Application Note

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Analysis of DNA integrity and stability using digital PCR

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Introduction

Digital PCR (dPCR) represents an optimal technology for quantifying DNA molecules with unprecedented precision and a high tolerance towards inhibitors. dPCR enables absolute quantification without reliance on references or standard curves. When analyzing multiple targets within a single DNA molecule, it may be crucial not only to quantify but also to assess the proportion of intact DNA present in the sample.

Traditionally, genome integrity and linkage analyses have been determined via agarose gel electrophoresis and Southern blot. Next-generation sequencing approaches have been used for characterization purposes as well. High resolution and accuracy, as well as repeatability and high-throughput capabilities, are crucial for integrity determination. Lately, multiplex digital PCR has gained acceptance and is utilized for genome integrity and linkage analyses (Prantner & Maar, 2023; Tereshko et al., 2023).

Applications in which it is essential to assess DNA quality and integrity are diverse, and there are many challenges. DNA molecules can break during freeze-thaw cycles. Additionally, nucleic acid storage can contribute to the reduction of DNA template quality. Furthermore, various physical, biochemical (e.g., restriction enzymes) and chemical treatments can result in shortened DNA molecules. It is important to note that while PCR-based quantification of single targets may not be affected, the integrity of the whole molecule may be compromised. A comprehensive analysis of DNA stability and integrity is therefore needed.

In cell and gene therapy applications, it is essential to minimize the proportion of molecules that do not contribute to therapeutical efficacy. The development of safe and effective cell and gene therapies is key to potentially treating a broad spectrum of diseases. Viral vectors have become powerful delivery vehicles for gene therapies. Adeno-associated virus (AAV) vectors have become primary modalities for efficient gene therapy applications thanks to their lack of pathogenicity and persistent transgene expression (Issa et al., 2023).

Errors made during the replication and packaging process of recombinant AAVs can lead to heterogenous viral vector populations with a direct impact on their efficacy and safety. Current purification workflows can efficiently separate empty capsids from full capsids (e.g., via anion exchange chromatography and affinity chromatography). However, capsids carrying partial or truncated genomes, as well as capsids packaged with host cell or plasmid DNA, are challenging to separate and can be present in the viral vector product after purification.

Here, we describe a rapid dPCR approach for characterizing genome integrity of in-process and purified AAV samples using the same primers and probes that have been optimized for vector genome titration. The underlying Poisson distribution of dPCR enables the assessment of genome integrity over a broad dynamic range by differentiating between physically linked and unlinked targets. As the calculation estimates the concentration for all groups of template molecules included in a ▷

sample individually, it can be used for other applications, such as determining the integrity and stability of DNA molecules after certain processing procedures (e.g., by restriction enzyme digestion) or storage. We show that up to 5 targets can be analyzed simultaneously, increasing the precision and reproducibility of the analyses.

Material and Methods

Integrity analysis using the QIAcuity® dPCR System and the QIAcuity Software Suite

Software integrity feature

The QIAcuity Software Suite version 2.5. (or higher) enables calculating the percentage of intact (physically linked) molecules among non-intact (physically unlinked) molecules in a given well. This functionality works for a multiplexing grade of up to 5 targets. After the threshold settings per channel have been set, automatically or manually, the QIAcuity Software Suite offers a download option for a multiple occupancy CSV file, which includes the percentage of intact or linked targets. Based on the number of channels or targets selected during data analysis, calculated values for the percentage of intact/linked molecules and dPCR-specific data, such as λ and λ error for each well, are shown and may be used for further result interpretation. If the replicate and/or hyperwell feature is enabled within the QIAcuity Software Suite, the multiple occupancy CSV file contains the calculated values for the percentage of intact/linked molecules per replicate group and/or hyperwell(s).

Mathematical background and formulas

When using dPCR, mathematical approximations based on the underlying Poisson distribution-related statistics can be used to estimate the percentage of intact/linked molecules among all other non-intact/unlinked molecules. A DNA template molecule comprising three targets addressed by three assays labeled with three different dyes (Figure 1) is used to show the mathematical background and formulas for integrity analyses implemented in the QIAcuity Software Suite version 2.5 and higher.

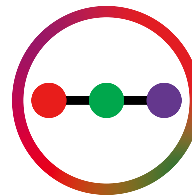


Figure 1. Fully intact/linked DNA template molecule comprising three targets. The 5' target, 3' target and gene of interest addressed by assays labeled with a red, crimson and green dye, respectively. A partition in the QIAcuity Nanoplate containing a fully intact DNA molecule will result in positive signals for all three channels.

An intact/linked template molecule can be detected as a non-intact/unlinked template molecule for several reasons:

- Incomplete synthesis
- Breakage resulting from storage, chemical, biochemical and physical processing
- Mutation events

In these cases, specific targets on the DNA template might no longer be amplified, leading to a lack of positive signal in the corresponding channel(s). Fragmentation can also lead to the distribution of the non-intact/unlinked DNA template to different partitions, resulting in partitions that are no longer positive for all three channels. In the triplex example, some partitions might be positive in only one channel, while others can be positive in two channels (Figure 2).

Depending on the quality of the DNA template molecules, the ratio of fully intact/linked versus non-intact/unlinked template molecules may vary.

To calculate the percentage of intact/linked molecules, the QIAcuity Software Suite performs mathematical calculations and summarizes them in the multiple occupancy CSV output file. For simplicity reasons, the subsequent section describes those mathematical calculations stepwise, even though they are done in parallel during computation.

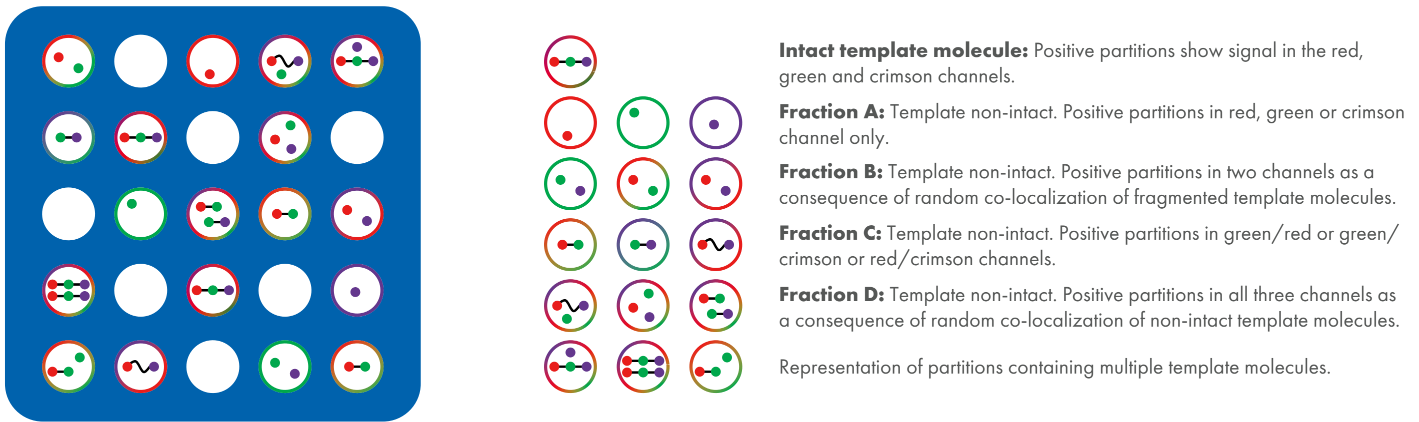


Figure 2. QIAcuity Nanoplate partitions containing one or more template molecules that are either intact/linked or non-intact/unlinked (not all possible scenarios are shown).

Here are the step-by-step calculations as outlined in Figure 2:

- **First step:** Calculate the share of partitions with non-intact/unlinked template molecules showing positive signals in just one channel and estimate the number of respective template molecules (Figure 2, Fraction A).
- **Second step:** The non-intact/unlinked molecule counts estimated in the previous step are used to calculate the number of partitions with positive signals in two channels resulting from random co-localization of template molecules from Fraction A (Figure 2, Fraction B).
- **Third step:** The residual number of partitions containing only partially linked template molecule(s) can be calculated, and with that, the overall number of respective template molecules can be estimated (Figure 2, Fraction C—total number of molecules).

Based on the number of template molecules from Fraction A and Fraction C, the number of partitions with positive signals in all three channels that do not contain fully linked template molecules can be estimated (Figure 2, Fraction D).

From the known number of partitions with positive signals in all three channels coming from random co-localization of non-intact/unlinked template molecules, the number of partitions containing at least one fully intact/linked molecule can be determined. This allows for the estimation of the overall number of fully intact template molecules in a given sample.

The same methodology is applicable to higher multiplexing grade PCR reactions. Up to 5 channels can be used for integrity/linkage analyses, and concentrations of all groups of template molecules can be obtained using the QIAcuity Software Suite (refer to Appendix for more details).

The percentage of intact/linked DNA molecules, as well as the associated integrity error (refer to **QIAcuity User Manual**), is calculated using the following formula and included in the multiple occupancy CSV file:

$$\% \text{ intact} = \frac{[\text{intact/linked template molecules}]}{\sum[\text{non-intact/unlinked template molecules}^{\#}] + [\text{intact/linked template molecules}]} \times 100$$

[#] For the 3-plex scenario described above, “non-intact/unlinked template molecules” are the sum of the concentrations of fractions A and C (illustrated in Figure 2).

The integrity algorithm used to obtain the concentrations for all types/groups of template molecules and the count of the intact/linked vs. non-intact/unlinked template molecules was extensively tested. Using Monte-Carlo simulation, the reliability of the algorithm was challenged, and the boundary areas were identified. The algorithm produced balanced estimates within a total λ (= sum of all λ for all groups) range of 0.02 and 5 (refer to Appendix section “Robust integrity determination over a broad input range”). ▷

There are alternative methods for calculating the number of intact/linked DNA molecules within a sample (Regan et al., 2015; Furuta-Hanawa et al., 2019; Prantner & Maar, 2023). The widely used method only considers events originating from purely intact/linked DNA molecules without considering that non-intact/unlinked molecules, which were never intact/linked at any point, such as incomplete DNA syntheses or DNA molecules resulting from error-prone packaging of AAV particles, can also be analytes of interest.

$$\text{Integrity \%} = \frac{[\text{linked}]}{([\text{Target 1}] + [\text{Target 2}])/2} \times 100$$

Note: This formula (Regan et al., 2015; Prantner & Maar, 2023) has not been implemented within the QIAcuity Software Suite version 2.5.

Experimental design and setup

Detailed information on experimental setup and materials used for the experiments is provided in the "Supplementary Protocols" section in the Appendix.

Results

DNA integrity determination enables the monitoring of template quality

Determination of integrity is a critical quality attribute (CQA) for AAV analytics (Tereshko et al., 2023). Using the QIAcuity dPCR system and software, viral genome templates of different fragmentation states can be easily analyzed without further processing or dilutions.

An AAV transfer plasmid containing a CMV promoter (CMVp), GFP and WPRE was analyzed for quantity and integrity. Subsequently, different concentrations of a second plasmid containing only the CMVp were added (Figure 3A). This introduced unlinked CMVp molecules into the reactions, progressively shifting the integrity value towards non-intact/unlinked. Analyses were conducted using both QX200™ ddPCR™ and QIAcuity dPCR systems. Integrity calculation using QIAcuity Software Suite version 2.5 yielded integrity scores consistent with the expected values. However, values obtained from ddPCR diverged significantly, attributed to differences in the calculation methods (Figure 3B, C). Please refer to "Mathematical background and formulas" for detailed information on the QIAcuity dPCR calculations.

Titer determination using multiple assays gives additional insights into genome integrity

Besides the requirement to accurately and reproducibly quantify viral vector genome titers, it is essential to determine the intactness of the viral vector genomes for a safe, stable and effective therapy. Adeno-associated virus (AAV) particles are not always perfectly packaged due to several factors inherent to the biology of AAVs and the processes involved in their production. Some common issues with AAV particle packaging include incomplete packaging, overpackaging as well as genomic rearrangements (Asokan et al., 2012, Wang et al., 2020, Gao et al., 2005).

Digital PCR enables the analysis of the viral vector's physical titer and genome integrity in a single experiment without the need for additional data collection using other methods.

An AAV2 reference standard was processed and quantified in a 5-plex dPCR reaction (Figure 4). Despite the comparable quantification observed across all five non-ITR targets, the integrity of the AAV genome did not attain 100%. An overall integrity level of 36% was achieved when quantifying all five targets. However, when restricting the calculation to only the two outermost targets (CMVe and hGHpA analyzed as 2-plex), integrity rises to 46%, as existing DNA fragments between the two outermost targets are missed in the integrity calculation. In summary, covering a larger sequence range and using higher multiplexing grade reactions within the analysis results in a more accurate and precise the integrity calculation, especially when the expected integrity value is lower than 90% (Figure 3, Figure 4).

Figure 3. Template integrity can be accurately and precisely analyzed using QIAcuity dPCR. A An AAV transfer plasmid containing an upstream and downstream ITR, CMV promoter (CMVp), GFP and WPRE as targets was linearized using *EcoRI* (between CMVp and GFP) and analyzed by digital PCR. The targets were amplified using the QIAcuity CGT dPCR Assays. The integrity of the linearized plasmid was analyzed on a QIAcuity dPCR System using the QIAcuity Software Suite version 2.5 and on a QX200 ddPCR System using the linkage value provided by the QX Manager Software 2.1. and the calculation described in "Mathematical background and formulas" and in Figure 5. The targets CMVp (FAM), GFP (Cy5) and WPRE (HEX) were amplified in technical triplicates in a 3-plex (dPCR) and a 2-plex (ddPCR) reaction (CMVp + WPRE), respectively. The linearized plasmid without spike-in was quantified, and the integrity was determined. A second plasmid containing only the CMVp region was stepwise added to the PCR reaction. 250 copies/µL (1x), 500 copies/µL (2x), 750 copies/µL (3x) and 1000 copies/µL (4x) were added. Expected integrity values (%) are indicated. **B** dPCR and ddPCR quantification of the CMVp-GFP-WPRE plasmid with and without CMVp spike-in is shown. dPCR was performed on 8.5K 96-well Nanoplates using the QIAcuity Probe PCR Kit. ddPCR was performed according to the manufacturer's recommendations. **C** Normalized deviations from expected integrity scores are indicated for both dPCR systems.

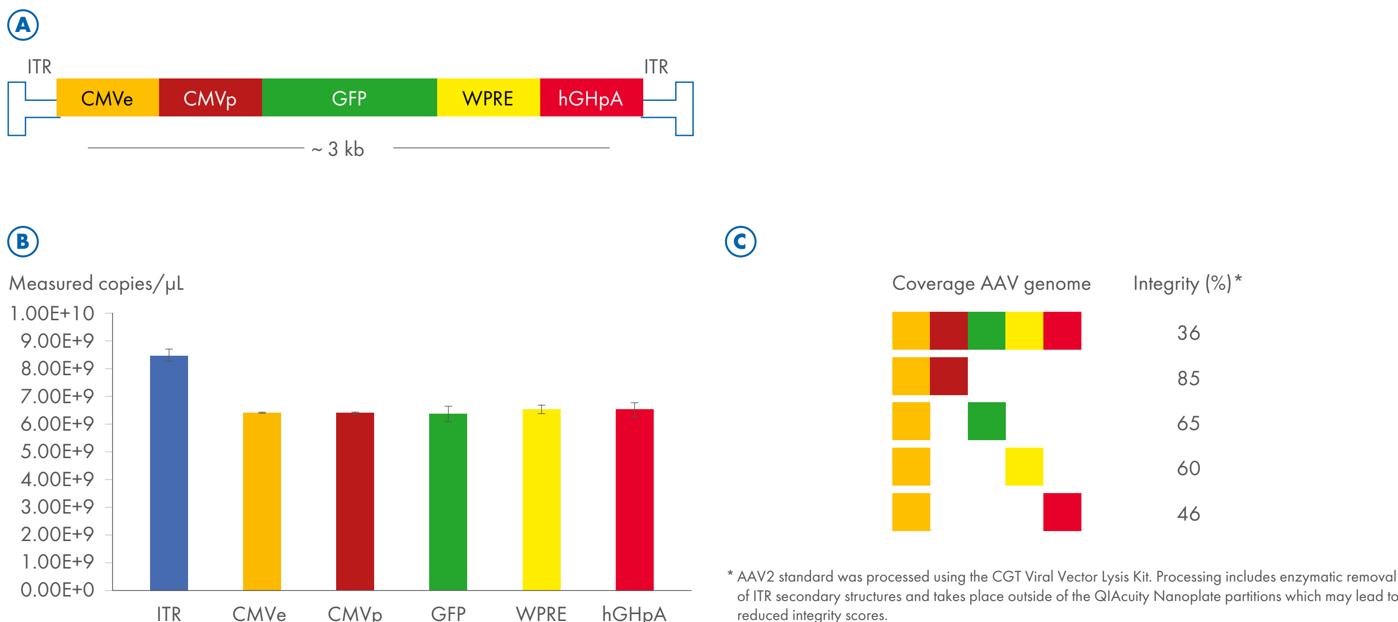


Figure 4. Target quantification does not necessarily reflect the status of genome integrity. A The AAV2 reference standard material (supplier P) contains ITRs upstream and downstream of the 3 kb genome. The gene of interest, GFP (FAM), is under the control of the CMV enhancer (Atto550) and CMV promoter (Cy5). WPRE (HEX) and hGHpA (TexasRed) are present as additional regulatory sequences. **B** The standard was processed using the CGT Viral Vector Lysis Kit and quantified on a QIAcuity dPCR System using 8.5K Nanoplates and QIAcuity CGT dPCR Assays. Concentrations were measured in technical triplicates. **C** AAV2 genome integrity was analyzed using the QIAcuity Software Suite version 2.5. Integrity was calculated for the 5-plex reactions, including all non-ITR assays used, as well as pairwise combinations throughout the genome. Integrity values (%) are indicated for all analyzed combinations.

In general, the question arises as to how many targets need to be examined on a template to obtain reliable information regarding integrity. The QIAcuity system can analyze five targets simultaneously in one reaction, whereas the QX200 ddPCR System is limited to a 2-plex reaction.

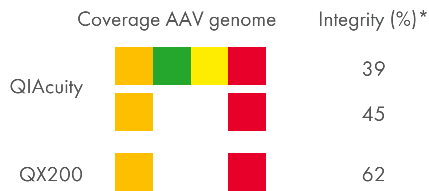
As depicted in Figure 5, the integrity of an AAV2 sample was determined to be 39% in a 4-plex reaction. When considering only the outermost targets in the analysis, the integrity increases to 45%. This can be attributed to the intermediate targets (GFP and WPRE) not being included in the calculation. A similar observation is made in the ddPCR calculation, where an integrity of 62% was computed. The difference between this value and the value obtained using dPCR, in turn, is attributable to the manner of calculation (Figure 5).

Genome integrity determination benefits from higher multiplexing capabilities, especially in the case of fragmented templates. For nearly intact samples such as plasmids, the difference between lower and higher multiplexing grade analyses is less pronounced (see Appendix Figure 2 for additional information).

Additional information on assessing each parameter in the multiple occupancy CSV file from the QIAcuity Software Suite can be found in the Appendix. It provides a step-by-step demonstration of how each value is derived and evaluated, giving insights into why running integrity analyses with more than two assays seems better suited in most cases.

Viral vector genome titer and integrity determination is compatible with purified and in-process AAV samples

AAV samples are purified along the manufacturing process. As shown in Figure 4 and Figure 5, purified AAV reference samples can be processed via direct lysis and analyzed using dPCR. Unpurified samples such as in-process AAVs carry over a complex matrix that can potentially interfere with the analysis. However, assessing the quality of AAV particles before extensive purification may be beneficial. This allows for the identification of manufacturing challenges and the need for optimization of the process at an earlier stage.



Conversion of QX200 linkage values to integrity %:

$$\text{Integrity \%} = \frac{[\text{linked}]}{([\text{Target 1}] + [\text{Target 2}])/2} \times 100$$

Regan et al., 2015; Prantner & Maar, 2023

* AAV2 standard was processed using the CGT Viral Vector Lysis Kit. Processing includes enzymatic removal of ITR secondary structures and takes place outside of the QIAcuity Nanoplate partitions which may lead to reduced integrity scores.

Figure 5. Genome integrity determination benefits from higher multiplexing capabilities. The AAV2 reference standard material (supplier P) contains ITRs upstream and downstream of the 3 kb genome. The gene of interest, GFP (FAM), is under the control of the CMV enhancer (Cy5) and CMV promoter (data not shown). WPRE (HEX) and hGHpA (TexasRed) are present as additional regulatory sequences. The standard was processed using the CGT Viral Vector Lysis Kit and quantified in 4-plex reactions on a QIAcuity dPCR System using 8.5K Nanoplates and QIAcuity CGT dPCR Assays. Concentrations were measured in technical triplicates. The processed samples were additionally analyzed via ddPCR using the QX200 System (CMVe (HEX) + hGHpA (FAM) in a 2-plex reaction) following manufacturers' settings and recommendations. AAV2 genome integrity was analyzed in the dPCR setup using the QIAcuity Software Suite version 2.5. The integrity values were calculated for a 4-plex and 2-plex reaction via dPCR and a 2-plex reaction via QX200 ddPCR. Genome integrity for the ddPCR run was analyzed using the Bio-Rad QX Manager Software version 2.1.

An in-process AAV sample, solely extracted from the producer cell line, was lysed, and the concentration and integrity across the entire length covered by the assays of interest were determined in a 3-plex dPCR reaction.

Additionally, the lysate was serially diluted. As depicted in Figure 6, quantification was linear over the four orders of magnitude, and the calculated integrity did not significantly differ across dilutions.

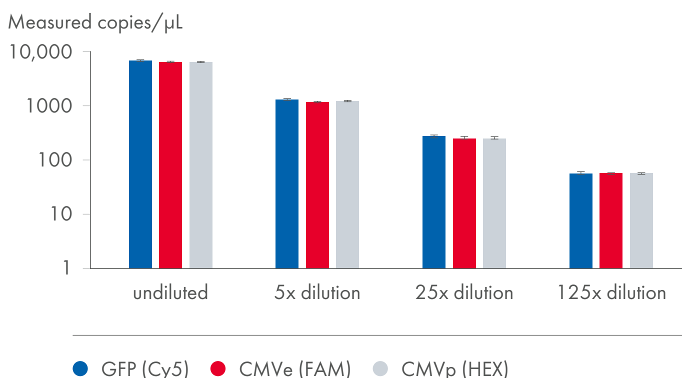
As long as the complex matrix does not interfere with dPCR amplification, the determination of integrity is not compromised. The integrity analysis is robust over a broad dynamic range (total λ of 0.02 and 5). This is particularly important for samples with unknown concentrations.

Additional information can be found in the Appendix Figure 3.

The QIAcuity software integrity feature provides insights into AAV lysate stability

The storage conditions of different samples can influence the DNA integrity, which becomes particularly important when an analysis needs to be paused or when the reproducibility of an analysis is of interest without starting the whole procedure from scratch.

AAV lysates were generated using the CGT Viral Vector Lysis Kit and either frozen at −20°C for one week or ▷



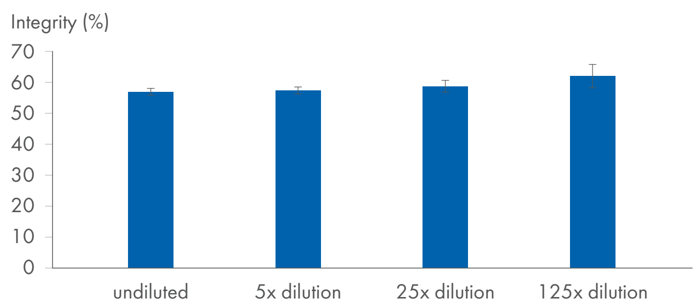


Figure 6. QIAcuity dPCR integrity analyses are compatible with unpurified AAV in-process samples. An AAV2 in-process sample was processed and serially diluted directly after the harvest step using the CGT Viral Vector Lysis Kit and quantified in 3-plex reactions on a QIAcuity dPCR System using 8.5K Nanoplates and QIAcuity CGT dPCR Assays. Concentrations and integrity values were determined in 4 technical replicates. Genome integrity was analyzed using the QIAcuity Software Suite version 2.5.

stored at 4°C for the same duration. Afterwards, titer and integrity values were analyzed via digital PCR. The QIAcuity CGT dPCR Assays were used for the amplification of 3 AAV targets.

For comparison, integrity was assessed before storage.

It can be noted that storage at 4°C can significantly contribute to preserving the integrity of the AAV lysates over at least one week (Table 1).

Since storage conditions are critical, a QIAcuity integrity analysis can help identify the optimal storage conditions.

Not only storage but also various treatments such as chemical, enzymatic and physical processing can negatively affect DNA integrity. Additional information can be found in the Appendix.

Table 1. Integrity values of fresh lysates and lysates stored under different conditions

Samples	Integrity (%) Lysate fresh	Integrity (%) Lysate 1x freeze-thaw	Integrity (%) Lysate storage at 4°C (1 week)
AAV2 (Supplier P)	65	21	not measured
AAV2 in-process sample	60	not measured	59

Conclusion

The QIAcuity dPCR System, complemented by the QIAcuity Software Suite version 2.5 and higher, offers a powerful solution for determining DNA integrity and stability. The ability to analyze up to 5 targets simultaneously enables accurate and precise integrity assessment. Additionally, integrity can be calculated over a broad dynamic range, which allows to skip extensive dilutions to obtain very low λ values.

The importance of determining genome integrity becomes particularly evident in the analysis of viral vectors such as AAVs, which are known for their susceptibility to packaging errors.

Moreover, dPCR is a valuable tool for assessing DNA stability, providing valuable insights into storage and processing impacts.

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See the detailed description and discussion of the integrity calculation, supplementary data and protocols presented in this application note. Download the appendix:
www.qiagen.com/qiacuitysoftware-integrityfeature-appendix

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