Utility of ICE COLD-PCR coupled to Sanger Sequencing or Next Generation Sequencing for increased mutation detection sensitivity during patient monitoring from circulating free DNA

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Introduction and Background:

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 is 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, we show correlation data between solid tumor (standard analysis) and matched plasma using ICP coupled to Sanger sequencing. In addition, we show the advantages of using ICP as a pre-enrichment step for NGS.

Longitudinal Assessment of cfDNA *BRAF* & *KRAS* Mutations

Patient #	Disease	Tissue Mutation	Treatment prior to baseline cfDNA	Baseline cfDNA Mutations	Follow up cfDNA Mutation	Treatment Response*
5	Erdheim-Chester	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
6	Papillary Thyroid Cancer	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
2	Melanoma	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
3	Melanoma	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
4	Melanoma	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
1	Melanoma	*BRAF* V600E	Yes	Wild-Type	*BRAF* V600E	PD
7	Appendiceal Carcinoma	*KRAS* G13D	Yes	Wild-Type	*KRAS* G13D	PD
8	Colorectal Carcinoma	*KRAS* G12D	Yes	Wild-Type	*KRAS* G12D	PD

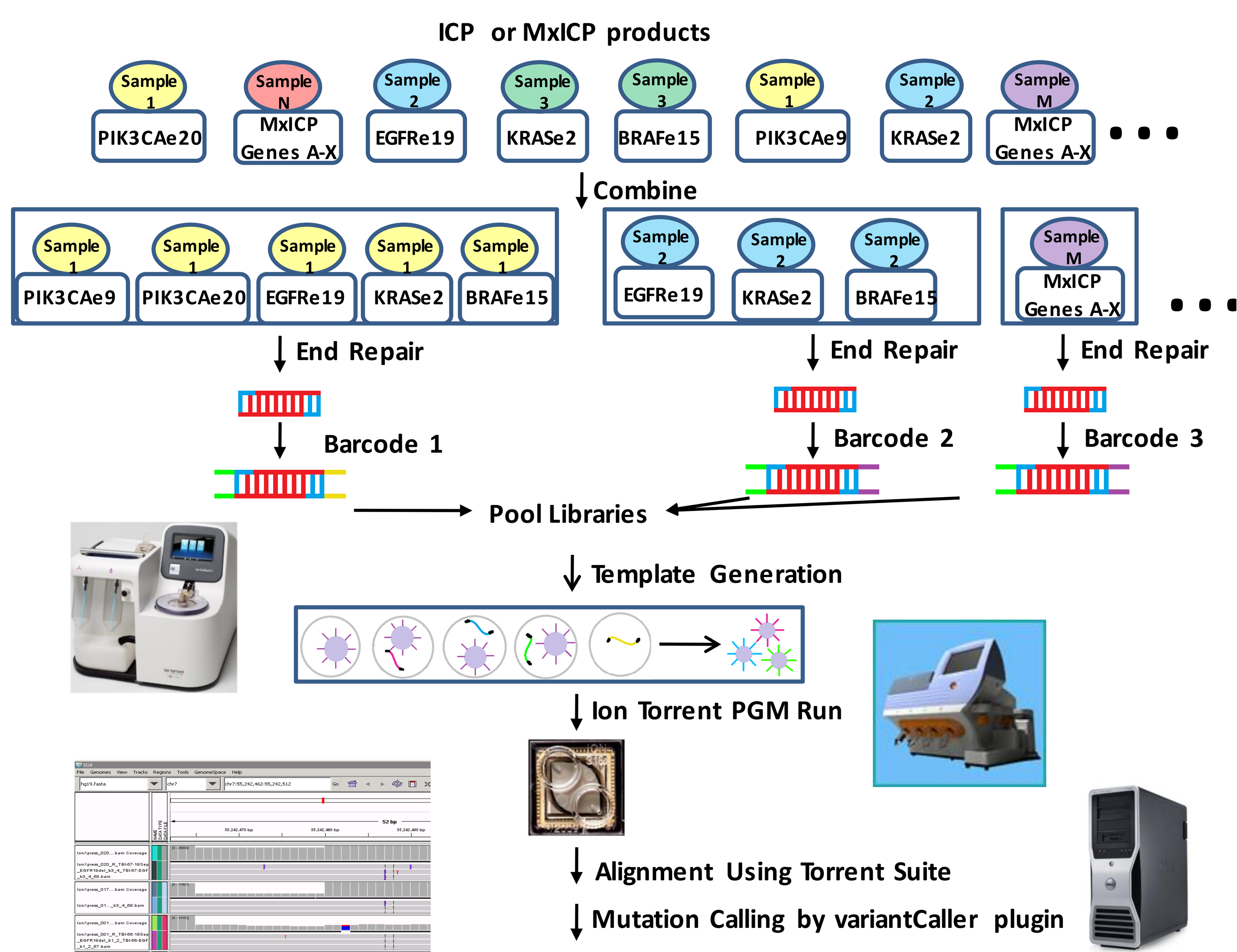
*SD = Stable disease; PD = Progressive disease

Concordance Study (ICP + Sanger): Tumor vs cfDNA, *BRAF* & *KRAS*

TESTED (N=31)	*BRAF* mutation CLIA	*BRAF* wild-type CLIA
BRAF mutation PLASMA	20	0
BRAF wild-type PLASMA	10	1
Observed agreements	21 (68%)	

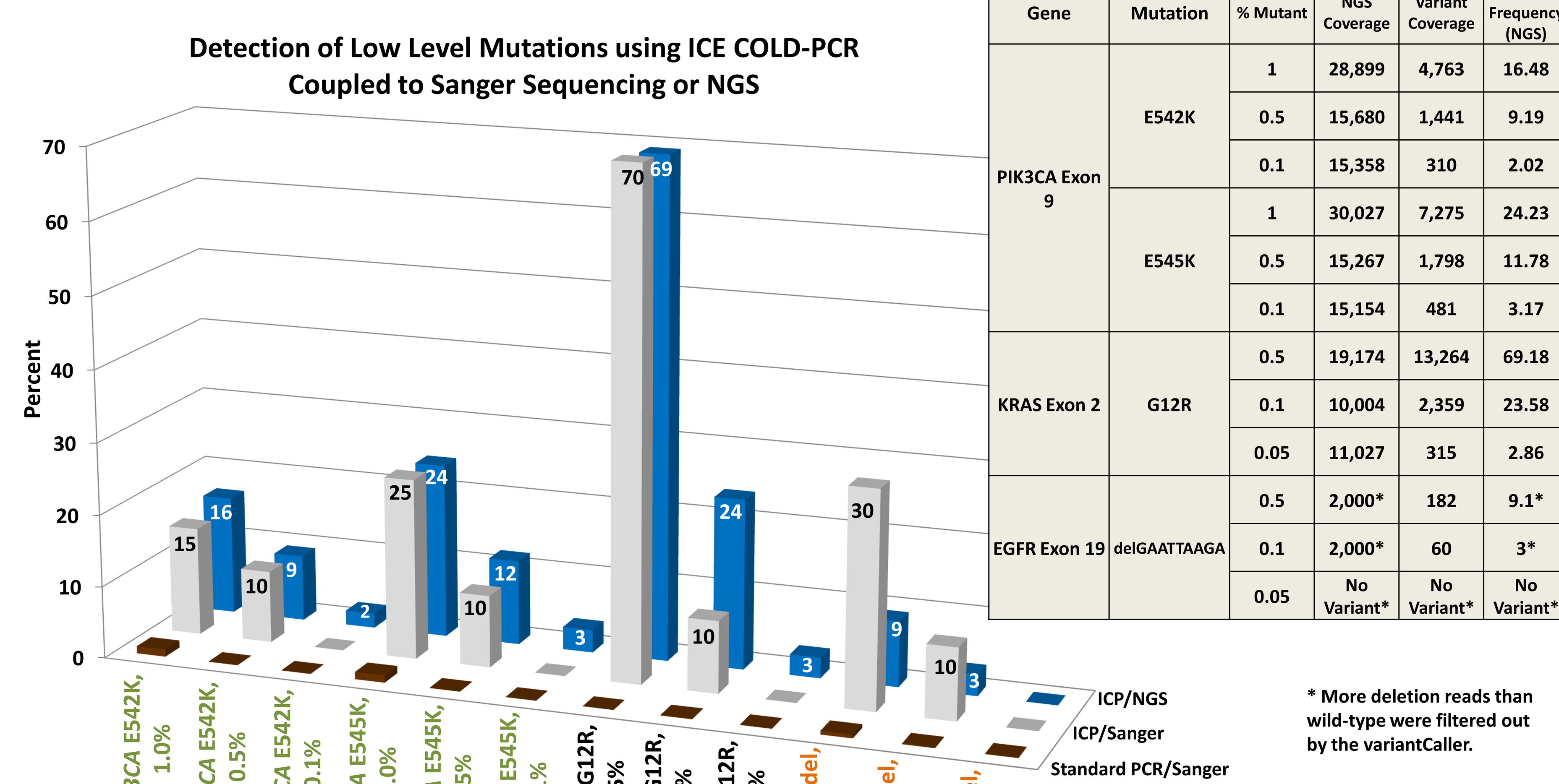
TESTED (N=29)	*KRAS* mutation CLIA	*KRAS* wild-type CLIA
KRAS mutation PLASMA	22	2
KRAS wild-type PLASMA	4	1
Observed agreements	23 (79%)	

ICP-NGS (ICP prior to NGS analysis): Workflow



High Sensitivity Molecular Landscaping for Multiple Patient Samples

ICP-NGS: Proof-of-concept study

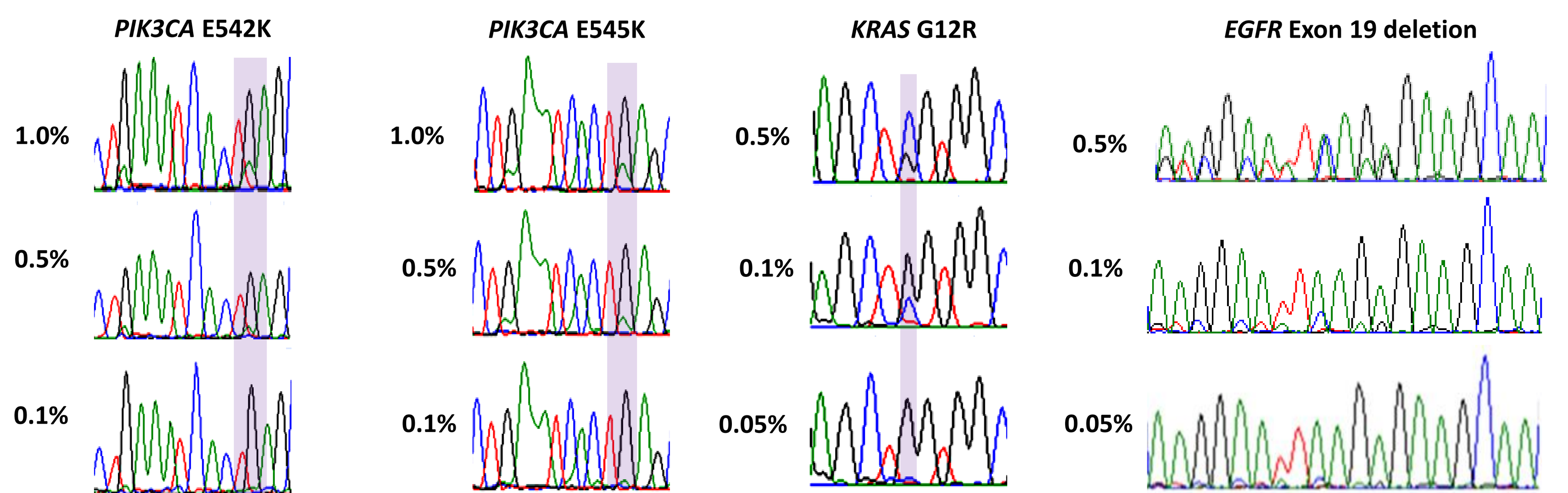


Gene	Mutation	% Mutant	NGS Coverage	Variant Coverage	% Variant Frequency (NGS)
PIK3CA Exon 9	E542K	1	28,899	4,763	16.48
		0.5	15,680	1,441	9.19
		0.1	15,358	310	2.02
	E545K	1	30,027	7,275	24.23
		0.5	15,267	1,798	11.78
		0.1	15,154	481	3.17
KRAS Exon 2	G12R	0.5	19,174	13,264	69.18
		0.1	10,004	2,359	23.58
		0.05	11,027	315	2.86
EGFR Exon 19	delGAATTAAGA	0.5	2,000*	182	9.1*
		0.1	2,000*	60	3*
		0.05	No Variant*	No Variant*	No Variant*

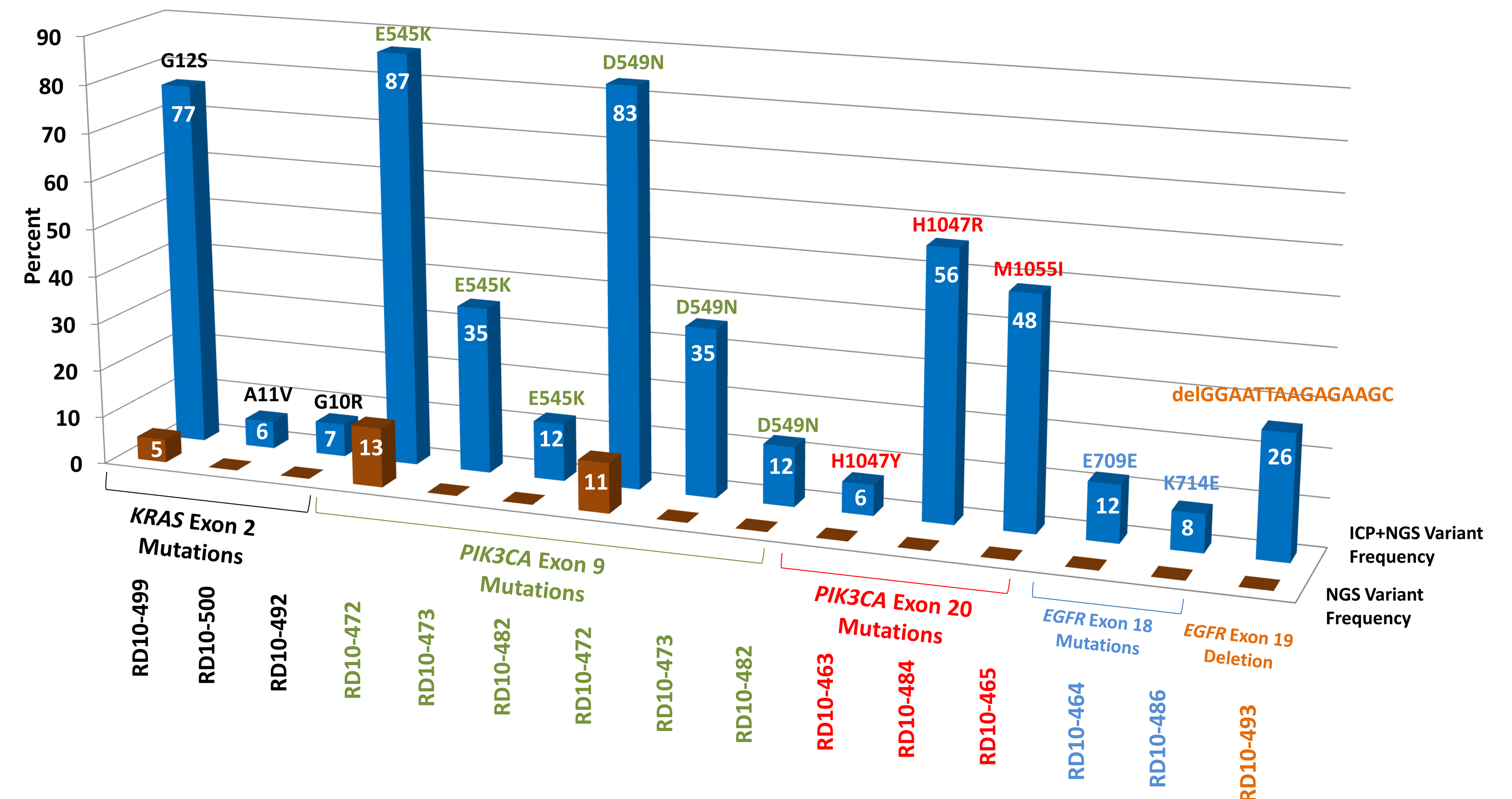
* More deletion reads than wild-type were filtered out by the variantCaller.

Starting Percentage of Mutations in *PIK3CA*, *KRAS* and *EGFR*

Mutants enriched and detected **individually** using ICP-Sanger sequencing



NGS Analysis of cfDNA with and without ICE COLD-PCR Enrichment



Conclusions:

- ✓ Longitudinal Assessment Section:
  - ✓ ICP enrichment of mutations present in cfDNA provides:
    - ✓ A sensitive methodology for use in determining patient treatment options when tumor tissue is not available or difficult to obtain.
    - ✓ A monitoring capability to assess treatment effectiveness.
- ✓ Sanger Concordance Study Section:
  - ✓ ICP enrichment of mutations followed by Sanger sequencing show promising concordance with mutations detected in the tumor.
- ✓ ICP-NGS Section Workflow for multi-gene, high-throughput mutational analysis of mutations present in cfDNA:
  - ✓ ICP assays can be run individually and then combined and sequenced on NGS platforms.
  - ✓ ICP assays can be multiplexed and sequenced on NGS platforms.
- ✓ ICP-NGS Proof of Concept Section:
  - ✓ ICP prior to NGS analysis results in at least a 100-fold increase in the sensitivity of NGS alone.
  - ✓ Deletions are detected, but bioinformatics analyses for the deletions need refinement.
- ✓ NGS Analysis with and without ICP:
  - ✓ Enrichment of mutations by ICP prior to sequence analysis on NGS shows significant increases in mutation detection as compared to mutation detection using NGS alone.
- ✓ Next Steps:
  - ✓ Multiplex ICP for gene panels
  - ✓ Re-evaluate the concordance data using ICP to NGS.

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