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Detecting aquatic pathogens with field-compatible dried qPCR assays

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Abstract:

Field-ready qPCR assays with a long 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.

Keywords: lyophilization, qPCR, air-dried assay, environmental diagnostics, aquatic pathology, eDNA

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 crayfish pathogen oomycete *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 more than 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 a quick and relatively inexpensive method for monitoring and detecting 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 worked upon reconstitution after drying.

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 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. 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: 1x; forward and reverse primer: 0.75 µM; probe: 0.25 µM. Total volume in molecular-grade water: 18 µl) were frozen at -80ºC 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: 1x; 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 5µl 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).

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
Gs (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,	Lyophilization	Yes

						1 min 60°C)		
Aa (Vrålstad et al., 2009)	AphAstITS-39F (1.2 µM)	AphAstITS-97R (1.2 µM)	AphAstITS-60T (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
Bd (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

Table 1. qPCR assays evaluated in this study.

[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

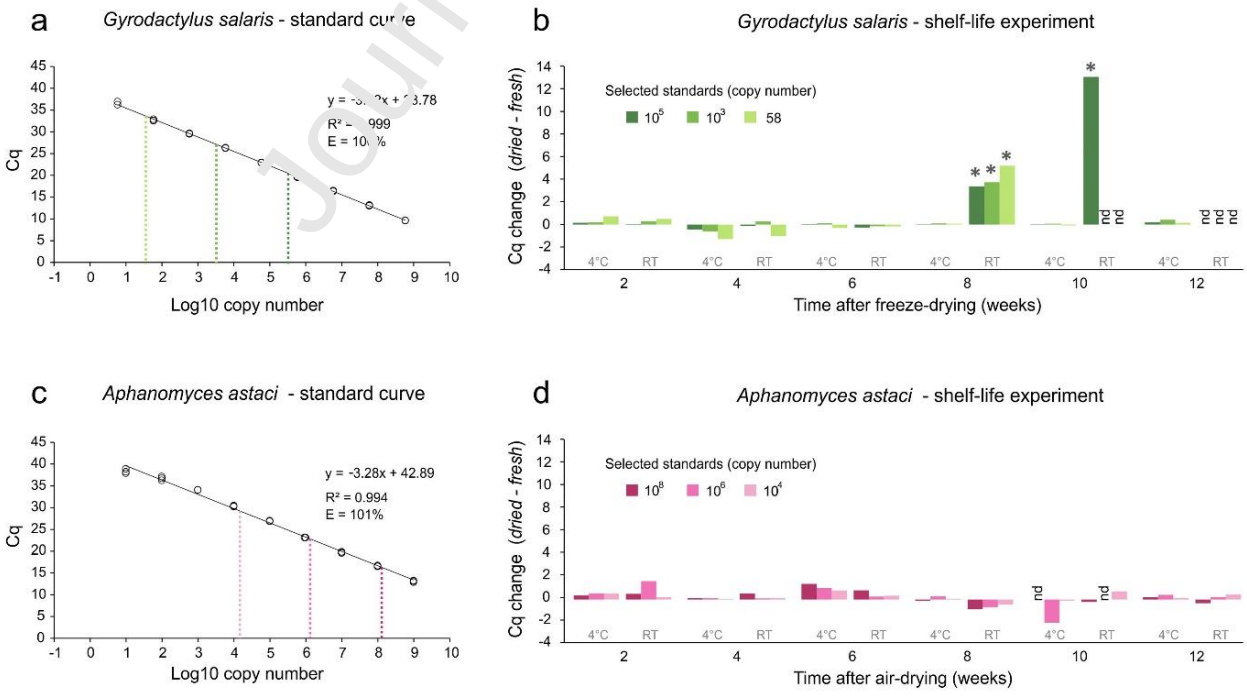

Figure 1. Validation and stability results for the two dried assays. (a + c) Standard curves of TaqMan-based qPCR amplification of *Gyrodactylus salaris* (*Gs*; a) and *Aphanomyces astaci* (*Aa*; c) using fresh assays and gBlocks fragments. Standard curves were plotted using all three replicates for 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 (y-axis = Cq dried – Cq fresh); where a positive Cq means the sample amplified after the control and a negative Cq the sample amplified before 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 quantification range. Asterisks indicate the main Cq changes associated with the degradation of the assays (see details in Fig. 2). nd: non-detected qPCR signals.

We find that in three of four conditions (i.e., *Gs*: 4°C, *Aa*: 4°C, RT), dried assays perform equally well as fresh assays even after 12 weeks (3 months) of storage. *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 flourescence) should be 25±2, as shown in Fig. 2b for the assays stored at 4°C and fresh controls. While at week 10, only the highest concentration could be detected, 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 for the same duration. In a diagnostic setting, however, often, a combination of storage options is possible, where assays can be stored for a longer time at 4°C and used or stored at RT for field-based studies (<6 weeks) when cold storage is not possible. 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 in 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 if there was not equal airflow across all samples (communication with the company), a future issue that would need to be addressed.

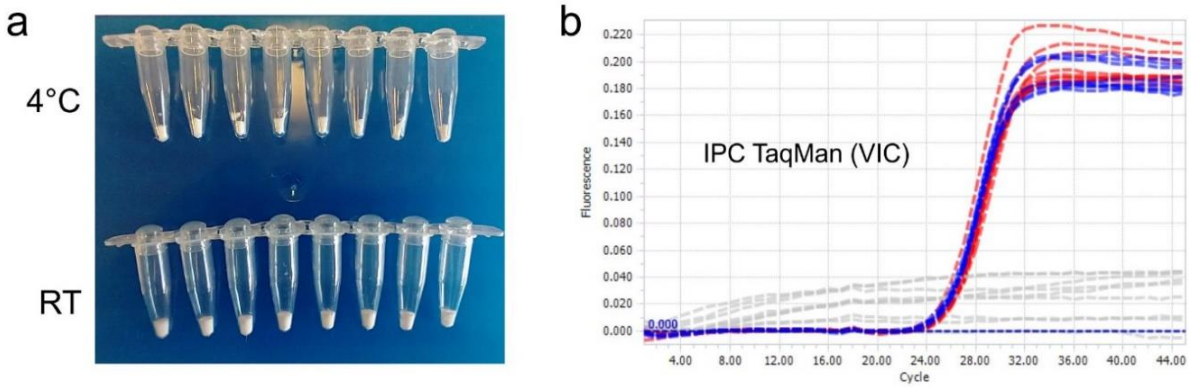

Figure 2. Partial degradation of the freeze-dried *Gyrodactylus salaris* qPCR assays. (a) Aspect of qPCR reagents. (b) Amplification curves for the internal positive controls (IPC; VIC signals). Note the poor performance of the dried assays stored at room temperature (grey IPC curves) compared to those stored at 4°C (red) and fresh controls (blue).

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 easy transferability to developing (semi)-automatic microfluidic devices. A possible method for ease of transferability would be to follow 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 template. All assays would be suitable for field-based conservation monitoring programs.

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Author statement

Rieder: conceptualization, methodology, validation, writing - original draft, and writing – review & editing

Martin-Sanchez: conceptualization, methodology, investigation, writing - original draft, and writing –

review & editing

Osman: methodology, writing – review & editing

Adrian-Kalchhauser: funding acquisition, supervision, writing – review & editing

Eiler: conceptualization, funding acquisition, supervision, writing – review & editing

Declaration of competing interest

The authors declare no conflict of interest.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Development of field-ready qPCR assays for detecting aquatic pathogens
- Lyophilization and air-drying methods are suitable for drying qPCR assays
- The development of dried assays is vital for monitoring and conservation programs