

# Validation of a Polymerase Chain Reaction (RT-qPCR) Method to Quantify a Codon-optimized Human Cystic Fibrosis Transmembrane Conductance Regulator mRNA (CO-hCFTR)

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## Introduction

Translate Bio is developing a novel therapeutic messenger RNA (mRNA) designed to enable the *in vivo* production of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein as a treatment for cystic fibrosis. This approach uses a codon-optimized human CFTR mRNA (CO-hCFTR) to restore healthy levels of CFTR. An RT-qPCR method was designed and developed to detect and quantitate the CO-hCFTR mRNA in lung tissue and whole blood, and validated per ICH Harmonized Tripartite Guidelines.

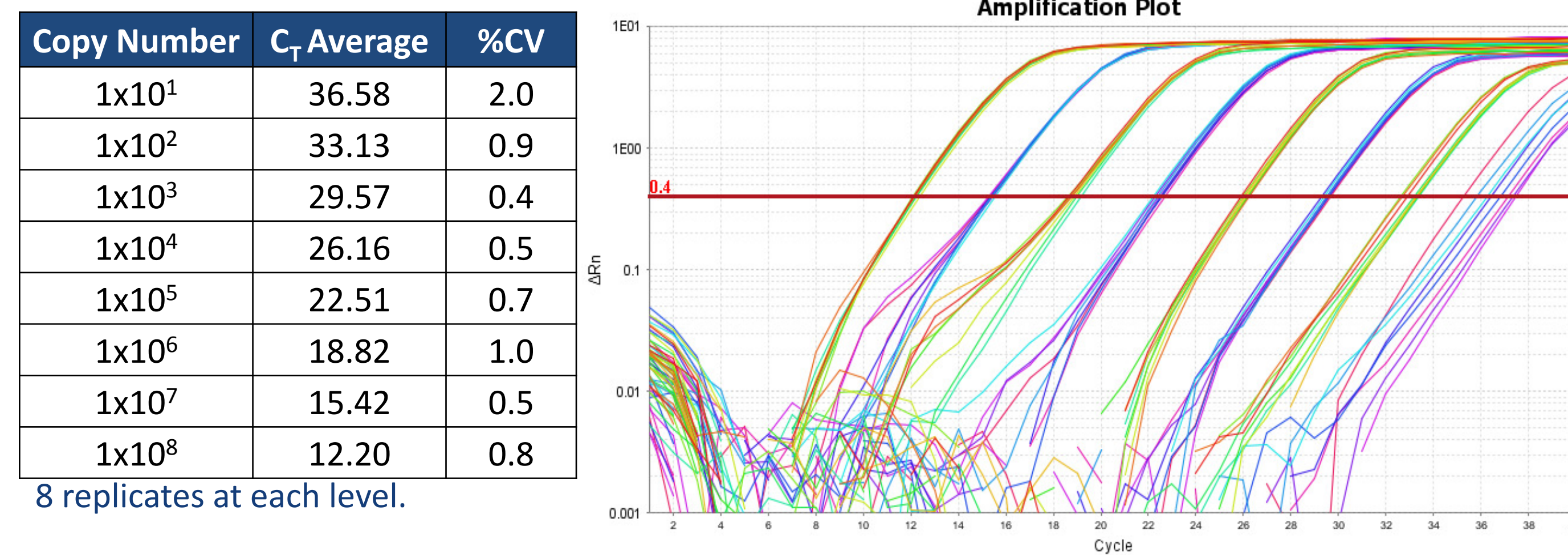
## Materials and Method Development

The mRNA standard was prepared via *in vitro* transcription from a synthesized runoff oligonucleotide template with quantity and quality assessment on the Nanodrop 8000 (Thermo Fisher Scientific) and the 2100 BioAnalyzer (Agilent). RT-qPCR was performed on the QuantStudio™ 7 Real-Time PCR System (Thermo Fisher Scientific).

Four primer/probe sets were designed. Assay selection and optimization was performed by testing 45 combinations of forward and reverse primers and probe concentrations with one input amount (e.g.  $1 \times 10^5$  copies) of the transcribed mRNA standard for each of the four designs.

The primer/probe combination having the lowest Cycle threshold ( $C_T$ ), the highest  $\Delta RN$  (change in fluorescence), and no detectable amplification (within 40 cycles) in the negative controls was chosen to advance to further method development and validation.

Table 1 and Figure 1. Initial Standard Curve of Chosen Assay



An initial standard curve of 10 to  $1 \times 10^8$  copies was tested to determine detection and quantitation limits.

## Specificity Results

Table 2. Primers/Probe Specificity

Assay specificity to the exogenous target mRNA transcript was confirmed by analyzing 100 ng of endogenous total RNA and genomic DNA from various species using the optimized assay concentrations. In all cases, the assay did not amplify the negative controls or background DNA/RNA.

Matrix	Assay Specificity
TE pH 8.0	Negative
Nuclease Free Water	Negative
100 ng Yeast tRNA	Negative
100 ng Lambda DNA-HindIII Digest	Negative
100 ng Cynomolgus Genomic DNA	Negative
100 ng Cynomolgus Total RNA	Negative
100 ng Human Placental DNA	Negative
100 ng Human Placental RNA	Negative
100 ng Rat Genomic DNA	Negative
100 ng Rat Total RNA	Negative

Negative = No amplification in 8 out of 8 wells

Additional specificity testing was performed against human, mouse, rat, and cynomolgus monkey liver, human lung tissue, and stabilized whole blood (RNAprotect® for animals and Paxgene® for human). Total RNA was extracted from tissue and stabilized whole blood (RNAprotect®) using the automated QIASymphony SP or the Promega Maxwell RSC®, respectively. Tissue lysates were prepared by QIAGEN TissueLysers and total RNA was extracted on the QIASymphony SP.

RNA concentration and purity were determined using the Nanodrop 8000. Up to  $1 \mu g$  of purified RNA was analyzed on the QuantStudio™ 7 Flex Real-Time PCR System via RT-qPCR for absolute quantitation with a standard curve using the optimized assay conditions.

Table 3. CO-hCFTR Assay Specificity in Blank Liver and Stabilized Whole Blood Matrices

Species	Matrix Type	Sex	Average Mass of Tissue (mg) or Volume of WB (mL)	Average RNA Concentration (ng/ $\mu$ L)	A260/A280	Copies of Exogenous CO-hCFTR per mg
Mouse C57-Bl/6	Liver	M	53.1	853.8	2.0	BQL
Mouse CD-1	Liver	F	53.0	858.1	2.1	BQL
Rat - Sprague Dawley	Liver	M	52.0	1038	2.1	BQL
Rat - Sprague Dawley	Liver	F	52.8	1035	2.1	BQL
Cynomolgus Monkey	Liver	M	53.1	250.6	2.1	BQL / 82*
Human	Liver	M	53.0	565.0	2.0	BQL
Human	Whole Blood	M	2.5	43.51	2.0	BQL
Human	Whole Blood	F	2.5	42.60	2.1	BQL
Cynomolgus Monkey	Whole Blood	M	0.5	24.36	1.8	BQL
Cynomolgus Monkey	Whole Blood	F	0.5	36.79	1.9	BQL
Mouse CD-1	Whole Blood	M	0.5	242.5	2.1	BQL
Mouse CD-1	Whole Blood	F	0.5	83.29	2.1	BQL
Rat - Sprague Dawley	Whole Blood	M	0.5	791.6	2.1	BQL
Rat - Sprague Dawley	Whole Blood	F	0.5	761.4	2.1	BQL

\*One extraction replicate out of six resulted in non-BQL result due to incidental touch contamination.

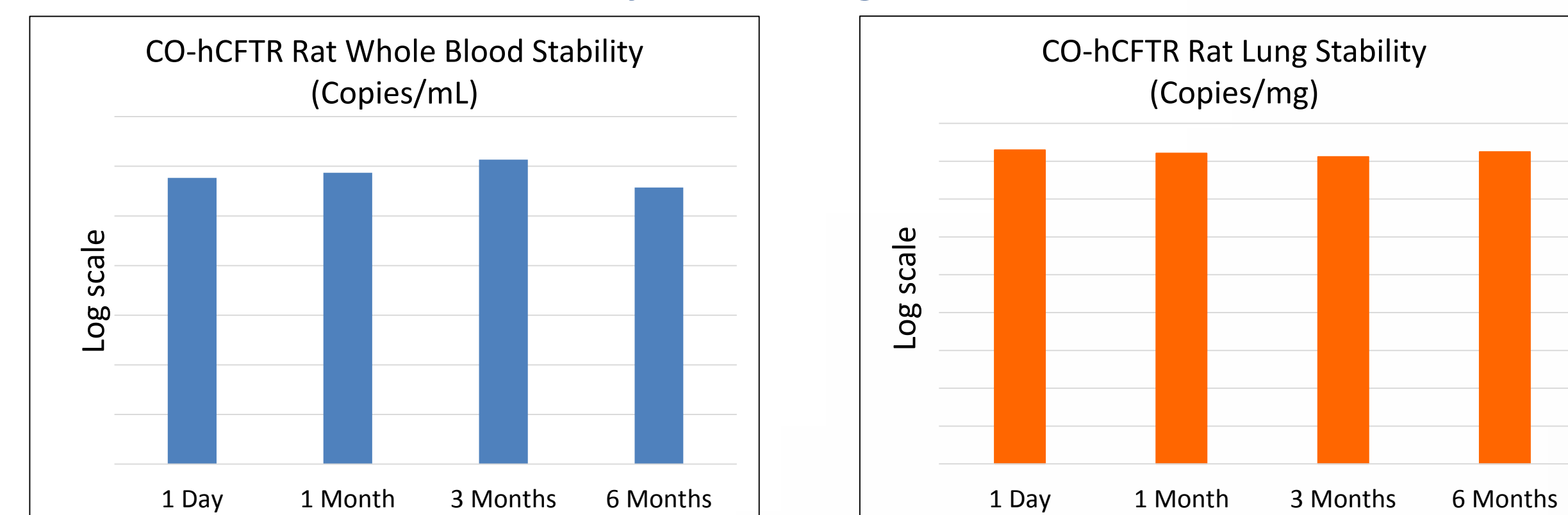
In addition, an RT-qPCR quantitation method was established for human endogenous CFTR mRNA. Assay specificity was verified and there was no cross-talk between the CO-hCFTR mRNA and endogenous hCFTR mRNA quantitation methods.

Table 4. Endogenous CFTR Assay Specificity against Human Lung Tissue

Species	Matrix Type	Sex	Donor No.	Average Mass of Tissue (mg) N=4	Average RNA Concentration (ng/ $\mu$ L)	A260/A280	Detection of Endogenous CFTR per mg N=4 (*)
Human	Lung	M	1	23.6	127.5	2.1	Detected
Human	Lung	M	2	24.2	50.1	2.0	Detected
Human	Lung	M	3	22.4	95.4	2.0	Detected
Human	Lung	M	4	22.8	60.8	2.0	Detected
Human	Lung	M	5	23.5	46.6	2.0	Detected

(\*) Detected = Detected in significant amounts within the assay range. Actual copies/mg excluded for confidentiality.

Figure 2. *in vivo* CO-hCFTR Stability in Rat Lung and Whole Blood



## Validation Results

Table 5. RT-qPCR Method Validation Acceptance Criteria

Parameter	Acceptance Criteria
Specificity	Confirmed by meeting acceptance criteria stated in System Suitability.
Linearity and Range of the Standard Curve	Slope: $\leq -3.1$ and $\geq -3.6$ and Correlation Coefficient: $ r  \geq 0.980$ (or $r^2 \geq 0.96$ ). Standards: Accuracy of valid wells will be $\leq 0.3$ for the $\log_{10}$ concentrations. The %CV of Precision will be $\leq 6.5$ % for the $C_T$ values. A minimum of 5 standards must be used for the standard curve.
Precision (Repeatability)	The %CV of Intra-Assay Variability $\log_{10}$ will be $\leq 20$ % for Precision Controls.
Intermediate Precision	The %CV of Inter-Analyst/Inter-Day $\log_{10}$ will be $\leq 20$ % for Precision Controls. The %CV of Inter-Reagent/Equipment $\log_{10}$ will be $\leq 20$ % for Precision Controls.
Accuracy	The $\log_{10}$ for the Precision Controls will be $\leq 0.50$ from the expected value.
Detection Limit (DL) and Above Detection Limit (ADL) Precision	Two of three sample wells must show amplification ( $C_T$ value $< 40$ ). Inter-Analyst/Inter-Day: $\geq 95$ % of DL and ADL samples must show amplification ( $C_T$ value $< 40$ ). Inter-Reagent/Equipment: $\geq 95$ % of DL and ADL samples must show amplification ( $C_T$ value $< 40$ ).
System Suitability	Two of three No Template Control (NTC) samples must have a $C_T$ value of Undetermined. Two of three NEG wells must have a $C_T$ value of Undetermined or have a $C_T$ greater than the $C_T$ . The DL must be greater than or equal to the average $C_T$ value for the ADL by a minimum of 2 $C_T$ s. A minimum of 5 standard levels of standard curve must remain after outlier removal.

Run acceptance is based upon Standard Curve and System Suitability performance.

Table 6. Validation Standard Curve Performance

Based on the Method Development results, the Detection Limit (DL) was estimated to be 25 copies, and a standard curve range from 25 to  $25 \times 10^6$  copies was evaluated.

Run	Slope	$r^2$	y-intercept	No. of Standards	NTC <sup>a</sup>	NEG <sup>a</sup>
3	-3.419	0.9994	39.44	7	---	---
2	-3.446	0.9990	39.67	7	---	---
1	-3.423	0.9993	39.74	7	---	---

<sup>a</sup> --- indicates 3 out of 3 wells had  $C_T$  value of "undetermined."

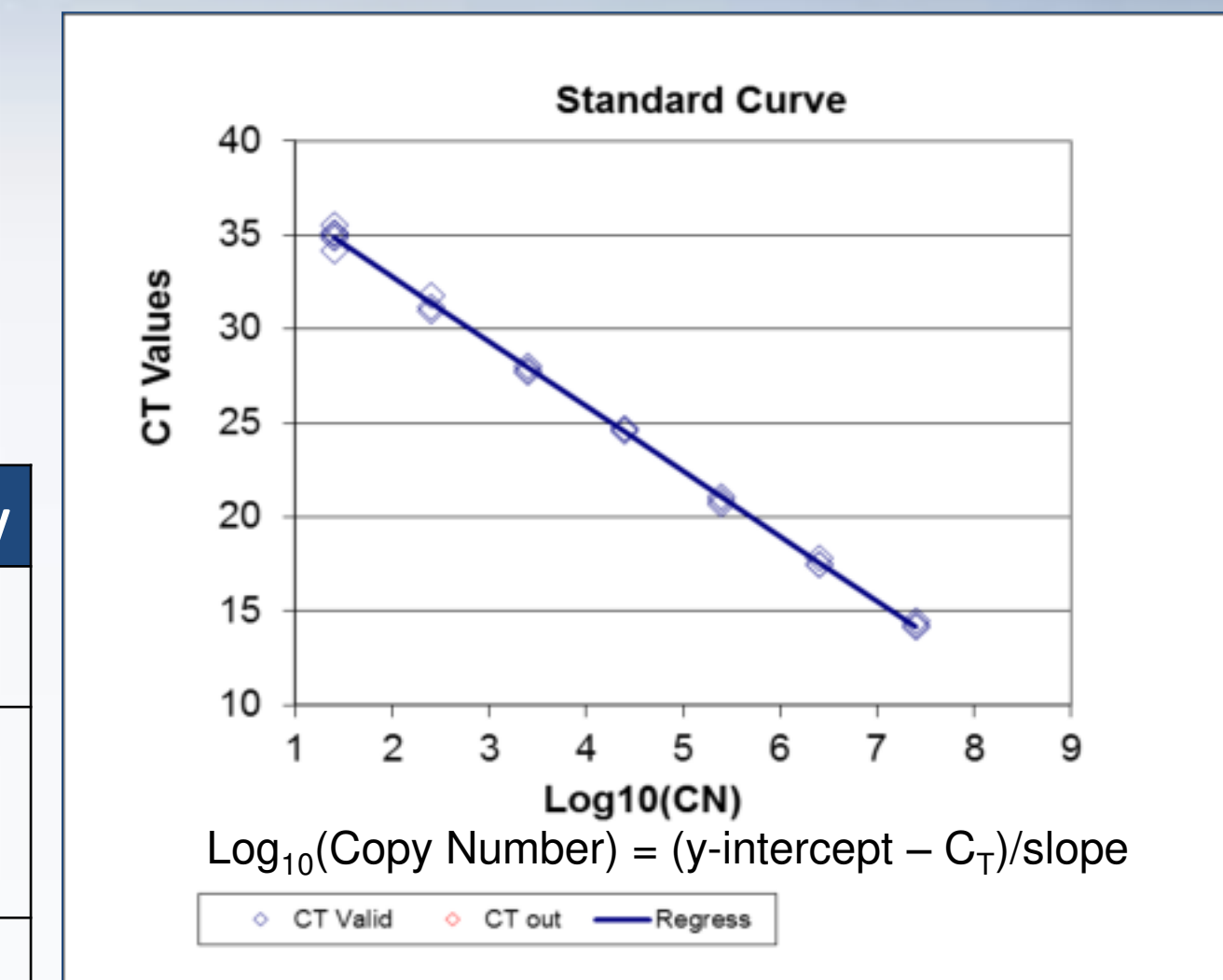


Table 7. Intermediate Precision: Inter-Reagent/Equipment and Inter-Day/Analyst

Precision Controls (PCs) of template mRNA prepared in yeast tRNA/TE at concentrations of  $5 \times 10^1$ ,  $5 \times 10^3$ , and  $5 \times 10^5$  copies/ $\mu$ L.

Inter-Reagent/Equipment Log (CN)				Inter-Analyst/Day Log (CN)			
Run	PC1 $5 \times 10^1$	PC2 $5 \times 10^3$	PC3 $5 \times 10^5$	Run	PC1 $5 \times 10^1$	PC2 $5 \times 10^3$	PC3 $5 \times 10^5$
2	1.8	3.8	5.8	1	1.8	3.7	5.8
2	1.7	3.8	5.8	1	1.8	3.7	5.8
2	1.8	3.8	5.8	1	1.8	3.8	5.7
3	1.5	3.5	5.7	2	1.8	3.8	5.8
3	1.5	3.5	5.7	2	1.7	3.8	5.8
3	1.5	3.6	5.7	2	1.8	3.8	5.8
%CV	9.2	4.1	1.0	%CV	2.3	1.4	0.7

Table 8. Accuracy of Quantitation

Run	PC1		PC2		PC3	
	$\log_{10}$ (CN)	Log <sub>10</sub> (CN) [Expected-Calculated]	$\log_{10}$ (CN)	Log <sub>10</sub> (CN) [Expected-Calculated]	$\log_{10}$ (CN)	Log <sub>10</sub> (CN) [Expected-Calculated]
3	1.5	0.5	3.5	0.5	5.7	0.3
	1.5	0.5	3.5	0.5	5.7	0.3
	1.5	0.5	3.6	0.4	5.7	0.3
2	1.8	0.2	3.8	0.2	5.8	0.2
	1.7	0.3	3.8	0.2	5.8	0.2
	1.8	0.2	3.8	0.2	5.8	0.2
1	1.8	0.2	3.7	0.3	5.8	0.2
	1.8	0.2	3.7	0.3	5.8	0.2
	1.8	0.2	3.8	0.2	5.7	0.3

Expected  $\log_{10}$  (CN) = 2.0    Expected  $\log_{10}$  (CN) = 4.0    Expected  $\log_{10}$  (CN) = 6.0

Expected  $\log_{10}$ (CN) calculations:  
PC1 is at  $1 \times 10^2$  CN (2  $\mu$ L at  $5 \times 10^1$  copies/ $\mu$ L).  $\log_{10}(1 \times 10^2) = 2.0$   
PC2 is at  $1 \times 10^4$  CN (2  $\mu$ L at  $5 \times 10^3$  copies/ $\mu$ L).  $\log_{10}(1 \times 10^4) = 4.0$   
PC3 is at  $1 \times 10^6$  CN (2  $\mu$ L at  $5 \times 10^5$  copies/ $\mu$ L).  $\log_{10}(1 \times 10^6) = 6.0$

## Conclusions

- An RT-qPCR assay method was designed, developed and validated for quantifying an mRNA therapeutic for cystic fibrosis.
- Method Validation Acceptance criteria was met for Specificity, Linearity and Range, Precision, Intermediate Precision, Accuracy, and System Suitability.
- Because specificity was demonstrated against endogenous total RNA (inclusive of endogenous CFTR mRNA) extracted from naïve human, rat, monkey, and mouse liver tissue, human lung, and stabilized whole blood, this assay may be used in any of these sample types.
- With test article stability established up to 6 months in stabilized whole blood and flash frozen tissue, this method may be used to measure CO-hCFTR in pre-clinical and clinical trials for toxicology, pharmacokinetics and biodistribution.