

# Automated Media Exchange of Non-Adherent Cells and Spheroids



## Introduction

Media exchange is an essential element of successful long-term cell culture in both high- and low-throughput experiments. Automated Media Exchange streamlines this process by reducing workflow duration, increasing reproducibility, and saving time and labor. However, some cell culture systems are sensitive to the disruptive effects of media exchange and require specialized liquid handling equipment with optimized parameters for successful exchange. Agilent BioTek automated media exchange (AMX) module, available on the Agilent BioTek MultiFlo FX multimode dispenser, is designed to gently and reliably exchange media for biology that is sensitive to the disruptive forces of fluid movement. The AMX module uses two peristaltic pumps to gently aspirate and dispense media from up to eight wells simultaneously.

This technical overview demonstrates the ability of the AMX module to gently perform media exchange for nonadherent T cells, as well as 3D spheroids. The AMX module is able to exchange media with no discernable displacement of the biology, as well as complete retention of suspension cells. Technical tips regarding media exchange settings and guidelines for assay optimization using the MultiFlo FX with AMX are provided.

## Hardware and software

The Agilent BioTek MultiFlo FX multimode dispenser with automated media exchange (AMX) module. The Agilent BioTek Cytation 5 cell imaging multimode reader.

## Procedures

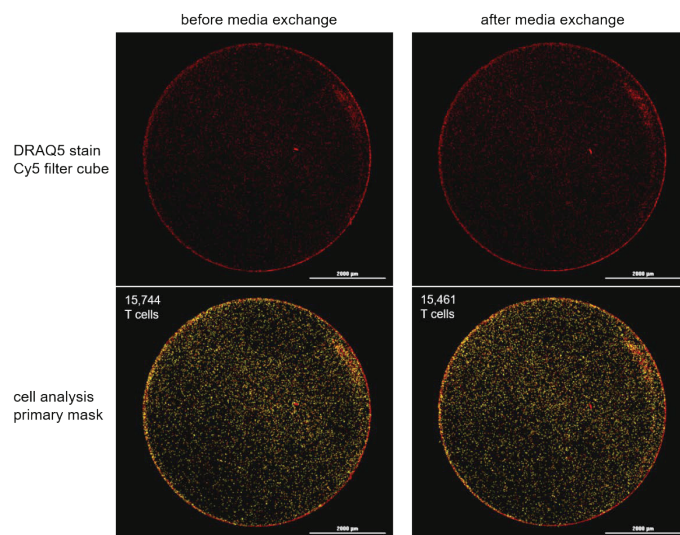
### T cell AMX media exchange in 96-well, flat-bottom microplate

#### 1) Define the aspiration height for the vessel.

All vessels, including 96-well plates, vary in dimensions between manufacturers, so it is important that the **aspirate** and **dispense** settings on the MultiFlo FX are configured to optimize the media exchange and washing processes. When washing suspension cells, it is important to leave a substantial remaining media volume after aspirating to avoid disturbing cells with fluid mixing forces. Leaving 100  $\mu$ L volume in a standard 96-well flat-bottom plate, 50% volume exchange, results in undisturbed T cells. The exact aspiration height required to leave behind a similar volume in an experiment is best determined empirically using the intended microplate and media. Media exchange settings for use with a Corning Costar 96-well, ultra-low attachment (ULA) microplate (part number 3474) are shown in Table 1. These steps were repeated five times for complete media exchange.

#### 2) Use the *slower carrier mode* feature for washing and imaging of suspension cells.

Normal handling, including transporting a microplate, can disturb nonadherent cells. The slower carrier feature, available in both Agilent BioTek Liquid Handling Control (LHC) software and Agilent BioTek Gen5 microplate reader and imager software, ensures slow, smooth plate movements throughout the liquid handling and imaging processes. These features were designed to minimize disruption of sensitive cells and structures. T cells imaged using a fluorescent nuclear stain show virtually undetectable movement following repeated media exchanges and imaging (Figure 1).



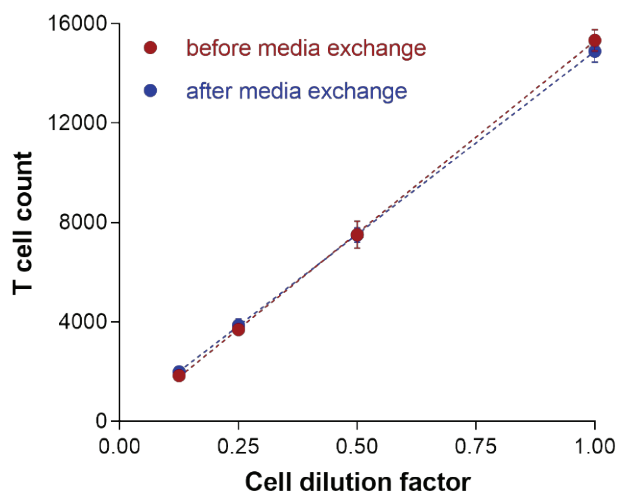
**Figure 1.** Whole-well imaging and cell count. A single whole-well image (4 × 5 montage) of T cells stained with DRAQ5 nuclear marker (red, top panels) from a 96-well plate was captured using the Agilent BioTek Cytation 5 cell imaging multimode reader. Images were processed, stitched, and T cell counts determined in Agilent BioTek Gen5 microplate reader and imager software using a primary mask (mask outline shown in yellow, bottom panels). T cells were imaged and counted before (left column) and then again after media exchange (right column) with the Agilent BioTek automated media exchange (AMX) module. Well media was exchanged using five cycles, 50% volume exchange, with parameters shown in Table 1. Scale bar indicates 2,000  $\mu$ m.

#### 3) Determine the aspiration and dispense rates.

The AMX module takes advantage of two peristaltic pumps: one for aspiration and one for dispensing. Flow rates can be individually optimized for each step. Begin using the slowest aspiration and dispense rates to verify that the biology is undisturbed. This procedure demonstrates successful T cell media exchange using the slowest aspiration and dispense rates. T cells were labeled with the fluorescent nuclear marker DRAQ5 (Thermo Fisher Scientific, Waltham, MA) for visualization and cell count. Images were taken before and after media exchange, and image analysis of cell counts validated T cell retention following repeated media exchange (Figure 2).

**Table 1.** Media exchange settings for Corning Costar 3474 96-well microplate.

Step	Volume ( $\mu$ L)	Flow Rate ( $\mu$ L/sec)	Z Offset	X Offset	Y Offset
pW - Aspirate	105	10	208 steps (8.45 mm above carrier)	50 steps (2.29 mm right of center)	0 steps (center of well)
pW - Dispense	100	10	230 steps (10.52 mm above carrier)	50 steps (2.29 mm right of center)	0 steps (center of well)



**Figure 2.** Comparison of T cell counts before and after media exchange. A dilution series of T cells demonstrates excellent retention of T cells after 5x media exchange protocol with the Agilent BioTek automated media exchange (AMX) module. Linear analysis of average T cell counts for six replicate wells before (red) and after (blue) media exchange.

#### 4) Dispense near the well edge.

The position of the dispense and aspiration pins within the wells are customizable using LHC software. Dispensing media near the well edge allows a liquid bridge to form against the sidewall thus avoiding droplets hitting the well media surface that may introduce mixing forces.

#### 5) Minimize the distance between the dispense needle and the final liquid surface.

Dispense pin height is customizable in LHC software. Dispensing from far above the surface interface, however, encourages droplet formation and increases mixing forces. The dispense height was optimized to just above the final well volume of 200  $\mu$ L for this 96-well vessel.

### 3D Spheroid media exchange in 96-well, round-bottom ultra-low attachment microplate

Visualization of this process is depicted in a short video.<sup>1</sup>

#### 1) Define the aspiration height for the vessel.

Careful empirical experimentation must be performed when working with suspension or nonadherent cells, such as during the formation of spheroids in ULA microplates. The plate dimensions are required to determine a starting point for both aspiration and dispense heights, as well as the requirement for substantially more residual volume to be left in the well in comparison to adherent cell assays. The composition of the spheroids, and subsequent level of cellular cohesion between cells in the spheroid, will determine the residual volume requirement. A 50% residual volume is a good starting point in a standard 96-well microplate format. For tightly formed, structurally stable spheroids in a round-bottom plate, up to ~75% of media can be exchanged per cycle. Parameters used for 50% media exchange of 200  $\mu$ L volume in a cell-repellent (ULA), round-bottom Greiner CELLSTAR 96-well microplate (part number 650979) are shown in Table 2. These steps were repeated five times for a complete media exchange.

#### 2) Use the *slower carrier mode* feature for washing and imaging of suspension cells.

The use of slower carrier speed reduces the chance of disruption during both media exchange and imaging.

#### 3) Determine other aspiration and dispense parameters.

The aspirate and dispense parameters will need to be optimized for the plate type and biological system. The slowest setting for spheroid media exchange is used. For round-bottom, ULA microplates, it is recommended to aspirate directly above spheroids (center of well) when spheroid positioning needs to remain constant. Media dispense can be performed near the edge of the well, above the final volume, to prevent disruption.

**Table 2.** Media exchange settings for Greiner CELLSTAR 96-well microplate, part number 650979.

Step	Volume ( $\mu$ L)	Flow Rate ( $\mu$ L/sec)	Z Offset	X Offset	Y Offset
pW - Aspirate	110	10	222 steps (9.02 mm above carrier)	0 steps (center of well)	0 steps (center of well)
pW - Dispense	100	10	250 steps (11.43 mm above carrier)	50 steps (2.29 mm right of center)	0 steps (center of well)

### 3D Spheroid with T cells: Directed T cell activation media exchange in 96-well, flat-bottom ULA plate

Visualization of this process is depicted in a short video.<sup>2</sup>

#### 1) Define the aspiration height for the vessel.

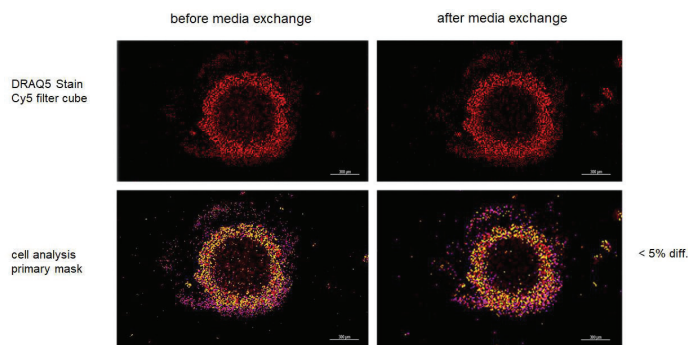
Determine the best protocol parameters for each individual assay, as previously described in this technical overview. Similar to isolated cultures of suspension cells, cocultures of 3D spheroids and T cells require that a substantial residual volume be left during media exchange. A 50% residual volume (~100  $\mu$ L) works well in a standard 96-well, flat-bottom microplate format for cocultured suspension cell media exchange.

#### 2) Use the *slower carrier mode* feature for washing and imaging of suspension cells.

The use of slower carrier speed reduces the chance of disruption during both media exchange and imaging. It is important not to interrupt the interaction between T cells and the spheroid, as this interaction is essential to the directed activation of the T cells against the spheroid cells.

#### 3) Determine other aspiration and dispense parameters.

As with the isolated suspension cell cultures, the slowest aspiration and dispense rates are used for the cocultured suspension cell media exchange. The parameters used during the media exchange are shown in Table 1. Imaging was used to confirm preservation of both the T cell numbers, as well as the T cell/spheroid interaction zone. Spheroids consisted of 75% MDA-MB-231 epithelial adenocarcinoma cells (ATCC, Manassas, VA) and 25% human neonatal dermal fibroblast cells (Angio-Proteomie, Boston, MA) formed with ~2,000 total cells. Spheroids were formed by transfecting each cell line with magnetic particles (NanoShuttle) and using magnetic drive plates (Greiner Bio-One, Monroe, NC) to encourage spheroid formation. T cells were added after spheroid formation. Before media exchange, all cells were labeled with the fluorescent nuclear marker DRAQ5 (Thermo Fisher Scientific, Waltham, MA) for visualization and cell count. Images were taken before and after media exchange for validation of T cell retention following five cycles



**Figure 3.** Whole-well imaging and cell count. A single whole-well image ( $4 \times 5$  montage) of T cells and spheroids stained with DRAQ5 nuclear marker (red, top panels) from a 96-well plate was captured using the Agilent BioTek Cytation 5 cell imaging multimode reader. Images were processed, stitched, and T cell counts determined in Agilent BioTek Gen5 microplate reader and imager software using a primary mask (mask outline shown in yellow, bottom panels) and subpopulation analysis (T cell subpopulation mask outline shown in purple, bottom panels). T cells were imaged and counted before (left column) and then again after media exchange (right column) with the Agilent BioTek automated media exchange (AMX) module. Visual inspection confirms T cell/spheroid interaction region remains undisturbed. Well media was exchanged using five cycles, 50% volume exchange, with parameters shown in Table 1. Scale bar indicates 300  $\mu$ m.

## Best practices and tips

A short list of tips to consider when setting up the AMX module for media exchange of suspension cells.

#### 1) Start with the slowest possible aspiration and dispense settings, and modify as needed.

If total washing time is a factor for the experiments, increase dispense or aspiration speeds incrementally and always verify cell retention.

#### 2) Allow nonadherent cells to settle to the bottom of the well before washing.

Ensure that cells have had adequate opportunity to settle to the bottom of the vessel before attempting a media exchange to avoid directly aspirating cells near the top of the liquid column. Typically, a media exchange will occur days after initial plating of cells, so most nonadherent cells will be well settled to the bottom of the vessel. This study found that T cells settled within as little as a few hours of plating.

### 3) Validate the exchange settings using relevant experimental liquids.

Although tempting to use less expensive and more abundant liquids such as saline or water, liquids demonstrate different liquid handling characteristics due to differences in physical properties such as surface tension. Therefore, the use of the final experimental liquid is recommended for validating settings, typically complete media.

### 4) Leave more media in the well, and run more wash cycles.

As a general principle, to avoid disturbing the biology at the bottom of the well, minimize the amount of media aspirated per wash cycle. Simply repeat the wash cycle more times to achieve a complete media exchange.

## References

1. [https://download.chem.agilent.com/videos/Automated-Gentle-Media-Exchange-for-Spheroid-Assays-using-AMX-for-MultiFlo-FX\\_05\\_optimized.mp4](https://download.chem.agilent.com/videos/Automated-Gentle-Media-Exchange-for-Spheroid-Assays-using-AMX-for-MultiFlo-FX_05_optimized.mp4)
2. <https://download.chem.agilent.com/videos/amx-of-suspension-cells-using-mfx.mp4>