

Isolation of total RNA with the Amersham RNAspin 96 Isolation Kit

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Isolation of total RNA with the illustra RNAspin 96 Isolation Kit

Key words: *illustra • total RNA • microarray • quantitative reverse transcription polymerase chain reaction (QRT-PCR) • primer extension • Northern blot • sample preparation • high-throughput*

The illustra™ RNAspin 96 Isolation Kit can be used to reproducibly (well-to-well, plate-to-plate) isolate total RNA from cultured cells, tissue, bacteria or yeast. It allows for the simultaneous processing of 96 samples via either centrifugation or vacuum-based protocols that can easily fit onto existing automated systems. The isolated RNA is of a high enough quality and quantity (up to 100 µg) for multiple downstream experiments, including sensitive enzymatic applications like quantitative reverse transcription polymerase chain reaction (QRT-PCR), primer extension and RNase protection assays. Thus, the RNAspin 96 kit is a flexible, high-throughput solution for total RNA isolation. This application note describes results obtained from using both the manual centrifugation as well as an automated vacuum workflow to isolate total RNA from several types of source material and their subsequent use in QRT-PCR.

Materials

Products used

illustra RNAspin 96 Isolation Kit	28-0500-74
RNAspin 96 Filter Plate	25-0500-88
CyScribe™ GFX™ Purification Kit	RPN5660X
Hybond™N Membrane	RPN82N

Other materials required

Agilent 2100 bioanalyzer (Agilent)
Tecan Genesis 150 Liquid Handling System
(Tecan Trading AG)

RNAlater™-ICE buffer (Ambion)

SYBR™ Green (Molecular Probes)

MultiProbe™ II HT Liquid Handling System (Perkin Elmer)

Biomek™ FX Liquid Handling System (Beckman Coulter)

ABI 7900 instrument (Applied Biosystems)

TaqMan™ Reverse Transcription Reagents (Applied Biosystems)

TaqMan TAMRA primer/probe sets (Applied Biosystems)

LightCycler™ Detection System (Roche Applied Science)

Protocol

The RNAspin 96 RNA Isolation Kit was used to purify total RNA for several different applications according to the protocol illustrated in Figure 1. To reduce the risk of clogging the RNA-binding plate wells and the potential loss in sample yield and purity, the lysates were prefiltered using the optional RNA Filter Plate. Incubation with DNase I—included in the kit—was efficiently carried out on-column in conditions optimized by the Membrane Desalting Buffer.

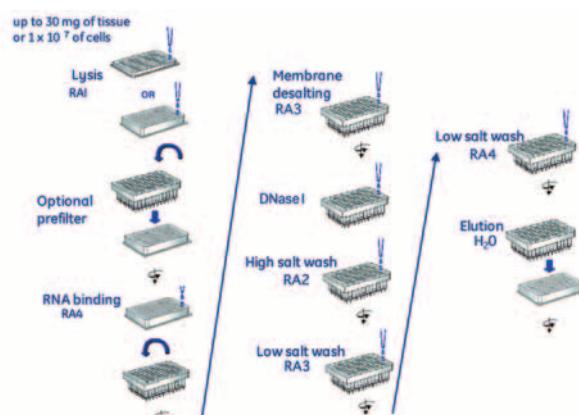


Fig 1. Schematic representation of the RNAspin 96 kit centrifugation protocol



Isolation of total RNA

Experiment 1: Frozen rat liver tissue was stored in RNAlater-ICE buffer and then aliquoted into several 20-mg samples. Total RNA was extracted from several of these aliquots, over three different 96-well plates, using the centrifugation protocol of the RNAspin 96 kit. Each RNA sample was eluted with 100 μ l of RNase-free water. A microliter of each eluate from nine of these samples (different wells of the RNAspin 96 preparation over 3 plates) was ran on the Agilent 2100 bioanalyzer.

Experiment 2: HeLa cells (5×10^5) from a homogeneous cell culture, grown according to standard procedures (1), were pelleted in each well of a 96-well, cell-culture plate. Total RNA was isolated according to the RNAspin 96 kit as adapted for the Tecan Genesis 150 automated system. Each eluate (20 μ l from a 100- μ l elution volume) was subjected to denaturing gel electrophoresis (1). Each sample (15 μ l) was run on a 1% denaturing gel and subjected to Northern blot analysis using the Hybond-N nylon membrane. GAPDH was used as the radioactively labeled probe.

Experiment 3: 48 \times 10-mg mouse liver tissue samples were manually processed via the RNAspin 96 kit (vacuum protocol). The tissue was homogenized in RA1 buffer under liquid nitrogen and the lysate was clarified using the RNAspin Filter Plate. Yield and purity were determined using usual spectrophotometric methods (1) for all the 48 samples.

Experiment 4: 30 wells containing 10 mg of mouse liver tissue each were processed for RNA isolation using the RNAspin 96 kit on a Biomek FX vacuum automation system. The RNA was eluted with 80 μ l of RNase-free water, and 1 μ l of this was used for QRT-PCR. cDNA was synthesized using GAPDH primers and SYBR Green. The level of GAPDH transcript was measured with the LightCycler detection system according to the manufacturer's protocol.

Isolation of total RNA from a relatively large amount of cells (1×10^7 of HeLa cells)

HeLa cells were grown in culture according to standard procedures (1) and diluted to the indicated final cell numbers. Total RNA was isolated from this range of HeLa cells (5×10^5 to 1×10^7) using the RNAspin 96 kit with the vacuum protocol. Yield and purity were measured by spectrophotometry at A_{260} and A_{280} .

Isolation of total RNA from a relatively small amount of cells (≤ 10 of HeLa cells)

HeLa cells were grown in culture according to standard procedures (1) and diluted to the indicated final cell numbers. Total RNA was extracted from these HeLa cell aliquots using the RNAspin 96 kit with the vacuum protocol

in an automated format on the MultiProbe II HT platform. The cell numbers ranged from $1-2 \times 10^6$. The presence of amplifiable RNA was evaluated by RT-PCR using 2 μ l of the 100- μ l eluate and GAPDH primers.

Application of the total RNA product in quantitative RT-PCR

A 100-mg aliquot of rat liver tissue was frozen and subsequently stored in RNAlater-ICE buffer. Total RNA was extracted using the RNAspin Mini kit. cDNA was generated using TaqMan Reverse Transcription Reagents and TaqMan TAMRA primer/probe sets with 1 μ g of the RNA templates (1). The probe/primer set targeted the rat thyroid hormone sulfotransferase (*ST1B1*) gene. GAPDH was used as a control transcript. The reactions were then purified using the CyScribe GFX Purification Kit. The purified cDNA (4.0 ng, 0.4 ng, and 0.04 ng) were used for QRT-PCR with the HotStarTaqTM Kit (Qiagen) on the ABI 7900 instrument.

Comparison of the vacuum and centrifugation protocols

Total RNA was isolated from 8×10 mg of mouse liver tissue aliquots as input material for either the RNAspin 96 manual centrifugation or the automated vacuum method. For both methods, the total RNA product was eluted with 100 μ l of RNase-free water. The eluted volume was 80 μ l and 100 μ l for the vacuum and centrifugation protocols, respectively. A sample (20 μ l) of each eluate was subjected to denaturing gel electrophoresis.

Results

Consistent isolation of high-quality RNA

Reproducibility is key to the success of any high-throughput process. In this section, we describe several RNA isolation experiments that highlight the consistency of RNA yield and quality well-to-well, plate-to-plate. A common method for measuring total RNA quality and purity is to determine the A_{260}/A_{280} ratio associated with a specific total RNA sample. The A_{260}/A_{280} ratio for all the RNA samples described in this section were between 1.9-2.2. An important aspect of total RNA purity is whether or not it is contaminated with genomic DNA post-isolation. The membrane desalting step of the RNAspin 96 kit creates an optimal chemical environment for an efficient in-process DNase I digest. Thus, the isolated total RNA is free of genomic DNA (gDNA). This is especially critical for downstream applications, such as cDNA synthesis, that involve enzymatic reactions, where contaminating gDNA may interfere and cause spurious product formation and/or low product yield. The efficient in-process DNase I digest of the RNAspin 96 kit effectively eliminated genomic DNA contamination in the preparation of rat liver RNA; hence, the absence of genomic DNA contaminants in the samples shown in Figure 2.

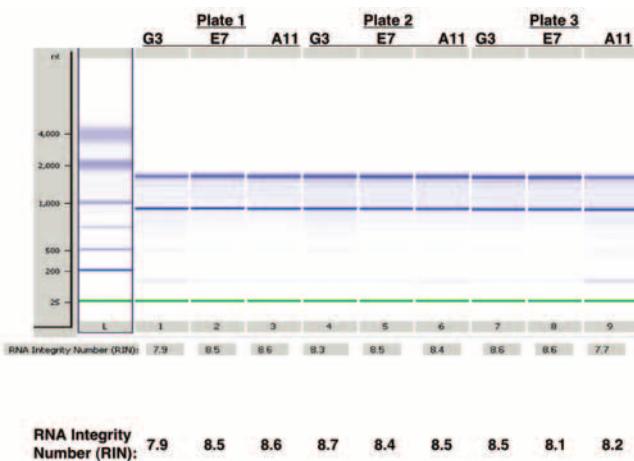


Fig 2. The RNAspin 96 kit yields consistent, high-quality total RNA within and across plates. A microliter of RNA sample (out of a total eluate of 100 μ l) was analyzed on the Agilent 2100 bioanalyzer.

Where the RNA quality was also assessed using the Agilent 2100 bioanalyzer, RNA Integrity Number (RIN) values were on average, greater than 8.0 thereby indicating the presence of high RNA integrity and purity (Fig 2). The rRNA bands were sharp with no visible signs of degradation and the 28S band was about twice as intense as the 18S band (Figs 2 and 3). Vital parameters such as RNA yield, purity, and integrity were all highly reproducible as shown in Figure 4 in which 48 mouse liver samples were prepared in parallel and the yields were $41.0 \pm 2.4 \mu\text{g}$ (%CV of 5.7), and the A_{260}/A_{280} ratios were 2.0 ± 0.1 .

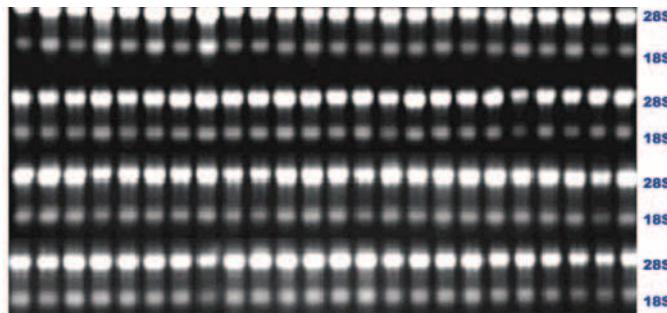


Fig 3. Well-to-well reproducibility is maintained using the RNAspin 96 kit for RNA isolation. Twenty microliters of each HeLa cell culture RNA eluate (100 μ l elution volume) of a 96-well plate was ran in each lane on a denaturing gel.

For qualitative analysis, the isolated RNA was used in a number of rigorous downstream applications: 30 separate, 10-mg mouse liver samples (individual wells) were prepared using the RNAspin 96 Kit. Each sample was then used to interrogate the GAPDH transcript. The LightCycler results shown in Figure 5 indicate that the Cycle Thresholds (CT) were also highly reproducible, with a mean $CT = 22.0 \pm 0.2$ with a %CV of 0.95 (Fig 5).

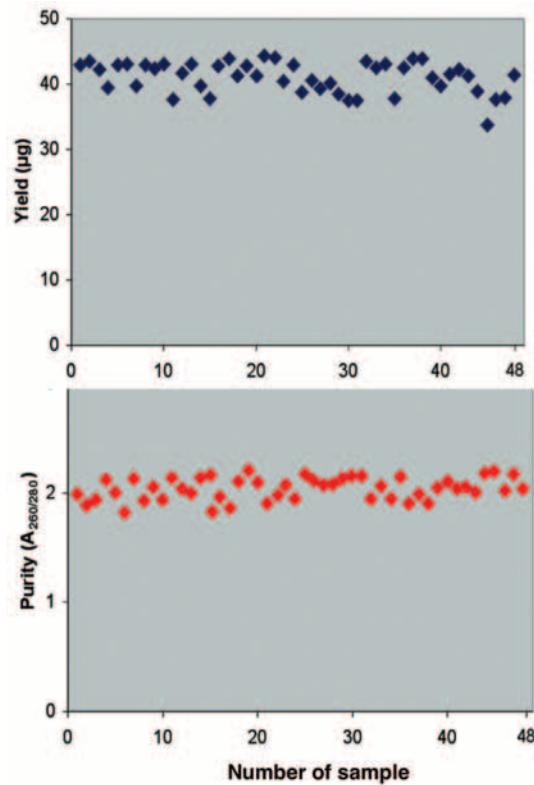


Fig 4. Highly reproducible total RNA yield and purity as determined from 48 \times 10-mg mouse liver tissue samples.

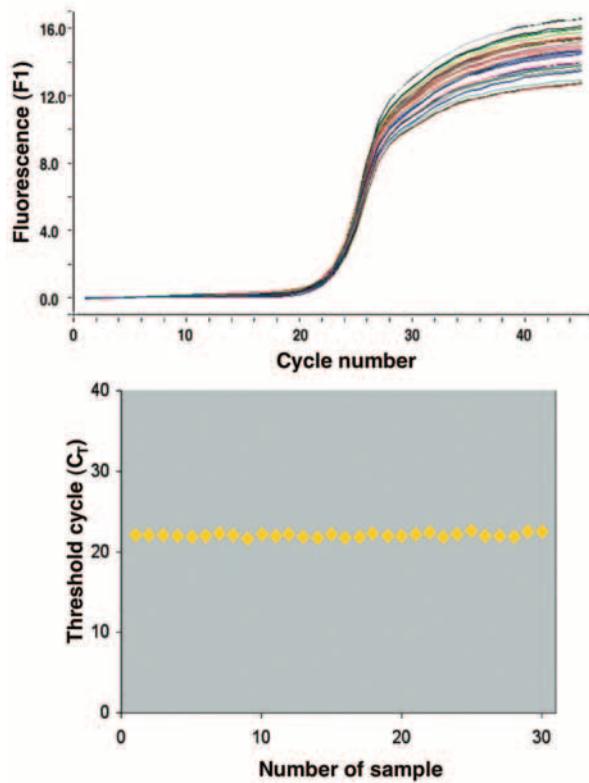


Fig 5. The total RNA produced from the RNAspin 96 kit yields consistent performance in sensitive downstream applications. The LightCycler CT values from 30 separate total RNA samples are shown.

Purification of total RNA from large amounts of cells (1×10^7 of input cells)

For many RNA isolation protocols, the processing of large amounts of input cells compromises yield and purity. However, this limitation does not apply to the RNAspin 96 kit because of effective homogenization and the removal of cellular debris with the RNAspin 96 Filter Plate. Thus, the RNAspin 96 kit can be used to process as much as 1×10^7 cells with high yield and purity: the A_{260}/A_{280} ratio for all the RNAspin samples was between 1.94 and 1.96 (Fig 6).

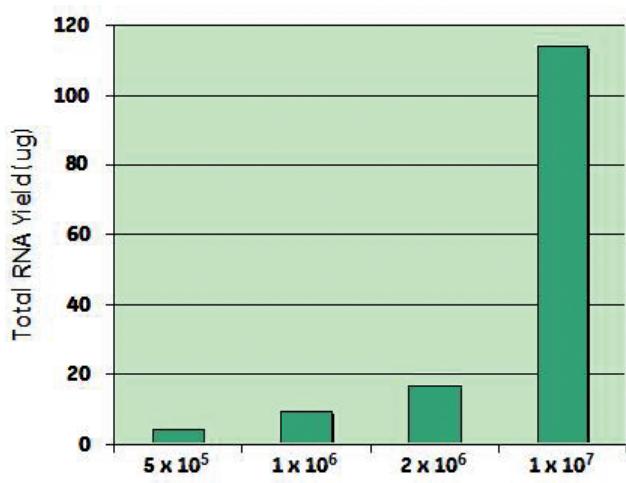


Fig 6. Successful isolation of total RNA with the RNAspin 96 kit from up to 1×10^7 for HeLa cells.

Purification of total RNA from a relatively small amount of cells (≤ 10 cells)

RT-PCR results using total RNA isolated from a range of HeLa cell amounts are shown in Figure 7. The GAPDH RT-PCR product was easily detected from as a low a sample size as 10 HeLa cells. This makes the RNAspin 96 kit particularly suitable for the isolation of high-quality RNA from very small amounts of input cells.

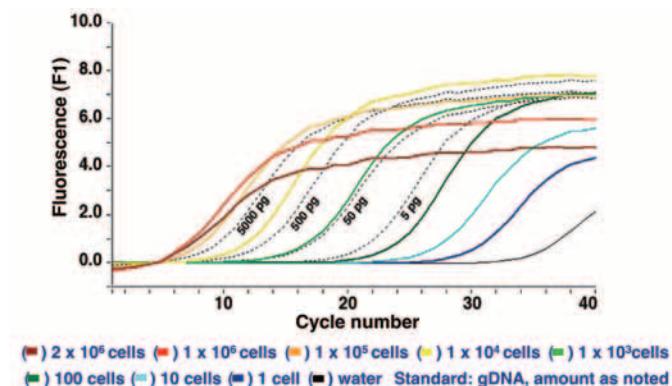


Fig 7. The efficient isolation of RNA with the RNAspin 96 kit allows for the detection of amplifiable RNA from very small amounts of cells.

Detection of gene expression by Northern analysis

GAPDH gene expression is often used as a positive expression control in gene expression profiling. Therefore, GAPDH is expected to be expressed at a detectable level in many cell types, including cultured HeLa cells. The known pattern of GAPDH gene expression in cultured HeLa cells is shown for 48 separate, 96-well culture samples (Fig 8). This data highlights the reproducibility aspect of the RNAspin 96 kit and also shows that it can be used to isolate sufficient, high-quality total RNA for Northern blot analysis.

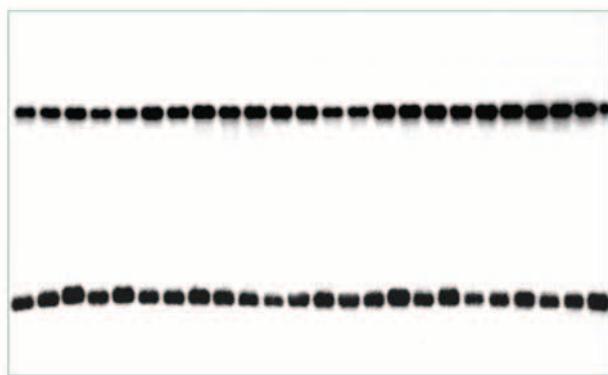


Fig 8. Northern blot analysis of the GAPDH transcript in 48 separate HeLa cell cultures. Fifteen microliters of each HeLa cell culture RNA eluate from a 96-well plate was ran per lane (see Fig 3 legend for additional details).

Detection of RNA transcripts by QRT-PCR

The rat thyroid hormone sulfotransferase, encoded by the *ST1B1* gene, is an important regulator of thyroid hormone metabolism. As with other important transcripts, measurement of the relative expression level in a given tissue (liver in this case) is important for understanding its biological function. The data presented here shows that the quality of the RNA generated from the RNAspin 96 kit is high enough for sensitive downstream applications like QRT-PCR (Fig 9).

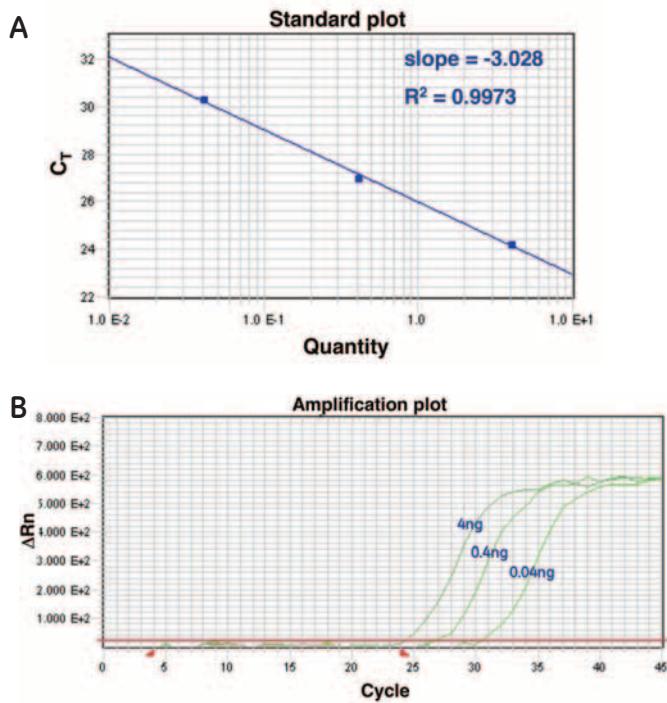


Fig 9. Total RNA from the RNAspin 96 kit performed well in sensitive downstream applications like QRT-PCR. Titration of an RNAspin 96 kit rat liver total RNA sample (NAP303) with TAQ100 primer/probe set [rat thyroid hormone sulfotransferase (*ST1B1*) gene] (A) QRT-PCR standard curve indicating the appropriate 10-fold differences in the reaction dilutions; (B) QRT-PCR CT curves showing typical response curves for clean RNA template.

A comparative analysis of centrifugation and vacuum methods

The ability to use the RNAspin 96 kit with either a benchtop centrifuge or on an automated platform enhances the flexibility of the kit. A comparison of the RNA output (Fig 10) from both the centrifugation and vacuum methods indicates that although the RNA yields were similar, the centrifugation method produced slightly higher yields (about an 8% increase, on average).

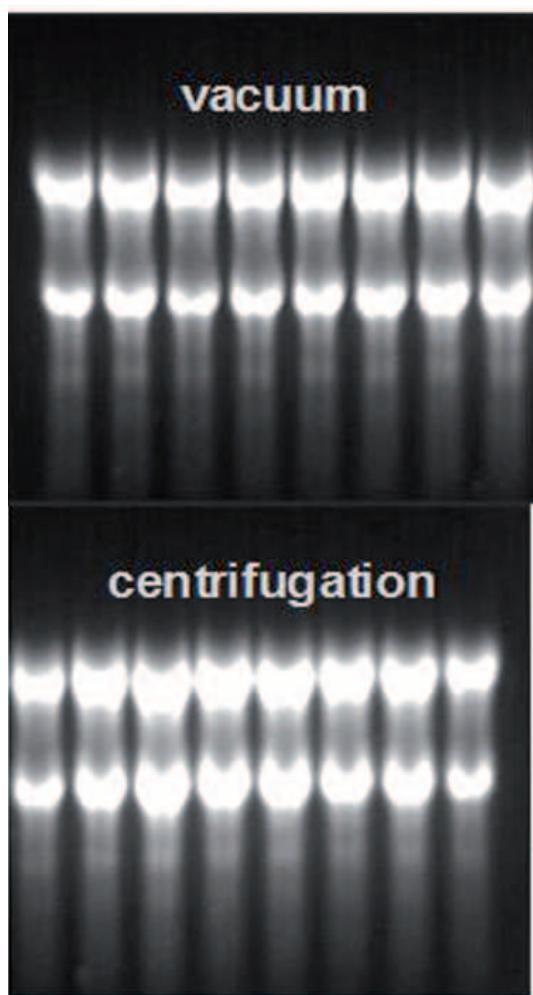


Fig 10. Comparative analysis of isolated total RNA via centrifugation and vacuum protocols on a denaturing gel with 20 μ l of each eluate per lane.

Conclusions

The RNAspin 96 RNA Isolation kit reproducibly (well-to-well, plate-to-plate) yields total RNA of high quality and integrity with little or no contaminating genomic DNA. The RNA product can be purified in varying amounts from a diverse range of sample types including samples that are difficult to process. Typical input tissues include rat and mouse tissue (liver, kidney, brain), different human cell lines, and *Streptococci*, *Actinobacillus pleuropneumoniae* and *Pseudomonad* species. The quality, integrity, and yield of total RNA is high enough for the most sensitive of downstream applications such as QRT-PCR and Northern Blot.

Reference

1. Ausubel, F.A., ed., *Short protocols in molecular biology: a compendium of current protocols in molecular biology*, John Wiley and Sons, Inc. Indianapolis, IN (2002).

Asia Pacific	Tel: +85 65 62751830	Fax: +85 65 62751829
Australasia	Tel: +61 2 8820 8299	Fax: +61 2 8820 8200
Austria	Tel: 01 /57606 1613	Fax: 01 /57606 1614
Belgium	Tel: 0800 73 890	Fax: 02 416 8206
Canada	Tel: 1 800 463 5800	Fax: 1 800 567 1008
Central & East Europe	Tel: +43 1 972 720	Fax: +43 1 972 722 750
Denmark	Tel: +45 70 25 24 50	Fax: +45 45 16 2424
Eire	Tel: 1 800 709992	Fax: +44 1494 542010
Finland & Baltics	Tel: +358 9 512 3940	Fax: +358 9 512 39439
France	Tel: 01 69 35 67 00	Fax: 01 69 41 98 77
Germany	Tel: 0800 9080 711	Fax: 0800 9080 712
Greater China	Tel: +852 2100 6300	Fax: +852 2100 6338
Italy	Tel: 02 26001 320	Fax: 02 26001 399

Japan	Tel: 81 3 5331 9336	Fax: 81 3 5331 9370
Korea	Tel: 82 2 6201 3700	Fax: 82 2 6201 3803
Latin America	Tel: +55 11 3933 7300	Fax: +55 11 3933 7304
Middle East & Africa	Tel: +30 210 96 00 687	Fax: +30 210 96 00 693
Netherlands	Tel: 0800-82 82 82 1	Fax: 0800-82 82 82 4
Norway	Tel: +47 815 65 777	Fax: +47 815 65 666
Portugal	Tel: 21 417 7035	Fax: 21 417 3184
Russia, CIS & NIS	Tel: +7 495 956 5177	Fax: +7 495 956 5176
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Switzerland	Tel: 0848 8028 10	Fax: 0848 8028 11
UK	Tel: 0800 515 313	Fax: 0800 616 927
USA	Tel: +1 800 526 3593	Fax: +1 877 295 8102

www.gehealthcare.com/nap

GE Healthcare UK Limited
 Amersham Place
 Little Chalfont
 Buckinghamshire
 HP7 9NA
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