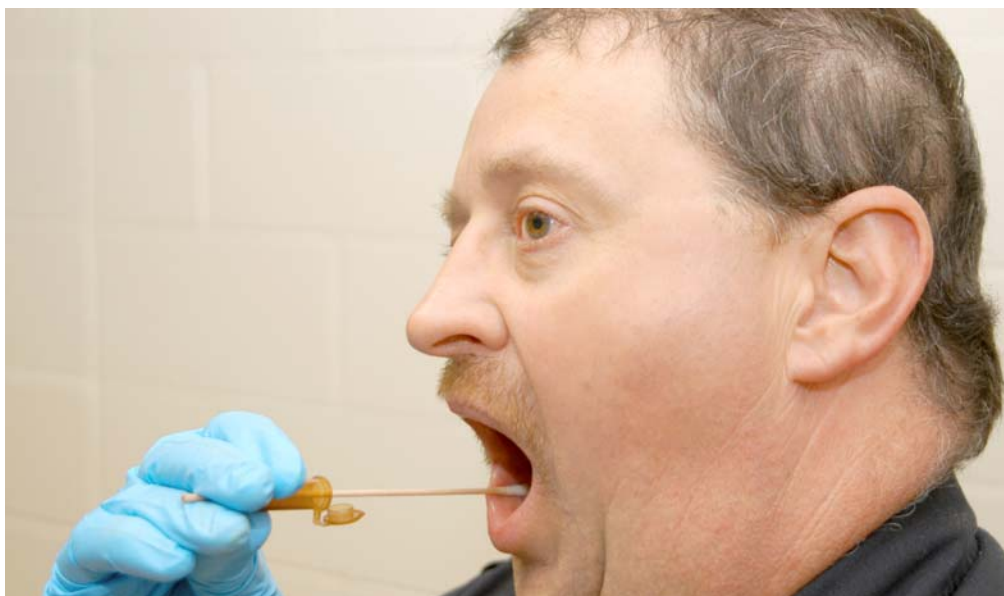


## Using superconvective DNA amplification to speed up Sanger sequence analysis of forensic mitochondrial DNA samples



Quick and simple preparation/amplification of DNA samples, combined with fast DNA sequencing, is of great interest to the forensic genetics community. In this study the QuanTyper™-48 instrument has been used to speed up the amplification steps involved in Sanger DNA sequencing. This method provides the possibility to perform a full DNA-sequencing analysis within two hours. This could be particularly useful in criminal investigations, for example, as a screening test to quickly clear innocent suspects.

### Introduction

Over the last decade forensic genetics has made tremendous progress in the analysis of nuclear DNA (nDNA) through a combination of new analysis tools and improved databases. However, situations still occur when the nDNA in a sample is either too degraded or is not present in sufficient amounts to allow analysis. In such cases the analysis of mitochondrial DNA (mtDNA) can serve as a very useful alternative (1).

The copy-number for human mtDNA is in the range of 100-1000 copies per cell,

which makes it possible to analyze forensic DNA samples even when only a few cells are present in the sample. The drawback with mtDNA is that its discriminatory power is just a fraction of that which is possible to obtain with nDNA, due to the fact that the mitochondrial genome is much smaller and uniparentally inherited. However, analysis of mtDNA is still very useful as circumstantial evidence in certain situations, e.g. to match minute amounts of DNA found at a crime scene against DNA sampled from suspects. Furthermore, analysis of mtDNA is also

useful in situations where the nDNA is too degraded, as was the case in the disaster victim investigations related to the terrorist attack on the World Trade Center (2) in September 2001 and the 2004 Asian tsunami.

Sanger DNA sequencing is regarded as the 'gold standard' in DNA analysis since it gives the highest resolution possible of any DNA analysis; i.e. the actual DNA-sequence. The downside is that the analysis procedure is regarded as being slow and complicated. Here we present data where QuanTyper-48, a superconvective (3,4) real-time PCR instrument, has been used to dramatically shorten the time it normally takes to perform the two amplification steps involved in the complete sequence analysis, i.e. PCR\* and cycle sequencing (CS).

By combining the speed of QuanTyper-48 with new developments in sample clean-up procedures, and with the speed of capillary electrophoresis (CE) using short capillaries, it is now possible to obtain DNA sequencing results within two hours of starting the initial PCR amplification of a DNA sample.

## Materials and Methods

Human DNA was prepared from tested whole blood provided by Uppsala University Hospital. The prepared DNA (containing both nDNA and mtDNA) was diluted to a concentration of 26 ng/ $\mu$ L and stored at  $-20^{\circ}$  C. This preparation was then used as template DNA in the PCR amplification step performed in QuanTyper-48.

PCR primers F-15971 and R-16410 were used to amplify a 440 bp fragment of the HVI region while primers L-15 and R-429 amplified a 415 bp fragment of the HVII region. Both HVI and HVII are hyper variable regions within the non-coding mtDNA D-loop.

After performing real-time PCR amplification QuanTyper-48 was also used for the CS amplification step. One microlitre of the raw/unpurified PCR product was used as template DNA in the CS reaction. To add specificity to the CS reaction internal sequencing primers were used (one primer specific for HVI and one primer specific for HVII respectively).

Prior to loading the samples into the sequencing instrument a clean-up procedure was performed to remove the unincorporated BigDye<sup>®</sup> Terminator nucleotides used in the CS reaction. Traditionally, the most commonly used method for this procedure utilizes ethanol and centrifugation to precipitate the DNA and then simply wash away the excess nucleotides dissolved in the ethanol supernatant. Although cost efficient, it takes roughly two hours to complete the whole procedure, which also includes an evaporation step, (to evaporate residual ethanol), and the step to dissolve the final DNA pellet.

In this study, instead of using an ethanol precipitation method, we utilized a small 'ready-to-use' gel-matrix column (AmpliPurifi ExTerminator Kit from AME Bioscience) to perform the whole clean-up procedure within 10 minutes. The purified DNA sample was eluted in a solution that could then be directly analyzed using an ABI 3730 XL DNA Analyzer.

**Table 1. PCR protocol**

|                                | <u>Final conc.</u> |
|--------------------------------|--------------------|
| DNA template                   | 26 ng              |
| <b><i>HVI fragment</i></b>     |                    |
| primer F-15971                 | 0.2/0.4 $\mu$ M    |
| primer R-16410                 | 0.2/0.4 $\mu$ M    |
| <b><i>HVII fragment</i></b>    |                    |
| primer L-15                    | 0.2/0.4 $\mu$ M    |
| primer R-429                   | 0.2/0.4 $\mu$ M    |
| MgCl <sub>2</sub> <sup>1</sup> | 2.5 mM             |
| 10x Buffer <sup>1</sup>        | 1x                 |

<sup>1</sup> From Invitrogen.

|  |                     |
|--|---------------------|
| Taq polymerase <sup>1</sup>            | 0.1 U/ $\mu$ L      |
| SYBR Green <sup>1,2</sup>              | 0.125 X             |
| dNTPs                                  | 0.2 mM <sup>3</sup> |
| dH <sub>2</sub> O to a final volume of | 50 $\mu$ L          |

*PCR cycling parameters*

95° C for 90 s followed by 35 cycles of [94° C for 0 s; 60° C for 8 s; 72° C for 30 s].

*Process time: 30 minutes using QuanTyper-48.*

**Table 2. Cycle sequencing (CS) reaction protocol**

|  | Amount      |
|--|-------------|
| Unpurified PCR product                     | 1 $\mu$ L   |
| Sequencing primer (5 $\mu$ M) <sup>4</sup> | 0.8 $\mu$ L |
| Ready Reaction Premix <sup>5</sup>         | 2.5 $\mu$ L |
| Sequencing Buffer (5x) <sup>5</sup>        | 5.0 $\mu$ L |
| dH <sub>2</sub> O to final volume of       | 25 $\mu$ L  |

*Cycle sequencing parameters*

95°C for 10 s followed by 25 cycles of [95° C for 5 s; 54° C for 5 s; 65° C for 20 s].

*Process time: 19 minutes using QuanTyper-48.*

**Post CS clean up procedure**

The clean-up procedure was done according to the protocol provided with the AmpliPurifi ExTerminator kit. The purified sequencing sample was eluted in a final volume of 25  $\mu$ L of water.

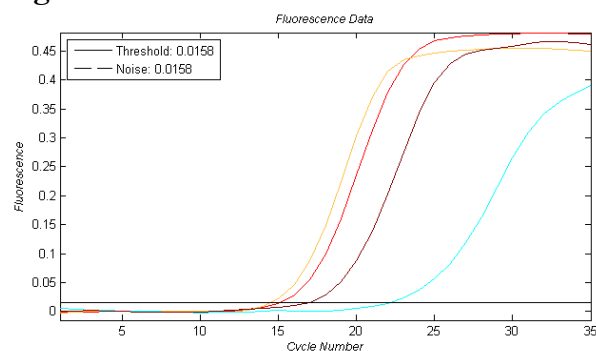
*Process time: 10 minutes to complete the clean-up procedure.*

<sup>2</sup> Invitrogen cat nr: 11744-100, diluted 80,000x.

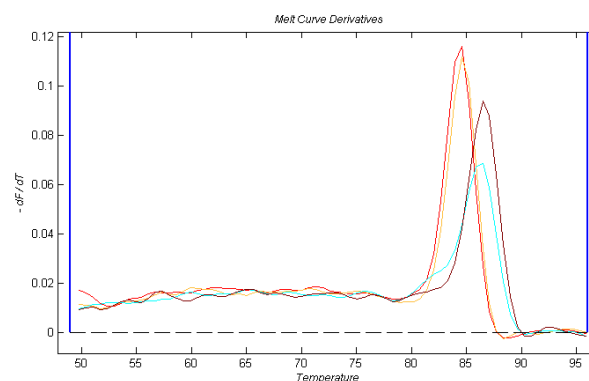
<sup>3</sup> Each dNTP at 0.2 mM final conc. dNTPs from GE Healthcare.

<sup>4</sup> Sequencing primer F-15971 was used for the HVI fragment while sequencing primer HVII F was used for the HVII fragment

<sup>5</sup> ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit.

**Results****Figure 1 Real-time PCR**

| Sample | Name                      | Type    | Channel 1 |      |
|--------|---------------------------|---------|-----------|------|
|        |                           |         | Conc      | Ct   |
| 2      | HVI, 0.2 $\mu$ M primers  | Unknown | -         | 15.1 |
| 4      | HVI, 0.4 $\mu$ M primers  | Unknown | -         | 14.5 |
| 6      | HVII, 0.2 $\mu$ M primers | Unknown | -         | 22.1 |
| 8      | HVII, 0.4 $\mu$ M primers | Unknown | -         | 17.0 |

**Figure 2 Melting point analysis**

| Sample | Tm (49-96°C) |
|--------|--------------|
| 2      | 84.4         |
| 4      | 84.6         |
| 6      | 86.2         |
| 8      | 86.6         |

The result from the real-time PCR analysis (Figure 1) shows that fast (<30 minutes) and efficient amplification of mtDNA samples is possible to obtain using QuanTyper-48. The melting curve analysis (Figure 2), following the amplification step, detected no formation of spurious by-products in the amplified samples. The unpurified PCR-products were then directly used as templates in the CS-reaction.



As illustrated in Figures 3 and 4, excellent sequencing data was obtained with only minor increases in the background signal in certain regions of the HVI and HVII sequences. In essence, the whole sequence was read to the PCR run-off on both templates (HVI and HVII).

The two serial amplification procedures (PCR and CS) followed by the post-CS clean up procedure took less than one hour to perform. This method followed by CE in short capillaries, means that it is possible to obtain mtDNA sequencing results within two hours, starting from a purified DNA sample. The potential to dramatically shorten the sequencing analysis of mtDNA using this procedure should be of great interest to the forensic genetic community. This method could prove particularly useful as a quick pre-screening assay, for example, to exclude suspects involved in a criminal investigation.

## Conclusions

- Superconvective PCR technology enables efficient DNA amplification with fast temperature ramping both in PCR and cycle sequencing amplification procedures.
- The procedure described in this study, followed by CE in short capillaries, makes it possible to obtain mtDNA sequencing results within two hours starting from a purified DNA sample.

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**All primers kindly provided by Dr. Marie Allen, Dep. Of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Sweden**

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