# Advancing forensic RNA profiling

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# Chapter 3

## Advancing forensic RNA profiling: Preventing noise signals in RNA profiling by adding the multiplex buffer last

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# Abstract

We noticed variation in the level of background noise when using custom-made fluorescently labelled primer mixes during PCR amplification. Various aspects that may affect the level of noise generated during PCR set-up were assessed. Best results are obtained when the multiplex buffer is added as a last component.

# Introduction

Custom-made fluorescently labelled primer mixes, such as RNA typing multiplexes, are increasingly used in forensic analyses. These multiplexes are regularly used in combination with commercially available multiplex amplification chemicals, for example the QIAGEN multiplex kit. We experienced variation in the occurrence of background noise, and noticed higher noise levels when more samples were analysed. This background noise also observed in blank PCR controls suggested that it is caused during PCR set-up. Various causes and solutions were considered to reduce the level of background signals.

# Materials and methods

PCR amplification and product detection was performed according to standardized protocols [1], which uses a master mix of 12.5 µL 2x QIAGEN multiplex reaction mix supplemented with 5 µL primer mix for 19 amplicons based on [2], followed by the addition of the appropriate amount of cDNA sample and/or water that together make a volume of 7.5 µL. PCR products were purified [1] prior to detection using a 3130XL Genetic Analyzer (Life Technologies), POP-4 (Life Technologies) separation matrix and 3 kV, 10 s injection settings. Various aspects were assessed using NTCs (no template controls or blank consisting of nuclease-free water) for PCR amplification. We evaluated the use of QIAGEN multiplex *Plus* buffer, Q-solution (2.5 µL/reaction), increased annealing temperature (from 60 °C to 64 °C) and using a multiplex with reduced complexity (from 19 to 11 labelled primers).

# Results and discussion

An overlay electropherogram of a skin sample amplified using the standard amplification protocol is shown in Figure 1A. Visible is the background noise, which severely interferes with the interpretation of amplified peaks (*id est* skin and housekeeping markers) and may lead to false positive scoring of absent markers. We noticed that more noise occurred with larger sample sizes, that it also occurred for the blank control sample that was pipetted last and that it occurred more frequent with less experienced laboratory personal. Thus we hypothesized that the time that buffer and primer mix remain mixed in concentrated form may have a role in the formation of noise signals (note that the four components of the reaction – buffer, primer mix, water and cDNA – are not pipetted per sample but each component

is pipetted for the full sample series). A time series was performed varying the time that multiplex buffer and primer mix stand combined before adding water. Results for the 60,12 and 0 min incubation are shown in Figure 1B. A decrease in background signals is observed with reduced time intervals. Similar time series experiments were repeated to assess the effect when the buffer and primer mix stand combined in less concentrated forms, that is after adding water to the reaction volume of 25 µL. Results are shown in Figure 1C and a clear reduction of background noise was observed compared to the 60 min profile shown in Figure 1B. The use of QIAGEN multiplex *Plus* buffer was compared to the use of the regular QIAGEN multiplex kit (both use a HotStartTaq DNA Polymerase to prevent polymerase activity at room temperature; the *Plus* buffer has a reduced activation time), but this did not reduce the level of background noise (data not shown). Addition-ally, Q-solution was assessed as this is described to improve amplification of difficult template by modifying the melting behaviour of the template. However, the addition of Q-solution led to drop-out of various markers while background signals remained visible (data not shown). Increased annealing temperatures and a reduced complexity of the primer mix also failed to reduce background noise (data not shown). Most promising results are obtained when adding the multiplex buffer as a last component, visible in Figure 1D.

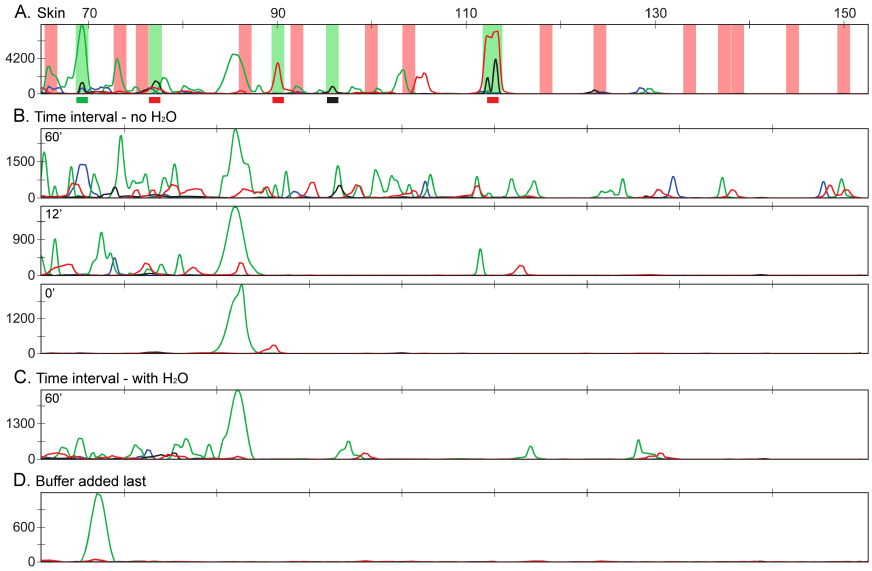


Figure 1. Overlay electropherograms. A: A skin sample amplified showing background noise. Red markers bins indicate locations for which no signals are expected; light green marker bins those of skin and housekeeping markers. The bars below the green bins indicate the fluorophore colour of the expected signal. B: Blank controls amplified after primer mix and multiplex buffer stood combined in concentrated (no water added) for 60, 12 or 0 minutes. C: Blank control amplified after primer mix and multiplex buffer were combined in diluted form (water added) for 60 minutes. D: Blank control amplified when the multiplex buffer was added last, and the PCR was started immediately.

## Concluding remarks

We infer that, when using custom-made fluorescently labelled primer mixes, background noise increases when primer mix and multiplex buffer stand combined in concentrated form. By adding the QIAGEN multiplex reaction mix as a last component during PCR set-up of RNA profiling multiplexes, the occurrence of background noise is reduced. Since noise occurs without the presence of template DNA, we assume the noise is caused by destabilisation or aggregation of the fluorescently labelled primers. However, as the composition of the commercial buffer is unknown, we cannot speculate what components may cause this instability. In our laboratory several assays use this multiplex buffer but since these assays do not use fluorescently labelled primers, this background noise is not observed.

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