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1 **Detecting aquatic pathogens with field-compatible dried qPCR assays**

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18 Abstract:
19 Field-ready qPCR assays with a long shelf-life support monitoring programs for emerging aquatic
20 pathogens and enable quick conservation and management decisions. Here, we develop, validate, and
21 test the shelf-life of qPCR assays targeting *Gyrodactylus salaris* and *Aphanomyces astaci* with
22 lyophilization and air-drying.
23

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

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

37 The analysis of environmental DNA (eDNA) is an emerging approach for quick, relatively
38 inexpensive monitoring and detection of aquatic pathogenic organisms (Amarasiri et al., 2021). As a
39 result, scientists, governmental agencies, and companies are increasingly incorporating eDNA methods
40 into (semi)-automatic sampling machines coupled to portable real-time quantitative PCR (qPCR)
41 thermocyclers for continuous on-site pathogen monitoring of waterways (Thomas et al., 2020;
42 Sepulveda et al., 2019, 2020). However, a remaining challenge is the requirement of cold storage for
43 key reagents, which prohibits their use in field-operating machinery. Reagents that can be dried and
44 are stable at room temperature (RT) from are commercially available. However, they have not been
45 independently evaluated for their applicability and true shelf-life regarding eDNA monitoring of
46 pathogens.

47 This study describes field-ready storable dried qPCR assays for three aquatic pathogens, *Gs, Aa,*
48 and *Bd*. The dried *Bd* assay was not evaluated for shelf-life, so results are not shown but worked upon
49 reconstitution after drying. For *Gs* and *Aa* assays, we compared two different drying methods,
50 lyophilization and air-drying, and amplification efficiency of dried assays across a time series (Table 1).

51 All three assays targeted the rDNA internal transcribed spacer 1 (ITS1) region and were evaluated
52 for reproducibility and sensitivity in a wet, freshly-made state. The standard curves were generated
53 using serial dilutions of synthetic double-strain DNA fragments (gBlocks, Integrated DNA Technologies,
54 Inc., Belgium) encompassing the target regions of the three assays (Table 1; Fig.1a,c; Supp. Material

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55 Fig. S1).

56 After generating baseline data for the wet assays, the efficiency and shelf-life of dried assays for *Gs*
57 and *Aa* were evaluated with a 12-week time-series experiment. The *Gs* assays were prepared using
58 SensiFAST Lyo-Ready (Meridian Biosciences, Bioline Assays Ltd, UK) with an exogenous internal
59 positive control (IPC; Applied Biosystems), which allows for the assessment of both the overall integrity
60 of assays and the potential false negatives (PCR inhibition) in future environmental analyses. *Gs* assays
61 were frozen at -80ºC for 24h and then lyophilized at -50°C and <0.1 mbar for 4h with a FreeZone 2.5
62 Liter Benchtop (Labconco, USA). *Aa* assays were prepared with Air-Dryable qPCR Mix (Meridian
63 Biosciences, Bioline Assays Ltd, UK) and air-dried at 65°C for 65 min using a drying oven with a fan
64 speed of 100%; no IPC was used (Table 1). Both assays were vacuum-sealed in bags with silica beads,
65 placed in darkness, and stored at either 4°C or RT (21°C ±1°C). qPCR analyses comparing dried vs.
66 fresh assays were conducted every two weeks post-drying. Three different concentrations of the
67 gBlocks fragments were used as standards for *Gs* (5.8×10^5, 5.8×10^3 and 58 copies of Gs_124-289)
68 and *Aa* (1.9×10^8, 1.9×10^6 and 1.9×10^4 copies of Aa_1-152) (Fig. 1).

69

Target[a]	Forward primers (conc.)	Reverse primers (conc.)	TaqMan probe (conc.)	IPC[b]	gBlocks fragments of 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 (10s 95°C, 1 min 60C)	Lyophilization	Yes
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 (5s 95°C, 20s 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	Lyophilization	No

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						60C)		

70 **Table 1**. qPCR assays evaluated in this study.

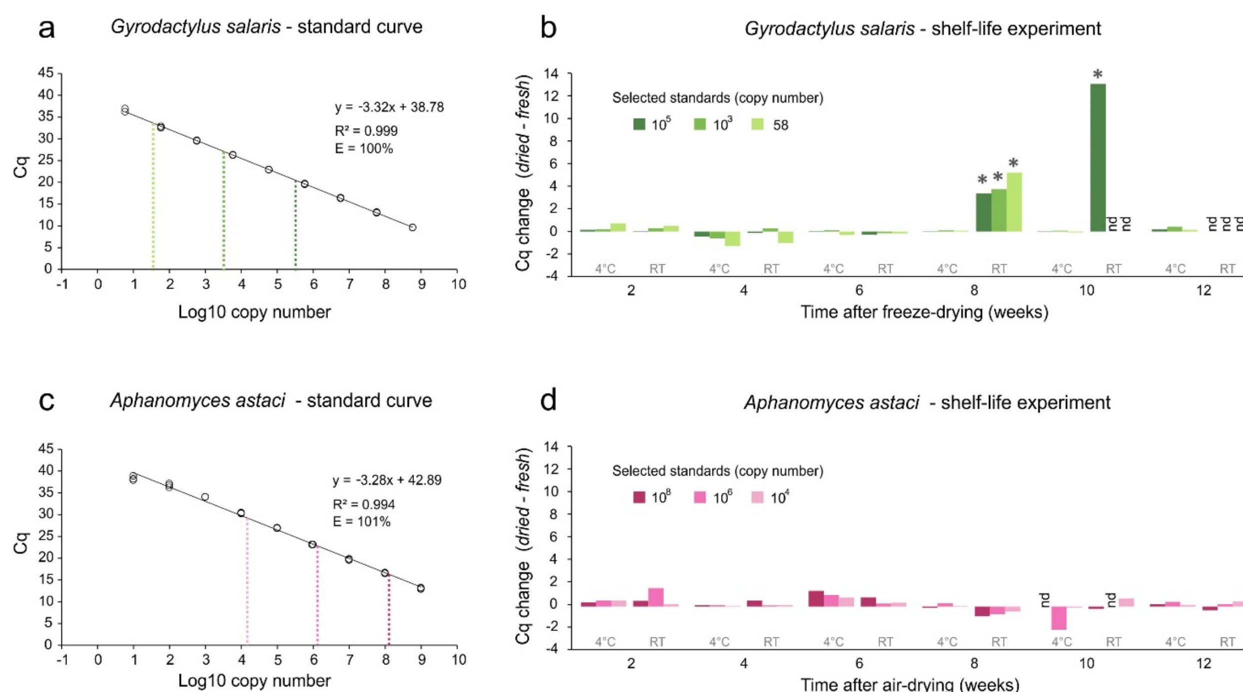
71 [a] *Gs*: *Gyrodactylus salaris*; *Aa*: *Aphanomyces astaci*; *Bd*: *Batrachochytrium dendrobatidis*

72 [b] IPC: Internal Positive Control including a TaqMan probe plus its complementary standard DNA.

73 [c]: gBlocks names refer to the selected positions in the corresponding reference sequences, whose

74 GenBank Accession numbers are detailed

75



76

77 **Figure 1**. Validation and stability results for the two dried assays. (a + c) Standard curves of TaqMan-

78 based qPCR amplification of *Gyrodactylus salaris* (*Gs*; a) and *Aphanomyces astaci* (*Aa*; c) using fresh

79 assays and gBlocks fragments. Standard curves were plotted using all three replicates for each serial

80 dilution. The dotted lines represent the three concentrations used in each shelf-life experiment. (b + d)

81 Shelf-life experiment results for *Gs* (b) and *Aa* (d) over 12 weeks, testing three concentrations and two

82 different storage temperatures (4°C and room temperature - RT). These results are shown as changes

83 in Cq values compared to fresh assay (Cq dried – Cq fresh). Concentrations for each assay were

84 selected within the quantification range. Asterisks indicate the main Cq changes associated with the

85 degradation of the assays (see details in Fig. 2).

86

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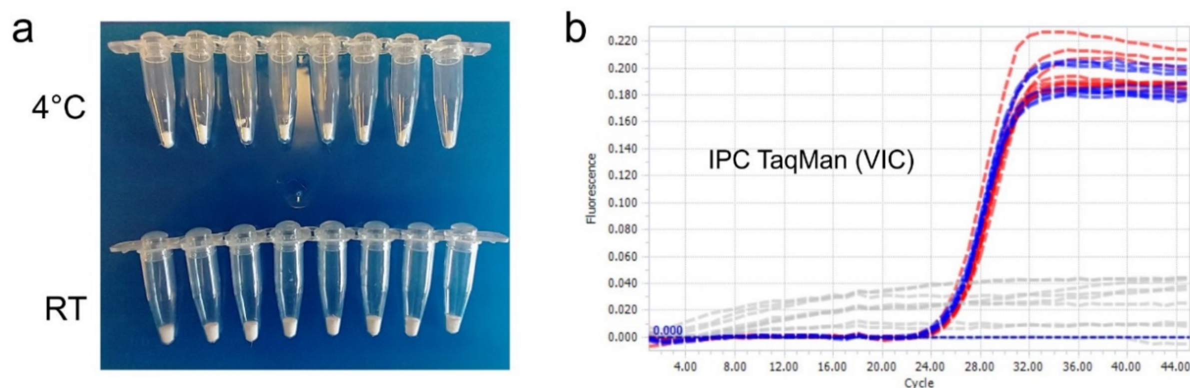
87 We find that in three of four conditions (i.e., *Gs*: 4°C, *Aa*: 4°C, RT), dried assays perform equally
88 well as fresh assays even after 12 weeks (3 months) of storage. Only *Gs* assays stored at RT declined
89 in performance at week 8, with increased Cq values and anomalous IPC signals (Fig. 1b, indicated by
90 the asterisks; Fig. 2). In optimum conditions, the Cq values for IPC (VIC) should be 25±2, as shown in
91 Fig. 2b for the assays stored at 4°C and fresh controls. While at week 10, only the highest concentration
92 could be detected, by week 12, all concentrations were undetectable (Fig. 1b). Since the aim was to
93 develop *Gs* assays stable at RT, further optimization is required to make this assay stable at RT for the
94 same duration. In a diagnostic setting, however, often a combination of storage options is possible,
95 where assays can be stored for a longer time at 4°C and used or stored at RT for field-based studies
96 (<6 weeks) when cold storage is not possible. Air-dried *Aa* assays were stable until the end of the
97 experiment at all concentrations and in both storage conditions. An anomaly occurred in week 10 when
98 the highest concentration of the 4°C stored group and the medium concentration of the RT stored group
99 were not detected. Since results in the following timepoint, in week 12, were on par with the control
100 group, we assume that this anomaly was human or machine-based.

101



102 *Gyrodactylus salaris* - 8 weeks post-drying

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

107

108 The development of field-ready diagnostic assays is vital for detecting and controlling emerging

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109 diseases quickly on site. Here, we provide proof-of-concept data for field-ready qPCR assays that could
110 be further coupled with portable field-use qPCR machines to detect and monitor aquatic pathogens.
111 Additional steps include further optimization to increase shelf-life and easy transferability to developing
112 (semi)-automatic microfluidic devices. A possible method for ease of transferability would be to follow
113 Xu et al. (2021), where the addition of liquid nitrogen to the master mix formed a transferable ball.

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

117 This work was supported by the Norwegian Environment Agency (Auto e-DNA project).

118

119 **Author statement**

120 Rieder: conceptualization, methodology, validation, writing - original draft, and writing – review & editing
121 Martin-Sanchez: conceptualization, methodology, investigation, writing - original draft, and writing –
122 review & editing
123 Osman: methodology, writing – review & editing
124 Adrian-Kalchhauser: funding acquisition, supervision, writing – review & editing
125 Eiler: conceptualization, funding acquisition, supervision, writing – review & editing

126

127 **Declaration of competing interest**

128 The authors declare no conflict of interest.

129

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