



Increased precision of microbial RNA quantification using NASBA with an internal control

Stacey S. Patterson, Erica T. Casper, Luis Garcia-Rubio,
Matthew C. Smith, John H. Paul III*

College of Marine Science, University of South Florida, 140 7th Ave., South, St. Petersburg, FL 33701, United States

Received 17 September 2004; received in revised form 14 October 2004; accepted 14 October 2004

Available online 11 November 2004

Abstract

Detection and quantification of low abundance target RNA has wide utility in the fields of clinical diagnostics, environmental monitoring, gene expression analysis, and biodefense. Nucleic acid based sequence amplification (NASBA) is an isothermal amplification method that provides the sensitivity needed for these applications. However, the requirement for three separate enzymes in NASBA often results in a greater variability between replicate samples than that seen in PCR-based assays. To overcome this problem, we have adapted the bioMérieux Nuclisens Basic Kit and Nuclisens EasyQ Analyzer along with the introduction of a synthetic internal control RNA (IC-RNA) for quantification of potentially any RNA sequence. Using the *rbcL* gene from the Florida red tide organism *Karenia brevis* as our target, we describe a simple method to accurately quantify the native target by computing the ratio of the time to positivity (TTP) values for both the wild-type and IC-RNA, and plotting this ratio against the starting number of target molecules or cells. By utilizing this simple method, we have significantly increased our accuracy and precision of prediction over the standard TTP calculations.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Quantitative NASBA; *Karenia brevis*; IC-NASBA

1. Introduction

Nucleic acid sequence-based amplification (NASBA) is an isothermal method able to specif-

ically amplify target RNA in a DNA background (Compton, 1991). By combining NASBA amplification with molecular beacon probes, this assay becomes a real-time analysis tool that offers faster results than quantitative PCR (QPCR). This, along with its isothermal nature, makes NASBA ideal for remote monitoring and/or high throughput applications (Leone et al., 1998). Like QPCR, quantification of unknown samples using real-time NASBA

* Corresponding author. Tel.: +1 727 553 1168; fax +1 727 553 1189.

E-mail address: jpaul@marine.usf.edu (J.H. Paul).

has typically been performed by direct comparison of the time to positivity (TTP) for the unknown sample to an external known standard curve (Polstra et al., 2002). This type of analysis assumes equal amplification efficiency in all standards and samples and requires a standard curve for each experiment. Because NASBA amplification involves three separate enzymes each with their own kinetic parameters, the variability between replicate samples is often greater than that seen in PCR-based assays. Furthermore, samples extracted from different sources may introduce inhibitors to the reaction that are not accounted for by separate standards (Stocher and Berg, 2002).

For end-point electrochemiluminescent (ECL) NASBA applications, internal amplification calibrators have been used to normalize enzyme efficiency differences between reactions (Peter and Sevall, 2004). Weusten et al. (2002a,b) were the first to describe a mathematical model for RNA amplification of both target and internal calibrator RNA in a molecular beacon-based NASBA reaction. However, the description of this model did not include all of the essential parameters needed to operate the model. Consequently, analysis using this model requires software calibrated to each target and is commercially available for only a few specific targets. In an attempt to improve quantitative NASBA for use with any target, we have evaluated a simple TTP ratio calculation that reduces the variability between replicates and increases the precision and accuracy for predicting unknown concentrations of target RNA.

To test this method, an IC-RNA molecule for the Gulf of Mexico red tide organism *Karenia brevis* was generated. We have previously shown that NASBA amplification enabled detection of less than one *K. brevis* cell while unknown concentrations from bloom samples could be predicted within an order of magnitude using simple TTP measurements (Casper et al., 2004b). With the addition of an IC-RNA molecule and application of the IC-NASBA method described here, we have further increased our precision for predicting unknown concentrations of *K. brevis*. This method could easily be adapted for use with any target and should aid in broadening the utility of quantitative NASBA efforts.

2. Materials and methods

2.1. *In vitro* transcript

In vitro transcript was generated as described by Gray et al. (2003). Briefly, degenerate primers were designed to amplify a 554-base region of the *rbcL* gene of *K. brevis* (Gray et al., 2003). The reaction product was TA TOPO cloned into the pCRII cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. White colonies were chosen and subsequently grown in Luria broth supplemented with 50 µg of kanamycin and ampicillin per ml. Plasmid DNA was isolated using Wizard Midi-plasmid purification kit (Promega, Madison, WI) and sequenced in both directions using the vector M13 priming sites. The plasmid was linearized by digestion with *Hind*III, the enzyme removed using the Wizard DNA clean-up kit (Promega), and run-off transcripts generated from an upstream T7 promoter using the Riboprobe *in vitro* transcription kit (Promega). The transcripts were purified by the RNeasy spin protocol (Qiagen, Valencia, CA) and quantified with a Ribogreen RNA quantification kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Following quantification, the transcript RNA was mixed 1:1 in an RNA storage buffer (8M guanidinium isothiocyanate, 80mM Tris-HCl (pH 8.5), 24mM MgCl₂, 140mM KCl) and frozen at -80°C until use.

2.2. Synthesis of internal control (IC) RNA

The IC-RNA molecule was designed to include the exact base composition as the 87 base amplicon of the *rbcL* gene from the *K. brevis* NASBA assay (Casper et al., 2004b) with the exception that the original beacon site was removed and replaced with the beacon site from our enterovirus NASBA assay (Casper et al., submitted for publication) (Table 1; Fig. 1). To generate the IC-RNA, two oligonucleotides were designed that spanned the entire internal control sequence and contained a 20-base overlap with each other (Fig. 2). The oligos (serving as both template and primers) were placed in a PCR reaction (100pmol each) and run under standard PCR conditions with an annealing step of 55°C for 20 cycles. Reaction products were visualized on a 1% agarose gel stained with ethidium

Table 1
K. brevis NASBA primer and molecular beacon sequences

Primer name	Sequences (5' to 3')
Kb forward	ACGTTATTGGGTCTGTGTA
Kb reverse	AATTCTAATACGACTCACTATAGGGAGAAGGTACACACTTTCGTAACCTA
<i>K. brevis</i> molecular beacon	[6-FAM]CGATCGCTTAGTCTCGGGTTATTTTTTCGATCG-[DABCYL]
IC-RNA molecular beacon	[6-ROX]CGATCGTGGCTGCTTATGGTGACAATCGATCG-[DABCYL]

bromide. Amplicons were TA TOPO cloned into the pCRII cloning vector according to the manufacturer's instructions (Invitrogen). Clones were checked for directionality by PCR screening using an internal upstream M13 reverse primer site located on the plasmid and the downstream *K. brevis* NASBA primer. Two clones appearing to have the insert in the correct orientation were grown overnight in LB supplemented with 50 µg/ml of kanamycin and the plasmid was extracted using the Wizard Midi-plasmid purification kit (Promega). Plasmids were sequenced using the vector M13 reverse priming site to insure the insert's integrity. The plasmid was linearized by digestion with *NotI*, the enzyme removed using the Wizard DNA clean-up kit (Promega), and run-off transcripts generated from an upstream SP6 promoter using the Riboprobe in vitro transcription kit (Promega) (Fig. 2). RNA was quantified and stored as described above.

2.3. *K. brevis*

K. brevis cultures were provided courtesy of Karen Steidinger of the Florida Fish and Wildlife Research Institute (FMRI). A strain isolated from Piney Island off the coast of Florida (Piney Island B4) was used in all studies herein and was routinely grown in the appropriate media at 24 °C at FMRI. Before RNA extraction, 1 ml of culture was examined in triplicate

to obtain the cell number. Cell counts were made by epifluorescence microscopy as described by Vernet et al. (1990) and were used to construct cell standard curves for quantitative analysis.

2.4. RNA extraction from *K. brevis*

The appropriate number of cells was filtered onto a 25 mm, 0.45 µm Durapore (Millipore) filter by vacuum filtration. The filter was then removed and placed directly into 500 µl of RLT lysis buffer (Qiagen) plus 0.01% 2-mercaptoethanol. Following a 10-min incubation at room temperature, 350 µl of 100% EtOH was added. The RNA purification was then performed on an RNeasy spin column according to the manufacturer's recommendations (Qiagen).

2.5. NASBA assay

NASBA was performed using the Nuclisens Basic Kit (bioMérieux, Durham, NC) and an EasyQ incubator and detection system (bioMérieux). Each NASBA assay included a deionized H₂O blank as a negative control. Titrations of IC-RNA (between 10⁴ and 10⁷ copies) and *K. brevis* RNA were performed to determine the optimal amount of competitor to generate the greatest dynamic range for the assay (data not shown). As a result, each reaction was run

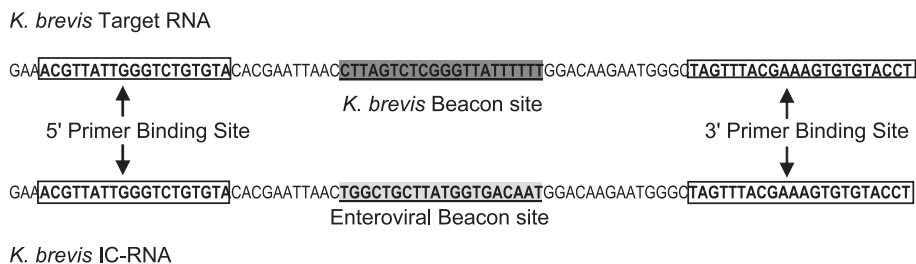


Fig. 1. Sequence of *K. brevis* target RNA versus the synthetic IC-RNA molecule.

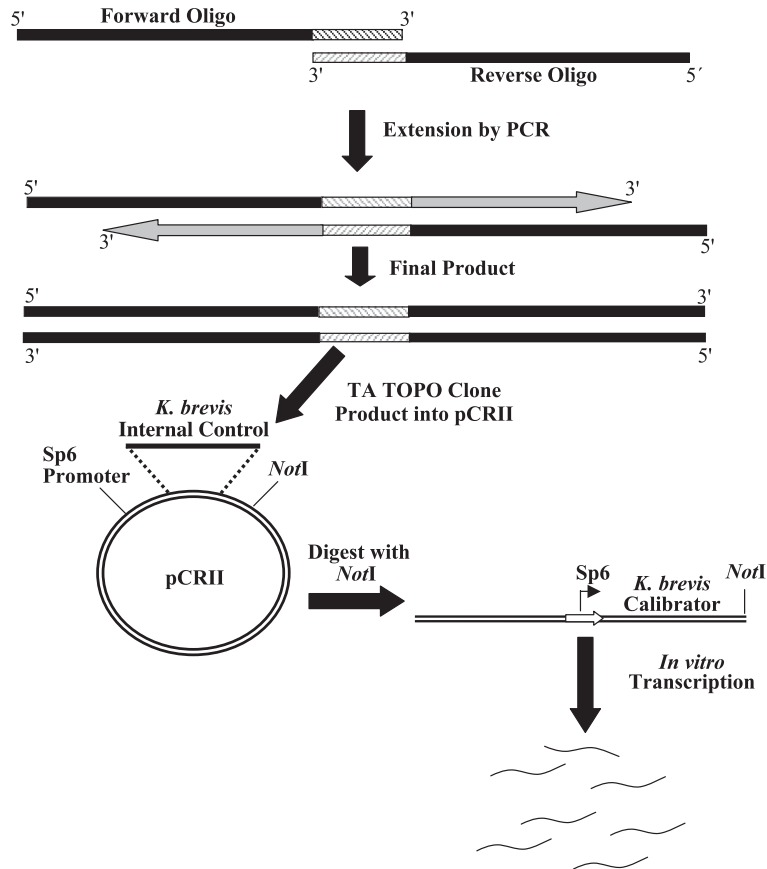


Fig. 2. Schematic representation of the steps to make the synthetic internal control molecule (IC-RNA).

with the addition of 10^6 copies of the IC-RNA. Cell and/or transcript standards for each experiment typically spanned four orders of magnitude, and were run in triplicate reactions at each concentration. Standard curves were created using the standard TTP method as well as a ratio of the TTP for the wild-type RNA (*K. brevis*) and the IC-RNA signals (IC-NASBA).

To maximize the number of reactions per Nuclisens Basic kit, each sample was run in a 10 μ l NASBA reaction (half the volume recommended by the bioMérieux protocol), consisting of 5 μ l NASBA reagent/primer/IC-RNA mix (80mM KCl), 2.5 μ l target RNA template, and 2.5 μ l enzyme mix (Nuclisens Basic Kit, bioMérieux). HPLC-purified primers and beacon were obtained from Qiagen, and were diluted to final concentrations of 400nM for primers and 100nM for each beacon per NASBA reaction.

The *K. brevis* beacon was labeled with 6-carboxy fluorescein (6-FAM) at its 5'-end and quencher DABCYL at its 3'-end while the IC beacon contained a 6-carboxy-X-rhodamine (6-ROX) at its 5'-end and a DABCYL quencher at the 3'-end (Table 1).

2.6. Time to positivity (TTP) ratio

Following amplification, a specific fluorescence value was chosen as a positive signal (threshold level). Typically, this value was 0.25 fluorescence units above the final value of the FAM curve for the negative control reaction (generally 1.3–1.5 in this assay). Using this value for all reactions in one experiment, the time that the target and IC amplification curves reached the threshold level was recorded as the time to positivity (TTPs) (Fig. 3). The ratio of TTP for the target to IC was regressed against the

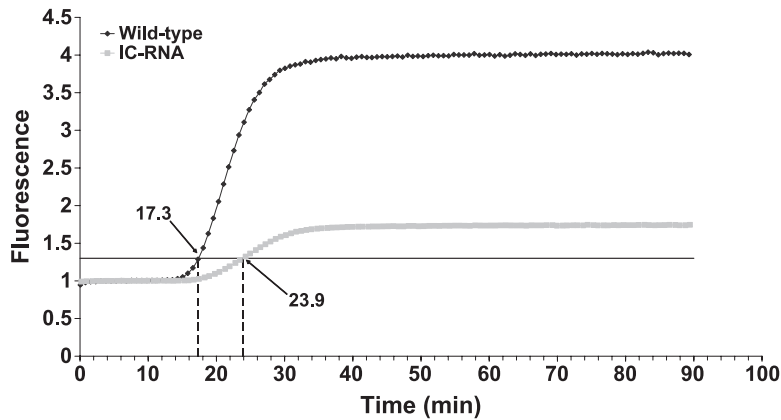


Fig. 3. Typical IC-NASBA plot. The TTP for both the target and IC-RNA amplification curves are identified by a specific fluorescence value. The IC-NASBA value is calculated by taking the ratio of these two values.

original standard curve to generate a predicted amount of target RNA.

2.7. Statistical analysis of the TTP and IC-NASBA

An *F*-test was used to evaluate whether there were significant differences between predicted concentrations using the standard TTP versus the IC-NASBA method. A Student's *t*-test was used to define differences in the mean of predicted concentrations.

For this analysis, it was important to evaluate the standard TTP method and the proposed IC-NASBA method on the basis of the uncertainty associated with the prediction of the concentration (x). As illustrated in Figs. 4 and 5, both quantification methods can be calibrated using a linear relationship with the logarithm of the concentration. In other words, the standard curve for both methods is given by:

$$y_{ik} = a_k + b_k \log(x_i) \quad (1)$$

where y_{ik} represents the i th observation for the k th method, and a_k and b_k represent the corresponding regression coefficients. In the case of the standard method, the observation variable y_{i0} is equal to the TTP for the target, whereas for the IC-NASBA method $y_{i1} = y_{i0}/y_{i2}$ where y_{i2} corresponds to the corresponding TTP for the internal control. The concentration estimates at each sample point can be obtained using Eq. (1).

Application of the propagation of error formula (Garcia-Rubio et al., 1984) to linear equations under

the assumptions of independent measurements and negligible correlation in the regression parameters yields for the standard method:

$$\text{var}(x_{i0}) = \left(\frac{1}{b_0}\right)^2 \exp\left(2\frac{y_{i0}-a_0}{b_0}\right) \times \left[\sigma_{y_{i0}}^2 + \sigma_{a_0}^2 + \left(\frac{y_{i0}-a_0}{b_0}\right)^2 \sigma_{b_0}^2\right] \quad (2)$$

And for the IC-NASBA method:

$$\text{var}(x_{i1}) = \left(\frac{1}{b_1}\right)^2 \exp\left(2\frac{\left(\frac{y_{i0}}{y_{i2}}\right) - a_1}{b_1}\right) \times \left[\left(\frac{1}{y_{i2}}\right)^2 \sigma_{y_{i0}}^2 + \left(\frac{y_{i0}}{y_{i2}^2}\right)^2 \sigma_{y_{i2}}^2 + \sigma_{a_1}^2 + \left(\frac{\left(\frac{y_{i0}}{y_{i2}}\right) - a_1}{b_1}\right)^2 \sigma_{b_1}^2\right] \quad (3)$$

where $\text{var}(x_{i0})$ and $\text{var}(x_{i1})$ are the variance estimates for the i th concentrations calculated from the standard and the IC-NASBA methods; $\sigma^2(y_{ik})$ are the variance estimates for TTP replicate measurements for the target and for the internal control; $\sigma^2(a_k)$ and $\sigma^2(b_k)$ correspond to the variance associated with the regression parameters of the standard curves for the direct and the IC-NASBA methods. Note that Eq. (3) includes the additional uncertainty associated with the internal control TTP measurements.

3. Results

3.1. Internal Control titrations

To determine the appropriate amount of IC-RNA needed for optimal dynamic range for the competitive amplification, a titration series of IC-RNA to *K. brevis* target was tested. It was determined that 10^6 copies of the IC were optimal to obtain amplification of both target and IC over an environmentally relevant concentration of *K. brevis* (data not shown). Therefore, 10^6 copies of IC-RNA were added to each reaction for amplification.

3.2. Standard curves based on IC-NASBA or TTP alone

The coefficient of determination (r^2) values describing the negative linear relationship of TTP or IC-NASBA to transcript copy number were 0.84 and 0.97,

respectively (Fig. 4). Similarly, cell standard curves also resulted in a better linear fit from the IC-NASBA method with r^2 values increasing from 0.80 to 0.98 (Fig. 5).

3.3. Predictions of RNA concentrations

To determine if one IC-NASBA standard curve could serve to predict unknown concentrations from several NASBA assays while using different lots of NASBA enzymes and reagents, the TTP and IC-NASBA values from different experiments were used to calculate concentrations using a single standard curve. The predictions obtained using a single standard curve were similar to those where individual standard curves were run with each batch using the IC-NASBA method. The mean predicted concentration was not significantly different between these treatments (Table 2). The variance was also not significantly different, except for the 10^7 copy concentration where

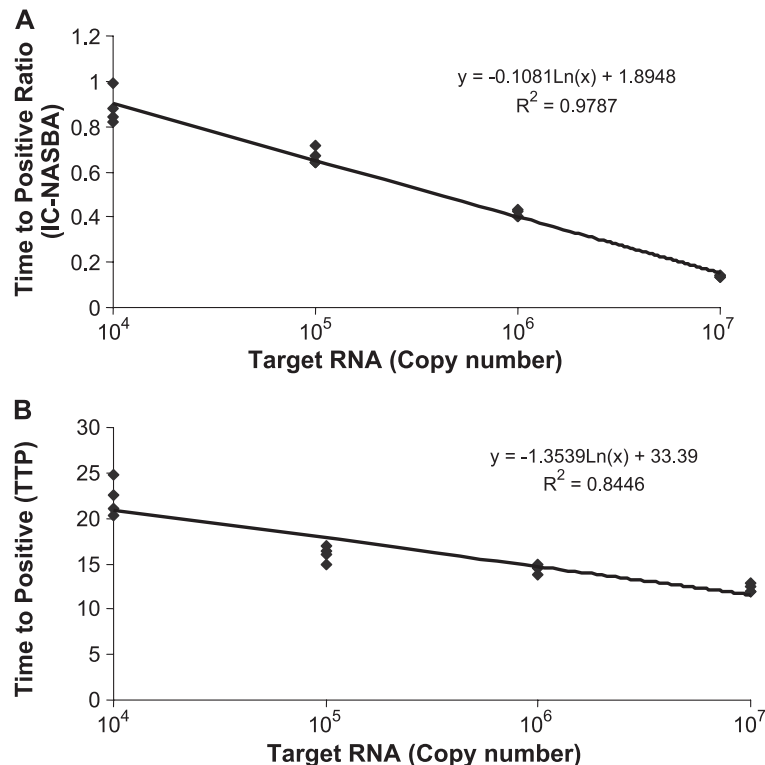


Fig. 4. (A) IC-NASBA standard curve using 10^4 – 10^7 copies of target *K. brevis* RNA transcript and 10^6 copies of *K. brevis* IC-RNA. (B) TTP standard curve using 10^4 – 10^7 copies of target *K. brevis* RNA transcript.

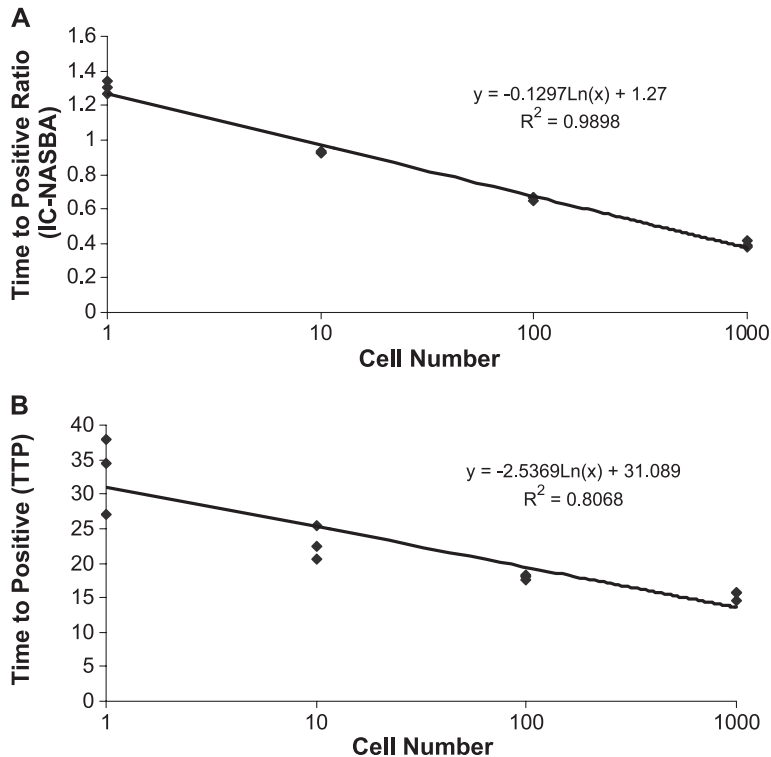


Fig. 5. (A) IC-NASBA standard curve using 1–1000 *K. brevis* cells and 10^6 copies of *K. brevis* IC-RNA. (B) TTP standard curve using 1–1000 *K. brevis* cells.

the variance was significantly lower when a single standard curve was used ($p < 0.01$). On the other hand, when one standard curve was used to predict concentrations using TTP alone, the calculated variance in these predictions was significantly increased for every concentration tested ($p < 0.05$) (Table 2).

The accuracy of prediction was improved as determined by the magnitude of the range of values at any one concentration. Using IC-NASBA, individual predictions were made that ranged from 38% to 269% of the actual known concentration (Table 2). These predictions were based on a single standard curve. Using the TTP method, the predictions ranged from 6% to 853% of the actual concentration when a standard curve was run with each batch and became even less accurate when a single standard curve was applied, ranging from 6% to 5090% of the measured concentration (Table 2).

To further evaluate the precision, the overall variance of each method was calculated for the standard TTP and IC-NASBA methods. Application

of Eqs. (2) and (3) to the data reported herein clearly demonstrates that the IC-NASBA method results in smaller variance estimates for the predicted concentrations (Fig. 6).

3.4. Predicting *K. brevis* cell number

The overall variance of the predictions of cell number was calculated at each concentration when using the standard and IC-NASBA method. The variance was significantly higher in all cell concentrations tested when predictions were made with a TTP calculation alone versus predictions made using IC-NASBA (Table 3). The accuracy of the method was, again, increased. Predictions made by IC-NASBA ranged from 59% to 740% of the actual cell number (based on direct cell count standard curve). Using the standard TTP method resulted in a less accurate calculation and ranged from 6% to 3820% of the known cell concentration (Table 3). Although the largest deviations occurred in the lowest concentration

Table 2
Average concentration (copy number) and data range (% of known) predicted using the standard TTP method versus IC-NASBA

Starting number of target molecules	Multiple standard curves ^a				Single standard curve ^b			
	TTP ^a	Magnitude of prediction (%)	IC-NASBA ^a	Magnitude of prediction (%)	TTP ^b	Magnitude of prediction (%)	IC-NASBA ^b	Magnitude of prediction (%)
1 × 10 ⁷	9.2 × 10 ⁶ ± 5.47 × 10 ⁶	40–208	1.46 × 10 ⁶ ± 3.02 × 10 ⁶	66–118	6.12 × 10 ⁶ ± 3.00 × 10 ⁶	14–122	1.03 × 10 ⁷ ± 1.61 × 10 ⁶	66–120
1 × 10 ⁶	1.24 × 10 ⁶ ± 6.02 × 10 ⁵	75–192	7.26 × 10 ⁵ ± 2.15 × 10 ⁵	58–101	2.54 × 10 ⁶ ± 1.14 × 10 ⁶	79–433	7.15 × 10 ⁵ ± 3.21 × 10 ⁵	38–140
1 × 10 ⁵	2.48 × 10 ⁵ ± 2.72 × 10 ⁵	24–853	7.56 × 10 ⁴ ± 2.39 × 10 ⁴	47–110	6.63 × 10 ⁵ ± 2.98 × 10 ⁵	181–1230	1.06 × 10 ⁵ ± 5.33 × 10 ⁴	47–130
1 × 10 ⁴	1.40 × 10 ⁴ ± 1.47 × 10 ⁴	6–418	1.65 × 10 ⁴ ± 7.05 × 10 ³	40–269	1.02 × 10 ⁵ ± 1.69 × 10 ⁵	6–5090	1.85 × 10 ⁴ ± 7.47 × 10 ³	42–269

^a Predictions made using a separate standard curve with each run.

^b Predictions made using a single standard curve for all assay runs ($n=54$).

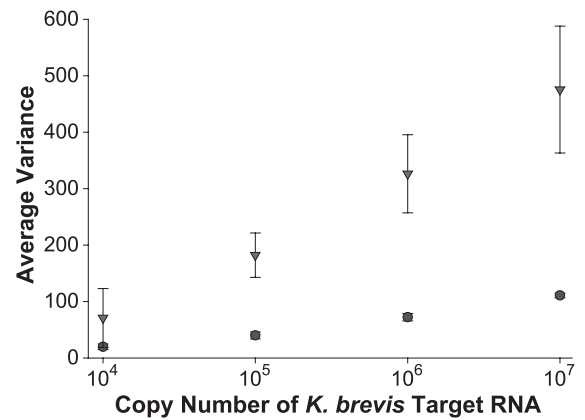


Fig. 6. Estimation of variance within the method using IC-NASBA versus the standard TTP method at each concentration. Black triangles=TTP method; black circles=IC-NASBA.

tested (one cell), the TTP method was less accurate at all concentrations tested.

4. Discussion

To accurately quantify samples using nucleic acid-based amplification methods, the quality of the sample and tube to tube variations need to be assessed. Previously reported RNA quantifications using NASBA have been made by simply comparing the TTP measurement to external reference standards (Polstra et al., 2002; Casper et al., 2004b; Goncalves et al., 2002; Hibbitts et al., 2003; Lanciotti and Kerst, 2001; Gore et al., 2003). However, the inherent variability associated with the kinetics of an assay involving three enzymes makes the TTP assay semi-quantitative. It has been shown that tube to tube variation within NASBA can be normalized with the addition of as many as three internal IC-RNAs in each reaction and

Table 3

Average cell number and data range for predictions made using the standard TTP method versus IC-NASBA (based on one standard curve for all samples)

Target cell number	TTP	Magnitude of prediction (%)	IC-NASBA	Magnitude of prediction (%)
1000	677 ± 209	42–84	920 ± 118	70–96
100	303 ± 157	160–550	125 ± 28	79–165
10	104 ± 108	90–2290	28 ± 19	130–740
1	58 ± 143	6–38,200	3 ± 2.5	59–400

subsequent detection using an ECL probe (Peter and Sevall, 2004; Schallig et al., 2003). Nevertheless, this method lacks the quantitative capabilities that are offered with a molecular beacon and real-time measurement approach.

Weusten et al. (2002a,b) suggested that RNA levels in internally calibrated real-time NASBA reactions could be quantified by modeling the fluorescence within the reaction curves. Unfortunately, we found that this model lacks all of the necessary parameters needed to evaluate a data set. Specifically, the α_3 value for the model is not fully defined (Weusten et al., 2002a,b). Although the value, as defined, should be the same for the wild-type and IC curves, in our data sets this is not always the case and, therefore, the model assumptions do not fit.

Here we describe an alternative method for normalizing NASBA data in the presence of an internal control that should be useful for any RNA target. Our ability to predict concentrations of both in vitro generated transcript and *K. brevis* cells was significantly increased with the IC-NASBA method over the TTP method. Using the IC-NASBA method, we were able to predict copies of transcript within 2.69 times of the known concentration whereas the TTP method was generally within an order of magnitude at the three highest concentrations and >50 times the lowest concentration. This pattern was reflected in the subsequent experiments involving *K. brevis* cells. In this case, again the IC-NASBA method resulted in far greater accuracy and precision for predicting cell number.

We have demonstrated that predictions of unknown concentrations of target RNA could be made using a single standard curve and that a new standard curve run with each batch may not be necessary for every assay. In fact, for our highest concentration tested (10^7 copies of target) the variability was reduced using a single standard curve. By eliminating the need to run batch standards each time, the cost efficiency of NASBA would be considerably increased.

Although not addressed directly in this study, the presence of inhibitors could also be inferred from the data using the IC-NASBA method. The presence of inhibitor substances is a known problem in amplification methods (Stocher and Berg, 2002). With the

introduction of a competitive internal control molecule, false negative results due to reaction failure can be identified. This attribute is important for both the analysis of environmental samples (as in this paper) and in clinical samples.

While 10^6 copies of the internal control RNA were optimal for the assay reported here, this concentration is assay-dependent. Experimental determination of this value would be required for each individual target over the relevant ranges in target concentration. The optimal concentration for the IC molecule should be determined as the concentration providing the greatest dynamic range of amplification of both the target and internal control RNA.

In conclusion, we describe a simplified method of calculating unknown concentrations of target RNA using an internally calibrated NASBA assay (IC-NASBA). This method allowed for greater precision and accuracy for predicting both transcript copy number and *K. brevis* cell number over the standard TTP analysis. Furthermore, the addition of an internal control allows the user to monitor sample quality and identify false negative results due to reaction failure. This approach should prove useful for the enumeration of any RNA target amplified by NASBA.

Acknowledgements

The authors would like to thank Bill Richardson and Karen Steidinger at the Florida Marine Research Institute for kindly providing cultures of *K. brevis* for these studies. Nuclisens Basic Kits were supplied by bioMérieux. We would also like to thank Sherryl Gilbert and Steve Meyers for their help with the interpretation of the Weusten et al.'s model. This work was funded in part by the Office of Naval Research and the National Science Foundation Biocomplexity Program.

References

- Casper, E.T., Patterson, S.S., Smith, M.C., Paul, J.H., 2004a. Development of a method to detect and quantify enteroviruses using NASBA and internal control RNA. *Journal of Virological Methods* (In press).
- Casper, E.T., Paul, J.H., Smith, M.C., Gray, M., 2004b. Detection and quantification of the red tide dinoflagellate *Karenia brevis*

- by real-time nucleic acid sequence-based amplification. *Applied and Environmental Microbiology* 70 (8), 4727–4732.
- Compton, J., 1991. Nucleic acid sequence-based amplification. *Nature* 350, 91–92.
- Garcia-Rubio, L.H., Talatinian, A.V., MacGregor, J.F., 1984. Propagation of errors in polymer measurement equations and the estimation of second order effects. *Proceedings of the Symposia of Quantitative Characteristics of Plastics and Rubber*. McMaster University, pp. 37–55.
- Goncalves, M.C., Klerks, M.M., Verbeek, M., Vega, J., van den Heuvel, J.F.J.M., 2002. The use of molecular beacons combined with NASBA for the sensitive detection of sugarcane yellow leaf virus. *European Journal of Plant Pathology* 108, 401–407.
- Gore, H.M., Wakeman, C.A., Hull, R.M., McKillip, J.L., 2003. Real-time molecular beacon NASBA reveals *hblC* expression from *Bacillus* spp. in milk. *Biochemistry and Biophysics Research Communication* 311 (2), 386–390.
- Gray, M., Wawrik, B., Paul, J., Casper, E., 2003. Molecular detection and quantification of the red tide dinoflagellate *Karenia brevis* in the marine environment. *Applied and Environmental Microbiology* 69 (9), 5726–5730.
- Hibbitts, S., Rahman, A., John, R., Westmoreland, D., Fox, J.D., 2003. Development and evaluation of NucliSens Basic Kit NASBA for diagnosis of parainfluenza virus infection with 'end-point' and 'real-time' detection. *Journal of Virological Methods* 108, 145–155.
- Lanciotti, R.S., Kerst, A.J., 2001. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. *Journal of Clinical Virology* 39 (12), 4506–4513.
- Leone, G., van Schijndel, H., van Gemen, B., Kramer, F.R., Schoen, C.D., 1998. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Research* 26, 2150–2155.
- Peter, J.B., Sevall, J.S., 2004. Molecular-based methods for quantifying HIV viral load. *AIDS Patient Care and STDS* 18 (2), 75–79.
- Polstra, A.M., Goudsmit, J., Cornelissen, M., 2002. Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes. *BMC Infectious Diseases* 2 (1), 18.
- Schallig, H.D., Schoone, G.J., Lommerse, E.J., Kroon, C.C., de Vries, P.J., van Gool, T., 2003. Usefulness of quantitative nucleic acid sequence-based amplification for diagnosis of malaria in an academic hospital setting. *European Journal of Clinical Microbiology and Infectious Diseases* 22 (9), 555–557.
- Stocher, M., Berg, J., 2002. Normalized quantification of human cytomegalovirus DNA by competitive real-time PCR on the LightCycler instrument. *Journal of Clinical Microbiology* 40 (12), 4547–4553.
- Vernet, M., Mitchell, B.G., Holm-Hansen, O., 1990. Adaptation of *Synechococcus* in situ determined by variability in intracellular phycoerythrin-543 at a coastal station of the Southern California coast, USA. *Marine Ecological. Progress Series* 63, 9–16.
- Weusten, J.J.A.M., Wouters, P.A.W.M., vanZuijlen, M.C.A., van de Wiel, P.A., 2002a. Stochastic processes defining sensitivity and variability of internally calibrated quantitative NASBA-based viral load assays. *Nucleic Acids Research* 30 (24), e137.
- Weusten, J.J.A.M., Carpay, W.M., Oosterlaken, T.A.M., vanZuijlen, M.C.A., van de Wiel, P.A., 2002b. Principles of quantitation of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Research* 30 (6), e26.