Appendix S1

Buffer system optimization. The reaction buffer used was based on a previous study of duplex-crossed allele-specific-PCR from our group (28), in which TPMT*3B and *3C were identified successfully using a Platinum® Quantitative PCR Supermix-UDG (Applied Biosystems; Thermo Fisher Scientific, Inc.) and their corresponding primers and plasmids. When Platinum® Quantitative PCR Supermix-UDG was used as a premix in the system, no amplification curves were observed in a TPMT*3B wild-type (WT), mutant (MT) or mixed plasmid reaction system (Fig. S5A) and the same result was obtained from TPMT*3C (Fig. S5B). This indicated that the Platinum® Quantitative PCR Supermix-UDG was not suitable for TPMT*3B or TPMT*3C detection. In order to explore the effects of the reaction agents on the amplification curves, two other reagents were used for the reaction system. One was Premix Ex Taq™ (Probe qPCR; Takara Biotechnology Co., Ltd.), which was used in genotyping of TPMT*2 with CRAS-PCR (19), whereas the other was Premix Ex Taq™ Hot Start (Takara Biotechnology Co., Ltd.). As expected, amplification curves appeared in the Premix Ex Taq™ reagent system, but non-specific amplification also occurred. However, genotypes could still be determined based on the results of amplification curves. Subsequently, Premix Ex Taq™ Hot Start was used to investigate non-specific amplification in the system. In the system containing WT primers, WT, MT or mixed plasmids were added, but no amplification was observed (Fig. S6A-D). In the system containing MT primers, WT, MT or mixed plasmids were added; while the WT plasmid had no amplification, the MT and mixed plasmids had good amplification but no specific amplification (Fig. S6B-E). In the MIX system containing WT and MT primers, WT, MT or mixed plasmids were added, but there was no amplification (Fig. S6C-F). Therefore, gene polymorphisms could not be distinguished from a single reaction tube. To ensure implementation of the experiment, Premix Ex Taq™ (Probe qPCR) was used as the system premix reagent.

In this experiment, three master mixes [1X Platinum® Quantitative PCR Supermix-UDG; 1X Premix Ex Taq™ Hot Start Version and Premix Ex Taq™ (Probe qPCR)] were used for preparation of the reaction system. Distinct experimental results were obtained for the three premix reagents. With 1X Platinum® Quantitative PCR Supermix-UDG, there were no amplification curves that appeared in the reaction system. It was hypothesized that this was because Supermix-UDG can selectively cleave the glycosidic bond of the dU-base, and the Supermix-UDG might not inactivate completely; thus, it was recommended to add uracil glycosylase inhibitors to prevent degradation of the amplification products. In the comparison of Premix Ex Taq™ Hot Start Version with Premix Ex Taq™ (Probe qPCR), the Premix Ex Taq™ (Probe qPCR) contained Tli RNaseH, which is specific for the probe method quantitative PCR (qPCR) reaction. The results revealed that Premix Ex Taq™ (Probe qPCR) had higher amplification efficiency and sensitivity. Non-specific amplification may be due to the thermodynamic driving force of thermophilic DNA polymerase, and a single, base terminal mismatch between the primer and template could easily trigger the non-specific amplification of input DNA with an opposite genotype. With sufficient time and energy, the cause of non-specific amplification could be studied in detail and eliminated from further experiments.

Figure S1. Sequencing chromatograph of wild-type-quality control plasmid for TPMT*3B. The underlined bases show the codon containing TPMT*3B, and the arrow indicates the location of TPMT*3B. TPMT, thiopurine S-methyltransferase.

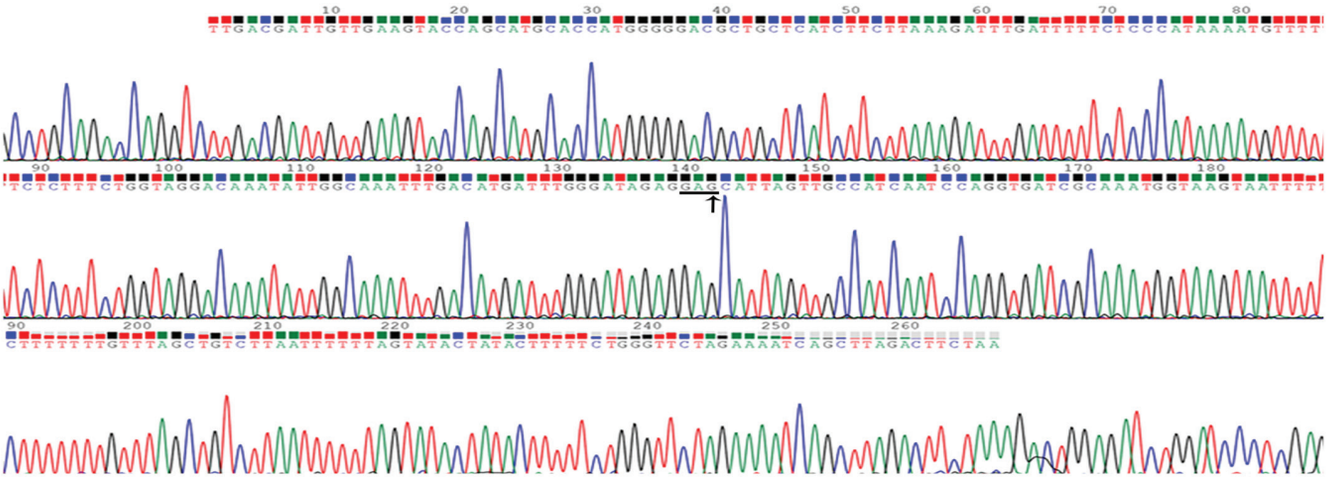

Figure S2. Sequencing chromatograph of mutant-quality control plasmid for TPMT*3B. The underlined bases show the codon containing TPMT*3B, and the arrow indicates the location of TPMT*3B. TPMT, thiopurine S-methyltransferase.

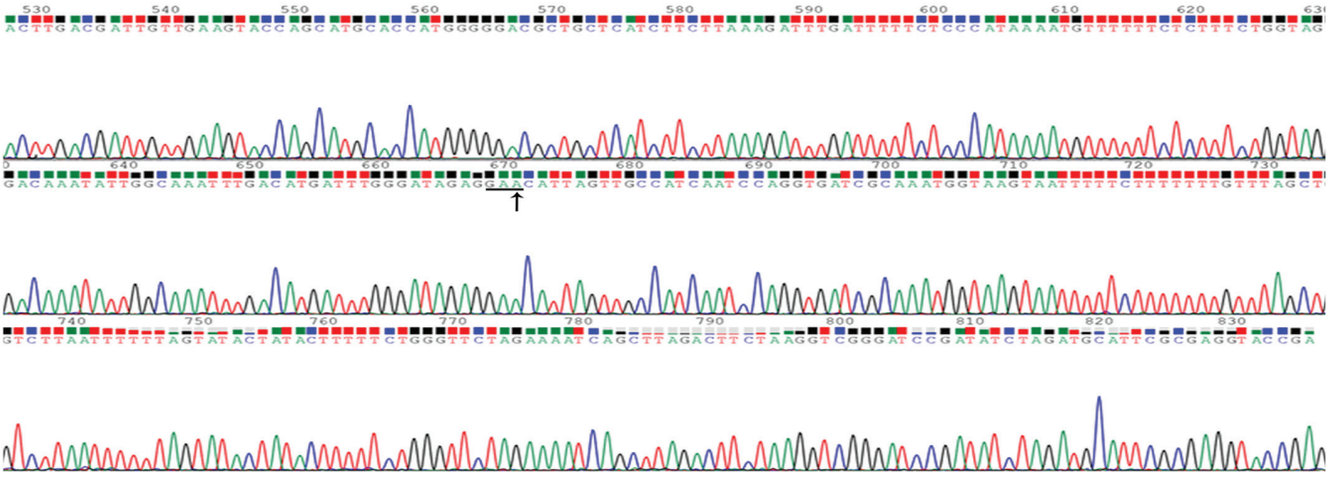

Figure S3. Sequencing chromatograph of wild-type-quality control plasmid for TPMT*3C. The underlined bases show the codon containing TPMT*3C, and the arrow indicates the location of TPMT*3C. TPMT, thiopurine S-methyltransferase.

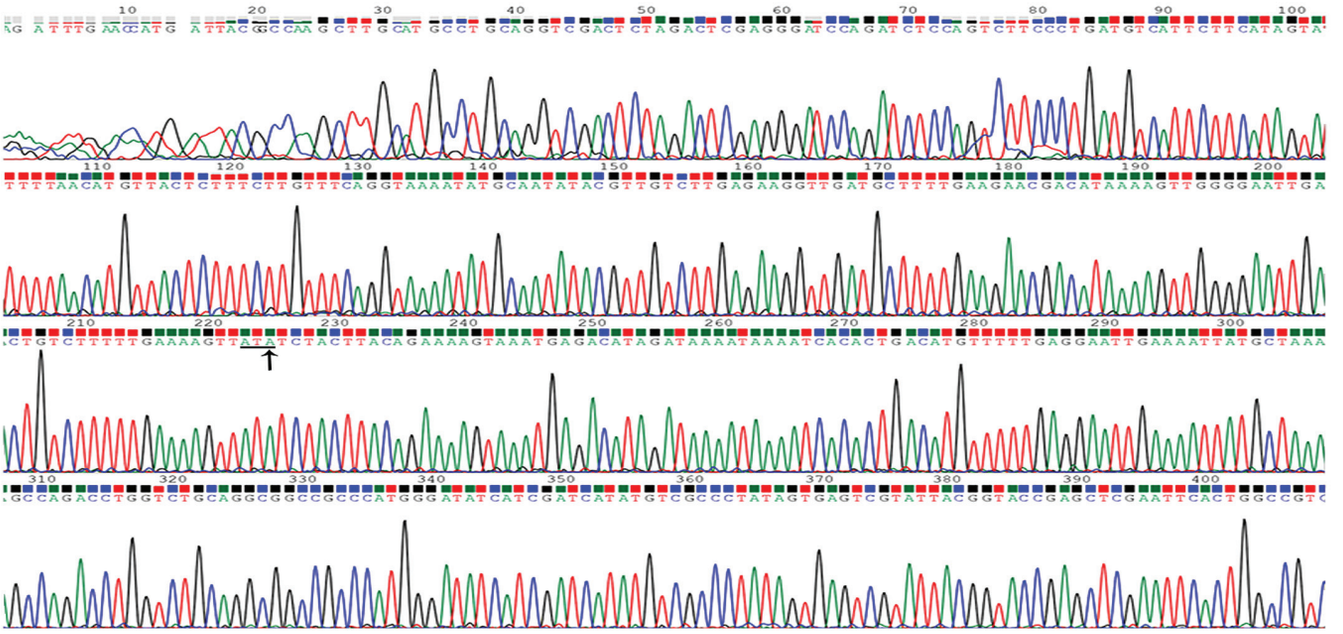

Figure S4. Sequencing chromatograph of mutant-quality control plasmid for TPMT*3C. The underlined bases show the codon containing TPMT*3C, and the arrow indicates the location of TPMT*3C. TPMT, thiopurine S-methyltransferase.

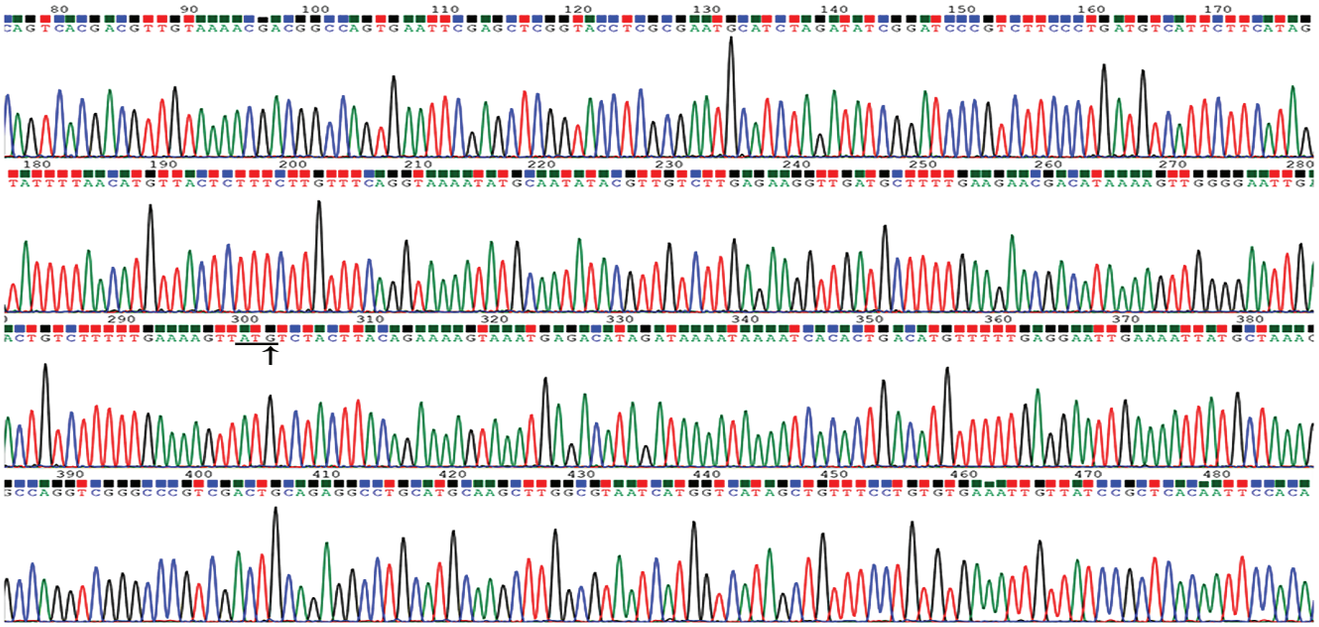

Figure S5. Platinum® Quantitative PCR Supermix-UDG was used as a premix for (A) TPMT*3B and (B) TPMT*3C. TPMT, thiopurine S-methyltransferase.

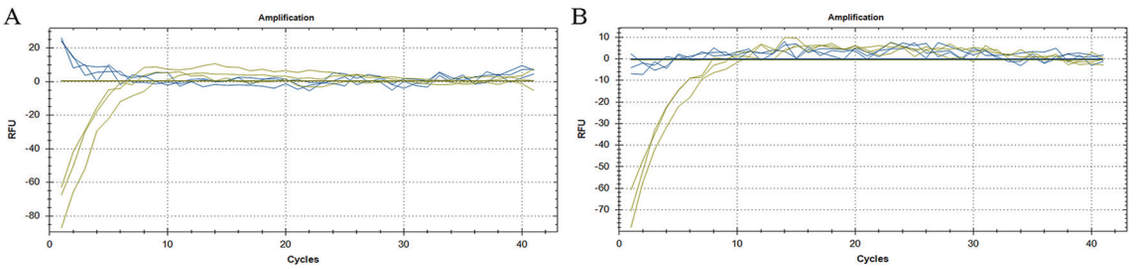

Figure S6. Premix Ex Taq™ Hot Start Version was used as a premix for (A-C) TPMT*3B and (D-F) TPMT*3C.

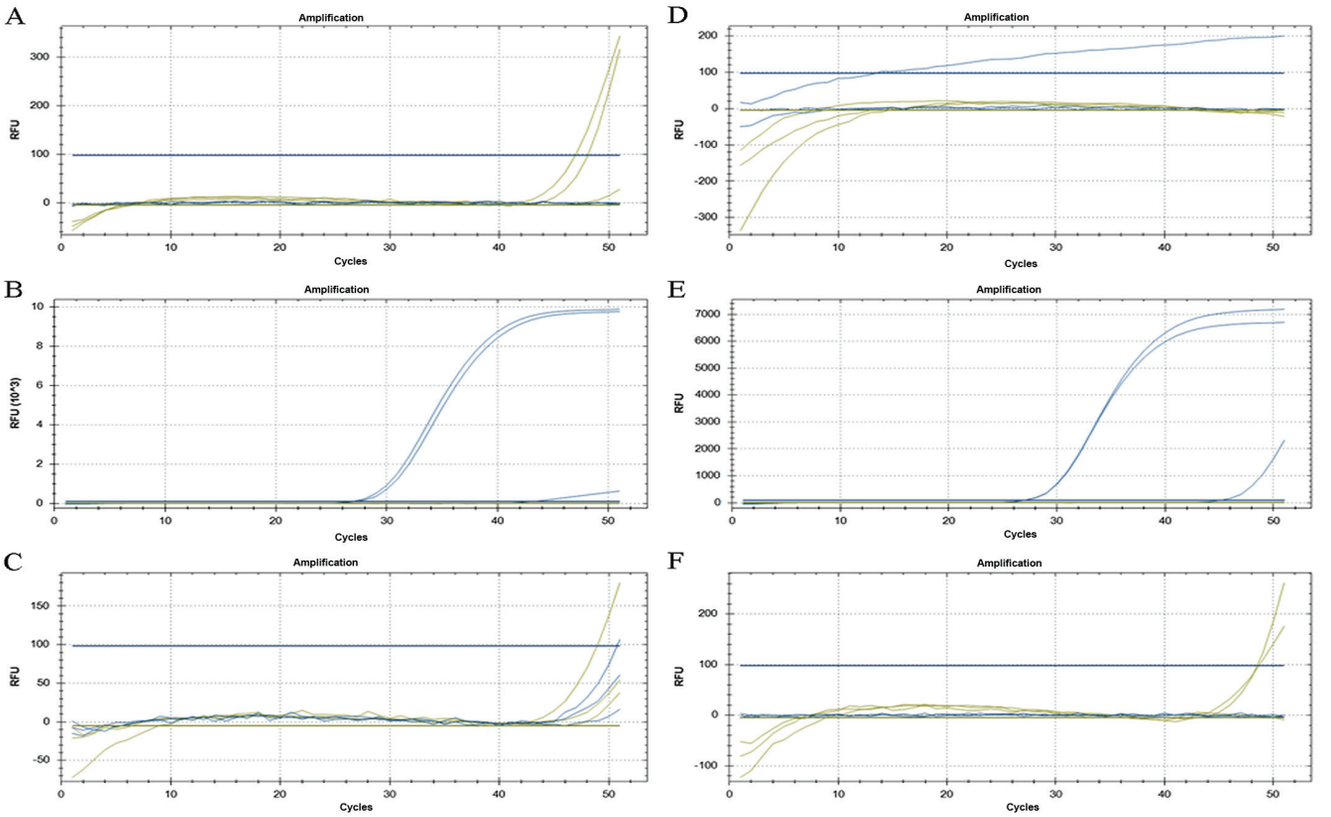

Table SI. Reaction system of TPMT*3B.

Reagents	Final concentration
Premix Ex Taq	1X
HQ-1742 (WT-ASF, VIC)	300 nM
HQ-1743 (MT-ASF, FAM)	300 nM
HQ-1608 (COR)	600 nM
Template	N/A
ddH_2O	N/A

For TPMT*3B, WT-QC, MT-QC or MIX-QC (an equal ratio of WT- and MT-QC plasmids), was used with a total of $1x10^6$ copies. TPMT, thiopurine S-methyltransferase; WT, wild-type; QC, quality control; MT, mutant; ASF, allele-specific forward; COR, common reverse primer.

Table SII. Reaction system of TPMT*3C.

Reagents	Final concentration
Premix Ex Taq	1X
HQ-1744 (WT-ASR, VIC)	300 nM
HQ-1745 (MT-ASR, FAM)	100 nM
HQ-1609 (COF)	400 nM
Template	N/A
ddH_2O	N/A

For TPMT*3C, WT-QC, MT-QC or MIX-QC (an equal ratio of WT- and MT-QC plasmids) was used with a total of $1x10^6$ copies. TPMT, thiopurine S-methyltransferase; WT, wild-type; QC, quality control; MT, mutant; ASR, allele-specific reverse; COF, common forward primer.