A Novel cMET and EGFR Copy Number Variation and cMET Gene Expression Profiling Single-Tube Assay

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Abstract (revised)

Objective:
We report the development and verification of the ICEPlex* cMET CNV assay, a multiplex and multimodal PCR assay, which combines cMET/EGFR copy number variation (CNV) and cMET expression in a single reaction on the ICEPlex® System.

***Clinical Relevance*:**
cMET is a proto-oncogene encoding the Hepatocyte Growth Factor Receptor, a receptor tyrosine kinase, which plays an essential role in normal cellular function and oncogenesis. Recent studies have indicated cMET as biomarker for various cancers as well as for drug resistance in the case of anti-EGFR therapy. The protein highly overexpresses in cancer cells by several mechanisms, one of which is the increase in cMET gene copy number.

Methodology:
A 5-gene, 18-target multimodal assay was constructed for both mRNA and gDNA target detection. For cMET/EGFR CNV and cMET expression profiling three amplicons per target were designed to increase the precision of target quantification, and two reference genes each for mRNA and gDNA target normalization. Another reference gene was included to monitor chromosome 7 polysomy (Ref 3-C). The assay is designed to allow amplification from FFPE tissue with template sizes of <100 bp nucleotides. Multiplexed reactions were optimized for target identification and amplification performance on the ICEPlex System using total nucleic acids (NA) from various cell lines.

Validation:
We developed a 18-target, multimodal assay that detects cMET/EGFR CNV and cMET expression in a single well on the ICEPlex System. Performance testing using total nucleic acids from cell lines with known cMET CNV and overexpression as well as chromosome 7 polysomy matched published results. The assay allowed discrimination of approximately two- to four-fold changes in target copy number levels. As little as 1 ng of total nucleic acid was sufficient to determine cMET/EGFR CNV and cMET expression changes.

Conclusions:
We have developed a novel 18-target quantitative, multimodal assay that allows determination of both CNV and expression profiling for multiple targets. This assay has the potential to provide rapid and accurate information on cMET CNV and expression status in a clinical setting with advantages over standard FISH and PCR approaches.
* ICEPlex System 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).

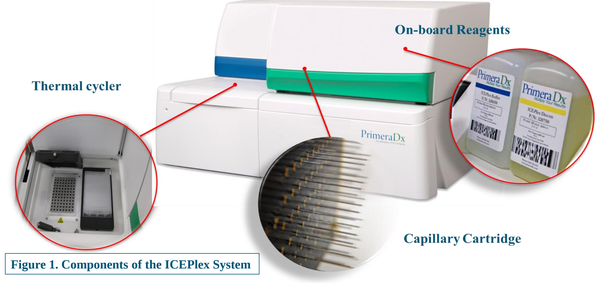

Figure 1. Components of the ICEPlex System

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 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).

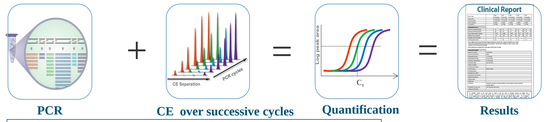

Figure 2. Multiplex real-time PCR detection on the ICEPlex System

Materials and Methods

Target selection and primer design: Reference genes were selected using public data, based on minimal variability in expression and CNV in target tissues. Primers for each target were designed using Geneious™ Pro software (Auckland, New Zealand) and Primer3 (Dice Holding, Inc.) to detect spanning exon-exon junctions for RNA targets and intron regions for DNA targets. The core amplicon sizes were less than 100bp to allow amplification of fragmented nucleic acids from FFPE specimen. Primers were screened for target specificity using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (Bethesda, MD) and for potential primer-primer interactions using a PrimeraDx developed software (Cross-Hyb, PrimeraDx, Inc., Mansfield, MA).

Reverse transcription (RT) and PCR setup: Twenty microliter RT reactions were carried out using SuperScript ®III Reverse Transcriptase (Life Technologies, Grand Island, NY) according to manufacturer's recommendations containing gene-specific primers (IDT, Inc., Coralville, IO) and total NA. The PCR reactions contained: PrimeraDx proprietary multiplex buffer with 2.5U of Apta Taq Δexo DNA polymerase (Roche Diagnostics, Indianapolis, IN), gene-specific forward and reverse primers (IDT, Inc., Coralville, IO), of which one was labeled with either FAM or TYE fluorescent dye, 0.25x of the ICEPlex Calibrator 1 (PrimeraDx, Inc., Mansfield, MA), SNU-1, SNU-5 or A549 cell line NA (ATCC, Manassas, VA) and 5 microliters of the RT reaction. Twenty five microliter multiplex PCR reactions were subjected to thermocycling in a standard 96-well PCR plate on the ICEPlex System.

PCR setup and amplification conditions: 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

Results

Multiplex and Multimodal Detection of cMET/EGFR CNV and cMET mRNA Targets in Gastric Carcinoma Cell Lines

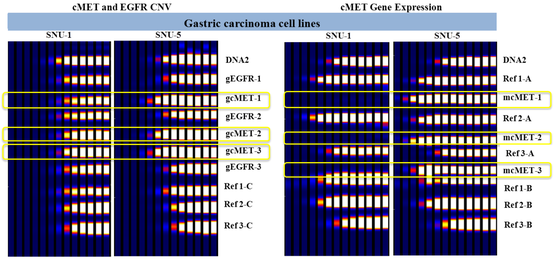

Figure 3. Representative electropherograms of cMET/EGFR CNV and cMET Gene Expression Assay generated on the ICEPlex System.
Nucleic acids from two gastric carcinoma cell lines (SNU-1 and SNU-5) were tested in the cMET/EGFR CNV and cMET Gene Expression Assay. The RNA targets were detected in the FAM channel (right panels), while the DNA targets were detected in the TYE channel (left panels). The assay is designed to detect multiple amplification products per target. The ICEPlex results confirmed published data that SNU-1 has no detectable cMET expression and no CNV, and SNU-5 shows high cMET expression and CNV.

Lower Target Quantification Limits of cMET/EGFR CNV and cMET Gene Expression Assay

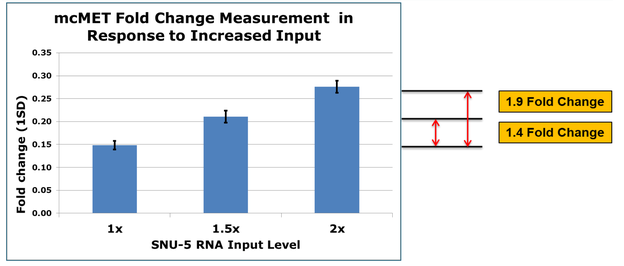

Figure 4. Lower detection limits of relative quantification of cMET/EGFR CNV and cMET Gene Expression Assay. Relative concentration of cMET mRNA was measured by using total NA from SNU-1 to which different amounts of mRNA from SNU-5 was added. The baseline (1x) comprised of 2 ng of SNU-1 and 0.02 ng of SNU-5. The lower target quantification limits were evaluated by testing of 1.5x and 2x of SNU-5 mRNA above the baseline. The output of the cMET/EGFR CNV and cMET Gene Expression Assay reflected a matching fold change detection in relative cMET mRNA concentration of 1.4x and 1.9x accordingly.

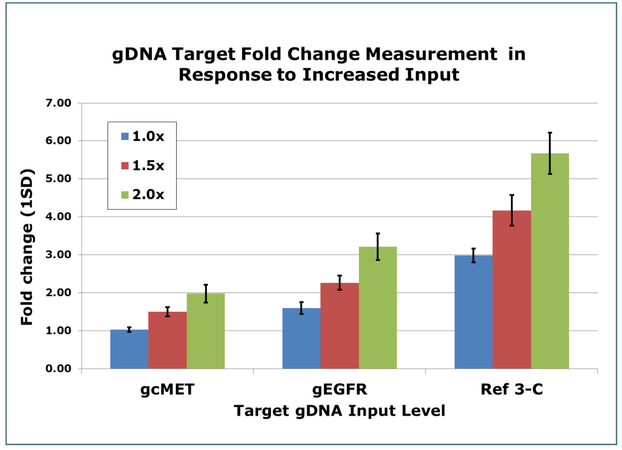

Figure 5. Lower detection limits of cMET/EGFR CNV and chromosome 7 polysomy (Ref 3-C). Relative concentration of DNA targets cMET, EGFR and Ref 3-C were measured similarly to mcMET but using synthetic template added to SNU-1. The synthetic template was designed to contain a single copy of each DNA target sequences. The concentration of the synthetic template was normalized based on Ct values to match 2ng of SNU-1 NA. Corresponding amounts of synthetic template were subsequently spiked into 2ng of SNU-1 total NA (set as 1x baseline for cMET, EGFR and Ref 3-C) and assayed to achieve an increase by 1.5x and 2x. The cMET/EGFR CNV and cMET Gene Expression Assay output showed a fold change detection in relative concentration that is close to the expected value for all three targets.

Precision of cMET/EGFR CNV and cMET Gene Expression Assay

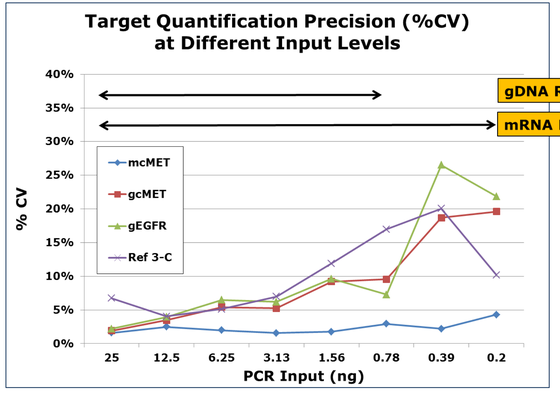

Figure 6. cMET/EGFR CNV and cMET Gene Expression Assay precision at lower quantification range. Dilution series of lung cell line A549 total NA (moderate cMET expression, no known cMET/EGFR CNV, no known chromosome 7 polysomy), ranging from 0.2ng to 25ng were assayed twice on two different ICEPlex Systems with 3 replicates per run. Results for cMET mRNA quantification showed a very low variability across the input range of more than 2 logs. For gDNA targets quantification was consistent between 1-25ng.

Conclusions

- An 18-target, single-well multimodal assay designed to detect cMET and EGFR genes, expression of cMET and polysomy of chromosome 7 has been developed on the ICEPlex System
- The results from testing of the gastric carcinoma cell lines confirmed published reports on cMET overexpression and CNV
- The assay demonstrated reliable two fold discrimination for CNV and cMET gene expression targets
- Accurate and precise quantification was established down to 1ng of total nucleic acid

ICEPlex System and cMET/EGFR CNV and cMET Gene Expression Assay have not been approved by the FDA for IVD.
This information is for demonstration purposes only.