An Evaluation of eDNA Technology to Detect Clandestine Human Activity at Sequoia and Kings Canyon National Parks

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Background

In collaboration with Sequoia and Kings Canyon National Parks, we tested several techniques for detecting trace amounts of human DNA in stream samples that could indicate the presence of illegal marijuana gardens in backcountry areas. The major challenge of this approach is that the detection method needs to be highly sensitive to detect rare DNA particles from stream samples, which means that it is difficult to prevent similar levels of trace human DNA from contaminating the samples through sample collection and processing. While forensic methods to detect crimes focus on the nuclear genome to identify individual people, environmental DNA (eDNA) detection from water samples uses markers from mitochondrial DNA, which is much more abundant, to identify species.

Methods

Three batches of samples were collected by park personnel and analyzed in the eDNA lab at Washington State University. We conducted DNA extractions and qPCR setup in a restricted-access room, where personnel are required to shower and change clothes before entering after having been in a lab with high concentration DNA samples or PCR product. All samples were analyzed in triplicate using the mtND1 Taqman-MGB qPCR assay of Timkin et al. (2005). We duplexed this assay with the internal positive control (IPC) from AppliedBiosystems to test for inhibition. We used 3 µl of DNA extract in each reaction and ran each qPCR reaction in triplicate using 1X QuantiTect Multiplex PCR Mix with 0.2 µM of each primer and 0.2 µM of the probe (Qiagen). To run all reactions, we used a cycle of 15 minutes at 95°C followed by 50 cycles at 94°C for 60 seconds and 60°C for 60 seconds. We used an extracted DNA sample from a mouthswab of CSG for the standard curve in a duplicated serial dilution of 10^{-3} through 10^{-6} (coded as 100-0.1) to encompass the range of values expected from eDNA samples. All extractions were conducted creating at least one extraction negative to test for lab contamination.

Batch 1. The first batch consisted of 29 samples and was received September 16, 2015.

Protocol test 1.1 (September 2015) We extracted half of each of these filters using our standard DNeasy/Qiashredder protocol (Goldberg et al. 2011).

Protocol test 1.2 (October 2015) We replaced the standard extraction kit with a QIAamp UCP (Ultraclean Production) Pathogen Mini Kit and extracted half of the remaining filter portion without using the Qiashredder.

Protocol test 1.2a (November 2016) We tested 8 variations of Protocol 1.2 on extraction negatives: 1) new ethanol, freshly UV sterilized 1.5 pop-top tubes, and new sealed boxes of tips; 2) same as 1 but with general use ethanol (that had been previously opened); 3) same as 1 but with pop-top tubes from general use jar (previously UV-sterilized); 4) same as 1 but with tips in reloaded boxes; 5-8) same as above but with closing the tube for the first night of the extraction rather than leaving open as is done with samples to evaporate ethanol.

Protocol test 1.3 (February 2016) We obtained a positive pressure HEPA-filtered UV-sterilized workstation and extracted the last portion of each filter in this hood using Protocol 1.2. Prior to sample extraction, we extracted four negative control samples with this protocol to determine if additional protocol modifications were necessary. Results were delivered on February 29, 2016.

Batch 2. The second batch consisted of 20 samples and was received April 22, 2016. We analyzed this batch of samples with Protocol 1.3 except that we changed the material that the filter was handled on from paper towels to laboratory kimwipes to increase quality control. However, the kimwipes were not certified DNA-free (note that the paper towels were not either). Results were delivered May 2, 2016.

Batch 3. The third batch consisted of 17 samples and was received October 30, 2016. We analyzed this batch using a QIAamp DNA Investigator Kit, certified to be free of human DNA, with the Qiashredder as recommended in the kit. We used UV-sterilized weigh boats as the handling surface. Results were delivered on December 2, 2016.

Results

Protocol test 1.1 All samples tested positive including all three wells of the extraction negative, indicating failure to distinguish contamination from signal in the samples.

Protocol test 1.2 In this test, one well of the extraction negative tested positive and we used that to create two criteria for testing positive. The only field negative collected (S99B) also tested positive in one well. The count criterion was that a sample needed to test positive in more than one well to be considered positive. The quantitative criterion was that the sample had to have a quantitative value greater than the extraction negative to be considered positive. Note that the average value is unitless and only meaningful as a comparison within this study.

Table 1. Results from extraction protocol test 1.2 – test of Ultraclean extraction kit.

Sample	Average value	Result - quantitative criterion	Result- count criterion
S1	2.80	Positive	Positive
S10	0.69	Positive	Positive
S11	3.90	Positive	Positive
S12	0.23	Positive	Positive
S13	0.15	Below threshold	Positive
S14	1.25	Positive	Positive
S18	11.72	Positive	Positive
S19	0.27	Positive	Positive

S2	0.07	Below threshold	Positive
S21	0.68	Positive	Positive
S22	0.30	Positive	Positive
S23	0.27	Positive	Positive
S24	8.01	Positive	Positive
S26	0.22	Positive	Positive
S27	0.00	Negative	Negative
S28	0.48	Positive	Positive
S29	0.20	Positive	Positive
S31	0.83	Positive	Positive
S4	0.16	Positive	Positive
S5	0.62	Positive	Positive
S6	0.77	Positive	Positive
S7	0.13	Below threshold	Positive
S8	2.07	Positive	Positive
S99	0.10	Below threshold	Positive
S99B	0.12	Below threshold	Below threshold
W1	0.79	Positive	Positive
W6	0.04	Below threshold	Below threshold
W7	0.08	Below threshold	Positive
W8	11.61	Positive	Positive

Protocol test 1.2a. Samples 3,5, and 6 tested positive in 2 of 3 replicates. Samples 1, 2, 6, and 8 tested positive in 1 of 3 replicates. The only sample testing negative used tips in reloaded boxes and an open tube on day one, indicating that none of the attempted measures to prevent contamination were improvements on the previous protocol.

Protocol test 1.3 Of the initial four extraction negatives, only one of the 12 wells tested positive for human DNA. We determined that this was successful enough to proceed. In the sample extraction, one of the triplicate wells for the extraction negative tested positive and again this was used for criteria to consider samples positive. The field negative (S99B) in tested negative in this set.

Table 2. Results from extraction protocol test 1.3 – test of Ultraclean extraction kit in positive pressure UV-sterilized workstation.

Sample	Average Value	Result - quantitative criterion	Result- count criterion
S1	2.30	Positive	Positive
S10	0.15	Below threshold	Positive
S11	0.11	Below threshold	Below threshold
S12	0.37	Below threshold	Positive
S13	0.55	Positive (weakly)	Positive

S14	0.50	Positive (weakly)	Positive
S18	0.32	Below threshold	Positive
S19	0.29	Below threshold	Positive
S2	0.21	Below threshold	Positive
S20	0.24	Below threshold	Positive
S22	0.19	Below threshold	Below threshold
S23	0.15	Below threshold	Positive
S24	0.31	Below threshold	Positive
S26	0.11	Below threshold	Below threshold
S27	0.15	Below threshold	Positive
S28	24.09	Positive	Positive
S29	0.00	Negative	Negative
S31	0.40	Below threshold	Positive
S4	0.28	Below threshold	Positive
S5	0.84	Positive (weakly)	Positive
S6	0.32	Below threshold	Positive
S7	0.43	Below threshold	Positive
S8	1.72	Positive	Positive
S99	1.03	Positive	Positive
S99B	0.00	Negative	Negative
W1	0.00	Negative	Negative
W6	0.10	Below threshold	Positive
W7	0.17	Below threshold	Positive
W8	7.37	Positive	Positive

The lab was blind to which samples in this set were duplicates and blanks, but feedback from the park to this set (from Protocol 1.3) was highly positive. From Don Seale (hydrologist) on March 1, 2016: “These results have really piqued our interest. The positives are all downstream of known or logical sites and the low values are in some vary remote places. Quasi-duplicated give very similar results and the blank came back as "0". We can get into the details later and we need more testing, but for now all signs are encouraging -- Tell you team "Good Job". We will give careful consideration to our remaining samples and we will be seeking additional funding.”

Batch 2. In this batch, we found less overall variation between samples but feedback from the park indicated more variation between duplicates. Some of this concern stemmed from a misunderstanding that we would expect eDNA samples to be consistent in samples taken at the same location, as expected with water quality parameters (temperature, pH, DO, etc.). However, eDNA travels as a particle and it is very common to get widely different readings from replicate samples (see Pilliod et al. 2013 for examples). In this set, we could see that the quantitative criterion effectively identified the blanks, while the count criterion did not. However, the

quantitative criterion may have been too strict, as it only identified three sites as positive, and both blanks tested positive in more than one well.

Table 3. Batch 2 results. Samples with a D indicate second samples taken at the same site and those with a B indicate blanks

Sample	Average Value	Result - quantitative criterion	Result- count criterion
S02	3.70	Positive	Positive
S02D	0.38	Below threshold	Positive
S04	0.23	Below threshold	Positive
S05	0.17	Below threshold	Positive
S06	0.24	Below threshold	Positive
S08	0.26	Below threshold	Positive
S09	0.11	Below threshold	Below threshold
S09B	0.11	Below threshold	Positive
S09D	0.05	Below threshold	Positive
S10	0.40	Below threshold	Positive
S11	0.39	Below threshold	Positive
S15	0.40	Below threshold	Positive
S15D	0.19	Below threshold	Positive
S16	0.50	Positive (weakly)	Positive
S17	0.58	Positive (weakly)	Positive
S17B	0.09	Below threshold	Positive
S17D	0.07	Below threshold	Positive
S29	0.22	Below threshold	Positive
S29D	1.22	Positive	Positive
S50	0.14	Below threshold	Positive

Batch 3. For Batch 3, again one well of the three for the extraction negative tested positive. Of the blank samples, only S17B tested positive above the criterion set by the extraction negative (in two wells) and so the criteria were applied with each as a threshold (Table 4). Only one of the four field negatives tested completely negative.

Table 4. Results from Batch 3 testing for human mtDNA in water samples with a forensic-grade kit.

Sample	Average Value	Result - quantitative criterion (extraction negative)	Result- count criterion (extraction negative)	Result - quantitative criterion (B samples)	Result- count criterion (B samples)
S02	0.37	Positive	Positive	Below threshold	Positive
S02D	1.25	Positive	Positive	Positive	Positive
S06	0.02	Below threshold	Below threshold	Below threshold	Below threshold

S08	0.06	Below threshold	Below threshold	Below threshold	Below threshold
S08D	1.22	Positive	Positive	Positive	Positive
S11	0.22	Positive	Positive	Below threshold	Below threshold
S11D	0.20	Positive	Positive	Below threshold	Below threshold
S12	0.00	Negative	Negative	Negative	Negative
S12B	0.13	Below threshold	Below threshold	Below threshold	Below threshold
S16	0.01	Below threshold	Below threshold	Below threshold	Below threshold
S16B	0.11	Below threshold	Below threshold	Below threshold	Below threshold
S17	0.38	Positive	Positive	Below threshold	Positive
S17B	0.54	Positive	Positive	Below threshold	Below threshold
S17D	0.71	Positive	Positive	Positive	Positive
S29	0.06	Below threshold	Below threshold	Below threshold	Below threshold
S29B	0.00	Negative	Negative	Negative	Negative
S29D	0.22	Positive	Positive	Below threshold	Positive

Conclusions

Detection of trace human DNA in water samples is challenging due to the required high sensitivity of analysis. Trace levels of human DNA are ubiquitous in lab settings as well as lab supplies, and we found that despite extensive protocol development we could not exclude low-level false positive results in our extraction controls. Because of this, we could not identify whether the human DNA signal from the field negatives originated in the field or the lab.

Standard DNA extraction kits are not free of human (or mouse) DNA (Erlwein et al. 2011). Even with human DNA free certified supplies, UV sterilization of all surfaces and tubes, and a positive pressure hood, extraction negatives still showed a slight bit of contamination that did not improve between clean kits (UCP vs. Investigator) or with the use of the clean hood. Additional tests could be conducted to determine if the inclusion of the Qiashredder column (often used in forensic settings) in the analysis of the final sample set introduced trace human DNA into the samples.

If the level of trace human DNA cannot be further improved, the sensitivity of the detection method technique could be reduced (e.g., by reducing the number of qPCR cycles or targeting nuclear DNA). However, this would likely lead to false negatives, and it may be more useful to take a threshold approach as applied here, rather than a binary (positive/negative) result. One challenge for this approach is that eDNA samples are often below the limit of accurate quantification for qPCR and estimates vary between replicates at these low amounts due to sampling stochasticity (i.e. the probability that a copy of trace DNA will be in the 3 µl included in the replicate). Therefore, the relative amounts of eDNA in a sample should be taken more as indicative of amount on an ordinal scale (a little, some, a lot) rather than a precise measure. Given these caveats, this could be a useful approach for a first alert to detecting clandestine activity or human presence in unpopulated areas.

Literature cited

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