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# Extraction of both RNA and DNA from formalin-fixed paraffin embedded tissue cores

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| **Abstract:** | Formalin-fixed paraffin embedded tissue (FFPET) represents a valuable, well-annotated substrate for molecular investigations. The utility of FFPET in molecular analysis is complicated both by heterogeneous tissue composition and low yields when extracting nucleic acids. A literature search revealed a paucity of protocols addressing these issues, and none that showed a validated method for simultaneous extraction of RNA and DNA from regions of interest in FFPET. This method addresses both issues. Tissue specificity was achieved by mapping cancer areas of interest on microscope slides and transferring annotations onto FFPET blocks. Tissue cores were harvested |

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| | from areas of interest using 0.6 mm microarray punches. Nucleic acid extraction was performed using a commercial FFPET extraction system, with modifications to homogenization, deparaffinization, and Proteinase K digestion steps to improve tissue digestion and increase nucleic acid yields. The modified protocol yields sufficient quantity and quality of nucleic acids for use in a number of downstream analyses, including a multi-analyte gene expression platform, as well as reverse transcriptase coupled real time PCR analysis of mRNA expression, and methylation-specific PCR (MSP) analysis of DNA methylation. |
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**TITLE:**
Extraction of both RNA and DNA from formalin-fixed paraffin embedded tissue cores

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**SHORT ABSTRACT:**
This modified extraction protocol improves RNA and DNA yields from more precisely targeted regions of interest in histopathologic tissue blocks.

**LONG ABSTRACT:**
Formalin-fixed paraffin embedded tissue (FFPET) represents a valuable, well-annotated substrate for molecular investigations. The utility of FFPET in molecular analysis is complicated both by heterogeneous tissue composition and low yields when extracting nucleic acids. A literature search revealed a paucity of protocols addressing these issues, and none that showed a validated method for simultaneous extraction of RNA and DNA from regions of interest in FFPET. This method addresses both issues. Tissue specificity was achieved by mapping cancer areas of interest on microscope slides and transferring annotations onto FFPET blocks. Tissue cores were harvested from areas of interest using 0.6 mm microarray punches. Nucleic acid extraction was performed using a commercial FFPET extraction system, with modifications to homogenization, deparaffinization, and Proteinase K digestion steps to improve tissue digestion and increase nucleic acid yields. The modified protocol yields sufficient quantity and quality of nucleic acids for use in a number of downstream analyses, including a multi-analyte gene expression platform, as well as reverse transcriptase coupled real time PCR analysis of mRNA expression, and methylation-specific PCR (MSP) analysis of DNA methylation.

**INTRODUCTION:**
Genomic biomarker research seeks to identify molecular correlates that accurately and reliably reflect disease status, and do so in a clinically useful manner.[1] Biomarker development is reliant on retrospective analysis of well-annotated tissue samples. Diseased and normal tissue samples are stored either as fresh-frozen tissue in specialized biobanks or as formalin-fixed paraffin-embedded tissue (FFPET) blocks in clinical archives. Fresh-frozen tissue allows for the extraction of high-quality nucleic acids and has been widely used in genomic biomarker discovery studies.[2,3] However, fewer tissue samples are available in biobanks and studying such tissue introduces a bias towards larger samples, unusual categories of disease, and patients seen at specialized centers with greater abilities to bank tissue.[4] FFPET, in contrast, is the default storage method for diseased human and animal tissues. While FFPET blocks maintain cellular morphology, the fixation process cross-links other cellular constituents to nucleic acids. Cross-linked RNA and DNA are recoverable, but only in degraded, highly fragmented forms.[5,6] However, these DNA and RNA fragments are amenable to analysis by an expanding array of assays, including mRNA expression, DNA hypermethylation, and targeted sequencing.[7,8] To exploit this opportunity in the large quantity and variety of FFPET available for research, there is a need for an efficient and reliable extraction protocol.

A large proportion of biomarker research in tissue focuses on cancer. Like other types of diseased tissue, cancer tissue often shows significant regional heterogeneity in cell preservation and cell type. Since biomarker research relies on the ability to correlate constituents of diseased tissue with molecular features, a critical step of this process is the precise harvesting of tissue that is well preserved and enriched for the disease under study. In FFPET, two enrichment techniques are often utilized: laser capture microdissection (LCM), and microtome sectioning. LCM enables highly focused tissue harvesting and can be used to isolate specific, well-preserved cell types in heterogeneous tissues.[9,10] However, LCM requires expensive equipment and is prohibitively time consuming for large numbers of samples. Microtome sectioning is a more widely-used process where thin sections are cut from FFPET blocks.[11,12] Microtome-cut sections often include tissue that is heterogeneous in cell preservation (e.g., necrotic vs. well-preserved) and composition (e.g., cancer vs. benign parenchyma), and hence may lead to the homogenization of molecular features best investigated separately. Thus, there is a need for a high throughput method that enriches for cells of interest. A third method, isolation of nucleic acids from FFPET cores, provides this enrichment, is suitable for high throughput protocols, and has been used by others to isolate RNA or DNA from separate tissue cores.[7,13,14]

A number of published protocols specify methods of extracting nucleic acids from FFPET (Table 1). However, protocols where RNA and DNA are extracted from the same tissue have been optimized for microtome tissue sections, but not for tissue cores.[15,16] Similarly, published protocols which offer increased tissue specificity, either through tissue cores or slide microdissections, specify procedures for extraction of DNA, but not RNA.[7,17] Here, an optimized protocol for dual extraction of both DNA and RNA from the same tissue core is demonstrated. Tissue cores are harvested by inserting tissue microarray (TMA) punches into regions of interest mapped onto FFPET blocks. The mapping is performed by annotating a microscope slide with a marker pen and transferring the annotation to the surface of the corresponding FFPET block (Figure 1).

Prior work that led to the development of this protocol included a comparison of several commercially available nucleic acid extraction systems. In this comparison, modifications to commercial protocols, as described below provided the highest DNA and RNA yields and quality (Selvarajah et al., *In Prep*). Tissue cores are thicker than the 5-10 µm micron sections typically used in FFPET extraction protocols[11,12,14,18–20], and may contain more variable amounts of paraffin. To compensate for this, deparaffinization was enhanced by repeating xylene and ethanol treatments and by introducing a motorized homogenization step (Figure 1). Furthermore, proteinase K digestion times were lengthened to increase DNA yield. Overall, this protocol is cost-effective and enables the establishment of linkages between molecular and histopathologic features of disease in large, well characterized populations. The protocol in its entirety can be carried out reliably within 2 days, including 3 hours of hands-on time, with little need for specialized or expensive equipment.

The step-by-step protocol is hereafter as a modified version of the manufacturer's protocol[21].

Please see Table of Materials/Equipment for specific reagents, equipment, and manufacturers.

**PROTOCOL:**

1. **Tissue Coring**

1.1) Review the microscope slide and outline the region(s) of interest using a fine-point permanent marker. Cut out a section of paraffin film large enough to cover the region of interest on the microscope slide. Place film firmly on slide and wrap film over edges to keep the film from slipping. Using a fine-point permanent marker, outline the entire tissue and the region(s) of interest within the tissue, keeping the outline touching – but outside of – the region(s).

1.2) Remove the film and transfer it to the corresponding tissue block. Orient the film by flipping or rotating it so that the outline of the entire tissue matches the observed shape of the tissue in the block (Figure 1). Press the section of film firmly to the surface of the block to prevent slippage.

1.3) Using the tip of the permanent marker, make shallow but visible (~0.2 mm) indentations along the outline of the region(s) of interest, then remove the film. Load 1 mL of bleach, 70% ethanol, and water into separate 1.5 or 2.0 mL microcentrifuge tubes.

1.4) Clean the receptor (red) punch from the 0.6 mm punch set by sliding the punch up and down several times while the tip is submerged into the tube containing bleach. Repeat the above step with 70% ethanol and then water (critical to ensure that bleach is removed).

1.5) Press the punch into the tissue, inside the region of interest to a depth of 3 mm and withdraw the punch. Release the core into a low binding 1.5 or 2 mL tube by pushing it out of the punch with the stylus. Store the cores at -20 °C (long-term) or 4 °C for short-term use.

1.6) Clean the punch according to step 1.4 and continue with the next regions or sample.

**2. Deparaffinize the FFPE tissue cores**

2.1) Carryout deparaffinization in 1.5 or 2 mL tubes by adding 1 mL xylene to the tissue core and vortexing vigorously for 10 sec. Heat for 3 min at 50 °C.

2.2) Centrifuge for 2 min at room temperature (RT) and maximum speed (21,130 x g) and place tube on ice for 5 min (allows the waxy residue to solidify on the top).

2.3) Carefully remove paraffin accumulated around meniscus with supernatant using a pipette tip and repeat xylene treatment (steps 2.1 – 2.2).

2.4) Add 1 mL of ethanol (100%) and vortex vigorously for 10 sec. Centrifuge for 2 min at RT (maximum speed), and carefully discard the ethanol. Repeat the above step once.

**3. Homogenization of the deparaffinized cores**

3.1) Resuspend the cores in 700 µL of ethanol (100%) prior to homogenization. Using a motorized tissue homogenizer, grind the cores into fine tissue particles (~1 min on medium setting). Clean the homogenizer probe between each sample to minimize carry-over contamination.

3.1.1) Fill 15 mL tubes with ~10 mL of bleach, RNase neutralizing solution and 70% ethanol. After sample homogenization, wash the homogenizer probe in each of the cleaning solutions in the order stated above. Run the homogenizer on the highest speed during the washing stage.

3.1.2) Wipe the probe with tissue and allow probe to dry completely before homogenizing the next sample. Visually inspect the probe blades for residual tissue pieces. If found, clean the probe again. Change the cleaning solutions (bleach, ethanol, and RNase neutralizing solution) daily.

3.2) Following homogenization, bring the sample volume to 1 mL by adding more 100% ethanol (~300 µL). Centrifuge at maximum speed for 15 min, carefully aspirate the ethanol and air dry pellet for approximately 15-20 min before proceeding with RNA extraction.

**4. Digestion with Proteinase K**

4.1) Resuspend the pellet in 150 µL Buffer PKD and flick tube to loosen the pellet. Add 10 µL of temperature-stable proteinase K and mix by flicking (do not vortex the tube). Incubate the content in the tube at 56 °C for 15 min with mild agitation.

4.2) Allow tube to incubate on ice for 3 min. Complete cooling is important for efficient precipitation in the following step. Centrifuge for 15 min at maximum speed.

**5. Separate RNA from DNA**

5.1) Carefully transfer the supernatant, without disturbing the pellet, to a new 1.5 mL for RNA purification.

5.2) Keep the pellet for DNA purification (pellet can be stored for 2 hr at RT, for up to 1 day at 2 - 8 °C, or for longer periods at -20 °C).

**6. RNA purification**

6.1) Incubate the RNA-containing supernatant at 80 °C for 15 min (do not exceed this time). Next, briefly centrifuge the tube to collect drops from the inside of the lid.

6.2) Add 320 µL Buffer RLT to adjust binding conditions, and mix by pipetting. Next, add 720 µL ethanol (100%), and vortex.

6.3) Transfer 600 µL of the sample, including any precipitate that may have formed, to RNA spin column (supplied in the kit) placed in a 2 mL collection tube and set aside the remaining content. Centrifuge for 15 sec at ≥ 8,000 x g, discard the flow-through and reuse the collection tube.

6.4) Transfer remaining sample onto a column, including droplets that may have accumulated in the lid of the tube, centrifuge for 15 sec at ≥ 8,000 x g, and discard the flow-through.

6.5) Add 350 µL Buffer FRN to the spin column and centrifuge for 15 sec at ≥8,000 x g, discard the flow-through and reuse collection tube.

6.6) Gently mix 10 µL DNase I stock solution with 70 µL Buffer RDD, add directly to the spin column membrane, and incubate at RT for 15 min.

6.7) Add 500 µL Buffer FRN to the spin column, centrifuge for 15 sec at ≥ 8,000 x g and save the flow-through for use in the next step. To enhance recovery of small RNAs, place the spin column in a new 2 mL collection tube and apply the flow-through from the previous step to the spin column.

6.8) Centrifuge for 15 sec at ≥8,000 x g, discard the flow-through and reuse the collection tube in next step. Add 500 µL Buffer RPE to the spin column and centrifuge for 15 sec at ≥ 8,000 x g, discard the flow-through and reuse the collection tube in the next step.

6.9) Add 500 µL Buffer RPE to the spin column and centrifuge for 15 sec at ≥ 8,000 x g and discard the collection tube with the flow-through.

6.10) Place the spin column in a new 2 mL collection tube, open the lid and centrifuge at maximum speed for 5 min. Discard the collection tube with the flow-through.

6.11) Place the spin column in a new 1.5 mL collection, add 20 µL of RNase-free water directly onto the spin column membrane, and incubate the tube for 1 min at RT. Centrifuge at maximum speed for 1 min to elute the RNA. Store the eluted RNA sample at -80 $^{o}$C.

## 7. DNA purification

7.1) Resuspend the pellet obtained during the RNA extraction by stepwise addition of 45 µL of proteinase K buffer (400 mM Tris 7.5, 400 mM NaCl, 3 mM $MgCl_2$, 4% SDS); 45 µL $H_2O$; and 400 µg of high potency Proteinase K.

7.2) Incubate the above solution at 56 °C for 24 hr (recommended) or overnight. Perform incubation at 90 °C for 2 hr without agitation and briefly centrifuge the microcentrifuge tube to collect drops from inside of the lid.

7.3) Allow the sample to cool to RT and then add 4 µL RNase A (100 mg/mL). Incubate the sample for 2 min at RT.

7.4) Add 200 µL Buffer AL to the sample, and mix thoroughly by vortexing. Next, add 200 µL of 100% ethanol, and mix thoroughly by vortexing. Transfer the entire sample to the provided spin column, place in a 2 mL collection tube, and centrifuge for 1 min at ≥ 8,000 x g.

7.5) Discard the collection tube with the flow-through and place the spin column in a new 2 mL collection tube. Add 700 µL Buffer AW1 to the spin column, centrifuge for 15 sec at ≥8,000 x g, discard the flow-through and reuse the collection tube.

7.6) Add 700 µL Buffer AW2 to the spin column, centrifuge for 15 sec at ≥8,000 x g, discard the flow-through and reuse the collection tube. Next, add 700 µL of 100% ethanol to the spin column, centrifuge for 15 sec at ≥8,000 x g and discard the collection tube with the flow-through.

7.7) Place the spin column in a new 2 mL collection tube, open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.

7.8) Place the spin column in a new 1.5 mL collection tube and add 25 µL of heated nuclease-free water (50 °C). Incubate column and tube at 50 °C for 10 min. Centrifuge for 1 min at maximum speed, add 25 µL of nuclease-free water (RT) to column and incubate for 1 min at RT.

7.9) Centrifuge for 1 min at maximum speed (21,130 x g) and harvest flow-through containing genomic DNA (approximately 50 µL of DNA in total). Store the column at -20 °C (in case another elution is required later).

**REPRESENTATIVE RESULTS:**

This protocol represents an optimized method for recovering DNA and RNA from tissue cores, using modifications of a commercial extraction system designed for tissue sections. Optimization included the introduction of tissue homogenization, utilization of more potent Proteinase K for DNA extraction, and extension of tissue digestion time. Graphs and statistical analyses included 2-way ANOVA, linear regression and correlation.

**Optimizations of proteinase digestion**

The commercial kit included a room temperature stable proteinase K solution which was substituted with a more potent proteinase K, resulting in higher DNA yield (Figure 2A). To further increase the DNA yields, digestion was extended from 2 to 24 hours. No significant differences were seen between the two time points, but the 24 hour digestion appeared to provide more consistent yields across samples. However, further incubation to 48 hours did not

further improve DNA recovery (Figure 2B; *p=0.74*).

**Typical DNA recovery from FFPE prostate cancer tissue samples**

Using the optimized protocol, RNA and DNA were co-extracted from 333 prostate cancer FFPET samples ranging from 3 to 14 years in sample age. From each sample, 3 tissue cores (average total tissue volume of 0.95 ± 0.13 $mm^3$) were used as input. While there are other microfluidic based gel-electrophoresis methods which can estimate concentrations and provide evaluations of the size distribution of nucleic acids molecules, such methods do not provide reproducible nucleic acids quantification, and cannot distinguish between RNA and DNA as flourometrically-based assays do[22]. And, because microfluidic based gel-electrophoresis results are not reliable for fragmented nucleic acids derived from FFPET[23], nucleic acid yields were measured fluorometrically (see reagent list for details). The average yield was 2,270 ng of RNA and 820 ng of DNA (Figure 3A). Approximately 90% of all FFPET samples analysed in this study yielded ≥100 ng of DNA and ≥500 ng of RNA. Interestingly, there was no significant correlation between the age of the FFPET sample and nucleic acid recovery (Figure 3B). Overall, RNA and DNA yields were correlated across samples ($R^2$ = 0.39; *p<0.0001*), although more than twice as much RNA than DNA was recovered from each sample (Figure 3C).

As the pilot and optimization work was performed on prostate tissues, the next step was to investigate the performance of this protocol on a few additional types of archival tissue. Starting with surgically removed and autopsy FFPET samples representing benign liver (1 sample from 1 case), cancers of the brain (8 samples from 1 case), urinary bladder (2 samples from 2 cases), and breast (3 samples from 3 cases), the protocol yielded >100 ng of DNA and RNA from 90% of samples (Figure 3D). While nucleic acid yields were lower in autopsy tissues than in surgical tissues, representative results indicate that the protocol produces similar yields across cancers derived from different sites.

**Assessment of RNA and DNA integrity and their representative performance in downstream analysis**

RNA expression analysis of 47 genes in 8 selected FFPET prostate cancer samples and a fresh PC-3 prostate cancer cell line sample (as a positive control) was performed using a commercial multianalyte gene expression platform that is optimized for FFPET. The mRNA counts in PC3 were typically higher than those from FFPET samples (Figure 4A). However, comparing relative expression of all genes, FFPET prostate cancer samples showed similar expression profiles to PC-3 RNA, indicating that both sources of RNA are suitable for RNA expression profiling.

To demonstrate performance of genomic DNA extracted with this protocol, bisulfite-converted DNA extracts from FFPET samples were amplified by methylation specific PCR (MSP)[24]. MSP analysis of ALU repetitive elements, highly methylated regions present in millions of copies in the human genome[25], was used as a genomic methylation control, and expected to show minimal variations between samples. As shown in Figure 4B, there was little to no variation seen between different samples in ALU MSP methylation levels. Further, MSP assays based on GSTP1, a gene known to be hypermethylated in prostate cancer but not in benign samples[26], showed no detectable amplifications in DNA from benign samples. As expected, lower qPCR

cycle threshold values were detected in DNA from cancer tissues, indicating enrichment of methylated GSTP1 copies. The utility of nucleic acids recovered by this protocol was further tested in typical downstream assays, using nucleic acids recovered from benign liver and from a brain (post-mortem) and from two surgically removed breast cancer samples. Both RT-qPCR based expression and MSP assays performed well on breast cancer and liver FFPET, but the RT-PCR assay failed to amplify a highly expressed mRNA from the post-mortem brain tumor sample (Figure 4C), suggesting that RNA had degraded, likely due to delayed tissue fixation.

**Figure 1**

**Overview of the extraction procedure for FFPET samples.** The figure illustrates how an area of interest in a tissue block is mapped based on histopathologic selection from a microscope slide. Three 0.6mm tissue cores are then obtained from each tissue area by using biopsy punches, homogenized together and then subjected to extraction of both RNA and DNA.

**Figure 2**

**Nucleic acids (DNA and RNA) yields in ng/mm$^3$ of FFPET from two proteinase K (temperature stable and high potency enzymes), and tested across a range of incubation times for the latter.** A) Performance of Proteinase K from different suppliers. DNA extractions were performed on a representative FFPET sample using temperature stable enzyme supplied with the kit versus a more potent enzyme from another manufacturer. B) Determining optimum Proteinase K incubation time to maximize the DNA yield. Performance of high concentration Proteinase K digestion was evaluated at three different incubation periods using 3 FFPET samples. Error bars represent standard error of mean (SEM).

**Figure 3**

**Nucleic acids (DNA and RNA) yields in ng/mm$^3$ of FFPET in total, across sample years and representative tissue types.** A) Total recovered nucleic acids from formalin-fixed paraffin-embedded tissue. The nucleic acids quantities presented are based on the extractions of 333 FFPET samples using the optimized protocol. B) Correlation plot between recovered total DNA and RNA and the age of FFPET samples. The extracted FFPET samples used were obtained from the years 2000 to 2012. C) Correlation between yields from concurrently extracted DNA and RNA from 333 prostate samples. There is a positive correlation between DNA and RNA yields. D) Demonstration of the protocol using additional archival tissue types. The optimized protocol was used to extract nucleic acids from 14 cancer (breast, bladder and brain) and normal (liver) samples. Error bars represent SEM.

**Figure 4**

**Performance of RNA and DNA co-extracted from tissue cores in downstream applications.** A) mRNA counts for FFPE prostate cancer tissues and for fresh PC-3 cell line control. Each point represents the average of 3 technical replicates extracted separately. Fresh PC-3 cell line RNA values are illustrated by yellow bars and FFPET tissue values are represented by coloured dots. B) Methylation specific PCR assays on DNA of FFPET prostate cancer samples. Cycle threshold values were obtained for 10 samples performed as expected using 50 ng/reaction of bisulfite converted DNA. ALU MSP assays from all FFPET samples had similar cycle threshold values

(*p>0.67)*. GSTP1 MSP assays showed higher methylation (lower cycle threshold) levels in prostate cancer than in benign prostate. C) Assessment of DNA and RNA quality from additional tissue types. HPRT1 gene expression and Alu gene methylation assays were performed on nucleic acids (RNA and DNA respectively) extracted from normal liver (autopsy), and brain (autopsy) and breast cancers (all FFPET). Results from prostate are shown for comparison. Note: similar results were observed from each tissue type, except for failed mRNA amplification from one autopsy sample. Each point or bar represents a sample, and error bars represent SEM.

**DISCUSSION:**

For successful extraction of DNA and RNA from tissue regions of interest, accurate coring is critical. This protocol describes the use of a tissue punch to isolate 0.6 mm diameter cores and outlines the process for transferring notations from microscope slides to corresponding FFPET blocks. Modifications to the manufacturer's protocol were required to efficiently extract nucleic acids from cores, which are approximately 50 times thicker than the microtome sections for which the protocol was intended. Since the cores may contain more paraffin wax relative to tissue sections, effective deparaffinization of cores through repeated xylene and ethanol treatment steps were required. The success of the post-deparaffinization steps depended on proper mechanical tissue cores homogenization and efficient proteinase K digestion. Further optimization of the proteinase K digestion can be performed.

It is worth mentioning that this method identifies areas of interest on the surface of the block, as identified in corresponding histopathology slides. As the core harvests tissue that may be 3 or 4 mm deep, users of this protocol may be concerned about what cells or tissues lay beneath the block surface. While this is a valid concern, multiple studies (reviewed in[27]) have demonstrated that tissue cores faithfully represent the histologic and molecular features of pathologic tissue blocks, particularly when duplicate or triplicate cores are sampled from the area of interest.

As the modified commercial extraction kit adopted in this protocol enables concurrent extraction of both DNA and RNA from the same tissue, the protocol saves precious biological material and allows a direct comparison between the two resulting nucleic acids from the same sample. Concurrent extraction of RNA and DNA cuts down labor and tissue depletion by half, and enables precise integrated analysis of gene expression, as well as epigenetic and genetic features found in DNA. Since the yields of both RNA and DNA from these representative tissue cores typically exceed 600 and 300 ng, respectively, and since most current PCR and next generation sequencing applications typically require 10-100 ng, most of the samples purified by this protocol should provide adequate material for several downstream assays. This protocol has been shown to be reproducible across independent laboratories (Selvarajah et al., *In Prep.*). RNA from this protocol was of sufficient quality for gene expression analysis using either RT-PCR or a popular multianalyte platform, and DNA performed well in methylation specific PCR assays. Future studies aimed at assessing the utility of recovered nucleic acids in next generation sequencing are warranted.

Thus, several modifications were made to a commercially available protocol, designed for thin FFPET sections, rendering it suitable for the co-extraction of RNA and DNA from 0.6 mm FFPET

cores. The protocol demonstrated consistently high yields in a large cohort of prostate cancer samples and in a limited set of samples from cancers of the breast, brain and bladder. Overall, the protocol should enable users to carry out targeted gene-based analyses of large well-annotated tissue collections. Importantly, the protocol enables efficient focused sampling of regions of interest in FFPET, relatively little hands-on time, and high enough yields for most downstream applications.

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The authors declare no competing financial interests.

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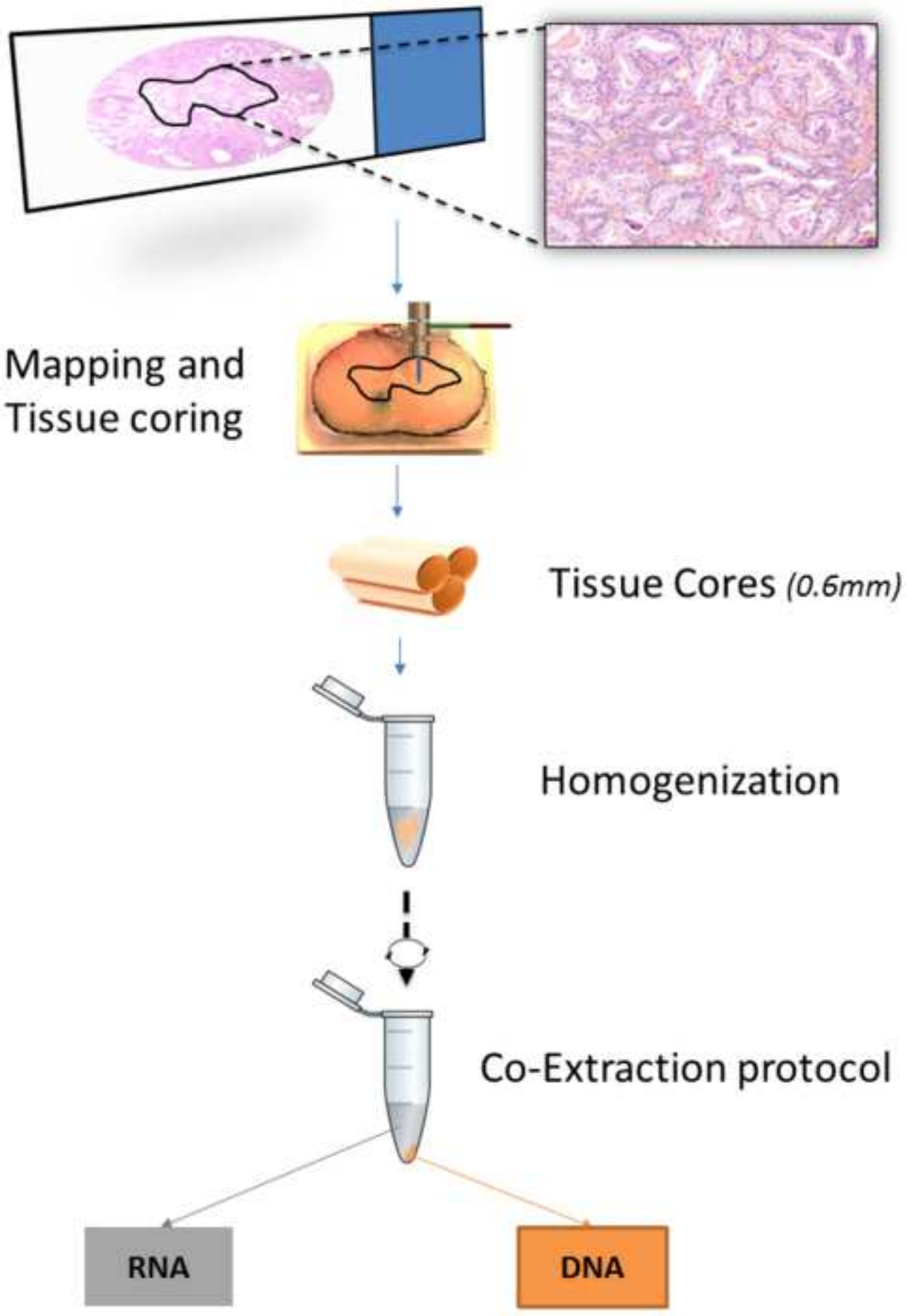

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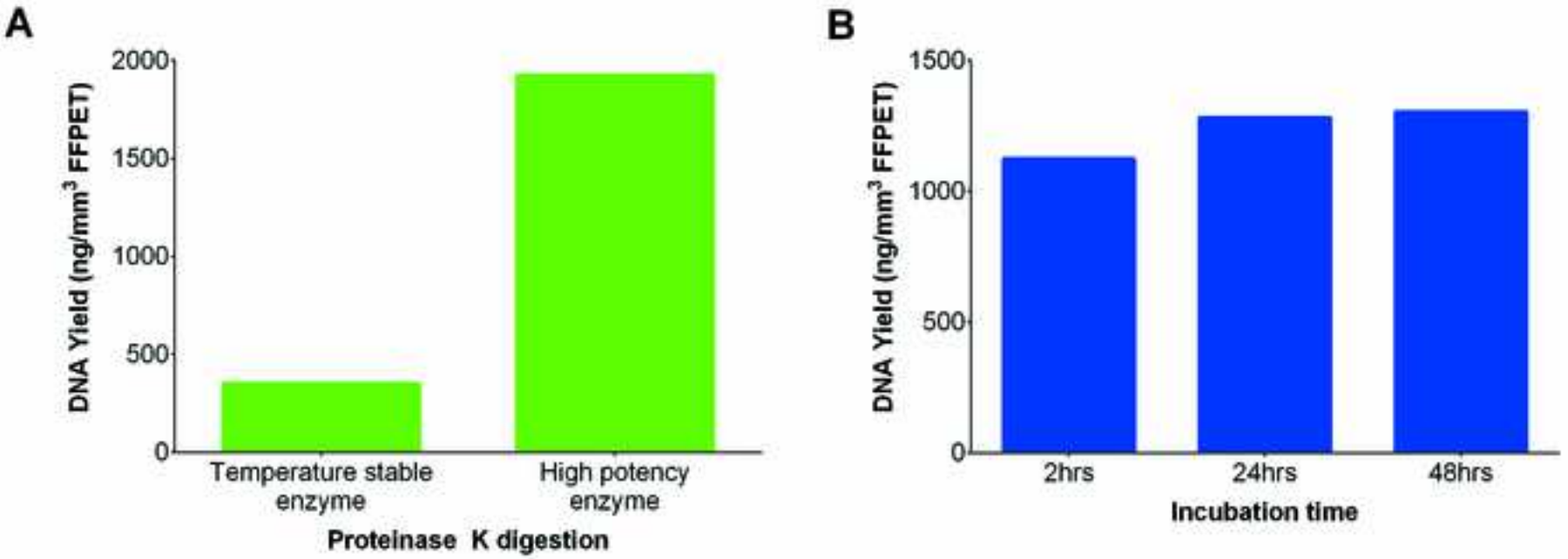

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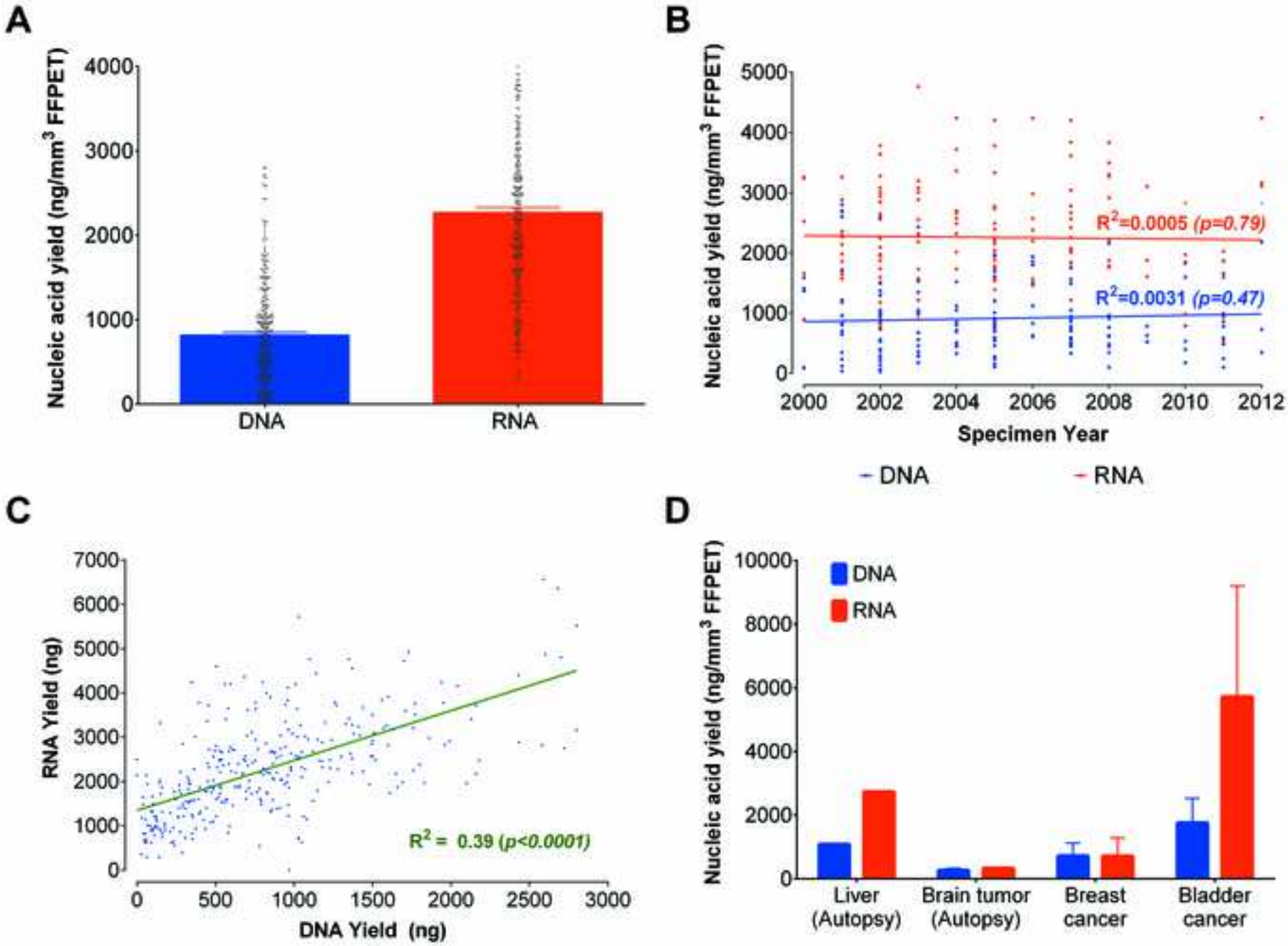

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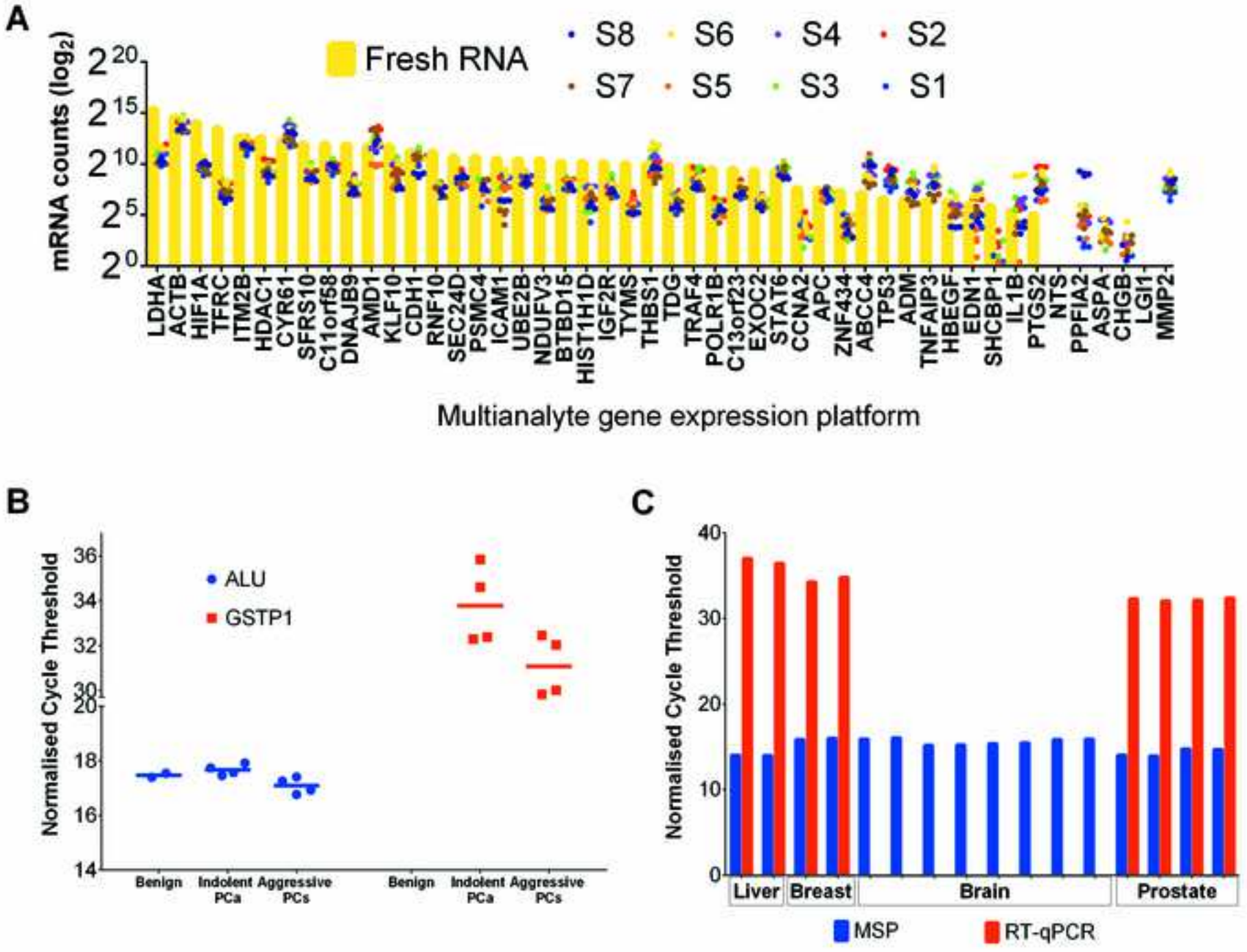

Table 1



| Tissue Cores | | | | | |
|---|---|---|---|---|---|
| | Tissue Input (mm3) | Extracted Nucleic Acid | Validation | | Quality che |
| | | | (# of samples) | Age of Sample (years) | Total Yield (ng) |
| Pikor et al. | 43 - 129 | DNA | No data | No data | No data |
| Montaser-Kouhsari et al | 18 - 29.5 | RNA | 763 | 0 - 25 | |
| This paper | 1.71 | DNA and RNA | >350 | 3 - 12 | 820 |
| **Tissue Sections** | | | | | |
| | Tissue Input | Extracted Nucleic Acid | Validation | | Quality che |
| | | | (# of samples) | Age of Sample (years) | Total Yield (ng) |
| Heikal et al. | 5 x 5 um | DNA | 12 | 7 - 22 | 88 - 300 |
| Chung et al. | 1 x 20 um | RNA | 9 | > 5 | |
| Antica et al. | 2 x 4 um | RNA | 18 | No data | |
| Ghatak et al. | 5 x 5 um | DNA and RNA | 5 | 1 | 14 256 |
| Hennig et al. | 1 x 10 um | DNA and RNA | 210 | 1 - 25 | No data |
| **Laser Capture Microdissecti** | | | | | |
| | Tissue Input (mm2) | Extracted Nucleic Acid | Validation | | Quality che |
| | | | (# of samples) | Age of Sample (years) | Total Yield (ng) |
| Snow et al. | 1 - 2 | DNA | 110 | 0 - 2 | 430 |

| ck: DNA | | Quality check: RNA | | | |
|---|---|---|---|---|---|
| Fragment size (bp) | PCR | Total Yield (ng) | Fragment size (bp) | PCR | NanoString |
| No data | | | | | |
| | | 843 | No data | | |
| 100 - 500 | ✓ | 2270 | 100-500 | ✓ | ✓ |

| ck: DNA | | Quality check: RNA | | | |
|---|---|---|---|---|---|
| Fragment size (bp) | PCR | Total Yield (ng) | Fragment size (bp) | PCR | NanoString |
| 103 - 351 | ✓ | | | | |
| | | 16 000 - 23 000 | 100 - 200 | ✓ | |
| | | Unknown (621 ng/uL) | 80 - 202 + | ✓ | |
| < 1030 | ✓ | 16 000 | 109 - 400 + | ✓ | |
| No data | ✓ | No data | No data | ✓ | |

ion

| ck: DNA | | Quality check: RNA | | | |
|---|---|---|---|---|---|
| Fragment size (bp) | PCR | Total Yield (ng) | Fragment size (bp) | PCR | NanoString |
| No data | ✓ | | | | |

| Name of Material/ Equipment | Brand/Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Plastic paraffin film, "Parafilm 'M'" | Bemis | RK-06720-40 | Any generic paraffin film wi |
| Sodium Hypochlorite, "Ultra Bleach" | Likewise | 53-2879-2 | Any generic bleach will wor |
| Molecular biology grade absolute ethanol | Fisher BioReagents | BP2818-500 | Sigma-Aldrich E7023 suffice |
| Molecular Grade H2O | G-Biosciences | 786-293 | Sigma W4502 suffices, as w |
| 0.6mm Punch Set for Beecher Instruments | Estigen | MPO6[Yellow] | Make sure to use the red re |
| Fine point permanent marker | Sharpie | 10365796S | Using the marker on FFPE t |
| FFPE tissue block | | | |
| Stained tissue slide corresponding to FFPE block | | | |
| 1.5 mL Micro-Centrifuge Tubes | Fisher BioReagents | 05-408-137 | |
| 2.0 mL Low binding tubes (LoBind Micro-Centrifuge Tubes) | Eppendorf | 22431048 | |
| 1.5 mL Low binding tubes (LoBind Micro-Centrifuge Tubes) | Eppendorf | 22431021 | |
| Histology Xylene | VWR | CA 95057-822 | Fisher Scientific X5-500 suff |
| Molecular Biology Grade 2-Propanol | Sigma | I9516 | |
| AllPrep FFPE DNA/RNA Kit | Qiagen | 80234 | Prepare buffers accodring t |
| Buffers: RLT, FRN, RPE, ATL, AL, AW1, AW2, DNaseI solution | Qiagen | 80234 | Prepare buffers accodring t |
| Temperature stable proteinase K | Qiagen | 80234 | |
| High potency proteinase K | Invitrogen | 25530-049 | Invitrogen 25530-015 suffic |
| RNAse neutralizing solution (Rnase AWAY) | Molecular BioProducts | 7003 | |
| RNaseA 100mg/mL | Qiagen | 19101 | |
| BD Integra Syringe 3mL 21G x 1/2 | BD | 305274 | |
| Motorized tissue homogenizer (TissueRuptor) | Qiagen | 9001271 | Fisher Scientific 14-261-29 s |
| -20$^{o}$C and -80$^{o}$C Laboratory Freezer | | | |
| Micro-Centrifuge with rotor for 2mL tubes | | | |

| | | |
|---|---|---|
| Digital Vortex Mixer | | |
| Pipettes and filter tips | | |
| Heating blocks or water baths | | |
| Tris Hydrochloride | Amresco | 0234 |
| Sodium Chloride | Amresco | 0241 |
| Anhydrous Magnesium Chloride | Sigma | M8266 |
| Sodium Dodecyl Sulfate | Sigma | L4509 |
| Acrodisc 25mm syring filters with 0.45 um Supor membrane | Pall | PN 4614 |
| Syringe with retracting BD PrecisionGlide needle 3mL | BD Integra | 305274 |
| Hydrochloric Acid | BDH | 3026 |
| Multianalyte gene expression platfrom (nCounter ® CAE codeset and Nanostring nCounter platform) | Nanostring nCounter platform, Nanostring | |
| Fluorometric nucleic acid quantification (Qubit dsDNA HS Assay Kit and Qubit® RNA BR Assay Kit) | Invitrogen | |

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ell as any other brand of molecular grade H2O

eceiver punch from the set

issues causes it to dry out quickly, so several may be required

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Changes made by the Science Editor:

1. There have been edits made to the manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- The manuscript has been edited and grammatical errors corrected as seen in track changes.

2. Please verify the in step protocol references.

- The references have been verified and a few errors corrected as seen in track changes.

3. Formatting: Short Abstract – Unless the validation steps are detailed in the protocol, the second sentence should be removed.

- Since the validation steps are not detailed in the protocol, the second sentence has been removed as suggested.

4. Grammar:
-Line 156 – "protocol" should be plural

- Corrected as suggested.

-1.1 – Please correct grammar in the first sentence, which is a run-on.

- A period introduced in the sentence to avoid the run on error; splitting the sentence into two as shown in track changes.

-1.3 – The last sentence is incomplete.

- A period has been introduced at the end of this sentence, and the hanging word "and" deleted.

-6.6 – Last sentence should end with a period.

- Done.

5. Visualization: Step 1.5 must be highlighted. It is unclear why this entire section except for the main action is highlighted.

- Highlighted as suggested.

6. Unnecessary branding should be removed: The last column of Table 1 can be deleted due to branding.

- The last column in Table 1 has been deleted.

7. Results: All figure legends should have a title and a brief description. Figure 1 lacks a description and Figures 2 & 3 lack titles.

- We have made changes to the figure legends, and each now has a title and a short description.

8 Discussion: Please discuss potential modifications and troubleshooting that can be performed with this protocol rather than modifications on the manufacturer's protocol.

- We have revised the discussion to include potential adaptation of this protocol for LCM and cut-section samples as well as its implementation on automatic robotic extraction systems.

Reviewers' comments:

**Reviewer #1:**
*Manuscript Summary:*
This article focuses on the extraction of RNA and RNA from FFPE tissue cores. The protocol is very detailed yet easy to follow. The Authors also present an excellent analysis of analyte yields based on a variety of factors. This article will be an excellent reference for those working with these analytes as FFPE banks represent rich sources of patient tissue. The accompanying video will be of great benefit for guiding investigators new to these techniques.

- We are gratified by the reviewer's endorsement of this work, and will work with the JoVE Editorial team to ensure the key steps in the protocol are captured in the accompanying video.

**Reviewer #2:**

*Manuscript Summary:*
This is a well described method for the extraction of RNA and DNA simultaneously from FFPE tissue cores from samples. The use of modified commercially available kits/protocols should put this methodology within reach of any laboratory with a reasonable amount of expertise in standard molecular biology techniques. It should be noted that this work still requires the expertise of an experienced pathologist as selection of appropriate regions for nucleic acid purification remains the most important step in this process. The figures are clear and adequately show the utility/yield of this procedure for samples dating back up to 15 years. The detailed reagent list is helpful. While not specifically tested for this paper, the authors describe nucleic acid yields that should be useful for next-generation sequencing experiments and other downstream applications.

- We thank reviewer #2 for the positive evaluation of our manuscript. We look forward to utilizing this protocol in next generation sequencing studies.

*Minor Concerns:*
-Utility is somewhat limited using brain tissue, likely due to the high lipid concentration of this tissue, but this limitation is clearly presented in the text and figures.

- We concur with this observation.

-Margins of table one are cut off in my downloaded version of this text and should be verified.

- We will work with the editors to ensure that the final document is properly formatted. We note that complete images could have been viewed by clicking the appropriate links in the pdf version of the manuscript.

-Error bars should be added to figures 2 and 4c. They are discussed in the figure legends, but not visible on the graphs.

- Unfortunately, error bars would not be applicable here since the figures in question are showing individual sample/data points.

**Reviewer #3:**
*Manuscript Summary:*
An excellent and well written manuscript that explains in detail the rationale for using the extraction method as well as all the steps that are part of the process.

- We thank reviewer # 3 for this positive feedback.

**Reviewer #4:**
*Manuscript Summary:*
The manuscript described the use and optimization of a commercially available kit for simultaneous extraction for RNA and DNA from formalin-fixed paraffin-embedded (FFPE) tissues. The authors describe three modifications, including homogeneization in ethanol, use of

an "improved" protein K, and digest time extension from 2h to 24h. The protocol is clearly described step by step and the authors use clinical specimens from gene expression and methylation analyses.

*Major Concerns:*

There are a couple major concerns with the manuscript: 1- In the abstract and later in the introduction statements relating to the lack of "validated methods for simultaneous extraction of RNA and DNA.." are inaccurate. The method developed by Kotorashvili et al. 2012 describes an improved approach to extract RNA and DNA simultaneously from tissue sections, and thus is most likely applicable to the extraction of tissue cores. This method stems from the protocol developed by Loudig O et al. 2007 initially developed with tissue cores that underwent homogeneization.

- We thank the reviewer for pointing out these additional methods paper and have improved our manuscript by citing them and their contributions in the text and in Table 1. We point out that neither of these published protocols addresses the main purpose of the method reported in this manuscript: To simultaneously extract both RNA and DNA from FFPE tissue cores.

2- The use of tissue cores represent a major issue for collection of heterogeneous cells as cells below the surface of the tumor or lesions may vary and allow introduction of normal surrounding tissue. The use of LCM is indeed time consuming and expensive, but the use of tissue core, at a time when tumor cell heterogeneity is being explored represents a global and potentially error prone approach. Comparisons between microdissected cells from randomly selected tumors and a tumor cores obtained from these tumors should be performed to determine the gene expression and genomic DNA methylation profile differences.

- We thank the reviewer for this observation and have addressed it in a new section in the revised discussion starting on line 495. In essence, this is a valid concern, but one that has been widely addressed in the tissue microarray literature. In the revised manuscript, we provide a reference to a pertinent review on the topic, along with an alert to the reader to consider this issue.

*Minor Concerns:*

There are actual concerns with the development of the method:

1- The use of a motorized homogenizer will allow for tissue particles to remain trapped in the instrument. A comparison between different homogenizations approaches and non-homogenization would provide a better sense on the improvement.

- We thank the reviewer for bringing up this concern. We submit that homogenization is a common practice in molecular biology, and that we not only include multiple wash steps and inspection steps, but also repeatedly alert the user to pay careful attention to avoid this pitfall.

2- The use of ethanol during homogenization may not be adaptable to tissue sections or selected tissue lesions from sections or LCM, for RNA purification.

- We agree with the reviewer on this issue and go back to the first "Major" point the reviewer brought up. This protocol is not meant to inform nucleic acid purification from LCM or sectioned samples.

3- The purification of RNA from the ethanol fraction recovered after homogeneization is somewhat puzzling as FFPE RNA has been shown to be cross-linked to proteins, hence the use of proteinase K for improved recovery. Once again an extraction comparison with and without proteinase K, followed by RTqPCR for a few genes, would allow to determine the amount of information lost or tissue expression specificity retention, as well as the amount of RNA lost. It is known that miRNAs will not cross-link in FFPE tissues and will wash off especially during FISH experiments, and thus will provide most of the RNA fraction recovered during homogeneization. Messenger RNAs however may remained trapped in the tissue and thus may not be extracted efficiently in this way. A Bioanalyzer profile observing RNA purified in both ways would somehow provide details on the selectivity of this ethanol based purification.

- We thank the reviewer for raising this issue. These are valid points about RNA quality from FFPET, but we submit that the "proof is in the pudding." Indeed, in other work, we and others have shown that RNA from FFPET is highly fragmented, and cannot be reliably analyzed by Bioanalyzer. We therefore used quantification by a sensitive and specific fluorometric assay, as well as endpoint analysis (RT-PCR and NanoString) to validate the suitability of the RNA. In the revised manuscript we clarify this issue (line 380) and provide a relevant reference. .

4- The use of a high potency proteinase K that displays such difference with the commercially available enzyme is striking, but no information on its origin, purification (grade), concentration is provided, which leaves room for questions. This enzyme alone may account for most of the improvement alone. More detailed comparative measures between the pK provided with the All Prep kit and the high potency pK should be given.

- We agree that the information requested is important. Due to editorial instructions from the Journal, we have excluded this information from the main text, but include only what is allowed in the reagent list.

5-The difference between 2h and 24h of pK treatment does not appear to be significant, and no repeated measures can provide such consensus. Repeats should be provided.

- We thank the reviewer for pointing out this issue and have revised the text to downplay any differences between the 2 hour and 24 hour time points (line 374).

6- The methods used for quantification of RNA and DNA are not provided. It is noteworthy that amounts determined with nanodrop or other types of spectrophotometers are somehwhat inaccurate, especially working with FFPE DNA. The Qubit apparatus is a better and more accurate instrument.

- Nucleic acids were quantified flourometrically using the Qubit system. As instructed by the Journal, brand names are included in the reagents section. In the revised manuscript, we alert the reader to look in this section for details.

*Additional Comments to Authors:*

Considering that this study highlights the improvement of an already existing commercial kit, side by side comparisons with the initial protocol should be performed to provide users with a better sense of the improvements.

- We thank the reader for this suggestion and submit that our presentation would be more streamlined and useful for a user/reader than one that tried to compare tissue sections to cores.