Multiplexed ICE COLD-PCR Provides High Concordance in Clinical Testing

Amy Kruempel, Jaclyn Pope MS, Emily McCutchen MS, Paula Bartlett, Jason Stoddard, Stephanie Peterson MS, Benjamin Legendre Jr. PhD, Transgenomic, Inc., Omaha, NE

Matched tumor and plasma samples for twenty-two patients with late stage CRC were analyzed via Transgenomic’s Multiplexed ICE COLD-PCR (MX-ICP) for KRAS Exon 2 mutation status. The use of MX-ICP resulted in an 83% (10/12) concordance rate between the FFPE and plasma in mutation positive samples as compared to a concordance rate of 58% (7/12) using traditional PCR. Taking into account all samples, the concordance for ICE COLD-PCR was 91% (20/22) vs. 77% (17/22) for traditional PCR. This concordance rate supports the clinical utility of liquid biopsies for patient monitoring and the increase in mutation detection highlights the excellence of MX-ICP technology.

Introduction
In the recent past, the limited quantity of circulating tumor DNA (ctDNA) recoverable from a blood draw has required mutation detection limits far below those achievable by the available technologies. The development of highly sensitive molecular techniques, such as Transgenomic’s Multiplexed ICE COLD-PCR (MX-ICP), now support the use of liquid biopsies, such as blood or plasma, as an ideal sample type for non-invasive clinical monitoring and theranostics of cancer patients.

Approximately 35-40% of colorectal tumors have been found to be KRAS Exon 2 (codons 12 and 13) mutation positive. These mutations are predictive of resistance to EGFR-specific tyrosine kinase inhibitors (TKI), including the commonly prescribed cetuximab and panitumumab. Recently, it was determined that mutations in KRAS Exons 3 and 4 as well as NRAS Exons 2, 3, and 4 were also predictive of poor response. This study compares matched tumor and plasma samples of colorectal cancer (CRC) stage III/IV patients to investigate the levels of concordance of mutation status between the two sample types. MX-ICP technology was used in the analysis of these samples.

MX-ICP is a two-stage assay that entails an initial multiplexed amplification (MX PCR) of extracted ctDNA followed by a targeted ICE COLD-PCR (Improved and Complete Enrichment CO-amplification at Lower Denaturation temperature) amplification.

In its current format MX PCR targets amplification of the following genes/exons in a single pre-amplification step: KRAS Exons 2, 3 & 4; NRAS Exons 2, 3 & 4; BRAF Exons 11 and 15; PIK3CA Exons 9 & 20; EGFR Exons 12, 18, 19, 20 & 21. These exons are regions of oncogenes that have been determined to be predictive and/or prognostic for the treatment of many types of cancers.

Following the initial MX PCR, Transgenomic’s exclusively licensed ICE COLD-PCR technology preferentially enriches DNA sequence alterations when they are present in an excess of wild-type DNA (Figure 1). The use of an oligonucleotide complementary to wild-type sequence (RS-Oligo) is the key element in the process that suppresses PCR amplification of wild-type sequences while allowing unobstructed amplification of DNA containing any altered sequence in the region covered by the RS-Oligo.

Experimental Methods
Matched tumor and pre-surgery plasma samples from twenty-two chemonaive stage III/IV CRC patients were collected. The tumor samples underwent pathological review and were macrodissected to enrich for the tumor area. The extraction was completed using the QIAamp® DNA FFPE Tissue Kit (QIAGEN®, Hilden, Germany) and the eluted DNA was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, MA). ctDNA was extracted from 4 mL of each plasma sample via the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) and the eluted ctDNA was quantified using a Qubit™ dsDNA HS Assay Kit (Thermo Scientific, Waltham, MA) on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Selective Amplification of Mutant DNA in ICE COLD-PCR

![Figure 1. ICE COLD-PCR technology preferentially enriches mutant DNA sequences in an excess of wild-type DNA through selective amplification of the mutant DNA. This results in the capability of detecting mutations down to 0.01%, or 5 copies in 50,000 genomic equivalents.](image-url)
When available, 150 ng of DNA was used as template for the MX PCR reaction. If 150 ng was not available, as seen in twenty of the twenty-two plasma samples, 20 µl of sample at stock concentration was used. Following MX PCR amplification, gel electrophoresis was used as a quality control measure to ensure contamination free PCR and the MX PCR product was diluted 1:200 with nuclease-free water.

Following MX PCR dilution, all products were subjected to two separate PCR amplifications. One amplification was performed using Transgenomic’s validated KRAS Exon 2 specific ICE COLD-PCR technology. The second amplification was identically analyzed, but without the addition of an RS-Oligo. Ultimately, this second amplification, referred to as “non-enriching”, resulted in a nested KRAS Exon 2 specific PCR reaction that did not preferentially amplify mutant populations.

Gel electrophoresis was used as a quality control measure for amplification and Sanger sequencing was used for sequence analysis of both secondary amplifications.

Results

Analysis of matched tumor and plasma samples from twenty-two patients using MX-ICP resulted in an overall concordance of 91% for KRAS Exon 2 (20/22). This is a 14% increase compared to analysis via non-enriching PCR methods (17/22). In addition, a total of 12 FFPE samples harbored a KRAS Exon 2 mutation after ICE COLD-PCR, while the corresponding mutation was only observed in 10 of the samples using traditional, non-enriching PCR. Therefore, the mutation enrichment generated by ICE COLD-PCR resulted in 17% (2/12) more mutations detected in the gold standard FFPE samples when compared to plasma, with five unique mutations identified within KRAS codons 12 and 13 (Figure 2).

Using non-enriching PCR analysis, mutations were identified in tumor samples from ten patients; however, mutations were only detected in seven of the matched patient plasma samples (70% mutation positive concordance). Using MX-ICP analysis, mutations were detected in the tumor FFPE samples from two additional patients, resulting in a total of 12 tumor mutation positive patients. Ten of these patients showed concordance between plasma and tumor sample types (83% mutation positive concordance vs. 58% for non-enriched analysis). For the two patients determined to be non-concordant following MX-ICP, no mutation was detected in either tumor or plasma when also analyzed via non-enriching PCR methods. The two additional mutations observed in the MX-ICP analysis were detected only in tumor samples. The DNA input for the two associated plasma samples was relatively low due to poor extraction yield.

References


Figure 2. MX-ICP and non-enriching PCR sequencing electropherograms for matched tumor/plasma samples from three stage III/IV CRC patients are shown for three of the five different mutations detected in codons 12 or 13 for KRAS Exon 2. Mutation enrichment can be seen in the increase in mutation percentage visualized following Sanger sequencing when ICE COLD-PCR technology is implemented during amplification steps.

Transgenomic is a registered trademark and the helix logo, Advancing Personalized Medicine and the multiplexed ICE COLD-PCR logo are trademarks of Transgenomic, Inc.
All other trademarks are the property of their respective owners.
© Transgenomic, Inc.