

eDNA qPCR Report – Wood E & I Solutions, Inc. (Lassen Volcanic National Park – Cascades Frog and its Potential Predators)

Report Contents

1. REPORT INFORMATION	1
2. SAMPLE SUBMISSION TABLE.....	2
3. METHODS	5
3.1 qPCR Assay Design	5
3.2 Sample preservation and transport.....	5
3.3 eDNA Extraction from Filters	5
3.4 Fluorometric Quantification of DNA Yield	5
3.5 qPCR for the Detection of eDNA from <i>Rana cascadae</i> , <i>Haemopsis marmorata</i> , <i>Oncorhynchus mykiss</i> , <i>Salvelinus fontinalis</i> , and <i>Salmo trutta</i>	5
4. RESULTS	6
4.1 DNA Extraction Yield	6
4.2 Results of qPCR Analysis.....	6
5. APPENDIX I: <i>Rana cascadae</i> qPCR ASSAY	15
6. APPENDIX II: <i>Haemopsis marmorata</i> qPCR ASSAY	17
7. APPENDIX III: <i>Salvelinus fontinalis</i> qPCR ASSAY	19
8. APPENDIX IV: <i>Salmo trutta</i> qPCR ASSAY	21
9. APPENDIX V: <i>Oncorhynchus mykiss</i> qPCR ASSAY	23
6. APPENDIX VI: GLOSSARY	25
7. APPENDIX VII: REFERENCES	26
8. DISCLAIMER	27

Report Issued:

Prepared By: Steve Crookes	Date: March 25th, 2022	Email: steve.crookes@precisionbiomonitoring.com
Validated by: Melissa MacLeod-Bigley	Date: April 1 st , 2022	Email: melissa.macleodbigley@precisionbiomonitoring.com

1. REPORT INFORMATION

Prepared for

Wood Environmental & Infrastructure Solutions, Inc.
104 West Anapamu Street, Suite 204A (Main Floor)
Santa Barbara, CA 93101, USA

Contact Name	Email Address	Phone
Dawn Johnson	dawn.johnson@woodplc.com	+1 805 252 4370
Kevin Stolzenbach	kevin.stolzenbach@woodplc.com	

Sample Information (Table below)

Sample Collection Conducted by: Kate Buckley/ Kevin Stolzenbach
Sampling Period: 22nd June – 1st July 2021
Storage condition before shipment: NA
Sample Shipment Format: 1.2 um and 0.45 um Smith-Root self-preserving filters
Date Shipped: 2nd July 2021
Date Received: Week of 5th July 2021
Samples Received By: Emily McLeod, Christina Rusu and Grace Stelter
Condition of Samples Upon Receipt: Intact
DNA Extracted by: Emily McLeod, Christina Rusu and Grace Stelter
Sample Extraction: Qiagen 'DNeasy Blood and Tissue' and 'Qias shredder' kits.

Analysis

Analysis applied: qPCR for cascades frog (*Rana cascadae*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), and the parasitic leech *Haemopsis marmorata*.

Analysis Conducted By: Melissa MacLeod-Bigley

Analysis Location: PBI Laboratory

Date of qPCR Analysis: March 2022

2. SAMPLE SUBMISSION TABLE

Client Sample ID	PBI Sample ID	Target(s):	Filter Size (µm)	DNA Yield (ng/mL)
CAR-M-C1	W1	qPCR for 5 target species (see Analysis)	0.45	10800
CAR-M-C2	W2	qPCR for 5 target species (see Analysis)	1.2	1630
CAR-M-C3	W3	qPCR for 5 target species (see Analysis)	0.45	638
CAR-M-C4	W4	qPCR for 5 target species (see Analysis)	1.2	738
Neg Control 1	W5	qPCR for 5 target species (see Analysis)	0.45	<10
44-S Archive	W6		0.45	3080
44-S	W7	qPCR for 5 target species (see Analysis)	0.45	1540
43-M-C-Archive	W8		1.2	1760
43-M-C	W9	qPCR for 5 target species (see Analysis)	1.2	2280
42-S	W10	qPCR for 5 target species (see Analysis)	0.45	1230
42-S Archive	W11		0.45	1350
48-L Archive	W12		1.2	2040
48-L	W13	qPCR for 5 target species (see Analysis)	1.2	1030
36-S-C	W14	qPCR for 5 target species (see Analysis)	0.45	1310
36-S-C Archive	W15		0.45	616
45-M-C Archive	W16		1.2	644
45-M-C	W17	qPCR for 5 target species (see Analysis)	1.2	1680
37-M-C	W18	qPCR for 5 target species (see Analysis)	0.45	2040
37-M-C Archive	W19		0.45	1560
8-M-C	W20	qPCR for 5 target species (see Analysis)	1.2	1220
8-M-C-Archive	W21		1.2	1360
7-M-C Archive	W22		0.45	696
7-M-C	W23	qPCR for 5 target species (see Analysis)	0.45	998
27-L	W24	qPCR for 5 target species (see Analysis)	1.2	3660
27-L Archive	W25		1.2	3580
20-L	W26	qPCR for 5 target species (see Analysis)	0.45	3400
20-L Archive	W27		0.45	2440
38-S	W28	qPCR for 5 target species (see Analysis)	0.45	2660
38-S Archive	W29		0.45	1970
39-S-Archive	W30		1.2	1130
39-S	W31	qPCR for 5 target species (see Analysis)	1.2	994
27-L-P	W32	qPCR for 5 target species (see Analysis)	1.2	11100
27-L-P Archive	W33		1.2	5860
21-S	W34	qPCR for 5 target species (see Analysis)	0.45	8760
21-S Archive	W35		0.45	4420
26-P	W36	qPCR for 5 target species (see Analysis)	1.2	>12000
26-P Archive	W37		1.2	10400
24-L	W38	qPCR for 5 target species (see Analysis)	1.2	1170
24-L Archive	W39		1.2	1360

28-L Archive	W40		0.45	2300
28-L	W41	qPCR for 5 target species (see Analysis)	0.45	2100
28-L-P Archive	W42		1.2	1240
28-L-P	W43	qPCR for 5 target species (see Analysis)	1.2	4860
22-L	W44	qPCR for 5 target species (see Analysis)	1.2	1830
22-L Archive	W45		1.2	1510
23-P	W46	qPCR for 5 target species (see Analysis)	1.2	3520
23-P Archive	W47		1.2	4080
25-P Archive	W48		0.45	8320
25-P	W49	qPCR for 5 target species (see Analysis)	0.45	8200
30-S	W50	qPCR for 5 target species (see Analysis)	0.45	1250
30-S Archive	W51		0.45	1510
35-S Archive	W52		0.45	1250
35-S	W53	qPCR for 5 target species (see Analysis)	0.45	1200
40-M	W54	qPCR for 5 target species (see Analysis)	1.2	4660
40-M Archive 1	W55		1.2	6000
40-M Archive 2	W56		0.45	3560
19-L	W57	qPCR for 5 target species (see Analysis)	1.2	5260
19-L Archive	W58		1.2	2820
18-L	W59	qPCR for 5 target species (see Analysis)	1.2	4640
18-L Archive	W60		1.2	5220
33-M-C	W61	qPCR for 5 target species (see Analysis)	0.45	2920
33-M-C Archive	W62		0.45	1060
31-M-C	W63	qPCR for 5 target species (see Analysis)	0.45	1690
31-M-C Archive	W64		0.45	1240
34-S	W65	qPCR for 5 target species (see Analysis)	0.45	2700
34-S Archive	W66		0.45	2340
3-S	W67	qPCR for 5 target species (see Analysis)	0.45	1490
3-S Archive	W68		0.45	1550
1-S Archive	W69		0.45	8920
1-S	W70	qPCR for 5 target species (see Analysis)	0.45	8240
1-S-2	W71	qPCR for 5 target species (see Analysis)	0.45	5960
1-S-2 Archive	W72		0.45	8020
4-S	W73	qPCR for 5 target species (see Analysis)	0.45	1790
4-S Archive	W74		0.45	1680
32-S	W75	qPCR for 5 target species (see Analysis)	0.45	964
32-S Archive	W76		0.45	864
16-L	W77	qPCR for 5 target species (see Analysis)	1.2	1700
16-L Archive	W78		1.2	2140
15-L	W79	qPCR for 5 target species (see Analysis)	1.2	1870
15-L Archive	W80		1.2	1400
17-L Archive	W81		1.2	5240
17-L	W82	qPCR for 5 target species (see Analysis)	1.2	5080

46-S	W83	qPCR for 5 target species (see Analysis)	0.45	4000
46-S Archive	W84		0.45	3240
12-P	W85	qPCR for 5 target species (see Analysis)	1.2	3760
12-P Archive	W86		1.2	3620
11-L	W87	qPCR for 5 target species (see Analysis)	0.45	3380
11-L Archive	W88		0.45	4840
51-L	W89	qPCR for 5 target species (see Analysis)	1.2	2300
51-L Archive	W90		1.2	2260
14-L	W91	qPCR for 5 target species (see Analysis)	1.2	3620
14-L Archive	W92		1.2	1310
9-M-C Archive	W93		1.2	1160
9-M-C	W94	qPCR for 5 target species (see Analysis)	1.2	1930
5-S	W95	qPCR for 5 target species (see Analysis)	0.45	3000
5-S Archive	W96		0.45	1510
6-S	W97	qPCR for 5 target species (see Analysis)	0.45	2280
6-S Archive	W98		0.45	2180
Neg Control 2	W99	qPCR for 5 target species (see Analysis)	0.45	<10
47-S	W100	qPCR for 5 target species (see Analysis)	0.45	6040
47-S Archive	W101		0.45	2520
52-S	W102	qPCR for 5 target species (see Analysis)	0.45	4320
52-S Archive	W103		0.45	1560
50-S	W104	qPCR for 5 target species (see Analysis)	0.45	2460
50-S Archive	W105		0.45	2200
49-L	W106	qPCR for 5 target species (see Analysis)	1.2	4260
49-L Archive	W107		1.2	4240

3. METHODS

3.1 qPCR Assay Design

TripleLock™ qPCR assays, designed and validated to detect the following species: cascade frog (*Rana cascadae*), brown trout (*Salmo trutta*), and brook trout (*Salvelinus fontinalis*) were deployed on samples provided by Wood Plc. Additionally, A brand new de novo TripleLock™ qPCR assay was commissioned to detect the parasitic leech *Haemopsis marmorata*. Finally, we adapted and validated a rainbow trout (*Oncorhynchus mykiss*) qPCR assay from the literature (Wilcox *et al.* 2015) to check for compatibility with PBI standards. For complete assay design and validation methodology see [Appendices I, II, III, IV and V](#).

3.2 Sample Preservation and Transport

A combination of Smith-Root self-preserving filters with pore sizes 0.45 µm and 1.2 µm were used to collect eDNA from water samples (See Sample Submission Table). Filters were preserved using silica gel desiccant during transport. Samples that consisted of multiple filters were combined during extraction to yield a single eDNA extract.

3.3 eDNA Extraction from Filters

eDNA was extracted from filters using a validated protocol for eDNA. eDNA was extracted using the DNeasy Blood and Tissue kit and the Qiashredder kit (Qiagen). Individual filters from each sample envelope were subject to overnight digestion in lysis buffer ATL (Qiagen) and proteinase K before the lysate from each was pooled into single tubes representing each sample at the post-Qiashredder stage. eDNA extracts from any pooled filters were immediately stored at 4° C until further analysis.

3.4 Fluorometric Quantification of DNA Yield

DNA yields per sample was measured by fluorometric quantification on a Qubit Flex™ Spectrophotometer (Thermofisher). This measure quantifies total eDNA (including non-target DNA) captured on the filter papers and extracted into the final elution solution during DNA extraction. This quality control step is used to control against filter and extraction malfunctions to ensure that sufficient DNA was extracted for qPCR analyses.

3.5 qPCR for the Detection of eDNA from *Rana cascadae*, *Haemopsis marmorata*, *Oncorhynchus mykiss*, *Salvelinus fontinalis*, and *Salmo trutta*

qPCR was conducted using a CFX96 Real-Time Detection System (BioRad) thermocycler with optimized thermal cycling conditions (see Appendices for cycling conditions for each assay). All qPCRs performed in triplicate per sample (N = 3). Positive amplification controls (PAC) consisting of reactions containing the target species' DNA fragment were included in each qPCR run to verify qPCR assay performance. No-template controls (NTC) were included in each qPCR run to detect the potential presence of sample or reagent contamination during analysis. Each qPCR reaction was 20 µL, consisting of 15 µL of customized master mix and 5 µL of eDNA extract. Prior to target species analysis, each sample was tested for PCR inhibition by comparing the C_q value of each eDNA extract from the expectations of uninhibited samples in which TE buffer is added as a template

(the NTC). If the Cq value of the samples are delayed by at least one Cq value relative to the IPC control, then the sample is diluted with ultrapure water and this diluted extract is subject to target species qPCR.

Any positive Cq values were converted to copy number per reaction by applying the following equation:

$$E_{AMP}^{(b-Cq)}$$

where E_{AMP} is the amplification efficiency of the assay conveyed as a fraction of the doubling number (2), as determined by a standard curve, b is the y-intercept of the slope calculated from the same standard curve, and Cq is the Cq value derived per sample from the survey's qPCR reaction.

4. RESULTS

4.1 DNA Extraction Yield

All samples – to be analysed with qPCR now and for archiving - yielded greater than 600 ng/mL DNA (See [Sample Submission Table](#)), with the exception of the two field negative blanks, which were below the detectability threshold of the Qubit Flex™, as expected.

The average DNA concentration across all samples was 3096.65 ± 2527.41 ng/mL (standard deviation). Excluding the negative controls, the average yield rises slightly to 3155.45 ± 2514.74 ng/mL.

4.2 Results of qPCR Analysis

All NTCs displayed no amplification for any of the target species, indicating no contamination during the PCR preparation and analytical stages. All IPCs amplified in the NTCs providing the baseline mean IPC value against which all sample IPC values will be measured to determine the presence of PCR inhibitors. Mean NTC IPC (= baseline uninhibited IPC level) was calculated as 35.20 ± 0.60 Cq units. Of the 56 eDNA extracts analyzed, 14 showed no signs of PCR inhibition (see Tables 1, 2 and 3). The remaining 42 samples were subject to both 1:10 and 1:20 dilutions in TE buffer, and the IPC analysis was rerun. All samples exhibited IPC values akin to the baseline NTC IPC level with 1:20 dilutions, but only one sample did not with a 1:10 dilution (sample W75 – denoted with a double-asterisk). Thus, qPCR for the five target species were conducted on a 1:20 dilution of sample W75, but on a 1:10 dilution of all the other samples in which the undiluted samples showed initial PCR inhibition.

For all species, positive amplification controls (PACs) amplified 100% across replicates: *R. cascadae* (Mean Cq = 19.19 ± 0.88); *H. marmorata* (Mean Cq = 18.97 ± 0.86); *S. fontinalis* (Mean Cq = 21.83 ± 0.66); *O. mykiss* (Mean Cq = 29.78 ± 0.26); *S. trutta* (Mean Cq = 24.56 ± 0.64). Successful PAC amplification renders any risk of false negative detection from degraded reagents or suboptimal or defective PCR cycling conditions as negligible.

qPCR results delivered as Cq values can be viewed in Table 1 for *R. cascadae* and *H. marmorata*, and in Table 2 for the predatory fishes. For, *R. cascadae*, 100 % amplification of each of three technical replicates was observed for samples W3 and W4, both known positive samples (Table 1). However, samples W1 and W2, also from a site of known occupancy, were negative. Both these samples were analysed undiluted. As a secondary test, we performed a further set of triplicate qPCR assays for both 1:10 dilutions of W1 and W2. However, this secondary analysis also failed to turn-up any *R. cascadae* amplification (results not tabulated).

Table 1: Results of qPCR analysis for the detection of *R. cascadae* and *H. marmorata* DNA from aquatic environmental samples. qPCR results are reported as a Cq value. A lower Cq values indicates greater levels of target DNA in the sample. IPC Cq is reported for this assay only (but applies to all target species qPCR results as each species-specific PCR is conducted on the same sample DNA extract). Sample numbers with an asterisk were diluted by 1:10; samples with a double asterisk were diluted by 1:20. All IPC Cq values are reported for the neat extract, or for the final diluted sample at which the IPC was recovered, establishing the mitigation of PCR inhibitors.

Sample	<i>R. cascadae</i> qPCR (Cq)			<i>H. marmorata</i> qPCR (Cq)			IPC Cq
W1	-	-	-	-	-	-	35.13
W2	-	-	-	-	-	-	35.92
W3	33.41	33.75	33.88	-	-	-	35.67
W4*	36.29	37.12	38.44	-	-	-	35.59
W5*	-	-	-	-	-	-	35.82
W7*	-	-	-	-	-	-	36.00
W9*	-	-	-	-	-	-	34.92
W10*	-	-	-	-	-	-	35.73
W13*	-	-	-	-	-	-	34.23
W14*	-	-	-	-	-	-	35.14
W17*	-	-	-	-	-	-	34.39
W18*	-	-	-	-	-	-	34.04
W20*	-	-	-	-	-	-	35.09

Sample	<i>R. cascadae</i> qPCR (Cq)			<i>H. marmorata</i> qPCR (Cq)			IPC Cq
W23*	-	-	-	-	-	-	34.97
W24*	-	-	-	-	-	-	34.78
W26*	-	-	-	-	-	-	35.14
W28*	-	-	-	-	-	-	34.19
W31*	-	-	-	-	-	-	35.18
W32*	-	-	-	-	-	-	36.65
W34*	-	-	-	-	-	-	33.40
W36*	-	-	-	-	-	-	34.83
W38*	-	-	-	-	-	-	33.44
W41*	-	-	-	-	-	-	34.58
W43*	-	-	-	-	-	-	35.53
W44*	-	-	-	-	-	-	35.55
W46*	-	-	-	-	-	-	34.33
W49*	-	-	-	-	-	-	35.34
W50*	-	-	-	-	-	-	35.08
W53*	-	-	-	-	-	-	35.06
W54*	-	-	-	38.13	37.62	37.92	35.69
W57*	-	-	-	-	-	-	36.13
W59*	-	-	-	-	-	-	35.88
W61*	-	-	-	-	-	-	34.46
W63*	-	-	-	-	-	-	34.15
W65*	-	-	-	-	-	-	35.67
W67*	-	-	-	-	-	-	34.83
W70*	-	-	-	-	-	-	35.09
W71*	-	-	-	-	-	-	35.17
W73*	-	-	-	-	-	-	34.76
W75**	-	-	-	-	-	-	35.67
W77	-	-	-	-	-	-	35.23
W79	-	-	-	-	-	-	34.14
W82	-	-	-	-	-	-	34.34

Sample	<i>R. cascadae</i> qPCR (Cq)			<i>H. marmorata</i> qPCR (Cq)			IPC Cq
W83	-	-	-	-	-	-	34.34
W85	-	-	-	-	-	-	34.14
W87	-	-	-	-	-	-	35.28
W89	-	-	-	-	-	-	34.54
W91	-	-	-	-	-	-	34.54
W94	-	-	-	-	-	-	34.14
W95*	-	-	-	-	-	-	35.38
W97*	-	-	-	-	-	-	36.78
W99	-	-	-	-	-	-	34.56
W100	-	-	-	-	-	-	34.78
W102*	-	-	-	-	-	-	34.11
W104*	-	-	-	-	-	-	34.68
W106*	-	-	-	-	-	-	34.50

-: Indicates that no Cq value was detected.

Low technical qPCR replication effort may explain continued non-detection in samples W1 and W2, especially for a rare species, despite its presence being known. Without further information on the numbers of individuals present and the water chemistry and quality of the surveyed site (which may impact the removal/degradation of eDNA in situ), it is difficult to make concrete conclusions. However, converting the Cq values to copy number per μL of extracted DNA (Table 3), one can begin to make meaningful comparison between samples. The mean copy numbers of *R. cascadae* genetic markers detected for W3 and for W4 are 106.23 ± 17.87 and 10.89 ± 6.95 , respectively, suggesting higher loads of *R. cascadae* eDNA in sample W3 over sample W4, possibly reflecting a higher density population where W3 was sampled. No formal conclusions can be made, however, except that the assay was shown to amplify target DNA in 100% of samples in which a detection was observed, with the caveat that further field optimization, with controlled water chemistry and site characteristic measurements, be employed if one assumes, *prima facie*, a false negative (Type II) error of 0.5 (albeit tempered by performing only 12 PCR reactions in total for the positive controls).

Samples W1 – W4 were in fact negative for all the other four species, except for a singleton detection of *O. mykiss* in sample W3. This inverse pattern of frog and predator detection is displayed in broader array in the remainder of the samples (W5 – W106) in which zero detection of the frog was observed, but large amounts of predatory fish eDNA was qualified, often in the same samples, and in significant concentrations.

Table 2: Results of qPCR analysis for the detection of the three putative fish predators of *R. cascadae*: *S. fontinalis*, *O. mykiss*, and *S. trutta*. qPCR results are reported as a Cq value. A lower Cq values indicates greater levels of target DNA in the sample. Sample numbers with an asterisk were diluted by 1:10; samples with a double asterisk were diluted by 1:20.

Sample	<i>S. fontinalis</i> qPCR (Cq)			<i>O. mykiss</i> qPCR (Cq)			<i>S. trutta</i> qPCR (Cq)		
W1	-	-	-	-	-	-	-	-	-
W2	-	-	-	-	-	-	-	-	-
W3	-	-	-	38.94	-	-	-	-	-
W4*	-	-	-	-	-	-	-	-	-
W5*	-	-	-	-	-	-	-	-	-
W7*	-	-	-	38.42	-	-	-	-	-
W9*	-	-	-	-	-	-	-	-	-
W10*	-	-	-	35.16	35.03	34.91	36.15	36.29	36.35
W13*	-	-	-	-	-	-	-	-	-
W14*	-	-	-	-	-	-	-	-	-
W17*	34.41	34.43	35.02	-	-	-	-	-	-
W18*	34.03	34.58	34.63	-	-	-	-	-	-
W20*	35.22	35.46	35.83	-	-	-	-	-	-
W23*	34.65	34.90	36.39	-	-	-	-	-	-
W24*	-	-	-	-	-	-	-	-	-
W26*	-	-	-	-	-	-	-	-	-
W28*	36.58	37.26	38.05	34.92	34.62	34.36	34.77	35.28	35.37
W31*	-	-	37.92	39.15	38.20	37.04	37.10	37.24	37.35
W32*	-	-	-	-	-	-	-	-	-
W34*	32.71	32.87	32.95	34.16	34.13	34.12	37.44	37.97	38.03
W36*	-	-	-	-	-	-	-	-	-
W38*	-	-	-	-	-	-	-	-	-
W41*	-	-	-	-	-	-	-	-	-
W43*	-	-	-	-	-	-	-	-	-
W44*	-	-	-	-	-	-	-	-	-

Sample	<i>S. fontinalis</i> qPCR (Cq)			<i>O. mykiss</i> qPCR (Cq)			<i>S. trutta</i> qPCR (Cq)		
W46*	-	-	-	-	-	-	-	-	-
W49*	-	-	-	-	-	-	-	-	-
W50*	36.70	37.65	38.24	34.57	34.19	33.34	35.54	36.00	36.52
W53*	-	38.57	38.65	36.48	34.44	34.24	36.18	36.24	36.46
W54*	-	-	-	-	-	-	-	-	-
W57*	-	-	-	-	-	-	-	-	39.28
W59*	-	-	-	-	-	-	-	-	-
W61*	32.78	33.11	33.07	-	-	-	-	-	-
W63*	31.64	31.49	31.16	-	36.80	-	-	-	-
W65*	35.05	34.46	34.70	37.45	36.82	37.36	35.71	36.12	36.17
W67*	-	-	-	-	-	-	-	-	-
W70*	36.43	36.35	35.86	39.21	38.50	38.19	33.30	34.29	34.81
W71*	33.58	33.79	33.45	39.01	-	-	31.20	31.45	31.59
W73*	-	-	-	-	-	-	-	-	-
W75**	-	36.46	36.53	-	-	-	-	-	-
W77	-	-	-	-	-	-	-	-	-
W79	-	-	-	-	-	-	-	-	-
W82	-	-	-	-	-	-	-	-	-
W83	-	-	-	-	-	-	-	-	-
W85	-	-	-	-	-	-	-	-	-
W87	-	-	-	-	-	-	-	-	-
W89	-	-	-	-	-	-	-	-	-
W91	-	-	-	-	-	-	-	-	-
W94	-	-	-	-	-	-	-	-	-
W95*	32.33	32.00	32.10	-	-	-	-	-	-
W97*	33.29	33.25	33.51	-	-	-	-	-	-
W99	-	-	-	-	-	-	-	-	-
W100	-	-	-	-	-	-	-	-	-
W102*	-	-	-	-	-	-	-	-	-
W104*	34.26	34.35	33.59	36.07	36.23	37.04	36.70	37.29	37.30

Sample	<i>S. fontinalis</i> qPCR (Cq)			<i>O. mykiss</i> qPCR (Cq)			<i>S. trutta</i> qPCR (Cq)		
W106*	-	-	-	37.24	38.36	38.63	37.30	38.12	38.45

In more detail (Table 2), positive *S. fontinalis* detection was observed in 18 individual samples, encompassing 50/177 qPCR reactions (28.24% test positivity rate); positive *O. mykiss* detection was observed in 14 individual samples encompassing 31/177 qPCR reactions (17.51% test positivity rate); and positive *S. trutta* detection was observed in 12 individual samples encompassing 34/177 qPCR reactions (19.21% test positivity rate). In terms of copy numbers of target genetic markers per sample (Table 3), across all positive PCR reactions, *S. fontinalis* eDNA was most prevalent of the three fishes (mean copy number = 130.17 ± 9.42), followed by *O. mykiss* (mean copy number = 28.88 ± 11.43), and *S. trutta*, respectively (mean copy number = 7.82 ± 1.81). Elevated copy number may be related to the abundance of the target species, but may also be influenced by shedding rates, which may differ allometrically among species and life-history stages, among other factors. Thus, any interpretation of the ‘strength of eDNA’ signal should be used cautiously and should be corroborated by other sources of information.

Finally, only one sample was positive for the parasitic leech, *H. marmorata* (Tables 1 and 3), and although this one sample had a 100% test positivity rate, copy numbers were very low. Aquatic invertebrates are harder to detect using eDNA methods than larger vertebrates, even free-swimming invertebrate species, due to orders-of-magnitude lower shedding rates per unit of biomass, among other possibly influential factors related to allometric-scaling of physiology and inherent metabolic differences between vertebrate and invertebrate taxa. Further corroborate work and known positive samples for the leech will enable further optimization of invertebrate-specific protocols to maximize the probability of detecting invertebrate DNA from aqueous environmental samples.

Table 3: Copy number analysis (copies per µl of eDNA extract) for each target species analyzed for all qPCR positive replicates. Cq values were converted to copy number for each sample using $E_{AMP}^{(b-Cq)}$, where “E_{AMP}” and “b” were derived from the standard curve analyses for each species assay (see Appendix I – V).

Sample	<i>R. cascadae</i>			<i>H. marmorata</i>			<i>S. fontinalis</i>			<i>O. mykiss</i>			<i>S. trutta</i>		
W3	126.3	100.4	92.0	-	-	-	-	-	-	3.1	-	-	-	-	-
W4	18.1	10.33	4.24	-	-	-	-	-	-	-	-	-	-	-	-
W7	-	-	-	-	-	-	-	-	-	4.4	-	-	-	-	-
W10	-	-	-	-	-	-	-	-	-	36.3	-	-	2.3	2.1	2.0
W17	-	-	-	-	-	-	80.7	79.6	53.9	-	-	-	-	-	-
W18	-	-	-	-	-	-	103.7	72.1	69.8	-	-	-	-	-	-
W20	-	-	-	-	-	-	47.3	40.3	31.6	-	-	-	-	-	-
W23	-	-	-	-	-	-	68.9	58.4	21.8	-	-	-	-	-	-
W28	-	-	-	-	-	-	19.3	12.3	7.3	42.4	51.5	60.9	5.9	4.2	3.9
W31	-	-	-	-	-	-	-	-	8.0	2.7	5.1	10.7	1.2	1.1	1.0
W34	-	-	-	-	-	-	247.8	222.9	211.5	-	70.7	71.15	0.9	0.7	0.6
W50	-	-	-	-	-	-	17.8	9.5	6.4	53.15	68.0	118.0	3.5	2.5	1.8
W53	-	-	-	-	-	-	-	5.2	4.9	15.4	57.8	65.8	2.2	2.2	1.8
W54	-	-	-	0.1	0.2	0.1	-	-	-	-	-	-	-	-	-
W57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2
W61	-	-	-	-	-	-	236.6	190.3	195.4	-	-	-	-	-	-
W63	-	-	-	-	-	-	502.1	554.4	689.3	-	12.5	-	-	-	-

W65	-	-	-	-	-	-	52.9	78.1	66.6	8.2	12.4	8.7	3.1	2.3	2.3
W70	-	-	-	-	-	-	21.3	22.4	31.0	2.6	4.2	5.1	16.3	8.2	5.8
W71	-	-	-	-	-	-	139.5	121.5	152.0	3.0	-	-	69.2	58.2	52.9
W75	-	-	-	-	-	-	-	20.8	19.9	-	-	-	-	-	-
W95	-	-	-	-	-	-	318.4	395.9	370.6	-	-	-	-	-	-
W97	-	-	-	-	-	-	169.0	173.5	146.1	-	-	-	-	-	-
W104	-	-	-	-	-	-	89.07	83.9	138.6	20.1	18.1	10.7	1.6	1.0	1.0
W106	-	-	-	-	-	-	-	-	-	9.4	4.6	3.8	1.0	0.6	0.5

5. APPENDIX I: *Rana cascadae* qPCR ASSAY

The Precision Biomonitoring TripleLock™ cascades frog (*Rana cascadae*) qPCR assay for eDNA is a quantitative real-time PCR (qPCR) assay, designed for the detection of *R. cascadae* environmental DNA (eDNA) obtained from filtered water samples.

Technical Specifications

The TripleLock™ *R. cascadae* qPCR assay was validated in accordance with parameters outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). The *R. cascadae* assay consists of a proprietary set of oligonucleotide primers and hydrolysis probe, and qPCR Master Mix, for which reactions are run using optimized thermal cycling conditions. Optimized thermal cycling conditions were validated using a magnetic induction thermal cycler (MIC, Bio Molecular Systems) (see Table A1).

Table A1: Optimized thermal cycling conditions of the *R. cascadae* assay

Step	Time (s)	Temperature (°C)
1. Initial Denature	180	95
<i>Followed by 40x cycles of steps 2 and 3</i>		
2. Denature	15	95
3. Anneal	30	62.8

The performance of the *R. cascadae* assay was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment of which the copy number is known. The qPCR efficiency, limit of detection (LOD), and limit of quantification (LOQ) were interpreted from the results of the standard curve (see Table A2). The LOD is the lowest concentration of target for which positive amplification is observed in 95% of the replicates. The LOQ is defined as the lowest concentration of target for which 100% of replicates amplify and that can be accurately quantified with a coefficient of variance below a threshold of ≤35% obtained from back-calculated copy number for replicates in a standard curve (Forootan *et al.*, 2017). The standard curve was run on a BioRad CFX96 thermocycler.

Table A2: Validated *R. cascadae* assay parameters

Parameter	Validated Result
qPCR Efficiency	96.35%
LOD	1.55 copies/μL
LOQ	30.90 copies/μL
R ²	0.999
Equation	y = -3.410x + 40.581

Specificity Testing

The specificity of the *R. cascadae* assay was tested using genomic DNA extracted from specimens of sympatric species (see Table A3). Based on these results the assay was determined to be species specific.

Table A3: Results of *R. cascadae* Assay Specificity Testing

Species	No. of samples	Detection Result
<i>Rana cascadae</i> (target)	1	Positive
<i>Lithobates catesbeianus</i>	1	Negative
<i>Lithobates septentrionalis</i>	1	Negative
<i>Lithobates pipiens</i>	1	Negative
<i>Lithobates palustris</i>	1	Negative
<i>Lithobates sylvaticus</i>	1	Negative
<i>Rana draytonii</i>	1	Negative
<i>Rana aurora</i>	1	Negative
<i>Rana boylei</i>	1	Negative
<i>Rana luteiventris</i>	1	Negative
<i>Rana muscosa</i>	1	Negative
<i>Rana pretiosa</i>	1	Negative

References

- Bustin, S.A., Benes, V., Garson, J.A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622.
<https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantif. 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>

Disclaimer

For Research Use Only. Not for use in human or veterinary diagnostic procedures. The performance characteristics of this product have not been established. Precision Biomonitoring products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or to provide a service to third parties without written approval of Precision Biomonitoring, Inc.

6. APPENDIX II: *Haemopsis marmorata* qPCR ASSAY

The Precision Biomonitoring TripleLock™ *Haemopsis marmorata* qPCR assay for eDNA is a quantitative real-time PCR (qPCR) assay, designed for the detection of *H. marmorata* environmental DNA (eDNA) obtained from filtered water samples.

Technical Specifications

The TripleLock™ *H. marmorata* qPCR assay was validated in accordance with parameters outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). The *H. marmorata* assay consists of a proprietary set of oligonucleotide primers and hydrolysis probe, and qPCR Master Mix, for which reactions are run using optimized thermal cycling conditions. Optimized thermal cycling conditions were validated using a magnetic induction thermal cycler (MIC, Bio Molecular Systems) (see Table A4).

Table A4: Optimized thermal cycling conditions of the *H. marmorata* assay

Step	Time (s)	Temperature (°C)
1. Initial Denature	180	95
<i>Followed by 40x cycles of steps 2 and 3</i>		
2. Denature	15	95
3. Anneal	30	59.4

The performance of the *H. marmorata* assay was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment of which the copy number is known. The qPCR efficiency, limit of detection (LOD), and limit of quantification (LOQ) were interpreted from the results of the standard curve (see Table A5). The LOD is the lowest concentration of target for which positive amplification is observed in 95% of the replicates. The LOQ is defined as the lowest concentration of target for which 100% of replicates amplify and that can be accurately quantified with a coefficient of variance below a threshold of ≤35% obtained from back-calculated copy number for replicates in a standard curve (Forootan *et al.*, 2017). The standard curve was run on a BioRad CFX96 thermocycler.

Table A5: Validated *H. marmorata* assay parameters

Parameter	Validated Result
qPCR Efficiency	98.26%
LOD	0.2 copies/μl
LOQ	1.5 copies/μl
R ²	0.9994
Equation	y=-3.3643x+35.062

Specificity Testing

The specificity of the *H. marmorata* assay was tested using genomic DNA extracted from the target species and on synthetic homologs of the target gene fragment in related species (see Table A6). Based on these results the assay was determined to be species specific.

Table A6: Results of *H. marmorata* Assay Specificity Testing

Species	No. of samples	Detection Result
<i>Haemopsis marmorata</i> (target)	1	Positive
<i>Haemopsis terrestris</i>	1	Negative
<i>Haemopsis elegans</i>	1	Negative
<i>Whitmania pigra</i>	1	Negative
<i>Whitmania laevis</i>	1	Negative

References

- Bustin, S.A., Benes, V., Garson, J.A., Helleman, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622. <https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantif. 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>

Disclaimer

For Research Use Only. Not for use in human or veterinary diagnostic procedures. The performance characteristics of this product have not been established. Precision Biomonitoring products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or to provide a service to third parties without written approval of Precision Biomonitoring, Inc.

7. APPENDIX III: *Salvelinus fontinalis* qPCR ASSAY

The Precision Biomonitoring TripleLock™ brook trout (*Salvelinus fontinalis*) qPCR assay for eDNA is a quantitative real-time PCR (qPCR) assay, designed for the detection of *S. fontinalis* environmental DNA (eDNA) obtained from filtered water samples.

Technical Specifications

The TripleLock™ *S. fontinalis* qPCR assay was validated in accordance with parameters outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). The *S. fontinalis* assay consists of a proprietary set of oligonucleotide primers and hydrolysis probe, and qPCR Master Mix, for which reactions are run using optimized thermal cycling conditions. Optimized thermal cycling conditions were validated using a magnetic induction thermal cycler (MIC, Bio Molecular Systems) (see Table A7).

Table A7: Optimized thermal cycling conditions of the *S. fontinalis* assay

Step	Time (s)	Temperature (°C)
1. Initial Denature	120	95
<i>Followed by 40x cycles of steps 2 and 3</i>		
2. Denature	15	95
3. Anneal	45	60

The performance of the *S. fontinalis* assay was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment of which the copy number is known. The qPCR efficiency, limit of detection (LOD), and limit of quantification (LOQ) were interpreted from the results of the standard curve (see Table A8). The LOD is the lowest concentration of target for which positive amplification is observed in 95% of the replicates. The LOQ is defined as the lowest concentration of target for which 100% of replicates amplify and that can be accurately quantified with a coefficient of variance below a threshold of ≤35% obtained from back-calculated copy number for replicates in a standard curve (Forootan *et al.*, 2017). The standard curve was run on a MIC (Bio Molecular Systems).

Table A8: Validated *S. fontinalis* assay parameters

Parameter	Validated Result
qPCR Efficiency	93.50%
LOD	13.4 copies/μl
LOQ	13.4 copies/μl
R ²	0.99
Equation	Y=-3.493x+41.061

Specificity Testing

The specificity of the *S. fontinalis* assay was tested using genomic DNA extracted from specimens of sympatric species (see Table A9). Based on these results the assay was determined to be species specific.

Table A9: Results of *S. fontinalis* Assay Specificity Testing

Species	No. of samples	Detection Result
<i>Salvelinus fontinalis</i> (target)	5	Positive
<i>Oncorhynchus tshawytscha</i>	5	Negative
<i>Oncorhynchus keta</i>	5	Negative
<i>Oncorhynchus kisutch</i>	5	Negative
<i>Oncorhynchus gorbuscha</i>	5	Negative
<i>Oncorhynchus nerka</i>	5	Negative
<i>Oncorhynchus mykiss</i>	5	Negative
<i>Salmo salar</i>	5	Negative
<i>Lota lota</i>	1	Negative
<i>Esox lucius</i>	1	Negative
<i>Catostomus commersonii</i>	1	Negative
<i>Cottus bairdii</i>	1	Negative

References

- Bustin, S.A., Benes, V., Garson, J.A., Helleman, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622.
<https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantif. 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>

Disclaimer

For Research Use Only. Not for use in human or veterinary diagnostic procedures. The performance characteristics of this product have not been established. Precision Biomonitoring products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or to provide a service to third parties without written approval of Precision Biomonitoring, Inc.

8. APPENDIX IV: *Salmo trutta* qPCR ASSAY

The Precision Biomonitoring TripleLock™ brown trout (*Salmo trutta*) qPCR assay for eDNA is a quantitative real-time PCR (qPCR) assay, designed for the detection of *S. trutta* environmental DNA (eDNA) obtained from filtered water samples.

Technical Specifications

The TripleLock™ *S. trutta* qPCR assay was validated in accordance with parameters outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). The *S. trutta* assay consists of a proprietary set of oligonucleotide primers and hydrolysis probe, and qPCR Master Mix, for which reactions are run using optimized thermal cycling conditions. Optimized thermal cycling conditions were validated using a magnetic induction thermal cycler (MIC, Bio Molecular Systems) (see Table A10).

Table A10: Optimized thermal cycling conditions of the *S. trutta* assay

Step	Time (s)	Temperature (°C)
1. Initial Denature	120	95
<i>Followed by 40x cycles of steps 2 and 3</i>		
2. Denature	15	95
3. Anneal	45	60

The performance of the *S. trutta* assay was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment of which the copy number is known. The qPCR efficiency, limit of detection (LOD), and limit of quantification (LOQ) were interpreted from the results of the standard curve (see Table A11). The LOD is the lowest concentration of target for which positive amplification is observed in 95% of the replicates. The LOQ is defined as the lowest concentration of target for which 100% of replicates amplify and that can be accurately quantified with a coefficient of variance below a threshold of $\leq 35\%$ obtained from back-calculated copy number for replicates in a standard curve (Forootan *et al.*, 2017). The standard curve was run on a BioRad CFX96 thermocycler.

Table A11: Validated *S. trutta* assay parameters

Parameter	Validated Result
qPCR Efficiency	99.10%
LOD	1.06 copies/μl
LOQ	1.06 copies/μl
R ²	0.9966
Equation	Y=-3.343x+37.352

Specificity Testing

The specificity of the *S. trutta* assay was tested using genomic DNA extracted from specimens of sympatric species (see Table A12). Based on these results the assay was determined to be species specific.

Table A12: Results of *S. trutta* Assay Specificity Testing

Species	No. of samples	Detection Result
<i>Salmo trutta</i> (target)	5	Positive
<i>Salmo salar</i>	5	Negative
<i>Oncorhynchus tshawytscha</i>	5	Negative
<i>Oncorhynchus kisutch</i>	5	Negative
<i>Oncorhynchus gorbuscha</i>	5	Negative
<i>Oncorhynchus keta</i>	5	Negative
<i>Oncorhynchus mykiss</i>	5	Negative
<i>Coregonus clupeaformis</i>	5	Negative
<i>Prosopium cylindraceum</i>	5	Negative
<i>Salvelinus fontinalis</i>	5	Negative

References

- Bustin, S.A., Benes, V., Garson, J.A., Helleman, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622.
<https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol. Detect. Quantif. 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>

Disclaimer

For Research Use Only. Not for use in human or veterinary diagnostic procedures. The performance characteristics of this product have not been established. Precision Biomonitoring products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or to provide a service to third parties without written approval of Precision Biomonitoring, Inc.

9. APPENDIX V: *Oncorhynchus mykiss* qPCR ASSAY

The Rainbow trout (*Oncorhynchus mykiss*) qPCR assay is a quantitative real-time PCR (qPCR) assay, published by Wilcox et al., 2015, for the detection of *O. mykiss* (including steelhead types) environmental DNA (eDNA).

Technical Specifications

The TripleLock™ *O. mykiss* qPCR assay was validated in accordance with parameters outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The *O. mykiss* assay consists of a proprietary set of oligonucleotide primers and hydrolysis probe, and qPCR Master Mix, for which reactions are run using optimized thermal cycling conditions. Optimized thermal cycling conditions were validated using a magnetic induction thermal cycler (MIC, Bio Molecular Systems) (see Table A13).

Table A13: Optimized thermal cycling conditions of the *O. mykiss* assay

Step	Time (s)	Temperature (°C)
1. Initial Denature	120	95
<i>Followed by 40x cycles of steps 2 and 3</i>		
2. Denature	15	95
3. Anneal	60	60

The performance of the *O. mykiss* assay was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment of which the copy number is known. The qPCR efficiency, limit of detection (LOD), and limit of quantification (LOQ) were interpreted from the results of the standard curve (see Table A14). The LOD is the lowest concentration of target for which positive amplification is observed in 95% of the replicates. The LOQ is defined as the lowest concentration of target for which 100% of replicates amplify and that can be accurately quantified with a coefficient of variance below a threshold of ≤35% obtained from back-calculated copy number for replicates in a standard curve (Forootan et al., 2017). The standard curve was run on a MIC (Bio Molecular Systems).

Table A14: Validated *O. mykiss* assay parameters

Parameter	Validated Result
qPCR Efficiency	91.2 %
LOD	10 copies/reaction
LOQ	586 copies/reaction
R ²	0.994
Y-intercept	40.7

Specificity Testing

Specificity testing was robustly tested in the original paper (Wilcox *et al.* 2015).

References

- Bustin, S.A., Benes, V., Garson, J.A., Helleman, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55, 611–622.
<https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol. Detect. Quantif.* 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>
- Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., Schwartz, M.K., 2015. The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *Oncorhynchus*. *PLoS One* 10, 1–13.
<https://doi.org/10.1371/journal.pone.0142008>

Disclaimer

For Research Use Only. Not for use in human or veterinary diagnostic procedures. The performance characteristics of this product have not been established. Precision Biomonitoring products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or to provide a service to third parties without written approval of Precision Biomonitoring, Inc.

6. APPENDIX VI: GLOSSARY

C_q: quantification cycle; the PCR cycle at which the target is considered positively amplified in a given sample.

Internal positive control (IPC): used to detect PCR inhibition in a reaction that may result from inhibitory substances carried through the sampling and extraction process.

Limit of detection (LOD): the lowest concentration of DNA that can be detected by a given qPCR assay 95% of the time, typically expressed as target copies/μL (Bustin *et al.* 2009).

Limit of quantification (LOQ): the lowest concentration of target that can be accurately quantified with a coefficient of variance below a threshold of ≤35% obtained from replicates in an assay specific standard curve (Forootan *et al.* 2017). Typically expressed as target copies/μL.

No template control (NTC): omits any DNA template from a reaction and serves as a control for extraneous nucleic acid contamination.

Positive Amplification Control (PAC): used to verify that a qPCR assay is performing properly during analysis. Amplification controls consist of the target DNA and should always return a positive amplification with an expected C_q value for a given assay.

Quantitative Real-time PCR (qPCR): a highly sensitive polymerase chain reaction procedure which monitors the amplification and detection of a targeted DNA molecule in real time.

qPCR Assay: the collection of primers, hydrolysis probe, IPC, master mix, and cycling conditions on a specific thermal cycler designed and optimized to amplify and detect target DNA.

qPCR efficiency: determined from the slope of the log-linear portion of the standard curve. High qPCR efficiency is indicative of precise and robust qPCR assay performance.

TaqMan™: a type of qPCR assay which employs fluorescent DNA probes to increase specificity.

Target: universal term for the nucleic acid sequence to be amplified.

Technical replicate: replicates used to perform the same test multiple times on a single sample.

7. APPENDIX VII: REFERENCES

- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* 55, 611–622. <https://doi.org/10.1373/clinchem.2008.112797>
- Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol. Detect. Quantif.* 12, 1–6. <https://doi.org/10.1016/j.bdq.2017.04.001>
- Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K., Schwartz, M.K., 2015. The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *Oncorhynchus*. *PLoS One* 10, 1–13. <https://doi.org/10.1371/journal.pone.0142008>

8. DISCLAIMER

REPORTS FROM PRECISION BIOMONITORING INC. ("The Company") ARE ISSUED SUBJECT TO THE FOLLOWING DISCLAIMERS:

1. Reports are issued free of any alterations or additions. The Company does not accept any liability whatsoever for the tampering or any unlawful alteration of documents sent via any electronic transmission media.
2. Reports or any attachments shall not be reproduced, except in full, without the prior written consent of The Company. Reports and any attachments by The Company are e-mailed in pdf format to the customer.
3. Reports relate only to the samples as received from the client and are issued in good faith. Every reasonable care is taken to ensure that the reports are accurate. Regarding the conclusions and/or opinions drawn from the results of these tests or investigations, The Company does not accept responsibility for any matters arising or consequences from the further use of these reports by third parties.
4. While every effort is taken by The Company and its employees to ensure that results/reports are timely presented to clients, The Company is not responsible for any late delivery of services because of circumstances beyond its control. In no event will The Company be liable for damages of any kind, including without limitation, direct, incidental or consequential damages (including, but not limited to, damages for lost profits, business interruption and loss programs or information) arising out of the use of or inability to use The Company's testing results and/or because of a delay in The Company providing test results, or in the test results or claims attributable to errors, omissions or other inaccuracies in the interpretations thereof. The customer agrees to indemnify, defend and hold The Company harmless from and against all losses, expenses, damages and costs, including reasonable attorney fees arising out of or relating to any misuse by the customer of the content and/or services provided by The Company.
5. Any possible infringement of any patent rights of formulations or processes or any other patent rights is the sole responsibility and liability of the customer.
6. If the Company or its employees or agents are required to give expert evidence in any litigation arising from the reports, then the client will be charged for such services at the prevailing rate of The Company.
7. All services rendered by The Company are treated as strictly confidential.
8. Any samples which remain after testing, will be retained for a period of 60 days only, and any documents arising from the service rendered will be retained for a period of two (2) years, unless otherwise agreed to by and between the customer and The Company in writing.
9. A legal contract between the customer and The Company will be deemed to have been constituted upon the receipt by the customer of services/reports.
10. The Company will not disclose information or test results to anyone other than the customer without the customer's written authorization and consent.
11. In the event of that, if any, of these terms or conditions are found to be invalid, unlawful, or unenforceable, such terms will be severable from the remaining terms, which will continue to be valid and enforceable.
12. Any legal costs and reasonable attorney fees incurred by The Company in enforcing any aspect of this agreement including legal or reasonable attorney fees will be the sole responsibility of the customer and will be payable by the customer to The Company on demand.