DOCKETED	
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TECHNICAL MEMORANDUM

DATE: January 16, 2020

TO: John Kuba, ConnectGen Operating LLC

FROM: Kori Hutchison and Andrea Chatfield, WEST, Inc.

RE: 2019 eDNA Surveys for Foothill Yellow-legged Frog at the Fountain Wind Project, Shasta County, California

INTRODUCTION

In September 2019, Western EcoSystems Technology, Inc. (WEST) performed aquatic environmental DNA (eDNA) surveys to assess presence/absence of foothill yellow-legged frog (FYLF; *Rana boylii*) at the proposed Fountain Wind Project (Project) in Shasta County, California. The eDNA surveys at the Project build upon the previous FYLF habitat assessment and visual encounter surveys (VES) conducted in 2018 and 2019, the methods and results of which are presented in Chatfield and Hutchison 2019. VES conducted at the Project in 2018 and 2019 yielded no detections of FYLF. Nonetheless, California Department of Fish and Wildlife (CDFW) biologists recommended presence/absence surveys using eDNA methodology to supplement the previous VES efforts at the Project. The eDNA survey results for FYLF were negative. The following memorandum summarizes the methods and results of eDNA surveys conducted at the Project in September 2019.

eDNA BACKGROUND

Environmental DNA is genetic material that has been released by an organism into its environment in the form of sloughed cells, feces, or other exogenous processes, and can be detected in air, water, or soil (Laramie et al. 2015; Carim et al. 2016). The collection and analysis of eDNA can be an efficient method of determining presence of aquatic organisms, and is an effective alternative to standard survey methods that are logistically difficult or have low probabilities of detection (Laramie et al. 2015; Carim et al. 2016; Goldberg et al. 2016; 2017). Environmental DNA methodology can be especially useful in determining presence of rare and sensitive species, as it is capable of detecting species presence at very low densities (Goldberg et al. 2013; 2016; 2017). Avoidance of contamination during sample collection, transportation, and analysis is of utmost importance, given the high sensitivity of eDNA methods and generally very low concentrations of aqueous DNA (Goldberg et al. 2016).

PROJECT AND SURVEY AREA

The Project is located on privately-owned, commercial timberlands in central Shasta County, California. The dominant vegetation type in and around the Project is mixed coniferous forest (post-fire and unburned), with smaller amounts of mixed montane chaparral and mixed montane riparian forest/scrub. The primary land use in this area is commercial timber production, which has resulted in a highly fragmented landscape across much of the area. Dominant overstory species include a combination of white fir (*Abies concolor*), Douglas fir (*Pseudotsuga menziesii*), incense cedar (*Calocedrus decurrens*), ponderosa pine (*Pinus ponderosa*), sugar pine (*P. lambertiana*), and California black oak (*Quercus kelloggii*).

For the purpose of determining eDNA sampling locations, an updated Project Site (i.e., the area in which Project facilities could be sited) was provided in a Geographic Information System (GIS) format by the Project proponent in September 2019 (Figure 1). The Project Site consists of approximately 4,463 acres (ac; 1,806 hectares [ha]) including all facilities included in the site plan and an appropriate buffer to capture areas where permanent and temporary disturbance could occur (e.g., newly proposed roads, roads that may be expanded, turbine pads, and underground collection lines) to provide for some flexibility in final project micrositing. For comparison purposes, previous development corridors (dated May 2018 and May 2019) used for the 2018 and 2019 FYLF habitat assessment and VES (see Chatfield and Hutchison 2019) are also depicted in Figure 1. The current (dated September 2019) Project Site largely overlaps the previous 2018 and 2019 development corridors, with the exception that the survey corridor around each turbine was increased from 500 feet to 700 feet resulting in approximately an additional 800 ac (324 ha). The additional 800 ac consists of mostly upland environments. The previous FYLF habitat assessment and VES, therefore, included the majority of the current Project Site. In combination with the 2019 eDNA surveys, which included the entirety of the Project Site, the FYLF studies conducted to date provide comprehensive coverage of all potential FYLF habitats within the current Project Site.

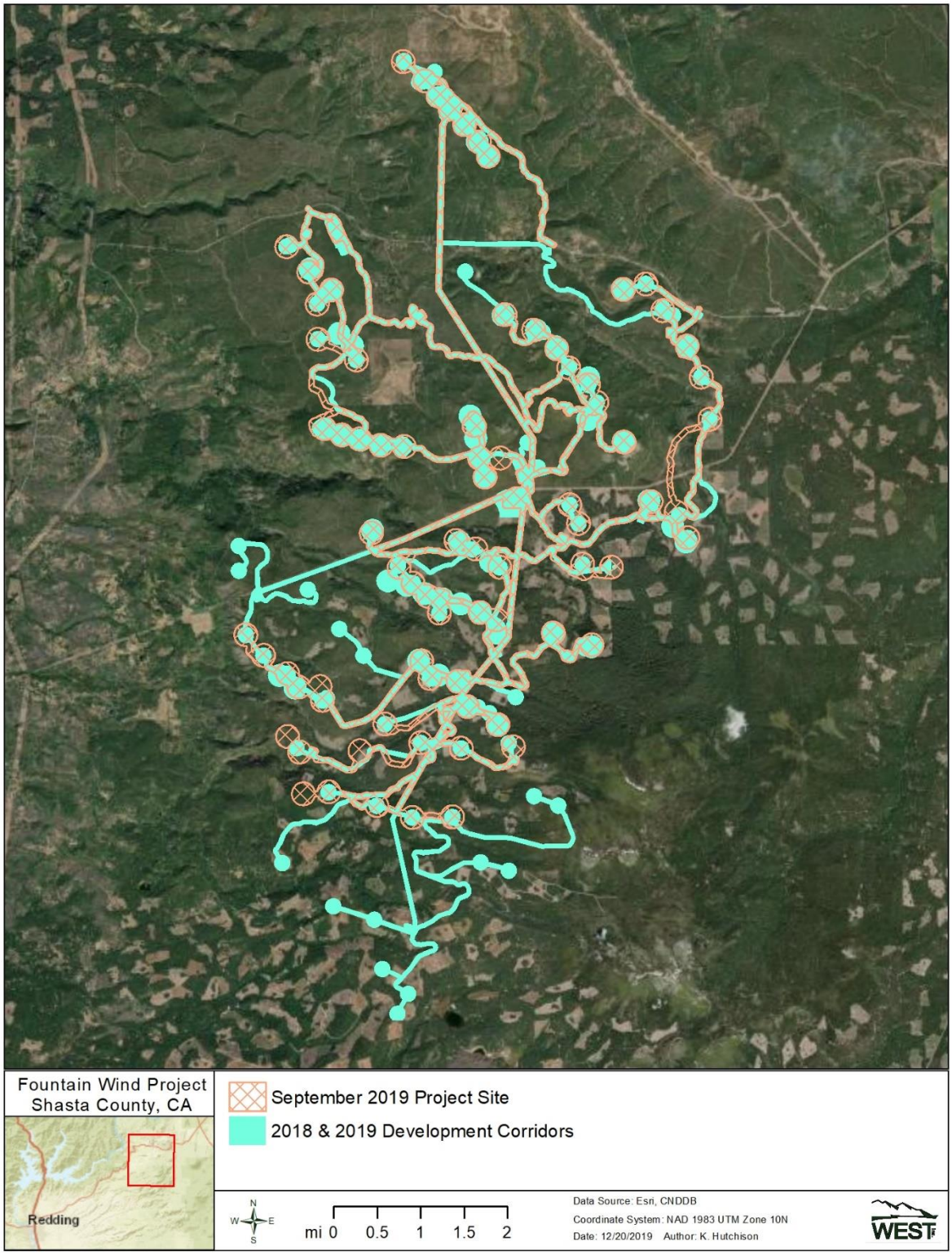

Figure 1. Current (September 2019) Project Site evaluated during the 2019 environmental DNA survey effort, in relation to the previously proposed development corridors evaluated during the 2018 and 2019 FYLF habitat assessment and visual encounter surveys (VES).

METHODS

Determining Sampling Locations

Geographic information system (GIS) data from the United States Geological Survey (USGS) National Hydrography Dataset (NHD; USGS 2019) and examination of aerial imagery were used to conduct a desktop assessment of potentially suitable stream crossings within the Project Site. Following the desktop assessment, a WEST biologist with training in FYLF survey methods conducted a field verification to determine the suitability of identified stream crossings for conducting eDNA surveys. Criteria considered during the field verification included whether stream crossings had sufficient surface water to conduct sampling (i.e., >3 liters [L]), and whether stream crossings would be directly affected by temporary or permanent ground disturbance (i.e., roads, turbine pads, underground collection lines). Streams that crossed below long spans of overhead collection lines were not surveyed. Additional crossings identified during the field verification were also evaluated for inclusion.

Sampling Equipment

In order to simplify sample collection and reduce the possibility of contamination and DNA degradation, eDNA surveys were conducted using Smith-Root's eDNA Sampler Backpack, a fully-integrated, purpose-built aquatic eDNA sampling system (Thomas et al. 2018; Smith-Root 2019a). This system was designed to minimize the risk of sample contamination in the field, using single-use, self-preserving filters so that the sample is never handled until it is received by the lab (Smith-Root 2019b). The eDNA Sampler Backpack uses a smart pump system that monitors the rate at which water is pumped to minimize clogging from suspended sediments, standardizing flow and pressure, and making it easy to accurately reach target volume (Smith-Root 2019a).

eDNA Field Surveys

Environmental DNA field surveys were conducted on September 25-27, 2019, consistent with recommendations and guidelines described in Goldberg et al. (2016; 2017) and methodology used in Carim et al. (2016). Surveys were conducted in all suitable stream crossings within the Project Site, targeting the potential fall distribution of FYLF. Samples were taken on the downstream side of each stream crossing, or on both sides when there was little to no connectivity between water on each side of the crossing (i.e., rocks, woody debris, or other materials interrupting stream flow). Three replicate samples were taken at each sampling station to safeguard against collection error and to detect any potential contamination (Goldberg et al. 2016). A new single-use filter was used for each of the three samples at each station. Given that each sample station was relatively clear of debris, the target volume of 1 L of water was filtered for each individual sample (Goldberg et al. 2017; J. Ponce, Technical Sales Associate and Biologist, Smith-Root, Inc., personal communication). Filters were placed at the end of a telescoping pole, and submerged just below the surface of the water without surveyors having to enter the stream. When the pump reached 1 L, the filter was removed and returned to its original re-sealable bag for transport to the laboratory. Time, water temperature, and geographic location of each sample were recorded, along with an identifying sample number.

Positive control samples were taken at a site known to have recent FYLF detections along the Trinity River, north of the junction with Ramshorn Creek in Trinity County, California (41.181002, -122.658809). Adult and subadult life stages of FYLF were observed when collecting positive control samples. Negative control samples were taken outside of the FYLF range on the Willamette River in Benton County, Oregon.

Sample Analysis

Laboratory-based qPCR analysis of the eDNA samples was performed by Precision Biomonitoring Inc. in Guelph, Ontario, using a qPCR assay adapted from Goldberg et al. (2016) and validated on a BioRad CFX96 PCR thermocycler to detect FYLF eDNA. The methods of this analysis are described in detail in the Environmental DNA Analysis Report prepared by Precision Biomonitoring Inc. (2019) and included as Appendix A.

RESULTS AND CONCLUSIONS

Results from the desktop assessment yielded 47 potential stream crossings within the Project Site suitable for eDNA sampling (Figure 2). During field verification of these locations, 29 crossings were determined to be unsuitable for sampling; 23 crossings were dry streambeds with no surface water present and six crossings did not cross the Project Site in areas that would be subject to ground disturbance (e.g., long spans of overhead distribution lines). An additional six crossings were identified as suitable during field verification, including one crossing that necessitated two sampling stations due to a lack of surface water connectivity between upstream and downstream flows. Together, the desktop assessment and field verification resulted in a total of 24 stream crossings determined to be suitable for eDNA sampling and a total of 25 sampling stations.

Environmental DNA samples were taken at each of the 25 sampling stations on September 25-27, 2019, with three water samples taken at each station. Twenty-one (21) of the 25 samples yielded quantifiable levels (>0.5 ng mL^{-1}; Crookes 2019) of DNA isolated from filters. Each of the five samples without quantifiable levels of DNA had corresponding replicates that did contain quantifiable levels of DNA. There were no positive detections of FYLF eDNA identified in the analysis. A detailed description of analysis results is provided in Crookes (2019; Appendix A).

The results of the 2019 eDNA surveys for FYLF are consistent with previous VES conducted at the Project in 2018 and 2019 (Chatfield and Hutchison 2019) which have found no evidence of FYLF presence within the Project Site. Results of the studies conducted at the Project to date indicate no impacts to FYLF resulting from Project development.

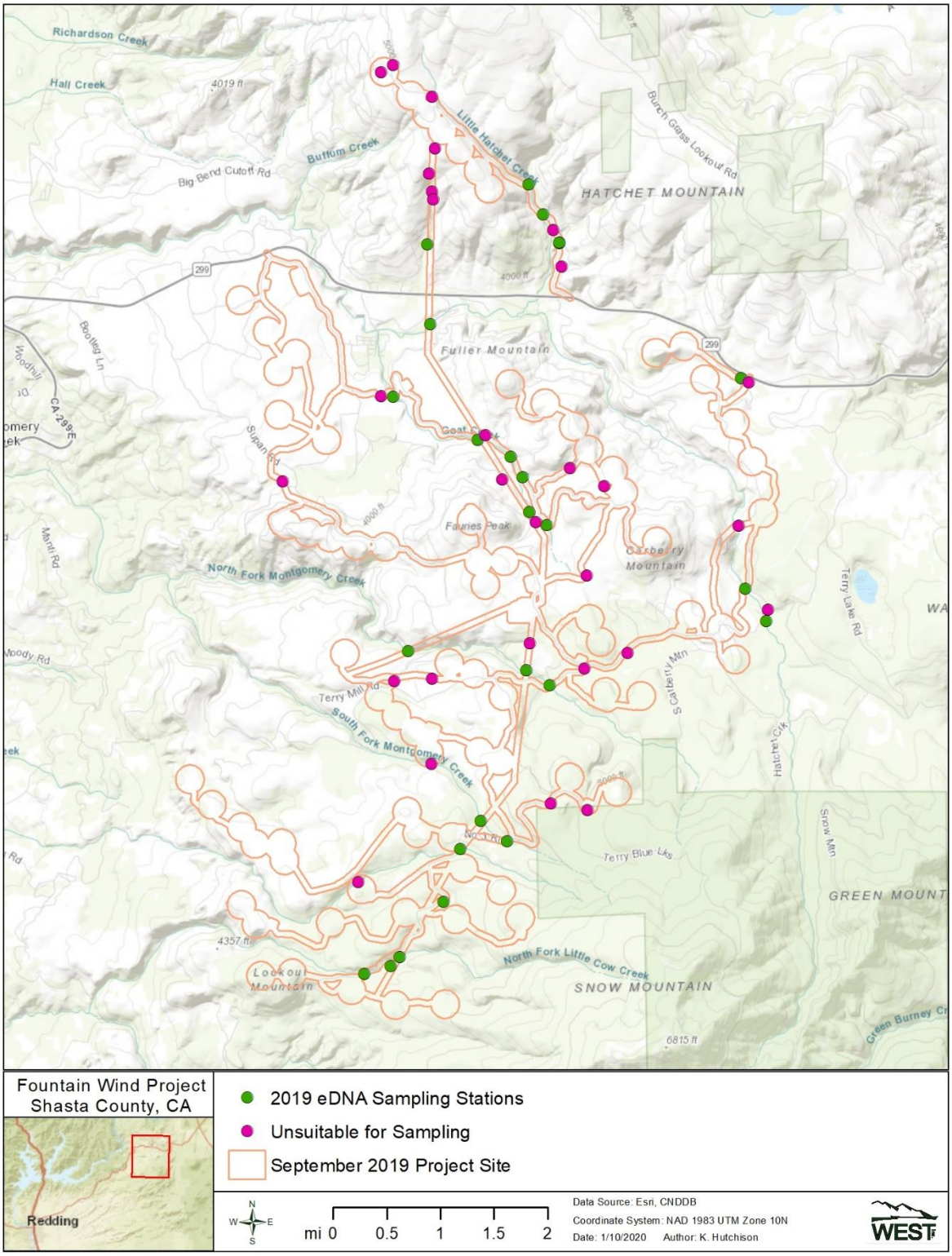

Figure 2. 2019 environmental DNA (eDNA) sampling stations with the Fountain Wind Project. Also shown are locations determined to be unsuitable for sampling based on field evaluation.

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APPENDIX A – ENVIRONMENTAL DNA ANALYSIS REPORT

Precision Biomonitoring Inc.
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Environmental DNA Analysis Report

Report Contents

Report Issued: 08/11/2019

Prepared By: Steve Crookes	Date: 08/11/2019	Email: steve.crookes@precisionbiomonitoring.com
Approved By: Mario Thomas	Date: 08/11/2019	Email: mario.thomas@precisionbiomonitoring.com

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1. REPORT INFORMATION

Prepared for

Smith-Root, Inc.,

16603 NE 50th Avenue, Vancouver,

Washington 98686

Contact Name	Email Address	Phone
Austen Thomas	a.thomas@smithroot.com	360 573 0202 x 2143

Sample Information (For additional sample information see Sample Information Table below.)

Project Reference: Unknown

Sample Collection Conducted by: West Inc.

Date Shipped: 10/2019

Sample Shipment Format: Filters (pore size = 5 µm), self-preserving .

Date Received: 10/2019

Samples Received By: Steve Crookes

Condition of Samples Upon Receipt: Intact, ambient temperature.

Sample Extraction: Qiagen DNeasy Blood and Tissue Kit and Qiashredder

Analysis

Analysis applied: qPCR to detect foothills yellow-legged frogs (*Rana boylii*) eDNA, fluorometric quantification of eDNA yield.

Analysis Conducted By: Steve Crookes

Analysis Location: Precision Biomonitoring Laboratories

Date(s) of Analysis: 22/10/2019 – 07/11/2019

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2. SAMPLE INFORMATION TABLE

Sample	Date of Filtration	Date of Sample Extraction	Date of DNA Yield Analysis	Date of qPCR Analysis
003	25/09/2019	22/10/2019	22/10/2019	07/11/2019
004	25/09/2019	22/10/2019	22/10/2019	07/11/2019
005	25/09/2019	22/10/2019	22/10/2019	07/11/2019
006	25/09/2019	22/10/2019	22/10/2019	07/11/2019
007	25/09/2019	22/10/2019	22/10/2019	07/11/2019
008	25/09/2019	22/10/2019	22/10/2019	07/11/2019
013	25/09/2019	22/10/2019	22/10/2019	07/11/2019
015	25/09/2019	22/10/2019	22/10/2019	07/11/2019
016	25/09/2019	22/10/2019	22/10/2019	07/11/2019
017	25/09/2019	22/10/2019	22/10/2019	07/11/2019
018	25/09/2019	22/10/2019	22/10/2019	07/11/2019
020	25/09/2019	22/10/2019	22/10/2019	07/11/2019
021	25/09/2019	22/10/2019	22/10/2019	07/11/2019
022	25/09/2019	22/10/2019	22/10/2019	07/11/2019
023	25/09/2019	22/10/2019	22/10/2019	07/11/2019
024	25/09/2019	22/10/2019	22/10/2019	07/11/2019
025	25/09/2019	22/10/2019	22/10/2019	07/11/2019
026	25/09/2019	22/10/2019	22/10/2019	07/11/2019
027	25/09/2019	22/10/2019	22/10/2019	07/11/2019
028	25/09/2019	22/10/2019	22/10/2019	07/11/2019
029	25/09/2019	22/10/2019	22/10/2019	07/11/2019
030	25/09/2019	24/10/2019	24/10/2019	07/11/2019
031	25/09/2019	24/10/2019	24/10/2019	07/11/2019
032	25/09/2019	24/10/2019	24/10/2019	07/11/2019
033	26/09/2019	24/10/2019	24/10/2019	07/11/2019
034	26/09/2019	24/10/2019	24/10/2019	07/11/2019
035	26/09/2019	24/10/2019	24/10/2019	07/11/2019
036	26/09/2019	24/10/2019	24/10/2019	07/11/2019
037	26/09/2019	24/10/2019	24/10/2019	07/11/2019
038	26/09/2019	24/10/2019	24/10/2019	07/11/2019

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Sample	Date of Filtration	Date of Sample Extraction	Date of DNA Yield Analysis	Date of qPCR Analysis
039	26/09/2019	24/10/2019	24/10/2019	07/11/2019
040	26/09/2019	24/10/2019	24/10/2019	07/11/2019
041	26/09/2019	24/10/2019	24/10/2019	07/11/2019
042	26/09/2019	24/10/2019	24/10/2019	07/11/2019
043	26/09/2019	24/10/2019	24/10/2019	07/11/2019
044	26/09/2019	24/10/2019	24/10/2019	07/11/2019
045	26/09/2019	24/10/2019	24/10/2019	07/11/2019
046	26/09/2019	24/10/2019	24/10/2019	07/11/2019
047	26/09/2019	24/10/2019	24/10/2019	07/11/2019
048	26/09/2019	24/10/2019	24/10/2019	07/11/2019
049	26/09/2019	28/10/2019	28/10/2019	07/11/2019
050	26/09/2019	28/10/2019	28/10/2019	07/11/2019
051	26/09/2019	28/10/2019	28/10/2019	07/11/2019
052	26/09/2019	28/10/2019	28/10/2019	07/11/2019
053	26/09/2019	28/10/2019	28/10/2019	07/11/2019
054	26/09/2019	28/10/2019	28/10/2019	07/11/2019
055	26/09/2019	28/10/2019	28/10/2019	07/11/2019
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057	26/09/2019	28/10/2019	28/10/2019	07/11/2019
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061	26/09/2019	28/10/2019	28/10/2019	07/11/2019
062	26/09/2019	28/10/2019	28/10/2019	07/11/2019
063	27/09/2019	28/10/2019	28/10/2019	07/11/2019
064	27/09/2019	28/10/2019	28/10/2019	07/11/2019
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066	27/09/2019	28/10/2019	28/10/2019	07/11/2019
067	27/09/2019	28/10/2019	28/10/2019	07/11/2019
068	27/09/2019	28/10/2019	28/10/2019	07/11/2019
069	27/09/2019	29/10/2019	29/10/2019	07/11/2019

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Sample	Date of Filtration	Date of Sample Extraction	Date of DNA Yield Analysis	Date of qPCR Analysis
070	27/09/2019	29/10/2019	29/10/2019	07/11/2019
071	27/09/2019	29/10/2019	29/10/2019	07/11/2019
072	27/09/2019	29/10/2019	29/10/2019	07/11/2019
074	27/09/2019	29/10/2019	29/10/2019	07/11/2019
075	27/09/2019	29/10/2019	29/10/2019	07/11/2019
076	27/09/2019	29/10/2019	29/10/2019	07/11/2019
077	27/09/2019	29/10/2019	29/10/2019	07/11/2019
078	27/09/2019	29/10/2019	29/10/2019	07/11/2019
079	27/09/2019	29/10/2019	29/10/2019	07/11/2019
080	27/09/2019	29/10/2019	29/10/2019	07/11/2019
081	27/09/2019	29/10/2019	29/10/2019	07/11/2019
082	27/09/2019	29/10/2019	29/10/2019	07/11/2019
083	27/09/2019	29/10/2019	29/10/2019	07/11/2019
084	27/09/2019	29/10/2019	29/10/2019	07/11/2019
085	27/09/2019	29/10/2019	29/10/2019	07/11/2019
086	27/09/2019	29/10/2019	29/10/2019	07/11/2019
087	27/09/2019	29/10/2019	29/10/2019	07/11/2019
088	27/09/2019	29/10/2019	29/10/2019	07/11/2019
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090	27/09/2019	30/10/2019	30/10/2019	07/11/2019
091	28/09/2019	30/10/2019	30/10/2019	07/11/2019
092	28/09/2019	30/10/2019	30/10/2019	07/11/2019
093	28/09/2019	30/10/2019	30/10/2019	07/11/2019
095	2/10/2019	30/10/2019	30/10/2019	07/11/2019
096	2/10/2019	30/10/2019	30/10/2019	07/11/2019
097	2/10/2019	30/10/2019	30/10/2019	07/11/2019

N/A: Not Applicable

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3. METHODS

3.1 qPCR Assay Design

A qPCR assay was adapted from Goldberg et al. and validated on a BioRad CFX96 PCR thermocycler to detect *Rana boylii* eDNA. For assay details and validation methodology see Appendix i.

3.2 Sample preservation and transport

Filters were preserved using SRI's self-preserving filter cartridges during transport..

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 Qia-shredder kit (Qiagen). eDNA extracts were aliquoted and those aliquots assigned for downstream qPCR were immediately stored at 4° C, otherwise aliquots were stored at – 20 ° C.

3.4 Fluorometric Quantification of DNA Yield

DNA yields per sample were measured by fluorometric quantification on a Qubit 2.0™ 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 Detection of *R. boylii* eDNA

qPCR was conducted using a CFX96 Real-Time Detection System (BioRad) with optimized thermal cycling conditions (see Appendix i). All qPCRs performed in triplicate per sample. Positive amplification controls (PAC) consisting of reactions containing the target DNA fragment were included in each qPCR run to verify qPCR assay performance. In this case, the PAC consisted of *R. boylii* synthetic gBlock of the target locus (ND2), loaded at 162,000 copies per PCR reaction. No-template controls (NTC) were included in each qPCR run to detect the potential presence of sample or reagent contamination during analysis. In the final run, two replicates of an extraction blank were also run to determine if there was contamination during the DNA extraction phase. Each qPCR reaction was 20 µL, consisting of 11.2 µL of customized 2 x master mix and 8.8 µL of eDNA extract. All qPCR reactions included an internal positive control (IPC), which is used to test for the presence of PCR inhibition. The presence of PCR inhibition stemming from environmental samples must be tested for, which when not identified can lead to false negatives. The IPC is set up such that a delay in the mean quantification cycle (Cq) value, of 1 or more, for a reaction containing eDNA extract, relative to NTC reactions containing pure water is indicative of PCR inhibition.

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4. RESULTS

4.1 DNA Extraction Yield

All but five samples (samples: 003, 004, 006, 007 and 008) yielded quantifiable levels of DNA isolated from filters indicating effective extraction performance (Table 1). The concentration of DNA in the five low-DNA samples fell below the lower limit of detection of the Qubit 2.0™ Spectrophotometer. DNA yields across samples were highly variable (mean yield = 339.83 ng mL^{-1}; standard deviation = 843.58 ng mL^{-1}).

4.2 Results of qPCR Analysis

Of the 261 total qPCR tests for the filter-extracted eDNA samples, only nine amplified to detectable levels during the 50 cycles of the *R. boylii* assay thermocycle (Table 1). However, these tests represent three contiguous samples (samples 91 through 93) and each technical replicate amplifed. Of these nine reactions, five were above or just below the LOD. Per reaction copy numbers ranged between 1.628 – 16.28 copies per reaction.

All NTC reactions were negative for *R. boylii* DNA indicating no contamination during qPCR analysis (Table 1), as were the extraction blank qPCR reactions. Further, no IPC Cq values were delayed relative to the mean IPC Cq value of the NTC (mean NTC IPC = 30.212), indicating the absence of qPCR inhibition (Table 1). The PAC reactions were positive with Cq values consonant with the number of target copies of the frog's gBlock, indicating optimal assay performance.

Table 1: Results of qPCR and DNA yield analysis. Results for qPCR are reported as Cq, and where calculable, copy number of frog target DNA per PCR reaction. Copy number is reported by reference to the standard curve used to validate the assay. A lower Cq value is indicative of higher levels of target DNA. Mean IPC Cq is reported across technical replicates for each sample. DNA yields are reported within the capable range of the Qubit 2.0™ Fluorometer, 10 – 12000 ng mL^{-1}.

Sample	qPCR Cq Value			qPCR Copy #			Mean IPC Cq	DNA Yield (ng mL^{-1})
003	0	0	0	0	0	0	29.128	< 0.5
004	0	0	0	0	0	0	29.488	< 0.5
005	0	0	0	0	0	0	28.951	14.2
006	0	0	0	0	0	0	29.838	< 0.5
007	0	0	0	0	0	0	29.404	< 0.5
008	0	0	0	0	0	0	28.935	< 0.5
013	0	0	0	0	0	0	29.162	20
015	0	0	0	0	0	0	29.175	25
016	0	0	0	0	0	0	29.527	53
017	0	0	0	0	0	0	29.109	24.4

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Sample	qPCR Cq Value			qPCR Copy #			Mean IPC Cq	DNA Yield (ng mL^{-1})
018	0	0	0	0	0	0	29.189	107
020	0	0	0	0	0	0	29.213	478
021	0	0	0	0	0	0	29.111	22.4
022	0	0	0	0	0	0	28.919	45.2
023	0	0	0	0	0	0	29.184	36
024	0	0	0	0	0	0	29.335	22.6
025	0	0	0	0	0	0	29.381	90.4
026	0	0	0	0	0	0	28.796	51.6
027	0	0	0	0	0	0	28.617	196
028	0	0	0	0	0	0	29.238	96
029	0	0	0	0	0	0	28.458	280
030	0	0	0	0	0	0	28.506	382
031	0	0	0	0	0	0	28.631	230
032	0	0	0	0	0	0	29.044	318
033	0	0	0	0	0	0	28.874	33.2
034	0	0	0	0	0	0	28.469	28
035	0	0	0	0	0	0	28.814	91.6
036	0	0	0	0	0	0	29.067	26.2
037	0	0	0	0	0	0	29.463	81
038	0	0	0	0	0	0	29.339	36.6
039	0	0	0	0	0	0	29.173	40.4
040	0	0	0	0	0	0	29.610	27.2
041	0	0	0	0	0	0	39.776	55.8
042	0	0	0	0	0	0	29.659	169
043	0	0	0	0	0	0	29.719	77.6
044	0	0	0	0	0	0	29.659	28.4
045	0	0	0	0	0	0	29.036	27.4
046	0	0	0	0	0	0	29.035	29.6

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Sample	qPCR Cq Value			qPCR Copy #			Mean IPC Cq	DNA Yield (ng mL^{-1})
047	0	0	0	0	0	0	29.357	33.4
048	0	0	0	0	0	0	29.146	123
049	0	0	0	0	0	0	28.491	202
050	0	0	0	0	0	0	29.234	194
051	0	0	0	0	0	0	29.467	240
052	0	0	0	0	0	0	29.269	120
053	0	0	0	0	0	0	29.247	167
054	0	0	0	0	0	0	29.228	100
055	0	0	0	0	0	0	29.385	168
056	0	0	0	0	0	0	29.030	115
057	0	0	0	0	0	0	28.955	226
058	0	0	0	0	0	0	28.538	117
059	0	0	0	0	0	0	29.189	284
060	0	0	0	0	0	0	29.140	185
061	0	0	0	0	0	0	28.587	165
062	0	0	0	0	0	0	28.617	86
063	0	0	0	0	0	0	29.985	64.2
064	0	0	0	0	0	0	29.324	137
065	0	0	0	0	0	0	28.917	154
066	0	0	0	0	0	0	28.881	45.6
067	0	0	0	0	0	0	28.996	91
068	0	0	0	0	0	0	29.650	216
069	0	0	0	0	0	0	28.911	5660
070	0	0	0	0	0	0	29.077	230
071	0	0	0	0	0	0	30.046	292
072	0	0	0	0	0	0	29.712	175
074	0	0	0	0	0	0	29.801	216
075	0	0	0	0	0	0	30.010	324

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Sample	qPCR Cq Value			qPCR Copy #			Mean IPC Cq	DNA Yield (ng mL^{-1})
076	0	0	0	0	0	0	29.622	105
077	0	0	0	0	0	0	29.504	54.6
078	0	0	0	0	0	0	29.505	96.8
079	0	0	0	0	0	0	29.700	212
080	0	0	0	0	0	0	29.806	16.6
081	0	0	0	0	0	0	29.436	142
082	0	0	0	0	0	0	29.286	106
083	0	0	0	0	0	0	29.600	128
084	0	0	0	0	0	0	29.400	55.4
085	0	0	0	0	0	0	29.231	56.4
086	0	0	0	0	0	0	29.611	94.6
087	0	0	0	0	0	0	29.042	64.4
088	0	0	0	0	0	0	29.366	576
089	0	0	0	0	0	0	29.059	586
090	0	0	0	0	0	0	29.277	584
091	45.75	48.72	40.47	*	*	1.628	29.185	3580
092	42.35	41.51	38.24	*	1.628	16.28	29.287	1630
093	38.74	38.09	37.52	16.28	16.28	16.28	29.219	3860
095	0	0	0	0	0	0	29.511	1290
096	0	0	0	0	0	0	28.917	1353
097	0	0	0	0	0	0	29.079	1600
Blank	0	0	0	0	0	0	29.342	< 0.5

0: No Cq observed. *: Uncalculable – Cq value is > intercept of standard curve (Appendix i; essentially, there are inferred to be fewer than one copy of target DNA per reaction as calculated by standard curve) and below LOD.

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5. APPENDIX I: *R. BOYLII* qPCR ASSAY

The Precision Biomonitoring foothills yellow-legged frog (*Rana boylii*) qPCR assay for eDNA is a quantitative real-time PCR (qPCR) assay designed by Bedwell (2018), but validated and optimized for the detection of *R. boylii* environmental DNA (eDNA) obtained from filtered water samples.

Technical Specifications

The *R. boylii* 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. boylii* assay thermocycler conditions and reagent concentrations were derived from Bedwell (2008), and optimized to a BioRad CFX96 Real-Time Detection System (see Table A1).

Table A1: Optimized Thermal Cycling Conditions of the *R. boylii* Assay.

Step	Time (s)	Temperature (°C)
1. Initial Denature	120	95
Followed by 50x cycles of steps 2 and 3		
2. Denature	15	95
3. Anneal	60	60

The performance of the *R. boylii* assay on this system was verified by constructing a standard curve using serial 10-fold dilutions of synthetic target fragment (gBlock) 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 MIC thermal cycler (Biomolecular systems).

Table A2: Validated *R. boylii* Assay Parameters.

Parameter	Validated Result
qPCR Efficiency	96.5%
LOD	1.85 copies/µL
LOQ	1.85 copies/µL
R^2	0.993
Equation	y = -3.408x + 41.30

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Specificity Testing

The specificity of the *R. boylii* assay was assessed by Bedwell (2018). Based on these results the assay was determined to be species specific.

Table A3: *R. boylii* Assay Specificity

Species	No. of samples	Detection Result
Foothills yellow-legged frog (*Rana boylii)*	10	Positive
Cascades frog (*Rana cascadae*)	5	Negative
Bullfrog (*Lithobates catesbeianus*)	5	Negative
Western toad (*Anaxyrus boreas*)	5	Negative
California red-legged frog (*Rana draytonii*)	5	Negative
Sierran treefrog (*Pseudacris sierrae*)	5	Negative

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

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6. APPENDIX II: GLOSSARY

Cq: 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 Cq 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.

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7. APPENDIX III: 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

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