Development of a Sensitive and Robust qPCR Method to Quantify siRNA Against HBV in Human Plasma

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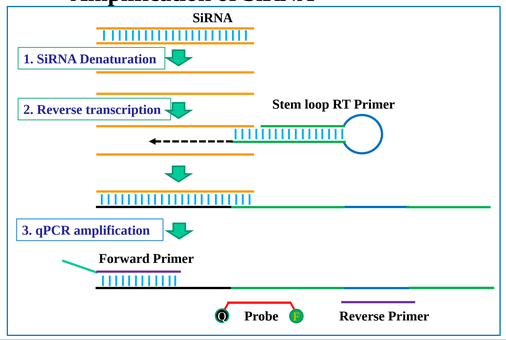
Introduction

Small interfering RNAs (siRNA) are powerful tools to control RNA metabolism via RNA interference mechanism and become promising therapeutic modality. After 20 years' development, three siRNA based drugs have been approved so far in the United States and European Union, and more candidates are in different stages of pre-clinical and clinical trials. To perform bioanalysis of siRNA based drugs in pharmacokinetics studies, different techniques such as LC-MS/MS, HPLC-fluorescence detection and qPCR have been applied. Among them, LC-MS/MS has high accuracy and the advantage to determine siRNA metabolite. However, the sensitivity of LC-MS/MS method is usually at the level of 5-10 ng/mL; HPLC-fluorescence could detect less amount of siRNA and reach to slightly higher sensitivity. Beyond that, qPCR technique has the characteristics of great sensitivity, broad measure range, high throughput performance, ideally for determining siRNA concentration in biological samples. To apply qPCR technique for quantifying siRNAs, there are still technical challenges such as matrix interference of samples, difficulty to obtain efficient primer/probe to amplify the short-length siRNA molecules et al.

Assay Design

Fig.1 Schematic Presentation of qPCR Amplification of SiRNA



Method

In this work, we try to establish a qPCR method to measure the concentration of siRNA product against HBV, we tried several different strategies to optimize siRNA RT-PCR/qPCR procedures including changing the detergent types and concentrations, testing different dilutions of plasma samples, changing primer/probe sequences and concentrations or using different RT-PCR/qPCR commercial kits. Finally, we identified that a higher dilution of plasma sample and optimized RT-PCR primer/probe sequence and concentration could significantly improve the assay performance and lead to high sensitivity of siRNA detection in plasma samples. Based on the optimized procedure, we conducted accuracy and precision runs, carried out selectivity tests in healthy, hemolyzed and lipemic human plasma, performed dilution linearity and robustness assays.

Experiment procedure

siRNA was spiked into plasma, and diluted at certain ratio (e.g. 1:100) with dilution buffer (0.25% Triton X-100 in PBS)

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The diluted siRNA/plasma samples were heated at 95℃ for 10min, then cooled to 4 ℃ and spinned at 20000g for 20min.

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Standard samples (STD), QC samples and validation samples were prepared

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STD, QC and validation samples were denatured and cooled to 4℃

↓

Reverse transcription

↓

qPCR amplification

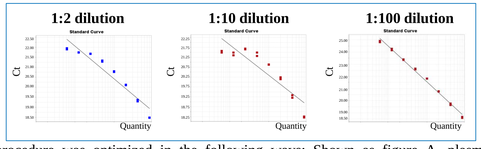
Instruments and Kits

Instrument	ABI QuantStudio 7 Flex
RT-PCR kit	Thermo, TagMan MicroRNA Reverse Transcription kit
qPCR Kit	Tsingke, 2xT5 qPCR mix (Probe）

Results

Fig 2. SiRNA qPCR Assay Optimization

A



Assay procedure was optimized in the following ways: Shown as figure A, plasma samples containing siRNA were diluted at the ratio of 1:2, 1:10 and 1:100 separately, the amplified standard samples Ct and quantity were plotted and displayed as figure A, indicating 1:100 plasma dilution improved assay performance. Different sets of RT primers and qPCR primers/probes and different concentrations of RT primers (0.1μM, 1μM and 10μM) were tested, and other optimizations such as different concentrations of detergents in dilution buffer, different qPCR kits were also examined. Eventually, a better assay condition (1:100 plasma dilution and 1μM RT primer) was selected for siRNA qPCR method development.

Results

Fig 3. Typical Standard Curve

Standard Samples	Theoretical Concentration (pg/mL)	Determined Concentration (pg/mL)	CV (%)	Bias (%)
STD01	102.40	109.81	8.5	7.2
STD02	25.60	25.79	7.5	0.7
STD03	6.40	6.34	3.8	-0.9
STD04	3.20	2.92	3.6	-8.8
STD05	1.60	1.49	1.9	-6.9
STD06	0.80	0.78	3.6	-2.5
STD07	0.40	0.42	3.4	5.0
STD08	0.20	0.22	6.4	10.0

- SiRNA stock were spiked in human plasma, then were diluted 100 fold to generate siRNA working solution (20000pg/mL) . SiRNA standard samples were prepared by serial dilution of the siRNA working solution with the dilution solution (0.25% Triton x-100 in PBS containing 1% human plasma).

Fig 4. Data Summary of A&P runs

Run No.	Parameter	LLOQ (20.0pg/mL)	LQC (100.0pg/mL)	MQC (500.0pg/mL)	HQC (2500.0pg/mL)	ULOQ (10240.0pg/mL)
Run1	Intra-run Mean(pg/mL)	20.3	93.3	468.7	2428.3	9675.3
	Intra-run %CV	2.8	2.2	4.1	6.4	4.1
	Intra-run %Bias	1.5	-6.7	-6.3	-2.9	-5.5
Run2	Intra-run Mean(pg/mL)	19.0	97.7	492.3	2492.7	10156.0
	Intra-run %CV	6.1	3.6	2.4	6.1	4.5
	Intra-run %Bias	-5.0	-2.3	-1.5	-0.3	-0.8
Run3	Intra-run Mean(pg/mL)	20.7	99.0	482.3	2445.0	10286.3
	Intra-run %CV	5.6	2.0	3.3	5.4	7.5
	Intra-run %Bias	3.5	-1.0	-3.5	-2.2	0.5
	Total n	9	9	9	9	9
	Inter-run Mean(pg/mL)	20.2	96.7	481.1	2455.3	10039.2
	Inter-run %CV	4.8	3.5	3.6	5.3	5.6
	Inter-run %Bias	1.0	-3.3	-3.8	-1.8	-2.0

- Three sets of A&P run were conducted by two analysts in two days.
- The concentration of QC samples are adjusted concentration (MRD 1:100).
- All of the intra-run and inter-run CV% and Bias% passed the acceptance criteria (CV%≤25% and Bias% are within ±25%)

Fig 5. Data Summary of Selectivity Assay

	HQC level 2500.0pg/mL					
	HQC-S1	HQC-S2	HQC-S3	HQC-S4	HQC-S5	HQC-S6
Adjusted Mean Concentration(pg/mL)	2472.0	2414.0	2431.0	2433.0	2409.0	2473.0
CV (%)	1.0	5.6	2.2	3.3	2.6	5.0
Bias (%)	-1.1	-3.4	-2.8	-2.7	-3.6	-1.1

	LQC level 100.0pg/mL					
	LQC-S1	LQC-S2	LQC-S3	LQC-S4	LQC-S5	LQC-S6
Adjusted Mean Concentration(pg/mL)	95.0	97.0	93.0	94.0	93.0	94.0
CV (%)	12.7	2.2	3.8	2.3	5.3	1.5
Bias (%)	-5.0	-3.0	-7.0	-6.0	-7.0	-6.0

	Blank-S1	Blank-S2	Blank-S3	Blank-S4	Blank-S5	Blank-S6
Adjusted Concentration(pg/mL)	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

- Selectivity samples were prepared by spiking siRNA stock into 6 individual human plasmas.
- Matrix effect were evaluated at HQC and LQC levels as well as unspiked samples.
- All CV% and Bias% passed the acceptance criteria. All the blank samples are BLQ.
- No obvious matrix interference was observed.
- No obvious matrix interference were observed for hemolyzed and lipemic samples (Data not shown here)

Results

Fig 6. Data Summary of Dilution Linearity Assay

	Set1			Set2			Set3		
Sample	Theoretical Conc. (pg/mL)	Adjusted Determined Conc.(pg/mL)	CV %	Bias %	Adjusted Determined Conc. (pg/mL)	CV %	Bias %	Adjusted Determined Conc. (pg/mL)	CV %	Bias %
D0	4000.0	ALQ	NA	NA	ALQ	NA	NA	ALQ	NA	NA
D1	4000.0	3660.6	3.1	-8.5	3297.2	0.9	-17.6	3324.2	3.6	-16.9
D2	400.0	347.1	0.8	-13.2	346.9	0.1	-13.3	324.0	3.0	-19.0
D3	40.0	32.4	5.0	-19.0	32.8	1.3	-18.0	31.9	1.6	-20.3
D4	4.0	BLQ	NA	NA	BLQ	NA	NA	BLQ	NA	NA

	Set4				Set5		
Sample	Theoretical Conc. (pg/mL)	Adjusted Determined Conc. (pg/mL)	CV (%)	Bias(%)	Adjusted Determined Conc. (pg/mL)	CV (%)	Bias(%)
D0	40000.0	ALQ	NA	NA	ALQ	NA	NA
D1	4000.0	3337.7	3.3	-16.6	3261.6	4.4	-18.5
D2	400.0	330.7	7.9	-17.3	313.9	5.9	-21.5
D3	40.0	30.9	2.5	-22.8	29.8	0.7	-25.5
D4	4.0	BLQ	NA	NA	BLQ	NA	NA

- Dilution linearity was assessed by serially diluting the template plasmid with a factor of 10.
- Dilution linearity assay results met the acceptance criteria.

Conclusion and Novelty

- We identified that a higher dilution of plasma sample and optimized RT-PCR primer/probe sequence and concentration could significantly improve the assay performance and sensitivity of siRNA using RT-PCR/qPCR technique.
- We conducted AP run, selectivity and dilution linearity assay. They all passed the acceptance criteria.
- In the end, we developed a high sensitive and robust siRNA qPCR method to measure the HBV siRNA concentration in plasma.

References

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2. Castellanos-Rizaldos, E., et al. Reverse Transcription Quantitative Polymerase Chain Reaction Methods to Support Pharmacokinetics and Drug Mechanism of Action to Advance Development of RNA Interference Therapeutics. Nucleic Acid Ther 30, 133-142 (2020).