HAL
open science

Increased sensitivity of KRAS mutation detection by High-Resolution Melting analysis of COLD-PCR products

Lasse Sommer Kristensen, Iben Lyster Daugaard, Mariann Christensen, Stephen Hamilton-Dutoit, Henrik Hager, Lise Lotte Hansen

To cite this version:

Lasse Sommer Kristensen, Iben Lyster Daugaard, Mariann Christensen, Stephen Hamilton-Dutoit, Henrik Hager, et al.. Increased sensitivity of KRAS mutation detection by High-Resolution Melting analysis of COLD-PCR products. Human Mutation, 2010, 31 (12), pp.1366. 10.1002/humu.21358 . hal-00588662

HAL Id: hal-00588662

https://hal.science/hal-00588662v1

Submitted on 26 Apr 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Human Mutation

Increased sensitivity of KRAS mutation detection by High-Resolution Melting analysis of COLD-PCR products

Journal:	*Human Mutation*
Manuscript ID:	humu-2010-0267.R2
Wiley - Manuscript type:	Methods
Date Submitted by the Author:	13-Aug-2010
Complete List of Authors:	Kristensen, Lasse; Aarhus University, Institute of Human Genetics Daugaard, Iben; Aarhus University, Institute of Human Genetics Christensen, Mariann; Aarhus University Hospital, Department of Pathology Hamilton-Dutoit, Stephen; Aarhus University Hospital, Department of Pathology Hager, henrik; Aarhus University Hospital, Department of Pathology Hansen, Lise Lotte; Aarhus University, Institute of Human Genetics
Key Words:	Cancer, KRAS mutations, COLD-PCR, High-Resolution Melting

SCHOLARONE™
Manuscripts

Increased sensitivity of *KRAS* mutation detection by High-Resolution Melting analysis of COLD-PCR products

Running head: *KRAS* mutation detection by HRM analysis of COLD-PCR products

Authors: Lasse S. Kristensen[1], Iben L. Daugaard[1], Mariann Christensen[2], Stephen Hamilton-Dutoit[2], Henrik Hager[2], and Lise Lotte Hansen[1]

[1]Institute of Human Genetics, University of Aarhus, The Bartholin Building, Wilhelm Meyers Allé 4, DK-8000 Aarhus C, Denmark

[2]Department of Pathology, Aarhus University Hospital, Nørrebrogade 45, DK-8000 Aarhus C, Denmark

Corresponding author: Lasse Sommer Kristensen, Institute of Human Genetics, Aarhus University, Wilhelm Meyers Allé, Bygn. 1242 , Universitetsparken, Aarhus C, 8000, Denmark. Tel: (+45-89) 42 16 59, Fax: (+45-86) 12 31 73, Email: lasse@humgen.au.dk

Keywords: Cancer, *KRAS* mutations, COLD-PCR, High-Resolution Melting

Abbreviations: EGFR, epidermal growth factor receptor; HRM, high-resolution melting; COLD-PCR, coamplification at lower denaturation temperature-PCR; T_m, melting temperature; FFPE, formalin fixed paraffin embedded; PB, peripheral blood; dNTP, deoxynucleoside triphosphate; T_c, critical denaturation temperature.

For Peer Review

Abstract

Considerable effort has been invested in the development of sophisticated technologies enabling detection of clinically significant low-level tumor specific *KRAS* mutations. Coamplification at lower denaturation temperature-PCR (COLD-PCR) is a new form of PCR that selectively amplifies mutation-containing templates based on the lower melting temperature of mutant homoduplexes versus wild type homoduplexes. We have developed a fast COLD-PCR and High-Resolution Melting (HRM) protocol to increase the sensitivity of *KRAS* mutation detection. The clinical applicability of COLD-PCR for *KRAS* mutation detection was assessed by analyzing 61 colorectal cancer specimens, for which *KRAS* mutation status has been evaluated by the FDA approved TheraScreen® *KRAS* mutation kit. The sensitivity was increased by 5 to 100 fold for melting temperature decreasing mutations when using COLD-PCR as compared to standard PCR. Mutations, undetectable by the TheraScreen® kit in clinical samples, were detected by COLD-PCR followed by HRM and verified by sequencing. Finally, we have observed a previously undescribed low prevalence synonymous mutation (*KRAS* c.39C>T, codon 13) in colorectal cancer specimens and in the peripheral blood from an unaffected individual. In conclusion, COLD-PCR combined with HRM is a simple way of increasing the sensitivity of *KRAS* mutation detection without adding to the complexity and cost of the experiments.

For Peer Review

Introduction

Activation of the epidermal growth factor receptor (EGFR; MIM# 131550) stimulates proliferation, angiogenesis, invasion, and survival of cancer cells. Since EGFR is frequently overexpressed in a range of solid tumors it represents an important target for cancer treatment. Monoclonal antibodies targeting EGFR such as panitumumab and cetuximab have proved to significantly increase survival rates of a subgroup of metastatic colorectal cancer patients. However, immunohistochemical staining for the EGFR protein has been found to be a poor biomarker for predicting response to EGFR inhibition (Chung, et al., 2005). In contrast, activating mutations in the *KRAS* gene (KRAS; MIM# 190070) have proven to be a biomarker for non-reponse to EGFR blockage, and both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency require that *KRAS* mutation status is determined prior to anti-EGFR treatment. The biological explanation is that KRAS is part of a mitogenic signaling cascade normally triggered by binding of EGF or other ligands to EGFR, and that oncogenic KRAS is able to transmit the signal independent of EGFR. Screening for *KRAS* mutations is not only clinically relevant in metastatic colorectal cancer. The detection of *KRAS* mutations may be used for monitoring residual disease after treatment in lymphoma patients (Cikota, et al., 2007), and evidence is also present that *KRAS* mutation status is highly predictive of response to anti-EGFR treatment in lung cancer (Massarelli, et al., 2007).

In general, tumor heterogeneity as well as contamination with normal cells in clinical samples often results in clinically relevant mutations being diluted in wild type sequences below the detection limit of conventional PCR based assays. Furthermore, highly sensitive methods are required for the detection and identification of somatic mutations in early tumorigenesis and the emergence of resistance mutations (Milbury, et al., 2009b).

For Peer Review

Traditional Sanger sequencing has been considered the gold standard for detecting and identifying genetic alterations. However, this approach suffers from a relatively low sensitivity as mutant alleles must be present in a proportion of at least 10-20% to be reproducibly detected (Vogelstein and Kinzler, 1999).

A range of different PCR-based technologies have been developed with the goal of increasing the analytical sensitivity of mutation detection (Do and Dobrovic, 2009; Newton, et al., 1989; Ogino, et al., 2005; Takeda, et al., 1993; Ward, et al., 1998). However, increased sensitivity usually comes with the cost of lower accuracy, increased complexity, and higher costs (Milbury, et al., 2009b). When applying a particular technology for routine diagnostic testing it must achieve a sufficient sensitivity without compromising accuracy, while being convenient and cost-efficient.

High-resolution melting (HRM) analysis is a convenient and cost-effective way of analyzing genetic as well as epigenetic variations (Kristensen and Hansen, 2009; Kristensen, et al., 2008; Krypuy, et al., 2007; Krypuy, et al., 2006; Ma, et al., 2009; Polakova, et al., 2008; Takano, et al., 2007; Wittwer, 2009; Wittwer, et al., 2003; Wojdacz, et al., 2008). However, the sensitivity of HRM analysis of standard PCR products has generally been found to be limited to mutant alleles present at 5%-10% in a background of wild type DNA (Krypuy, et al., 2007; Krypuy, et al., 2006; Polakova, et al., 2008; Takano, et al., 2007). Coamplification at lower denaturation temperature-PCR (COLD-PCR) is a new form of PCR that significantly increases the sensitivity of mutation detecting assays without adding to the costs and complexity of the experiment (Li, et al., 2008). However, fast COLD-PCR does not enrich for mutations that retain or increase the melting temperature (T_m) of the amplicon (Luthra and Zuo, 2009), unless the protocol is adjusted to facilitate the formation of mutation/wild type heteroduplexes. This approach is called full COLD-PCR and significantly adds to the time it takes to perform the experiments while it may only provide a moderately increased sensitivity when applied to *KRAS* mutation detection (Zuo, et al.,

For Peer Review

2009). In general, mutation enrichment can be expected to be 5-8 fold for melting temperature retaining mutations and 3-5 fold for melting temperature increasing mutations when using full COLD-PCR as opposed to standard PCR. A thorough description of the differences between fast and full COLD-PCR can be found in (Li, et al., 2008). In the human genome approximately 70% of all encountered mutations, and 84% of the somatic mutations found in colon cancer, are melting temperature decreasing (Li, et al., 2008).

We have applied fast COLD-PCR and HRM for the analysis of *KRAS* exon 2 codon 12 and 13 mutations. By using five different mutation-containing cell lines serially diluted into wild type DNA we have compared the sensitivity of the assay with HRM following standard PCR. Furthermore, we have analyzed a cohort comprising 61 colorectal cancer specimens that have been screened for *KRAS* mutations using the FDA approved TheraScreen® *KRAS* mutation kit, and directly compared the results obtained using HRM after standard PCR and after COLD-PCR, respectively. Sanger sequencing was used to confirm the HRM results. In addition, we have applied a two round COLD-PCR protocol to verify subtle deviations in melting profiles.

For Peer Review

Materials and Methods

Nucleotide and Codon Numbering

Nucleotide numbering reflects the cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference GenBank sequence NM_033360.2, according to the Journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon number 1.

Samples and DNA Extraction

Formalin-Fixed Paraffin Embedded (FFPE) blocks from surgical biopsies of 61 patients diagnosed with adenocarcinoma in colon were selected from the archives at the Institute of Pathology, Aarhus University Hospital. The specimens were between 2 and 10 years old. However, we have previously shown that PCR and HRM analysis can be successfully performed using FFPE specimens aged up to 30 years (Kristensen, et al., 2009). Specimens containing vital tumor cells were chosen by an experienced pathologist and no microdissections were performed prior to DNA extraction. For each sample, six tissue sections of 10 µm were transferred into a micro centrifuge tube and incubated in 1 ml of xylene in a thermomixer at 45°C and 800 rpm for 20 min. After centrifugation at 12,200 rpm for 5 min at room temperature, the supernatant was removed. This step was repeated once. Subsequently, the tissue samples were washed in 1 ml of 99% ethanol using a thermomixer at 60°C and 800 rpm for 20 min. After centrifugation at 12,200 rpm for 5 min the supernatant was removed. The washing procedure was repeated once. The samples were air dried in the thermomixer at 60°C for 20 min after adding one drop of acetone to each sample, and incubated with 150 µl Proteinase K (10 mg/ml) in the thermomixer at 55°C and 1,000 rpm over night. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (QIAGEN) according to the manufacturer's instructions.

DNA from peripheral blood (PB), obtained from medical students of both sexes in their first year of Medical school, was extracted following a modified salt precipitation protocol as previously described (Hansen, et al., 1998).

The Local Ethical Committee, Aarhus County, Denmark, approved this study.

Cell lines and dilution series

For Peer Review

Five cell lines containing different *KRAS* mutations were used in this study; A549 (c.34G>A, codon 12), DLD-1 (c.38G>A, codon 13), LS174T (c.35G>A, codon 12), RPMI-8226 (c.35G>C, codon 12), and SW480 (c.35G>T, codon 12). The cell lines were cultured in RPMI 1640 culture media (Invitrogen, Breda, The Netherlands) and supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 10,000 IU/ml penicillin, and 0.1 mg/ml streptomycin (both Leo Pharma, Copenhagen, Denmark) antibiotics. The cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were harvested by scraping and DNA extracted as described for PB with slight modifications; the centrifugation steps were carried out for 5 min at 13000 rpm instead of 15 min at 3000 rpm, and 1 volume of isopropanol was added instead of two volumes of ice cold ethanol.

DNA from each of the *KRAS*-mutant cell lines was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and serially diluted into wild type DNA obtained from PB from medical students to the following fractions (100%, 50%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, and 0.1%).

Primer design

The *KRAS* (GenBank accession number NM_033360.2 [http://www.ncbi.nlm.nih.gov/GenBank/]) primers were designed to immediately flank codon 12 and 13 of exon 2, to ensure that sequence variations outside codon 12 and 13 did not give rise to aberrant melting curves. The primers used were: 5'-gaatataaacttgtggtagttggagct-3' (forward primer) and 5'-atcgtcaaggcactcttgcctac-3' (reverse primer) giving an amplicon size of 56 bp. To confirm that the primers were unable to amplify from the *KRAS1P* pseudogene on chromosome 6 (GenBank accession number NC_000006.11 [http://www.ncbi.nlm.nih.gov/GenBank/]), SNP analysis of rs4516942:G>A were performed using

For Peer Review

primers specific for the pseudogene: 5'-agcggctcccagacctgct-3' (forward primer) and 5'-ggaattctctattgttggatcatattgg-3' (reverse primer).

Standard PCR and HRM Conditions

PCR cycling and HRM analysis were performed on the Rotor-Gene 6000™ (Corbett Research, Sydney, Australia). SYTO® 9 (Invitrogen) was used as the intercalating dye. The final reaction mixtures consisted of 50 ng of DNA, 1x PCR buffer, 2.5 mmol/L $MgCl_2$, 200 nmol/L of each primer, 200 µmol/L of each dNTP, 5 µmol/L of SYTO® 9, 0.5U of HotStarTaq (QIAGEN) (5U/µL) in a volume of 20 µL. The cycling protocol was initiated by one cycle at 95°C for 15 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, 72°C for 10 s, and one cycle at 95°C for 1 min. HRM was performed from 65°C to 95°C, with a temperature increase at 0.1°C/s. Samples were analyzed in triplicates (cell line experiments) or in duplicates (colorectal cancer specimens).

COLD-PCR and HRM Conditions

The COLD-PCR cycling conditions were experimentally determined by adjusting the number of initial standard PCR cycles and the critical denaturation temperature (T_c) to ensure strong mutation enrichment while maintaining adequate amplification of all dilutions.

The COLD-PCR experiments were done using the same instrument and reaction conditions as the standard PCR and HRM protocol. The cycling protocol was initiated with one cycle at 95°C for 15 min, followed by 15 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s, 30 cycles of 80.5°C for 10 s, 60°C for 10 s, and 72°C for 10 s, and one cycle at 95°C for 1 min. HRM was performed

For Peer Review

from 65°C to 95°C, with a temperature increase at 0.1°C/s. All samples were analyzed in triplicates (cell line experiments) or in duplicates (colorectal cancer specimens).

Data analysis

The Rotor-Gene 6000 Series Software version 1.7.87 supplied with the instrument was used to analyze the data. This was done by looking at the derivative of the raw data (Melting curve analysis), the normalized HRM and difference graphs (High resolution melting analysis). For the difference graphs a wild type sample was selected as reference.

Mutation analysis using the TheraScreen® *KRAS* mutation Kit

All samples were analyzed using the TheraScreen® *KRAS* mutation kit (DxS Diagnostic Innovations). This kit analyze the mutation status for the seven most commonly found *KRAS* exon 2 mutations (see Table 1) by a technology that combines ARMS® (allele specific PCR) with Scorpions® real-time PCR. The manufacturer has reported the sensitivity to be 1% mutant alleles in a wild type background if sufficient DNA input is used.

Two-round COLD-PCR

A two-round COLD-PCR protocol was developed to confirm mutations of very low prevalence. The COLD-PCR products from the first round of COLD-PCR were diluted 100 fold and used as template in a second round of COLD-PCR using the same primers and conditions as for the first round of COLD-PCR except no standard PCR cycles were used. The comparisons of COLD-PCR

For Peer Review

and HRM with the TheraScreen *KRAS* mutation kit were performed without the use of two-round COLD-PCR.

Sanger sequencing

Sequencing was performed on the ABI Genetic Analyzer 3130 XL (Applied Biosystems). To create a longer product of the *KRAS* codon 12 and 13 containing template, which could be sequenced successfully, a second amplification was performed with M13 tagged *KRAS* primers. These products were sequenced using the BigDye terminator kit v1.1 (Applied Biosystems) according to the manufactures' instructions with slight modifications. The single stranded PCR was performed using 1 µL of the BigDye terminator in a final volume of 10 µL. Sequencing of the amplicon generated by the primers specific for the *KRAS1P* pseudogene was done directly.

Results

The analytical sensitivity of COLD-PCR versus standard PCR

We have compared the analytical sensitivity of the COLD-PCR and HRM assay with standard PCR followed by HRM using serial dilutions of mutant DNA derived from cell lines into wild type DNA. Five different *KRAS* mutations were analyzed; c.34G>A, codon 12 (A549), c.35G>A, codon 12 (LS174T), c.35G>T, codon 12 (SW480), c.35G>C, codon 12 (RPMI-8226), c.38G>A, codon 13 (DLD-1). Three different T_c's were tested (80°C, 80.5°C, and 81°C) and we found 80.5°C to provide the overall strongest selectivity for mutant alleles. The sensitivity was increased by up to 100 fold for melting temperature decreasing mutations when using COLD-PCR as compared to

For Peer Review

standard PCR, whereas no increase in sensitivity was observed for the c.35G>C, codon 12 melting temperature retaining mutation. The results are listed in Table 2. The A549 and SW480 cell lines are homozygous for the *KRAS* mutations whereas all the other cell lines are heterozygous for the respective *KRAS* mutations. To facilitate direct comparisons between the cell lines, sensitivities in Table 2 are given as percentages of mutant alleles (assuming all cells contained the mutation) and not as percentages of cell line DNA. Figure 1 shows HRM and sequencing results derived from standard PCR and COLD-PCR amplifications of the A549 cell line dilutions.

The reproducibility of the COLD-PCR and HRM assay

The reproducibility of the COLD-PCR and HRM assay was assessed by repeating the A549 cell line dilution experiment six times in triplicate for each dilution point. The fraction of the replicates for which the mutation could be detected for each dilution point for each of the experiments is shown in Supp. Table S1. At least two of three replicates containing 0.1% mutant alleles were detectable in four out of six experiments.

Detection of *KRAS* mutations in clinical samples

We have analyzed the *KRAS* mutation status in 61 colorectal cancer specimens using the FDA approved TheraScreen® *KRAS* mutation kit, standard PCR followed by HRM, and COLD-PCR followed by HRM, respectively. Sequencing was used to confirm selected HRM results. In two cases (sample 49-50) mutations were only detectable by the TheraScreen® kit and COLD-PCR, and in four cases (sample 51-54) mutations were only detectable by COLD-PCR. These mutations were all melting temperature decreasing mutations as expected. In two cases (sample 55-56) mutations

For Peer Review

were only detectable by the TheraScreen® kit. These mutations were temperature retaining mutations, which could also be expected. Finally, in five cases (sample 57-61) discrepancies were observed between the TheraScreen® kit and the other methods. In three of these cases (sample 57-59) mutations were observed by HRM and sequencing after standard PCR as well as after COLD-PCR. In the remaining two cases (sample 60-61) temperature decreasing mutations were detected by the TheraScreen® kit but not by HRM or sequencing after standard PCR or COLD-PCR. An overview of the results can be found in Table 3, and Figure 2 shows selected HRM and sequencing results from cases for which the three different methods showed contradicting results.

Furthermore, when using COLD-PCR a synonymous c.39C>T, codon 13 mutation, which to our knowledge has not been described before, was detected in 14 of the 61 colorectal cancer specimens. Therefore, additional samples deviated from wild type when using COLD-PCR as opposed to standard PCR.

The COLD-PCR primers do not amplify the *KRAS1P* pseudogene

We have confirmed that the COLD-PCR primers were unable to amplify the *KRAS1P* pseudogene at chromosome 6. This was done by SNP analysis using HRM and sequencing with pseudogene specific primers to establish the genotype of rs4516942:G>A located only within the pseudogene at position 1 of codon 13 analogue to *KRAS*. The genotype frequencies for the SNP in the European group are; A/A: 0.450, A/G: 0.417, and G/G: 0.133. Fifteen of the colorectal cancer specimens were genotyped for rs4516942 and six were homozygous for the A allele (0.400), two were homozygous for the G allele (0.133), and seven were heterozygous (0.467). Since this mutation is melting temperature decreasing and not detected with the COLD-PCR primers we can conclude that the COLD-PCR primers do not amplify the *KRAS1P* pseudogene.

For Peer Review

Two-round COLD-PCR

A two-round COLD-PCR protocol was developed to evaluate whether melting profiles with subtle deviations from wild type were due to intrinsic variation of the instrument or due to the presence of low-level mutations. Mutations that were initially discarded as intrinsic variation in the HRM analysis or as background in the sequencing analysis could be confirmed as real mutations by applying an extra round of COLD-PCR. This was possible as melting profiles of mutation containing samples are much easier to distinguish from wild type melting profiles after two rounds of COLD-PCR. DNA from A459 (c.34G>A, codon 12) diluted into wild type DNA was used for these experiments. Figure 3 shows representative HRM profiles and sequencing results derived after one and two rounds of COLD-PCR respectively. It can be observed that the G>A mutation was not detected in one of the 0.5% dilutions indicating that minor variation from reaction to reaction occur. Finally, these experiments also showed that the synonymous c.39C>T, codon 13 mutation detected in 14 of the 61 colorectal cancer specimens also was present in PB from an unaffected individual.

For Peer Review

Discussion

Changing a single parameter of the PCR, the denaturation temperature, can potentially increase the sensitivity of subsequent mutation detection assays by up to a hundred fold. This has been shown for traditional Sanger sequencing, pyrosequencing, MALDI-TOF, and RFLP based detection (Li, et al., 2008), for HRM analysis (Milbury, et al., 2009a), and for TaqMan-based real-time PCR (Li, et al., 2009b). The denaturation temperature of COLD-PCR is referred to as the critical denaturation temperature (T_c) and the precise determination of this temperature has been considered to be

"tricky" (Luthra and Zuo, 2009). We found that selecting a T_c one degree below the melting temperature of the wild type PCR amplicon provided maximum sensitivity, and thus, in this case, confirmed what has been considered a rule of thumb for T_c selection (Milbury, et al., 2009a). The number of initial standard PCR cycles is also important for optimal sensitivity. We found that approximately 10 cycles before the quantification cycle (C_q) ensured robust amplification while maintaining strong mutation enrichment.

When comparing COLD-PCR to standard PCR, a drastically increased sensitivity was observed for all melting temperature decreasing mutations. The sensitivity ranged from 0.1% to 1% mutant alleles in a wild type background when using an input of 50 ng DNA in the reactions. However, some run to run variation as well as replicate to replicate variation were observed when repeating the A549 cell line experiments (Supp. Table S1). Furthermore, the benefit from using COLD-PCR as opposed to standard PCR was highly dependent on the specific mutation (Table 2). In contrast, the sensitivity was not increased for the melting temperature retaining mutation (c35G>C, codon 12) present in the RPMI8226 cell line. This was expected as fast COLD-PCR protocols do not facilitate the formation of mutation/wild type heteroduplexes. The full COLD-PCR approach described earlier enriches all types of mutations based on the lower melting temperature of mutation/wild type heteroduplexes. This approach, however, requires substantial optimization, mutation enrichment may be modest, and a lengthy hybridization time is needed.

The superior sensitivity of COLD-PCR versus standard PCR was also reflected by our analysis of colorectal cancer specimens. In six of 61 cases (sample 49-54), mutations were detected by HRM and sequencing after COLD-PCR but not after standard PCR. All six mutations were melting temperature decreasing. The FDA approved TheraScreen® *KRAS* mutation kit is more sensitive than standard PCR followed by HRM and sequencing. However, in four cases (sample 51-54) mutations detected by COLD-PCR followed by HRM and sequencing were missed by the TheraScreen® kit.

For Peer Review

These mutations were also melting temperature decreasing, and not detected by standard PCR and HRM. In two cases (sample 55-56), mutations were only detected by the TheraScreen® kit. These mutations were, as expected, melting temperature retaining. The sensitivity of the TheraScreen® kit is reported by the company to be 1% depending on the total amount of DNA used (http://www.dxsdiagnostics.com). However, it has been reported that only a minority of diagnostic samples derived from FFPE tissues can be analyzed at this level of sensitivity (Kotoula, et al., 2009).

Another interesting aspect of this study is that discrepancies that could not be explained by the relative sensitivities of the methods were observed between the TheraScreen® kit and the other two methods in five cases (sample 57-61). In three of these cases (sample 57-59), scored as wild type by the TheraScreen® kit, mutations were observed by HRM and sequencing after standard PCR as well as after COLD-PCR. Nevertheless, these mutations were detected by the TheraScreen® kit, but at too high C_q-values to meet the threshold for scoring them as positives (according to the manufactures instructions). In the remaining two cases (sample 60-61) temperature decreasing mutations were detected by the TheraScreen® kit but not by HRM or sequencing after standard PCR or COLD-PCR.

Concordance between standard PCR and COLD-PCR was observed in 55 of 61 cases (90%), between standard PCR and the TheraScreen® kit in 52 of 61 cases (85%), and between COLD-PCR and the TheraScreen® kit in 50 of 61 cases (82%). Concordance between all three methods was observed in 48 of 61 cases (79%).

Similar degrees of disconcordance between the TheraScreen® kit and a range of other methods including, Sanger sequencing, pyrosequencing, and HRM after standard PCR, and another FDA approved *KRAS* mutation kit, the TIB Molbiol *KRAS* mutation detection kit (LightMix Kit, TIB

For Peer Review

Molbiol) have been reported in a large multicenter blinded study (Whitehall, et al., 2009). In this study, high false-positive rates were also observed for the TIB Molbiol kit when analyzing FFPE samples (Whitehall, et al., 2009).

It can be observed from Table 3 that most of the discrepancy between the methods appear to be caused by the c.38G>A, codon 13 mutation. However, there is no obvious explanation for this since the difference in sensitivity between standard PCR and COLD-PCR is less for this mutation compared the other mutations. In addition, this mutation was detected in one sample by the TheraScreen® kit and not by HRM after standard PCR and COLD-PCR, but for two other samples the situation was the opposite.

All together, the results reflect the order by which each method meet their sensitivity limits and suggest an advantage of using COLD-PCR. That the frequency of detected *KRAS* mutations in clinical samples is influenced by the sensitivity of the method has previously been noted (Kobunai, et al., 2010). However, clinical decisions based upon very low prevalence mutations are controversial.

The costs and labor associated with each method are also important factors for routine clinical testing. HRM is a rapid, high-throughput, and inexpensive method that has performed well in other studies for both frozen and FFPE samples (Whitehall, et al., 2009). However, Sanger sequencing is more expensive and a necessary quality control step for HRM based assays. Nevertheless, HRM followed by sequencing is still less expensive and less time consuming than the commercially available TheraScreen® and TIB Molbiol kits (Whitehall, et al., 2009). The switching from standard PCR to COLD-PCR does not add to the costs of the assay.

When using COLD-PCR we have detected a synonymous c.39C>T, codon 13 mutation in 14 of 61 colorectal cancer specimens. Therefore, more samples deviated from wild type when using COLD-

For Peer Review

PCR as opposed to standard PCR, and thus, the use of COLD-PCR would increase the sequencing load in clinical settings. This synonymous mutation is probably not clinically relevant, and may be a result of 5-methyl cytosine deamination since it is part of a CpG site. Such mutations are the most commonly found mutations in the human genome. We have also detected the synonymous c.39C>T, codon 13 mutation in PB from an unaffected individual, which suggests that it is not tumor specific. However, in most of the samples carrying this mutation, a small peak was observed in the electropherogram after sequencing, suggesting that the mutation often is present at very low level.

We developed a two-round COLD-PCR protocol to confirm that the synonymous C>T mutation, and other cases where mutations were present at low levels, were not merely background. First of all, we have observed that very subtle deviations of melting profiles are often reflected by very small peaks in the electropherograms, which in many cases would have been attributed to background. By applying an extra round of COLD-PCR it was confirmed that these subtle deviations actually reflected low-level mutations. After the second round of COLD-PCR melting profiles of mutation containing samples were much easier distinguished from wild type melting profiles, and the peaks in the electropherograms were clearly distinguishable from background. Two-round COLD-PCR has previously been described by Li *et al* (Li, et al., 2009a). However, they have used a second set of primers for the second round of COLD-PCR. By using the same primers as for the first round, we did not need to optimize an extra assay, and no initial standard PCR cycles were necessary.

The use of fast COLD-PCR and HRM for the detection of *KRAS* mutations in clinical samples has been evaluated in a parallel publication by Mancini and colleagues (Mancini, et al., 2010). However, a different primer design was used and the sensitivity of their assay was only evaluated using a single cell line (CCRF-CEM) containing the c.35G>A mutation and reported to be 0.8%

For Peer Review

mutant alleles. Furthermore, the clinical samples were analyzed by PCR and COLD-PCR followed by HRM and sequencing, and no comparisons with the TheraScreen® kit were provided. Finally, a two round COLD-PCR protocol to confirm very low prevalence mutations was not established, which in combination with a lower sensitivity may explain why the c.39C>T, codon 13 mutation was not detected by (Mancini, et al., 2010).

In conclusion, COLD-PCR combined with HRM is a simple way of increasing the sensitivity of *KRAS* mutation detection without adding to the complexity and cost of the experiments. However, clinically irrelevant mutations present at very low levels may be detected, which emphasizes the need for subsequent confirmation by sequencing.

For Peer Review

Acknowledgements

Tina Kjeldsen and Tina Hindkjær are acknowledged for excellent technical assistance. Professor Torben Falck Ørntoft, Professor Peter Andreasen, and Dr. Bo Sandahl Sørensen kindly donated the cell lines used in this study. This study was funded by the Danish Cancer Society, the Lundbeck Foundation, the Foundation of A. P. Møller and Chastine McKinney Møller, and Max and Inge Wørzners Memorial Foundation.

References

Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L and others. 2005. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23(9):1803-10.

Cikota BM, Tukic LJ, Tarabar OT, Magic ZM. 2007. Detection of t(14;18), P53 and RAS gene mutations and quantification of residual disease in patients with B-cell non-Hodgkin's lymphoma. J Exp Clin Cancer Res 26(4):535-42.

Do H, Dobrovic A. 2009. Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low level mutations in cancer biopsies. Mol Cancer 8:82.

Hansen LL, Andersen J, Overgaard J, Kruse TA. 1998. Molecular genetic analysis of easily accessible breast tumour DNA, purified from tissue left over from hormone receptor measurement. APMIS 106(3):371-7.

Kobunai T, Watanabe T, Yamamoto Y, Eshima K. 2010. The frequency of KRAS mutation detection in human colon carcinoma is influenced by the sensitivity of assay methodology: a comparison between direct sequencing and real-time PCR. Biochem Biophys Res Commun 395(1):158-62.

Kotoula V, Charalambous E, Biesmans B, Malousi A, Vrettou E, Fountzilas G, Karkavelas G. 2009. Targeted KRAS mutation assessment on patient tumor histologic material in real time diagnostics. PLoS ONE 4(11):e7746.

Kristensen LS, Hansen LL. 2009. PCR-based methods for detecting single-locus DNA methylation biomarkers in cancer diagnostics, prognostics, and response to treatment. Clin Chem 55(8):1471-83.

Kristensen LS, Mikeska T, Krypuy M, Dobrovic A. 2008. Sensitive Melting Analysis after Real Time-Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. Nucleic Acids Res 36(7):e42.

Kristensen LS, Wojdacz TK, Thestrup BB, Wiuf C, Hager H, Hansen LL. 2009. Quality assessment of DNA derived from up to 30 years old formalin fixed paraffin embedded (FFPE) tissue for PCR-based methylation analysis using SMART-MSP and MS-HRM. BMC Cancer 9:453.

Krypuy M, Ahmed AA, Etemadmoghadam D, Hyland SJ, DeFazio A, Fox SB, Brenton JD, Bowtell DD, Dobrovic A. 2007. High resolution melting for mutation scanning of TP53 exons 5-8. BMC Cancer 7:168.

Krypuy M, Newnham GM, Thomas DM, Conron M, Dobrovic A. 2006. High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. BMC Cancer 6:295.

Li J, Milbury CA, Li C, Makrigiorgos GM. 2009a. Two-round coamplification at lower denaturation temperature-PCR (COLD-PCR)-based sanger sequencing identifies a novel spectrum of low-level mutations in lung adenocarcinoma. Hum Mutat 30(11):1583-90.

Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM. 2008. Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 14(5):579-84.

Li J, Wang LL, Janne PA, Makrigiorgos GM. 2009b. Coamplification at Lower Denaturation Temperature-PCR Increases Mutation-Detection Selectivity of TaqMan-Based Real-Time PCR. Clinical Chemistry 55(4):748-756.

Luthra R, Zuo Z. 2009. COLD-PCR finds hot application in mutation analysis. Clin Chem 55(12):2077-8.

Ma ES, Wong CL, Law FB, Chan WK, Siu D. 2009. Detection of KRAS mutations in colorectal cancer by high-resolution melting analysis. J Clin Pathol 62(10):886-91.

Mancini I, Santucci C, Sestini R, Simi L, Pratesi N, Cianchi F, Valanzano R, Pinzani P, Orlando C. 2010. The Use of COLD-PCR and High-Resolution Melting Analysis Improves the Limit of Detection of KRAS and BRAF Mutations in Colorectal Cancer. J Mol Diagn.

Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, Bekele BN, Herbst RS, Wistuba, II. 2007. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 13(10):2890-6.

Milbury CA, Li J, Makrigiorgos GM. 2009a. COLD-PCR-enhanced high-resolution melting enables rapid and selective identification of low-level unknown mutations. Clin Chem 55(12):2130-43.

Milbury CA, Li J, Makrigiorgos GM. 2009b. PCR-based methods for the enrichment of minority alleles and mutations. Clin Chem 55(4):632-40.

For Peer Review

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 17(7):2503-16.

Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M, Fuchs CS. 2005. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 7(3):413-21.

Polakova KM, Lopotova T, Klamova H, Moravcova J. 2008. High-resolution melt curve analysis: initial screening for mutations in BCR-ABL kinase domain. Leuk Res 32(8):1236-43.

Takano T, Ohe Y, Tsuta K, Fukui T, Sakamoto H, Yoshida T, Tateishi U, Nokihara H, Yamamoto N, Sekine I and others. 2007. Epidermal growth factor receptor mutation detection using high-resolution melting analysis predicts outcomes in patients with advanced non small cell lung cancer treated with gefitinib. Clin Cancer Res 13(18 Pt 1):5385-90.

Takeda S, Ichii S, Nakamura Y. 1993. Detection of K-ras mutation in sputum by mutant-allele-specific amplification (MASA). Hum Mutat 2(2):112-7.

Vogelstein B, Kinzler KW. 1999. Digital PCR. Proc Natl Acad Sci U S A 96(16):9236-41.

Ward R, Hawkins N, O'Grady R, Sheehan C, O'Connor T, Impey H, Roberts N, Fuery C, Todd A. 1998. Restriction endonuclease-mediated selective polymerase chain reaction: a novel assay for the detection of K-ras mutations in clinical samples. Am J Pathol 153(2):373-9.

Whitehall V, Tran K, Umapathy A, Grieu F, Hewitt C, Evans TJ, Ismail T, Li WQ, Collins P, Ravetto P and others. 2009. A multicenter blinded study to evaluate KRAS mutation testing methodologies in the clinical setting. J Mol Diagn 11(6):543-52.

Wittwer CT. 2009. High-resolution DNA melting analysis: advancements and limitations. Hum Mutat 30(6):857-9.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49(6 Pt 1):853-60.

Wojdacz TK, Dobrovic A, Algar EM. 2008. Rapid detection of methylation change at H19 in human imprinting disorders using methylation-sensitive high-resolution melting. Hum Mutat 29(10):1255-60.

Zuo Z, Chen SS, Chandra PK, Galbincea JM, Soape M, Doan S, Barkoh BA, Koeppen H, Medeiros LJ, Luthra R. 2009. Application of COLD-PCR for improved detection of KRAS mutations in clinical samples. Mod Pathol 22(8):1023-31.

For Peer Review

Figure legends

Figure 1. Selected HRM and sequencing results from Table 2. The sensitivity was increased by approximately 100 fold for the c.34G>A, codon 12 mutation present in the A459 cell line when using COLD-PCR instead of standard PCR. Selected replicates are shown. **A.** HRM and sequencing results after standard PCR. **B.** HRM and sequencing results after COLD-PCR.

Figure 2. Selected HRM and sequencing results from Table 3. A. HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay and COLD-PCR. **B.** HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by COLD-PCR. In this case, the synonymous c.39C>T, codon 13 mutation was also detected. **C.** HRM and sequencing results after standard PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay. **D.** HRM and sequencing results after COLD-PCR for a specimen for which disconcordance between the TheraScreen® kit and the other two methods could not be explained by the relative sensitivities of the methods.

Figure 3. Two-round COLD-PCR improves the separation between wild type and mutation containing samples. The COLD-PCR products from the first round of COLD-PCR were diluted 100 fold and used as template in a second round of COLD-PCR. Sequencing results of selected amplicons are shown in the inserts. In addition to the c.34G>A, codon 12 mutation a synonymous c.39C>T, codon 13 mutation was observed in several of the standards as well as in one assumed wild type derived from peripheral blood DNA from a healthy individual. DNA from the A459 cell line diluted into DNA derived from peripheral blood from a healthy individual was used for these experiments. **A.** HRM analysis and sequencing results after one round of COLD-PCR. **B.** HRM analysis and sequencing results after two rounds of COLD-PCR.

For Peer Review

Table 1. KRAS exon 2 mutations detected by the TheraScreen® *KRAS* mutation kit.

***KRAS* hotspot mutations**	
Mutation	**Amino acid change**
c.35G>C	Gly12Ala
c.35G>A	Gly12Asp
c.34G>C	Gly12Arg
c.34G>T	Gly12Cys
c.34G>A	Gly12Ser
c.35G>T	Gly12Val
c.38G>A	Gly13Asp

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference GenBank sequence (NM_033360.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

For Peer Review

Table 2. Sensitivity of standard PCR versus COLD-PCR.

The percentages refer to mutant alleles in a wild type background using 50 ng DNA input in the reactions. Three different T_c's were tested when using COLD-PCR.

Cell line		Standard PCR	COLD-PCR		
Name	**Mutation**		**80**°C	**80.5**°C	**81**°C
A549	c.34G>A	10%	0.5%	0.1%	2%
RPMI8226	c.35G>C	5%	25%	5%	5%
SW480	c.35G>T	5%	2%	0.2%	0.5%
DLD-1	c.38G>A	5%	1%	1%	5%
LS174T	c.35G>A	10%	1%	0.5%	2.5%

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference GenBank sequence (NM_033360.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

For Peer Review

Table 3. *KRAS* mutation analysis of 61 colorectal cancer specimens. Consensus groups: *A*, complete agreement between all three assays. *B*, mutation only detectable by the TheraScreen® assay and COLD-PCR. *C*, mutation only detectable by COLD-PCR. *D*, mutation only detectable by the TheraScreen® assay. *E*, Disconcordance between the TheraScreen® kit and the other two methods not explained by the relative sensitivities of the methods. The asterisk indicates that a synonymous c.39C>T, codon 13 mutation was detected.

Sample no.	TheraScreen®	Standard PCR		COLD-PCR		Consensus group
		HRM	Sequencing	HRM	Sequencing	
1	c.35G>A	Mutation	-	Mutation	-	*A*
2	c.34G>T	Mutation	-	Mutation	-	*A*
3	c.35G>A	Mutation	c.35G>A	Mutation	c.35G>A	*A*
4	c.35G>T	Mutation	-	Mutation	-	*A*
5	c.34G>T	Mutation	-	Mutation	-	*A*
6	Wild type	Wild type	-	Wild type	Wild type	*A*
7	c.34G>C	Mutation	-	Mutation	-	*A*
8	Wild type	Wild type	-	Wild type	Wild type	*A*
9	Wild type	Wild type	-	Wild type	Wild type	*A*
10	Wild type	Wild type	-	Wild type	-	*A*
11	Wild type	Wild type	-	Wild type	-	*A*
12	c.35G>A	Mutation	-	Mutation	-	*A*
13	Wild type	Wild type	-	Wild type	Wild type	*A*
14	c.35G>A	Mutation	-	Mutation	-	*A*
15	Wild type	Wild type	-	Wild type	Wild type	*A*
16	c.35G>T	Mutation	-	Mutation	-	*A*
17	Wild type	Wild type	-	Wild type	Wild type	*A*
18	Wild type	Wild type	-	Wild type	-	*A*
19	Wild type	Wild type	-	Mutation*	Wild type*	*A*
20	c.34G>A	Mutation	-	Mutation	-	*A*
21	Wild type	Wild type	-	Mutation*	Wild type*	*A*
22	c.34G>T	Mutation	-	Mutation	-	*A*
23	c.35G>C	Mutation	-	Mutation	c.35G>C	*A*
24	c.35G>C	Mutation	-	Mutation	-	*A*
25	c.34G>T	Mutation	-	Mutation	-	*A*
26	c.34G>C	Mutation	-	Mutation	c.34G>C	*A*
27	c.35G>A	Mutation	-	Mutation	-	*A*
28	c.35G>C	Mutation	c.35G>C	Mutation	c.35G>C	*A*

29	c.35G>T	Mutation	-	Mutation	c.35G>T	*A*
30	c.35G>T	Mutation	-	Mutation	-	*A*
31	Wild type	Wild type	-	Wild type	Wild type	*A*
32	c.35G>A	Mutation	-	Mutation	-	*A*
33	Wild type	Wild type	-	Mutation*	Wild type*	*A*
34	c.35G>T	Mutation	-	Mutation	c.35G>T	*A*
35	c.35G>T	Mutation	-	Mutation	-	*A*
36	Wild type	Wild type	-	Mutation*	Wild type*	*A*
37	c.35G>C	Mutation	-	Mutation*	c.35G>C*	*A*
38	c.38G>A	Mutation	-	Mutation	-	*A*
39	c.35G>A	Mutation	-	Mutation	c.35G>A	*A*
40	Wild type	Wild type	-	Mutation*	Wild type*	*A*
41	c.35G>A	Mutation	-	Mutation	-	*A*
42	c.35G>T	Mutation	-	Mutation	-	*A*
43	Wild type	Wild type	-	Wild type	Wild type	*A*
44	c.35G>T	Mutation	-	Mutation	-	*A*
45	c.35G>T	Mutation	-	Mutation	-	*A*
46	Wild type	Wild type	Wild type	Mutation*	Wild type*	*A*
47	Wild type	Wild type	-	Mutation*	Wild type*	*A*
48	Wild type	Wild type	-	Mutation*	Wild type*	*A*
49	c.35G>T	Wild type	Wild type	Mutation	c.35G>T	*B*
50	c.35G>A	Wild type	Wild type	Mutation	c.35G>A	*B*
51	Wild type	Wild type	-	Mutation	c.34G>A	*C*
52	Wild type	Wild type	-	Mutation*	c.38G>A*	*C*
53	Wild type	Wild type	-	Mutation*	c.38G>A*	*C*
54	Wild type	Wild type	-	Mutation	c.35G>T	*C*
55	c.34G>C	Wild type	Wild type	Mutation*	Wild type*	*D*
56	c.35G>C	Wild type	Wild type	Mutation*	Wild type*	*D*
57	Wild type	Mutation	c.38G>A	Mutation	c.38G>A	*E*
58	Wild type	Mutation	c.34G>C	Mutation	c.34G>C	*E*
59	Wild type	Mutation	c.35G>T	Mutation	c.35G>T	*E*
60	c.38G>A	Wild type	Wild type	Mutation*	Wild type*	*E*
61	c.35G>T	Wild type	Wild type	Wild type	Wild type	*E*

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference GenBank sequence (NM_033360.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

For Review Only

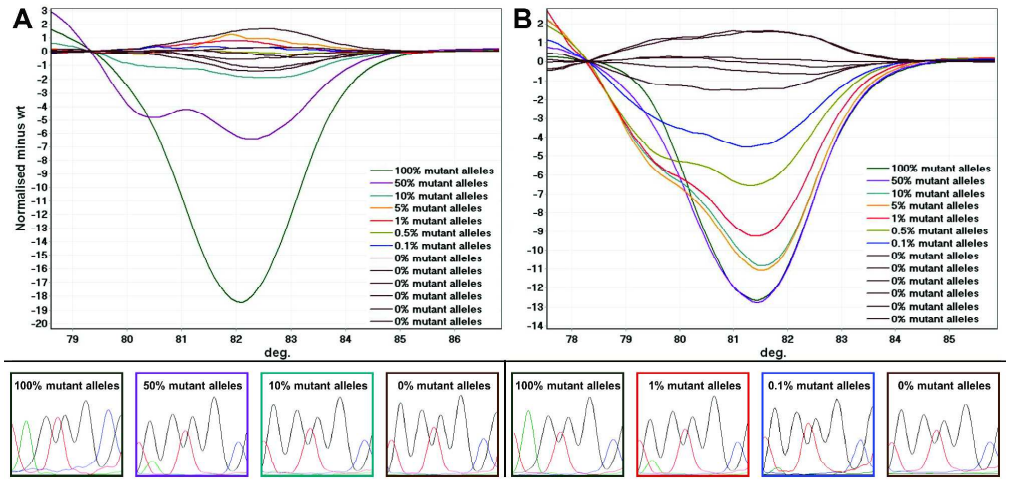

Figure 1

Figure 1. Selected HRM and sequencing results from Table 2. The sensitivity was increased by approximately 100 fold for the G>A mutation present in the A459 cell line when using COLD-PCR instead of standard PCR. Selected replicates are shown. A. HRM and sequencing results after standard PCR. B. HRM and sequencing results after COLD-PCR.
254x190mm (600 x 600 DPI)

view

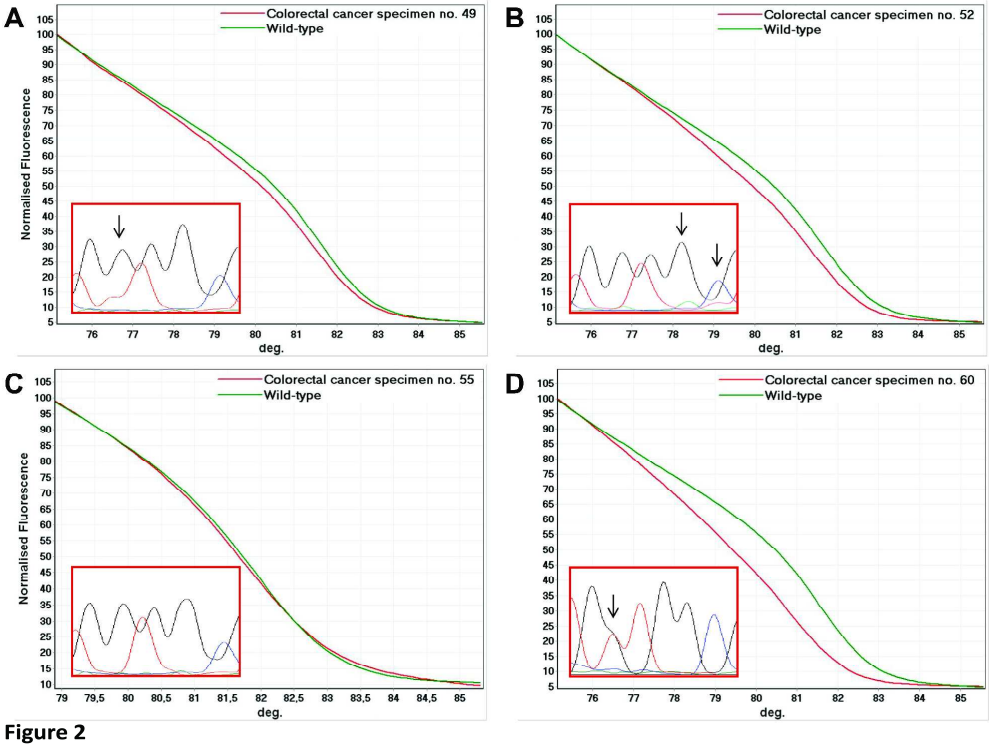

Figure 2

Figure 2. Selected HRM and sequencing results from Table 3. A. HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay and COLD-PCR. B. HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by COLD-PCR. In this case, the synonymous codon 13 GGC>GGT mutation was also detected. C. HRM and sequencing results after standard PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay. D. HRM and sequencing results after COLD-PCR for a specimen for which the mutation a suspected false negative result by the TheraScreen® assay.
254x190mm (600 x 600 DPI)

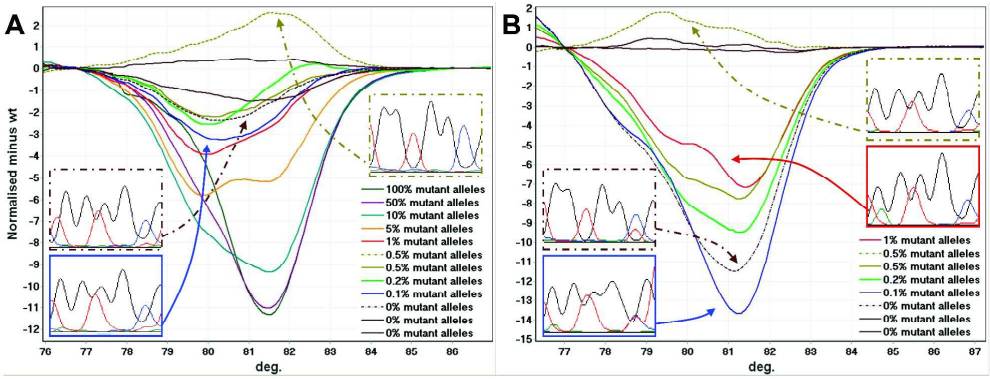

Figure 3

Figure 3. Two-round COLD-PCR improves the separation between wild type and mutation containing samples. The COLD-PCR products from the first round of COLD-PCR was diluted 100 fold and used as template in a second round of COLD-PCR. Sequencing results of selected amplicons are shown in the inserts. In addition to the codon 12 G>A mutation a synonymous codon 13 C>T mutation was observed in several of the standards as well as in one assumed wild type derived from peripheral blood DNA from a healthy individual. DNA from the A459 cell line diluted into DNA derived from peripheral blood from a healthy individual was used for these experiments. A. HRM analysis and sequencing results after one round of COLD-PCR. B. HRM analysis and sequencing results after two rounds of COLD-PCR.
254x190mm (600 x 600 DPI)

Supplementary Table S1. The reproducibility of the COLD-PCR and HRM assay as assessed by repeating the A549 cell line dilution experiment six times in triplicates for each dilution. The number of replicates for which the mutation could be detected is shown for each dilution point for each of the six experiments.

Experiment number	Analytical sensitivity				
	0.1%	**0.2%**	**0.5%**	**1%**	**2%**
1	2/3	No data	3/3	3/3	3/3
2	3/3	3/3	3/3	3/3	3/3
3	2/3	3/3	2/3	2/3	3/3
4	0/3	0/3	0/3	3/3	3/3
5	2/3	3/3	3/3	3/3	3/3
6	0/3	0/3	1/3	3/3	3/3
In total	9/18	9/15	12/18	17/18	18/18

For Peer Review

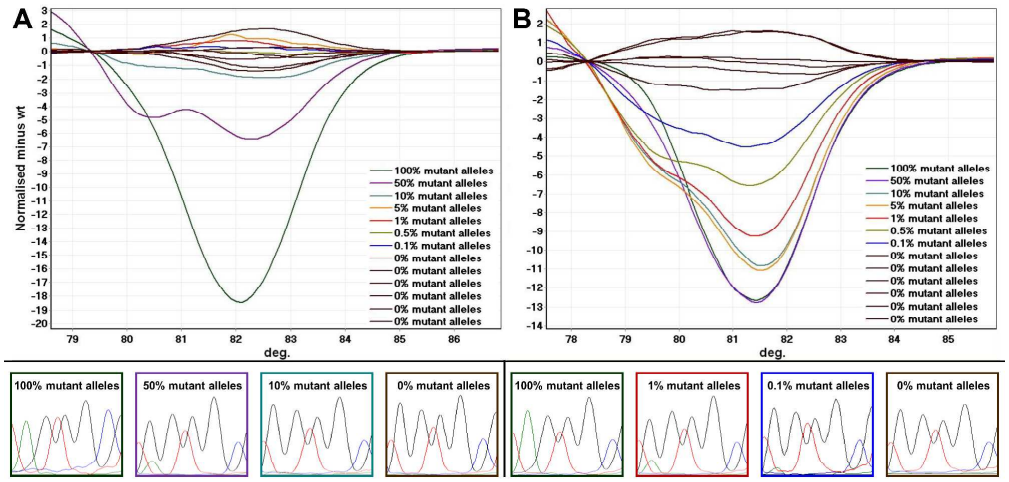

Figure 1

Figure 1. Selected HRM and sequencing results from Table 2. The sensitivity was increased by approximately 100 fold for the c.34G>A, codon 12 mutation present in the A459 cell line when using COLD-PCR instead of standard PCR. Selected replicates are shown. A. HRM and sequencing results after standard PCR. B. HRM and sequencing results after COLD-PCR.
254x190mm (600 x 600 DPI)

view

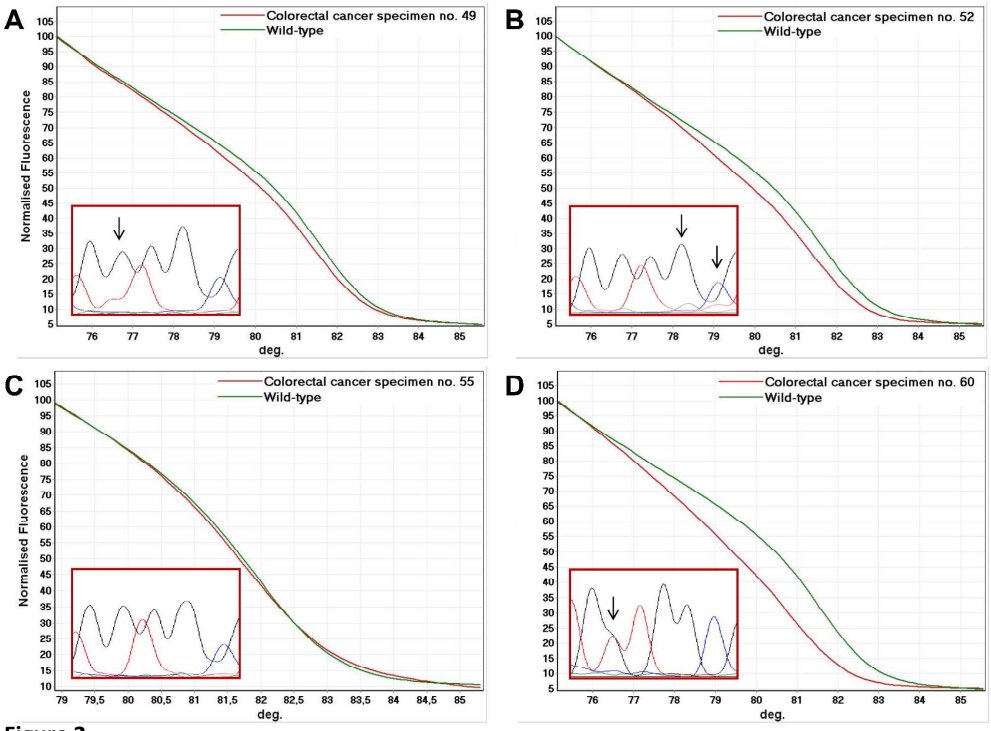

Figure 2

Figure 2. Selected HRM and sequencing results from Table 3. A. HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay and COLD-PCR. B. HRM and sequencing results after COLD-PCR for a specimen for which the mutation was only detectable by COLD-PCR. In this case, the synonymous c.39C>T, codon 13 mutation was also detected. C. HRM and sequencing results after standard PCR for a specimen for which the mutation was only detectable by the TheraScreen® assay. D. HRM and sequencing results after COLD-PCR for a specimen for which disconcordance between the TheraScreen® kit and the other two methods could not be explained by the relative sensitivities of the methods.
254x190mm (600 x 600 DPI)

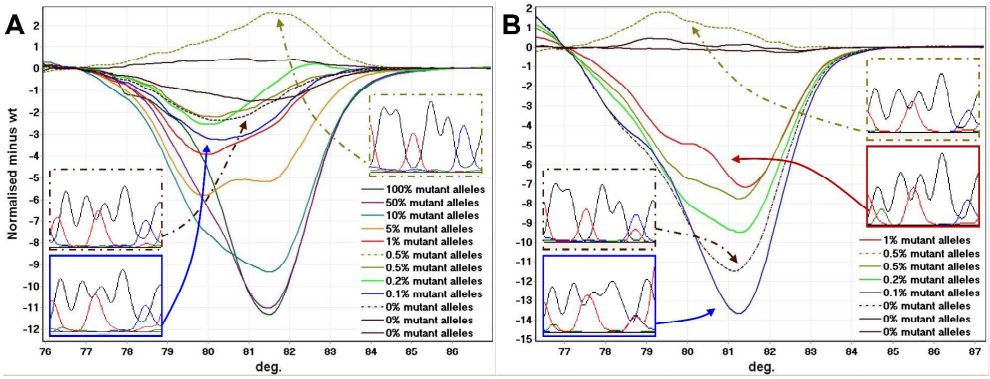

Figure 3

Figure 3. Two-round COLD-PCR improves the separation between wild type and mutation containing samples. The COLD-PCR products from the first round of COLD-PCR was diluted 100 fold and used as template in a second round of COLD-PCR. Sequencing results of selected amplicons are shown in the inserts. In addition to the c.34G>A, codon 12 mutation a synonymous c.39C>T, codon 13 mutation was observed in several of the standards as well as in one assumed wild type derived from peripheral blood DNA from a healthy individual. DNA from the A459 cell line diluted into DNA derived from peripheral blood from a healthy individual was used for these experiments. A. HRM analysis and sequencing results after one round of COLD-PCR. B. HRM analysis and sequencing results after two rounds of COLD-PCR.
254x190mm (600 x 600 DPI)