Short Communication

Nobody Is Perfect: Comparison of the Accuracy of PCR-RFLP and KASP™ Method for Genotyping. *ADH1B* and *FTO* Polymorphisms as Examples

(genotyping / PCR-RFLP / KASP™ genotyping assay / accuracy)

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Abstract. DNA genotyping is among the most common analyses currently performed in scientific research. Two high-throughput genotyping techniques are widely used – the "classic" PCR-RFLP and probe-based methods such as TaqMan® PCR assay or KASP™ genotyping. The probe-based techniques are claimed to be more accurate than PCR-RFLP; however, the evidence for this claim is sparse. We have directly compared results of genotyping of two SNPs (rs1229984 and rs17817449) obtained by the PCR-RFLP and KASP™ in 1,502 adult Caucasians. The results were identical in 97.3 % and 95.9 % cases, respectively. Discrepancies (either different results or result obtained with one but not with the other method) were addressed by confirmatory analysis using direct sequencing. The sequencing revealed that both methods can give incorrect results, but the frequency of incorrect genotyping of rs1229984 and rs17817449 was very low for both methods – 0.1 % and 0.5 %, respectively, for PCR-RFLP and 0.1 % and 0.3 %, respectively, for KASP™. These results confirm that the KASP™ technique is slightly more accurate, but it achieves slightly lower call rates than PCR-RFLP. When carefully set up, both PCR-RFLP and KASP™ could have accuracy of 99.5 % or higher.

Introduction

The analysis of single-nucleotide polymorphisms (SNPs) is among the most common laboratory procedures currently used in molecular genetic research. The methods for genotyping have developed distinctly over the last few decades. The radioactively labelled probes (Southern blotting) (Southern, 1975) were very expensive and the whole procedure was time- and material-consuming. When the polymerase chain reaction (PCR) method was described (Saiki et al., 1985), it became the gold standard for all subsequent genotyping approaches. The first, and probably still the most commonly used method, is restriction of the PCR product with exact bacterial endonucleases, the polymerase chain reaction – restriction fragment length polymorphism method (PCR-RFLP) (Shi et al., 1999). More recently, "modern" real-time PCR methods, using different modifications of probes hybridizing to the PCR product, are rapidly expanding (Shi et al., 1999; Jenkins and Gibson, 2002). Commonly cited is the TaqMan® PCR assay (Dušátková et al., 2013; Maubaret et al., 2013). Other methods, such as high-resolution melting (Obeidová et al., 2012; Šafaříková et al., 2013), NanoChip electronic microarray (Schrijver et al., 2003) or direct sequencing (Ohmoto et al., 2014; Tomašov et al., 2014) are also based on the analyses of PCR products. These methods are sometimes cheaper (although analysers are usually more expensive), may be quicker (depending on the number of samples and equipment) and are often claimed to be much more accurate than the "classic" PCR-RFLP (Ali et al., 2010). However, exact assessments of the methods' accuracy are sparse and inconclusive (Johnson et al., 2004; Bianchi et al., 2010; Osaki et al., 2011). The PCR-RFLP method remains widely accepted in impact journals (for example Hubáček et al., 2013; Bloudíčková et al., 2014; Drogari et al,. 2014;

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Abbreviations: ADH1B – alcohol dehydrogenase, EDTA – ethylenediamine tetraacetic acid, FTO – fat mass and obesity related protein, PCR – polymerase chain reaction, RFLP – restriction fragment length polymorphism, SNP – single-nucleotide polymorphism.

Ergen et al., 2014; Holmes et al., 2014; Zheng et al., 2013; Yenmis et al., 2015), but most recent meta-analyses or consortia seem to prefer probe-based methods of genotyping (see e.g. Patel et al., 2014).

More recently, the KASP method for genotyping has become popular. The method is based on competitive allele-specific PCR amplification with one universal primer and two allele-specific, differentially labelled primers (FAM™ and HEX™).

In this investigation, we compared the accuracy of these two types of genotyping in a large group of adult individuals, using two SNPs: rs1229984 (G>A; Arg47>His exchange with a significant effect on the activity of the enzyme and associated with alcohol consumption) within the alcohol dehydrogenase gene (*ADH1B*, OMIM acc. No. 103720) and rs17817449 (G>T substitution within the 1st intron of the gene, with a strong impact on body weight; each G allele is associated with a mean of about 1.2 kg of body weight growth) within the fat mass and obesity associated gene (*FTO*, OMIM acc. No. 610966).

Material and Methods

Two polymorphisms (rs1229984 and rs17817449) were genotyped in 1,502 individuals, a random subsample of the Czech branch of the HAPIEE study (Peasey et al., 2006), independently by two methods: PCR-RFLP (for exact details see Hubacek et al., 2008, 2012) and KASP™ genotyping assay (LGS Genomics, Heidelberg, Germany).

Briefly, primers 5′ ACA ATC TTT TCT GAA TCT GAA CAG CTT CTC and 5′ TTG CCA CTA ACC ACG TGG TCA TCT GCG) were used to amplify a 97 bp fragment of the *ADH1B* gene containing the rs1229984 polymorphism. The PCR product was cut with restriction enzyme Hin6I; restriction fragments of 65 bp and 27 bp refer to the common G allele, while the uncut PCR product is characteristic for the allele A.

For PCR-RFLP analysis of the *FTO* rs17817449 variant, primers 5′ GGT GAA GAG GAG GAG ATT GTG TAA CTG G and 5′ GAA GCC CTG AGA AGT TTA GAG TAA ATT GGG were used. This fragment (198 bp) was cut with restriction enzyme AlwNI (the uncut PCR product of 198 bp represents allele G, and restriction fragments of 99 bp and 99 bp allele T).

In both cases, PCR products had not been purified before the restriction, and restriction fragments were separated in 10% polyacrylamide gel using the MADGE electrophoresis (Day and Humphries, 1994).

All used chemicals were produced by Fermentas, Burlington, Canada. Restrictions were performed according to the conditions as recommended by the manufacturer using untreated PCR products.

For the KASP™ genotyping assay, the universal KASP Master mix was added to the DNA samples (http://www.lgcgroup.com/products/kasp-genotyping-chemistry/#.VT-sCmdO7Z4). For the allele-specific amplification of rs1229984 SNP, the common primer 5’ GKT TGC CAC TAA CCA CGT GGT CAT was used with either 5’ ATG GTG GCT GTA GGA ATC TGT CA (allele A specific) or 5’ GGT GGC TGT AGG AAT CTG TCG primer (allele G specific).

For the allele-specific amplification of rs17817449 SNP, the common primer 5’ CTT TGT GTT TCA GCT TGG CAC ACA GAA was used with either 5’ AAG GAG CTG GAC TGT TAA ATT AAA ACC (allele G specific) or 5’ AAA GGA GCT GGA CTG TTA AAT TAA AAC A primer (allele T specific).

Results obtained by different genotyping methods were compared and in the case of discrepancy or when only one method was successful, the PCR product was re-amplified, treated with MinElute PCR purification Kit (Qiagen, Hilden, Germany) and custom-sequenced by GATC Biotech (Konstanz, Germany). Oligonucleotides used for sequencing were as follows: 5′ ACA ATC TTT TCT GAA TCT GAA CAG CTT CTC for *ADH1B* polymorphism and 5′ GGT GAA GAG GAG GAG ATT GTG TAA CTG G for the *FTO* polymorphism. Sequencing results were then compared with the original results.

Results and Discussion

For both polymorphisms, both methods achieved call rates between 97.5 % and 99.3 %. Surprisingly, slightly higher call rates were observed using the PCR-RFLP method for both SNPs.

In the case of the *ADH1B* gene (rs1229984), 97.3 % of the samples were genotyped with identical results (Table 1). For two samples results could not be obtained with any method. Out of the five cases where discrepancies were observed, in four cases direct sequencing confirmed the KASP™ genotyping results and in one case the PCR-RFLP was correct. Eight samples were genotyped successfully with KASP™ but not with PCR-RFLP; of these, four were correct and in four cases sequencing failed. Among 17 cases where the KASP™ genotyping assay failed but which were successfully genotyped by PCR-RFLP, 13 were genotyped correctly, no sample was misgenotyped, and in four cases the sequencing failed.

In the case of the *FTO* gene (rs17817449), 95.9 % of samples were genotyped with identical results (Table 2). For two samples results were not obtained with either

Table 1. Comparison of the results obtained by PCR-RFLP and KASP™ genotyping assay; rs1229984 polymorphism within the ADH1B gene

KASP™	**PCR-RFLP**				
ADH genotype	**AA**	**AG**	**GG**	**Result unclear**	**total**
AA	**4**	0	0	0	4
AG	0	**153**	2	1	156
GG	0	3	**1313**	7	1323
Result unclear	0	11	6	2	19
Total	4	167	1321	10	1502

Table 2. Comparison of the results obtained by PCR-RFLP and KASP™ genotyping assay; rs17817449 polymorphism within the FTO gene

KASP™	PCR-RFLP				
FTO genotype	**GG**	**TG**	**TT**	**Result unclear**	**Total**
GG	**302**	5	0	3	310
TG	1	**693**	2	5	701
TT	1	3	**444**	4	452
Result unclear	1	34	2	2	39
Total	305	735	448	14	1502

method. The results were discrepant in 12 cases; among these, sequencing confirmed that the KASP™ genotyping assay was correct in nine cases and PCR-RFLP was correct in one case (however, see paragraph below). One sample was incorrect both when genotyped by KASP™ and PCR-RFLP, and in one case the sequencing failed. Of 12 samples genotyped successfully with KASP™ genotyping assay but not with PCR-RFLP, four were correct, two were misgenotyped, and in six cases sequencing was not successful. Among 37 cases where the KASP™ genotyping assay failed but which were successfully genotyped with PCR-RFLP, 27 were genotyped correctly, eight were misgenotyped and in two cases sequencing did not provide clear results.

To detect the possible mistakes caused by the "human factor", we searched the archives for results where PCR-RFLP outcomes and KASP™ genotyping assay/sequencing were not identical. We detected one mistake in *ALD1B* and five mistakes in the *FTO* procedures where DNA was correctly genotyped by PCR-RFLP but incorrectly entered in the database (despite that the database was checked three times by three different members of the staff).

The final frequency of incorrect genotyping was very low for both methods – 0.1 % and 0.5 %, respectively, for PCR-RFLP and 0.1 % and 0.3 %, respectively, for KASP™. The total numbers of correct, incorrect and not verified/unclear results are summarized in Table 3 and Fig. 1.

In all cases of discordance between the results, subsequent electrophoretic analysis (0.7% agarose in Tris-EDTA buffer) revealed that all these DNA samples were partially degraded. This suggests that careful processing, storage and/or selection of DNA prior to analysis can further minimize the risk of false results for any genotyping method. In the case of the PCR-RFLP method, a careful quality control through manual allelic detection is particularly important. We hypothesize that some of the discordant results reported in the literature (Johnson et al., 2004; Bianchi et al., 2010; Osaki et al.. 2011) could have been caused by suboptimal PCR or restriction analysis conditions.

Based on the results of this investigation, we conclude that the relatively "old fashioned" PCR-RFLP method is well suited for genotyping DNA samples with very high accuracy, comparable with the "modern" KASP™ genotyping assay.

Table 3. Final summary of correct results obtained by PCR-RFLP and KASP™ genotyping assay. Percentages are calculated for genotyped samples/total number of examined samples.

		Correct		Misgenotyped		Unclear/failed	
		n	**%**	**n**	**%**	**n**	**%**
PCR-RFLP	*ADH1B*	1,485	99.9/98.9	2	0.1/0.1	15	0.0/1.0
	FTO	1,472	99.5/98.0	13	0.5/0.9	17	0.0/1.1
KASP™	*ADH1B*	1,478	99.9/98.4	1	0.1/0.1	23	0.0/1.5
	FTO	1,452	99.7/96.7	4	0.3/0.3	46	0.0/3.1

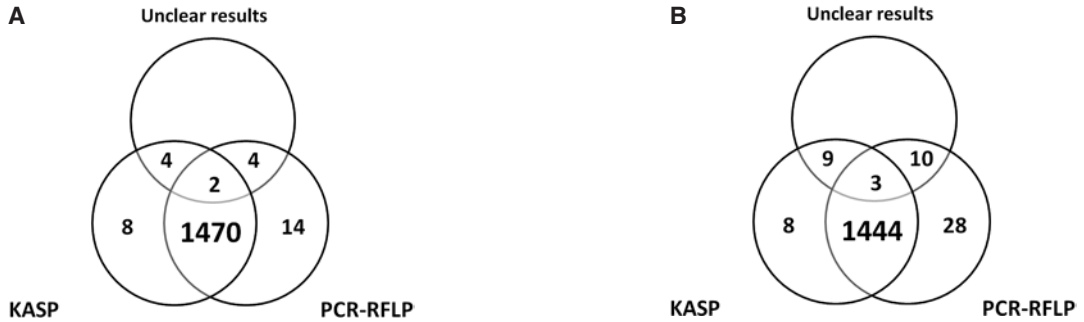

Fig. 1. Summary of the results. Total numbers of correctly genotyped samples by both methods; results confirmed by at least one method and unclear results are summarized under a) for *ADH1B* rs1229984 polymorphism and under b) for *FTO* rs17817449 polymorphism.

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