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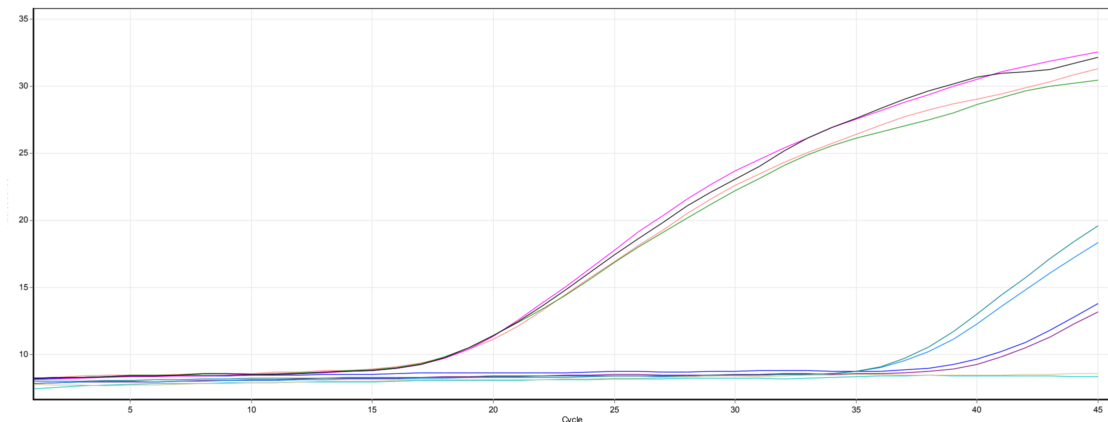
Development and validation of hydrolysis assays for seven species of exotic fish



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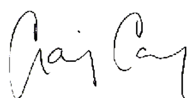

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Executive summary

Determining the distribution and presence of exotic taxa are significant challenges for eradication efforts and for restoration ecology. One method to detect the presence of introduced species that has shown promise is the use of environmental DNA - DNA obtained from an environmental sample such as soil or sediment rather than directly from a tissue sample. As part of Intermediate Output 2 (IO2) of the Lake Ecosystem Restoration New Zealand project, we were asked to develop hydrolysis assays (commonly called Taqman assays) to detect the presence of seven species of introduced fish. Here we report the sequences of the primers and the hydrolysis probe for assays for catfish, *Ameiurus nebulosus*; mosquito fish, *Gambusia affinis*; goldfish, *Carassius auratus*; koi carp, *Cyprinus carpio*; perch *Perca fluviatilis*; rudd, *Scardinius erythrophthalmus* and tench, *Tinca tinca*. We identified assay conditions that resulted in true-negative results by testing for cross reactivity against the six species of exotic fish that were not the target species. Additionally, we compared the effect of adding different amounts of DNA to the assays and found that adding more DNA template can result in false-negative results (DNA present but not detected) possibly due to the concentration of inhibitory substances reaching concentrations high enough to inhibit the assays.

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Introduction

Determining the distribution and presence of exotic taxa, especially elusive taxa such as fish, are significant challenges for restoration ecology. One method that has shown promise for detecting introduced species is the use of environmental DNA - DNA obtained from an environmental sample such as soil or sediment rather than from a tissue sample (Ficetola et al. 2008; Jerde et al. 2011; Dejean et al. 2012; Mahon et al. 2013). To date, environmental DNA (eDNA) has been used to detect species in a diverse range of environments and sample types such as soils (Hofreiter et al. 2003), ice cores (Willerslev et al. 2007) and faeces (Banks et al. 2009). For freshwater vertebrates, DNA from material such as sloughed skin cells or gut epithelium is likely to be present in the environment and has been used to infer the range of introduced Asian carp (*Hypophthalmichthys molitrix*), bighead carp (*H. nobilis*) (Jerde et al. 2011), frogs and salamanders (Goldberg et al. 2011), as well as six species of fish in the Yura River, Japan (Minamoto et al. 2012).

The use of eDNA for detection of a taxon is dependent on the availability of species-specific PCR assays. Quantitative PCR assays are preferred to endpoint PCR-based assays as they have increased sensitivity and specificity, and can quantify the DNA in a sample (VanGuilder et al. 2005). Hydrolysis probe PCR assays, like other PCR-based assays, use “forward” and “reverse” primers - short sequences of DNA referred to as oligonucleotides - that are complementary to a portion of the species’ genome. Hydrolysis probe-based assays use a third oligonucleotide (the “probe”) that binds to part of the DNA sequence (amplicon) produced by the two primers. The use of the probe increases the specificity of the assays. The probe differs from a primer by having a fluorescent reporter chemical (fluorophore) on the 5’ end and a “quencher” molecule on the 3’ end. The quencher prevents fluorescence by

fluorescent resonant energy transfer (FRET). As the *Taq* enzyme synthesises new DNA downstream from the primer, it eventually reaches the probe bound to the amplicon. The exonuclease activity of the *Taq* cleaves the probe, separating the fluorophore from the quencher resulting in increased fluorescence in response to excitation from the thermal cycler's light source. As the reaction proceeds, fluorescence is proportional to the extent of amplification which is in turn proportional to the amount of starting template (Wilcox et al. 2013). Production of amplicons by PCR is generally quantified by comparing fluorescence at a particular threshold value during the exponential phase of the amplification.

Successful primer design is critical for PCR success, and primer efficiency is dependent on a number of factors including: 1) optimal thermal cycler conditions; 2) design of primers without significant secondary structure that can prevent binding to targets; and 3) primer binding specificity. Software packages (e.g., http://sg.idtdna.com/scitools/Applications/RealTimePCR/) are available to aid primer design. However, testing assays for amplification of non-target species is still necessary. Once primers are designed, the annealing temperature (the temperature step in the PCR at which the primers bind to the target sequence) is the variable that is most commonly manipulated to increase specificity. Selection of the optimal annealing temperature is a balance point between a low temperature which may allow primers to bind to regions with base mismatches resulting in non-target amplification, and a high temperature which may result in insufficient primer binding for target amplification to occur.

Here, we report on the development and validation of seven hydrolysis probe-based quantitative polymerase chain reaction (qPCR) assays to detect the following introduced fish:

catfish, *Ameiurus nebulosus*; mosquito fish, *Gambusia affinis*; goldfish, *Carassius auratus*; koi carp, *Cyprinus carpio*; perch *Perca fluviatilis*; rudd, *Scardinius erythrophthalmus* and tench, *Tinca tinca*. We tested the assays developed for these species against DNA extracted from tissue samples to assess the reaction conditions necessary for assay specificity and the rate of false positives (a positive result from DNA of the non-target species). The goal of the study was to identify the lowest annealing temperature at which the assays did not amplify non-target exotic fish species. We also report on the efficiency of the koi carp assay with two concentrations of template DNA extracted from koi carp holding tank water.

Methods

Development of hydrolysis assays

Sequences for the mitochondrial cytochrome b gene (*cytb*) of the seven fish species of fish were downloaded from GenBank. The cytochrome b gene was chosen because: we have found it to generally show the most variation among fish species increasing the probability of assay specificity; the large number of mitochondrial genomes in each cell is likely to increase sensitivity of the assay compared with assays that target nuclear genes; and the circular genome of mitochondria is likely to increase the durability of the genetic material in the environment.

Sequences for *cytb* were then loaded into the IDT PrimerQuest^{TM} software (http://sg.idtdna.com/PrimerQuest/Home/Index) for designing primers and hydrolysis probes. PrimerQuest^{TM} returned several potential primer and probe sequences for the assays. These suggested sequences were then compared with the sequences for the other six exotic fish

species to assess their similarity to non-target DNA. The primer and probe sequences most dissimilar to non-target species were selected for synthesis as IDT mini-assays (Table 1). We also tested the specificity of a koi carp qPCR assay developed by Takahara et al. (2012).

The DNA extracted from the fish tissues was used as a template in each of the seven assays to test for specificity. Where assays amplified the DNA of non-target species, the annealing temperature of each assay was increased until there was no amplification of DNA from any of the other six non-target species.

Table 1 Primer and hydrolysis probes selected for testing as pest fish detection assays.

Assay	Primer and Probe	Sequence
Ameiurus	Primer 1	TATCACGGCTGTCCTCCTATTA
	Primer 2	GGGTCAAAGAATGTGGTGTTTAAG
	Probe	56-FAM/TCTCCCAGT/ZEN/CTTAGCGGCTGGTAT/3IABkF/
Gambusia	Primer 1	GCAGTAAGAGACCACCATCAG
	Primer 2	GGACCTGAAATAGGAGCCAAA
	Probe	56-FAM/AGC ACA CGG /ZEN/TGA ACT ATT CCT GGC /3IABkFQ'
Goldfish	Primer 1	TCCTTGAATGACTCAACTAAGGTTT
	Primer 2	CCACAGTTAGTGTCCTTGATTCC
	Probe	56-FAM/ATGTAGTAA/ZEN/GAAACCACCAACCAGT/3IABkFQ
Koi (Takahara et al. 2012)	CpCyB_573R	GGCGGCAATAACAAATGGTAGT
	CpCyB_496F	GGTGGTTCTCAGTAGACAATGC
	CpCyB-550 Probe	56-FAM/CACTAACAC/ZEN/GATTCTTCGCATTCCACTTCC/3IABkFQ
Koi JCB	Fwd	TCCTATCTGCCGTACCATACA
	Rev	GTAGGAAGTGGAATGCGAAGAA
	Probe (anti sense)	56-FAM/TGTTGCATT/ZEN/GTCTACTGAGAACCCACC/3IABkFQ
Perch	Primer 1	CCCTCTATTCGTATGAGCTGTG
	Primer 2	CCGGTCTGTGAGAAGCATTGTA
	Probe	56-FAM/CTCTCACTT/ZEN/CCTGTTCTTGCCGCT/3IABkFQ
Rudd	Primer 1	GGTAACAGCTGTCCTTCTACTTC
	Primer 2	CTGGGTCGAAGAATGTGGTATT
	Probe	56-FAM/TCACTACCA/ZEN/GTTCTAGCTGCCGGA/3IABkFQ
Tench	Primer 1	TTAGCCTCTTCTGGTGTTGAG
	Primer 2	CCTGCTAGGTGAAGTGAGAAA
	Probe	56-FAM/ACCCACCAC/ZEN/TCGCAGGTAATCTTG/3IABkFQ

DNA was extracted from tissue samples taken from catfish, *Gambusia*, goldfish, koi, perch rudd, and tench using the Extract N Amp kit (Sigma-Aldrich, St Louis, Cat. n.o. XNAT-1KT). Fish specimens were obtained opportunistically from a variety of sources. An aliquot of extracted DNA was subsequently diluted 1:10 with DNAse RNAse free water (Gibco, Grand Island, Cat. No. 10977) and the diluted fish DNA was used as the template for assay optimisation.

Quantitative PCR assays used 4 µL of q-probe 5X master mix (Qarta Bio, Fremont) 2µL of Prime time mini assays (IDT, Coralville) containing the primers and probe listed above, DNA as specified below and HPLC grade water to make a final volume of 20 µL. Reactions were conducted on a Rotor-Gene Q thermal cycler (Qiagen, Venlo) with a temperature profile of 2 minutes at 50°C, 2 minutes at 94°C followed by 50 cycles of 15 seconds at 94°C and 1 minute at temperatures specified in Tables 2 - 9. Reaction progress was quantified by fluorescence at 510 nm in response to exposure to 470 nm wavelength light. Reactions were conducted at each temperature steps until specificity was achieved.

Optimisation of template concentration

We tested the effect of adding differing amounts of DNA template to the koi qPCR assay. Two 30 L aliquots (samples 3A and 3B) of water from a 6880 L tank containing a 1.616 kg koi carp were pumped through glass wool aquarium filter pads (2500 1W, Aqua One, Ingelburn). DNA was extracted from the filters using a Mo-Bio Power Water kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's protocol. DNA was re-suspended in 100 µL of DNA extraction buffer. A blank (no template DNA) extraction on an unused filter was performed at the same time as the template extractions as a control. Quantitative PCRs using the koi carp JCB primers were made up as described above except

that 1 µL, 5 µL or 10 µL of DNA template was added to each assay. The quantity of water added was varied appropriately to obtain a final reaction volume of 20 µL. Thermal cycler conditions were as described above with an annealing temperature of 68°C. We also compared the sensitivity of the koi carp assay on 5 µL of the two eDNA templates at 65°C and 68°C.

Effect of temperature on PCR amplification

The effect of increased annealing temperature was tested on the tank water extracts 3A and 3B using the thermal cycler profile described above. Two annealing temperatures of 65°C or 68°C were trialled.

Results

Optimising annealing temperatures

Each of the eight assays was conducted with DNA extracted from each of the seven pest fish species as the template at each annealing temperature as listed in Tables 2 to 9. The optimal annealing temperature (i.e., the lowest temperature at which target amplification occurred without non-target amplification occurring) for each assay is listed in Table 10.

Table 2. Annealing temperature at 60°C. +++ denotes strong amplification, + weak amplification , - no amplification after 45 cycles.

				Assay				
Species	*Ameiurus*	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	-	-	-	++	-	-	+++	+
2 Goldfish	-	-	-	+/-	-	-	+	-
3 Koi	-	-	-	+++	+++	-	-	-
4 Goldfish	-	-	-	++	++	-	+	-
5 Catfish	+++	+	-	++	++	-	-	-
6 Gambusia	-	+++	-	+	-	-	-	-
7 Perch	+	++	-	-	++	+++	+	-
8 Gambusia	+	+++	-	-	++	-	+	-
9 Catfish	-	++	-	-	++	-	+	-
10 Tench	-	++	-	-	++	-	-	+++

Table 3 Results: annealing @ 65°C +++ denotes strong amplification, +/- denotes amplification only after 40 cycles, - denotes no amplification after 45 cycles. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	-	ND	-	-	-	ND	+++	-
2 Goldfish	-	ND	-	-	-	ND	+	-
3 Koi	-	ND	-	-	+++	ND	+	-
4 Goldfish	-	ND	-	++	++	ND	+	-
5 Catfish	++	ND	-	++	++	ND	-	-
6 Gambusia	++	ND	-	+	+	ND	-	-
7 Perch	++	ND	-	-	+	ND	+	-
8 Gambusia	-	ND	-	++	++	ND	+	-
9 Catfish	ND	ND	-	-	+	ND	+	-
10 Tench	ND	ND	-	-	++	ND	-	+++

Table 4 Annealing temperature at 68°C. +++ denotes strong amplification, +/- weak amplification at CT > 40 cycles, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	ND	ND	ND	+/-	-	ND	ND	ND
2 Goldfish	ND	ND	ND	-	-	ND	ND	ND
3 Koi	ND	ND	ND	+++	+++	ND	ND	ND
4 Goldfish	ND	ND	ND	+/-	+/-	ND	ND	ND
5 Catfish	ND	ND	ND	+/-	+/-	ND	ND	ND
6 Gambusia	ND	ND	ND	-	+/-	ND	ND	ND
7 Perch	ND	ND	ND	-	+/-	ND	ND	ND
8 Gambusia	ND	ND	ND	+/-	+/-	ND	ND	ND
9 Catfish	ND	ND	ND	-	-	ND	ND	ND
10 Tench	ND	ND	ND	-	-	ND	ND	ND

Table 5. Results: annealing at 67°C. Rudd assay specific at 67°C. +++ denotes strong amplification, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	ND	+/-	ND	ND	ND	ND	++	-
2 Goldfish	ND	-	ND	ND	ND	ND	-	-
3 Koi	ND	-	ND	ND	ND	ND	-	-
4 Goldfish	ND	-	ND	ND	ND	ND	-	-
5 Catfish	ND	-	ND	ND	ND	ND	-	-
6 Gambusia	ND	++	ND	ND	ND	ND	-	-
7 Perch	ND	-	ND	ND	ND	ND	-	-
8 Gambusia	ND	++	ND	ND	ND	ND	-	-
9 Catfish	ND	-	ND	ND	ND	ND	-	-
10 Tench	ND	-	ND	ND	ND	ND	-	-

Table 6. Results: annealing at 70°C. Catfish specific at 70°C, Gambusia specific at 70°C. +++ denotes strong amplification, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	-	-	-	-	-	ND	-	ND
2 Goldfish	-	-	-	-	-	ND	-	ND
3 Koi	-	-	-	-	-	ND	-	ND
4 Goldfish	-	-	-	-	-	ND	-	ND
5 Catfish	++	-	-	-	-	ND	-	ND
6 Gambusia	-	+++	-	-	-	ND	-	ND
7 Perch	-	-	-	-	-	ND	-	ND
8 Gambusia	-	+++	-	-	-	ND	-	ND
9 Catfish	++	-	-	-	-	ND	-	ND
10 Tench	-	-	-	-	-	ND	-	ND

Table 7. Goldfish: annealing at 55°C. +++ denotes strong amplification, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Ameiurus	Gambusia	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	ND	ND	-	ND	ND	ND	ND	ND
2 Goldfish	ND	ND	-	ND	ND	ND	ND	ND
3 Koi	ND	ND	-	ND	ND	ND	ND	ND
4 Goldfish	ND	ND	+	ND	ND	ND	ND	ND
5 Catfish	ND	ND	-	ND	ND	ND	ND	ND
6 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
7 Perch	ND	ND	-	ND	ND	ND	ND	ND
8 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
9 Catfish	ND	ND	-	ND	ND	ND	ND	ND
10 Tench	ND	ND	-	ND	ND	ND	ND	ND

Table 8. Goldfish: annealing @ 50°C. +++ denotes strong amplification, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	ND	ND	+/-	ND	ND	ND	ND	ND
2 Goldfish	ND	ND	+	ND	ND	ND	ND	ND
3 Koi	ND	ND	-	ND	ND	ND	ND	ND
4 Goldfish	ND	ND	++	ND	ND	ND	ND	ND
5 Catfish	ND	ND	-	ND	ND	ND	ND	ND
6 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
7 Perch	ND	ND	-	ND	ND	ND	ND	ND
8 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
9 Catfish	ND	ND	-	ND	ND	ND	ND	ND
10 Tench	ND	ND	-	ND	ND	ND	ND	ND

Table 9. Annealing Goldfish @ 53°C. +++ denotes strong amplification, - no amplification. ND denotes assay not trialled at that temperature.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
1 Rudd	ND	ND	-	ND	ND	ND	ND	ND
2 Goldfish	ND	ND	+	ND	ND	ND	ND	ND
3 Koi	ND	ND	-	ND	ND	ND	ND	ND
4 Goldfish	ND	ND	++	ND	ND	ND	ND	ND
5 Catfish	ND	ND	-	ND	ND	ND	ND	ND
6 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
7 Perch	ND	ND	-	ND	ND	ND	ND	ND
8 Gambusia	ND	ND	-	ND	ND	ND	ND	ND
9 Catfish	ND	ND	-	ND	ND	ND	ND	ND
10 Tench	ND	ND	-	ND	ND	ND	ND	ND

Table 10. Optimal annealing temperatures for each assay.

	Assay							
	Catfish	*Gambusia*	Goldfish	Koi Takahara	Koi JCB	Perch	Rudd	Tench
Annealing temperature	70°C	70°C	53°C	68°C	68°C	60°C	67°C	63°C

DNA concentration.

Increasing the volume of DNA extracted from the water sampled from the tank containing a koi carp to the PCR reduced the yield of amplification product as assessed by the Ct scores (Table 11).

Table 11. The effect of increased template volume on PCR amplification with a 68°C annealing temperature. Values are Ct scores.

	Volume of template DNA added			
DNA extract	1 µL	5 µL	10 µL	14 µL
3A	33.9	No amplification	No amplification	No amplification
3B	34.2	43.7	No amplification	No amplification

Annealing temperature

Increasing the annealing temperature from 65°C to 68°C reduced the yield from the PCR (Table 12). The template used was the DNA extracted from the water sampled from the tank containing a koi carp.

Table 12. The effect of increased annealing temperature on PCR amplification. Values are Ct scores.

DNA extract	Annealing temperature 65°C	68°C
3A	28.9	No amplification
3B	30.2	43.7

Discussion

We designed hydrolysis probe assays for the seven exotic fish species that are of interest to the project. Several of the assays amplified non-target species initially, although increasing the annealing temperature of the reactions reduced non-target amplification. Wilcox et al. (2013) showed that the specificity of assays could also be increased by synthesising primers that bound species-specific regions of the genome and that probe specificity was less important in producing non-target amplification. Thus, if the current assays are not sufficiently sensitive at the annealing temperatures needed to ensure specificity, it will be possible to design alternative primers that are less likely to bind and amplify DNA from non-target species.

We found the sensitivity of the koi carp assay decreased with increased amounts of DNA template extracted from the tank samples suggesting that the reactions were inhibited. If the inhibition is due to the co-extraction of inhibitory compounds with the DNA, dilution of the template to retain the target DNA at concentrations sufficiently high enough for amplification to occur but reduce the effect of inhibitory compounds may be successful (King et al. 2009). However, as the concentration of target DNA from environmental samples is likely to be already low, dilution may reduce the target to below detectable amounts resulting in false negative results (target present but not detected). Thus, it would be useful to confirm

inhibition. One method to test for the presence of inhibitory compounds is to add DNA from a second species during the extraction as an internal positive control. Inhibition can be detected by a change in the Ct values for an assay specific to the internal control DNA (King et al. 2009). Strategies to reduce the effect of inhibitors include the addition of BSA (bovine serum albumin) or increasing the concentration of *Taq* polymerase in the reactions (King et al. 2009).

Other factors that can be manipulated to improve assay sensitivity include the addition of Mg^{2+} and/or re-suspending the DNA in water rather than the elution buffer supplied with the DNA extraction kits. Higher concentrations of DNA are known to reduce Mg^{2+} and, as Taq polymerase is a magnesium-dependent enzyme (Markoulatos et al. 2002), increased Mg^{2+} concentration may counteract the effect of increased DNA. Additionally, buffers used to elute the DNA from the spin columns in the DNA extraction kits generally contain EDTA (ethylenediaminetetraacetic acid) to stabilise DNA for storage. EDTA is a chelating agent and can remove Mg^{2+} from solutions, thus re-suspending DNA in water may increase reaction success rates.

The reliability of the assays could be further increased by incorporating a high resolution melt curve analysis as a final step of the assay. High resolution melt curve analysis is performed by adding a fluorescent dye such as Sybr Green (Life Technologies, after the completion of the PCR. The dye fluoresces when light at the excitation wavelength of the dye is shone on the reaction. Fluorescence is measured by the thermalcycler. The PCR product is then heated incrementally and the fluorescence measured at each temperature step. Double stranded DNA separates (or “melts”) in to single stranded DNA at a temperature that is correlated with the

sequence of the PCR product. Once the PCR product separates in to single stranded DNA the dye stops fluorescing in response to excitation and, by measuring the change in fluorescence, different PCR products (for example from a non-target species of fish) can be identified.

The seven species-specific assays for introduced fish will ultimately need to be field-tested on environmental samples (water and sediment). Additionally, understanding the sensitivity of each of the assays under different environmental parameters such as increased turbidity and current flow is necessary to generate confidence intervals around negative results.

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