Determining the Copy Number of Genes Using Real-Time Quantitative PCR

Program #1037/W

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ABSTRACT

Polymorphisms have been classically identified as SNPs, MNPs, indels and STRs. Gene deletion and gene duplication are genomic variations that can also affect protein function or phenotype. Deletions and duplications in drug metabolism genes have been associated with phenotypic variation, for example, they can characterize a phenotype as poor (PM), intermediary (IM), extensive (EM), and ultrarapid metabolizer (UM) [1]. Polymorphisms such as SNPs, MNPs, indels and STRs can be detected using one of many detection methods available (TaqMan® hybridization arrays, allele-specific PCR, sequencing, etc.) Gene copy number, or dosage, can be readily quantitated using real-time quantitative PCR. The method involves relative quantification of the gene of interest versus a reference gene known to be single copy. Relative quantity is determined by the ΔΔCt method [2], where the calibrator is a sample used as the basis for comparative results. Gene copy number is 2 x relative quantity. We have developed assays to measure gene dosage in a variety of genes, including drug metabolism genes such as CYP2D6, CYP2E1, GSTM1 and GSTT1.

INTRODUCTION

During the validation of our TaqMan® Drug Metabolism Genotyping Assays, we observed that SNP assays for some genes had reproducible outlier DNAs or some gDNAs consistently clustered with the no-template-controls (NTCs). Outlier samples can result from other SNPs under the primers or probes, or gene copy number variations. Sequencing of these samples ruled out the presence of underlying SNPs, thus we suspected that the outliers were caused by a gene duplication effect, and samples clustering with the NTCs were caused by a null allele. Gene dosage assays utilizing real-time quantitative PCR on the Applied Biosystems 7900HT Fast Real-Time PCR System and SDS 2.1 software were used to confirm a gene dosage effect by measuring gene copy number in the outlier samples and all the other samples from the SNP assay experiments.

MATERIALS AND METHODS

Primers and probes were designed on genomic DNA template sequence. Ninety-one African American and Caucasian gDNAs (Coriell) were used in the gene dosage assays. These were the same DNAs used in our SNP genotyping studies. Each 20 µl assay contained 10ng of gDNA, 900 nM each of forward and reverse primers for the reference gene (RNaseP) and for the target gene, 250 nM each of the VIC® dye (reference) and FAM™ dye- (target) labeled gene-specific probe in 1X TaqMan® Universal Master Mix. Individual samples were run in quadruplicate. Thermal-cycling conditions (7900HT) were: 2 mins at 50°C, 10 mins at 95°C, followed by 40 cycles of 15 secs at 92°C and 60 secs at 60°C. Real-time data was collected by the SDS 2.1 software. Each replicate was normalized to RNaseP to obtain a ΔCt (FAM dye Ct - VIC dye Ct), and then an average ΔCt for each sample (from the 4 replicates) was calculated. All samples were then normalized to a calibrator sample to determine ΔΔCt. Relative quantity (RQ) is $2^{-\Delta\Delta Ct}$, and copy number is 2 X RQ.

RESULTS

Figure 1. Calculations for determining CYP2D6 copy number

DNA ID	Ave ΔCt	ΔΔCt	RQ ($2^{-\Delta\Delta Ct}$)	copy # (2 x RQ)
NA17120	-1.22	0	1.00	2
NA17104	-1.84	-0.62	1.54	3
NA17107	-0.38	0.84	0.56	1

Figure 1. Copy number is determined by a series of calculations. ΔCt is calculated by subtracting the RNaseP Ct from the CYP2D6 Ct for each replicate. The average ΔCt from the 4 replicates is then calculated. NA17120 was used as the calibrator for the CYP2D6 gene dosage assay, so ΔCt's from all other DNA samples were normalized to NA17102 to determine the ΔΔCt. Relative quantity (RQ) is $2^{-\Delta\Delta Ct}$, and copy number is 2 X RQ.

Figure 2. CYP2D6*17, 1023C>T, T107I (AB Assay ID C__2222771_10)

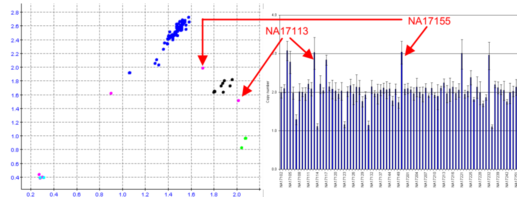

Figure 2. Two DNA samples were consistent outliers in the CYP2D6*17 1023C>T SNP genotyping assay. Primers and probes for the gene dosage assay target exon 9. The real-time assay not only confirms that NA17113 and NA17155 have 3 copies of CYP2D6, but also illustrates that other samples in the DNA panel have 3 copies of CYP2D6. These other 3-copy individuals do not show up as outliers in the genotyping plot because they are homozygous for either allele C or T. Likewise, individuals with 1 copy of CYP2D6 also fall into one of the two homozygous clusters. Four African American DNAs and one Caucasian DNA have one copy of the CYP2D6 gene with the C (major) allele at position 1023. This hemizygous situation may indicate a poor metabolizer phenotype, even with the major allele genotype at the SNP [3]. This exemplifies the need to understand both the SNP genotype and copy number to predict a phenotype.

Figure 3. CYP2E1*7A, -333T>A, rs2070673 (AB Assay ID C___2431871_30)

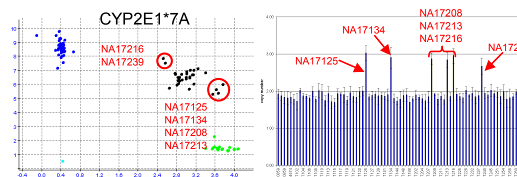

Figure 3. Six DNA samples were consistent outliers in replicate runs of the CYP2E1*7A SNP genotyping assay. In addition, these DNA samples were outliers in the other CYP2E1 SNP genotyping assays. The target for the gene dosage assay was intron 6. The 6 DNAs showing 3 copies in the dosage results were the same samples that were outliers in the SNP assay. Two other CYP2E1 dosage assays that targeted the promoter and exon 4 produced the same gene copy number results (data not shown), thus we believe the entire CYP2E1 gene is amplified.

Figure 4. GSTM1, rs2071487 (AB Assay ID C__26020680_10)

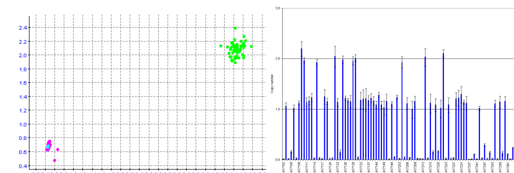

Figure 4. 46% of the Caucasian DNAs and 22% of the African American DNAs from our panel did not amplify in replicate runs of this GSTM1 SNP genotyping assay. The gene dosage assay targeting the intron 6 and exon 7 boundary confirmed that these individuals were homozygous for the deletion. The frequencies for deletion are close to those reported in literature, 54% and 28% for Caucasians and African Americans, respectively [2]. Note that the majority of the individuals we tested have only 1 copy of the GSTM1 gene, and thus all are homozygotes.

Figure 5. GSTT1; 505C>T, I169V (AB Assay ID C___8717770_20)

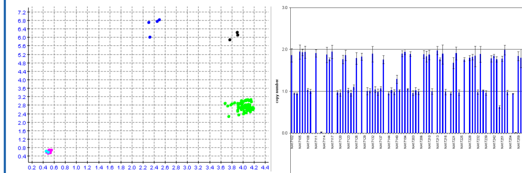

Figure 5. 20% of the Caucasian DNAs and 16% of the African American DNAs (16 individuals total) did not amplify for this GSTT1 SNP assay. A gene dosage assay in exon 3 was designed and results were as predicted – these 16 individuals were homozygous for the deletion. Our results are similar to those found in literature, 24% for Caucasians and 15% for African Americans [4].

CONCLUSIONS

TaqMan® genotyping assays with off-cluster heterozygotes or samples consistently clustering with the NTCs may be predictive of a gene dosage polymorphism. Quantitative real-time PCR to determine gene copy number can be used to confirm a gene copy number polymorphism. We are currently in the process of developing real-time assays to measure copy number. Genes we are developing assays for include the 4 described here and others.

REFERENCES

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TRADEMARKS/LICENSING

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