Novel DNA purification method facilitates the bioanalysis of DNA-based gene therapeutics in whole blood samples.

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INTRODUCTION

- DNA-based therapeutics (etc., plasmid) have experienced remarkable growth and are represented as an attractive approach for gene therapy or DNA vaccines. DNA integration is a significant safety concern for such drugs; therefore, a long-term and careful adverse effect evaluation is crucial.
- The robust and susceptible quantification method, which measures DNA-based therapeutics with the LLOQ (lower limit of quantitation) of less than 50 copies per µg of genomic DNA in different biological matrixes, is required. However, method validation parameters are difficult to meet the acceptance criteria when genomic DNA is purified by conventional purification methods such as Tris-saturated phenol or column-based assay in whole blood.
- Here, we developed a novel DNA purification approach for DNA-based therapeutics quantification in whole blood samples. A mimic DNA-based gene therapeutics (a commercialized plasmid) was evaluated as an example.

NOVEL ASPECTS

- A novel DNA purification method was developed to quantify DNA-based gene therapeutics (plasmid as an example). A head-to-foot evaluation method of the new DNA extraction method in whole blood, covering concentrations as well as the overall performance in RT-qPCR[1].
- Using rat whole blood matrix as an example, compared with the traditional DNA purification method, the newly developed method aids in building a quantification qPCR method with superior sensitivity, specificity, and robustness.
- We offer novel considerations when developing and validating DNA-based gene therapeutics qPCR methods. In contrast to the published protocols and papers, the new extraction procedure has been well-optimized as evidenced by both recovery efficiencies of around 100% and double amount of DNA isolated quantity.
- Compared with other methods, the new method saves time (1.5~2 hours), cost (less than 2 dollar for 1 mL of whole blood) and effort (no work gain for extraction volume expansion)[2][3].

METHOD(S)

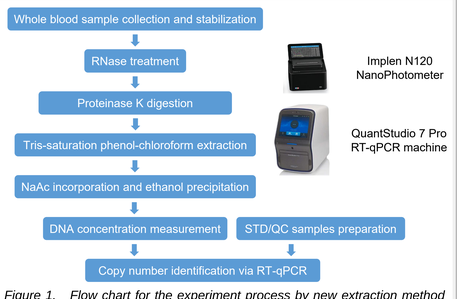

Figure 1. Flow chart for the experiment process by new extraction method and absolute quantitation RT-qPCR method for a mimic mRNA therapeutic (a commercialized plasmid) quantification in whole blood.

RESULT(S)

The DNA concentrations were measured by Implen NanoPhotometer after being extracted from whole blood samples with column-based method and novel extraction method (Figure 2). After that, two sets of primer/probe were designed and synthesized, which have been utilized in further RT-qPCR experiments. The result showed that the novel extraction method got lower signals in the blank whole blood samples, allowing higher sensitivity for the further RT-qPCR quantitation assay(Table 1).

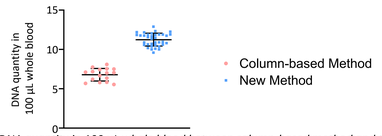

Figure 2 . The comparison of DNA quantity in 100 µL whole blood between column-based method and the new method.

Table 1 . The comparison of Ct values for blank DNA samples between column-based method and the new method modified from CTAB method.

		Column-based Method		New Method	
		Sample #1	Sample #2	Sample #3	Sample #4
Primer/Probe Set 1	Well #1	32.62	32.60	Undetermined*	Undetermined
	Well #2	32.47	33.50	Undetermined	Undetermined
Primer/Probe Set 2	Well #1	32.72	32.34	Undetermined	Undetermined
	Well #2	33.21	32.86	Undetermined	Undetermined

Undetermined* means no obvious signal produced after forty-five cycles in whole blood samples without plasmid spiking.

We evaluate the method validation parameters with DNA extracting from the novel method: sensitivity, assay range, inter/intra accuracy and precision (A&P), selectivity, stability and extraction recovery[4][5]. For sensitivity parameter and assay range, the RT-qPCR method's lower limit of quantification (LLOQ) is 50 copies/µg whole blood genomic DNA and the assay range is $5x10^1$-$5x10^8$ copies/µg whole blood genomic DNA (covering 8 logs of linearity) (Figure 3).

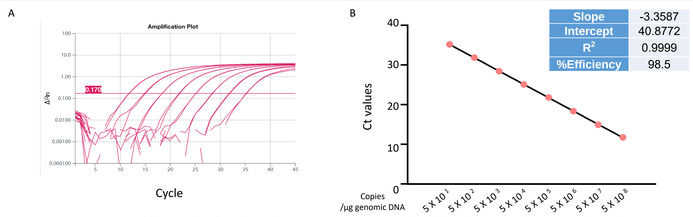

Figure 3. A. RT-qPCR amplification plot for plasmid quantitation in SD-1 rat whole blood. X-axis is the qPCR cycle number ; Y-axis is the ΔRn value. Curves indicate the standard sample from STD01 to STD08 (corresponding concentration shown in panel B); B. RT-qPCR method standard curve for plasmid quantitation. X-axis from right to left indicates STD01-STD08 samples concentration (copies/µg whole blood genomic DNA). Y-axis is the RT-qPCR Ct values.

Amplification efficiency and regression parameters are also crucial for absolute quantitation RT-qPCR method. Eight batches were evaluated with the addition of DNA isolated with novel method, showing that the DNA qualify are good enough to slightly or even no influence on the RT-qPCR reaction (Table 2).

Table 2 . The parameters for eight batches of standard curves to evaluate the influence on the RT-qPCR after incorporation the DNA isolated with novel method.

Batch	Slope	Intercept	R^2	Amplification Efficiency(%)
Acceptance criteria	-3.100 ≤ Slope ≤ -3.600	Not defined	≥0.9800	90.0 ~ 110.0
1	-3.3587	40.8772	0.9999	98.5
2	-3.3459	41.0176	0.9997	99.0
3	-3.3147	40.2781	0.9989	100.3
4	-3.3266	39.7070	0.9998	99.8
5	-3.3153	38.6954	1.0000	100.3
6	-3.3105	38.4322	0.9999	100.5
7	-3.3761	38.8612	0.9999	97.8
8	-3.3616	39.0170	0.9998	98.4

The inter/intra accuracy and precision evaluation for RT-qPCR spiked with DNA isolated with new method shown below. Each run has one set of standard sample (STD) and three sets of quality control (QC) samples in duplicate wells (Figure 4 A-D). The result exhibited that all the points meet the acceptance criteria well.

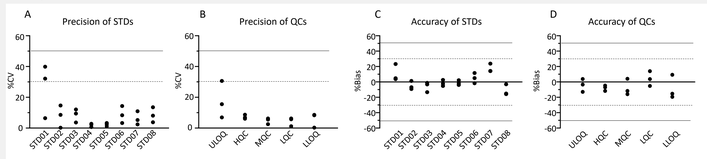

Figure 4. A, C. show RT-qPCR method %CV (precision) and %Bias (accuracy) for standard samples for each run, standard samples concentration are $5x10^1$, $5x10^2$, $5x10^3$, $5x10^4$, $5x10^5$, $5x10^6$, $5x10^7$, $5x10^8$ copies/µg whole blood genomic DNA, respectively. Lines indicate the acceptance criteria for each level, which also apply to other panels; B, D. show RT-qPCR method %CV and %Bias for quality control samples for each run. Quality control samples concentration are $5x10^1$, $2.5x10^2$, $2.5x10^4$, $2.5x10^6$, $5x10^8$ copies/µg whole blood genomic DNA, respectively.

Selectivity: Ten individuals were tested in the plasmid-spiked or unspiked whole blood DNA samples. At least 80% normal individual DNA samples met the acceptance criteria: the Ct values for unspiked DNA samples were "undetermined" or Ct higher than LLOQ samples; the plasmid-spiked samples met the acceptance criteria of precision and accuracy parameters (Table 3).

Table 3 The selectivity result for identification plasmid concentration in SD-1 Rat whole blood from 10 individuals.

Individuals	Unspiked samples	Spiked at LLOQ level			Spiked at ULOQ level		
		Back-calculated concentration	%CV	%Bias	Back-calculated concentration	%CV	%Bias
Male1	Undetermined	4.98E+01	3.7	-0.3	5.85E+08	9.3	17.1
Male2	Undetermined	3.56E+01	27.7	-28.9	5.26E+08	9.9	5.1
Male3	Undetermined	5.52E+01	18.5	10.3	5.30E+08	3.8	5.9
Male4	Undetermined	5.86E+01	17.1	17.2	5.51E+08	29.4	10.1
Male5	Undetermined	4.38E+01	4.6	-12.3	4.89E+08	8.4	-2.2
Female1	Undetermined	6.19E+01	1.7	23.9	4.69E+08	6.0	-6.1
Female2	Undetermined	4.55E+01	17.5	-9.0	4.55E+08	40.4	-9.0
Female3	Undetermined	5.62E+01	9.6	12.4	4.77E+08	4.5	-4.6
Female4	Undetermined	4.66E+01	4.2	-6.9	4.48E+08	2.6	-10.4
Female5	Undetermined	6.08E+01	24.7	21.7	5.07E+08	4.5	1.5
Pooled	Undetermined	4.59E+01	13.6	-8.3	5.67E+08	17.3	13.5

Stability: Three sets of stability samples for the two levels were prepared independently and analyzed. At least 2/3 stability samples met the acceptance criteria. It showed that bench-top stability (2-8℃, room temperature), thaw-freeze cycles (3 times and 5 times) and long-term stability all passed (Table 4).

Table 4 . The stability result for identification plasmid concentration in SD-1 Rat whole blood from different storage conditions.

	Freeze-Thaw Cycle (3 Cycles)	Freeze-Thaw Cycle (5 Cycles)	2–8℃ for 24 hours	Room Temperature for 24 hours
Stability samples at HQC level	Pass	Pass	Pass	Pass
Stability samples at HQC level	Pass	Pass	Pass	Pass

Three different levels of plasmid-spiked samples were extracted with novel extraction method. The %Recovery is defined as the ratio between the extracted plasmid-spiked samples and the same concentration without extraction. Surprisingly, the %recovery is between 106.0% ~118.0% across high level and low level samples (Table 5).

Table 5 . The extraction recovery result for the new extraction method of SD-1 Rat whole blood spiked plasmid at high/middle/low level.

	Nominal Conc. (copies/µg genomic DNA)	Without Extraction		Extraction Samples		%Recovery
		Measured Conc. (copies/µg genomic DNA)	%CV	Measured Conc. (copies/µg genomic DNA)	%CV	
Extraction Recovery Samples at high level	2.50E+07	2.01E+07	9.9	2.13E+07	2.2	106.0
Extraction Recovery Samples at middle level	2.50E+05	2.57E+05	8.0	3.03E+05	5.2	118.0
Extraction Recovery Samples at low level	2.50E+03	2.51E+03	1.2	2.80E+03	2.4	111.6

CONCLUSION(S)

- We hereby developed a novel DNA isolation approach to facilitate the quantification of DNA-based therapeutics in the whole blood sample, covering the evaluation of the new extraction method and absolute quantification of plasmid in whole blood samples.
- Apart from being labor-, money-, and time-efficient, the new method enables satisfying the acceptance criteria of the parameters required for the method validation of the absolute quantitation in whole blood samples.
- Thus, compared with traditional DNA isolation methods such as Tris-saturated phenol and column-based extraction methods, the new method could generate high-quality of DNA extraction for whole blood samples, which is more suitable for the downstream bioanalysis of DNA-based therapeutics.

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