Flow Cytometric Analysis of Cell Therapy Products

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Overview

• cGMP and cGTP requirements
• CAP Flow Cytometry requirements
• PACT and the UPMC HSCLab
• Two typical GTP applications
cGMP General Requirements

- Personnel
- Quality Control
- Facilities
- Equipment
- Control of Components
- Production and Documentation
- Laboratory Controls
- Container Closure and Labeling
- Distribution
- Recordkeeping
Laboratory Controls

• Tests conducted using established written procedures under controlled conditions
• Scientifically sound analytical procedures
• Properly calibrated and maintained analytical lab equipment
• Perform stability studies to support use of product in clinical investigation
CAP Flow Cytometry requirements

- PROFICIENCY TESTING
- QUALITY MANAGEMENT AND QUALITY CONTROL
- PROCEDURE MANUAL
- SPECIMEN COLLECTION AND HANDLING
- REAGENTS
- CONTROLS AND STANDARDS
- INSTRUMENTS AND EQUIPMENT
  - Flow Cytometers
  - Temperature-Dependent Equipment
  - Thermometers
  - Centrifuges
- PROCEDURES AND TEST SYSTEMS
- IMMUNOPHENOTYPING
  - Blood Lymphocyte Subset Enumeration
  - CD34 Stem Cell Enumeration
  - Leukemia and Lymphoma
- DNA CONTENT AND CELL CYCLE ANALYSIS
- PERSONNEL
- PHYSICAL FACILITIES
- LABORATORY SAFETY
Cytometer QC

**Daily**

- Alignment and Fluidic Verification with the Flowcheck and Flowset beads
- PMT Voltages standardized with Flowset beads
- Standard sample run to verify assay is in range

**Weekly**

- Full Matrix Compensation performed using FITC, PE Calibrite beads, ECD, PC5, and PC7 antibody-stained Ig capture beads
- Settings confirmed with 5-color verify tube
QC of cytometer optics and fluidics

Flow Check Fluorospheres

- [Ungated] SS Lin/FS Lin - ADC
  - A: 80.1%

- (5000) [A] FS Lin - ADC
  - B: 99.9%
  - C: 99.9%

- [A] FL1 Lin - ADC

- [Ungated] SS Lin/FS Lin Region X-Mean X-Mode X-CV HP X-CV
  - A: 502.8 493.0 3.7 2.4

- (5000) [A] FS Lin Region X-Mean X-Mode X-CV HP X-CV
  - B: 533.9 534.0 1.6 1.4
  - C: 530.4 531.0 1.5 1.1

- [A] FL2 Lin - ADC
  - D: 94.5%

- [A] FL3 Lin - ADC
  - E: 99.9%

- [A] FL4 Lin - ADC
  - F: 93.9%

- [A] FL2 Lin Region X-Mean X-Mode X-CV HP X-CV
  - D: 564.8 560.0 1.9 1.1

- [A] FL3 Lin Region X-Mean X-Mode X-CV HP X-CV
  - E: 619.0 610.0 1.5 1.1

- [A] FL4 Lin Region X-Mean X-Mode X-CV HP X-CV
  - F: 591.1 589.0 1.5 1.2
QC of cytometer electronics

Flow-Set Fluorospheres

[Ungated] FS Lin - ADC

[A] SS Lin - ADC

(10000) [A] FL1 Log - ADC

[Ungated] FS Lin
Region Number %Gated X-Mean X-Mode
A 9400 94.00 147.5 140.0

[A] SS Lin
Region Number %Gated X-Mean X-Mode
B 3952 41.69 412.5 393.0

(10000) [A] FL1 Log
Region Number %Gated X-Mean X-Mode
C 9211 97.10 72.5 71.5

[A] FL2 Log - ADC

[A] FL3 Log - ADC

[A] FL4 Log - ADC

[Ungated] FS Lin
Region Number %Gated X-Mean X-Mode
D 9192 96.96 69.6 68.9

[A] FL2 Log
Region Number %Gated X-Mean X-Mode
E 9167 96.70 73.1 76.1

[A] FL4 Log
Region Number %Gated X-Mean X-Mode
F 9286 97.95 69.2 70.2
Determination of the optimal PMT voltage on unstained peripheral blood lymphocytes
QC of cytometer linearity with 8-peak beads
Color Compensation

Measure spillover coefficients in single-stained cells or beads:
15% of FITC spills over into FL2, 2% of PE spills over into FL1
Solve for True FITC fluorescence in FL1 and True PE fluorescence in FL2 (2 simultaneous equations, 2 unknowns)
Understanding Spillover Coefficients

FITC  43.9
PE    5.37
ECD   2.35
PE-Cy5 1.28
PE-Cy7 0.64

\[
\frac{5.37}{43.9} = 12.2\%
\]
\[
\frac{2.35}{43.9} = 5.4\%
\]
\[
\frac{1.28}{43.9} = 2.9\%
\]
\[
\frac{0.64}{43.9} = 1.5\%
\]
QC standards: Stabilized human blood

Reference values for percent positive and absolute count
Controls for reagent, sample prep, operator, and instrument variability
Interpreting Levey-Jennings Plots

0.5 * 0.5 * 0.5 = 0.125
A run of 3 values above the mean happens 12.5% of the time by chance alone

A value ≥2 SD below the mean happens 2% of the time by chance alone
Flow Cytometry as a Potency Surrogate

CD34+ CD38- cells/Kg b.w.

Use of Flow to Assess Purity/Potency

2.96% of WBC

100% CD34 Viability

99.8% WBC Viability

Pre Separation, Cy G-CSF mobilized PB
Use of Flow to Assess Purity/Potency

Isolex 300i V 2.5 +/- Separation

98.5% purity, 99% viability
Translational and Clinical Projects at the UPMC HSCLab

• Radically T-depleted peripheral HPCA (CD34 positive selection followed by CD3- selection) for autologous transplant in systemic sclerosis (Medsger, Agha)

• Cardiac repair during LVAD placement, in Patients with chronic angina, in patients receiving CABGs (Kormos, Patel, Lee).

• Esophageal repair (Luketich, Badylak)

• Chronic wound repair by adipose-derived stem cells (Rubin, Marra)
NIAMS-funded study Auto HSCT in rapidly progressing SSc

CY (2g/m² over 24 hours), G-CSF (10 mg/kg/day sq) mobilization until WBC>2500

Apheresis, T-depletion by Isolex 300i
  – Positive selection on CD34
  – Negative selection by CD3 depletion on CD34+ population

Cryopreserve, infuse after immunoablative ablative therapy (Cytoxan, Fludarabine, ATG)
Baxter Isolex 300i

Illustration Courtesy of Baxter
Paramagnetic CD34 Positive Cell Selection

MNC Fraction Containing CD34+ Stem Cells

Anti-CD34 mAb

Paramagnetic bead

SAM Ig antibody

PR34+ Release Agent

Purified CD34+ Cells

Additional Step with anti-CD3 followed by beads
Collect non-adherent fraction

Illustration Courtesy of Baxter
Isolated CD34+ PBPC
Cell Selection: CliniMACS

**Ag/Aby Binding**
Mouse Anti-Human CD 3-
Ferromagnetic particles
(50 nM)

**Immunomagnetic Capture**

Source: Jeff Miller, M.D.
PBMC Apheresis

- CD3+ cell depletion

Activation/Expansion

- IL-2/X-VIVO-15

Harvest

- Suspend in 5% HSA

Infuse

37% NK cells

Source: Jeff Miller, M.D.
**Performance Qualification Acceptance Criteria Summary Report**

**Allogeneic Natural Killer Cells: CD3 Depleted/CD56 Enriched**

<table>
<thead>
<tr>
<th>Product Date/#</th>
<th>NC Dose (NC/Kg)*</th>
<th>LAL</th>
<th>CD3+ Dose (per Kg)*</th>
<th>%CD3-/CD56+</th>
<th>Viability</th>
<th>Sterility</th>
<th>Gram Stain</th>
<th>Accept?</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-14-2004</td>
<td>1.96E+06</td>
<td>1.96E+06</td>
<td>&lt; 0.5EU/mL</td>
<td>&lt; 0.2EU/mL</td>
<td>&lt; 3.00 E+04</td>
<td>6.26 E+03</td>
<td>≥70%</td>
<td>93%</td>
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<td>095W00010</td>
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<td>10-26-2004</td>
<td>1.00E+06</td>
<td>1.00E+06</td>
<td>&lt; 0.5EU/mL</td>
<td>&lt; 0.2EU/mL</td>
<td>&lt; 3.00 E+04</td>
<td>5.80 E+03</td>
<td>≥70%</td>
<td>84%</td>
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<td>R04792</td>
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</tr>
<tr>
<td>11-11-2004</td>
<td>3.91E+06</td>
<td>3.91E+06</td>
<td>&lt; 0.5EU/mL</td>
<td>&lt; 0.2EU/mL</td>
<td>&lt; 3.00 E+04</td>
<td>2.20 E+04</td>
<td>≥70%</td>
<td>98%</td>
</tr>
<tr>
<td>R04798</td>
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</tr>
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</table>

*Dose is calculated using a body weight of 80 kg.

Source: Jeff Miller, M.D.
Development of a Product Purity Assay from a Research Assay

(Rare event detection of circulating T cells after lymphoablation)

- Adapted from BC Stem-Kit CD34 determination
- CD45-FITC/CD3-PE/7AAD/FlowCount beads
- Single platform lyse/no wash protocol
- Replicate tubes (3-9) plus carryover & isotype control
- Exhaustive acquisition (18 min/tube)
- Assay dynamic range from >1,000 to 0.1 T cells/μL

<table>
<thead>
<tr>
<th>WBC</th>
<th>Est %Ly</th>
<th>Vol/tube uL</th>
<th>Tubes</th>
<th>Est absT</th>
<th>Total WBC/tube</th>
<th>Est T events/tube</th>
<th>Est T events (all tubes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>5.0%</td>
<td>100</td>
<td>3</td>
<td>15.00</td>
<td>100000</td>
<td>1500</td>
<td>4500</td>
</tr>
<tr>
<td>250</td>
<td>5.0%</td>
<td>200</td>
<td>6</td>
<td>3.75</td>
<td>50000</td>
<td>750</td>
<td>4500</td>
</tr>
<tr>
<td>10</td>
<td>5.0%</td>
<td>200</td>
<td>9</td>
<td>0.15</td>
<td>2000</td>
<td>30</td>
<td>270</td>
</tr>
<tr>
<td>5</td>
<td>5.0%</td>
<td>200</td>
<td>9</td>
<td>0.08</td>
<td>1000</td>
<td>15</td>
<td>135</td>
</tr>
<tr>
<td>2</td>
<td>5.0%</td>
<td>200</td>
<td>9</td>
<td>0.03</td>
<td>400</td>
<td>6</td>
<td>54</td>
</tr>
</tbody>
</table>
Single Platform Absolute T-cell Count Day +3

CAL BEADS (UNGATED)

WBC (NOT R1)

CD3+ (R2 NOT R1)

CD3+ CD45HI (R2 R3 NOT R1)

STEM COUNT

SS

CD 45 FITC

SS

CD3 PE

CD 45 FITC

CD3+CD45HI FSINT (R2 R3 R4 NOT R1)

CD3+ VIABILITY (R2 R3 R4 R5 NOT R1)

TOTAL CD3+ VIABILITY (R2 R3 R4 NOT R1)

CD45 VIABILITY (R2 NOT R1)

0.655% of WBC
7.86 CD3/μL

99.3% CD3 Viability

99.0% WBC Viability

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Single Platform Absolute T-cell Count Day 0

CAL BEADS (UNGATED)

WBC (NOT R1)

CD3+ (R2 NOT R1)

CD3+ CD45HI (R2 R3 NOT R1)

CD3+CD45HI FSINT (R2 R3 R4 NOT R1)

CD3+ VIABILITY (R2 R3 R4 R5 NOT R1)

TOTAL CD3+ VIABILITY (R2 R3 R4 NOT R1)

CD45 VIABILITY (R2 NOT R1)

0.028% of WBC
0.99 CD3/μL

100% CD3 Viability

99.7% WBC Viability
Autologous HSCT for SSc

Product Release Criteria

- Endotoxin negative (Endosafe PTS)
- Sterile (14 day bacterial and fungal)
- CD34 dose ($5 \times 10^6$/kg)
- CD34 purity (>85%)
- CD34 viability (>92%)
- CD3 depletion (> 4 logs reduction)
- Satisfactory freeze curve
- Alternatives if not met:
  - Additional Leukapheresis
  - Medical exception (life saving product)
Adaptation to cGMP Product Testing

• Purity: CD34 content, CD3 depletion
• Potency: Viable CD34
• Non x-reactive CD3 clone (test PBMC pre-incubated with OKT3 depleting clone)
• Instrument QC (FlowCheck, FlowSet, Compensation)
• Validation (known controls, Streck CDChex Plus)
• ASR labeling of results (CAP requirement)
• GMP reagent handling (quarantine, overlap testing)
• Deviation management
Mobilized Leukapheresis Product
Isotype Control Pre-Isolex

Note: Tight Gates, ISHAGE-type gating strategy, beads for absolute count
Mobilized Leukapheresis Product
CD3 determination Pre-Isolex

Note: Identical gates, approximately same number of cells acquired, high sample viability
Cal factor*Cells/Beads = 1714 CD3/μL
Mobilized Leukapheresis Product
Isotype Control Post-Isolex

Note: Very homogeneous with respect to CD45, FSc & SSc, CD3 gate avoids noise
Mobilized Leukapheresis Product
CD3 determination Post-Isolex

Note: Identical gates, most CD3 signal falls outside normal CD3 and CD45 space (defined by pre-sample) and within isotype control space, Events within normal CD3/CD45 space have lymphoid light scatter and high (90%) viability. This is one of six replicate determinations.

0.45 CD3/μL

Beads
Cells
CD3 Enumeration Report Form

University of Pittsburgh Medical Center  
University of Pittsburgh Cancer Institute/Children's Hospital of Pittsburgh  
HSC Laboratories

CD3 CELL ENUMERATION REPORT FORM

UPN

Recipient Name
Hospital Number
Donor Name
Hospital Number
Physician

Processing Performed:
Body weight used for calculations
Cell goal
Reference Blood Types
Recipient
Donor
Other

PRODUCT
TYPE
HPC-A/M/C

UNIQUE COMPONENT NUMBER

PRODUCT VOLUME

Flow Cytometry
WBC/ml

Flow Cytometry
TOTAL WBC

CD3+/%

CD3+/Kg

TOTAL CD3+/Kg

TECH

BAGS FROZEN

VOLUME FROZEN

TECH

TOTAL

* This test was developed and its performance characteristics determined by the HSCLab. It has not been cleared or approved by the US Food and Drug Administration. The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the CLIA-88 as qualified to perform high complexity clinical laboratory testing.

COMMENTS :

DIRECTOR REVIEWED : ___________________________ DATE : Slide 37
The Analyte Specific Reagent Disclaimer

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COMMENTS:

____________________________________________________

____________________________________________________

____________________________________________________

DIRECTOR REVIEWED: ________________________________  DATE: ________
**Autologous HSCT for SSc**

CD3 depletion by positive CD34 selection/ negative CD3 selection

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD34 Dose/kg</th>
<th>CD34 Purity</th>
<th>CD3 Dose/kg</th>
<th>Log Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.18E+07</td>
<td>99.7%</td>
<td>9.76E+02</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>9.46E+06</td>
<td>96.4%</td>
<td>5.71E+01</td>
<td>6.3</td>
</tr>
<tr>
<td>3</td>
<td>1.30E+07</td>
<td>97.4%</td>
<td>4.54E+03</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>4.80E+06</td>
<td>98.9%</td>
<td>6.34E+02</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>1.07E+07</td>
<td>97.4%</td>
<td>4.85E+02</td>
<td>5.3</td>
</tr>
<tr>
<td>Mean</td>
<td>9.94E+06</td>
<td>98.0%</td>
<td>1.34E+03</td>
<td>5.2</td>
</tr>
<tr>
<td>SD</td>
<td>3.16E+06</td>
<td>1.3%</td>
<td>1.82E+03</td>
<td>0.6</td>
</tr>
</tbody>
</table>
## Heterogeneity of Bone Marrow Progenitor Cells

<table>
<thead>
<tr>
<th>Type</th>
<th>CD45+/CD34+/CD38- or CD34+/CD133+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC</td>
<td>CD45+ CD34+, lineage marker+ (CD14, CD15, Glycophorin A, CD41a, CD3)</td>
</tr>
<tr>
<td>Endothelial</td>
<td>CD45- CD34+, CD133+, VEGFR2+, VE-cadherin+</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td>CD45- CD34-, CD73+, CD105 (endoglin)+</td>
</tr>
</tbody>
</table>
Heterogeneity of Bone Marrow Progenitor Cells
Non-hematopoietic Progenitors in SVF can be Characterized and Isolated

Gating Strategy

Non-hematopoietic Progenitors in SVF can be characterized and isolated.

Endothelial Mature

CD31+ CD34-

Endothelial Progenitor

CD31+ CD34+

Pericyte

CD31- CD146+

Preadipocyte

CD31- CD34+ CD146-
Challenges

• Beyond CD34 as metric for stem cell content
• Challenges of intraoperative separation of bone marrow derived stem cells for tissue repair
• Rapid testing of product for release criteria
• Adaptation of multiparameter flow including stem cell functions (aldehyde dehydrogenase, MDR dye exclusion) as measures of potency
Acknowledgements

Linda Moore, Heather Stanczak, Eileen Koch, Maria Dantella, Debe Griffin, The PACT team & The AVDLab tissue acquisition and processing team