UK NEQAS CD34+ Stem Cell Enumeration Programme Findings

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UK NEQAS History

- In 1962, 50 leading laboratories were provided with an identical blood sample
- Returned haemoglobins ranged from 120 to 170 g/L
- This highlighted the need for interlaboratory quality assessment
- From this the foundations of UK NEQAS were then laid in 1969 by Professor Tom Whitehead in Clinical Chemistry & Dr. Mitchell Lewis in Haematology

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UK NEQAS : Objectives

The mission of UK NEQAS is to ensure the provision of an appropriate, responsive & high standard of EQA

To provide laboratories with:

- an assessment of intra and inter laboratory performance
- relative performance of available kits and methods
- factors associated with good and poor performance
- a tool to monitor and improve the between-laboratory agreement

The primary role of UK NEQAS is educational

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$CD34^+$ Stem Cell Enumeration

- Routinely performed to optimize timing of peripheral stem cell collections
- EQA important as test is a go/no go test
- ISHAGE protocol most widely employed
- Currently 292/315 (93%) participants in the UK NEQAS $CD34^+$ stem cell enumeration programme indicate that this is their chosen protocol

Trial Operation

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- $CD34^+$ stem cell samples procured from consented patients undergoing stem cell harvest
- Samples stabilised and pooled to produce blood samples of differing $CD34^+$ stem cell counts
- Samples suitable for use with whole blood lysis techniques and sequential gating strategies on all platforms

Stability of Samples

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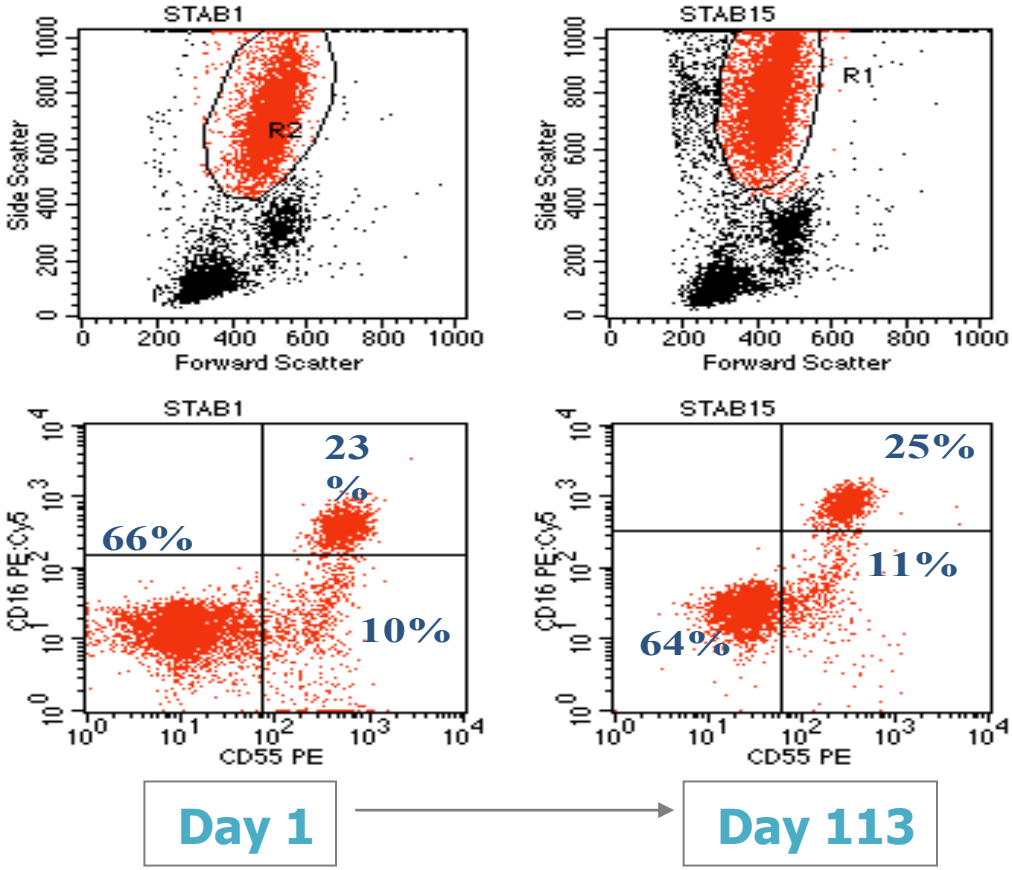
Stabilised PNH Granulocytes

Developed biological control material for internal & external quality assurance
with Dr S Richards, HMDS, Leeds



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Trial Operation

- 2 samples issued
- 3 week trial window
- $CD34^{+}$ enumeration performed (Single platform ISHAGE is recommended)
- Results are returned via the web site www.ukneqasli.co.uk
- Report produced

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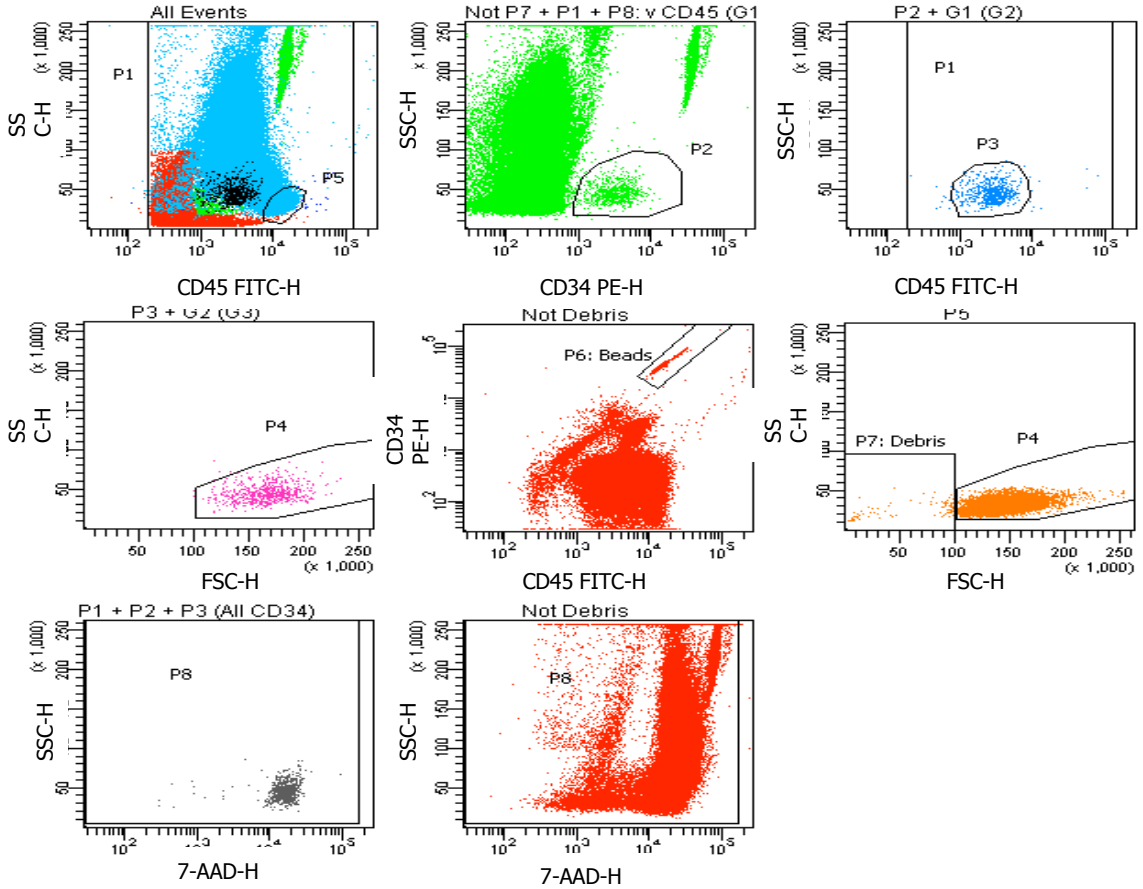
ISHAGE Protocol

- Sutherland D.R, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for $CD34^+$ cell determination by flow cytometry. Journal of Hematotherapy 1996; 5: 213-226

CD34 Gating – ISHAGE Protocol



- Boolean gating strategy
- Uses Forward and Side scatter characteristics and antigen expression

D.R. Sutherland Toronto, Canada

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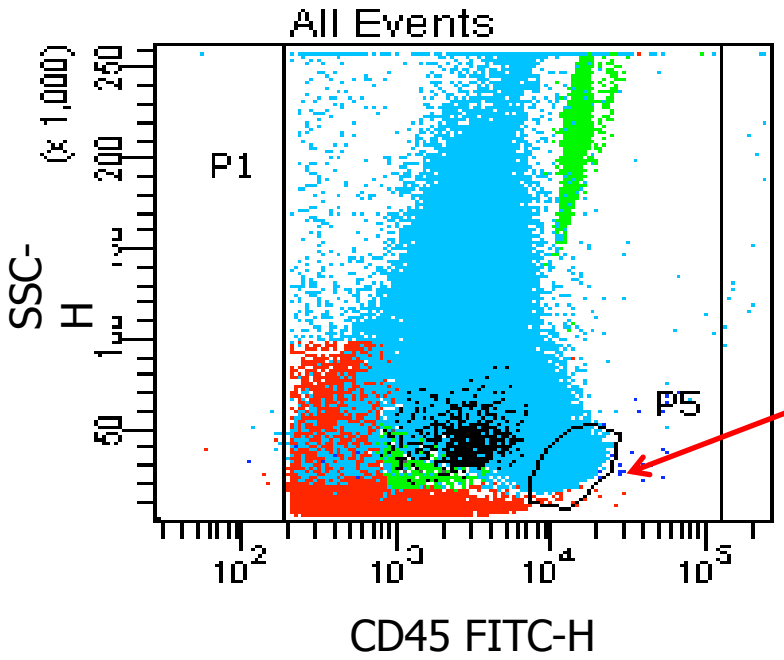
Plot 1



- Region 1 contains all CD45$^+$ events
- NOTE P5 GATE

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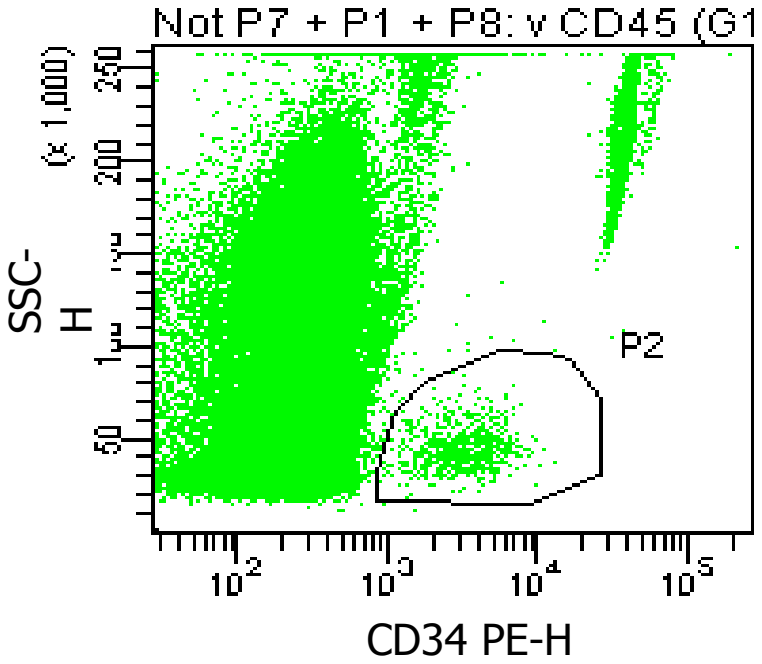
Plot 2



- Events from P1 displayed on SSC/CD34 plot
- P2 includes $CD34^+$ events

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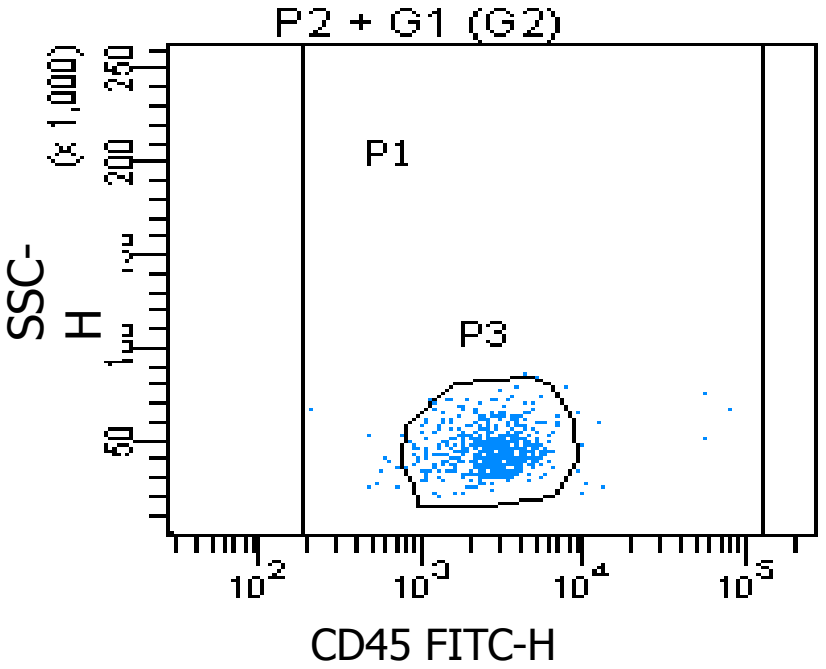
Plot 3



- CD45$^+$ and CD34$^+$ events from P1 and P2 displayed on SSC/CD45 (P3)
- Shows cells with characteristic low SSC and dim CD45 fluorescence

Plot 4 And Plot 5

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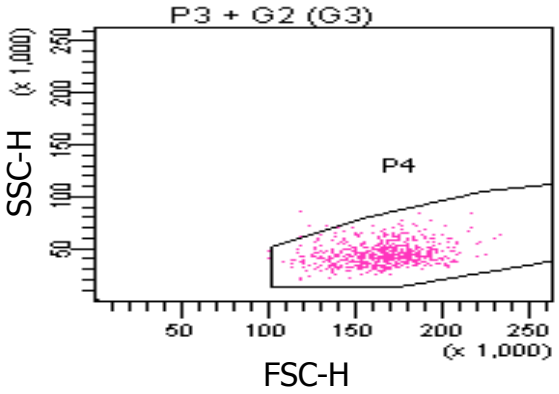
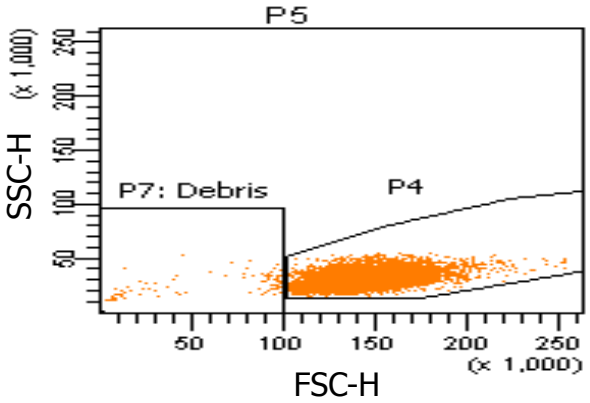
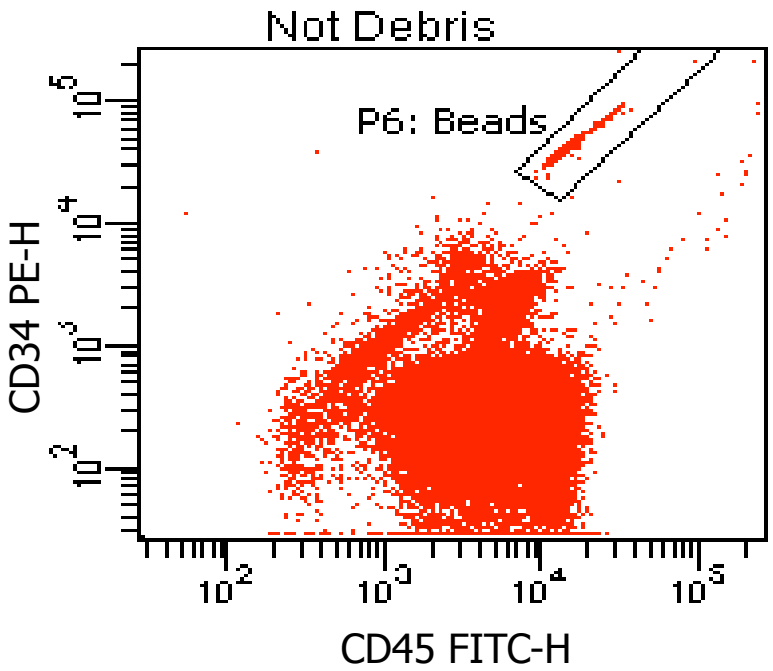




- Events from P1, P2 and P3 are displayed on a SSC/FSC plot to confirm the selected events fall into a generic ‘lymph-blast’ region – P4
- P4 is set precisely to include lymphocytes from plot 1(P5) as shown in plot 5
- Any cells falling outside region P4 – excluded from final calculation

Plot 6



- Ungated data displayed on a CD34/CD45 histogram
- Assists in positioning of CD45 dim gate in plot 3
- Also useful for identifying bead population for single platform

Plot 7 And Plot 8

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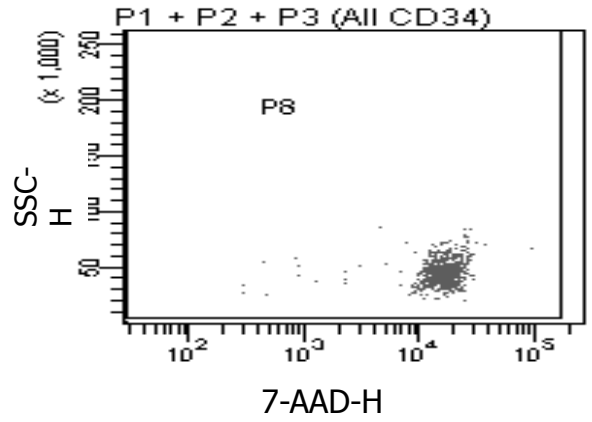

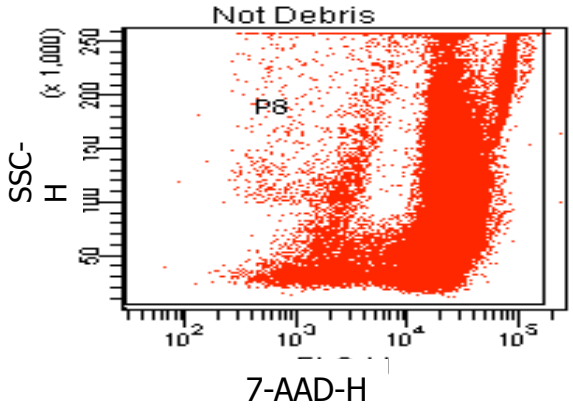

- These plots show viable cells
- Important in removing dead cells present particularly in thawed samples
- When using stabilised cells either increase this gate to include all cells or remove this stage completely

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Gating Strategy Study

- Study carried out in response to poor performance caused by incorrect gating strategy
- Results published 2012 - Whitby A, Whitby L, Fletcher M, Reilly JT, Sutherland DR, Keeney M, Barnett D. ISHAGE protocol: Are we doing it correctly? Cytometry Part B 2012; 82B: 9-17
  - Second most downloaded paper in Cytometry Part B for the year 2013

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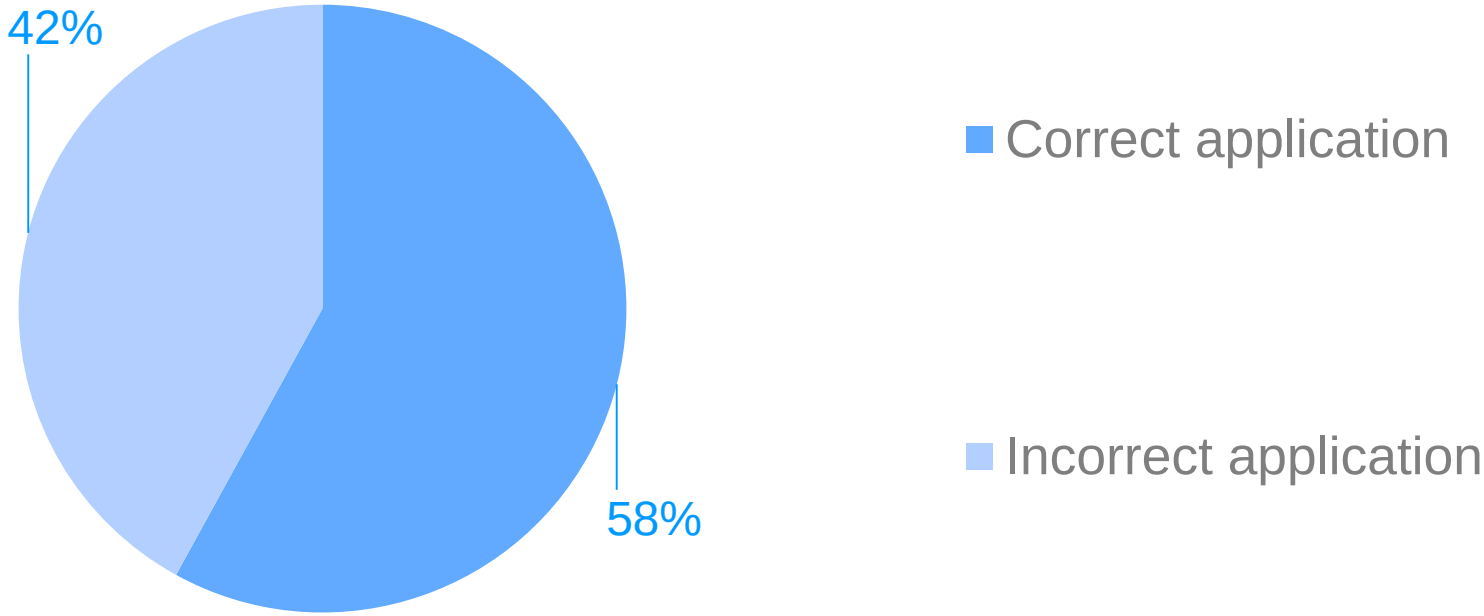
Gating Strategy Study

- Labs were asked to submit their dot plots (103/255 submitted)
- These were compared to the stated protocol used and whether it was correctly or incorrectly implemented
- 81% who returned dot plots stated that they were using ISHAGE

Results of ISHAGE Gating Strategy Analysis



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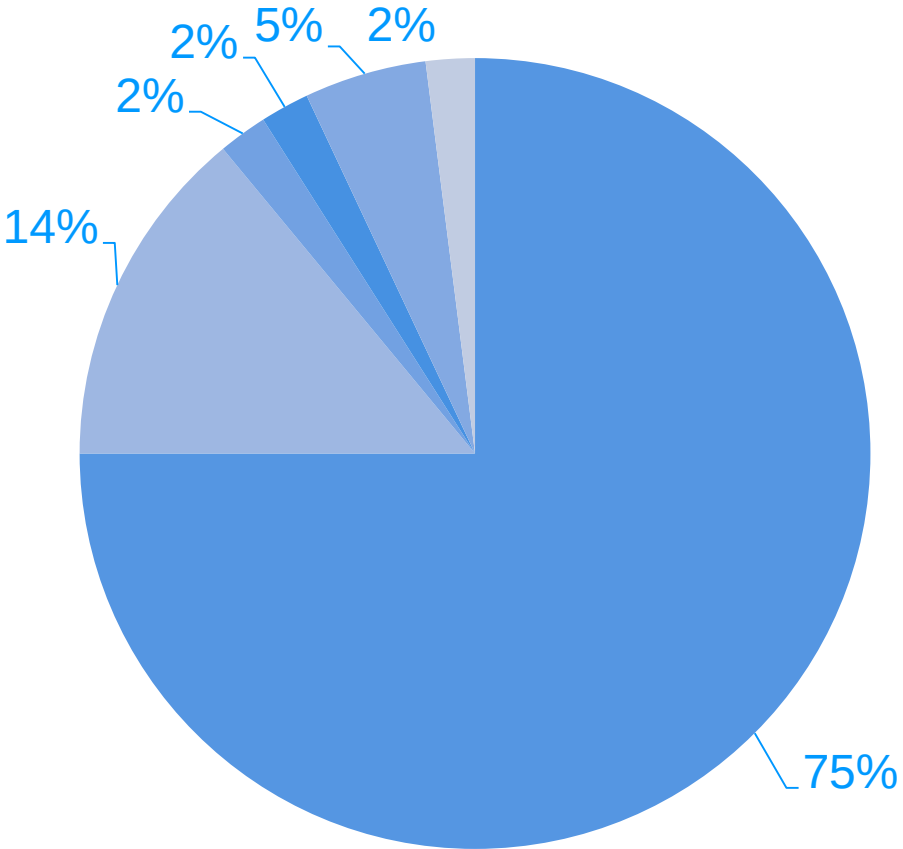
Breakdown Of Incorrect Application



- Omission of P5 region in P1 region - 75%
- Omission of Low SSC/CD45 dim plot (plot 3) - 14%
- Wrong parameter - 2%
- Wrong antibody - 2%
- Non-use of ISHAGE gating strategy - 5%
- Non-use of any previously published gating strategy - 2%

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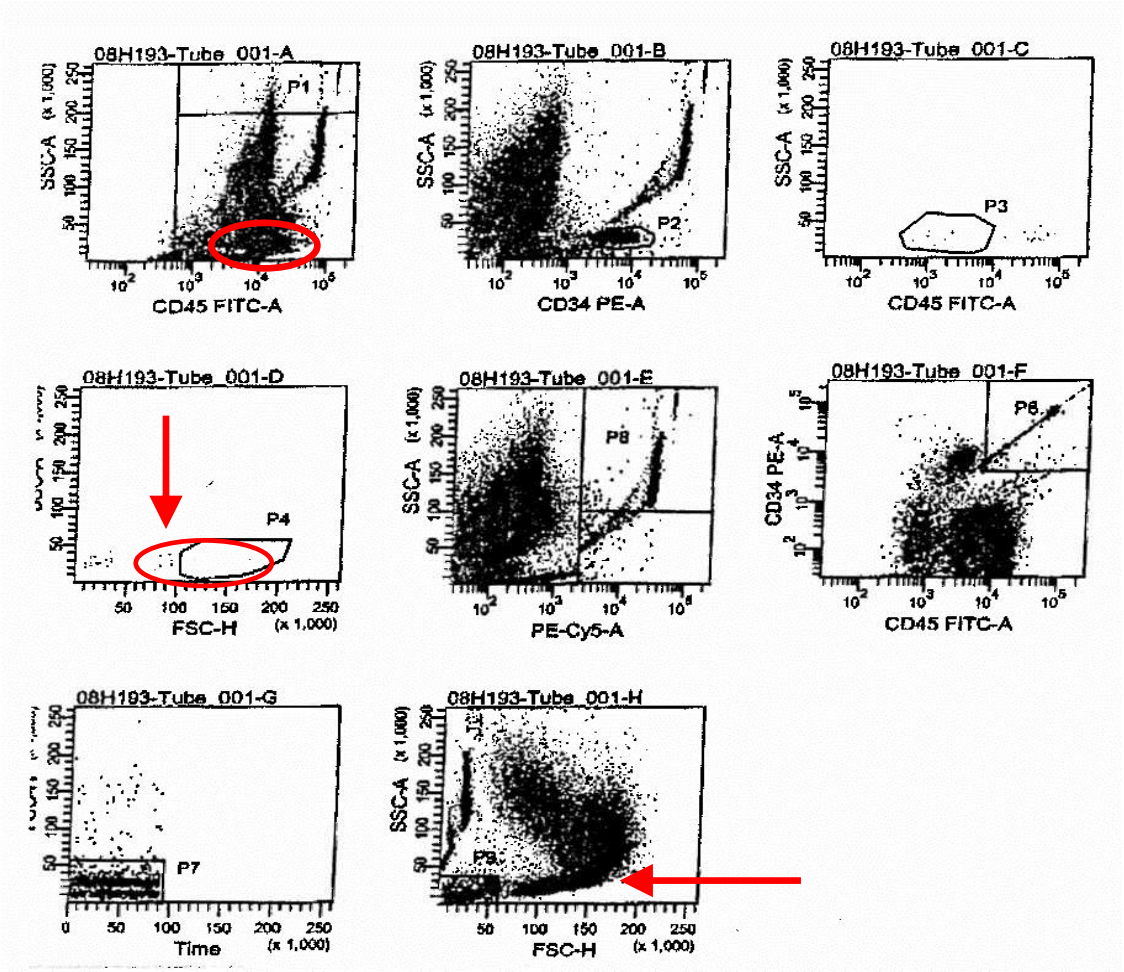
Missing Gate



- No R5 gate in region 1 showing position of lymphocytes to satisfy R4 lymphocyte check

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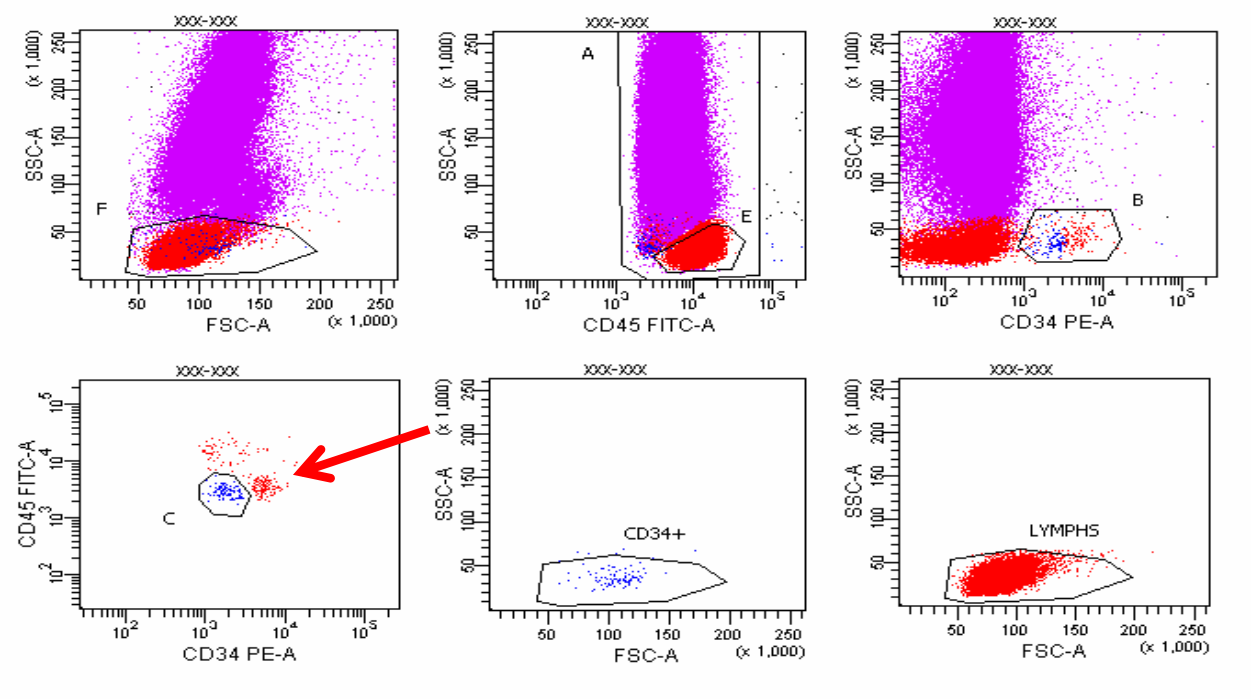
Wrong Parameter



- CD34/CD45 plot used to gate on CD45 dim instead of the correct SSC/CD45 plot

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Participant Survey 2015

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Survey Rationale

- Dedicated questionnaire issued to all participating centres (n=1587) to survey current and planned changes in flow cytometric techniques
- Questionnaire featured several sections, each related to different aspects of flow cytometry
- Issued to 310 participants in $CD34^+$ stem cell enumeration programme
- 148 returned results (48%) for the $CD34^+$ stem cell enumeration section were downloaded and analysed

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Best Practice CD34

- According to the BCSH guidelines (1999)
  - Use ISHAGE protocol
  - Use single platform
  - Use electronic pipette
  - Use reverse pipetting
  - Count a minimum 50000 events or 100 $CD34^+$ events

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Best Practice CD34

A total of 148 laboratories returned information on their flow cytometry technique for CD34 $^{+}$ stem cell enumeration

Recommendations	Number of Laboratories (n=148)
ISHAGE	
Single Platform	
Electronic Pipette	
Reverse Pipetting	
50000 Events/100 CD34^{+} events	
*Pipette Serviced Annually	
*Flow Cytometer Serviced Annually	

*Good laboratory practice

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Best Practice CD34

A total of 148 laboratories returned information on their flow cytometry technique for $CD34^+$ stem cell enumeration

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50000 Events/100 $CD34^+$ events	12 (8%)
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Participant Survey Results – CD34 Methodology

- Events counted ranged from
  - 10000 (n=1) to 1000000 (n=3) total events
  - 50 (n=3) to 500 (n=3) $CD34^+$ (BCSH Guidelines recommend minimum of 100 $CD34^+$ cells or 50000 events)
- Limits for harvest varied from 5 to 64 $CD34^+$ cells/uL
- Reporting units are cells/uL, cellsx10^6/L and cellsx10^9/L

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Conclusion

- Survey data shows only 7% laboratories testing are performing the assay correctly
- Previous publication shows only 58% laboratories applying ISHAGE gating strategy correctly
- These figures combined give a theoretical worst case of only 4% of centres performing the assay correctly
- Urgent need for laboratories to ensure correct performance of $CD34^+$ stem cell enumeration assay
- Imperative that laboratories ensure the correct application of the ISHAGE gating strategy

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Summary- So What!

- Team GB - British Cycling team were once described as a laughing stock or also-rans
- Sir Dave Brailsford transformed British Cycling
  - 22 gold medals at the last 3 Olympic games
  - 4 Tour de France winners in the last 5 years
- Used the theory of marginal gains
  - Break a process into small segments and make 1% improvement in each area
  - These marginal gains accumulate
- “They’re tiny things but if you clump them together it makes a big difference” – Sir David Brailsford, 2012

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What Should Labs Do?

- There are options available to ensure correct methodologies are in place
- Refer to publications
  - Whitby A, Whitby L, Fletcher M, Reilly JT, Sutherland DR, Keeney M, Barnett D. ISHAGE protocol: Are we doing it correctly? Cytometry Part B 2012; 82B: 9-17
  - D. Barnett, G. Janossy, A. Lubenko, E. Matutes, A. Newland, J. T. Reilly. Guideline for the flow cytometric enumeration of $CD34^+$ haematopoietic stem cells. Clin. Lab. Haem 1999; 21: 301-308
  - Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for $CD34^+$ cell determination by flow cytometry. Journal of Hematotherapy 1996; 5: 213-226
- UK NEQAS is here to help.
  - Tel - +44 (0) 114 267 3600
  - Email – alison.whitby@ukneqasli.co.uk