**RESULTS and DISCUSSION**

To understand the difference in between traditional RAPD-PCR and real-time RAPD, it is important to understand what happens during a PCR reaction. A basic PCR run can be broken up into three phases: Exponential, Linear, and Faint. In plateau phase, reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade. Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample. The plateau phase is where traditional PCR and real-time HRM analysis take its measurement, also known as end-point detection.

**RAPD-PCR screening can have many end-point measures such as variation in band intensity, band appearance and disappearance, identifying the precise reasons for the changes is one of the major challenges. Different types of DNA lesions and mutations can induce the same type of alterations in RAPD profiles**

At the end of traditional and real-time PCR, amplification efficiency is not exponential because double-stranded DNA is an excellent inhibitor of DNA polymerase. The reason why duplex DNA inhibits DNA polymerase is that the polymerase binds to the duplex rather than binding to the small quantity of duplex arising from the primers binding to target strands during the early cycles of PCR.

Application of the Inhibition Principle to real-time HRM assisted RAPD-PCR Design

The concept of amplicon inhibition of PCR is particularly important for multiplex PCR design and in our real-time RAPD-PCR approach we exploit this fact of real-time PCR. Consider a RAPD reaction in which there are plenty of JNTPs available. It is expected that if one of the amplicons is produced more efficiently than the others, then it will reach saturation and inhibit the polymerase from subsequently amplifying the other amplicons. Here we do not need to introduce the idea of different targets as it can be optimized in traditional RAPD-PCR, but we let the real-time conditions choose the appropriate amplicon to amplify efficiently and reproducibly to further the evaluation of variation from this selected amplicon's window with HRM analysis and real-time PCR's selection dynamic and even to produce SCAR markers. To achieve uniform amplification of the different targets, the RAPD primers must be screened to bind with high efficiency to their preferred target. Binding with high efficiency means with compromise to the requirements of real-time machine, real-time detection criteria, real-time PCR chemistry and HRM chemistry and thermodynamic parameters and any accounting for the effects of competing equilibria.

HRM analysis represents a simple and cost-effective screening method for the detection of genetic variation within RAPD-PCR amplicons. HRM-based post-screening assay could easily be integrated into the traditional RAPD-PCR routine. Using HRM, a large number of RAPD primers can be screened simultaneously for identification of real-time selected amplicons of interest for further DNA sequencing analysis and SCAR marker production. As this is a proof of concept study, more work will be done to further test, and prove the limits and the benefits of the procedure especially in producing useful SCAR markers to detect further variation.

**REFERENCES**


**FROM TRADITIONAL RAPD-PCR TO NEXT GENERATION HRM ASSISTED REAL-TIME RAPD PCR**

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**ABSTRACT**

High Resolution Melting (HRM) Analysis is a powerful technique for the detection of mutations, polymorphisms and epigenetic changes through differences in melting plots of amplicons by measuring the melting temperature of amplicons in real time. In this study, a novel RAPD-PCR based method will be discussed to detect various levels of genetic variation exploiting the advantages of various next-generation qPCR applications such as new dye technologies and HRM analysis.

**INTRODUCTION**

Traditional random amplified polymorphic DNA (RAPD) technique is based on the amplification of DNA fragments using a short arbitrary primer that anneals multiple locations on the genomic DNA. This is followed by separation of amplified fragments based on their sizes using gel electrophoresis. Samples are identified by comparing the DNA bands of fingerprints, which are expected to be consistent for the same primer, DNA and experimental conditions used.

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases from a specific proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. In addition, fluorescence data can be collected directly from a real-time PCR instrument avoiding the drawbacks of end-point detection.

High Resolution Melting (HRM) is a novel, homogenous, close-tube, post-PCR method. HRM allows to study the thermal denaturation of a double-stranded DNA in much more detail and with much higher information yield than ever before. HRM characterizes nucleic acid samples based on their dissociation (melting) behavior. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified.

The most important HRM application is gene scanning - the search for the presence of unknown variations in PCR amplicons prior to or as an alternative to sequencing. Mutations in PCR products are detectable by HRM because they change the shape of DNA melting curves. A combination of new-generation DNA dyes, high-end instrumentation and sophisticated analysis software allows to detect changes and to derive information about the underlying sequence constellation. With HRM, these and other applications are done using low-cost generic dyes where previously custom labelled probes such as TaqMan® or fluorescence resonance energy transfer (FRET) probes were required. HRM is thus a simpler and much more cost-effective way to characterize samples.

**Traditional random amplified polymorphic DNA (RAPD) technique**

When RAPD is used, it is necessary to be as careful as possible by (i) optimising RAPD reactions, (ii) knowing the optimal variation among control samples, (iii) using two DNA concentrations that differ at least by a factor 2 (when variation is observed or not), and (iv) interpreting the changes in RAPD profiles (bearing in mind that most of the time diverse factors can have an impact on the RAPD assay).

HRM analysis differs from standard melt curve analysis in three ways:

1. Chemistry—HRM analysis uses brighter dyes at higher concentrations.
2. Instruments—HRM analysis requires instruments that collect fluorescence data at finer temperature resolution.
3. Software—HRM analysis requires more sophisticated software which uses new fluorescent scaling algorithms and plots.

**HRM data normalization—shape & shift**

There are two ways HRM curve plots can discriminate between samples, by “Shape”, i.e. using detail in the shape of the melt curve itself and by “Shift”, i.e. the thermal offset of a curve from other curves. Before HRM curve plots are plotted, the raw data is first normalised: Melt curves are normally plotted with fluorescence on the Y axis and temperature on the X axis. This is similar to real-time PCR, in which samples are run under constant/variable/forcing as a common optical detector. This is seemingly ideal for HRM as thermal or optical variation between samples is insignificant. The result is that the Rotor-Gene HRM performance closely matches the HRF-1 benchmark with the compromise that samples are not arranged in a conventional array format (as they are in block-based instruments) but are instead arranged around the perimeter of a spinning rotor.

Optimized conditions to produce good quality HRM assisted Real-time RAPD PCR.

**MATERIALS and METHODS**

HRM and HRM analysis was performed on a Rotor-Gene 6000, by using variable real-time kits. The Rotor-Gene 6000 is the first of the multi-well instruments capable of both thermal cycling and HRM. This dual capability enables pre-amplification and HRM analysis consecutively in the one run. A major advantage of this is that amplification plots can be used to help interpret RAPD results since abrupt amplification plots (i.e. that amplification differently to what was expected) also produce aberrant HRM data. In this way compromised samples can be easily identified and removed from downstream HRM analysis. The main advantage of the Rotor-Gene for HRM stems from its rotary design, in which samples spin under centrifugal force past a common optical detector. This is seemingly ideal for HRM as thermal or optical variation between samples is insignificant. The result is that the Rotor-Gene HRM performance closely matches the HRF-1 benchmark with the compromise that samples are not arranged in a conventional array format (as they are in block-based instruments) but are instead arranged around the perimeter of a spinning rotor.

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