A Study of Pre-Analytical Variables and Optimization of Extraction Method for Circulating Tumor DNA Measurements by Digital Droplet PCR

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Abstract

Background: Circulating free DNA (cfDNA) is an exciting novel method to diagnose, monitor, and predict resistance and response to cancer therapies, with the potential to radically alter the management of cancer patients. To fulfill its potential, greater knowledge about preanalytical variables is required to optimize and standardize the collection process, and maximize the yield and utility of the small quantities of cfDNA extracted.

Methods: To this end, we have compared the cfDNA extraction efficiency of three different protocols, including a protocol developed in house (Jewish General Hospital). We evaluated the impact on cfDNA levels of preanalytical variables including speed and timing of the second centrifugation and the use of k-EDTA and CTAD blood collection tubes. Finally, we analyzed the impact on fractional abundance of targeted pre-amplification and whole genome amplification on tumor and circulating tumor DNA (ctDNA) from patients with breast cancer.

Results: Making use of a novel protocol for cfDNA extraction we increased cfDNA quantities, up to double that of commercial kits. We found that a second centrifugation at 3,000 g on frozen plasma is as efficient as a high-speed (16,000 g) centrifugation on fresh plasma and does not affect cfDNA levels.

Conclusions: These results allow for the implementation of protocols more suitable to the clinical setting. Finally, we found that, unlike targeted gene amplification, whole genome amplification resulted in altered fractional abundance of selected ctDNA variants.

Impact: Our study of the preanalytical variables affecting cfDNA recovery and testing will significantly enhance the quality and application of ctDNA testing in clinical oncology.

Introduction

The presence of circulating tumor DNA (ctDNA) in blood provides an accessible source of genetic material from solid tumors and holds great promise for the development of clinical tools for less invasive diagnostic testing in cancer patients (1–3). Owing to the relative ease of access and the minimally invasive nature of blood collection, the detection of tumor genomic modifications in ctDNA with highly sensitive sequencing methods has demonstrated great potential to provide sensitive biomarkers for non-invasive diagnosis, prognosis, prediction and monitoring of treatment response, and acquired resistance in cancer patients (4–7). More importantly, evidence suggests that ctDNA measurement may provide a more comprehensive picture of tumor heterogeneity than tumor biopsies and allow the detection of genomic alterations in tumors present at different locations throughout the body, thus providing a more accurate prediction of patient’s tumor burden (8, 9). The clinical applicability and utility of ctDNA is highly promising, and the recent approval by the FDA of the Cobas EGFR mutation test to guide therapy in patients with non-small cell lung carcinoma opens a new era in clinical liquid biopsy testing (10, 11). Nonetheless, many challenges remain to be overcome for ctDNA testing to be adopted as a routine clinical tool (12, 13), and for now, the use of tissue biomarkers remains the gold standard to guide therapy for most cancer patients. The major limiting factor comes from the fact that ctDNA represents only a small fraction of the total amount of circulating cell free DNA (cfDNA; refs. 1, 5). Therefore, even though ctDNA contains genetic modifications suitable to be tested as potential cancer biomarkers its use and reliability is hampered by its relative scarcity. In fact, although some have reported difficulties in detecting tumor-derived mutations in plasma (14), others have been able to isolate ctDNA in both metastatic and nonmetastatic patients (14–16). These inconsistencies are likely due not only to the inherent variability of the fractional abundance of tumor-derived circulating DNA (from <0.01% to >90%), but also to the different sensitivity of methodologies used for mutation detection and
quantification (17–20). Furthermore, it has been shown that the amount of isolated cfDNA depends on the efficiency of the extraction method (21–23) and can be affected by pre-analytical variables including type of blood collection tube, centrifugation speed, and storage temperature (23–28). These pre-analytical variables can also impact on the release of nonmutated DNA from leukocytes that result in the dilution of the cfDNA fraction. In this work, we aimed at optimizing the yield of cfDNA and studied the effect of different pre-analytical variables on cfDNA detection using digital droplet PCR (ddPCR). We developed an improved protocol for cfDNA extraction from plasma, which formed the basis for the investigation of other variables such as the type of anticoagulant in blood collection tubes, and centrifugation parameters. Finally, to obtain a larger amount of DNA for downstream applications, we also tested if pre-amplification of cfDNA would affect the fractional abundance of ctDNA. Our findings contribute to the pre-analytical optimization of cfDNA processing which is required for precise cfDNA results, thus strengthening the potential biomarker value of cfDNA measurements in cancer patients.

Materials and Methods

Samples

Cell lines. BT-20 and MDA-MB-436 from American Type Culture Collection (ATCC) were cultured according to ATCC recommendation. Cells were authenticated using array comparative genomic hybridization (aCGH) and mycoplasma testing confirming the cells were mycoplasma-free. Cells were grown to 70% to 80% confluence and harvested for DNA isolation using QIAamp DNA Mini Kit (Qiagen; Catalog No. 51304).

Human samples. Blood was collected from healthy control volunteers, and plasma and tumor DNA were obtained from patients with breast cancer taking part in the Q-CROC-03 (NCT01276899) clinical trial. All participants provided informed consent and the study was conducted in accordance with recognized ethical guidelines (e.g., Declaration of Helsinki), and approved by the Jewish General Hospital Ethics Committee Review Board (JGH; protocols 05-006 and A04-M31-12B).

Blood collection and plasma processing

Plasma processing for cfDNA extraction optimization. Blood from a healthy volunteer was collected by venipuncture in 2 × 4 mL BD Vacutainer K₃ EDTA tubes (referred to as EDTA Tubes in text; BD#366643; Becton Dickinson). Tubes were inverted ten times and immediately centrifuged at 1,500 × g for 10 minutes at room temperature (RT). The plasma fraction was carefully collected and a second centrifugation at 16,000 × g was carried out at RT for 10 minutes. The supernatant was collected and 1 mL aliquots were either centrifuged at low (3,000 × g) or high (16,000 × g) centrifugal force, respectively. Samples were centrifuged for 10 minutes at RT and 1 mL of supernatant from each condition was collected for cfDNA extraction (Fig. 1B).

Plasma processing in EDTA and CTAD tubes. Peripheral blood from seven (n = 7) healthy volunteers was collected by venipuncture in 2 × 4 mL EDTA tubes and 2 × 4.5 mL BD Vacutainer CTAD Tubes (referred to as CTAD Tubes in text; BD#367599; Becton Dickinson). Tubes were inverted 10 times and immediately centrifuged at 1,500 × g for 10 minutes at RT. The plasma fraction was carefully collected and a second centrifugation at 16,000 × g was carried out at RT for 10 minutes. The supernatant was collected and 1 mL aliquot was used for cfDNA extraction (Fig. 1C).

Plasma processing Q-CROC-03 patients. As part of the Q-CROC-03 trial, patients provided blood at different time points while undergoing neoadjuvant chemotherapy for breast cancer. Blood was collected in 2 × 4.5 mL CTAD tubes and centrifuged within 30 minutes of collection following standard operating procedures (SOP) submitted to the NCi-SOP database (https://brd.nci.nih.gov/brd/sop/show/1961): samples were centrifuged at 1,500 × g for 15 minutes at RT and plasma immediately aliquoted and stored at –80°C. For this study, aliquots were thawed and a second centrifugation at 16,000 × g was performed for 10 minutes at RT prior to analysis.

Breast cancer tumor DNA

Patients in the Q-CROC-03 trial provided tissue samples prior to or following standard neoadjuvant chemotherapy. DNA was extracted from tumor biopsies as per reported SOPs (29). DNA from tumor samples and cell lines underwent whole exome sequencing (WES) and mutation calling at the McGill University Genome Center (Supplementary methods). Data have been deposited in the European Nucleotide Archive database with study primary accession number PRJEB30048.

Generation of spiked DNA samples

We selected genes with single nucleotide variants (SNV) present in cell lines and one Q-CROC-03 patient: TP53 (BT20), CDH5 and MAP1LC3B (MDA-MB-436), ROBO2 and PARK2 (patient Neo-05). Targeted pre-amplification of the genomic regions containing specific SNVs in breast cancer cell lines or patient tumor DNA was performed with primers listed in Supplementary Table S1. DNA was quantified with Qbit dsDNA HS Assay Kit (Catalog No. Q3285; Life Technologies) and targeted pre-amplification of mutated DNA fragments (MUT DNA) was performed with Biorad SsoAdvanced PreAmp Kit (Catalog No. 172-5160; Bio-Rad) as per the manufacturer’s instructions. DNA copies generated from the amplification reaction were quantified by QX200 ddPCR with primers and probes (Integrated DNA Technologies) and known amounts of mutated DNA copies were spiked into 1 mL of control human plasma (Supplementary Table S1; Fig. 1A).

cfDNA extraction optimization

For the comparison of different circulating DNA extraction methods, extraction was performed from 1 mL of plasma spiked with known amounts of DNA copies as described above. We tested the QIAamp DSP Kit (Qiagen; Catalog No. 61504), the
QIAamp Circulating Nucleic Acid Kit (Qiagen; Catalog No. 55114), and a modified protocol developed in house combining components from both kits (referred to as modified hybrid JGH protocol). Both QIAamp kits were used in accordance with the manufacturer’s instructions. For the modified hybrid JGH protocol, we used the QIAamp Circulating Nucleic Acid Kit handbook instructions with the following modifications (see Supplementary Table S2).

We added 125 µL QIAGEN Proteinase K/mL of plasma and 1 mL of buffer AL (from DSP Kit) containing carrier RNA (concentration of carrier RNA was established according to QIAGEN Circulating DNA kit handbook instructions) per 1 mL plasma. The lysate mixture was vortexed for 30 seconds and incubated at 56°C for 15 minutes with occasional agitation. 1.2 mL of AGB buffer/mL of plasma was added to the lysate mixture, vortexed for 30 seconds, and incubated for 5 minutes on ice. The lysate was applied onto the QIAamp MinElute column provided in the QIAGEN DSP Kit and centrifugations were performed instead of using a vacuum pump. The lysate was applied in several elutions of 650 µL each followed by centrifugation at 10,000 rpm for 15 seconds, after applying the last 650 µL of lysate the column was centrifuged for 2 minutes. The flow-through from the collection tube was discarded and 600 µL of AW1 buffer (from DSP Kit) was added to the column, incubated for 5 minutes at RT and then centrifuged at 10,000 rpm for 1 minute. After discarding the AW1 eluate, 750 µL of AW2 buffer (from DSP Kit) was added to the column, incubated for 5 minutes at RT and then centrifuged at 14,000 rpm for 2 minutes. After the flow-through was discarded, 750 µL of EtOH (95%-100%) was applied to the column, incubated for 5 minutes at RT and then spun at 14,000 rpm for 2 minutes. After the centrifugation, the column was placed in a new collection tube, centrifuged for 3 minutes at 14,000 rpm and incubated at 56°C for 10 minutes to clear the column of interfering substances and improve the quality of DNA to be eluted in the next step. DNA elution consisted in the addition of 25 µL of carrier RNA to the center of the membrane and after incubation at RT for 3 minutes, the column was spun at 14,000 rpm for 2 minutes to collect the eluate, which was transferred to a clean 1.5 mL Eppendorf tube. The elution step was repeated twice.

Whole genome amplification

The IlluCentralPrep kit for use with whole genome amplification (WGA) reactions using genomic DNA from tumor tissue or ctDNA from plasma obtained from patients with breast cancer. It is based on phi29 DNA polymerase and was carried out in a 20 µL reaction volume according to manufacturers’ protocols with a 3-hour incubation time at 30°C.

Development of ddPCR assays

We designed a set of primer-probe combination for each gene investigated (Supplementary Tables S1 and S3). Primers were analyzed for specificity using the electronic PCR (In-silico PCR) tool (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). ddPCR conditions were optimized to identify the optimal annealing temperature for each assay.

ddPCR analysis

ddPCR was performed on a QX200 ddPCR system (Bio-Rad). Probes for mutated variants were labeled with FAM fluorescent dye and probes for wt variants were labeled with HEX fluorescent dye. PCR reactions were prepared with ddPCR Supermix (Catalog No. 1863024; Bio-Rad) and partitioned into droplets in a QX200 droplet generator, emulsified PCR reactions were performed with C-1000 touch thermal cycler (Bio-Rad) according to the manufacturer’s instructions. Plates were read in the FAM and HEX channels on a Bio-Rad QX200 droplet reader using QuantaSoft software from Bio-Rad (version 1.7.4.0917) to assess the number of droplets positive for mutant (MUT) DNA, wt DNA, both, or neither. At least two negative control wells with no DNA were included in every run.

To measure efficiency, copies of spiked DNA recovered from plasma were measured with ddPCR using probes labeled with FAM on at least three replicates for each gene. The recovery fraction was calculated as the number of MUT DNA copies extracted from 1 mL of plasma over the number of MUT DNA copies spiked in 1 mL of plasma. ctDNA levels we measured the number of wt TP53 DNA copies present in 1 mL of plasma using primers and probes against the wt region of TP53 labeled with HEX.

To measure the fractional abundance of mutated TP53 after targeted or WGA, we used the formula: no. of copies of mutated TP53/no. of copies wtTP53 + no. of copies mutated TP53.

Statistical analyses

All statistical analyses reported were performed using R (R Development Core Team; ref. 30).

Results

Improved ctDNA extraction efficiency with a novel hybrid extraction protocol

The quantity of ctDNA that can be extracted from plasma can vary depending on the efficiency of the extraction method chosen (21, 22). For the first objective of this study, we aimed at optimizing the ctDNA extraction efficiency from plasma. We tested two commercially available DNA extraction kits, the QIAGEN Circulating Nucleic Acid Kit (CNA) and the QIAGEN DSP Virus Kit (DSP) as well as a modified hybrid protocol developed in house (JGH) and described above. To compare the three extraction protocols, we spiked 1 mL of human plasma with a known number of reference DNA copies with specific SNVs (Fig. 1A; Supplementary Table S1) and measured ctDNA recovery by ddPCR. As shown in Fig. 2, the highest
Extraction efficiency was obtained with the JGH hybrid protocol with an average percent recovery (all variants combined) of 89% compared with 46.9% and 75.4% for the CNA and DSP protocols, respectively ($P < 0.001$), that is, a doubling of cfDNA yield compared with the CNA protocol. When we look at each variant separately, we observe that recovery efficiency and variability vary for each gene tested independently of the protocol of extraction. Nevertheless, the hybrid JGH protocol resulted in more consistent recovery (>79% for all variants) and less variability compared with the other two protocols (Fig. 3). For all subsequent experiments, we adopted the hybrid JGH protocol for cfDNA extraction.

Impact of plasma processing on cfDNA levels

One of the major limitations in ctDNA testing is the fact that ctDNA gets diluted in a pool of genomic cfDNA, which in turn masks the detection of tumor-specific mutated DNA. The source of nonmutated genomic DNA is for the most part circulating lymphocytes and its release can be triggered during blood collection and plasma processing procedures (31, 32). In general, protocols to process plasma recommend a first centrifugation of blood at low speed to enable the separation of plasma from blood cells, followed by a second high-speed centrifugation to minimize the contamination of plasma with nucleic acids from cellular debris (33, 34). However, performing the second high-speed centrifugation is not always suitable in the clinical setting due to the lack of ultra-high-speed centrifuges in hospitals and also to the increased burden that it represents for clinical research associates and nurses. To evaluate the impact of the second centrifugation on plasma cfDNA levels, we tested if different speeds (3,000 $g$ vs. 16,000 $g$) or time of centrifugation (immediately on fresh plasma or two weeks later on frozen plasma) could affect the amount of cfDNA detected by ddPCR (Fig. 1B). We measured the levels of cfDNA by counting the number of copies of wt TP53.

Figure 1.
Schematic of plasma processing conditions tested. cfDNA extraction protocol optimization with spiked in samples (A), evaluation of impact of different centrifugation conditions (B), and type of anti-coagulant (C) on cfDNA levels.

Figure 2.
Median average percent recovery for all five genes measured ($n = 3$–$7$ replicates) with each protocol tested. The JGH method shows the highest percent recovery of reference DNA compared with the other two commercial protocols. Unpaired $t$ test (**, $P < 0.01$; ****, $P < 0.001$).
in plasma processed with the different conditions. As shown in Fig. 4, cfDNA levels were not affected by any of the conditions tested (P = 0.932), suggesting that the speed and the timing of the second centrifugation do not influence the amount of cfDNA purified from plasma and thus should not decrease the sensitivity of ctDNA detection.

We also analyzed two types of blood collection tubes, EDTA tubes which are the standard of blood collection for biobanking purposes and CTAD tubes, which contain buffered sodium citrate and additives (theophylline, adenosine, and dipyridamol) that have been shown to inhibit ex vivo platelet activation (35). With increasing proteomics studies being performed on clinical samples, CTAD tubes are being more frequently utilized for biobanking so as to minimize platelet discharge of protein biomarkers during blood collection. We therefore tested whether there were differences on cfDNA detection by ddPCR in plasma collected in CTAD compared with EDTA tubes. Despite a slight increase in average cfDNA levels in samples collected in EDTA tubes (mean = 905.62 copies/mL ± 468.49) compared with CTAD tubes (mean = 805.83 copies/mL ± 413.32), the difference observed was not statistically significant (P = 0.477; Supplementary Fig. S1).

Targeted pre-amplification of cfDNA does not modify the fractional abundance of mutated variants in plasma

Because cfDNA quantities are often limited, it may be advisable to perform a DNA pre-amplification step to increase DNA starting material for subsequent analysis. However, the concern is that this pre-amplification step may introduce a bias and alter the fractional abundance of cfDNA relative to wt DNA. The third part of our study thus aimed at evaluating if the fractional abundance of mutated DNA could be affected by either "targeted" or "whole genome" DNA pre-amplification reactions. We first analyzed a reference DNA sample containing a serial dilution of TP53 mutant DNA. Targeted pre-amplification was carried out and TP53 variant allele frequency (VAF) was correlated with non-pre-amplified samples. The linearity of the TP53 dilution was maintained with an excellent correlation (R² = 0.9982, P value < 0.001) in VAF for non-amplified versus pre-amplified samples (Supplementary Fig. S2). Targeted pre-amplification was also tested on tissue DNA and serial plasma cfDNA samples from patients with breast cancer harboring mutations in the TP53 gene (Supplementary Table S3). A perfect correlation in VAF between non-amplified and targeted pre-amplified samples was again
obtained for both tissue \( r^2 = 1 \) and plasma samples \( r^2 = 0.993; \) Fig. 5A and B. These results confirm that allele frequency is well preserved following targeted pre-amplification reactions.

Finally, we performed WGA on DNA from tissue and plasma samples from the same patients as above. We found that WGA does not alter the fractional abundance of mutated TP53 in tissue samples \( r^2 = 0.9938 \), however, allele frequency was not preserved in plasma samples as shown with the poor correlation between the VAF of non-amplified versus amplified plasma DNA samples \( r^2 = 0.4337; \) Fig. 6A and B. Therefore, WGA appears to introduce a bias when performed on cfDNA but not on tissue DNA.

**Discussion**

Currently, liquid biopsy shows considerable potential in capturing the genomic profile of solid tumors. For instance, several groups have reported the reliable measurement of somatic mutations in KRAS and ERBB2 amplification in cfDNA \( 36, 37 \). One of the main limitations in using cfDNA as a biomarker in the clinical setting is the lack of analytical validation which starts with the standardization of sample processing methods, storage conditions, and protocols for both cfDNA extraction and quantification. Although most of the variation observed between different individuals will be biological, there are many technical factors that could cause a wide range of measurement inconsistencies. Indeed, several reports have highlighted the lack of standardization \( 13, 33, 34 \) and the effect of different extraction methods on cfDNA yields \( 21–23 \).

While assessing cfDNA extraction efficiency obtained using different components of commercial kits for downstream ddPCR applications, we developed our own in-house hybrid protocol (IGH protocol), which produced significantly higher yields of cfDNA than the original commercial kits. We were able to show that the protocol we developed was able to recover nearly the totality of spiked DNA in plasma \( 80\%–100\% \) with minimal variability. As previously reported by others, our results further confirm that one of the causes of variability in cfDNA measurements comes from the different isolation methods used.

Currently, there is no consensus protocol for the pre-analytical processing of blood for isolation of cfDNA and it is known that different pre-analytical factors can influence its stability and levels. For instance, apoptosis or necrosis of peripheral blood mononuclear cells in the blood collection tube may lead to the release of genomic DNA, explaining the observed increase of cfDNA when the blood sample is stored for several hours before processing \( 23, 38 \). Ideally, the entire procedure from blood draw until plasma separation, should be performed within a couple of hours \( 33, 39 \). As rapid and well-controlled blood processing is not always possible in the clinical setting and blood samples may lie unprocessed for long periods of time, blood collection tubes with stabilizing agents that minimize the release of contaminating DNA in plasma have been developed. Streck cfDNA BCTs tubes are commonly used and have been reported to prevent the lysis of white blood cells, and therefore any dilution of ctDNA with wt genomic DNA \( 28, 31, 32 \). Although EDTA is the anticoagulant of choice for most biological analyses, blood is also collected in CTAD tubes especially for proteomics studies. Until now, no study had evaluated the effect of CTAD additives on cfDNA levels. Our results concluded that cfDNA concentration is not significantly altered when extracted from plasma collected in both EDTA and CTAD anticoagulant tubes.

**Figure 5.** Correlation of mutated TP53 VAF measured by ddPCR in tissue \( n = 4 \) patients; A) and plasma ctDNA \( n = 4 \) patients; B) after targeted pre-amplification and without pre-amplification. Between one and nine technical replicates were performed for each patient sample. Spearman's correlation coefficient was used to correlate the two variables.

**Figure 6.** Correlation of mutated TP53 VAF measured by ddPCR in tissue \( n = 4 \) patients; A) and plasma ctDNA \( n = 4 \) patients; B) after whole genome pre-amplification and without pre-amplification. Between one and nine technical replicates were performed for each patient sample. Spearman's correlation coefficient was used to correlate the two variables.
Published protocols for centrifugation of blood samples typically use two sequential spins to separate plasma from blood cells. Different groups recommend a second centrifugation at different speeds, with the highest speed reported at 16,000 × g (40, 41). Because most laboratories can centrifuge blood at low speed, we evaluated the influence of the second centrifugation at low (3,000 × g) and high (16,000 × g) speed. We also evaluated the impact of delaying the second centrifugation following plasma storage at −80°C for 2 weeks, or performing it immediately on fresh plasma. Our results confirm that proceeding with the second centrifugation at low or high speed and storing plasma samples for as long as two weeks at −80°C before the second spin does not alter cfDNA levels. These observations may facilitate the adoption of plasma processing protocols that are more accommodating to the clinical setting either by allowing a second centrifugation at low speed with standard centrifuges or by allowing the shipment of frozen plasma samples to undergo high-speed centrifugation in the laboratory where ctDNA testing is performed.

Finally, we also tested the reliability of pre-amplification strategies, including targeted and WGA. Pre-amplification reactions are a useful strategy when the amount of DNA is limited. WGA could theoretically enable the testing of different variants in the same sample whereas targeted gene-specific amplification could enable technical replication of the assay in conditions of limited cfDNA. First, we needed to ensure that the process of pre-amplification did not alter allele frequency ratios. We found that the fractional abundance of DNA was preserved when using targeted pre-amplification of known mutated SNVs. Interestingly, these results were obtained when the fractional abundance of the mutated molecules was more than 0.05%. We were not able to obtain consistent fractional abundances for samples with fractional cfDNA abundances under 0.05%. However, using WGA, we were not able to generate DNA with the same fractional abundance from cfDNA. This can be caused by the fact that cfDNA is composed of DNA fragments of different length which can affect the uniformity of WGA in cfDNA samples (42). We therefore recommend caution when applying WGA to ctDNA analysis.

In summary, protocol harmonization and the use of consistent and reproducible extraction and processing methods are required for ctDNA testing to enter the clinical setting. This study allowed us to optimize cfDNA extraction efficiencies with a novel and significantly more efficient protocol and allowed us to better understand the impact of pre-analytical factors on ctDNA analyses. Our results will be important to consider while designing future clinical studies. Development of reliable and robust non-invasive diagnostic platforms using standardized protocols for ctDNA measurement will ensure that this biomarker fulfills its promise to add tremendous value to diagnosis and monitoring of treatment response of cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: L. Cavallone, M. Aldamry, A. Aguilar-Mahecha, M. Basik
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Pre-Analytical Protocol Optimization for ctDNA Testing

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