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366.1	**Determining the total cell count and ratios of high and low nucleic acid content cells in freshwater using flow cytometry**

Table of contents

I Summary

Title	Determining the total cell count and quantitative ratios of high and low nucleic acid content cells in freshwater using flow cytometry
Measurement principle	Flow cytometry
Method number	366.1
Components	Total cell count and basic microbiological structure of a water sample
Matrix	Freshwater (e.g. groundwater, spring water, drinking water, mineral water, surface water etc.)
Abstract	A fluorescent stain is added to a water sample and penetrates the cells after a short incubation time, binding to the DNA and RNA. The stained water sample is then analysed with a flow cytometer. The total cell count is registered by measuring the fluorescence signals of each cell. The basic microbiological structure of the sample is analysed by differentiating between the fluorescence intensity and light scattering signals of low nucleic acid content (smaller) and high nucleic acid content (larger) microbial cells (mostly bacteria).
Measurement range	1.0×10^3 – 2.0×10^5 cells/ml (samples with higher cell concentrations are diluted with the media mentioned under *4.3*)
Detection limit	1.0×10^2 cells/ml [1;2]
Quantification limit	1.0×10^3 cells/ml [1;2]
Validation specifications	Yes
Comment on validation	External and internal comparative tests
Method status	Recommended
Latest update	2012
Method based on	–
Comments	Method: Eawag, Dept. Environmental Microbiology, Dübendorf

II Method description

1 Aim

Flow cytometry allows precise and rapid detection of the total microbial cell count of a water sample after staining with a fluorescent stain that binds to nucleic acids, as well as the differentiation between small, weak fluorescent (low nucleic acid, LNA) and large, strong fluorescent (high nucleic acid, HNA) bacterial cells that can occur in natural waters as well as drinking water [5; 6; 7; 8; 9; 10; 11].

The main areas of application cover everything from drinking water production, drinking water distribution networks, through to household installations [8; 12; 13; 14; 15; 16; 17; 18].

The method allows determination of microbial bulk parameters and delivers detailed information on the general microbial state and possible existing problems that can be verified with further specific analysis.

2 Symbols and acronyms

FC	flow cytometry, flow cytometer
DMSO	Dimethyl sulfoxid
G-FL	Green fluorescence
R-FL	Red fluorescence
SSC	Side scatter
TCC	Total cell count
LNA	Low Nucleic Acid
HNA	High Nucleic Acid
{G1}	Gate 1
{G2}	Gate 2
{G3}	Gate 3

3 Principle

The cells in a water sample are stained with a fluorescent stain (SYBR Green I) aliquot and incubated at 37°C (± 2°C) for 13 minutes in the dark to allow optimal penetration and binding of the stain. The sample is then analysed with the FC, where the cells are hydrodynamically focussed in a glass capillary and scanned one-by-one with a laser (488 nm). Each cell that passes the laser causes light to scatter and the emission of fluorescent light depending on the fluorescent stain used. The emerging scattered light and fluorescent light signals are recorded by separate detectors following wavelength specific filtration. The analysis software coupled to the FC can assign a specific scatter light and fluorescence signal to each particle or cell. Through the intensity of the recorded signals, small LNA content cells (weak fluorescence) can be discerned from larger HNA content cells (strong fluorescence), since binding of the fluorescent stain is proportional to the amount nucleic acids in microbial cells [19; 20]. This flow cytometric method allows the detection of hundreds of particles or cells per second. A very small sample volume (as little as 1 ml) is needed for the analysis.

4 Chemicals

Note: The chemicals used here could alternatively be acquired from other producers/suppliers as long as the criteria for the substances mentioned here are met.

4.1 Fluorescent stain

- SYBR® Green I Nucleic Acid Gel Stain, 10.000x concentrated in DMSO
  Invitrogen/Molecular Probes, Cat. Nr.: S7563

4.2 Dilution medium for the fluorescent stain

- Dimethyl sulfoxid (DMSO) 500 ml, CAS-Nr. 67-68-5
  Fluka, Cat. Nr.: 41644

4.3 Dilution medium for the water samples

- Evian® mineral water
- Vittel® mineral water

Note: Either Evian® or Vittel® can be used for dilution, but both must be filtered before use. The pore size for filtration is 0.22 µm.

- Ultra-pure water (e.g. NANOpure®)

4.4 Sheath fluids for the particle stream

The sheath fluids delivered by the producer with the flow cytometer in use

- *alternative*: ultra-pure water (e.g. NANOpure®)
- *alternative*: ultra-pure water (e.g. NANOpure®) with 0.05% Tween80® (Polysorbat 80, CAS-Nr. 9005-65-6)

Note: The quality of the ultra-pure water is influenced by the maintenance of the purification unit and if not done properly, can adversely affect measurements.

5 Equipment and consumables

Note: The specific flow cytometers and consumables (e.g., filters, vials, etc.) used during the validation of this method are not binding. Alternative instruments and consumables can be used, as long as they comply with the specifications required by the method described here (see also 5.1.1: Information on equipment requirements).

5.1 Flow cytometer

(Listing of the equipment used for the validation of this method)

– Partec PAS III	Partec GmbH, Münster (D)
– Partec Cyflow-Series	Partec GmbH, Münster (D)
– Accuri C6	BD Biosciences, 2350 Qume Drive, San Jose, California USA, 95131

5.1.1 Equipment requirements

- Laser: 488 nm, at least 20 mW
- Scatter light detectors: forward scatter (FSC) and side scatter (SSC)
- Fluorescence sensitivity: <100 MESF fluorescein isothiocyanate; <50 MESF phycoerythrin (MESF: Molecules of Equivalent Soluble Fluorochrome)
- Emission filters: 520 – 530 nm (green fluorescence); 610 – 670 nm (red fluorescence)
- Minimum particle size for SSC: 0.2 µm – 0.5 µm

5.1.2 Autoloader

Note: The use of an autoloader with the flow cytometer is possible for this method.

- Robby (PAS III) — Partec GmbH, Münster (D)
- Robby Well (Cyflow-Series) — Partec GmbH, Münster (D)
- CSampler — BD Biosciences, 2350 Qume Drive, San Jose, California, USA, 95131

5.2 Filter for DMSO

- IC Millex – IG 0.2 µm — Millipore, Cat.-Nr.: SLLGC25NS

5.3 Filter for the dilution medium mineral water

- Millex GV 0.22 µm (low throughput) — Millipore, Cat.-Nr.: SLGV033RB
- Millex GP 0.22 µm (high throughput) — Millipore, Cat.-Nr.: SLGP033RB

5.4 Storage of the stain aliquots

- Glass vial 2 ml — Supelco, Cat.-Nr.: 27267-U
- Septum 10 mm, PTFE/Silicon — Supelco, Cat.-Nr.: 27277
- Polypropylene screw cap — Supelco, Cat.-Nr.: 27271

5.5 Containers for the analysis on Partec instruments

- Tubes, 3.5 ml — Sarstedt, Ref.-Nr.: 55.484

6 Method execution

6.1 Preparation of the fluorescent stain

- The fluorescent stain SYBR Green I (10’000x) is diluted 1:100 in 0.2 µm filtered DMSO.

Note: The prepared stain aliquots can be stored at -20 °C in glass vials over a long time period (also see *5.4*). Protective gloves should be worn while handling the stain.

6.2 Staining the samples

- The stain is added in a ratio of 1:100 to the sample (for example: 10 µl SYBR Green I 100x in DMSO *(6.1)* in 1000 µl sample).
- The sample is mixed on the vortex for 5 seconds.
- The stained sample is incubated in a heating block at 37°C (± 2°C) for a minimum of 13 minutes in the dark.
- In this manner, many samples can be prepared simultaneously and stored in the dark until needed.

Note: In laboratories, samples are usually stored at 4 °C. With a sample volume of 1 ml, three minutes are necessary to increase the core temperature of the sample from 4 °C to 37 °C. Based on this the 13-minute incubation time was calculated - 3 minutes to reach core temperature and 10 minutes incubation time.

6.3 Sample dilution and measurement range

- During the measurement, an upper limit of approximately 1’000 counts/second must not be exceeded (depending on specific FC instrument specifications). This represents a cell count in the sample of approximately $1.0 \times 10^5 – 2.0 \times 10^5$ cells/ml (depending on the sample background signals). Otherwise, the sample should be diluted with the dilution media listed under *4.3* until a value below the upper detection limit is reached.

Note: It is recommended to dilute the samples with too high cell concentrations before staining and incubation. The stain does not bind irreversibly to the cell DNA, and can diffuse out of the cell when diluted after staining, which will diminish the signal.

6.4 Sample analysis

6.4.1 Controls

- The flow cytometer should be tested routinely with the appropriate calibration beads supplied by the producer for this purpose. Individual laboratories can change or improve this test if needed.
- It is recommended, that the dilution medium (see *4.3*) be used as a blind sample or negative control. Stain (see *6.1*) is added to the blind sample and treated as described under *6.2*.

6.4.2 Measurement

- The “trigger”-setting on the analysis software of the flow cytometer, must be set on green fluorescence (G-FL).
- The optimal “gain”-setting for Partec instruments should be determined for each instrument separately. When the optimal settings are determined, they should (and must) not be changed again.
- The „log“-setting for Partec instruments should be chosen as follows:

	Option 1 (recommended)	**Option 2**
SSC	log 3	log 3
G-FL	log 3	log 4
R-FL	log 3	log 4

- For Partec instruments (PAS III and Cyflow-Series), a nominal flow rate („speed“) of ≤ 5 (corresponding to ≤ 5 µl/s) should be chosen.
- For Accuri C6 instruments, a nominal flow rate („speed“) of 35 µl/min (corresponding to the setting „medium“ in CFlow-software) is recommended.

Note: For software different from the here-mentioned instruments (Partec, Accuri), functions such “gain“, “trigger“, “log“ or “speed“ could have different descriptions.

7 Processing

For the processing of the measurements the following signal combinations are required:

- G-FL/R-FL density plot
- G-FL/SSC density plot
- G-FL histogram

7.1 Determining the TCC

Determining the TCC {G1} is done using the gating option in the G-FL/R-FL density plot.

The software indicates the current concentration for {G1} in counts/volume (counts/ml or counts/µl) (*Fig. 1*).

7.2 Determining the LNA/HNA ratios

Option 1:

Determining LNA {G2} and HNA {G3} is done using the gating option in the G-FL/SSC density plot. It is done as follows:

The TCC-gate {G1} is transferred to the G-FL/SSC density plot.

→ Gates 2 {G2} and 3 {G3} are then defined in the G-FL/SSC density plot.

The software calculates the concentrations for {G2} and {G3} in counts/volume (counts/ml or counts/µl). The gates {G2} and {G3} are then calculated percentagewise (*Fig. 1*).

Option 2:

Determining LNA {G2} and HNA {G3} is done using the gating option in the G-FL histogram. It is done as follows:

The TCC-gate {G1} is transferred to the G-FL histogram.

→ Gates 2 {G2} and 3 {G3} are then defined in the G-FL histogram.

The software calculates the concentrations for {G2} and {G3} in counts/volume (counts/ml or counts/µl). The gates {G2} and {G3} are then calculated percentagewise (*Fig. 1*).

Note 1: For Accuri instruments it is recommended to determine the LNA/HNA with Option 2.

Note 2: For detailed information regarding the determination of TCC and LNA/HNA ratios, please see "*Methodenhandbuch*" (a.t.m. in german language only)

Example (Option 1 and Option 2 are calculated identically. The results differ insignificantly.):

LNA {G2} = 5.05 x 10^4 cells/ml

HNA {G3} = 3.65 x 10^4 cells/ml

Percentagewise:

$$\frac{\text{LNA }\{G2\}}{(\text{LNA }\{G2\}+\text{HNA }\{G3\})} \times 100 = \text{LNA in \%, here } 58\% \text{ LNA}$$

$$\frac{\text{HNA }\{G3\}}{(\text{LNA }\{G2\}+\text{HNA }\{G3\})} \times 100 = \text{HNA in \%, here } 42\% \text{ HNA}$$

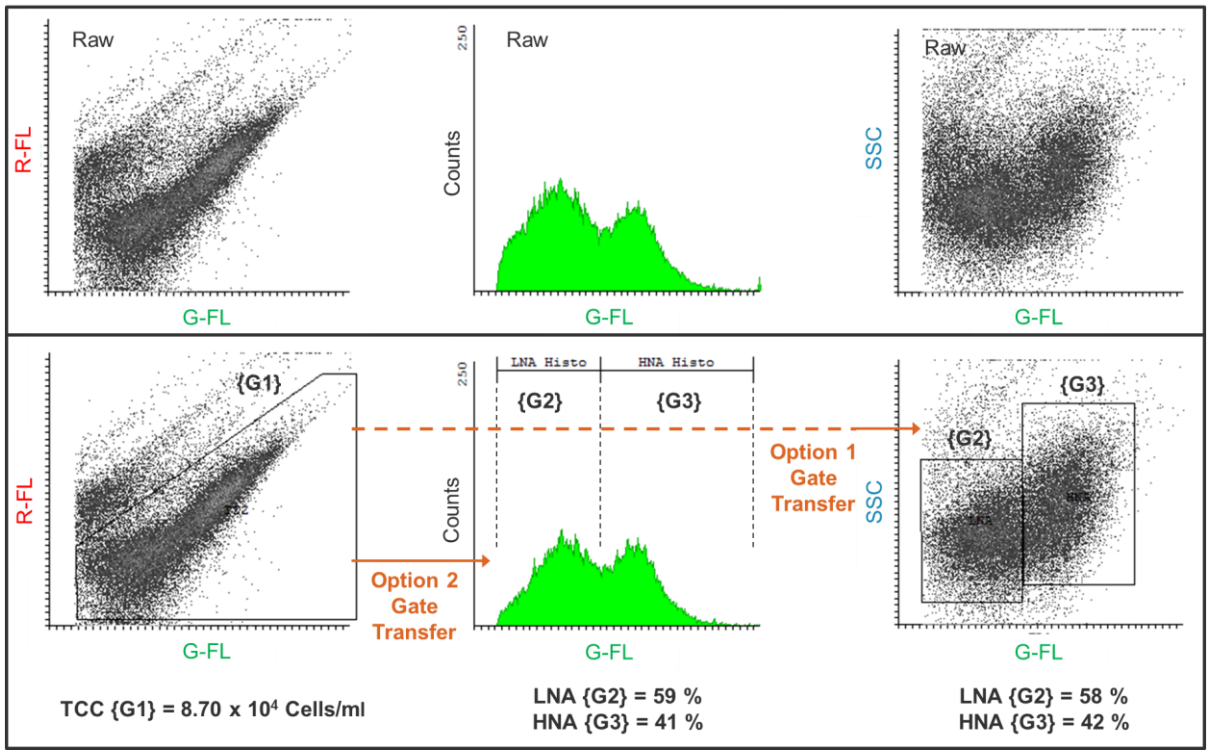

Figure 1: Example for determining the TCC {G1} in the G-FL/R-FL density plot. *Option 1*: Gate-transfer from the G-FL/R-FL density plot {G1} to the G-FL/SSC density plot. *Option 2*: Gate-Transfer from the G-FL/R-FL density plot {G1} to the G-FL histogram. Both options are used to calculate the numbers for LNA {G2} and HNA {G3}.

8 Validation

The validation was done according to BAG guidelines [21].

Information on the measurement range, analysis and quantification limit as well as accuracy can be found in the method summary. The purpose of the validation presented here was to verify that applying the method in different laboratories will lead to comparable results. To this aim, an external comparative study was done with active participation and under guidance from Eawag, Department of Environmental Microbiology, Dübendorf. The reproducibility (R), repeatability (r) and measurement uncertainty (RSD) of the method in this comparison were recorded.

The financing of the comparative study was provided by the Commission for Technology and Innovation (CTI), Project number 12121.1 PFIW-IW. The project to adapt the method for the SLMB was provided by the Forschungsfond Wasser (FOWA) of the Swiss Society for Gas and Water (des Schweizerischen Vereins des Gas- und Wasserfachs (SVGW)), Project number 5221.00532.

Group 1

Participants: 7 external, 4 internal

Parameters: Analysis of water samples to determine the bead count, the total cell count and the LNA/HNA-ratios in triplicate

Sample treatment:

- The samples were fixed with glutaraldehyde (5.6 M, 50 %)) for the purpose of this study.
- Dilution 1:1000 (1 ml glutaraldehyde in 1000 ml sample). Samples fixed in this manner could be sent for analysis without cooling.
- All 45 samples were analysed directly after sampling to determine the untreated original state. A control analysis with the glutaraldehyde-fixed samples showed that the fixation had no influence on the TCC or LNA/HNA-ratios.

- The samples were filled in 40 ml glass vials with Teflon lids and sent to the external participants (listed in *Table 2*).
- Prior to sending, the 40 ml glass vials were burned clean at 500 °C for 3 hours.
- During the delivery period, reference samples were stored at room temperature in the Eawag laboratory to ensure comparable experimental conditions for all the samples.

Group 2

Participants: 2 external, 2 internal

Parameters: Analysis of water samples to determine the bead count, the total cell count and the LNA/HNA-rations in triplicate

Sample treatment:

- The samples were cooled to 8 °C.
- All 20 samples were analysed directly after sampling to determine original state.
- The samples were then filled in 40 ml glass vials with Teflon lids and sent cooled with cooling packs to the external participants (listed in *Table 2*).
- Prior to sending, the 40 ml glass vials were burned clean at 500 °C for 3 hours.
- During the delivery period, reference samples were stored at 8 °C in the Eawag laboratory to ensure comparable experimental conditions for all the samples.

Table 1: List of all the samples sent out for the comparative study, according to their origin, total cell count and LNA/HNA-ratios. AVE = average; n = number of measurements.

Sample origin	**Name**	**AVE bead count & AVE TCC in counts/ml (n=3)**	**AVE LNA in % (n=3)**	**AVE HNA in % (n=3)**
Calibration-Beads Partec (d = 3 µm) REF: 05-4018	Beads (3 µm)	1.08×10^4	-	-
Groundwater Hardhof Zürich	Sample 1	3.43×10^4	61	39
Spring water Hardhof Zürich	Sample 2	6.60×10^4	51	49
Drinking water after treatment Lengg Zürich	Sample 3	1.13×10^5	48	52
Drinking water Oetwil a. See (household installation after rinsing)	Sample 4	1.35×10^5	36	64

Note: For the comparative study, the calibration beads were diluted, mixed thoroughly and from this reference solution evenly filled in the individual sample vials. The beads were used to control and compare instrument performance and accuracy. None of the samples in the comparative study received any kind of network treatment (e.g. chlorine) or were treated with any disinfecting agent (e.g. UV) after sampling. No further dilution of the samples was necessary for analysis. The participants received aliquots from the original sample to ensure that the cell count for all the samples was as identical as possible.

Table 2: List of the participating institutions, the number of active participants and the **instruments** used. The individual **instruments** are numbered (in brackets) for easy reference.

Participating Institutions Group 1	**Abbreviation**	**Number of participants**	**Instruments**
Swiss Federal Institute of Aquatic Science and Technology	Eawag	4	Partec SL (1) Partec Space (2) Partec Space (3)
Cantonal Laboratory Zürich	KLZ	1	Accuri C6 (4)
Wasserversorgung Zürich	WVZ	1	Partec Space (5)
Federal Office of Public Health	BAG	1	Partec ML (6)
Labor Spiez	-	1	Partec SL (7)
Technical University Hamburg-Harburg / DVGW Forschungsstelle	TUHH-DVGW	1	Partec SL (8)
European Aeronautic Defense and Space Company / Innovation Works	EADS / IW	1	Partec SL (9)
Technical University Dresden / Professur Wasserversorgung	TU Dresden	1	Accuri C6 (10)
Participating Institutions Gruppe 2			
Swiss Federal Institute of Aquatic Science and Technology	Eawag	2	Partec SL (1) Partec PAS III (11)
Industrielle Werke Basel	IWB	1	Partec SL (12)
Bachema AG	-	1	Partec SL (13)

8.1 Comments on the validation

The results of the comparative study, with the aim of validating the method, show that flow cytometry is a robust method to determine the microbial state of freshwater and therewith also drinking water. Flow cytometry delivers microbial bulk parameter information rapidly and in detail.

The comparative study was done with active participation and under guidance of the Swiss Federal Institute of Aquatic Science and Technology (Eawag), Department of Environmental Microbiology, Dübendorf. The Eawag laboratory was responsible for sampling, preparation and distribution of the samples to the participants. The 15 test participants were divided over 10 different institutions in Switzerland and Germany.

Thirteen flow cytometers were used in this study. For this comparative study, the participants were divided into two groups.

Group 1 received samples fixed in glutaraldehyde, while Group 2 received samples that were not fixed but cooled at ~ 8 °C. Both groups received a bead sample.

The parameters determined by both groups were the bead count, the TCC and the LNA/HNA-ratio.

The data for the total of 655 generated data points (including the LNA/HNA-values) for this comparative study showed very little variance.

The relative standard deviation for the bead samples for Group 1 were 6.74 % (*Fig. 2*) and for Group 2 3.33 % (*Fig. 4*) For the water samples, the average for the relative standard deviation (RSD) for the TCC was 6.88 % for Group 1 (*Fig. 2*) and 5.72 % for Group 2 (Fig. 4).

Below are given the individual relative standard deviation values:

Group 1, TCC (see *Fig. 2*):

„Sample 1“ = 4.56 %, „Sample 2“ = 7.97 %, „Sample 3“ = 6.92 % and „Sample 4“ = 8.08 %

Group 2, TCC (See *Fig. 4*):

„Sample 1“ = 4.76 %, „Sample 2“ = 11.43 %, „Sample 3“ = 3.37 % and „Sample 4“ = 3.33 %

The LNA- and HNA-values determined for Group 1 had an average relative standard deviation (RSD) of 12.53 % for the LNA-values and an average of 16.16 % for the HNA-values.

Below are given the individual relative standard deviation values (see *Fig. 3*):

Group 1, LNA-values:

„Sample 1“ = 7.81 %, „Sample 2“ = 13.68 %, „Sample 3“ = 15.22 % and „Sample 4“ = 13.42 %

Group 1, HNA-values:

„Sample 1“ = 17.73 %, „Sample 2“ = 14.44 %, „Sample 3“ = 15.86 % and „Sample 4“ = 16.85 %

Group 2 generated similar values. The average relative standard deviation (RSD) for the LNA-values was 9.79 % and for the HNA-values 12.53 %.

Below are given the individual relative standard deviation values (see *Fig. 5*):

Group 2, LNA-values:

„Sample 1“ = 8.70 %, „Sample 2“ = 10.17 %, „Sample 3“ = 10.15 % and „Sample 4“ = 10.14 %

Group 2, HNA-values:

„Sample 1“ = 10.00 %, „Sample 2“ = 23.15 %, „Sample 3“ = 5.56 % and „Sample 4“ = 11.40 %

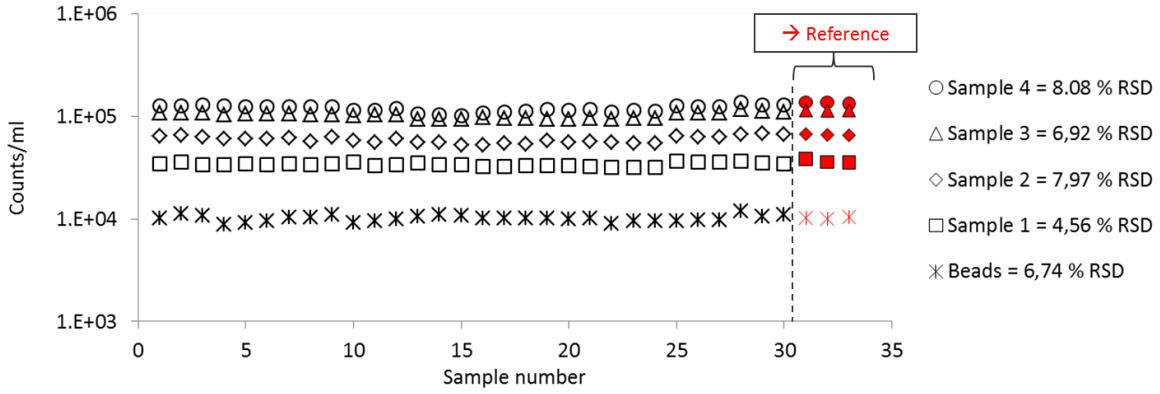

Figure 2: Overview of the results of the comparative test for Group 1 (fixed samples) showing the bead count and total cell count and their relative standard deviation (RSD).

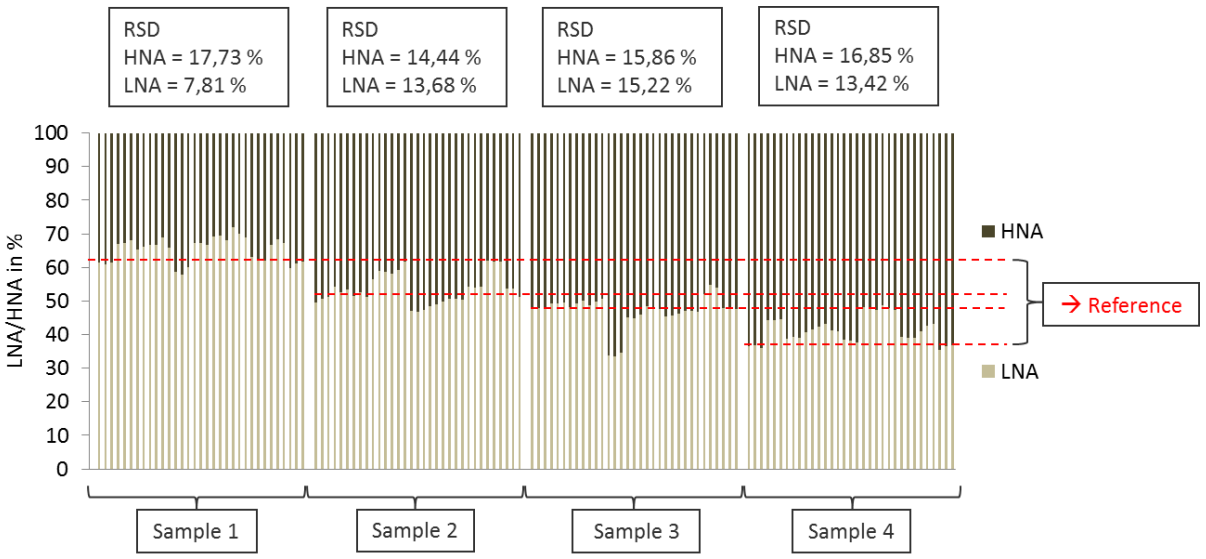

Figure 3: Overview of the results of the comparative test for Group 1 (fixed samples) showing the LNA- and HNA-values and their relative standard deviation (RSD) reported as percentage ratios to each other (LNA/HNA in %).

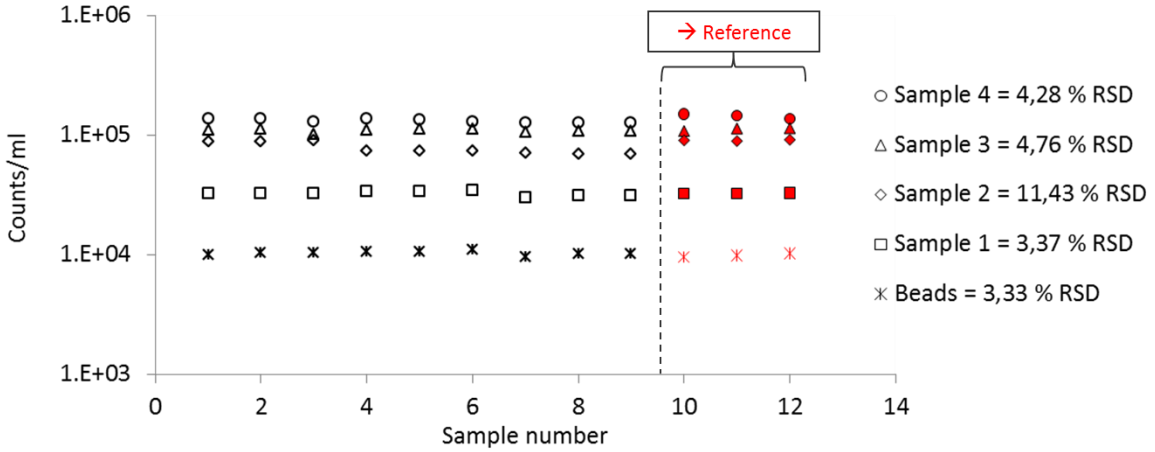

Figure 4: Overview of the results of the comparative test for Group 2 (non-fixed samples) showing the bead count and total cell count and their relative standard deviation (RSD). Regarding sample 2, a slight increase in cell numbers was observed despite cooling.

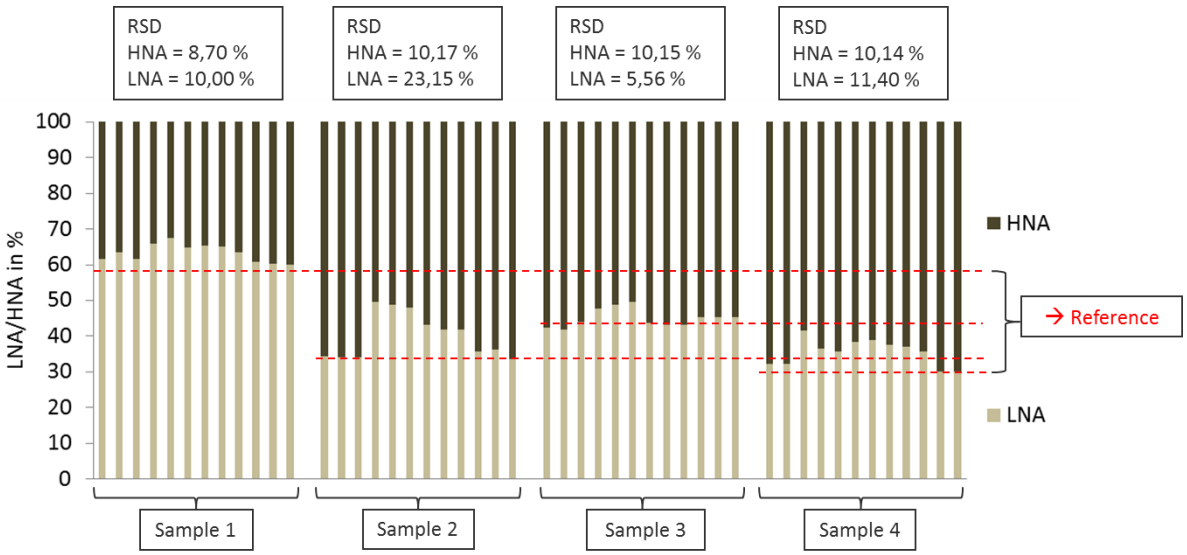

Figure 5: Overview of the results of the comparative test for Group 2 (non-fixed samples) showing the LNA- and HNA-values and their relative standard deviation (RSD) reported as percentage ratios to each other (LNA/HNA in %). Regarding sample 2, a slight change in LNA/HNA-ratio was observed despite cooling.

Table 3: Comparative results for the bead samples (3 µm), Group 1, for the number of beads

Participant / Instrument	**AVE Beads (3 µm) in counts/ml (n=3)**	**Beads repeatability (r) in counts/ml (n=3)**	**Beads measurement Uncertainty RSD in % (n=3)**
*Participant 1 / Partec SL (1)	1.01E+04	1.48E+03	5.21
* Participant 2 / Partec Space (2)	8.85E+03	8.82E+02	3.56
* Participant 3 / Partec SL (1)	1.04E+04	1.04E+03	3.58
* Participant 4 / Partec Space (3)	1.02E+04	6.02E+02	2.12
Participant 5 / Accuri C6 (4)	9.22E+03	8.98E+02	3.48
Participant 6 / Partec Space (5)	1.04E+04	7.02E+02	2.4
Participant 7 / Partec ML (6)	1.00E+04	2.52E+02	0.9
Participant 8 / Partec SL (7)	9.58E+03	3.02E+02	1.13
Participant 9 / Partec SL (8)	1.00E+04	4.11E+02	1.46
Participant 10 / Partec SL (9)	8.94E+03	9.39E+02	3.75
Participant 11 / Accuri C6 (10)	1.19E+04	2.05E+03	6.15
Beads reproducibility (R) in Counts/ml (n=33)	1.91E+03		
Beads measurement uncertainty RSD in % (n=33)	6.74		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 4: Comparative results for sample "1", Group 1, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 1 / Partec SL (1)	3.42E+04	2.70E+03	2.83
*Participant 2 / Partec Space (2)	3.38E+04	1.01E+03	1.07
*Participant 3 / Partec SL (1)	3.45E+04	1.31E+03	1.36
*Participant 4 / Partec Space (3)	3.81E+04	4.08E+03	3.82
Participant 5 / Accuri C6 (4)	3.60E+04	4.00E+03	3.97
Participant 6 / Partec Space (5)	3.47E+04	1.77E+03	1.82
Participant 7 / Partec ML (6)	3.22E+04	1.75E+03	1.94
Participant 8 / Partec SL (7)	3.64E+04	1.19E+03	1.17
Participant 9 / Partec SL (8)	3.28E+04	1.70E+03	1.86
Participant 10 / Partec SL (9)	3.14E+04	6.65E+02	0.76
Participant 11 / Accuri C6 (10)	3.64E+04	2.98E+03	2.92
TCC reproducibility (R) in counts/ml (n=33)	4.36E+03		
TCC measurement uncertainty RSD in % (n=33)	4.56		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 5: Comparative results for sample "2", Group 1, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 1 / Partec SL (1)	6.35E+04	2.31E+03	1.3
*Participant 2 / Partec Space (2)	6.05E+04	1.01E+03	0.6
*Participant 3 / Partec SL (1)	6.16E+04	8.72E+03	5.06
*Participant 4 / Partec Space (3)	6.70E+04	2.56E+03	1.36
Participant 5 / Accuri C6 (4)	5.76E+04	6.71E+03	4.16
Participant 6 / Partec Space (5)	5.55E+04	5.01E+03	3.22
Participant 7 / Partec ML (6)	5.22E+04	3.62E+03	2.48
Participant 8 / Partec SL (7)	6.39E+04	1.91E+03	1.06
Participant 9 / Partec SL (8)	5.77E+04	2.46E+03	1.52
Participant 10 / Partec SL (9)	5.54E+04	1.90E+03	1.22
Participant 11 / Accuri C6 (10)	6.70E+04	3.19E+03	1.7
TCC reproducibility (R) in counts/ml (n=33)	1.33E+04		
TCC measurement uncertainty RSD in % (n=33)	7.97		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 6: Comparative results for sample "3", Group 1, for the total cell count.

Participant / Instrument	AVE TCC in counts/ml (n=3)	TCC repeatability (r) in counts/ml (n=3)	TCC measurement uncertainty RSD in % (n=3)
*Participant 1 / Partec SL (1)	1.08E+05	1.95E+03	0.65
*Participant 2 / Partec Space (2)	1.03E+05	2.92E+03	1.02
*Participant 3 / Partec SL (1)	1.06E+05	3.06E+03	1.03
*Participant 4 / Partec Space (3)	1.14E+05	8.88E+02	0.28
Participant 5 / Accuri C6 (4)	1.01E+05	2.84E+03	1.0
Participant 6 / Partec Space (5)	9.43E+04	1.89E+03	0.72
Participant 7 / Partec ML (6)	9.80E+04	4.45E+03	1.62
Participant 8 / Partec SL (7)	1.08E+05	1.04E+03	0.34
Participant 9 / Partec SL (8)	9.42E+04	4.39E+02	0.17
Participant 10 / Partec SL (9)	9.31E+04	4.20E+03	1.61
Participant 11 / Accuri C6 (10)	1.16E+05	1.01E+04	3.11
TCC reproducibility (R) in counts/ml (n=33)	1.99E+04		
TCC measurement uncertainty RSD in % (n=33)	6.92		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 7: Comparative results for sample "4", Group 1, for the total cell count.

Participant / Instrument	AVE TCC in counts/ml (n=3)	TCC repeatability (r) in counts/ml (n=3)	TCC measurement uncertainty RSD in % (n=3)
*Participant 1 / Partec SL (1)	1.27E+05	3.88E+03	1.09
*Participant 2 / Partec Space (2)	1.27E+05	5.56E+03	1.56
*Participant 3 / Partec SL (1)	1.24E+05	1.91E+03	0.55
*Participant 4 / Partec Space (3)	1.37E+05	7.39E+03	1.93
Participant 5 / Accuri C6 (4)	1.13E+05	7.29E+03	2.3
Participant 6 / Partec Space (5)	1.05E+05	5.12E+03	1.73
Participant 7 / Partec ML (6)	1.08E+05	4.80E+03	1.58
Participant 8 / Partec SL (7)	1.26E+05	4.32E+03	1.23
Participant 9 / Partec SL (8)	1.17E+05	3.29E+03	1.0
Participant 10 / Partec SL (9)	1.09E+05	6.82E+03	2.23
Participant 11 / Accuri C6 (10)	1.36E+05	1.17E+04	3.08
TCC reproducibility (R) in counts/ml (n=33)	2.72E+04		
TCC measurement uncertainty RSD in % (n=33)	8.08		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 8: Comparative results for sample "1", Group 1, for the LNA/HNA-ratios.

Participant / Instrument	AVE LNA in counts/ml (n=3)	LNA repeatability (r) in counts/ml (n=3)	LNA measurement uncertainty RSD in % (n=3)	AVE HNA in counts/ml (n=3)	HNA repeatability (r) in counts/ml (n=3)	HNA measurement uncertainty RSD in % (n=3)	AVE LNA + AVE HNA in counts/ml	LNA fraction in %	HNA fraction in %
*Participant 1 / Partec SL (1)	2.32E+04	2.24E+02	0.34	1.45E+04	4.53E+02	1.11	3.78E+04	61	39
*Participant 2 / Partec Space (2)	2.26E+04	5.83E+02	0.92	1.11E+04	8.08E+02	2.60	3.36E+04	67	33
*Participant 3 / Partec SL (1)	2.31E+04	1.19E+03	1.84	1.22E+04	7.71E+02	2.25	3.51E+04	66	34
*Participant 4 / Partec Space (3)	2.07E+04	8.88E+02	1.53	1.39E+04	1.19E+03	3.04	3.44E+04	61	39
Participant 5 / Accuri C6 (4)	1.75E+04	8.54E+02	1.74	8.74E+03	1.46E+03	5.96	2.59E+04	67	33
Participant 6 / Partec Space (5)	2.04E+04	1.39E+03	2.43	1.44E+04	1.45E+03	3.60	3.40E+04	59	41
Participant 7 / Partec ML (6)	2.16E+04	3.23E+01	0.05	1.05E+04	3.60E+02	1.22	3.23E+04	67	33
Participant 8 / Partec SL (7)	2.24E+04	1.49E+03	2.37	1.31E+04	2.03E+02	0.55	3.49E+04	62	38
Participant 9 / Partec SL (8)	2.04E+04	1.56E+03	2.74	9.11E+03	6.03E+02	2.36	2.99E+04	69	31
Participant 10 / Partec SL (9)	2.22E+04	5.74E+02	0.92	8.60E+03	1.80E+03	7.47	3.14E+04	70	30
Participant 11 / Accuri C6 (10)	2.32E+04	3.02E+03	4.64	1.16E+04	2.28E+03	7.00	3.28E+04	67	33
Reproducibility (R) in counts/ml (n=33)	4.68E+03			5.71E+03					
Measurement uncertainty RSD in % (n=33)	7.81			17.73					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 9: Comparative results for sample "2", Group 1, for the LNA/HNA-ratios.

Participant / Instrument	AVE LNA in counts/ml (n=3)	LNA repeatability (r) in counts/ml (n=3)	LNA measurement uncertainty RSD in % (n=3)	AVE HNA in counts/ml (n=3)	HNA repeatability (r) in counts/ml (n=3)	HNA measurement uncertainty RSD in % (n=3)	AVE LNA + AVE HNA in counts/ml	LNA fraction in %	HNA fraction in %
*Participant 1 / Partec SL (1)	3.09E+04	1.09E+03	1.26	3.13E+04	1.80E+03	2.05	6.21E+04	51	49
*Participant 2 / Partec Space (2)	3.28E+04	2.10E+03	2.29	2.75E+04	1.01E+03	1.31	5.99E+04	53	47
*Participant 3 / Partec SL (1)	3.04E+04	3.17E+03	3.72	2.86E+04	5.32E+03	6.64	5.76E+04	52	48
*Participant 4 / Partec Space (3)	3.68E+04	5.28E+03	5.13	3.16E+04	3.06E+02	0.35	6.72E+04	53	47
Participant 5 / Accuri C6 (4)	2.51E+04	3.92E+03	5.57	1.95E+04	3.00E+03	5.49	4.51E+04	58	42
Participant 6 / Partec Space (5)	3.23E+04	1.13E+03	1.25	2.32E+04	4.71E+03	7.26	5.47E+04	60	40
Participant 7 / Partec ML (6)	2.46E+04	1.48E+03	2.15	2.76E+04	2.18E+03	2.82	5.32E+04	47	53
Participant 8 / Partec SL (7)	3.48E+04	1.22E+03	1.25	2.94E+04	9.19E+02	1.12	6.34E+04	54	46
Participant 9 / Partec SL (8)	2.48E+04	1.23E+03	1.77	2.63E+04	7.33E+02	1.00	5.11E+04	49	51
Participant 10 / Partec SL (9)	2.64E+04	9.26E+02	1.25	2.58E+04	6.97E+02	0.96	5.27E+04	51	49
Participant 11 / Accuri C6 (10)	3.59E+04	2.14E+03	2.13	2.21E+04	1.65E+03	2.67	5.92E+04	62	38
Reproducibility (R) in counts/ml (n=33)	1.17E+04			1.07E+04					
Measurement uncertainty RSD in % (n=33)	13.68			14.44					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 10: Comparative results for sample "3", Group 1, for the LNA/HNA-ratios.

Participant / Instrument	AVE LNA in counts/ml (n=3)	LNA repeatability (r) in counts/ml (n=3)	LNA measurement uncertainty RSD in % (n=3)	AVE HNA in counts/ml (n=3)	HNA repeatability (r) in counts/ml (n=3)	HNA measurement uncertainty RSD in % (n=3)	AVE LNA + AVE HNA in counts/ml	LNA fraction in %	HNA fraction in %
*Participant 1 / Partec SL (1)	4.92E+04	6.45E+02	0.47	5.34E+04	1.09E+03	0.73	1.02E+05	48	52
*Participant 2 / Partec Space (2)	5.04E+04	1.91E+03	1.35	5.19E+04	1.22E+03	0.84	1.04E+05	49	51
*Participant 3 / Partec SL (1)	4.89E+04	2.16E+03	1.58	5.24E+04	3.17E+03	2.16	1.01E+05	49	51
*Participant 4 / Partec Space (3)	5.45E+04	7.21E+02	0.47	5.92E+04	1.23E+03	0.74	1.14E+05	48	52
Participant 5 / Accuri C6 (4)	3.36E+04	1.90E+03	2.02	3.55E+04	2.19E+03	2.21	6.92E+04	50	50
Participant 6 / Partec Space (5)	3.19E+04	2.00E+03	2.24	6.24E+04	1.82E+03	1.04	9.36E+04	34	66
Participant 7 / Partec ML (6)	4.41E+04	2.25E+03	1.82	5.39E+04	3.30E+03	2.19	9.62E+04	45	55
Participant 8 / Partec SL (7)	4.94E+04	1.14E+03	0.83	5.59E+04	6.58E+02	0.42	1.05E+05	47	53
Participant 9 / Partec SL (8)	4.20E+04	1.85E+03	1.57	4.45E+04	9.75E+02	0.78	8.61E+04	48	52
Participant 10 / Partec SL (9)	4.13E+04	2.89E+03	2.50	4.95E+04	2.17E+03	1.56	9.27E+04	46	54
Participant 11 / Accuri C6 (10)	4.46E+04	1.32E+03	1.05	4.11E+04	6.46E+03	5.62	8.29E+04	54	46
Reproducibility (R) in counts/ml (n=33)	1.90E+04			2.24E+04					
Measurement uncertainty RSD in % (n=33)	15.22			15.86					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 11: Comparative results for sample "4", Group 1, for the LNA/HNA-ratios.

Participant / Instrument	AVE LNA in counts/ml (n=3)	LNA repeatability (r) in counts/ml (n=3)	LNA measurement uncertainty RSD in % (n=3)	AVE HNA in counts/ml (n=3)	HNA repeatability (r) in counts/ml (n=3)	HNA measurement uncertainty RSD in % (n=3)	AVE LNA + AVE HNA in counts/ml	LNA fraction in %	HNA fraction in %
*Participant 1 / Partec SL (1)	4.92E+04	6.45E+02	0.47	5.34E+04	1.09E+03	0.73	1.02E+05	48	52
*Participant 2 / Partec Space (2)	5.04E+04	1.91E+03	1.35	5.19E+04	1.22E+03	0.84	1.04E+05	49	51
*Participant 3 / Partec SL (1)	4.89E+04	2.16E+03	1.58	5.24E+04	3.17E+03	2.16	1.01E+05	49	51
*Participant 4 / Partec Space (3)	5.45E+04	7.21E+02	0.47	5.92E+04	1.23E+03	0.74	1.14E+05	48	52
Participant 5 / Accuri C6 (4)	3.36E+04	1.90E+03	2.02	3.55E+04	2.19E+03	2.21	6.92E+04	50	50
Participant 6 / Partec Space (5)	3.19E+04	2.00E+03	2.24	6.24E+04	1.82E+03	1.04	9.36E+04	34	66
Participant 7 / Partec ML (6)	4.41E+04	2.25E+03	1.82	5.39E+04	3.30E+03	2.19	9.62E+04	45	55
Participant 8 / Partec SL (7)	4.94E+04	1.14E+03	0.83	5.59E+04	6.58E+02	0.42	1.05E+05	47	53
Participant 9 / Partec SL (8)	4.20E+04	1.85E+03	1.57	4.45E+04	9.75E+02	0.78	8.61E+04	48	52
Participant 10 / Partec SL (9)	4.13E+04	2.89E+03	2.50	4.95E+04	2.17E+03	1.56	9.27E+04	46	54
Participant 11 / Accuri C6 (10)	4.46E+04	1.32E+03	1.05	4.11E+04	6.46E+03	5.62	8.29E+04	54	46
Reproducibility (R) in counts/ml (n=33)	1.90E+04			2.24E+04					
Measurement uncertainty RSD in % (n=33)	15.22			15.86					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 12: Comparative results for the bead samples (3µm), Group 2, for the number of beads.

Participant / Instrument	**AVE Beads (3 µm) in counts/ml (n=3)**	**Repeatability (r) in counts/ml (n=3)**	**Measurement uncertainty RSD in % (n=3)**
*Participant 12 / Partec SL (1)	9.57E+03	9.30E+02	3.47
*Participant 13 / Partec PAS III (11)	9.66E+03	8.19E+02	3.03
Participant 14 / Partec SL (12)	9.31E+03	9.11E+02	3.49
Participant 15 / Partec SL (13)	1.03E+04	6.86E+02	2.38
Beads reproducibility (R) in counts/ml (n=12)	1.21E+03		
Beads measurement uncertainty RSD in % (n=12)	4.28		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 13: Comparative results for sample "1", Group 2, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 12 / Partec SL (1)	3.23E+04	7.23E+02	0.80
*Participant 13 / Partec PAS III (11)	3.24E+04	5.25E+02	0.58
Participant 14 / Partec SL (12)	2.98E+04	2.25E+03	2.70
Participant 15 / Partec SL (13)	3.38E+04	1.15E+03	1.21
TCC reproducibility (R) in counts/ml (n=12)	4.31E+03		
TCC measurement uncertainty RSD in % (n=12)	4.76		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 14: Comparative results for sample "2", Group 2, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 12 / Partec SL (1)	9.03E+04	3.17E+03	1.25
*Participant 13 / Partec PAS III (11)	8.68E+04	8.62E+02	0.35
Participant 14 / Partec SL (12)	6.87E+04	1.48E+03	0.77
Participant 15 / Partec SL (13)	7.15E+04	1.54E+03	0.77
TCC reproducibility (R) in counts/ml (n=12)	2.42E+04		
TCC measurement uncertainty RSD in % (n=12)	11.43		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 15: Comparative results for sample "3", Group 2, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 12 / Partec SL (1)	1.08E+05	9.19E+03	3.03
*Participant 13 / Partec PAS III (11)	1.08E+05	1.43E+04	4.73
Participant 14 / Partec SL (12)	1.03E+05	3.64E+03	1.26
Participant 15 / Partec SL (13)	1.07E+05	5.84E+03	1.95
TCC reproducibility (R) in counts/ml (n=12)	1.01E+04		
TCC measurement uncertainty RSD in % (n=12)	3.37		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 16: Comparative results for sample "4", Group 2, for the total cell count.

Participant / Instrument	**AVE TCC in counts/ml (n=3)**	**TCC repeatability (r) in counts/ml (n=3)**	**TCC measurement uncertainty RSD in % (n=3)**
*Participant 12 / Partec SL (1)	1.50E+05	1.97E+04	4.68
*Participant 13 / Partec PAS III (11)	1.37E+05	1.30E+04	3.40
Participant 14 / Partec SL (12)	1.27E+05	3.98E+02	0.11
Participant 15 / Partec SL (13)	1.36E+05	1.02E+04	2.69
TCC reproducibility (R) in counts/ml (n=12)	1.23E+04		
TCC measurement uncertainty RSD in % (n=12)	3.33		

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 17: Comparative results for sample "1", Group 2, for the LNA/HNA-ratios.

Participant / Instrument	**AVE LNA in counts/ml (n=3)**	**LNA repeatability (r) in counts/ml (n=3)**	**LNA measurement uncertainty RSD in % (n=3)**	**AVE HNA in counts/ml (n=3)**	**HNA repeatability (r) in counts/ml (n=3)**	**HNA measurement uncertainty RSD in % (n=3)**	**AVE LNA + AVE HNA in counts/ml**	**LNA fraction in %**	**HNA fraction in %**
*Participant 12 / Partec SL (1)	2.04E+04	1.61E+03	2.82	1.31E+04	1.17E+03	3.18	3.33E+04	60	40
*Participant 13 / Partec PAS III (11)	2.05E+04	9.39E+02	1.63	1.28E+04	9.10E+02	2.54	3.33E+04	62	38
Participant 14 / Partec SL (12)	1.84E+04	6.17E+02	1.20	9.73E+03	1.11E+03	4.09	2.86E+04	65	35
Participant 15 / Partec SL (13)	2.23E+04	8.41E+02	1.35	1.16E+04	1.55E+03	4.78	3.41E+04	66	34
Reproducibility (R) in counts/ml (n=12)	5.01E+03			3.20E+03					
Measurement uncertainty RSD in % (n=12)	8.70			10.00					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 18: Comparative results for sample "2", Group 2, for the LNA/HNA-ratios.

Participant / Instrument	AVE LNA in counts/ml (n=3)	LNA repeatability (r) in counts/ml (n=3)	LNA measurement uncertainty RSD in % (n=3)	AVE HNA in counts/ml (n=3)	HNA repeatability (r) in counts/ml (n=3)	HNA measurement uncertainty RSD in % (n=3)	AVE LNA + AVE HNA in counts/ml	LNA fraction in %	HNA fraction in %
*Participant 12 / Partec SL (1)	3.25E+04	1.67E+03	1.84	5.82E+04	7.74E+03	4.75	9.12E+04	35	65
*Participant 13 / Partec PAS III (11)	3.05E+04	5.88E+02	0.69	5.81E+04	9.24E+02	0.57	8.88E+04	34	66
Participant 14 / Partec SL (12)	2.77E+04	1.97E+03	2.53	3.67E+04	1.77E+02	0.17	6.36E+04	42	58
Participant 15 / Partec SL (13)	3.55E+04	1.05E+03	1.05	3.59E+04	2.40E+03	2.39	7.21E+04	49	51
Reproducibility (R) in counts/ml (n=12)	8.88E+03			3.10E+04					
Measurement uncertainty RSD in % (n=12)	10.17			23.15					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 19: Comparative results for sample "3", Group 2, for the LNA/HNA-ratios.

Participant / Instrument	**AVE LNA in counts/ml (n=3)**	**LNA repeatability (r) in counts/ml (n=3)**	**LNA measurement uncertainty RSD in % (n=3)**	**AVE HNA in counts/ml (n=3)**	**HNA repeatability (r) in counts/ml (n=3)**	**HNA measurement uncertainty RSD in % (n=3)**	**AVE LNA + AVE HNA in counts/ml**	**LNA fraction in %**	**HNA fraction in %**
*Participant 12 / Partec SL (1)	3.25E+04	1.67E+03	1.84	5.82E+04	7.74E+03	4.75	9.12E+04	35	65
*Participant 13 / Partec PAS III (11)	3.05E+04	5.88E+02	0.69	5.81E+04	9.24E+02	0.57	8.88E+04	34	66
Participant 14 / Partec SL (12)	2.77E+04	1.97E+03	2.53	3.67E+04	1.77E+02	0.17	6.36E+04	42	58
Participant 15 / Partec SL (13)	3.55E+04	1.05E+03	1.05	3.59E+04	2.40E+03	2.39	7.21E+04	49	51
Reproducibility (R) in counts/ml (n=12)	8.88E+03			3.10E+04					
Measurement uncertainty RSD in % (n=12)	10.17			23.15					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

Table 20: Comparative results for sample "4", Group 2, for the LNA/HNA-ratios.

Participant / Instrument	**AVE LNA in counts/ml (n=3)**	**LNA repeatability (r) in counts/ml (n=3)**	**LNA measurement uncertainty RSD in % (n=3)**	**AVE HNA in counts/ml (n=3)**	**HNA repeatability (r) in counts/ml (n=3)**	**HNA measurement uncertainty RSD in % (n=3)**	**AVE LNA + AVE HNA in Ccounts/ml**	**LNA fraction in %**	**HNA fraction in %**
*Participant 12 / Partec SL (1)	3.25E+04	1.67E+03	1.84	5.82E+04	7.74E+03	4.75	9.12E+04	35	65
*Participant 13 / Partec PAS III (11)	3.05E+04	5.88E+02	0.69	5.81E+04	9.24E+02	0.57	8.88E+04	34	66
Participant 14 / Partec SL (12)	2.77E+04	1.97E+03	2.53	3.67E+04	1.77E+02	0.17	6.36E+04	42	58
Participant 15 / Partec SL (13)	3.55E+04	1.05E+03	1.05	3.59E+04	2.40E+03	2.39	7.21E+04	49	51
Reproducibility (R) in counts/ml (n=12)	8.88E+03			3.10E+04					
Measurement uncertainty RSD in % (n=12)	10.17			23.15					

*=Eawag internal, AVE = average, n = number of measurements, RSD = relative standard deviation

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10 Glossary

Autoloader	–	Automatic sample loader for the flow cytometer
Beads	–	Calibration beads for flow cytometry
Counts	–	Number of cells or particles counted
Density plot	–	Dot diagram for analysis of flow cytometer data
Gain	–	High voltage setting of the photomultiplier
Gate	–	Defined area in the density plot
Gate-Transfer	–	Transfer of an area defined by a gate to another histogram or density plot
Sheath fluids	–	Fluid for sample movement through the flow cytometer
Speed	–	nominal flow rate of the sample stream
Staining	–	Staining of a sample for analysis
Trigger	–	defined main parameters

III Chronicle of changes

Overview

Version	Comment
366.1	Erstfassung English translation