

Correcting False Gene Expression Measurements From Degraded RNA Using RTQ-PCR

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Abstract: This paper describes a method allowing correcting false gene expression measured on highly degraded RNA using real-time quantitative reverse transcription-polymerase chain reaction (RTQ-PCR). RNA was isolated from different models (in vitro cell lines, in vivo models of human and dog) and different tissue types. In vitro RNA degradation and modeling of in vivo degradation were applied on intact and degraded total RNA. Gene expression (eg, Bcl-2, GAPDH, PGK, PSME3, RAB2, BAX) was measured using RTQ-PCR. 18S rRNA proved to be the most constant house-keeping gene. Less than 10-fold degraded RNA can be quantified correctly when using 18S rRNA for normalization purposes. Higher-fold degraded RNA can be quantified correctly up to a precision that is comparable to RTQ-PCR measurements on intact RNA when simulating the RNA-species and tissue-specific degradation kinetic.

Key Words: RNA degradation, RTQ-PCR, correction of false gene expression measurements, house-keeping genes, simulation of RNA degradation

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With the advent of molecular biologic methods, it became desirable to apply these potent methods to archival paraffin-embedded tissue samples. Isolation of RNA in particular seemed to be interesting, because expression alterations caused by gene abnormalities like deletions, structural alterations, rearrangements, and amplifications are associated with certain diseases such

as cancer.¹ The recovery of RNA from paraffin-embedded tissue samples and the development of the polymerase chain reaction (PCR) method have significantly expanded the opportunity for gene expression analysis not only of paraffin-embedded tissue samples but also of those that are fresh and frozen.

It is widely known that RNA and DNA extracted from paraffin-embedded tissue blocks are of poor quality and degraded to various degrees compared with those from fresh and frozen tissues.^{2–6} Our own experiences with RNA isolated from paraffin-embedded tissue samples of different type (thyroid cancers, testis tumors and normal testis tissues, lymph nodes, kidney, liver) and origin (human, dog, mice) always lacked in the successful extraction of intact RNA, which is in line with the literature. Unfortunately, degradation of RNA can be also detected in frozen tissues sometimes caused from warm ischemia before extraction.⁷ However, when using state of the art technologies, such as immediate transfer of the fresh tissues excised during the operation into a solution that inhibits RNases and stabilizes RNA-species (eg, RNA-later, Qiagen), degraded RNA was usually absent after RNA isolation.⁸

Nevertheless, the usability of RNA from archival tissues has been reported for investigating gene expression.^{9–11} These and other groups reduced their research on the detection of the presence or absence of a certain RNA-species (yes or no answer). When considering the well-accepted experience of RNA degradation taking place especially in paraffin-embedded tissues, the question arises whether the lack in detection of a certain RNA-species related, for example, to oncogenesis might be caused by either degradation or the absence of this RNA-species. These considerations point to the need to develop methods that allow correction of false gene expression measured on degraded RNA while taking into account the degradation process.

It is the purpose of the newly developed method described in this manuscript not only to focus on the detection of certain RNA-species, but to *quantify* gene expression after degradation occurred. With the method shown herein, quantification on degraded RNA can be performed with a precision resembling real-time quantitative reverse transcription-polymerase chain reaction (RTQ-PCR) measurement on intact RNA.

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MATERIALS AND METHOD

Cells and Cell Culture

Human mammary carcinoma (MCF-7), mouse fibroblast (L929), and human leukemia (HL-60) cells (DSMZ, Braunschweig, Germany) were grown either as a monolayer culture (MCF-7, L929) or in cell suspension (HL-60) and subcultured twice per week. MCF-7 and HL-60 cells were maintained in RPMI 1640 medium (Sigma, Deisenhofen, Germany) and L929 cells in DMEM medium (Sigma, Deisenhofen, Germany). Media were supplemented with a 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Mannheim, Germany). Additionally, MCF-7 cells received 10 µg insulin/mL medium (Sigma, Deisenhofen, Germany). Culture conditions were 37°C in a humidified atmosphere buffered by 5% CO₂ in the air and hydrogen carbonate (Merck, Darmstadt, Germany), at pH 7.4.

Radiation Conditions

Twenty-four hours after passaging, exponentially growing cell cultures were irradiated at room temperature with single doses of 240 kV x-rays (Isovolt 320/10, Seifert, Ahrensberg, Germany) filtered with 3 mm Be. The absorbed dose was measured with a Duplex dosimeter (PTW, Freiburg, Germany). The dose rate was ~1 Gy/min at 13 mA. MCF-7 cells were irradiated with 2 and 6 Gy and L929 cells with 6 Gy.

Tissue Samples and Histologic Examination

Testicular tumor biopsies from 37 patients (age range 19 to 45) were analyzed. TNM classification (tumor, node, metastasis, see Ref. 12), ranged from T1N0M0 to T3 N2 M1; 29 pure seminomas and 8 nonseminomas (mixed nonseminomatous germ cell tumors) were examined. In addition, 37 biopsies of unaffected sites of the resected testis representing normal tissue were analyzed. Tissues were fixed in RNA-later solution (Qiagen, Hilden, Germany) immediately after surgery. However, some of these tissues contained degraded RNA. All human samples were obtained with informed consent.

Human RNA isolated from 3 organs, namely liver, kidney, and colon, of 9 different individuals (4 females, 5 males, age range: 30 to 82 y) were commercially available (Stratagene Europe, Amsterdam, Netherlands and BD Bioscience, Heidelberg, Germany).

Tissue samples were taken from lymph nodes, skin, liver, and spleen of 2 female dogs aged 1 and 15 years. Tissues were immediately fixed in RNA-later solution.

RNA Isolation and Quality Control

Tissue was homogenized (homogenizer, Omni, Warrenton) and digested (proteinase K, 20 mg/mL; Invitrogen, Karlsruhe, Germany), total RNA was isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany), and remaining DNA digested (RNase free DNase Set, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Exponentially growing cell cultures were trypsinized if necessary, and the total RNA was isolated

applying 7×10^6 cells per column using the RNeasy Mini Kit and DNA digestion as described above.

Absorption of total RNA at A260/A280 (ratio had to be >1.8), and quantification was examined with spectral photometry (Nano Drop, PeqLab, Erlangen, Germany). Agarose gel electrophoresis allowed differentiation between degraded (smear) and intact RNA (ratio 28S/18S rRNA ribosomal band ~2:1). DNA contamination of the probes was checked with conventional PCR (40 cycles using β-actin primers).

Release of Tissue-specific RNases

Biopsies were stored in RNA-later solution. Pieces of these biopsies were cut and weighed after being squeezed manually to remove the liquid. About 25 mg was given into a tube (Eppendorf, Weikersheim, Germany) containing 1 mL of 50% ethanol (diluted in aqua dest) and stored over 1 hour at room temperature. After removal of the tissue, this solution was immediately used for RNA degradation purposes or stored at -20°C. In certain cases the solution was diluted 10-fold with aqua dest to decrease the fast enzymatic digestion of RNA-species.

RNA Degradation

In vitro degradation was performed with RNase A (Sigma Deisenhofen, Germany). A sequential 10-fold dilution in $0.2 \times$ SSC (according to the guidance of the manufacturer Roche) of stock RNase A (10 mg RNase A/10 mL aqua dest) led to solutions containing 1 to 10^{-4} ng RNase A/µL. Typically, a certain volume of precooled $0.2 \times$ SSC and 10 µL of the precooled RNase-solutions were filled into a 0.5-mL tube and carefully shaken. Finally, 20 µg of precooled RNA were added. The total volume was 50 µL. Enzymatic reaction was run at 4°C and 10°C over 1 to 10 minutes to decrease the fast enzymatic digestion of RNA-species. Reaction was stopped by adding RLT buffer and running the conventional RNA-isolation cleanup protocol as recommended by the manufacturer (Qiagen).

In vivo degradation was performed with the ethanol-resistant RNase-cocktails isolated from the biopsies. Typically, in a 0.5-mL tube 15 to 25 µg of RNA (which equals about 15 to 25 µL) were added to the same volume of the RNase-cocktail and incubated at 30°C over 1 to 30 minutes. The reaction was stopped by adding 1 volume of RNA-later solution and stored on ice before the RNA-isolation cleanup according to conventional protocols (Qiagen) was driven. Spectral photometric quantification of RNA followed the next day.

RTQ-PCR

Aliquots of total RNA (1 µg) were reverse transcribed using Multiscribe reverse transcriptase and thermal cycled according to a 2-step PCR protocol (TaqMan Gold RT-PCR Kit). For reverse transcription, the volumes of RNA-eluates of degraded RNA had to be equal to the volumes of intact RNA. The resulting cDNA in general was diluted in water (1 µg/µL), stored at

–20°C, and used as a template for subsequent PCR reactions. Seven primers and probes (GAPDH, Bcl-2, Rab2, CDK4, PCNA, HPRT, and PGK) were commercially available as so-called assays on demand. One predeveloped assay for detection of 18S rRNA (used for normalization purposes) was used. Two self-designed primers and probes (PSME3 and PKCI) were used as described before.¹³ The PCR reaction typically included 0.5 volume of the 2 × concentrated TaqMan Master Mix containing hot start AmpliTaqGold DNA polymerase, 300 nM forward and 300 nM reverse primer, 200 nM FAM labeled probe, and 10 ng cDNA. RNase-free water was added to a final volume of 30 µL per reaction. However, with higher degree of degraded RNA the amount of template had to be increased from 10 ng of cDNA up to 500 ng of cDNA. A hot-start Taq-Polymerase was used and activated at 95°C over 10 minutes. Forty PCR cycles were driven, with annealing and elongation of primers and probes occurring at 60°C over 1 minute followed by a denaturation step at 95°C over 1 minute. A relative standard curve derived from sequential 8-fold dilutions of stock cDNA of known quantity (lasting from 0.5 ng cDNA until 7.6 fg cDNA per reaction) was used for a linear regression analysis of unknown samples, thus allowing conversion of the so-called threshold-cycles (C_T) of the PCR-reaction of unknown samples into ng cDNA. The dynamic range of linearity lasted over 6 log units. The slope of the standard curves was almost constant throughout the experiments (range 3.4 to 3.6, which corresponds to a PCR efficiency > 90%). Analysis of gene expression was generated using a GeneAmp 5700 Sequence Detection System (SDS, Version 1.3, TaqMan), which uses the 5' nuclease activity of Taq DNA polymerase to cleave a TaqMan probe during PCR. All materials used for RTQ-PCR were ordered from Applied Biosystems, Weiterstadt, Germany.

Statistics

The gene expression analysis of genes in general was done in triplicate and duplicate, employing RTQ-PCR. The means, SEM, and minimum and maximum values were visualized with the aid of graphical software (Sigma Plot 2000, Jandel, Erkrath, Germany). Every experiment was performed at least 3 times. However, in some cases due to restricted material these conditions had to be changed which is mentioned in the legend.

RESULTS

House-keeping Genes

Comparison of 3 common house-keeping genes in 3 human tissue types of 9 individuals revealed mean differences for absolute 18S rRNA gene expression of < 0.2-fold (20%) among the 3 organs and among the same organs of 3 individuals (Fig. 1). In contrast, organ-specific mean differences in absolute gene expression were 10-fold and 2-fold for PGK and HPRT (Fig. 1). Absolute gene expression of both genes typically differed 100% among the same organs of 3 individuals. Moreover,

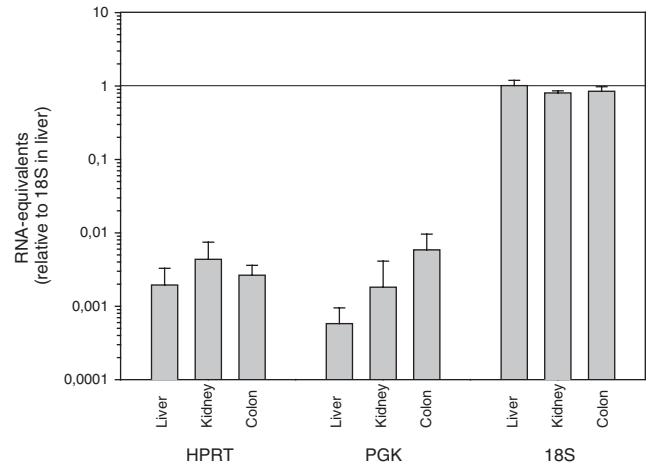


FIGURE 1. Comparison of absolute gene expression of 3 house-keeping genes in 3 tissue types of 9 human individuals. Bars represent the mean calculated from 3 individuals. Error bars show the SEM, $n=3$.

absolute gene expression of 18S rRNA was 2 log scales higher compared with PGK and HPRT.

Examination of absolute 18S rRNA gene expression in different species (mouse, human, dog), organs (liver, kidney, colon), testis tumors and normal testis tissues, in vitro cell lines (MCF-7, L929), radiation doses (2 and 6 Gy) and certain time points after irradiation (up to 72 h) showed almost comparable values for gene expression within each model system (Fig. 2A). In contrast, up to 10-fold differences in absolute 18S rRNA gene expression could be found, for example, among L929 cells and human RNAs isolated from liver, kidney, and colon of 9 individuals or dog tissues. Unirradiated (control) values of 18S rRNA were in the same order of magnitude compared with the irradiated probes. The same pattern could be found for testis tumors and their corresponding normal tissues (controls), all of which revealed lower absolute 18S rRNA gene expression compared with dog or other human organ tissues. Therefore, absolute gene expression of 18S rRNA relative to appropriate control values compensated model-specific differences in absolute gene expression and demonstrated an almost homogeneous and radiation dose, time, cell, tissue, and species-independent height of differential 18S rRNA gene expression with a variance of 0.5 (Fig. 2B).

Certain RNAs (marked with an asterisk in Fig. 2B) showed up to 15-fold differences in absolute gene expression of 18S rRNA. Aliquots of these RNAs were taken and diluted to search for substances present in the probes which might inhibit the PCR-reaction. When plotting absolute gene expression of 18S rRNA equivalents versus C_T -values for all 4 diluted probes, a linear regression with almost similar slopes (3.4 to 3.5) and comparable efficiencies (93% to 96%) of the PCR-reaction evolved (Fig. 2C). This was indicative for the absence of inhibitory substances in the RNA probes.

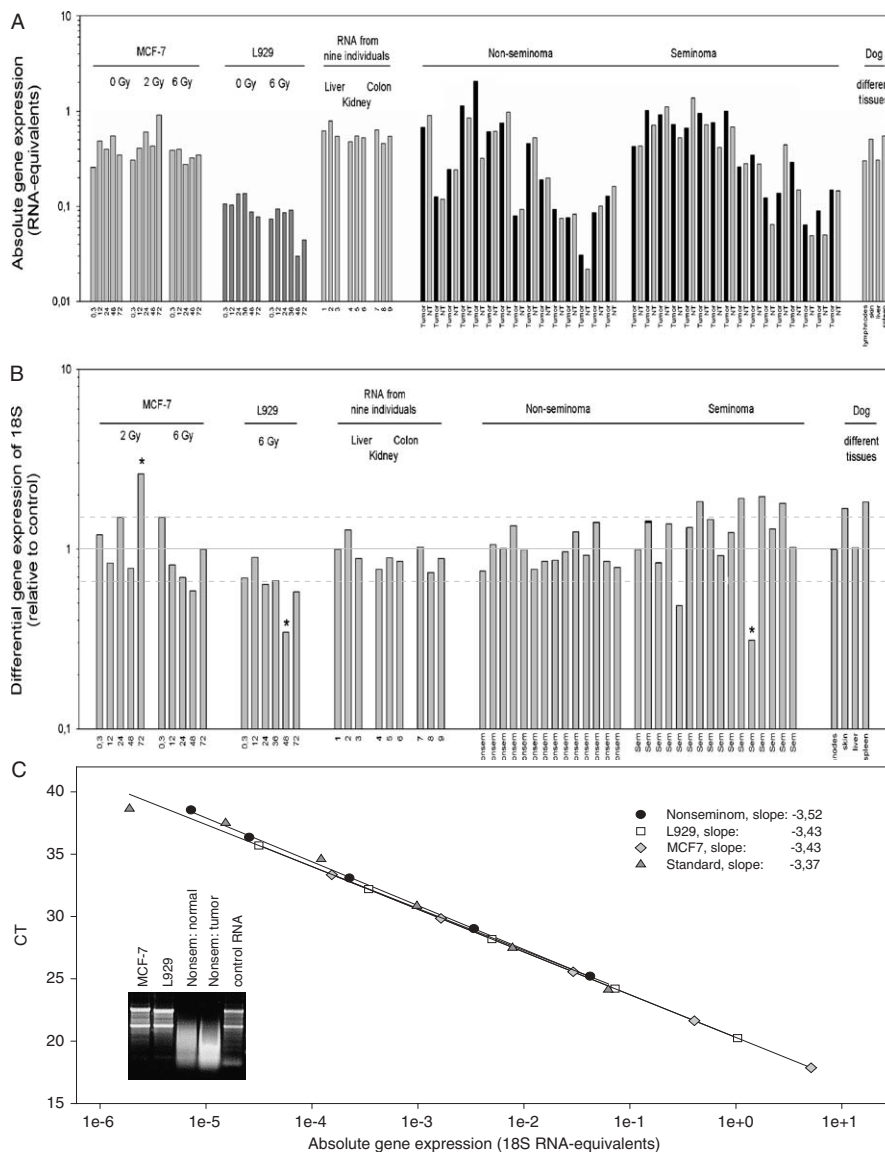


FIGURE 2. A, Absolute gene expression of 18S rRNA was measured for each PCR-reaction containing 10 ng RNA equivalents. Measurements were performed in different models as outlined in the graphs. Depicted *in vitro* values reveal a representative selection of 12 further experiments not shown herein. B, The abundance of the gene of interest under certain conditions relative to the abundance of the same gene under control conditions was built. This ratio was called “differential gene expression.” C, Three cDNA-probes (marked with an asterisk in Fig. 1B) were diluted, and 18S RNA equivalents measured. Slope values for the selected 4 probes are depicted in the corresponding legend. Gel electrophoresis revealed degraded testis tissue (inset in Fig. 2C).

Gel electrophoresis revealed degraded RNA in the nonseminoma normal and tumor tissue (Fig. 2C, inset), which had no influence on the differential gene expression as shown in Figure 2B.

In Vitro Degradation Kinetic and Normalization

Degradation of human MCF-7 RNA with RNase A reduced absolute gene expression of 18S rRNA over almost 5 log scales, thus leading to a degree of degradation covering nearly 5 log scales (Fig. 3, white columns in the left panels). This was comparable with GAPDH degradation, which showed a similar decrease

(Fig. 3, gray columns, left upper panel). However, PSME3 and PKCI revealed only a decrease of their absolute gene expression over 2 log scales (Fig. 3, left middle and lower panel). After normalizing the 3 genes versus 18S rRNA, a differential gene expression relative to the intact RNA-species (degree of degradation equals 1) was calculated (Fig. 3, right panels). Differential gene expression increased with increasing degree of degraded RNA. As a tendency it could be shown that lower degraded RNA showed differential gene expression comparable with undigested RNA. For instance, at a degradation degree of 6 (one-sixth of intact molecules are

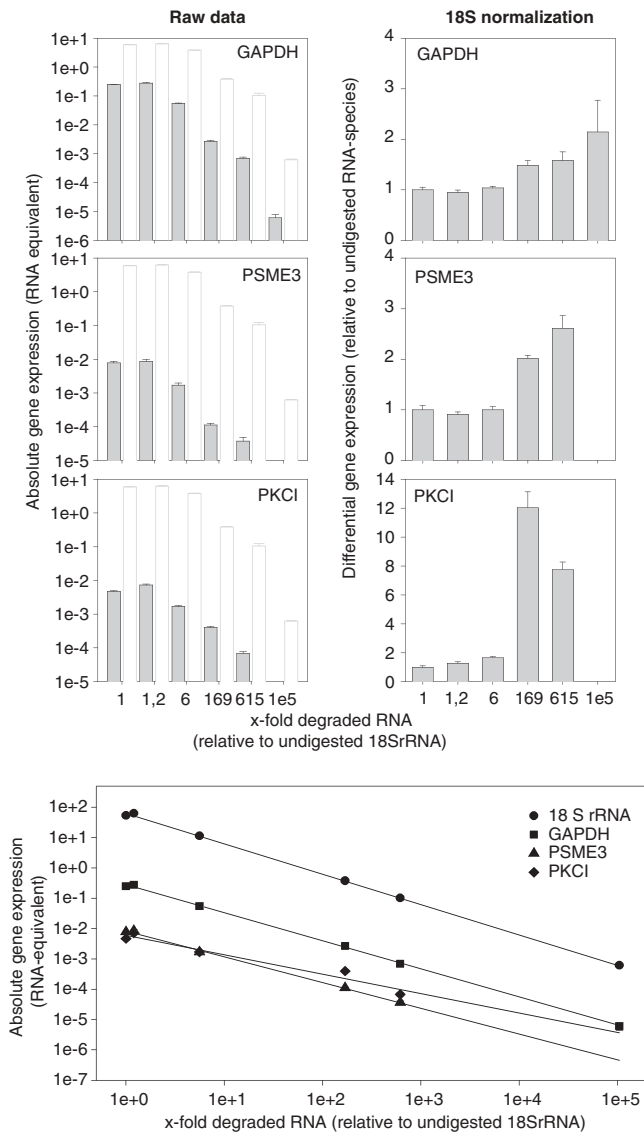


FIGURE 3. The left panels of the upper graph depict the individual decrease in absolute gene expression of 3 genes (gray columns) and 18S rRNA (white column), whereas the human RNA was in vitro degraded using RNase A. After normalization (18S rRNA), differential gene expression was calculated relative to the undigested RNA-species (equals 1-fold degradation). The lower graph reflects the degradation kinetics of the 4 RNA-species examined. Absolute gene expression of degraded RNA-species was plotted versus the x-fold degraded RNA-species, which was calculated relative to absolute gene expression of undigested 18S rRNA.

present, which equals 17%), the differential gene expression measured was 1.04, 1.00, and 1.7 for GAPDH, PSME3, and PKCI, respectively. With further increase in degraded RNA differential gene expression of the genes was up to 12-fold (PKCI) over undigested conditions (Fig. 3, right lower panel). The in vitro degradation kinetics of different genes were always linear, but the slopes of the 3 genes regression curves were lower

(slopes: GAPDH -0.93 , PSME3 -0.85 , PKCI -0.65) compared with 18S rRNA (slope: -1.00 , Fig. 3, lower graph) so that the plots converged against 18S rRNA with higher degraded RNA. These degradation kinetics allowed us to calculate an almost correct differential gene expression for the 3 genes at a degree of degradation equal to 10 (one-tenth of intact molecules are present, which equals 10%). The 18S rRNA normalized differential gene expression relative to the corresponding undigested values was 1.2, 1.6, and 1.9 for GAPDH, PSME3, and PKCI, respectively. Moreover, the known RNA-specific degradation kinetic represented by appropriate regression models allowed to extrapolate from the degraded RNAs to the correct intact RNA expression values (Fig. 3, lower graph).

In Vivo Degradation

Release of Tissue-specific Ethanol Resistant RNases

Pieces of the same tumor biopsy were incubated in an ethanol 50% solution (diluted in aqua dest) over 30, 60, 90, and 120 minutes. Isolated RNA from these pieces run in an agarose gel showed a disappearance of the 28/18S bands while a smear appeared, which became more prominent with prolonged incubation time of the tissues in the alcohol solution. Concomitant the amount of RNA decreased (Fig. 4, black circles, upper graph). This degradation process could be avoided when diluting ethanol not in water but in RNA-later solution (Fig. 4, white rectangles, upper graph). Up to 120 minutes intact 28/18S bands and no smears were visible (Fig. 4, inset upper graph), although spectral photometry measurements suggested a decrease in RNA amount taking place at 120 minutes. Aliquots of these solutions presumably containing RNases were transferred in tubes to digest intact human RNA of other origin (HL-60 cells). The 28/18 S bands disappeared after a 10-minute incubation. This was associated by a decrease in the amount of RNA (Fig. 4, lower graph, upper inset, and black circles). No degradation of HL-60 RNA was found after incubation in an RNase-free ethanol 50% solution (Fig. 4, lower graph, lower inset, and white rectangles).

Degradation Kinetic

A piece of not degraded testis biopsies stored in RNA-later was taken and transferred into 50% ethanol. The RNA isolated from the remaining tumor was incubated in aliquots of the 50% ethanol solution over several times. The absolute gene expression of 3 genes (Bcl-2, CDK4, Rab2, PCNA) and 18S rRNA showed a reduction over almost 4 log scales for 18S rRNA (Fig. 5, left panel, upper graphs). The decrease in absolute gene expression appeared over 2 log scales for Bcl-2, Rab2, and PCNA, whereas CDK4 showed a decrease over about 1 log scale. Moreover with 106-fold to 676-fold degradation no further reduction in absolute gene expression was found for CDK4, Rab2, and PCNA. After normalization versus 18S rRNA for 3 genes differential gene expression similar to undigested RNA-species was found up to a

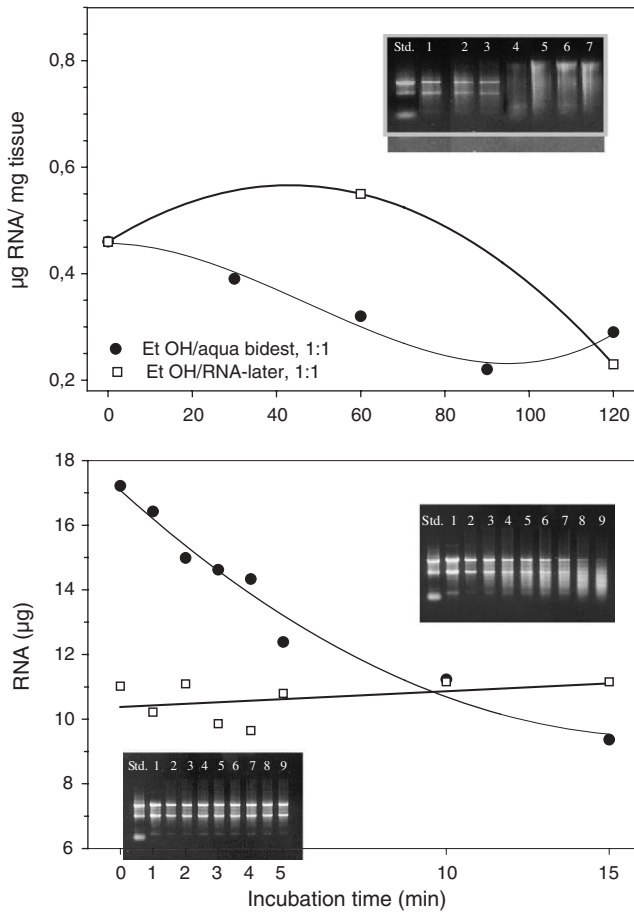


FIGURE 4. RNA isolated from pieces of tumor biopsies that were previously incubated in 50% ethanol (diluted in aqua bidest) were degraded versus time (shown by gel electrophoresis, line 4 to 7 inset upper graph). This was associated with a decrease in the amount of RNA (black circles, upper graph). Neither smear (line 2 and 3 inset upper graph) nor decreased amounts of RNA were found when diluting ethanol in RNA-later solution (white rectangles, upper graph). However, at 120 minutes spectral photometry measurements suggested a decrease in RNA amount. Symbols represent mean values of measurements done in triplicate. These representative experiments were repeated more than 3 times and performed on other tissues. Intact human RNA was incubated into a 50% ethanol-solution (diluted in aqua bidest), which previously contained a piece of tumor tissue. The degradation of the RNA increased with incubation time (line 1 to 9 upper inset lower graph). This degradation could not be observed after incubation of RNA in a clean 50% ethanol-solution.

13-fold degraded RNA (Fig. 5, right panels upper graph). However, CDK4 showed a differential gene expression being 3 times over values of intact RNA. Greater 13-fold degraded RNA in most cases showed a several-fold higher differential gene expression compared with the undigested RNA-species measured. The in vivo degradation kinetics of the 4 RNA-species was characterized by equations of second or third order (Fig. 5, lower graph).

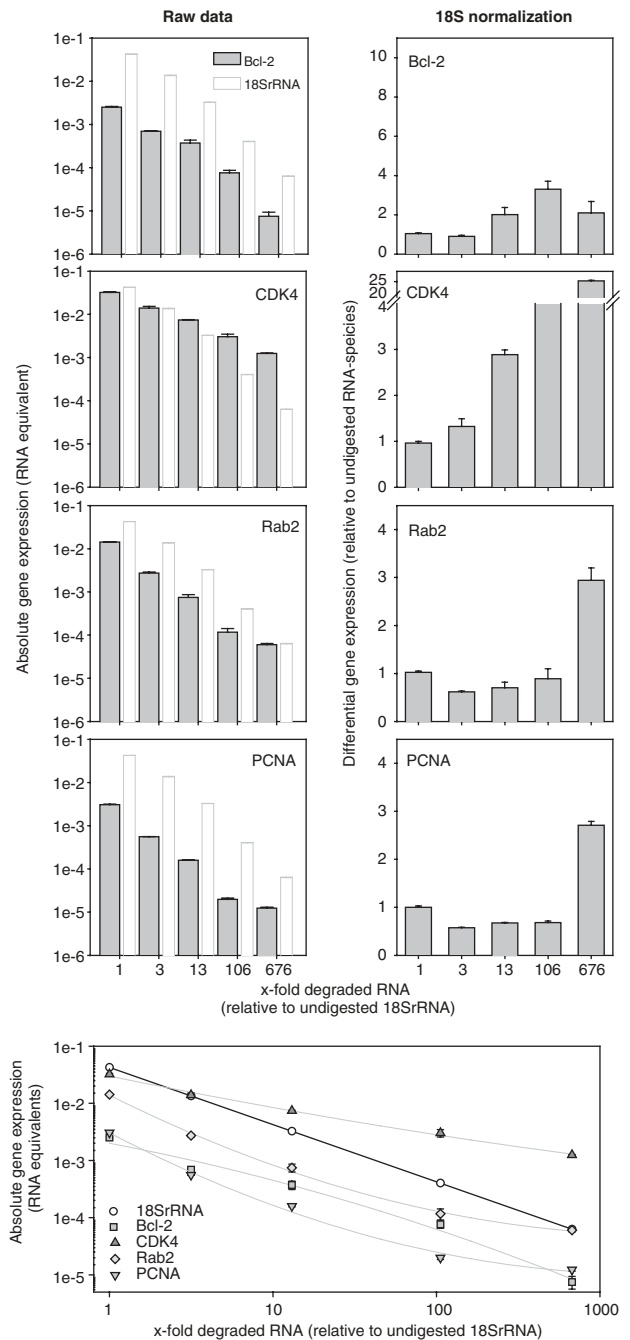


FIGURE 5. The left panels of the upper graph depict the individual decrease in absolute gene expression of 3 genes (gray columns) and 18S rRNA (white column), whereas the human testis-RNA was degraded using an ethanol-solution previously containing a piece of testis tumor. After normalization (18S rRNA) differential gene expression was calculated relative to the undigested RNA-species (equals 1-fold degradation). The lower graph reflects the degradation kinetics of the 4 RNA-species examined. Absolute gene expression of degraded RNA-species was plotted versus the x-fold degraded RNA-species, which was calculated relative to absolute gene expression of undigested 18S rRNA. These representative results were performed in duplicate at 3 different tissues. Error bars represent the SEM.

The kinetic of every RNA-species appeared individually different. The known RNA-specific degradation kinetic allowed to extrapolate from the degraded RNAs to the correct values of intact RNA with the aid of the regression curves (Fig. 5, lower graph).

Degradation kinetic of the 4 RNA-species was examined after incubating the isolated RNA of 3 individuals into their corresponding ethanol solutions, which previously contained a piece of the same tissue biopsy. While Bcl-2 and CDK4 degradation appeared

almost linear, a linear quadratic degradation characteristic could be shown for Rab2 and PCNA (Fig. 6A). Additionally, these degradation kinetics appeared dependent on the biopsy/ethanol solution. For instance, depending on the biopsy absolute gene expression of CDK4 showed a more than 15-fold difference at a 1000-fold degradation.

However, after digestion of 3 other tissues but in the same ethanol solution that previously contained a piece of a biopsy, the individual plots of the biopsies discovered

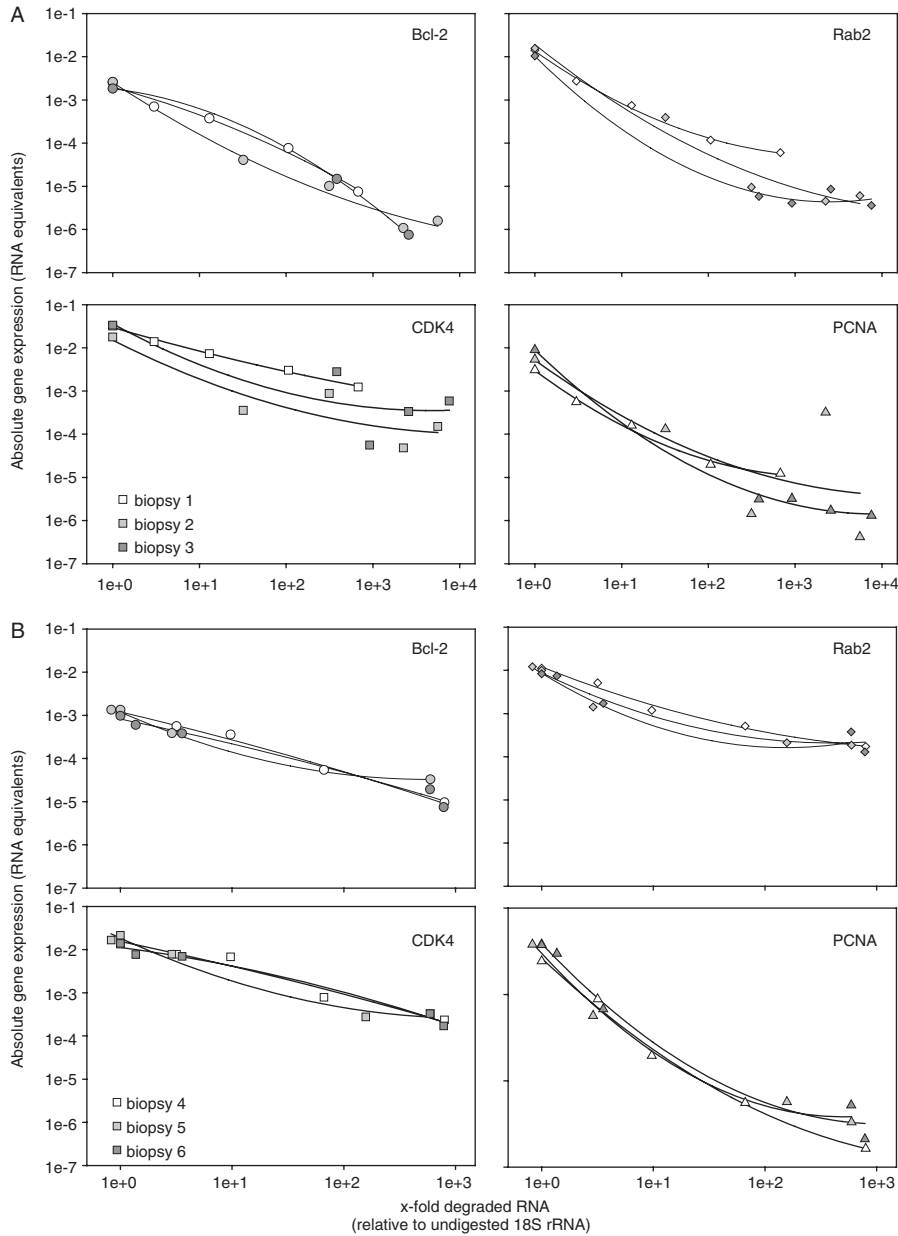


FIGURE 6. A, Intact RNA was isolated from 3 tumors and partly digested in ethanol-solutions that previously contained the corresponding tumor tissues. Absolute gene expression of 4 genes was measured in RNA-probes characterized by increased degradation. B, The measurements shown in A were performed on intact RNA of 3 other tumors, but these RNAs were partly digested in the same ethanol-solution that contained a tumor tissue previously. These representative results were performed as single measurements and using 9 different tissues.

before converged to 1 plot in all 4 RNA-species examined (Fig. 6B). For instance, at a 1000-fold degradation the difference in CDK4 absolute gene expression between the 3 biopsies was negligible (Fig. 6B).

Precision of the Method

Gene expression of 3 genes (PCNA, CCND2, and Bax) were measured in not degraded tumor biopsies (3 individuals), their corresponding normal tissues (to calculate differential gene expression), and on intact RNA isolated from HL-60 cells. Then aliquots of intact RNA isolated from these tumor biopsies and HL-60 cells were digested up to 3000-fold in the tumor-specific RNases released by incubation of a piece of the tumor biopsy in ethanol (see above). Intact RNA and degraded gene expression values of the 3 genes measured at RNA isolated from HL-60 were fitted with an appropriate regression curve (Fig. 7, triangles) and plotted versus the degree of 18S rRNA degradation. Under consideration of the same fold-18S rRNA-degradation, this calibration

curve was moved into the data points of the degraded tumor biopsies (Fig 7, gray vertical arrow). The intersection where the regression line crossed the vertical long dashed gray line (marker for intact RNA, which in the graph equals the value 1) represented the recalculated gene expression before the process of degradation occurred and could be read from the y-axis. On the not degraded testis biopsies the differential gene expression of PCNA, CCND2, and Bax relative to the corresponding normal tissues (controls) could be calculated. For the biopsies shown in Figure 7 differential gene expression were 0.6, 1.9, and 3.9 for PCNA, CCND2, and Bax, respectively. When using the method as described above, differential gene expression on degraded tumor RNA could be calculated with the help of the regression lines. Then the ratio between the known (undigested biopsies) and the recalculated differential gene expression was performed (Fig. 7, lower right graph). A ratio of 1 was indicative for a similar result. For 27 measurements (3 genes measured in 3 individuals and using 3 stages of

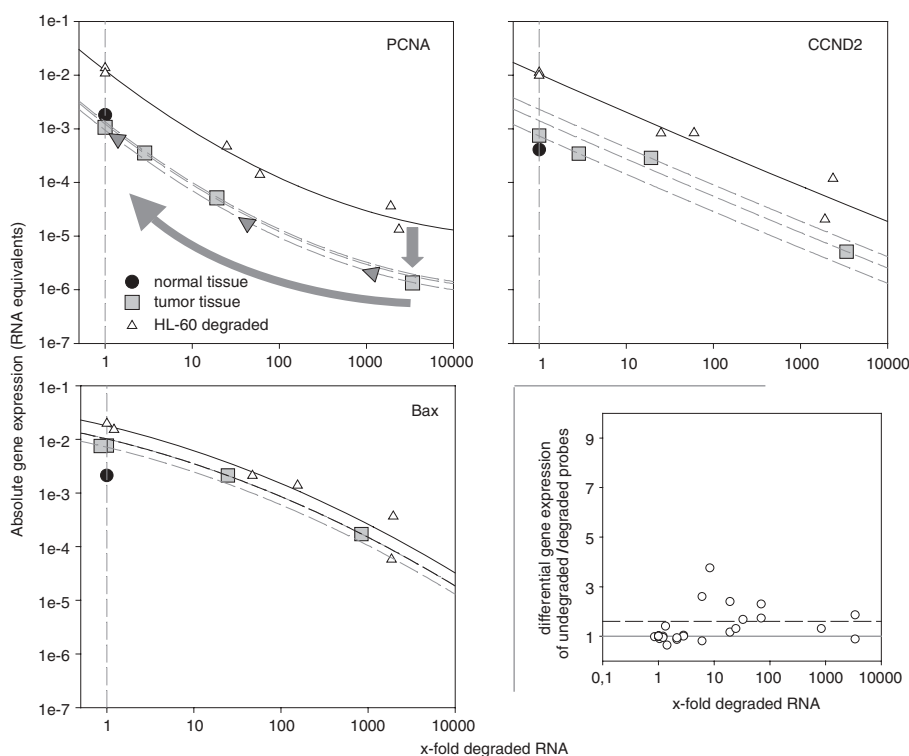


FIGURE 7. Intact RNA was isolated from tumor tissue and the corresponding normal tissue. Aliquots of intact tumor RNA were also digested in RNase-cocktails released from individual pieces of tumor tissues incubated in ethanol-solutions. In parallel, intact HL-60 RNA was also digested in these RNase-cocktails. Absolute gene expression of PCNA, CCND2, and Bax was measured in duplicate in 3 patients. Representative results from 1 patient are shown. Regression curves of HL-60 RNA were moved into the data points measured for degraded tumor tissues (exemplified in left upper graph with vertical arrow). Then the regression curves allowed the reading of predicted absolute gene expression values before the degradation of RNA occurred (exemplified in left upper graph with curved arrow). Differential gene expression of intact RNA isolated from not degraded tumor tissues relative to normal tissue was performed. Then the predicted gene expression values of degraded RNAs of the same tumor were used to calculate differential gene expression. The differential gene expression of intact tumor RNA was then compared with the differential gene expression measured on degraded tumor RNA of the same tumor using the method as described above. The results of 27 measurements (3 stages of degradation measured for 3 genes in 3 patients) are depicted in the lower right graph. Dashed lines represent the 99% confidence level.

RNA degradation), it could be shown that the geometric mean ratio was 1.2 with an SD of 0.7. Furthermore, up-regulated or down-regulated genes always appeared similarly up-regulated or down-regulated in the degraded RNAs after recalculation as described above.

Application of the Method on Degraded Biopsies

RNA was isolated from 5 degraded testis tumor biopsies and their corresponding intact normal tissues (Fig. 8A). Five genes (PCNA, Fas-Ligand, Clusterin, Clusterin,

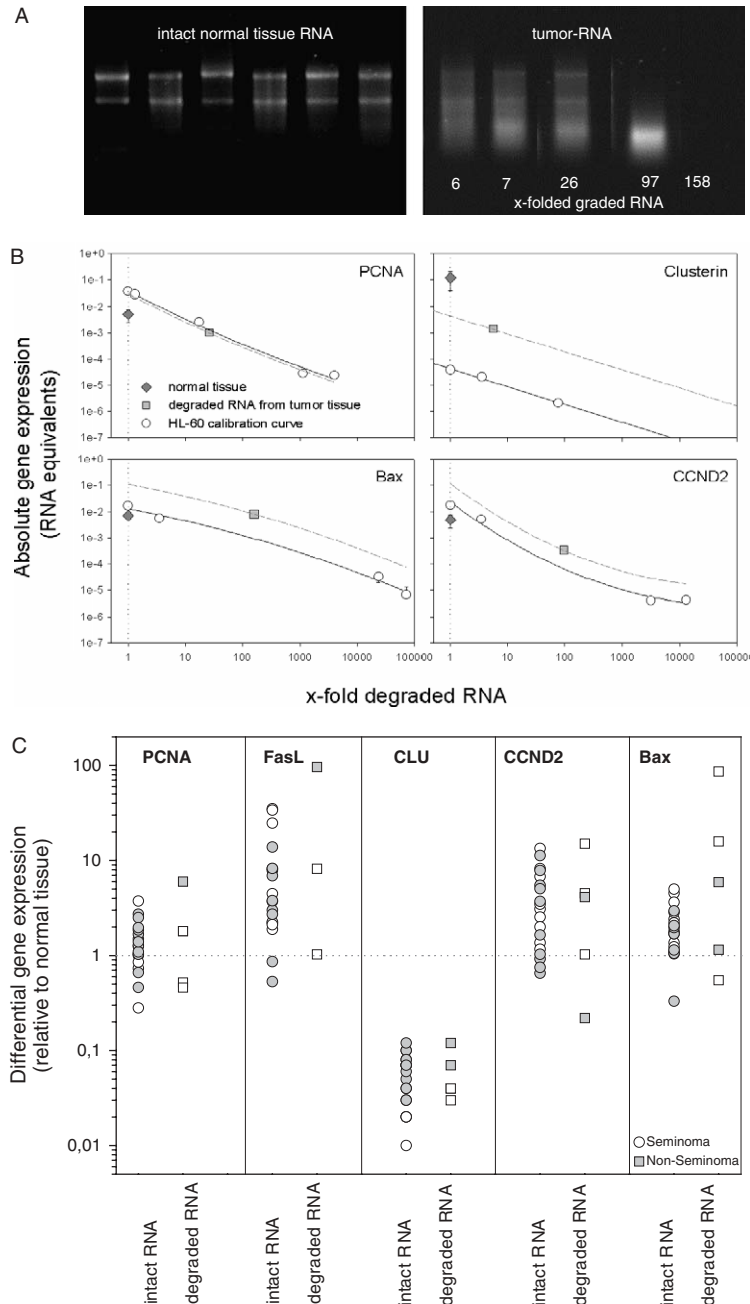


FIGURE 8. RNA was isolated from 5 degraded testis tumor biopsies and their corresponding intact normal tissues (A). The degree of 18S rRNA degradation is depicted in (A). Four of the 5 genes, in which gene expression was measured in the testis biopsies, are shown (B). Aliquots of RNA isolated from HL-60 cells were digested up to 10,000-fold in the tumor-specific RNase-cocktails released from tumor biopsies. Gene expression of genes was plotted versus degree of 18S rRNA degradation of HL-60 RNA and connected with a regression curve (B, black line). On the basis of these calibration curves the absolute gene expression before degradation of RNA occurred was calculated (B), and these values were taken for calculation of differential gene expression. In parallel, differential gene expression was also calculated on a second cohort of 20 testis tumors and normal tissues with intact RNA (C).

CCND2, and Bax) were measured (Fig. 8B reveals representative results of 4 genes) in the testis biopsies. Aliquots of RNA isolated from HL-60 cells were digested up to 10,000-fold in the tumor-specific RNases released by incubation of a piece of the tumor biopsy in ethanol (see above). Gene expression of the same 5 genes was plotted versus degree of 18S rRNA degradation of HL-60 RNA and connected with a regression curve (Fig. 8B, black line). As shown above (section Precision of the Method), this biopsy and gene-specific regression curves were taken to estimate the absolute gene expression in the tumors before the degradation of RNA occurred (Fig. 8B). In parallel, differential gene expression was also calculated on a second cohort of 20 testis tumors and normal tissues with intact RNA. When comparing the recalculated differential gene expression of the degraded tumor tissues with another cohort of tumors characterized by intact RNA, no significant differences could be detected (Fig. 8C).

DISCUSSION

To correct false gene expression measurements in degraded tumors, it is necessary to quantify the height of degradation. In the first step, we searched for an adequate house-keeping gene with almost constant copy number in tissues of different origin. It could be shown that the 18S rRNA gene expression in opposite to other genes (PGK, HPRT) appeared almost constant within a certain cell/tissue type provided an equal amount of the template cDNA was given into the PCR-reaction (Fig. 1). 18S rRNA absolute gene expression differed up to 15-fold between different cell lines or tissue types (Fig. 2A). Within these models, control probes and the altered probes showed similar 18S rRNA abundance so that differential gene expression (relative to the adequate control values) between different models appeared comparable (Fig. 2B). The differences in 18S rRNA absolute gene expression could be caused by inhibitory substances present in the isolated total RNAs and characteristic of the cell and tissue types examined. The total RNAs of 3 cell and tissue types and standard total RNA were diluted over 6 log scales, and the PCR efficiency was calculated. The linear regressions and the equal slopes of all regressions corresponding to PCR efficiencies of 93% to 96% were indicative for the absence of differences in the amount of inhibitory substances among these RNA probes (Fig. 2C).

An *in vitro* degradation model using the commercially available RNase A allowed to examine the degradation kinetic among different RNA-species more carefully (Fig. 3). Degradation kinetic in this model for each RNA-species was characterized by a first order equation, but the slopes differed among the RNA-species and were slightly smaller compared with 18S rRNA (Fig. 3, lower graph). Hence, when correcting absolute gene expression of the RNA-species by 18S rRNA absolute gene expression, differential gene expression of the RNA-species increased with the height of degradation

(Fig. 3, right panels of upper graph). However, up to a 10-fold degradation differential gene expression of the RNA-species was comparable to the control values (intact RNA-species). Furthermore, when knowing the height of degradation and the RNA-species' individual degradation kinetic, a corrected expression of RNA-species could be estimated (Fig. 3, lower graph). To develop the degradation kinetic, large amounts of RNA isolated from tissues would be necessary, but tissue material is generally limited. Commercially available kits allow amplification of total RNA. However, in previous experiments we showed that amplification rate was dependent on the RNA-species and increased with the height of degraded total RNA (data not shown). Therefore, this approach seemed inappropriate. Recently, it was suggested that the detection system used must fit the length of the RNA-fragment of degraded RNA-species.¹⁴ Besides the fact that it will become difficult to manage this, we recently demonstrated that depending on the amplicon size the detected amount of 18S rRNA decreased in parallel with almost similar slopes (data not shown). When calculating differential gene expression relative to control values gene expression of both probes will become altered in the same order of magnitude. As a result, differential gene expression will be equal although the absolute amount of RNA-species would be smaller in the probes.

Often total RNA isolated from biopsies not stored in RNA-later solution appeared degraded (own experience). When we searched for the reason, it became obvious that during the conventional dehydration steps of the tissue preparation taking place before paraffin embedding, ethanol-resistant RNases were released (Fig. 4, upper graph); this might also explain the results of Perlmutter et al.¹⁵ Their enzyme activity could be inhibited by dilution of ethanol in RNA-later (Fig. 4, upper graph). Active ethanol resistant RNases could be only isolated when using 50% to 70% ethanol; at higher ethanol concentration, no RNA degradation could be found, thus suggesting that released RNases become denatured with higher concentrated ethanol (data not shown). Therefore, it might be advisable to perform the dehydration procedure with > 70% ethanol solutions. These RNase-cocktails could also be used to degrade RNA of other origin (Fig. 4, lower graph). With this in mind ethanol-resistant RNases were released from pieces of testis tumors or normal testis tissues. Corresponding intact total RNAs isolated from the remaining pieces of these tissues were incubated into the appropriate solutions. Even *in vivo*, with an up to a 10-fold degradation, differential gene expression of the RNA-species was comparable to the control values (intact RNA-species), but increased with further degradation of RNA (Fig. 5 upper graphs). The degradation kinetic was characterized by equations of second or third order and more complex compared with the *in vitro* experiments (Figs. 3, 5, lower graphs). RNA-species digested in their corresponding RNase-cocktails released from the same tissue showed regressions characteristic for the RNA-species and differed between the tissues (Fig. 6A). This suggested

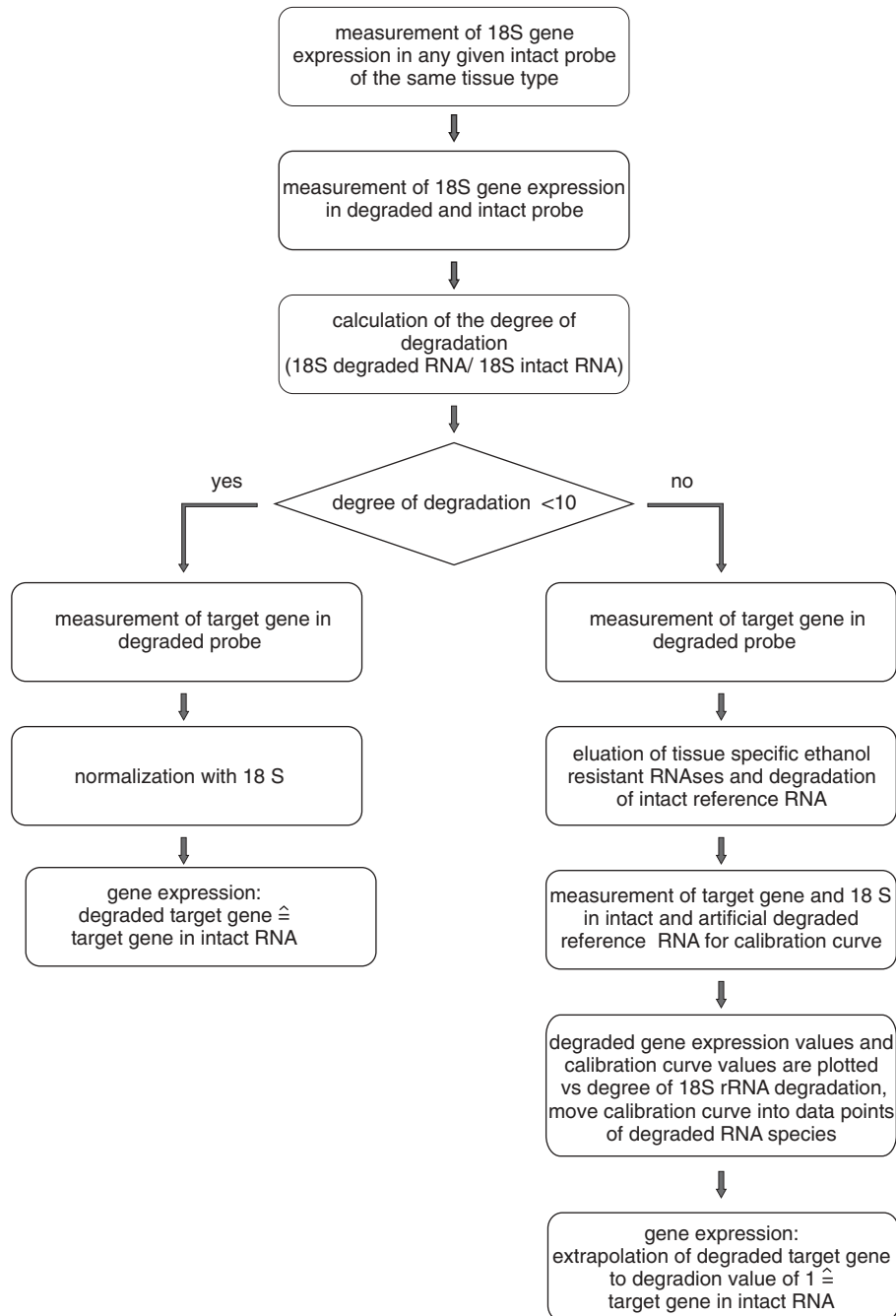


FIGURE 9. Schematic representation of the technique applied to correct false gene expression measurements from degraded RNA using RTQ-PCR. For a degree of degradation smaller than 10, normalization with 18S is sufficient; for a higher degree of degradation, an *in vitro* simulation of the degradation leads to a precision that is comparable to RTQ-PCR measurements on intact RNA.

that the released RNase-cocktails were tissue specific. These differences vanished when digesting RNA from different tissues but in the same RNase-cocktail (Fig. 6B). With the knowledge of the height of degradation and the RNA-species' individual degradation kinetic, corrected gene expression on degraded RNA-species could be estimated (Figs. 5, 6).

Because RNA from different origin becomes degraded similarly when using the same RNase-cocktail (Fig. 6B), intact human HL-60 RNA (which can be isolated in the desired amounts *in vitro*) was used to create biopsy (or RNase-cocktail) and gene-specific calibration curves. These calibration curves were used to estimate the precision of a method for recalculation of

gene expression before degradation of RNA occurred but considering the biopsy and gene-specific degradation kinetics (Fig. 7). Therefore, intact RNA was isolated from 3 tumor tissues and corresponding normal tissues, thus allowing us to calculate differential gene expression before degradation occurred (depicted as x -fold degradation of 1 in Fig. 7). From pieces of tumor tissues, individual ethanol-resistant RNA-cocktails were isolated and aliquots of intact tumor RNAs and intact HL-60 RNA digested over up to 4 log scales. When moving the calibration curves into the false gene expression results of the degraded tumor RNAs but considering the same degree of 18S rRNA degradation it could be shown that the calculated geometric mean was closed to the expected value (1.2 vs. 1). With an SD of 0.7, the precision of the method lies within the recommendations of the manufacturer regarding the precision of RTQ-PCR: up to 2-fold differences in gene expression are expected to represent variations inherent to the method (manual of the 5700 RTQ-PCR instrument, Applied Biosystems).

These results forced us to apply the method on degraded tumor tissues that previously could not be examined (Fig. 8). When comparing the recalculated values of differential gene expression with another cohort of biopsies characterized by intact RNA similar values could be shown (Fig. 8C), which supports the significance of the method applied.

Recently Antonov et al¹⁶ could show that based on short amplicon design and normalization using the mean of several control genes as proposed by Vandesompele et al,¹⁷ meaningful results could be measured in degraded RNA extracted from formalin-fixed, paraffin-embedded tissues. Nevertheless, this special normalization is not necessary if the degradation grade is smaller than 10. However, in case of degraded RNA > factor 10 according to the proposed method the calculated differential gene expression can be 10 to 15 times different from that what would be expected. Furthermore, for the method proposed here only 1 gene, namely 18S, is needed for normalization purposes.

In summary, RNA degradation depends on the RNA-species and on the tissue. After calculation of the height of degraded RNA with the aid of the housekeeping gene 18S rRNA an up to 10-fold degradation will be automatically considered. However, with higher degraded RNA both factors have to be taken into account by simulation of the degradation kinetic as described above (overview see Fig. 9). This newly

developed method leads to a precision that is comparable to RTQ-PCR measurements on intact RNA.

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