Poster#107

Multiplexed ICE COLD-PCR: A Mutation Detection Methodology for Achieving Sensitivities of <0.01% using either Sanger or NGS

Abstract # 227

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ABSTRACT:

Background: Blood-based mutation analysis from circulating free DNA (cfDNA) is becoming very important for molecular demographics and diagnostics where no tumor is available as well as in the pharmacodynamic monitoring of the patient during therapy. ICE COLD-PCR technology is capable of high sensitivity detection for both point mutations and insertion/deletions through unbiased enrichment of relevant gene regions. This method preferentially amplifies low levels of mutant DNA in a sample containing a vast excess of wild-type DNA.

Materials and Methods: In order to increase throughput as well as address the limited amounts of DNA present from cfDNA sources, a multiplex approach for ICE COLD-PCR has been developed. Horizon Cell line DNA with digital PCR verified mutation percentages was first amplified using singleplex PCR for a single region of interest or multiplex PCR for several regions of interest. The advantage of the multiplex PCR is to allow simultaneous amplification of all targets with the same input DNA. The digitally-verified DNA was used for the LOD dilutions where the starting mutation percentage was below 1%. The amplified DNA can then be used in multiple singleplex or multiplex ICE COLD-PCR reactions. A constraint of all ICE COLD-PCR reactions is the optimal thermal cycling parameters needed for mutation enrichment and this critical temperature (Tc) is dependent on the sequence context. A Veriti thermal cycler was used for ICE COLD-PCR analysis of *EGFR* Exons 19 and 20, *KRAS* Exons 2 and 3, and *NRAS* Exons 2 and 3 because it can simultaneously perform thermal cycling at 6 different Tc's on a single 96-well plate. This was followed by hemi-nested PCR using a single thermal cycling program if required to provide sufficient sample for Sanger sequencing and NGS using an Ion Torrent.

Results: Limits of detection experiments using the Horizon Cell Line DNA and serial dilution of this DNA indicated that samples containing 0.01% mutation in the starting material were easily confirmed using both Sanger and NGS sequencing platforms. This was true for the point mutations as well as the EGFR Exon 19 E746_A750delGGAATTAAGAGAAGC. Concordance of cfDNA and matched FFPE tumor DNA is also presented.

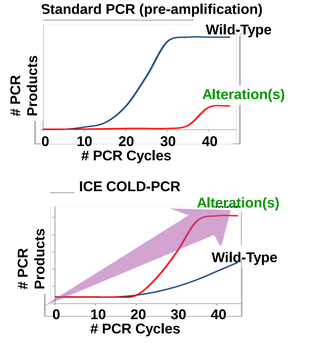
Conclusion: ICE COLD-PCR can be used in a multiplex fashion for the sensitive detection of all mutations in a region. This is important when investigating regions with multiple mutations such as EGFR Exon 19 deletions. The sensitivities achieved indicate that ICE COLD-PCR is an ideal tool for detection of low level mutations found in cfDNA and potentially circulating tumor cells. The ability to confirm these mutations by either Sanger of NGS platforms allows flexibility in rapid confirmation when few or many gene regions need to be interrogated.

BACKGROUND/METHODS:

ICE COLD-PCR (ICP) technology enables high sensitivity detection by Sanger and NGS sequencing of both point mutations and insertions/deletions through unbiased enrichment of relevant gene regions. The use of a reference sequence oligonucleotide (RS-oligo) complementary to one of the wild-type strands results in linear amplification of the wild-type sequences but exponential amplification of any the sequence alterations that are present in the sample.

In order to increase throughput (turn-around time) as well as address the limited amounts of DNA present from cfDNA samples, Transgenomic has developed a multiplex approach for ICP using multiplexed ICE COLD-PCR (MX-ICP) which consists of a multiplexed PCR (MX-PCR) reaction coupled to ICP. The use of a Veriti Thermal cycler allows PCR amplification of up to 6 different Tc 's. The determination of the appropriate RS-oligos for each ICP reaction has been ascertained in order to ensure that they work within these 6 constrained temperature ranges. This allows the simultaneous amplification of many different ICP assays using one PCR instrument.
Development of the Transgenomic's proprietary MX-ICP assay provides an enrichment process that can be used upstream of methodologies used for analysis of mutations, insertions/deletions or other sequence alterations.

Overview: MX-ICP Enrichment



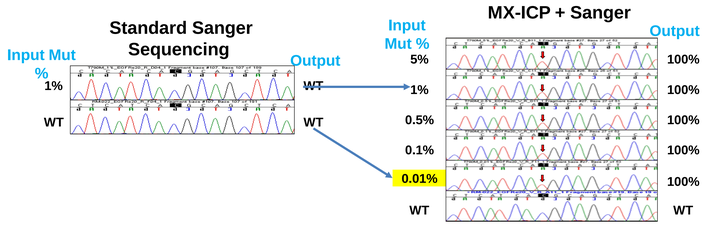
Process Used to Generate Results

1. Use of a Veriti Thermal cycler with Transgenomic's proprietary MX-ICP was used for amplification of DNA from:
    1. Constructed plasmids
    2. Cell Line DNA
    3. FFPE
    4. cfDNA
2. Following the MX-ICP reactions, PCR products enriched for sequence alterations were analyzed for the presence of sequence alterations:
    1. Sanger Sequencing
    2. Ion Torrent PGM NGS Instrument

RESULTS: (digitally verified cell line DNA)

EGFR Exon 20 T790M Mutation

- Digitally verified Horizon Discovery Cell lines were diluted to provide a range of concentrations
- DNA was amplified using Transgenomic's proprietary MX-PCR assay
- The MX-PCR product was used in an MX-ICP assay for EGFR Exon 20
- Sequence analysis was performed using Sanger sequencing and NGS.

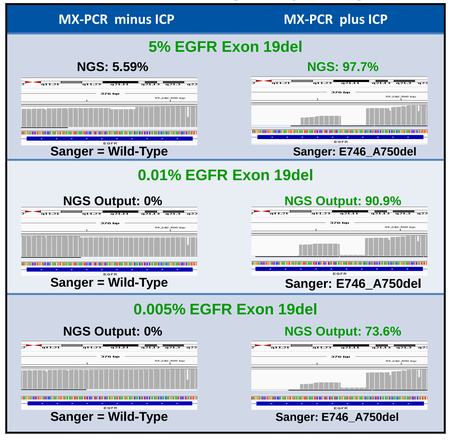


Sample Name	Type	Ref	Variant	Var Freq	Coverage	Var Cov
RM-022 EGFR Ex 20 WT Ctrl	NVD		NVD	0		
5% EGFR Ex 20 T790M	SNP	C	T	100	17211	17204
1% EGFR Ex 20 T790M	SNP	C	T	100	14945	14933
0.5% Ex 20 T790M	SNP	C	T	100	11432	11423
0.1% Ex 20 T790M	SNP	C	T	99	6245	6209
0.01% Ex 20 T790M	SNP	C	T	99	8706	8616
RD10-018 EGFR Ex 20 WT	NVD		NVD	0		
RD10-471 EGFR Ex 20 WT	NVD		NVD	0		

EGFR Exon 19 deletion: ΔE746-A750del (GGAATTAAGAGAAGC)

- Digitally verified Horizon Discovery Cell lines were diluted to provide a range of concentrations (only the 5%, 0.01% and 0.005% are presented here)
- DNA was amplified using Transgenomic's proprietary MX-ICP assay.
- Sequence analysis used Sanger sequencing and NGS.

IGV view of NGS and Sanger Sequencing Results

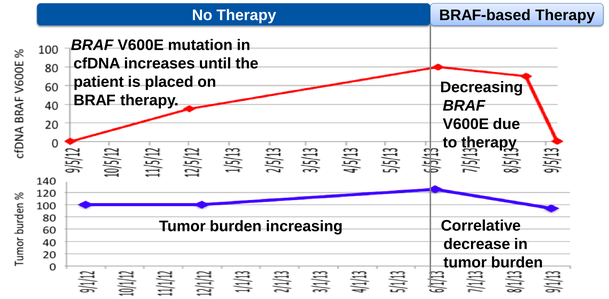


Sample Name		Type	Ref	Var Freq	Coverage	Var Cov
RM-022 EGFR Ex 19 WT Ctrl		NVD		0		
5% EGFR Ex 19 del	Without MX-ICP	DEL	AGGAATTAAGAGAAGC	5.59	715	40
0.01% EGFR Ex 19 del	Without MX-ICP	NVD		0		
0.005% EGFR Ex 19 del	Without MX-ICP	NVD		0		
5% EGFR Ex 19 del	With MX-ICP	DEL	AGGAATTAAGAGAAGC	97.7	6443	6293
0.01% EGFR Ex 19 del	With MX-ICP	DEL	AGGAATTAAGAGAAGC	90.9	7205	6550
0.005% EGFR Ex 19 del	With MX-ICP	DEL	AGGAATTAAGAGAAGC	73.6	3218	2368

RESULTS: (cfDNA from patients)

Collaboration with Dr. Filip Janku and MD Anderson

Utility in Monitoring cfDNA during patient treatment



Feasibility of ICP for Mutation Determination in cfDNA: Treatment Selection Treatment Monitoring Tissue/Plasma Concordance

	Patient #C	Disease	Tissue Mutation	Treatment prior to baseline cfDNA	Baseline cfDNA Mutations	Follow up cfDNA Mutation	Treatment Response
Cancers without Available Tissue	5	Erdheim-Chester[1]	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
	6	Papillary Thyroid Cancer[1]	*BRAF* V600E	No	*BRAF* V600E	Wild-Type	SD
Concordance with Tissue Samples	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
Monitoring & correction due response to drug	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

[1]Diseases with limited Tumor Tissue Abbreviations: SD, Stable Disease; PD, Progressive Disease

CONCLUSIONS:

Transgenomic'c proprietary MX-ICP is designed as an upstream process that can be used with various sequencing platforms for the simultaneous detection of:
Multiple Sequence Alterations (all known and unknown mutations in one reaction)
Multiple Genes (MX-ICP provides sufficient sample for targeted resequencing)
Multiple Samples (NGS allows simultaneous screening of many patients)

The Veriti Thermal Cycler is useful for MX-ICP for 6 different Tc's.

MX-ICP provides a PCR product that can be used on different sequence analysis platforms

Transgenomic's MX-ICP assay was used in conjunction with downstream sequencing platforms for the detection of the EGFR Exon 20 T790M point mutation and the common EGFR Exon 19 deletion at levels ≤ 0.01%
- ✓ Sanger sequencing was able to detect the low level mutations for T790M but not the EGFR Exon 19 deletion.
- ✓ NGS was able to detect the T790M mutations at these low levels when the MX-PCR was followed by ICP.
- ✓ NGS was able to detect the EGFR Exon 19 deletion at the 0.01% and the 0.005% levels when the MX-PCR was followed by ICP.

Analysis of cfDNA from patients showed :
- ✓ Utility of ICP for patient treatment decision when tumor tissue is unavailable
- ✓ Useful of ICP for monitoring patient treatment

IN SUMMARY:
Transgenomic's MX-ICP in combination with NGS provides an ideal process for the simultaneous detection of multiple sequence alterations in multiple genes for multiple samples. It is especially suited for mutational analysis of cfDNA.