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Feasibility of using tissue microarray cores of paraffin-embedded breast cancer tissue for measurement of gene expression: a proof-of-concept study

Suzanne Drury,[1] Janine Salter,[1] Frederick L Baehner,[2] Steven Shak,[3] Mitch Dowsett[1]

[1]Royal Marsden Hospital and The Breakthrough Breast Cancer Research Centre, London, UK
[2]Departments of Pathology and Laboratory Medicine, University of California, San Francisco, California, USA
[3]Management Team, Genomic Health Inc, Redwood City, California, USA

Correspondence to
Suzanne Drury, Academic Department of Biochemistry, 4th Floor Wallace Wing, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, UK; suzanne.drury@icr.ac.uk

SD and JS contributed equally to this paper.

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ABSTRACT

Aims To determine whether 0.6 mm cores of formalin-fixed paraffin-embedded (FFPE) tissue, as commonly used to construct immunohistochemical tissue microarrays, may be a valid alternative to tissue sections as source material for quantitative real-time PCR-based transcriptional profiling of breast cancer.

Methods Four matched 0.6 mm cores of invasive breast tumour and two 10 μm whole sections were taken from eight FFPE blocks. RNA was extracted and reverse transcribed, and TaqMan® assays were performed on the 21 genes of the Oncotype DX® Breast Cancer assay. Expression of the 16 recurrence-related genes was normalised to the set of five reference genes, and the recurrence score (RS) was calculated.

Results RNA yield was lower from 0.6 mm cores than from 10 μm whole sections, but was still more than sufficient to perform the assay. RS and single gene data from cores were highly comparable with those from whole sections (RS $p=0.005$). Greater variability was seen between cores than between sections.

Conclusions FFPE sections are preferable to 0.6 mm cores for RNA profiling in order to maximise RNA yield and to allow for standard histopathological assessment. However, 0.6 mm cores are sufficient and would be appropriate to use for large cohort studies.

INTRODUCTION

Interest in measuring gene expression to aid the tailoring of treatment of patients with malignant disease is increasing,[1] and breast cancer has been the subject of particularly extensive investigation.[2–4] Formalin-fixed paraffin-embedded (FFPE) material is the most readily available tissue resource, from which H&E-stained sections allow for assessment of routine histopathological metrics, and immunohistochemistry (IHC) is routinely performed to assess for protein expression to guide clinical decision making. Until recently, molecular analysis was hindered by the fact that RNA from FFPE tissue is extensively degraded. Formalin fixation cross-links nucleic acids and proteins, making the molecules rigid and susceptible to shearing, and extracted RNA is highly fragmented.[5] However, technological developments have allowed gene expression profiling to be conducted on FFPE material, and quantitative real-time PCR (qRT-PCR) is currently the most widely used method. Use of carefully designed primers that target short amplicons at the 3′ end of the gene[6] and selecting appropriate reference genes[7] can minimise the effects of RNA degradation on gene expression measurements.[8] This has unlocked the molecular potential of vast numbers of archival tissues.

Quantitative real-time PCR has been applied successfully to develop a 21-gene signature (the Oncotype DX® Breast Cancer Assay; table 1) that has been shown to predict distant recurrence in tamoxifen-treated and anastrozole-treated oestrogen-receptor (ER) positive breast cancer from archival tissue samples.[2] The assay uses 10 μm FFPE sections to produce a recurrence score (RS) from 0 to 100 that categorises patients according to their risk of disease recurrence at distant sites. Low risk is determined by a RS <18, intermediate risk by RS 18–30 and high risk by RS >30.

A common format for high-throughput IHC analysis is tissue microarray (TMA), where 0.6 mm diameter cores of enriched tumour, generally three per tumour, are taken from a donor block and placed in a recipient block, enabling hundreds of samples to be processed simultaneously. Such cores can also be easily stored in microfuge tubes for molecular analysis. At least two 0.6 mm cores are generally required for IHC analysis due to protein expression heterogeneity in fixed paraffin-embedded tumour tissues.[9] Here, we describe a feasibility study to determine whether sufficient RNA can be extracted from 0.6 mm TMA cores and whether cores can be used as an alternative to whole tissue sections for gene expression studies. We also explored the observed variability in quantitative gene expression between cores and whole sections. This was tested by comparative analysis of RNA extracted and analysed from cores and sections using the Oncotype DX® Breast Cancer Assay (Genomic Health, Redwood City, California, USA).

MATERIALS AND METHODS

Paraffin-embedded tumour blocks from eight unique patients (by IHC: five ER positive, four progesterone receptor (PgR) positive and three HER2 positive) were selected for analyses. One 4 μm and two 10 μm sections were cut serially from the blocks under RNAse-free conditions. The 4 μm sections were H&E stained and marked for TMA tumour core selection by a highly experienced technician. Initially, one 0.6 mm TMA core was taken (core 0) from tumour areas and placed in an individual 1.5 ml microcentrifuge tube. Three further cores (cores 1, 2 and 3) were taken at a later date. Samples were shipped to Genomic Health under ambient conditions. All four cores were extracted and analysed.

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Table 1 The 21 genes included in the Oncotype DX® Breast Cancer Assay

Recurrence-related genes		Reference genes
BAG1	*GSTM1*	*β-ACTIN*
BCL2	*HER2*	*GAPDH*
CCNB1	*KI-67*	*GUS*
CD68	*MYBL2*	*RPLPO*
SCUBE2	*PgR*	*TFRC*
CTSL2	*STK15*	
ER	*STMY3*	
GRB7	*SURV*	

Table 2 RNA yields from matched cores and whole sections

	RNA yield (ng)			
	Core (n=4)		Section (n=2)	
Patient	Mean	SEM	Mean	SEM
3	1156	91	11 756	124
47	989	165	17 306	2795
51	1261	250	15 403	669
63	777	84	3419	75
71	2342	361	22 403	583
80	818	106	7836	78
87	569	40	1726	78
104	786	225	3251	109

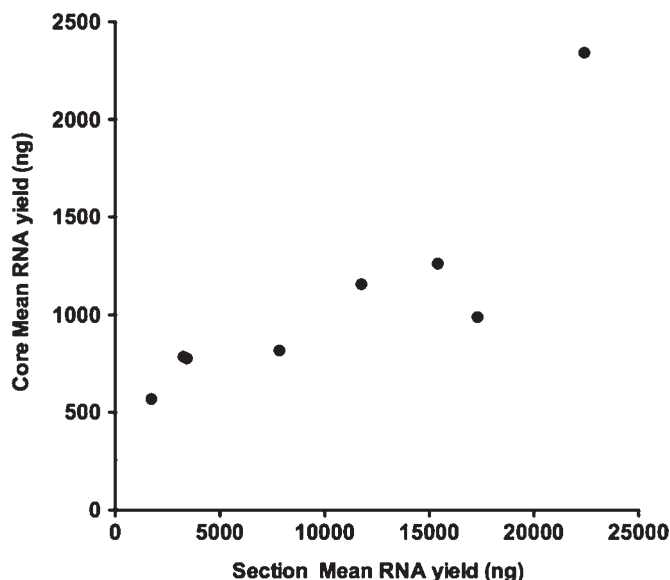

Figure 1 Correlation between the RNA yield from sections (n=2) and cores (n=4). Spearman r=0.91; p=0.005.

According to Genomic Health Inc standard operating procedures, the H&E-stained sections were reviewed microscopically and, when necessary, marked for manual microsdissection (where tumour tissue comprised <50% of the metabolically active tissues). Whole sections from four patients (3, 80, 87 and 104) required microdissection to enrich tumour content.

RNA was extracted using the method described by Cronin *et al*[8] and the standard Oncotype DX® Breast Cancer Assay was performed using 200 ng RNA. Briefly, paraffin was removed from the specimens by xylene extraction, followed by ethanol washes. RNA was isolated from the pellet using the MasterPure™ Complete DNA and RNA purification kit (Epicentre Biotechnologies, Madison, Wisconsin, USA). Total RNA content was measured by Ribogreen® RNA-specific quantitation kit (Invitrogen, Carlsbad, California, USA). Residual genomic DNA contamination was assayed by quantitative TaqMan® PCR assay for β-actin (Applied Biosystems, Foster City, California, USA).

Reverse transcription of purified RNA was carried out with Omniscript RT kit (Qiagen, Valencia, California, USA) using simultaneous random hexamer and gene specific priming TaqMan® reactions using the ABI Prism 7900HT (Applied Biosystems). Quantitative real-time PCR for the 21 genes was conducted in triplicate.

Expression of the 16 recurrence-related genes was normalised to a set of five reference genes (table 1); reference normalised expression ranged from 0 to 15 units where each 1-unit increase reflected approximately a twofold increase in RNA and recurrence score calculated. ER, PgR and HER2 status were assigned as measured by previously defined cut-offs (6.5, 5.5 and 11.5 respectively).[10–13]

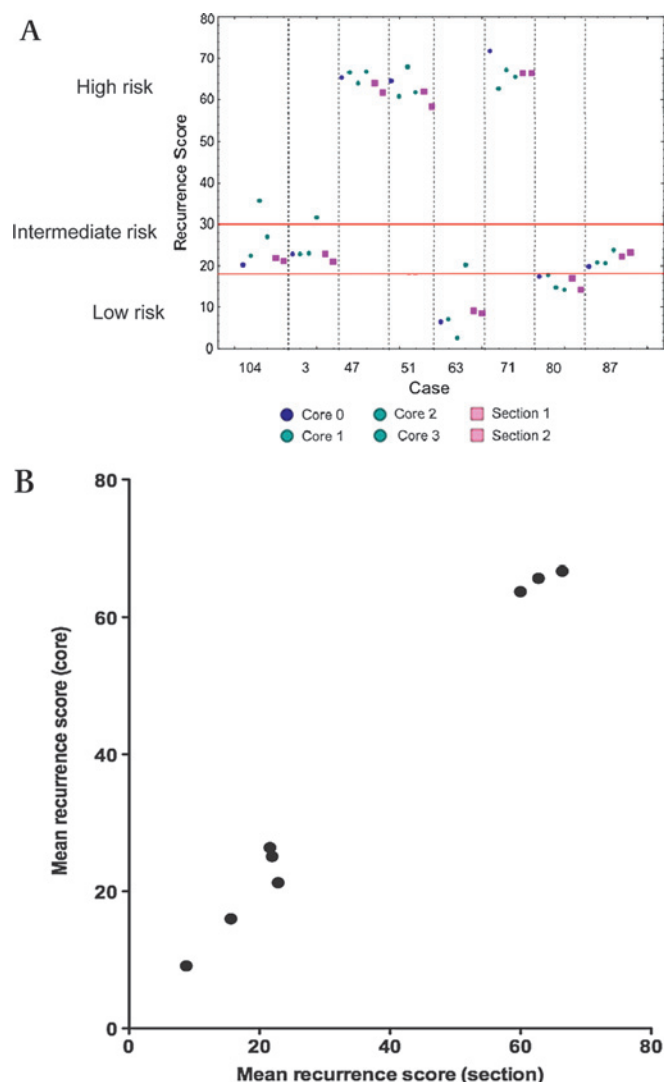

Figure 2 Recurrence scores of individual whole sections (n=2) and cores (n=4) for eight patients. (A) Overall, recurrence score (RS) from sections and cores were within the same category, with the exception of one core from each of patients 3, 63 and 104. (B) Correlation between RS from cores and sections (Spearman r=0.91; p=0.005).

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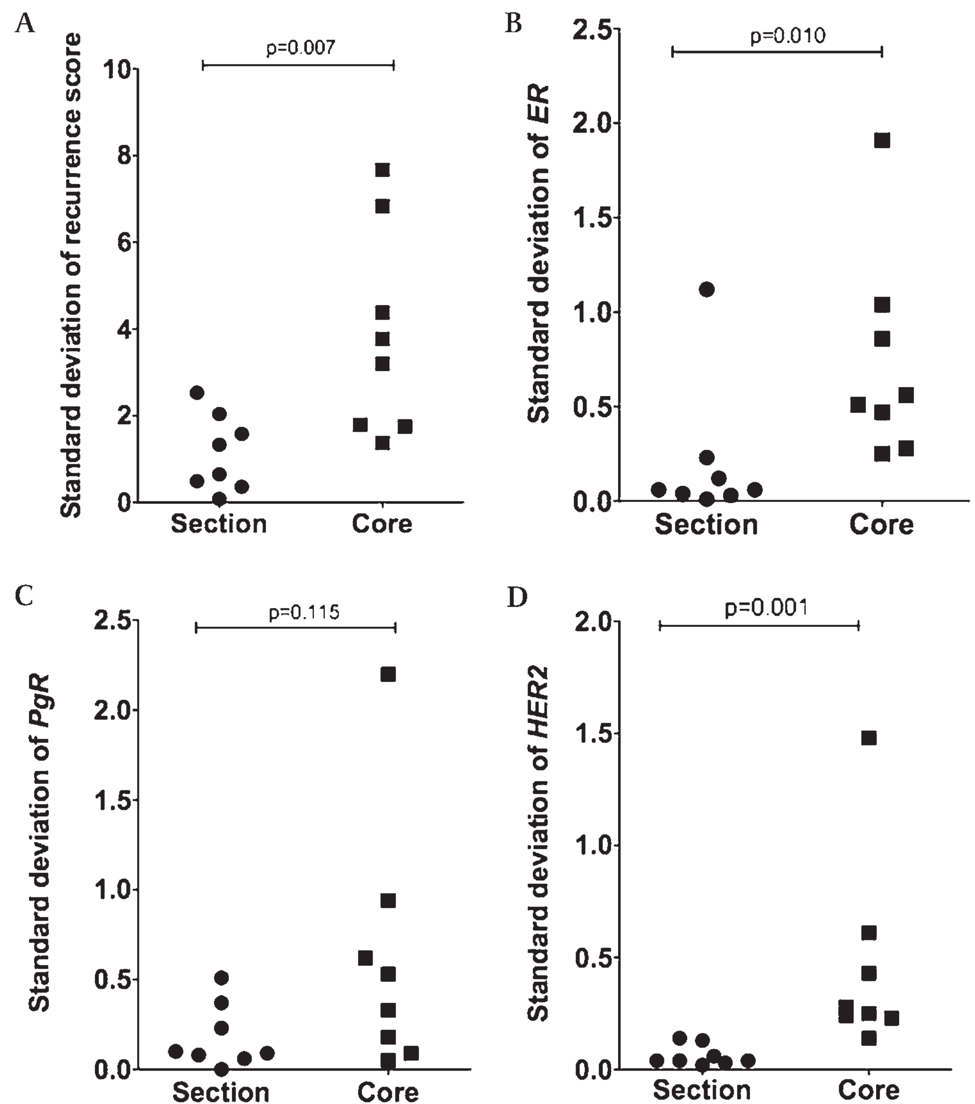

Figure 3 SD for pairs of sections and sets of cores of (A) recurrence score, (B) *ER*, (C) *PR* and (D) *HER2*. There was greater variation across cores than sections in all instances except for *PR*.

RESULTS

Cores taken in quadruplicate from eight tumours yielded a mean of 0.6–2.3 μg of RNA/core (table 2).

All 32 cores yielded more than the 0.38 μg required for the Oncotype DX® assay. The mean yield of RNA from the pairs of sections ranged from 1.7 to 22.4 μg RNA/section. The overall means were 1.1 μg/core and 10.4 μg/section. Mean yield/core was correlated significantly with mean yield/section (figure 1; Spearman r=0.91; p=0.005).

Using the Oncotype DX® assay, three patients were classified as high risk, three as intermediate risk and two as low risk, based on either the overall mean RS/core or the RS/section (figure 2A).

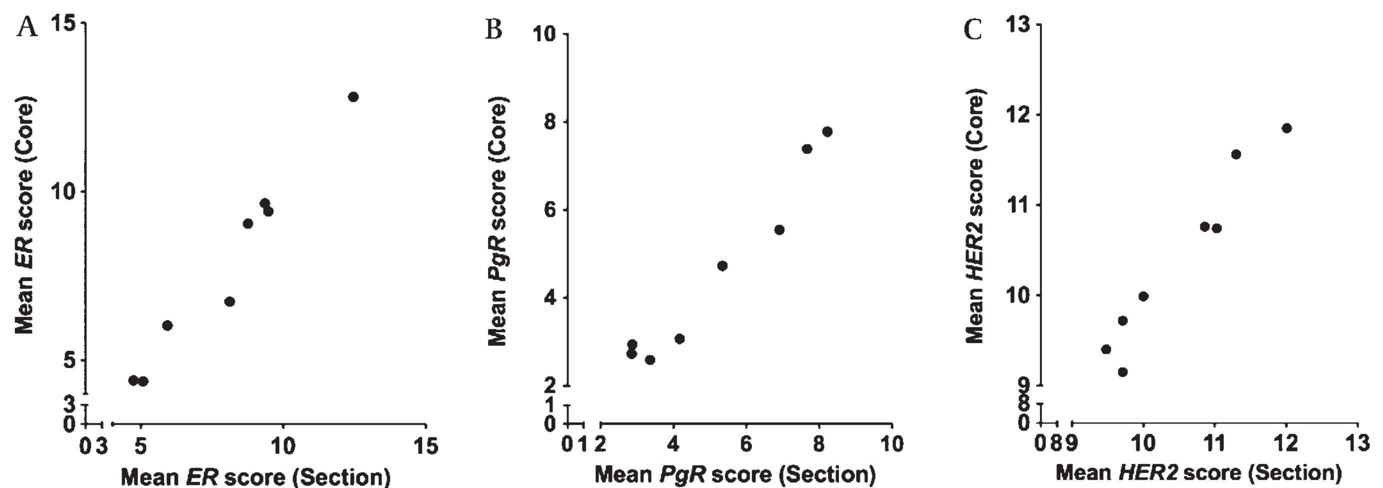

Figure 4 Individual gene scores for (A) *ER*, (B) *PgR* and (C) *HER2*. There was an excellent correlation between cores and sections for each of these genes.

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In most cases, the RS values clustered closely irrespective of a core or section being the source material and there was a significant correlation of section RS with core RS (figure 2B; Spearman r=0.91; p=0.005).

However, variability of RS was greater between replicate cores than between sections (p=0.007, Mann–Whitney test, figure 3A), and in samples from three patients (3, 63 and 104) the variability between the RS of cores was sufficient for one out of the four cores to result in a higher risk category than the other three (figure 2A). For patient 104, it was noted retrospectively that cores were not taken from areas of enriched tumour, and in this case marked differences in tumour content were likely to have contributed to the differences in score. However, the SD of RS from the sample from this patient (SD 6.83) was similar to those of patient 3 (SD 4.38) and patient 63 (SD 7.67) that did have cores taken from tumour areas.

Individual *ER*, *PgR* and *HER2* scores showed excellent correlation between the averages of the four cores and of the whole sections (figure 4, table 3).

In all eight cases for each of the three genes, the RNA levels from paired sections were consistently above or below the Oncotype DX® positive/negative cut-off (figure 5A–C). However, one of the eight cases showed positive–negative discordance when RNA from cores was used (patient 104 was discordant for *ER* and *PgR*, patient 3 was discordant for *HER2*). Variability of *ER*, *PgR* and *HER2* was greater between replicate cores than between sections. This was significant for *ER* and *HER2* (p=0.010 and p=0.001 respectively), but not for *PgR* (figure 3B–D).

DISCUSSION

High-throughput technologies and genetic analysis have contributed enormously to understanding the molecular characteristics of breast cancer. Current technologies have enabled gene expression analysis to be extended to FFPE tissue. In some cases, such as the Oncotype DX® 21-gene breast cancer assay, validated tests have been derived from FFPE tissues that aid prediction of patient outcome. The taking of 0.6 mm cores, as used for TMA construction, for nucleic acid analysis would be a very convenient approach to storage of tissue samples.

As well as aiding the management of individual patients, tests using FFPE material have allowed research on collections of tissues from clinical trials and from the vast collections of fixed tissues in histopathology archives. Such studies are generally performed using whole sections but the current research practice of creating TMAs for IHC studies might be usefully extended to provide a TMA core or cores for RNA analysis if comparability with thick sections could be demonstrated.

While the sample size of this study was small, it was sufficient to investigate the main issues. It was clear that the yield from cores was consistently lower than that from sections: on average 9.5-fold less. The smaller volume of the core may in part account for this. For a tissue section having an area of $1 cm^2$ and with a thickness of 10 µm, the tissue volume would be $1 mm^3$. A core of 0.6 mm diameter has a surface area of $0.28 mm^2$ and with a thickness of 2 mm, a volume of $0.57 mm^3$. So in such a case nearly double the amount of DNA would be expected from the sections, although tumour area and core thickness will clearly vary markedly between tumours. This volumetric difference does not, however, explain the near 10-fold difference. It is possible that the greater yield could be due to digestion of the tissue, which may be compromised in the thicker core.

Table 3 *ER*, *PgR* and *HER2* RNA scores

	RNA score					
	Core		Section		Correlation	
Gene	Mean	95% CI	Mean	95% CI	Spearman	p Value
ER	7.81	5.35 to 10.26	7.99	5.80 to 10.18	0.95	0.001
PgR	4.60	2.83 to 6.37	5.18	3.34 to 7.00	0.93	0.002
HER2	10.40	9.57 to 11.23	10.51	9.75 to 11.28	0.93	0.002

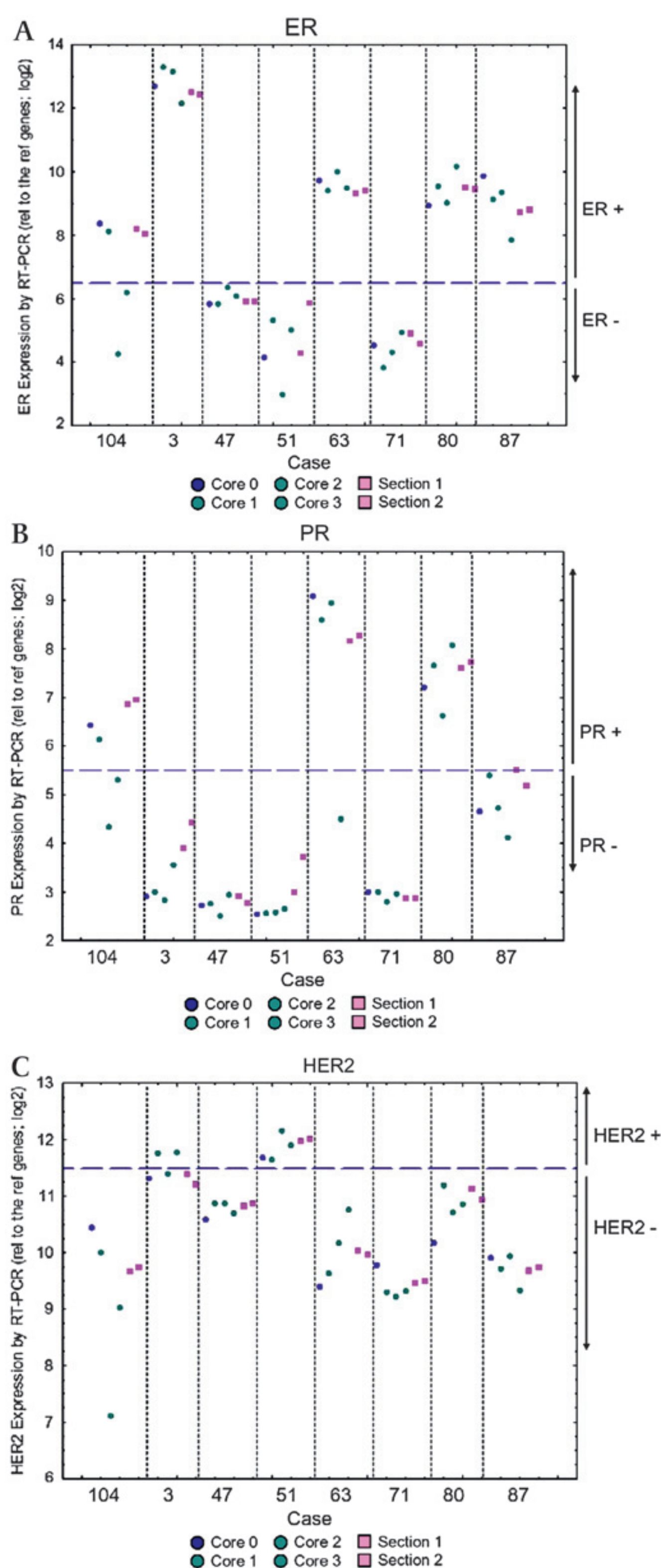

Figure 5 (A) *ER* (B) *PgR* (*PR*) and (C) *HER2* mRNA measured by Oncotype DX in cores and sections for eight patients.

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Take-home messages

- RNA yield was less from 0.6 mm formalin-fixed paraffin-embedded (FFPE) cores than from 10 μm FFPE sections, but was still more than sufficient for performing the Oncotype DX® Breast Cancer Assay.
- Individual gene scores and overall recurrence score were highly comparable between cores and sections, but variation was greater between cores.
- FFPE cores of 0.6 mm are viable material for RNA analysis for cohort studies. However, whole sections allow histological analysis and provide less variable results, making sections preferable for diagnostic studies.

Despite the marked difference in yield, agreement between the RS and the quantitative assessment of individual genes, as determined in sections versus that in cores taken from representative areas of invasive carcinoma, was generally good, with few disagreements between the categorisation of single gene values and RS.

The variability between cores was greater than between sections. This was the case for three important individual genes as well as for the RS. This may be due to microscopic assessment of whole sections and the use of microdissection for tumour tissue enrichment from four sets of whole sections. This is clearly not possible with cores, despite attempts to take the cores, using H&E slides as a guide, from areas of known malignant tissue at the face of the block, as unknown admixtures of benign epithelial tissue or stroma may be contained deeper. Thus for clinical analysis of individual patient tumours, the use of whole sections is preferred. For large-scale clinical studies where absolute precision in each case is often not critical, the slightly greater variability with cores will frequently be acceptable.

In summary, 0.6 mm cores are an entirely viable option for RNA analysis of FFPE material for cohort studies. However, whole sections provide greater yields, less variable results, allow for detailed histological analysis and the potential for microdissection, making them preferable for diagnostic studies.

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Competing interests Dr Rick Baehner is Director of Pathology for Genomic Health Inc. Dr Steve Shak is Chief Medical Officer for Genomic Health Inc.

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