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Comparison of Branched DNA (B-DNA) and Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) Method Validation for the Quantification of mRNA Therapeutics in Plasma

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PURPOSE

The therapeutic use of mRNAs holds great promise for combating a wide range of diseases. The rapid development of biotechnology in recent years has made it possible to produce almost any functional proteins in the human body by using mRNA as a therapeutic agent [1]. There are many ongoing mRNA vaccine/therapeutic research pipelines. To better understand the mRNA's efficacy and safety properties, especially for the pharmacokinetics (PK), toxicokinetic (TK), tissue distribution, and pharmacodynamics (PD), it is critical to quantify the level of mRNA in the system circulation and different tissues.

RT-qPCR platform is widely considered the gold standard for the quantification of mRNA-based therapeutics. Recently, the B-DNA (Branched-DNA) platform is emerging as a unique complement to the RT-qPCR platform. Here we compare the method validation performance on these two platforms for a mimic mRNA therapeutic bioanalysis (commercial LNP coated EGFP mRNA) in Sprague Dawley rat (SD rat) plasma. These evaluations provide some hands-on experiences for researchers who plan to do therapeutic mRNA bioanalysis.

OBJECTIVE

- Compare RT-qPCR assay and B-DNA assay for the quantification of LNP-mRNA drug in biological matrix
- Evaluate method validation parameters of RT-qPCR and B-DNA method. Summarize the strength and weakness for both ways.

METHOD(S)

- For RT-qPCR assay, we use a modified sample preparation method that can directly lyse the plasma without the enrichment of mRNA, then followed by a two-step Taqman RT-qPCR analysis.
- For B-DNA assay, we use a modified protocol previously designed to measure endogenous mRNA.
- The workflow of the two methods are shown in Figure 1.

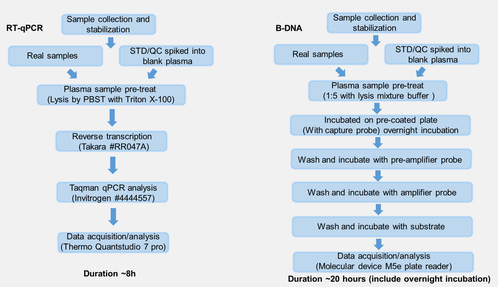

Figure 1. *Flow chart for the experiment process by RT-qPCR method and branched DNA method for a mimic mRNA therapeutic (commercial LNP coated EGFP mRNA) quantification in plasma.*

RESULT(S)

The RT-qPCR primer and probe for EGFP were designed by Primer-blast software. Forward primer sequence is GAGCGCACCATCTTCTTCAA, reverse primer sequence is TGATGCCGTTCTTCTGCTTG. TaqMan probe sequence is ACAAGACCCGCGCCGAGGTG labelled with 5'Fam - 3'Tamra. For the B-DNA assay, using a commercialized QuantiGene™ kit (Thermo, QS0013) and B-DNA EFGP Probe set (Thermo, SF-10256). The incubation temperature calibration was using the QuantiGene™ Temperature Validation Kit (Thermo, QS0517).

We evaluate each method's sensitivity, assay range, inter/intra accuracy and precision **(A&P), endogenous mRNA interference, selectivity, and stability** [2][3].

Table 1. *The method validation evaluation items and corresponding acceptance criteria for both RT-qPCR method and B-DNA method.*

Evaluation Items	RT-qPCR method Acceptance Criterial	B-DNA method Acceptance Criterial
Standard calibrators	≥6 non-zero calibrators should meet acceptance criteria Ct %CV ≤2.0%; %CV≤50% for ULOQ/LLOQ, %CVs≤30% for other points; %Bias±50% for ULOQ/LLOQ, %Bias±30% for other points. All NTC wells below LOD.	≥6 non-zero calibrators should meet acceptance criteria %CVs≤25% for ULOQ/LLOQ, %CVs≤20% for other points; %Bias±25% for ULOQ/LLOQ, %Bias±20% for other points.
Standard curve linearity	Linear regression: E = [10(-1/Slope)-1); 90–110% efficiency; Slope: -3.1 ≤ slope ≤ -3.6; R2 ≥0.98.	Logistical regression using 4pl/5pl parameters.
Quality controls	5 levels of QCs; Ct %CV ≤2.0%; %CVs≤50% for ULOQ/LLOQ, %CVs≤30% for other points; %Bias±50% for ULOQ/LLOQ, %Bias±30% for other points.	5 levels of QCs; %CVs≤25% for ULOQ/LLOQ, %CVs≤20% for other points; %Bias±25% for ULOQ/LLOQ, %Bias±20% for other points.
Selectivity	At least 10 individual matrix samples, tested unspiked and spiked with target standard for at LLOQ and HQC. At least 80% of spiked samples should have acceptable accuracy and precision, unspiked matrix should be BQL.	At least 10 individual matrix samples, tested unspiked and spiked with target standard for at LLOQ and HQC. At least 80% of spiked samples should have acceptable accuracy and precision, unspiked matrix should be BQL.
Stability	Assess for each matrix using spike in controls and processing, QC Samples (bench top, storage, and 3 freeze thaw cycles) at least low and high QC levels. %RE relative to freshly prepared QCs.	Assess for each matrix using spike in controls and processing, QC Samples (bench top, storage, and 3 freeze thaw cycles) at least low and high QC levels. %RE relative to freshly prepared QCs.
Critical reagents	Master mix; Primers/probes; Calibrator standards; Reagent kits for amplification.	B-DNA probes; Reagent kit for quantification; Sample processing kit.
Replicates per sample	2 replicates of STD/QC;2 replicates of real samples; 3 replicates of NTC.	2 replicates of STD/QC;2 replicates of real samples.

For sensitivity parameter, the RT-qPCR method's lower limit of quantification (LLOQ) is 1pg/mL. The B-DNA method shows a comparable LLOQ at 2pg/mL. For assay range, the RT-qPCR method shows a very broad linear range from 1pg/mL to 1ug/mL (covering 6 logs of linearity). In compare, the B-DNA method have much narrower assay range from 2 pg/mL to 250pg/mL, due to the maximal chemiluminescence capacity (Figure 2 & 3).

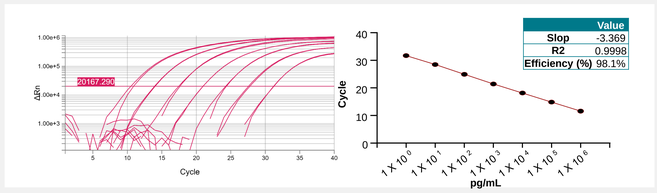

Figure 2. *RT-qPCR amplification plot for EGFP mRNA quantification in SD-1 rat plasma. X-axis is the qPCR cycle number; Y-axis is the ΔRn value. Curves indicate the standard sample from STD01 to STD07 (corresponding concentration shown in panel B); B. RT-qPCR method standard curve for EGFP mRNA qualification. X-axis from right to left indicates STD01-STD07 samples concentration (pg/mL). Y-axis is the qPCR cycle ct value.*

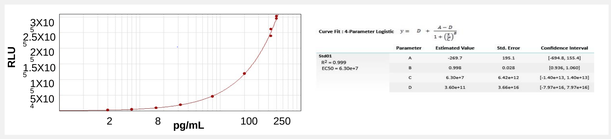

Figure 3. *B-DNA method standard curve for EGFP mRNA qualification, X-axis from left to right indicates STD01-STD08 samples concentration (pg/mL). Y-axis is the relative light unit (RLU) value.*

The inter/intra accuracy and precision evaluation for both RT-qPCR and B-DNA method shown below. Each run has one set of standard sample (STD) and three sets of quality control (QC) samples in duplicate wells.

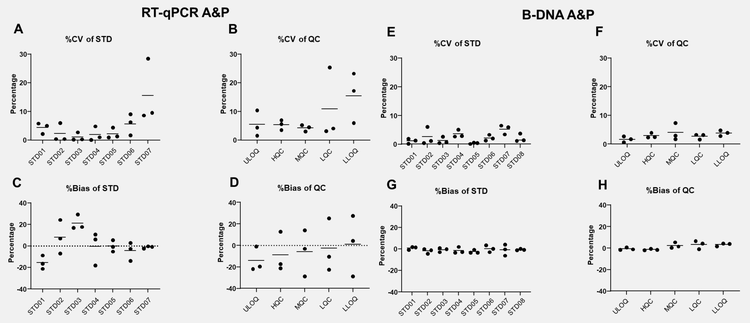

Figure 4. *A, C. show RT-qPCR method %CV and %Bias for standard samples for each run, standard samples concentration are $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$, $1x10^0$ pg/mL respectively. Lines indicate the average value of 3 runs, which also apply to other panels; B, D. show RT-qPCR method %CV and %Bias for quality control samples for each run. Quality control samples concentration are $1x10^6$, $5x10^5$, $5x10^3$, $5x10^1$, $1x10^0$ pg/mL, respectively; E, G. show B-DNA method %CV and %Bias for standard samples for each run, standard samples concentration are 250, 212.5, 100, 40, 16, 8, 4, 2 pg/mL respectively; F, H. show B-DNA method %CV and %Bias for quality control samples for each run. Quality control samples concentration are 250, 200, 30, 6, 2 pg/mL, respectively.*

The B-DNA method shows much tighter back calculated %CV and %Bias for accuracy and precision evaluation. The general %CV and %Bias for B-DNA method were less than 10%. However for the RT-qPCR method the %CV and %Bias are much higher, epically for the lower range of quantification. For the B-DNA method, the total error (%CV plus |%Bias|)of quality control (QC) samples (ULOQ, HQC, MQC, LQC, LLOQ) are 2.8%, 4.4%, 6.5%, 6.7%, and 7.1%, respectively. In contrast, for RT-qPCR, the values are 19.6%, 22.5%, 19.5%, 30.2%, and 35.6%, respectively.

Surprisingly, the RT-qPCR method could tolerate a higher level of endogenous mRNA interference (extracted from liver tissue in SD rat and spiked in the reaction). Based on the signal recovery assay, the HQC/LQC samples from RT-qPCR method could tolerate high endogenous mRNA concentration up to 25ug/reaction. However for the B-DNA method, the tolerance is much worse, 1ug/reaction mRNA already could suppress the signal already.

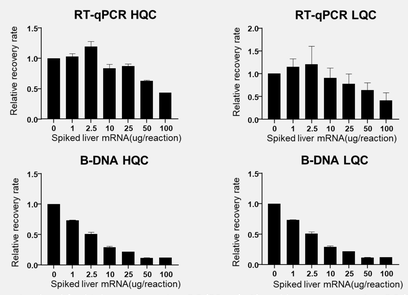

Figure 5. *Shows the signal suppression level for high concentration QC (80pg/mL) and low concentration QC (2.4pg/mL) samples. For both RT-qPCR and B-DNA method. X-axis indicates the spiked liver mRNA level, Y-axis indicates the relative back-calculated concentration recovery rate. The un-spiked sample is considered as 1 (100%).*

For selective evaluation, both methods show comparable selectivity at different concentrations (ULOQ, HQC, and LLOQ) in different SD rat individual plasmas for both sexes. For the RT-qPCR method, 100% ULOQ/HQC samples, 94% LLOQ samples passed the selectivity evaluation. As the B-DNA method, 100% ULOQ/HQC/LLOQ samples passed. And both methods show good stability for the test article at different conditions, including five freeze/thaw cycles, room temperature incubation for 6 hours, and 4℃ incubation for 24 hours. The |%Bias| between the stability sample and baseline samples are all less than 20% (30% for RT-qPCR).

CONCLUSION(S)

In summary, this comparison of both methods' validation for the therapeutic mRNA quantification in plasma shows that:

- With an optimized sample preparation procedure, the B-DNA method could achieve a comparable lower limit of quantification (2 pg/mL) with the RT-qPCR method (1 pg/mL) but with a much narrower assay range (2 - 250pg/mL for B-DNA compared with RT-qPCR of 6 logs of linear range).
- The B-DNA method has much tighter %CV and %Bias compared with the RT-qPCR method in accuracy and precision evaluation.
- B-DNA method is less tolerant to endogenous mRNA interference. This assessment is more informative for the bioanalysis of tissue samples, since the tissue samples will have much more endogenous mRNA than plasma.
- Both methods have comparable selectivity, and stability performance.
- The primer/probe design and optimization are critical for both methods. The proper design could significantly improve the assay performance.

REFERENCES

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[3] Bioanalytical Method Validation Guidance for Industry, FDA 2018.