Automated Target Preparation for Microarray-Based Gene Expression Analysis

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Automated Target Preparation for Microarray-Based Gene Expression Analysis

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DNA microarrays have rapidly evolved toward a platform for massively paralleled gene expression analysis. Despite its widespread use, the technology has been criticized to be vulnerable to technical variability. Addressing this issue, recent comparative, interplatform, and interlaboratory studies have revealed that, given defined procedures for "wet lab" experiments and data processing, a satisfactory reproducibility and little experimental variability can be achieved. In view of these advances in standardization, the requirement for uniform sample preparation becomes evident, especially if a microarray platform is used as a facility, i.e., by different users working in the laboratory. While one option to reduce technical variability is to dedicate one laboratory technician to all microarray studies, we have decided to automate the entire RNA sample preparation implementing a liquid handling system coupled to a thermocycler and a microtiter plate reader. Indeed, automated RNA sample preparation prior to chip analysis enables (1) the reduction of experimentally caused result variability, (2) the separation of (important) biological variability from (undesired) experimental variation, and (3) interstudy comparison of gene expression results. Our robotic platform can process up to 24 samples in parallel, using an automated sample preparation method that produces high-quality biotin-labeled cRNA ready to be hybridized on Affymetrix GeneChips. The results show that the technical interexperiment variation is less pronounced than with manually prepared samples. Moreover, experiments using the same starting material showed that the automated process yields a good reproducibility between samples.

DNA microarray technology allows for massively paralleled monitoring of gene expression levels, as well as the study of gene regulation and gene interactions at a global level. In food research, a major objective of employing DNA microarrays is to perform nutritional intervention studies to elucidate the impact of nutrients on gene expression. These experiments are key elements of the discipline nutrigenomics. Nutrigenetics investigates the inverse relationship, i.e., how individual genetic predisposition affects susceptibility to diet.

Despite a 10-year history of DNA microarray analysis, the technology still suffers from limitations such as interexperiment variability and difficulties in identifying and measuring low expressed genes. For example, the regulation of gene expression by nutritional intervention is often difficult to detect, because the dietary change is an indirect intervention compared to, for example, drug application and may therefore result in many small rather than a few large effects: many low-abundant genes may be only subtly regulated but can make a difference in a concert.[1] Consequently, microarray experiments in nutritional research are particularly prone to low signal-to-noise levels. Furthermore, interstudy comparisons may become problematic, even if performed on the same microarray platform, especially when different operators conduct the experiments.[2−8] The difficulties in comparing gene expression data can be deduced to be the lack of a standard format for exchanging microarray data between laboratories and—most importantly—to be the insufficient definition of rules for execution and description of microarray experiments. Interlaboratory reproducibility improves significantly with standardized protocols being applied to RNA labeling and hybridiza-

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tion, microarray processing, data acquisition, and normalization. To address this need for standardization, the Minimum Information About a Microarray Experiment (MIAME) XML-based standard for describing a microarray experiment is being adopted by many journals as a requirement for the submission of papers incorporating microarray results.[9] However, standardization of procedures remains a challenge when using different microarray platforms. Indeed, the differences in terms of probe design and specificity, labeling strategies, and analysis techniques are great barriers to interplatform comparisons.

Summarizing, while technical differences may influence gene expression, standardized procedures, high-quality microarrays, and appropriate data collection and transformation are able to generate reproducible and comparable results across experimental replicates and even laboratories, especially if a common platform and a joint set of procedures are used.[10] It is therefore essential to reduce technical variability during sample preparation, to reduce interference with (a) contextually meaningful biological gene expression variation and, most importantly, (b) the "true" gene expression signals specific to the nutritional intervention.

We have implemented the Affymetrix GeneChip platform (Affymetrix, Santa Clara, CA). Affymetrix microarrays consist of on-chip synthesized oligonucleotide probes used for hybridization to biotin-labeled cRNA targets prepared from RNA samples.[11] The Affymetrix standard protocol is well established for the preparation of cRNA targets from microgram amounts of total RNA. This procedure is composed of a large number of molecular biology steps, which involve pipetting of small volumes (1−10 μL). Significant differences from one experimenter to another regarding the yield and quality of the generated labeled cRNA are often observed.

Therefore, standardization of sample preparation and interstudy consistency have to be improved. We have equipped our laboratory with a Hamilton Microlab Star robot programmed to fulfill all requirements of the microarray target preparation. The automated method follows in principle the manual procedure regarding enzymatic reactions as recommended by Affymetrix, but with substantial technical adaptations for the robot and the external devices. Thus, and in contrast with previous attempts to automate microarray sample preparation,[12] we implemented a complete method, from the total RNA starting material to the final hybridization mix ready for chip application.

In this study, we have examined the reproducibility and quality of the automated RNA target preparation. We present data, which illustrate good RNA quality and high reproducibility of the automated method. The variations observed for cRNA yields, cRNA quality, and hybridization intensities across automated target preparations are so low that they suggest the implementation of our robotic method and equipment for microarray analyses in general and on a routine basis. Most importantly, we show that the technical interexperiment variation is less pronounced than with manually prepared samples. The low variability and the high reproducibility result in a standardized and fully integrated protocol for microarray experiments.

EXPERIMENTAL SECTION

RNA Preparation. To assess the reproducibility of the automated method, a single extraction of total RNA from one mouse liver was performed (Macherey Nagel−Nucleospin RNA II). After homogenization, this RNA sample was divided into 24 aliquots, which were equalized to a concentration of 5 μg in 11 μL of RNase-free water. We used 8 of these samples for the manual processing and 16 for the robotic procedure.

We also processed 48 RNA samples from an internal study to demonstrate the performance of the automated method in a real-life situation. These samples were derived from eight dogs, which provided six tissues each (liver, kidney, brain, skin, muscle, heart). Tissues were obtained at necropsy of the dogs, following euthanasia for clinical health reasons.

Target Preparation. Synthesis of Biotin-Labeled cRNAs. All cRNA targets were synthesized, labeled, and purified according to the Affymetrix protocol. Briefly, 5 μg of total RNA was used to produce double-stranded cDNA, followed by an in vitro transcription, and by cRNA labeling with biotin. This method is based on the Eberwine T7 procedure.[13] The entire sample processing is summarized in Figure 3, which also depicts the major changes between the automated and the manual target preparation method from Affymetrix.

(1) Labeled cRNA Hybridization. Prior to the hybridization on the gene array, 15 μg (volume dependent on cRNA concentration) of fragmented cRNA was added to the hybridization mix, which contained control oligonucleotide B2, hybridization controls (bioB, bioC, bioD, cre), herring sperm DNA, acetylated BSA, hybridization buffer, and water. Since the amine functions of proteins may bind to nucleic acids, herring sperm DNA is added to block this nonspecific hybridization by competition. The control oligonucleotide B2 generates a control hybridization pattern on the border of the array used to assess the array quality.

(2) Nucleic Acid Quantification: Ribogreen Assay. The most commonly used technique for measuring nucleic acid concentrations is the determination of absorbance at 260 nm (A260). The major disadvantages of the A260 method are (a) the large contribution of proteins and free nucleotides to the signal, (b) the interference of contaminants commonly found in nucleic acid preparations, and (c) the poor sensitivity of the assay (an A260 value of 0.1 corresponds to 4 μg/mL RNA). The use of fluorescent nucleic acid stains avoids these problems. Thereby, the Ribogreen RNA quantitation assay (Molecular Probes) can quantify RNA from 1 ng/mL to 50 μg/mL. Since the Ribogreen reagent also binds to DNA, pretreatment of mixed samples with DNase allows us to generate RNA-selective assays.[14]

The Ribogreen assay was performed in a 96-well plate. Using a universal standard RNA, a five-point standard curve was established, each point representing 0, 2, 10, 50, and 100 ng of RNA, respectively.

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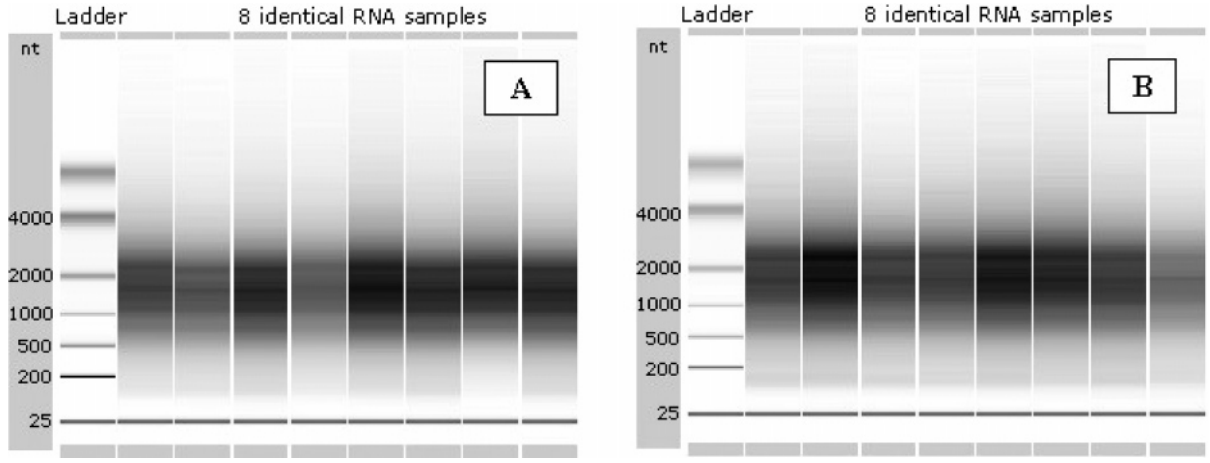

Figure 1. Synthesis of 16 cRNAs from the same mouse liver total RNA. Gel view of Bioanalyzer results. Both manual (A) automated (B) target preparations produce cRNAs with good quality (cRNA fragment size ~1800 bp).

Subsequently, 1 μL of each RNA sample was loaded on the same reader plate with 99 μL of TE buffer and 100 μL of Ribogreen reagent. A fluorescence microplate reader calibrated with standard fluorescein wavelengths (excitation 485 nm, emission 538 nm) was used to measure the signal in each well.

Fluorescence signals of the five points were used to calculate the equation of the standard curve trend line. The resulting formula is employed to determine the quantity of RNA for each sample depending on their signal values.

The Ribogreen assay offers the advantage of measuring RNA quantities with high precision, and the 96-well plate format provides an elevated throughput.

(3) RNA Quality Control: Agilent Bioanalyzer 2100. The quality of synthesized cRNAs was evaluated with the Bioanalyzer 2100 (Agilent). This microfluidic instrument utilizes capillary electrophoresis for RNA analysis. As an alternative to labor-intensive gel electrophoresis techniques, the RNA 6000 Nano Chips kit determines the distribution of RNA fragments size, allowing for total RNA and cRNA quality assessment.[15] As this step involves special equipment, the RNA quality control step appeared to be difficult to automate. It was thus the only remaining manual operation throughout the otherwise fully automated target preparation.

Automation. The automated method was implemented on a Microlab Star robot from Hamilton. This robot is a liquid handling system for the automation of laboratory processes involving sample pipetting (from 1 to 1000 μL), transport, mixing, and incubation.

For our experiment, the Microlab Star was combined with a thermocycler equipped with a heated lid (TRobot, Whatman Biometra), and a microtiter plate reader (SpectraMax M2, Molecular Devices) as external devices. Both instruments are fully integrated into the Microlab Star platform, and the Hamilton Vector software controls the whole system managing instrument-dedicated libraries and algorithms. A graphical interface serves to (a) define the robotic configuration (deck layout), (b) set up each piece of equipment (labware), and (c) identify liquids by their physicochemical properties (liquid classes). The labware definition is flexible enough to accommodate several different configurations, with adaptable carrier types and locations.

Our deck layout thus included 10 positions for conductive tips (small, medium, and large volume), 10 positions for 96-well plates (U- and V-well shapes), several reagent and buffer containers, and two racks for 32 1.5-mL tubes. A special, custom-made adaptor allowed for the employment of a magnetic bead-based kit for nucleic acid purification (Agencourt). A cooling carrier kept at 4 °C was used to store sensitive reagents during the entire sample preparation process, and a Peltier heating−cooling element was installed for sample shaking under controlled temperature.

The whole automated procedure is divided into two main methods: (1) cDNA synthesis and (2) labeled cRNA synthesis. They were developed to process up to 24 samples in parallel. Required reagent volumes, plates, and vial quantities were calculated in an MS Excel worksheet. Therefore, the volumes of the different solutions depended on sample number, reagent viscosity, container type, and pipetting mode.

Automation methods followed the manual procedure regarding enzymatic reactions as recommended by Affymetrix (Figure 2) but with significant technical adaptations. Thus, all incubations were performed in the integrated thermocycler, and both cDNA and cRNA purification steps were completed using the magnetic bead-based kit from Agencourt.

Starting with 5 μg of total RNA, the cDNA synthesis method encompassed complete cDNA synthesis and cleanup. After the labeled cRNA synthesis method had accomplished the cRNA synthesis and cleanup, the Microlab Star robot directly prepared the Ribogreen assay to quantify cRNAs and stored a 1:10 dilution of each sample for the off-line, manual quality control (Agilent total RNA nanoassay). After verification of the good cRNA quality, the method continued with sample normalization, required for the fragmentation, adjusting the concentration to 0.5 μg/μL. Then, the Microlab Star completed the RNA fragmentation step and the hybridization mix preparation. The method finished by producing 300-μL ready-to-use hybridization mixes.

For normalizations, the Ribogreen assay was fully integrated into the automated process to measure up to 24 samples in duplicates, and the cRNAs were quantified by a five-point calibration curve. The Vector software subsequently retrieved cRNA concen-

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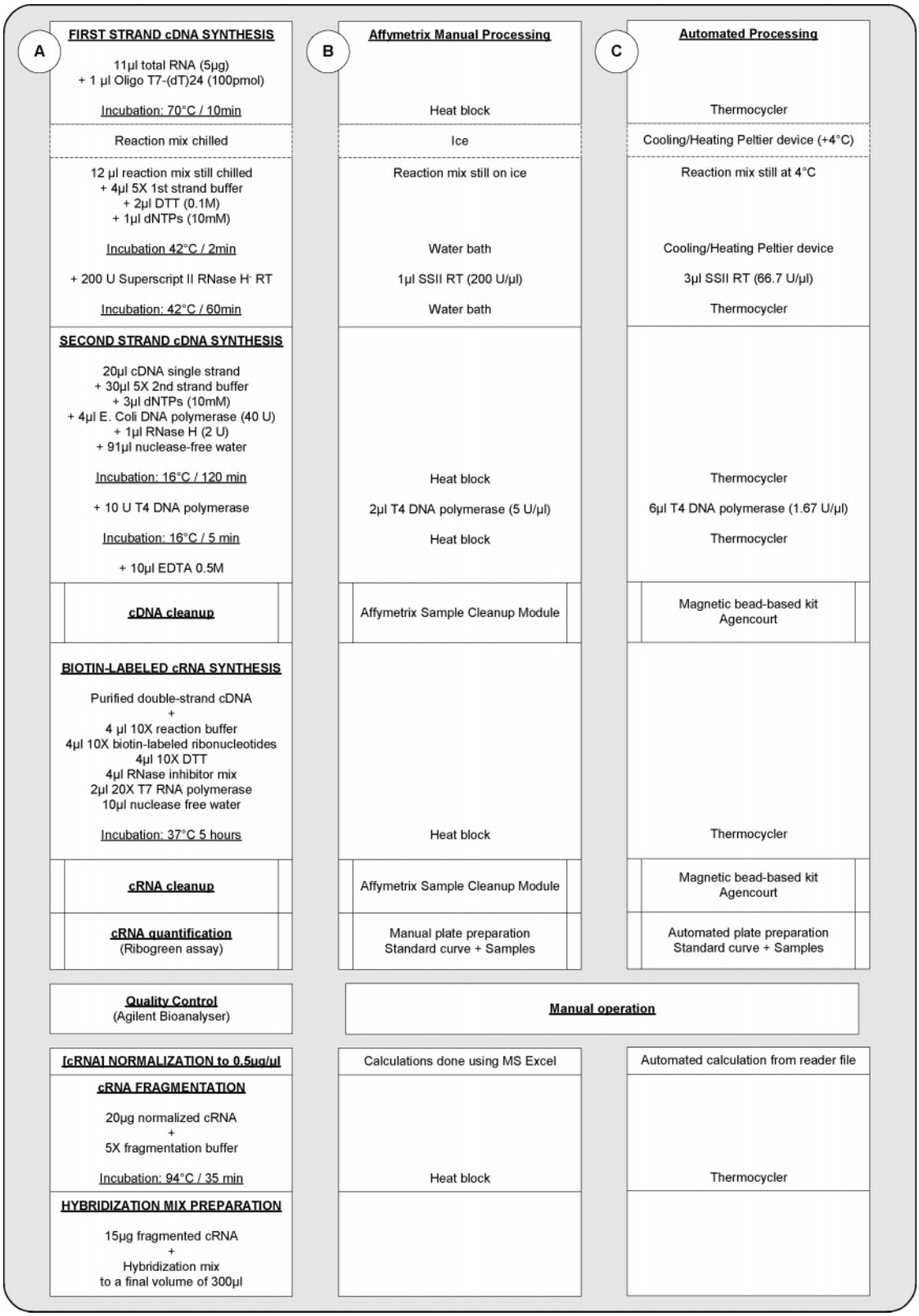

Figure 2. Microarray target preparation workflow (A). Specifications for the Affymetrix manual sample processing (B) and adaptations for the robotic procedure (C).

trations directly from the microtiter plate reader, and an algorithm normalized the cRNA concentrations for the fragmentation step. All calculations were done automatically "on the fly", i.e., during the robotic procedure.

Modifications with Regard to the Manual Method. *Concentrations and Volumes of Enzymatic Reactions.* Because of their high viscosity and their small dispense volume, original solutions of the enzymes SSII RT and T4 DNA polymerase were diluted

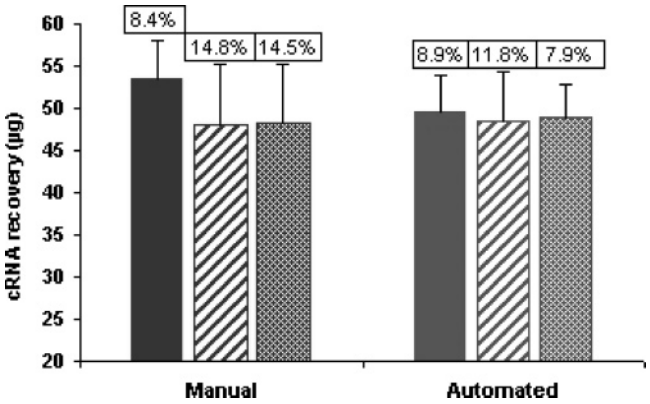

Figure 3. Target preparation. Three series of eight identical mouse RNA samples (5 μg) were processed both manually and automatically. While both methods show comparable yields, the automated procedure induces a reduced variability across target preparations.

three times in nuclease-free water prior to their use on the robot. Indeed, pipetting 3 μL ensured higher precision and reproducibility compared to 1-μL pipetting of viscous solutions. Moreover, increasing this volume prevented the waste of enzymes by reducing dead volumes and improved the mixing of the reaction solution. Although the reaction volumes were 2 μL larger for SSII RT step and 4 μL larger for the T4 DNA polymerase step, the yield of the reaction was not affected by the enzyme dilution.

Thermocycler and Peltier Element Incubations. According to Enzo (IVT reagent provider), the 5-h incubation during IVT was done without any shaking step, which contrasts the Affymetrix recommendation. This modification did not reduce the final cRNA yield compared to the manual procedure.

Magnetic Bead-Based Nucleic Acid Purification. The filter columns in the 96-well format combined with a vacuum system for nucleic acid purification resulted in poor and unreliable DNA/RNA yields with up to 80% loss of the reaction material (data not shown). Therefore, the Agencourt RNA clean magnetic bead kit was implemented, which resulted in better yields and higher reproducibility of the nucleic acid cleanup. After purification, yield and quality of recovered cRNA equalled the manual method, and showed a reduced technical variability (see Results and Discussion).

Array Hybridization and Processing. We used 20 μg of fragmented labeled-cRNA for hybridization on microarrays. Each mouse liver sample was hybridized on one Affymetrix MOE430A array, while dog samples from the internal study were hybridized on a Nestlé custom-built canine microarray (NesChip).

The NesChip comprises mRNA sequences present in GenBank (Benson et al. 2004; July 2003 version) and the EST sequences present from the ZooSeq database (Incyte Corp., Wilmington, DE; September 2002 update). The libraries were generated from sequences isolated from 13 different organs from beagle.[16]

All arrays were scanned on the Affymetrix GeneChip scanner 7G, and images were quantified using the Affymetrix GCOS software.

RESULTS AND DISCUSSION

Our main objective was to develop and implement a reproducible, medium-throughput, and fully automated robotic method for microarray sample preparation. The performance of the automated method was compared to and benchmarked against the manual target preparation of a thoroughly trained and highly experienced laboratory technician, who is considered as our internal "gold" standard.

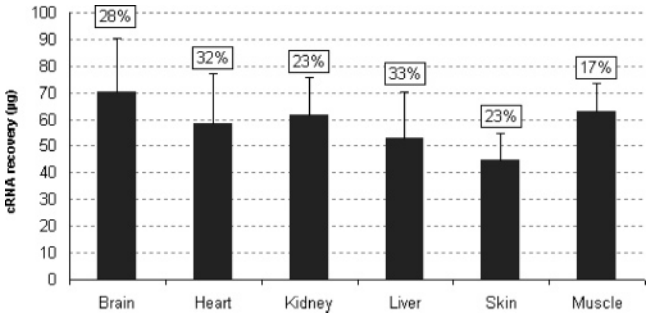

Figure 4. Robotic target preparation. Average of cRNA yields (±%CV) from multiple tissues starting with 5 μg of total RNA.

As we modified the original Affymetrix protocol for the purpose of automation, especially regarding two enzyme dilutions and the cleanup of nucleic acids (Figure 3), the comparison "automation versus manual" has been done including those modifications in the manual protocol. This ensured that differences possibly observed between robot and technician could exclusively be attributed to the automation of liquid and plate handling and not to any other technical modification.

Target preparation. Regarding cRNA synthesis, the automated method was found to be robust and reproducible over time and between experimental repeats. As shown in Figure 3, starting with 5 μg of the same mouse total RNA material, the robot was able to produce cRNAs with yields comparable to the manual processing and with slightly lower yield variability.

The efficiency and robustness of the automated procedure was confirmed in a case study, in which six different tissues from eight different animals were analyzed. Those 48 samples have been processed in two separated robotic runs of 24 samples each. The higher variability in yield for these samples could be due to the percentage of mRNA differing between animals and tissues (Figure 4).

All target preparations produced sufficient amounts of cRNA (>40 μg) to enable on-chip hybridization.

Hybridization Quality Control. We assessed the quality of the automated procedure by comparing the average intensity and the number of genes called "present" in both methods. Call values are used to determine whether genes exhibit reliable signals. As shown in Table 1, samples synthesized automatically demonstrated performance equivalent to the manual ones regarding percentage and signal intensities of present calls. We also compared the 3′/5′ ratios for β-actin and GAPDH genes. Such ratios for housekeeping genes illustrate the overall quality of the transcripts, and a value of 1 would signify a perfect quality without any degradation of the mRNA transcripts. Table 1 indicates that the robotic protocol ensured a better target quality with less variability across the eight samples.

Expression Data. Signal intensities for each probe were normalized using the RMA algorithm[17] (robust multiarray analysis)

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Table 1. Performance of Hybridization and Target Quality[a]

protocol	*n*	% present calls	signal present calls	3′/5′ ratio β-actin	3′/5′ ratio GAPDH
manual (± SD)	8	44.6 ± 1.9	610.6 ± 11.01	2.0 ± 0.3	1.2 ± 0.1
automated 1st (± SD)	8	44.8 ± 1.5	574.9 ± 9.99	1.3 ± 0.09	0.9 ± 0.03
automated 2nd (± SD)	8	43.9 ± 1.8	569.3 ± 9.40	1.4 ± 0.1	0.9 ± 0.04

[a] Values averaged across eight identical samples. 'Signal present calls' indicates the average intensity of detected transcripts after background reduction.

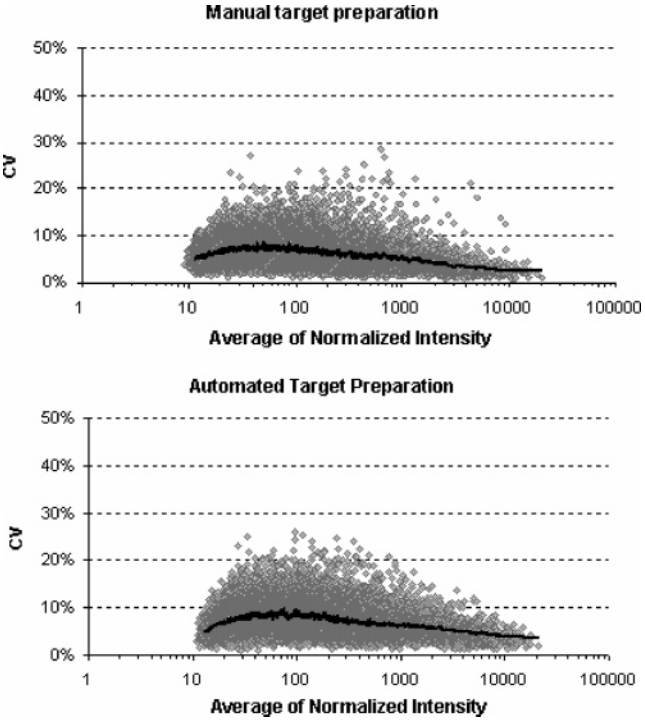

Figure 5. Coefficients of variation for each target preparation method as a function of intensity across all replicates ($n = 8$). The black line represents the 100 point moving average.

provided by the ArrayAssist Lite 3.4 software (Stratagene). These normalized intensities were recruited to perform several comparisons between manual and automated methods. We evaluated the coefficient of variation for each probe signal across eight samples, and we plotted these values against the average intensity. As shown in Figure 5, manual and automated procedures produced comparable results with a better distribution for the automated one. This is confirmed by the average CV calculated for all probes, which is less important in the automated procedure (7.0 vs 8.4%) with a majority of CVs below 15%.

The array-to-array reproducibility for each method is represented in Figure 6. These typical scatterplot charts, which reveal the correlation between two replicates, showed equivalent distribution of intensity values in both methods. Moreover, we calculated the Pearson correlation coefficients for each pairwise comparison (Table S-1, Supporting Information). This evaluation shows a very good linear correlation between hybridization intensities within either the manual or the automated preparations.

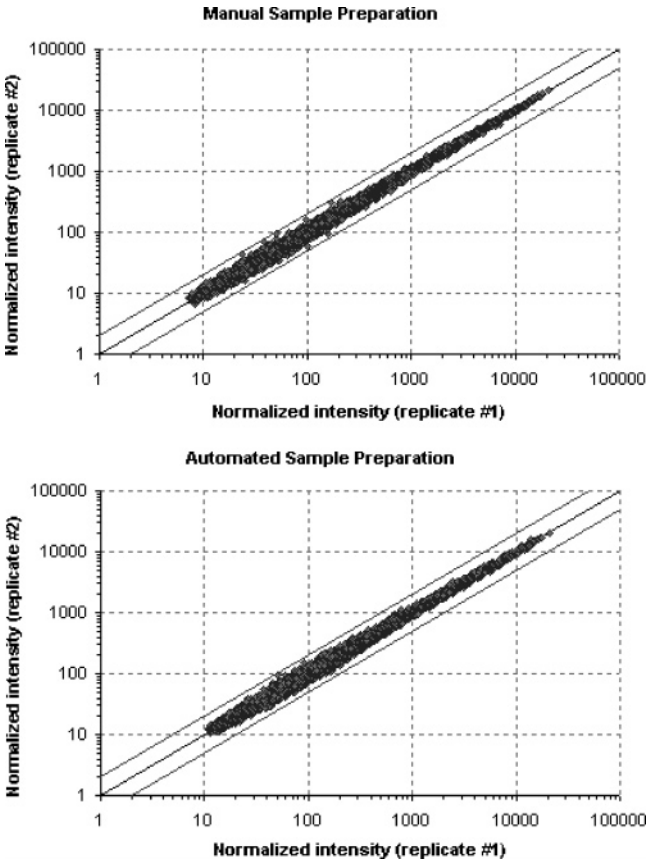

Figure 6. Pairwise array precision of manual and automated methods. The three lines represent the 2-fold limit (−2; +2), and the 1:1 equality of gene expression.

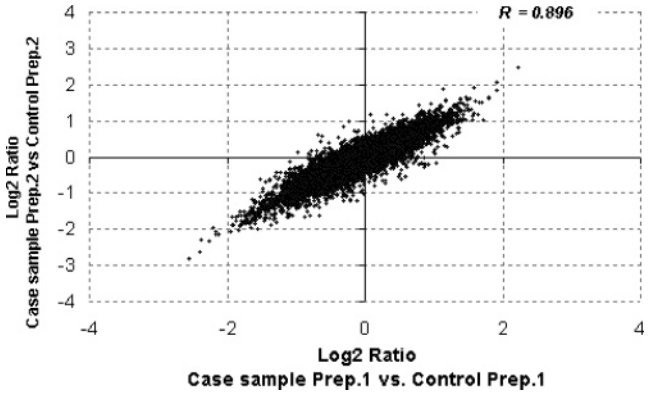

Figure 7. Target preparation variability. Correlation of differential expression ratios between two different samples processed in two automated target preparations. A perfect preparation would result in an *R* value equal to 1.

To further assess the performance of the robotic target preparation in a real-life case study, we processed RNA samples from canine tissues. These were derived from eight dogs, which provided six tissues each (liver, kidney, brain, skin, muscle, heart). Tissues were obtained at necropsy of dogs, following euthanasia for clinical health reasons. We assessed the correlation of differential expression ratios between two automated preparations. Therefore, the Microlab Star robot processed two replicates each of one case and one control sample, but in two independent processes (preparation 1 and preparation 2). A plot of the expression ratio changes (log2 transformed data) showed a good reproduc-

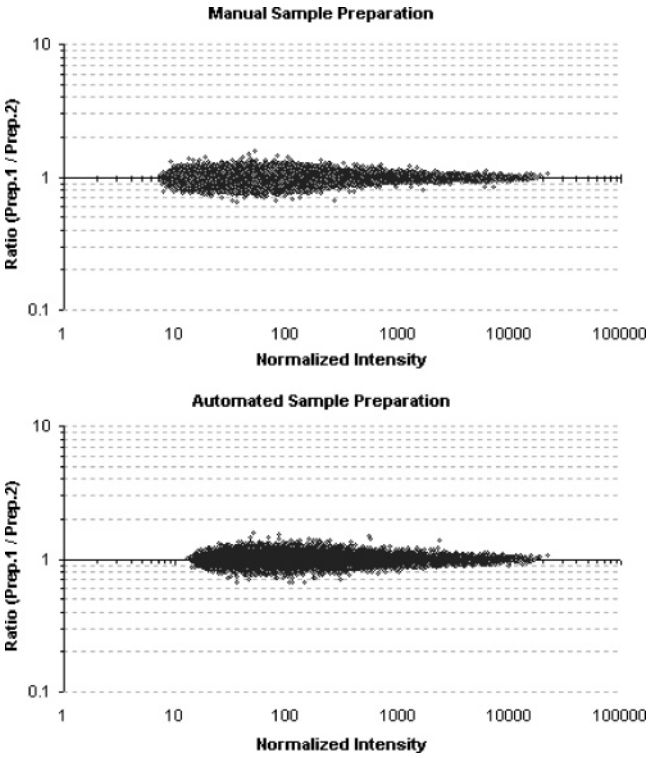

Figure 8. Target preparation variability. The signal intensity ratio for each gene between two preparations is plotted against the average signal intensity.

ibility (Pearson correlation coefficient $R = 0.896$) between preparation 1 and preparation 2 (Figure 7). In the case study, we also compared both the manual and the automated methods. We determined the expression ratio for each transcript between two preparations of the same sample, and we plotted these ratios as a function of the signal intensity. While a perfect preparation would give expression ratios equal to 1, we observed an equivalent distribution of ratio values between both methods with a slightly lower variability for the automation (Figure 8). The case study therefore confirms the good reproducibility of the automated preparation regarding determined gene fold changes.

CONCLUSIONS

Microarray-based gene expression studies, in particular those employing the Affymetrix platform, are widely used to investigate gene regulation and interaction at global scale. Nevertheless, gene chip platforms have been criticized for being susceptible to experimental variability. Poor overlap of regulated gene sets between platforms and laboratories investigating the same context has been reported. More recently, it has been shown that interplatform and interlaboratory comparability of transcriptomic studies can be greatly improved when adhering to defined protocols for sample handling, platform operation, and data processing. The minimization of experimental "noise" and its distinction from biological (interindividual) variability and true-positive gene regulation is particularly challenging in nutrigenomics, i.e., when the effect of nutrients on gene expression is under scrutiny: nutritional intervention studies typically reveal many genes regulated with small fold changes at individual gene level. However, the concert of these many subtle alterations in gene expression may account for a big difference in terms of response to diet.

In view of these challenges, we have developed and implemented a reproducible, medium-throughput, and fully automated robotic method for RNA sample preparation. The method results in high and reproducible yields of labeled target cRNA. Its performance is comparable to the manual target preparation of a thoroughly trained and highly experienced laboratory technician, our performance gold standard to date. The automated protocol results in convenient target preparation from 1 to 24 samples in parallel within 12 h. The method has been tested and validated using standardized RNA as starting material as well as in a medium-scale real-life study with hybridization onto 48 mammalian chips. The latter application revealed high RNA quality. At global expression level, we observed good-to-excellent reproducibility of gene fold changes between robotic replicates and equally satisfying correspondence to gene expression results obtained from the same mammalian samples but processed manually. Therefore, we recommend the broad implementation of such automated target preparations for chip-based transcriptomics in order to improve result quality, raise throughput, "delegate" repetitive laboratory work, and improve interplatform, interlaboratory and, eventually even interstudy comparison of gene expression results.

Finally, from a financial point of view, within one study it is important to process as many samples in parallel as possible (24 in our case). With elevated parallel processing, fewer reagents are wasted, especially because of the dead volumes during automated pipetting. Our method, currently designed for 24 samples in parallel, can readily be adapted to simultaneous processing of 96 samples. However, in such case, we would recommend the switch from an 8-channel to a 96-format pipetting head in order to speed up and homogenize liquid handling.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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