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Rapid Quantitation of Gene Therapy Specific *CFTR* Expression Using the Amplification Refractory Mutation System

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ABSTRACT

Gene therapy offers the potential of correcting genetic disorders such as cystic fibrosis (CF). By complementing the non-functional endogenous cystic fibrosis transmembrane conductance regulator (*CFTR*) gene with a functional transgene, we anticipate it may alleviate the disease phenotype. All approaches to CF gene therapy rely upon sensitive assays to monitor delivery, expression and maintenance of *CFTR* vectors. Here, we describe the adaptation of the amplification refractory mutation system (ARMS) to discriminate between different forms of *CFTR*. A LightCycler® PCR machine allows real-time continuous fluorescence monitoring of rapid-cycle PCR. We show quantitation of and discrimination between expression of endogenous (wild-type or mutant) *CFTR* and the introduced transgene.

INTRODUCTION

Cystic fibrosis (CF) gene therapy is being pioneered in the lung because in addition to being the organ most severely affected by CF, it is directly accessible to aerosolized reagents. While showing promise, current vectors only show partial and transient correction of the CF phenotype (see Reference 1 and references therein). Therefore studies are aimed at improving the existing gene therapy protocols and designing new ones to overcome the physical and biological blocks to gene therapy (for review see Reference 3).

To facilitate bacterial cloning of cystic fibrosis transmembrane conductance regulator (*CFTR*), Gregory et al. (5) introduced a neutral mutation into the *CFTR* gene (936T→C) that silences

a cryptic bacterial promoter. This derivative of *CFTR* (936C) is used in most current CF gene therapy vectors, and thus the single-base substitution (936T→C) potentially allows discrimination between transgene and endogenous *CFTR*. By designing primers for the polymerase chain reaction (PCR) that will specifically amplify either the 936C or 936T forms of *CFTR*, an amplification refractory mutation system (ARMS) assay (11) (also known as allele-specific PCR; see References 4, 12, 13 and 15) can be used to discriminate these two species of template (see Figure 1). An attraction of such an assay is that it can be used on transfected samples from any source, expressing either wild-type or mutant *CFTR*, irrespective of the particular *CFTR* mutations present. We describe the development and use of an ARMS assay to distinguish endogenous (936T) from

transgene (936C) *CFTR* using a LightCycler™ Instrument (14), allowing rapid real-time quantitation of the specific reverse transcription (RT)-PCR products.

MATERIALS AND METHODS

Template DNA for the ARMS assay was prepared either by linearizing plasmids encoding *CFTR* cDNA or from RNA isolated from transiently transfected cultured cells, which had been DNase-treated (Boehringer Mannheim, East Sussex, UK) to remove contaminating gene therapy vector. cDNA synthesis was performed using a Random Primer cDNA Synthesis Kit (Boehringer Mannheim) following the manufacturer's instructions.

The ARMS assay consists of two separate amplification reactions for

Figure 1. The amplification refractory mutation system. (a) The primers used for the ARMS assay are shown along with a diagram of their hybridization positions on *CFTR* cDNA. ARMS-G and ARMS-A differ by only a single base at their 3' ends, which hybridizes to the 936 position of *CFTR* (ARMS-G identical to 936C and ARMS-A identical to 936T). S1U hybridized to both forms of *CFTR*. (b) The two *CFTR* cDNAs are represented along with their specific ARMS primers. ARMS-G specifically amplifies transgene (936C), whereas ARMS-A specifically amplifies endogenous (936T) *CFTR*.

Table 1. Quantitation of Endogenous and Transgene *CFTR* Using the ARMS Assay

Sample No.	No. of Transgene <i>CFTR</i> Template Molecules ^a Added	Assay's Predicted No. of Transgene <i>CFTR</i> Molecules ^b	Assay's Predicted No. of Endogenous <i>CFTR</i> Molecules ^c
1	10	5.9×10^2	2.7×10^4
2	10^2	1.6×10^3	2.7×10^4
3	10^3	9.5×10^2	2.9×10^4
4	10^4	1.1×10^4	2.8×10^4
5	10^5	1.7×10^5	2.6×10^4
6	10^6	8.8×10^5	3.3×10^4
7	10^7	3.3×10^7	2.2×10^4
8	10^9	1.2×10^9	3.7×10^6
Mean squared error ^d	-	1.38	1.83
A fixed amount of endogenous <i>CFTR</i> (936T) cDNA is seeded with the following:			
^a Known amounts of transgene <i>CFTR</i> (936C) and the samples analyzed in two separate reactions.			
^b The assay measures the amount of transgene present using the ARMS-G and S1U primers as described—the predicted amounts are shown.			
^c ARMS-A and S1U primers are used to quantify endogenous <i>CFTR</i> , and the predicted amounts are shown. Note that in Sample 8, the excess of transgene <i>CFTR</i> present (10^9 copies) causes an apparent increase in endogenous <i>CFTR</i> .			
^d The mean squared error (of concentration) from the standard curve is shown for each of the two reactions (see Figure 2, for details on standard curve and mean squared error).			

each sample: one specific for transgene, and the other specific for endogenous *CFTR*. Primers used were S1U, ARMS-G and ARMS-A [Figure 1; Genosys BioTechnologies (Europe) Ltd., Cambridge, England, UK]. One microliter of the cDNA template was mixed with 9 μ L of a mixture, producing final concentration of reagents as follows: 50–200 μ M dNTPs, 500 nM primers, 3 mM $MgCl_2$, 50 mM Tris-base (pH 8.3), 1 mg/mL bovine serum albumin (BSA), 0.1 U *Taq* DNA Polymerase and 1 in 10^4 dilution of SYBR® Green I (all reagents supplied by Bio-Gene, Kimbolton, Cambs, UK). Five microliters of the mixtures were then placed in a reaction capillary and amplified in a Light-Cycler (supplied by Bio-Gene for Idaho Technology, Idaho Falls, ID, USA).

Temperature profiles for amplification were optimized for each set of primers: (i) For ARMS-A, 95°C for 8 s followed by 60 $\times$ (94°C for 0 s, 56°C for 0 s and 74°C for 4 s). The temperature ramp rate was 20°C/s, except for the transition from 56° to 74°C, which was 6°C/s. (ii) For ARMS-G, the conditions are the same as for ARMS-A with the exception that the annealing temperature was 58° not 56°C, and the ramp rate from 58° to 74°C was 8°C/s. At the end of each extension step (74°C), the fluorescence of each sample was measured to allow quantitation of the PCR. After the PCR was complete, the products were subjected to a temperature gradient from 74° to 95°C at 0.2°C/s with continuous fluorescence monitoring to produce a melting profile of the

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products. Quantitative analysis was performed on the LightCycler data according to manufacturer's instructions; standard controls of known concentration were included in each run to produce a standard curve, allowing quantitation of templates. Identical template concentrations are detected amplifying exponentially at equivalent number of cycles in separate reactions (data not shown). However, to minimize errors, a standard curve was produced for each separate reaction to allow quantitation of the unknown samples.

RESULTS

The assay was used to quantitate either endogenous or transgene *CFTR* cDNA. cDNA samples from cells expressing endogenous human *CFTR* were quantified using primers S1U and ARMS-A. Figure 2 shows quantitation

of a cDNA sample of endogenous *CFTR* relative to known controls. The single unknown sample in this reaction was predicted to contain 2.1×10^6 copies of endogenous *CFTR* (mean squared error of concentration of 1.45). After the conclusion of the quantitative amplification reactions, the products were melted (as outlined in Materials and Methods). The melting is monitored with fluorescence, which is lost when the double-strand products melt apart. As different DNA products melt at different temperatures, distinguishing genuine products from primer dimers or nonspecific products is possible. The assay could distinguish as few as 10^2 – 10^3 copies of *CFTR*, below which nonspecific amplification of primer-dimers was seen (for example, see Table 1).

The main strength of this assay however is not in its sensitivity but in its abil-

ity to distinguish 936C (transgene) from 936T (endogenous) *CFTR*. Figure 3 shows an example of this. *CFTR* (10^5 copies) was amplified with primers (S1U and ARMS-G) specific for transgene *CFTR*. One sample contains 10^5 copies of 936C transgene *CFTR*, while the other had an identical concentration of 936T endogenous *CFTR* (two H_2O controls are included). Only the transgene *CFTR* is amplified, showing that the PCR is specific for transgene sequence.

The assay could be used to analyze a mixture of both endogenous and transgene *CFTR*, such as we might find in a cDNA sample from a clinical trial. To model this situation, samples of a fixed concentration of endogenous *CFTR* cDNA were seeded with known concentrations of transgene *CFTR*. The absolute quantities of both species were assayed with their respective specific

Figure 2. Quantitation of endogenous *CFTR*. A screen of the LightCycler quantitation software for six different amounts of endogenous *CFTR*; also included is a sample of unknown concentration. The primers used (ARMS-A and S1U) are specific for the endogenous *CFTR* (936T). The panels are labeled (from top left clockwise): 'Files', the particular reaction selected. 'Samples to Graph', samples are as follows: samples 1–6 contain various control quantities of endogenous *CFTR*, presented as absolute numbers of *CFTR* molecules; sample 1 = 10^9 , sample 2 = 10^8 , sample 3 = 10^7 , sample 4 = 10^6 , sample 5 = 10^5 and sample 6 = 10^4 ; sample 8 contains an unknown quantity of endogenous *CFTR* (sample 7 is not shown). 'Concentration', the predicted concentration based upon the standard curve (bottom left) for all samples, including those of known concentration; sample 8 is predicted to contain 2.1×10^6 copies of *CFTR*. The two graphs demonstrate the following: (i) 'Flo v. Cycle #' shows the SYBR Green I fluorescence (relative units) on the y-axis and PCR cycle number on the x-axis; and (ii) 'Log Flo vs. Cycle #' shows log fluorescence against PCR cycle number. 'Crossing Point vs. Log (Concentration)', the standard curve for this data with the six known controls; the actual data points are shown in red, and the standard curve is shown in green. The mean squared error of log (concentration) of the data vs. the standard curve is shown in red immediately right of the graph (in this case 0.16; hence, the mean squared concentration error is 1.45). The 'Temperature Chart', the temperature profile of the entire PCR.

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Figure 3. Distinguishing transgene from endogenous *CFTR*. This figure shows the 'Flo vs. Cycle #' section of the quantification software (compare with Figure 2). SYBR Green I fluorescence (in relative units) is plotted on the y-axis against PCR cycle number on the x-axis. The primers used in this reaction are ARMS-G and S1U; hence, this reaction is specific for the transgene (936C) *CFTR*. Four samples are shown: two are H₂O controls, and these seem to amplify (primer-dimer amplification) at approximately the same position as 10⁵ copies of endogenous *CFTR*; whereas 10⁵ copies of transgene amplify over 10 cycles earlier in the reaction. Thus the assay discriminates between transgene and endogenous *CFTR*.

primers. Table 1 shows the results of the assay relative to the known concentrations used. This demonstrates the ability of our assay to compare levels of cDNA in a mixture of the two species (endogenous and transgene).

DISCUSSION

Detection of *CFTR* transcripts using RT-PCR is a standard method for assessing the efficiency of gene therapy. In experiments using CF mouse models, the primers can be designed specifically for the human sequence, thereby eliminating problems associated with endogenous *CFTR*. In human cell lines or in clinical trials, endogenous and exogenous *CFTR* will be co-amplified, as the template *CFTR* sequences are identical. Mayall and Coutelle (10) exploited the presence of the simian virus 40 (SV40) small T-antigen intron in their gene

therapy vector to distinguish transgene signal from both DNA and endogenous message in RT-PCRs; however, such mechanisms limit vector choice and rely on the presumption of efficient splicing. The presence of the T→C base change, at position 936 in the cDNA, allows another way of discriminating between genomic- and vector-derived expression by virtue of the fortuitous creation of a *FokI* site in 936C *CFTR*. Hence, appropriate PCR products can be digested with *FokI*, and the resulting fragments quantified to determine the ratio of genomic-to-vector transcript. However, this is laborious and relies on full cleavage of the 936C product. A different restriction assay developed by Hart et al. (6), introduced two silent mutations into *CFTR* to allow restriction digests of PCR products for quantitation. Proof-reading PCR (2) could also be used to make the discrimination; although, variation in efficiency of polymerase proof-reading could compromise quantitation.

The ARMS assay makes use of the single base pair difference engineered into transgene *CFTR* cDNA (5) in a simple and direct way to prevent amplification of the nonspecific product. An excess of 10³ copies of one template species over the other could be detected, beyond which, mis-amplification of the nonspecific template was seen. This is in agreement with published mis-incorporation rates of *Taq* DNA polymerase (7,8). Fluorescent monitoring of PCR can be optimized to enable detection of a single-template molecule (9); although, the sensitivity of our assay is limited because of constraints in primer design and in our use of SYBR Green I for PCR product detection. However, it provides a quantitative assessment of transgene mRNA levels both in absolute terms and in comparison with the level of endogenous *CFTR* in the range and relative ratio likely to be encountered experimentally and in clinical trials. The analysis of 24 samples (including controls) takes less than 20 min, allowing a simple and rapid way to quantify *CFTR* cDNA from in vitro and in vivo experiments.

REFERENCES

1. Alton, E.W.F.W., D.M. Geddes, D.R. Gill, C.F. Higgins, S.C. Hyde, J.A. Innes and D.J. Porteous. 1998. Towards gene therapy for cystic fibrosis: a clinical progress report. *Gene Ther.* 5:291-292.
2. Bi, W. and P.J. Stambrook. 1998. Detection of known mutation by proof-reading PCR. *Nucleic Acids Res.* 26:3073-3075.
3. Davies, J.C., D.M. Geddes and E.W.F.W. Alton. 1998. Prospects for gene therapy for cystic fibrosis. *Mol. Med. Today* 4:292-299.
4. Ehlen, T. and L. Dubeau. 1989. Detection of ras point mutations by polymerase chain reaction using mutation specific, inosin-containing oligonucleotide primers. *Biochem. Biophys. Res. Commun.* 160:441-447.
5. Gregory, R.J., S.H. Cheng, D.P. Rich, J. Marshall, S. Paul, K. Hehir, L. Ostedgaard, K.W. Klinger, M.J. Welsh and A.E. Smith. 1990. Expression and characterization of the cystic fibrosis transmembrane regulator. *Nature* 347:382-386.
6. Hart, S.L., E. Mayall, M. Stern, F.M. Munkonge, A. Frost, L. Huang, M. Vasilou, R. Williamson, E.W.F.W. Alton and C. Coutelle. 1995. The introduction of two silent mutations into a *CFTR* cDNA construct allows improved detection of exogenous mRNA in gene transfer experiments. *Hum. Mol. Genet.* 4:1597-1602.
7. Huang, M.M., N. Arnheim and M.F. Goodman. 1992. Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acids Res.* 20:4567-4573.
8. Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson and J.J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type I model studies. *Nucleic Acids Res.* 18:999-1005.
9. Lockey, C., E. Otto and Z. Long. Real-time fluorescence detection of a single DNA molecule. *BioTechniques* 24:744-746.
10. Mayall, E.S. and C. Coutelle. 1997. RT-PCR method specific for the detection of transgenic *CFTR* mRNA in the presence of transgene plasmid DNA and endogenous *CFTR* mRNA. *Gene Ther.* 4:875-878.
11. Newton, C.R., A. Graham, L.E. Heptinstall, S.J. Powell, C. Summers, N. Kalsheker, J.C. Smith and A.F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* 17:2503-2516.
12. Okayama, H., D.T. Curiel, M.L. Brantly, M.D. Holmes and R.G. Crystal. 1989. Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J. Lab. Clin. Med.* 114:105-113.
13. Ugozzoli, L. and R.B. Wallace. 1991. Allele-specific polymerase chain reaction. *Methods: A Companion to Methods Enzymol.* 2:42-48.
14. Wittwer, C.T., K.M. Ririe, R.V. Andrew, D.A. David, R.A. Gundry and U.J. Balis. 1997. The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. *BioTechniques* 22:176-181.
15. Wu, D.Y., L. Ugozzoli, B.J. Pal and R.B. Wallace. 1989. Allele-specific enzymatic amplification of β -globin genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl. Acad. Sci. USA* 86:2757-2760.

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