Application Note

Overcoming RT inhibition in multiplex RNA analysis with QIAcuity® OneStep High Multiplex PCR Kit and dPCR RNA-Plex Assays

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Introduction

Reliable RNA quantification can be challenging when residual substances from complex samples or nucleic acid purification interfere with reverse transcription, PCR amplification or fluorescence detection. The reverse transcription (RT) reaction is particularly susceptible to inhibition, making the efficiency and robustness of this step critical for reliable RNA quantification. Such inhibitors can reduce reaction efficiency, delay or suppress signal generation and compromise the accuracy and reproducibility of gene expression and RNA quantification measurements.

In quantitative reverse transcription PCR (RT-qPCR), target abundance is inferred from fluorescence measured during the exponential phase of amplification. Consequently, inhibitor-induced changes in reverse transcription or amplification efficiency can shift quantification cycle values and bias the calculated target quantities. This susceptibility is particularly relevant for multiplex assays, in which inhibitors may affect individual targets differently and compromise analyses relying on relative comparisons between targets.

Digital PCR (dPCR) combines sample partitioning with endpoint detection and determines target concentration by counting positive and negative partitions. Because quantification does not rely on real-time amplification kinetics, dPCR can remain more robust when moderate inhibition alters PCR reaction efficiency, provided that sufficient endpoint signal is generated to distinguish positive from negative partitions. However, in one-step RNA analysis, inhibitors may also affect the preceding reverse transcription (RT) reaction. The robustness of the RT-PCR chemistry is therefore an additional critical determinant of inhibitor tolerance and overall workflow performance in inhibitor-containing RNA samples.

In this application note, we compare the inhibitor tolerance of one-step multiplex RNA quantification by dPCR using the new QIAcuity OneStep High Multiplex PCR Kit together with dPCR RNA-Plex Assays and by qPCR using a multiplex RT-qPCR master mix. Humic acid, ethanol and heparin were added at increasing concentrations to represent inhibitors associated with environmental matrices, sample preparation carryover and biological specimens, respectively. By assessing the effects of these substances on multiplex target detection and quantification, we demonstrate that the complete dPCR setup workflow provides superior inhibitor tolerance compared to the corresponding qPCR workflow, maintaining more robust and reliable quantification under inhibitor-rich conditions. Additionally, we show that the superior performance in the dPCR workflow is primarily due to greater inhibitor tolerance within the RT reaction.

Materials and methods

Workflows and experimental setups

RNA quantification was performed using one-step RT-dPCR and one-step RT-qPCR workflows. RNA was combined with QuantiNova Internal Control (QNIC) DNA, which served as a PCR positive control in the one-step reactions to distinguish RT-specific inhibition from general PCR inhibition. Varying amounts of three common inhibitors – humic acid, ethanol and heparin – were added separately to each reaction. Reactions were set up as 5-plex assays, with each of the four RNA-targeting assays analyzed together with the QNIC-targeting assay, performed in triplicate. This setup enables direct comparison of normalized quantification measurements for each target across reactions with increasing inhibitor concentrations and the no-inhibitor reference reaction.

Templates

A mixture of QIAGEN Human XpressRef Universal Total RNA (cat. no. 338112) and QNIC DNA template was used in each reaction. Input amounts were adjusted according to the respective reaction format, with 1 ng RNA used in 12 µL dPCR reactions and 1.67 ng RNA used in 20 µL qPCR reactions. QNIC DNA input amount was 10,000 copies per reaction. The resulting template mixture was diluted in water before use and served as the template for all qPCR and dPCR reactions, providing equivalent template inputs for comparisons between inhibitor concentrations and PCR technologies.

Assays

Four dPCR RNA-Plex assays (cat. nos. 250336, 250337) were used together with an assay targeting QuantiNova Internal Control (QNIC) DNA. The selected dPCR RNA-Plex Assays targeted *CDH1* (Cadherin 1), *DNAJB4* (DnaJ Heat Shock Protein Family Hsp40 Member B4), *HPRT1* (Hypoxanthine Phosphoribosyltransferase 1), and *NRGN* (Neurogranin). These genes were selected because they are commonly used as reference genes in gene expression studies and exhibit stable expression across a broad range of tissue types. The four dPCR RNA-Plex Assays and the QNIC DNA assay were combined in a 5-plex reaction for both qPCR and dPCR measurements.

Inhibitors

Humic acid (9, 18, 36 and 48 ng/µL), ethanol (3%, 4%, 8% and 10%), and heparin (0.05, 0.1, 0.3 and 0.5 µM) were added separately to represent inhibitors originating from environmental matrices, purification carryover and biological specimens, respectively. These substances can interfere with PCR through distinct mechanisms, leading to reduced sensitivity or false-negative results (1–3). The selected ranges resemble inhibitor burdens reported or plausibly encountered in real sample types, including humic-substance-rich environmental extracts, nucleic acid eluates containing residual alcohol from purification and specimens collected or processed with heparin.
Because the actual amount transferred into a PCR depends strongly on the sample matrix, extraction method, elution volume and template input, the inhibitor concentration series spanned over a range from representative to severe contamination, rather than a universal concentration.
This concentration range was used to compare progressive inhibition of the qPCR and dPCR workflows where reactions without added inhibitor served as the reference condition.

qPCR and dPCR analysis

All dPCR experiments were performed on the QIAcuity Digital PCR System using QIAcuity Nanoplate 8.5K 96-well (cat. no. 250021) and the QIAcuity OneStep High Multiplex PCR Kit (cat. no. 250135, 250136). Parallel qPCR experiments were performed on a Bio-Rad® CFX Opus 96 instrument using 96-well plates and a multiplex RT-qPCR master mix from supplier A. The qPCR and dPCR runs, including thermal cycling conditions, were performed according to the respective manufacturer's instructions. qPCR data were analyzed using CFX Maestro Software, whereas dPCR data were analyzed using QIAcuity Software Suite version 3.5.
For each 5-plex reaction, the quantity measured for each target was normalized to that obtained in the corresponding no-inhibitor reference reaction. For qPCR, efficiency-corrected initial target quantities were determined in accordance with the MIQE guidelines (4). Raw fluorescence-versus-cycle data without prior baseline correction were analyzed using LinRegPCR via RDML tools (5).
The software performs baseline correction for each reaction, identifies the window of linearity within the exponential amplification phase and applies linear regression to the selected data points. Reaction-specific amplification efficiency was derived from the slope of the regression line, which is extrapolated to cycle 0 to estimate the initial target quantity (N_0) in relative fluorescence units. For dPCR, target concentrations in copies/µL were obtained directly by endpoint analysis of positive and negative partitions.
Inhibitor-dependent effects were assessed by comparing each inhibitor-containing reaction with the corresponding no-inhibitor reference. For dPCR, measured target concentrations in copies/µL were expressed relative to the concentration in the reference reaction. For qPCR, efficiency-corrected initial target quantities (N_0) were expressed relative to the corresponding reference value. Results for both methods were reported as percentages of the no-inhibitor reference.

Results and discussion

One-step RT-dPCR maintains RNA quantification across inhibitor-rich conditions

Across all three inhibitor classes, one-step RT-dPCR demonstrated superior inhibitor resistance during target quantification compared to one-step RT-qPCR (Figure 1). In the presence of low-to-moderate concentrations of humic acid or heparin, dPCR produced RNA-target values comparable to those of the inhibitor-free reference sample, whereas, qPCR showed significantly reduced quantifications at these same inhibitor levels (Table 1). With higher inhibitor concentrations, dPCR showed decreased quantification, whereas qPCR often failed to detect RNA targets altogether.
Because measurements of the QNIC DNA control remained stable under these conditions, the reduced RNA recovery is consistent with inhibition occurring predominantly during reverse-transcription rather than during PCR amplification.
Compared to humic acid and heparin, ethanol showed a different response pattern. Within the lower tested range (3–4%), dPCR preserved both the RNA targets and the QNIC control, whereas qPCR already showed strong RNA-target inhibition without decreased QNIC recovery. This indicates that the lower ethanol concentrations primarily affected the RT reaction within the qPCR workflow. At 8–10% ethanol, however, both the RNA targets and QNIC were undetectable in both workflows, demonstrating that high ethanol concentrations inhibit the PCR step itself rather than selectively affecting reverse transcription. Thus, the combined evaluation of RNA targets and the DNA control distinguished RT-specific inhibition from general PCR inhibition.

The QIAcuity OneStep High Multiplex PCR Kit combined with dPCR RNA-Plex Assays provides a robust one-step dPCR workflow for multiplex RNA quantification in inhibitor-containing samples. Across the tested humic acid, heparin and ethanol conditions, the dPCR workflow preserves substantially more target signal and maintains quantification over a broader inhibitor range than the qPCR workflow, which shows pronounced signal loss or complete target non-detection at comparably lower inhibitor concentrations.

This increased tolerance reduces the risk of inhibitor-driven target underestimation within gene expression and false-negative results, supporting a more dependable analysis of challenging RNA samples. In contrast to qPCR, which relies on amplification kinetics and is therefore highly sensitive to inhibitor-induced changes in PCR efficiency, dPCR uses partition-based endpoint detection to maintain reliable target quantification despite moderate reaction inhibition.

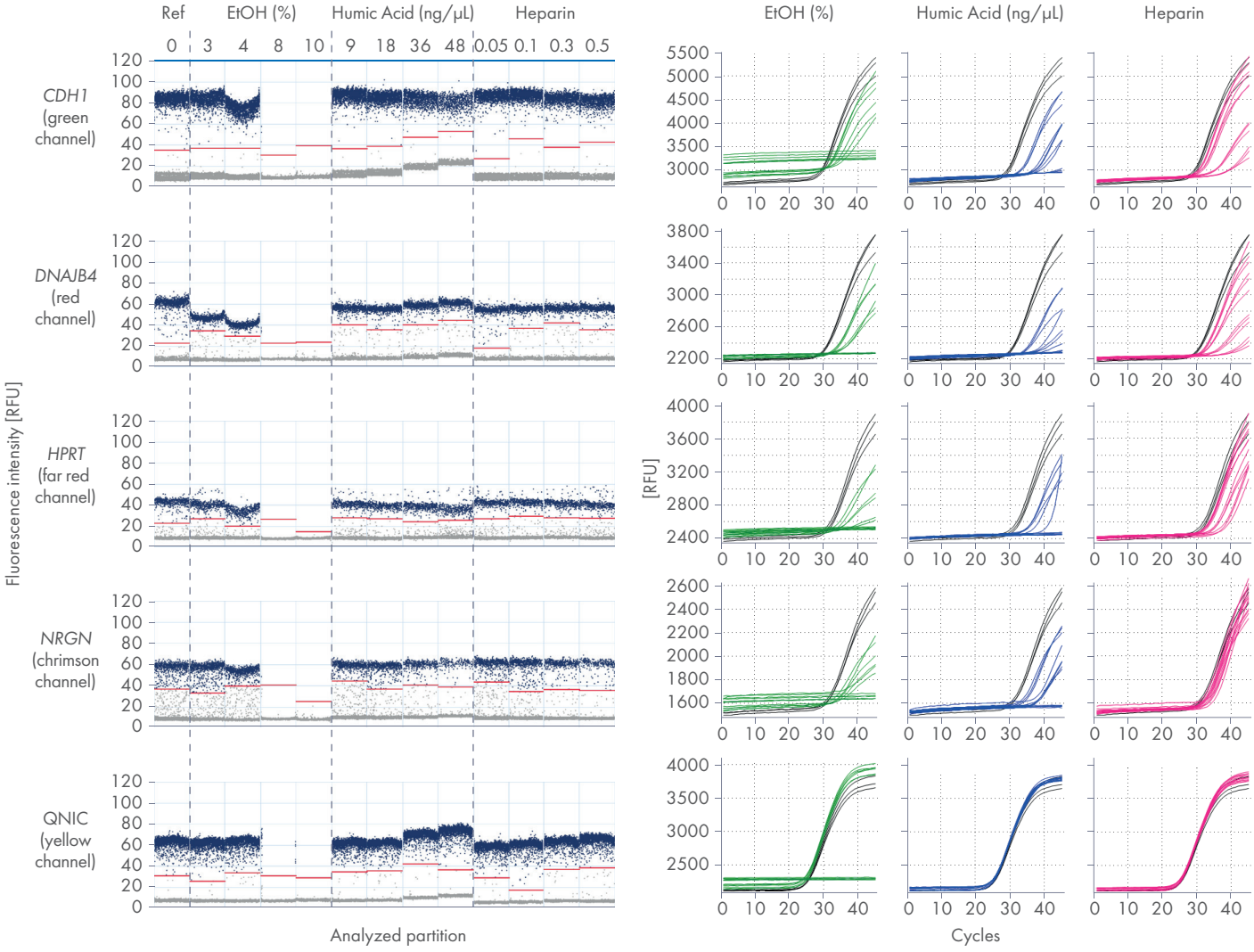


Figure 1. Inhibitor spike-ins have more notable effects on RT-qPCR than RT-dPCR performance.

Representative dPCR 1D scatterplots and qPCR amplification curves show the effects of increasing concentrations of ethanol, humic acid and heparin on multiplex RNA detection. In the dPCR 1D scatterplots, each point represents the endpoint fluorescence of an individual partition. Positive and negative partition populations are visualized for the no-inhibitor reference and inhibitor-spiked reactions. The dPCR workflow maintains detectable positive populations and clear partition classification across a broader inhibitor range, although increasing inhibitor concentrations can reduce endpoint fluorescence and positive-partition separation. In contrast, the qPCR curves show inhibitor-dependent delays, reduced amplification slopes and plateaued fluorescence or complete loss of amplification. In the qPCR plots, black curves represent samples without added inhibitor, whereas the colored curves represent samples containing increasing amounts of inhibitor. The QNIC DNA control supports differentiation between inhibitor effects on reverse transcription versus direct inhibition of PCR amplification.

Table 1. One-step multiplex RT-dPCR exhibits higher inhibitor tolerance than RT-qPCR.

			EtOH (%)				Humic acid (ng/µL)				Heparin (µM)			
		No Inhibitor	3	4	8	10	9	18	36	48	0.05	0.1	0.3	0.5
dPCR	*CDH1*	100%	100%	104%	0%	0%	99%	103%	63%	39%	102%	103%	88%	74%
	DNAJB4	100%	98%	102%	0%	0%	101%	103%	99%	92%	103%	105%	96%	95%
	HPRT1	100%	96%	102%	0%	0%	100%	101%	95%	74%	106%	101%	97%	91%
	NRGN	100%	117%	110%	0%	0%	97%	93%	48%	26%	91%	92%	66%	41%
	QNIC*	100%	101%	103%	1%	0%	100%	101%	105%	102%	98%	102%	101%	104%
qPCR	*CDH1*	100%	37%	16%	0%	0%	16%	3%	2%	0%	55%	33%	7%	2%
	DNAJB4	100%	24%	7%	0%	0%	15%	0%	0%	0%	47%	23%	7%	0%
	HPRT1	100%	23%	5%	0%	0%	11%	6%	1%	0%	54%	38%	13%	4%
	NRGN	100%	16%	0%	0%	0%	15%	1%	0%	0%	58%	40%	29%	16%
	QNIC*	100%	124%	125%	0%	0%	107%	107%	105%	106%	110%	110%	109%	116%

* Values represent the measured quantity for each RNA target and the QNIC DNA control relative to the corresponding no-inhibitor reaction, which was set to 100%. A value of 0% indicates that no target quantity was obtained under the tested condition. The QNIC DNA control is included to distinguish between RT-specific inhibition and general PCR inhibition.

Conclusion

The QIAcuity OneStep High Multiplex PCR Kit combined with dPCR RNA-Plex Assays enables robust one-step multiplex RT-dPCR across inhibitor-challenged conditions. Compared with RT-qPCR, the RT-dPCR workflow maintains reliable RNA quantification over a broader range of humic acid, heparin and ethanol concentrations, reducing inhibitor-driven target underestimation and false-negative results. A dPCR workflow, therefore, provides a dependable solution for multiplex gene expression analysis of challenging RNA samples.

Ordering Information

Product	Contents	Cat. no.
QIAcuity One, Four or Eight Platform System	Digital PCR instrument for detecting up to 5 fluorescent dyes or up to 8 fluorescent dyes operating under software version 3.1 or higher, enabling up to 12-plex analysis	911021, 911042, 911052
QIAcuity Nanoplate 8.5k 96-Well	10 QIAcuity Nanoplate 8.5k 96-well, 11 Nanoplate Seals	250021
dPCR RNA-Plex Assays	One tube with lyophilized assay, dye/fluorophore configurable, 250 to 750 reactions (12 µl reaction in Nanoplate 8.5K)	250336, 250337
QIAcuity OneStep High Multiplex PCR Kit	x OneStep High Multiplex PCR Master Mix, 100x OneStep High Multiplex RT Mix, Enhancer GC, RNase-free water, QN Internal Control RNA; for 100 (or 500) reactions in Nanoplate 26K and 333 (or 1666) reactions in Nanoplate 8.5K	250135, 250136

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