Quantitative real-time PCR for gene dosage determinations in microdeletion genotypes

Jonathan Covault, Christine Abreu, Henry Kranzler, and Cheryl Oncken
University of Connecticut School of Medicine, Farmington, CT, USA

BioTechniques 35:594-598 (September 2003)

Phase I and II metabolic enzymes are involved in the metabolism and elimination of a variety of compounds, including potentially harmful components of tobacco smoke. Null deletion mutations of genes encoding two phase II enzymes, glutathione S-transferase θ 1 (GSTT1, 22q11.2), and glutathione S-transferase μ 1 (GSTM1, 1p13.3) are common, with up to 20% and 50% of subjects, respectively, homozygous for deletions at each locus (1). Subjects that are homozygous for deletions of either *GSTM1* or *GSTT1* have been reported to be at an increased risk for several cancers (2–6) and other smoking-related illnesses (7). The prevalence of the heterozygous condition for these loci (i.e., deletion on a single chromosome) has not typically been reported because the nonquantitative end point PCR assays used to investigate these deletions do not differentiate the homozygous-present from heterozygote genotypes (1–7).

Wiebel et al. (8), using a gel-based semiquantitative PCR assay of the *GSTT1* deletion, observed that in vitro *GSTT1* enzyme activity was quantitatively related to gene dosage in a large pedigree. This observation is consistent with measurements of *GSTT1* enzyme activity in larger samples that show subjects clustering into three groups: high conjugators, low conjugators, and non-conjugators (9). Together, these results suggest that the ability to differentiate the homozygous-present genotype (high activity) from the heterozygote genotype (low activity) might be important for studies that correlate clinical phenotypes with *GSTT1* and *GSTM1* genotypes (e.g., for assessing health outcomes related to a variety of environmental exposures including tobacco smoke). Here we report the use of a TaqMan® (Applied Biosystems, Foster City, CA, USA)-based quantitative PCR assay to differentiate

0, 1, or 2 copies of the *GSTT1* or *GSTM1* genes. The method can be readily applied to genotype other similar gene deletions and should be useful for the quantitative investigation of a variety of deletion or trisomy genetic conditions. This method has been previously reported to be capable of identifying transgene copy numbers in transformed plants (10).

Quantitative real-time PCR was carried out using the TaqMan (5'-nuclease) assay system (11–13) with signal from a *GSTT1*- or *GSTM1*-specific probe that is normalized to the signal for a reference autosomal gene [cannabis receptor (*CNI*) or β -2-microglobulin (*B2M*)] using DNA samples from 270 Caucasian subjects of European-American descent. The DNA samples used in this study were originally obtained as controls for genetic studies of substance dependence. All subjects provided IRB-approved written informed consent at the time of blood sample collection for the genetic analysis of purified DNA.

TaqMan probes and primers were designed using the Primer Express® software package (Applied Biosystems) and genomic sequences from the November 2002 freeze of the human genome database (<http://www.genome.ucsc.edu>). We compared primer and probe sequences for target and reference genes to avoid cross-hybridization between oligonucleotides. A Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA) was used to eliminate the selection of products with significant sequence similarity at other genetic loci. Finally, because the utility of the assay depends on similar efficiencies of amplification of the target and reference genes, we verified that the slope of Δ cycle threshold (ΔC_T) versus input DNA was <0.1 over a 100-fold genomic DNA dilution series.

To assay *GSTT1* copy number

relative to the reference gene *CNI*, triplicate PCRs (20 μ L) containing 2 ng genomic DNA, 300 μ M each *GSTT1* primers (forward, 5'-CTGTGGTCC-CCAAATCAGATG-3' and reverse, 5'-TTGGGTCGGCCTTCGAA-3'), *CNI* primers (forward, 5'-CACAGCCATC-GACAGGTACATATC-3' and reverse, 5'-CCTTGGGCCTGGTGACAA-3'), and fluorescent oligonucleotide probes specific for each product containing a 3'-quencher [TaqMan Minor Groove Binder (MGB); *GSTT1*, FAM-TGCC-CTCACAACCAT-MGB (60 nM) and *CNI*, VIC-CCTGGCCTATAAGAGG-MGB (120 nM)] were prepared in 1 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems). Thermal cycling was carried out in 96-well plates with real-time fluorescence detection using an ABI PRISM® 7700 thermal cycler (Applied Biosystems). The samples were incubated for 10 min at 95°C and then cycled 35 times at 95°C for 20 s, followed by 60°C for 60 s. To assay *GSTM1* copy number relative to the reference gene *B2M*, we used 300 μ M each gene-specific *GSTM1* primers (forward, 5'-AAGATTCGT-GTGGACATTTTGG-3' and reverse, 5'-TCTGGATTGTAGCAGATCATGCC-3'), *B2M* primers (forward, 5'-TTGTTTCACTGTCCTGAGGAC-TATTTAT-3' and reverse, 5'-ATGTTA-CTCTGTCAATGTTCTCCACAT-3'), and fluorescent product-specific probes *GSTM1* (FAM-CAGACCATGGACA-ACCA-MGB; 120 nM) and *B2M* (VIC-CTCTAACATGATAACCCTCAC-MGB; 30 nM) using identical thermal cycling conditions as for *GSTT1* primers.

Log-normalized change in fluorescence intensity versus cycle number amplification plots was generated using the ABI PRISM 7700 Sequence Detection Systems (SDS) 1.7 software (Applied Biosystems), and a fluorescence intensity threshold was manually selected in the middle portion of the exponential PCR amplification phase for each assay plate. The cycle number at which the change in fluorescence intensity reaches this threshold is then automatically identified by the software for FAM (*GSTT1* or *GSTM1*) and VIC (*CNI* or *B2M*) fluorescence for each well. Figure 1 illustrates representative TaqMan amplification plots for three samples, each in triplicate, using this assay. Figure 1A contains the amplification profiles for the VIC-*B2M* reference gene

product, and Figure 1B contains the amplification profiles of the FAM-GSTM1 product. The reference gene amplification curves overlap, which indicates nearly equal amplification efficiency and input DNA amount for each well. In contrast, the *GSTT1* curves separate into three distinct groups consistent with 2N-homozygote, 1N-heterozygote, and null genotypes for *GSTM1*. A pattern of poorly clustered triplicates without a sharp exponential rise are typical of TaqMan reactions for samples with very low or no input product targets. The increase in fluorescence seen in late cycles for the null genotypes (Figure 1B) represents nonspecific products generated under the high Mg concentrations used in the TaqMan buffer system to drive high-efficiency amplification. Samples that do not demonstrate a sharp exponential rise in the reference gene (VIC) fluorescence before cycle 26 were considered failed amplifications and repeated. The average ΔC_t (e.g., CN1-GSTT1) was calculated for each triplicate from the C_t values for each well. Results for each plate were plotted in a ΔC_t versus ΔC_t graph, generating three distinct clusters of ΔC_t samples. ΔC_t values were scaled for each assay plate to give an average ΔC_t of 1 for heterozygotes (intermediate ΔC_t cluster) to plot results from several plates on a common graph. For *GSTT1*, the raw heterozygote ΔC_t mean ($\bar{x} \pm SD$) for nine

plates was -0.33 ± 1.29 . To assay small sets of unknown samples, a set of known DNAs can be included with each plate to identify the ΔC_t cluster values to facilitate genotype assignment.

Figure 2A is a dot plot of ΔC_t values using this method for *GSTT1* deletion genotyping of personally de-identified genomic DNA from 270 healthy control European-American subjects from Connecticut. Heterozygous *GSTT1* deletion and homozygous *GSTT1*-present DNA samples cluster in two groups with a difference in mean ΔC_t of 0.9, which is consistent with one versus two copies of *GSTT1* target because during the exponential phase, there is approximately a doubling of target sequence for each PCR cycle. Heterozygote samples (1N copy number) reach cycle threshold one cycle later on average compared with homozygous-present samples (2N copy number) (Figure 1B, green vs. red curves), while the average cycle threshold for the reference gene is unchanged. Subjects with the deletion of both *GSTT1* alleles fail to generate an increase in FAM fluorescence until later cycles, if at all (e.g., Figure 1B, blue curves), despite normal amplification of the reference gene target, producing ΔC_t s from -4 to -12. The mean ($\bar{x} \pm SD$)-normalized ΔC_t values for the group of 270 subjects examined were homozygous-present with 1.9 ± 0.14 ; heterozygotes with 1.0 ± 0.15 ;

and double deletions with -10.4 ± 3.9 . The frequencies for the three genotypes in this sample were 0.285, 0.570, and 0.144, respectively, which are consistent with Hardy-Weinberg equilibrium for the observed frequency of the *GSTT1* allele deletion of 0.43 ($\chi^2 = 3.9$; $P = 0.14$).

To assay *GSTM1* copy number, a second reference gene, *B2M*, was used to demonstrate that the assay could be used with alternative reference genes. Figure 2B shows a dot plot of ΔC_t values for *GSTM1* deletion genotyping for the sample of European-American subjects examined for the *GSTT1* deletion (Figure 2A). ΔC_t values again clustered into three groups: homozygous-present with $\Delta C_t = 1.90 \pm 0.15$; heterozygotes with $\Delta C_t = 0.98 \pm 0.17$; and double deletions with $\Delta C_t = -9.1 \pm 2.3$. The frequencies for these three genotypes in the subject sample were 0.054, 0.414, and 0.532, respectively, which are consistent with

Figure 1. Log fluorescence intensity change versus PCR cycle number for the TaqMan quantitative real-time PCR assay. (A) The *B2M* reference gene (VIC probe) amplification profiles from triplicate wells for three DNA samples, illustrating nearly identical cycle thresholds, with average C_t s of 23.4, 23.6, and 23.3 for red, green, and blue tracings, respectively. (B) The corresponding same-well *GSTM1* (FAM probe) amplification profiles, with average C_t s of 22.8, 23.8, and 33.4 for the red, green, and blue tracings. The corresponding ΔC_t values are +0.64, -0.22, and -10.1, respectively, indicating a relative copy number of $2^{\Delta C_t}$ or 1.8, 1, and 0.001 for the three samples, which is consistent with 2N homozygote (red), 1N heterozygote (green), and null genotypes (blue). The dark horizontal line at the midpoint of exponential amplification indicates the threshold intensity value for this plate.

Figure 2. Pooled quantitative PCR data from nine assay plates. (A) Dot plot of the difference in cycle threshold number for *GSTT1* or (B) *GSTM1* relative to a reference gene amplified in the same well (CN1 for panel A or B2M for panel B). Higher concentrations of target DNA generate threshold fluorescence signal after fewer cycles, and lower ΔC_t values (reference gene C_t - target gene C_t) indicate low levels of *GSTT1* or *GSTM1* relative to the reference gene. Heterozygous subjects (1 allele deleted) have a ΔC_t average of 1; homozygous-present subjects have ΔC_t s of 1.6–2.2; while homozygous deletion subjects have ΔC_t values in the -4 to -16 range. The frequency of deleted alleles was 43% for *GSTT1* and 74% for *GSTM1* for this sample of European-American subjects.

Hardy-Weinberg equilibrium for the observed frequency of *GSTM1* allele deletion of 0.74 ($\chi^2 = 0.8$; $P = 0.68$). To validate our results, we also compared them with the currently used method of genotyping *GSTT1* and *GSTM1* null deletions. End point (35 cycles) PCR products from 10 subjects for each of the three *GSTT1* and *GSTM1* genotypes (i.e., homozygous-present, heterozygous, and null samples) were analyzed using ethidium bromide stained gels. All samples that had been assigned the null genotype using the TaqMan assay failed to generate a *GSTT1*- or *GSTM1*-specific PCR product despite co-amplification of the reference gene product (data not shown). The samples that were identified as heterozygote or homozygous-present with the quantitative TaqMan assay gave similar results, each showing a *GSTT1*- or *GSTM1*-specific band as well as reference gene products. The relative intensity of bands for heterozygotes versus homozygous-present samples was not sufficiently different to distinguish

between these two genotypes.

In summary, the closed-tube quantitative dual-label TaqMan PCR assay with same-well normalization to a reference gene provides a robust method for the discrimination of one versus two gene copies at the two loci examined. Only a small number of samples required re-amplification to obtain satisfactory amplification (10 of 270 samples for *GSTT1* and 7 of 270 samples for *GSTM1*). Repeat analysis of 30 randomly selected samples each for the *GSTT1* and *GSTM1* showed a 100% concordant replication. The same assay should be useful for genotyping at other loci where there are deletions and to differentiate two from three gene copies in trisomy conditions by using an increased number of replicates to further reduce the between-sample variation in ΔC_T . Compared to other techniques that have been used to assay relative genetic copy number—Southern blot analysis, competitive PCR, and fluorescent in situ hybridization (FISH)—the quantitative TaqMan assay is technically less demanding, involves less time to complete, and can be easily scaled to examine a few or hundreds of samples. We are currently exploring the use of this method to identify the ends of disease-associated deletions as part of positional cloning strategies in family-based genetic studies.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grant nos. M01-RR06192 [General Clinical Research Center at the University of Connecticut Health Center (UCHC)], 2P50-AA003510-26 (Alcohol Research Center at UCHC), K24-AA13736 (to H.K.), and R01-DA15167 (to C.O.).

REFERENCES

1. Garte, S., L. Gaspari, A.K. Alexandrie, C. Ambrosone, H. Autrup, J.L. Autrup, H. Baranova, L. Bathum, et al. 2001. Metabolic gene polymorphism frequencies in control populations. *Cancer Epidemiol. Biomarkers Prev.* 10:1239-1248.
2. Zhong, S., A.H. Wyllie, D. Barnes, C.R. Wolf, and N.K. Spurr. 1993. Relationship between the *GSTM1* genetic polymorphism and susceptibility to bladder, breast and colon cancer. *Carcinogenesis* 14:1821-1824.
3. Kelsey, K.T., M.R. Spitz, Z.F. Zuo, and J.K. Wiencke. 1997. Polymorphisms in the glutathione S-transferase class mu and theta genes interact and increase susceptibility to lung cancer in minority populations (Texas, United States). *Cancer Causes Control* 8:554-559.
4. Taioli, E., L. Gaspari, S. Benhamou, P. Boffetta, J. Brockmoller, D. Butkiewicz, I. Cascorbi, M.L. Clapper, et al. 2003. Polymorphisms in CYP1A1, *GSTM1*, *GSTT1* and lung cancer below the age of 45 years. *Int. J. Epidemiol.* 32:60-63.
5. Yuille, M., A. Condie, C. Hudson, Z. Kote-Jarai, E. Stone, R. Eeles, E. Matutes, D. Catovsky, et al. 2002. Relationship between glutathione S-transferase M1, T1, and P1 polymorphisms and chronic lymphocytic leukemia. *Blood* 99:4216-4218.
6. Duell, E.J., E.A. Holly, P.M. Bracci, M. Liu, J.K. Wiencke, and K.T. Kelsey. 2002. A population-based, case-control study of polymorphisms in carcinogen-metabolizing genes, smoking, and pancreatic adenocarcinoma risk. *J. Natl. Cancer Inst.* 94:297-306.
7. Wang, X., B. Zuckerman, C. Pearson, G. Kaufman, C. Chen, G. Wang, T. Niu, P.H. Wise, et al. 2002. Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight. *JAMA* 287:195-202.
8. Wiebel, F.A., A. Dommermuth, and R. Thier. 1999. The hereditary transmission of the glutathione transferase hGSTT1-1 conjugator phenotype in a large family. *Pharmacogenetics* 9:251-256.
9. Hallier, E., R. Jager, S. Deutschmann, H.M. Golt, and H. Peter. 1990. Glutathione conjugation and cytochrome P-450 metabolism of methyl chloride *in vitro*. *Toxicol. In Vitro* 4: 513-517.
10. Ingham, D., S. Beer, S. Money, and G. Hansen. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* 31: 132-140.
11. Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-----3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276-7280.
12. Livak, K.J., S.J. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4:357-362.
13. Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams. 1996. Real time quantitative PCR. *Genome Res.* 6:986-994.

Received 22 May 2003; accepted 26 June 2003.

Address correspondence to Jonathan Covault, Department of Psychiatry, MC 2103, UCHC, Farmington, CT 06030-2103, USA. e-mail: covault@psychiatry.uchc.edu