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Full Length Original Research Article

**Detection and Quantification of the Toxic Microalgae *Karenia brevis* using Lab on a Chip mRNA Sequence-Based Amplification**

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**Abstract**

Now and again, the rapid proliferation of certain species of phytoplankton can give rise to Harmful Algal Blooms, which pose a serious threat to marine life and human health. Current methods of monitoring phytoplankton are limited by poor specificity or by the requirement to return samples to a highly resourced, centralised lab. The Lab Card is a small, microfluidic cassette which, when used in tandem with a portable Lab Card Reader can be used to sensitively and specifically quantify harmful algae in the field, from nucleic acid extracts using RNA amplification; a sensitive and specific method for the enumeration of potentially any species based on their unique genetic signatures. This study reports the culmination of work to develop a Lab Card-based genetic assay to quantify the harmful algae *Karenia brevis* using mRNA amplification by the Nucleic Acid Sequence Based Amplification (NASBA) method. *K. brevis* cells were quantified by amplification of the *rbcL* gene transcript in nucleic acid extracts of *k. brevis* cell samples. A novel enzyme dehydration and preservation method was combined with a pre-existing reagent Gelification method to prepare fully preserved Lab Cards with a shelf-life of at least six weeks prior to use. Using an internal control (IC), the Lab Card-based *rbcL* NASBA was demonstrated for the quantification of *K. brevis* from cell extracts containing between 50 and 5,000 cells. This is the first demonstration of quantitation of *K. brevis* using IC-NASBA on a Lab Card.

**Introduction**

Nucleic acid sequence amplification is a state of the art analytical tool for the identification and enumeration of microorganisms in the ocean including the specific recognition of species that pose a threat to human and animal health. However, this can require the retrieval of samples from remote or hazardous off-shore locations, prior to analysis in a centralised lab, leading to delayed results, increased risks and high economic costs. Accordingly, there is an increasing demand for autonomous systems that can make accurate (laboratory standard) nucleic acid-based measurements *in situ*, using portable or deployable self-powered instrumentation 1. These systems could be particularly useful for the measurement and surveillance of harmful algal blooms (HABs), which result from the rapid proliferation of certain species of phytoplankton, and which can occur stochastically and offshore 2, 3. The incidence of HABs has been increasing globally for some time 4 and there is a demand for new tools to provide a more reliable early warning of their occurrence 5.

Existing lab-based methods for the measurement of phytoplankton including harmful algae are often based on various forms of nucleic acid analysis (molecular methods) 6-8, flow cytometry 9, 10 or microscopy 11. In addition, multispectral satellite imaging can be used for the indirect (based on radiance / ocean colour) measurement of phytoplankton *in situ* 12, 13, but is non-specific and limited by a low spatial resolution and high limit of detection (LOD). Deployable flow cytometers such as the CytoBuoy 14, CytoSub 15 and Imaging FlowCytobot 16, 17 can measure phytoplankton cells directly in seawater, but these instruments cannot always reliably discriminate HAB species from morphologically similar, benign cell types. Nucleic acid amplification has the principle advantages of high sensitivity and specific recognition and discrimination of the target species based on unique genetic signatures, even from a complex, mixed species sample. For example, a number of nucleic acid-based techniques have been demonstrated for the selective enumeration of *Karenia brevis* 18, 19, a bio-toxin producing microalga which periodically gives rise to HABs known colloquially as “red tides”, and which pose a severe threat to wildlife, and can cause respiratory irritation in humans 20-25. Specifically, *K. brevis* cells can be quantified from genetic extracts of seawater samples using Nucleic Acid Sequence Based Amplification (NASBA) of the *rbcL* gene transcript 19. This encodes a large subunit of the *K. brevis* RuBisCo enzyme, which catalyses the first step in CO2 fixation and has been employed as a proxy for the detection and analysis of phytoplankton groups in environmental samples 26. NASBA is an RNA amplification technique which features a practical LOD of 10 cells per litre of processed water 27 and, due to the highly labile nature of mRNA, it preferentially measures viable, transcriptionally active cells over dead cells 28.

The potential to use NASBA for the quantification of *K. brevis* from nucleic acid extracts of cell samples *in situ* has been demonstrated using a miniature Lab on a Chip (LOC), known as a ‘Lab Card’, and a small, battery powered ‘Lab Card Reader’ 29, developed as part of the European LABONFOIL project (www.labonfoil.eu). Although the Lab Card Reader does not feature nucleic acid extraction capability, it could be combined with any of the large number of portable ‘extraction’ devices currently under development 30, 31 such that all steps required for *in situ* nucleic acid analysis could be performed in the field. The structure and composition of a Lab Card and Lab Card Reader has been described in detail by Tsaloglou *et al* 29, and summarised here, shown in Figure 1. The Lab Cards are disposable (single use) plastic, credit-card-sized microfluidic assemblages featuring two micro-chambers (approx. 10 μL in volume) in which (i) RNA sample is mixed with heat-tolerant NASBA reagents (a pH-buffered solution of co-factor ions and nucleotides) and denatured before passing to a second chamber where (ii) the mixture is combined with heat-sensitive enzymes and incubated at a stable, low temperature (41oC) to achieve the amplification of the target sequence. The ‘Lab Card Reader’ is a small, portable instrument, which features peristaltic pumps and disc valves to actuate the Lab Card microfluidics, heaters to control the temperature of each micro-chamber and a fluorescence optics module which measures in real-time the emission of multi-wavelength fluorescence. An autonomous workflow can be configured and initiated via an interface with a Tablet computer.

In addition to the Lab Card system, Tsaloglou *et al* 29 have reported that *K. brevis* *rbcL* mRNA can be amplified on a Lab Card in a multiplexed reaction featuring a co-amplified ‘internal control’ construct of known concentration. This could theoretically enable accurate quantitation of *rbcL* without the need to run multiple standards in parallel or replicate reactions, using a method invented by Weusten *et al* 32, 33 and adapted for the real time quantification of *K. brevis* by Casper *et al* 34 and Patterson *et al* 35. Furthermore, the reactions could be performed on a Lab Card using NASBA reagents that were pre-prepared and preserved using a proprietary Gelification technology, however wet enzyme mixtures had to be prepared and added manually. In the present study, we report the development of fully pre-prepared and preserved (enzymes and reagents) Lab Cards that can perform all stages of *rbcL* NASBA in an autonomous workflow and we demonstrate for the first time the use of the system to quantify *K. brevis* from RNA extracts of samples containing a range of *K. brevis* cell concentrations spanning two orders of magnitude.

**Materials and Methods**

***Karenia brevis* Culture**

*K. brevis* (Strain CCMP2228) was obtained from the Provasoli-Guillard National Centre for the Culture of Marine Phytoplankton (CCMP), and maintained in static cultures at 19 ± 1°C in L1 medium 36, with a 12 hour photoperiod using cool fluorescent light. Cell counts were performed using a Sedgewick Rafter counting chamber (Fisher Scientific, UK) and an inverted microscope after fixation and staining in Lugol’s Iodine.

**The Lab Card System**

Full details of the Lab Card system and Lab Card Reader instrument have been described in previous work by Tsaloglou *et al* 29, but a brief overview of the system is illustrated in Figure 1. The Lab Cards are fabricated by injection moulding Cyclic Olefin Copolymer (COC) to form a series of microfluidic channels (approx. 250 μm wide x 450 μm deep) and two reaction chambers (approx. 10 μL volume). The COC is laminated between self-adhesive polypropylene film to seal the features. Luer connectors are used to add RNA sample and recover waste. When inserted into a Lab Card Reader, the microfluidic outlets are connected to peristaltic pumps to actuate fluid flow, which is directed through the Lab Card using Cyclic Olefin Polymer (COP) disc valves controlled by linear actuators. The two chambers sit adjacent to resistive micro-heaters and the second chamber (where NASBA occurs) is covered by a two-channel fluorescence reader. Prior to assembly, each Lab Card was decontaminated by immersion in RNaseZap® (Sigma-Aldrich, UK) and cleaned using an ultrasonic bath containing sterile, nuclease-free water. The Lab Card Reader was controlled through an interface with a tablet computer.

**RNA Isolation**

Total RNA was isolated from 1 mL samples of 50 mL cultures containing exponentially dividing *K. brevis* cells. Each sample was mixed with 1 mL of a custom lysis buffer containing 100 mM Tris-HCl (pH 7.5), 4 M GuSCN, 500 mM LiCl, 10 mM EDTA and 1% (v/v) Triton X-100. Cell lysis proceeded at room temperature for 10 minutes, followed by the addition of a 50 μL colloidal suspension of magnetic beads (bioMérieux UK Ltd). After 10 minutes each sample was centrifuged at 1,500 g for two minutes and the supernatant was discarded. For the preparation of RNA samples containing an Internal Control (IC), a known quantity of IC RNA construct was added to the lysis buffer prior to the addition of the magnetic beads. After centrifugation the beads were washed twice in 500 μL of a custom wash buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM LiCl and 1 mM EDTA using a NucliSENS® miniMAG® (bioMérieux UK Ltd). RNA was eluted from the beads after suspension in 10 mM Tris-HCl (pH 7.5) and heating to 60°C for five minutes with agitation at 1,200 rpm using an Eppendorf Thermomixer (Eppendorf, USA). The beads were removed by centrifugation, and the supernatant containing the eluted RNA was stored at -20°C.

**Nucleic Acid Sequence Based Amplification (NASBA)**

NASBA was carried using out using reagents and enzymes supplied in the NucliSENS® EasyQ Basic Kit V2 (bioMérieux UK Ltd), and custom oligonucleotide primers and molecular beacon probes obtained from Eurofins (Eurofins Genomics, Germany), shown in Table 1. NASBA was performed using either commercially available laboratory equipment (an EasyQTM fluorescence micro-plate reader; bioMérieux UK Ltd), referred to herein as ‘bench-top NASBA’, or the portable Lab Card system according to the method described by Tsaloglou *et al* 29, with modifications for the use of preserved reagents and internal control-based quantification as described below. The oligonucleotide primers directed the amplification of an 87 nucleotide sequence within the *K. brevis* *rbcL* gene transcript, or in the case of IC-NASBA, a synthetic RNA construct of the same length (Table 1). The amplification was measured in real time using the fluorescence emission of dual molecular beacon probes specific for either the *rbcL* transcript or IC, and conjugated to unique fluorophore / quencher pairs (AF488 / BHQ1 and Cy5 / BHQ2 respectively; Table 1). NASBA amplification followed a two-step procedure. First, the RNA template was mixed with a reaction buffer containing the oligonucleotide primers and molecular beacon probes, and heated to 65°C for two minutes (bench-top) or five minutes (Lab Card) for optimal primer annealing. Then, the reaction mixture was cooled to 41°C and mixed with a heat-labile enzyme mixture. Amplification occured at 41°C for 90 minutes.

**Internal Control**

An Internal Control (IC) construct for *K. brevis* was prepared as described by Tsaloglou *et al* 29, and contained the same primer annealing sites as the *rbcL* target sequence, but a unique molecular beacon binding site. The IC sequence is given in Table 1. To demonstrate quantitative NASBA on the Lab Cards, samples were spiked with 3,000 IC copies following cell lysis and total RNA isolation. Quantification was achieved by comparing the amplification of the *rbcL* transcript and IC, which occurred simultaneously in each NASBA reaction, and which were measured independently using dual molecular beacons with different emission profiles. This comparison was made either based on the time taken for each amplification plot to reach a threshold fluorescence value (the Time To Positivity; TTP) 34, 35, or based on the ratio of a ‘Quantitation Variable’ derived from a non-linear fit to each curve according to Weusten *et al* 32, 33.

**Table 1. Oligonucleotides and Target Sequences for *K. brevis* IC-NASBA.**

|  |  |
| --- | --- |
| **Name** | **Sequence (5’ – 3’)** |
| ***rbcL* Forward Primer** | ACGTTATTGGGTCTGTGTA |
| ***rbcL* Reverse Primer** | AATTCTAATACGACTCACTATAGGGAGAAGGTACACACTTTCGTAAACTA |
| **rbcL Molecular Beacon** | [AF488]GAGTCGCTTAGTCTCGGGTTATTTTTTCGACTC[BHQ1] |
| **IC Molecular Beacon** | [CY5]ACGGAGTGGCTGCTTATGGTGACAATCTCCGC[BHQ2] |
| **rbcL Target Sequence** | GAAACGTTATTGGGTCTGTGTACACGAATTAACCTTAGTCTCGGGTTATTTTTTGGACAAGAATGGGCTAGTTTACGAAAGTGTGTACCT |
| **IC Sequence** | GAAACGTTATTGGGTCTGTGTACACGAATTAACTGGCTGCTTATGGTGACAATGGACAAGAATGGGCTAGTTTACGAAAGTGTGTACCT |

**Gelification of NASBA Reagent Mixtures**

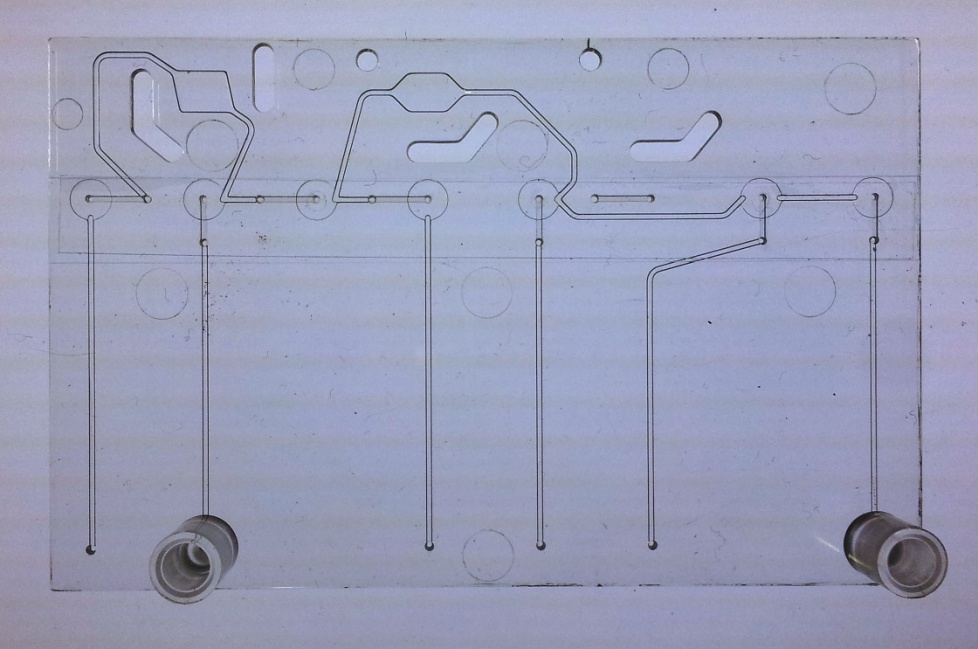
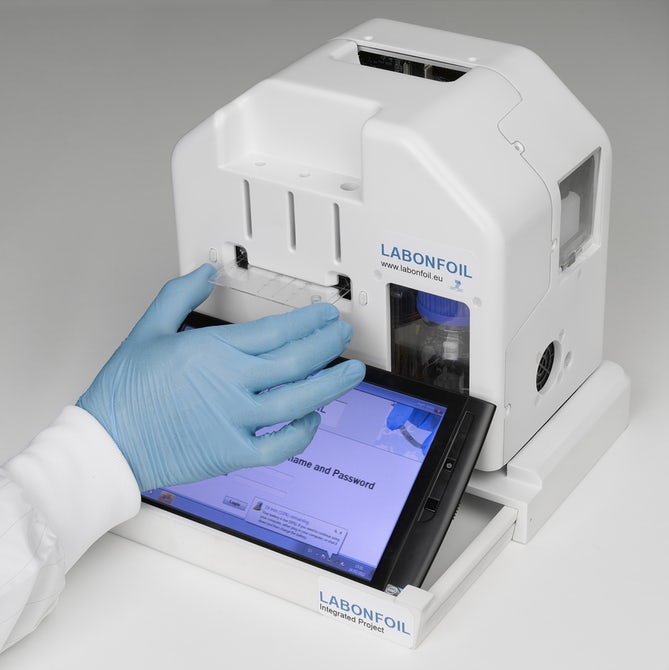
NASBA reagents were prepared from lyophilised stocks supplied in the NucliSENS® EasyQ Basic Kit V2 (bioMérieux UK Ltd). The reagents were supplemented with 800 nm of each oligonucleotide primer, 200 nM of each molecular beacon probe, 200 nM of KCl and 160 mM of a proprietary reagent stabilisation (Gelification) component (BioTools B&M Labs S.A., Madrid, Spain); the final volume of each batch was 100 μL. The mixture was added to either a sterile, nuclease free 0.2 mL polycarbonate tube or a Lab Card, and dehydrated at 35°C under low pressure (30 mbar) for 35 minutes.

**Dehydration of NASBA Enzyme Mixtures**

NASBA enzyme mixtures were prepared from lyophilised stocks supplied in the NucliSENS® EasyQ Basic Kit V2 (bioMérieux UK Ltd). The enzyme mixtures were hydrated according to the manufacturers recommendation, and supplemented with a sugar solution containing 50% (w/v) each of sucrose and trehalose in sterile, nuclease-free water. Each batch of enzyme-sugar mixture contained 10% (w/v) of each disaccharide in a total volume of 50 μL. The mixture was either added to a sterile, nuclease free 0.2 mL poly-carbonate tube or a Lab Card and dehydrated at 21°C, under low pressure (30 mbar) for 16 hours.

**Results and Discussion**

Previously, a real time NASBA assay was developed to quantify the harmful microalga *K. brevis*, based on the amplification of *rbcL* (RuBisCo) mRNA, on a disposable microfluidic Lab Card 29. Two key developments are reported herein: (1) the use of fully pre-prepared and preserved reaction mixtures including dehydrated enzymes and (2) the first demonstration of Lab Card NASBA for the enumeration of *K. brevis* from nucleic acid extracts of cells over a range of environmentally relevant concentrations spanning two orders of magnitude. The culmination of this work is proof of concept that *K. brevis* mRNA can be quantified using the battery-powered Lab Card system without a centralised laboratory or bulky apparatus.

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**B**

**A**

**Figure 1.** An overview of the Lab Card system, adapted from Tsaloglou *et al* 29. Lab Cards are credit-card sized, injection moulded COC cartridges featuring microfluidic channels and micro-chambers (left panel). Lab Cards are inserted into the Lab Card Reader (right panel). Once inserted, peristaltic pumps actuate the movement of an RNA sample into the first Lab Card chamber (A), where it re-hydrates a mixture of preserved (Gelified) NASBA reagents and oligonucleotides. Heating units on the Lab Card Reader raise the temperature of the chamber to 65oC for 5 minutes, after which the fluid is moved into a second Lab Card chamber (B), were it re-hydrates and activates a mixture of preserved NASBA enzymes. This chamber is heated to 41oC for 90 minutes during which the RNA sample is amplified resulting in the emission of fluorescence from molecular beacons. Amplification is measured in real-time based on the fluorescence emission using a two channel optics module. The process is configured and controlled via an interface between the Lab Card Reader and a tablet computer.

**Enzyme preservation**

Standard NASBA enzyme mixtures contain T7 RNA Polymerase (RNAP), Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and RNase H. Hydrated enzymes are unstable at room temperature and must be refrigerated or frozen, thus dry preservation is a principle requirement for the development of field-based assays where refrigeration is unavailable. Accordingly, a method was developed for preparing stabilised, dehydrated enzyme mixtures, which could be stored for long periods (weeks to months) at ambient temperatures in a disposable Lab Card. To do this, enzyme mixtures were prepared using the NucliSENS® EasyQ Basic Kit V2 (bioMérieux UK Ltd) according to the manufacturers recommendations, but including a final concentration of 10% (w/v) sucrose and trehalose sugars, and then dehydrated under low pressure. Sucrose and trehalose interact with the enzymes in a manner which stabilises their tertiary structure during dehydration. The precise mechanism by which these sugars preserve protein structure is still not fully understood, but prevailing theories include the displacement and substitution of the protein’s hydration shell such that the removal of water does not change the molecular structure 37. The preservation of the enzymes using this method did not alter their ability to amplify *K. brevis rbcL* mRNA using NASBA, as shown in Figure 2. The NASBA reactions were measured in real-time using a molecular beacon, indicating that the presence of sucrose and trehalose did not significantly change the total yield of NASBA product (i.e. total fluorescence), but was associated with a small decrease in the rate of amplification. In contrast, the dehydration of the enzymes in the absence of sucrose and trehalose led to a complete loss of enzyme activity (not shown). The reduction in amplification efficiency probably reflects some degradation of the enzymes during the dehydration process rather than any unfavourable interactions with the sugars; the addition of sugars to freshly prepared enzyme stocks had no effect on amplification (not shown). The preservation of the enzymes also increased the variability between replicate NASBA reactions, as shown in Figure 2, reflecting differences in the preparation of each batch; however the influence of this variability on NASBA-based quantification of the target sequence is removed using an internal control, as described below. Other suitable methods for the dry preservation of proteins include encapsulation by Pullulan, a polysaccharide that dries to form an oxygen impermeable film, but which is not currently widely available 38. Alternatively, trehalose-containing protein samples have been efficiently preserved directly onto fibrous substrates including glass fibre 39, however the incorporation of insoluble material into the chambers of a Lab Card could lead to blockage of the microfluidics. Therefore these methods were not considered further.

Figure 1.tif

**Figure 2.** NASBA of *K. brevis* *rbcL* mRNA was measured using the fluorescence emission of a *rbcL*-specific molecular beacons containing a Cy5 fluorophore. The NASBA was carried out using freshly prepared enzymes (positive control) or enzymes that had undergone a dehydration and preservation process and which contained a disaccharide mixture. No amplification was measured in the absence of mRNA (negative control). The error bars represent the standard deviation from the mean (n=3).

Figure 2_2.tif

**Figure 3.** NASBA of *k. brevis* *rbcL* mRNA was measured using the fluorescence emission of a *rbcL*-specific molecular beacon containing Cy5. The NASBA was carried out using reagents that had been partially dehydrated and preserved by a Gelification process, and stored for six months, and enzymes that had been dehydrated and preserved with a disaccharide mixture, and stored for up to six weeks. No amplification was measured after extended storage of the reagents for eleven months. The error bars represent the standard deviation from the mean (n=3).

**Testing of Fully Pre-prepared and Preserved NASBA Reactions**

Fully preserved NASBA reactions were prepared using the aforementioned enzyme dehydration process in combination with a NASBA reagent ‘Gelification’ process previously described in earlier work by Tsaloglou *et al* 29. These components (enzymes and reagents) must be preserved separately because the enzymes are sensitive to heat and must be added to the reaction after a high temperature (65oC) primer annealing stage. The NASBA reagent mixture contains a buffered solution of inorganic co-factors, a reducing agent (dithiothreitol; DTT), nucleotide triphosphates (NTPs) and ribonucleotide triphosphates (rNTPs) as well as a sequence-specific oligonucleotide primers and molecular beacon probes; these components determine the specificity of the NASBA assay and could be interchanged for alternative target sequences. The hydrated reaction mixture can degrade by a number of processes including the disproportionation of NTPs and rNTPs into di- and tetra-phosphate derivatives, and therefore preservation is essential. However complete dehydration of the mixture was found to be inappropriate for use with Lab Cards due to difficulty in re-hydrating the preparation into a homogeneous suspension in a microfluidic system. Gelification is a process of encapsulating compounds in a protective matrix, which offers a compromise, enabling a reagent mixture to be partially dehydrated to extend its lifetime, but also more easily solubilised. A propriety, patented Gelification reagent was provided by BioTools B&M Labs S.A., and added to the NASBA reagent mixtures, which were partially dehydrated under low pressure. The long-term stability of both dry enzyme / sugar mixtures and partially dehydrated (Gelified) reagent mixtures when stored at room temperature (21oC) was tested by hydrating the mixtures using a *K. brevis* mRNA sample and heating to initiate NASBA reactions. For convenience, these tests were carried out using conventional ‘bench-top’ NASBA apparatus rather than Lab Cards, but were later applied to the Lab Card system. The Gelified reagent mixtures were stored for six months, and combined with dry enzyme mixtures stored for between 24 hours and six weeks. The stored mixtures could be used to amplify *K. brevis rbcL* mRNA, and with equivalent yield when compared to reactions prepared from fresh mixtures (without preservation), but with a subtle reduction in amplification rate, as shown in Figure 3. Contrary to expectation, the reactions prepared using six-week old enzymes produced an increased rate of amplification over enzymes stored for just one week. This difference, although minimal, may reflect differences in the conditions under which the preserved enzymes were prepared or batch-to-batch variations in the EasyQ kit from which they were sourced. Longer storage of the mixtures (enzymes up to 5 months and reagents up to 11 months) resulted in unsuccessful NASBA, indicating substantial degradation of their components.

**Quantification of *K. brevis* mRNA Using ‘Fully Preserved’ Lab Cards**

‘Fully preserved’ Lab Cards (in which both the NASBA reagents and NASBA enzymes were preserved) were prepared using the enzyme and reagent preservation processes described above. Aliquots of Gelified reagent mixtures and enzyme-sugar mixtures were added to separate chambers of the Lab Cards (see Figure 1) and dehydrated under low pressure. The fully preserved Lab Cards were sealed and stored at ambient temperature for 28 days prior to use, during which they were shipped internationally between Spain, Denmark and the UK using standard courier freight; this provided a suitable test of their durability. The reagent mixtures contained oligonucleotide primers and an *rbcL*-specific molecular beacon for the amplification and real-time detection of *K. brevis* *rbcL* mRNA. In addition, the mixtures contained a second molecular beacon with a unique fluorophore, which was specific for a synthetic RNA construct, known as an internal control (IC) for quantitation according to a method invented by Weusten *et al* 32, 33 and previously adapted for the *K. brevis rbcL* NASBA assay 34, 35. A detailed description of this method is provided by Patterson *et al* 35, and described briefly here. The internal control (IC) is a short, synthetic sequence of RNA which is mostly identical to the target (‘wild-type’) sequence and therefore, when added to a the wild-type RNA sample, both are co-amplified using the same primer pool, with the exception that the IC contains a unique molecular beacon annealing site. Thus, for internally controlled NASBA (IC-NASBA) two beacons are employed in tandem with different fluorescence emission wavelengths; one of these is used to plot the amplification of the mRNA sample and the other is used to simultaneously plot the amplification of the internal control. In this case, both are measured using the two-channel optics of the Lab Card Reader. The IC is added to the cell lysate during the nucleic acid extraction procedure, and therefore any variation in the efficiency of the extraction and subsequent amplification will affect the IC and wild-type in tandem. Comparison of wild-type and IC amplification in each reaction can be used to reduce variation between runs and obviates the need to run standard curves for each experiment or replicate reactions 34. A quantification curve is prepared prior to the measurement of unknowns in which mRNA extracts from a dilution series of known cell numbers, which have been ‘spiked’ with an appropriate concentration of IC following cell lysis, are amplified using NASBA. Then, the same concentration of IC is added to unknown cell samples prior to extraction and NASBA. The comparison between wild-type and IC can be made based on either the ‘Time to Positivity’ (TTP) 19, 34, 35, defined as the time taken for the amplification, measured using molecular beacon fluorescence, to reach a specified threshold, or ‘Quantitation Variable’ (QV), derived from non-linear fit of the two amplification profiles 29, 32, 33 using Equation 1.

Equation 1:

Here, Y(t) is the fluorescence emission from a molecular beacon as a function of time, Y0 is the background fluorescence emission at the zero time point, λY0 is the maximum fluorescence value, α1α2 represents the shape of the curve, α3 is the curve location relative to time and K1 is a reaction rate constant 29, 32, 33.

In this study, IC-NASBA on a Lab Card was carried out using *K. brevis* mRNA extracted from 5,000, 500, and 50 cells, spiked with 3,000 IC RNA copies, using a Lab Card Reader as described by Tsaloglou *et al* 29. The detection of low cell numbers (~ 50 cells per sample) is essential for the early warning of *K. brevis* blooms before they can proliferate to critical levels, whilst the enumeration of higher cell numbers (~ 5,000 cells per sample) can be used to inform upon which intervention should be enacted (e.g. in the State of Florida, USA a *K. brevis* concentration of more than 5,000 cells per litre of seawater requires Shellfish bed closures and a period of continuous monitoring). Each *K. brevis* mRNA sample was quantified twice using different Lab Cards, by comparing the amplification of the unknown (wild-type mRNA) and known (IC RNA) templates using TTP and QV analysis. As shown in Figure 4, amplification curves for the same RNA sample, but run on different Lab Cards, were highly variable, reflecting differences in the assembly of each unit, which are currently prepared by hand. Nonetheless, since this variability was applied to both mRNA and IC RNA we expect quantification to be consistent from run to run. The results, shown in Figure 5, demonstrate a clear trend between the ratio of wild-type to IC, based on either TTP or QV, and cell number. A linear fit of the data sets indicated an R-squared value of 0.996 for the TTP-based analysis and 0.968 for the QV-based analysis, suggesting TTP is a more accurate metric by which to quantify mRNA copy number. However, the QV-based analysis had a greater slope (0.637) than the TTP equivalent (0.160) indicating greater resolution in cell number. Additionally, the standard deviation (error bars) from TTP-based analysis increased in line with cell number, reaching a value of ±0.067 at 5,000 cells. In contrast, the standard deviation recorded from the QV-based analysis was overall significantly smaller indicating that it may provide increased precision for cell quantification.

This study follows previous work by Tsaloglou *et al* 29 to develop a portable and autonomous system for the measurement of *K. brevis* using IC NASBA. Here, we have demonstrated that the Lab Card system can be used to measure *K. brevis* from nucleic acid extracts over two orders of magnitude using fully preserved (i.e. reagents and enzymes) microfluidic cartridges which can be stored for up to six weeks and are suitably robust to withstand international freight shipping. With the addition of effective enzyme preservation, and proof of concept over a wide range of target cell concentrations (two orders of magnitude), this system could be used improve current surveillance of *K. brevis* and other HABs by providing specific measurements, which don’t rely upon a highly resourced laboratory and could be performed in the field. However, true *in situ* measurement is still dependent upon the provision of portable nucleic acid extraction methods of which there are many under development. Nonetheless, the Lab Card system and others like it are likely to become increasingly important as changes to the global ocean drive the demand for high resolution algal surveillance data. Due to their unparalleled specificity, nucleic acid analysis (molecular) methods are likely to remain at the forefront of ocean observing systems for marine microbiology.

Figure 3 complete.tif

Figure 4. IC-NASBA of *K. brevis* *rbcL* mRNA using Lab Cards featuring preserved enzyme and reagent mixtures, and which had been stored for four weeks including international shipping. The reactions were monitored in real-time using the Lab Card Reader to measure the fluorescence emission of two molecular beacons, specific for the target (wild-type) and internal control sequence. The RNA samples were prepared from 5,000 (a), 500 (b), or 50 (c) *K. brevis* cells and spiked with 3,000 IC copies following cell lysis. The error bars represent standard deviation between duplicate Lab Cards used to measure each sample.

Figure 4_2hr.tif

Figure 5. Quantification curves for *K. brevis* using the Lab Card *rbcL* NASBA assay data shown in Figure 4. RNA was isolated from either 50, 500 or 5,000 *K. brevis* cells and spiked with 3,000 internal control (IC) copies. The quantification curve was prepared by comparing the amplification of wild-type and IC RNA from each sample based on either the Time To Positivity (TTP; left panel) or Quantitation Variable (ln(k1a1a23 ratio). The error bars represent standard deviation between the duplicate Lab Cards run using each sample. A linear fit to each data set shows how, despite run-to-run variability between Lab Cards, the use of an internal control can reduce the standard deviation and produce a strong linear relationship over samples containing *K. brevis* cells spanning two orders of magnitude. Details of the linear relationship are shown inset.

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