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The application of CRISPR-Cas for single species identification from environmental DNA

Using CRISPR-Cas to detect species from eDNA

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Abstract

We report the first application of CRISPR-Cas technology to single species detection from environmental DNA (eDNA). Organisms shed and excrete DNA into their environment such as in skin cells and faeces, referred to as environmental DNA (eDNA). Utilising eDNA allows non-invasive monitoring with increased specificity and sensitivity. Current methods primarily employ PCR-based techniques to detect a given species from eDNA samples, posing a logistical challenge for on-site monitoring and potential adaptation to biosensor devices. We have developed an alternative method; coupling isothermal amplification to a CRISPR-Cas12a detection system. This utilises the collateral cleavage activity of Cas12a, a ribonuclease guided by a highly specific single CRISPR RNA. We used the target species *Salmo salar* as a proof-of-concept test of the specificity of the assay among closely related species and to show the assay is successful at a single temperature of 37°C with signal detection at 535 nM. The specific assay, detects at attomolar sensitivity with rapid detection rates (<2.5 h). This approach simplifies the challenge of building a biosensor device for rapid target species detection in the field and can be easily adapted to detect any species from eDNA samples from a variety of sources enhancing the capabilities of eDNA as a tool for monitoring biodiversity.

Introduction

Environmental DNA (eDNA) offers a new opportunity for biologists and conservationists to monitor biodiversity and track invasive species from the organic material that they leave behind. The urgency of biodiversity monitoring is at an all-time high with the latest WWF Living Planet Index showing an overall decline of 60% in wildlife population sizes since 1970, rising to 83% for freshwater organisms (WWF, 2018). An organism can provide a rich source of eDNA in both soil and water through the cells and waste that they shed and excrete including faeces, mucus, gametes, hair and skin (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). As well as

retrieving samples directly from the environment such as fresh or sea water, eDNA can also be collected from longer term deposits such as sediment and ice cores (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Jerde, Mahon, Chadderton, & Lodge, 2011; Thomsen, Kielgast, Iversen, Møller, et al., 2012; Turner, Uy, & Everhart, 2015; Willerslev et al., 2007). eDNA will improve biodiversity monitoring by providing data on the variety, geographic range (Beans, 2018) and potentially the abundance of species enabling greater ecosystem protection (Guisan et al., 2013). Species monitoring has traditionally relied on the sighting and often the capture of an organism of interest. The monitoring of fish in aquatic environments is challenging, requiring the collection of fish using electricity, the placement of fish traps, nets or rod catches provided by anglers (The Standing Scientific Committee on Salmon, 2016). These techniques can be expensive, labour intensive and potentially harmful to the species of interest (Snyder, 2003). Moreover, organisms present in low abundance are often missed by these methods due to their low capture probabilities (Magnuson, Benson, & Mclain, 1994).

The coupling of eDNA with contemporary molecular methods allows biologists to either generate a biodiversity profile of an ecosystem using metabarcoding (Deiner et al., 2017) or to target and monitor a particular species using specific eDNA assays. Next generation sequencing technologies are driving developments in DNA metabarcoding (Shokralla, Spall, Gibson, & Hajibabaei, 2012) and allow the potential identification of multiple species across all taxa from microbes to higher vertebrates and from either contemporary or ancient eDNA samples (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). This includes the possibility of identifying microbes that previously would have required cultivation on an artificial medium or more critically could not be grown outside of their host and hence not amenable to identification using conventional methods (DeLong, 2005).

Quantitative PCR (qPCR) and digital droplet (dd) PCR are currently the main methods used to detect eDNA of focal species (Baker, Steel, Nieukirk, & Klinck, 2018; Boothroyd, Mandrak, Fox, & Wilson, 2016; Carlsson et al., 2017; Dejean et al., 2011; Gargan et al., 2017; Rusch et al., 2018;

Thomsen, Kielgast, Iversen, Møller, et al., 2012; Uthicke, Lamare, & Doyle, 2018). However, the need to cycle from 95°C to lower temperatures during the PCR process makes the adaptation of PCR-based techniques to a portable biosensor device challenging (Zhang & Xing, 2007). All eDNA assays reported to date need to be processed in a specialised molecular laboratory with trained personnel, which excludes their use in the field. In seeking to develop a device for rapid, on-site monitoring of a target species using eDNA, our aim was to develop a molecular diagnostic technique that does not require PCR-based temperature cycling.

Here, as a proof of concept, CRISPR/Cas technology (Chen et al., 2018; Gootenberg et al., 2017), is deployed to identify *Salmo salar* from eDNA samples collected in Irish rivers, where presence or absence had been previously confirmed using conventional field sampling (Atkinson et al., 2018). The assay uniquely requires incubation at 37°C using fluorescence detection at 535 nM. Not only does employing CRISPR/Cas dramatically simplify the next challenge of building a biosensor device, it enhances the differential detection of closely related species and can be easily adapted to detect any species from eDNA samples from a variety of sources. To the best of the authors knowledge, this is the first application of this technology to eDNA.

Materials and Methods

Target site selection. In order to select a target site for the assay, mitochondrial sequences (SI Table S1) were obtained from GenBank Release 229.0 (NCBI) for *Salmo salar, Salmo trutta* (brown trout) and *Salvelinus alpinus* (Arctic char). These three species are members of the Salmonidae family which are indigenous to Ireland and are closely related to *S. salar*. Multiple sequence alignment of the complete mtDNA genome was performed using ClustalW alignment algorithm (Geneious 11.1.5). The alignment was visually scanned for regions whereby only *S. salar* contains the Cas12a 5'-TTTV-3' PAM site (Zetsche et al., 2015). Areas adjacent to appropriate PAM sites were scanned to ensure greatest number of mismatches between the *S.*

salar sequence and the sequences of *S. trutta* and *S. alpinus*. The final target site selected resides within the NADH dehydrogenase subunit 5 gene. Intraspecific polymorphisms were assessed using single nucleotide polymorphism (SNP) data obtained from dbSNP Build 151 (NCBI) to ensure the assay would not be compromised by a polymorphism within the *S. salar* population. Selected primer and guide RNA sequences were subject to a BLAST 2.8.0 search (NCBI) to ensure they were specific to *S. salar.*

Recombinant DNA Cloning of salmonid sequences. PCR assays that target the mtDNA gene NADH dehydrogenase subunit 5 were designed and optimised for both *S. salar* and *S. trutta.* Our *in silico* analysis showed considerable differences at the target site in *S. alpinus* compared to *S. salar* and *S. trutta* and therefore, *S. alpinus* was excluded from further experimentation. Specific PCR products (SI Table S4) of 205 bp and 225 bp in size respectively, were cloned into a pUC19 vector using FastCloning (Li et al., 2011) with custom primers (SI Table S4). The FastCloning amplified and *Dpn* I digested products were transformed into OneShot® Top 10 chemically competent *Escherichia coli* cells (Invitrogen, USA) as per manufacturer's protocol. Recombinant clones were confirmed by Sanger cycle sequencing (Source Biosciences, IRL) using a custom primer, FastCloneCheck_F (SI Table S4).

Tissue DNA extraction. DNA was extracted from tissue samples from commercially available *S. salar* using DNeasy Blood and Tissue Kit (Qiagen, UK) following manufacturer's instructions. DNA from *S. trutta* (Burren River, Ireland) was extracted as previously described (Atkinson et al., 2018). To confirm species identity, the mitochondrial DNA Cytochrome Oxidase subunit I (COI) region was amplified and confirmed following Sanger sequencing (Source Biosciences, IRL) using previously published generic fish primers (Fish F1 and Fish R1) and conditions (Ward, Zemlak, Innes, Last, & Hebert, 2005).

Environmental DNA preparation. Water samples from four Irish rivers were collected for eDNA analysis. Filtered eDNA extracts including negative field controls from Atkinson et al., (2018) were made available from the Burren, Dalligan and Delour rivers. At the Srahrevagh

river, 550 ml water samples were collected in sterilised 1 L Nalgene bottles and filtered through sterile 47 mm cellulose nitrate filters (0.45 µm) using a vacuum. One negative control consisting of distilled water exposed to air was also filtered for each sampling session. Filters were cut in two and stored at -20 °C. Environmental DNA was extracted from the filter using the Qiagen DNeasy Blood and Tissue kit with some modifications as in Renshaw, Olds, Jerde, McVeigh, & Lodge (2015) and eluted in 50 µl Buffer AE.

All work with eDNA was carried out in a dedicated Low Copy DNA laboratory to minimise potential contamination.

Cas12a detection assays. Alt-R Acidaminococcus sp. BV3L6 (A.s) Cas12a nuclease and crRNA (both synthesised commercially and purchased from IDT) complexes were preassembled by incubating 2.52 µM A.s Cas12a with 3.2 µM of a *S. salar* targeting crRNA in PBS at room temperature for 20 min. Recombinase Polymerase Amplification (RPA) products were generated using TwistAmp Basic (TwistDx). Briefly, 50 µl reactions containing plasmid DNA at concentrations ranging from 8.4 x 10^{-7} – 84 ng/µl, 4 ng/µl tissue DNA or 1 - 20 ng/µl eDNA sample, 0.48 µM forward and reverse primer, 1x rehydration buffer (TwistDx), 14 mM magnesium acetate and RPA reaction pellet (TwistDx) were incubated at 37 °C for 20 min with manual mixing after 4 min. Fluorescence assays were set up by diluting Cas12a-crRNA complexes to a final concentration of 50 nM Cas12a: 62.5 nM crRNA in a solution containing 1x Binding Buffer (20 mM Tris-HCL, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5 % glycerol, 50 µg/ml heparin), 50 nM ssDNA-FQ reporter 5'-/56-FAM/TTATT/3IABkFQ/-3' (synthesised commercially and purchased from IDT) and either 2 µl of RPA product or varying concentrations of plasmid DNA. Reactions (20 µl, 96-well plate format) were incubated in a LightCycler 480 instrument (Roche) for 120 min at 37 °C with fluorescence measurements taken every 30 sec (ƛex = 485 nm, ƛem = 535 nm).

Statistical Analysis. Background-corrected fluorescence values were calculated by subtracting fluorescence values obtained from reactions carried out in the absence of target DNA. A

threshold value for fluorescence was calculated as three standard deviations above background fluorescence, obtained from reactions carried out in the absence of target DNA (n=3). Background noise may be present in the sample due to incomplete quenching of the fluorescent reporter. Only fluorescence values greater than this threshold value are considered as positive detection of target. A two-sample t-test assuming unequal variances was carried out to compare the fluorescence output of the test samples to the fluorescence values of the negative control at 120 minutes. Results are reported to indicate the relative level of the p-value whereby "*" indicates $p<0.05$, "**" indicates $p<0.01$ and "***" indicates $p<0.001$.

Results & Discussion

CRISPR/Cas mediated species detection from eDNA

The adaptation of CRISPR-Cas to a range of applications is expanding rapidly and is driven in the main by the identification and further characterisation of Cas nucleases and equivalent endonuclease enzymes to perform functions which go beyond genome editing (Barrangou & Doudna, 2016). Recent studies have exploited the functionality of Cas12a (Barrangou & Doudna, 2016). Cas12a, an RNA guided ribonuclease, functions similarly to the original genome editing Cas9 enzyme in that it binds a guide RNA that directs it to a specific sequence of DNA in order to catalyse a double stranded break. The guide RNA consists of a single crRNA region which is complementary to the target DNA (Fonfara, Richter, Bratovič, Le Rhun, & Charpentier, 2016) and a scaffold region which interacts with the nuclease (Yamano et al., 2016). The Cas12a nuclease requires a T-rich protospacer adjacent motif (PAM) site (Zetsche et al., 2015) succeeding the crRNA target site in order to bind and cleave the target sequence. The additional functionality of Cas12a is that once it is bound to its target sequence, it gains an indiscriminate single stranded DNase activity that can be exploited for diagnostics (Chen et al., 2018). The collateral cleavage activity can be utilised for target detection through incorporation of a single stranded DNA fluorophore quencher (ssDNA-FQ) molecule with fluorescence only released upon cleavage by the Cas12a-crRNA-target site complex (Chen et al., 2018). This system of

detection has proven to be a valid approach for the detection of clinical pathogens, but has not yet been considered for eDNA, despite offering the sensitivity, specificity and assay simplicity required for the detection of specific species from eDNA samples in a biosensor device. By coupling an isothermal amplification method known as Recombinase Polymerase Amplification (RPA) to a CRISPR-Cas detection system, both SHERLOCK (Gootenberg et al., 2017) and DETECTR (Chen et al., 2018) enable nucleic acid detection with attomolar sensitivity for clinical applications. This system also provides the potential for adaptation to a simplistic device with Gootenberg et al. (2018) developing a lateral flow system for visual readout of viral DNA detection.

Our application of CRISPR-Cas technology (DETECTR) for specific species detection from eDNA required the selection of a target species followed by assay design and the subsequent demonstration of this proof-of-concept. RPA-CRISPR-Cas technology can differentiate between closely related species due to three layers of sequence complementarity required. These include the primers for RPA, the binding sequence of the crRNA itself and the requirement for a T-rich sequence known as PAM that must proceed the crRNA target site for binding to occur. These three different sequence requirements adds an additional level of species specificity which qPCR may lack between sympatric taxa (Wilcox et al., 2013). Our target species was *Salmo salar*, and the overall concept for the assay is described in Figure 1. We chose *S. salar* given its ecological and economic importance. *S. salar* have a high level of protection and, for example, are designated under Annex II of the European Union Water Framework Directive legislation. As a consequence, both the fish and its environment demands a high degree of assessment and reporting in respect of status (Chaput, 2012). Despite substantial controls on exploitation and increased environmental protection, numbers of wild *S. salar* continue to decline (Friedland et al., 2008). *S. salar* is also the primary farmed fish in Europe in terms of biomass and economic value (Dalvin, Glover, Sørvik, Seliussen, & Taggart, 2010). *S. salar* often co-occur in waters with the closely related brown trout, *S. trutta* (Macqueen et al., 2017) so a highly specific assay is required for accurate detection from eDNA samples collected from their natural aquatic

environments. The majority of eDNA studies to date target mitochondrial DNA (mtDNA) due to its high copy number compared to nuclear DNA (Rees, Maddison, Middleditch, Patmore, & Gough, 2014) as well as its ability to resist degradation in the environment (Foran, 2006). Additionally, eDNA studies target short amplicons (90-120 base pairs (bp)) (Rees et al., 2014) increasing target detection when DNA may be highly degraded. For these reasons, our CRISPR-Cas assay design also targets *S. salar* mtDNA. The first experiment was to prove that our species-specific crRNA in combination with the Cas12a enzyme can differentiate between *S. salar* and *S. trutta*.

Cas21a can distinguish between *Salmo salar* and *Salmo trutta* in species specific recombinant DNA sequences.

We tested whether our *S. salar* Cas12a-crRNA could distinguish between *S. salar* and the closely related species *S. trutta* using recombinant versions of their respective DNA sequences. A 20bp *S. salar* target sequence in the mtDNA gene NADH dehydrogenase subunit 5, next to a 5'-TTTC-3' PAM site was selected, conforming to the consensus Cas12a 5'-TTTV-3' PAM site (Zetsche et al., 2015). In order to select the crRNA targeting region, complete mitochondrial sequences from *S. salar, S. trutta* and *S. alpinus* (SI Table S1) were aligned and visually scanned for regions whereby only *S. salar* contained the Cas12a specific T-rich PAM site. These species are closely related so assay design required maximum number of nucleotide differences. This specific PAM site was absent in *S. trutta* and *S. alpinus* and the subsequent target sequence conserved in *S. salar,* varied by two base pairs between *S. salar* and *S. trutta.* (Fig 2a). *S. salar* or *S. trutta* specific DNA containing plasmids were incubated with Cas12a-crRNA targeting the *S. salar* fragment and a single stranded DNA fluorophore-quencher (ssDNA FQ) reporter. Only upon target recognition and subsequent collateral cleavage by Cas12a nuclease should the reporter produce a signal. We show that our *S. salar* specific Cas12a-crRNA only produces a fluorescent signal when exposed to the recombinant *S. salar* DNA and not *S. trutta* DNA (Fig 2b). *S. salar* specific

signal detection above background was detected down to a plasmid DNA concentration of $\geq 10^{-7}$ nM (Fig 2b).

Cas12a coupled with RPA reaches attomolar sensitivity for its target *S. salar* sequence

It is necessary to increase the sensitivity of the assay in order to achieve the detection of target species from eDNA. As previously reported (Chen et al., 2018; Gootenberg et al., 2017), the inclusion of a pre-amplification step can increase the sensitivity by several orders of magnitude. A major challenge with the adaptation of qPCR assays to a portable device is the need for cyclical high temperatures (Zanoli & Spoto, 2013). Isothermal amplification methods, however, enable exponential amplification of DNA molecules at a single temperature. One isothermal method developed is RPA which uses enzymatic activity to amplify small amplicons (<1.5Kb) rapidly and at low temperatures (Piepenburg, Williams, Stemple, & Armes, 2006). This technique shows potential for on-site amplification of eDNA molecules due to the isothermal nature of the reaction creating a system which is easy to operate and requires less energy than PCR methods (Zanoli & Spoto, 2013). Although RPA alone has been shown to be extremely sensitive (Wand, Bonney, Watson, Graham, & Hewson, 2018), it can tolerate mismatches (Daher, Stewart, Boissinot, Boudreau, & Bergeron, 2015) and therefore is not an optimal method to distinguish between closely related species such as *S. salar* and *S. trutta*. For this reason, it is important to couple such technology to a highly specific detection system.

We designed *S. salar* specific primers for RPA and coupled it with the *S. salar* Cas12a-crRNA detection assay. The additional pre-amplification step was tested on the *S. salar* specific recombinant DNA plasmids and showed an increase in sensitivity down to attomolar range (Fig 2c).

Sensitivity and specificity maintained in the context of whole *S. salar* genome complexity

The sensitivity and specificity of this assay needed to be tested in a more complex genomic context i.e. using the entire *S. salar* and *S. trutta* genome as a template rather than a simple recombinant plasmid. Using DNA extracted from the tissue of *S. salar* and *S. trutta* we show that RPA coupled to CRISPR-Cas12a detected down to a concentration of 10^{-5} ng/µl in whole genomic *S. salar* DNA and showed no fluorescent signal with *S. trutta* genomic DNA (Fig 3). This demonstrates that the assay performance in a whole genomic context is both sensitive and specific for *S. salar* with no signal detection when *S. trutta* is used as template. The capability of the assay in its current form is to detect the presence or absence of the target species. Further work is required to explore the quantitative capabilities of the assay which involves optimising the concentrations of all molecular reagents at each step of the process and consideration of the kinetics of the Cas12a nuclease.

RPA coupled to CRISPR-Cas12a can distinguish *S. salar* presence or absence from eDNA samples confirmed by qPCR and electrofishing

The final test of this assay was to assess if it would work on eDNA samples acquired in the field that may contain amplification inhibitors and consist of a substantial proportion of degraded DNA (Taberlet et al., 2012). We used eDNA extracted from Irish freshwater river samples with known presence and absence of *S. salar,* confirmed previously using qPCR (SI Table S3) and electrofishing surveys (SI Table S2). Within a short-time frame (<2.5 hr) *S. salar* was successfully detected from eDNA extracted from water samples with known presence of *S. salar* (Fig 4). Importantly, no fluorescence signal was seen in environmental samples where *S. salar* was not detected using electrofishing above migration barriers but known to contain *S. trutta* eDNA (SI) (Fig 4). While the quantitative aspects of this assay need to be explored further, our data in SI Table S3 indicates that we can detect the presence of *S. salar* DNA at equivalent concentrations to qPCR. The successful detection of *S. salar* in these samples shows the promise for using RPA coupled to CRISPR-Cas12a as an eDNA detection technique. Through careful design of species-specific primers (forward and reverse RPA primers) and a crRNA, the assay

may be applied to any target organism to monitor species distribution (SI RPA-CRISPR-Cas12a Guide). This will make this assay a powerful tool for biodiversity monitoring in natural environments.

In summary, eDNA has already been used to monitor biodiversity in ancient and modern environments (Thomsen & Willerslev, 2015) but the development of simplistic, specific and sensitive methods for on-site detection is needed to extend the application of eDNA. The next challenge is to adapt the assay to a device for on-site monitoring and a recently developed hand-held fluorescent monitor for bacterial detection may prove suitable (Heery et al., 2016). One major recognised threat to biodiversity in aquatic systems is that of invasive species (Molnar, Gamboa, Revenga, & Spalding, 2008). Detection of these species using traditional methods may be slow, enabling their establishment prior to detection. This threatens native biodiversity (Mooney & Cleland, 2001) and can cause native species extinction (Gurevitch & Padilla, 2004). The use of eDNA has already been shown to improve detection of invasive species (Dejean et al., 2012) due to the ability to detect organisms of low abundance. However, the development of RPA-CRISPR-Cas12a detection with an improved capability to differentiate closely related species and the potential for adaptation to a biosensor device, enhances the capabilities and utility of eDNA as an 'early warning' system for detection and management of any valuable, invasive or rare species and extends the reach of CRISPR-Cas technology to environmental monitoring.

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Data Accessibility

All data pertaining to this work is contained in the main text and the Supplementary Information file.

Author contributions: MAW, FR, JOG, PMcG, EJ, APMCD designed the research; MAW performed the experiments and analysed the data; BB, JC provided eDNA samples, qPCR and electrofishing data; EdeE provided data and fieldwork support; MAW, APMCD interpreted data and wrote the paper; all authors contributed to and approved the final draft.

The authors declare no conflict of interest

Figure Legends

Figure 1: Overview of RPA-CRISPR-Cas12a detection. Water sample collected and filtered before DNA extraction. Target DNA is amplified using isothermal Recombinase Polymerase Amplification (RPA) prior to CRISPR-Cas12a mediated fluorescence detection of target using a ssDNA fluorophore-quencher (FQ) Reporter. PAM, Protospacer Adjacent Motif; NTS, Non-Target Strand; TS, Target Strand

Figure 2: Species specific crRNA proceeded by a 5'-TTTV-3' PAM site enables detection of recombinant target DNA. (a) Alignment of crRNA targeting site in *S. salar* and *S. trutta* showing PAM sequence, base pair differences and polymorphic bases. (b) Background subtracted fluorescence time course of Cas12a preassembled with a crRNA targeting *S. salar* in the presence of a recombinant plasmid containing a *S. salar* or *S. trutta* DNA fragment and a ssDNA FQ reporter. Fluorescence measurements were taken every 30 seconds for 2 hours at 37°C. Error bars are mean ± standard deviation, where $n = 3$ (c) Titration of *S. salar* recombinant plasmid detected with Cas12a alone (without pre-amplification of target DNA) and with RPA-CRISPR-Cas12a. Alone Cas12a shows detection above background at 10^{-7} nM while coupling CRISPR-Cas12a to RPA achieved attomolar sensitivity detecting down to 10^{-13} nM. Error bars are mean ± standard deviation, where $n = 3$. Threshold value is 3 x standard deviation of background fluorescence (samples with no DNA template added).

Figure 3: Sensitivity and specificity of assay is maintained in a whole genomic context. The coupled RPA-CRISPR-Cas12a assay was applied to a dilution series of DNA extracted from *S. salar* tissue (4 - 4 x 10^{-6} ng/µl) and DNA extracted from *S. trutta* tissue (5.75 ng/µl). The assay detected *S. salar* down to a concentration of 10^{-5} ng/µl and importantly showed no detection of *S. trutta*. Error bars are mean ± standard deviation, where $n = 3$. Threshold value is 3 x standard deviation of background fluorescence (samples with no DNA template added). Level of significant difference from background, based on a one tailed t-test assuming unequal variances where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$

Figure 4: RPA coupled to CRISPR-Cas12a enables detection of *S. salar* from eDNA extracted from freshwater samples. *S. salar* presence confirmed in rivers Burren, Delour and downstream Srahrevagh. *S. salar* absence confirmed in Dalligan and upstream Srahrevagh. Note: *S. trutta* was present in all samples but not detected. Positive control: *S. salar* DNA from tissue

extract. Negative control: no DNA template added. (+) *S. salar* confirmed present by electrofishing surveys and qPCR. (-) *S. salar* confirmed absent by electrofishing and qPCR. Error bars are mean ± standard deviation, where n = 3. Threshold value is 3 x standard deviation of background fluorescence (samples with no DNA template added). Level of significant difference from background, based on a one tailed t-test assuming unequal variances where * $p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$