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Supplemental Appendix

Development of quantitative nested AS-PCR assays for detection of *BTK^{Cys481}* variants.

Highly sensitive nested-PCR assays were developed to detect known *BTK^{Cys481}* mutations that included *BTK$^{Cys481Ser(c.1635G>C\ and\ c.1634T>A)}$* and *BTK$^{Cys481Arg(c.1634T>C)}$*. A 382 bp fragment with the mutation site was amplified by the primers used in the cloning and sequencing study, and the amplified PCR product was used as template for detection of the *BTK^{Cys481}* mutations by real-time AS-PCR. Three allele specific forward primers were designed to differentiate the nucleotide positions corresponding to the mutant and wild-type alleles for *BTK$^{Cys481Ser(c.1635G>C\ and\ c.1634T>A)}$* and *BTK$^{Cys481Arg(c.1634T>C)}$*. To optimize the specificity, an internal mismatch in the second position from the 3'-end was introduced. The three pairs of AS-PCR primers and the reference primers are listed in **Supplementary Table 1**.

Quantitative detection of the BTK^{Cys481} mutations was achieved using the above described primers with Power SYBR® Green PCR Master Mix used in accordance with manufacturer's instructions for the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR experiments were performed as previously described.[18-20] PCR reactions were performed in a final volume of 25 µL with 25 nM of each primer and 50 ng DNA. Thermal cycling conditions were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each sample was assayed in triplicate. The standard curve for *MYD88^{L265P}* was generated by a serial dilution of the mutant DNA with the wild-type DNA.

The three BTK mutations are all at the site of BTK^{Cys481}. To eliminate the possibility of non-specific cross-reaction, we further tested the three assays using the plasmid template with specific BTK mutations (isolated from the cloning and sequencing study). No cross-reaction was observed for any of the assays.

The real-time AS-PCR developed for $BTK^{Cys481Ser(c.1635G>C)}$ detected this mutation at a dilution of 0.1% with a $\geq$ 2 cycle difference from the wild-type DNA background. The correlation coefficient of the standard curve for this assay was 0.998 and demonstrated a slope value of -3.69. The melting curve analysis revealed that the $BTK^{Cys481Ser(c.1635G>C)}$ mutant-specific amplicon melted at 82.3^0C (**Supplementary Figure 1**).

For $BTK^{Cys481Ser(c.1634T>A)}$, the real-time AS-PCR detected this mutation at a dilution of 0.1% with $\geq$ 2 cycle difference from the wild-type DNA background. The correlation coefficient of the standard curve for this assay was 1.000 with a slope value of -3.92. The melting curve analysis revealed that the $BTK^{Cys481Ser(c.1634T>A)}$ mutant-specific amplicon melted at 81.9^0C (**Supplementary Figure 1**).

For $BTK^{Cys481Arg(c.1634T>C)}$, the real-time AS-PCR detected this mutation at a dilution of 0.8% with $\geq$ 2 cycle difference from the wild-type DNA background. The correlation coefficient of the standard curve for this assay was 0.995 with a slope value of -3.73. The melting curve analysis revealed that the $BTK^{Cys481Arg(c.1634T>C)}$ mutant-specific amplicon melted at 82.8^0C (**Supplementary Figure 1**).

Legend

Supplementary Table 1. AS-PCR primers used for *BTK*^{Cys481} quantitative nested AS-PCR assays.

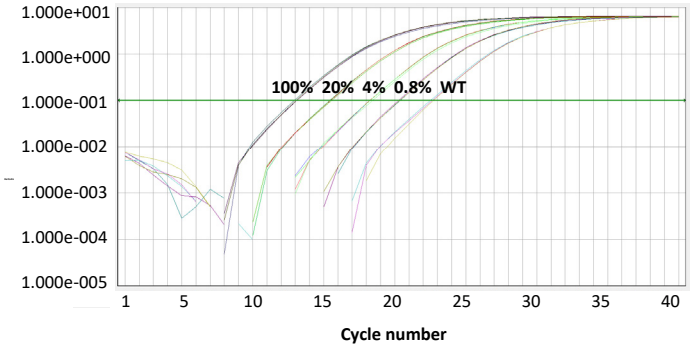
Supplementary Figure 1. Sensitivity and specificity plots of the quantitative nested AS-PCR assays. The amplification plots, dissociation curves, and standard curves are presented for *BTK*$^{Cys481Arg(c.1634T>C)}$(**A**), *BTK*$^{Cys481Ser(c.1634T>A)}$ (**B**), and *BTK*$^{Cys481Ser(c.1635G>C)}$(**C**). Serial dilutions of DNA from the plasmids with mutant *BTK*^{Cys481} were made at the concentrations indicated in the amplification plots. The dissociation curves show the specificities of the assays. The correlation coefficients and slope values for the assays are also presented.

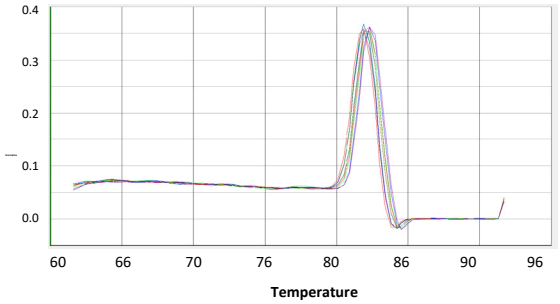
Supplementary Table 1.

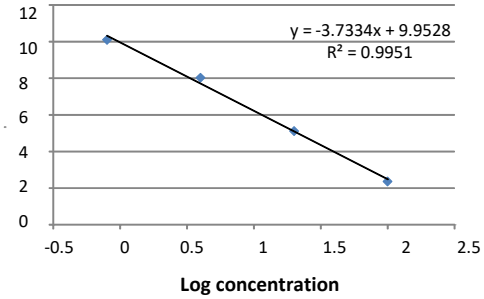
BTK mutations	**AS-PCR primers**
BTK$^{Cys481Arg(c.1634T>C)}$	Forward: 5-ATCACTGAGTACATGGCCAATGGaC-3
	Reverse: 5-TGTCCTAGGCCAATCCTTCTAAG-3
BTK$^{Cys481Ser(c.1634T>A)}$	Forward: 5-CACTGAGTACATGGCCAATGGaA-3
	Reverse: 5-TGTCCTAGGCCAATCCTTCTAAG-3
BTK$^{Cys481Ser(c.1635G>C)}$	Forward: 5-ACTGAGTACATGGCCAATGGCaC-3
	Reverse: 5-TGTCCTAGGCCAATCCTTCTAAG-3
Reference	Forward: 5-CTTCATCATCACTGAGTACATGGC-3
	Reverse: 5-TGTCCTAGGCCAATCCTTCTAAG-3

Supplementary Figure 1.

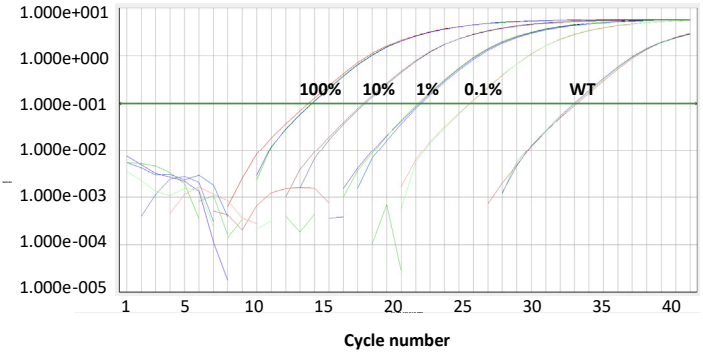
A. ***BTK$^{Cys481Arg(c.1634T>C)}$***

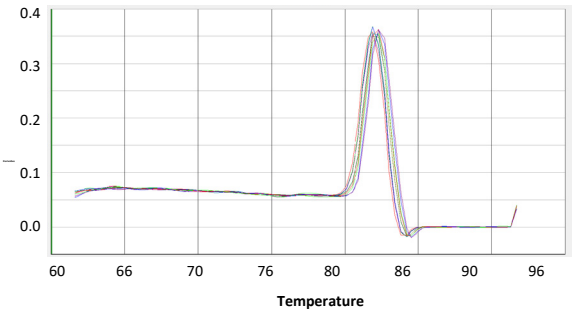


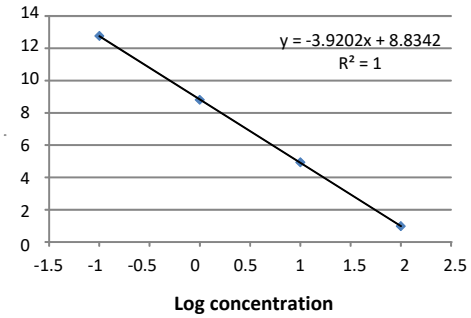




B. ***BTK$^{Cys481Ser(c.1634T>A)}$***







C. ***BTK$^{Cys481Ser(c.1635G>C)}$***