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.

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