A Highly Specific & Sensitive Single-Well Multiplex KRAS/BRAF Mutation Assay

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Abstract

Anti-EGFR monoclonal antibodies have been used or under trials for treating metastatic colorectal cancer (mCRC), non-small cell lung cancer (NSCLC), and other cancers. However, some KRAS/BRAF activating mutations can render these monotherapies ineffective. A well designed assay for determination of the KRAS & BRAF mutation status is thus critical. Designed around the ICE*Plex* platform*, a multiplex assay (ICE*Plex* KRAS/BRAF Mutation Assay*) was developed, where all primers for detection of the major KRAS (G12S, G12R, G12C, G12D, G12A, G12V, G13S, G13R, G13C, G13D, G13A, G13V) and BRAF (V600E/D) mutations, along with primers for DNA quality, extraction, and internal controls, are included in a single-well format. To reduce the common non-specific and cross-reactive issues with allele-specific PCR, primers were designed with two domains: 1) target-specific core sequences at the 3' end and 2) heterogeneous tail sequence at the 5' end both for increasing the specificity and size differentiation by capillary electrophoresis. To test the assay specificity, individual cell-line DNAs (KRAS G12S, G12C, G12D, G12A, G12V, G13C, and G13D, BRAF V600E, and wild type) or synthetic mutant DNAs (KRAS G12R, G13R, G13S, G13A, and G13V) were included in the assay. All the tested mutants were specifically detected while the wild type was positive only for the DNA-quality control reactions. To test the sensitivity (selectivity), mutant cell-line DNAs were individually tested in the background of wild-type cell-line DNA at a 1:100 mass ratio. Each individual mutant signal was specifically and simultaneously generated with the DNA-quality control signal. Preliminary verification was performed with FFPE specimens with or without information on the mutation status. Evaluation using plasma and serum ('liquid biopsy') specimens is underway. In summary, we have developed a highly specific and sensitive one-well multiplex PCR assay to accurately detect the major KRAS and BRAF mutations. The results presented here demonstrate many benefits of the single-well multiplex format when coupled with automated detection, including conservation of precious clinical specimens, saving on the cost and labor, increase in assay throughput, and reduction in turn-around time.

Technology Overview

ICE*Plex* integrates a PCR thermal cycler that accommodates a standard 96-well PCR plate, a capillary electrophoresis (CE) system with a replaceable CE cartridge, and fluidic pumps with on-board reagents. Equipped with two solid state lasers (488 nm & 639 nm Ex) and a spectrophotometer with a CCD camera, the ICE*Plex* System is capable of detecting two different dyes simultaneously in single well. (Figure 1)

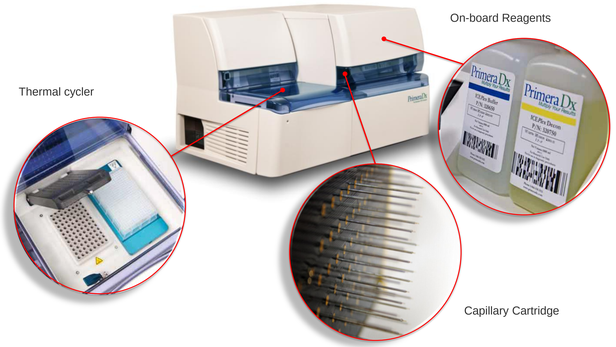

Figure 1. Components of the fully automated ICE*Plex*® real-time PCR/CE system

Unlike other real-time PCR technologies, STAR (**S**calable **T**arget **A**nalysis **R**outine) technology detects and quantifies fluorescently labeled PCR products with unique sizes on the ICE*Plex* System by sequential sampling and separation on a CE system in real time. Multiple quality controls and calibrators can be embedded in the same PCR reaction to ensure the assay quality. The built-in software automatically generates amplification curves by converting the fluorescent signals corresponding to the detected PCR amplicons once the run on ICE*Plex* is complete. The threshold cycle numbers (C_t) for all PCR targets are reported in the final report (Figure 2).

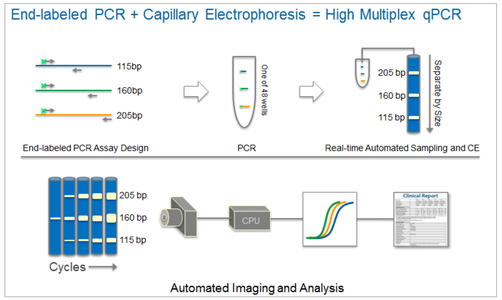

Figure 2. Principles of the multiplex real-time PCR detection on the ICE*Plex* System

Materials & Methods

Primer design: The primers for KRAS and BRAF mutations were designed based on sequence of NT_009714.17 and NT_007914.15, respectively, from the GenBank database of the NCBI. *In silico* specificity of the primers was then performed with Primer-BLAST from NCBI. Tag sequences were designed at the 5' ends by using the following guidelines: (1) the tag sequences should not have homology to the target sequences, and (2) for closely related primers, the tag sequences should not have continuous homology of more than six nucleotides (patent pending). A plug-in program (Cross-Hyb, PrimeraDx, Inc., Mansfield, MA) for the Geneious™ Pro software (Auckland, New Zealand) was used to carry out the primer-primer interaction analysis. Unlabeled and FAM- or TYE-labeled PCR primers were synthesized by IDT (Coralville, IA).

DNAs and FFPE specimens: Cell-line DNA harboring the KRAS G12S (CCL255), G12C (HTB54), G12D (CRL2558), G12A (CCL155), G12V (CCL228), or G13C (CRL5891) mutation, or the BRAF V600E (HTB38) mutation was obtained from ATCC (Manassas, VA), whereas DNA carrying the KRAS G13D mutation (HCT116) or the wild type (K562) was obtained from Zymo Research (Irvine, CA) and Promega, Inc. (Madison, WI), respectively. For mutant targets (KRAS G12R, G13R, G13S, G13A, and G13V) without a cell line source, DNAs containing each respective mutations, along with the Extraction Control DNA, were custom-synthesized at IDT. The Universal Calibrators (Internal Controls) is a product from PrimeraDx. FFPE section samples were obtained from AcroMetrix (LifeTechnologies, Grand Island, NY), Cureline, Inc. (South San Francisco, CA), or OriGene Technologies (Rockville, MD). DNA was extracted from FFPE samples with QIAamp DSP DNA FFPE Tissue Kit from Qiagen (Valencia, CA).

PCR setup: The PCR reactions were conducted with the FastStart Taq kit from Roche, (Indianapolis, IN) and contained 1X Buffer, 1.7 mM $MgCl_2$, 0.8 mM dNTPs, varied primer concentrations, 0.25X Universal Calibrators, and FastStart Taq DNA polymerase (1.6U per 50 µl reaction). The PCR reactions were subjected to thermocycling in a standard 96-well PCR plate on the ICE*Plex* System.

BRAF Mutant & PCR Primers	
Braf-600-R4	CCTATCGAATCAGTGGAAAAATAGC
V600E-AF2	CCGCATTTTGGTCTAGCTACAGA
KRAS Mutant and PCR Primers	
Kras-12-13-F3	CGCCTAGTGTATTAACCTTATGTGTG
G12R-CR2	TCGCTGCCTACGCCACG
G12C-TR	CATTTGTTTGCCTACGCCACA
G12S-AR	CATTATACGAGTTGCCTACGCCACT
G12D-AR	CAATATCTAATGTCCTTGCCTACGCCAT
G12A-CR	ACTATCGACGTCTTGCAACTTGCCTACGCCAG
G12V-TR	CATATAACGGTCGAGGACTCTACTTGCCTACGCCAA
G13R-CR	CTTCGACTTCAACGAAGTTGATGTCTCTTGCCTACGCG
G13S-AR	CAATAATTAATTAACGTATAAATTCCCACTCTTGCCTACGCT
G13C-TR3	CTGTGACAACTTCATTGATCTGTTAAGTATTTCTCTTGCCTACGCA
G13A-CR3	CATCTCACTCATCATTACTAACTAGATAAACTTTAACTCTTGCCTACGG
G13D-AR5	CAAATTCTTTCATAAAATTCAATCTAAGTCATCTTTAACACTCTTGCCTACGT
G13V-TR2	CAATACTTTAGCTTGTCGATACTCAGTACAAATAGTGTTTAGCACTCTTGCCTACGA
Wild-type KRAS Ref Gene Primers	
Kras-Ctrl-F1	CTACACTTCATTAAGATTGGATCCAC
Kras-Ctrl-R1-204	CTCATTCCAAGTGTGTACTACTCCCAA

Table 1. Primer sequences for the KRAS/BRAF mutations and DNA-quality control in the ICE*Plex* KRAS/BRAF Mutation Assay.

Red: Point mutation base; Green: Target-specific sequence; Blue: Tag sequence.

The PCR amplification conditions were as follows:

- 96 ° C for 360 sec.
- **4 cycles**
  - 98° C for 3 sec.
  - 48° C for 10 sec.
  - 68° C for 10 sec.
- **17 cycles**
  - 98° C for 3 sec.
  - 53° C for 10 sec.
  - 72° C for 5 sec.
- **19 cycles**
  - 98° C for 3 sec.
  - 53° C for 5 sec.
  - 72° C for 224 sec (the CE separation stage)

Results

High Specificity of the ICE*Plex* KRAS/BRAF Mutation Assay

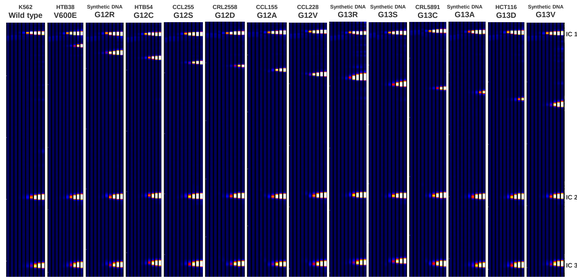

Figure 3. Wild-type or mutant DNA was tested individually with the ICE*Plex* KRAS/BRAF Mutation Assay, where all the 13 mutant and control PCR primers are included in single-tube format. Cell-line DNAs tested include K562, HTB54, CCL255, CRL2558, CCL155, CCL228, CRL5891, HCT116, and HTB38 for wild type, G12C, G12S, G12D, G12A, G12V, G13C, G13D, and V600E, respectively. Synthetic DNAs for G12R, G13R, G13S, G13A, and G13V were also used for the testing since no cell-line DNA could be obtained for these mutations. All the mutant targets were detected from each corresponding mutant DNAs (2 ng per 50 µl reaction) but not from the wild-type DNA even at a significantly higher input (100 ng per 50 µl reaction).

High Sensitivity of the ICE*Plex* KRAS/BRAF Mutation Assay

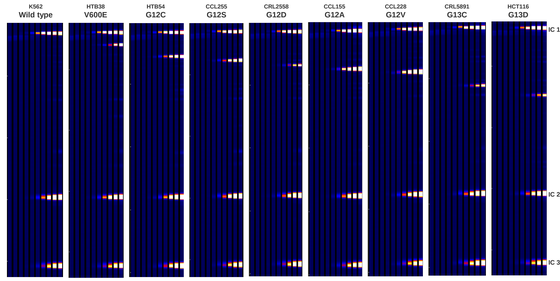

Figure 4. Individual mutant DNA (2 ng) was mixed with the K562 wild-type DNA (100 ng) and tested in 50 µl PCR with the ICE*Plex* KRAS/BRAF Mutation Assay. All mutations were detected at the 1:100 (mutant:wt) mass ratio (since all but one mutant cell line, CCL228, are heterozygous). Based on the C_t values, sensitivity for some targets could be even higher than 1:100.

*** The ICE*Plex* system and ICE*Plex* KRAS/BRAF Mutation Assay have not been cleared or approved for IVD use; information presented is for demonstration only.**

Performance of the ICE*Plex* KRAS/BRAF Mutation Assay on FFPE Specimens

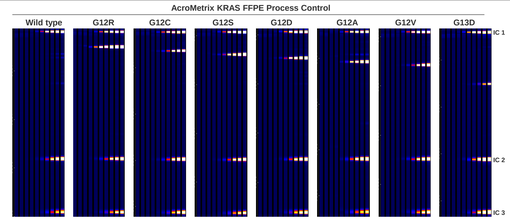

Figure 5. FFPE sections with known KRAS mutation status were obtained from AcroMetrix. After extraction, a portion (5 µl) of the eluate (100 µl) was used per 50 µl PCR for testing on the ICE*Plex* KRAS/BRAF Mutation Assay. All AcroMetrix mutant targets were detected in concordance as expected.

Embedded Controls in the Same Reaction

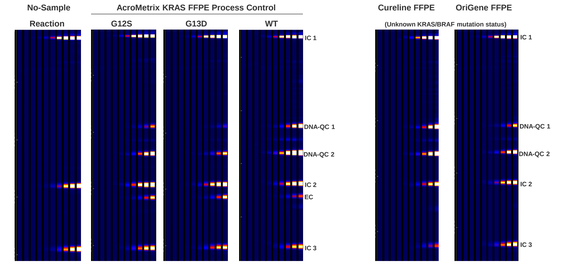

Figure 6. Three kinds of controls are included in the ICE*Plex* KRAS/BRAF Mutation Assay on the TYE Channel (vs. KRAS/BRAF targets on the FAM channel): internal controls (IC, i.e. Universal Calibrators of PrimeraDx) to monitor potential PCR inhibition, DNA quality controls (DNA-QC) to monitor extracted DNA quality (i.e., DNA fragmentation/amplifiable DNA), and extraction control (EC) to monitor the purification process. As expected, only IC amplicons were obtained from the No-Sample control reaction, whereas the EC signal was obtained only when EC DNA was included in the purification process (AcroMetrix FFPE, but not Cureline or OriGene FFPE samples). Intensity of the DNA-QC controls can be used to gauge the amplifiable DNA input.

Summary

- **A multiplex PCR assay performed in a single well (ICE*Plex* KRAS/BRAF Mutation Assay) has been developed for detection of point mutations on the KRAS (G12R, G12C, G12S, G12D, G12A, G12V, G13R, G13C, G13S, G13D, G13A, and G13V) and BRAF (V600E/D) oncogene biomarkers.**
- **The high multiplex ICE*Plex* KRAS/BRAF Mutation Assay includes not only all target KRAS/BRAF mutant PCR primers but also DNAs & PCR primers for various process/assay controls (internal, extraction, and DNA-quality controls) in the same reaction, which can be used to monitor the assay validity.**
- **Pre-verification was performed by using cell-line, synthetic, and FFPE-extracted DNAs. As presented here, the results demonstrate that the ICE*Plex* KRAS/BRAF Mutation Assay is both highly specific and sensitive for discrimination of all the targeted KRAS/BRAF mutations in a high multiplex single-tube format.**

Preliminary evaluation with plasma & serum ('liquid biopsy') specimens has provided excellent results.

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