Molecular analysis of a collection of clinical specimens stored at 4˚C as an alternative to snap-freezing

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Abstract. It is critical for both basic and clinical translational cancer research to use high quality DNA, RNA and proteins from specimens with clinical outcome in order to validate novel diagnostic biomarkers and to monitor successful treatments for patients. However, using current standard procedures, the collection of specimens is often limited by the availability of liquid nitrogen in some hospitals and liquid nitrogen can be hazardous to transport. These problems would be eased if the tissue could be stored unfixed at 4˚C, conditions that are readily available in hospitals. Thus the effect of storing tissue specimens at 4˚C on the quality of DNA, RNA and protein has been examined. Clinical tissue samples were halved and kept either at 4˚C for up to 24 h or snap-frozen in liquid nitrogen within 30 min of removal from the patient. The results show that the quality of RNA, DNA and protein isolated from the specimens stored at 4˚C up to overnight is equal to that obtained from snap-frozen material. In conclusion, simplifying the collection procedure may allow for greater flexibility of conducting studies in units where liquid nitrogen is not readily available.

Introduction

Human cancer is a significant public health problem in the world (1). In 2006, in Europe alone, there were >3 million cancer cases diagnosed and >1.7 million deaths from cancer (2). The major cause of death from cancer is the metastatic spread of the primary tumour to distant sites in the body. Modern biotechnology such as gene expression profiling and proteomics have generated a dramatic amount of data that provides an opportunity to identify gene products that play key roles in cancer development and to develop novel therapies to target cancer cells. It is essential to validate the gene products identified from such studies as potential diagnostic/prognostic and therapeutic targets and monitor the response and resistance to new drug treatments. However, both basic research and translational research studies often depend on the availability of high quality DNA, RNA and protein isolated from clinical specimens from patients with cancer along with related clinical information and large studies are needed for correlations with clinical outcome.

With the advent of cancer tissue banks containing large number of specimens, analysis of DNA, RNA and protein from clinical human carcinoma specimens is becoming an increasingly important feature of clinical cancer research, and the collection, manipulation, and storage conditions of the tissue are thought to be the most critical aspects determining the quality of DNA, RNA and protein. In order to preserve DNA and particularly RNA in the specimen, it has been standard procedure to snap-freeze the tissue in liquid nitrogen as soon as possible after its removal from the patient. However, the use of liquid nitrogen in an operating theatre in some local hospitals can cause logistical problems, in addition to the hazards of transporting samples in liquid nitrogen between the surgical centre and the pathology laboratory; the provision of liquid nitrogen is often not suitable when specimens become available out of normal working hours, thus valuable specimens could be lost. Especially some clinical trials often involve large national and international networks linked with local hospitals. These problems would be eased if the tissue could be stored at 4˚C, conditions that are readily available in all the hospitals, however, the effect of storing tissue at 4˚C on the quality of DNA, RNA and protein is largely unknown. Thus the effect of storing tissue specimens at 4˚C on the quality of DNA, RNA and protein subsequently isolated from the tissue has been examined. The results show that the quality of RNA, DNA and protein isolated from the specimens stored at 4˚C up to overnight is not appreciably different to that obtained from snap-frozen material.

Materials and methods

Tissue specimens. Thirty-six breast carcinomas and normal breast tissue samples were obtained with full informed consent from patients at the Liverpool Women's Hospital, Liverpool, UK. Each specimen was divided into two parts and one part

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Table I. Primer sequences used to amplify hypoxanthine phosphoribosyl transferase (HPRT) and S100A4 genes.

Primer name and sequences	Region amplified[a] (size of PCR product)	Cycling profile
HPRT 5' U 5' CGCCTCCTCCTCTGCT 3' L 5' CTCCCATCTCCTTCATCACAT 3'	15 to 260 (246 bp)	94°C 5 min X1; 94°C 30 sec, 60°C 30 sec, 72°C 45 sec X35, 72°C 5 min X1
HPRT U1 U 5' CTATTGTAATGACCAGTCAACAGGG 3' L 5' AACTCAACTTGAACTCTCATCTTAGGC 3'	397 to 763 (367 bp)	94°C 5 min X1; 94°C 30 sec, 64°C 30 sec, 72°C 45 sec X35, 72°C 5 min X1
HPRT U2 U 5' GTGTTGGATATAAGCCAGACTTTGTT 3' L 5' AACTCAACTTGAACTCTCATCTTAGGC 3'	597 to 763 (167 bp)	94°C 5 min X1; 94°C 30 sec, 64°C 30 sec, 72°C 45 sec X35, 72°C 5 min X1
HPRT 3' U 5' TACCACCGTGTGTTAGAAAAG 3' L 5' GGGAACTGCTGACAAAGAT 3'	1132 to 1297 (166 bp)	94°C 5 min X1; 94°C 30 sec, 53°C 30 sec, 72°C 45 sec X35, 72°C 5 min X1
TATA box region of S100A4 gene U 5' CCTGTCACCCACCACCCCCT 3' L 5' CCTCCCCTTCTCCTTCCCCA 3'	320 to 792 (473 bp)	94°C 2 min X1; 94°C 50 sec, 62°C 50 sec, 72°C 2 min X45, 72°C 5 min X1
1st intron of S100A4 gene U 5' CAGCTTCTCTTCCAACCCTT 3' L 5' CACTACCCACTCAC	1175 to 1830 (656 bp)	94°C 2 min X1; 94°C 50 sec, 60°C 50 sec, 72°C 2 min X45, 72°C 5 min X1

[a]Numbering according to GenBank Accession No. NM_000194.1 (HPRT) and Accession No. Z33457 (S100A4). U, Upper primer sequence and L, lower primer sequence.

was stored in a refrigerator at 4˚C for 18-24 h before being used for the isolation of DNA, RNA and protein. The other part was snap-frozen in liquid nitrogen within 30 min of removal from the patient and stored at -140˚C until used for the isolation of DNA, RNA and protein. Local Ethics Committee Approval was obtained and the patient data were anonymized.

RNA, DNA and protein extraction. Total RNA, genomic DNA and protein were isolated from both the snap-frozen in liquid nitrogen and 4˚C sets of carcinomas and normal tissue using the guanidinium isothiocyanate/CsCl method, as described previously (3). Yields of RNA and DNA were determined by optical density measurement at 260 nm and the yield of protein was determined using the Bradford Coomassie dye-based assay (Bio-Rad Laboratories, Hercules, USA).

Estimation of the quality of RNA, DNA and protein. In order to find out whether the RNA preparations were at all degraded, total RNA was assessed using standard Northern blotting and hybridisation techniques following denaturing agarose gel electrophoresis, in which the 4˚C and the snap-frozen samples were compared on the same gels. The resulting blotted filters were incubated with a cDNA corresponding to the constitutively-expressed human acidic ribosomal phosphoprotein mRNA, 36B4 (GenBank Accession No. M17885) (4), as described previously (5).

Quality of genomic DNA isolated from the specimens was compared using the polymerase chain reactions (PCR) using primers that amplify the upstream TATA regulatory region and the intronic region of the human metastasis-inducing S100A4 gene (6,7) (GenBank accession No. Z33457) (Table I), yielding fragments of 473 and 656 bp, respectively. The cycles used were 94˚C for 2 min for 1 cycle, 94˚C for 50 sec, 62˚C (TATA region) or 60˚C (intronic region) annealing for 50 sec and 72˚C extension for 2 min, cycled 45 times. A final cycle of 72˚C for 5 min completed any unfinished extensions. Amplified DNA was analysed by agarose gel electrophoresis using standard procedures (8).

In order to assess the quality of isolated protein, 10 μg of protein extract from each of the specimens was subjected to SDS-polyacrylamide gel electrophoresis, as previously described (9) and stained with Coomassie blue to visualise the separated protein bands.

Reverse Northern hybridisation. cDNAs from RNAs isolated from specimens which had been stored at 4˚C or snap-frozen in liquid nitrogen were compared for the distribution of target cloned cDNA sequences using hybridisation to two separate cDNA arrays on nylon filters. To ensure accurate loading of the targets, PCR products were subjected to agarose gel electrophoresis and the bands of DNA, stained with ethidium bromide, were scanned and accurately quantitated using Image Analysis software (Image, NIH), prior to being spotted onto the nylon membranes.

One duplicate set of filters (PCR product filters) contained 19 separate PCR products of cloned cDNA inserts of known origin (Table II). After experiments to determine the amount of target and RNA probe required for optimal detection, precisely 100 ng of each target cDNA was immobilised onto

Table II. Cloned cDNAs used in array experiments.

cDNA	Approx. mRNA size	Area under scanned peak with 4˚C probe	Area under scanned peak with snap-frozen probe	Exposure time
36B4	1.5 kb	37757	43672	4 h
GAPDH	1.0 kb	0	0	4 days
Estrogen receptor	2.0 kb	6024	5749	4 days
c-erbB-2	4.0 kb	2639	1833	4 days
Osteopontin	1.5 kb	0	0	4 days
Cyclin D1	1.3 kb	51120	41139	4 h
CDK4	1.4 kb	2975	3346	4 h
H-ras	2.0 kb	0	0	4 days
c-myc	1.5 kb	4101	1579	4 days
Non-muscle myosin B	7.0 kb	0	0	4 days
pS2	0.5 kb	5562	3747	4 h
S100A4	0.58 kb	6515	5632	4 h
HPRT 5'	1.3 kb	2334	1374	4 days
HPRT U2	1.3 kb	0	0	4 days
HPRT U1	1.3 kb	0	0	4 days
HPRT 3'	1.3 kb	1734	2090	4 days
Porphobilinogen deaminase 5'	1.4 kb	0	0	4 days
Porphobilinogen deaminase 3'	1.4 kb	0	0	4 days
Glucose-6-phosphate dehydrogenase	~2.2 kb	0	0	4 days

Hybond N Nylon filters (GE Healthcare, Amersham, UK) using a 48-well slot blot apparatus. An independent second set of duplicate arrays (subtracted library filters) was also used, which consisted of 186 PCR products of cloned cDNA inserts on two filters. The cDNAs were derived from a subtracted library which contained cDNAs corresponding to mRNAs from an estrogen receptor-positive human mammary cell line, MCF-7, that had been subtracted with cDNA from an estrogen receptor-negative benign human mammary cell line, Huma 123 (10). For this array, 50 ng of each cDNA was immobilised to the filters using a 96-well Bio-Dot blotting apparatus (Bio-Rad Laboratories). Three control cDNAs, the 'housekeeping' GAPDH and 36B4 and the estrogen responsive cDNA, pS2, were included on each blot for normalisation purposes.

One tissue sample was selected from the experimental group and 5 μg of total RNA isolated from each of the 4˚C and snap-frozen pieces were radioactively labelled by reverse transcription with RNase H-free, Murine Moloney Leukaemia Virus (M-MLV)-reverse transcriptase (Promega, Madison, WI, USA) in the presence of [^{32}P]dCTP, according to the manufacturer's instructions, to generate cDNAs from the RNA templates. Unincorporated nucleotides were removed using ProbeQuant G-50 Micro Columns (GE Healthcare). The specific activities of the labelled probes were between $1.1x10^9$ and $1.2x10^9$ dpm/μg DNA.

Membranes were prehybridised in 25 ml of hybridisation buffer [5X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 5X Denhardt's reagent, 0.5% (w/v) SDS and 100 μg/ml denatured, sonicated salmon sperm DNA] for 2-4 h at 68˚C. Denatured probes were added to 5-10 ml hybridisation buffer containing 10% (w/v) dextran sulphate and hybridisations were performed overnight at 68˚C. Hybridised reverse Northern membranes were washed in 0.1% (w/v) SDS, 0.1 x SSPE (1X SSPE = 0.15 M sodium chloride, 10 mM sodium dihydrogen phosphate, 1 mM EDTA, pH 7.4) for 30 min at room temperature, then for 2 h at 68˚C. Membranes were exposed to X-O-MAT AR film (Kodak, Rochester, NY, USA) for 4 h and for either 4 days (PCR product filters) or 7 days (subtracted library filters). Individual slot or dot intensities were quantified using NIH Image Analysis software. The images of individual spots were scanned three times and the mean values calculated. Two housekeeping genes, 36B4, GAPDH and an estrogen responsive gene, pS2, gave values that were not statistically significantly different between all pairs of filters hybridised, indicating that the pairs of probes were not of significantly different specific activities, thus filter to filter normalisation was not applied.

For all spots, the $\log_{10}$ of the mean values (11) corresponding to spot intensity values on one filter, obtained using a probe derived from 4˚C stored tissue, was plotted against the spot intensity values obtained on the duplicate filter using a probe derived from snap-frozen tissue. Statistical analyses were carried out using the StatsDirect statistical package (StatsDirect Ltd. UK, http://www.statsdirect.com).

RT-PCR. Total RNA (1 μg) from each of the 4˚C and snap-frozen pieces of samples was used to confirm that cDNA from the entire length of mRNA molecules was being made in the reverse transcription reactions using M-MLV-reverse transcriptase (Promega). The resulting reverse transcripts were then amplified in PCR reactions using a Taq polymerase

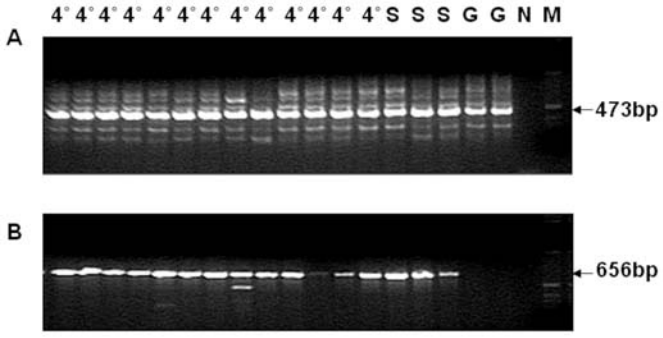

Figure 1. Amplification by PCR of the S100A4 gene from genomic DNA isolated from breast carcinoma specimens. DNA was isolated from breast carcinoma specimens stored at 4°C (4°), from snap-frozen specimens (S) or from positive control genomic DNA isolated from tissues unrelated to this experiment, but previously shown to be positive with this PCR (G). A 473 bp region surrounding the TATA box (A) and a 656 bp region of the first intron (B) of the S100A4 gene were amplified by polymerase chain reaction, the products were subjected to agarose gel electrophoresis alongside a 1 kbp DNA ladder (M) and the DNA visualised using ethidium bromide. There were no amplification products when DNA was omitted from the PCR reactions (N). The arrows point to the position of the PCR products of the correct size.

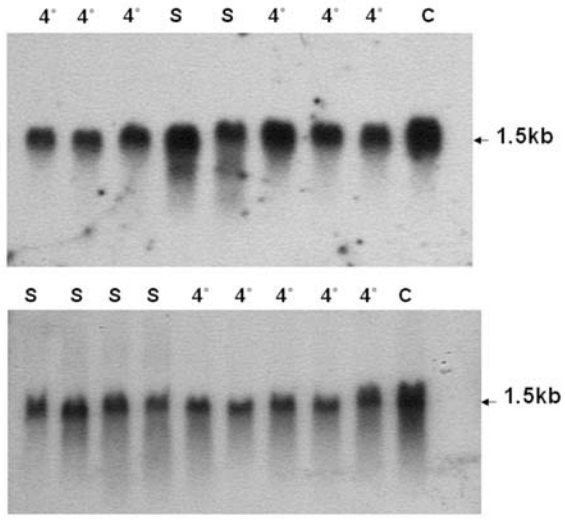

Figure 2. Northern hybridisation of RNA isolated from breast carcinoma specimens. Total RNA was isolated from eleven 4°C-stored (4°) and 6 snap-frozen (S) breast carcinoma specimens and from the malignant breast carcinoma cell line, MCF-7 (C). The RNA was subjected to agarose gel electrophoresis, Northern blotting and hybridisation with a probe to constitutive human acidic ribosomal phosphoprotein, 36B4 mRNA. The arrow points to the position of the band of hybridisation of the 1.5 kb 36B4 mRNA on the filters. The two panels are from separate experiments.

kit (Invitrogen, Paisley, UK). Each cDNA was amplified with four sets of primers in separate reactions that together amplified the entire length of the mRNA for the housekeeping gene, hypoxanthine phosphoribosyl transferase (HPRT) (Table I). The 4 regions amplified are named H5', U1, U2 and H3'. Each PCR reaction contained 1X PCR buffer (Invitrogen), 10 pmol of one set of primers, 0.02% (w/v) detergent W^{-1} and 0.2 mM dNTPs. 1.5 mM, 3 mM, 2.5 mM and 3 mM Mg^{2+} were used in the H5', U1, U2 and H3' reactions, respectively, with 2.5 units of Taq polymerase. The PCR reactions were carried out on the entire reverse transcription reaction mix using the appropriate cycling profiles as shown in Table I.

Results and Discussion

DNA isolated from carcinoma specimens was amplified by PCR, using primers specific for the TATA box or intronic region of the S100A4 gene. Amplification of genomic DNA isolated from the specimens stored at 4°C yielded a pattern of PCR products that was identical to the pattern obtained with snap-frozen specimens (Fig. 1), indicating that DNA isolated from carcinomas treated in either way was suitable for amplification to produce PCR products in the 473-656 bp size range. Furthermore, the SDS gel electrophoretic pattern of proteins obtained from the specimens stored at 4°C overnight was identical to the pattern obtained from snap-frozen specimens (not shown).

A more stringent test of any differences between cold-stored and snap-frozen tissue is the quality of RNA extracted. The mean yield of RNA from the 4°C stored samples (0.74±0.51 SD μg/mg tissue) was not significantly different from the average yield (1.03±0.9 SD μg/mg tissue) obtained from the snap-frozen specimens (P=0.19, two-tailed Mann-Witney U test).

Northern blots of these samples incubated with a probe representing the constitutively expressed housekeeping gene, human acidic ribosomal phosphoprotein, 36B4, revealed an intact message size of 1.5 kb in RNA from both the stored and snap-frozen samples. The quality of the 4°C stored RNA as judged by the degree of lower molecular weight smearing of the hybridisation band is equal to, or better than, RNA from the snap-frozen specimens, based on visual appearance (Fig. 2) or quantitatively by scanning the lanes (not shown).

In order to test whether storage at 4°C affected the relative levels of individual mRNAs in the RNA preparations, reverse Northern filter array hybridisations were carried out. Initial experiments using RT-PCR showed that the cDNA probes were not confined to the 3' end of mRNAs (not shown). Such probes were used to test three separate sets of duplicate filters containing cloned cDNAs. The first consisted of a panel of 19 cDNAs (Table II) and when probed with radioactive cDNA from the cold-stored and snap-frozen specimens, the same 10 target mRNAs, namely ribosomal phosphoprotein 36B4, hypoxanthine phosphoribosyl transferase HPRT 5', HPRT 3', estrogen receptor, c-*erb*B-2, cyclin D1, CDK4, c-*myc*, pS2 and S100A4, yielded a positive signal, irrespective of whether the filters were probed with cDNAs arising from the cold-treated or snap-frozen specimens (Fig. 3A). The failure to obtain a signal on the remaining 9 cDNAs, GAPDH, osteopontin, H-ras, non-muscle myosin heavy chain, HPRT U2, HPRT U1, porphobilinogen deaminase (PBD) 5', PBD 3' and glucose-6-phosphate dehydrogenase (G6PD) when screened with cDNA from either snap-frozen or 4°C-stored specimens probably reflects a limitation of the sensitivity of the filter array methodology. When the quantitated hybridisation signals arising from the probes from 4°C-stored and snap-frozen tissue for each cloned cDNA were plotted against one another, the hybridisation intensities were clustered around the identity line, y=x (Fig. 3A), representing equal intensities in the two probes. Nine cloned cDNAs fell within two standard deviations of the identity line (calculated using the residual of each spot

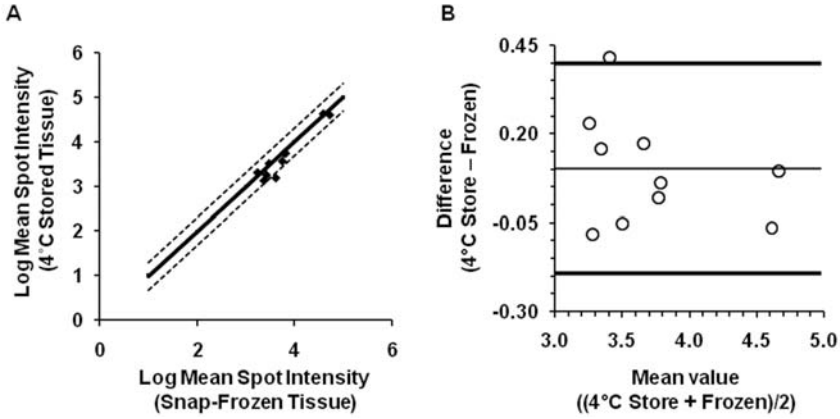

Figure 3. Array hybridisation of known breast cancer-associated cDNAs. (A) Equal weights of nineteen cDNAs (see text) were spotted onto duplicate Nylon filters using a slot-blotting apparatus. The filters were hybridised either to a [^{32}P]-labelled cDNA probe corresponding to mRNA from samples of a carcinoma specimen which had been stored either at 4˚C or snap-frozen in liquid nitrogen as described in Materials and methods. The resulting regions of hybridisation were scanned three times and the $\log_{10}$ of the mean values were plotted (♦) for each cDNA. The identity-line (y=x) is shown as a solid line and the dotted lines indicate ±2 SDs of the sample values calculated from the residual of each spot. For paired t-test of these data, 95% CI of the mean difference = -0.014664 to 0.206079; 95% limits of agreement of individual differences 0.206692 to 0.398107, P=0.0814. (B) The corresponding Bland-Altman plot shows that the differences in values between 4˚C stored and snap-frozen in liquid nitrogen are not dependent on the mean of the differences (thin line).

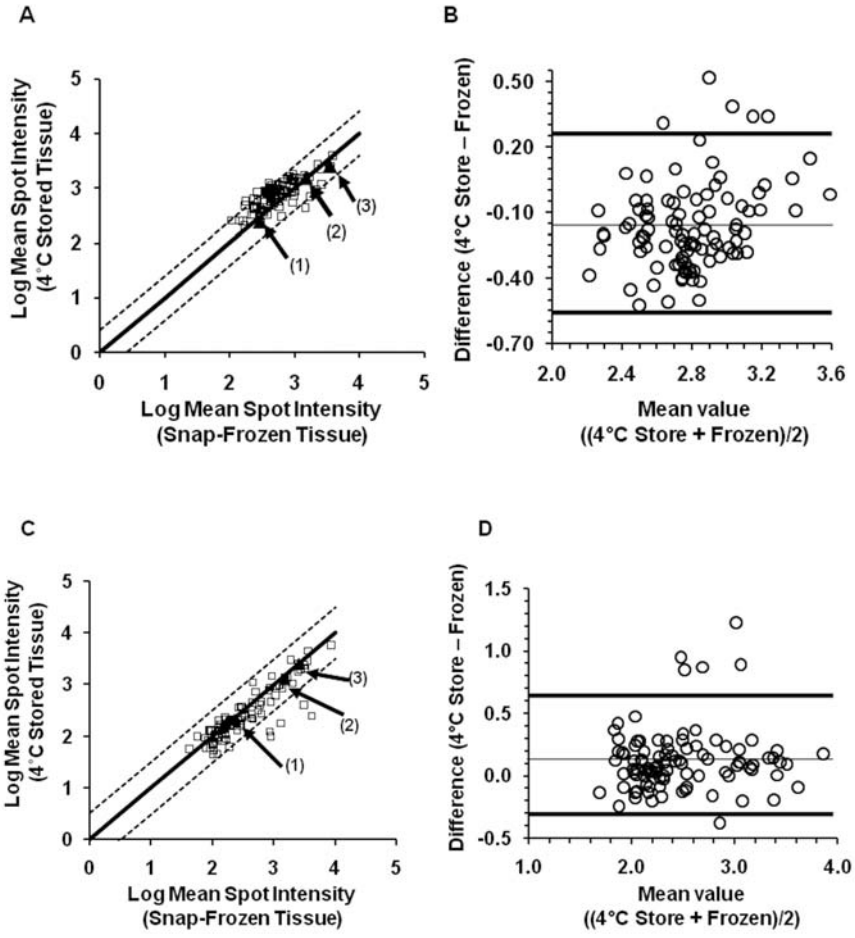

Figure 4. Array hybridisation of a panel of differentially-expressed cDNAs. (A and C). cDNAs (186) were spotted in equal amounts onto 2 nylon filters (93 per filter) using a dot blotting apparatus. Duplicate sets of filters were prepared. Each filter also contained spots of control cDNAs (triangle), 36B4 (1), GAPDH (2), and pS2 (3). The filters were incubated with a [^{32}P]-labelled DNA probe corresponding to mRNAs from samples of a carcinoma specimen which had been stored at 4˚C or to a similar probe prepared from a sample snap-frozen in liquid nitrogen, as described in Materials and methods. The resulting regions of hybridisation were scanned three times and the $\log_{10}$ of the mean values from the 4˚C stored and frozen sample hybridisations were plotted against each other (square) for each cDNA. The identity-line for each filter (y=x) is shown as a solid line, with the signals for three control cDNAs indicated by the arrows. The dotted lines show ±2 SDs of the sample values calculated from the residual of each spot. For paired t-tests of these data, 95% CI of the mean difference = -0.202612 to -0.121073, P<0.0001 (A) and 0.068082 to 0.170764, P<0.0001 (C); 95% limits of agreement of individual differences -0.556215 to 0.23253 (A) and -0.377209 to 0.616054 (C). (B and D) The corresponding Bland-Altman plots show that the bias evident in the paired t-tests is not dependent on the mean of the differences (thin line).

to the identity line), however, hybridisation to one clone, c-*myc*, fell outside and this outlier exhibited a signal that was lower in the specimens stored at 4˚C (Fig. 3A).

Two further filter arrays were used, consisting of a total of 186 cDNAs (93 per filter) which had been selected on the basis of being differentially expressed in malignant relative to benign breast tumour cells (10). These filters were hybridised in duplicate with probes derived from RNA of a carcinoma specimen, part of which had been 4˚C stored and part of which had been snap-frozen in liquid nitrogen (Fig. 4A and C). Hybridisation signals for the 186 cloned DNAs also clustered around the identity line (Fig. 4A and C). Only 11/186 separate cDNAs fell outside 2 standard deviations, but all of these outlying samples arose from leakage of the cDNA during production of one of the filters.

Pair-wise comparison of the hybridisation intensities of all the spots between 4˚C stored and frozen samples for the three filters indicated significant mutual dependence of the intensities between the two treatments at a 95% confidence level (Kendall's Rank Correlation test, two-tailed $P=0.0046$, $P<0.0001$, $P<0.0001$ for hybridisations shown in Figs. 3A and 4A and C, respectively). Paired t-tests at the 95% confidence level indicated a marginal systematic bias of the data in Fig. 3A ($P=0.0814$) and opposite systematic biases for the data in Fig. 4A and C ($P<0.0001$). Bland-Altman (agreement) plots (12) of the difference between the two measurements vs. the mean (Fig. 3B; Fig. 4B and D) do not show any dependence of the bias on the mean value, confirming that paired *t* (H_0: no bias vs. H_A: a constant bias) is the appropriate test to apply.

The results of the array hybridisations taken together show that the relative levels of at least 190 individual mRNAs do not differ statistically between breast carcinoma specimens stored at 4˚C for 18-24 h and samples of the same specimens snap-frozen within 30 min of excision. Collectively, the results show that storage of the breast carcinoma specimens at 4˚C for up to 24 h affects neither the yield, the extent of degradation nor the distribution of 190 individual mRNAs. Thus, in some situations, storage of tissue specimens at 4˚C for up to at least 18 h is a satisfactory alternative to snap-freezing tissue in liquid nitrogen. The results indicate that storage of clinical tissue specimens at 4˚C as an alternative means to simplifying the standard procedure may improve the feasibility of conducting studies in units where liquid nitrogen is not readily available.

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