Chapter 5

Sensitive and Specific KRAS Somatic Mutation Analysis on Whole Genome Amplified DNA from Archival Tissues

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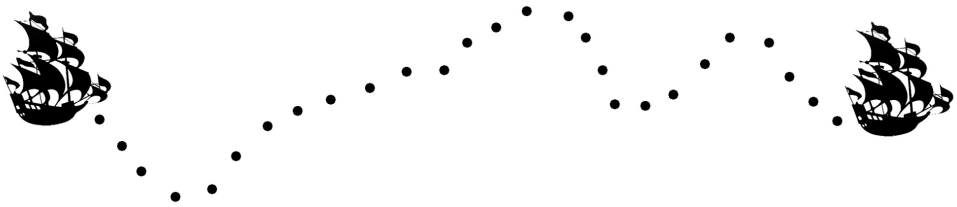

Abstract
KRAS is a small GTPase that plays a key role in Ras/MAPK signaling; somatic mutations in *KRAS* are frequently found in many cancers. The most common *KRAS* mutations result in a constitutively active protein. Accurate detection of *KRAS* mutations is pivotal to the molecular diagnosis of cancer and may guide proper treatment selection.
We describe a two-step *KRAS* mutation screening protocol that combines whole genome amplification (WGA), high resolution melting analysis (HRM) as a prescreen method for mutation carrying samples, and direct Sanger sequencing of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue, from which limited amounts of DNA are available. We developed target-specific primers, thereby avoiding amplification of homologous *KRAS* sequences. The addition of Herring Sperm DNA facilitated WGA in DNA samples isolated from as few as 100 cells.
We show that *KRAS* mutation screening using HRM on wgaDNA from FFPE tissue is highly sensitive and specific; additionally, this method is feasible for screening of clinical specimens, as illustrated by our analysis of pancreatic cancers. Furthermore, PCR on wgaDNA does not introduce genotypic changes, as opposed to unamplified genomic DNA. This method can, after validation, be applied to virtually any potentially mutated region in the genome.

Introduction
Kirsten RAS *(KRAS)* is a member of the *Ras* gene family, which encodes small G proteins with intrinsic GTPase activity. These proteins play a key role in Ras/MAPK signaling, which is involved in multiple pathways including proliferation, differentiation, and apoptosis. It has been suggested that *KRAS* mutations are related with a random CpG island methylation pattern which may lead to CpG island methylator phenotype (CIMP)-low tumors (1). *KRAS* is an important etiological factor in many cancers. Somatic mutations in *KRAS* are found in 75-90% of pancreatic adenocarcinomas, 35-50% of colorectal carcinomas, and 30% of lung adenocarcinomas. In other cancers, *KRAS* mutations are less frequent or only present in specific subsets, such as subsets of bladder, endometrial, thyroid, and liver cancers (2-5). Mutations in *KRAS* negatively predict success of anti-EGFR therapies. Gain-of-function *KRAS* mutations lead to EGFR independent activation of intracellular signaling pathways, resulting in tumor cell proliferation, protection against apoptosis, increased invasion and metastasis, and activation of tumor induced angiogenesis (6).
The most common *KRAS* mutations are found in exon 2 (codons 12 and 13) and, more rarely, in exon 3 (codons 59 and 61). These mutations alter the conformation of *KRAS,* causing impaired GTPase activity that results in constitutive activation of the protein (7). Accurate detection of *KRAS* mutations is pivotal to the molecular diagnosis of cancer and may guide proper treatment selection. *KRAS* mutation analysis has been shown to be important for disease stratification in clinical trials of EGFR inhibitors (8;9), and for the detection of *MUTYH* mutants after *KRAS* mutation pre-screening (10). In the near future, it is expected that at least 50% of all recurrent colorectal tumors will be screened for *KRAS* mutations.

Various methods have been described for the detection of *KRAS* mutations, such as a mutagenic PCR assay (11), pyrosequencing (12), and real time PCR (13); however, Sanger sequencing on PCR products remains the golden standard. (6;14;15) Recently, high-resolution melting analysis (HRM) was added as a method for mutation scanning and genotyping (16-18), including analysis of *KRAS* mutations in heterogenic tumor populations. This method is a valuable addition to Sanger based sequencing, as it detects heterozygous genetic changes in samples containing only 10% of mutant cells (19-21), whereas direct Sanger sequencing requires the mutation to be present at a level of 20% of the sample. (20) HRM has also been described for methylation detection and the detection of internal tandem duplications (22;23). In addition, HRM has a high sensitivity and specificity for the detection of variants in a background of normal DNA (24;25).
For mutation analysis, the majority of tissues are available as formalin-fixed, paraffin-embedded (FFPE) material. The genomic DNA (gDNA) that can be isolated from FFPE tissue is usually fragmented due to formalin fixation. At the same time, for most cases, including pre-operative biopsies, the available (FFPE) tissue, and thus gDNA, is limiting. As a result, the number of genetic assays that can be performed is restricted (26;27).
One approach designed to overcome this limitation is whole genome amplification (WGA), which ideally generates a new whole genome sample of amplified DNA (wgaDNA) that is indistinguishable from the original, but with a higher DNA concentration (28). We used a primer extension pre-amplification (PEP) method that has been successfully applied to formalin-fixed paraffin-embedded (FFPE) tissue (29;30). We have studied HRM as a pre-screening method for somatic mutation detection in combination with WGA on gDNA from FFPE tissue. This approach is sensitive and specific and can open the archives for large scale mutation analysis (31-34).

Materials and methods

Samples

We previously performed somatic *KRAS* mutation analyses in a series of colorectal cancers (35). A subset of 60 tumors (14 FF and 46 FFPE) was used to determine the sensitivity and specificity of the assay on FF and FFPE tissue. The tumor cell percentage in the series was 50-80%. Additionally we isolated gDNA from five pre-operative biopsies from pancreatic adeno-carcinomas and three 0.3 mm tissue punch cores that were isolated from matching resection specimens. Guided by an H&E stained section, the extremely small tumor fields were dissected from the biopsies.
DNA was extracted using a standard proteinase K method as described elsewhere (36). All samples were handled according to the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (www.federa.org, accessed June 2, 2009).

gDNA concentrations were measured using PICOgreen (Invitrogen/Molecular Probes, Leiden, The Netherlands) according to the manufacturer's protocol. For WGA and PCR, gDNA is brought to a standard concentration of 5 ng/µL in 10 mM Tris, pH 8.0, 0.1 mM EDTA, and is stored in 2D bar-coded sample tubes (Thermo Fisher Scientific, NH, USA) for process standardization and robotic analysis.

Whole genome amplification

Primer extension pre-amplification (PEP) WGA using thermo stable DNA polymerases (Kbioscience, UK) was carried out according to the manufacturer's protocol using 25 ng gDNA in a final reaction volume of 25 µL. For FFPE samples and other samples from which limited gDNA was available, herring-sperm DNA (Promega) was added to a final concentration of 2 ng/µL per reaction. Thermal cycling was performed in a Biorad I-cycler. After an initial denaturation step of 10 minutes at 94^{o}C, 40 cycles of 30 seconds at 94^{o}C, 30 seconds at 37^{o}C, and ramping at a speed of 0.1^{o}C /sec to 55^{o}C, and 4 minutes at 55^{o}C were performed.

Mutation scanning and detection

Oligonucleotides were obtained from Operon (Germany). The primer sequences for the amplification of *KRAS* codons 12 and 13 were KRAS_C1213_M13F 5'-(*TGT AAA ACG ACG GCC AGT*-TCG ACC CAG GAT CCA ACT T-**GCT GAA AAT GAC TGA ATA TAA ACT TG)-3'** and KRAS_C1213_M13R 5'-(*CAG GAA ACA GCT ATG ACC ATG A*-TCC AGT ACT TGA GAG AAT TCC ATC-**TAG CTG TAT CGT CAA GGC ACT C)-3'**. Stuffer sequences (underlined), were added between M13 tails (*in italics*) and the *KRAS*-specific part (**bold**) of the primer. The total length of the amplicon, inclusive of the M13 tails and stuffers, is 166 base pairs.

Duplicate PCR reactions were carried out in 10 µL reactions in white 96 well plates (AB0800/W, ABgene) that are suitable for HRM. The reactions included iQ Supermix (Bio-Rad, cat nr 170-8860), 2 pmol primers and 1 µM SYTO9 (Invitrogen). PCR reactions were performed with an initial denaturation step of 10 minutes at 95^{o}C, followed by 40 cycles of 5 seconds at 95^{o}C, 10 seconds at 60^{o}C, and 10 seconds at 72^{o}C, and a final elongation step of 10 minutes at 72^{o}C. Sanger DNA sequencing was performed on gDNA and wgaDNA at the sequence core of the Leiden Genome Technology Center, using the same PCR products as those submitted to HRM. Prior to Sanger sequencing PCR fragments were purified using a filter system according to the manufacturer's protocol (Montage, Millipore). DNA was eluted in 25 µL of sterile water. Sanger sequencing was subsequently performed with 5-10 ng of DNA and 6 pmol of an M13 primer (PR_M13F TGTAAAACGACGGCCAGT and PR_M13R CAGGAAACAGCTATGACC) on an ABI 3700 DNA Analyzer using Big Dye Terminator Chemistry (Applied Biosystems). All sequences were visually analyzed with Mutation Surveyor^{TM} DNA variant analysis software (version 2.61 Softgenetics, State College, PA).

HRM was performed in a LightScanner (Idaho Technology) after the addition of 15 µL of mineral oil (Sigma); Light Scanner software (version 1.1.0.566, Idaho Technology) was used for analysis. High salt addition (24); 1.5 µL of 1M KCl

and 0.5M Tris-HCl, pH 8.0, was added post-PCR to the 10 µL PCR products followed by 4 additional temperature cycles (30 seconds 94˚C, 30 seconds 72˚C). The sensitivity and specificity of the HRM were calculated. The sensitivity was determined as the number of true positives divided by the sum of the true positive and false negative samples. The specificity of the samples was determined as the number of true negatives divided by the sum of the true negatives and false positives. Mineral oil overlay, high salt addition, and PCR product purification was performed in a post-PCR setting.

Results

Detection of *KRAS* mutations in genomic DNA using HRM

KRAS PCR and HRM analyses were performed on gDNA in duplicate before and after the addition of high salt. All duplicate samples with an aberrant melting pattern were identified as carrying a possible mutation. The data were compared with the Sanger sequencing results. An overview of the results is shown in Table 1. `Six FFPE samples (5%) failed to give an interpretable HRM pattern in one of the duplicates. None of these gave contradictory sequence results and were included in further analysis. (Supplementary table 1 at http://jmd.amjpathol.org)
In the set of 60 tumors we observed an overall sensitivity of 100% (33/33) and a specificity of 81% (22/27) for the detection of *KRAS* codon 12 or 13 variations. The specificity of gDNA from FFPE tissue (75%; 15/20) was lower than in FF tissue (100%; 7/7), probably as a result of poor gDNA quality intrinsic to the material (Table 2).

HRM on whole genome amplified (WGA) DNA from archival tissues

To evaluate the sensitivity and specificity of HRM on WGA-treated DNA from tumor specimens of different origins and quality, we performed WGA on the gDNA samples with known *KRAS* mutation status. To assess the DNA quality pre- and post-WGA, a multiplex PCR containing 3 fragments (150, 255, and 511 base pairs) was carried out. In all of the samples at least the 255-bp band was visible (data not shown).
Herring Sperm DNA was added up to 50 ng to all samples prior to WGA as driver DNA to prevent the amplification of excess small random PCR products
After WGA, 1:1, 1:5, and 1:10 dilutions of the wgaDNA were made to determine the amount of input wgaDNA that was required for HRM PCR. The addition of 2 µl of the 1:5 dilution to a 10 µl PCR gave the best results and we were able to detect the different mutations in control samples (Figure 1).
We combined 25 ng gDNA with 25 ng Herring Sperm DNA in each WGA reaction for the subset of 60 tumors. In these experiments, the researchers were blinded to the HRM and Sanger sequence results obtained for the gDNA samples. Subsequently, *KRAS* PCR was performed in duplicate, and HRM analysis was performed before and after high salt addition. Three samples (2.5%) failed to give an interpretable HRM pattern in one set of the duplicates. For each product, one of the duplicates was purified and Sanger sequenced. HRM and re-sequencing of the WGA PCR samples revealed no discrepancies with the original samples

Table 1. False positives and false negatives in *KRAS* (wga) HRM and sequencing. Overview of false positives (FP) and false negatives (FN) in HRM performed with or without WGA. In the wgaDNA column, the HRM results on samples without the addition of high salt are shown between brackets. HRM results after the addition of a high salt solution on samples performed in duplicate (results for samples marked with * are based on a single result). The last column shows the concordant gDNA and wgaDNA results of Sanger sequencing on one of the duplicates. The complete overview of all tested samples is listed in Supplementary Table 1. FF; Freshly Frozen tissue, FFPE; formalin fixed paraffin embedded tissue, Var; KRAS-variant, WT; KRAS wild-type.

ID	Type	gDNA	wgaDNA (no salt)	(g-wga)DNA Sequencing
972	FF	Var	Var (FN)	c12. GGT>GTT
755	FF	WT	FP (WT*)	WT
819	FF	WT	WT (FP)	WT
826	FF	WT	WT (FP)	WT
977	FFPE	Var	Var (FN)	c13. GGC>GAC
998	FFPE	Var	Var (FN*)	c13. GGC>GAC
811	FFPE	Var	Var (FN)	c12. GGT>GAT
761	FFPE	Var	Var (FN)	c12. GGT>GTT
768	FFPE	Var	Var (FN)	c12. GGT>GTT
785	FFPE	Var	Var (FN)	c12. GGT>GTT
806	FFPE	Var	Var (FN*)	c12. GGT>GTT
013	FFPE	FP	WT (WT)	WT
023	FFPE	FP	WT (WT)	WT
750	FFPE	FP*	WT (FP)	WT
958	FFPE	FP	WT (WT)	WT
975	FFPE	FP*	WT (WT)	WT

Table 2. Sensitivity and specificity in (wga)HRM. Sensitivity and specificity calculated for HRM in FFPE, Freshly Frozen (FF) and combined FFPE and FF gDNA and wgaDNA samples in the presence of high salt. In the wgaDNA columns, HRM results on samples without the addition of high salt are shown between brackets. FP, false positives; FN, false negatives; Var, KRAS-variant; WT, KRAS wild-type.

	FFPE	FFPE	FF	FF	Com - bined	
DNA type	**gDNA**	**wgaDNA**	**gDNA**	**wgaDNA**	**gDNA**	**wgaDNA**
WT	15	20 (19)	7	6 (5)	22	26 (24)
Var	26	26 (19)	7	7 (6)	33	33 (25)
FN	0	0 (7)	0	0 (1)	0	0 (8)
FP	5	0 (1)	0	1 (2)	5	1 (3)
overall	46		14		60	
% Sensitivity	100	100 (73)	100	100 (86)	100	100 (76)
% Specificity	75	100 (95)	100	86 (71)	81	100 (89)

(Table 1 and Supplementary table 1 at http://jmd.amjpathol.org). The sensitivity and specificity of the HRM was calculated. WGA-HRM proved to be 100% (33/33) sensitive and 96% (26/27) specific in the presence of post-PCR high salt. Without high salt, the sensitivity and specificity were lower (76% (25/33) and 89% (24/27), respectively).(Table 2)

Detection limits for HRM of wgaDNA

To determine the limits of HRM to detect a possible *KRAS* mutation in wgaDNA from FFPE tissue, we performed WGA on a two-fold serial dilution of gDNA carrying a g.35GGT>GTT (p.12G>V) mutation. The gDNA input ranged from 10 ng to 0.08 ng, the latter corresponding to the gDNA equivalent of approximately 10-12 cells. After WGA, HRM was performed in duplicate on WGA samples diluted 1:5. A mutant allele was detected in WGA products corresponding with 600 pg (equivalent to approximately 100 cells) or higher. HRM on wgaDNA originating from lower input gDNA resulted in low fluorescence of the mutant allele, thus impairing the analysis and interpretation. We performed direct Sanger sequencing on all wgaDNA samples. The *KRAS* mutation was found in all dilutions, although the mutant allele was difficult to identify due to background noise in samples with lower than 600 pg input gDNA. *KRAS* mutations were easily detected in sequences from WGA PCR isolates corresponding to 100 cells or more (Figure 2) (29).

***KRAS* mutation detection in pancreatic adenocarcinomas**

We further evaluated *KRAS* HRM and Sanger sequencing in wgaDNA from archived clinical specimens. gDNA from pancreatic adenocarcinomas was isolated from a single slide (samples 1a-4a) and from four combined slides (1b-4b). For comparison, we also isolated gDNA from three 0.3 mm tissue punch cores that were isolated from matching resection specimens. After gDNA isolation, this sample was split into a minor (5f) and major (5p) fraction; the major fraction contained a 6-fold higher gDNA concentration. As expected, the gDNA concentrations of the isolates were low or not measurable (Table 3). Subsequently, WGA was performed on the fractions, and, HRM and Sanger sequencing were performed on the wgaDNA. For the samples 1b and fp, gDNA was also tested.

WGA was performed on 10 ng gDNA and adjusted to 50 ng with Herring Sperm DNA. For samples where the gDNA concentration was not measurable 15 µl of the raw isolate was added to the WGA reaction together with 50 ng with Herring Sperm DNA. HRM revealed concordant *KRAS*-variant curves in samples 5f and 5p, while samples 1a and 1b gave contradictory results for HRM. The variant curves for sample 3b were interpretable, while the signals for samples 2a, 2b, 3a, 4a, and 4b were low and could not be interpreted. Purification and re-sequencing of all of the HRM PCR products was possible in all cases, and revealed that samples 2 and 5 carried the g.35G>A (p.G12D) mutation, sample 3 was a g.35G>T (p.G12V) mutant, and samples 1 and 4 were wild-type. These results indicated that the HRM results for sample 1a were false positive (Table 3).

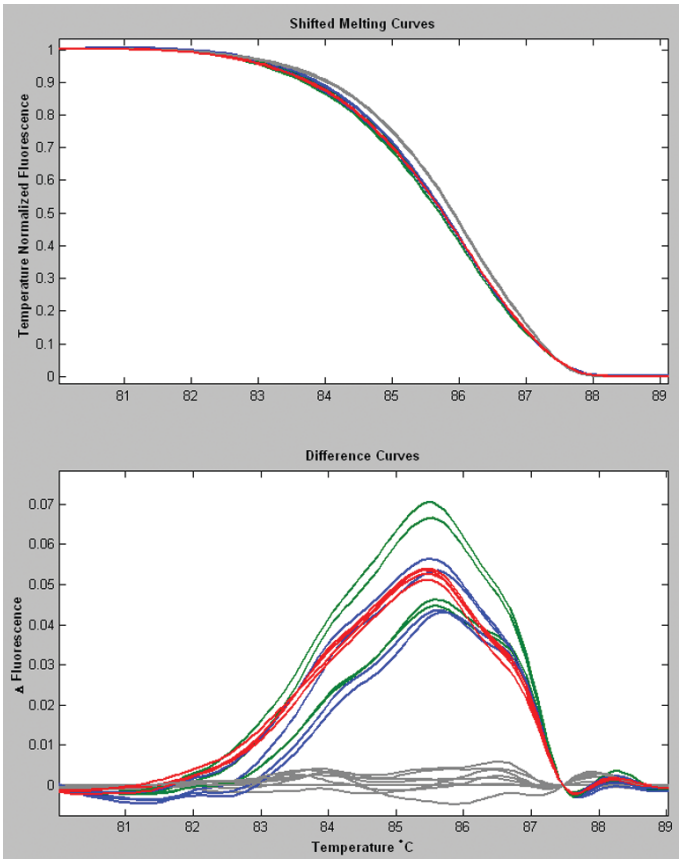

Figure 1. HRM curves in samples treated with or without WGA. HRM shifted melting curves and difference curves of 3 different *KRAS* codon 12 and 13 mutations in duplicate, with or without WGA treatment. g.35G>A (p.12G>D) DNA lower green, wgaDNA upper green; g.38G>A (p.13G>D) DNA lower blue, wgaDNA upper blue; g.35G>T (p.12G>V) red; and a series of 8 wild-type samples (gray).

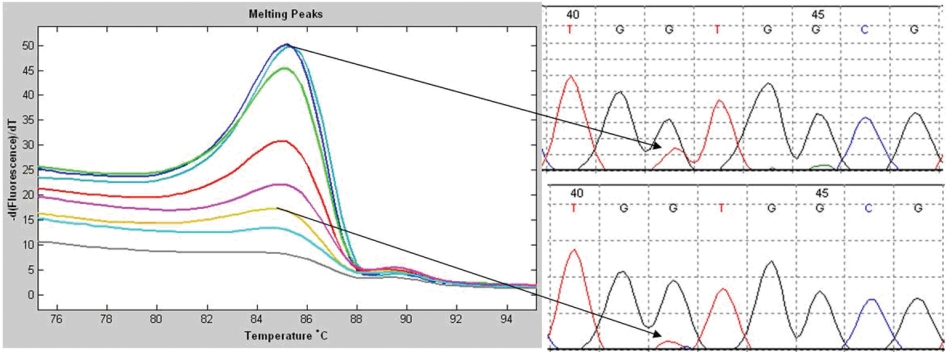

Figure 2. Detection limit of *KRAS* mutations in a PCR of serial diluted wgaDNA. Left panel: 2-fold serial dilution of gDNA starting with 0.08 ng input (lowest curve) to 10 ng (upper curve) shows the detection limit for *KRAS* mutations in a PCR on wgaDNA. Upper right panel: Sanger sequence on wgaDNA corresponds with 10 ng gDNA input in the WGA. Lower right panel: Sanger sequence on wgaDNA corresponds with 0.625 ng (+/- 100 cells) gDNA input in the WGA.

Table 3. WGA HRM results in histological specimens from pancreatic adenocarcinomas. Concentration measurements are performed on gDNA isolates from a single slide (samples 1a-4a), from four combined slides (samples 1b-4b), and from a matching resection specimen with minor (5f) and major fractions (5p). For a number of samples the gDNA concentration was not measurable (low). WGA is performed on all samples. HRM results in the presence of high salt. Samples marked Low Signal had an unclear HRM pattern. The last column shows the *KRAS* Sanger sequencing results on the purified HRM sample.

Sample	Tumour Sections	Concentration gDNA (ng/µl)	wgaDNA HRM+HighSalt	wgaDNA Sequence
1a	1	2.2	VAR	WT
1b	4	9.9	WT	WT
2a	1	<2	Low Signal	c12. GGT>GAT
2b	4	2.9	Low Signal	c12. GGT>GAT
3a	1	<2	Low Signal	c12. GGT>GTT
3b	4	3.3	VAR	c12. GGT>GTT
4a	1	<2	Low Signal	WT
4b	4	<2	Low Signal	WT
5f	fraction	<2	VAR	c12. GGT>GAT
5p	3 punches	12.1	VAR	c12. GGT>GAT

Discussion

Accurate detection of *KRAS* mutations is pivotal to the molecular diagnosis of cancer and may guide proper treatment selection. We have developed a standard WGA and PCR protocol for *KRAS* mutation detection in gDNA derived from FFPE tissue, for which limited amounts of gDNA are available. High resolution melting analysis (HRM) is used for mutation prescreening and Sanger sequencing is used for mutation detection.

The most frequently mutated codons, 12 and 13, in exon 2, are located in a region that is highly homologous to regions on chromosomes 6 and 16. To prevent the amplification of pseudogene sequences we used primers based on non-homologous nucleotides (10;37;38). Addition of universal M13 tails to the primers increased the specificity and fidelity of the PCR (10;39) and allows the use of uniform sequencing primers. Since the *KRAS* amplicon length is only 75 nucleotides and short PCR fragments can be difficult to sequence, we added additional stuffer sequences to the primers, between the M13 tails and the *KRAS*-specific part of the primer. These stuffers bear no homology to any known human sequence. Since the amplicons are very small, the length of each step of the PCR cycle was shortened, resulting in a 40 cycle PCR that lasted just over 1 hour.

We evaluated LCGreen and SYTO9 for HRM and found that both dyes were capable of detecting different *KRAS* variations. SYTO9 has been reported to have some advantages in terms of dye stability, dye-dependent PCR inhibition, and selective detection of amplicons during DNA melting curve analysis of multiplex

PCR (40). In our experiments, SYTO9 appeared to improve the quality of the melting curves, since the fluorescence signal was approximately 50% higher and the duplicate curves fit more tightly together. Therefore, we decided to use SYTO9 in subsequent experiments (Supplementary Figure S1 http://jmd.amjpathol.org). It has been reported that high-resolution analysis of amplicon melting is limited by any T_m variance, including differences in salt concentrations (arising from evaporation during processing or differences in buffers used for DNA preparation), and any variation in instrument temperature (41). Furthermore, poor DNA quality, low input, and positional effects of the samples on the microtiter plate might lead to false positive or false negative HRM measurements (42). Therefore, all samples were analyzed in duplicate. For calibration, at least two wild-type samples were analyzed in each experiment.

We observed an overall sensitivity of 100% (33/33) and a specificity of 81% (22/27) for the detection of *KRAS* codon 12 or 13 variations. However the specificity of gDNA from FFPE tissue (75%,15/20) was lower than in FF tissue (100%, 7/7), probably as a result of poor gDNA quality intrinsic to the material (Table 2). From the melting curve behavior, it was not possible to determine the specific mutation, likely due to tumor percentage and tumor heterogeneity. Consequently, all samples with possible mutations were Sanger sequenced in order to identify the nature of the mutation. Sanger sequencing was directly performed on the purified HRM PCR product without repeating the PCR, which is time, and costs saving.

KRAS mutations are frequently found in ductal pancreatic cancers (4) making it important in clinical practice to identify *KRAS* mutations in cytological, pancreatic juice with only minimal amounts of cells and limiting (FFPE) gDNA (26;27;43). One approach designed to overcome the limited number of assays possible on this material is whole genome amplification (WGA). Different types of WGA methods are available. Strand displacement amplification (SDA or MDA) has been described as most reliable for genotyping, giving highest call rates, best genomic coverage and lowest amplification bias. However this method has the disadvantage that the specific performance largely depends on input DNA quality making it less suitable to efficiently amplify DNA extracted from FFPE material (44). Primer extension pre-amplification PEP based WGA has been successfully applied to FFPE tissue. (29;30) although some bias as result of the WGA has been observed (12). We used a PEP based WGA method that is known to have high sequencing accuracy and is less dependent upon the quality of the input DNA (45) however we observed that, with low amounts of input (< 10 ng) gDNA and with poor quality FFPE samples, the random primers in the WGA produce excess random wgaDNA and primer dimer products. These additional products impaired HRM and subsequent Sanger sequencing. Therefore, we added non homologous Herring Sperm to the human DNA, for all samples prior to WGA to suppress primer dimer formation. The combined human and herring DNA input in the WGA reaction was approximately 50 ng. Herring Sperm DNA has low homology to human *KRAS* and we did not detect any PCR product with the described primers in a control PCR. Therefore, herring sperm DNA addition should not interfere with *KRAS* HRM or Sanger sequencing. We compared the Sanger

sequencing results on gDNA and wgaDNA. This did not reveal any discrepancies and showed that *KRAS* mutation screening using HRM on wgaDNA from FFPE tissue is concordant with the non-wgaDNA results. With this approach, we were able to WGA minimal amounts of gDNA with reliable results. We observed that with less than 2 ng of FFPE-derived gDNA input in the WGA reaction HRM on wgaDNA becomes unreliable. The PCR products still produced reliable Sanger sequences. This was also demonstrated by the pancreatic adenocarcinomas which failed for HRM but gave good Sanger sequence results. This showed that HRM is very sensitive for DNA variants, however, to obtain interpretable results higher amounts of DNA are required in the HRM than in Sanger sequencing (Table 3).

We optimized HRM in wgaDNA by the post-PCR addition of high salt (24). This resulted in enhanced heteroduplex formation, better discrimination of the mutation carrier during analysis, and 100% sensitivity and 96% specificity. Cho et al 2008 (34) reported that HRM on wgaDNA results in a higher fals positive rate and reduced sensitivity and specificity. We show that high-salt addition prior to analysis overcomes this problem, thereby making this approach suitable for high throughput mutation pre-screening.

A potential disadvantage of our method could be that PCR plates need to be opened prior to analysis, to add mineral oil and high salt solution, although opening the plates for PCR cleanup prior to sequencing is standard practice. To minimize the chance of PCR amplicon contamination pre, and post PCR rooms were strictly separated and we used a direct PCR reaction to avoid pseudogene amplification rather than nested PCR for *KRAS* mutation detection (37;46).

Another factor to consider is tumor heterogeneity. In samples with lower tumor percentage and low amount of the mutated allele, automated Sanger sequence analysis could miss variants while HRM could still detect mutations in gDNA and wgaDNA from samples with low tumor percentages. Vossen et al show that DNA variations could be detected in sample mixtures with as little as 5% variation fraction, although 30% and higher gave more reliable results in HRM (24). Because HRM in samples with unknown tumor percentage is limited in predicting the exact *KRAS* variant, samples with HRM variations have to be sequenced for variant determination. A combination of low tumor percentage and low input concentrations in the WGA might cause HRM dropout or contradictory results in the duplicate reactions, making careful (re)analysis of the Sanger sequencing indispensable (Figure 2). For this type of samples alternative mutation detection after HRM pre-screening should be considered such as pyrosequencing which is known to be more sensitive than Sanger sequencing (12). Allele specific real time PCR is also a very sensitive method but it has the disadvantage of detection known (*KRAS*) variants only while our approach envisions application for denovo mutation screening (47). For this type of samples sensitivity and specificity have to be calculated in relation to results obtained with pyrosequencing or real time PCR.

One can argue that the majority of laboratories can obtain a sufficient amount of DNA for *KRAS* mutation screening from even tiny biopsies without the WGA step.

This assertion might be true in some cases. However, in a time when personalized medicine is the norm, *KRAS* mutation detection may be one test in a series of many and in that respect, WGA may be an excellent method by which to increase the initial amount of DNA that can be used for the analysis of any potentially mutated region in the genome. (48)
Finally, the required equipment for this approach is limited to two standard thermal cyclers (one dedicated to WGA and another in a separate room for PCR), dedicated HRM equipment, and a sequencing facility. HRM on wgaDNA from FFPE origin can be a cost effective pre-screening method, since only potential variants found after HRM need re-sequencing. Therefore, HRM, in combination with WGA and sequencing, is a strong tool for *KRAS* mutation screening of samples with partially degraded or low yield DNA, as is often found in pathology archives.

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