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Detecting aquatic pathogens with field-compatible dried qPCR assays

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ABSTRACT

Field-ready qPCR assays with extended shelf-life support monitoring programs for emerging aquatic pathogens and enable quick conservation and management decisions. Here, we developed, validated, and tested the shelf-life of qPCR assays targeting *Gyrodactylus salaris* and *Aphanomyces astaci* with lyophilization and air-drying.

Pathogenic microorganisms are a major threat to aquatic and terrestrial ecosystems. Globalization (international trade, transportation, and urbanization) and anthropogenic global changes have fostered the spread of pathogens (McIntyre et al., 2017; Guenard, 2021), resulting in biodiversity decline and economic losses. Three relevant aquatic pathogens with negative economic and ecological implications are: (i) the monogenean salmon parasite *Gyrodactylus salaris* (*Gs*) that colonizes the skin, gills, and fins of salmon and has caused widespread losses in both wild and farmed Atlantic salmon (Bakke et al., 1992; Rusch et al., 2018), (ii) the oomycete crayfish pathogen *Aphanomyces astaci* (*Aa*) that elicits crayfish plague in native European, Asian, and Australian crayfish species and causes massive population die-off events (Martín-Torrijos et al., 2021), and (iii) the amphibian-targeting panzootic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which originated in Asia, spread globally because of amphibian trade, and has decimated >500 amphibian species over the past half-century (Fisher and Garner, 2007, 2020; Scheele et al., 2019).

The analysis of environmental DNA (eDNA) is an emerging tool for quick and relatively inexpensive monitoring and detection of aquatic pathogenic organisms (Amarasiri et al., 2021). As a result, scientists, governmental agencies, and companies are increasingly incorporating eDNA methods into (semi)-automatic sampling machines coupled to

portable real-time quantitative PCR (qPCR) thermocyclers for continuous on-site pathogen monitoring of waterways (Thomas et al., 2020; Sepulveda et al., 2019, 2020). However, a remaining challenge is the requirement of cold storage for key reagents, which prohibits their use in field-operating machinery. Reagents that can be dried and stable at room temperature (RT) are commercially available. However, they have not been independently evaluated for their applicability and true shelf-life regarding eDNA monitoring of pathogens.

This study describes field-ready storable dried qPCR assays for three aquatic pathogens, *Gs*, *Aa*, and *Bd*, all based on previously published and optimized primers and probes (Table 1). For *Gs* and *Aa* assays, we compared two different drying methods, lyophilization and air-drying, respectively, and the amplification efficiency of dried assays across a time series (Table 1). The dried *Bd* assay was not evaluated for shelf-life, so results are not shown, but it was tested at several time points after drying and showed promising results.

All three assays targeted the ribosomal DNA internal transcribed spacer 1 (ITS1) region and were evaluated for reproducibility and sensitivity in a wet, freshly-made state. The standard curves were generated using serial dilutions of synthetic double-stranded DNA fragments (gBlocks, Integrated DNA Technologies, Inc., Leuven, Belgium) encompassing the primer/probe target regions of the three

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Table 1
qPCR assays evaluated in this study.

Target ^a (Reference)	Forward primers (conc.)	Reverse primers (conc.)	TaqMan probe (conc.)	IPC ^b	gBlocks / reference sequences (Acc. No) ^c	qPCR program	Drying method	Shelf-life tested
<i>Gs</i> (Rusch et al., 2018)	Gsal-208F (0.75 μM)	Gsal-149R (0.75 μM)	Gsal-188P-MGB2 (0.25 μM)	Yes	Gs_124–289 (DQ898302)	2 min 95 °C; 45 cycles (10 s 95 °C, 1 min 60 °C)	Lyophilization	Yes
<i>Aa</i> (Vrålstad et al., 2009)	AphAstITS-39F (1.2 μM)	AphAstITS-97R (1.2 μM)	AphAstITS-60 T (0.3 μM)	No	Aa_1–152 (AM947023)	2 min 95 °C; 45 cycles (5 s 95 °C, 20 s 60 °C)	Air drying	Yes
<i>Bd</i> (Boyle et al., 2004)	ITS1–3 Chytr (0.9 μM)	5.8S-Chytr (0.9 μM)	Chytr-MGB2 (0.25 μM)	Yes	Bd_26–271 (AY598034)	2 min 95 °C; 50 cycles (10 s 95 °C, 1 min 60 °C)	Lyophilization	No

^a *Gs*: *Gyrodactylus salaris*; *Aa*: *Aphanomyces astaci*; *Bd*: *Batrachochytrium dendrobatidis*.

^b IPC: Internal Positive Control including a template DNA and its complementary TaqMan probe and primers.

^c gBlocks names refer to the selected positions in the corresponding reference sequences, whose GenBank Accession numbers are detailed.

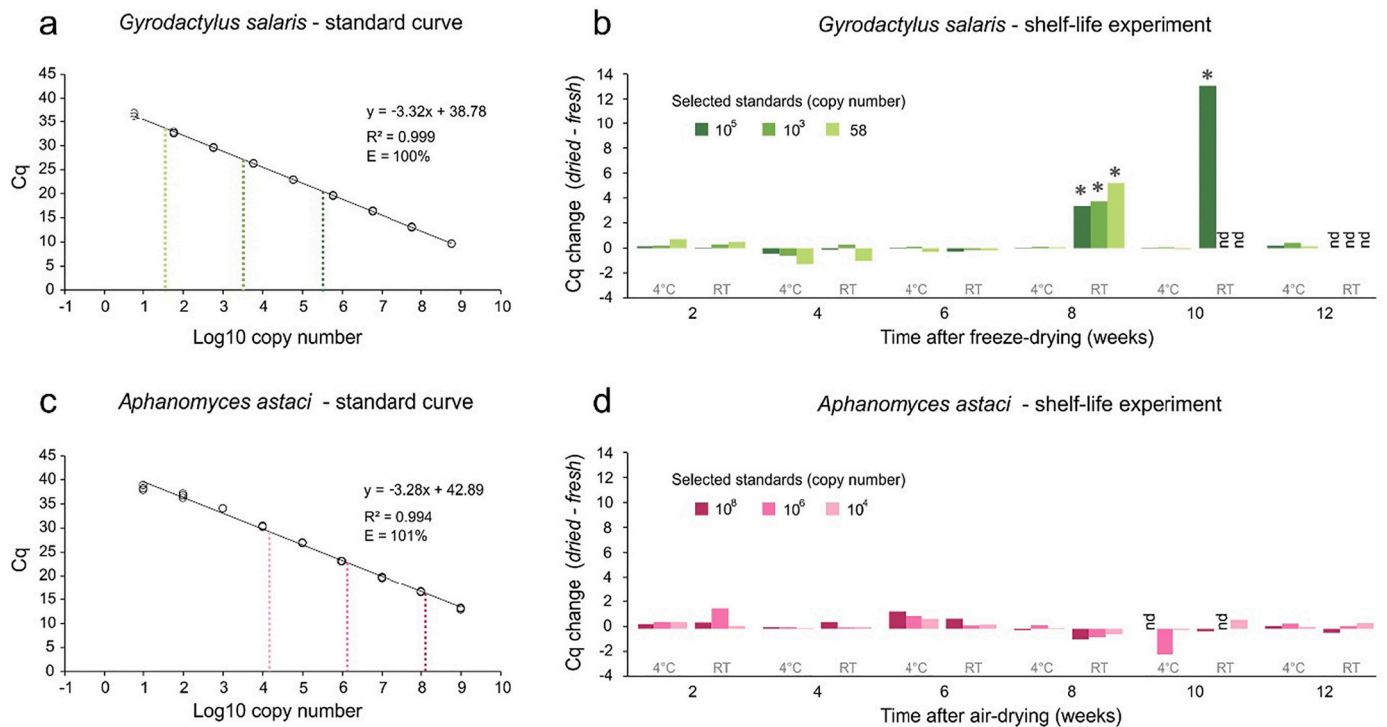


Fig. 1. Validation and stability results for the *Gyrodactylus salaris* (*Gs*) and *Aphanomyces astaci* (*Aa*) dried assays. (a + c) Standard curves of TaqMan-based qPCR amplification of *Gs* (a) and *Aa* (c) using fresh assays and gBlocks fragments. Standard curves were plotted using all three replicates for each serial dilution. The dotted lines represent the three concentrations used in each shelf-life experiment. (b + d) Shelf-life experiment results for *Gs* (b) and *Aa* (d) over 12 weeks, testing three concentrations and two different storage temperatures (4 °C and room temperature - RT). These results are shown as changes in Cq values compared to fresh assay controls (y-axis = Cq dried – Cq fresh); where a positive Cq indicates that the sample amplified less well than the control, and a negative Cq indicates that the sample amplified better than the control; a perfect match in Cq values of the fresh and dried assays is indicated by a zero. Concentrations for each assay were selected within the linear quantification range of the standard curve. Asterisks indicate Cq changes associated with the degradation of the assays (see details in Fig. 2). nd: non-detected qPCR signals.

assays (Table 1; Fig. 1a,c; Supp. Material Fig. S1).

After generating baseline data for the wet assays (Fig. 1a,c), the efficiency and shelf-life of dried assays for *Gs* and *Aa* were evaluated with a 12-week time-series experiment (Fig. 1b,d). The *Gs* assays were prepared using SensiFAST Lyo-Ready Mix (Meridian Biosciences, Bioline Assays Ltd., London, UK) with an exogenous internal positive control (IPC; Applied Biosystems, Waltham, MA, USA), which allows for the assessment of both the overall integrity of assays and the potential false negatives (PCR inhibition) in future environmental analyses. The IPC kit includes a synthetic template DNA with its corresponding primers and TaqMan probe (VIC-labeled probe, in contrast to the FAM-labeled probes used for the three target assays). *Gs* assays (final drying concentrations: qPCR Mix: 1×; forward and reverse primer: 0.75 μM; probe: 0.25 μM. Total volume in molecular-grade water: 18 μl) were frozen at –80 °C for 24 h and then lyophilized at –50 °C and < 0.1 mbar for 4 h with a FreeZone 2.5 Liter Benchtop (Labconco, Kansas City, MO, USA).

Aa assays were prepared with Air-Dryable qPCR Mix (Meridian Biosciences, Bioline Assays Ltd) (qPCR Mix: 1×; forward and reverse primer: 1.2 μM; probe: 0.3 μM. Total: 15 μl) and air-dried at 60 °C for 60 min using a drying oven (Memmert UE 200–800; Memmert GmbH, Schwabach, Germany) with a fan speed of 100% (drying time and temperature optimization, not shown); no IPC was used (Table 1). Both assays were vacuum-sealed in bags with silica beads, placed in darkness, and stored at either 4 °C or RT (21 °C ± 1 °C). qPCR analyses comparing dried vs. fresh assays were conducted every two weeks post-drying. The dried *Gs* assays were reconstituted with 18 μl of molecular-grade water and 2 μl of gBlocks, while the dried *Aa* assays were reconstituted with 15 μl of molecular-grade water and 5 μl of gBlocks. Three different concentrations of the gBlocks fragments were used as standards for *Gs* (5.8×10^5 , 5.8×10^3 and 58 copies of *Gs*_124–289) and *Aa* (1.9×10^8 , 1.9×10^6 and 1.9×10^4 copies of *Aa*_1–152) (Fig. 1).

We find that in three of four conditions (i.e., *Gs*: 4 °C, *Aa*: 4 °C, RT),

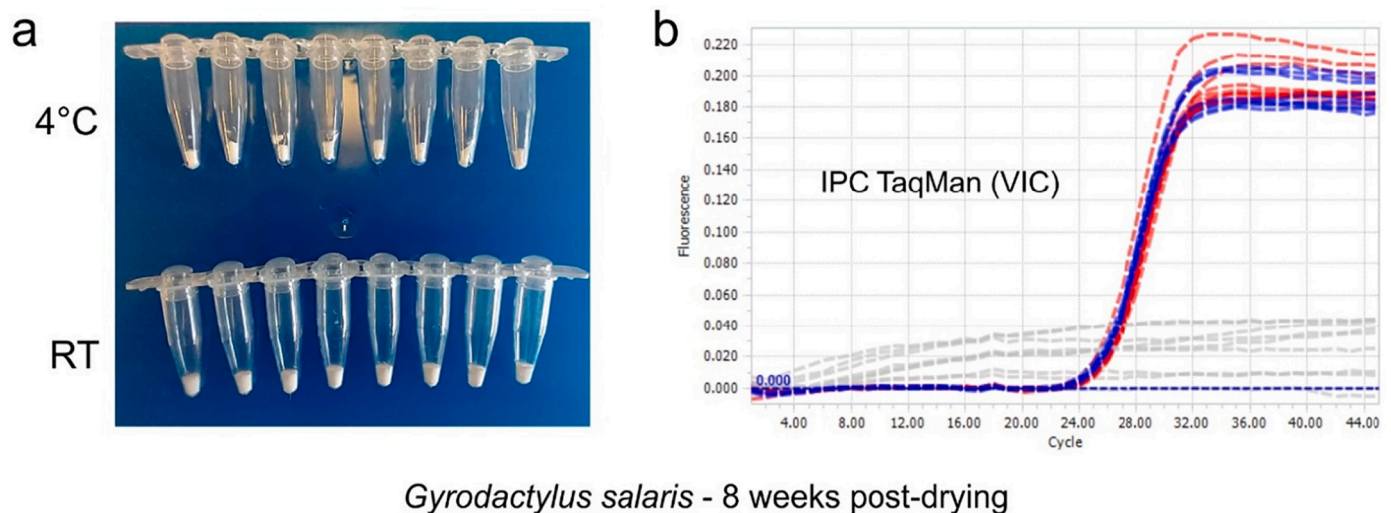


Fig. 2. Partial degradation of the freeze-dried *Gyrodactylus salaris* qPCR assays. (a) Representative of dried qPCR reagents. (b) Amplification curves for the internal positive controls (IPC; VIC signals). Note the poor performance of the dried assays stored at room temperature (grey IPC curves) compared to those stored at 4 °C (red) and fresh controls (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dried assays perform equally well as fresh assays even after 12 weeks (3 months) of storage. However, the *Gs* assays stored at RT declined in performance at week 8, with increased Cq values compared to the control and anomalous IPC signals (Fig. 1b, indicated by the asterisks; Fig. 2). In optimum conditions, with stable reagents and lack of PCR inhibitors (often present in environmental DNA samples), the Cq values for IPC (VIC fluorescence) should be 25 ± 2 , as shown in Fig. 2b for the assays stored at 4 °C and fresh controls. At week 10, only the highest concentration could be detected, and by week 12, all concentrations were undetectable (Fig. 1b). Since the aim was to develop *Gs* assays stable at RT, further optimization is required to make this assay stable at RT beyond week 8. However, the assay may be suitable for settings that allow for a combination of storage conditions, e.g., long-time storage at 4 °C and short-term exposure to RT during field-based studies. Air-dried *Aa* assays were stable until the end of the experiment at all concentrations and in both storage conditions. An anomaly occurred in week 10 when the highest concentration of the 4 °C stored group and the medium concentration of the RT stored group were not detected. Since results at the following timepoint, in week 12, were on par with the control group, we assume that this anomaly was likely a result of the drying position in the oven, possibly because of unequal airflow across all samples, which can affect drying efficiency (communication with the company); a future issue that would need to be addressed.

The development of field-ready diagnostic assays is vital for detecting and controlling emerging diseases quickly on-site. Here, we provide proof-of-concept data for field-ready qPCR assays that could be further coupled with portable field-use qPCR machines to detect and monitor aquatic pathogens. Additional steps include further optimization to increase shelf-life and to enable transferability to (semi)-automatic microfluidic devices. A possible method for the latter is proposed by Xu et al. (2021), where the addition of liquid nitrogen to the master mix formed a transferable ball.

We demonstrate the feasibility of preparing dried, long-term stable qPCR reactions that can be reconstituted with water and a DNA template. All assays would be suitable for field-based conservation monitoring programs.

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CRediT authorship contribution statement

Jessica Rieder: Conceptualization, Methodology, Validation,

Writing – original draft, Writing – review & editing. **Pedro M. Martin-Sanchez:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Omneya A. Osman:** Methodology, Writing – review & editing. **Irene Adrian-Kalchauer:** Funding acquisition, Supervision, Writing – review & editing. **Alexander Eiler:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2022.106594>.

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