RNA Isolation from 3D Microtissue Cultures: A Comparison of Manual and Automated Methods

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Materials Used:

- ReliaPrep™ RNA Cell Miniprep System (Cat.# Z6010, Z6011, Z6012)
- Maxwell® 16 LEV simplyRNA Cells Kit (Cat.# AS1270)
- Maxwell® 16 Instrumentt (Cat.# AS2000)
- QuantiFluor® RNA System (Cat.# E3310)
- GloMax® Discover System (Cat.# GM3000)
- GravityPLUS™ 3D Culture and Assay Platform (InSphero Cat.# CS-06-001)
- HCT116 cells–human colon epithelial cell line
- Agilent 2100 Bioanalyzer
- Agilent RNA 6000 Pico Kit (Agilent Cat.# 5067-1513)

Protocols:

- *ReliaPrep™ RNA Cell Miniprep System Technical Manual,* #TM370.
- *Maxwell® 16 LEV simplyRNA Cells Kit Technical Manual*, #TM351
- *Maxwell® 16 Instrument Technical Manual,* #TM295

Here, we demonstrate the performance of manual and automated methods for extraction of RNA from 3D micro-tissue cultures.

Introduction

Regulation of gene expression is an area of high interest for both academic researchers and biopharmaceutical companies. There are many methods for determining up-regulation or suppression of gene expression, most of which begin with RNA purification.

Traditional 2D tissue culture methods have been used routinely for most biochemical and molecular research studies. They provide accessibility to biological materials and highly reproducible results, and are adaptable to high-throughput analysis formats. However 2D cultures poorly represent the tissue microenvironment, and are being replaced with 3D culture methods that enable formation of intercellular contacts, allowing establishment of signaling pathways and gene expression patterns characteristic of tissues in vivo. In this report, we demonstrate the performance of two different RNA isolation methods using 3D microtissue cultures developed using the InSphero hanging-drop method.

RNA Isolation Methods

We evaluated one manual method (ReliaPrep™ RNA Cell Miniprep System) and one semi-automated method (Maxwell® 16 LEV simplyRNA Cells Kit) for RNA extraction. The ReliaPrep™ RNA Miniprep Systems provide a fast and simple way to prepare intact total RNA from cultured cells or tissues in about 30 minutes. The column/binding matrix efficiently captures RNA from very small amounts of input material and the RNA is eluted in a minimal volume (less than 15µl). For the semi-automated method, we used the Maxwell® 16 LEV simplyRNA Cells Kit with the Maxwell® 16 Instrument to evaluate extraction from microtissues. In this method, microtissue sample lysates are added directly to a prefilled reagent cartridge, which is then processed by the Maxwell® Instrument using an automated method.

Methods

HCT116 cells were harvested, counted and diluted to 6.4 x 10^5 cells/ml in filtered McCoy's medium with 10% FBS. The cells were then serially diluted in McCoy's medium in a twofold dilution series from 6.4 × 10^5 cells/ml to 5.0 × 10^3 cells/ml. GravityPLUS™ Hanging-Drop Plates were prepared as described in the GravityPLUS™ kit manual. Forty microliters of each cell dilution was added to the plate at 200, 400, 800, 1,600, 3,200, 6,400, 12,800, and 25,600 cells/well and the plate was placed in a humidity controlled, 37°C/5% CO_2 incubator for 4 days. Microtissue formation was monitored daily using an Olympus CKX41 microscope with a

10X objective. After 4 days incubation, the cells were transferred to the GravityTRAP™ Plate as described in the GravityPLUS™ kit manual. Microtissue size was determined using cellSens® Software and an Olympus CKX41microscope with a 4X objective.

RNA Isolation

For samples processed using the **Maxwell® 16 LEV simplyRNA Cells Kit**, 25µl of medium and microtissues were transferred from the GravityTRAP™ Plate to individual 1.5ml microcentrifuge tubes. Two hundred microliters of chilled 1-Thioglycerol/Homogenization Solution was added to each microtissue and vortexed until the microtissue was dispersed and lysed. Lysis Buffer (200µl) was added to each of the lysed microtissues and the lysates vortexed vigorously for 15 seconds to mix. All ~400µl of lysate was then transferred to well #1 of the Maxwell® 16 LEV Cartridge and 5µl of DNase I solution was added to well #4 of each cartridge. On the Maxwell® 16 Instrument, "RNA" was selected, followed by "simplyRNA". The Maxwell® 16 Instrument, reagents, and cartridges were prepared as described in the *Maxwell® 16 LEV simplyRNA Cells Kit Technical Manual*, TM351.

For samples processed using the **ReliaPrep™ RNA Cell Miniprep System**, 25µl of medium and microtissues were transferred from the GravityTRAP™ Plate to individual 1.5ml microcentrifuge tubes. One hundred microliters of BL+TG buffer was added directly to the 25µl medium/microtissue and vortexed to disperse the microtissue. Isopropanol (35µl) was then added and vortexed for 5 seconds. The protocol provided in the ReliaPrep™ RNA Cell Miniprep System Technical Manual was followed for all column binding and wash steps, and RNA was eluted in 30µl water. All reagents were prepared as described in the *ReliaPrep™ RNA Cell Miniprep System Technical Manual*, TM370.

Concentration and Yield

The QuantiFluor® RNA dye, 1X TE buffer, and high concentration standards were prepared as described in the *QuantiFluor® RNA System Technical Manual*, TM377.

On hundred microliters of the standards were added in duplicate into a black 96-well plate and 96µl of 1X TE buffer added to all reaction wells. RNA eluate (4µl) was then added to the 96µl of 1X TE buffer.

One hundred microliters of the RNA dye (diluted 1:200) was added to all reaction and standards wells, and the plate shaken for 1 minute at 600 rpm to mix. The plate was then placed in a GloMax® Discover System and fluorescence determined using the QuantiFluor® RNA protocol after a 5-minute incubation.

Data were analyzed using the QuantiFluor® Dye Systems Data Analysis Workbook (available at: www.promega.com/resources/tools).

Quality-RIN Value

The Bioanalyzer and the RNA 6000 Pico kit were prepared as described in the instrument and kit technical manuals. RNA eluates were heated to 70°C for 2 minutes, and then transferred to ice. One microliter of each sample was added to the RNA 6000 Pico chip.

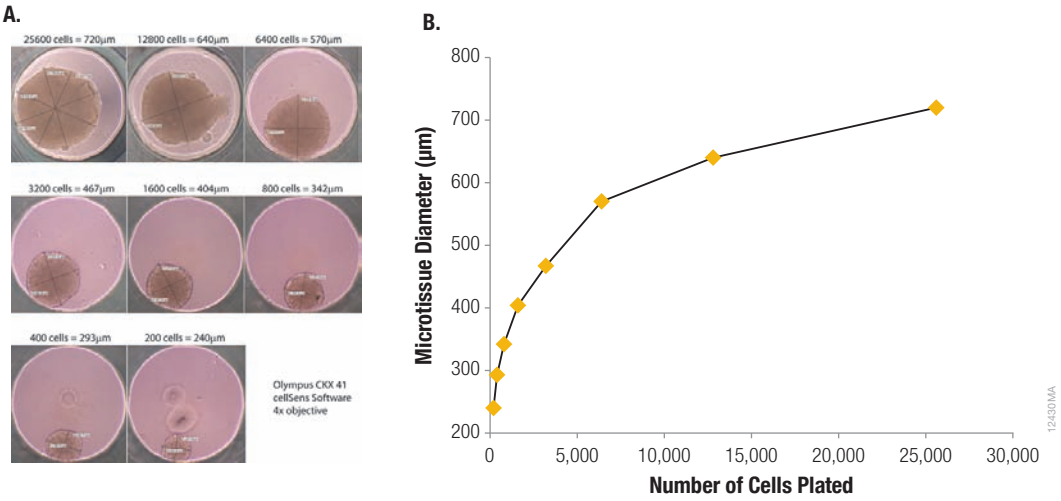

Figure 1. Microtissue size vs. number of cells plated. Panel A. HCT116 microtissues created using the Insphero GravityPLUS™ 3D Culture and Assay Platform. Size was determined using an Olympus CKX41 microscope with cellSens® Software. The number of cells plated/40µl and the diameter of the microtissue are indicated. **Panel B.** The relationship between microtissue size and number of cells plated.

Results

Microtissue Size Analysis

Olympus cellSens® software was used to determine the size of microtissues grown in a GravityTRAP™ Plate (Figure 1). The sizes in Figure 1 represent an average of three microtissues, which were seeded at the same cell number. As the number of seeded cells increased, the size of the microtissue increased. The curvilinear relationship between microtissue diameter and seeding cell number reflects different proliferation rates during the microtissue formation process.

RNA Isolation and Yield

The average final amount of eluate was 40µl for the Maxwell® method and 28µl for the ReliaPrep™ method. RNA concentration was determined using the QuantiFluor® RNA System, and the concentration value multiplied by the average eluate volume to determine total yield (Figure 2). As expected, as microtissue size increased, the RNA yield also increased. The linear fit r^2 value was near 0.989, and yields were similar with both methods. The largest microtissues (720µm), gave yields of ~700ng RNA, and the smallest microtissues (200µm), gave yields of ~50ng RNA.

RNA Quality

RNA integrity numbers (RIN values) are used to indicate RNA quality, the highest quality RNA having a RIN Value of 10 and the most degraded or lowest quality RNA having a RIN value near 1.0. RIN values were determined for the RNA isolated from each microtissue size using the Agilent 2100 Bioanalyzer with the RNA 6000 Pico Kit (one sample per value; Figure 2). All the RIN values for both systems were above 9.0, indicating high-quality RNA with little degradation.

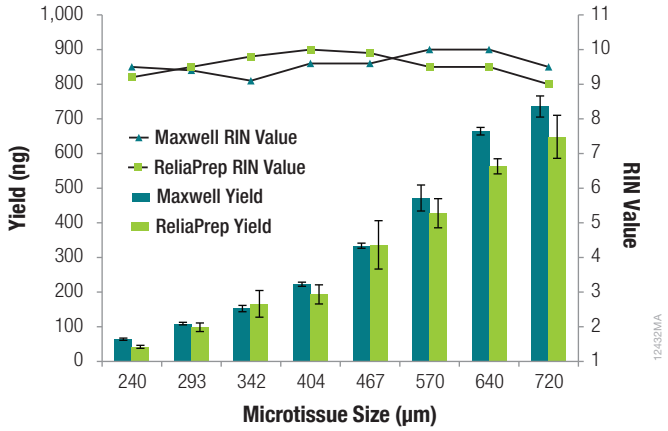

Figure 2. RNA yield from microtissues. RNA yield isolated from variously sized microtissues using the Maxwell® 16 LEV simplyRNA Cells Kit and the ReliaPrep™ RNA Cell Miniprep System. Yield was determined using the QuantiFluor® RNA System. Data represent the mean and standard errors from three replicates. This graph also shows the RIN values (quality number) as determined by the 2100 Bioanalyzer/RNA 6000 Pico System.

Summary

The Maxwell® 16 LEV simplyRNA Cells Kit and the ReliaPrep™ RNA Cell Miniprep System gave similar RNA yields. With the largest microtissues (720µm), ~700ng of RNA was isolated. With small microtissues (200µm), ~50ng of RNA was isolated.

All the RIN values for RNA isolated with both systems were above 9.0, indicating high quality RNA with little degradation.

Ordering Information

Product	Cat.#
ReliaPrep™ RNA Cell Miniprep System*	Z6011
Maxwell® 16 LEV simplyRNA Purification Kits	AS1270
Maxwell® 16 Instrument	AS2000
QuantiFluor® RNA System	E3310
GloMax® Discover System	GM3000

*Additional sizes available.