Mutant Enrichment Using ICE COLD-PCR Paired to Next-Generation Sequencing Enables High Sensitivity and High Throughput Detection of Cancer Biomarkers in Patient Samples

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Abstract

Blood-based mutation analysis from serum and plasma is becoming very important for molecular diagnostics where no tumor is available as well as in the pharmacodynamic monitoring of the patient during therapy. Longitudinal monitoring from these liquid biopsies for the early detection of activating mutation recurrence or the emergence of resistance mutations is integral for ensuring proper treatment and best overall outcome for the patient. For monitoring purposes, a highly sensitive and rapid throughput technique is required.

ICE COLD-PCR (ICP) technology is capable of high sensitivity detection for both point mutations and indels through unbiased enrichment of relevant gene regions. Mutation detection in circulating cell-free DNA (cfDNA) is becoming increasingly important for monitoring treatment and resistance responses to various cancer drugs. Since the amount of cfDNA in plasma is limited, a methodology, such as ICP, that provides increased sensitivity for detecting these informative mutations in needed.

Next-Generation Sequencing (NGS) allows high throughput analysis of cancer mutations using target resequencing of a set/panel of genes, thus a broad mutation signature of tumors can be determined. However, the level of detection is ~2-4% for mutations unless a higher depth of coverage is used; this comes at the price of reducing the number of samples that can be analyzed on a chip. ICP enrichment of mutations prior to NGS mimics an increased “depth of coverage” without reducing the throughput per chip.

In this study, ICP products from low frequency mutation mixtures were enzymatically treated, ligated to the Ion Torrent adapters and analyzed by the Ion Torrent Personal Genome Machine (PGM) and the MiSeq NGS platforms. The ICP-NGS analysis of low level mutations provided a virtual increase in depth of coverage ahead of NGS analysis that increased the sensitivity of NGS detection after ICP enrichment.

In conclusion, we have established a platform of mutant enrichment by ICE COLD-PCR followed by NGS analysis that is capable of high sensitivity and high throughput detection of cancer genetic biomarkers. ICP-NGS has the potential to detect and monitor patients’ genetic biomarkers in real-time and provide guidance for personalized cancer treatments.

Methods

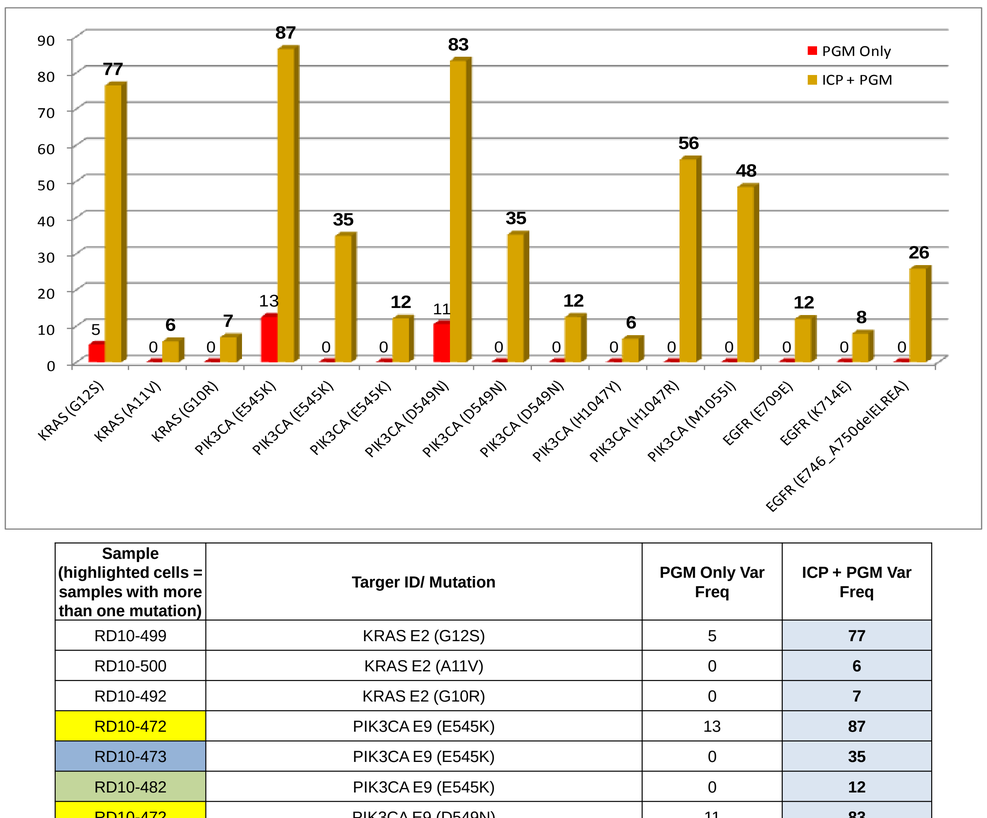
A proof of concept experiment was performed using low level of mutations. The DNA mixtures were enriched for mutants by ICP reactions followed by Sanger sequencing. After ICP enrichment of mutants, samples from different amplicons were mixed and enzymatically treated before ligating to the Ion Torrent adapters. Libraries with adapters were processed for template generation and subsequently run on the PGM. Torrent Suite was used for alignment and the variantCaller plugin for mutant calls. The BAM files generated from the alignment were uploaded to Integrative Genome View (IGV; Broad Institute) for NGS data analysis and viewing.

For the cfDNA ICP-NGS study, 12 DNA samples were harvested from plasma of patients with carcinomas and analyzed for ICP for *PIK3CA* exons 9 and 20, *EGFR* exons 18 and 19, and *KRAS* exon 2, followed by NGS analysis. For comparison, the 12 DNA samples were also analyzed by NGS without prior ICP enrichment.

For the *EGFR* Exon 19 deletion analyses, wild-type and deletion plasmids were mixed, resulting in a 5% mixture of deletion to wild-type. These low level mixtures of deletions were analyzed by NGS with and without prior ICP enrichment. For these studies, only one ICP assay is used for the enrichment of all the deletions.

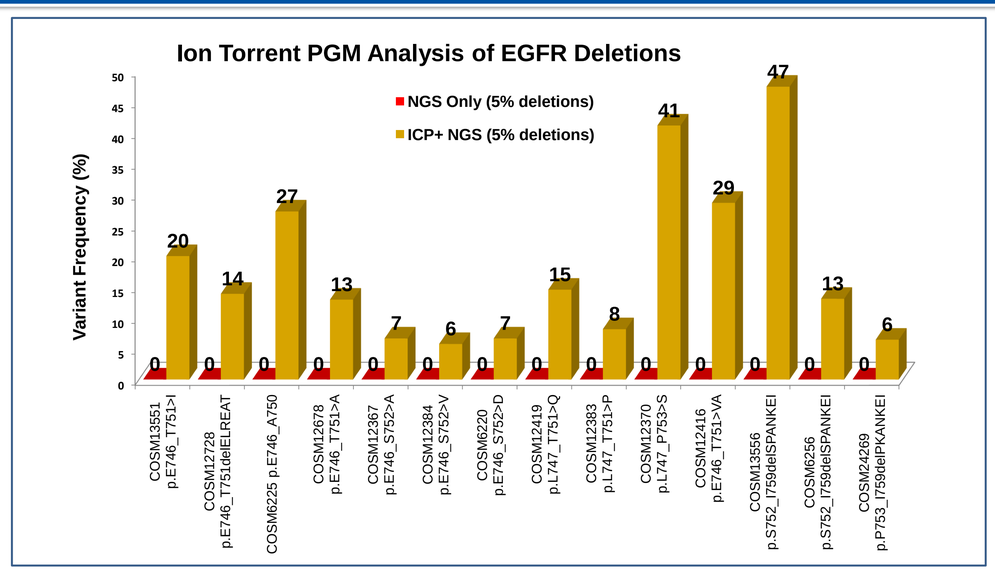
For MiSeq analysis, 10 ng PCR products were converted to NGS libraries using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB). Libraries passing quality control were diluted to 4 nM using sterile water and then sequenced on the MiSeq (Illumina) at a final concentration of 18 pM on a paired end flowcell with 150 sequencing cycles in each direction, following manufacturer protocols.

ICP-NGS: Detection of Mutations in cfDNA

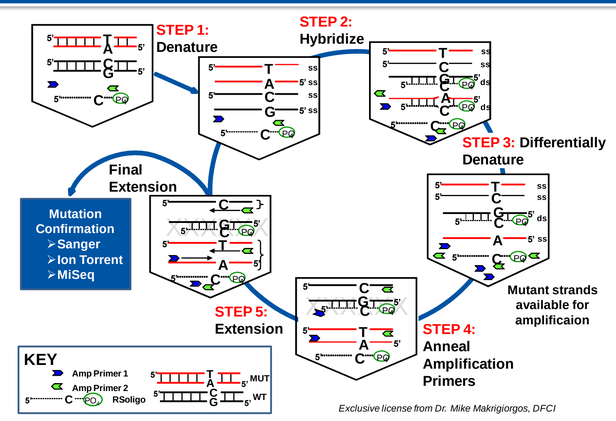


Sample (highlighted cells = samples with more than one mutation)	Targer ID/ Mutation	PGM Only Var Freq	ICP + PGM Var Freq
RD10-499	KRAS E2 (G12S)	5	**77**
RD10-500	KRAS E2 (A11V)	0	**6**
RD10-492	KRAS E2 (G10R)	0	**7**
RD10-472	PIK3CA E9 (E545K)	13	**87**
RD10-473	PIK3CA E9 (E545K)	0	**35**
RD10-482	PIK3CA E9 (E545K)	0	**12**
RD10-472	PIK3CA E9 (D549N)	11	**83**
RD10-473	PIK3CA E9 (D549N)	0	**35**
RD10-482	PIK3CA E9 (D549N)	0	**12**
RD10-463	PIK3CA E20 (H1047Y)	0	**6**
RD10-484	PIK3CA E20 (H1047R)	0	**56**
RD10-465	PIK3CA E20 (M1055I)	0	**48**
RD10-464	EGFR E18 (E709E)	0	**12**
RD10-486	EGFR E18 (K714E)	0	**8**
RD10-493	EGFR E19 (E746_A750delELREA)	0	**26**

Detection of Multiple EGFR Deletions



ICP (ICE COLD-PCR): Methodology



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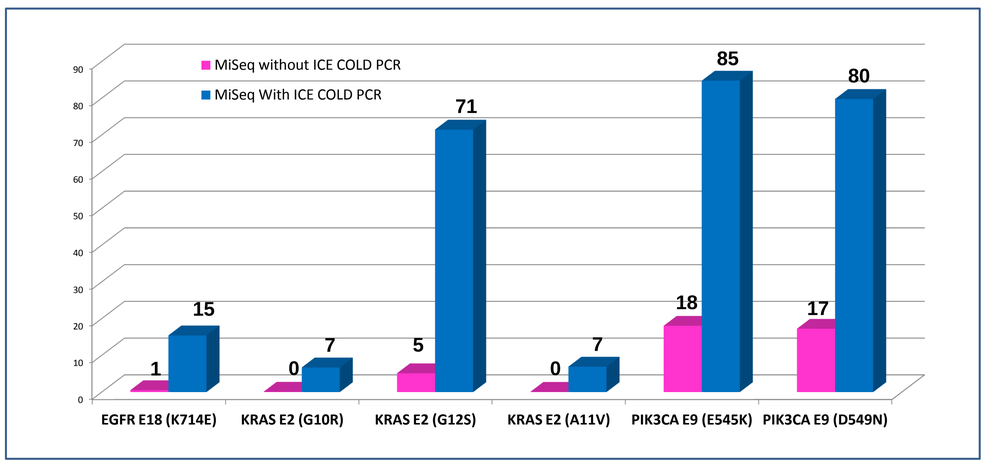
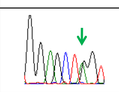
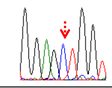
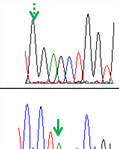
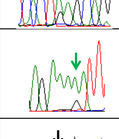
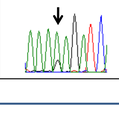
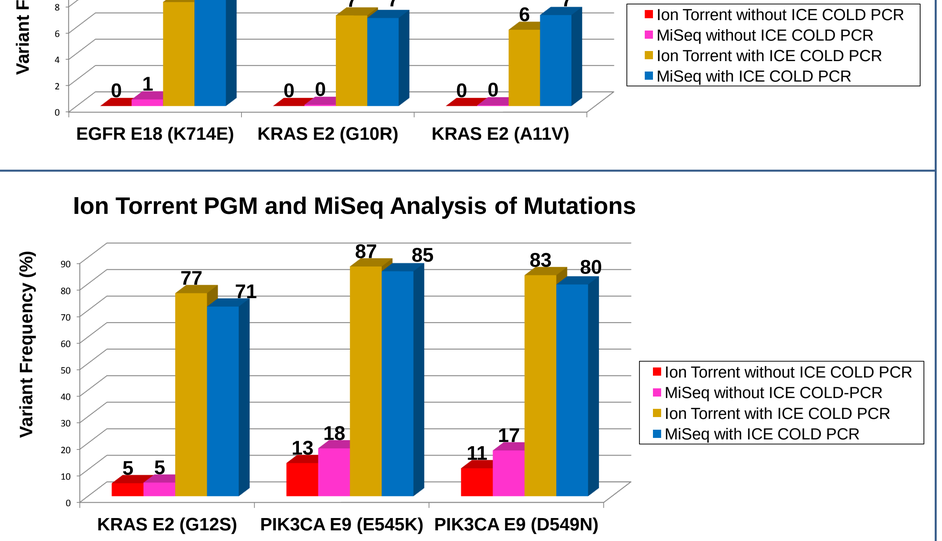
Mutation Detection: NGS +/- ICP: Ion Torrent vs. MiSeq



Sample	Target ID/ Mutation	MiSeq Only Var Freq	ICP + MiSeq Var Freq	ICP + Sanger
RD10-499	KRAS E2 (G12S)	5.12	71	
RD10-500	KRAS E2 (A11V)	0.06	7	
RD10-492	KRAS E2 (G10R)	0.09	7	
RD10-472	PIK3CA E9 (E545K)	18.03	85	
RD10-472	PIK3CA E9 (D549N)	17.24	80	
RD10-486	EGFR E18 (K714E)	0.50	15	



Conclusions

- ICP-NGS platform combines ICE COLD-PCR and NGS technologies and allows high sensitivity detection of mutations from multiple genes from multiple samples, and with added advantage of higher sensitivity for detecting substitution mutations.
- Use of ICP in front of NGS detected mutations in circulating cfDNA that were undetectable without ICP enrichment, suggesting ICP-NGS can be utilized for monitoring patient’s mutation in cfDNA and subsequent management of cancer treatment.
- A single ICP assay was used to enrich for a set of *EGFR* Exon 19 deletions, both common and uncommon deletions.
  - ICP paired to NGS was able to detect all the *EGFR* Exon 19 deletions tested.
  - NGS alone was not able to detect any of the deletions.
- Comparable results of ICP-NGS were obtained using Ion Torrent PGM system and Illumina MiSeq system, suggesting ICP-NGS for high throughput and high sensitivity mutant detection is NGS platform independent.