Library Methods for Production of Soluble and Crystallisable Proteins and Domains

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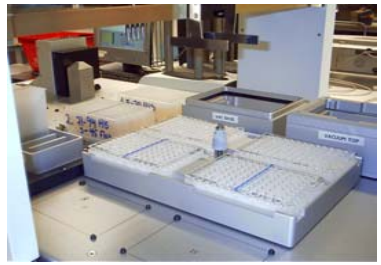
”Our simple (and affordable…) philosophy”

- **Get the maximum out of E.coli - the perfect host when it works !**

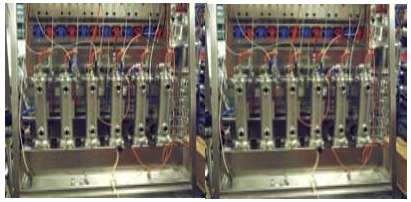
- **Appropriate benchmarking of technologies - large resources are wasted based on rumors and oversold technologies !**

Some platforms established in Stockholm

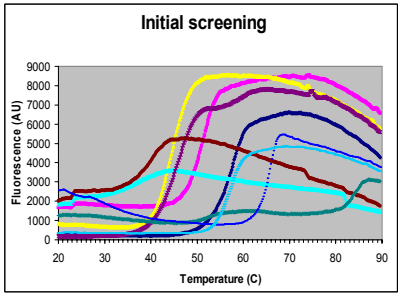
- **Parallel cloning and expression screening** => Gateway cloning, FiDo-screen =>



- **Parallel scale-up** => Belach, Greta Parallel fermentor =>



- **HTP-stability screen** => Thermoflour, TSA =>



Library technologies – a magic box or an ugly sink for resources ?

- **Difficult proteins can often be produced in *E.coli* after exhaustive and sequential efforts. Can take years...**

- **Simultanious screening of large libraries of expression variants could potentially constitute a magic ”one shot solution” ?**

- Different length constructs

- Random mutations

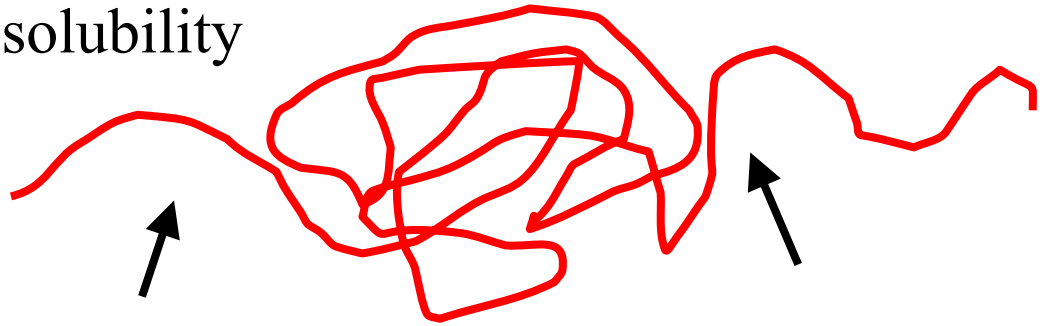
- Mixed pools of vectors, strains etc

- Mixed pools of genes/orthologes etc

Screening construct libraries - Why bother ?

The "right domain borders" can: .

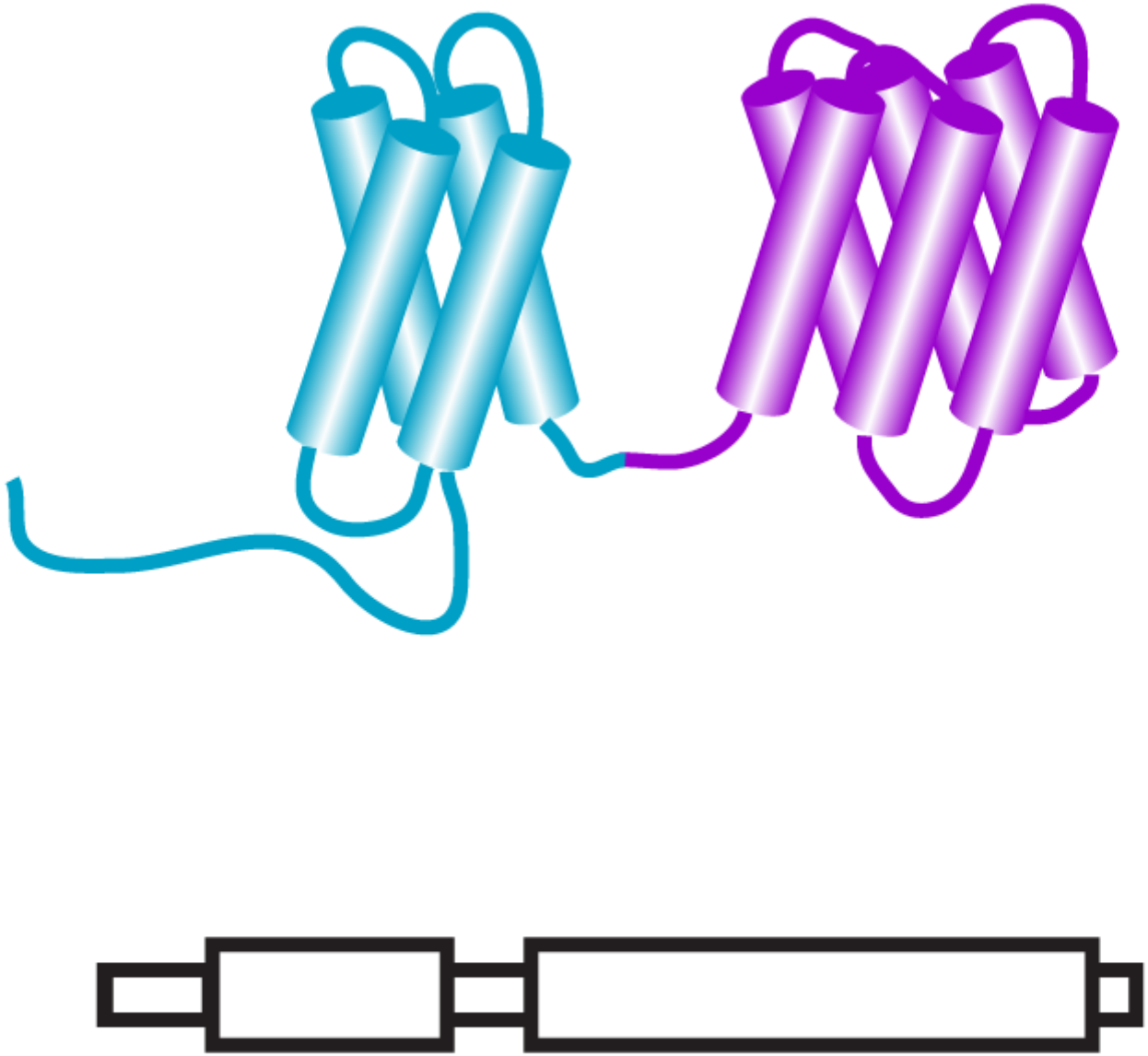
- improve expressibility and solubility
- improve crystallisibility

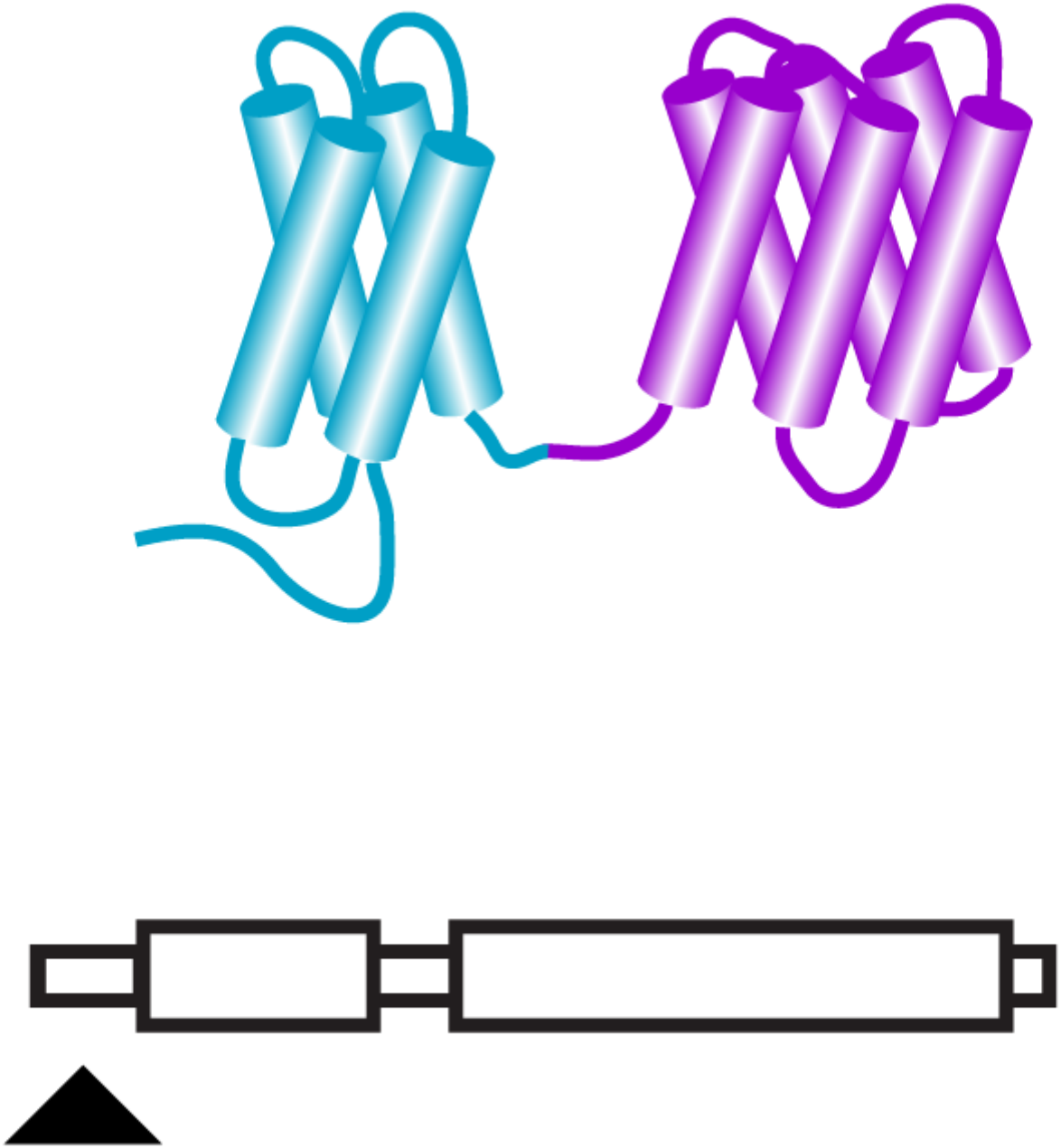


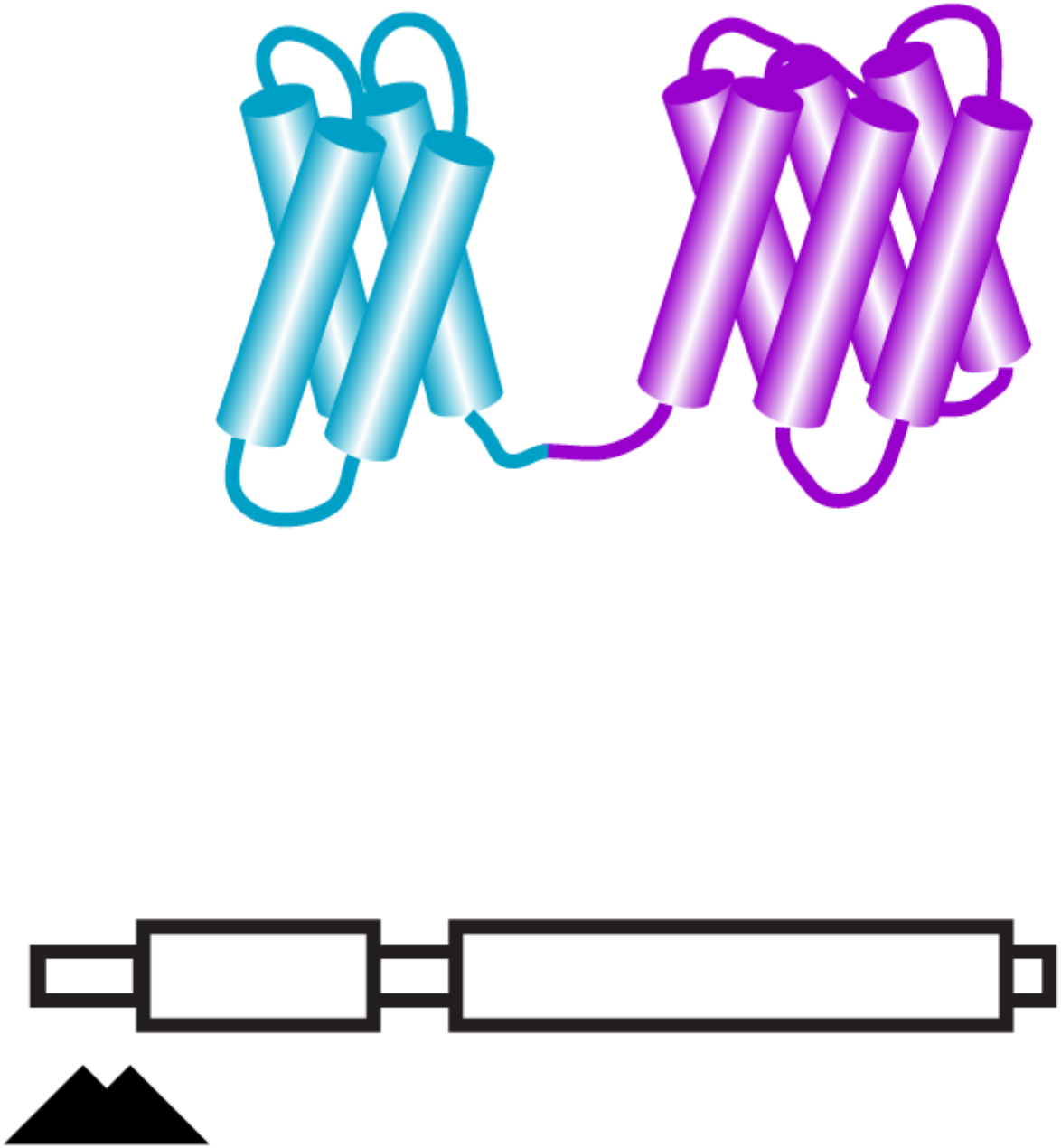
Current main strategies:

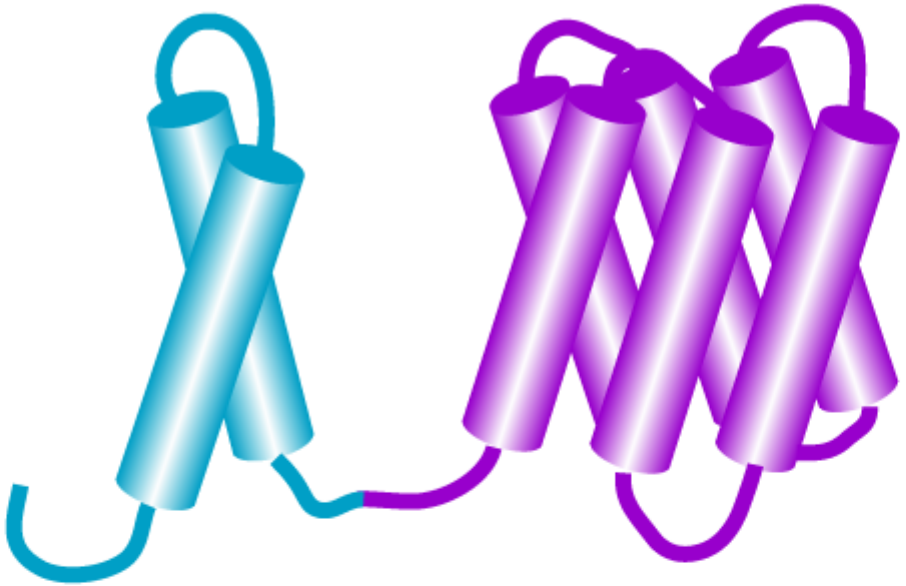
- Bioinformatics based domain border analysis
- Partial proteolysis => Mass Spec => re-cloning
- Often try 5-15 constructs of each protein

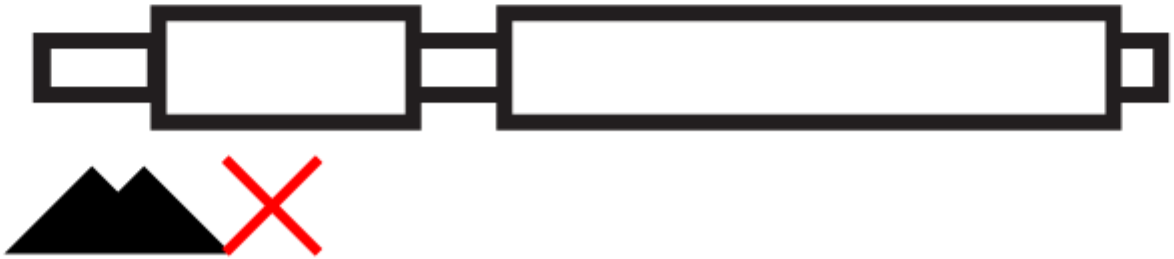
Let's test an N-terminal deletion construct library strategy !

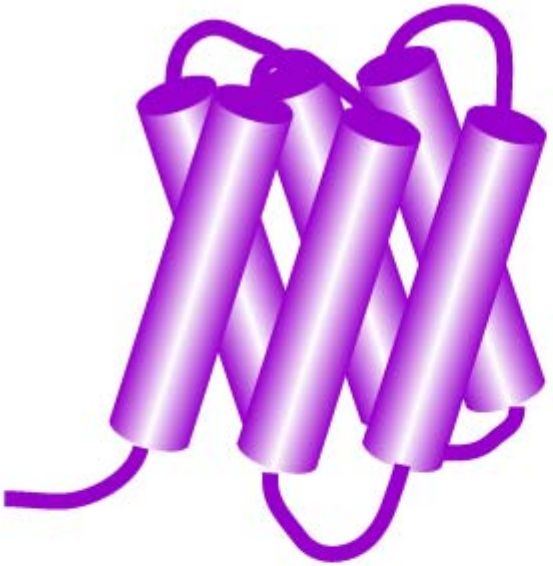


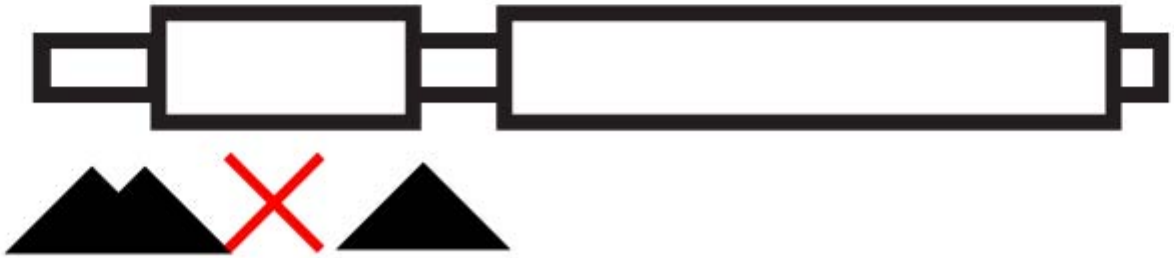


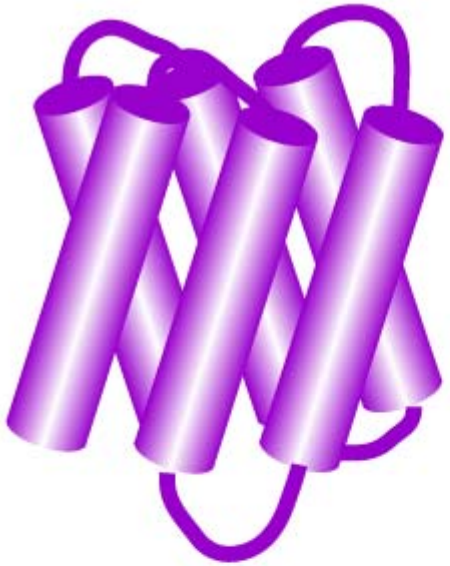


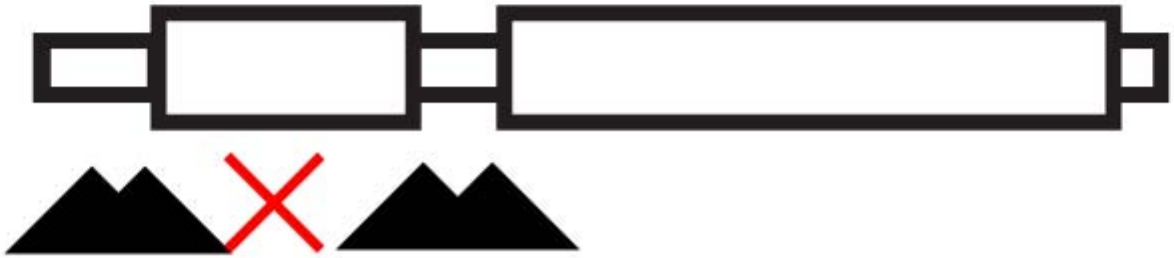


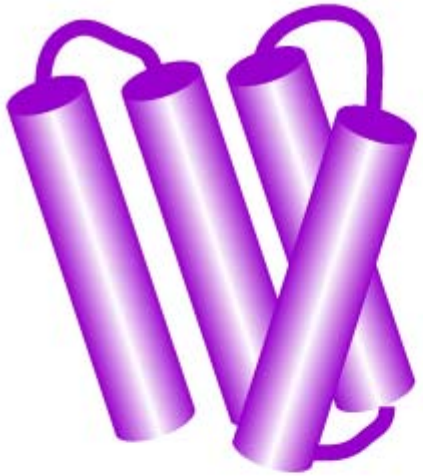


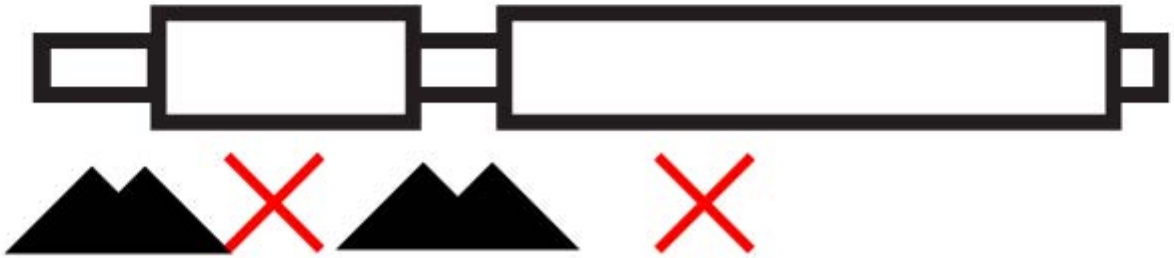


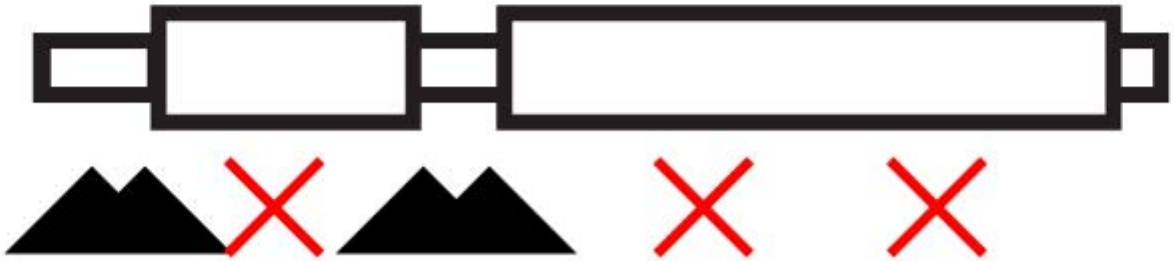




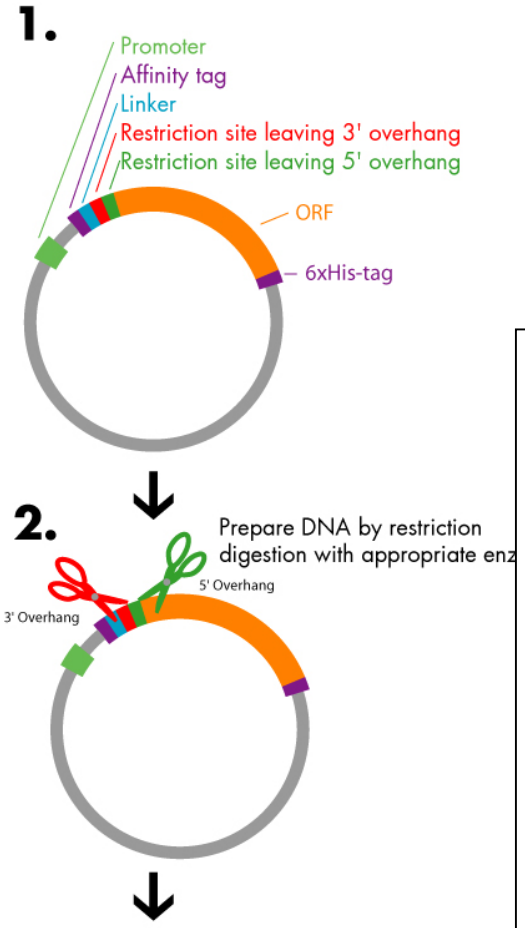


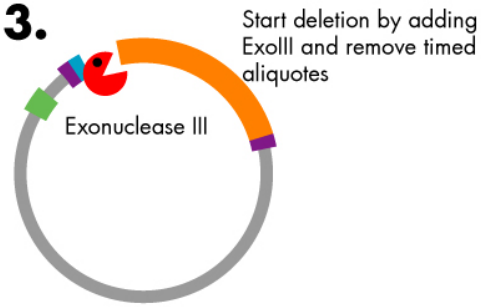




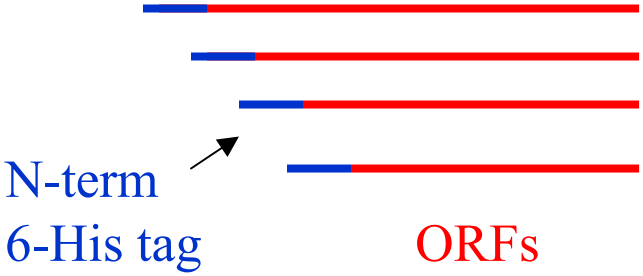


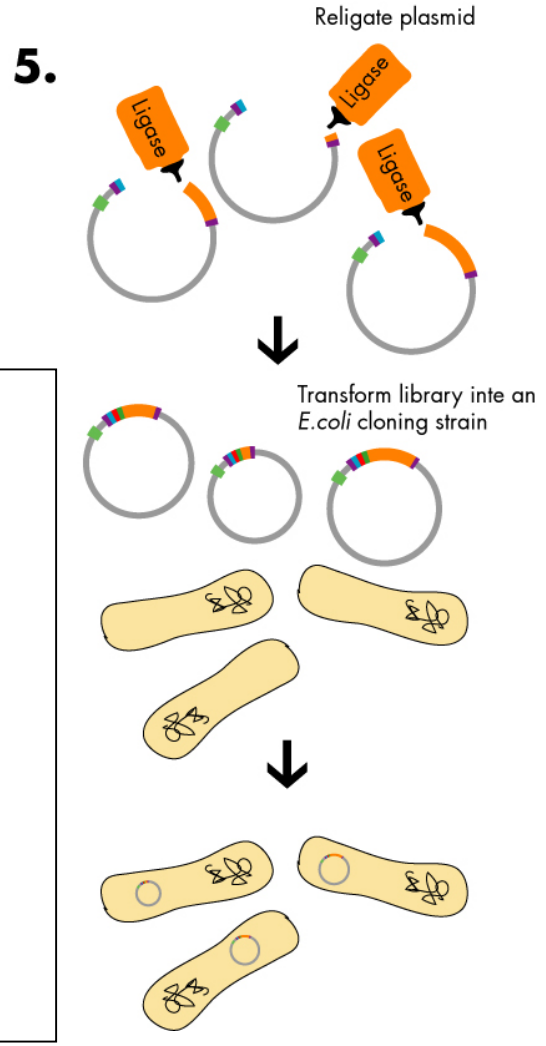
Erase-a-Base (E-a-B) for N-terminal deletion library generation





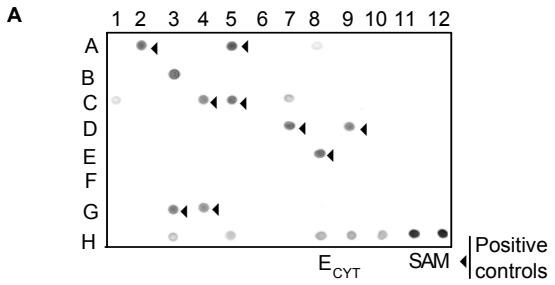
Generate a set of plasmids expressing ORFs with different N-term start



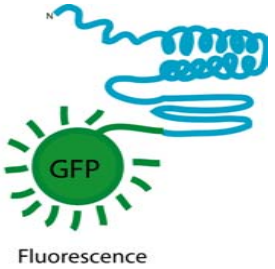


HTP solubility screening methods

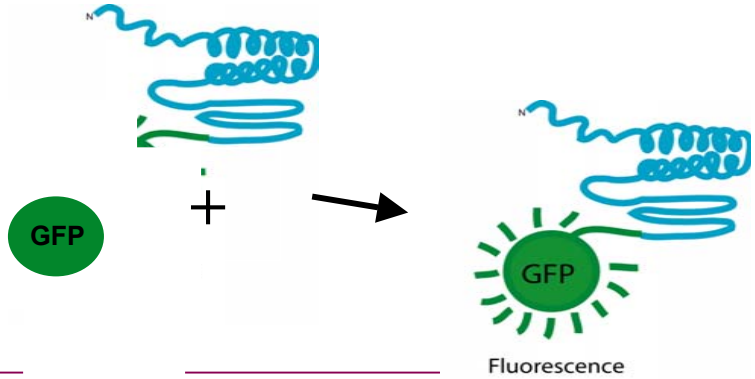
- **Direct small culture 96-well based methods**
  e.g. Ni-NTA affinity, FiDo-screen etc
  Problems:
  - Limited numbers of clones
  - Expensive and requires automation



- **Direct C-terminal fusion proteins as a folding markers**
  e.g GFP, CAT
  Problems:
  - Change solubility of target
  - Solubility can change upon lysis
  - Fusions have to be removed

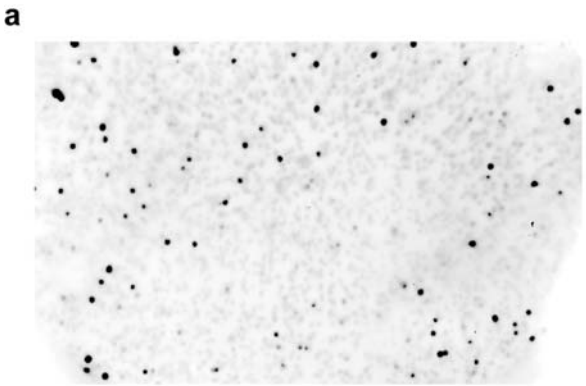


- **Split C-terminal fusion proteins**
  e.g. GFP, Lac-Z
  Problems:
  - Solubility can change upon lysis

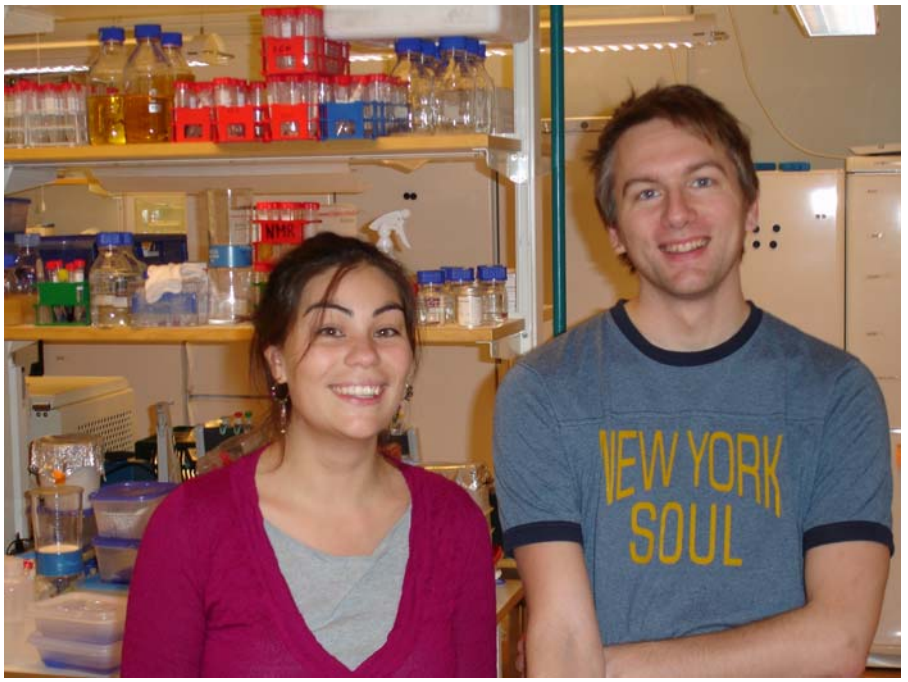


The Colony Filtration blot

Soluble proteins detected on colony level by applying 2-d filtration separation step followed by blotting of His-tag



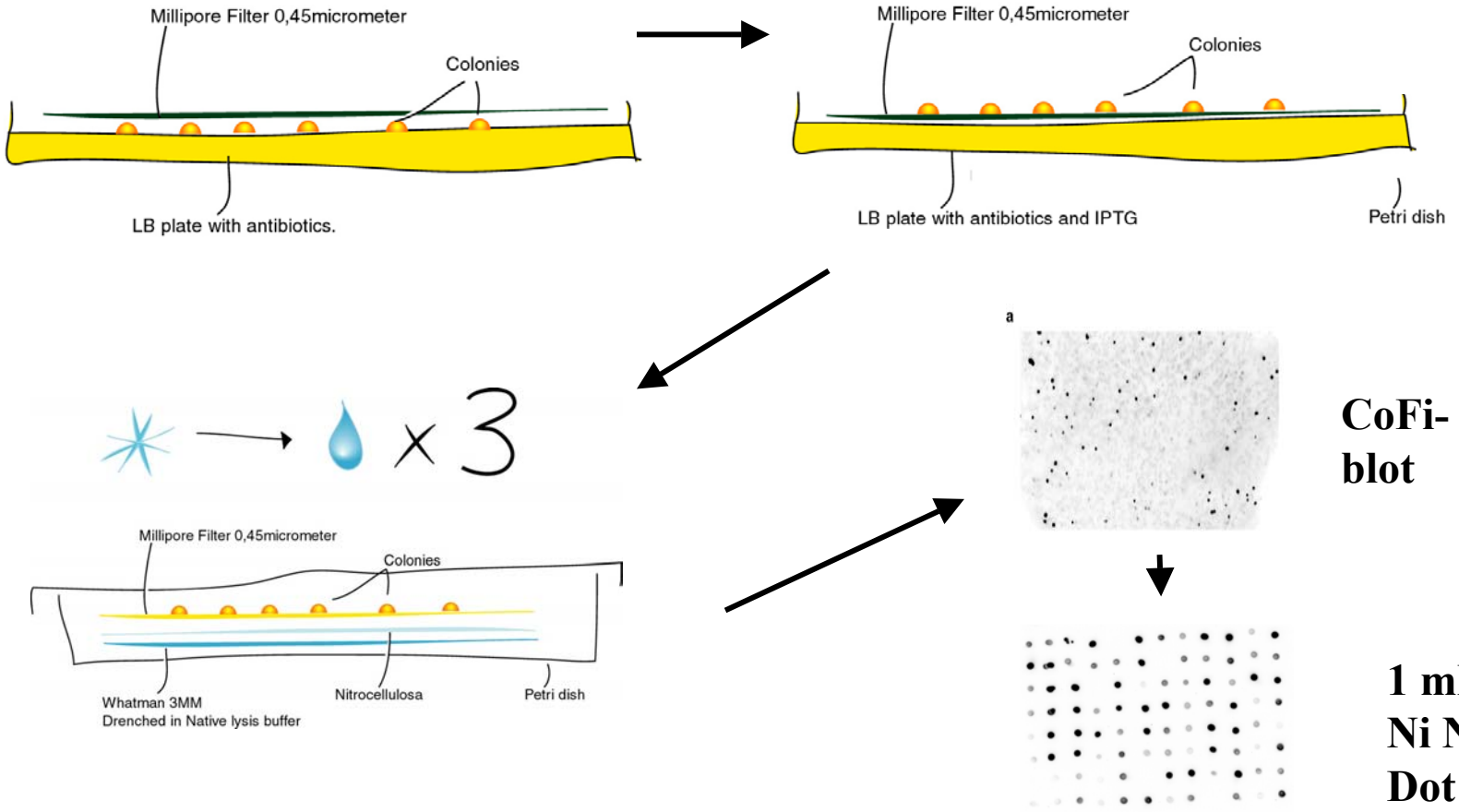
CoFi-blot of a ”deletion library” (Patent pending, www.Evitra.se)



Some applications of CoFi- blots

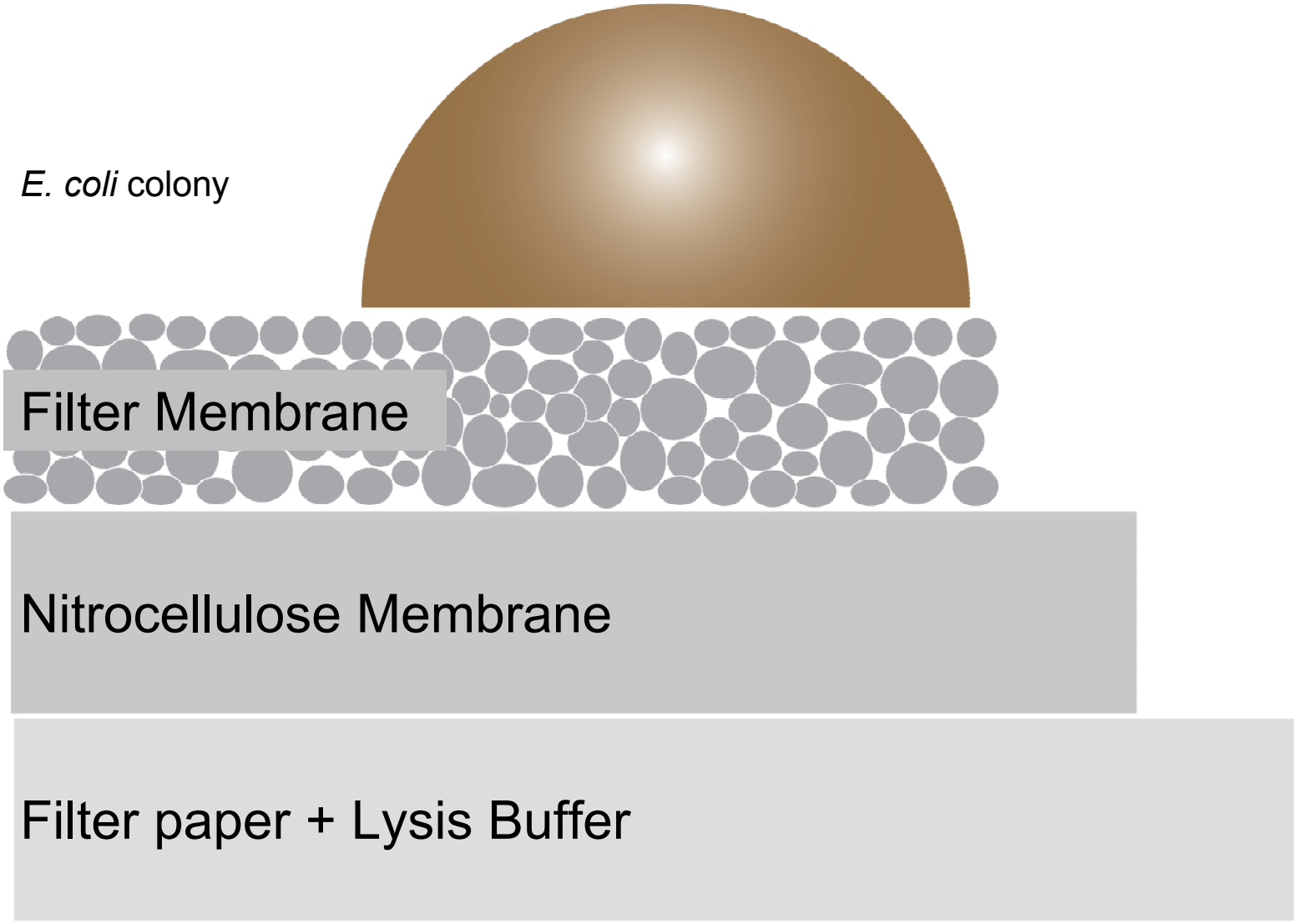
- Deletion libraries
- Random mutagenesis libraries
- Membrane proteins (deCoFi-blot)

The craft of the CoFi blot



CoFi-blot showing colonies potentially express soluble protein.

Colony Filtration (CoFi) Blot



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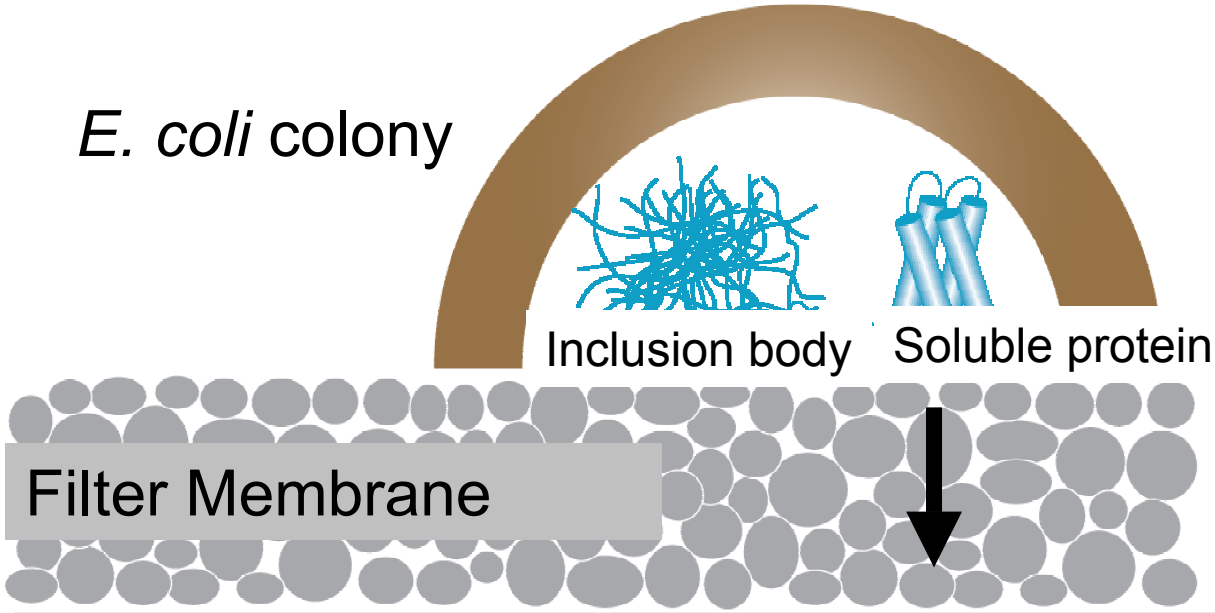
Lysis and filtration



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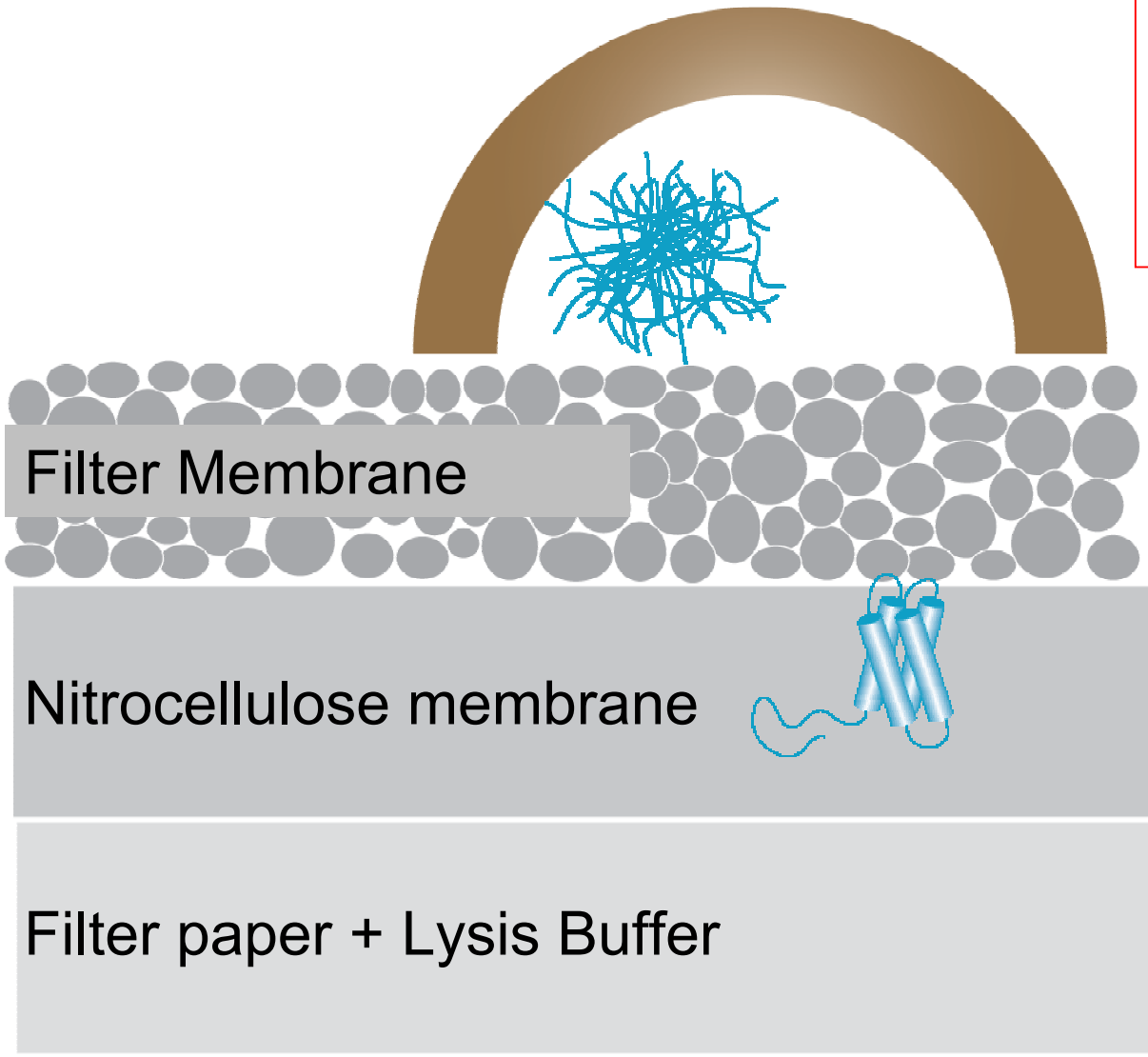
Lysis and filtration

The lysis step and a Ni-conjugate probe simulate the scale-up process !



Nitrocellulose membrane is probed with antibody or Ni conjugate to identify colonies that express soluble protein

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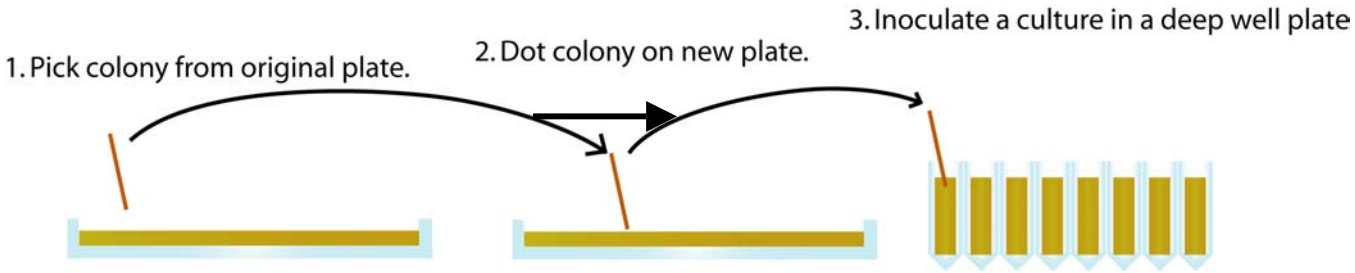
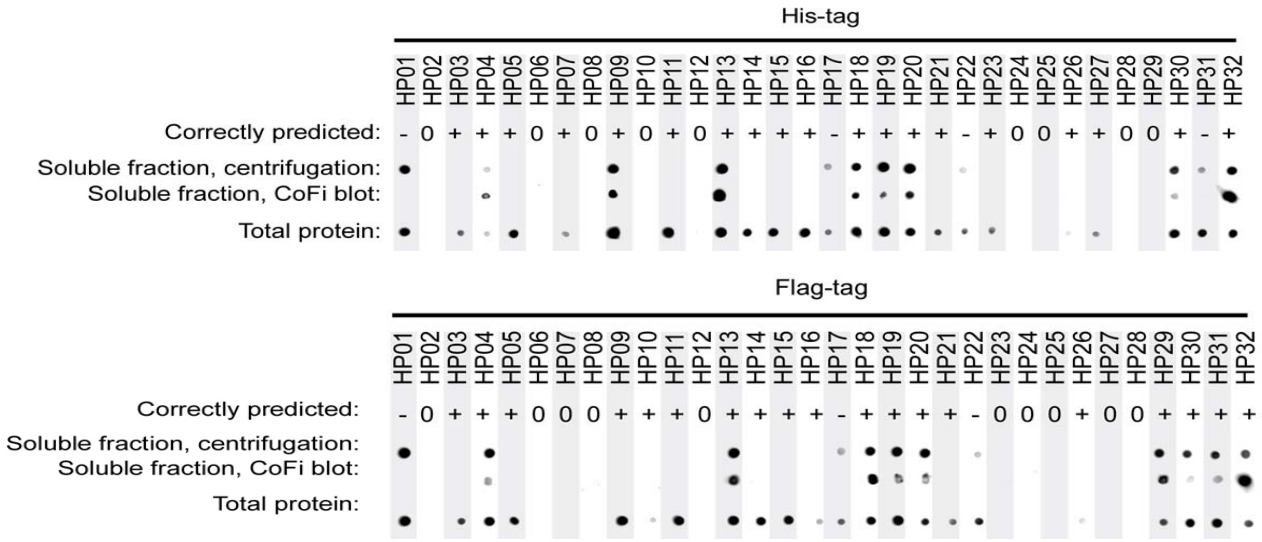
Comparison of CoFi-blot and Centrifugation





Detection using INDIA HIS HRP

- 83 % (38 of 45) proteins with total expression are in agreement between the two methods! In all cases CoFi-blot was a "more stringent criteria" !

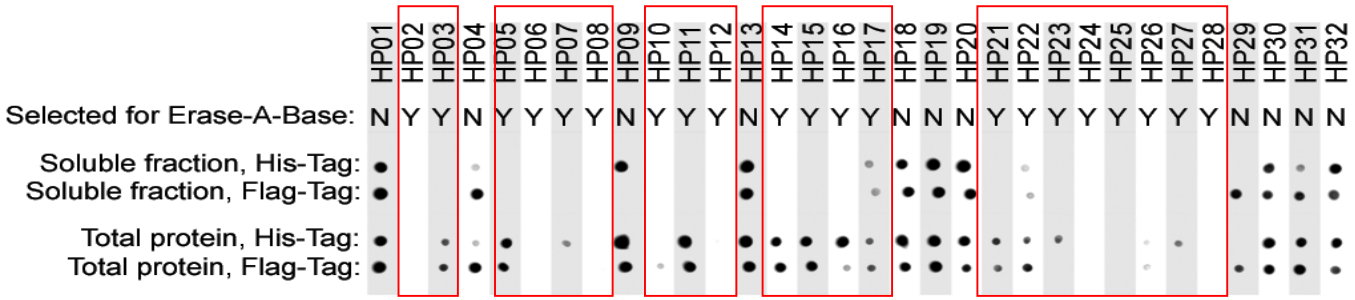
Benchmarking of the E-a-B => CoFi-blot strategy

By Tobias Cornvik
Sue-Li Dahlroth
Audur Magnusdottir
Victoria Lieu &
Monika Ekberg

Success rates before E-a-B => CoFi-blot:

- 32 Human/Mouse proteins used to benchmark the technology

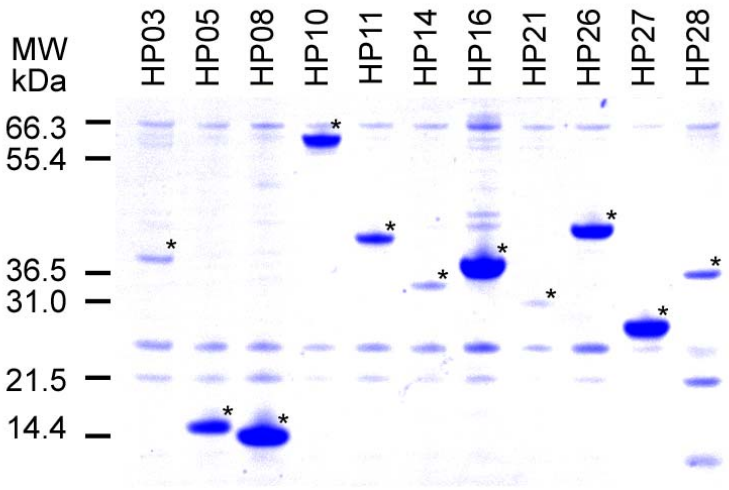
- 19 of 32 proteins do still not express soluble protein in 2 different vectors (N-term Flag and His) - i.e. ”hard to express proteins”



After E-a-B => CoFi-blot – many "hard to express proteins" can now be purified

- 11 of 19 "hard to produce" proteins could be "rescued" by the erase-a-base trick - at least one domain
4 more could be purified at lower level.

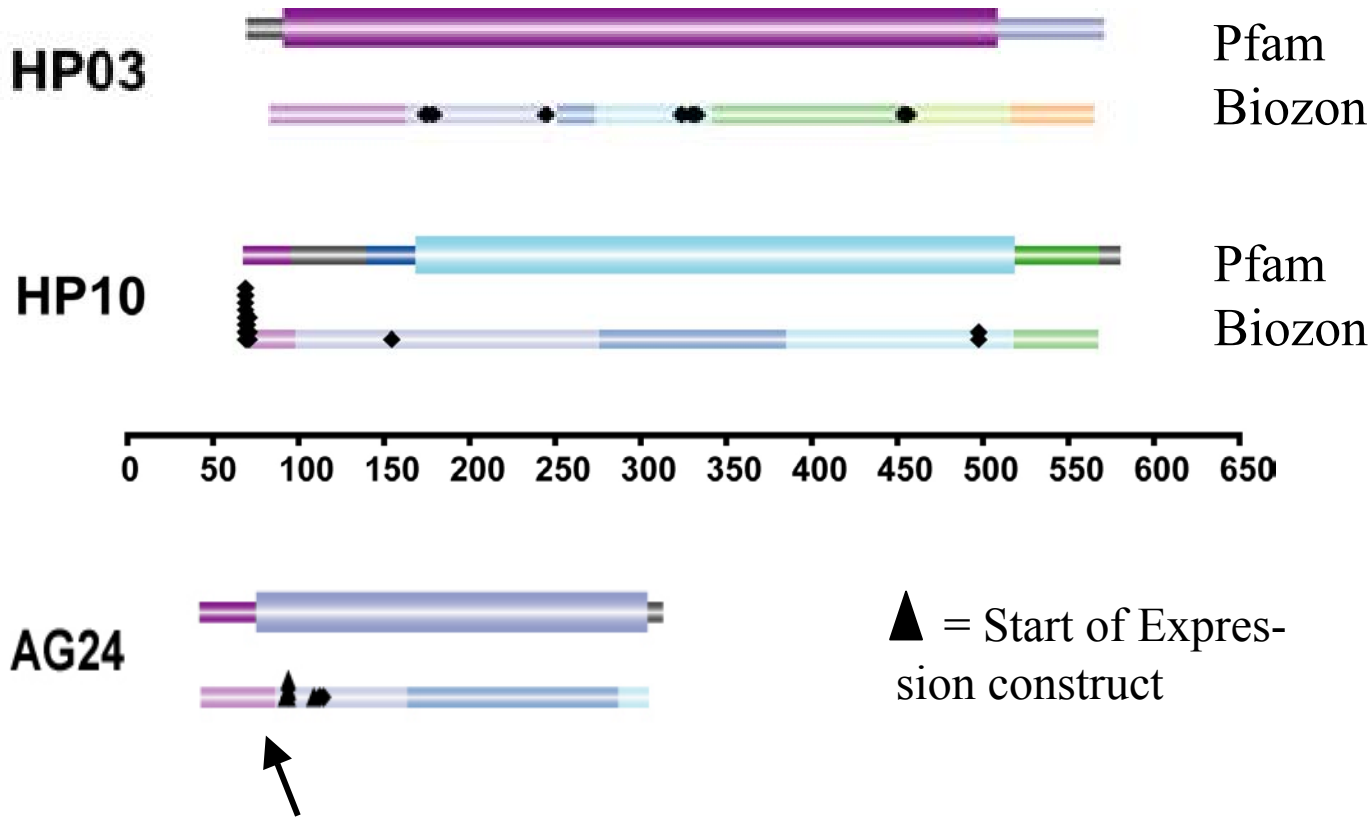
-



After a small-scale IMAC purification

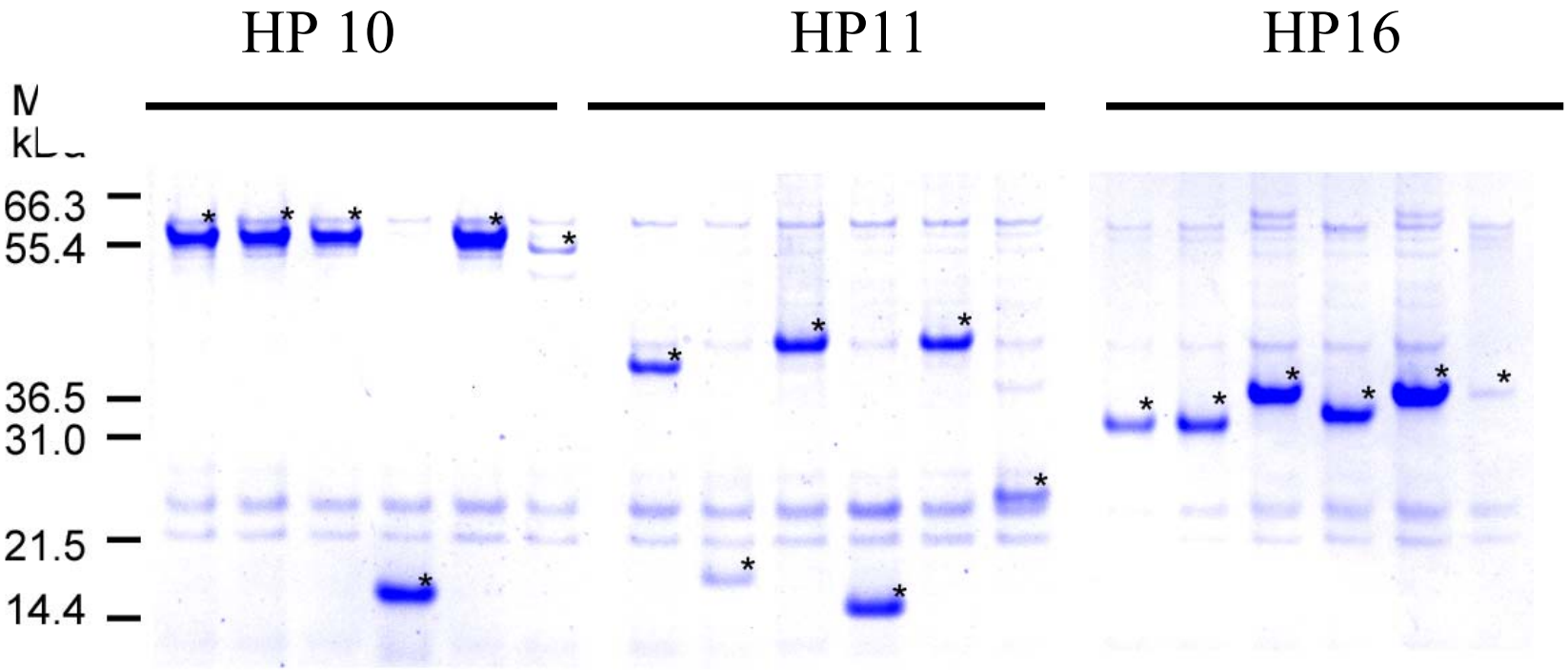
- Purified constructs have no/little proteolysis problems

E-a-B => CoFi-blot - a generic experimental domain foot-printing strategy



N-terminal mitochondrial signalling sequence is removed

A number of different length constructs are directly produced



- ”orthogonal constructs” will improve probability for crystallisation

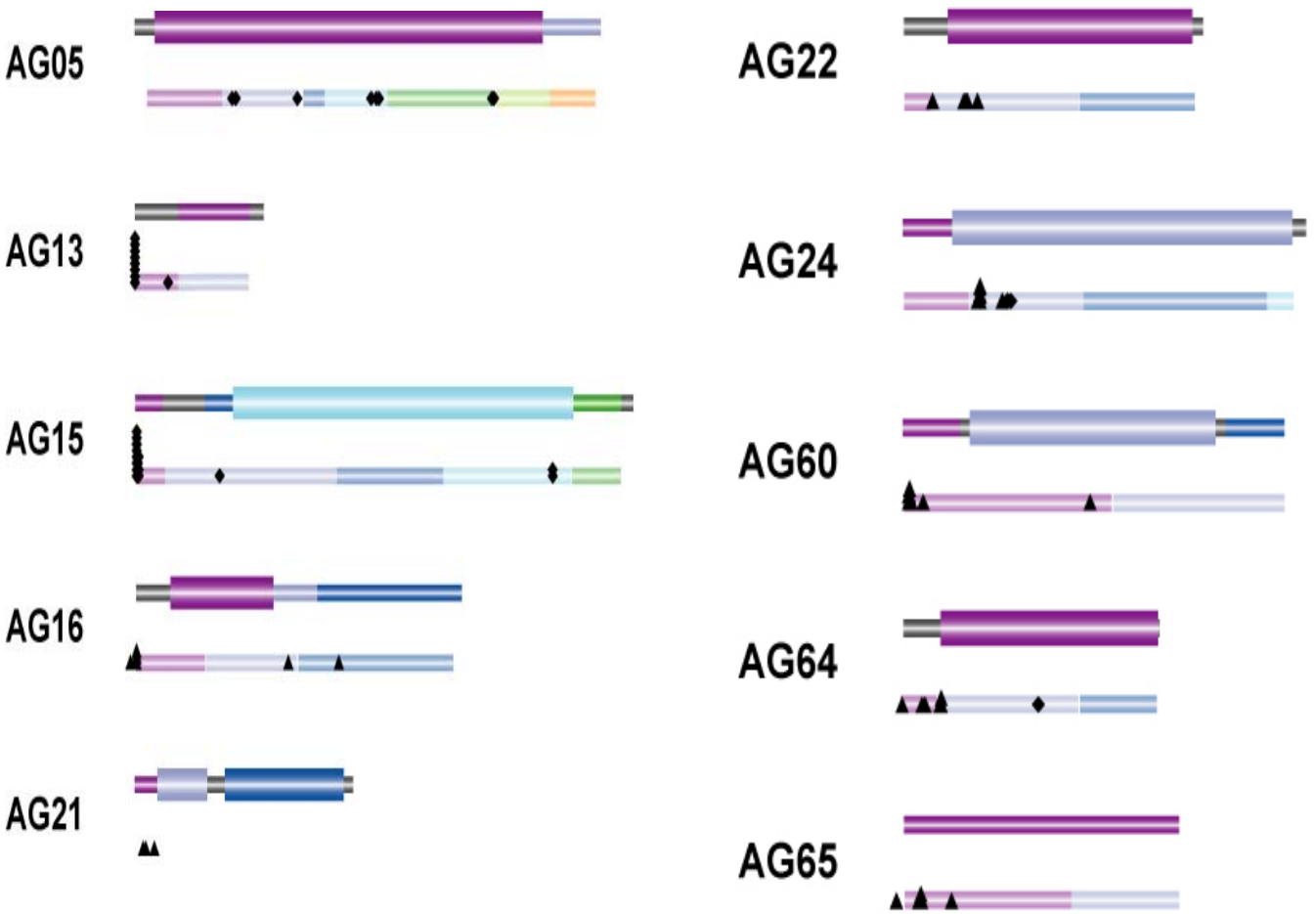
How useful are multi construct for crystallisation ?

SGC-Stockholm study in press in Prot Expr.& Purif (Gräslund et al)

- ~10 different constructs approach approach ⇔ rational 1-2 construct
- 15 proteins with diffracting crystals ⇔ 65 proteins with diffraction crystals

A limited number of construct taken into crytallisation trials give diffracting crystals for > 4 times more proteins

Most Human/Mouse proteins can be expressed close to full-length



Summary of Benchmarking - E-a-B constructs => CoFi-blot strategy

- **> 70 % of the starting 32 Human/Mouse proteins could be purified from *E.coli*.**
- **Most proteins express as nearly full length**
- **Comparison to Multi-Construct methods not made, but the E-a-B => CoFi-blot strategy is likely to work better for less characterised protein families.**
- ***E.coli* can potential produce many more eukaryotic proteins than we have anticipated**

Selection of an intriguing expression construct with a translational frame shift

Karolinska Institutet

By Martin Moche

DNA sequencing and N-terminal peptide sequencing of a positive construct of protein X selected with the E-a-B => CoFi-blot strategy

⇒ 4 bases are not translated by *E.coli* !

ATG AGA GGA TCG CAC CAC CAC CAC CAC G TGG CTG GTC ---
Met Arg Gly Ser His His His His His Trp Leu Val
R G S H H H H H L V

- Yields active enzyme with activity profile equal to wt enzyme !
(In frame reading yields 52 residue peptide)

The detergent adapted CoFI-blot - Selection of ”purifiable domains” of IMPs

Issues:

- How do you define a domain of an IMP ?

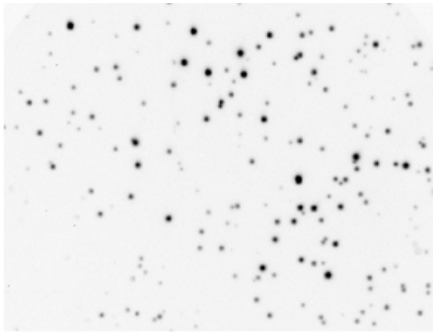
- What does a purifiable domain means when it is covered/protected by a detergent micelle?

Possible domain definitions:

- Single TM-helix

- Compact/”globular” domains

- Set of TM-helices which are “insertion competent”



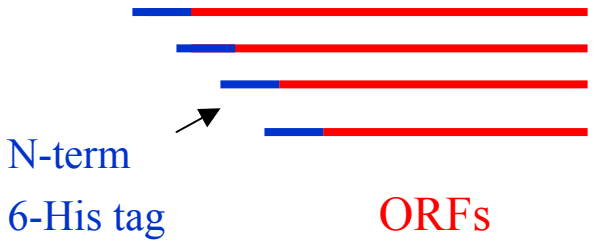
Detergent adapted CoFi-blot devel-oped by Marina I Sabet

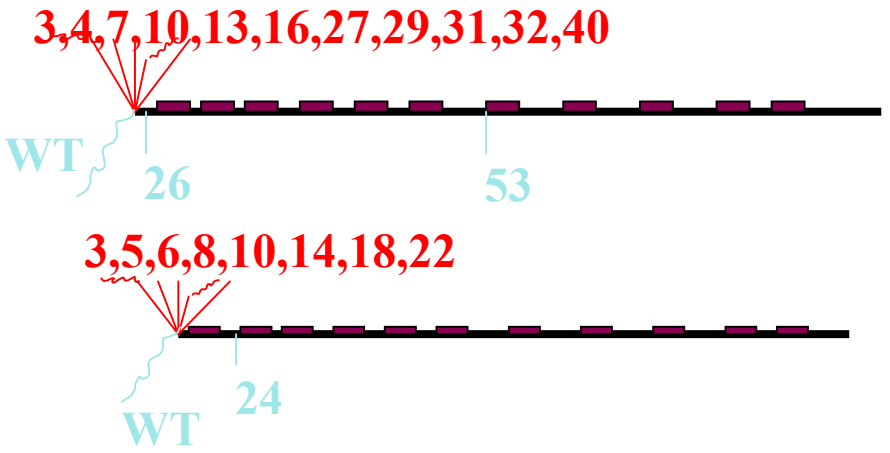
Construct library screening using the detergent adapted CoFi-blot

Karolinska Institutet

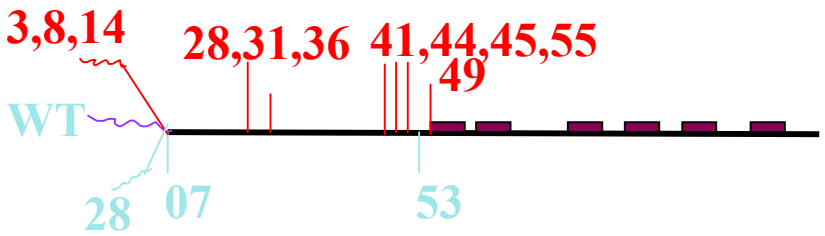
By Marina Igantuschencho-Sabet

Deletion library – ORFs with different N-termal start points



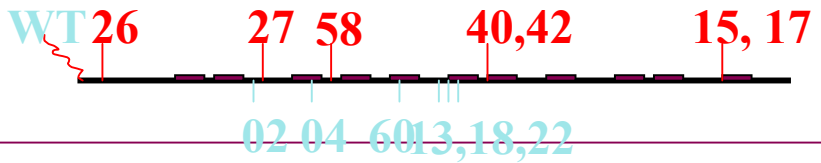


Purifiable IMP constructs selected from large libraries



- **Better expressors**
- **Worse expressors**

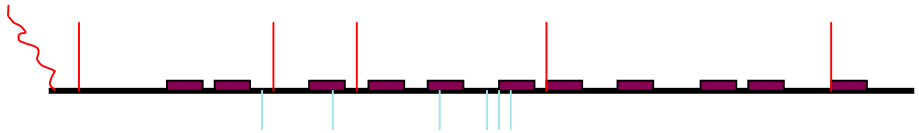
Constructs selected inbetween helices => membrane inserted proteins



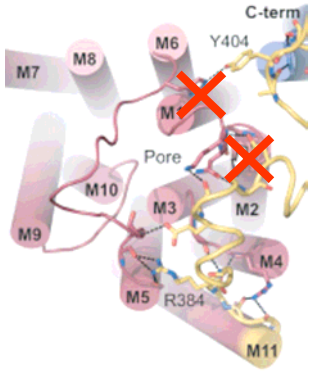
Selected domains of AmtB

WT D0 D1+ D2+ D3+ D4+

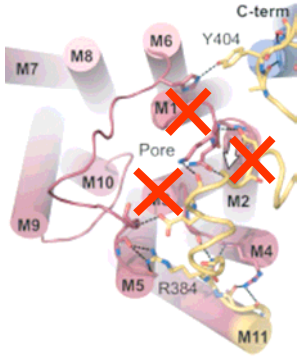
AmtB



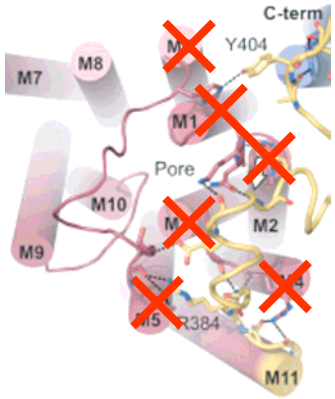
= Deleted helix



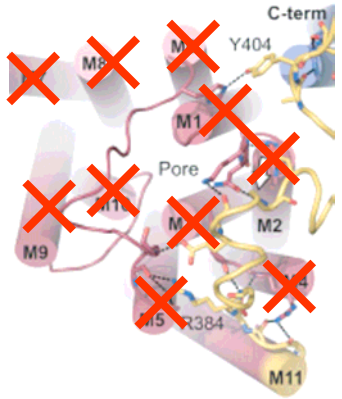
D1+



D2+



D3+

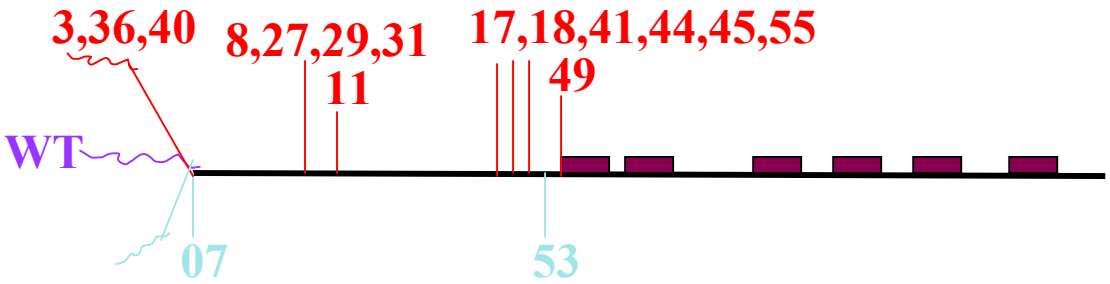


D4+

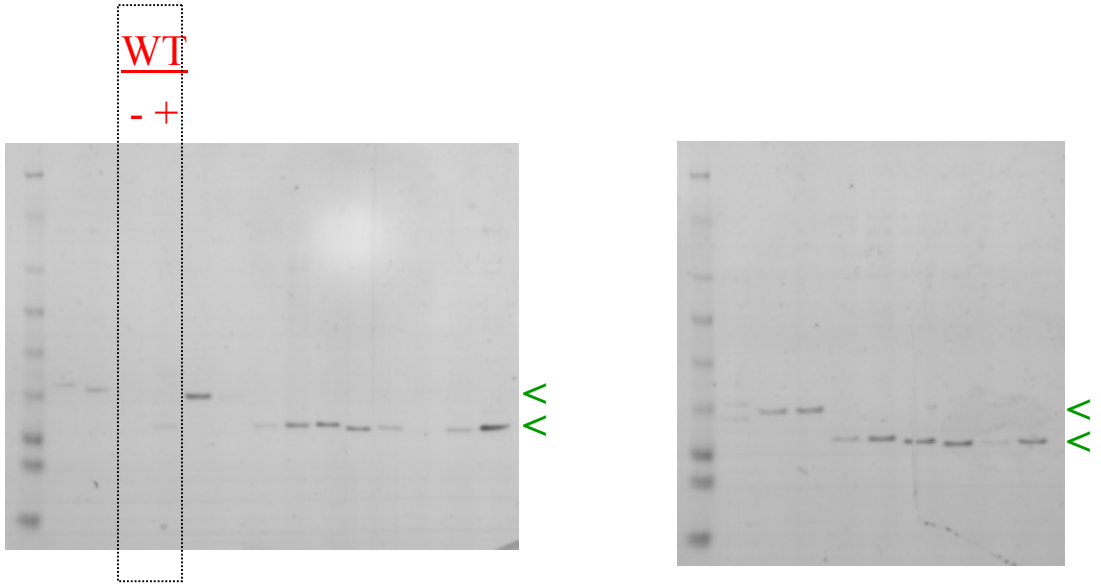
Pictures from
Conroy et al PNAS

- Selected domains show ”some tedency” to be ”globular” as defined from structure?
- Insertion might not be critical factor

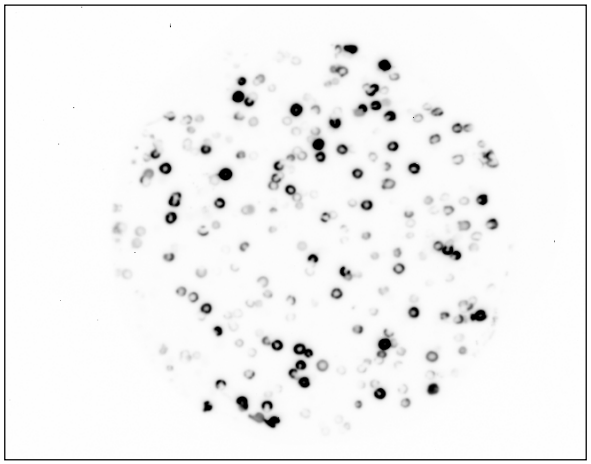
Example: EM29 and *E.coli* transporter



SDS-page from 1 ml cultures after IMAC purification



Rapid screening of solubilizing detergents of a GPCR library using the CoFi-blot



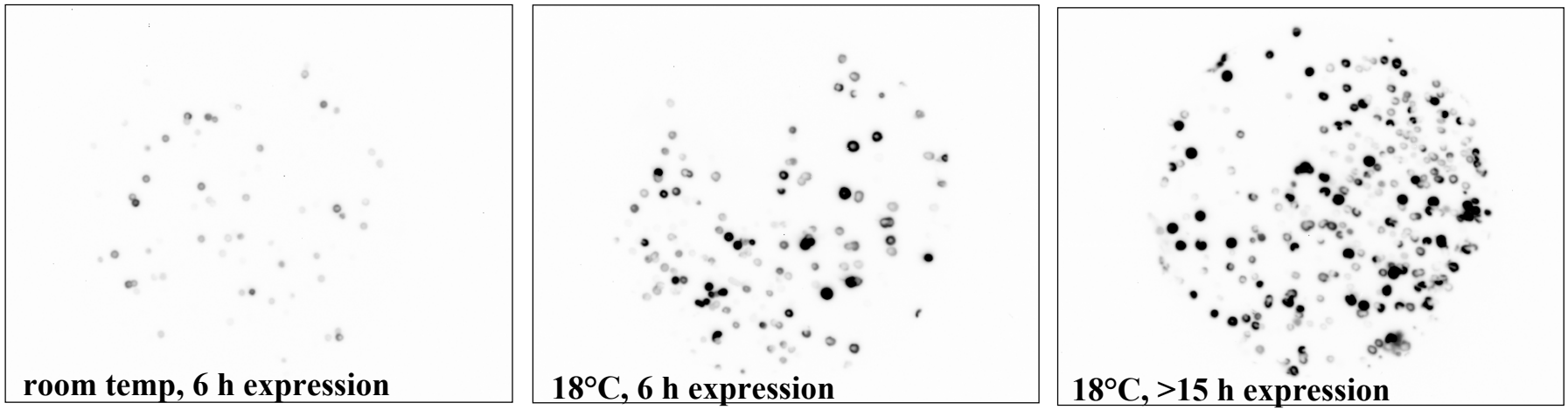
FC12 18°C expression

DM 18°C expression

CHAPS 18°C expression

Effects of different detergents

Rapid screening of expression conditions of a GPCR library in *E. coli*



FC12 in the lysis-buffer

Selection from random mutation libraries – mechanisms for potential improvments

- **Transcription/mRNA – e.g mRNA stability**
- **Translation - e.g. minimize RNA hairpins**
- **Folding - e.g. timing of folding events**
- **Protien stability – e.g. extra salt links**
- **Protein aggregation - e.g hydrophobic surfaces**
- **Protease resistance – e.g mutations in recognition site**

Test of a random mutagenesis => selection approch for IMPs

Karolinska Institutet

By Daniel Martinez-M Tobias Cornvik & Marina Ignatushchenko

- **9 IMPs used in a feasability study:**

  - 3 Ecoli IMPs expressing at medium level

  - 5 Ecoli IMPs expressing at low level or not at all

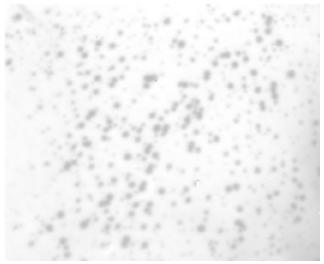
  - 1 Human MGST family member, medium level

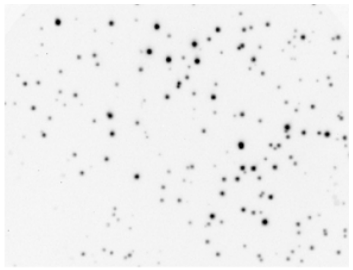
- **Random libraries were generated - 0.6 mutations/ 100 aa**

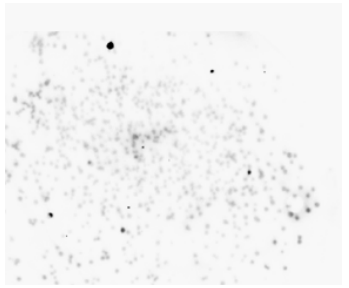
- **Selection made using a detergent adapted CoFi-blot**

Examples of detergent adapted CoFi-blots of IMPs

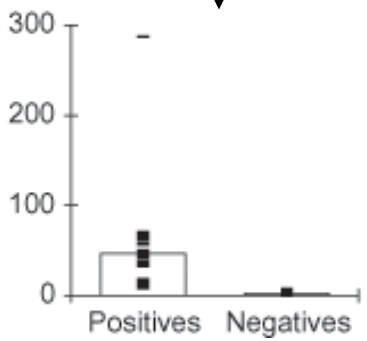
IMP1 **IMP4** **IMP5** **IMP7**

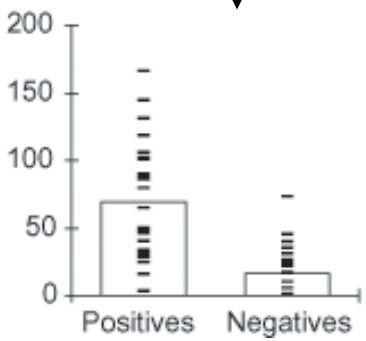


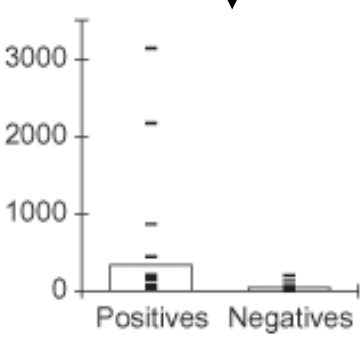




No positive colonies







- Quantitatification of expression using Small-scale IMAC- purification

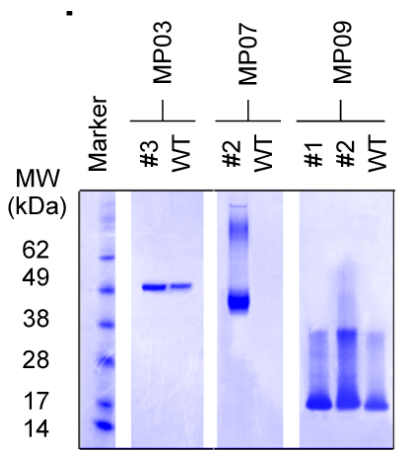
Result - feasability study

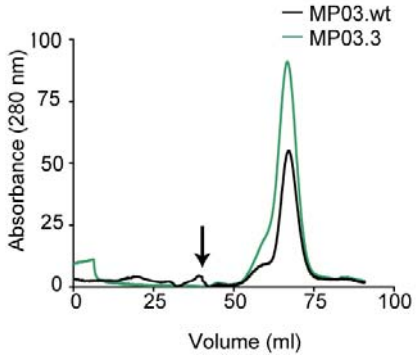
Low/Non expressors:

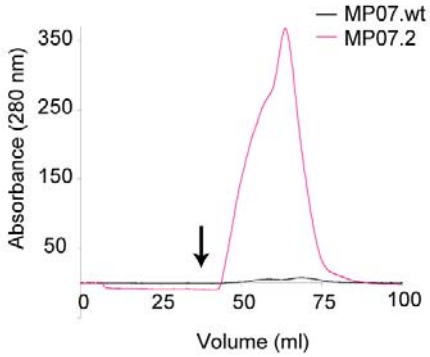
4 No positive colonies
1 Increased expression 4000% fold

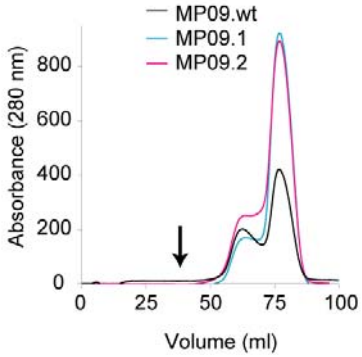
Medium expressers;

5 Increased levels of 40-300 % (one Human)





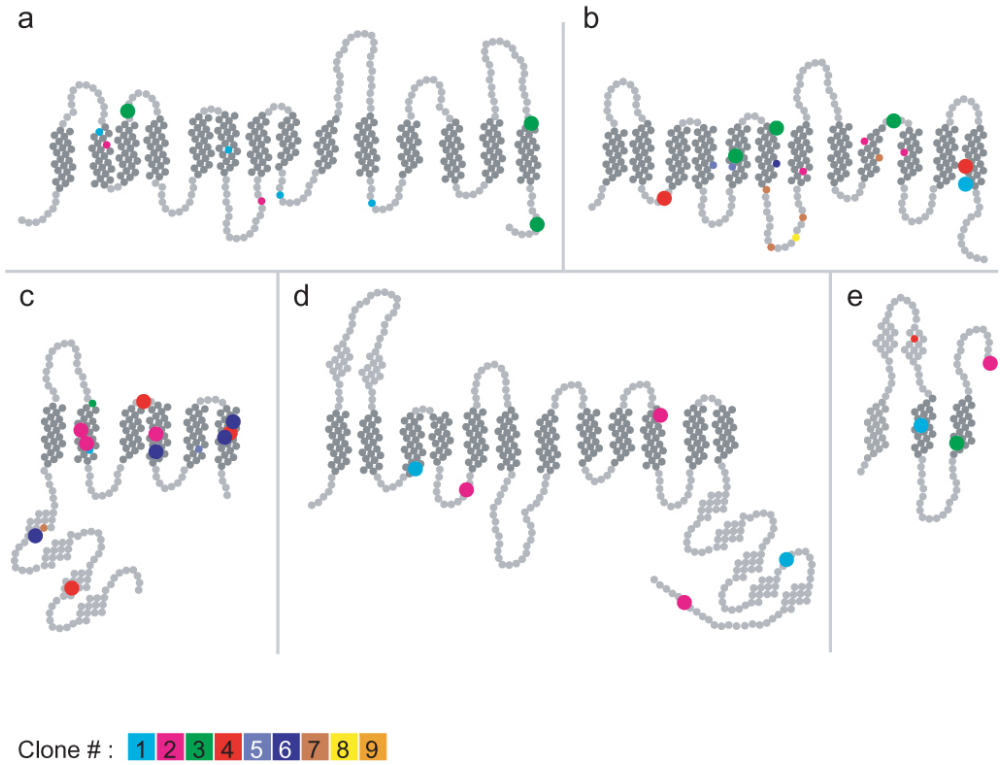


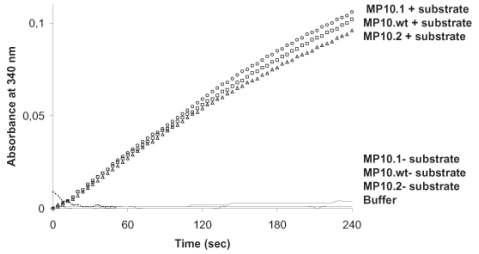


Examples of scale-up for three selected IMPs

What caused the improvment in expression levels ?

- **No strong positional preference for selected mutations**



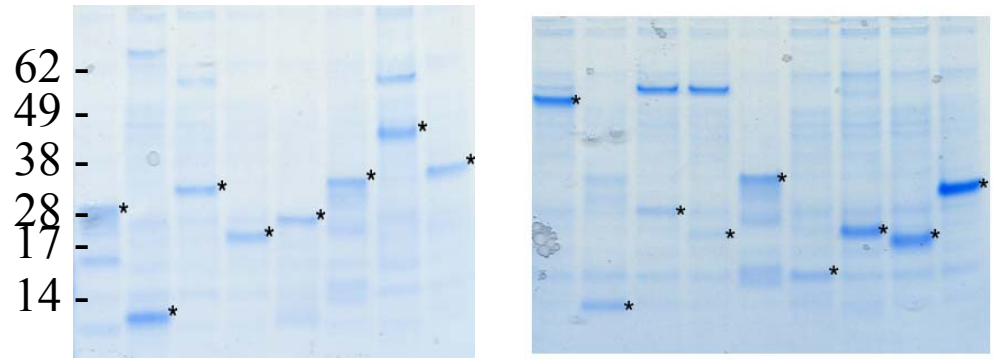


Evolution do not effect activity of the Human MGST family member

EaB + CoFi-blot (= Spotlight) is a generic, robust and relatively fast procedure

Human and mouse proteins	**> 80 processed**	**- > 15 ongoing**
VIZIER – RNA-virus proteins	**> 25 processed**	**- > 60 ongoing**
VIRCIR - Herpes virus proteins	**> 80 processed**	**- > 100 ongoing**

1 ml scale Herpes protein expression after IMAC purification



Summary – library work

- **The CoFi-blot constitutes an efficient HTP tool for screening for soluble protein expression**
- **Requires no automation – ”in everyone's hands”**
- **CoFi-blot ”simulate scale-up” in contrast to fusion reporter blots**
- **Screening of N-terminal expression libraries, yields dramatic improvements of success rates for mammalian proteins (intra cellular)**
- **CoFi-blot works for IMPs !**
- **Allows larger numbers of proteins to be screened => applicable ”genome wide studies”.**

Evitra

Karolinska Institutet

- Offers licensing or Contract Research

- Have completed successful projects for several major pharma and biotech companies.

- Spotlight – soluble proteins
- SpotlightM – membrane proteins

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