the samples and hemolysis can be responsible for LD measurements with poor precision in addition to higher LD activity values *(7, 8)*. To investigate these effects, we performed LD measurements with serum-gel (Sarstedt Monovette prod. no. 02.1388), lithium-heparin-gel (same as given above), and lithium-heparin tubes (Sarstedt Monovette prod. no. 01.1604.400). Blood samples were collected from the same person in five different tubes and centrifuged differently to obtain various degrees of platelet contamination. Serum-gel, lithium-heparin-gel, and lithium-heparin samples were centrifuged for 10 min at 3000*g* or for 5 min at 500*g*. Platelet counts were done for all samples (Max M; Beckman-Coulter). IFCC-recommended serum samples had very low platelet contamination, even after reduced centrifugation (see Table 1). Standard-centrifugation lithium-heparin samples contained platelet-poor plasma, whereas reduced centrifugation produced platelet-rich plasma (Table 1). LD activity was measured (n = 77) for each of the five types of sample.

The higher platelet contamination of the samples in case of reduced centrifugation caused only a small increase of the within-run CV: 2.8% (142.9 U/L) and 3.2% (141.8 U/L) compared with 1.1–1.6% (133.9–165.2 U/L) for samples with low platelet contamination (Table 1). The mean LD activity for standard-centrifugation lithium-heparin-gel samples was higher than the activity for the corresponding serum-gel samples (mean difference, 28.8 U/L) and the corresponding reduced-centrifugation lithium-heparin-gel samples (Table 1). This may be the result of platelet destruction with subsequent release of intracellular LD. Lithium-heparin samples showed the same LD activity as lithium-heparin-gel samples despite higher platelet contamination. These results demonstrate that very high platelet contamination may have a small influence on the performance of the LD assay, but again, it would not account for the reported high frequency of duplicate errors.

Bakker et al. *(1)* found different frequencies of duplicate errors for heparin-plasma samples with (19%) and without separator (35%), and they could show that after heparin-plasma samples were transferred to secondary tubes (efficient mixing), the frequencies of duplicate errors dropped to 1.1%. We speculate that in the case of the Becton Dickinson heparin-plasma tubes used, both partial instability of the gel as well as inhomogeneities attributable to platelets and platelet aggregation might have caused the described problems. In combination with the specific sampling tubes, different variables, including blood sample collection, time between blood draw and centrifugation, reduced centrifugation, temperature, or specific analyzer features (e.g., rinsing program, sample and reagent volumes, or timing) could also contribute to the high frequency of duplicate errors.

We conclude that the IFFC method from Roche for LD measurement in heparin plasma is reliable, at least when performed under the conditions described here with Sarstedt Monovette tubes.

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Comparison of Gene Expression Profiles in Laser-Microdissected, Nonembedded, and OCT-Embedded Tumor Samples by Oligonucleotide Microarray Analysis,

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In the study and interpretation of expression profiles in tumor samples, the quantity and quality of the RNA and the heterogeneity of the tissue specimen from which it is extracted are both important. Optimization of amplification protocols has allowed researchers to use fewer tumor cells to obtain expression profiles for some clinical samples *(1, 2)*. Assessment of RNA quality control has been improved by the use of highly sensitive gel analysis *(3)*, and flow cytometry and laser microdissection techniques allow isolation of tumor cells from nondiseased connective tissue as well as inflammatory infiltrates *(4, 5)*. Several groups have reported the use of gene expression profiles from laser-microdissected tumors *(6–8)*. However, the extent of the effect of the laser beam on the quality of the RNA and on the expression profile results remains unclear. Because the amount of RNA obtained after laser microdissection is generally low, opportunities for additional quality control and validation are limited *(9, 10)*. These issues led us to investigate the potential differences in gene expression profiles among samples obtained by different sample manipulation procedures.

We compared the gene expression profiles of nonmicrodissected (NE) samples with those from manually microdissected OCT blocks (OCT) and laser capture-microdissected sections (LASER; obtained with a PixCell microdissector) from aliquots of tumors obtained from the same patients. Four bladder transitional cell carcinomas and one breast carcinoma were evaluated. The expression profiles of the 15 tumor samples obtained with oligonucleotide HG-133A arrays containing 22 283 probes (Affymetrix) were analyzed by means of a *t*-test and hierarchical clustering *(11, 12)*. We estimated the effect of laser microdissection on gene profiling as well as potential sources of differences and criteria that may enable characterization of a sample as suitable for gene expression analysis. A detailed description of the materials and methods used and complementary information are provided as a Data Supplement available with the online version of this Technical Brief at http://www.clinchem.org/content/vol49/issue12/.

We first evaluated the quality of the total RNA by means of absorbance ratios, gel analysis, and hybridization on a GeneChip test 3 array before hybridization on the HG-133A oligonucleotide arrays. The total RNA obtained from the 15 samples was sufficient (concentrations >0.1 $\mu g/\mu L$) and of adequate quality (based on $A_{260\ nm/280\ nm}$ ratios >1.8) for these analyses. We analyzed the gel images of the total RNA obtained by the Agilent 2100 Bioanalyzer for each sample (Fig. 1A) and observed that certain samples had similarly high total RNA quality, e.g., cases 2 and 102769. Conversely, other samples displayed lower total RNA quality, such samples 3- and 6-LASER or the three specimens from case 11. To assess the quality of the total RNA, we analyzed the characteristics of the probe obtained in the GeneChip test 3 array. The ratios of the signal intensities of the probe sets for β-actin and glyceraldehyde-3-dehydrogenase (GAPDH) on the GeneChip test 3 arrays were used as hybridization controls (see the online Data Supplement). Samples 3- and 6-LASER, as well as the three samples from case 11, showed a 3′/5′ ratio for GAPDH >5 and a 3′/5′ ratio for β-actin >65. This latter ratio was at least four times higher than the ratios for any of the 10 remaining specimens, suggesting that the extracted RNA was of poorer quality. For the LASER samples with poorer quality RNA (cases 3 and 6), the percentages of genes on the GeneChip were lower than for their corresponding OCT and NE aliquots. We found no remarkable differences in noise and background (web site), but the scaling factor was higher in the laser-microdissected samples compared with the nonmicrodissected samples, with means of 6.4 for the LASER samples, 4.3 for the OCT, and 4.7 for the NE (see the online Data Supplement). Overall, the RNA spectra obtained with highly sensitive gel analyses and the 3′/5′ ratios of the housekeeping genes GAPDH and β-actin on specific GeneChips identified samples with high-quality RNA before hybridization on HG-133A expression arrays. A major difference between our study and previous reports on gene profiling of laser-microdissected tumors was that no additional amplification protocol was used for the laser-microdissected samples *(5–10)*. The standard hybridization protocol was modified to reduce the amount of starting RNA material from 10–15 μg to 1.5 μg. Because we used the same hybridization protocol for the OCT, NE, and LASER samples obtained from the same case, any difference in the gene expression profiles among samples could be attributed to the handling protocols or the RNA quality in each sample.

The issues of sample replication/validation are often raised for microarray experiments. Our study design dealt with that important analytical aspect; we conducted both biological and technical validation studies. The biological validation was conferred by the analysis of three distinct samples from each patient included in the study. The technical validation was conferred by evaluation of the variability of the hybridization of multiple probes representing the same gene or expressed sequence tag on the HG-U133A array. We calculated the variation in the hybridization signal for each probe among the three samples, NE, OCT, and LASER, for each case. The mean, SD, and CV for each probe for each of the three samples from each case as well as for the groups of several probes representing a specific gene (ordered by its gene symbol) are available in the online Data Supplement. We decided not to run the same sample more than once on different arrays. Because there were several probes representing each gene/expressed sequence tag, redundancy of results did not warrant a technical GeneChip duplicate.

Hierarchical clustering was used to evaluate the association of the gene profiles of the samples under study *(11)*. To assess the robustness of the clustering analysis, we applied a bootstrap resampling technique *(12)*. All clusters provided nearly 100% confidence after 1000 iterations (Fig. 1B). Hierarchical clustering grouped together LASER samples with their respective OCT and NE specimens in certain cases, such as for cases 2 and 102769. Samples 6- and 3-LASER clustered together and were separated from their respective OCT and NE samples. The gene expression profiles of the LASER samples from case 11 were more similar to those of the OCT than to the NE samples. The expression profiles of laser-microdissected tumors 6 and 3 clustered together with the three specimens from case 11. The gene expression profiles of laser-microdissected sections with high-quality total RNA grouped with their respective nonmicrodissected samples. Gene expression profiles of laser-microdissected sections of poorer quality RNA clustered together, but separately from their nonmicrodissected specimens. Overall, the application of bootstrapping techniques on hierarchical clustering grouped tumor specimens depending on sample handling and RNA quality, evaluated by means of absorbance ratios, gel analysis, and GeneChip test 3 array hybridization. Inclusion of a breast tumor as an internal control showed that the difficulty in obtaining high-integrity RNA was related to tissue handling rather than tissue type.

We then compared the number of differentially expressed genes among the samples obtained from the same case by the different procedures. We compared the ex-

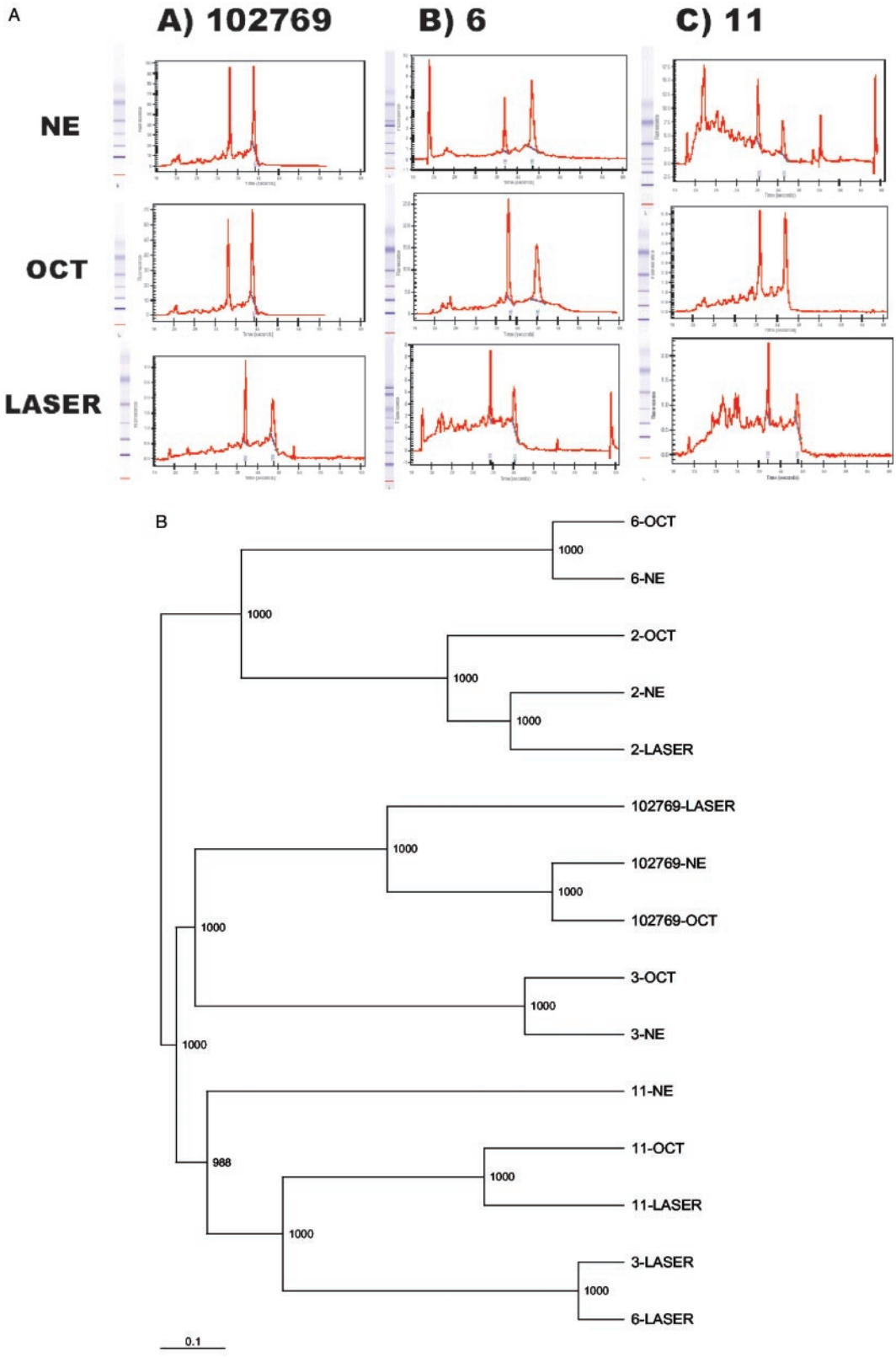

Fig. 1. Examples of quality-control results for total RNA extraction (*A*), and hierarchical clustering analysis of the 15 specimens analyzed (*B*).

(*A*), total RNA was extracted with the Agilent 2100 Bioanalyzer. The specimens displayed distinct degrees of RNA degradation, as seen by the number of peaks observed in the *left portion* of each spectrum. In case 102769 (*spectrum A*), the three aliquots displayed similar high RNA quality (similar to case 2; not shown). In case 6 (*spectrum B*), OCT and NE samples showed higher RNA quality compared with the laser specimen (similar to case 3; not shown). In case 11 (*spectrum C*), the three samples showed poor RNA quality. (*B*), application of bootstrapping techniques confirmed the robustness of the relatedness of the expression profiles of the samples under study. Tumor specimens were grouped depending on sample handling and RNA quality.

pression profiles of the LASER vs the OCT and the NE samples. The number of genes differentially expressed were recorded; the cutoff criteria were two- or threefold changes, with a P value ≤ 0.001 considered significant (Table 1 in the online Data Supplement). We observed that only one gene, topoisomerase (DNA) I, was commonly overexpressed in three of the four bladder OCT samples compared with the respective NE samples when we used the conservative cutoff of a fold change >2. We found that the number of genes differentially expressed between OCT and NE samples was smaller than the number of genes differentially expressed between the LASER and either the OCT or NE samples. An important observation of the present study is the relatively small differences in gene expression between OCT and NE tumor samples. Thus, molecular profiling of OCT-embedded and fresh-frozen tumors could be considered comparable.

We also searched for common genes differentially expressed among laser-microdissected and non-laser-beam-exposed samples in the bladder tumors under evaluation. We observed very few genes commonly over- or underexpressed in these comparisons among different experiments (see the online Data Supplement). This observation suggests that these genes were the products of different amounts of smooth muscle, mesenchymal fibroblasts, and endothelial cells contaminating tumor samples. This pattern was not observed in samples from patient 11, which showed the poorest RNA quality, even in the NE aliquot. Interestingly, we observed a lower number of genes differentially expressed among LASER samples with high-quality RNA compared with their respective OCT and NE samples. When we used the cutoff based on threefold difference in expression, the number of genes differentially expressed among the experiments decreased considerably compared with the twofold cutoff (Table 1). The low number of common over- and underexpressed genes among the laser-microdissected bladder cancer specimens compared with their non-laser-beam-exposed counterparts could be attributed to the fact that the laser-microdissected samples were enriched with tumor cells compared with the non-laser-microdissected samples. However, these bladder cancer cases presented the same histopathology (pT3G3) and were selected based on displaying similar percentages of nonneoplastic tumor cells (see the hematoxylin and eosin-stained sections in the online Data Supplement). Minimum variation was observed between LASER and nonmicrodissected aliquots in samples of high quality, based on two different RNA extraction protocols. This observation reveals that the quality of the RNA in the sample and not the extraction protocol used is a major cause of experimental differences. Overall, the results suggest that the genes identified more represent RNA quality (independent of methodology) and sample-handling-dependent effects.

Once we observed that different numbers of genes were expressed among the samples as a result of RNA quality and sample-handling procedures, we were interested in identifying the biological processes of those genes differentially expressed. Because one of the main objectives of this study was to evaluate whether LASER samples were representative of the tumors from which they were obtained, we searched for the common biological functional annotations of the probes differentially expressed among samples with high and low RNA integrity. We focused on comparing the expression profiles of samples 3- and 6-LASER with the profiles of the remaining 13 samples. Because all three samples from case 11 showed poor RNA quality, we also compared the gene expression profiles of samples 3- and 6-LASER together with the three samples from case 11 vs the remaining 10 samples (online Data Supplement). The hypergeometric method was applied to detect the combination of genes that could genetically define these four groups identified by multidimensional analysis, hierarchical clustering, and RNA quality analysis *(13–15)*. These over- and underexpressed genes were grouped according to the biological processes in which they are involved according to GO functional annotation (see the online Data Supplement).

Two main groups of functional annotations were noted. On one hand, variability of the cell cycle-related genes among samples of different RNA integrity could also be attributed to the presence of different percentages of stromal cells together with cancer cells in the non-laser-microdissected samples. Thus, these functional processes might reveal differential enrichment of cancer cell populations in the different samples. This observation could explain the differential expression of known cancer-related genes in the laser-microdissected material from cases 3 and 6 compared with the remaining experiments.

Table 1. Number of genes differentially expressed among differently handled specimens from the same tumors.[a]

		No. of genes differentially expressed, up/down				
Comparison	Fold change	Tumor 102769 (breast)	Tumor 2 (bladder)	Tumor 3 (bladder)	Tumor 6 (bladder)	Tumor 11 (bladder)
OCT vs NE	>2	38/10	108/555	366/131	371/413	1224/962
	>3	1/18	14/208	130/46	80/98	415/210
LASER vs OCT	>2	533/323	131/434	1167/1894	1231/1330	482/152
	>3	170/76	19/122	758/1199	235/590	189/14
LASER vs NE	>2	638/389	213/962	1094/1604	891/1198	1596/1378
	>3	225/77	19/413	673/912	511/709	642/349

[a] Cutoff criteria included significant differences by *t*-test ($P < 0.001$) and fold changes >2 or 3.

The significant differences in cancer-related genes among these groups of specimens under comparison support the potential clinical utility of laser microdissection for the analysis of cancer biological processes. Caution is necessary when interpreting results comparing dissected and nonmicrodissected samples.

A second type of annotation differentially expressed among the groups under study was related to RNA integrity. Analysis of the genes differentially expressed in samples 3- and 6-LASER, the samples with low RNA integrity, revealed higher overexpression of RNA-processing genes and underexpression of protein biosynthesis-related genes. Genes related to RNA and ribosome processing were among the top differentially overexpressed genes, whereas protein biosynthesis-related genes were among the most underexpressed genes in the two laser-microdissected samples of poorer quality and in the tissue specimen (case 11) displaying lower total RNA integrity. Genes related to functional annotation of protein biosynthesis, protein folding, transcription regulation, or RNA and ribosome processing showed lower expression in these poorer quality samples. These genes could be considered as potential candidates for assessing the integrity of total RNA in laser-microdissection-related experiments before hybridization to GeneChips or other microarrays of interest. Differences in expression observed between samples with poor- and high-quality RNA might also depend on the probe set design and the relative position of the probe set to the polyA tail. If a probe set is close to the 3′ end of a gene, no differences between poor- and high-quality RNA would be detected. Probe sets designed far from the 3′ might allow identification of cRNAs not present in the poor-quality samples; differences in expression could then be detected. It is beyond the scope of this study to evaluate whether these might be more susceptible to modification by the effect of the laser beam during laser microdissection.

In summary, this study represents a technical exercise that deals with differences in gene expression profiles associated with RNA integrity in laser-microdissected and nonmicrodissected samples. The expression profiles of total RNA extracted from OCT-embedded and immediately frozen tumors were comparable. It was possible to obtain RNA of high quality for gene expression analyses with the PixCell microdissector, especially if samples displayed high quality in the OCT or NE aliquots. The generated expression profiles of samples of low RNA integrity after laser microdissection might differ from the profiles for non-laser-microdissected specimens. Laser-microdissected samples of low RNA quality can be identified before hybridization to expression arrays. High-sensitivity gel analysis and GeneChip test 3 arrays are valuable for assessing the quality of the RNA obtained from clinical specimens before hybridization to the microarrays. Analysis of the identified genes that are more likely to display differential expression between specimens of high and low RNA quality represents an additional tool to control the lack of total RNA integrity before gene expression analysis. Overall, control of RNA quality in laser-microdissected samples can avoid misinterpretation of gene expression results derived from analysis of clinical tumor samples.

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