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High Throughput Protein Expression & Purification a new CSIRO capability for structural genomics

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Australian Bioprocessing Network, Brisbane 1st October 2009



AIMS

- Develop higher throughput using laboratory automation
- Increase number of projects in the pipeline
breadth and depth
- Increases our outcomes in structural genomics

FACILITY



Tecan Liquid Handling Robot

Avastin C5 cell homogeniser

Chromatography workstations

- ÄKTExpress (x 2)
- ÄKTAbasic
- Biorad Duoflow (x 2)
- Biorad Profinia

Invitrogen Midi electrophoresis system

STRATEGY

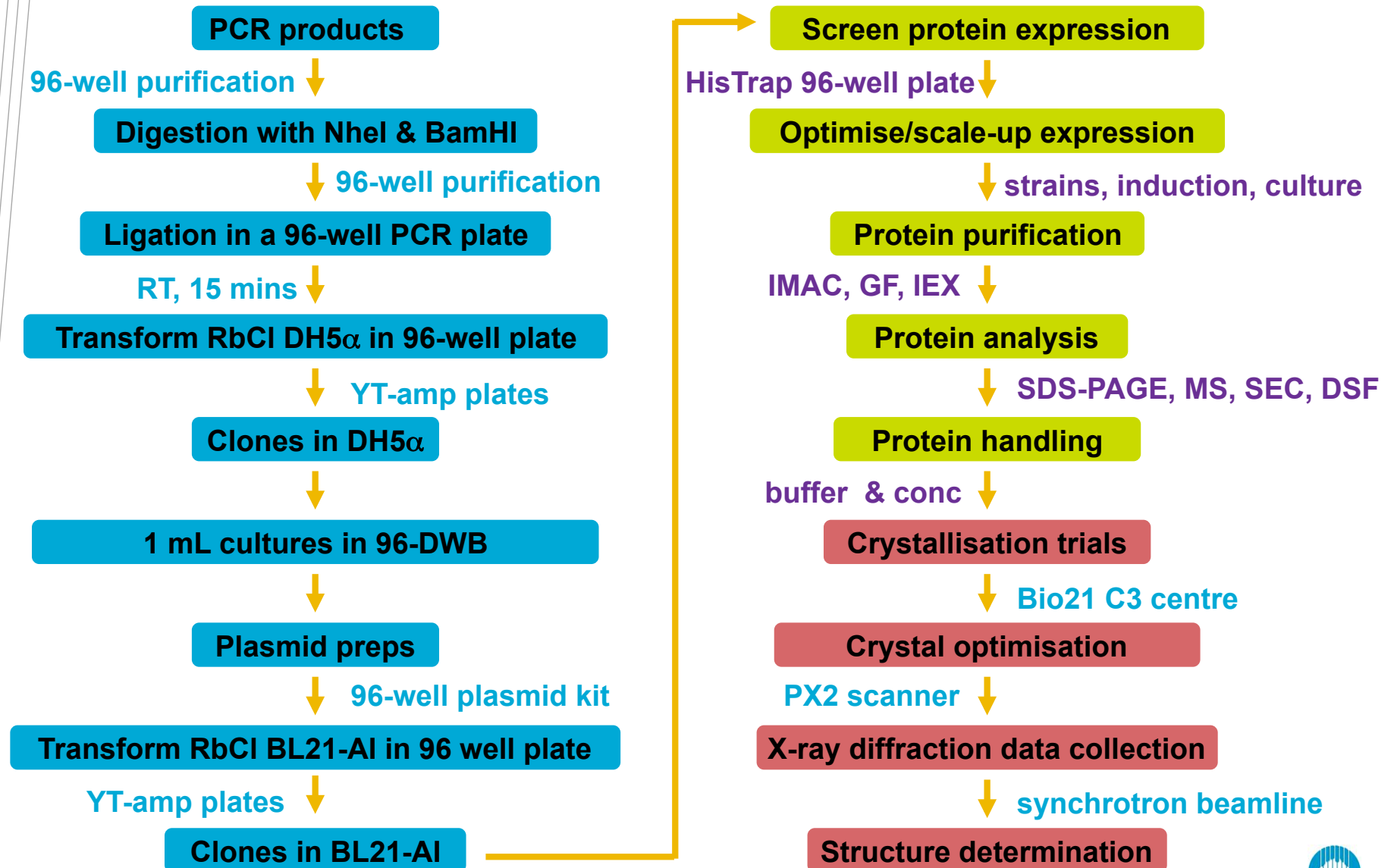
- Ligation Dependent Cloning
- Expression Vectors – modified pET43
 - N-terminal 6xHis tag



- C-terminal 6xHis tag

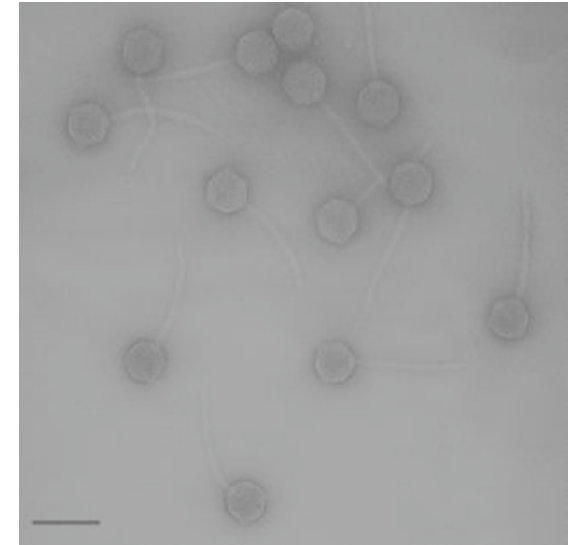


WORKFLOW



PILOT STUDY - PHAGENOMICS

- Bacteriophage infecting *Lactococcus lactis* disrupt dairy fermentation processes
- Genomic sequencing of 50 isolates (sizes ranging from 22 kb (c2 phage) to 32 kb (936 phage) each)
- Genomes are poorly annotated
- Many genes code for closely related proteins
- High percentage of genes are Function UNknown
- Semi-automated cloning, expression and purification
- Structural biology
 - identify function of unknown phage proteins
 - small changes in genes of closely related phage proteins might alter protein function



Siphoviridae sp.

BACTERIOPHAGE PROTEIN FAMILY PROPERTIES

ORF	Function	MW (kDa)	pI	Identity (%)
1	Terminase Nu1	21.1 - 21.2	5.5 - 5.8	96 - 99
2	FUN	10.2 - 10.5	4.3 - 4.8	69 - 98
3	Terminase A	64.1 - 64.2	6.1 - 6.4	97 - 98
7	Capsid	44.5 - 44.9	5.8 - 6.2	95 - 99
12	Amidase	80.5 - 98.2	5.2 - 5.4	30 - 98
14	Tail	33.5 - 33.8	5.3 - 5.5	91 - 96
17	Tape measure protein	96.1 - 106.5	8.9 - 9.2	91 - 97
18	Fibre protein	35.6 - 35.9	5.7 - 5.9	87 - 94
19	FUN	43.7 - 44.0	5.3 - 5.9	89 - 94
21	Receptor binding protein 1 & 2	29.0 - 30.9	6.0 - 7.9	45 - 99
23	Lysin	26.6 - 28.9	6.3 - 7.8	93 - 98
24	Transglycosylase	21.7 - 22.3	5.2 - 9.5	67 - 100
27	FUN	14.6 - 14.9	6.4 - 9.3	76 - 91
28	FUN	10.6 - 10.9	6.4 - 9.2	29 - 97
33	FUN	13.3 - 15.5	4.9 - 5.4	80 - 98
41	FUN	21.2 - 22.2	9.3 - 9.6	86 - 98
44	FUN	14.2 - 14.4	6.1 - 6.7	89 - 99
45	FUN	22.5 - 25.6	6.1 - 6.3	57 - 96
47	FUN	17.3 - 17.4	9.3 - 9.7	83 - 95
53	DNA polymerase	31.9 - 36.8	6.1 - 6.6	95 - 98
62	Holliday Junction Endonuclease	19.2 - 19.4	6.5 - 7.1	93 - 98

ANALYTICAL PROTEIN EXPRESSION

Clones grown O/N, 96-DWB containing 2YT + amp at 37°C, 1000 rpm

1:100 dilution of O/N culture into a fresh 96-DWB (1.5 mL)

Induce expression OD600 ~0.8 with 1 mM IPTG/ 0.025% arabinose, 37°C, 4 hr, 1000 rpm

Harvest cells -1000 x g 10 min, store -20°C

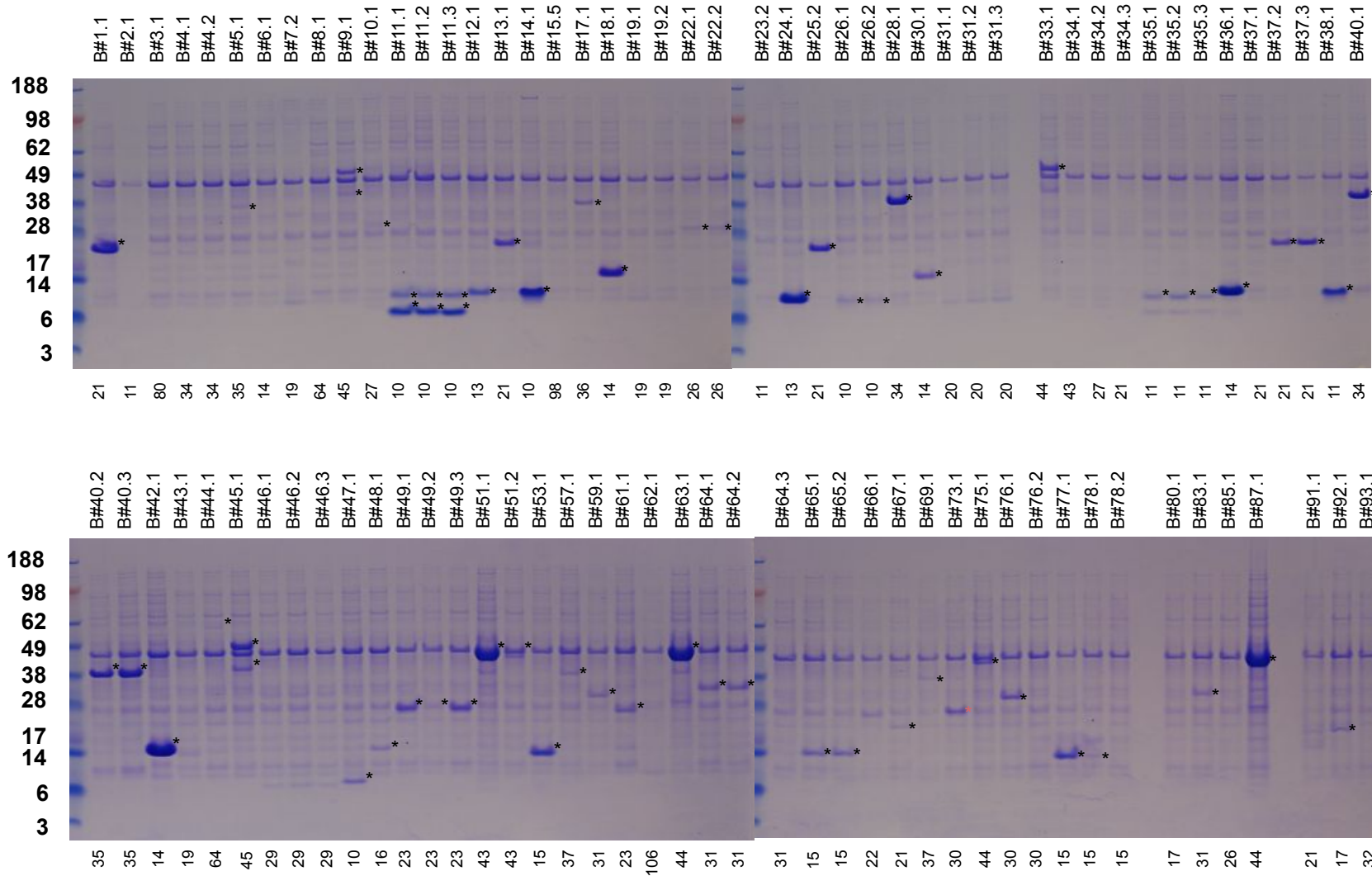
Lysis : Bugbuster containing benzonase

HisTRAP plate

wash 1	TBS + 350 mM NaCl, 5% glycerol, 5 mM DTT, 10 mM imidazol
wash 2	TBS + 350 mM NaCl, 5% glycerol, 5 mM DTT, 50 mM imidazol
elution	TBS + 350 mM NaCl, 5% glycerol, 5 mM DTT, 250 mM imidazol

SDS-PAGE analysis (4-12% NuPAGE BT gel / MES electrophoresis buffer)

SCREENING FOR PROTEIN EXPRESSION





Scaling-up Protein Expression and Purification for Crystallisation Trials

EXPRESSION AND PURIFICATION

- 2L shake flasks or 10 L fermentor (2YT + amp)
- Induction: 0.5 mM IPTG / 0.025 % arabinose, 30°C, 4h, 120 rpm
- Lysis Buffer: TBS + 150 mM NaCl, 10 mM imidazol, 5 mM DTT, 1 mM PMSF, PI tabs, 2 mM MgCl₂, benzonase, 0.5 mg/mL lysozyme
- Homogenisation: Avestin C5 cell crusher 15,000 psi (x3)
- Clarification: 20,000 rpm, 4°C, 20 min; 5.0 µm filtration
- Purification: AKTÅxpress; 1 mL HisTRAP FF, 16/60 Superdex 200 pg
Binding buffer: TBS + 150 mM NaCl, 5 mM DTT, 10 mM imidazol
Wash buffer: TBS + 150 mM NaCl, 5 mM DTT, 20 mM imidazol
Elution buffer: TBS + 150 mM NaCl, 5 mM DTT, 250 mM imidazol
- SDS - PAGE analysis, mass spec, N-terminal sequencing
- Protein concentration (Amicon Ultra (10,000 MWCO))

B#42.1 (FUN 44)

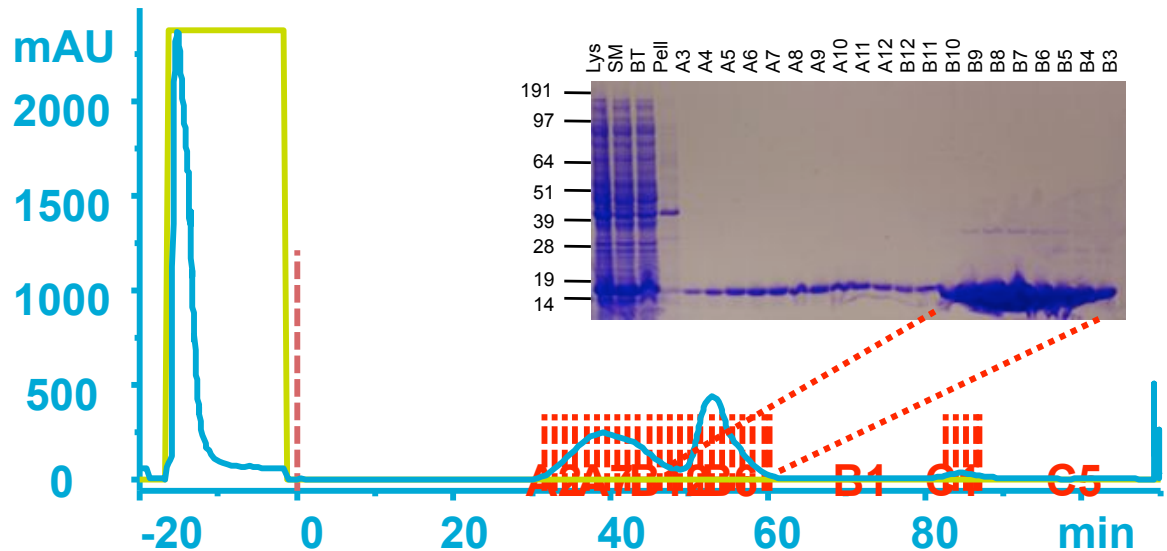
```

Clone_18 MAIITVTAQANEKNRTRTVSTAKGDKKIIISVPLFEKEKGS SVKVAYGSAFLPDFIQLGDTV 60
Clone_42 MAIITVTAQVNEKNRTRTVNTAKGDKKIIISVPLFEKEKGS NVKVAYGSAFLPDFIQLGDTV 60
Clone_6 MAIITVTAQANEKNRTRTVSTAKGDKKIIISVPLFEKEKGS NVKVAYGSAFLPDFIQLGDIV 60
Clone_39 MAIITVTAQANEKNRTRTVNTAKGDKKIIISVPLFEKEKGS NVKVAYGSAFLPDFIQLGDIV 60
***** .***** .*****.*****.*****
  
```

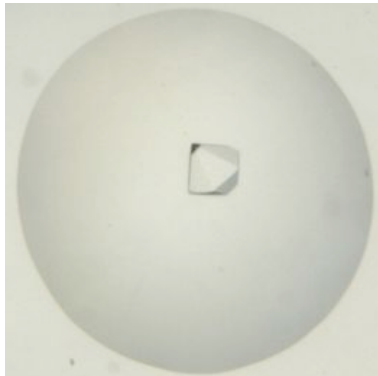
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Clone_18 TVSGRVQAKESGEYVNYNFVFP TVEKVF IHNDNSSQSQAKQDLFGGSEPIEINTEDLPF 119
Clone_42 MVSGRVQAKESGEYVNYNFVFP AVEKVF IPNDNNSQSQAKQDLFGKSEPIEINTEDLPF 119
Clone_6 TISGRVQAKESGEYVNYNFVFP AVEKVF IPNDNSKQSQAKQDLFGGSEPIEVHELGLPF 119
Clone_39 TISGRVQAKESGEYVNYNFVFP AVEKVF IPNDNSKQSQAKQDLFGGSEPIEVHELGLPF 119
:*****:***** **..***** *****:..**
  
```

clone: **B#42.1**
 mw: **14.4 kDa**
 pl: **6.28**
 Eo: **4470**
 cys: **0**
 met: **3**

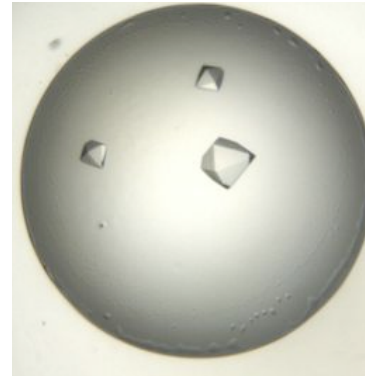


B#42.1 (FUN 44) Crystallisation



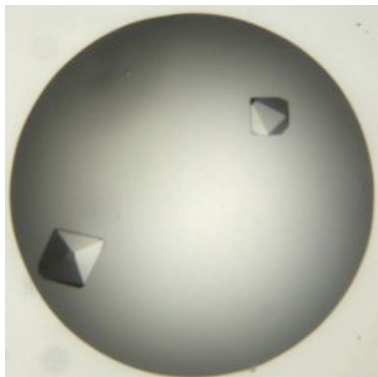
2.1 M malic acid pH 7.0
(JCSG Screen)

20°C
20 mg/mL



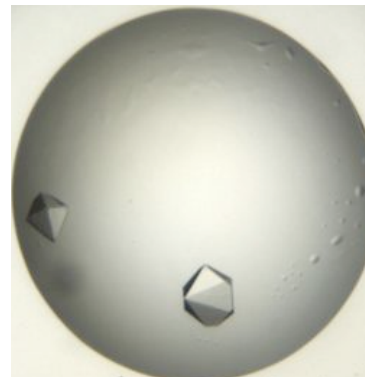
2.0 M Na malonate pH 7.0
100 mM Na acetate pH 4.5
(PS gradient)

20°C
20 mg/mL



2.0 M $(\text{NH}_4)_2\text{SO}_4$
(PS gradient)

20°C
20 mg/mL



2.0 M $(\text{NH}_4)_2\text{SO}_4$
100 mM Na citrate pH 5.5
(PS gradient)

20°C
20 mg/mL

B#1.1 (Terminase Nu1)

```

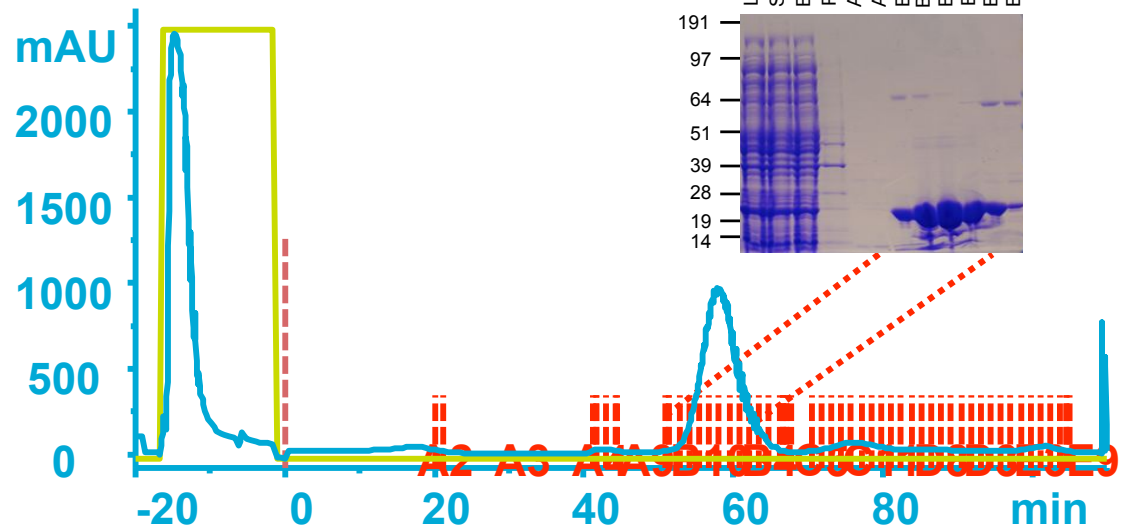
Clone_13 MAIITVTAQANEKNRTRVSTAKGDKKIISVPLFEKEKGSVVKVAYGSAFLPDFIQLGDTV 60
Clone_37 MAIITVTAQVNEKNRTRVNTAKGDKKIISVPLFEKEKGSNVKVAYGSAFLPDFIQLGDTV 60
Clone_1  MAIITVTAQANEKNRTRVSTAKGDKKIISVPLFEKEKGSNVKVAYGSAFLPDFIQLGDIV 60
Clone_25 MAIITVTAQANEKNRTRVNTAKGDKKIISVPLFEKEKGSNVKVAYGSAFLPDFIQLGDIV 60
*****.*****.*****.*****.*****
  
```

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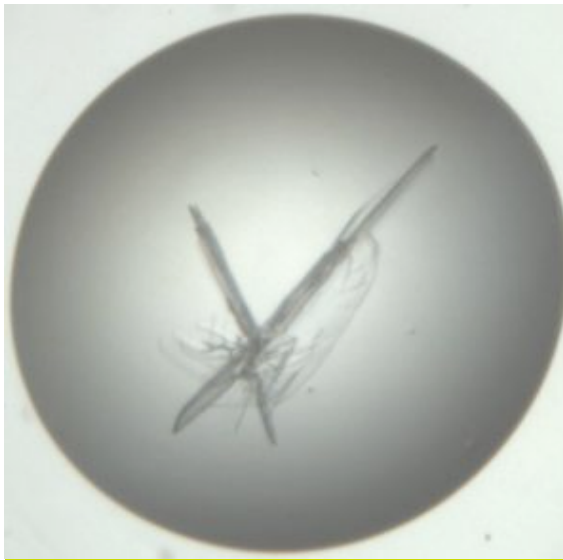
Clone_13 TVSGRVQAKESGEYVNYNFVFP TVEKVF I HNDNSSQS QAKQDLFGGSEPIEINTEDLPF 119
Clone_37 MVSGRVQAKESGEYVNYNFVFP AVEKVF I PNDNNSQS QAKQDLFGKSEPIEINTEDLPF 119
Clone_1  TISGRVQAKESGEYVNYNFVFP AVEKVF I PNDNSKQS QAKQDLFGGSEPIEVHELGLPF 119
Clone_25 TISGRVQAKESGEYVNYNFVFP AVEKVF I PNDNSKQS QAKQDLFGGSEPIEVHELGLPF 119
:*****:*****:***** **..***** *****:; .***
  
```

clone: **B#1.1**
 mw: **21.2 kDa**
 pl: **5.52**
 Eo: **21555**
 cys: **2**
 met: **5**

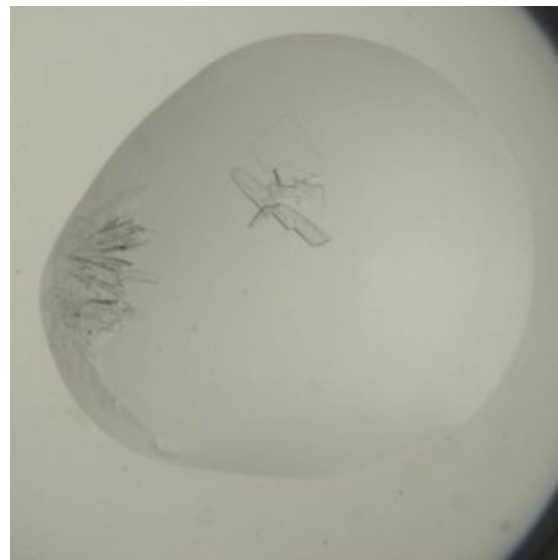
Highly soluble



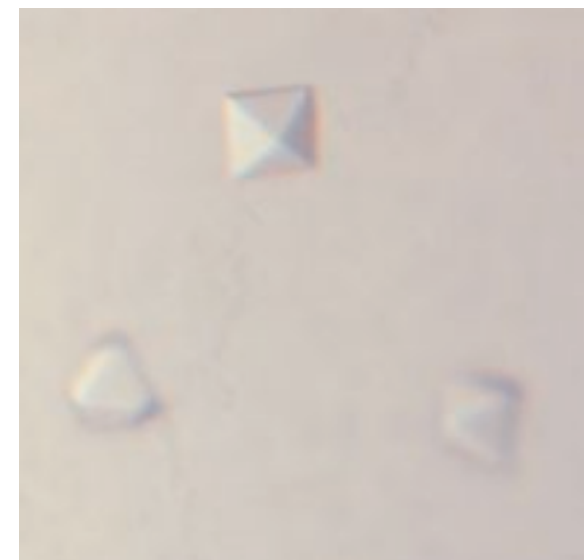
Crystal optimisation of B#1.1



100 mM BTP pH 6.65
18 % PEG 3350
150 mM MgCl₂
45 mM KNO₃
8°C
22.5 mg/mL



100 mM phos-citrate pH 4.2
40 % MPD
20°C
26.2 mg/mL

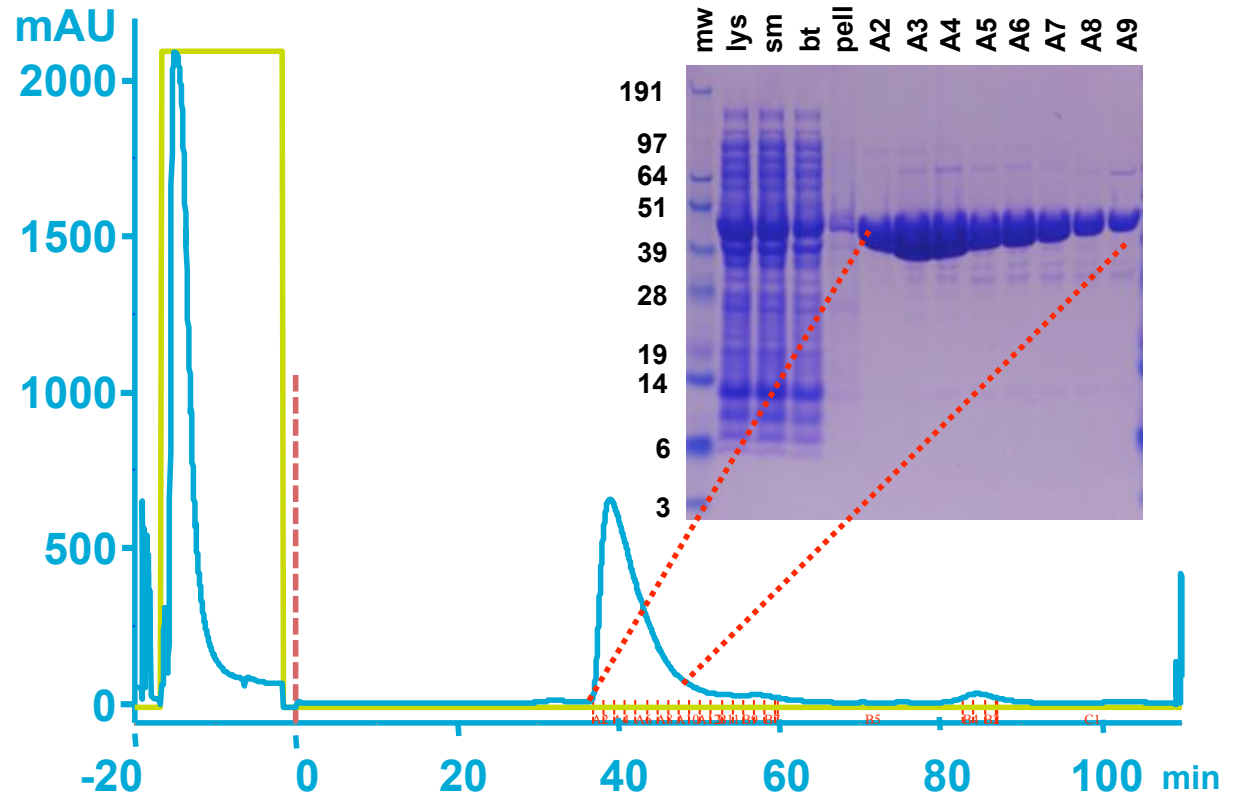


100 mM phos-citrate pH 4.2
36 % MPD
200 mM NDSB 221
20°C
26.2 mg/mL

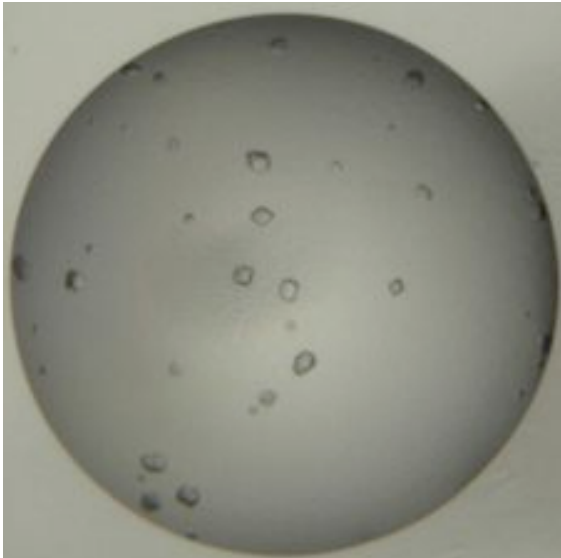
B#51.1 (FUN 19)

clone: **B#51.1**
mw: **43.7 kDa**
pl: **5.94**
Eo: **53290**
cys: **1**
met: **8**

**Concentration
& solubility issues**

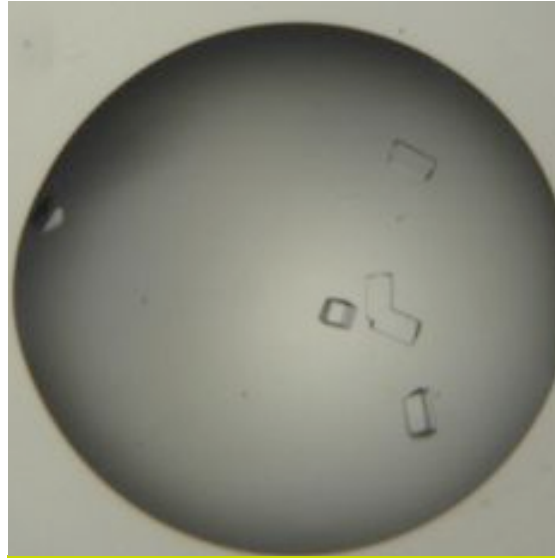


Crystal optimisation of B#51.1



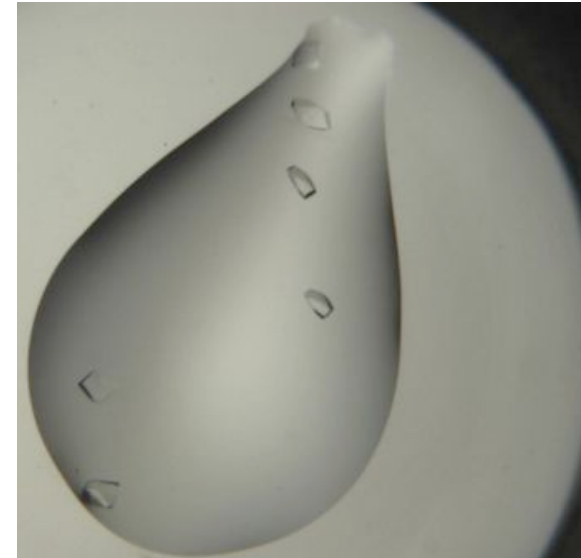
10 % malate-MES-TRIS pH 7.0
500 mM Na malonate pH 7.0

20°C
4.04 mg/mL (high salt)



100 mM Bis-Tris pH 6.34
960 mM sodium nitrate

20°C
4.04 mg/mL (high salt)



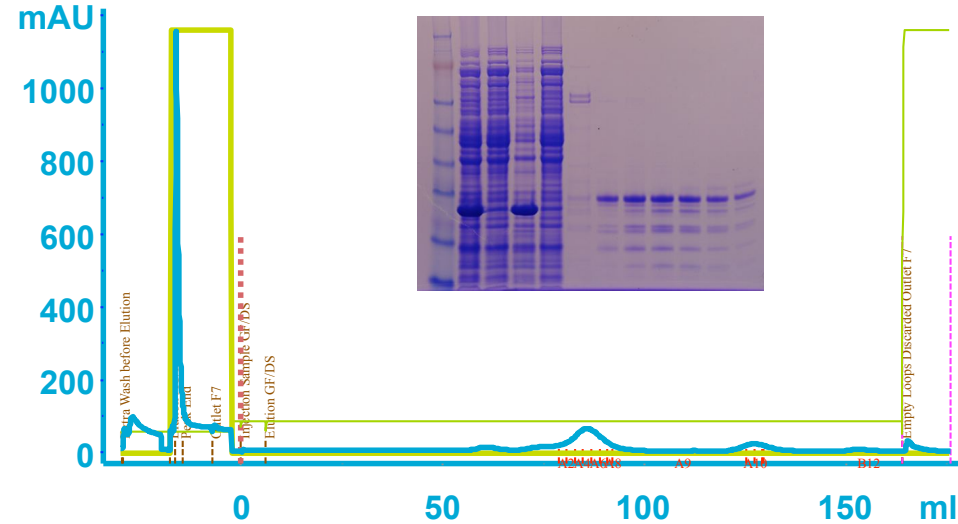
100 mM Bis-Tris pH 7.02
330 mM sodium nitrate

20°C
4.04 mg/mL (high salt)

Experiments that did not work

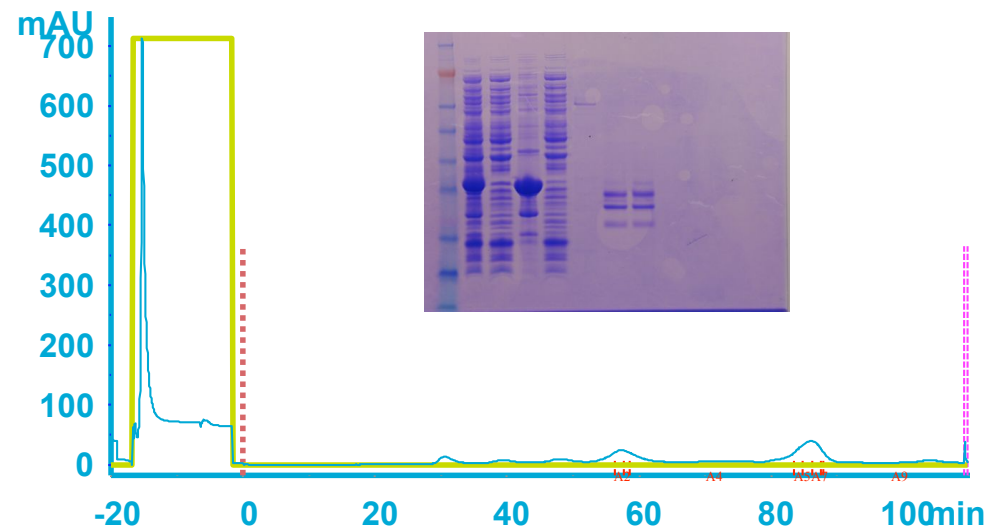
clone: **B#55.2 (FUN 41)**
mw: **22.1 kDa**
pl: **9.32**
Eo: **23950**
cys: **0**
met: **4**

Insoluble



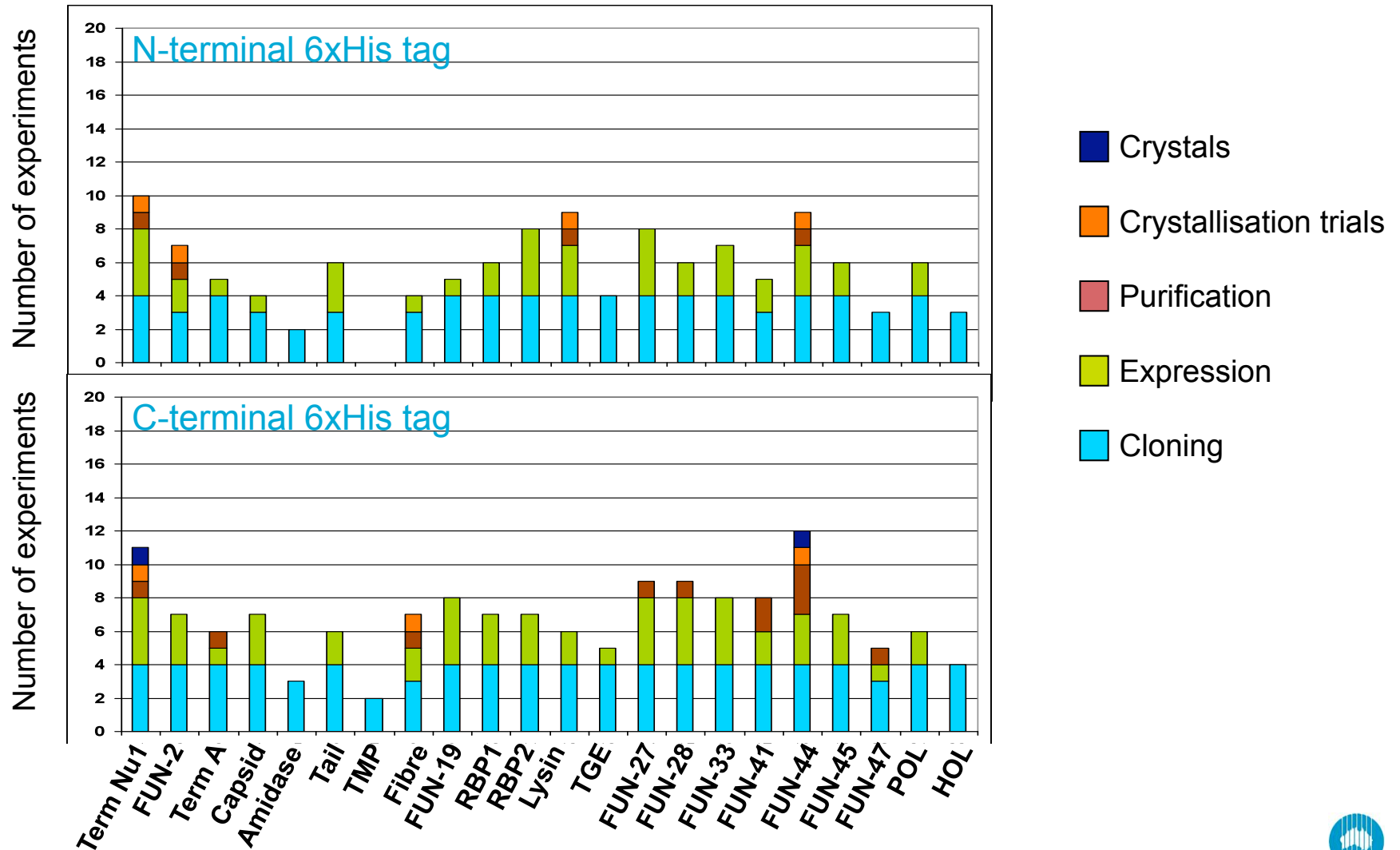
clone: **B#88.1 (RBP1)**
mw: **29.1 kDa**
pl: **6.75**
Eo: **40910**
cys: **0**
met: **5**

Insoluble



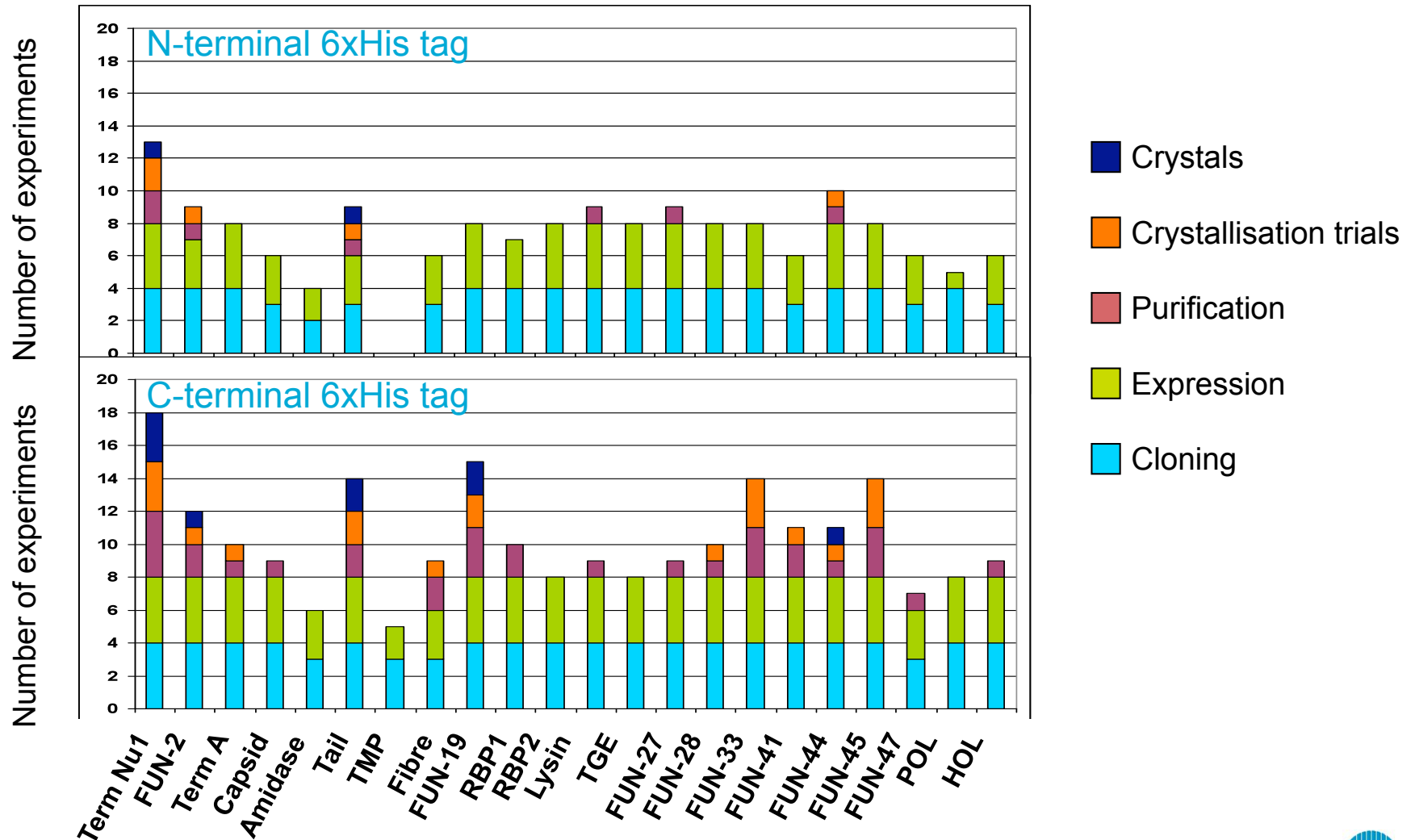
ANALYSIS - Phagenomics

JUNE 2009

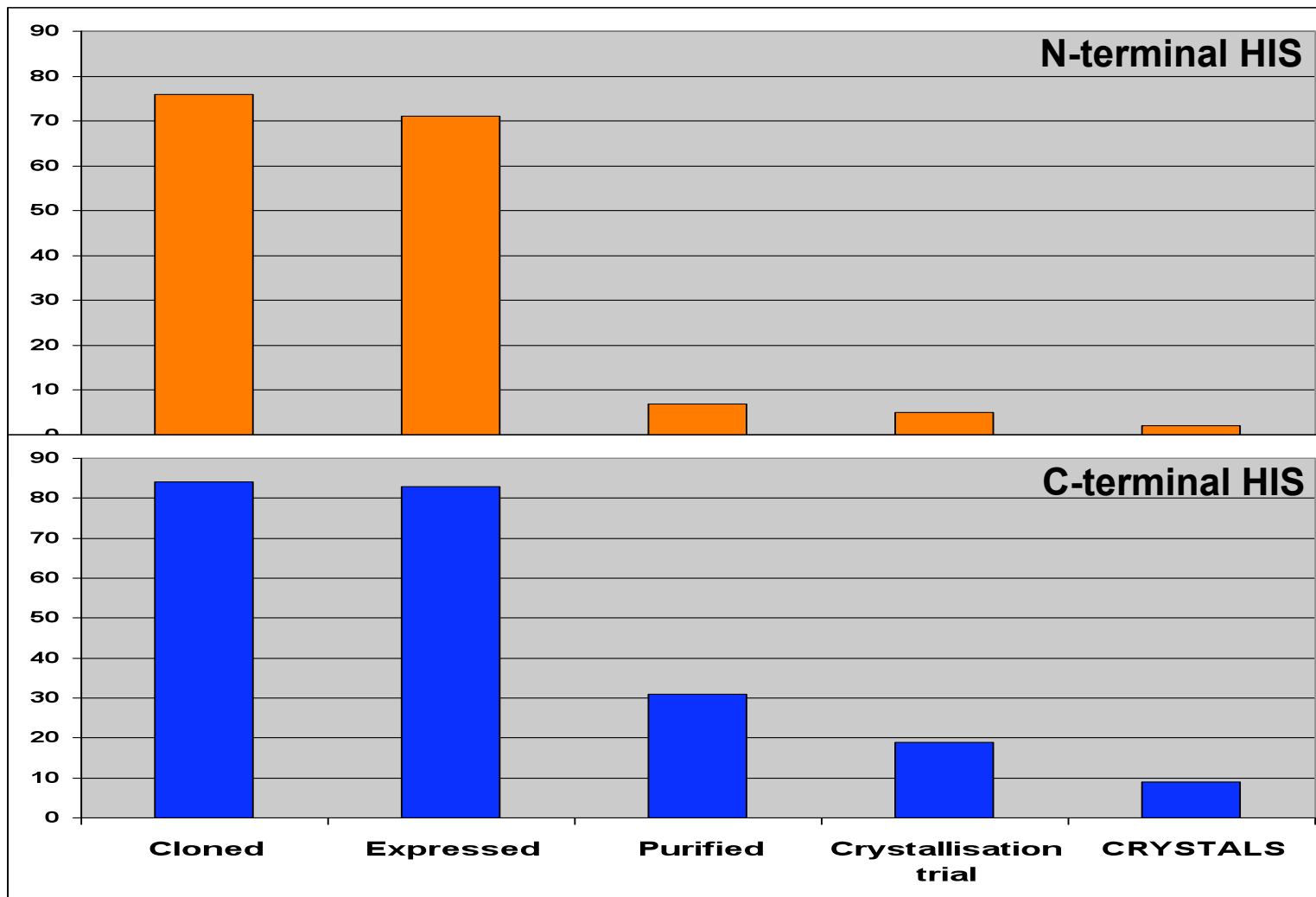


ANALYSIS - Phagenomics

SEPTEMBER 2009



Summary of Cloning, Purification and Crystallisation of N and C-terminal Constructs – September '09



FUTURE DIRECTIONS

- Continue large scale expression and purification of proteins for crystallisation trials in Bio21 C3 facility.
- Utilise NCRIS facility for fermentation and producing selenomethionine derivatives of FUN proteins.
- Identification of binding partners to FUN proteins from *Lactococcus lactis* lysates.
- Macromolecular complexes of FUN proteins with their binding partners for crystallisation trials.

FUTURE DIRECTIONS – NEW PROJECTS

- **Functional Genomics – Transformational Biology**

30 – 70% of proteins in any given genome have no known function

Acid resistance in *Escherichia coli*

Salmonella sp. genomics

Metagenomics

Bat Genomics

Characterisation of novel viral genes

Host/pathogen interactions

- Structural biology approaches to determine the 3D structure
- Produce purified proteins for functional assays and antibody production

- **Functional Genomics – One CSIRO**

- CFNS (Phil Hendry) - Genomics of microflora from oil reservoirs
- CMAR (Malcolm Brown, Stan Robert) - Genomics of algae
- Entomology (John Oakschott) - Genomics of Cotton Boll Worm

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CMHT - Clayton

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Thank you

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