High-Throughput Automated Extraction of RNA Using the Aurum™ Total RNA 96 Kit

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Introduction

Laboratories involved in drug screening, diagnostics, or other medium- to high-throughput methodologies increasingly rely on automation to reduce time to completion, technical errors, labor, and costs. RNA extractions for use in qPCR assays are labor-intensive, time-consuming procedures that are now amenable to automation. This raises the question of whether commercially available RNA extraction kits that have been designed for low- to medium-throughput extractions using centrifugation or filtration-based methods can be used in automated high-throughput procedures.

The Aurum total RNA 96 kit was adapted to a centrifugation method that allowed simultaneous extraction of two 96-well plates in the time required to complete a single plate using the standard protocol. In this tech note, we evaluate the use of the Aurum total RNA 96 kit in a fully automated extraction process using the KingFisher and BioSprint magnetic extraction platforms that capture targets on magnetic beads through an automated rod assembly that functions in a 96-well format (Fang et al. 2007). We adapted the Aurum total RNA 96 kit to extract RNA from human cells expressing hepatitis C virus (HCV) replicons as part of ongoing drug screening for compounds active against HCV. The same protocol was employed to help address the recent influx of clinical material associated with the H1N1 swine flu pandemic.

Methods

Cell Lines and Sample Processing

HuH-7 cells harboring an HCV replicon that expresses secreted alkaline phosphatase (SEAP) (Bourne et al. 2005) were aliquoted into 96-well plates. At selected time points after drug application, the cells were lysed using 100 µl of Aurum total RNA 96 kit–provided lysis buffer (Bio-Rad Laboratories, Inc.) containing 1% β-mercaptoethanol and stored at –80°C until use. Lysates were thawed on ice before RNA extraction. Serial 10-fold dilutions of the lysates were performed in lysis buffer.

RNA Extraction

Manual RNA extraction was performed following the Aurum total RNA 96 kit instructions with slight modifications. The DNase I digestion was increased to 20 min and samples were eluted in a volume of 150 µl. To increase throughput, the kit-provided protocol was modified by replacing the vacuum filtration steps with short spin centrifugation. This allowed two plates to be extracted at the same time. The plates were spun using a Sigma 4–15 centrifuge with a 2 x 96-well plate rotor (QIAGEN). During centrifugation, the filter plates were supported by the 96-well "grow blocks" supplied in the Aurum kit. For each wash step, the plates were spun at 4,000 rpm for 3 min. The elution step was done twice (2 x 75 µl) by spinning at 1,400 rpm for 1 min each.

Automated RNA extraction was carried out using KingFisher 96 (Thermo Fisher Scientific Inc.) or BioSprint 96 (QIAGEN) magnetic bead processing instruments fitted with deep-well magnetic head configurations in conjunction with platform-specific software. Plastic consumables, necessary for automated extraction, were purchased from VWR International. MyOne SILANE magnetic Dynabeads were obtained from Invitrogen Corporation. The computerized script to perform the Aurum kit–based 96-well extraction will be provided upon request.

Reverse Transcription (RT)–qPCR

cDNA was synthesized using an iScript™ cDNA synthesis kit (Bio-Rad). Reactions (10 µl) were assembled in 96-well PCR plates through the addition of 2 µl iScript reaction mix, 6.5 µl nuclease-free water, 0.5 µl reverse transcriptase, and 1 µl RNA. RT was completed using a C1000™ thermal cycler (Bio-Rad) by performing the following incubation steps: 5 min at 25°C, 30 min at 42°C, and 4 min at 85°C.

cDNA was evaluated using qPCR with primers amplifying targets within the HCV 5' non-translated region and the human glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) (Bourne et al. 2005). TaqMan probes were used to track the specific amplification in each duplex. Each 25 µl reaction contained 12.5 µl iQ™ supermix (Bio-Rad), 1 µl (5 µM) of each of the primers, 0.5 µl (7.5 µM) HCV-FAM probe, 0.5 µl (15 µM) *GAPDH*-Texas Red probe, 6.5 µl nuclease-free water, and 1 µl cDNA template. qPCR was completed in

a C1000 thermal cycler equipped with a CFX96™ optical reaction module (Bio-Rad) using the following cycling parameters: 95°C for 1.5 min, followed by 40 cycles of 95°C for 20 sec and 62°C for 1 min. Data were collected at the end of each extension step. Starting quantity values were extrapolated from standard curves of plasmids harboring the PCR target generated in parallel for each run. Mathematical analyses were performed using Excel spreadsheet software (Microsoft Corporation) or Prism v4.0 analysis software (GraphPad Software Inc.).

Results

Medium-Throughput Centrifugation-Based RNA Extractions Using the Aurum Total RNA 96 Kit

The modification of the protocol from vacuum to centrifugation allowed parallel extraction of two 96-well plates in ~1.5 hr, the same amount of time required to process a single plate using the vacuum method. In order to validate the centrifugation method, sample lysates were divided equally between two 96-well plates — one was processed using vacuum and the other using centrifugation. After cDNA production, qPCR for HCV and *GAPDH* was performed on both sets. Analysis of the qPCR results revealed a correlation of 98% between the vacuum and centrifugation protocols, indicating that centrifugation was indistinguishable from the vacuum method for RNA production.

Development of an Automated Method for RNA Extraction Using the Aurum Total RNA 96 Kit

Before testing the Aurum total RNA 96 kit's compatibility with magnetic bead processing, an automated procedure was developed. The assay was designed to run on either a KingFisher 96 or BioSprint 96 instrument and utilized a custom protocol generated by the associated software for each platform. The method involved sequential movement of magnetic beads via a 96-rod magnetic head between assay plates containing previously loaded Aurum kit reagents. Loading of these plates was accomplished in 15 min using a multichannel pipetor or in 6 min with a Tecan 8-tip liquid handler (Tecan Group Ltd.).

Assay plates for automated RNA extraction were prepared in advance and the KingFisher or BioSprint magnetic rods were sheathed in a disposable plastic tip comb that provided an RNase-free protective barrier during processing. These items were loaded into the instrument prior to initiating the run (Table 1). The sample plate was prepared by the addition of 100 µl of lysate (created by adding kit-provided lysis buffer to the monolayer or cell pellet) plus an equal volume of 70% RNase-free ethyl alcohol (EtOH) in addition to indicated volumes of magnetic beads. Next, 350 µl of kit-supplied low-stringency wash solution was added to each well of two deep-well plates (Table 1, Steps 3 and 4). The remaining assay plates will be described in the steps in which they were utilized below.

RNA in each well of the sample plate was first bound to the magnetic beads through automated mixing using the magnetic rods at room temperature for 5 min. The beads then were transferred to the first low-stringency wash plate. After incubation with automated mixing, the beads were transferred to the second low-stringency wash plate. This second wash ensured complete removal of protein and lysis solution chemicals before incubation with DNase I. The RNA-loaded beads were lifted out of the wash solution and air-dried for 1 min before transfer to a plate pre-loaded with DNase I solution (Table 1, Step 6). The DNase I solution was prepared by diluting 2.5 µl of reconstituted DNase I into 77.5 µl of DNase dilution buffer in each well. DNase I digestion was carried out for 20 min at room temperature. The success of DNase I digestion was evaluated by amplifying human *GAPDH* in the eluted RNA (no RT). The results showed only occasional DNA contamination (<100 copies/sample), similar to levels seen with the vacuum method (data not shown).

Because the conditions used for DNase I digestion could cause release of RNA from the magnetic beads, four volumes of a 1:1 ratio of Aurum total RNA lysis solution (Bio-Rad) and 70% RNase-free EtOH were manually added to the DNase I

Table 1. Protocol for Aurum automated procedure.

Step	Plate	Release Magnetic Beads	Parameter	Magnetic Rods Mixing Speed	Step Duration	Collect Magnetic Beads	
1. Load tip plate	Tip plate	–	–	–	–	–	
2. Lysis/binding	Sample lysis	Yes	Mix	Slow	5 min	Yes	
3. Low-stringency wash 1	Low-stringency wash 1	Yes	Wash	Fast	30 sec	Yes	
4. Low-stringency wash 2	Low-stringency wash 2	Yes	Wash	Fast	30 sec	Yes	**32 min***
5. Dry beads	N/A	No	Dry	N/A	1 min	N/A	
6. DNase I digestion	DNase I treatment	Yes	Mix	Very slow	20 min	No	
7. Pause/add rebinding solution	DNase I treatment	N/A	N/A	N/A	N/A	N/A	
8. Rebind RNA	DNase I treatment	Yes	Mix	Fast	5 min	Yes	
9. High-stringency wash	High-stringency wash	Yes	Wash	Very fast	30 sec	Yes	
10. Low-stringency wash 3	Low-stringency wash 3	Yes	Wash	Very fast	30 sec	Yes	**22 min***
11. Dry beads	N/A	No	Dry	N/A	1 min	N/A	
12. Heated elution	Elution	Yes	Mix/70°C	Slow	4 min	Yes	

* Cumulative assay time includes step time, collection of magnetic beads, and user addition of rebinding buffer.

plate after digestion to favor binding of RNA to magnetic beads prior to the wash steps (Table 1, Steps 7 and 8). Subsequent high-stringency and low-stringency washes were performed as suggested in the Aurum kit protocol (Table 1, Steps 9 and 10), including an additional air-dry step (Table 1, Step 11). Elution was accomplished by incubation with agitation of the beads for 4 min in elution buffer pre-warmed to 70°C using the internal heating block in the KingFisher (or BioSprint) device (Table 1, Step 12). Using this method, RNA extraction was completed in 54 min.

Evaluation of Use of the Aurum Kit with Magnetic Bead Automated Processing

To assess the efficiency of automated RNA extraction, we utilized a high-throughput cell culture–based drug-screening assay utilizing HCV replicons previously developed in our laboratory (Bourne et al. 2005). RNA was extracted from HuH-7 cells expressing HCV replicons (n = 11 replicate wells). A final well without cells served as an extraction control (lysis buffer only) using the automated procedure. qPCR for both HCV and *GAPDH* was then performed as described (Bourne et al. 2005). Results of this experiment indicated the presence of RNA for both qPCR targets at levels indistinguishable from the standard vacuum or centrifugation extraction methods (HCV = 5 x 10^5, *GAPDH* = 2 x 10^6 RNA copies). No amplification was observed in the RNA (no RT) controls indicating a lack of measurable DNA contamination. These initial results indicated that the Aurum total RNA 96 kit was compatible with automated magnetic bead extraction.

Optimization of the Automated RNA Extraction Procedure

To further validate the automation methodology and optimize the procedure, we compared automated extraction to the standard manual vacuum-based method. Sample lysates were created and then diluted to represent an input concentration of 10^5 cells/well in a 96-well plate prior to parallel RNA extraction by either the automated or vacuum protocols. Based upon the initial success with automation, we sought to minimize costs without compromising RNA recovery by identifying the optimal amount of magnetic beads to add to each sample. When 12.5 µl or more of magnetic beads were added to the lysates, amounts of total RNA recovered were comparable to those obtained by the manual method (Figure 1), while the use of 6 µl of magnetic beads significantly decreased the RNA yield. From this experiment, 12.5 µl was identified as the optimal volume of magnetic beads to use.

Next, the dynamic range of the automated method was evaluated through a series of 10-fold serial dilutions starting with lysates of 10^5 HuH-7 replicon cells (Figure 2). The diluted lysates were divided evenly for extraction using manual vacuum or magnetic bead automation (using the optimal 12.5 µl of magnetic beads/extraction). The first dilution tested

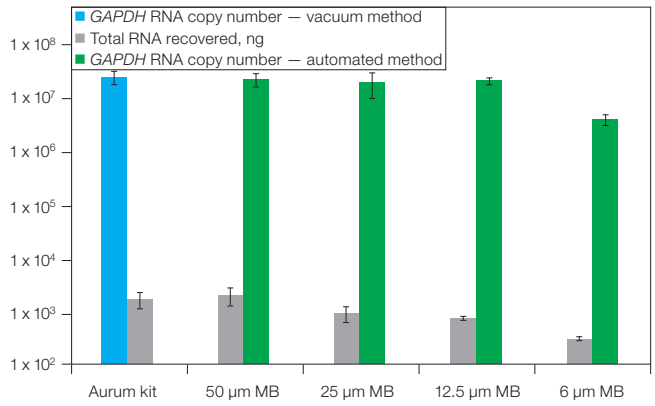

Fig. 1. Optimization of magnetic bead concentration. RNA from replicate cell lysates was extracted using the automated procedure with the addition of various quantities of magnetic beads (MB) or via the standard vacuum method (Aurum). RNA was quantified by spectrophotometry. *GAPDH* was evaluated by qPCR (RNA copy number). Error bars: 1 SD, n = 3, except 25 µl MB, n = 6.

was expected to yield ~5 x 10^6 copies of HCV and ~5 x 10^7 copies of *GAPDH*, with successive dilutions providing 10-fold fewer copies of each target. As anticipated, the vacuum method provided RNA copies consistent with past studies. Comparison of the results revealed a >99% relationship (correlation coefficient r^2 = 0.993) between manual and automated extractions with a lower limit of 10^2 (HCV) or 10^3 (*GAPDH*) across the 5 replicate extractions completed for each dilution. Together, these results showed the automated assay equated or exceeded the RNA binding capacity of the standard method over a dynamic range of approximately 10^7 to 10^2 RNA copies (Figure 2).

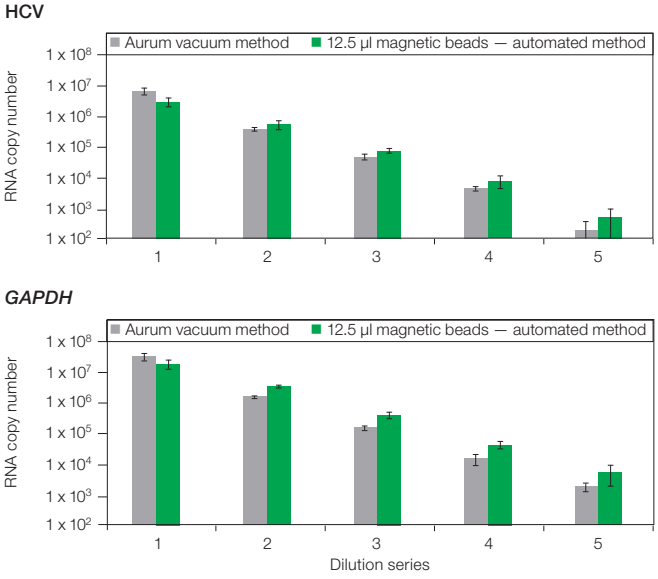

Fig. 2. Comparison of manual and automated extraction using the Aurum total RNA 96 kit. Serial dilutions of cell lysates (1–5) were matched for extraction using either the vacuum method or by automated extraction using 12.5 µl of magnetic beads. Quantity of purified RNA was evaluated via qPCR targeting HCV or *GAPDH*. Error bars: 1 SD, n = 3.

Application of the Automated Procedure

To demonstrate a relevant use of the automated assay, HuH-7 HCV replicon cells were evaluated by qPCR subsequent to treatment with a dilution series (0–500 µM) of a candidate antiviral designated test compound B. Triplicate wells of cells were subjected to vehicle or the selected concentrations of test compound B in 96-well plate formats. After 48 hr of treatment, RNA was extracted from cells using a standard vacuum- or magnetic bead–based protocol. HCV and *GAPDH* levels were evaluated by qPCR, then RNA levels, as well as the outcome of effective concentration 50% (EC50) calculations for compound B from each extraction method, were statistically compared. The EC50 values represented the concentration of drug needed to reduce the HCV RNA copy number to 50% of the untreated wells. Both methods provided statistically indistinguishable outcomes with calculated EC50 values of 0.8 µM (Figure 3, HCV). *GAPDH* levels also were consistent across both data sets and did not substantially decrease, indicating compound B was not cytotoxic (Figure 3, *GAPDH*).

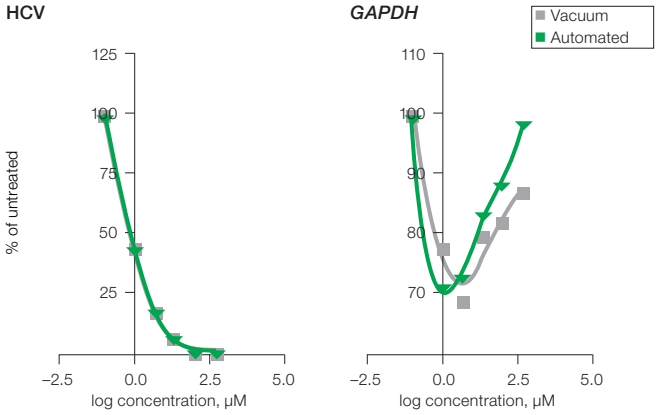

Fig. 3. Evaluation of HCV inhibition by experimental antiviral compound using manual and automated Aurum RNA extraction. HuH-7 HCV replicon cells were treated with a dilution series of an experimental antiviral compound B (0–500 µM). RNA was subsequently extracted by the standard vacuum or optimized automated protocols and qPCR for HCV and *GAPDH* was performed. To establish EC50 values, fourth-order polynomial curves were compared. *GAPDH* served as an indicator of potential test compound B cytotoxicity and allowed comparison of the procedures. Compound B HCV EC50 = 0.8 µM for both extraction methods, n = 3.

Discussion

The ability to automate extraction of nucleic acids in a high-throughput modality has the potential to greatly expand the breadth and reduce the costs of biomedical research. For many ongoing projects, it is important to be able to utilize existing kit-based systems that have been validated using lower-throughput methods. To this end, we were able to adapt the Aurum total RNA 96 kit to both medium- and automated, high-throughput platforms via optimized centrifugation or magnetic bead–based extraction protocols. For the magnetic bead–based automation, a KingFisher 96 or BioSprint 96 instrument equipped with a deep-well magnetic head with accompanying software was employed to develop the assay. These magnetic bead–based extraction instruments are becoming common in research and diagnostic labs and greatly increase throughput while reducing labor and technical errors.

The medium-throughput centrifugation protocol requires access to a centrifuge outfitted with a rotor capable of accommodating 96–deep-well blocks and achieving a force of ~2,600 x g. The 96-well "grow blocks" provided in the kit served to catch the flowthrough and were emptied between spins to reduce plastic requirements without causing cross-contamination. After optimization, the centrifugation protocol provided RNA from 192 samples in the time required to manually extract a single plate of 96 samples, reducing labor costs and increasing throughput. For higher throughput, we developed a protocol that took advantage of magnetic bead–based processing while successfully utilizing the reagents provided with the Aurum kit. In initial experiments, automation of the method did not result in sample cross-contamination, leading us to optimize and then validate the magnetic bead–based protocol by comparison to standard vacuum extraction.

After optimization, we validated the automated system under a practical application of the technology that involved the screening of experimental compounds from a small molecule library that provided anti-HCV activity. In this study, the automated extraction protocol yielded indistinguishable activities for the selected test compound compared to the manual extraction method completed in parallel. We note that the automated extraction conditions reported here proved optimal for these experiments, but would expect that other protocols may require empirical re-optimization of magnetic bead concentration.

Conclusions

In summary, we found that increased throughput could be easily achieved with the Aurum total RNA 96 kit and determined that the optimized centrifugation and automation protocols yielded more reliable outcomes, particularly in high sample volume circumstances. Importantly, the automated Aurum kit extraction protocol took 54 min to complete, during which time a technician could prepare other reagents or additional experimental steps (for example, cDNA production, qPCR, etc.). Together, these results established the medium- and high-throughput capability of the Aurum total RNA 96 kit to simultaneously extract RNA from viral and eukaryotic sources with outcomes comparable to the standard vacuum method.

References

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