Quantitative RT-PCR to Evaluate In Vivo Expression of Multiple Transgenes Using a Common Intron

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J. Fairman, L. Roche, I. Pieslak, M. Lay, S. Corson, E. Fox, C. Luong, G. Koe, B. Lemos, R. Grove, L. Fradkin and J. Vernachio
Valentis, Burlingame, CA, USA

INTRODUCTION

Many techniques have been developed for the quantitative analysis of DNA and RNA (7,9,10). However, most of these techniques lack the sensitivity and reproducibility for routine analysis. These procedures also require considerable time, sample size and extensive manipulation of the samples, increasing the possibility of contamination or degradation of the sample. Reverse transcription polymerase chain reaction (RT-PCR) has proven to be a powerful tool for quantitative RNA analysis (1,6), even in the case of very low copy number messages in a considerable background of endogenous RNA.

The TaqMan[®] methodology offers a high-throughput, sensitive and accurate PCR-based assay system that is useful for monitoring in vivo therapeutic gene delivery and expression. The system is based on the 5' exonuclease activity of the *Taq* DNA polymerase for detection of PCR products (3,4). The detection is accomplished by using a nonextendable oligonucleotide probe that hybridizes to the sequence undergoing amplification. The probe is labeled with a reporter dye [FAM (6-carboxy-fluorescein)] at the 5' end and a quencher dye [TAMRA (6-carboxy-tetramethyl-rhodamine)] at the 3' end. When the probe is intact, fluorescent resonance energy transfer to TAMRA quenches the FAM emission. During the extension phase of PCR, the 5' nuclease activity of the *Taq* DNA polymerase cleaves the hybridization probe at the 5' end and releases the reporter dye. The resulting relative increase in

fluorescence emission of reporter vs. quencher is monitored in real time during the PCR amplification. The PCR cycle at which the fluorescence attains a point above a user-determined threshold is defined as the threshold cycle (C_t). The C_t is inversely proportional to the log of the starting copy number in the PCR (4).

A major limitation in the analysis of RNA expression of transfected genes is the recognition of endogenous mRNA identical to the transgene target sequence. Traditionally, recognition of endogenous RNA is overcome by designing amplification primers such that one primer recognizes the vector backbone while the other is gene specific (5). Although this strategy allows for transgene specificity, a distinct primer set needs to be designed and tested for each gene of interest. We have devised a quantitative (q)RT-PCR method that allows us to monitor the expression of any transgene within a common expression vector without interference from endogenous sources. In the assay scheme, the PCR product is contained entirely within the 5' untranslated region (5' UTR) portion of the expression construct. Targeting the non-coding portion of the mRNA eliminates false signals due to endogenous RNA, while allowing universal recognition of any promoter/transgene combination cloned into the expression vector. In this study, we describe the development of this quantitative RT-PCR assay, illustrate its use in measuring transgene expression levels after systemic plasmid delivery in mice and compare this method vs. traditional methods of monitoring transgene

ABSTRACT

An assay measuring RNA expression levels of a gene-encoded therapeutic must distinguish between endogenous mRNA and mRNA transcribed from the transgene. Specificity for the delivered transgene is especially critical when the treatment involves genes that are expressed in the target tissue. To facilitate uniform detection of transgene RNA without interference from endogenous mRNA, we have engineered expression vectors that include a 5' untranslated region (5' UTR) containing a synthetic intron (PGL3). The synthetic intron splice junction was the target sequence for a quantitative reverse transcription (RT)-PCR assay utilizing TaqMan[®] technology. In this study, we demonstrate that a quantitative RT-PCR assay designed to recognize an engineered intron splice site in the 5' UTR of expression constructs effectively measures the expression level of in vivo-delivered gene therapeutics.

expression. We believe this method will be widely applicable for monitoring viral or nonviral therapeutic gene expression vectors *in vivo*.

MATERIALS AND METHODS

Formulation

For lipid DNA formulation MB134, the plasmid DNA was prepared in a 10 mM solution of Tris-HCl, pH 7.0 (Sigma), (2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride), pH 8.0 at 5.0 mg/mL. Liposomes were prepared by dissolving cholesterol (Sigma, St. Louis, MO, USA) and the cationic lipid, MBN275 (Valentis, Burlingame, CA, USA) in chloroform (EM Science, Gibbstown, NJ, USA) at equimolar concentrations in a 3-L round bottom flask. The clear solution was rotated on a Rotovapor R-134 (Büchi, Zurich, Switzerland) at 30°C for 30 min and hydrated in 5% wt/vol dextrose (McGraw, Irvine, CA, USA) to produce a nominal concentration of 40 mM. The liposome dispersion was extruded through successive filters (0.4, 0.2, 0.1 and 0.05 μ m) to obtain a vesicle size of approximately 100 nm as measured by a Nicomp C-370 Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). The stock DNA solution was diluted to 0.625 mg/mL with 5% wt/vol dextrose (D5W). DNA/liposome complexes were prepared by the addition of the DNA solution to an equivalent volume of liposomes at a total liposome concentration of 7.5 mM. For lipid/ DNA formulation MB127, the final DNA liposome complexes consist of a 1:6 ratio of DNA to cationic lipid (mg DNA to μ mol cationic lipid) and a DNA concentration of 312.5 μ g/mL DNA and lipid concentrations of 1.875 mM MBN275/1.875 mM cholesterol. Lipid/DNA formulation MB112 (DOTIM/cholesterol) was prepared as reported previously (8).

Expression Vectors for e-NOS, G-CSF and VEGF

The expression plasmid contains the cDNA for the gene of interest, the cytomegalovirus (CMV) promoter/enhancer region, the simian virus 40 (SV40) early poly(A) site, the cDNA

for the kanamycin resistance gene and the 5'UTR and PGL3 intron sequences.

Total RNA Isolation

Unless otherwise noted, reagents for this protocol are from the FastRNA™ Kit (Bio 101, Vista, CA, USA). Initial RNA pellets were obtained as directed by the manufacturer (Bio 101). The total RNA pellets were dissolved in 50 μ L diethyl pyrocarbonate (DEPC)-treated 50 mM Tris-HCl, pH 7.0, 10 mM MgCl₂ with 10 U RNase free DNase (Roche Molecular Biochemicals, Indianapolis, IN, USA). Samples were incubated for 1 h at 37°C. After incubation, 450 μ L of CRSR green was added and extracted with 500 μ L of chloroform. After centrifugation, the aqueous phase was removed, and 500 μ L of isopropanol were added. The solution was mixed briefly and centrifuged at 18 000 $\times$ g for 5 min to precipitate RNA. The resulting pellet was washed with 500 μ L of 70% ethanol and air-dried for 5–10 min. Samples of total RNA were resuspended in 50 μ L TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.001% DEPC).

Preparation of RNA Standard

The standard was prepared from a plasmid (pGEM-3Z; Promega, Madison, WI, USA) containing the PCR product of the analyte. A synthetic RNA transcript was prepared by *in vitro* transcription using either T7 or SP6 polymerase depending upon the orientation of the PCR product in the vector. Transcription was performed as described in the protocol supplied with the Riboprobe® *in vitro* Transcription Systems (Promega). Steps included linearization of the plasmid with a restriction enzyme downstream of the polymerase binding site and the cloned PCR product, purification of the DNA template by phenol:chloroform extraction, a large-scale transcription reaction using supplied components and removal of DNA template by addition of supplied DNase, followed by a phenol:chloroform extraction. Unincorporated nucleotides were removed by resin capture as described in the QIAGEN® Nucleotide Removal Kit (Qiagen, Valencia, CA, USA) using

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DEPC-distilled (d)H₂O for elution. The concentration in micrograms per milliliter of the purified, synthetic RNA standard was determined by the Ribo-green® RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) and converted to copy number/μL by using a conversion factor based on the molecular base composition of the transcript.

5'UTR Assay

Expression of the gene of interest was monitored by RT-PCR using the ABI PRISM® 7700 Sequence Detection System (PE Biosystems, Foster City, CA, USA). Amplification of all test samples was compared to amplification of an absolute standard curve of known copy number. The RNA copy number standard was diluted 1:10 in RNase-free TE from a stock solution of 1×10^5 copies/μL. Aliquots of 5 μL of each dilution yielded duplicate PCR standards with 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 and 5×10^1 copies of mRNA. All RT-PCRs were performed with an initial RT step at 48°C for 30 min followed by an initial denaturation step of 95°C for 10

min. PCR was performed for 40 cycles of (95°C for 15 s and 60°C for 1 min 30 s). Components for the RT-PCR are as follows: 8% glycerol (Sigma), 1× Taq-Man Buffer (PE Biosystems), 5.5 mM MgCl₂, 300 μM dATP, 300 μM dCTP, 300 μM dGTP, 300 μM dTTP, 1.25 U AmpliTaq Gold™ DNA Polymerase (PE Biosystems), 12.5 U MuLV (Life Technologies, Gaithersburg, MD, USA), 20 U RNase inhibitor (PE Biosystems), 150 nM PGL3 forward-1 5'-GCTAC-AGAAGTTGGTCGTGA-3', 150 nM PGL3 reverse-1 5'-GCGGCTGCTTAGAGCTGTA-3' (Operon Technologies, Alameda, CA, USA), 200 nM 5'UTR probe 6FAM-5'-TGGGCAGG-TGTCCACTCCCAGGTT-3'-TAMRA (PE Biosystems).

Actin Assay

Expression of murine actin was monitored by RT-PCR using the ABI PRISM 7700 Sequence Detection System. Amplification of all test samples were compared to the amplification of an absolute standard curve of known copy number. The actin RNA copy number standard,

RT-PCRs and components were as previously described except for: 150 nM mouse actin forward-2 5'-AGGTCA-TCACTATTGGCAACGA-3', 150 nM mouse actin reverse-2 5'-CACTTCATGATGGAATTGAATGTAGTT-3' (Operon Technologies), 200 nM actin probe 6FAM-5'-TGCCACAGGATTCCATACCCAAGAAGG-3'-TAMRA (PE Biosystems).

Animal Dosing Regimen

Mice (ICR; CD-1) were injected intravenously via the tail vein with 200 μL of lipid DNA formulations MB134, MB127 or MB112 with the expression vectors for e-NOS, G-CSF and VEGF, respectively. Lungs were harvested at the indicated time post-injection, and total RNA was prepared. Samples were analyzed in the 5'UTR and actin assay.

Northern Blot

Messenger RNA was prepared as directed by manufacturer (Cat. No. 200347-1; Stratagene, La Jolla, CA, USA). Equivalent amounts of mRNA (1

Figure 1. 5'UTR amplification scheme. Plasmid is transcribed into processed mRNA. A reverse transcriptase reaction produces a cDNA of the 5'UTR region. During the process of PCR, the FAM is cleaved from the probe by the 5' exonuclease activity of the *Taq* DNA polymerase. The probe spans the intron splice sites, with 7 bp on the 5' side and 15 bp on the 3' side.

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Table 1. Test for Cross-Reactivity of Plasmid DNA

Increase in Signal	Plasmid Copy No. Spiked into 5×10^2 Copies RNA Standard						
	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2
	100 000	10 000	10 000	60	10	2	1

Increasing amounts of plasmid DNA were spiked into 5×10^2 copies of the RNA standard for the 5'UTR assay.

μg) were loaded in each lane of an agarose gel. Transfer and northern blot were performed as directed by manufacturer of the northern blot kit (Cat. No. 1940; Ambion, Austin, TX, USA).

RESULTS

Assay Design

The primers were designed to flank the PGL-3 intron/exon splice junction. The probe spans this junction, with 9 bp on the 5' side of the splice site and 15 bp 3' of the intron/exon splice junction (Figure 1). The expression of the transgene was normalized against the expression of murine actin. Expression levels of the transgene and murine actin are determined by comparison with an absolute standard curve. The expression levels of the gene of interest in a

given sample are then normalized to the amount of actin mRNA and are reported as the number of target mRNA copies per 1000 copies of actin mRNA. The size of the target sequence necessitates the use of probe discrimination instead of primer discrimination. Placing the primer over the splice site would yield a product of approximately 65 bp, which is significantly below the recommended product size for this type of assay.

Specificity of the Assay

A source of concern is the detection of the plasmid DNA used to transfect the cells in vivo. This is a possible source of false positives in the assay because expression constructs are delivered in high copy number to selected organs. The signal from plasmid DNA should be minimized in the RT-PCR by

Table 2. Test of Treatments to Reduce Non-specific Signal Due to High Concentrations of Plasmid DNA

Treatment	Increase in Signal
No Treatment	120 000
<i>EagI</i>	120 000
64°C	40 000
DNAse	0

Figure 2. To determine the accuracy of the 5'UTR assay, known quantities of 5'UTR template were spiked into 100 and 200 ng of total RNA prepared from control mouse lung tissue. The number of copies was then measured by qRT-PCR.

Figure 3. Transgene expression in lung after intravenous administration of a lipid/DNA complex. Control animals were injected with a similar amount of 5% dextrose. (A) Time course of expression of G-CSF after dosing with 100 μg DNA. (B) Time course of expression of e-NOS after dosing with 92 μg DNA. (C) Time course of expression of VEGF after dosing with 94 μg DNA.

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Table 3. 5'UTR Assay Variability

Copies of RNA	Mean Copy No.	Standard Deviation	Coefficient of Variance	Median Copy No.	MAD ^a
5e5	520 000	96 000	0.19	510 000	56 000
5e4	52 000	9600	0.18	51 000	6200
5e3	5000	1500	0.30	4700	860
5e2	500	200	0.39	460	120
5e1	63	26	0.42	58	16
(n = 488)					
^a median absolute deviation					

Table 4. Actin Assay Variability

Copies of RNA	Mean Copy No.	Standard Deviation	Coefficient of Variance	Median Copy No.	MAD ^a
5e5	480 000	81 000	0.17	480 000	40 000
5e4	54 000	11 000	0.20	54 000	6700
5e3	5300	1100	0.20	5200	670
5e2	520	120	0.24	510	80
5e1	48	16	0.32	47	8
(n = 276)					
^a median absolute deviation					

the targeting of a sequence that spans an intron splice junction (Figure 1). To test this assumption, 5×10^2 to 5×10^8 copies of plasmid DNA were spiked into 5×10^2 copies of sequence-specific RNA. The magnitude increase in signal was from $0-1 \times 10^5$ (Table 1). The increase in signal due to the plasmid DNA was unexpected, given the assay design presented in Figure 1.

Subsequently, we examined techniques to remove contaminating DNA from the total RNA samples. We investigated three different methods to achieve this: (i) digestion with *EagI*, (ii) increasing the annealing temperature from 60° to 64°C, and (iii) digestion with DNase. Table 2 shows the result of using each of these three strategies. Digestion with *EagI* restriction enzyme did not decrease the plasmid-dependent background. Increasing the annealing temperature to 64°C decreased the non-specific signal by more than 50%. Treatment of the sample with DNase eliminated the nonspecific signal.

With these findings, we chose to treat our samples with DNase I to eliminate false positives due to DNA contamination.

As an added precaution, every sample is tested in a reaction that omits reverse transcriptase to confirm the complete degradation of any contaminating plasmid DNA. The presence of a reverse transcriptase signal will allow us to identify any contamination problem.

Accuracy of the Assay

To evaluate the possibility of decreased efficiency of the PCR in a more complex RNA sample, total RNA samples were spiked with known amounts of target RNA. As shown in Figure 2, the assay values correlated with the known amount of target RNA. The results indicate that in the presence of nonspecific RNA (up to 200 ng), the matrix had no effect on the quantitative range of the assay, and that the assay was accurate.

Precision and Reproducibility of the Assay

The precision of the 5'UTR and actin assays were assessed with multiple standard curves. Over 244 replicate standard curves for the 5'UTR assay

Table 5. Expression Levels of G-CSF mRNA by qRT-PCR in Samples Analyzed by Northern Hybridization (Figure 4)

Hours Post-Injection	5'UTR/1 k Actin
6	560
6	600
6	430
6	1000
6	1200
6	820
12	2000
12	1300
12	1100
12	1500
12	1300
12	910
24	350
24	1000
24	570
24	950
24	580
24	950
48	2.5
48	13
48	13
48	10
48	6
48	4.2
D5W	0
D5W	0
D5W	0

and 138 for the actin assay were performed. These replicates were performed on two ABI 7700 Sequence Detection Units using multiple lots of reagents and multiple operators over a twelve-month period. Tables 3 and 4 show the variability of the 5'UTR and actin assays. The variability was calculated by using the standard curve and calculating the value for each individual point along the curve. As would be

expected, the variability for both assays increased as the amount of analyte decreased. The coefficient of variation (standard deviation divided by mean) showed similar trends in both the 5'UTR and actin assays, with the actin being less variable at the lower levels of quantitation. The actin assay was not regulated by the administration of DNA/lipid complexes (data not shown).

Quantifying Transgene Expression In Vivo

In vivo transfections with three plasmids containing different cDNAs were used to illustrate the utility of the 5'UTR assay. All three plasmids contained the PGL3 intron in the 5'UTR region that is targeted in the quantitative RT-PCR assay. Animals were in-

jected intravenously with cationic lipid/DNA formulations, and lung tissue was harvested at different times post-injection. Total RNA prepared from the tissues was analyzed for both the target transcript and murine actin. As shown, transcripts from all three expression constructs could be detected (Figure 3, A–C). The data demonstrates that the assay has sufficient sensitivity and specificity to detect time-dependent changes in expression following in vivo transfection.

Comparison to Traditional Northern Blot Using In Vivo Samples

To further qualify the qRT-PCR technique for use in vivo, traditional northern hybridization techniques were used to quantitate expression levels in a

Figure 4. Northern blot to determine transgene expression. One microgram of poly(A)⁺ RNA from each sample was analyzed. Probe sequences were complimentary to the human G-CSF coding region of the expression vector.

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number of samples and compared with qRT-PCR (Figure 4 and Table 5). The qualitative measurement obtained by northern hybridization confirmed the values obtained by qRT-PCR.

DISCUSSION

There are few alternatives to quantitatively measure expression of gene-based therapeutics in vivo. One option to ensure recognition of only the transgene is to design amplification primers that recognize a combination of the vector and gene of interest (5). A significant amount of time would be required to develop and characterize assays that are specific to each gene of interest. An alternative is to design primers from within the coding region, and assume that the expression of the endogenous gene is constant or nonexistent (3). The disadvantage of this approach is that the administration of

cationic lipid/DNA complex might regulate cytokine expression in vivo (2). This may not be an advantage in certain therapeutic applications, such as autoimmunity and inflammation.

We chose to use a synthetic sequence in the 5'UTR of the expression construct that does not interfere with protein expression or function. This sequence was used to design PCR primers and a probe that can be used with TaqMan methodology to quantitatively measure RNA levels. The region was chosen because it is outside of the cloning regions for the promoter and gene of interest, allowing a single assay to be applicable to any expression construct with the target sequence. The assay evaluates expression without the modification of the coding sequence, and yet retains the ability to distinguish between endogenous and exogenous gene expression. Because this quantitative RT-PCR assay system is designed using a common platform, it eliminates the need for assay development in the evaluation of different gene products or promoters. Furthermore, one assay validation package can be used for multiple regulatory submissions.

To demonstrate that the assay detects levels of expression after in vivo transfection, experiments were conducted to monitor the kinetics of expression of three different transgenes. Changes in expression over time could be monitored with the assay. We have also demonstrated that the assay is consistent with a traditional method of determination of transgene expression, the northern blot.

The use of such a universal assay system would minimize the chance of systematic error that could be an issue in the comparison of expression, using gene-specific assay systems. We believe that assays such as this will assist in the identification and characterization of plasmid delivery systems and their ability to target specific tissues. This assay is currently being used to correlate mRNA expression with preclinical efficacy in multiple animal models.

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Address correspondence to:

Isabella Pieslak
Valentis, Inc.
863A Mitten Road
Burlingame, CA 94101, USA
Internet: ipieslak@valentris.com

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