Ultrasensitive Detection of Unknown Colon Cancer-Initiating Mutations Using the Example of the Adenomatous Polyposis Coli Gene

Christian Gerecke1, Conny Mascher1, Uwe Gottschalk2, Burkhard Kleuser1, and Bettina Scholtka1

Abstract
Detection of cancer precursors contributes to cancer prevention, for example, in the case of colorectal cancer. To record more patients early, ultrasensitive methods are required for the purpose of noninvasive precursor detection in body fluids. Our aim was to develop a method for enrichment and detection of known as well as unknown driver mutations in the Adenomatous polyposis coli (APC) gene. By coupled wild-type blocking (WTB) PCR and high-resolution melting (HRM), referred to as WTB-HRM, a minimum detection limit of 0.01% mutant in excess wild-type was achieved according to as little as 1 pg mutated DNA in the assay. The technique was applied to 80 tissue samples from patients with colorectal cancer (n = 17), adenomas (n = 50), serrated lesions (n = 8), and normal mucosa (n = 5). Any kind of known and unknown APC mutations (deletions, insertions, and base exchanges) being situated inside the mutation cluster region was distinguishable from wild-type DNA. Furthermore, by WTB-HRM, nearly twice as many carcinomas and 1.5 times more precursor lesions were identified to be mutated in APC, as compared with direct sequencing. By analyzing 31 associated stool DNA specimens all but one of the APC mutations could be recovered. Transferability of the WTB-HRM method to other genes was proven using the example of KRAS mutation analysis. In summary, WTB-HRM is a new approach for ultrasensitive detection of cancer-initiating mutations. In this sense, it appears especially applicable for noninvasive detection of colon cancer precursors in body fluids with excess wild-type DNA like stool. Cancer Prev Res; 6(9); 898–907. ©2013 AACR.

Introduction
Improving prevention and early detection of cancer is a defined goal of the World Health Organization based on the observation that removal of cancer precursor lesions has been shown to reduce cancer incidence, for example, in the case of colorectal cancer (1). In particular, there is a need to provide those people who refuse screening colonoscopy with highly sensitive alternative tests. The main problems in the attempt to establish molecular assays for noninvasive detection of early cancer stages or even of cancer precursors are the choice of the appropriate biomarkers, the high background of wild-type DNA in biologic specimens such as stool or other body fluids, and the difficulty to detect mutations of unknown type and position, for example, in genes with several possible mutation sites or large mutation cluster regions (MCR).

Mutations in APC, KRAS, or BRAF genes have been classified as colorectal cancer-initiating events and thus represent promising biomarkers for molecular detection of beginning cancer and its precursors (2–5). APC mutations have been described in early cancerous lesions as well as in cancer precursors like adenomas (6, 7) and even in aberrant crypt foci (8, 9). These mutations mimic an active Wnt signaling and are assumed to be initiating at least for traditional colon cancers developing along the adenoma-carcinoma sequence (10). KRAS and BRAF mutations, both resulting in a constitutively active Ras-Raf-MEK-MAPK signaling pathway, were described in earliest lesions of tumors following the so-called serrated path (3, 11, 12). KRAS mutations are furthermore present in some of the above-mentioned traditional tumors as well as in tumors combining features of both pathways in a so-called fusion pathway (13).

In addition, APC and KRAS are the most frequently mutated genes in sporadic colorectal cancer being affected in about 40% (KRAS) and up to 60% (APC) of patients with colorectal cancer, respectively (14–17). Commercially available kits for KRAS and BRAF mutation analysis (e.g., Signature KRAS/BRAF Mutations assay, Asuragen) possess an average detection limit of 1% mutated DNA in mixtures with wild-type DNA which is satisfactory for prognostic
purposes rather than for early cancer screening, when taken into account that the ratio of mutated human DNA to total feces DNA is assumed to be in the range of 1:10,000–1:100,000 (18–20).

Low-level detection of unknown mutations is a formidable challenge. The paramount example is the APC gene. A large mutation cluster region of almost 1 kb being frequently hit during sporadic colon carcinogenesis has been defined in exon 15 of APC (21, 22). Surroundings of codons 1309, 1450, 1465, and 1556 represent mutational hotspots within the MCR (23, 24). From our recent work and from the literature, it is known that different mutation types (insertions, deletions, or base substitutions) can occur in one and the same mutational hotspot, aggravating the detection. For instance, the codon 1309 hotspot which contains repetitive sequences spanning codons 1306–1311, might be affected by a 5 bp deletion leading to a frameshift or by a G>T substitution resulting in a stop codon, whereas the codon 1556 hotspot (codons 1553–1556) most often exhibits a 1 bp insertion of an adenine but can also be affected by a G>T substitution resulting in a stop codon (4, 5). For this reason, detection methods using mutant-specific probes are inappropriate in this case and although APC is the most frequently mutated gene in colorectal cancer, there is no sensitive commercial test for APC mutation analysis available up to now.

We hypothesized that combined enrichment and ultra-sensitive detection of carcinogenic driver mutations could represent a tool to sense not only more carcinomas but also more colorectal cancer precursors like advanced adenomas in body fluids. For this purpose, we developed a two-step method consisting of wild-type blocking (WTB)-PCR for suppressing wild-type DNA by use of locked nucleic acid (LNA) blocker molecules, followed by further enrichment and detection of mutations using high-resolution melting (HRM) analysis. The efficacy of this new approach, referred to as WTB-HRM, to detect low-level mutations in APC whose type or position inside the large MCR is not exactly predictable was compared with direct sequencing. The applicability on human colon tissue and feces analysis is shown and the transferability of the ultrasensitive method to other genes is proven using the example of KRAS.

Materials and Methods

Cell lines, human tissue, and feces

DNA from human colorectal cancer cell lines with known mutated or wild-type status of the examined genes was used as control for mutation analysis. For the analysis of the mutational hotspots around codons 1309 and 1556 of APC as well as in codon 1367 of APC, cell lines LS1034 (APC c.3927_3931del, p.E1309fs), Colo678 (APC c.4666_4667insA, p.T1556fs), and Caco-2 (APC c.4099C>T, p.Q1367*), respectively, served as positive controls. For KRAS codon 12 analysis, LS174T (KRAS c.35C>A, p.G12D) was applied. HCEC DNA was used as wild-type reference for APC as well as for KRAS analysis. Cells were purchased in 2009 from American Type Culture Collection (LS1034) and from DSMZ/German Collection of Microorganisms and Cell Cultures (Colo678) and were cultured according to protocols of the suppliers. LS174T, Caco-2, and HCEC were obtained as generous gifts from A. M. Otto (Technical University of Munich, Munich, Germany), R. Brigelius-Flohé [German Institute of Human Nutrition (DifE), Nuthetal, Germany], and the Nestlé Ltd. Research Centre (Lausanne, Switzerland), respectively. For authentication, sanger sequencing was conducted to ensure the wild-type or mutated state of both, APC and KRAS genes, in every cell line. Furthermore, all cell lines were tested negative for mycoplasma infection within 6 months before use.

Eighty human colon tissue and 31 associated feces specimens from subjects that had undergone routine colonoscopy and histopathologic assessment had been obtained freshly frozen from the Maria Heimsuchung Caritas Hospital Pankow (Berlin, Germany). DNA from human specimens and from cell pellets was extracted within 2 weeks after harvesting by standard methods (QIAamp DNA Mini Kit and QIAamp DNA Stool Mini Kit, Qiagen). The APC mutation status of the colon tissues was known from a previous study (5) in which APC had been analyzed by direct sequencing. The study was carried out in compliance with the Declaration of Helsinki. Written informed consent was received from participants before inclusion in the study. Permission for the study was given by the Ethics Commission of the University of Potsdam, Germany (Decision 3/25 taken on November 03, 2008).

Site-directed mutagenesis and cloning

A positive control mutated in the APC codon 1465 mutation hotspot (APC c.4393_4394del, p.S1465fs) was generated by site-directed mutagenesis and subcloned into pGEM-T Easy vector (Promega). The frequent APC codon 1450 mutation (APC c.4348C>T, p.R1450*) was subcloned into pGEM-T Easy from a colon carcinoma previously analyzed for use as positive control.

Wild-type blocking-PCR

Design and calculation of melting conditions of LNA/DNA oligomers, referred to as LNA blockers, were conducted by using the online available LNA Oligo design tools (Exiqon). LNA blockers hybridized exclusively with wild-type DNA in the space of the respective mutational hotspots enclosed in corresponding gene segments. One LNA blocker for suppressing wild-type DNA amplification was included in every reaction (Supplementary Table S1). For APC analysis, the MCR was subdivided into four overlapping segments of 200 to 300 bp each that were analyzed separately (Supplementary Fig. S1). These segments are referred to as APC1 (codons 1228–1328, including the codon 1309 mutational hotspot), APC2 (codons 1328–1452, including the frequently mutated codon 1467), APC3 [codons 1448–1509, including the codon 1450 mutational hotspot (APC3.1) and the frequently mutated codon 1465 (APC3.2)], and APC4 (codons 1482–1568, including codon 1556 mutational hotspot). For KRAS analysis, a gene segment encompassing the complete exon 1 (with codons 12 and 13 mutational hotspots included) was amplified.
Oligonucleotide primers for both genes were the same as reported earlier (25). WTB-PCR reactions for APC analysis contained 1× AmpliTaq Reaction Buffer (Applied Biosystems), 4 mmol/L MgCl₂, 0.2 mmol/L of each of the 4 deoxynucleoside triphosphates (dNTP), 0.2 μmol/L of each primer, and 1 U/μL AmpliTaq DNA polymerase Stoffel Fragment (Applied Biosystems) in a 25 μL reaction volume. For KRAS amplification, 2.5 mmol/L MgCl₂ was used instead. Template amount was either 10 ng of control DNA or 50 ng of tissue DNA. WTB-PCR was conducted on a Mastercycler gradient (Eppendorf). APC amplification of tissue DNA was carried out for 5 minutes at 95°C followed by 18 cycles of 30 seconds at 95°C, 30 seconds at the respective annealing temperature Ta (Supplementary Table S1), 45 seconds at 72°C, and final extension was conducted for 10 minutes at 72°C. For APC amplification of fecal DNA, a touchdown-PCR protocol was run for 5 minutes at 95°C, 10 cycles of 30 seconds at 95°C, 30 seconds at the respective Ta (Supplementary Table S1) + 10°C, reduced by 1°C per cycle, and 40 seconds at 72°C. Subsequently, 15 cycles of 30 seconds at 95°C, 30 seconds at the appropriate Ta, and 45 seconds at 72°C were conducted, followed by a final extension for 10 minutes at 72°C. The KRAS protocol was as follows: 7 minutes at 94°C initial denaturation, 25 cycles of 94°C for 45 seconds, 79°C for 45 seconds, 58°C for 45 seconds, 72°C for 1 minute, and final extension at 72°C for 10 minutes. Amplicons obtained from each initial WTB-PCR reaction were diluted 10 times and used as template for subsequent HRM analysis.

High-resolution melting

For the purpose of further enrichment and detection of mutations in specimens previously amplified by WTB-PCR, HRM was carried out on a LightCycler 480 II instrument (Roche Diagnostics). All samples were examined in triplicates. Oligonucleotide primers used in the real-time amplification step associated with HRM (Supplementary Table S2) were seminested in relation to the WTB-PCR reaction (Supplementary Fig. S1). For APC analysis, the HRM reaction volume of 10 μL contained 1× LC480 HRM Master Mix (Roche Diagnostics), 1 μL of diluted template, 4 mmol/L MgCl₂, and 0.4 μmol/L of each PCR primer. For KRAS analysis, 2 μL of diluted template, 6.5 mmol/L MgCl₂, and 0.1 μmol/L of each PCR primer were used instead. APC amplification was conducted at 95°C for 10 minutes followed by 45 to 55 cycles of 95°C for 5 seconds, Ta (Supplementary Table S2) for 10 seconds, and 72°C for 10 seconds. The KRAS amplification protocol consisted of: 95°C for 5 minutes followed by 50 cycles of 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 10 seconds. A constant temperature increase of 4.4°C per second from 60°C to 95°C with an acquisition of fluorescence of 25 per second was directly attached. The LC 480 Gene Scanning Software Version 1.5.0 (Roche Diagnostics) was applied for HRM analysis. Normalized and temperature-adjusted melting curves of test samples and wild-type controls were compared and automatically grouped by the software. Curves of samples matching the wild-type were figured in the same color as the wild-type control, whereas mutated samples were assigned to mutant control or to further groups as depicted by different colored melting curves. To validate LNA blocker efficiencies, the respective real-time PCR runs were analyzed by the Advanced Relative Quantification Software—Version 1.5.0 (Roche Diagnostics).

Control sequencing

WTB-HRM mutation analysis results were controlled by sequencing. HRM products were cleaned from a 2% agarose gel by use of Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. Sanger sequencing of cleaned fragments was conducted by GATC Biotech. Sequencing results were analyzed by means of the DNASTAR Lasergene 9 Core Suite software (DNASTAR).

Results

WTB-HRM is suitable to detect low-level gene mutations in large mutation cluster regions

The objective to provide a highly sensitive method applicable for the detection of unknown mutations in genes with more than a few possible mutation sites was pursued using the example of APC as an initially mutated gene in colorectal cancer. The intended mutation enrichment as a precondition for highly sensitive low-level mutation detection in biologic specimens could be achieved by the developed two-step method involving WTB-PCR upstream of HRM. Four overlapping segments of the complete MCR of APC (APC1-APC4) were analyzed separately in presence of one LNA blocker in every analysis. In total, 5 different LNA blockers were used to hybridize wild-type APC in the region of mutational hotspots around codons 1309 (located inside APC1), 1367 (APC2), 1450 (APC3.1), 1465 (APC3.2), and 1556 (APC4). Specificity of every LNA blocker to suppress completely wild-type but not mutant APC amplification could be confirmed by conducting WTB-PCR on each APC segment (Fig. 1A). The respective blocking efficiency was apparent from the ratio of relative product formation in assays being run with and without added LNA blocker as determined by real-time quantitative PCR and depended on position and number of LNA molecules in the blocker as well as on sequence context (Table 1). The highest blocking efficiency was achieved by a blocker traversing the codon 1556 mutational hotspot in segment APC4 suppressing wild-type amplification even by more than 7,000-fold compared with the amount of product being formed without use of LNA blocker. In contrast, amplification of control DNA, carrying mutations within APC mutational hotspots, was nearly unaffected in presence of the respective LNA blocker molecules (Table 1). Detection limits for selected APC mutations, located in the space of the mutational hotspots, were determined by applying serial dilutions of mutated in wild-type control DNA to WTB-HRM. Exemplarily shown is the representative HRM curve chart of a 1 bp insertion (ins A) in segment APC4 with a minimum detectable mutant level of 0.01% in the template composite according to a detection limit of 1 pg.
mutated DNA in the assay (Fig. 1B). Regarding the remain-
der MCR segments, mutation detection limits differed
between 1 pg and 100 pg mutated APC in the template
composite, depending on the mutation type (Table 2).

Improvement in mutation enrichment and detection by
using LNA blockers
The benefit of mutation enrichment using LNA blockers
was verified by repeating APC analysis on mixed mutant and
wild-type control DNA comparatively by conducting con-
ventional PCR instead of WTB-PCR during the first step. By
applying WTB-HRM on each of 4 overlapping segments
(APC1–APC4) of the complete MCR, any deletions, inser-
tions, and base substitutions contained in control DNA
could be enriched and discriminated from wild-type DNA
background in mixed samples. Related detection limits
of each fragment analysis are summarized in Table 2.
WTB-PCR improved mutation detection considerably com-
pared with conventional PCR when coupled with HRM in
each case. For 3 of 5 examined mutant controls, the detec-
tion limit could be even lowered by 5,000-fold when using
WTB-PCR.
Detection of different mutation types in human colon tissue DNA

To test the feasibility of APC mutation analysis by WTB-HRM in human specimens, the method was applied to a total of 80 colon tissues with known mutation status due to previous direct sequencing. Exemplary HRM curve charts for APC segments 1 and 2 are shown in Fig. 2A and B, respectively. Segment APC3 containing two different mutational hotspots was analyzed by the use of two separate LNA blockers APC3.1 and APC3.2 (Fig. 2C and D, respectively). A representative HRM analysis of segment APC4 is shown in Fig. 2E. Tissues exhibiting wild-type APC clustered around the baseline, whereas HRM curves of specimens carrying an APC mutation within the enclosed mutational hotspot matched the positive control curve and peaked either below (Fig. 2A–C and E) or above (Fig. 2D) the baseline. Either kind of mutation, deletions, insertions, and substitutions were detectable.

Control sequencing of specimens displaying a melting curve different from both wild-type and mutant controls revealed further mutations that were detectable as well (Fig. 2A, B and E). For example, on analysis of segment APC1, a c.3925G>T, p.E1309*/C3 substitution was found within the codon 1309 mutational hotspot apart from the more common c.3927_3931del, p.E1309fs deletion at this site (Fig. 2A). In segment APC4, a 1 bp deletion (c.4666del, p.T1556fs) was clearly distinguishable from the c.4666_4667insA, p.T1556fs mutated tissue specimens and mutant control (Fig. 2E). Thus, WTB-HRM was able to detect different mutation types within APC mutational hotspots by using only one LNA blocker, hybridizing wild-type APC in the respective region of the gene.

In total, the MCR of APC was found to be mutated in 41 of the 80 (51%) analyzed colon tissues by use of WTB-HRM compared with 28 mutations (35%) detected by previous direct sequencing (Supplementary Table S3). Referred to histopathologic findings WTB-HRM detected mutations in 9 of 17 carcinoma, 27 of 50 adenoma, and 5 of 7 serrated lesions. No APC mutation was found in normal colon mucosa. All mutations previously detected by direct sequencing could be confirmed by WTB-HRM except for 2 adenoma samples (C80 and C91). However, WTB-HRM was vastly superior to direct sequencing detecting 9 instead of 5 mutations in carcinoma, 27 instead of 21 mutations in adenoma, and 5 instead of 2 mutations in serrated lesions. All mutations identified by means of aberrant melting curves could be confirmed by subsequent control sequencing of HRM products.

The analyses of colon tissues show that different types of APC mutations like insertions, deletions, and substitutions

### Table 1. Efficiency of LNA blockers used in WTB-PCR

<table>
<thead>
<tr>
<th>APC Segment (hotspot enclosed)</th>
<th>Relative product formation (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT/(WT + LNA blocker)</td>
</tr>
<tr>
<td>APC1 (cd 1309)</td>
<td>732</td>
</tr>
<tr>
<td>APC2 (cd 1367)</td>
<td>315</td>
</tr>
<tr>
<td>APC3.1 (cd 1450)</td>
<td>442</td>
</tr>
<tr>
<td>APC3.2 (cd 1465)</td>
<td>977</td>
</tr>
<tr>
<td>APC4 (cd 1556)</td>
<td>7,109</td>
</tr>
</tbody>
</table>

**NOTE**: Indicated is the ratio of APC product formation without LNA blocker versus product formation in presence of blocker for wild-type (WT) and mutant (MT) APC, respectively.

**Abbreviations**: RFU, relative fluorescence units; cd, codon.

### Table 2. Mutation detection limits of conventional PCR/HRM and of WTB-HRM

<table>
<thead>
<tr>
<th>Mutational hotspot of APC (MT control)</th>
<th>Mutation detection limit in excess wild-type (pg)</th>
<th>Fold improvement of WTB-HRM versus conventional PCR/HRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>cd 1309 (c.3927_3931del, p.E1309fs)</td>
<td>5,000</td>
<td>100</td>
</tr>
<tr>
<td>cd 1367 (c.4099C&gt;T, p.Q1367*)</td>
<td>1,000</td>
<td>10</td>
</tr>
<tr>
<td>cd 1450 (c.4348C&gt;T, p.R1450*)</td>
<td>5,000</td>
<td>1</td>
</tr>
<tr>
<td>cd 1465 (c.4393_4394del, p.S1465fs)</td>
<td>5,000</td>
<td>1</td>
</tr>
<tr>
<td>cd 1556 (c.4666_4667insA, p.T1556fs)</td>
<td>5,000</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE**: Indicated is the minimum amount of mutated (MT) DNA being detectable in mixed templates containing 10 ng of wild-type (WT) DNA each.

**a**Nomenclature of mutations according to guidelines of the Human Genome Variation Society.
being localized within mutational hotspots can clearly be distinguished from wild-type samples in a highly sensitive manner by conducting WTB-HRM.

Detection of unknown APC mutations situated outside mutational hotspots

On analysis of segment APC2 in colon tissue specimens, two divergent mutations, c.4147_4148del, p.M1383fs and c.4057G>T, p.E1353*, were noticed besides the APC c.4099C>T, p.Q1367* mutant control (Fig. 2B). Both of these unexpectedly detected mutations affected different codons compared with the mutant control. On the basis of this finding, the putative detection of mutations being situated outside that DNA section, which is hybridized by the respective LNA blocker, was further examined for segment APC2 as an example. A total of eight selected tissue samples...
specimens with known different mutations (deletions and substitutions) situated in segment APC2 but outside the LNA blocker-binding site around codon 1367 were subjected to WTB-HRM. All melting curves of these samples clearly differed from wild-type APC2 curves as well as from curves of the control being mutated within the associated hotspot (Fig. 2F). In one case (patient no. K40), the software classified the melting curve as wild-type, whereas subsequent control sequencing of the corresponding HRM product clearly revealed the mutated state. Hence, WTB-HRM is principally able to detect different mutation types with unknown position being situated outside the LNA blocker-binding site.

**Detection of APC mutations in feces DNA**

The applicability of WTB-HRM for noninvasive stool screening was examined by analyzing the available corresponding fecal DNA of 22 patients (3 carcinoma, 17 adenoma, 2 serrated lesions) whose tissue analysis had been positive for APC mutations in one of the four investigated MCR sections. Furthermore, stool DNA of 9 patients with mutation-negative tissue (2 carcinoma, 3 adenoma, 1 serrated lesion, 3 normal) was analyzed. As exemplary depicted for segment APC3.1, WTB-HRM was able to detect APC mutations matching the mutant control as well as different mutations from fecal DNA (Fig. 3). In total, mutations could be recovered in stool DNA of all but one adenoma patient. By stool analysis of patients with mutation-negative tissues, the wild-type status for APC was confirmed (Supplementary Table S3).

**Transferability of the method to other genes**

Applying WTB-PCR on KRAS analysis, the associated LNA blocker was proven to suppress amplification of the wild-type control completely, whereas the positive control, carrying the frequent KRAS c.35G>A, p.G12D mutation, was still amplified in presence of the blocker (Fig. 4A). Real-time quantitative PCR revealed 1,700-fold stronger blocking of wild-type than of mutated KRAS by means of the respective LNA blocker (not shown).

When using serial dilutions of mutant in wild-type control DNA, WTB-HRM was able to discriminate a 0.05% KRAS c.35G>A, p.G12D mutant level from excess wild-type as can be clearly seen in the difference plot of melting curves (Fig. 4B). This is consistent with a detection limit of 5 pg mutated KRAS in the template composite. The curve shape of mutated KRAS at a level of 0.01% (1 pg) differed slightly from wild-type KRAS but was too close for being discriminated reliably (Fig. 4B).

To examine the applicability of the method on biologic specimens, a test set of 20 human colon tissues was analyzed for KRAS mutations by use of WTB-HRM. Samples mutated in KRAS could be clearly differentiated from wild-type control by their melting curves, whereas curve charts of samples exhibiting the wild-type shaped around the baseline (Fig. 4C). In total, five specimens of the test set were found to carry mutations in KRAS codon 12 which could be confirmed by subsequent control sequencing of HRM products (Supplementary Table S4).

**Discussion**

The data presented here show for the first time an ultra-sensitive method for mutation analysis of the mutational cluster region of APC being able to detect mutations of unknown type and position in up to 10,000 times excess wild-type DNA. Furthermore, it is shown that by analyzing fecal DNA for cancer-initiating mutations, a sensitive non-invasive detection of colon cancer precursors becomes feasible. By the especially developed WTB-HRM method, a considerable enhancement of detection frequencies of carcinoma, adenoma, and serrated lesions, compared with direct sequencing, was achieved. The method was proven to
detect not only those gene mutations expected at particular sites but also unknown mutations, even those being situated outside mutational hotspots, in excess wild-type DNA at a ratio present in body fluids like feces.

The extraordinary high sensitivity of WTB-HRM, being able to detect 0.01% (APC) and 0.05% (KRAS) mutant levels in excess wild-type, respectively, according to 1–5 pg of mutated DNA in the template composite, was achieved by a two-step protocol including mutation enrichment preceding the detection step. Suppression of wild-type DNA amplification by use of LNA blockers was mandatory for the excellent sensitivity of the method and was advantageously complemented by HRM.

Figure 4. Analytic sensitivity of KRAS mutation analysis. A, complete suppression by LNA blocker of wild-type (WT) but not of mutated (MT) KRAS amplification. WTB-PCR was conducted on the respective control DNA in presence (+) or absence (−) of LNA blocker as indicated. Ten microliter of the PCR products were separated in a 2% agarose gel and visualized under UV light after ethidium bromide staining. B, detection limit of WTB-HRM for KRAS mutations. The MT control was serially diluted (10 ng–1 pg) in 10 ng WT DNA. The WT was set as baseline. Melting curves representing mutated DNA may proceed below or above the baseline. Percentage and amount of MT DNA in mixed templates as indicated. C, KRAS mutation screening of colon tissue specimens derived from patients with carcinomas or adenomas using WTB-HRM. The curve shape of the 0.05% MT level according to the detection limit was taken as reference for assessment of the mutated state of human colon samples. Results of control sequencing of HRM products are shown in brackets. Cellular genomic DNA of HCEC (KRAS WT) and LS174T (KRAS c.35G>A, p.G12D) was used as control, respectively.
composite (26). Regarding HRM, KRAS mutations at a ratio of 5% were necessary to be detectable (27, 28). A related method, the Digital Melt Curve (DMC) assay, was conducted as a two-step method enriching target genes by sequence-specific capture before combined digital PCR and HRM (29). By DMC, 0.1% levels of KRAS mutations and 0.6% levels of APC mutations were detected when applying 1,000 copies of mutant DNA (29). Deep-sequencing technology was reported to achieve a detection limit of 0.2% mutant DNA (30). In contrast, conventional PCR/HRM detected mutations only at a level of 2%–10%, whereas mutation enrichment by Co-amplification at lower denaturation temperature PCR (COLD-PCR) coupled with HRM resulted in a detection limit of 0.1%–1% mutant DNA as was shown using the example of the TP53 gene (31). The aforementioned examples usually require nanogram amounts of mutated DNA. Altogether, these data support the need of an additional enrichment step to reach high sensitivity as was already postulated (32).

The extent of wild-type suppression by an LNA blocker is dependent on the number and position of LNA molecules contained as well as on the sequence context (33, 34). This explains why in this study the same substitution of C>T resulted in a different detection limit when either codon 1367 or codon 1450 of APC is affected. We tried just one LNA blocker for every mutational hotspot. Hence, there might be some scope for influencing blocker efficiency by ingenious design.

Detection of the frequent APC c.3927,3931delp. E1309fs hotspot mutation exhibited the poorest detection limit because of its location within a repetitive sequence. A long LNA blocker for specific recognition of the wild-type was required in this case. As differences in melting temperatures of LNA oligomers decrease with increasing duplex length (34), the respective blocker was less effective in binding. This indicates that the detection of long deletions or insertions within repetitive sequences might be at the limits of the technique. Anyway, it was possible to detect said mutation not only in tissue but also in feces DNA from carcinoma as well as from patients with adenoma.

Two of the previously by direct sequencing detected mutations were not recovered by WTB-HRM analysis (samples no C80 and C91). In the case of sample number C91, this can be explained by the experimental design. For WTB-HRM, seminested PCR reactions were conducted. While the outer primers pairs for each segment produce overlapping amplicons, the inner primers leave small gaps between APC segments 1 and 2 as well as between segments 2 and 3. In the special case of sample number C91, the rare mutation APC c.4340delp. Q1447fs was located outside the sequence which is amplified by use of the second primer pairs of segments 2 and 3. For a gapless analysis of this area, it would be necessary to move the primers a bit.

As seen by direct sequencing, sample no C80 contained a single-nucleotide polymorphism (SNP) affecting the third position of codon 1493. Although a substitution at this position has no effect the SNP should be principally detectable by means of WTB-HRM (like in sample no C92). However, the HRM chart of sample C80 differed only slightly from the wild-type curve. So it was classified as wild-type by the software. Resequencing of the HRM product revealed the coexistence of both possible nucleotides (G and A) in sample C80.

Because of its exceptionally low detection limit, the WTB-HRM method could facilitate noninvasive screening for early colorectal cancer as well as for precursor lesions by detecting cancer initiating gene mutations in body fluids like stool. In this context, it would be also interesting to answer the question whether those mutations indicating cancer precursor lesions can be detected at similar levels in plasma compared with stool. The data should be verified next by determining sensitivity and specificity of WTB-HRM in a representative contingent of patients. It can be expected that besides APC and KRAS, the technique is transferable to any other gene being mutated during carcinogenesis. Hence, WTB-HRM could be a valuable tool in cancer prevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: C. Gerecke, B. Scholtka
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Gerecke, U. Gottschalk, B. Scholtka
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C. Gerecke, C. Mascher, B. Kleuser, B. Scholtka
Writing, review, and/or revision of the manuscript: C. Gerecke, B. Kleuser, B. Scholtka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Gerecke, C. Mascher, B. Scholtka
Study supervision: B. Scholtka

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Ultrasonic Detection of Cancer-Initiating Mutations

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Ultrasensitive Detection of Unknown Colon Cancer-Initiating Mutations Using the Example of the Adenomatous Polyposis Coli Gene

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