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

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Biologics Research, Centocor R&D

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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.

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

Structures total: 70

Crystallized without MMS: 32
- Fabs 18
- Complexes 3
- Others 11

MMS crystallization: 38
- Fabs 17
- Complexes 15
- Others 6

Crystallized from:
- in house screens hits 61
- other screens hits 9
## In house crystallization screens and hit frequency

### Screen 1:

<table>
<thead>
<tr>
<th>pH precipitate</th>
<th>3.5</th>
<th>4.5</th>
<th>5.5</th>
<th>6.5</th>
<th>7.5</th>
<th>8.5</th>
<th>9.5</th>
<th>10.5</th>
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<tbody>
<tr>
<td>PEG 8000</td>
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<tr>
<td>AmmSul</td>
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</tbody>
</table>

Color intensity - hit frequency

* no hits

**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:

<table>
<thead>
<tr>
<th>salts/ pH</th>
<th>4.5</th>
<th>6.5</th>
<th>7.5</th>
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<tbody>
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<td>AmmAcet</td>
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<td>NaForm</td>
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<tr>
<td>LiCl 1 M</td>
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<tr>
<td>NaAcet 1 M</td>
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<tr>
<td>MgAc</td>
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<tr>
<td>NaSCN</td>
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</table>

**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)

<table>
<thead>
<tr>
<th>VH</th>
<th>VL</th>
<th>B3</th>
<th>A27</th>
<th>L6</th>
<th>O12</th>
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<td>1-69</td>
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<td>3-23</td>
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<td>3-53</td>
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<tr>
<td>5-51</td>
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</table>

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

<table>
<thead>
<tr>
<th>Crystallization without MMS optimization</th>
<th>MMS</th>
<th>MMS cross-seeding</th>
<th>No crystallization hits</th>
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<tbody>
<tr>
<td>Crystallization without MMS optimization</td>
<td></td>
<td>MMS</td>
<td>MMS cross-seeding</td>
</tr>
</tbody>
</table>
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 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)
MMS optimization with seed dilution

Crystals used for seed stock preparation → MMS screening → 50x diluted seeds

undiluted 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

↓
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

30% PEG 4000
0.2M 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

Seed stock 2

Different MW PEGs, pH 6.5
Crystallization optimization

MMS to narrow crystallization conditions

- Ammonium sulfate
- PEG 4000
- PEG 4000, 0.2 M Na Phosphate

Complex prep with Fab1

MMS PEG/salts conditions

A100/Fab1 complex Mono S purification test

Fractions pooled for crystallization

A100 Mono S purification

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

The most promising initial crystallization screening result
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

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
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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