

# Ultrasonic Decalcification Offers New Perspectives for Rapid FISH, DNA, and RT-PCR Analysis in Bone Marrow Trephines

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**Abstract:** The requisite analyses on bone marrow biopsies are increasing: Molecular analyses such as fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), and reverse transcriptase (RT)-PCR are demanded in addition to morphology and immunohistochemistry to improve diagnostic accuracy. Moreover, analysis of certain molecular prognostic or predictive biomarkers is increasingly mandatory in the assessment of hematologic diseases. In some circumstances, only formalin fixed, bone-containing tissue is available for molecular analysis. Because various fixation and decalcification procedures can impair DNA and RNA quality, there is an urgent need for standardized decalcification protocols which allow FISH and PCR analysis. In this study we developed a routinely applicable decalcification protocol to optimize the molecular analysis method although preserving morphology and immunohistochemical results. Therefore, we compared 2 different approaches including ultrasonic decalcification versus nonultrasonic procedures and ethylenediaminetetraacetate-based reagents versus acid-based ones. In our hands, the combined use of ultrasound and ethylenediaminetetraacetate-based reagents permits successful interphase FISH, PCR, and RT-PCR analysis whereas concomitantly preserving morphology and antigenicity.

**Key Words:** bone marrow trephine, decalcification, ultrasound, molecular analysis, FISH, PCR

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Pathologic examination of bone marrow trephines is of utmost importance for the classification and staging of hematologic disorders or for the diagnosis of metastatic disease.<sup>4,5,7</sup> Besides morphology and immunophenotyping, molecular analysis in bone marrow became a standard practice in many settings.<sup>2–4,8</sup> In the current

World Health Organization classification, they are integral part of the classification of many hematologic neoplasms.<sup>7</sup> Moreover, there is increasing evidence, that molecular alterations such as for example, chromosomal translocations show prognostic and predictive significance and hence are considered for planning of individual therapeutical strategies.<sup>1,6,11</sup>

In the last decades, immunophenotypic and particularly molecular analysis on bone marrow trephines were hampered by impaired DNA and RNA quality. This resulted mainly from decalcification procedures in acidic compounds, a prerequisite for proper sectioning. It is well known, that decalcifying reagents like formic or hydrochloric acid have negative effects on antigenicity of proteins and integrity of nucleic acids.<sup>12,13</sup> To overcome these technical restrictions, the aim of our study was to establish a routinely applicable and standardized protocol for the processing of bone marrow trephines which fulfills the requirements for contemporary bone marrow diagnostics.

## MATERIALS AND METHODS

### Tissue Samples

This study was performed in compliance with regulations decreed by the local ethics committee. To obtain sufficient material for comparable standards, postmortem bone marrow trephines were performed from the vertebral body of 3 patients. In total, 25 specimens were investigated. Biopsies of an average length of 1 cm were taken by a “Jamshidi Crown Bone Marrow Biopsy Needle” (Cardinal Health, OH) and fixed in 4% buffered formalin for 12, 24, 48 hours and 1 week. Sixteen samples from the same patient (patient 1) fixed for 24 and 48 hours were subjected to the various decalcification methods (Table 1). In addition, we fixed 1 sample from patient 1 for 1 week for DNA analysis. The sample of patient 2 was fixed for 24 hours and subjected for 2 hours to ultrasonic decalcification (Table 1). To confirm the minimal fixation time, we fixed 1 specimen for 12 hours. Six samples (patient 3) fixed in 4% buffered formalin for 3 weeks were used to assess the optimal decalcification time for both methods and for DNA evaluation.

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**TABLE 1.** Eight Trephine Biopsies per Fixation Time (24 and 48 h) From Patient 1 Where Subjected to the Following Decalcification Protocols

	Time of Ultrasonic Decalcification in Hours (h)		Time of Decalcification Without Ultrasonic Treatment (h)
USEDECALC	2*	3	2
EDTA 10%	2*	3	2
USERAPID	nd	nd	2
Nitric acid 5%	nd	nd	2

\*Treatments were repeated for patient 2 (fixation time 24 h).  
nd indicates not done.

**Methods**

Two different decalcification approaches were compared (Figs. 1A, B): (1) A commercially available ultrasonic decalcifier (USE33, Medite, Burgdorf, Germany) was used in the first approach. This is an ultrasonic decalcifying automate with an output of 200 W ultrasonic power with an integrated temperature controller and a programmable timer (USE33, Medite, Burgdorf, Germany). Fixed trephines were placed in the ultrasonic decalcifier for 2 to 3 hours (Fig. 1A), during which the biopsies were submerged either in a commercially available ethylene diaminetetraacetate (EDTA)-containing reactant USEDECALC (Medite, Burgdorf, Germany), or in a 10% EDTA solution (pH 7.4). Thereafter, the samples were dehydrated and embedded into paraffin. (2) A direct decalcification step of paraffin-embedded tissue without using an ultrasonic decalcifier was tested in the second approach. Therefore, the trephines were first dehydrated and then paraffin embedded, before the decalcification procedure. For the subsequent decalcification step of these paraffin blocks,

we compared 4 different reactants either on a EDTA or an acid base (Fig. 1B) using: (a) USEDECALC (Medite, Burgdorf, Germany), (b) the 10% EDTA solution, (c) the acid containing USERAPID (Medite, Burgdorf, Germany), and (d) 5% nitric acid. For this second approach, paraffin blocks were immersed in the respective solution for 2 hours.

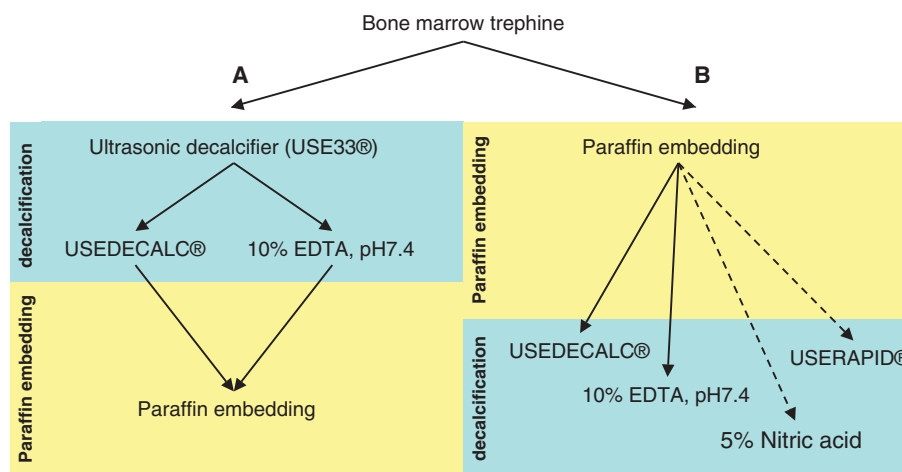
**Immunohistochemistry**

Immunohistochemical staining was carried out on an automated stainer (Ventana Medical System, Tucson, AZ). Antibodies included anti-CD20, CD3, GlycophorinA, and Mib1 (Dako, Glostrup, Denmark).

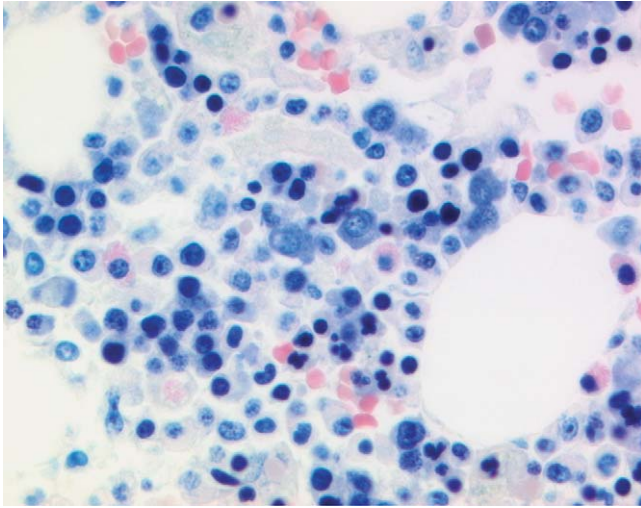
**Molecular Analysis**

For fluorescence in situ hybridization (FISH), a double color, double fusion probe specific for the translocation t(11;14) (LSI IGH/CCND1 XT, Abbott/Vysis Molecular Diagnostics, IL) was used according to the manufacturer’s instructions with minor modifications. These included tissue preincubation with 30 mg of pepsin (Sigma Buchs, Switzerland) at 37°C for 25 minutes. Two signals split by a distance of more than one signal diameter were considered to be germline. We set the cut-off level for translocation (fusion signals) at 9.4% (mean ± 3 SD) in negative control tissues (tonsils) without hematologic disease. FISH signals of at least 100 nuclei were evaluated for each trephine, using a fluorescence microscope (Olympus BX61) with filters 4’,6-Diamidino-2-phenylindole, Spectrum Orange and Spectrum Green. The images were acquired and stored by a computed picture-analysis system (AnalySIS<sup>D</sup>, Muenster, Germany).

For the extraction of DNA and RNA from paraffin-embedded tissue, between five and eight 10-µm thick sections were suspended in extraction buffer and incubated for 10 minutes at 95°C. Extraction buffers were as



**FIGURE 1.** Schematic representation of the different decalcification methods applied in the current study: A, Ultrasonic decalcification in EDTA-based liquids followed by paraffin embedding. B, Paraffin embedding before decalcification in either EDTA-based solutions (blackened arrows) or acid-based solutions (dotted arrows).



**FIGURE 2.** Giemsa stain showing morphologic details of a trephine decalcified by ultrasound and a 10% EDTA solution.

follows, for DNA: 50 mM Tris/1 mM EDTA/0.5% Tween 20; and for RNA: 20 mM Tris, pH 7.5/20 mM EDTA/1% sodium dodecyl sulfate. Centrifugation for 15 minutes with decreasing temperature to 4°C resulted in a solid paraffin layer on the top of the solution, which was removed with a sterile pipette tip. Digestion was performed for 24 hours (DNA) or 48 hours (RNA) at 55°C by the addition of 0.3 µg/µL of proteinase K (Roche Diagnostics, Basel, Switzerland). The crude DNA extract was used directly as polymerase chain reaction (PCR) template, whereas the RNA extract was additionally purified using Trizol LS reagent (Invitrogen, Basel, Switzerland). PCR amplification with versican specific primers was carried out using the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Rotkreuz, Switzerland) at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for a total of 40 cycles. The OneStep reverse transcriptase- (RT-PCR) Kit (Qiagen) was used for reverse transcription (30 min at 50°C) and PCR amplification (1 min at 95°C, 1 min at 57°C, and 1 min at 72°C for 40 cycles) of  $\beta$ -actin mRNA. Primer sequences are

available on request. Both PCR and RT-PCR products were analyzed by gel electrophoresis (2% MetaPhor agarose gel, Cambrex, Rockville) and visualized under ultraviolet-light after staining with ethidium bromide.

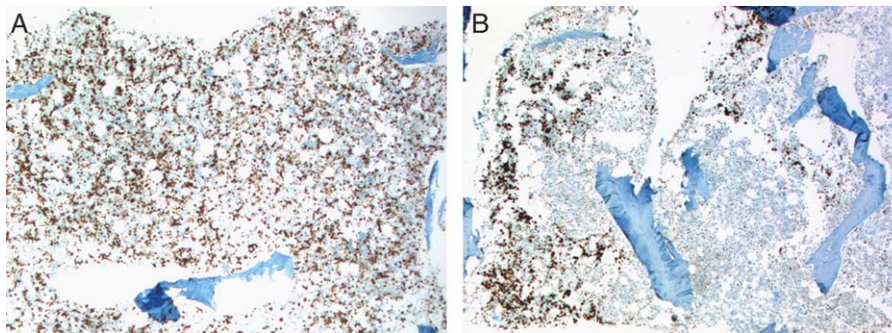
## RESULTS

All methods were compared regarding preservation of morphology, maintenance of antibody reactivity and ability to perform molecular analyses. Altogether, microscopic examination revealed a good morphology with all the decalcification procedures applied with only negligible differences among them (Fig. 2). In our hands, the main requisite to obtain a good morphology is a fixation time of 24 hours. Moreover, an ultrasonic-based decalcification approach allowed easily to cut several serial sections after a 2-hour decalcification period. This was advantageous to the second decalcification approach, where additional decalcification steps frequently became necessary after the initial sections were cut.

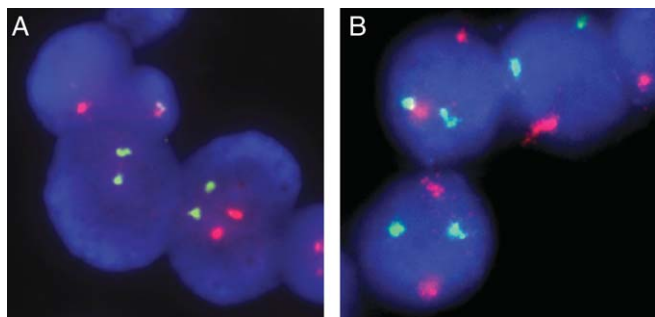
Whereas morphology was not much influenced by different decalcification procedures, antigen preservation was impaired in acid-decalcified samples. By immunohistochemistry we observed weaker and inhomogenous staining intensities. The difference was less striking in cytoplasmic antigens (CD20, CD3, and GlycophorinA), than in nuclear antigens (Mib1). Nuclear Mib1 staining was strong and uniform after decalcification by EDTA (Fig. 3A), whereas the staining pattern was weaker and patchy in the acid-decalcified samples (Fig. 3B).

FISH signals were similar in intensity and frequency in the ultrasonic-decalcified trephines compared with those in noncalcified control tissue (Fig. 4). A higher background and fewer signals were observed in samples, subjected to an acid-decalcification treatment.

For PCR, we were able to amplify DNA fragments from 290 to 590 bp (Fig. 5). The yield of longer DNA fragments was influenced by the fixation time (1) and the mode of decalcification (2): (1) after a fixation period of more than 1 week we observed a decline in the yield of longer DNA amplicates. Moreover, after a fixation time of 3 weeks, we were unable to amplify any DNA at all, (2) after a minimal decalcification period of 2 hours in nitric



**FIGURE 3.** A, Homogenous Mib1 staining in an EDTA-decalcified sample. B, Heterogenous Mib1 staining in an acid-decalcified sample of the same patient.



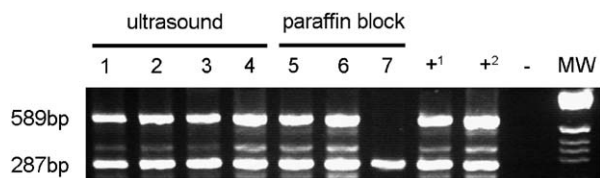
**FIGURE 4.** Comparison of FISH-analysis for translocation t(11;14) on decalcified bone marrow trephines (A) and formalin-fixed lymph node (B). Germ line configuration with 2 green dots for the IgH locus on chromosome 14 and 2 red dots for the CCND1 locus on chromosome 11.

acid, we were able to amplify short DNA fragments of 290 bp. In contrast, DNA fragments of 590 bp were obtained in samples decalcified on an EDTA base, but not in the acid-decalcified ones. This indicates that the acid pretreatment compromised DNA integrity. These differences were independent whether tissue was decalcified by ultrasound or on the paraffin block.

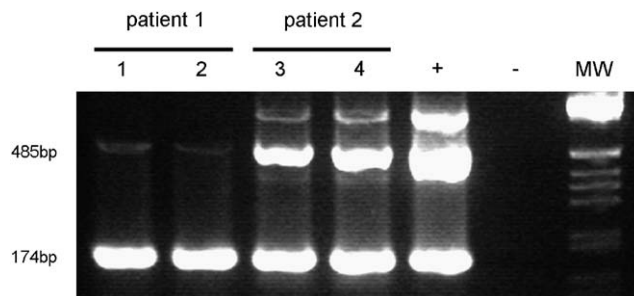
Further, we were able to reverse transcribe and PCR amplify RNA fragments for  $\beta$ -actin of 174 bp in EDTA and acid-decalcified samples. However, longer fragments of 485 bp could only be amplified in specimens decalcified by ultrasound in USEDCALC and 10% EDTA, but not in those decalcified on an acid base (USERAPID or 5% nitric acid) (Fig. 6).

### CONCLUSIONS

Molecular analysis of bone marrow trephines is frequently hampered by impairment of DNA quality owing to decalcification procedures.<sup>9</sup> Strong decalcifiers such as nitric or formic acid allow rapid decalcification, but lead to degradation of DNA, fragmentation of RNA,



**FIGURE 5.** Influence of decalcification-period, -method and -solution: PCR products of DNA amplification for Versican. Each sample was fixed for 24 hours, and the same amount of DNA was employed in the PCR: (1) USEDCALC, ultrasound treatment (UT) for 2 hours (h); (2) 10% EDTA, UT for 2 hours; (3) USEDCALC, UT for 3 hours; (4) 10% EDTA, UT for 3 hours, (5) decalcification for 2 hours in USEDCALC without UT; (6) decalcification for 2 hours in 10% EDTA without UT; and (7) decalcification for 2 hours in 5% nitric acid without UT.<sup>+1</sup> DNA from paraffin-embedded tissue as PCR positive control. <sup>+2</sup> DNA from fresh-frozen tissue as PCR-positive control. – water as negative control.



**FIGURE 6.** Samples of 2 patients (patient 1: No. 1 and 2; patient 2: No. 3 and 4) fixed in 4% formalin for 24 hours and decalcified by ultrasound and EDTA-based solutions showing variable amounts of amplified RNA for  $\beta$ -actin. No. 1 and 3 = USEDCALC; No. 2 and 4 = 10% EDTA. RNA from paraffin-embedded tissue as PCR positive control. – water as negative control.

and to deterioration of stainability, particularly of nuclear chromatin.<sup>3,14</sup> Although, decalcification by a chelating agent such as pH-neutral EDTA circumvents the negative effects of acids on DNA and RNA quality, it has the disadvantage of being slow-acting, requiring a decalcification time of up to 2 days. Oscillation in form of ultrasound, accelerates the penetration of chelating agents into bone tissue, leading to a rapid destruction of crystalline structures like calcium phosphate, magnesium phosphate, and calcium carbonate.<sup>10</sup>

In our hands, fixation in buffered formalin, followed by an EDTA-based ultrasonic decalcification, significantly accelerates the processing of bone marrow biopsies. The exclusion of strong acidic compounds and the shortened incubation time in an EDTA solution conserves DNA quality, enabling interphase FISH analysis, and amplification of long DNA and RNA fragments while preserving morphology and antigens for immunohistochemistry. According to our experience in the daily routine work, the ultrasound decalcification procedure of bone marrow trephines allows an excellent integrative diagnostics of hematopoietic disorders involving bone marrow. Furthermore, it provides perspectives for an extended application in surgical pathology.

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