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Maintaining Breast Cancer Specimen Integrity and Individual or Simultaneous Extraction of Quality DNA, RNA, and Proteins from Allprotect-Stabilized and Nonstabilized Tissue Samples

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The Saint James's Hospital Biobank was established in 2008, to develop a high-quality breast tissue BioResource, as a part of the breast cancer clinical care pathway. The aims of this work were: (1) to ascertain the quality of RNA, DNA, and protein in biobanked carcinomas and normal breast tissues, (2) to assess the efficacy of AllPrep® (Qiagen) in isolating RNA, DNA, and protein simultaneously, (3) to compare AllPrep with RNEasy® and QIAamp® (both Qiagen), and (4) to examine the effectiveness of Allprotect® (Qiagen), a new tissue stabilization medium in preserving DNA, RNA, and proteins. One hundred eleven frozen samples of carcinoma and normal breast tissue were analyzed. Tumor and normal tissue morphology were confirmed by frozen sections. Tissue type, tissue treatment (Allprotect vs. no Allprotect), extraction kit, and nucleic acid quantification were analyzed by utilizing a 4 factorial design (SPSS PASW 18 Statistics Software®). QIAamp (DNA isolation), AllPrep (DNA, RNA, and Protein isolation), and RNeasy (RNA isolation) kits were assessed and compared. Mean DNA yield and $A_{260/280}$ values using QIAamp were 33.2 ng/μL and 1.86, respectively, and using AllPrep were 23.2 ng/μL and 1.94. Mean RNA yield and RNA Integrity Number (RIN) values with RNeasy were 73.4 ng/μL and 8.16, respectively, and with AllPrep were 74.8 ng/μL and 7.92. Allprotect-treated tissues produced higher RIN values of borderline significance ($P = 0.055$). No discernible loss of RNA stability was detected after 6 h incubation of stabilized or nonstabilized tissues at room temperature or 4°C or in 9 freeze-thaw cycles. Allprotect requires further detailed evaluation, but we consider AllPrep to be an excellent option for the simultaneous extraction of RNA, DNA, and protein from tumor and normal breast tissues. The essential presampling procedures that maintain the diagnostic integrity of pathology specimens do not appear to compromise the quality of molecular isolates.

Introduction

Cancer tissue biobanking involves striking a balance between maintaining the diagnostic integrity of surgical specimens while simultaneously preserving RNA, DNA, and proteins.[1,2] The size of a sample allocated to a biobank is variable and depends on cancer type, size, stage, and proximity to resection margins. This is particularly relevant in breast cancer where screening and mammography programs have led to increased rates of detection, earlier diagnosis, improved prognosis, and smaller tumors.[3,4] Zabicki et al.[5] reported a significant decrease in tumor size from 3 cm (1980) to 1.84 cm (2002) for women between 50 and 60 years of age. This potentially reduces the amount of tissue and number of cases that can be safely biobanked for research without compromising diagnostic integrity, and limits the quantity of research material available to researchers in the years to come.

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The preparation of pure, high-quality, and concentrated molecular isolates is essential for downstream applications.[6] Numerous commercial kits are available for RNA, DNA, and protein extraction.[7–15] Failure to isolate quality molecular isolates has negatively impacted on the ultimate reliability of research results. Morrow[16] reported numerous instances where candidate biomarkers and "therapeutic targets" were subsequently discredited and attributed the errors to poor-quality RNA. The quality of DNA, miRNA, and mRNA expression profiles and reverse transcription polymerase chain reaction (RT-PCR) data are greatly influenced by the quality of the starting tissue from which these molecular components are isolated.[17–22] Total RNA degradation is reported to affect miRNA expression profiles.[19] The assembly of large RT-PCR amplicons (over 400 bp) is strongly influenced by RNA quality and requires an RNA Integrity Number (RIN) of 5 or higher.[6] Becker et al.[18] confirmed these results and reiterated the importance of high-quality RNA for reliable and reproducible gene expression profiles. An RIN of ≥ 7.8 is now regarded as a nominal requirement for microarray analyses.[23] This value was suggested after analyses pointed to an increase in false positive microarray results where samples with RINs <7 had been included.

The Saint James's Hospital Biobank (SJHB) seeks to ensure that each tissue aliquot is utilized to the utmost. AllPrep® (Qiagen), a kit designed to simultaneously isolate RNA, DNA, and protein from individual tissue samples, offers obvious advantages. Cancers may show pronounced heterogeneity, even in samples from adjacent areas. Extraction kits that isolate RNA, DNA, and proteins from the same sample provide an opportunity to follow oncogenic mutations from genome through to ribosome and proteome, and increase the likelihood of identifying robust new biomarkers. We, therefore, investigated the performance of AllPrep in conjunction with a new stabilization medium, Allprotect® (Qiagen). Allprotect is designed to stabilize RNA, DNA, and protein concurrently, a potential advantage over RNAlater®.

The aims of this study were (1) to ascertain the quality of a representative portion (10%) of samples biobanked at SJHB utilizing a combination of quality control approaches utilized by established international biobanks,[24–28] (2) to assess the capacity of AllPrep (Qiagen) to simultaneously isolate RNA, DNA, and protein from tumor and normal breast tissues, (3) to compare AllPrep with dedicated RNA and DNA extraction kits, that is, RNEasy® (Qiagen, RNA isolation) and QIAamp® (Qiagen, DNA isolation), (4) to evaluate the effectiveness of Allprotect, a new RNA, DNA, and protein stabilizer, and (5) to assess the impact (if any) of presampling measures taken to maintain the pathological specimen's diagnostic integrity on the ultimate quality of RNA, DNA, and protein isolates. This analysis was very timely given the imminent expansion of the Irish hospital biobank network.

Patients and Methods

Ethics approval

Tissue samples were collected from 13 patients in whom invasive ductal carcinoma of the breast had previously been diagnosed (by fine needle aspiration). The collection and analysis of biobanked material was approved by St. James Hospital and The Adelaide and Meath Hospital, Dublin, incorporating the Research Ethics Committee of the National Children's Hospital. Wide local excision specimens and mastectomy specimens were transported immediately post-operatively, reaching the laboratory within 20–40 min. A pathologist examined each specimen and where possible took samples of tumor and normal tissue for biobanking (Table 1). This procedure was expedited to minimize RNA degradation.

Tissue processing

Sample preservation was completed within 20–40 min of arrival. Tissue samples were further subdivided to permit multiple sampling approaches. First, tissues were orientated in cryomolds, submerged in optimal cutting temperature (OCT) (both Tissue-Tek), and snap frozen in liquid nitrogen. Fresh tissue was also snap frozen in liquid nitrogen (fresh frozen; FF). Allprotect (Qiagen) stabilization was achieved by suspending tissues in a 15:1 (v:w) ratio of Allprotect to tissue. Samples were then equilibrated overnight at 4°C and transferred to a fresh cryovial before snap freezing (Allprotect stabilized tissue; AP). All tissue samples were stored in an ultra-low temperature freezer (-80°C). The relative compatibility of each tissue type (tumor, tumor-Allprotect, normal, and normal-Allprotect) to 3 Qiagen kits (QIAamp, AllPrep, and RNeasy) was compared. Tumor (T) and Normal

Table 1. Key Elements in Developing a High-Quality Breast Tissue BioResource

Element		*Optimal practice*
Transport	From theatre to pathology	<30 min
Examination of surgical resection specimen by pathologist[29]	Tumor identification, measurement, weighing, inking of margins etc	Mammography and ultrasound information, especially useful for detecting multifocal tumors in large mastectomy specimens
Frozen sections	To confirm presence of normal ducts and acini, determine percentage of tumor cells, identify necrosis etc	
Tissue processing time	Time from arrival in pathology laboratory to liquid nitrogen/stabilization medium (Allprotect)	<30 min
Annual quality control	Assessment of DNA, RNA, protein yields, and quality	1%–5% of biobanked specimens
Homogeneous cell population	Lazer capture microdissection[30]	

(N) tissue morphology was confirmed by using cryostat frozen sections (5 μm) stained with hematoxylin and eosin (Table 1). DNA, RNA, and protein quality control analysis was conducted in accordance with protocols from 3 international biobank networks: Wales Cancer Bank, C.N.I.O. (Spain), and CTRNet (Canada).[26–28]

DNA, RNA, and protein isolation

The samples of invasive ductal carcinoma were randomly selected from the SJH biobank and represented 10% of the total number of samples in the breast biobank at that time. Tumor, normal, tumor-Allprotect, and normal-Allprotect tissue samples were collected from each patient and subsequently committed to DNA, RNA, and protein isolation by utilizing 3 commercial kits. In total, molecular isolates (DNA/RNA/Protein) were extracted from 111 individual breast tissue samples: 32 samples were selected for DNA extraction (QIAamp); 47 samples for the simultaneous isolation of DNA, RNA, and protein (AllPrep); and 32 samples for RNA extraction (RNeasy). Ten milligrams of tissue was homogenized during three 2 min cycles at 30 Hz (TissueLyser I, 5 mm Stainless Steel Beads, both Qiagen). Tissues were incubated on ice between cell lysing cycles.

DNA analysis

DNA was quantified using Qubit [also known as Quant-iT™ dsDNA HS (High Sensitivity) Assay Kit; Invitrogen], Nanodrop 2000™, Thermo Scientific (designated Nanodrop1), and NanoDrop ND-1000™, NanoDrop Technologies (designated Nanodrop2), as per manufacturer's instructions. Both the ratio of absorbance at 260 and 280 nm ($A_{260/280}$) and gel electrophoresis were utilized to ascertain the quality and purity of DNA isolates. $A_{260/280}$ values were determined by Nanodrops 1 and 2. DNA isolates from QIAamp and AllPrep kits were subjected to electrophoresis (1.0% agarose). One hundred nanograms of DNA was mixed in a 2:1 ratio with Loading dye (Sigma). DNA isolates were visualized using Syber Safe (10,000×; Invitrogen) and awarded DNA Quality Scores. DNA isolates were scored as follows: 1 = High Quality (discrete band, no smear), 2 = Good Quality (broad band, no smear), 3 = Poor Quality (unaffixed band, pronounced smear), and 4 = Complete Degradation (No band, complete smear).

RNA analysis

RNA was quantified by using Nanodrop1 and the Agilent 2100 Bioanalyzer® (in conjunction with RNA 6000 Nano LabChip®) according to manufacturer's specifications. The Bioanalyzer was also utilized to determine the quality of isolated RNA. The Bioanalyzer utilizes lab-on-a-chip technology and algorithms that assign RINs based on 28s and 18s ribosomal profiles (Electropherograms). An algorithm assigned RIN numbers based on the 28s/18s peak ratio information gleaned from the electrophoretic trace. RIN scores between 1 and 10 were awarded based on RNA quality where 1 represented degraded RNA and 10 represented very high-quality, intact RNA. Electrophoresis-like images were also produced. All breast tissue samples fell within the kit limitations (5–500 ng/μL). RNA stability was evaluated by incubating isolates at room temperature, and at 4°C for 6 h, and subjecting aliquots to 9 freeze-thaw cycles.

RT-PCR of glyceraldehyde-3-phosphate

RNA concentrations were normalized to 10 ng/μL. Fifty nanograms of RNA was retro-transcribed to form cDNA by using Qiagen's LongRange 2Step RT-PCR kit®. Briefly, samples were incubated for 90 min at 42°C, followed by incubation at 85°C for 5 min. Fifteen micrograms of cDNA was amplified for a 198 bp transcript of the glyceraldehyde-3-phosphate (GAPDH) gene (forward primer GGA AAT CCC ATC ACC ATCT, reverse primer TTC ACA CCC ATG ACG AAC AT) by using Qiagen's HotStart PCR kit® [95°C for 6 min, 35 cycles of denaturation at 95°C (30 s) followed by annealing at 53.3°C for 30 s and elongation at 72°C for 1 min]. PCR products were loaded onto a 10% acrylamide gel and run at 150 V for about 45 min to confirm GAPDH amplification.

Protein analysis

The Pierce 660 nm Protein Assay® (Thermo Scientific) was employed to quantify AllPrep-isolated proteins. AllPrep's ALO protein re-suspension buffer was found to inhibit the Pierce reagent. A number of buffers were appraised before a 9M Urea, 2% Chaps, and 50 mM DTT were selected, because they optimized protein solubility while maintaining Pierce reagent stability. A standard curve was generated by using bovine serum albumin (2500–50 μg).

Western blot

One hundred micrograms of protein per sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels at 40 mA per gel, constant current. Proteins were subsequently transferred onto a 0.22 μm nitrocellulose membrane at a constant voltage of 100 V for 1 h. Nitrocellulose membranes were blocked in 5% milk and incubated with an actin antibody (Sigma) to assess protein expression and integrity.

Analysis of results

This study was an example of a 4 factorial design. The 4 factors were (1) tissue type (T/N), (2) tissue treatment (FF/AP), (3) method of DNA/RNA/protein quantification, and (4) extraction kit choice. Interactions between each of the 4 factors were also examined, and, therefore, the simple t-test was insufficient for statistical analyses. Results were analyzed by Repeat Measurement analysis of variances (ANOVAs) and Two-way ANOVAs using SPSS PASW 18 Statistics Software®.

Results

Figure 1 shows frozen section images of breast carcinoma and normal breast tissue. AllPrep was compared with dedicated DNA and RNA extraction kits (QIAamp and RNeasy, respectively). The yield and quality of DNA extracted using AllPrep were compared with those of QIAamp, and AllPrep RNA yields and quality were compared with those of RNeasy.

DNA isolation using the QIAamp Kit

The QIAamp kit was employed to extract DNA from tumor and normal breast tissues. Qubit ds DNA High

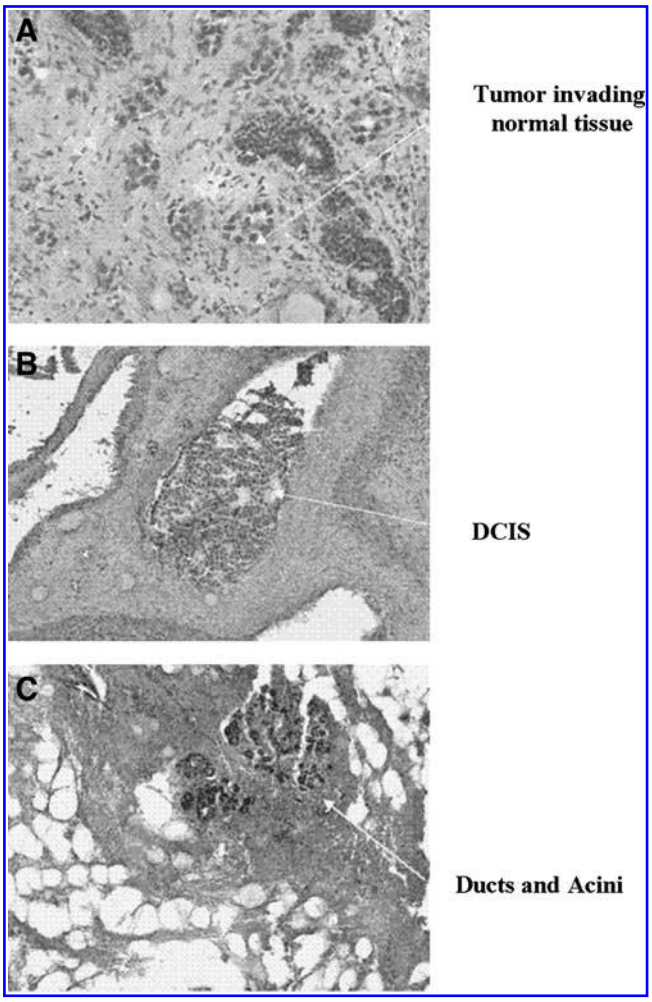

FIG. 1. **(A–C)** Hematoxylin and eosin-stained frozen sections (5 μm): **(A)** Invasive ductal carcinoma invading normal breast tissue (X 200). **(B)** Ductal carcinoma *in-situ* (X 100). **(C)** Normal breast tissue (X 200).

Sensitivity Assay, Nanodrop1, and Nanodrop2 were then utilized to quantify DNA yields. Qubit quantifications were significantly lower than Nanodrops 1 and 2 quantifications [$F(1,28)=12.611$, $P=0.001$ and $F(1,28)=8.242$, $P=0.008$, respectively]. This was unsurprising, as Nanodrops quantify both single- and double-stranded nucleic acids, whereas Qubit specifically quantifies double-stranded DNA. A Bland-Altman Plot (Fig. 2) illustrated that Qubit and Nanodrop (1 and 2) concentrations correlated closely below 50 ng/μL, whereas above this concentration, a proportional error ensued. Allprotect-treated tissues produced significantly lower DNA yields [$F(1,28)=4.680$, $P=0.039$] compared with FF tissues (Table 2).

The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) was utilized to assess the purity of isolated DNA. $A_{260/280}$ values were not influenced by Allprotect treatment [$F(1,28)=3.177$, $P=0.086$] or tissue type (tumor/normal) [$F(1,28)=0.194$, $P=0.663$]. The mean $A_{260/280}$ value, 1.86, indicated that DNA was pure and of good quality (ie, $A_{260/280} \geq 1.8$) (Table 2). DNA Quality Scores determined that 50% of scored DNA samples were of good quality, and 40% were of high quality; 10% were estimated to be of poor quality.

DNA, RNA, and protein isolation using the AllPrep Kit

DNA. AllPrep was utilized to extract DNA from tumor and normal breast tissues. Qubit quantifications were again significantly lower than Nanodrops 1 and 2 [$F(1,43)=62.833$, $P=0.000$ and $F(1,28)=107.919$, $P=0.000$, respectively]. Tumor tissue DNA yields were significantly greater than normal tissues [Qubit; $F(1,43)=53.122$, $P=0.000$, Table 2]. Allprotect tissues did not exhibit reduced DNA yields [$F(1,28)=0.229$, $P=0.635$], that is, in contrast to QIAamp (Fig. 4).

Neither Allprotect stabilization nor tissue type affected $A_{260/280}$ values significantly [$F(1,43)=0.976$, $P=0.329$ and $F(1,43)=3.118$, $P=0.085$, respectively]. This was reflected in similar mean values (Table 2). The mean $A_{260/280}$ value, 1.94, indicated that DNA was pure and of good quality. The majority of scored DNA isolates obtained high DNA Quality Scores (54%), and 46% obtained good quality scores.

RNA. Bioanalyzer and Nanodrop1 were employed to quantify RNA isolates. Bioanalyzer RNA quantifications were significantly lower than Nanodrop1 values [$F(1,43)=14.974$, $P=0.000$]. Tumor tissue RNA yields were significantly higher than normal tissues [Bioanalyzer; $F(1,43)=17.423$, $P=0.000$ and Nanodrop1; $F(1,43)=19.352$, $P=0.000$]. Allprotect stabilization did not significantly influence RNA yields [Bioanalyzer; $F(1,43)=0.300$, $P=0.587$ and Nanodrop1; $F(1,43)=1.139$, $P=0.292$].

The Bioanalyzer also assigned RINs according to the 28s and 18s ribosomal profiles of RNA isolates (Electropherograms). Each region of the electropherogram provided information on the integrity of the RNA (Fig. 3b). Tumor tissue RIN values were significantly higher than normal tissues [$F(1,25)=7.498$, $P=0.011$, $T\bar{x}=8.5\pm1.4$, $NT\bar{x}=7.2\pm1.4$, Table 2]. Allprotect-stabilized and FF tissue RNA isolates produced comparable RINs [$F(1,25)=0.280$, $P=0.601$].

Protein. The Pierce 660 nm Protein Assay was utilized to quantify AllPrep-isolated proteins. Protein yields were significantly higher for tumor tissues than for normal tissues [$F(1,40)=6.150$, $P=0.017$]. This was unsurprising given the difference in cellularity and dramatically different means [$TT\bar{x}(\mu g/\mu L)=121.7\pm77.6$, $NT\bar{x}(\mu g/\mu L)=67.2\pm78.2$]. Allprotect treatment did not significantly influence protein yields [$F(1,43)=2.757$, $P=0.105$]. Western blot analysis confirmed the presence of actin in AllPrep protein isolates (Fig. 3d).

RNA isolation using the RNeasy Kit

The Bioanalyzer and Nanodrop1 were employed to quantify RNA yields. Bioanalyzer RNA quantifications were significantly lower than Nanodrop1 [$F(1,43)=12.993$, $P=0.001$]. Tumor tissue RNA yields were significantly greater than normal tissues [$F(1,28)=17.278$, $P=0.000$, Table 2]. FF tissues exhibited significantly higher RNA yields [Bioanalyzer; $F(1,28)=4.426$, $P=0.044$]. Allprotect treatment did not influence RNA quality [$F(1,14)=0.430$, 0.522]. RIN values from tumor tissues were significantly greater than normal tissues [$F(1,14)=0.430$, 0.051, Table 1].

DNA yield and quality–QIAamp vs. AllPrep

QIAamp yields were significantly greater than AllPrep yields [Qubit; $F(1,28)=4.683$, $P=0.039$, Table 2]. FF and

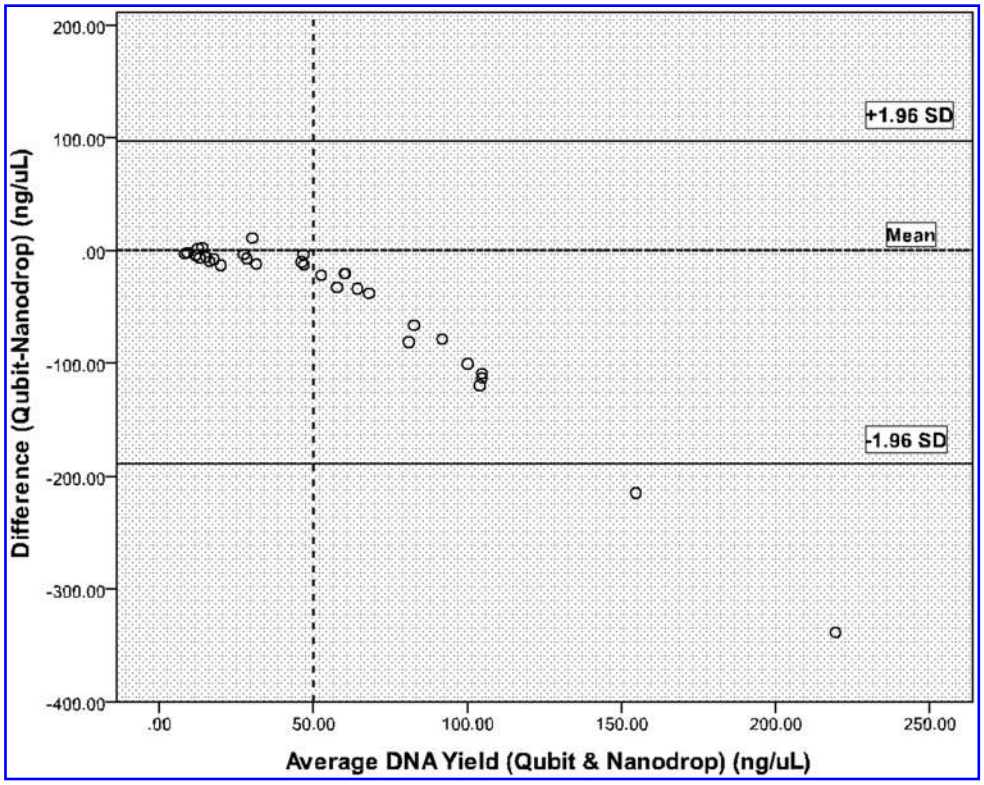

FIG. 2. Bland-Altman Plot of Difference (Qubit-Nanodrop) (ng/μL) vs. average DNA yield (mean of Qubit and Nanodrop).

TABLE 2. QUANTITY AND QUALITY OF DNA, RNA, AND PROTEIN ISOLATED UTILIZING QIAGEN'S QIAAMP, ALLPREP, AND RNEASY KITS

Extraction Kit	*Tumor tissue mean (T)*	*Normal tissue mean (N)*	*Fresh-frozen tissue mean (FF)*	*Allprotect-treated tissue mean (AP)*	*Overall mean (T, N, FF, AP)*	P *value* *QIAamp vs. AllPrep*	P *value* *RNEasy vs. AllPrep*
QIAamp							
DNA							
Qubit (ng/μL)	42.5 ± 11.6	29.0 ± 17.0	37.8 ± 13.9	25.5 ± 18.3	33.2 ± 16.5		
NanoDrop1 (ng/μL)	162.6 ± 124.3	48.6 ± 35.5	106.4 ± 104.2	47.2 ± 45.3	84.2 ± 90.7		
NanoDrop2 (ng/μL)	143.3 ± 118.3	42.6 ± 34.5	94.5 ± 96.6	40.0 ± 43.8	74.1 ± 84.3		
A260/280	1.89 ± 0.13	1.85 ± 0.15	1.87 ± 0.10	1.83 ± 0.20	1.86 ± 0.14		
AllPrep							
DNA							
Qubit (ng/μL)	44.1 ± 15.6	13.7 ± 16.1	28.2 ± 21.3	14.9 ± 19.1	23.2 ± 21.2	0.039	
NanoDrop1 (ng/μL)	169.3 ± 80.6	32.7 ± 52.8	104.3 ± 98.1	94.2 ± 96.2	99.6 ± 96.3		
NanoDrop2 (ng/μL)	124.1 ± 51.8	60.2 ± 39.0	91.2 ± 46.0	91.6 ± 65.9	91.4 ± 55.6		
A260/280	1.91 ± 0.01	1.95 ± 0.10	1.93 ± 0.06	1.97 ± 0.11	1.94 ± 0.08	0.004	
RNA							
NanoDrop1 (ng/μL)	197.0 ± 163.9	19.2 ± 26.7	107.3 ± 145.9	20.6 ± 35.1	74.8 ± 123.7		0.418
Bioanalyzer (ng/μL)	136.0 ± 131.7	20.0 ± 27.2	75.4 ± 110.3	24.3 ± 35.0	56.2 ± 92.3		
RIN	8.47 ± 1.37	7.17 ± 1.38	7.71 ± 1.61	8.57 ± 0.90	7.92 ± 1.47		0.055
Protein							
Protein yield (mg/ml)	1.22 ± 0.78	0.67 ± 0.78	0.80 ± 0.49	1.13 ± 1.05	0.96 ± 0.82		
RNeasy							
RNA							
NanoDrop1 (ng/μL)	193.2 ± 127.1	19.0 ± 10.0	92.3 ± 119.7	41.9 ± 76.7	73.4 ± 107.2		
Bioanalyzer (ng/μL)	149.9 ± 124.8	18.6 ± 30.1	77.6 ± 113.0	29.7 ± 40.8	59.6 ± 94.7		
RIN	8.94 ± 0.36	7.06 ± 2.79	8.02 ± 2.24	8.47 ± 0.84	8.16 ± 1.96		

Numbers in this table were calculated by using repeat measurement ANOVAs. Values differ from those of 2-way ANOVAs, as repeat measurement ANOVAs require pair-wise comparisons between variables, whereas 2-way ANOVAs are comparisons within a variable (eg, QIAamp Qubit quantifications within group analysis would include T vs. N and FF vs. AP). QIAamp and AllPrep DNA yields and quality were compared (red and blue *P* values, respectively). The quantity and quality of RNA RNeasy and AllPrep isolates were also compared (green and bold black *P* values, respectively).

ANOVA, analysis of variance; RIN, RNA Integrity Number.

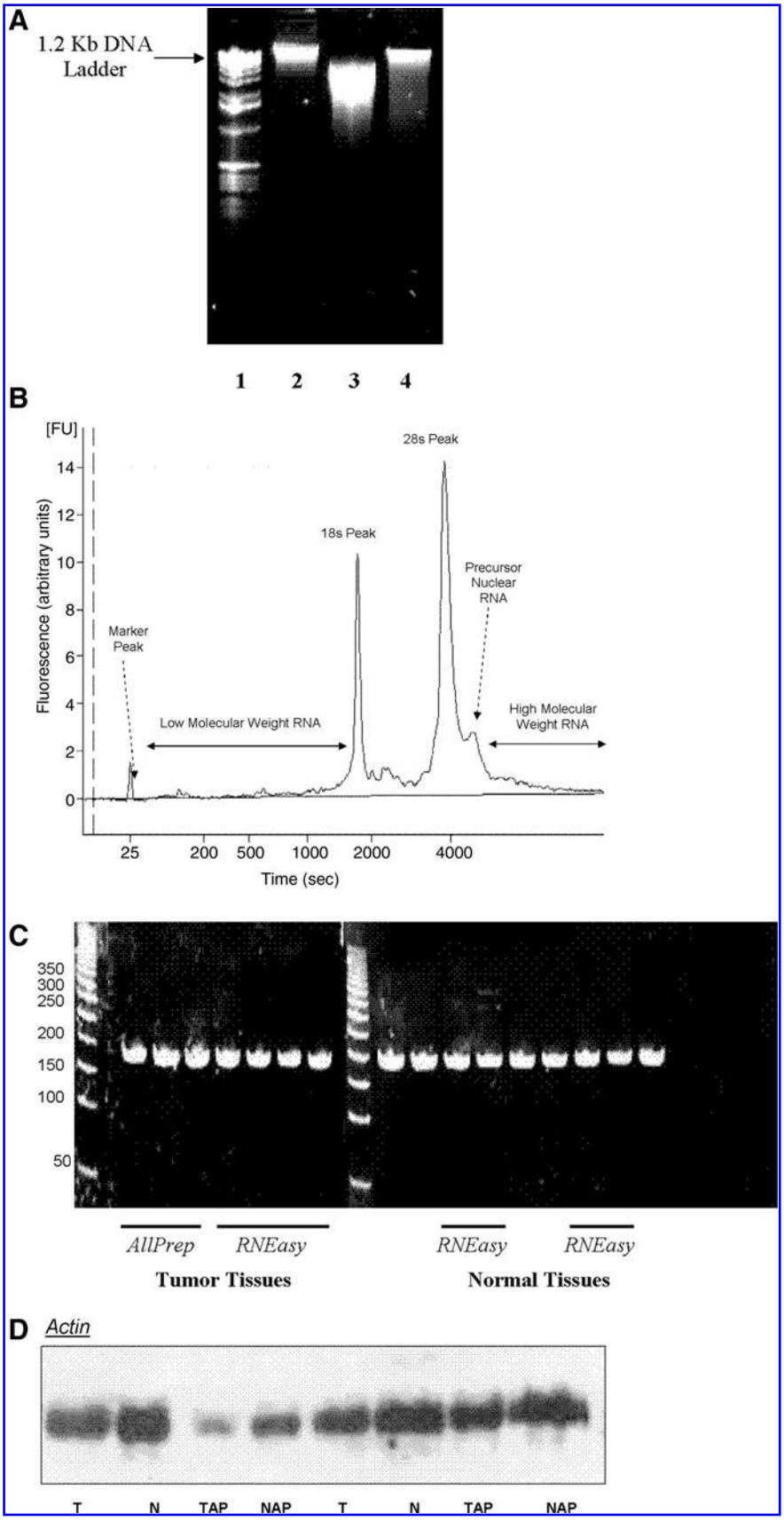

FIG. 3. **(A)** Agarose gel electrophoresis of DNA isolated from tumor and normal breast tissues (100 ng). Lane 1, 1.2 Kb DNA ladder; lane 2, normal breast tissue (QIAamp isolate); lane 3, normal breast tissue (AllPrep isolate), and lane 4, Allprotect treated tumor breast tissue (QIAamp Isolate). **(B)** Interpretation of breast tumor bioanalyzer electropherogram (RNA Integrity Number, RIN 9.4). **(C)** Acrylamide gel electrophoresis (10%) of glyceraldehyde-3-phosphate reverse transcription polymerase chain reaction gene products from tumor and normal breast tissue RNA isolates (100 ng). RNEasy and AllPrep RNA isolates were compared and showed no discernible differences. **(D)** Western blot analysis of beta-actin in T (tumor), N (normal), TAP (Allprotect stabilized tumor), and NAP (Allprotect stabilized normal) breast tissue samples (100 μg). Actin was present in all samples tested.

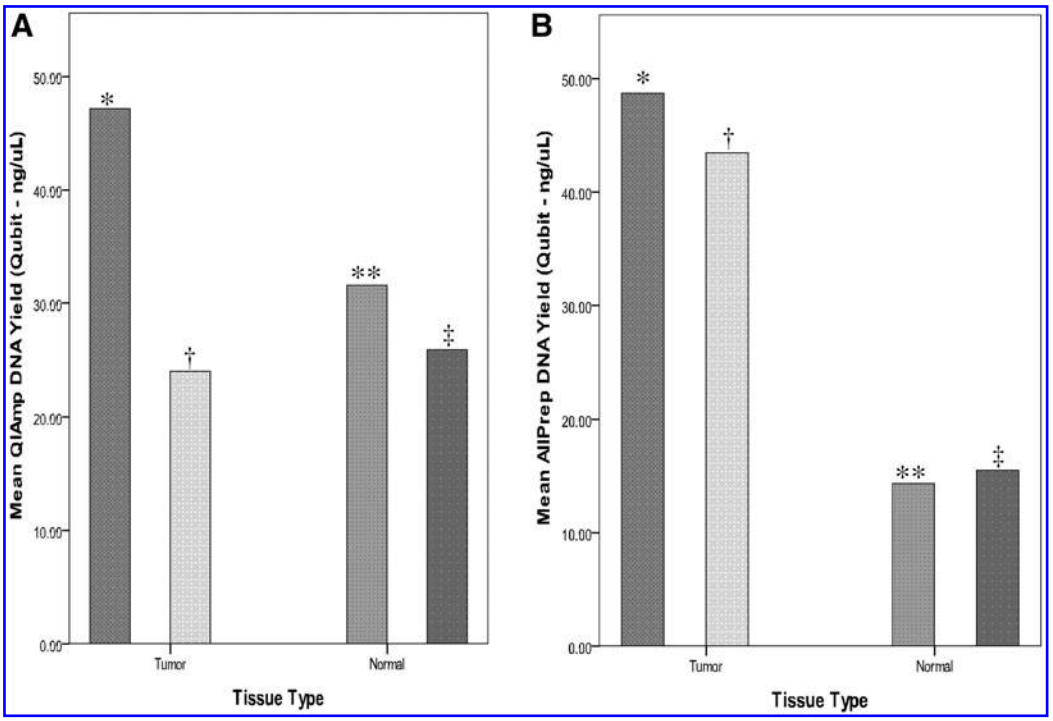

FIG. 4. **(A, B)** Qubit DNA yields for fresh frozen tumor tissue (*), Fresh frozen normal tissue (**), Allprotect-treated tumor tissue, and Allprotect-treated normal tissue isolated using **(A)** Qiagen's QIAamp kit **(B)** Qiagen's AllPrep kit.

tumor tissues produced significantly higher DNA yields across both kits [$F(1,28) = 5.149$, $P = 0.031$, and $F(1,28) = 6.230$, $P = 0.019$, respectively]. QIAamp was most compatible with FF tissue (see Fig. 4) [significant 2 way interaction; $F(1,28) = 6.543$, $P = 0.016$].

AllPrep produced DNA isolates with significantly greater $A_{260/280}$ values than QIAamp [$F(1,28) = 9.706$, $P = 0.004$, Table 2]. Neither Allprotect treatment nor tissue type significantly influenced $A_{260/280}$ values [$F(1,28) = 1.195$, $P = 0.284$ and $F(1,28) = 0.776$, $P = 0.386$, respectively]. In contrast, DNA Quality Scores for QIAamp and AllPrep were comparable ($Z = -1.584$, $P = 0.113$).

RNA yield and quality–RNeasy vs. AllPrep

RNeasy and AllPrep RNA yields were comparable [Bioanalyzer; $F(1,28) = 0.003$, $P = 0.958$ and Nanodrop1; $F(1,28) = 0.676$, $P = 0.418$]. Both kits demonstrated significantly higher RNA yields from tumor tissues [Bioanalyzer; $F(1,28) = 5.043$, $P = 0.033$ and Nanodrop 1; $F(1,28) = 21.281$, $P = 0.000$, Table 2]. FF tissues produced significantly higher RNA yields [Bioanalyzer; $F(1,28) = 10.800$, $P = 0.003$].

RIN values were also comparable across the 2 kits [$F(1,8) = 0.000$, $P = 0.999$]. Tumor tissue RINs were significantly higher than normal tissue RINs for both RNeasy and AllPrep [$F(1,8) = 8.754$, $P = 0.018$, Table 2]. RINs were higher for Allprotect-stabilized tissues, though this effect was of borderline significance [$F(1,8) = 5.026$, $P = 0.055$].

RT-PCR of GAPDH also produced similar amplification profiles for RNeasy and AllPrep RNA isolates (Fig. 3c). Beta-2-microglobulin (β2-MG), Abelson (ABL), and Breakpoint Cluster Region (BCR) genes were RT-PCR amplified from both AllPrep and RNeasy isolates (data not shown).

RNA stability

There were no significant changes in RNA quality (RINs) during 6 h incubations of RNA isolates at room temperature [$F(1,12) = 1.524$, $P = 0.257$) or at 4°C [$F(1,12) = 0.778$, $P = 0.481$]. Allprotect did not influence RNA stability at room temperature [$F(1,12) = 0.566$, $P = 0.582$] or at 4°C [$F(1,12) = 0.877$, $P = 0.441$]. RNA integrity appeared unaffected by 9 freeze-thaw cycles [$F(1,24) = 0.671$, $P = 0.578$]. A significant protective effect for Allprotect was again undetected [$F(1,24) = 0.759$, $P = 0.528$].

Discussion

The hallmark of a good cancer biobank is the quality of its samples. Tissue samples should be obtained from the patient donor's surgical resection specimen by the pathologist without jeopardizing full pathological examination, which forms the basis of further treatment. The results presented here clearly show that the essential teamwork and procedures introduced for breast biobanking in this hospital-based biobank did not adversely affect breast tissue sample quality (Table 1 and Fig. 3). Microscopic examination of frozen sections predicted the approximate yield of nucleic acids (data not shown). Tumors are hypercellular as a result of deregulated cell proliferation,[11] and, thus, tumor tissues exhibited consistently greater RNA, DNA, and protein yields than normal breast tissues. This was not unexpected. In mastectomy specimens in particular, normal breast tissue is nearly all fat, with a few or no ducts or acini, which contain the normal "control" epithelial cell analogs of cancer cells.[7] Samples vary considerably in the volume of tumor cells they contain, and the extent of fibrosis or necrosis present. Certain samples biobanked as either normal or cancer will contain a

mixture of cancer cells and normal breast ducts (Fig. 1). The yield of nucleic acids from normal "control" breast tissue might conceivably be increased by sampling below the nipple and areola, where there is a concentration of normal large ducts. However, sampling normal breast ducts remains a problem in wide local excision and small breast cancer specimens. Downstream amplification may be required to increase nucleic acid yields from normal breast tissues.

This study failed to demonstrate an improvement in the quality of molecular isolates that would justify the extra cost of using Allprotect as a stabilizing medium. Allprotect was initially thought to produce a protective effect, because stabilized tissues had higher RIN values than FF tissue. However, this was of borderline significance only ($P = 0.055$, Table 2). Future evaluation of Allprotect should include comparisons of gene expression profiles in FF and Allprotect-treated tissues. Chowdary et al.[12] determined that a 95% correlation existed between RNAlater-stabilized and snap-frozen tissues. Allprotect should attain a similar level of correlation to be considered a viable substitute for RNAlater.

In our hands, AllPrep proved very effective for the simultaneous isolation of RNA, DNA, and proteins, from both tumor and normal breast tissues, providing that starting breast tissue material did not exceed 10–20 mg. AllPrep isolated DNA and RNA in concentrations [DNA (Qubit) T = 4.4 μg, N = 1.4 μg and RNA (Bioanalyzer) T = 8.2 μg, N = 1.2 μg] conducive to downstream applications such as cDNA microarrays. Earlier, researchers considered that the adipose tissue-rich nature of normal breast tissue precluded the use of conventional extraction kits.[31,32] AllPrep will continue to be utilized by the SJHB: in theory, molecular isolates from breast tissues should correlate more closely when a single extraction kit is employed. Importantly, the simultaneous isolation of RNA, DNA, and proteins will potentially increase the number of studies in which a single sample can be included, thus ensuring that the information gleaned from each valuable tissue sample is maximized. However, the assessment of quality is, nonetheless, a surrogate measure for potential downstream applications, such as expression arrays, PCR, and genotyping. Though measures of quality do not predict success, they constitute a useful guide for determining the appropriateness of samples for inclusion in specific assays.

AllPrep DNA yields (23 ng/μL) were 30% lower than those with QIAamp (33 ng/μL), (Table 2). However, in terms of quality, AllPrep's $A_{260/280}$ values were significantly higher than QIAamp's (1.94 and 1.86, respectively, Table 2). DNA Quality Scores failed to detect a significant difference between the kits, despite the fact that 54% of AllPrep DNA isolates obtained high-quality scores compared with 40% with QIAamp. However, awarding scores based on the appearance of DNA bands on agarose gels is clearly a more subjective assessment.

RNeasy and AllPrep RNA yields were similar (Table 2) and produced RNA isolates of comparable quality (RIN: 7.9 and 8.2, respectively). GAPDH, β2-MG, ABL, and BCR genes were RT-PCR amplified from both AllPrep and RNeasy isolates (data not shown). The presence of actin was confirmed in AllPrep protein isolates (Fig. 3d). Notably, AllPrep isolated up to 2.41 mg/ml of protein from 10 mg of starting tissue. This is important, as tissue proteomics is central to the discovery of biomarkers and potential therapeutic targets, and the improved isolation of proteins is essential for the future of personalized medicine.[33]

To date, AllPrep has received mixed reviews. Radpour et al.[34] reported that DNA, RNA, and protein were successfully isolated from a "single sample aliquot" but noted that modifications to Qiagen's original protocol were required to optimize yields. Gross et al.[35] reported several drawbacks including a low capacity to bind RNA. Significantly, Gross employed PC12 cells rather than tissue to assess AllPrep, and may have employed a different AllPrep kit to the one utilized in this study.[35]

Xu et al.[17] reported that AllPrep extracted significantly less RNA and DNA than PicoPure (RNA) and QIAamp (DNA) (62% and 10%, respectively). Those authors also found that DNA was consistently contaminated with a 28s ribosome peak.[17] A possible explanation is that they may have overloaded the column, because the quantity of tissue applied to the column was never weighed. In addition, tissue was resuspended in 50 μL guanidine thiocyanate buffer (RLT) and not the recommended 300–600 μL. Several agarose gels were inspected during the present research, and no contaminating 28s ribosomal peak was detected. Qiagen warn against overloading AllPrep's column, stating that doing so may reduce RNA yields and cause RNA and DNA co-elution.[36] Xu et al. also noted that AllPrep and QIAamp DNA isolates produced similar amplification profiles.[17] Those profiles differed considerably from Trizol amplification profiles: larger fragments were identified only in Trizol extracts. In contrast, Ennis et al.[37] reported that Trizol isolated RNA in greater quantities, but of poorer quality than that from Qiagen's RNeasy kit. De Cecco et al.[38] utilized Trizol to investigate changes in breast cancer expression profiles as a function of time (at room temperature), and reported that 0.76% of genes were modified after 2 h and 0.98% after 6 h. However, their electropherograms display low-molecular-weight components of degradation not evident in our study, and the average tumor RIN value of 7.55 was low compared with AllPrep and RNeasy RIN values recorded herein (8.47 and 8.94, respectively). This might suggest that Trizol produced RNA of suboptimal quality, but other factors may be involved.[38] Understanding the relationship between post-excision modifications and alterations in gene expression profiles is a crucial prerequisite to the incorporation of gene expression analysis into clinical practice.

Our RNA isolates displayed surprising levels of stability. RNA quality was unchanged after 6 h incubations (4°C and room temperature) and successive freeze-thaw cycles. Other researchers have also failed to detect RNA degradation after 1, 3, and 16 h incubations at room temperature.[8,10] However, isolated RNA cannot be compared with cellular RNA, which exists in a dynamic environment,[7,8] and RNA stability results should be interpreted with caution. Freeze-thaw cycles are potentially more damaging to RNA integrity than fixed temperature incubations.[23] Further, although the Bioanalyzer is a recognized technique for ascertaining RNA quality and less subjective than gel electrophoresis, its limitations—sensitivity, linearity, and reproducibility between chips—are often overlooked in lieu of convenience. An alternative platform (Experion) is reported to show superior reproducibility and quantity assessment.[9,39,40]

The storage and release of quality samples are the *sine qua non* of a modern biobank, and biobank personnel assume this responsibility on behalf of patient donors, funders, and researchers, including those who ultimately develop new patient interventions. Biospecimen science focuses on

improving the quality of a biobank's unique human sample resource, and will henceforth ensure that fewer research studies are discredited because of substandard samples.[41–47] These principles are being applied to the expansion of the Irish biobank network.

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Author Disclosure Statement

The authors declare that they have no competing interests.

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