Development and Verification of a Multiplex SNP Assay for Detection of 13 cMET Mutations on the ICEPlex System in a Single Reaction

Kiran Madanahally Divakar, Smriti Gupta, Vanessa Scialabba, Jork Nolling, Jessica Riley, and Lilly Kong. PrimeraDx, Mansfield, MA 02048

PrimeraDx
The Multiplex PCR Company

Abstract (revised)

Objective:
Here, we report the development and verification of the ICEPlex* cMET SNP panel, a multiplex PCR assay, which can detect 13 most important cMET mutations using nucleic acid extracted from FFPE samples, in a single reaction on the ICEPlex® System.

Clinical Relevance:
cMET is a proto-oncogene that encodes the Hepatocyte Growth Factor Receptor, a receptor tyrosine kinase, which plays an essential role in normal cellular function and oncogenesis. In cancer cells, cMET has been implicated in cellular proliferation, cell survival, invasion, cell motility, metastasis and angiogenesis. Recent studies have indicated cMET as a biomarker for various cancers as well as for drug resistance in the case of anti-EGFR therapies. The cMET protein highly overexpresses in cancer cells by several mechanisms. One of the mechanisms is via acquired point mutations in the tyrosine kinase domain.

Methodology:
cMET SNP 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 and wild type cell line gDNA and DNA extracted from FFPE samples. Reaction conditions were optimized using proprietary PCR chemistry on the ICEPlex System.

Validation:
The single-reaction ICEPlex cMET SNP Panel targets thirteen most important mutations in cMET gene. The assay includes an internal control, which is used as DNA fragmentation control and for calculation of a ΔCt to determine mutation status; and calibration controls to determine the size of amplicons. Analytical studies demonstrates the assay is selective/sensitive (mutant to wild type ratio), and specific (relative to wild type genomic DNA background).

Conclusions:
We have developed a novel multiplex assay, the ICEPlex cMET SNP Panel, capable of detection of 13 cMET SNP mutations on the automated ICEPlex System in one single reaction. The ICEPlex cMET SNP Panel will be a useful molecular tool for accurate diagnosis of cMET SNP mutations in clinical specimens, which will help in personalized patient management.
***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).

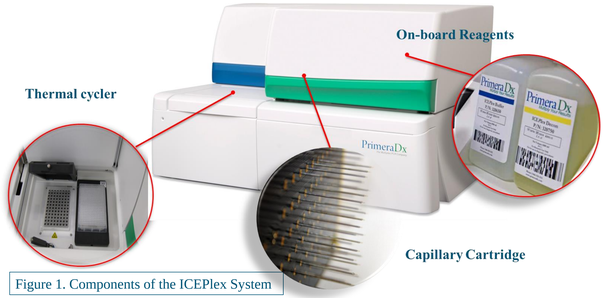

Figure 1. Components of the ICEPlex System

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).

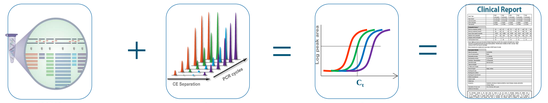

Figure 2. Multiplex real-time PCR detection on the ICEPlex System

Materials and Methods

Primer design: **To enable detection of point mutation from FFPE materials, core amplicon sizes of all the amplicons were less than 110bps. Geneious™ Pro software (Auckland, New Zealand) was used to design reverse or common primers. Primer-primer interaction analyses among different primers *in silico* was carried out using Cross-Hyb (Primera Dx, Inc., Mansfield, MA), and cross-reactivity among primers was determined in silico using ThermoBLAST (DNA Software, Inc.)**

PCR setup and amplification conditions: **The PCR reactions contained: PrimeraDx's proprietary multiplex buffer, 1 U of Apta Taq Δexo DNA Polymerase (Roche Diagnostics, Indianapolis, IN), equimolar concentration of each gene-specific forward and reverse primer pairs (IDT, Inc., Coralville, IO), of which one is labeled with FAM dye, and 0.35 X of the ICEPlex Calibrator 1 (PrimeraDx Inc., Mansfield, MA); and point mutation specific gBlock or ultramer™ templates (IDT, Inc.). Twenty five microliter multiplex PCR reactions were subjected to thermocycling in a standard 96-well PCR plate on the ICEPlex System. PCR amplification conditions were as follows:**

- **98° C for 10 minutes**
- **2 cycles at 54° C for 45 sec., 72° C for 45 sec. and 96° C for 20 sec.**
- **16 cycles at 64° C for 45 sec., 72° C for 45 sec. and 98° C for 5 sec.**
- **20 cycles at 64° C for 45 sec., 72° C for 220 sec., 96° C for 10 sec**

List of targets in the ICEPlex CMET SNP Panel

Amino Acid Change	CDS Mutation	Amino Acid Change	CDS Mutation
S1058P	c.3172T>C	V1206L	c.3616G>T
V1110I	c.3328G>A	L1213V	c.3637C>G
H1112Y	c.3334C>T	V1238I	c.3712G>A
H1124D	c.3370C>G	D1246N	c.3736G>A
G1137V	c.3410G>T	Y1248C	c.3743A>G
M1149T	c.3446T>C	K1262R	c.3785A>G
M1268T	c.3803T>C		

Results

Representative PCR Amplification Curves for cMET Mutations Targets Generated on the ICEPlex System in a Single Reaction

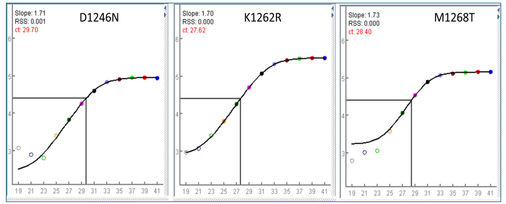

Figure 3. Representative PCR amplification curves and Ct calling generated on ICEPlex System for cMET point mutation targets, D1246N, K1262R, and M1268T in multiplex PCR reactions. ICEPlex software automatically generated PCR amplification curve, and calculated Ct and slope values, which were used in data analysis.
ICEPlex cMET SNP Panel can detect all 13 important point mutation in a single reaction. A known amount of each mutation specific synthetic template was spiked into 50ng of K562 genomic DNA, separately, and detected using cMET point mutation panel.

Representative Dynamic Range and PCR efficiency

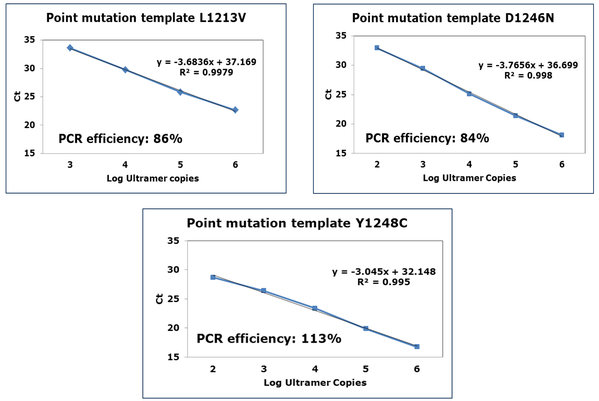

Fig 4. Serial dilutions of synthetic templates were tested to demonstrate the dynamic range of the assay and to calculate the PCR efficiency. ICEPlex cMET SNP Panel showed a dynamic range of 3-5 logs, and with PCR efficiency ranging from 80% to 120%.

Determination of Limit of Blank (LOB) Using FFPE and K562 Cell Line DNA

cMET Mutation targets	Δ Ct Cut-off
Ref-1058P	-9.1
Ref-V1110I	-10
Ref-H1112Y	-6.2
Ref-1124D	-10
Ref-G1137D	-10
Ref-1149T	-10
Ref-1206L	-10
Ref-1213V	-10
Ref-1238I	-10
Ref-1246N	-10
Ref-1248C	-10
Ref-1268T	-10
Ref-1262R	-10

Table 1. Determination of Limit of blank (LOB).
LOB was determined using the breakthrough Ct values, and defined as the difference between lowest target Ct observed from all wild type FFPE and cell line DNA tested in the sample set in relation to an internal reference gene (Ref). For this purpose, 100ng of known wild type FFPE DNA or cell line DNA was used, and breakthrough amplifications were noted. A ΔCt between reference gene and breakthrough values were calculated. The results showed breakthrough ΔCt values lower than -9.1 for 12 targets. For H1112Y target, breakthrough ΔCt was lower than -6.2. To achieve a higher selectivity/sensitivity lower breakthrough ΔCt values are preferred. Lower ΔCt indicated a potential selectivity/sensitivity of 0.1% to 5%, depending on the target.

Selectivity of ICEPlex cMET SNP Panel

Point Mutation Targets	% Detection (100 ng input)	ΔCt = Ref Ct – Target Ct
S1058P	<10%	-3.0
V111OI	10%	-6.7
H1112Y	<10%	-1.9
H1124D	<10%	-0.5
G1137V	<10%	-0.6
M1149T	<10%	-3.9
V1206L	10%	-7.6
L1213V	10%	-7.8
V1238I	10%	-4.1
D1246N	10%	-5.5
Y1248C	10%	-5.6
K1262R	10%	-4.6
M1268T	10%	-4.5

Table 2. Selectivity of the ICEPlex cMET SNP Panel for all targets was less than 10%. The testing was performed using 3,000 copies of synthetic templates, spiked in 100 ng of wild type genomic DNA (30,000 copies)

Interfering Substances Study

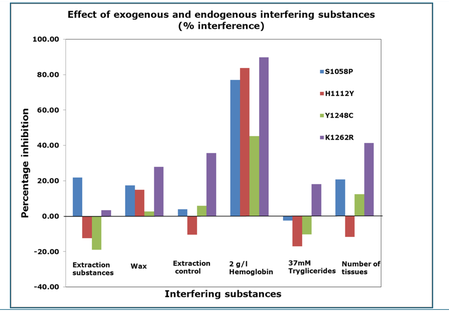

Figure 5. Interfering substances study was carried out on four representative mutations and the results indicated that:
- **All four point mutation targets were affected by hemoglobin, 46-90% depending on the target**
- **No other significant interference was detected in this study**

Conclusions

- **A robust multiplex ICEPlex cMET SNP Panel to detect 13 important point mutations for FFPE samples has been developed**
- **The results showed 3-4 log dynamic range and PCR efficiency ranging from 80-120%**
- **The breakthrough ΔCt for most targets were lower than -9.1 indicating a potential sensitivity of 0.1% to 5%, depending on the target**
- **The results from the study indicated 10% selectivity for all the targets**
- **Hemoglobin has a negative effect on ICEPlex cMET SNP Panel sensitivity and therefore should be removed effectively during the nucleic acid extraction and purification processes from FFPE**

ICEPlex System and cMET SNP Panel have not been approved by the FDA for IVD. This information is for demonstration purposes only.