DRY BEER, ICE BEER AND ISHAGE: THE EVOLUTION OF BEER AND CD34+ CELL ENUMERATION

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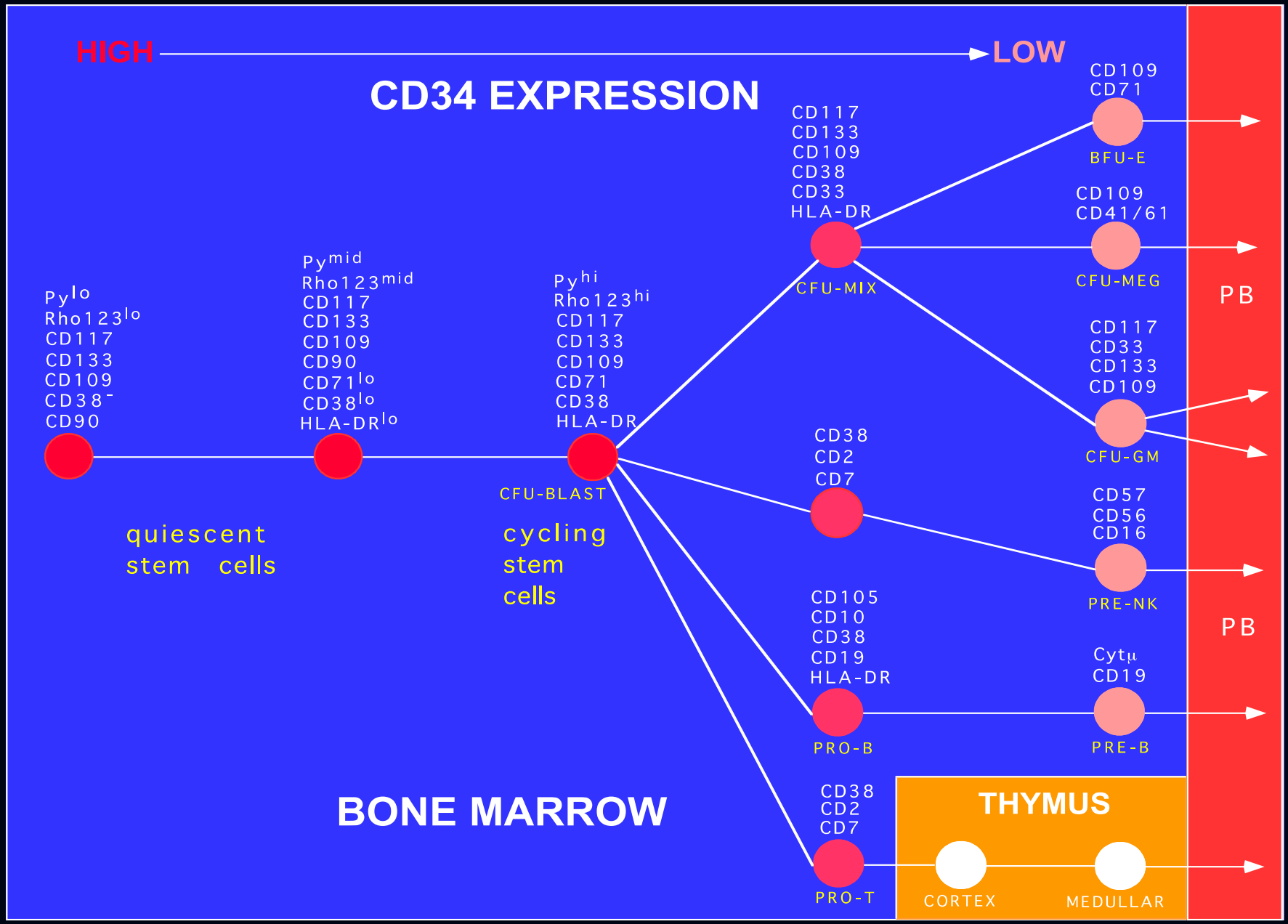
MetroFlow: NY/NJ Flow Cytometry Users Group
October 24, 2016

Learning Objectives

- Why do we count CD34+ cells?
- How do we count CD34+ cells?
- How do we develop International Guidelines

- Importance of a Quality Assurance program

WHY IS CD34 IMPORTANT?



Sources of Hematopoietic Stem Cells

BONE MARROW

Thomas et al, 1957

PERIPHERAL BLOOD (PB)

McCreadie et al, 1971, Korbling et al, 1980

CHEMOTHERAPY-MOBILIZED PB

Juttner et al, 1985, Reiffers et al, Korbling et al, Kessinger et al, 1986

CYTOKINE MOBILIZED PB

Siena et al, 1989, Chao et al, 1993

CORD BLOOD

Christenson et al, 1987, Gluckman et al, Broxmeyer et al, 1989

Cord Blood as a Source of HSCs

- **Advantages**
  - Availability
  - Reduced viral transmission
  - Reduced Graft vs Host Disease (GVHD)

- **Disadvantages**
  - Low CD34+ cell counts
  - Prolonged engraftment period/graft failure
  - Quality of units stored in cord banks

Assessing Graft Adequacy in mobPBSC: What do we need from an assay?

NUCLEATED CELL COUNT

- does not correlate well with engraftment potential

CFC ASSAYS FOR PROGENITOR CELLS

- Takes 10 - 14 days for assay read-out
- Assay measures 'late' progenitors only (CFU-GM)
- Assay is almost impossible to standardize

CD34+ CELL ENUMERATION BY FLOW CYTOMETRY

- Milan Protocol (Sienna et al Blood 77:400, 1991)

mononuclear cells (MNCs), Simple light scatter, isotype controls and single parameter flow analysis; measures '%CD34+ events'

- Two platform absolute counting (flow cytometer plus hematology analyzer) 'CD34+ cells/Kg'

Counting CD34+ Cells provides critical information to the Transplant Physician

Number of CD34+ cells in peripheral blood after mobilization with cytokines and/or chemotherapy predicts 'yield' of CD34+ cells in apheresis product

AND:

Number of CD34+ cells collected predicts time to engraftment after autologous or allogeneic HSC transplantation

BUT:

The use of mobilized peripheral blood for HSCT initially evolved without a consensus means to assess the engraftment potential of the HSC product

Flow Assay Development Considerations Before you Start: General Issues

Identify Target cells and Target Structures

- Q: What are the target populations in Hematopoietic Stem Cell enumeration?
- A: Primitive ‘blast’ cells that express the CD34 antigen
- Is anything known about the structural characteristics of the CD34 molecule(s) to be targeted?
- Will this constrain the choice of MAb clone or specific conjugates?
- Are the MAb clones available in desired conjugated form?
- Will the selected conjugates/cocktails work across different platforms?

Gather Scientific Knowledge

What Flow Cytometric methods are available?
What is the science behind them?
What is the basis of antibody conjugate selection?

What are the requirements of the assay?

- Simple methodology
- Suitable for all sources of HSCs (BM, PB, CB etc)
- Suitable for all Flow Cytometers with 3 or more PMTs
- Rapid
- Accurate at level of clinical decision-making (5-10 cells/μL)

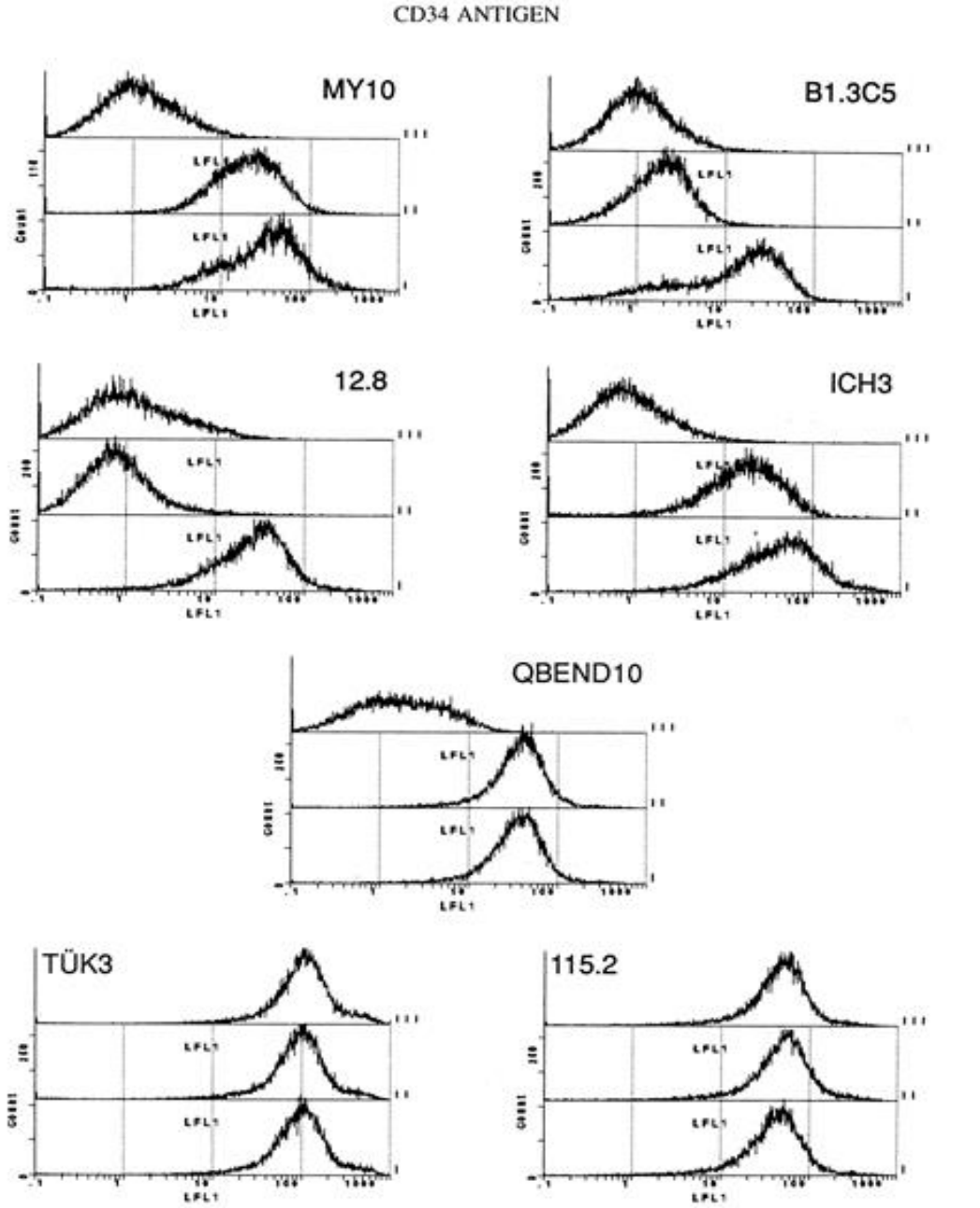
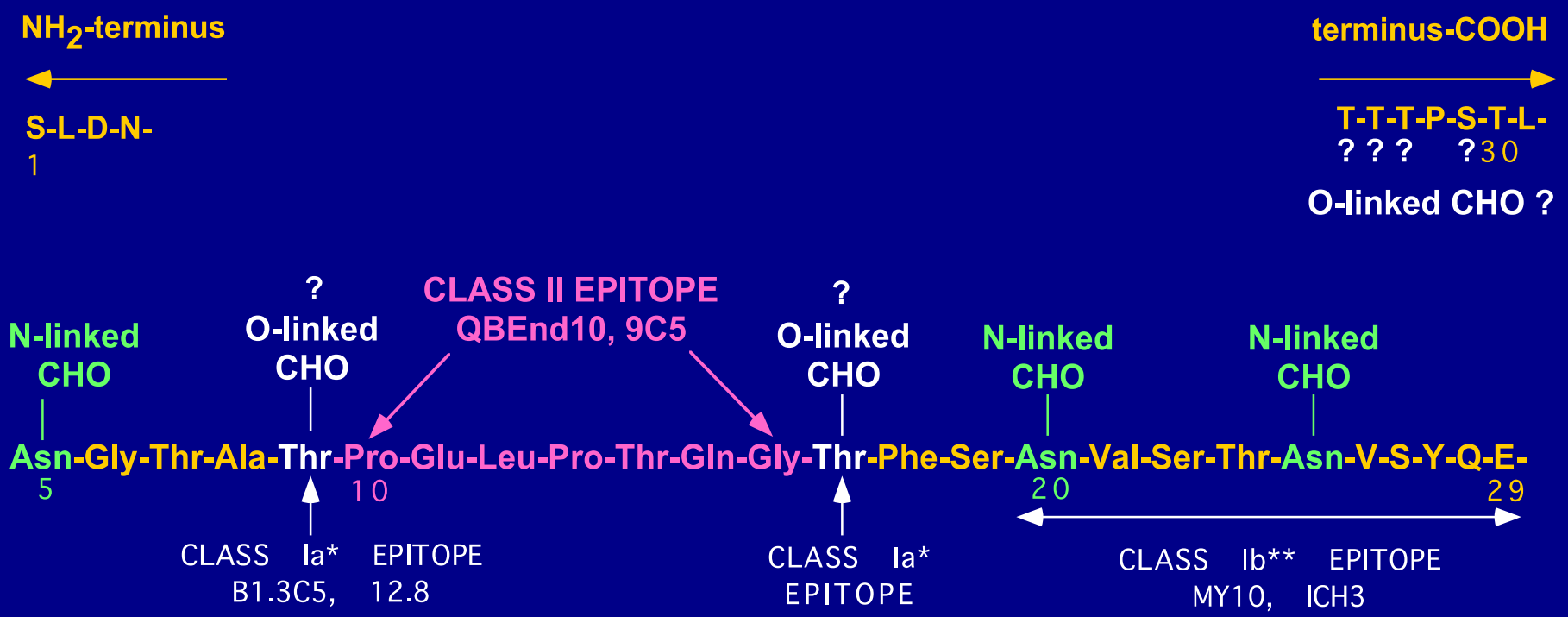


FIG. 1. Effects of neuraminidase and *Pasteurella haemolytica* glycoprotease cleavage on CD34 epitopes. KG1 cells were stained with anti-CD34 antibodies as indicated in Sutherland *et al.* (1992b) and analyzed by flow cytometry. For each antibody, the lower histogram (i) represents the staining of untreated cells, the middle histogram (ii) represents the staining of neuraminidase-treated cells, and the upper histogram (iii) represents the staining of the glycoprotease-treated cells.

3-class CD34 epitope classification based on sensitivity to sialidase and *P. haemolytica* O-sialoglycoprotease

Sutherland DR, Marsh JCW, Davidson J, Baker MA, Keating A, and Mellors A. Differential sensitivity of CD34 epitopes to cleavage by *Pasteurella haemolytica* glycoprotease: implications for purification of CD34-positive progenitor cells. Experimental Hematology 20: 590-599,1992.

CD34 ANTIGEN: AMINO-TERMINUS SEQUENCE



* Class Ia: complete sialic acid dependence (O-linked specific?)

** Class Ib: partial sialic acid dependence (N-linked specific?)

Sutherland et al. Structural and partial amino acid sequence analysis of the human haemopoietic progenitor cell antigen CD34. Leukemia 2:793, 1988

Sutherland et al. Differential sensitivity of CD34 epitopes to *P. haemolytica* glycoprotease: implications for purification of CD34-+ cells. Exp Hematol 20:590 1992

Simmons et al. Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. Immunology 148:267, 1992

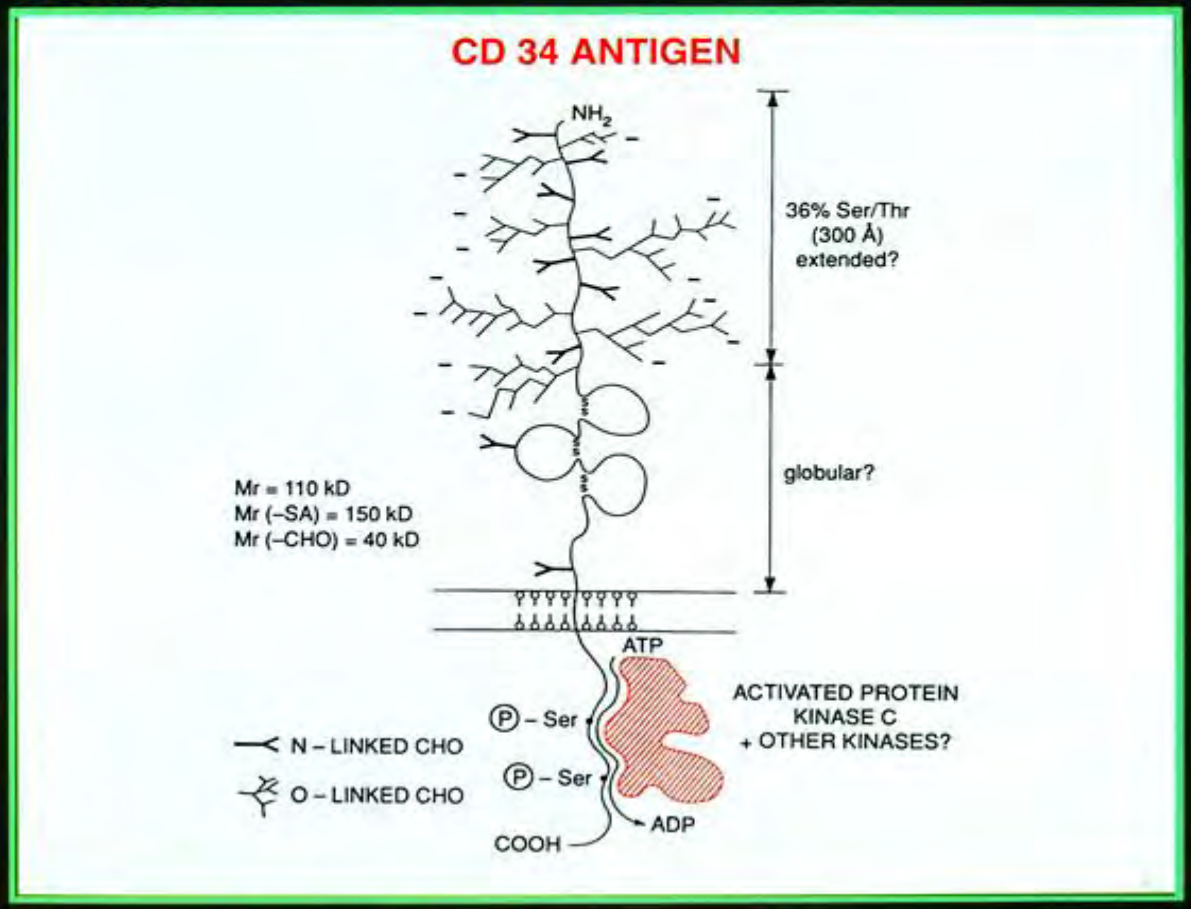
Ramanathan et al. Epitope mapping and binding affinity analysis of CD34 monoclonal antibodies (MABS). Blood 86:305a, 1995

Unverzagt. et al. Epitopes of CD34 identified by QBEnd10 and 9C5 monoclonal antibodies. Blood 90:3539a, 1997

Jones et al. In: Epitope mapping CD34 monoclonal antibodies using an immobilized peptide array. Leukocyte Typing VI, pp 977, 1998

Lanza, Healy, Sutherland. Structural and functional features of the CD34 antigen: an update. J Biological Regulators and Homeostatic Agents 15:1, 2001

The Journal of
BIOLOGICAL REGULATORS
& Homeostatic Agents
JBRHA



Volume 15 - Number 1 January - March 2001

Lanza F, Healy L, Sutherland DR. Structural and functional features of the CD34 antigen: An update. J Biol Regulators and Homeostatic Agents 15: 1-13, 2001.

CD34 Antigen: Epitope Considerations

Not all CD34 monoclonal antibodies detect all Glycoforms of CD34 Antigen

CD34 Epitopes:

CLASS I (MY10, B1.3C5, 12.8, ICH3)
- neuraminidase and O-sialo-glycoprotease sensitive

CLASS II (QBEnd10, 9C5, 11.A.10)
- O-sialo-glycoprotease sensitive

CLASS III (TUK3, 8G12, 581)
- Insensitive to both enzymes

Greaves MF, Titley I, Colman SM, Buhring H-J, Campos L, Castoldi GL, Garrido F, Gaudernack G, Girard J-P, Ingles-Esteve J, Invernizi R, Knapp W, Lansdorp PM, Lanza F, Merle-Beral H, Parravicini, C, Razak K, Ruiz-Cabello F, Springer TA, van der Schoot CE, Sutherland DR. Report on the CD34 cluster workshop. In: Leukocyte Typing V; Proceedings of the Vth HLDA Workshop (Schlossman S., et al eds.) Oxford University Press, Oxford pp 840-846, 1995.

CD34 Antibodies: Conjugate Considerations

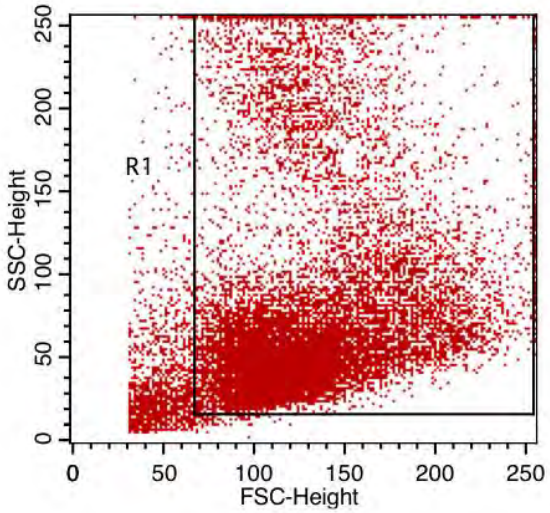
- Class I antibodies fail to detect all glycoforms of CD34
- Class I antibodies conjugated with negatively-charged fluorochromes e.g. FITC lose binding efficiency

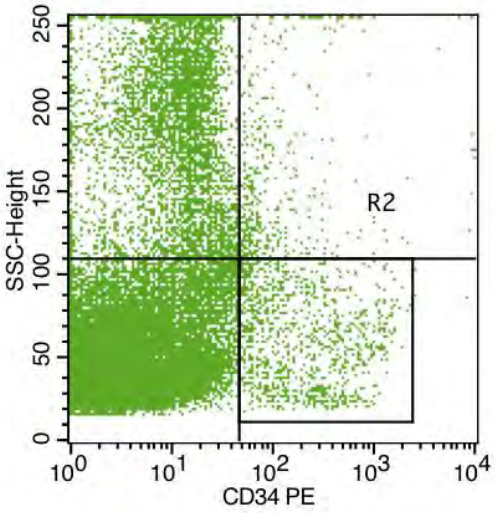
- Class II antibodies detect all glycoforms of CD34
- Class II antibodies conjugated with negatively-charged fluorochromes e.g. FITC lose binding efficiency

- Class III antibodies detect all glycoforms of CD34
- Class III antibodies still fully functional regardless of conjugated form

Sutherland DR, et al. The ISHAGE Guidelines for CD34+ cell determination by Flow Cytometry. J Hematotherapy 3:213-226, 1992

BM: Milan versus Bender Feb 1993





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Total Events: 50000

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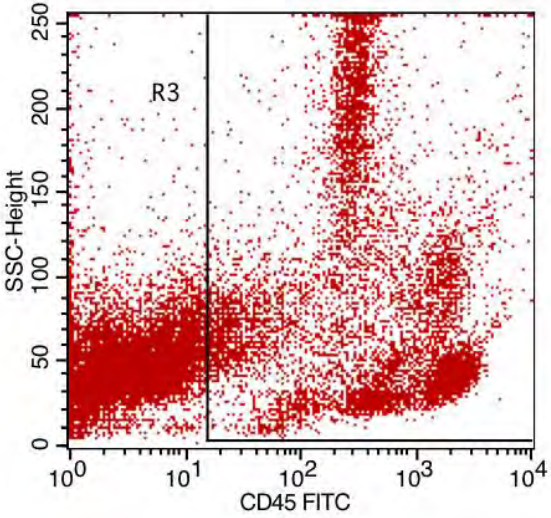
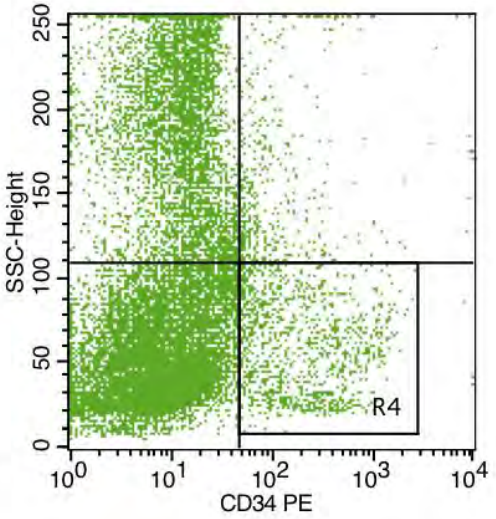
G2	1175	2.52
G3	20899	44.81
G4	1135	2.43

Milan protocol 1992





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Total Events: 50000

Gate	Events	% Gated
G1	20899	96.31
G2	1193	5.50
G3	21700	100.00
G4	1159	5.34

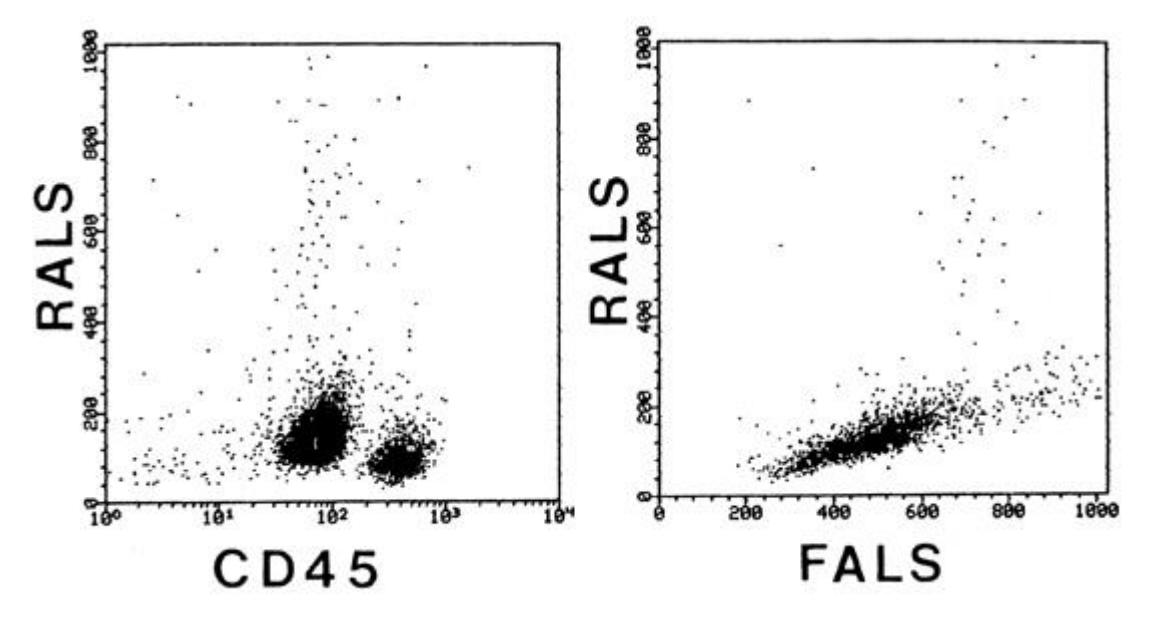
Bender et al 1994

Original Article

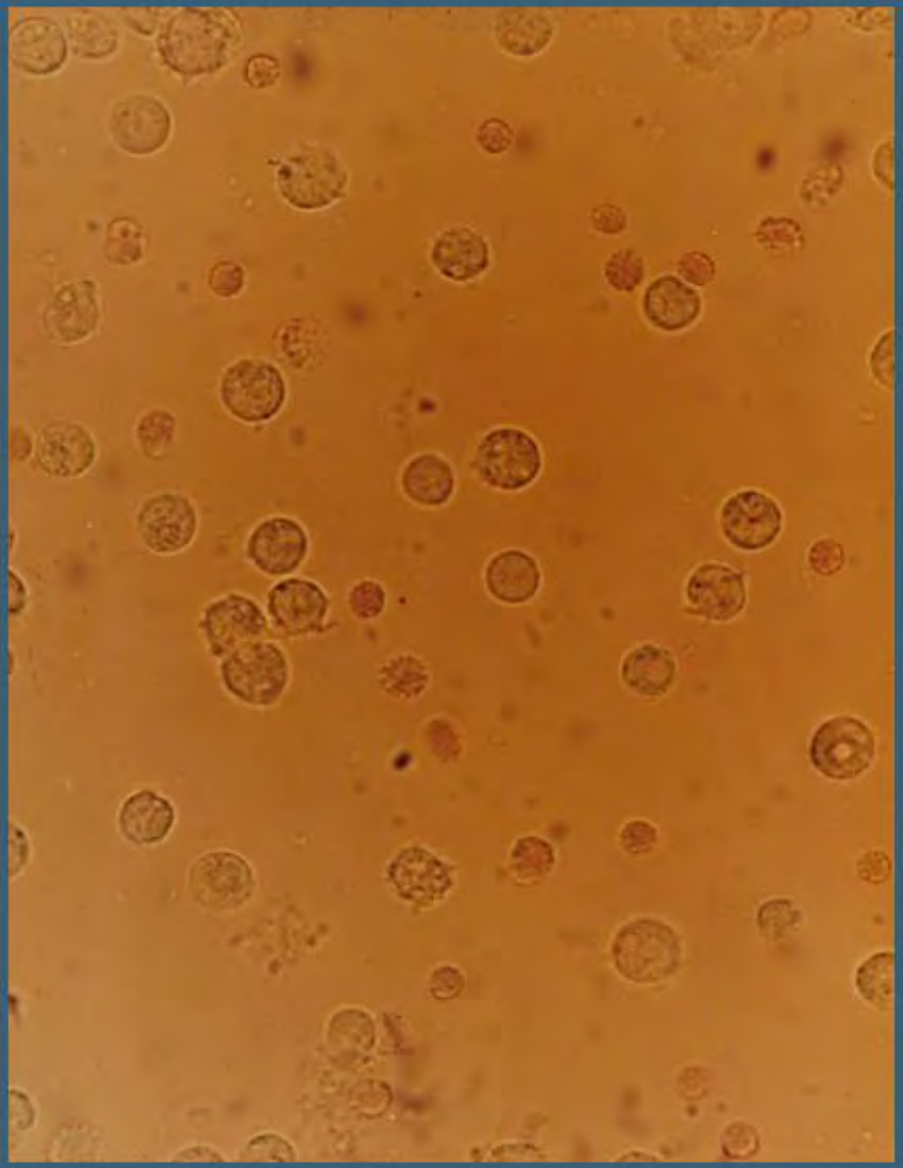
Immunophenotyping of Acute Leukemia by Flow Cytometric Analysis

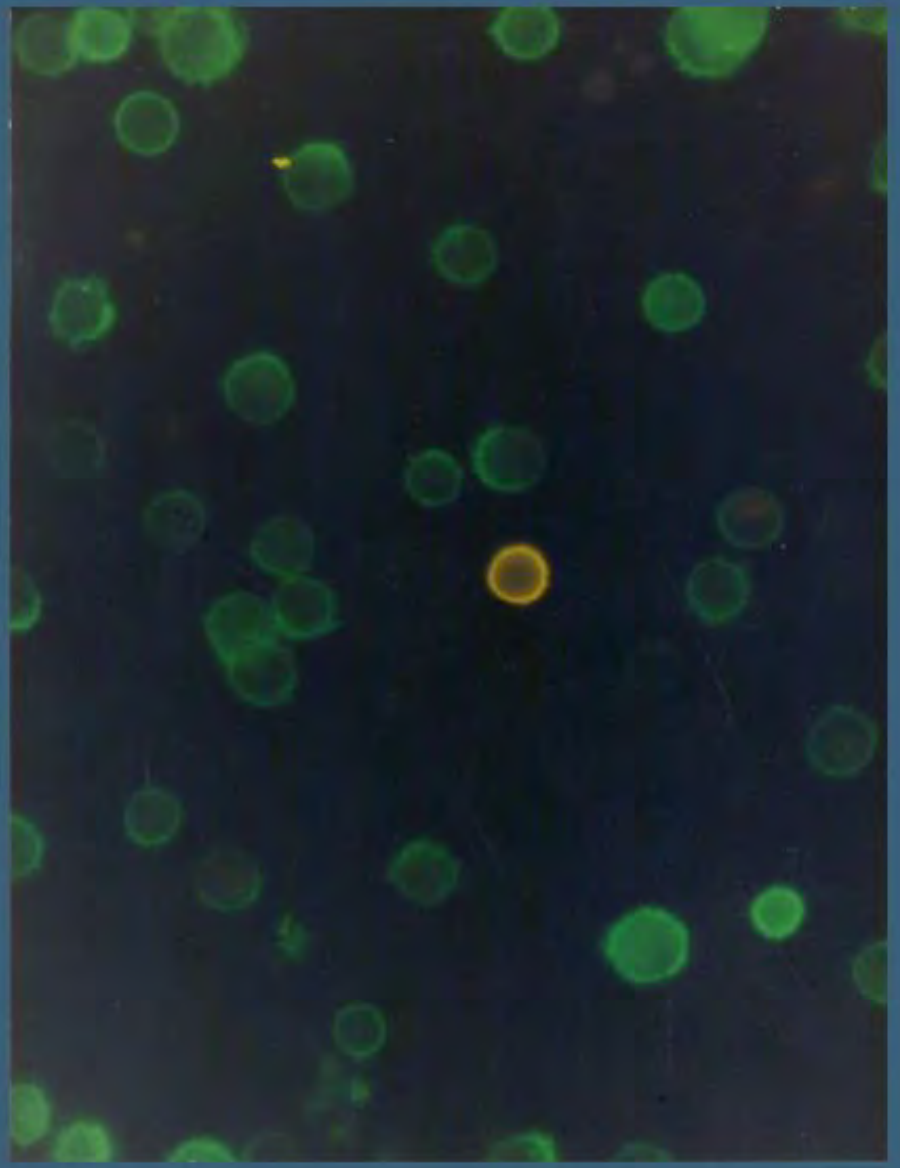
Use of CD45 and Right-Angle Light Scatter to Gate on Leukemic Blasts in Three-Color Analysis

MICHAEL J. BOROWITZ, MD, PHD,[1] K. LYNN GUENTHER, MT (ASCP),[1]
KEITH E. SHULTS, BS,[2] AND GREGORY T. STELZER, PHD[2]



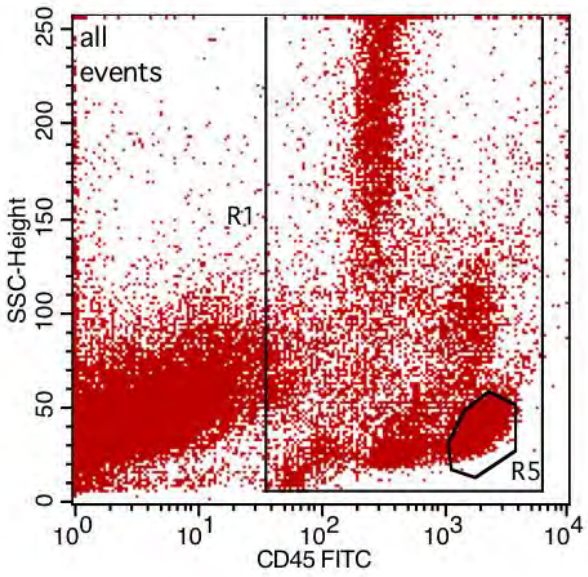
American Journal of Clinical Pathology 100: 534-540, 1993

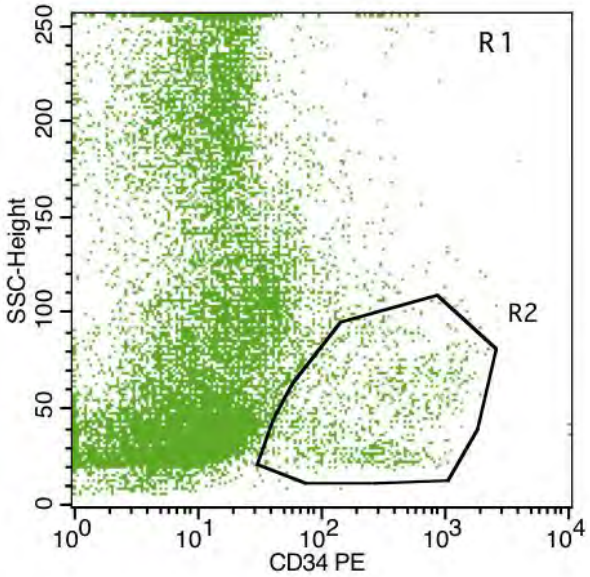




Need a ‘pan’ CD45 conjugate detecting all CD45 isoforms/glycoforms

BM: CD34/CD45 & Boolean gating Feb 1993





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Total Events: 50000

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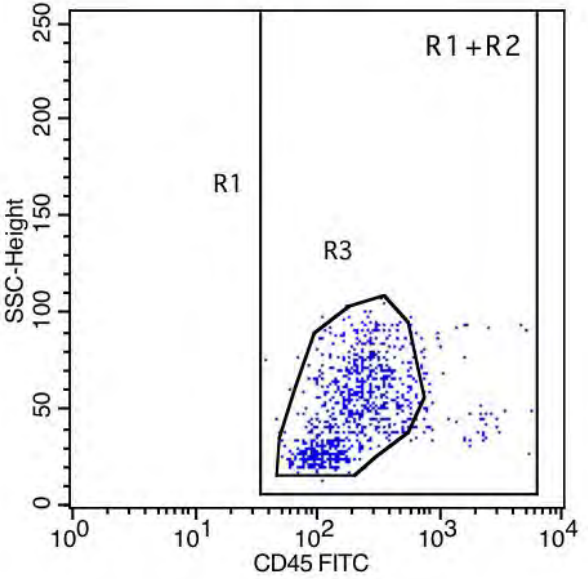
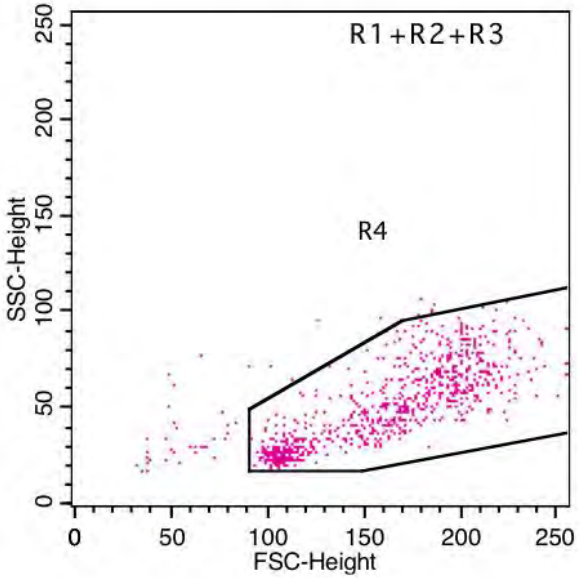
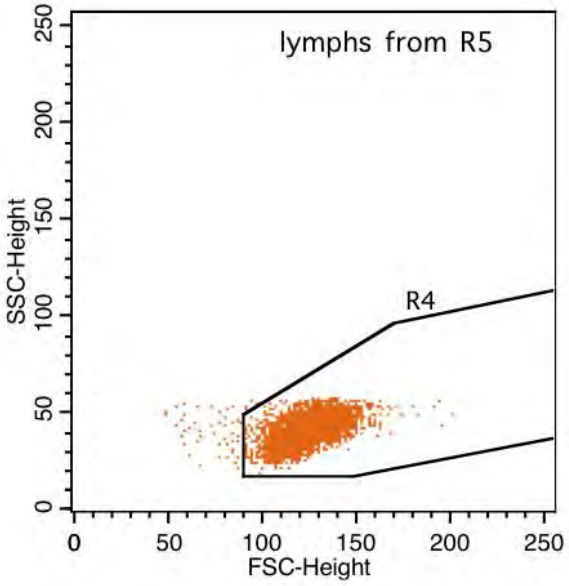
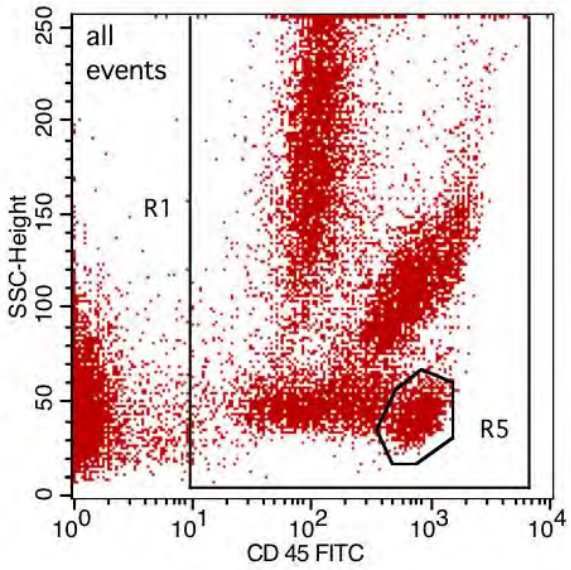
G2	909	4.62	1.82
G3	830	4.22	1.66
G4	787	4.00	1.57

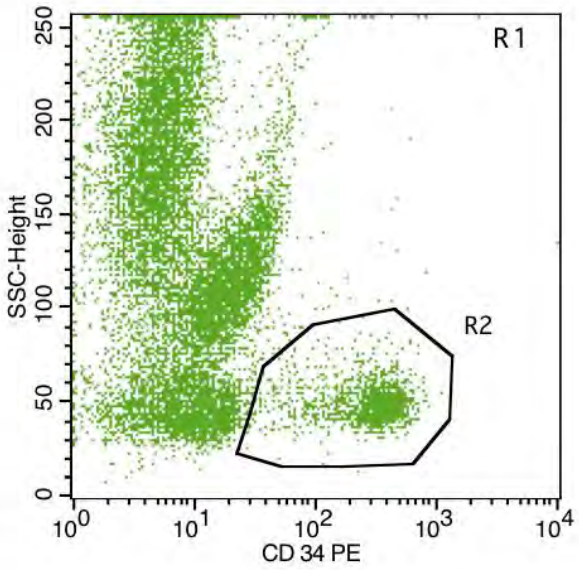






PBSC: CD34/CD45 & Boolean gating Jan 1993





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Total Events: 30000

Gate	Events	% Gated	% Total
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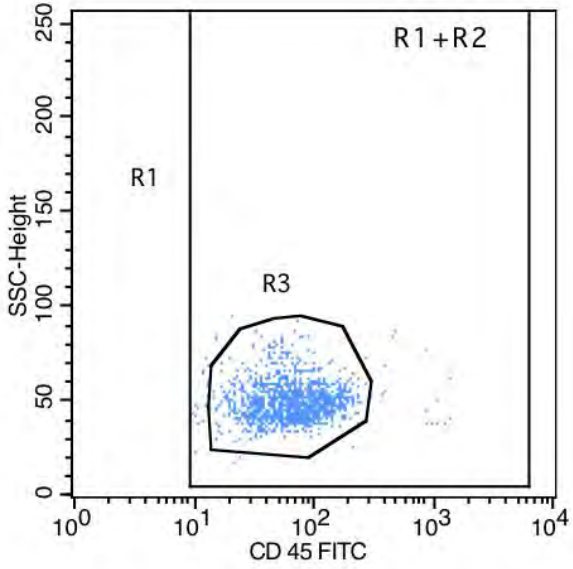
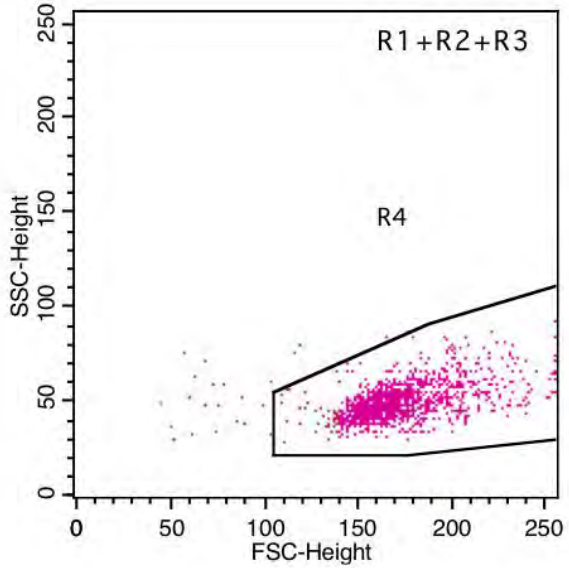
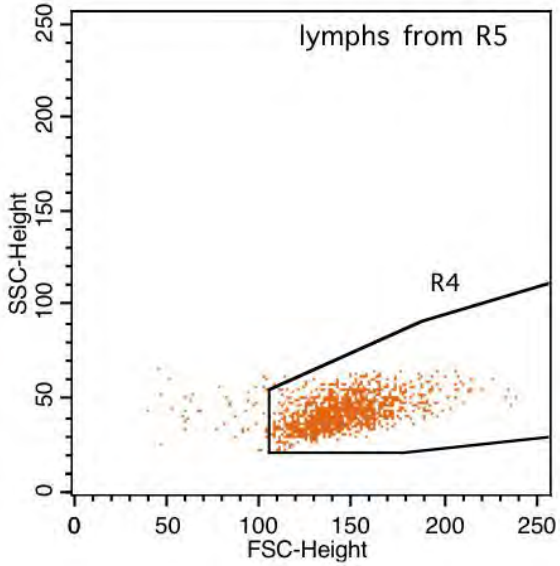
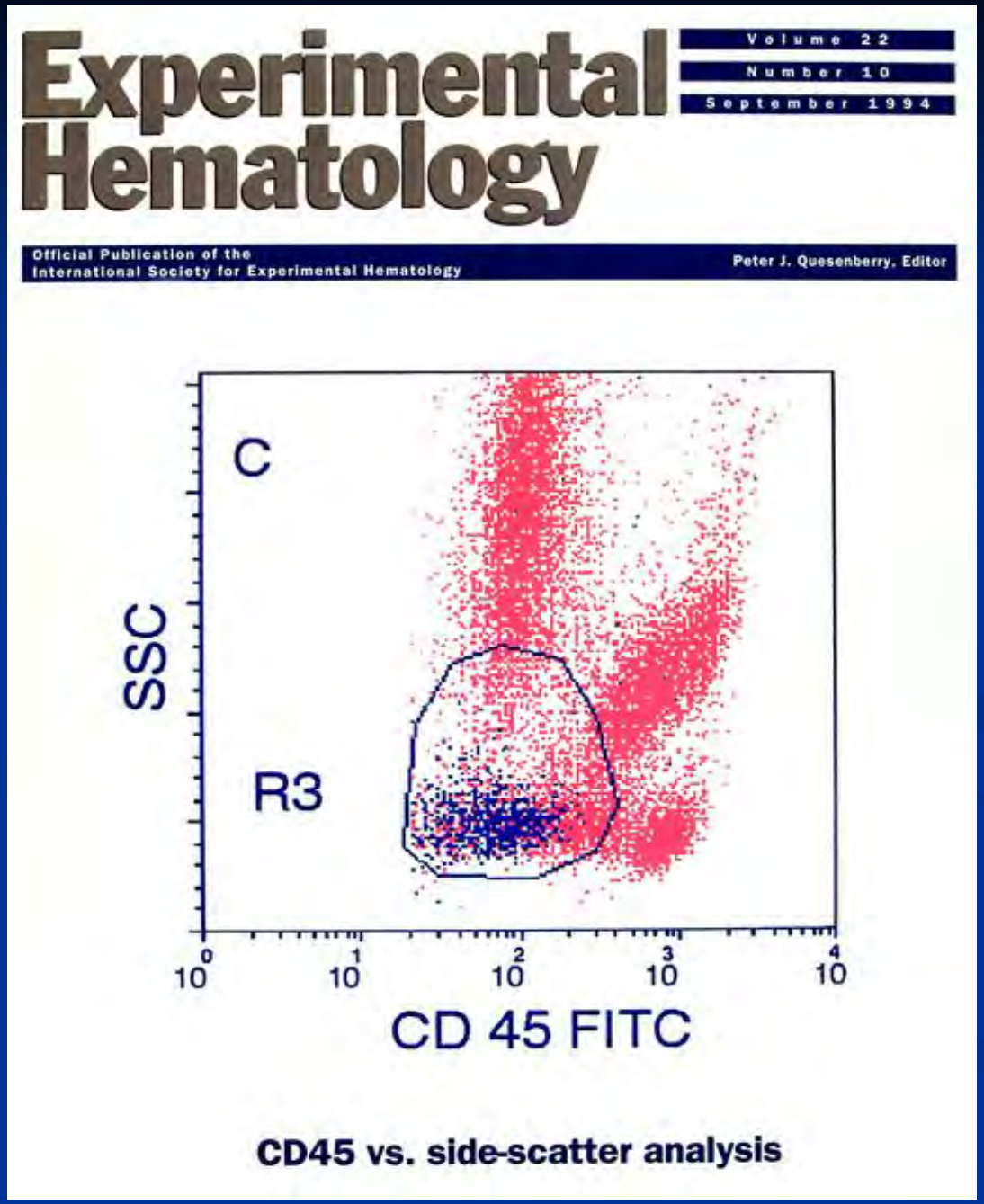
G2	1590	10.30	5.30
G3	1549	10.04	5.16
G4	1527	9.89	5.09









Sutherland DR, Keating A, Nayar R, Anania S, and Stewart AK.

Sensitive detection and enumeration of CD34+ cells in peripheral and cord blood by flow cytometry.

Exp Hematol 22:1003-1010, 1994.

A LIFE-CHANGING EVENT!!

JOURNAL OF HEMATOTHERAPY 5:213–226 (1996)
Mary Ann Liebert, Inc.

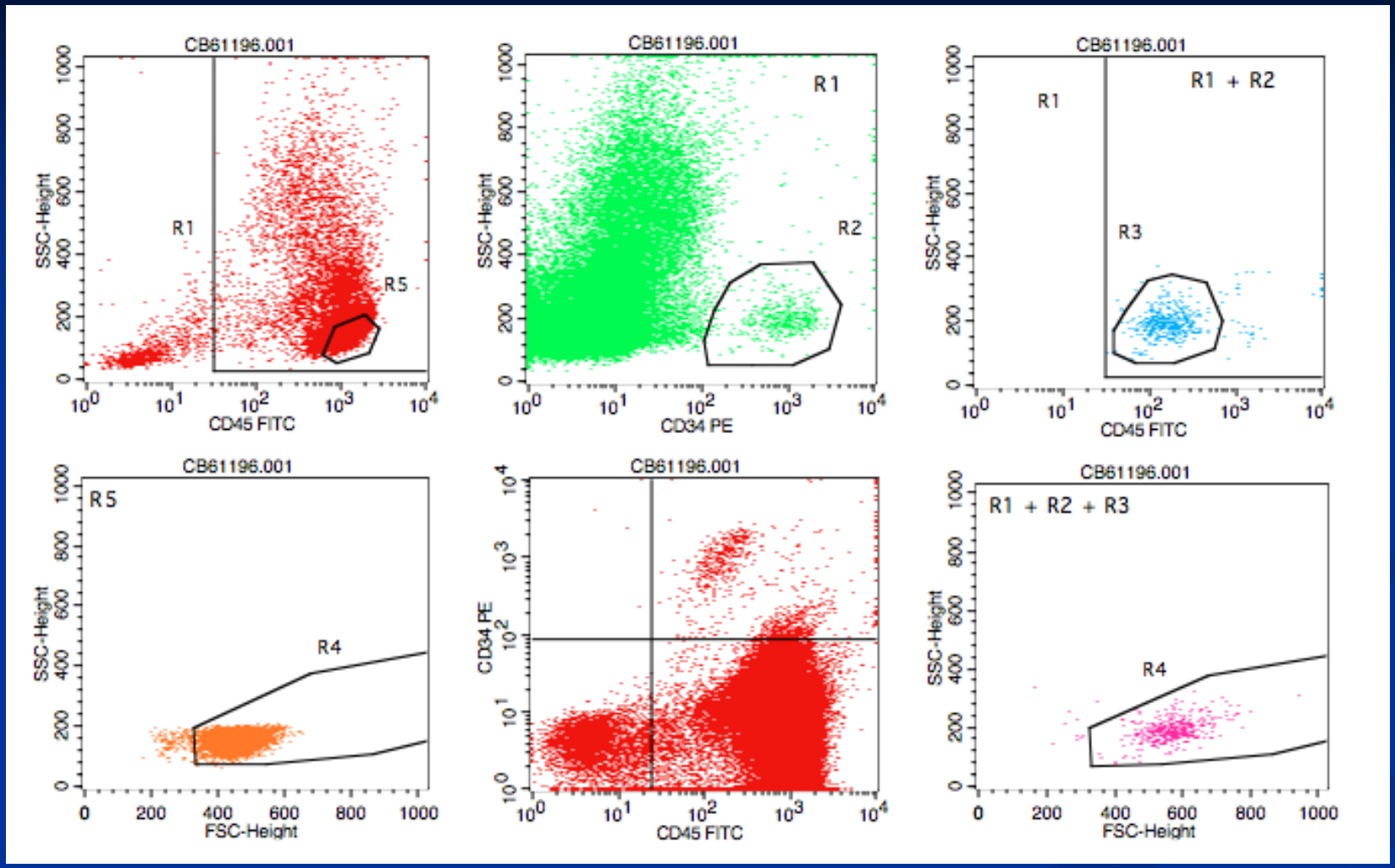
The ISHAGE Guidelines for CD34+ Cell Determination by Flow Cytometry

D. ROBERT SUTHERLAND,[1] LORI ANDERSON,[2] MICHAEL KEENEY,[2] RAKASH NAYAR,[1] and IAN CHIN-YEE[2]

ABSTRACT

The increased use of Peripheral Blood Stem Cells (PBSC) to reconstitute hematopoiesis in autotransplant and, more recently, allotransplant settings has not been associated with a consensus means to quality control the PBSC product. Since the small population of cells that bear the CD34 antigen are thought to be responsible for multilineage engraftment, graft assessment by flow cytometric quantitation of CD34+ cells should provide a rapid, reliable, and reproducible assay. Unfortunately, although a number of flow cytometric assays for CD34 enumeration have been described, the lack of a standardized method has led to the generation of widely divergent data. Furthermore, none of these assays has been validated as to interlaboratory reproducibility and suitability for widespread clinical application. In early 1995, the International Society of Hematotherapy and Graft Engineering (ISHAGE) established a Stem Cell Enumeration Committee, the mandate of which was to validate a simple, rapid, and sensitive flow cytometric method to quantitate CD34+ cells in peripheral blood and apheresis products. We also sought to establish its utility on a variety of flow cytometers in clinical laboratories and its reproducibility between transplant centers. Here, we describe the four-parameter flow methodology adopted by ISHAGE for validation in a multicenter study in North America.

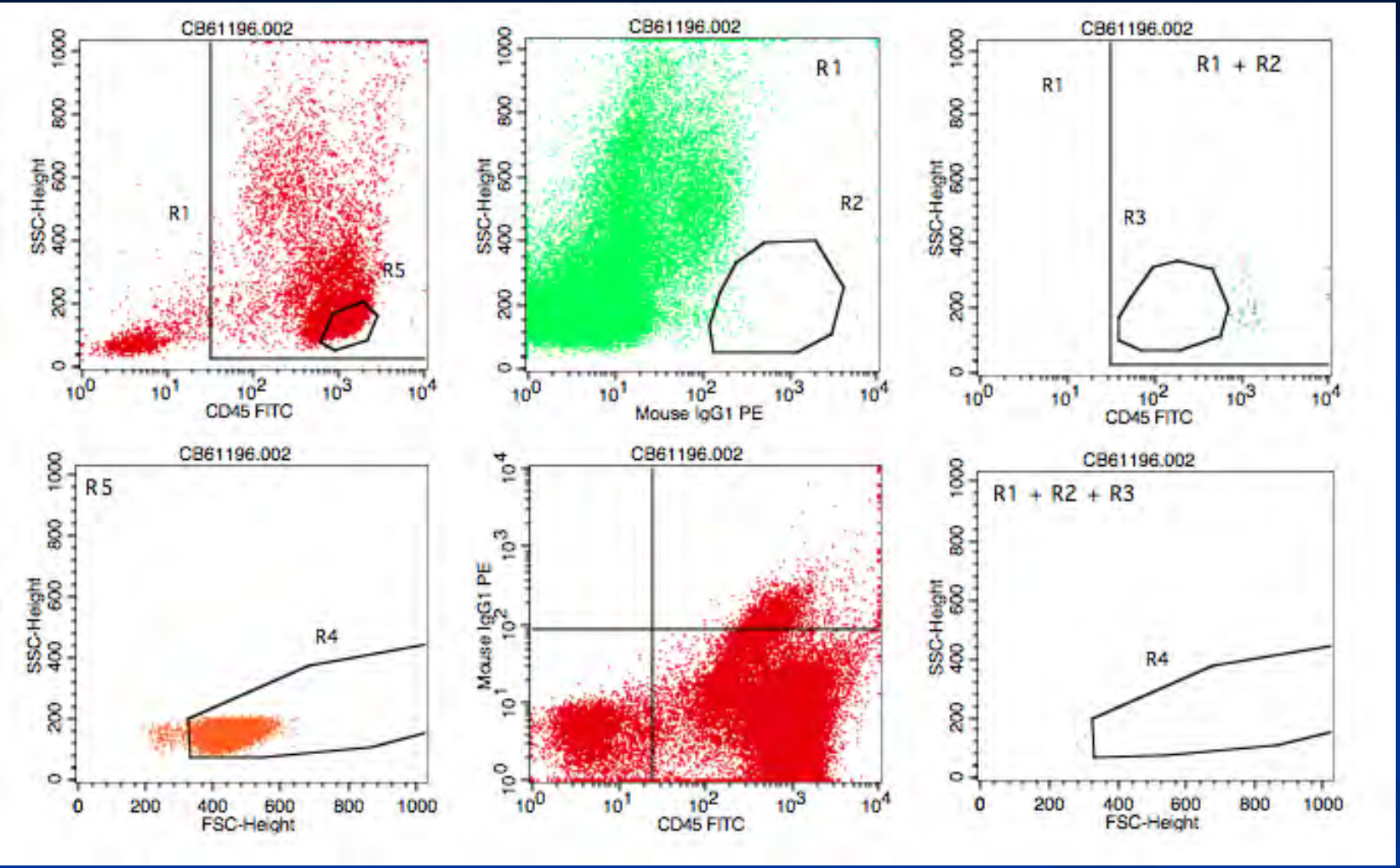
ISHAGE Guidelines 1996: Dual Platform



Fresh Cord Blood sample CD45/CD34

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I.
The ISHAGE Guidelines For CD34+ Cell Determination By Flow Cytometry.
J. Hematotherapy 3:213-226, 1996.

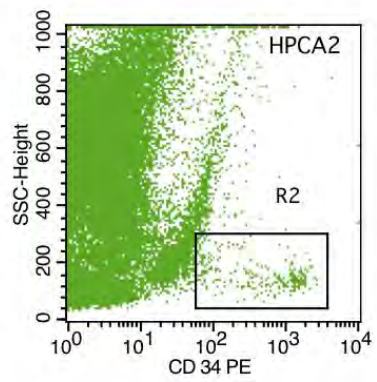
ISHAGE Guidelines 1996: Dual Platform

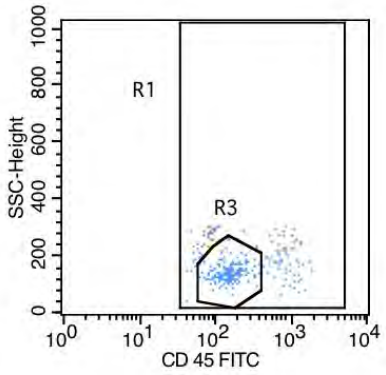


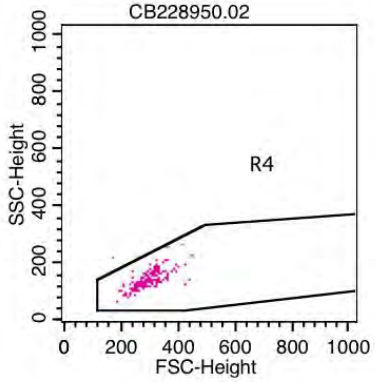
CD45FITC/IgG1PE: Isotype controls Useless!

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I.
The ISHAGE Guidelines For CD34+ Cell Determination By Flow Cytometry.
J. Hematotherapy 3:213-226, 1996.

Isotype controls: Useless AND Dangerous







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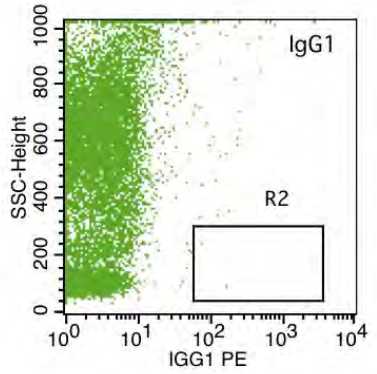
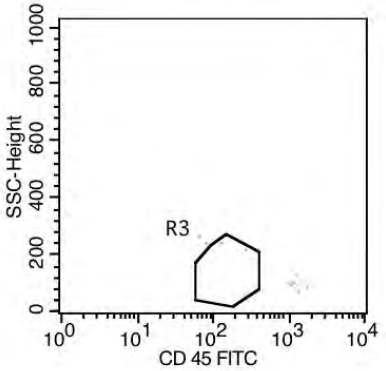
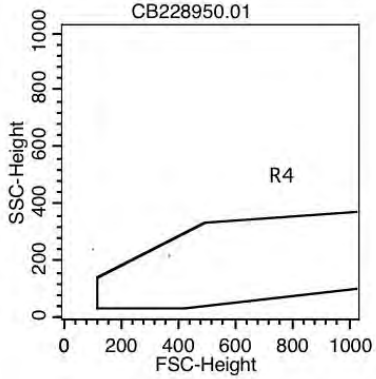
G3	186	0.37
G4	185	0.37







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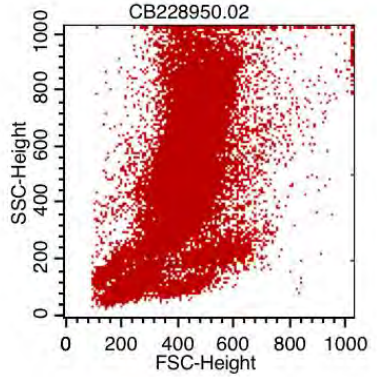
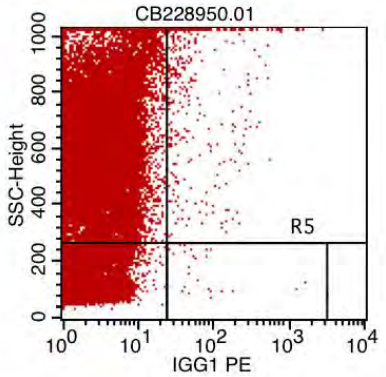
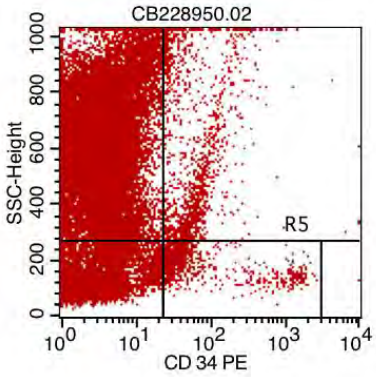
G3	2	0.00
G4	1	0.00







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Gate	Events	% Gated
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G2	302	0.60
G3	186	0.37
G4	185	0.37
G5	1026	2.05

Isotype Controls

- Do not aid in analysis of CD34+ cells
- May lead to inaccurate estimation of true CD34+ cells
- Boolean gating strategy obviates requirement for ‘negative reagent’ controls in ISHAGE protocol
- Isoclonic control – 50-part excess of unlabeled CD34: one part CD34PE

  The ‘Perfect Negative’

  - irrelevant to test sample and totally useless!

Keeney M, Chin-Yee I, Gratama JW, Sutherland DR. Perspectives: Isotype controls in the analysis of lymphocytes and CD34+ stem/progenitor cells by flow cytometry - Time to let go! Cytometry (Comm in Clin Cytom) 34: 280-283, 1998.

Hulspas R, O’Gorman MRG, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in Clinical Flow Cytometry. Cytometry Part B 76B: 355-364, 2009.

Dual platform to Single Platform and other Refinements (1998)

Eliminate redundant isotype control

Add a fluorescent counting beads to make the method single platform

Add a viability dye (7-AAD)

Single platform absolute counting of viable CD34+ cells in 45 minutes

Automate ?

(Coulter® EPICS® XL™ and Beckman Coulter Cytomics FC 500)

Cytometry (Communications in Clinical Cytometry) 34:61–70 (1998)

Original Articles

Single Platform Flow Cytometric Absolute CD34+ Cell Counts Based on the ISHAGE Guidelines

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In concert with the International Society of Hematotherapy and Graft Engineering (ISHAGE), we previously described a set of guidelines for detection of CD34+ cells based on a four-parameter flow cytometry method (CD45 FITC/CD34 PE staining, side and forward angle light scatter). With this procedure, an absolute CD34+ count is generated by incorporating the leukocyte count from an automated hematology analyser (two-platform method). In the present study, we modified the basic ISHAGE method with the addition of a known number of Flow-Count™ fluorospheres. To reduce errors inherent to sample washing/centrifugation, we implemented ammonium chloride lyse, no-wash no-fix sample processing. These modifications convert the basic protocol into a single-platform method to determine the absolute CD34 count directly from a flow cytometer and form the basis of the Stem-Kit from Coulter/Immunotech. A total of 72 samples of peripheral blood, apheresis packs, and cord blood were analysed and compared using the ISHAGE protocol with or without the addition of fluorescent microspheres. Comparison of methods showed a high correlation coefficient ($r = 0.99$), with no statistically significant difference or bias between methods ($P > 0.05$). Linearity of the absolute counting method generated an R^2 value of 1.00 over the range of 0–250/μl. Precision of the absolute counting method measured at three concentrations of CD34+-stabilised KG1a cells (Stem-Trol, COULTER®) generated a coefficient of variation (C.V.) ranging from 4% to 9.9%. In a further modification of the single-platform method, the viability dye 7-amino actinomycin D was included and demonstrated that both viable and nonviable CD34+ cells could be identified and quantitated. Together, these modifications combine the accuracy and sensitivity of the original ISHAGE method with the ability to produce an absolute count of viable CD34+ cells. It is the accurate determination of this value that is most clinically relevant in the transplant setting. These modifications may improve the interlaboratory reproducibility of CD34 determinations due to the reduction in sample handling and calculation of results. Cytometry (Comm. Clin. Cytometry) 34:61–70, 1998.

Single Platform CD34 Enumeration

Single-Platform ISHAGE Protocol

- Any clinical cytometer with 3 or more PMTs
- Pan-CD45 FITC (all isoforms and glycoforms)
- Pan-CD34 PE (class III)
- Viability dye (7-AAD)
- Flow-Count™ Fluorospheres or Trucount tubes
- Reverse-pipetting of sample (and beads) mandatory
- Sequential boolean gating strategy

  - to identify 'true' $CD34^{+}$ cells:

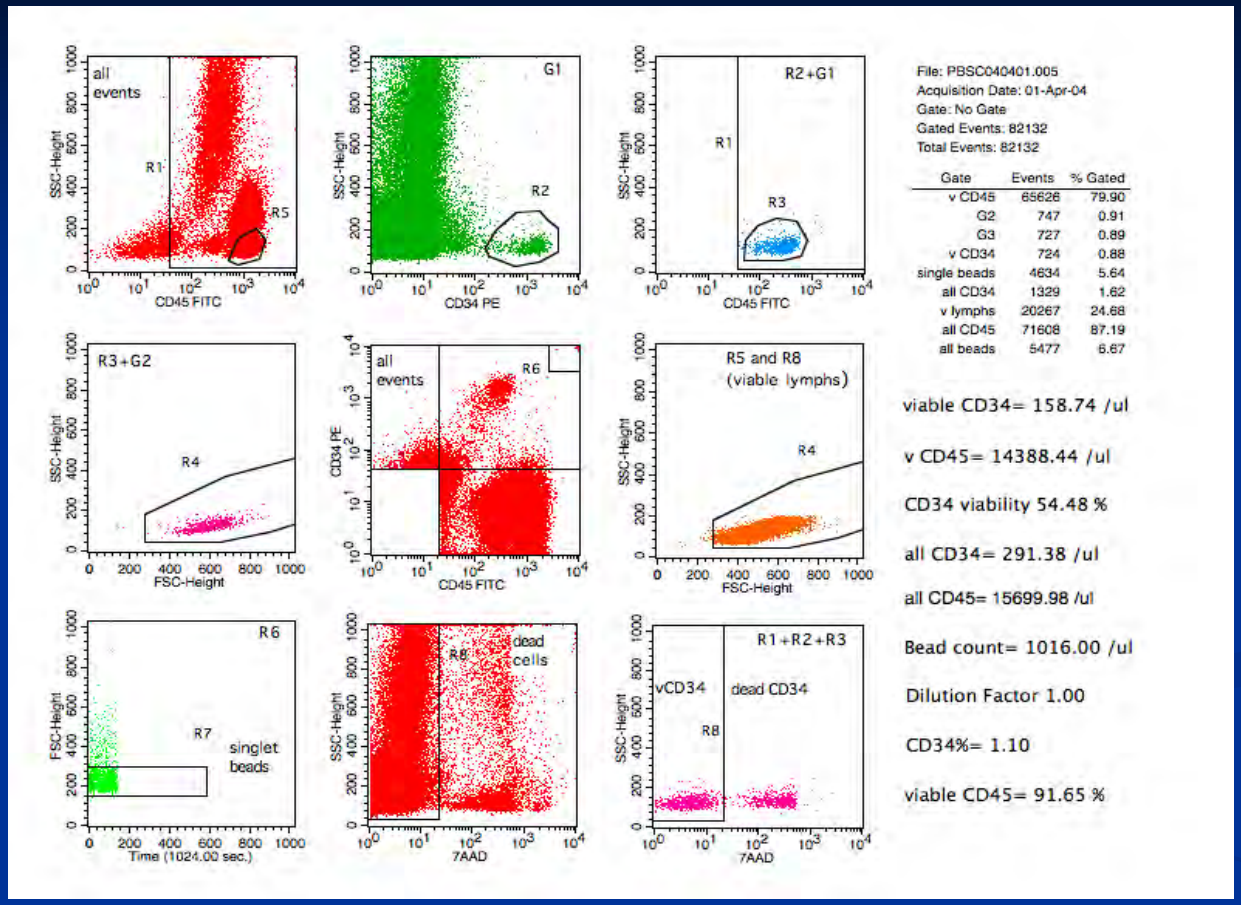
    - $CD34^{+}$, $CD45^{dim}$, $SSC^{low/int}$, $FSC^{low/int}$

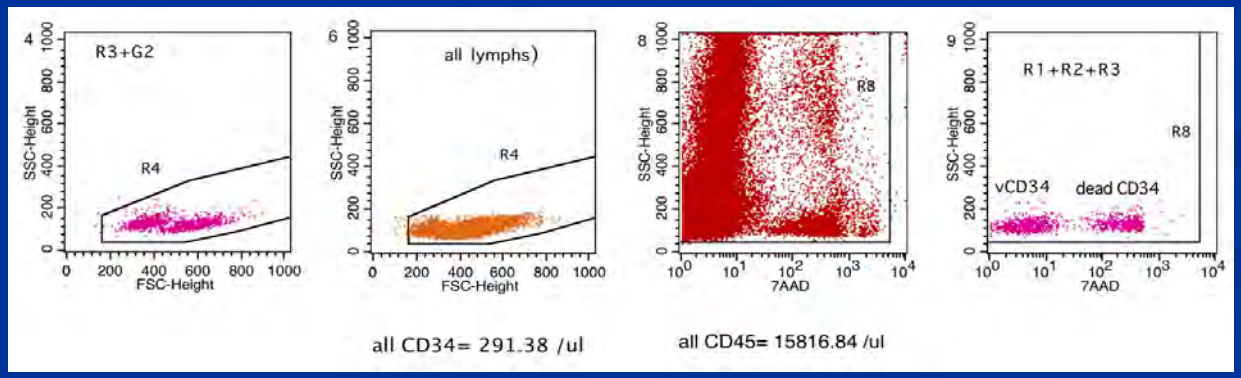
Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR.
Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE Guidelines.
Cytometry 34: 61-67, 1998

Evolution Of Flow Cytometric Methods For CD34 Enumeration

- Milan/Nordic - Single color, isotype controls, 2 platform (1992)
- ISHAGE - Dual color, sequential gating, 2 platform (1994-1996)
- BD ProCount™ - 3 color, sequential gating, single platform (1995)
  – no viability dye but automated software on FACSCalibur
- Single Platform (SP) ISHAGE - 3/4 color, with viability (1998)
- Stem-Kit™ Reagents (Beckman Coulter) (1999)
  – commercial variant of SP ISHAGE
  – automated software for EPICS-XL and FC500
- CD34 Count Kit™ DAKO
  – commercial variant of SP ISHAGE (Europe 2004)
- Stem Cell Enumeration Kit™ BD Biosciences
  – commercial variant of SP ISHAGE using TruCount™ tubes (2008)

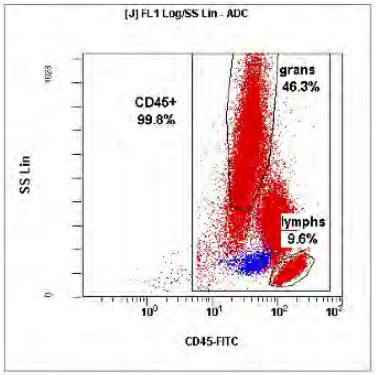
Stem-Kit on BD FACSCalibur

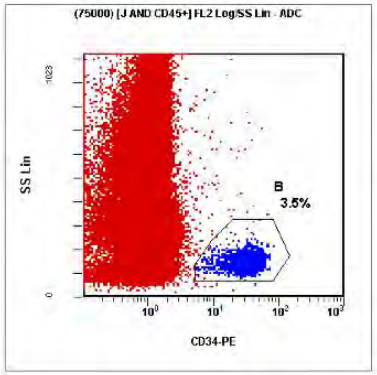


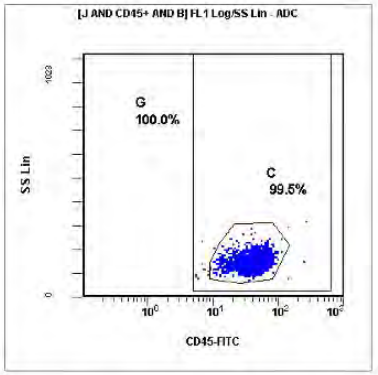


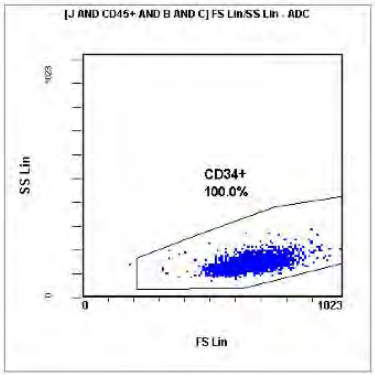
Importance of viability assessment
Viable cells only (7-AAD-negative)

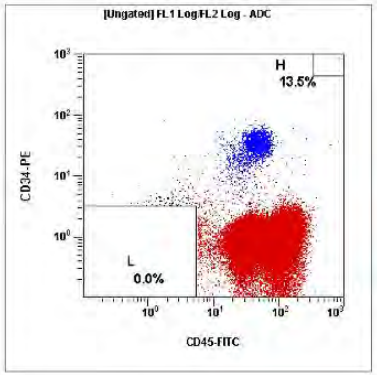
All cells: (live plus dead)

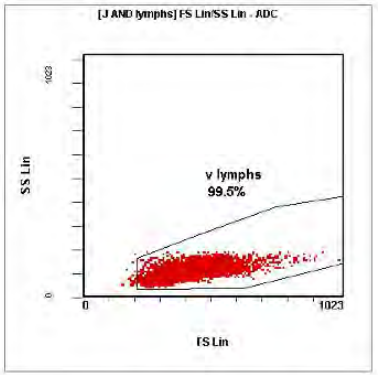


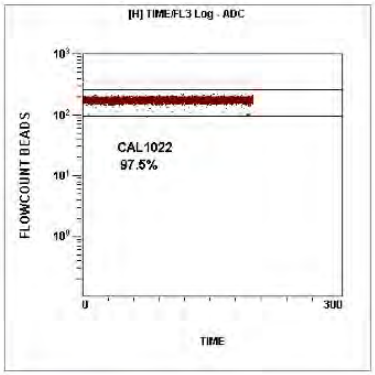


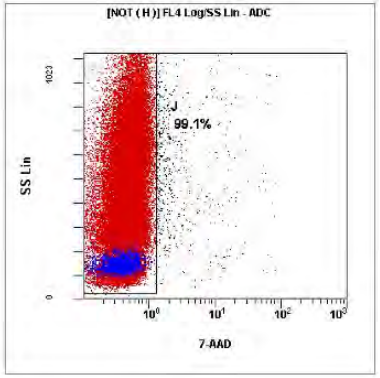


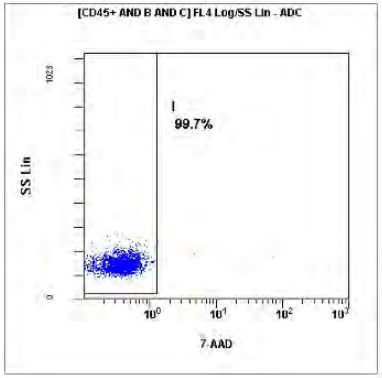








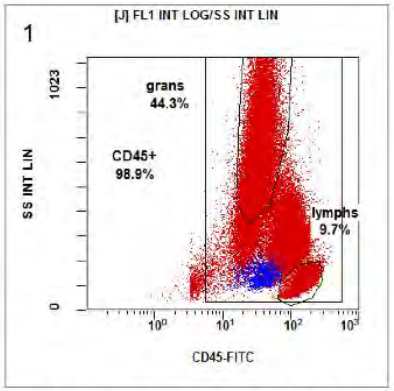


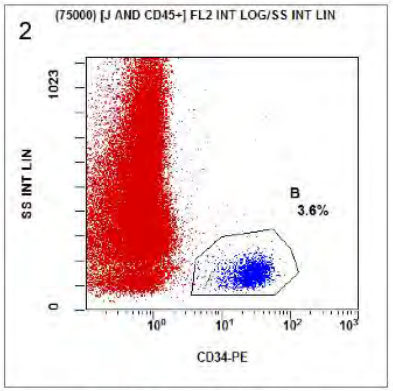


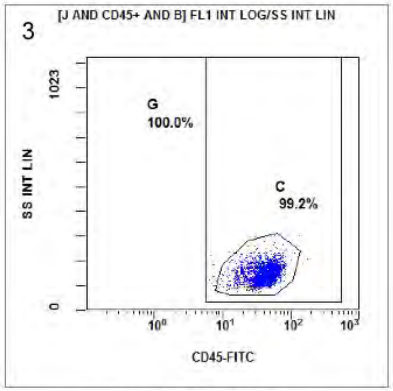
[Ungated] Legend

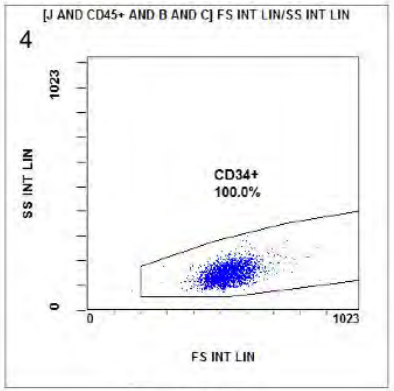
Color	Name	% Gated	% Total	Number	Cells/µL
	v CD34+	3.00	3.00	2653	233.02
	ALL CD34+ (C)	3.01	3.01	2662	233.81
	v CD45+	85.53	85.53	75564	6636.85
0x0100001A	ALL CD45+	86.49	86.49	76416	6711.68
	CAL	13.17	13.17	11636	1022.00

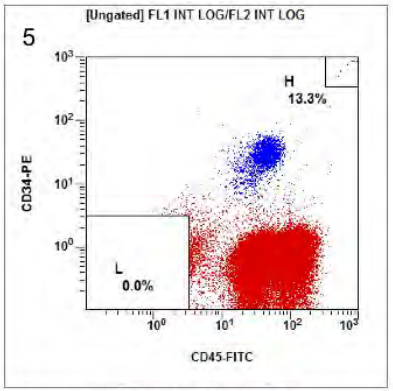
Stem-Kit™
ISHAGE manual protocol
Beckman Coulter FC500™

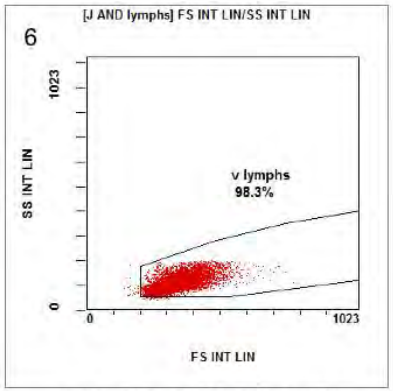


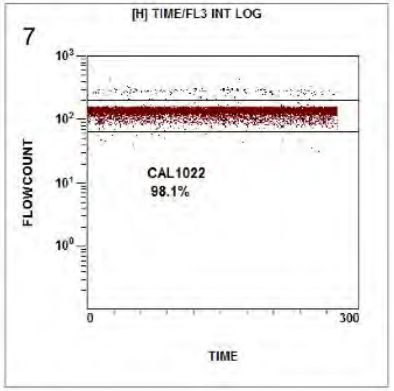


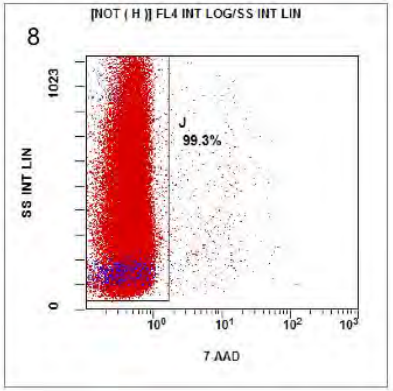


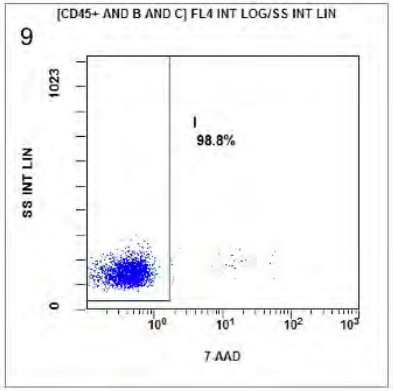










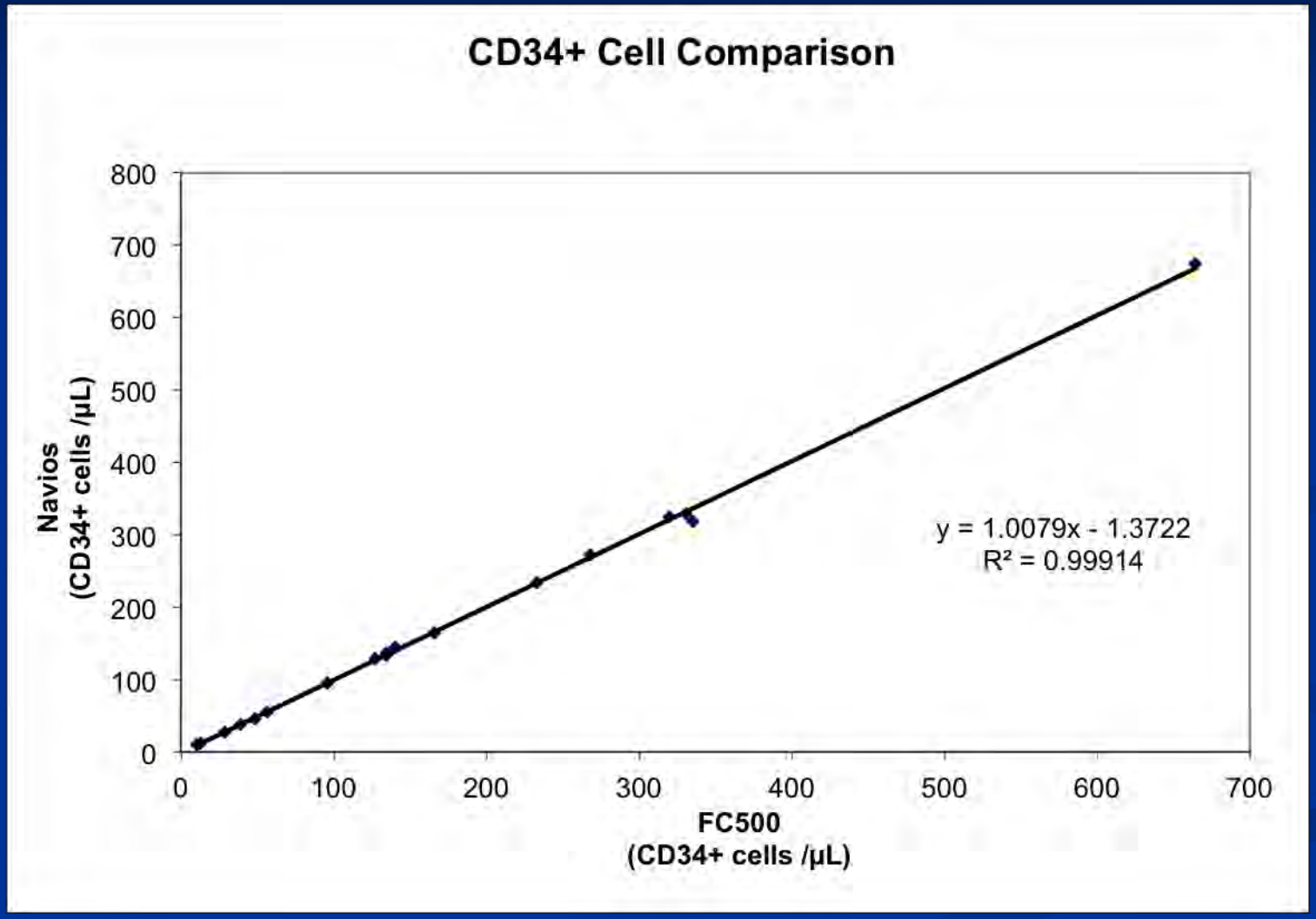


[Ungated] Legend

Color	Name	% Gated	% Total	Number	Cells/μL
	v CD34+	3.00	3.00	2644	234.32
	ALL CD34+	3.04	3.04	2676	237.16
	v CD45+	85.15	85.15	75011	6647.70
	ALL CD45+	86.65	86.65	76336	6765.12
	CAL	13.09	13.09	11532	1022.00

Stem-Kit™
ISHAGE manual protocol
Beckman Coulter Navios™

ISHAGE manual protocol: FC500 versus Navios



20 fresh PB or PBSC samples acquired on both instruments

ISSUES IN CD34+ CELL ENUMERATION

ISHAGE protocols are the most widely used methods to identify and enumerate CD34+ cells in both auto- and allo-transplant settings (3 or more ISHAGE-based kits).

Single Platform ISHAGE with viability assessment (7-AAD) is most accurate for fresh samples (PB, PBSC, CB and BM)

- reverse-pipetting mandatory!!

Samples must be lysed with NH_4Cl-based lysing agents

10 minutes at room temperature followed by immediate data acquisition

(or samples on melting ice for maximum 60 minutes to reduce lysing agent-induced death and apoptosis)

CB CD34+ cells less sensitive to the toxic effects of NH_4Cl

SINGLE PLATFORM ISHAGE WITH VIABILITY (7-AAD) ASSESSMENT

Non-fresh samples must be analyzed only with Single Platform ISHAGE

- shipped, CD34-selected, purged or otherwise manipulated

Post-thawed samples must be analyzed only with SP ISHAGE because the '%CD34' value in DP ISHAGE increases due to loss of most granulocytes post thaw

If pre-freeze WBC count is used with post thaw '%CD34+' in DP ISHAGE, more than 100% recovery of CD34+ cells is common

Hematology analyzers not accurate for post-thawed samples

ISSUES IN ENUMERATING VIABLE CD34+ CELLS IN POST-THAWED SAMPLES

How are samples processed/frozen?- many differences

How are samples thawed?

If samples diluted post thaw,

- Which diluent and how much?
- How is it done; drop-wise/dump?

Is centrifugation/washing employed after dilution?

How is staining performed;

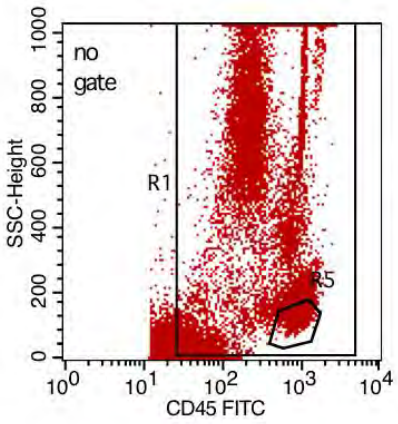
- In the cold or room temperature
- How long?

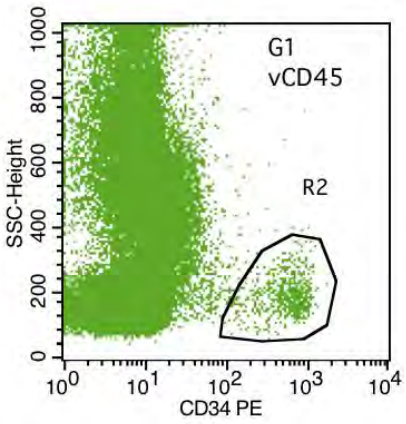
After staining, is lysing agent used?

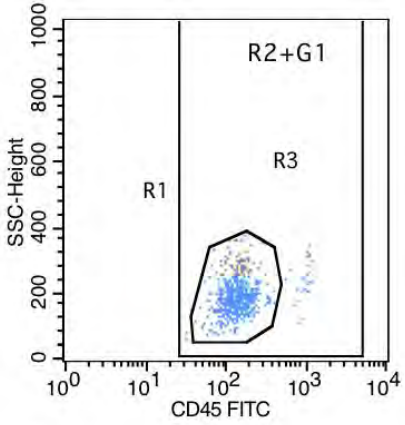
- Lysing agents increase prep time by 10 minutes at room temp during which cell death and apoptosis are increased

Despite Kit manufacturers' recommendations, lysing agents ARE NOT recommended by authors of ISHAGE protocols!!

POST-THAWED CORD BLOOD; NH_4Cl LYSE







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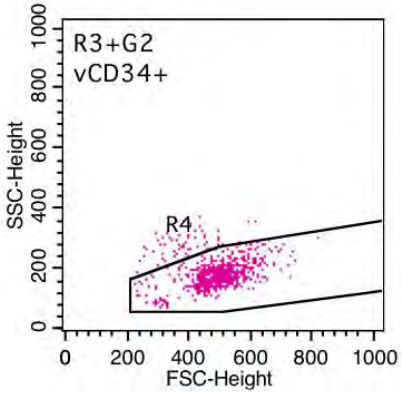
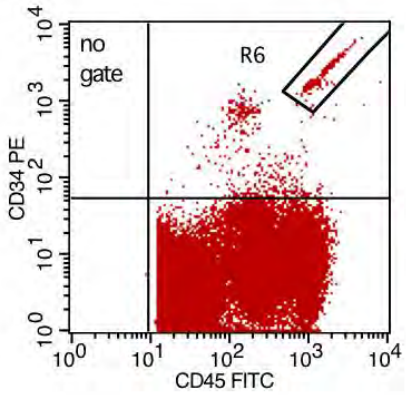
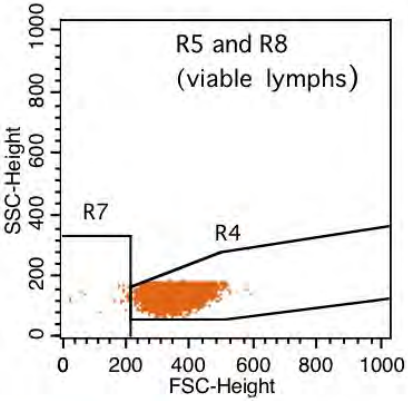
Sample ID: PW-A lyse
Acquisition Date: 10-Mar-06
Gate: No Gate
Gated Events: 280825
Total Events: 280825

Gate	Events	% Gated
v CD45	61100	21.76
G2	665	0.24
G3	636	0.23
v CD34	573	0.20
Beads	16868	6.01
all CD34	662	0.24
lymphs	19296	6.87
all CD45	99663	35.49
Debris	164087	58.43
R9 beads	29	0.01







viable CD34= 33.83 /ul

all CD34= 39.09 /ul

CD34 viability= 86.56 %

viable CD45= 3607.75 /ul

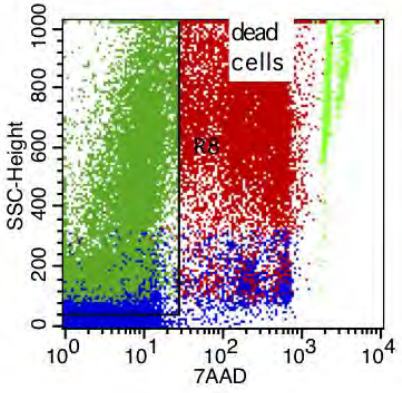
all CD45= 5884.77 /uL

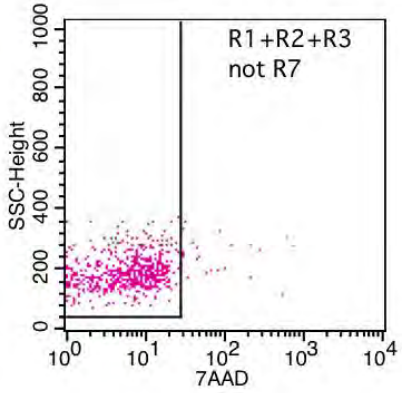
% Viable CD45= 61.31

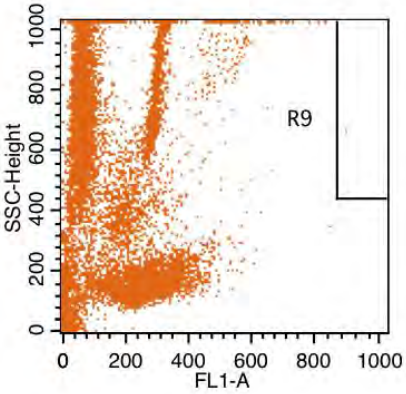
bead count = 49800.00

dilution factor 1.00

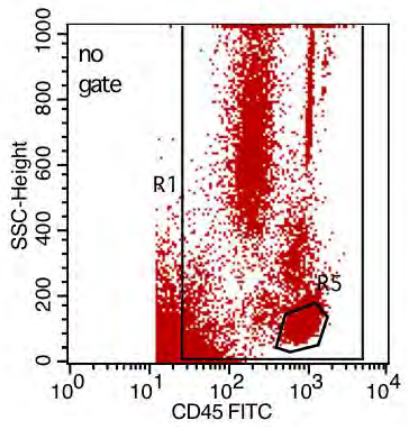
Sample volume 50.00 ul

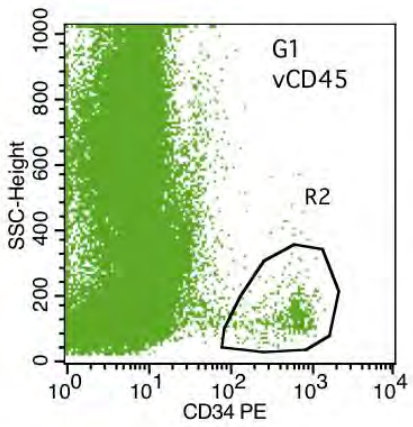


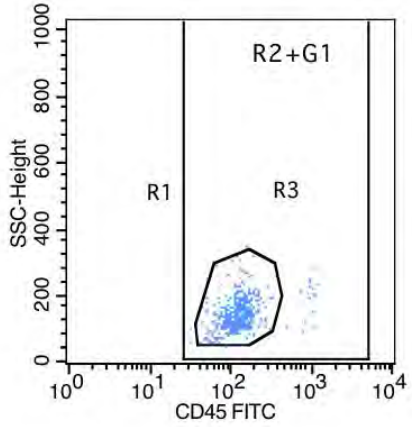




POST-THAWED CORD BLOOD; NO LYSE







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Acquisition Date: 10-Mar-06
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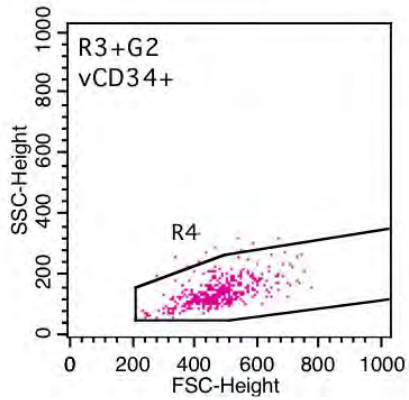
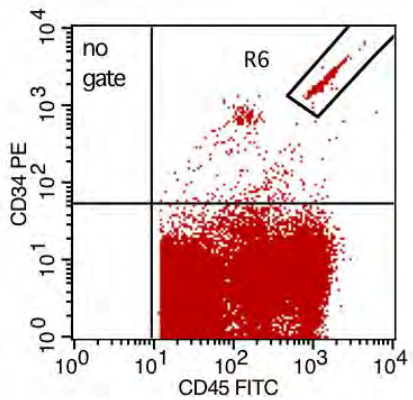
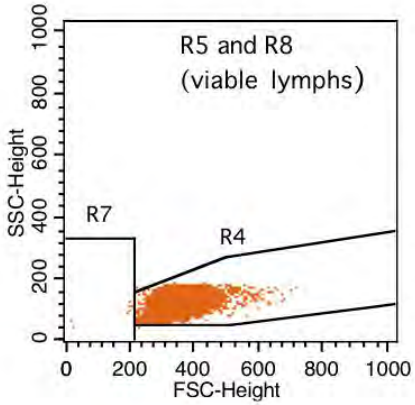
Gated Events: 189427
Total Events: 189427

Gate	Events	% Gated
v CD45	65093	34.36
G2	475	0.25
G3	451	0.24
v CD34	440	0.23
Beads	10795	5.70
all CD34	469	0.25
lymphs	17547	9.26
all CD45	78115	41.24
Debris	92965	49.08
R9 beads	28	0.01







viable CD34= 40.60 /ul

all CD34= 43.27 /ul

CD34 viability= 93.82 %

viable CD45= 6005.80 /ul

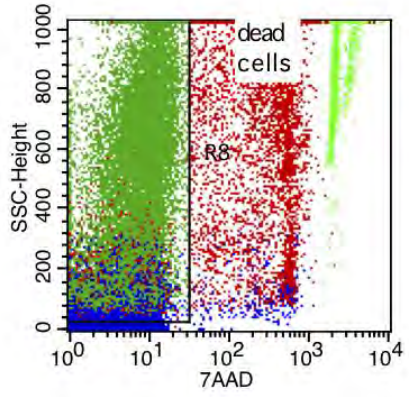
all CD45= 7207.28 /uL

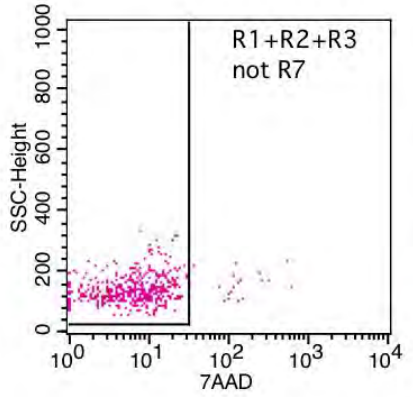
% Viable CD45= 83.33

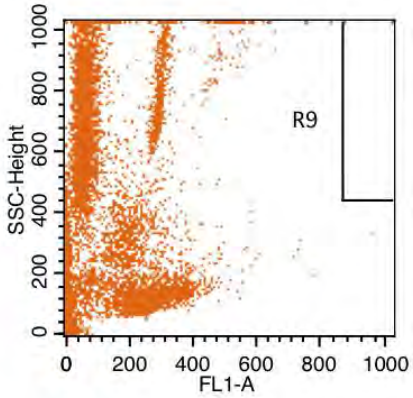
bead count = 49800.00

dilution factor 1.00

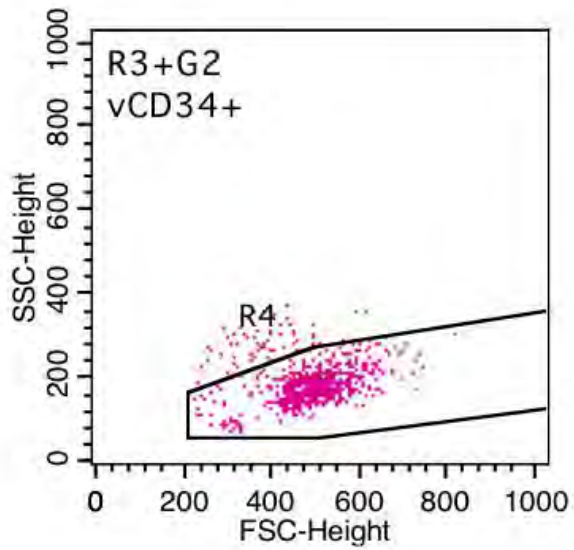
Sample volume 50.00 ul







NH_4CL-LYSE
10 min @ RT



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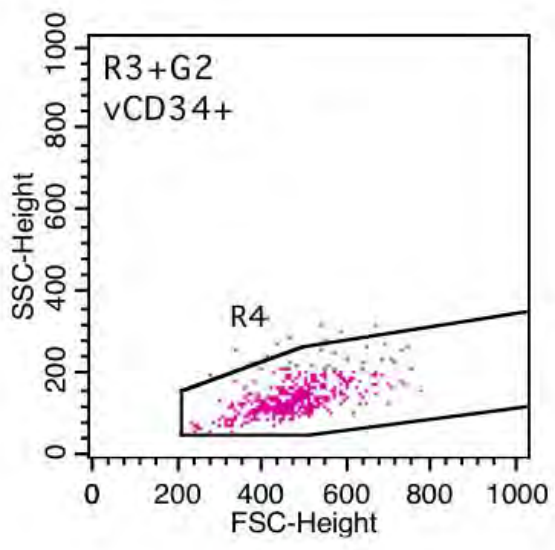
viable CD45= 3607.75 /ul

all CD45= 5884.77 /uL

% Viable CD45= 61.31

No LYSE
Acquired ASAP



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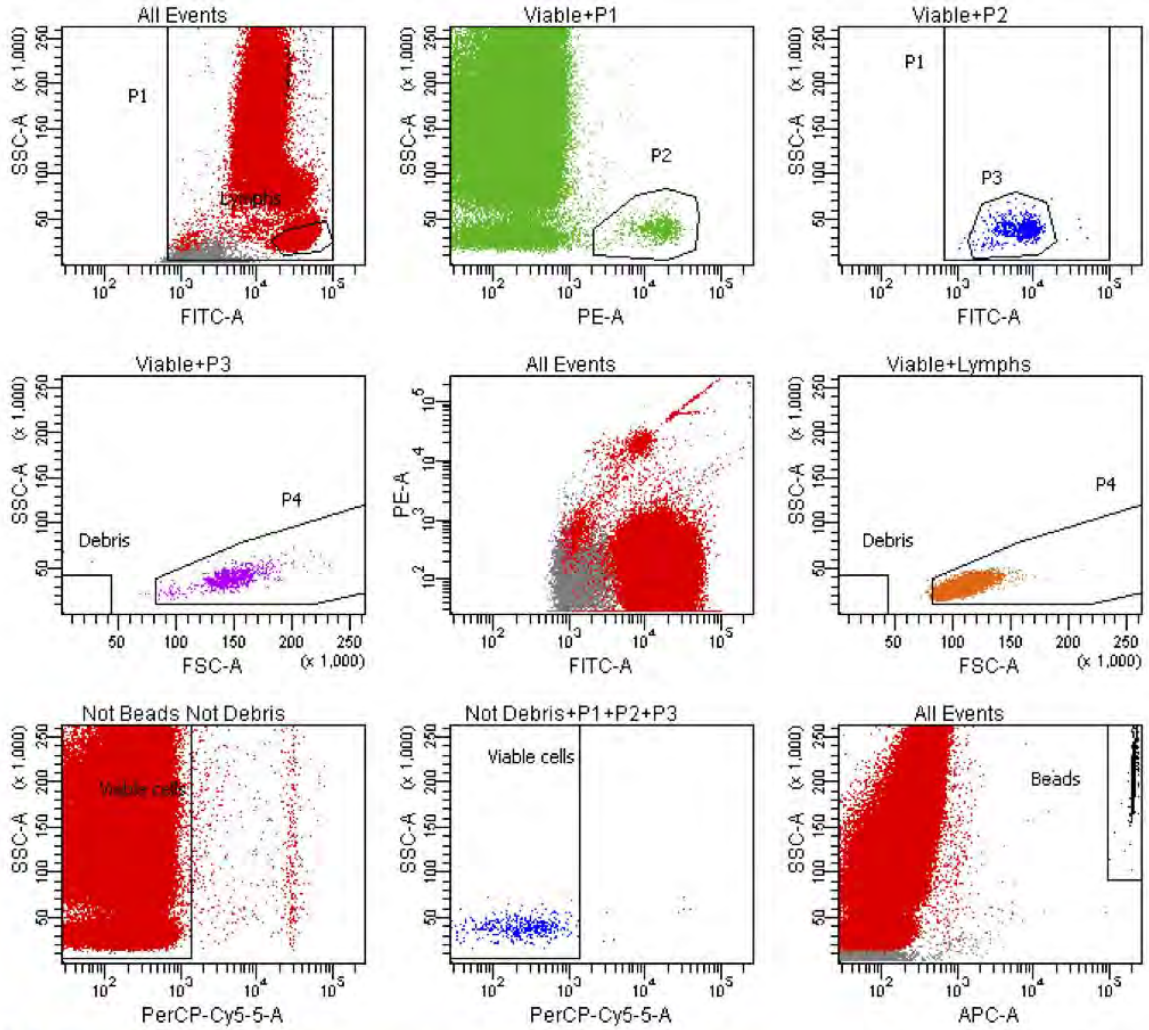
% Viable CD45= 83.33

TruCount-ISHAGE (SCE-Kit™) Canto II



Specimen Name: 96 hours
Tube Name: GC05-1-96
Record Date: Apr 19, 2013 12:06:02 PM

Tube: GC05-1-96

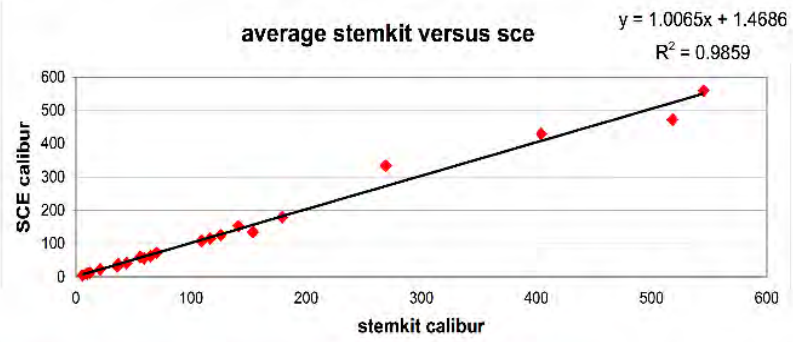
Population	#Events	%Total
All Events	295,771	100.0
Debris	18,769	6.3
Not Debris	277,002	93.7
Beads	3,724	1.3
Not Beads	273,278	92.4
P1	273,262	92.4
P2	766	0.3
P3	749	0.3
P4	738	0.2
Lymphs	30,826	10.4
Viable cells	272,518	92.1
Viable Lymphs	30,811	10.4
Viable P1	272,515	92.1
Viable P2	750	0.3
Viable P3	742	0.3
Viable P4	737	0.2

Viable CD34 = 98.84/ul
Viable CD45 = 36548.76/ul
Total CD34 = 100.45/ul
Total CD45 = 36648.95/ul
CD34 Viability = 98.39%
CD45 Viability = 99.72%

Calculations
Viable CD34 cells/ul = ([Viable P4] x Bead Count x DF) / (beads x SV)
Viable CD45 cells/ul = ([Viable P1] x Bead Count x DF) / (beads x SV)
Total CD34 cells/ul = ([P3] x Bead Count x DF) / (beads x SV)
Total CD45 cells/ul = ([P1] x Bead Count x DF) / (beads x SV)
CD34 Viability = [Viable P4] / [P3] x 100
CD45 Viability = [Viable P1] / [P1] x 100

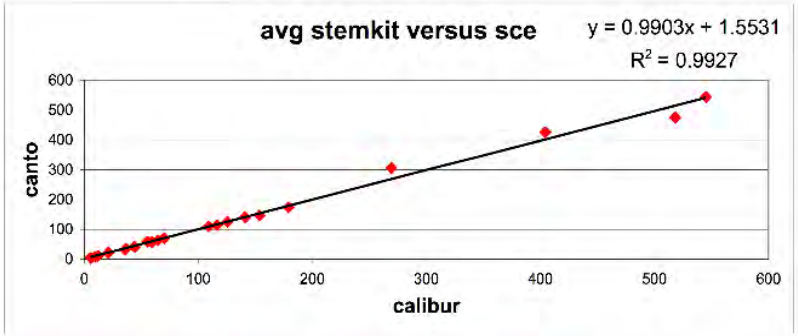
Bead Count: 49945
Sample Volume (SV): 100
Dilution Factor (DF): 1
Bead Lot#

Stem-Kit vs SCE on FACSCalibur



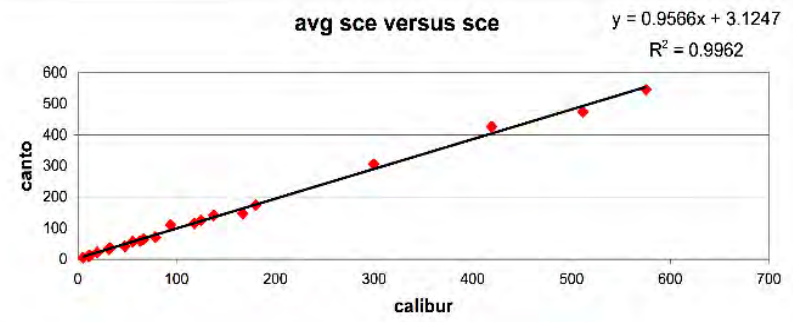
Data points represent the means of duplicate samples stained with Stem-Kit and the BD SCE reagent set and analysed on FACSCalibur

Stem-Kit on FACSCalibur vs SCE on FACSCanto



Data points represent the means of duplicate samples stained with Stem-Kit and analysed on FACSCalibur, or with the SCE reagents and analysed on a FACSCanto

SCE on FACSCalibur vs SCE on FACSCanto



Data points represent the means of duplicate samples stained with the BD SCE reagent set and analysed on both FACSCalibur and FACSCanto

Sutherland DR, Nayyar R, Acton E, Giftakis A, Dean S, Mosiman V.
Comparison of Two Single Platform ISHAGE-based CD34 Enumeration Protocols on FACSCalibur™ and FACSCanto™ Cytometers.
Cytotherapy 11: 595-605, 2009

Important to Monitor Viability

- Overnight storage and/or shipping of product may lead to cell death
- Purging, T cell depletion or other manipulations may negatively impact viability
- Cord blood and bone marrow contain a variable percentage of dead cells
- 7-AAD - viability dye added to single platform ISHAGE method allows direct assessment of cell viability

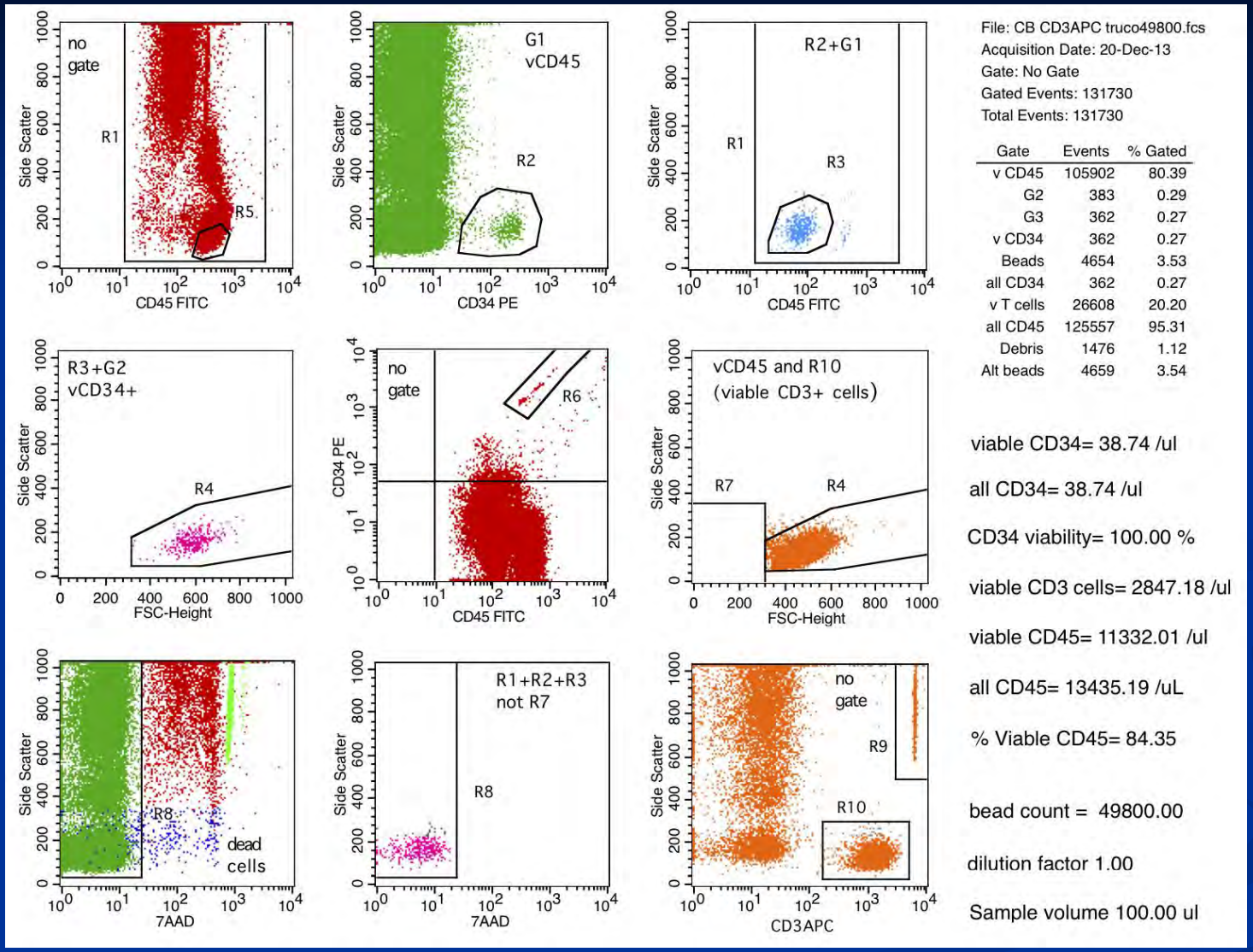
When Should CD34+ Cell Viability Be Assessed?

- Fresh blood and PBSC under 4 hours old
  - probably not required (CAP)

- *Single Platform With Viability Assessment Essential*
  - Cord Blood
  - Bone Marrow
  - PBSC stored overnight – unless validated
  - Post-cryopreserved samples*

  *CFC assays also recommended for post-thawed CB samples

ISHAGE Single Platform Protocol for Allograft Assessment



File: CB CD3APC truco49800.fcs
Acquisition Date: 20-Dec-13
Gate: No Gate
Gated Events: 131730
Total Events: 131730

Gate	Events	% Gated
v CD45	105902	80.39
G2	383	0.29
G3	362	0.27
v CD34	362	0.27
Beads	4654	3.53
all CD34	362	0.27
v T cells	26608	20.20
all CD45	125557	95.31
Debris	1476	1.12
Alt beads	4659	3.54

viable CD34= 38.74 /ul

all CD34= 38.74 /ul

CD34 viability= 100.00 %

viable CD3 cells= 2847.18 /ul

viable CD45= 11332.01 /ul

all CD45= 13435.19 /uL

% Viable CD45= 84.35

bead count = 49800.00

dilution factor 1.00

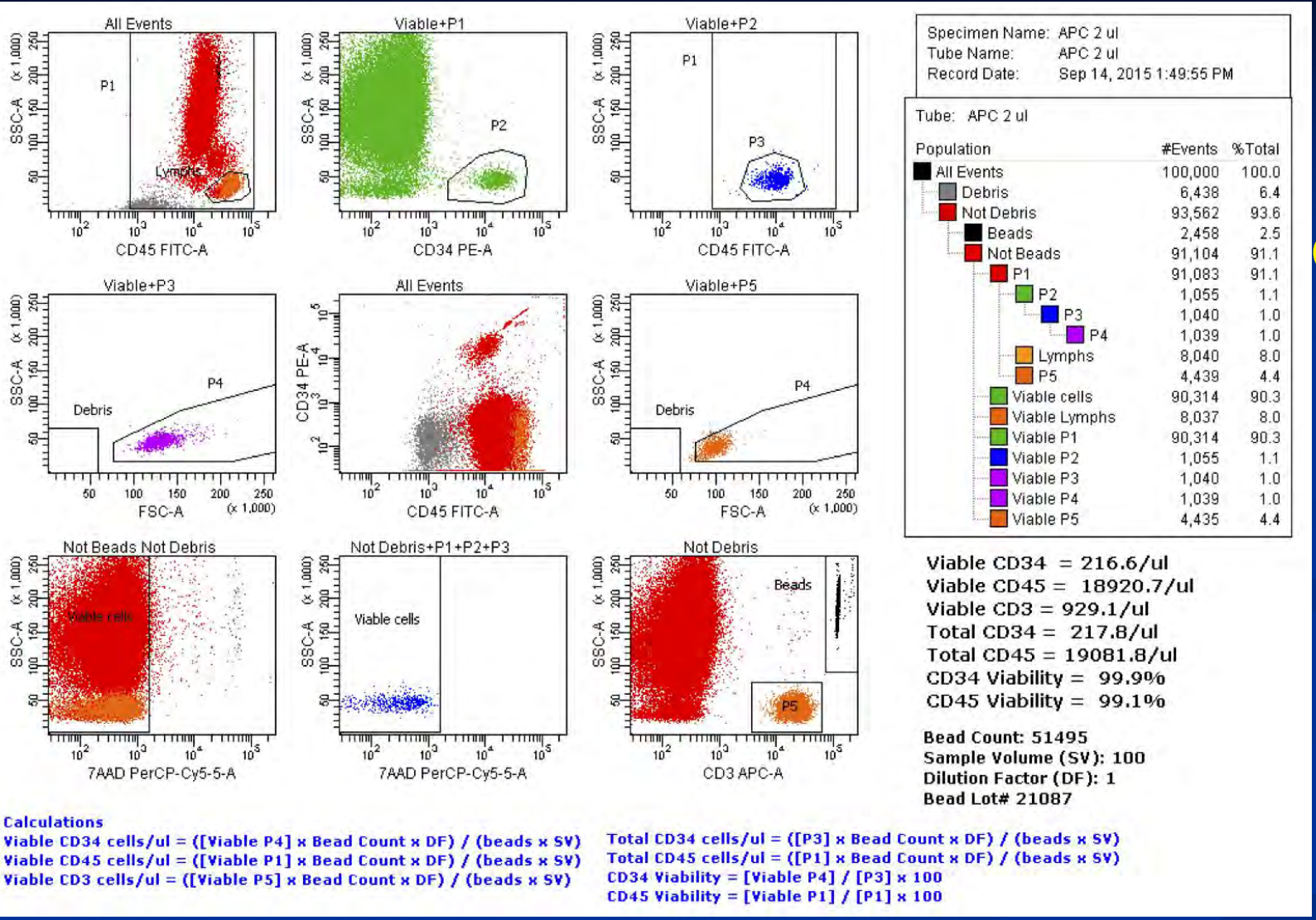
Sample volume 100.00 ul

CD45-FITC
CD34-PE
7-AAD
CD3-APC
Trucount

FACSCalibur

Enumerate viable CD34+ and CD3+ cells for allo-transplants, assess CD3+ cell content in selected CD34+ cell preps for non-matched transplants

ISHAGE Single Platform Protocol for Allograft Assessment



Specimen Name: APC 2 ul
Tube Name: APC 2 ul
Record Date: Sep 14, 2015 1:49:55 PM

Tube: APC 2 ul

Population	#Events	%Total
All Events	100,000	100.0
Debris	6,438	6.4
Not Debris	93,562	93.6
Beads	2,458	2.5
Not Beads	91,104	91.1
P1	91,083	91.1
P2	1,055	1.1
P3	1,040	1.0
P4	1,039	1.0
Lymphs	8,040	8.0
P5	4,439	4.4
Viable cells	90,314	90.3
Viable Lymphs	8,037	8.0
Viable P1	90,314	90.3
Viable P2	1,055	1.1
Viable P3	1,040	1.0
Viable P4	1,039	1.0
Viable P5	4,435	4.4

Viable CD34 = 216.6/ul
Viable CD45 = 18920.7/ul
Viable CD3 = 929.1/ul
Total CD34 = 217.8/ul
Total CD45 = 19081.8/ul
CD34 Viability = 99.9%
CD45 Viability = 99.1%

Bead Count: 51495
Sample Volume (SV): 100
Dilution Factor (DF): 1
Bead Lot# 21087

Calculations

Viable CD34 cells/ul = ([Viable P4] x Bead Count x DF) / (beads x SV)
Viable CD45 cells/ul = ([Viable P1] x Bead Count x DF) / (beads x SV)
Viable CD3 cells/ul = ([Viable P5] x Bead Count x DF) / (beads x SV)

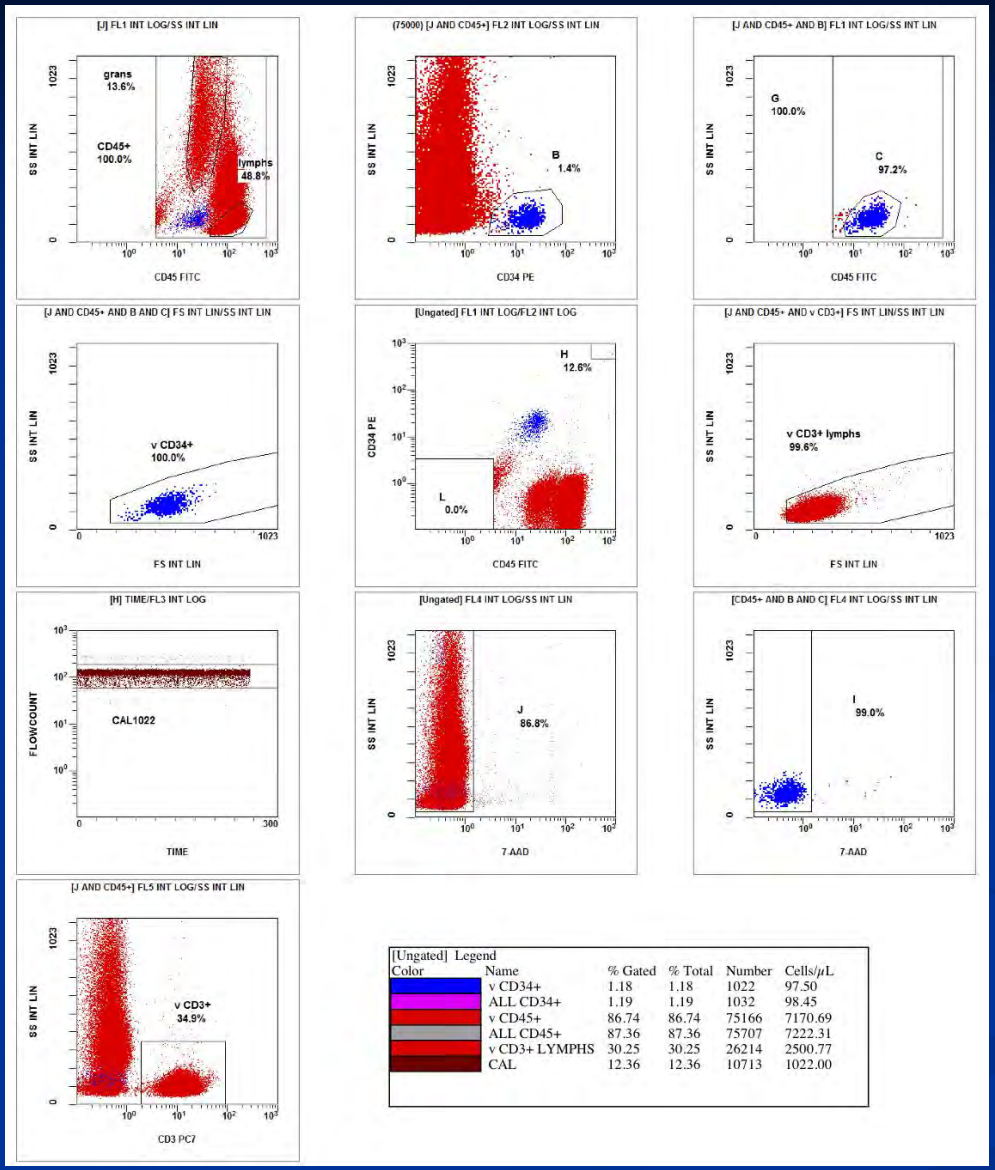
Total CD34 cells/ul = ([P3] x Bead Count x DF) / (beads x SV)
Total CD45 cells/ul = ([P1] x Bead Count x DF) / (beads x SV)
CD34 Viability = [Viable P4] / [P3] x 100
CD45 Viability = [Viable P1] / [P1] x 100

CD45-FITC
CD34-PE
7-AAD
CD3-APC
Trucount

Canto II

Enumerate viable CD34+ and CD3+ cells for allo-transplants and assess CD3+ cell content in selected CD34+ cell preps for non-matched transplants
Count viable CD3+ cells for Donor Lymphocyte Infusions

ISHAGE Single Platform Protocol for Allograft Assessment

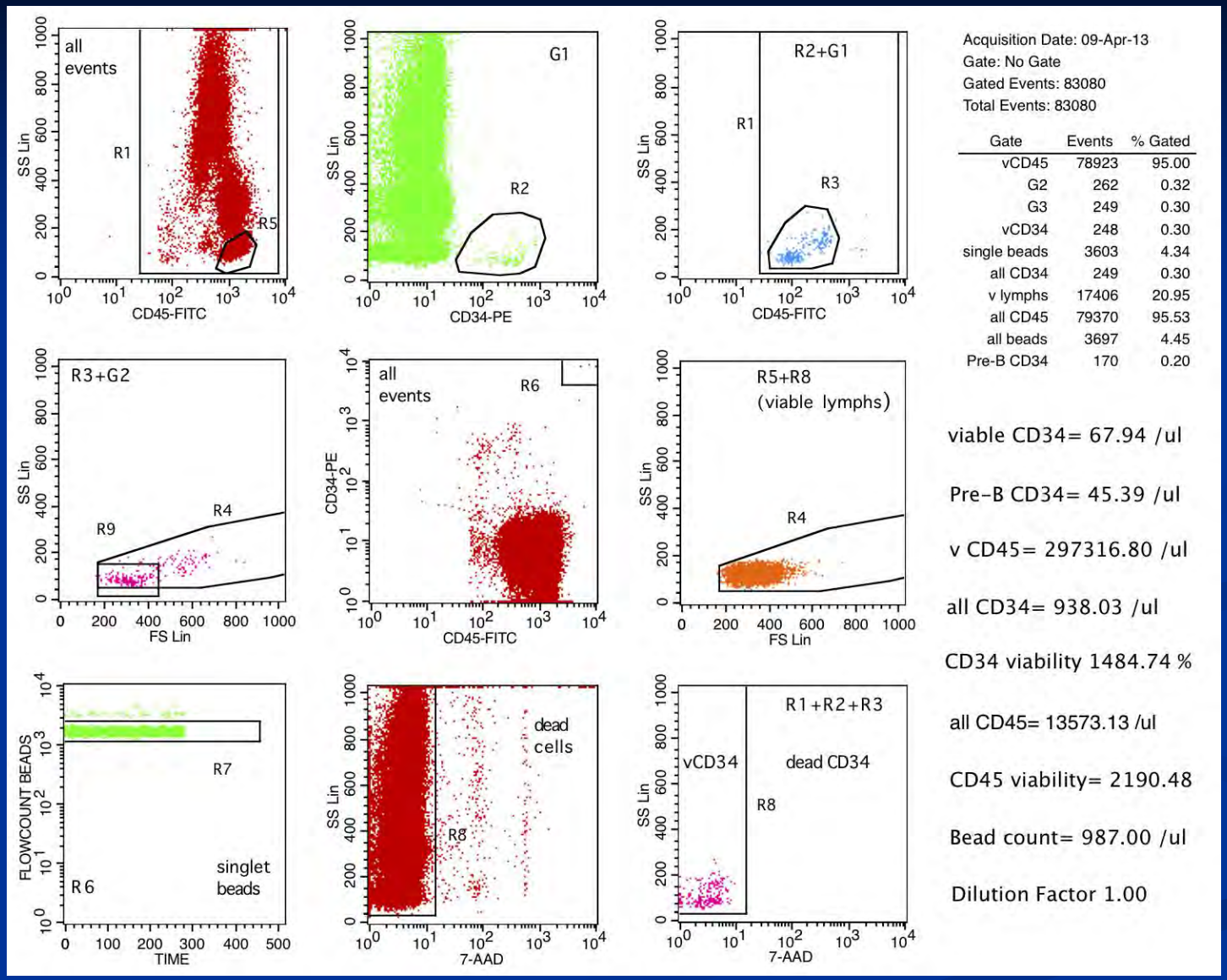


CD45-FITC
CD34-PE
FlowCount
7-AAD
CD3-PEC7

Navios

Enumerate viable CD34+ and CD3+ cells for allo-transplants, assess CD3+ cell content in selected CD34+ cell preps for non-matched transplants

Mobilized PBSC with Hematogones



Acquisition Date: 09-Apr-13
Gate: No Gate
Gated Events: 83080
Total Events: 83080

Gate	Events	% Gated
vCD45	78923	95.00
G2	262	0.32
G3	249	0.30
vCD34	248	0.30
single beads	3603	4.34
all CD34	249	0.30
v lymphs	17406	20.95
all CD45	79370	95.53
all beads	3697	4.45
Pre-B CD34	170	0.20

viable CD34= 67.94 /ul

Pre-B CD34= 45.39 /ul

v CD45= 297316.80 /ul

all CD34= 938.03 /ul

CD34 viability 1484.74 %

all CD45= 13573.13 /ul

CD45 viability= 2190.48

Bead count= 987.00 /ul

Dilution Factor 1.00

Thiago LS and Sutherland DR. CD34+ B-cell progenitors in Mobilized Peripheral Blood Apheresis Collections: Implications for flow cytometric assessment of graft adequacy. Cytotherapy 2015; 17: 689-691.

Enumerating CD34+ Cells With ISHAGE Protocols

Detect CD34+ cells in normal and abnormal samples and discriminate specific from non-specific staining without isotypic controls

Discriminate live CD34+ cells from dead and apoptopic CD34+ cells
- CD34+ cells in freeze - thawed samples
- CD34+ cells in post-purged samples

Generates absolute viable CD34+ cell count in 45 minutes

Works on all clinical cytometers tested

Commercial variants: Stem-Kit™, CD34Count™, SCE-Kit™

Simultaneously enumerate viable T cells and viable CD34+ cells in single tube in combination with counting beads and viability dyes

Enumerate 'key' CD34+ subsets:
- candidate stem cell subsets (e.g. CD34+/CD90+)

Gain Consensus – the Hard Part!

Take the show on the road

- identify key groups
- take the message to them
- answer the hard questions
- accept criticism (gracefully if possible):
    - this is what good science is all about!
- teach workshops
- do talks
- visit MetroFlow

Develop and publish Consensus Guidelines

CORRESPONDENCE ARISING FROM THE ISHAGE GUIDELINES PUBLICATIONS

Sutherland DR. Assessment of Peripheral Blood Stem Cell Grafts by CD34+ Cell Enumeration: Towards a standardized flow cytometric approach. J. Hematother. 5: 209-210, 1996 (Editorial).

Knape CC. Standardisation of absolute CD34 cell enumeration. Letter to Editor. J. Hematother. 5: 211-2, 1996.

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE Guidelines For CD34+ Cell Determination By Flow Cytometry. J. Hematother. 5: 213-226, 1996.

Weinberg DS and Benjamin RJ. QBEnd10 (CD34) antibody is unsuitable for routine use in the ISHAGE CD34+ cell determination assay. Letter to Editor. J. Hematother 6: 599-603, 1996.

Sutherland DR, **Anderson L, Keeney M, Nayar R, Chin-Yee I.** re: QBEnd10 (CD34) antibody is unsuitable for routine use in the ISHAGE CD34+ cell determination assay. J. Hematother. 5: 601-603, 1996.

Johnsen HE. Toward a worldwide standard for CD34+ enumeration? Letter to Editor. J Hematother. 6:83-84, 1996

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. Re: Toward a worldwide standard for CD34+ enumeration. J Hematotherapy 6: 85-89, 1996.

Marti GE, Johnsen HE, Sutherland DR, Serke S. A convergence of methods for a world wide standard for CD34+ cell enumeration. Letter to the Editor. J. Hematotherapy. 7:105, 1998.

Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE Guidelines. Cytometry 34: 61-7, 1998.

Serke S. CD34+ guidelines between science and commerce. Letter to Editor. Cytometry 34:286-288, 1998.

Keeney M, Chin-Yee I, Sutherland DR. re: CD34+ guidelines between science and commerce. Cytometry 34:287-288, 1998.

Serke S, van Lessen A, Pardo I, Huhn D. Selective susceptibility of CD34-expressing cells to acquire flow cytometric features of apoptosis/necrosis on exposure to an ammonium chloride-based red blood cell lysing reagent. J Hematotherapy 8:315-318, 1998.

Keeney M, Chin-Yee I, Nayar R, Sutherland DR. Effect of Fixatives on CD34+ cell enumeration. J. Hematother & Stem Cell Res 8: 327-329, 1999.

Johnsen HE. The real CD34+ events: simplicity or complexity? Lettere to Editor. Exp. Hematol. 26:550-551, 1998

Gratama JW, Keeney M, Sutherland DR, Papa S. The real CD34+ events: simplicity versus accuracy and flexibility. Letter to editor, response to Johnsen HE. Exp. Hematol. 27: 975-977, 1999.

Chang A, and Ma DD

The influence of gating strategy on the standardization of CD34+ cell quantitation: An Australian multicenter study.

J Hematotherapy 5:605, 1996.

24 labs analysed list mode data files from 2 PBSC samples

When all labs used the ISHAGE gating strategy, reproducible results obtained and results from all centres within +/-10% of the median

When different gating strategies used, significantly different results obtained ($p<0.006$)

"the FCM gating strategy was a critical issue for standardization...
the (ISHAGE) guideline utilizing FSC, SSC, CD34 and CD45 gave most reproducible results"

FLOW CYTOMETRIC ENUMERATION OF CD34$^+$ HEMATOPOIETIC STEM AND PROGENITOR CELLS

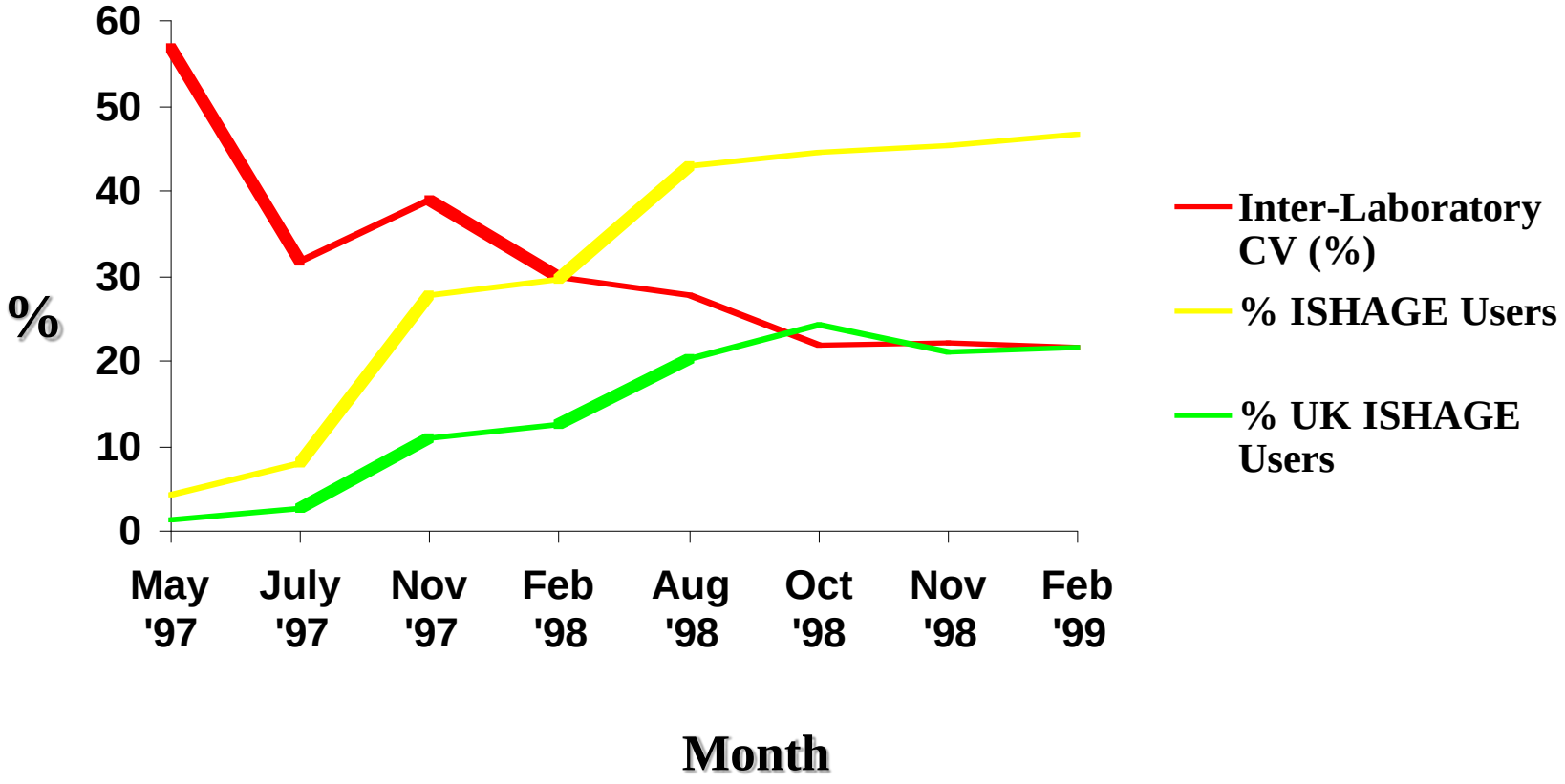
From The European Working Group On Clinical Cell Analysis

J.W. Gratama, A. Orfao, D. Barnett, B. Brando, A. Huber, G. Janossy, H.E. Johnsen, M. Keeney, G. E. Marti, F. Preijers, G. Rothe, S. Serke, D. R. Sutherland, C. E. Van der Schoot, G. Schmitz, S. Papa

1. Bright conjugates (PE) of class II or class III monoclonal antibodies that detect all glycoforms of CD34
2. Use of a vital nucleic acid dye to exclude platelets, unlysed red cells and debris, or use of 7-amino actinomycin D (7-AAD) to exclude dead cells during data acquisition
3. CD45 staining to be included in the definition of HPC
4. Use of Boolean gating to resolve the CD34$^+$ HPC from irrelevant cells based on low levels of CD45 expression/low side scatter
5. Inclusion of CD34^{dim} and CD34^{bright} CD34$^+$ cells
6. Omission of the negative control staining (isotypic or isoclonic)
7. For apheresis products, enumeration of at least 100 CD34$^+$ cells to ensure a 10% precision

Cytometry (Communications in Clinical Cytometry). 34: 128, 1998

Relationship of Inter-Laboratory CV and ISHAGE Sequential Gating Strategy



Courtesy D. Barnett. UK NEQAS. Sheffield UK

EWGCCA CD34 Task Force Quality Assurance Study

3 send outs of stabilized blood specimens

24 labs over a period of 6 months (11/98-4/99)

Method used - EWGCCA standard protocol (ISHAGE single platform)

Wet workshop, coordinating centres, standard reporting format

EWGCCA QC Study - Conclusions

By the third send out 16 (70%) and 19 (83%) of labs had intra-lab C.V.s of <5%, using Flow-Count and Trucount respectively (no statistical difference)

C.V. improved over time

target C.V. of 10% by >2/3 of participating labs met

Conclusions:

Stabilized samples, targeted training and technical support led to improved CVs compared to UK NEQAS survey

Single platform confirmed as the most reproducible method

Barnett D, et al, British Journal of Hematology 108:784, 2000

Write Consensus Guidelines

Gratama JW Keeney M, and Sutherland DR. Enumeration of $CD34^+$ Hematopoietic Stem and Progenitor Cells.
In: Current Protocols in Cytometry Unit 6.4.1 - 6.4.22, 1999

Sutherland DR, Keeney M, and Gratama JW. Enumeration of CD34+ Hematopoietic Stem and Progenitor Cells.
In: Current Protocols in Cytometry: Unit 6.4.1 - 6.4.23, 2003 (update soon?)

Gratama JW, Kraan J, Keeney M, Mandy F, Sutherland DR, Wood BL
Clinical Laboratory Sciences Institute (CSLI)
H42-A2 Volume 27 Number 16. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition 2007

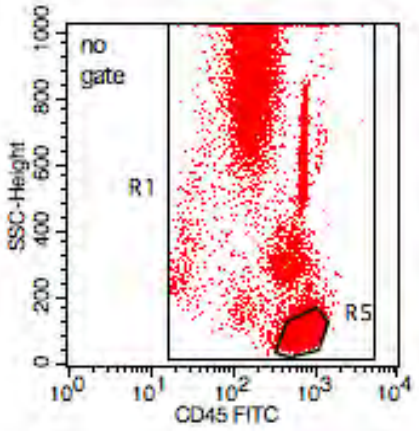
Keeney M, Sutherland DR.
Current methods for identification of hematopoietic stem and progenitor cells in the clinical laboratory.
In: Flow Cytometry in Clinical Diagnosis (4^{th} Edition) (Keren DF, McCoy JP Jr, Carey JL Eds). ASCP Press Chicago Illinois USA. Chapter 16 pp 321-344, 2007

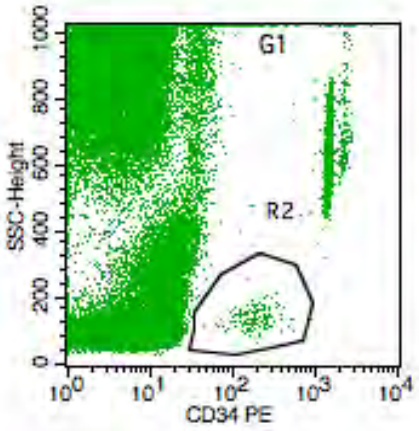
Sutherland DR, Keeney M. Enumeration of CD34+ cells by Flow Cytometry.
In: Aremen EM, Loper K, eds, Cellular Therapy: Principles, Methods and Regulations. An American Association of Blood Bankers Cell Therapy Technical Manual, Bethesda MD pp 538 – 54, 2009
And Second Edition, Chap 56, 558-569 and Method 56-1, 809-823, 2016

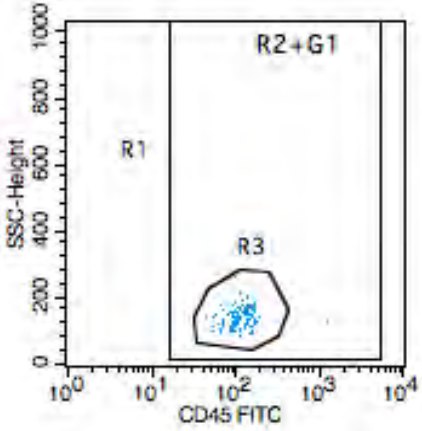
Quality Assurance

- External quality assurance is an essential part of clinical testing (not just CD34!!)
- Several commercial products are available for CD34 QA
- All current QA products are stabilized cells
- Analysis with 7-AAD requires that all events LIVE and DEAD are included

- See following slide

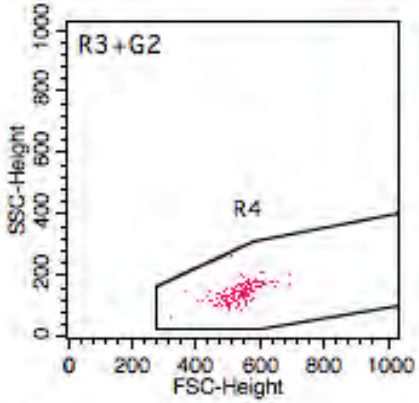


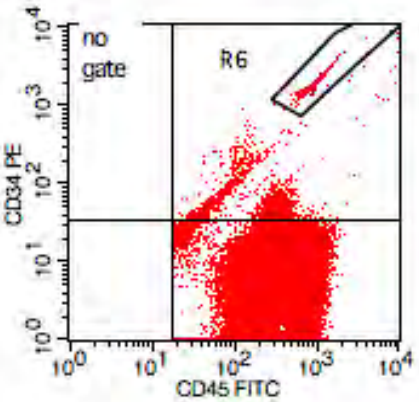


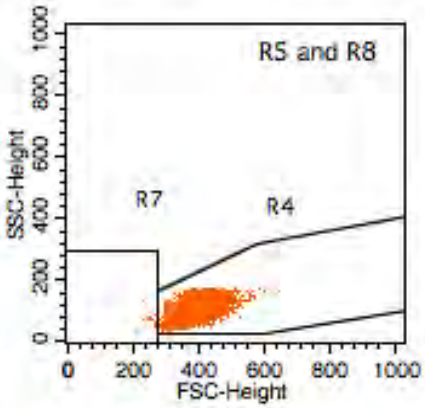


Gate: No Gate
Gated Events: 75040
Total Events: 75040

Gate	Events	% Gated
v CD45	74542	99.34
G2	138	0.18
G3	137	0.18
v CD34	137	0.18
Beads	5447	7.26
all CD34	137	0.18
lymphs	22218	29.61
all CD45	69152	92.15
Debris	441	0.59







viable CD34= 12.56 /ul

viable CD45= 6834.96 /ul

CD34 viability= 100.00 %

all CD34= 12.56 /ul

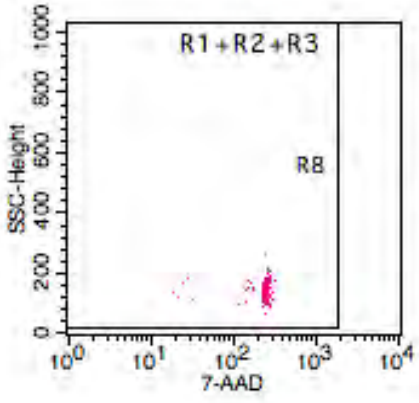
all CD45= 6340.73 /uL

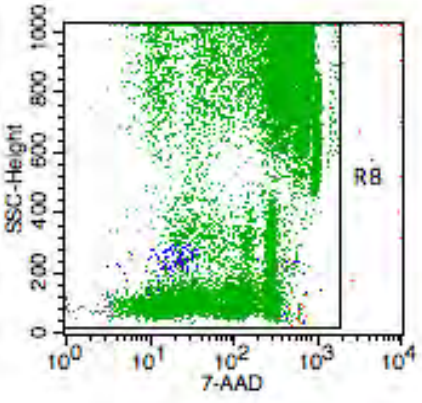
% Viable CD45= 107.79

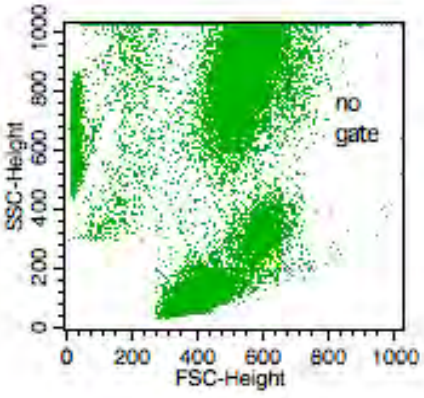
bead count = 49945.00

dilution factor 1.00

Sample volume 100.00 ul







Open viability gate (R8) when analyzing stabilized control/EQA samples

Conclusions (1)

- CD34+ cell transplantation is an important treatment option in hematological, genetic and in other malignant/non-malignant conditions
- The Flow Lab plays a CRITICAL ROLE in the monitoring and assessment of HSC product collection and manipulation
- If viability is an issue, method must contain a viability dye to exclude dead cells
- Standardized methods of enumeration have lead to reduced variability between laboratories in EQA schemes

Conclusions (2)
Standard Development and Acceptance

Start with the BEST SCIENCE when adopting a methodology

Use workshops to disseminate the method

GAIN CONSENSUS – no matter how long it takes

Develop CONSENSUS GUIDELINES

DEVELOP A QC PROGRAM

Monitor performance and provide educational feedback

Travel to MetroFlow Users Group Meeting!!

FIND GREAT COLLABORATOR - Mike Keeney

ISSUES IN CD34 ENUMERATION

Michael Keeney[1], D. Robert Sutherland[2]

London Health Sciences Centre[1]
London Ontario

Toronto General Hospital/University Health Network[2]
Canada

http://www.cytometry.org/public/educational_presentations/Keeney-Sutherland-CD34.pdf

CD34+ CELL ENUMERATION: FAQ

Michael Keeney and D. Robert Sutherland

http://www.cytometry.org/public/index.php

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CD34+ CELL ENUMERATION: FAQ
http://www.cytometry.org/public/index.php

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Acknowledgments

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Rakesh Nayyar Erica Acton

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Jan Gratama Stefano Papa Jaco Kraan

UK NEQAS
David Barnett Alison Whitby