DEVELOPMENT OF AN ULTRASENSITIVE QPCR METHOD TO IDENTIFY AND QUANTIFY POINT MUTATION IN CIRCULATING TUMOR DNA

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Shenglei Yuan[a], Nan Jia[a], Jinping Lai[b], Lan Li[a], Chenpu Zhang[a], Jianbo Diao[a]

[a]WuXi AppTec (Shanghai) Co., Ltd., 288 FuTe Zhong Road, Shanghai, 200131, China

[b]XenoBiotic Laboratories, Inc. 107 Morgan Lane, Plainsboro NJ 08536, USA

PURPOSE

Circulating tumor DNA (ctDNA), belonging to cell-free DNA (cfDNA), refers to tumor-derived DNA fragments found in body fluids. Tracking changes in ctDNA levels in plasma samples of cancer patients enables the assessment of therapeutic outcomes. FDA has recommended the application of ctDNA as a biomarker in clinical trials of cancer patients. However, developing a method with high sensitivity and specificity to discriminate mutant DNA from more abundant wild-type DNA is still challenging. This limits the clinical application of ctDNA quantitative assays.

The purpose of this study is to develop a highly sensitive and specific method for ctDNA analysis and render this method meeting the reliability, accuracy and precision requirement of the bioanalysis in clinical trials. In the present work, we established an ultrasensitive and reliable qPCR method to quantify ultra-low (0.0025%) mutant allele frequencies and provided a data-driven example for future experimental designs on ctDNA bioanalysis of clinical samples.

METHODS

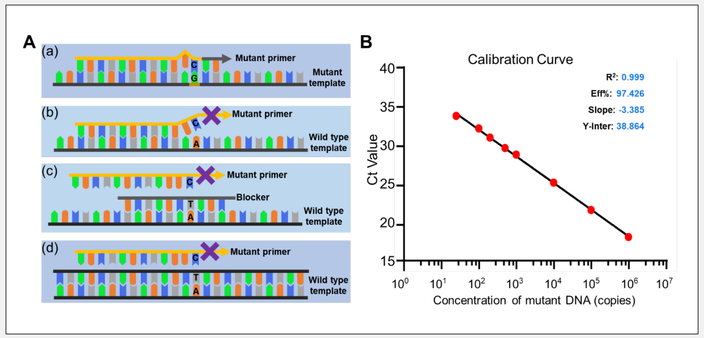
In this assay, the sequence of EGFR with T790M point mutation was used as the target to develop a quantitative method. To achieve accurate quantification, linear DNA with 200 base pairs were designed as the reference standard to prepare calibration standards and quality control samples (QCs). Primers, probes, and blockers were designed specifically against the region of EGFR T790M (Mutation, MT) and EGFR (Wild type, WT). We combined multiple PCR techniques to improve the sensitivity of our assay, including applying mismatch PCR primers, adding WT sequence-specific blockers, and tuning PCR denature temperatures (Figure 1A). The optimized reaction condition was used for testing essential bioanalytical parameters, including accuracy and precision, selectivity, specificity, dilution linearity and stability.

Each reaction contained 1,000,000 copies of WT DNA as the background, except no template control (NTC). A calibration curve consisted of eight concentration levels of MT DNA. NTC and WT-only controls were used to monitor environmental contamination and nonspecific amplification, respectively. Accuracy and precision was evaluated by analyzing 5 QC levels. Selectivity and specificity were evaluated by spiking the mutant DNA into cell-free DNA isolated from six individual plasmas. Stability and dilution linearity were also evaluated.

RESULTS

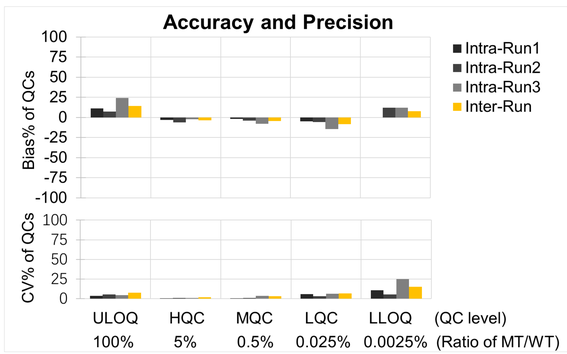
Concentrations of calibration standards range from 25 (LLOQ) to 1,000,000 (ULOQ) copies of MT DNA per 1,000,000 copies of WT DNA. As shown in Figure 1B, the coefficient of determination R2 of the standard curve was 0.999, and the PCR amplification efficiency was 97.426%. As shown in Figure 2, intra- and inter-run accuracy and precision validation reveals a Bias% of within ±25% and a low CV% of less than 25%. The sensitivity defined by LLOQ can reach 0.0025% (the lowest detected ratio of MT DNA in WT DNA). Selectivity and specificity assays indicate that the present method is able to detect MT DNA accurately without matrix inference. Stability tests show that the MT DNA analyte is stable under freeze-thaw and short-term stress conditions. Dilution linearity assay confirms that our method is also applicable for MT DNA of high concentrations.

Figure 1. *The qPCR method scheme of point mutation analysis at high sensitivity and a standard curve*



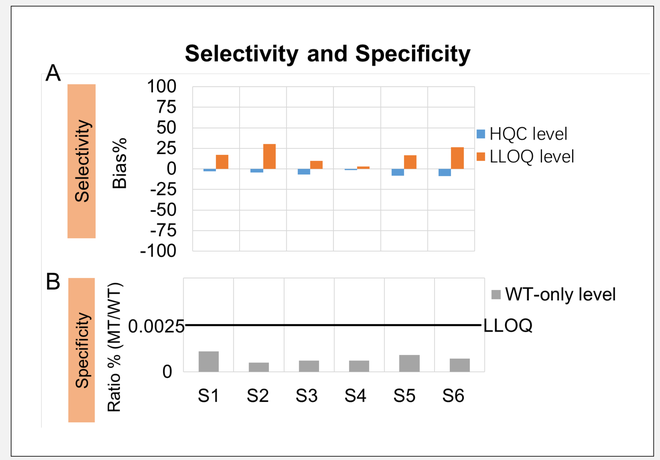
(A) *The qPCR method scheme of ctDNA point mutation analysis at high sensitivity: (a) Mismatch mutant primer could amplify well on mutant template; (b) Mismatch mutant primers could not amplify well on wild type template; (c) Adding wild template specific blocker to block the primer annealing on wild type template; (d) Tuning PCR denature temperature to decrease separation of double strand DNA of wild type template.* ***(B)*** *A standard curve for qPCR amplification of MT DNA: Concentration of standard samples ranged from 25 to 106 copies MT DNA per 106 copies WT DNA. The standard samples were tested in duplicate. The standard curve shows a coefficient R2=0.999, and the PCR amplification efficiency is 97.426%. WT: wild type; MT: mutant.*

Figure 2. *A summary of intra- and inter-assay CV% and Bias% in Accuracy and Precision runs*



Five levels of quality control samples (ULOQ, HQC, MQC, LQC, and LLOQ) were prepared by spiking different levels (from 25 copy to 106 copies) of mutant DNA fragments into 106 copies wild type DNA. Three sets of quality control samples were tested for each of the three Accuracy and Precision runs. Bias% of back calculated concentration of intra- and inter-run are within ±25%. CV% of back calculated concentration of intra- and inter-run are less than 25%. WT: Wild type; MT: Mutant. ULOQ: Upper limit of quantification; HQC: High level quality control; MQC: Medium level quality control; LQC: Low level quality control; LLOQ: Lower limit of quantification.

Figure 3. *A summary of selectivity and specificity assay data*



cfDNA was isolated from 6 individual plasma (S1~S6). ***(A)*** *Selectivity was evaluated by testing spiked MT DNA at HQC and LLOQ levels in cfDNA. The accuracy of MT DNA in presence of WT DNA was within ±10% of the nominal values at HQC level, and within ±35% of the nominal values at LLOQ level.* ***(B)*** *Specificity was evaluated by testing spiked WT DNA (106 copies) in cfDNA using mutant DNA primers. The response of 6 cfDNA samples spiked with WT DNA were all below the LLOQ. cfDNA: cell-free DNA; WT: wild type; MT: mutant. HQC: High level quality control; LLOQ: Lower limit of quantification.*

CONCLUSIONS

The analysis of ctDNA remains challenging as it requires a highly selective, specific and reliable method and lacks detailed regulatory guidance. In the present work, we have demonstrated an ultra-sensitive method using qPCR for the detection of the EGFR T790M mutation. The reliability of the method was validated by performing accuracy and precision, selectivity, specificity, dilution linearity, and stability assays. Our workflow contributes to the development of ctDNA bioanalytical assays, which could facilitate the implementation of ctDNA analysis into clinical diagnosis.