Combination of DISSECT[a] and COLD-PCR Technologies for Ultra-Sensitive Detection of Mutations Using Either Sanger or Next Generation Sequencing Methodologies

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[a]Patent pending

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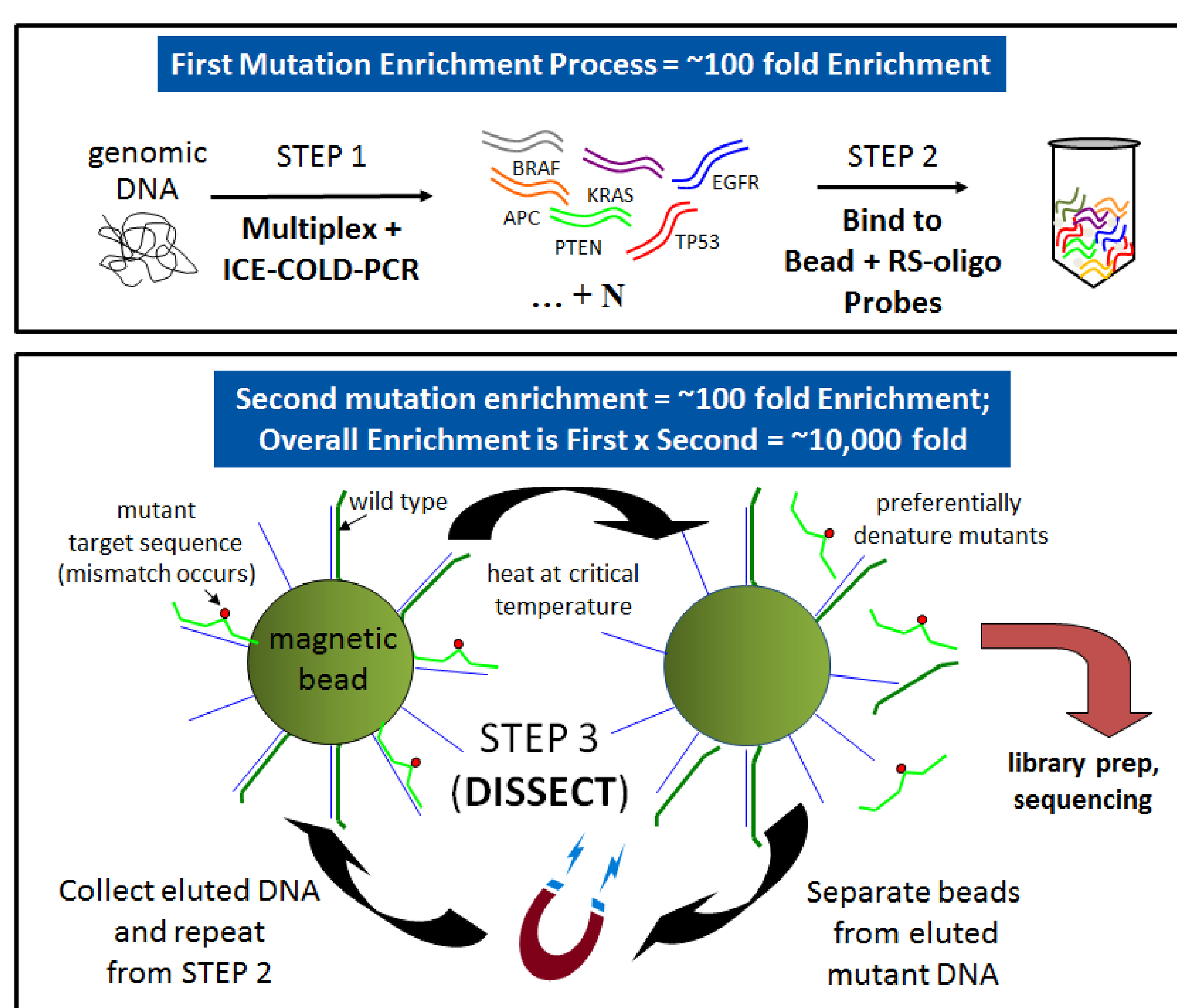
BACKGROUND

- Low abundance mutations are particularly important for early cancer detection, assessment of residual disease post surgery or radiation, disease staging, and monitoring of therapy following remission/relapse (Milbury *et al.* 2009).
- The ICE COLD-PCR technology is capable of simultaneous enrichment of mutations targeted by a ~50 base reference oligonucleotide in a modified PCR reaction followed by DNA sequencing using Next Generation Sequencing (NGS). This workflow results in a 50 to > 100 fold increase in the sensitivity compared to NGS alone.
- Attaining a sensitivity of 0.01% is critical for the accepted use of mutational analysis in circulating free DNA (cfDNA) for cancer treatment monitoring, surveillance and management.
- Other platform such as BEAMing, digital PCR, CastPCR and the OnTarget methodology developed by Boreal Genomics, claim to detect mutations at the 0.01% level, however all of these methodologies rely on some type of an allele-specific probe or capture probe. Therefore for each new mutation discovered, a new probe has to be made and validated.
- Differential Strand Separation at Critical Temperature (DISSECT) uses magnetic beads to enrich for mutation strands in the absence of an enzyme, relying mostly on the denaturation properties of the DNA heteroduplex.
  - The DISSECT methodology provides further enrichment of any mutation present in the ICE COLD-PCR amplified DNA.
  - DISSECT uses the same RS-oligo as used in the initial ICE COLD-PCR reaction, but now uses that RS-oligo to preferentially bind up the wild-type DNA so that the DNA used for NGS is further enriched for the mutations without using additional PCR cycles.

METHODS



- Plasmid DNA containing *KRAS* mutations was mixed with WT plasmid DNA resulting in serial dilutions of the mutations.
  - A single *KRAS* Exon 2 ICE COLD-PCR assay was used to enrich all mutations.
  - Mutations were confirmed using Sanger and Next Generation Sequencing.
- Samples of the ICE COLD-PCR product were sent to the DFCI for capture by biotinylated probes coupled to streptavidin-coated magnetic beads by a stepdown anneal procedure, DISSECT.
  - DISSECT preferentially releases single-stranded mutant DNA from the beads by denaturation at a critical temperature (T_c) followed by magnetization.
  - DISSECT-enriched mutations were analyzed using Sanger and Next Generation Sequencing.

RESULTS

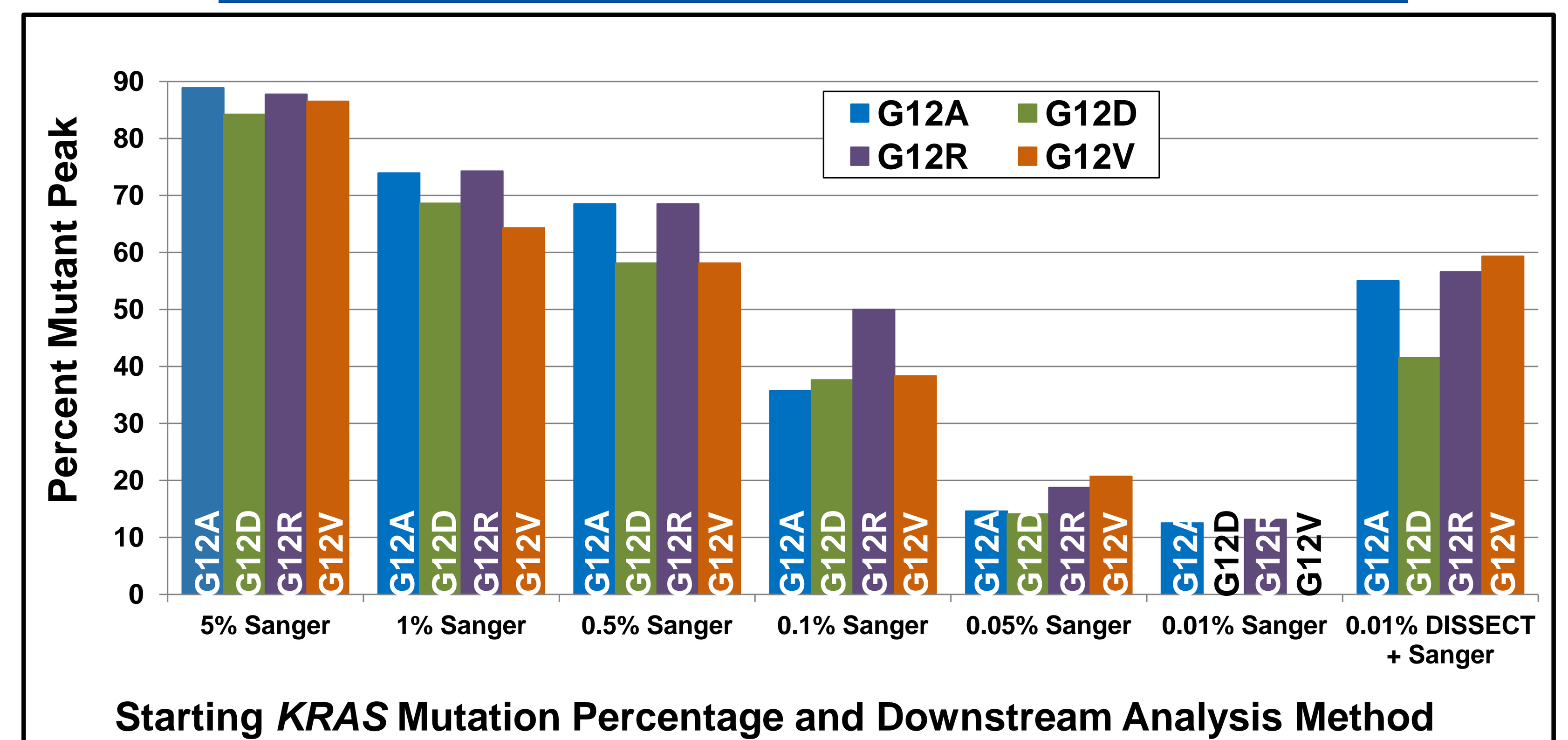
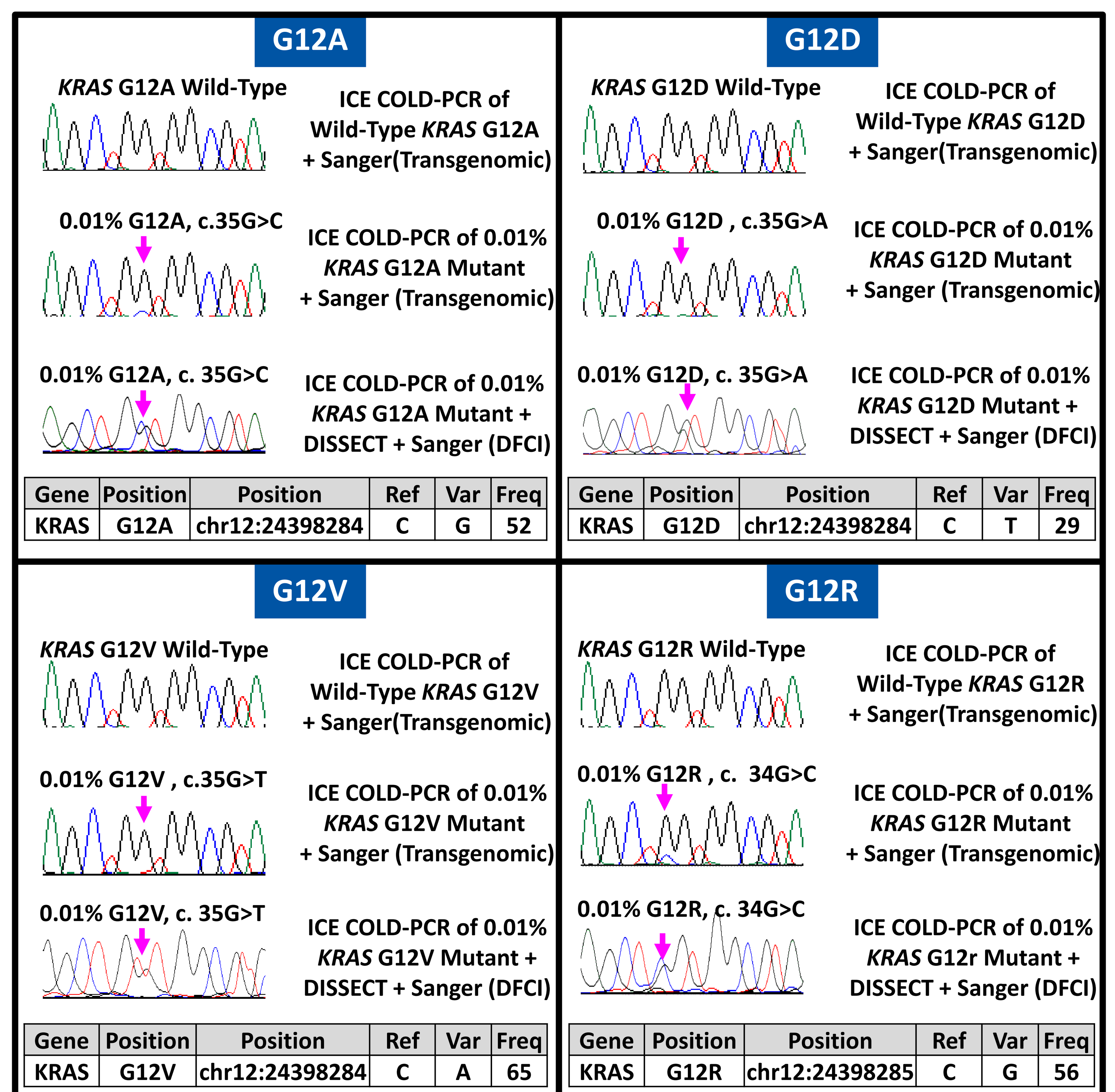


Figure 1: Serial dilutions (5, 1, 0.5. 0.1. 0.05 and 0.01%) of *KRAS* mutations were enriched using one ICE COLD-PCR assay. The samples were analyzed by Sanger sequencing and the relative percent of the mutant peak was determined. The 0.01% mutant samples were sent to the Dana Farber for DISSECT and Sanger sequencing. The last set of bars shows the relative percent mutant peak of each of the 0.01% mutants when the ICE COLD-PCR reactions were subjected to DISSECT prior to Sanger sequencing.



Gene	Position	Position	Ref	Var	Freq
KRAS	G12A	chr12:24398284	C	G	52

Gene	Position	Position	Ref	Var	Freq
KRAS	G12D	chr12:24398284	C	T	29

Gene	Position	Position	Ref	Var	Freq
KRAS	G12V	chr12:24398284	C	A	65

Gene	Position	Position	Ref	Var	Freq
KRAS	G12R	chr12:24398285	C	G	56

Figure 2: The Sanger sequencing electropherograms of *KRAS* exon 2 mutations following ICE COLD-PCR at Transgenomic and then analysis of the same ICE COLD-PCR products further enriched for mutations by the use of DISSECT followed by Sanger performed at the DFCI. Samples that had undergone the DISSECT methodology after ICE COLD-PCR were also analyzed for mutations using the Ion Torrent PGM instrument. The NGS results are displayed as Excel data.

CONCLUSIONS

- The use of the ICE COLD-PCR RS-oligo in DISSECT allows Sanger sequencing for detection of *KRAS* mutations when present in the starting material at 0.01%
  - Further dilutions need to be tried to determine the LOD of ICE COLD-PCR followed by DISSECT
- Samples analyzed using NGS showed detection of the 0.01% mutation after DISSECT
  - This indicates that a multiplex panel of mutations can be analyzed from cfDNA using one ICE COLD-PCR assay paired with DISSECT
- When limited numbers of cfDNA samples and limited numbers of gene regions need to be analyzed, the ICE COLD-PCR, DISSECT methodology coupled to Sanger sequencing provides a cost-effective alternative to NGS
- Several important lung cancer gene regions are being tested for simultaneous enrichment using ICE COLD-PCR and DISSECT.