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Evaluation of ctDNA extraction methods and amplifiable copy number yield using standardized human plasma-based ctDNA control materials

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ABSTRACT

Introduction: The use of cell-free circulating tumor DNA (ctDNA) for non-invasive cancer testing has the potential to revolutionize the field. However, emergence of an increasing number of extraction methods and detection assays is rendering laboratory workflow development much more complex and cumbersome. The use of standardized, well characterized ctDNA control materials in human plasma could facilitate the evaluation of extraction efficiency and assay performance across platforms. In this study, we use a full process ctDNA quality control material in true human plasma to demonstrate the variability of extraction yield between different ctDNA extraction kits. We also examine the correlation between the amplifiable copy number and DNA concentration post-extraction.

Methods: DNA materials that carry cancer hotspot mutations were spiked into the NIST Genome in a Bottle (GM24385) reference gDNA as background DNA at various allelic frequencies. The DNA was then fragmented and spiked into AcroMetrix™ Normal Human Plasma matrix to mimic human samples, enabling full process evaluation from extraction to data analysis. Nucleic acids were extracted using 3 different commercially available kits to evaluate extraction efficiency. DNA concentration was determined with Qubit® 3.0 instrument using dsDNA HS assay kit. The absolute copy number of ctDNA was evaluated using Bio-Rad® droplet digital PCR system.

Results: The bead-based MagMAX™ nucleic acid isolation kit resulted in up to 97% recovery efficiency while some of the column-based QIAamp® circulating Nucleic Acid kit resulted in >100% recovery rate. The combination of bead- and column-based QIAamp® MinElute® ccfDNA mini kit showed similar recovery rate to the MagMAX™ extraction kit, but different from the result of QIAamp® circulating Nucleic Acid kit. The amplification efficiency of 7 commercially available assays showed significant differences among three extraction platforms, which suggests that the extraction method could impact assay performance. No extracted samples showed changes in allelic frequency, which indicates that the extraction step does not preferentially select the wild-type or mutant DNA.

Conclusions: This study demonstrated that the commercially available ctDNA extraction kits have various extraction efficiencies, and yield different correlations between DNA input and amplifiable copy number. The ctDNA quality control material in normal human plasma with known DNA input serves as a useful tool for evaluation of different extraction kits and assay performance.

INTRODUCTION

- Hotspot DNA: Single to highly multiplexed (550+) variants (SNV, MNV, Indel) using MegaMix™ technology enables more data in fewer runs
- gDNA: well-characterized GM24385 allows understanding of ground truth

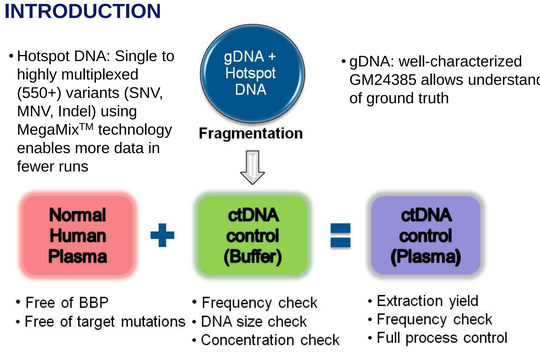

Figure 1. Workflow for manufacturing and qualifying ctDNA reference materials: Fragmented DNA ~160 bp is spiked into normal human plasma to act as a full process control. Synthetic DNA with target mutation(s) is mixed with NIST GIAB GM24385 genomic DNA at target frequency. DNA was fragmented and checked for allelic frequency, concentration and size before spiking into normal human plasma to mimic patient sample. Normal human plasma was tested to ensure that it is free of blood-borne pathogens (BBP) and target mutations.

- Built on NIST Genome in a Bottle GM24385
- Variants confirmed by Sanger sequencing
- Platform agnostic
- Highly multiplexed control

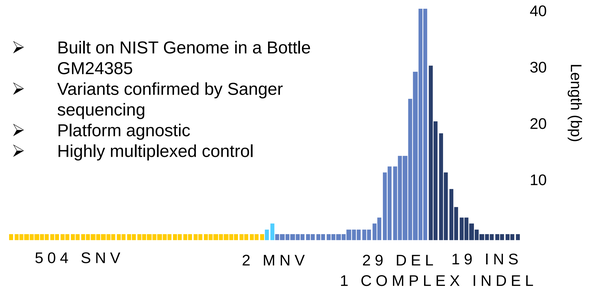

Figure 2. AcroMetrix™ Oncology Hotspot Control: Built on AcroMetrix™ MegaMix™ Technology + NIST Genome in a Bottle. 550+ common variants confirmed by Sanger sequencing.

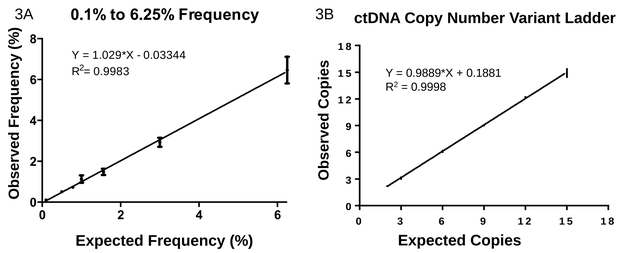

Figure 3. Dilution of 555-Hotspot Frequency Ladder and *MET* Copy Number Variant (CNV) ctDNA Ladder. (A) All 555 variants were targeted to target frequencies of 6.3%, 3.1%, 1.5%, 0.5%, 0.1%. (B) The MET CNV ladder was diluted with target copy number of 2, 3, 6, 9, 12 and 15 copies and fragmented to ~160 bp. Dilutions were made in GM24385 Genome in a Bottle gDNA.

Gene	Mutation	CDS mutation	COSMIC ID
AKT1	E17K	c.49G>A	33765
BRAF	V600E	c.1799T>A	476
EGFR	T790M	c.2369C>T	6240
EGFR	E746_A750 del	c.2235_2249del15	6223
EGFR	L858R	c.2573T>G	6224
KRAS	G12D	c.35G>A	521
PIK3CA	H1047R	c.3140A>G	775

Sample	1	2	3	4	5
Mutation (AF%)	5%	2.5%	1%	0.1%	0%

Table 1. List of mutations and target AF% used in the study.
7 different COSMIC variants at 5 different target allelic frequencies were used to evaluate the extraction platforms and amplification efficiency.

	QIAamp® MinElute ccfDNA kit	QIAamp® circulating nucleic acid kit	MagMAX™ Cell-Free total nucleic acid extraction kit
Extraction principle	Bead + Column	Column-based	Bead-based
Carrier RNA Requirement	N	Y	N
Required Equipment	• Magnet • Heat block • Shaker • Centrifuge	• Vacuum pump • QIAvac 24 Plus • Centrifuge	• Magnet • Centrifuge
Replicates	10	10	10
Total DNA input	130 ng/2 mL	130 ng/2 mL	130 ng/2 mL

Table 2. Comparison of three ctDNA extraction platforms.

RESULTS

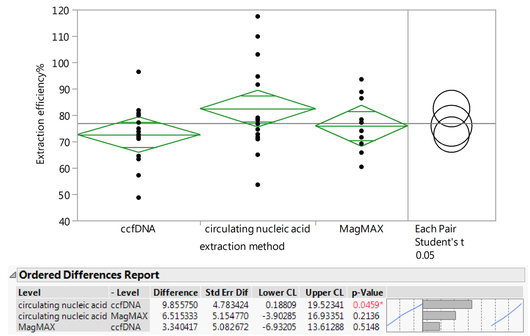

Figure 4. Comparison of DNA yield from ctDNA QC samples using different ctDNA extraction kits. 10 ctDNA QC samples with DNA spike-in and 2 normal human plasma samples were used to evaluate each of the three commercially available ctDNA extraction kits. The DNA concentration was determined using Qubit 3.0® instrument, and the extraction yield was subtracted by the endogenous DNA in the normal human plasma matrix. The recovery rate was calculated by (DNA output/DNA input) *100%. The results were analyzed using JMP13. Student's t-test analysis showed a significant difference between the mean extraction yield of circulating nucleic acid kit vs. ccfDNA kit (alpha=0.05; Prob > |t| is less than 0.0459).

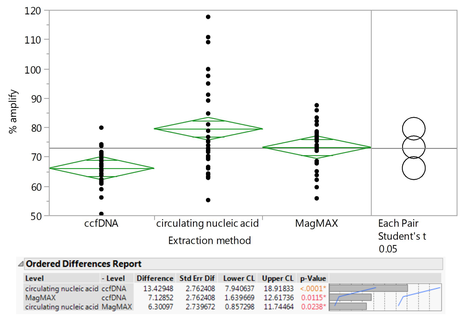

Figure 5. Use of ctDNA QC materials to evaluate amplification efficiency across different extraction kits. Amplifiable copy number showed significant difference (p-value<0.05) between three extraction methods. % amplification was determined by observed copy number divided by nominal copy number (calculated by DNA input, assuming 1 ng DNA = 300 copies of each gene) times 100%. Significant difference was observed in amplifiable copy number when using DNA from three extraction platforms. This study suggests that the extraction methods could affect the amplification efficiency even using commercially available assays.

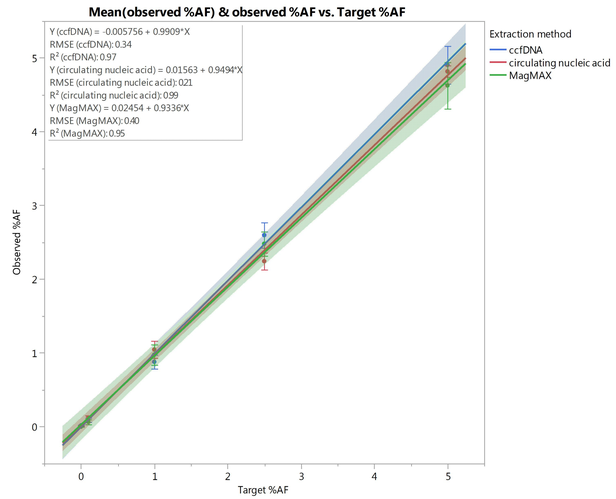

Figure 6. Allelic frequency does not vary with extraction platforms. The allelic frequency of each target was determined (observed) %AF, and plotted as a function of target %AF using DNA extracted by three different methods. All of the coefficient of determinations were ≥0.95. No significant difference between the linear regressions generated based on three extraction methods (F factor=0.4310), suggesting that the extraction methods does not impact the final AF% calculation.

CONCLUSIONS

- Full-process ctDNA QC materials allow for efficient evaluation of different commercially available extraction platforms, facilitating liquid biopsy assay optimization.
- Amplification efficiency may be affected by different extraction methods, which suggests that proper evaluation of the extraction platform for ctDNA study is essential.
- No AF% change due to different extraction methods, suggesting that the impact of extraction methods to each amplicon target is consistent.

ACKNOWLEDGEMENTS

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TRADEMARKS

The control is currently in development and not available for use.

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