Full automation of CD34+ cells enumeration by flow cytometry: from low-throughput single tubes to high-throughput 96-well plates

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INTRODUCTION

CD34+ cell enumeration in whole blood is an important pharmacodymic biomarker for hematopoietic stem cells mobilization, e.g. following recombinant G-CSF treatment. Quasi-quantitative CD34+ cell enumeration is most commonly performed using single-platform two-color (CD45-FITC and CD34-PE antibodies) flow cytometry following the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines. Despite improvements in the ISHAGE protocol and availability of commercial kits, the method is suboptimal for clinical samples bioanalysis for two major reasons: sample stability and low-throughput. We previously demonstrated that the use of a formaldehyde-containing whole blood stabilizer allows the long-term storage (at least 6 months) of CD34+ clinical samples at -80°C without negatively affecting CD34+ enumeration (Muruganandham A. et al., 2019). Here we demonstrate the successful transfer of a manual low-throughput tube-based commercial assay (BD Stem Cell Enumeration Kit) to a fully automated high-throughput 96-well plate-based assay.

METHOD AUTOMATION

Automation was performed with a Hamilton MICROLAB STARlet pipetting robot, and acquisition with a LSR Fortessa flow cytometer with a high throughput sampler (HTS) unit. Figure 1 illustrates the setup of the manual Stem Cell Enumeration Kit vs. the automated procedure. 7-AAD live/dead stain was not used due to the use of a 20% formaldehyde-containing stabilizer in samples (K3 EDTA whole blood).

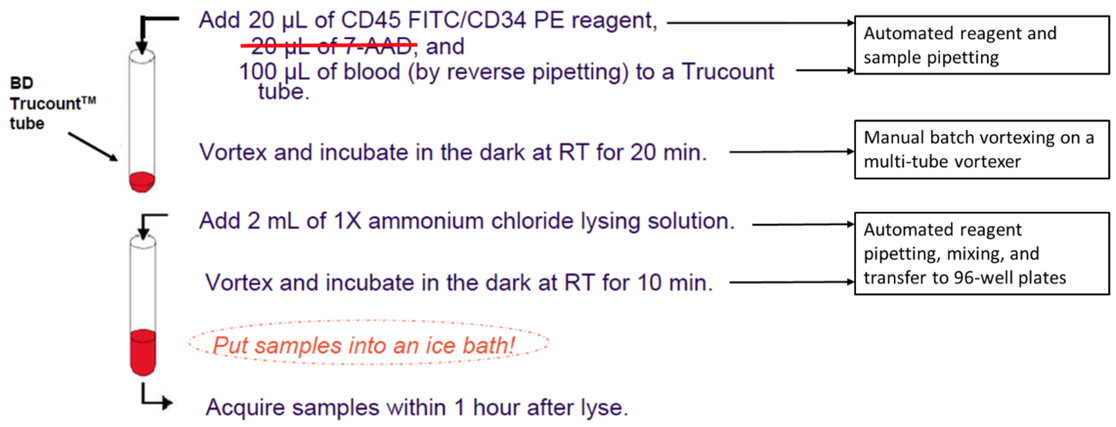

Figure 1: BD Stem Cell Enumeration Kit manual procedure and automatic procedure adaptations.

RESULTS

HTS acquisition volume

As HTS acquisition from 96-well plates was limited to 200 µL/well, acquisition of the same manually processed samples (3x commercial QCs, 1x G-CSF-mobilized sample) in tube vs. plate mode at low speed were compared over several runs to assess the precision and the relative accuracy to the original tube-based method. Precision was assessed by %CV, and relative accuracy by a paired t-test. Results are presented in Table 1 as CD34+ cells/µL. As the %CV was similar for almost all samples, and there was no significant difference in the paired t-test between the two conditions for any of the samples, we concluded that HTS acquisition of a smaller volume does not negatively impact the assay precision.

Table 1: Comparison of tube vs. plate (HTS) acquisition mode

	Commercial HQC		Commercial MQC		Commercial LQC		G-CSF-mobilized sample	
	tube (0.5 µL/sec)	HTS (1 µL/sec)	tube (0.5 µL/sec)	HTS (1 µL/sec)	tube (0.5 µL/sec)	HTS (1 µL/sec)	tube (0.5 µL/sec)	HTS (1 µL/sec)
	97.06	108.80	28.82	21.15	7.16	7.28	120.43	122.85
	99.54		23.03		8.70		157.63	156.96
	109.34	114.73	41.16	44.09	11.27	8.08	108.36	
	119.89	82.93	29.29	36.72	13.20	9.56	107.21	112.15
	124.10	162.57	37.09	29.41	11.09	9.13	125.09	112.70
	117.09		26.11	32.73	10.69	11.87	101.64	101.25
			33.80	40.01	11.40	9.83	112.19	126.31
					11.91	11.21	125.59	109.42
average	**111.17**	**117.26**	**31.33**	**34.02**	**10.68**	**9.57**	**119.77**	**120.23**
%CV	**9.98**	**28.32**	**20.29**	**24.00**	**17.77**	**16.93**	**14.70**	**15.15**
paired t-test		0.78		0.67		0.08		0.78
vendor range	98.1-138.1		29.2-43.2		8.8-16.8			

HTS acqusition speed and incubation times

To asses what is the highest HTS acquisition speed that can be used, 6 different manually processed samples (3x commercial QCs, 1x G-CSF-mobilized sample, 2x non-mobilized samples) were pipetted across a 96-well plate to evaluate plate homogeneity. Acquisition was performed at RT using 4 different speeds in independent runs (1.0 µL/sec = ~5.5h/plate, 1.5 µL/sec = ~4h/plate, 2.0 µL/sec = ~3h/plate, 2.5 µL/sec = ~2.5h/plate), while the reference tubes were stored at 5°C. Precision was assessed by %CV of all columns, and relative accuracy by %CV of the reference tube. Representative results for 1.5 µL/sec and 2.0 µL/sec are presented in Tables 2 and 3 respectively. Prolonged RT incubation times during HTS acquisition at low speeds (<2.0 µL/sec) increased unspecific staining, CD34+ cells/µL calculations, and %CV across the plate and in comparison to the reference tube for all samples, with non-mobilized samples and commercial HQC and MQC affected the worst (Table 2). This effect was partially ameliorated by using higher HTS acquisition speeds (≥2.0 µL/sec), which reduced RT incubation times and unspecific staining (Table 3).

Table 2: Full plate homogeneity with 1.5 µL/sec HTS acqusition speed

	1	2	3	4	5	6	7	8	9	10	11	12		mean row	%CV all columns	%CV col. 1 vs.12	%CV col 12 vs. Tube	tube	range
HQC	113.17	144.91	163.13	168.82	178.53	167.24	180.76	201.67	179.03	190.23	186.75	179.94		171.18	13.60	32.22	31.64	114.14	98.1-138.1
MQC	35.61	38.78	40.03	49.88	49.75	42.61	47.76	49.33	58.89	51.13	54.54	51.24		47.46	14.46	25.47	29.33	33.64	29.2-43.2
LQC	6.43	13.05	12.26	11.25	15.32	12.42	12.79	13.05	15.77	13.75	12.80	14.88		12.81	18.81	56.03	29.08	9.80	8.8-16.8
D																			
G-CSF-mobilized	122.86	128.74	127.89	136.73	138.31	157.82	154.52	133.87	163.73	146.78	168.16	162.52		145.16	10.87	19.65	18.95	124.12	
Non-mobilized 1	2.39	1.41	4.79	1.21	3.30	4.73	3.55	5.48	6.72	4.73	3.51	3.62		3.79	42.80	28.86	78.95	1.03	
Non-mobilized 2	2.08	3.09	3.63	3.40	3.92	5.14	4.30	4.50	6.60	6.17	3.54	4.77		4.26	30.11	55.60	51.70	2.22	

Table 3: Full plate homogeneity with 2.0 µL/sec HTS acqusition speed

	1	2	3	4	5	6	7	8	9	10	11	12		mean row	%CV all columns	%CV col. 1 vs.12	%CV col 12 vs. Tube	tube	range
HQC	139.71	152.93	149.00	155.92	154.22	158.95	156.28	153.19	166.10	152.23	154.08	160.87		154.46	4.18	9.96	18.18	124.22	98.1-138.1
MQC	39.28	47.50	44.33	39.54	42.64	44.07	41.13	45.90	41.95	41.02	47.79	41.27		43.04	6.72	3.50	9.87	35.89	29.2-43.2
LQC	11.46	12.32	12.27	13.36	12.65	11.41	9.04	12.17	10.44	15.41	11.36	12.50		12.03	12.92	6.15	9.49	10.93	8.8-16.8
D																			
G-CSF-mobilized	129.34	133.64	105.67	123.07	123.06	110.20	131.75	152.11	144.71	131.68	136.51	119.97		128.48	10.28	5.31	10.76	103.01	
Non-mobilized 1	3.71	3.25	2.01	4.14	4.36	3.35	4.46	4.98	6.06	6.01	4.45	5.39		4.35	27.14	26.05	92.98	1.11	
Non-mobilized 2	2.77	2.77	2.74	4.01	2.64	2.63	3.09	3.26	6.52	4.62	3.36	4.91		3.61	33.24	39.32	68.34	1.71	

To further reduce RT incubation times, the experiment was repeated using half 96-well plates and high HTS acquisition speeds (2.0 µL/sec = ~1.5h/half plate, 2.5 µL/sec = ~1h15min/half plate). Results for 2.5 µL/sec are presented in Table 4. All samples except the non-mobilized showed reduced unspecific staining, CD34+ cells/µL calculations, and %CV across the plate and in comparison to the reference tube. Non-mobilized samples showed high random variability, as low precision is expected for very low cell concentrations in quasi-quantitative flow cytometry assays. Therefore, the combination of only using half a plate and a high HTS acqusition speed was able to minimize RT incubation times and unspecific staining effects.

Table 4: Half plate homogeneity with 2.5 µL/sec HTS acqusition speed

	1	2	3	4	5	6		mean row	%CV all columns	%CV col. 1 vs.6	%CV col 6 vs. Tube	tube	range
HQC	124.98	118.61	121.57	129.26	138.29	137.22		128.32	6.34	6.60	10.87	117.63	98.1-138.1
MQC	39.29	34.65	36.57	37.90	38.32	46.28		38.84	10.26	11.55	17.09	36.30	29.2-43.2
LQC	10.89	13.38	12.00	11.41	10.59	9.58		11.31	11.49	9.06	7.75	10.69	8.8-16.8
blank													
G-CSF-mobilized	104.27	121.76	124.38	130.34	120.89	119.94		120.26	7.22	9.89	2.80	115.29	
Non-mobilized 1	2.12	2.14	N/AV	3.52	4.80	3.71		3.26	34.95	38.42	18.65	2.84	
Non-mobilized 2	2.27	1.93	1.58	3.57	3.26	6.37		3.16	55.29	67.07	49.40	3.07	

Automation processing and incubation times

Automated sample processing was assessed 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 half plate with dummy samples in between. In order to assess only the effect of robot sample processing without the effect of prolonged HTS acquisition (2.5 µL/sec) at RT, the acquisition of the dummy samples was skipped. No high %CVs, no negative effects of automated processing, and no negative effects of prolonged incubation during processing were observed (Figure 2A).

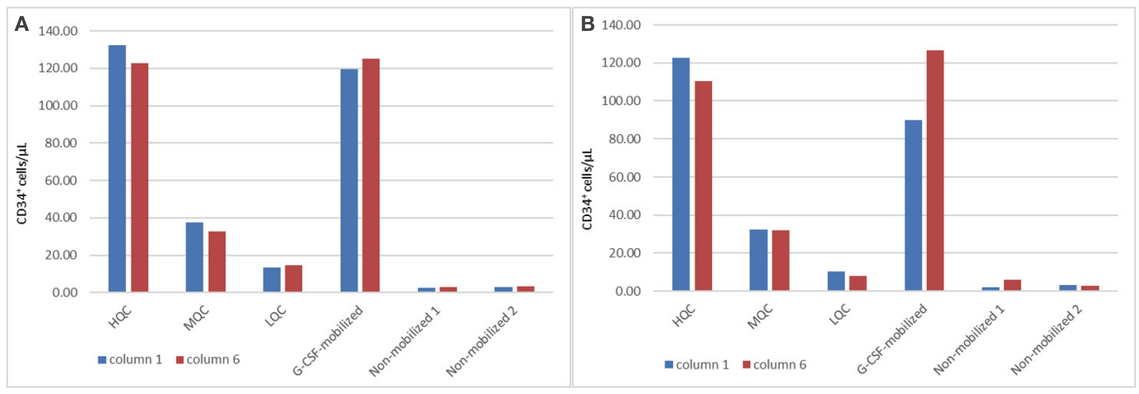

Figure 2:
A) Automated processing without HTS acquisition incubation times, B) Automated processing with HTS acquisition incubation time

To assess the combination of robot sample processing and HTS acquisition incubation times, the experiment was repeated with acquisition of the dummy samples (Figure 2B). No high %CVs and no negative effects of prolonged incubation times were observed, except for much lower CD34+ cells/µL calculations for the G-CSF-mobilized sample in the first column. We hypothesized that this imprecision was due to a much shorter and insufficient CD45-FITC/CD34-PE antibody incubation of the first column samples (~10 min) compared to the last column samples (~30 min) and the kit recommended incubation time (20 min). To minimize this difference in incubation times, a timer was added to the automation procedure to incrementally delay sample processing such that all samples are incubated with the CD45-FITC/CD34-PE antibodies for 30 min. After this adjustment, the automation experiment assessing the combination of robot sample processing and HTS acquisition incubation times with dummy acquisition was repeated (Figure 3). No high %CVs (except for low-precision non-mobilized samples), no negative effects of automated processing, and no negative effects of prolonged incubation during processing and HTS acquisition were observed.

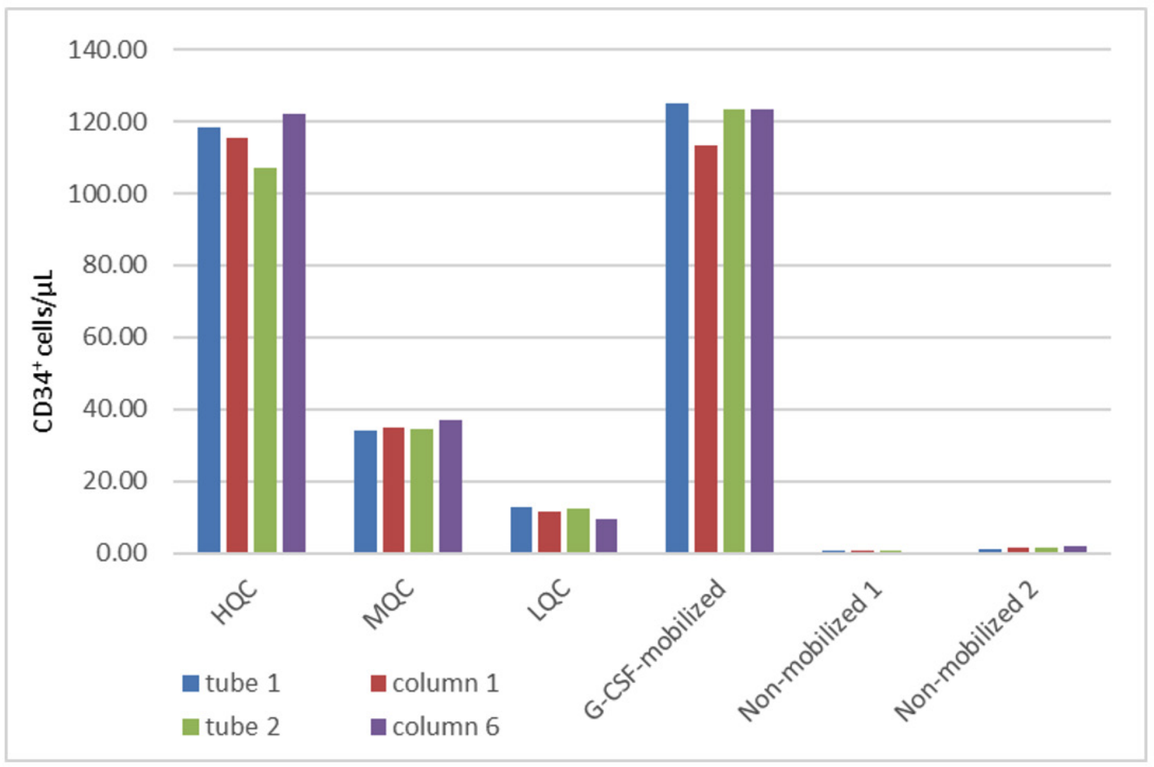

Figure 3: Automated processing with HTS acquisition incubation times – final setup

METHOD QUALIFICATION

Method qualification was successful, with <20% inter-and intra-run precision of G-CSF-mobilized samples and commercial QCs (Table 5), 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 at 5°C of G-CSF-mobilized samples and commercial QCs.

Table 5: Method qualification – inter- and intra-run precision

	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		

DISCUSSION & CONCLUSIONS

We demonstrate that it is possible to automate and transfer a low-throughput tube-based commercial assay to a high-throughput 96-well plate-based format with excellent precision, sufficient freeze-thaw and post-processing stability, and no instrument carryover. Together with the previously demonstrated -80°C long-term stability of stabilized whole blood, our fully automated CD34+ enumeration method allows the transport, long-term storage, and high-throughput bioanalysis of CD34+ samples, and is therefore able to support large clinical studies including G-CSF-based biosimilar studies.

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