Multiplexed ICE COLD-PCR (MX ICP): A Highly Sensitive Mutation Detection Methodology that can Achieve Sensitivities <0.01% on Sanger and NGS on a Single Plate

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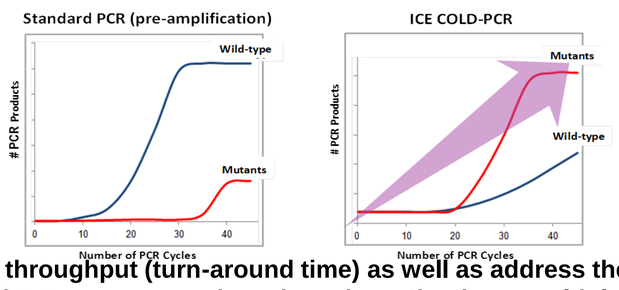
BACKGROUND

Blood-based mutation analysis from circulating free DNA (cfDNA) 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.

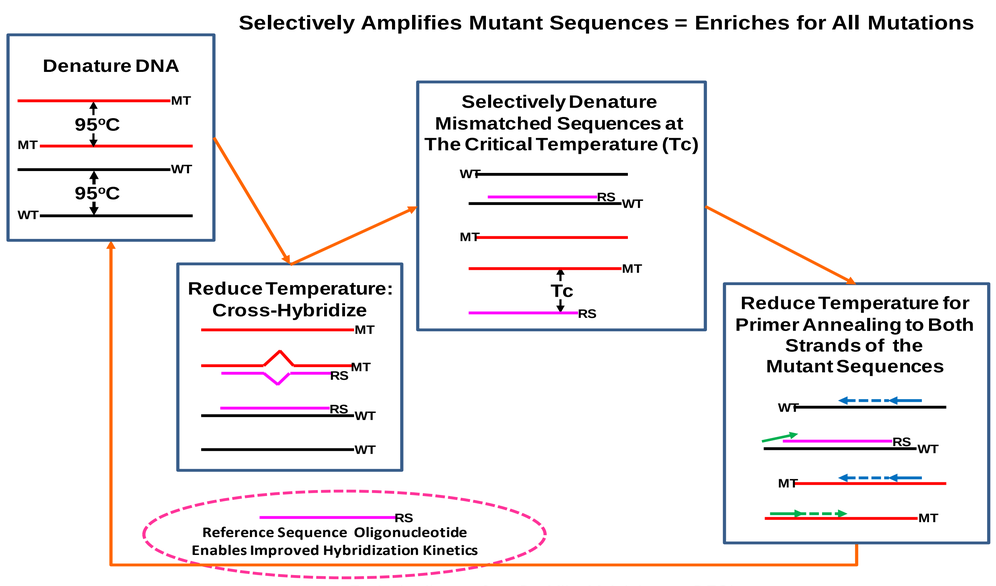
ICE COLD-PCR (ICP) technology is capable of high sensitivity detection for both point mutations and insertion/deletions through unbiased enrichment of relevant gene regions. ICP is a method that preferentially amplifies low levels of mutant DNA in a sample containing a vast excess of wild-type DNA. The 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 mutant sequences present.



In order to increase throughput (turn-around time) as well as address the limited amounts of DNA present from cfDNA sources, we have been investigating a multiplex approach for ICP (MX ICP). The first step is to harmonize all ICP reactions. Given that optimal thermal parameters for individual ICP reactions are different at this stage, we investigated using a Veriti thermal cycler to simultaneously amplify samples for 1st round ICP and then a 2nd round nested PCR (to provide enough product for sequencing) . In this proof of concept study, we performed a multiplex ICP analysis of *EGFR* Exons 19 and 20, *KRAS* Exons 2 and 3, and *NRAS* Exons 2 and 3 followed by Sanger sequencing and Ion Torrent NGS.

METHOD

ICE COLD PCR Overview:



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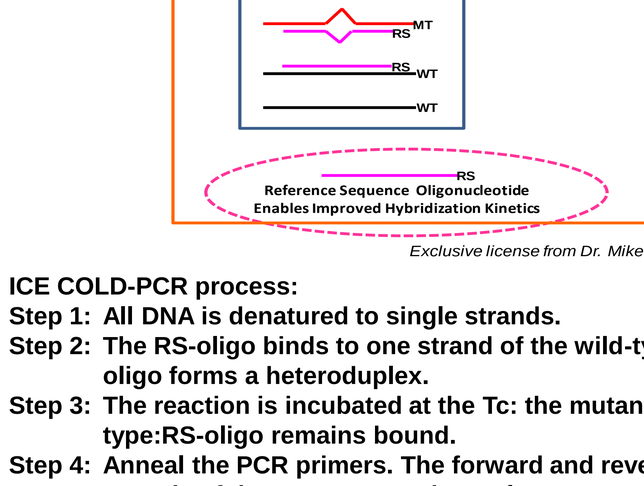
ICE COLD-PCR process:

Step 1: All DNA is denatured to single strands.
Step 2: The RS-oligo binds to one strand of the wild-type and mutant sequences: mutant:RS-oligo forms a heteroduplex.
Step 3: The reaction is incubated at the Tc: the mutant:RS-oligo denatures but the wild-type:RS-oligo remains bound.
Step 4: Anneal the PCR primers. The forward and reverse PCR primers will bind to both strands of the mutant DNA, but only one strand of the wild-type.
Step 5: Extension of the PCR primers along the mutant and wild-type DNA sequences. The mutant sequence will undergo exponential amplification while the amplification of the wild-type sequence will be linear.
Step 6: Perform standard Sanger Sequencing reactions.
Step 7: Analyze using an DNA sequencer.

ICE COLD-PCR using Veriti Thermal Cycler:



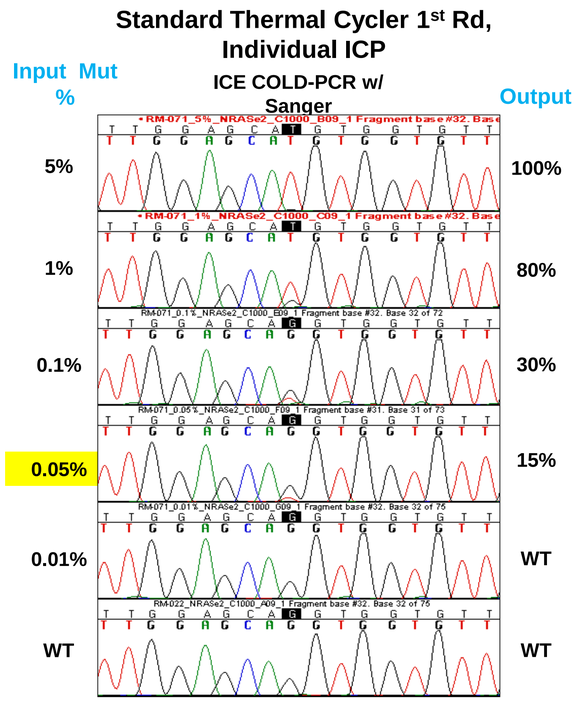
96-Well Layout for Proof of Concept

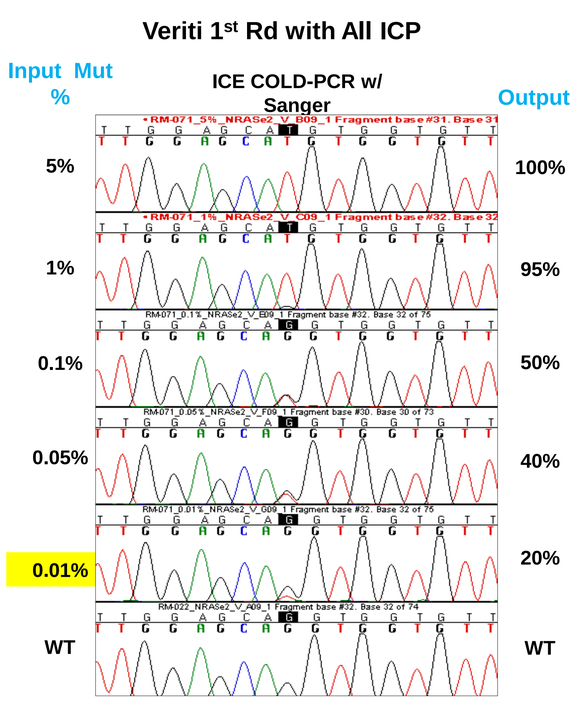
Temperature Profiles					
EGFR 19	EGFR 20	KRAS 2	KRAS 3	NRAS 2	NRAS 3
RS_{A1}	RS_{A2}	RS_{A3}			
TC_1	TC_2	TC_3	TC_4	TC_5	TC_6
P_{A1}	P_{A2}	P_{A3}	P_{A4}		P_{A5}

The Veriti thermal cycler allows 6 zones for varying temperature profiles within a thermal cycler run. By harmonizing the number of cycles as well as the times of the cycles, multiple ICE COLD-PCR reactions for different amplicons possessing different thermal profiles can be performed on a single thermal cycler.

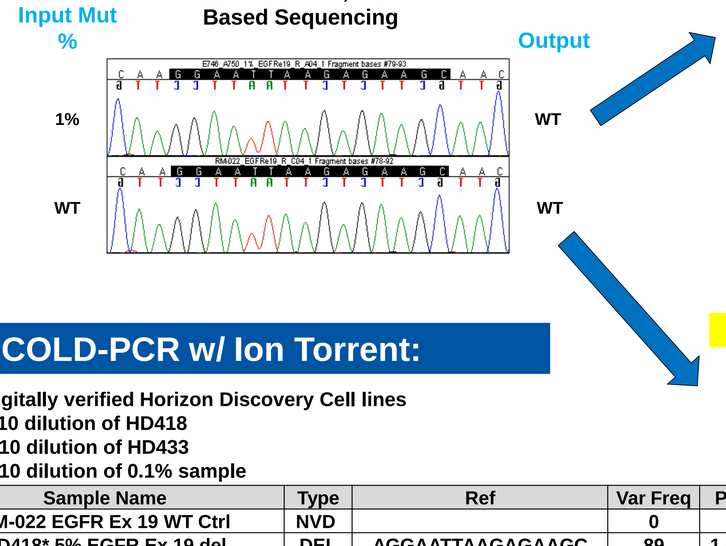
RESULTS:

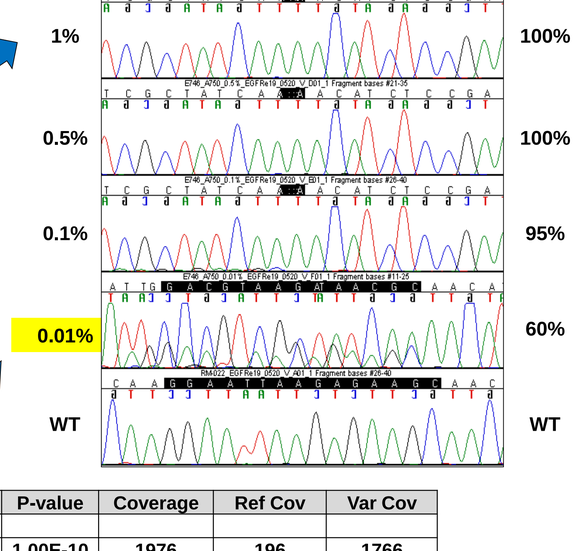
NRAS Exon 2 (G12C) LOD:





EGFR Exon 19 (ΔE746-A750 delGGAATTAAGAGAAGC) LOD, Veriti:





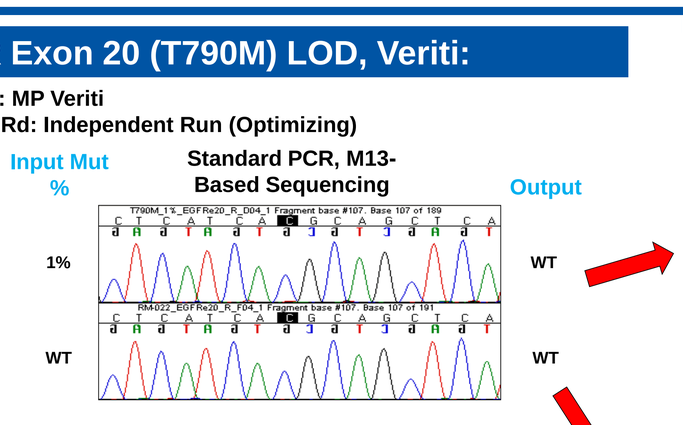
ICE COLD-PCR w/ Ion Torrent:

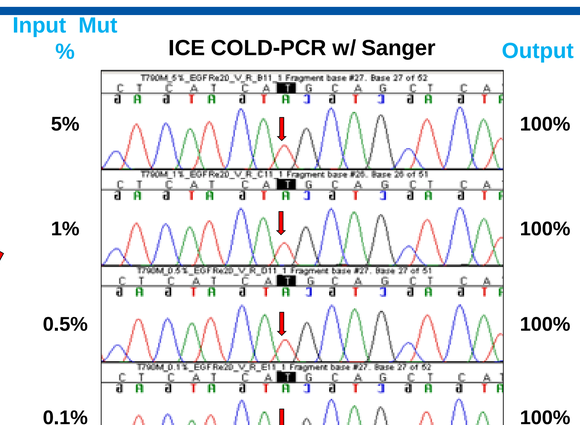
*** Digitally verified Horizon Discovery Cell lines**
[1] 1:10 dilution of HD418
[2] 1:10 dilution of HD433
[3] 1:10 dilution of 0.1% sample

Sample Name	Type	Ref	Var Freq	P-value	Coverage	Ref Cov	Var Cov
RM-022 EGFR Ex 19 WT Ctrl	NVD		0				
HD418* 5% EGFR Ex 19 del	DEL	AGGAATTAAGAGAAGC	89	1.00E-10	1976	196	1766
HD433* 1% EGFR Ex 19 del	DEL	AGGAATTAAGAGAAGC	80	1.00E-10	1962	358	1569
0.5% EGFR Ex 19 del [1]	DEL	AGGAATTAAGAGAAGC	87	1.00E-10	1987	248	1724
0.1% EGFR Ex 19 del [2]	DEL	AGGAATTAAGAGAAGC	62	1.00E-10	1934	720	1193
0.01% EGFR Ex 19 del [3]	DEL	AGGAATTAAGAGAAGC	18	1.00E-10	1967	1598	345
RD10-475 EGFR Ex 19 WT rep 1	NVD		0				
RD10-475 EGFR Ex 19 WT rep 2	NVD		0				
RD10-475 EGFR Ex 19 WT rep 3	NVD		0				

EGFR Exon 20 (T790M) LOD, Veriti:

First Rd: MP Veriti
Second Rd: Independent Run (Optimizing)





ICE COLD-PCR w/ Ion Torrent:

*** Digitally verified Horizon Discovery Cell lines**
[1] Frequency below significance
[2] 1:10 dilution of HD420
[3] 1:10 dilution of HD435
[4] 1:10 dilution of 0.1% sample

Sample Name	Type	Ref	Variant	Var Freq	P-value	Coverage	Ref Cov	Var Cov
RM-022 EGFR Ex 20 WT Ctrl[1]	SNP	C	T	2	7.94E-06	7862	7668	194
HD420* 5% EGFR Ex 20 T790M	SNP	C	T	100	1.00E-10	17211	7	17204
HD435* 1% EGFR Ex 20 T790M	SNP	C	T	100	1.00E-10	14945	12	14933
0.5% Ex 20 T790M[2]	SNP	C	T	100	1.00E-10	11432	9	11423
0.1% Ex 20 T790M[3]	SNP	C	T	99	1.00E-10	6245	36	6209
0.01%Ex 20 T790M[4]	SNP	C	T	99	1.00E-10	8706	90	8616
RD10-018 EGFR Ex 20 WT			NVD	0				
RD10-471 EGFR Ex 20 WT			NVD	0				

CONCLUSIONS

We have shown the utility of using the Veriti thermal cycler for 6 Multiplexed ICE COLD-PCR (MX ICP) reactions within a single thermal cycling run on a single plate.

Sensitivities with 6 MX ICP reactions within a single thermal cycler run on a single plate comparable to individual ICP reactions on a standard thermal cycler (6 separate reactions and plates).

Sensitivities <0.01% for the MX ICP data shown using the Veriti thermal cycler.

Ramp rates and other thermal cycler parameters need to be investigated. Some optimization is still required.

Next steps include:

1. **Test true multiplex pre-amplification PCR as precursor to MX ICP for amplicons of interest. This will allow testing of all amplicons from a single aliquot of original sample.**
2. **Test MX ICP on Veriti thermal cycler.**