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Application of COLD-PCR for improved detection of *KRAS* mutations in clinical samples

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***KRAS* mutations have been detected in approximately 30% of all human tumors, and have been shown to predict response to some targeted therapies. The most common *KRAS* mutation-detection strategy consists of conventional PCR and direct sequencing. This approach has a 10–20% detection sensitivity depending on whether pyrosequencing or Sanger sequencing is used. To improve detection sensitivity, we compared our conventional method with the recently described co-amplification-at-lower denaturation-temperature PCR (COLD-PCR) method, which selectively amplifies minority alleles. In COLD-PCR, the critical denaturation temperature is lowered to 80°C (*vs* 94°C in conventional PCR). The sensitivity of COLD-PCR was determined by assessing serial dilutions. Fifty clinical samples were used, including 20 fresh bone-marrow aspirate specimens and the formalin-fixed paraffin-embedded (FFPE) tissue of 30 solid tumors. Implementation of COLD-PCR was straightforward and required no additional cost for reagents or instruments. The method was specific and reproducible. COLD-PCR successfully detected mutations in all samples that were positive by conventional PCR, and enhanced the mutant-to-wild-type ratio by >4.74-fold, increasing the mutation detection sensitivity to 1.5%. The enhancement of mutation detection by COLD-PCR inversely correlated with the tumor-cell percentage in a sample. In conclusion, we validated the utility and superior sensitivity of COLD-PCR for detecting *KRAS* mutations in a variety of hematopoietic and solid tumors using either fresh or fixed, paraffin-embedded tissue.**
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KRAS, a member of the RAS oncogene family, plays a key role in RAS/MAPK signaling, which is involved in multiple cellular processes, including proliferation, differentiation and apoptosis.[1] Activating mutations of the *KRAS* gene impair GTPase activity resulting in constitutive activation. Up to 30% of all human tumors harbor an activating mutation in this gene, most commonly in lung, colon, and pancreatic cancers, and hematopoietic neoplasms.[2] Screening tumors for possible *KRAS* mutation has multiple clinical applications and is gaining increasing attention in clinical practice. For instance, in lymphoma patients with KRAS mutations quantitative assessment of mutation levels in follow-up specimens provides an effective way to monitor and quantify residual disease and to detect relapse.[3] In lung cancer patients, *KRAS* mutation is an adverse prognostic factor.[4,5] Accumulating evidence has shown that *KRAS* mutation status can be highly predictive for patient response to anti-epidermal growth factor receptor (EGFR) antibody therapies, such as cetuximab and panitumumab, in colorectal cancer, lung, and head and neck cancers.[6–9] These therapies are exclusively effective in tumors with wild-type *KRAS*.[10–13] In colorectal cancer patients, in particular, *KRAS* mutation testing has quickly become standard-of-care.[14] In the era of targeted therapy and personalized medicine, requests for *KRAS* mutation testing will increase, and are likely to do so exponentially. In the molecular diagnostic laboratory at our institution, a doubling of requests for *KRAS* mutation occurred in the year 2008.

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Currently, the gold standard for *KRAS* mutation detection is conventional PCR amplification followed by direct sequencing.[15] Most other detection methods reported in literature[16–19] also involve PCR. PCR amplification enables mutation detection from a very small amount of samples. However, conventional PCR methods do not selectively amplify the mutant sequences in tumor specimens, which is needed for optimal sensitivity in specimens with a low tumor-cell count or high non-neoplastic cell content. On the contrary, in conventional PCR the predominant wild-type sequences have a competitive advantage over mutant sequences in being amplified, leaving the mutant sequences more difficult to be detected by downstream methods. To improve the sensitivity of mutation detection, tumor-cell enrichment methods, such as cell sorting or microdissection, are commonly required before DNA extraction for PCR. However, tumor-cell enrichment is not always achievable, particularly in samples with abundant desmoplastic stroma surrounding a minority of tumor cells and abundant inflammatory cells, or in scanty tumor cells after chemotherapy or radiation therapy in the follow-up setting.[20,21] As a result, conventional PCR-based assays are limited in their ability to identify low levels of mutation-bearing tumor cells and this may greatly effect on therapeutic decisions.

Co-amplification-at-lower denaturation-temperature PCR (COLD-PCR) is a novel modification of the conventional PCR method that selectively amplifies minority alleles from a mixture of wild-type and mutant sequences irrespective of the mutation type or position within the sequence.[22] This method is based on the observation that there is a critical denaturation temperature (Tc) for each DNA sequence, which is lower than its melting temperature (Tm). PCR amplification efficiency for a DNA sequence drops abruptly if the denaturation temperature is set below its Tc.

In this study, we compared COLD-PCR with the conventional PCR for the detection of *KRAS* mutations in 50 clinical samples. The data show that COLD-PCR is up to four times more sensitive than the conventional PCR method, able to detect 1.5% of tumor cells with KRAS mutation. COLD-PCR was also equal to the conventional PCR method in its reproducibility and did not require additional costs in either reagents or instruments.

Materials and methods

Clinical Samples and Genomic DNA Extraction

Clinical specimens were obtained from archival DNA in the Clinical Molecular Diagnostics Laboratory at The University of Texas MD Anderson Cancer Center. A total of 50 samples formed the study group, including DNA extracted from 20 fresh bone-marrow aspirate specimens involving hematopoietic neoplasms, and DNA extracted from 30 solid tumor specimens that were routinely fixed in formalin and paraffin embedded. The clinical characteristics of the patients from whom these specimens were derived are listed in Table 1. All samples had been tested earlier for *KRAS* codon 12 and 13 mutations as part of their clinical work up using our routine pyrosequencing analysis following conventional PCR. Genomic DNA from fresh bone-marrow aspirate specimens was extracted using the Gentra AUTOPURE LSTM automated DNA extraction system (Qiagen Systems, Valencia, CA, USA). The percentage of tumor cells in each specimen was obtained from a review of the manual differential counts listed in the pathology reports associated with each case. For solid tumor specimens, the hematoxylin–eosin stained slides were reviewed, and the percentage of tumor was estimated as a percentage of tumor cells to non-neoplastic cells by visual inspection. Using the hematoxylin–eosin-stained slide as a guide, tumor areas on the unstained formalin-fixed paraffin-embedded (FFPE) sections were manually micro-dissected and DNA was extracted using the QIAmp DNA mini kit (Qiagen Systems).

Detection of *KRAS* Mutation by Conventional PCR and Pyrosequencing

KRAS mutation testing has been routinely carried out in our laboratory using conventional PCR followed by pyrosequencing. An amplicon of 98 bp is amplified using a forward primer, 5′-TATAA ACTTGTGGTAGTTGG-3′, and a reverse biotinylated primer, 5′-biotin-ATTGTTGGATCATATTCGT-3′. The PCR master mix contains the forward and reverse primers (each 0.2 μmol/l), 250 μmol/l of dNTP mix, 2.5 mmol/l $MgCl_2$, 1 × PCR buffer (Applied Biosystems, Carlsbad, CA, USA), 1 U of AmpliTaq Gold and 200 ng of sample genomic DNA in a total volume of 50 μl. PCR-cycling conditions consist of initial denaturing at 95°C for 2 min; 49 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 10 min. The reactions are carried out on an ABI 2720 Thermocycler (Applied Biosystems). All samples are run in duplicate and patient samples are processed along with a positive control sample (positive for the presence of a GGT-to-GCT mutation at codon 12), a negative control sample and a reagent control. The PCR products are electrophoresed in an agarose gel to confirm successful amplification of the 98-bp PCR product before pyrosequencing. The PCR products (each 15 µl) are then sequenced by the Pyrosequencing PSQ96 HS System (Biotage AB, Uppsala, Sweden), following the manufacturer's instructions using the pyrosequencing primer, 5′-CTTGTGGT AGTTGGAGCT-3′. Each sample is sequenced with two separate programs of nucleotide dispensation orders: 5′-TACGACTGC-3′, designed for detecting mutations at codon 12; and 5′-TGTATCGATCGT-3′, designed for detecting mutations at codon 13. This

Table 1 Clinical characteristics of the 50 patients in the study group

No.	*Age (59.52 ± 14.81 years)*	*Gender (M:F = 1.27)*	*Sample type*	*Diagnosis*
1	57	F	BM	MDS
2	84	M	BM	AML (M2)
3	48	M	BM	MDS
4	33	F	BM	AML
5	63	M	BM	MDS
6	60	M	BM	AML (M4)
7	50	F	BM	AML (M2)
8	57	M	BM	CMML
9	35	F	BM	AML (M5)
10	51	F	BM	AML
11	31	F	BM	AML (M5)
12	78	M	BM	CMML
13	78	M	BM	AML
14	72	M	BM	MDS
15	77	M	BM	MDS
16	79	M	BM	AML (M4)
45	39	F	BM	AML (M4)
46	77	M	BM	MDS
47	39	M	BM	ALL-B
48	49	F	BM	AML (M2)
17	66	F	Bladder	Colon cancer metastasis
18	45	F	Liver	Colon cancer metastasis
19	54	M	Colon	Colon cancer metastasis
20	67	F	Rectum	Rectal cancer metastasis
21	75	F	Rectum	Rectal cancer metastasis
22	45	F	Liver	Colon cancer metastasis
23	77	M	Colon	Colon cancer metastasis
24	54	F	Liver	Colon cancer metastasis
25	46	F	Lung	Colon cancer metastasis
26	66	F	Liver	Rectal cancer metastasis
27	72	F	Colon	Adenocarcinoma
28	37	F	Colon	Adenocarcinoma
29	66	M	Rectum	Adenocarcinoma
30	76	M	Colon	Adenocarcinoma
31	76	M	Rectum	Adenocarcinoma
32	69	M	Colon	Adenocarcinoma
33	76	F	Colon	Adenocarcinoma
34	55	F	Colon	Adenocarcinoma
35	45	M	Colon	Adenocarcinoma
36	78	F	Colon	Adenocarcinoma
37	76	M	Colon	Adenocarcinoma
38	57	M	Colon	Adenocarcinoma
39	63	M	Lung	Adenocarcinoma
40	38	M	Rectum	Adenocarcinoma
41	45	M	Colon	Adenocarcinoma
42	65	F	Colon	colon rectal cancer
43	65	M	Liver	Adenocarcinoma
44	45	M	Liver	Adenocarcinoma
49	69	M	Retroperitoneal	Pancreatic adenocarcinoma
50	51	M	Colon	Adenocarcinoma

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome.

primer and the dispensation orders enable us to capture all possible mutations of the wild-type sequence of GGT GGC at codons 12 and 13 of *KRAS*.

Cold-PCR for *KRAS* Mutation Detection

We applied the COLD-PCR assay for detecting *KRAS* mutations based on the methodology described in the literature,[22] with several modifications. A key consideration in designing a COLD-PCR assay that selectively amplifies the minority mutant alleles is to determine a new reduced denaturation temperature for the reaction. Ideally, this reduced denaturation temperature allows mainly the heteroduplexes to be denatured and amplified, and leaves the homoduplexes double-stranded and not amplified efficiently. We used the same primers as used in our conventional PCR assay, which produces an amplicon of 98 bp with 41.8% of GC content and a Tm of 70.9°C. Using the Poland algorithm,[23] we plotted Tm profiles of the wild-type sequence along with those of mismatched sequences at each base pair within *KRAS* codons 12 and 13 (Figure 1). On the basis of this information, we set the reduced denaturation temperature of the COLD-PCR reaction at 80°C. The

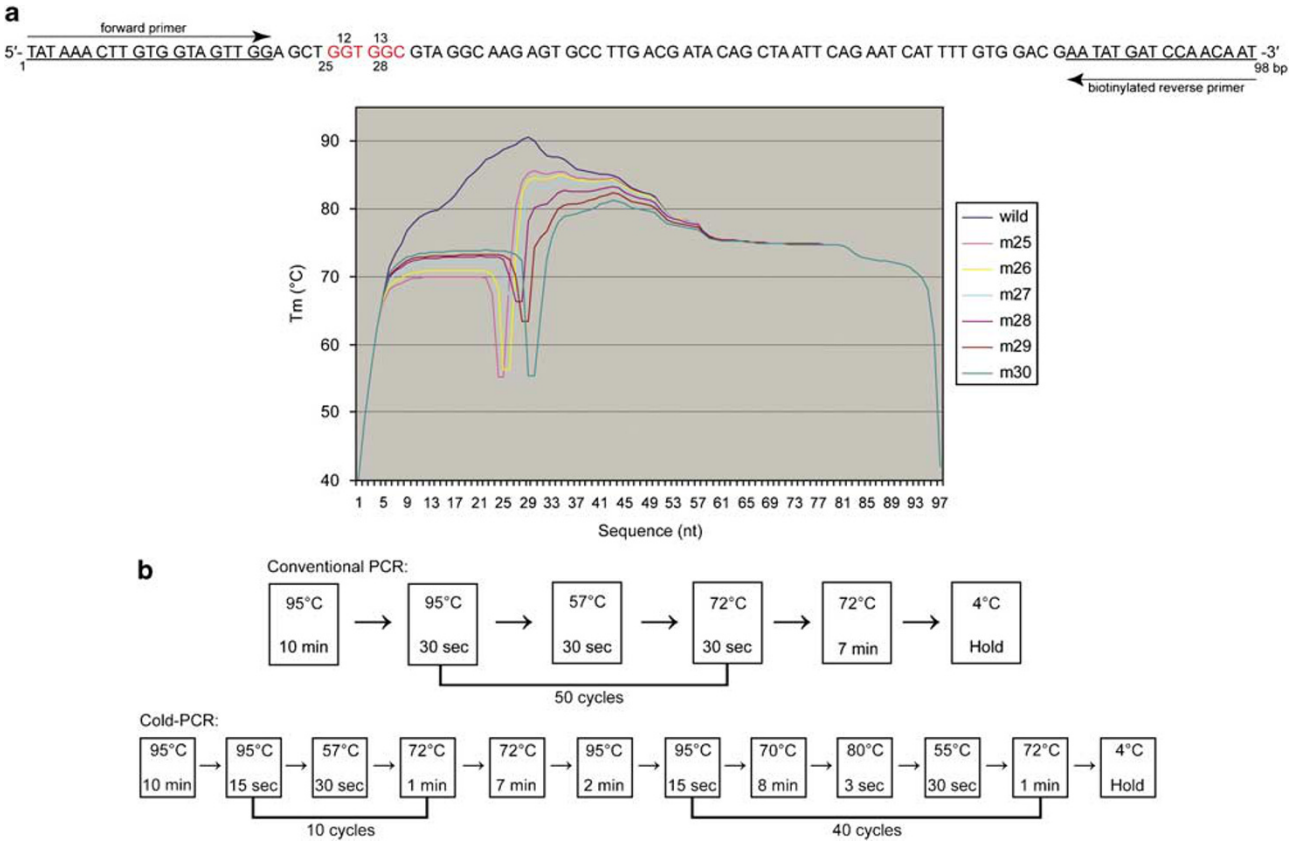

Figure 1 Design of the full co-amplification at lower denaturation temperature PCR COLD-PCR assay for *KRAS* mutations detection. (**a**) Nucleotide sequence of the 98-bp PCR product and melting temperature (Tm) profiles of the wild-type and mutant sequences at each of the six nucleotides of codons 12–13. Arrows indicate primer sequences (underlined). Sequence of codons 12–13 is indicated in red. Figure legends indicate Tm profiles for wild-type ('wild') and mutant ('m' and nucleotide number, eg 'm25') sequences. (**b**) Description of the PCR reaction conditions used in this study for conventional PCR and COLD-PCR protocols.

reaction protocol started with 10 cycles of the conventional PCR amplification for an initial build-up of all amplicons, followed by 40 COLD-PCR cycles to selectively enrich for mutant sequences. The initial conventional PCR cycling conditions are summarized as follows: 95°C for 10 min; 10 cycles at 95°C for 15 s, 57°C for 30 s, and 72°C for 1 min; 72°C for 7 min; and 95°C for 2 min. Then 40 cycles of COLD-PCR were performed at 95°C for 15 s, 70°C for 8 min, 80°C for 3 s, 55°C for 30 s, and 72°C for 1 min (Figure 1). Full COLD-PCR reactions were performed on an ABI 2720 Thermocycler (Applied Biosystems). The COLD-PCR products were electrophoresed in agarose gels to confirm successful amplification of the 98-bp PCR product prior to pyrosequencing as described above. As with our routine sequencing assay, a positive control, a negative control and a reagent control were included in each run. All samples were run in duplicate.

Comparison of Sensitivities of Cold-PCR and Conventional PCR

We performed a serial dilution study to examine the enhancement of the sensitivity of mutation detection by COLD-PCR in comparison with conventional PCR. A patient DNA sample containing a GGT to GCT mutation at codon 12 was used as the source of the mutant allele. This mutation-containing DNA sample was serially diluted with a wild-type DNA sample to obtain 1:2, 1:4, 1:8, 1:16, 1:32, 1:40 and 1:50 mutant-to-wild-type mixtures. All of the DNA mixtures were simultaneously subjected to conventional PCR and COLD-PCR followed by pyrosequencing, as described above. All samples were run in duplicate. The mutant-to-wild-type ratio was defined as the ratio of the peak height of a single-mutant nucleotide over the peak height of a single wild-type nucleotide on pyrograms. The enhancements were represented as fold changes of the average mutation-to-wild-type ratios of the same dilution between COLD-PCR and conventional PCR amplifications.

Statistical Analysis

Student *t*-tests were carried out wherever applicable. A *P*-value of <0.05 was considered statistically significant. Associations between two variables were determined by calculating the Pearson correlation coefficient.

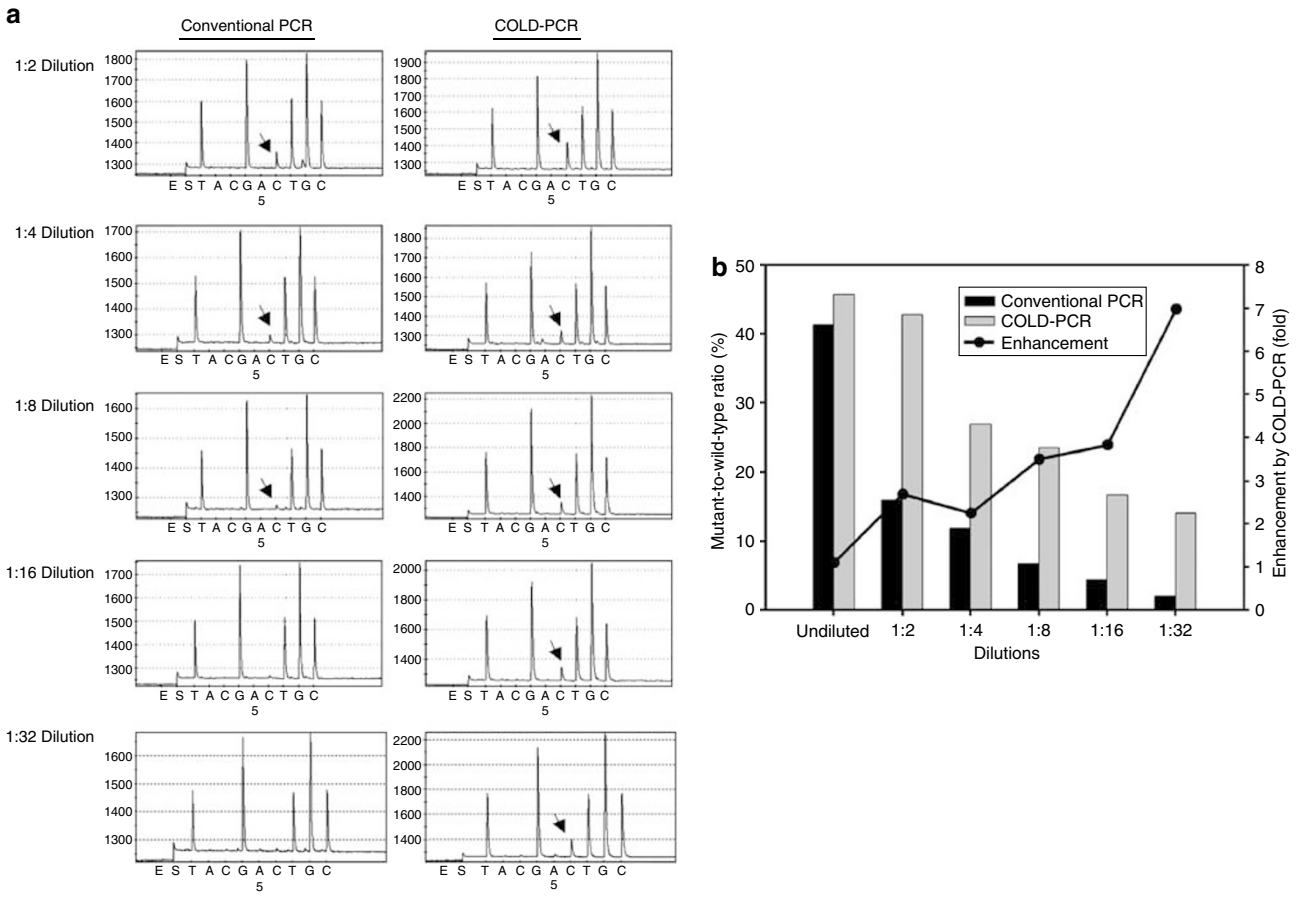

Figure 2 Sensitivity determined by dilution study. (**a**) Representative pyrograms of serial-dilutions study with conventional PCR and co-amplification-at-lower denaturation-temperature PCR (COLD-PCR). (**b**) Comparison of pyrosequencing results from undiluted to1:32 dilution after conventional PCR and COLD-PCR (bars), and enhancement of COLD-PCR in serial dilutions (line).

Results

Determination of the Sensitivity with Dilution Study

Figure 2a shows the pyrograms from the dilution study. With the conventional PCR amplification, the mutant-nucleotide peak on the pyrogram became indistinguishable at 1:8 dilution, whereas it was clearly present at 1:32 dilution with COLD-PCR. As the mutant sample we used for the dilution study was heterozygous at *KRAS* codon 12, a 1:32 dilution translates to approximately 1.5% of the detection sensitivity, which is four times better than the 6% sensitivity obtained with the conventional PCR method.

As shown in Figure 2b, with increasing serial dilutions, the mutation-to-wild-type ratio decreased proportionally in a first-order kinetics manner in both conventional PCR and COLD-PCR. The enhancement of mutation detection by COLD-PCR increased exponentially with increasing dilution. With the original specimen, which contains approximately 50% mutant allele, COLD-PCR and conventional PCR had roughly the same efficiency in amplifying mutant alleles. At 1:32 dilution, COLD-PCR showed a sixfold greater efficiency in amplifying mutant allele compared with conventional PCR.

Within the detectable dilution range, all duplicate analyses of COLD-PCR products produced consistent pyrosequencing results. Different runs from the same sample also generated very similar results (correlation coefficient, 0.923).

Comparison of *KRAS* Mutation Detection Using Conventional PCR and COLD-PCR in Patient Samples

With the sensitivity of the COLD-PCR method obtained from the dilution study above, we further examined the potential enhancement of *KRAS* mutation detection by COLD-PCR over conventional PCR in different types of clinical samples. A total of 50 patient samples were tested, including 20 fresh bone-marrow aspirate samples from patients with acute leukemia (mostly myeloid with one lymphoblastic) and myelodysplastic syndromes (MDS) and 30 fixed paraffin-embedded samples of adenocarcinomas arising in the colon, rectum, liver, lung, bladder and pancreas (primary neoplasms or sites of metastasis; Table 1). The results using conventional PCR and COLD-PCR are summarized in Tables 2 and 3.

Table 2 Comparison of mutant-to-wild-type-ratio by conventional PCR and COLD-PCR in fresh bone-marrow aspirate samples

No.	*Age*	*Diagnosis (FAB classification)*	*Mutation type*	*Nucleotide change*	*Tumor cell count (%)*	*Ratio by conventional PCR (%)*[a]	*Ratio by COLD-PCR (%)*	*Enhancement by COLD-PCR (fold)*
1	57	MDS	G12D	GGT to GAT	4	1.7	1.8	1.0
2	84	AML (M2)	G12D	GGT to GAT	31	23.7	55.3	2.3
3	48	MDS	G13S	GGC to AGC	2	6.2	12.1	1.9
4	33	AML	G12V	GGT to GTT	83	28.9	51.6	1.8
5	63	MDS	G12S	GGT to AGT	4	57.7	67.7	1.2
6	60	AML (M4)	G12D	GGT to GAT	52	2.4	3.1	1.3
7	50	AML (M2)	G12D	GGT to GAT	40	47.2	66.0	1.4
8	57	CMML	G12D	GGT to GAT	10	64.1	68.0	1.1
9	35	AML (M5)	G12V	GGT to GTT	75	39.4	57.0	1.5
10	51	AML	G12D	GGT to GAT	36	20.7	31.2	1.5
11	31	AML (M5)	G12D	GGT to GAT	80	19.7	23.3	1.2
12	78	CMML	G12R	GGT to CGT	14	50.3	54.5	1.1
13	78	AML	G12D	GGT to GAT	90	44.7	40.6	0.9
14	72	MDS	G12R	GGT to CGT	8	16.5	21.9	1.3
15	77	MDS	G12R	GGT to CGT	80	32.5	35.0	1.1
16	79	AML (M4)	G12D	GGT to GAT	35	24.7	38.8	1.6
48	49	AML (M2)	G12D	GGT to GAT	3	0.0	8.1	

AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; COLD-PCR, co-amplification-at-lower denaturation-temperature PCR; MDS, myelodysplastic syndrome.
[a]Mean tumor cell count = 38.06%.

In all 35 samples were shown to harbor *KRAS* mutations at codon 12, 2 had mutations of codon 13, and 13 were negative for mutation.

In most cases, COLD-PCR enhanced *KRAS* mutation detection by up to 4.74-fold, with an average enhancement of 1.55-fold ± 0.76. Moreover, in a patient with acute myeloid leukemia after chemotherapy, in a follow-up bone marrow sample that tested negative for *KRAS* mutations by conventional PCR, COLD-PCR showed a GTT–to-GAT mutation at codon 12 (Figure 3b). This same mutation was present at the time of an initial diagnosis, three months earlier.

Mutation detection in fixed, paraffin-embedded specimens of solid tumors (1.67 ± 0.95) was also enhanced by COLD-PCR. In particular, COLD-PCR showed significant higher enhancement in the solid tumor samples, in which the mutant-to-wild-type ratios by conventional PCR were less than 50% (1.94 ± 0.93 *vs* 0.86 ± 0.36, $P = 0.022$). Neither location or type of solid tumors nor the type of hematopoietic neoplasms had any effect on enhancement. The tumor-cell count or percentage did not correlate with the mutant-to-wild-type peak ratio in pyrograms using either conventional PCR or COLD-PCR (correlation coefficient of 0.452 and 0.244, respectively). However, lower tumor-cell content in the specimens was significantly associated with greater enhancement; the average enhancement for samples with 50% or less tumor cells was 1.89 ± 0.88, *vs* 1.34 ± 0.26 in samples with higher than 50% tumor cells ($P = 0.012$). In four cases in which COLD-PCR failed to improve the mutation detection (enhancement <1), the tumor cell percentages were over 80%.

A total of eight different types of KRAS point mutations at codons 12 or 13 were detected in these samples by pyrosequencing (Table 2). All but two of these mutations were at codon 12. These mutations involved four G–A transitions (G12D, G12S, G13D, G13S), two G–T transversions (G12V, G12C) and two G–C transversions (G12A, G12R). The frequency of each type of mutation at codon 12 (listed in decreasing order) and its percentage in all positive samples from this study were: G12D (GAT, 49%) >G12V (GTT, 24%) >G12R (CGT, 11%) >G12S (AGT, 5%) >G12A (GCT, 3%) or G12C (TGT, 3%). The G12D mutation was most frequent in acute myeloid leukemias (seven of nine positive cases), whereas the G12D and G12V mutations were most frequent in colorectal carcinoma (nine and seven in 19 positive cases, respectively). The two cases with mutations at codon 13 were a case of MDS (G13S) and a case of colon carcinoma (G13D). Enhancement by COLD-PCR among different mutation types (nucleotide exchanges and locations) did not show any significant differences.

Discussion

The main purpose of this study was to evaluate the potential of COLD-PCR to enhance the detection of *KRAS* mutations in clinical samples. The current sensitivity of the *KRAS* mutation test in our laboratory using conventional PCR methods followed by pyrosequencing is approximately 10%. This requires that specimens submitted for testing have approximately 20% tumor cells, accounting for the fact that each neoplastic cell may be

Table 3 Comparison of mutant-to-wild-type-ratio by conventional PCR and COLD-PCR in formalin-fixed paraffin-embedded tissue samples

No.	*Age*	*Sample type*	*Diagnosis*	*Mutation type*	*Nucleotide change*	*Tumor percentage*[a]	*Ratio by conventional PCR (%)*	*Ratio by COLD-PCR (%)*	*Enhancement by COLD-PCR (fold)*
17	66	Bladder	Colon cancer metastasis	G12D	GGT to GAT	60	38.0	45.2	1.2
18	45	Liver	Colon cancer metastasis	G12V	GGT to GTT	30	20.2	40.0	2.0
19	54	Colon	Colon cancer metastasis	G12A	GGT to GCT	10	1.4	6.4	4.7
20	67	Lymph node	Rectal cancer metastasis	G12V	GGT to GTT	70	42.0	56.0	1.3
21	75	Colon and rectum	Rectal cancer metastasis	G12D	GGT to GAT	80	43.0	46.5	1.1
22	45	Liver	Colon cancer metastasis	G12C	GGT to TGT	80	53.2	53.7	1.0
23	77	Colon	Colon cancer metastasis	G12V	GGT to GTT	90	52.9	17.2	0.3
24	54	Liver	Colon cancer metastasis	G12S	GGT to AGT	80	48.0	46.3	1.0
25	46	Lung	Colon cancer metastasis	G12D	GGT to GAT	80	58.6	64.7	1.1
26	66	Liver	Rectal cancer metastasis	G12D	GGT to GAT	50	35.8	76.2	2.1
27	72	Colon	Adenocarcinoma	G12D	GGT to GAT	90	69.6	47.8	0.7
28	37	Colon	Adenocarcinoma	G12V	GGT to GTT	50	42.6	90.1	2.1
29	66	Rectum	Adenocarcinoma of rectum	G12D	GGT to GAT	5	17.2	45.7	2.7
30	76	Colon	Adenocarcinoma	G13D	GGC to GAC	50	43.3	56.1	1.3
31	76	Rectum	Adenocarcinoma	G12R	GGT to CGT	50	17.5	37.0	2.1
32	69	Colon	Adenocarcinoma	G12V	GGT to GTT	40	19.2	49.0	2.6
33	76	Colon	Adenocarcinoma	G12V	GGT to GTT	80	30.8	51.7	1.7
34	55	Colon	Adenocarcinoma	G12V	GGT to GTT	60	42.5	60.0	1.4
35	45	Colon	Adenocarcinoma	G12D	GGT to GAT	70	52.2	61.0	1.2
36	78	Colon	Adenocarcinoma	G12D	GGT to GAT	80	48.4	88.6	1.8

COLD-PCR, co-amplification-at-lower denaturation-temperature PCR.
[a]Mean tumor percentage = 60.25%.

heterozygous and thus may carry only one mutated allele. In our laboratory, solid tumor samples are routinely micro-dissected to enrich tumor-cell content, but this approach can be challenging when using small biopsy specimens. Enhancing the sensitivity of the KRAS assay potentially eliminates this tumor-cell enrichment step, saving manpower and time.

Our dilution study demonstrated that COLD-PCR increased the KRAS mutation detection sensitivity from 1:8 to 1:32 (fourfold), and that this approach is both reliable and reproducible. Adoption of the method was straightforward, requiring no additional cost for reagents and instruments. With its selective enhancement of minority mutant-allele amplification, the resultant increased sensitivity also could potentially shorten the overall turnaround time by reducing the repeats needed when results are equivocal using conventional PCR methods.

In this study, we identified a total of eight different point mutations, six at codon 12 and two at codon 13. Mutations at codon 12 were by far the most common, found in 35 of the 37 mutation-positive samples. There was roughly an equal frequency of transition and transversion mutations identified in these samples, even though transition mutations have been found to be about an order of magnitude more common than transversions among all point mutations.[24] Mutations that switch nucleotide types may also slightly affect the melting temperature,[25,26] which might change the Tc of the COLD-PCR reaction. However, using a fixed Tc for all types of mutations in this study, we did not observe a significant difference in the enhancements by COLD-PCR among mutation types, suggesting that this effect may be insignificant.

As the dilution study results indicated, enhancement by COLD-PCR was non-linear and more prominent when the concentrations of mutation-containing sequences were lower. The most likely explanation is that during each cycle of COLD-PCR, only the heteroduplexes are denatured and subsequently amplified. In an ideal situation, with sufficient amplification cycles, the amplification product at the end of the reaction will contain almost equal amounts of mutant and wild-type sequences. Therefore, the maximum mutant-to-wild-type ratio in COLD-PCR products should be close to 1.

The lower enhancement by COLD-PCR observed in our study (<2-fold on average) as compared with the 3–12-fold enhancement reported earlier by others may be explained by decisions we routinely make before analysis.[22] First, we require all submitted samples to contain at least 20% tumor cells, and we routinely carry out micro-dissection on fixed, paraffin-embedded samples to enrich tumor-cell content. Second, our conventional PCR reactions comprise 50 cycles compared with 40 cycles in a study reported earlier. Both of these steps boost the concentration of mutation-containing sequences,

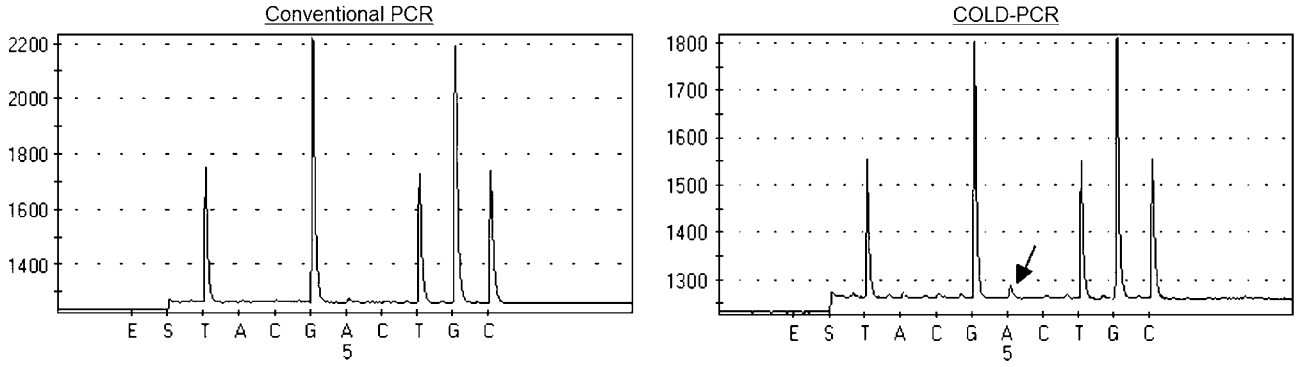

Figure 3 Comparison of pyrograms of sample no.48 after conventional PCR and co-amplification-at-lower denaturation-temperature PCR (COLD-PCR) amplification. The arrow indicates a G12D mutation identified by COLD-PCR, which is not visible in conventional PCR.

and therefore minimize the potential for enhancement of mutation by COLD-PCR to further enrich the mutant sequence. In addition, a whole genome amplification (WGA) step was applied to all the specimens before COLD-PCR in the reported study. It is also possible that WGA further dilutes the mutant allele concentrations.

An intriguing issue with increasing the sensitivity of *KRAS* mutation detection is understanding what level of mutation has clinical significance. Will the ability to detect very low levels of mutation, on the order of 1–2%, be as significant as a high level? Although it has been shown that patients with carcinomas that have *KRAS* mutations as well as patients with KRAS wild-type tumors do not benefit from anti-EGFR therapy, one may argue that patients with a low level of *KRAS* mutations could still benefit from the therapy. Clinical trials are necessary to assess the quantitative effects of *KRAS* mutation on patient response to EGFR antibody therapy.

In conclusion, our findings confirm that the COLD-PCR method can increase the sensitivity of *KRAS* mutation detection in various clinical samples, including DNA extracted from either fresh or fixed, paraffin-embedded tissue specimens. COLD-PCR can be a good strategy for mutation detection in specimens with high non-neoplastic cell content, in small specimens in which neoplastic cells are difficult to micro-dissect and therefore enrich, and whenever a mutation is suspected to be present but is undetectable using conventional PCR and sequencing methods.

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Conflict of interest

The authors declare no conflict of interest.

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