**Application Note**

# Ensuring viral safety through multiplexed adventitious virus detection with QIAcuity dPCR

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## Introduction

Testing for adventitious viruses, including human viral contaminants, is critical to ensure the safety and integrity of all biotechnology products derived from cell lines. The risk of viral contamination must be minimized, as such events can lead to serious clinical consequences. Regulatory bodies such as the EMA and FDA adopted the guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin, known as ICH Q5A(R2). This document emphasizes the need for sensitive and validated methods to detect both known and unknown viral agents that may be introduced from the original source of the cell lines or during production (*International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2023*).

Adventitious virus testing is a key component of viral safety evaluation and helps manufacturers demonstrate process control and product safety throughout biopharmaceutical development. As part of a risk-based approach and in alignment with ICH Q5A(R2), Next-Generation Sequencing (NGS) is encouraged as a powerful tool for broad, untargeted and targeted screening, and may supplement or replace traditional *in vivo* and *in vitro* assays for adventitious virus detection. While NGS is well-suited for detecting unexpected viral sequences, its use for routine or selective quality control (QC) testing may vary depending on specific program needs, testing goals, available resources and timelines.

In this context, digital PCR (dPCR) offers an efficient alternative. It can be used to screen for known viral targets in a focused panel format or to confirm NGS findings with high specificity. For QC labs and other viral safety testing contexts, dPCR provides several practical advantages:

- Rapid turnaround for decision-critical workflows
- Multiplexing capabilities that allow simultaneous detection of multiple viral targets with minimal sample input
- High sensitivity and precision for low-level viral detection
- Robust performance across diverse sample types and matrices
- Cost-efficiency compared to sequencing-based methods

Together, these attributes make dPCR an ideal tool for routine targeted adventitious virus testing, enabling reliable detection of human viral contaminants in biologics manufacturing environments. This application note demonstrates the feasibility of dPCR for multiplexed detection of a subset of relevant human viruses.

### Performance evaluation

To demonstrate the feasibility of this approach, a panel targeting selected human viruses relevant to biologics manufacturing was tested using the QIAcuity Digital PCR System. The panel was comprised of 13 viral targets, organized into two independent multiplex reactions: one for RNA viruses (including RNAseP as a human sampling control, where applicable) and one for DNA viruses. These represent a diverse set of virus families, including ▷

*Herpesviridae*, *Retroviridae*, *Flaviviridae* and *Polyomaviridae*, which are commonly referenced in regulatory guidance and biosafety testing frameworks.

The ready-to-use assays are available through the GeneGlobe Design & Analysis Hub, enabling streamlined setup and integration into digital PCR workflows using the QIAcuity system (Table 1).

This human virus panel was assembled to demonstrate the potential of digital PCR for multiplex detection of representative human viral contaminants in an adventitious virus testing context.

The testing strategy used combines classical fluorophore-based multiplexing with amplitude-based multiplexing. This strategy enables the simultaneous detection of multiple viral targets within the same optical channel by adjusting probe concentrations to generate distinct signal intensities. With the QIAcuity High Multiplex Probe PCR Kit, it is possible to perform higher-order multiplexing (up to 12 targets) within a single reaction on the existing QIAcuity hardware. This approach efficiently uses limited sample input and minimizes errors while maintaining high specificity and clear signal separation. In validation experiments using synthetic DNA targets, each assay demonstrated robust performance across both 5-plex (RNA panel) and 9-plex (DNA panel) configurations (Figure 1).

This multiplexing strategy illustrates the flexibility of the QIAcuity platform for high-multiplex applications. By combining classical with amplitude-based signal separation, the workflow achieves precise discrimination of multiple viral targets without compromising assay accuracy or throughput.

Each assay was tested in both singleplex and multiplex reactions. The results demonstrated comparable quantification across both formats, confirming that multiplexing does not compromise assay performance. This consistency highlights the robustness and reliability of digital PCR for high-multiplex applications in adventitious virus testing, where maintaining sensitivity and specificity across multiple targets is essential (Figure 1, Table 2).

Furthermore, non-template controls (NTCs) consistently showed no signal, confirming the absence of contamination and highlighting assay specificity. Together, these findings support the suitability of the QIAcuity dPCR platform for viral detection workflows in QC and biosafety settings.

**Table 1. Human virus panel for adventitious agents detection**

| Target Name | Acronym | GeneGlobe ID | Dye | Assay Concentration | RNA/DNA panel |
|---|---|---|---|---|---|
| Human betaherpesvirus 6A | HHV-6A | DMA00837 | FAM* | 1x | DNA |
| Human betaherpesvirus 6B | HHV-6B | DMA00838 | FAM* | 1.5x | DNA |
| Hepatitis B | HBV | DMA00808 | HEX* | 0.5x | DNA |
| Human parvovirus B19 | B19V | DMA00862 | HEX* | 1x | DNA |
| Betapolyomavirus macacae (SV40) | SV40 | DMA00936 | TAMRA | 1x | DNA |
| Human betaherpesvirus 7 | HHV-7 | DMA00839 | ROX* | 1x | DNA |
| Human gammaherpesvirus 8 | HHV-8 | DMA00836 | ROX* | 1.5x | DNA |
| Human gammaherpesvirus 4 | HHV-4 (EBV) | DMA00840 | Cy5 | 1x | DNA |
| Human betaherpesvirus 5 | HHV-5 (HCMV) | DMA00835 | ATTO700 | 2x | DNA |
| Hepatitis C | HCV | DMA00809 | FAM | 1x | RNA |
| Hepatitis A | HAV | DMA00807 | HEX | 1x | RNA |
| Human T-cell leukemia virus type I | HTLV-1 | DMA00867 | TAMRA | 1x | RNA |
| Human T-cell leukemia virus 2 (2) | HTLV-2 | DMA00870 | ROX | 1x | RNA |
| RNAseP(g) | - | DMA00720 | ATTO700 | 2x | RNA ** |

* Amplitude-based multiplexing
** Added to the RNA panel as a human sampling control target

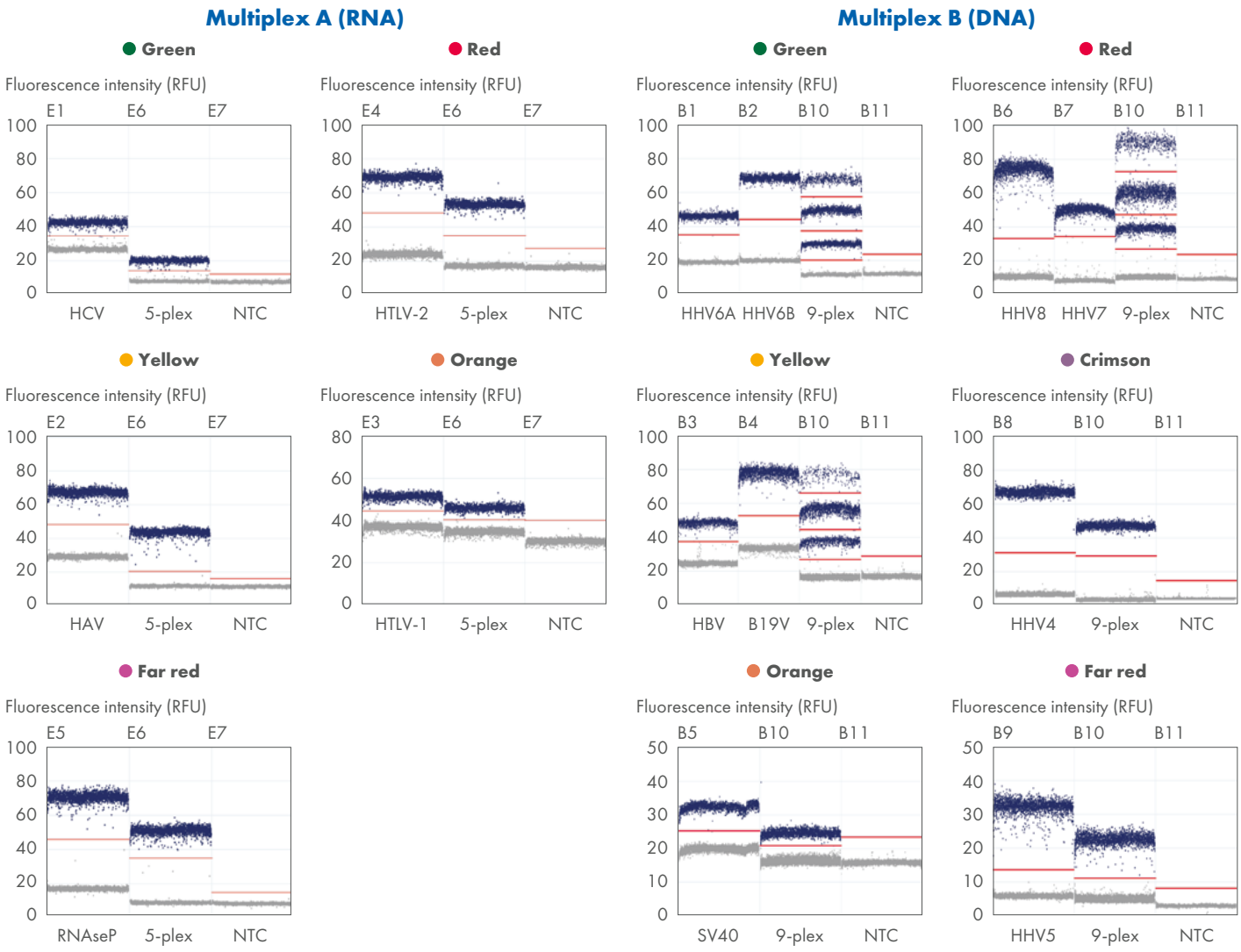


**Figure 1. The multiplexing capabilities of the QIAcuity High Multiplex Probe PCR Kit, combined with the QIAcuity Software Suite 3.1, provide significant benefits for virus detection.** dPCR Microbial DNA Detection Assays were used in a 5-plex reaction for relevant adventitious RNA viruses (Multiplex A) and a 9-plex reaction for DNA viruses (Multiplex B). Each multiplex assay setup was used to detect an individual synthetic target DNA or a pool of all synthetic target DNAs on an 8.5k nanoplate. Specific amplification of each target was observed in both singleplex and multiplex reactions. The no template control (NTC) reactions were consistently negative. Amplitude-based multiplexing allowed for the detection of two distinct targets within the Green, Yellow and Red fluorescence channels by modulating probe concentrations. For these channels, multiple thresholds (red lines) can easily be set, resulting in clearly distinguishable signal clusters. Imaging settings were adjusted to increase fluorescence intensities. A custom crosstalk matrix was applied.

**Table 2. Highly concordant quantification between singleplex and multiplex reactions.** Absolute quantification (copies/µL) of human virus targets measured on the QIAcuity dPCR System shows comparable results between singleplex and multiplex reactions

| Target | Singleplex (copies/µL) | RNA Multiplex (copies/µL) |
|---|---|---|
| HCV | 819 | 840 |
| HAV | 1294 | 1308 |
| HTLV-1 | 921 | 938 |
| HTLV-2 | 986 | 986 |
| RNAseP | 1145 | 1148 |

| Target | Singleplex (copies/µL) | DNA Multiplex (copies/µL) |
|---|---|---|
| HHV-6A | 1134 | 1117 |
| HHV-6B | 950 | 1009 |
| HBV | 558 | 541 |
| B19V | 1050 | 932 |
| SV40 | 1155 | 1060 |
| HHV-7 | 1183 | 1008 |
| HHV-8 | 1116 | 1116 |
| HHV-4 (EBV) | 1218 | 1140 |
| HHV-5 (HCMV) | 1102 | 1069 |

## Conclusion

The tested panel effectively demonstrates the capability of dPCR for multiplexed detection of human viruses in the context of adventitious virus testing. The human virus panel described here is available for easy ordering and convenient integration into digital PCR workflows as two assay bundles (DNA and RNA) on the GeneGlobe platform, with additional human viral targets of interest on the bundle page.

As outlined in ICH Q5A(R2), the type and extent of testing depend on a risk assessment considering the specific risk factors associated with the cell substrate and the manufacturing process. In line with this principle, users are encouraged to adopt a mix-and-match approach, selecting viral assays that best reflect the risk profile of their product and production environment.

Additional common human viral targets, such as those targeting HSV-1 and HSV-2 (Assay IDs: DMA00724 and DMA00725), HIV (Assay ID: DMA00811), HPV 16 and 18 (Assay IDs: DMA00882 and DMA007883), BK (Assay ID: DMA00894) and JC (Assay ID: DMA00861) and others, are also available through the dPCR Microbial DNA Detection Assays page as part of a wet-lab tested assay portfolio that continues to expand to support evolving biosafety and quality control needs. Furthermore, for targets not currently included in the portfolio, users can leverage the easy-to-use Custom dPCR Microbial Assays design tool. This tool allows the creation of tailored assays for any pathogen of interest, including but not limited to human viruses, supporting diverse biosafety and microbial testing needs. It is important to note that multiplexing configurations involving additional targets require testing and optimization to ensure reliable performance. Additionally, regulatory guidelines require these methods to be validated for their intended use, based on product-specific risk assessment. Performance characteristics, including sensitivity, specificity and robustness, should be demonstrated through appropriate validation studies.

In conclusion, dPCR is valuable for targeted screening, or confirmatory testing of adventitious agents. This initiative is part of our broader commitment to supporting biopharmaceutical development with high-performance molecular testing solutions, including assays, instruments and digital tools tailored to the needs of regulated environments.

The combined use of wet-lab tested assays, customizable assay design and scalable multiplexing capabilities positions the QIAcuity Digital PCR System as a robust and adaptable platform for advancing adventitious virus testing in modern viral safety workflows.

**References**

**International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (November 1, 2023)**. *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (ICH Q5A(R2))* [Guideline]. https://database.ich.org/sites/default/files/ICH_Q5A%28R2%29_Guideline_2023_1101.pdf

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**http://www.qiagen.com/qiacuitydpcr**