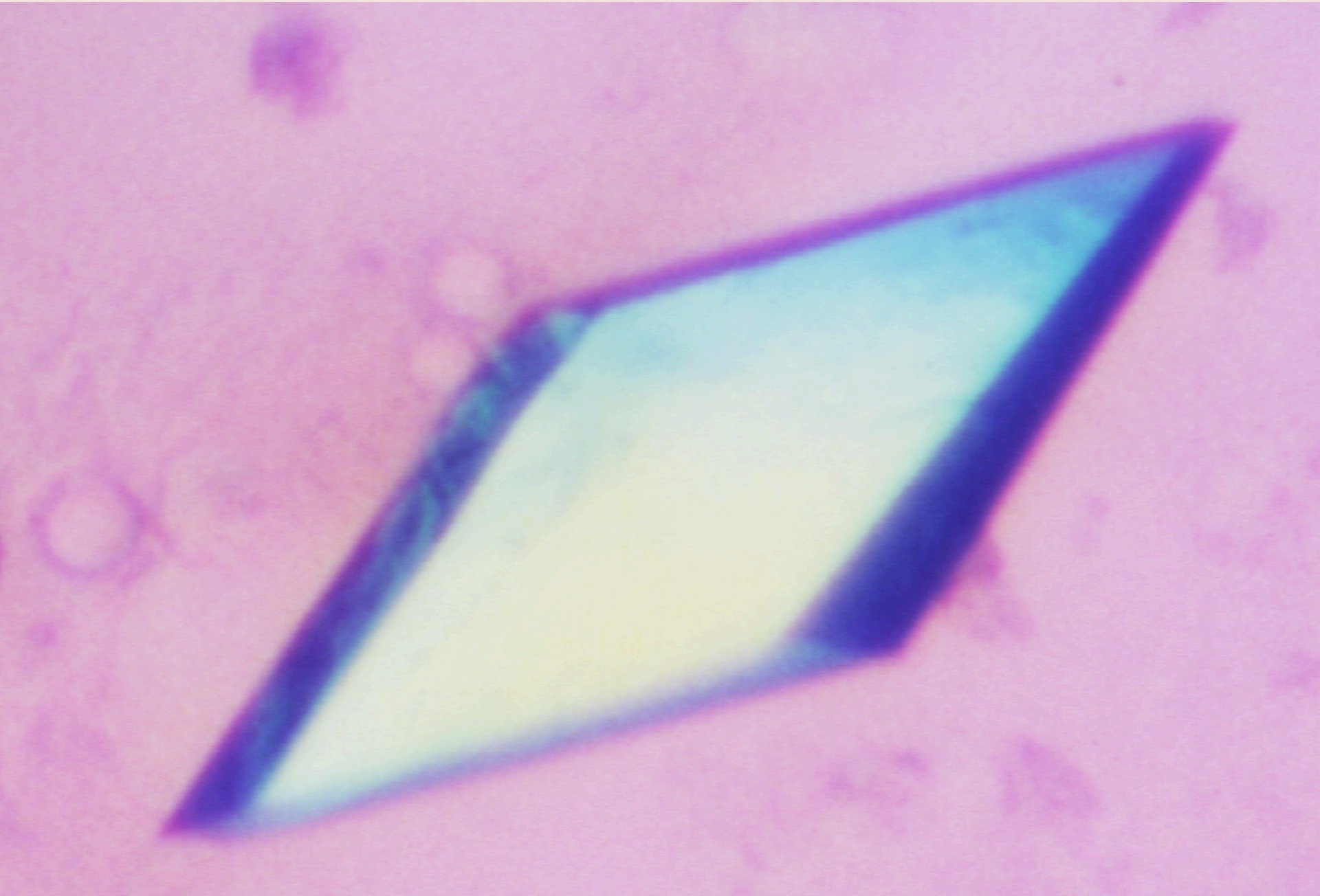
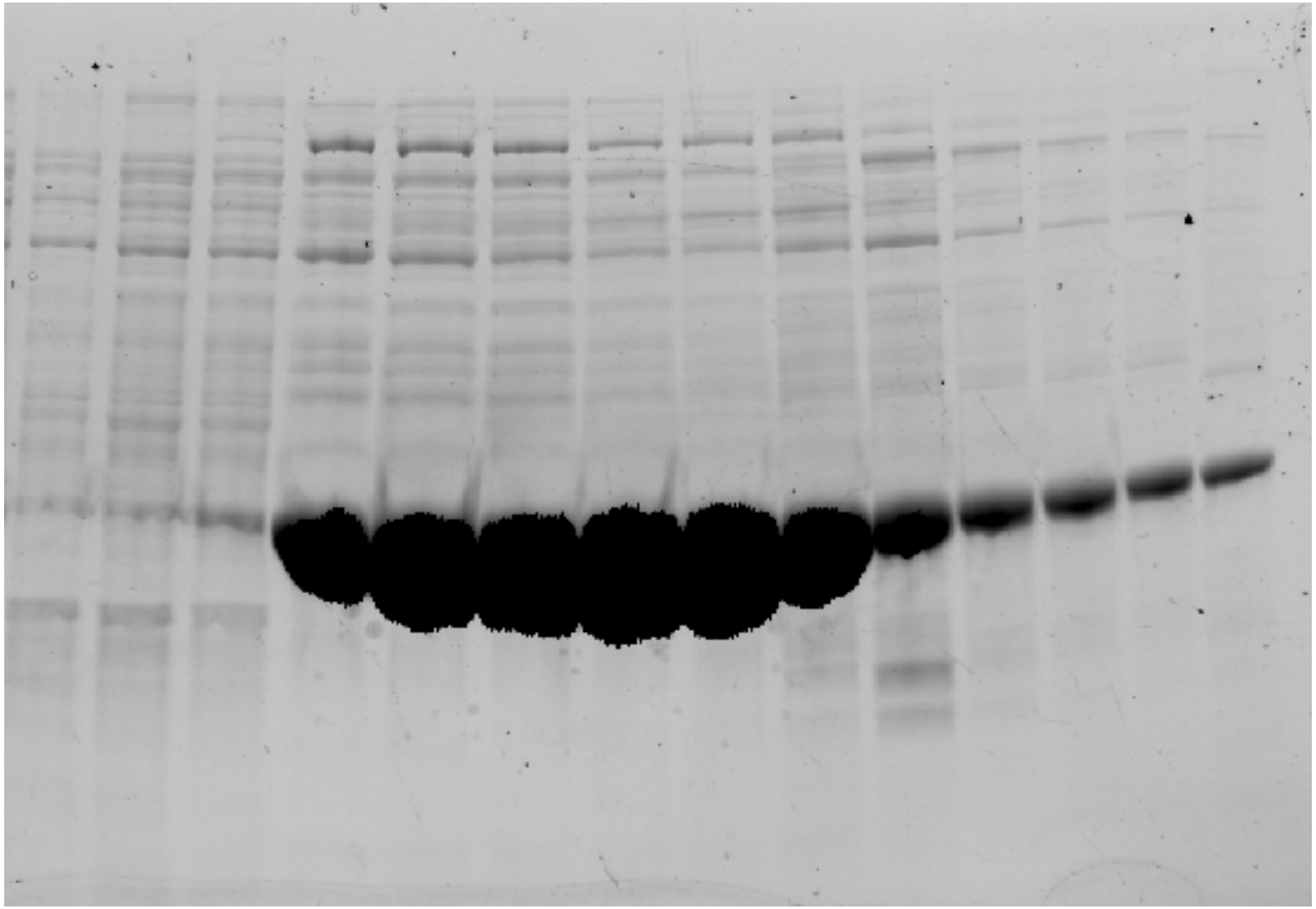
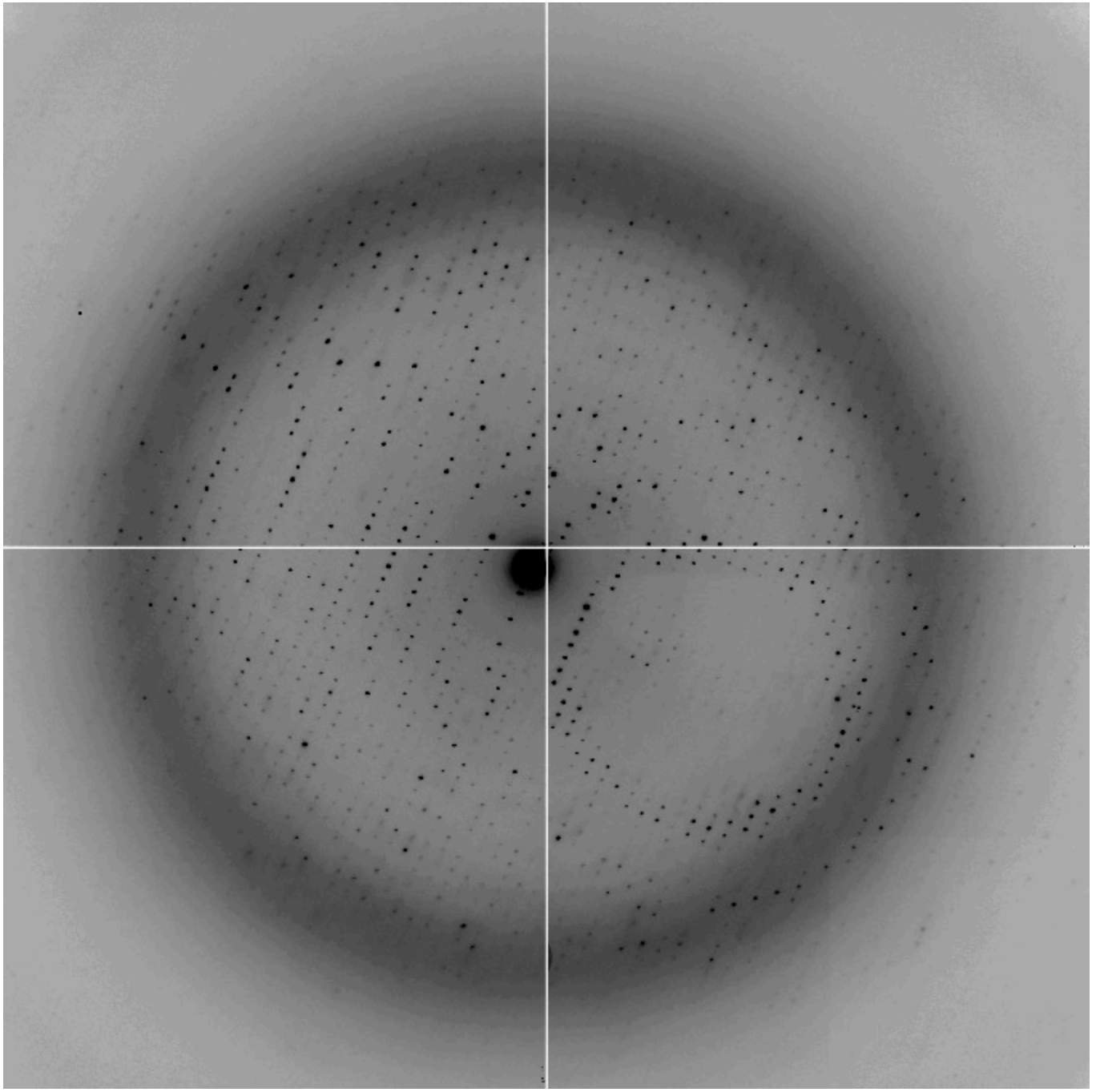
# Expression, purification, characterization, and crystallization







# So how does one go about solving a crystal structure?

| | | |
|---|---|---|
| formulate question | very hard! | Boss |
| make sample | cloning, expression, purification | you |
| make crystal | screening, optimisation | you |
| collect diffraction data | synchrotron, integration, scaling | Post-doc |
| solve phase problem | MR, SIRAS, MIRAS, SAD, MAD, hybrid | Post-doc/Randy/Phil |
| build model | manual or autotracing | Post-doc/you |
| refine model | agreement of model and data | Post-doc/Garib |
| interpret model | very hard! back to top? | Boss |

… and might fail at any step!

**This is the part where YOU make all the difference!!**

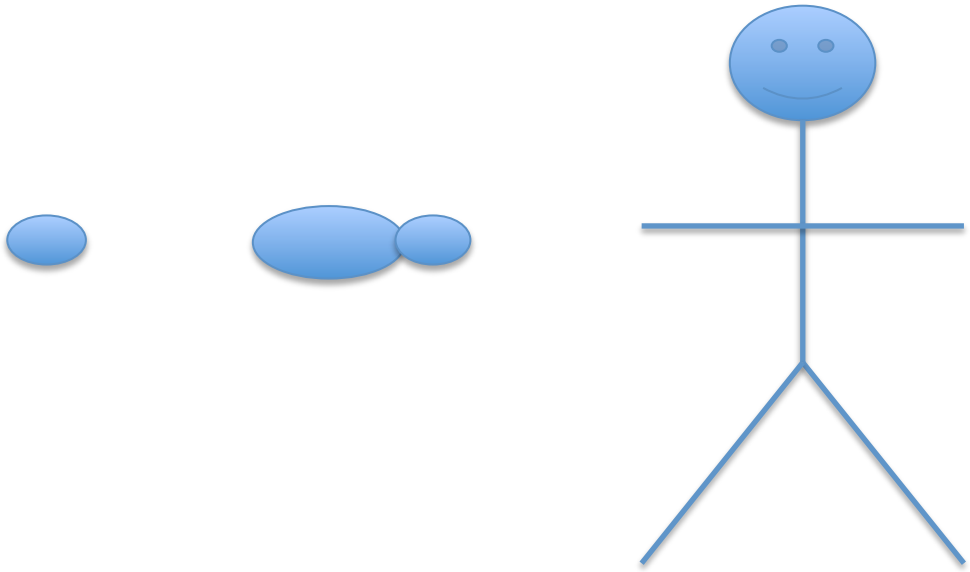
**Disclaimer:**
**This is a limited (i.e. my) view of how to do crystallography**

**You have to find out what works for you**

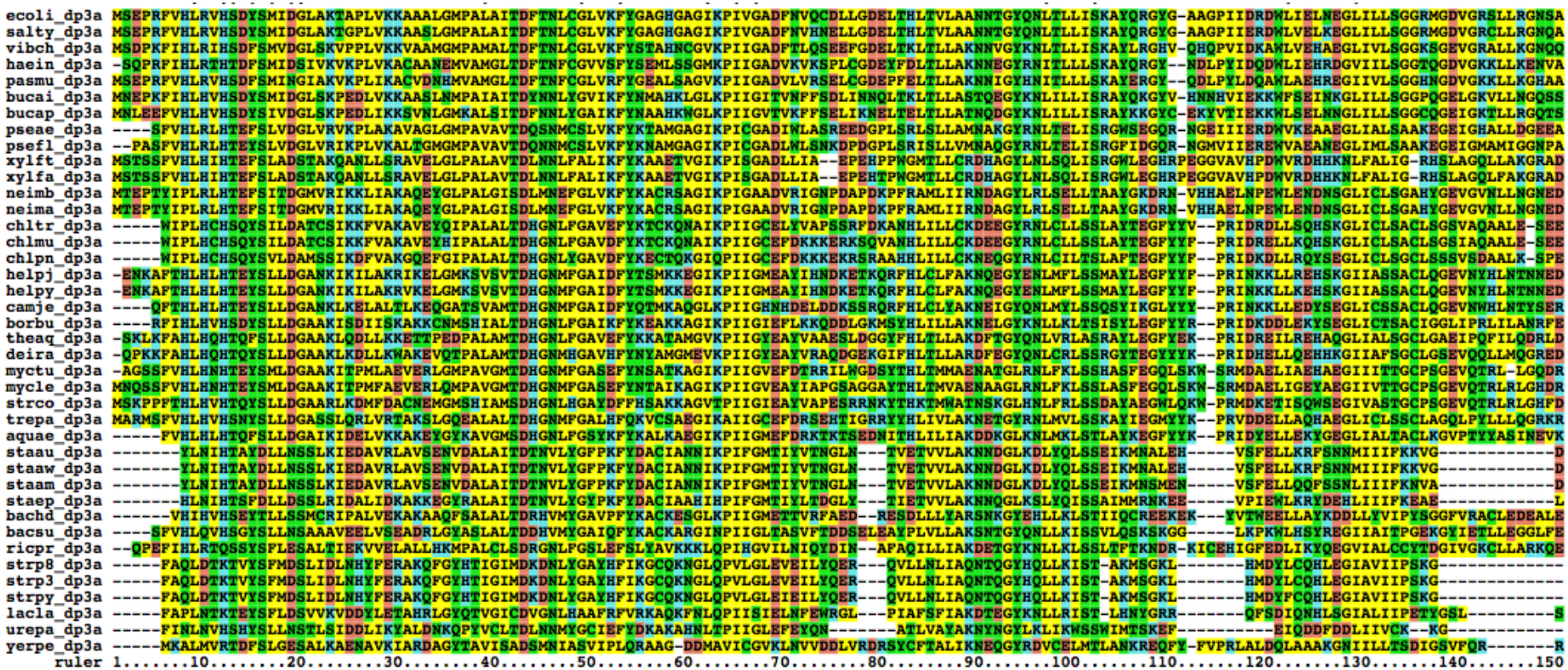
**(But some of this might be useful)**

# What to crystallize: Different species

| | |
|---|---|
| Bacterial: | Great diversity, Easy to work with |
| Archeal: | Half way between bacterial and eukaryotic |
| Eukaryotic: | more difficult, but sometimes essential |



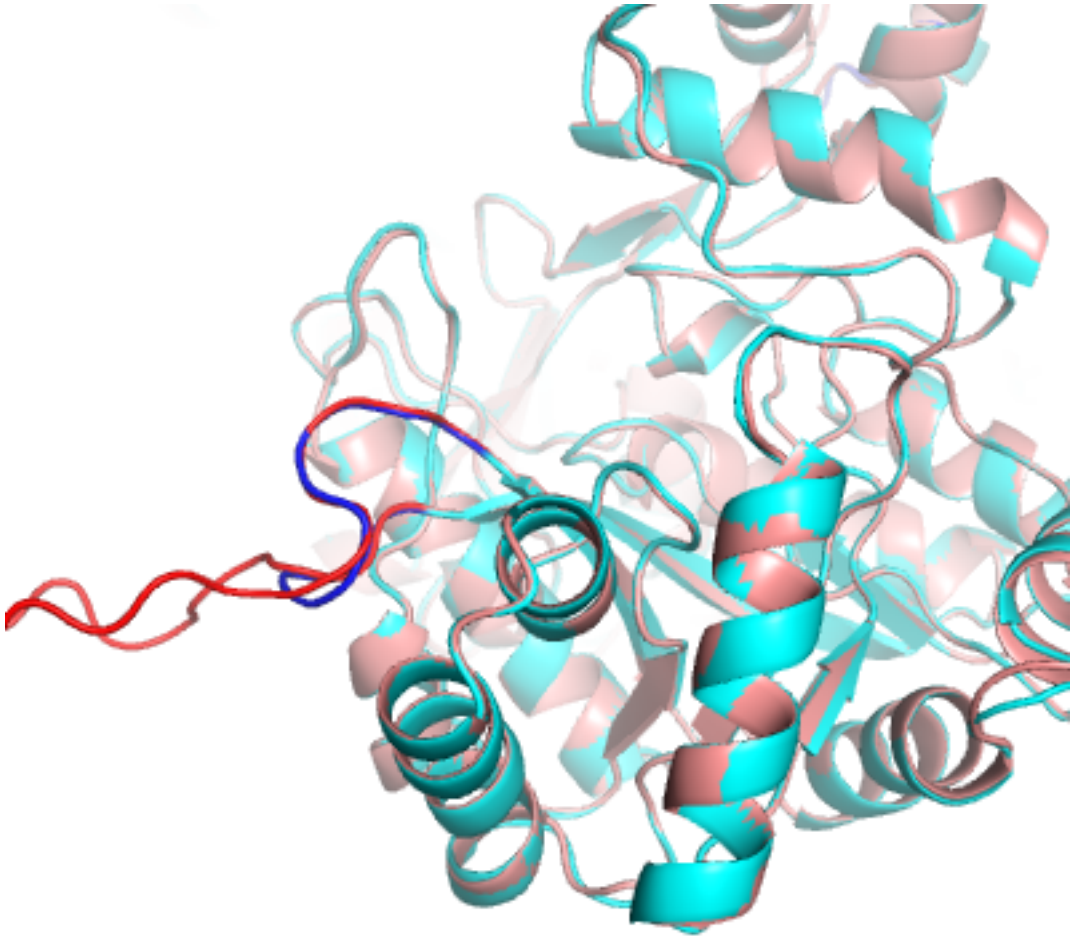
# What to crystallize: Different species



# What to crystallize: Different constructs


Max Planck Institute Teubingen toolkit => sequence searches, 2ndary structure prediction
Clustal (alignments) Jalview (view/edit)

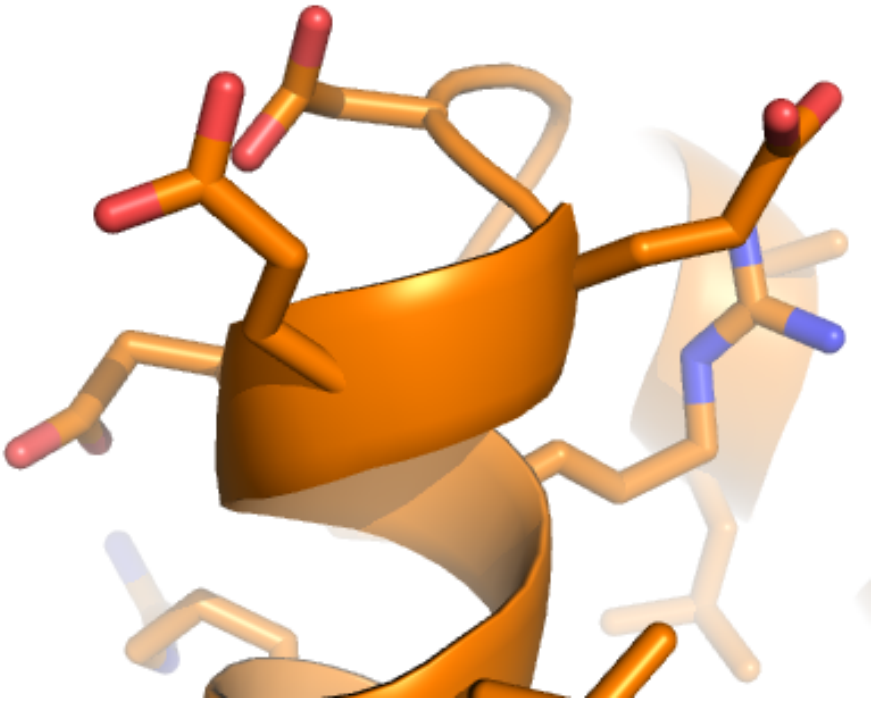
# What to crystallize: Different constructs

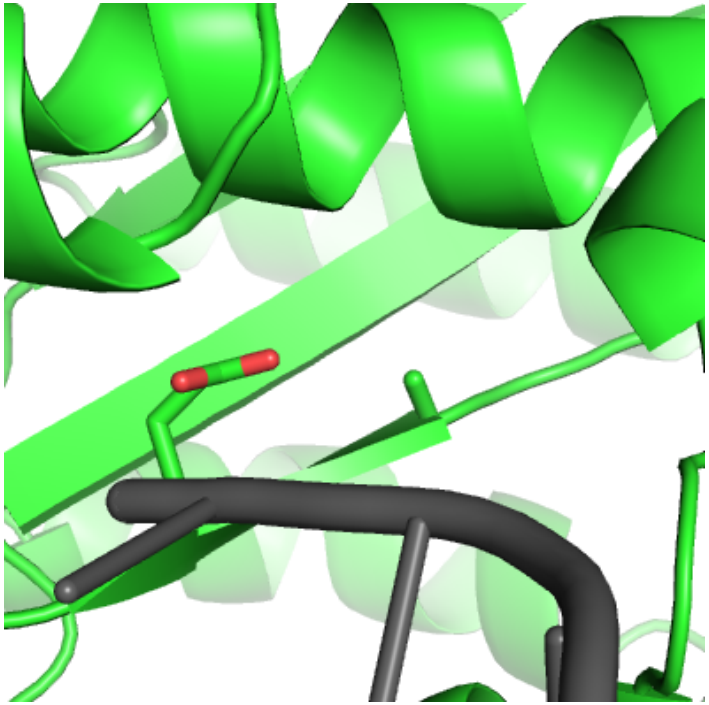


Modeller (Andrej Sali, UCSF)

# What to crystallize: make mutants

Surface engineering:  hydrophylic residues at loops into hydrophobic residues
Mutate active site residues: catch it in the act





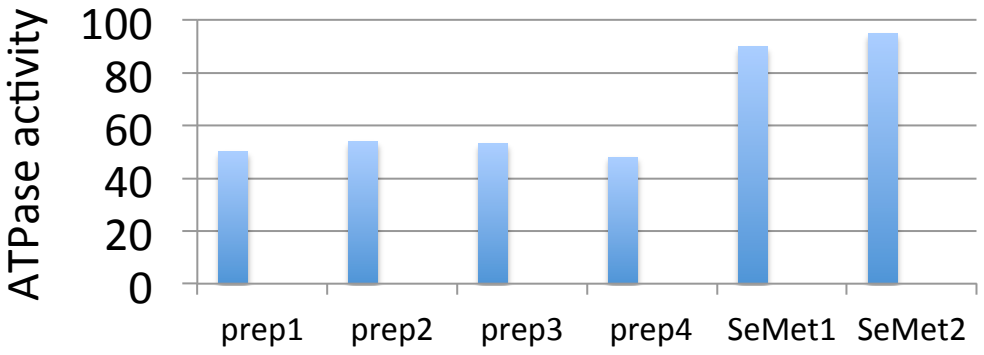
Derewenda ZS. Application of protein engineering to enhance crystallizability and improve crystal properties. Acta Crystallogr D Biol Crystallogr. 2010 May;66(Pt 5):604-15. PubMed PMID: 20445236

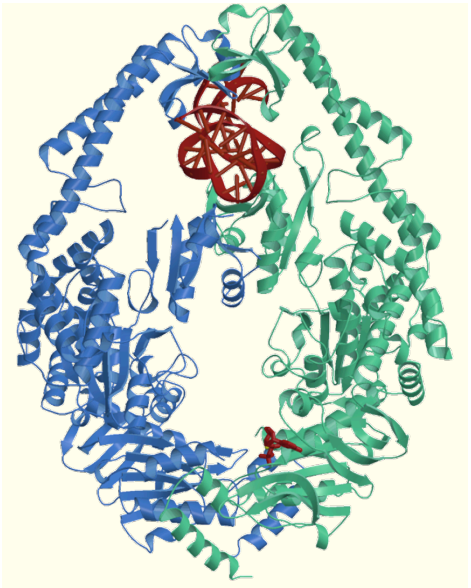
## What to crystallize: add partner protein and/or substrate! (or anything else that will stabilize your protein)

Partner protein (if complex)
Peptide
DNA/RNA
ATP/GTP (or non-hydrolysable analogue)
Inhibitor
…

But how do you know what to add??

## Study your protein!



# … or cross your fingers and hope for the best

**X family**

| | |
|---|---|
| **Pol β** | **GCCGCGGGAAA** |
| (Pelletier ’94) | **CGGCGCCC** |
| **Pol β** | **CGACTACGCGACAGCC** |
| (Batra ’06) | **GCTGATGCGC GTCGG** |

**A family**

| | |
|---|---|
| **Bacillus** | **CGTACTACGAGAGA** |
| (Kiefer ’98) | **GCATGATGC** |
| **T7 phage** | **GCTTTTGCTGCCGG TCACGGTTCCCC** |
| (Doublie ’98) | **CGAAAACGACGGCCAGTGCCAAG** |
| **Taq** | **CTGGTGCCGCGGGAAA** |
| (Li ’98) | **GACCACGGCGCCC** |

**B family**

| | |
|---|---|
| **RB69** | **GCGCCTGACGAATGGACA** |
| (Franklin ’01) | **GCGGACTGCTTACC$_d$T** |

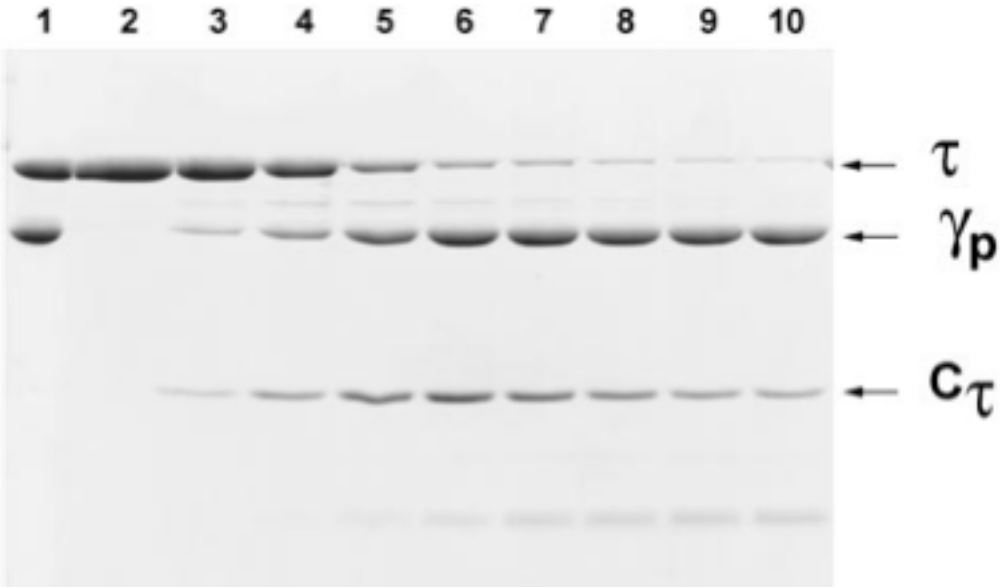
**Y family**

| | |
|---|---|
| **Dpo4** | **CCCCCTTCCTGATTACTT** |
| (Ling ’01) | **GGGGGAAGGACTAA** |

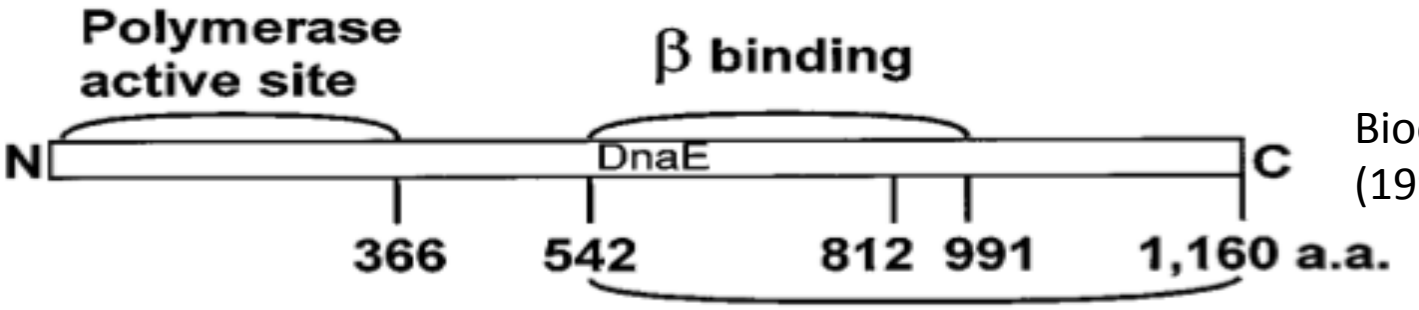
**A = added during crystallization**
**A = not visible in structure**

# What to crystallize: DO NOT…

..do limited proteolysis




.. believe everything that is published

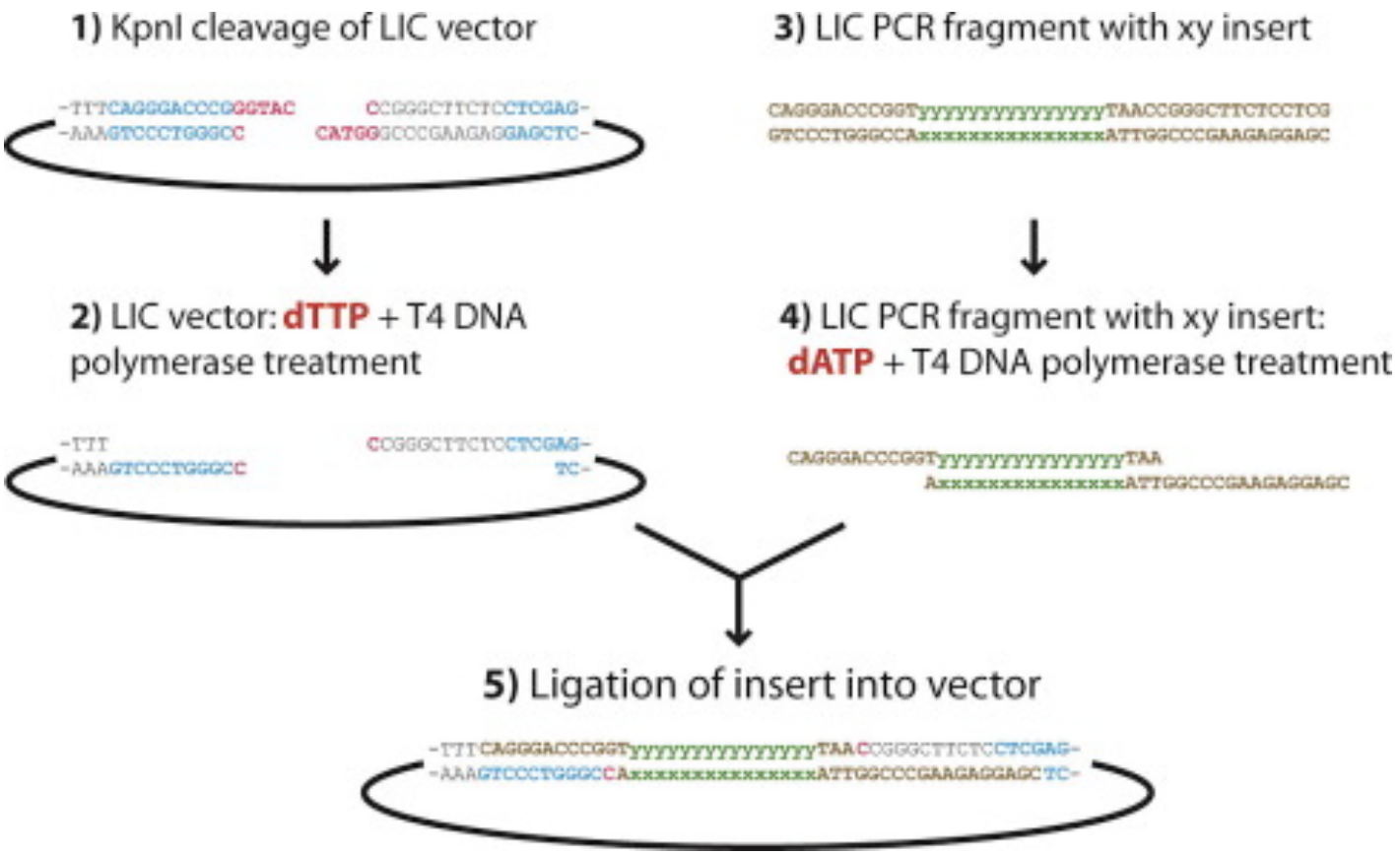



Biochemistry (1996)

Crystal Structure (2006)

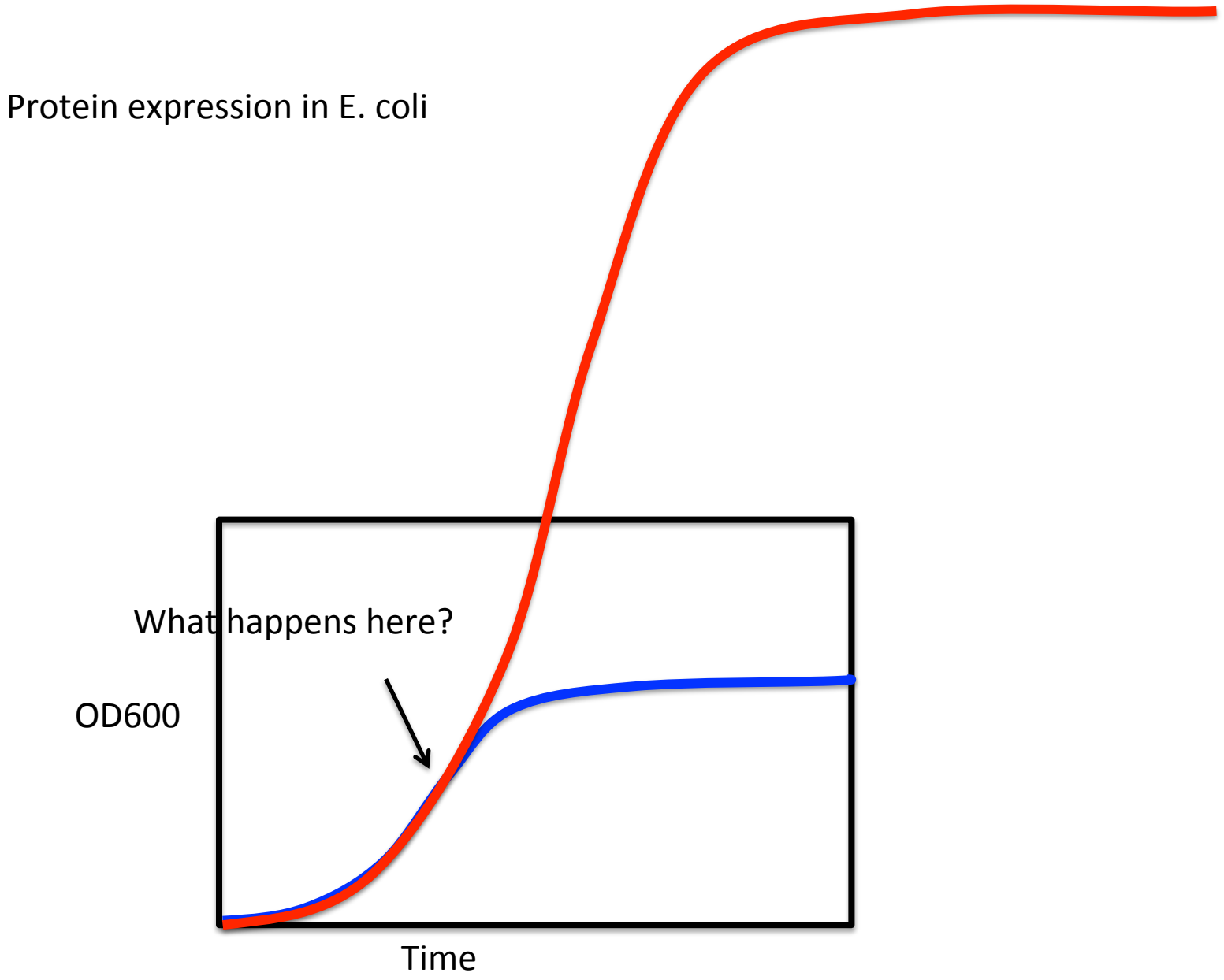
Active site: 401/403 β-bind: 920-924 τ-bind: 1072-1160

# What to crystallize: how to clone




http://www2.lmb.internal/wiki/index.php/Lamers_lab -> cloning

Aslanidis C, de Jong PJ. Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. 1990 Oct 25;18(20):6069-74. PubMed PMID: 2235490


Protein expression in E. coli
What happens here?
OD600
Time

Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41,** 207–234 (2005).

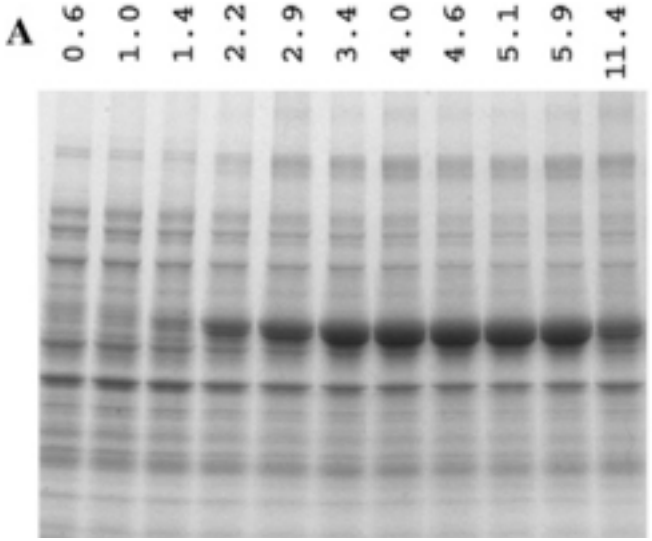
Table 10
Effect of magnesium on saturation density in 2×YT and terrific broth (TRB)

| Growth medium | Source | $A_{600}$ | pH |
|---|---|---|---|
| ZYM-505 | Local | 12.0 | 7.05 |
| 2×YT | Local | 5.7 | 8.37 |
| 2×YT + 2 mM $MgSO_4$ | Local | 8.3 | 8.44 |
| TRB | Gibco/BRL | 3.6 | 7.73 |
| TRB + 2 mM $MgSO_4$ | Gibco/BRL | 18.6 | 8.21 |
| TRB | Local | 12.5 | 8.06 |
| TRB + 2 mM $MgSO_4$ | Local | 18.1 | 8.18 |

**E. coli is easily satisfied…**

Need enough food: 2xTY (or even better: terrific broth)
Need Mg: 1mM $MgSO_4$
Need lots of $O_2$:use  baffled flasks (Thomson UtraYield)
Prevent leaky expression: 1% glucose (and pLysS plasmid)
Don’t like extra baggage (i.e your plasmids): do NOT do starter culture

# Max protein production in 1-2 hrs…




**Fool proof protocol:**

Transform enough cell to plate out on 6 plates & grow overnight

Scrape ALL cells and inoculate 6 x 0.5 Ltr 2xTY + 1mM MgSO4, 1% Glucose, Antibiotic

Grow 2-3 hrs at 37$^{o}$C to $OD_{600}$=3-6 in BAFFLED UltraYield flasks (2.5 Ltr)

Add 1 volume of RT 2xTY (+Mg, Gluc, Antib, IPTG)

Express protein for 1-2 hrs @ 30$^{o}$C

Harvest cells and freeze

=> 70-100g cells => 80-100mg protein (need ~10 mg to set up 2000 drops)

Sorry: I don't know much about protein expression in yeast, baculovirus or other systems

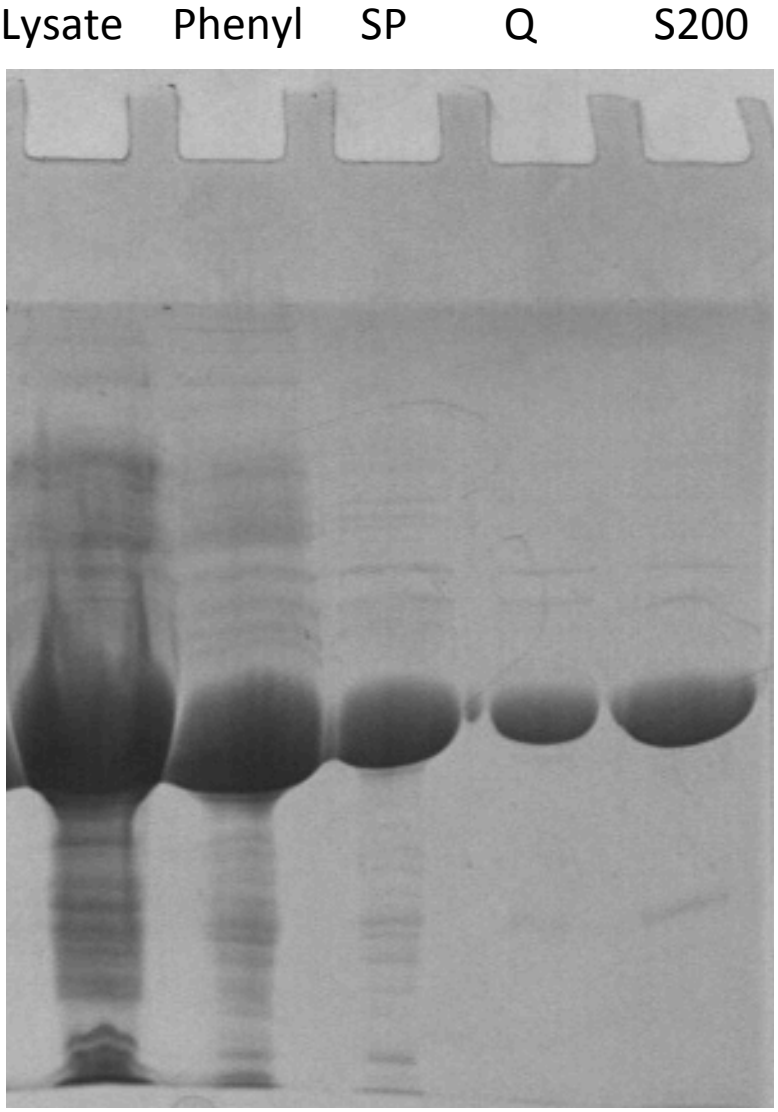
Don't ever say (or even think): "but this is how everyone does it"

Andrew Carter: Yeast to OD600=80
Imre Berger: multi protein expression in baculovirus
Wayne Hendrickson: SeMet expression for phasing
William Studier: using T7 phages for protein expression in E. coli

# Protein purification

Tags
Hydrophobic
Ion-exchange
Affinity
Size exclusion
Buffer exchange
Protein storage

www.gelifesciences.com
**> Service & support**
**> Handbooks**

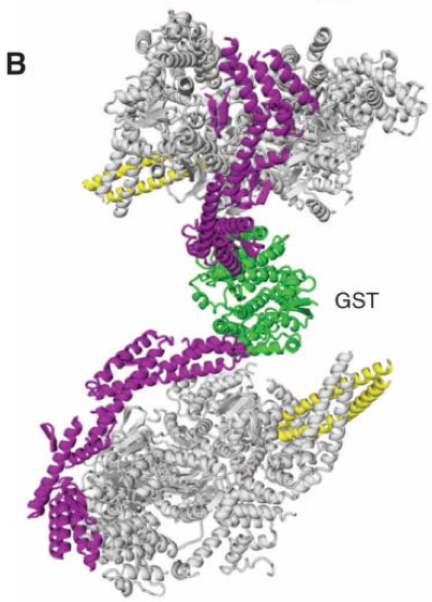


# Protein purification

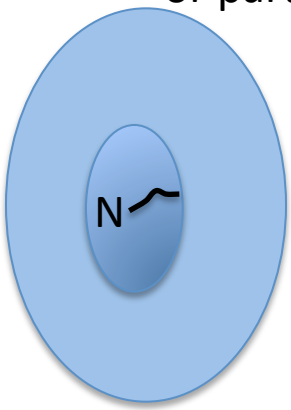
**Tags**

Hydrophobic
Ion-exchange
Affinity
Size exclusion
Buffer exchange
Protein storage

GST is a dimer!




N-terminus may be buried or part of active site!




Histrap columns can leak Ni into your protein

Ni Ni Ni Ni Ni Ni Ni

# Protein purification

Tags
Hydrophobic
Ion-exchange
Affinity
**Size exclusion**
Buffer exchange
Protein storage

Do you really need to a run a gel filtration run??
Only good if:

- You have lots of aggregates
- You have degradation products

⇒ Concentrating afterwards can do more harm then good

# Protein purification

Tags
Hydrophobic
Ion-exchange
Affinity
Size exclusion
**Buffer exchange**
Protein storage

Instead of dialyzing try:
* 5-10 fold dilution
* Desalting column (30 minutes)
* Changing pH by adding HCl or NaOH (within buffer capacity)

# Protein purification

Tags
Hydrophobic
Ion-exchange
Affinity
Size exclusion
Buffer exchange
**Protein storage**

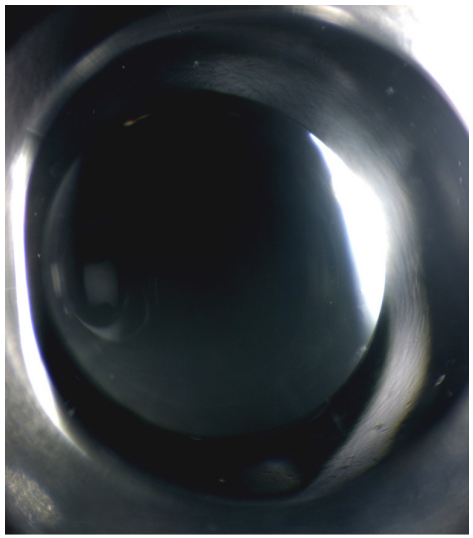
If you can, freeze your protein
Flash freeze protein in LN2. Use PCR tubes if needed
Thaw in hand, then store on ice
Find optimal storage buffer (use solubility screen)

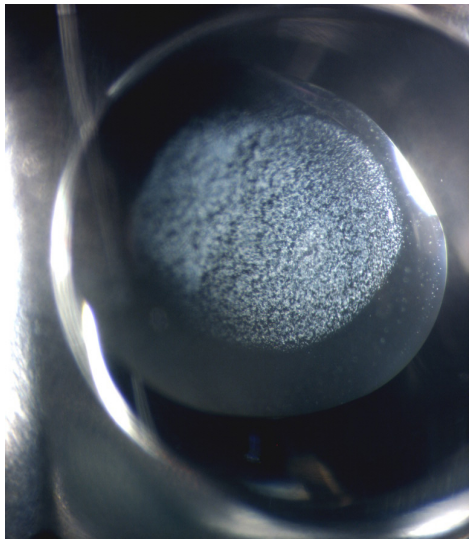
Deng, J. *et al.* An improved protocol for rapid freezing of protein samples for long-term storage. *Acta Crystallogr. D Biol. Crystallogr.* **60,** 203–204 (2004).

# Protein characterization

1-10mg/ml
100nl/drop
Incubate 1-24 hrs

Solubility Screen



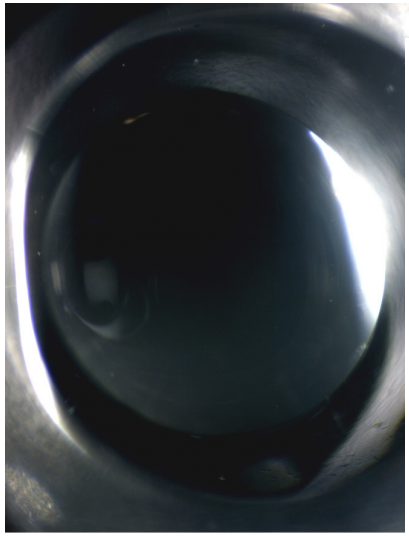


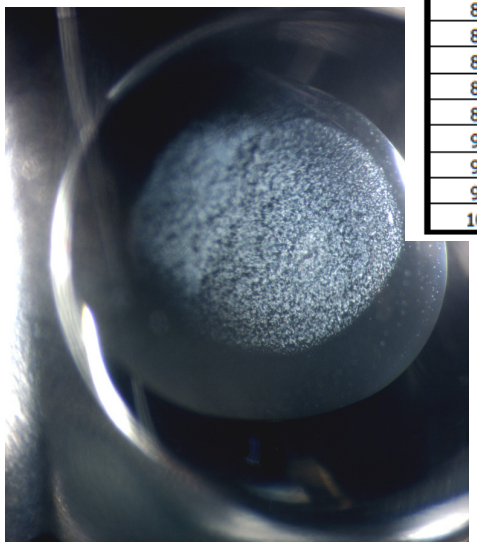
| | | Well | | | |
|---|---|---|---|---|---|
| | | A1-B12 | C1-D12 | E1-F12 | G1-H12 |
| pH | Buffer (100mM) | No Additive | NaCl (150mM) | Glycerol (5%V/V) | NaCl & Glyc |
| 5.0 | K Acetate | A1 **20% TCA** | C1 | E1 | G1 |
| 5.5 | MES | A2 | C2 | E2 | G2 |
| 6.0 | ADA | A3 | C3 | E3 | G3 |
| 6.5 | MES | A4 | C4 | E4 | G4 |
| 6.5 | BisTris | A5 | C5 | E5 | G5 |
| 6.5 | $KHPO_4$ | A6 | C6 | E6 | G6 |
| 7.0 | PIPES | A7 | C7 | E7 | G7 |
| 7.0 | Imidazole | A8 | C8 | E8 | G8 |
| 7.0 | ADA | A9 | C9 | E9 | G9 |
| 7.0 | MOPS | A10 | C10 | E10 | G10 |
| 7.5 | HEPES | A11 | C11 | E11 | G11 |
| 7.5 | $KHPO_4$ | A12 | C12 | E12 | G12 |
| 7.5 | DIPSO | B1 | D1 | F1 | H1 |
| 7.5 | Tricine | B2 | D2 | F2 | H2 |
| 8.0 | HEPES | B3 | D3 | F3 | H3 |
| 8.0 | Bicine | B4 | D4 | F4 | H4 |
| 8.0 | Tris | B5 | D5 | F5 | H5 |
| 8.5 | Tris | B6 | D6 | F6 | H6 |
| 8.5 | TABS | B7 | D7 | F7 | H7 |
| 8.5 | TAPS | B8 | D8 | F8 | H8 |
| 9.0 | CAPSO | B9 | D9 | F9 | H9 |
| 9.0 | CHES | B10 | D10 | F10 | H10 |
| 9.5 | Glycine | B11 | D11 | F11 | H11 |
| 10.0 | CAPS | B12 | D12 | F12 | H12 **EMPTY** |

# Protein characterization

1-10mg/ml
100nl/drop
Incubate 1-24 hrs

Solubility Screen





| | | Protein 1 (4.5 mg/ml) | | | |
|---|---|---|---|---|---|
| pH | Buffer (100mM) | No Additive | NaCl (150mM) | Glycerol (5%V/V) | NaCl & Glyc |
| 5.0 | K Acetate | A1 | C1 | E1 | G1 |
| 5.5 | MES | A2 | C2 | E2 | G2 |
| 6.0 | ADA | A3 | C3 | E3 | G3 |
| 6.5 | MES | p | p | E4 | p |
| 6.5 | BisTris | p | C5 | E5 | G5 |
| 6.5 | $KHPO_4$ | p | C6 | E6 | G6 |
| 7.0 | PIPES | p | C7 | p | G7 |
| 7.0 | Imidazole | p | C8 | p | G8 |
| 7.0 | ADA | A9 | p | E9 | G9 |
| 7.0 | MOPS | p | C10 | p | G10 |
| 7.5 | HEPES | p | p | p | G11 |
| 7.5 | $KHPO_4$ | p | C12 | E12 | p |
| 7.5 | DIPSO | p | D1 | p | H1 |
| 7.5 | Tricine | p | p | p | H2 |
| 8.0 | HEPES | p | D3 | p | H3 |
| 8.0 | Bicine | p | D4 | p | H4 |
| 8.0 | Tris | p | p | p | H5 |
| 8.5 | Tris | p | D6 | p | H6 |
| 8.5 | TABS | p | D7 | p | p |
| 8.5 | TAPS | p | D8 | p | H8 |
| 9.0 | CAPSO | p | D9 | p | H9 |
| 9.0 | CHES | p | p | p | p |
| 9.5 | Glycine | p | p | p | H11 |
| 10.0 | CAPS | p | D12 | p | H12 |

| Protein 2 (10 mg/ml) | | | |
|---|---|---|---|
| No Additive | NaCl (150mM) | Glycerol (5%V/V) | NaCl & Glyc |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| p | p | p | p |
| B1 | p | p | H1 |
| p | p | p | H2 |
| p | p | p | H3 |
| p | p | p | H4 |
| B5 | p | p | H5 |
| B6 | D6 | F6 | H6 |
| B7 | D7 | F7 | H7 |
| B8 | D8 | F8 | H8 |
| B9 | p | p | H9 |
| B10 | p | F10 | H10 |
| B11 | p | F11 | H11 |
| B12 | p | F12 | H12 |

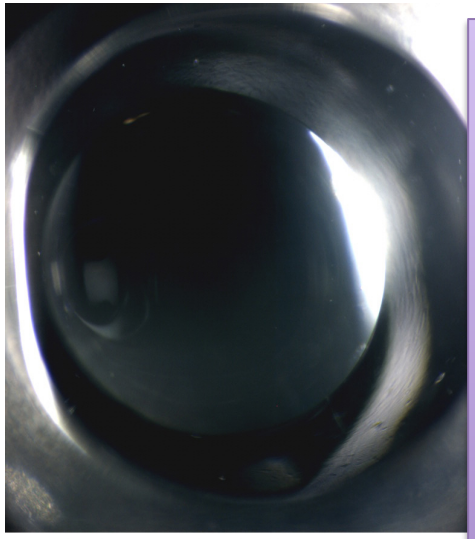
| Protein 3 (15 mg/ml) | | | |
|---|---|---|---|
| No Additive | NaCl (150mM) | Glycerol (5%V/V) | NaCl & Glyc |
| p | p | p | p |
| p | p | p | p |
| p | C3 | p | G3 |
| p | C4 | p | G4 |
| A5 | C5 | p | G5 |
| A6 | C6 | E6 | G6 |
| A7 | C7 | E7 | G7 |
| p | C8 | p | G8 |
| A9 | C9 | E9 | G9 |
| p | C10 | p | G10 |
| p | C11 | p | G11 |
| A12 | C12 | E12 | G12 |
| B1 | D1 | F1 | H1 |
| p | p | p | H2 |
| B3 | D3 | F3 | H3 |
| p | D4 | p | H4 |
| B5 | D5 | F5 | H5 |
| B6 | D6 | F6 | H6 |
| B7 | D7 | F7 | H7 |
| B8 | D8 | F8 | H8 |
| B9 | D9 | F9 | H9 |
| B10 | D10 | F10 | H10 |
| B11 | D11 | p | H11 |
| B12 | D12 | F12 | H12 |

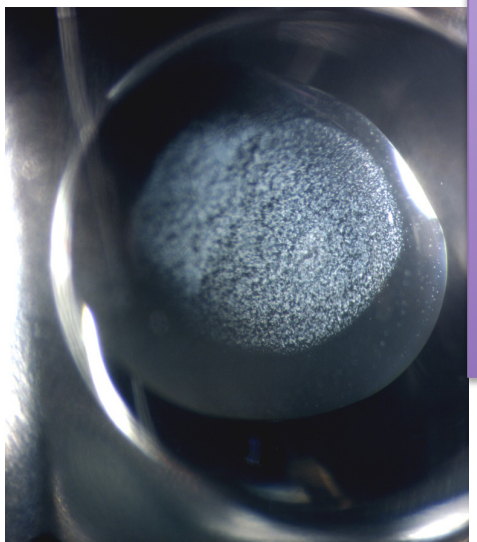
See: http://www2.lmb.internal/wiki/index.php/Lamers_lab
> Crystallography > Solubility screen
Or Jena Bioscience JBScreen Solubility HTS

## Protein characterization

1-10mg/ml
100nl/drop
Incubate 1-24 hrs

Solubility Screen





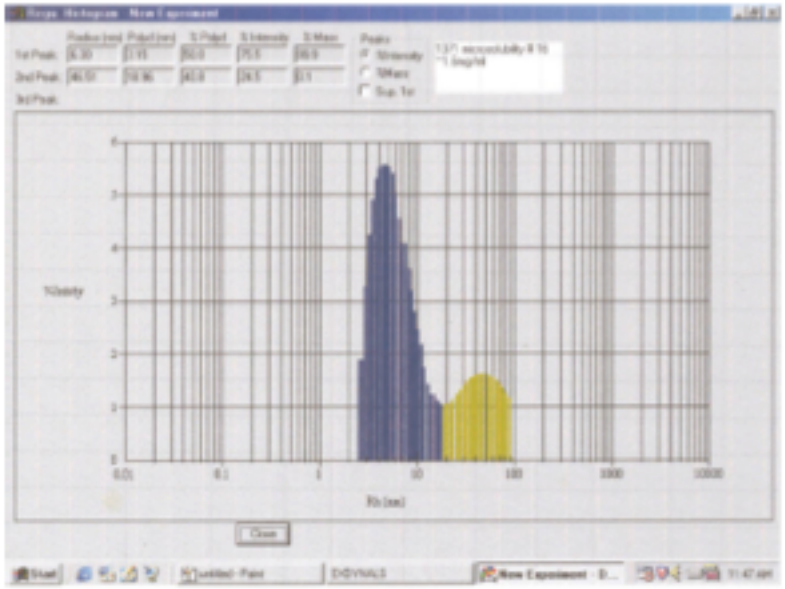
| | | Well | | | |
|---|---|---|---|---|---|
| | | A1-B12 | C1-D12 | E1-F12 | G1-H12 |
| pH | Buffer (100mM) | No Additive | NaCl (150mM) | Glycerol (5%V/V) | NaCl & Glyc |
| 9.0 | CAPSO | B9 | D9 | F9 | H9 |
| 9.0 | CHES | B10 | D10 | F10 | H10 |
| 9.5 | Glycine | B11 | D11 | F11 | H11 |
| 10.0 | CAPS | B12 | D12 | F12 | H12 EMPTY |

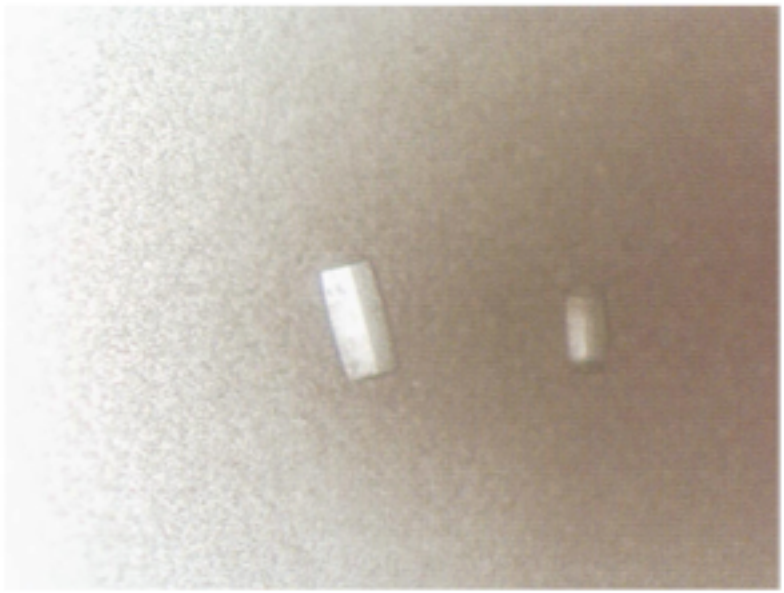
Now also available for:
activity assays
protein labeling
cross-linking
freezing

# Protein characterization

Dynamic Light Scattering

5uL, 1-5mg/ml





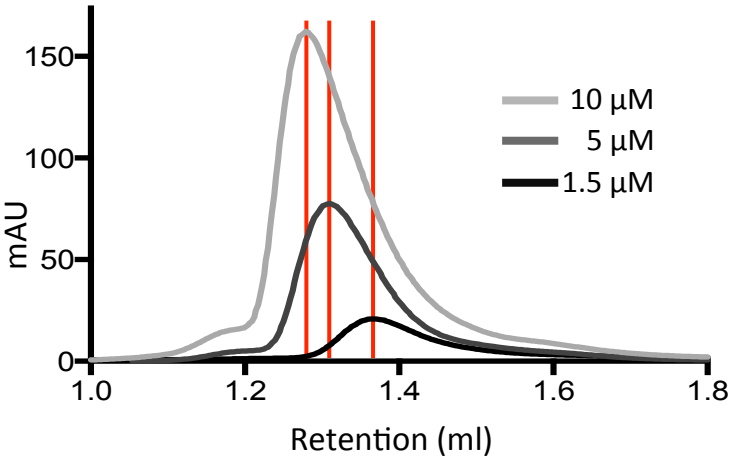
(d)

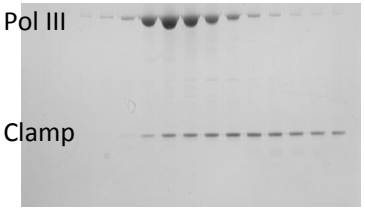
**Monodisperse proteins give more crystal hits**

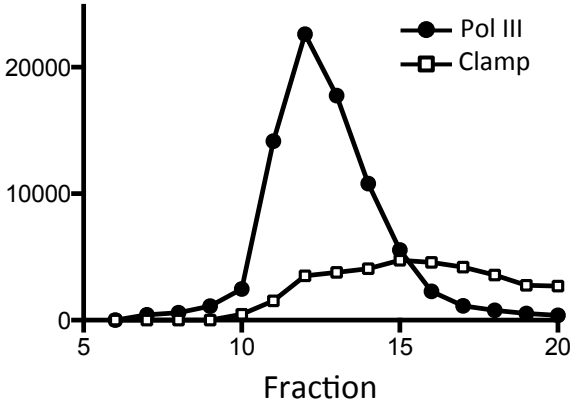
Jancarik, J., Pufan, R., Hong, C., Kim, S.-H. & Kim, R. Optimum solubility (OS) screening: an efficient method to optimize buffer conditions for homogeneity and crystallization of proteins. *Acta Cryst (2004). D60, 1670-1673* (2004)

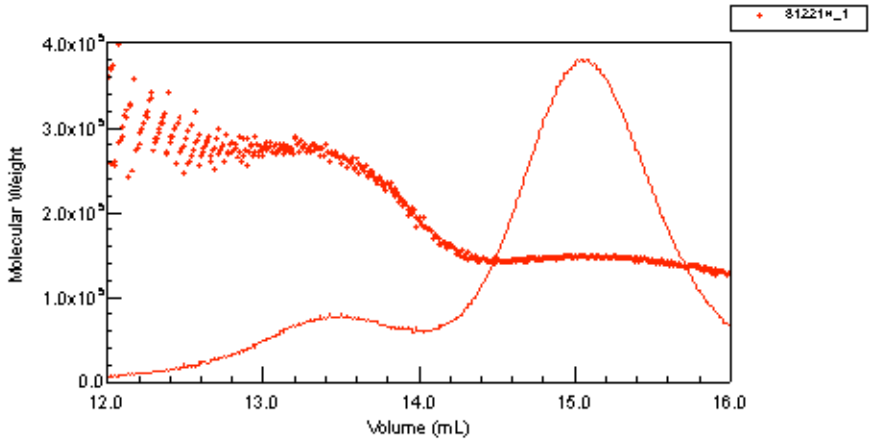
# Protein characterization

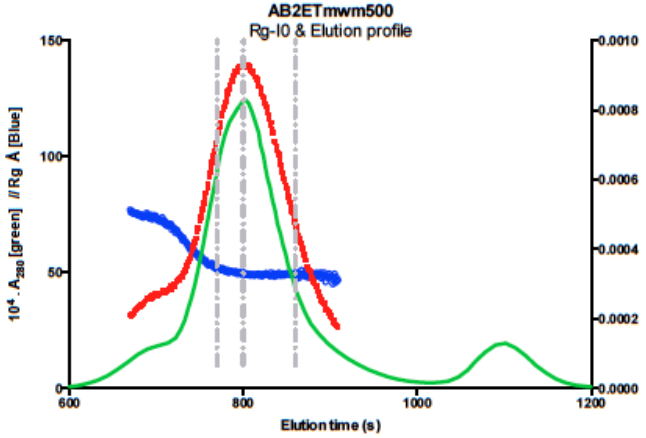
Analytical gel filtration
SEC-MALS
SEC-SAXS



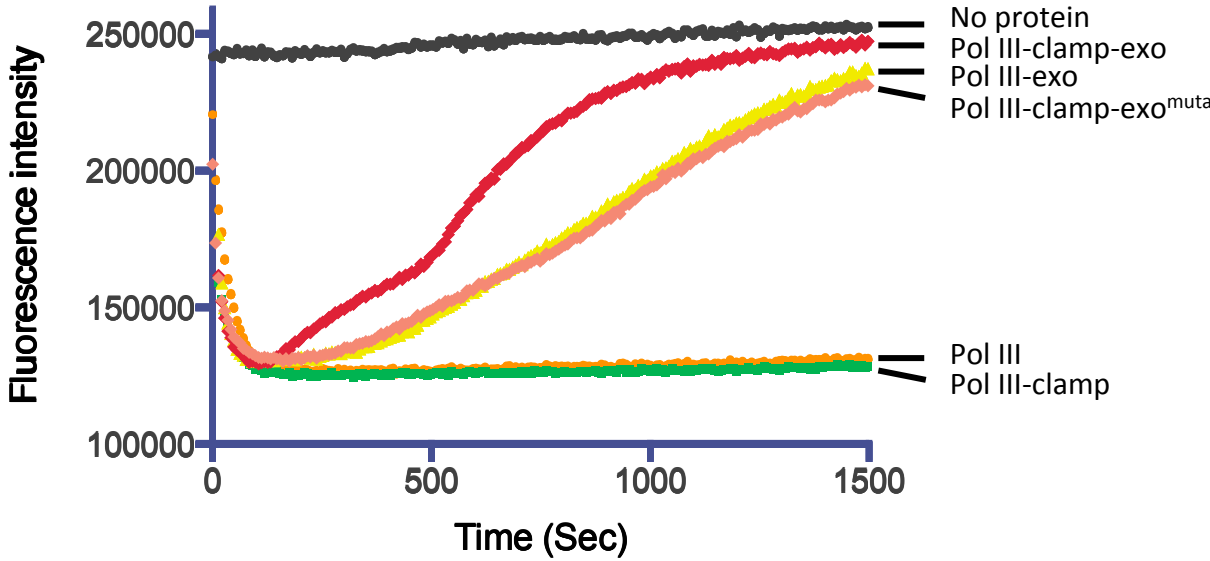



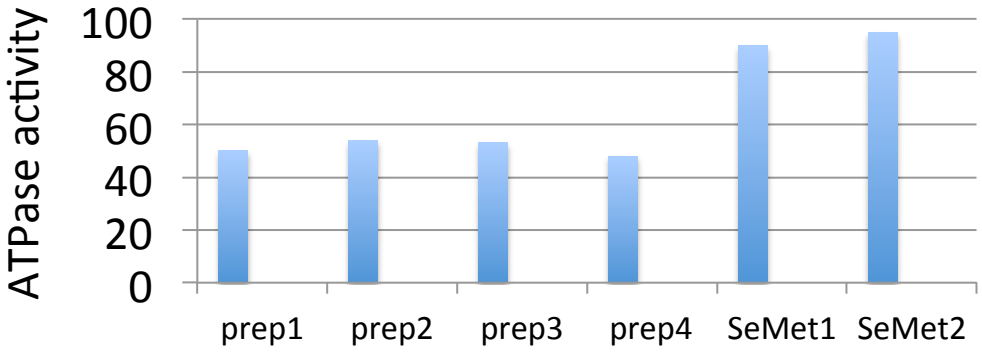



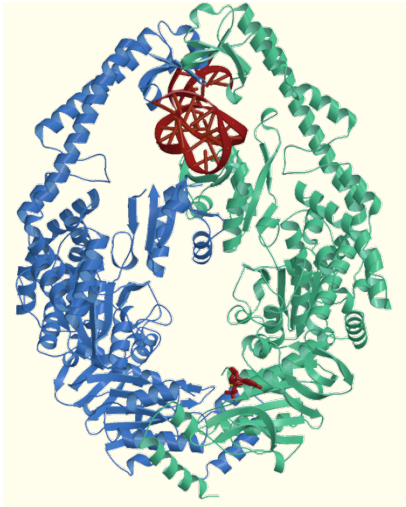



# Protein characterization

Activity assay (Semet MutS)

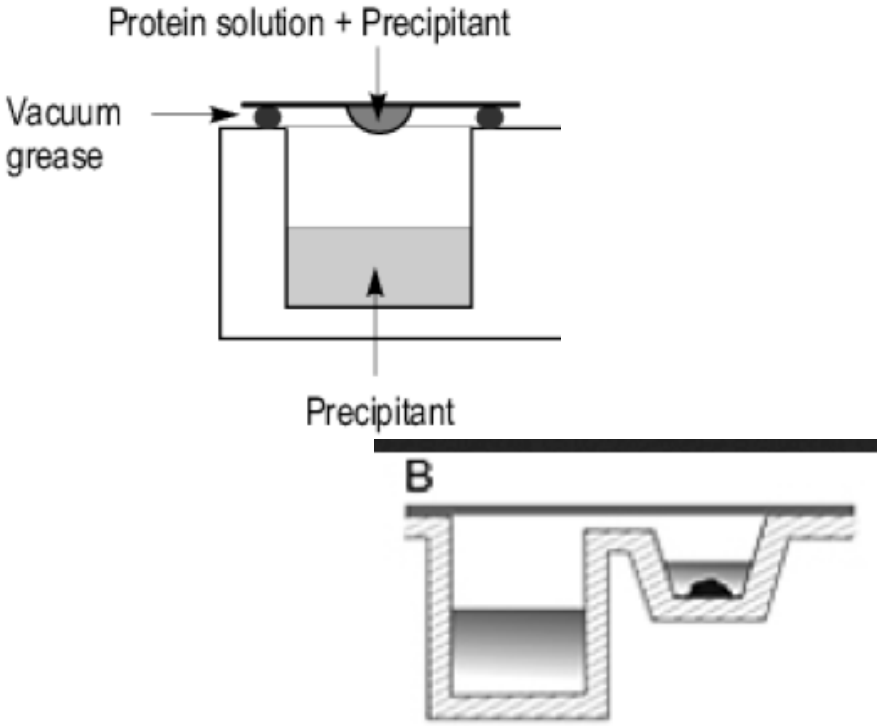


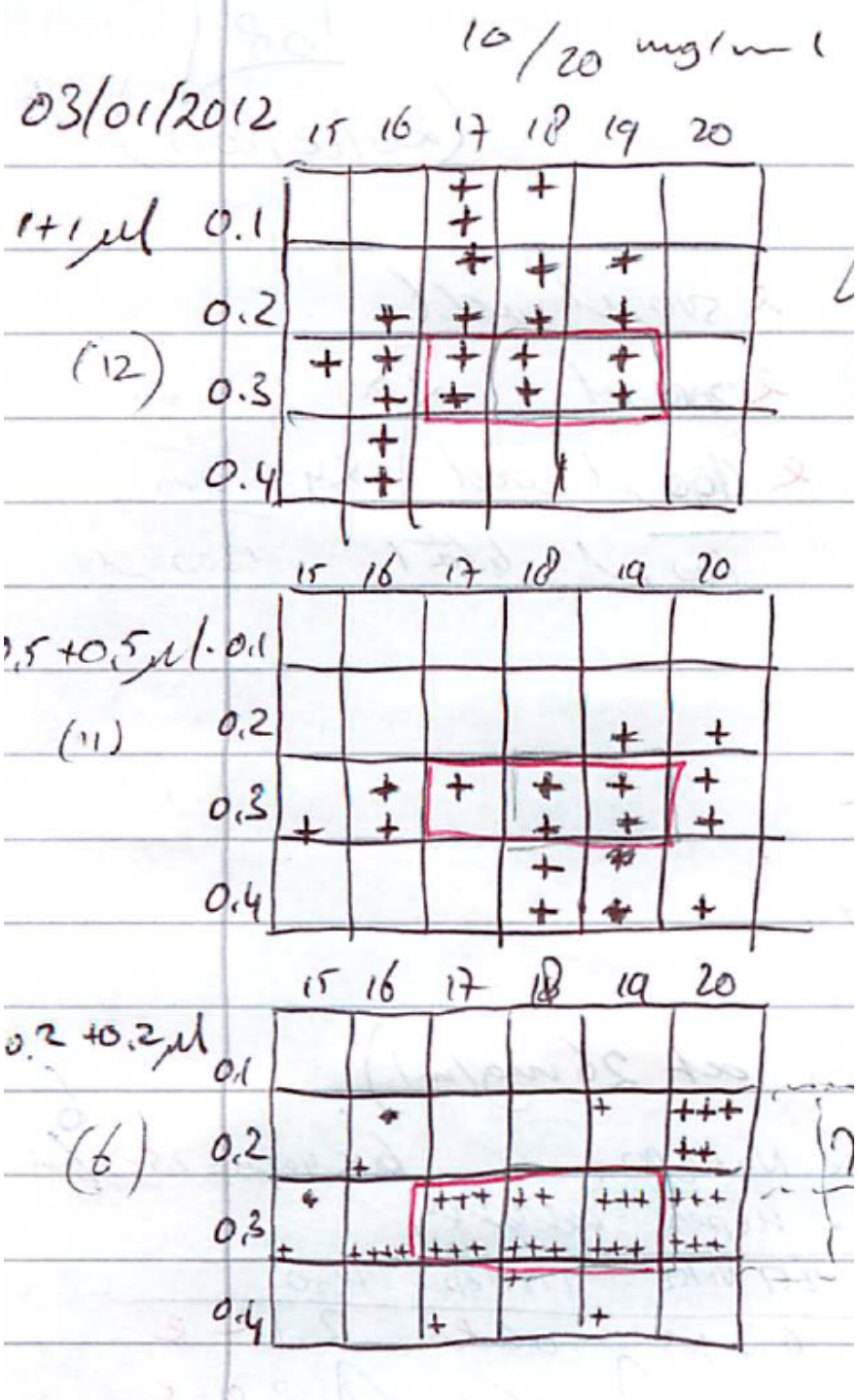



# Protein crystallization

Are robotic approaches the best way?

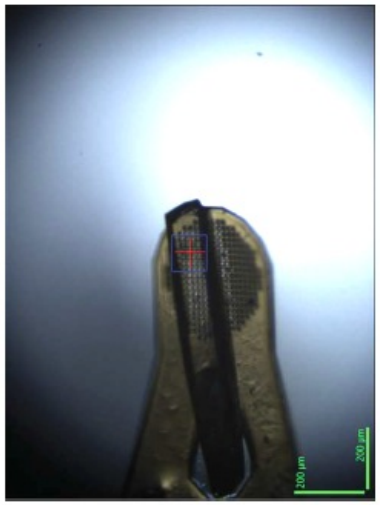


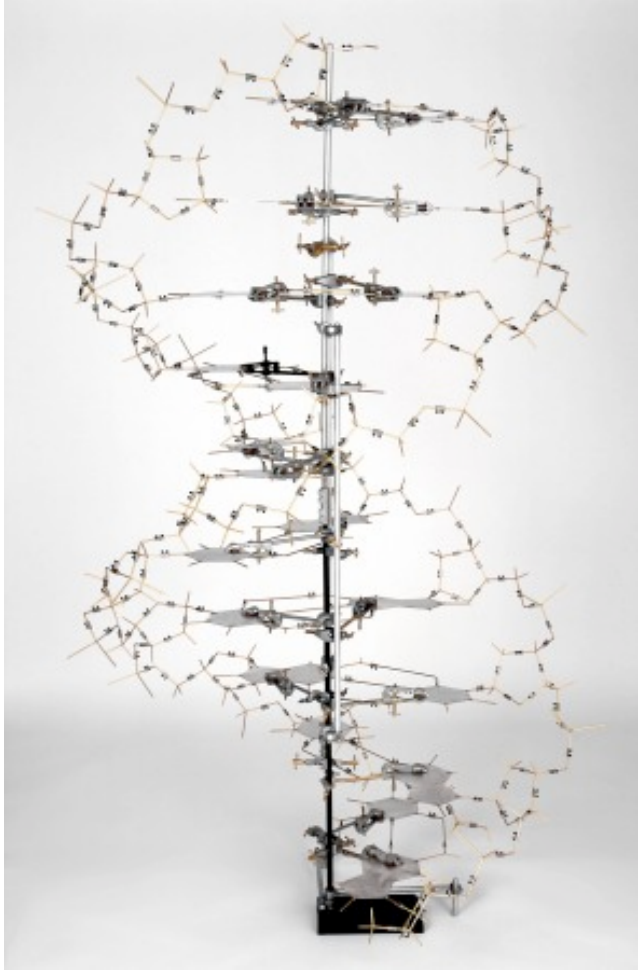

Diller DJ, Hol WG. An accurate numerical model for calculating the equilibration rate of a hanging-drop experiment. Acta Crystallogr D Biol Crystallogr. 1999 Mar;55(Pt 3):656-63. PubMed PMID: 10089462.

# Cryocrystallography (freezing)

Principles of cryocrystallography (1)
Freeze in liquid nitrogen or gas stream?
Beware of the layer of cold air over the liquid nitrogen (2)
Test crystal at room temp: Mitegen crystal sleeve: MicroRT
Test different cryos & precipitant concentrations
Remove any mother liqour (3)
Dehydrate with PEGs
Dehydrate with controlled humidified air (4)




1. Garman, E. F. & Schneider, T. R. Macromolecular Cryocrystallography. *J. Appl. Cryst (1997). 30, 211-237*
2. Warkentin, M., Berejnov, V., Husseini, N. S. & Thorne, R. E. Hyperquenching for protein cryocrystallography. *J Appl Crystallogr* **39,** 805–811 (2006).
3. Pellegrini, E., Piano, D. & Bowler, M. W. Direct cryocooling of naked crystals: are cryoprotection agents always necessary? *Acta Cryst (2011). D67, 902-906*
4. Russi, S. *et al.* Inducing phase changes in crystals of macromolecules: status and perspectives for controlled crystal dehydration. *J. Struct. Biol.* **175,** 236–243 (2011).



First DNA structure, 1953
(Watson & Crick)

First protein structure: Haemoblobin 1959
(Perutz & Kendrew)

In a difficult project you're not going to succeed because you do the same as everyone else, but because you do something **different**…

# Recommended reading:

**Ligation Independent Cloning**
Aslanidis C, de Jong PJ. Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. 1990 Oct 25;18(20):6069-74. PubMed PMID: 2235490

**Protein expression**
Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41,** 207–234 (2005).

**Protein enginering**
Derewenda ZS. Application of protein engineering to enhance crystallizability and improve crystal properties. Acta Crystallogr D Biol Crystallogr. 2010 May;66(Pt 5):604-15. PubMed PMID: 20445236
SERP server http://services.mbi.ucla.edu/SER/

**Protein purification**
www.gelifesciences.com > Service & support > Handbooks

**Hanging drop diffusion**
Diller DJ, Hol WG. An accurate numerical model for calculating the equilibration rate of a hanging-drop experiment. Acta Crystallogr D Biol Crystallogr. 1999 Mar;55(Pt 3):656-63. PubMed PMID: 10089462

**Cryo crystallography**
Garman, E. F. & Schneider, T. R. Macromolecular Cryocrystallography. *J. Appl. Cryst (1997). 30, 211-237*

Warkentin, M., Berejnov, V., Husseini, N. S. & Thorne, R. E. Hyperquenching for protein cryocrystallography. *J Appl Crystallogr* **39,** 805–811 (2006)

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*Russi, S et al.* Inducing phase changes in crystals of macromolecules: status and perspectives for controlled crystal dehydration. *J. Struct. Biol.* **175**, 236-243 (2011)

## When all goes well…