B61 Optimization of Alternative Mini-Primers for the Amplification of Ancient Mitochondrial DNA

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The goal of this presentation is to identify alternative mini-primers for use in amplification of ancient skeletal remains that may have primer-binding site issues due to polymorphism contained within the primer binding site.

An accepted strategy within the forensic community for the identification of ancient skeletal remains is the isolation and sequencing of mitochondrial DNA (mtDNA) hypervariable regions one and two (HV1 and HV2). The high copy number of mtDNA genomes contained within the cytoplasm allows for identification of individuals where little to no nuclear DNA would be isolated. This strategy allows the mtDNA section of the Armed Forces DNA Identification Laboratory to accomplish its primary mission to aid Central Identification Laboratory, Hawaii, in the identification of human skeletal remains recovered from World War II, the Korean War, and the Vietnam Conflict. Currently HV1 (nt15989-16410) and HV2 (nt15-389) regions are amplified with one of four primers sets (PS1–PS4); or for highly degraded or inhibited case samples one of eight more sensitive mini-primer sets (MPS 1A – MPS 4B) are used (Gabriel et al., 2001). In experiments described below, increased knowledge of mutational hotspot locations within the mtDNA control region is utilized to identify and optimize alternative mini-primers for the amplification and sequencing of mtDNA HV1&2 regions where these hotspots fall within the primer binding sites.

Initially, primer R16237 (MPS1B) was investigated which has mutational hotspots in its binding-site at nt16223 and 16224. In an attempt to detect these polymorphisms, the use of a previously validated PS 1 primer R16251 was used to amplify and sequence. Experiments were carried out using known DNA (200pg/10ul) and several reported case and research extracts that have known nt16223 and 16224 polymorphisms. Amplification and sequencing assays demonstrated that R16251 had equivalent sensitivity to R16237 at 100, 10, and 1 pg of template DNA and that both R16237 and R16251 gave the correct sequence for their respective amplicons. Thus, either primer can be used in amplification and sequencing, however, use of R16251 generates an 11 base-pair longer amplicon that allows conformation of the 16223 and 16224 polymorphisms as well as an overlap with other mini-primer sets.

The authors next examined why, on rare occasions and with certain case extracts, R16322 (nt16303-16322) appears to flip in sequencing reactions to give a forward sequence instead of a reverse sequence. It is believed this flipping event may be a regional issue, not a template concentration issue since an amplification dilution assay of the positive control DNA from 100pg to 1fg with R16322 (MPS2A nt16268 - 16322) failed to exhibit the same phenomenon. To address the regional issue, the research department designed a new primer R16353 to be used as an alternative primer for amplification when this phenomenon occurs. Amplification and sequencing reactions comparing R16322 and R16353 showed that R16353 had the same sensitivity and generated the same sequences as R16322, but with an additional 30 base pair read.

Finally, a commonly observed issue was addressed with MPS2B (F16268/R16410) where, in addition to the expected amplicon product, there is also a less prominent higher MW band that can lead to unusable mixed sequencing results. An alternative forward primer [F16222 (nt16222-16239)] was developed and even though it has potential binding-site issues at nt16223 and 16224, case extracts that did and did not contain these polymorphisms showed a reduction in the amount of double banding as well as reportable non-mixed sequencing results. Therefore, F16222 should be used as an alternative amplification primer for MPS 2B when amplified case extracts demonstrate this higher MW banding.

In addition to addressing mini-primer set amplification issues, sequencing issues with MPS4B (F220/R377), where R377 gave ambiguous base calls along with truncated sequencing reads were also addressed. Previously reported case extracts were amplified with F220 and either R377 or R389 and, depending on the extract, results demonstrated a 2-8-fold increase in product yield when amplified with R389 as well as decreased ambiguous base calls and full length reads when sequenced with R381 instead of R377.

The continued identification and optimization of alternative miniprimers allows the primary mission of identifying human remains to be more efficiently accomplished by increasing productivity and decreasing the turn around time of case samples.

Alternative Mini-Primers, Polymorphism, Ancient DNA

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