DEVELOPMENT OF QUANTITATIVE TAQMAN RT-PCR FOR THE EVALUATION OF NON-VIRAL MEDIATED GENE TRANSFER TO THE AIRWAYS

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Overview.

- Gene therapy is being developed for the treatment of Cystic Fibrosis.
- Pre-clinical and clinical testing requires sensitive cellular and molecular assays.
- TaqMan RT PCR is widely used as clinical endpoint assay (Hyde *et al*., 2000).
- Absolute quantification of RT-PCR requires production of synthetic 'RNA mimics'.
- TaqMan assays have been designed for non-viral vectors and CFTR (Figure 1).
- DNA templates have been produced based on these mRNA sequences (Figure 2).
- *In-vitro* transcription used to produce RNA mimics (Figure 3).

Pre-clinical development of non-viral gene therapy for CF.

- A range of different GTAs are being tested in pre-clinical models.
- These models include:- Normal mouse nose and lung.
  CF mouse nose and lung.
  Sheep lung
- TaqMan RT-PCR assays developed to detect mRNA from different sources:-
  A range of different plasmid vectors
  Species specific CFTR mRNA

Figure 1. Design of pre-clinical TaqMan assays.

Design Overview.

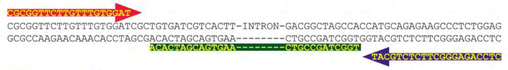
- TaqMan primers and probes were designed using Primer Express (Applied Biosystems).
- Discrimination between DNA and mRNA was achieved by spanning an intron with a primer or probe sequence.
- Probes were labelled with different fluorescent reporters to allow multiplex detection in the same reaction.

Assay 1. pCI mRNA detection.

Detects mRNA from any plasmid containing the CMVIE promoter and hybrid intron from Promga's pCI vector series.

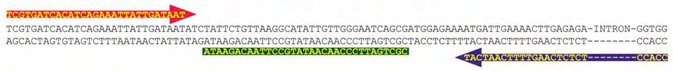
Assay 2. UbC mRNA detection.

Detects CFTR mRNA from plasmids containing the human Ubiquitin C promoter.



Assay 3. Murine CFTR mRNA detection.

Reverse primers spans the intron between exon 5 and exon 6 of murine CFTR.



Assay 4. Ovine CFTR mRNA detection.

Probe spans the intron between exon 6a and exon 6b of ovine CFTR.

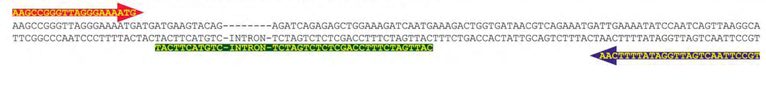
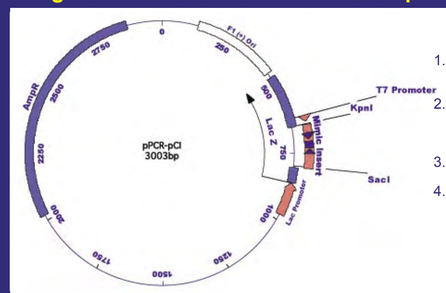
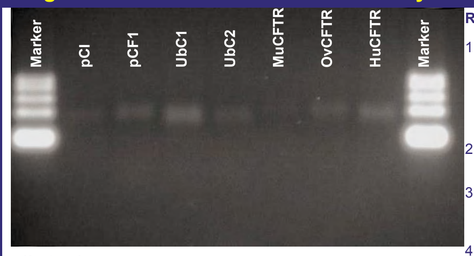


Figure 2. Construction of mimic templates.



1. DNA templates for TaqMan assays produced by overlapping PCR (GeneArt, Regensburg, Germany).
2. Fragments cloned into pCR-Script (Stratagene, Cambridge, UK) as *Kpn*I - *Sac*I fragments downstream of T7 promoter.
3. Plasmids linearised by digestion with *Sac*I.
4. Templates purified by phenol chloroform extraction and ethanol precipitation.

Figure 3. Production of RNA mimics by in-vitro transcription.



Key to gel.
Marker = dsRNA 500 bp, 400 bp, 300 bp, 200 bp & 100 bp.
pCI, pCF1, UbC1 & UbC2 = RNA mimic sequences for potential clinical trial vectors.
Mu, Ov & Hu CFTR = RNA mimics for mouse, sheep and human CFTR mRNA.

RNA Mimic Production Protocol.

1. 500 ng of *Sac*I linearised pDNA template was used as a template in in-vitro transcription reactions using the Ambion Competitor Construction kit (Ambion, Huntingdon, UK). Reactions were incubated at 37 °C for 18 hours.
2. 1 µl DNase was added and incubated at 37 °C for 1 hour.
3. Reactions were purified by acid phenol chloroform extraction (pH 4.5) (Ambion) followed by ethanol precipitation.
4. Mimic templates were resuspended in 300 µl nuclease free water (Promega, Southampton, UK).

Figure 4. Quantification of RNA mimics and creation of standard curve.

1. The MW of each RNA mimic was determined using the following formula:-

$$MW\ RNA\ mimic = \left((A_n \times 382.2) + (U_n \times 305.2) + (C_n \times 304.2) + (G_n \times 344.2)\right) + 159$$

2. The mass of 1 copy and the concentration required to yield 1 copy/µl can be derived as follows:-

$$Mass\ of\ 1\ copy = MW / 6.0223 \times 10^{23}$$

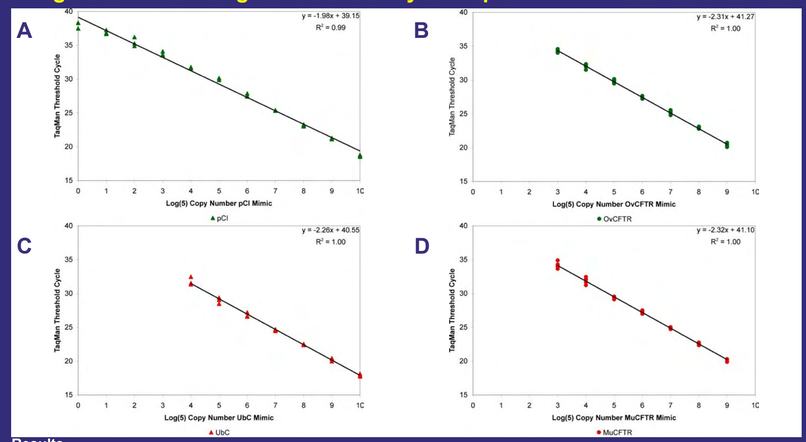
$$Mass\ of\ 1\ copy\ in\ 1\ \mu l = 1\ copy/\mu l$$

3. The RNA concentration was determined using the RiboGreen RNA Quantitation Kit (Invitrogen, Paisley, UK) and serial dilutions were made in 5 ng/µl yeast total RNA (Ambion) to create a 5 fold standard curve from 5^{12} to 5^{0}

Reverse Transcriptase Reaction.
(All reagents, Applied Biosystems, Warrington, UK)
Reverse transcriptase reactions were set using known starting quantities of RNA mimic and containing, 1 x PCR Buffer II, 5.5 mM MgCl2, 500 µM dNTPs, 1.25 U/µl MultiScribe Reverse Transcriptase, 0.4 U/µl RNase Inhibitor and 0.4 mM target -specific reverse primers to a final volume of 17.5 µl. Reactions without RT and RNase inhibitor were also set up as controls. Samples were incubated in a GeneAmp 9700 thermocycler at 48 °C for 30 min, 90 °C for 5 min and held at 4 °C until needed.

TaqMan Quantitative PCR.
5 µl of RT reaction mixture was added to each of 3-6 wells of a 96-well reaction plate containing 20 µl PCR mix, containing final concentrations of 1 x PCR MasterMix, and various optimal concentrations of primers and probes (Figure 1). PCR conditions were 50 °C 2 min, 95 °C 10 min followed by 40 cycles of 95 °C 15 sec 62 °C 1 min. Samples were analysed using the ABI Prism 7700 Sequence Detector and accompanying software.

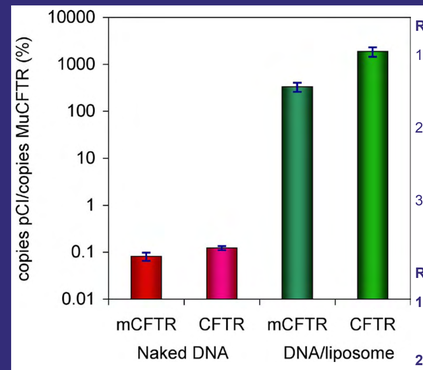
Figure 5. Linear range and sensitivity of TaqMan RT-PCR standard curves.



Results.

A. The pCI assay is sensitive in the linear range between 5^0 and 5^{10} copies per reaction.
B. The Ovine CFTR assay is sensitive in the linear range between 5^3 and 5^9 copies per reaction.
C. The UbC assay is sensitive in the linear range between 5^4 and 5^{10} copies per reaction.
D. The MuCFTR assay is sensitive in the linear range between 5^3 and 5^9 copies per reaction.

Figure 6. Detection of pCI specific mRNA in the murine lung.



Representative mouse lung data.

1. Plasmid DNA expressing murine and human CFTR under the control of the CMV promoter was delivered intransally to the mouse lung as either naked pDNA or pDNA/ liposome complex (n=6 per group).
2. Total RNA was extracted from the mouse lung 24 hours later and TaqMan RT-PCR was performed to detect the copy numbers of vector derived pCI mRNA and endogenous MuCFTR.
3. The number of pCI molecules detected was normalised against the number of MuCFTR molecules detected and expressed as a percetage of endogenous MuCFTR. Error = 1 SEM.

Results.

1. **Delivery of naked pDNA resulted in very low levels of pCI specific mRNA in the mouse lung (~0.1 % endogenous MuCFTR).**
2. **Delivery of pDNA liposome complexes resulted in up to 20 copies of vector derived mRNA per copy of endogenous MuCFTR at 24 hours post-dosing.**

Conclusions.

- Truly quantitative TaqMan RT-PCR assays have been developed.
- Copy number of endogenous and vector derived mRNA can be determined in a range of pre-clinical models.
- Increases the sensitivity of TaqMan RT-PCR for clinical end point assays.
- Direct comparison between different vectors is possible.

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