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 insertions/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 of 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:

- Selectively Amplifies Mutant Sequences = Enhances for All Mutations
- Denature DNA
- Reduce Temperature: Cross-Hybridization
- Selectively Denature Mutated Sequences at The Critical Temperature (Tc)
- Reduce Temperature for Primer Annealing to Both Strands of the Mutant Sequences

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 a DNA sequencer.

ICE COLD-PCR using Veriti Thermal Cycler:

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.

ALEX

80% 90% 100% 100% 100%

90% 80% 70% 60% 50%

70% 60% 50% 40% 30%

60% 50% 40% 30% 20%

50% 40% 30% 20% 10%

40% 30% 20% 10% 0%

30% 20% 10% 0% 0%

20% 10% 0% 0% 0%

10% 0% 0% 0% 0%

0% 0% 0% 0% 0%


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 precurser 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.

CONCLUSIONS