

Microseed Matrix Screening Crystallization of Antibody Fragments and Antibody-Antigen Complexes

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Microseed-Matrix Screening (MMS) is an extension of conventional seeding techniques where crystals grown in one set of conditions are systematically seeded into new conditions as part of the screening or optimization procedure.

Ireton, G. C. & Stoddard, B. L. (2004). *Acta Cryst.* D60, 601-605

D'Arcy, A., Villard, F., Marsh M. (2007). *Acta Cryst.* D63, 550-554

Outline

Centocor R&D:

3D structure applications
crystallization instrumentation

In-house screens and MMS

MMS applications

Project 1: human germline Fabs

Project 2: Fab-antigen complex

Project 3: TLR3 quaternary complex with 3 Fabs

Summary

3D structure applications in antibody drug design:

- antibody engineering:
 - structure based antibody humanization and affinity maturation
 - developability
- understanding mechanism of action
- epitope mapping

Centocor crystallization instrumentation



Optimization screens preparation



Plate re-formatting

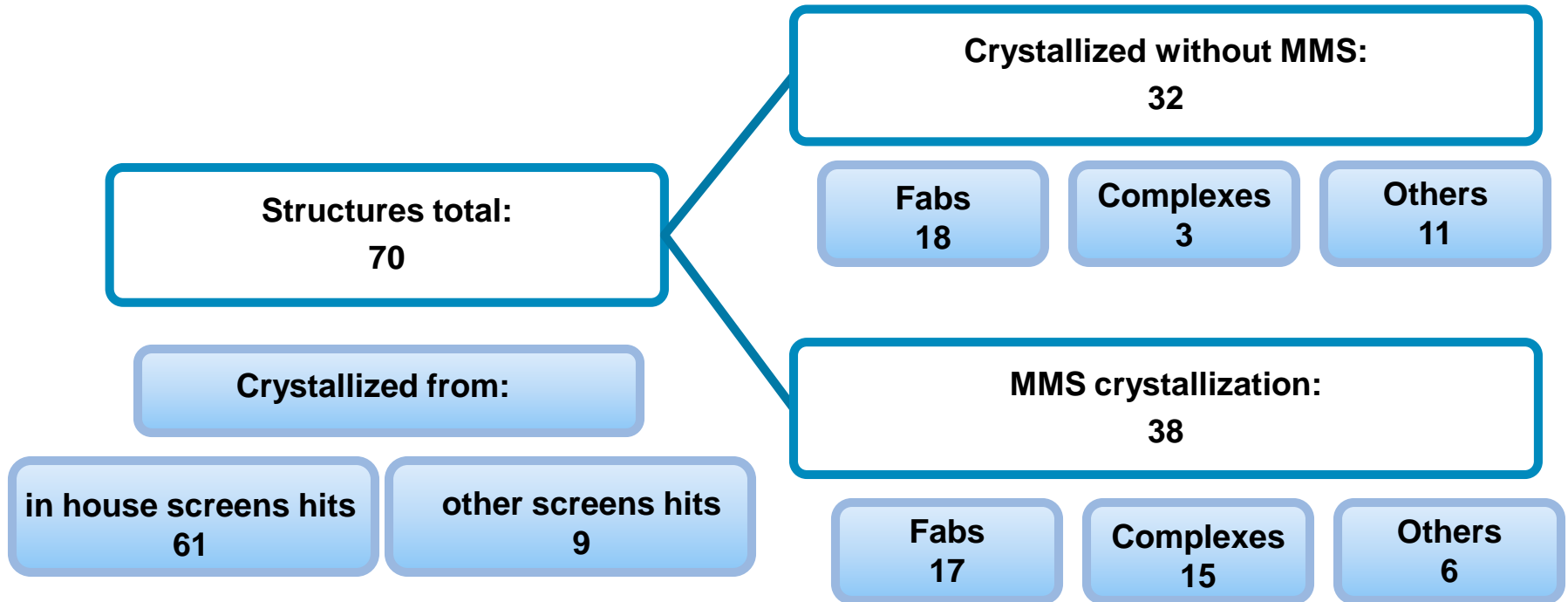


Crystallization set ups



Incubation / imaging

Centocor Crystallization Summary



In house crystallization screens and hit frequency

Screen 1:

pH precipitate	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5
PEG 8000							*	
AmmSul								
AmmSul/Add								
NaForm								
NaForm/Add								

Color intensity - hit frequency

* no hits

Screen 2:

salts/ pH	4.5	6.5	7.5	8.5		No buffer
AmmAcet			*	*	ZnAcet	
NaAcet					CaAcet	
AmmCl			*	*	AmCitr	
LiCl					K/NaCitr	
LiSul			*		AmmTart	
AmmSul			*		K/NaTart	
MgCl					Phosphates	
NaForm			*		MgAc	*
LiCl 1 M					NaSCN	
NaAcet 1 M	*					

Screen 1:

PEG 8000: 18-25-30 % w/v
 Amm Sulfate: 1.5 -2.0-2.4 (2.8) M
 NaFormate: 2.5-5.8 M
 Additives MPD, PEG 400, isopropanol

Screen 2:

PEG 3350: 18-25% w/v
 Salts: 0.2 M
 NaAcetate, LiCl 0.2 M & 1 M

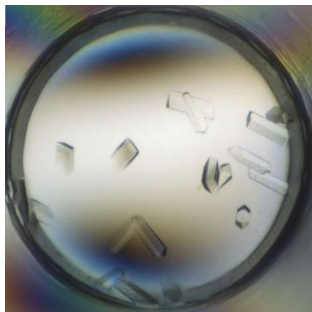
In-house screens: multiple AmSO4 conditions increase success of MMS

Fab 5-51/A27 MMS cross seeding results:

In house screen 1:

CHES pH 9.5, AmmSO4

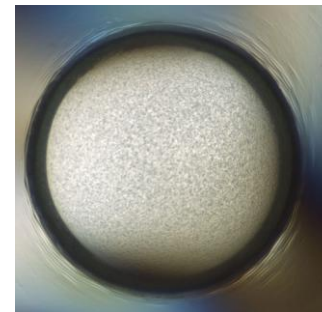
2.0 M



2.4 M



2.8 M



Crystallization of Fab fragments with human germline sequences (HGS)

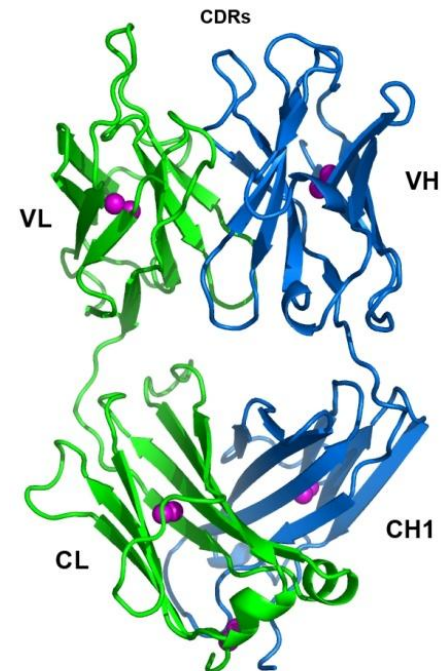
VH \ VL	B3	A27	L6	O12
1-69	Yellow	Grey	Yellow	Grey
3-23	Yellow	Teal	Yellow	Magenta
3-53	Teal	Magenta	Teal	Magenta
5-51	Teal	Teal	Magenta	Magenta

- Crystallization without MMS optimization
- MMS
- MMS cross-seeding
- No crystallization hits

Project to provide template structures to support antibody modeling and humanization

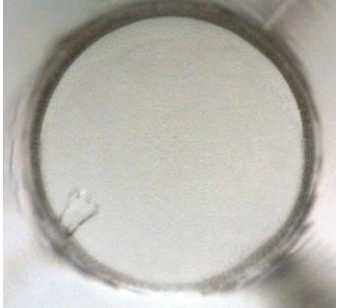
Fab fragments:

- V_H/V_L : human germline sequences
- V_H -CDR3: fixed sequence
- Constant domains: hIgG1/k isotype



MMS crystallization optimization

Fab 3-53/B3



Screen: in house 2 (PEG/salts)

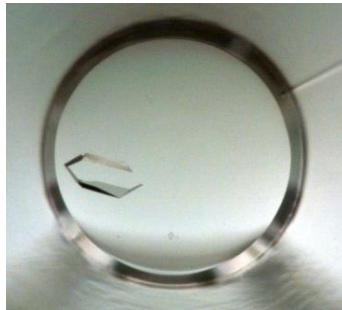
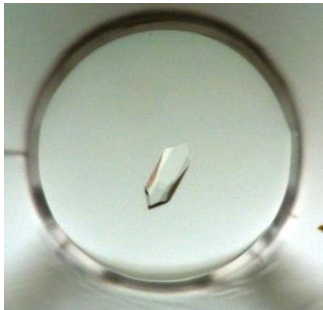
— **Screening results:** clusters

Seeds: mixed crystals from:

pH 6.5- 7.5

PEG 3350

0.2 M NaAcetate, LiCl, and Na Formate



MMS optimization screen

X-ray quality crystals were obtained from:

MES pH 6.5

16% PEG 3350

0.2 M NaFormate

MMS improvement of crystal morphology

Fab 5-51/B3

Screens: in house 1&2

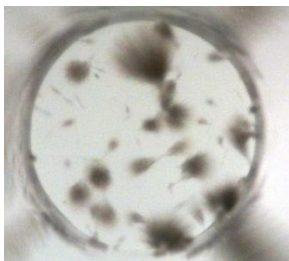
— **Screening results:** needles

Seeds mix

pH 6.5, 8.5

PEG 3350 and PEG 8000

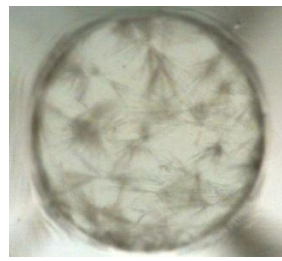
0.2 M AmmAcetate, LiSO₄ or LiCl



pH 8.5 PEG 3350
0.2 M AmmAcetate

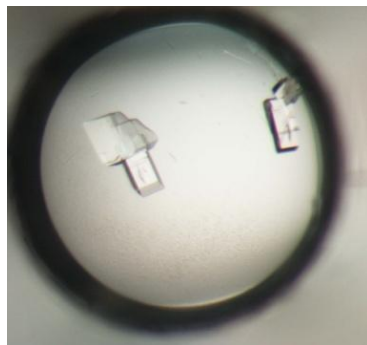


pH 6.5 PEG 8000



pH 8.5 PEG 3350
0.2 M LiSO₄

MMS optimization with in house screen 2 (PEG/salts)



pH 6.5 PEG 3350 0.2 M AmmAcetate

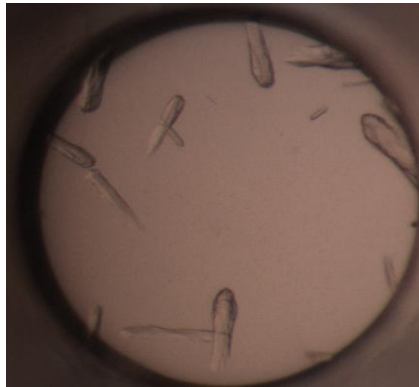


pH 8.5 PEG 3350 0.2 M AmmAcetate



pH 7.5 PEG 3350 0.2 M AmmCl

MMS optimization with seed dilution



Crystals used for seed stock preparation

MMS screening
→
undiluted seeds



50x diluted seeds
→



Oryx 2 drops set up – “effective” seeds dilution

Fab 3-23/A27

Drop 1: protein + reservoir + seed stock

Drop 2: protein + reservoir + residual seeds (“effective” seed dilution)

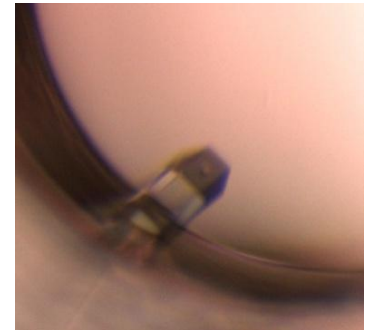
Without seeding



Drop 1: seeding



Drop 2: “seed dilution”



MMS cross-seeding: multiple seeds

Fab 5-51/O12

Ammonium sulfate derived seeds (AS):

Fab 3-23/L6 2.4 M AmmSO₄, Acetate pH 4.5
Fab 3-53/A27 2.0 M AmmSO₄, Acetate pH 4.5
Fab **5-51**/A27 2.0 M AmmSO₄, Tris pH 8.5, 5% PEG 400

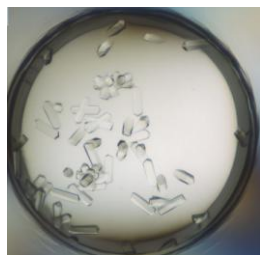
PEG derived seeds:

Fab 3-53/B3 16% PEG 3350, MES pH 6.5, 0.2 M NaFormate
Fab 5-51/B3 20% PEG 3350, Tris pH 8.5, 0.2 M AmmAcetate
Fab 3-23/A27 18% PEG 3350, MES pH 6.5, 0.1 M LiCl

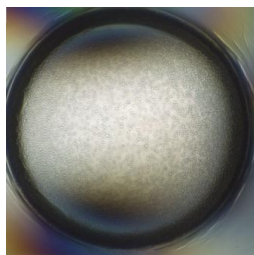
Crystallization conditions:

AmmSO₄, CHES pH 9.5, 5% dioxane

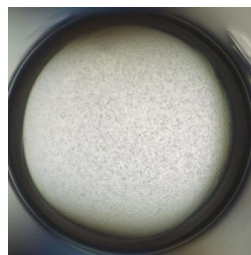
Cross-seeding with multiple seeds:



AS seed mix

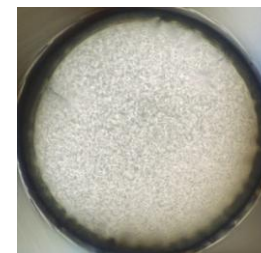


AS & PEG seed mix

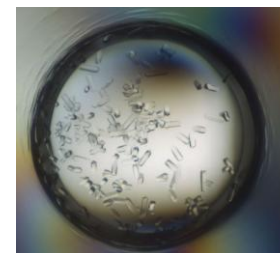


AS seed mix w/o
Fab 5-51/A27

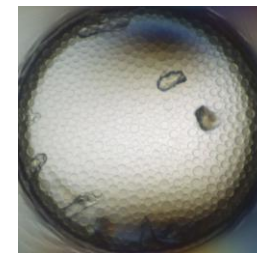
Without seeding



Fab 5-51/A27 seeds



Fab 5-51/A27 seeds 3x diluted with
20% PEG 3350, Tris pH 8.5



Cross-seeding: MMS stimulated hit formation

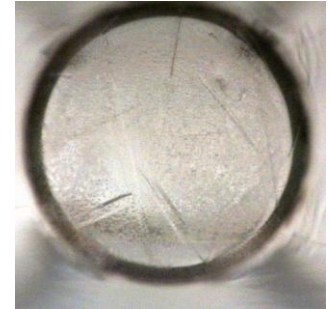
Fab 3-53/A27

Cross – seeding:

seeds Fab 3-53/B3 – the same V_H - no hits

seeds Fab 3-23/A27 – the same V_L - one hit – Hampton #C7

30% PEG 4000
0.2M AmmSO4



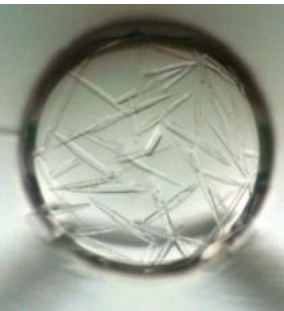
New self-seed stock



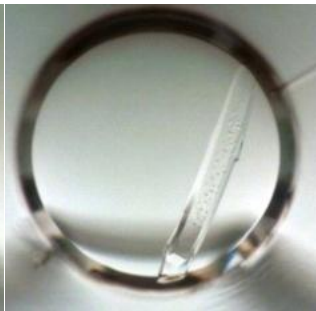
MMS, repeated with the same screens



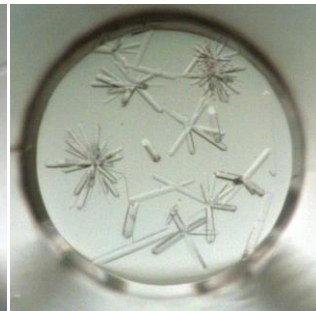
Results: new hits



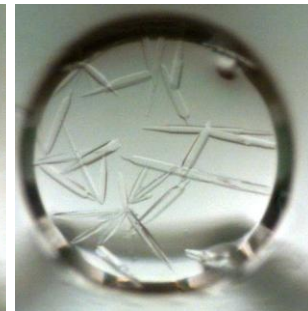
IH1: pH 3.5 PEG 8000



IH1: pH 7.5 PEG 8000



IH2: pH 4.5 PEG 3350
0.2 M AmmSO4



IH2: pH 4.5 PEG 3350
0.2 M LiCl



IH2: PEG 3350
1 M LiCl



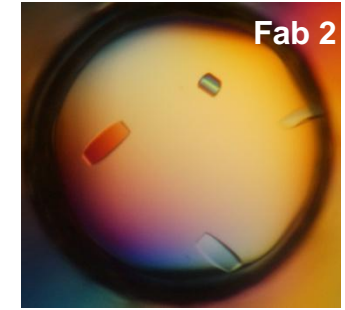
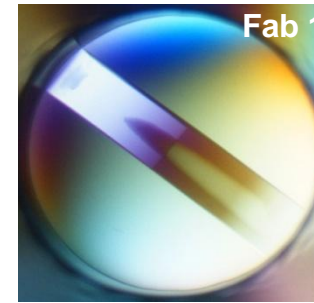
Hampton HT #C2:
pH 4.5 AmmSO4

Antigen / Fab co-crystallization

Antigen characteristics: highly glycosylated human antigen. Baculovirus expression to eliminate O-glycosylation.

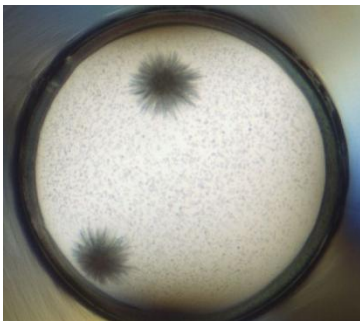
3 C-terminal truncations: A85, A100 (one site with N-glyc.), A110 (2 sites with N-glyc.)

3 Variants with 2 non-competing Fabs.
7 out of 9 possible combinations tested



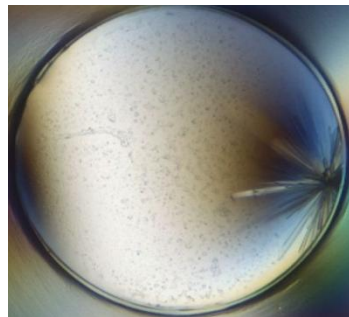
X-ray crystal structures of 2 Fabs solved.

A-100 / Fab 1 crystallization hits from initial screening

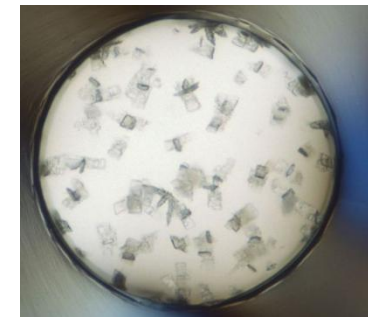


Ammonium sulfate,
pH 6.5 or 9.5

Seed stock 1



PEG 3350, 0.2 M
different salts

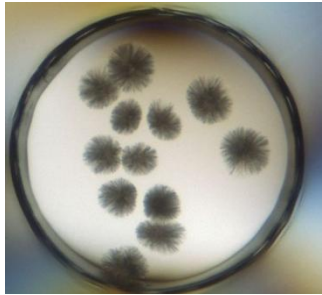


Diferent MW PEGs,
pH 6.5

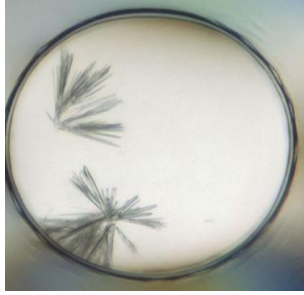
Seed stock 2

Crystallization optimization

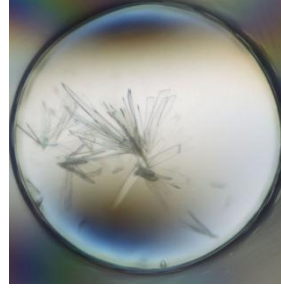
MMS to narrow crystallization conditions



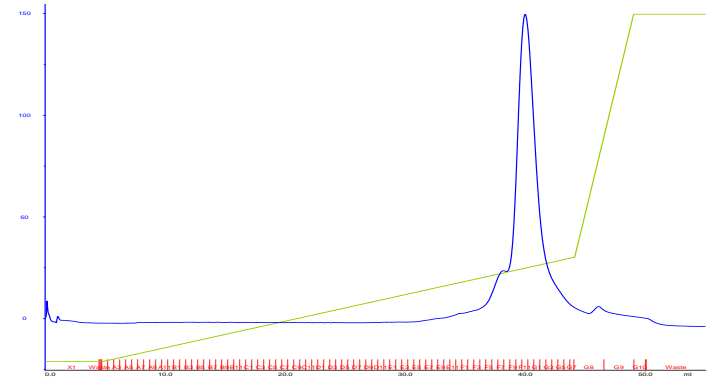
Ammonium sulfate



PEG 4000

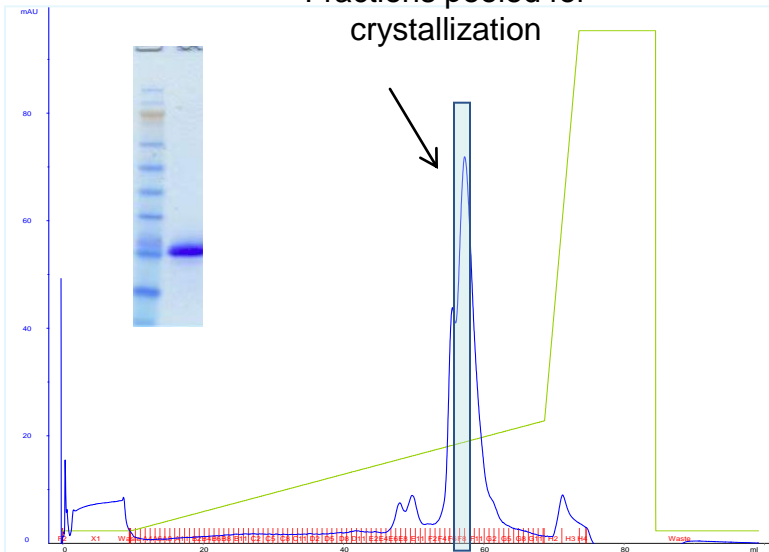


PEG 4000, 0.2 M Na Phosphate



A100/ Fab1 complex Mono S purification test

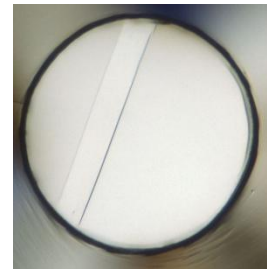
Fractions pooled for crystallization



A100 Mono S purification

Complex prep with Fab1

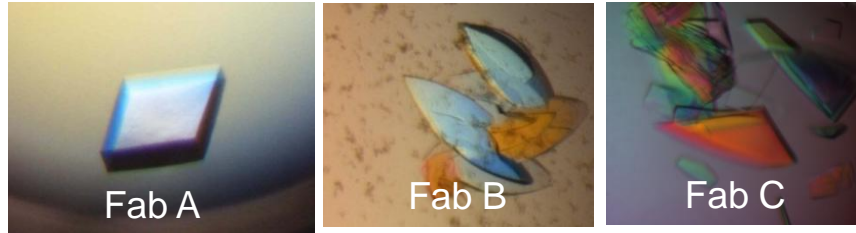
MMS
PEG/salts conditons



A100/Fab1 crystal
20% PEG 3350, 0.2 M Ammonium Citrate

TLR3 ECD/ anti TLR3 Fabs co-crystallization

High resolution X-ray crystal structures of 3 non-competing anti-TLR3 Fabs



TLR3 ECD source: baculovirus/Sf9 expression.

Systematic search for crystallizable TLR3 complex included:

Complex formation

SEC purification

Crystallization screening

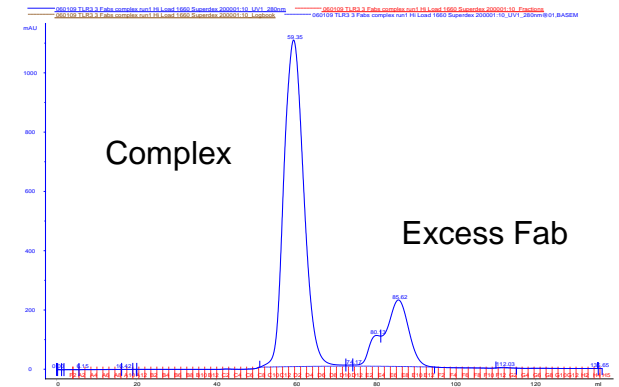
Variants tested:

TLR3 ECD/ one Fab – 3 combinations

TLR3 ECD/ two Fabs - 3 combinations

TLR3 ECD/FabA/FabB/FabC

SEC purification of TLR3/Fabs complexes

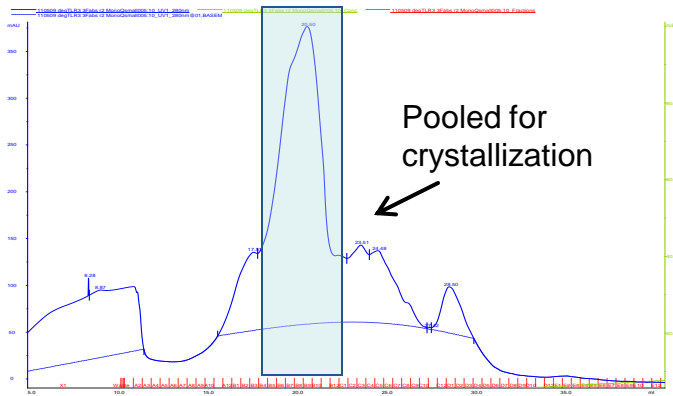


The most promising initial crystallization screening result

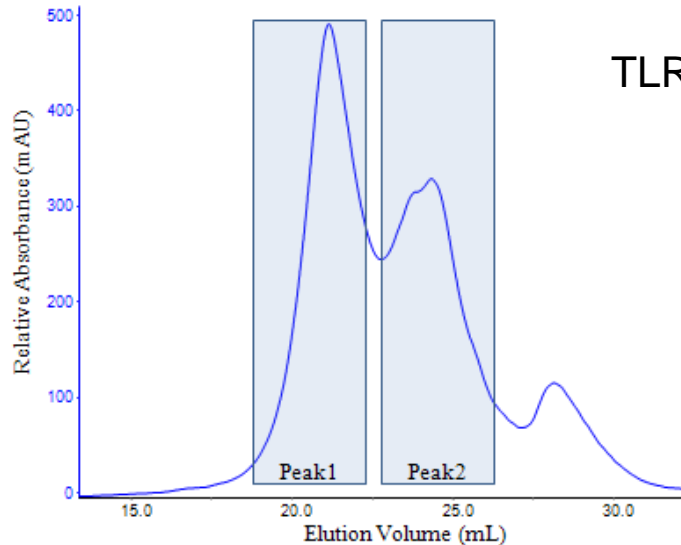
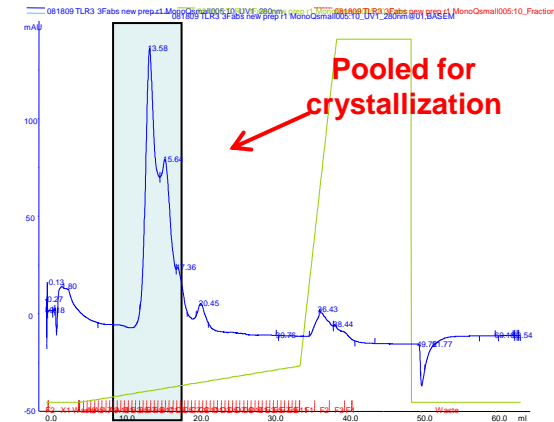
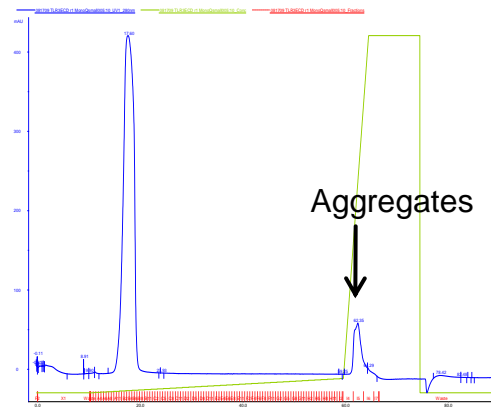
TLR3 ECD/ three Fabs

Purification as a refinement tool for TLR3/Fab crystallization.

TLR3 deglycosylation, complex preparation, IEX



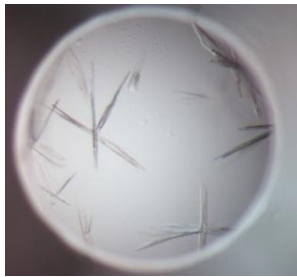
TLR3 IEX, complex preparation, IEX complex purification



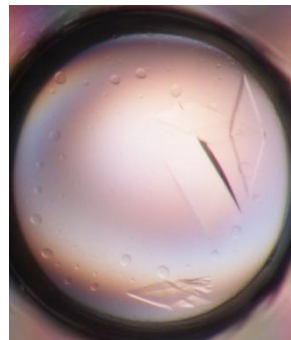
TLR3+3 Fab complex purification by anion exchange with a shallow gradient

Peaks 1 and 2 were pooled, concentrated and setup in crystallization trials separately.

TLR3 + 3 Fabs crystallization refinement



MMS refinement



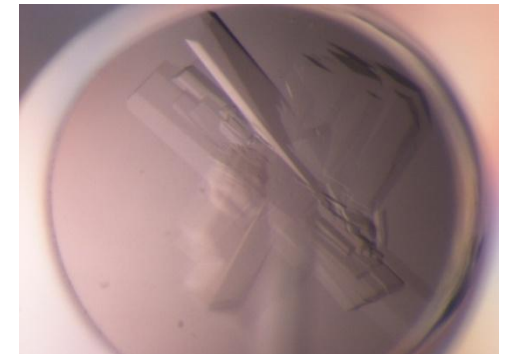
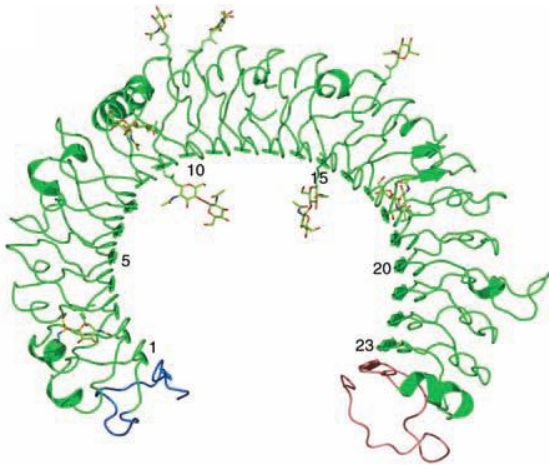
TLR3+3Fab peak1 crystal: weak diffraction
Acetate pH 4.5, 26% PEG 3350, 1 M LiCl



Peak1 seeding and additive screening
(Hampton additive screen, selected conditions)

TLR3+3Fab first crystals:
Acet pH 4.5, 2.4 M AmmSO₄,
5% PEG400

Crystals of TLR3+3Fab peak1 complex:
0.1 M sodium acetate pH 4.5, 28% PEG
3350, 1 M LiCl, and 30 mM Gly-Gly-Gly.



Ribbon representation of the TLR3 structure

Choe, J., Kelker, M. S. & Wilson, I. A. (2005). Science 309, 581.

Summary

Microseed matrix seeding:

Increase the number of hits

Improve crystal morphology and diffraction quality

Minimize optimization time

X-ray quality crystals often can be obtained without additional optimization

Seeding (cross-seeding) can be fully automated

Highly reproducible

Acknowledgements

Thomas Malia

Protein expression & purification

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Juan Carlos Almagro

Ray Sweet

Gary Gilliland