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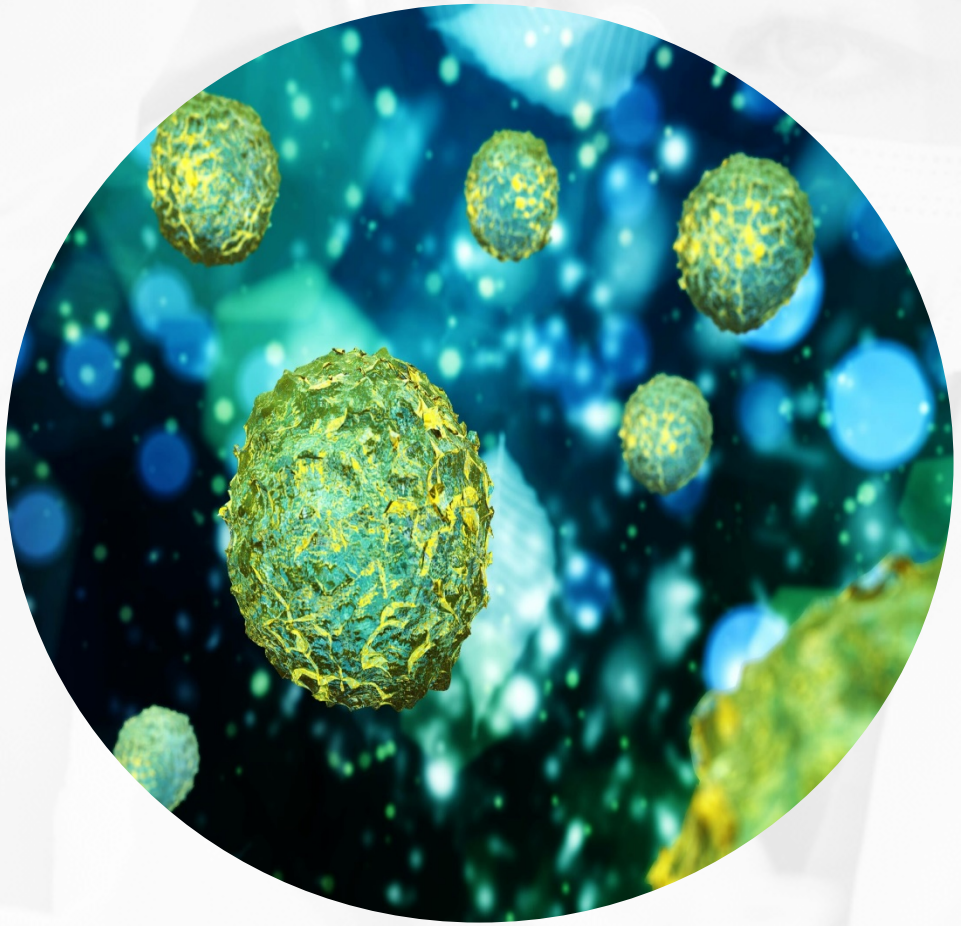
Translating Science to Medicine

A Fully Automated Method for CD34+ Cells Enumeration by Flow Cytometry in Stabilized Whole Blood



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Lead Scientist Molecular and Cell Biology

25-Nov-2021, EBF Open Symposium

CD34+ Hematopoietic Stem Cells

- 2%-4% of bone marrow cells express the cell surface marker CD34 and are capable of reconstituting long-term multilineage hematopoiesis, i.e. have the properties of hematopoietic stem and progenitor cells

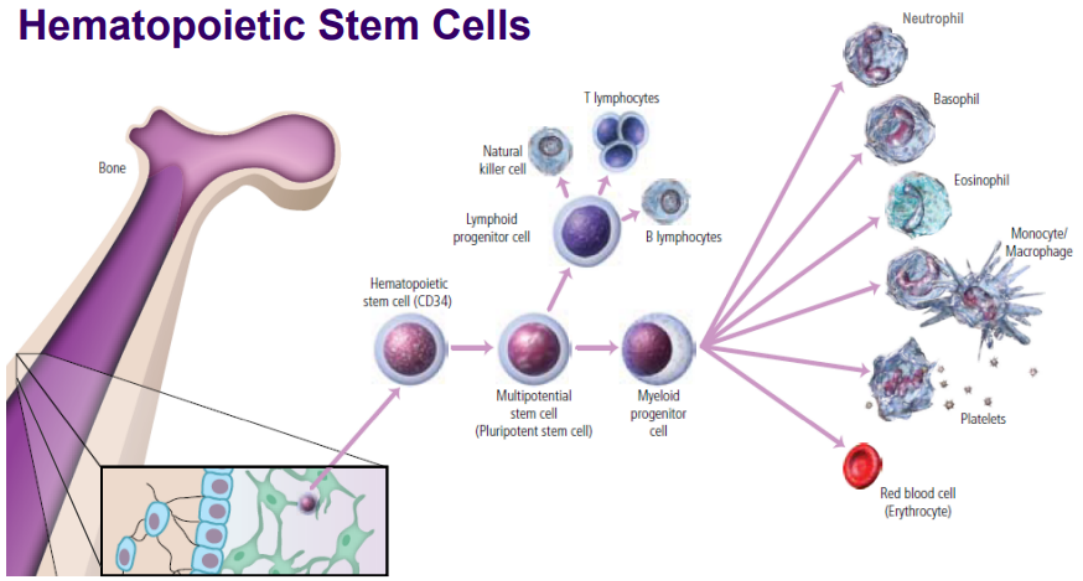
  CD34 is a hematopoietic stem cell biomarker

- CD34 positive (CD34+) cells are also found in the peripheral circulation of normal individuals but are extremely rare (approx. 0.01%-0.05% of nucleated cells, or <5 CD34+ cells/µL)

  CD34+ rare event analysis is possible by flow cytometry

- CD34+ cells can be mobilized from the bone marrow to the peripheral circulation in higher numbers (approx. 0.1% – 2% of nucleated cells, between 5-200 CD34+ cells/µL) by cytotoxic drugs or hematopoietic cytokines

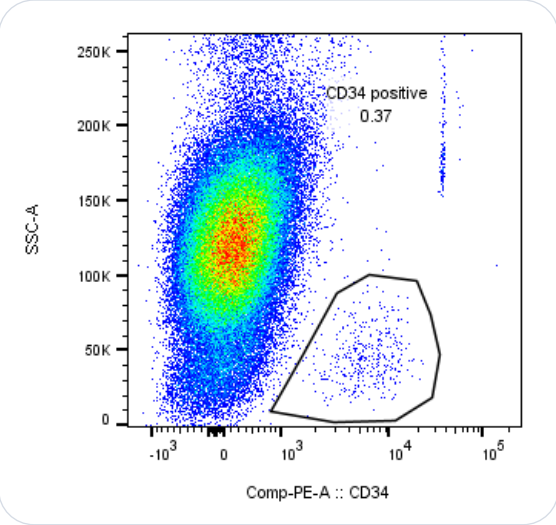
  CD34+ is a secondary PD endpoint in recombinant G-CSF treatment



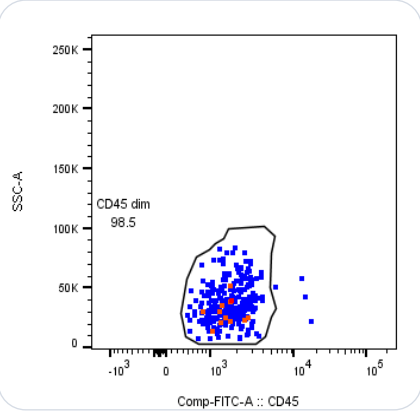
Source: Ellen Meinelt, BD, 2012

CD34+ Enumeration: ISHAGE Guidelines

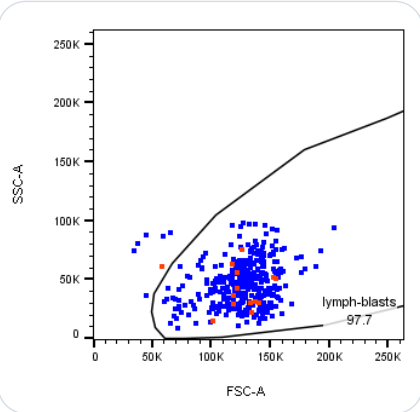
- Two-color (CD34-PE and CD45-FITC) flow cytometry assay
- Hematopoietic stem cells are **CD34+/CD45dim/SSClow/FSClow-med**
- **First ISHAGE gate:** select all CD45+ cells (can be substituted by an acquisition threshold on the CD45-FITC channel)
- **Second ISHAGE gate:** select all CD34+ cells from the first gate



- **Third ISHAGE gate:** select CD45dim cells from the second gate



- **Fourth ISHAGE gate:** select cells with «lymph-blast» properties (SSClow / FSClow-med) from the third gate



CD34⁺ Enumeration: Current State-of-the-art

- ISHAGE guidelines: sequential gating approach ensures specificity and eliminates the need for isotype controls
- Single-platform: fluorescent counting beads allow for absolute cell quantification (quasi-quantitative assay)

$$\text{Absolute cell counts} = \frac{\text{Number of CD34 Cell Events} * \text{Trucount Bead Count} * \text{Dilution Factor}}{\text{Number of Trucount Bead Events} * \text{Sample Volume}}$$

- Commercial QCs and IVD-quality commercial kits available, simple protocol
- Very sensitive and precise method when at least 75 000 - 100 000 CD45⁺ cells are acquired, according to literature capable of detecting 100 CD34⁺ cells with a ~10% CV

Limitations:

- Stability of fresh whole blood is very limited (recommended <24h) → impossible to store and ship samples from large clinical studies to a single laboratory for analysis
- Manual tube-based method → extremely low-throughput

CD34+ Enumeration: Whole Blood Stability

- It is possible to freeze and store whole blood at -80°C for CD34+ enumeration when stabilized immediately upon collection with a formaldehyde-containing stabilizer/fixative → cannot use the 7-AAD live/dead stain anymore
- 6 months of storage at -80°C validated for CD34+ enumeration

Allows the storage and shipping of samples from large clinical studies to a single laboratory for analysis.

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Bioanalysis

Approaching stability challenges for flow cytometry in a regulated bioanalytical environment

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Stability of samples for flow cytometry is a critical parameter since storage period of samples is restricted to only a limited period after collection. For most studies, clinical samples have to be shipped to a testing laboratory, in contrast to preclinical samples, which can be analyzed on-site or off-site. Therefore, evaluating stability is critical to provide flexibility on testing of samples to obtain reliable data. A wide variety of factors contributes to establishing stability from sample collection through acquisition. We provided suggestions for experimental and stability parameters to be taken into consideration when designing a flow cytometry method. The case studies presented represent how certain stability issues were overcome to perform flow cytometry assays in a regulated bioanalytical environment.

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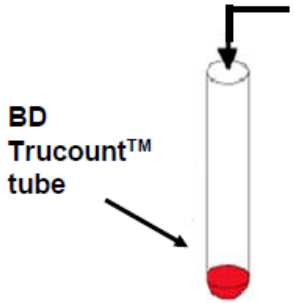
CD34+ Enumeration: Automation of BD Stem Cell Enumeration Kit



Add 20 µL of CD45 FITC/CD34 PE reagent,
~~20 µL of 7-AAD~~, and
100 µL of blood (by reverse pipetting) to a Trucount tube. → **contains a predefined number of fluorescent beads**

Vortex and incubate in the dark at RT for 20 min.

Add 2 mL of 1X ammonium chloride lysing solution.

Vortex and incubate in the dark at RT for 10 min.

Put samples into an ice bath!

Acquire samples within 1 hour after lyse.

Automated reagent and sample pipetting

Manual batch vortexing on a multi-tube vortexer

Automated reagent pipetting, mixing, and transfer to 96-well plates

AIM: Automate the BD Stem Cell Enumeration Kit procedure and transfer to 96-well plate format to allow automatic acquisition with LSR Fortessa High Throughput Sampler (HTS).

CD34+ Enumeration: Automation Concerns

Hamilton MICROLAB STARlet pipetting robot and LSR Fortessa flow cytometer with HTS:

Will automated pipetting affect cell integrity and Trucount beads concentrations?

= will the precision and the relative accuracy to the manual method be acceptable?

Will prolonged incubation times have a negative effect on enumeration?

= will the precision and the relative accuracy to the manual method be acceptable?

LSR Fortessa HTS can aspirate a maximum volume of 200 µL/well → is this a sufficient volume to acquire at least 75 000 CD45+ events?

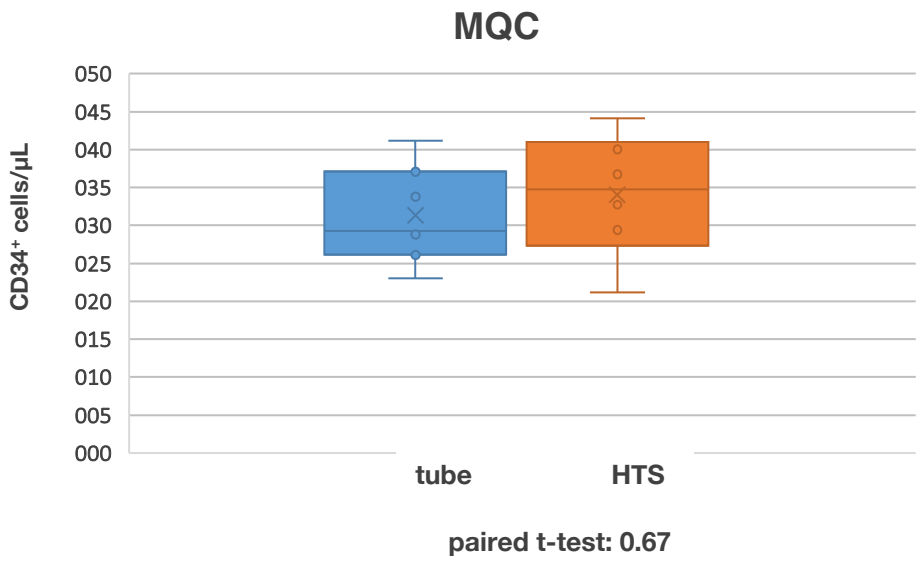
= will the precision and the relative accuracy to the manual method be acceptable?

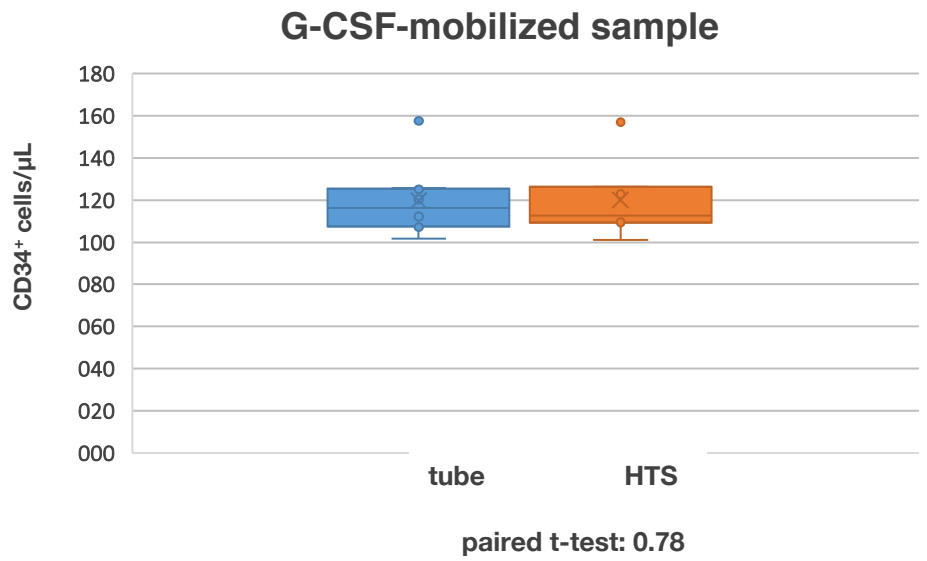
What is the highest HTS acquisition speed that can be used?

= will the precision and the relative accuracy to the manual method be acceptable?

CD34+ Enumeration: HTS Volume

- *Samples prepared manually, acquired in both tube and HTS mode in >5 independent runs*
- *3x commercial QCs (CD-Chex CD34 Level 1-3), 1x G-CSF-mobilized sample, 2x non-mobilized samples*
- *Acquisition performed at low speed (0.5-1.0 µL/sec)*



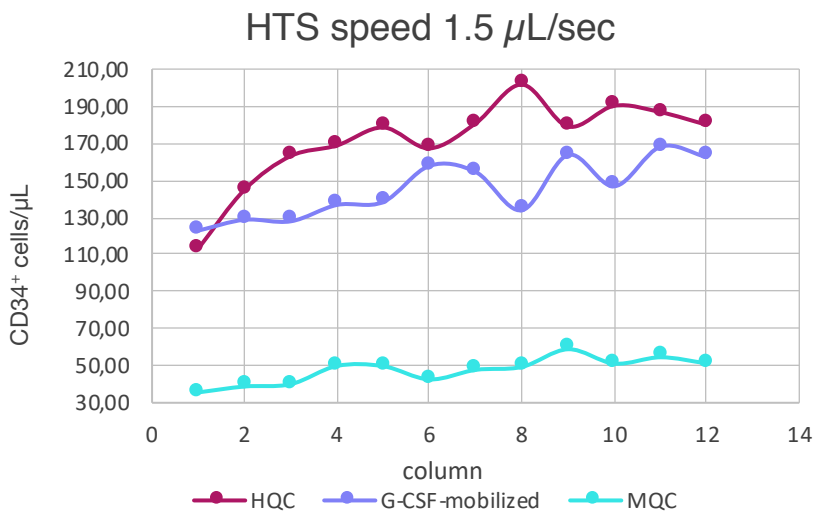


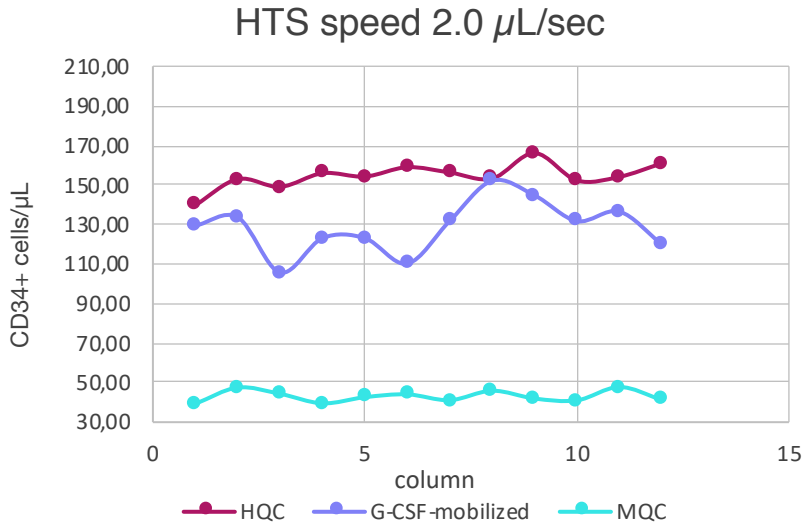
No major difference in «relative accuracy» and precision when using HTS acquisition for all samples, QCs within vendor range

Acquisition of 200 µL/well is sufficient

CD34+ Enumeration: HTS Speed – Full 96-well Plate

Assessed HTS plate homogeneity using 6 different samples (3x commercial QCs, 1x G-CSF-mobilized sample, 2x non-mobilized samples) processed manually and then pipetted across a 96-well plate. Acquisition performed at RT using 4 different speeds (1.0 - 2.5 µL/sec).



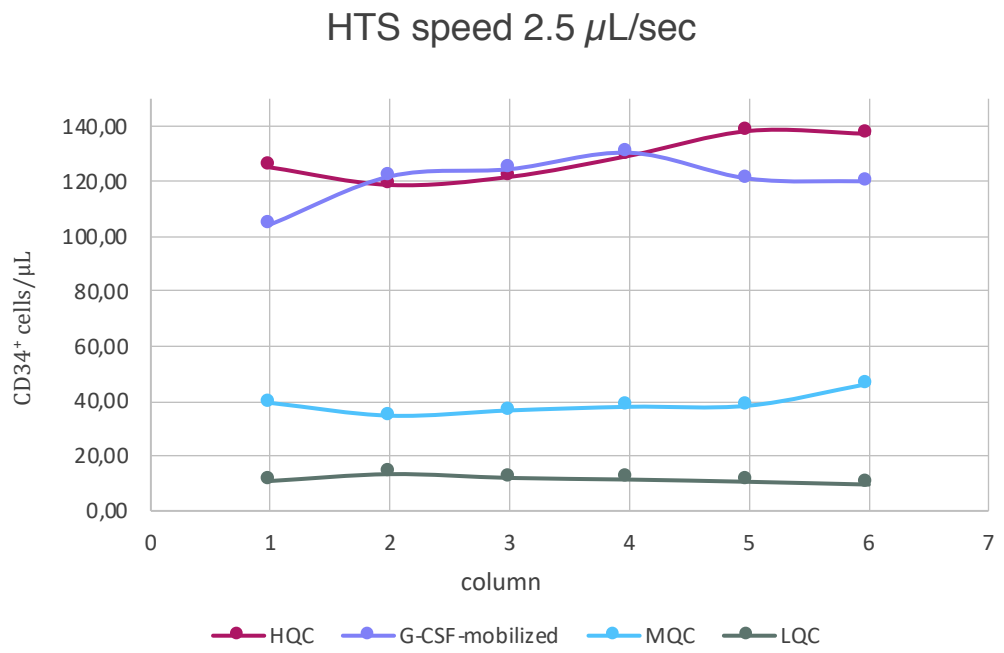


- ***All samples:*** *increased CD34+ calculations across plate with lower HTS acquisition speeds (< 2.0 µL/sec), due to increased unspecific staining during prolonged acquisition at RT*
- ***G-CSF-mobilized sample:*** *20% CV between columns 1 and 12*
- ***MQC:*** *25% CV between columns 1 and 12, above vendor range*
- ***HQC:*** *32% CV between columns 1 and 12, above vendor range*

- ***G-CSF-mobilized sample: improved to ~5% CV when using higher acquisition speeds (>2.0 µL/sec)***
- ***MQC: partially improved (~5% CV but some values above range) when using higher acquisition speeds (>2.0 µL/sec)***
- ***HQC: partially improved (~10% CV but still mostly above range) when using higher acquisition speeds (>2.0 µL/sec)***

CD34+ Enumeration: HTS Speed – Half 96-well Plate

Assessed HTS plate homogeneity using 6 different samples (3x commercial QCs, 1x G-CSF-mobilized sample, 2x non-mobilized samples) processed manually and then pipetted across HALF a 96-well plate. Acquisition performed at RT using 2 different speeds (2.0 µL/sec = ~1.5h, 2.5 µL/sec = ~1h15min).



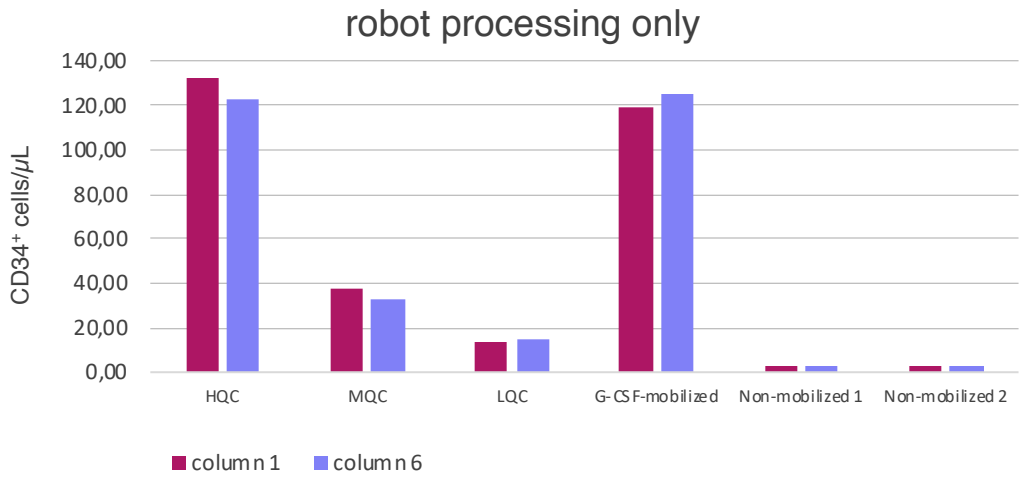
- ***G-CSF-mobilized sample:*** *~7% CV across half plate*
- ***Non-mobilized samples:*** *random high variability, >20 %CV*
- ***LQC:*** *~12% CV across half plate, within vendor range*
- ***MQC:*** *improved by the combination of half plate and high acquisition speed (>2.0 µL/sec), within vendor range, ~10% CV across half plate*
- ***HQC:*** *improved by the combination of half plate and high acquisition speed (2.5 µl/sec), within vendor range, ~6% CV across half plate*

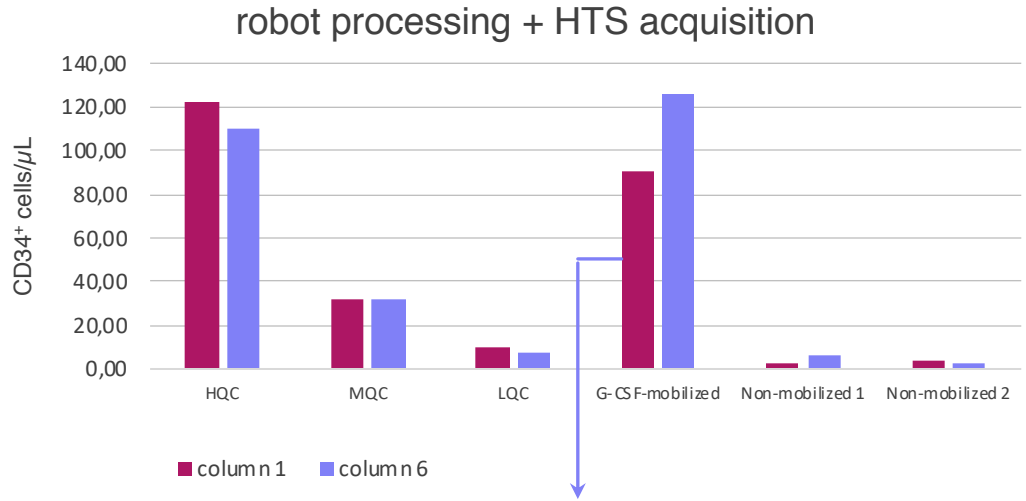
Continue with HTS speed 2.5 µL/sec and half 96-well plates

CD34+ Enumeration: Automation

Assessed automated sample processing using 6 different samples (3x commercial QCs, 1x G-CSF-mobilized sample, 2x non-mobilized samples) processed at the beginning and at the end of the run (columns 1 and 6), with dummy samples in between.

1. Acquisition skips dummy samples, acquired immediately after processing → ***assess only the effect of robot sample processing without the effect of prolonged HTS acquisition***
2. Acquisition of entire half plate, acquired immediately after processing → ***assess the combination of robot sample processing and prolonged HTS acquisition***





- QCs within vendor range, no high %CVs between columns
- No negative effects of automated processing
- No negative effects of prolonged incubation during processing

Shorter Ab incubation times in first column = lower CD34+ cells/µL calculation for G-CSF-mobilized sample in first column?

CD34+ Enumeration: Automation Incubation Times – Before

15 20 25 30 35 40 45 50 55 60 65 70 75

Ab Incubation for First Sample STARTS

Ab Incubation for Last Sample STARTS

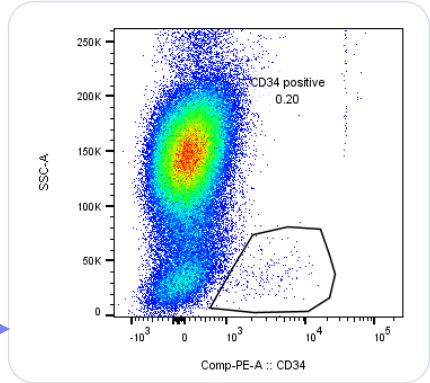
Lysis Incubation for First Sample STARTS

Lysis Incubation for Last Sample STARTS

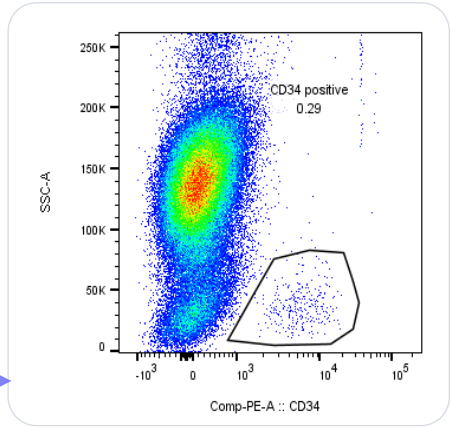
HTS Acquisition for First Sample STARTS

5 min unload, mix, scan and load

10 min Ab incubation for **first** sample



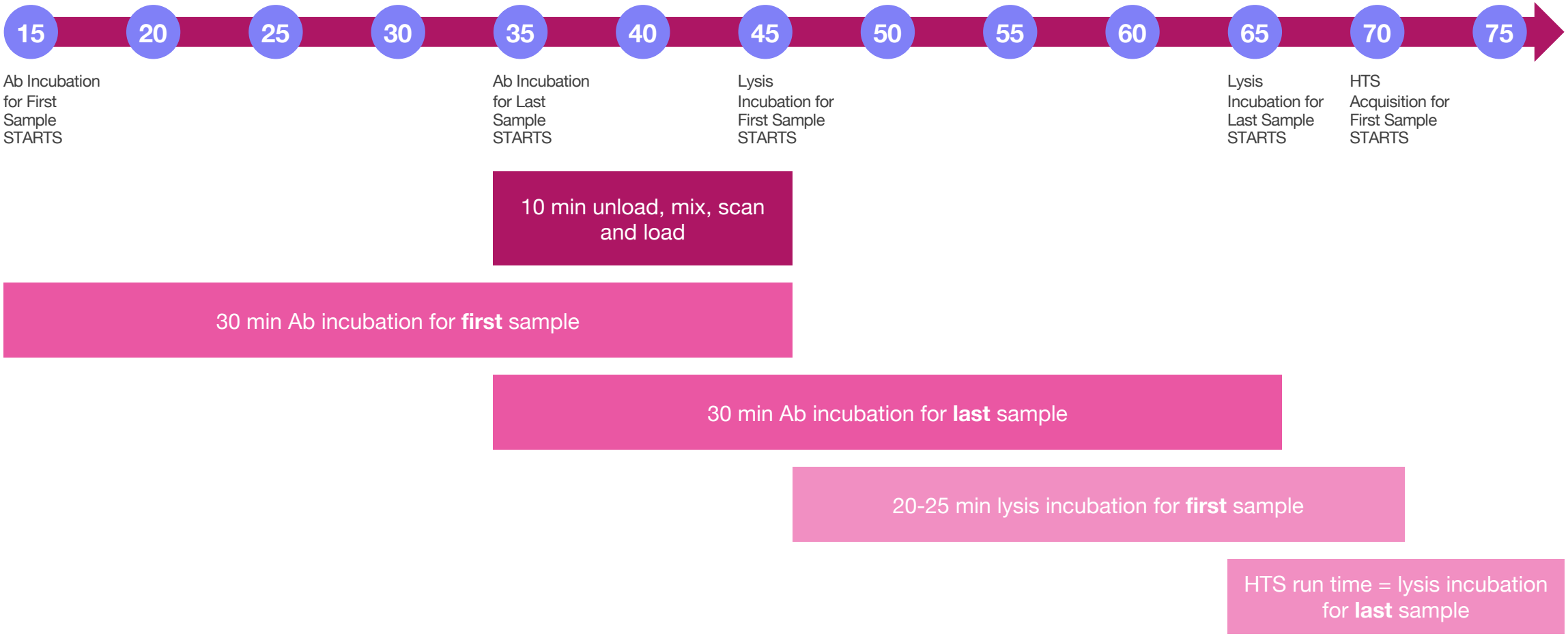
30 min Ab incubation for **last** sample



25-30 min lysis incubation for **first** sample

HTS run time = lysis incubation for **last** sample

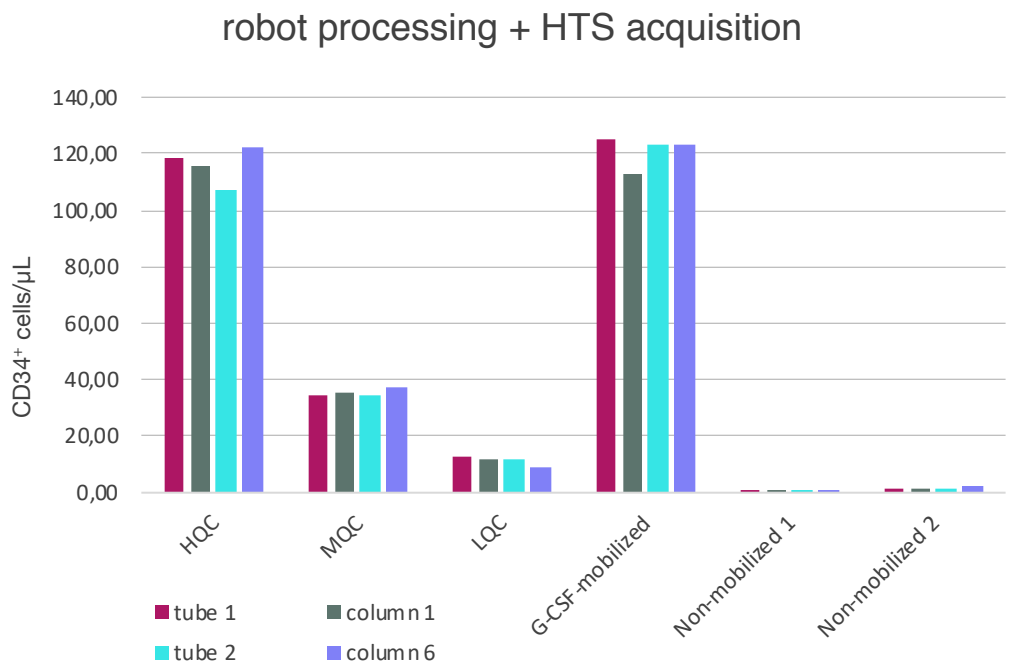
CD34+ Enumeration: Automation Incubation Times - After



CD34+ Enumeration: Automation Final Setup

Assessed automated sample processing using 6 different samples (3x commercial QCs, 1x G-CSF-mobilized sample, 2x non-mobilized samples) processed at the beginning and at the end of the run (columns 1 and 6) with dummy samples in between, with acquisition of entire half plate, acquired immediately after processing

Assess the combination of robot sample processing and prolonged HTS acquisition



- ***QCs within vendor range, <10-15 %CVs (except for non-mobilized)***
- ***No negative effects of automated processing***
- ***No negative effects of prolonged incubation during processing and HTS acquisition at 2.5 µL/sec***

Qualification: Biomarker Quasi-Quantitative Assay

Parameter	*Result*	*Conclusion*
Inter- and intra-run precision	• 2-4x replicates in 3x independent runs • <20% CV for QCs and two G-CSF-mobilized samples	• Pass
Stress test / carryover	• 0 $CD34^+$ events in PBS samples tested in an alternating pattern with HQC	• Pass
Freeze-thaw stability	• 2x and 3x freeze-thaw cycles within 80%-120% of reference for two G-CSF-mobilized samples	• Pass
Post-processing stability (1h, 3h, 24h at 5°C)	• QCs and two G-CSF-mobilized samples within 80%-120% of reference at all timepoints	• Pass
Dilution linearity	• 1:2 dilution within 80%-120% of reference for two G-CSF-mobilized samples • 1:4 dilution only 13%-60% of reference for the same two G-CSF-mobilized samples	• 1:2 dilution pass • 1:4 dilution didn't pass
Sensitivity (LOB, LOD, LLOQ)	• Not assessed • LOB ($mean_{blank}$ + 1.645xSD_{blank}) to be assessed in validation	• N/AV

Conclusions

It is possible to freeze and store whole blood at -80°C for CD34⁺ enumeration when stabilized immediately upon collection with a formaldehyde-containing stabilizer/fixative.

Method qualification was successful, with <20% inter- and intra-run precision of G-CSF-mobilized samples and commercial QCs, no carryover, 3x freeze-thaw cycles of G-CSF-mobilized samples, 1:2 dilution linearity of G-CSF-mobilized samples, and approximately 24h post-processing stability of G-CSF-mobilized samples and commercial QCs.

It is possible to automate and transfer a low-throughput tube-based commercial assay to a high-throughput 96-well plate-based format.

Method allows the transport, long-term storage, and high-throughput bioanalysis of CD34⁺ samples, and is therefore able to support large clinical studies, e.g. G-CSF-based biosimilar studies.

Acknowledgements

- ***Rebeca Schibli**, Team Leader Automation & ELN*
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- ***Petra Struwe**, Executive Director Bioanalytical Services*

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Thank You

Back-up Slides

History of CD34 Enumeration Protocols

1985 Siena, et al.

Milan Protocol
- Dual platform
- FSC/SSC
- 50k or 50 $CD34^+$ events
- Class III CD34 clone (1991)

1994 Bender

- CD45

1995 Owens and Loken

- 7-AAD viability dye

1994, 1996 Sutherland, et al.

Original ISHAGE Protocol
- Dual platform
- 4 Parameter, 2 Colors:
  - FCS / SSC
  - CD34 PE / CD45 FITC
- Requires isotype control

1998 BD Procount Kit

Single platform with BD™ Trucount beads
- Nucleic acis staining
- Isotype control included
- Lyse/no wash methodology
- Leukopheresis and Peripheral Blood

1998 Keeney, et al.

Modified ISHAGE Protocol
- Single platform method with counting beads
- 75K or 100 CD34
- 7-AAD viability dye
- 4 Parameter, 2 Colors:
  - FSC / SSC
  - CD34 PE / CD45 FITC
- Sequential gating eliminates use of isotype control.

Qualification: Inter- and Intra-run Precision

2-4x replicates in 3x independent runs

	HQC	*MQC*	*LQC*	*GCSF-mobilized 1*	*GCSF-mobilized 2*
n	10	10	10	6	7
mean	**119.61**	**36.27**	**11.37**	**113.55**	**185.41**
SD	11.16	2.59	0.39	17.26	23.72
inter-assay precision	9.33	7.15	3.47	15.20	12.79
intra-assay precision	7.11	6.35	15.72	5.79	8.58
manufacturer range	87.4-127.4	25.0-39.0	7.2-15.2		

Establishing QC acceptance criteria for new lots:

1. For each QC level, mean concentration (cells/µL) is experimentally obtained in at least 3 independent precision runs including at least 3 replicates of each QC level. Gating analysis can be adjusted accordingly to accommodate lot-to-lot variation.
2. If the mean concentration of each QC level is within the respective expected range provided by the vendor, the lot is suitable to be used as a quality control sample. The experimentally obtained mean concentration is then used as the nominal value.
3. The accepted ranges for each QC level are calculated as 3x SD of the experimentally obtained mean concentration.