

Fit for Purpose Frozen Tissue Collections by RNA Integrity Number-Based Quality Control Assurance at the Erasmus MC Tissue Bank

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About 5000 frozen tissue samples are collected each year by the Erasmus Medical Center tissue bank. Two percent of these samples are randomly selected annually for RNA isolation and RNA Integrity Number (RIN) measurement. A similar quality assessment was conducted during centralization of a 20-year-old tissue collection from the cancer institute, a 15-year-old liver sample archive (-80°C), and a 13-year-old clinical pathology frozen biopsy archive (Liquid Nitrogen). Samples were divided into either high-quality (RIN ≥ 6.5) or low-quality overall categories, or into four “fit-for-purpose” quality groups: RIN < 5 : not reliable for demanding downstream analysis; $5 \leq \text{RIN} < 6$: suitable for RT-qPCR; $6 \leq \text{RIN} < 8$: suitable for gene array analysis; and RIN ≥ 8 : suitable for all downstream techniques. In general, low RIN values were correlated with fatty, fibrous, pancreatic, or necrotic tissue. When the percentage of samples with RIN ≥ 6.5 is higher than 90%, the tissue bank performance is adequate. The annual 2011 quality control assessment showed that 90.3% ($n=93$) of all samples had acceptable RIN values; 97.4% ($n=39$) of the cancer institute collection had RIN values above 6.5; and 88.6% ($n=123$) of samples from the liver sample archive collection had RIN values higher than 6.5. As the clinical pathology biopsy collection contained only 58.8% ($n=24$) acceptable samples, the procurement protocols used for these samples needed immediate evaluation. When the distribution of RIN values of the different collections were compared, no significant differences were found, despite differences in average storage time and temperature. According to the principle of “fit-for-purpose” distribution, the vast majority of samples are considered good enough for most downstream techniques. In conclusion, an annual tissue bank quality control procedure provides useful information on tissue sample quality and sheds light on where and if improvements need to be made.

Introduction

EVER SINCE THE HUMAN GENOME PROJECT¹ was completed and high-throughput genomics became available, the demand for high-quality human samples for medical research increased. In the case of rare diseases, research teams joined their efforts in consortia, sharing samples² to form large enough cohorts for solid statistical analysis.³ In general, professional biobanking of various samples such as blood, serum, urine, and tissue increased. Soon after formation of the first international cooperative groups, inter-center variation (institutional bias) due to lack of standardization of sample procurement protocols was observed, and standardization of sample procurement procedures became desirable to enhance exchangeability and comparability of samples. This need for harmonization and standardization led to the establishment of the Best Practices for Repositories by the International Society for Biological and

Environmental Repositories (ISBER).⁴ While biorepositories evolved from activities within clinics or pathology laboratories into professional institutes, the need to maintain appropriate levels of sample quality also grew. Quality control (QC) and quality assurance (QA) schemes were designed⁵ and are now embedded in the routine procedures of many biobanks.

The main benefit of these QC (measurement) and QA (improvement) exercises is to establish an efficient and constant tissue bank workflow in which all protocols are designed to deliver high-quality samples. This automatically implies that tissue banks need dedicated personnel who are not only skilled in procuring samples the right way, but who can also, on a multi-disciplinary level, improve the sample quality. Tissue biobank personnel should give clinicians directions on how to send in their specimens under the specific conditions needed for optimal tissue sample procurement. In addition, biobank personnel must

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be able to facilitate clinical trials, which may require specialized tissue procurement protocols. Furthermore, biobank personnel must also be able to perform the QC and subsequent QA to determine systematic errors and non-compliance with Standard Operating Procedures (SOPs). There are several steps involved in the process of QC, ranging from traceability of samples to RNA integrity measurement. The kind of QC performed by a biobank depends on the kinds of samples preserved and in what way. For instance, a biobank with blood and serum samples will most probably focus on the quality of DNA and serum proteins,⁶ while a tissue biobank will also have to assess tissue morphology (percentage of tumor or necrosis in a sample),⁵ and molecular integrity. The latter will be described in this article.

At our facility, the accuracy of sample storage (i.e., the position of a sample in the freezer as recorded in the tissue bank database) is assessed by randomly selecting 2% of samples,⁷ and confirming in the database whether the numbers of the tubes match the contents of the tube. For this reason, frozen hematoxylin and eosin (H&E) sections are prepared from the randomly selected samples and compared to a formalin-fixed and paraffin-embedded H&E section of an adjacent piece of tissue that was taken and processed during sample procurement. In this way, the tissue and disease type can be determined and compared to the description in the biobank database to confirm proper annotation and storage position. In addition, the quality of frozen tissue samples is assessed by RNA isolation and RNA Integrity Number (RIN) measurement. In order to achieve reliable QC results, biobank personnel must know how to isolate RNA in the most reproducible and accurate manner. If the RNA isolation is done with sub-standard materials or following protocols that lead to RNA degradation, the RIN values may be lower than expected for technical reasons. This would result in misleading performance indicators because they would be based on technical shortcomings rather than true tissue quality. The ISBER RNA Proficiency Testing Program confirms that the method used for assessment of RNA quality is accurate.⁸ The QC and QA programs allow tissue bank personnel to trust their product and their skills and then provide advice to researchers who want to do high-quality research on tissues. Because of the high-quality standards pursued by the central tissue bank, many researchers have transferred their private collections into the biobank in order to obtain and maintain a higher level of sample quality, and to take advantage of the tissue bank's expertise and expanding facilities.

In this article we describe how and what we have learned about our frozen tissue sample collections during our standard QC and QA processes. Tissue bank performance was assessed by measuring RIN values of a random selection of samples that were acquired over the past 6 years; this was primarily to determine systematic errors or noncompliance with SOPs. In addition, the quality of several centralized frozen tissue collections, which had been received from researchers from several sites within our institute, was assessed. Samples in these collections had been procured using different protocols and/or were stored under different conditions. These assessments gave insight into the quality of the collections considering the different procurement and storage conditions, and the influence of these conditions on RNA, and therefore sample integrity.

Materials and Methods

Quality assurance procedures and quality criteria

The Erasmus Medical Center tissue bank QC procedure is based on the fact that RNA is unstable and therefore sensitive to pre-analytical factors. RNA integrity can be reliably measured using microcapillary gel electrophoresis from which the RIN value can be calculated. In the literature, RNA with RIN values <5 have been deemed to be of a quality that is too low for demanding downstream RNA analysis; RIN values higher than 8 have been considered to be perfect.⁹ The cut-off RIN values for samples used in gene expression arrays appear to be between 6 and 7.^{10,11} Considering these literature references, we used a RIN value of 6.5 as a cut-off for RNA quality and therefore, tissue quality. In other words, if RNA with RIN values >6.5 can be derived from a tissue sample, the tissue sample is assumed to also provide DNA and proteins of sufficient quality for demanding techniques.

The assessment of tissue bank performance can be determined by RIN values, but RIN values can be influenced by tissue type. A low RIN value is not always due to procurement errors; certain tissue types notoriously yield too little RNA for exact RIN measurement, or contain naturally degraded RNA. This is why frozen sections are also made during RNA isolation for morphological assessment. "Badly-procured" samples are those with low RIN values even though the cells are morphologically viable and there is enough RNA for measurement. Fatty tissue has a low cell density and contains lipids that interfere in the RNA isolation protocol (low RNA yield). Pancreatic tissue contains high levels of endogenous RNase in combination with proteolytic enzymes, which can cause RNA to be degraded when the isolation is not performed fast enough. Fibrous and muscle tissues are difficult to disrupt and do not contain that much RNA. And necrotic tissue does not contain viable cells.¹²

Tissue bank performance is more accurately assessed by omitting from the calculations the results from notoriously low-yielding tissues. By taking into account only those tissue types where it is possible to collect good quality samples routinely (and true "badly-procured" samples can therefore be recognized) can tissue bank performance be calculated as the percentage of "badly-procured" samples. It has been suggested that if this percentage is below 10%,¹³ the tissue bank can be deemed to function well. As soon as this percentage rises above 10%, it suggests that systematic errors may have occurred or that there has been noncompliance with tissue bank protocols. In that case, the tissue bank protocols and procedures, as well as the practice of individual tissue bank technicians, must be evaluated to improve the overall quality.

To gain additional insight into the scientific value of the samples, they were divided into four different "fit-for-purpose" quality groups based on information from the literature:⁹⁻¹¹ RIN <5: not reliable for demanding downstream analysis; 5 ≤ RIN <6: suitable for RT-qPCR only; 6 ≤ RIN <8: suitable for RT-qPCR and gene expression arrays; and RIN ≥ 8: suitable for all downstream techniques. This approach provides a more comprehensive "fit-for-purpose" quality assessment of the biobank, since a single RIN cut-off may lead to underestimation of tissue sample quality and thus the scientific value of the biobank. The

notoriously low-yielding tissues were also omitted from these calculations. This way, only the true “badly-procured” samples are taken into account.

The average number of frozen tissue samples collected annually in the Erasmus MC tissue bank has grown to about 5000 and each year approximately 2% are randomly chosen for QC.⁷ In the case of rare tumors, other samples from the same acquisition date were selected. In the earlier years, lower percentages were used. Recently, the tissue bank has assumed and centralized three peripheral tissue collections and the same QC scheme was used to assess their value.

Tissue collections

Annual QC of centralized tissue bank. Tissues of various organs and disease types (mostly tumors) were collected during routine macroscopic examination of the specimens. The transport time from the operating theater to the pathology laboratory varied between 30 min and 2 h; the longer time was due to batching of specimens for more cost efficient transport. Samples were placed on a piece of filter paper covering a cork sheet for orientation reasons. The tissues were snap frozen in pre-cooled isopentane and placed in pre-cooled vials with screw lids (3 mL cryo tubes,

Sanbio B.V., The Netherlands). The samples were stored in liquid nitrogen for a year before the QC procedure took place. For the 2011 QC RNA isolation procedure, 103 samples (2.1% of the 4394 collected samples) were randomly picked from the biobank database (Table 1). Samples collected during autopsy were not selected for RNA isolation. QC assessments for the preceding years (2006–2010), were performed on 1.3% (33 of 2631), 0.7% (17 of 2316), 0.5% (18 of 3484), 1.7% (67 of 3905), and 2.2% (98 of 4458) of the samples, respectively, using comparable tissue types (no further details shown).

Cancer institute archive collection. Tissues of various organs and disease types (mostly tumors) were collected during routine macroscopic examination of the specimen. The transport time was between 5 and 30 min, due primarily to the short distance between the operating theater and the pathology laboratory. Samples were directly frozen in liquid nitrogen and stored in the same type of containers as described above. The samples had been collected and stored in mechanical –80°C freezers for over 20 years before they were transferred to the central tissue bank in 2005. Forty-eight (48) samples (2% of the approximately 2500 collected) were randomly selected for the QC RNA isolation procedure (Table 2).

TABLE 1. AVERAGE RIN VALUES OF SELECTED TISSUE FROM THE ERASMUS MC TISSUE BANK*

<i>Tissue type</i>	<i>Average RIN</i>	<i>Standard deviation</i>	<i>N</i>	<i>N with RIN < 6.5</i>	<i>Tissue type</i>	<i>Average RIN</i>	<i>Standard deviation</i>	<i>N</i>	<i>N with RIN < 6.5</i>
Abdomen	7.80		1		Penis	7.60		1	
Adnex	6.90		1		Peritoneum	7.80	0.42	2	
Adrenal gland	8.03	0.76	3		Prostate	7.95	0.21	2	
Bladder	8.07	0.60	3		Rectum	7.77	1.81	3	1
Breast	8.45	0.07	2		Skin	4.95	3.46	2	1
Cervix	9.10		1		Soft Tissue	8.00	0.42	5	
Colon	7.44	0.80	5		Spleen	5.70		1	1
Esophagus	7.83	1.01	3		Stomach	7.60	1.13	3	1
Ethmoid	8.80		1		Testis	7.80	0.71	2	
Gall bladder	7.10		1		Thorax	7.90		1	
Kidney	8.17	0.25	3		Thymus	8.05	0.78	2	
Larynx	8.77	0.21	3		Thyroid	8.20	0.26	3	
Liver	8.09	0.76	7		Tongue	7.40		1	
Lung	6.99	0.60	7	1	Tonsil	7.03	1.52	3	1
Lymph node	7.75	1.00	6		Tuba	7.75	0.35	2	
Nose	8.30		1		Ureter	7.90		1	
Ovary	5.33	2.78	3	2	Uterus	5.50	4.38	2	1
Pancreas	7.97	0.29	3		Vulva	8.05	0.35	2	
Parotid	8.90		1						

LOW YIELDING TISSUE SAMPLES EXCLUDED FROM ANALYSIS

<i>Tissue type</i>	<i>RIN</i>	<i>Reason for exclusion</i>
Adrenal gland	5.6	partially necrotic
Adrenal gland	N/D	totally necrotic
Bladder	N/D	TUR heat damage**
Muscle	N/D	fibrosis
Nerve	N/D	low cellular content
Omentum	5.2	fatty
Ovary	N/D	partially necrotic
Soft Tissue	1.1	fatty
Bladder	4.8	TUR heat damage**
Omentum	5.8	fatty

*From 2011 annual QC evaluation; ** TUR, Trans Urethral Resection (with electric scalpel); N/D, Not determined.

TABLE 2. AVERAGE RIN VALUES OF SELECTED TISSUE FROM THE CANCER INSTITUTE ARCHIVE COLLECTION

Tissue type	Average RIN	Standard deviation	N
Breast	8.20		1
Colon	8.20	1.27	2
Larynx	8.15	1.48	2
Liver	8.23	0.71	4
Lung	8.50		1
Lymph node	7.63	0.92	15
Mouth	7.70		1
Omentum	6.20		1
Ovary	7.25	0.35	2
Pancreas	8.30		1
Penis	7.40		1
Rectum	7.70	1.13	2
Salivary gland	8.67	0.38	3
Thymus	6.90		1
Thyroid	8.80		1
Tonsil	6.90		1

LOW-YIELDING TISSUE SAMPLES EXCLUDED FROM ANALYSIS

Tissue type	Average RIN	Standard deviation	N	Reason for exclusion
Soft tissue	2.40		1	necrosis
Colon	2.40		1	necrosis
Skin	2.60		1	necrosis
Lymph node	3.83	1.08	3	fatty
Liver	5.50		1	partially necrotic
Breast	3.00		1	fatty
Pancreas	3.70		1	fatty

Of all RIN measurements, the best of two assessments are shown in this table.

Liver sample archive collection. Liver samples were collected at the clinic (needle biopsies), the operating theater (wedge biopsies of liver transplantation patients), or during routine macroscopic examination of the specimen. The transport time of these samples was not recorded but was known to vary between 30 min and several hours. The longer waiting time was one reason to take over this collection and to expand it under control of the biobank. There is no record of how the samples were frozen during the first 5 to 7 years, but from 2003 till the present, samples were snap frozen in pre-cooled isopentane. Samples were stored in a -20°C freezer for several weeks to months before being transferred to a -80°C freezer where they were stored for at least 15 years before transfer of the collection to the central liquid nitrogen facility in 2007. One hundred and twenty-three (123) samples (2% of the approximately 5000 collected) were randomly selected for QA RNA isolation (Table 3). Since most of the samples were very small, morphology was not assessed.

Clinical pathology frozen biopsy archive collection. Biopsy samples of various tissue types were sent to the pathology laboratory from the outpatient clinics. Sample transport could have taken place under a variety of conditions (e.g., on ice, at room temperature, dry, on moist gauze, in salt solution). Unfortunately, there is no record of this. Since unfixed biopsies are treated with high priority by clinicians, the transport from the outpatient clinic to the pa-

TABLE 3. AVERAGE RIN VALUES OF SELECTED TISSUES FROM THE LIVER SAMPLE ARCHIVE COLLECTION

Diagnosis	Average RIN	Standard deviation	N	N RIN < 6.5
Abcess	9.10		1	
Adenoma	7.44	1.06	6	1
Alcohol hepatitis	6.30	1.41	2	1
Carcinoma	8.80		1	
Cellular decay	5.90		1	1
Cholestasis	8.00		1	
Chronic hepatitis	7.85	0.35	2	
Circulation disorder	8.28	0.59	5	
Cirrhosis	7.51	0.95	9	2
Fibrosis	8.90		1	
FNH	7.00		1	
HBV	7.35	1.66	17	2
HCC	7.70	0.69	10	
HCV	7.46	0.91	19	2
Hemangioma	7.40		1	
Inflammation	7.77	1.74	18	1
Ischemia	7.63	0.87	3	
Metastasis	7.84	0.65	8	
NEC	8.60		1	
Necrosis	3.00		1	1
Normal	8.20	0.68	4	
PSC	7.20	0.66	3	
Pseudo tumor	7.80		1	
Rejection	7.60	0.46	3	
Fatty liver	8.20	0.71	2	
Storage disorder	6.90		1	
Toxic reaction	5.30		1	1

FNH, focal nodular hyperplasia; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NEC, neuroendocrine carcinoma; PSC, primary sclerosing cholangitis.

thology laboratory never took longer than 30 minutes. The samples were frozen in pre-cooled isopentane and stored in liquid nitrogen. Interestingly, these biopsies were collected for histotechnical procedures that required fresh frozen (i.e., not formalin-fixed) tissue. Nineteen of these biopsies were used for cutting frozen sections and were put back after use. The archive dates back 13 years and samples were centralized in 2011. Of this relatively small collection (approximately 1000 samples), 24 samples (2%) were randomly taken for RNA isolation for QC (Table 4). Since samples were very small, morphology was not assessed.

RNA isolation, RIN measurement, and frozen tissue morphology

All QC assessments. A small drop of OCT (Sakura TissueTec, KliniPath, The Netherlands) was used to mount the selected tissue samples on the cryostat microtome object holder. The samples were not submerged in OCT to avoid interference with the RNA isolation procedure.

2006–2008 QC assessments. Frozen sections of $20\ \mu\text{m}$ thickness were cut and placed frozen in pre-cooled tubes with 1 mL RNA-Bee (Amsbio, Oxon, UK). The sections were disrupted by shaking the tube vigorously for about 5 sec. Chloroform ($200\ \mu\text{L}$) was added, the mixture was shaken for 15 sec and placed on ice for 5 min. After centrifugation at $12,000\ \text{g}$ at 4°C , the upper aqueous

TABLE 4. AVERAGE RIN VALUES OF SELECTED TISSUE FROM THE CLINICAL PATHOLOGY BIOPSY ARCHIVE COLLECTION

<i>Tissue type</i>	<i>Average RIN</i>	<i>Standard deviation</i>	<i>N</i>	<i>N with RIN < 6.5</i>	<i>Used for histology</i>
Cervix	8.10		1		no
Colon	4.00		1	1	yes
Conjunctiva	9.50		1		yes
Esophagus	7.60		1		no
Nasopharynx	7.90		1		no
Palate	2.20		1	1	yes
Rectum	8.43	0.68	3		yes
Skin	7.63	1.68	11*	4**	10 yes/1 no
Small intestine	7.90	1.41	2		yes
Stomach	8.00		1		no
Stomach/esophagus	5.80		1	1	no

*For two samples, no RIN values were available; these were excluded from the calculation of the average RIN value.

**Two samples with RIN values lower than 6.5 and two samples where no RIN value could be determined.

phase was transferred to a clean tube. An equal volume of cold (-20°C) isopropanol was added and the RNA was allowed to precipitate for 30 min in the -20°C freezer. After centrifugation for 30 min at 12,000 g, the supernatant was decanted and the RNA pellet washed with 80% ethanol. The RNA solution was centrifuged for 8 min at 12,000 g and the supernatant decanted. The RNA pellet was air dried and subsequently dissolved in 100 µL nuclease-free water.

2009–2011 QC assessments. Frozen sections of 10 µm thickness were cut and placed in 700 µL Qiazol (Qiagen, Germany). The tissue sections were disrupted by shaking the tubes vigorously for about 5 sec. To avoid chemical degradation of RNA by the Qiazol cell lysis solution, no more than six samples were isolated at once. Total RNA was isolated according to the protocol supplied with the miR-Neasy kit (Qiagen, Germany). The RNA samples obtained were placed on ice for approximately 30 min prior to RIN value measurement with RNA Nano Chips (Bio Analyzer 2100, Agilent, California). After the 10 µm sections for RNA isolation were cut, a 4 µm section was cut and mounted on a slide for H&E staining (LiniStainer, Leica, Germany).

Results

Annual QC

Of the 103 samples tested, 19 (18.4%) had a RIN value lower than 6.5 or the RIN value could not be determined (Table 1). Of these 19 samples, 9 (9.68%) had RIN < 6.5, and 10 were comprised of notoriously low-yielding tissue types—fibrous, fatty, or necrotic tissues, or tissues with otherwise low cellular content (see Fig. 1 for photographs of some typical examples).

According to the principal of “fit-for-purpose” distribution, the annual QC of the Erasmus tissue bank in 2011 ($n=93$ after correction for notoriously low yielding tissue types) showed that 4.3% ($n=4$) of samples were unreliable for demanding downstream procedures (RIN < 5); 3.2% ($n=3$) of the samples should only be used for RT-qPCR ($5 \leq \text{RIN} < 6$); 49.5% ($n=46$) of the samples can be used for gene array work ($6 \leq \text{RIN} < 8$); and 43% ($n=40$) of samples are considered fit for all demanding downstream techniques (RIN ≥ 8) (Fig. 2). The numbers in Table 1 reflect average

RIN values of N samples, while the numbers cited here reflect sample counts based on individual RIN values.

Results of the preceding QC assessments (2006–2010) were analyzed in the same fashion (data not shown). The changes in “fit-for-purpose” distribution (Fig. 3A), as well as the tissue bank performance (Fig. 3B) from 2006–2010 are shown in Figure 3. The number of samples unreliable for demanding downstream analysis decreases, while the number of samples suitable for RT-qPCR increases. In the high RIN value side of the spectrum, in general, the number of samples suitable for gene array analysis increase, while there is a small decrease in the number of samples suitable for all genomic analyses (Fig. 3A). To give a clearer presentation of deterioration or improvement of the tissue bank performance, the percentages of “badly-procured” samples (RIN < 6.5) from all annual QC assessments were converted to negative figures (Fig. 3B). This shows a 3-year period of substandard performance (2007–2009), which ultimately resolved during the last 2 years (2010–2011).

Cancer institute archive collection

Out of 48 tested samples, only 28 showed a RIN value above 6.5 after the first RNA isolation (Table 2). RNA isolation was repeated for 20 samples that showed low RIN values. After this re-assessment, RIN values improved and only 10 samples (20.8%) scored RIN values below 6.5. For 9 of these 10 samples, the low RIN values could be explained by tissue type and these were excluded from the analysis: five samples showed necrosis or a low number of cells, and the other four had good to fair morphology but it is notoriously difficult to obtain reasonable amounts of RNA from these tissue types (1 pancreatic tumor, 2 fatty lymph nodes, and 1 fatty breast).^{5,12} One sample had a RIN value lower than 6.5 (2.6%, $n=39$) that could not be explained by morphology. The numbers in Table 2 reflect average RIN values of N samples, while the numbers cited here reflect sample counts based on individual RIN values.

The “fit-for-purpose” distribution of the cancer institute collection ($n=39$ after correction for notoriously low-yielding tissue types) shows that none of the samples are unreliable for demanding downstream procedures (RIN < 5); no samples should only be used for RT-qPCR ($5 \leq \text{RIN} < 6$); 59% ($n=23$) of the samples can be used for

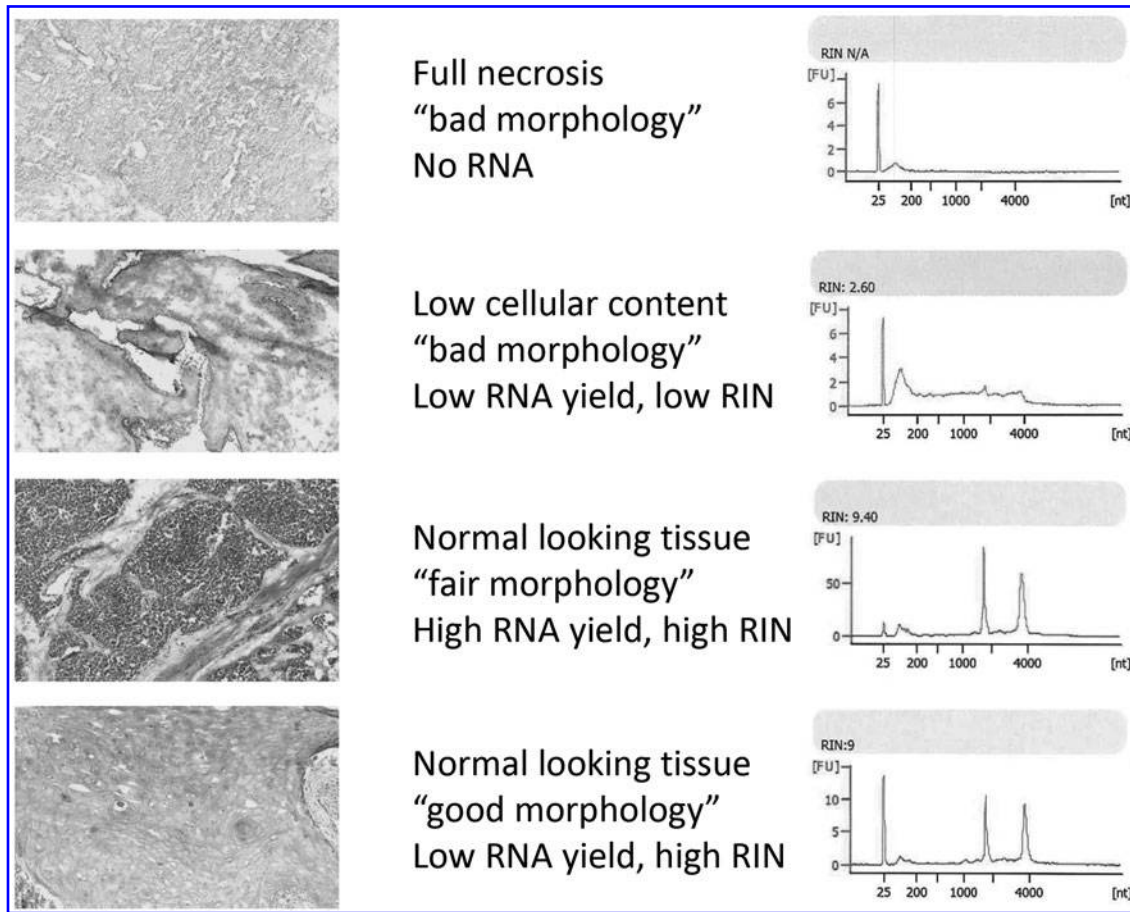


FIG. 1. Morphology scores and possible RNA yield and quality. Low RIN values are mostly correlated with morphology. Samples comprised of completely necrotic tissue (*top panel*) mostly do not yield RNA at all. When tissue is comprised of structures with low cell density (*second from top*) and the morphology shows either freeze artifacts or an abundance of noncellular matrix, RNA yield and integrity is mostly low. Normal looking tissue with fair morphology (*third from top*) is defined as tissue with high cellular content, absence of freeze artifacts, and relatively low amounts of fatty, necrotic, or fibrous tissue components. In some cases, excellent morphology and high cellular content does not correlate with high RNA yield. In the squamous cell carcinoma shown in the *bottom panel*, the RNA yield is low because the cells are hard to disrupt during RNA isolation; the RNA integrity, however, is high.

gene array work ($6 \leq \text{RIN} < 8$); and the remaining 41% ($n=16$) of samples are considered fit for all demanding downstream techniques ($\text{RIN} \geq 8$) (Fig. 2).

Liver sample archive collection

Out of 123 tested samples, 12 samples (9.8%) had RIN values below 6.5 (Table 3). The relatively high percentage of "badly-procured" samples cannot be correlated with morphology since morphology was not assessed for this collection. The "fit-for-purpose" distribution of the liver sample collection shows that 2.4% ($n=3$) of samples are unreliable for demanding downstream procedures ($\text{RIN} < 5$); 4.9% ($n=6$) of the samples should only be used for RT-qPCR ($5 \leq \text{RIN} < 6$); 53.7% ($n=66$) of the samples can be used for gene array work ($6 \leq \text{RIN} < 8$); and the remaining 39% ($n=48$) of samples are considered fit for all demanding downstream techniques ($\text{RIN} \geq 8$) (Fig. 2). The numbers in Table 3 reflect average RIN values of N samples while the numbers cited here reflect sample counts based on individual RIN values.

Clinical pathology frozen biopsy archive collection

Out of 24 tested samples, 7 samples (41.2%) had RIN values lower than 6.5 (Table 4). Nineteen biopsies were previously used to cut frozen sections for diagnostic reasons. Pearson Chi-Square analysis showed the use for histology was not correlated to low RIN value. In the diagnostic pathology reports, we did not find morphological characteristics that could explain low RIN values. The "fit-for-purpose" distribution of the clinical pathology biopsy sample collection shows that 20.83% ($n=5$) of samples are unreliable for demanding downstream procedures ($\text{RIN} < 5$); 8.33% ($n=2$) of the samples should only be used for RT-qPCR ($5 \leq \text{RIN} < 6$); 25% ($n=6$) of the samples can be used for gene array work ($6 \leq \text{RIN} < 8$); and the remaining 45.83% ($n=11$) of samples are considered fit for all demanding downstream techniques ($\text{RIN} \geq 8$) (Fig. 2). The numbers in Table 4 reflect average RIN values of N samples, while the numbers cited here reflect sample counts based on individual RIN values.

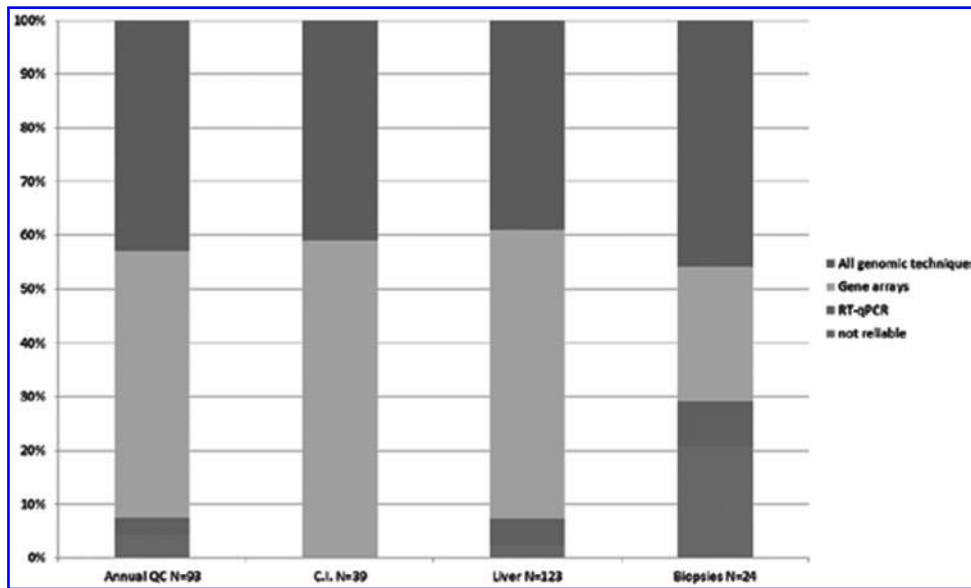


FIG. 2. Fit-for-purpose distribution of all assessed tissue collections. Samples were divided by RIN values. Samples with $RIN < 5$ are not reliable for demanding downstream genomic techniques; samples with $5 \leq RIN < 6$ are only reliable for RT-qPCR; samples with $6 \leq RIN < 8$ are reliable for RT-qPCR and gene array analysis; and samples with $RIN \geq 8$ are reliable for all imaginable genomic techniques. The latter two categories combined generally represent the tissue quality level that is likely reliable for most other “omics” research such as proteomics and metabolomics. C.I. = Cancer Institute.

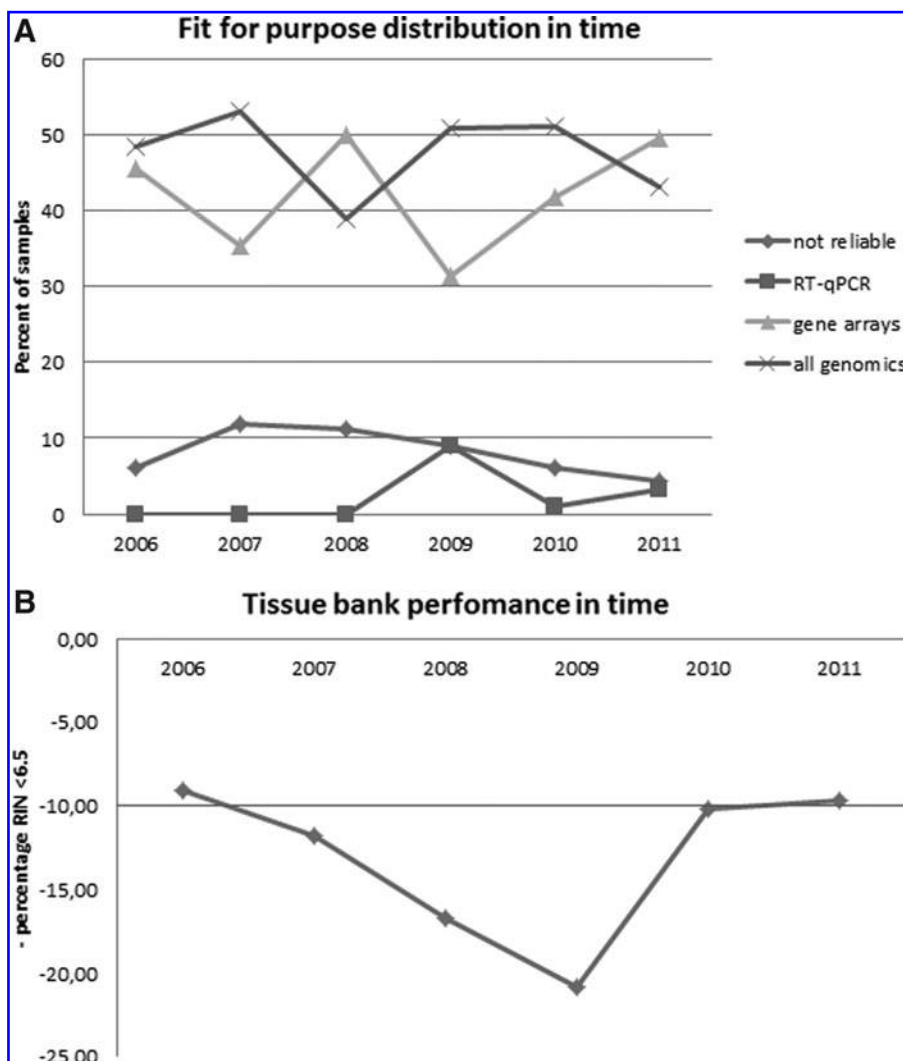


FIG. 3. Fit-for-purpose distribution and tissue bank performance over the past 6 years. (A) shows how the fit-for-purpose distribution of quality assessed tissue bank samples has changed over the past 6 years. The percentage of samples that are deemed unreliable for downstream techniques has decreased steadily since 2008. The percentage of samples that are deemed perfect for all genomic techniques also decreased, while the percentage of samples suitable for gene array analysis increased. (B) shows how the tissue bank performance level (displayed as a negative percentage of samples with $RIN < 6.5$) has improved after being substandard for 3 years. Tissue bank performance is considered adequate when the percentage of samples with $RIN \geq 6.5$ exceeds 90% and thus -10% is considered the cut-off.

We performed statistical analysis of RIN vs. sample type (biopsy vs. excision); RIN vs. storage type (-80°C vs. liquid nitrogen); and RIN vs. storage time (annual QC samples stored for 1 year vs. long-term stored collections). None of the analyses showed significant differences in RNA integrity.

Discussion and Conclusions

RNA isolation and RIN measurement were used as tools for tissue quality assessment. We designated a cut-off RIN value of 6.5 as an indicator of adequate tissue quality. It is not standard practice to assess the applicability of demanding proteomic or metabolomic techniques using RIN values, but it is safe to assume that, as RNA is an unstable tissue derivative that can be reliably tested, RNA RIN values can be used as a surrogate indicator of good tissue quality. When RIN values lower than 6.5 were found, in most cases this could be correlated with overall low cellular content or with notoriously low-yielding tissue types.⁵ For some specific tissue types, such as pancreas and fatty (adipose) tissue, special RNA isolation procedures¹⁴ and kits (Qiagen, RNeasy Lipid Tissue Mini Kit) are available. It may be useful to use these specialized kits in future tissue bank QC exercises, especially when a research group works with these tissue types. When these tissue types are left out of the QC exercise, the results are a better indicator of the tissue bank's performance.

Tissue bank performance was above the threshold standard of 10% of samples with RIN < 6.5 (10% RIN < 6.5) for 2 years (see Fig. 3B). In the first 3 years, during the developmental phase of our QC procedure, we aspired to gain all necessary information by testing as few samples as possible ($n=20$) rather than a percentage of all samples. The old RNA isolation protocol was very time consuming and RIN values were still a novelty. In 2009, when it was repeatedly observed that the amount and quality of RNA isolated from tissue depended heavily on tissue type, we decided to take more samples for QC purposes. From 2010 onward, we have been taking 2% of all annually collected samples for QC. This also became more feasible due to the use of the RNeasy kits, which made it possible to isolate RNA in under 1 hour.

In the tissue bank QC reports before 2009 there was also no mention of the 10% RIN < 6.5 standard performance cut-off. That makes sense because the cut-off was first described in the ISBER Best Practices document of 2008. Nevertheless, in retrospect, the tissue bank performance in 2009 was substandard compared to earlier years and may have been due to major reconstruction of the pathology cutting room. This reconstruction forced the pathology laboratory, as well as the tissue bank facility, to move the entire cutting room to the morgue, which is situated far away from the rest of the pathology department. Although most of the logistics, such as transport of tissue specimens from the clinic to the morgue facility, remained the same as before, pathologists had to walk a fair distance to examine fresh specimens, from which samples were then taken for the tissue bank. This caused a batching effect and thus longer cold ischemia periods occurred during which RNA could possibly have degraded more.

The "fit-for-purpose" distribution over the course of 6 years (Fig. 3A) shows that the number of unreliable samples

decreased while the number of samples useful for RT-qPCR increased. This implies that the low performing end of the sample spectrum improved. On the other hand, the opposite is observed in the high performing end of the spectrum, where fewer samples became fit for all genomic techniques and more samples became fit for use in gene array studies. The decrease in the number of top-quality specimens may also be due to financial reorganizations within the pathology department, especially after the temporary move to the morgue in 2010/2011. The tissue bank strives to collect and freeze samples within 30 min after specimens are taken from the patient at the operating theater. But due to budget cuts, the specimen transport frequency was decreased and specimens arrived at the pathology laboratory often after 2 hours of waiting in refrigerators in the clinic.

In the case of the clinical pathology frozen biopsy collection, a correlation between tissue type and low RIN values could not be established. Since these samples were collected for histological purposes and would have been disposed of after 10 years, the procurement protocols did not anticipate use of these samples for future molecular research. Another reason for the high number of samples with low RIN values in this collection could be that after sectioning for histological procedures, the tissue was thawed while taking it off the cryostat microtome object holders. The clinical pathology biopsies are now handled by the central tissue bank in a standardized manner. However, the biopsies are still used for diagnostic purposes before storage in the tissue bank, so education of technicians on how to handle this tissue properly (e.g., don't let the samples thaw while putting them back in the vials) is necessary to preserve these useful samples for future research. Again, this emphasizes the need for dedicated biobank personnel who can educate others on how to collect high-quality samples that are also suitable for medical research purposes.

RIN measurements have always been performed using Agilent Bio Analyzer RNA Nano Chips. In some cases, a proper RIN value could not be established because the RNA yield was too low. Although the amount of tissue used for RNA isolation was always the same (the area and number of sections were observed and accounted for (e.g., 10 sections of 1 cm^2 area or 20 sections of 0.5 cm^2 area were cut), the use of RNA Pico Chips could give a better impression of RNA quality when low RNA yields are obtained. However, the occurrence of low RNA yield is small and the majority of the Pico Chips would expire before being put to use. Therefore, the use of these chips is financially unfeasible in the QC procedure.

We have also noticed that when tissue morphology would predict a higher-than-measured RIN value, it is best to repeat the RNA isolation. It is possible that something can go wrong at any time during the RNA isolation procedure. Although the pre-PCR laboratory where RNA isolation is performed is clean, an airborne speck of dust may contaminate the sample, causing RNA degradation by nucleases.

For the 2011 QC report, only the cases where no valid explanation for low RIN values could be determined (i.e., high enough RNA yield and tissue types not suspected to yield low RNA quality, but RIN value lower than 6.5) were accounted as "badly-procured" samples. Therefore, the biobank does not need evaluation of the methods used since the performance is good, with only 9.68% insufficiently

procured samples. Of course, only 2% of all samples were actually tested. Before using the remaining 98% untested samples for further genomic analysis, it is advisable to measure RNA integrity in order to be able to take corrective measures during data analysis.

By doing QC we showed that long-term storage of various tissue types in either liquid nitrogen or in -80°C freezers preserves tissue well enough to achieve a high proportion with RIN values that we have characterized as good for most downstream “omic” techniques. Furthermore, our results suggest that the mechanical freezers used to store these collections have not failed and the samples did not thaw accidentally, since this would likely have led to significantly lower average RNA RIN values. However, more than 10% of the liver samples had RIN values that suggested that they had been badly procured; these numbers cannot be explained by morphological features, as morphology was not assessed. Temporary storage (weeks to months) of liver samples at -20°C may have harmed RNA integrity in some of these cases. However, when statistical analysis was performed and different aspects of the collections (sample type, storage temperature, and procurement method) compared, no significant correlation between RIN values and these factors were found. Therefore it seems that sample storage (time or temperature) does not significantly influence RNA integrity.

When samples were divided into four “fit-for-purpose” groups, it was observed that the majority of samples can be designated as useful for downstream techniques varying from RT-qPCR and gene expression array analysis to next generation sequencing, based on cut-off RIN values found in the literature.^{9–11} This “fit-for-purpose” distribution can be helpful for researchers who need samples for certain downstream techniques. It would facilitate making statistical calculations on the number of samples needed for a study, and the expected availability of those samples in a tissue bank. There is no known correlation between RIN value and proteomic, DNA, or metabolomics research performance. Some reports describe the simultaneous isolation of RNA, DNA, and proteins.^{13,15–17} There seems to be a correlation between RNA, DNA, and protein quality when different sample types such as paraffin embedded tissue and serum are considered. However, this does not say anything about an eventual correlation between RIN value and DNA/protein quality within one specific sample type group, namely frozen tissue. The amount of information found in these articles on RIN vs DNA/protein quality is too little (either all RIN values are high or RIN isn’t measured at all) to draw final conclusions. So, although the RIN value should provide an indication for overall tissue quality, these “fit-for-purpose” groups only apply for RNA-based genetic research. It is recommended that for all downstream tests an appropriate “fit-for-purpose” test should be considered, especially where signals of very unstable components need to be determined.

The minority of samples labeled as “not reliable for downstream techniques” can still be valuable, albeit for less demanding techniques. Ongoing research at our laboratory shows that RNA with RIN values as low as 1.5 can be used for RT-qPCR with 530 bp amplicons (data not shown). This implies that these samples could also be suitable for gene array analysis, a technique which uses smaller probes on chips or beads. Viljoen et al.¹⁸ recently showed that statis-

tical correction of gene array data can be used to correct for low RIN or degraded RNA samples, making the worst samples seem useful, even for (allegedly) demanding downstream analyses. The same goes for high quality samples to some extent. For now, we maintain a cut-off of $\text{RIN} \geq 8$ as fit for all downstream techniques, but future research (with new techniques) may very well show that all downstream techniques can be performed on tissues with lower RIN values. Thus, the cut-off values used here are not necessarily permanent. Since RNA quality is not yet correlated with performance of “omic” techniques outside the RNA spectrum, it is impossible to even imply that low RIN tissue would also be unsuitable for proteomic or metabolomics techniques. Therefore, it is necessary to develop easy to assess tissue quality markers which are tailored to these techniques.

In conclusion, the annual QC, as well as incidental quality assessments of peripheral frozen tissue collections, can provide useful information on how tissue procurement and storage can influence tissue quality. It will eventually lead to improvement of procurement protocols and guarantee a high level of sample quality, which will instill confidence for both the tissue banker and the researchers who use the samples.

Author Disclosure Statement

No competing financial interests exist.

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