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Technical Advance

Quantitation of DNA Extracted after Micropreparation of Cells from Frozen and Formalin-Fixed Tissue Sections

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Quantitation of DNA from microdissected fresh-frozen or paraffin-embedded tissue sections would be not only a valuable tool for ensuring optimum reaction conditions for many types of qualitative polymerase chain reaction (PCR) analyses, but also a prerequisite for any kind of subsequently performed genetic analyses aimed at the absolute quantitation of target sequences. The present study describes the quantitation of DNA after microdissection and extraction of cells with the PicoGreen fluorescence method. The limits of detection and of quantitative determination, respectively, have been determined by measuring dilutional series of three different DNA extractions, using either a medium-scale preparation from a solid tissue specimen or a known number of leukocytes or microdissected cells from frozen tumor sections. As corresponding limits of detection, 26, 24, and about 40 diploid genomes, and as limits of quantitative determination, 80, 73, and about 120 diploid genomes were obtained. Furthermore, it was shown that formalin fixation as well as hematoxylin staining of frozen sections with Delafield's and Mayer's alum or Weigert's iron hematoxylin before microdissection significantly diminishes the amount of extractable DNA and may lead to less reliable results, even of qualitative PCR analysis. In conclusion, the PicoGreen method allows precise quantitation of DNA corresponding to a minimum of about 120 diploid cells. It provides the basis for reliable qualitative analyses as well as the precondition for further quantitative genetic measurements from microdissected frozen or formalin-fixed and paraffin-embedded tissue sections. ***(Am J Pathol 2000, 156:1189–1196)***

Microdissection of histologically characterized cells from fresh-frozen or paraffin-embedded tissue sections has become an important technique,[1–7] particularly for the analysis of genetic alterations occurring in heterogeneous tumors such as premalignant and primary lesions.[1,8–10] The subsequently performed analyses of nucleic acids are usually carried out by polymerase chain reaction (PCR)-based methods. PCR-directed amplifications, however, require a careful control of reaction parameters, such as quality and quantity of the DNA template, to ensure reliable results.[11] In contrast to the analysis of DNA that has been extracted from tissue specimens in medium scale, an accurate quantitation of template DNA obtained by microdissection before PCR analysis has so far been made difficult by the low amounts of DNA available for measurement. Although the amount of DNA extracted from microdissected cells can seemingly be estimated by counting the absolute number of dissected cells, significant deviations from the expected results may occur. Apart from deviations due to specific effects characteristic for the tissue investigated, eg, mitotic activity, degree of poly- or aneuploidy in neoplastic cells, and variations regarding the thickness of tissue sections, significant negative effects of tissue fixation on the extractable amount and the quality of DNA, caused, for example, by formalin, have been reported.[12–15] Moreover, reaction conditions and duration of formalin fixation may vary between individual specimens, hence altering the efficiency of DNA extraction from an individual specimen in a specific way.[12] Consequently, it is not clear how close the quantity of template DNA obtained by microdissection does correlate with the number of cells visually determined during microdissection. On the other hand, the reliability of certain PCR analyses

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might significantly benefit from a previous quantitation of the template DNA, in particular if only low genome copy numbers are available and a reliable routine analysis is demanded.[16] It is obvious that all investigations aimed at the absolute quantitation of target sequences present within microdissected cells require a precise quantitation of the template DNA as an exclusive precondition. Accurate quantitation of DNA from microdissected cells, therefore, would provide the basis for both reliable qualitative and quantitative measurements of histologically defined cell populations from fresh-frozen or paraffin-embedded tissue sections. In the course of a project that leads to the need for a quantitative detection of viral DNA in sections of prostate cancer specimens,[17,18] we have investigated whether the PicoGreen fluorescence DNA quantitation method is sufficient for quantitation of DNA from microdissected tissue sections with standard fluorimeter equipment. Herein we show that the method offers an accurate and efficient way of quantitation of microextracted DNA that could also be of benefit for qualitative PCR analyses. It is further demonstrated that the effect of routine staining and fixation on the efficiency of DNA microextraction can now be precisely measured, a finding that has led to the observation that hematoxylin staining of sections seriously interferes with the extraction of DNA.

Materials and Methods

For preparation of DNA standard solutions, medium-scale DNA extractions from a peritumoral renal cancer tissue and a benign prostatic hyperplasia specimen were carried out with an extraction kit (RotiExtract T; Roth, Karlsruhe, Germany). Concentrations of reference DNA solutions were determined spectrophotometrically. High-sensitivity DNA quantitation using the PicoGreen reagent was performed according to the manufacturer's protocol (Molecular Probes, Eugene, OR), using a spectrofluorimeter (RF-5001PC; Shimadzu) and a total sample volume of 800 μl. Fluorescence was excited at 480 nm, and the intensity of emission was detected at 532 nm. Frozen sections (6-μm) were cut from snap-frozen, fresh tissue specimens with a Microm HM 500 OM cryostat. After sectioning, the slides were air-dried and fixed by acetone. The rat kidneys were fixed in unbuffered 4%-formalin in distilled water. Sections from the formalin-fixed and paraffin-embedded tissues were dewaxed before proteinase K digestion. Microdissection was carried out as described previously.[19] Briefly, uncovered and (to avoid a potential interference of staining procedures with DNA extraction) unstained 6-μm tissue sections were first soaked with buffer (10 mmol/L Tris/HCl (pH 7.6), 1 mmol/L EDTA). Micropreparation of target cell areas was then carried out by hand processing under microscopic control (Diavert; Leitz, Germany; magnification ×63), using sterilized, disposable insulin drain tubes (O 0.45, Microlance). After microdissection the sections were hematoxylin stained, and the area of microdissected cells was measured using a video image capture and an image analysis system (Optimas; Bioscan Edmonds, WA). Reference leukocyte preparations were obtained from citrated whole blood samples, using Ficoll centrifugation and a Neubauer counting chamber. To ensure comparability with cells microdissected from fixed fresh frozen sections, isolated leukocytes were also fixed by incubation in acetone. Total DNA was extracted from reference leukocyte preparations or microdissected cells, using enzymatic digestion (50 mmol/L Tris/HCl (pH 7.6), 1 mmol/L EDTA, 500 μg/ml proteinase K, 180 minutes at 50°C, 10 minutes at 80°C), in a maximum total volume of 20 μl. Note that the efficiency of enzymatic digestion may vary, depending on fixation, type of tissue, and thickness of section. Thus specific optimization of digestion parameters is usually required.

For removal of high-molecular-weight RNA, DNA extractions were treated with DNase free RNase (final concentration 1 μg/μl; Boehringer Mannheim, Mannheim, Germany). Sensitivity parameters were calculated as limit of detection (L_D) and limit of quantitative determination (L_Q), according to the concept of Currie.[20] First, the 3.29-fold (L_D) and 10-fold (L_Q) of the SD of the multiple interassay blank measurement were calculated. Second, the corresponding difference of diploid genomes that can be reliably detected (L_D) or precisely quantitated (L_Q) was calculated using the relationship between signal and the input variable, represented as the mass of DNA or the number of leukocytes or the volume of microdissected tissue. Each specific relationship was given by the slope of the corresponding regression line (compare Figures 1, 2, and 4 and Table 1). The number of diploid genome equivalents present within a sample was calculated using the mass of DNA determined by fluorimetrical measurement and a mass of 6.57 pg genomic DNA per single diploid cell (M_R 660 per bp and 3×10^9 bp per haploid genome).[21] The values obtained were not corrected for slight systematic deviations due to the measurement of mitochondrial DNA. Dye solutions used for histological stainings were Delafield's hematoxylin (stock solution 0.6% w/v, diluted to 1:4 before use; Chroma, Köngen, Germany), Mayer's hematoxylin (0.1% w/v; Merck, Darmstadt, Germany), Weigert's iron hematoxylin (0.5% w/v; Sigma, Deisenhofen, Germany), and kernechtrot (0.1% w/v; Merck). Hematoxylin stainings of sections were carried out using either incubation periods according to routine protocols of about 7 minutes for intensive staining or shortened incubation periods of about 20 seconds for less intensive stainings. Nested PCR amplification of the exon 5 sequence of the *p53* tumor suppressor gene was carried out as described previously.[19]

Results

Determination of the Sensitivity Parameters Limit of Detection (L_D) and Limit of Quantitative Determination (L_Q), using Purified Genomic DNA and a Defined Number of Leukocytes

To assess the limits of the quantitation of microextracted DNA that can be achieved with our experimental setup in

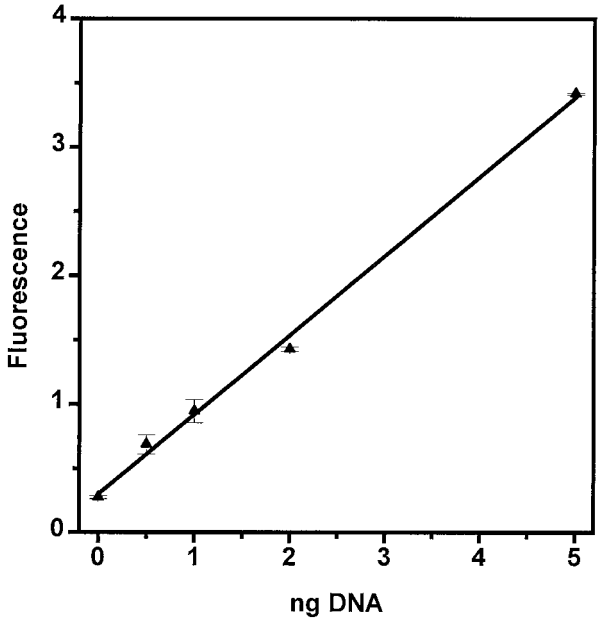

Figure 1. Sensitivity of the PicoGreen assay using medium scale-purified genomic DNA. The indicated amount of DNA was incubated in quadruplicate with the PicoGreen reagent in a total volume of 800 μl, and the fluorescence emission was measured at 532 nm (excitation wavelength 480 nm). To calculate the sensitivity parameters the slope of the regression line was determined (see Table 1). Note that some of the bars indicating the positive and negative SD fall within the data symbols.

practice, the L_Q and L_D of the PicoGreen method were first determined using medium-scale purified and spectrophotometrically quantitated genomic DNA. A 10-fold interassay measurement of the reagent blank value yielded a SD of 6%. Together with the slope of 0.74 fluorescence units/ng of DNA, as determined on average from DNA standard curves

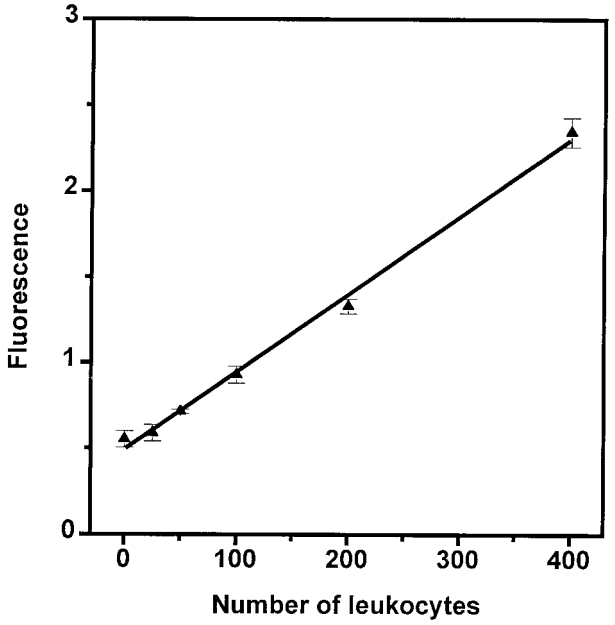

Figure 2. Sensitivity of the PicoGreen assay using a dilutional series of total DNA extracted from a known number of leukocytes. The amount of DNA corresponding to the indicated number of cells was incubated in quadruplicate with the PicoGreen reagent. For calculation of the sensitivity parameters, the slope of the indicated regression line was determined (see Table 1).

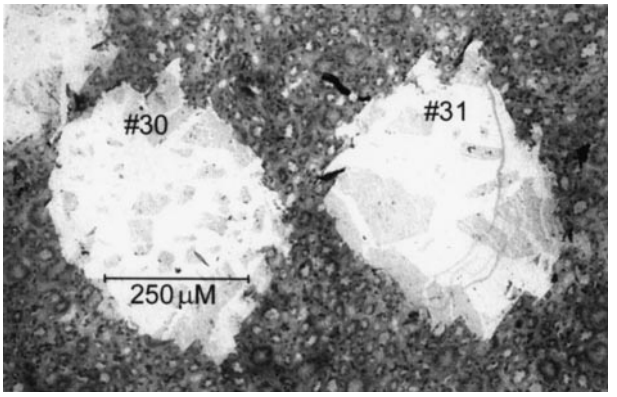

Figure 3. Microdissections of a fresh frozen section of a renal cancer specimen (original magnification ×63). Note that hematoxylin staining of sections was carried out after the microdissection of cells. The numbers of microdissection (30 and 31) refer to the sensitivity analysis of microdissections shown in Figure 4.

(one shown in Figure 1), a L_D of 174 pg (26 diploid genomes) and a L_Q of 529 pg DNA (80 diploid genomes) per 800 μl were calculated (see Table 1). To directly determine the assay sensitivities L_D and L_Q, a dilutional series of DNA extracted from a defined number of isolated leukocytes was also measured (Figure 2). Performing the analogous calculation as described above, a limit of detection L_D of 24 diploid cells and a limit of quantitative determination L_Q of 73 diploid cells per 800 μl were obtained. Both values agree well with those obtained by the measurements of purified genomic DNA (see Table 1).

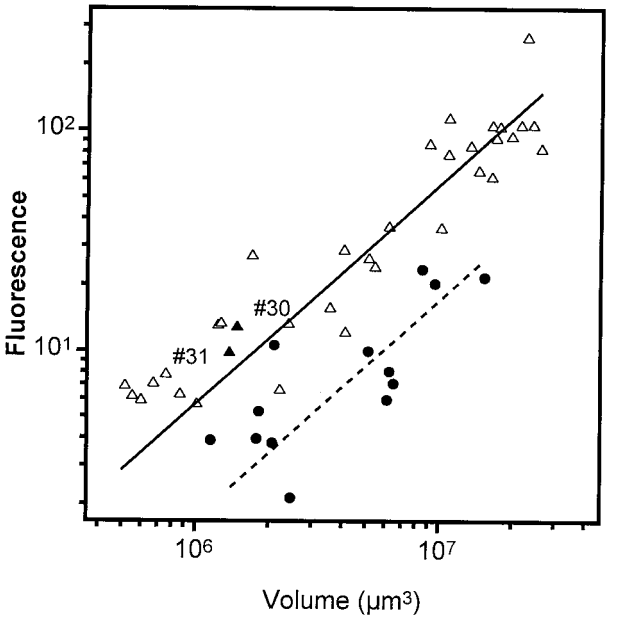

Figure 4. Sensitivity analysis using DNA from microdissections of renal cell cancer (△) and cervical cancer specimens (●). The amount of DNA extracted from each microdissection was assayed by PicoGreen fluorescence. Fluorescence signals were plotted against the volume of microdissected cells as calculated from the measured microdissected areas and the thickness of the section. The slope of the regression line together with the blank value variation was used for calculation of the sensitivity parameters (see Table 1). ▲, Microdissections 30 and 31, as shown in Figure 3.

Table 1. Calculation of the Sensitivity Parameters L_D and L_Q for Reference DNA Preparations and Microdissections from Renal Cell Cancer and Cervical Cancer, Respectively

	Purified DNA	Leukocytes	Renal cell cancer	Cervical cancer
No. of samples	20*	24*	35	13
Slope of regression line	0.74† (FU/ng)	4.5×10^{-3}† (FU/Leuk.)	5.7×10^{-6} (FU/μm^3)	1.7×10^{-6} (FU/μm^3)
Correlation	$R = 0.998$*	$R = 0.998$*	$R = 0.82$	$R = 0.83$
p value	<0.0001	<0.0001	<0.0001	<0.001
Sensitivity	L_D = 174 pG L_Q = 529 pG	L_D = 24 Leuk. L_Q = 73 Leuk.	L_D = 33,624 μm^3 L_Q = 102,201 μm^3	L_D = 119,926 μm^3 L_Q = 340,200 μm^3
Average volume per diploid genome	—	—	856 μm^3	2782 μm^3
Sensitivity (Diploid genomes)	L_D = 26 L_Q = 80	L_D = 24 L_Q = 73	L_D = 39 L_Q = 119	L_D = 40 L_Q = 122

Note that all given sensitivities refer to a total volume of 800 μl. For calculation of sensitivity parameters a uniform interassay standard deviation of 5.95%, as determined from multiple blank value measurements, has been used. The corresponding ΔFU values for L_D and L_Q were $\Delta FU = 0.19$ (3.29-fold standard deviation of mean blank value) and $\Delta FU = 0.58$ (10-fold standard deviation of mean blank value), respectively.

*Values refer to one representative measurement.

†Slopes of regression lines were determined by multiple interassay measurements.

L_D, limit of detection; L_Q, limit of quantitative determination; FU, arbitrary fluorescence units; R, coefficient of correlation.

Quantitation of DNA from Microdissected Cells and Determination of the Sensitivity Parameters L_D *and* L_Q

The basis of determination of L_D and L_Q as described above is to measure the fluorescence signal as a function of the input variable given by the mass of DNA or the number of extracted diploid genomes. In contrast, neither the mass nor the number of input DNA molecules are easily available for measuring DNA from microdissected cells. Therefore, the volume of microdissected cells (Figure 3) was calculated after determination of each microdissected area by video image analysis and used as an input variable. Figure 4 shows the relationship obtained between PicoGreen fluorescence of DNA extracted from 39 and 13 microdissections of unstained renal carcinoma and cervical carcinoma cryosections, respectively, and the corresponding volume of each microdissection. Although some divergence is observed with respect to individual microdissections and the expected number of diploid genome equivalents, as they can directly be calculated from the signal intensity of fluorescence, the correlation between fluorescence of microextractable DNA and microdissected volume is clearly indicated in both cases by the regression analysis (see Table 1). Using the slope of the regression lines, sensitivities of about 40 (L_D) and 120 (L_Q) diploid genome equivalents per 800 μl were calculated (see Table 1).

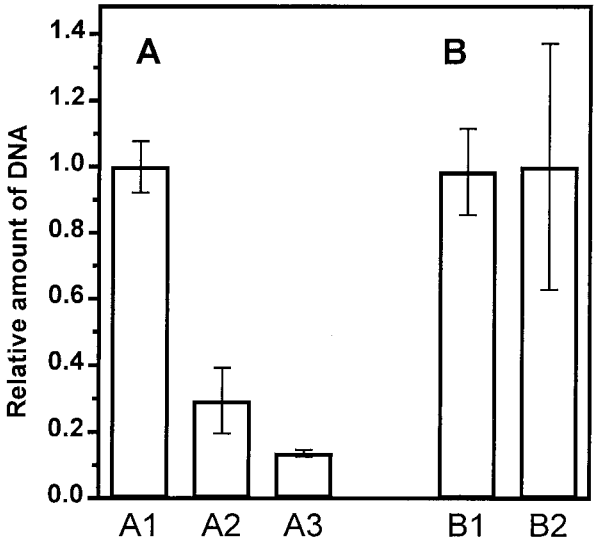

Figure 5. A: Effect of formalin treatment on DNA extraction efficiency of complete serial cryosections from a snap-frozen rat kidney, each experiment carried out in triplicate. **A1:** Control, untreated cryosections; **A2:** 10-minute treatment of cryosections with 4% (w/v) unbuffered formalin; **A3:** extraction of sections of the corresponding contralateral formalin-fixed and paraffin-embedded rat kidney specimen. **B:** Control incubation of human placental DNA with formalin. **B1:** No formalin; **B2:** 4% (w/v) formalin. The error bars indicate the positive and negative standard deviations, respectively.

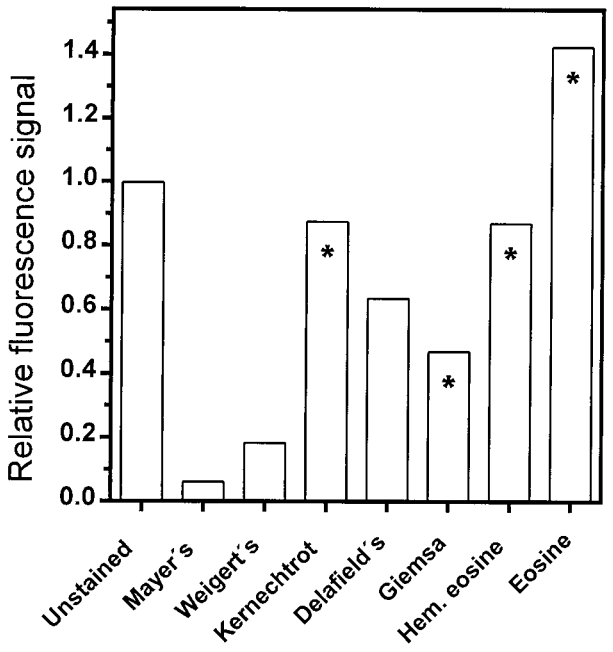

Figure 6. Screening for effects of various histological stainings on the amount of DNA that can be extracted with a uniform Proteinase K digestion procedure and serial cuttings of a prostatic hyperplasia specimen. *Fluorescence signals that might be biased because of an interference with fluorescence detection (see Table 2).

Table 2. Fluorescence of Various Dye Solutions at Detection Wavelength of PicoGreen Fluorescence Emission

Dye solution	Concentration* (%)	Fluorescence† (480/532 nm)
Delafield's hematoxylin	0.5	8.70
Mayer's hematoxylin	0.5	0.27
Weigert's iron hematoxylin	0.5	1.02
Eosin	0.5	≅250
Kernechtrot	0.5	≅450
Giemsa	0.5	≅110

*Concentration refers to the concentration of solutions ready for use, as used for staining.
†Arbitrary units.

Effect of Formalin Fixation and Paraffin Embedding of Sections on the Yield of DNA Microextraction

To investigate the effect of formalin fixation and paraffin embedding on the yield of microextractable DNA, a rat kidney was divided symmetrically into two pieces and then either subjected to shock freezing in liquid nitrogen or fixed in formalin and embedded in paraffin. For exclusive measurement of a formalin-caused effect, first serial cuttings of the fresh frozen specimen were prepared and then alternately subjected to DNA extraction in total or incubated before extraction in a formalin solution (Figure 5). The effect of both formalin fixation and paraffin embedding was measured by extracting multiple serial cuttings of the correspondingly treated second part of the rat kidney (Figure 5, column A3). As can be seen, the yield of standard DNA microextraction is significantly diminished, by about 70%, when only formalin fixation has been applied to the specimen beforehand (Figure 5, column A2), whereas a control incubation of purified DNA with formalin alone demonstrated no significant effect (Figure 5, part B). However, it is not clear whether paraffin embedding causes an additional, distinct effect (Figure 5, column A3), because the results of formalin-fixed, fresh-frozen sections show slight variations (Figure 5, column A2).

Effect of Staining of Sections on the Yield of DNA Microextraction and on PCR Amplification

During the initial microdissections it was observed that various histological stainings might interfere with DNA microextraction as measured by PicoGreen fluorescence. The effects of common staining methods on the results of fluorescence measurement are comprehensively depicted in Figure 6. To systematically investigate these effects, direct fluorescence measurements of different staining solutions were carried out in advance. As expected, the eosin dye, which derives chemically from fluorescein, as well as the kernechtrot dye, demonstrated a strong fluorescence signal at the wavelength of PicoGreen fluorescence emission detection (see Table 2). Consequently, the relatively increased fluorescence signals observed for the corresponding sections, as compared to their counterparts without eosin or kernechtrot staining, can be explained (Figure 6, columns marked with asterisks). On the other hand, only minimum fluorescence signals were detectable when Delafield's, Weigert's iron, or Mayer's hematoxylin solutions were measured at the PicoGreen excitation and emission wavelength, thus showing that no direct interference with the fluorimetrical detection should occur (Table 2). Even so, staining of serial sections in quadruplicate using Delafield's, Mayer's, and Weigert's hematoxylin according to a routine histological staining protocol and subsequent DNA extraction of the complete sections demonstrated that a significantly decreased fluorescence signal is obtained, indicating a lower amount of extractable DNA (Figure 7). Note that for DNA extraction experiments shown in Figure 6 a less intensive staining of sections such as is performed for counterstaining of immunostained slides has been carried out. Further experiments demonstrated that neither the fluorimetric inner filter effect nor a simple direct quenching of PicoGreen fluorescence influence fluorescence measurements to an extent that would explain the observed phenomenon (data not shown). Interestingly, subsequently performed investigations revealed that many of the protocols used for histological stainings of nuclei, including those shown in Figure 7, cause the formation of comparatively insoluble precipitates of nucleic acids, depending on the concentration of the staining solutions, thus leading indirectly to a significantly diminished fluorescence signal. (Serth et al, manuscript in preparation). To directly demonstrate the effect of hematoxylin staining of sections before DNA extraction on the efficiency of subsequent PCR amplifications, the SD regarding the amount of total extractable DNA from complete serial sections was first determined: this was about 5% when three consecutive sections of a human renal cell carcinoma were used. Two identical

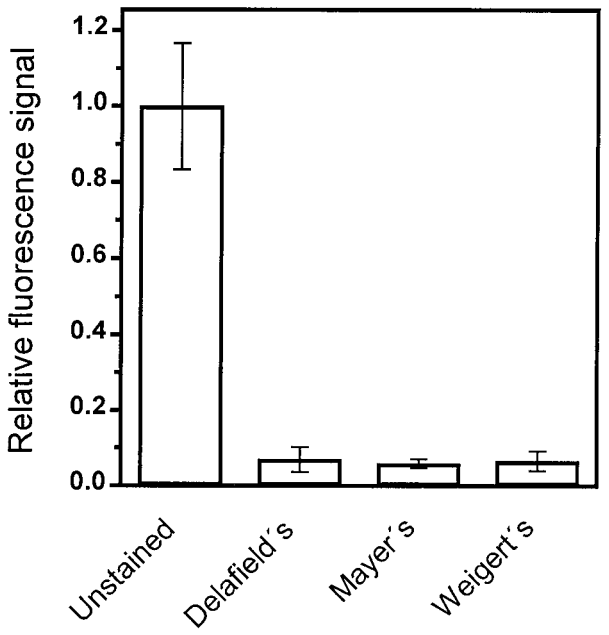

Figure 7. Effect of Delafield's, Weigert's iron, and Mayer's hemalum stainings on the yield of DNA extraction from serial cuttings of a rat kidney in comparison to unstained reference sections; all stains have been carried out in quadruplicate. The bars indicate the positive and negative SD, respectively.

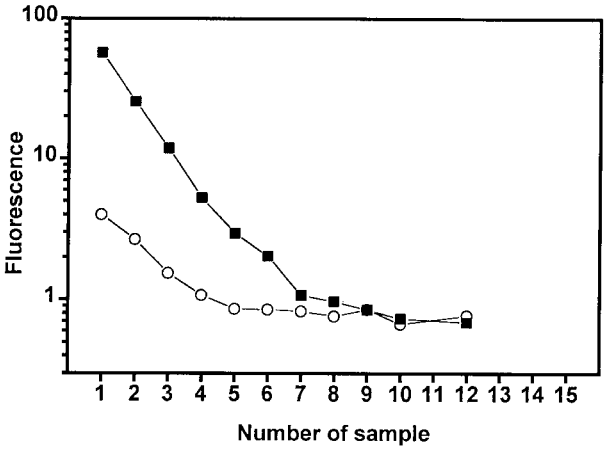

Figure 8. Fluorescence quantitation of identical dilutional series of complete DNA extractions obtained from two serial sections, either hematoxylin stained (○) or unstained (■). Numbers 1–15 refer to dilutional steps, each of a constant factor of 2.153 corresponding to a dilutional factor of one order of magnitude per four lanes.

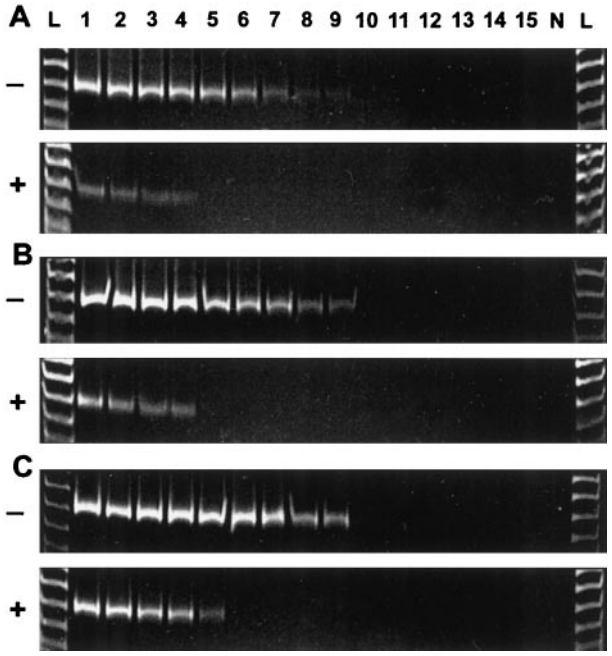

Figure 9. Nested PCR amplification of the dilutional series described in Figure 8. PCR of dilutional series deriving from the hematoxylin-stained section (+) or unstained control section (−) were analyzed by PAGE after 25 (**A**), 27 (**B**), and 29 (**C**) cycles of the second-round PCR. Designation of lanes is the same as described in Figure 8. L and N refer to the length markers and negative controls, respectively. Note that for reasons of comparability some overloading of the (−) samples was necessary.

dilutional series of genomic DNA were then prepared, using in each case a complete serial section, either unstained, for reference purposes, or stained before extraction with hematoxylin according to a routine protocol. This approach was chosen instead of analyzing a multiple of distinct microdissections to avoid deviations in DNA yield, which otherwise would have been unavoidable because of tissue heterogeneity (compare Figure 4). Aliquots of both dilutional series were then subjected to fluorescence quantitation (Figure 8) and nested PCR amplification of an exon 5 sequence of the *p53* tumor suppressor gene (Figure 9 A-C). It is evident that hematoxylin staining leads to a significantly diminished DNA yield, as is clearly demonstrated by the observed correlation of fluorescence and PCR signals (compare Figures 8 and 9, A–C). Fluorimetical quantitation of both undiluted DNA extractions indicated about a 14-fold better yield for the extraction from the unstained section. This roughly correlates with the results of our semiquantitative evaluation of the corresponding PCR data, which exhibits a factor of about 21 between the amount of template DNA obtained from unstained and stained sections. This can be estimated from both the cycle-dependent accumulation of PCR products (Figure 9, A–C) and the approximate end point of amplification (Figure 9 C), if one considers that a difference in the appearance of signals of about four lanes between the two series corresponds to a dilutional factor of 2.153,[4] which is a factor of about 21.

Discussion

Whereas quantitations of extracted DNA samples are generally recommended before a subsequent qualitative PCR analysis to ensure optimal reaction conditions, they are an exclusive precondition for investigations aimed at the absolute quantitation of particular nucleic acids within DNA samples of unknown concentration. The PicoGreen method can be used for high-sensitivity DNA quantitation[22,23] and has been applied to the quantitation of genomic DNA obtained from buccal scrapes preceding a qualitative PCR analysis.[16] In the present paper the PicoGreen quantitation of DNA obtained by microdissection of histological sections before qualitative and quantitative PCR analysis is discussed.

Initially, the sensitivity of the method using the Currie concept,[20] which permits calculation of the sensitivity parameters rather than a mere estimation of sensitivity, was determined. Therefore, technical equipment different from that used in the present investigation can easily be evaluated to determine whether it is of sufficient sensitivity for the described analysis or whether it perhaps performs an even more sensitive analysis. Interestingly, nearly identical values were obtained for the limits of detection L_D (26 and 24 diploid cells) and the limits of quantitative determination L_Q (80 and 73 diploid cells) when we compared the measurements of dilutional series of medium-scale extracted DNA and of DNA extracted from a known number of leukocytes (Figures 1 and 2 and Table 1). For detection of DNA from microdissected cells of unstained renal and cervical cancer sections, comparatively higher values for L_D (39 and 40 diploid cells) and L_Q (119 and 122 diploid cells) were obtained. The simplest explanation for this result is that DNA extraction was incomplete, whereby a decreased sensitivity in terms of higher L_D and L_Q values would be the direct outcome, as theoretical considerations show. In contrast to the measurements of macroscopically extracted DNA or DNA from leukocytes, it becomes obvious that individual microdissections frequently demonstrate significant deviations of fluorescence signals with respect to the regres-

sion lines (Figure 4). This, however, was expected because of the histological heterogeneity of the sections and could be overcome for the sensitivity analysis by increasing the number of microdissections. Most important, our sensitivity analysis of microdissections clearly demonstrates that the sensitivity of DNA quantitation of microdissections is independent of the type of tissue subjected to microdissection. So nearly identical results were obtained for the micropreparations from renal cell cancer and cervical cancer sections (Table 1), though both regression lines, which characterize the relationship between DNA-dependent fluorescence signal and microdissected volume, obviously exhibit differing slopes (Figure 4). Consequently, our experiments show that precise quantitations of as little as about 120 diploid genomes from microdissections, independent of the type of tissue, are feasible for a standard fluorimeter. Even if one takes into account that the overall sensitivity is decreased, depending on the amount of DNA and the number of necessary repetitions that are required for each analysis, this sensitivity should be sufficient for the majority of qualitative and quantitative PCR analyses of microdissected cells.

In addition, we have performed PicoGreen quantitations to measure the efficiency of DNA extractions from tissue sections after formalin fixation and different staining procedures. Interestingly, we have observed that short-term fixation using formalin (Figure 5), three hematoxylin staining procedures (Figure 7), and even a temporary drying of fresh-frozen sections (data not shown) significantly impair the yield of microextraction. Regarding the tissue fixation by formalin, it is known that denaturation and modification of macromolecules by formalin (eg, alkylating and cross-linking of functional groups) leads to an insolubilization of the macromolecular network,[24] thereby minimizing the loss of nucleic acids from fixed tissues.[25] On the other hand, the solubilization of DNA from formalin-fixed specimens is negatively correlated with the duration of formalin treatment,[12–14] and the yield of DNA extractions may be seriously reduced when compared to an unfixed specimen.[26] As a result DNA extraction from tissues after various fixation procedures requires prolonged or modified proteinase K digestion.[15,26,27] Hence, our finding that even short-term treatment of sections with formalin causes a significantly decreased DNA solubility agrees well with the results described above. Moreover, in view of the fact that duration of fixation and tissue-specific factors might individually affect the efficiency of DNA extraction[12] and that less reliable results have been obtained when DNA from microdissected formalin-fixed sections has been used,[2] a control of both quality and quantity of microextracted DNA appears to be recommended before routine qualitative PCR analyses are performed. Whereas DNA quality is amenable by PCR amplification of control amplicons of varying length,[12] so far the DNA quantity of each microdissected sample could not be assayed. Thus PicoGreen quantitation could provide the basis for more reliable results, particularly if "problematic" specimens with an unknown history of fixation have to be investigated.

Interestingly, not only fixation but also different hematoxylin stainings of sections, such as by Delafield's, Mayer's alum hematoxylin, and Weigert's iron hematoxylin, demonstrated significantly diminished yields of DNA extraction when compared to the unstained slides (Figure 7). These results could not be explained by direct interference of the histological dyes with the fluorimetrical measurements. Rather, further investigations revealed that the respective staining formulas cause formation of insoluble precipitates consisting of the nucleic acids and the respective histological dye (Serth et al, manuscript in preparation). The main question arising from these findings is whether this effect is of any consequence for PCR analysis of DNA extracted from histochemically stained tissue sections. On the one hand it is evident that any kind of absolute quantitative analysis requires precise quantitation of the amount of extracted DNA, a quantitation that obviously cannot be carried out by counting microdissected cells from an intensively hematoxylin-stained tissue section. On the other hand, intensive hematoxylin staining according to routine protocols of sections before microdissection and DNA extraction obviously may also affect qualitative PCR analyses, as demonstrated by the seminested amplification of a *p53* tumor suppressor gene sequence that shows that the PCR efficiency of amplifying DNA from hematoxylin-stained sections is significantly diminished (Figure 9, A–C). Taking into account that the efficiency of these PCRs correlates well with the corresponding fluorescence analyses (compare Figures 8 and 9), we conclude that the observed hematoxylin effect is largely due to the diminished yield of the initial DNA extraction rather than to an inhibition of PCR, such as by possibly soluble hematoxylin-DNA complexes. Moreover, from a theoretical point of view, a potential interference that is beyond the mere aspect of DNA quantity cannot be ruled out so far when analyzing hematoxylin-stained microdissections by PCR, even if they are corrected for decreased amounts of template DNA. Because of the possibility that any differences in intracellular hematoxylin concentration during the staining process (eg, due to differing cellular permeabilities) could give rise to a bias in DNA extractions, a bias of PCR results could also occur. Therefore, a comparison of microdissected cell numbers as visually estimated during microscopy and cell numbers as determined by fluorescence measurements of extracted DNA could provide additional information for the pathologist as to whether PCR signals generated are representative.

However, as indicated by a comparison of DNA yields obtained from either less intensive or regularly stained sections (Figures 6 and 7), in practice, fluorescence quantitation of DNA before PCR analysis provides an efficient way of identifying the staining intensity needed on the one hand for precise microdissection and on the other hand for an efficient, unbiased recovery of DNA.

In view of the fact that genetic analysis of cells isolated by microdissection is of increasing importance, our results clearly demonstrate that a precise control of the extraction efficiency has to be strongly recommended, particularly if a tissue section has undergone steps of routine histological fixation or staining before molecular

pathological analysis. In conclusion, quantitation of microextracted DNA templates offers both an efficient and accurate way to ensure optimal reaction conditions for qualitative PCR analysis of DNA from microdissections and provides the basis for subsequent absolute quantitations of target sequences within histopathologically defined cells.

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