Application Note

Robust **Clara® & Air-Dryable Inhibitor-Tolerant Probe Mixes** tolerate a wide range of inhibitors

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Introduction

PCR inhibition can be a serious challenge for molecular biologists. While in most cases good practice during sample extraction and PCR setup can minimise or eliminate inhibition, there are many situations where this can remain an issue. For example, certain sample types are rich in inhibitory compounds (like those containing blood, tissues, or cellular debris). In other cases, sample preparation has not been optimally performed, leaving behind some chemical used in the process. Additionally, there is an increased interest in conducting PCR directly on crude samples, which therefore will contain PCR-inhibiting compounds. Clinical diagnostics, forensic testing, and environmental monitoring are just few of the situations requiring PCR reagents that can withstand these molecules in order to provide reliable results.

Inhibitor-tolerant qPCR mixes are therefore key in overcoming this challenge. 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 challenged our inhibitor-tolerant chemistry, using Clara® Inhibitor-Tolerant Probe 1-Step Mix as a test-case, in the presence of a wide range of clinical, laboratory, plant and other chemical inhibitors. Inhibitor classes tested include:

- Laboratory / chemical reagents (*e.g.*, ethanol, SDS, guanidine, phenol)
- Phenolic / plant compounds (*e.g.*, tannic acid, quercetin, catechin, chlorophyll)
- Soil inhibitors / plant polysaccharides (*e.g.*, humic acid, xylan, pectin, cellulose)
- Proteinaceous / biological inhibitors (*e.g.*, hemoglobin, lactoferrin, IgG)
- Clinical / sample-derived pigments and anticoagulants (*e.g.*, bilirubin, melanin, hematin, sodium citrate, heparin).

Similar results can also be obtained using our Air-Dryable Inhibitor-Tolerant formulations for the same mix.

These results highlight the broad-spectrum tolerance of the mixes and their suitability for diverse workflows, from environmental testing and plant biology studies to diagnostic assays using minimally processed biological samples.

Materials & Methods

Reaction setup

Reactions were performed in a 20 µL total volume using 5 µL of 4× Clara® Inhibitor-Tolerant Probe 1-Step Mix. Primers and probes were added in concentrations recommended in the product manual (400 nM primers, 200 nM probe). A fixed amount (400 copies per reaction) of template RNA was included to ensure robust amplification in inhibitor-free controls. Inhibitors were spiked at increasing concentrations (as shown in results figures), spanning physiologically or environmentally relevant ranges where these were known or relevant to an inhibitor. Amplification was monitored on a real-time qPCR instrument (Bio-Rad CFX Opus) using standard (47 ˚C for 10 min, 95 ˚C 2 min, 50x (95 ˚C 15 sec/ 60 ˚C 30 sec)) cycling conditions. The same conditions were used for competitor comparisons. Competitor mixes tested include: Air-Dryable Direct RNA/DNA qPCR Saliva and Inhibitor-Tolerant RT-qPCR Mix from Meridian, One Step PrimeScript III RT-PCR Kit from Takara, and TaqPath 1-Step Multiplex Mastermix from Thermo Fisher.

Data Analysis

Relative activity (%) was calculated by comparing amplification (Cq) in inhibitor-spiked reactions to inhibitor-free controls. IC_{50} values (concentration causing 50% PCR inhibition) were determined via dose–response curve fitting using standard pharmacodynamic tools. Error bars represent standard deviations of replicate reactions ($n = 3$).

Results & Discussion

Clara® Inhibitor-tolerant Probe 1-Step Mix showed varying level of tolerance to different pure inhibitors. The full list of tested inhibitors and IC_{50} for each one are listed in Table 1. Below is a summary of performance across inhibitor classes. Values reported in the table and throughout the text refer to the final concentration of these compounds in the reaction well.

Inhibitor	IC_{50}	Units	Comment
Pigments			
Bilirubin	2.3	µg/mL	
Hemin	6.44	µM	
Hematin	NA		No inhibition up to 45 µM
Melanin	35	µg/mL	
Proteins			
Lactoferrin	0.01	mg/mL	
IgG	NA		No inhibition up to 3.5 mM
Hemoglobin	0.96	mg/mL	
Chemical reagents			
Heparin	0.01	mg/mL	
Sodium Citrate	NA		No inhibition up to 2 mM
Urea	NA		No inhibition up to 400 mM
Phenol	24.9	mM	
SDS	0.01	%	
ETOH	4.64	%	
Guanidine	0.82	%	
Plant & environmental compounds			
Humic Acid	NA	µg/mL	No inhibition up to 1.5 µg/mL
Quercetin	114	µM	
Catechin	NA		No inhibition up to 200 µM
Tannic Acid	8.4	µM	
Chlorophyl-b	14.1	ng/µL	
Xylan	9.20	mg/mL	
Cellulose*	NA		Was inhibited by 125 ng/ µL
Pectin	10.1	ng/µL	

*Cellulose was insoluble in the acqueous reaction buffers used

Table 1: IC_{50} of inhibitors tested.

1. Clinical, proteinaceous and anticoagulant inhibitors

To assess the robustness of our inhibitor-tolerant chemistry, a range of clinically relevant proteinaceous and anticoagulant inhibitors were evaluated. These compounds are frequently encountered in blood, serum, or diagnostic matrices and are known to interfere with nucleic acid amplification through enzyme binding, sequestration of essential cofactors, or direct reaction with nucleic acids.

Several clinically significant pigments were assessed to evaluate tolerance in diagnostic workflows involving minimally processed biological materials (Table 1, Pigments). Bilirubin, a breakdown product of haemoglobin and common serum pigment, caused only mild inhibition, with an $IC_{50} \approx 2.3$ µg/mL. In contrast, hematin, another haem-derived compound, showed minimal inhibitory effect up to 45 µM (the highest concentration tested). Melanin, a known PCR inhibitor present in pigmented tissues and melanoma samples, showed progressive inhibition with increasing concentration, resulting in an $IC_{50} \approx$ 35 µg/mL.

Among the proteinaceous inhibitors tested (Table 1, Proteins), haemoglobin displayed moderate inhibition, with a steady decline in activity as concentration increased ($IC_{50} \approx 0.96$ mg/mL). Lactoferrin, an iron-binding glycoprotein commonly present in secretions such as saliva, tears, and milk, showed strong inhibition at comparatively low concentrations, with an $IC_{50} \approx 0.01$ mg/mL. In contrast, immunoglobulin G (IgG), a major class of serum antibodies, was only weakly inhibitory even at high concentrations (3.5 mM), suggesting limited interaction with the polymerase or reaction cofactors.

Overall, these results demonstrate that PCR Biosystems' Inhibitor-Tolerant Probe Mixes perform robustly in the presence of typical blood serum pigments and proteins, maintaining high activity at concentrations relevant to crude blood or plasma-derived samples.

To ensure these results are clinically relevant, Clara® Inhibitor-Tolerant Probe 1-Step Mix was further tested with two common anticoagulants (Table 1, Chemical reagents). Heparin, a strongly anionic anticoagulant widely used in clinical sampling, exhibited potent inhibition even at low concentrations ($IC_{50} \approx$ 0.014 mg/mL), confirming its well-documented impact on polymerase activity. The chelating agent sodium citrate, often present in plasma samples as an anticoagulant, had negligible impact on amplification efficiency (up to 2 mM, the highest concentration tested).

Together, these results confirm that PCR Biosystems' Inhibitor-Tolerant Probe Mixes are highly resilient to the complex mix of proteins, pigments, and anticoagulants typically present in clinical and diagnostic samples. Their performance across these inhibitor classes underscores their suitability for direct amplification workflows, where minimal sample purification is possible, without compromising assay reliability. Although use of heparin-containing samples may inhibit amplification. In this case, given the high sensitivity of the mixes, diluting the sample(s) prior to amplification is recommended, when extraction is not an option.

2. Laboratory / Chemical reagent inhibitors

To investigate the extensive use-cases of our inhibitor-tolerant chemistry, additional chemical reagents were also investigated. Laboratory reagents commonly found in nucleic acid extraction and other regularly used solutions showed potent inhibition (Table 1, Chemical reagents). Urea, the major form of excreted nitrogen in humans is present in urine and other bodily fluids, and also a common laboratory chemical, did not cause inhibition up to 400 mM, which is well above the biologically relevant range in clinical samples (160-330 mM in urine). Phenol, which is often used for RNA/DNA extractions showed moderate inhibition, with $IC_{50} \approx 24.9$ mM. Guanidine caused strong inhibition at 1%, with an $IC_{50} \approx 0.82$ %. While the highly denaturing detergent, SDS, showed the greatest inhibition of any of tested compound, with an IC_{50} of 0.01%. Finally, ethanol had an $IC_{50} \approx 4.6$%. These results show that while certain lab reagents are manageable, strong chaotropes and detergents remain challenging even for inhibitor tolerant mixes.

These results demonstrate that PCR Biosystems' inhibitor-tolerant mixes can overcome trace contamination with these inhibitory reagents, particularly ethanol and phenol, which can occur with classic guanidine/phenol/chloroform-type nucleic acid extraction protocols. However, complete removal of such reagent contaminants is strongly advised, because they remain potent PCR inhibitors.

Inhibitor-tolerant probe mixes for DNA targets are less susceptible to this inhibition than 1-step mixes, and can potentially tolerate higher concentrations of these inhibitors. This should be tested on a case-by-case basis.

3. Environmental and plant-derived compounds

Similarly, environmental and plant-derived compounds with known, or hypothesised inhibition of polymerases were also tested with varying results. Humic acid, which is abundant in some types of soil and water samples, showed minimal inhibition up to the highest tested concentration (with no inhibition observed up to 1.5 µg/mL, the highest concentration used in this experiment).

Plant-derived compounds showed varying levels of inhibition. Plant phenolic compounds showed a wide range of effects, with quercetin showing moderate to low inhibition ($IC_{50} \approx 114$ µM) and catechin having negligible inhibition even at 200 µM, while tannic acid being a relatively strong inhibitor ($IC_{50} \approx 8.4$ µM). Chlorophyll b, one of the most abundant plant specialised metabolites in vegetative tissues, showed strong inhibition at very high concentrations and had an $IC_{50} \approx 14$ ng/mL.

Another class of plant-derived PCR-inhibitory compounds are polysaccharides, which can sequester DNA polymerase in PCR reactions, preventing or reducing successful amplification. Xylan had strong inhibition, with an $IC_{50} \approx 9.2$ mg/mL. Cellulose, which was insoluble in the aqueous PCR buffer, showed no effects on amplification up to 12.5 ng/mL but complete inhibition at 125 ng/mL; Similarly, pectin showed an IC_{50} of approximately 10 ng/µL, indicating very strong inhibition.

This data demonstrates that environmental and plant inhibitors vary in impact, with humic acid and catechin being very well tolerated, while other phenolics and polysaccharides have a potent inhibitory effect. Thus, PCR Biosystems' inhibitor-tolerance mixes can enable robust performance in the presence of some plant-derived inhibitors and potentially on crude plant nucleic acid extracts, whereas others (e.g., polysaccharide-rich tissues) will be more challenging. As such, performance will be species- and tissue-specific and should be tested on a case-by-case basis in each study.

4. Challenging competitor mixes with inhibitors

To further investigate how our inhibitor-tolerant chemistry performs compared to similar competitor mixes, which claim broad inhibitor tolerance, we tested their performance in parallel with Clara® Inhibitor-Tolerant Probe 1-Step Mix against some of our previously tested compounds (Figure 1). All mixes performed similarly in the presence of increasing

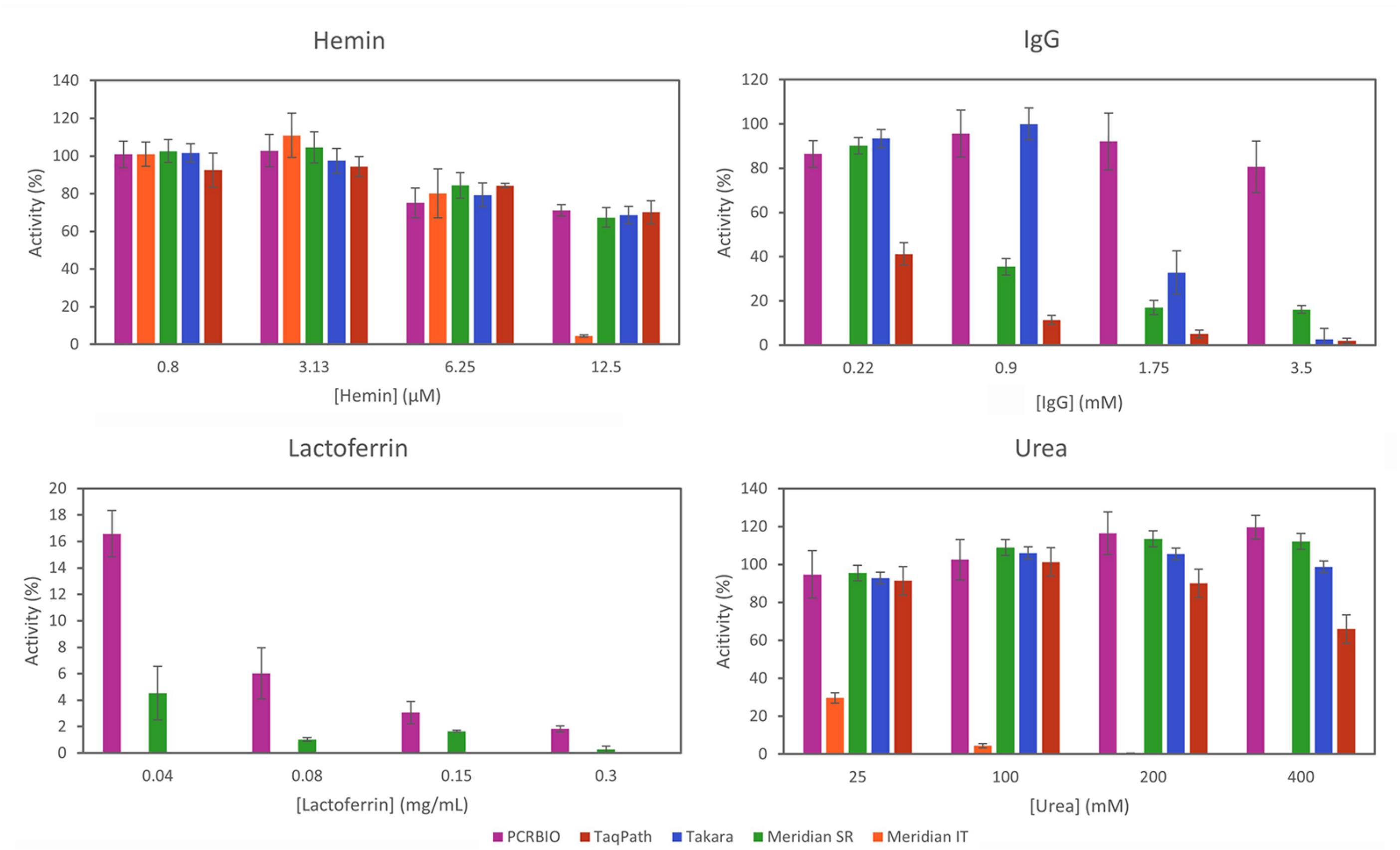

Figure 1. Performance of Clara® Inhibitor-Tolerant Probe 1-Step Mix compared to competitor mixes in the presence of various inhibitors.

Clara® Inhibitor-Tolerant Probe 1-Step Mix (PCRBIO, purple), Inhibitor-Tolerant RT-qPCR Mix (Meridian IT, orange), Air-Dryable™ Direct RNA/DNA qPCR Saliva-resistant (Meridian SR, green), One Step PrimeScript III RT-PCR Kit (Takara, dark blue), TaqPath™ 1-Step RT-qPCR Master Mix (Thermo, red) were used to amplify the sample amount of RNA template in the presence of increasing amounts (indicated on the X-axis in each graph) of hemin, immunoglobulin G (IgG), lactoferrin, and urea. Percent (%) inhibition (Y-axis) was calculated from corresponding uninhibited reactions for each mix. Three technical replicates were run for all reactions.

concentrations of hemin, except for Meridian's Inhibitor-Tolerant Mix, whose activity dropped to near 0% at 12.5 µM hemin.

Clara® Inhibitor-Tolerant Probe 1-Step Mix showed reliable amplification in the presence of IgG, with about 80%-90% activity at all the tested concentrations. Meridian's Inhibitor-Tolerant Mix was unable to amplify the target at any concentration, while their Saliva Resistant Mix had strong amplification at the lowest IgG concentration, but dropped significantly at higher concentrations. The Takara mix enabled robust amplification up to 0.9 mM IgG, but dropped to ~30% activity at 1.75 mM and to negligible activity at higher concentration. While TaqPath from Thermo also showed significant inhibition at all concentrations.

All mixes were greatly inhibited in by lactoferrin, with Clara® Inhibitor-Tolerant Probe 1-Step Mix having the best performance overall, although activity dropped from 16% at 0.04 mg/mL lactoferrin to roughly 2% at 0.3 mg/mL, Meridan Saliva resistant showing trace amounts of activity and all other mixes failed to amplify entirely.

Finally, Clara® Inhibitor-Tolerant Probe 1-Step Mix, Meridian Saliva Resistant and Takara mixes performed well at all tested concentrations of urea. Thermo's TaqPath showed moderate resistance, only dropping to 60% activity at the highest tested concentration, whereas Meridian's Inhibitor Tolerant mix was greatly inhibited.

Cumulatively, these results indicate that PCR Biosystems' inhibitor-tolerant chemistry offers the most robust broad-spectrum inhibitor tolerance compared to the tested competitor mixes on the inhibitors shown and therefore constitutes a sound first choice when working with crude samples or samples potentially tainted with inhibitors.

Practical Considerations

When using crude or even purified templates from inhibitor-rich samples testing on a case-by-case basis is recommended. For samples likely containing strong inhibitors (*e.g.*, phenolics, polysaccharides, anticoagulants), pre-testing or dilution may be warranted. It is also important to consider that real samples contain multiple inhibitor types, and as such, combined effects may differ from single-compound data.

In cases where samples display strong or moderate PCR inhibition, sample dilution can be a first-choice solution, because small dilution factors (1.5×–2×) often restore amplification. Additionally, target and cycle optimisation can be key in reducing inhibition further, with shorter amplicons and optimised cycling improving results with inhibitor-rich samples. Accurate screening during assay or product development can indicate if sample dilution and cycling condition alteration may be required.

Summary & Conclusion

The Clara® and Air-Dryable Inhibitor-Tolerant Probe Mixes exhibit exceptional robustness across diverse inhibitor categories, spanning laboratory reagents, plant-derived compounds, environmental and proteinaceous inhibitors, and clinically relevant pigments and anticoagulants. Crucially, Air-Dryable formulations maintain equivalent inhibitor tolerance post drying and reconstitution.

This wide inhibitor tolerance means these mixes are suitable for:

- DNA and RNA amplification from many crude or minimally processed biological matrices,
- PCR in the presence of a range of environmental or plant extracts rich in phenolics or polysaccharides,
- Clinical diagnostics involving blood, serum, or tissue samples,
- Field-deployable and air-dryable qPCR assays.

Together, these results underscore the versatility of PCR Biosystems' inhibitor-tolerant formulations for reliable amplification under even the most challenging conditions.

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 2 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 2: Pack sizes and catalogue numbers of Air-Dryable and Clara® Inhibitor-Tolerant Probe & Probe 1-Step Mixes.