WHITE PAPER No. 35 I November 2016

# Five Challenges in Plate Assays that Can Be Mastered by the Right Choice of Pipetting Tool

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## Executive Summary

Five major challenges arise in every plate assay: precision and accuracy, (high) liquid diversity, differing concentrations of additives, potential contamination, and the turnaround time of the assay. These points are not only influenced by the experimental setup, but also by the liquid handling tool used for assay execution. With that said, choosing a dispenser instead of a multichannel pipette can reduce the risk of cross contamination. For time sensitive enzymatic assays, a device with 96 channels is more beneficial.

When it comes to the addition of different concentrations of additives, an electronic pipette beats a manual pipette. For demanding liquids like glycerol, a manual pipette with the correct pipetting technique can be as dependable as a positive displacement system. In general, considering the correct liquid handling tool and proper operation are essential to improve plate assays and can make the difference between good and bad results.

## Introduction

Plate-based assays are performed on a daily basis in laboratories worldwide. The main executing research fields are cell biology, microbiology, and immunology. Of course industrial companies active in food & beverage and pharmaceutics also apply plate assays. However, every laboratory employee faces the same challenges and needs to consider some important aspects when performing these assays.
The five main points that influence to high reproducibility during plate assays are:

1. High precision and accuracy
2. Variable liquid properties of chemicals
3. Addition of different concentrations of compounds to the plate assay
4. Potential contamination with bacteria, viruses, or mycoplasma
5. Improvement and acceleration of time critical assays

The reproducibility of results is of paramount importance in each experiment. It guarantees a stable, reliably working system that enables the researcher to interpret the results and publish the data. A non-reproducible test can neither be justified in scientific nor industrial communities. Proof is always needed to convince the scientific community as well as the customers. Reproducibility of results in plate assays is influenced by multiple factors; one of these is the repetitive pipetting task that has to be performed inevitably when using 96- or 384-well plates.

When developing plate assays, you have to consider all the different liquids that are used during your experiment. The liquids used vary greatly depending on the application and may not have the same properties as water [1]. Each application has its own tripping hazard, e.g., viscous liquids like glycerol for stock cultures, foam formation when using cell culture medium, or aerosol formation when pipetting warm liquids.

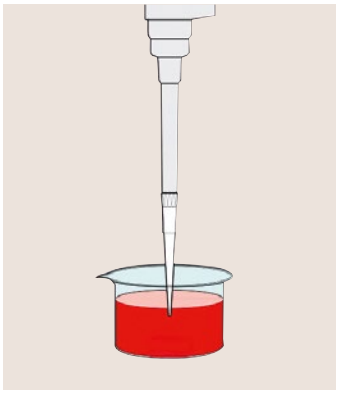
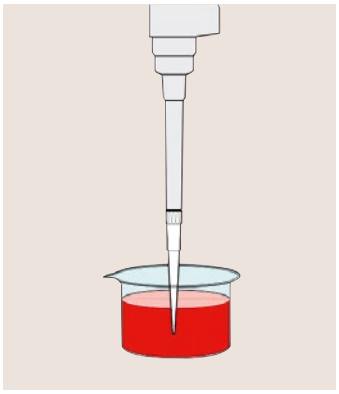
The addition of different concentrations of antibiotics or compounds of any kind is essential for most plate assays and influences your results. This step should be quick and easy to execute in order to avoid the next big danger in plate assays: contamination. This everyday threat caused by mycoplasma in cell culture, bacteria or viruses, DNA and RNA, cross contamination, and contamination introduced by the operators themselves can destroy plate assays you have been working on for weeks or even months [2, 3, 4, 5]. This major threat can be reduced significantly when considering some main points during the pipetting of your plate assay.

Speed is another important consideration when working with plates. The time needed for tasks such as reagent addition to replicates should be kept as short as possible to avoid airborne contamination during long periods of opened plates. Furthermore, each minute outside the incubator can have an impact on your results and lead to slower cell or bacterial growth, or even cell death. Additionally, the risk of making mistakes rises when many plates have to be processed in a short time. Not to mention that potential time gradients between the first and last sample can skew the results. Not only your assay and laboratory setup influence these points, but also the liquid handling tool used for your plate assay has a tremendous impact on the assay itself. Two main pipetting systems exist on the market, air cushion system and positive displacement system. In air cushion systems, often also referred to as "classic pipettes", air is between the sample and the piston of the pipette. This is advantageous for aqueous solutions such as water or salt solution. But the air also reacts like an elastic spring when liquids of a higher density and viscosity such as glycerol are pipetted. Furthermore, volatile liquids such as ethanol evaporate due to their high vapor pressure, and dripping out of the pipet tip is the result. This leads to a decrease in precision and accuracy if the wrong pipetting technique is applied. Positive displacement systems lack the air cushion and therefore pipetting liquids apart from water does not lead to this effect. Here we present solutions by choosing the correct pipetting system and technique for mastering challenging plate assays, assuring you that there is a solution to match you and your lab.

## Solutions and Benefits

**1. High precision and accuracy**

Guaranteed reproducibility in plate assays is essential for a stable process. It depends not only on the test itself, but also on the pipetting instrument used and the operator performing the pipetting tasks. The addition of 10 µL of a compound to an entire 96 well plate is a good example to compare the reproducibility of different pipetting tools. The sample volume is low, so high precision is required. Using a mechanical, single-channel pipette with the same tip for pipetting all wells of that plate is recommended to assure consistent pipetting results. But during manual pipetting of that many wells, not every aspirating and dispensing event can be exactly the same; in most cases, 8- or 12-channel mechanical pipettes are used. This increases the reproducibility within each column, respectively. Still, the aspiration and dispensing volume, as well as the speed, are mostly under user control. A perfectly vertical pipetting angle and shallow immersion depth are essential during aspiration to achieve accurate volume delivery into the pipette tip (figure 1). Otherwise, the systematic and random pipetting errors grow and lead to non-reproducible and inaccurate pipetting results. One essential step to increase the precision and accuracy of pipetting is pre-wetting of the pipette tip. Once the tip is immersed into the liquid one should aspirate and dispense 2-3 times into the liquid to saturate the air cushion. This is mandatory for volumes below 10 µL and recommended for each pipetting step.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|---|---|---|---|
| **Holding angle** | 90° | 90° | >30° |
| **Immersion depth** | 2 - 3 mm | 3 cm | 3 - 4 cm |
| **Systematic measurement deviation** | 0.2 - 0.4 % | 0.6 - 0.8 % | 1 - 1.2 % |
| **Additional volume when 10 µL are set** | 0.02 - 0.04 µL | 0.06 - 0.08 µL | 0.1 - 0.12 µL |

**Figure 1:** The systematic measurement deviation increases when a pipette is not held vertical and the immersion depth is too deep during liquid aspiration. This results in higher volume than actually set on the pipette and constantly wrong pipetting results. When dispensing liquid an angle of 20-45° with contact to the vessel wall is recommended.

Additionally, the user must take care to hit the well and dispense in contact to the vessel wall. The reproducibility can vary between each well and, in the worst case, all 96 wells can show different results.
A more mind-relaxing option is the usage of a dispenser or stepper. These instruments can aspirate the whole volume for all 96 wells into one tip and then dispense it in multiple steps. In this case, 960 µL of the additive would be aspirated to dispense 10 µL in 96 steps. Each dispensing step is exactly the same and more accurate than using a mechanical pipette since no repeated aspirating and dispensing cycles need to be performed. In electronic dispensers the dispensing speed and stroke is under the instrument's control; the user only has to take care to precisely hit the well. Additionally, the aspirating and dispensing angle does not have to be considered, which increases the reproducibility and speed of plate assays. The fastest option for adding sample liquids to a 96 well plate is for sure an instrument that can handle 96 channels at the same time, like the ep*Motion*® 96. Each of the 96 channels aspirates 10 µL of your sample and dispenses the volume in all wells at the same time. This is particularly advantageous when performing enzymatic plate assays where the start and stop time point of a reaction is important. Furthermore, the electronic instrument takes over the aspirating and dispensing speed. The platform secures a tight fit of the plate and guarantees that each pipette tip is aligned perfectly straight and hits its target. Only the height of the plate is under user control. Therefore the reproducibility of plate assays using an electronic 96-channel instrument is the highest [6].

## 2. Variable liquid properties of chemicals

Almost every experimental setup contains multiple liquids. These often vary in their properties and differ significantly from the properties of water. One example is glycerol, a commonly used additive for stock cultures, PCR master mix, and enzyme storage buffers. It has a higher density than water, which leads to viscosity and a bad flow behavior with air cushion pipettes. But why?

Firstly, the general differences between the two pipette types must be clarified. Air cushion pipettes are the best known pipette type. These are characterized by a cushion of air that is present between the sample liquid and the piston inside the instrument. The piston is moved up during pipetting which leads to less air inside the pipette tip. The volume of liquid sample aspirated equals the air volume that has been pushed out before (figure 2, left). Air cushion pipettes are calibrated to water and work best with aqueous solutions. The second pipette type is the positive displacement pipette. Here the liquid sample is aspirated directly into a tip that has an integrated piston and no air is present in between (figure 2, right).

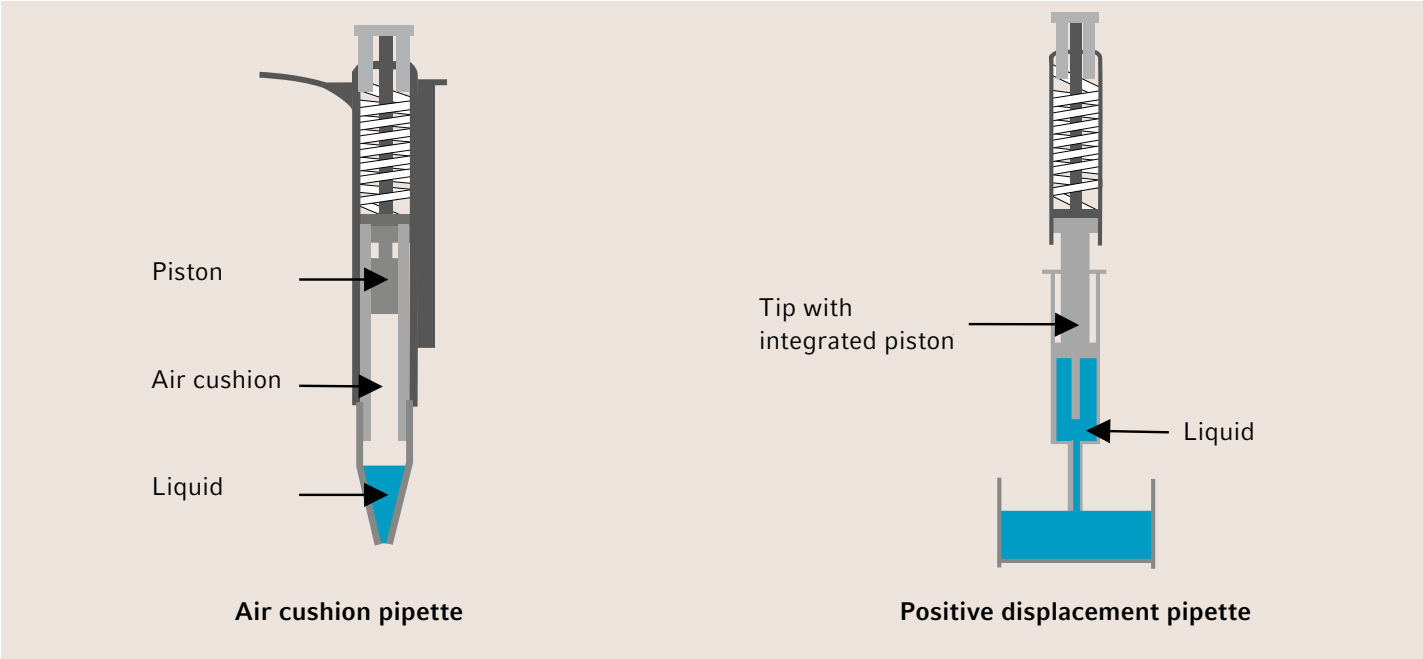


**Figure 2:** Two pipetting systems exist on the market: air cushion systems with air in between the liquid and the piston of the instrument (left side) and positive displacement pipettes, where no air is present (right side).

Back to our example with glycerol that is highly viscous: it shows a poor flow behavior with air cushion pipettes because the density is much higher than that of water. Glycerol cannot be aspirated and dispensed without a special technique, so called reverse pipetting, and a lot of patience. Another common problem is the formation of foam or bubbles during pipetting protein-containing solutions like cell culture medium or Bovine Serum Albumin (BSA) containing buffer. This phenomenon occurs only with air cushion pipettes and the technique of reverse pipetting needs to be applied to counter it. Volatile liquids, like ethanol, acetone, and chloroform, are also used on a regular basis in laboratories. They lead to liquid dripping out of the pipette tip because the high vapor pressure simply presses the liquid out of the tip again.

One strategy to avoid this is pre-wetting of the pipette tip. This means aspirating and dispensing the liquid 2-3 times before actually aspirating the sample volume that shall be used for the experiment.

As described manual pipettes that work according to the air cushion principle (Eppendorf Research® plus, Eppendorf Reference® 2) reach their limit with these demanding liquids. So using these liquids with air cushion pipettes can lead to inaccurate and imprecise pipetting results. Careful consideration of the pipetting technique, as well as the pipetting tool to use with these liquids is necessary.

One option is the usage of an electronic pipette in which one can choose the liquid type, like the Eppendorf Xplorer® plus. For example, it is possible to select 50 % glycerol as the sample and the pipette adjusts itself to the liquid properties to guarantee accurate and precise pipetting results.

Nevertheless, the systems which are most dependable are positive displacement systems, such as Multipette®/Repeater® M4, Multipette®/Repeater® E3/E3x and Biomaster® 4830.

These systems can deal with most of the liquid types without further consideration of adjusting your pipetting technique. The piston is integrated in the instrument's tip and no air cushion is present between the sample and the piston. This principle has the effect of eliminating problems occurring due to an air cushion, so that liquid dripping, bad flow behavior, and foam formation can be excluded.

**3. Addition of different concentrations of compounds to the plate assay**

Often during plate-based screenings, one needs to apply different concentrations of additives in each row or column of a plate. Sometimes even in each well. It is time-consuming work to change the volume on manual pipettes after each pipetting step. Some electronic pipettes and dispensers offer a mode called "Sequential Dispensing".

In this mode one can program different volumes for each dispensing step e.g., step 1 = 20 µL, step 2 = 33 µL, step 3 = 51 µL and so on. Thus, not only the time for setting the volume is reduced, but also the overall time needed for pipetting since a large volume of additive is aspirated at once and then dispensed in multiple steps. Instruments offering this convenient method are the Eppendorf Xplorer plus or Multipette/Repeater E3x.

**4. Potential contamination with bacteria, viruses or mycoplasma**

A major threat in plate-based assays, and especially in cell-based assays, is the contamination of the plate. This ruins the whole experiment and possibly spreads over to colleagues' plates to ruin their experiment, too. Good laboratory practice is mandatory when working with cell-based assays in plates. Not only the surroundings and the working material need to be sterile, but also the operators themselves have to take care not to introduce contamination into the plate. Most of the threats actually stem from handling mistakes or incomplete disinfection by the operators. Usually, the pipetting tool is not taken into account when contamination occurs in a laboratory. But let us have a look on the different threats brought in by pipetting tools. One major issue is cross contamination via aerosols. This could happen by e.g., warm cell culture medium that evaporates into the pipette cone. There, a nice habitat for bacteria and mycoplasma develops and these might be transferred into the next sample during following pipetting steps. One way to prevent aerosols is using a filter tip for each pipetting step in cell-based assays when air cushion pipettes like Eppendorf Research plus, Reference 2 or Eppendorf Xplorer are used. Hereby one should choose two layered filter tips that retain aerosols, splashes, biomolecules like DNA and RNA, as well as bigger particles, such as bacteria and viruses. Another contamination source can be the pipette itself, especially when it is used by multiple users. Therefore it is recommended to autoclave and de-contaminate pipettes on a regular basis. All manual Eppendorf pipettes (Eppendorf Research plus and Eppendorf Reference 2) can be autoclaved without having to be disassembled.

Contamination-free work with infectious samples can be guaranteed by using positive displacement systems. The sample is safely enclosed between the sealing lip of the piston inside the tip and the tip opening without any air and without any possibility of aerosol formation. Furthermore, the instrument is perfectly protected from the sample since it is only present inside the tip and no connection between the sample and the handheld device is possible.
Additionally, positive displacement tips are available in a very high purity grade, called Biopur®, where each tip is sterile, single- wrapped, free of DNA, RNA, DNase, ATP, PCR inhibitors, and pyrogens. So using the Multipette/Repeater M4 or Multipette/Repeater E3/E3x in combination with Combitips advanced® in Biopur quality is an ideal solution to avoid contamination in plate assays.

**5. Improvement and acceleration of time-critical assays**

Speeding up time-sensitive plate assays can have both advantages and disadvantages for the experiment. Working too fast may lead to mistakes, while working too slow leads to misleading, false positive or false negative results. Especially in enzymatic applications, time is critical. Starting and stopping multiple reactions at the same time is essential for achieving comparable, reproducible results. Improving your time-critical enzymatic assays means high precision in pipetting with a pipetting tool that covers as many wells as possible at the same time.

Simultaneous addition of enzymatic solutions is not possible using manual single-channel pipettes. Even with multi-channel pipettes, only one row or column of the plate can be handled and the pipetting speed of the user mainly influences the experiment. So it is impossible to add the solution to the whole plate at the same time point. Some researchers are using a stopwatch to record the time differences between each row. This method is widely used, but not precise enough. A reliable solution for time-critical enzymatic assays can only be provided by systems that apply the enzymatic solution onto the whole 96-well plate at once. Therefore, a 96-channel pipette, like the ep*Motion* 96 is ideal (figure 3).

**Time needed to fill one 96 well assay plate with different liquid handling tools**

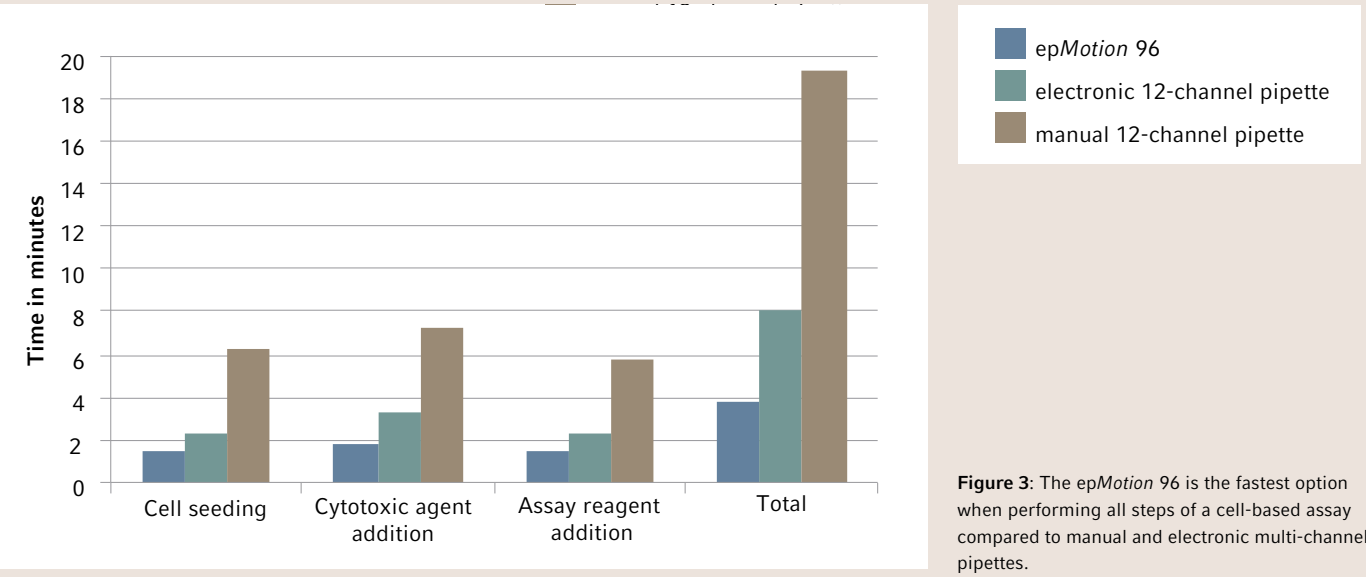


**Figure 3**: The ep*Motion* 96 is the fastest option when performing all steps of a cell-based assay compared to manual and electronic multi-channel pipettes.

This device showed to be not only advantageous for enzymatic assays and simultaneous addition of compounds, but also the fastest option for cell applications compared to manual multichannel and electronic multichannel pipettes [6]. Additionally all steps of a cell-based assay, like cell seeding and reagent addition, can be performed with the 96-channel pipette head which leads to most reliable and reproducible results. Increasing the speed during enzymatic assays can also be achieved by using the ep*Motion* 96 2-position slider. This upgrade of the 1-position instrument offers the possibility to work with a reservoir and a plate at the same time. It reduces the time needed to switch between vessels.

## Summary

It is important to consider the different steps of a plate assay when choosing the correct tool to provide optimal performance for pipetting accuracy and precision. Careful consideration of the pipetting technique, such as a vertical angle and low immersion depth, can determine the success or failure of an experiment. Examples like challenging liquids with properties differing from water have been discussed, showing that either a special pipetting technique with air cushion pipettes or a different tool, like a positive displacement system, lead to the desired result. While for general washing steps or time-critical assays where the whole plate needs to be implemented at once, a 96-channel instrument is beneficial. Preventing contamination is always mandatory and can be achieved by using either air-cushion pipettes with filter tips or positive displacement systems. So for every plate assay an ideal pipetting tool is offered by Eppendorf.

If you have further questions about which pipetting tool is ideal for your application, please contact your local sales rep for expert guidance.

## References

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| Eppendorf Reference® 2, 10 – 100 µL yellow | 4920 000.059 | 4920000059 |
| Eppendorf Reference® 2, 20 – 200 µL yellow | 4920 000.067 | 4920000067 |
| Eppendorf Reference® 2, 100 – 1,000 µL blue | 4920 000.083 | 4920000083 |
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| Eppendorf Xplorer® plus, 5 – 100 µL yellow | 4861 000.716 | 4861000716 |
| Eppendorf Xplorer® plus, 15 – 300 µL orange | 4861 000.724 | 4861000724 |
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| Multipette®/Repeater® E3, with charging cable and 2 Combitips advanced® assortment packs, 1 µL - 50 mL | 4987 000.010 | 4987000010 |
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| ep*Motion*® 96, semi-automated electronic pipette for parallel 96 channel microplate pro-cessing, (without iPod® controller), 100 - 240 V ±10 %/50 - 60 Hz ±5 %, 0.5 - 300 µL | 5069 000.012 | 5069000012 |
| ep*Motion*® 96, with 2-position slider, semi-automated electronic pipette for parallel 96 channel microplate processing, (without iPod® controller), 100 - 240 V ±10 %/50 - 60 Hz ±5 %, 0.5 - 300 µL | 5069 000.101 | 5069000101 |
| **Upgrade kit,** 2-position slider | 5069 074.008 | 5069074008 |

*The Eppendorf Research® plus and Reference® 2 are available as single channel pipette with fixed and variable volume as well as 8 and 12 channel pipette.
** The Eppendorf Xplorer® and Xplorer® plus are available as single channel, 8 and 12 channel pipette in multiple variable volumes.

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