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# Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology

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## Abstract

High-throughput screening (HTS) can efficiently assay multiple discrete biological reactions using multi-well microplates. The choice of microplate is a crucial yet often overlooked technical decision in HTS and quantitative biology. This chapter reviews key criteria for microplate selection, including: well number, well volume and shape, microplate color, surface treatments/coatings, and considerations for specialized applications such as high-content screening. This chapter then provides important technical advice for microplate handling including mixing, dispensing, incubation, and centrifugation. Special topics are discussed, including microplate surface properties, plate washing, well-to-well contamination, microplate positional effects, well-to-well and inter-lot variability, and troubleshooting. This information and best practices derived from academic, government, and industry screening centers, as well as microplate vendors, should accelerate assay development and enhance assay quality.

## Key Abbreviations

| | |
|---|---|
| ADE | acoustic droplet ejection |
| ALPHA (“Alpha”) | amplified luminescent proximity homogeneous assay |
| COC | cyclic olefin copolymer |
| COP | cyclic olefin polymer |
| ELISA | enzyme-linked immunosorbent assay |
| FRET | fluorescence/Förster resonance energy transfer |
| HCS | high-content screening |

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|---|---|
| HTS | high-throughput screening |
| NA | numerical aperture |
| PP | polypropylene |
| PS | polystyrene |
| RFMP | radio frequency microwave plasma |

# Introduction to Microplates in High-Throughput Screening and Quantitative Biology

The discovery of the microtiter plate, as we know it today, was first conceived and designed in the laboratory of Dr. Gyula Takatsy in 1950 in Hungary (1). The original plate, made of plexiglass with 6 x 12 "cups" or wells (72 total wells), was intended for serological testing of the influenza virus. This plate design and the spiral loop as a quantitative liquid transfer device (a precursor to micropipettes) were seminal developments at that time because the intent was to semi-automate serial dilutions of viruses to determine titer and potency, and to test drugs that inhibit viral activity. In 1955, Dr. Takatsy redesigned the plate with 8 x 12 wells (96 total wells) to accommodate the original loop handles for liquid delivery with knitting needles which was more adaptable for use by technicians. In 1961, Dr. John Sever at the National Institutes of Health widely adopted this technique for serological investigations. He reported the use of the 96-well plate along with a "modified pipette dropper" and a centrifuge carrier for the plate, further popularizing the use of microplates for biological testing (2). In the 1970s, the 96-well microtiter plates were adapted for immunological testing assays (i.e., ELISA) followed by subsequent commercialization and standardization of the plate dimensions, materials, and well designs. As this format became extensively utilized for HTS and automated drug discovery methodologies in the 1990s, the applications for biological measurements rapidly expanded. The detailed history has been elegantly described in articles by Tom Astle and Roy Manns (3,4).

Further refinement of the microplate design took place to standardize its use for HTS and encourage all plate and instrument manufacturers to agree to specified dimensions for compatibility with screening instruments and automation. This effort was pioneered by the Society for Biomolecular Screening in 1998 in collaboration with American National Standards Institute (ANSI) which became the SBS/ANSI standard (Figure 1). This ensured that microplates will have standardized footprint dimensions, plate height, flange dimensions, chamfers, side wall rigidity, and well positions. Some commonly modified plate dimensions include flange height, well number, and well depth. Despite this standardization, for many instruments (microplate readers, robotic handlers, pin tools, acoustic dispensing) users still may need to specify *exact* plate dimensions for optimal use. Most manufacturers can provide specific technical blueprints for microplates.

From a practical standpoint, microplates are also important because they can represent the largest portion of a HTS budget, though this can be offset by expensive reagents. From a budgetary perspective, multiple competing factors must be considered when selecting the final microplate. While many institutions have preferred vendors with lower negotiated prices, a more expensive but optimal microplate may actually enable less reagent per well, yielding more cost savings when all assay costs are considered. An Excel template is available to assist with microplate cost estimation.

The cost of a microplate is typically determined by several factors: material, type of plate bottom (solid, clear, or permeable), necessary molds and overall manufacturing process, well density, barcodes, and finally any surface treatments/coatings. This combination of factors can determine if a microplate has a retail cost of around $4 for a standard 6-well cell culture plate, to $50 per plate for a 1536-well plate with a collagen coating, to over $300 per plate for a 96-well permeable support plate. Although increasing plate density drives the cost of the part higher (injection molding fixtures become more complex and therefor more expensive), it is still typically more cost

effective to miniaturize an assay as the overall cost per well will decrease as less reagents will be required due to the lower well volume and less plates will be required to run an experiment as more samples can be placed within the plate. We note the microplate manufacturing process can be the primary cost factor. For example, the process of applying a permeable membrane to the bottom of a 96-well support structure is incredibly difficult and laborious, driving the cost of the part far higher as compared to a solid-bottom 96-well plate manufactured via injection molding. Although cost will always be a factor when determining which well plate to use for an assay, the primary factor should always be to make a selection based upon the requirements of the assay and the desired measurement technique to be utilized. *In our experience, assay performance and optimized microplate measurement should rarely if ever be sacrificed due to the cost the plate*.

## Desired microplate properties

Microplates to be used for biological assays should have certain properties:

- Dimensional stability under multiple temperature and humidity conditions
- Flatness
- Chemical and biological compatibility with assay reagents (DMSO-stable, does not denature proteins)
- Low-binding surfaces/surface energy (lower adsorption of chemicals or biologicals)
- Low autofluorescence
- Supports cell viability, attachment, and growth
- Supports relevant optical detection modes
- No leaching of solvents, metals, or chemicals (e.g., bisphenol A, heavy metals from catalysts, solvents)

## Microplate manufacturing

It can be helpful to understand some of the basic microplate manufacturing processes. For many microplates, the manufacturing process involves an injection of liquid polymer into a mold ("injection molding"). Depending on the plate type, several pieces can be fused together, such as a glass bottom and the main polymer frame. For clear-bottom microplates, the main polymer frame are injected molded and fused with a pre-made clear bottom film in a process known as *overmolding*. Incomplete fusing and poor quality control has been known to create conduits between adjacent wells (unpublished observations; see also subsequent section Troubleshooting and Special Topics – Well-to-Well Contamination). Pigments to color the microplate are often added by the manufacturers. The raw materials used to create microplates are often an overlooked source of microplate performance by the end-user scientists. Manufacturers will usually acquire raw materials from an outside vendor, and any changes in this sourcing or the manufacturing techniques are not usually provided to consumers. In our experience, we have encountered significant changes in assay behavior for a given microplate across different manufacturing lots that can reasonably be attributed to a seemingly innocuous change in the manufacturing process. Raw materials for microplates can have highly variable quality and characterization, and while medical-grade plastics have higher quality standards, these are often not used for microplates.

## Section summary

Microplates are a critical yet often overlooked component for enabling robust, reproducible biological assays. This chapter will describe key technical factors in microplate selection, certain technology-specific guidelines (notably high-content imaging), best practices for microplate usage in biological assays, troubleshooting and special topics intended to maximize microplate usage. The goal of this chapter is to disseminate high-yield common as well as tribal (tacit) knowledge from microplate manufacturers and academic/industry/government experts, which should enhance the quality of biological assays performed with microplates.

*Authors' note: While microplates are also used for applications besides biological assays (compound storage, sample storage, sample purification, etc.), for brevity such applications will not be discussed.*

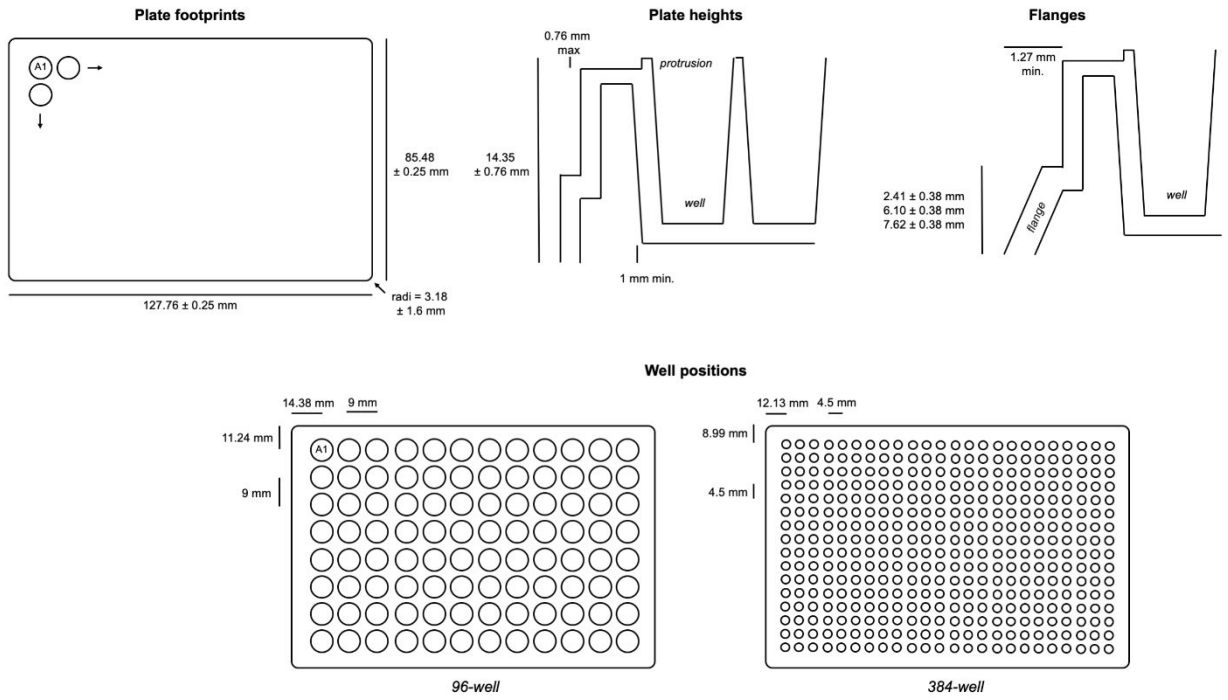



**Figure 1.** Schematic of standard microplates. Standard microplates used in quantitative biology will typically conform to the following dimensions. Adapted fromANSI/SLAS standards (www.slas.org/resources/information/industry-standards, accessed 14 May 2019). Scientists should consult the technical drawings from plate manufacturers for specialized applications such as incorporation of automation and microplate reader optimization.

# Technical Factors in Microplate Selection

This section describes major decision points in microplate selection for *in vitro* assays. Once a particular assay technology and approach is chosen, scientists should select microplates that meet the specific technical requirements of their project.

## Overview of the microplate selection process

The selection of an optimal microplate for an assay can be a tortuous process due to multiple options (and competing trade-offs) in microplate choices at each step of the assay development workflow. Not only does the assay development scientist have a series of decisions to make on plate choices based on the biology, assay technology, and desired throughput, but there are multiple microplate manufacturers offering similar products where differences in the microplate plastic, quality control of a coating, and even the "whiteness" of the plate can significantly affect assay performance. A query with "Select Sciences" (search date 21 Oct 2019) resulted in approximately 700 microplate types across 26 vendors (5). Many HTS laboratories will have a preferred vendor and as a result, have several different microplate options readily available in the lab which can serve as starting points. In addition, high-quality publications and protocols are careful to detail the specifications of the microplate (catalog and lot numbers) and detection systems, which can be a critical pairing in order to achieve and maintain the desired assay performance metrics.

The following section highlights one path for the selection of microplates that has been helpful for several of our labs with diverse discovery approaches (Figure 2). *In practice, there are likely other optimal decision trees for microplate selection*. The basic process will be outlined, and many of the individual criteria will be discussed in more detail in subsequent sections. Microplate selection is an iterative process with multiple comparisons between plates often needed before selecting an optimal plate. Many screening labs have minimum quality

criteria' using parameters such as Z-score, Robust Z-score, B-score, Z-factor, Z'-factor, signal-to-noise, signal-to-background, and percent CV to characterize the assay performance and maintain it at a level necessary to increase the possibility of identifying the desired chemical matter (6-8). In practice, a balance must be struck between understanding the quality of an assay system at each stage of assay development or validation and the time to complete the microplate selection. Using one lot/batch of a microplate for the selection process can be critical to mitigate lot-to-lot variances.

In our experience, deciding on a cell-free or cell-based assay is the highest-yielding initial decision point during the microplate selection process (Figure 2). Microplates supporting cellular assays are typically tissue culture treated (sometimes abbreviated "TC treated" in catalogs), sterilized, and might have requirements for clear-bottomed wells and special coatings aimed at visualization and enhancing or preventing cell attachment. Cell-free assays, on the other hand, can require a range of surface conditions from non-binding surfaces which minimize protein adsorption (e.g., enzymatic, homogenous proximity assays) to high-binding surfaces which enhance adsorption (e.g., ELISA). Another key consideration for identifying the optimal microplate is the assay/detection technology, where multiple plate types can be tested against multiple assay/detection technologies using the performance criteria as part of the decision matrix. The expected throughput of the assay must also be factored and while the preferred density for screening assays might be 1,536-well (Figure 2), assay performance or automation may only support 384- or 96-well formats. In these situations, alternative strategies for prosecuting a screening deck might be necessary (9). The assessment of plate density can also raise questions about the well shape and volume, which will also impact the shaking efficiency and well meniscus that can be critical components in certain detection systems.

Whether the well density has been established by good performance statistics or performance is sub-optimal with the preferred plate density, consideration should be given to the physical components of the microplate itself. For example, the color of white is not standardized in opaque microplates and there are blue-white and grey-white hues. The hue of the color can make a difference in the performance of an assay and often varies from vendor to vendor and sometimes even from lot-to-lot by the same vendor. Additionally, there are several options for plate material, including polystyrene (PS), polypropylene (PP), cyclic olefin copolymer (COC), and cyclic olefin polymer (COP). The assay biology itself and sometimes the preferred detection system might influence the choice of microplate material.

The microplate surface treatment must be well understood to maximize the overall success of an assay and to minimize variability and misleading data interpretations. There are numerous microplate surface modifications available to enhance a preferred biological feature, such as cell attachment for a cell-based assay or surface adsorption of a capture molecule for an ELISA. Alternatively, surface modifications can be selected to minimize an effect, such as protein adsorption by an enzyme or other assay component. One critical point to be aware of while investigating surface treatments is the within-well, intra-plate, and inter-plate uniformity of the surface treatment. It is recommended to have a QC workflow either within the assay development lab or with the vendor to ensure that the variability of the surface coatings are understood and within the tolerance limits. Such a workflow can include an investigation of uniformity (see also Assay Guidance Manual chapter HTS Assay Validation), reference compound performance, and assay robustness metrics (e.g., Z', signal-to-background ratios).

There are additional components of the assay workflow that can have an impact on the selection of an optimal microplate for an assay and screen. Two of the most impactful components of an assay system are the assay temperature sensitivity and the microplate lid. To minimize rework of the microplate selection process, it is recommended to decide on the temperature at each step of the assay and the acceptable tolerances before beginning the selection process. The same recommendation is made for the selection of a microplate lid. There are a variety of lids available on the market, not all of which are compatible with all plate types and with all types

of automation. It is also a good idea to select the lid type with the end automation platform in mind to avoid costly delays in revalidating/redeveloping an assay due to an incompatible lid type.

Finally, many microplate vendors provide plate selection guides to facilitate choosing an optimal microplate within their product line. These guides typically provide a detailed overview of the plate types, surface treatments, and typical applications.

## Microplate well density

Microplates for HTS applications are available with various numbers of individual wells, usually from 96 to 1,536 wells per plate, each with distinct advantages and disadvantages (Figure 3, Table 1). Microplates with 6, 12, 24, and 48 wells per plate are also used for certain lower-throughput and/or specialized applications including HCS (e.g., *ex vivo*) and experiments which may require manual manipulations of the microplate well contents. In general, the reaction volume per well decreases with increasing numbers of wells per plate. Overall, the cost per well is relatively constant as higher-density microplates are more expensive (Table 1). However, miniaturization reduces the quantity of reagents required for each well, which reduces the cost per test point. Additionally, working with higher-density plates reduces the total number of plates required to perform a screen, which decreases the labor required, processing time, as well as wear and tear of the equipment. Thus working with higher-density plates can be a cost-effective strategy. However, miniaturization can present new technical challenges for the assay as well as the need for instrumentation capable of dispensing compounds and reagents into higher-density formats. The overall throughput will ultimately depend on a variety of technical factors including instrumentation (e.g., plate reader speed, with well-by-well reads slower than whole-plate reads) and properties of the assay itself such as readout stability and reagent availability (e.g., precious or difficult to culture cells).

*Micro-surfaces*. Note that surface area only refers to gross surface area and does not account for microscopic effects due to surface asperities (i.e., unevenness of surface, roughness, ruggedness). Some of these asperities are due to the microplate production process (molds for microplates are machined so that the microplates will retain the inverse of the mold surface features). It has been observed that such surface asperities can affect certain biological assays. For more information, see the subsequent section “Troubleshooting and Special Topics – Microplate Surfaces and Associated Properties”.

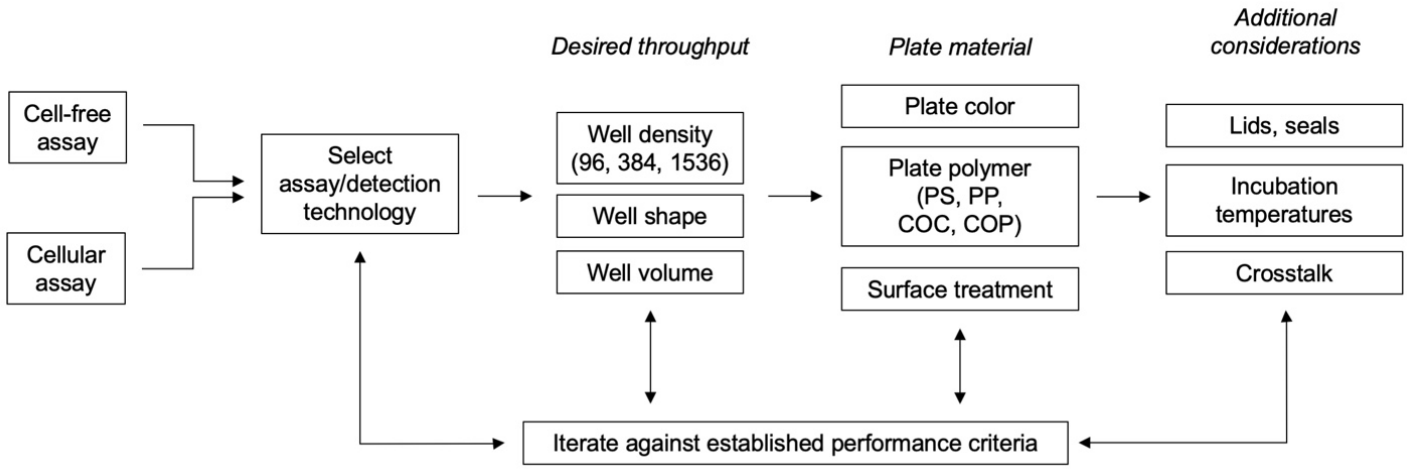



**Figure 2.** Flowchart for a microplate selection process. This flowchart provides a potential workflow to identify an optimal assay microplate for screening. In practice, the selection of a microplate is usually an iterative process that must balance competing factors such as automation compatibility, cost, availability, and performance.

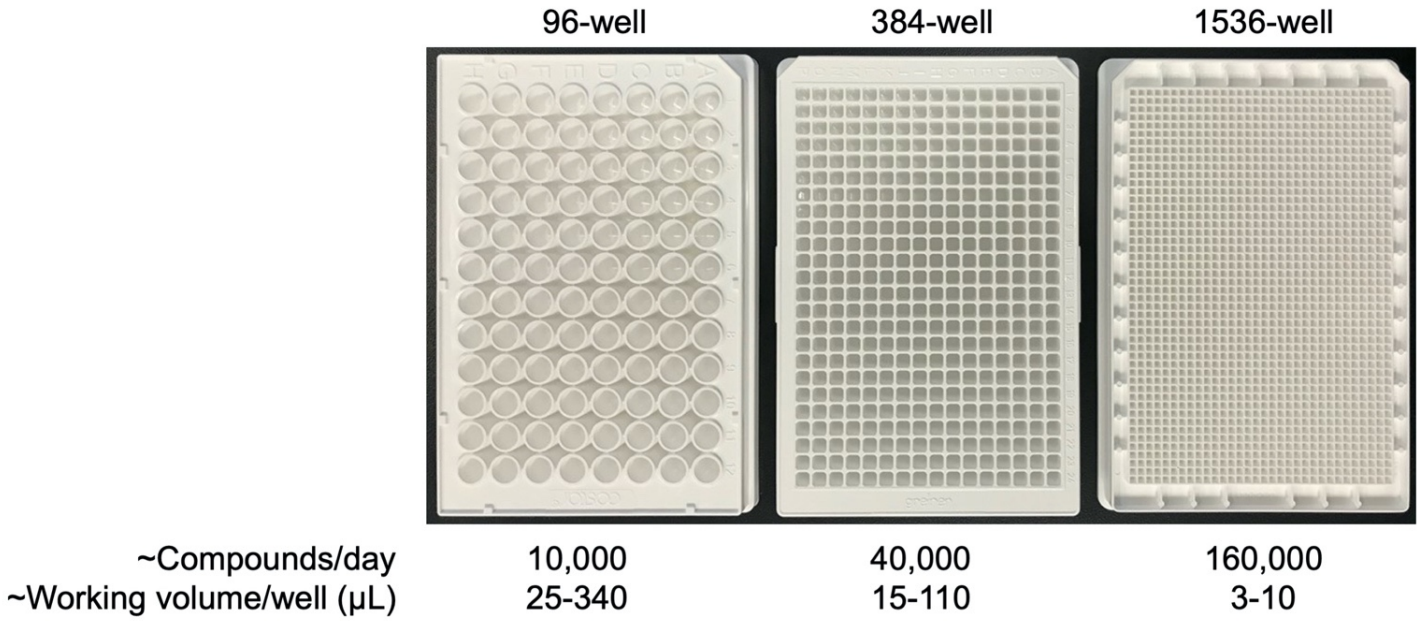



**Figure 3.** Typical *in vitro* assay microplate densities. Examples of the plate density of 96-, 384-, and 1,536-well microplates. Per microplate, increasing well density enables more compounds to be tested per experiment.

**Table 1.** Comparison of microplates with circular, F-bottom wells. Exact characteristics will vary based on individual microplates. For sample comparison of cost per microplate well, data was taken from a major microplate manufacturer for otherwise identical black, F-Bottom, TC-treated, sterile plates with lids.

| Number of wells | Typical gross well surface area (GWSA, $mm^2$)[a] | Typical working surface area (WSA, $mm^2$)[b] | Typical well volumes (µL) | Typical well working volumes (µL) | Typical working volume/ plate (mL) | Cost/ microplate (USD) | Cost/ well (USD) | Pros | Cons |
|---|---|---|---|---|---|---|---|---|---|
| 96 | 259 | 34 | 250-382 | 25-340 | 12-33 | $2.74 | $0.03 | Supported by most instruments and manufacturers; adaptable to both manual and automated workflows | Increased reagent per well; required reagent volumes can present challenges for supply |
| 96 half-area | 173 | 15 | 125-175 | 15-75 | 1.5-17 | | | Reduction in reagent use compared to conventional 96-well microplates | More limited product catalog |
| 384 | 140 | 10 | 100-152 | 15-145 | 6-56 | $5.43 | $0.02 | Supported by most instruments and manufacturers; adaptable to both manual and automated workflows | Overall volumes can be limiting and expensive for custom reagents for uHTS |
| 384 low-volume | 48 | 5 | 10-30 | 4-25 | 1.5-9.6 | | | Reduction in reagent use compared to conventional 384-well microplates | More limited product catalog |
| 1,536 | 26 | 2 | 10-15 | 3-10 | 5-15 | $20.20 | $0.01 | Cheapest per well<br>Least reagent per well | Specialized instrumentation<br>Mixing can be limited to diffusion<br>More limited product catalog<br>Difficult to work with in a manual mode |

[a] Does not consider micro-surface areas; spherical well shape using formula GSWA = $2\pi rh + \pi r^2$
[b] Does not consider micro-surface areas; spherical well shape using formula WSA = $\pi r^2$

## Microplate color

The choice of microplate color and base material is usually based on the type of assay readout, most often to maximize the signal-to-background ratio (Table 2). Microplates generally come in four colors: clear, white, grey, and black:

- Clear microplates are typically used for absorbance (colorimetric)-based readouts.

    - Note that most conventional polystyrene microplates are not suited for UV light transmission. UV-transparent microplates are often made from COC or COP which can typically measure wavelengths as low as 230 nm.
- White microplates are most often used for luminescence-based readouts. The white color (often from titanium dioxide, $TiO_2$) can reduce well-to-well crosstalk, while enhancing the luminescence signal by better reflecting the light.
    - Example assay technologies include AlphaScreen (and related technologies such as AlphaPlex, AlphaLISA; PerkinElmer), time-resolved fluorescence (TRF), time-resolved fluorescence-resonance energy transfer (TR-FRET), homogeneous time-resolved fluorescence (HTRF; Cisbio), and bioluminescence resonance energy transfer (BRET; e.g., NanoBRET, Promega).
    - Can be used with fluorophores that have longer half-lives (µsec to msec), as the background autofluorescence has usually diminished when the assay readout is measured.
    - White microplates can be "dark-adapted" to reduce background phosphorescence by the plate materials. This is typically done by placing the microplate in reduced light conditions for ten minutes before reading the microplate.
- Black microplates are well-suited for fluorescence-based readouts, which have higher signal intensities than luminescence and therefore do not need the reflective contributions from the white color. Similar to white microplates, the black color (often from carbon) can reduce well-to-well crosstalk, while also reducing background autofluorescence and phosphorescence from test compounds, reaction mixture components, and the microplate itself.
    - Example technologies include fluorescence intensity (FI), FRET, and fluorescence polarization (FP).
- Other less common colors include grey and yellow microplates for specific assay technologies. Grey is an attempt to compromise between white and black microplates, and proprietary grey plates have been developed for certain applications such as Alpha technology. Yellow plates have been developed for certain TRF applications to reduce background autofluorescence. An intra-run comparison between specialized and conventionally-colored microplates is usually the best approach to objectively determine if there are any benefits to such boutique microplates.
- Suboptimal choice of microplate color will often manifest as [1] lower signal-to-background ratios compared to the optimal microplate color, and/or [2] well-to-well crosstalk when highly active and inactive samples are adjacent to one another.

Microplates can also have clear-bottom wells to facilitate optical measurements from the bottom, including high-resolution microscopy. For many cell-based assays, it can be highly advantageous to use a clear-bottom microplate to enable spot-checking of the cells for proper cell density and morphology.

**Table 2.** Comparison of microplates by color. The optimal choice of microplate color is usually based on the assay technology to maximize the signal-to-background ratio.

| Microplate color | Recommended assay formats – top read | Recommended assay formats – bottom read | Notes |
|---|---|---|---|
| Clear | Colorimetric/Absorbance | Colorimetric/Absorbance | Consider COC/COP when needing near-UV light transmission |
| White | Luminescence<br>AlphaScreen/AlphaPlex/ AlphaLISA<br>Time-resolved fluorescence (TRF) | | Reduces well-to-well crosstalk<br>Enhances luminescence by reflecting light |
| White with clear bottom | | Colorimetric/Absorbance<br>Luminescence | Enables cell visualization in cell-based normal and confocal assays |

*Table 2. continued from previous page.*

| Microplate color | Recommended assay formats – top read | Recommended assay formats – bottom read | Notes |
|---|---|---|---|
| Black | Fluorescence intensity (FI)<br>Fluorescence-resonance energy transfer (FRET)<br>Fluorescence polarization (FP) | Fluorescence intensity (FI)<br>Fluorescence-resonance energy transfer (FRET) | Quenches background/nonspecific fluorescence<br>Reduced well-to-well crosstalk |
| Black with clear bottom | | Fluorescence intensity (FI)<br>Fluorescence-resonance energy transfer (FRET) | Enables cell visualization in cell-based normal and confocal assays |
| Grey | AlphaScreen/AlphaPlex/ AlphaLISA | | Reportedly optimized for Alpha-based assays |

## Microplate well shape

The selection of a microplate well shape usually follows the choice of assay technology. Microplate wells typically come in round, square, or rounded-square geometries (Figure 4).

- Round wells have less total area compared to square wells, which can be advantageous for minimizing reaction volumes. Also, the shape of a round well promotes mixing in a circular vortex, whereas vortex mixing in a square well (described below) is not optimal. The round corner-less shape may reduce certain intra-well effects and the lack of corners eliminates wicking effects (see below).
- Square-shaped wells with flat bottoms maximize the area for light transmission and hold a larger volume of liquid than round wells. Depending on the surface tension and viscosity of the liquid sample dispensed into a square well, the geometry can result in a slow wicking of liquid up along the 90° angles of the well corners towards the top of the plate. For example, organic solvents including methyl *tert*-butyl ether, acetonitrile, and methanol are known to be susceptible to wicking. This can result in sample accumulation near the top of the wells, facilitating evaporation and possibly cross-contamination between wells. Wicking can also make it difficult to retrieve the entire volume of liquid within a well. The use of square wells with rounded corners (Figure 4) can reduce the phenomenon of wicking, which does not occur in round wells (10). See also subsequent section Troubleshooting and Special Topics – Well-to-Well Contamination.

The bottoms of wells can be flat (F-bottom), conical (V-bottom), rounded/spherical (U-bottom) or flat with a rounded edge (C-bottom):

- Flat-bottom wells (F-bottom) allow for the maximum transmission of light and are well-suited for bottom-reading applications, including total fluorescence intensity measurements from plate readers or high-content imaging from automated microscopes and screening systems. Additionally, flat bottom wells are ideal for adherent cell cultures grown as monolayers.
- Conical bottom (V-bottom) wells enable maximal retrieval of small sample volumes or the concentration of small particles. This well shape was originally designed for hemagglutination assays, where a small pellet of red blood cells could be concentrated at the tip of the V-shaped cone (11).
- Rounded/spherical bottom (U-bottom) wells are intended to facilitate mixing, washing and coating as well as the retrieval of solutions from wells. Rounded wells may also decrease trapped air at the bottom of the well, enabling a more consistent meniscus level from well-to-well. This well shape is often used with suspension cells and spheroid or organoid cultures (12).
- Curved bottom (C-bottom) wells combine features of both the flat bottom and round bottom wells, with a flat bottom suitable for optical measurements, but with rounded corners to facilitate mixing and washing (11). Also, this well shape is well-suited for use with suspension cells, spheroids, or organoid cultures (12).

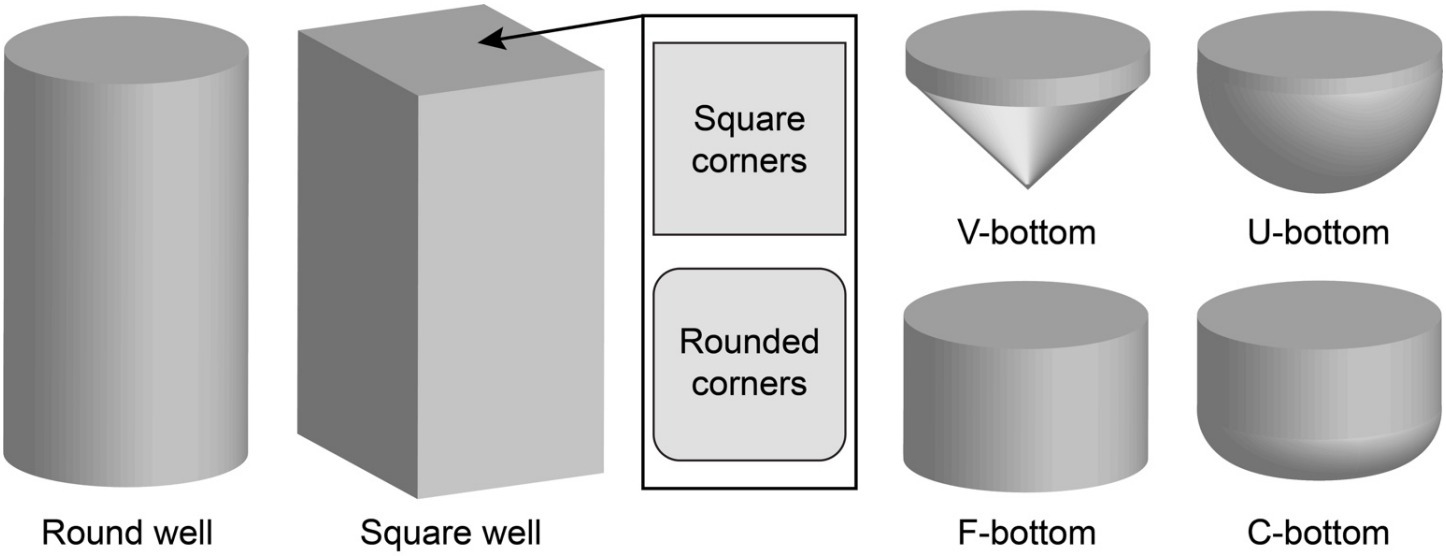



**Figure 4.** Schematic of microplate well shapes. Microplate wells generally have either a round or square shape. Square-shaped wells can have either straight or rounded corners. Wells can have a variety of bottom shapes including conical (V-bottom), rounded/spherical (U-bottom), flat (F-bottom) and flat with rounded corners or curved bottom (C-bottom) that endow the well with different properties.

## Microplate well volume

Microplates wells have a usable "working volume" and are manufactured as normal and low-volume variants (Figure 5). At a certain point, assay performance declines by simply reducing the reaction volume and specialized microplates can enable a lower working volume. Lower-volume variants are usually constructed by tapering the well along the vertical dimension, and/or reducing the well depth. When utilizing low-volume microplates (especially those with reduced well depths), users should carefully ensure their compatibility with instrumentation. For example, pin tools would need to have their depth reduced for low-volume microplates to avoid damaging the pins, and certain microplate readers may benefit from different read heights to accommodate altered sample heights.

## Microplate polymers

The most common microplate materials are the polymers polystyrene (PS), polypropylene (PP), COP, and COC (Figure 6). Each of these polymers has advantages and disadvantages (Table 3). For example, COP has many favorable properties including low autofluorescence, UV transparency, and mechanical stiffness (13).

The choice of microplate polymer can impact assay quality due to properties such light transmission, autofluorescence, water absorption, and gas exchange. Light absorption by a polymer decreases sensitivity in fluorescence assays by attenuating the intensity of the excitation illumination, while autofluorescence from a polymer can decrease sensitivity and dynamic range by contributing additional light to the assay emission signal. The common polymer PS is generally not suitable for transmitting UV light, while COC/COP are often used for such purposes. The autofluorescence from a microplate can also vary depending on the plate material will also vary based on the pigments used in generating the color.

Polymers can also have different propensities to absorb water and exchange gases (13). Finally, polymers have different mechanical and thermal properties (Table 3). The mechanical properties are important considerations during the manufacturing process, but more relevant for end-users, day-to-day handling where overly brittle or flexible polymers can crack or warp with aggressive handling, respectively. Thermal properties can play a role when sealing plates with heat sealers, as excessive or prolonged heating can result in warping of the plate (small

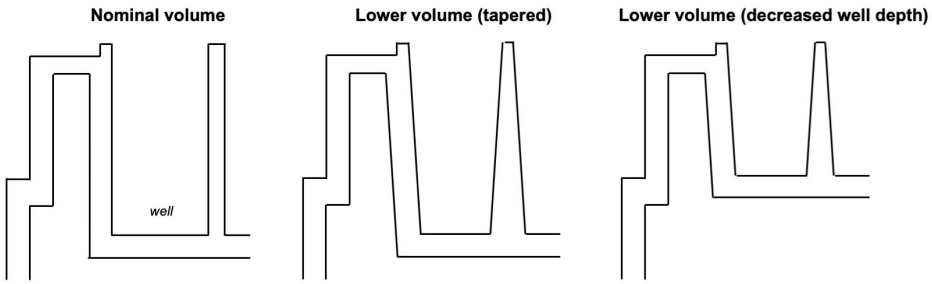



**Figure 5.** Comparison of microplate volumes. Lower-volume variants are available from some plate manufacturers. Volume reduction is usually obtained by tapering the well shape along the vertical plate axis and/or reducing the well depth.

degrees of bending or torsion can cause significant perturbations) and also issues with imaging or handling of the plates by automated systems.

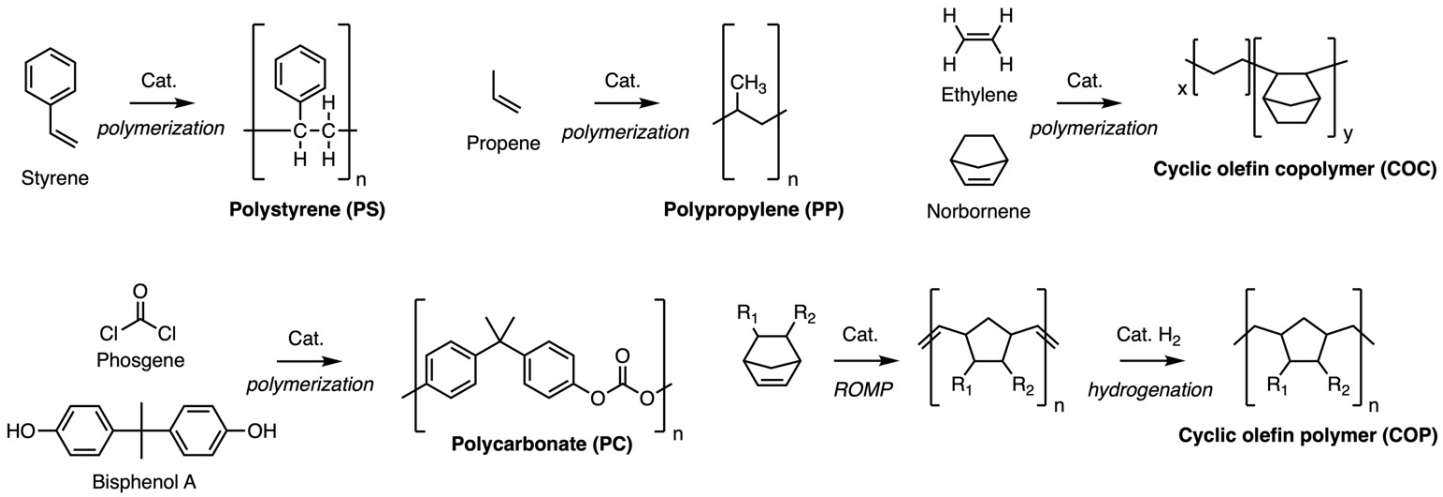



**Figure 6.** Chemical structures and general syntheses of common microplate materials. For the most common microplate materials, monomers are polymerized with the aid of catalysts to yield the desired polymer.

**Table 3.** Comparison of thermal and mechanical properties of microplate polymers. Note that COP behaves mechanically similar to PS during molding. Adapted from Niles and Coassin (13). COP, Zeonor 1420R; PS, high-impact, injection mold-grade.

| | COP | PS | PP |
|---|---|---|---|
| **Flexural modulus (GPa)** | 2.2 | 3.3 | 0.55-2.41 |
| **Flexural strength (Mpa)** | 94 | 103 | 23-50 |
| **Tensile modulus (Gpa)** | 2.4 | 3.4 | 0.5-7.6 |
| **Tensile strength (Mpa)** | 61 | 80 | 12-43 |
| **Glass transition temperature (°C)** | 136 | 90-104 | 44-148 |

## Microplate surfaces and treatments

The surface properties of microplates are fundamentally determined by the chemical composition of the plate polymer. Olefins like PP, COP, and COC consist of saturated hydrogen and carbon only. These materials tend to be very hydrophobic and present high contact angles with water when untreated by standard plasma and corona discharge systems (reminder: hydrophilic surfaces have lower contact angles with water droplets, while hydrophobic surfaces repeal water droplets and produce higher contact angles). These hydrophobic surfaces have a low surface energy and low protein adsorption. PS is an example of an unsaturated hydrocarbon-based material that has aromatic substituents which confer partial positive and negative charges, and as a result, untreated PS will adsorb more protein to the surface than untreated olefins. Polycarbonate microplates (now rarely used due to high leachables like bisphenol A ("BPA") and heavy metals; and high fluorescence backgrounds) are an example of a polymer with aromatic functionality and heteroatom contributions due to the presence of oxygen in the polymer.

The primary purpose of cell culture treatment to microplates is to make the microplate surface more amenable to mammalian cell culture. Enhancing wettability and providing surface charge enables cell adhesion molecules to "attach" firmly to the surface *via* charge neutralization. There are two primary techniques that have been used for decades to surface-modify plastics: [1] radio frequency microwave plasma (RFMP) and [2] corona discharge *via* high voltage in regular atmosphere. Both of these processes generate covalently-modified surfaces. The RFMP technique is generally preferred for scientific applications because of the uniformity of the surface treatment. RFMP is a vacuum-based process whereby electric fields create plasma (charged gas) from a carrier

gas (commonly $CO_2$, $O_2$, or $NO_2$). These gases are released at very low pressures into a reaction chamber, and collisions between the charged gas molecules and the microplate surface result in covalent attachments of the gas to the surface, effectively creating chemical functionality. If $CO_2$ is used as the carrier gas, a carboxyl surface with a negative charge is generated; if $O_2$ is used, an ether and hydroxide surface that is polar but neutral is generated; and if $NO_2$ or $NH_3$ are used, an amine surface is generated with a positive charge. The $CO_2$ or $NH_2$ that are incorporated into polymer surfaces can also be exploited to attach molecules with an appropriate conjugation chemistry. This primary surface treatment is essential if a secondary treatment is necessary.

Additional chemical functionality can be conferred to the surface with the use of special chemicals and modified plasma instruments (Table 4). Epoxide groups, tri-amino-hydrocarbons, non-fouling (e.g., resists protein adsorption) low-adhesion molecules, diethyl ether, and azides are a few relevant examples. It should be noted that surface treatments alter the surface but not the bulk polymer. These treatments are durable but have limitations and must be protected from excessive heat. It can sometimes be helpful to think of the polymer as a very viscous liquid that can slowly rotate and invert the charged functionality away from the microplate surface, which can effectively eliminate the original surface treatment modification (14).

Secondary treatments can typically include liquid-based biologicals such as poly-D-lysine (PDL), poly-L-lysine (PLL), laminen, collagen, hydrogels, or cellular extracts. These surface treatments are used because they can enhance adherence for specialized cells and can be critical for more biologically relevant morphology, growth, migration, and differentiation of many cell types. In practice, these biological materials do not adsorb well to untreated polyolefin or polystyrene plates. Therefore, when commissioning a secondary treatment (often done for special cell-based assay applications), it is important to make sure that the original microplate product is "TC-treated" first. Another important consideration is whether to have the secondary treatment performed locally or by a manufacturer.

There are anecdotal reports that fresher secondary treatments perform better, presumably because over time the microplate reverts to the neutral/unmodified polymer surface (PA Coassin, unpublished observations). As this phenomena has not been widely documented (i.e., treatments most susceptible to degradation, magnitude of degradation), scientists should define early-on in the project the acceptable performance criteria for surface-treated microplates (e.g., less than 20% deviation of historical max control signal), and then include periodic experiments to detect shifts in assay performance over time. Approaches for detecting performance shifts and drifts are discussed in subsequent sections ("Special Topics and Troubleshooting – Microplate Lots).

**Table 4.** Comparison of microplate surface treatments. For cell-free assays, surfaces can be treated to enhance or attenuate biomolecule binding, as well as functionalized with reactive functional groups (e.g., maleimides, amines) or affinity reagents (e.g., streptavidin) to anchor specific biomolecules. For cell-based assays, surfaces must be tissue-culture treated to functionalize the surface; following this, additional secondary treatments such as the addition of extracellular matrix (ECM) coatings can be performed to enhance adherence for specialized cell types.

| Surface treatment | Recommended assay formats | Notes |
|---|---|---|
| Non-treated (medium binding) | Immunoassays | Usually PS which has a hydrophobic surface that passively/nonspecifically adsorbs biomolecules |
| Non-binding surfaces | Homogenous assays (including enzymatic assays) | Treated PS with nonionic, hydrophilic surface to reduce polymer-biomolecule interactions; net effect is the reduction of nucleic acid and protein binding |
| High-binding | Immunoassays | Suitable for binding biomolecules with ionized groups |
| Streptavidin | ELISA | Contains streptavidin which can bind biotin-containing reagents |
| Sulfhydryl-binding surfaces | | Contains thiol-reactive maleimides on polymer surface |

*Table 4. continued from previous page.*

| Surface treatment | Recommended assay formats | Notes |
|---|---|---|
| Tissue culture (TC)-treated | General adherent cell culture | Creates a net-negative surface charge (*via* carboxyl groups) |
| TC-treated,<br>Poly-(D ± L)-lysine (PDL, PLL) | Specialized adherent cell cultures | Non-biological reagent, therefore does not significantly modulate surface receptors<br>Creates a net-positive surface charge; can be useful for difficult-to-attach cell lines (e.g., neuronal) |
| TC-treated,<br>ECM coatings | Specialized adherent cell cultures | Critical for biologically relevant adherence, morphology, growth, migration, and differentiation in many cell types<br>Collagen I: HUVEC, hepatocytes, muscle cells, osteoclasts |

## Barcoding

Depending on the expected throughput, plates can be labelled manually or with barcodes. Many modern screening instruments are equipped with barcode readers. Barcoding enables unambiguous plate identification and reduces errors from manual labeling methods. Barcodes are typically placed on a short side of the microplate and can be left-, center-, or right-aligned. Modern barcoding can consist of an alphanumeric text, linear barcode, or matrix (2D) barcode. Many vendors offer pre-barcoded plates, which can be especially advantageous for microplates to be used in sterile conditions. Otherwise, microplates must be removed from the sterile packaging in order to be barcoded. Barcoding can also be performed on-site with appropriate instrumentation, but is often nonsterile.

Additional considerations for barcodes include:

- When using any barcode system, ensure that the barcode format is readable by the relevant instruments.
- Stability of the barcode to storage and handling conditions. Barcodes might not adhere to microplates in excessive heat or cold, or if exposed to certain solvents which might dissolve the adhesive. A common mistake is to attach non-cryogenic labels to a batch of microplates prior then place them into a freezer, only to lose all barcoding information.

## Microplate covers (lids, seals)

During various assay steps, microplates can be sealed, covered with a lid, or left uncovered. Microplate covers are generally made of clear polystyrene, while plate seals are typically made of aluminum or polyolefin acrylates. A special lid used on GNF robotic systems is designed from a solid block of stainless steel and fitted with an autoclavable sealing rubber gasket designed to increase the plate contact area with the weight of the steel lid ensuring even contact across the plate edges (15). Seals can be generated by an adhesive (e.g., silicone), or by heat (e.g., Agilent PlateLoc). Usual advantages of microplate covers are the prevention of evaporative losses, well-to-well contamination (*via* spillage or wicking), and biological and environmental contamination. Disadvantages of using seals/lids include additional costs and the procedural steps to accommodate their placement and removal. Therefore, the use of lids or seals for microplates must consider multiple factors:

- *Length of incubation time*. If assays are performed in a short time-scale (minutes to several hours), microplate covers might be superfluous as there would be minimal evaporation.
- *Reaction volume*. When using lids or seals, generally avoid filling microplate wells with greater than 50% of the well volume, which mitigates contact between the well contents and the lid.
- *Condensation*. The well contents may condense at the top of lids or seals, which can affect assay performance and even facilitate well-to-well contamination. A brief centrifugation can usually recover condensates from lids or seals prior to their removal. For certain applications, such as live-cell imaging

where lids are used, condensation can interfere with image acquisition (Figure 7A). This can be mitigated with humidified incubators within the instrument combined with a short (30 - 60 min) equilibration period prior to imaging.

- *Number and timing of assay steps*. If using lids or seals, carefully consider the timing of the placement and removal steps. For biochemical assays, it might only be necessary to cover microplates after the initiation of the biological reaction, whereas for cell-based assays it is generally recommended to cover microplates whenever there is not active dispensing.
- *Compatibility with automation and instrumentation.* Certain instruments might be able to remove and replace microplate lids. This is generally a manual process for microplate seals.
- *Detection.* For certain bottom-reading applications, the presence of lids or seals might not interfere with the readout detection (e.g., fluorescence imaging), whereas it may interfere with other applications that require light transmission between the top and bottom of the microplate (e.g., phase-contrast microscopy).
- *Sealants*. Microplate sealants have the potential to chemically modulate bioactivity or interfere with light-based readouts (Figure 7B).
- *Continuous versus end-point assay formats.* Continuous or kinetic assays with multiple detection steps might necessitate the repeated removal and addition of microplate covers, especially if multiple plates are being analyzed in a given experiment. To prevent this logistical burden, consider bottom-read options.
- *Prevention of biological contamination.* Depending on the assay, biological contamination might not be a major concern, such as simple biochemical assays. However, for cell-based assays, covering microplates is practically essential.
- *Prevention of environmental contamination.* Environmental contamination from airborne particulates (dust) might be a concern for certain light-based readouts (see also subsequent section, "Special Topics – Environmental Contaminants").
- *Permeability.* When sealing microplates, consider whether there is a need for gas and/or water permeability. Certain materials designed for such permeability, while other seals might prevent adequate gas exchange between the incubator and the microplate wells, which can modulate cell functions by perturbing media pH and oxygen content.
- *Lid stability*. Note that certain lid designs might not be as secure as others and therefore might be inadvertently opened (i.e., important for sterility) if not handled with care (Figure 7C).
- *Photo protection*. For light-sensitive applications (such as labile fluorophores or AlphaScreen), seals or opaque lids can be used to protect the well contents from light in addition to the usual light-reduction strategies.

### Section summary

A key factor in obtaining robust assay performance is the optimal choice of a microplate. During assay development and optimization, scientists should select microplates that meet their technical requirements. Key parameters in microplate selection include microplate well number, well shape, plate color, plate material, and surface coatings or treatments.

## Technology-Specific Considerations – High-Content Screening

When selecting microplates, consideration must be given to the specific assay technology. This section describes detailed considerations for high-yield specialized applications, specifically high-content imaging. *Other specialized microplate applications will be described in updated versions of this chapter or in relevant Assay Guidance Manual chapters.*

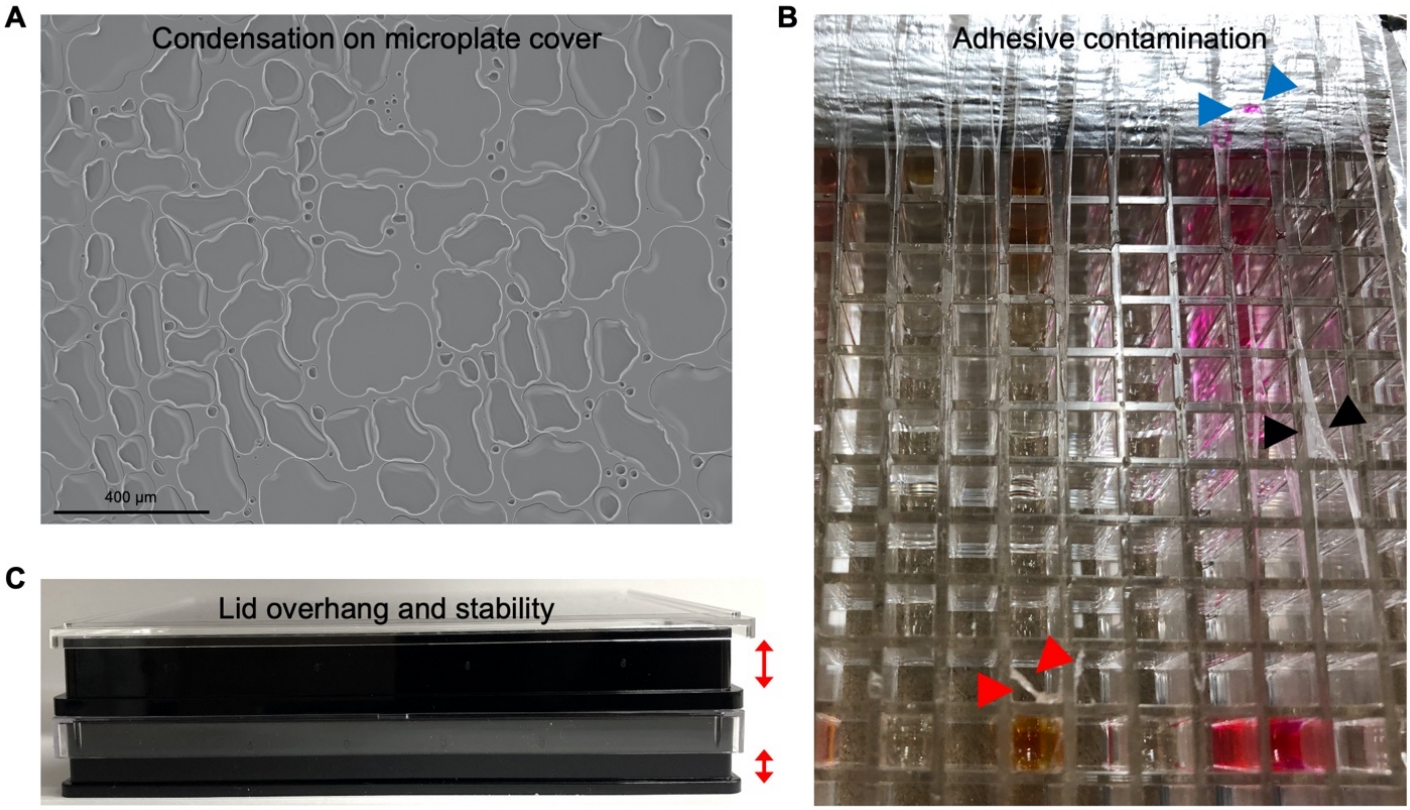



**Figure 7.** Microplate covering considerations. (A) Condensation interference. Water condensation can occur on microplate covers, which can interfere with certain imaging applications requiring light transmission, as in this case of live-cell imaging. Condensation can be mitigated by adequate equilibration (30 - 60 min) in a humidified incubator prior to image acquisition. (B) Sealant interference. When utilizing microplate seals with adhesives, be aware that sealants can adhere to the microplate (between black arrowheads) and even contaminate well contents (between red arrowheads). Also note that the well contents can adhere to the interior surface of the seal, even after a brief centrifugation (between blue arrowheads). Shown is a clear 384-well polystyrene microplate sealed for six months (RT, desiccator) with an aluminum-based adhesive seal. (C) Microplate lid stability. When utilizing some microplate lid designs, be aware that the lids may not descend as completely toward the microplate flange (see top plate, gap indicated by red arrows), which can make the lids susceptible to inadvertently opening if not handled carefully.

## Considerations for choosing microplates in high-content screening applications

High-content screening (HCS, also used interchangeably with high-content imaging (HCI), high-content analysis (HCA), or image cytometry), encompasses a platform of automated computer-assisted fluorescent and transmission-light microscopy to rapidly capture images of cells or objects (16). Most HCS experiments begin with the process of seeding cells into microplate wells but may also include microscope slides, petri dishes, or other specialized vessels. Generating reliable and reproducible HCS data requires high-quality images, therefore careful considerations when selecting microplates is a key driver during experimental design.

There are several commercial choices for "image compatible" microplates for HCS applications, with most microplates constructed with black walls to reduce fluorescent signal crosstalk from adjacent wells and fabricated with an optically clear bottom (COC/COP, glass, or quartz) for transmission of fluorescent light to the cells or objects of interest when using inverted microscope systems. The ideal microplate bottom surface should be uniform with a bottom thickness of approximately ~170 µm (equivalent to number 2 cover slip) to match microscope objective lenses with high numerical apertures (NA; e.g., 0.17 mm). While imaging-compatible microplates are readily available from commercial suppliers, there remain a few key considerations when

selecting microplates for HCS applications that include the cell model being investigated, the microplate type, design and composition materials as previous described in the chapter, and the optics on HCS imager including the autofocus mechanism to identify cells or objects in the microplate wells. The *biology* is the most important criteria of HCS experimental design, and this will dictate which cell model is used and the subsequent microplate selection, both of which may or may not be ideal for HCS imaging, therefore understanding the limitations and challenges to generate high-quality images is important to gain insights into the biological question.

Even with the advances in manufacturing and fabrication of microplates and improved HCS imaging instrument technologies, there are still challenges and obstacles to consider when matching microplates with the optics of the HCS imagers. Ultralow attachment (ULA) microplates are designed to force cells into the center of the well bottom to physically form 3D spheroid-like structures (12). One of the challenges in ULA microplate use is not all cells form spheroid-like structures and may not remain in the center of the well bottom due to manipulations or handling microplates during treatment, staining, or other automation manipulations. The outcome may lead to subsequent challenges in image capture of objects in ULA microplates versus flat-bottom microplates (Figure 8).

## HCS cell models

Since the cell model is likely the most important criteria of the biological experimental design to study disease or address a biological question, the selection of the microplate type often is usually not considered early during the experimental design process. Furthermore, not all cell types are amendable to miniaturization in microplate format or can even be sustained on conventional microplate materials. In HCS applications, cell models are categorized as adherent, suspension, microtissue, *ex vivo*, or even small organisms (e.g., *Caenorhabditis elegans*, *Danio rerio*). Some cell models may require additional growth enhancements for attachment, survival, or to promote a biological response in the artificial environment of microplates. These enhancements may include growth factors, PDL, ECM, or other matrices. Both 2D and 3D cell models are widely used in HCS imaging applications and although the selected microplate dictates the type of assay, the choice of microplate will have a direct consequence on how the cell model is captured and interrogated by HCS imaging system.

*Adherent* immortalized cell lines are the most widely used cell model in HCS today. Stem cells and primary cells alone or combined with more sophisticated complex cell model systems (including mixed cell cultures such as primary human tumoroids and other normal and diseased tissue cell models) are also being used by HCS practitioners. After the selection of the microplate and secondary surface treatments (if applicable), the cell seeding process is an essential step to allow cells to naturally adhere, spread, proliferate, or remain in stasis over hours to days in optimized media and environmental conditions. For basic 2D imaging, the "flatter" the cell in z-dimension and wider in x- and y-dimensions (e.g., U-2 OS cells), the easier it is for an HCS imaging device to find and focus on the cells during the automated image acquisition step.

When using *suspension* cells in HCS applications, there are two methods to consider when choosing microplates: [1] the coating on bottom of the plate to enhance cell binding, and [2] the geometric shape of the microplate wells. The simplest coating method is PDL, PLL, or equivalent; these synthetic polymer agents help but do not guarantee cell adherence to the bottom of the microplate well. Adherence can be assisted by gentle centrifugation (i.e., 5 min, less than 1000 rpm, greater than room temperature) and is recommended during any subsequent staining process to minimize cell loss. There are other sources of chemical treatments offered by commercial microplate suppliers that have the promise to enhance cell binding such as covalently-bound coatings (e.g., streptavidin, nickel chelate, protein A, lectin wheat germ agglutin, etc.), but these need to be carefully evaluated to make sure they do not adversely perturb the relevant biology. The microplate design can also assist in forcing suspension cells to the bottom of the microplate well; U-bottom and V-bottom microplates are recommended for labeling suspension cells and for preparing higher-throughput flow cytometry experiments. Standard U-bottom and V-bottom microplates are not recommended for HCS imaging due to the thickness at the bottom of

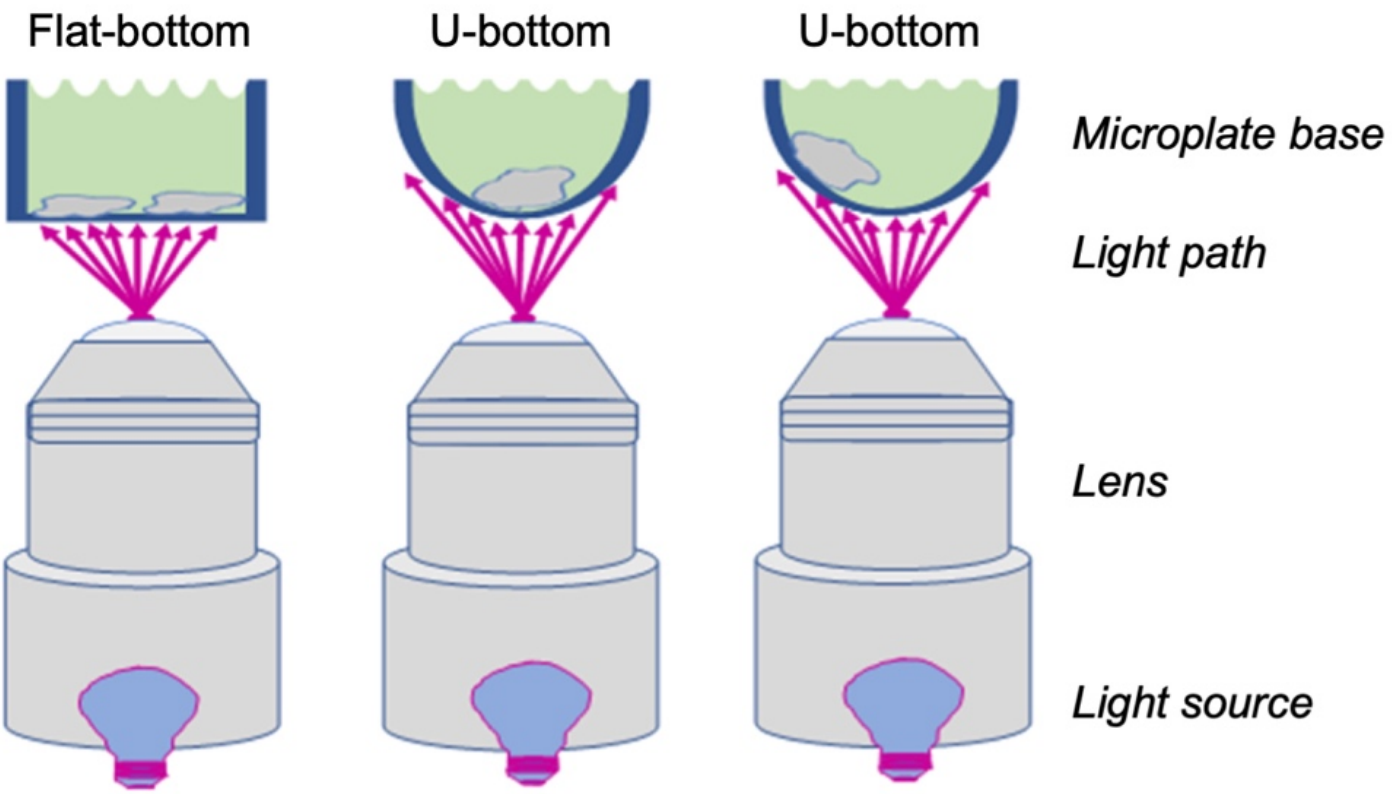



**Figure 8.** Objective lens light scatter on microplate well bottoms. Left: cellular objects in flat-bottom microwell plate; light scatter detects all objects within the well's field of view. Center: cellular objects in the center of the U-bottom microplate well; light scatter detects objects only in the center of the well. Right: cellular objects on the left curvature part of the U-bottom microplate well; due to the position, light scatter results in an out-of-focus image.

the well and the curved shape are not ideal for microscope objective lens to capture high-quality images of cell objects to allow proper image segmentation (Figure 8). Additionally, there are a few other specialized microplates with geometric designs to force suspension or adherent cells into designated growth areas including micropatterning (e.g., CYTOO and ForCyte Biotechnologies).

The use of microtissue, organoids, and spheroids has grown and gained traction in the field to better represent and recapitulate an *in vivo* response within microplate well environment (17). Microtissues typically require ECM substrates or matrices to support and maintain growth and typically form 3D structures or scaffolds. The microplate type and design may vary from ultralow attachment (ULA) to flat-bottomed microplates containing hydrogel or Matrigel matrices. *Ex vivo* tissue is typically comprised of whole intact tissue slices from organs. While this cell model should best represent the biological function of the organ, the longevity outside of the body is only a few hours or days before succumbing to cell death in the artificial microplate environment. This is not necessarily the fault of the microplate, but rather due to the lack of nutrients and other signaling cascades to sustain tissue viability outside of the body. *Ex vivo* tissue such as brain or liver slices are typically plated into 6-well, 12-well, or 24-well microplate formats due to their size and because higher-throughout experiments are often not feasible. When moving to these larger microwell plate formats, the properties of the microplate construction changes from thin bottom thicknesses (as available in 96-well and 384-well formats) to thicker bottom thicknesses that are not compatible with higher NA objective lenses (e.g., 0.17 mm). If using a higher magnification objective lenses with high NA with microplates having thick bottoms, then the image quality is less optimal, which can manifest later as poorer object segmentation and "blurrier" images (Figure 9).

*In vivo* imaging of small organisms such as *C. elegans* and *D. rerio* are imageable in microplate wells. One of the biggest challenges using adult organisms in HCS is the reduction of organism movement during image acquisition. Several different methodologies have been used to solve this technical challenge, from chemical treatment to specialized microplates to force organisms into compartments (so they remain upright or semi-stationary). Some specialized microplates have reflective mirrors built into the wells to allow investigators to observe the transverse side of the organism (e.g., ZF microplate, Funakoshi, Japan; PSI SideView microplate, ViewPoint, France).

## General considerations when matching a cell model with microplates for HCS imaging

1. When possible, use optically-clear, thin-bottom microplates.
2. Determine the resolution (magnification) requirements to answer the biological question.
3. Decide if cells are "plateable" – do they readily attach?
4. Are PDL, ECM, or other matrices required for cell attachment, for cell health, or for the biological process being studied?
5. Beware of new or undefined microplate composition materials modulating cellular health (e.g., proliferation, cytotoxicity) or relevant bioactivity.
6. Do the microplate well geometric dimensions affect the biology being studied? Consider surface area to support growth and gas exchange needs.
7. Are cells or cell objects imageable by HCS imagers? Does the microplate well have clear bottoms for inverted microscope HCS imagers to transmit light?
8. Are the cell objects plated in a manner to allow adequate image analysis segmentation?

## Microplate type and design for HCS imaging

Commercially available HCS imagers are equipped with a microscope stage that supports ANSI/SLAS standard microplate formats. To determine if a given microplate is compatible with a particular HCS imager, practitioners can consult the microplate manufacturers (often posted on microplate supplier website) to determine more exact microplate specifications such as the length, width, and height of the microplate; the well positions; well bottom elevation; the microplate shirt height; outside flange dimensions; bottom thickness; well volume; and distance between wells. These standardized microplate dimensions are critical for proper alignment to capture images of cells or other objects within the microplate well, as this information is applied to the HCS imager instrument software setup to optimize autofocusing and image capture. Some of the most important ANSI/SLAS standard microplate dimensions for microplate HCS imaging instruments are the bottom thickness and skirt height (Figure 10). The bottom thickness informs the selection of the microscope objective lenses, both the numerical aperture and working distance. The microplate skirt height determines if the outside well boundaries from the microplate geometry will physically interfere with the microscope objective lens.

### Autofocus mechanism and fluorescent probe effects in HCS imaging

1. *Autofocusing*. HCS imaging instruments use an autofocusing mechanism to determine the location of the cells or objects in the microplate well for rapid image capture. Often this is at the bottom of the microplate wells. The two most common methods of HCS autofocusing are [1] image-based autofocus, otherwise known as contrast-based image focus, and [2] laser-based autofocus (see also Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging). Both methods are dependent on the microplate dimensions, the type of materials used in the fabrication of the microplate, the liquid media interface within wells, and the preparation of microplates for the HCS assay for the following reasons: [1] some polymer based plastics may reflect light differently than the glass – the gold standard for optical systems; and [2] contaminates or impurities in plastics or well artifacts can modulate biological affects or identify objects that are not of biological

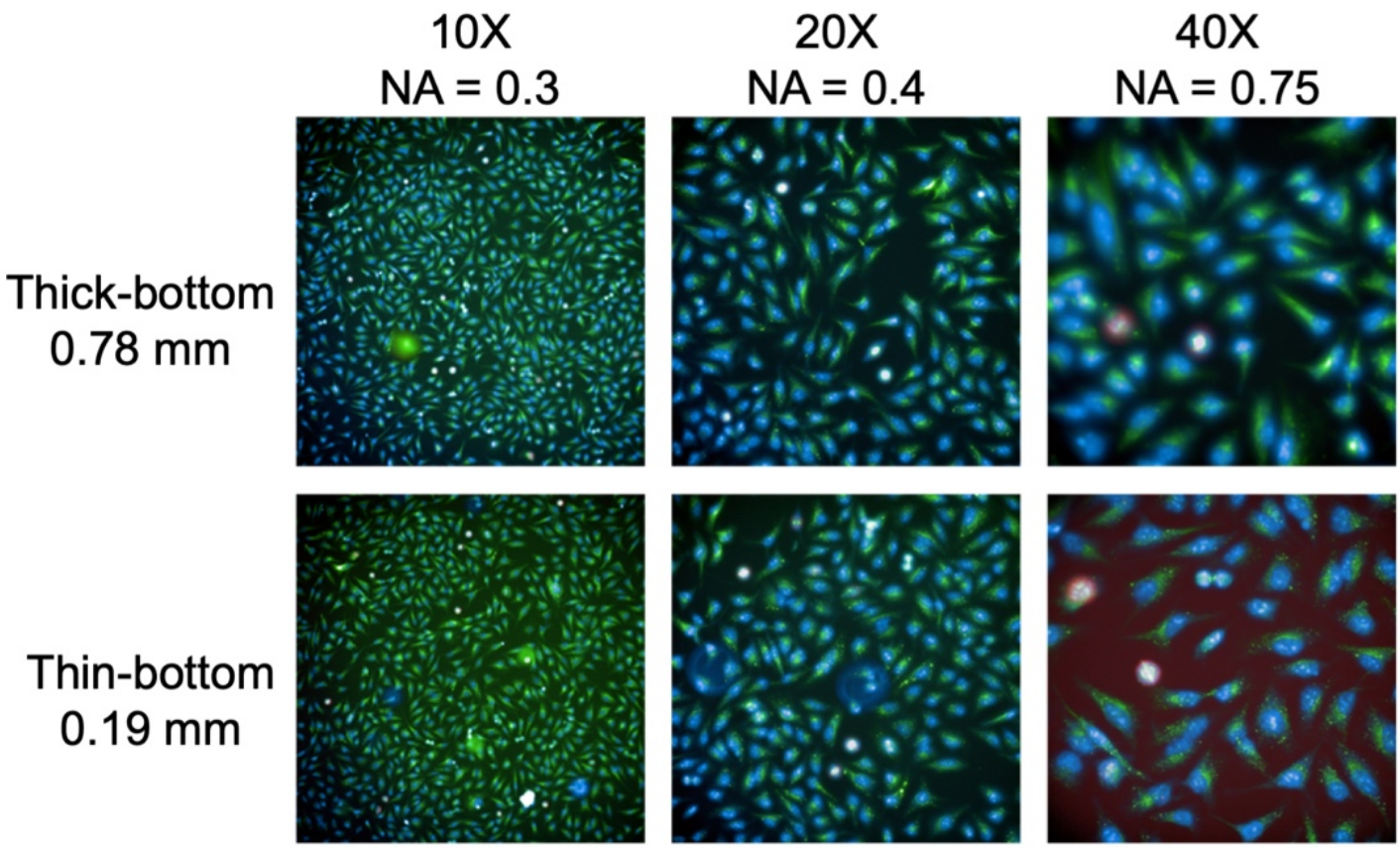



**Figure 9.** Microplate well bottom thickness compatibility with microscope air objective lenses. HCS images of cells seeded into thick (0.78 mm) and thin (0.19 mm) microplate bottoms. Left: Plan-Neofluar objective lens with a 0.3 NA with the recommended 0.17 mm coverslip thickness/microplate well bottom. However, due to the low magnification, the images of both thin and thick bottom microplate wells are indistinguishable. Center: LD Plan-Neofluar with correction collar has a 0.4 NA with coverslip thickness/ microplate well bottom of 0.55 mm. Note adjusting the correction collar moves the barrel lens elements inside the objective lens to the appropriate setting for proper focus of the image which is ideal for thin and thick microplate bottom thickness. The caveat is correction collar lenses have lower NA, thus lower light throughput. Right: Plan-Neofluar with a 0.3 NA with the recommended 0.17 mm coverslip thickness / microplate well bottom. Note the higher NA with fixed thickness of 0.17 mm allows higher light throughput for reduced exposure times and higher resolution on HCS imaging device. In this example, 190 µm microplate bottom-thickness images (bottom) are in focus and the 780 µm microplate bottom-thickness images (top) are out of focus. Therefore, match the objective lens coverslip thickness setting with a compatible microplate bottom thickness.

interest (18-20). Image-based autofocus, unlike laser-based autofocusing, is more likely to fail when objects are not detectable in the field of view or from artifacts in different z-plane space. However, image-based autofocusing is capable of finding objects when present anywhere in z-dimensional space to the limits of the working distance of the objective lens.

2. *Fluorescent probes*. Unless the fluorescent probe is contaminated, falls out of solution, or forms precipitants, rarely do fluorescence probes interfere with autofocusing process in labeled cells in microplates. However, HCS practitioners need to be cautious of possible interference in unique cases (e.g., fluorescent compounds or lint in bottom of microwell microplate; Figure 11).

## HCS imaging instrument optics and microplate specifications

Microplates selection should always be matched with the objective lens numerical aperture, working distance, depth of focus requirements, and minimal planes for z-steps to identify objects in the microplate well. Note some of these details can be found on the microscope objective lens (Figure 12A). HCS imaging instruments are

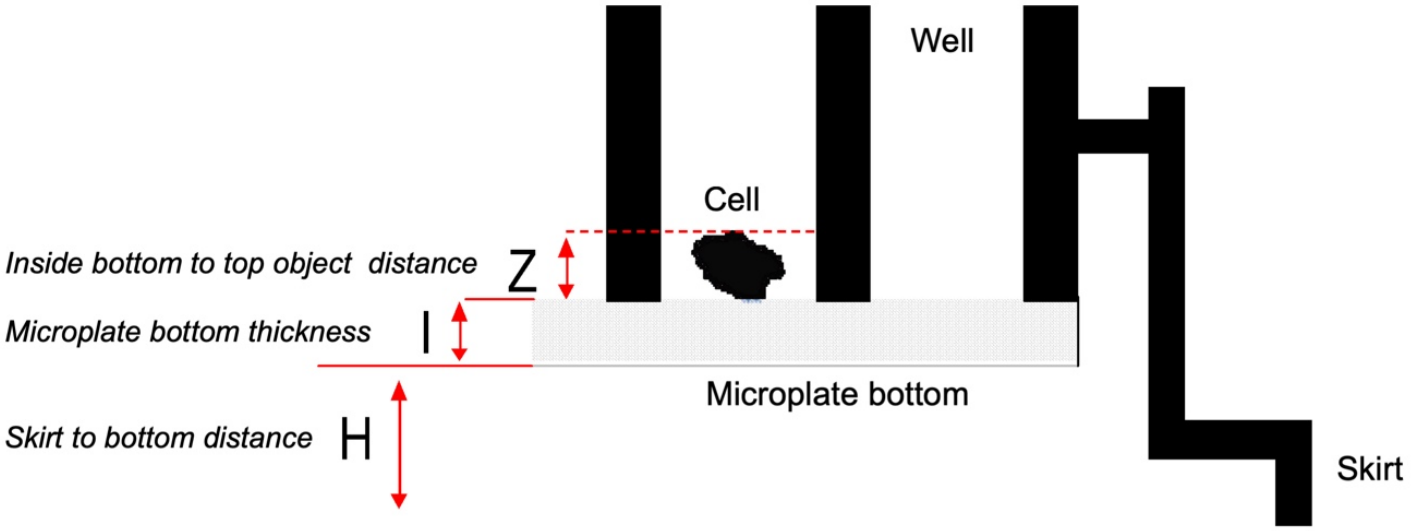



**Figure 10.** Illustration of the cross-section of a black wall HCS imaging microplate wells. "H" is the distance from the skirt to the microplate well bottom; "I" is the thickness of the microplate well bottom; Z" is the distance from the inside bottom surface of the microplate well to the top of the cellular object inside the well. The combination of H + I will determine if high numerical aperture objective lenses can be used; and the combination of H + I + Z will determine the recommended working distance for the objective lens.

commonly equipped with commercially available microscope objective lenses (e.g., Leica, Nikon, Olympus, Zeiss) to enable multiplexing fluorescent probes. These objective lenses can also reduce chromatic aberrations using plan achromat lenses that use normal working distances. However, HCS instruments can also include long-working objective lens equipped with correction collars to more flexibly dial-in and match the microplate bottom thickness.

Designing, developing, and validating experiments need to consider all aspects of the microplate specification along with these five key features of the objective lens (Figure 12A):

1. *Magnification power*. Selection of the objective lens magnification power enlarges the object size, but *it does not necessarily improve the resolution power*.
2. *Numerical aperture (NA)*. The NA of the objective lens is an important consideration because it indicates the resolving power of a lens (e.g., the size of the finest detail which is directly dependent on the proportional to λ/NA, where λ is the wavelength of the light transmitted). The higher the NA, the more light that enters through the objective lens to the specimen, resulting in a signal increase (intensity) that can be detected by a camera, a photomultiplier tube, or another detection device. Reducing the exposure times helps to reduce photobleaching and phototoxicity. *A lens with a larger NA will be able to visualize finer details than a lens with a smaller NA*.
3. *Working distance(WD)*. The WD is commonly referred to the distance between the interface of the objective lens and the in-focused objects (usually cells) in the microplate well. *As the WD decreases, the magnification power of the objective lens increases.*
4. *Depth of field*. Depth of field (a.k.a. depth of focus) is the tolerance or range of the optical path to keep objects in focal clarity within the desired image plane(s) captured. *High NA objective lenses have a smaller depth of focus range than low NA objective lenses.*
5. *Minimal Z-plane interval spacing*. Lens and HCS instrument manufacturers provide specifications of the minimal z-axial intervals for resolving power based on the Nyquist theorem. Do not over-sample 3D objects; only use the minimal or higher z-intervals to capture objects. In HCS confocal and widefield systems, the spatial resolution of a 3D object (voxel) is typically about one-half of the x-y lateral

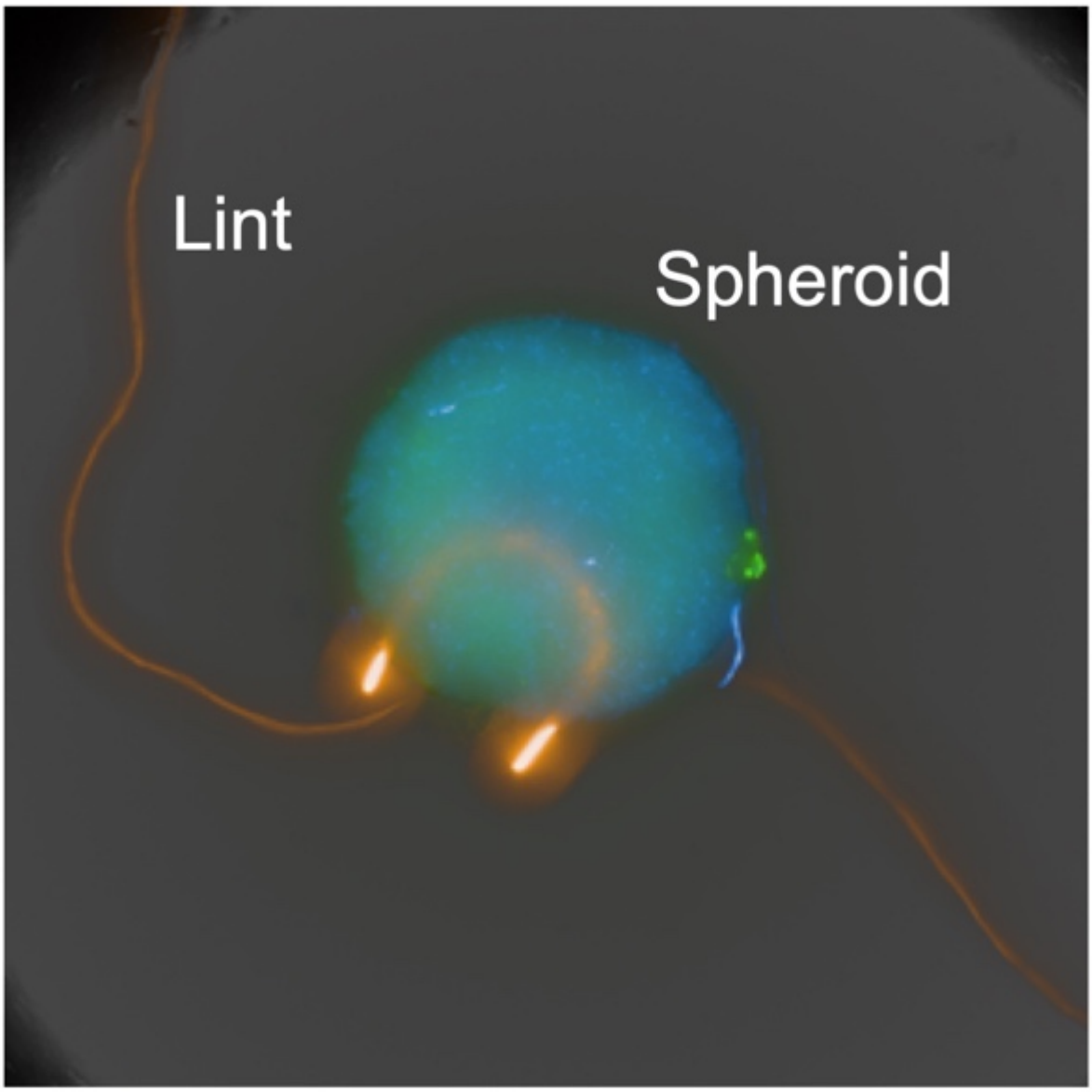



**Figure 11.** Laboratory contaminant interference with HCS autofocusing. 3D spheroid image from 96-well ULA microplate well labeled with Hoechst nuclear dye (blue) captured with 5X objective lens. Artifact lint is pseudocolor in orange.

resolution. *Use the recommended minimal (or higher) z-plane intervals to best capture objects in 3D.* Acquiring images with lower z-plane intervals will not improve the overall rendering of large 3D objects, and can be counter-productive because it will create longer acquisition times, increase the potential for phototoxicity or photobleaching, generate unnecessary data, and slow down the subsequent image analysis process. For example: if the minimal z-plane specification of the objective is 1 µm, then use a 1 µm or higher step size; adjusting the image acquisition to 0.5 microns with the intention of capturing a better image by over-sampling will *not* improve image analysis segmentation. Caution: oversampling images greater than the volume of a 3D object could result in out of focus image planes, which may affect subsequent image analysis segmentation.

Overall, high NA objective lenses are designed and engineered with short working distances (WD). The most commonly used high NA objective lenses equipped on HCS imaging instruments are engineered with 0.17 mm working distances, which are ideal match for number 1.5 microscope coverslip equivalence or microplates with approximately 170 µm bottom thicknesses. Some objective lenses are engineered with a correction collar with a

tunable adjustment on the lens to correct for microplates bottom thicknesses. However, the NA in these lenses are typically lower, and these adjustable microscope objective lenses are useful for both thin bottom or thicker microplate bottoms that exceed 500 µm. In cases where microplate bottom thickness are unknown or outside of the specifications of the objective lens, then it is recommended that all microplates or objective lenses be tested for overall compatibility. Objective lenses should be appropriately paired with numerical aperture to match the microplate bottom thickness (Figure 12B).

### Interference from foreign materials

Since HCS imaging instruments are equipped with very sensitive charge-coupled devices (CCDs) or complementary metal–oxide–semiconductor (CMOS) camera detectors to capture dim fluorescent signals, HCS practitioners should be cautious of any debris, lint, or other foreign matter that may obscure the light path and the autofocus processes. Such contaminants can be introduced during screening operations from "dirty" work areas and from liquid handing devices during compound/reagent delivery, microplate washing, or other workflow processing steps. This usually occurs when dispensers inadvertently inject salt solutions to the bottom of the microplate, or when concentrated salt solutions form as a result of well-to-well contamination. Interferences may also be introduced while performing live-cell imaging when plate seals are not used, and media or buffer overflows the edge of the well height and spills onto side or bottom of microplate (Figure 13). Salts and other foreign materials on the bottom of HCS microplates can interfere with image acquisition and even damage lenses when immersion-based objective lenses are used. Another type of interference that can affect the optical path is scratches on the bottom surface of the microplate wells. This is more common in microplates with low or non-existing skirt heights, or glass bottom microplates that are more prone to scratching then polymer-based plastic microplate bottoms (n.b., recall discussion of hardness related to COC/COP). The interior well bottom can also be damaged by processes such as the scratch wound assay, micropatterning, or other automation manipulations that employ apparatuses in close proximity to the bottom surface. The effects of such artifacts can be mitigated by [1] maintaining a clean work environment, [2] systematically examining the bottom of microplates by visual inspection, [3] gently removing dust and lint with air blowing, and [4] for salt residue contamination, applying copious amount of $dH_2O$ to the area and drying with a clean lint-free paper cloth (e.g., Kimwipe).

## Microplate Usage

In addition to the choice of microplate, another key aspect of robust assays is determining the optimal use of microplates. In other words, even with the perfect microplate, assays will only be as robust as the experimental design and processes they are subjected to during an experiment. This section describes several topics in microplate usage, including microplate storage, mixing, dispensing, wicking, and other miscellaneous best practices.

### Microplate storage and shelf-life

Microplates are generally very stable laboratory consumables. In order to maximize the shelf-life of microplates, they should not be exposed to extreme conditions, such as extreme heat or cold. Always consult the vendor documents for recommended storage conditions. Key issues to consider include:

- *Sterility*. Plates used for tissue culture should be stored such that the sterile packaging is preserved.
- *Warping*. Over time, especially when subjected to temperature extremes or mechanical stresses, microplates may warp, which can adversely affect their performance. For example, warped plates may interfere with automation processes or assay detection if the microplate wells have even slightly-altered positions (different read heights, x-y coordinates).
- *Degradation*. Certain microplates with special chemical treatments have limited stability. In such cases, assays should be timed such that they can be performed within the validated stability period. If new

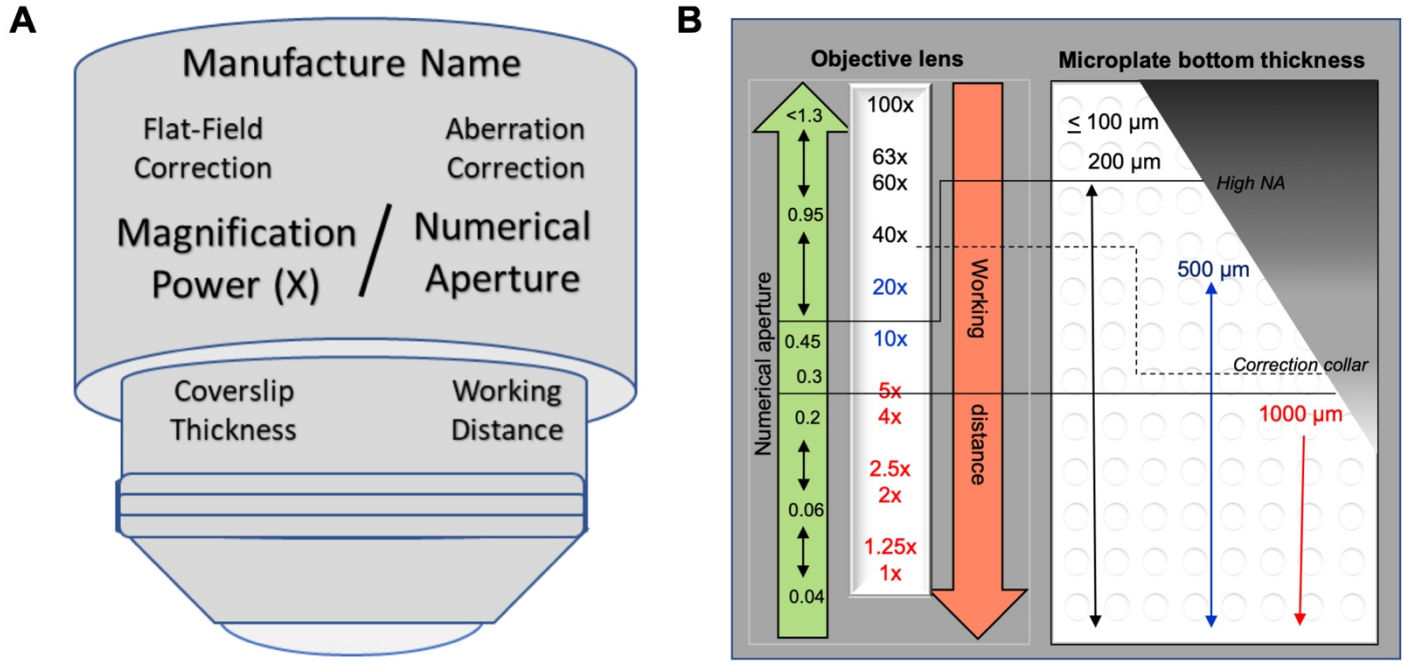



**Figure 12.** Lens-microplate compatibility considerations for HCS applications. (A) Outside anatomy of microscope objective lens. The information is generic placeholder standard for commercial suppliers. The objective lens is labeled with information about the lens including the magnification power, numerical aperture, and coverslip (microplate bottom) thickness, and working distance. (B) Guidelines to match HCS instrument objective lens with the microplate bottom thickness.

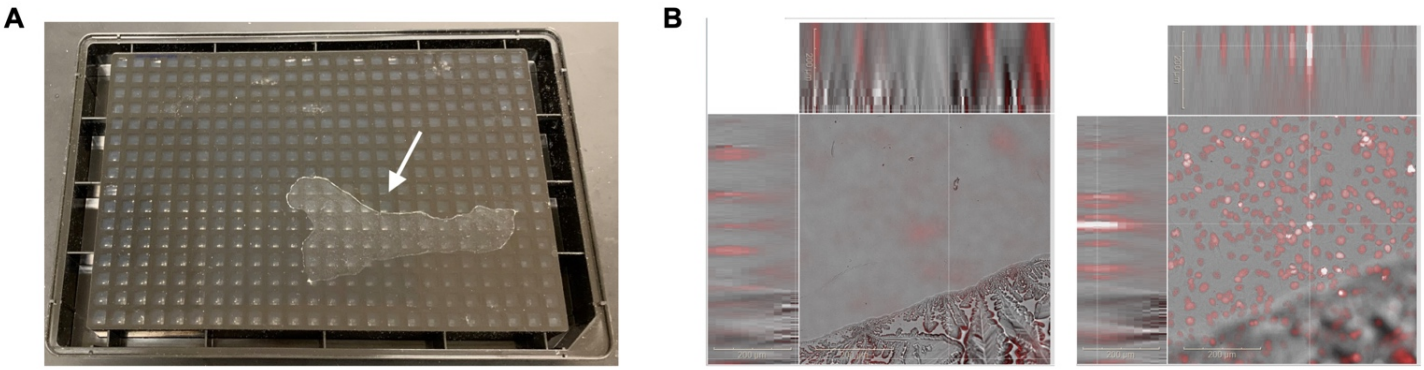



**Figure 13.** Examples of salt residue from PBS interfering with HCS image quality. (A) Salt residue area from buffer on bottom surface of the 0.19 mm-bottom microplate (B) Brightfield and fluorescent 3D image captured with 10X objective lens. Left: 3D image showing z-position of salt residue in focus at bottom of the microplate well. Right: cells labeled with DRAQ5 (red) showing in-focus z-position.

microplate lots are used, procedures should be in place to identify and account for batch effects. Such batch effects can be detected by using reference compounds in every experiment and comparing assay performance metrics (e.g., Z', signal-to-background ratios, max and min signal), along with specialized data analyses such as control charting (see also "Special Topics and Troubleshooting – Microplate Lots" section for suggested processes to detect batch effects or changes in assay performance over time).

- Certain specialty treatments might require specialized storage conditions, such as refrigeration or protection from light.

- *Assay support*. When performing HTS and structure-activity relationship (SAR) support, consider the length of time the microplate-based assay will need to be performed, and whether it is feasible to maintain the same lot or supply of a given microplate.
- *Inspection*. Carefully inspect microplates from a new case for acceptability, checking for warping (lay microplate on flat surface and check for any wobbling) and cracks (often from shipping and handling).

## Mixing

For most assays performed in microplates, several dispensing steps are required to combine the assay reagents and test compounds. These various components must be equilibrated to produce robust data. For example, most compounds are delivered into aqueous solutions from DMSO stocks. DMSO droplets tend to sink to the bottom of microplate wells and slowly diffuse without agitation.

Mixing is *occasionally* performed for microplate-based assays, and this section is included for completeness should mixing be necessary depending on the nature of the bioassay (e.g., simple diffusion might not be an acceptable mixing strategy). Whereas individual microcentrifuge tubes can be inverted or vortexed at the benchtop, such practices are typically avoided with microplates (even sealed microplates) given the number and proximity of samples. The design of the microplate and the desire for throughput necessitates some special considerations with respect to mixing microplate well contents (21).

There are several important considerations with respect to mixing in microplates. The reaction volume is one primary consideration for microplate mixing. Standard 96- and 384-well plates can often be adequately mixed with simple orbital shakers. With 1,536-well plates, the capillary-like dimensions of the wells do not always create a robust vortex. However, the small volumes are more amenable to simple diffusion. For example, sodium ions can diffuse at approximately 0.5 mm in under two minutes (with 100% water and no other ions). Assuming a similar diffusion rate for other assay components, standard pre-incubation times might be sufficient for high-density microplates. Another important consideration is the time and desired throughput. Depending on the protocol, mixing may take several minutes. Users should carefully consider the nature of the assay to determine what lengths of mixing time are acceptable for a given workflow. For instance, kinetic assays with early time-points might place a premium on fast mixing after a reaction is initiated. By contrast, endpoints after several hours of incubation might be more tolerant to longer mixing protocols. *In general, mixing is not done with cell-based assays*, as it may perturb cell function or even disperse adherent cells. For certain cell-free systems, assay components such as detergents and highly proteinaceous solutions have the tendency to form bubbles, which can interfere with some light-based readouts.

There are several technologies available to mix microplate contents, including:

- *Orbital shaking*. Orbital plate shakers or mixers operate by producing hundreds-to-thousands of small-diameter revolutions per minute, effectively creating a vortex within each microplate well. Orbital mixers are available that can fit multiple plates at once, or single plates that can be fitted into automation lines. When using these devices, users should carefully validate mixing protocols to ensure no spillage and adequate mixing.
- *Pipetting*. With this method, a percentage of the well contents can be aspirated and re-dispensed. This approach is straightforward but requires consumables (i.e., each well requires a new pipette tip or thoroughly-washed tip) and certain compounds may preferentially adhere to pipette tips.
- *Diffusion*. In this method, well contents are allowed to diffuse over time unperturbed. This is the slowest but simplest mixing method.
- *Reagent addition*. Additional media/buffer (which may include additional reagents) can be added *after* compound addition to mix compounds. Efficient mixing typically occurs when added volumes are at least 25% of the final assay volume.

## Dispensing

There are a variety of approaches for dispensing reagents, cells, and test compounds into microplate wells. The technical details of dispensing assay components into microplates are non-trivial, as imprecise or inaccurate dispensing can reduce the assay performance. In general, care should be taken to minimize the time between dispensing components into the first and last wells.

For reagent and cell dispensing, the main approaches are single- or multi-channel pipetting, single-well dispensers (e.g., BioRAPTR), and multi-well dispensers (e.g., MultiDrop). Each approach has pros and cons:

- *Manual pipetting*. This enables the greatest customization or flexibility, but can be susceptible to user errors and is also the slowest method for dispensing into microplates. Manual pipetting is generally not appropriate for low microliter volumes.
- *Single-well dispensers.* Dispensing into one well at a time allows for more customization of plate maps compared to multi-well dispensers, but at the expense of time. Also, the time delay between dispensing into the first and last well might introduce systematic errors. However, dispensing a reagent from a single tip compared to multiple tips increases the precision and minimizes the variability among dispenses.
- *Multi-well dispensers.* Dispensing into multiple wells at a time (usually column-wise) increases the overall throughput, but can restrict plate layouts such that any given row or column must contain the same reagents. Also, each tip will dispense volumes with a slightly different accuracy, which can cause striping patterns in the data.

For compound dispensing, the main approaches are single- or multi-channel pipetting, noncontact dispensing, and pin tool transfer. The choice of a compound delivery method for microplates can have substantial ramifications on the apparent bioactivity and false-negative rates (22). Each approach has pros and cons:

- *Pin tool transfer.* Utilizes pins to directly transfer a fixed volume (usually 20 - 200 nL) from a compound source plate to an assay plate already containing reagents (23). The advantages of this method include: its speed of dispensing and acceptable precision, and the reduction in consumables (pipette tips) by the reuse of pins. Disadvantages include: [1] fixed-volume dispensing with a given pin type; [2] the destination plate layout is dependent on the source plate layout; [3] the need for washing steps and solvents, as well as the risk of compound carryover or contamination from incompletely washed pins; [4] the need to carefully tune methods based on plate geometry, compound volume, and solvent; and [5] the requirement to deliver compounds into destination wells with solvent ("wet dispense").
- *Non-contact dispensing.* Usually performed by acoustic ejection or piezo sample distribution (24,25). With acoustic droplet ejection (ADE) technology, nanoliter volume droplets are ejected from a source plate to a destination plate, with significant advantages being [1] its ability to deliver compound to multiple destination wells, [2] the elimination of consumables (pipette tips) and contamination sources, and [3] the ability to deliver compounds into destination wells without solvent ("dry dispense") to generate assay-ready plates (ARPs) prior to performing an assay. Disadvantages include: [1] the need for specialized source plates; [2] decreased speed relative to pin tools as only one well is dispensed at a time; [3] introduction of compound-solvent-microplate interactions by virtue of dry-dispensing (n.b., can be avoided with modified protocols utilizing solvent-loaded destination plates). The size and appearance of compound droplets can vary due to the volume transferred, excipients, microplate type, and storage conditions (temperature, humidity, time), which can thereby affect assay performance (25,26).

ADE dispensing requires the assay plate to be inverted to receive the nanoliter acoustic-ejected drop from the source plate. When the source plate contains compounds dissolved in DMSO, several items need to be considered, particularly in cell-based assays. Although cell-based assays have been enabled with ADE technology using assay ready plates, many assays with adherent cell lines require prior seeding of the plates with cells before compound addition (27). The challenges involved with using ADE technology in a direct manner

have been reviewed (28). The main issue is wicking or dripping of cell media from the wells upon inversion which is not an issue for high-density 1536-well plates where the capillary forces are very high (Figure 14). The severity of the liquid loss or wicking upon inversion of lower well-density plates (96-, 384-well) can depend on the well shape, coating, as well as media composition. Direct addition of compounds to 96-well plates has been enabled using a workflow where 70% of the well volume is aspirated before the acoustic dispense to prevent the media from draining out of the wells upon inversion (30 µL was left in the well). In another case, as most of the remaining liquid pooled to the corners of the well, a dispense protocol (using an Labcyte Echo acoustic dispenser) was implemented where the 2.5 nL DMSO droplets were ejected towards the side of the well (less than 1 mm from the side of the well wall), which preserved the cell monolayer (29). Media was then added back following this acoustic dispensing step (29,30). It has also been noted that acoustic addition immediately after media exchanges where the sides of the well walls may still be wet can lead to similar issues as observed in 96-well plates (31). In general, it is important to monitor this during assay development of cell-based assays to ensure the dispensing of compounds by ADE is not adversely affecting the cells.

Compound-solvent-microplate interactions can have substantial impacts on assay performance. Pin tool dispensing into microplates appears to result in more validated hits compared to ARPs, while freezing ARPs (-20 °C) and longer storage times can result in lower hit confirmation rates compared to refrigeration (4 °C) and "just-in-time" use in one study utilizing a biochemical luciferase assay (22). Furthermore, even the choice of microplate material can affect the false-negative rate, as COP results in fewer false-negative hits compared to PS (22). There are likely several important chemical principles explaining these observations. Compounds directly applied to microplate polymers can be potentially sequestered by microplate plastics or precipitated due to a combination of organic solvent evaporation and water uptake from ambient air. We note there may be additional significant relationships between (a) assay performance, (b) compound dispensing methods, (c) assay format (i.e., cell-based versus biochemical, buffer/media composition), and (d) other microplate properties (e.g., other polymers, surface treatments) that have not been reported in the literature.

Some other miscellaneous points with respect to dispensing assay components into microplate wells include:

- *Dispensing speed.* For automated dispensers, note that the dispensing speed can have several effects on assay performance, including mixing. For cell-based assays, excessive flow rates may damage cells by turbulence.
- *Location of dispensing flow.* Contents can be added to microplate wells at either the center of the well or towards a side/corner. The specific location may need to be adjusted to optimize reagent mixing, minimize disruption to cells, or to prevent intra-well effects.
- *Bubbling.* Poorly optimized dispensing can create bubbles, which can adversely affect many light-based readouts. Bubbling most often occurs with detergent-containing solutions and highly proteinaceous solutions, in combination with excessive flow rates and poorly-directed flow directions.
- *Calibration of microplate dispensers*. Any instrument that dispenses a liquid including compounds or reagents (e.g., buffer, media, cells) should be calibrated for accuracy and precision. For the dispensing of low volumes (e.g., pin tool, acoustic dispenser) this is most practically accomplished by dispensing known amounts of fluorescent or colored dyes into each well by the instrument in question and back-calculating the dye concentrations, with the concentration differences being attributable to errors in the dispensing volume (e.g., ARTEL system). For higher volume liquid dispensers commonly used to deliver reagents, this is typically accomplished by a gravimetric analysis. By verifying the accuracy and precision of liquid handlers, scientists can better exclude liquid handling as a source of systematic bias when there are apparent positional effects. In general, such calibration should occur *at least* annually (preferably quarterly with high-use instruments) given the importance of accurate and precise dispensing in microplate-based assays. Users should consult instrument manuals for recommended calibration frequency.

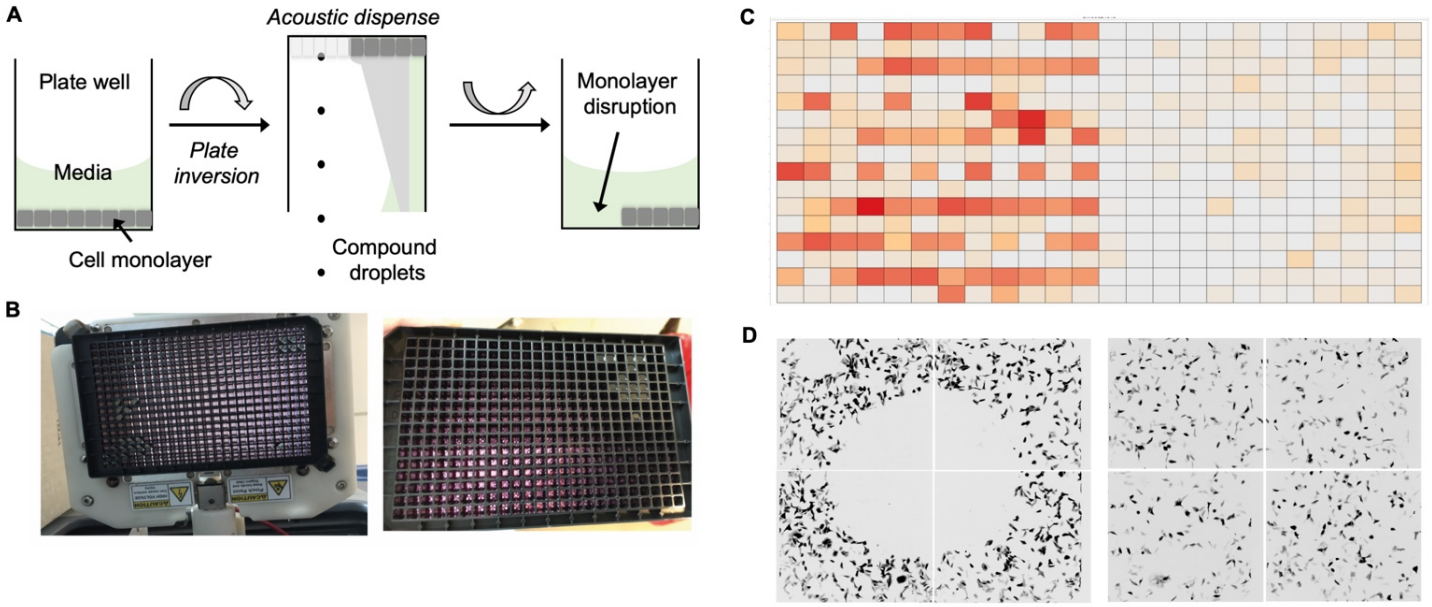



**Figure 14.** Special considerations for using ADE with cell-based microplate assays. (A) Illustration of the ADE dispense artifact. Plates are inverted for acoustic dispensing which may lead to displacement of the well liquid toward the top of the well. Acoustic dispensing of nanoliter DMSO droplets into the nearly-dry well disrupts the cell monolayer in the dispense area. The plate is reinverted and the cells are lost within the dispense area. (B) Photo of inverted plate showing wicking wells on a Labcyte Echo acoustic dispenser (left) and example of what is often observed once the plate is finished. (C) Heatmap showing wells where holes in the cell monolayer were detected by imaging (analysis of the cell monolayer was performed by Cell Profiler; red wells show loss of cells in wells). The left half of the plate used acoustic dispensing from a DMSO source plate (90% DMSO/10% $H_2O$, v/v), while the right half was not dispensed by ADE. The plate was imaged immediately after the acoustic dispense. (D) Example of a hole in the cell monolayer caused by the ADE in a wicking well (left) and an undisturbed cell monolayer (right).

## Centrifugation

Centrifugation can have a place in various steps of a screening platform. It is a critical component in compound source plate management where a spin before removal of the microplate seal reduces the potential for well-to-well cross contamination. For cell and biochemical microplate assay systems, centrifugation has been used as a surrogate for mixing, especially in the 1536-well format, where well volumes are small and the physics of the well sizes themselves challenge a successful mix with 'traditional' mix strategies such as shaking (linear, orbital, double orbital) or pipetting.

Centrifugation of microplates can be incorporated into experimental protocols at various steps, with some potential advantages (Figure 15). Briefly centrifuging microplates can [1] eliminate dead space at the bottom of the well when the dispensed reagents do not completely settle; [2] eliminate bubbles which can interfere with imaging and light-based readouts (32,33); [3] recover droplets adhering to the side and lids of microplates; [4] pellet suspended particles which may interfere with top-read applications; and [5] enhance settling of adherent or suspension cells. Each of these benefits can increase the overall well-to-well consistency in certain settings.

Centrifugation has also been used as a 'time zero' timestamp for compound/enzyme preincubations in biochemical assay systems as it enables immediate and consistent contact of compound and enzyme solutions across a microplate without loss of reagent. Depending on the detection technology, centrifugation can also play a critical step in reducing variability; for example in radioactive filter binding assays, where scintillant addition is the final step before detection, a centrifugation step before the read ensures contact of the scintillant with the filter and thereby reduces well to well variability due to uneven scintillant dispensing. *However, in some detection technologies, centrifugation will increase variability by creating uneven menisci across the microplate* (MJ Wildey

and colleagues, unpublished observations). This can be difficult to predict *a priori* based on the assay technology and experimental design, but in general it is found in top-read assays. If one canvases the literature, one will find a wide range of recommendations on the value and use of centrifugation as part of *in vitro* microplate-based assay systems. As with many components of our screening toolbox, validation of the impact of centrifugation in your specific automated workflow is critical and highly recommended (e.g., compare performance of two plate groups, one centrifuged and one not centrifuged).

Some technical considerations for centrifuging microplates include:

- Adding centrifugation increases the number of steps in an assay protocol and can decrease the overall throughput. Centrifuging after the final dispensing step (rather than after each dispensing step) might be sufficient, which should be experimentally validated.
- Most larger laboratory centrifuges can accommodate microplates with special holders or buckets. Note that most centrifuges can only accommodate 10 to 20 microplates at a given time, which might necessitate batching and adjustments to optimize the automation and workflow.
- Microplates are generally spun at weaker *g*-forces than tubes, usually less than 400 x *g* for only 1 - 2 min.
- Note the temperature of the centrifuge. Some laboratories may have the default temperature set to 4 °C, which can adversely affect certain reagents or cells. For most applications, the centrifuge should be set at 25 °C.
- Note that the centrifuge chamber is non-sterile and might contain airborne particles. Therefore, utilize lids or seals for microplates to prevent their contamination.

## Incubation practices

Depending on the assay design and operation, microplates may need to be incubated at specific temperature and atmospheric conditions. For assays that require incubation at above-ambient temperatures, it must be noted that heating and is hardly instantaneous and not necessarily uniform even in an incubator (Figure 16A). Depending on the amount of thermal mass in the microplate, the heating gradient, and surface contacts between the microplate and temperature sources, it might take several hours to achieve a temperature equilibrium. An example illustrating the importance of surface contact is PCR microplates, which feature separate V-wells that can individually contact the PCR heating block for rapid and uniform heating and cooling. In large oven incubators, there is the potential for a significant heating gradient whereby microplates closest to the heated air flow or higher vertical positions are at higher temperatures than more distal microplates.

The practical implication is heating gradients and incubation practices can contribute to systematic biases (34,35). To mitigate systematic biases related to microplate incubation, there are several recommended strategies and considerations:

- During assay development and operational validation, investigate the effect of plate positioning on the assay readout using uniformity plates positioned at different orientations. The presence of gradients that respond to plate positioning and orientation is suggestive of a bias.
- Minimize the opening and closing of incubators whenever possible to prevent perturbations in heating and atmospheric conditions.
- Avoid simply stacking microplates on top of one another when incubating for prolonged periods of time, as this can prevent airflow along the top and bottom surfaces of the interior microplates and create an uneven heating gradient.
    - A simple and economic solution for smaller labs that do not have industrial-scale plate incubators might be the use of 3D-printed “plate hotels” which can separate microplates but enable easy handling and close proximity within standard incubator ovens (Figure 16B).
- For cell-based experiments, utilize continuous temperature and $CO_2$ monitoring.

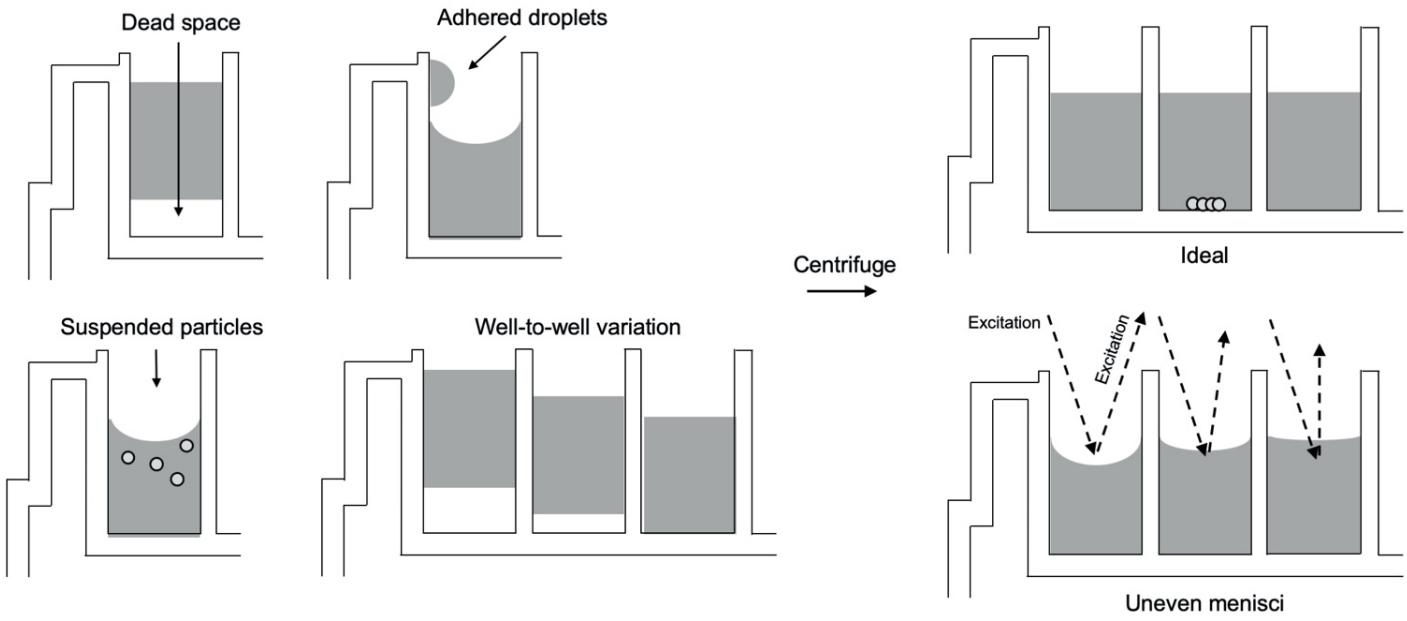



**Figure 15.** Potential benefits of centrifugation. Briefly centrifuging plates can eliminate dead space, recover droplets from the microplate walls, pellet suspended particles, and create more uniform reactions. Note that in certain cases, centrifugation can produce uneven menisci, which can be counter-productive as it can introduce additional variability (e.g., certain top-read assays).

- Depending on the assay, consider periodically rotating the position and/or orientation of microplates (this is most amenable with prolonged incubations), or randomizing the position of the microplates within incubators with respect to plate order. Regardless, it is prudent to note the location of each plate in case systematic biases need to be investigated after-the-fact.

### Section summary

Often overlooked, robust assays depend on the proper use of microplates. During assay design and validation, scientists should consider the use of microplate handling including centrifugation, mixing, dispensing, and incubation steps.

## Troubleshooting and Special Topics

This section describes special topics relating to microplates including batch-to-batch variability, well-to-well contamination (e.g., wicking), edge effects, microplate surfaces, and plate washing.

### Microplate lots

It is tribal knowledge amongst seasoned screeners that microplates are susceptible to variations among batches that can add variability to assay results. This usually occurs when the manufacturer changes something between batches, such as the raw materials (different composition or even as subtle as a different source of the same material), the microplate mold, or some variable during the manufacturing process. One practical approach to mitigate this batch-to-batch variability is to purchase microplates from the same batch or lot whenever possible (a similar tactic is used for cell culture media, where scientists often purchase bulk animal serum from the same lot). Scientists should calculate the expected number of plates for assay optimization, screening, follow-up assays, and SAR support.

After the assay plate has been selected, the best practices are to secure enough plates to complete the project (such as a screen or SAR support) for at least one year. It is also recommended that a set (case/sleeve) of these

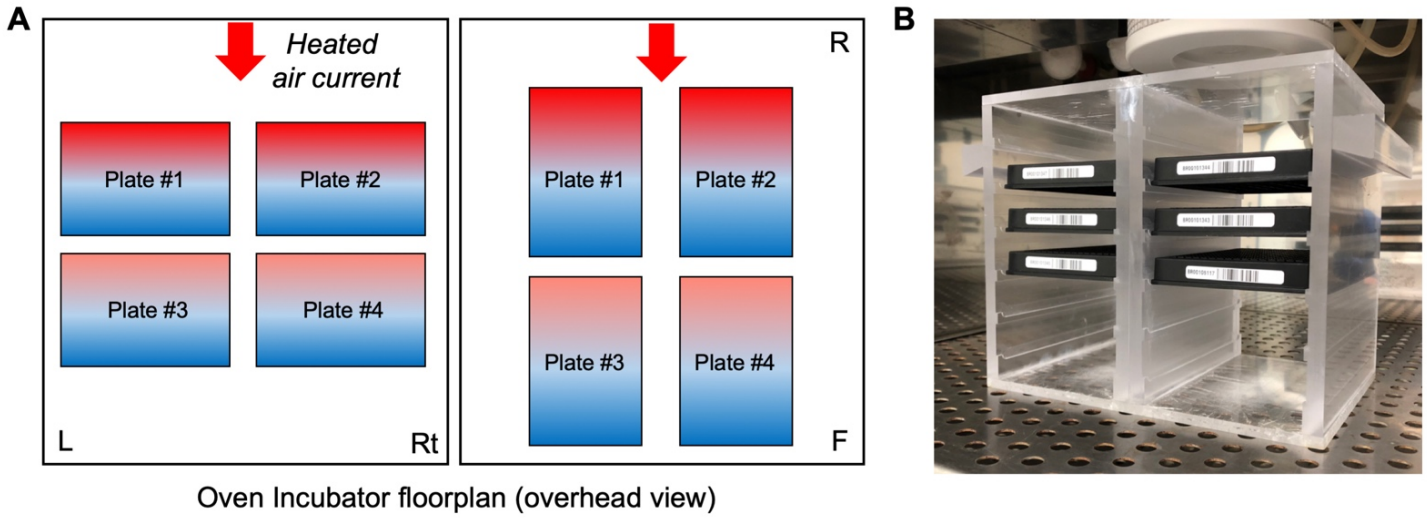



**Figure 16.** Effects of microplate positioning on systematic biases during incubation. (A) In oven incubators, the positioning and orientation of microplates can introduce systematic biases due to heating gradients. Both individual plates and the wells within these plates, might experience different temperatures depending on their proximity to heating elements. This temperature differential might perturb the assay readout (e.g., alter cell growth, denature or enhance enzymes). A bias that shifts upon microplate rotation (compare left and right panels) or re-orientation is suggestive of an incubation-based systematic error. (B) A microplate hotel for compact storage and uniform microplate incubation. Microplate hotels can be assembled by 3D printing. Within the hotels, microplates can be placed in close-proximity inside standard oven incubators while enabling airflow between the plates.

validated plates be set aside for comparison testing with future plate orders. The Replicate-Experiment format as described in the HTS Assay Validation AGM chapter is one tool that can be used to compare the performance of a new batch of microplates to an existing validated batch when using identical compounds and reagents on each microplate batch tested in parallel. The testing-mode minimum significant ratio (MSR; potency analysis) should be used for SAR support and minimum significant difference (MSD; efficacy analysis) for single-concentration screening (36,37). An Excel template is available to simplify the data analysis. Other approaches to detecting performance drift due to batch effects or degradation include control charting (e.g., Levey-Jennings charts) of assay metrics such as signal strength, signal-to-background ratios, and Z'.

## Well-to-well contamination (including wicking)

Due to the proximity of samples within a microplate, well-to-well contamination can be a significant and frustrating source of error. An otherwise perfectly-optimized assay can fail to reliably interrogate bioactivity if well-to-well contamination occurs. The consequences of such contamination are assay-specific, and depend on the amount of contaminants, chemical identity of contaminants, plate layout, and assay technology. For example, a cross-contamination between two adjacent vehicle control wells (DMSO-treated for example) might have minimal consequences to the assay readout. By contrast, the contamination of an otherwise non-bioactive well with even a small amount of highly potent bioactive compound from an adjacent well can produce a false-positive readout.

Cross-contamination can occur by several mechanisms, the most common culprits being [1] simple spillage or overflow from handling or dispensing, and [2] capillary-like effects ("wicking") (Figure 17A,B). Simple spillage can arise from a variety of sources:

- *Non-optimized liquid dispensing to microplates*. Excessive flow rates or poorly-aimed liquid streams can eject contents into adjacent wells.
- *Indelicate microplate handling procedures*. Like any experimental vessel, microplates subjected to course handling procedures (i.e., sudden, jerky movements) can cause contents to spill from well to well.

- *Non-optimized handling of a lid or seal*. Well contents can occasionally adhere to the interior surfaces of microplate lids or seals. Cross-contamination between wells can occur when the contents are not removed from the interior surface prior to opening (e.g., by simple centrifugation) and/or indelicate cover handling.

Wicking occurs by a capillary-like mechanism such that liquid from one well ascends along the vertical well dimension and eventually mixes with the liquid from an adjacent well (Figure 17B). Factors that influence wicking include:

- *Well geometry*. Wells with sharp corners can create a capillary-like conduit for liquids.
- *Laboratory environment*. Static charges can have the effect of accelerating wicking. Non-humid laboratories are often associated with pro-static conditions (e.g., laboratories in northern climates during the winter).
- *Assay buffer components and microplate surfaces*. Buffer components such as organic solvents and detergents can alter the surface tension and adherence to specific microplate surfaces.

For optimizing microplate handling and automation, troubleshooting can be done by mimicking the assay protocol with colored dyes and assay buffer. This approach can often identify cases of gross spillage, such as misaligned liquid dispensers or sloppy lid/seal removal. Non-conspicuous sources of well-to-well contamination can be investigated by utilizing highly potent controls in wells adjacent to inactive controls (Figure 17C). In this straightforward setup, even small amounts of cross-well contamination (e.g., 0.1% well volume) from the bioactive control should produce a measurable change in the negative control well.

If a source of cross-contamination is identified, one should still consider a variety of suggested practices to mitigate well-to-well contamination:

- *Avoid maximal well volumes when possible*. Wells that are filled near the maximal volume have less vertical distance to traverse before spilling into adjacent wells.
- *Utilize microplate wells with rounded corners*. Wells with right angles can act as capillaries that enable fluid to ascend along the microplate well. Wells with rounded corners can mitigate this capillary-like phenomenon (38).
- *Centrifuge microplates*. Incorporating brief centrifugation steps can return well contents to the bottom of wells. See also the "Centrifugation" section.
- *Avoid excessively dry conditions*. Incubator ovens can be humidified with water pans at the bottom. Controlling the humidity of an entire laboratory is more difficult, especially in northern climates during the winter.
- *Utilize microplate wells with ridges or chimney construction*. Some microplates have built-in ridges and valleys between the wells. Other plates feature chimney wells, in which each microplate well exists as a freestanding structure connected to adjacent wells only at the microplate base (Figure 17D).
- *Experiment with different surface treatments and buffer compositions*. Some surface treatments might repel buffer components, possibly accelerating a wicking-like effect.
- *Carefully apply and remove lids or seals*. When removing seals, make sure the removed portion does not re-contact the microplate (i.e., peel "away").
- *Prevent formation and discharge of static electricity.* As is the practice in several industries, labs can incorporate air knifes and de-ionizers, as well as alcohol mists, to mitigate static electricity. Some manufacturers also package microplates in anti-static packaging.

## Positional effects ("plate effects")

Microplates can exhibit systematic bias as a function of well position within a given microplate, so-called "plate effects" (positional effects) of which there are several examples in HTS and clinical assays (39-42). Plate effects

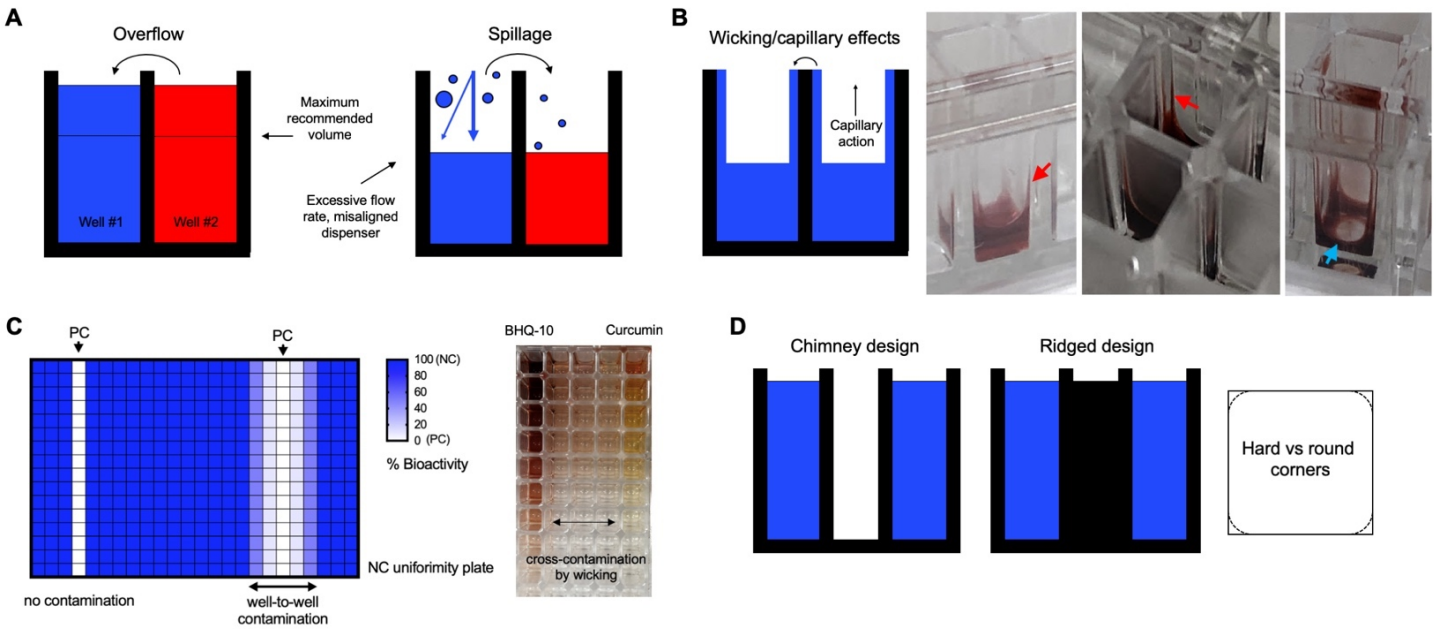



**Figure 17** Well-to-well contamination in microplates. (A). Common sources of well-to-well contamination. Simple spillage or overflow is related to over-filled wells and poorly optimized microplate handling (automation and procedures). Wicking can be caused by ambient laboratory conditions (usually dry air), microplate construction (sharp corners), and microplate surface-buffer interactions. (B) Examples of wicking phenomenon in a 384-well PS plate with non-rounded corners. Note the presence of colored compound (BHQ-10 carboxylic acid as 0.5 mM PBS solution) ascending along the corners (red arrows) and adhering to the edges (blue arrows), forming pronounced menisci. (C) Troubleshooting well-to-well contamination using bioactive controls. Sources of contamination can be quantified by assaying highly-active controls adjacent to negative control wells. In this picture DMSO stock solutions of BHQ-10 and curcumin were separated by DMSO wells, and allowed to incubate overnight. Note the colored wells, indicative of well-to-well contamination *despite the use of adhesive seals*. (D) Chimney wells, ridged wells, and rounded corners are specific microplate design features to mitigate well-to-well contamination.

can arise from several sources, including manufacturing (injection mold defects, non-uniform irradiation or surface treatments), incubation effects (evaporation, heating gradients), reagent and compound dispensing, and microplate readers (43).

One of the unique sources of experimental error associated with microplates are edge effects, which is when wells along the perimeter show substantial differences in assay activity relative to more interior wells (Figure 18A) (44). Unlike the interior wells, perimeter wells are not surrounded on all sides by other wells and therefore have at least one side bordering the external environment, which results in different thermal conditions. Edge effects can be significant if they are not mitigated, because they can introduce additional analytical uncertainty. One of the main sources of so-called edge effects is evaporation along the exterior edges of the microplate (45-48). The functional consequence of edge effects (increase or decrease in signal) is assay-dependent. There are several strategies that have been proposed to reduce the impact of edge effects, including:

- Ensure adequate humidity within incubators (by saturating the atmosphere with water so that there is no driving force) (49). In many settings, this is done by placing a water tray at the bottom of the incubator.
- Utilize microplates with lid-lock designs (Figure 18B).
- Utilize microplates with dummy perimeter wells (Figure 18C).
- Fill perimeter wells with assay buffer or media but omit them from analyses.
- Edge effects in cellular assays requiring media changes may also be reduced with centrifugal plate washers (50).

A related source of systematic errors affecting microplates are row and column effects in which there is a systematic bias with respect to the microplate rows and/or columns. Row and column effects are usually caused by instrumentation (dispensers or readers), rather than a specific property of the microplate. Examples include:

- *Dispensing procedures*. Reagents dispensed column-wise (dispense column 1, then column 2, etc.) may have a bias reflecting the difference in dispense timing between the first and last columns. Similarly, reagents dispensed along rows might result in different assay signals that are proportional to the difference in time at which they were dispensed. Cassette-based dispensers, which dispense simultaneously from multiple tips can deliver volumes unevenly or even have a clogged tip, both of which can result in striped patterns. A striped pattern can also result from incomplete priming of the dispenser or adsorption of assay reagents to the dispenser tubes.
- *Detectors*. Microplate readers that read row-wise (i.e., read row 1, then row 2, etc.) may have a bias reflecting the difference in read timing between the first and last rows. Also, the position of a microplate within a reader can cause an apparent signal gradient across the plate if the distance between the detector and plate change during the readout process (51).
- *Temperature-sensitive assays and plate effects*. Occasionally one encounters significant temperature gradients when performing the plate detection step. As an example, there have been assays run in the past that require plates of cells to incubate at 37°C after addition of a detection reagent, followed by a brief incubation at ambient temperature before being placed into a luminescent plate detector. Once read, it became obvious that there was an artifact of some sort as a full plate view showed an oval pattern in the middle of the plate being present, with the values within the oval being higher and those outside of it being lower. This artifact was attributed to the cooling pattern of the plate once it was removed from 37°C and brought to ambient temperature, with the outer edges of the plate cooling faster than the inner portion, leading to a measurable difference due to the nature of the detection reagent being used (S Michael, personal observation). It is important to note that it is very common for an assay biologist to immediately think she has done something wrong when artifacts or inconsistent assay results present themselves, potentially leading to rounds of unnecessary biological troubleshooting where the problem may not be on her side at all.

Positional effects and other systematic errors in microplates can be corrected with appropriate statistical tools (52-56). *This being said, we do not recommended correcting for plate patterns involving greater than 30% change in signal*. One pragmatic strategy is to use uniformity plates (which should in principle produce the same readout in every well). By making the assumption that edge/row/column signals are constant between plates, perimeter wells can be corrected by this strategy. Another strategy involves utilizing plate layouts with controls along an entire row and column, though this comes at a sacrifice to the number of compounds that can be tested on a given plate. For additional discussions on validation and corrections of microplate positional effects, including for HCS data, refer to the AGM chapters, HTS Assay Validation and Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis.

For cell-based assays, another consideration is the homogeneity of the well contents ("well effects"; Figure 18D). Some practical tips include:

- Incubators can create vortex-like effects that force cells towards the edges of individual microplate wells. For cellular assays, pre-incubation of the newly seeded plates in ambient conditions rather than direct placement in a $CO_2$ incubator has been proposed (57).
- Briefly centrifuge freshly-seeded cells to facilitate adherence to the bottom of the microplate wells (e.g., 100 *g*, 2 min).
- Limit physical disturbances near incubators, including heavy foot traffic, unnecessary open and closing of incubator, etc.

- Image several fields per well. Compare edges, corners, and center. Determine the optimal number of fields per well that best represents the intra-well variability.

*Predictive analytics to reduce plate effects.* Statistical analysis of repeated measures designs offers the ability to partition out variability due to repeating individual differences in measurements. In the context of microplates, one can measure the differences between individual wells in a uniformity plate whereby all wells should report identical assay results. Predictive analytics using machine learning can reveal slight differences between the wells that are non-random. Well-to-well differences in optimized assays contribute to about 30% of the overall imprecision (P. Hensley, unpublished results). Incorporating predictive analytics in the post-assay processing step has the potential to correct this sizeable contribution to imprecision, without changing the assay method, lab equipment, or analytical measurement. This can have a substantial impact, as lowering the CV can reduce the statistical threshold for identifying "active" compounds from a HTS, and enhance confidence in ranking compounds. Post-processing with predictive analytics can accommodate other correction factors routinely for parallax, other optical/mechanical repeating biases, pipetting effects, and others. The standard deviation of a given assay can be reduced approximately 30% for both cell-based assays and biochemical assays (Figure 19). Such methods usually require only two or three teaching cycles with uniformity plates (i.e., plates prepared for uniform high signal), with incremental improvement with more teaching cycles.

## Well-to-well variance

Well-to-well shapes and volumes can vary within a given microplate, albeit these differences are often relatively very small in magnitude (one non-peer-reviewed study suggests intra-plate well volumes are within 5%) (58). The same study suggests that individual well dimensions are relatively constant between plates of the same plate. This well shape variation can arise because each well is molded from a different feature of the microplate mold. Because molds are machined, wall surfaces are not perfectly smooth and as a result, the surface area of each well can vary and ultimately affect the quality of some assays. Besides gross well shape and volume, wells within a plate can have *different surface properties* that can dramatically alter assay results, and in some instances, this variation can eclipse edge effects in terms of magnitude (41).

## Microplate surfaces and associated properties

The uniformity and properties of microplate surfaces are often overlooked, but hardly trivial in that they can dramatically affect assay results. One explanation for differences in microplate performance metrics are microplate surface properties, including surface "roughness", chemical composition, and wettability (i.e., hydrophobicity/hydrophilicity), which can now be analyzed by a variety of sophisticated analytical techniques. Scanning electron micrographs (SEMs) and atomic force microscopy (AFM) have recently been used to assess microplate surface roughness. In one study, SEM identified four distinct problems with some 1,536-well microplates: surface debris, roughness, inclusions (holes into the surface), and exclusions (material extending into the well from the wall) (Figure 20A-F). No brand that was tested seemed better or worse than the other brands (P. Hensley, unpublished observations). Microplate surfaces can vary significantly within a given plate. Contact angle measurements suggest the wettability of individual wells can vary within a given microplate, and have the potential to affect assay results (e.g., higher wettability variability was associated with higher CVs) (39). Even within the same well, there can be patches of hydrophobic contaminants that alter protein binding (59).

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has also been utilized to analyze microplate surface chemicals (Figure 20G). Of special interest were the chemicals used in production to initialize and control the polymerization rate, control primary and secondary oxidation, improve polymer characteristics (including melting temperature and flow), and mold-release agents. In one analysis, very few ions were unique to a single brand; which is to say almost all were common to every brand tested (P. Hensley, personal observation).

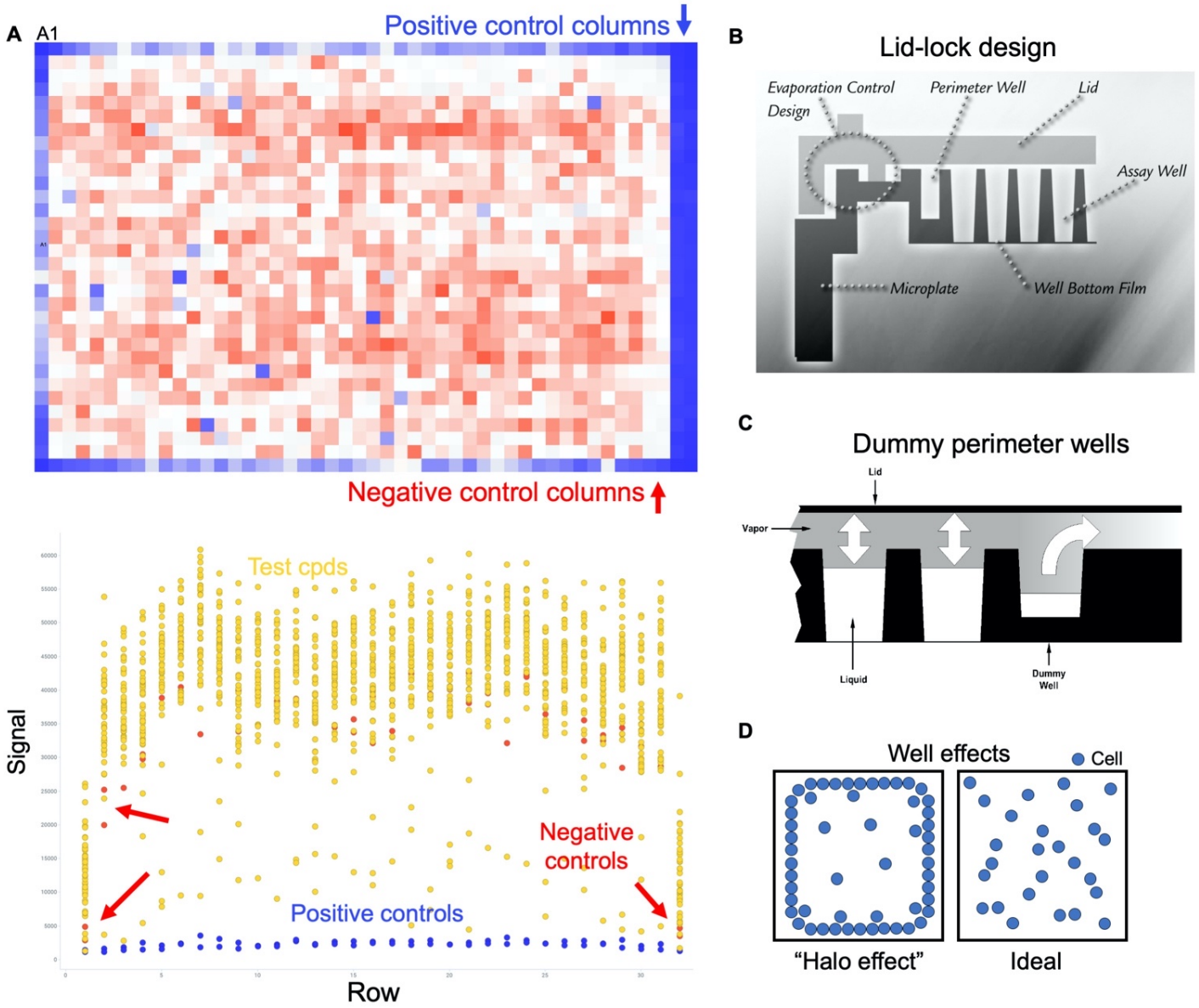



**Figure 18.** Microplate positional and well effects. (A) Example of microplate edge effects in a 1536-well antagonist assay (red, not active; blue, active). Compared to the interior wells, the perimeter wells show a decreased readout (blue), and note the significant bias of perimeter negative controls (arrows). (B) Microplates and lids can incorporate interlocking designs to mitigate the escape of vapors from within the microplate. (C) Microplates can incorporate dummy perimeter wells that can be filled with media, buffer, or water that will be preferentially evaporated given the proximity to the microplate edge, effectively sparing evaporation from interior wells. (D) Schematic of microplate well effects. In an ideal assay, cells are plated uniformity across the well; well effects can manifest as a "halo"-like effect, whereby cells have a propensity to settle along the microplate edges.

In another study with ELISA plates in a clinical assay for plasma *N*-terminal pro-urocortin 2, SEM revealed particle-like structures on opaque (white) microplate surfaces, which energy-dispersive X-ray (EDX) spectroscopy suggest are $TiO_2$ remnants from the manufacturing process (39). In the same study, sterilized microplates had higher oxygen content than comparable non-sterilized microplates, thought to be due to γ-irradiation (60,61). Notably, higher oxygen content can confer increased wettability.

*Surface area of polymers.* Understanding polymer surfaces is importance because they can directly affect assay performance. Determining the surface area of what appears to be a flat polymer surface is a complex process that only results in an approximated value. As there are chemicals on or embedded near the surface of the polymer,

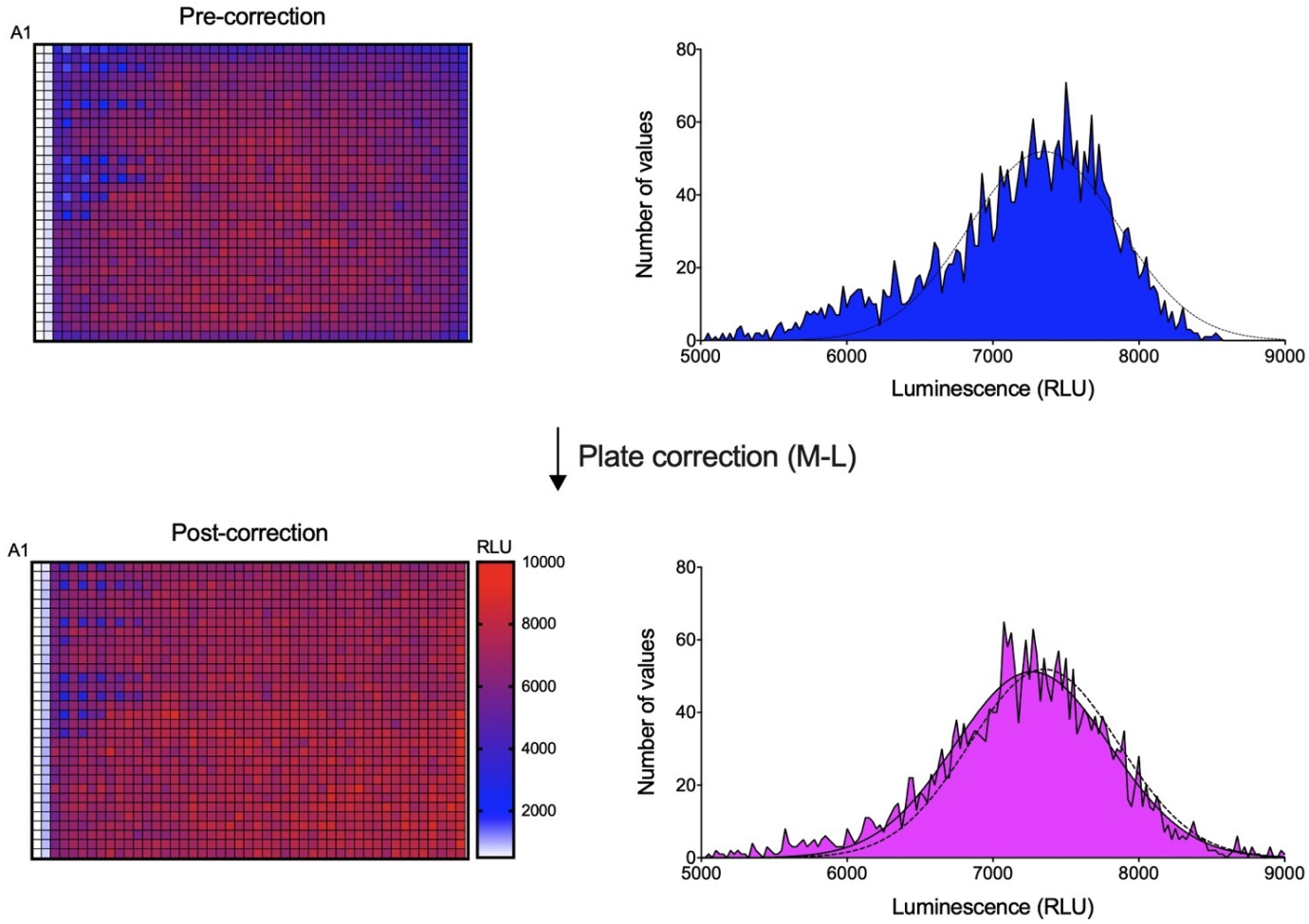



**Figure 19.** Correction of well-to-well variation using predictive analytics. (Top) Heatmap and corresponding distribution histogram of a uniformity plate read by a ViewLux (PerkinElmer), a microplate reader with a known edge effects. (Bottom) The heatmap and distribution histogram of the same microplate after processing with machine-learning to correct for well-to-well variation. The standard deviation was reduced slightly more than 30% using predictive analytics, as well as a more Gaussian distribution pattern (Assay Analytics, IonField Systems).

understanding the mechanisms of how the chemicals migrate from the polymer to the solvent are critical. The factors related to the polymer surface are: [1] the interface between the surface and the solvent, [2] the solubility of the chemicals into the solvent, and [3] the physical changes to the surface resulting from interfacial forces due to contact with the solvent. A cursory review of the high-magnification SEMs (Figure 20A-F) demonstrates that the surface area of microplate wells is vastly greater than the geometric dimensions. Water comes in direct contact with virtually every polymer molecule on the surface area (Figure 20A-F, note each 'tic' mark on the x-axis is the length of 40,000 water molecules). Using AFM, one study showed pigmented plates had greater roughness compared to non-pigmented plates, and greater roughness in γ-irradiated compared to non-irradiated plates (39). While the increased oxygen content from irradiation can increase wettability, this can be mitigated by increased roughness associated with irradiation.

*Physical changes to microplate surfaces/surface dynamics.* A logical implication from the ToF-SIMS data is that chemicals added to the polymer mix during production can eventually dissolve into microplate well solutions. Generally, it is the negative ion of the chemical that is the active part of the molecule in starting and controlling the polymerization process. The positive ion is selected for its charge being appropriate or ease of synthesis. The findings from ToF-SIMS is that the positive ion species used are bioactive and as ions all are soluble in water.

Studies of polymer surface properties, specifically the measuring of the contact angle with water, show that the contact angle initially is high (indicating the surface when dry is hydrophobic). If the surface is covered with water and allowed to stay wet and then the contact angle is re-measured, it decreases, indicating that the surface becomes more hydrophilic. These changes in "wettability" are hypothesized to reflect molecular motions in the polymer layer that contacts water (14). Specifically, nonpolar sections of the microplate polymer molecule that are outward facing (to the well contents) when the surface is dry will reverse position with polar sections of the molecule that are inward facing when the surface is in contact with water, resulting in a reduction in contact angle. This phenomenon is commonly seen at the benchtop when one re-pipettes aqueous solutions with the same tip multiple times; with microplates this process is somewhat slower but the contact angle does decrease over time. A practical implication of this molecular movement is that it can expose chemicals in the polymer matrix to the assay solution itself.

## Leachable chemicals within microplates can affect assay results

An increasingly acknowledged problem is the ability of chemicals to leach from the microplate material into the microplate well solution. These leachable chemicals and other contaminants can originate from the manufacturing process, such as volatile residues, plastic enhancers, and mold-flow and mold-release agents (59). This can be a significant problem, as some of these chemicals can significantly modulate bioactivity, as shown by a prominent example where the biocide di(2-hydroxyethyl)methyldodecylammonium chloride (DiHEMDA) and the slip agent oleamide inhibited human monamine oxidase-B (Figure 21) (18). Other examples of leachables and assay modulation have also been described (62,63). Metals from polymerization catalysts and impurities (e.g., Zr, Al, V, Cr, Fe, Mg, Ti, Zn, Os, Pd, Ru, Ni, Pb) can also leach and may perturb assay readouts (13).

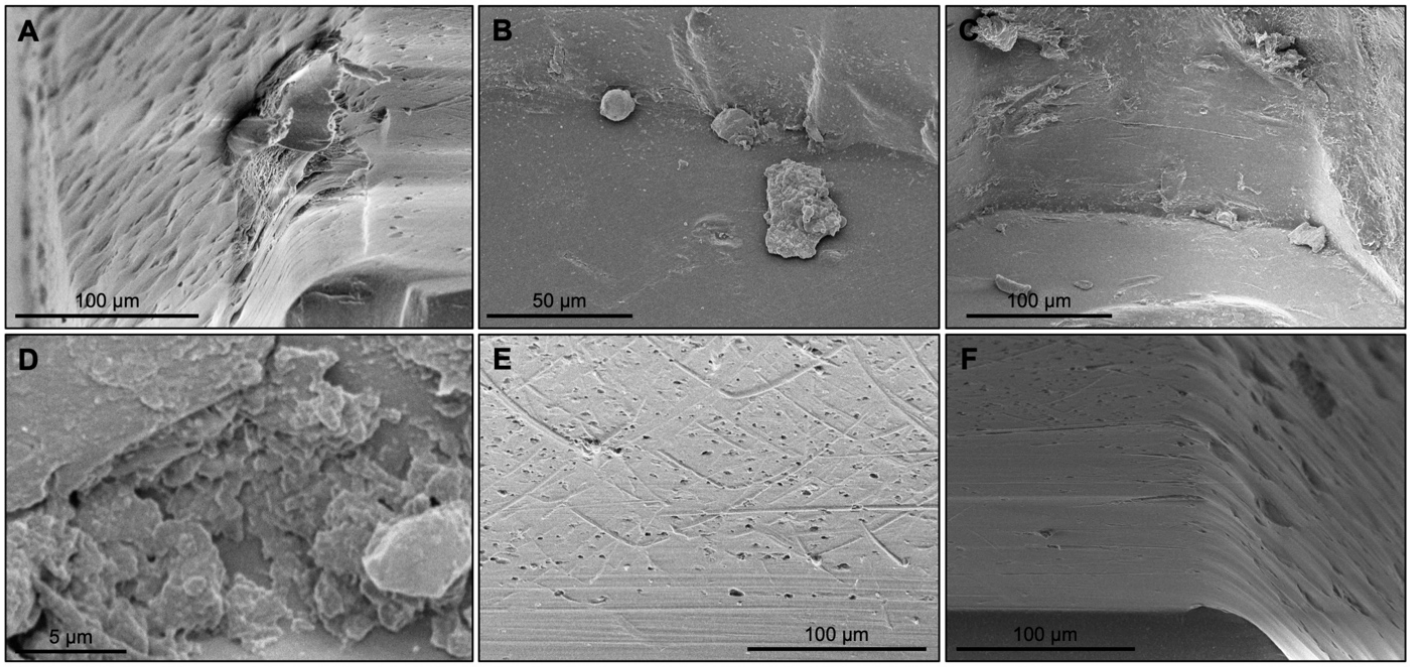
There can also be differences in cell growth rates between comparable microplate brands due to the presence or absence of surface chemicals, as demonstrated by CellTiter-Glo studies with Be2C cells grown in similar microplates with different ToF-SIMS signatures (64). Therefore, assessing cell growth rates and cell health when selecting between microplates might be prudent. Variations in cell growth rates within the same brand can be attributed to well-to-well differences, both chemical and physical, while the variation between brands of cell growth rates is attributed to the effect of chemical and surface differences between brands. Notably, cell growth rates cluster by brand, and all brands in this study showed a skewing toward low values due to edge effect parallax from the ViewLux plate reader used in this testing, which is not microplate related.

## Plate washing and reuse

Interest in cleaning microplates to enable their reuse started immediately after the development of microplates as a commercial product. A basic approach involves washing plates with a conventional benchtop plate washer, then centrifuging the microplates with their wells facing "out" and paper towels on the bottom of the microplate holder to adsorb residual assay material and wash solution. The first commercial product intended to clean microplates was the SQUIRT (Matrical), which mechanically inverts a microplate onto a seal and pressure rinses the microplate wells, followed by a blow-drying step.

Through a collaboration between NCATS/NIH and industry (SBIR, HHSN271201400051C), there have been some recent advances in plate washing, including a systematic analysis of washing steps and the application of plasma treatment after plate rinsing. Several critical parameters of an effective microplate wash were identified in this collaboration:

- Fluid pressure: there is a narrow band of pressure for optimal effective cleaning
- Angle of the fluid entering a microplate well: a critical setting as it determines the flow pattern in the well
- Distance of the pressure spray head from the microplate: has a narrow optimized range of maximum effectiveness as the width of the fluid stream changes with time after leaving the spray head

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(+) ToF-SIMS intensity (relative, per ion)

| Individual microplate | Na (23) | Al (27) | Si (28) | Ca (40) | $C_3H_5$ (41) | $C_2H_3O$ (43) | Ti (48) | $C_3H_3O$ (55) | Zn (64) | $C_5H_7$ (67) | $C_4H_{12}N$ (74) | $C_7H_7$ (91) | $C_8H_{20}N$ (130) | $Si_2OC_5H_{15}$ (147) | $C_{11}H_{22}N$ (168) | Pb (208) | $C_{20}H_{40}NO$ (310) | $C_{23}H_{38}N$ (328) | $C_{42}H_{64}PO_3$ (648) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 16 | 79 | 2 | 12 | 41 | 2 | 100 | 2 | 100 | 9 | 1 | 61 | 1 | 3 | 12 | 9 | 100 | 7 | 100 |
| 2 | 2 | 4 | 2 | $6\times10^{-2}$ | 100 | 100 | 1 | 100 | 1 | 100 | 100 | 100 | 100 | 100 | 100 | 82 | 2 | 25 | 33 |
| 3 | 100 | 100 | 14 | $4\times10^{-1}$ | 44 | 27 | 18 | 21 | 6 | 16 | 16 | 48 | 30 | 37 | 19 | 100 | $3\times10^{-1}$ | 11 | 33 |
| 4 | 7 | 16 | 2 | $3\times10^{-1}$ | 11 | 1 | 14 | 1 | 41 | 2 | 1 | 87 | 1 | 7 | 2 | 9 | 0 | 2 | 0 |
| 5 | 6 | 32 | 3 | $2\times10^{-1}$ | 8 | $2\times10^{-1}$ | 19 | 1 | 41 | 1 | $2\times10^{-1}$ | 23 | $1\times10^{-1}$ | 3 | 1 | 7 | 0 | 0 | 33 |
| 6 | $4\times10^{-1}$ | $8\times10^{-2}$ | 2 | 4 | 76 | 33 | 1 | 74 | $4\times10^{-1}$ | 74 | 39 | 80 | 83 | 54 | 56 | 80 | 2 | 5 | 100 |
| 7 | $2\times10^{-1}$ | $1\times10^{-1}$ | 100 | $1\times10^{-1}$ | 73 | 24 | 1 | 50 | 1 | 68 | 85 | 68 | 96 | 45 | 5 | 64 | 2 | 7 | 33 |
| 8 | 3 | 6 | 7 | 100 | 26 | 5 | 2 | 5 | 15 | 8 | 33 | 59 | 29 | 16 | 6 | 80 | 1 | 31 | 67 |
| 9 | 4 | 2 | 5 | 81 | 50 | 15 | 1 | 13 | 10 | 15 | 89 | 27 | 53 | 31 | 6 | 93 | 1 | 100 | 33 |

Ion (*m/z*)

**Figure 20.** Microplate surfaces. (A-F) Representative SEM of microplate surfaces. Microplates were frozen with liquid nitrogen, then fractured to expose well walls. (A) Polymer pulled from wall; (B) small loose particles; (c) surface scales; (d) surface tear; (e) surface bubbles; and (f) random non-flat surfaces. (G). ToF-SIMS analyses of microplate surfaces. Using positive ion analysis, ToF-SIMS revealed a large number of positive-ion species either on or within 1 nm of the microplate surfaces. Microplates were tested from three brands. All had at least 27 "non-polymer" positive ions, which one would anticipate based on a review of the literature in this field. This analysis was performed to identify potential differences between the brands. Very few ions were unique to a single brand and almost all were common to every brand tested. However, there were variations in concentration by brand. Examples are that $Na^+$ varied 498-fold and $Ca^{2+}$ 2860-fold. Typically there were minimal differences for trace ions, such as $Pb^{2+}$ and $Ag^+$. Labs performing assays known to be sensitive to trace chemicals should check the list of chemicals shown in product attachments and consider testing the microplates they intend to use.

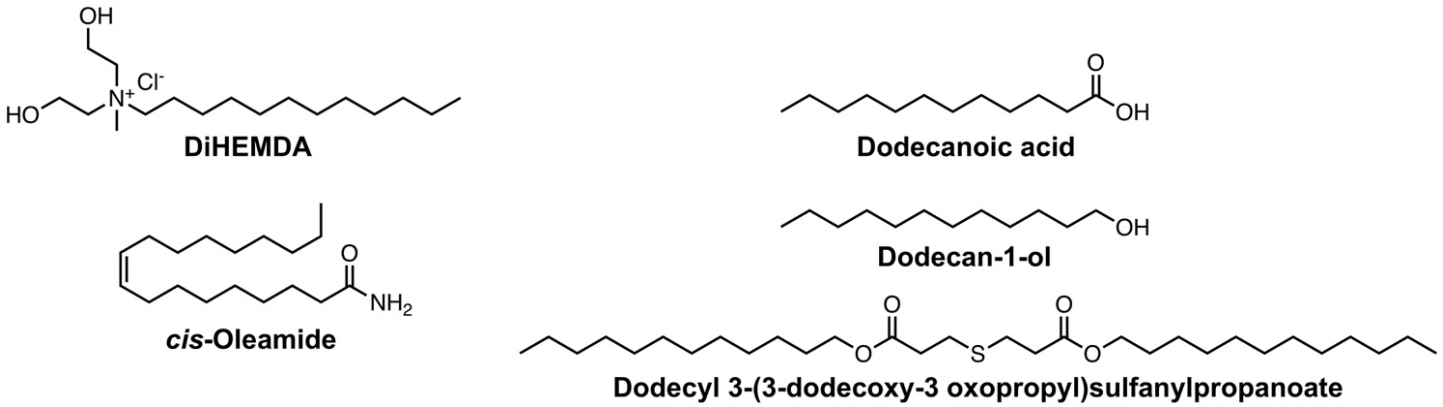


**Figure 21.** Examples of leachable chemicals from microplates. Chemicals such as the biocide DiHEMDA and the slip agent oleamide from the microplate manufacturing process can leach and dissolve into microplate well solutions, and potentially modulate bioactivity.

- Speed of the microplate as it moves under the pressure spray head: demonstrated a harmonic effect between speed and the width of the fluid

Using conventional plate washers plus centrifugation methods, washing did not consistently reach a 1,000-fold reduction benchmark (the limit of cleaning effectiveness of most serial dilution cleaning processes). Seemingly minor differences in plate handling lead to a variation in cleaning results, even with controlled conditions. With optimized conditions, cleaning effectiveness can be reliably increased to over 10,000-fold.

Considerable research was also done on how to break droplets off a polymer surface, as micro-droplets may contribute to carryover when microplates are reused. Adding a small amount of alcohol to the washing pressure fluid reduced surface tension and improved droplet release, but did not entirely resolve all droplet formation issues. Notably, the biggest improvement in microdroplet removal occurred with the use of a high-performance centrifuge (as opposed to air drying) that are highly precise and allow extremely high rates of rotor acceleration and de-acceleration (0.7 s to reach 1500 rpm, 3.6 s at speed, 0.7 s to stop). The acceleration/de-acceleration rates when centrifuging microplates is a key factor in droplet removal, with extended spin times having minimal additional effects. The "at speed time" is the least amount of time needed for air flow in the centrifuge to clear droplets and aerosol, an important safety consideration.

Another recent advance in microplate washing is the application of atmospheric-pressure plasma (gas excited by electrical current) which is directed into the microplate wells. High-energy plasma causes the breakdown of organic molecules on contact by an oxidative process without the production of liquid or solid waste. Another key technical advance in microplate washing from this public-private collaboration is the importance of a low pressure micro-dispensing process with a trace amount of a solvent mix to enhance residual compound removal. By serendipity, visual inspection of washed clear microplates revealed visible "spots" on the bottom of wells that previously contained high concentrations of certain test compounds (Figure 22A). These spots were presumed to be residual compound, and are significant because they have the potential to perturb bioactivity in subsequent assays if they are not removed. Experimentally it was determined that the solvent mix solubilized both types of residual compound in 3 - 5 s when followed by a high-pressure wash. This approach completely eliminates dried compound as a source of carryover (Figure 22B). In summary, plate washing has advanced from the crude centrifugation of plates to a highly optimized system of solvents, fluid washes, timing, centrifugal force (i.e., precise assay- and microplate-specific application of said force), and plasma treatments.

There are several notable considerations with plasma-based microplate washing (while Microplate Cleaning System powered by PlasmaKnife Technology is specifically discussed, many of these concepts can apply to other systems):

- Applicable to: SBS/ANSI-compliant microplates; multiple microplate heights; 96-, 384-, and 1536-wells; all polymers; glass or polymer clear bottom.
- The plasma cleaning process leaves a trace amount of nitric acid ($HNO_3$) on the microplate surface. If an assay is pH-sensitive, then post-processing application of a neutralizing buffer is recommended.
- Microplates that have been allowed to completely dry after the assay are less likely to be successfully cleaned for reuse.
- Cell-based assays that include a lysing step with detergents (e.g., Triton X-100) will deposit membrane proteins and lipids on the bottom of the microplate well. In such cases, these microplates must be pre-washed immediately with Tergazyme post-assay while this cellular debris is still wet before commencing plasma-based microplate cleaning.
- Most secondary surface treatments are removed by the plasma cleaning process. Surface treatments can then be reapplied after washing.
- Treatments like the conventional TC process are replaced by the plasma process. In virtually all testing, TC-treated microplates perform better after plasma cleaning (P Hensley, unpublished observations).
- The plasma cleaning does not restore the giga ohm surface properties of patch clamp microplates (e.g., NanIon).

## Environmental contaminants

Another factor inherent to microplate-based assays is the effect of environmental contaminations such as airborne particulates ("dust") and even lint from cotton-based laboratory coats on assay performance (65). Microplate-based assays are particularly prone to airborne particle effects because of their relatively large surface compared to vials or microcentrifuge tubes, they are often exposed to air when lids are removed for dispensing and reading steps, and because they may be moved many times during the experimental procedure. Light-based readouts are particularly sensitive to anomalous results from dust.

There are several recommended tactics to mitigate the impact of airborne impurities, including dust:

- Pre-read plates
- Work in clean environments (clean all work articles before placing on systems)
- Keep paper out of screening areas (boxes, packaging)
- Utilize disposable plastic coats to minimize lint contamination (65)

## Sterility

For cell-based assays in microplates, it is imperative to maintain sterility to avoid contamination by laboratory microorganisms such bacteria (including *Mycoplasma*) and fungi (yeasts, molds). Most manufactures sterilize microplates by γ-irradiation, which interestingly can have effects on microplate surface properties including wettability, surface roughness, and well uniformity (39). Several best practices are recommended in the context of microplates (in addition to general good laboratory and cell culture processes). These include:

- Refrain from opening plastic sleeves of sterile microplates until prior to experimentation.
- Barcode microplates prior to de-contaminating (autoclaving, etc.); this can often be done by request from microplate manufacturers. This avoids the need to expose opened microplates to barcoding instruments which are often in general laboratory areas and are not sterile.
- Minimize the frequency and duration of lid or seal removal from microplates.
- Whenever possible, dispense reagents (buffer, cells, compounds) into microplates within a tissue culture hood or sterile environment. Some plate dispensers (e.g., NanoDrop, MultiDrop) can fit within standard tissue culture hoods. This minimizes the chances of airborne contaminants from settling into microplate wells when the lids must be inevitably removed for dispensing.

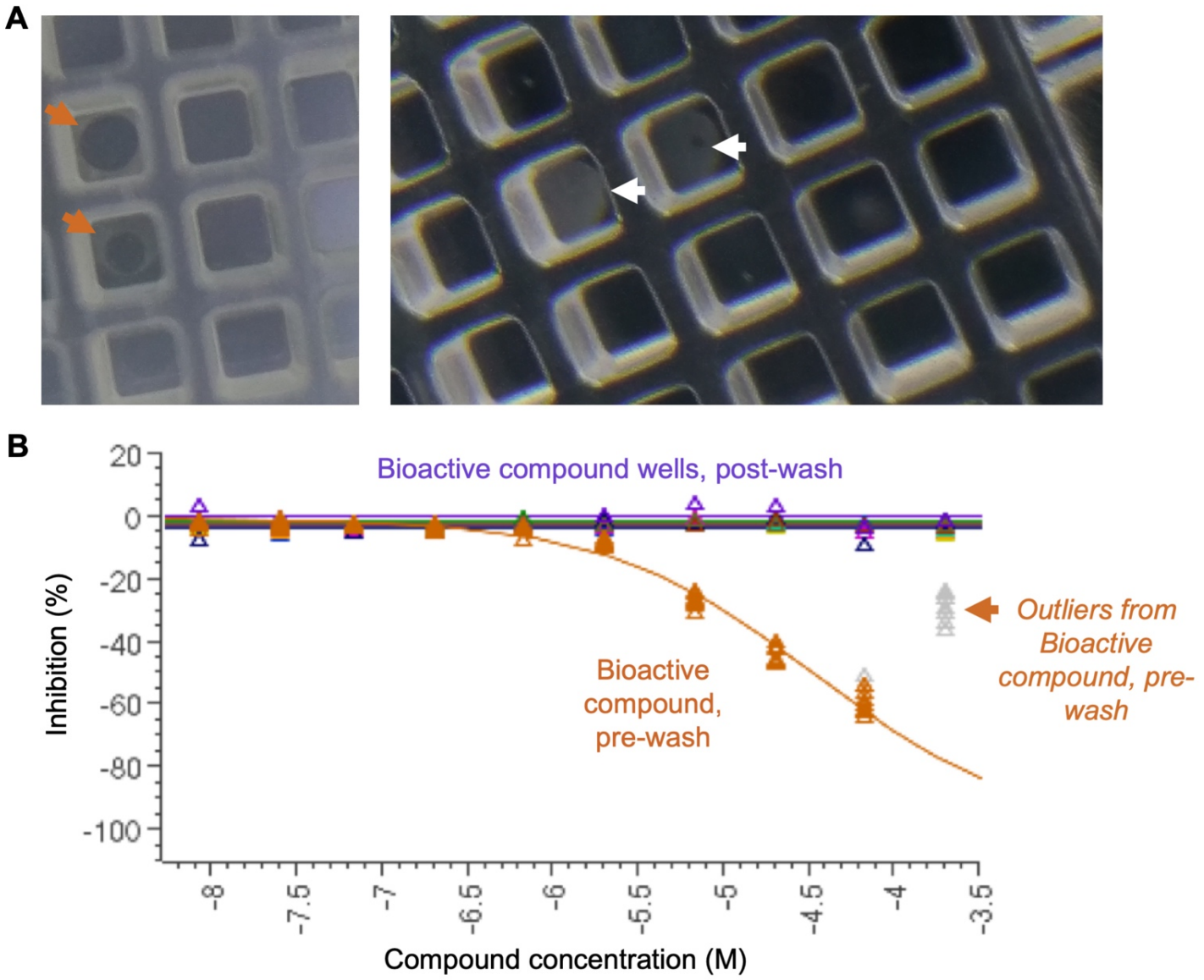


**Figure 22.** Compound carryover in non-optimized plate washing protocols. (A) Left; circles having a clear area surrounded by a hazy area defining the circle (orange arrows). Right; circles having a hazy area defining the circle with the no haze outside the circle (white arrows). The difference between the two lead to the conclusion that two different phenomena are shown. On the left, compound that "missed" the center droplet dried and once dry would not go into solution when the assay liquid was added. On the right, most of the compound did go into the assay solution but some precipitated and is stuck to the surface of the plate. (B) Effectiveness of microplate washing by incorporation of plasma treatment. A bioactive compound (orange, same as the compound in Figure 22A, left panel) shown was testing using a standard 10-point serial dilution by a homogenous assay. The microplate was then cleaned using a Microplate Cleaning System powered by PlasmaKnife Technology or MCS (ionField Systems). The assay was then repeated in the same wells without test compound. Note that the same wells did not produce significant bioactivity after microplate washing. Data are five technical replicates on a single microplate.

- Sterilize or clean any tissue component that will come into contact with microplates or assay reagents. For example, cassettes for liquid dispensers can be autoclaved and primed with 70% ethanol and then sterile PBS prior to running cells or reagents through.
- Depending on the assay requirements, antibiotics can be included in culture media (e.g., penicillin/streptomycin combination).

- Include control wells or plates without cells and carry it through the assay protocol. Examine these controls for signs of microbial growth. This might be useful for applications not including antimicrobials in culture media.

## Chemical compatibility

Some solvents or reagents might not be compatible with a given microplate polymer (Table 5). Perhaps the most important compatibility in the context of biological assays is DMSO, as most test compounds are prepared as DMSO stock solutions, though the total concentration is typically less than 1%. Chemical compatibility is often a more significant issue when certain quenching, detection, or extraction reagents are employed that utilize organic solvents or markedly acidic/basic solutions. Such chemicals can react with the microplate polymers (i.e., oxidization, reaction with functional groups, catalyze de-polymerization), or be absorbed into the bulk microplate material and soften/swell the microplate. It is sound practice to verify the chemical compatibility of assay reagents with microplates in such cases.

**Table 5.** Generalized compatibility of microplate polymers with select chemicals. Note that compatibility can be concentration- and/or time-dependent. Compatibilities are often assessed using high concentrations of chemicals (e.g., > 10% v/v for most solvents). +, generally compatible; -, generally incompatible; +/-, exercise caution/verify.

| | | Microplate polymer | | |
|---|---|---|---|---|
| | | COP | PP | PS |
| Alcohols | Ethanol | + | + | +/- |
| | Isopropanol | + | + | + |
| | Methanol | + | + | +/- |
| Other organic solvents | Acetone | + | + | - |
| | Dimethylformamide (DMF) | +/- | + | - |
| | Dimethyl sulfoxide (DMSO) | + | + | +/- |
| Acids | Acetic acid | + | + | +/- |
| | Hydrochloric acid (HCl) | + | + | +/- |
| | Sulfuric acid ($H_2SO_4$) | +/- | - | - |
| Bases | Sodium hydroxide (NaOH) | + | + | + |
| Other | Formaldehyde | + | + | - |
| | Hydrogen peroxide ($H_2O_2$) | +/- | + | + |

## Optical isotropy

An optically isotropic material has the same optical properties in all directions. In other words, light rays behave the same in all directions of propagation and polarization when traveling through an optically isotropic material. By contrast, in anisotropic materials, light transmitted through the material is dependent on the ray direction and polarization. Certain microplate plastics (such as polystyrene) can become more anisotropic with mechanical stress from molding procedures. Certain polymers such as COP have more isotropic properties (13).

Optical isotropy is an important consideration for imaging-based assays and those involving polarized light because anisotropic materials can produce certain light-based interferences. Anisotropic materials that affect light polarization can diminish illumination intensity by polarized light sources in fluorescence polarization, FRET, and time-resolved FRET assays. In such cases, the material undesirably depolarizes the polarized incident light, which effectively attenuates the transmitted and emitted light.

## Section summary

The optimal use of microplates requires knowledge of special topics including batch-to-batch variability, well-to-well contamination, plate effects, microplate surfaces, and plate washing.

# Conclusions

Robust HTS and quantitative biology can be significantly influenced by careful microplate selection and employing best practices. Along with an appreciation that microplate surfaces are non-uniform and can dramatically influence assay readouts, the choice of microplate should not be seen as an afterthought. Particular attention should be made to key parameters such as well number, microplate color, well volume, well shape, and microplate surface coatings. Scientists should carefully optimize experimental procedures including incubation and compound/reagent dispensing to maximize microplate performance. Careful validation processes, plate layouts, and statically tools can help correct for imprecision. Variability in microplates is often be attributed to positional effects, which can be introduced by manufacturing processes and experimental conditions. This information and best practices should therefore enable higher-quality bioassays.

# Suggested Readings (alphabetical order)

Knight S, Plant H, McWilliams L, Murray D, Dixon-Steele R, Varghese A, Harper P, Ramne A, McArdle P, Engberg S, Bennett N, Blackett C, Wigglesworth M. Enabling 1536-well high-throughput cell-based screening through the application of novel centrifugal plate washing. SLAS Discov. 2017;22(6):732–742. PubMed PMID: 28027450.

Describes the application of centrifugal plate washing to reduce edge effects in microtiter cellular assays, including those with complex protocols and media changes.

Lilyanna S, Ng EMW, Moriguchi S, Chan SP, Kokawa R, Huynh SH, Chong PCJ, Ng YX, Richards AM, Ng TW, Liew OW. Variability in microplate surface properties and its Impact on ELISA. J Appl Lab Med. 2018;2(5):687–699.

An illustrative case study highlighting the importance of microplate surface properties on assay performance. Includes excellent examples of biophysical and chemical characterization of microplate surfaces including SEM, contact angles, AFM, and elemental analysis.

Smith T, Ho PI, Yue K, Itkin Z, MacDougall D, Paolucci M, Hill A, Auld DS. Comparison of compound administration methods in biochemical assays: effects on apparent compound potency using either assay-ready compound plates or pin tool-delivered compounds. J Biomol Screen. 2013;18(1):14–25. PubMed PMID: 22904199.

An excellent study comparing the performance of compound dispensing technologies and microplate materials in HTS.

Trask OJ. Guidelines for microplate selection in high content imaging. Methods Mol Biol. 1683;2018:75–88. PubMed PMID: 29082488.

A detailed discussion of factors related to microplate selection in high content and phenotypic screening.

# Glossary

**column/row effects** – a type of systematic signal differential in microplates in which rows and/or columns display a bias. Such a bias can be caused by temperature gradients, liquid dispensers and detection instrumentation.

**contact angle** – the angle where a liquid–vapor interface meets a solid surface; this quantifies the wettability of a solid surface by a liquid

**edge effects** – a type of systematic signal gradient often observed in microplates in which the perimeter wells have a different readout compared to the interior wells. This is often caused by a temperature gradient and evaporation.

**flange** – a projecting rim along the base of microplates that serves to maintain plate positioning with various instruments and automation devices

**microplate** – any type of flat plate containing multiple wells that serve as individual chambers for biological assays; designed for the purpose of increasing throughput

**optical isotropy** – a material property in which light transmitted through the material behaves the same regardless of its direction and polarization

**uniformity plate** – a microplate containing the same reagents in every well, usually a positive control achieving maximal assay signal, to estimate systematic biases such as edge, column, and row effects

**well effects** – a type of systematic signal gradient often observed in microplates in which the signal within a microplate well has a different readout compared to another portion of the same microplate well.

**wettability** – the ability of a liquid to maintain contact with a microplate surface

**wicking** – the vertical movement of liquid towards the top of a square microplate well due to capillary-like effects along the right angle edges of the well, often leading to evaporation and well-to-well contamination

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## Conflict of Interest Statement

The authors declare no conflicts of interest.

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