Benchmark

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DNA recovery from Droplet Digital™ PCR emulsions using liquid nitrogen

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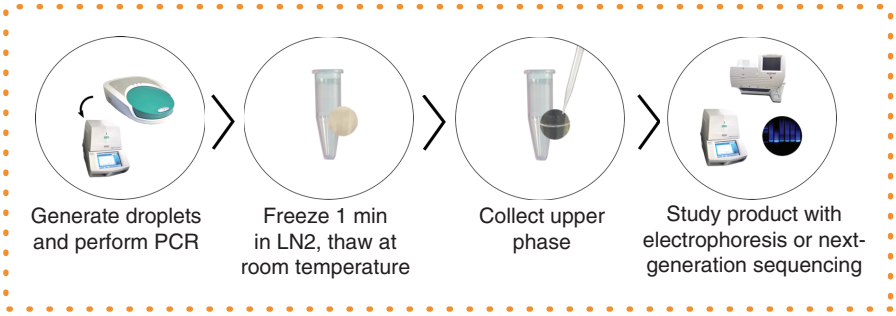
ABSTRACT

Droplet microfluidics is a technology that enables the production and manipulation of small volumes. In biosciences, the most popular application of this technology is Droplet Digital™ PCR (ddPCR™), where parallel nanoliter-scale PCR assays are used to provide a high sensitivity and specificity for DNA detection. However, the recovery of PCR products for downstream applications such as sequencing can be challenging due to the droplets' stability. Here we compared five methods for disrupting the droplets to recover DNA. We found that rapid freezing in liquid nitrogen results in a clear phase separation and recovery of up to 70% of the DNA content. Liquid nitrogen freezing can thus offer a simple and environmentally friendly protocol for recovering DNA from ddPCR.

METHOD SUMMARY

The coalescence of the Droplet Digital™ PCR emulsion droplets is achieved by rapid freezing in liquid nitrogen. Thawing of the frozen emulsion forms separate oil and aqueous phases; the latter contains the DNA, which is readily available for secondary PCR reactions and other downstream applications (see Graphical abstract).

GRAPHICAL ABSTRACT



KEYWORDS:

amplicon recovery • breaking droplets • Droplet Digital™ PCR (ddPCR™) • droplet microfluidics • emulsion PCR

Droplet microfluidics (DM) technology has proved to be a unique and versatile tool for a broad range of biological assays. It relies on the physicochemical properties of two immiscible liquids and their manipulation through interconnected microfluidic channels, enabling the generation and manipulation of small and separated volumes [1–3]. The properties of the droplets, such as size and stability, can be adjusted by altering the microfluidic channel geometry, flow rate and reagent composition [1–3]. As a result, droplets can be manipulated in a controlled manner for different ends.

DM has been used for various purposes ranging from molecular analyses to the cultivation of individual microbes [3,4]. The replication of microbes inside droplets has revolutionized the study of metabolism, antibiotic resistance and enzyme activity [4,5]. Methods have also been developed to select and cultivate combinations of organisms for studying cell–cell interactions [6]. Lately, DM has even been explored for studying single cells [7,8]. Nonetheless, the most popular and well-established use of DM has been for nucleic acid detection and quantitation.

The Droplet Digital™ PCR (ddPCR™) system is a droplet-based PCR platform commercialized by Bio-Rad (CA, USA) and has been widely used for pathogen detection [9,10], food quality analysis [11], environmental studies [12] and medical research [13]. ddPCR relies on DM's generation of numerous nanoliter-sized droplets, inside which independent DNA amplification reactions are carried out. As with quantitative PCR, the increase in DNA copy number due to specific amplification is revealed via fluorescent dye. However, ddPCR analysis

Table 1. Summary of methods used for breaking the ddPCR emulsion.

Method	Protocol	Procedure	Ref.
Chemical	Standard chloroform protocol (Bio-Rad)	i) Remove excess oil ii) Add 20 μl of TE buffer iii) Add 70 μl of chloroform iv) Homogenize by vortexing at top speed for 1 min v) Centrifuge at 16,000 × *g* for 10 min vi) Collect the aqueous phase	[16]
	n-Octane protocol	i) Remove excess oil ii) Add 20 μl of TE buffer iii) Add 23.3 μl of n-Octane iv) Homogenize by vortexing at top speed for 1 min v) Centrifuge at 16,000 × *g* for 10 min vi) Collect the aqueous phase	
	1H,1H,2H,2H-Perfluoro-1-octanol protocol	i) Remove excess oil ii) Add 20 μl of TE buffer iii) Add 23.3 μl of PFO iv) Homogenize by vortexing at top speed for 1 min v) Centrifuge at 16,000 × *g* for 10 min vi) Collect the aqueous phase	
Physical	Silica column (GeneJET PCR purification kit, Thermo Scientific)	i) Add 70 μl of binding buffer ii) Add 70 μl of isopropanol iii) Homogenize by vortexing at top speed for 1 min iv) Transfer to silica column and proceed according to manual	[17]
	Thermal	i) Remove excess oil† ii) Add 20 μl of TE buffer† iii) Freeze for 1 min iv) Thaw at room temperature v) Collect the aqueous phase	

Volumes are adjusted per single emulsions (20 μl of PCR reaction and 70 μl of Droplet Generation Oil) processed by the QX200 Droplet Generator.
†Optional step.
PFO: 1H,1H,2H,2H-perfluoro-1-octanol; TE buffer: Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

is based on the end-point fluorescence intensity measured for each separate droplet, thereby increasing method sensitivity. After the ddPCR process, the droplets are usually discarded, despite their potential as material for downstream genetic studies. For instance, DNA recovered from droplets has been used for production of next-generation sequencing libraries for single-cell genetics [8,14] and transcriptomics studies [15]. The lack of efficient methods for breaking the ddPCR emulsion forms a major challenge to using the PCR products in downstream analysis.

In this study we addressed the challenge of recovering DNA from droplets generated by the QX200™ ddPCR System (Bio-Rad, CA, USA). Different chemical and physical methods were compared: chloroform, n-Octane and 1H,1H,2H,2H-perfluoro-1-octanol (PFO) as chemical treatments, and freeze-thawing and silica columns as physical methods (Table 1).

The ddPCR emulsion samples were prepared with QX200 ddPCR EvaGreen Supermix and QX200 Droplet Generation Oil for EvaGreen according to the manufacturer's instructions. To assess the efficiency of DNA recovery, DNA fragments up to 1 kb were added to the ddPCR master mix as input. Initially, GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, MA, USA) was used as the DNA control, containing fragment sizes ranging from 100 to 1000 bp. Later, purified PCR amplicons or Qubit® dsDNA HS Standard #2, of sizes 263 bp and 1 kb, respectively, were chosen. Up to three independent emulsion samples were combined into one tube to form a single replicate; the number of replicates per protocol in each experiment varied from one to seven. The initial and recovered DNA was quantified using the Qubit dsDNA high-sensitivity assay (Thermo Fisher Scientific, MA, USA). The method efficiency was calculated as the percentage of DNA recovered against the initial DNA input for each sample (Supplementary Table 1).

Our reference method for DNA retrieval was the chloroform protocol described in the Bio-Rad ddPCR application guide [16]. Briefly, this protocol consists of combining the emulsion replicates, removing excess oil and homogenizing the sample with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and chloroform (Sigma-Aldrich, MO, USA). These steps were followed by a high-speed centrifugation to separate the phases into a lower oil phase and an upper aqueous phase, with the latter containing the DNA.

We tested the efficiency of the standard protocol with several chloroform-to-oil ratios (Chl:O), which were achieved by increasing or decreasing the chloroform volume and keeping other conditions identical. A Chl:O of close to 2 was the protocol recommendation, for which 70 μl of chloroform was added to the combined emulsion sample after removing the excess oil. Higher ratios (Chl:O >2) were achieved by adding twice the volume and lower ratios (Chl:O <2) were achieved by adding a third of the chloroform volume. The average recovery of DNA using the standard protocol (Chl:O ratio ≈ 2) was $45 \pm 4\%$. Chl:O >2 yielded $34 \pm 18\%$ of the initial DNA, and Chl:O <2 yielded $61 \pm 4\%$ recovery (Figure 1). Despite the differences in the DNA recovery, the results presented here do not demonstrate which chloroform-to-oil ratio could improve the outcome. Whereas Chl:O <2 provided higher apparent recovery, in some samples Chl:O >2 was necessary for a clear phase separation.

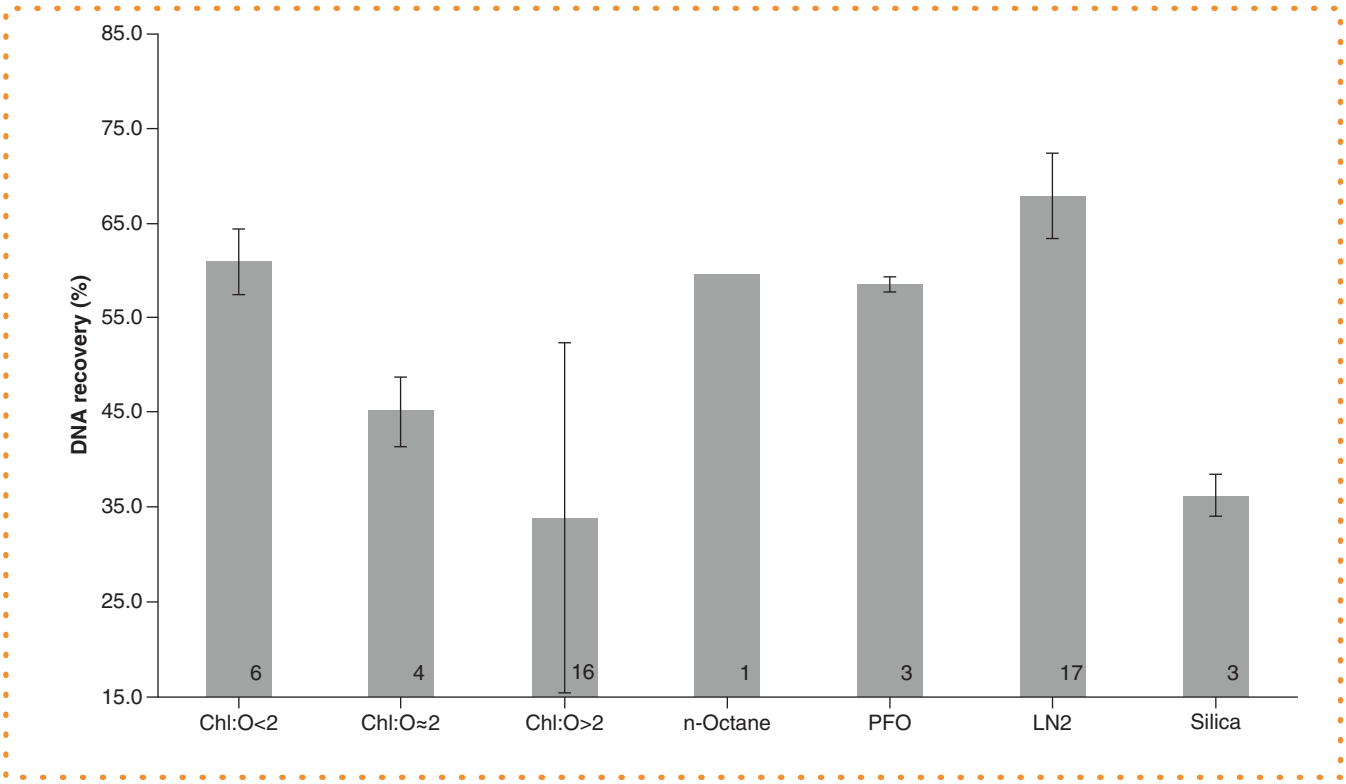


Figure 1. Efficiency of DNA recovery from ddPCR droplets. Three solvents were tested to break the droplets in ddPCR emulsion: chloroform, n-Octane and 1H,1H,2H,2H-perfluoro-1-octanol. Adjustments of chloroform-to-oil ratio (Chl:O) were tested. In addition, two physical methods were tested: freezing-thawing in liquid nitrogen and purification with silica columns. The number of replicates used for each method is shown at the bottom of the respective column and the bars represent the standard deviation of the recovery percentage.
LN2: Liquid nitrogen; PFO: 1H,1H,2H,2H-perfluoro-1-octanol.

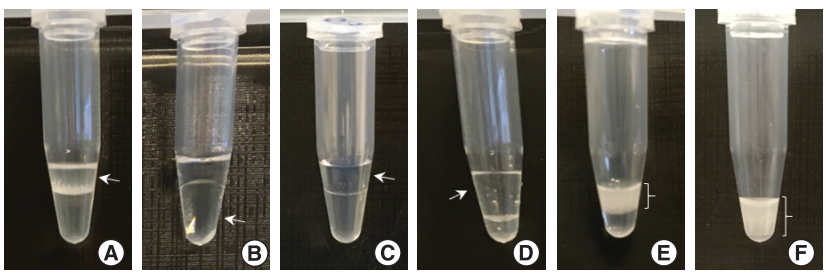


Figure 2. Phase separation after breaking the ddPCR emulsion. The photo panel shows the phase separation by using **(A)** chloroform, **(B)** n-Octane, **(C)** 1H,1H,2H,2H-perfluoro-1-octanol and **(D)** liquid nitrogen. The arrows indicate the aqueous phase. In the tubes on the right, the intact emulsion sample is shown before **(E)** and after **(F)** removing the excess oil, with the emulsion layer indicated by the white brackets.

In addition to chloroform, two other chemical compounds, n-Octane and PFO (both Sigma-Aldrich), were compared. The use of low-density n-Octane resulted in an undesirable reversal of the phases when compared with other treatments (Figure 2A–D), making the now lower aqueous part hard to retrieve. DNA was successfully recovered from only one of the three replicates using n-Octane. For PFO, the average recovery of DNA was 59 $\pm$ 1% (Figure 1). A comparison of the DNA yields between the alternative chemical methods showed that PFO and n-Octane were as good as Chl:O $<$2. Nonetheless, with regard to phase separation, PFO appeared to perform better than n-Octane or chloroform; PFO resulted in a clear upper aqueous phase, while n-Octane resulted in inverted phases and chloroform left a cloudy layer between the two phases (Figure 2A).

Two physical methods were also investigated to explore alternative means for recovering DNA from the emulsion. The GeneJET PCR Purification Kit (Thermo Fisher Scientific, MA, USA) was tested as a physical silica column extraction method for recovering DNA

from the droplets. The emulsion was homogenized with the binding buffer and added to the silica columns for binding, washing and recovery, according to the manufacturer's instructions (including isopropanol addition, as recommended for short fragments) [17]. The silica columns from the GeneJET PCR Purification Kit were able to recover 36 ± 2% of the DNA from the emulsion.

As a second physical method, we developed a temperature-based protocol for breaking the emulsion samples. Initially, the freezing temperature of the oil was investigated by storing 250-µl aliquots at -20°C (freezer), -80°C (dry ice/deep freezer) or -195°C (liquid nitrogen [LN2]) for up to 24 h. Incubation at -20 and -80°C did not result in state change, while samples in LN2 were quickly frozen. In further experiments, samples were cooled in LN2 for 1 min, followed by thawing at room temperature. Initial tests showed that freeze-thawing was enough to coalesce the droplets (Figure 2D–F). The average DNA recovery from the aqueous phase of LN2-frozen emulsions was up to 68 ± 5% of the total DNA. Minor changes to the procedure – for example, keeping or removing the excess oil or adding Tris-EDTA buffer to the emulsion – did not have a significant effect on the yield. Phase separation was successfully achieved with emulsions prepared with both kits commercialized by Bio-Rad: ddPCR for EvaGreen® and ddPCR for Probes (Supplementary Figure 1). When compared with the other methods, the LN2 protocol was able to provide the highest recovery rate with minimal sample manipulation steps. This approach differs from the rest likely due to two simultaneous processes: first, the rapid expansion of the water droplets increases the liquid–liquid boundary surface area, lowering the concentration of adsorbed, stabilizing surfactant; and second, the buildup of additional surfactant may be limited by the low temperature [18].

In conclusion, we show that our novel LN2-based methodology is a simple and an efficient way to recover DNA from ddPCR emulsions. Chloroform and fluorinated solvents pose a safety risk and generate hazardous waste; LN2 is available in most research institutes and can provide a safer and greener alternative. In addition, the recovery rate achieved using LN2 presents less variation when compared with other protocols and prevents contamination risk by minimizing the need for manipulation and addition of reagents in the sample. This method can support the use of the ddPCR system, not only as a tool for quantitative analysis, but also as a sample preparation process for downstream applications such as secondary PCR and sequencing. The suggested method also provides an alternative way to handle fluorinated oil-based emulsions in general and to collect the encapsulated contents for many types of applications.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0076

Author contributions

L Dutra and M Tiirola conceived the idea. L Dutra and O Franz carried out experiments. V Puupponen helped in interpreting the temperature results. L Dutra analyzed results and wrote the first version of the manuscript; all authors provided critical comments. The final version of the manuscript was reviewed by all authors.

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Financial & competing interests disclosure

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