# On Slide Extraction of Biomolecules from FFPE Tissue Specimens

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## ABSTRACT

Almost all current methods for molecule extraction from tissue specimens require tissue homogenization. We have developed a simple, on-slide, non-destructive molecule extraction (NDME) technology to extract proteins and nucleic acids directly from tissue sections on microscopic slides. The NDME technology eliminates the need of scraping tissue samples off the slides, a procedure that is tedious and time-consuming and may lead to contaminations. The NDME system consists of an incubation device for temperature control and exertion of physical forces during incubation, snap-on slide chambers for applying a small volume of extraction buffer on tissue sections, and an extraction buffer set. In the NDME procedure, only the upper surface of the tissue section is exposed to the extraction buffer, so biomolecules are extracted controllably layer by layer into the extraction buffer. We have demonstrated that by changing extraction conditions (e.g. buffer stringency, physical force, and incubation time) the NDME procedure can achieve two distinct results depending on the experimental needs: 1) a controllable partial extraction of biomolecules while keeping the tissue morphology intact for histopathology studies; 2) a complete molecule extraction while the tissue morphology is abolished. We showed that NDME produced better extraction efficiency than conventional FFPE tissue extraction methods and that proteins and nucleic acids extracted are of good quality and suitable for most downstream molecular assays.

## INTRODUCTION

With the advent of personalized medicine, obtaining proteins and nucleic acids in soluble form from tissue specimens will be increasingly required for proteomic and genomic analyses for cancer diagnostic and prognostic purposes.

Pathological diagnosis is made possible due to tissue preservation techniques. Formalin (10% buffered formaldehyde) fixation followed by paraffin embedding (FFPE) has been a standard procedure used in over 90% of clinical tissue treatment. Formalin preserves tissue morphology by cross-linking proteins and nucleic acids causing them insoluble under physiological conditions.

Currently clinical molecular assays are severely hampered for FFPE tissue specimens because cross-linked biomolecules in FFPE tissue specimens are difficult to be extracted. However, due to the widespread use of FFPE tissues, any application of molecular pathological technologies should be in compliance with FFPE tissue specimens in order to gain clinical acceptance.

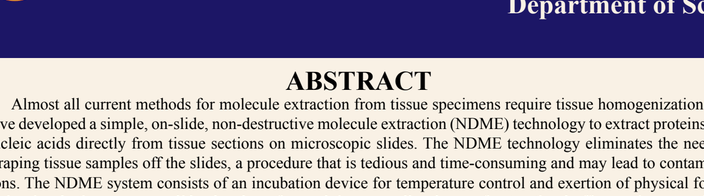
The first problem encountered when applying proteomic and genomic study to FFPE tissues is how to release cross-linked proteins and nucleic acids from fixed state into soluble form in buffer solutions. In theory, extraction of a specific biomolecule type from tissue section is a process of reaching equilibrium between the molecules being released to extraction buffer and those remaining in the tissue section.

The existing protocols for molecule extraction from FFPE tissues at least have the following 3 limitations: 1) they need long processing time, especially for nucleic acids extraction; 2) they need tissue homogenization or scraping tissue sections off slides; 3) they need relatively large amount of tissues.

We have developed the on-slide NDME technique to address the above limitations of conventional tissue extraction methods. The NDME technology provides a method to achieve control of molecule extraction from tissue section. Harsher extraction buffer and prolonged heat incubation time (45 min to 2 hours) is usually required for complete molecule extraction and the tissue section is destroyed.

Controlled partial NDME under mild conditions can simultaneously extracts molecules and achieve antigen retrieval (AR) or pretreatment for IHC or ISH on a single tissue slide section. Using the right buffer conditions, the NDME extracts can be made compatible with downstream detection platforms, such as PCR, RT-PCR, Western blot, protein array, and various high throughput multiplex assays without further purification.

NDME makes it possible for molecular fractionation to be performed to cells in tissue sections and cytology smears.




### Protein Extraction and Tissue Morphology Preservation

The amount of extracted proteins increased with increases in extraction time. Longer extraction may dissolve too much structural proteins, causing loss of tissue morphologic details. As depicted in Fig. 1A, IHC staining against CD5 on consecutive FFPE tissue sections after increasing length of extractions was compared. Without extraction, no IHC signal appeared as expected due to antigen masking in FFPE tissues. IHC signal and detailed antigen location were more clearly observed with increasing extraction time. Proteins extracted at each time point were analyzed on a SDS PAGE (Fig. 1B). As expected, protein yields increased gradually with increasing extraction time. Proteins of low MW appeared to be extracted earlier than the proteins of High MW.

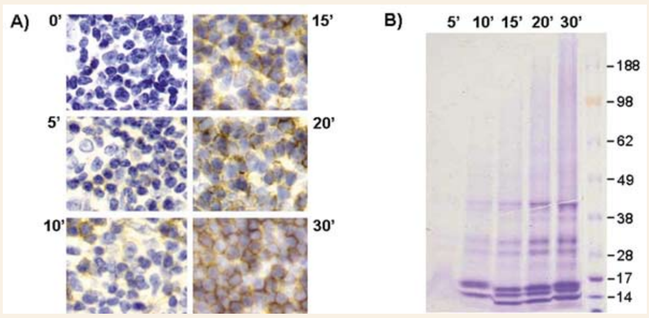



**Figure 1.** A time course study of extracted proteins and tissue morphology after NDME treatment with Buffer B for 5, 10, 15, 20, and 30 minutes at 100° C on FFPE lymph node tissue specimen. A) IHC staining of tissue sections after NDME treatment with anti-CD5 antibody. B) SDS-PAGE analysis of proteins extracted by NDME from tissue sections in Panel A.

### Nucleic Acid Extraction and Tissue Morphology Preservation

DNA and RNA of good quality and integrity were extracted by Buffer X after 30 min NDME and the extracts could be directly amplified by PCR and RT-PCR (Fig. 2A, 2C) without further purification. Extract from a single section of 30-year-old archived FFPE retinal tissue could generate RT-PCR amplicons of 367 bp in 4 out of 6 reactions (Fig. 2A). For DNA, PCR products of 1,309 bp could be obtained from the tissue extract (Fig. 2C). Furthermore, tissue sections after NDME could be used for on-slide nucleic acid hybridization, such as ISH (BioGenex) and CISH (Zymed) (Figs. 2B and 2D). There was no detectable difference in hybridization pattern and intensity before and after NDME treatment, except a decrease in counter-staining intensity following NDME.

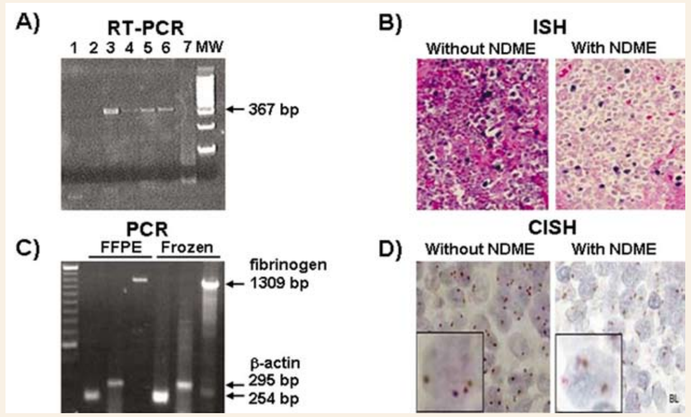



**Figure 2.** A) RNA extracted by NDME from 6 cases (2-7, #1=water control) of 30-year FFPE retinal sections, generating RT-PCR amplicons of 367 bp of β-actin gene. M=100bp DNA ladder. B) RNA-ISH of consecutive sections of lymph node with infectious mononucleosis. Blue signals show Epstein-Barr virus early RNA (EBER) hybridization. C) PCR of NDME extracts from FFPE and frozen tissue sections generated DNA of up to 1,309 bp. D) CISH detection of the c-Myc translocation in Burkitt's lymphoma tissue sections with/without NDME. Inset showed translocation in one cell. The c-Myc translocation was obvious in Burkitt's lymphoma tissue sections before and after NDME.

### HER2 Extraction and IHC

A FFPE breast tissue section with 3+ HER2 overexpression was extracted by controlled partial NDME. The extract was subjected to SDS PAGE and Western blot and the treated tissue sections on slides were stained by routine HER-2 IHC assay (HercepTest™, DAKO). The 180-kDa HER2 protein could be detected by Western blot (Figure 3A). The IHC staining result for the tissue section treated with NDME as antigen retrieval was indistinguishable from the one using the routine AR by the standard protocol (Figure 3B). Although more studies need to be done, this experiment supports our suggestion that NDME can replace routine AR or pretreatment in important clinical histopathology assays.

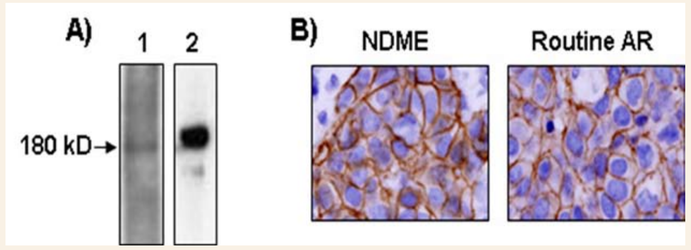



**Figure 3.** A) Western Blots for HER2 protein extracted by a 30-min NDME. Lanes 1 and 2 each represents the HER2 protein from a breast cancer tissue section with a score of 3+ and from the cell line T47D serving as a positive control. B) IHC staining for HER2 protein on the same section after NDME and another section treated by routine AR.

### NDME Extracts Analyzed by Reverse Phase Protein Arrays

Proteins extracted from various tissues by NDME procedure were sequentially diluted by 2 folds with Buffer B and applied onto nitrocellulose-coated glass slides to make reverse-phase protein lysate microarrays. Proteins were readily extracted from ethanol- and formalin-fixed tissues as well as from frozen tissue by NDME. While the expression level of common keratin proteins did not vary very much in various tissue types, higher amount of prostate-specific proteins PSA (prostate-specific antigen) and PAP (prostatic acid phosphatase) were expressed only in all 3 prostate tissues (No. 8-No. 10).

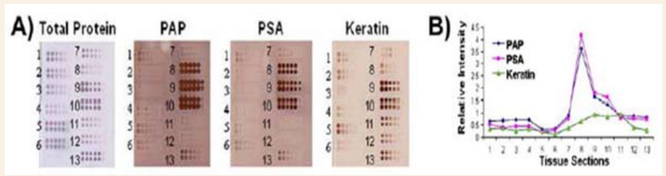



**Figure 4.** Reverse phase protein microarray for NDME extracts. A) Microarray analysis of NDME extracted proteins from frozen (1), ethanol-fixed (3), and formalin-fixed (the rest) tissue sections. Specific proteins expressed to different levels in various tissues. 1=frozen lymph node (LN), 2=LN, 3=ethanol-fixed LN, 4-6=kidney, 7=spleen, 8-10=prostate, 11-12=breast, 13=lung. B) Relative protein amount, Average intensity of 12 sample dots adjusted by that of total proteins.

### NDME is More Efficient than Conventional Protein Extraction Method

We compared our modified Buffer B with the Qproteome™ buffer using both the on-slide and scraping procedures. We saw a 2-3 fold higher signal intensity for extracts using our buffer than that by the Qproteome buffer (Figure 5 A). For both buffers, a 45-min incubation by NDME already reached maximum extraction (lanes 1 and 4) since it produced same intensities as by 2 hr incubation with NDME (lanes 2 and 5) and scraping methods (lanes 3 and 6). The tissue/cell morphology was completely destroyed by the 2 hr NDME with Buffer B, only residual connective tissues left on slide (Figure 5 B). There was cell morphology left on slide after the 2 hr NDME with the Qproteome Buffer (Figure 5 B).

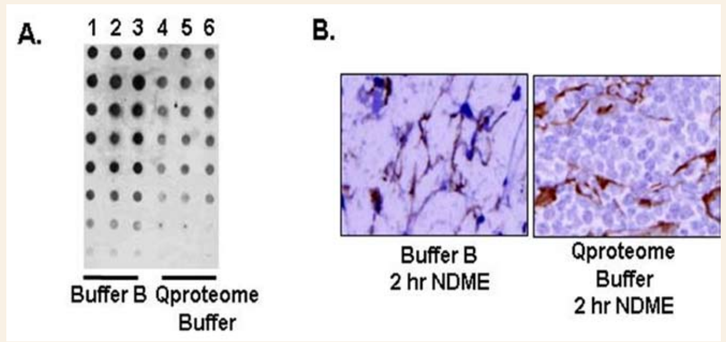



**Figure 5.** Comparison of extensive extractions between NDME and conventional FFPE tissue extraction methods. A) Dot blot analysis at 2-fold serial dilutions of tissue extracts from tonsil tissue sections by NDME (columns 1, 2, 4, and 5) and the conventional method by scraping tissue sections off slides (columns 3 and 6). Columns 1, 4 were derived from extracts by 45 min NDME. Columns 2, 5 were derived from extracts by 2 hr NDME. Columns 3 and 6 were derived from extracts by scraping method and 2 hr incubation at 100° C. B) IHC staining on tissue section after the 2 hr NDME. Buffers used in the experiment are listed below the corresponding picture. Primary antibody used in both dot blot and IHC was anti-human Phospho-p42/44 antibody.

### NDME is More Efficient than Conventional RNA Extraction Method

Two parallel extensive extraction procedures using the NDME/Buffer X and the RecoverALL™ Total Nucleic Acid Isolation Kit for FFPE (Ambion) were performed separately. In the Ambion approach, tissue sections were scraped off slide and subjected to multiple steps of incubation and a final purification column. In the NDME, we used two incubation steps and no further purification of the final extracts was done before RT-PCR reaction. As determined by 3'/5' ratio of β-actin real time RT-PCR assay, all RNA samples were of good quality. Figure 6 shows the real-time RT-PCR results from the NDME/Buffer X extracts. Higher initial RNA copy numbers were obtained by the NDME approach than by the Ambion kit (data not shown).

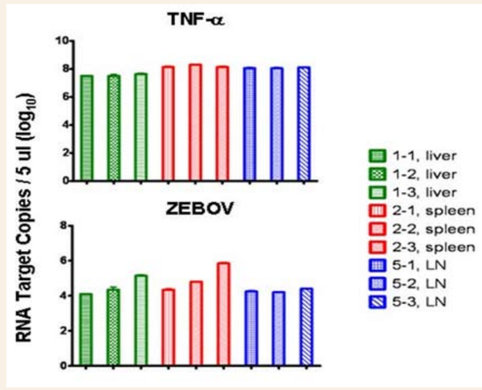



**Figure 6.** Real-time RT-PCR on extracts prepared by 30 min NDME with Buffer X on monkey tissue sections. The monkey tissues were fixed for 3 weeks in formalin.

## SUMMARY

1. It is practiced for almost a century in modern pathology that a tissue specimen is sectioned and put on glass slides for microscopic examinations. Tissue sections are generally cut to 3-5 microns in thickness which is about a quarter to a half of the diameter of an average mammalian cell. All cells in a tissue section on slide are cut open and laid out in a single layer.
2. Although it is common to place a small volume of solution on a tissue section on slide for binding interactions, the seemingly obvious concept of extracting biomolecules on-slide is novel since all existing tissue extraction protocols resort to homogenization or scraping tissue sections off slides for incubation.
3. We assume that extracting directly from tissue sections on slides is more efficient than homogenizing tissue chunks or scraping tissue sections off slides since the latter two approaches lead to clumping of cell debris and reduce surface area exposed to the extraction buffer. We have developed the on-slide non-destructive molecule extraction (NDME) technology based on this assumption.
4. In the NDME procedures, biomolecules are released controllably layer by layer from the upper surface of the tissue section into the extraction buffer while the lower surface is protected by attachment to the slide.
5. We have demonstrated that NDME extracted high quantity and wide spectrum of macromolecules suitable for various downstream molecular analyses from both FFPE and frozen tissue sections. The tissue sections after controlled partial NDME extraction can be used for histological, immunostaining, and in situ hybridization analyses.