Conservation Genet Resour (2014) 6:283–284
DOI 10.1007/s12686-013-0113-4

TECHNICAL NOTE

A blocking primer increases specificity in environmental DNA detection of bull trout (*Salvelinus confluentus*)

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Received: 22 December 2013 / Accepted: 25 December 2013 / Published online: 4 January 2014

Abstract Environmental DNA (eDNA) is increasingly applied as a highly sensitive way to detect aquatic animals non-invasively. However, distinguishing closely related taxa can be particularly challenging. Previous studies of ancient DNA and genetic diet analysis have used blocking primers to enrich target template in the presence of abundant, non-target DNA. Here we apply a blocking primer to increase the specificity of a TaqMan assay for eDNA detection of rare and endangered bull trout (*Salvelinus confluentus*) in the presence of the closely related (*Salvelinus namaycush*). We found that addition of a blocking primer substantially increased assay specificity without compromising sensitivity or quantification ability.

Keywords Environmental DNA · Bull trout · Blocking primer · Complex templates

Environmental DNA (eDNA) detection of aquatic animals uses water samples containing feces, tissues, and other cell debris to determine species presence without directly observing the organism (Jerde et al. 2011; Wilcox et al. 2013). This has proven to be a powerful tool for detection of rare taxa. However, a particular challenge for many PCR-based methods is target species specificity and sensitivity in the presence of very closely related species (Darling and Mahon 2011). Because eDNA collection cannot target a particular species, mixtures are the norm and, consequently, fluorescence associated with DNA amplification of a rare target species can be depressed through primer competition when mixed with large quantities of eDNA from a closely related non-target species (Wilcox et al. 2013).

Studies of ancient DNA (Boessenkool et al. 2012) and diet analysis (Vestheim and Jarman 2008; Deagle et al. 2010) have used blocking primers to enrich for target templates by preventing the amplification of abundant non-target templates. Here we developed and apply a blocking primer to increase the specificty of a TaqMan MGB assay (BUT1) for detection of endangered bull trout (*Salvelinus confluentus*). The BUT1 primer has previously been found to perform well, but lacked the specificity to exclude closely related lake trout (*Salvelinus namaycush*) (Wilcox et al. 2013). The blocking primer (Integrated DNA Technologies) hybridizes to lake trout DNA at the same *cyt b* locus as the reverse primer of the BUT1 assay reverse primer, and has a 3' C3-amine modification which prevents elongation (5′-TAGTATAGCTACGAAGCCGAGGAGG-c3-3′).

Caudal fin tissues were collected from bull trout and lake trout and DNA was extracted using the DNeasy Tissue and Blood Kit (Qiagen, Inc.) according to the manufacturer's specifications. The resulting DNA solutions were quantified on a NanoPhotometer (IMPLEN) and diluted to a concentration of 10 ng/μl in TE (10 mM Tris pH 8.0, 0.1 mM EDTA; Integrated DNA Technologies). We then diluted bull trout DNA 1:100 into lake trout DNA to create a mixed sample where lake trout was the predominant template.

We tested 0, 0.45, 0.9, 1.8, 4.5, and 9 μM concentrations of blocking primer in our qPCR reactions (0X, 0.5X, 1X, 2X, 5X, and 10X the final concentration of the assay primers in the reaction). We obtained the BUT1 assay from

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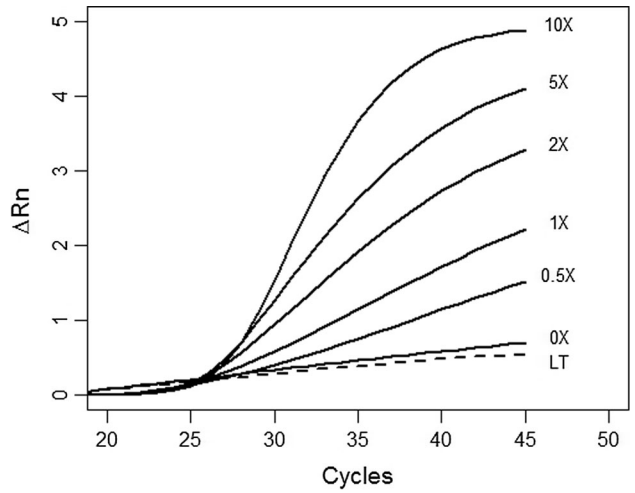

Fig. 1 The amplification curve of a mixed bull trout and lake trout sample becomes steeper with increasing concentration of the blocking primer. The *x-axis* shows the number of PCR cycles, and the *y-axis* shows the change in normalized fluorescence. The *dashed curve* shows amplification of lake trout DNA alone (LT), which is not clearly distinguishable from the mixed sample without addition of the blocking primer (0X). All *curves* are averaged across triplicate reactions

Applied Biosystems (Life Technologies), which contains a primer set and a FAM-labeled minor groove binding, non-fluorescent quencher (MGB-NFQ) probe. All experiments were run in 20 µl volumes with 4 µl of template, 10 µl 2X TaqMan Environmental Master Mix (Applied Biosystems), 1 µl assay (primers each at 18 µM, probe at 5 µM), and 4 µl diH_2O following standard cycling conditions [95 °C/10 m (95 °C/15 s, 60 °C/60 s) × 45 cycles] on a StepOne Plus real-time PCR instrument (Applied Biosystems). All reactions were run in triplicate.

Without the blocking primer, amplification curves (which show level of fluorescence from the hydrolysis probe proportional with the accumulation of target amplicon) from the mixed bull/lake trout DNA solution were nearly linear and not clearly distinguishable from that of lake trout alone. Addition of the blocking primer increased endpoint fluorescence of the amplification curve of the mixed bull/lake trout DNA solution, yielding an unambiguous positive result for bull trout at a 10X concentration of blocking primer (Fig. 1).

To determine if addition of the blocking primer had an effect on PCR efficiency, the bull trout DNA solution was further diluted to a series of 10, 1, 0.1, 0.01, and 0.001 ng/µl in TE. This dilution series was then run in reactions without the blocking primer and with the blocking primer at a 10X concentration. Addition of the blocking primer at a 10X concentration did not decrease PCR efficiency. The standard curves of five dilutions with and without the blocking primer showed no shift in cycle threshold of the amplification curves (Ct) and the slopes of the standard curves indicated a PCR efficiency of 108 % and 107 % respectively (R^2 = 0.999 for both standard curves).

Previous research has suggested that inclusion of base-pair mismatches in the middle of a primer can be used to increase specificity of qPCR assays (Wright et al. 2013), but this may not always be desirable because it increases the risk that a rare polymorphism in the target species will result in a false negative result. Additionally, placement of a mismatch on the 3' ends of primers may not always be possible while meeting the stringent requirements of an efficient qPCR assay. Our results show how blocking primers may be used in concert with TaqMan assays for eDNA when it is difficult or impossible to design assay primers that are specific against all taxa that may be present.

Acknowledgments The authors thank the McCutheon Lab at the University of Montana for use of their spectrophotometer. TW is supported by a NSF Graduate Research Fellowship (Grant No. DGE-1313190).

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