**Introduction**

We have identified GL67A as the leading gene transfer agent for the next phase of the UK Cystic Fibrosis Gene Therapy consortium clinical programme. Our preclinical data in mice have shown sustained CFTR expression from pGM169, our CpG-free vector, when complexed with GL67A liposomes and delivered by aerosol. pGM169-derived CFTR expression in nasal and bronchial brushing samples, as measured by quantitative RT-PCR, is one of the primary outcome measures for our clinical programme. The aim of this study is to optimise the division of limited brushing samples between various molecular assays, and the handling and processing of the samples.

**Key concept - Residual sensitivity**

- The key limiting factors in successful quantitative RT-PCR are the concentration of the total RNA in the samples and absolute sensitivity of the assays used.
- We used residual sensitivity (RS - threshold level of % pGM169 vector-derived mRNA / endogenous CFTR mRNA (%VE), above which a given sample has to be, in order to be reported as positive and quantifiable by the assay) as an outcome measure in our process development.
- **IN A GIVEN SAMPLE, THE LOWER THE RS, THE MORE LIKELY IT IS THAT pGM169 VECTOR-DERIVED mRNA WILL BE DETECTABLE.**

**Initial assay performance**

- An initial sample validation study (SV1) was performed using nasal brushing (NBr), bronchial brushing (BBr) and bronchial biopsy (BBx) samples taken from CF (n=15-16) and non-CF (n=6-22) volunteers.
- Levels of total RNA and endogenous CFTR mRNA were measured and RS determined.
- For BBr and BBx, RS is lower in non-CF than CF samples. For NBr RS is similar in CF and non-CF.
- Importantly, endogenous CFTR mRNA detection was unreliable in BBx samples, thus we subsequently focused on BBr and NBr clinical sample collection.

**Sensitivity of RT-PCR assays**

- We have previously shown how primer combinations were optimised to increase the sensitivity of the pGM169 TaqMan assay to as few as 2.5 copies per µl RNA (input into cDNA synthesis reaction).
- Further optimisation of the pGM169 vector-derived and endogenous CFTR mRNA assays was performed. Briefly, incremental increases in RS were achieved by:
  - separating the assays rather than multiplexing them,
  - increasing the input of total RNA to cDNA synthesis reactions,
  - increasing the input of cDNA into TaqMan PCR reactions,
  - critically evaluating primer concentrations, enzymatic components and reaction conditions.
- Crucially this still enables us to carry out a 3-step (nested) TaqMan on remaining cDNA for pGM169 mRNA (for further details see Poster 271).

**Vector DNA delivery**

- In parallel we also optimised sample processing and handling to facilitate parallel detection of pGM169 DNA in brushing samples. We found the QIAGEN AllPrep method of simultaneous DNA and RNA purification to be the most reliable and high yielding of methods available to us.
- Compared to mRNA detection and quantification, determining levels of vector-derived and endogenous CFTR DNA is trivial in pre-clinical samples.

**Current assay performance**

- Applying our incremental advances in RNA yield from brushing samples and the performance of our TaqMan RT-PCR assays has resulted in a significant improvement in RS for both NBr and BBr types.
- In our most recent samples from CF patients (SV2), RS for NBr has reached 0.14% (as opposed to 0.4% in SV1), and for BBr, RS is now at 0.09% from 3.86% in SV1 (medians).

**Conclusions**

- We have fine-tuned processing and analysis procedures to maximise the probability of detecting and quantifying pGM169-derived CFTR expression in nasal and bronchial brushings from CF patients receiving our gene therapy formulation.
- This has led to the current sample handling cascade for the single dose clinical trial, as shown below:

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