Development of a highly sensitive direct qPCR method for cell therapy products quantification in non-clinical biodistribution studies

○Yoichi Naritomi, Yuka Sato, Kumiko Goto, Yuriko Takeno, Hironobu Kimura, Kouichi Tamura

HEALIOS K.K.

Purpose

Non-clinical biodistribution studies are essential in the development of cell therapy products (CTPs). The studies are typically performed using qPCR with DNA extraction. However, the DNA extraction step is complicated and time-consuming. In contrast, direct qPCR, in which lysed blood or tissue samples are directly quantified by qPCR, is a simple method that does not require DNA extraction. However, quantitative evaluation of biodistribution of CTPs using direct qPCR is limited. In this study, we evaluated sensitivity, precision, and accuracy of the direct qPCR method for CTPs biodistribution studies.

Methods

【Model cells for evaluating the direct qPCR method】

HLCN061, gene engineered human iPS cell-derived NK cells (eNK) expressing NKG2D, IL-15, CD16 (F176V), CCL19, and CCR2B for the treatment of refractory solid tumors

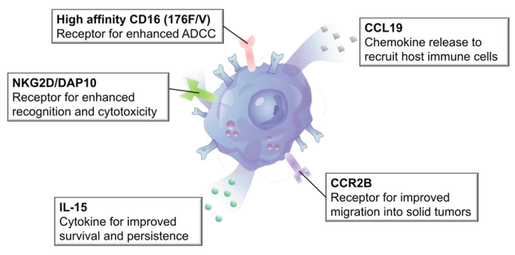


Fig. 1 HLCN061

【Experimental animals】

Female NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}/ShiJic（NOG）mice

【Development of the direct qPCR method】

The concentrations of HLCN061 cells in liver, kidney, heart, lung, spleen, ovary and blood of female NOG mice were estimated.

① HLCN061 were added to organ homogenates or blood and lysed in lysis buffer and proteinase K at 65 ℃ for 2 hours.

② The lysed samples were then diluted 10~60-fold with distilled water and subjected to real-time PCR.
The concentrations of HLCN061 cells per mg weight of organ or µL blood were determined by qPCR using a human-specific Alu primer set.

DirectAce qPCR mix (Nippon Gene, Tokyo, Japan) was used for PCR reactions. The reagent suppresses the effects of PCR inhibitors including surfactants used for cell lysis.

For comparison, a conventional master mix (PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA) was also evaluated.

Accuracy and precision for the direct qPCR method was estimated by measuring quality control (QC) samples.

Organ homogenates or blood
MagMAX™ Cell and Tissue DNA Extraction Buffer
Blank or HLCN061* in MagMAX™ Cell and Tissue DNA Extraction Buffer　　* : Standard or QC
MagMAX™ DNA Multi-Sample Ultra 2.0 Enhancer Solution
MagMAX™ DNA Multi-Sample Ultra 2.0 Proteinase K

↓ 65℃, 2hr

Crude sample

↓ Dilution with distilled water (x10～60)

Real-time PCR (QuantStudio 5)
30 µL/well
Alu primer set
DirectAce qPCR mix
or a conventional master mix

［PCR condition］
95 ℃　10 or 3 min (conventional master mix)
95 ℃　15 sec
60 or 61 ℃ (lung)　60 sec　40 cycles

Scheme 1 Procedure of the direct qPCR method

【Condition setting of the direct qPCR method】

The direct qPCR method conditions were set to meet the following criteria.

- ✓ The linearity of the calibration curve: $R^2 \geq 0.99$
- ✓ PCR amplification efficiency: 90 - 110%
- ✓ Relative error (RE) value: within ±25%
- ✓ Coefficient of variation (CV) value: within 25%

Calibration curve ⇒ $R^2 \geq 0.99$, 90% ≤ Efficiency ≤ 110%

QC ⇒ RE < |±25|%, CV < 25%

Considerations：
- Organ weight or blood volume/sample
- Crude sample volume
- LLOQ
- Dilution ratio of a crude sample
- Annealing temperature

[QC]
HQC: 80 % of HLOQ
MQC: 1/10 of HQC
LQC: 2- or 3-fold of LLOQ
LLQC: LLOQ
n=4

Scheme 2 Condition setting of the direct qPCR method

Table 1 Nominal concentrations of standard and QC samples

Liver, kidney, heart, lung

Nominal concentration (cells/mg)	Standard or QC
500	S1
400	HQC
150	S2
50	S3
40	MQC
15	S4
5	S5
0.75	LQC
0.5	S6
0.25	LLOQ, LLQC

Spleen, ovary

Nominal concentration (cells/mg)	Standard or QC
1000	S1
800	HQC
300	S2
100	S3
80	MQC
30	S4
10	S5
3	S6, LQC
1	LLOQ, LLQC

Blood

Nominal concentration (cells/µL)	Standard or QC
200	S1
160	HQC
60	S2
20	S3
16	MQC
6	S4
2	S5
0.6	S6, LQC
0.3	LLOQ, LLQC

S1～S6, LLOQ: standard
HQC, MQC, LQC, LLQC: QC

【*In vivo* study】

HLCN061 were administered iv to female NOG mice at a dose of 5 × 10^6 cells/mouse.
Blood and organs were collected at 5 min, 1 hour, and 24 hours after administration.
The blood and organ concentrations of HLCN061 cells were determined by the direct qPCR method.

Results and Discussions

【Comparison of DirectAce qPCR mix and a conventional master mix 】

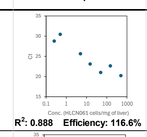
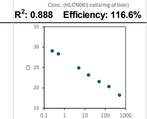
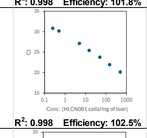
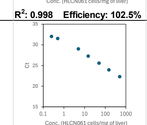
Dilution	DirectAce qPCR mix	Conventional master mix
x3	 R^2: 0.888 Efficiency: 116.6%	No amplification
x10	 R^2: 0.998 Efficiency: 101.8%	No amplification
x30	 R^2: 0.998 Efficiency: 102.5%	R^2: 0.933 Efficiency: 97.6%
x100	 R^2: 0.994 Efficiency: 115.5%	 R^2: 0.999 Efficiency: 106.9%

Table 2 Effects of dilution ratios on calibration curves in liver samples

Compared to a conventional master mix, DirectAce qPCR mix enabled quantification with samples at lower dilution ratios.
⇒ Improved quantitative sensitivity

【Conditions of the direct qPCR method】

Table 3 Conditions of the direct qPCR method in organ and blood samples

	Liver	Kidney	Heart	Lung	Spleen	Ovary	Blood
mg of organ or µL blood/sample	20	20	10	10	6	5	50
Crude sample volume (µL)	560	560	280	280	336	280	560
LLOQ (cells/mg or µL)	0.25	0.25	0.25	0.25	1	1	0.3
Dilution ratio of crude sample	10	10	10	20	30	30	60
Annealing temperature (℃)	60	60	60	61	60	60	60

Optimization was necessary for each organ and blood.

【Method validation】

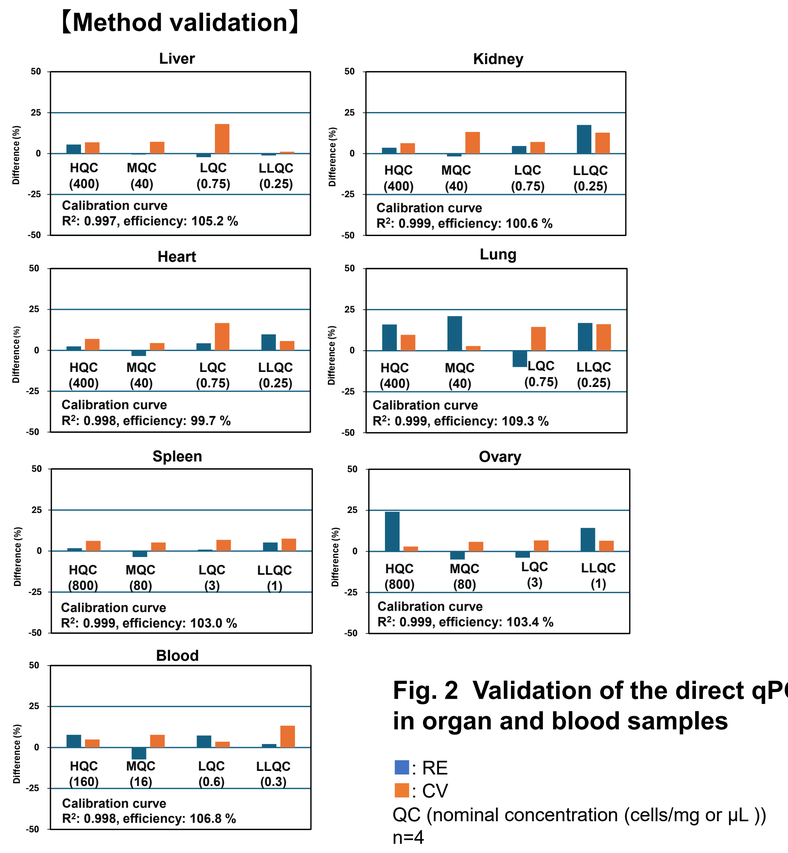


Fig. 2 Validation of the direct qPCR method in organ and blood samples

■: RE
■: CV
QC (nominal concentration (cells/mg or µL))
n=4

The good linearities of the calibration curves, very high sensitivity, and excellent precision and accuracy were observed.

【Specificity】

Table 5 Concentrations of human cells in blank and HLCN061-spiked samples

	Nominal concentration at LLOQ (cells/mg or µL)	Blank (cells/mg or µL)	LLOQ (cells/mg or µL)
Liver	0.25	0.043	0.24, 0.25, 0.25, 0.25
Kidney		0.034	0.28, 0.35, 0.26, 0.28
Heart		0.036	0.28, 0.27, 0.29, 0.25
Lung		0.062	0.30, 0.36, 0.25, 0.27
Spleen	1	0.11	1.0, 1.1, 1.1, 0.94
Ovary		0.082	1.2, 1.2, 1.1, 1.1
Blood	0.3	0.049	0.25, 0.31, 0.32, 0.34

The direct qPCR method enabled to specifically quantify HLCN061.

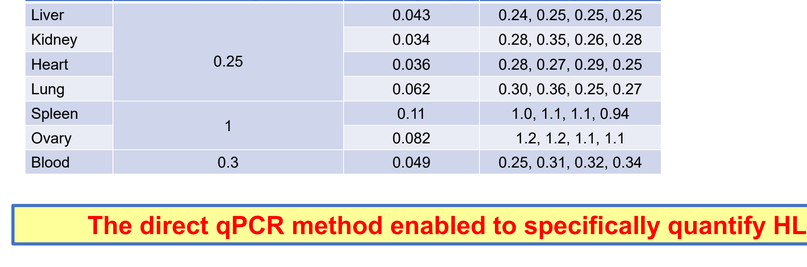
【Selectivity】

Table 6 Concentrations of human cells in blank and HLCN061-spiked individual blood samples

	Nominal concentration at LLOQ (cells/µL)	Blank (cells/µL)	LLOQ (cells/µL)
Mouse No. 1	0.3	0.066	0.31
Mouse No. 2		0.091	0.27
Mouse No. 3		0.080	0.39
Mouse No. 4		0.080	0.34
Mouse No. 5		0.084	0.33
Mouse No. 6		0.084	0.44
Mean		0.081	0.35
RE (%)		-	16.0
CV (%)		10.3	17.2

The direct qPCR method enabled to selectively quantify HLCN061 in the presence of other mouse matrix components.

【*In vivo* study (5x10^6 cells/mouse iv) 】



Ovary: pooled sample

Fig. 3 Concentrations of HLCN061 in organs and blood of female NOG mice

The direct qPCR method enabled to evaluate HLNC061 biodistribution in female NOG mice.

Conclusions

In this study, we developed a highly sensitive direct qPCR method for CTPs quantification in non-clinical biodistribution studies.

[Acknowledgement]

We are grateful to Akatsuki Therapeutics Inc. and AMED (Grant Number JP24be0904017) for supporting this research.