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

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centocor research & development
a division of Johnson & Johnson Pharmaceutical Research & Development, L.L.C.

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

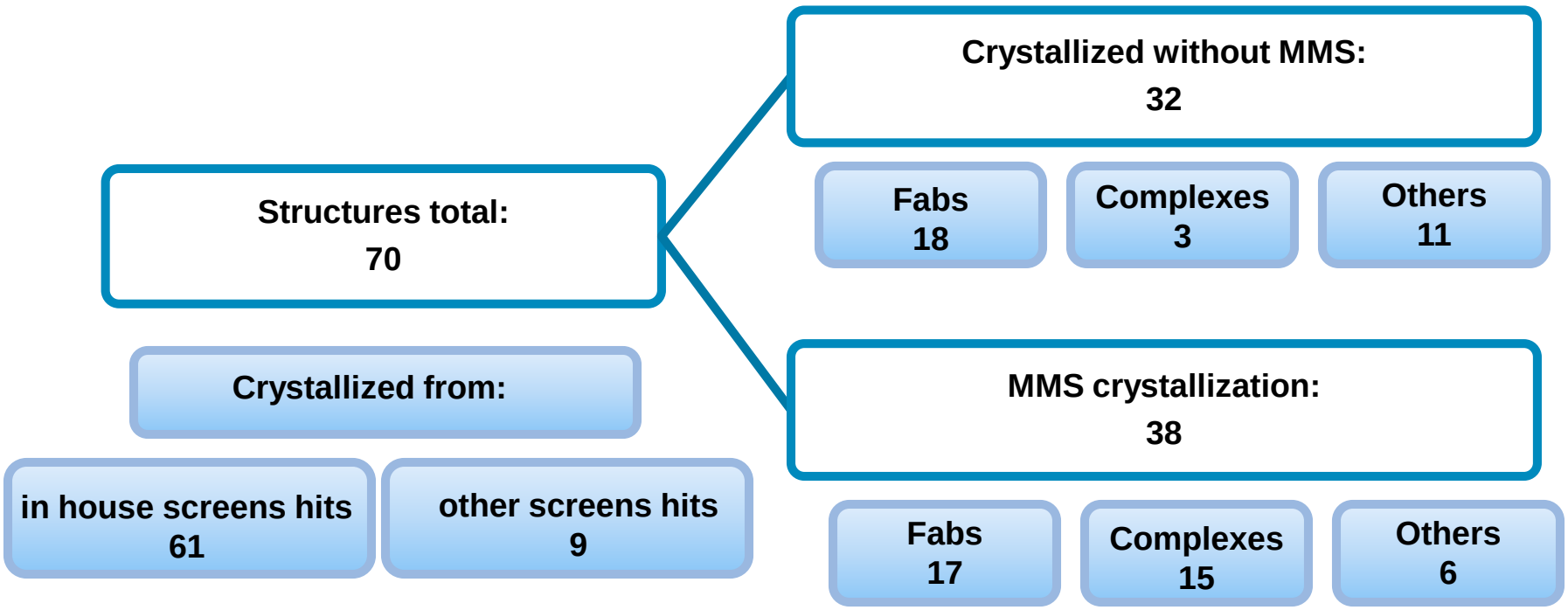
Optimization screens preparation

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
AmmAcet			*	*
NaAcet				
AmmCl			*	*
LiCl				
LiSul			*	
AmmSul			*	
MgCl				
NaForm			*	
LiCl 1 M				
NaAcet 1 M	*			

	No buffer
ZnAcet	
CaAcet	
AmCitr	
K/NaCitr	
AmmTart	
K/NaTart	
Phosphates	
MgAc	*
NaSCN	

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

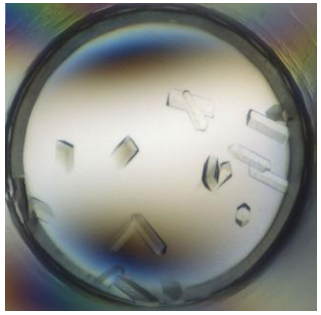
In-house screens: multiple AmSO4 conditions increase success of MMS

Fab 5-51/A27 MMS cross seeding results:

In house screen 1:

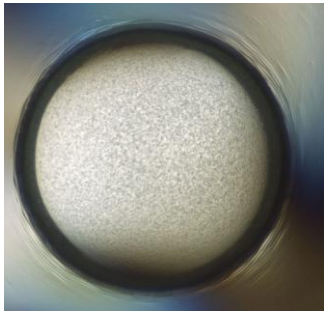
CHES pH 9.5, AmmSO4

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				
3-23				
3-53				
5-51				

Crystallization without MMS optimization

MMS

MMS cross-seeding

No crystallization hits

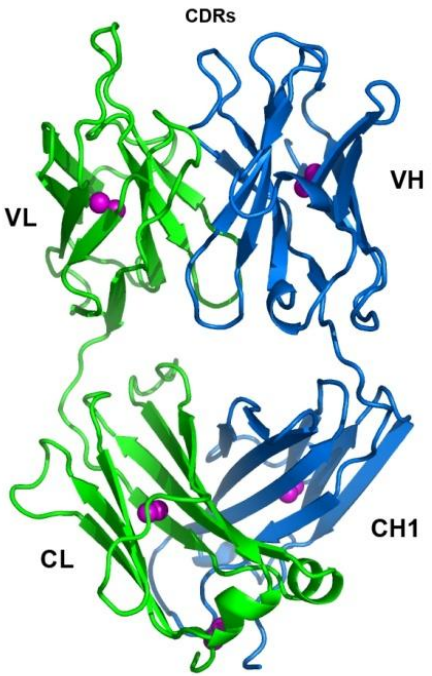
Project to provide template structures to support antibody modeling and humanization

Fab fragments:

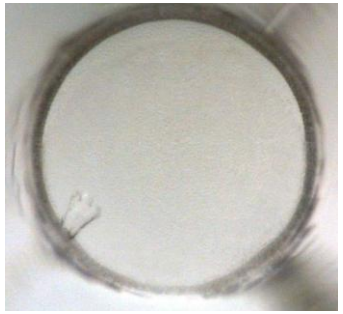
V_H/V_L: human germline sequences

V_H-CDR3: fixed sequence

Constant domains: hIgG1/k isotype



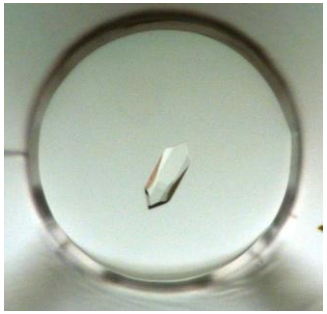
MMS crystallization optimization
Fab 3-53/B3

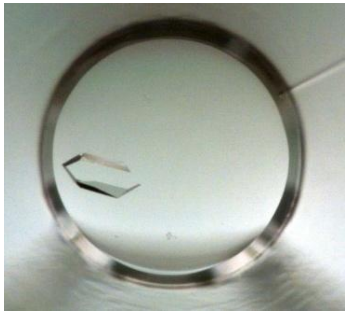


Screen: in house 2 (PEG/salts)

Screening results: clusters

Seeds: mixed crystals from:
pH 6.5- 7.5
PEG 3350
0.2 M NaAcetate, LiCl, and Na Formate

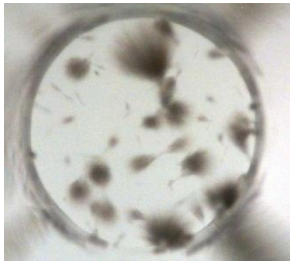




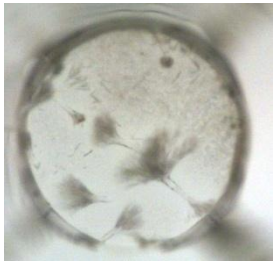
MMS optimization screen

X-ray quality crystals were obtained from:
MES pH 6.5
16% PEG 3350
0.2 M NaFormate

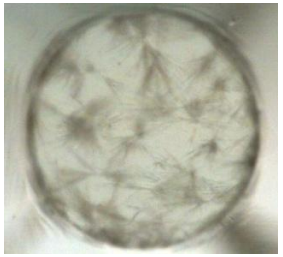
MMS improvement of crystal morphology
Fab 5-51/B3



pH 8.5 PEG 3350
0.2 M AmmAcetate



pH 6.5 PEG 8000



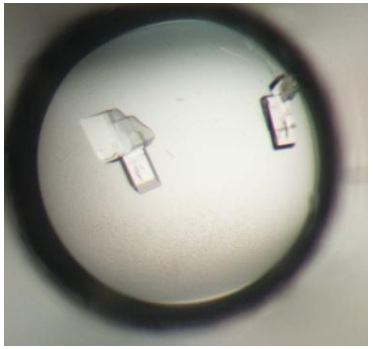
pH 8.5 PEG 3350
0.2 M LiSO4

Screens: in house1&2

Screening results: needles

Seeds mix
pH 6.5, 8.5
PEG 3350 and PEG 8000
0.2 M AmmAcetate, LiSO4 or LiCl

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

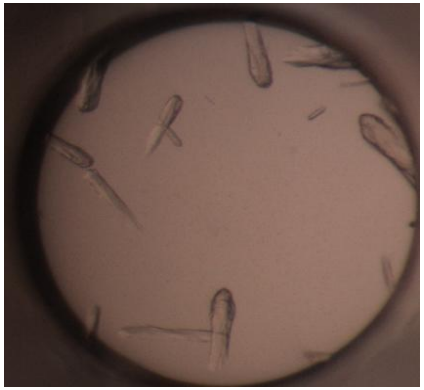


pH 6.5 PEG 3350 0.2 M AmmAcetate

pH 8.5 PEG 3350 0.2 M AmmAcetate

pH 7.5 PEG 3350 0.2 M AmmCl

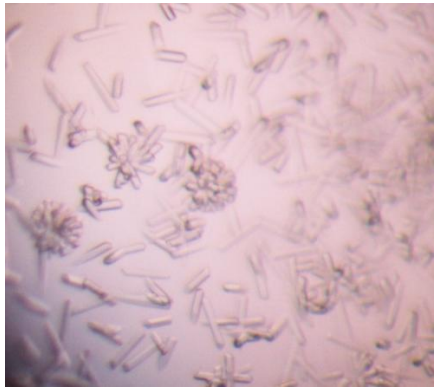
MMS optimization with seed dilution



Crystals used for seed stock preparation

MMS screening

undiluted seeds



50x diluted seeds

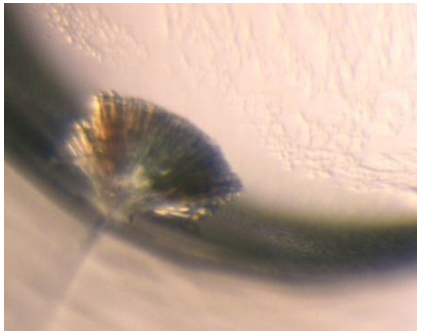
Oryx 2 drops set up – “effective” seeds dilution

Fab 3-23/A27

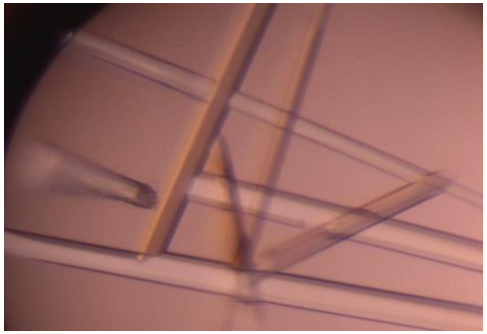
Drop 1: protein + reservoir + seed stock

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

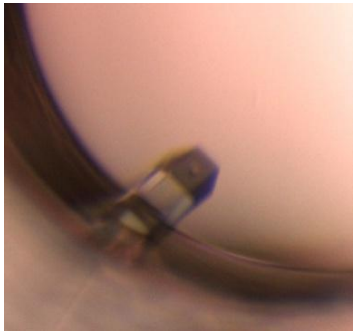
Without seeding



Drop 1: seeding



Drop 2: “seed dilution”



MMS cross-seeding: multiple seeds
Fab 5-51/O12

Ammonium sulfate derived seeds (AS):

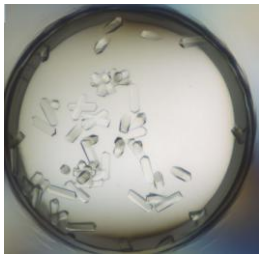
Fab 3-23/L6	2.4 M AmmSO4, Acetate pH 4.5
Fab 3-53/A27	2.0 M AmmSO4, Acetate pH 4.5
Fab **5-51**/A27	2.0 M AmmSO4, Tris pH 8.5, 5% PEG 400

PEG derived seeds:

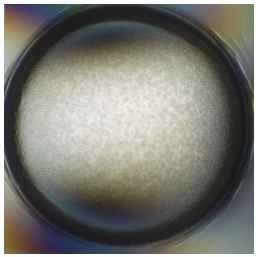
Fab 3-53/B3	16% PEG 3350, MES pH 6.5, 0.2 M NaFormate
Fab 5-51/B3	20% PEG 3350, Tris pH 8.5, 0.2 M AmmAcetate
Fab 3-23/A27	18% PEG 3350, MES pH 6.5, 0.1 M LiCl

Crystallization conditions:
AmmSO4, CHES pH 9.5, 5% dioxane

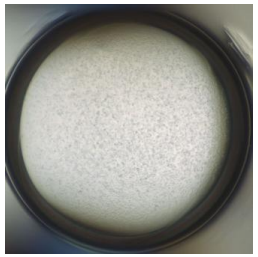
Cross-seeding with multiple seeds:



AS seed mix

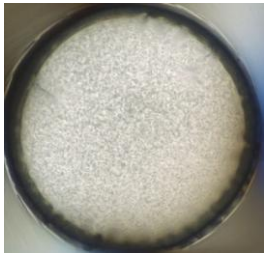


AS & PEG seed mix



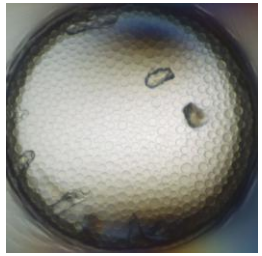
AS seed mix w/o Fab 5-51/A27

Without seeding



Fab 5-51/A27 seeds

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



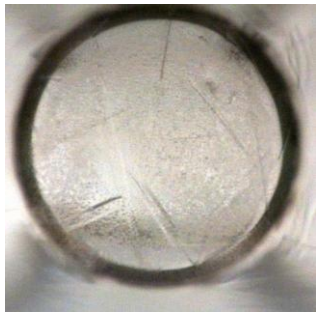
Cross-seeding: MMS stimulated hit formation
Fab 3-53/A27

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



↓

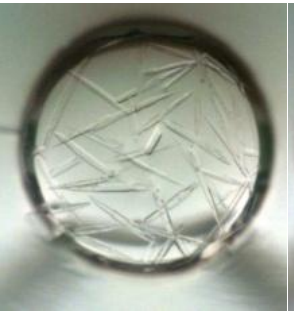
New self-seed stock

↓

MMS, repeated with the same screens

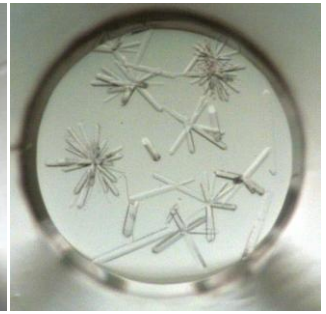
↓

Results: new hits



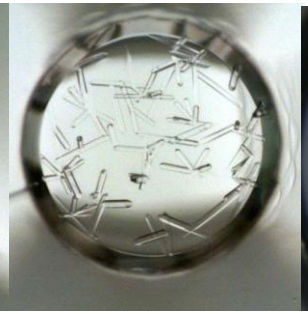
IH1: pH 3.5 PEG 8000

IH1: pH 7.5 PEG 8000

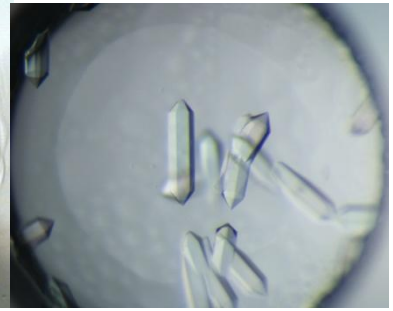


IH2: pH 4.5 PEG 3350
0.2 M AmmSO4

IH2: pH 4.5 PEG 3350
0.2 M LiCl



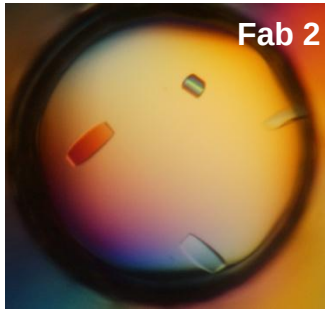
IH2: PEG 3350
1 M LiCl



Hampton HT #C2:
pH 4.5 AmmSO4

Antigen / Fab co-crystallization

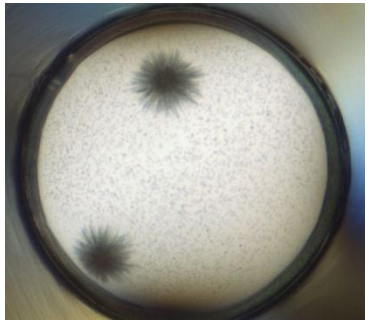
Antigen characteristics: highly glycosylated human antigen. Baculovirus expression to eliminate O-glycosylation.
3 C-terminal truncations: A85, A100 (one site with N-glyc.), A110 (2 sites with N-glyc.)



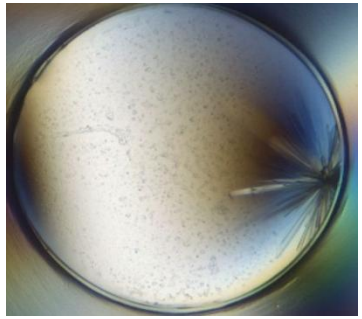
3 Variants with 2 non-competing Fabs.
7 out of 9 possible combinations tested

X-ray crystal structures of 2 Fabs solved.

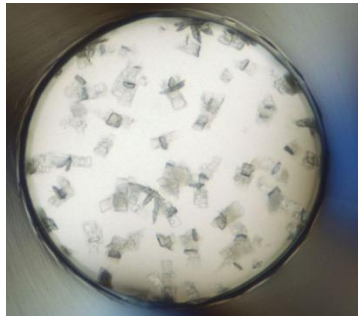
A-100 / Fab 1 crystallization hits from initial screening



Ammonium sulfate,
pH 6.5 or 9.5



PEG 3350, 0.2 M
different salts



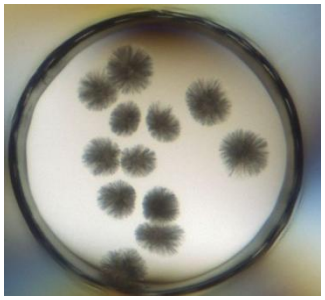
Diferent MW PEGs,
pH 6.5

Seed stock 1

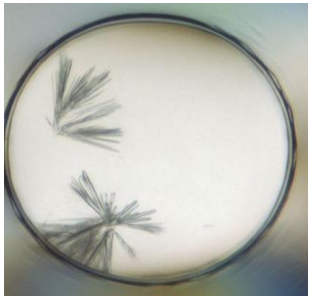
Seed stock 2

Crystallization optimization

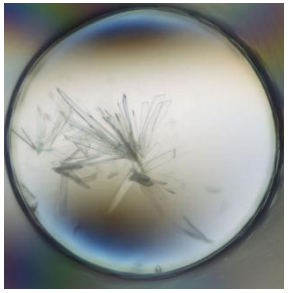
MMS to narrow crystallization conditions



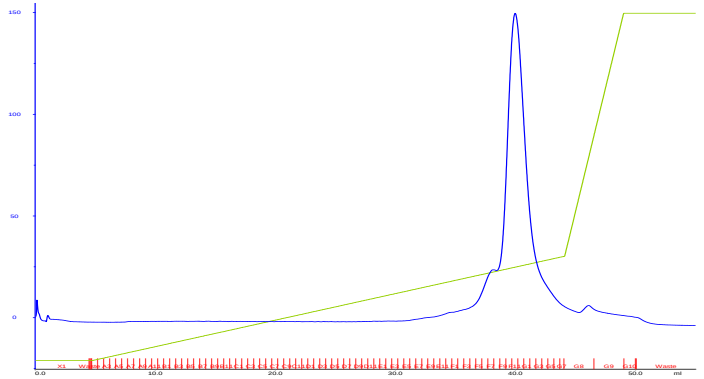
Ammonium sulfate



PEG 4000

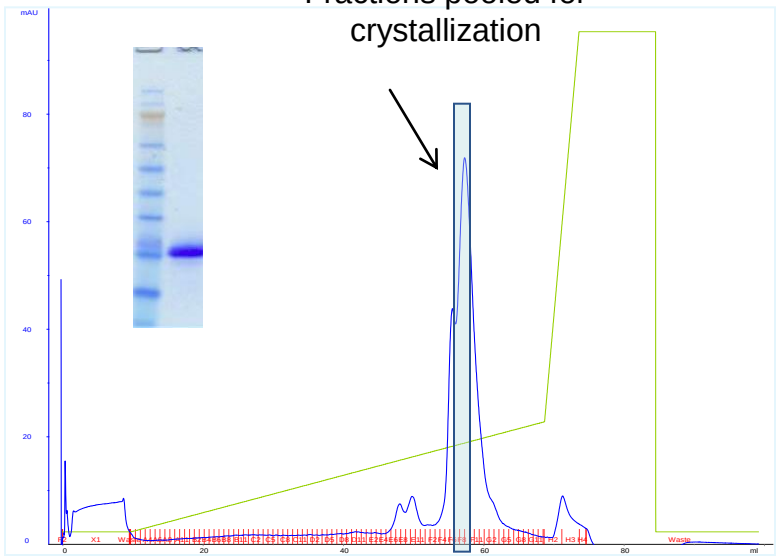


PEG 4000, 0.2 M Na Phosphate



A100/ Fab1 complex Mono S purification test

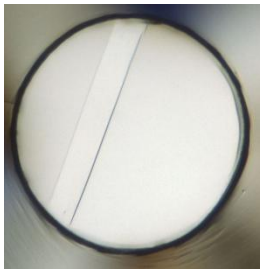
Fractions pooled for crystallization



A100 Mono S purification

Complex prep with Fab1

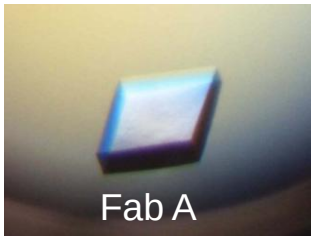
MMS PEG/salts conditons

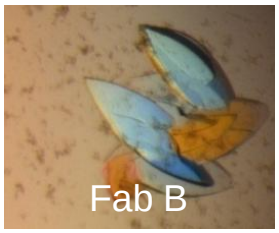


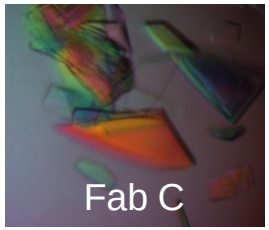
A100/Fab1 crystal
20% PEG 3350, 0.2 M Ammonium Citrate

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.

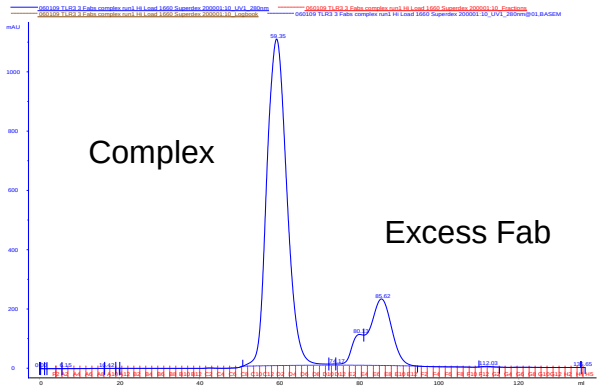
Systematic search for crystallizable TLR3 complex included:
Complex formation
SEC purification
Crystalization screening

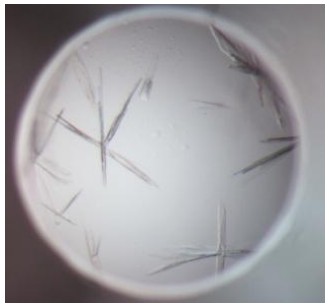
Variants tested:

TLR3 ECD/ one Fab – 3 combinations

TLR3 ECD/ two Fabs - 3 combinations
TLR3 ECD/FabA/FabB/FabC

SEC purification of TLR3/Fabs complexes



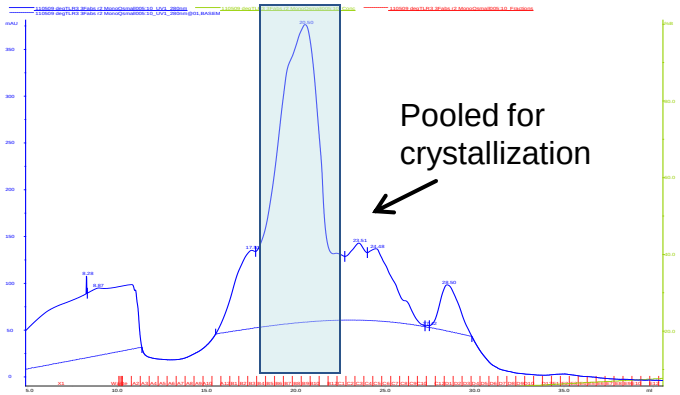


The most promising initial crystallization screening result

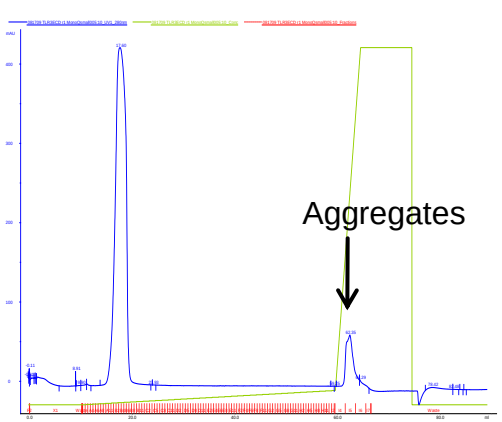
TLR3 ECD/ three Fabs

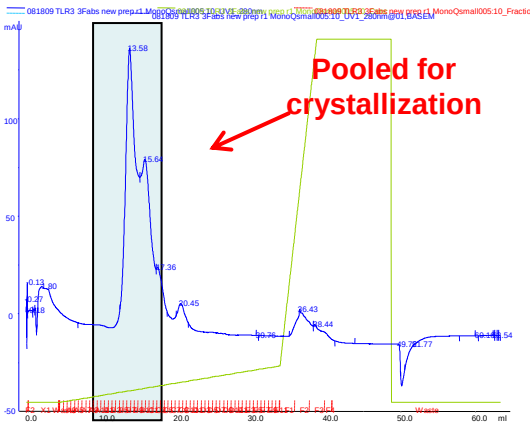
Purification as a refinement tool for TLR3/Fab crystallization.

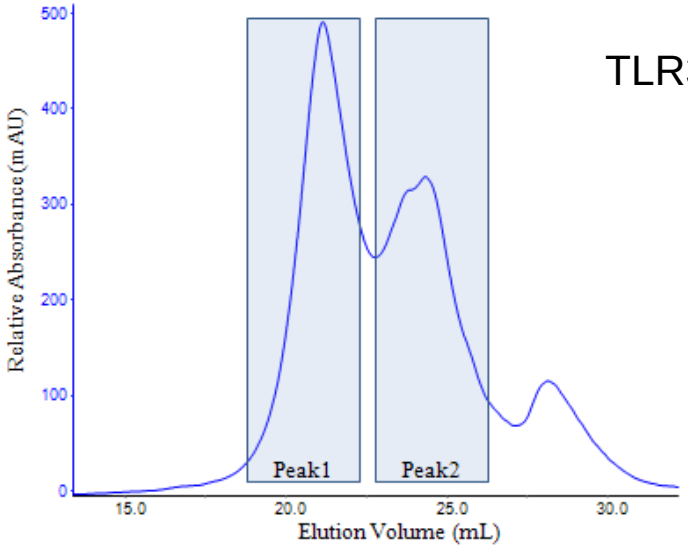
TLR3 deglycosylation, complex preparation, IEX



TLR3 IEX, complex preparation, IEX complex purification



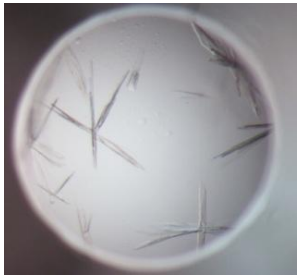




TLR3+3 Fab complex purification by anion exchange with a shallow gradient

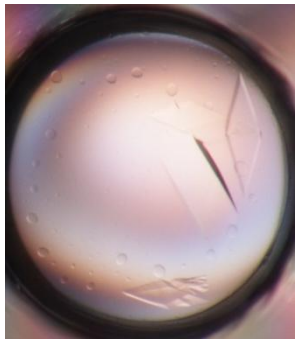
Peaks 1 and 2 were pooled, concentrated and setup in crystallization trials separately.

TLR3 + 3 Fabs crystallization refinement



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

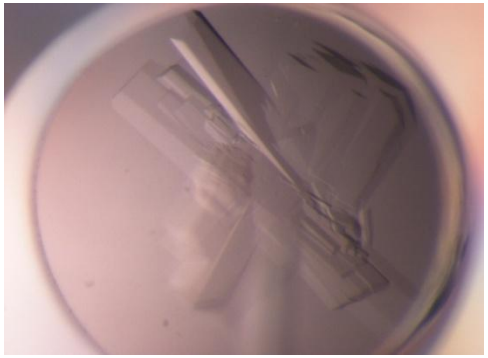
MMS refinement

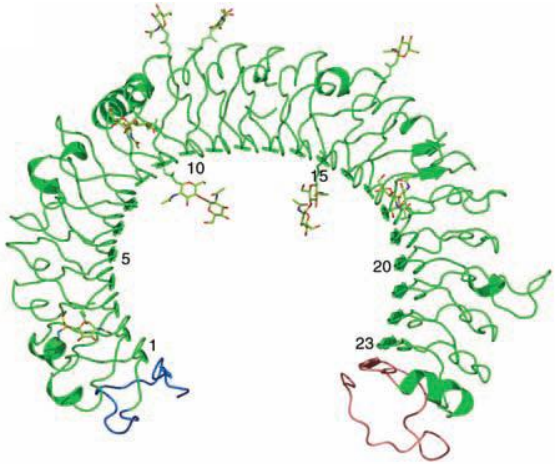


TLR3+3Fab peak1 crystal: weak diffraction
Acetate pH 4.5, 26% PEG 3350, 1 M LiCl

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

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

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