Application Note

Using the Q-Solution Kit to mitigate the negative effects of highly structured DNA templates and inhibitors on PCR efficiency

Introduction

For most applications on the QIAcuity® Digital PCR System, the uniquely formulated QIAcuity digital PCR assays and kits can be used straight out of the box. However, in certain instances, such as when highly structured DNA templates are used or the presence of PCR inhibitors is suspected, adjusting the QIAcuity reaction chemistry may be necessary to achieve optimal performance. For these cases, we have developed the Q-Solution Kit (cat. no. 210220), which contains 5x Q-Solution and a standalone solution of 25 mM magnesium chloride ($MgCl_2$).

Q-Solution is an innovative and versatile PCR additive that works in part by lowering the melting temperature of DNA. Adjusting template melting properties can be useful when interrogating DNA with high-GC content or with complex secondary structures, such as adeno-associated virus (AAV) genomes. Q-Solution has additional properties that can be used to mitigate the carryover of various PCR inhibitors.

In contrast to Q-Solution, the $MgCl_2$ supplied with the kit stabilizes double-stranded DNA structures, including those formed between primers and the template. If weak primer or probe binding is suspected of causing poor PCR performance, $MgCl_2$ may be used to boost oligonucleotide binding affinity. $MgCl_2$ is also an essential cofactor for DNA polymerase function.

Q-Solution and $MgCl_2$ can be used alone or in combination. When higher concentrations of Q-Solution are used (e.g., >1x), adding $MgCl_2$ to the reaction may be necessary to achieve best results. This application note will highlight a few examples of how the Q-Solution Kit can be used in QIAcuity workflows. The utility of the Q-Solution Kit is not limited to the use cases presented here. Please note that the concentrations of Q-Solution and $MgCl_2$ used in the examples shown here may not work for all applications.

Using the Q-Solution Kit to address challenging templates

Complex DNA structures can hinder PCR efficiency and ultimately lead to inaccurate quantification or PCR failure. AAVs are one example of samples whose genomic structure is a significant obstacle for PCR amplification. At the 5′ and 3′ termini of the single-stranded AAV genomes, sequences known as inverted terminal repeats (ITRs) form stable double-stranded DNA loops that greatly impede the amplification of targets in their proximity. Thus, AAVs provide an ideal model system for showing how the Q-Solution Kit can be used to overcome the difficulties that certain DNA templates pose for PCR amplification.

To ensure optimal PCR efficiency and accurate quantification of AAVs, restriction enzymes are typically used to release the inhibitory secondary structures in AAV genomes. Compared to AAVs digested with the restriction enzyme MspI/HpaII, untreated AAVs samples can be underquantified by a degree of 25% with the SV40 polyA (Figure 1A) and SV40 promoter (Figure 1B) assays from the QIAcuity Cell and Gene Therapy (CGT) dPCR Assays portfolio.

Here we show that improvements in PCR efficiency equivalent to those following restriction enzyme digestion can be achieved through the combined action of Q-Solution and $MgCl_2$ found in the Q-Solution Kit. ▷

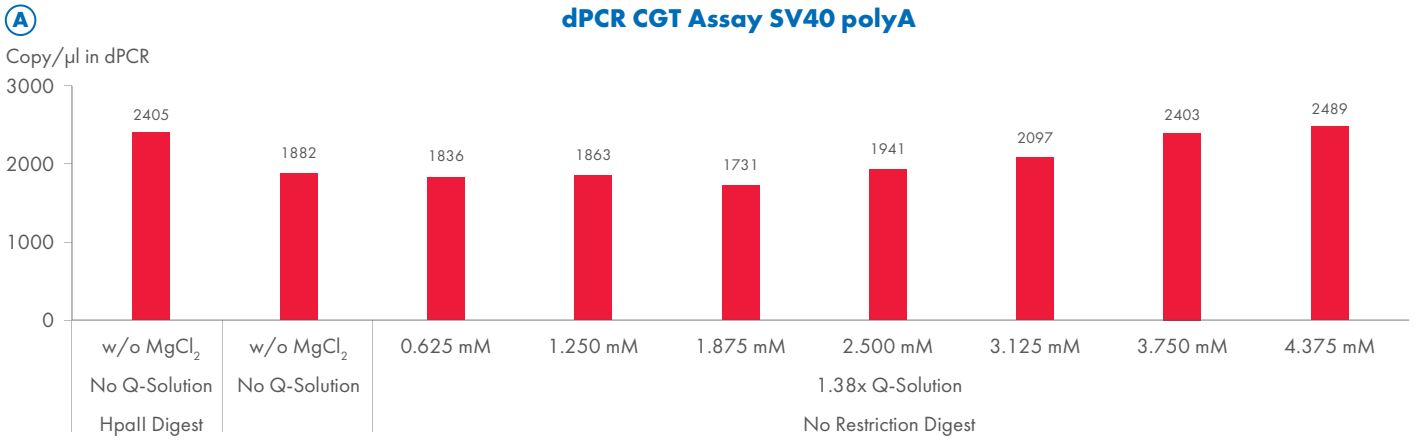
When QIAcuity Probe PCR Kit reactions contain 1.38x Q-Solution and 4.375 mM of added $MgCl_2$, quantification of AAV genomes with the SV40 polyA and SV40 promoter assays was equivalent to that of AAVs treated with the restriction enzyme HpaII (Figure 1A, B). For the SV40 promoter assay, addition of Q-Solution alone was sufficient to obtain quantification equivalent to restriction digest. In contrast, the SV40 polyA assay required the addition of $MgCl_2$ at a concentration of 4.375 mM to show equivalent quantification.

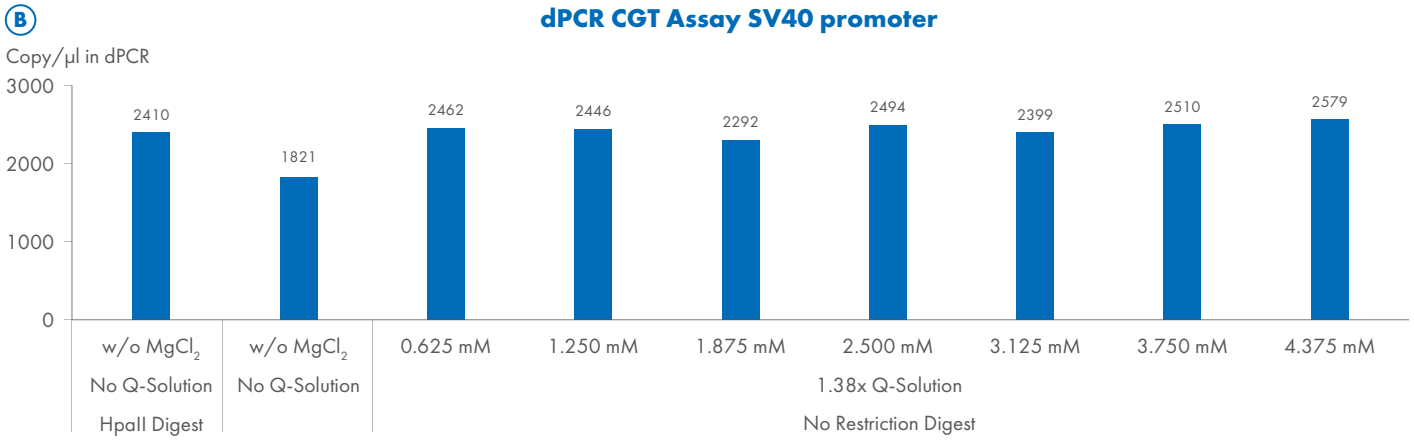
In addition to improving quantification accuracy, adding Q-Solution and $MgCl_2$ to reactions improved the amplification of the SV40 polyA assay in other appreciable ways. When reactions contained Q-Solution and extra $MgCl_2$, a stronger fluorescent signal was generated from the SV40 polyA assay, such that a larger separation between the negative and positive partitions could be observed in the 1D scatterplots (Figure 1C).

The fluorescent signal generated with the SV40 promoter assay was unaffected by the addition of Q-Solution and $MgCl_2$ (Figure 1D).

The data generated with AAVs and the Q-Solution Kit demonstrate the capacity of PCR additives to mitigate the challenges posed by highly structured templates. Importantly, the response of the SV40 promoter and SV40 polyA assays to the modified reaction chemistries underscore two important points that must be considered when using the Q-Solution Kit:

- Each individual assay will react differently to the addition of Q-Solution and $MgCl_2$
- Titration experiments with Q-Solution and $MgCl_2$, will most likely be required to obtain best results





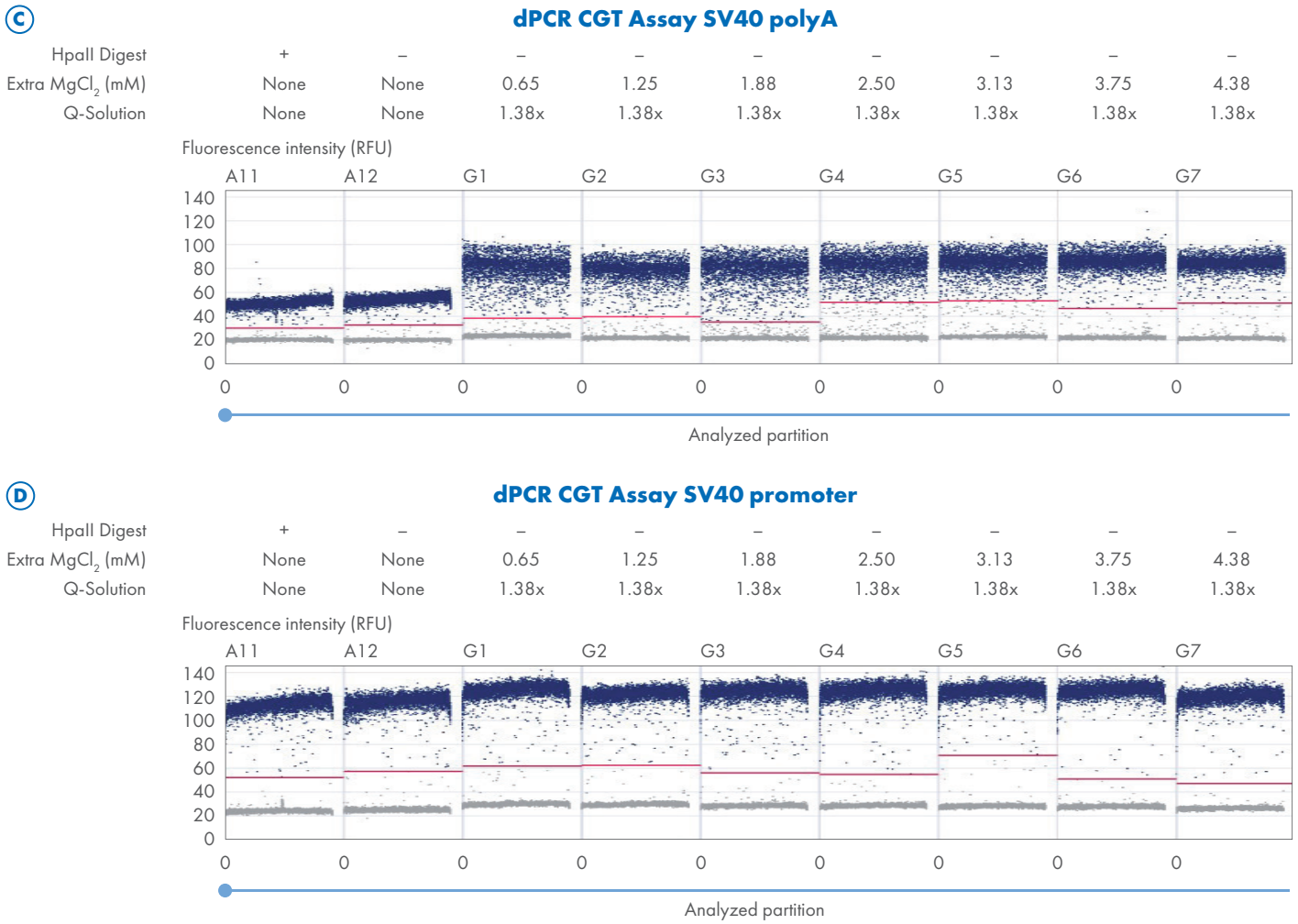


Figure 1. Defined concentrations of Q-Solution and $MgCl_2$ make use of restriction enzymes for AAV genome titration redundant. AAV2 (unpurified, produced in-house) samples were processed using the QIAGEN Cell and Gene Therapy Viral Vector Lysis Kit. Lysates were added to QIAcuity Probe PCR reactions containing 1.38x Q-Solution and different concentrations of $MgCl_2$ ranging from 0.625 mM to 4.38 mM. These samples were not treated with HpaII restriction enzyme digestion. For comparison, a control reaction using HpaII restriction enzyme digestion was also assembled. All samples (with and without restriction enzyme) were incubated for 10 minutes at room temperature before transfer to an 8.5k Nanoplate. Quantification was performed using the QIAcuity Cell and Gene Therapy (CGT) dPCR Assays in a multiplex reaction (SV40 promoter-FAM, ITR-HEX, SV40pA-Cy5) following the recommended cycling and imaging conditions.

Mean target quantification of 3 replicates per condition for SV40pA (**A**) and SV40 promoter (**B**) shows that quantification of AAVs is the same between reactions treated with HpaII and reactions to which Q-Solution and $MgCl_2$ were added. 1D scatterplots for the assay SV40 polyA (**C**) show that addition of $MgCl_2$, even at the lowest show that the addition of Q-Solution and $MgCl_2$ increases the fluorescence signal generated by the reaction. For the assay SV40 promoter (**D**), addition of Q-Solution and $MgCl_2$ had no impact on the fluorescent signal.

Using the Q-Solution Kit to address PCR inhibitors

Carryover of inhibitors into digital PCR set-ups can lead to suboptimal amplification efficiencies or complete reaction failure. In some instances, suboptimal workflow design or poor sample handling can lead to the carryover of salts, detergents and alcohols that greatly impede PCR. In other cases, such as DNA extracted from environmental samples, the presence of inhibitors like tannic acid is intrinsic to the sample type. For either scenario, we demonstrate that Q-Solution is an effective option for counteracting the presence of diverse PCR inhibitors.

A potent PCR inhibitor that may be carried over from sample preparation methods is ethanol, which is routinely used in wash steps during nucleic acid extraction protocols. PCR efficiency is greatly reduced when 3.5% ethanol is present in QIAcuity Probe PCR Kit reactions quantifying the wildtype BRAF locus with the dPCR LNA Mutation ▷

Assay BRAF 476 Human (GeneGlobe ID DMH0000004). This manifests as 1D scatterplots that appear as U-shapes (Figure 2A). When ethanol is present at even higher concentrations, such as 5%, the PCR reaction fails (Figure 2A).

Adding 0.25x Q-Solution to QIAcuity Probe PCR Kit reactions contaminated with 3.5% ethanol is sufficient to greatly diminish the severity of U-shapes in the 1D scatterplots, while adding 0.5x to 1.5x Q-Solution completely restores PCR reaction efficiency. Compared to control reactions without ethanol, quantification of the wildtype BRAF locus was unaffected by the presence of 3.5% alcohol, even without the addition of Q-Solution (Figure 2B). This is because a clear threshold could be placed between the positive and negative populations, despite the obvious signs of PCR inhibition.

When greater amounts of ethanol are present in QIAcuity Probe PCR Kit reactions, a higher concentration of Q-Solution is needed to overcome PCR inhibition and must be adjusted accordingly. Reactions containing 5% ethanol still exhibit PCR inhibition when 0.25x or 0.5x Q-Solution is added, as indicated by severe to mild U-shapes in the 1D scatterplots. The inhibitory effects of 5% ethanol are only resolved when Q-Solution is added at a concentration of 1x or 1.5x (Figure 2A). Nevertheless, accurate quantification could already be achieved when 0.5x Q-Solution was added (Figure 2B).

The ability of Q-Solution to mitigate the effects of ethanol carryover into PCR reactions does not require supplemental magnesium. Adding magnesium at concentrations of 3.4 mM or 4 mM did not improve the ability of Q-Solution to rescue PCR reactions contaminated with ethanol. In fact, the addition of magnesium appeared to exacerbate the deleterious effects of ethanol in PCR reactions.

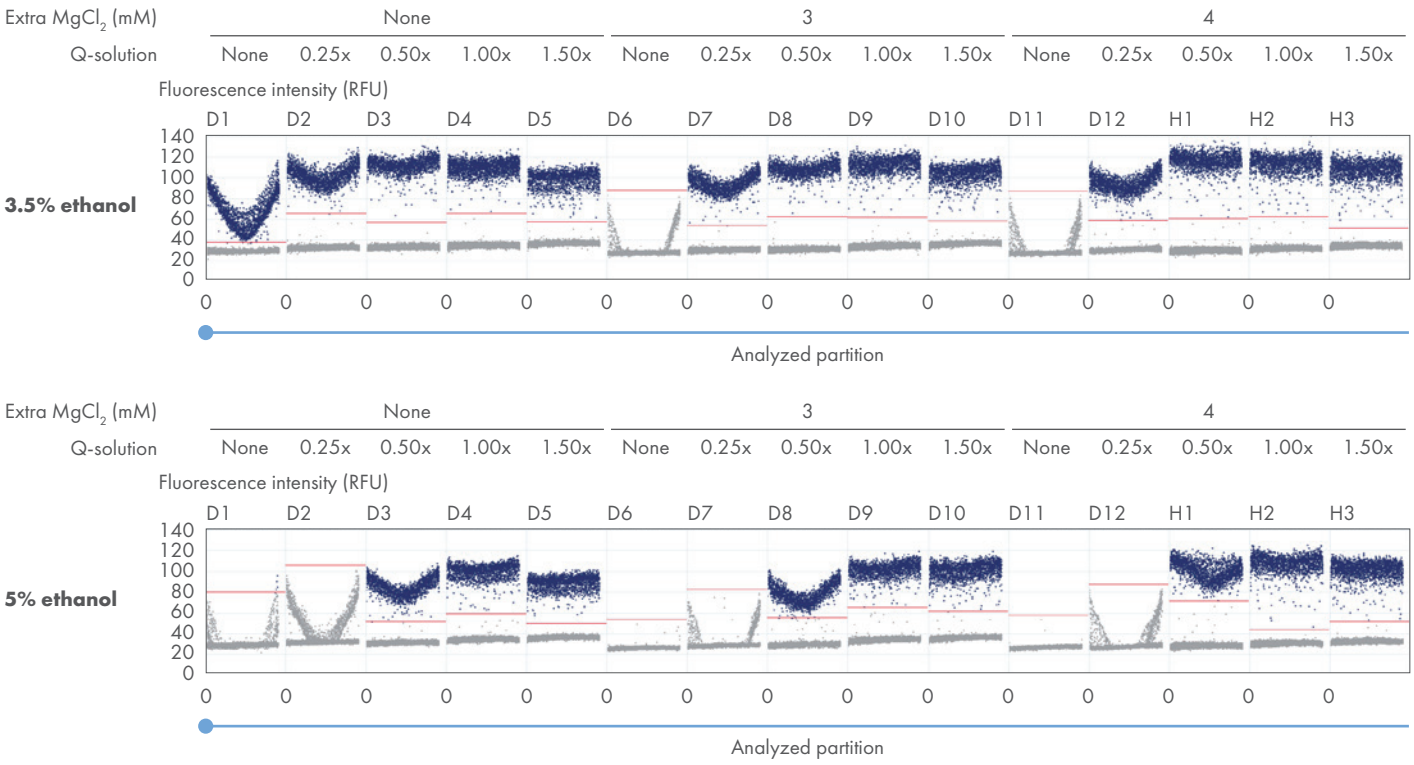
Besides ethanol, Q-Solution can neutralize the effects of additional PCR inhibitors. Using the QIAcuity OneStep Advanced Probe Kit, we tested the ability of 0.5x Q-Solution to offset the effects of a variety of PCR inhibitors on the amplification of DNA targets (Figure 3A). Q-Solution was able to improve or completely restore the efficiencies of PCR reactions containing guanidinium thiocyanate (GITC), humic acid, heparin, isopropanol, sodium dodecyl sulfate (SDS), sodium chloride and tannic acid at specific concentrations. This could be observed in the improved shapes of 1D scatterplots (Figure 3A) as well as in target quantification compared to reactions without inhibitors (Figure 3B).

The ability of Q-Solution to rescue PCR reactions eventually broke down above certain inhibitor concentrations. Moreover, Q-Solution could not address the effects of all PCR inhibitors tested. When present at concentrations leading to the first signs of PCR inhibition, the effects of bile salts (1.5 µg/µl), hematin (40 µM) and urea (100 mM) could not be mitigated by the addition of Q-Solution.

Taken together, these data underlie a few important points that users should consider when using the Q-Solution Kit to offset the effects of PCR inhibitors:

- Q-Solution does not mitigate the negative effects of all PCR inhibitors
- The required dose of Q-Solution will likely vary according to the concentration of PCR inhibitor in the reaction.
- While the appearance of U-shaped 1D scatterplots reflects PCR inhibition, this will not automatically lead to inaccurate quantification. This is especially true for U-shaped 1D scatterplots with very clear separation between positive and negative populations.
- The use of Q-Solution Kit components will not always be beneficial, as evidenced by the ability of additional magnesium chloride to increase the PCR inhibitory effects of ethanol.

A



B

		Q-Solution concentration				
		0x	**0.25x**	**0.5x**	**1x**	**1.5x**
No ethanol	0.0 mM $MgCl_2$	100%	90%	102%	99%	95%
	3.4 mM $MgCl_2$	103%	101%	95%	97%	100%
	4.0 mM $MgCl_2$	96%	101%	99%	100%	102%
3.5 % ethanol	0.0 mM $MgCl_2$	103%	102%	94%	102%	99%
	3.4 mM $MgCl_2$	0%	104%	95%	97%	98%
	4.0 mM $MgCl_2$	0%	103%	98%	93%	99%
5.0 % ethanol	0.0 mM $MgCl_2$	0%	0%	95%	93%	97%
	3.4 mM $MgCl_2$	0%	0%	98%	99%	105%
	4.0 mM $MgCl_2$	0%	0%	101%	98%	101%

Figure 2. Q-Solution counteracts the effects of ethanol carryover in QIAcuity dPCR reactions. QIAcuity Probe PCR Kit reactions quantifying the wildtype BRAF locus with the dPCR Mutation Assay BRAF 476 Human were inhibited when 3.5% or 5% ethanol were present in the reaction, as indicated by the U-shapes in the 1D scatterplots (**A**) and quantification relative to control reactions without ethanol (**B**). Adding Q-Solution at concentrations of 0.25x (3.5% ethanol) or 0.5x (5% ethanol) was able to greatly reduce the inhibition. Adding magnesium chloride to PCR reactions exacerbated the effects of ethanol inhibition. Quantification is based on the mean target quantification of 3 replicates per condition.

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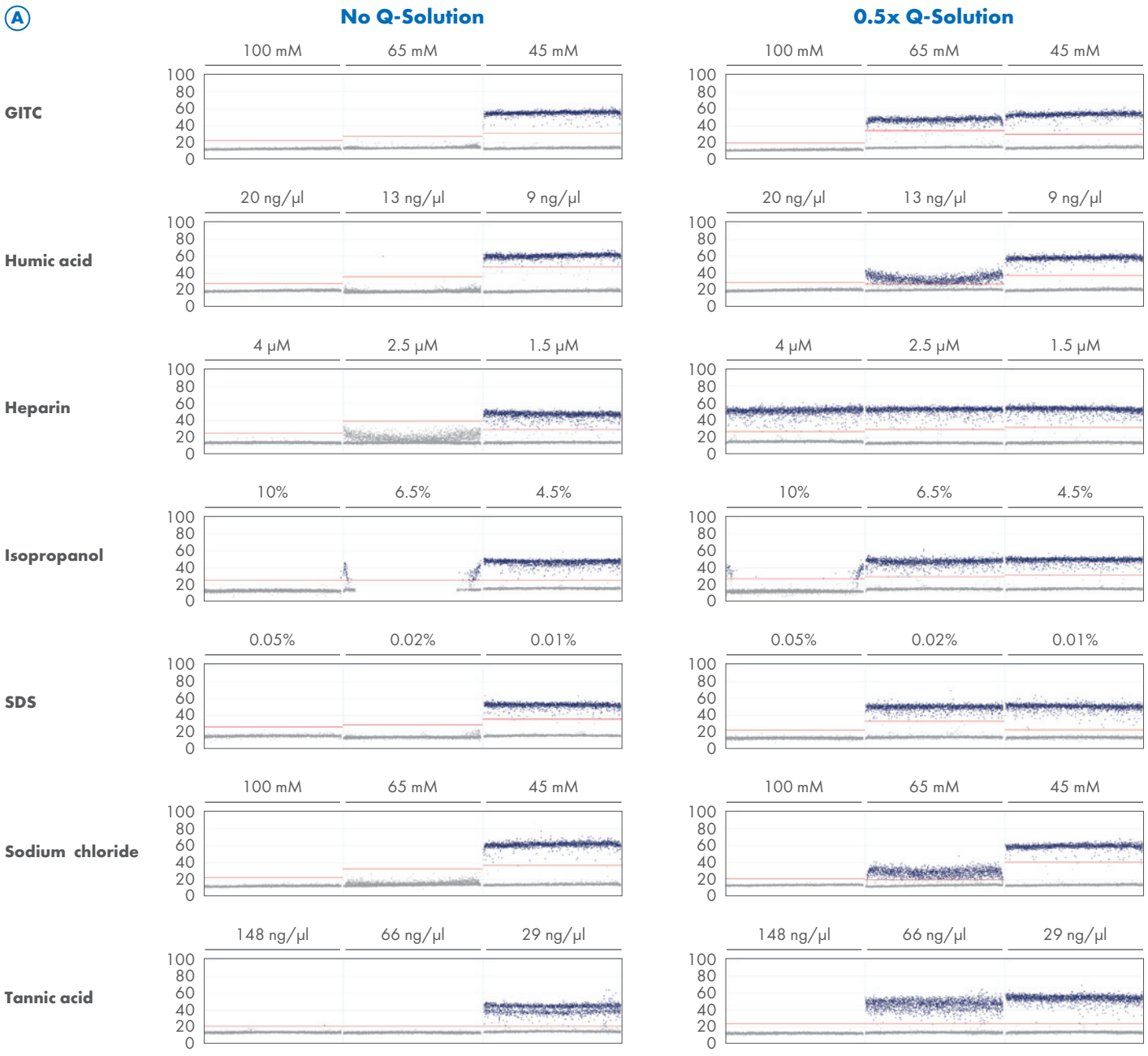
No Q-Solution
0.5x Q-Solution
GITC
100 mM
65 mM
45 mM
Humic acid
20 ng/µl
13 ng/µl
9 ng/µl
Heparin
4 µM
2.5 µM
1.5 µM
Isopropanol
10%
6.5%
4.5%
SDS
0.05%
0.02%
0.01%
Sodium chloride
100 mM
65 mM
45 mM
Tannic acid
148 ng/µl
66 ng/µl
29 ng/µl
100
80
60
40
20
0

B

Quantification relative to no inhibitor control

GITC	**Inhibitor concentration**	**100 mM**	**65 mM**	**45 mM**
	No Q-Solution	0%	0%	102%
	0.5x Q-Solution	0%	97%	99%
Humic acid	**Inhibitor concentration**	**20 ng/µl**	**13 ng/µl**	**9 ng/µl**
	No Q-Solution	0%	0%	100%
	0.5x Q-Solution	0%	84%	99%
Heparin	**Inhibitor concentration**	**4 µM**	**2.5 µM**	**1.5 µM**
	No Q-Solution	0%	0%	101%
	0.5x Q-Solution	97%	98%	99%
Isopropanol	**Inhibitor concentration**	**10%**	**7%**	**5%**
	No Q-Solution	0%	32%	101%
	0.5x Q-Solution	5%	100%	100%
SDS	**Inhibitor concentration**	**0.05%**	**0.02%**	**0.01%**
	No Q-Solution	0%	0%	98%
	0.5x Q-Solution	0%	95%	97%
Sodium chloride	**Inhibitor concentration**	**100 mM**	**65 mM**	**45 mM**
	No Q-Solution	0%	0%	100%
	0.5x Q-Solution	0%	90%	99%
Tannic acid	**Inhibitor concentration**	**148 ng/µl**	**66 ng/µl**	**29 ng/µl**
	No Q-Solution	0%	0%	101%
	0.5x Q-Solution	0%	95%	100%

Figure 3. Q-Solution can mitigate the effects of multiple PCR inhibitors. Various PCR inhibitors were added individually to QIAcuity OneStep Advanced Probe Kit reaction mixes across a range of concentrations. The 1D scatterplots (**A**) and quantification of DNA targets relative to inhibitor-free control reactions (**B**) were compared between reactions with or without 0.5x Q-Solution. QuantiNova IC Probe Assay (200) (cat. no. 205813) with QN IC DNA template was used for tests with heparin, SDS and tannic acid. dPCR LNA Mutation Assay FLT3 28042 Human (GeneGlobe ID DMH0000281) and a synthetic DNA template containing the 1352C>T mutation was used for tests with GITC, humic acid, isopropanol and sodium chloride. Quantification is based on the mean target quantification of 3 replicates per condition.

Summary

The Q-Solution Kit is an effective measure against challenging templates and PCR inhibitors in PCR reactions. Using the Q-Solution Kit with highly-structured DNA templates, such as AAV genomes, is effective at improving PCR efficiency without the need for restriction digestion. Data also shows that Q-Solution is an effective PCR additive for counteracting the negative effects of various PCR inhibitors, including ethanol, GITC, humic acid, heparin, isopropanol, SDS, sodium chloride and tannic acid.

Individual assays are apt to react differently to Q-Solution and $MgCl_2$, so titration experiments are recommended to achieve optimal results. Taken together, the Q-Solution Kit is a simple and effective approach for users interested in extending the reach of the QIAcuity Digital PCR System to more challenging applications.

For more information on QIAcuity digital PCR products, visit: **www.qiagen.com/applications/digital-pcr/products**

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.