Universal rapid RNA-based quantification of toxigenic *Alexandrium* species (Dinophyceae) using quantitative recombinase polymerase amplification

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Abstract

Harmful algal blooms caused by toxigenic *Alexandrium* species pose recurrent risks to coastal ecosystems and public health, yet current monitoring approaches rely on microscopy and laboratory-based toxin analysis with limited capacity for rapid, functional early warning. Here, we present a universal quantitative reverse-transcriptase recombinase polymerase amplification (qRT-RPA) assay targeting the *sxtA4* transcript, an essential gene in saxitoxin biosynthesis. The RPA chemistry is isothermal with a low running temperature, making it suitable for portable, on-site testing. The assay was designed against a conserved *sxtA4* region and validated using synthetic templates, isolated amplicons and total RNA from multiple *Alexandrium* species. Using *Alexandrium minutum* as a reference, the assay achieved a limit of detection below 10^3 synthetic RNA copies and 0.1 ng total RNA per reaction, with uniform amplification kinetics across species and a runtime of less than 15 minutes. The assay selectively detected *sxtA4* transcripts from toxigenic *Alexandrium* strains, showed no cross-reactivity with non-target phytoplankton, and retained it's LoD in complex RNA matrices. These results establish qRT-RPA as a rapid, RNA-

26 based, functionally informative molecular tool that provides a foundation for portable, early-
27 warning monitoring of toxigenic *Alexandrium* blooms.

28

29 **Keywords**

30 *Alexandrium*, saxitoxin, *sxt*A4 RNA, recombinase polymerase amplification, harmful algal
31 blooms

1 Introduction

Harmful Algal Blooms (HABs) are posing increasing ecological and socioeconomic pressure in coastal areas worldwide. Their global expansion is attributed to anthropogenic nutrient enrichment and ocean warming (Dai et al., 2023; Gobler et al., 2017; Griffith et al., 2019; Hayashida et al., 2020; Rabalais et al., 2007; Yu et al., 2023). Warming temperatures can promote bloom initiation (Anderson et al., 2021a) accelerated cell growth rates and prolonged bloom seasons (Anderson et al., 2021a; Gobler et al., 2017; Griffith et al., 2019) especially in temperate regions (Dai et al., 2023), whilst also allowing their intrusion into previously unaffected polar areas (Anderson et al., 2021a). The consequences of HABs range from ecosystem-scale deoxygenation to the accumulation of potent toxins that threaten food security and human health (Anderson et al., 2021a; Anderson et al., 2021b). Despite progress in ocean observing systems, early-warning capacity often remains limited, particularly in regions where toxic events develop rapidly or surveillance infrastructure is sparse.

Most regulatory monitoring programs still rely on a dual structure: (i) microscopic identification and enumeration of phytoplankton in water samples, and (ii) chemical toxin analysis in shellfish tissue [EC 853/2004; Turner 2014; Parks 2020]. The first assesses putatively toxic cell abundance, while the second measures saxitoxin and congeners which are converted to toxicity equivalence factors (TEFs) (Commission Implementing Regulation (EU) 2021/1709, (Commission., 2021)). Concerned countries like the UK follow this regime with practical thresholds of 400 STX eq. (Turner et al., 2014) in shellfish flesh or 40 - 100 *Alexandrium* cells L^{-1} of water triggering intensified monitoring frequency (Parks et al., 2020; Senovilla-Herrero et al., 2025). While these methods underpin long-established monitoring frameworks, they present significant operational constraints. Microscopy cannot reliably distinguish between toxic and non-toxic strains of morphologically similar taxa and provides no functional information on toxin biosynthesis. Meanwhile, toxin chemical analyses by HPLC (Standard EN 14526) requires centralized laboratories, limiting sampling frequency and increasing turnaround time to minimum of 3 days. Together, these limitations hinder early warning and intervention, increasing the likelihood of food contamination and ecological damage.

62 Molecular amplification-based represent an alternative to microscopy analysis, offering higher
63 sensitivity, faster time-to-result, species-level resolution and quantification of specific cellular
64 functions (John et al., 2007; Kon et al., 2015; Murray et al., 2019). A wealth of DNA-based qPCR
65 assays to enumerate specific toxic species (Dai et al., 2020; Erdner et al., 2010; Gao et al., 2015;
66 Garneau et al., 2011; Hatfield et al., 2019; Ruvindy et al., 2018; Toebe et al., 2013; Vandersea et
67 al., 2017). Most assays target the small or large ribosomal genes of specific *Alexandrium* species
68 to provide abundance measurements, but are not able to assess the populations' toxigenic potential.
69 Several qPCR assays also target genes of the saxitoxin cluster, to directly assess toxigenic potential
70 (Gao et al., 2015; Han-Sol et al., 2024; Murray et al., 2019; Murray et al., 2011; Penna et al., 2015;
71 Savela et al., 2016; Stüken et al., 2013). Nevertheless, the transition from traditional light
72 microscopy to molecular monitoring for HAB surveillance has been slow to materialize. While
73 qPCR is the gold standard of molecular detection due to its cost-effectiveness and high throughput,
74 it is limited to a laboratory setting due to the requirement for high-power consuming thermal
75 cycling. Therefore, it does not overcome the need for centralized analytical facilities and
76
77 HAB surveillance will significantly benefit from on-site or *in situ* monitoring offering real-time
78 detection. In contrast to qPCR, isothermal amplification can achieve molecular quantification in
79 remote field settings, using portable battery-powered analyzers (e.g. Pebble R, BIOPIX-T;
80 TwistAmp, TwistDx). In particular, recombinase polymerase amplification (RPA) operates at a
81 low amplification at a low, stable temperature (37 – 42 ºC), has high tolerance to impurities and a
82 fast time-to-result in less than 15 minutes (Tan et al., 2022). RPA chemistry can be used for
83 qualitative detection using lateral flow strips (Liu et al., 2023; Yao et al., 2024; Yu et al., 2025)
84 and quantitative analysis by incorporating fluorescent probes into the reaction, with run times
85 below 15 minutes (Tan et al., 2022). Also, RNA can be amplified in one step simply by adding
86 reverse transcriptase in the RPA reaction. These features make RPA a versatile and fast
87 amplification technology that is suitable for portability, that can be applied to quantify functional
88 genes in field-monitoring applications.
89
90 Here, we use the HAB forming, saxitoxin-producing dinoflagellate genus *Alexandrium* as a case-
91 study, to develop and evaluate a qRPA assay for RNA-based quantification of metabolically active
92 toxigenic cells. *Alexandrium* species are globally distributed and responsible for recurrent paralytic

shellfish poisoning (PSP) outbreaks (Anderson et al., 2012; Gribble et al., 2025; Lewis et al., 2018; Suleiman et al., 2017). They produce an array of toxins as secondary metabolites, being most well-known for the production of saxitoxins that cause Paralytic Shellfish Poisoning (PSP). Saxitoxin biosynthesis in dinoflagellates is linked to a family of genes homologous to the cyanobacterial *sxt* cluster (Kellmann et al., 2008; Stüken et al., 2011). The *sx*tA4 domain is recognised as essential for toxin production and strongly correlated with transcript abundance and intracellular toxin content (Geffroy et al., 2021; Murray et al., 2015). This makes the *sxt*A4 gene domain an appropriate target for quantifying toxic populations rather than simply detecting genus presence.

In this study, we present the development, optimization and performance testing of a universal *Alexandrium sxt*A4 RNA-based qRT-RPA assay. To our knowledge, this is the first report of an RNA-based quantitative RPA assay for a harmful algal bloom species. We present optimization strategies for qRT-RPA and quantify analytical sensitivity using synthetic and total RNA templates. We establish realistic limits of detection and standard curves for sample quantification using three *A. minutum* strains of differing size, RNA content and *sxt*A4 transcript content. We demonstrate the assay performance using mock samples, prepared by spiking known amounts of target RNA into background RNA from natural samples. We therefore provide a methodological foundation for transitioning HAB molecular detection into portable-ready, rapid-response monitoring, positioning RPA as a robust approach for HAB surveillance and early warning.

2 Materials and Methods

2.1 Culture maintenance and growth experiments

Dinoflagellate species were maintained in L1 culturing media (CCAP, UK) in polypropylene flasks (Starstedt), at 18°C, with a light : dark cycle of 16 : 8 hours and an irradiance of approximately 100 µmol m^{-2} s^{-1}. Cells were kept in exponential growth phase by frequent subculturing and monitoring the cell concentration using a Sedgewick-Rafter counting chamber and a brightfield microscope (Olympus CX-43).

2.2 Nucleic acid isolation

121 Cell material was collected for nucleic acid extraction by either pelleting by centrifugation at
122 4000g for 10 mins, or by vaccum filtration onto a 3.0 µm pore size polycarbonate membrane
123 (Nuclepore, Whatman, USA). Samples were flash frozen in liquid nitrogen and stored at -80 C
124 until further processing. Immediately prior to nucleic acid isolation, cells were disrupted using
125 liquid nitrogen and grinding with a pestle. DNA was extracted using the DNeasy Plant Mini Kit
126 (Qiagen, Netherlands). RNA was extracted using the GRS Total RNA kit – plant (GRiSP,
127 Portugal), residual DNA was removed from RNA using Turbo DNAse treatment (Thermo Fisher
128 Scientific, USA) and re-purified using the Monarch RNA cleanup kit (NEB, USA). DNA and RNA
129 purity was assessed spectrophotometrically (Nanodrop One, Thermo Fisher Scientific, USA) and
130 quantity was measured using a Qubit 4 instrument (Thermo Fisher Scientific, USA). Additionally,
131 RNA integrity was also assessed using a Bioanalyzer 2100 with Plant RNA pico reagents (Agilent,
132 USA) electrophoresis.

133

134 2.3 RPA primer and probe design

135 *Sxt*A4 gene sequences from all dinoflagellate species were assembled and aligned through NCBI.
136 For consensus sequence was generated for each species EMBOSS cons
137 (https://www.ebi.ac.uk/jdispatcher/msa/emboss_cons). The most conserved regions for assay
138 design covering all of *Alexandrium* species were initially identified manually.

139 RPA primers (Eurofins, Germany and IDT, USA) were designed according to TwistDx's RPA
140 assay design manual (GC content: 20–70%, length: 30–35 nt, Tm: 50–100 °C), using Primer-
141 BLAST on *Alexandrium minutum sxtA4* gene (accession number KM438016.1). Primer candidates
142 were evaluated for 1) potential self- and cross-dimer formation using the Multiple Primer Analyzer
143 (Thermo Fisher Scientific), 2) stable secondary structures using Mfold (unafold.org) and 3) cross-
144 reactivity using Blastn. Where potential primer dimers were detected at the 5' or 3' end, the primer
145 sequence was truncated by 1-2 nucleotides.

146

147 The RPA probe was designed according to TwistDx's RPA assay design manual and produced by
148 Biomers (Germany). The design criteria were expanded based on the literature to specifically
149 minimize the G-content of the probe and reduce the distance between fluorophore and quencher

150 to a minimum (Liu et al., 2019). A custom script identified regions of 47-nucleotides on both the
151 forward and reverse strands of the target sequence, ensuring a thymine was at position 31 as
152 recommended by the manufacturer. Probes predicted by the Multiple Primer Analyzer to form
153 dimers on the abasic site were excluded to prevent non-specific fluorescence emission. The final
154 selected primers and probe are shown in Table 1.
155

156 2.4 Identification and sequencing of *Sxt*A4 from cultured isolates

157 To confirm the presence of the *sxtA4* gene *Alexandrium* cultures, we used touch-up PCR protocol
158 utilizing *sxtA4* specific primers (Stüken et al., 2011) (**Table 1**). All PCRs were run at 25 µL
159 reaction volumes containing 1x GoTaq G2 green Master Mix (Promega, USA), 5% v/v DMSO,
160 0.4 µM Forward and Reverse primers. PCR cycling was carried out as follows: 95°C for 3 minutes,
161 5 x (95°C for 30s, 58°C for 45s, and 72°C for 50s), 5 x (95°C for 30s, 60°C for 45s, and 72°C for
162 50s), 27 x (95°C for 30s, 62°C for 45s, and 72°C for 50s), 72°C for 5 minutes. Results were
163 visualized by electrophoresis on a 1.5% agarose gel.
164

165 **Table 1.** Primer and probe sequences used in this study.

Primers/Probe	Amplification	Sequence (5'-3')	Source
AlexU_SxtA4_3256F	RPA	AGACGGCMGCCAGGCTCTGCAAGAAGGG	This study
AlexU_SxtA4_3301F	RPA	TGAAGCACAACGACACGGAGCAGCTYGAG	This study
AlexU_SxtA4_3565R	RPA	CCCGGCGTAGATGATGTTGTTCTCCGCGT	This study
AlexU_SxtA4_3355Pr	RPA	GGGCCGACATCACCTACGTGTGCGACGGCG[FAM-dT][dSpacer]T[BMN-Q3535]ACTCCACGGACGGAG[Spacer C3]	This study
AlexU_SxtA4_3396Pr	RPA	GACGGAGAGCTCGCCGACTTGCCCGCCATA[dT-FAM][dSpacer][BMN-Q3535]TGCTTGTTTGAGGCC[Spacer C3]	This study
AlexU_SxtA4_3264F	qPCR	GCCAGGCTCTGCAAGAAGGG	This study
AlexU_SxtA4_3410R	qPCR	CACATATGGCGGGCAAGTCG	This study

sxt007	PCR	ATGCTCAACATGGGAGTCATCC	(Stüken et al., 2011)
sxt008	PCR	GGGTCCAGTAGATGTT GACGATG	(Stüken et al., 2011)

166

167 Where *sxt*A4 was detected, we repeated the PCR in triplicate with a high-fidelity polymerase to
168 recover the amplicons for sequencing. All PCRs were run in 25 µL reaction volumes containing
169 1x KAPA HiFi HotStart ReadyMix (Roche, Switzerland), 5% v/v DMSO, 0.4 µM Forward and
170 Reverse primer and water adjusted for the volume of the template. The PCR cycle was: 95°C for
171 3 minutes, 35 x (95°C for 20s, 60-65°C for 15s, and 72°C for 50s), 72°C for 2 minutes. After gel
172 electrophoresis the bands were excised, purified using the NucleoSpin Gel and PCR Clean-up kit
173 (Macherey-Nagel, Germany), both strands were sequenced by Genewiz (Azenta Life Sciences,
174 USA) and the sequences were validated using NCBI's blastn.

175

176 2.5 Recombinase Polymerase Amplification

177 Quantitative RPA and Reverse Transcriptase-RPA (qRT-RPA) reactions were performed
178 following the manufacturer's instructions for the TwistAmp Exo kit (TwistDx, Cambridge, UK).
179 Real-time, exonuclease-assisted RPA reactions were run at 40ºC, at a final volume of 25 µL per
180 reaction and detected on a CFX Opus 96 Real-Time qPCR system (BioRad, USA) at 15 second
181 intervals. Briefly, for the reaction setup, a rehydration master mix was prepared containing 29.5
182 µL rehydration buffer, 1.2 µL of 10 µM forward primer, 2.1 µL of 10 µM reverse primer, 0.75 µL
183 10 µM probe and 1 µL Superscript IV for RNA templates (Thermo Fisher Scientific, USA). The
184 freeze-dried enzyme pellet was reconstituted with rehydration master mix and split into two
185 individual 25 µL reactions. After template addition, 1.25 µL of 280mM MgOAc were added to the
186 flat cap of each reaction. The introduction of MgOAc into each reaction and the subsequent mixing
187 were performed by simultaneously spinning down, vortexing and spinning down again all
188 reactions together. Measurements of Time to Positivity (TTP) were calculated as the timepoint at
189 which the fluorescent signal crossed the fluorescence threshold, using OriginPro's (2024) Intersect
190 Gadget. The threshold for each reaction was set as the mean fluorescence signal plus three times
191 the standard deviation of all the negative control measurements.

192

193 2.6 Determination of *sxt*A4 transcripts per cell in *A. minutum* strains

194 We determined the number of *sxt*A4 transcripts per cell under nutrient replete condition in three
195 cultured strains with different cell volume and growth rate profiles (strains VGO 657, CCAP
196 1119/48 and FE126). Growth rates were calculated by bi-daily counting of triplicate culture flasks
197 in the middle of the light phase when cells were not dividing. The specific growth rate μ (cells day^{-}
198 ^{1}) during the exponential phase was calculated using the formula $\mu = (\ln(N_1)-\ln(N_0)) / (t_1 - t_0)$
199 (Anderson et al., 1990), where N denotes cell concentration and t denotes day of culture between
200 two timepoints. Cells were then kept in exponential growth phase for at least 10 generations for
201 stable physiology. Cells were collected for RNA extraction in mid-exponential phase using 5 µm
202 polycarbonate filters to exclude bacterial cells (Cytiva, US). Cell size of the 3 strains was also
203 measured using an Operetta CLS High-Content Analysis System (Revvity).
204
205 From these samples we determined total RNA cell^{-1} as well as *sxt*A4 trancripts per cell using both
206 our qRT-RPA assay as described above and qRT-PCR primers that we specifically designed for
207 this purpose. The qRT-PCR primers uSxtA4_3264F - uSxtA4_3410R, overlap with the RPA probe
208 and reverse primer for comparability with the RPA assay, and amplify with 86% efficiency based
209 on a standard curve analysis. The 20 µL reactions volumes contained 1x GoTaq qPCR Master Mix
210 (Promega, UK), 1x GoScript™ RT Mix (Promega, UK), 200nM each primer and 2µL template.
211 The cycle was: 1x (42°C for 15 minutes), 1x (95°C for 10 minutes), 40x (95°C for 30s, 64°C for
212 30s, and 72°C for 30s) on a CFX Opus 96 Real-Time qPCR system (BioRad, USA).
213

214 2.7 Sensitivity and quantitative range across species

215 Firstly, the assay's performance was tested across species using the partial *sxt*A4 amplicons
216 generated initially four sequencing as well as synthetic DNA of the target region plus 20 bp
217 flanking either side (IDT, USA). The amplicon concentrations were determined
218 spectofluorometrically (Qubit, Invitrogen, USA) and serially diluted for testing over 5 orders of
219 magnitude. The assay's quantitative range was then tested using RNA of *A. minutum*, as a
220 representative *Alexandrium* species. Initially, this was done using dilution series of synthetic RNA

221 that corresponded to the region of the synthetic DNA (Genewiz), followed by total RNA extracted
222 from *Alexandrium minutum* VGO657.
223

224 2.8 Assay inclusivity and specificity

225 Inclusivity was tested using four strains of *Alexandrium minutum* and another 4 species of
226 *Alexandrium* that are known to produce toxins and one that is putatively non-toxic. Because
227 *Alexandrium* genomic DNA was not suitable for amplification by q-RPA, the sequenced served as
228 a template for testing with q-RPA. Specificity to the genus was tested against another SXT
229 producing dinoflagellate, two putative SXT producing cyanobacteria, and four representative non-
230 dinoflagellate phytoplankton species. For these non-dinoflagellate species, genomic DNA was
231 tested directly at a concentration of 10 ng reaction^{-1} .

232

233 2.9 Assay performance with increasing sample complexity

234 To test whether non-target RNA affected amplification behaviour, we created mock samples where
235 the target was spiked into a non-target background matrix. Initially, *A. minutum* total RNA from
236 strains FE126, CCAP1119/48, VGO657 at 1 ng reaction^{-1} was spiked into a mixture of non-target
237 RNA from cultured cells provided by colleagues (*Bathycoccus sp., Emiliania huxleyi,*
238 *Phaeodactylum trichornutum* and *Thalassiossira pseudonana*). The background matrix was
239 composed of equal amounts of these four species at a fixed concentration of 10 ng reaction^{-1}.
240 Finally, a dilution series of *A. minutum* CCAP 1119/48 total RNA was performed using RNA from
241 a natural seawater sample that did not contain *Alexandrium* cells. A 1.5 L water sample was
242 collected from Karteros Beach, 10km east of Heraklion, Crete, Greece and filtered with a 3.0µm
243 polycarbonate (Cytiva, US) and snap frozen with liquid nitrogen and stored at -80 C until the RNA
244 extraction. Again, the background matrix was kept at a fixed concentration of 10 ng reaction^{-1}.
245

3 Results

3.1 Primer and probe design

The assay design was targeted to the initial ~400bp part of the *sxt*A4 gene which contains a highly conserved region without nucleotide polymorphisms among *Alexandrium* species. This is key for a truly "universal" RPA probe design, as even a single mismatch can reduce or preclude target amplification. Mismatches are better tolerated in the primers than in the probe (Liu et al., 2019). Nevertheless, we avoided regions with polymorphisms at the 3' end of the primer or used degenerate bases to encompass possible sequence variation (**Figure 1**). We tested a total of 2 combinations of primer pairs and 2 probes. By using amplicons of the *sxt*A4 region from four different species, we could compare amplification based solely on sequence variation, by conducting tests at a single template input concentration of 10^6 copies per reaction.

Despite mismatches in the 3' end of the forward primer AlexU_SxtA4_3301F, *sxt*A4 amplified from all *Alexandrium* species. However, TTP differed among species and those with no mismatches amplified faster. Furthermore, Probe 1, did not produce any fluorescence signal in the amplification, possibly due to cross-reactions with the reverse primer. The primer and probe combination AlexU_SxtA4_3256F / 3565R / 3396Pr amplified a 309 bp region from all species with an equal TTP. This was selected as the Universal qRT-RPA assay and taken through further optimization.

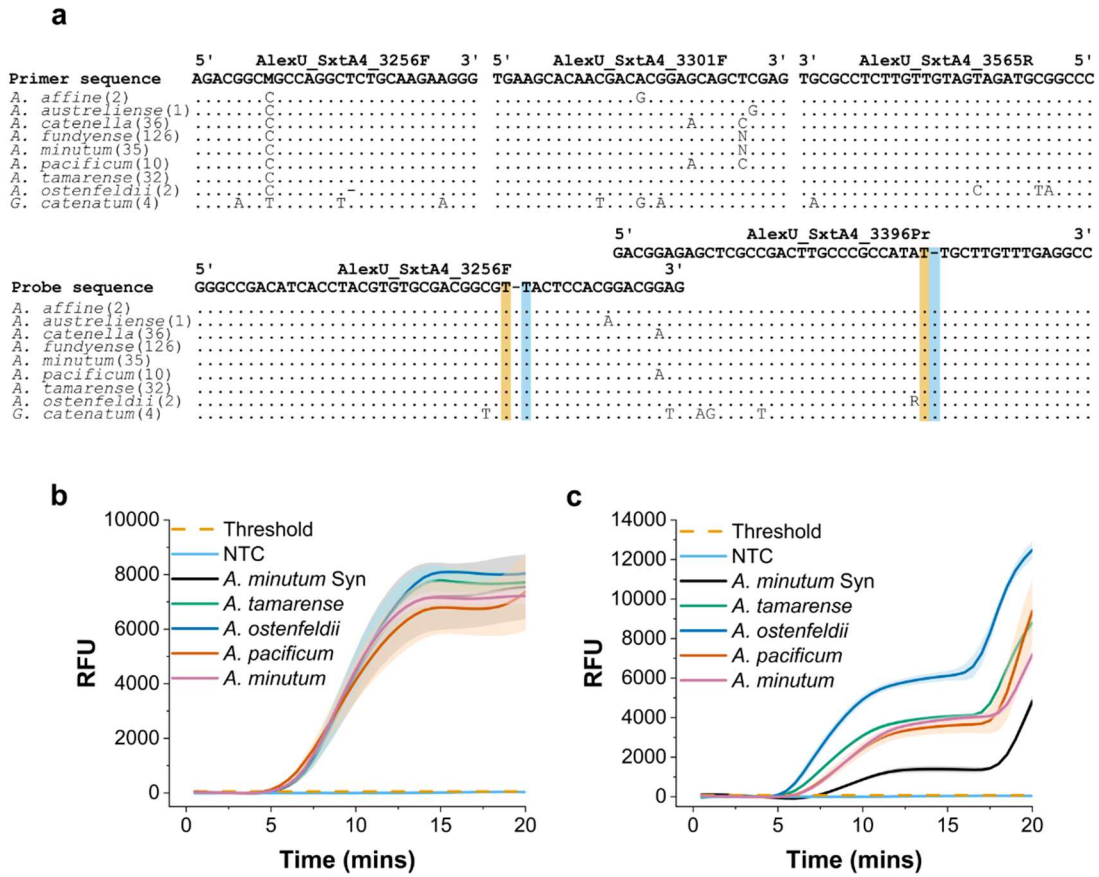


Figure 1. qRT-RPA assay design and testing. a) Nucleotide alignment of *Alexandrium* and *Gymnodinium sxt*A4 sequences from NCBI, along with the tested universal *sxt*A4 primers and probes. Numbers in brackets denote the number of sequences used to generate the consensus sequence for each species. Multiple alleles are represented as degenerate bases. The probe fluorophore and quencher positions are highlighted in orange and blue, respectively; the dash represents the abasic site. b) qRT-RPA amplification curves using Probe 1 and AlexU_SxtA4_3256F / AlexU_SxtA4_3565R. c) qRT-RPA amplification curves using Probe 1 and AlexU_SxtA4_3330F / AlexU_SxtA4_3565R. The notation Syn denotes synthetic RNA generated based on the *A. minutum* sequence. All amplification tests were done using amplicons of the target regions at 10^6 copies reaction^{-1}.

277 ## 3.2 qRT-RPA assay optimization

278 To maximise assay sensitivity and speed, we performed optimization tests for the reaction
279 temperature, primer and probe concentrations, and MgOAc levels, based on the ranges of tolerance
280 suggested by the manufacturer. The template for all tests was synthetic *Alexandrium minutum*
281 *sxt*A4 DNA at a concentration of 10^4 copies per reaction, which was near the initial limit of
282 detection using RPA's default recommended parameters.

283 We performed optimization tests for 5 parameters using a sequential approach instead of a multi-
284 parameter matrix. At each step we selected the optimal condition and used it in the next
285 optimization step – the conditions were tested in the order they are presented in **Figure 2**. The final
286 optimized assay consisted of the highest recommended probe concentration for a maximum
287 fluorescence signal, the lowest possible primer concentrations that did not compromise
288 amplification signal to minimize primer-dimer formation, the default MgOAc concentration a
289 temperature of 40 °C which is favorable for the reverse transcription enzyme.

290 We chose to use maximum signal intensity as the optimization strategy, rather than the quickest
291 TTP. This accounts for the need to maintain a detectable signal at low concentrations for enhanced
292 sensitivity. Also, it enables the assay to be run on portable devices that have less sensitive
293 fluorescence detectors than laboratory-based qPCR cyclers (Figure 2 f or g). These changes
294 resulted in the TTP becoming faster by 2.5 mins and a 3.3-fold increase in the maximum signal
295 intensity.

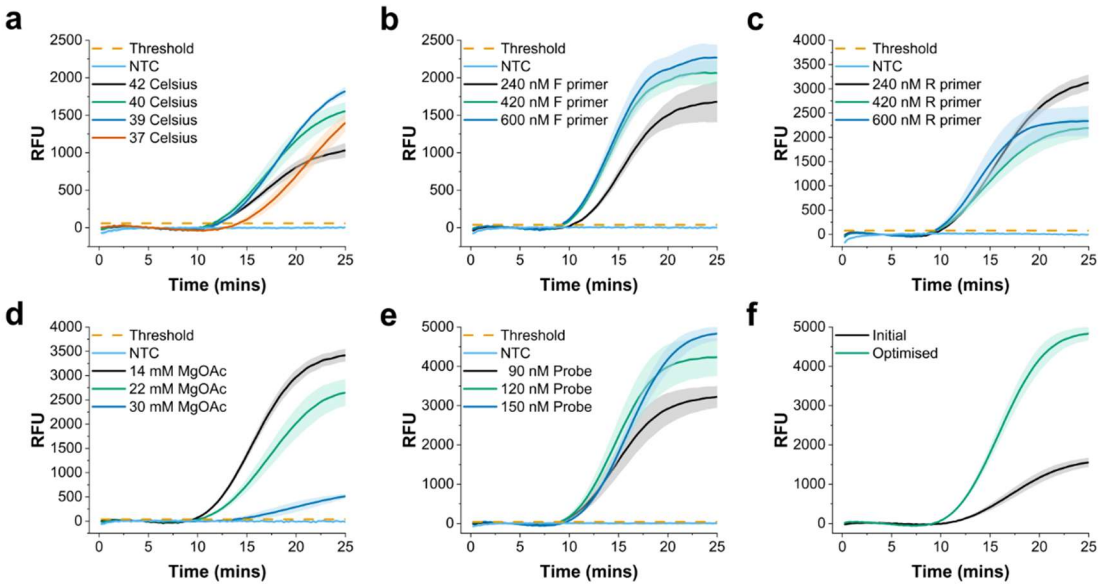


Figure 2. Universal assay optimization using 10^4 copies of *Alexandrium minutum* synthetic DNA. (a) Temperature (b) Forward primer concentration (c) Reverse primer concentration (d) Magnesium acetate concentration (e) Probe concentration (f) Comparison between initial and optimized assay conditions. The coloured halo of each curve represents the SD from 3 technical triplicates.

3.3 Performance of the assay with different template types

We next evaluated the performance of the assay with different templates types to determine a) any potential effect of reverse transcription step in a one-pot reaction on assay speed and sensitivity, and b) whether the assay can be used for both genomic DNA as a direct cell abundance proxy despite the high GC-content of the gene (Figure S1). Genomic DNA showed very poor amplification in qRPA on genomic DNA, with a low signal intensity and resolution among different template concentrations. Normally, amplification of the *sxt*A4 gene by PCR or by basic, non-quantitative RPA, requires the addition of DMSO due to the high template GC-content. However, this is not with qRPA chemistry (exo RPA kit) and is the most probable reason for inefficient amplification. We therefore concluded that genomic DNA is an unsuitable target for *sxt*A4 qRT-RPA, and focused solely on RNA amplification.

314

315 ## 3.4 Sensitivity and quantitative range across multiple species

316 The optimized assay was then evaluated using isolated *sxt*A4 amplicons from four *Alexandrium*
317 species (**Figure 3**). This approach enabled direct comparison of amplification efficiency based
318 solely on sequence variation. Under optimized conditions, the assay showed uniform amplification
319 kinetics across four species and over four orders of magnitude, retaining quantitative performance
320 in serial dilutions. The LoD was 10^3 copies per reaction with the exception of *A. tamarense* which
321 failed to amplify below 10^4 copies per reaction. Overall, these results demonstrate robust, cross-
322 species quantitative performance over a wide dynamic range.
323 Following the initial assay optimization using synthetic and amplicon DNA templates, the
324 remaining assay characterization was performed on RNA templates. We assessed analytical
325 sensitivity for RNA using two approaches: i) a 10-fold, 6-point dilution series (10^8-10^3 copies per
326 reaction) using synthetic *sxtA4* RNA and ii) a 10-fold, 4-point dilution series (0.1-100 ng per
327 reaction) of total RNA, isolated from *A. minutum* VGO 657 (**Figure 3**). The assay's quantification
328 range spans at least 4 orders of magnitude and the limit of detection is 10^3 synthetic RNA copies
329 and 0.1 ng of *A. minutum* VGO 657 total RNA. Furthermore, the assay run time is fast, with the
330 lowest concentrations of total RNA amplifying in under 12 minutes.
331
332

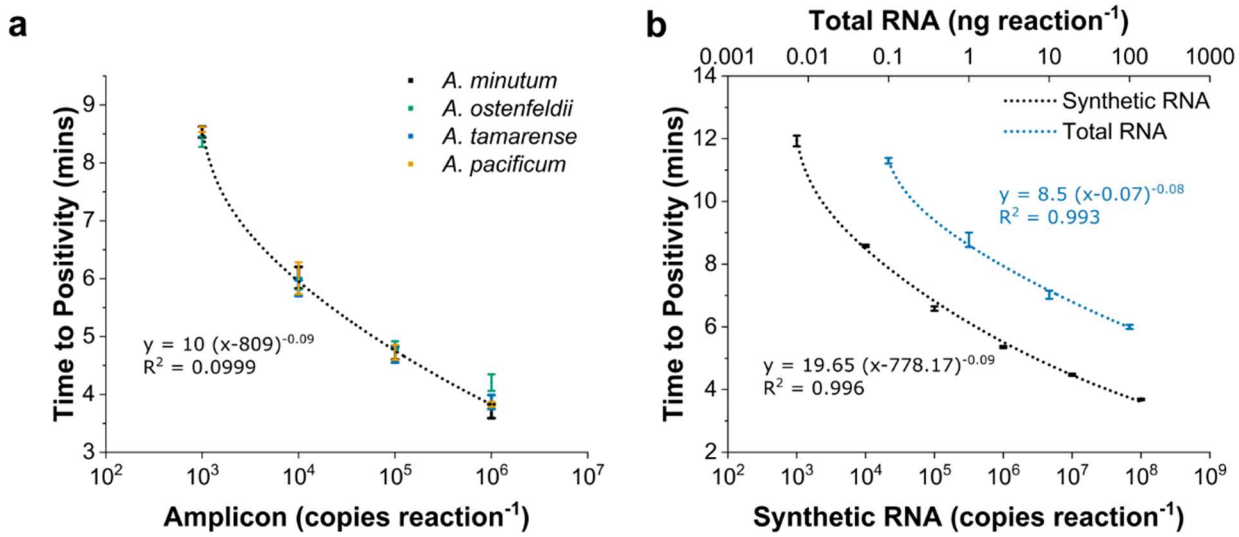


Figure 3. Characterization of the *sxt*A4 qRT-RPA universal assay. (a) Serial dilutions of isolated amplicons from four different *Alexandrium* species (*A. tamarense, A. ostenfeldii, A. pacificum, A. minutum*). (b) Standard curves of *A. minutum* synthetic sxtA4 RNA (black) and *A. minutum* VGO657 total RNA (blue). Error bars represent the standard deviation of 3 technical triplicates.

3.5 Inclusivity and specificity

To test inclusivity and specificity of the assay we used a panel of marine dinoflagellates of the target genus as well as a non-target saxitoxin-producing dinoflagellate, two putative saxitoxin-producing freshwater cyanobacteria, and other non-target cosmopolitan microalgal species (**Table 2**). The assay successfully detected *sxt*A4 in RNA from all 5 toxic *Alexandrium* strains, as well as *A. tamutum* which is generally regarded as non-toxic. We did not detect *sxt*A4 in toxic *Gymnodinium catenatum* neither by RT-qRPA nor by PCR; this species is generally regarded as toxic, but toxicity in this particular strain had not been assessed by the provider. Of the cyanobacteria species, *Planktothrix agardhii* is known to produce saxitoxins and the *Anabaena* genus contains saxitoxin-producing species, although the toxicity profile of the *A. cylindri*ca strain we used was not tested by the provider. The sxtA4 gene was not detected in neither of these strains, nor in any other non-target phytoplankton species.

353 **Table 2.** *Alexandrium sxt*A4 assay inclusivity and specificity.

Species	Strain ID	Phylum	sxtA4 DNA	sxtA4 RNA
Alexandrium andersonii	RCC10728	Dinoflagellata	-	n.a.
Alexandrium minutum	VGO657 CCAP119/48 FE126 VGO650	Dinoflagellata	+	+
Alexandrium ostenfeldii	CCAP 1119/53	Dinoflagellata	+	n.a.
Alexandrium pacificum	CCAP 1119/52	Dinoflagellata	+	n.a.
Alexandrium tamarense	RCC292	Dinoflagellata	+	n.a.
Alexandrium tamutum	RCC4860	Dinoflagellata	+	n.a.
Gymonodinium catenatum	VGO1486	Dinoflagellata	-	n.a.
Anabaena cylindrica	CCAP 1403/2A	Cyanobacteria	-	n.a.
Planktothrix agardii	CCAP 1459/21	Cyanobacteria	-	n.a.
Bathycoccus sp.	RCC4222	Chlorophyta	n.a.	-
Emiliania huxleyi	RCC6241	Haptophyta	n.a.	-
Phaeodactylum trichornutum	CCAP 1055/1	Heterokontophyta	n.a.	-
Thalasiossira pseudonana	CCMP 1355	Heterokontophyta	n.a.	-

354

355 ## 3.6 Relationship of *sxt*A4 transcripts to cell abundance

356 As trigger limits for HAB monitoring programs generally refer to specific cell concentrations, we
357 equated our RNA-based assay results to cell concentrations by determining the number of *sxt*A4
358 transcripts per cell. We used *A. minutum* as a representative species and compared characteristics
359 across three strains with differing morphology and growth rate. Strains VGO657, CCAP 1119/48

360 and FE 126 were analyzed for cell size, total RNA yield cell^{-1} and transcript number cell^{-1} using
361 qRT-RPA (**Table 3, Figure S1**). Cell diameter and volume varied 1.8-fold and 3.4-fold,
362 respectively. The total RNA content, transcripts pg^{-1} RNA and transcripts cell^{-1}, all increased with
363 increasing cell size. Considering the qRT-RPA assay LoD of 0.01 ng reaction^{-1} *A. minutum*
364 VGO657 total RNA, this equates to the detection of 6 cells reaction^{-1}.

365

366 **Table 3.** Size, total RNA yield and *sxt*A4 transcript analysis based on qRT-RPA for three *A.*
367 *minutum* strains. Standard deviation is based on 3 replicates.

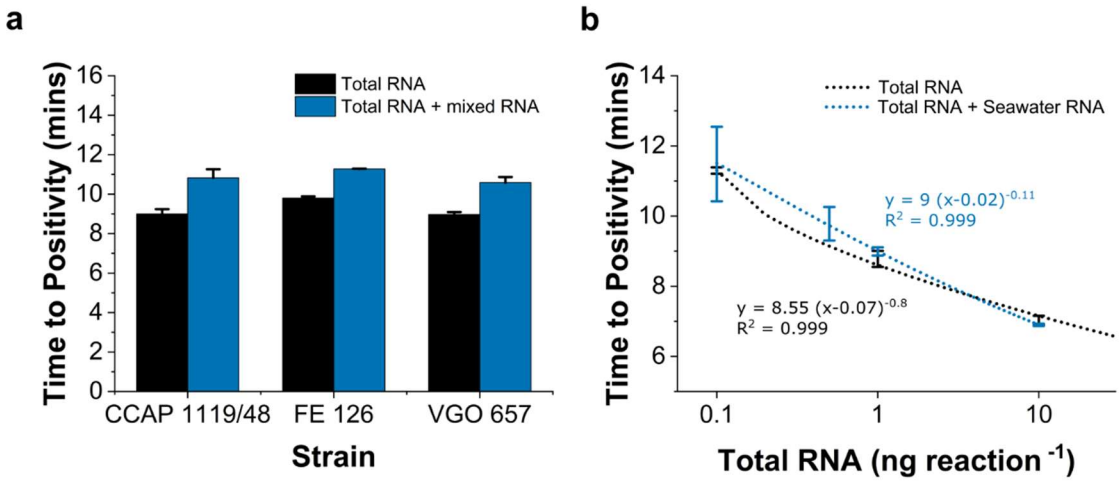
Strain	Cell diameter (µm)	Cell volume (µm^3)	Growth rate (day^{-1})	Total RNA (pg cell^{-1})	Transcripts pg^{-1}	Transcripts cell^{-1}
FE126	14.7 ± 1.7	1701 ± 557	0.19	5.1 ± 0.68	3.7 ± 2.2	19 ± 1
CCAP1119/48	16.8 ± 2.4	2625 ± 1025	0.16	9.1 ± 0.52	6.8 ± 1.2	62 ± 11
VGO657	22.0 ± 3.2	5718 ± 2226	0.15	15.9 ± 1.96	7.0 ± 0.7	112 ± 11

368

369 3.7 Assay performance with increasing sample complexity

370 We evaluated the influence of background RNA on *sxt*A4 RNA quantification using two types of
371 mock samples (**Figure 4**). In the first test, 1 ng RNA from three *A. minutum* strains was spiked
372 into 10 ng background RNA from a mixture of phytoplankton species (**Figure 4a**). These tests
373 showed that there was a degree of inhibition delaying the TTP by 1.7 minutes, on average for the
374 3 strains. Based on the equations of synthetic and total RNA standard curves (Figure 3b), this
375 would cause a quantitative underestimation of one order of magnitude (i.e. it would appear as 0.11
376 ng if the input was unknown). To further test this the effect of inhibition by more complex
377 background RNA, we used 10 ng total RNA from a natural sample, spiked with *A. minutum* total
378 RNA and over a wide range of concentrations (**Figure 4b**). The natural seawater sample contained
379 several non-target dinoflagellate species including *Peridiniales*, *Gymnodinium*, *Tripos spp.*,
380 *Prorocentrales*, *Protoperidinium* and *Gonyaulax,* as well as other phytoplankton and
381 microzooplankton. For the spike we used total RNA from *A. minutum* CCAP 1119/48, as this was

382 the representative strain with average size and *sxt*A4 transcript content. In this test, no inhibitory
383 effect was observed near the assays LoD at 0.1 ng, however, amplification was more variable
384 resulting in a higher standard deviation. The final LoD of the assay in complex samples was
385 therefore set as 0.1 ng reaction^{-1} of *A. minutum* CCAP 1119/48 total RNA which is equivalent to
386 11 *A. minutum* cells reaction^{-1}.
387



388
389 **Figure 4.** Assay performance in complex samples. 4a) qRT-RPA of total RNA from *three A.*
390 *minutum* strains (FE126, CCAP1119/48, VGO657) in the presence and absence of 10 ng
391 background total RNA 4b) qRT-RPA of total RNA from *A. minutum* CCAP 1119/48 in the
392 presence of 10 ng total RNA isolated from a natural seawater sample. In both cases, the
393 amplification curve from synthetic RNA is used for comparison (black).

394 # 4 Discussion

395 ## 4.1 Methodological advance

396 This study demonstrates that qRT-RPA can robustly quantify *sxt*A4 transcripts across multiple
397 *Alexandrium* species, with comparable amplification kinetics over several orders of magnitude
398 despite interspecific sequence variation. This assay design ensures applicability across different
399 geographic locations, where the causative microorganism might vary or where multiple saxitoxin
400 producing species may coexist. Furthermore, as qRT-RPA is compatible with simple portable
401 devices, our assay will enable RNA-based testing for toxigenic *Alexandrium* species in the field.

402 By validating assay performance using isolated *sxt*A4 amplicons rather than genomic DNA, we
403 were able to separate sequence-dependent amplification behavior from confounding effects related
404 to genome size and variable gene copy number, which are known to complicate molecular
405 ecological analyses of dinoflagellates (Ruvindy et al., 2023). This approach revealed that probe–
406 template complementarity, rather than copy number variation, is the primary determinant of cross-
407 species performance in probe-based RPA, consistent with previous studies on mismatch sensitivity
408 in fluorescent RPA chemistries (Liu et al., 2019).

409 Targeting RNA enables preferential detection of metabolically active cells with the capacity for
410 toxin production, while also improving analytical sensitivity by exploiting the higher abundance
411 of transcripts relative to genomic copies (Geffroy et al., 2021; Murray et al., 2015). Furthermore,
412 the poor performance of genomic DNA in *sxt*A4 q-RPA reinforces RNA as the appropriate target
413 when DMSO-dependent amplification is incompatible with assay chemistry. Importantly, the
414 RNA-based assay achieved robust quantitative performance in less than 15 minutes, significantly
415 beyond conventional qPCR-based laboratory workflows. This demonstrates the suitability of
416 isothermal functional gene monitoring for early-warning HAB surveillance.

417

418 4.2 Performance characteristics

419 The qRT-RPA assay exhibited a quantitative range spanning four orders of magnitude, with limits
420 of detection of approximately 10^3 synthetic *sxt*A4 RNA copies reaction^{-1} and 0.1 ng total
421 *Alexandrium minutum* RNA reaction^{-1}, corresponding to approximately 10 cell equivalents
422 depending on strain. These sensitivities are comparable to those reported for qPCR-based saxitoxin
423 gene assays (Gao et al., 2015; Han-Sol et al., 2024; Murray et al., 2019; Yu et al., 2025) while
424 being achieved with substantially shorter runtimes. Cross-species testing using *sxt*A4 amplicons
425 revealed uniform amplification kinetics across most *Alexandrium* species, with a lower sensitivity
426 only seen for *A. tamarense* despite no mismatches with the probe and primer sequences indicating
427 a potentially strain-specific result. On the whole, our results indicate that the assay design
428 successfully minimizes sequence-dependent bias.

429 Inclusivity testing confirmed *sxt*A4 detection in all tested toxic *Alexandrium* strains and in *A.*
430 *tamutum*, a species previously reported to express *sxt*A transcripts despite the absence of
431 measurable saxitoxin production (Vingiani et al., 2020). This observation is consistent with earlier
432 work showing that *sxt*A4 expression is necessary but not sufficient for toxin accumulation and
433 reinforces the need to interpret transcript-based signals as indicators of toxigenic potential rather
434 than confirmed toxicity (Geffroy et al., 2021). In contrast, no amplification was observed for
435 *Gymnodinium catenatum*, despite its established toxigenicity and presence of *sxt*A4 (Muhammad
436 et al., 2025). This is consistent with the four mismatches within the probe-binding region that result
437 in amplification failure and make the assay specific to the genus *Alexandrium.*

438 Background non-target nucleic acids are known to have an inhibitory effect on RPA, most likely
439 due to non-specific primer binding at the low running temperature (Munawar, 2022; Rohrman and
440 Richards-Kortum, 2015). In our assay, we found moderate inhibition in the presence of complex
441 background RNA from cultured phytoplankton. This was expected as one of the primers is
442 degenerate, however, the probe design is considered more important for specificity (Liu et al.,
443 2019). The quantitative underestimation equated to less than one order of magnitude. However,
444 this effect did not persist when a more complex natural sample was used as the background RNA.
445 Perhaps a larger diversity of sequences causes a dilution of potentially interfering sequences. Our
446 results highlight that standard curves with natural background RNA are more representative for
447 future quantification of the target in unknown samples, and should be adopted as a standard
448 practice for qRT-RPA assays. Importantly, reliable detection at low target concentrations equating
449 to as little as 11 cell equivalents reaction^{-1} was retained under these conditions. To achieve
450 quantification of 100 *Alexandrium* cells L^{-1}, this would require that RNA from 1L of a water
451 sample be extracted and eluted into 50 µL water, of which 5 µL can be used for amplification. This
452 is in line with standard molecular biology protocols, making the assay highly suitable for HAB
453 monitoring programmes.

454

455 ## 4.3 Comparison with qPCR- and isothermal-based approaches

456 Quantitative PCR assays for *Alexandrium* are well established and have demonstrated high
457 sensitivity and strong correlations with toxin concentrations and microscopy, particularly when
458 targeting functional genes of the saxitoxin biosynthesis pathway rather than ribosomal markers.
459 Early LSU-based assays achieved very low detection limits but could not distinguish toxic from
460 non-toxic strains (Garneau et al., 2011), motivating the development of qPCR assays targeting *sxt*
461 genes. Universal and species-inclusive *sxt*A4 qPCR assays have since shown detection limits on
462 the order of 30–50 cell equivalents L^{-1} and tighter correlations with toxin levels than rDNA-based
463 assays (Murray et al., 2019; Savela et al., 2016; Stüken et al., 2013), while other toxin-gene targets
464 such as *sxt*A1 and *sxt*B have similarly improved correspondence between molecular signal and
465 toxicity at low cell abundances (Kim et al., 2024; Penna et al., 2015). Despite these strengths,
466 qPCR and RT-qPCR workflows remain constrained to laboratory settings due to thermal cycling,
467 multi-step processing and longer time-to-result, limiting their suitability for rapid, on-site decision
468 making.

469 A growing body of work has therefore explored isothermal amplification strategies for HAB
470 detection, primarily aiming at field deployability. Most reported approaches rely on DNA-based
471 detection with qualitative or endpoint readouts, including RPA or LAMP coupled to lateral flow
472 dipsticks or CRISPR–Cas signal amplification for species identification rather than functional risk
473 assessment (e.g. haptophytes, *Karenia mikimotoi*, *Pseudo-nitzschia multiseries* (Wang et al., 2023;
474 Yao et al., 2024; Yu et al., 2025). A notable exception is the use of RPA coupled to Nanopore
475 sequencing, which enables portable, high-resolution taxonomic profiling but remains non-
476 quantitative and DNA-based (Hatfield et al., 2020). Among isothermal methods, the only study to
477 date targeting a toxin biosynthesis gene rather than a ribosomal marker is a LAMP assay targeting
478 *dab*A in *Pseudo-nitzschia multistriata*, demonstrated on a custom-made field-deployable device;
479 however, it is also DNA-based and lacks detailed quantitative and specificity characterization
480 (Alrefaey et al., 2026). In contrast, the qRT-RPA assay presented here uniquely combines
481 isothermal amplification, transcript-level detection and real-time quantification, making it well
482 suited for early-warning applications where speed, functional relevance and portability are
483 prioritized.

4.4 Conclusion

This study establishes the analytical performance of a universal qRT-RPA assay targeting the *sxt*A4 transcript, enabling rapid, cross-species detection of putatively toxigenic *Alexandrium* with functional relevance and short time-to-result. Validation under controlled conditions, including sequence diversity and increasing matrix complexity, provides a solid methodological foundation for RNA-based isothermal monitoring. As with any emerging molecular approach, targeted field testing with natural bloom samples and direct comparison to qPCR, microscopy and toxin measurements will be required to define operational performance and establish informed risk thresholds. With full compatibility to low-power portable fluorescence readers and sensor-integrated platforms, qRT-RPA is well positioned as a rapid early-warning tool in HAB monitoring programmes.

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