APPLICATION NOTE

Maximizing cell wash performance with AquaMax 2000/4000 Microplate Washers and cell wash heads

Introduction

Many cell-based applications, including calcium flux assays and cell-based ELISAs, require gentle but thorough washing of cells in microplates in order to minimize cell loss and ensure cell viability and optimal assay performance. Critical wash parameters such as aspiration and dispense rates, as well as aspiration height, must be adjustable to allow users to customize wash conditions for the specific cell types used. The angle of fluid delivery to the microplate wells is also important, as dispensing directly onto a cell monolayer tends to disrupt weakly adherent cells, which are then lost during aspiration. Criteria for effective cell washing include:

- High rate of cell retention after washing
- Good wash efficiency (removal of unwanted material)
- Optimal performance of washed cells in assays

Cell wash heads for AquaMax® 2000/4000 Microplate Washers from Molecular Devices use angled dispense pins to dispense wash fluids with minimal cell disturbance. Adjustable dispense rate and aspiration height allow users to minimize disruption of even weakly adherent cells. We demonstrate excellent retention and viability of weakly adherent HEK293 cells on a standard, non-coated tissue culture microplate surface after washing with the AquaMax Microplate Washer and cell wash head. High wash efficiency is achieved with the cell wash head, enabling excellent response of washed CHO-M1 cells in fluorescent calcium flux assays performed on the FlexStation® 3 Multimode Microplate Reader.

Materials

Cell retention tests

- AquaMax 4000 Microplate Washer (Molecular Devices Cat. #0310-5227)
- 96-well cell wash head (Molecular Devices Cat. # 0310-5217T)
- 384-well cell wash head (Molecular Devices Cat. # 0310-5218T)
- HEK293 cells
- Growth medium for HEK293 cells: MEM + 10% FBS + 1% pen/strep
- Black-wall, clear-bottom 96-well (Corning Cat. #3904) and 384-well (Corning Cat. #3912) tissue culture microplates
- Calcein AM (Invitrogen Cat. #C3100MP)
- 1X Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium
- ImageXpress® Velos™ Laser Scanning Cytometer (Molecular Devices)

Wash efficiency

- Solid black 96-well microplates (Greiner Cat. #655076)
- Fluorescein sodium salt (Sigma-Aldrich Cat. #F6377)

Calcium flux assays

- CHO-M1 cells: CHO cells stably transfected with M1 muscarinic receptor
- Growth medium for CHO-M1 cells: Ham's F12 + 10% FBS + pen/strep + 50 µg/mL G418
- Calcium assay wash buffer: HBSS/20 mM HEPES/2.5 mM probenecid (Sigma Cat. #8761)
- Fluo-4 AM (Invitrogen Cat. #F23917)
- Acetylcholine chloride (Sigma Cat. # A6625)
- Carbachol (Sigma Cat. #C4382)
- FlexStation 3 Multimode Microplate Reader (Molecular Devices Cat. #Flex3)

Benefits

- **Angled dispense pins for gentle cell washing**
- **Cell retention >95% after washing**
- **Improved assay performance compared to manual cell washing**

Methods

Cell retention measurements

HEK293 cells were plated at 10,000 cells per well in 96-well or 5000 cells per well in 384-well tissue culture-treated, non-coated microplates. Calcein AM was added to wells for a final concentration of 0.5 µM, and cells were incubated at 37°C for 30 minutes. Excess calcein AM dye was removed with a gentle pre-wash prior to test washes (Table 1). Test washes were performed using the wash programs outlined in Table 2. For comparison, some cells were washed with comparable settings using a 96-well microplate wash head with straight dispense pins. Plates were scanned on an ImageXpress Velos laser scanning cytometer before and after washing to determine the area covered by cells in each well of the microplate. The ImageXpress Velos instrument scans the entire microplate, so complete data for all wells were obtained.

Wash efficiency

To test wash efficiency, 1 µM fluorescein in cell culture medium was added to a solid black 96-well microplate at 100 µL per well. The microplate was read on a SpectraMax® M5 Multimode Microplate Reader to determine initial fluorescence level. The microplate was then washed with PBS using a 96-well cell wash head. A wash consisted of four or six aspirate-dispense cycles, and the same dispense and aspirate settings outlined in Table 2 above. A 3.0-mm aspirate height suitable for weakly adherent cells was chosen. After washing, the microplate was re-read on the SpectraMax M5 Multimode Microplate Reader to determine post-wash fluorescence level.

Post-wash fluorescence values were compared to pre-wash values to determine wash efficiency.

Calcium flux assays

To assess the performance of cells washed using the 96- and 384-well cell wash heads, calcium flux assays were run using a fluorescent calcium dye, Fluo-4. CHO-M1 cells were plated at a density of 50,000 cells per well (96-well) or 12,500 cells per well (384-well) the day prior to assay. Cells were loaded with 2 µM Fluo-4 for one hour. Cells were washed three times with calcium wash buffer using an AquaMax 4000 Microplate Washer with 96- or 384-well cell wash head. Wash programs

Step	Action	Settings, 96 cell wash head	Settings, 384 cell wash head
1	Aspirate	Rate = 5 Descent speed = Fast Dwell time = 2.0 sec Probe height = 5.0 mm	Rate = 5 Descent speed = Fast Dwell time = 0 sec Probe height = 5.0 mm
2	Dispense	Rate = 1 Volume = 200 µL	Rate = 5 Volume = 80 µL
3	Aspirate	(Same as Step 1)	(Same as Step 1)
4	Repeat	1 time from Step 2	1 time from Step 2

Table 1. Pre-wash settings for cell retention tests.

Step	Action	Settings, 96 cell wash head	Settings, 384 cell wash head
1	Aspirate	Rate = 5 Descent speed = Fast Dwell time = 2.0 sec Probe height = 3.0 mm	Rate = 5 Descent speed = Fast Dwell time = 0 sec Probe height = 3.0 mm
2	Dispense	Rate = 1 or 2 Volume = 300 µL	Rate = 5 Volume = 80 µL
3	Aspirate	(Same as Step 1)	(Same as Step 1)
4	Repeat	2 times from Step 2	2 times from Step 2

Table 2. Wash settings for cell retention tests.

Step	Action	Settings, 96 cell wash head	Settings, 384 cell wash head
1	Aspirate	Rate = 5 Descent speed = Fast Dwell time = 2.0 sec Probe height = 1.0, 2.0, or 3.0 mm	Rate = 5 Descent speed = Fast Dwell time = 0 sec Probe height = 2.0 mm
2	Dispense	Rate = 1 or 2 Volume = 300 µL	Rate = 5 Volume = 80 µL
3	Aspirate	(Same as Step 1)	(Same as Step 1)
4	Repeat	1 time from Step 2	1 time from Step 2
5	Dispense	Rate = 1 or 2 Volume = 300	Rate = 5 Volume = 80
6	Aspirate	Rate = 5 Descent speed = Fast Dwell time = 2.0 sec Probe height = 4.5 mm	Rate = 5 Descent speed = Fast Dwell time = 0 sec Probe height = 4.5 mm

Table 3. Wash settings for calcium flux assay*.

*Note: Programming the washer with a final aspirate height of 4.5 mm left a residual volume of 100 µL in each well of a 96-well plate or 25 µL for a 384-well plate.

consisted of a series of programmed aspirate and dispense steps (Table 3). Some 96-well plates of cells were washed manually for comparison. Note: attempts at manually washing in 384-well format proved extremely cumbersome, so that data is not presented.

Calcium flux was measured with a FlexStation 3 Microplate Reader using the Flex (fast kinetic) read type. Two different agonists, acetylcholine and carbachol, were tested. Cells were stimulated with acetylcholine concentrations ranging from 15 pM to 300 nm, or carbachol concentrations ranging from 0.5 nM to 10 µM. Agonist was delivered to assay wells using the FlexStation 3 System's integrated pipettor, and fluorescence reads were taken at 1.6-second (96-well format) or 2.5-second (384-well format) intervals for 90 seconds. For each well's kinetic read, the minimum fluorescence value (RFU) was subtracted from the maximum RFU value to obtain reduced data values for graphing. All data were acquired, analyzed, and graphed using SoftMax® Pro Software.

Results

Cell retention

When HEK293 cells were washed using the AquaMax 4000 microplate washer and 96-well cell wash head, using the wash conditions outlined above, the percentage of cells retained in the microplate wells after washing exceeded 95%. This was in stark contrast to cells washed using the same instrument settings and a 96-well microplate wash head with straight dispense pins, which had an average retention rate of about 44% (Figure 1). With the 384-well cell wash head, cell retention rates exceeding 95% were likewise achieved using the wash conditions described (Figure 2).

Other instrument settings that had a critical effect on washed cell retention were aspirate height and dispense rate. Aspirate height, the height of the aspirate pins above the bottom of the microplate wells during liquid aspiration, had a significant impact on cell retention. A minimal aspirate height of 3.0 mm yielded higher cell retention rates than lower aspirate heights and is thus recommended for loosely adherent cells like HEK293 (Table 4).

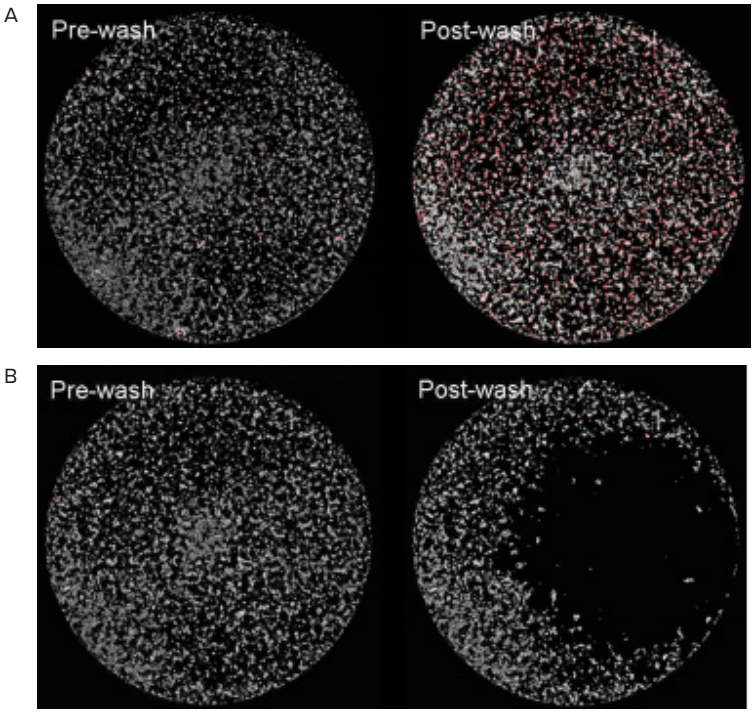

Figure 1. 96 cell wash head retention of washed HEK293 cells. Images of HEK293 cells washed with **(A)** 96-well cell wash head showing cell retention >95% and **(B)** 96-well microplate wash head showing cell retention at 44%. In each case, the images on left and right are of the very same well before and after washing. (Red-colored areas on the images indicate fluorescent signal levels at 50% of the maximum.)

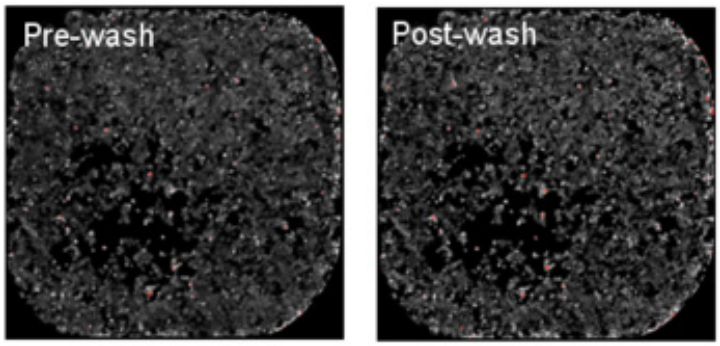

Figure 2. 384 cell wash head retention of washed HEK293 cells. Images of HEK293 cells washed with 384-well cell wash head showing cell retention >95%. Images on left and right are of the very same well before and after washing. (Red-colored areas on the images indicate fluorescent signal levels at 50% of the maximum.)

Replicate	Aspirate height 2 mm	Aspirate height 2.5 mm	Aspirate height 3.0 mm	
			Dispense rate 1	Dispense rate 2
Plate 1	87.6	94.5	96.2	98.0
Plate 2	80.6	98.5	98.6	100
Plate 3	88.7	96.2	95.7	97.1

Table 4. Percent cell retention values for HEK293 cells with different aspirate heights and dispense rates using the 96-well cell wash head.

Previous studies with the 96-well cell wash head determined that dispense rates of 1 or 2 promoted the highest cell retention rates (data not shown). Data from cells washed using a dispense rate of 1 or 2 shows that there was a minimal difference in results between the two rates (Table 4).

For the 384-well cell wash head, dispense and aspirate rates must be set at maximal or near-maximal values to ensure even dispensing and aspiration across the plate. With this wash head, it is therefore never recommended to use slow or intermediate dispense or aspiration rates. The angle of the pins ensures that even with fast dispense rates, cell layers are not disturbed even after multiple aspirate-dispense cycles. Setting the aspirate dwell time to zero seconds was also essential to optimal cell retention. Even a small increase in dwell time, from 0 seconds to 1 second, led to significant aspiration of cells from the wells (Table 5).

Replicate	Dwell time (sec)	% Cell retention
Plate 1	1.0	89.5
Plate 2	0	95.7
Plate 3	0	95.7

Table 5. Percent cell retention values for HEK293 cells with different aspirate dwell times using the 384-well cell wash head.

Number of aspirate-dispense cycles	Average fluorescence (RFU) before wash	Average RFU after wash	% Fluorescence removed by washing
4	1958.5	6.9	99.6
4	2108.2	6.5	99.7
6	2214.9	1.9	99.9
6	2028.3	2.2	99.9

Table 6. Wash efficiency values for 96-well cell wash head*.

*Note: Fluorescence was detected using a SpectraMax M5 multimode plate reader. Settings for aspirate and dispense steps were the same as those outlined in Table 2.

Wash efficiency

Washing microplates containing 100 µL of 1 µM fluorescein per well through a series of four or six aspirate-dispense cycles with PBS removed greater than 99% of fluorescence from the wells (Table 6). The average RFU detected after washing was reduced about three-fold when the number of aspirate-dispense cycles was increased from four to six. Average background for wells containing PBS only was about 0.3 RFU.

Calcium flux assays

To assess the performance of cells washed using the 96-well and 384-well cell wash heads, calcium flux assays were run using Fluo-4 fluorescent calcium dye. With Fluo-4, excess dye must be washed off prior to running the assay in order to minimize background fluorescence.

CHO-M1 cells washed with the AquaMax 4000 Microplate Washer and 96-well or 384-well cell wash head yielded excellent assay results for the agonists tested, with wide dynamic range and good Z' factors at the EC_{80} values for the assays (Figures 3-5). Compared to cells washed manually with a multi-channel pipettor, cells washed with the AquaMax plate washer showed larger calcium assay windows (Figures 3 and 4).

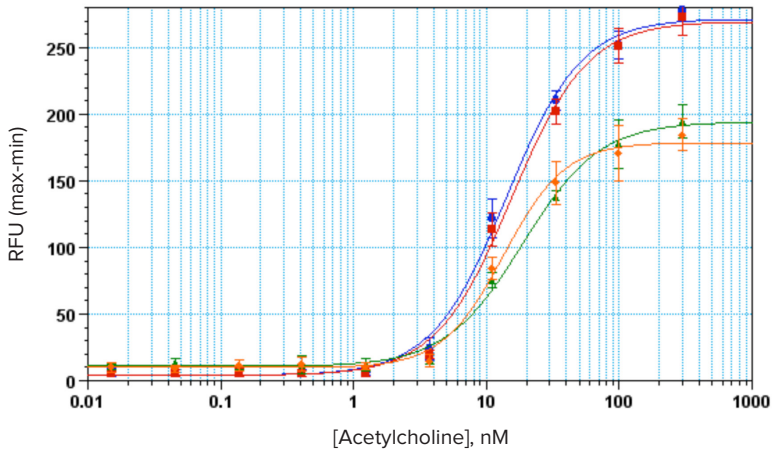

Figure 3. Acetylcholine concentration response curves for CHO-M1 cells in 96-well assay format. Blue and red plots represent two separate assays using cells washed using the AquaMax microplate washer with cell wash head. Green and orange plots represent two separate assays using cells washed manually using a multichannel pipettor. Z' factors at EC_{80} for AquaMax-washed assays were 0.87 and 0.82, and EC_{50} values were 14.2 and 15.4 nM. For manually washed assays Z' factors at EC_{80} were 0.83 and 0.59, and EC_{50} values were 18.7 and 13.5 nM.

Conclusion

The data presented here demonstrate the ability of the new 96-well and 384-well cell wash heads for the AquaMax 2000/4000 Microplate Washers to wash cells efficiently while minimizing cell loss and enabling optimal assay performance. Greater than 99% removal of fluorescent material from wells was observed even when using wash conditions suitable for weakly adherent cells (data shown for 96-well cell wash head). HEK293 cells plated on regular tissue culture-treated microplates were retained at greater than 95% when wash conditions were optimized for 96- and 384-well wash heads. Finally, Fluo-4 calcium assays performed on cells washed with the cell wash heads yielded Z' factors comparable to or better than those obtained with manual cell washing, with similar EC_{50} values regardless of wash method.

The angled dispense pins of the cell wash heads deliver wash solutions to the sides of microplate wells, avoiding cell blow-off that can decrease assay signal and well-to-well variability. Adjustable dispense rate, aspirate rate, and aspirate height settings on the AquaMax 2000/4000 Microplate Washer let users choose the best wash settings for even problematic cell types with weak adherence. A 96-well or 384-well cell wash head can be added to any existing AquaMax 2000 or 4000 Microplate Washer and installed in seconds with the flip of a lever.

Benefits of the AquaMax 2000/4000 Microplate Washers

- Wash heads are fully interchangeable and can be installed in seconds without tools, creating a modular system with a wide range of plate and cell washing applications.
- Simultaneous well washing without plate indexing or quadrant processing provides faster wash times
- Microplate washers are completely programmable via touch screen interface, with no external computer required.
- No external pump means bench space is saved.
- Comprehensive, automated cleaning utilities allow easy maintenance with less down time.

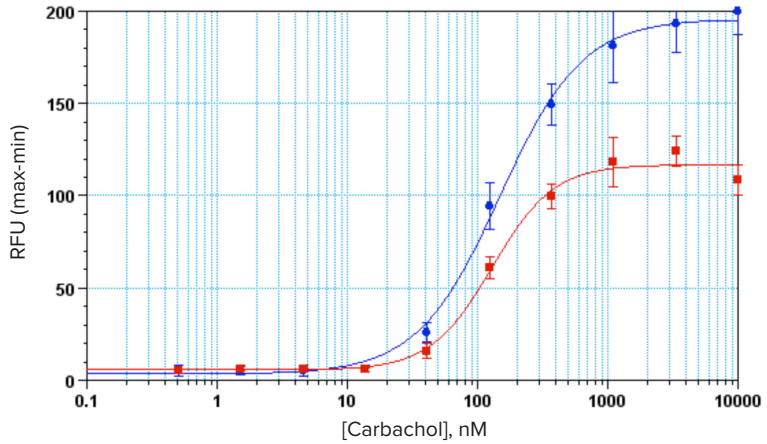

Figure 4. Carbachol concentration response curves for CHO-M1 cells in 96-well assay format. The blue plot represents cells washed with the AquaMax microplate washer and cell wash head; the red plot is for manually washed cells. Z' factor at EC_{80} for an AquaMax-washed assay was 0.73, and EC_{50} value was 148 nM. For the manually washed assay Z' factor at EC_{80} was 0.75, and EC_{50} value was 129 nM.

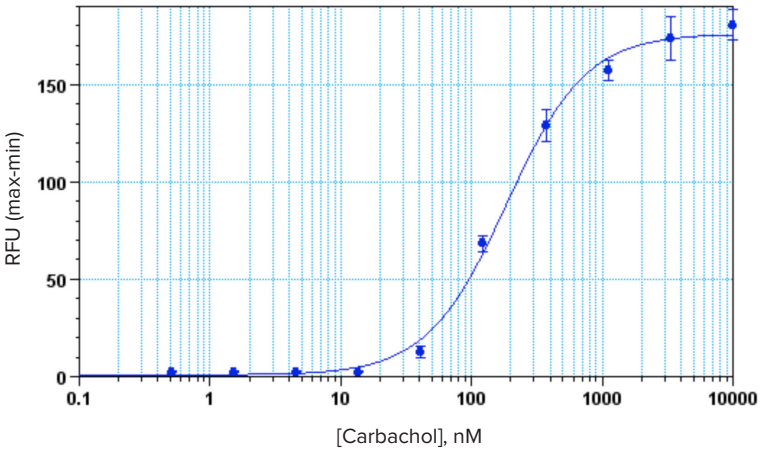

Figure 5. Carbachol concentration response curve for CHO-M1 cells in 384-well assay format. The Z' factor at EC_{80} was 0.79, with an EC_{50} value of 185 nM.

- Robotics-friendly design allows integration with all major automation systems, as well as Molecular Devices' StakMax® Microplate Stacker.

Additional solutions

AquaMax Sterilant Kit

The AquaMax Sterilant Kit (Molecular Devices Cat. #R8156) provides users with a complete cleaning solution for the AquaMax Microplate Washers to ensure trouble-free operation.

StakMax Microplate Stacker

The StakMax Microplate Stacker is an easy-to-set-up, powerful, and reliable microplate stacking solution for Molecular Devices microplate readers and washers. The system provides options for 20-, 40-, and, 50-plate magazines, as well as barcode reading.

Product videos

To view AquaMax Microplate Washer videos and more, visit us at www.youtube.com/MolecularDevicesInc.

Contact Us

Phone: +1-800-635-5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

6/16 2033A
Printed in USA

MOLECULAR DEVICES