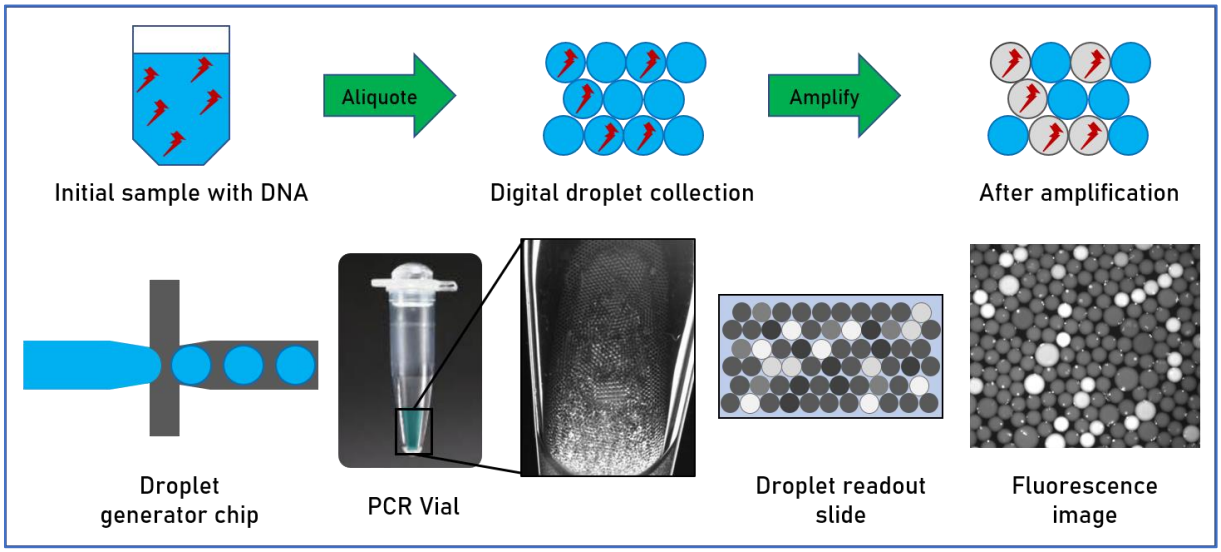
Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

Application Note

Analysis of a commercial surfactant for digital PCR

Anett Reichert, Daniel Kraus, Theresa Heckmann, Karina Weber, Thomas Henkel
Leibniz-Institute of Photonic Technology, Albert-Einstein-Str. 9, 07745 Jena, Germany



Abstract

Digital droplet-based assays offer promising opportunities for the absolute quantitation of low concentration analytic species. During the last decade digital-PCR (dPCR) became one of the most prominent assays for this class of analytical methods. For performing the assay, the sample volume is split into multiple droplets in such a way that each droplet contains either one or none of the target DNA molecules. Due to the small droplet volume, the PCR reaction runs very efficiently even from a single molecule. During amplification, a fluorescent dye is formed or activated. The positive droplets become fluorescent. Absolute quantitation of the number of target molecules is simplified to the count of fluorescence active droplets in the generated droplet collection. Not regarding the simplicity of the approach, its technical implementation is challenged by stabilizing the droplets collected over the complete assay avoiding unwanted droplet coalescence or crosstalk between the droplet ingredients. This has been solved by utilizing perfluorinated mineral oils as the carrier oil in combination with advanced perfluorinated surfactants, which stabilize the emulsion and avoid crosstalk and DNA exchange between the individual droplets. In this application note we are investigating the usability of the commercially available surfactant dSurf for an exemplary digital PCR-assay.

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

Introduction

Reliability of digital PCR assays is strongly influenced by the quality of the utilized perfluorinated surfactants, which are responsible for preserving the droplet integrity during the whole dPCR protocol workflow. Unless many self-made compositions are reported in the literature [1-3]. The utilization of commercial-grade detergents with uniform characteristics, long term availability and minimal batch to batch variability becomes crucial for the sustainability of the developed dPCR assays. Recently, the Fluigent company has launched the dSurf surfactant. Therefore, this surfactant has been compared for their compatibility with an existing dPCR assay for the detection of the variable region of the bas-C gene of Acinetobacter baumannii. The dPCR-process itself can be performed with chip components from stock or, for better performance, with digital PCR system setups, available from an ongoing number of commercial system providers.
In our application note we are analyzing the commercially available surfactant dSurf. For the experiments we are utilizing microfluidic chips from Fluigent, which are commercially available, in order to force the reproduction of our results and the broad application of this powerful method by the community. In the end, we confirm that the dSurf surfactant works at the same quality level as comparable commercial surfactants under identical test conditions and buffer compositions and can therefore be easily substituted in existing assays.

Materials and Methods

For experiments the droplet kit from Fluigent was used. The droplets were generated with the Fluigent EZ Drop chip (with 3 microfluidic droplet generators designs on each chip). 2m PEEK 1/32" tubing OD x .010" and 2x sleeves 1/16" OD x .033" ID x 1.6" were used for the interconnecting the chip with the fluid reservoirs. All further information and the dimension of the droplet generation chip can be found on Fluigent website. Pressure-driven flow control was managed utilizing a "Fluigent-MFCS™-EZ" pressure control system. A thermocycler "Eppendorf Mastercycler® Gradient" (Eppendorf AG, Hamburg, Germany) was used for DNA amplification. For optical readout the droplets were loaded into a disposable 10 µl cell counting chamber "Countess™" (EVETM NanoEnTek, Seoul, South Korea). The counting chamber used had no grid.

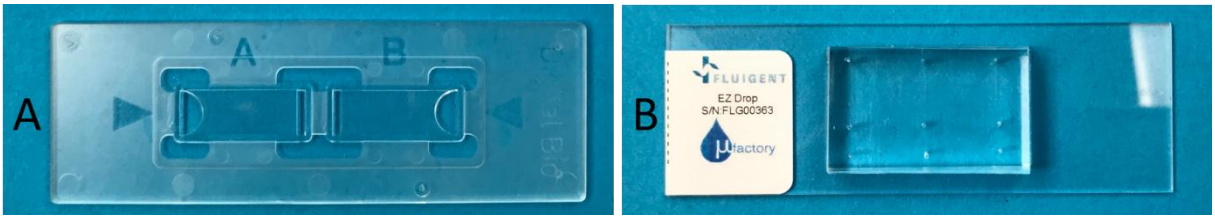


Figure 1: *Utilized chip devices: A) 10 µl cell counting chamber B) Fluigent PDMS EZ Drop chip*

Image acquisition was performed with a standard fluorescence microscope (Axiovert-MAT-M, Carl Zeiss AG, Germany) utilizing a Zeiss Fluar 10x magnification NA 0.5 objective, HBO 100 light source, FITC-filter set and a Andor-Neo sCMOS camera (Oxford Instruments, Abingdon, UK) with 5 s exposure time for the fluorescence images.

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

Reagents

Continuous phase reagent:

Novec HFE-7500 containing 2% dSurf (Fluigent).

PCR reaction composition:

1x PCR Buffer without surfactants
dNTP-Mix (0.2 mmol/l for each dNTP)
0.02 Units / µl innuTaq DNA Polymerase (Hot-Start)
2.5 mmol/l $MgCl_2$
Intercallator dye EvaGreen at 1x concentration
Forward and reverse primer at concentration of 0.2 µmol/l
Template DNA

Methods

Droplets were generated at a working pressure of 240 mbar for the dSurf and 140 mbar for the PCR-Mix. The chip was connected with PEEK 1/32" tubing OD x .010" and 2x sleeves 1/16" OD x .033" ID x 1.6", tubing length: 200 mm. Generated droplets were collected into a 0.2 ml PCR vial. Amplification was performed in a conventional thermocycler with the following settings:

Step	***Parameters***
Initial denaturation and hot-start Taq-Polymerase activation	*94°C / 5min*
Number of Cycles	*40*
Annealing *Extension* *Denaturation*	*58°C / 15 s* *72°C / 22s* *94°C / 15s*
Final cooling to	*4°C*

For readout, the amplified droplet collection was transferred into a cell counting capillary slit chamber for subsequent brightfield and fluorescence image acquisition. For readout the results, the droplets should arrange as a monolayer inside the readout chamber. This can be forced by loading 10 µl of the droplet suspension into a pipette tip and allowing the droplets to rise in the tip. The complete volume will be loaded into the chamber beginning with the pure fraction of the continuous phase in order to force the injection of the droplets into the partially pre-filled chamber. After loading, the rear slit of the chamber is sealed with adhesive tape to reduce the evaporation of the perfluorinated fluid. This sealing was confirmed to reduce droplet motion and droplet rearrangement in the capillary slit chamber during the readout procedure.

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

Results

Droplet generation for dPCR sample has been performed with the parameter settings at the Fluigent-MFCS™-EZ" pressure control system as given in the Materials and Methods section. For the investigated surfactant droplet generation characteristics in terms of droplet generation regime, droplet size and frequency were observed as given in Figure 2. The average droplet size was 70 µm with a volume of 180 pl.

Droplet Generation process

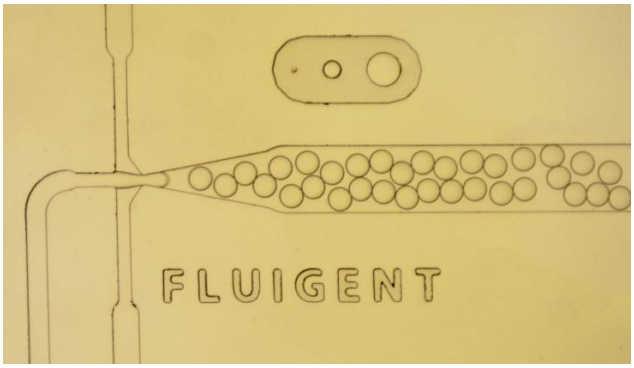
Surfactant	Characteristics of droplet generation
dSurf™ *2% in Novec HFE-7500* *Sample Fluid:* *PCR Reaction Mix*	

***Figure 2**: Droplet generating process. The droplet generator operating in transition mode between dripping and jetting. No significant differences in the mode of operation as in the droplet sizes and size uniformity can be recognized. The middle circle above the droplet channel has a diameter of 50µm.*

The droplet generation process itself has been run on a microfluidic workstation (Figure 3) with the opportunity for process monitoring by video microscopy. In terms of quality management this was used to confirm the correct operation of the droplet generation process during the whole experiment.

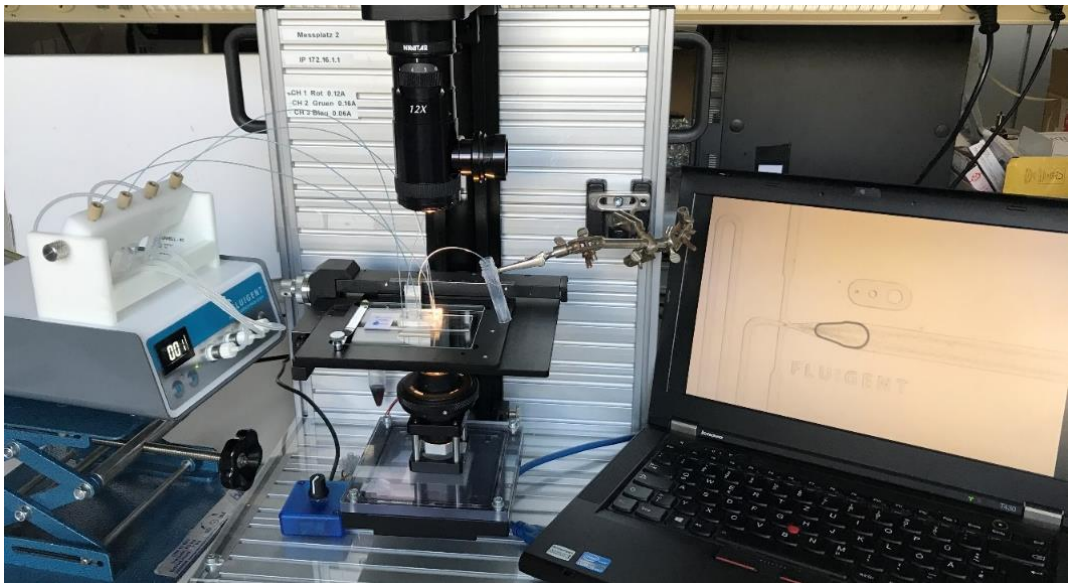


***Figure 3:** Microfluidic workstation utilized for droplet generation with quality monitoring by video microscopy.*

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

For the evaluation of the results a brightfield and fluorescence image is acquired from the same region of interest (ROI) (Figure 4). The brightfield image highlights the droplet contours. The fluorescence image provides the droplet internal fluorescence intensities. For each counting chamber multiple image pairs were acquired for improving statistical significance and precision of the readout.
Data analysis has been performed by an in-house dPCR data analysis software which recognizes the droplets, measures the fluorescence intensities for each droplet and creates an initial clustering hypothesis utilizing a non-supervised automated classification into positive and negative droplets. The droplets are assigned the same number in the brightfield and fluorescence image. Thus, images can be superimposed. Results and a subset of the generated data plots are given in this report. Alternatively, the both images can be manually aligned with standard image processing software and manual counting can be performed.

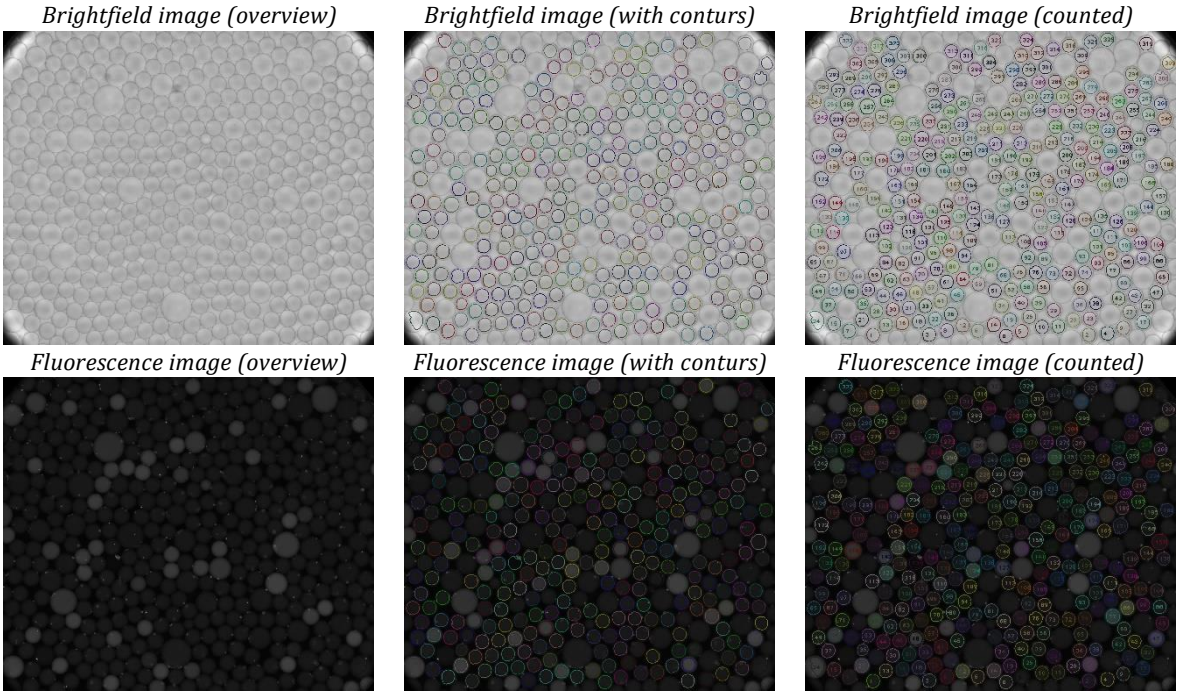
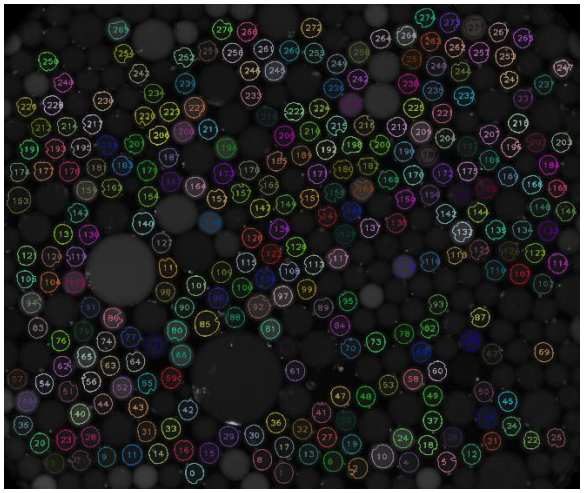


***Figure 4**: Evaluation procedure for the dPCR. Starting point are brightfield and fluorescence images of the generated droplets. The processing is done by a self-developed software to detect and evaluate the intensity of the droplets. The parameters for detecting the droplet contours are defined in advance. Only droplets of a valid size are included in the result.*

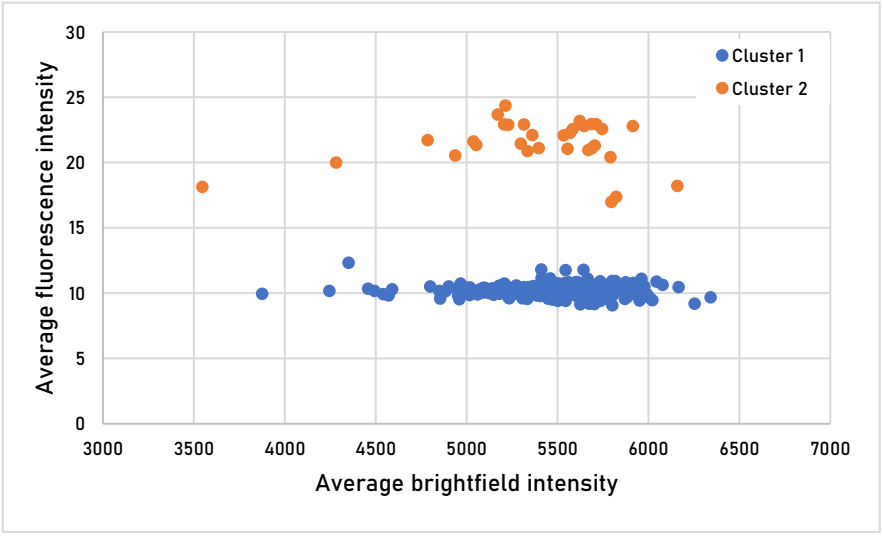
The dPCR sample images and data plots are given in Figure 5. In the first row (A) a fluorescence image from the final readout is shown. The detected measurement spots for each droplet are added. Only droplets with valid sizes were included in the analysis. Faulty droplets with significantly larger (merged droplets) and smaller droplets (satellite droplets) were excluded. The classification plot is given in the second row (B). Both clusters – positive and negative droplets are clearly and reliably separated. For data analysis, the sample is measured at multiple positions. In the third row (C) the droplet counts of positive and negative droplets are given for all measured fluorescence images in the sample.

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

A



B



C

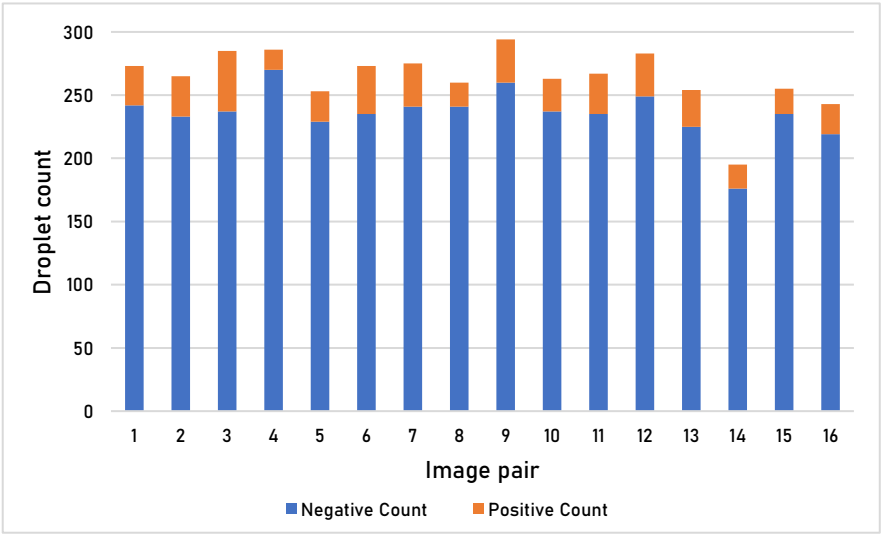


***Figure 5**: Digital PCR results compilation. A) Fluorescence readout image example with spots, used for droplet measurements. B) Clustering scatter plot, showing the separation of the positive and negative droplets based on their*

Leibniz Institute of Photonic Technology
Microfluidics Group
Albert-Einstein-Straße 9, 07745 Jena, Germany
+49 (0) 3641 206 307
thomas.henkel@leibniz-ipht.de

fluorescence intensity. In both cases positive and negative droplets are from clearly separated clusters. C) Droplet statistics over all analyzed image pairs of a sample.

Conclusion

The experiments have shown that the dSurf surfactant is suitable for scientific as well as routine dPCR applications. The generated droplets were homogeneous in shape and size. Superior droplet stability of the dSurf surfactant system was observed during the amplification process. A few droplets have dissipated during the experiments, but this can be neglected. The reproducibility of the experiments was also confirmed. Droplet generation with identical parameters leads to identical droplet size and quality. Summarily, dSurf can be employed as a surfactant composition for digital droplet-based assays.

References

1. *Pohl, G. and I.-M. Shih, Principle and applications of digital PCR. Expert review of molecular diagnostics, 2004.* ***4****(1): p. 41-47.*
2. *Huggett, J.F., S. Cowen, and C.A. Foy, Considerations for digital PCR as an accurate molecular diagnostic tool. Clinical chemistry, 2015.* ***61****(1): p. 79-88.*
3. *Quan, P.-L., M. Sauzade, and E. Brouzes, dPCR: a technology review. Sensors, 2018.* ***18****(4): p. 1271.*