

# A TaqMan<sup>®</sup> Reverse Transcription Polymerase Chain Reaction (RT-PCR) In Vitro Potency Assay for Plasmid-based Vaccine Products

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**Abstract** A TaqMan<sup>®</sup>-based reverse transcription polymerase chain reaction (RT-PCR) assay has been developed as an in vitro potency assay to measure the most immediate biological activity of plasmid DNA (pDNA)-based products. The assay measures transgene-specific messenger RNA (mRNA) from cultured cells transfected with VCL-CB01, a bivalent pDNA-based human cytomegalovirus (CMV) vaccine. The forward and reverse primers have been designed to make the RT-PCR reaction selective for plasmid-derived mRNA and to allow discrimination of expression levels of individual plasmids in a multivalent pDNA vaccine. The relative potency of a vaccine lot is assessed by transfecting reference and test samples into cultured cells in parallel and analyzing total RNA from the cells by RT-PCR. Statistical analysis of dose response data from reference material supports a parallel-line model for calculating relative potency. Preliminary data demonstrate the ability of this assay to distinguish product potencies at 50, 75, 150, and 200% of the reference material. In addition, forced degradation of pDNA demonstrates that a decrease in relative potency as measured by the RT-PCR assay in vitro correlates well with a decrease in CMV DNA vaccine-mediated humoral immune responses in mice injected with the same material.

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## Introduction

DNA-based vaccines present a new paradigm in the field of vaccine development. Unlike traditional vaccines which deliver target antigens directly (in the form of either purified or recombinant proteins or as part of attenuated or inactivated pathogens), DNA-based vaccines deliver the genes encoding the immunogens. Generation of a protective immune response is dependent on cellular uptake and expression of the delivered genes preceding the immunologic presentation of target antigens. In this context, DNA-based products, such as plasmid DNA (pDNA) vaccines, can be viewed as pre-biologics. This distinction between the product and the intended biological response presents a unique challenge in measurement of the potency of DNA-based drug vaccines.

Potency is described in Title 21 of the Code of Federal Regulations (CFR) Part 600, Subpart 3, as the specific ability or capacity of the product to elicit a given result (21 CFR 600.3). Traditional potency assays for vaccines with preformed immunogens include in vivo demonstration of a prospectively determined level of immunogenicity to ensure product consistency. Unlike traditional vaccines, the most immediate result of pDNA vaccine administration is the expression of gene-specific messenger RNA (mRNA). Gene expression is followed by intracellular production, and then intra- or extra-cellular trafficking of the immunogens, which, in turn, induce the desired immune responses [1–3]. While immunogenicity of a vaccine product can only be measured in an immune competent animal model, transcriptional potency of pDNA is largely a

function of basic cellular machinery and is likely to be highly similar in transfected cultured cells *in vitro* as in cells of injected tissue *in vivo*. Therefore, measurement of gene-specific mRNA levels may be a reasonable *in vitro* surrogate for measurement of immune responses in determining pDNA vaccine potency for lot release, and for assessment of product stability and lot-to-lot consistency. Consequently, we believe that development of potency assays for pre-biologics such as pDNA vaccines should focus on *in vitro* assays particularly since *in vitro* responses are less variable than *in vivo* responses, tend to have a greater dynamic range, are more amenable to high-throughput formats, and can provide meaningful information about product consistency in a much shorter time frame.

We have developed an *in vitro* TaqMan<sup>®</sup>-based reverse transcription polymerase chain reaction (RT-PCR) potency assay designed to measure the level of mRNA expressed from a clinical lot of vaccine upon transfection of cultured cells relative to mRNA levels expressed from a reference standard lot. The well-established sensitivity, specificity, precision, accuracy and reproducibility of real time PCR technology allow an accurate measurement of the most immediate functional product of a pDNA vaccine, mRNA [4–6]. The versatility of this approach allows analysis of mRNA expression of individual genes encoded in a multivalent pDNA vaccine.

Quantitation of infectious potency of many live virus vaccines is currently determined using traditional infectivity assays (i.e., plaque or TCID<sub>50</sub> assays) in cultured cells [7–9]. To overcome some of the limitations of these types of assays, researchers have begun to apply more sensitive and selective approaches using quantitative polymerase chain reaction (PCR) assays to measure vaccine potency. While the increased sensitivity of PCR-based assays allows for a more rapid determination of viral efficacy, the specificity of the PCR reagents allows for the determination of infectivity of individual components of a multivalent viral vaccine. Viral titers of new lots of product have been determined either by PCR measurement of total viral DNA in infected tissue culture cells [10, 11] or by quantitation of virus-specific RNA produced upon infection [12–16]. The development of these new methods of determining vaccine potency is supported by the correlation of results with those obtained using traditional methods.

As pDNA-based vaccine products continue to show promise in human clinical trials [17, 18], regulatory agencies appear to be responsive to evolving regulatory approaches towards rationally designed assays to measure vaccine potency. Contrary to the old paradigm of potency as the most important characteristic to ensure human efficacy of new vaccine lots, the current regulatory approach acknowledges vaccine potency as one of several

parameters available to “ensure that a manufacturing process yields immunobiologics of quality consistent with that of lots proven efficacious.”<sup>1</sup>

Vical is currently conducting a Phase 2 clinical trial evaluating the safety and efficacy of a pDNA-based CMV immunotherapeutic vaccine (VCL-CB01) in donors and recipients undergoing hematopoietic cell transfer (HCT). The purpose of this trial is to evaluate incidence rates of CMV infection, disease, and other complications from immunosuppression and/or transplantation in recipients following administration of the vaccine to donors prior to peripheral blood stem cell (PBSC) donation and to CMV-seropositive recipients just before and after transplantation. VCL-CB01 is a bivalent, poloxamer-formulated pDNA vaccine composed of two plasmids (VCL-6365 and VCL-6368 at 1:1 mass ratio) expressing CMV antigens [19]. VCL-6365 encodes a secreted form of the CMV glycoprotein B (gB), while VCL-6368 encodes a modified form of the CMV tegument phosphoprotein 65 (pp65).

A key component of any product development plan is a potency assay to release clinical product and monitor long-term stability and lot-to-lot consistency. Presented are data describing the development of the *in vitro* RT-PCR-based relative potency assay currently being used for lot release of the VCL-CB01 vaccine. The specificity, selectivity, reproducibility, dose-response range, and accuracy of the assay are described. Statistical analysis of RT-PCR data obtained from multiple transfections of the assay reference was used to develop a parallel-line model for assessing relative potency of a test sample when analyzed in parallel with a reference standard. Statistical analysis further defined the assay parameters including dose range, suitability criteria, and replicate number necessary for employing the assay to accurately measure potency. A preliminary study using heat-degraded VCL-CB01 samples of varying potencies to demonstrate correlation between *in vitro* measured potency and vaccine immunogenicity in mice further supports the use of the *in vitro* RT-PCR assay as a valid measure of vaccine potency.

## Materials and Methods

### VCL-CB01

VCL-CB01 comprises two plasmids (VCL-6365 and VCL-6368 at 1:1 mass ratio) expressing CMV antigens. VCL-6365 encodes a secreted form of the CMV gB protein,

<sup>1</sup> Bruce D. Meade and Juan L. Arciniega, “Assays and laboratory markers of immunological importance” Laboratory of Methods Development and Quality Control, Office of Vaccines Research and Review, CBER, FDA, February 2001 ([www.fda.gov/ohrms/dockets/ac/06/slides/2006-4205S1\\_3.ppt](http://www.fda.gov/ohrms/dockets/ac/06/slides/2006-4205S1_3.ppt)).

while VCL-6368 encodes a modified form of CMV pp65. Both antigens are cloned into Vical's mammalian expression plasmid, VR10551, which contains the CMV IE promoter, CMV intron A, and a synthetic rabbit beta-globulin (RBG) consensus poly A signal. Processing of the transcript by the precise removal of the intron to generate transgene-specific mRNA is a critical step in the effective expression of the antigens. Cloning of plasmids and preparation of poloxamer formulation is described in detail elsewhere [19].

Single-use aliquots of unformulated individual bulk pDNA as well as formulated final product were prepared and stored at  $-70^{\circ}\text{C}$  for use as reference material in our assays.

### Cell Transfection and RNA Isolation

RNA expression analysis was set up using transient transfection of a murine melanoma cell line (VM92). Cells were seeded in 24-well plates at  $4 \times 10^4$  cells/well 24 h prior to transfection. When cells reached a confluency of approximately 70%, monolayers were incubated with 250  $\mu\text{l}$  of VCL-CB01 complexed with a transfection reagent (DMRIE/DOPE) following standard transfection procedures [20]. In most cases, duplicate transfections of each dose were carried out.

Twenty-four hours post-transfection cells were lysed and total RNA isolated from individual wells using the QIA-Shredder and RNeasy Mini Kits (Qiagen, Inc., Valencia, CA). RNA was isolated following the manufacturer's instructions. Total RNA was eluted in approximately 50  $\mu\text{l}$  of RNase and DNase-free water (Invitrogen, Carlsbad, CA) and nucleic acid concentration was determined spectrophotometrically ( $A_{260}$ ). Ten nanograms of total RNA from each transfection well were used as a template for a TaqMan<sup>®</sup>-based one-step RT-PCR assay as described below.

### Primer and Probe Design, Optimization, and RT-PCR

Primers were designed to take advantage of the presence of the intron in the expression cassette to make the assay specific for the transgene mRNA. The DNA sequence for the 5' forward primer was 5'-CCG TGC CAA CAC TGA CTC ACC-3'. The forward primer spans the intron region, with 16 bases homologous to sequences upstream of the intron and five bases that bind to sequences downstream of the intron splice site. The primer is able to initiate amplification only if the message has been correctly processed and the intron spliced out.

A forward primer specific for a site within the intron was designed to assay for the presence of pDNA in the RNA samples (5'-GCG GTG CTG TTA ACG GTG GAG-3').

The reverse primer and probe were designed by using Primer Express Software 2.0 (Applied Biosystems, Foster

City, CA) according to the manufacturer's recommendations. The software was used to identify a reverse primer and TaqMan<sup>®</sup> probe compatible with the forward primer. The size limit of the desired amplicon was increased from the default setting to 650 bp, to accommodate the distance of the intron splice sites from the gene-specific regions of the CMV plasmids. The DNA sequences for the 3' gB and pp65 reverse primers were GCA GAC TAC CAG GCA CCA GAT C and GGG TAT CGC CGC GAC TAA A, respectively. The DNA sequence for the TaqMan FAM-labeled probe was FAM-ATA TCG AAT TCC GTA CGA CGC GTG GTT-TAMRA. The probe hybridizes to a common expressed region of both plasmids, while the reverse primer provides the specificity needed to distinguish gB from pp65 messages in a mixed transfection. These primers resulted in amplicons of 119 bp for gB and 184 bp for pp65. The reverse primer served an additional function as a cDNA primer during the reverse transcription step in the one-step RT-PCR reaction.

RT-PCR reactions were carried out using the TaqMan<sup>®</sup> One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA). Primer and probe concentrations were optimized according to the manufacturer's recommendations. The combination of conditions of 300 nM of forward primer, 900 nM of reverse primer and 100 nM of probe resulted in the highest  $\Delta\text{Rn}$  and the lowest Ct values in the optimization runs (data not shown).

The RT-PCR reaction was set up using 50- $\mu\text{l}$  reactions with three replicates of 10 ng of total RNA each in a 96-well optical plate and assayed in an Applied Biosystems 7900HT instrument (Applied Biosystems, Foster City, CA). As per the manufacturer's recommendations, a three-step thermal cycling program was run: (1) 30-min soak at  $48^{\circ}\text{C}$  for the reverse transcription; (2) 10-min soak at  $95^{\circ}\text{C}$  to inactivate the reverse transcriptase and activate the Taq polymerase; (3) a 40-cycle step of alternating  $95^{\circ}\text{C}$  strand melting (15 s) and  $60^{\circ}\text{C}$  annealing and extension for amplification (60 s). The fluorescent signal detected for each well was analyzed by Applied Biosystems' Sequence Detection Software (SDS), and the mean Ct of the three replicates was determined for each sample. The Ct values of reference and test samples were plotted against pDNA transfection dose and the curves compared to determine equivalence of expression.

### Heat-stressed VCL-CB01 Samples

One milliliter of VCL-CB01 at 0.2 mg/ml pDNA was aseptically aliquoted into glass vials. Vials were placed in a Hybaid oven set at  $80^{\circ}\text{C}$  (ambient air) for 0, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, and 87 h. Vials were removed from the oven and placed at room temperature for 0.5 h, transferred to ice for 0.5 h, then frozen at

–70°C. Vials were then thawed and thermally cycled four times between room temperature and 4°C to ensure that physical formulation characteristics were similar to pre-treatment conditions.

Aliquots of samples from each thermal degradation time-point were tested by agarose gel electrophoresis. Fifty nanograms of each sample was loaded onto a 1% agarose TAE-buffered gel to separate pDNA conformers. After separation, gels were stained with SYBR<sup>®</sup> gold nucleic acid gel stain at a 1:10,000 dilution in 1× TAE buffer and imaged on a FluorImager instrument (Molecular Dynamics, Sunnyvale, CA) equipped with an argon ion laser (488-nm excitation wavelength) and 530-nm emission filter.

Selected samples were tested by *in vitro* RT-PCR for relative potency and by injection into mice to measure their ability to induce immunogen-specific serum antibodies.

### Mouse Immunizations and Serum Collection

For RT-PCR/immunogenicity correlation studies, frozen untreated and heat-treated VCL-CB01 samples were thawed and diluted in PBS to a final concentration of 0.1 mg/ml and mixed by inverting the vials.

Immunogenicity studies were performed in female BALB/c mice (Jackson Laboratories, Bar Harbor, ME). Animals were separated into groups of eleven, with each group receiving bilateral injections of 50 µl per injection (5 µg per injection for a total of 10 µg per animal) in the *rectus femoris* on Days 0 and 14. Blood was collected from each animal prior to the first injection and on Day 26 via orbital sinus puncture.

All animal procedures were approved by the Vical Institutional Animal Care and Use Committee (IACUC) and complied with the standards set forth in the Guide for the Care and Use of Laboratory Animals (ILAR, 1996) and the Animal Welfare Act and Animal Care Regulations (see <http://www.aphis.usda.gov/ac/publications.html>).

### CMV gB ELISA

CMV gB-specific IgG antibody responses in the mice were measured by ELISA. Ninety-six-well plates were coated

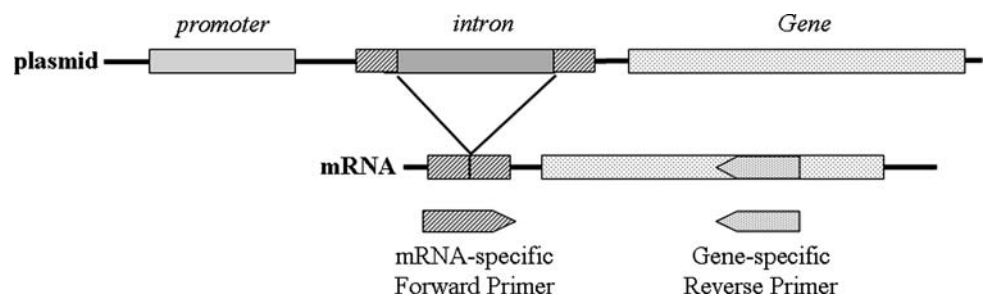
with recombinant gB (Austral Biologicals, San Ramon, CA) at a concentration of 2 µg/ml. Individual test sera from immunized mice were serially diluted and added to the gB-coated wells. After a 2-h incubation at room temperature, the plates were washed and AP-conjugated goat anti-mouse IgG Fcγ-fragment-specific antibody (Jackson ImmunoResearch, West Grove, PA, 1:2,000) was added. The plates were incubated for 1 h at room temperature, the plates were washed, and colorimetric substrate (pNPP AP substrate; Calbiochem, San Diego, CA) was added to detect bound antibodies. After color development, sample absorbance at 405 nm was read. Antibody activity was determined by comparison with a Reference Standard: a pool of serum with well-documented potency. Antibody levels were reported in ELISA units/ml (EU/ml).

## Results

### Primer Selectivity and Specificity

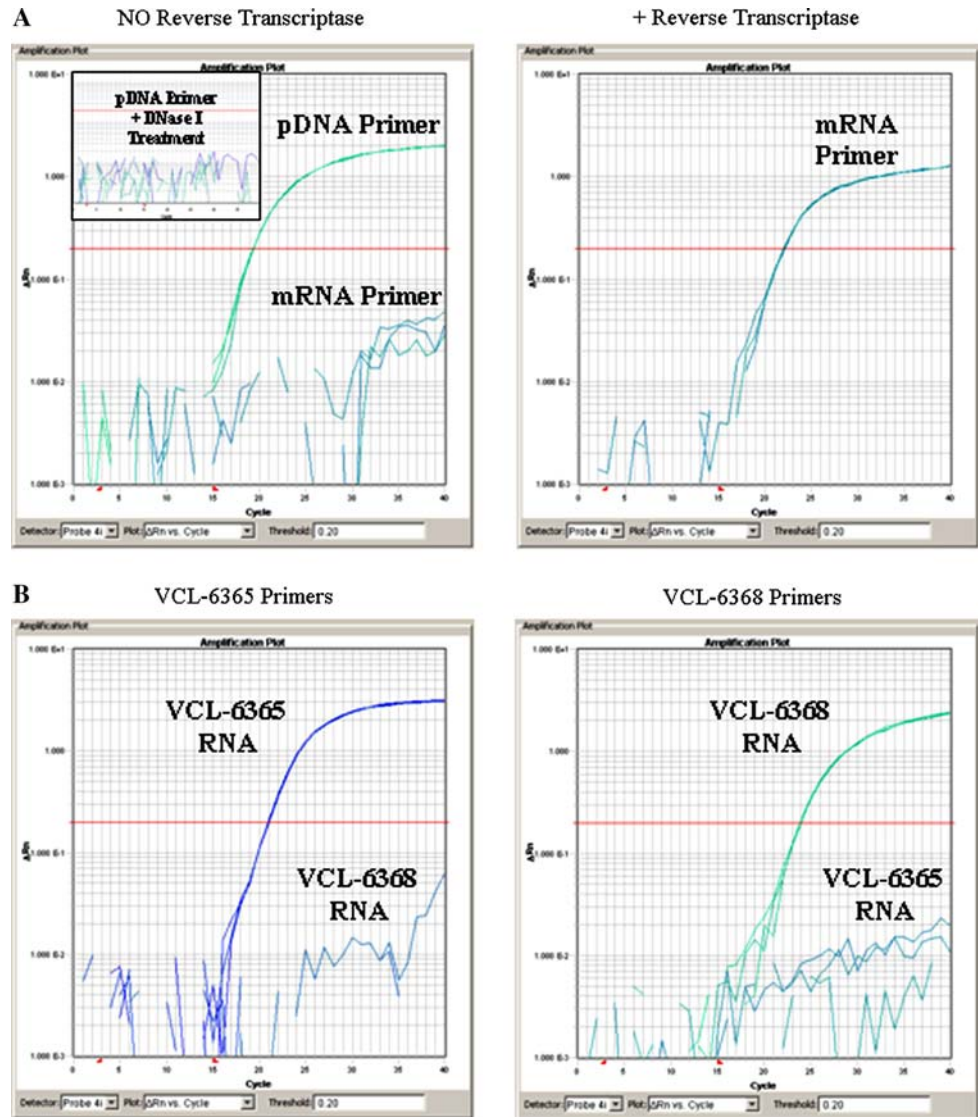
A critical feature for the effectiveness of this assay was the development of reagents with the specificity for individual components of a multivalent vaccine coupled with the selectivity to be able to target transgene mRNA alone in a complex mixture of nucleic acids. The selectivity of the assay is provided by the intron-spanning forward primer, while the specificity for transgene message comes from the gene-specific reverse primer (Fig. 1). To demonstrate the ability of the primers to recognize mRNA alone, cells were transfected with VCL-6365 plasmid, lysed, and total RNA isolated as described. Total RNA was then assayed using the RT-PCR primers but in the absence of reverse transcriptase enzyme. If the forward primer was able to hybridize to the plasmid template, the TaqMan<sup>®</sup> reaction would have resulted in measurable amplification. Figure 2a demonstrates that the forward primer does not bind in the absence of reverse transcription and PCR amplification does not occur. Amplification of the same RNA sample with a pDNA-specific forward primer hybridizing to a backbone region of the plasmid resulted in measurable amplification (Fig. 2a, left panel), demonstrating the

**Fig. 1** Design of RT-PCR primers. The 5' forward primer is designed to recognize sites flanking both ends of the intron. The 3' reverse primer gives specificity for the gene by priming the reverse transcription step as well as the subsequent PCR amplification step





**Fig. 2** (a) Specificity of the intron-spanning forward primer for mRNA. Total RNA isolated from transfected cells was analyzed with mRNA- and pDNA-specific primers in the absence (left panel) and presence (right panel) of reverse transcriptase enzyme. DNA-specific primers demonstrate the presence of a significant level of pDNA co-purifying with the total RNA, emphasizing the need for selective primers. Additional treatment of the RNA sample with DNase I removes all traces of DNA-specific amplification (left panel, inset). In the absence of reverse transcription there is no amplification, demonstrating the inability of the mRNA-specific primer to bind pDNA. (b) Specificity of the gene-specific reverse primers for their target mRNA. Reverse primer specificity was evaluated on RNA samples from cells transfected separately with each individual pDNA. RT-PCR primers are able to amplify only when run with RNA from cells transfected with their target plasmid



presence of residual plasmid in the total RNA sample and reinforcing the requirement for uncompromising selectivity of the reagents. Treatment of the RNA sample with DNase I prior to PCR abolished pDNA-specific amplification (Fig. 2a, left panel, inset). As intended, analysis of the sample with the RT-PCR-specific primers in the presence of reverse transcriptase enzyme resulted in measurable amplification (Fig. 2a, right panel).

The specificity of the gB and pp65 primers is demonstrated in Fig. 2b. Bulk VCL-6365 and VCL-6368 plasmids were transfected individually and the total RNA from each set was analyzed with both sets of primers (Fig. 2b). As expected, gB-specific primers result in measurable amplification only from RNA samples from gB-transfected cells, while pp65-specific primers result in measurable amplification only on pp65-specific RNA.

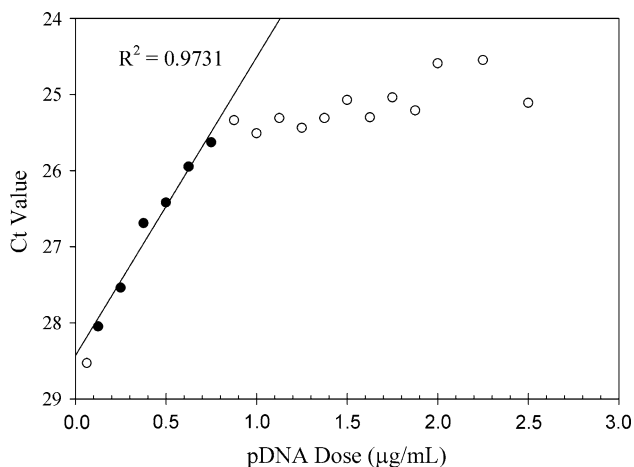
All subsequent studies were conducted using our bivalent vaccine product (VCL-CB01). In all cases, total RNA

from transfected cells was analyzed in parallel with reagents for both VCL-6365 and VCL-6368. For simplicity, all further graphical representation of experiments is based on data generated with the VCL-6365-specific reagents. All the data presented were similar to data generated with the VCL-6368 reagents.

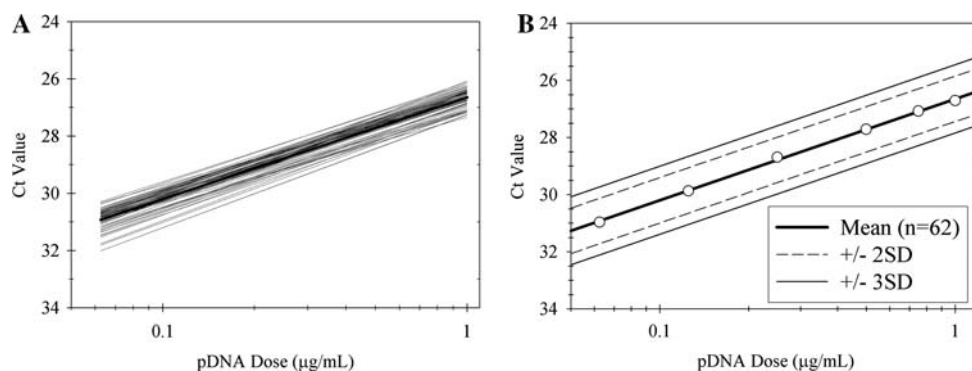
#### Linear Range

To establish the linear dose range for transfection, reference VCL-CB01 material at concentrations ranging from 0.0625 to 2.5  $\mu\text{g}/\text{ml}$  was transfected and the harvested RNA analyzed with the RT-PCR assay. The Ct values for each concentration were plotted against the transfected dose (Fig. 3). The linear range was determined by measuring the increase in  $R^2$  value of a linear fit obtained with different sets of six or more concentration points. While the optimal linear range for both VCL-CB01

plasmids was determined to be from 0.125 to 0.75  $\mu\text{g/ml}$  (Fig. 3, filled circles;  $R^2$  value of 0.9731), concurrent development of the RT-PCR assay for additional vaccine products revealed a consensus optimal linear range from 0.0625 to 1.0  $\mu\text{g/ml}$  (data not shown). The revised six-point dose range (0.0625, 0.125, 0.25, 0.5, 0.75, and 1.0  $\mu\text{g/ml}$ ) was further tested with VCL-CB01 and showed a linear fit consistent with the one determined previously ( $R^2$  value was 0.9748 for VCL-6365 and 0.9738 for VCL-6368;  $n = 21$ ). For consistency of assay format across products, the dose range for initial development of the VCL-CB01 potency assay was expanded to include the 0.0625 and 1.0  $\mu\text{g/ml}$  dose points.



**Fig. 3** Determining the linear transfection dose range. Several concentrations of VCL-CB01 were transfected into VM92 cells seeded 24 h prior in a 24-well plate (250  $\mu\text{l}$  per well). Total RNA was isolated, analyzed with the RT-PCR assay, and the resulting Ct values plotted against the pDNA concentration (circles). Solid line indicates the six-dose points (filled circles) providing the best linear fit. Ct values are displayed in reverse order to emphasize that smaller Ct values indicate increased expression



**Fig. 4** Characterization of reference variability. (a) A single lot of cGMP manufactured VCL-CB01 was designated as reference material and stored in single-use aliquots. Multiple dose response curves, covering the dose range identified earlier, were analyzed by several operators on different days using different lots of critical reagents.

## Reference Characterization

Initial assay characterization was performed by running multiple reference curves ( $n = 62$ ) over a 6-week period. Either two or four reference curves were run in parallel per experimental run (one replicate well per dose). Performing the assay under identical assay parameters, different operators, cell passage numbers, 7900HT instruments, and lot numbers of critical reagents were evaluated. All the reference curves had an  $R^2$  value greater than 0.90 and 58 of the 62 reference curves had an  $R^2$  value greater than 0.95. A plot of all 62 trend lines is shown in Fig. 4a. Figure 4b shows a trend line for the mean of all curves (bold solid line), bracketed by trend lines for the mean  $\pm 2$  (dotted lines) and  $\pm 3$  (solid lines) standard deviations.

## Statistical Analysis/Modeling

The semi-log plots of Ct versus dose suggested that the data fit a parallel-line model [21]. Therefore, if the slopes of the curves for a reference sample and a test sample run in the same assay are equivalent, the relative potency of the test sample can be computed in relation to the reference sample. Equivalence is defined through acceptance limits on the difference between the two slopes. For each reference assayed, the intercept, slope, and root mean square error (RMSE) were compiled for determination of assay acceptance criteria. Averaging over assays, the mean, standard deviation, and 2 and 3 standard deviation confidence bounds were calculated for the three-assay acceptance criteria: Slope, Y-intercept, and RMSE. Only upper bounds are calculated for the RMSE because a very small RMSE indicates an exceptionally good fit, which is acceptable. Together the bounds define the acceptance criteria within which future assay reference results must fall. The data were further analyzed to evaluate the

Concentration of pDNA for transfection is plotted against Ct value. Trend lines for VCL-6365 mRNA expression levels are shown ( $n = 62$ ). (b) Graph shows the trend lines for the mean (bold line) and the ranges for  $\pm 2$  times the standard deviation ( $\pm 2$  SD, dotted line) and for  $\pm 3$  times the standard deviation ( $\pm 3$  SD, solid line)

feasibility of reducing the linear range to a four-point dose set. The pDNA concentration set of 0.0625, 0.125, 0.25, and 0.75 µg/ml was selected as the optimal dose set with minimal standard deviations for the assay reference suitability criteria for both plasmids (data not shown).

For reference characterization, one dose response curve within each experimental run was randomly assigned to be a “Reference” sample, while another was defined as a “Test” sample (reference versus reference pair). Sixty-two assays for the VCL-6365 plasmid generated 31 reference versus reference pairs for analysis. The reference measurements were randomly paired within each operator, assay date, run number, and curve number set. In total, 31 VCL-6365 and 30 VCL-6368 reference pairs were analyzed.

Relative potencies were calculated for each reference vs. reference pair. A key assumption used in statistical analysis of the reference characterization was that a “test” sample was as potent as the “reference sample.” Although there is no way to directly test this assumption, all samples were aliquots of the same reference material and were treated identically in the assays, therefore, any differences in performance criteria (i.e., slope, Y-intercept, etc.) can be assumed to reflect intra-assay variability.

Means, standard deviations, and confidence limits were calculated for the set of relative potencies as a measure of how well the assay measured 100% relative potency. Relative potency was calculated using the following equations (adapted from Finney [22]):

$$M = \frac{Y_T - Y_R}{b} \quad \text{and} \quad RP = 10^M$$

where  $Y_R$  and  $Y_T$  are the Ct values for a given pDNA concentration for the Reference and Test assays, respectively,  $b$  is the common slope for the parallel-line fit, and RP is the relative potency [22].

The confidence limits were calculated around “ $M$ .” The upper and lower limits were then anti log transformed to obtain results in units of relative potency. As stated before, similar data were collected and analyzed for both plasmids in the bivalent vaccine. For plasmid VCL-6365, mean RP was 100.3% with 2 standard deviation (SD) bounds of 73.2–137.6% and the 3 SD bounds of 62.5–161.2%. Therefore, with 3 SD bounds a sample that measures within 62.5–161.2% of reference is statistically equivalent to the reference, or 100% relative potency. For plasmid VCL-6368, mean RP was 102.4% with the 2 SD bounds of 72.9–143.7% and the 3 SD bounds of 61.5–170.3%. Table 1 summarizes reference suitability criteria, assay acceptance criteria, assay precision, and sample size determinations. Due to a limited data set early in product development, three SD limits were used for all assay acceptance criteria to release clinical trial material. As the variability of critical assay parameters are further characterized, and the robustness of the assay further tested a new statistical analysis data will be carried out and the release criteria will be made more stringent in preparation for assay validation.

#### Potency Determination

Once an equation for determining the relative potency was identified, experiments were performed to define the accuracy and range of the assay. VCL-CB01 reference

**Table 1** Summary of statistical analysis results

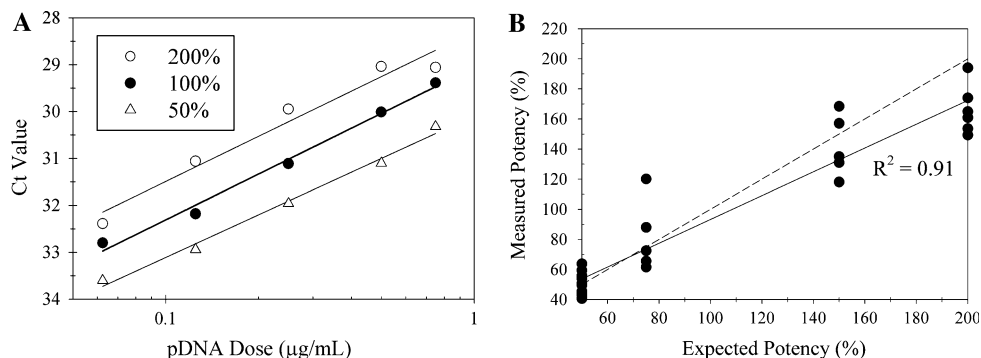
	VCL-6365	VCL-6368
Criteria		
Number of reference assays	62	60
Number of reference/reference pairs	31	30
Concentration response model	Parallel lines, log transformed concentration	Parallel lines, log transformed concentration
Linear range	Ct between 1 and 0.06 µg/ml	Ct between 1 to 0.06 µg/ml
Concentrations to run assay (µg/ml)	0.0625, 0.125, 0.25, 0.75	0.0625, 0.125, 0.25, 0.75
Reference assay suitability		
Slope (Ct/log(µg/ml))	−4.39 to −2.87	−4.66 to −2.74
Intercept (Ct)	25.60–27.71	25.73–28.96
RMSE (Ct)	≤0.57	≤0.61
Test sample acceptance		
CI on difference between slopes (Ct/log(µg/ml))	−2.99 to +2.99	−2.79 to +2.79
Assay precision		
Relative potency (±3 SD)	62.5–161.2%	61.5–170.3%
Standard deviation of M	0.07	0.07

material was prepared at 50, 75, 100, 150, and 200% of pDNA mass used for the 10  $\mu\text{g}/\text{ml}$  reference stock and run as unknowns. Test samples were prepared by altering the concentration of the starting material while treating the samples for transfection as if they were at 10  $\mu\text{g}/\text{ml}$ . Therefore, the 50% sample was diluted to 5  $\mu\text{g}/\text{ml}$ , but it was processed for transfection as if it were at 10  $\mu\text{g}/\text{ml}$ .

For this experiment, the range of the reference sample consisted of five pDNA concentrations from 0.75 to 0.0625  $\mu\text{g}/\text{ml}$ . The potency test samples were diluted and transfected in the same way as the reference sample. RT-PCR was performed as before and the data curves were plotted as Ct versus pDNA concentration (Fig. 5). Relative potency was calculated using the Finney equation described above. The potency calculations for the various samples are plotted in Fig. 5b against the expected results. Based on the similarity between trend lines of the experimentally derived potency values (Fig. 5b, solid line) and optimal 100% accuracy values (Fig. 5b, dotted line), the experimentally calculated relative potency values are in good accordance with the theoretical potency values and reinforce the validity of this *in vitro* assay to measure differences in sample potency.

#### In Vitro–In Vivo Correlation

To support the use of an *in vitro* assay to measure the potency of pDNA vaccines, a preliminary study was performed to establish a correlation of *in vitro* determined potency with *in vivo* biological activity. For this analysis, we generated samples of varying potencies by heat treating VCL-CB01 and evaluating selected samples in parallel in the *in vitro* RT-PCR relative potency assay and in a mouse immunogenicity model.



**Fig. 5** Measurement of sample potency with the RT-PCR assay. (a) Samples of varying potency were prepared by adjusting the concentration of VCL-CB01 material and running them as unknowns against VCL-CB01 reference. Graph shows examples of a dose response with 200% samples (○) and 50% samples (△) run in parallel against a 100% reference (●). If the slopes of the trend lines from each curve are equivalent to the slope of the reference trend (bold line), then a

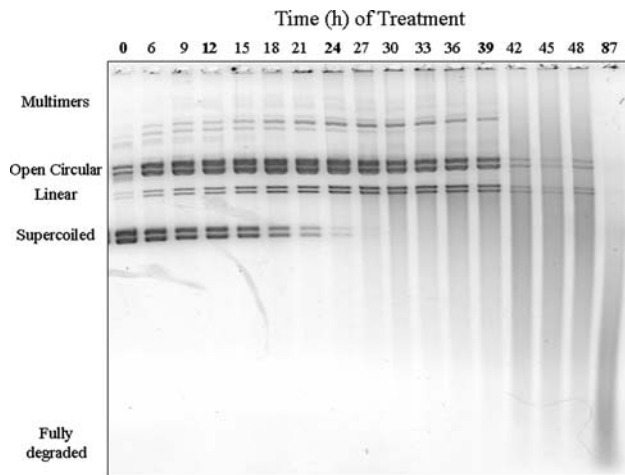
Heat-treated samples were prepared as described in the Material and Methods section. Physical changes of the samples were visualized by agarose gel electrophoresis (Fig. 6). Degradation of supercoiled pDNA into open circular, and linear conformers, and then eventually DNA fragments, was proportional to incubation time at 80°C.

Selected heat-degraded VCL-CB01 samples were analyzed by the RT-PCR relative potency assay. The data show that the duration of heat treatment correlates with a decrease in percent relative potency as measured by the RT-PCR assay (Table 2). Data for both VCL-6365 and VCL-6368 are shown. The correlation holds for both plasmids which clearly trend together in their loss of potency with increasing heat-induced degradation. When plotted as relative percent potency versus time (data not shown), the trend follows a third order polynomial fit with an  $R^2$  value of 0.9783. This pattern of potency reduction clearly mirrors the overall physical change of pDNA forms observed in Fig. 6.

Of the samples analyzed by RT-PCR, five were chosen as representative samples of the various degradation stages to test for immunogenicity in mice (Table 2, samples marked with an asterisk). Mice were injected with 10  $\mu\text{g}$  of VCL-CB01 from each of the test samples at Day 0 and Day 14 and serum was collected on Day 26. For simplicity, immunogenicity of only VCL-6365 pDNA, encoding the gB protein, was evaluated. Antibodies specific for gB were measured by ELISA. The trend of antibody responses across the various samples tested was very similar to the measured potencies from the RT-PCR assay. Figure 7 shows an overlay of the relative potency values obtained for VCL-6365 (left axis) and the antibody levels (right axis) from the same samples. As expected, the graph shows that the *in vivo* and the *in vitro* assays correlate very well

potency value can be calculated. (b) Accuracy of potency measurement. Measured potency values calculated for 50, 75, 150, and 200% samples were plotted against expected values. The similarity of the linear fit trend line (solid) to the theoretical optimal trend line (dotted) supports the ability of the assay to accurately measure sample potencies





**Fig. 6** Forced thermal degradation of VCL-CB01. Aliquots of VCL-CB01 were incubated at 80°C for varying amounts of time. Heat-degraded pDNA samples were loaded onto a 1% agarose TAE-buffered gel (50 ng per lane) and electrophoresed (numbers above lanes indicate hours of treatment). The gel was stained with SYBR<sup>®</sup> gold to visualize pDNA conformers. The different populations of pDNA visible are described in the legend to the left. The doublets observed for the higher order structures represent the two plasmids present in VCL-CB01

**Table 2** Relative potency values for VCL-CB01 heat-stressed material

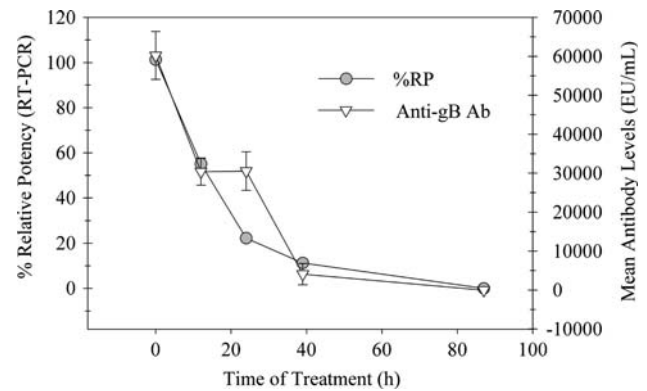
Test sample hours at 80°C	VCL-6365 results	VCL-6368 results
0*	107.1	94.9
6	72.2	71.2
9	52.7	46.2
12*	54.2	55.5
18	39.1	35.4
24*	25.1	19.1
33	19	14.4
39*	13.2	9.3
42	2.1	1.2
45	1.7	0.9
87*	0	0

\* Samples used in mouse immunogenicity study

in their ability to distinguish relative potencies of the various degraded samples.

## Discussion

We are developing an assay platform to measure the potency of pDNA-based products. The TaqMan<sup>®</sup>-based RT-PCR assay described here provides a sensitive, reproducible, and accurate measure of the transcriptional potency of a pDNA-based vaccine, VCL-CB01. As demonstrated, the specificity of the assay is built into the design



**Fig. 7** In vivo and in vitro potency of heat-stressed VCL-CB01 samples. Selected samples from the heat-treatment time course were used to correlate relative potency measurements using the in vitro RT-PCR assay (●) with in vivo potency measurements, as assessed by ability of the samples to elicit anti-gB antibodies in mice injected with 10 µg of each sample (▽). Data show mean potency values ( $n = 3$  replicate transfections) plotted alongside mean antibody titers ( $n = 11$  for all groups except 87 h,  $n = 5$ ). Error bars indicate  $\pm$ SEM of antibody titers

of the RT-PCR primers which makes the assay selective for transgene-expressed mRNA while allowing for the specific detection of individual transgenes in a multivalent product. Statistical characterization of assay performance supports a parallel-line model for a relative potency measurement which can measure both hyper- and hypo-potent samples when transfected in parallel with an appropriate reference standard. In further support of the use of this in vitro assay as a reliable measure of vaccine product potency, an in vivo–in vitro correlation study demonstrates striking agreement between in vitro measured relative potency of heat-stressed pDNA vaccine and the immunogenicity of the same samples following injection into mice.

Potency assays for traditional vaccines have relied primarily on the measurement of immunological potency in animal models. However, the unique mechanism of action of pDNA-based vaccines makes an in vitro evaluation of product potency a reasonable surrogate. In fact, since the most immediate result for pDNA vaccines is the expression of transgene-mRNA, transcriptional potency of pDNA is arguably more relevant than immunogenicity in measuring lot-to-lot manufacturing consistency. While immunogenicity is dependant on a fully functioning immune system found only in animal models, transcriptional potency, as characterized by the cellular uptake and expression of pDNA-specific mRNA, can be measured equally effectively in transfected tissue culture cells.

In general, in vitro assays are simpler to develop and implement than in vivo assays. This allows for potentially more replicates per routine analysis than are practically feasible for most in vivo-based assays. In addition, the well-characterized nature of most in vitro systems allows

for better control of assay parameters, leading to the generation of more interpretable and reproducible results.

While the accuracy and sensitivity of real time PCR technologies are well documented [6], the RT-PCR assay described here offers additional benefits for measuring pDNA vaccine potency, particularly over protein-based in vitro assays. Foremost is the availability and consistency of product-specific reagents. RT-PCR oligonucleotides can be designed as soon as the pDNA product sequence configuration is known. Unlike antibody reagents for many protein-based assays, customized oligonucleotides are readily available, are stable for long periods of time, and exhibit minimal lot-to-lot variability.

Furthermore, this nucleic acid-based assay complements one of the strengths of pDNA vaccines. In the current environment of heightened concern over unpredictable public health threats, such as bioterrorism and emerging or recurring infectious diseases, pDNA-based technologies offer the flexibility of rapidly developing vaccine candidates as soon as sequence information on target immunogens becomes available. Specific RT-PCR reagents can then be designed and assay development for product release can be initiated immediately, without having to wait for the development, characterization, and general distribution of antibody reagents.

In the context of standard vaccine product development assays, it is important to recognize that the RT-PCR-based potency assay described here constitutes part of the overall characterization and quality control release testing for pDNA vaccines. One parameter that could potentially affect the potency of pDNA-based products is the sequence integrity of the pDNA. Any mutation within the expression cassette, whether large (i.e., insertions or deletions) or small (i.e., point mutations), could influence potency of the product by altering levels of protein expression. A key requirement of large-scale pDNA manufacturing processes is that the process itself should not alter the genetic integrity of the product. Molecular analytics (i.e., restriction digests and DNA sequencing) commonly used in the release of manufactured pDNAs have confirmed the fidelity of the manufacturing process. Hypothetically, if a pDNA-based product produced using the same manufacturing process can be demonstrated to be genetically stable, then it follows that there will be no lot-to-lot variability of primary nucleotide sequence or of primary, secondary, or tertiary protein structure. Although there is always a certain probability that a mutant clone might be propagated during the manufacturing process, preliminary studies at Vical (unpublished) have shown that there is less than 10% prevalence of mutations following our current manufacturing process. Therefore, the only potential lot-to-lot variability of the drug substance is strength (as measured

by concentration) or higher order DNA structure (i.e., plasmid forms).

While the design of the RT-PCR assay does not measure the full intact message expressed from each target plasmid, it is sufficient for measuring the transcriptional potential of the particular plasmid lot. Additional analytical tests, such as Western blot analysis, HPLC, DNA sequencing, particle sizing (for formulated products), and others, complement the relative potency assay by providing a detailed characterization of the overall potency, strength, and lot-to-lot consistency of pDNA vaccine products.

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