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Application Note

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How to Pipette PCR Master Mix for Increased Accuracy in qPCR Results

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Abstract

Sensitivity of Quantitative Polymerase Chain Reaction (qPCR) based assays is one of the most important reasons for their success and abundant use in scientific laboratories. However, many factors including pipetting technique can influence qPCR assay results. PCR Master mix is routinely used during qPCR set-up but can be challenging to pipette accurately. In this study, we determined the best pipetting technique for pipetting Master mix in qPCR assays. We tested forward and reverse pipetting techniques, the type of pipette tips, pre-wetting of the pipette tip, and the use of electronic pipettes. The test was done with Sartorius Tacta® mechanical pipettes and Sartorius Picus® electronic pipettes. We demonstrate that Master mix can be pipetted to obtain good precision and accuracy using low retention pipette tips and forward pipetting technique, or standard pipette tips with reverse pipetting technique. Use of electronic pipettes ensured both speed and reproducibility of the results. We conclude that for pipetting Master mix, it is important to focus on good pipetting techniques and selection of the right consumables to obtain reproducible and reliable results when performing PCR-based assays.

Introduction

PCR-based applications have become pivotal in biopharmaceutical process, clinical diagnostics, and academic research. In the biopharmaceutical industry, sensitivity of PCR-based applications, such as qPCR or Next Generation Sequencing, enables testing for residual viral material in monoclonal Ab drug products, or provides proof of human-DNA-free products that meets the regulatory standards. The Limit of Detection (LoD, LoQ) of qPCR-based diagnostic tests is important in clinical diagnostics.[1] However, variability in assay results can be a problem when performing Quantitative PCR, qPCR.[2]

Master mix is a challenging reagent to pipette during qPCR set-up. Typically, Master mixes contain polymerase, dNTPs, $MgCl_2$ in buffers that may contain Tween and glycerol.[3] Nowadays, Master mixes are commercially available as ready-to-use solutions. They are slightly viscous and cold since they must be kept on ice. These properties make it difficult to pipette correct volumes. In literature searches, opposing recommendations on handling Master mixes were found with no clear direction for the best practices. In many laboratories, when pipetting Master mix, particularly in qPCR assays that involve large numbers of samples and replicates, the common practice has been to pipette Master mixes without pre-wetting the pipette tips in order to speed the time for assay set-up, and because pre-wetting of pipette tips before pipetting Master mix for each PCR tube is a challenging endeavor in large experiments which also leads to creation of bubbles.

According to ISO-8655, the pipette tip should be pre-wet before pipetting, especially for viscous liquids. For viscous liquids, pre-wetting acts similarly to the extra sample volume aspirated in reverse pipetting technique (described below), and compensates for sample loss because of the tendency of viscous liquids to stick to the standard plastic pipette tip during dispensing. Currently, researchers have the option to use low retention pipette tips instead, which significantly reduces the residual sample left in the tip when pipetting viscous liquids.

In this application note, we provide guidelines and best pipetting practices for reproducibly pipetting qPCR Master mix.

In this application note, we tested Master mix for:

- Forward pipetting vs. reverse pipetting
- Type of pipette tip
- Pre-wetting
- Benefits of electronic pipette use

Methods

Pipetting Techniques

The pipette is a precision instrument and the pipette and tip combination acts as a system. Correct pipetting technique was adhered to throughout this experiment to avoid data variation due to errors from poor pipetting. In brief, proper tip sealing was ensured between the pipette and pipette tip by using manufacturer's tips on the manufacturer's pipette (Sartorius). The pipette was kept vertical during aspiration. Tip immersion during aspiration was kept at 2 mm into the Master mix to avoid aspirating excess volume and to avoid excess Master mix sticking on the outside of the pipette tip. During dispensing, the pipette was angled at 45 degrees and the pipette tip was touched to the inner side of the PCR tube. Master mix was pipetted slowly because of its viscous nature. Forward pipetting and reverse pipetting techniques are shown in Figure 1.

qPCR Setup

Sartorius pipettes (Tacta® mechanical pipettes and Picus® Nxt electronic pipettes), Sartorius Safetyspace filter tips, and Low Retention filter tips were used for qPCR set-up. Lo-Bind EP tubes (Eppendorf) were used for DNA sample preparation and Master mix preparation. A stock PCR Master mix for all tests was prepared using Maxima SYBR Green qPCR Master mix (without ROX) (Thermo Fisher Scientific), primers for *E. coli uidA* gene and nuclease-free water. PCR primers UAL 5'–TGGTAAT-TACCGAC-GAAAACGGC (Sigma-Aldrich) and UAR 5'–ACGCGTGGTTA-CAGTCTTGCG (Sigma-Aldrich) amplify a 147 bp segment of the *uidA* gene in genomic *E. coli* DNA. *E. coli* strains contain a single copy of the *uidA* gene.[4] Eight 15 µL replicates of Master mix were pipetted into wells of the PCR plate for each condition tested. Non-template control (NTC) samples did not contain

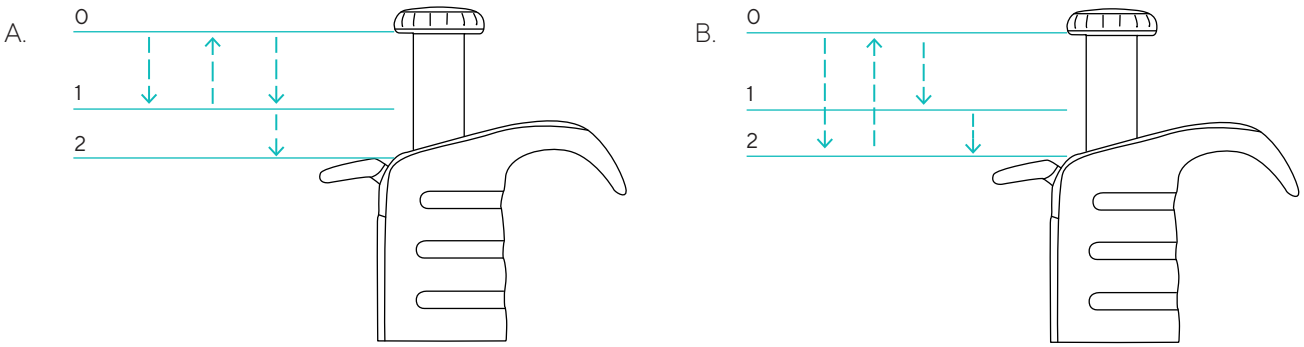

Figure 1: (A) Forward pipetting sequence and (B) Reverse pipetting sequence. For more information on these two pipetting techniques: https://www.youtube.com/watch?v =oH-5hwC8q0, https://www.youtube.com/watch?v=6ZvIVNTu7Xk

E. coli genomic DNA and received 5 µL of nuclease-free water. Serially diluted standards containing 5 µL of *E. coli* gDNA containing 1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 copies | reaction were pipetted similarly. Each test well contained 5 µL of *E. coli* gDNA containing 1×10^3 copies | reaction. All DNA samples were added to the PCR tubes in the same manner using multi-dispense program on Picus® Nxt electronic pipette and Low Retention filter tips. qPCR was performed using LightCycler® 480 qPCR instrument (Applied Biosystems, Foster City, CA). The cycling parameters were as follows: pre-incubation at 95° C for 10 min, 40 cycles of 95° C for 10 sec, 55° C for 10 sec and 75° C for 15 sec, extension for 75° C for 10 sec. SYBR green fluorescence emission was quantified in standards, controls, and samples. Cycle of quantification (Cq) values and actual copy numbers were determined using LightCycler® 480 software and MS Excel was used to analyze the results.

Data Analysis

The systemic error during pipetting is a measure of accuracy—it tells how close the obtained result is to the true value. %Systemic error (%S) of Cq values reflects the error in Cq value of the instrument system (pipette and tip system) for handling Master mix. Random error during pipetting is a measure of the precision of the results, and reflects the variance between replicates in the experiment. %Random error (%R) of Cq values reflects the reproducibility of the results and could be influenced by the experimenter's pipetting. The %Uncertainty value accounts for both accuracy (%Systemic error) and the precision (%Random error) of the results.

Results

Forward Pipetting and Reverse Pipetting

Forward pipetting of Master mix was compared to reverse pipetting, and pre-wetting before forward pipetting was compared to reverse pipetting technique, using Tacta® mechanical pipettes, standard Safetyspace filter tips, and Low Retention (LR) filter tips. As shown in Figure 2, forward pipetting with low retention filter tips gave the best results (%Systemic error of Cq = 0.02, %Random error of Cq = 0.46 and %Uncertainty of Cq = 0.9). The second best method was reverse pipetting with standard filter tips (%Systemic error of Cq = 0.22, %Random error of Cq = 0.50 and %Uncertainty of Cq = 1.2). Thus, forward pipetting with low retention pipette tips or reverse pipetting with standard pipette tips are good methods for accurately and precisely pipetting Master mix. This result also suggests that the low retention properties of the low retention pipette tip eliminate the need for the excess sample in reverse pipetting technique or in pre-wetting technique which compensates for the stickiness of viscous liquids on the standard pipette tips.

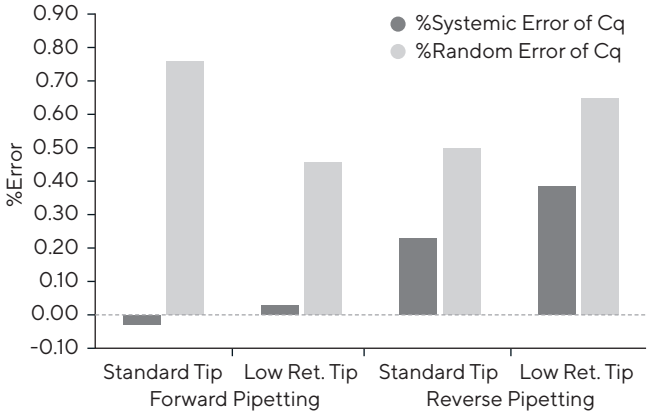

Figure 2: %Error of Cq (cycle of quantification) for forward and reverse pipetting of Master mix. %Systemic error of Cq (cycle of quantification) and %Random of Cq are shown. For each data point, n = 8. Forward pipetting gives a low systemic error compared to reverse pipetting (gray bars). Standard Tip—Sartorius Safetyspace Filter Tips, Low Ret. Tip—Sartorius Safetyspace Low Retention Tips.

Pre-Wetting of Pipette Tips Before Pipetting Master Mix

Pre-wetting conditions the air column of the air displacement pipette, as well as coats the inside of the pipette tip with some excess sample, in order to improve the reproducibility of the results (precision, %Random error). Pre-wetting of the pipette tip 5 times before pipetting Master mix was tested using Tacta® mechanical pipette and standard Safetyspace filter tips. As shown in Figure 3, consistent with ISO-8655, for Master mix with standard filter tips, pre-wetting before forward pipetting slightly improved reproducibility of the results (%Random error of Cq = 0.7) compared to no-pre-wetting before forward pipetting (%Random error of Cq = 0.8). However, reverse pipetting technique with standard filter tips gave better reproducibility of the results (%Random error of Cq = 0.5) than pre-wetting with forward pipetting (%Random error of Cq = 0.7). As expected, pre-wetting of standard filter tips before reverse pipetting is not necessary as it did not have a significant effect on reproducibility of the results (%Random error of Cq = 0.5) compared to not-pre-wetting before reverse pipetting (%Random error of Cq = 0.5). It is important to note that low retention filter tips with forward pipetting gave better reproducibility for pipetting Master mix (%Random error of Cq = 0.5) compared to pre-wetting before forward pipetting (%Random error of Cq = 0.7). This result is also consistent with previous findings that for cold liquids, imprecision caused by pipetting cold liquids with room temperature pipettes and tips can be reduced by not pre-wetting the pipette tip.

Low Retention Pipette Tips and Standard Pipette Tips

The type of pipette tip that is best for pipetting Master mix was tested using Sartorius Low Retention filter tips and Safetyspace filter tips, and Tacta® mechanical pipettes. For the preparation of DNA samples and DNA standards, and for pipetting of primers, low retention filter tips were used since the benefit of low retention plastics in preventing DNA adherence to plastic has been well established.[5] For pipetting Master mix, as shown in Figure 4, using forward pipetting technique, low retention filter tips gave better reproducibility and lower uncertainty in Cq values (%Random error of Cq = 0.5, %Systemic error of Cq = 0.02, %Uncertainty = 0.9) compared to standard filter tips (%Random error of Cq = 0.8, %Systemic error of Cq = -0.03, %Uncertainty = 1.6%). This result suggests that low retention tips are best for handling PCR Master mix.

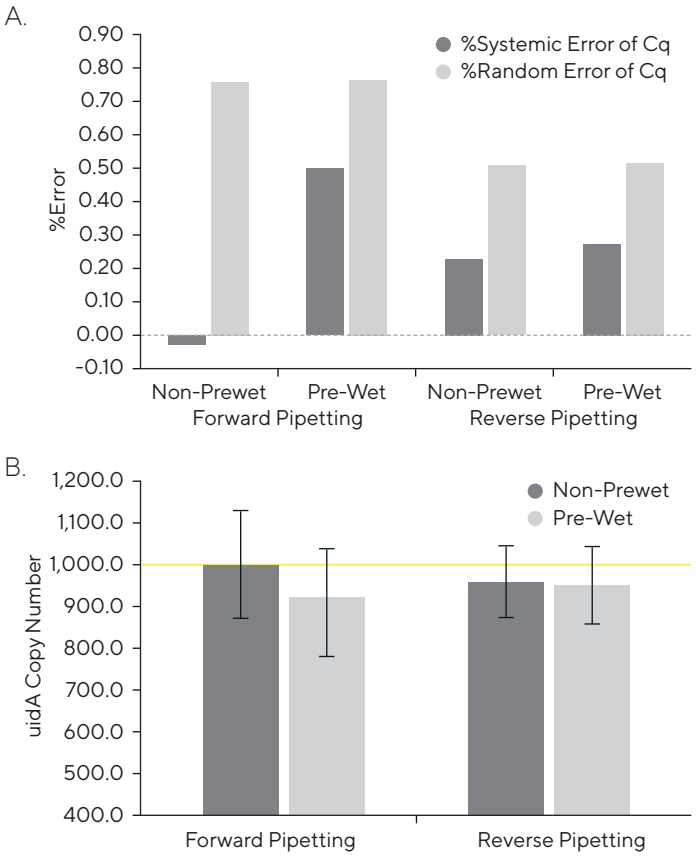

Figure 3: (A) %Error of Cq (cycle of quantification) for Pre-wet and Non-prewetting of pipette tips when pipetting Master mix. %Systemic error of Cq (cycle of quantification) and %Random error of Cq are shown. For each data point, n = 8. (B) Quantified *E. coli uidA* copy number. Yellow line indicates actual target amount. Non-prewet with forward pipetting gives the lower systemic error compared to pre-wetting. Standard Tip—Sartorius Safetyspace Filter Tips.

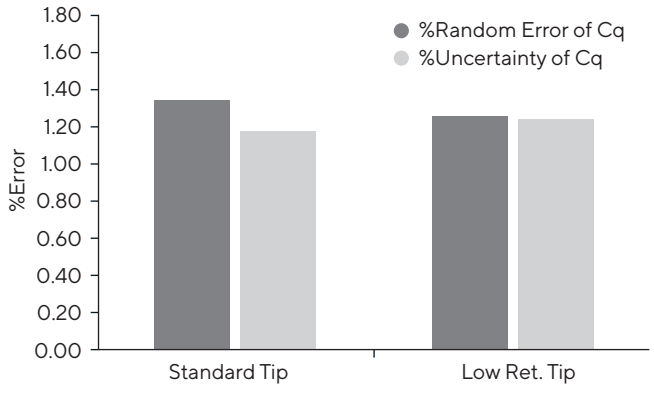

Figure 4: Percent Error for standard tips and low retention tips for pipetting of Master mix. %Random error of Cq and %Uncertainty of Cq (%Random error and %Systemic error) are shown. Forward pipetting technique was used. For each data point, n = 8. %Uncertainty for low retention tips is lower compared to standard tips. Standard Tip—Sartorius Safetyspace Filter Tips, Low Ret. Tip—Sartorius Safetyspace Low Retention Tips.

Electronic Pipettes

Electronic pipette use for pipetting of Master mix was compared to mechanical pipette using Sartorius Picus® Nxt electronic pipette, Sartorius Tacta® mechanical pipette, and Low Retention filter tips. The multi-dispensing mode of the electronic pipette was used. For mechanical pipettes, forward pipetting technique was used. As shown in Figure 5, when low retention filter tips were used to pipette Master mix, multi-dispense mode of electronic pipette gave Cq = 24.54 ± 0.09, %Systemic error of Cq = 0.12, %Random error of Cq = 0.4 and %Uncertainty of Cq = 0.9 compared to forward pipetting on mechanical pipette (Cq = 24.52 ± 0.11, %Systemic error of Cq = 0.02, %Random error of Cq = 0.5 and %Uncertainty of Cq = 0.9). The use of electronic pipette gave good reproducibility of the results (%Random error) and kept the overall %Uncertainty in Cq values at low levels similar to that of mechanical pipette. The multi-dispensing mode of the electronic pipette ensured that with one aspiration, Master mix was dispensed into all eight replicate wells sequentially, increasing the speed of pipetting significantly, as well as reducing the number of pipette tips used, making it more ecologically friendly and reducing the tip-to-tip variance.

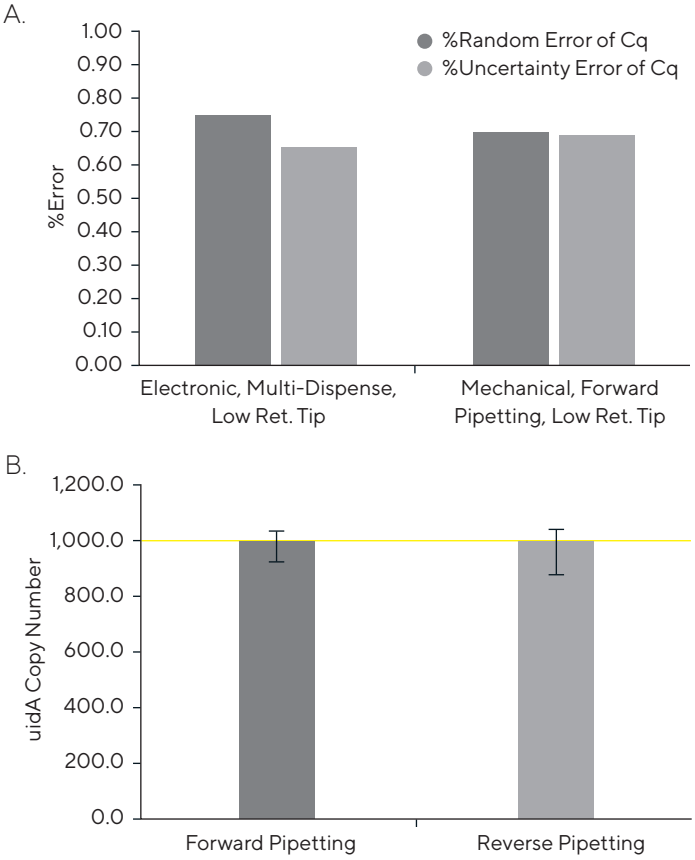

Figure 5: (A) %Error for pipetting of Master mix with electronic or mechanical pipette. %Random error of Cq and %Uncertainty of Cq (%Random error and %Systemic error) are shown. Forward pipetting was used for mechanical pipette and multi-dispensing mode for Electronic pipette. (B) Quantified *E. coli uidA* copy number. Yellow line indicates actual target amount. For each data point, n = 8. Low Ret. Tip—Sartorius Safetyspace Low Retention Filter Tips.

Discussion

Pipetting is fundamental for PCR-based assays. In this study, the pipetting of Master mix, an important component of PCR, was investigated in order to determine the best pipetting techniques and conditions necessary for the accuracy and precision. Here, we have demonstrated that forward pipetting technique using low retention filter tips is best. For laboratories which still use standard pipette tips for pipetting Master mixes, reverse pipetting gives the next best reproducibility (precision) of results. We demonstrated that electronic pipettes ensured high accuracy and precision, and additionally increased the speed to complete the assay, making it a more ergonomic option since it reduced the amount of time spent pipetting. Thus, it would reduce the chances of Repetitive Strain Injury (RSI) for the laboratory worker, make the experiment less error prone, and it is also the more environmentally friendly option since it uses less pipette tips for the same experiment.

Conclusion

We concluded from this study that the best practices for pipetting Master mix for PCR-based assays is to use the optimal pipette-and-pipette-tip combination (mechanical pipette or electronic pipette, with low retention pipette tips) and the correct pipetting technique (forward pipetting with low retention tips, or reverse pipetting with standard pipette tips). These recommendations are key to ensuring that Master mix is accurately and precisely pipetted for minimal variability in assay results. The differences between pipetting techniques and tip types also stress that, in order to get good and reproducible results, it is important not to change tip types or techniques between experiments. The results of this study are especially relevant for people in assay development, diagnostics and quality control who need to report the CV% and Z-factors of their assays to be able to offer the best specifications to their end users. These guidelines are also important for individuals performing quantitative assays, such as determination of intestinal microbiota characterizations in which the presence and quantities of bacteria are determined by qPCR, or measuring bacterial contamination in cell culture supernatants and cell media components in research and development, or for regulation conform testing, i.e., according to EP | USP | JP, using qPCR based kits such as Microsart® Research Bacteria kit (Sartorius).

References

1. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G.L., Vandesompele, J., & Wittwer, C.T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry, 55*(4), 611-22.
2. Murphy, J., & Bustin, S.A. (2010). Real-time quantitative PCR as a bioanalytical tool in the pharmaceutical industry. *International Drug Discovery,* Feb March.
3. Yang, J., Kemps-Mols, B.M., & Spruyt-Gerritse, M.J. (2016). The source of SYBR green master mix determines outcome of nucleic acid amplification reactions. *BMC Res Notes, 9*(1), 292.
4. Martins, M.T., Rivera, I.G., Clark, D.L., Stewart, M.H., Wolfe, R.L., & Olson, B.H. (1993). Distribution of uidA gene sequences in Escherichia coli isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbellifer-yl-beta-D-glucuronide media. *Appl Environ Microbiol, 59*(7), 2271–2276.
5. Lewis, L.K., Robson, M., Vecherkina, Y., Ji, C., & Beall, G. (2010). Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes. *Biotechniques, 48*(4), 297–302.

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