Applications

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Discrimination of microsatellite repeat polymorphisms of the *SLC11A1* promoter by melting curve analysis using the Eppendorf Mastercycler® ep *realplex*

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Abstract

During early macrophage activation the solute carrier family 11A member 1 (SLC11A1) plays an important role. A range of association studies, in humans, have produced conflicting results regarding the association of specific *SLC11A1* $(GT)_n$ promoter polymorphisms with the incidence of infectious and autoimmune diseases. This may be attributable to the small sample sizes investigated due, in large part, to the use of expensive, non-optimal and time-consuming genotyping methods. The completion of sufficiently large association studies that are required to determine the role of SLC11A1 in disease makes the development of a reliable genotyping methodology necessary. Here we show that melting curve analysis, using the Mastercycler ep *realplex*, is a fast and sufficiently sensitive method to differentiate the most common *SLC11A1* $(GT)_n$ promoter polymorphisms.

Introduction

Solute carrier family 11A member 1 (SLC11A1) is a divalent cation transporter that is important for the early activation of macrophages and has been linked to susceptibility to both infectious and autoimmune diseases. Expression of *SLC11A1* is modulated by a complex polymorphic $(GT)_n$ microsatellite repeat within the gene promoter. Currently there are 9 identified $(GT)_n$ promoter polymorphisms; designated alleles 1-9. Allele 2 and allele 3 are the most commonly occurring polymorphisms and differ in length by a single dinucleotide GT repeat. Allele 2 has 10 GT repeats [t(gt)5ac$(gt)_5$ac (gt)10ggcaga$(g)_6$] while allele 3 has 9 GT repeats [t(gt) 5ac$(gt)_5$ac(gt)9ggcaga$(g)_6$]. Individuals homozygous or heterozygous for alleles 2 or 3 account for greater than 95 % of all *SLC11A1* $(GT)_n$ genotypes.

Conventional methods for genotyping using real-time PCR include the use of allele specific fluorescence detection using fluorescently labelled primers, probes, or molecular beacons. However, these methods require expensive, fluorescently-labelled oligonucleotide probes or primers. In the case of genotyping microsatellites, the use of sequence specific probes is not optimal due to the repetitive nature of the DNA, which may cause non-specific binding of the probe. Cloning and sequencing is the most sensitive and accurate methodology for genotyping complex polymorphic microsatellite repeats. However, cloning and sequencing of polymorphic microsatellites is labour intensive, time consuming and expensive.

Therefore cloning and sequencing methodologies are not amenable for high-throughput large scale genotyping studies that are required for association studies attempting to correlate the presence of specific alleles with disease incidence.

Melting curve (MC) analysis is a simple and cost effective real-time PCR technique with a range of applications, including genotyping and mutation discovery. MC analysis allows the characterisation of post-PCR DNA using unlabelled oligonucleotides coupled with an inexpensive saturating DNA intercalating dye. The dissociation curve is obtained after PCR amplification, by monitoring the fluorescence of the saturating intercalating dye as the temperature passes through the denaturation temperature of the PCR product. Upon denaturation, the intercalating dye is released resulting in a rapid loss of fluorescence (Figure 1a). Wittwer et al. [1] was the first to report that PCR products could be differentiated in a closed system using their melting temperatures. Because the denaturation temperature of a product is dependent upon its GC content, length and sequence composition, PCR products with slightly different base compositions or lengths have different melting characteristics and, therefore, have different melting temperatures. This phenomenon can be exploited to distinguish genotypes or to screen for gene variants.

MC analysis has been most commonly used in the detection of single nucleotide polymorphisms and can differentiate both homozygous and heterozygous samples (Figure 1b). Homozygous samples result in the formation of homoduplexes, and, due to different base compositions, different homozygous samples have different denaturation temperatures (resulting in altered melt curves). Heterozygous samples contain two different alleles, which results in the formation of both homoduplexes and heteroduplexes. Heteroduplexes arise from the annealing of non-complementary strands of DNA, which form during the fast cooling of the samples. The mis-paired regions of the heteroduplexes cause 'bulges' in the DNA making the heteroduplexes less stable, and therefore they dissociate at a lower temperature. The lower dissociation temperature produces a different melt curve profile, allowing heterozygous samples to be differentiated from homozygous samples.

In this paper we show that discrimination of the most common genotypes of the *SLC11A1* promoter polymorphism is achievable with MC analysis using the Mastercycler ep *realplex*. This methodology has been validated and will now enable larger association studies to be conducted to determine more clearly the association of specific *SLC11A1* $(GT)_n$ promoter polymorphisms with the incidence of infectious or autoimmune/inflammatory diseases.

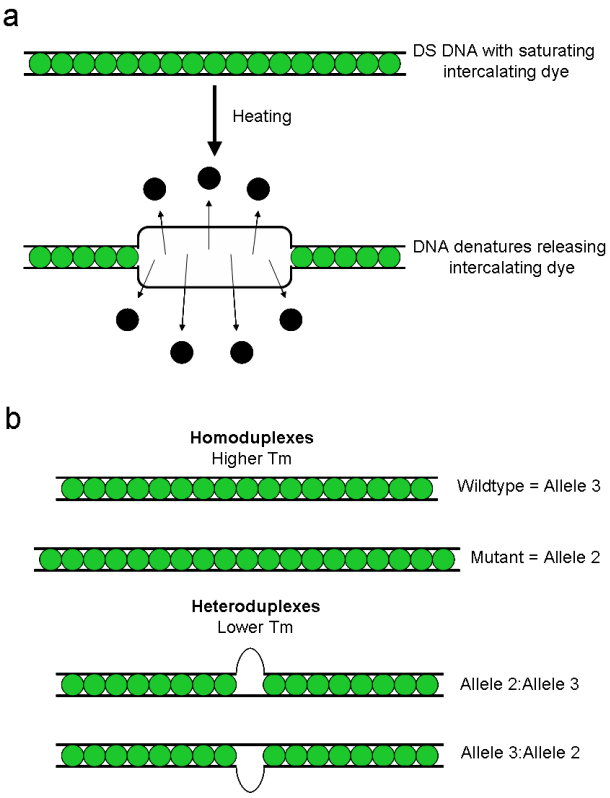

Figure 1: Molecular mechanism of melting curve analysis.
(a) A double stranded DNA intercalating dye (green circles) binds between the DNA bases. The sample is heated at a fixed rate and denaturation of the double stranded (DS) DNA results in a rapid loss of fluorescence.
(b) Molecular species formed during melting curve analysis. Homozygous samples result in the formation of homoduplexes while heterozygous samples result in the formation of a mixture of homoduplexes and herteroduplexes. Because heterduplexes contain bulges, or regions of mispairing of the bases, the denaturation temperature is lower than that of the homoduplex samples.

Materials and Methods

Three oligonucleotide primer pairs that flanked the *SLC11A1* $(GT)_n$ promoter polymorphism were initially designed. These primer sets allowed the production of a range of fragment sizes, from 127–208 bp, in which the polymorphic $(GT)_n$ repeat was located within different regions of the amplicon. PCR conditions for the generation of each amplicon were optimised and the amplicon producing the most consistent MC results was selected for use in genotyping of the $(GT)_n$ promoter alleles.

Results and Discussion

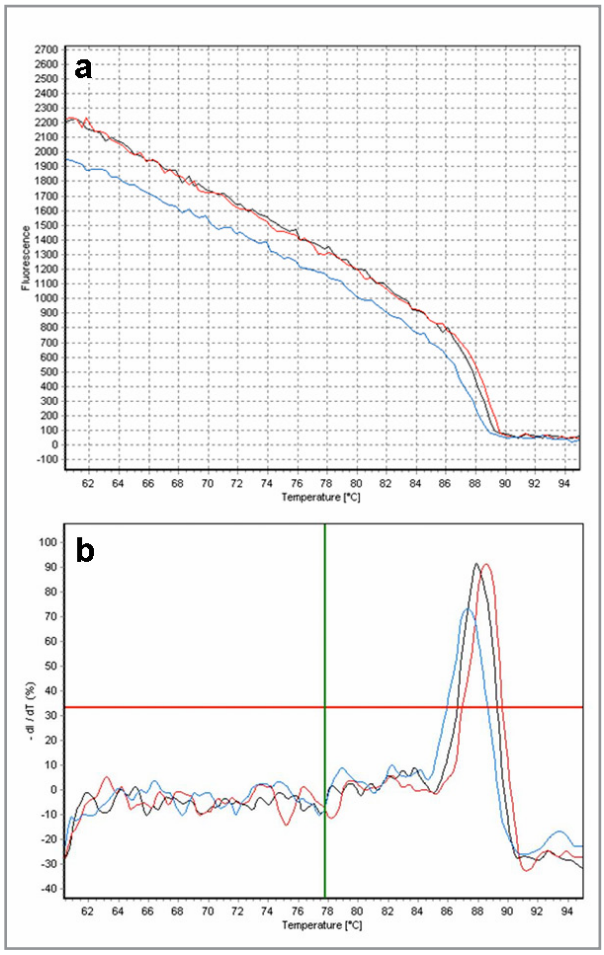

Fig. 2: Melting profiles for the most common *SLC11A1* $(GT)_n$ promoter genotypes. (a) Raw melting data of the 127bp amplicon. (b) Peak curves (negative first derivative of the raw data). Each *SLC11A1* $(GT)_n$ promoter genotype has a different peak maximum. Red - homozygous allele 3, black - homozygous for allele 2 and blue - heterozygous alleles 2 and 3.

All real-time PCR reactions were carried out using the Eppendorf twin.tec PCR Plates 96, skirted. PCR was carried out in a 25 µL reaction volume, which contained 1.0 U Platinum *Taq* polymerase (Invitrogen, USA); 1X LCGreen I Mix (1X LCGreen I, 0.25 mg/mL BSA, 0.2 mM dNTPs and 1–3 mM Mg Buffer) (Idaho Technology Inc., USA), 0.5–20 µM of each forward and reverse primer and 0.2 pg plasmid DNA. All PCR reactions included a no template control in which plasmid DNA was replaced with water. The plates were sealed using Eppendorf Heat Sealing Film and PCR reactions were mixed at 650 rpm for 30 sec using the Eppendorf MixMate.

A plasmid clone of allele 3 of the *SLC11A1* $(GT)_n$ promoter polymorphisms, which had been previously cloned and sequenced, was used to optimise the parameters of the genotyping methodology. PCR was carried out on the Eppendorf Mastercycler gradient to determine the optimal annealing temperature of the primers and the optimal magnesium concentration. The annealing temperature was determined with the saturating intercalating dye LCGreen I, as the dye stabilises double stranded DNA, thereby increasing the annealing temperature by 5–10 °C. The annealing temperatures of the gradient ranged from 56–68 °C. The $MgCl_2$ concentration was also determined using a gradient from 1.0–3.0 mM. PCR products were resolved on 1.4 % agarose gels (Sigma-Aldrich, USA). Optimal forward and reverse primer concentrations were determined using a primer matrix in which the forward and reverse primer concentrations were varied from 0.5–9.0 µM. The forward and reverse primer combinations were tested in triplicate and the quantification plots were analysed to determine optimal primer concentrations. These were the reactions in which the C_t was low and the fluorescence at the end of the amplification cycles was high, with the absence of an early plateau phase.

Run No.	Replicates	Homozygenous Allele 2		Homozygenous Allele 3		Homozygenous Allele 2 & 3	
		Temp	Range	Temp	Range	Temp	Range
1	6	88.0	(87.9-88.2)	88.3	(88.2-88.4)	87.6	(87.6-87.8)
2	5	88.0	(87.9-88.2)	88.4	(88.3-88.5)	87.7	(87.5-87.8)
3	5	88.0	(88.0-88.1)	88.4	(88.3-88.5)	87.6	(87.5-87.8)
4	5	88.0	(87.9-88.0)	88.2	(88.1-88.4)	87.6	(87.4-87.8)
5	5	88.0	(87.9-88.0)	88.4	(88.1-88.4)	87.6	(87.4-87.8)
6	5	88.4	(88.4-88.5)	88.9	(88.8-89.0)	87.9	(87.8-88.1)
7	5	87.9	(87.8-88.0)	88.3	(88.3-88.5)	87.5	(87.3-87.7)

Table 1: Genotyping and melting temperature determination of the most common *SLC11A1* $(GT)_n$ promotor polymorphisms.

Once optimised, all PCRs were conducted on the Eppendorf Mastercycler ep *realplex*. PCR was initiated by an initial denaturation (95 °C, 5 min), followed by 40 cycles of denaturation (95 °C, 15 sec), primer annealing (56–68 °C, 15 sec) and extension (72 °C, 15 sec). This was followed by a dissociation step consisting of 95 °C for 15 sec, 60 °C for 15 sec, heating at a rate of 0.4 °C resolution followed by 95 °C for 15 sec. Real-time PCR amplification was assessed using the quantification plot and the melting curves.

Samples that underwent MC analysis on the Eppendorf Mastercycler ep *realplex* were re-tested on a dedicated high resolution melt curve instrument, the HR-1 (Idaho Technology), to compare performance of the Mastercycler ep *realplex* and also to validate results.

Optimisation

Assay design and optimisation are essential for the accurate discrimination of different genotypes using MC analysis, as the double stranded intercalating dye used is non-specific and will bind to any double stranded DNA present in the reaction. Therefore, any non-specific amplification, primer dimers or contaminating DNA will bind the intercalating dye, lowering the resolution and sensitivity of the melting profile, thereby preventing the differentiation of alleles.

Several studies suggest that shorter amplicons allow for better discrimination of alleles as the polymorphic region then accounts for a larger portion of the amplicon [2-4]. However, there are several other studies which show that in certain circumstances longer PCR products may be more beneficial [5,6]. Thus, the primer sets used were designed to be interchangeable to allow the production of a range of different amplicon sizes (127-208 bp) to determine the optimal amplicon length for this application.

The shortest amplicon size (127 bp), in which the $(GT)_n$ repeat was located centrally, gave the most consistent results and the best discrimination between the different genotypes. The optimal primer annealing temperature and magnesium concentration were determined to be 64.5 °C and 2 mM, respectively. From the primer matrix experiments, it was determined that the optimal primer concentration for the 127 bp amplicon was 6.0 µM for the forward primer and 9.0 µM for the reverse primer. The annealing and extension times were initially 30 sec, but were subsequently shortened to 15 sec as there was no qualitative difference in the amplification achieved by using the shorter time interval. This also significantly reduced the length of time required to perform a real-time PCR experiment, which further facilitates the development of a high-throughput genotyping methodology

***SLC11A1* Genotyping using Melting Curve Analysis**

After the parameters of the PCR reaction were optimised, the reproducibility of the methodology was assessed. The three most common *SLC11A1* promoter genotypes (homozygous allele 2 or allele 3, or heterozygous), which account for greater than 95 % of the total promoter allele frequencies, were simulated using previously cloned and sequenced plasmid DNA samples containing either allele 2 or allele 3. These were used individually to mimic homozygosity for alleles 2 or allele 3, and the plasmids were mixed to produce a sample that represented the heterozygous genotype. Representative melting curve profiles for the genotypes tested are presented in Figure 2. To allow the maximum intensity changes to be identified more easily, the raw melting profiles (Figure 2a) were converted to peak curves (Figure 2b). The peak curves are the negative first derivative of the melting curves as a function of temperature. It can be clearly seen from Figure 2b that each of the *SLC11A1* promoter genotypes have different peak maxima.

MC analysis of the 127 bp amplicon of allele 2 and allele 3, using the optimised methodology, was completed in seven individual experiments, in which each of the most common promoter genotypes were tested with a minimum of five replicates (Table 1). The MC genotyping methodology was consistently able to discriminate each of the *SLC11A1* promoter genotypes. There was no overlap between the melting temperature ranges of the different genotypes within each experiment. While the intra-assay variability was very low allowing for the discrimination of the different genotypes, there were however, slight variations between each of the individual experiments (inter-assay variability).

The samples amplified and melted using the Eppendorf Mastercycler ep *realplex* were subsequently analysed using the HR-1 instrument (Idaho Technologies), which is a dedicated high resolution melting instrument. The samples were melted on the HR-1 at a rate of 0.4 °C/s with a 100 % correlation between the grouping of the *SLC11A1* promoter genotypes obtained using the Eppendorf Mastercycler ep *realplex* to those obtained with the HR-1.

While the Mastercycler ep *realplex* is not a dedicated high resolution melt instrument, like the HR-1 or Lightcycler (Roche), we have shown that the instrument has the sensitivity to detect subtle changes of a few bases between PCR samples. In the current experiment, the Mastercycler ep *realplex* was able to discriminate between the different SLC11A1 $(GT)_n$ genotypes where the amplicons differ by only a single 2 base pair GT repeat unit. The use of MC technology using the Eppendorf instrument may therefore be used to study other microsatellite, insertion deletion polymorphisms or complex genetic regions in a rapid and high throughput fashion that does not require post-PCR analysis. The Eppendorf Mastercycler ep *realplex* is able to discriminate between amplicons that differ by only 2 bases, which also suggests that the instrument is likely capable of having the sensitivity of discriminating single nucleotide polymorphisms. The MC genotyping methodology designed to genotype the *SLC11A1* promoter polymorphism can discriminate greater than 95 % of the promoter genotypes and provides a means for rapid, high throughput and sensitive analysis of these polymorphisms in association studies that will help elucidate the role of *SLC11A1* in the susceptibility to disease.

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Ordering Information Eppendorf

Product	Description	Order no. international	Order no. North America
Mastercycler® ep *realplex*^{4} S	with silver block and 4 emission filters	6302 000.601	950020318
Mastercycler® gradient		5331 000.010	950000015
MixMate®	incl. 3 tube holders: PCR 96, 0.5 mL, 1.5/2.0 mL	5353 000.014	022674226
Heat Sealer		5390 000.024	951023078
twin.tec PCR Plate 96, skirted	25 pcs.	0030 128.648	951020401
Heat Sealing Film	100 units	0030 127.838	0030127838

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