



Accurate and reproducible SNP analysis using ABsolute™ QPCR mixes

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on behalf of ABgene®

SNP Analysis: Application Note

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Introduction

Single Nucleotide Polymorphisms (SNPs) are mutations that have accumulated throughout our genetic history and are passed onto following generations. Many human disorders and genetic predispositions originate from combinations of such mutations. Identifying these polymorphisms and grouping them has therefore been a major focus in industry and medicine over the last decade.

SNP analysis for known polymorphisms can be performed in numerous ways. A popular technique that facilitates high throughput screening is the 5' nuclease (TaqMan®) assay. Two fluorogenic probes, consisting of sequence-specific oligonucleotides labelled with fluorescent reporter dyes (a different one for each allele), are coupled with quencher dyes and included in the PCR reaction. Each oligonucleotide is designed to be complementary to either the SNP (Probe 1) or the wild type (Probe 2). Amplification of the probe-specific product causes cleavage and degradation of the probe, separating the fluorophore from the quencher and generating an increase in fluorescence. Mismatched probes may still bind loosely to the sequence and will be displaced by the polymerase activity, but they are less likely to actually be degraded and, as a result, less fluorescence will be detected. Therefore alleles with known SNPs can be distinguished from the wild type.

SNP data generated using TaqMan® probes is usually expressed as a scatter plot with the fluorescence intensity of Probe 1 on the x axis and that of Probe 2 on the y axis. A homozygous template containing a SNP on both alleles will produce high fluorescence from Probe 1, whereas a wild type homozygote will produce high fluorescence from Probe 2. A heterozygous template will produce an equal level of fluorescence from each probe. As a result, there will be three distinct clusters representing each genotype

on the scatter plot. As up to 384 samples can be analysed in one run, it is essential that each result falls clearly into one of these groups. The composition of the QPCR Master Mix is essential in producing tight distinct clusters. High levels of dNTPs, MgCl₂ and PCR enhancers are included in ABgene®'s ABsolute™ QPCR Mix to maximise SNP distinction even in GC rich templates. A proprietary additive is also included to facilitate amplification of complex templates such as plant DNA.

Oxagen, a biopharmaceutical company focused on building a novel drugs pipeline based on target identification, compared the performance of ABgene®'s ABsolute™ QPCR ROX Mix with mixes from 3 other suppliers. 10 SNP assays were selected representing allele frequencies of between 2 and 46%. The success rates, allele frequencies and Hardy-Weinberg issues were analysed.

Methods

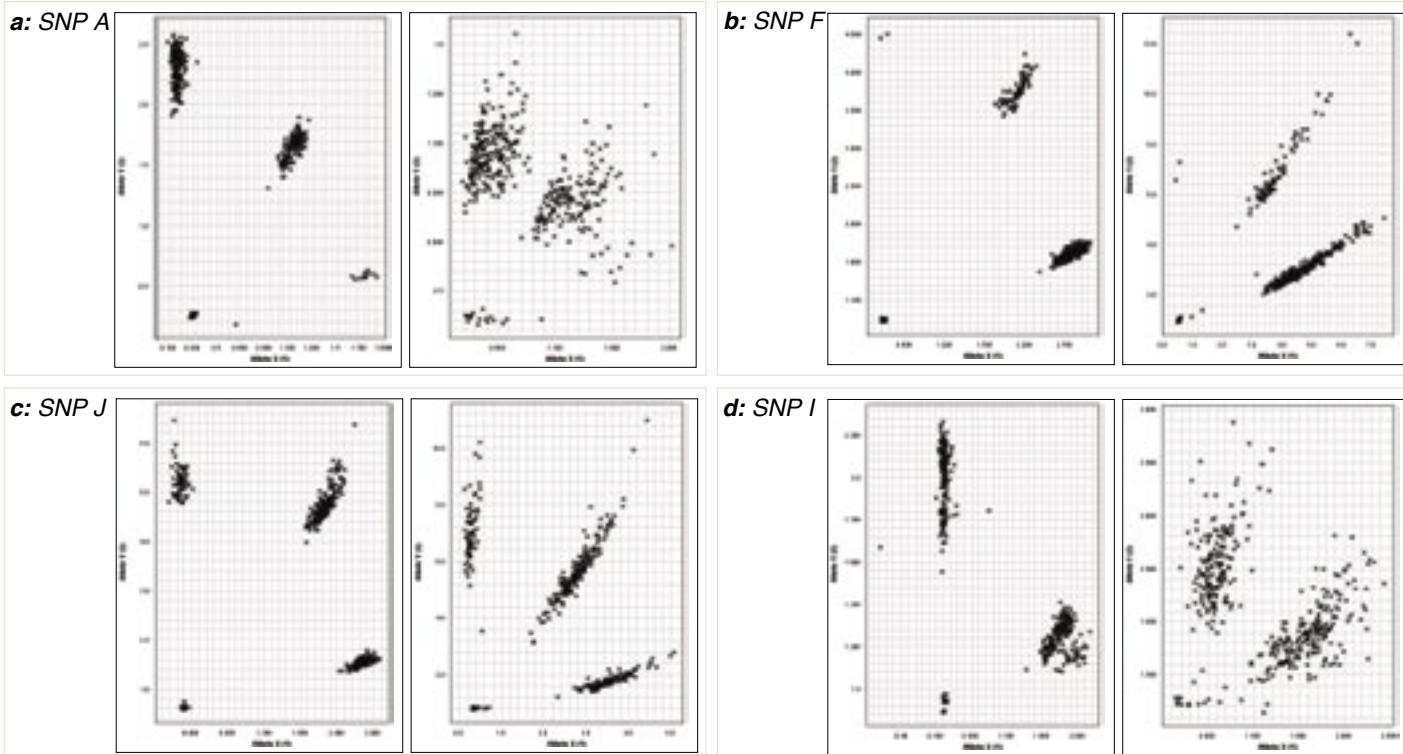
The suppliers chosen for this validation were ABgene®, Supplier 1 and Supplier 2. After the analysis stage of the first run, the master mix from Supplier 1 was excluded due to poor performance and replaced in the second run with the mix from Oxagen's current Supplier A. The data was then compared.

10 assays were selected (Assays-by-Design, Applied Biosystems) representing allele frequencies of between 2 and 46%. These 10 assays were then amplified across 1x 384 well plate containing DNA from a European Caucasian control population using the 3 chosen master mixes for each run (10 plates per run). PCR was carried out using MJ Tetrads following a TaqMan® 2-step protocol. Readings were then taken on the Applied Biosystems 7900HT and the results analysed using SDS 2.0 software. Success rates, allele frequencies and Hardy-Weinberg issues were analysed using the in-house BIAS software.

Table 1: SNP assay data. Allele frequencies and success rates are averages of 4 identical reactions carried out in 4 quadrants of the 384-well PCR plate. Grey cells indicate failed assays.

Assay	Supplier 1		Supplier 2				Supplier A		ABgene®			
	FIRST RUN		FIRST RUN		SECOND RUN		SECOND RUN		FIRST RUN		SECOND RUN	
	Success	Allele Freq	Success	Allele Freq	Success	Allele Freq	Success	Allele Freq	Success	Allele Freq	Success	Allele Freq
A			96.47%	22.16%	99.73%	22.89%	96.74%	22.05%	99.72%	22.90%	98.64%	22.73%
B	98.64%	43.29%	97.28%	42.48%	99.73%	42.90%	98.37%	43.35%	98.37%	42.98%	97.01%	43.25%
D	99.45%	26.77%	99.46%	26.78%	99.18%	26.71%	94.92%	26.59%	97.56%	26.44%	98.10%	26.85%
E	100%	35.70%	99.18%	36.11%	99.73%	35.83%	99.46%	35.92%	99.46%	32.89%	98.64%	35.54%
F	99.19%	11.09%	99.73%	11.03%	99.18%	10.95%	98.92%	11.00%	99.46%	11.08%	99.73%	11.17%
G			99.19%	3.97%	98.10%	3.87%	98.10%	4.13%	97.01%	3.36%	100%	4.08%
H	98.37%	47.38%	98.91%	47.39%					99.46%	47.67%	99.73%	47.55%
J			99.45%	43.44%	98.64%	43.67%	98.91%	43.15%	99.46%	43.45%	100%	43.35%
Av. success over 8 assays	88.68%		98.71%		99.18%		97.32%		98.80%		98.98%	
Av. success between runs	88.68%		98.95%				97.32%		98.89%			

Figure 1: Analysis of four SNPs, each pair of graphs comparing master mixes from ABgene® (left) and Supplier A (right).



Results

Assay Variance – The protocol was carried out on 2 separate occasions and the results compared to check assay variance. Little difference was noted between the 2 runs.

Accuracy – Oxagen’s pass criterion for SNP genotyping is 99% identical genotypes. By using the Duplicate Check function within BIAS it was seen that no contradictory data was generated for any of the assays. However, some genotypes were lost for individual assays based on a slight variation in success rates. Minor allele frequency was also monitored. Any significant change in allele frequency would also indicate genotyping errors.

Recovery – Oxagen’s pass criterion for SNP genotyping is a pass rate of 90% for each SNP assay. In Table 1 the grey boxes represent failed assays. Supplier 1 had three fails; Supplier 2 and Supplier A each had one fail. All other assays gave a success rate of between 94 and 100%.

Two out of the ten assays (SNP C and SNP I) are excluded from Table 1 for the following reasons: First, SNP C is a low frequency SNP and higher than expected readings from negative controls made it difficult to decide where the cut-off lay between negative and low-intensity positive results. Second, SNP I is a high frequency SNP for which only 2 clusters were visible in previous assays using Supplier A’s mix. This result implied that there were no mutant homozygotes in the test population. However, when these assays were run with the ABgene® master mix, three clusters were seen (figure 1d). This was also seen in the second run of Supplier 2’s master mix, demonstrating that there are in fact three genotypes present. Although this 3rd cluster was obviously visible using the ABgene®

mix, it was very close to the heterozygote cluster and, as it was impossible to genotype it accurately, SNP I was excluded.

Of the 8 remaining assays in Table 1, Supplier 1’s Master Mix showed the poorest performance as only 5 out of 8 assays successfully fulfilled the pass rate criterion in the first run. This master mix was subsequently dropped from the validation experiment and Supplier A’s mix was used for the second run.

Both Supplier 2’s Master Mix and ABgene®’s ABsolute™ QPCR ROX Mix performed well in both stages of the validation, but Supplier 2’s mix had one additional assay fail in the 2nd round as compared to the ABgene® mix. The success rate of ABgene®’s ABsolute™ QPCR ROX Mix was 98–99% compared to a 97% success rate from Supplier A. It was also clearly shown that both ABgene®’s and Supplier 2’s mixes showed better clustering than Supplier A’s mix, as they had fewer points outside the clusters.

Summary

From this experiment it can be seen that both the ABgene® and Supplier 2 master mixes provide highly accurate and successful SNP results. Both mixes give better results than Supplier A in terms of cluster quality and differentiation. The ABsolute™ QPCR ROX Mix in particular is better able to distinguish between close clusters, and ABgene® has been chosen as Oxagen’s preferred supplier of QPCR mix for SNP analysis.

Cat. No.	Description	Quantity
AB-1139	ABsolute™ QPCR ROX Mix	200 x 50µl rxns (5ml vial)
Other formats and pack sizes are available - please contact ABgene®.		

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