Estimation of Extraction Efficiency by Droplet Digital PCR

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Forensic DNA typing requires a specific quantity of input DNA (typically 0.5 - 1.0 nanograms) to generate an optimal short tandem repeat (STR) profile. For reference samples, the amount of DNA collected on a standard buccal swab or blood punch is generally more than that which is needed for testing (on the order of hundreds of nanograms (ng)). Typically, extraction efficiency is evaluated by determining the number of samples that produce a full STR profile divided by the total number of samples processed. Less attention has been paid to the amount of DNA unrecovered during the extraction process. The importance of evaluating the theoretical yield versus the functional yield is in cases when the amount of available DNA is low. In these cases, it would be beneficial to obtain an extraction recovery that is closer to the theoretical yield than the functional yield. Evaluating the amount of unrecovered DNA could lead to more efficient methods to recover higher percentages of DNA from the extraction and purification processes.

Extraction efficiency experiments were conducted to evaluate the percentage of DNA recovered through three extraction methods: a manual phenol-chloroform method, the use of the Qiagen EZ1 Advanced XL Extraction robot, and with Qiagen QIAamp DNA Mini extraction kit. Three DNA sources (cells, blood, extracted DNA) were tested at varying known concentrations. Extracted samples were quantified with the use of droplet digital PCR with nuclear DNA assays designed and optimized in-house. Results indicated that the observed recovery value range was lower than many reported extraction efficiency calculations using the number of full STR profiles produced.

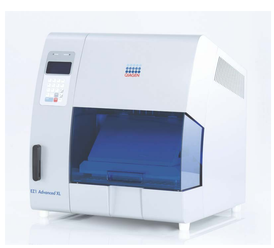
What is Extraction Efficiency?

$$\frac{\text{Amount of DNA recovered post-extraction}}{\text{Original amount of DNA pre-extraction}} = \text{Extraction Efficiency}$$

Knowing the original amount of DNA in the extraction process allows for the **comparison of extraction protocols** and methods to accurately determine the efficiency of the extraction process. **Digital PCR** offers ***absolute quantitation*** without the need of a calibration standard curve for determining the amount of recovered DNA post extraction.

Extraction Methods

Qiagen EZ1 Advanced XL



Materials were extracted according to manufacturer's recommended protocols for the DNA Investigator kit [1]. This kit utilizes silica covered magnetic particles for DNA purification.

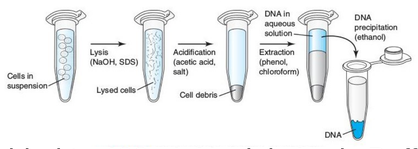
50 µL sample was added to 140 µL G2 Buffer and 10 µL Proteinase K and **incubated at 56 °C in a thermomixer for one hour**. Purification on the EZ1 Advanced XL instrument with the DNA Investigator kit. Samples **were eluted in 50 µL TE^{-4}**

Qiagen QIAamp Spin Columns

Materials were extracted according to manufacturer's recommended protocols for the QIAamp DNA Mini extraction kit following the Purification from Blood or Body Fluids Spin Protocol [2].

Samples were **normalized to a volume of 200 µL** and added to 200 µL Buffer AL and 20 µL Proteinase K. Samples were **incubated at 56 °C in a thermomixer for ten minutes**. Purification took place in the QIAamp silica spin columns with Qiagen Buffers. Samples were **eluted in 200 µL AE Buffer**.

Phenol-Chloroform (Organic)



100 µL of sample was added to 400 µL Nucleic Lysis Buffer, 4 µL 10 % SDS, and 10 µL Proteinase K and incubated overnight at 37 °C.

After incubation, an equal volume of phenol chloroform (514 µL) was added and vigorously mixed before **adding solution to a phase lock light tube** (Quantabio, VWR, 10847-800) for complete separation of the two phases. The phase lock light tubes were centrifuged at 14,000 x g for 15 minutes to separate the layers. The aqueous layer was transferred to a fresh tube where 2x the volume of ethanol was added.

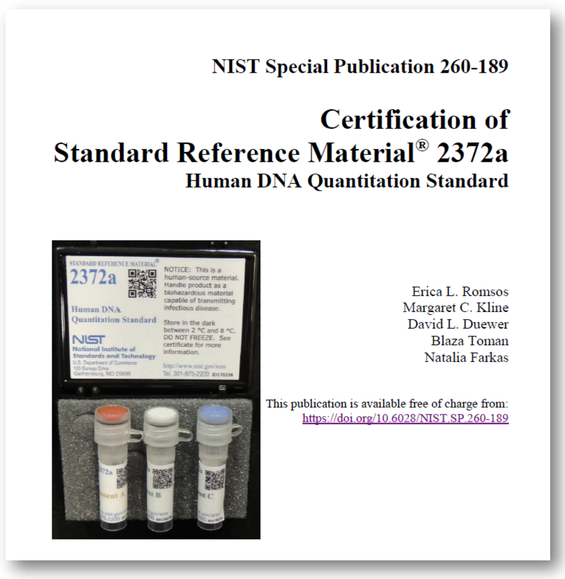
After gently mixing the aqueous layer with the ethanol, the tubes were centrifuged at 12,000 x g for 5 minutes. The ethanol was carefully removed from the tube and the DNA was allowed to dry overnight. The DNA **was resolubilized with 50 µL TE^{-4}.**

Droplet Digital PCR

BIO-RAD QX200

In ddPCR

- Sample/mastermix is placed in a droplet generator
- Individual droplets in an oil emulsion are formed
- PCR amplification is performed (end point)
- Droplets are read as being positive or negative
- Counting of positive droplets can be converted into sample concentration



NIST Special Publication 260-189

Certification of Standard Reference Material® 2372a Human DNA Quantitation Standard

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ddPCR allows for absolute quantitation without the need of a standard curve or calibrant

What if I don't have access to digital PCR?

Calibration of qPCR standards to NIST Standard Reference Material 2372a: Human DNA Quantitation Standard will help with accuracy of qPCR measurements to assess extraction efficiency. SRM 2372a was certified with ddPCR. This will allow for comparison of qPCR plates over time and reduce bias from commercial qPCR kit standards.

Component	Copy Number (per nL)	DNA (ng/µL)
A (red cap)	15.1 ± 1.5	49.8 ± 5.0
B (white cap)	17.5 ± 1.8	57.8 ± 5.8
C (blue cap)	14.5 ± 1.5	47.9 ± 4.8

SRM 2372a allows for more accurate and precise qPCR measurements and the ability to compare data over time, operators, and qPCR plates.

References:
1. Qiagen EZ1 DNA Investigator Handbook. July 2014.
2. QIAamp DNA Mini and Blood Mini Handbook. May 2019.
3. Romsos, E.L., Kline, M.C., Duewer, D.L., Toman, B., Farkas, N. (2018) Natl. Inst. Stand. Technol. Spec. Publ. 260-189, DOI: 10.6028/NIST.SP.260-189

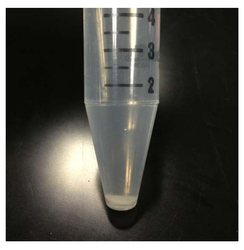
DNA Sources

DNA was extracted from three sources

Extracted DNA
Component A of SRM 2372a: Human DNA Quantitation Standard

Known concentration of 49.8 ng/µL
Determined by ddPCR

Cell suspension in PBS
Normal Fibroblast Cells



Known cell count of 1x10^6 per mL
Determined by flow cytometry

Whole Blood
Freshly collected

Known White Blood Cell Count of 4.6 x10^6 per mL
WBC reported by blood bank

Original DNA input into extraction was determined by the measured starting concentration of each DNA source

Four DNA input amounts were tested in replicates of five for each extraction method

	Amount (ng)	# of Cells	Uncertainty (± # Cells)	# of Replicates	Total Samples (Per Extraction Method)
Extracted DNA	50	8,333	833	5 per amount	20
	20	3,333	333		
	10	1,667	167		
	5	781	78		
Cells	38	6,250	313	5 per amount	20
	19	3,125	156		
	9	1,563	78		
	5	781	39		
Blood	276	46,000	2,300	5 per amount	20
	138	23,000	1,150		
	28	4,667	233		
	14	2,333	117		

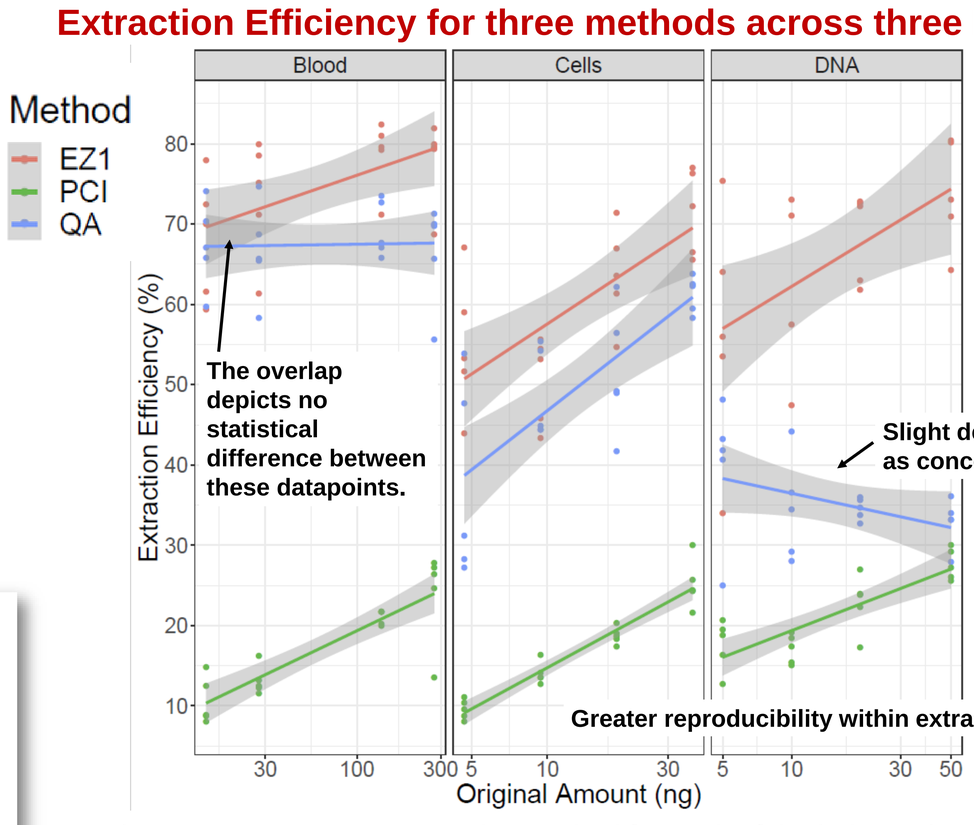
60 Samples per DNA Source

60 Samples per Extraction Method

Extraction Efficiency

The extraction efficiency is the ratio of the amount of DNA recovered post extraction (quantity) to the original amount of DNA pre-extraction (known). The recovered amount of DNA was determined through absolute quantitation via ddPCR.

Extraction Efficiency for three methods across three DNA Sources



The efficiency of each DNA extraction method is consistent across DNA sources

Each color represents an extraction method for each of the independent DNA sources. The individual points represent the extraction replicates for each DNA input amount. The slope of the line represents a change in efficiency dependent on original DNA input amount. Only a **slight increase in efficiency is observed across increasing DNA input amounts**, for all extraction methods ***with the exception of DNA with the QIAamp spin columns*.**

The gray bars around each line represents the 95 % confidence interval for each point on the line. Where there is overlap, it there is no statistical difference between the two overlapping points. The **efficiency of the extraction method is different between methods, but *similar across DNA sources*** for each method.

The EZ1 demonstrates the highest extraction efficiency in this set of experiments. The Organic PCI results are the least variable between extraction replicates.

Conclusions

Different extraction methods yield different efficiencies, but were **relatively consistent across different DNA sources**. The **amount of DNA originally added** into the extraction method showed a **trend in increased efficiency for increased amounts of DNA** for all except DNA and Blood with the QIAamp Mini Spin Columns. Organic extraction demonstrated the lowest efficiency for all of the methods tested, but was the most reproducible among extraction replicates. **Additional experiments need to be conducted to confirm the repeatability of these measurements.**

Future Work

- Additional operators (Reproducibility and Repeatability)
- Examination of alterations in extraction incubation times
- Addition of carrier RNA for lower DNA inputs
- Use of Microcon Centrifugal Filters for Organic extraction
- Altering elution buffer volume
- Examination of additional extraction chemistries, methodologies, kits, and techniques
- Addition of a swab/substrate to the extraction process

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A copy of this poster is available at: http://strbase.nist.gov/NISTpub.htm#Presentations

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