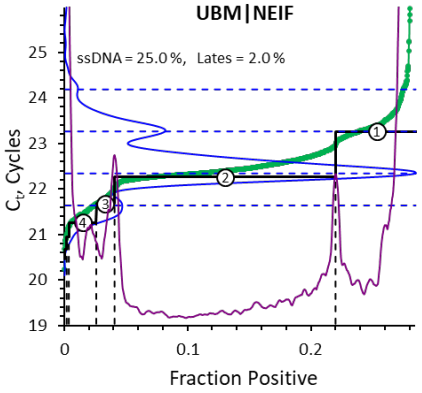
NIST Special Publication 1200-28

Evaluating Digital PCR for the Quantification of Human Nuclear DNA: Factors Influencing Target Strandedness



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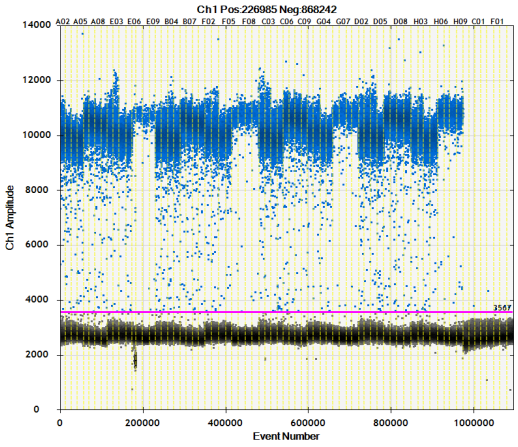

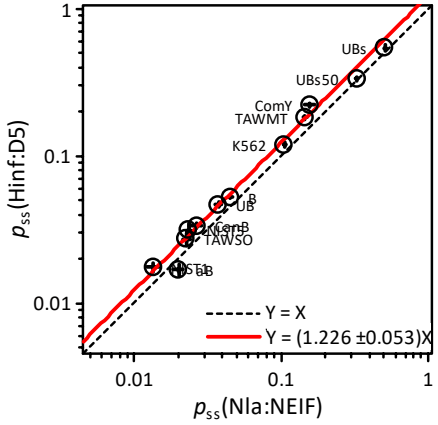

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Evaluating Digital PCR for the Quantification of Human Nuclear DNA: Factors Influencing Target Strandedness

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February 2021

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National Institute of Standards and Technology Special Publication 1200-28
Natl. Inst. Stand. Technol. Spec. Publ. 1200-28, 25 pages (February 2021)
CODEN: NSPUE2

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.1200-28

Abstract

The presence of single stranded DNA (ssDNA) in an extract of nominally double stranded DNA (dsDNA) can lead digital polymerase chain reaction (dPCR) measurements to overestimate the mass concentration of DNA in a sample by up to a factor of two. Motivated by measured ssDNA proportions of nearly 40 % in commercially obtained extracts, we have investigated some of the possible drivers of high ssDNA content in these materials to inform the development of future DNA reference materials. Our primary focus has been on the extraction methods: aqueous solution salt out, silica column, magnetic particles, and ion exchange resin. While many studies have compared various implementations of these methods with regard to the resources required and the quantity, purity, and amplifiability of the extracted DNA, none have compared the strandedness of extracts produced nor have they examined the impact of the methods on the ratio between the human mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) entity content, mtDNA/nDNA. In our hands, salt-out extraction of DNA from human blood-related tissue induces less dsDNA strand separation than the silica column and magnetic particle methods evaluated. Salt-out is also more efficient at extracting nDNA. It is therefore our method of choice for producing human-source nDNA reference materials for evaluation by and use with dPCR techniques. However, salt-out may be less efficient for and/or more damaging to mtDNA then are the silica column and magnetic particle methods. Further characterization is required before the accuracy of mtDNA/nDNA ratio measurements can be assured.

Key words

digital polymerase chain reaction (dPCR); double stranded DNA (dsDNA); mitochondrial DNA (mtDNA); nuclear DNA (nDNA); salt out extraction; single stranded DNA (ssDNA).

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Glossary

A_{260}	absorbance at 260 nm
cdPCR	chamber digital PCR
ddPCR	droplet digital
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA.
DTT	dithiothreitol
FAM	6-carboxyfluorescein dye
GC	guanine-cytosine pair
Hinf	*Hinf*III endonuclease
$\lambda(.)$	copy number concentration in a specified sample
mtDNA	mitochondrial DNA
Na_2EDTA	ethylenediaminetetraacetic acid disodium salt
nDNA	nuclear DNA
Nla	*Nla*I endonuclease
PCR	polymerase chain reaction
PBS	phosphate buffered saline
$p_{ss}(.)$	proportion of ssDNA in a specified mixture of dsDNA and ssDNA
qPCR	quantitative PCR
RFLP	restriction-fragment length polymorphism
SDS	sodium dodecyl sulfate
SRM®	Standard Reference Material®
ssDNA	single stranded DNA
Sty	*Sty*I endonuclease
TE^{-4}	10 mmol/L Tris-HCl, 0.1 mmol/L Na_2EDTA, pH8.0 buffer
Tris-HCl	2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride
Xcm	*Xcm*I endonuclease

1. Introduction

The presence of single-stranded DNA (ssDNA) in an extract of nominally double-stranded DNA (dsDNA) can bias digital polymerase chain reaction (dPCR) measurements [1]. The potential bias is up to a two-fold overestimate because: 1) in dPCR, amplification begins after the PCR target-containing entities are dispersed into individual reaction volumes and 2) each dsDNA entity that has denatured to ssDNA provides two dPCR-countable entities. While recognizing that the presence of ssDNA must be considered when using dPCR measurements to value-assign the DNA mass concentration ([DNA]) of reference materials, we and others did not previously regard it as a significant bias for fresh extracts [2]. Since pertinent literature [1,3,4,5] did not describe practical measurement methods for determining the ssDNA proportion in extracts of human tissue, this assertion was based on the absence of significant spectroscopic change over time rather than direct evidence [6].

Motivated by discrepant dPCR and spectroscopic estimates of [DNA] in a commercial extract, we recently developed an enzyme digest/dPCR-based method for determining ssDNA proportions, p_{ss}.[7]. By this assay ≈18 % of the target-containing entities in the commercial extract were ssDNA, resolving the ≈9 % motivating discrepancy. All three components of Standard Reference Material® (SRM®) 2372a Human DNA Quantitation Standard [6] were determined to contain less than 3 % ssDNA, indicating potential biases of less than 1.5 % – well within the 10 % expanded uncertainties assigned on the basis of between-PCR assay variability.

In contrast, measurements on a series of commercial standards intended for calibration of routine quantitative PCR (qPCR) assays revealed p_{ss} of from 10 % to nearly 40 %. While the presence of ssDNA in a calibrant or test sample is not pertinent for qPCR (qPCR amplification takes place in a common reaction volume after all target entities have been thermally denatured), we have investigated some of the possible drivers of high ssDNA content in these materials to inform development of future DNA reference materials intended for use with dPCR technologies.

Our primary focus has been extraction methods. While there exist a multitude of commercial and literature variants, a 2014 review of extraction techniques [8] identified five major approaches: aqueous solution salt-out, silica column extraction, magnetic particle-based purification, chelating resin (Chelex) extraction, and organic (phenol-chloroform) extraction. We have examined variants of the first four of these methods; for human and environmental safety concerns we do not use any chloroform-phenol based methods.

A number of studies have compared various implementation of these methods with regard to the resources required and the quantity, purity, and amplifiability of the extracted DNA [9,10,11,12]. However, none have considered the strandedness of extracts produced by methods that do not involve sample boiling nor have they examined the impact of the methods on the ratio between the human mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) entity content, mtDNA/nDNA.

2. Methods and Materials

2.1. Samples

Commercially-obtained blood or blood-derived tissue is the most practical source of human DNA for providing the forensic and clinical communities with DNA reference materials having representative genomic content. To evaluate extraction methods, we used archived units of component #15 Male Cell Pellet (3×10^6 cells) of the discontinued SRM 2390 DNA Profiling Standard that was designed for use with the obsolete restriction-fragment length polymorphism (RFLP) technology. These units had been stored by us at –80 °C since their purchase in the late 1980s. They were considered suitable for this study because 1) they were commercially prepared in fairly large number from purified peripheral blood mononuclear cells provided by a single healthy male donor, 2) the analogous extracted material, component #16 Male Undigested DNA (200 ng/μL) (referred to as "NIST1" in prior publications [7,13] but here termed "N1-Com"), has been stored under identical conditions for the same length of time and contains less than 1.5 % ssDNA, and 3) they were available in sufficient quantities. The cell pellets are here referred to as "N1" and the extracts derived from them as "N1-X" where "X" reflects the method used.

The DNA stock solution used to prepare component B of SRM 2372 Human DNA Quantitation Standard [14], was selected as the test material to evaluate post-extraction factors. This material was extracted in 2006 from commercially-sourced buffy coat white blood cells provided by multiple anonymous female donors, diluted with 10 mmol/L 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (Tris-HCl), 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), pH 8.0 buffer (TE^{-4}) to an approximate [DNA] of 100 ng/μL, and stored at 4 °C in a polytetrafluoroethylene container. This material was used for this phase of our studies because: 1) previous studies had demonstrated that this material contained about 6 % ssDNA, about twice that of other materials that we have extracted, 2) the ssDNA proportion in it was more easily increased by experimental manipulation than in our other materials, and 3) it was available in suitable quantity. This material is referred to as "UB" here and in a prior publication [7].

2.2. Extraction Methods

After extraction, all extracts were stored in TE^{-4} buffer at (2 to 8) °C.

2.2.1. Aqueous solution salt-out

For the basic salt-out protocol [15] we evaluated two variants: 1) our usual method where the cell pellets were covered in a lysis buffer containing proteinase K and incubated in a heated thermal mixer at 31 rad/s (300 rpm) for 2 h at 56 °C and 2) covering the pellets in a lysis buffer containing dithiothreitol and sodium dodecyl sulfate in addition to the proteinase K and incubated in the mixer at 31 rad/s for 18 h at 37 °C.

2.2.1.1. Reagents

DTT:	dithiothreitol (MilliporeSigma, Burlington, MA,USA)
Lysis Buffer:	(1 mg DTT, 250 μL 10 % SDS) per 1 mL Nuclei Lysis Buffer
Na_2EDTA:	ethylenediaminetetraacetic acid disodium salt (MilliporeSigma, Burlington, MA,USA)

Nuclei Lysis Buffer:	10 mmol/L Tris-HCl, 400 mmol/L NaCl, 2.0 mmol/L Na_2EDTA in deionized water.
Protease K Solution:	2 mg proteinase K in 1 mL of 2.0 mmol/L Na_2EDTA and 1 % SDS in deionized water.
Proteinase K	a broad-spectrum serine protease (MilliporeSigma, Burlington, MA,USA)
SDS:	sodium dodecyl sulfate (MilliporeSigma, Burlington, MA,USA)
TE^{-4} Buffer:	10 mmol/L Tris-HCl, 0.1 mmol/L Na_2EDTA, pH 8.0 (Affymetrix, Inc, Cleveland ,OH USA)
Tris-HCl:	2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (MilliporeSigma, Burlington, MA,USA)

2.2.1.2. 56 °C Incubation Protocol

In the original cell pellet 1.5 mL polypropylene microfuge tube: add 300 µL Nuclei Lysis Buffer and 100 µL of Protease K Solution. Vortex the mixture for 15 s then microfuge briefly (just until all liquid collects at the bottom of the tube). Incubate at 56 °C in a heated-thermal mixer at 31 rad/s (300 rpm) for 2 h.

2.2.1.3. 37 °C Incubation Protocol

In the original cell pellet 1.5 mL polypropylene microfuge tube: add 300 µL Lysis Buffer, and 100 µL of Protease K Solution. Vortex the mixture 15 s and microfuge briefly. Incubate at 37 °C in a heated-thermal mixer at 31 rad/s overnight (≈18 h).

2.2.1.4. Extraction Protocol

After incubation add 100 µL of saturated ammonium acetate to the tube. Vortex vigorously for 30 s then centrifuge at 262 rad/s (2500 rpm) for 5 min. Transfer the supernatant to a new 1.5 mL tube. Add 800 µL room-temperature absolute ethanol and invert gently for (1 to 2) min. Centrifuge at (1050 to 1260) rad/s (10 000 rpm to 12 000 rpm) for 5 min. Discard the supernatant. Wash pellet with 225 µL of 70 % ethanol/30 % water (volume fractions). Centrifuge at (1050 to 1260) rad/s for 5 minutes. Discard the supernatant.

Place open tube in a laminar flow hood to let the ethanol evaporate. When dry add 100 µL TE^{-4} buffer pre-heated to 60 °C and allow DNA pellet to solubilize at room temperature for 1 min. Vortex for 15 s and microfuge briefly. Store DNA solution at (2 to 8) °C.

2.2.2. Silica column extraction

Using the Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA USA) [16], we evaluated three silica column protocols. All protocols use the reagents supplied with the kit, and the incubated material was processed through a silica-based column using a chaotropic salt.

2.2.2.1. Cell Culture Incubation Protocol

This protocol is recommended for (1×10^4 to 5×10^6) cells.

In the original cell pellet 1.5 mL polypropylene microfuge tube: add 100 µL of cold phosphate buffered saline (PBS) pipetting up and down 5 to 10 times to resuspend the cells.

Dilute the provided Proteinase K stock solution, 5 µL Proteinase K to 15 µL PBS; mix by vortexing briefly. Pipet 5 µL of the diluted Proteinase K into each tube, mix by vortexing briefly. Add 100 µL Cell Lysis Buffer and vortex immediately and thoroughly, microfuge briefly. Incubate at 56 °C in a heated-thermal mixer at 147 rad/s (1400 rpm) for 10 min. Add 400 µL gDNA Binding Buffer to the sample and mix thoroughly by pulse-vortexing for (5 to 10) s. Place a gDNA Purification Column into a collection tube; transfer the mixture to the column.

2.2.2.2. 56 °C Tissue Incubation Protocol

In the original cell pellet 1.5 mL polypropylene microfuge tube: add 3 µL provided Proteinase K and 200 µL of provided Tissue Lysis Buffer; mix immediately by vortexing, microfuge briefly. Incubate at 56 °C in a heated-thermal mixer at 147 rad/s for 1 h. Add 400 µL gDNA Binding Buffer; pulse vortex for (5 to 10) s to mix thoroughly. Place a gDNA Purification Column into a collection tube; transfer the mixture to the column.

2.2.2.3. 37 °C Tissue Incubation Protocol

Same protocol as the 56 °C Tissue protocol except incubate at 37 °C in a heated-thermal mixer at 31 rad/s overnight.

2.2.3. Magnetic particle extraction

We evaluated results from the EZ1 DNA Tissue Kit (Qiagen) magnetic-particle technology using the EZ1 Advanced XL DNA Tissue Card [17]. The cells were suspended in a proprietary buffer with proteinase K, incubated in a heated thermal mixer at 147 rad/s for 1 h at 56 °C, then transferred to the EZ1 instrument. All reagents were supplied with the kit.

In the original cell pellet 1.5 mL polypropylene microfuge tube: add 190 µL proprietary G2 buffer and 10 µL provided proteinase K, mix immediately by vortexing, microfuge briefly. Incubate in a heated thermal mixer at 147 rad/s for 1 h at 56 °C, microfuge briefly, then transfer to a 2 mL sample tube (provided) prior to loading on to the EZ1 instrument with the EZ1 Advanced XL DNA Tissue Card installed. Select to elute in 200 µL elution buffer.

2.2.4. Ion Exchange (Chelex 100)

The basic Chelex 100 [18] protocol involved covering the sample in distilled water and incubating in a boiling water bath for 8 min. It is well known that this method converts dsDNA to ssDNA [18,19].

In the original cell pellet 1.5 mL polypropylene microfuge tube: pipet 1 mL sterile distilled water into the tube. Incubate 15 min at room temperature, occasionally mixing by gentle vortexing, Centrifuge 3 min at (100 to 150) N (10 000 g_n to 15 000 g_n). Remove all but (20 to 30) µL of supernatant. Add 200 µL of a 5 % (mass fraction) Chelex 100 in sterile distilled water suspension. Vortex gently and microfuge briefly. Incubate in a boiling water bath for 8 min. Vortex at high speed for (5 to 10) s. Centrifuge for 3 min at (100 to 150) N. Pipet supernatant into a new tube and store at (2 to 8) °C

2.3. Post-Extraction Factors

We examined several post-extraction sample treatments, including silica column clean-up of extracted DNA and mechanical shaking at various intensities, temperatures, duration, and container fill volumes.

2.3.1. Silica Column Cleanup

Figure 1 outlines the experiment that explored whether passage through the silica purification column increased the ssDNA proportion.

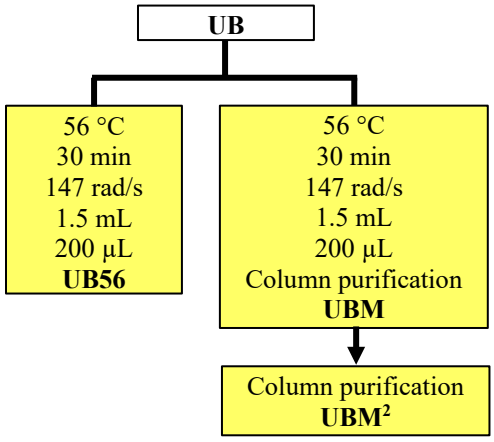

Figure 1. Influence of Passage Through Silica Purification Column

The following treatment protocol was used for all column purifications.

Close the cap of the gDNA Purification Column and centrifuge for 3 min at 10 N (1000 g_f) to bind the DNA to the column. Centrifuge again for 1 min at >118 N (>12 000 g_f). Discard the flow through and collection tube. Transfer the column to a new collection tube and add 500 µL Wash Buffer. Close the cap and invert so the wash buffer reaches the cap. Centrifuge for 1 min at >118 N. Discard the flow through and invert the collection tube on a paper towel to remove residual buffer. Re-insert the column in the collection tube and add 500 µL Wash Buffer. Close the cap and invert so the wash buffer reaches the cap. Centrifuge for 1 min at >118 N. Discard the collection tube and flow through. Place the Column in a new 1.5 mL microfuge tube. Add 100 µL TE^{-4} buffer preheated to 60 °C to the column, close the cap and incubate at room temperature for 1 min. Centrifuge for 1 min at >118 N to elute the DNA. Remove the tube and store at (2 to 8) °C until use.

2.3.2. Mixing Vigor and Temperature During Incubation

Figure 2 outlines the experiments that explored the effect of mixing vigor and temperature during incubation.

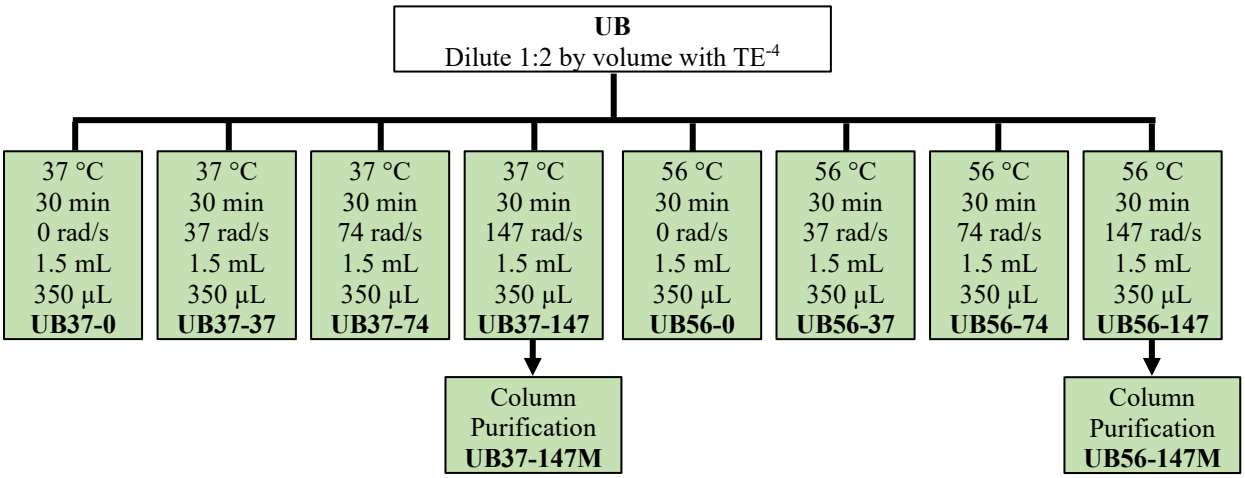

Figure 2. Influence of Rotational Speed and Temperature

2.3.3. Tube Size and Fill Volume During Incubation

Figure 3 outlines the experiments that explored the storage tube size and fill volume during incubation.

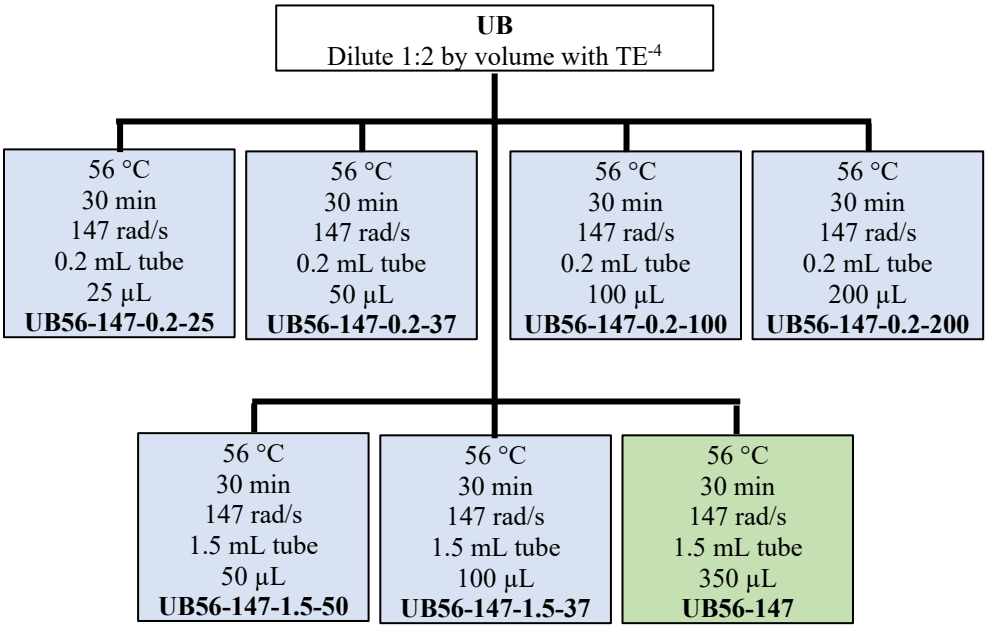

Figure 3. Influence of Tube and Fill Volumes

2.3.4. Incubation Duration

Figure 4 outlines the experiments that explored the impact of the duration of incubation.

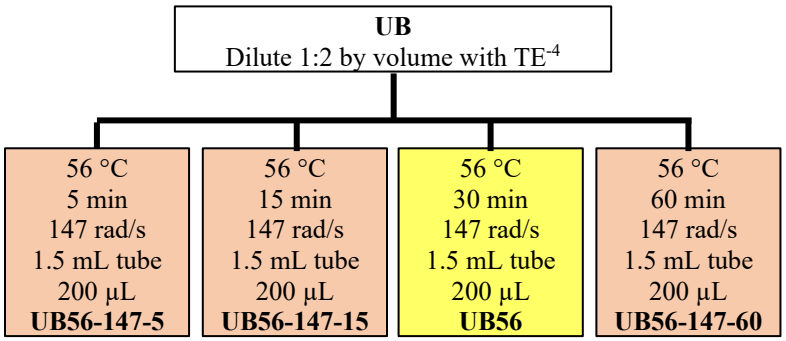

Figure 4. Influence of Treatment Duration

2.4. Human DNA Assays

Table 1 lists the probes, forward and reverse primers, and mastermix compositions for the D5, HBB1, NEIF, and POTP human nuclear DNA (nDNA) and mtND1 human mitochondrial DNA (mtDNA) assays used in this study. The NEIF assay has an average guanine-cytosine (GC) content of ≈ 30 % in the near-region of its target sequence, D5 and HBB1 assays have ≈ 40 %, and POTP ≈ 60 % [7].

Table 1. Human nDNA and mtDNA Assays

nDNA Assay Target	Chromosome Band Accession #	Primers and Probe [a]		Amplicon Length, bp
NEIF Gene EIF5B	Chr 2 p11.1-q11.1 NC_000002.12	F	GCCAAACTTCAGCCTTCTCTTC	67
		R	CTCTGGCAACATTTCACACTACA	
		P^{B+}	TCATGCAGTTGTCAGAAGCTG	
POTP STR TPOX	Chr 2 p25.3 NC_000002.12	F	CCACCTTCCTCTGCTTCACTTT	60
		R	ACATGGGTTTTTGCCTTTGG	
		P^{T}	CACCAACTGAAATATG	
D5 STR D5S2500	Chr 5 q11.2 NC_000005.10	F	TTCATACAGGCAAGCAATGCAT	75
		R	CTTAAAGGGTAAATGTTTGCAGTAATAGAT	
		P^{T}	ATAATATCAGGGTAAACAGGG	
HBB1 Gene HBB	Chr 11 p15.5 NC_000011.10	F	GCTGAGGGTTTGAAGTCCAACTC	76
		R	GGTCTAAGTGATGACAGCCGTACCT	
		P^{T}	AGCCAGTGCCAGAAGAGCCAAGGA	

mtDNA Assay	Location	Primers and Probe [a]		Length, bp
mtND1	3485:3504 3533:3553 3506:3522	F	CCCTAAAACCCGCCACATCT	69
		R	GAGCGATGGTGAGAGCTAAGGT	
		P^{T}	CCATCACCCTCTACATC	

a) F = Forward primer, R = Reverse primer,
P^{B+} = Blackhole Plus quencher probe, P^{T} = TaqMan MGB probe.
A = adenine, C = cytosine, G = guanine, T = thymine

Primers were purchased from Eurofins Operon (Huntsville, AL USA). TaqMan probes were purchased from Thermo Fisher (Waltham, MA USA) and 6-Carboxyfluorescein (FAM) labeled. Blackhole Quencher+ probes were purchased from LGC Biosearch Technologies (Novato, CA).

2.5. ssDNA Analysis

Two dPCR-based methods were used in this study, one using a Fluidigm BioMark (South San Francisco, CA USA) real time chamber dPCR (cdPCR) system with BioMark 48.770 digital arrays and the other using a QX200 droplet dPCR (ddPCR) system (Bio Rad, Hercules, CA USA). These and the other methods we have investigated for determining the proportion of ssDNA, p_{ss}, are documented in detail elsewhere [7,20].

2.5.1. cdPCR Staircase

Given a suitable PCR assay, the shape of the cumulative distribution (ogive) of the amplification cycle when fluorescence intensity in each chamber crosses a threshold enables direct visual evaluation of p_{ss}. The method requires that the signals in all chambers that contain the same number of amplifiable entities cross the threshold in synchrony, resulting in an ogive with a staircase-like profile. This requires that the assay be very efficient; of the ten nDNA assays we have routinely used [6,20], only NEIF and POTP reliably provide ogives with fairly vertical risers and fairly flat steps.

While requiring relatively little sample and little preparation beyond dilution to an appropriate [DNA], cdPCR Staircase analysis has a large "background" signal that limits its quantitative utility for p_{ss} less than about 6 % ssDNA; however, the method works well for samples having larger proportions of ssDNA. The background may arise from mechanical shearing of dsDNA entities during passage through the digital array's microfluidic channels.

Table 2 details the experimental cdPCR setup per reaction, using the proprietary Taqman Gene Expression Master mix from Thermo Fisher, Waltham, MA, USA.

Table 2: Mastermix Setup for Human Nuclear DNA cdPCR Assays

Concentration	nDNA Mastermix	Microliters per Reaction
2X	TaqMan Gene Expression Master Mix	2.5
10 µmol/L	Forward primer	0.19
10 µmol/L	Reverse primer	0.19
10 µmol/L	Probe (FAM)	0.13
20X	GE loading buffer	0.5
	PCR Grade water	1.0
	DNA, diluted to provide $\lambda \approx 0.4$	0.5
	Total Volume	5.0

2.5.2. ddPCR Enzyme

Use of type II restriction endonucleases that selectively cut dsDNA within an assay's target sequence enables quantification of low and moderate ssDNA proportions. For each sample of interest, four test portions from each sample aliquot are prepared to have the same final nDNA content: 1) native, 2) enzyme digested, 3) heat denatured at 96 °C for 1 min, and 4) heat denatured followed by enzyme digestion. Using ddPCR, the copy number concentration, λ, is determined for each test portion: λ(native), λ(enzyme), λ(denatured), and

λ(denatured-enzyme). The p_{ss} is equal to {λ(enzyme)/λ(native)}/{λ(denatured-enzyme)/λ(denatured)}. Since ssDNA can form local structures that are recognized and cut by (nominally) dsDNA-specific enzymes, the results for the two heat-denatured portions enable an estimate of the proportion of target-containing ssDNA entities in the native portion that were inactivated by cutting.

We have used the (enzyme, nDNA assay) combinations (*Hinf*III, D5), (*Sty*I HF, HBB1), (*Nla*I, NEIF) and (*Xcm*I, POTP) in this study; all enzymes purchased from New England BioLabs Inc. We refer to these assays as Hinf:D5, Sty:HBB1, Nla:NEIF, and Xcm:POTP.

Table 3 details the ddPCR setup per reaction, using the proprietary Supermix for Probes (no dUTPs), lot numbers 64191911 and 64268278, from Bio Rad, Hercules, CA USA.

Table 3. Mastermix Setup for Human Nuclear DNA ddPCR Assays

Concentration	nDNA Mastermix	Microliters per Reaction
2X	Supermix for Probes (no dUTPs)	12.5
10 µmol/L	Forward primer	0.94
10 µmol/L	Reverse primer	0.94
10 µmol/L	Probe (FAM)	0.63
	PCR Grade Water	7.5
	DNA, diluted to provide $\lambda \approx 0.4$	2.5
	Total Volume	25.0

2.6. Mitochondrial/Genomic Ratio

Mitochondrial DNA measurements were initially accomplished by diluting the sample used in the nDNA analysis 100-fold with TE^{-4}, however, after determining the general mtDNA to nDNA of N1 was lower than 100, additional dilutions of a 60-fold dilution or a 5-fold dilution were used to increase the mtDNA λ. The 100-fold dilution was accomplished with two serial 10-fold dilutions (5 µL solution combined with 45 µL TE^{-4}) to ensure accuracy, while the 60-fold dilution was accomplished with a 6-fold dilution (5 µL solution combined with 25 µL TE^{-4}) followed by a 10-fold dilution. The 5-fold dilution was a simple 10 µL solution combined with 40 µL TE^{-4}. The nDNA and mtDNA assays were run in parallel. The mtDNA/nDNA ratio was calculated as $D\lambda$(mtDNA)/λ(nDNA), where D is the fold dilution factor.

Table 4 details the ddPCR setup per reaction, using the same lots of the proprietary Supermix for Probes (no dUTPs) used in the nDNA assays.

Table 4. Mastermix Setup for Human Mitochondrial DNA ddPCR Monoplex Assays

Concentration	mtDNA Mastermix	Microliters per Reaction
2X	Supermix for Probes (no dUTPs)	12.0
10 µmol/L	Forward Primer	0.9
10 µmol/L	Reverse Primer	0.9
10 µmol/L	Probe (FAM)	0.6
	PCR Grade Water	7.2
	DNA, diluted to provide $\lambda \approx 0.4$	2.4
	Total Volume	24.0

2.7. DNA Mass Concentration

The [DNA] for most extracts were estimated from absorbance measurements at 260 nm (A_{260}) provided by a Nanodrop (Thermo Fisher Scientific*)* microvolume UV/Vis spectrophotometer. The [DNA] of the Chelex extracts was estimated from ddPCR λ measurements using the conversion equation described in [2] with an estimated droplet volume of 0.74 nL, divided by 2.0 to account for the nominally complete conversion of dsDNA to ssDNA. The droplet volume derived from measurements made by NIST researchers [21].

3. Results and Discussion

Table 5 lists the proportion of ssDNA in all of the N1 extracts. Due to resource limitations, not all extracts were evaluated with all assays. To provide a reliable comparison, all assay values were transformed to the scale of the ddPCR Hinf:D5 enzyme assay, p_{ss}(Hinf:D5). While p_{ss} values provided by different assays are systematically different, results between assays are linearly related [7].

Table 5. Influence of Extraction Method
Proportion of ssDNA, mitochondrial/nuclear DNA Ratio, and Amount of DNA Extracted

		p_{ss}(Hinf:D5)[a]			mtDNA/nDNA			DNA, µg/pellet		
Extract	Method	n_a[b]	Mean	SD	n_a[b]	Mean	SD	n_p[c]	Mean	SD
N1-Com[d]	Commercial salt-out	4	2.3	0.8	1	69.6		N/A		
N1-SO56	Salt-out @ 56 °C	4	3.1	0.8	2	49.7	3.5	4	25.5	5.3
N1-SO37	Salt-out @ 37 °C	2	3.3	0.9				6	24.4	2.4
N1-MP	Magnetic particle	1	12.1[e]		1	62.4		3	19.1	1.0
N1-ST56	Silica Tissue @ 56 °C	4	16.0	1.1	2	63.4	1.4	6	19.2	4.9
N1-SC	Silica Cell				2	60.4	8.6	3	13.6	0.9
N1-ST37	Silica Tissue @ 37 °C	2	19.4	0.9				4	19.9	2.1
N1-CX	Chelex 100	1	98[e]		2	13.6	2.3	2	22.1[a]	0.6

a Assay results transformed to the p_{ss}(Hinf:D5) scale
b Number of independent assays
c Number of one-pellet extractions
d Commercial extract, stored at -80 °C since ≈1988;
e Estimated from cdPCR Staircase result

Table 2 lists the transformation parameters used to convert the p_{ss}(Nla:NEIF), p_{ss}(Sty:HBB1), p_{ss}(Xcm:POTP), and p_{ss}(Staircase) assay results to p_{ss}(Hinf:D5) values. The parameter values and their standard uncertainties were estimated using the FREML errors-in-variables software [22].

Table 6. Assay Transformations: p_{ss}(Hinf:D5) = β(X- α)

Assay X	β	α	Reference
p_{ss}(Nla:NEIF)	1/(0.89 ± 0.02)	0	[7: Fig. S6-F]
p_{ss}(Sty:HBB1)	0.97 ± 0.02	0	[7: Fig. S6-D]
p_{ss}(Xcm:POTP)	1.57 ± 0.04	0	[7: Fig. S6-B]
p_{ss}(Staircase)	(1.57 ± 0.04)/(1.34 ± 0.06)	0.057 ± 0.006	[7: Figs. 7, S6-B]

The commercially-obtained N1-Com extract has the lowest p_{ss}(Hinf:D5), followed closely by salt-out extracts N1-SO56 and -SO37. While the detailed history of N1-Com is unknown since the method was proprietary, it was purchased from a company that preferentially used a salt-out extraction method. The lower p_{ss}(Hinf:D5) of the commercial extract may be related to its higher [DNA] (200 ng/µL rather than ≈ 50 ng/µL) and/or use of a Tris-EDTA buffer with higher EDTA content (0.2 mmol/L rather than 0.1 mmol/L).

While 4-fold higher than in the salt-out extracts, the estimated p_{ss}(Hinf:D5) of the magnetic particle extract N1-MP is 1.5-fold lower than those provided by the silica column methods. The increased p_{ss}(Hinf:D5) in N1-ST37 over that in N1-ST56 suggests that the duration of incubation rather the temperature or speed of rotation is a determining factor.

Nearly all of the nDNA in the Chelex-extracted N1-CX was ssDNA; heat-treating the extract completed the conversion. The unexpected presence of a small proportion of dsDNA in the Chelex extract suggests that some ssDNA may renature to dsDNA following the boiling step.

Table 1 also lists the mtDNA/nDNA ratios for most of the extracts and the mass of DNA recovered per pellet for the different methods and their variants. The two salt-out methods recovered ≈ 30 % more DNA per pellet than did the magnetic particle and silica tissue methods and ≈ 80 % more than the silica cell method; however, they may not extract mtDNA and nDNA with the same efficiency. Chelex extraction recovered somewhat more DNA per pellet than the silica tissue variants, but Chelex extraction either is much less efficient at extracting mtDNA or renders a much larger proportion of the mtDNA targets non-amplifiable or inaccessible.

The low p_{ss}(Hinf:D5) provided by the salt-out variants for the N1 pellets strongly suggests that the commercial qPCR standards we evaluated were not produced using a salt-out method. While both silica column and magnetic particle are candidates for the method(s) used to produce standards having 10 to 20 % ssDNA, they are not by themselves candidate methods for standards having 30 to 40 %. We therefore investigated the impact of post-extraction processes using our UB extract. This 2006 material has about twice the p_{ss}(Hinf:D5) content as "fresh" salt-out extracts [7,20], suggesting that its dsDNA strands are less tightly paired and therefore should be relatively responsive to insults.

Table 7 lists the samples, treatments, and measurement results from our investigations of treatment factors: agitation vigor, temperature, duration, collection tube volume, fill volume, and number of purification passages. The row colors correspond to the treatments diagramed in Figure 1 through Figure 4. Figure 5 provides a graphical summary of these results.

Table 7: Samples, Treatments and Measurements

							p_{ss} [b]						
	Treatments [a]						Hinf:D5		Xcm:POTP		Combined		
Sample	°C	rad/s	min	#	mL	µL	*x*	*u*(*x*)	*x*	*u*(*x*)	*n*	*x*	*u*(*x*)
UB	20	0	30	0	1.5	200	4.6	0.4	3.5	0.6	2	4.0	0.8
UB56	56	147	30	0	1.5	200	23.0	1.5	22.1	0.8	2	22.6	0.6
UBM	56	147	30	1	1.5	200	20.4	0.8	23.7	0.9	2	22.1	2.3
UBM[2]	56	147	30	2	1.5	200	20.9	1.3	23.6	1.3	2	22.2	1.9
UB37-0	37	0	30	0	1.5	350	3.3	0.3	3.8	0.6	2	3.5	0.3
UB37-37	37	37	30	0	1.5	350	3.9	0.3	3.5	0.3	2	3.7	0.3
UB37-74	37	74	30	0	1.5	350	3.3	0.4	3.6	0.3	2	3.5	0.2
UB37-147	37	147	30	0	1.5	350	6.8	0.4	8.0	0.5	2	7.4	0.9
UB37-147M	37	147	30	1	1.5	150	12.8	0.8	15.1	1.4	2	13.9	1.6
UB56-0	56	0	30	0	1.5	350	3.7	0.5	3.8	0.3	2	3.7	0.0
UB56-37	56	37	30	0	1.5	350	3.6	0.4	3.6	0.5	2	3.6	0.0
UB56-74	56	74	30	0	1.5	350	3.9	0.5	3.5	0.2	2	3.7	0.3
UB56-147	56	147	30	0	1.5	350	12.0	0.5	11.9	0.9	2	12.0	0.0
UB56-147M	56	147	30	1	1.5	150	14.8	1.1	17.1	0.8	2	16.0	1.6
UB56-147-0.2-25	56	147	30	0	0.2	25			4.9	0.2	1	4.9	
UB56-147-0.2-50	56	147	30	0	0.2	50			5.0	0.3	1	5.0	
UB56-147-0.2-100	56	147	30	0	0.2	100			6.1	0.6	1	6.1	
UB56-147-0.2-200	56	147	30	0	0.2	200			5.2	0.5	1	5.2	
UB56-147-1.5-50	56	147	30	0	1.5	50	6.9	0.5	6.4	0.9	2	6.7	0.3
UB56-147-1.5-100	56	147	30	0	1.5	100	16.8	0.6	18.2	1.1	2	17.5	1.0
UB56-147-5	56	147	5	0	1.5	200			11.3	0.9	1	11.3	
UB56-147-15	56	147	15	0	1.5	200			16.8	0.8	1	16.8	
UB56-147-60	56	147	60	0	1.5	200			35.2	2.5	1	35.2	

a °C: Incubation temperature
rad/s: Thermal mixer rotational speed
min: Incubation duration
#: Number of passages through silica purification column
mL: Volume of microfuge polyethylene tube
µL: Volume extract in the tube during treatment

b p_{ss}: Proportion of ssDNA
Hinf:D5: p_{ss}(Hinf:D5) produced by the *HinfI*-cut D5 enzyme assay
Xcm:POTP: p_{ss}(Xcm:POTP) produced by the *XcmI*-cut POTP enzyme assay, transformed to the Hinf:D5 scale
Combined: Equal-weighted mean of p_{ss}(Hinf:D5) and the transformed p_{ss}(Xcm:POTP)
x: p_{ss} value
u(*x*): Standard uncertainty of the p_{ss} value
n: Number of assays combined

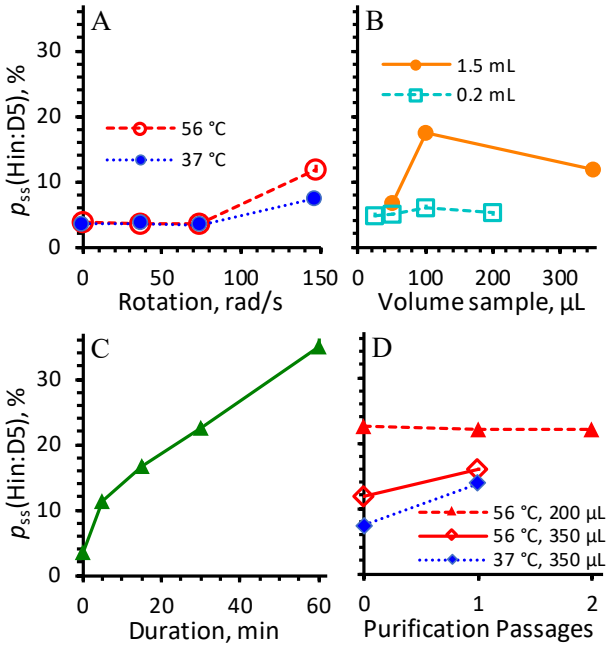

Figure 5. Post-Extraction Influence Factors

Each symbol represents the estimated p_{ss}(Hinf:D5) in the UB extract after being subjected to one or more treatments; in all cases, error bars representing measurement standard uncertainty are covered by the symbol. A) The effect of mixer rotational speed at 37 °C and 56 °C. The mixing duration was 30 min, with 350 µL of the UB extract in 1.5 mL tubes. B) The effect of tube and sample fill volumes. The mixer rotational speed was 147 rad/s (1400 rpm) held at 56 °C for 30 min. C) The effect of treatment duration. The mixer rotational speed was 147 rad/s held at 56 °C, with 200 µL of the UB extract in 1.5 mL tubes. D) The effect of passage through a silica column as a purification step following post-extraction treatment at three combinations of temperature and sample volume in 1.5 mL tubes. The mixer rotational speed was 147 rad/s for 30 min.

Figure 5A reports the effect of thermal mixture rotational speed on 350 µL of the UB extract contained in sterile polypropylene 1.5 mL tubes. At both 37 °C and 56 °C, the proportion of ssDNA remained unchanged by 30 min at low to moderate agitation with the maximum change at the higher temperature with the mixer's maximum rotation of 147 rad/s (1400 rpm). In consequence, subsequent investigations were carried out at 56 °C and 147 rad/s.

The results displayed in Figure 5B reveal that rather than rotational speed it is the vigor of the "slosh" of the material within its tube that drives strand separation. Regardless of fill volume, liquid in a 0.2 mL polypropylene PCR tube does not move much as the mixer rotates. With the 1.5 mL tubes, small fills stay confined in the tapered tip while vortices can form with larger fills. We assume that strand separation is driven by interactions between the swirling extract and the tube wall. Figure 5C shows that whatever causes the strand separation, once initiated the magnitude of the effect increases linearly with the length of the treatment time.

Figure 5D reports the effect of multiple passes through the silica column purification process [16]. For treated extracts with relatively low p_{ss}(Hinf:D5), binding the DNA to a column and eluting it increases the proportion of ssDNA. However, as the p_{ss}(Hinf:D5) of the treated extract increases the effect of binding and eluting appears to decrease. At 20 % p_{ss}(Hinf:D5), two sequential purification passages had essentially no impact on the ssDNA proportion. This suggests that when dsDNA is shaken apart as far as it wants to go under a given treatment, passage through the purification process does not cause further separation; however, passage can completely separate strands that have only been loosened by the treatment.

The information provided in Table 8 suggests that the impact of purification on DNA recovery is less complex: each purification passage reduced the mass of DNA recovered by ≈ (30 to 50) % without much change to the spectrophotometric quality metrics.

4. Summary

In our hands, salt-out extraction of DNA from human blood-related tissue induces less dsDNA strand separation than the silica column and magnetic particle methods evaluated. Salt-out is also more efficient at extracting nDNA. It is therefore our method of choice for producing human-source nDNA reference materials for evaluation by and use with dPCR techniques. However, salt-out may be less efficient for and/or more damaging to mtDNA then are the silica column and magnetic particle methods. Further characterization is required before the accuracy of mtDNA/nDNA ratio measurements can be assured.

While the (10 to 20) % p_{ss}(Hinf:D5) produced by our silica column and magnetic particle methods is compatible with the observed ssDNA content of some qPCR calibration standards, other commercial extracts appear to have been subjected to less gentle treatment during or after extraction.

Table 8: DNA Loss on Passage Through a Silica Purification Column

		Volume [a]			Spectrophotometric Information [b]						[DNA] [c]		DNA [d]		Loss [e]		Mean Loss [f]	
		µL			A_{260}		A_{260}/A_{280}		A_{260}/A_{230}		ng/µL		ng		%		%	
Status [g]	Sample	x^h	u^i	n^j	x^h	u^i	x^h	u^i	x^h	u^i	x^h	u^i	x^h	u^i	x^h	u^i	x^h	u^i
Before	UB37-147	350	7	2	1.002	0.002	1.900	0.014	2.905	0.021	50.1	0.1	17518	351	48.2	1.4	38.0	8.8
After	UB37-147M	150	3	2	1.211	0.007	1.880	0.000	2.680	0.552	60.6	0.4	9083	189				
Before	UB56-147	200	2	2	2.112	0.012	1.851	0.001	2.272	0.007	105.6	0.6	21116	438	35.1	1.2		
After	UB56-147M	100	1	2	2.743	0.045	1.852	0.003	2.449	0.006	137.1	2.3	13713	355				
Before	UB56	200	2	2	2.153	0.048	1.846	0.004	2.269	0.022	107.7	2.4	21534	646	40.7	2.9		
After	UBM	100	1	6	2.554	0.158	1.862	0.008	2.409	0.071	127.7	7.9	12769	832				
Before	UBM	100	2	6	2.554	0.158	1.862	0.008	2.409	0.071	127.7	7.9	12769	832	27.9	2.5		
After	UBM[2]	100	1	6	1.841	0.107	1.850	0.010	2.477	0.013	92.0	5.4	9203	566				

a Volume sample in µL, uncertainty from pipette specification.
b Spectrophotometric absorbance at specific nm wavelengths are symbolized as "A_{wl}", where wl is the wavelength in nm. The mass concentration of an aqueous solution of dsDNA, [DNA], is conventionally estimated as $50A_{260}/L$, where L is the pathlength in cm. Protein-free DNA is expected to have a A_{260}/A_{280} ratio of 1.8 to 2.0. The A_{260}/A_{230} ratio helps evaluate the level organic contamination; as a guideline this ratio should be above 1.5. For further information see [S5].
c Conventional estimate of the mass concentration of dsDNA expressed in ng/µL: $50(A_{260})$.
d Mean ng of DNA: (Volume)([DNA])
e Loss in DNA quantity due to passage through a silica column, expressed as a percentage: 100(1-(ng after passage)/(ng before passage))
f Summary of loss-on-purification results
g Status of extract: before purification passage or after passage
h Mean value
i Standard deviation or standard combined uncertainty
j Number of cell pellets extracted

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