Using real-time (Taqman®) RT-PCR to measure gene expression – a core facility of the UK Cystic Fibrosis Gene Therapy Consortium

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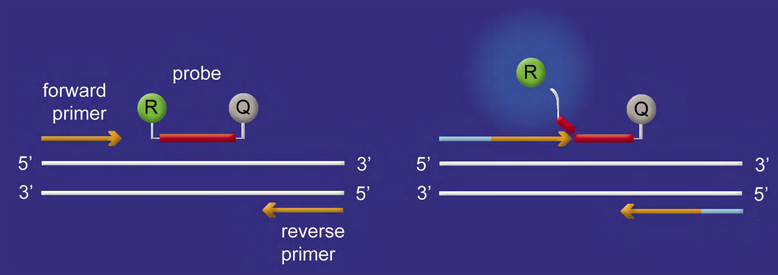
Introduction

- Gene therapy for cystic fibrosis has been predominantly aimed at correcting the chloride ion transport defect in the lung epithelia.
- Mouse and sheep lung models are used to assess the effectiveness of gene transfer vectors and to test delivery methods for use in the clinic.
- Testing in these animal models requires a sensitive, fast and accurate way of measuring gene transfer.
- Routine protein expression studies using reporter genes are of limited use due to the sensitivity required to detect low levels of expression that may still be therapeutic.
- At the John Radcliffe Hospital in Oxford, we have established a Core Testing Facility using the ABI 7700 Sequence Detector (Taqman®) to quantify vector delivery (DNA) and gene expression (mRNA).
- Measurement of gene transfer using the reverse transcriptase polymerase chain reaction (RT-PCR) in real time with the Taqman® can be performed in a range of pre-clinical and patient samples including blood, tissue biopsies, airway brushings and bronchial washings.

Figure 1. ABI 7700 Sequence Detector 'Taqman'

- Samples are collected in RNAlater® stabilisation solution (Ambion).
- Either DNA is extracted to measure vector delivery (using the Qiagen DNeasy® Tissue Kit)
- Or RNA is extracted to measure gene expression (using the Qiagen RNeasy® Mini Kit).
- Extracted nucleic acids are quantified using a spectrophotometer and diluted as appropriate.
- A reverse transcriptase step is performed to synthesize cDNA from sample RNA.
- The resultant cDNA is amplified using the Taqman® instrument shown in Figure 1.
- Each sample is analysed in triplicate.
- Controls include known positive samples at different levels, no template controls (NTC) and reactions containing no reverse transcriptase (RT-).

Figure 2. Taqman Chemistry



- Oligonucleotide primers bind to specific DNA sequences in the target gene, and probes dual-labelled with fluorogenic reporters and quenchers bind in between pairs of primer binding sites.
- DNA polymerase amplifies the fragments, detaching the probes. The reporter becomes separated from the quencher, allowing it to fluoresce.
- The instrument measures the fluorescence of each sample at every amplification cycle, detecting the change as more fragments are amplified.
- The difference between initial and final fluorescence (ΔRn) is calculated.
- A 'housekeeping' gene, typically the ribosomal RNA gene, is analysed at the same time as the transgene target, either by multiplexing or by assaying for the endogenous gene separately.
- Standards with known levels of both endogenous and target expression are used to calculate a relative expression or delivery level.

Figure 3. Raw data

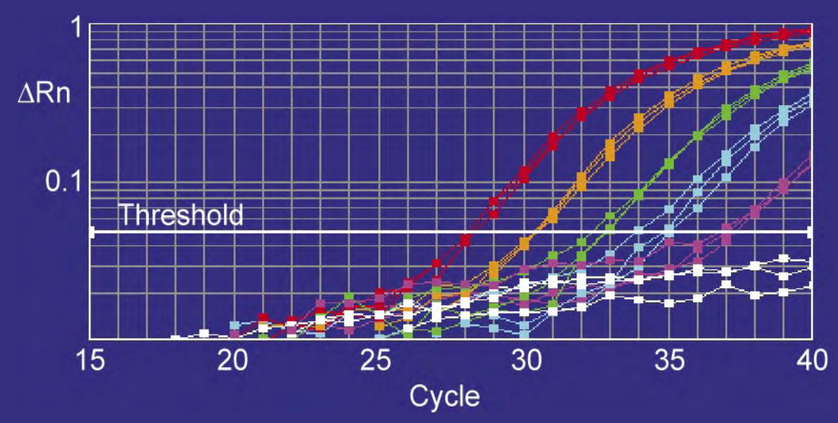
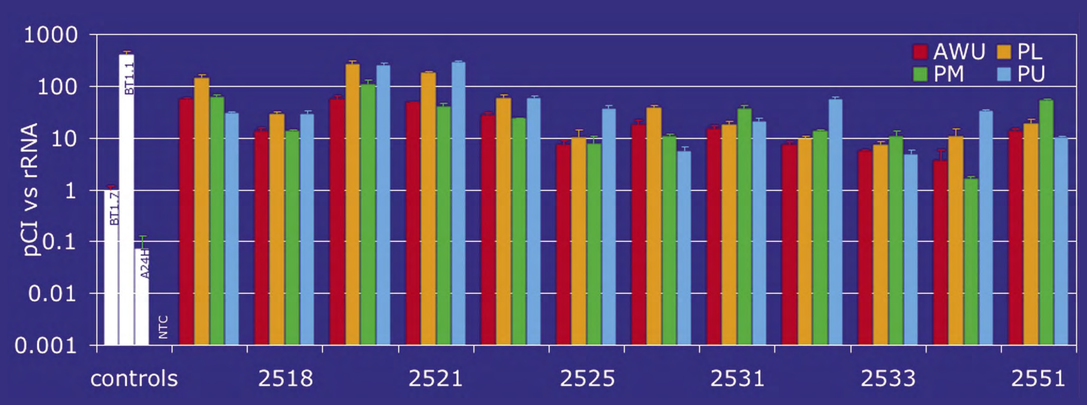


Figure 3 shows a graph of raw results from the Taqman® instrument of DNA at varying concentrations. Samples with higher levels of target sequence cross the threshold earlier than those with lower levels. The NTC negative controls, shown in white, never cross the threshold at all.

Figure 4. Final results



- Figure 4 shows tissue RNA samples from 4 different lung segments of each of 12 sheep dosed by aerosol with pCI plasmid vector (Promega). Samples were analysed for pCI and normalised to rRNA. Samples were taken by Gerry McLachlan at the Edinburgh site of the Consortium.
- Controls: BT1.7 is RNA from airway tissue dosed with 1 mg pCI vector; BT1.1 2 mg; A24H 0.1 mg; NTC is a no-template negative control.
- The sensitivity of the assay is 10^2 target RNA molecules in 100 ng of RNA, with a minimum of 25 ng total RNA or 10 pg DNA necessary for reliable detection.
- The assay is highly specific both between targets and between species.
- Intra-assay variability is typically less than 2%.
- The final results are presented as a graph for ease of interpretation and emailed to the sample sender along with spreadsheets of raw and analysed data.
- Turnaround time is usually less than 7 days, and can be as low as 36 hours.
- This is a highly efficient service enabling Consortium members throughout the UK to benefit from real-time vector delivery and expression analysis.

Conclusions

- Use of RT-PCR in real time is reliable and sensitive
- It can be used to measure both vector delivery (DNA) and gene expression (RNA)
- It is recommended as an end-point assay for clinical assessment of gene transfer

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