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APPLICATION NOTE No. 490

No Trespassing for Bio-Aerosols! Approved Protection of Pipettes with ep Dualfilter T.I.P.S.® Filter Pipette Tips.

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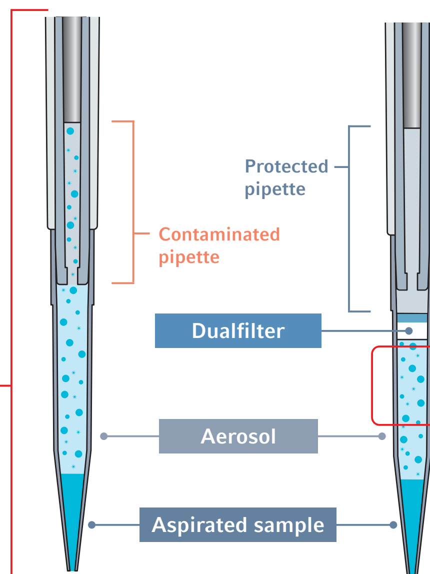
Abstract

Aerosol formation during pipetting with air cushion pipettes is a major concern in laboratories due to the risk of contamination which can lead to incorrect results. This study investigates the formation of aerosols with various biological samples including DNA, viruses, mycoplasma and bacteria, and the resulting contamination risk during pipetting. The findings confirm that aerosols are generated from all tested sample types, and that they infiltrate and contaminate the pipette lower part, even by regular pipetting. The use of ep Dualfilter T.I.P.S.® filter pipette tips and ep Dualfilter T.I.P.S. SealMax® with a two-layered filter was found to be highly effective in preventing such contamination. Regardless of the sample type, they efficiently blocked aerosols from entering the pipette shaft, ensuring contamination-free pipetting. These results highlight the critical role of high-quality filter pipette tips for reliable and reproducible results.

Sample mixing

Aerosol formation above sample



Aerosols

> Form after one mixing step

> Form over every sample type

> Contain the sample:
- DNA
- Virus
- Living mycoplasma
- Living bacteria

> Contaminate the pipette shaft

> Are blocked efficiently by ep Dualfilter T.I.P.S®

Graphical abstract.

Introduction

An aerosol is a colloid of fine solid particles or liquid droplets in a gas. Besides evaporation and condensation, aerosols can form through physical processes such as spraying, bubbling, splashing, or even simple liquid movement. Consequently, normal pipetting or mixing of a liquid sample with an air-cushion pipette and the corresponding pipette tip can lead to aerosol formation by the sample [1], [2]. This poses a significant contamination risk, as parts of the sample could infiltrate the pipette, leading to (cross-)contamination and subsequently to inaccurate results after pipetting the following samples.
To mitigate this risk, it is a common practice to use positive displacement systems or, in case of the commonly used air-cushion system, filtered pipette tips. The latter contain a small filter to prevent aerosols from entering and contaminating the pipette. Filter pipette tips optimized for the use with Eppendorf pipettes, ep Dualfilter T.I.P.S., are pipette filter tips that feature a two-layered filter. These filter tips demonstrate a filter efficiency of ISO 25 E in accordance with DIN EN ISO 29463-5 or higher [3], [4]. Filter efficiency measurements further highlight that these tips offer the highest efficiency among twelve filter pipette tip manufacturers according to ISO guidelines and even reach the HEPA-filter level [5].
However, questions remain: Do aerosols form during standard pipetting? Does a contaminated pipette transfer contamination to subsequent samples? Do filter pipette tips truly block aerosols generated during pipetting? Is it essential to use filter pipette tips to prevent contamination of the pipette?
This study investigates the formation of aerosols with various biological samples and the resulting contamination risk during pipetting. In addition, it compares the efficacy of filtered versus non-filtered pipette tips in preventing contamination.

Results and Discussion

Aerosols form during pipetting

First, it was investigated whether aerosols *containing the sample* form above a sample that is mixed by simple up and down pipetting. We used the very common pipette and tip combination of an Eppendorf Research® plus 100 - 1000 µL mechanical pipette and a non-filtered pipette tip epT.I.P.S.® 50 - 1000 µL. Genomic λDNA was mixed once or 20 times, and the air space above the filling level inside the tip was then sampled to detect a potentially contaminated aerosol formed during mixing.
After only mixing once, four out of ten replicates contained detectable fragments of gλDNA, indicating aerosol formation in 40% of the cases. After 20 mixing cycles, a gλDNA-contamination of the air space was detectable in every experiment, indicating that in every case, an aerosol forms above the sample. The amount within the aerosol was determined to approx. 10^3 copies of the DNA. The experiment was repeated with the produced PCR product, the DNA amplicon, as the sample. With this sample containing much smaller DNA molecules (107 bp) in a higher concentration, mixing only once was enough to create an aerosol containing the sample and thus detectable contamination of the air space above the sample. The amount within the aerosol was determined to approx. 10^4 copies of the DNA amplicon. The results show that in any case, even after just one pipetting step, detectable contamination was found in the air space above the sample, indicating aerosol formation and the risk of contamination (Figure 1).

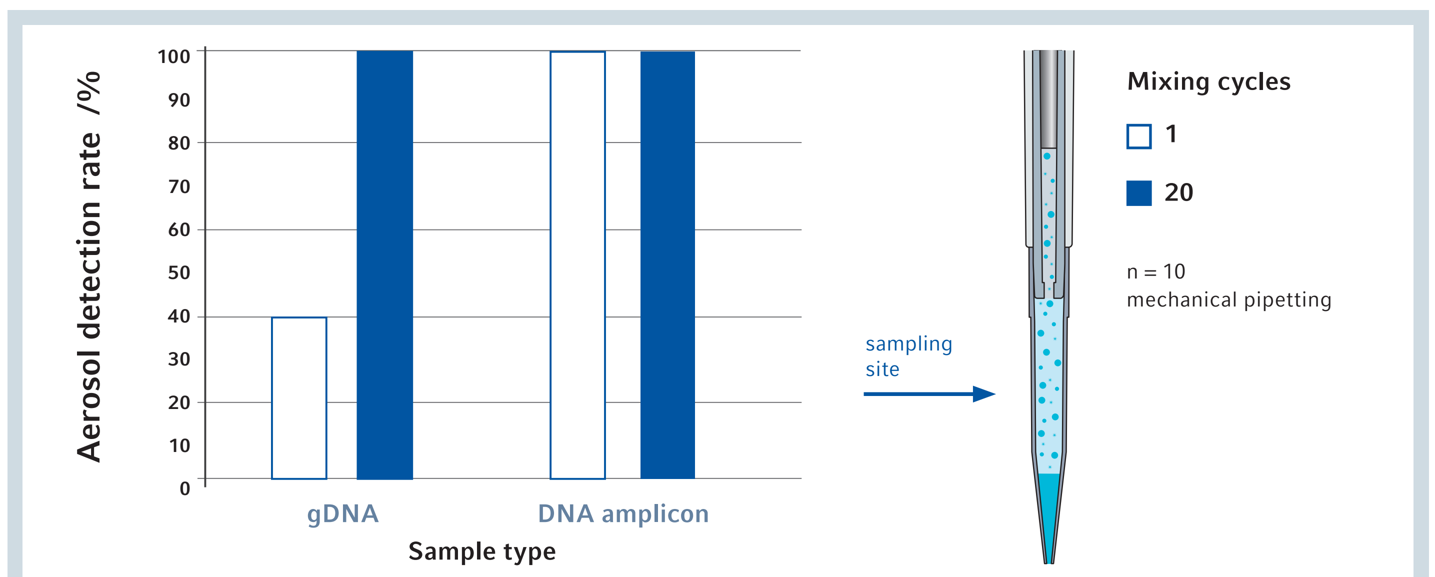


Figure 1: Aerosol detection rate (%) after mixing gDNA (left) or a DNA amplicon (right) once (open bars) or twenty times (closed bars). A mechanical Eppendorf Research plus 100 - 1000 µL pipette and corresponding non-filtered epT.I.P.S. were used, n = 10.

Aerosols form among different sample types

It was then investigated whether aerosols containing the sample are also formed from other sample types during pipetting. To standardize the method, an Eppendorf Xplorer® plus 50 - 1000 µL electronic pipette was used.
The λDNA-PCR-amplicon was sampled again as a reference point. In addition, three more sample types were tested: a bacterial culture, a viral suspension, and a mycoplasma culture. As a bacterial sample, *Pseudomonas aeruginosa* was selected, as recommended by the American Type Culture Collection to investigate bio-aerosols [6]. As a viral sample, we used living, attenuated yellow fever virus. As a mycoplasma sample, we used *Acheloplasma laidlawii*, one of the most common mycoplasma species contaminating cell-culture assays [7]. These four sample types vary in size to represent different particle sizes of samples that could be found in generated aerosols (Figure 2), while also representing the most common sample types in molecular biology and cell culture labs.

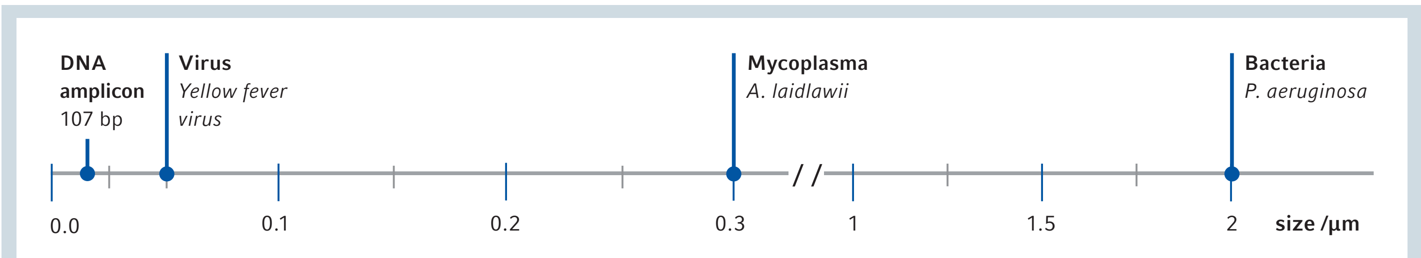


Figure 2: Sizes (smallest approx. diameter) of sample types tested within this study: DNA amplicon, virus, mycoplasma, and bacteria. All sizes depict an aqueous suspension or solution of the stated sample type. For the DNA amplicon, the theoretical hydrodynamic radius is displayed.

We applied the same protocol of mixing twenty times to all samples and evaluated how often an aerosol was detected in the air space above the sample.
It was observed that using an electronic pipette, an aerosol formed with the DNA amplicon, too, with a 50% detection rate. All other tested challenging samples were also detected in the air space above the sample. The aerosol detection rates for the bacteria culture were 20 % (4 out of 20 replicates), for the mycoplasma culture it was 38% (8 out of 21 replicates) and for viral suspension 100% (9 out of 9 replicates). The number of replicates was adapted to the frequency an aerosol was detected to make the data reliable. This resembles a clear indication of the formation of aerosol with each sample (Figure 3). It is noteworthy that some assays are more sensitive than others (e.g., qPCR is more sensitive than selective growth, see Materials and Methods). This means that the data indicates the bare minimum of chances that an aerosol forms and that depending on operator (skill level, technique), sample type (size, density, concentration, etc.), pipette and tip (pipetting speed, shape of air cushion, etc.), the aerosol formation rate might be higher.

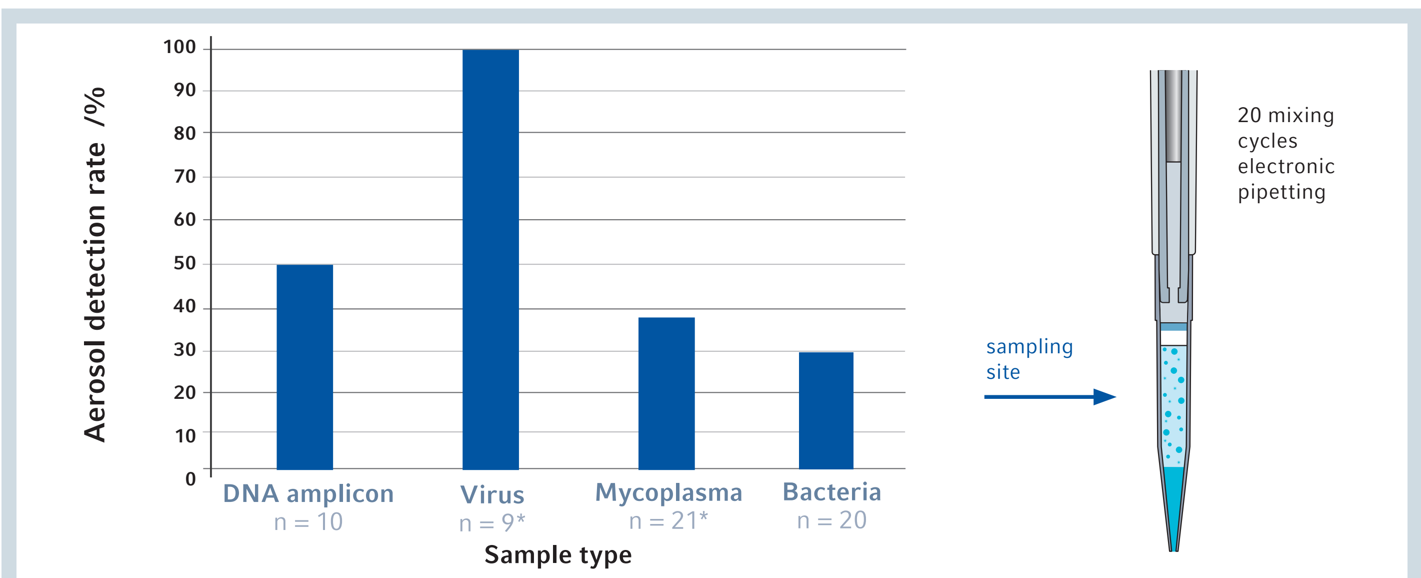


Figure 3: Aerosol detection rate (%) after mixing the stated sample type twenty times with an electronic Eppendorf Xplorer plus 50 - 1000 µL pipette used with the corresponding ep Dualfilter T.I.P.S. *Replicates were performed over three independent biological replicates.

Aerosols formed during pipetting are efficiently blocked by the two-layered Dualfilter

Next, it was checked whether the detected aerosols can be blocked with the two-layered Dualfilter inside the ep Dualfilter T.I.P.S. pipette filter tips. To do so, a sample was taken above the filter inside the pipette tip after the described mixing experiments. The results were clear: regardless of the type of mixed sample, no contaminations were detected above the filter (Figure 4). The same was checked and observed on a random basis with the self-sealing ep Dualfilter T.I.P.S. SealMax (data not shown). This vividly shows that the Dualfilter inside ep Dualfilter T.I.P.S. prevents aerosols that are created by standard pipetting from entering the pipette and thus prevents (cross-)contamination.

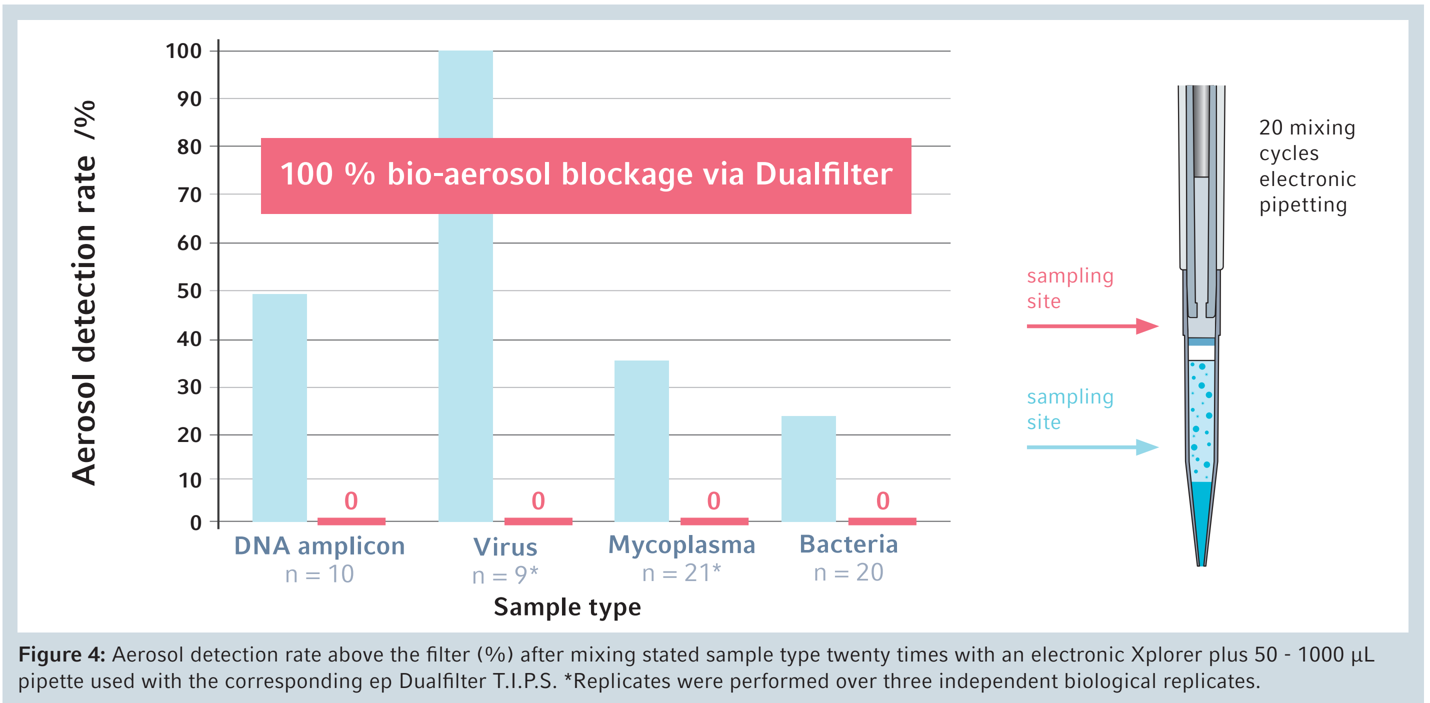


Figure 4: Aerosol detection rate above the filter (%) after mixing stated sample type twenty times with an electronic Xplorer plus 50 - 1000 µL pipette used with the corresponding ep Dualfilter T.I.P.S. *Replicates were performed over three independent biological replicates.

Aerosols infiltrate the pipette shaft

The next question was whether the detected aerosols could penetrate the pipette shaft and thus lead to contamination of the lab equipment. To investigate this, the bacterial sample and mycoplasma sample were mixed with the same electronic pipetting system using a non-filtered pipette tip. After mixing, samples were taken within the pipette shaft. Indeed, samples of living bacterial and mycoplasma cells were detected inside the pipette lower part after mixing (Figure 5). For the bacteria culture, in three out of seven replicates the inner pipette lumen was found to be contaminated with living organism. For the mycoplasma sample, in six of nine cases, the living sample was detectable inside the pipette cone shaft and thus infiltrated the device demonstrably (aerosol detection rate of 43 and 67% respectively). These results not only demonstrate that various sample types infiltrate the pipette lumen during simple pipetting, but that other biological sample types are likely to do so, too (like the previously tested DNA and virus samples, where aerosol formation was detectable inside the pipette tip, above the filling level) when using non-filtered pipette tips. And, they also indicate that using a non-filtered tip led to higher aerosol formation than using filtered tips (Figure 3, Bacteria: 25%, Mycoplasm: 38%), most possibly due to a change in the flow behavior of the air in the air cushion due to the missing filter, or due to differences of the sampling site.

Accuracy and precision of the pipetting system however were not impaired by the switch from standard to filter tips and the pipetting systems error lay within ISO 8655:2022 and the devices limits (data not shown).

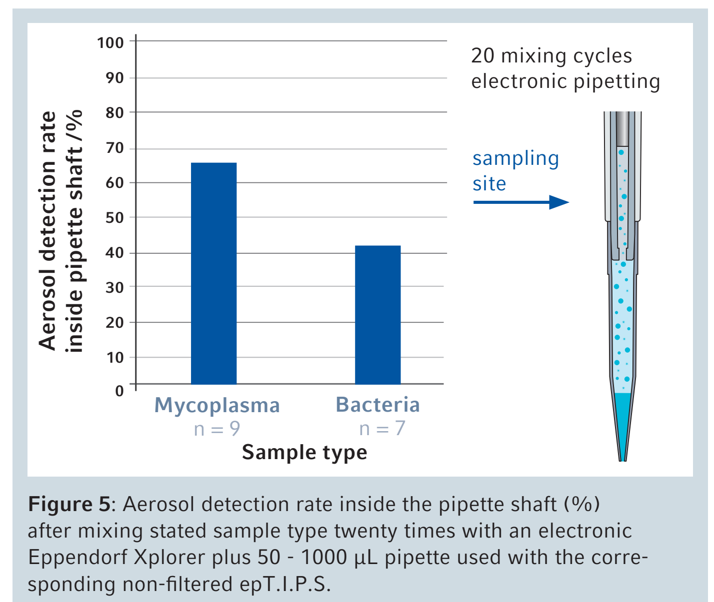


Figure 5: Aerosol detection rate inside the pipette shaft (%) after mixing stated sample type twenty times with an electronic Eppendorf Xplorer plus 50 - 1000 µL pipette used with the corresponding non-filtered epT.I.P.S.

Mycoplasma contamination stays inside pipette for six weeks

To clean the lower part of the pipette, the lower sections of the pipette from each replicate of the mycoplasma samples (Figure 6) in the non-filtered tip experiment were disassembled and wiped with 70% isopropanol. **Despite this, contamination was still detectable in one-third of the pipette cones after six weeks.** Soaking the entire lower part in 70% isopropanol also failed to fully eliminate the contamination. Complete decontamination was achieved only through autoclaving the lower parts. This highlights the necessity of using filter pipette tips to prevent initial contamination, of the autoclavability of pipette lower parts, even from electronic versions, and the importance of establishing proper decontamination protocols when working with living organisms or following a mycoplasma contamination [8]. Without it, a contamination by mycoplasma cells has drastic effects on numerous cell-culture assays [9].

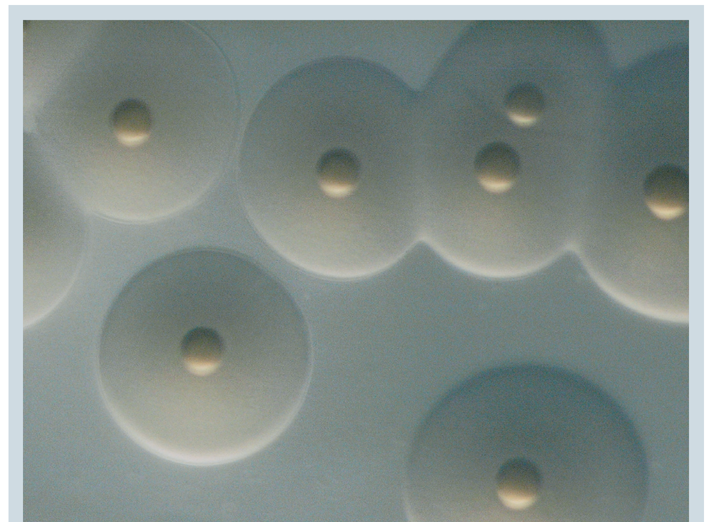


Figure 6: Growth of the mycoplasm species *A. laidlawii* used for the mycoplasm mixing experiments. 10^{-6} dilution, 25x magnification.

Conclusion

This study highlights the significant risk of aerosol formation during pipetting and the contamination of pipettes and subsequent samples. Aerosols were found to form with all tested samples during pipetting and to carry contaminants like DNA, viruses, mycoplasma, and bacteria. Our experiments confirmed that these aerosols can indeed contaminate the pipette, posing a serious threat of cross-contamination. These contaminants of living organisms were found to stay inside the pipette lower part for at least six weeks if not decontaminated properly.

The use of ep Dualfilter T.I.P.S. filter pipette tips by Eppendorf was found to be highly effective in preventing such contamination. Regardless of the type of sample – whether DNA, virus, mycoplasma, or bacteria – the ep Dualfilter T.I.P.S. efficiently blocked aerosols from entering the pipette lower part.

In conclusion, the findings of this study demonstrate that aerosols consistently form above various biological sample types and highlight the critical role of using high-quality filter pipette tips like ep Dualfilter T.I.P.S., in maintaining contamination-free laboratory environments. By efficiently preventing aerosol contamination of the pipette, ep Dualfilter T.I.P.S. two-layered filter pipette tips ensure the reliability and integrity of sensitive molecular biology workflows, making them an essential tool for a laboratory concerned with contamination control, which safes a lot of time and money in the end by avoiding unreproducible results due to unintended and/or undetected contamination.

Materials and Methods

Determination of aerosol formation

The general protocol for aerosol formation determination went as follows: 600 µL of the challenging solution were pipetted up and down ("mixing") 20 times using an Eppendorf Xplorer plus 50 - 1000 µL electronic pipette (art. no. 4861 000 732) at speed 8 employing ep Dualfilter T.I.P.S. 50 - 1000 µL (art. no. 0030 078 578). This resulted in an air space volume between the liquid filling-level and the filter inside the tip of approx 580 mm^3. The full air-cushion size, in which the aerosol can be formed during pipetting, is approx. 2970 mm^3 when using a standard tip, and approx 3220 mm^3 when using a filtered pipette tip. All tips for one experiment were from one lot. Experiments were carried out at a room temperature of (20 ± 3) °C. For mechanical pipetting, all repetitions of an experiment were pipetted by the same person with fast, consistent speed to avoid variances due to technique or varying pipetting speed. Samples of aerosol formation were collected at up to three sites per mixing-experiment (Figure 7): in the air space above the sample, below the filter (1), above the filter within the tip (2) or inside the pipette shaft lumen (3).

DNA samples were probed using a syringe to extract the air space where indicated. To do so, a sterile syringe was pierced through the tip after mixing and dispensing. It was inserted above the filling level and the full air cushion was extracted. In order to be able to detect living organisms transmitted via aerosol, too, sampling was adapted for other sample types: In these cases, the tip or pipette shaft were swept with a sterile swab after dispensing (see individual assay types). To reach inside the tip with a swab below the filter, the liquid filling level was marked, and the tip was cut at this level after mixing and dispensing to allow for sampling swabs to enter. An optical inspection ensured that no splashes contaminated the part to be sampled. To observe the influence of mixing cycles, mixing was adjusted to only one step where indicated in the results. To examine differences in pipetting techniques, the mechanical Eppendorf Research plus 100 - 1000 µL (art. no 3123 000 063) was used where indicated in the results. To investigate differences according to the pipette tip, ep Dualfilter T.I.P.S. SealMax 50 - 1000 µL (art. no. 0030 078 748) or epT.I.P.S. 50 - 1000 µL (art. no. 0030 073.851 and 0030 075 250) were employed where indicated in the results. When tips without filters were used, the lower part of the pipette was removed and replaced with a new, sterile and pure spare part (art. no. 3120 620 001) to ensure no cross-contamination. Different sample types were analyzed when mixed individually within the pipette tips to examine the aerosol formation behavior: a DNA solution, a virus suspension, a mycoplasma suspension, and a bacteria suspension. In the following text, each sample preparation and read out is described in detail.

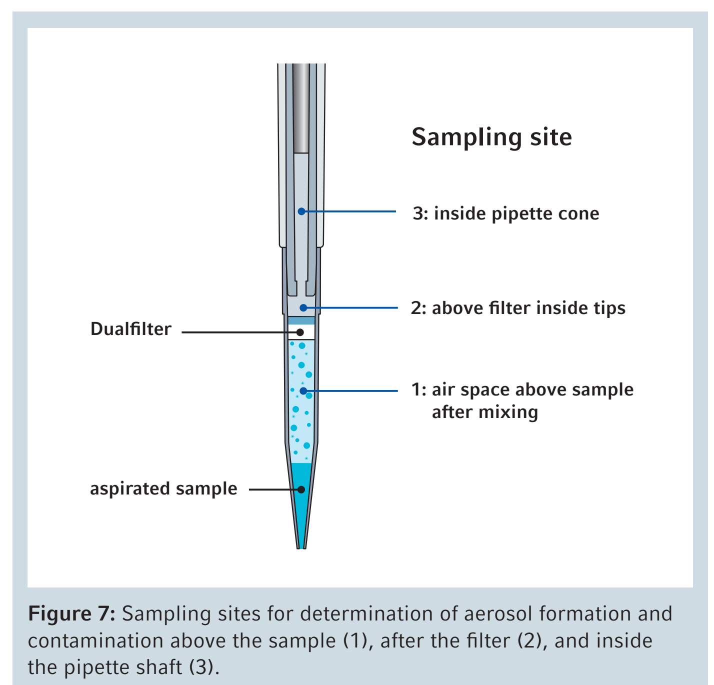


Figure 7: Sampling sites for determination of aerosol formation and contamination above the sample (1), after the filter (2), and inside the pipette shaft (3).

DNA samples

Genomic lambda Phage DNA (gλDNA, Sigma-Aldrich®, art. no. 10745782001) was diluted to $5 \cdot 10^9$ copies μL^{-1} and mixed according to the described protocol for aerosol formation determination. Samples were taken extracting the full gaseous phase using a syringe (TERUMO, art. no. SS05LE1). To detect DNA in the collected sample, a qPCR assay for λDNA was developed using the Kapa SYBR® Fast qPCR Kit (Kapa Biosystems, art. no. KK4601) and specific primers (Eurogentec®, forward: 5'-CGC ACA GGA ACT GAA GAA TG-3', reverse: 5'-CCG TCG AGA ATA CTG GCA AT-3'). Each 20 µL reaction contained 10 µL of Kapa SYBR Fast qPCR Master Mix, 0.6 µL of each primer (10 µM), 3.8 µL of water, and 5 µL of sample. For the aerosol sample, 5 µL of water was added to the qPCR Master Mix, and air from the tip was injected into the mix. The thermal conditions were 3 min at 95 °C, followed by 40 cycles of 95 °C for 3 seconds and 60 °C for 60 seconds. For DNA amplicon samples, the same protocol was used with the amplicon from one reaction as a sample with a concentration of $3 \cdot 10^{12}$ copies μL^{-1}. Aerosol detection was read positive when the qPCR resulted in a sigmoidal curve with a Cq between 10 and 35. Diluted gλDNA served as a positive control, water as a negative control. With a serial dilution of gλDNA the concentration of DNA in the aerosol could be determined. The dimension of the DNA amplicon was calculated using the radius of gyration, where the persistence length was set to $5 \cdot 10^{-2}$ µm, the approximate distance between base pairs to $3.4 \cdot 10^{-4}$ µm and the hydrodynamic factor to 0.662.

Bacteria samples

A high titer (approx. 10^9 CFU mL^{-1}), 24 hour actively growing culture of *P. aeruginosa* (DSMZ, art. no. 50071) was mixed according to the protocol described for aerosol formation. Samples (Figure 7) were collected using a swab (Copan Sterile swabs in tubes, VWR™, art. no. 710-0457) and placed on McConkey agar plates (NutriSelect™ Basic, Sigma, art. no. M7408-250G). For reliable identification of the growing cells, API® 20 NE strips (bioMerieux, art. no. 20050) were employed following the manufacturer's instructions. After 24 hours of incubation at 29 °C, results were interpreted according to the reading table. Additionally, an oxidase test (bioMerieux, art. no. 55635) was performed as recommended in the manual. Bacterial identification was completed using the API® 20 NE identification table.
An aerosol detection was read positive when cells grew on McConkey agar and the corresponding species identification correlated to *P. aeruginosa*.

Virus samples

Lyophilized live-attenuated yellow-fever vaccine (Stamaril®, Sanofi Pasteur) was resuspended with the accompanying solvent to at least 1000 LD_{50} units and filtered through a 0.45 µm filter for sterilization. This formed the challenging solution and was mixed according to the described protocol for aerosol formation determination. Samples (Figure 7) were taken with a swab (Copan Sterile swabs in tubes, VWR, art. no. 710-0457). After sample collection, swabs were mixed in 200 µL PBS. The PBS-solution was used directly for kit-based RNA extraction and purification according to the instructions (QIAmp viral RNA MINI kit instructions, QIAGEN®, art. no. 52904).

The quantification of potentially purified RNA was performed using a kit-based RT-qPCR according to instructions (Altona Diagnostics, RealStar® Yellow Fever Virus RT-PCR kit 1.0, art. no. 671013). For each condition, 10 µL of the sample were mixed with 20 µL of Master Mix. The plates were sealed (Eppendorf Masterclear® real-time PCR film, art. no. 0030 132 947) and centrifuged for 30 seconds at 500 rpm. The RT-qPCR was conducted following the kit instructions. An aerosol detection was read positive when the qPCR delivered a sigmoidal curve with a Cq between 10 and 35. Water was used as a negative control and the positive control was provided with the kit.

Mycoplasma samples

A high titer (around 10^9 CFU mL^{-1}), 24 hour actively growing culture of *A. laidlawii* was mixed according to the described protocol for aerosol formation. Samples (Figure 7) were taken using a swab (TSC sterile swabs, 216-24-0848 TF1), and transferred to 5 mL Mycoplasma Liquid Medium (European Pharmacopoeia. 2.6.7). All samples were incubated at 36 °C for 14 days with a liquid to solid subculture after seven days on Mycoplasma Solid Medium (European Pharmacopoeia. 2.6.7). All agar plates were incubated in 95 % N_2 5 % CO_2 at 36 °C for seven days. An aerosol detection was considered positive when the liquid culture showed a color change due to the media's indicator. Additionally, if subcultures from these cultures also showed growing cells, their correct phenotype was confirmed under the microscope.

Literature

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[9] **Article** Eppendorf Lab Academy. How does Mycoplasma contamination affect cell cultures?

Ordering Information

Product	Order no.
ep Dualfilter T.I.P.S.®, Racks, 50-1000 µL PCR clean and sterile	0030 078 578
ep Dualfilter T.I.P.S.® SealMax®, Racks 50-1000 µL, PCR clean and sterile	0030 078 748
epT.I.P.S.® Racks, 50-1000 µL, Biopur®	0030 075 250
Eppendorf Xplorer® plus, 50-1000 µL, blue	4861 000 732
Eppendorf Research® plus, 100-1000 µL, blue	3123 000 063

Eppendorf's pipette filter tips ep Dualfilter T.I.P.S.® are available in volume variants from 0.1 µL to 10 mL. They are also available as ep Dualfilter T.I.P.S.® BioBased.

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