STR analysis and mitochondrial DNA sequence analysis are two important techniques that are widely used in forensic casework where nuclear DNA is extensively degraded or is limited in concentration. In contrast to the high resolving power of the STR techniques in cases containing DNA mixtures, mtDNA sequence analysis is not useful in resolving DNA mixture situations. To expand on this limitation, the following strategy was developed. To minimize the level of contamination, cellular material from semen stain evidence was differentially enriched for male and female fractions using physical methods for separating spermatozoa from female epithelial cells. DNA mixtures from the enriched female fraction were amplified for each of the two mtDNA regions and sequenced. As expected, at nine different polymorphic positions (compared to Anderson standard sequence) mix bases were found. Separation of the amplified DNA mixtures into their individual components was achieved by sub-cloning and sequencing of resultant clones. Twenty eight-clones with positive inserts were identified. Sequencing of these clones correctly matched with the polymorphic positions originally identified in the sequenced DNA mixture. Furthermore, mtDNA sequences obtained from the clones were identical to either the mtDNA sequence established for the victim or the suspect. The presence of the victim’s and the suspect’s DNA in the mixture was positively identified and confirmed by STR analysis. The presented DNA evidence was successfully used by court to convict the suspect.