Targeted Gene-Expression Analysis by Genome-Controlled Reverse Transcription-PCR

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Background: For gene-expression analysis, which is anticipated to play an important role in classification of tumors and premalignant conditions, PCR-based quantitative assays must have increased diagnostic quantitative accuracy and reproducibility and enable analysis of gene expression in formalin-fixed paraffin-embedded (FFPE) tissue samples.

Methods: We developed a reverse transcription–PCR-based quantitative assay that modifies the cDNA sequence to increase the melting temperature of short (56–64 bp) PCR amplicons, enabling their quantification in-tube by homogeneous melting-curve analysis. We used this method to analyze the expression of 8 genes, 7 potential colon cancer markers, and 1 control in samples obtained from 3 colon carcinoma cell lines, endoscopic biopsy from 8 patients undergoing gastroscopy for Barrett esophagus, and archival FFPE and frozen tissue from 20 patients who underwent surgery for colon carcinoma.

Results: The detection limit of the assay, when optimized for FFPE samples, was 100 copies of cDNA, and the dynamic range was 3 orders of magnitude. A prototype assay containing a panel of 8 genes displayed good reproducibility compared with the commercially available TaqMan® assay (interassay CVs, 5%–20% vs 7%–43%, respectively). Gene-expression analysis was performed successfully in 26 (96%) of 27 endoscopic biopsy specimens, 30 (86%) of 35 archival FFPE samples, and 20 (100%) of 20 archival frozen samples.

Conclusions: This new technology combines the reproducibility of competitive PCR with accurate quantitative detection by in-tube melting-curve analysis, enabling efficient analysis of mRNA profiles in samples with small numbers of cells or small amounts of tissue, as well as in archival FFPE tissues.

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High-density microarrays are extensively used to define subsets of differentially expressed genes suitable for classification of various forms of cancer (1–3). Reverse transcription–PCR (RT-PCR)10 has been successfully used for studying the expression of a limited number of genes in a large number of clinical samples to evaluate the clinical relevance of candidate genes (4–6). Increasing the usefulness of RT-PCR technology in this area, however, requires the achievement of sufficient quantitative accuracy and reproducibility for diagnostic purposes and development of assays for analysis of gene expression in formalin-fixed paraffin-embedded (FFPE) tissue samples (7, 8).

The first truly quantitative gene-expression assays were based on competitive coamplification by RT-PCR of the wild-type cDNA template with an in vitro-generated RNA (9, 10) or DNA (11) internal standard and, later, universal internal standards containing concatenated primer sequences for multiple genes (12). The use of competitive RT-PCR decreased in favor of real-time PCR (13, 14) until Sequenom® Corporation launched its quantitative gene-expression application for the MassARRAY® platform (15). We have shown that highly accurate endpoint detection can be achieved in a competitive RT-PCR

10 Nonstandard abbreviations: RT-PCR, reverse transcription–PCR; FFPE, formalin-fixed paraffin-embedded; gDNA, genomic DNA.

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system with highly homologous competing templates (16), provided that the amplification reaction originates from a sufficient amount of cDNA molecules (17).

Although shorter amplicons are essential for analysis of the fragmented mRNA in FFPE samples (4), RT-PCR amplicons are generally designed to span at least 1 exon–exon boundary to enable separation of the mRNA-derived amplicons from genomic DNA (gDNA), complicating the design of PCR amplicons shorter than ~100 nucleotides. Different approaches have been used to enable differential competitive amplification of templates derived from cDNA and gDNA within the boundaries of a single exon, including a technique for measuring the amount of a specific mRNA transcript in relation to the amount of gDNA (18) and a similar approach to enable differential amplification of viral RNA and DNA lacking introns (19). Taking this concept further, we developed an RT-PCR technique for quantitative measurement of the relative amounts of multiple mRNA transcripts in a cell or in a tissue sample containing as few as 1000 cells (20). We analyzed the expression of 8 genes. Seven of these genes, trypsinogen (Try or PRSS1, protease serine 1)11, matrix-metalloproteinase-2 (MMP2), MMP7, MMP9, urokinase-type plasminogen activator (uPA or PLAU, plasminogen activator urokinase), uPA receptor (uPAR or PLAUR, plasminogen activator urokinase receptor), and mutS homolog 2 (MSH2) are potential markers for colon cancer (21–24). Glyceraldehyde-3-phosphate dehydrogenase (GAPD) was included in the assay as a positive control.

Materials and Methods

CELL CULTURE
COLO 205, DLD-1, and HT-29 cells were obtained from American Type Culture Collection (Rockville, MD). The cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. COLO 205 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100,000 units/L penicillin, and 100 mg/L streptomycin. DLD-1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100,000 units/L penicillin, 100 mg/L streptomycin, 14 mmol/L sodium bicarbonate, 25 mmol/L glucose, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. HT-29 cells were maintained in McCoy 5a medium supplemented with 10% heat-inactivated fetal bovine serum, 1.5 mmol/L L-glutamine, 100,000 units/L penicillin, and 100 mg/L streptomycin.

PATIENT SAMPLES
We obtained endoscopic biopsy samples from 8 patients undergoing gastroscopy for intestinal metaplasia of the lower esophagus (Barrett esophagus) at Helsinki University Central Hospital. Biopsy specimens ~1–5 mm³ in size were taken from the unaffected upper part of the esophagus, the area of intestinal metaplasia in the lower part of the esophagus, and from the corpus of the stomach. We also used archival FFPE and frozen tissue samples obtained during 2000–2004 from 20 patients who underwent surgery for colon carcinoma. The study was carried out with the approval of the ethics committee of the Helsinki University Central Hospital. Informed consent was obtained from all study patients.

Tissue specimens were macroscopically divided in the operating room into parallel samples for freezing and formalin fixation. The frozen tissue samples were primarily snap frozen in liquid nitrogen and stored at −80 °C for 5 years. The corresponding FFPE samples were primarily fixed in formalin, embedded in paraffin, and stored at room temperature for the same time period. RNA was extracted from 10 8-μm FFPE whole sections and 10 10-μm frozen sections, and tissue morphology was evaluated from an adjacent hematoxylin and eosin–stained section (FFPE samples) or toluidine blue–stained section (frozen samples).

IN VITRO SYNTHESIS OF cRNA
We obtained full-length Try-2 and GAPD cDNA clones from American Type Culture Collection and MMP2, MMP7, MMP9, uPA, uPAR, and MSH2 clones from the Incyte Genomics IRAL cDNA clone library (Incyte Genomics). We confirmed sequences of the cDNA clones by sequencing from both ends on an ABI Prism 310 genetic analyzer (Applied Biosystems) with the ABI Prism dye terminator cycle sequencing core method and AmpliTaq DNA polymerase. With the cDNA clones as templates, we generated cRNA by in vitro transcription with a MEGAscript High Yield Transcription Kit and purification with MEGAclear (Ambion).

RNA EXTRACTION FROM CULTURED CELLS,
ENDOSCOPIC BIOPSY SPECIMENS, AND FROZEN TISSUE SECTIONS
With the RNeasy Mini Kit (Qiagen), used according to the manufacturer’s instructions, we extracted total RNA from the cultured cells, the 1–5 mm³ endoscopic biopsies, and 10 10-μm sections of each frozen sample. Total RNA extracted from cultured cells was DNase-treated by adding to 10 μg of RNA, 5 μL 10× DNase buffer (100 mmol/L Tris, pH 7.5, 25 mmol/L MgCl₂, 5 mmol/L CaCl₂), and 10 units of DNase I (Roche Applied Science) and incubating for 30 min at 37 °C, after which the DNase was inactivated for 5 min at 70 °C. RNA was then precipitated with 5 μL of 2 mol/L sodium acetate (pH 4.0) and 140 μL of 100% ethanol overnight at −20 °C, after which the sample was centrifuged at 5200g for 30 min at 4 °C. The supernatant

11 Human genes: Try, trypsinogen; MMP2/7/9, matrix-metalloproteinase-2/-7/-9; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; MSH2, Mut S homolog 2; GAPD, glyceraldehyde-3-phosphate dehydrogenase.
was removed carefully and the RNA pellet was washed with 200 μL of 70% ethanol, after which the sample was centrifuged at 5200g for 20 min at 4 °C. The supernatant was removed, and the RNA was dissolved in 20 μL of RNase-free water. RNA extracted from endoscopic biopsies and frozen sections was DNase-treated during the extraction on the membrane of the RNeasy column, according to the manufacturer’s instructions.

RNA EXTRACTION FROM FFPE TISSUE SAMPLES
RNA was extracted from 10 8-μm FFPE tissue sections of each sample. Paraffin was dissolved in octane (Sigma) for 2 × 10 min at 37 °C and then washed for 2 min with absolute ethanol. After centrifugation at 2000g for 1.5 min, we added 300 μL of 4 mol/L guanidium isothiocyanate buffer and 1 μg of yeast tRNA (Sigma). The samples were incubated at 95 °C for 30 min, followed by centrifugation at 5200g for 2 min (25). Total RNA was extracted by use of a modification of the protocol of Chomczynski and Sacchi as follows (26). Thirty μL of 2 mol/L sodium acetate and 300 μL of water-saturated phenol were added to the sample, after which the sample was mixed thoroughly by repeated inversion and incubated on ice for 30 min. We transferred the sample to a prespun Phase Lock Gel 2 mL Heavy tube (Eppendorf), added 90 μL of chloroform–isoamyl alcohol (49:1), and mixed by repeated gentle inversion. After centrifugation at 5200g for 5 min to separate the phases, we added 300 μL of phenol–chloroform–isoamyl alcohol (50:49:1) to the aqueous phase and mixed thoroughly. After centrifuging at 5200g for 5 min, the resulting aqueous phase was collected and the RNA was precipitated by adding a double volume of absolute ethanol and incubating at –70 °C overnight. The precipitate was washed twice with 500 μL of 70% ethanol and centrifuged at 5200g for 10 min at 4 °C to repellet the sample. After the final wash, the pellet was dried at room temperature and dissolved in 20 μL of RNase-free water. The extracted total RNA was then DNase-treated as described for cultured cells.

REVERSE TRANSCRIPTION
We performed reverse transcription with the DyNAmo SYBR Green 2-step qRT-PCR Kit (Finnzymes) according to the manufacturer’s instructions. Briefly, 1 μg of total RNA from cultured cells, 0.5–1 μg of total RNA from endoscopic biopsies, 60–200 ng of total RNA from frozen sections, or 5 μL of FFPE-derived RNA (the concentration of which could not be directly measured) was transcribed in a 20-μL reaction volume containing 1× reverse transcription buffer, 10 pmol of each of the sequence-modifying antisense primers (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue11), and 2 μL of M-MuLV RNase H+ reverse transcriptase. The reverse transcription reaction was incubated at 42 °C for 50 min, and then the reverse transcriptase was inactivated for 5 min at 85 °C.

EXONUCLEASE I TREATMENT
After reverse transcription primers were degraded by exonuclease I (New England Biolabs) in a 25-μL reaction volume in 1× restriction buffer (67 mmol/L glycine-KOH, 6.7 mmol/L MgCl2, 10 mmol/L β-mercaptoethanol, pH 9.5) and with 2 units of exonuclease I at 37 °C for 60 min, followed by denaturation at 80 °C for 20 min.

POLYMERASE CHAIN REACTION
We amplified 2.5 μL of the exonuclease I-treated reverse transcription products together with 2 ng (FFPE samples) or 20 ng (cultured cells and endoscopic biopsies) of human gDNA (Roche Applied Science) in gene-specific amplification reactions with the DyNAmo SYBR Green 2-step qRT-PCR Kit (Finnzymes) according to the manufacturer’s instructions. For PCR primers used, see Table 1 in the online Data Supplement. We used locked nucleic acids to optimize the melting temperature of some primers (27). We tested primer pairs by analyzing the amplification products by standard agarose gel electrophoresis and by melting-curve analysis, as described. The accepted primer pairs showed no nonspecific or dimer amplification on an agarose gel and no nonspecific peaks in the temperature range of the specific cDNA and gDNA peaks in the melting-curve analysis. PCR was performed on an ABI 7500 Fast (Applied Biosystems) instrument for 30 cycles (cultured cells and endoscopic biopsies) or 35 cycles (FFPE samples), with denaturation at 95 °C (15-s hold) and annealing at 57 °C (1-min hold). Fluorescence was measured at the end of annealing.

MELTING CURVE ACQUISITION AND ANALYSIS
Immediately after amplification, we detected and quantified amplification products by melting-curve analysis on the ABI 7500 Fast instrument, with an additional denaturation at 95 °C and continuous melting curve acquisition from 57 °C to 98 °C with a 0.1 °C/s ramp rate. We obtained a derivative melting curve plot by use of the default settings of the instrument. We determined background fluorescence for the 1st peak by forward baseline extension and for the 2nd peak by backward extension from a 5 °C temperature range adjacent to the peak. The areas of the cDNA- and gDNA-derived melting curve peaks were calculated after subtraction of the background, by integrating the negative derivative raw data measuring points at 0.3 °C intervals over a temperature range of 5 °C. The cDNA:gDNA ratio was calculated from these peak areas.

DETERMINATION OF THE DYNAMIC RANGE OF THE ASSAY
The measuring range of the assay was determined by analyzing a 10-fold dilution series of sequence-modified cDNA reverse-transcribed from 1010 copies of cRNA of single genes in relation to the reference DNA template
containing 20 ng of human gDNA corresponding to ~10^4 copies of each gene.

DETERMINATION OF THE IMPRECISION OF THE GENOME-CONTROLLED RT-PCR AND TAQMAN® ASSAYS
To determine the intra- and interassay CVs of the genome-controlled RT-PCR assay, we used 20 ng of gDNA as an internal calibrator and performed 8 parallel multiplexed cDNA synthesis reactions from total RNA extracted from COLO 205, HT-29, and DLD-1 cells. To determine intraassay variation, we divided pooled cDNA synthesis products treated with exonuclease I into 8 parallel sets of gene-specific amplification reactions [2.5 µL (10^4 cells) per reaction]. Each set contained gene-specific amplification reactions for each of the analyzed genes. For determining interassay variation, 8 separate exonuclease I-treated cDNA synthesis products were transferred to 8 parallel sets of amplification reactions. With 2 ng of gDNA as an internal calibrator, we determined the interassay variation of the genome-controlled RT-PCR assay for FFPE samples by performing 8 separate multiplexed cDNA synthesis reactions from total RNA extracted from 8 FFPE samples. Each sample consisted of 10 8-µm sections from the same archival colon cancer paraffin specimen.

To determine imprecision of the TaqMan assay, we used the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s instructions to perform single (intraassay) or 8 parallel (interassay) cDNA synthesis reactions with random hexamers. To amplify cDNA reverse transcribed from 10^4 cells, we used TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instruction and inventory TaqMan assays Hs00605631_g1 for Try-1, Hs00234422_m1 for MMP2, Hs00159163_m1 for MMP7, Hs00234579_m1 for MMP9, Hs00170182_m1 for uPA, Hs00182181_m1 for uPAR, Hs0079887_m1 for MSH2, Hs99999905_m1 for GAPD (as an external standard), and GAPD endogenous control (VIC/TAMRA Probe).

STATISTICAL ANALYSIS
We used the Mann–Whitney U-test (2-sided) to estimate the statistical significance of the differences in the expression of the analyzed genes.

RESULTS
GENOME-CONTROLLED RT-PCR ASSAY PROCEDURE
The 3-step analytical procedure is shown in Fig. 1 (20). The structure of the sequence-modifying reverse transcription primer is shown in Fig. 2. Detection of the coamplified sequence-modified cDNA and gDNA templates by melting-curve analysis is shown in Fig. 3.

MEASURING RANGE AND DETECTION LIMIT
For analysis of fresh tissue samples or cultured cells with 20 ng of gDNA (containing ~10^4 copies of each gene) as an internal standard, the linear range of the assay was ~3 orders of magnitude (interval, 10^3 to ~10^6 copies of target cDNA) (Fig. 4). The detection limit of the assay was 1000 copies of target cDNA, corresponding to a cDNA:gDNA ratio of 0.1. Above this limit, the CVs of the assay were ≤20% for all the analyzed genes (Figs. 4 and 5).

When we analyzed FFPE samples, we decreased the amount of internal standard to 2 ng of gDNA (containing ~10^3 copies of each gene). This decrease was necessary because the RNA yield from FFPE samples was lower than that from fresh biopsy samples. Thus, the linear interval of the assay was decreased to 10^2 to 10^5 copies of target cDNA. The detection limit was 100 copies of target cDNA, corresponding to a cDNA:gDNA ratio of 0.1. As a trade-off, the imprecision of the assay was slightly increased, with CVs ≤34% for all of the analyzed genes.

IMPRECISION
For the genome-controlled RT-PCR technique, the intra- and interassay CVs were 5%–15% and 5%–20%, respec-
The intraassay CV of the TaqMan assay was 15%–45% with GAPDH as an external standard and 10%–42% with GAPDH multiplexing. The corresponding interassay CVs were 14%–43% and 7%–43%, respectively. The correlation between the different techniques was good; discrepant results occurred only for the expression amounts of the MSH2 gene. MSH2 expression was considerably higher when analyzed by the TaqMan technique, but the CV was high (43%), suggesting a technical problem in the TaqMan assay (Fig. 5).

When we analyzed 8 replicate FFPE samples with 2 ng of gDNA as an internal standard, we observed expression above the detection limit (cDNA:gDNA ratio, >0.1) for GAPDH, Try, MMP7, MMP9, uPA, and uPAR, whereas MMP2 and MSH2 were expressed below the detection limit. For these genes, the interassay variation of the genome-controlled RT-PCR assay, including deparaffinization and RNA extraction steps, was 11%–34%. When we analyzed the same samples with the TaqMan assay, expression of MMP9, uPAR, MMP2, and MSH2 was detected, with interassay CVs of 63%, 44%, 130%, and 110%, respectively. Expression amounts of MMP2 and MSH2 were below the lowest standard used in TaqMan assay, i.e., 100 copies of mRNA. For FFPE sample analysis, however, Try, MMP7, uPA, and GAPDH did not amplify in the TaqMan assay, possibly because of the relatively long PCR amplicons for these genes (interval, 101–122 bp). In the genome-controlled RT-PCR assay the amplicon length of the expressed genes was 56–64 bp, whereas in the TaqMan PCR assay it was 54–87 bp.

**ANALYSIS OF BIOPSY SAMPLES AND ARCHIVAL FFPE AND FROZEN SPECIMENS**

To demonstrate genome-controlled RT-PCR expression profiling, we analyzed fresh endoscopic biopsy samples from 8 patients undergoing follow-up for intestinal metaplasia of the lower esophagus (Barrett esophagus) (n = 27), and 1–5-year-old archival FFPE surgical specimens of colon carcinoma (n = 20) and healthy colonic mucosa (n = 15). The correlation between FFPE and frozen archival samples was tested in samples (n = 10) from 5 patients undergoing surgery for colon carcinoma.

We obtained sufficient amounts of mRNA from 26 of 27 single endoscopic biopsies. Distinct patterns of gene expression were observed. For example, in colon carcinoma samples, we noted upregulation of certain genes (e.g., uPA, MMP7) and downregulation of others (e.g., GAPDH, MSH2) compared to healthy control specimens.

**Fig. 2.** Structure of the sequence-modifying reverse transcription primers.

The sequence-modifying primers comprise 3 functional segments: a 5′-terminal segment containing the entire nucleotide sequence of the antisense primers used in subsequent amplification reactions, a central segment containing 5 or 6 A or T to C or G substitutions, giving rise to a 4–5 °C increase in melting temperature of the subsequent cDNA-derived PCR amplicon, and a 3′-terminal segment complementary to the mRNA sequence of the corresponding gene to ensure specific annealing to the target mRNA.

**Fig. 3.** Amplification and melting-curve analysis of a 10:1 dilution series of cDNA reverse transcribed from 10^10 copies of Try-1 cRNA.

(A), real time amplification curves from 8 separate gene-specific amplification reactions containing 10-fold dilutions, corresponding to 10–10^8 copies of Try cRNA, and 20 ng of gDNA. (B), melting curves obtained from the same reactions after amplification. A temperature shift of 4–5 °C allowed for sufficient separation of the gDNA- and cDNA-derived PCR amplicons for all of the analyzed genes.
expression were observed in different anatomic regions: GAPD was consistently highly expressed in all regions. Samples of esophageal tissue displayed low expression of the rest of the analyzed genes; intestinal metaplasia tissue displayed consistent expression of uPAR and occasional expression of MMP-2, MMP-7, and uPA; and stomach tissue displayed relatively high expression of Try and uPAR (data not shown).

We obtained sufficient amounts of mRNA from 18 of 20 of the carcinoma FFPE tissue samples and from 12 of 15 healthy FFPE tissue samples. Carcinoma samples displayed variable expression of the analyzed genes, with significantly increased expression of MMP9 \((P = 0.003)\) and uPA \((P = 0.02)\) compared with healthy samples. Healthy colonic mucosa displayed high expression of GAPD and low expression of the rest of the analyzed genes.

We obtained sufficient amounts of mRNA from all 20 frozen carcinoma samples. We analyzed 15 colon cancer tissue specimens with similar morphology to test correlations between archival frozen tissue and FFPE specimens. The correlation between frozen and FFPE tumor tissue samples was good (Fig. 6).

**Discussion**

Genome-controlled RT-PCR allowed accurate measurement of the relative expression amounts of any mRNA species in small cell and tissue samples. This technique has several advantages over conventional RT-PCR. The assay is robust and reproducible because competitive coamplification of the gDNA internal standard occurs in every amplification reaction for any amount of target cDNA template. The detection limit and imprecision of the assay can be controlled by adjustment of the amount of internal standard. Differentiation between expressed and genomic sequences is achieved by modification of the cDNA transcript and does not rely on intronic sequences. This characteristic facilitates use of PCR amplicons short enough for analysis of FFPE samples and allows use of the untranslated regions for differential analysis of closely related genes. Carryover of gDNA from the RNA extraction step remains unmodified during reverse transcription and thus does not lead to false-positive results. Quantitative detection of the PCR products is done in-tube by melting-curve analysis of the PCR amplicons with a saturating DNA dye, such as SYBR Green or LC Green, lowering the costs of high-throughput applications.

The primer pairs for genome-controlled RT-PCR, in contrast to those of conventional RT-PCR (including real-time and competitive PCR), are designed to generate PCR amplicons within the boundaries of a single exon. The cDNA- and gDNA-derived amplicons are equal in length and differ only by a few nucleotide substitutions that produce a slight but sufficient difference in melting temperatures of the respective amplicons, ensuring truly competitive amplification of cDNA and gDNA templates with a single pair of primers. Short PCR amplicons are a prerequisite for melting-curve analysis of the PCR amplicons (28) and are also essential for analyzing mRNA in archival FFPE samples, in which most of the RNA is degraded (4). We used PCR amplicons of only 56–64 bp, a length that was efficient for amplifying the mRNA in FFPE samples. When we analyzed these samples with the TaqMan assay, we detected expression only for genes with <100 bp amplicons. With short PCR amplicons, melting temperature differences of ~4–5 °C yielded ex-
cellent discrimination power in the melting-curve analysis, with a dynamic range of ~3 orders of magnitude. The melting curve analyses were done on an ABI 7500 instrument, but we obtained comparable results with a Roche LightCycler. The 96-well format of these instruments allows PCRs to be performed on affordable benchtop thermal cyclers and uses the capacity of the detection instrument for melting curve analyses in high-throughput applications.

In the genome-controlled RT-PCR technique, the sequence, and thus the melting temperature, of the cDNA-derived amplicons is modified in the reverse transcription step. When the reactions are performed under nondenaturing conditions, any gDNA present in the reaction remains unmodified because the reverse transcriptase enzyme cannot use double-stranded DNA as a template (18, 19). In malignant tissues, the gDNA is often aneuploid, and the genetic aberrations vary from sample to sample. Therefore, we used as internal standard a fixed amount of diploid gDNA, in which the relative copy number of the analyzed genes was constant. In theory, carryover of aneuploid gDNA from the RNA isolation step could lead to analytical inaccuracy by skewing the relative copy number of the genes in the gDNA internal standard. This problem was eliminated by DNase digestion of the extracted RNA, which decreased DNA carryover to a degree that did not affect the quantitative analysis. For analysis of nonmalignant cells, RNA and DNA can be coisolated, and the gDNA contained in the analyzed cells can be used as an internal standard (18). In such a setup, the gene-specific cDNA:gDNA ratios will directly reflect the number of mRNA copies per genome, thus allowing for absolute quantification of mRNA production in the analyzed cells. This approach can also be

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**Fig. 5.** Imprecision of the genome-controlled RT-PCR assay and the commercially available TaqMan gene-expression assay (Applied Biosystems). A panel of 7 genes was analyzed in 8 repetitive experiments on 3 different colon carcinoma cell lines (COLO 205, DLD-1, and HT-29), and the imprecision of the genome-controlled RT-PCR assay (left panels) and the TaqMan assay (right panels) was compared. A cutoff of 0.1, corresponding to 1000 copies of cDNA, was used to define the detection limit of the genome-controlled RT-PCR assay. The TaqMan assay was multiplexed with GAPD to obtain optimal reproducibility. The correlation between the 2 different techniques was good, showing a discrepant result only in the expression levels of the MSH2 gene. The mean (error bars, SD) values of the cDNA/gDNA ratios are shown. The interassay CVs are displayed above the diagram for each of the analyzed genes. Values for genes expressed above the detection limit (genome-controlled RT-PCR) or above the lowest standard (TaqMan) are shown in boldface.
used for analyzing changes in RNA production in cultured cells, e.g., after knockdown with short inhibitory RNAs, when possible aberrations in the genome of the analyzed cells remain constant.

In conclusion, genome-controlled RT-PCR enables highly reproducible and cost-efficient analysis of mRNA production in small samples, such as needle and endoscopic biopsy specimens and FFPE tissue specimens. This technique provides an efficient research tool for large-scale analysis of mRNA profiles in archival FFPE samples, as well as for monitoring changes in entire gene-expression pathways and networks in cell cultures. Because assay imprecision can be controlled by adjusting the amount of internal standard, in the future this technique may be used for diagnostic gene-expression analysis.

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