Matrices 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 x 10^8 copies) of the transcribed mRNA standard for each of the four designs. The primer/probe combination having the lowest baseline threshold (Ct), the highest ΔRn (change in fluorescence), and no detectable amplification (within 40 cycles) in the ΔRn was analyzed on the QuantStudio™ Real-Time PCR System (Thermo Fisher Scientific). A minimum of 5 standards must be used for the standard curve.

Validation Results

An initial standard curve of 10^1 to 10^9 copies was tested to determine detection and quantitation limits.

Validity 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.

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

RNA concentration and purity were determined using the Nanodrop 8000. Up to 1 µg of purified RNA was analyzed on the QuantStudio™ 7 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 against Human Lung Tissue

Table 4. Endogenous CFTR Assay Specificity against Human Lung Tissue

Table 5. RT-qPCR Method Validation Acceptance Criteria

Parameter | Acceptance Criteria
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Specificity | Conformed by meeting acceptance criteria stated in System Suitability
Linearity and Range of the Standard Curve | Slope: -1 ± 0.1 (r ≥ 0.99) and Correlation Coefficient: r ≥ 0.99 (r ≥ 0.90). Standards: Accuracy of validation will be ≥ 95.0 for the low end concentration. The CV% of Precision will be ≤ 10.0 for the high end concentration.
Intermediate Precision | %CV of between-day/within-run Log10(CN) = 5.9% for Precision Controls, %CV of between-run/within-day Log10(CN) = 20.0% for Precision Controls.
Assay | The log10 for the Precision Controls will be ≤ 0.50 from the expected value.
 Detection Limit (DL): The DL is defined as the lowest known concentration that can be detected with acceptable accuracy and precision using the RT-qPCR method. Inter-Assay/Inter-Day: A 95% C.I. of DL and ADL samples must show amplification (Ct > 40 cycles).

Conclusions

- An RT-qPCR assay method was designed, developed and validated for quantifying an mRNA therapeutic for cystic fibrosis.
- Method Validation Acceptance criteria were met for Specificity, Linearity and Range, Precision, Intermediate Precision, Accuracy, and System Suitability.
- Because specificity was demonstrated against endogenous total RNA (nullis of endogenous CFTR mRNA) extracted from naive human, rat, mouse and cynomolgus monkey liver tissue, human lung tissue, 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 frozen tissue, this method may be used to measure CO-hCFTR in pre-clinical and clinical trials for toxicology, pharmacokinetics and biodistribution.