Highly Sensitive and Specific Single-Tube SNP Assay for Simultaneous Detection of *NRAS* and *BRAF* Mutations

PrimeraDx
The Multiplex PCR Company

Kiran Madanahally Divakar, Vanessa Scialabba, Shuhong Li, Sergey Voronov, Mary Schwab, Gregory Tsongalis, Jessica Riley, Lilly Kong. PrimeraDx, Mansfield, MA 02048, Department of Pathology, Geisel School of Medicine at Dartmouth and Norris Cotton Cancer Center , Hanover, NH 03755

Abstract (revised)

Objective:
Here, we report the development of the *NRAS/BRAF* Point Mutation Analysis Panel, a multiplex PCR assay, which can detect the 12 most clinically important *NRAS* mutations along with 4 other *BRAF* mutations using nucleic acid samples, in a single reaction on the ICEPlex* System.

Clinical Relevance:
The *RAS* genes are proto-oncogenes that are frequently mutated in human cancers and are encoded by three ubiquitously expressed genes: *HRAS*, *KRAS* and *NRAS*. These *RAS* genes have GTP/GDP binding and GTPase activity, and their proteins may be involved in the control of cell growth. *RAS* proteins exhibit isoform-specific functions and in *NRAS*, gene mutations which change amino acid residues 12, 13 or 61 activate the potential of the encoded protein to transform cultured cells with implications in a variety of human tumors, particularly cancers of the skin, blood and lymphoid tissue.

Methodology:
NRAS/BRAF Point Mutation Analysis Panel detection primers were designed using proprietary technology from PrimeraDx. All primers were analyzed *in silico* for primer-primer interaction. Cross-reactivity was determined using the ThermoBlast program, wild type cell line gDNA, and synthetic DNA templates. Reaction conditions were optimized using proprietary PCR chemistry on the ICEPlex® System.

Validation:
The single-reaction *NRAS/BRAF* Point Mutation Analysis Panel targets the 16 most clinically important mutations in the *NRAS* and *BRAF* genes. The assay includes a Reference Gene Controls (RGCs), which serves as the DNA fragmentation control and for calculating of a delta Ct to determine mutation status; and Calibration Controls (C1-3) to determine the size of PCR amplicons. We demonstrate that ICEPlex *NRAS/BRAF* SNP Panel is specific to intended targets.

Conclusions:
The *NRAS/BRAF* Point Mutation Analysis Panel detects the mutation status in a single well reaction. Adoption of this automated multiplex assay may provide a valid tool for future applications in the detection of genomic mutations in cancers.
*ICEPlex is for Research Use Only. Not for clinical diagnostic use.

Technology

The ICEPlex System is a fully automated real time PCR platform that combines an amplification module (thermocycler) and a detection module (a capillary electrophoresis cartridge, two solid state lasers with excitation maximum at 488 nm and 639 nm and a spectrophotometer with CCD camera). All ICEPlex System reagents are kept on board of the platform enabling an easy consumable maintenance (Figure 1).

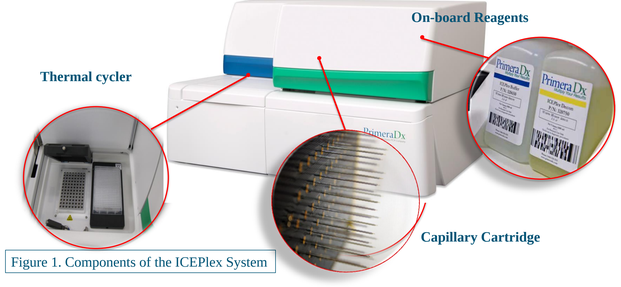


Figure 1. Components of the ICEPlex System

The ICEPlex System generates fluorescently labeled PCR products (amplicons) which are separated based on their different sizes by capillary gel electrophoresis (CE). Amounts of the fluorescent amplicons are monitored in real time by ICEPlex System's software that converts the fluorescent signal into amplification curves and calculates cycle thresholds (Cts) for all PCR targets. The combination of PCR and CE enables simultaneous detection and quantification of multiplex targets in 48 individual reactions in the same manner as traditional real-time PCR methods (Figure 2).

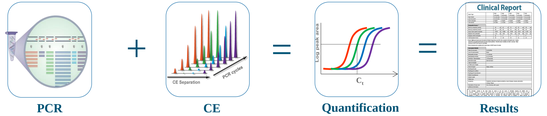


Figure 2. Multiplex real-time PCR detection on the ICEPlex System

Materials and Methods

Primer design: Primers were designed based on the *NRAS* gene sequence and *BRAF* gene sequence available from NCBI. Primers were tested in silico using PrimerBlast (NCBI). Tag sequences were added to the mutant primers such that the tag should not have homology to the target sequence and have less than 6 nucleotide homology to closely related primers (patent pending). Primers were tested for primer-primer interaction in multiplex using the Cross-Hyb plug-in (PrimeraDx Inc., Mansfield, MA) for the Geneious Pro software. (Biomatters Ltd., Auckland, New Zealand). Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA).

Table 1. List of primers designed to generate different sizes amplicons on ICEPlex System

Primer Name	Primer Sequences
NRAS-G12S	5'-TAGGATGGCCAATATTTTTGGTGGTTGGAGCAA-3'
NRAS-G12D	5'-AAGCTTCGTGATTAATAAATTAATGGTGGTTGGAGCAGA-3'
NRAS-G13R	5'-ATCGGACTTCTTAAAATAATAAATTAATTGGTTGGAGCAGGTC-3'
NRAS-G13A	5'-AACTTCTGGGATTTAAAAATATTTTATAATATATGGTTGGAGCAGGTGC-3'
NRAS-G13D	5'-ATCTATATAAAAAAATTAAAAATTAATAAATATTAATAAGGTTGGAGCAGGTGA-3'
NRAS-G13V	5'-ACCATGGTTTTTATTTATTAATATTATTTATTTAATTTTTATAAGGTTGGAGCAGGTGT-3'
NRAS-G13C	5'-TGAGTTACCAAATATTTTATTTATAATATAAAAATTATTAAAATAATATAGGTTGGAGCAGGTT-3'
NRAS-12-13-Intron-R	/56-FAM/AGACAGGATCAGGTCAGCGG
NRAS-Q61K	5'-TCAGAAGGACAATAAAAATACTGGATACAGCTGGAA-3'
NRAS-Q61L	5'-TGGCAGTAGGATAATAATATTAATACTGGATACAGCTGGACT-3'
NRAS-Q61R-1	5'-TGTGGAGATTTAATAATTTTAATATAAATACTGGATACAGCTGGACG-3'
NRAS-Q61R-2	5'-AGAAGGACCGATTAATTAAAATTTTTATATTTATACTGGATACAGCTGGACGG-3'
NRAS-Q61H-1	5'-AAACGCACAATAATTAATAATAAAATAATTTAATTATATACTGGATACAGCTGGACAC-3'
NRAS-61-Intron-R	/56-FAM/AGATCATCCTTTCAGAGAAAATAATGC
V600D-TG/AT-F	5'-GCATATCACATTTTGGTCTAGCTACAGAT-3'
V600E-T/A-F	5'-CCGCATTTTGGTCTAGCTACAGAG-3'
V600E-TG/AA-F	5'-CATACATAGATACATATAAATTTTGGTCTAGCTACAGAA-3'
V600K-GT/AA-F	5'-CATCATGATCAATTGATTTTGGTCTAGCTACAAA-3'

PCR setup and amplification conditions: PCR reactions were carried out in proprietary 1X Multiplex PCR Buffer (PrimeraDx Inc., Mansfield, MA); 0.2uM each primer; 0.25X ICEPlex Calibrator 1 (PrimeraDx Inc., Mansfield, MA); and 1U of Apta Taq Δexo DNA Polymerase (Roche Diagnostics, Indianapolis, IN). Total reaction volume was 25uL and reactions were carried out on the ICEPlex System. PCR amplification conditions were as follow: 96˚ C for 6 minutes, 2 cycles at 50˚ C for 10 sec., 68˚ C for 20 sec. and 98˚ C for 5 sec., 19 cycles at 55˚ C for 10 sec., 72˚ C for 20 sec. and 98˚ C for 5 sec., 19 cycles at 53˚ C for 5 sec., 72˚ C for 230 sec., 98˚ C for 3 sec.

The ICEPlex NRAS/BRAF Panel detects:

CDS Mutation	Amino Acid Residue Change	CDS Mutation	Amino Acid Residue Change
NRAS c.35 G>A	G12D	NRAS c.182 A>G (R1)	Q61R
NRAS c.34 G>A	G12S	NRAS c.182_183AA>GG (R2)	Q61R
NRAS c.38 G>C	G13A	NRAS c.182 A>T	Q61L
NRAS c.38 G>A	G13D	NRAS c. 181 C>A	Q61K
NRAS c.37G>C	G13R	BRAF c.1799 1800 TG>AT	V600D
NRAS c.38 G>T	G13V	BRAF c.1799 T>A	V600E
NRAS c.37 G>T	G13C	BRAF c.1799 1800 TG/AA	V600E
NRAS c.183 A>C(H1)	Q61H	BRAF c.1798 1799 GT>AA	V600K

Results

Multiplex detection of *NRAS/BRAF* Targets on ICEPlex System

4 *BRAF* targets detected in the TYE channel

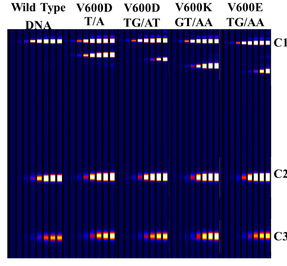
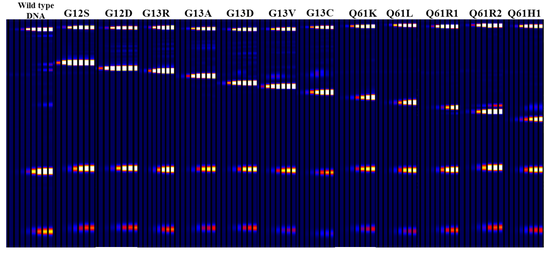


Figure 3. The *NRAS/BRAF* Point Mutation Analysis Panel is capable of simultaneously detecting and differentiating 16 *NRAS/BRAF* mutations. The ICEPlex System can detect PCR amplicons labeled with two different dyes (FAM and TYE). The built-in Calibration Controls (C1, C2 and C3) were used for sizing of the targets in the assay.

12 *NRAS* targets detected in the FAM channel



Development of Reference Gene Controls to be Used as Internal DNA Fragmentation Control

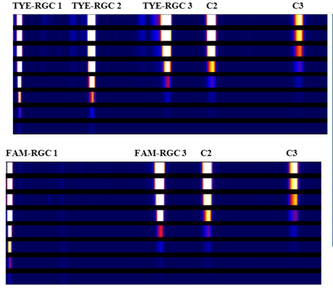


Figure 4. We have developed a Reference Gene Controls (RGCs) for determining the quality of the DNA starting material as well as for calculating the delta Ct. The assay results are based on the delta Ct values.
- Three pairs of primers amplify the Reference Gene Controls on the NRAS gene
  - 3 targets (TYE-RGC 1, 2 and 3) in the TYE channel
  - 2 targets (FAM-RGC 1, 3) in the FAM channel.
- The Reference Gene Controls are used to determine the quality of amplifiable DNA in the sample. They are intended to be included in the same multiplex reaction.

NRAS/BRAF Point Mutation Analysis Panel, Results from Cross Reactivity Study

Synthetic template added to the multiplex PCR reactions	Target detected	Crossreactive on other assay targets
G12D	Detected	Not detected
G12S	Detected	Not detected
G13A	Detected	Not detected
G13C	Detected	Not detected
G13D	Detected	Not detected
G13R	Detected	Not detected
G13V	Detected	Not detected
Q61H1	Detected	Detected at delayed Cts
Q61K	Detected	Not detected
Q61L	Detected	Not detected
Q61R1	Detected	Not detected
Q61R2	Detected	Detected at delayed Cts
V600D TG/AT	Detected	Not detected
V600E T/A	Detected	Not detected
V600E TG/AA	Detected	Not detected
V600K GT/AA	Detected	Not detected
Wild type genomic DNA from cell line K562 (Negative control)	N/A	Not detected

Table 2. The results from the study demonstrated high target specificity of the *NRAS/BRAF* Point Mutation Analysis Panel. Cross reactivity was observed for only two targets.

Conclusions

- A *NRAS/BRAF* Point Mutation Analysis Panel has been developed for the detection of point mutations in the *NRAS* and *BRAF* oncogene biomarkers
- The high multiplex *NRAS/BRAF* Point Mutation Analysis Panel detects not only all target *NRAS/BRAF* mutations but also Reference Gene Controls for DNA fragmentation and delta Ct calculation and Calibration Controls for sizing
- The results from the studies demonstrate that the *NRAS/BRAF* Point Mutation Analysis Panel is highly specific for discrimination of all the targeted *NRAS/BRAF* mutations in a high multiplex single-tube format

ICEPlex System and single-tube *NRAS/BRAF* Point Mutation Analysis Panel have not been approved by the FDA for IVD.
This information is for demonstration purposes only.