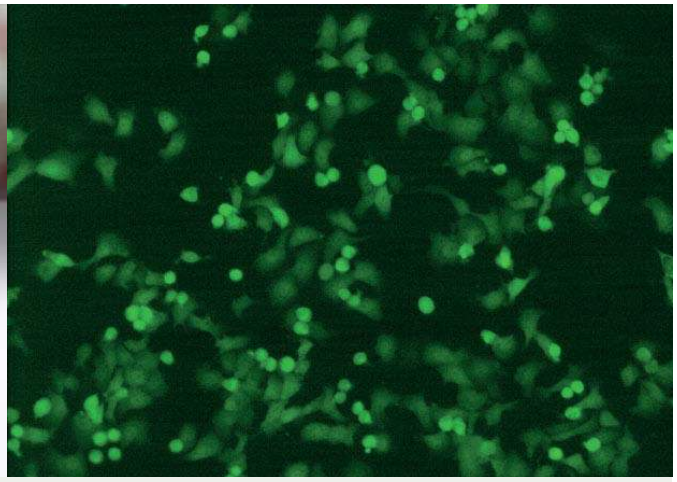

Extra gentle washing of weakly adherent cells

using Tecan's HydroSpeed™ plate washer with Cell Protection™ wash settings

Introduction

This application note describes the outcome of a successful evaluation study of Tecan's new HydroSpeed plate washer, using Cell Protection wash settings for extra gentle washing of adherent and weakly adherent cells in 96- and 384-well plate formats.

Tecan's new HydroSpeed plate washer offers advanced features for a range of applications including washing of cell-based assays and ELISAs, magnetic bead separation and filter washing via vacuum filtration.

The HydroSpeed provides individual control of critical wash parameters – such as the aspiration power, speed settings for gentle dispensing and wash head positioning – enabling fine-tuning of the wash protocol to suit the characteristics of a broad range of different cell types.

For optimized wash results, especially with weakly adherent cell types, Cell Protection wash settings offer improved cell recovery rates; combining very gentle drop-wise dispensing with fine-tuning of the dispense position relative to the rising liquid level using the Move function.

Furthermore, the HydroSpeed provides full control of the aspiration rate via tuneable vacuum settings, allowing extra gentle aspiration to remove assay reagents from the wells with little or no influence on cell viability.

To determine the wash efficiency and viability of different cell lines, experiments with adherent cells (A431) and very weakly adherent cells (P815) were performed in this study. In addition, the cell layers were monitored before and after washing to visually confirm the gentle wash procedure.

Materials and methods

Instruments

- HydroSpeed plate washer equipped with the 96 indexing wash head (96i), suitable for gentle cell washing, magnetic bead washing and ELISA washing in 96- and 384-well plates (Figure 1). High throughput wash heads for 96- and 384-well plates (96HT & 384HT) were also used for this study.
- Infinite® M200 PRO multimode reader equipped with Quad4 Monochromators™ technology

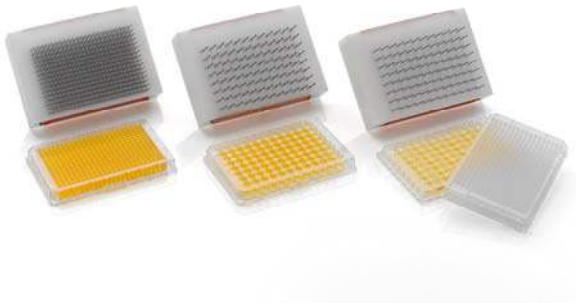

Figure1 Wash heads available for Tecan's HydroSpeed plate washer (from left to right): high speed 384-channel wash head (384HT), dedicated 96-channel wash head (96HT) and universal 96-indexing wash head (96i)

Microplates for cell culture

- 96-well, transparent, flat bottom plate (Greiner, Bio-One)
- 384-well, transparent, flat bottom plate (Greiner, Bio-One)

Reagents

- Dulbecco's modified Eagle's medium (DMEM, PAA Laboratories)
- PBS wash buffer
- MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide)
- Trypan blue (TB)

Cell culture and assay procedures

Human epidermoid carcinoma (A431) and mouse mastocytoma (P815) cells were grown in DMEM supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM Na-pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5 % (v/v) fetal calf serum (FCS) (all from PAA Laboratories, Austria) in a humid atmosphere at 37 ^{o}C and 5% CO_2.

Very weakly adherent P815 cells were selected by a series of washing procedures over a period of two weeks, where the supernatant (containing the suspended cells) was replaced by fresh DMEM supplemented as listed above.

For all assays, either 2 x 10^4 cells of each cell line in 100 µl of supplemented DMEM, or 7 x 10^3 cells in 50 µl of supplemented DMEM were seeded into 96-well and 384-well microplates respectively.

The wash efficiency of the HydroSpeed plate washer was determined by monitoring the removal of a colored solution. For this study, either 20 µl of 0.05% TB for a 96-well plate, or 10 µl of 0.05% TP for a 384-well plate were added to each well, and the absorbance at 565 nm was measured with an Infinite M200 PRO multimode reader before washing. After washing, the remaining amount of TB was determined using an absorbance measurement at 565 nm. Please note that not all P815 cells seeded into microplates become loosely adhered to the bottom of the wells. P815 cells which remain floating in the wells are typically lost during manual or automated washing, and this has been incorporated into the calculation of the cell recovery rate.

To determine the cell viability, the MTT assay was performed before and after washing. This assay works on the principle that a metabolically active, viable cell reduces the soluble yellow tetrazolium salt (MTT) to an insoluble dark formazan by the mitochondrial dehydrogenase activity. The resulting formazan can be measured by direct absorbance and used to determine the number of viable cells (1).

Cell washing using a dilution wash protocol

Dilution wash protocols consist of a sequence of aspiration and dispense steps, typically starting with an aspiration step. For optimized cell recovery, the HydroSpeed plate washer offers extra gentle settings for aspiration and dispensing steps, including the option to individually define the aspiration height for each aspiration step within a wash protocol, allowing the user to leave a defined residual volume per well.

Wash programs
The HydroSpeed wash programs used during this evaluation study were optimized to achieve both good cell viability and high wash efficiency (for details see Table 1 and Table 2).

All wash results obtained with the HydroSpeed plate washer were compared with the corresponding manual wash procedure, which used a handheld dispenser for buffer dispensing and a glass tube with an angled tip connected to a vacuum pump for aspiration. The aspiration position used for manual washing was set at the bottom of the wells, with no option for controlling the residual volume per well. The manual wash protocol consisted of a single wash cycle using either 100 µl or 50 µl of buffer for 96-well and 384-well plates respectively.

HydroSpeed wash protocol for 96-well plate format
Cells were seeded into uncoated 96-well tissue culture microplates and incubated for 16 hours at 37 °C, using 100 µl of DMEM growth medium per well.

To increase the efficiency of the first program cycle, the wash protocol started with an initial dispense step (with activated Move function) to top up the partly filled wells with 150 µl of PBS buffer prior to aspiration, as indicated in Table 1.
Extra gentle aspiration settings were used to optimize cell recovery, including an aspiration rate setting of 1 and a single point of aspiration in the centre of each well.

Wash program	Parameters
CYCLE 1 (optional)	**# of cycles: 1**
Dispense	Custom z-pos: 8 mm with Move funct.
	Volume: 150 µl; Disp. rate: 2 (90 µl/s)
CYCLE 2	**# of cycles: 2**
Aspirate	Mode: normal Custom z-pos: 6.8 mm
	Aspiration time: 1 s
	Head speed: 2 mm/s
Dispense	Custom z-pos: 7 mm with Move funct.
	Volume: 300 µl; Disp. rate: 2 (90 µl/s)
CYCLE 3	**# of cycles: 1**
Aspirate	Mode: normal Custom z-pos: 8 mm
	Aspiration time: 1 s
	Head speed: 1 mm/s

Table 1 Dilution wash program for 96-well plate format using the HydroSpeed with 96i or 96HT wash heads

To minimize cell detachment using the 96i or 96HT wash heads, a dispense rate setting of 1 or 2 is recommended (corresponding to a dispense speed of approximately 70 µl/s to 90 µl/s).

HydroSpeed wash protocol for 384-well plate format
Cells were seeded into uncoated 384-well tissue culture microplates and incubated for 16 hours at 37 °C, using 50 µl of DMEM growth medium per well.

To increase the efficiency of the first program cycle, the wash protocol started with an initial dispense step (with activated Move function) to top up the partly filled wells with 50µl of PBS buffer prior to aspiration, as shown in Table 2.

For optimum wash results in 384-well plate formats, an aspiration rate setting of 1 and a single point aspiration per well were used.

Wash program	Parameters
CYCLE 1 (optional)	**# of cycles: 1**
Dispense	Custom z-pos: 7 mm with Move funct.
	Volume: 50µl; Disp. Rate: 2 (90 µl/s)
CYCLE 2	**# of cycles: 3**
Aspirate	Mode: normal Custom z-pos: 8.2 mm
	Aspiration time: 1 s
	Head speed: 1mm/s
Dispense	Custom z-pos: 8.5 mm with Move funct.
	Volume: 80 µl; Disp. rate: 2 (90 µl/s)
CYCLE 3	**# of cycles: 1**
Aspirate	Mode: normal Custom z-pos: 8 mm
	Aspiration time: 1 s
	Head speed: 1mm/s

Table 2 Dilution wash program for the HydroSpeed equipped with the 96i wash head for processing of 384-well plates

For gentle cell washing in 384-well plates, dispense rate settings of 2 (90 µl/s) and 3 are recommended for the 96i and 384HT wash heads respectively.

Recommended wash protocols for high throughput, parallel washing of P815 and A431 cell lines using the 384HT wash head can be obtained from Tecan.

Results

Microscope images of both adherent A431 (Figure 1) and very weakly adherent P815 (Figure 2) cells were taken before and after washing, to evaluate the wash performance of the HydroSpeed plate washer.

As these images show, no holes were visible in the cell layers after washing with the HydroSpeed, indicating a gentle wash performance with almost no cell detachment.

These results confirm that the HydroSpeed plate washer is well suited to gentle processing of both adherent and weakly adherent cell lines, ensuring cell layers remain intact after washing.

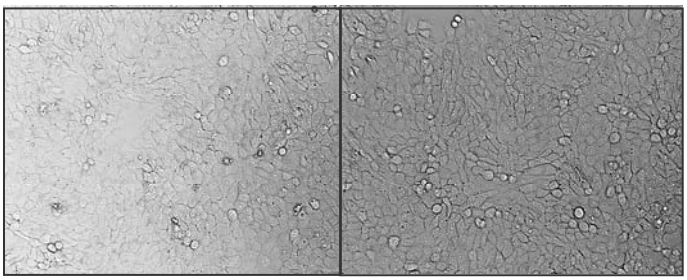

Figure 1 Adherent A431 cells before (left) and after washing with PBS (right)

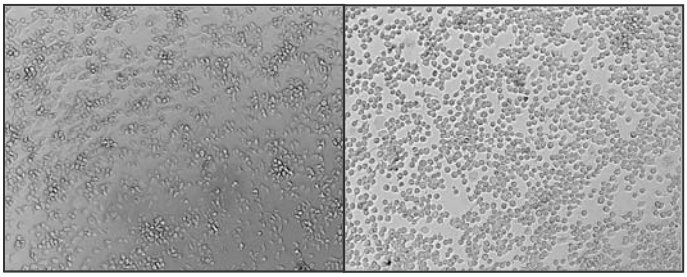

Figure 2 Very weakly adherent P815 cells before (left) and after washing with PBS (right)

Additionally, the cell viability and the wash efficiency using the HydroSpeed plate washer were compared to manual washing. Comparative results for the cell viability (MTT) and wash efficiency (TB) tests are shown in Table 3.

96-well plate washing

As shown in Table 3, the extra gentle Cell Protection wash settings of the HydroSpeed plate washer provide excellent washing performance compared to manual washing in 96-well plates.

Wash procedure	HydroSpeed plate washer with 96i head	Manual washing
A431 cells:		
Cell viability [%]	80.3	18.7
Wash efficiency [%]	95.6	98.9
P815 cells:		
Cell viability [%]	73.0	10.5
Wash efficiency [%]	95.4	97.3

Table 3 Comparative results for cell viability and wash efficiency of adherent (A431) and very weakly adherent (P815) cells using the HydroSpeed plate washer and manual washing

The recovery rates of viable cells after gentle and efficient washing with the HydroSpeed plate washer were nearly seven times higher for very weakly adherent P815 cells, and more than four times higher for adherent A431 cells, than after manual washing.

Results obtained with the 96HT wash head (not shown here) were comparable to the data obtained for the 96i wash head.

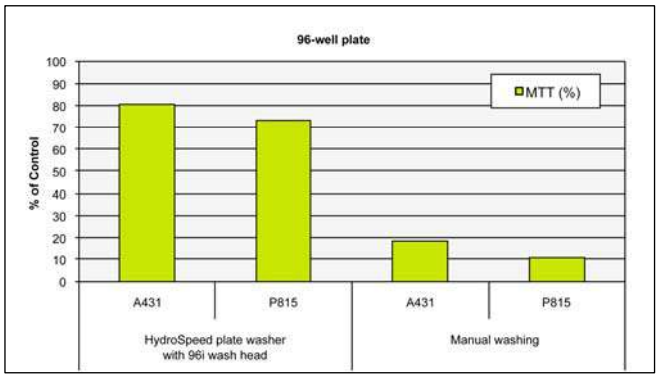

Figure 3 Comparison of cell viability (MTT) results relative to unwashed controls, obtained with the HydroSpeed plate washer (using program listed in Table 1) and manual washing in 96-well format.

384-well plate washing

As shown in Table 4, the extra gentle Cell Protection wash settings of the HydroSpeed plate washer provide excellent cell washing performance compared to manual washing techniques in 384-well plates.

Wash procedure	HydroSpeed plate washer with 96i head	Manual washing
A431 cells		
Cell viability [%]	60.8	32.0
Wash efficiency [%]	96.8	98.1
P815 cells		
Cell viability [%]	58.0	3.0
Wash Efficiency [%]	93.1	98.5

Table 4 Comparison of cell viability and wash efficiency results for adherent (A431) cells and very weakly adherent (P815) cells using the HydroSpeed plate washer and manual washing

The cell viability results after gentle and efficient washing with the HydroSpeed plate washer were more than fifteen times better for very weakly adherent P815 cells, and nearly twice as high for adherent A431 cells, than after manual washing.

Results obtained with the high throughput 384HT wash head (not shown here) showed viable cell recovery rates of over 77 % for both A431 and P815 cells.

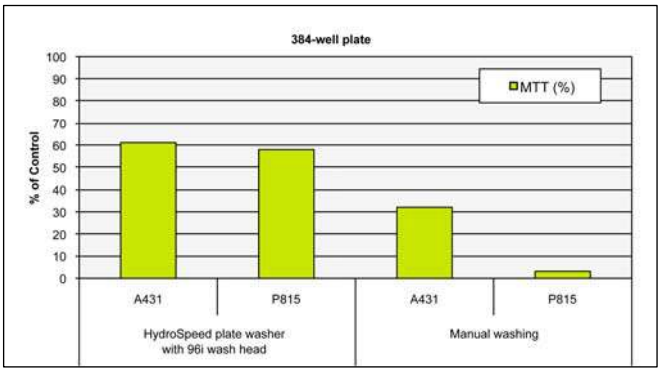

Figure 4 Comparison of cell viability (MTT) results relative to unwashed controls, obtained with the HydroSpeed plate washer (using program listed in Table 2) and manual washing in 384-well format.

Discussion

Gentle and efficient washing of weakly adherent cells is influenced by several critical wash parameters, such as the aspiration power, the aspiration height and the dispense rate.

The HydroSpeed plate washer provides advanced cell wash settings allowing the user to individually customize the aspiration height for each aspiration step. Together with the adjustable aspiration rate (aspiration power) this makes it possible to fine-tune the wash parameters of the HydroSpeed to the properties of the cell lines under evaluation.

In combination with the extra gentle drop-wise dispense speed and the Move function, the HydroSpeed plate washer provides full control of the critical wash parameters for optimized results, especially with loosely adherent cell lines.

According to the data obtained during this evaluation study, both the HydroSpeed dilution wash protocols and wash programs running in the ‘overflow’ position (results not shown) are highly effective for gentle cell washing in 96-well and 384-well plate formats.

The HydroSpeed plate washer provides excellent wash performance with both very weakly adherent P815 cells and adherent A431 cells, achieving good recovery of viable cells compared with manual washing.

The HydroSpeed plate washer equipped with the 96i wash head offers a very versatile solution that combines high flexibility with good wash performance for gentle washing of loosely adherent cells in 96-well and 384-well plates.

The optional 384HT wash head is recommended for high throughput applications, allowing fast, parallel washing of 384-well plates with no loss of performance for weakly adherent cells.

Conclusion

Tecan's new HydroSpeed plate washer has demonstrated excellent performance for automated washing of adherent and weakly adherent cells. It combines gentle, efficient washing with high cell retention rates and good cell viability.

The HydroSpeed plate washer provides advanced control of critical wash parameters such as aspiration rate, dispense speed and wash head position, allowing straightforward fine-tuning of wash conditions for a wide range of adherent and weakly adherent cell lines.

List of abbreviations

A431	human squamous epithelial carcinoma cells
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MTT	3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide
P815	mouse mastocytoma cells
PBS	phosphate buffered saline
TB	trypan blue

Literature

(1) Mosmann T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays; Journal of Immunological Methods, 1983 Dec. 16; 65(1-2):55-63.

Acknowledgements

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