


# Performance of Automated Dissection on Formalin-Fixed Paraffin-Embedded Tissue Sections for the 21-Gene Recurrence Score Assay

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## Abstract

This study aimed to compare the performance of MilliSect dissection and manual dissection. Twenty-five formalin-fixed paraffin-embedded (FFPE) breast cancer tissue blocks were selected for comparison. Specific areas of interest (AOIs) in invasive carcinoma on tissue sections were transferred to dissection slides by manual macrodissection or the MilliSect instrument. The comparison criteria were 1) the time required for dissection; 2) RNA concentration and purity; 3) RNA quantity of 5 house-keeping genes (by RT-qPCR); and 4) ER, PR, HER2, Ki-67 and recurrence score (RS) values (by the 21-gene assay). Then, tumor-adjacent tissues, including fibrocollagenous and epithelial tissues, from the same selected tissue blocks of 8 of 25 patients were scraped using the mesodissection method, and their RS values were assessed to evaluate the influence of tumor-adjacent tissues on the target AOIs. Ultimately, 4 AOIs of invasive ductal carcinoma (IDC) from 1 tissue block of another 4 patients with lymph node (LN) metastases each, LN tissue and a mixture of IDC and LN tissue from the other tissue block of the same 4 patients were mesodissected to evaluate the influence of infiltrating lymphocyte levels on the RS values of AOIs. In our experience, the MilliSect instrument, which provides process management documentation, required more time than manual macrodissection (on average, approximately 9.1 min per sample versus 5.8 min per sample, respectively). The RNA yield and quality of the dissected tissues were comparable for the 2 methods. However, the tumor-adjacent tissues of the AOIs may influence the RS to some extent. Tumor-infiltrating lymphocytes (TILs) can dramatically increase RSs, far exceeding the influence of tumor-adjacent fibrocollagenous and epithelial tissues. In conclusion, MilliSect mesodissection is comparable to manual dissection. This mesodissection tool may facilitate AOI alignment and the dissection process for the 21-gene RS assay. Samples whose adjacent tissues are intermixed with TILs warrant special attention.

## Keywords

microdissection, mesodissection, macrodissection, recurrence score, companion diagnostics

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## Introduction

Molecular genetic testing is increasingly being performed on nucleic acids or proteins extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections.<sup>1</sup> Generally, the specific areas of interest (AOIs) of tissue sections on a haematoxylin and eosin (H&E)-stained, cover-slipped glass slide are manually marked by a pathologist under microscopic guidance. Then, using the marked H&E slide as a guide, manual dissection, which involves the use of a scalpel or other cutting tools,<sup>2-6</sup> is performed on a second non-cover-slipped slide-mounted tissue section from the same tissue block without the aid of a

microscope. These methods are sufficient for most samples submitted for molecular testing at a low cost but provide limited

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resolution regarding tissue heterogeneity. In addition, non-tumor cells may hinder the detection of actionable and clinically relevant mutations or influence the accuracy of certain gene expression assays relative to the detection thresholds. Manual microdissection and laser capture microdissection (LCM) are used to address a lack of resolution, yet the dissection instruments are labor intensive, expensive, and sometimes dependent on the photo activation film or special slides. In addition, owing to the unstable nature of RNA, obtaining high-quality RNA using the LCM system is often challenging;<sup>7-9</sup> however, other researchers have shown that LCM samples present no such difficulties.<sup>10</sup> New developments in microdissection technology are still needed to make it more attainable to researchers in a time-sensitive and affordable manner.

The MilliSect mesodissection system, which utilizes a modified computer numerical control (CNC) milling machine, was recently introduced as an intermediate resolution method to dissect tissue from any glass slide, allowing the technology to be integrated easily into clinical laboratory workflows.<sup>11</sup> To some degree, Sequenom MassARRAY and more complex mutational assays typically benefit from this mesodissection method. However, to date, this microdissection method has not been tested in regard to whether it diminishes the yield and quality of the retrieved RNA from FFPE tissue sections.

Here, we compared the performance of the MilliSect instrument with that of the manual dissection method on a series of FFPE breast cancer specimens. The following criteria were applied to compare the 2 dissection methods: 1) time to perform the dissection; 2) RNA concentration and purity; 3) RNA quantity of 5 housekeeping genes (by RT-qPCR); and 4) ER, PR, HER2, Ki-67 and recurrence score (RS) values (by the 21-gene assay, which was developed to predict the likelihood of distant recurrence and chemotherapy benefit for breast cancer patients).<sup>12-16</sup> In our experience, the MilliSect instrument took slightly longer than manual macrodissection to perform tissue dissection. Despite being a more time-consuming method, mesodissection provides process management documentation, which is very important for the quality control of molecular testing. In addition, the RNA quantity and quality of the dissected tissue were similar for manual macrodissection and MilliSect instrument mesodissection. However, tumor-adjacent tissues of the AOIs may influence the RS to some extent. Tumor-infiltrating lymphocytes (TILs) can dramatically increase RS, and thus, close attention should be paid to samples whose adjacent tissues are intermixed with TILs. The mesodissection of FFPE sections from slides with the MilliSect instrument may assist in the dissection process for the 21-gene RS assay in the clinical laboratory setting, providing an economical, automated and robust platform.

## Materials and Methods

### *Case Selection, Evaluation of Tumor Content, and Slide Marking for Dissection*

The Fudan University Shanghai Cancer Center Institutional Review Board approved the study protocol (approval no.

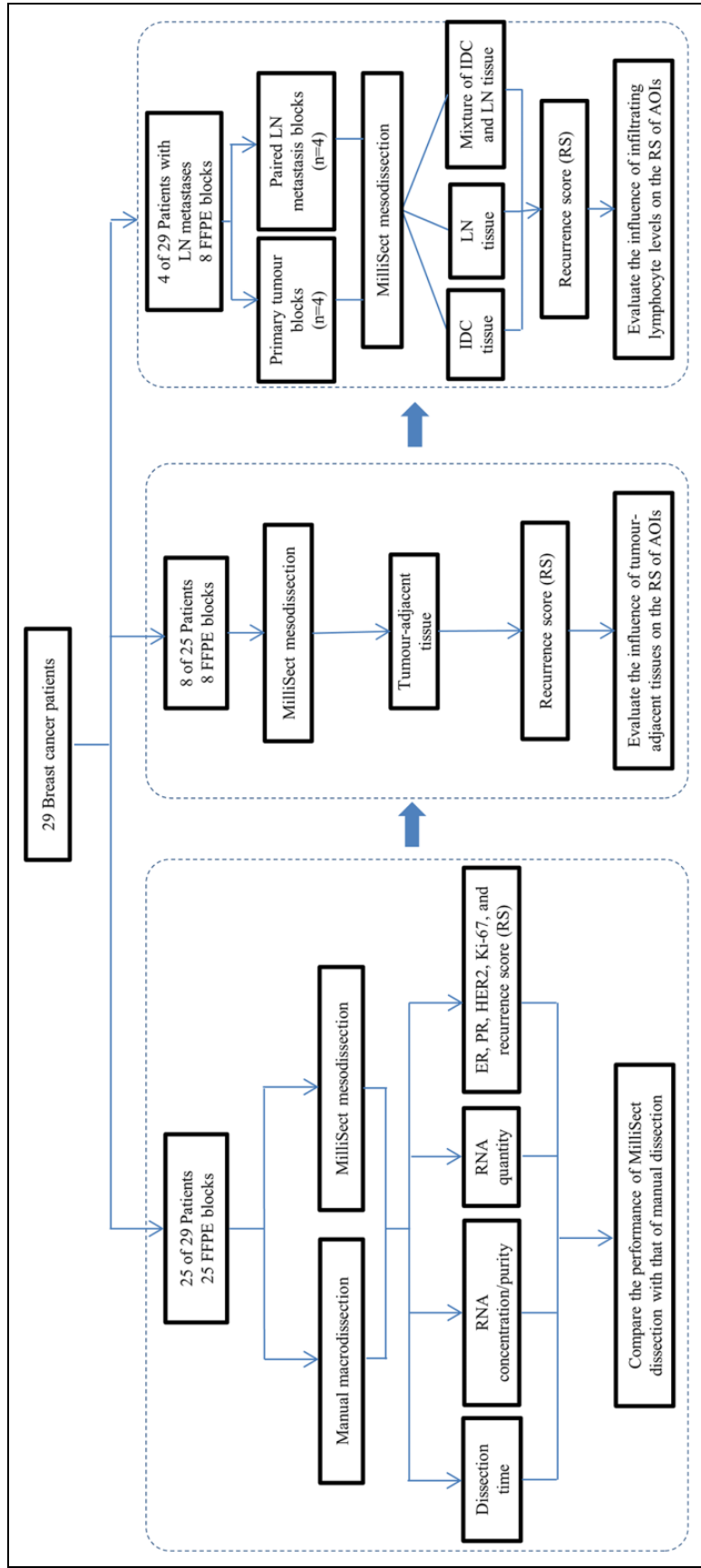
050432-4-1805C, Figure 1). A total of 29 patients provided written informed consent prior to enrolment in the study. First, representative ER-positive, HER2-negative newly produced breast cancer FFPE samples (n = 25) from the Department of Pathology archives, Fudan University Shanghai Cancer Center, were microscopically evaluated for specific AOIs, namely, invasive carcinoma areas, and tumor content by a pathologist using an H&E section. Prior to dissection either by hand or using the mesodissection system, eight 5- $\mu$ m sections were serially cut from the FFPE tissue blocks, mounted on adhesive glass slides, and deparaffinized. Then, tumor-adjacent tissues (tumor content < 5%), including fibrocollagenous and epithelial tissues, from the same selected tissue blocks of 8 of 25 patients were scraped using the mesodissection method, and their RS values were assessed to evaluate the influence of tumor-adjacent tissues on the target AOIs using the 21-gene RS assay. Ultimately, 4 AOIs of invasive ductal carcinoma (IDC, tumor content > 40%) from 1 tissue block of another 4 patients with lymph node (LN) metastases each, LN tissue and a mixture of IDC and LN tissue from the other tissue block of the same 4 patients were mesodissected to evaluate the influence of infiltrating lymphocyte levels on the RS values of the AOIs. No special criteria for the selection of the paired LN metastasis tissue block were applied, they were required to contain 2 areas: LN tissue and a mixture of IDC and LN tissue (IDC tumor content > 40%). The following data were also collected: age at initial diagnosis, pathology, histologic grade, T stage, N stage, PR status, the Ki-67 index and vascular invasion status. The tumor characteristics are shown in Table 1.

### *Slide Dissection*

The serial sections used for manual macrodissection or mesodissection were distributed equally on the adhesive glass slide. For manual macrodissection, the AOIs were manually scraped with a surgical scalpel. The AVENIO MilliSect system (Roche, Pleasanton, CA) was used for mesodissection (Figure 2). A pathologist can manually mark the AOIs under microscopic guidance and then save this reference image (HE) to a digital database. Marked reference images were manually aligned and resized to match the image of the dissection slide on the stage. The areas for dissection were transferred automatically from the reference slide to the dissection slides using a color selection tool in 2iD software, and a milling path was automatically generated. Mesodissection was performed using milling tips (Roche, Pleasanton, CA) loaded with proteinase K buffer. The milling tip with either a small or medium blade was used based on the estimated AOI to be dissected. All dissected tissue was recovered in RNase-free microfuge tubes.

### *RNA Isolation and Quantification*

RNA was extracted from annotated areas using an RNeasy FFPE Kit (Qiagen, Valencia, CA). Briefly, 10  $\mu$ l of proteinase K was added to 150  $\mu$ l of tissue lysis buffer mixture, and the mixtures were incubated on a heater/shaker at 56°C with



**Figure 1.** Workflow of the study. Twenty-nine patients were selected in this study. First, 25 formalin-fixed paraffin-embedded (FFPE) breast cancer tissue blocks were selected to compare the performance of MilliSect dissection with that of manual dissection. The applied comparison criteria were as follows: time for dissection, RNA concentration and purity, RNA quantity, and recurrence score (RS). Then, tumor-adjacent tissues, including fibrocollagenous and epithelial tissues, from the same selected tissue blocks of 8 of 25 patients were scraped using the mesodissection method, and their RS values were assessed to evaluate the influence of tumor-adjacent tissues on the target areas of interest (AOIs). Ultimately, 4 AOIs of invasive ductal carcinoma (IDC) from 1 tissue block of another 4 patients with lymph node (LN) metastases each, lymph node tissue and a mixture of IDC and lymph node tissue from the other paired lymph node metastasis tissue blocks of the same 4 patients were mesodissected to evaluate the influence of infiltrating lymphocyte levels on the RS values of the AOIs.

**Table 1.** Clinicopathologic Characteristics of the Patients.

Characteristic	No. (%)
Age (yr)	
≤50	15 (51.7)
>50	14 (48.3)
Pathologic type	
Ductal	22 (75.9)
Lobular	6 (20.7)
Mucinous	1 (3.4)
Tumor grade	
I	3 (10.3)
II	20 (69.0)
III	5 (17.2)
Unknown	1 (3.5)
T stage	
T1	18 (62.1)
T2	10 (34.5)
Unknown	1 (3.5)
N stage	
N0/N1mi	25 (86.2)
N1	4 (13.8)
PR	
Positive	26 (89.7)
Negative	3 (10.3)
Ki-67 (%)	
≤20	17 (58.6)
>20	12 (41.4)
Vascular invasion	
Yes	3 (10.3)
No	26 (89.7)
RS category	
Low risk	11 (37.9%)
Intermediate risk	12 (41.4%)
High risk	6 (20.7%)

regular vortexing for 40 min. Afterward, the tubes were heated to 80°C for 15 min to eliminate crosslinking and then incubated on ice for 3 min. The samples were centrifuged for 15 min to eliminate undigested particulate material. After the treatment, the supernatants were transferred to a Qiagen QIAcube robotic workstation, and RNA was ultimately eluted in 30 µl of nuclease-free water. RNA yield and purity assessment was performed using a Qubit RNA HS Assay Kit on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and a NanoDrop spectrophotometer (ND1000; Thermo Fisher Scientific, Carlsbad, CA, USA) for all samples simultaneously as a measure of the total sample recovered and for subsequent analysis. Each assessment was carried out in triplicate.

### Gene Expression Analysis

The 21-gene assay was performed as described by Paiket al.<sup>12</sup> In brief, after RNA content measurement and residual genomic DNA contamination assessment by a quantitative TaqMan<sup>®</sup> PCR assay, cDNA was generated, and the expression levels of 16 cancer-related genes (BAG1, BCL2, CCNB1, CD68, SCUBE2, CTSL2, ER, GRB7, GSTM1, HER2, Ki-67, MYBL2, PR, STK15, STMY3, and SURV) and 5 reference

genes ( $\beta$ -actin, GAPDH, GUS, RPLPO, and TFRC) were quantitatively analyzed by using RT-qPCR. The average Ct values of the 5 reference genes represent the RNA quality. A numerical score (the RS) ranging from 0 to 100 was computed and categorized into low-risk (score <18), intermediate-risk (score 18 to 30) or high-risk (score  $\geq$ 31) groups. The RS algorithm has been reported in detail.<sup>12</sup> Positive and negative controls were established in each 384-well plate.

### Assessment of TIL Levels

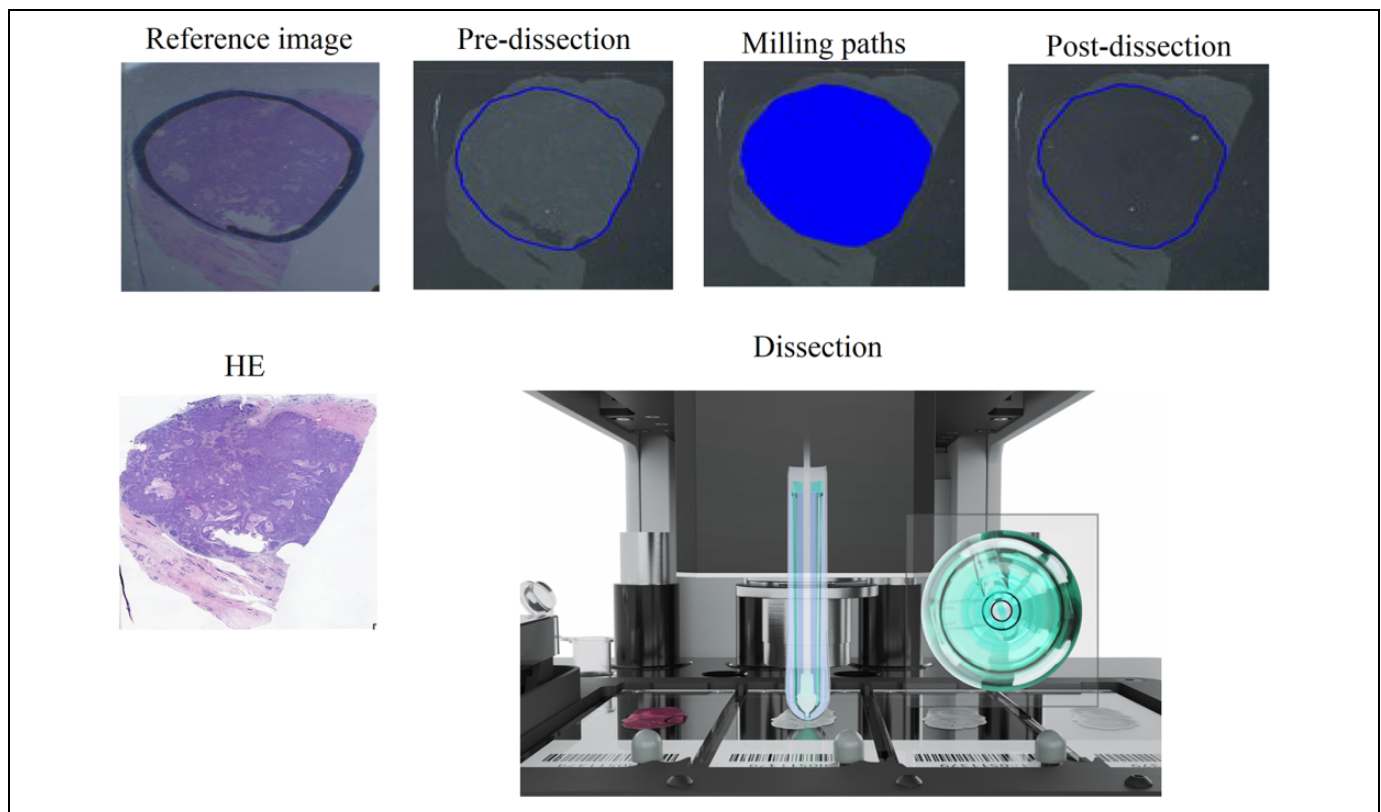
Histopathologic assessment of the percentage of TILs was performed on representative H&E sections of tumors using methods recommended by the International TILs Working Group 2014.<sup>17</sup> TILs were evaluated within the borders of invasive tumors (including the invasive borders). Briefly, the tumor area as defined by the presence of invasive tumor was evaluated, and all mononuclear cells, including lymphocytes and plasma cells but not polymorphonuclear leukocytes, were scored. Areas outside the tumor border, around the intraductal component, and normal lobules were excluded. Within the tumor border, TILs with crush artifacts and necrosis were excluded. For each case, 3 representative tumor areas were evaluated for TILs, and the average score was reported as a percentage.

### Statistical Analysis

The primary objective of this study was to compare the performance of MilliSect dissection and manual dissection. The RNA yield, RNA quality, and ER, PR, HER2, Ki-67 and RS values measured by the Qubit RNA HS assay and RT-qPCR, in addition to the time required for tissue dissection, were evaluated to be normally or non-normally distributed using the Shapiro-Wilk test. Then, a 2-tailed paired t-test or the Wilcoxon signed-rank test was performed to compare the data of 2 groups. Pearson's R was calculated to measure the correlations of each of the 5 reference genes, the average Ct values, and ER, PR, HER2, Ki-67 and RS values between the MilliSect dissection and manual dissection methods and the correlations between the RSs obtained from the mesodissection method and TIL levels. All calculations were performed using SPSS software (version 19.0, IBM Corp., Armonk, NY). A *P*-value of less than 0.05 (2-sided) was considered to indicate a significant result.

### Results

Twenty-nine patients were selected for this study (Table 1). The most common histologic subtype in this cohort was IDC ( $n = 22$ ; 75.9%), followed by invasive lobular carcinoma (ILC,  $n = 6$ ; 20.7%) and mucinous carcinoma (MC,  $n = 1$ ; 3.4%). The percentages of patients with grade I, II and III tumors were 10.3%, 69.0% and 17.2%, respectively. T1 and N0/micrometastatic disease in the LNs tumors accounted for 62.1% and 86.2% of all patients, respectively. The RS was distributed as



**Figure 2.** The MilliSect instrument software and workflow. An example of pre-dissection and post-dissection of an AOI from an FFPE tissue section off standard glass slides using the MilliSect instrument. A pathologist can manually mark the AOIs under microscopic guidance and then save this reference image (HE) to a digital database. The areas for dissection were transferred automatically from the reference slide to the dissection slides using a color selection tool in 2iD software, and a milling path was automatically generated. The area dissected is represented in blue (milling paths panel). Tissue was collected by mesodissection mediated by xScisor technology.

follows: low (score <18) = 11 (37.9%), intermediate (score 18 to 30) = 12 (41.4%), and high (score  $\geq$ 31) = 6 (20.7%).

First, 25 samples were selected for the manual macrodissection and mesodissection comparison study. We dissected the manually marked areas from additional serial sections using mesodissection to directly compare the RNA yield and quality of the dissected tissue. Figure 2 shows an example of pre-dissection and post-dissection of an AOI from an FFPE tissue section off standard glass slides using the MilliSect instrument. For the slide annotation process, a pathologist circled the tumor remotely through computer access, which allowed maximum flexibility, simplification of the workflow for slide annotation, the alignment of unstained sections, and ultimately dissection. Dissection time followed a skewed distribution. The MilliSect instrument took longer than manual macrodissection to perform tissue dissection (on average, approximately 9.1 min per sample versus 5.8 min per sample, respectively) ( $P < 0.05$ , Table 2). Despite being a more time-consuming method, mesodissection provides process management documentation, which is very important for the quality control of molecular testing. Process management documentation includes the time spent on each step of the whole process, the reference slide images, the dissection slide before and after dissection, and the dissected area dimensions.

The RNA amounts, followed a skewed distribution, ranged from 399 to 8600 ng for the manual macrodissection method and from 468 to 8480 ng for the mesodissection method. There was no statistically significant difference in the RNA yield or purity in this direct comparison ( $P > 0.05$ ). Because RNA is labile, the mesodissection system was subjected to RNA expression analysis, the 21-gene assay, to investigate its capability. All of the extracted RNAs were sufficient for this assay. The average Ct values of the 5 reference genes, followed a normal distribution and representing RNA quality, were highly similar between manual macrodissection and mesodissection ( $P = 0.388$ ), indicating that the recovered RNA was not impaired by the mesodissection method. In addition, we found strong significant correlations for the Ct values of each of the 5 reference genes and the average Ct values between manual macrodissection and mesodissection (all Pearson's  $R > 0.6$ ,  $P < 0.001$ ).

The ER, PR, HER2, Ki-67 and RS values, followed a normal distribution, were comparable between manual macrodissection and mesodissection for 25 patients (all  $P > 0.05$ ) or patients with different clinicopathologic characteristics according to age, tumor grade, T stage and pathologic type (all  $P > 0.05$ ). Strong significant correlations were also found for the ER, PR, HER2, Ki-67 and RS values between manual macrodissection

**Table 2.** RNA Recovery, CTs, RS Values, Time Spent and AOI Measurements for Each Sample Pair.

Patient ID	AOI (mm <sup>2</sup> )	Macrodissection										Mesodissection									
		RNA					time					RNA					time				
		recovery (ng)	purity (A260/280, A260/230)	average CT	RS	spent (min)	ER score	PR score	HER2 score	Ki-67 score	recovery (ng)	purity (A260/280, A260/230)	average CT	RS	spent (min)	ER score	PR score	HER2 score	Ki-67 score		
1	742	8600	1.86/1.94	27.9	39.5	7	8.1	4.1	8.6	5.3	8480	1.91/2.01	27.6	42.2	10	7.6	4.0	8.3	5.3		
2	114	399	1.88/2.01	28.1	30.2	3	8.0	6.3	7.0	4.1	468	1.82/1.91	28.2	26.6	5	8.1	6.7	7.2	3.4		
3	315	3030	1.92/2.03	27.1	38.7	7	6.6	5.9	8.7	3.9	3090	1.88/1.99	27.1	39.6	8	6.6	5.4	8.4	4.3		
4	620	4720	1.83/1.98	28.9	20.3	5	8.9	6.4	9.2	4.3	5040	1.95/2.05	28.4	20.1	8	8.6	7.1	9.7	3.4		
5	996	8580	1.95/2.03	26.7	22.2	4	11.1	6.0	7.8	5.1	8316	1.84/1.98	26.8	22.9	8	10.8	5.9	7.9	5		
6	110	2600	1.87/2.01	29.6	21.9	3	8.0	7.2	7.0	2.5	2638	1.89/1.96	30.1	20.0	5	8.1	8.0	7.8	3.1		
7	698	5310	1.82/1.93	27.9	15.4	8	9.7	6.9	7.9	4.4	5168	1.87/1.97	29.1	14.0	12	10.0	7.6	8.8	3.7		
8	237	1919	1.91/1.98	28.4	14.4	5	10.2	8.3	8.3	3.8	2034	1.82/1.94	28.5	13.1	7	11.0	8.9	8.6	4.6		
9	369	3357	1.83/1.95	28.9	30.4	6	8.6	4.1	8.2	5.0	3145	1.88/2.03	28.3	28.5	10	8.5	4.2	8.5	5.1		
10	401	3288	1.88/2.01	28.1	16.1	7	8.9	6.9	7.6	4.0	3348	1.82/2.01	28.3	17.7	11	9.1	6.8	7.5	3.6		
11	342	2291	1.86/1.96	29.0	10.8	7	11.4	8	8.4	3.1	2148	1.84/1.96	29.3	12.1	11	12.2	8.4	8.6	3.5		
12	189	1341	1.91/2.03	28.9	13.4	4	9.9	8.5	8.0	4.4	1217	1.83/1.94	29.9	14.1	6	9.6	8.9	8.3	3.5		
13	372	1941	1.82/1.91	32.1	27.9	7	8.1	5.7	7.6	4.2	1746	1.92/1.97	33.2	26.9	12	9.0	6.1	8.7	3.6		
14	391	1877	1.86/2.03	27.2	26.9	7	9.2	6.7	7.8	4.6	1937	1.84/1.98	27.8	26.4	12	9.6	7.0	8.1	5.1		
15	287	1779	1.92/2.06	27.8	10.4	7	9.1	8.9	6.5	4.3	1839	1.95/2.06	28.2	9.0	11	10.0	9.7	7.5	4.7		
16	598	4488	1.86/1.97	28.3	15.2	7	11.1	8.7	7.0	3.9	4241	1.81/1.93	28.9	14.5	11	11.7	9.3	7.6	2.9		
17	640	6150	1.85/1.99	28.4	26.9	7	10.8	2.6	7.6	5.3	5507	1.92/2.02	29.0	25.1	12	11.0	3.7	8.3	4.9		
18	177	1218	1.91/2.05	26.9	33.7	5	7.9	6.8	7.3	3.2	1306	1.82/1.97	26.8	33.3	7	7.7	7.7	7.4	2.7		
19	258	2139	1.88/1.98	28.1	16.1	6	8.9	6.9	7.6	4.0	2208	1.84/1.97	29.1	16.0	9	8.9	7.3	8.1	3.1		
20	449	3678	1.83/1.94	28.9	30.4	6	8.6	4.1	8.2	5	3518	1.92/2.02	28.3	30.1	10	7.6	3.8	8.1	4.6		
21	351	1509	1.84/2.01	28.6	16.4	8	8.4	9.7	7.5	4.5	1367	1.86/1.96	29.4	15.0	12	8.3	10.2	8.3	4.0		
22	407	2360	1.88/2.03	27.2	3.6	6	11.6	9.1	8.2	4.5	2157	1.82/1.98	27.2	5.3	11	11.3	9.3	8.4	4.6		
23	358	2291	1.82/1.92	28.5	12.6	5	8.8	8.9	7.7	5.1	2314	1.86/2.01	29.7	13.2	8	8.6	9.7	7.5	5.6		
24	224	1926	1.88/1.91	28.1	22.4	4	8.0	4.9	9.1	0.2	1821	1.82/1.99	27.7	23.2	6	7.8	4.7	8.9	0.4		
25	216	1685	1.92/2.05	28.2	31.7	4	9.7	1	6.7	4.7	1853	1.86/2.03	28.3	31.7	6	9.3	2.1	7.5	2.7		

CT: cycle threshold; RS: recurrence score.

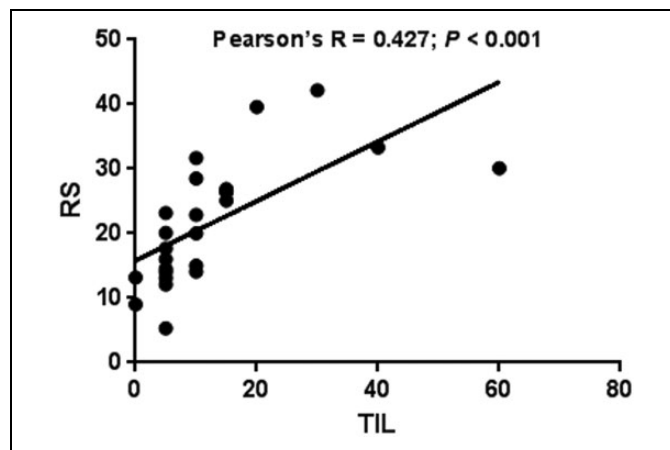
and mesodissection (all Pearson's  $R > 0.6$ ,  $P < 0.001$ ). The RSs of patients 1, 10, 11 and 22 were slightly higher with the mesodissection method, while the RSs of patients 2, 6, 9 and 17 were slightly higher with the manual macrodissection method. We believe that the tumor-adjacent tissue sections may have influenced the RSs of the AOIs. To test this possibility, we scraped tumor-adjacent tissues, including fibrocollagenous and epithelial tissues, from the same tissue blocks of these patients using the mesodissection method and assessed their RS values. As expected, the results indicated that the RSs of the tumor-adjacent tissues were slightly or substantially higher or lower than the RSs of the AOIs (Table 3). These results indicated that

tumor-adjacent tissue may slightly lower or increase the RS value of an AOI.

TILs in the microenvironment of breast cancer have been proposed to reflect the efficacy of immune therapy<sup>17</sup>. Many investigators have extensively studied the clinical value of TILs or RS in breast cancer; however, only a few studies address a possible association between RS and TILs in breast cancer. We analyzed those 25 samples selected for the manual macrodissection and mesodissection comparison to explore the correlation between continuous RS and TIL levels. We found a moderate but significant correlation between the RS obtained from the mesodissection method and TIL levels (Pearson's  $R = 0.427$ ,  $P < 0.001$ , Figure 3). To confirm this preliminary conclusion, we then evaluated the influence of TILs on patients with LN metastases. We scraped 1 IDC area (approximately 90% tumor content) from 1 tissue block obtained from patient 26 with the help of the MilliSect instrument, LN tissue and a mixture of IDC and LN tissue from the other paired LN metastasis tissue block of the same patient (Table 4). We found that the RS value of LN tissue was 44.4, which was much higher than that of IDC tissue. Naturally, the RS value of the mixture of IDC and LN tissue ( $RS = 27.2$ ) was between those of IDC tissue and LN tissue. We then scraped 9 different areas from an additional 6 tissue blocks obtained from 3 patients (patients 27, 28, and 29; Table 4) to confirm these results. All 3 patient samples demonstrated similar results (i.e., infiltrating lymphocyte levels may dramatically influence the RS value of the AOI, far exceeding the influence of adjacent fibrocollagenous tissue and epithelial tissue) (Table 4).

**Table 3.** Results of 21-Gene RS Analysis of Tumor-Adjacent Tissue for 8 Patients.

Patient ID	Tumor-adjacent tissue		
	tumor content (%)	average Ct value	RS
1	<5	27.6	37.0
2	<5	28.2	30.8
6	<5	29.4	25.2
9	<5	28.4	34.6
10	<5	28.5	10.3
11	<5	29.4	9.4
17	<5	28.6	29.4
22	<5	27.4	3.9



**Figure 3.** Scatter plots of continuous recurrence scores (RSs) and tumor-infiltrating lymphocytes (TILs) (Pearson's  $R = 0.427$ ,  $P < 0.001$ ).

## Discussion

The direct dissection of AOIs from slide-mounted FFPE sections is commonly used to obtain specific cell types for molecular genetic analysis. In most clinical laboratories, operators utilize manual dissection methods for cost and simplicity reasons. As early detection strategies have become more effective and less invasive needle biopsy strategies have been adopted, the need to process small specimens is increasing. In addition, the changing landscape of molecular genetic and genomic testing aims to preserve tissue and minimize the input of samples for testing in the future. However, processing small regions of tissue can be a challenge since samples can be lost during manual macrodissection. The alternative choice is LCM, which is highly precise but also complicated and costly. Due to the

**Table 4.** Results of 21-Gene RS Analysis of 4 Patients With Lymph Node Metastases.

Patient ID	invasive ductal carcinoma (IDC)			lymph nodes (LNs)			mixture of IDC and LNs		
	tumor content (%)	average Ct value	RS	tumor content (%)	average Ct value	RS	tumor content (%)	average Ct value	RS
26	90	25.0	19.4	-	26.6	44.4	70	25.3	27.2
27	60	27.2	28.8	-	26.8	54.2	50	27.0	32.5
28	60	25.3	33.4	-	24.9	51.1	60	25.2	37.1
29	50	26.5	45.8	-	25.8	59.7	40	25.3	50.4

disadvantages of LCM, new advances in microdissection technology are still needed to fulfill clinical requirements.

One such development in microdissection technology now available is an automated dissection system from Roche. For this technique, a machine is used to mill the annotated AOI and aspirate it into a mill bit.<sup>11</sup> Next, this material can be aspirated into a collection tube and used for subsequent applications.<sup>18-25</sup> The advantages of this technology are that it is significantly cost effective and time sensitive. More importantly, this system can improve the precision of certain molecular techniques, such as MassARRAY gene mutation analysis<sup>21</sup> and next-generation sequencing analysis.<sup>25</sup> For example, Geiersbach et al. found that the estimated neoplastic cellularity and KRAS mutant allele fraction were significantly higher in samples obtained by mesodissection, and 7 of the 32 samples (22%) showed a detectable mutation only with this technology.<sup>21</sup> Gustafson et al. found that the automated dissection of tumor AOIs could detect variants that would otherwise go undetected in 70% of cases, some of which, importantly, were clinically actionable mutations.<sup>25</sup> To date, other complex molecular tests, such as expression analysis, have not been assessed using this system. Since downstream molecular biology is expensive, improving the quality of the input sample may also be cost effective. In this study, we aimed to quantitate the degree of improvement in the precision of the 21-gene RS assay, which provides important information for predicting the benefit of adjuvant chemotherapy for early breast cancer patients.<sup>26</sup>

Each mesodissection took approximately 9 min, which was comparable to the time it took with manual dissection with a surgical scalpel. Mesodissection software allows a pathologist to annotate AOIs on a digital image, which can eliminate the need to send hand-annotated slides to a dissection laboratory, thus minimizing logistical issues. In addition, we found that milling solutions such as proteinase K buffer can be used as long as they can hold the tissue fragments in suspension and do not degrade the plastic xScissor. Finally, the software generates a digital document of the whole dissection process, which is very important for the quality control of molecular testing.

The RNA amounts and quantities were very similar for the manual macrodissection and mesodissection methods, indicating that RNA recovery is not impaired by the mesodissection method. This important finding shows that the samples benefit from a more precise dissection method and are often limited in overall tumor content and that the efficient recovery of RNA is critical. Often, only 1 or 2 slides are available for molecular testing after the primary diagnostic workup using H&E, immunohistochemistry, and special stains. Additionally, we used manual macrodissection and mesodissection methods to generate samples for 21-gene expression analysis applications. The results demonstrated that automated dissection samples are compatible with gene expression analysis using RT-qPCR. The gene scores of 16 cancer-related genes were comparable between manual macrodissection and mesodissection for 25 patients (available upon request). As for the RS value, most of the 25 samples showed very similar results ( $P > 0.05$ ), and the differences were less than 3. Strong significant correlations

were also found for the ER, PR, HER2, Ki-67 and RS values between manual macrodissection and mesodissection (all Pearson's  $R > 0.6$ ,  $P < 0.001$ ). By comparing specific AOIs in the same patient's FFPE tissue block (patients 1, 2, 6, 9-11, 17 and 22), we aimed to explore potential confounding factors, such as tumor-adjacent tissues, for the 21-gene expression analysis. Indeed, we confirmed that tumor-adjacent tissues, such as fibrocollagenous tissue and epithelial tissue, can result in slightly lower or higher RS values of AOIs.

There is accumulating evidence that TILs are an important immunological biomarker<sup>27</sup> and could predict the clinical response to chemotherapy and the prognosis in breast cancer.<sup>28,29</sup> A systematic review and meta-analysis of 13 neoadjuvant systemic therapy studies showed that HR-positive/HER2-negative tumors with higher TILs in the pre-treatment biopsy correlated with a higher probability of pathological complete response (pCR).<sup>30</sup> The 21-gene RS has been shown to predict the clinical benefit of chemotherapy for individuals with ER-positive/HER2-negative breast cancer.<sup>16</sup> Furthermore, previous studies have shown that tumors with a high RS have a higher rate of pCR in the neoadjuvant setting.<sup>30</sup> Both TIL levels and RS could serve as biomarkers associated with chemotherapy responsiveness in HR-positive/HER2-negative breast cancer. However, the relationship between the 2 markers has not been intensively examined. Previously, Ahn et al. compared TIL levels and RS in ER-positive/HER2-negative breast cancer.<sup>31</sup> They showed a weak correlation between continuous TIL levels and RS, and tumors with high TIL levels tended to have higher RS values. However, Krishnamurti et al. showed a negative correlation between TIL levels and RS,<sup>32</sup> implying that TIL is a favorable marker, in contrast to Ahn's results. Since the relationship between the 2 markers has not been intensively examined, we preliminarily analyzed the 25 samples selected for the manual macrodissection and mesodissection comparison. We found a moderate correlation between RS and TIL levels, which is in line with Ahn's findings. The reason may be that Krishnamurti et al. did not exclude HER2-positive tumors from ER-positive tumors, whereas we and Ahn et al. included only ER-positive/HER2-negative tumors because the RS assay is applied clinically for those specific tumors. Further studies with larger cohorts are required to determine the association between RS and TIL levels in breast cancer. To confirm this preliminary conclusion, we then evaluated the influence of lymphocyte levels on patients with lymph node metastases with the help of the MilliSect instrument. We confirmed that infiltrating lymphocyte levels dramatically influenced the RS values of the AOIs in patients 26-29. Attention should be paid to samples whose adjacent tissues are intermixed with TILs.

In conclusion, this study shows the highly selective and efficient collection of AOIs from slide-mounted FFPE tissue sections using an automated dissection platform that functions comparably to manual macrodissection. Importantly, this automated dissection system provides higher dissection resolution than manual dissection, resulting in highly confident molecular pathological results as reflected by adjacent tissues.



Maximizing the preservation of AOIs prior to molecular pathological testing not only reduces the need for additional biopsies for molecular testing but also diminishes possible inaccuracies. These results demonstrate that the MilliSect instrument may assist in the dissection process for RNA extraction from FFPE section slides and subsequent gene expression analysis in a clinical laboratory setting. FFPE blocks are available in most clinical laboratories; therefore, the technique described here can increase the knowledge obtained from currently unused samples. If this technology is restricted to samples deemed unsuitable for manual macrodissection, the additional information that this technique provides for these challenging samples could be useful in determining the benefit from investment.

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