

of 0.4 ng/L, upper reference limit (99th percentile) approximately 80 ng/L] (5).

The demographics of our study population presenting early with type 1 AMI are similar to those of the parent cohort (age 68.8 years, women 23.1%, primary symptom chest pain 84.6%, previous percutaneous coronary intervention 15.4%).

At each of the 3 time points we found a strong linear correlation between cMyC and cTnI, as we previously observed in an ambulatory population (5): Spearman  $\rho$  0.795 ( $P < 0.01$ ) at presentation, 0.902 ( $P < 0.01$ ) at 3 h, and 0.888 ( $P < 0.01$ ) at the late time point. Nonetheless, the ratio of cMyC to cTnI was the highest at presentation, thereafter decreasing significantly with time from presentation, mean ratio at presentation, 7.98 [median, 2.72; interquartile range (IQR), 3.48]; at 3 h, 2.67 (median, 1.83; IQR, 1.40); and at the late time point, 1.71 (median, 0.63; IQR, 1.09)—all  $P < 0.01$  by Friedman 2-way ANOVA by ranks. Furthermore, the ratio was also significantly greater than that we observed previously in a stable cohort without obstructive coronary artery disease and with a cTnT  $< 14$  ng/L (mean 1.97) (5).

Although the mean ratio of cMyC:cTnI was highest at presentation, there was substantial individual heterogeneity among the 26 patients with type 1 AMI (Fig. 1). The relative concentration of cMyC to cTnI at presentation soon after symptom onset in those with type 1 AMI was higher than in the same patients at later time points and in ambulatory patients at low risk. The more rapid rise of cMyC vs cTnI that we have observed in patients with type 1 AMI should enable their more rapid/accurate triage. However, the diagnostic performance of cMyC, with and without cTnI, needs further evaluation.

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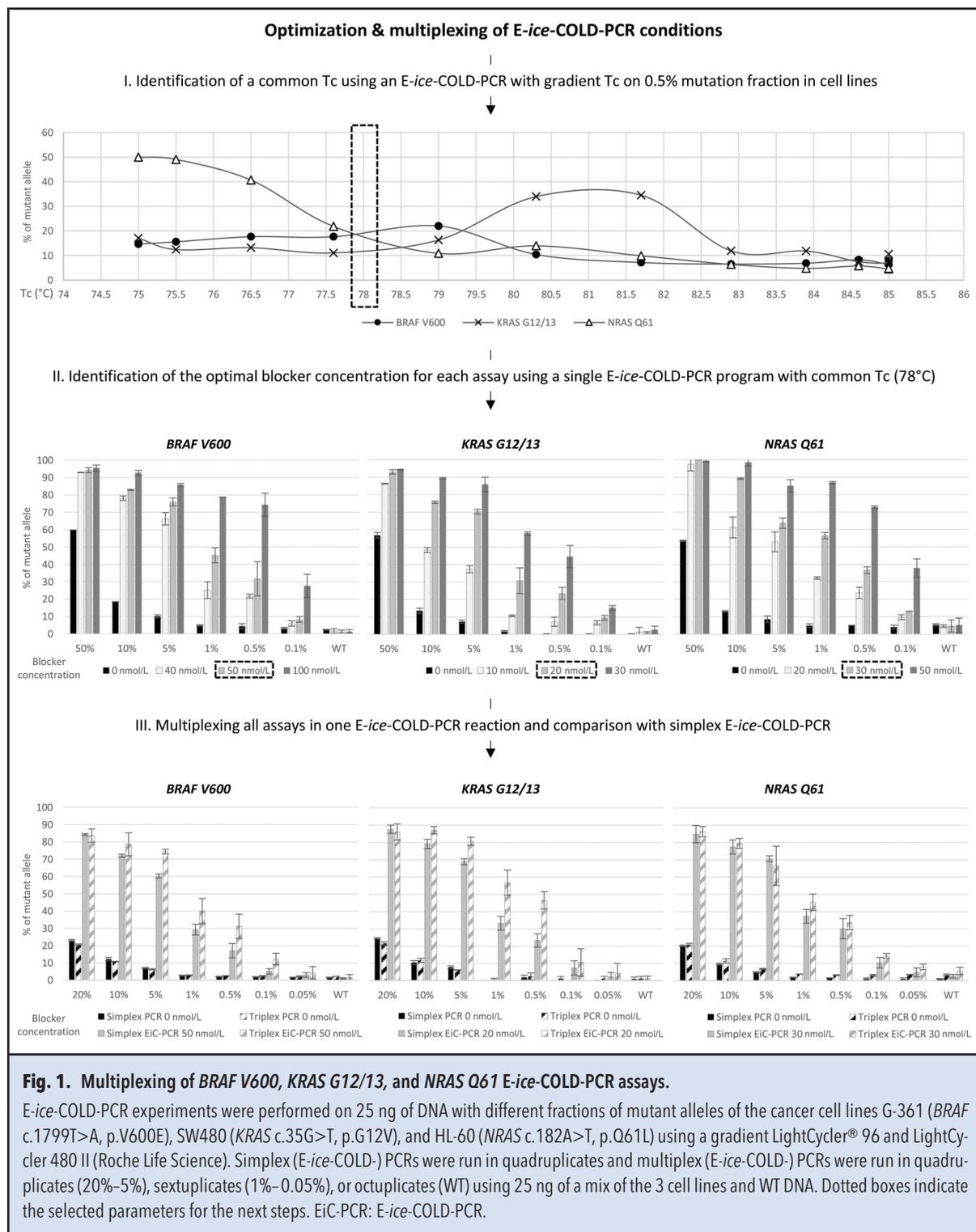
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## Multiplexing of E-ice-COLD-PCR Assays for Mutation Detection and Identification

### To the Editor:

Enhanced-ice-COLD-PCR allows the detection of mutations in a single mutation hotspot down to 0.05% of mutant alleles without prior knowledge of the mutated base, and their identification by subsequent (pyro)sequencing experiments (1). This approach is useful for diagnosis, choice of a personalized treatment, prediction, or the monitoring of treatment response, and has been successfully performed on fresh frozen and formalin-fixed, paraffin-embedded tissue, and circulating cell-free DNA (1, 2).



The method is a further development of *ice*-COLD-PCR (3) and based on the incorporation of locked-nucleic acid nucleotides in the blocker probe complementary to the wild-type (WT)<sup>1</sup> sequence (1). During each PCR cycle, denaturation is followed by hybridization at 70 °C, where WT-blocker and mutant-blocker heteroduplexes are formed, mutant-blocker heteroduplexes are denatured at a critical temperature (T<sub>c</sub>) higher than 75 °C, and primers are hybridized at the annealing temperature and elongated at 72 °C, leading to the enrichment of mutations underlying the blocker sequence (1).

Enhanced-*ice*-COLD-PCR can enrich all types of mutations, allows stronger mutation enrichment, and is less sensitive to variations of the T<sub>c</sub> ( $\pm 5$  °C) compared to *full*-, *fast*-, or *ice*-COLD-PCR, for which a slight variation (0.2 °C) can abolish mutation enrichment (1, 3, 4). Therefore, different *full*-, *fast*-, or *ice*-COLD-PCR assays might be less suitable for working under a single thermocycling program and even less suitable to multiplexing. For the specific case of T<sub>m</sub>-reducing mutations (where T<sub>m</sub> is the temperature at which 50% of double-stranded DNA or DNA/RNA hybrids is denatured) (C:G>A:T or >T:A), which represent 60%–70% of all mutations in cancer, these issues have been addressed by the development of temperature-tolerant (TT)-COLD-PCR, which relies on the use of a T<sub>c</sub> incremented by 0.3–0.5 °C for each defined number of cycles, leading to mutation enrichment when the T<sub>c</sub> falls in the right temperature window (5, 6).

To evaluate the potential of *E-ice*-COLD-PCR to multiplexing, 3 previously published assays were

selected: B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)<sup>2</sup> V600, KRAS proto-oncogene, GTPase (*KRAS*) G12/13, and neuroblastoma RAS viral oncogene homolog (*NRAS*) Q61 (1, 2, 7). A multiplex *E-ice*-COLD-PCR reaction was performed to amplify these genes in a single reaction followed by mutation detection in 3 different pyrosequencing assays.

The first step was the optimization of the amplification of the 3 hotspots using the same PCR conditions. The annealing temperature of each assay was set to 60 °C, which required the modification of the *NRAS* Q61 primers (forward: TTGTTGGA-CATACTGGATACAG; reverse: biotin-ATGACTTGCTATTATTG-ATGGC). To identify the best T<sub>c</sub> common to all assays, a gradient *E-ice*-COLD-PCR (T<sub>c</sub> 75–85 °C) was performed using cell lines bearing either *BRAF* V600E (G-361), *KRAS* G12V (SW480), or *NRAS* Q61L (HL-60) mutation at 0.5% abundance with a fixed quantity of blocker. 78 °C was chosen as the optimal T<sub>c</sub>, taking into account the mutation enrichment level, the yield of PCR product, and the absence of primer dimers (Fig. 1).

The determination of the optimal quantity of blocker was performed before multiplexing using serial dilutions of the 3 cell lines ranging from 50% to 0.1% abundance and different concentrations of blocker probes (Fig. 1). Thus, the concentrations selected for multiplex experiments were of 50 nmol/L for *BRAF*, 20 nmol/L for *KRAS*, and 30 nmol/L for *NRAS*.

Multiplex *E-ice*-COLD-PCR reactions were performed by reducing the quantity of each primer to half (100 nmol/L) on serial dilutions of a mix of the 3 cell lines with WT DNA. The results showed a good

performance and high specificity of the multiplex *E-ice*-COLD-PCR with the 3 assays, which in each case presented a stronger mutation enrichment compared to simplex PCR, probably due to the decrease in primer concentration (Fig. 1). This was confirmed by simplex *E-ice*-COLD-PCR experiments performed using either 100 or 200 nmol/L of primers, which resulted in a higher mutation enrichment for the reactions with the lower primer concentration (data not shown). The limit of detection was evaluated at 0.05% for *BRAF* ( $P = 0.004$ ) and 0.1% for *KRAS* and *NRAS* ( $P = 0.012$  and  $P = 0.002$ ) using a Wilcoxon rank-sum test. Due to the quantitative resolution of the pyrosequencer (7%–10%) and the low-level background signal present in some WT samples, we recommend a 10% mutation threshold to consider a sample as mutated (1, 2). Multiplexed *E-ice*-COLD-PCR was also evaluated on lower amounts of DNA and showed reproducible results for 2.5 ng of input DNA using, respectively, 20 nmol/L, 6.5 nmol/L, and 10 nmol/L of *BRAF* V600, *KRAS* G12/13, and *NRAS* Q61 blocker probes with a detection limit of 0.5% mutant allele, showing its potential application to cell-free circulating DNA (data not shown).

We have demonstrated that *E-ice*-COLD-PCR can overcome the problems of multiplexing encountered by most COLD-PCR methods without loss of sensitivity. This is principally due to the high flexibility of its T<sub>c</sub>, and this advantage should thus allow its use for massive multiplexing before next-generation sequencing experiments.

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<sup>1</sup> Nonstandard abbreviations: WT, wild-type sequence; T<sub>c</sub>, critical temperature; T<sub>m</sub>, temperature at which 50% of double-stranded DNA or DNA/RNA hybrids is denatured; TT, temperature tolerant.

<sup>2</sup> Human genes: *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *KRAS*, KRAS proto-oncogene, GTPase; *NRAS*, neuroblastoma RAS viral oncogene homolog.

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