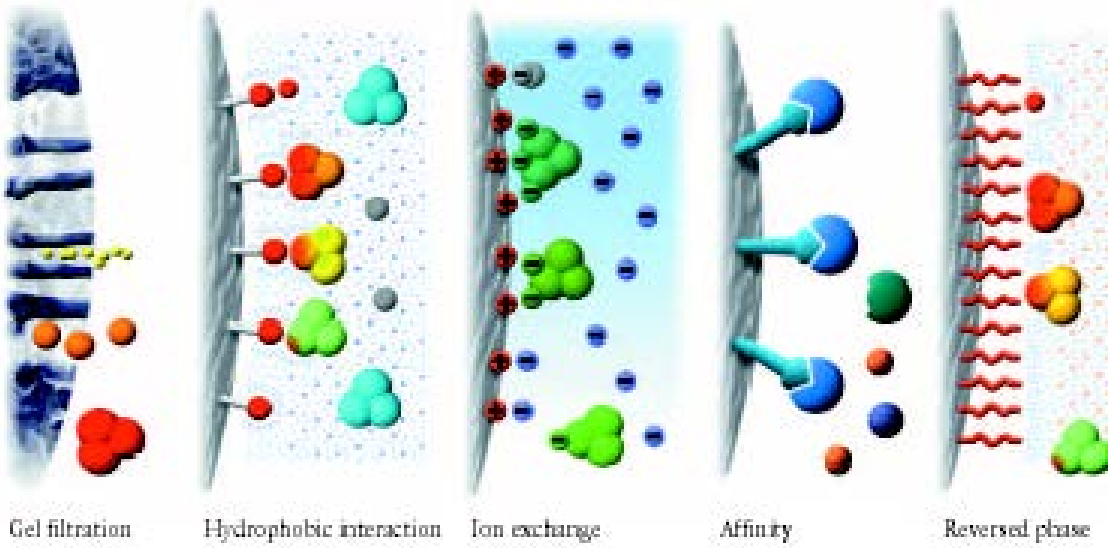
1. Physical means
1'. Sonication
1''. Osmotic shock

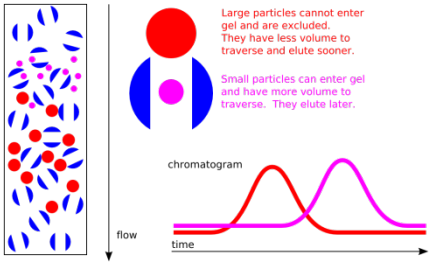
Centrifugation

Chromatographies

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

Concentration

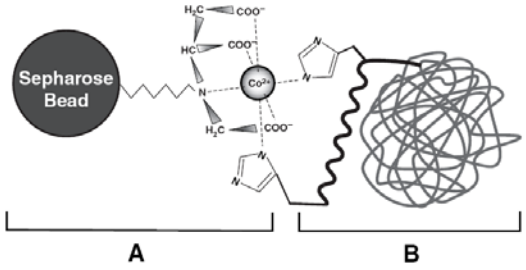


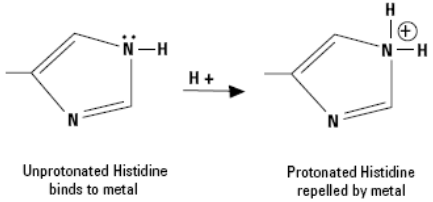


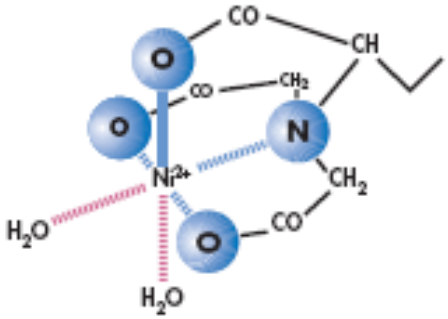
Most widely used tags: His, GST, strep, FLAG

His-tag

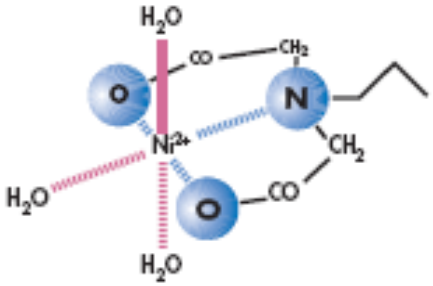
Immobilized Metal Chelate Affinity resin







Ni-NTA



Ni-IDA

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

10xHis

Ni, Co, Fe

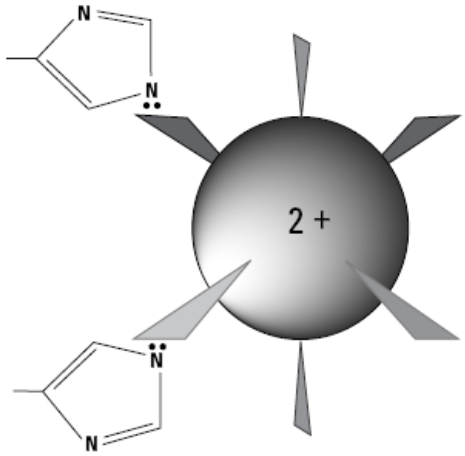
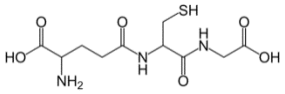
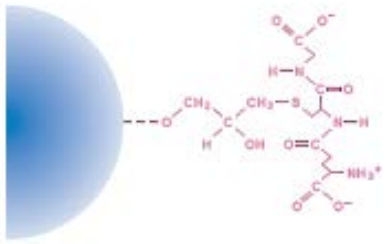
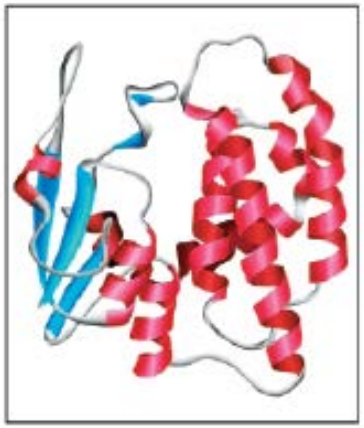


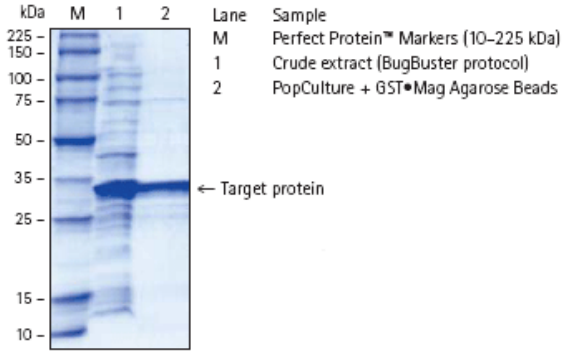
Figure 3. Binding of histidines to the TALON® Resin metal ion. Under conditions of physiological pH, histidine binds by sharing imidazole nitrogen electron density with the electron-deficient orbitals of the metal ion.

GST-affinity

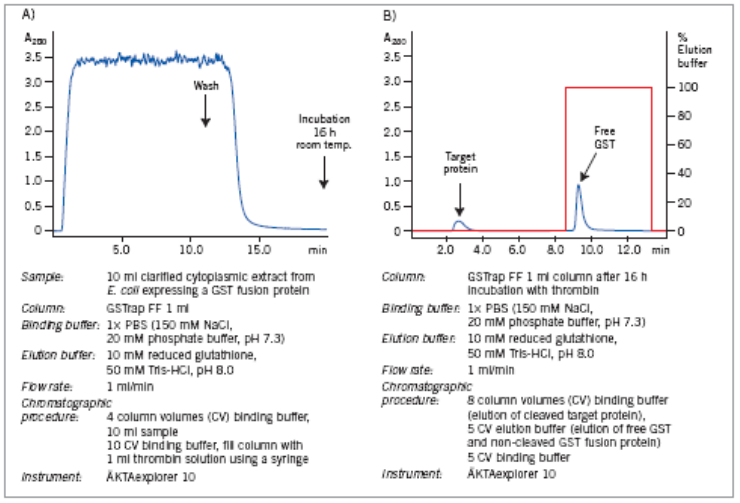








Lane	Sample
M	Perfect Protein™ Markers (10–225 kDa)
1	Crude extract (BugBuster protocol)
2	PopCulture + GST•Mag Agarose Beads



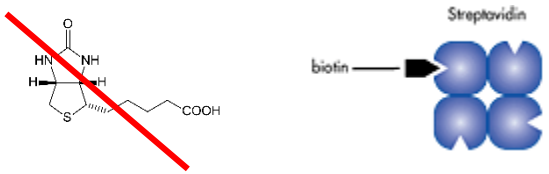
	A)	B)
Sample:	10 ml clarified cytoplasmic extract from *E. coli* expressing a GST fusion protein	
Column:	GSTrap FF 1 ml	GSTrap FF 1 ml column after 16 h incubation with thrombin
Binding buffer:	1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)	1× PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3)
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

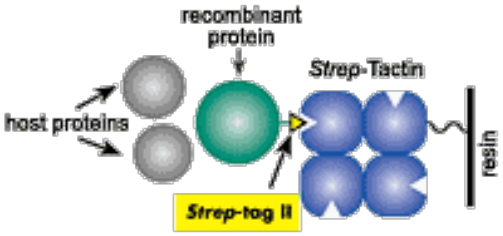
Fig 26. On-column thrombin cleavage of a GST fusion protein. A) Equilibration, sample application, and washing of a GST fusion protein on GSTrap FF 1 ml were performed using ÄKTAexplorer 10. After washing, the column was filled by syringe with 1 ml of thrombin (20 U/ml 1× PBS) and incubated for 16 h 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.

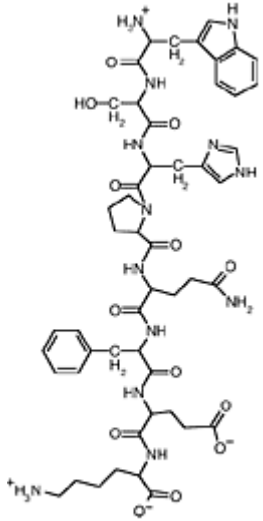
Strep tag-II

Derived from strepavidin-Biotin

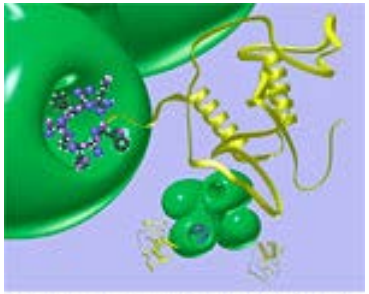




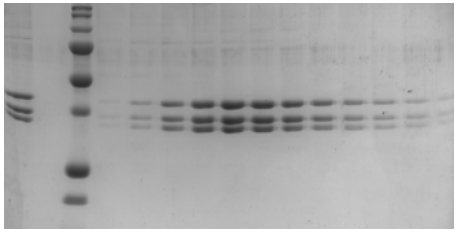
Elution with biotin analog: desthiobiotin or more recently Biotin (StrepTactin@XT)



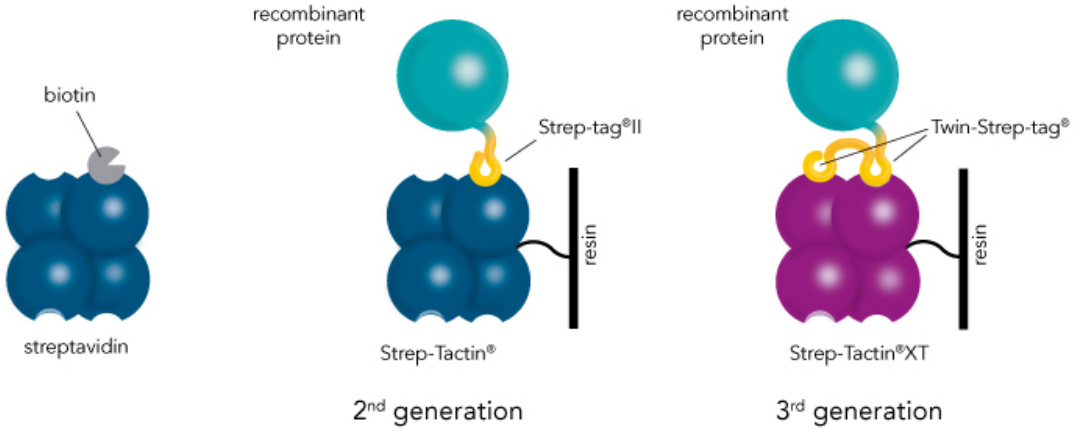
***Strep*-tag II**
NH_2 -WSHPQFEK-COOH



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



Recombinant Protein Purification using Strep-Tactin®XT



Strep-tactin®XT can be used in combination with the following tags:

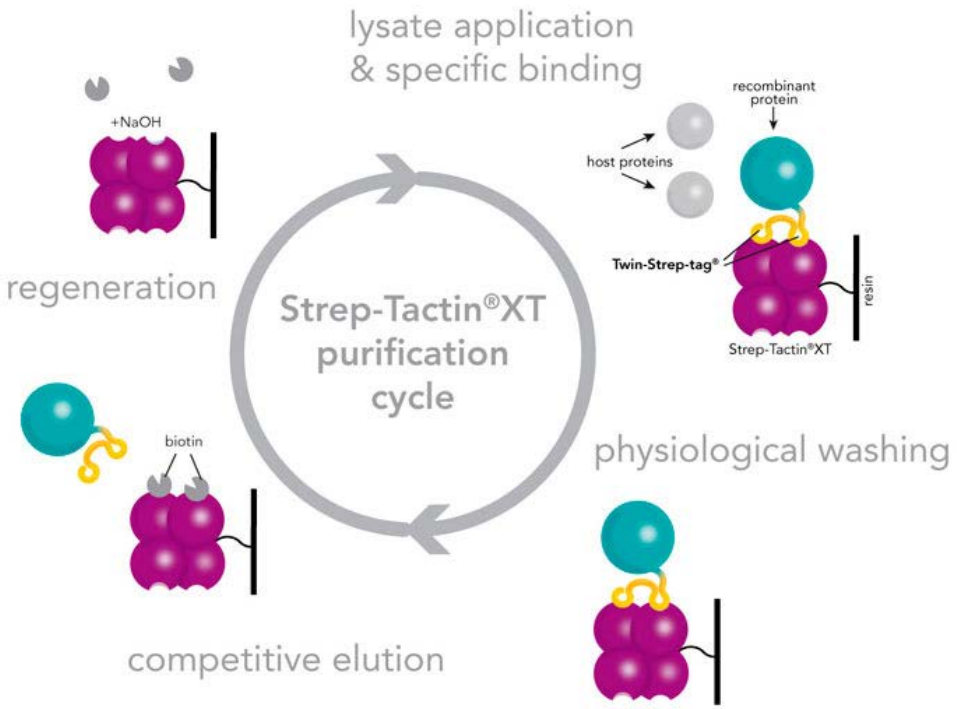
Strep-tag®II

8 amino acids
sequence: Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (WSHPQFEK)

Twin-Strep-tag® (in the low pM range)

28 amino acids (WSHPQFEK-GGGSGGGSGG-SA-WSHPQFEK)
Two Strep-tag®II motifs in series
Higher affinity than Strep-tag®II

Strep-Tactin®XT purification cycle

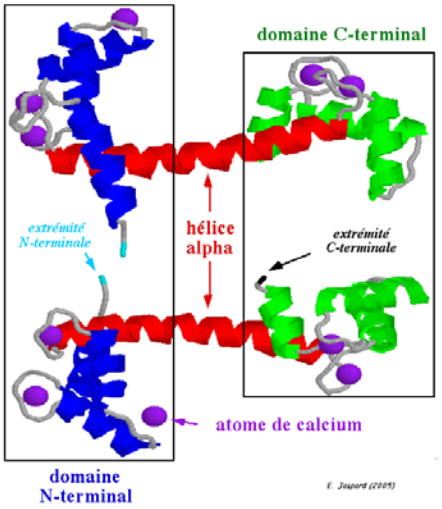


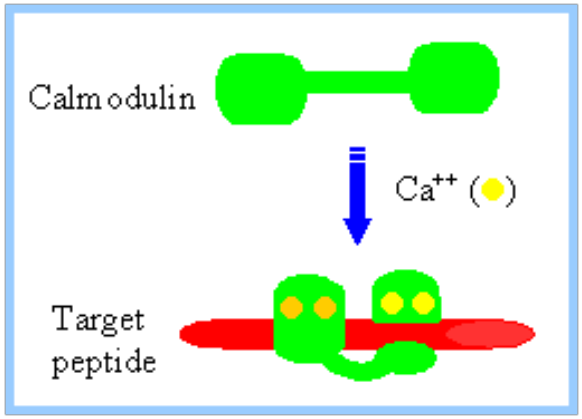
Elution with 50 mM Biotin

https://www.iba-lifesciences.com/tl_files/ProteinProductionAssays/3-Purification/Purification-Cycle-Strep-Tactin-XT-Twin-Strep-tag.jpg

CBP affinity

Calmodulin Binding Peptide interacts with Calmodulin coupled to an agarose resin





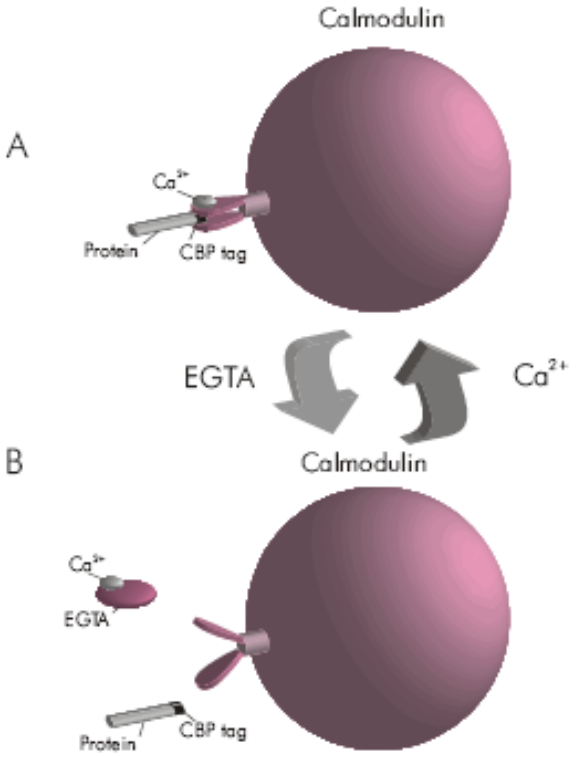
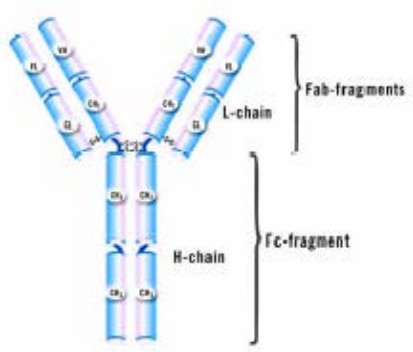
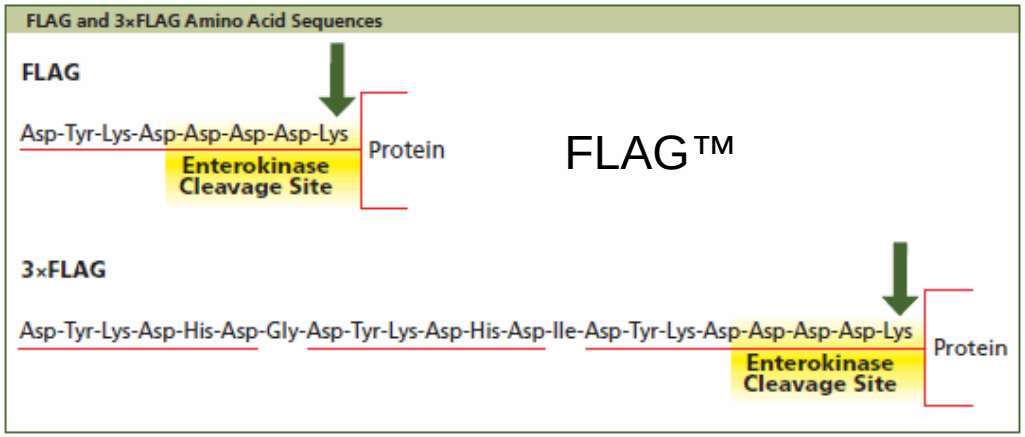
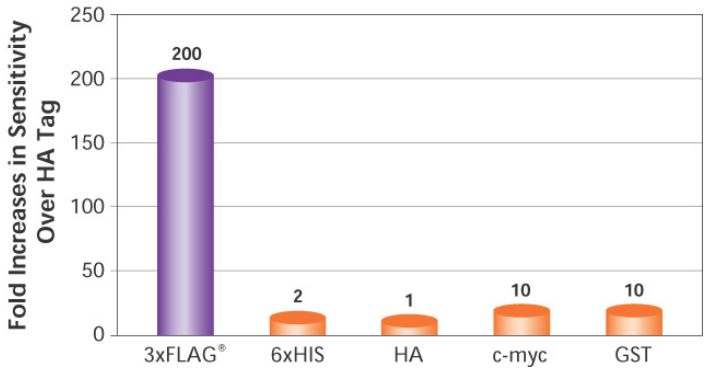


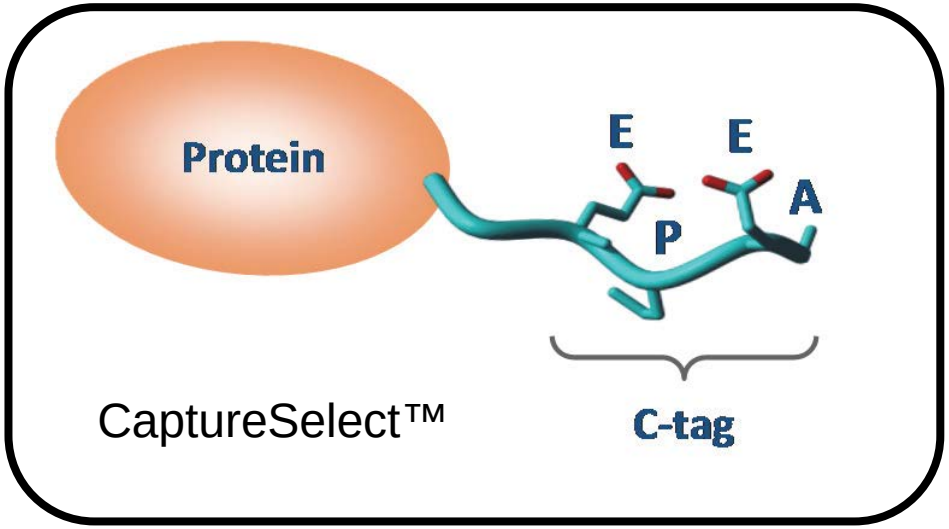
FIGURE 2 The Affinity protein expression and purification system. The highly conserved protein calmodulin binds to the CBP-tagged fusion protein in the presence of low concentrations of calcium at neutral pH (A). The fusion protein elutes from its ligand at neutral pH with 2 mM EGTA (B). The purified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

FLAG and Capture Systems



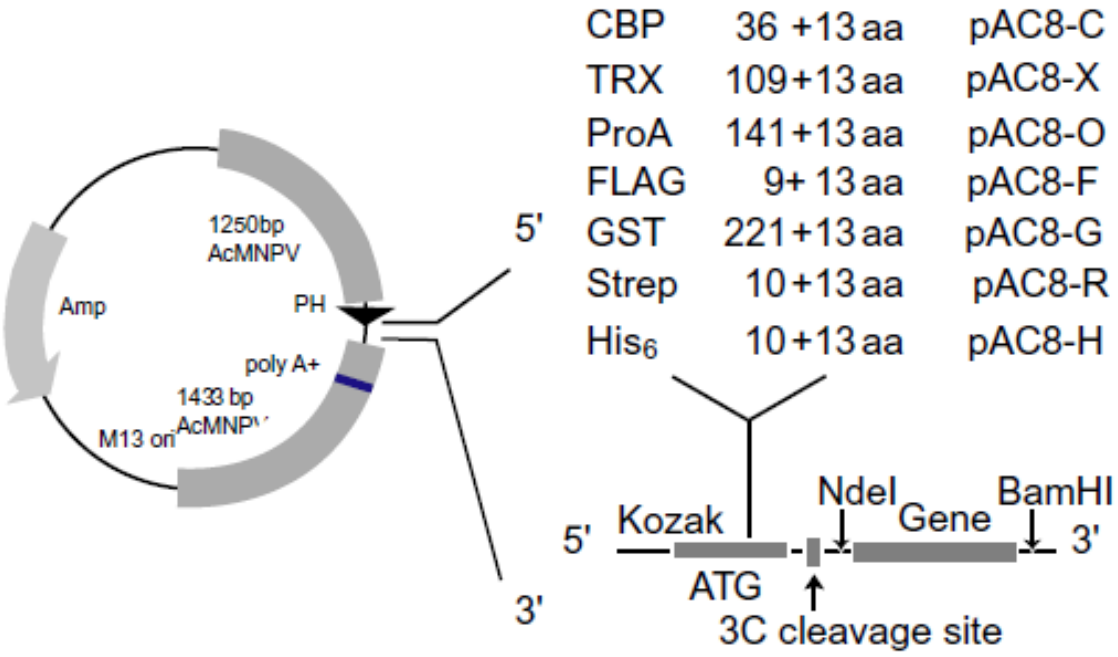






Transfer vectors for screening of affinity tags and parallel cloning of constructs

Standardize expression screening, enable constitent comparsons



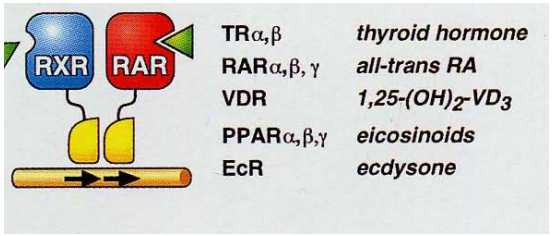
pBacPAK8 (Clontech) backbone
Restriction/Ligation/SLIC, or GW

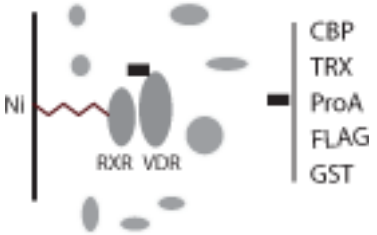
Abdulrahman, Anal Bioch, 2009

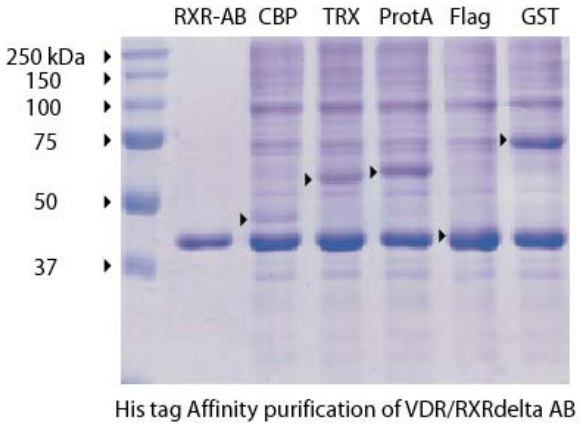
Protein A: Protein from Staphylococcus aureus that has affinity for immunoglobulins. Widely used for Ab pruifiction; Elution with pH shift or on collumn cleavage

The example of VDR

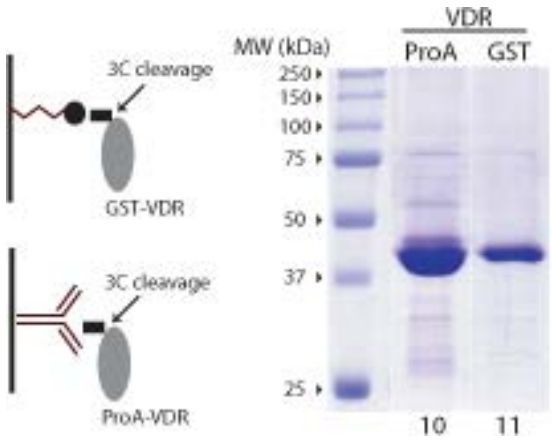
Expression screening to optimize expression of VDR (variant)







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 €

Preparation and characterisation of Eukaryotic macromolecular complexes

1/ What is a macromolecular complex?

2/ Recombinant production

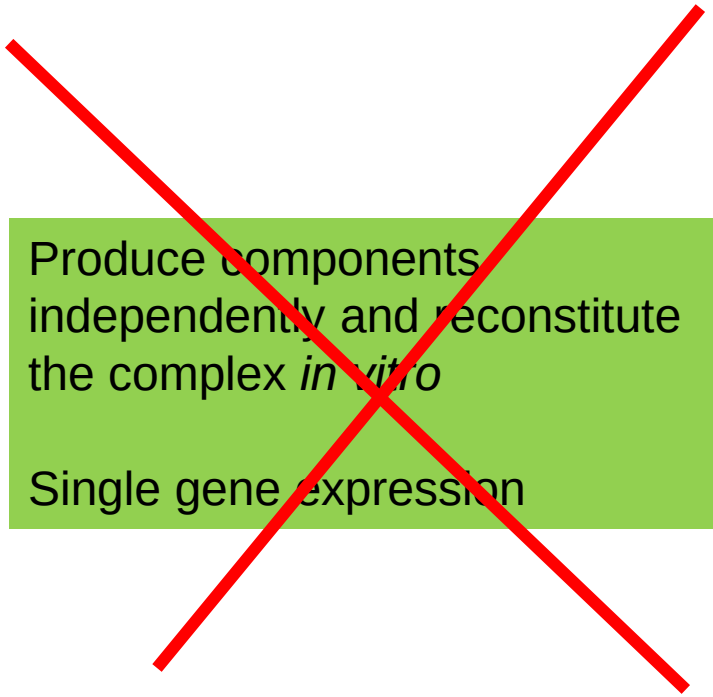
- The baculovirus expression system

- Purification strategies: tandem affinity

- Co-expression in insect cells

3/ Purification from endogenous sources

Obligatory complexes



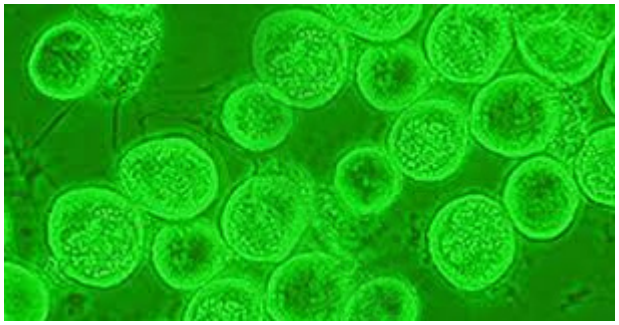
Produce components independently and reconstitute the complex *in vitro*

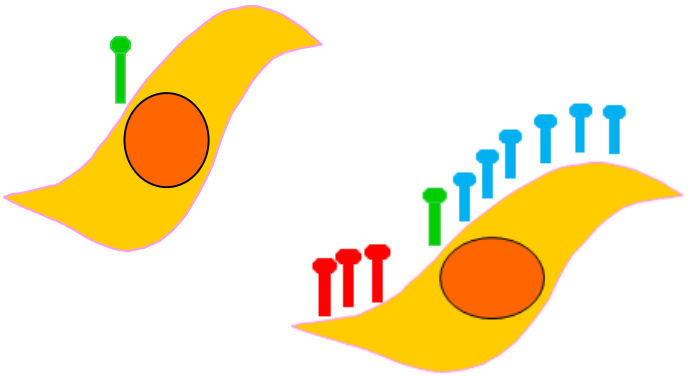
Single gene expression

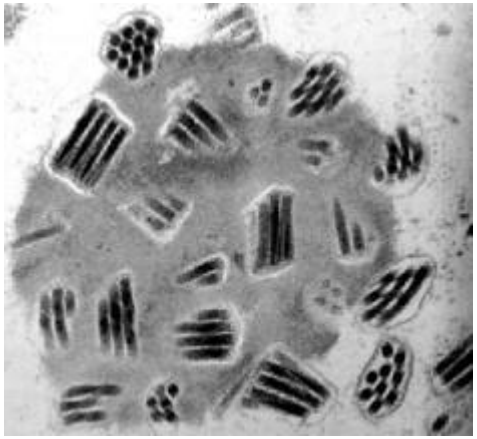
Produce components of the complex simultanously which are assembled *in vivo*

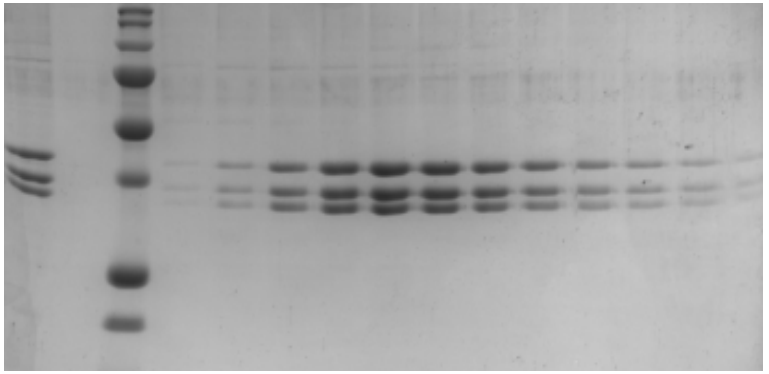
Co-expression

Co-expression in insect cells using the BVES

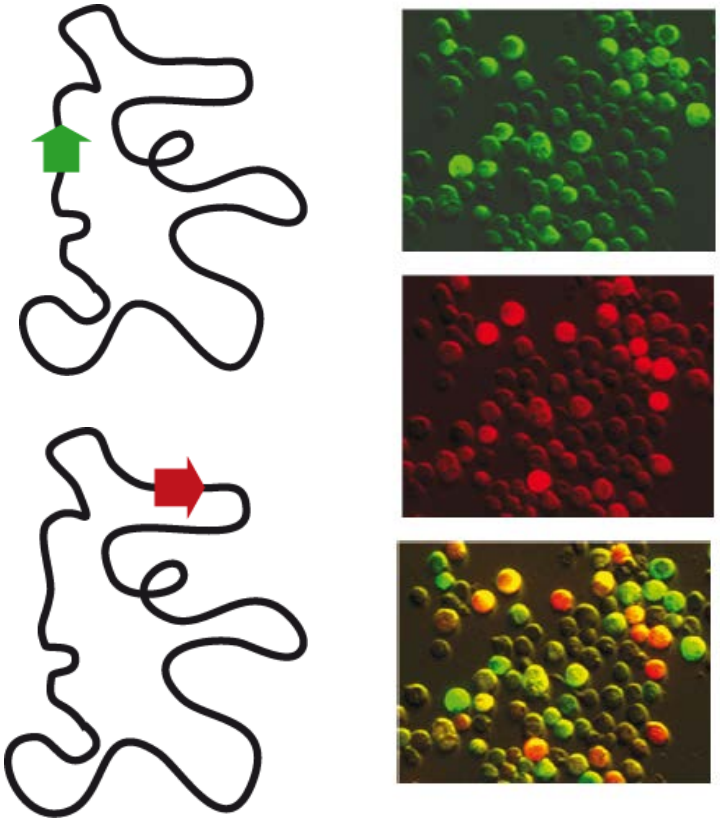




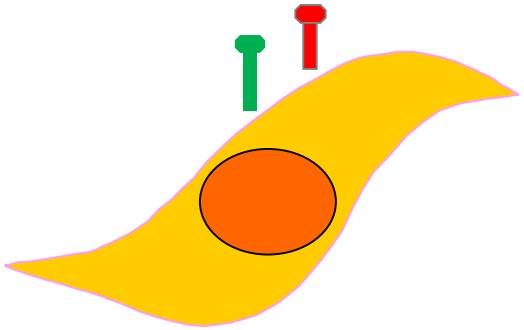




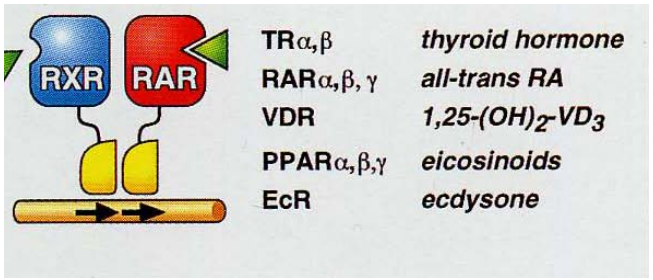
Co-infection: a simple way to co-express proteins

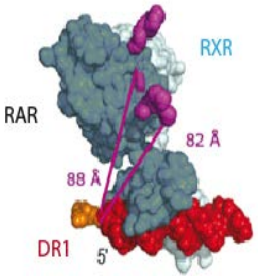


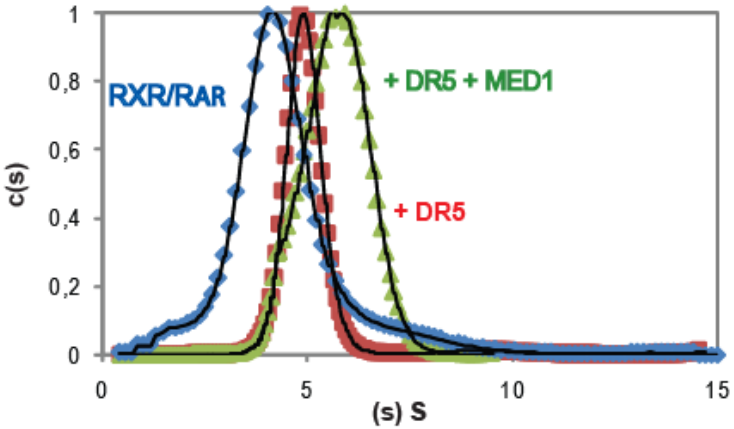
pH GFP+ p10 DsRed

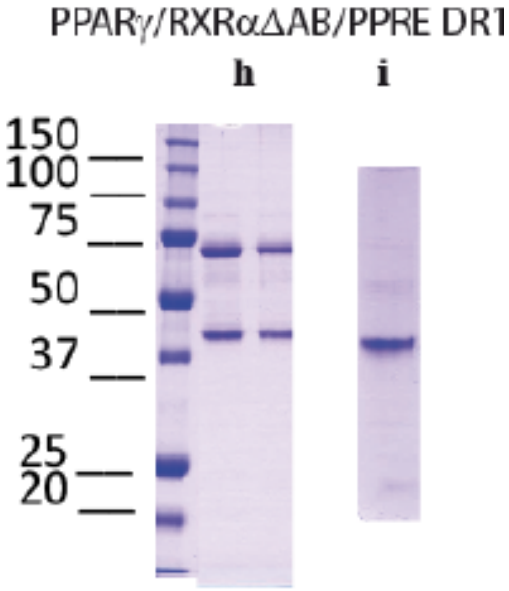


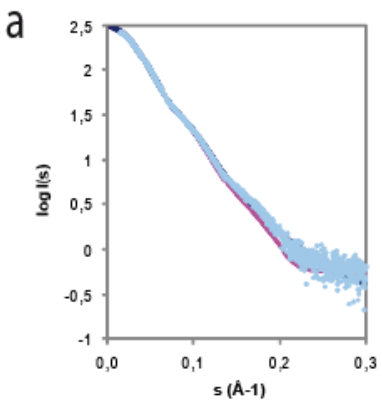
Production of nuclear hormone receptor complexes

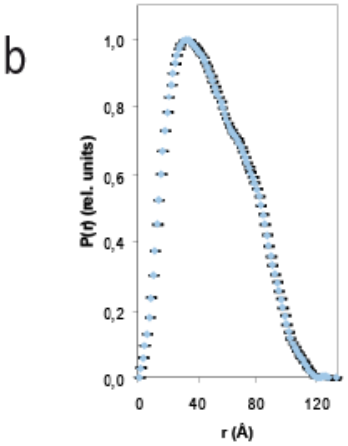








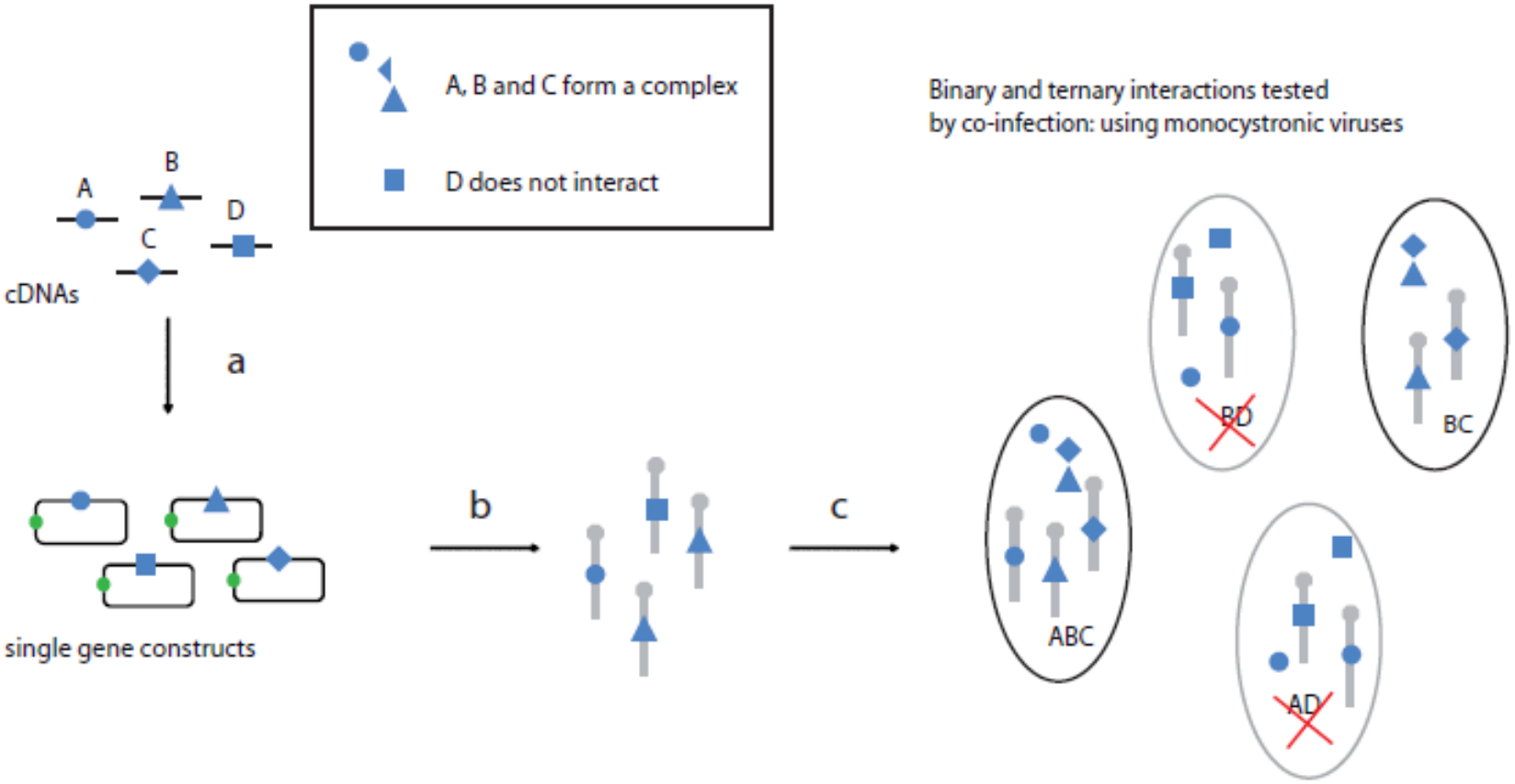




Rochel, et al. NSMB, 2011

Analysis of protein-protein interaction networks

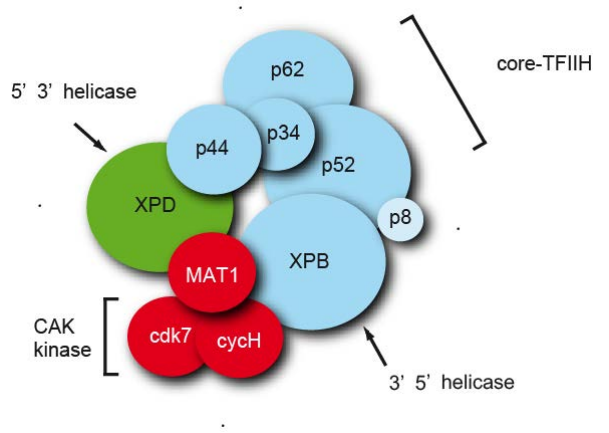
n proteins/subunits: do they form a stable complex ?

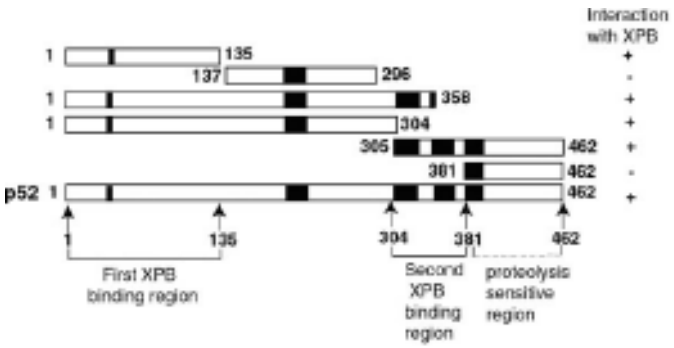


Systematic dissection of protein-protein interactions within a complex

Generate two sets of n viruses:typically the first with a FLAG epitope and the second with an 6His tag

Test all combinations of pair-wise interactions (Flag-protein x/His-protein y)



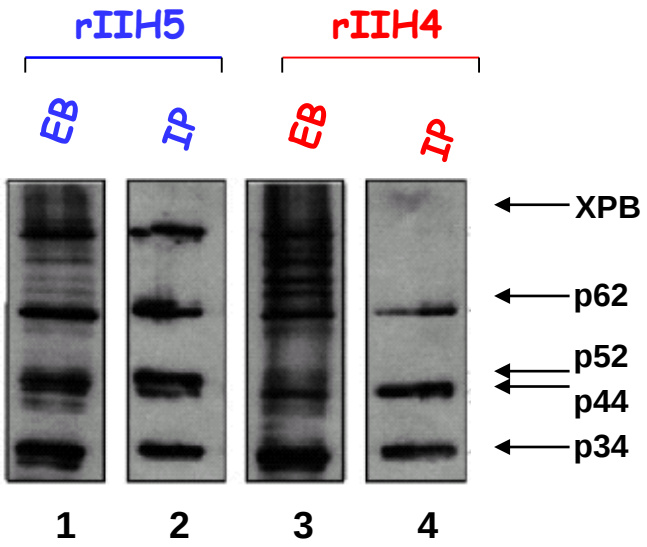


His-protein

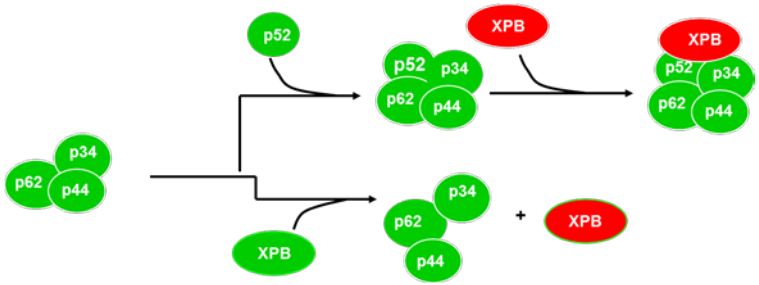
Flag-protein

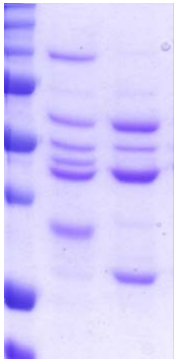
	p34	p44	p52	p62	XPB	XPD	MAT1
p34							
p44							
p52							
p62							
XPB							
XPD							

Systematic dissection of protein-protein interactions: deletion analysis



Analysis of the protein interaction network
Identification of key regulatory interactions



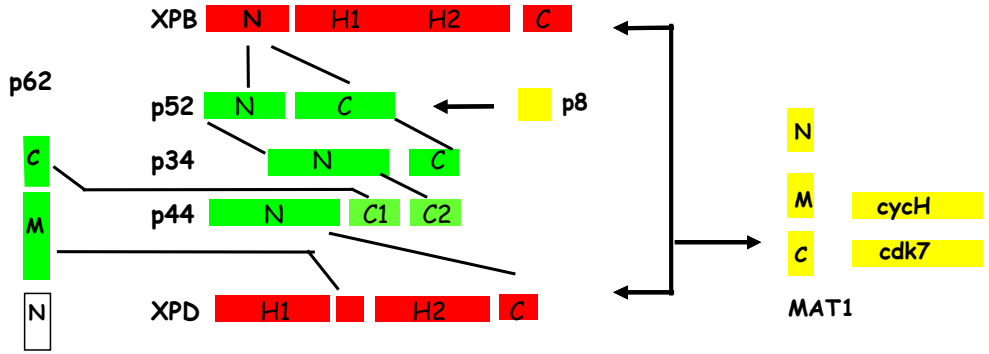


XPB

P62
*
P52,p44

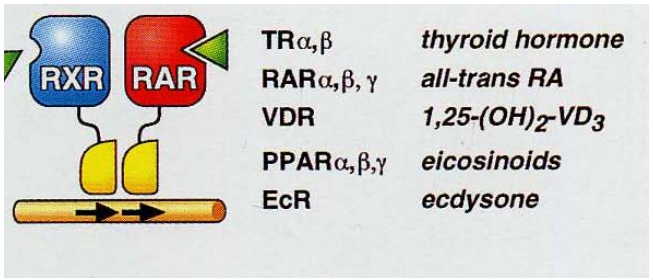
P34

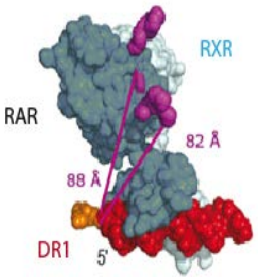
P34(1-233)

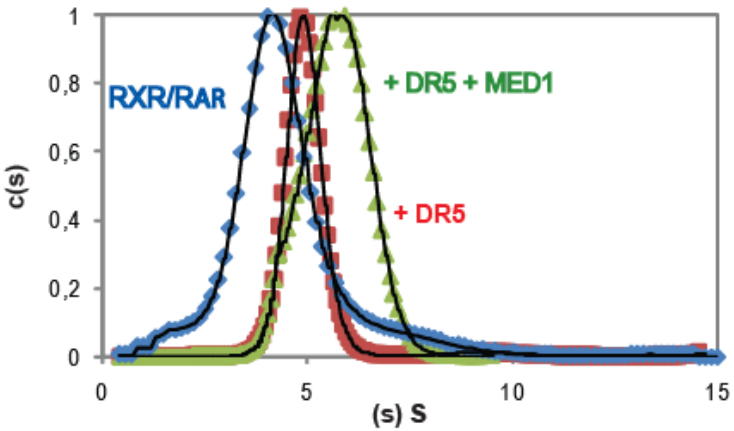


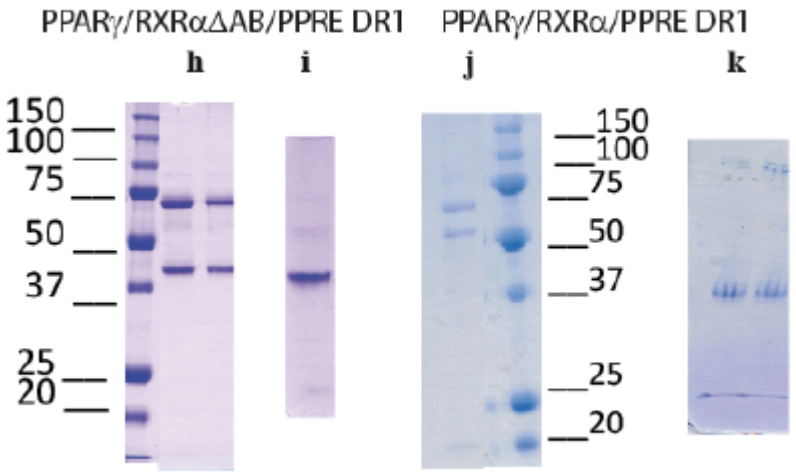
Jawahri at al. 2002, Radu et al, in prep

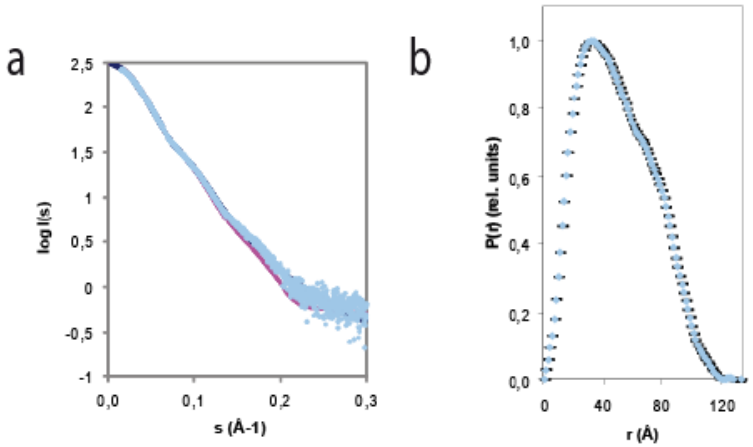
Production of nuclear hormone receptor complexes





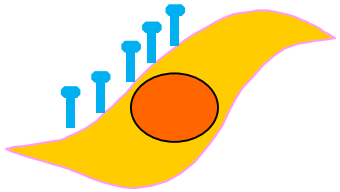


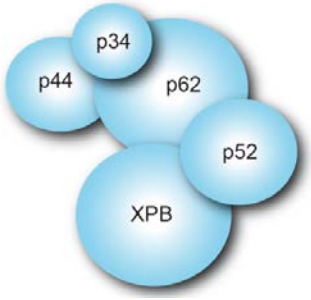




Rochel, et al. NSMB, 2011

Co-infection with multiple viruses for reconstitution of complexes



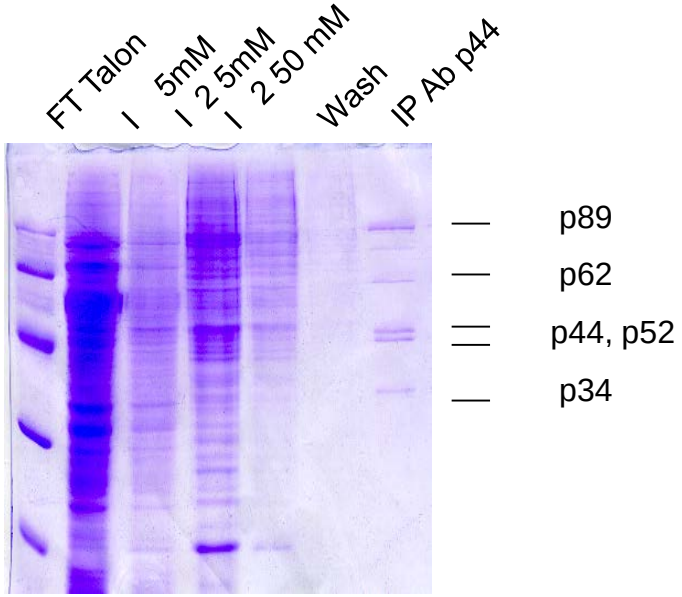


Cell extract

IMAC

Affinity purification

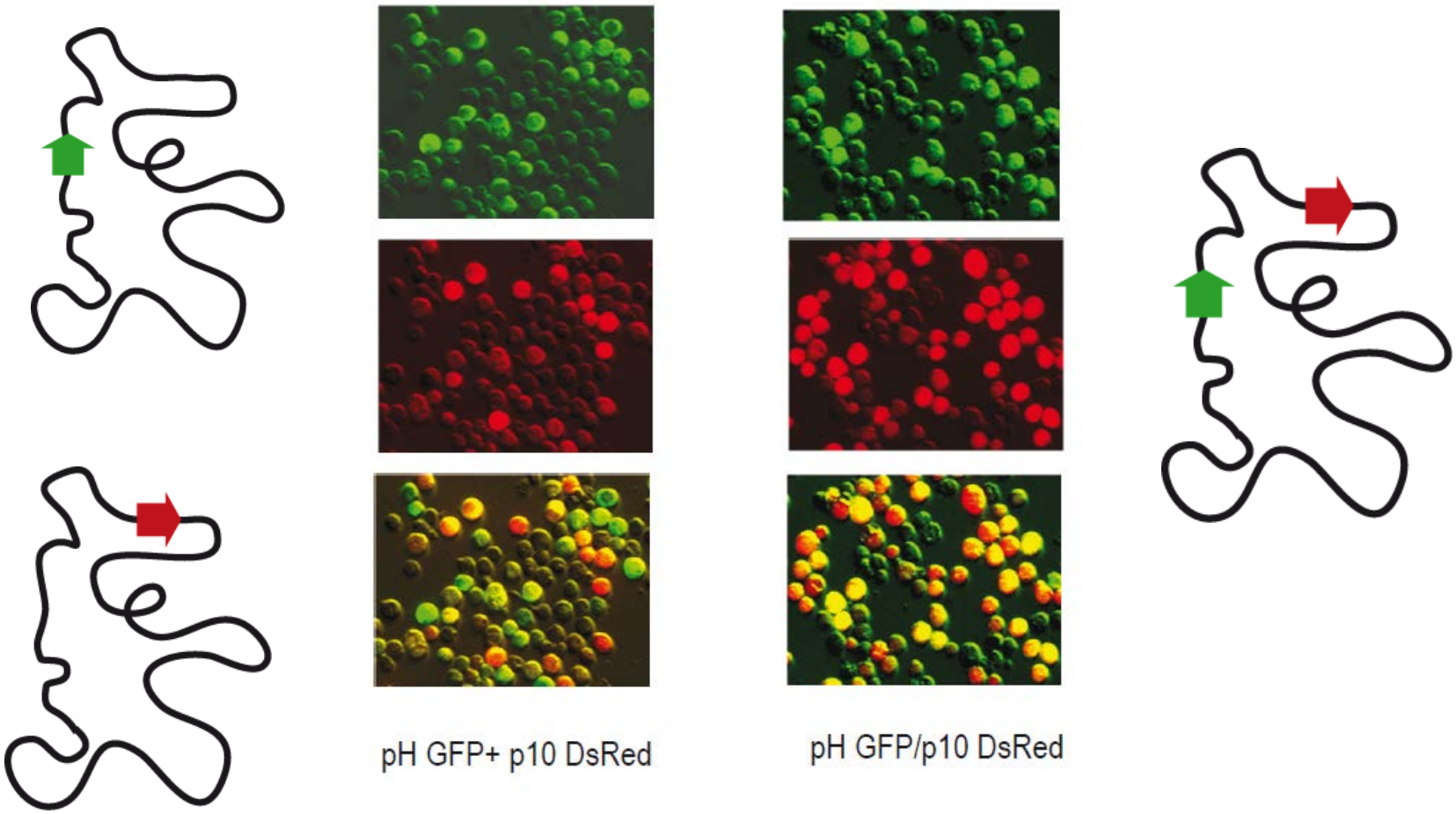
Size exclusion

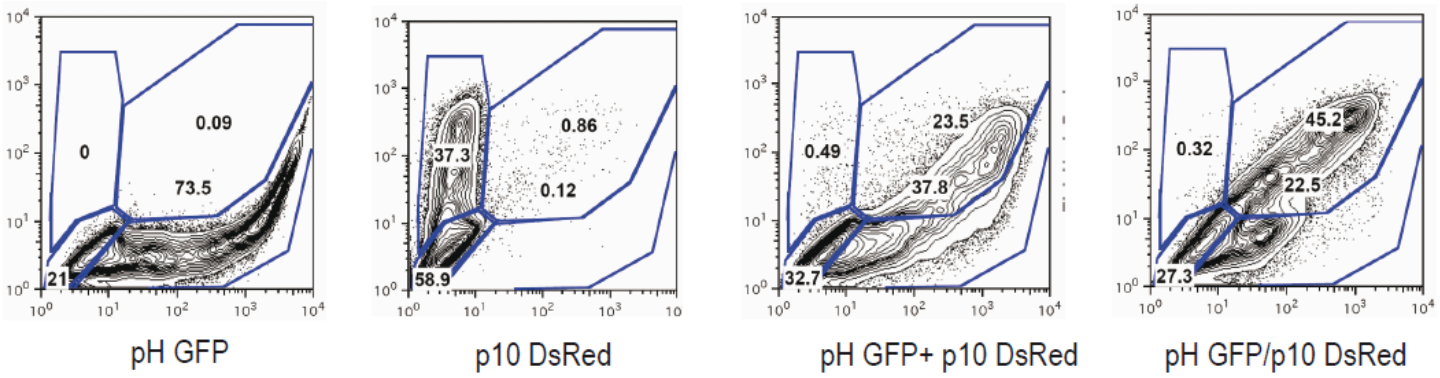


η # 50-100 μg/L

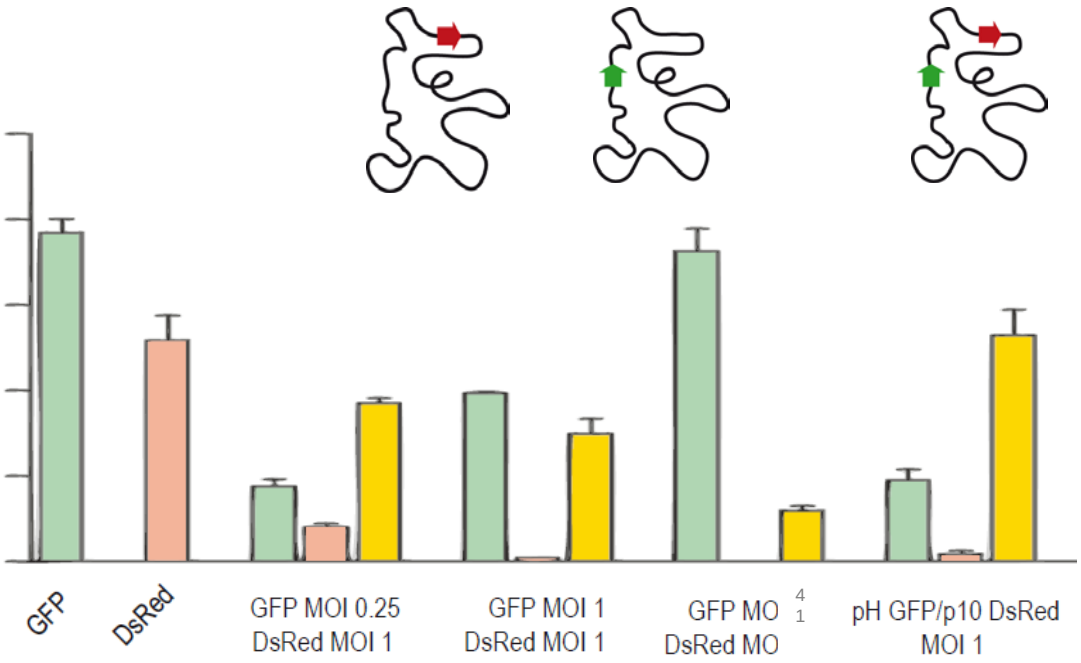
Low yields, labour intensive, poor reproducibility

Co-infection vs Multigene expression





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

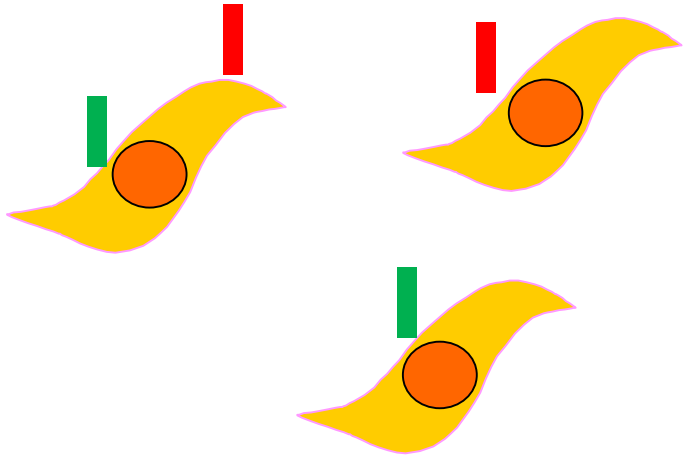


Idem for other MOIs

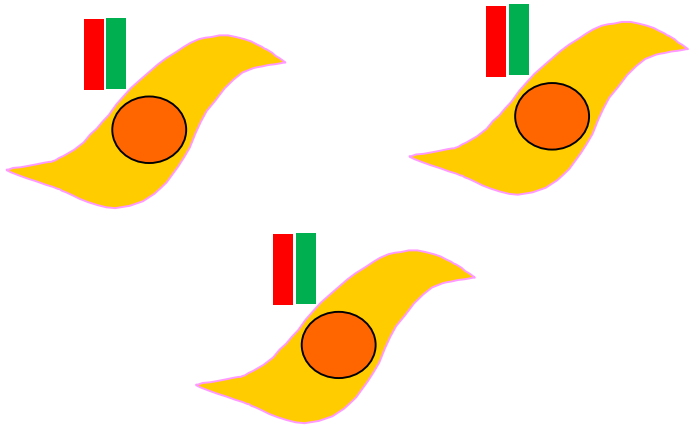
Co-infection vs Multigene expression

Two viruses encoding a single gene each

A single virus encoding the two genes



Co-infection

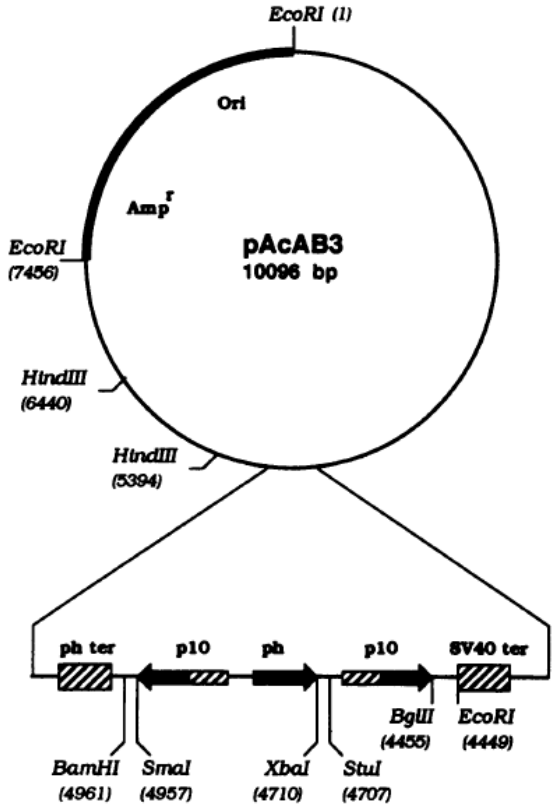


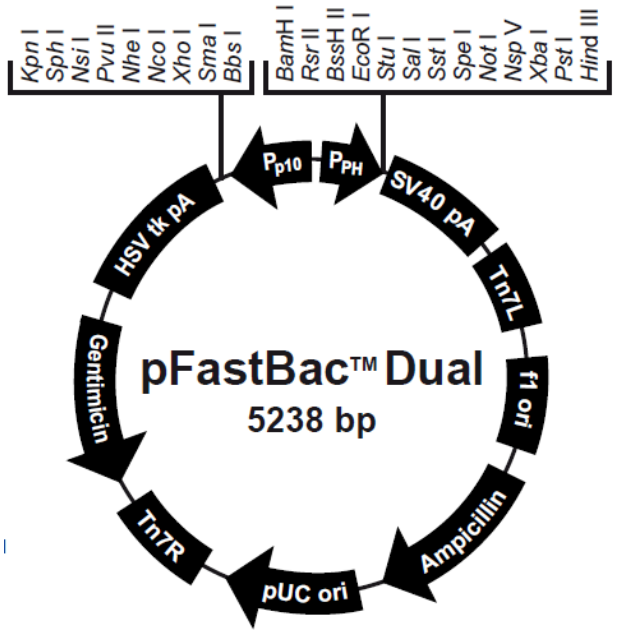
Infection by a single virus

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

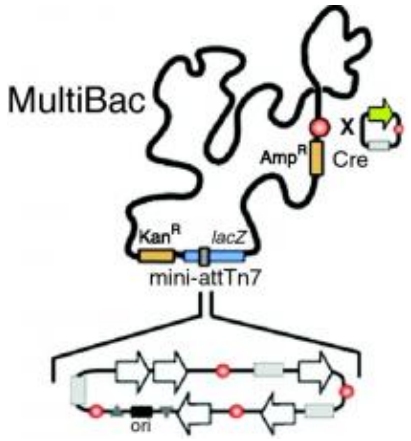
Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells

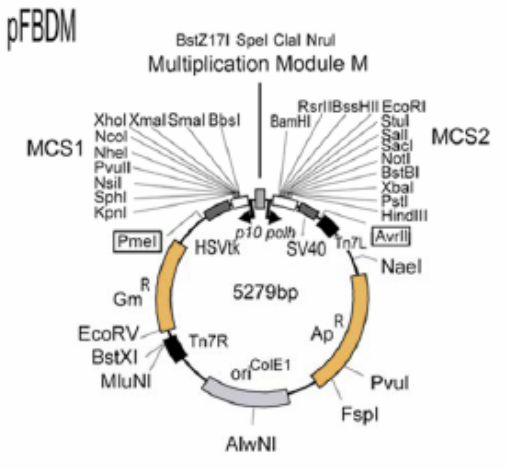
Alexander S.Belyaev[1] and Polly Roy[1,2,*]

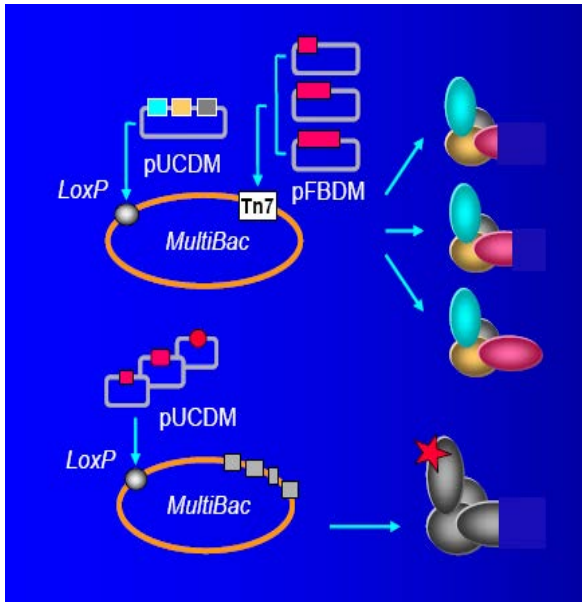




MultiBac technology: Combinatorial applications in protein expression



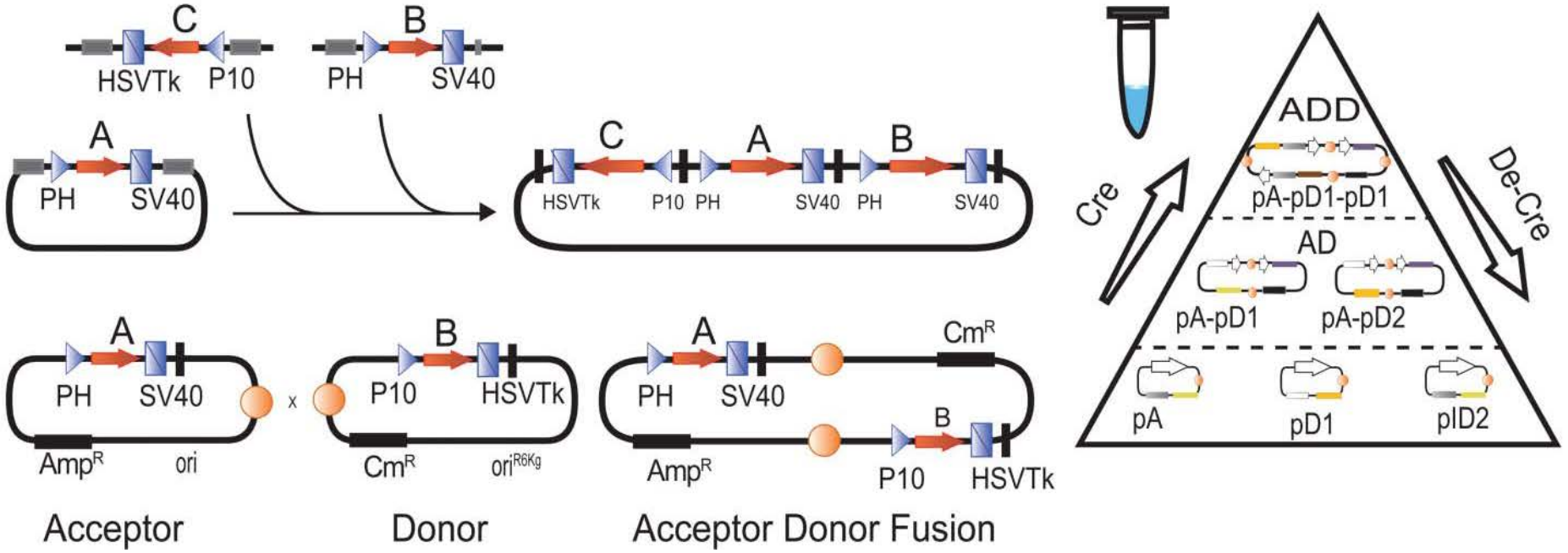




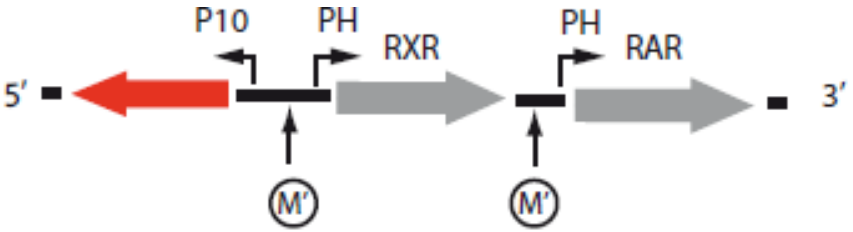
Tools to streamline design of multigene expression recombinnant baculoviruses

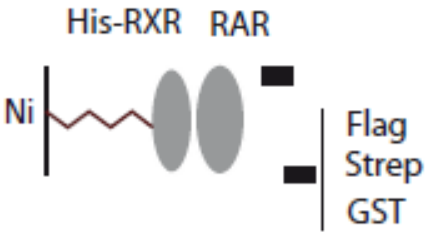
Imre BERGER, Bristol, UK

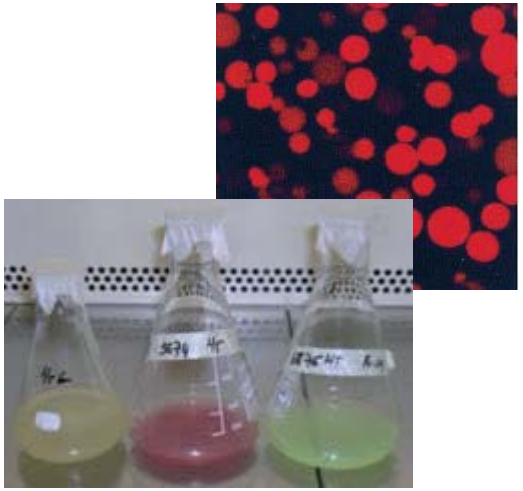
MultiBac technology: Combinatorial applications in protein expression

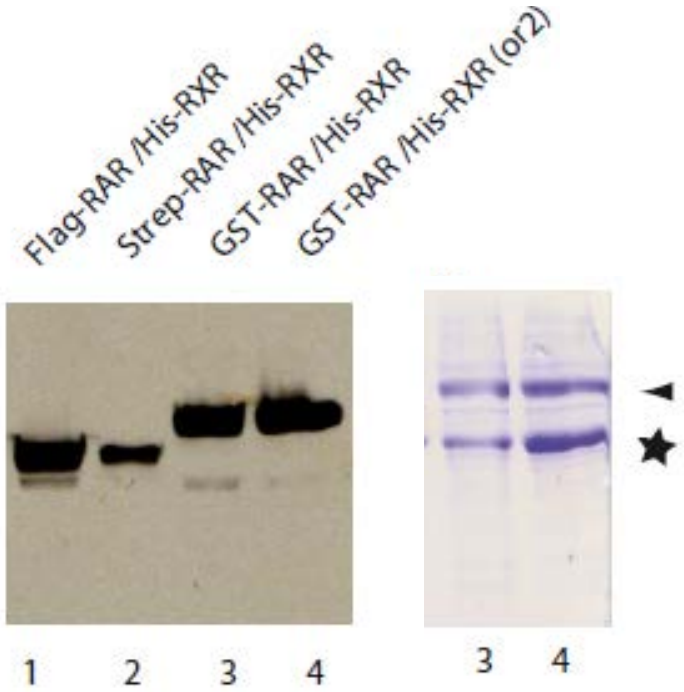


Insertion of an expression cassette into the multiplication module

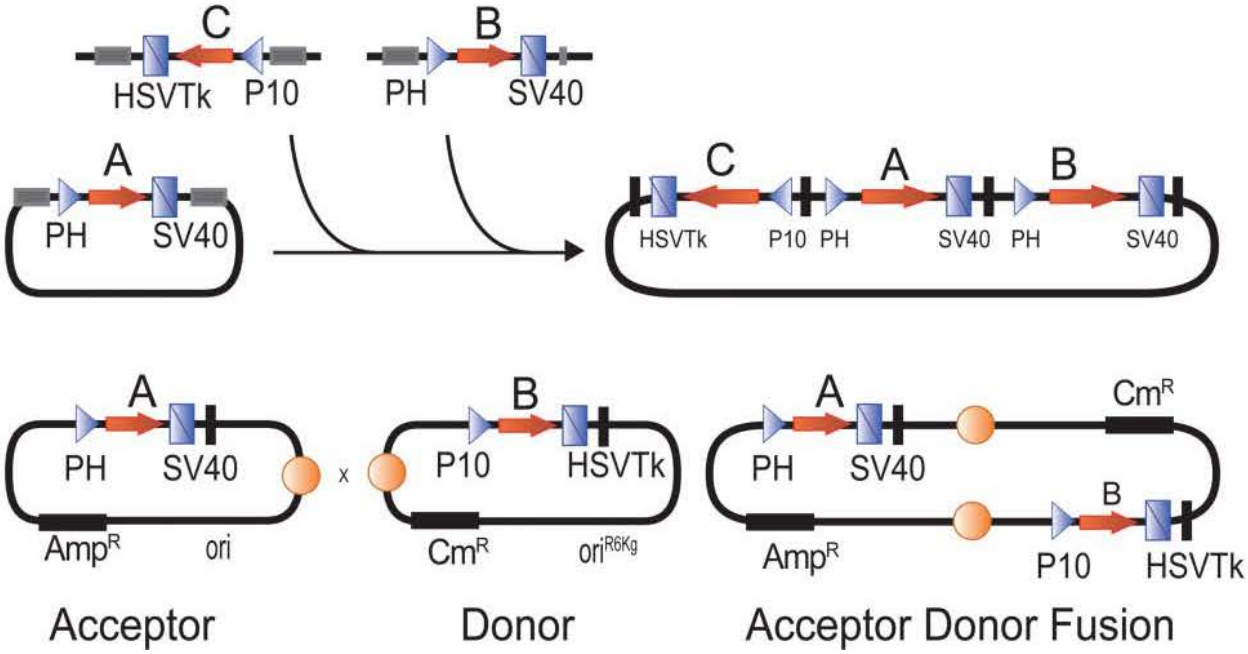


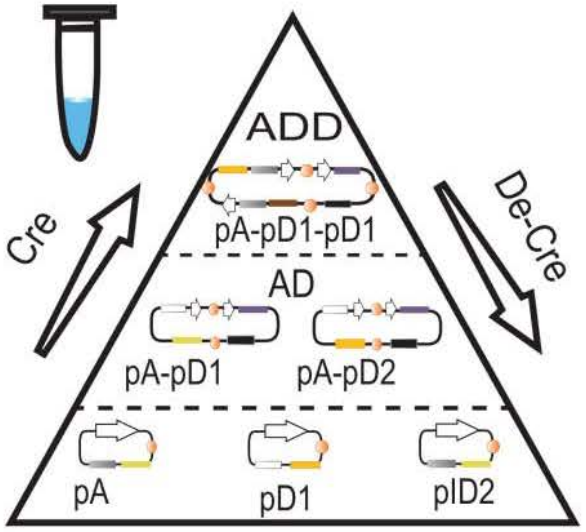






MultiBac technology: Combinatorial applications in protein expression





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.

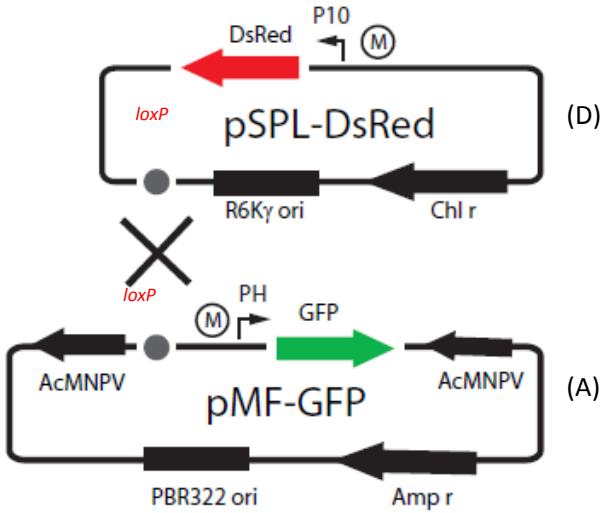
loxP sequence

8-bp overlap region

ATAACTTCGTATAGCATACATTATACGAAGTTAT

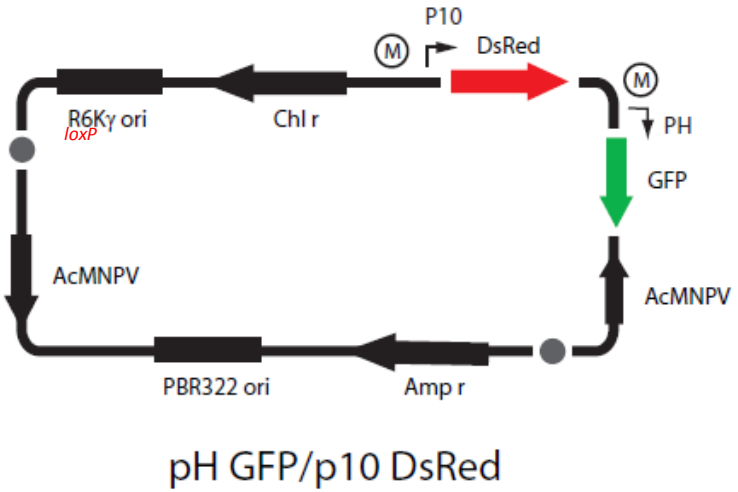
Inverted region Inverted region

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

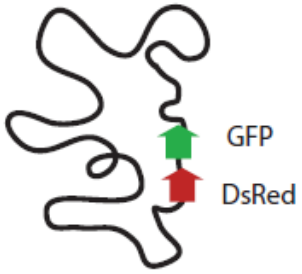


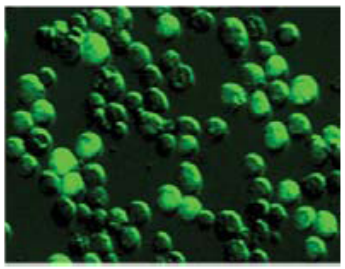
(D)

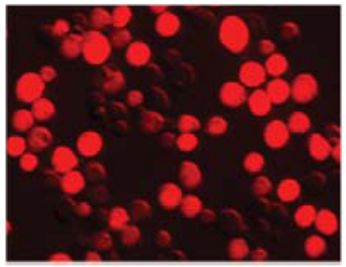
(A)

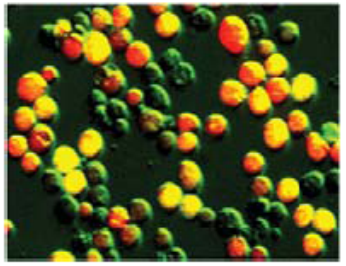


pH GFP/p10 DsRed









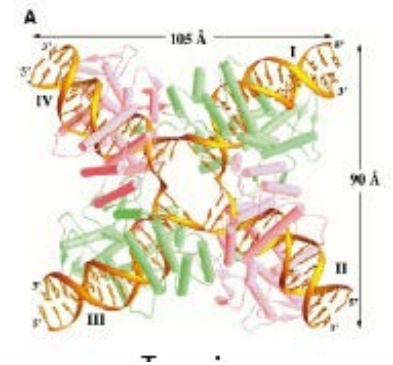
pSPL Multibac, (Fitzgerald, Nat Methods 2006)

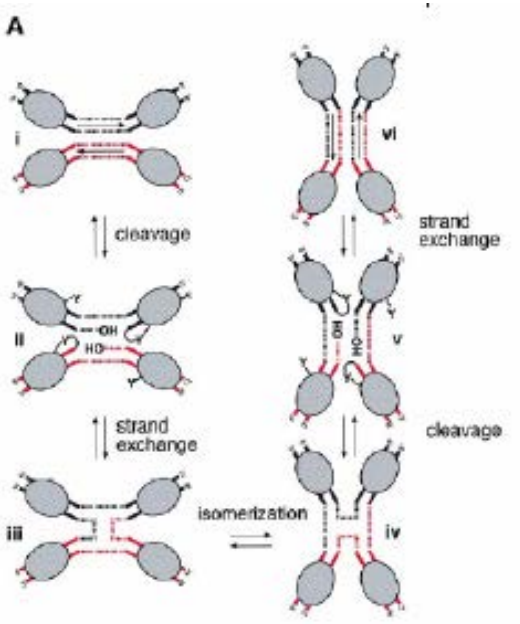
Site specific recombination cloning

- 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

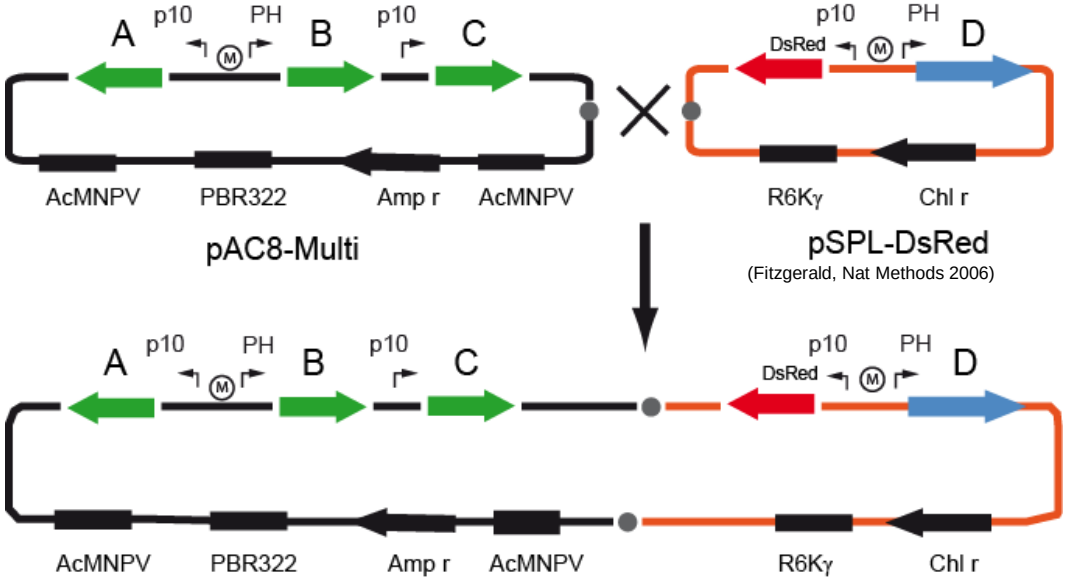
- MAIN APPLICATIONS
- *In VITRO*
  - Rapid restriction enzyme free subcloning
  - Plasmid excision
  - In vitro clone screening
- *In VIVO*
  - Genome rearrangements
  - Tightly regulated transcription

Cre-loxP recombination in bacteriophage P1





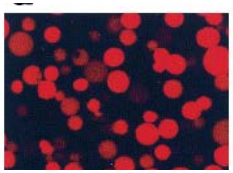
Expression of ternary and quaternary complexes with a single virus: a problem of DNA synthesis



pBac8 Multi-pSPL Fusion

A: strep-tag
D: Flag-tag

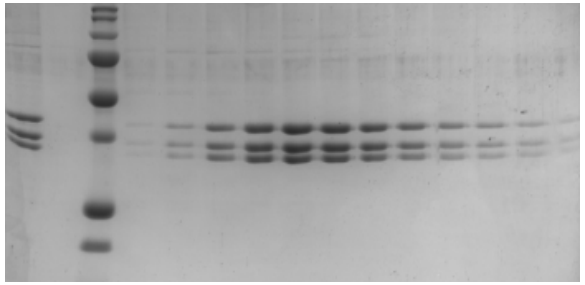
Flag-affinity



Elution with
Competitor-peptide

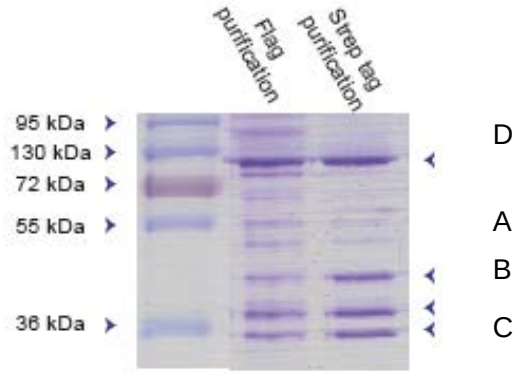
Strep-affinity

Elution with
Desthiobitin



A
B
C

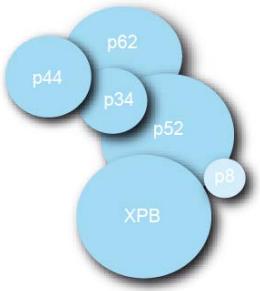
Ternary complex



Quaternary complex

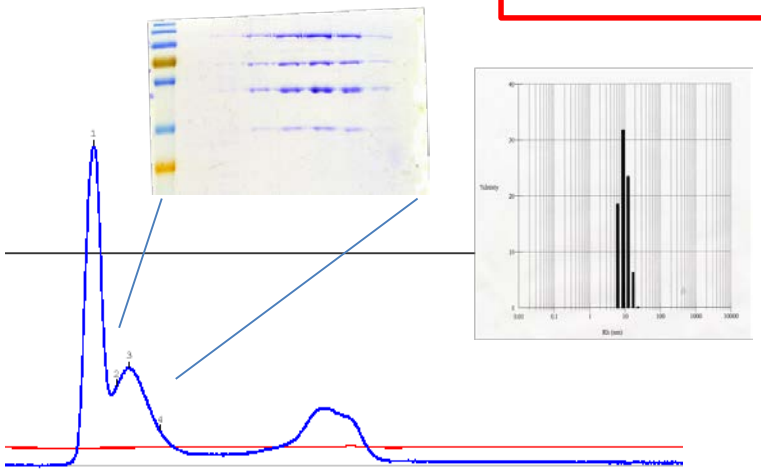
Production of core-TFIIH with a single virus

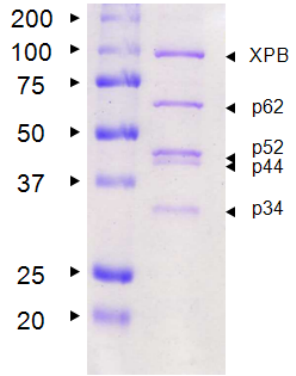
- 6 subunits: XPB, p62, p52, p44, p34, p8/TTDA (+ DsRed)
- Yield : 0.5 mg/L

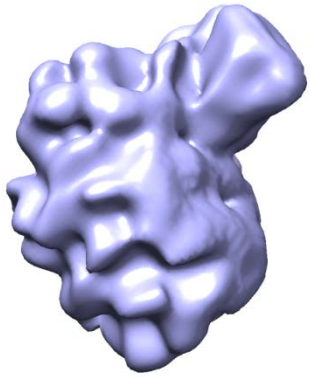


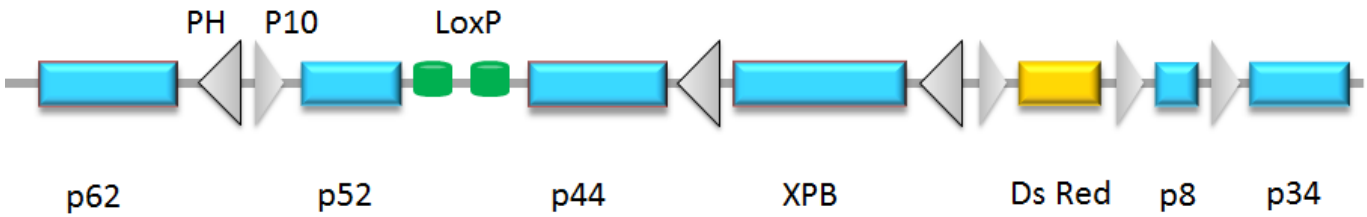
MS-cross linking, Cryo-EM and crystallization

Screening for XPB inhibitors



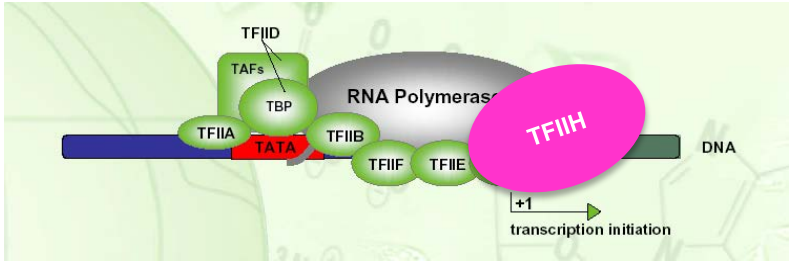


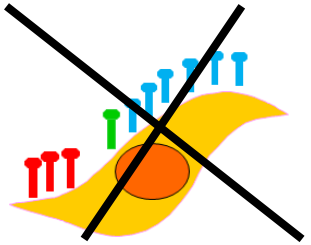


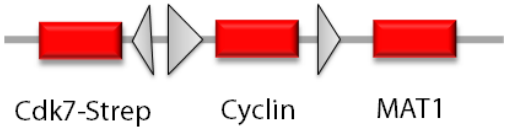


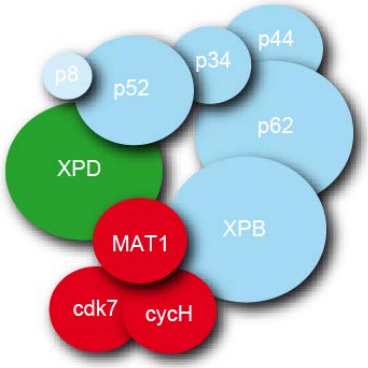
16 kbp

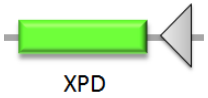
Reconstitution and in vitro assays

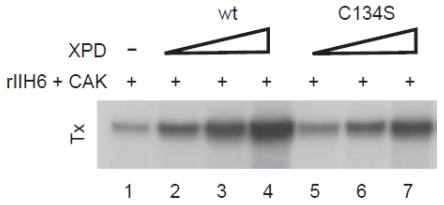


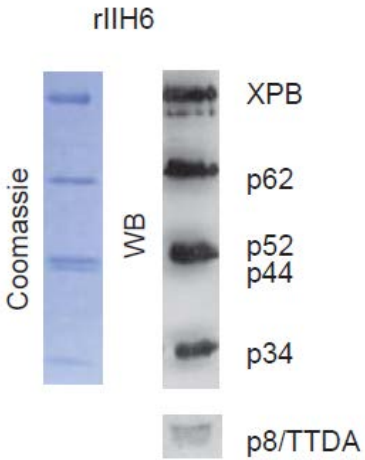


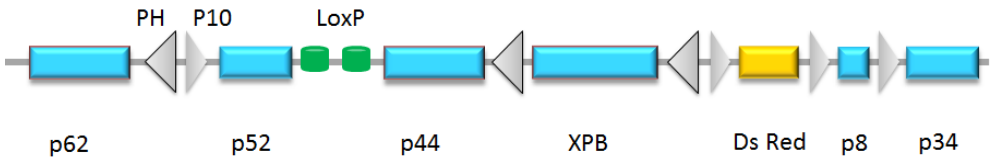






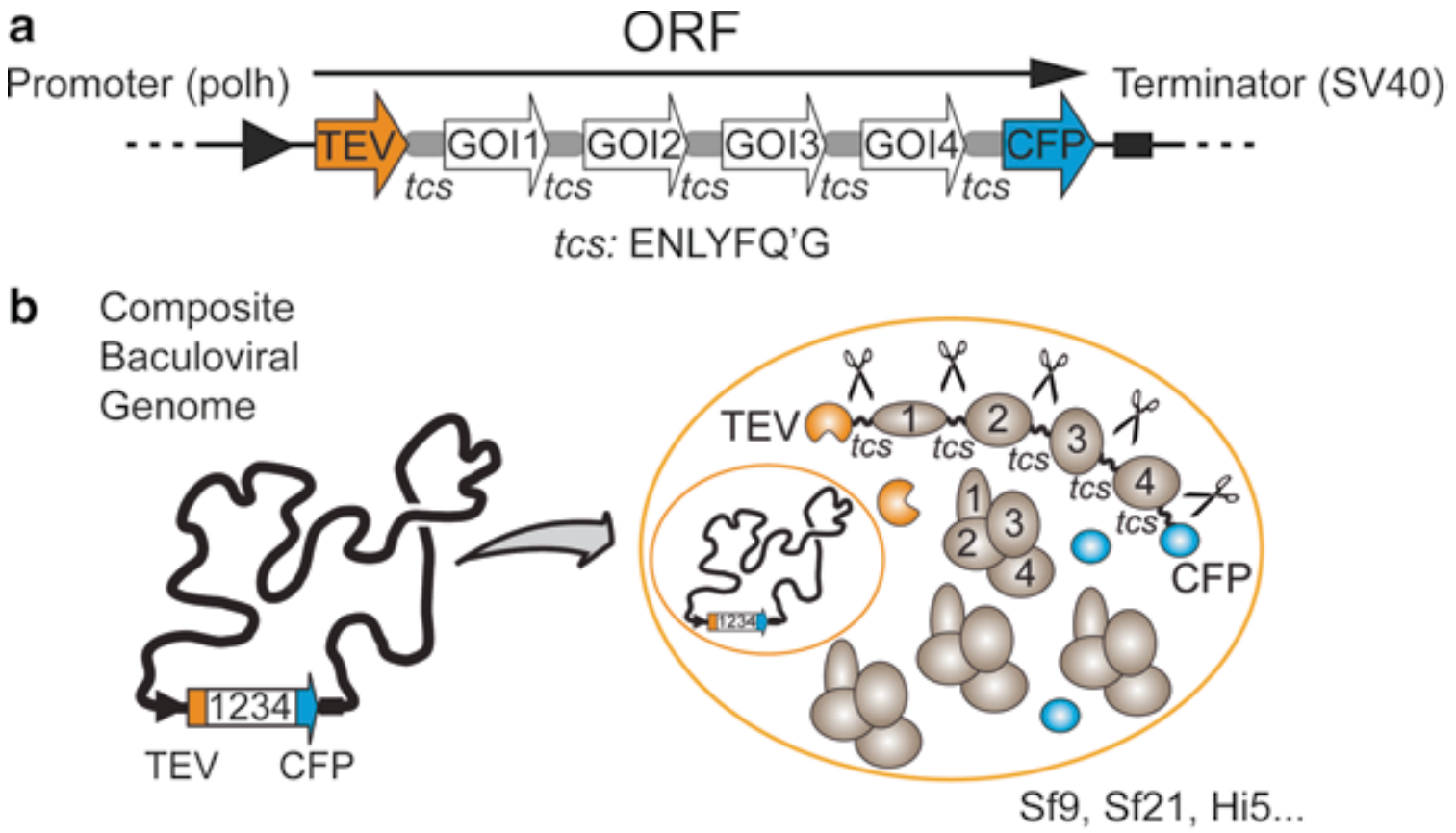




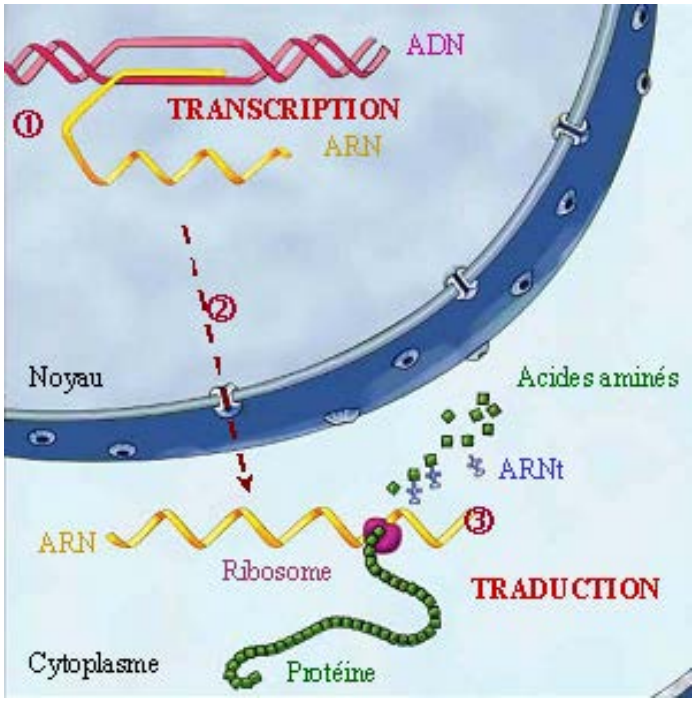


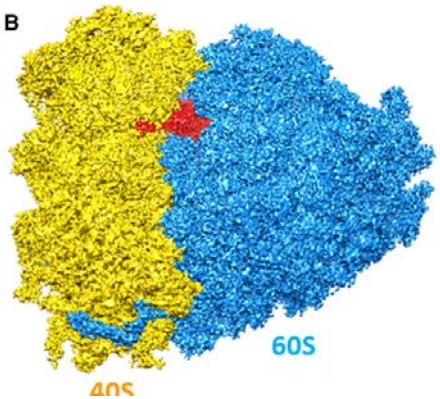
(Abdulrahman et al, PNAS 2013, Kupper et al., Plos Biology 2014, Radu NAR 2017)

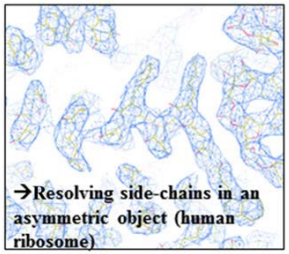
Polyproteins

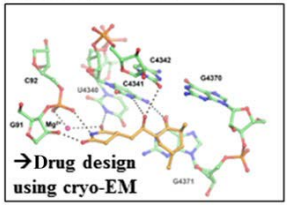


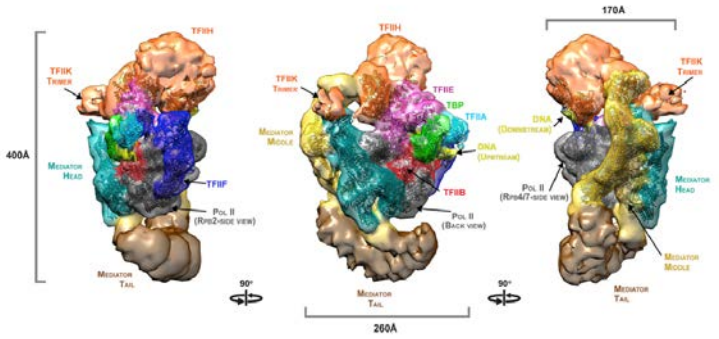
Preparation from endogenous sources











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
- Developed for genome wide protein-protein interactions studies and also used for structural biology applications

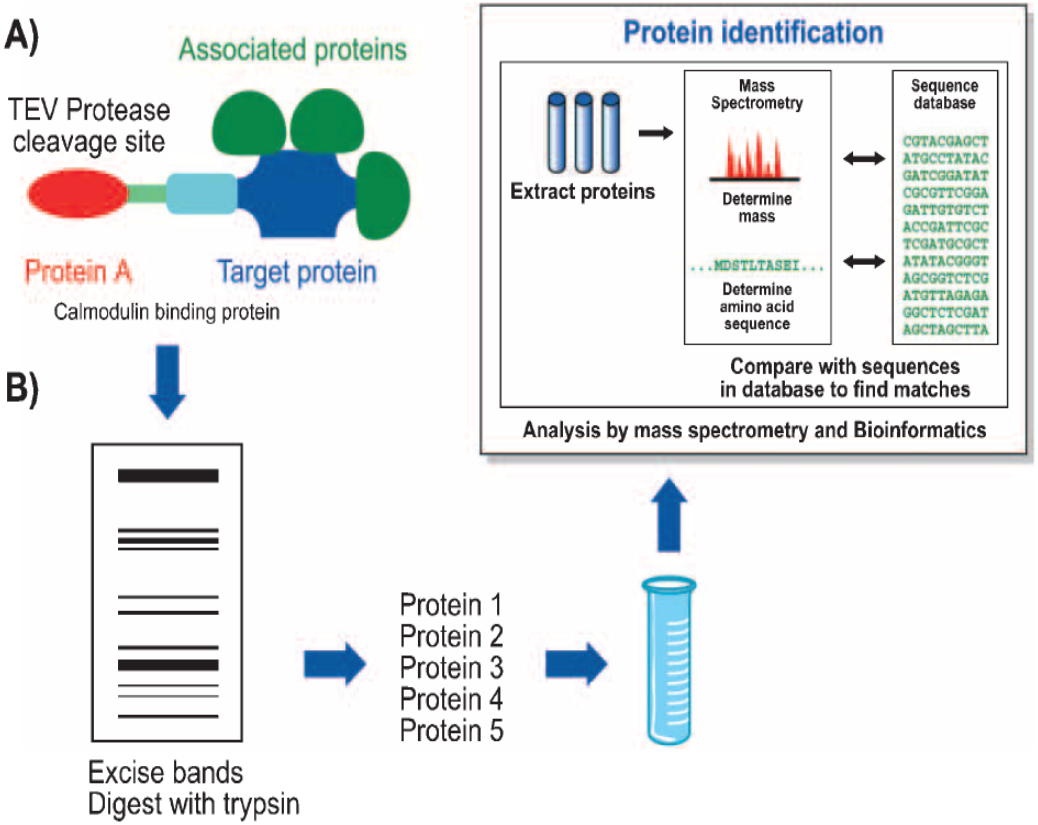
B Séraphin

TAP-MS

Target protein fused to a Protein A – Calmodulin binding protein

First step: an IgG column (TEV cleavage for elution)

Second step: a calmodulin beads (Ca°°/EGTA)



(Guillaume Rigaut, *et al.* 1999)

(Arnaud Droit, *et al.* 2005)

TAP-TAG in yeast

(Gavin, 2002)

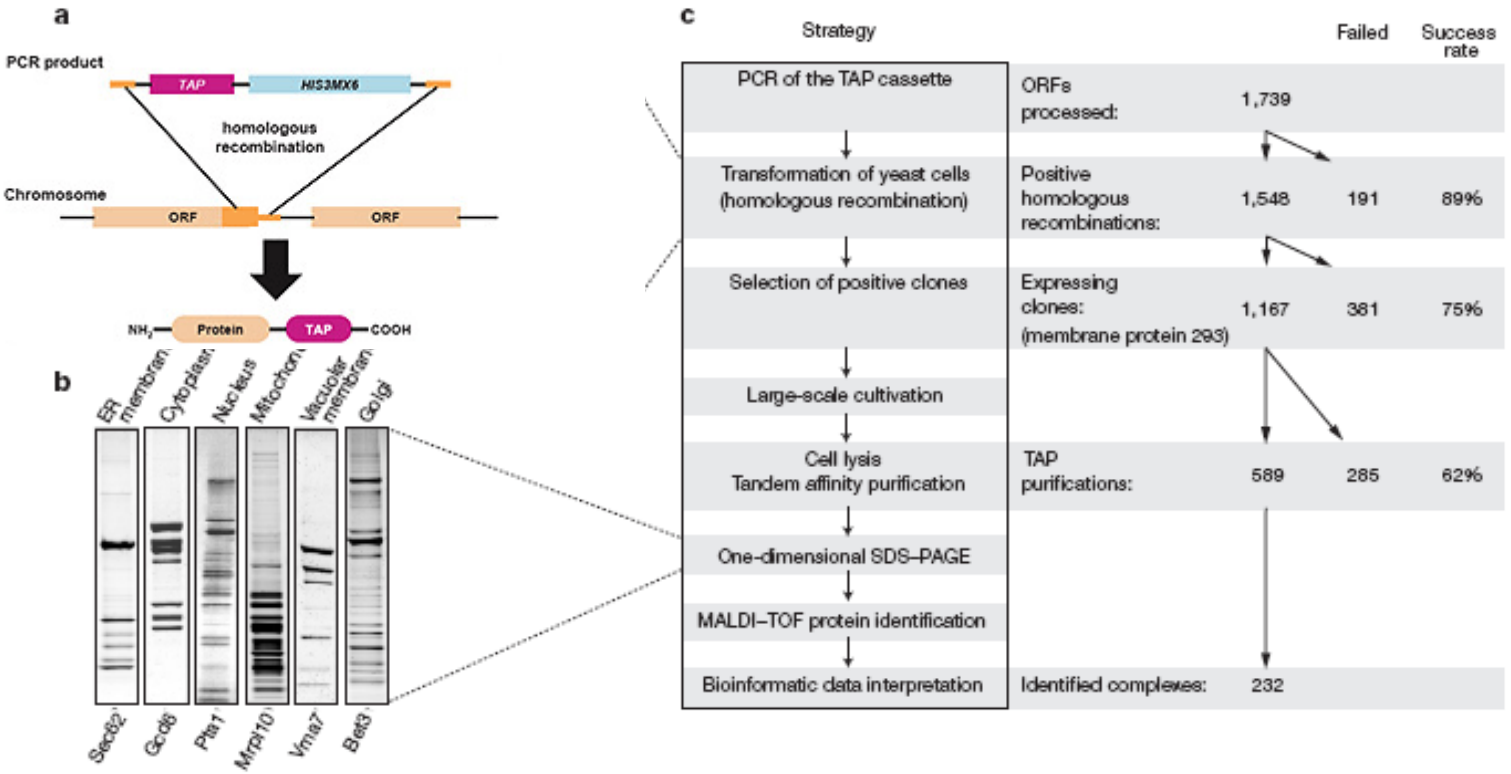
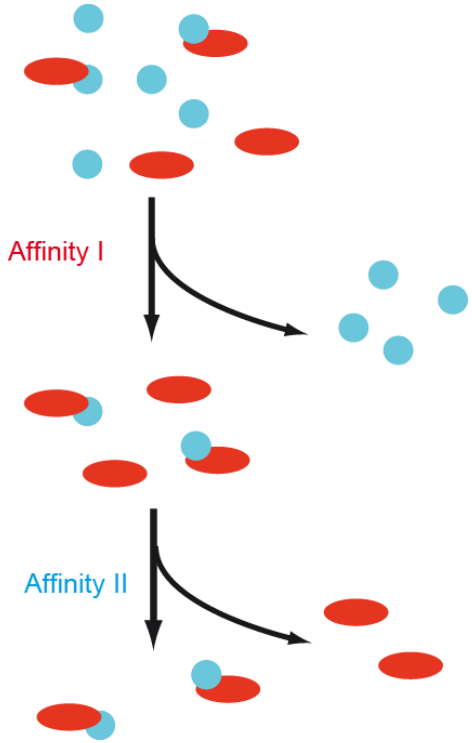


Figure 1 Synopsis of the screen. **a**, Schematic representation of the gene targeting procedure. The TAP cassette is inserted at the C terminus of a given yeast ORF by homologous recombination, generating the TAP-tagged fusion protein. **b**, Examples of TAP complexes purified from different subcellular 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).

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

Right tag on the right place

Tandem affinity purification



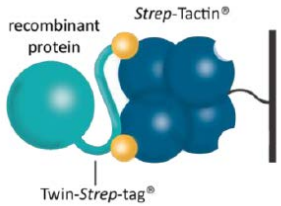
Which affinity tags?

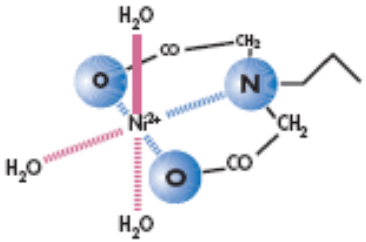
10His, Twin-strep, 3Flag, (HA, GFP, CBP)

The position maters

Two different subunits

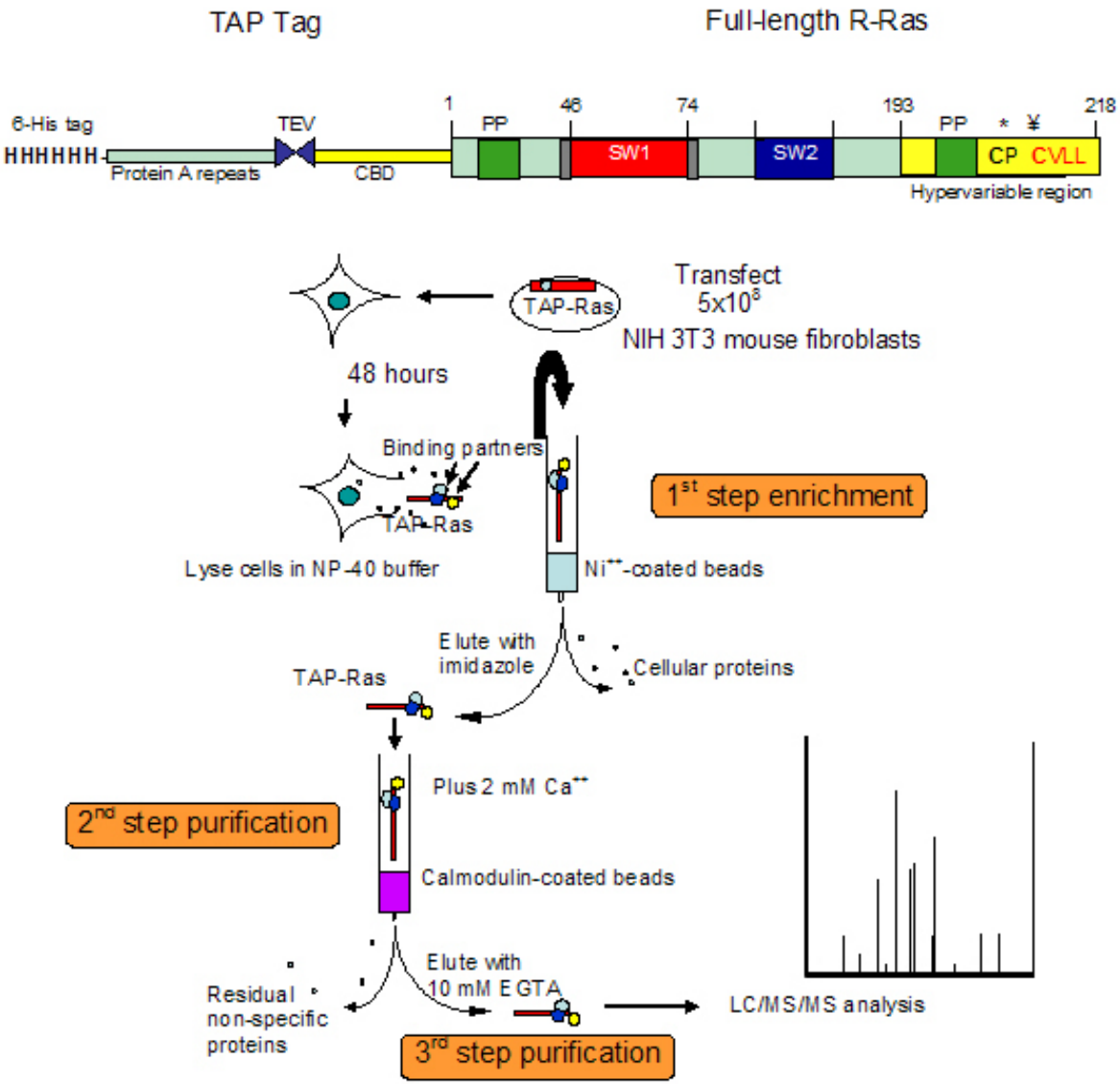
Location has tremendous impact





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

TAP-TAG in Mammalian cells



Engineering of Mammalian cell lines

Random integration (based on antibiotic selection)

Site specific integration by RMCE

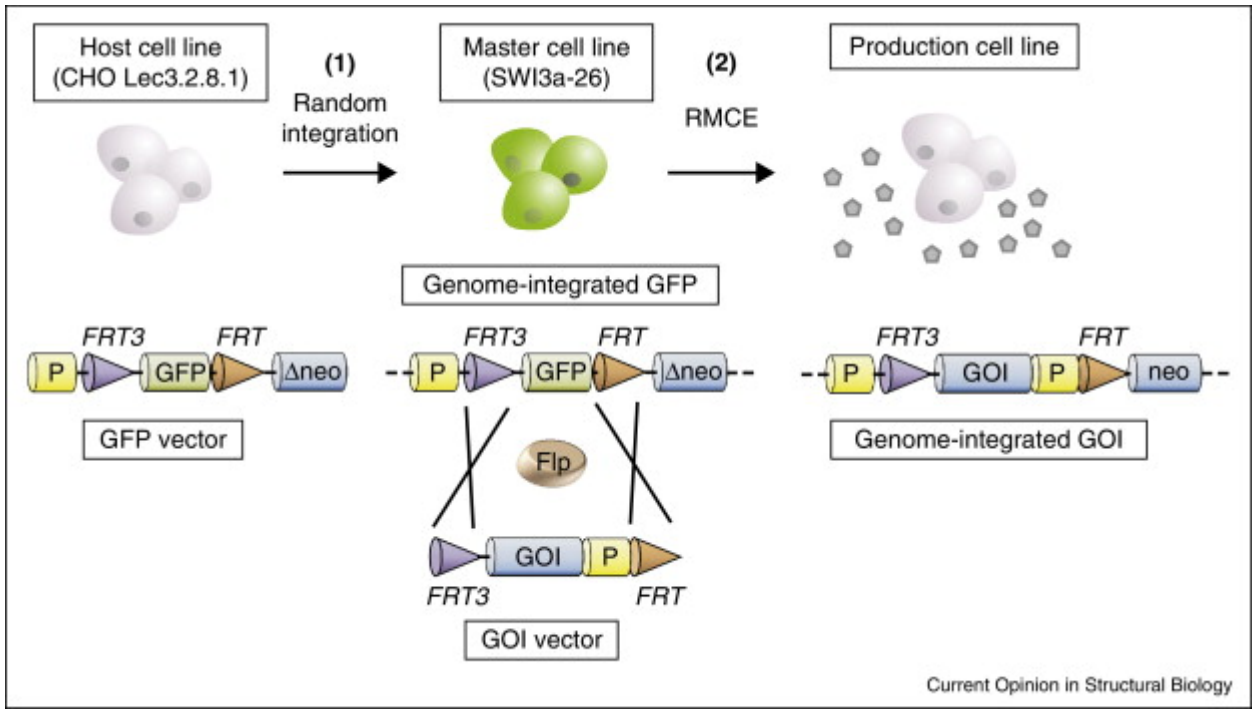
DNA Editing tools: Zinc-Fingers, Talen, CRISPR-Cas9

Recombinase Mediated Cassette Exchange (RMCE, Flp-IN)

Isogenic Expression Cell Lines

Master cell line containing a pair of genome integrated FRT sites (+ a GFP marker)

The FRT sites are used to replace the GFP gene by the GOI leading to a Genome-integrated GOI
- Reaction catalyzed by the FTR recombinase - Targeted integration

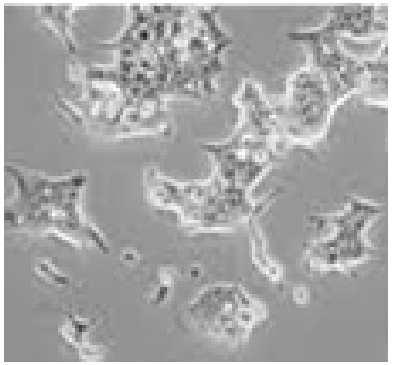


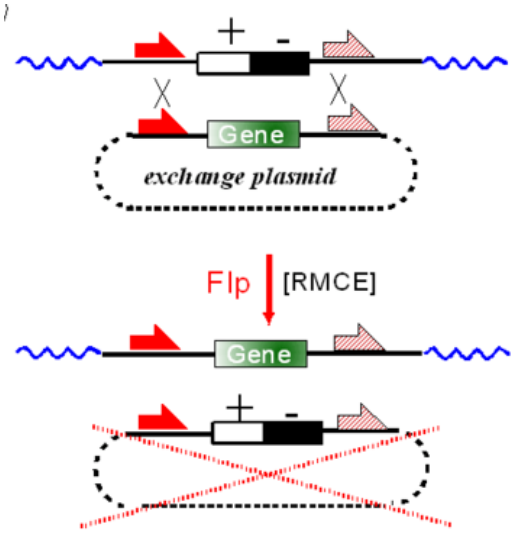
Recombinase Mediated Cassette Exchange (RMCE, Flp-IN)

Isogenic Expression Cell Lines

Targeted integration

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





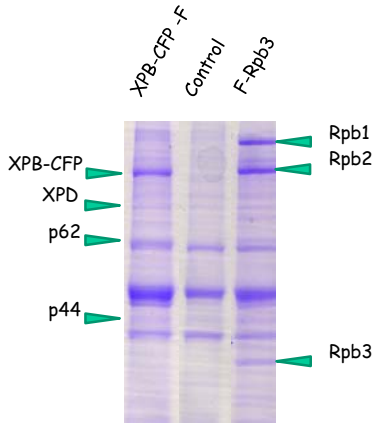
Clone the gene in pcDNA5FRT (1 week)

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

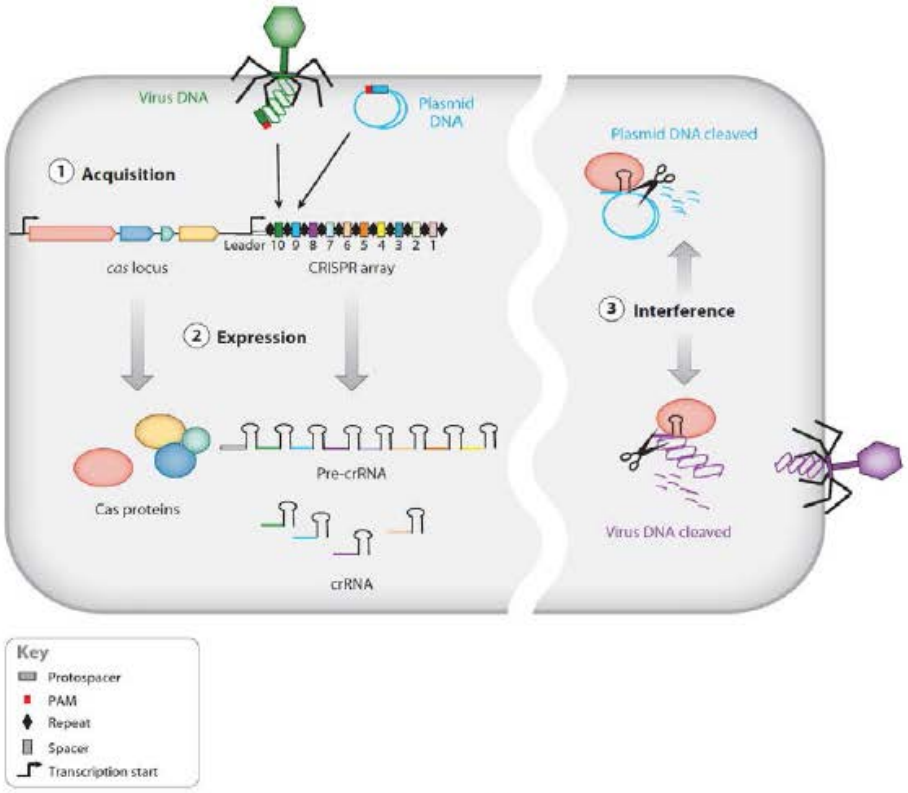
Establishment of a clonal population by limit dilution (3-4 weeks)

Clones screening by Western Blot (1-2 weeks)

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



CrispR-Cas 9 genome editing



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

Mechanism of adaptive immunity in bacteria and archaea

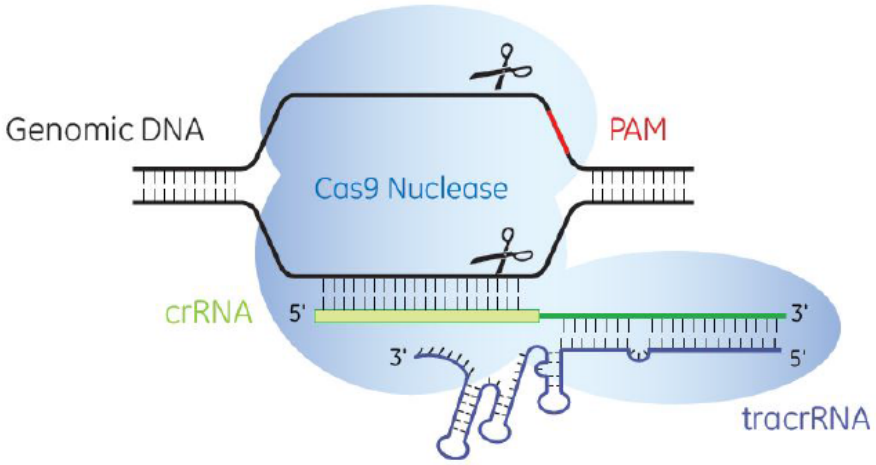
Evolved to adapt and defend against foreign genetic material (i.e., phage, horizontal gene transfer, etc.)

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cas: CRISPR-associated proteins

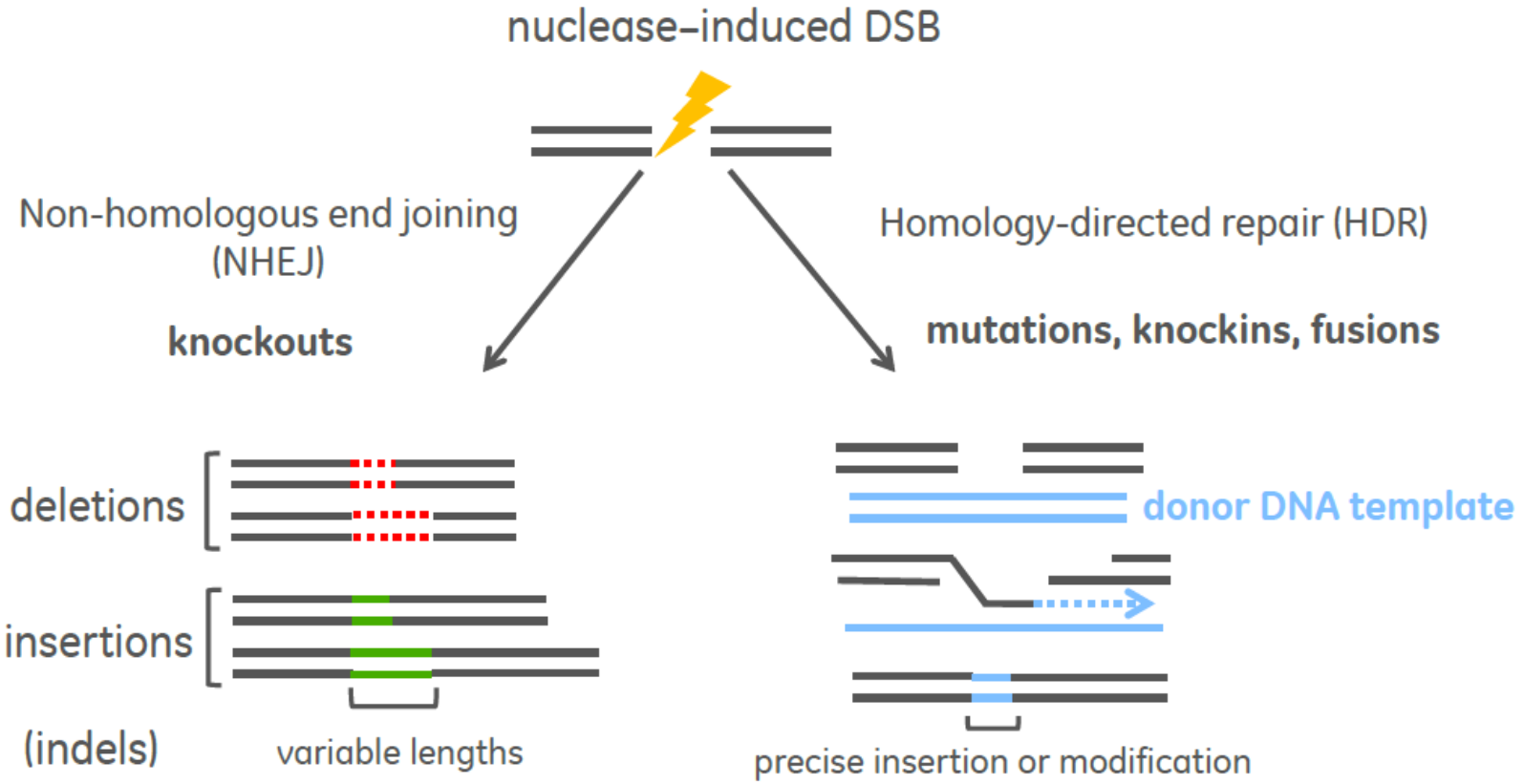
Required components for CRISPR-Cas9 gene knockout

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

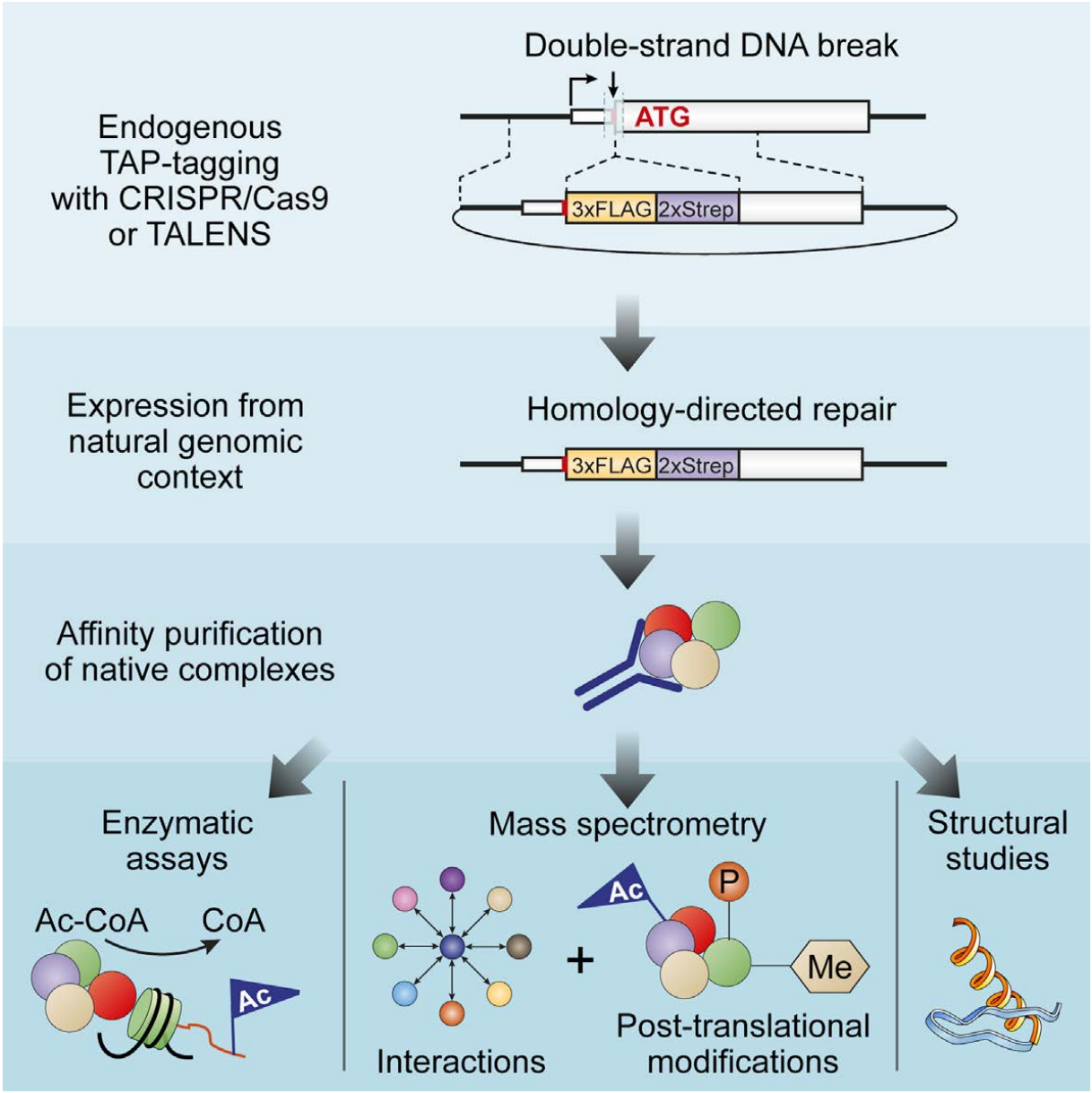


- **crRNA** – synthetic RNA comprising 20 nt target-specific sequence and fixed *S. pyogenes* repeat sequence
  - High-throughput synthesis to enable arrayed screening
- **tracrRNA** – Long synthetic RNA which hybridizes with crRNA

Editing by repair of double-strand breaks (DSB)

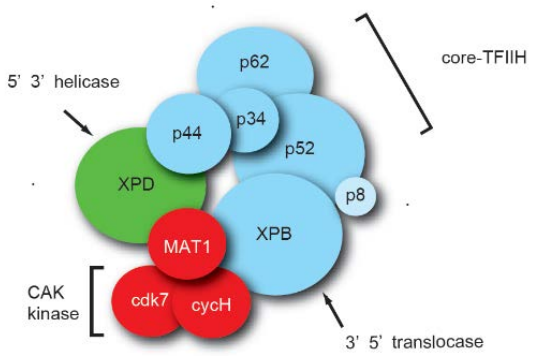


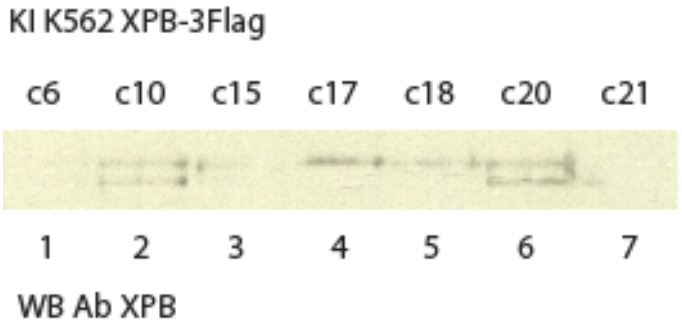
Donor DNA template : a double stranded DNA fragment or a single stranded DNA

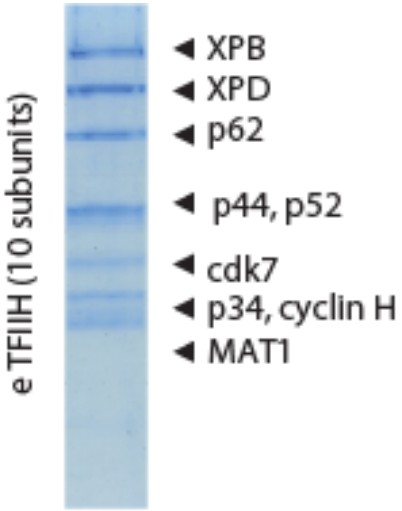


Dalvai, Cell reports, 2016

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