LITE 2000 vitamin B_{12} assay incorporates cobinamide in its formulation for the same reasons.

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Modification of the Standard Trizol-Based Technique Improves the Integrity of RNA Isolated from RNase-Rich Placental Tissue

To the Editor:

Preservation of RNA integrity is important in microarray techniques for identifying differentially expressed genes so that results reflect true biological differences and not differences in RNA degradation *(1)*. RNA degradation is usually low in RNA isolated from cultured cells *(2)*. When samples isolated from RNase-rich tissues are used, however, RNA degradation may introduce bias *(3, 4)*.

RNA extraction using Trizol (Invitrogen Life Technologies) is a common procedure in microarray experiments. Tissue homogenization at room temperature (15–25 °C), as specified in the manufacturer's protocol, generates heat that may increase RNase activity. When snap-frozen tissue pieces are processed, only the outer surfaces are initially in contact with Trizol, and RNase activity in the deeper regions could adversely affect RNA integrity. We modified the Trizol protocol by (*a*) performing homogenization of frozen placental specimens in cold Trizol on wet ice (0–4 °C), (*b*) limiting homogenization time to 30-s intervals for up to 1.5 min to minimize heat generation at the expense of complete tissue disruption, and (*c*) pelleting cellular debris at 12 000*g* for 10 min at 4 °C rather than at room temperature (see the Supplemental Methods section in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol52/issue1/).

Using capillary electrophoresis [RNA 6000 Nano LabChip (Agilent)] on an Agilent Bioanalyzer 2100 system *(5)*, we compared RNA obtained by the standard method and by our modified methods. RNA degradation, if present, would appear as a decrease in the 28S/18S ratio (Fig. 1A), with an increase in the small RNAs peak area (peak I) and the baseline signal (*bracket*). We analyzed capillary electrophoresis data with Degradometer Ver. 1.41 software (available at http://www.dnaarrays.org) *(2)*, which automatically rescaled the time axis so that the synchronization peak occurred at 23.0 s and the 28S peak occurred at 48.0 s. Rescaling allowed analysis of fixed time intervals: 30–41 s (degradation signal range), 41–42.5 s (18S peak range), and 48 s (28S peak range). The degradation factor (DF) was calculated as the ratio of the mean degradation signal value to the 18S peak value, expressed as a percentage. The 28S/18S rRNA ratio was also calculated. RNA degradation was categorized as follows: strong (DF >24%), severe (DF >16%), and detectable (DF >8%) *(2)*.

We evaluated 7 specimens from nonpathologic and pathologic placentas of various gestational ages (see Table 1 in the online Data Supplement). The 260/280 nm ratio for all samples was 1.97–2.13 and did not differ between the standard and modified methods. The mean (SE) total RNA yield (RNA weight/pla-

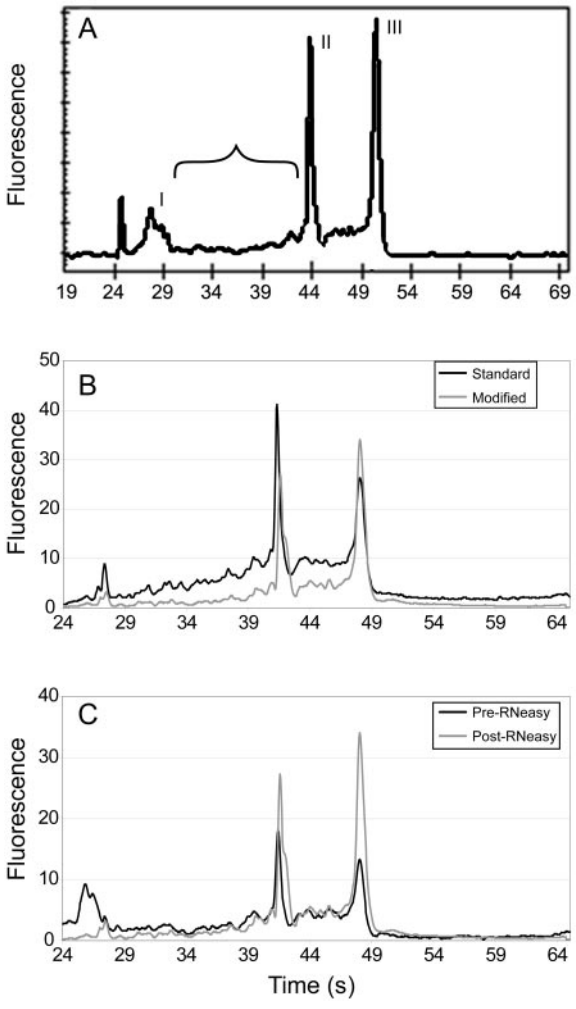

Fig. 1. Effect of RNA isolation method and RNeasy treatment on total RNA chromatograms.

RNA integrity was assessed by capillary electrophoresis with fluorescence detection (Agilent Bioanalyzer 2001). (*A*), prototype chromatogram of total RNA containing a marker peak at 24 s as well as 3 prominent peaks corresponding to small RNAs (*peak I*), 18S (*peak II*), and 28S (*peak III*) rRNA, along with faint signals from cellular RNAs with a broad range of molecular weights (*bracket*). (*B*), scaled capillary electrophoresis chromatograms of RNA from the same placenta (sample 1) isolated by the 2 methods, showing that the modified RNA isolation technique improved the RNA profile. Isolation under modified (cooled) conditions increased the 28S/18S rRNA ratio and decreased both the area of the small-RNA peak and peak signals in the region between the small-RNA and 18S peaks. *Black line*, RNA isolated at room temperature by the standard Trizol-based method; *gray line*, RNA isolated at 0–4 °C by the modified Trizol-based method. (*C*), comparison of RNA profiles of samples before and after the RNeasy cleanup. The post-RNeasy profile shows an increased 28S/18S rRNA ratio and considerably decreased peaks attributable to small RNAs (*arrow*). *Black line*, total RNA profile before RNeasy cleanup; *gray line*, total RNA profile after RNeasy cleanup.

cental tissue weight × 100) was 0.13 (0.03) with the standard Trizol method and 0.12 (0.04) with the modified technique. Incomplete homogenization with the modified method did not markedly affect the RNA yield ($P = 0.48$). The mean DF of RNA samples processed at room temperature was 14.8 (1.8)%, with a mean 28S/18S ratio of 0.76 (0.09). For RNA extraction under temperature-controlled conditions (0–4 °C), the mean DF decreased to 8.2 (1.6)% ($P = 0.0005$), and the 28S/18S ratio increased to 1.14 (0.2) ($P = 0.005$). Cooling produced a 44% decrease in the DF and a 32% increase in the 28S/18S ratio. During standard RNA isolation, the temperature of the Trizol reagent increased by 8 °C during homogenization, whereas the temperature remained constant during intermittent homogenization performed on ice. Chromatograms of total RNA from 1 placenta isolated by the 2 methods showed the enhancement of RNA quality with the modified Trizol method (Fig. 1B). With the modified protocol, all DFs were near or below 8%.

We evaluated the effect of RNeasy cleanup on RNA integrity. Total RNAs were processed with the RNeasy Minikit (Qiagen) according to the manufacturer's instructions. Using the Agilent RNA 6000 Nano LabChip assay as described above, we evaluated total RNA isolated from 4 placentas before and after RNeasy cleanup. The mean DF decreased 30% after RNeasy cleanup ($P = 0.14$). The height of the small RNAs peak, as expected, decreased ($P = 0.00001$; see Table 1 in the online Data Supplement), whereas the 28S/18S ratio unexpectedly increased by 23% ($P = 0.01$; Fig. 1C), suggesting that high–molecular-weight RNA (such as 28S rRNA) preferentially binds to the RNeasy columns. These data suggest that RNeasy treatment may artificially improve the RNA profile, leading to underestimation of RNA degradation. The reported DF should therefore be calculated before RNeasy clean-up.

Limiting RNA degradation during tissue processing and quantifying RNA degradation by DF are important, particularly when clinical samples are used, because time intervals from sample procurement to RNA preservation impact RNA integrity *(6)*. The use of RNA with comparable low DFs should provide microarray results that more accurately reflect biology rather than variable RNA degradation.

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First-Trimester Down Syndrome Screening

To the Editor:

The literature on invasive trophoblast antigen (ITA) as a Down syndrome marker in either maternal urine or serum has been remarkably inconsistent, and very few data on the utility of ITA as a first-trimester marker have been published. We therefore read with interest the study on maternal serum ITA and first-trimester Down syndrome screening by Palomaki et al. *(1)*. Unfortunately, we feel that this study does little to clarify the utility of ITA.

The dataset for the study by Palomaki et al. consists of 54 Down syndrome cases and 276 controls from a previously published NIH trial of first-trimester Down syndrome screening in which free β-human chorionic gonadotropin (hCG), intact hCG, and pregnancy-associated plasma protein-A (PAPP-A) were evaluated. ITA could therefore have been compared with the other serum markers in the same sample set, and because there is significant correlation among the hCG analytes, this would seem the ideal analysis. However, the data on free β-hCG, intact hCG, and PAPP-A obtained from this sample set were not used in the modeling. Rather, the required values for the modeling were taken either from the Serum, Urine, and Ultrasound Screening Study (SURUSS) trial (a study only slightly larger than the current study) or from an unpublished metaanalysis (which therefore cannot be evaluated). Indeed, exactly which parameters were used in the model was not at all clear because there was a discrepancy between the Material and Methods section in the report by Palomaki et al. *(1)*, which indicated that SURUSS parameters were used, and the footnotes to the tables, which indicated that meta-analysis was used. Furthermore, to