Rescue PCR: Reagent-rich PCR recipe improves amplification of degraded DNA extracts

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Abstract

Minimizing the inadvertent co-extraction of polymerase chain reaction (PCR) inhibitors and/or subduing their influence are two of the most pervasive challenges in the study of ancient DNA (aDNA). Some commonly employed methods to circumvent inhibition include dilution of DNA extracts and/or removal of inhibitors via silica-based treatments. While these methods have been shown to be effective, they may not be useful for all aDNA extracts. Samples with very low copy number, for instance, may not benefit from such methods, as dilutions lower DNA concentration in tandem with the inhibitors, and some DNA loss is expected to follow silica-based treatments. Therefore, the development of additional options to overcome PCR inhibition is at a premium. In this study, we present evidence that a reagent-rich PCR protocol, where all reagents are increased in equal relative proportion can increase amplification success when DNA concentration is reduced relative to inhibitors. The reagent-rich PCR recipe, termed *rescue PCR*, increased amplification success by 51% for the 112 extracts used in the study. Rescue PCR represents a simple and robust addition to the suite of options currently available to work with DNA in the presence of inhibition, especially ancient, degraded, and low copy number DNA extracts.

1. Introduction

Polymerase chain reaction (PCR), the *in vitro* process by which small amounts of template DNA can be copied and exponentially increased in copy number, has transformed molecular biology (Bartlett and Stirling, 2003, Palumbi, 1996). Despite several decades of refinement, some challenges remain. In particular, as PCR is dependent on enzymes, it is subject to inhibition. PCR inhibition is a failure to copy available DNA molecules due to the presence of some extraneous substance or substances, the inhibitor(s). Given that adequate DNA is present in an eluate, inhibition is one of the most frequent causes of PCR failure (Alaeddini, 2012). To this end, analysis of DNA derived from low copy number (LCN), ancient and/or degraded samples can be especially challenging, as such specimens have often spent time buried in the ground and/or in contact with environmentally-based inhibitory substances (Alaeddini, 2012, Kemp, et al., 2014a, Schrader, et al., 2012). While DNA recovered from these types of samples may be especially prone to PCR inhibition, this phenomenon has also been well documented in studies utilizing

clinical, food, and other contemporary sample sources (Al-Soud and Rådström, 2001, Alaeddini, 2012, Rådström, et al., 2004, Rossen, et al., 1992, Schrader, et al., 2012, Wiedbrauk, et al., 1995).

The list of compounds that can act as inhibitors to PCR is as long as it is diverse. Some inhibitors may be introduced during sample processing and/or DNA extraction. Types of such inhibitors include salts (e.g., sodium or potassium chloride), detergents, ethanol, isopropyl, phenol, and even powder from laboratory gloves (Burkardt, 2000, Demeke and Jenkins, 2010, Katcher and Schwartz, 1994, Schrader, et al., 2012, Weyant, et al., 1990, Wilson, 1997). In these cases, proper protocol selection and careful processing may be able to neutralize or minimize the effects of these inhibitors (Rådström, et al., 2008, Schrader, et al., 2012, Weyant, et al., 1990).

Naturally occurring environmental substances such as copper, humic acids, iron, lead, as well as substances that exist in the samples themselves (e.g., calcium and collagen in bone and/or connective tissue, melanin in hair and skin, hematin in blood, among others) can also inhibit PCR [for reviews see Alaeddini (2012), Kemp, et al. (2006), and Schrader, et al. (2012)]. Similarly, if present, exogenous non-target DNA can reduce the efficiency of PCR when present in high enough concentrations (Tebbe and Vahjen, 1993, Wilson, 1997). Inhibitors of these types are more difficult to exclude from processing as their sample-incorporated nature means they might be co-extracted with target DNA, despite even the most impeccable laboratory procedures.

The presence and effect of inhibitors has been well documented, but determining the actual mechanism of inhibition has proven more challenging (Alaeddini, 2012). Potential mechanisms include interference with cell lysing during DNA extraction as well as interference with polymerase, primer binding sites, and/or template DNA during PCR (Bickley, et al., 1996, Eckhart, et al., 2000, Opel, et al., 2010, Wilson, 1997). These mechanisms may be predictable for some specific inhibitors. For example, calcium, hematin, and tannic acid are indicated to act directly on polymerase, melanin appears to bind to DNA template molecules, and collagen exhibits both these behaviors (Opel, et al., 2010). Detailed mechanisms that drive inhibition are beyond the scope of this article, for in-depth information see Alaeddini (2012), Opel, et al. (2010), Schrader, et al. (2012), Wilson (1997). While these classifications may be informative, they are based on controlled experiments where known inhibitors, at known concentrations, are added to DNA standards and the outcomes observed. As such, they may lack direct application in the laboratory where, in any given DNA eluate, there can exist unknown concentrations of an unknown number of different inhibitory substances.

Practical investigations into inhibition have focused on strategies to remove inhibitors and/or subdue their influence. For example, removal of inhibitors can be accomplished via treatments in which DNA is bound to silica, thus allowing inhibitors, theoretically, to be washed away prior to releasing the DNA back into solution (Kemp, et al., 2006, Yang, et al., 1998). In fact, subjection of eluates to repeated rounds of silica extraction has been found to be particularly useful (Grier, et al., 2013, Kemp, et al., 2014a, Moss, et al., 2014). In addition to removal strategies, several methods for the circumvention of inhibition have been demonstrated. The most common is direct dilution of DNA extracts, which likely lowers the level of inhibitors below some “threshold” at which PCR can successfully copy DNA (Alaeddini, 2012, Kemp, et al., 2006). Modifications of

the PCR recipe have also been demonstrated as a means to amplify DNA in the presence of inhibitors. Adding protein-based facilitators such as bovine serum albumin (BSA) to PCR reactions may bind and inactivate some types of inhibitors (Juen and Traugott, 2006, Kreader, 1996). Increasing the concentration of polymerase and/or its magnesium cofactor (e.g., in the form of $MgCl_2$) may also aid in overcoming inhibition (Rådström, et al., 2008, Wilcox, et al., 1993). Additionally, alternating or blending multiple polymerases for use in PCR recipe has also been demonstrated to effectively overcome inhibition, as certain polymerases appear to have decreased susceptibility to specific types of inhibitors (Al-Soud and Rådström, 1998, Belec, et al., 1998, Eilert and Foran, 2009, Hedman, et al., 2010, Monroe, et al., 2013). Real-time PCR (qPCR) has also been used to study inhibition (Hudlow, et al., 2008, Kontanis and Reed, 2006, Opel, et al., 2010, Swango, et al., 2007, Swango, et al., 2006). Pairwise comparisons of qPCR results can be used to characterize, and then optimize for, specific PCR inhibitors (Opel, et al., 2010). However, these methods require repeated analysis for comparison, resulting in additional consumption of DNA template and may not be possible in all cases.

In practice, any DNA sample can be subject to a potential sundry of inhibitors, the outcomes of which can vary between samples, eluates, and PCR reactions (Huggett, et al., 2008). While methods exist to remove or dilute inhibitors, eluates with very low concentrations of DNA may not benefit from such applications. In the case of removal methods, for each treatment some loss of DNA will occur along with inhibitor removal, which is a particularly undesired outcome when processing eluates with low DNA concentrations (Barta, et al., 2014b, Kemp, et al., 2014b). Similarly, when eluates are diluted, the DNA concentration will be reduced in parallel with inhibitors, as such this also may not be an effective strategy for processing samples with low DNA concentrations (Alaeddini, 2012, Ye, et al., 2004). Thus, modifications to the PCR recipe may represent better options for PCR amplification from these types of samples. In any case, it offers and alternative approach that a researcher may elect to try. Here we present evidence that even low-levels of inhibition can produce false-negatives when DNA concentrations are reduced and, in turn, provide a simple and effective method to overcome such inhibition and obtain DNA amplification. In homage to Gilbert and Willerslev (2007) who suggested that new polymerases may help "rescue" ancient DNA, we term our new method "rescue PCR", a strategy based on a reagent-rich PCR recipe.

2. Materials and methods

All pre-PCR laboratory work (DNA extraction and PCR set-up) was conducted in the ancient DNA lab at Washington State University. Strict laboratory protocols are in place in this laboratory to closely monitor and minimize contamination to ensure the authenticity of results (Kemp and Smith, 2010).

2.1. Sample sources

A total of 227 fish vertebrae were acquisitioned from two archaeological collections. Collections were indicated to contain primarily salmonids (*Salmonidae*) along with minnows (*Cyprinidae*) and

suckers (*Catostomidae*). The first collection is comprised of materials from seven excavation locations coinciding with four contemporary dams along the Snake and Columbia Rivers (locations 1-4 depicted in Figure 1). The first site, Strawberry Island (45FR5) in the McNary Reservoir, is an excavated house pit village with materials dating to 2000 – 200 years before present (YBP) (Schalk, et al., 1983). The second site, Windust Caves (45FR46), is located near Ice Harbor Dam. These caves were used as ancient storage and camp shelters with materials dating 9000 – 200 YBP (Jenkins, 2011, Rice, 1965, Thompson, 1985). The caves were inundated by Lake Sacajawea in 1961, after the completion of the lock and dam. Three ancient house pit villages: Harder (45FR40), Hatiuhpuh (45WT134), and Three Springs Bar (45FR39) are located near the Lower Monumental Dam. Materials sampled from these sites date to ~1500 YBP (Harder), 4000 – 500 YBP (Hatiuhpuh), and 3000 – 200 YBP (Three Springs Bar) (Brauner, 1990, Browman and Munsell, 1969, Daugherty, et al., 1967, Funk, 1998, Hicks, 2004). Two sites, Granite Point (45WT41) and Wexpusnime (45GA61) are located near Lower Granite Dam. Granite Point is an ancient camp site with materials dating from 10000 – 200 YBP (Leonhardy, 1969). The Wexpusnime site is comprised of two components, a camp site with materials dated to pre-8000 YBP as well as a house pit village with materials dating to 500 YBP (Nakonechny, 1998). Samples from these seven locations will be collectively referred to as the *Snake River group*. The second sample of vertebrae originated from a collection of materials excavated at an ancient fishing site near the Spokane River (45SP266) (location 5 depicted in Figure 1) with two components, approximated at 2500 and 3250 YBP (Galm, 1994). These samples will be collectively referred to as the *Spokane River group*.

2.2. DNA extractions

Three hundred and thirty-four extractions were conducted from the 227 vertebrae using one of two methods. One hundred and fifty-five extractions were generated using the first method (henceforth referred to as *Extraction Method 1*) from approximately 7 - 48 mg of bone carefully removed from the whole. These portions of bone were submerged in 6% (w/v) sodium hypochlorite (bleach) for 4 min (Barta, et al., 2013) and the bleach poured off. The samples were then twice submerged in DNA-free water, with the water poured off following each submersion. Samples were transferred to 1.5 mL tubes, to which aliquots of 500 µL of ethylenediaminetetraacetic acid (EDTA) were added, and gently rocked at room temperature for >48 hours. Samples were extracted in batches of seven with one accompanying extraction negative control per batch. The negative control consisted of 500 µL of EDTA to which no bone sample was added. DNA was extracted following the WSU method described by Cui et al. (2013) and summarized here. Following EDTA treatment, three milligrams proteinase K was added and the samples were incubated for 3 hours at 65°C. To this digestion, 750 µL of 2% celite in 6 M guanidine HCl and 250 µL of 6 M guanidine HCl was added and the tubes vortexed numerous times over a 2-minute period. The mixtures were then pulled across Promega Wizard® Minicolumns via Luer-Lok syringes attached to a vacuum manifold. Syringes and columns were rinsed with 3mL DNA-free water prior to introducing the extraction mixture. Silica pellets were rinsed on the filter by pulling 3 mL of 80% isopropanol across the columns. Columns were transferred to 1.5mL tubes and centrifuged at 10,000 g for 2 minutes. The columns were then transferred to new 1.5 mL tubes to which 50 µL of 65°C DNA-free water was added. Columns

were incubated at room temperature for 3 minutes and then centrifuged at 10,000 g for 30 seconds. An additional 50 µL of 65°C DNA-free water was added and the incubation and centrifugation repeated, resulting in a final volume of 100 µL.

One hundred and seventy-nine extractions were generated with the second method (henceforth referred to as *Extraction Method 2*) from approximately 53 - 412 mg of bone carefully removed from the whole. These portions of bone were submerged in 6% (w/v) sodium hypochlorite (bleach) for 4 min (Barta, et al., 2013) and the bleach poured off. The samples were then twice submerged in DNA-free water, with the water poured off following submersion. Samples were transferred to 15 mL tubes, to which aliquots of 3 mL of EDTA were added, and gently rocked at room temperature for >48 hours. Samples were extracted in batches of seven with one accompanying extraction negative control per batch. The negative control consisted of 3 mL of EDTA to which no bone sample was added. DNA was extracted following a modified protocol of Kemp et al. (2007) described by Moss et al. (2014) and summarized here. Following EDTA treatment, three milligrams proteinase K was added and samples were incubated for 3 hours at 65°C. Following this, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the EDTA and briefly rocked to mix. Tubes were then centrifuged at 3,000 rpm for 5 min and the aqueous phase transferred to new tubes containing one volume phenol:chloroform:isoamyl alcohol (25:24:1). Tubes were vortexed and centrifuged as just described and the resulting aqueous phase was transferred to new tubes containing one volume chloroform:isoamyl alcohol (24:1). Tubes were vortexed briefly and centrifuged at 3,000 rpm for 3 min. The aqueous phase was transferred to a new tube to which one half volume of room temperature 5 M ammonium acetate one combined volume (equaling the aqueous phase and the ammonium acetate) of room temperature 100% isopropanol, as suggested by Hänni, et al. (1995). DNA was precipitated overnight at room temperature and then pelleted via centrifugation at 3,000 rpm for 30 minutes. The liquid was gently poured off and the inverted tubes air dried for 15 minutes. DNA pellets were washed with 1 mL of 80% ethanol and vortexed. DNA was re-pelleted by an additional centrifugation at 3,000 rpm for 30 minutes before gently decanting the ethanol and once again inverted and air dried for 15 min. Once completely dry, DNA was resuspended in 300 µL of 55°C DNA-free water. To this 750 µL of 2% celite in 6 M guanidine HCl and 250 µL of 6 M guanidine HCl were added and the tubes vortexed numerous times over a 2-minute period. The mixtures were then pulled across Promega Wizard® Minicolumns via Luer-Lok syringes attached to a vacuum manifold. Syringes and columns were rinsed with 3mL DNA-free water prior to introducing the extraction mixture. Silica pellets were rinsed on the filter by pulling 3 mL of 80% isopropanol across the columns. Columns were transferred to 1.5mL tubes and centrifuged at 10,000 g for 2 minutes. The columns were then transferred to new 1.5 mL tubes to which 50 µL of 65°C DNA-free water was added. Columns were incubated at room temperature for 3 minutes and then centrifuged at 10,000 g for 30 seconds. An additional 50 µL of 65°C DNA-free water was added and the incubation and centrifugation repeated, resulting in a final volume of 100 µL.

2.3. Initial evaluation for inhibition

All extracts were initially tested for inhibition following Kemp, et al. (2014a) (see schematic illustration in their Figure 1). In brief, PCRs were set-up with an aDNA control, one comprised of

pooled DNA extracted from ~3500-year-old northern fur seal (*Callorhinus ursinus*) remains (Barta, et al., 2014a, Barta, et al., 2013, Winters, et al., 2011). This pool was created using individual DNA extracts previously verified to yield 181 base pair (bp) amplicons of northern fur seal mitochondrial cytochrome B gene using the following primers: CytB-F 5'-CCAACATTCGAAAAGTTCATCC-3' and CytB-R 5'- GCTGTGGTGGTGTCTGAGGT-3' (with an annealing temperature of 60°C) (Moss, et al., 2006). This control PCR mix is then "spiked" with the DNA (2.5 µL added to 25 µL volume reactions) recovered from the fish vertebrae, that is DNA to be tested for the presence of sufficient inhibition to prevent amplification of northern fur seal mtDNA. One advantage of this approach to monitoring for the presence of PCR inhibitors is that the control is aDNA and exhibits characteristics common in ancient extracts (i.e., signatures of post-mortem chemical degradation, high levels of DNA fragmentation, and low concentrations) (Barta, et al., 2014a, Barta, et al., 2013, Winters, et al., 2011). Another advantage, given that the degree of PCR inhibition is directly related to the size of DNA to be amplified (McCord, et al., 2015), the northern fur seal mtDNA fragment size targeted by these reactions is similar to that targeted in fish (189 bp, see section 2.4 below). None of the ancient fish DNA extracts were shown to contain sufficient PCR inhibitors to cause amplification failure of the aDNA control.

2.4. "Standard" PCR

Except in one test (described below in section 2.5.), all PCRs targeted a 189 bp portion of the 12S mitochondrial gene using "universal" fish primers: OST12S-F 5'-GCTTAAAACCCAAAGGACTTG-3' and OST12S-R 5'- CTACACCTCGACCTGACGTT-3' (Jordan, et al., 2010). Note that Jordan et al. (2010) described the OST12S-R primer in the incorrect orientation. It has been corrected here. These primers can distinguish boney fish species and have been demonstrated to be especially effective in amplifying salmonid mtDNA, the sequences of which can be used to differentiate the Pacific salmonids and a variety of other fish to the to the species level (Grier, et al., 2013, Halffman, et al., 2015, Jordan, et al., 2010, Kemp, et al., 2014a).

Polymerase selection was based on results from Monroe, et al. (2013) indicating Klentaq LA was the least susceptible of nine polymerase or polymerase blends to inhibition associated with DNA obtained from prehistoric salmonid vertebrae recovered from two archaeological sites in the Pacific Northwest (DgRv-003 and DgRv-006). "Standard" 25 µL PCRs contained: 1X Omni Klentaq Reaction Buffer mix (containing a final concentration of $MgCl_2$ at 3.5 mM), 0.32 mM dNTPs, 0.24 µM each of forward and reverse primer, 0.3 U of Omni Klentaq LA polymerase, and 2.5 µL of template DNA. PCRs consisted of an initial three minute denaturation at 94°C, followed by sixty cycles of 94°C (denaturation, 15 s), 55°C (annealing, 15 s), and 68°C (extension, 15 s). This was followed with a final extension at 68°C for 3 minutes. Negative PCR controls and positive PCR controls (utilizing DNA extracted from contemporary Chinook salmon, added in the post-PCR lab prior to initiating PCR) accompanied all sets of standard PCRs and experimentally modified PCRs (described below). Negative controls, consisted of replacing DNA with an equal volume of DNA free-water. A minimum of one negative control for every 15 individual PCR reactions.

Successful amplification following standard PCR or any of the experimentally modified PCRs were confirmed via separation on a 4% agarose gel and approximate size was determined against a 20

bp ladder (Bayou BioLabs). Amplification outcomes were classified as either: 1) *successful* when a single, clear band of the correct size was observed, 2) *failure* when no band was seen, or 3) *non-target* (NT). Non-target classifications were further divided into two additional categories, those of the incorrect size (i.e., based on the relative position on the gel) (referred to, henceforth, as non-target size, or NT-S) and those that produced multiple bands (referred to, henceforth, as non-target multiple or NT-M).

2.5. Rescue PCR: Tests of varying percentage reagent increases (10%, 25%, and 50%)

Initial rescue PCRs conducted on thirty extracts consisted of increasing the buffer mix, dNTPs, primers, and polymerase in equal relative proportion (i.e., +10%, +25%, +50%) with the amount of water reduced to accommodate the increased reagent volumes. For example, in comparison to the "standard" PCR described in section 2.4, +50% rescue PCRs contained: 1.5X Omni Klentaq Reaction Buffer mix (containing a final concentration of $MgCl_2$ at 5.25 mM), 0.48 mM dNTPs, 0.36 µM each of forward and reverse primer, 0.45 U of Omni Klentaq LA polymerase, but maintained 2.5 µL of template DNA. Rescue PCR conditions using the 12S primers were as described in section 2.4.

In addition to the 12S primer set, an additional set of PCRs were used to account for any differential behavior of inhibitors on specific primers, as well as potential template/primer compatibility differences. These PCRs targeted a 193 bp portion of the mitochondrial control region (D-loop) with the following primers: 5'-GCTTTAGTTAAGCTACGCCAG-3' and reverse 5'-CCAGGAAGTTTCAAATCAGCA-3'. These reaction conditions were as described in section 2.4, with an annealing temperature of 58^{o}C for this primer set.

2.6. Effect of 25% increases of individual reagents (dNTP, buffer, polymerase, or primers) and combinations of those reagents

As the experiments described in section 2.5 demonstrate that +25% rescue PCR outperformed standard, +10% and +50% rescue PCRs (see results), to possibly determine the cause of the effect, we tested the efficacy of increasing individual reagents by 25%, as well as combinations of reagents. Sixteen PCR mixes were prepared, one using standard PCR (no increase in reagents), another using rescue PCR (all reagents increased by 25%), and the remaining fourteen using a 25% increase specifically of dNTPs, Omni Klentaq Reaction Buffer mix (including premixed $MgCl_2$), Klentaq LA polymerase, or primers, as well as all possible combinations of these four reagents. Each reaction mix was tested across twelve fish DNA extracts.

2.7. Comparisons of standard and rescue PCR across samples

Of the 334 extracts, neither standard or rescue PCR permitted amplification from 202 of them. Thus, we focused on the results from the remaining 132 extracts, 82 from the Snake River group and 50 from the Spokane River group (Table S1). From these 132 extracts, 423 PCR reactions were conducted using either standard (N = 268) or +25% rescue (N = 155) PCR recipes. Note that from this point forward, +25% rescue PCR will simply be referred to as "*rescue PCR*". Twenty

extracts (14 from the Snake River group and 6 from the Spokane River group) produced non-target DNA (indicated by a band of incorrect size present on the agarose gel, the presence of multiple bands on the agarose gel, or through direct sequencing; Table S1) and were omitted from further analyses. Results were tabulated in one of two ways. First, we established "application of method" for each sample by determining if a given method (rescue or standard PCR) could produce successful PCR amplification in any number of attempts. Second, we determined an "efficiency rate". Using the subset of samples that amplified using both rescue and standard PCR (N = 55), efficiency rate is based on the number of successful amplifications per PCR attempt.

2.8. Determining mechanism of rescue

Although all extracts passed the initial test for inhibition, as described above under section 2.3., it is possible that some level of inhibitors still exist, but at a threshold below that which would render PCR amplification of the aDNA positive control impossible. We hypothesized that in cases where only very small amounts of DNA are present in the fish DNA extracts, even undetectable low levels of inhibition might be sufficient to hinder amplification of the fish mtDNA. To investigate if rescue PCR is capable of overcoming this potential problem, we designed two complementary tests.

The first test was designed to simulate an incremental decrease of DNA concentration. Two pools of DNA were created from fish extracts (N = 24 for each pool) that individually amplified for the 12S fragment using standard PCR. These pools were then diluted 1:1, 1:5, 1:10, 1:25, 1:50, 1:75, 1:100, 1:150, and 1:200 with DNA-free water. Two replicates for each, as well as an undiluted pool (i.e., 1:0), were then subjected to standard and rescue PCR for each dilution value [undiluted (1:0) – 1:200] and checked for amplification (See Figure 2 for schematic illustration of the method). It is important to note that in this test inhibitors were diluted proportionally to that of the target DNA and, therefore, results would vary according to the effect of DNA concentrations, and not the relationship between inhibitors and the DNA, which here is a *constant*.

The second test was designed to simulate a decreasing concentration of target DNA relative to that of the inhibitors. We used a modification of the inhibition test described in Kemp, et al. (2014a). In our modified test, the aDNA control (northern fur seal DNA) was diluted 1:1, 1:5, 1:10, 1:25, 1:50, 1:75, 1:100, 1:150, and 1:200. These dilutions, along with an undiluted control (1:0), were then tested in replicates of four, each spiked with an individual fish extract. The fish DNA spike is added to all dilution treatments equally, introducing some level of additional inhibitors without increasing the amount of northern fur seal target DNA. Thus, each dilution treatment has a decreasing amount of target DNA relative to the amount of total inhibitors. Using primers specific to the aDNA control (described in section 2.3), amplification targeted the northern fur seal DNA. As such, the level of total inhibitors (from both the northern fur seal and fish DNA extracts) relative to the target DNA (northern fur seal) was *increased* across the dilutions. (See Figure 3 for schematic illustration of the method.) Each combination was tested in replicate for both standard and rescue PCR.

2.10. Statistical comparisons

Chi-square tests of independence were used to test for significant differences at the 0.05 level of probability between treatments. Significance was determined for differences in reagent concentration at the standard, rescue +10%, rescue +25%, and rescue +50% levels as well as across all attempts and when grouped by extraction method or geographical group, as well as for differences in efficiency rate between rescue and standard PCR.

2.5. Sequencing confirmation

Amplicons from 55 extracts, ones subjected to both standard and rescue PCR, were submitted for sequencing in both the forward and reverse directions using the same primers utilized for amplification. Product clean-up and sequencing were performed by Molecular Cloning Laboratories (South San Francisco, CA). Sequences were aligned and the priming regions were trimmed using Sequencher v 4.8 (Gene Codes; Ann Arbor, MI). Sequence quality scores were determined using data provided by Molecular Cloning Laboratories. Each base was assigned a score between zero and 60 as part of the sequencing process, with ranges of 20 for low, medium and high confidence. All bases scoring in the medium or high range (21-60) were combined to calculate the percent quality for the sequence as a whole. A sequence with a quality score of 75% indicates that 75% of the bases in the sequence were of medium to high confidence. All sequencing results were compared to the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST) to determine species and gene region.

3. Results and Discussion

3.1. Comparisons of rescue PCR at +10%, +25%, and +50% increases

In the test of standard PCR against rescue PCR using various levels of increased reagents (+10%, +25%, and +50%) results were tabulated for each primer set, as well as combined where a success was counted if an extract amplified for either primer set at a given increase (Table 1). For the D-Loop primers, of the 30 extracts tested, three amplified (extracts 9, 12, 26) using standard PCR, seven amplified (extracts 8, 9, 13, 19, 25-27) using the 10% increase, 16 amplified (extracts 1, 2, 3, 6, 8-10, 13, 20, 22-27, 30) using the 25% increase, and 13 amplified (extracts 1-3, 6, 8, 9, 13, 14, 19) using the 50% increase. Extracts 9, 13, 25, and 26 amplified across all rescue PCR treatments, but it is notable that extracts 13 and 25 did not amplify under standard PCR conditions. For the 12S primer set, eight amplified (extracts 12, 15, 22-27) using standard PCR, 12 amplified (extracts 9, 12, 15, 17, 20, 22-27, 30) using the 10% increase, 17 amplified (extracts 1, 3, 6, 9-12, 14, 16, 17, 21-23, 25-27, 30) using the 25% increase, and 14 amplified (extracts 2, 4-6, 9, 10, 12, 15, 19, 20, 22, 25, 26, 29) using the 50% increase in reagents. Here, extracts 9, 12, 22, 25, and 26 amplified across all rescue PCR treatments, of these five, extract nine did not amplify under standard PCR conditions. In the combined dataset, where a success was counted if a sample amplified for either primer set at a given increase, standard PCR amplified nine total extracts, the 10% increase amplified 15 extracts, a 25% increase amplified 22 extracts, and the 50% increase amplified 19 extracts. Both the +25% and +50% rescue PCR treatments resulted in

significantly more amplification over standard PCR ($P < 0.000$ and $P = 0.010$, respectively). There was no statistically significant difference indicated for differences between the other levels (standard vs. +10%, +10% vs. +25%, +10% vs. +50%, or +25% vs. +50%).

Based on the number of successful amplifications, all rescue PCR treatments (+10%, +25%, and +50%) outperformed standard PCR and the +25% rescue outperformed +10% and +50%. Despite a lower overall success rate, the 50% increase permitted amplification of four of the samples that could not be amplified using the lower increased reagent concentrations. However, this higher reagent concentration also resulted in three non-target (NT) amplifications (indicated by multiple bands) using the D-Loop primers (extracts 22, 27, 30) and two NT amplifications using the 12S primers (extracts 13, 27), for a total of four independent extracts producing non-target amplification (extracts 13, 22, 27, 30). In all four cases, lower reagent concentrations were able to amplify target DNA and the 50% increase resulted in multiple bands. Therefore, we conclude that a 50% reagent increase may be a good strategy to attempt on a set of samples if additional amplifications are desired after attempting rescue PCR at +25%.

3.2. Effect of 25% increases of individual reagents (dNTP, buffer, polymerase, or primers) and combinations of those reagents

In this test, two extracts (extract 1 and 2) failed to produce target DNA amplicons and none of the extracts benefitted from increasing single reagents (Table 2). For increases in combinations of two reagents, six extracts benefitted from an increase in dNTPs & $MgCl_2$ (extracts 3-8), four from $MgCl_2$ & polymerase (extracts 4-7), eight from $MgCl_2$ & primers (extracts 3, 5-11), one from dNTPs & polymerase (extract 5), five from dNTPs & primers (extracts 4-6, 9, 11), and six from polymerase & primers (extracts 4-7, 11, 12). Notably not a single extract benefited from all treatments. For combinations of three reagent increases, two extracts benefitted from increasing dNTPs & $MgCl_2$ & polymerase (extract 7, 8), eight from dNTPs & $MgCl_2$ & primers (extracts 4-7, 9-12), six from $MgCl_2$ & polymerase & primers (extracts 3, 5-7, 9, 11), and four from dNTPs & polymerase & primers (extract 4, 5, 8, 11). Notably here too, not a single extract benefited from the all treatments. Nine of the ten extracts that indicated amplification of target DNA for any treatment (i.e., not including extracts 1 and 2) benefitted from rescue PCR, or increasing all four reagents (extracts 3-5, 7-12).

Commonly employed methods to increase PCR success rates on difficult samples include increasing either the concentration of $MgCl_2$ or amount of polymerase (Alaeddini, 2012, Opel, et al., 2010, Schrader, et al., 2012). These strategies are designed to overcome inhibitory substances that act directly on the polymerase enzyme or that sequester the magnesium cofactor. However, it is possible that inhibitors could act on any component of PCR to prevent amplification (Alaeddini, 2012). This principal, and the great complexity of inhibitor action, is evidenced in our results. No single reagent increase resulted in amplification for any of the 12 extracts tested and results were mixed for combinations of two and three reagent increases. For example, amplification from Extract 3 was made possible by: 1) increased percent combination of dNTPs & $MgCl_2$, 2) increased percent combination of $MgCl_2$ & polymerase & primers, as well as 3) when all reagents were increased. Intriguingly, this same extract did not amplify with the

411 increased percent combination of dNTPs & $MgCl_2$ & polymerase. These incongruent
412 amplification results were seen throughout these experiments, highlighting the stochastic and
413 complex nature of PCR, especially when conducted with the presence of inhibitors.
414 Consequently, increasing only a portion of the PCR reagents may not provide a consistently
415 successful strategy. In the case of rescue PCR, which is an increase in all reagent components, it
416 is possible that inhibitory "combinations" are largely accounted for, resulting in a reduction of
417 stochastic effect, and an increase in overall amplification success. Other possibilities remain
418 which deserve further investigation.
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421 *3.3. Sequencing results*
422 For the subset of 55 extracts used for sequencing confirmation, 38 amplified with standard PCR
423 (11 that did not amplify with rescue PCR) and 44 with rescue PCR (13 that did not amplify with
424 standard PCR) (Table 3). All the standard PCR (N=38) amplifications were confirmed as target
425 DNA and 91% (40 of 44) of those generated from rescue PCR were confirmed as target DNA.
426 Four sequences generated using rescue PCR (extracts 52 – 55) matched to human DNA. Omitting
427 these four non-target amplifications, the average sequence quality score for each method was
428 approximately equal, with 80.1% and 82.8% confidence scores for standard and rescue PCR
429 generated sequences, respectively. In cases where templates generated under both standard
430 and rescue PCR were sequenced (extracts 1 – 27) the sequences generated using standard PCR
431 were identical to those generated using rescue PCR in every case.
432
433
434 *3.4. Amplification of non-target DNA*
435 Rescue PCR appears to be more prone to amplification of non-target DNA than is standard PCR.
436 Cases of non-target DNA, indicated by incorrect size or by the presence of multiple bands, were
437 noted throughout the tests in this study and have been observed as we continued to apply the
438 rescue method in the laboratory. Amplification of non-target DNA of a single-band of expected
439 size was also evidenced in the subset of samples that were selected for sequencing. In this
440 experiment, four amplifications were bands that appeared to be of the correct size but
441 sequencing results revealed the DNA as human in origin. As determined from the sequences, the
442 actual length of these amplicons ranged from 184 – 195 bp compared to the 189 bp expected
443 from target DNA. This points to a weakness in trying to estimate amplicons of 189 base pairs
444 from 4% agarose gels. Notably, all four non-target amplifications originated using rescue PCR on
445 extracts that failed to amplify with standard PCR.
446
447 This is not unexpected. Previous studies utilizing increased polymerase or $MgCl_2$ have indicated
448 that this modification can lead to increased non-specific binding of primers (Edwards, et al.,
449 2004). DNA extracted from degraded samples tends to be recovered in lower copy number and
450 is degraded with regards to strand length (Gilbert, 2006, Pääbo, 1989, Pääbo, et al., 1988). These
451 characteristics increase the susceptibility of degraded samples to contamination by endogenous
452 DNA sources (Kemp and Smith, 2010, Malmström, et al., 2005, Yang and Watt, 2005).
453 Endogenous DNA can be introduced to a DNA extract in many ways. Vectors include the
454 originating sample (e.g. through handling or contact with other sample sources) (Champlot, et

al., 2010, Kemp and Smith, 2005), laboratory personal (Champlot, et al., 2010), and from laboratory reagents and consumables (Evans, et al., 2003, Gefrides, et al., 2010, Leonard, et al., 2007, Shanks, et al., 2005).

In our study, we employed extensive negative controls in both the extraction and PCR processes. DNA introduced globally during extraction processing or from reagents or consumables may be detectable through some amount of positive amplification of these controls. However, no such amplification was indicated for any negative controls in the study. Further, amplification of non-target DNA was confined to a small number of extracts (Table S1) and repeated for those extracts in reactions prepared from independent PCR mixes. Although negative controls cannot detect every contamination event, the combined results and repeatability indicate that the endogenous DNA was likely co-extracted with the original sample and not introduced during subsequent processing or from reagents. Prior to extraction, all samples were decontaminated with bleach (see section 2.2) following Barta, et al. (2013). However, no protocol can guarantee complete decontamination. The risk of non-target amplification may be greater for our 12S primer set, as this region of mtDNA that is well conserved across species (Melton and Holland, 2007, Yang, et al., 2014). Conserved areas of the 12S portion of mtDNA have been noted for animal species including amphibians, fish, and mammals (Yang, et al., 2014). Less conserved genetic targets may lower the rate of non-target amplification. Cases of indicated non-target DNA amplification using +25% rescue PCR were only observed in extracts where standard PCR failed, indicating that rescue PCR is likely amplifying non-target DNA in the *absence of* target DNA, not *instead of* target DNA.

3.5. Application of method and efficiency rates between standard and rescue PCR

It was possible to amplify 49% (55 of 112) of the extracts in this study using either standard or rescue PCR (Table 4). The remaining 51% were amplifiable only with rescue PCR. The proportion of samples that were amplifiable using either standard or rescue PCR differed significantly ($P < 0.000$). To determine if extraction method or sample collection had an influence on the proportion of samples that could be amplified under either PCR recipe, we grouped extracts categorically and determined success rates for each method within the groupings. Standard PCR was used successfully to amplify 50% (24 of 48) of the samples generated using extraction method 1 and 48% (31 of 64) of the extracts generated using extraction method 2. Differences in the proportion of amplifications possible with standard PCR or rescue PCR was significant for both extraction methods ($P < 0.000$ in both cases). For extracts organized by geographical group, standard PCR was able to amplify 60% (41 or 68) of the Snake River extracts and 32% (14 or 44) of the Spokane River extracts. Statistical significance was indicated for the differences in amplification success rates between standard and rescue PCR in both the Snake and Spokane groups ($P < 0.000$ in both cases).

All PCR preparations are subject to some level of stochasticity. Each aliquot of a DNA extract will have varying amounts of inhibitors and DNA and, thus, mixed results may be seen across multiple PCR reactions from a single extract. In fact, this is a commonly cited observation in the laboratory; amplification of aDNA can be sporadic. Mixed outcomes are likely to occur frequently

in eluates where the concentration of target DNA and inhibitors exist near the threshold where amplification or failure are equally likely. By random chance one draw from the eluate may contain inhibitors above the threshold of amplification while the next has inhibitors below the threshold, allowing amplification to complete. This effect was commonly observed in our study, with multiple PCR reactions necessary to obtain amplification. We quantified this effect using the calculation of an efficiency rate, or the number of successful amplifications per PCR attempted. Standard PCR had an efficiency rate of 59% (58 of 98 attempts resulted in amplification) while rescue PCR was 88% (58 of 66 attempts resulted in amplification) (Table 4). Statistical significance was indicated ($P < 0.000$) for the differences in efficiency between the PCR types.

When the subset of amplicons was submitted for sequencing (section 3.3.) four of the 44 amplifications generated with rescue PCR appeared to be the correct size on the agarose gel, but were confirmed as human DNA (Table 3). This potential miscall rate (9%) was applied to the amplification counts for rescue PCR and reevaluated for statistical significance (Table 4). In all cases, significant differences between standard and rescue PCR were maintained.

In the present study, rescue PCR significantly outperformed standard PCR. This enhancement does not appear to be a function of the extraction methods employed here, indicating that success of rescue PCR is independent of how the DNA was extracted and purified. The benefit of rescue PCR was particularly evident in the case of the Spokane River collection. If processed using the standard PCR protocol these samples would have only produced 14 amplifications from the 44 DNA extracts in this group. However, rescue PCR permitted amplification from an additional 30 extracts, resulting in amplification from 100% of the samples.

3.6. Mechanism of rescue

There was no difference in the amplification success of standard and rescue PCR for any given dilution treatment of the pooled fish DNA (Figure 4). For fish pool 1 (FP1), at least faint amplification (even if inadequate for sequencing) was observed for the undiluted and 1:1 dilution using both standard and rescue PCR. As visually assessed, neither PCR method produced amplification at any of the further dilutions. For fish pool 2 (FP2), amplification was achieved from the undiluted samples through the 1:25 dilutions for both standard and rescue PCR treatments. No amplification was possible for either standard or rescue PCR at any further dilution.

For the test inducing increases of inhibitors relative to the amounts of northern fur seal DNA control, more striking differences are observed between the normal and rescue PCR results (Figure 5). The positive controls (aDNA control diluted but not spiked with fish DNA) indicate that both rescue and standard PCR can produce amplification up to the 1:150 dilutions. When additional inhibitors were introduced using the fish DNA spike, standard PCR could produce amplification up to the 1:10 dilutions. Rescue PCR was able to produce amplification through the 1:150 dilutions. Amplification strength does appear to drop off after the 1:25 dilutions but very faint amplification was indicated from the 1:50 – 1:150 dilutions.

Attempts to determine the mechanism of rescue PCR was based on two complementary tests focused on the concentration of target DNA and inhibitors. When the concentration of DNA was reduced in equal proportion to the inhibitors present, the amplified products (or lack thereof), appear consistent between standard and rescue PCR across all dilutions (1:0 – 1:200). Under these conditions, rescue PCR had no positive affect on amplification success. However, when the amount of target DNA was reduced at a rate different from that of inhibitors, rescue PCR was able to produce amplification when standard PCR could not. As the aDNA control sample is diluted, the target DNA and inhibitors from that sample are diluted at equal rates. In our tests, both standard and rescue PCR were able to amplify the target DNA up to a 1:10 level dilution. However, only rescue PCR was able to produce any amplification in the remaining dilutions. Unspiked controls were run for all dilutions indicating that sufficient DNA existed for amplification by both standard and rescue PCR up to the 1:150 dilutions and so amplification failure in these reactions is a likely a function of inhibitory action(s). Based on our results we conclude that rescue PCR circumvents the problem associated with overcoming the combination of reduced DNA concentrations relative to the amount of inhibitors present in a sample.

4. Conclusions

Despite wide-ranging efforts to remove inhibitors, it is likely that some amount of inhibitors is present in all DNA eluates. Samples with very low DNA concentrations are unlikely to benefit from some of the previously described methods to further reduce inhibitors. Specifically, direct dilution of sample reduces DNA along with inhibitors while each additional silica treatments will result in some loss of DNA. Samples of such low copy numbers may not be able to withstand such treatments. Notwithstanding the volume used to initiate Rescue PCR, our protocol provides an alternative means by which amplification is attempted without risking loss or dilution of target DNA in the extract.

Our results demonstrate a clear ability of reagent-rich PCR mixes to rescue DNA (i.e., rescue PCR). However, the application of this method should not be applied blindly or with the notion that it is the solution to all problems associated with the co-extraction of PCR inhibitors. Rescue PCR represents a simple and robust addition to the suite of options currently available to work with DNA in the presence of inhibition, especially ancient, degraded, and low copy DNA samples. This method appears to have particular value when applied to samples where the relationship between DNA and inhibitors concentration may be at an important crossroad, as it diminishing the effects of inhibitors without compromising the amount of DNA in an eluate.

Acknowledgements

This work was funded by a grant from Washington Sea Grant, University of Washington, pursuant to National Oceanic and Atmospheric Administration Award No. NA14OAR4170078-762754. The views expressed herein are those of the author(s) and do not necessarily reflect the views of NOAA or any of its sub-agencies. The authors thank Archaeological and Historical

Services, Stan Gough, and Sara Walker at Eastern Washington University as well as Mary Collins, Diane C. Curewitz, and Shannon Tushingham at Washington State University for providing the samples and archaeological information used in this manuscript. Thanks to Cara Monroe for assistance in the laboratory and to Nicole Fournier for editorial comments on a draft of the manuscript.

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Table 1. Results of PCR amplification tests for thirty DNA extracts against standard PCR (0% increase in reagents) as well as 10%, 25%, and 50% increases in reagents using two primer sets. Combined results indicate if a sample amplified using either primer set for a given PCR protocol. The symbol "+" indicates amplification of the target DNA occurred (inferred by band size), "-" indicates no amplification occurred, and "NT-M" indicates non-target DNA was amplified (multiple bands present on gel). No bands of incorrect size were seen in this experiment.

	Primer set 1 (D-Loop)				Primer set 2 (12S)				Combined			
	0	10%	25%	50%	0	10%	25%	50%	0	10%	25%	50%
Extract 1	-	-	+	+	-	-	+	-	-	-	+	+
Extract 2	-	-	-	+	-	-	-	+	-	-	-	+
Extract 3	-	-	+	+	-	-	+	-	-	-	+	+
Extract 4	-	-	+	-	-	-	-	+	-	-	+	+
Extract 5	-	-	-	-	-	-	-	+	-	-	-	+
Extract 6	-	-	+	+	-	-	+	+	-	-	+	+
Extract 7	-	-	-	-	-	-	-	-	-	-	-	-
Extract 8	-	+	+	+	-	-	-	-	-	+	+	+
Extract 9	+	+	+	+	-	+	+	+	+	+	+	+
Extract 10	-	-	+	-	-	-	+	+	-	-	+	+
Extract 11	-	-	-	-	-	-	+	-	-	-	+	-
Extract 12	+	-	-	-	+	+	+	+	+	+	+	+
Extract 13	-	+	+	+	-	-	-	NT-M	-	+	+	NT-M
Extract 14	-	-	-	+	-	-	+	-	-	-	+	+
Extract 15	-	-	-	-	+	+	-	+	+	+	-	+
Extract 16	-	-	-	-	-	-	+	-	-	-	+	-
Extract 17	-	-	-	-	-	+	+	-	-	+	+	-
Extract 18	-	-	-	-	-	-	-	-	-	-	-	-
Extract 19	-	+	-	+	-	-	-	+	-	+	-	+
Extract 20	-	-	+	-	-	+	-	+	-	+	+	+
Extract 21	-	-	-	-	-	-	+	-	-	-	+	-
Extract 22	-	-	+	NT-M	+	+	+	+	+	+	+	NT-M
Extract 23	-	-	+	-	+	+	+	-	+	+	+	-
Extract 24	-	-	+	+	+	+	-	-	+	+	+	+
Extract 25	-	+	+	+	+	+	+	+	+	+	+	+
Extract 26	+	+	+	+	+	+	+	+	+	+	+	+
Extract 27	-	+	+	NT-M	+	+	+	NT-M	+	+	+	NT-M
Extract 28	-	-	-	+	-	-	-	-	-	-	-	+
Extract 29	-	-	-	-	-	-	-	+	-	-	-	+
Extract 30	-	-	+	NT-M	-	+	+	-	-	+	+	NT-M
Negative control 1	-	-	-	-	-	-	-	-	-	-	-	-
Negative control 2	-	-	-	-	-	-	-	-	-	-	-	-
Target amplifications	3	7	16	13	8	12	17	14	9	15	22	19
Non-target amplifications	0	0	0	3	0	0	0	2	0	0	0	4

Table 2. Results of 16 PCR treatments testing the effect of increasing individual reagents as well as all possible combinations or reagents on 12 DNA extracts. Symbol "+" indicates amplification of the target DNA occurred (inferred by band size), "-" indicates no amplification occurred, non-target amplification is indicated by either "NT-S" (when inferred by size) or "NT-M" (when inferred by multiple bands).

	Reagent(s) increased by 25%															
dNTPs		✓				✓	✓	✓				✓	✓	✓		✓
$MgCl_2$ (+ buffer)			✓			✓			✓	✓		✓	✓		✓	✓
Polymerase				✓			✓		✓		✓	✓		✓	✓	✓
Primers					✓			✓		✓	✓		✓	✓	✓	✓
Extract 1	-	-	-	-	-	-	-	NT-S	-	NT-S	NT-M	-	NT-S	NT-S	NT-M	NT-S
Extract 2	-	-	-	-	-	NT-S	NT-M	NT-M	NT-M	NT-M	NT-M	NT-M	NT-M	NT-M	NT-M	NT-M
Extract 3	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+
Extract 4	-	-	-	-	-	+	NT-M	+	+	NT-M	+	NT-M	+	+	NT-M	+
Extract 5	-	-	-	-	-	+	+	+	+	+	+	NT-M	+	+	+	+
Extract 6	-	-	-	-	-	+	NT-M	+	+	+	+	NT-M	+	NT-M	+	NT-M
Extract 7	-	-	-	-	-	+	NT-M	-	+	+	+	+	+	NT-M	+	+
Extract 8	-	-	-	-	-	+	-	-	-	+	NT-S	+	-	+	NT-M	+
Extract 9	-	-	-	-	-	-	-	+	-	+	-	-	+	-	+	+
Extract 10	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
Extract 11	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+
Extract 12	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+
Negative control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Results from attempts to amplify and sequence 55 extracts using both standard and rescue PCR at +25%. Extracts that failed to amplify in this test are indicated by "NA", non-target DNA evidenced by multiple bands is indicated by "NT-M". All others were amplifications that appeared to be of the correct size and were submitted for sequencing. Raw sequence quality score and NCBI BLAST results including species and genetic region for match, number of matches expected by chance (E value), and percent similarity between the sequence generated and the BLAST match (Ident) are given for all attempts.

	Sequence quality		Results	Match quality	
	Standard	Rescue	Species (genetic region)	E value	Ident (%)
Extract 1	83.8	93.2	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 2	85.0	89.7	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 3	48.0	89.0	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 4	39.2	30.1	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 5	72.3	86.5	Oncorhynchus tshawytscha (12S)	4.00E-25	100
Extract 6	85.5	79.7	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 7	84.5	85.8	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 8	75.3	90.6	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 9	85.5	79.9	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 10	84.3	85.6	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 11	86.7	86.0	Catostomus catostomus (12S)	2.00E-68	99
Extract 12	87.9	85.8	Catostomus catostomus (12S)	2.00E-68	99
Extract 13	91.9	93.2	Ptychocheilus oregonensis (12S)	4.00E-90	99
Extract 14	83.8	86.2	Ptychocheilus oregonensis (12S)	4.00E-90	99
Extract 15	83.9	80.1	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 16	71.6	81.9	Catostomus catostomus (12S)	2.00E-68	99
Extract 17	87.9	83.9	Catostomus catostomus (12S)	3.00E-70	99
Extract 18	81.1	78.0	Catostomus catostomus (12S)	2.00E-68	99
Extract 19	84.2	81.4	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 20	95.2	87.8	Oncorhynchus kisutch (12S)	3.00E-70	100
Extract 21	73.4	80.8	Oncorhynchus kisutch (12S)	3.00E-70	100
Extract 22	79.3	78.8	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 23	82.3	85.5	Catostomus catostomus (12S)	2.00E-68	99
Extract 24	76.6	91.0	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 25	90.4	86.6	Oncorhynchus tshawytscha (12S)	2.00E-68	99
Extract 26	90.6	72.6	Catostomus catostomus (12S)	3.00E-70	99
Extract 27	93.4	88.6	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 28	52.2	NA	Oncorhynchus tshawytscha (12S)	2.00E-68	99
Extract 29	34.8	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100

Summary	Standard	Rescue
Attempted	55	55
Failed to amplify	17	9
Multiple bands on gel	0	2
Sequence attempted	38	44
Non-target DNA sequence	0	4
	0%	9%
Target DNA confirmed	100%	91%

Extract 30	82.8	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 31	89.7	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 32	81.4	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 33	89.2	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 34	84.0	NA	Catostomus catostomus (12S)	7.00E-67	99
Extract 35	85.1	NA	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 36	76.8	NA	Catostomus catostomus (12S)	2.00E-68	99
Extract 37	91.5	NT-M	Ptychocheilus oregonensis (12S)	4.00E-90	99
Extract 38	92.1	NT-M	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 39	NA	89.6	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 40	NA	88.8	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 41	NA	62.4	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 42	NA	71.1	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 43	NA	89.1	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 44	NA	87.7	Catostomus catostomus (12S)	3.00E-70	99
Extract 45	NA	81.3	Catostomus catostomus (12S)	3.00E-70	99
Extract 46	NA	83.3	Catostomus catostomus (12S)	3.00E-70	99
Extract 47	NA	81.2	Oncorhynchus tshawytscha (12S)	2.00E-68	99
Extract 48	NA	82.8	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 49	NA	88.2	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 50	NA	86.8	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 51	NA	81.7	Oncorhynchus tshawytscha (12S)	9.00E-71	100
Extract 52	NA	50.3	human (chromosomal)	4.00E-37	98
Extract 53	NA	93.0	human (chromosomal)	3.00E-25	94
Extract 54	NA	72.9	human (12S)	1.00E-64	99
Extract 55	NA	55.2	human (chromosomal)	9.50E-19	97.5

Table 4. Comparisons in application of method and efficiency between standard and rescue PCR at +25%. Successful application is based on the ability of standard and/or rescue PCR to generate amplification in a given extract. Calculated for all extracts as well as grouped by DNA extraction method and geography. Efficiency is the number of successful amplifications per attempted PCR across extracts that amplified using both PCR methods. Values for rescue PCR are give both as indicated by gel amplification and with a 9% reduction to account for miscalls of non-target DNA generating a band of the correct size.

	Application of Method										Efficiency	
	Method 1		Method 2		Snake River		Spokane River					
	Extracts	Amplified	Extracts	Amplified	Extracts	Amplified	Extracts	Amplified	Extracts	Amplified	Attempts	Amplified
Standard	112	55 (51%)	48	24 (50%)	64	31 (48%)	68	41 (60%)	44	14 (32%)	98	58 (59%)
Rescue (as indicated)		112 (100%)		48 (100%)		64 (100%)		68 (100%)		44 (100%)	66	88 (88%)
Rescue (9% reduction)		102 (91%)		44 (91%)		59 (91%)		62 (91%)		40 (91%)		80 (80%)
P value (as indicated)	<	0.000	<	0.000	<	0.000	<	0.000	<	0.000	<	0.000
P value (with reduction)	<	0.000	<	0.000	<	0.000	<	0.000	<	0.000		0.005

Figure captions

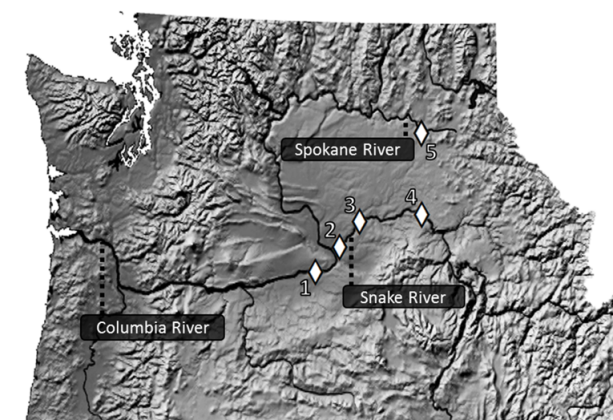
Figure 1. Excavation site locations and approximate ages for samples used in this study. Samples were comprised of two collections, those from in and near the Snake River basin (1 – 4) and those from a location near the Spokane River (5).

Figure 2. Schematic of experimental set-up to investigate effect of rescue PCR on decreasing concentrations of DNA. A pooled sample of fish DNA was subject to nine dilutions and amplification was attempted using standard and rescue PCR.

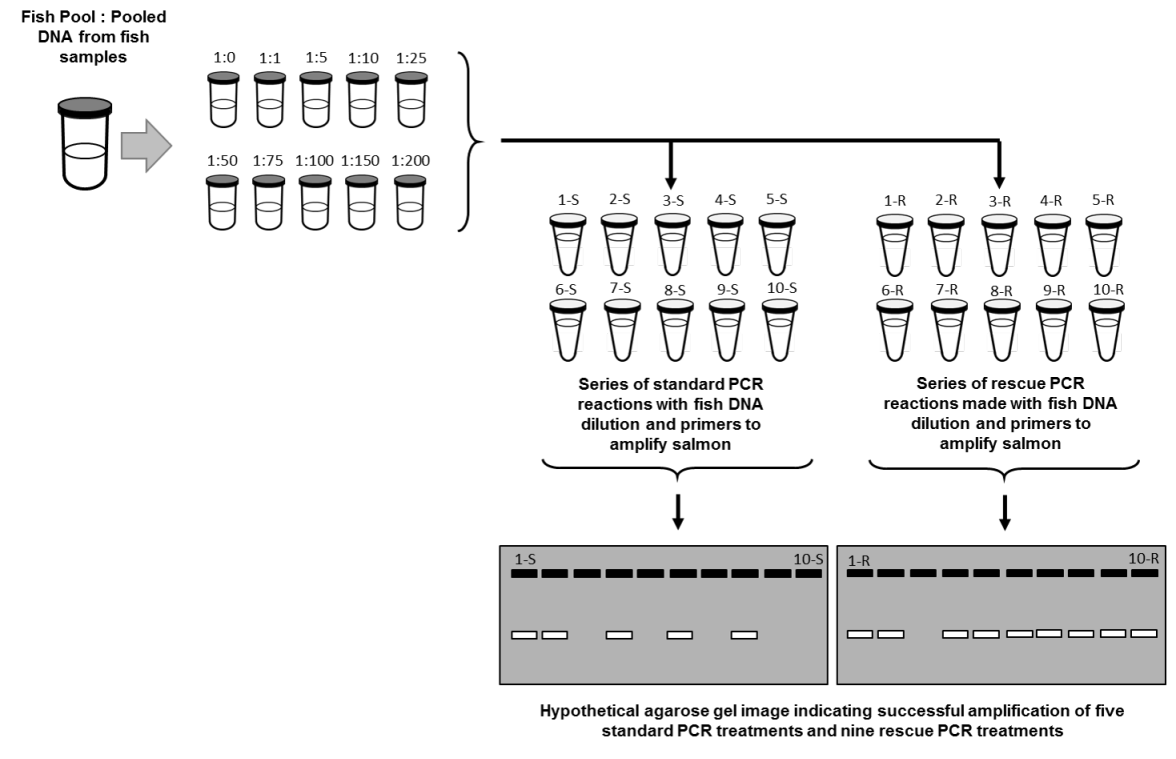
Figure 3. Schematic of experimental set-up to investigate the success of rescue PCR with changing DNA-inhibitor ratios. An aDNA control consisting of pooled DNA from northern fur seal was subject to nine dilutions; each dilution was then spiked with undiluted fish DNA. Amplification was attempted using standard and rescue PCR mixes created using primers designed to target the northern fur seal DNA and not fish DNA.

Figure 4. Amplification results for test inducing decreasing DNA concentrations. Two pools of fish DNA (FP1 and FP2) were diluted and amplification was attempted using standard and rescue PCR at +25%. Two replicates were run for each dilution of a DNA pool. Results indicate little difference between amplification capabilities of standard and rescue PCR. (Note: Results are from a single gel image that has been reordered to support interpretation, no other alterations were made to the images).

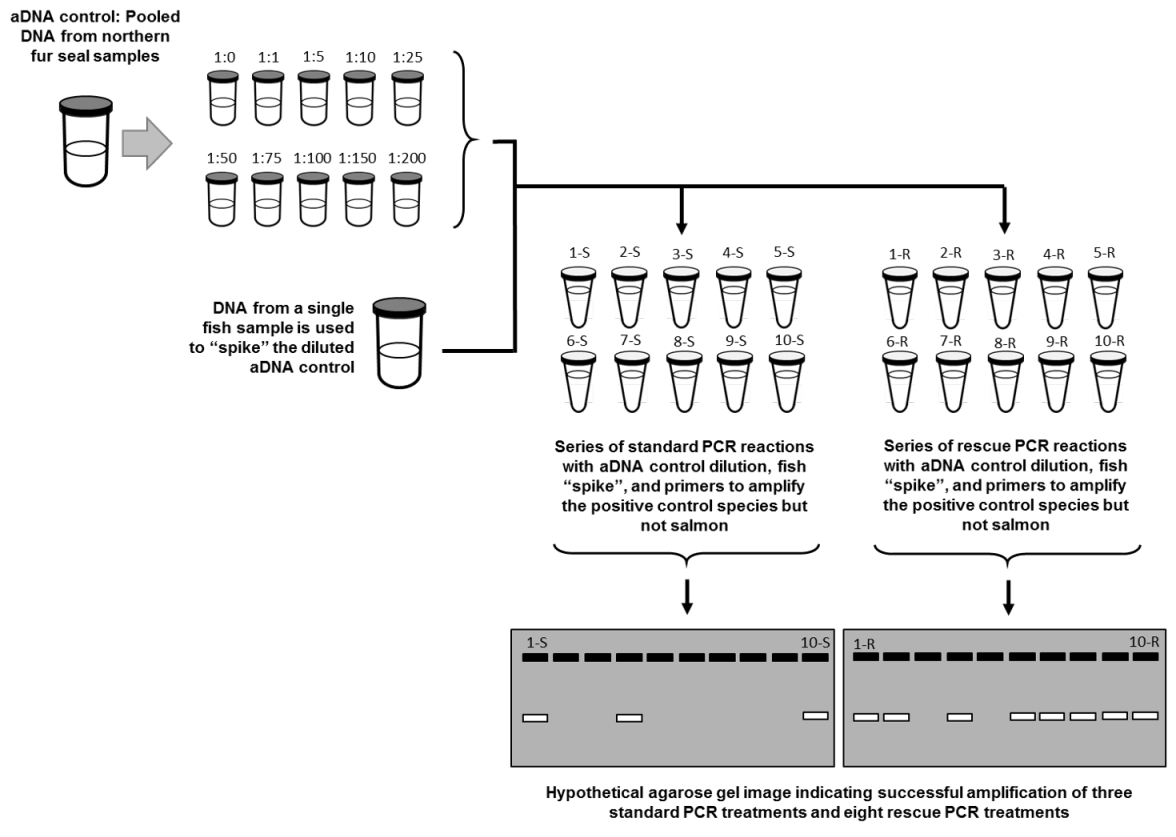
Figure 5. Amplification results for test inducing DNA-Inhibitor ratio changes. An aDNA positive control was diluted and then spiked with undiluted fish DNA (four distinct DNA samples, numbered 1 - 4). Primers used in the PCR target the aDNA control. Amplification was then attempted using standard and rescue PCR at +25%. Two replicates were done for each dilution/spike combination. "+C" shows the dilution treatment with no fish DNA spike. Results indicate amplification success of rescue PCR in cases where standard PCR fails. (Note: Results are from three gel images that has been combined and reordered to support interpretation, no other alterations were made to the images).



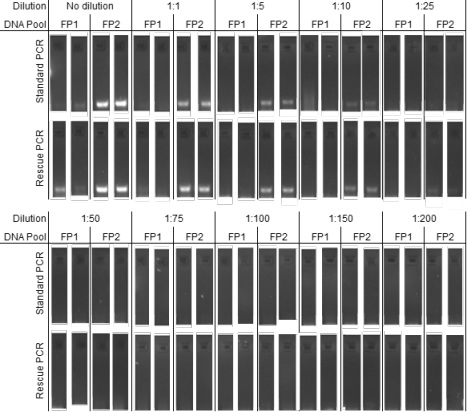
1] McNary Dam
2] Ice Harbor Dam
3] Lower Monumental Dam
4] Lower Granite Dam
5] Spokane

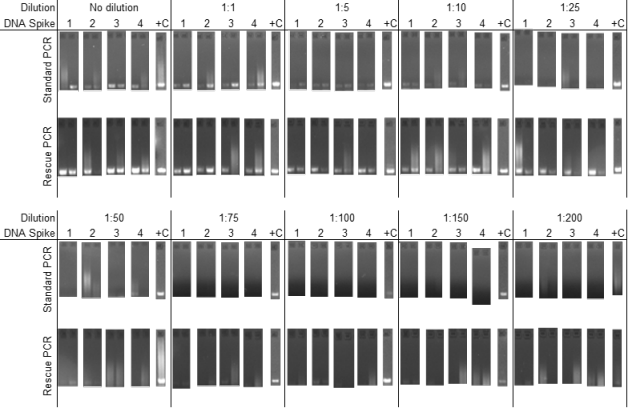


Hypothetical agarose gel image indicating successful amplification of five standard PCR treatments and nine rescue PCR treatments



Hypothetical agarose gel image indicating successful amplification of three standard PCR treatments and eight rescue PCR treatments


Dilution
No dilution
1:1
1:5
1:10
1:25
DNA Pool
FP1
FP2
Standard PCR
Rescue PCR
Dilution
1:50
1:75
1:100
1:150
1:200
DNA Pool
FP1
FP2
Standard PCR
Rescue PCR


Dilution
No dilution
1:1
1:5
1:10
1:25
DNA Spike
1 2 3 4 +C
Standard PCR
Rescue PCR
Dilution
1:50
1:75
1:100
1:150
1:200
DNA Spike
1 2 3 4 +C
Standard PCR
Rescue PCR