Robust sample recovery post-digital PCR for downstream genomic applications

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#2942

Abstract

Droplet microfluidics technologies, and particularly, digital PCR have provided highly precise and sensitive detection of genetic targets. There is a strong interest for researchers to reuse sample due to sample scarcity, as well as, to further characterize or validate their experimental findings. Historically, recovery of PCR products for downstream genomic testing applications has been challenging due to the stability of the sample post-reaction. Stilla Technologies has developed Crystal Digital PCR™ and the naica® system, a flexible and highplex droplet and imaging-based digital PCR platform that allows for multi-color detection of genetic targets with the ability to also recover samples after the reaction is performed.

This study shows how the 6-color naica® digital PCR system workflow can be combined with a simple protocol for sample recovery. DNA contained in the recovered emulsion is extracted using a standard chloroform protocol that takes approximately 1 hour. DNA recovery rate assessment showed high percentage recovery of droplet sample and on average, 70% of the total DNA present from the initial sample recovered. Recovered DNA from the droplet crystal was sequenced and the results were confirmed by qPCR.

The droplet and sample recovery protocol post-digital PCR has potential applications to support validation of Crystal Digital PCR™ results, concordance alongside a multitude of genomic platforms, and further characterization of biomarkers and mutations with profiling platforms such as NGS.

Sanger sequencing after droplet recovery, post Crystal Digital PCR™

The ability to Sanger sequence amplicons after droplet recovery and post Crystal digital PCR™ (cdPCR) was tested using an 81 basepair amplicon of the human Albumin gene (ALB) (Table1). Two validated PCR mixes, PerfeCTa Multiplex qPCR ToughMix from QuantaBio and the naica® multiplex PCR MIX from Stilla, were tested and cdPCR was performed in the Sapphire chip (Fig1). Once PCR was complete and positive droplets were visualized, the Sapphire chip was placed back into the Geode and the droplet recovery program was run. The blue caps of the Sapphire chip were removed, and all contents of the well (40-50ul) were pipet out (Fig 2). The emulsion was allowed to separate, and the bottom oil phase was removed. 20ul of TE and 70ul of chloroform were added to the aqueous phase and centrifuged at high speed for 10 minutes. The upper aqueous phase was removed and quantified. The recovery rate for this protocol was assessed by generating a droplet crystal containing known amounts of genomic DNA, counting the droplets in the Sapphire chips before and after recovery, extracting DNA from recovered droplets and quantifying the amount of DNA. We found 98% of the droplets were recovered from the Sapphire chips and on average, 70% of the total DNA present in the initial droplet crystal is recovered (Fig 3). The recovered DNA was then Sanger sequenced by GATC (Eurofinsgenomics, Cologne, Germany). Recovered DNA was premixed with the forward or reverse primer and sequenced by two methods, SupremeRun and LightRun (Fig 4A-B and 5A-B). SupremeRun is an optimized protocol for difficult templates, such as GC-rich sequences, hairpins and secondary structures that often inhibit sequencing reactions. Alignments were performed using the UCSC Genome Browser (Fig 4C and 5C).

Table 1: Sequence of Albumin amplicon and primers used for Sanger sequencing

ALB amplicon (81bp) chr4:73418213+73418293	5'TGAAACATACGTTCCCAAAGAGTTTaatgctgaaacattcaccttccat gcagatATATGCACACTTTCTGAGAAGGAGAG 3'

Figure 1 : Crystal Digital PCR™ workflow

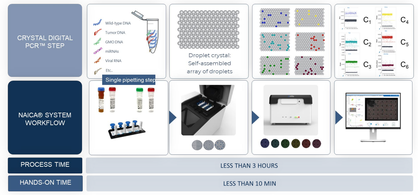


Figure 1. Crystal Digital PCR™ using the naica® system can be performed in under 3 hours with a single pipetting step required to load the Sapphire chip.

Figure 2 : Droplet Recovery from Sapphire chips

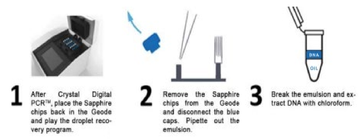


Figure 2. Overview of protocol for droplet recovery from Sapphire chips. The recovery and extraction procedure take approximately 1 hour for up to 24 samples.

Figure 3 : DNA Recovery Rate

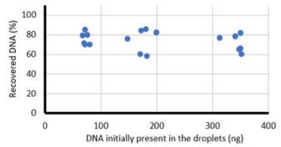
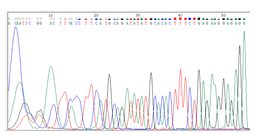


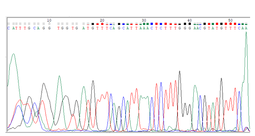
Figure 3. Graph of the percentage of DNA recovered from droplet crystals vs the amount of total DNA initially present in the droplet crystal (ng). Samples were assayed in duplicate, N=3.

Figure 4 : SupremeRun Sanger sequencing results

A) ALB-Forward sequencing results
5' ACAATTCGGACTTCACCTTCATGCAGAT**ATATGCACACTTTCTGAGAAGGAGAG**A 3'



B) ALB-Reverse sequencing results
3'CATTTGCAGGTGGTGATGTTTCAGCATT**AAACTCTTTGGGAACGTATGTTTCA**A 5'



C) Sequence alignment

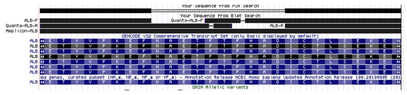
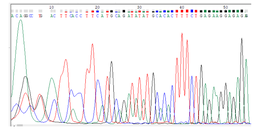


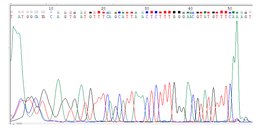
Figure 4. . (A) SupremeRun forward Sanger sequencing results performed by GATC (B) SupremeRun reverse Sanger sequencing results performed by GATC. Bolded sequences represent primers used in Crystal Digital PCR for target amplification. (C) Sequence alignment using UCSC Genome Browser.

Figure 5 : LightRun Sanger sequencing results

A) ALB-Forward sequencing results
5'ACAGGCCTGACTTCACCTTCATGCAGAT**ATATGCACACTTTCTGAGAAGGAGAG** 3'



B) ALB-Reverse sequencing resuts
3'TATGGCATGCAGTGATGTTTCAGCATT**AACTCTTTGGGAACGTATGTTTCA**AAGT 5'



C) Sequence alignment

Figure 5. (A) LghtRun forward Sanger sequencing results performed by GATC. (B) LightRun reverse Sanger sequencing results performed by GATC. Bolded sequences represent primers used in Crystal Digital PCR for target amplification. (C) Sequence alignment using UCSC Genome Browser.

Results and Conclusions

- Amplicons can be recovered from droplets post Crystal Digital PCR™.
- 98% of droplets are recovered and an average of 70% of the total DNA present in the initial droplet crystal is recovered.
- Recovered amplicons can be Sanger sequenced.
- Targets amplified by validated PCR mixes from QuantaBio and Stilla are compatible with Sanger sequencing after droplet recovery.

References

- Droplet Recovery Application note: https://www.stillatechnologies.com/wp-content/uploads/2019/12/AN_Naica_System_Droplet_compressed.pdf
- Sanger sequencing: https://eurofinsgenomics.eu/en/custom-dna-sequencing/gatc-services/

Targeted NGS library preparation using Crystal Digital PCR™ and droplet recovery

The Illumina® TruSight Tumor 15 library prep kit was used to generate libraries prepared using a protocol adapted for Crystal Digital PCR™ (cdPCR) and droplet recovery during the first amplification step and libraries created using the standard qPCR protocol (Fig 6). The TruSight Tumor 15 kit targets 15 Hotspots on 15 cancer-associated genes using 2 pools of primers, A and B (Table 2). A commercially available WT human genomic DNA and WT hgDNA spiked with a known concentration of commercially available mutant DNA with 11 mutations were used as the library starting material (Table 3). Six barcoded libraries were each generated by qPCR and by the cdPCR adapted protocol from WT hgDNA + mutant DNA (Fig 7). Two barcoded libraries were generated by qPCR and by the cdPCR adapted protocol from WT hgDNA (Fig 7). Libraries were pooled and quantified by Qubit and cdPCR and then sequenced on a MiSeq (Integragen, Evry, France) (Fig 8). The percentage of mutations detected from libraries created by qPCR and libraries created by cdPCR were reported compared to the theoretical percentage (Table 4).

Figure 6: Implementation of Crystal Digital PCR™ in NGS workflow

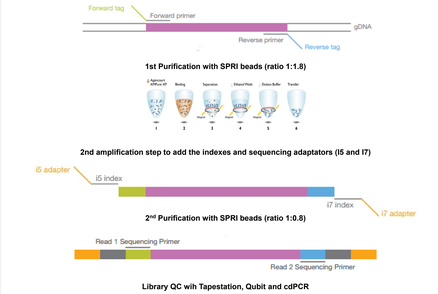


Figure 5. Illumina® TruSight Tumor 15 library prep workflow with the first amplification step adapted for Crystal Digital PCR™ and droplet recovery.

Table 2: Gene Content on TruSight Tumor 15

AKT1	ERBB2	GNAQ	MET	PIK3CA
BRAF	FOXL2	KIT	NRAS	RET
EGFR	GNA11	KRAS	PDGFRA	TP53

Table 3: Percentage of mutations spiked into WT hgDNA

Mutation	cp/µl stock	%	Mutation	cp/µl stock	%
TP53 R248W	151	5.5	PIK3CA E545K	8	0.3
BRAF V600E	120	4	PIK3CA H1047R	224	8
EGFR Del19	24	0.9	AKT1 E17K	2.1	0.1
EGFR L858R	10	0.4	KRAS G12V	71	2.6
EGFR G719S	27	1.0	NRAS Q61	3.5	0.1
EGFR T790M	9	0.3			

Figure 7: Pooled libraries created from WT DNA and WT + mutant DNA

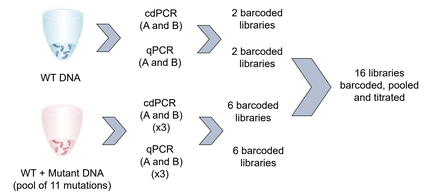


Figure 6. 16 pooled libraries created from WT DNA and WT + mutant DNA. Uising primer mixes A and B, libraries were created by the standard qPCR protocol and by the cdPCR adapted protocol. Mutant libraries were constructed in triplicate.

Figure 8: Quantitation of libraries

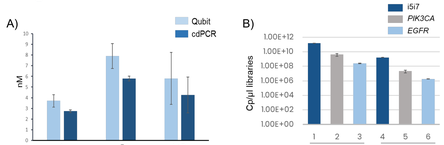


Figure 7. Quantitation in nM of library pools by Qubit (light blue) and cdPCR (dark blue) (Fig A). Two amplicons targeted by the TruSight kit were re-amplified from libraries created by cdPCR and by qPCR and quantitated by cdPCR (Fig B).

Table 4: Mean Sequencing Results (N=3)

Mutations		Theoretical (%)	qPCR A (%)	cdPCR A (%)
Pool A				
PIK3CA H1047R	c.3140A>G	8	8.97	9.77
TP53 R248W	c.742C>T	5.5	4.23	5.00
KRAS G12V	c.35G>T	2.6	ND	0.50
KRAS G13D	c.38G>A	Not expected	4.7	5.2
EGFR Del19		0.9	ND	ND
EGFR L858R	c.2573T>G	0.4	1.07	1.35
EGFR T790M	c.2369C>T	0.3	0.60	1.45
PIK3CA E545K	c.1633G>A	0.3	0.40	0.63
NRAS Q61H	c.183A>T	0.1	0.47	0.80
AKT1 E17K	c.49G>A	0.1	ND	ND
Pool B				
PIK3CA H1047R	c.3140A>G	8	10.10	8.13
TP53 R248W	c.742C>T	5	4.30	4.03
BRAF V600E	c. A>T	4	7.73	9.10
EGFR G719S	c.2155G>A	1.0	1.90	2.13
EGFR T790M	c.2369C>T	0.3	0.50	0.75

Results and Conclusions

- Crystal Digital PCR™ and droplet recovery can be used for the 1st amplification step of targeted NGS library preparation.
- Libraries created by cdPCR are similar in quantitation to libraries created by qPCR.
- The percentage of mutations from cdPCR libraries highly correlate to those from qPCR libraries and to the theoretical.

Acknowledgments

Special thanks to Jordan Madic who contributed to the adaptation of the NGS protocol for Crystal Digital PCR™.