Affimers – The Next Generation of Molecular Recognition Reagents

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ASTBURY CENTRE

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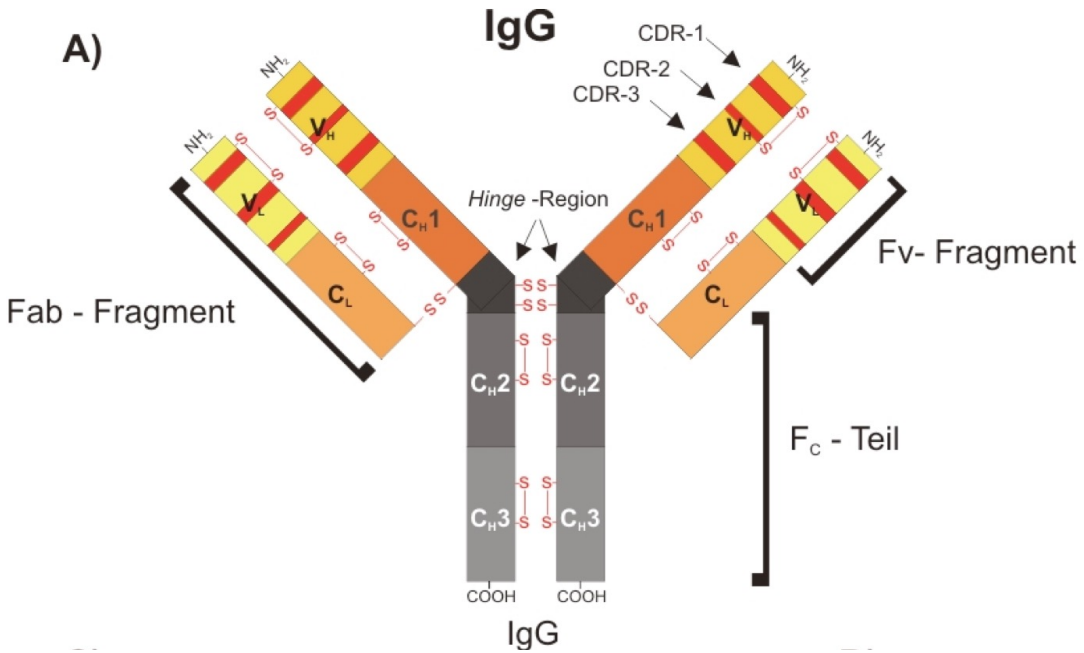
Gold Standard of Affinity Reagents

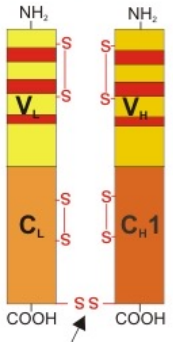
Antibodies: - Widely used in research, diagnostic and medicine.
- >80 therapeutic antibodies have been approved by the FDA.
- global sales about $122 billion (2018)



- Large (~1500 aa)
- Multi-subunit
  - 2 heavy & 2 light chains
- Disulphide bonds
- Can be unstable
- Difficult to generate in reproducible format
- Usually produced in animals

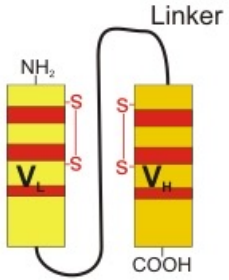
Range of Antibody Fragments

Fab - Fragment



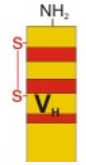
55 kDa

single-chain Fv (scFv) Fragment



25 kDa

Nanobody



12.5 kDa

What do we want?

An ideal scaffold should be:

- **Produced in animal free systems**
- **Easy to generate**
- **Available in large quantities**
- **Suitable for intracellular targets**
- **Suitable for oral administration**
- **Ideal for tissue penetration**
- **Able to distinguish between homologous proteins**
- **Free of IP restrictions**
- **Cheap**

But still challenges with the production…

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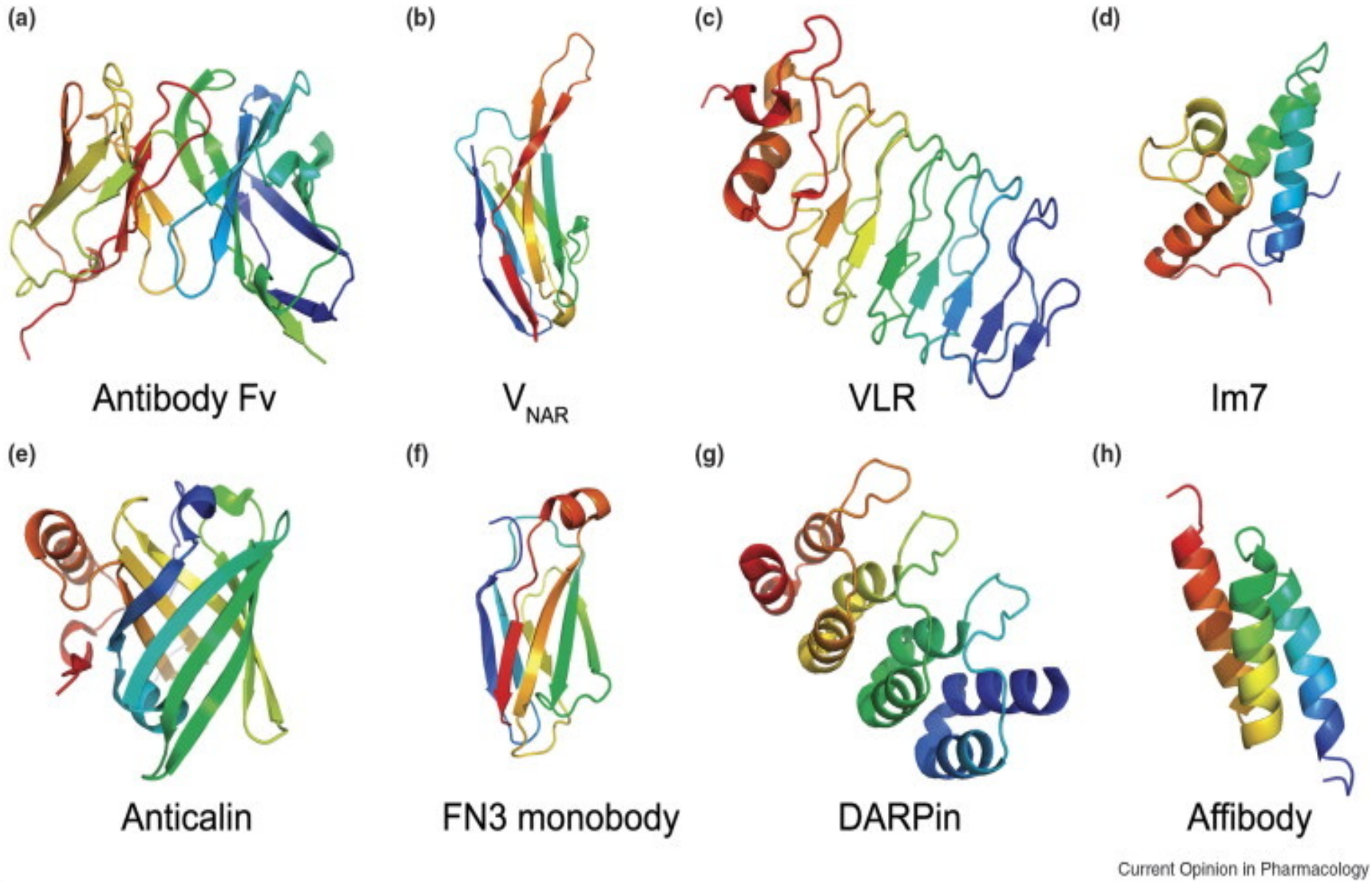
Artificial binding protein examples



Current Opinion in Pharmacology

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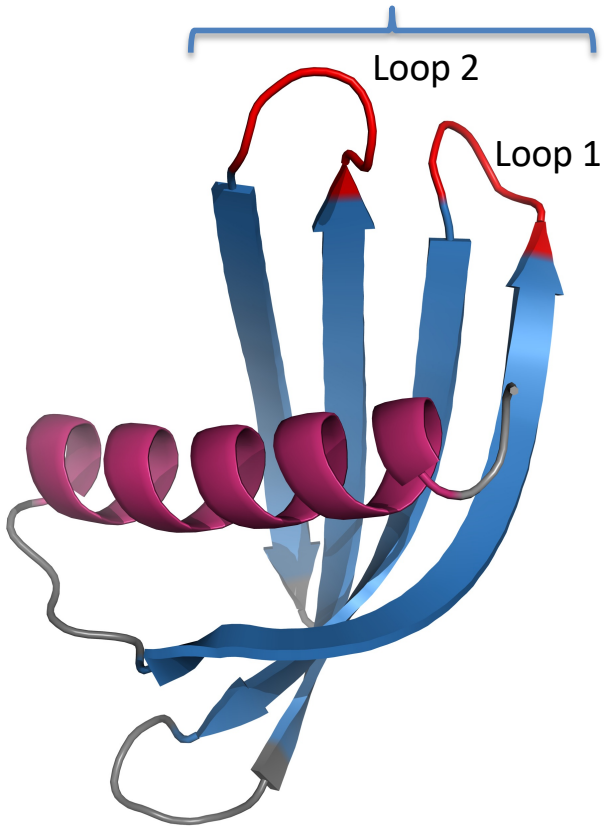
Affimer - Antibody Alternative protein

developed by the BioScreening Technology Group, University of Leeds

Very small
About 12 kDa

Extreme stability
-Up to 100 °C
-Stable in a broad pH range

Ideal for in vivo studies



Simple
Single chain, no S-S

Well expressed in *E.coli*

Easy to engineer
- Insertion of cysteines
- Multimers for multispecificity

Derived from the cystatin family protein fold
-consensus sequence of 57 plant phytocystatins
- Human scaffold available too

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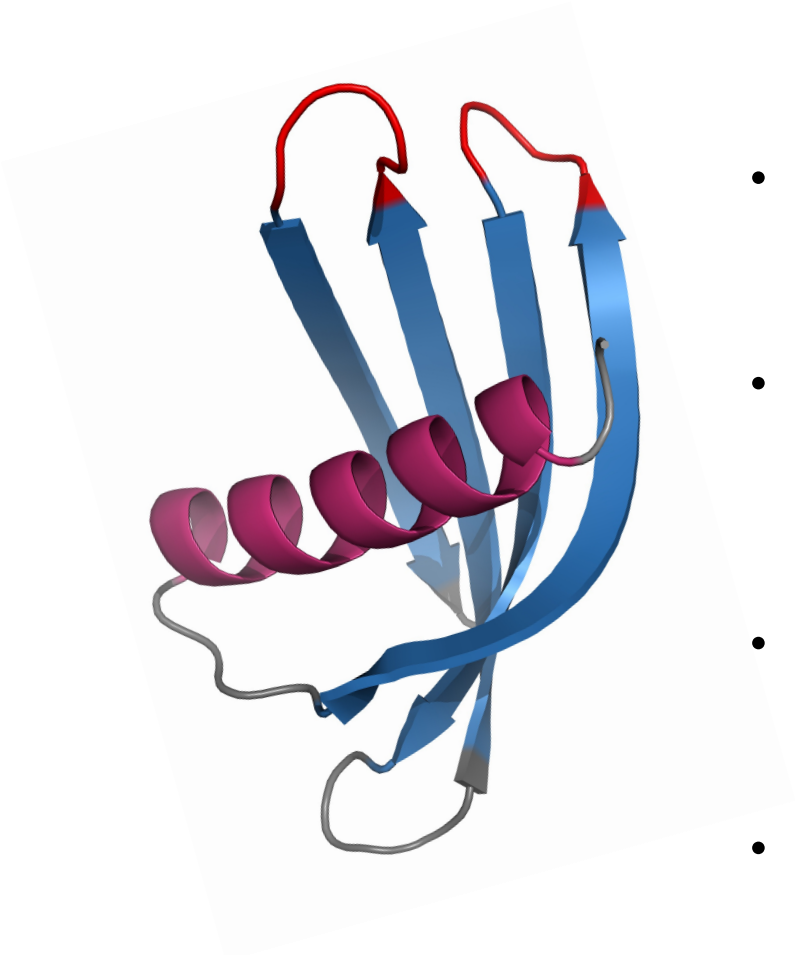
Affimer Advantages



- **Small, robust & stable**
  - increased packing density
  - improved tissue penetration
  - Super-resolution microscopy
- **Ease of cloning**
  - quick & cost effective library generation
  - insertion of cysteine
- **Fast selection**
  - 10 working days
  - controlled specificity
  - > 400 proteins, peptides and chemical compounds screened
- **High yields**
  - up to 250mg/L in *E. coli*
  - no batch-to-batch variation
- **High affinity**
  - typically 1-100 nM
- **Ideal for *in vivo* studies**

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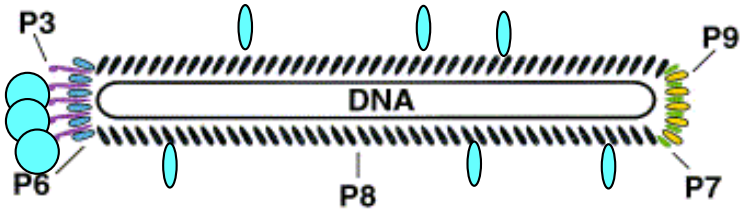
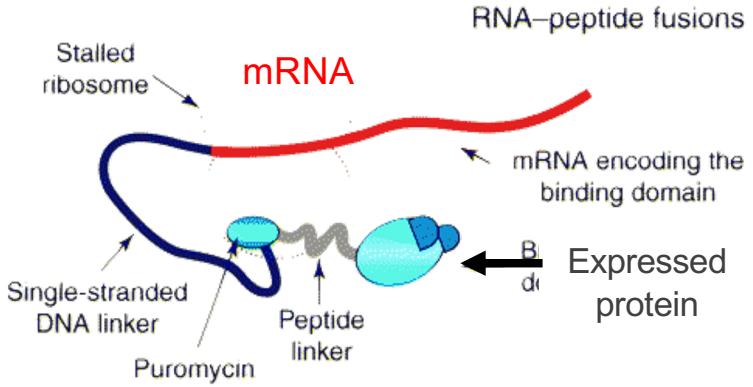
Protein/peptide display approaches

Phage

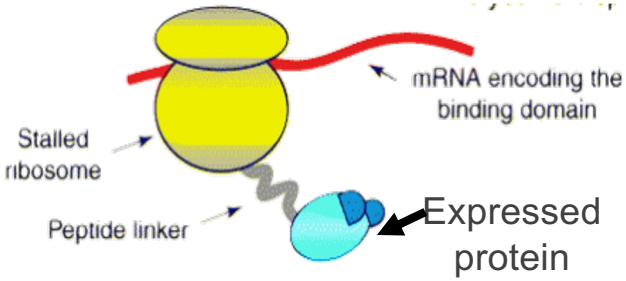


Smith, G.P., *Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface.* Science, 1985. **228**(4705): p. 1315-7.

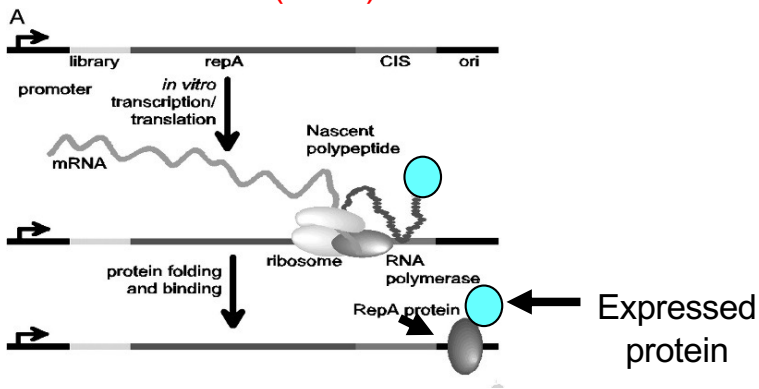
RNA–peptide fusions

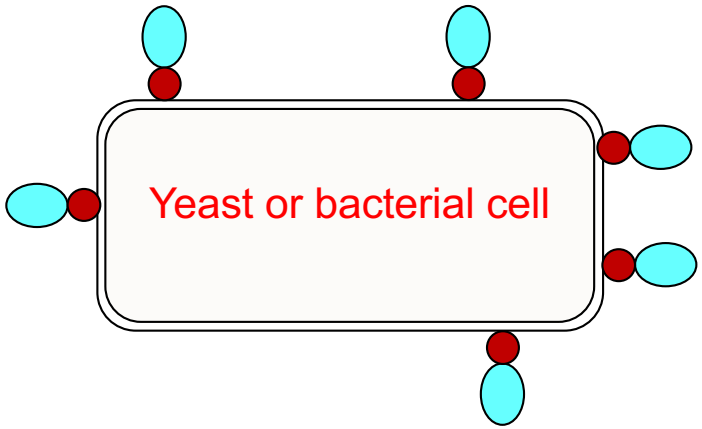


Ribosome



Cis (DNA)





Fitzgerald (2000) Drug Discovery Today, 5:253-258; Odegrip et al (2004) PNAS 101, 2806-2810; Reiersen (2005) Nucleic Acids Research 33:e10

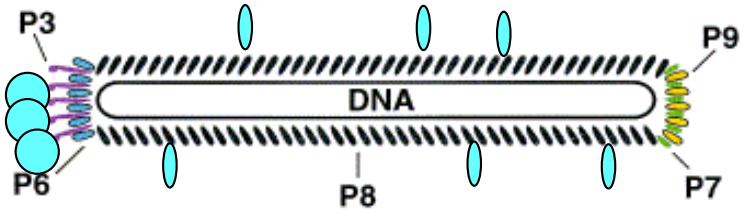
Greg Winter and George P. Smith won the Nobel Prize in Chemistry 2018 – Development of Antibody Phage Display

George P.Smith – awarded for his ground-breaking work on filamentous E. coli phage M13 and the fusion of peptides to the phage envelope proteins

Greg Winter - honoured for using that method for developing therapeutic antibodies

Protein/peptide display approaches

Phage



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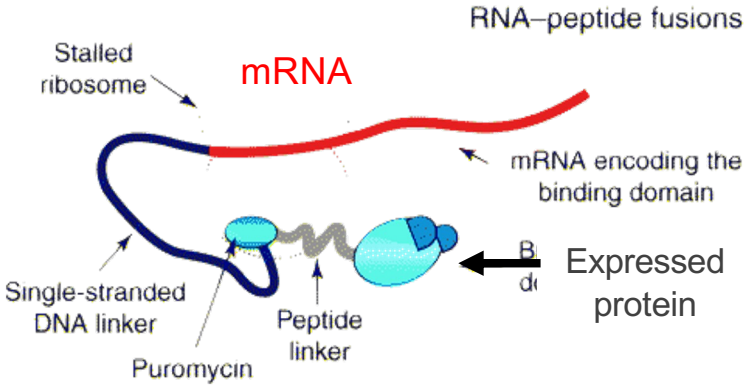
RNA–peptide fusions



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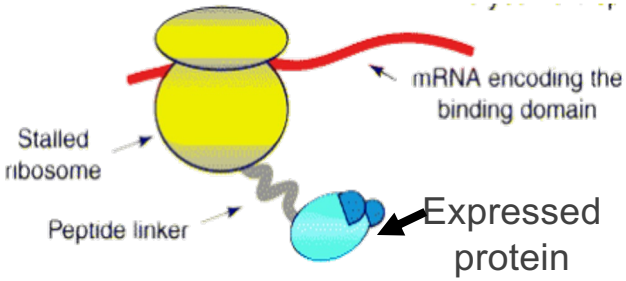

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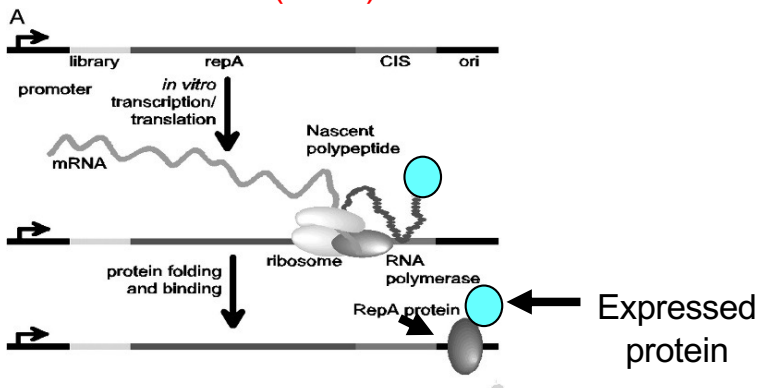
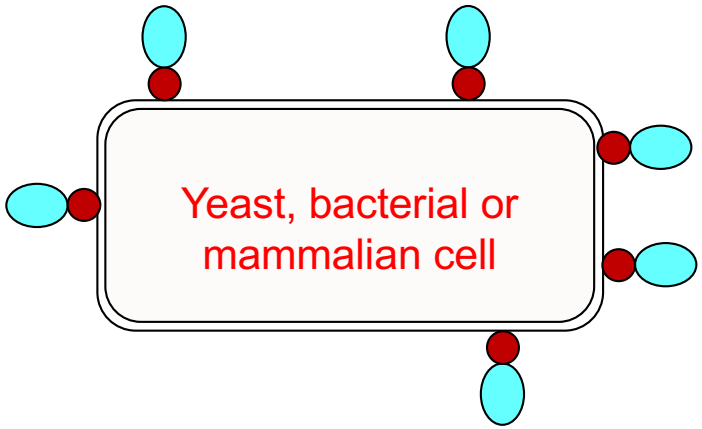




Fitzgerald (2000) Drug Discovery Today, 5:253-258; Odegrip et al (2004) PNAS 101, 2806-2810; Reiersen (2005) Nucleic Acids Research 33:e10

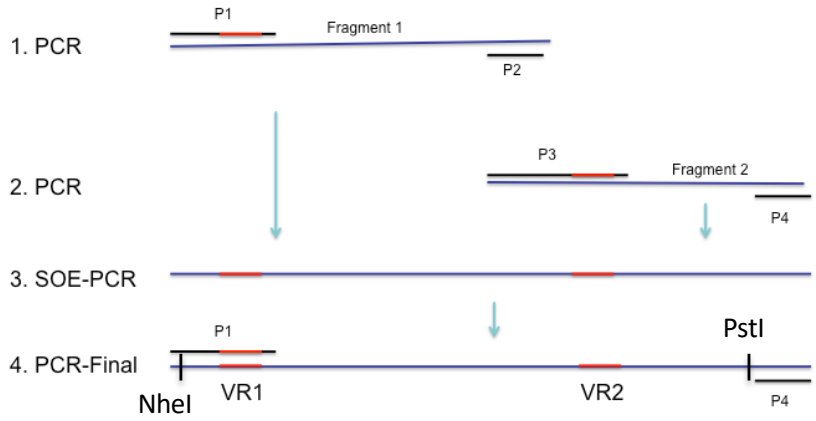
Building the library

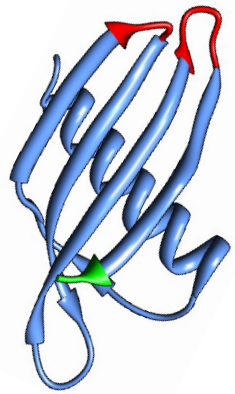
XXXXXXXXX

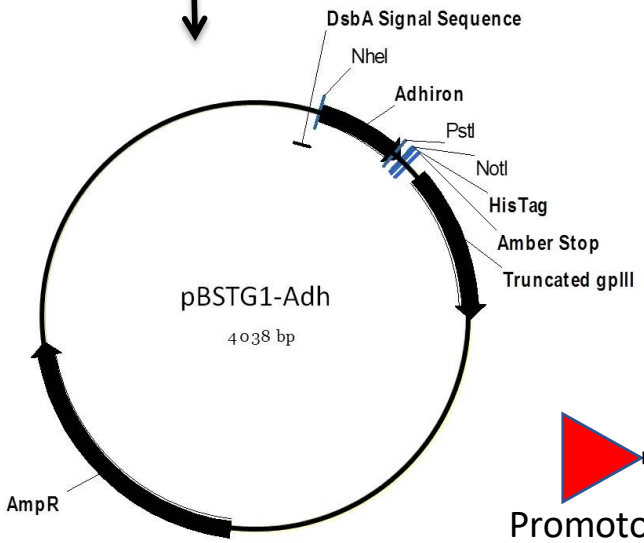
ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQVVAGTMYYLTL
EAKDGGKKKLYEAKVWVKPWENFKELQEFKPVGDA

XXXXXXXXX

Trimer	AA
AAA	Lys
AAC	Asn
ACT	Thr
ATC	Ile
ATG	Met
CAG	Gln
CAT	His
CCG	Pro
CGT	Arg
CTG	Leu
GAA	Glu
GAC	Asp
GCT	Ala
GGT	Gly
GTT	Val
TAC	Tyr
TCT	Ser
TGC	Cys
TGG	Try
TTC	Phe







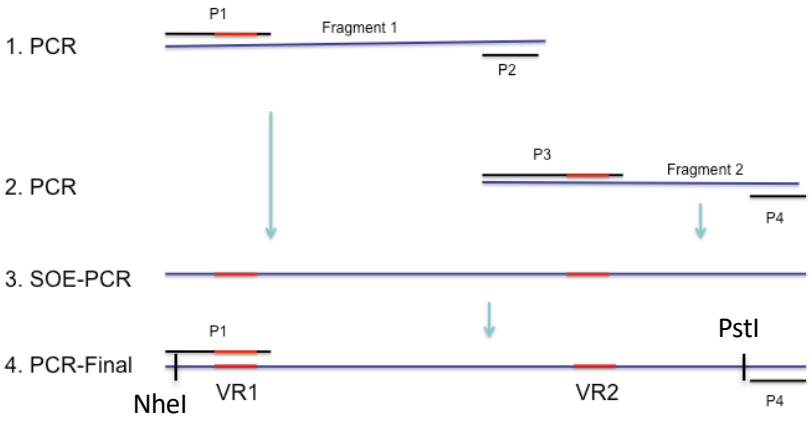
Building the library

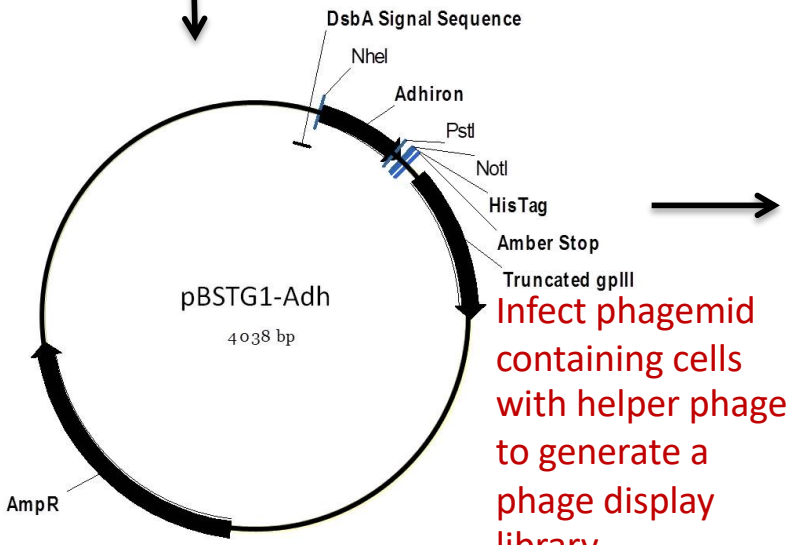
XXXXXXXXX

ATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQVVAGTMYYLTL
EAKDGGKKKLYEAKVWVKPWENFKELQEFKPVGDA

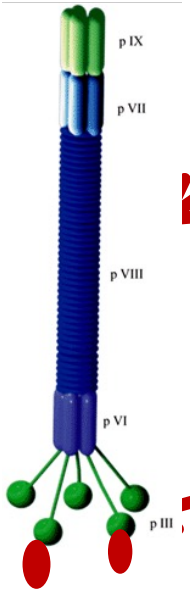
XXXXXXXXX

Trimer	AA
AAA	Lys
AAC	Asn
ACT	Thr
ATC	Ile
ATG	Met
CAG	Gln
CAT	His
CCG	Pro
CGT	Arg
CTG	Leu
GAA	Glu
GAC	Asp
GCT	Ala
GGT	Gly
GTT	Val
TAC	Tyr
TCT	Ser
TGC	Cys
TGG	Try
TTC	Phe





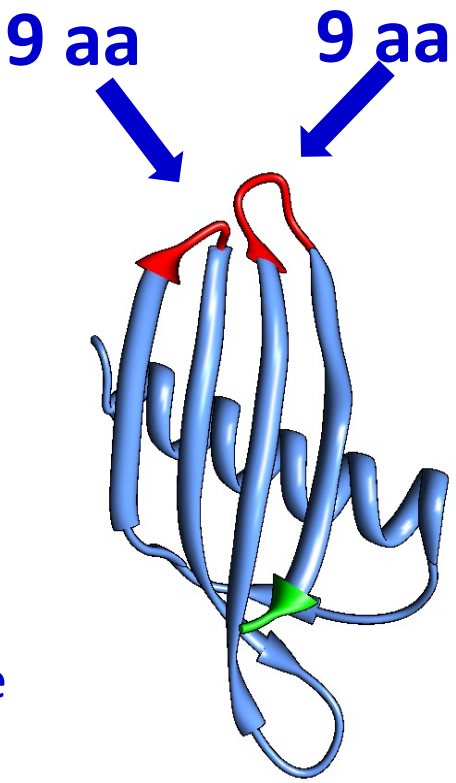
Infect phagemid containing cells with helper phage to generate a phage display library



genotype (the DNA that encodes the binding protein is encoded in the ss DNA)

phenotype (binding protein binds to a target)

Affimer library



How big is the library?

- 18 variable positions each with 19 possible amino acids (no cysteine)

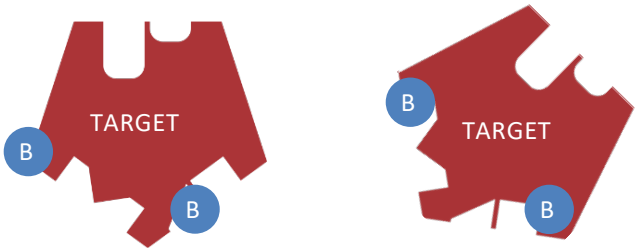
- Theoretical library size: $19^{18} = 1.04 \times 10^{23}$ molecules

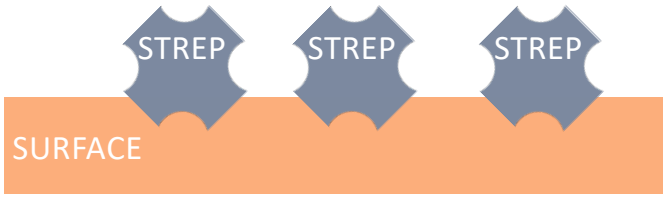
- Achieved library size: 1.3×10^{10} molecules, $1/3.5 \times 10^{12}$ of the theoretical library complexity

- Maximal achievable phage display library size: 1×10^{11} molecules

The panning process

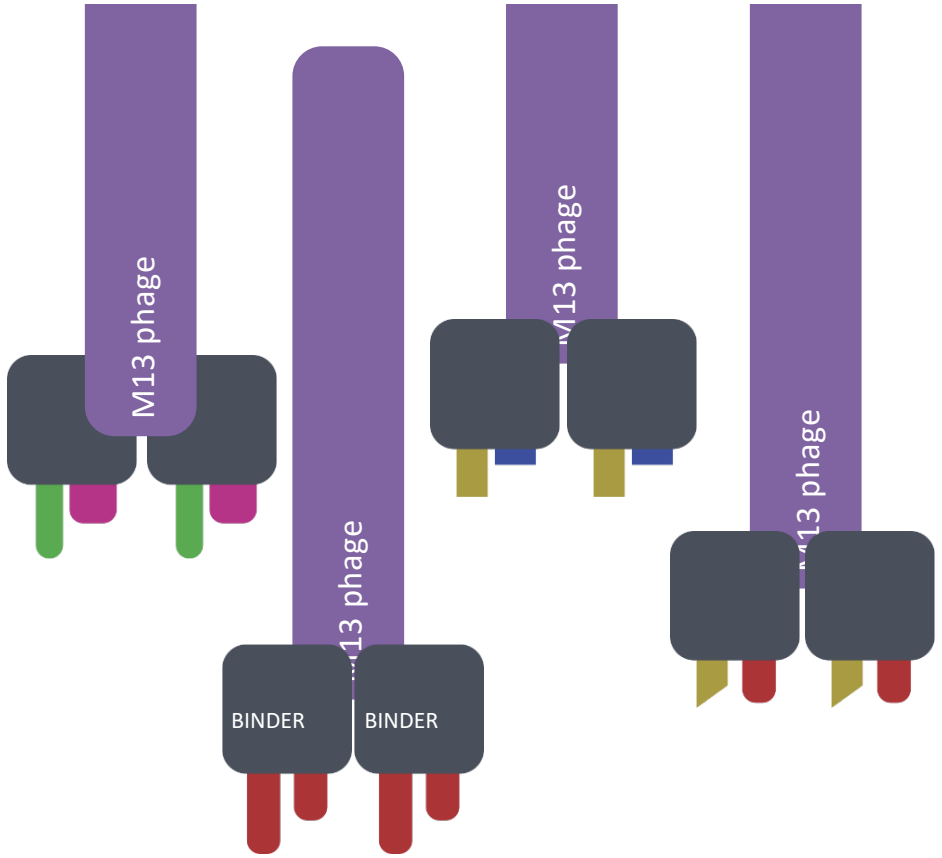
- Target molecules biotinylated, bound to surface

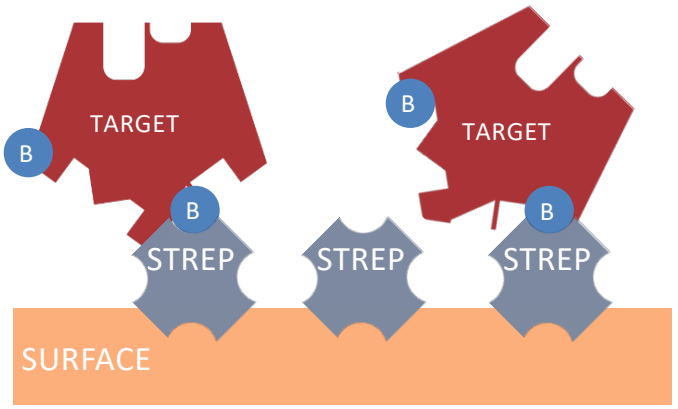




The panning process

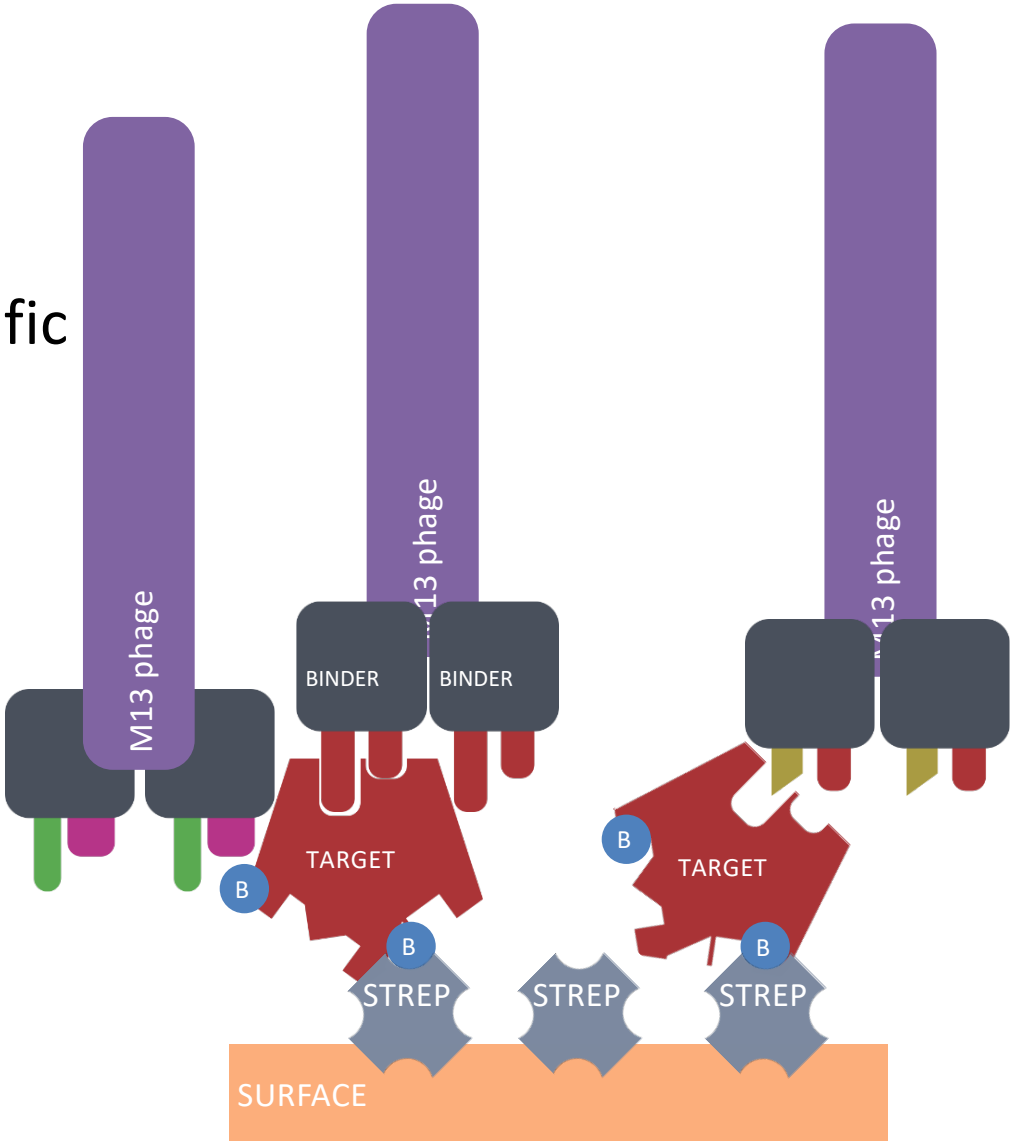
- Target molecules biotinylated, bound to surface
- Exposed to library of phage expressing Affimer mutants





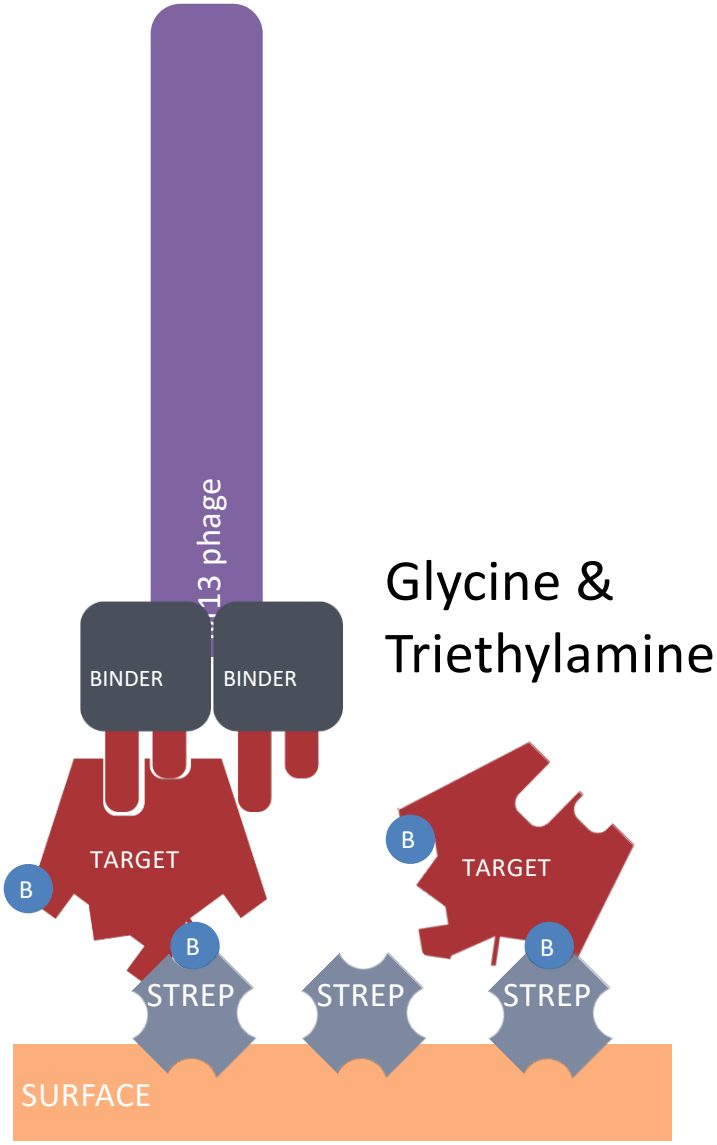
The panning process

- Target molecules biotinylated, bound to surface
- Exposed to library of phage expressing Affimer mutants
- Wash to remove weak/non-specific binding



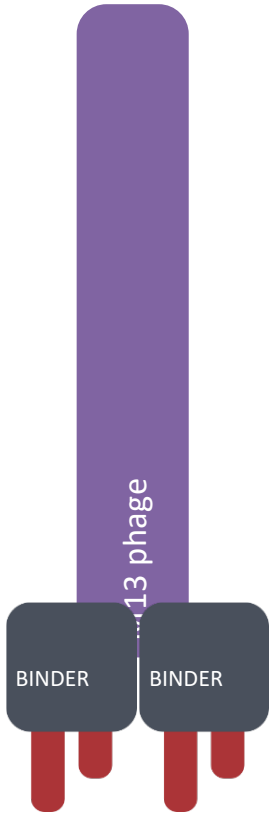
The panning process

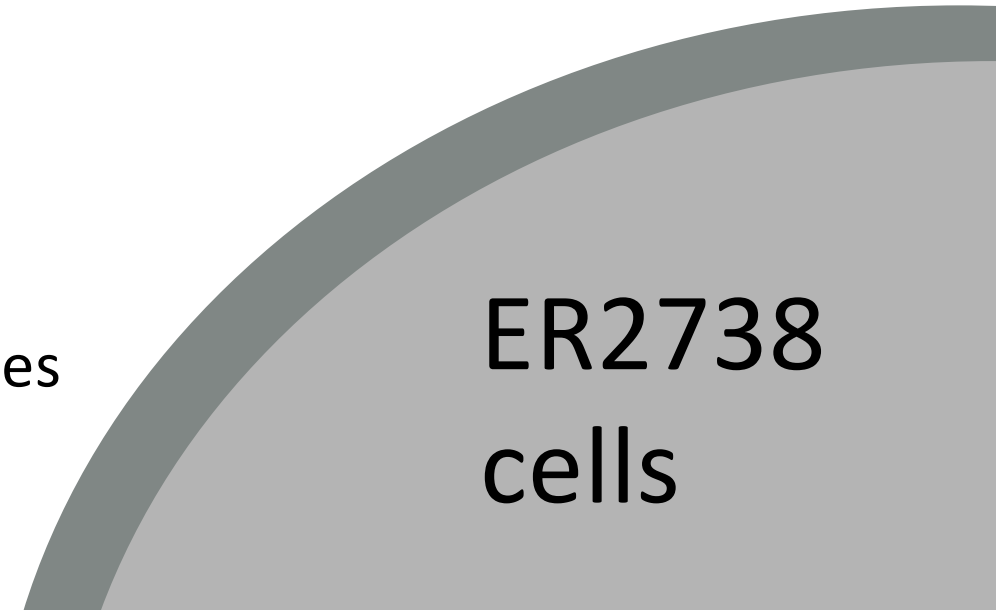
- Target molecules biotinylated, bound to surface
- Exposed to library of phage expressing Affimer mutants
- Wash to remove weakly bound, nonspecific binding
- Elute and recover binders



The panning process

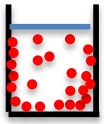
- Target molecules biotinylated, bound to surface
- Exposed to library of phage expressing Affimer mutants
- Wash to remove weakly bound, nonspecific binding
- Elute and recover binders
- Infect cells with phage, plate and amplify
- Repeat process 3 times:
  - After final pan, individual clones amplified for ELISA and sequencing





Phage Display

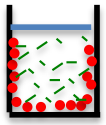
1. Immobilisation of biotin-target



- Streptavidin coated well
- 1µg biotin-target

Wash

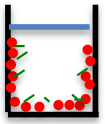
2. Incubation with phage library



- 2hr incubation
- Optional: counter selection

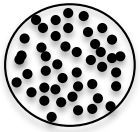
Wash

3. Phage elution



- 2 step elution

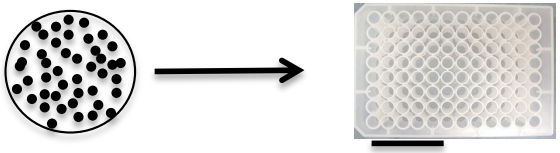
4. Infection of ER2738, plating & phage propagation



Biopanning 3 x rounds

Selection of individual Affimers – Phage ELISA

1. Phage prep of randomly picked colonies



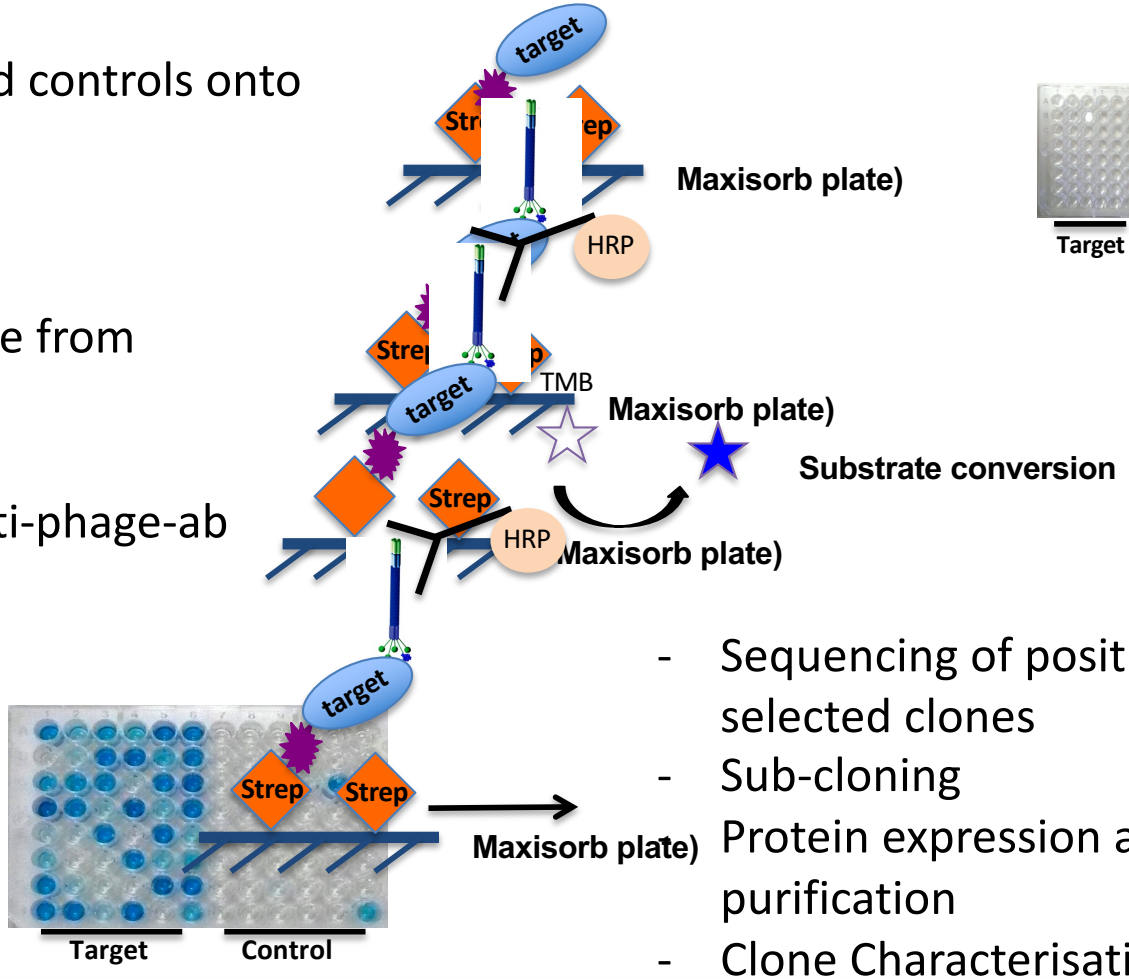
- Deep-well plate
- A1-H6 200µl each

2. Immobilisation of bio-target and controls onto streptavidin 96-well plates

3. Incubation with individual phage from step1

4. Detection with HRP-labelled anti-phage-ab

5. Adding TMB substrate



- Sequencing of positive selected clones
- Sub-cloning
- Protein expression and purification
- Clone Characterisation

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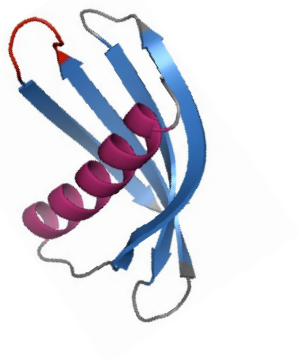
Remember: “You get what you screen for”

The screening design includes:

- choice of target protein source (eg prokaryotic or eukaryotic cells)
- mechanism of immobilization (random or oriented)
- Pre-blocking to prevent non-specific binding (plastic wells, magnetic beads)
- Pre-panning to prevent non-specific binding (plastic wells, magnetic beads)
- negative selection against similar molecules to enhance specificity
- Increase selectivity and binding properties
  - number and stringency of washes
  - competition with excess soluble target to select tighter binders
  - extended incubation times under competition (eg days)

Summary

- A novel affinity reagent named Affimer developed
- A direct replacement for antibodies
- Large phage display libraries up to $1.3x10^{10}$ generated
- Fast selection process (10 days) established
- High success rate (up to 90%) for recombinant proteins and small compounds
- Well expressed in *E.coli*
- High binding affinities observed (1-100nM)



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