Improved detection of expression from gene therapy plasmid pGM169 by 3-step TaqMan PCR

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Introduction
The UK Cystic Fibrosis Gene Therapy Consortium is currently conducting a Phase I clinical trial to evaluate the safety of aerosol delivery of a non-viral liposome formulation to the lungs of patients with Cystic Fibrosis (CF). Although the primary aim of the current clinical study is to evaluate safety, we are also taking the opportunity to measure gene expression in nasal and bronchial brushings.

When complexed with GL67A (a mixture of three lipids: GL67, DOPE and DMPE-PEG5000), plasmid DNA can be delivered to the lungs of mice and sheep via aerosol. The CpG-free plasmid pGM169 was selected for the clinic following demonstration of persistent, inflammation-free expression of human CFTR via the hCFI promoter in the mouse lung (Hyde et al. 2008, Nature Biotechnology 26:549).

To quantify transgene expression in clinical samples, traditional 2-step TaqMan RT-PCR is widely used as a reliable and sensitive method. Using this type of assay we are routinely capable of quantifying human CFTR mRNA and pGM169 mRNA down to 10 and 25 copies/µl total RNA respectively, typically reported as percentage of vector copies to endogenous copies detected.

Aim of this work
We wished to further improve the ability to detect vector-specific mRNA in clinical samples; thus we developed a 3-step TaqMan RT-PCR assay, which includes an additional nested PCR step. This approach was validated using RNA standards and two pre-clinical gene transfer models.

Assay design and methods
Our 2-step TaqMan pGM169 RNA assay is sensitive to a low copy number of mRNA molecules, has specificity for target mRNA and discriminates between DNA and RNA by the placement of the forward primer over the junction of two exons. The reverse primer used to make cDNA is placed downstream from the reverse primer used during the TaqMan PCR step:

We developed a 3-step TaqMan pGM169 RNA assay that uses the same primer set as the 2-step TaqMan assay, with an extra nested PCR step in between cDNA synthesis and TaqMan PCR:

Methods
Total RNA was prepared using AllPrep or RNaseFree columns (QIAGEN) with 2DNase steps and RNA concentration determined using RiboGreen Quant-iT™ (Invitrogen). 2-step and 3-step TaqMan PCR assays were performed using the ABI PRISM 7900 Sequence Detection System and Sequence Detection Software (Applied Biosystems), and Applied Biosystems TaqMan reagents. 20µl Reverse Transcription reactions were performed using 5µl total RNA and 15µl RT mastermix. 10µl TaqMan PCR was performed using 2µl cDNA + 8µl TaqMan mastermix. For 3-step reactions, 4µl cDNA (sheep samples) or 2µl cDNA (hALI samples) were pre-amplified by PCR in 20µl reactions; subsequently 2µl of the amplified product were analysed by TaqMan PCR as above. Reactions were performed in triplicate.

Proof of principle
The 3-step TaqMan assay resulted in improved sensitivity by facilitating detection of low concentration pGM169 mRNA standards that were previously undetectable by 2-step TaqMan RT-PCR. However, the addition of the nested PCR step resulted in the loss of the quantifiable aspects of qPCR.

Improved sensitivity in detection of pGM169 in sheep lungs
Subsequently we analysed RNA samples from outbred sheep dosed with 20ml cPEI/pGM169 (32mg pDNA). We deliberately chose 9 RNA samples with low (5/9 samples) or no detectable (4/9) levels of pGM169 mRNA. Encouragingly, all 5 samples containing low levels of pGM169 mRNA were more readily detected by 3-step than 2-step TaqMan. Importantly 1 of the 4 samples that contained no detectable pGM169 mRNA by 2-step TaqMan was found to contain detectable levels of pGM169 mRNA by 3-step TaqMan. Some amplification of pGM169 mRNA in 3-step TaqMan was noted in 4/9 naïve samples but at ~10³-fold lower levels (20 Ct).

3-step TaqMan PCR sensitively detects pGM169 RNA in human Air Liquid Interface cells (hALI)
Similarly, a comparison of the two assays was performed on RNA from hALIs (Epithelix, Switzerland) transfected with pGM169 (n=16) or untransfected (n=6). In the 2-step TaqMan assay 8 of the 16 pGM169-transfected samples had no detectable pGM169 mRNA. Of these, 5 had a strong positive pGM169 signal by 3-step TaqMan. Therefore the 3-step TaqMan assay showed detection of vector signal in 5 out of 8 samples that were previously determined as ‘negative’ by 2-step TaqMan.

Discussion
We have shown that the sensitivity of our clinical RT-qPCR assay can be increased by adding a nested PCR step on cDNA. This means weakly expressing samples that appear negative in 2-step TaqMan can be revealed in 3-step TaqMan. However this becomes a qualitative assay rather than quantitative, as the amplification of standards is not proportional to the input copy numbers and no reasonable standard curve can be obtained. To ensure that samples are not erroneously called positive by 3-step TaqMan PCR, negative controls are used to set a threshold below which samples cannot be called positive.

Conclusions
These results show improved detection of low concentrations of vector-specific mRNA in samples and indicate the suitability of this assay for measuring transgene expression in clinical samples, where sample availability is restricted.