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Practical Considerations of Liquid Handling Devices in Drug Discovery

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1. Introduction

Automated liquid handling has become an indispensable tool in drug discovery, particularly in screening campaigns ranging millions of compounds. Intense innovations of these devices go hand in hand with the progression towards assay miniaturization, accelerating dramatically the discovery of drug candidates and chemical probes for querying biological systems. The advancement in this technology is driven in large part by much impetus in cost reduction and efficiency. In addition to increased throughput, streamlining screening operations using automated fluid devices ensures consistency and reliability while avoiding human error.

In this chapter, we provide a general overview of existing liquid handlers, with emphasis on their strengths and limitations. Notably, we discuss practical considerations in the implementation of these devices, methods to discern performance quality and potential sources of error.

2. Types of liquid handling devices

A whole array of liquid handlers has been developed for every aspect of drug discovery. These instruments encompass different technologies for distinct purposes. In terms of application, they are broadly classified as bulk liquid dispensers, transfer devices and plate washers (Rudnicki and Johnston 2009).

Based on the way the reagent is being transferred, these instruments can follow two dispensing modes: contact or non-contact (Kong et al. 2012). Contact-based devices allow the fluid to be transferred to touch the surface of the destination container or solution, offering a simple and dependable alternative to sub-microliter fluid handling. Non-contact devices utilize additional force other than gravity to eject liquids, as minute volumes cannot be dispensed efficiently with gravity alone (Kong et al. 2012). The process is faster than using permanent tips or pins (Fig.1), because there is no washing step between delivery, while reducing cross-contamination and evaporation (Dunn and Feygin 2000).

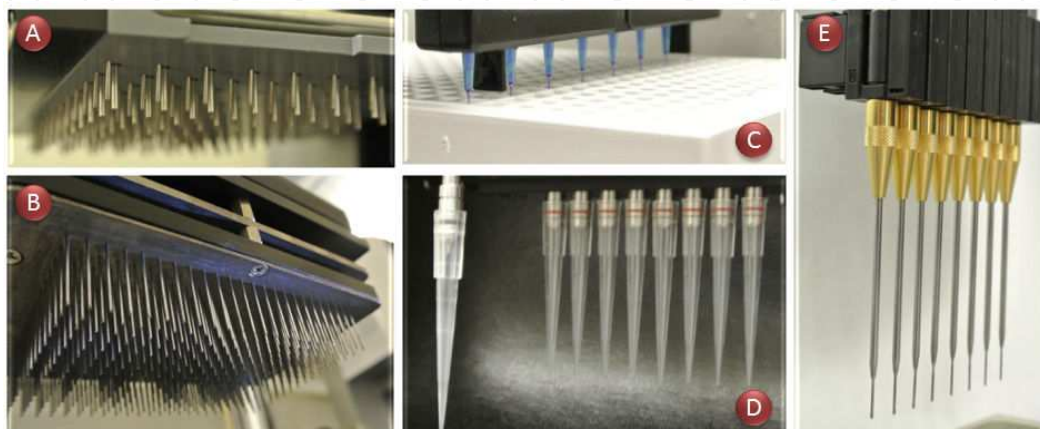


Figure 1. Various types of liquid handling tips, pins and heads from A) washer B) pintool C) peristaltic pump-based bulk dispenser D) liquid handler with single and 8-channel pipettors E) pipettor with 8-independent channels.

2.1. Peristaltic-based devices

The peristaltic pump is used for bulk reagent dispensing in conjunction with a nozzle head (Fig.1C) and a flexible tubing cartridge. The tubings stretch around a set of rollers connected to a motor. With the rotating motion of the motor, the rollers compress the tubings creating a continuous fluid motion due to positive displacement.

Typically, this type of dispenser is capable of handling volumes as low as 5 μL , offering a fast dispensing option for 96-/384-/1536-well plate formats. The disposable tubing cartridge is pre-sterilized, and the entire liquid path can be autoclaved. Additionally, these devices are normally equipped with programming capabilities that allow discrete column-wise dispensing, variable rolling speed settings and adjustable dispensing volume. The pump can roll both forward and backwards to execute priming and emptying functions, respectively. A major limitation is the lack of capabilities to dispense into individual wells.

2.2. Fixed-tip transfer devices

Fluid handlers that utilize fixed-tips (Fig.1E) are usually efficient at transferring relatively small volumes (100 μL or above) and have been largely used for compound pipetting (“cherry picking”) and serial dilutions. They incorporate 2-/ 4-/ 8-channel expandable liq-

liquid handling arms in addition to 96- and 384-channel heads. This type of liquid handling device functions based on air displacement mechanism. The dilutor or syringe plunger pulls system liquid from the pipette tubing to aspirate the sample, with an air gap separating both fluids. The plunger speed, syringe size and resolution are factors that affect pipetting flow rate.

2.3. Changeable-tip transfer devices

The use of disposable tips (Fig.1D) is a simple alternative to avoid washing steps required for fixed-tip based systems, while eliminating completely the risk of cross-contamination. These instruments employ a conventional air displacement mechanism. A wide array of commercially-available tip sizes, materials and molding qualities offers the scientist great flexibility. There are even specialized tips with nanoliter-scale transfer capabilities that can be used in any conventional pipettor (Murthy et al. 2011; Ramírez et al. 2008).

2.4. Pintool transfer devices

Pintool is a contact-based dispensing method widely used for handling volumes at the nanoliter scale (Cleveland and Koutz 2005). It consists of a set of stainless steel pins (Fig. 1B) carefully crafted for consistent dimensions. The bottom end of the pins can be solid, grooved or slotted, with the option of having a hydrophobic coating to prevent non-specific binding (Dunn and Feygin 2000; Rudnicki and Johnston 2009). Solutions are transferred through a combination of capillary action and surface tension, with the volume being highly dependent on the contact surfaces and solution properties (Dunn and Feygin 2000). The pin array is normally assembled in a floating pin cassette to ensure soaking of all the pins amid uneven surfaces, which also minimizes pin damage. After liquid transfer, the pins have to cycle through washing steps to prevent cross-contamination.

2.5. Piezoelectric devices

The piezoelectric dispenser is a non-contact technology, where solutions are delivered as multiple tiny drops of defined size (Niles and Coassin 2005). This technology has been utilized in contemporary inject printers and refined to be implemented in the biological sciences. Various biochemical solutions (DNA, RNA, proteins) and bacterial suspensions have been tested with no negative effects (Schober et al. 1993). The system is composed of a capillary tube made of quartz or steel, with one end connected to the reagent reservoir and the other end ending in an orifice from which droplets are ejected (Niles and Coassin 2005). A piezoelectric crystal collar is bound to the capillary, which is filled with solution. Upon voltage application, the piezoelectric element contracts causing pressure on the capillary to generate fine drops. The ejection is at high acceleration with minimal wetting of the nozzle (Schober et al. 1993). Several thousand drops can be dispensed per second, with attainable drop sizes spanning the picoliter and nanoliter range (Schober et al. 1993). Droplet volume depends on several factors, including bore diameter, solution viscosity and the voltage pulse amplitude and frequency (James and Papen 1998; Kong et al. 2012).

2.6. Solenoid-based devices

Solenoid-based devices are non-contact dispensers that use a positive displacement mechanism (Bateman et al. 1999). The flow of pressurized liquid is occluded by a solenoid valve, which is actuated by electric current to allow for liquid to pass through the valve. The dispensed volume is regulated by the fluid pressure, duration of the valve in the open position, solution properties and orifice diameter (Bateman et al. 1999; Niles and Coassin 2005). Depending on the time the valve stays in the open position, the device can eject droplets or a continuous stream (Niles and Coassin 2005).

2.7. Acoustic devices

Acoustic droplet ejection (ADE) is a recent touch-less technology that surges in popularity in recent years. It adopts acoustic energy to propel droplets from various types of solutions with good precision (Ellson et al. 2003; Harris et al. 2008; Rudnicki and Johnston 2009; Shieh et al. 2006). The source plate remains stationary as the transducer and destination plate shuffle to allow for solution transfer from any well in the source plate to any well in the destination plate, the latter one lying in an inverted position (Olechno et al. 2006). This system does not require any additional consumable other than microplates (Olechno et al. 2006), and it speeds up the process by avoiding washing steps and having the capability to prepare assay-ready plates (Turmel et al. 2010)

2.8. Microplate washers

Microplate washers are laboratory instruments designed to automate and expedite assay applications, where a washing step is essential. They play an important role in areas such as high-content screening and enzyme-linked immunosorbent assays (ELISA). In 1990, Stobbs developed the first multiple plate washer using readily available materials as a low cost alternative to the commercially available plate washers of the era (Stobbs 1990). Over the years, fully programmable plate washers have been developed with numerous features. The development of automated plate washers has decreased the time required for laborious washing steps involved in many screening assays and improved reproducibility through standardized plate handling across multiple wash cycles (defined as a single dispense and aspirate step per cycle).

The two most critical components of a plate washer are a plate carrier and a manifold containing a number of fixed stainless steel needle probes for solution dispensing (Fig.1A). This manifold (or a separate manifold depending on the design) aspirates the liquid from the wells after an optional soaking period, leaving a pre-defined residual volume in the wells. A third component is the vacuum/pump assembly, which supplies the necessary pressure differential to drive efficient aspiration. Sunghou Lee first developed an additional vacuum filtration system integrated with a conventional plate washer to speed up the wash process for applications involving filter plates (Lee 2006). Some plate washers have a built-in magnet or a vacuum filtration module for handling bead-based assays.

Microplate washers can be categorized into two types: strip washers, which wash a single column or row of a plate at a time, and full plate washers (Rudnicki and Johnston 2009). The availability of 8-/12-/16-channel manifolds for strip washers provides both single strip washing and full-plate washing capability in the same device, but at the cost of increased wash time for full plates. On the other hand, full plate washers with either a 96- or 384-channel manifold may be preferred for time-efficient wash operations (from a few seconds to a few minutes), but lack the flexibility of the 8-/12-/16-channel units.

The combination of plate washing and bulk dispensing features within the same device may be favored for a space-efficient solution. They are designed to dispense reliably low volumes and reduce prime volume (Rudnicki and Johnston 2009). A major advantage of the washer-dispenser combination comes into play with assay protocols that require the direct addition of fluid after or between the washing steps, such as cell fixation or microplate surface coating reagents.

3. Considerations for using liquid handling devices

3.1. Determination of quality assessment descriptors

Assessment of instrument performance has become important in order to minimize false-positive and false-negative rates in high-throughput screening (Taylor et al. 2002). One of the most important figures of merit in evaluating the performance of liquid handlers is accuracy, which is commonly reported as %bias (Rose 1999):

$$\%bias = 100 \times \left(\frac{V_M - V_T}{V_T} \right) \quad (1)$$

where V_M is the measured volume and V_T is the theoretical volume (desired). %bias represents the deviation from the desired volume, with a value of 0% indicating no deviation from the true value.

The precision, a measure of reproducibility, is calculated from the mean and standard deviation (SD) of a set of measurements, and it is reported as percent coefficient of variation (%CV) or relative standard deviation (RSD), as shown in Eq. 2. For most cases, it is adequate to have a bias value below 5% and a CV below 10% (Rose 1999).

$$\%CV = 100 \times \frac{SD}{\text{mean}} \quad (2)$$

There have been several approaches for volume verification, which typically consist of gravimetric or photometric methods. Gravimetric measurements utilize the mass and the density (ρ) of the dispensed solution to determine the volume. It has been used extensively to calibrate and verify the accuracy of liquid dispensers (Bergsdorf et al. 2006; Rhode et al. 2004; Taylor et al. 2002). Typically, the solution is dispensed across a pre-weighed microtiter

plate, which is weighed immediately after dispensing to prevent evaporation. %bias can be calculated based on the total weight of the dispensed solution (W_{total}) and the number of dispensed wells (n):

$$\% \text{bias per well (gravimetric)} = 100 \times \frac{\left(\frac{W_{\text{total}}}{n \times q}\right) - V_T}{V_T} \quad (3)$$

Environmental conditions (e.g. temperature and humidity) have major effects on the reliability of gravimetric methods, which facilitates evaporation and water uptake for hygroscopic solvents such as dimethyl sulfoxide (DMSO). These factors of variation can be minimized by placing gasketed lids on the microtiter plates immediately following dispense (Taylor et al. 2002).

Absorbance and fluorescence are the most common photometric methods utilized to test the accuracy and precision of the transferred volumes of a liquid handling device. In a study comparing the performance of the two methods on determining the precision in 96-/384-/1536-well plates, no significant difference was observed between the 96- and 384-well plates (Petersen and Nguyen 2005). However, to achieve similar results for both fluorescence and absorbance measurements in the 1536-well plate, a centrifugation step was required because of the irregular meniscus shape enhanced by the small well geometry. In another study performed on liquid handlers with two different mechanisms, absorbance was found to be a more reliable method as long as the pH stability of the dye-buffer solution is maintained (Rhode et al. 2004).

Fluorescence signal is also known to be susceptible to photobleaching, which can be prevented by shorter excitation times, suitable buffer solutions and adequate concentration of fluorophore (Diaspro et al. 2006; Harris and Mutz 2006). To overcome the problems encountered due to signal quenching in DMSO, sulforhodamine 101 was presented as an alternative fluorescence dye (Walling 2011). Fluorescein was found to be a suitable probe to use in liquid handling performance quantification as long as the DMSO concentration in the buffer solution does not exceed 1% and the stock solutions are stored in 70-100% DMSO in a dark environment (Harris and Mutz 2006). While photobleaching is not an issue in absorbance, the method is limited by high background levels and lower sensitivity compared to fluorescence (Bradshaw et al. 2007). Based on the physical characteristics of a transferred sample and the material of the consumables, unforeseen interactions may be observed influencing the assay results. Especially, DMSO-containing samples are highly affected by the hydroscopic properties of the solvent, which inflates sample volume (Berg et al. 2001).

3.2. Considerations for using bulk reagent dispensers: Peristaltic-based devices

A single screening experiment can be costly, requiring valuable compounds and biological reagents. Routine evaluation of liquid handlers, in particularly prior to each run, is a necessary mean for preventing disastrous outcomes. Simple procedures can be integrated to identify problems in a relatively short period of time, which in many instances, can be easily corrected. Routine analysis should be performed with the actual reagents, because there are

several factors that affect the dispensed volumes, including viscosity, density, and temperature (McGown and Hafeman 1998). General considerations to prevent undesirable dispensing performance and common sources of variations include:

3.2.1. Uneven dispensing

Tubings tend to stretch after certain period of use, affecting the intended volume to be delivered. When not in use, the cartridges should be placed in the “rest” position. In addition, autoclaving the cassettes should be minimized. Dispensing speed and the height of the tips in relation to the plate have to be optimized for the intended reagent, as viscous solutions could miss the targeted well at low dispensing speed and large spacing between the tips and microtiter plate. When working with cells, uneven dispensing can be reduced by increasing the prime volume, constant mixing/stirring the cell suspension source and minimizing cell clumps. Solutions should be dispensed in the center of the well, and plates have to be centrifuged when dispensing low volumes to force droplets at the walls to the bottom of the well. Cassettes should be calibrated regularly as recommended by the supplier and checked for tip clogging.

3.2.2. Protein binding

Protein binding to dispensing components is an important point to consider in the implementation of biochemical assays, particularly at low protein concentrations. In some instances, enzymes appear to be inactivated over time when dispensing multiple plates using a liquid handler, when in reality the enzymes have been depleted from the solution due to non-specific binding to plastic, silicone and other polymer-based surfaces. This effect is amplified when dispensing sizeable number of plates, as there is larger exposure time of the assay components to the surfaces of reagent reservoirs and dispensing cassette elements. In order to circumvent this problem, blocking reagents can be added to the buffer, plastic surfaces can be coated, or a combination of both. The two major types of blocking reagents are detergents and proteins. It is preferable to use non-ionic detergents such as Tween-20, Triton X-100 or Nonidet-P40. Among the most widely-used protein blockers are bovine serum albumin (BSA) and casein. Protein blockers are better suited for coating surfaces, as detergents can be easily washed away. Typical working concentrations for detergents range from 0.01 to 0.1%, while protein blockers are used between 0.1 to 3 %. The selection of the appropriate type of blocking reagent and concentration is central to a robust assay. Other less common blocking reagents include polyethylene glycol (PEG), polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP). Additionally, the use of glass reagent reservoirs is recommended.

3.2.3. Clogging

Particles can obstruct the flow of a dispensing cassette mainly by blocking the tips. Complete clogging is fairly easy to recognize, as the lack of fluid coming out of the tips can be visibly noticed. Depending on the degree of obstruction, partial clogging may not be easily perceived by the naked eye, and it is detected only by photometric or gravimetric testing. However, there are certain indications of partial clogging, such as slanted fluid spray or

drop formation at the tip. To prevent clogging, the tubing should be primed with deionized water shortly after use, especially prior to priming with alcohol, as salts in the buffer may precipitate and biological reagents may clump. When working with cells, it is recommended to wet the tubing with buffer or media before dispensing cells, and if possible, not to allow the cells to settle in the tubing by emptying the contents back to the reservoir immediately after dispensing (prime/empty cycle).

3.2.4. *Foaming*

Solutions with high protein content can cause frothing, including media containing serum and biochemical buffers with high percentage of BSA (used as blocking protein). To minimize frothing, it is recommended not to empty the tubing between dispensing (as ordinarily performed in fully automated platforms for large screenings). If tubing emptying is unavoidable, it is advisable to empty a volume smaller than the dead volume. Other means to reduce frothing involve decreasing dispensing speed and applying grease to the cassette tips. Torn or cracked tubing can pull air generating bubbles.

3.2.5. *Reservoir container*

The reservoir container is an important component of a liquid dispenser that is often neglected in troubleshooting. The material of the container can have a detrimental effect on the assay robustness, such as sticking of proteins to plastic surfaces. For peristaltic pump-based dispensers, we suggest using a jacketed glass flask connected to a water chiller (waterbath with adjustable temperature). Careful monitoring of the temperature in the flask using a thermometer is recommended, as the temperature set in the chiller is not always reflected in the container. Suspensions of cells, beads or nanoparticles have to be constantly stirred to prevent settling, which could result in uneven dispensing or clogging. The stirring speed needs to be optimized, as fast stirring can create bubbles and disturb biological components (cells). When working with large reagent volumes at the start of dispensing, the stirring may have to be reduced as the volume decreases to prevent foaming or bubble formation.

3.2.6. *Tubing extension*

Extensions can be implemented when the dispensing tubings cannot be immersed in the reservoir container because of its large dimensions. Some commercially available extensions allow for the 8 tubings of a standard cartridge to be coupled into single elongated tubing through metallic cannulas sticking out of a joint casing. For viscous solutions, these types of elongations can introduce bubbles due to the joint design, particularly during prime/empty cycles. The metallic cannulas can easily tear the tubing during fitting, which is ameliorated by using glycerol or alcohol to smoothen the surfaces. A better alternative is to build home-made extensions by attaching each of the new tubings to separate discarded tubings through connectors, which can be made by cutting the end of a pipette tip.

3.2.7. Routine quality assessment

During assay development and validation, factors affecting liquid dispenser performance are identified and corrected. However, setbacks can occur randomly regardless of detailed preparations ahead of the screens. For instance, torn tubing, tip blockage or incorrect cartridge setup cannot be prevented a priori. Therefore, it is recommended to rapidly monitor dispensing variations at the start of a screen, where problems encountered at this stage can be usually corrected fairly quickly.

We normally dispense a solution of fluorescein isothiocyanate (FITC) in PBS into a couple of 384-well plates. Fluorescence intensities are analyzed for signal variations corresponding to each cassette channel, as described by %CV and %bias' (Fig.2). Determination of %CV for the entire plate is frequently performed in many laboratories, but this approach cannot distinguish issues with individual channels. In addition, a flawed channel does not necessarily change drastically the %CV of the whole plate, as illustrated by Fig. 2A. The types of problems commonly associated to high %CV include improper cassette mounting, tubing stretching and damage.

There are instances when the tip is partially obstructed, leading to reduced volume delivered. Even when a channel displays low fluorescence counts, the signal can still have small %CV values (Fig. 2B). We have adapted the concept of %bias to detect significant deviations in signal intensity for each row (S_R) compared to that of the whole plate (S_T), resulting in %bias' (Eq. 4). Values lower than 10 %CV and 10 %bias' are acceptable.

$$\% \text{ bias ' } = 100 \times \left(\frac{S_R - S_T}{S_T} \right) \quad (4)$$

3.3. Considerations for using transfer devices: Pintool

The pintool has become a mature technology for transferring nanoliter to sub-microliter volumes. Even though the system is regarded as fairly simple and robust, there are a number of points to consider for a consistent and reliable performance:

3.3.1. Volume variation

The volume delivered by a pin can change due to a number of factors. To minimize volume variations, there should be consistency in immersion depth (Dunn and Feygin 2000). There is a minimum volume required in the source plate, and the destination plate should not be dry (Rudnicki and Johnston 2009). The dwell time that pins spend in the fluid and withdrawal speed from the liquid surface should be optimized for solutions of very different properties (e.g. viscosity).

The slot of a pin can be tainted by compound precipitation or formation of suspension deposits (Fig. 3B). Sufficient and robust washing and drying steps are effective in preventing deposition and being critical to avoid carry-over and cross-contamination. The pins can be physically damaged by dipping in highly uneven surfaces, particularly when using slotted

pins (Fig. 3C). Coated pins should avoid harsh washing procedures, such as going through powerful sonication washes.

A

	% CV	Raw signal	% bias		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
CHANNEL 1	3.4	2473	-1.9	A	2559	2616	2476	2547	2383	2532	2374	2516	2371	2497	2372	2516	2361	2517	2397	2508	2382	2520	2380	2522	2393	2561	2412	2627
	2.3	2511	-0.4	B	2630	2501	2574	2472	2555	2469	2534	2463	2543	2459	2574	2441	2527	2446	2556	2418	2561	2471	2549	2460	2569	2442	2562	2483
CHANNEL 2	1.9	2650	5.2	C	2576	2782	2712	2630	2691	2658	2583	2670	2699	2660	2647	2723	2599	2661	2594	2629	2645	2665	2570	2641	2601	2664	2610	2696
	1.6	2604	3.3	D	2639	2595	2677	2619	2684	2616	2585	2579	2621	2523	2609	2577	2627	2550	2588	2555	2598	2555	2622	2552	2643	2606	2609	2662
CHANNEL 3	2.3	2609	3.5	E	2780	2682	2684	2614	2699	2655	2603	2622	2599	2615	2589	2609	2567	2555	2553	2477	2545	2622	2586	2573	2630	2582	2589	2595
	1.6	2534	0.6	F	2559	2512	2514	2500	2566	2548	2523	2521	2588	2516	2550	2610	2516	2524	2491	2517	2515	2579	2510	2523	2501	2564	2437	2634
CHANNEL 4	4.0	2508	-0.5	G	2278	2584	2413	2573	2455	2608	2404	2587	2463	2629	2479	2624	2451	2616	2428	2574	2398	2612	2438	2550	2405	2596	2419	2620
	3.7	2503	-0.7	H	2619	2464	2601	2429	2579	2427	2565	2402	2613	2454	2617	2484	2613	2448	2602	2407	2551	2381	2547	2339	2533	2340	2581	2476
CHANNEL 5	4.1	2546	1.0	I	2523	2618	2525	2672	2488	2639	2414	2672	2468	2547	2499	2752	2533	2459	2460	2371	2472	2695	2459	2618	2463	2637	2428	2696
	4.4	2579	2.3	J	2704	2521	2716	2496	2749	2483	2662	2478	2722	2504	2714	2496	2686	2473	2669	2448	2689	2513	2669	2406	2637	2400	2562	2501
CHANNEL 6	17.9	2306	-6.5	K	2719	2090	2515	1967	2614	2040	2748	1950	2485	1946	2790	1946	2714	1929	2415	1999	2714	2084	2650	1922	2622	1928	2624	1933
	17.9	2281	-9.5	L	1979	2670	1923	2750	1703	2654	1616	2520	1720	2710	1611	2670	1905	2674	2009	2621	1983	2714	1915	2647	1954	2664	1965	2764
CHANNEL 7	4.2	2646	5.0	M	2669	2633	2792	2750	2714	2618	2689	2690	2700	2662	2661	2628	2576	2597	2714	2649	2524	2665	2602	2603	2371	2617	2574	2405
	3.4	2594	2.9	N	2755	2593	2673	2578	2683	2528	2611	2500	2647	2721	2652	2625	2597	2583	2697	2484	2592	2607	2464	2547	2460	2587	2670	2404
CHANNEL 8	5.4	2507	-0.5	O	2285	2590	2448	2739	2488	2680	2408	2626	2404	2672	2419	2616	2309	2637	2360	2595	2332	2555	2343	2555	2408	2626	2408	2662
	3.2	2468	-2.0	P	2514	2544	2634	2511	2374	2451	2516	2389	2517	2438	2382	2441	2652	2398	2479	2438	2560	2363	2441	2389	2511	2691	2459	2374
ENTIRE PLATE	7.3	2520																										

B

	% CV	Raw signal	% bias		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
CHANNEL 1	3.4	2473	-0.3	A	2559	2616	2476	2547	2383	2532	2374	2516	2371	2497	2372	2516	2361	2517	2397	2508	2382	2520	2380	2522	2393	2561	2412	2627
	2.3	2511	1.2	B	2630	2501	2574	2472	2555	2469	2534	2463	2543	2459	2574	2441	2527	2446	2556	2418	2561	2471	2549	2460	2569	2442	2562	2483
CHANNEL 2	1.9	2650	6.8	C	2576	2782	2712	2630	2691	2658	2583	2670	2699	2660	2647	2723	2599	2661	2594	2629	2645	2665	2570	2641	2601	2664	2610	2696
	1.6	2604	4.9	D	2639	2595	2677	2619	2684	2616	2585	2579	2621	2523	2609	2577	2627	2550	2588	2555	2598	2555	2622	2552	2643	2606	2609	2662
CHANNEL 3	2.3	2609	5.1	E	2780	2682	2684	2614	2699	2655	2603	2622	2599	2615	2589	2609	2567	2555	2553	2477	2545	2622	2586	2573	2630	2582	2589	2595
	1.6	2534	2.1	F	2559	2512	2514	2500	2566	2548	2523	2521	2588	2516	2550	2610	2516	2524	2491	2517	2515	2579	2510	2523	2501	2564	2437	2634
CHANNEL 4	4.0	2508	1.1	G	2278	2584	2413	2573	2455	2608	2404	2587	2463	2629	2479	2624	2451	2616	2428	2574	2398	2612	2438	2550	2405	2596	2419	2620
	3.7	2503	0.9	H	2619	2464	2601	2429	2579	2427	2565	2402	2613	2454	2617	2484	2613	2448	2602	2407	2551	2381	2547	2339	2533	2340	2581	2476
CHANNEL 5	4.1	2546	2.6	I	2523	2618	2525	2672	2488	2639	2414	2672	2468	2547	2499	2752	2533	2459	2460	2371	2472	2695	2459	2618	2463	2637	2428	2696
	4.4	2579	3.9	J	2704	2521	2716	2496	2749	2483	2662	2478	2722	2504	2714	2496	2686	2473	2669	2448	2689	2513	2669	2406	2637	2400	2562	2501
CHANNEL 6	3.2	2555	3.0	K	2639	2595	2677	2619	2694	2616	2585	2579	2621	2523	2574	2441	2527	2446	2556	2418	2561	2471	2549	2460	2393	2561	2630	2582
	2.4	2503	4.5	L	2639	2595	2677	2619	2694	2555	2469	2534	2463	2543	2550	2586	2516	2550	2599	2681	2594	2629	2630	2691	2658	2583	2609	2577
CHANNEL 7	4.2	2646	6.6	M	2669	2633	2792	2750	2714	2618	2689	2690	2700	2662	2661	2628	2576	2597	2714	2649	2524	2665	2602	2603	2371	2617	2574	2405
	3.4	2594	4.5	N	2755	2593	2673	2578	2683	2528	2611	2500	2647	2721	2652	2625	2597	2583	2697	2484	2592	2607	2464	2547	2460	2587	2670	2404
CHANNEL 8	7.1	1907	-22.8	O	1686	1990	1848	2130	1688	2080	1808	2026	1804	2072	1619	2016	1709	2037	1750	1995	1732	1955	1743	1955	1608	2026	1608	2052
	4.2	1968	-24.3	P	1914	1944	1934	1911	1774	1851	1916	1780	1917	1838	1782	1841	1952	1798	1879	1838	1950	1793	1841	1780	1911	2091	1859	1774
ENTIRE PLATE	9.9	2480																										

C

	1	2	3	4	5	6	7	8	9	10	11	12	
CHANNEL 1	A	5385520	5246640	4785600	4010320	3930240	3588880	3386240	3409360	2257840	1833440	1839200	1862640
	B	5159200	5311360	5430400	5021680	4097520	3672640	3767680	3306800	3129200	2183120	2146000	2101920
CHANNEL 2	C	4452480	5501120	5265600	5198480	4607280	3602240	4102480	3279440	3523280	3572560	2587040	2211120
	D	5697760	5619280	5633840	5183600	4588800	4400800	4148240	4322240	3471680	3494880	2361040	1954800
CHANNEL 3	E	5209600	5593920	4931120	4397120	4794000	4102320	3986400	4000800	4053760	4068800	3831440	2857280
	F	5838560	5511120	5694160	5386960	5189960	4600400	4711040	4990000	4393120	4330060	4025200	3537440
CHANNEL 4	G	8023800	5489860	8021200	5007800	5513280	5442160	5448960	5533440	3893200	3613520	3197920	3343920
	H	8538560	6358240	6344560	6116680	6442880	6913920	6372000	6091680	5124080	5039520	3948400	4302240
CHANNEL 5	I	5853760	5669120	4738400	5251360	5290160	3954960	3519200	3586320	3677280	2736320	2411920	1958880
	J	6194480	5629600	5933840	5663360	5771440	5247120	4340800	4513280	4100320	3046560	3047840	3140240
CHANNEL 6	K	5350880	4006320	5344080	4832880	5107360	4195540	3467840	2896880	2906960	2124560	2597840	2581680
	L	5641120	5504880	6313600	5208720	4428240	3940880	3537360	3011920	3131840	3018240	2783760	2598880
CHANNEL 7	M	5666480	5086240	5198400	4760400	4449200	3349440	3746400	2828880	2526640	2791920	2385600	2675280
	N	5707840	5489680	5857040	5331600	4843040	3897360	4529440	3117840	2447520	2352600	1849360	2253520
CHANNEL 8	O	5282160	4514800	4426440	4242320	3425360	3229760	3247600	2265360	2193360	2140000	1835040	1576000
	P	5285360	5593920	5518480	5210800	4177600	3192240	3452320	3141360	2691280	2512400	1821520	2310560

Figure 2. A-B) Delivery variation by a bulk reagent dispenser distributing a FITC solution into 384-well plates. Certain dispensing cassette channels display either higher %CV or %bias' values than the anticipated cut-off of 10%. C) Cell settling in the reagent reservoir when transferring to a microtiter plate using an automated pipetting system with an 8-channel head, with 1 min delay between transfers to each column. Cell settling is uneven due to the v-shaped bottom of the reservoir, causing the intensity pattern observed in the plate. The cells (HEK293T) were incubated with Cell-Titer-Glo® for 20 min prior to luminescence reading.



3.3.2. Carry-over

After transferring compounds from one plate to another, the pins are washed in DMSO, alcohol, water or a combination of these solutions. The pintool protocol involves dipping the pins in each solution bath certain number of times, at a particular speed and soaking time. The pins are then dried on lint-free blotting paper. Protocols of pintool devices used on robotic platforms are optimized for effectiveness in removing previous transfers while spending the minimum time between wash cycles. In many cases, the drugging (i.e., addition of compound to assay well) step using pintool becomes the bottleneck in a screening campaign, and the washing step accounts for most of the time consumed. However, certain assays can be very sensitive to compound carry-over, particularly if the compounds are very potent modulators and bind avidly to the pin surface. In such cases, increasing the number of dips and soaking time can improve cleanliness, albeit at the cost of increasing total transfer time.

Fig. 4 illustrates the effect of four different wash protocols in a kinase assay using staurosporine as the inhibitor. After compound transfer by pintool to the first assay plate, the pins are immersed in DMSO and isopropanol reservoirs, followed by drying on blotting paper. Subsequently, the pins are dipped in a second assay plate containing the kinase system. Residual staurosporine in the pins increases the signal variation as determined by %CV of a set of multiple wells. Protocol 1 has the least number of dips and soaking time per bath, resulting in the most dramatic signal variation due to carry-over. This general approach is recommended for detecting carry-over and selecting the appropriate pintool wash.

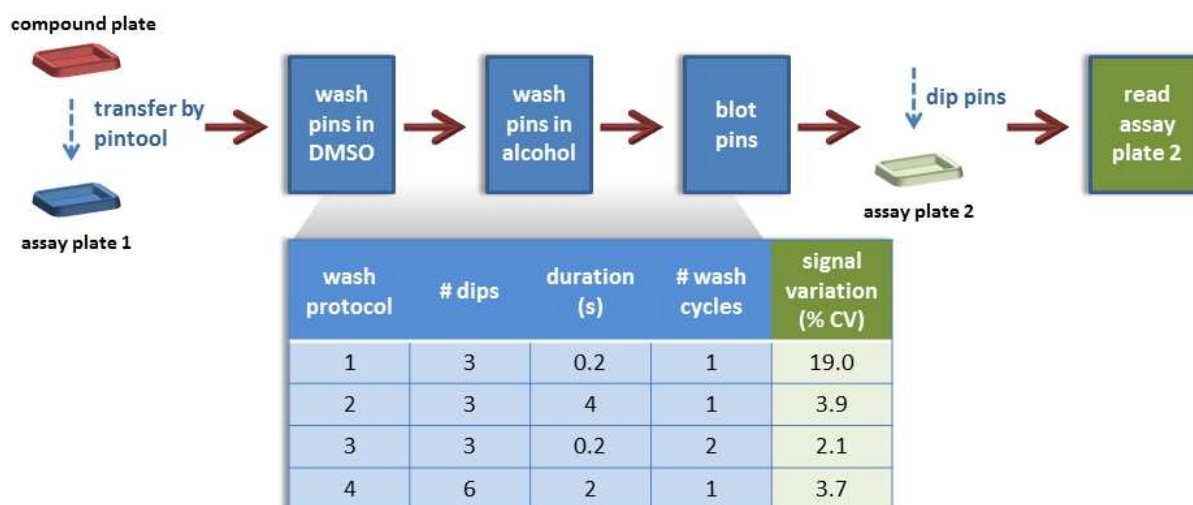


Figure 4. General approach to detect compound carry-over and optimize pintool washing. A single wash cycle consists of dipping the pins in DMSO and isopropanol baths, followed by blotting on lint-free paper.

3.3.3. Routine quality assessment

Regular pintool calibration and quality assessment can considerably improve data quality. In screening runs at a single compound concentration, well-maintained pins can lead to a

reduction of false negative hits, as damaged or dirty pins would usually deliver lower volumes than anticipated. In dose-response analysis, the quality of the curve fit is highly dependent on the variability of the data points.

A good quality control procedure should provide the transferred volume and the variation associated with the pin set. We implemented a relatively quick and simple procedure using a fluorescent dye (FITC). Prior to the test, the pins are washed as described above. A calibration curve is generated of fluorescence intensity as a function of FITC concentration. Using the pintool, FITC in DMSO is transferred from a source plate to several destination plates containing PBS (the use of 4 plates was shown to be sufficient). The average transferred volume per pin is calculated using the fluorescence signal of the destination plates and the calibration curve. Volume variation across the microtiter plate can be readily appreciated by plotting volume against well position (Fig. 5, top charts). The pink and green solid lines represent the upper and lower boundaries within 10% CV of the average volume, where outliers can be clearly identified. The frequency chart (Fig. 5, bottom chart) displays outliers present in 1, 2, 3 or all of the 4 destination plates, and it can be used to identify pins that consistently provide volumes outside a specified range. In the example shown in Fig. 5, pins corresponding to positions A13, B21, D8, F13, K1, N14 and P20 will have to be replaced. Depending on the need, stringency can be adjusted by changing the boundaries as specified by %CV. It is highly recommended to utilize the same freshly prepared fluorescent dye and buffer solutions in all aspects of the protocol. A template for data analysis can be easily created in conventional software such as MS-Excel.

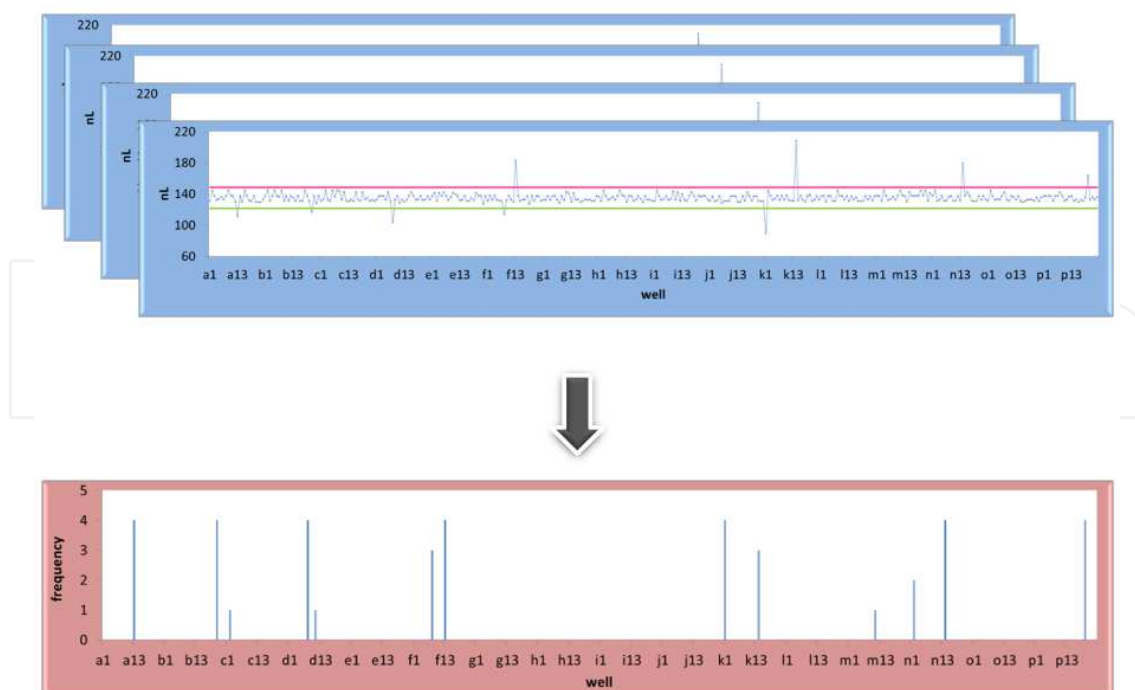


Figure 5. A simple and comprehensive approach to analyze pintool performance. Individual pins can be selected for replacement based on consistent variation across multiple transfers.

3.4. Considerations for using transfer devices: Pipettors

3.4.1. Pipette stations

The automation station is an integral part of any high throughput pipettor, regardless of the type of tips (fixed or disposable) it employs. It typically consists of ANSI/SBS standard compliant single or multiple deck positions on a stationary or moving platform to hold the lab-ware, with a moving arm situated above the platform containing the single- or multi-channel pipette head. A major advantage of automated pipettor devices over manual or electronic multichannel hand-held pipettes is the elimination of inconsistency in the transfer process by minimizing human intervention, which also enables high throughput applications that are not otherwise feasible. The three major tasks that can be performed with suitable hardware settings are liquid transfer, cherry-picking and serial-dilution.

For plate-to-plate liquid transfers, 96- or 384-well pipette heads are preferred to work with 96-/384-/1536-well microplates to speed up the process and increase the throughput. While 4-/8-/12-/16-pipette heads can also be used for direct transfer applications, they are primarily used to perform serial-dilutions. On the other hand, a single channel pipette tip is an essential component to accomplish cherry-picking tasks.

The speed of an automated pipettor is important for time-sensitive experiments. Especially when performing small volume transfers into microplates, the amount of time spent to transfer liquids in a column-by-column or row-by-row manner may be problematic due to quick evaporation. If the speed of transfer is too slow, some evaporation in the first column or row may be observed before dispensing to the last column or row, causing inconsistent volume across the plate. To avoid evaporation issues during liquid transfers, deck size, pipettor speed, head type and the transfer volume should be considered.

3.4.2. Tip contamination

Sample carry-over is a common problem in liquid handling tasks requiring sequential dipping steps into various sample reservoirs. With fixed-tips, an adequate cleaning step is essential between two transfer operations to prevent sample carry-over. An on-deck cleaning protocol often consists of immersion in a bath (DMSO, alcohol and/or water) with optional sonication step. The tips should be allowed sufficient drying time to prevent sample dilution in the following transfer phase. Appropriate wash solutions should be selected and the optimum length of washing time should be determined during the assay development stage. Although fixed-tips may have the risk of carry-over, they enable more accurate and precise transfers in smaller volume ranges (Felton 2003).

Contamination can also be associated with disposable tips, especially when sterile and nuclease-free assay conditions are required. The speed at which the pipette tips are removed from a sample fluid was found to correlate to the amount of macroscopic droplets stuck to the outer surface of the polypropylene tips, which contributed to cross-contamination (Berg et al. 2001). It was also reported that to decrease this form of cross-contamination, which is

influenced by the tip shape and the sample-polypropylene interactions, the removal speed should be slow enough to diminish droplet generation.

Impurities can also leach out of the disposable tips when in contact with solvents such as DMSO. Studies have shown that bioactive compounds released from plastic labware may interfere with assay readouts causing misleading experimental results (McDonald et al. 2008; Niles and Coassin 2008; Watson et al. 2009). Consumable materials, especially polypropylene tips, tend to adsorb certain compounds, leading to unreliable concentrations in the destination plates (Harris et al. 2010). Therefore, it is recommended to test and validate the influence of consumables on an assay during assay development and whenever there is a change in labware.

3.4.3. Foaming

Pipetting viscous and “sticky” samples is challenging due to bubble formation. Among the most important parameters to consider in avoiding these issues are the speed that the tips exit the sample fluid and the aspirate/dispense rates; they should be slow enough to avoid residuals at the inside and outside of the tips. Pre- and post-air pipetting options should be avoided.

3.4.4. Pipette behavior affecting dispensing variation

Most pipettor systems provide pre- and post-air aspiration functions to ensure accurate liquid transfers. Introduction of air into the tips before or after the aspiration of the sample liquid is recommended to improve volume accuracy by forcing all the liquid out of the tips. In a study performed to optimize the automated parameters to achieve a 10 μL transfer volume in a sequential transfer experiment, introduction of a 5 μL pre-air gap significantly reduced the relative volume inaccuracy along with the CV of the final transferred volume in a 96-well plate (Albert et al. 2007). While this method may help to achieve more precise results especially with small volume transfers, bubble formation in the destination wells may be inevitable unless preceded by a shaking or centrifugation step. Post-air aspiration may also be applied to create an air gap between liquids, preventing unsought contamination in the source reservoirs when multiple samples are picked up sequentially into a single tip before the delivery into the destination reservoir.

When small and repetitive volume transfers into multiple destinations are needed, it is a common practice to pick up a single large volume and deliver smaller amounts in a sequential mode. However, with this method, it is hard to achieve accurate delivery in each step. In a study of multi-sequential dispense accuracy, it was shown that the first and last dispense steps led to relatively higher and lower transferred volumes, respectively, in addition to increased relative inaccuracy (Albert et al. 2007). Therefore, it is recommended to dispense the first and last steps into the source reservoir to enhance the precision in the destination plate. Delivery performance of the dry versus pre-wetted tips may also exhibit differences in variability depending on the sample characteristics.

Droplet formation at the end of the pipette tips after a dispense action remains an issue for liquids with high viscosity or low densities. Besides the selection of the optimum dispense

speed, a “tip touch” function is a useful feature offered in some automated pipettors, where the tips contact the well wall at the end of a dispense step to force the release of the droplet. The path of the moving pipetting arm across the deck should be carefully determined to reduce the chance of contaminating other labware by hanging droplets.

Proper mixing of solutions in the source reservoir before aspiration and in the destination reservoir after dispensing may greatly affect the final assay quality due to the necessity of uniform sample concentrations. To avoid the formation of concentration gradient in wells, mixing can be performed by repetitive pipetting cycles. Mixing of the well contents by pipetting up and down is proven to be a quicker and more efficient method compared to free diffusion or shaking, which are not as successful due to the correlations between well size, content volume and the exerted capillary forces (Berg et al. 2001; Shieh et al. 2010; Travis et al. 2010). Mixing is necessary when dealing with suspensions (cells, beads, etc.). For instance, cell settling creates uneven cell density in the source reservoir, which would lead to aspiration of decreasing number of cells over time (Fig. 2C)

3.4.5. Routine quality assessment

Verification of transferred volumes and routine quality control (QC) are the most important and inevitable processes when working with liquid handling devices. While the verification method should be reliable enough to quantify the pipettor performance, it should also be easy and fast to be applied routinely. The performance assessment described for bulk liquid dispensers (section 3.2.7.) can also be applied to pipettors as long as the same volume is distributed throughout the plate for %CV and %bias' calculations.

As mentioned previously, liquid handlers are heavily used to perform serial dilutions, and suitable QC techniques should be employed to validate dilution performance, particularly when accurate compound potency is directly dependent on concentration accuracy. Dilution ratio, accuracy, precision and outlier distribution constitute the four major criteria that should be evaluated (Popa-Burke et al. 2009). Artel developed an approach to determine dilution and transferred volume accuracy by using dual-wavelength photometry, where two absorbance dyes with baseline resolved spectra are mixed at various ratios using a liquid handler (Albert 2007; Dong et al. 2007). This dual-dye ratiometric method can be applied by using a multichannel verification system (MVS) equipped with the necessary instrumentation and analysis (Bradshaw et al. 2005). Dual-dye photometry is also proven to be suitable to measure the efficiency of different mixing methods (Spaulding et al. 2006) and when pipetting non-aqueous solutions (Bradshaw et al. 2007).

3.5. Considerations for using microplate washers

3.5.1. General considerations

One of the major concerns with any high throughput microplate handler device is its compatibility with plates of various types and sizes. While most high throughput instruments

are designed to accommodate labware with dimensions conforming to ANSI/SBS standards, an ideal plate washer is also able to support flat, v-shaped and round-bottom plates.

Both the vacuum assembly and the bottle setup are also important aspects of the plate washer. Although most washers operate through changes in vacuum pressure, pump-based vacuum-free and pressure-free systems are also offered.

Plate washers functioning by positive displacement principle are also available, enabling non-contact washing with no residual volume (Rudnicki and Johnston 2009). For assays where more than one wash buffer may need to be used, plate washers with multiple dispense channels and automatic buffer switching capability are preferred to minimize both operation time and contamination. Examples of other optional features for safe instrument operation include waste liquid level sensors and plate detection sensors to avoid unwanted overflows and jams. For BSL2 or higher level experiments, a washer with aerosol cover should be chosen to prevent spread of the contagious material.

3.5.2. Washer performance

Although compatibility and control properties are important, plate washers are predominantly evaluated by their wash performance. Plate washers provide a range of user-defined dispense/aspirate heights, flow rates, and needle probe positioning in reference to the well walls. By adjusting these parameters for each step of the wash cycle, optimal wash performance can be ensured. On the other hand, an adequate wash quality needs to be reached to diminish extensive background signal and high signal variations amongst wells. This can be primarily achieved by minimizing the amount of liquid left inside each well at the end of the aspiration step. Besides their effects on wash power, the above-mentioned parameters also have an impact on the residual volume and need to be fine-tuned in conjunction with the vacuum/pump settings. Some plate washers may also provide multipoint, secondary, cross-wise or delayed aspiration modes aiming to deliver the best results. The number of wash cycles and the length of soaking time are other settings that can be modified to reduce background noise levels.

3.5.3. Washer maintenance

Since plate washers consist of tubing and needles which transport buffer solutions or waste liquid to or from the device, they require special cleaning processes as they are prone to be clogged by chemical residues such as salt and proteins from the wash liquids. Depending on the frequency of use, the fluid path may need to be rinsed daily to prevent blockage and contamination, especially if different buffers are being delivered through the same tubing. An efficient cleaning method alternates deionized water and a detergent such as Terg-a-Zyme®, which is highly recommended by plate washer manufacturers. Plate washers which provide an automatic cleaning feature or integrated ultrasonic washing technology are often easier to maintain. Models which do not contain built-in cleaning functionality are generally supplied with removable dispense/aspiration manifolds to ease the maintenance tasks.

Cleaning of the other detachable or fixed plate washer components should also be performed periodically.

3.5.4. Troubleshooting

Plate washers serve as an excellent alternative to time consuming manual wash procedures for many applications. Since all the wash parameters should be optimized for each specific application during the assay development stage, a tedious troubleshooting process may be inevitable while setting up wash protocols to meet specific assay needs. Table 1 presents a summary of wash parameters/components and their contributions to the wash performance along with various troubleshooting tips. Different assay types may require distinct considerations. With biochemical assays, minimizing the background signal and well-to-well variations are the most important tasks in the optimization process. Low background signal levels can be achieved by reducing the leftover liquid volume in each well. Decreasing the aspiration height and lowering the aspiration rate can greatly affect the residual volume leading to minimal liquid amounts in the wells. In order to prevent high standard deviations in the assay readouts, equal residual volumes should be attempted by optimizing the aspiration/dispense heights and rates. Depending on the viscosity of the wash buffer, high aspiration rates or low dispense rates may lead to unequal volumes. Inadequate priming volumes, unadjusted dispense or aspiration heights, clogged tubing, and physical misalignments between the manifolds and plate carrier should also be avoided to prevent high signal variations. The effect of the aspiration height on the final residual volume is presented in Fig. 6 for both 96- and 384-well black plates with clear bottom. The volume of the residual liquid (water) per well was measured with the gravimetric technique at several selected aspiration heights on a Biotek EL405 microplate washer, while all the other wash parameters were kept constant. A rising trend is observed in the final volume as the aspiration height is increased.

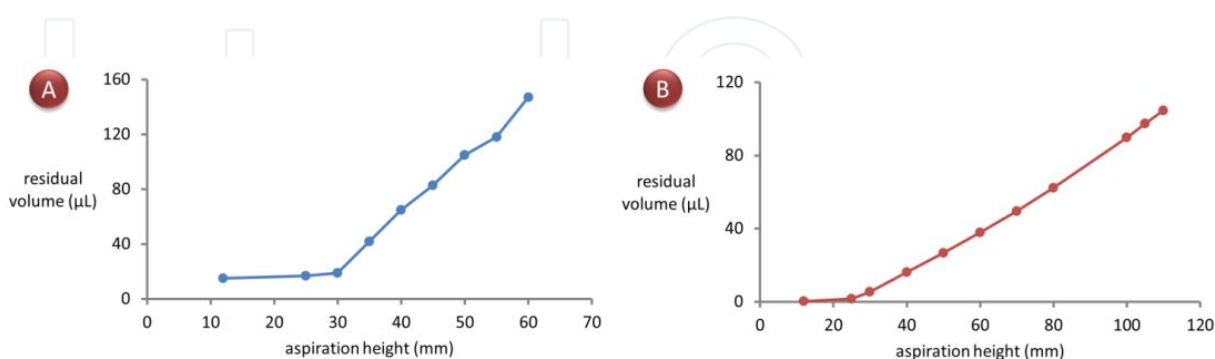


Figure 6. Effects of aspiration height on residual volume. Residual volume was measured in a) 96-well and B) 384-well plates at various aspiration heights. Residual volume was increased as the aspiration height from the bottom of the well was increased.

In cell-based assays, gentle cell washing is one of the most critical factors to produce reproducible assay results, and it can be controlled by several settings such as aspiration and dispense rates, heights and horizontal positions. For loosely-adherent cells, the cell layer attached to the bottom of the well may be easily disrupted by rigorous wash cycles, and the aspiration and dispense rates should be set low enough to prevent turbulence inside the wells. For the same purpose, wash fluid should be dispensed at a distance from the well bottom and may be even be aimed at the well walls when possible. To observe the consequences of inadequate washing and dispensing parameters on the cell layer endurance, a 3-cycle wash experiment was performed on HEK 293T cells, which are known for their low adherence and propensity to be frequently washed away in cell-based assays. The fixing solution was dispensed at medium speed, and the cells were washed before and after fixation. Representative images from wells containing an intact or damaged cell layer are presented in Fig. 7. When dealing with adherent cells, each step of the assay protocol should be optimized, including those involving other liquid handling devices such as bulk dispensers, pintools and pipettors.

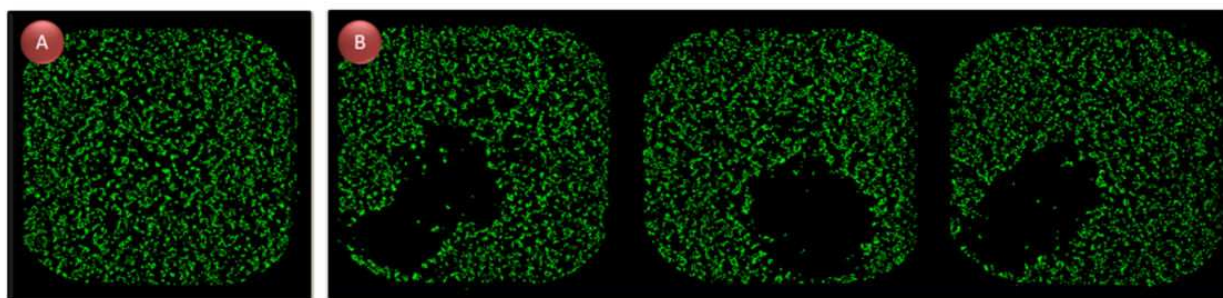


Figure 7. Effects of non-optimized dispensing and washing on low-adherent cells. HEK293T cells were fixed, stained with Hoechst 33258 and imaged with Acumen eX3 in a 384-well black clear bottom plates. The fixing solution was dispensed by a Thermo Scientific Matrix® Wellmate®. Representative images (shown here in false color green) of A) an intact cell layer and B) disrupted cell layers indicated cell loss due to harsh dispense and wash settings.

As with most high throughput instrument operations, it is a common practice to perform a periodic quality check on plate washers to assure a satisfactory wash performance at each use. It is important to perform these assessments with a wash buffer that has a similar viscosity to the buffers used in most of the applications. For evaluations on the residual volume, one can perform a mock wash with a dummy plate and measure the leftover liquid volume inside the wells with a single or multichannel manual pipettor. For more accurate results, gravimetric or colorimetric techniques can be used to calculate the average volume per well. This way, one can also test if dispensing/aspiration is consistent in all the probes, and if there is any physical failure with any of the device components.

parameter/component	effect	troubleshooting tips
prime	dispense performance	<ul style="list-style-type: none"> • prevent air bubble formation or no/uneven dispensing with adequate priming
aspiration rate	residual volume, gentle/rigorous washing	<ul style="list-style-type: none"> • higher residual volume if too fast • perturbed cell layer if too fast • uneven aspiration if too fast
aspiration height	residual volume, gentle/rigorous washing	<ul style="list-style-type: none"> • higher residual volume if too high • uneven aspiration if too low or too high • perturbed cell layer if aspiration probes touch the well bottom • undisturbed cell layer if high enough
horizontal aspirate position	gentle/rigorous washing	<ul style="list-style-type: none"> • prevent bead loss by offsetting the aspirate position (for magnetic bead assays)
dispense flow rate	dispense volume, gentle/rigorous washing	<ul style="list-style-type: none"> • uneven dispensing if too slow • fluid overflow if too slow or too fast • perturbed cell layer if too fast • air bubble formation if too slow
dispense height	dispense volume, gentle/rigorous washing	<ul style="list-style-type: none"> • uneven dispensing if too low or too high • fluid overflow if too high
horizontal dispense position	gentle/rigorous washing	<ul style="list-style-type: none"> • undisturbed cell layer if dispense position is offset to aim the well walls
assay buffer properties	residual volume, aspiration/dispense performance	<ul style="list-style-type: none"> • optimize for viscous/non-viscous buffer solutions • add surfactant to the buffer solution to reduce surface tension
vacuum/pump assembly	aspiration/dispense performance	<ul style="list-style-type: none"> • no/uneven aspiration with insufficient vacuum supply • no/uneven aspiration or leakage if tubing is defective, bent or clogged
plate carrier	aspiration/dispense performance	<ul style="list-style-type: none"> • uneven aspiration/dispense if plate carrier is not leveled or movement is blocked • plate is placed on the carrier with A1 in the correct position • enough plate clearance to prevent jams • higher throughput with lower plate clearance

Table 1. Wash parameters and troubleshooting advices

4. Conclusion

In order to fulfill the need for higher throughput options, the technology behind liquid handling devices is in constant progression, with systems capable of delivering smaller volumes at a faster rate with accuracy and precision. These developments should consider cost reduction by minimizing reagent and solvent expenditure, as well as reducing consumables.

The main concerns and limitations that liquid handling systems face are reproducibility and reliability. The devices should be robust to execute extensive experiments in a daily basis with minimal downtime and maintenance. However, as a single screen can generate thousands of data points, the user is required to ensure all the devices are functioning up to standards by implementing routine quality assessments. Regardless of the technological innovations and advancements, scientists are compelled to spend significant amount of time optimizing the liquid handling parameters to suit specific assay conditions. A thorough understanding of the principles, strengths and limitations of the instruments is advantageous in preventing undesirable results and facilitating troubleshooting.

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