Published in final edited form as:
Chem Res Toxicol. 2012 January 13; 25(1): 216–224. doi:10.1021/tx200457u.

Identification of the *GST-T1* and *GST-M1* Null Genotypes using High Resolution Melting Analysis

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Abstract

Glutathione S-transferases, including *GST-T1* and *GST-M1*, are known to be involved in the phase II detoxification pathways for xenobiotics as well as in the metabolism of endogenous compounds. Polymorphisms in these genes have been linked to an increased susceptibility to carcinogenesis and associated with risk factors that predispose to certain inflammatory diseases. In addition, *GST-T1* and *GST-M1* null genotypes have been shown to be responsible for interindividual variations in metabolism of arsenic, a known human carcinogen. To assess the specific *GST* genotypes in the Mexican population chronically exposed to arsenic, we have developed a multiplex High Resolution Melting PCR (HRM-PCR) analysis using LightCycler480 instrument. This method is based on analysis of the PCR product melting curve that discriminates PCR products according to their lengths and base sequences. Three pairs of primers that specifically recognize *GST-T1*, *GST-M1*, and *β-globin*, an internal control, to produce amplicons of different length were designed and combined with LightCycler480 High Resolution Melting Master Mix containing ResoLight, a completely saturating DNA dye. Data collected from melting curve analysis were evaluated using LightCycler480 software to determine specific melting temperatures of individual melting curves representing target genes. Using this newly developed multiplex HRM-PCR analysis we evaluated *GST-T1* and *GST-M1* genotypes in 504 DNA samples isolated from blood of individuals residing in Zimapan, Lagunera, and Chihuahua regions in Mexico. We found that Zimapan and Lagunera populations have similar *GST-T1* and *GST-M1* genotype frequencies which differ from Chihuahua population. In addition, 14 individuals have been identified as carriers of double null genotype, i.e. null genotypes in both *GST-T1* and *GST-M1* genes. Although this procedure does not distinguish between biallelic (+/+) and monoallelic (+/−) genotypes it can be used in an automated workflow as a simple, sensitive, time and money saving procedure for rapid identification of the *GST-T1* and *GST-M1* positive or null genotypes.

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DISCLOSURE: The Authors declare that they do not have any competing financial interests to disclose.

Keywords

Glutathione S-transferase; High Resolution Melting Analysis; Genotyping; Arsenic

INTRODUCTION

Glutathione S-transferases (GSTs) represent group of enzymes that are involved in the phase II detoxification reactions. Three families of GSTs have been described: the cytosolic GSTs, the mitochondrial GSTs, and the microsomal GSTs (membrane-associated proteins in eicosanoids and glutathione metabolism). These enzymes primarily catalyze the conjugation of reduced glutathione (GSH) to the electrophilic center of endo- or exogenous compounds (e.g., dopamine, prostaglandins, products of lipid peroxidation, chemotherapeuticals, environmental carcinogens). The conjugation with GSH increases solubility and facilitates excretion of these compounds.[1] Apart from GSH conjugation, several other biological functions have been described for different GSTs, such as involvement in biosynthesis of hormones, tyrosine degradation, peroxide breakdown, or dehydroascorbate reduction.[2] GSTs have also been shown to play a role in the metabolism of inorganic arsenic (iAs), a common drinking water contaminant and known human carcinogen. Four classes of cytosolic GSTs (GST-P1, GST-O1, GST-M1, and GST-T1) are suggested to take part in iAs metabolism. GST-O1 and GST-O2 have been shown to reduce monomethylarsonate.[3,4] An increased expression of GST-P1 has been observed in several arsenic-resistant cell lines and linked to the formation of arsenic-glutathione conjugates that are excreted from the cells by the multidrug resistant protein transporters.[5-9] Genetic polymorphism in *GST-T1* and *GST-M1* has been associated with distinct urinary profiles of arsenic metabolites in populations chronically exposed to iAs in drinking water.[10-13]

Epidemiological studies demonstrate that the absence or aberrant activities of GST-T1 and GST-M1 enzymes due to genetic polymorphism render a potential risk for development of multiple diseases such as cancer,[14-19] cardiovascular diseases[20] or diabetes[21,22]. *GST-T1* and *GST-M1* share a common polymorphism represented by a complete absence of the gene, the null genotype. This type of deletion leads to a complete loss of enzymatic activity and is associated with an increased susceptibility of individuals to genotoxic and carcinogenic agents plausibly due to an impaired detoxification capability.[23,24]

Studies examining gene-environment interactions or gene-disease associations require a simple, high-throughput, and reliable analysis of *GST*-null genotypes. As of today, two different procedures are used to identify *GST-T1* and *GST-M1* null genotypes. The most common method is a conventional multiplex PCR.[17,25,26] However, this technique that requires electrophoretic analysis of PCR amplicons is time consuming and cannot provide high throughput needed in large population studies. In addition, the poor detection limit for the ethidium bromide stained PCR amplicons (5-10 ng/band) may yield false-negative results, i.e., subjects can be falsely identified as the null genotype carriers. Another drawback is that ethidium bromide as a potent mutagen requires special handling and disposal. Recently, other techniques using real-time PCR for *GST-T1* and *GST-M1* null genotype evaluation have been implemented.[27-30] Gene specific hybridization probes have been used to identify the carriers of at least one functional *GST-T1* or *GST-M1* allele and to distinguish them from the individuals who are homozygous for the *GST-T1* or *GST-M1* null genotype.[27] A quantitative real-time PCR method has been developed to evaluate the copy number polymorphisms, a trimodal genotype (+/+, +/−, and −/−) for *GST-T1* or *GST-M1*.[28,29] However, like the method that uses gene specific hybridization probes,[27] this technique cannot analyze both genes, *GST-T1* and *GST-M1*, in one reaction vessel.

Recently, a multiplex assay for the simultaneous analysis of the *GST-T1* and *GST-M1* genotype has been described.[30] This technique is based on genotype discrimination by melting curve analysis that uses SYBR Green I, a fluorescence dye known to specifically bind double-stranded DNA (dsDNA). In general, SYBR Green I must be used at sub-saturating concentrations as it can inhibit PCR reaction.[31] Depending on PCR conditions, a low concentration of SYBR Green I may limit detection of multiple amplicons by melting peaks due to a low fluorescent signal[30] or dye migration from small amplicons to large ones ("dye jumping" - the process in which dye from a melted small DNA duplex may be reincorporated into regions of dsDNA which have not yet melted) affecting accuracy of the analysis.[32-35] Availability of completely DNA saturating dyes might overcome some limitations, especially when short fragments are to be amplified. We have developed a high-throughput multiplex genotyping analysis for *GST-T1* and *GST-M1* that is based on High Resolution Melting (HRM) analysis using a completely DNA saturating dye, ResoLight.[36] The HRM analysis is a powerful technique used for detection of mutations, polymorphisms and epigenetic differences in dsDNA samples with a high accuracy.[34,37] This newly developed technique was applied for assessment of specific *GST*'s genotypes in our ongoing cross-sectional studies in Mexico, where association between chronic exposures to iAs in drinking water and prevalence of diabetes is being examined.

MATERIALS AND METHODS

DNA Samples

To develop the HRM assay for *GST-M1* and *GST-T1* null genotypes, we used genomic DNA (gDNA) isolated from primary human hepatocytes obtained from CellzDirect (Invitrogen Corporation, Pittsboro, NC). The culture conditions for hepatocytes were described elsewhere.[38,39] Demographic description of the donors of human hepatocytes is provided in Table 1. DNA has been isolated and purified using QIAamp DNA Mini Kit from QIAGEN following the manufacturer's protocol. Briefly, cultured primary human hepatocytes were digested by Proteinase K. DNA was precipitated by ethanol and purified on silica-based membrane spin column. Quality and yield of DNA was verified on 1% agarose gel and by UV reading at 260 and 280 nm using Eppendorf BioPhotometer.

To validate the HRM assay and to assess its feasibility in population studies, we used gDNA isolated from the whole blood collected from 504 study subjects living in arsenicosis areas in Mexico (136 in Zimapan, 105 in Lagunera, and 263 in Chihuahua). Before blood collection all recruited study subjects were provided with details of the research project and gave written informed consent. All procedures were approved by the Institutional Review Boards of Cinvestav-IPN, Mexico City, Mexico, and University of North Carolina at Chapel Hill, North Carolina, USA.[40] DNA was extracted and purified using DNA Blood Mini Kit (QIAGEN). The final concentration and quality of DNA was determined by reading the absorbance at 260 and 280 nm using Eppendorf Biophotometer.

Conventional Singleplex and Multiplex PCR for GST-M1 and GST-T1 Genotypes

A conventional singleplex (one target gene) and multiplex (three target genes) PCR as described by Liu et al. (2010)[17] were used to determine *GST-M1* and *GST-T1* genotypes in primary human hepatocytes from seven donors and to validate results of the multiplex HRM analysis (95 samples randomly selected from population study representing 18.8% from total of 504 samples analyzed by HRM). The following sets of primers were used to amplify target genes (*GST-M1* and *GST-T1*) and internal control (*β-globin*): *GST-M1* forward primer 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and *GST-M1* reverse primer 5′-CTT GGG CTC AAA TAT ACG GTG G-3′; *GST-T1* forward primer 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and *GST-T1* reverse primer 5′-TCA CCG GAT CAT GGC CAG CA-3′; *β-*

globin forward primer 5′-CAA CTT CAT CCA CGT TCA CC-3′ and *β-globin* reverse primer 5′-GAA GAG CCA AGG ACA GGT AC-3′. The PCR mixture (50 μl total volume) consisted of 25 ng of gDNA, 200 μM of each dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, 0.5 U of Taq polymerase (Invitrogen), 20 mM Tris.HCl (pH 8.4) and 50 mM KCl. PCR reaction was performed using Eppendorf Gradient Thermal Cycler. PCR conditions were as follows: Initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min. The final amplicon extension has been performed at 72°C for 7 min. PCR amplicons were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. The length of amplicons was 215 bp for *GST-M1*, 480 bp for *GST-T1*, and 268 bp for *β-globin*. To ensure the authenticity of the PCR products, individual PCR reaction for each gene was performed under the PCR conditions described above and resulting amplicons were sequenced in the UNC-Chapel Hill Genome Analysis Facility using Big Dye Terminator chemistry. The sequences of PCR fragments were verified using the *GST*'s sequences accessed from NCBI database (NM_000561.3 for *GST-M1*; NM_000853.2 for *GST-T1*; NM_000518.4 for *β-globin*).

HRM Analysis of GST-M1 and GST-T1 Genotypes

Both, singleplex (one target gene) and multiplex (three target genes) HRM analyses were performed using LightCycler480 (Roche), a High Resolution Melting Master Mix (Roche) containing ResoLight (a completely saturating DNA dye), and HPLC purified primers. The primers for amplification of *GST-M1* and *β-globin* were those described above. However, new sets of *GST-T1* primers needed to be designed as the size of amplicon produced by primers used for conventional PCR was too long (480 bp) and in general unsuitable for HRM analysis. Using the Universal ProbeLibrary Assay Design Center (Roche;http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) we designed four new sets of primers for amplification of *GST-T1* which produced amplicons with lengths between 60 – 250 bp. This modification allowed production of a shorter *GST-T1* PCR fragment with lower melting temperature and with melting curve shape distinct from other two target genes. After testing different sets of *GST-T1* primers and several $MgCl_2$ concentrations the best conditions were applied to evaluate results from conventional multiplex PCR. For the amplification of *GST-T1* forward (5′-CCAGGATGTGAGGCTGTTCT-3′) and reverse (5′-GAAACCAACTAAACCTGCAACC-3′) primers producing a 69 bp long PCR fragment were used (Table 2). The PCR reaction (20 μl total volume) consisted of 25 ng of gDNA, 3.5 mM $MgCl_2$, 0.1 μM of each primer (forward and reverse) for *GST-M1* and 0.2 μM of each primer (forward and reverse) for *GST-T1* and *β-globin*, and 1X of LightCycler480 High Resolution Melting Master Mix. Gene Scanning 96-II LightCycler480 program with a touchdown PCR (covering a range of annealing temperature from 65 to 53°C with 0.5°C increments per cycle) and a high resolution melting curve analysis protocol were used to amplify the target genes. After PCR amplification and melting curve analysis, collected data were analyzed using the LightCycler480 "T_m calling" algorithm to determine the presence of melting peaks and their maxima, specific melting temperatures. DNA samples isolated from human hepatocytes with unique *GST's* genotype (Hu0503, Hu230, and Hu0534) were used as positive controls for HRM analysis of *GST-T1* and *GST-M1* genotypes in gDNA samples collected from Mexican study subjects.

RESULTS

Conventional Singleplex PCR Analysis of GST-T1 and GST-M1 Genotypes in Primary Human Hepatocytes

Genomic DNA (gDNA) isolated from primary human hepatocytes obtained from seven individuals (4 males and 3 females) were screened for the presence or absence of *GST-T1* and *GST-M1* genes by conventional singleplex PCR analysis. Demographic data (gender, ethnicity, and age) for all donors of hepatocytes are summarized in Table 1. The primers used for the PCR amplification and length of the amplicons are described in Table 2. The amplification of gDNA for individual target genes showed that only three donors were positive for *GST-M1* while four donors were carriers of null genotype (Fig. 1A). Five were positive for *GST-T1* and two were negative (Fig. 1B) indicating null genotype. The internal control, *β-globin*, was amplified for all donors (data not shown). Sequencing verified authenticity of the PCR products (amplicons) for all three target genes. The amplicon sequences were identical with the corresponding sequences in the NCBI database.

Conventional Multiplex PCR Analysis of GST-T1, GST-M1, and β-globin in Primary Human Hepatocytes

The multiplex PCR analysis was performed side by side with the individual (singleplex) PCR to assure sensitivity and reproducibility of the results and to confirm that no interference between primers would occur. Fig. 2 shows a representative images of multiplex PCR for donors who are carriers of both *GST-T1* and *GST-M1* genotypes (Fig. 2A), carriers of *GST-T1* gene and null in *GST-M1* gene (Fig. 2B), or carriers of *GST-M1* gene and null in *GST-T1* gene (Fig. 2C).

Singleplex and Multiplex HRM Analysis of GST-T1, GST-M1, and β-globin in Primary Human Hepatocytes

All seven samples of primary human hepatocytes were tested for the presence of the three target genes in a singleplex HRM-PCR reaction using the gene-specific sets of primers (Fig. 3A,B,C). A specific melting temperature (T_m) was determined for each gene PCR fragment as maximum of the negative first derivative of the change in fluorescence vs. temperature (−dF/dT, the rate of change of fluorescence). The singleplex HRM confirmed results obtained by the conventional PCR method. Among the seven donors of human hepatocytes, three were carriers of *GST-M1* gene, five were carriers of *GST-T1* gene and internal control, *β-globin*, was amplified for all donors. The calculated melting temperatures for PCR fragments were: 79.8 ± 0.1°C for *GST-T1*, 82.8 ± 0.1°C for *GST-M1*, and 87.3 ± 0.1°C for *β-globin*. Figures 3D, E and F show representative melting peak profiles for the donors who were carriers of both *GST-T1* and *GST-M1* genotypes (Fig. 3D), carriers of *GST-T1* gene and null for *GST-M1* gene (Fig. 3E), and carriers of *GST-M1* gene and null for *GST-T1* gene (Fig. 3F).

HRM Analysis of GST-T1 and GST-M1 Genotypes in the Mexican Population

The HRM analysis was used to genotype gDNA collected from 504 individuals living in the Zimapan, Lagunera or Chihuahua regions in Mexico. Based on our results, Zimapan and Lagunera populations have similar *GST-T1* and *GST-M1* genotype frequencies (Table 3) which differ from the frequencies found in Chihuahua population. The individual null genotypes for *GST-T1* and *GST-M1* are less frequent in residents from Chihuahua (62% and 34%, respectively) than in residents from Zimapan and Lagunera. Almost 5% of the Zimapan and Lagunera residents, but only 1% of the Chihuahua residents, were carriers of null genotypes for both *GST-T1* and *GST-M1*. Representative melting peak profiles obtained by the HRM analysis are shown in Figure 4. Fifteen percent of randomly selected samples

(75 in total) were analyzed also by singleplex HRM analysis (i.e., each target gene was evaluated separately under the same HRM-PCR cycling conditions described in Materials and Methods) to verify the results of multiplex HRM analysis. With exception of one sample results of the singleplex analysis were in agreement with results obtained by the multiplex HRM analysis. This one sample was positive for *GST-T1* and negative for *GST-M1* by the singleplex analysis, but no melting curves for *GST-T1* or *GST-M1* were present in the multiplex analysis. In addition, fourteen samples (out of 504 analyzed samples) that were identified by multiplex HRM analysis to be null for both *GST-T1* and *GST-M1* genotypes gave the same results after re-analysis by singleplex HRM. No PCR amplicons were obtained (no melting curves for *GST-T1*, *GST-M1*, and *β-globin* were present) from three samples of gDNA by either the multiplex or singleplex HRM analyses, suggesting that a poor gDNA quality or presence of an unknown inhibitor(s) might interfere with the PCR amplification. We also re-analyzed 95 samples (18.8% from total) using conventional multiplex PCR analysis. Results were then compared with the results of the multiplex HRM analysis. There were disagreements between these two methods for 24 samples, representing 25% of re-analyzed samples. The differences were due to the lack of amplification in conventional multiplex PCR resulting in missing signal for one or both target genes visualized by ethidium bromide in agarose gel.

DISCUSSION

Most epidemiological studies have assessed specific *GST-T1* and *GST-M1* genotypes using the conventional multiplex PCR technique that requires electrophoretic separation of PCR amplicons in agarose gel.[17,25,26] This approach is time consuming and increases handling errors as multiple steps from the beginning to the end of the process are required. It also produces a large amount of hazardous waste represented by agarose gel and buffers containing a carcinogen, ethidium bromide. Thus, this method is not environment friendly and makes a genotype screening in large-population studies difficult. The rapid development of real-time PCR has introduced multiple techniques and approaches that can be used to examine genetic polymorphisms, including the *GST-T1* and *GST-M1* null genotypes. However, these techniques require expensive hybridization probes or complex calculations of the copy-number variation and cannot determine *GST-T1* and *GST-M1* genotypes simultaneously in one reaction mixture.[27-29]

The High Resolution Melting method is a powerful technique used to analyze genetic variations (SNPs, mutations, DNA methylation) in PCR amplicons employing the melting curve data analysis.[34,37] This technique was developed in collaboration between University of Utah and Idaho Technology, Inc (Salt Lake City, UT) and introduced in 2003.[41] It is a close-tube system for genotyping and mutation analysis that does not require labeled oligonucleotides. The method uses a high data-density acquisition during the melting analysis of PCR fragments with a potential to detect small sequence differences. The HRM analysis requires a fluorescent dye to detect low T_m products and heteroduplexes in DNA during melting. The traditional fluorescent dye SYBR Green I has been also used for real-time PCR and melting analysis. However, it may not work well for the HRM analysis in situations when short PCR fragments are amplified as it results in a weak fluorescence emission, yielding small, hard to detect melting peaks.[30-35] Thus, “saturation dyes” such as LC Green Plus, SYTO9, Eva Green, or ResoLight have been developed to be used with the HRM technique.[33,36] These dyes do not inhibit PCR amplification even at concentrations that completely saturate DNA. The advantage of these dyes is that maximum fluorescence is achieved at the saturation point, providing high sensitivity of HRM analysis.

Here, we employed the principles of the HRM analysis to develop procedure for a simultaneous *GST-T1* and *GST-M1* genotyping in a single reaction vessel. We used

LightCycler480 system and High-Resolution Melting application available from Roche. This system uses ResoLight dye with homogenous staining properties in the target sequence that helps to generate a strong melting fluorescence signal. The overall advantages of this real-time PCR close-tube system is that it avoids end-product contamination, improves amplification efficiency, and provides high specificity and sensitivity. In addition, it allows a high-throughput analysis at a relatively low cost eliminating the need for a post-PCR evaluation of genotype by determining specific melting temperature using the "T_m calling" algorithm which is a part of the LightCycler480 instrument software. The drawback of this method is that it does not determine the copy-number of the target gene, i.e., does not distinguish between a homozygote (+/+) and a heterozygote (+/−).

Using the multiplex HRM analysis in our Mexican cohort, we found that only 8.9% of the study subjects are carriers of the *GST-T1* null genotype. This is one of the lowest frequencies reported for any ethnic group.[42,43] In contrast, the frequency of the *GST-M1* null genotype (34.7%) is similar to those previously reported for other ethnicities (30-70%).[44-47] Interestingly, we found that individuals living in Chihuahua region have significantly lower occurrence of null genotype in both genes than subjects from Zimapan and Lagunera regions. In particular, 5-times more subjects from Zimapan and Lagunera regions were carriers of the *GST-T1/GST-M1*-double null genotype than in Chihuahua. Some studies showed that the *GST-T1/GST-M1*-double null genotype may be associated with even higher risk for development of certain type of diseases (e.g., cancer, liver injury) than a single *GST*s null genotype.[48,49] The majority of Mexicans are represented by "Mestizos", i.e., people of mixed European and Native American ancestry while only ~7% belong to more conserved Mexican tribes.[50,51] Unfortunately, information about the ethnic origin is scarce in Mexico. Recent studies using autosomal Y-STRs (short-tandem repeats linked to the Y-chromosome) to identify human origin showed that Northwest Mexican territory (e.g., Chihuahua) displays European ancestry while Center and Southeast (e.g., Zimapan and Lagunera) Amerindian ancestry.[51-53] Considering the fact of admixed population, a certain level of genetic diversity in different regions of Mexico can be expected. These results suggest that admixture among Mexicans should be considered in studies where gene-environment interaction or gene-disease association is examined for *GST-T1* and *GST-M1* polymorphs.

The sensitivity of the multiplex HRM-PCR analysis is superior to that of the multiplex conventional PCR. When a subset of samples (95 samples in total) was re-analyzed by multiplex conventional PCR and compared with results obtained by the multiplex HRM-PCR analysis we observed 25% differences in genotype. Similar discrepancies between the conventional and real-time PCR (using SYBR Green I) methods were reported by Marin et al.[30] In our study the primer set used for *GST-T1* amplification by conventional multiplex PCR yields a larger PCR fragment (480 bp) than does the set of primers used for multiplex HRM-PCR (69 bp). Thus, reduced efficiency for the amplification of larger fragment might be responsible for falsely identified *GST-T1* null genotypes by conventional multiplex PCR. Even though the same amounts of gDNA were used for the amplification of all samples, the final amount of PCR product for *GST-T1* determined after ethidium-bromide staining in agarose gel vary among the samples (Fig. 1A). The variations may reflect different copy-number of the target gene. In addition, the limited sensitivity of ethidium bromide to visualize certain amount of final PCR product might influence the genotype identification. Similar explanation is possible for the misinterpretation of *GST-M1* genotype when multiplex conventional PCR method is used. However, an excellent agreement between the results obtained by singleplex and multiplex HRM-PCR analysis for DNA samples collected from Mexican population (only 1 sample out of 75 was in disagreement) suggests that multiplex HRM-PCR analysis is a suitable method for *GST-T1* and *GST-M1* genotypes evaluation and might become a method of choice.

Acknowledgments

We acknowledge Peter Bent and Dr. Rupal Cutting from Roche Applied Science in USA for their assistance and support. We also thank to Olivia Dong, a graduate student at the Department of Nutrition, UNC Gillings School of Global Public Health at Chapel Hill, NC, for helping with gel electrophoresis. This work has been supported in part by American Institute for Cancer Research/World Cancer Research Fund (AICR/WCRF) to ZD, by the U.S. EPA/ STAR grant No.832735 and the National Institute of Health grant R01 ES015326-01A2 to MS.

ABBREVIATIONS

HRM	High Resolution Melting
GST-T1	glutathione-S-transferase theta 1
GST-M1	glutathione-S-transferase mu 1
gDNA	genomic DNA
PCR	Polymerase Chain Reaction
dsDNA	double-stranded DNA
HPLC	High-Performance Liquid Chromatography
$\mathbf{T_m}$	melting temperature

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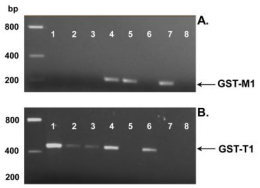

Fig. 1. *GST-M1* and *GST-T1* genotypes in primary human hepatocytes

Results of the conventional singleplex PCR analysis of *GST-M1* (A) and *GST-T1* (B) for seven donors of primary human hepatocytes: 1 – Hu228; 2 – Hu229; 3 - Hu-230; 4 - Hu0503; 5 – Hu0507; 6 – Hu0531; 7-Hu0534; 8 – Negative Control (no DNA template).

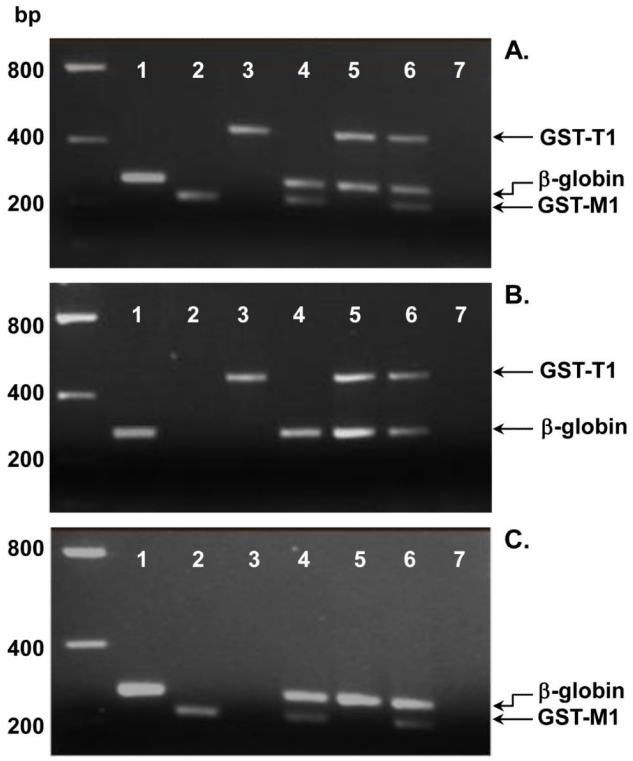

Fig. 2. The conventional singleplex and multiplex PCR

GST-M1 and *GST-T1* genotype in Hu0503 (A), Hu230 (B), and Hu0534 (C) primary human hepatocytes are shown as examples. The conventional PCR was performed using the following sets of primers with sequences described in Material and Methods and summarized in Table 2: 1 – *β-globin*; 2 – *GST-M1*; 3 – *GST-T1*; 4 - *β-globin* + *GST-M1* ; 5 – *β-globin* + *GST-T1*; 6 – *β-globin* + *GST-M1* + *GST-T1*; 7 - Negative Control (no DNA template).

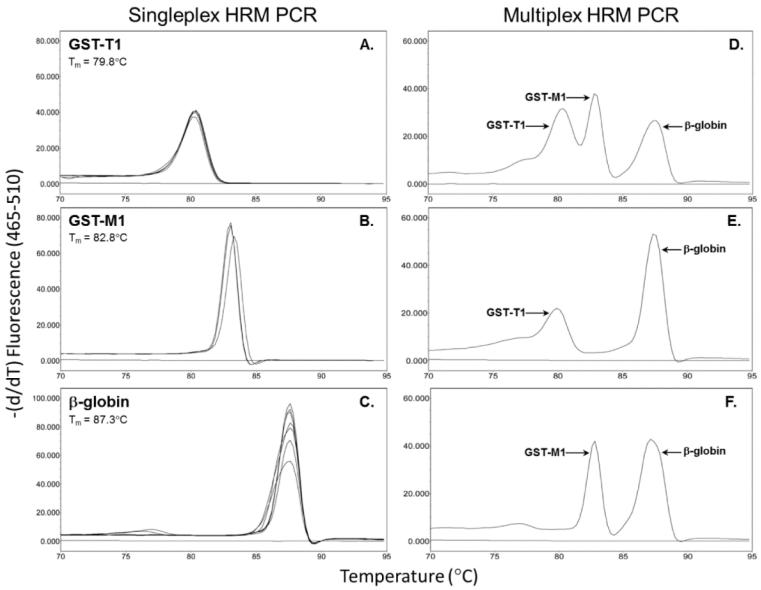

Fig. 3. The singleplex and multiplex High Resolution Melting analysis

Melting peak profiles and melting temperatures (T_m) as determined by "T_m calling" analysis of melting curve for seven donors of primary human hepatocytes. Left side; Singleplex HRM-PCR: (A) – Melting peaks for *GST-T1* with T_m = 79.8°C (N=5); (B) - melting peaks for *GST-M1* with T_m = 82.8°C (N=3); (C) - melting peaks for *β-globin* with T_m = 87.3°C (N=7). Right side; Multiplex HRM-PCR: (D) – melting peak profiles for Hu0503 donor; (E) - melting peak profiles for Hu230 donor; (F) - melting peak profiles for Hu0534 donor.

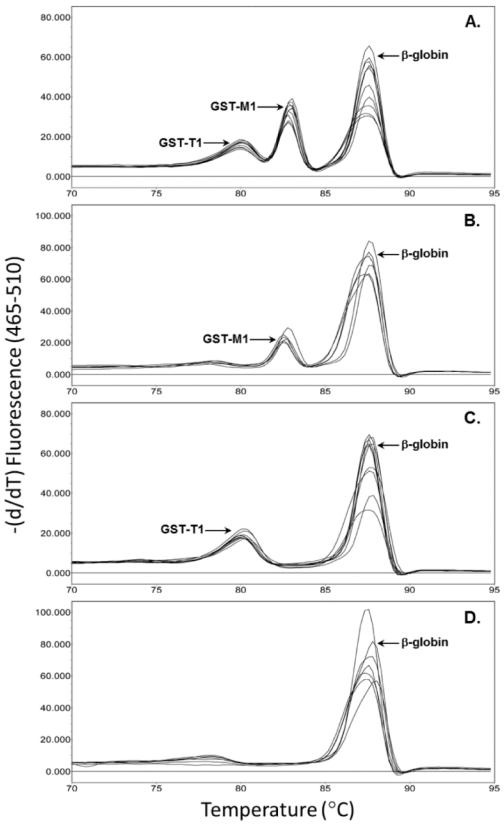

Fig. 4. Representative melting peak profiles for the multiplex HRM-PCR analysis of *GST-T1* and *GST-M1* genotypes in DNA samples collected from the Mexican study subjects

(A) Results for 10 randomly selected samples representing *GST-M1* and *GST-T1* positive genotypes and internal control (*β-globin*); (B) Results for 6 randomly selected samples representing *GST-M1* positive and *GST-T1* null genotype plus internal control (*β-globin*); (C) Results for 10 randomly selected samples representing *GST-T1* positive and *GST-M1* null genotype and internal control (*β-globin*); (D) Results for 7 randomly selected samples representing *GST-T1* and *GST-M1* null genotype and internal control (*β-globin*).

Chem Res Toxicol. Author manuscript; available in PMC 2013 January 13.

Table 1

Demographic description of primary human hepatocyte donors and their *GST-M1* and *GST-T1* genotypes

Human Hepatocytes	Gender	Ethnicity	Age	*GST-M1* genotype	*GST-T1* genotype
Hu228	Male	Caucasian	73	Negative	Positive*
Hu229	Female	Caucasian	39	Negative	Positive*
Hu230	Female	Caucasian	74	Negative	Positive*
Hu0503	Male	Caucasian	64	Positive*	Positive*
Hu0507	Male	Caucasian	51	Positive*	Negative
Hu0531	Female	Caucasian	70	Negative	Positive*
Hu0534	Male	Caucasian	56	Positive*	Negative

*Biallelic (+/+) or monoallelic (+/−) positive.

Table 2
Primers used for conventional and HRM PCR analyses

PCR Analysis	Targeted Gene	Primer Sequence	Size of Amplicon (bp)	Amplicon T_m (°C)
Conventional PCR	*GST-M1*	F: 5′-GAACTCCCTGAAAAGCTAAAGC-3′	215	
		R: 5′-CTTGGGCTCAAATATACGGTGG-3′		
	GST-T1	F: 5′-TTCCTTACTGGTCCTCACATCTC-3′	480	
		R: 5′-TCACCGGATCATGGCCAGCA-3′		
	β-globin	F: 5′-CAACTTCATCCACGTTCACC-3′	268	
		R: 5′-GAAGAGCCAAGGACAGGTAC-3′		
HRM Analysis	*GST-M1*	F: 5′-GAACTCCCTGAAAAGCTAAAGC-3′	215	82.8
		R: 5′-GTTGGGCTCAAATATAGGGTGG-3′		
	GST-T1	F: 5′-CCAGGATGTGAGGCTGTTCT-3′	69	79.8
		R: 5′-GAAACCAACTAAACCTGCAACC-3′		
	β-globin	F: 5′-CAACTTCATCCACGTTCACC-3′	268	87.3
		R: 5′-GAAGAGCCAAGGACAGGTAC-3′		

F – forward primer; R – reverse primer; bp – base pair; T_m – melting temperature.

Table 3
***GST-T1* and *GST-M1* genotype frequencies in Mexican populations**

Genotype	Zimapan % (N=136)	Lagunera % (N=105)	Chihuahua % (N=263)	Total % (N=504)
GST-T1 (−/−)	13.2 (N=18)	13.3 (N=14)	4.9 (N=13)	8.9 (N=45)
GST-T1 (+/+; +/−)	86.8 (N=118)	86.7 (N=91)	95.1 (N=250)	91.1 (N=459)
GST-M1 (−/−)	42.6 (N=58)	41.9 (N=44)	27.8 (N=73)	34.7 (N=175)
GST-M1 (+/+; +/−)	57.4 (N=78)	58.1 (N=61)	72.2 (N=190)	65.3 (N=329)
GST-T1 (−/−) GST-M1 (−/−)	5.1 (N=7)	4.8 (N=5)	1.1 (N=3)	3 (N=15)

"−/−" – null genotype; "+/+" – biallelic positive genotype; "+/−" – monoallelic positive genotype; N – number of subjects