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Application Note

Automation of Total RNA Purification from HeLa Cells and Mouse Liver Tissue with the CyBi®-RoboSpense Using MACHEREY-NAGEL NucleoSpin® 96 RNA Kit

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Abstract: The MACHEREY-NAGEL NucleoSpin® 8/96 RNA kit technology for purification of total RNA from various sample materials has been automated on the CyBi®-RoboSpense. The vacuum device, robotic microplate handler and shaker for microplates allowed performing a fully automated extraction procedure. Quality of extracted RNA was assessed by analysis of the samples on Agilent Bioanalyzer, on a denaturing agarose gel and in real time PCR. RNA was extracted with very good yields and excellent purity.

Introduction: The NucleoSpin® RNA kits are designed for total RNA isolation from cells or tissue samples. They are available with 8-well strips and 96-well plates providing a maximum of flexibility in sample throughput. The NucleoSpin® method is based on a bind-wash-elute procedure using silica membrane technology with a high binding capacity for RNA. With the NucleoSpin® 8/96 RNA kit, cells or tissue are lysed by incubation in a solution containing chaotropic salt. This lysis buffer immediately inactivates RNase and creates in combination with RA4 appropriate binding conditions which favour adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by DNase I which is directly applied onto the silica membrane during the preparation. Salts, metabolites and macromolecular cellular components are removed by simple washing steps with three different buffers. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water. The CyBi®-RoboSpense is an ideal robotic liquid handler for automating complex processes such as RNA extraction, due to its reliable liquid handling using fixed probes or disposable tips, its robotic microplate handler, and other specialized accessory modules. In this application note, we demonstrate as an example the fully automated NucleoSpin® 96 RNA extraction method on the CyBi®-RoboSpense. Programs are also available for the 8-well based NucleoSpin® 8 RNA kit.

Figure 1: CyBi®-RoboSpense for processing NucleoSpin® 8/96 RNA Kit

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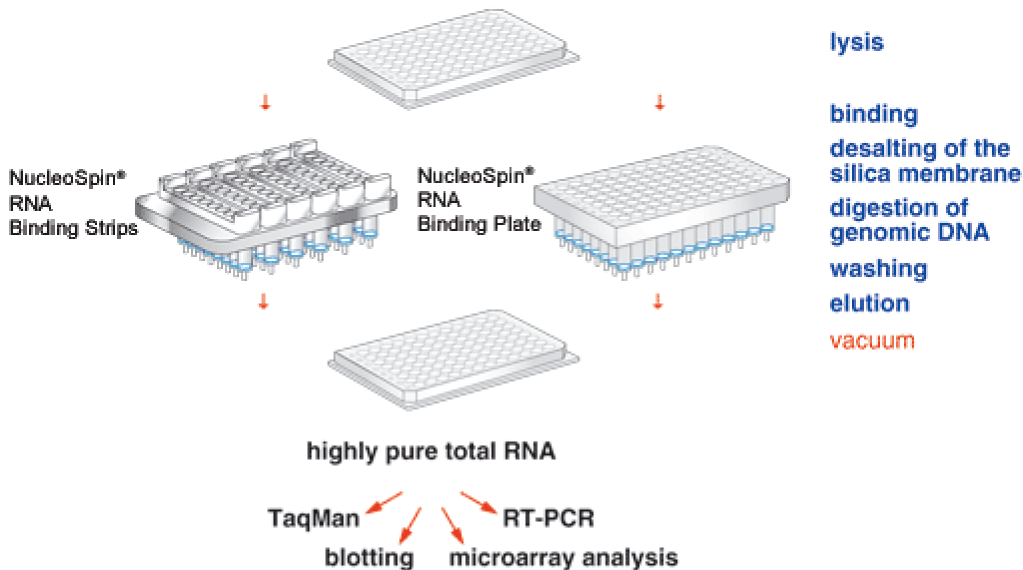

Figure 2: NucleoSpin® 8/96 RNA procedure

Methods: The MACHEREY-NAGEL NucleoSpin® 96 RNA kit (2x96 preps, MN Cat. No. 740709.2) extraction protocol was set up on a CyBi®-RoboSpense system consisting of a 35 position deck equipped with 8 liquid handling channels and disposable tips, 8 high precision pumps with a volume range from 1–1000 µL, a vacuum manifold, a shaker for microplates and a robotic microplate handler.
HeLa cells (7.5 x 10^5 cells per sample) and mouse liver (5 mg per sample) were used for verification of the automated method. The quality and integrity of the DNA obtained was analyzed by gel electrophoresis and using Agilent® 2100 Bioanalyzer (RNA 6000 Nano Chip). Yields and purity of extracted DNA were determined by measurement of absorbance at 260 nm and 280 nm with a Lambda Scan 200 microplate scanning spectrophotometer (MWG Biotech) and by calculation the $A_{260/280}$ ratio. Real time RT-PCR was performed with a Roche Lightcycler™ instrument using the Sigma SYBR Green Quantitative RT-PCR kit.

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Automated extraction procedure:

1. 300 µL of buffer RA1 / 1% ß-mercaptoethanol was added to 5 mg of mouse liver. Samples were manually homogenized in a Dispomix® (BioLab Products GmbH) and transferred into a 96-well deep-well microplate.
   Cells were grown in culture dishes. Following trypsine treatment and resuspension in PBS buffer aliquots of 7.5 x 10^5 cells were transferred into the wells of a 96-well deep-well microplate. Cells were pelleted by centrifugation (5 min, 500 x g) and supernatant was removed. 300 µL buffer RA1 / 1% ß-mercaptoethanol was added to the cells in each well. Cells have been lysed by pipetting up and down.
2. 300 µL binding buffer RA4 (with ethanol added) were added to wells, mixed by pipetting up and down 3 times and by shaking for 0.5 min at 1000 rpm.
3. The lysate was transferred to the NucleoSpin® RNA Binding Plate.
4. RNA is bound to the silica membrane under vacuum, 800 mbar for 1 min.
5. The silica membrane was desalted by adding 500 µL buffer RA3, followed by application of vacuum for 3 min at 600 mbar.
6. Contaminating DNA was removed by adding 30 µL of DNase I reaction mixture and an 15 minute incubation.
7. The silica membrane was washed three times by adding 500 µL wash buffer RA2, 800 µL wash buffer RA3 and 500 µL wash buffer RA4, followed by application of vacuum for 1 min at 600 mbar after each addition.
8. The wash plate was removed from the vacuum manifold with the robotic microplate handler disassembling and reassembling the vacuum manifold, and the membrane was dried using the automated column drying software mode with vacuum for minimum 12 min.
9. Purified DNA was eluted by adding 100 µL of RNase-free water. After a 2 min wait, vacuum was applied for 1 min at 800 mbar.

Results: RNA was purified from mouse liver lysates corresponding to 5 mg liver/sample or HeLa cell lysates corresponding to 7.5x10^5 cells/sample using the NucleoSpin® 96 RNA kit on the CyBi®-RoboSpense. For comparison, RNA from mouse liver was isolated manually using aliquots of the same lysate with a single-column kit (NucleoSpin® RNA II). RNA was purified with high yields (average: 23,40 µg from 5 mg mouse liver, 8.33 µg from 7.5x10^5 HeLa cells) and high purity (A260nm/280nm = 2.1). Comparable yield and integrity between manual and automated processing were observed as shown for the purification of RNA from mouse liver (Figure 3, Figure 4). Randomly selected RNA samples were analyzed on the Agilent Bioanalyzer 2100 using the RNA 6000 Nano Assay. Typical electropherograms are shown in Figure 5. High structural integrity of the purified RNA is indicated by the RNA Integrity Number (RIN) of >8.0 for the analyzed RNA isolated from tissue samples and >9.0 for the analyzed RNA samples isolated from HeLa cells. For further assessment of suitability in a typical downstream reactions RNA isolated from mouse liver was used in a SYBR-green RT-PCR reaction targeting the GAPDH gene. Randomly selected samples of the purified RNA were used undiluted and in a 1:2 dilution in water. All samples were amplified with good reproducibility (Figure 6). Average crossing points of 18.0 with a CV of 2.12% and 22.15 with a CV of 1.4% were obtained for the undiluted and 1:10 diluted RNA samples, respectively.

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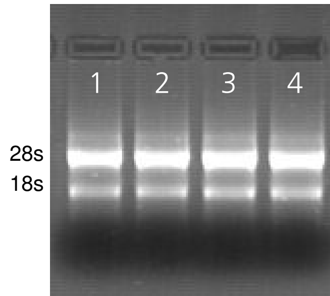

Figure 3: Manual RNA isolation with NucleoSpin® RNA II kit: 5 µL of purified RNA from mouse liver was loaded to each lane.

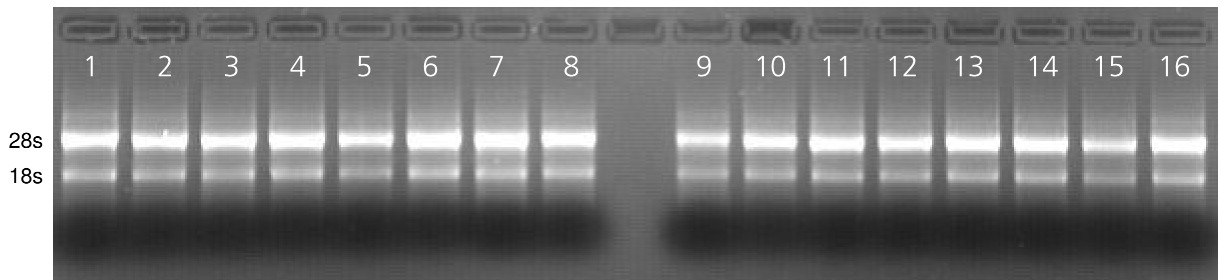

Figure 4: Automated isolation with NucleoSpin® 96 RNA kit : 5 µL of purified RNA from mouse liver were loaded to lanes 1-16. No samples were loaded on lane adjacent to 8. Consistent yield and integrity of RNA was observed. Comparable yields were obtained from manual and automated isolation of RNA.

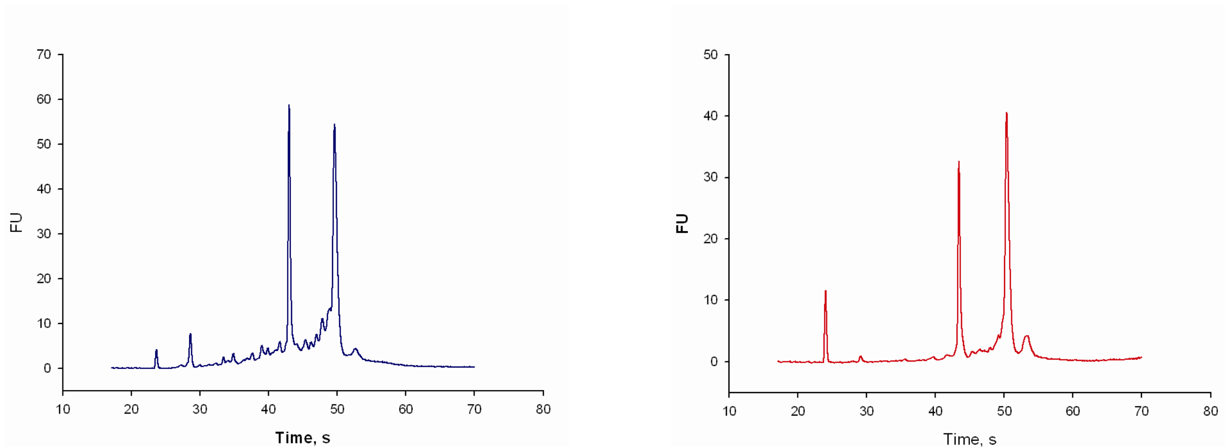

Figure 5: Typical electropherograms (Agilent 2100 Bioanalyzer, RNA 6000 Nano assay) of aRNA sample purified with the NucleoSpin® 96 RNA kit from mouse liver (5 mg per sample, blue) or HeLa cells (7.5 x 10^5 cells per sample, red). Sharp migrating bands of 28s RNA and 18sRNA and high calculated RNA integrity numbers (RIN >9 for Hela cells and RIN >8 for mouse liver samples) underline the excellent quality of purified RNA.

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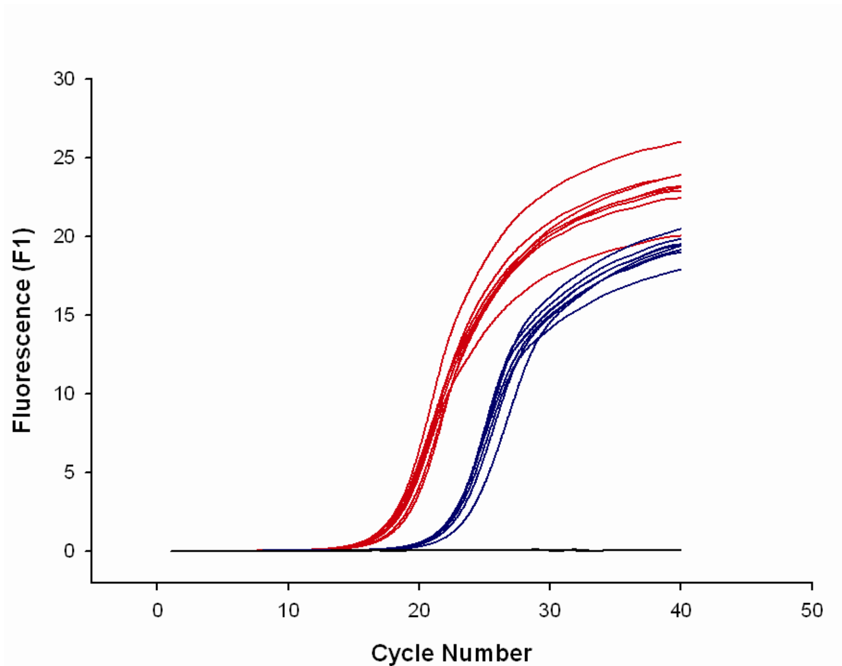

Figure 6: RT-PCR amplification of a 352 bp fragment from the GAPDH gene using 8 randomly selected samples (red) and 1:10 dilution (blue) of the same samples. All samples were amplified with high consistency. Amplification plots indicate the absence of PCR inhibitors.

Discussion: The NucleoSpin® 8/96 RNA kits can be used for automated total RNA purification from various sample materials on the CyBi®-RoboSpense as demonstrated for the RNA isolation from mouse liver and HeLa cells. The RNA can be purified with high and consistent yields. Purity as determined by an A260 nm/280 nm ratio of 2.1, was excellent. High structural integrity of the purified RNA samples was demonstrated by denaturating agarose electrophoresis and analysis of the purified RNA using the Agilent 2100 Bioanalyzer. High quality of RNA is further underlined by sharp migrating bands of 18s and 28 s RNA, lacking bands in the fast migration area and high RIN values. The resulting RNA can be used in a variety of downstream procedures e.g., real-time PCR. These findings demonstrate the robustness of the purification procedure for RNA using NucleoSpin® 8/96 RNA kits on the CyBi®-RoboSpense instrument. The specialized accessory modules such as the shaker for microplates and the vacuum filtration accessories, made it possible to implement the fully automated process on the CyBi®-RoboSpense with excellent results. Using the unique CyBio® EluteControl software in combination with an electronically controlled pump, the vacuum could be set specifically for every vacuum step as the application required. The CyBio® EluteControl software vacuum evaluation mode enabled the user to toggle between execute and edit modes during a run. This allows a simplified optimization of the individual vacuum filtration times.

Conclusion: The results demonstrate that the CyBi®-RoboSpense is very well suited for reliable automation of vacuum-based total RNA extraction from various sample materials using MACHEREY-NAGEL's NucleoSpin® 8/96 RNA kit technology, appling its specialized accessories for vacuum filtration and shaking, as well as optimization of pipetting parameters and vacuum settings.

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