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**Research Article** 

## Comparison of DNA Extraction Methods from Formalin-Fixed, Paraffin-Embedded Tissue and their Impact on Real-Time PCR-Based Mutation Assays

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#### Abstract

In this new era of personalized cancer care, molecular testing for somatic mutations plays an increasing role in treatment decisions for many targeted cancer therapeutics. Formalin-fixed paraffin-embedded tissue (FFPET) specimens remain the typical source of nucleic acid for such testing. Because formalin fixation can damage nucleic acids and many tumor specimens are small, obtaining a sufficient quantity of high-quality DNA for mutation testing can be challenging. Given these pre-analytic variables, the availability of a standardized and well-validated method to isolate DNA from such specimen types is critical. We compared two widely available commercial kits for DNA isolation (cobas DNA Sample Preparation Kit from Roche Molecular Systems, and QlAamp DNA FFPE Tissue Kit from Qiagen) using 120 FFPET specimens from a range of tumor types (melanoma, thyroid, colorectal, lung, breast, and ovarian cancer) and examined the effects of the different DNA isolation methods on the subsequent performance of real-time PCR-based assays for BRAF, KRAS and EGFR mutations. Although the two methods gave comparable nucleic acid quantities, the cobas method co-purified significantly less RNA (p<0.001) as determined by comparing DNA yields before and after RNase treatment. The presence of RNA in the extracted DNA was associated with delayed threshold cycle (Ct) in real-time PCR-based mutation tests. The cobas method consistently yielded a sufficient quantity of purified DNA across range of tumor types and specimen sizes for real-time PCR mutation detection tests without requiring an additional step of RNase treatment.

**Keywords:** Mutation detection; DNA extraction; Formalin-fixed paraffin-embedded tissue; PCR

**Abbreviations:** FFPET: Formalin-Fixed Paraffin-Embedded Tissue; NSCLC: Non-Small Cell Lung Cancer; H & E: Hematoxylin and Eosin; ANOVA: Analysis of Variance; EGFR: Epidermal Growth Factor Receptor

## Introduction

The role of companion diagnostic tests in drug development and patient management for targeted therapy has become an area of intensive cancer research [1,2]. Examples of recently approved targeted therapies that require companion diagnostic mutation testing include the selective BRAF inhibitor vemurafenib in malignant melanoma (BRAF V600 mutations) [3], anti-EGFR antibodies, such as cetuximab and panitumumab, in colorectal cancer (KRAS mutations) [4,5] and small-molecule anti-EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, in non-small cell lung cancer (EGFR mutations) [6,7].

Obtaining sufficient high-quality DNA to perform these types of mutation assays can be challenging, and there can be substantial variation in the yields of amplifiable DNA from specimen to specimen. That variability can be ascribed to a variety of pre-analytic factors. The prevailing specimen types are still formalin-fixed paraffin-embedded tissue (FFPET) specimens. Formalin fixation induces DNA-protein cross-links that can interfere with the amplifiability of the DNA in the specimen. The degree of fixation-induced DNA damage may be influenced by variables such as pH of the fixative and the duration of fixation [8]. A number of intrinsic attributes of the tumor specimen, such as tissue area, degree of necrosis and endogenous inhibitors such as melanin [9], can also contribute to the variability in yields of amplifiable DNA. Adding to these difficulties is the fact that many clinical specimens are small, and there are competing demands to utilize that limited tissue for an increasing number of other molecular and immuno-histochemical tests [10].

Another important pre-analytic variable can be the method used to isolate DNA from the tumor specimen. A few studies comparing the recovery of nucleic acid from FFPET specimens by various DNA extraction kits have reported data from a limited number of tumor types using specimens with relatively large amounts of tissue [11,12], without assessing the impact of different DNA isolation methods on the subsequent performance of real-time PCR-based mutation tests. A number of DNA isolation kits are commercially available and commonly used, but there are limited data comparing different DNA isolation methodologies and it is thus unclear which method can perform consistently across a spectrum of tumor types and sizes for molecular diagnostic applications.

In the present study, we compared the performance of two widely available commercial DNA isolation kits, using a panel of 120 clinical tumor specimens representing six different cancer types. The study examined the yields of nucleic acid isolated with the two methods from single 5-micron tissue sections, assessed the degree of RNA

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Figure 1 : Experimental design for comparison of DNA extraction methods from FFPET specimen. 'BRAF mutation test was performed using extracts with and without RNase treatment for both methods, † KRAS & ‡EGFR mutation tests were performed using extracts without RNase treatment for both methods.



yields (per mm<sup>2</sup> of tissue) from 120 FFPET specimens, with respect to tissue area. QIAamp = cobas depicting the difference between nucleic acid yields ≤ 5 ng/mm<sup>2</sup>

co-purification, and the impact of that RNA co-purification on the performance of three different real-time PCR mutation tests.

## Materials and Methods

## Tumor specimens and tissue processing

The 120 FFPET tissue blocks (all resected tumor specimens) used in this study included 20 blocks of each of six tumor types - melanoma, lung cancer, ovarian cancer, thyroid cancer, breast cancer and colorectal cancer with a range of tissue area and percent tumor content. All specimens were obtained by commercial vendors under informed consent with patient information de-identified.

A schematic diagram of the study design is depicted in figure 1. Ten consecutive 5 µm sections of each specimen block were prepared manually using high-profile steel blades on a Leica RM2245 semiautomated rotary microtome (Leica Microsystems, Bannockburn, IL). One slide from the middle of the ten sections was stained with hematoxylin and eosin (H & E). The H & E slides were evaluated by a board-certified pathologist (B.B.), who confirmed the diagnosis and assessed the tissue area and tumor content of each specimen.

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The tissue area for each specimen was measured in mm<sup>2</sup> by measuring lengths and widths of rectangular specimens or radii of ellipses and circles, or bases and lengths of triangular pieces by using a micrometer mounted on an Olympus microscope eyepiece. The tumor areas were then calculated according to standard geometric formulas based upon the tissue shape. Subtractions were made for cystic areas. The percent viable tumor was calculated for each specimen using the following formula: [Area of viable tumor (mm<sup>2</sup>)/Total tissue area  $(mm^2)] \times 100.$ 

Consecutive 5 µm sections from each specimen were placed on separate glass slides, air-dried for 5-10 minutes, and stored at room temperature. Deparaffinization was performed at room temperature by soaking tissue slides for 5 minutes in xylene followed by 5 minutes in 100% ethanol. Slides were then air-dried for 5-10 minutes.

### Genomic DNA extraction and quantification

Genomic DNA from the first slide-mounted section of each specimen was isolated using the cobas DNA Sample Preparation Kit (Roche Molecular Systems, Branchburg, NJ, USA, 'cobas method'), following the procedures described in the package insert for the cobas 4800 BRAF V600 Mutation test [13].

The QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA, 'QIAamp method') was used to extract genomic DNA from the second slide-mounted section of each specimen according to the Qiagen instruction handbook [14].

Nucleic acid concentration (ng/µL) and purity by OD ratio  $(\mathrm{A_{260}}/\ \mathrm{A_{280}})$  were determined by averaging two readings obtained from a NanoDrop UV-Vis Spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, DE) according to the manufacturer's instructions. Total nucleic acid yield per 5-micron section (µg) was calculated using the total volume (100  $\mu$ L) of eluate. The nucleic acid eluate was stored at 2°C to 8°C for up to two weeks or at -20°C for longterm storage.

#### Assessment of RNA co-purification in DNA extracts

The third and fourth slide-mounted sections of each specimen were extracted using the cobas and the QIAamp methods, respectively, with an RNase treatment step prior to binding and column purification, according to the Qiagen instruction handbook. RNase A (Qiagen) was used at the recommended volume of 2  $\mu$ L (100 mg/mL) for each sample. DNA was quantified as described above. Nucleic acid yields were compared to yields obtained from the nucleic acid isolation procedures without RNase treatment. The impact of the different DNA isolation methods on PCR-based mutation testing was then assessed using 3 different assays.

#### **BRAF** mutation testing

The cobas 4800 BRAF V600 Mutation Test ('BRAF Test'; Roche Molecular Systems, Branchburg, NJ, USA) is a real-time TaqMan PCR assay designed to detect the V600E (1799T>A) mutation in the BRAF gene in FFPET specimens of malignant melanoma [15]. The BRAF Test is a CE-marked and FDA-approved in vitro diagnostic

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test which is indicated for the selection of BRAF-mutant metastatic melanoma patients for treatment with the selective BRAF inhibitor vemurafenib [16]. BRAF mutation testing was performed on all 120 tumor specimens, following the instructions in the package insert [13].

#### EGFR mutation testing

The cobas EGFR Mutation Test ('EGFR Test'; Roche Molecular Systems, Branchburg, NJ, USA) is a CE-marked allele-specific real-time PCR assay designed to detect mutations in exons 18, 19, 20, and 21 of the *EGFR* gene in FFPET specimens of non-small cell lung cancer (NSCLC). The EGFR Test is a 3-tube assay designed to detect G719X mutations (G719A, G719C, and G719S) in exon 18; 29 deletions and complex mutations in exon 19; S768I, T790M, and insertions in exon 20; and L858R in exon 21. *EGFR* mutation testing was performed on 20 FFPET NSCLC specimens following the instructions in the package insert [17].

#### **KRAS** mutation testing

The cobas KRAS Mutation ('KRAS Test'; Roche Molecular Systems, Branchburg, NJ, USA) is a CE-marked real-time TaqMelt PCR assay designed to detect somatic mutations in codons 12 ,13 (exon 2) and 61 (exon 3) of the proto-oncogene KRAS [18]. KRAS mutation testing was performed on 20 FFPET colorectal tumor specimens, following the instructions in the package insert [19].

#### Instrumentation and software

All real-time PCR testing for BRAF, KRAS and EGFR mutations were carried out on a cobas' 4800 System v2.0 (Roche Molecular

Systems, Rotkreuz, SW). A proprietary software tool (Algorithm Testing Frameworks v2.0) was used to analyze threshold cycle ( $C_t$ ) results for the BRAF Test and EGFR Test and for melt curve analysis to generate peak heights for the KRAS Test.

#### Statistical analysis

The impact of co-purified RNA in the DNA isolate was evaluated in four ways: (1) by the reduction in the Nano-drop measured quantity of nucleic acid (ng/mm<sup>2</sup>) after RNase treatment; (2) by changes in  $C_t$ values observed in the BRAF test after RNase treatment; (3) by changes in  $C_t$  values observed in the EGFR test after RNase treatment and (4) by changes in peak heights for the KRAS test.

Statistical analyses were performed using R 2.12 for Windows software. Paired Wilcoxon signed rank tests (one-tailed) were performed to determine if significant differences were observed between the cobas and QIAamp methods in the reduction in yield and C<sub>t</sub> values for *BRAF*. Analysis of variance (ANOVA) was used to evaluate the statistical significance of differences in the EGFR C<sub>t</sub> values, using method effect (cobas or QIAamp methods) as one factor and tube effect (tubes 1, 2, or 3) as another factor. The sample was treated as a blocking factor (because each sample was tested for both kits and three tubes), generating a total of six outcomes per sample.

#### Results

#### Nucleic acid yields of the two DNA isolation methods

The range of tissue areas for the different tumor types, and the

Tumor type (N)	Melanoma (20)	Colorectal (20)	Lung (20)	Thyroid (20)	Breast (20)	Ovarian (20)
	Median/Mean (min-max)	Median/Mean (min-max)	Median/Mean (min-max)	Median/Mean (min-max)	Median/Mean (min-max)	Median/Mean (min-max)
Tissue area - mm <sup>2</sup> ( <i>range</i> )	66/78 (30-270)	85/96 (21-224)	128/145 (55-352)	40/43 (14-90)	162/160 (36-300)	99/129 (4-321)
Tissue area-% (range)	90/86 (10-100)	78/74 (35-100)	83/78 (40-98)	92/84 (0-100)	80/77 (40-98)	93/78 (20-98)
cobas Kit Standard method (Without RNase) NA yield - ng/mm² ( <i>range</i> )	42/43 (16-78)	40/49 (19-151)	23/26 (10-50)	42/46 (25-128)	13/20 (7-61)	27/45 (15-236)
QIAamp Kit Standard method (Without RNase) NA yield - ng/mm² ( <i>range</i> )	48/48 (15-97)	41/53 (16-133)	25/36 (8-158)	19/21 (6-48)	20/22 (2-54)	30/33 (12-74)
cobas Kit With RNase treatment NA yield - ng/mm² ( <i>range</i> )	28/33 (9-68)	38/46 (16-191)	19/22 (8-46)	41/43 (20-96)	15/20 (6-54)	24/35 (8-196)
QIAamp Kit With RNase treatment NA yield - ng/mm² ( <i>range</i> )	24/26 (6-65)	19/20 (2-53)	14/16 (4-34)	10/12 (3-33)	6/7 (2-16)	14/18 (5-63)

Table 1: Range of tissue areas, tumor content and nucleic acid (NA) yields (ng//mm² of tissue) before and after RNase treatment.

FFPET tumor type (N)	Total nucleic acid yield per 5-micron section* (μg) median(mean)		DNA yield (µg) <sup>+</sup> median(mean)		DNA (%) <sup>‡</sup> median(mean)	
	cobas	QIAamp	cobas	QIAamp	cobas	QIAamp
All 6 tumor types (120)	2.6 (2.9)	2.7 (3.3)	2.0 (2.5)	1.2 (1.4)	88 % (91%)	43 % (54%)
Melanoma (20)	2.9 (2.9)	3.2 (3.7)	2.0 (2.2)	1.7 (1.6)	70 % (75%)	52 % (43%)
Colorectal (20)	3.4 (3.5)	3.6 (4.1)	3.2 (3.3)	1.2 (1.5)	93 % (94%)	33 % (36%)
Lung (20)	3.2 (3.4)	4.0 (4.6)	3.2 (2.8)	1.7 (2.2)	99 % (83%)	43 % (49%)
Thyroid (20)	1.7 (1.7)	0.6 (0.8)	1.8 (1.6)	0.5 (0.5)	102 % (95%)	88 % (65%)
Ovarian (20)	3.2 (3.1)	3.1 (3.5)	2.2 (2.6)	1.4 (1.6)	69 % (83%)	45 % (45%)
Breast (20)	2.0 (2.7)	2.4 (3.2)	2.0 (2.7)	1.0 (1.0)	101% (100%)	42 % (32%)

\*NA Yield\_Std (DNA+RNA), †NA Yield\_RNase (Pure DNA obtained from post RNase treatment),

<sup>‡</sup>DNA (%) = <u>NA Yield\_RNase</u> x 100 NA Yield Std

Table 2: Total nucleic acid yields, DNA yields, and pure DNA of the two DNA isolation methods after RNase treatment.

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4800 BRAFV600 Mutation Test. C<sub>t</sub> reduction = C<sub>t</sub> (no RNase treatment) minus C<sub>t</sub> (with RNase treatment). Wilcoxon signed rank tests for paired samples yielded P values of 0.004 (mutants) and <0.001 (wild-type). Circle represents an outlier (>3x interquartile range).

nucleic acid yields (per mm<sup>2</sup> of tissue) from the two DNA isolation methods are depicted in table 1. The median tissue area was the lowest for the thyroid cancer specimens and the highest for the breast cancer specimens, but these differences were not statistically significant (N.S.). The nucleic acid yields per mm<sup>2</sup> of tissue (Table 1) and total nucleic acid yields per 5-micron section (Table 2) for the 2 methods prior to RNase treatment were comparable: the cobas method gave higher nucleic acid yields in 47% (56/120 specimens), and the QIAamp method gave higher yields in 53% (64/120). The nucleic acid purity by OD ratios (A  $_{\rm 260}$ / A  $_{\rm 280}$ ) was also similar between the two methods (median value 1.8 for the cobas method and 2.0 for the QIAamp method).

#### Nucleic acid yields within different tumor types

Using the cobas DNA isolation method, the median nucleic acid yields ranged from a low of 13 ng/mm<sup>2</sup> for breast cancer and a high of 42 ng/mm<sup>2</sup> for melanoma and thyroid cancer; using the QIAamp method, the median yields ranged from a low of 19 ng/mm<sup>2</sup> for thyroid cancer and a high of 48 ng/mm<sup>2</sup> for melanoma (Table 1). These differences were not statistically significant (N.S.). The median and mean nucleic acid yields were comparable between the 2 methods for most tumor types. However the mean nucleic acid yield in thyroid specimens was higher with the cobas method (46 vs. 21 ng/mm<sup>2</sup> – N.S.), and the mean yield in lung cancer specimens was higher with the QIAamp method (36 vs. 26 ng/mm<sup>2</sup> – N.S.).

#### Relationship of nucleic acid yield and tissue area

We assessed the relationship between nucleic acid yields (ng/mm<sup>2</sup>) and the estimated tissue area, across a range of areas of 4-352 mm<sup>2</sup> from all 120 specimens (Figure 2A). Compared to the QIAamp method, the cobas method tended to yield higher nucleic acid for small tissues and less for medium to large tissues. Overall, the cobas method gave higher yields in 37% (44/120) (blue circles) of the specimens (Figure 2A), the QIAamp method gave higher yields in 34% (41/120) (red circles), and both methods gave comparable nucleic acid yield in 29% (35/120) (black circles).

It is noteworthy that the cobas method yielded sufficient DNA from a single 5-micron section for all 120 specimens to provide sufficient DNA to perform the cobas *BRAF* mutation test. However in 6.6% (8/120) of cases, the DNA yield from a single section using the QIAamp Kit was insufficient to perform the assay. For each of these 8 samples, sufficient DNA was subsequently obtained after repeating the extraction from two to three 5  $\mu$ m sections.

# Assessing the degree of RNA co-purification with different DNA isolation kits

Since spectrophotometric measurement at 260 nm does not distinguish between DNA and RNA, the quantity of nucleic acids measured likely reflects both DNA and RNA. As a consequence, the median post-RNase nucleic acid yields per mm<sup>2</sup> tissue were uniformly higher for the cobas-extracted specimens than the QIAamp specimens for all 6 tumor types (Table 1). Similarly the median DNA per 5-micron section and the percentage of DNA was consistently higher with the cobas methods for all tumor types (Table 2). Based on the median values for all 120 FFPET specimens, the cobas method yielded 88% DNA purity vs. 43% for the QIAamp method. Details on the % DNA purity calculation are provided in table 2.

The relationship between tissue area and the nucleic acid yield as determined after RNase treatment is depicted in figure 2B. After RNase treatment, the cobas method yields were higher than those obtained with the QIAamp method for 70% (84/120) of the specimens (blue circles), while the QIAamp method gave higher yields for only 6% (7/120) (red circles), regardless of tissue area. Both methods gave comparable nucleic acid yields in 24% (29/120) of specimens (black circles).

## Effects of different DNA isolation methods on PCR-based mutation assays

We then investigated the impact of these variable degrees of RNA contamination on the subsequent PCR testing for BRAF mutations. The cobas-extracted specimens yielded valid BRAF test results in all 120 cases, both before and after RNase treatment; two QIAamp-extracted specimens gave invalid results. BRAF mutation calls were concordant for specimens isolated with the 2 methods (BRAF mutations were detected in 30/118 specimens).

In specimens that yielded valid test results, we measured C<sub>t</sub> differences of DNA specimens before and after RNase treatment. We anticipated that, if the QIAamp method co-purified more RNA than the cobas method, we would observe a greater C<sub>t</sub> reduction for the QIAamp method after RNase treatment. Among the mutation-positive specimens, higher C, values were observed for 87% (26/30) isolated with the QIAamp method and 10% (3/30) isolated with the cobas method. Across all tumor types, higher C, values for the wild-type allele were observed in 80% (94/118) of the QIAamp method extracts compared to 17% (20/118) from cobas method extracts across all tumor types. The median and mean reduction in mutant C<sub>t</sub> (Figure 3A; n=30) was 0.035 and -0.093 for the cobas method and 0.665 and 0.724 for the QIAamp method (p=0.004, paired Wilcoxon signed rank test, one-tailed). The wild-type signal median and mean reduction (Figure 3B; n=118) was 0.275 and 0.182 for the cobas method and 1.295 and 1.297 for the QIAamp method (p<0.001, paired Wilcoxon signed rank test, onetailed). These observations led us to perform similar experiments with two other real-time PCR-based assays for EGFR and KRAS mutations.

EGFR tests were performed on 20 FFPET lung tumor specimens.  $C_t$  results of the exon 28 internal controls indicate that QIAamp method extracts had delayed  $C_t$ s for 90% (18/20) of the specimens compared to the corresponding cobas method extracts (Figure 4). The mean  $C_t$ s were 26.7 for cobas and 27.7 for QIAamp. It is estimated that one

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cycle C<sub>t</sub> difference corresponds to an approximately two-fold increase in amplifiable DNA [8]. The 20 specimens tested (three tubes per specimen), generated 60 C<sub>t</sub> values per extraction method. The method effect was identified by ANOVA as significant (p<0.001).



KRAS tests performed using the extracts from the cobas and QIAamp methods from 20 FFPET colorectal tumor specimens gave concordant mutation calls for all specimens tested; 15% (3/20) of the specimens were reported as codon 12/13 mutations, and no codon 61 mutations were detected. Higher wild-type peak heights (indicating more amplifiable DNA) were observed with extracts from the cobas method for 90% (19/20) of the specimens. The differences in median peak heights for codon 12/13 (cobas method=0.079; QIAamp method=0.073) and codon 61 (cobas method=0.060; QIAamp method=0.056) produced p-values of 0.052 and 0.003, respectively, paired Wilcoxon signed rank test, one-tailed. Thus the results of the EGFR and KRAS mutation testing are consistent with those observed with the BRAF mutation testing, and indicate that the increased amounts of co-purified RNA in specimens isolated using the QIAamp method alters the apparent performance characteristics of 3 different types of PCR assays.

#### Discussion

Robust standardized methods are needed for rapid accurate detection of clinically important somatic BRAF, EGFR and KRAS mutations used in making targeted therapy treatment decisions. In clinical practice, these companion diagnostic tests must often be performed on relatively small biopsies and FFPET specimens that are typically utilized for an increasing number of histologic and immunohistochemical studies, thus limiting the tissue available for molecular studies.

Laboratory professionals focused on improving the quality of companion diagnostic testing and maximizing the use of available tissue to avoid the need for rebiopsy have emphasized the importance of standardized methodologies for DNA extraction and somatic mutation detection [20]. Determination of sensitivity, quality control and limitations of test methods are issues that should be considered when conducting mutation analysis for targeted therapies. Despite compelling clinical evidence for the relationship between certain biomarkers and targeted therapies, without the appropriate preanalytical methodologies for specimen selection and nucleic acid isolation and sensitive, specific mutation detection methods, the right patient may not be identified for these clinically beneficial therapies.

In this study we found that both DNA extraction kits yielded comparable quantities of nucleic acid; however, a substantial portion of the nucleic acid yield in the QIAamp method was attributed to RNA, which in turn affected the performance of 3 different RT-PCR based mutation assays. In recently published report on a multi-center validation study of nucleic acid extraction methods, the authors recommended adsorption column purification over precipitation methods for downstream applications where accurate DNA input is required [11]. In our study comparing two adsorption-based purification kits, we have found that the cobas method reduces co-purification of RNA significantly, resulting in more accurate quantification of DNA input for use in mutation detection methods.

It is noteworthy that authors of a recent study using the QIAamp method for DNA extraction concluded that the RNase treatment step was important for their protocol [21]. Without RNase treatment, *EGFR* and *KRAS* mutation detection was not as robust as expected and with RNase treatment, more FFPET sections were required to obtain a sufficient DNA concentration in the extracted solution. The authors proposed that RNA remaining in the non-RNase extraction solution likely led to an overestimation of the DNA concentration and that the actual amount of DNA may have been insufficient to allow detection of mutations. Their findings are consistent with the results of this study.

In this study, the cobas<sup>\*</sup> DNA Sample Preparation Kit yields a higher-quality and increased quantity of DNA, especially from tumor specimens with reduced tissue area compared to the commonly used QIAamp DNA FFPE Tissue Kit. The cobas method was associated with less co-purification of RNA, enabling more accurate quantification of DNA used for companion diagnostic tests of somatic mutations in BRAF, KRAS and EGFR.

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#### **Conflict of interest**

K.T. Malhotra is a full time employee of Roche Diagnostics International Ltd. In Rotkreuz, Switzerland and H.Y. Wu is a full time employee of Roche Molecular Systems in Pleasanton, CA, USA.

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