Application Note

Versatile **Clara® & Air-Dryable Inhibitor-Tolerant Probe Mixes** enable reliable target detection in crude saliva samples

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Introduction

The ability to perform sensitive and accurate PCR directly from crude biological samples, such as saliva, represents a major advancement for molecular diagnostics and research. Saliva is a non-invasive, easily accessible sample type that requires minimal collection equipment and is well-suited for high-throughput screening. However, its inherent complexity and high content in PCR inhibitors, including mucins, enzymes, food-derived compounds, varying pH levels, hormones, and bacteria, often compromise reaction efficiency and reliability. Overcoming these challenges is critical for expanding access to molecular testing in settings where sample purification may be impractical or time-consuming.

Applications of crude saliva PCR span numerous settings, from decentralised healthcare and field diagnostics to epidemiological surveillance and at-home testing. In clinical contexts, for example, rapid and robust detection of pathogens from saliva is essential for respiratory virus screening, including SARS-CoV-2, Influenza, and RSV, especially during seasonal outbreaks or public health emergencies. For researchers, working directly with saliva simplifies workflows, reduces costs and cross-contamination risks, and allows real-time monitoring of infection or biomarker expression without complex sample handling. Similarly, in point-of-care (PoC) scenarios and low-resource environments, using crude saliva as a direct input into PCR enables timely decision-making without the need for a cold chain or laboratory infrastructure.

PCR Biosystems' Clara® Inhibitor-Tolerant Probe and Probe 1-Step Mixes, and Air-Dryable Inhibitor-Tolerant Probe and Probe 1-Step Mixes, are engineered to address these needs. In this application note, we put these mixes to the test on crude saliva samples, demonstrating their exceptional inhibitor tolerance. This feature allows for dependable, high-sensitivity detection, even in unpurified saliva samples, both in standard and dryable formats, and offers streamlined, field-ready solutions for the most demanding PCR applications.

Materials & Methods

Clara® and Air-Dryable Inhibitor-Tolerant Probe 1-Step Mixes were evaluated against many different competitor mixes. These include Meridian Air-Dryable Direct RNA/DNA qPCR Saliva-resistant (Meridian), Quantabio PerfeCTa Multiplex qPCR ToughMix (Quantabio), Thermo TaqPath 1-Step RT-qPCR Master Mix (Thermo), SsoAdvanced Universal Probes Supermix (Bio-Rad), One Step PrimeScript III RT-PCR Kit (Takara). Clara® Inhibitor-Tolerant Probe Mix was also tested in the presence of saliva to evaluate its tolerance.

Reaction setup

For the crude saliva titration experiment, individual reactions were set up in a final volume of 20 µL to include 5 µL 4x Clara® Inhibitor-Tolerant Probe 1-Step Mix, 0.8 µL 4-plex primers/probes mix (the final concentrations in the reaction mix of each primer set and probe were 400 and 200 nM, respectively), and 5 µL of template (containing 400 target copies per reaction) and crude saliva appropriately diluted to achieve 0-10% final concentration in each reaction.

To investigate the performance of Clara® Inhibitor-Tolerant Probe Mix, four DNA targets, GEN (general MPX), β-microtubulin (B2M), CB (Congo Basin MPX), WA (West Africa MPX), were amplified, in the presence and absence of 2.5% crude human saliva, in a multiplex qPCR assay. Four template dilutions (10000, 1000, 100, and 10 copies for MPOX (MPX) viral targets, and 250, 25, 2.5, and 0.25 pg/ µL for B2M), with three technical replicates for each target were used in 20 µL reactions. Reactions contained 5 µL saliva diluted 1/10 in universal transport medium (MWE), corresponding to 2.5% human saliva per reaction.

To test the variation in samples from different donors, crude saliva samples from three different donors were diluted 1/10 in universal transport media. 5 µL of each diluted saliva sample were added per 20 µL reaction. Samples from different individuals were spiked with 400 copies of SARS-CoV-2 and four different viral RNA targets (E-gene, N1, N2 and RdRp) amplified in 4-plex RT-qPCR setup.

For the time course experiment, saliva samples were obtained daily from the same individual at arbitrary time-points, 400 copies of four different RNA viruses (SARS-CoV-2, Influenza-A, Influenza-B and Respiratory Syncytial Virus) were spiked in 5 µL of the diluted saliva. The four viral targets were amplified using a 4-plex RT-qPCR setup.

Reactions with Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix were set up against the same four viral RNA targets. SARS-CoV-2 E-gene (E-gene), Respiratory Syncytial Virus (RSV), Influenza-A (Inf-A), and Influenza-B (Inf-B) were amplified with human saliva in multiplex 1-step RT-qPCR reactions with Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix before and after drying (80 min at 40 ˚C). Four template dilutions (4000, 400, 40, and 4 copies) with three technical replicates for each target were used in 20 µL reactions. 5 µL of saliva diluted 1/10 in universal transport medium, corresponding to 2.5% human saliva, were added per reaction.

Reagent	Volume	Final conc.
4x Clara®/Air-Dryable Inhibitor-Tolerant Probe (1-step) Mix	5 µL	1x
Primer mix (10 µM)	0.8 µL	400 nM
Probe mix (10 µM)	0.4 µL	200 nM
Tempate (plus 0.5-2 µL crude saliva)	5 µL	RNA 4-4000 copies DNA 10-10000 copies*
PCR grade dH_2O	8.8 µL	–

*Except for cDNA dilutions for B2M which spanned a range of 0.25-250 pg/µL

Table 1: Reaction setup and composition

Table 1 summarises the final concentration of the components added to each reaction well in all the experiments described above. All reactions were set up using a Qiagen QIAgility robot.

Cycling conditions

Thermocycling was carried out on a Bio-Rad CFX96 Touch qPCR machine, with the cycling parameters outlined in Table 2. Fluorescence measurements were acquired at the end of each cycle. Cycling conditions for competitor mixes were the same for all mixes and were in line with the conditions recommended by all manufacturers.

Cycles	Temperature	Time	Notes
1	47 ˚C	10 minutes	Reverse transcription (RNA)
1	95 ˚C	2 minutes	Polymerase activation
50	95 ˚C	10/15 seconds	Denaturation (DNA/RNA)
	60 ˚C	20 seconds	Annealing/Extension

Table 2: Reaction setup and composition

Results

1. Inhibitor tolerance in the presence of increasing amounts of saliva

The ability of Clara® Inhibitor-Tolerant Probe 1-Step Mix to tolerate crude samples was tested in the presence of increasing concentrations of saliva and compared with five competitor mixes that feature saliva or general inhibitor resistance (TaqPath, BioRad, Takara, Quantabio, Meridian). To do so, multiplex reactions targeting four common respiratory viruses, SARS-CoV-2 E-gene (E-gene), Respiratory Syncytial Virus (RSV), Influenza A (Inf-A), and Influenza B (Inf-B), were set up with each mix in the presence of 0%, 2.5%, 5%, and 10% crude human saliva, in multiplex 1-step RT-qPCR reactions with Clara® Inhibitor-Tolerant Probe 1-Step Mix and each of the competitor mixes. Three technical replicates for each target were set up (Figure 1).

Clara® Inhibitor-Tolerant Probe 1-Step Mix exhibited the steepest amplification curves and highest fluorescence plateaus across all targets, at all saliva concentrations (except for detection of Inf-B with a Cy5-labelled probe at 5% and 10% saliva at which all mixes failed). It outperformed Takara and Meridian mixes by a few cycles on all targets, at all saliva concentrations. It also showed comparable results to TaqPath, BioRad and QuantaBio mixes without saliva

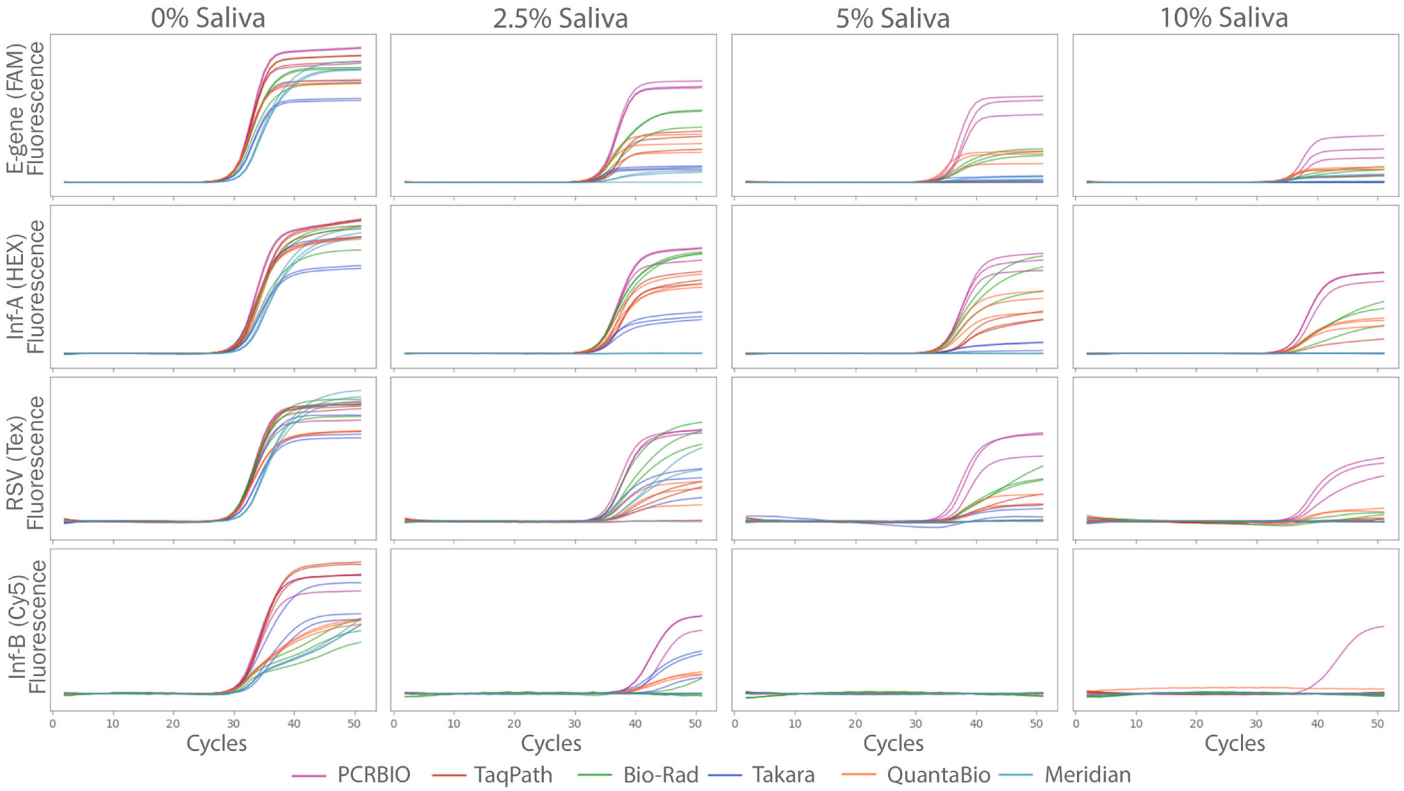

Figure 1. Improved detection of RNA viruses in crude saliva with Clara® Inhibitor-Tolerant Probe 1-Step Mix

Quadruplex amplification of RNA viruses, SARS-CoV-2 E-gene (E-gene), Influenza-A (Inf-A), Respiratory Syncytial Virus (RSV), and Influenza-B (Inf-B) in 2.5% crude saliva samples using Clara® Inhibitor-Tolerant Probe 1-Step Mix (PCRBIO, purple), Thermo TaqPath™ 1-Step RT-qPCR Master Mix (Thermo, red), SsoAdvanced Universal Probes Supermix (Bio-Rad, green), One Step PrimeScript III RT-PCR Kit (Takara, dark blue), Quantabio PerfeCTa Multiplex qPCR ToughMix (Quantabio, orange), Meridian Air-Dryable™ Direct RNA/DNA qPCR Saliva-resistant (Meridian, blue). Three technical replicates were run for each mix. 400 target copies were used for all reactions.

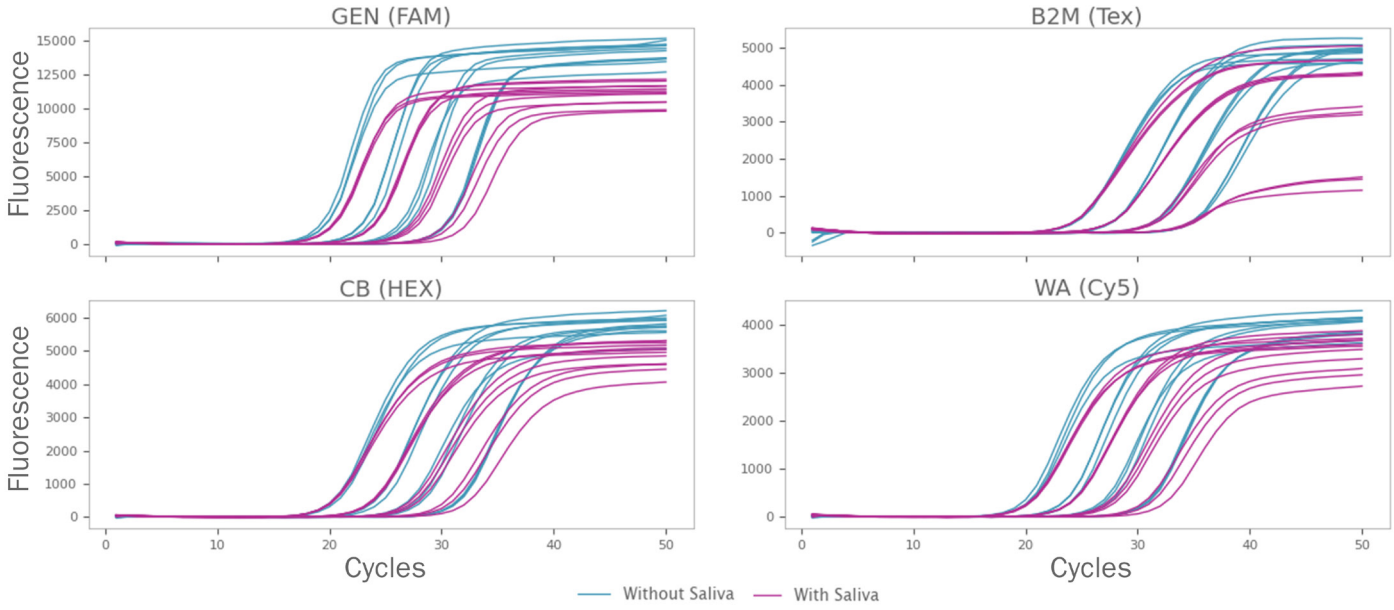

Figure 2. Fourplex detection of DNA targets with and without saliva

Four DNA targets, GEN (general MPX), β-microtubulin (B2M), CB (Congo Basin MPX), WA (West Africa MPX), were amplified, in the presence (purple) and absence (blue) of human saliva, in a multiplex qPCR assay with Clara® Inhibitor-Tolerant Probe Mix. Four template dilutions (10 000, 1000, 100, and 10 copies for the monkeypox (MPX) viral targets, and 250, 25, 2.5, and 0.25 pg/µL for B2M) with three technical replicates for each target were used in 20 µL reactions. Reactions with saliva contained 5 µL saliva diluted 1/10 in universal transport medium, corresponding to 2.5% human saliva per reaction. Clara® Inhibitor-Tolerant Probe Mix successfully amplifies DNA targets in multiplex setup even in the presence of human saliva.

and retained a higher plateau, greater reproducibility among technical replicates and slightly earlier Cq values than these competitors at higher saliva concentrations.

These results indicate superior tolerance of Clara® Inhibitor-Tolerant Probe 1-Step Mix versus competitors at up to 10% saliva per reaction.

2. Accurate detection of DNA in saliva samples

We additionally investigated the suitability of our inhibitor-tolerant chemistry for the detection of DNA targets in the presense of saliva. To this end, a 4-plex reaction was setup with Clara® Inhibitor-Tolerant Probe Mix to detect three MPOX viral targets and β-microtubulin cDNA.

The presense of saliva (Figure 2) did not impact detection over a range of target concentrations from 10 to 10 000 copies of viral DNA or from 0.25 to 250 pg/µL cDNA. Technical replicates showed high reproducibility with only minor delays of less than 1 Cq, if any, depending on the target and dilution. For example the GEN and WA MPX targets dected with a FAM- and Cy5-labeled probes, respectively, showed a minor delay of all replicates at all but the lowest dilutions, wereas B2M and CG, detected with Tex- and HEX-labelled probes, respectively, showed no delay in the presence of saliva at any dilution.

3. Variation across different donor samples

Saliva is a highly heterogeneous matrix. The composition of saliva, and therefore its inhibitory power highly change among individuals. To assess the performance of our inhibitor-tolerant chemistry, saliva samples were taken from three different donors.

Multiplex reactions against four SARS-CoV-2 targets, E-gene (FAM), N1 (HEX), RdRp (Tex), and N2 (Cy5) were set up with Clara® Inhibitor-Tolerant Probe 1-Step Mix in the presence of saliva samples from three donors. The ΔCq values (change in quantification cycle) compared to a control reaction without saliva were calculated (Figure 3). The patterns of inhibition were similar for each target, with donor 2 causing a markedly higher inhibition and donor 3 a smaller inhibition compared to the others in all the targets. Overall, the lowest inhibition was observed for donor 3 with a ~0.5 ΔCq in RdRp and the greatest inhibition of 3.5-4 ΔCq for donor 2 in E-gene amplification.

Detection was achieved in samples from all donors, indicating that Clara® Inhibitor-Tolerant Probe 1-Step Mix performs reliably despite donor-to-donor variability, ensuring good results in diverse sample sets, a key advantage for clinical and field applications.

4. Variation in inhibition by saliva samples over time

Saliva from the same donor might have very different composition depending on the moment the sample is collected, the time from the last meal, oral hygiene product residue and various other factors.

To examine how the level of inhibition of Clara® Inhibitor-Tolerant Probe 1-Step Mix can vary in an individual saliva sample over time, saliva from a single donor was collected over nine non-consecutive days (repeat sampling every day at arbitrary hours). ΔCq values for four targets, E-gene (FAM), RSV (Tex),

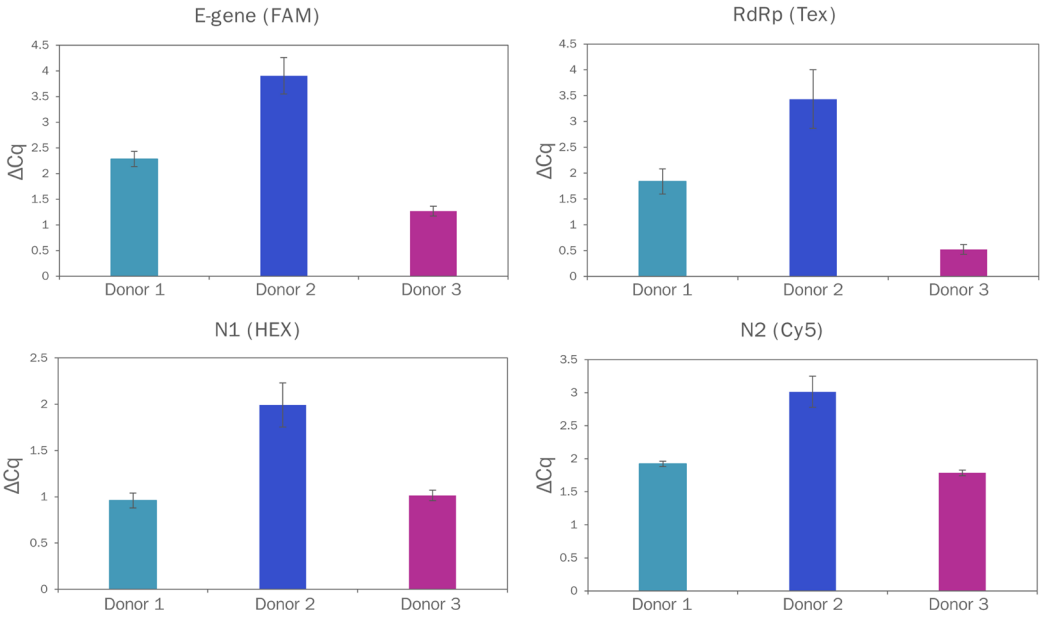

Figure 3. Successful target detection in spite of variable inhibition levels in saliva from different donors

Quadruplex amplification of SARS-CoV-2 RNA targets (E-gene, N1, N2 and RdRP, 400 copies each) using Clara® Inhibitor-Tolerant Probe 1-Step Mix in 2.5% crude saliva samples from three different donors. ΔCq values were calculated from corresponding uninhibited reactions that contained 400 target copies but no saliva.

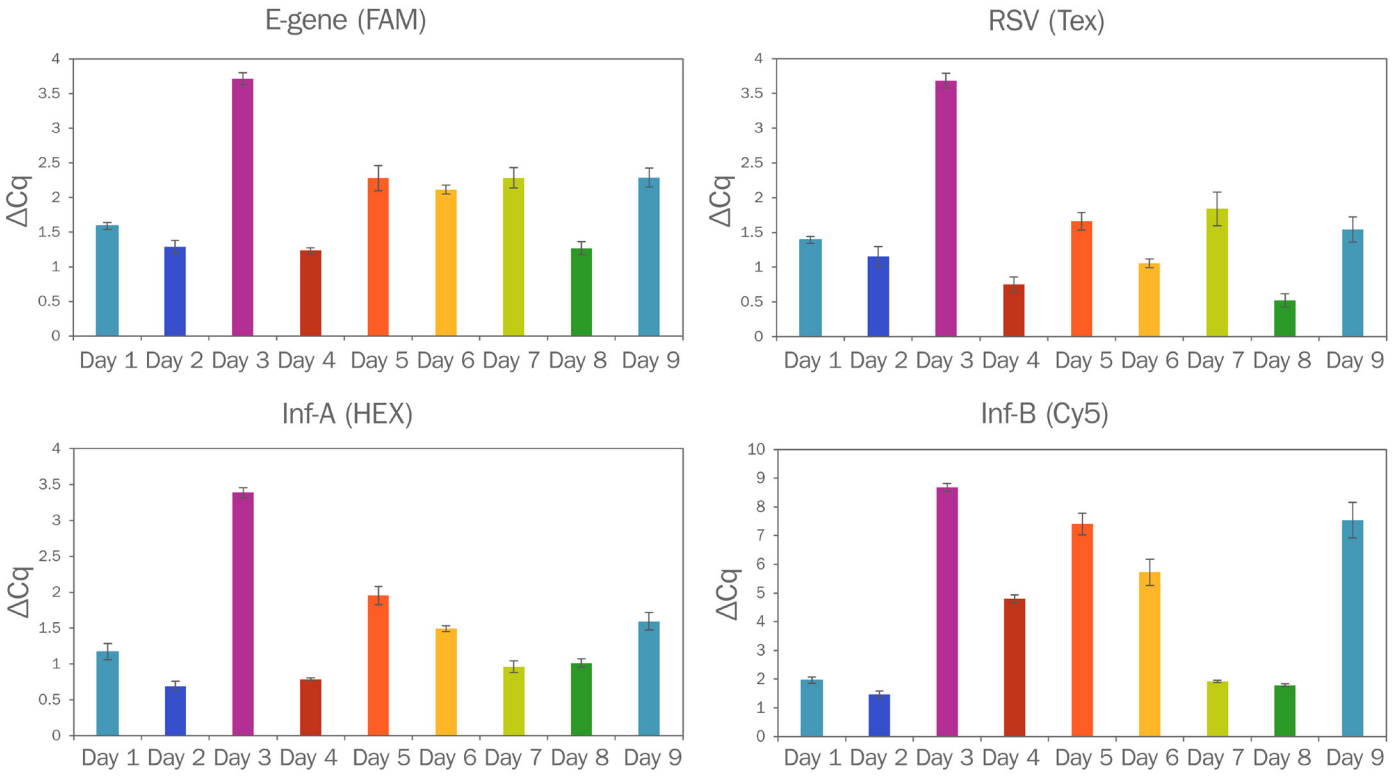

Figure 4. Successful target amplification despite variability in saliva samples collected at different time points

Quadruplex amplification of RNA viruses, SARS-CoV-2 E-gene (E-gene), Respiratory Syncytial Virus (RSV), Influenza-A (Inf-A), and Influenza-B (Inf-B) in 2.5% crude saliva samples using Clara® Inhibitor-Tolerant Probe 1-Step Mix. Saliva samples from the same donor were taken at arbitrary times in the day over a period of nine non-consecutive days and spiked with 400 target copies before being used as template for RT-qPCR reactions. ΔCq values were calculated from corresponding uninhibited reactions that contained the same target copy number but not saliva.

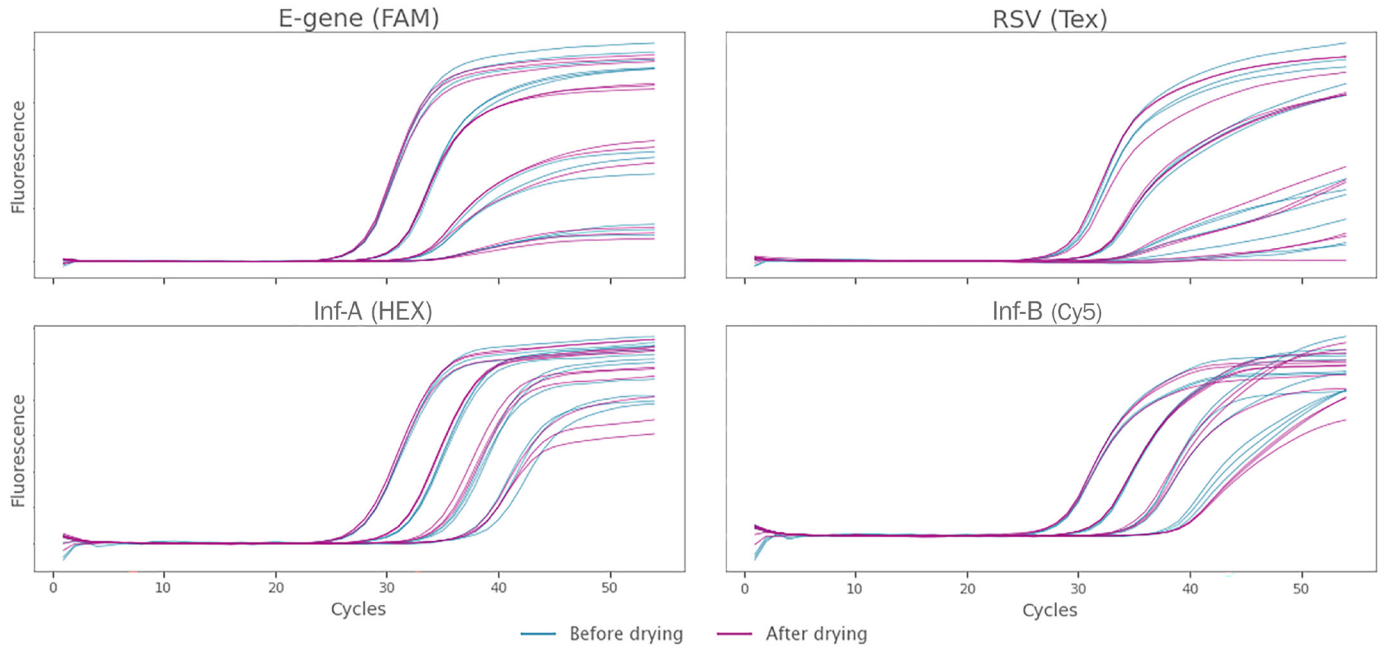

Figure 5: Reliable detection in saliva samples with Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix before and after air-drying

Four RNA targets, SARS-CoV-2 E-gene (E-gene), Respiratory Syncytial Virus (RSV), Influenza-A (Inf-A), and Influenza-B (Inf-B) were amplified, with human saliva, in multiplex 1-step RT-qPCR reactions with Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix before (blue curves) and after (purple curves) drying (80 min at 40 ˚C). Four template dilutions (4000, 400, 40, and 4 copies) with three technical replicates for each target were used in 20 µL reactions. 5 µL of saliva diluted 1/10 in universal transport medium, corresponding to 2.5% human saliva, were added per reaction.

Inf-A (HEX), and Inf-B (Cy5) were calculated based on a control reaction without saliva (Figure 4).

Inhibition levels varied considerably each day and by target. E-gene (FAM), RSV (Tex), and Inf-A (HEX) ΔCq values ranged from 1.0 to 3.5, with a maximum on Day 3. Inf-B (Cy5) ΔCq values showed greater variability from ~1.5 to 9.0, with the maximum on Day 3. These results again demonstrate Clara® Inhibitor-Tolerant Probe 1-Step Mix can detect targets in saliva samples despite large fluctuations in sample quality and levels of inhibition. This is a critical capability in a saliva-resistant inhibitor tolerant mix, particularly for longitudinal studies or applications where sample collection occurs over time.

5. Performance before and after drying

We tested the performance of Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix in a 4-plex assay on samples containing 2.5% crude saliva before and after air-drying (Figure 5). Targets selected were the same four respiratory viruses as before. Reactions mixes were either set up and dried, prior to reconstitution and addition of samples (purple) or were set up fresh without drying (blue) and both sets were run simultaneously. Amplification of all four targets remained unchanged, regardless of whether reactions had been dried or not, with Cq values and technical replicate reproducibility.

Overall, Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix maintained performance after air-drying. The ability to air-dry our inhibitor tolerant chemistry makes them ideal for applications requiring stable, transportable reagents, for point-of-care diagnostics.

Conclusion

PCR Biosystems' Clara® and Air-Dryable Inhibitor-Tolerant Mixes deliver unmatched performance in challenging qPCR scenarios. The data shown in this application note demonstrate their ability to outperform competitors in the presence of high-saliva concentration, and to adequately handle variability across different sample donors and over time. Additionally, Air-Dryable mixes maintain sensitivity after drying.

These features make our Inhibitor-Tolerant range a top choice for researchers and clinicians needing robust, versatile PCR solutions for a range of applications and workflows, including:

- Point-of-Care Testing: Air-dryable formats and inhibitor tolerance enable use in portable diagnostic kits.
- Multiplex Assays: Compatibility with multiple fluorophores supports high-throughput detection of viral and genetic targets.
- Clinical Diagnostics: Reliable performance with saliva samples enhances utility in non-invasive testing.
- Field Studies: Stability over time and after drying suits resource-limited or remote settings.

Ordering Information

Please reach out to our team with any queries or to get a quote for Air-Dryable or Clara® Inhibitor-Tolerant Probe & Probe 1-Step Mixes by email: info@pcrbio.com. Please refer to Table 3 below for available pack sizes and catalogue numbers.

Reactions	Presentation (reactions)	Cat. No.
Air-Dryable Inhibitor-Tolerant Probe Mix	600/2000/10000	PB90.41-01/03/50
Air-Dryable Inhibitor-Tolerant Probe 1-Step Mix	600/2000/10000	PB90.51-01/03/50
Clara® Inhibitor-Tolerant Probe Mix Lo-ROX	200/600/1000/10000	PB20.71-01/03/05/50
Clara® Inhibitor-Tolerant Probe Mix Hi-ROX	200/600/1000/10000	PB20.72-01/03/05/50
Clara® Inhibitor-Tolerant Probe Mix No-ROX	200/600/1000/10000	PB20.73-01/03/05/50
Clara® Inhibitor-Tolerant Probe Mix Separate-ROX	200/600/1000/10000	PB20.74-01/03/05/50
Clara® Inhibitor-Tolerant Probe 1-Step Mix Lo-ROX	200/600/1000/10000	PB25.91-01/03/05/50
Clara® Inhibitor-Tolerant Probe 1-Step Mix Hi-ROX	200/600/1000/10000	PB25.92-01/03/05/50
Clara® Inhibitor-Tolerant Probe 1-Step Mix No-ROX	200/600/1000/10000	PB25.93-01/03/05/50
Clara® Inhibitor-Tolerant Probe 1-Step Mix Separate-ROX	200/600/1000/10000	PB25.94-01/03/05/50

Table 3: Pack sizes and catalogue numbers of Air-Dryable and Clara® Inhibitor-Tolerant Probe & Probe 1-Step Mixes.