Analysis of DNA integrity and stability using digital PCR

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Ensuring genome integrity of recombinant AAV vectors using digital PCR

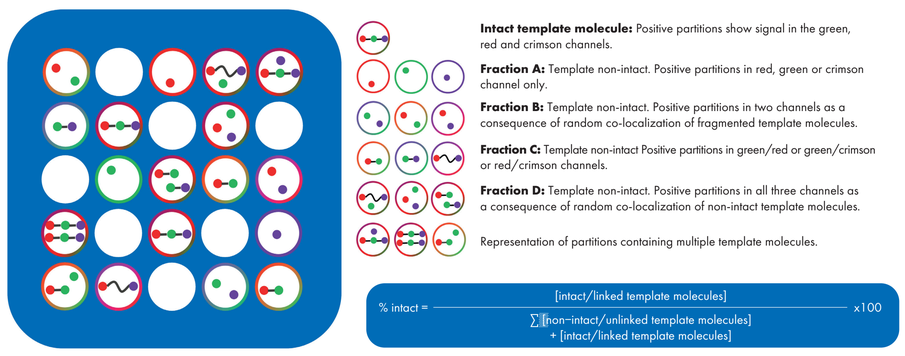
Development of safe and effective cell and gene therapies is key to potentially treating a wide spectrum of diseases. Viral vectors have become powerful delivery vehicles for gene therapies. Adeno-associated virus (AAV) vectors have turned into primary modalities for efficient gene therapy applications due to their lack of pathogenicity and persistent transgene expression.

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. Errors made during the replication and packaging process of recombinant AAVs can lead to heterogenous viral vector populations with direct impact on their efficacy and safety. Current purification workflows can efficiently separate empty from full capsids. However, removal of capsids carrying partial or truncated genomes, as well as capsids packaged with host cell or plasmid DNA, are difficult to separate and can be present in the viral vector product after purification. Traditionally, genome integrity has been determined via agarose gel electrophoresis and Southern blot. Next-generation sequencing approaches have also been used to characterize the capsid content. Nevertheless, high resolution and the accuracy needed for integrity determination, as well as repeatability and high-throughput capabilities, remain unmet needs. Of late, multiplex digital PCR (dPCR) has been adopted for genome integrity analyses. Digital PCR enables absolute quantification with unprecedented precision and a higher tolerance towards inhibitors without the need for standards.

Here, we propose 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 present groups of template molecules within a sample individually, it could also be used for other applications, such as determination of integrity and stability of DNA and plasmids after certain processing procedures (e.g., restriction enzyme efficiency) or storage. We show that up to five targets can be analyzed simultaneously, increasing precision and reproducibility of the analyses.

Analysis of physically linked and unlinked target molecules using QIAcuity digital PCR technology

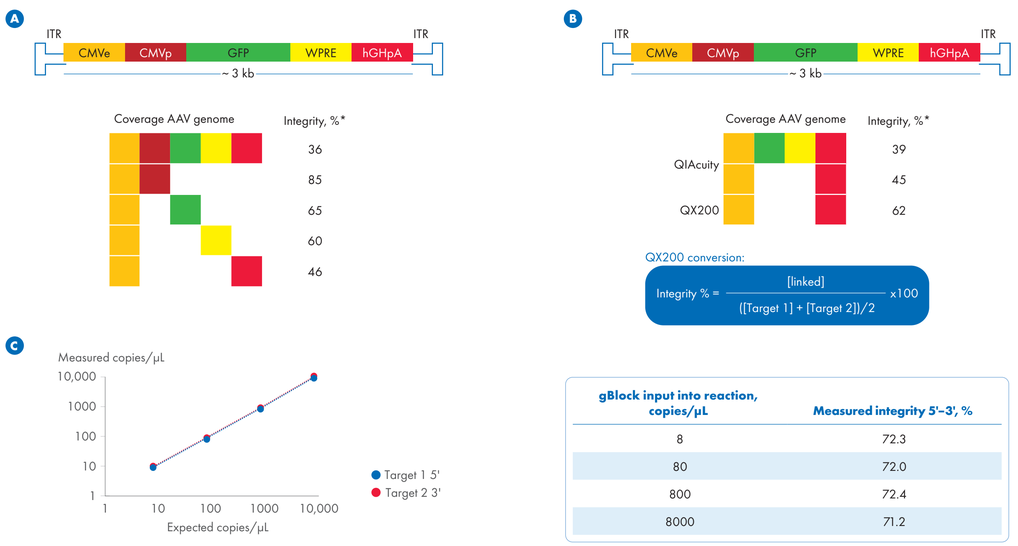
The QIAcuity dPCR System with QIAcuity Software Suite version 2.5 or higher offers the functionality to calculate the percentage of intact molecules within a sample containing a certain proportion of non-intact target molecules. Up to 5 targets can be analyzed at once on a template of interest in a single dPCR reaction.



Mathematical approximations for estimation of dPCR template integrity. Schematic representation of a QIAcuity nanoplate. Partitions containing one or more intact or non-intact template molecules are shown. The template contains 3 targets addressed in a triplex reaction within the green, red and crimson channels. Not all possible fragmentation and distribution scenarios are shown. % intact represents the calculated integrity of the analyte of interest.

Genome integrity determination benefits from higher multiplex capabilities

The determination of viral vector genome integrity is essential for safe, stable and effective therapies. Multiplex digital PCR has been adopted for genome integrity analyses. Up to 5 targets can be accurately and precisely analyzed in one reaction without extensive dilutions of the sample between a total λ of 0.02 and 5.



gBlock input into reaction, copies/µL	Measured integrity 5'–3', %
8	72.3
80	72.0
800	72.4
8000	71.2

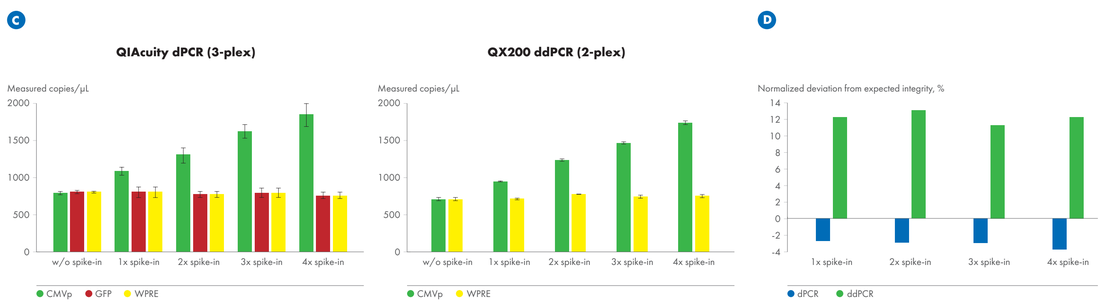
Accurate and precise integrity determination over a broad dynamic range. A The AAV2 reference standard (Supplier P) contains ITRs upstream and downstream of the 3 kb genome. The gene of interest GFP (FAM) is under the control of the CMV enhancer (Atto550) and CMV promoter (Cy5). WPRE (HEX) and hGHpA (Texas Red®) are present as additional regulatory sequences. The standard was processed using the CGT Viral Vector Lysis Kit and quantified in a 5-plex reaction on a QIAcuity dPCR System using 8.5k nanoplates and QIAcuity CGT dPCR Assays. Concentrations were measured in technical triplicates. Genome integrity was analyzed using the QIAcuity Software Suite version 2.5. Integrity was calculated for the 5-plex reaction including all non-ITR assays used, as well as pairwise combinations throughout the genome. Integrity scores (%) are indicated for all analyzed combinations. **B** The same processed AAV2 sample was additionally analyzed in a 4-plex and 2-plex reaction via dPCR and in 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. The linkage value was converted into an integrity score (%) by using the indicated formula. **C** QIAcuity dPCR quantification of a serial dilution of a gBlock containing 2 targets of interest is shown over the range 8 copies/µL up to 8000 copies/µL. Integrity values (%) are indicated for all dilution steps.

* 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.

Determination of DNA integrity enables monitoring of template quality

Determination of integrity is an important quality attribute. Higher multiplexing grade reactions enable accurate and precise integrity calculations. Templates of different fragmentation states can be easily analyzed without further processing and dilutions.

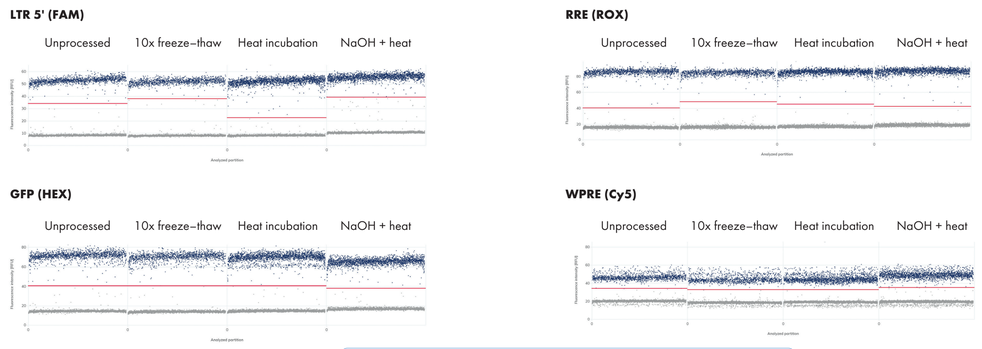
Accurate and precise integrity determination using digital PCR. A An AAV transfer plasmid containing an upstream and downstream ITR, CMV promoter, GFP and WPRE as targets was linearized using *EcoR*I (between CMVp and GFP) and analyzed by digital PCR. The targets were amplified using QIAcuity CGT dPCR Assays. **B** 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 QX Manager Software version 2.1. The targets CMVp (FAM™), GFP (Cy5®) and WPRE (HEX™) were amplified in a 3-plex (dPCR) and in a 2-plex (ddPCR) reaction in technical triplicates. The linearized plasmid without spike-in was quantified and the integrity 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.



dPCR and ddPCR integrity values do not necessarily match. C dPCR and ddPCR quantification of analyzed targets is shown. **D** Deviations from expected integrity scores are indicated for both PCR systems.

The QIAcuity Software integrity feature provides insights into DNA stability

Storage of nucleic acids or various treatments (e.g., chemical, enzymatic, physical) can affect intactness. Digital PCR enables the analysis of the integrity of DNA templates. The quantification may remain unchanged, but integrity can already be affected, due to storage or processing.



WHO standard processing	Integrity LTR-RRE-GFP-WPRE, %
Unprocessed	72
10x freeze–thaw cycles	58
Heat incubation	57
NaOH + heat incubation	54

Storage and processing can impact DNA integrity. The 1st international reference reagent for lentiviral vector integration site analysis (WHO, 18/144) was analyzed using dPCR in a 4-plex reaction. The 5' LTR (FAM), RRE (ROX™), GFP (HEX) and WPRE (Cy5) targets were analyzed on 8.5k nanoplates using the QIAcuity OneStep Advanced Probe Kit. 1D scatterplots for all analyzed targets are shown. The lyophilized reference reagent was reconstituted as recommended by the manufacturer. The reference reagent was digested with *Pvu*I before quantification. The resolved gDNA was either directly used for quantification in the dPCR (unprocessed) or further processed by either 10 freeze–thaw cycles, heat treatment for 10 min at 95°C, or treatment with 100 mM NaOH and subsequent heat treatment for 10 min at 95°C. Genome integrity was analyzed using the QIAcuity Software Suite version 2.5.

Conclusions

- The QIAcuity dPCR System with the QIAcuity Software Suite version 2.5 and higher enables determination of genome integrity. The feature supports the analysis of up to 5 targets in 5 channels over a broad dynamic range.
- The packaging of AAV particles is error-prone. The integrity status of AAV particles (purified or in-process samples) can be determined using digital PCR. The more targets are being covered in a multiplex reaction the better the calculation of integrity.
- The integrity analysis of a higher multiplexing grade reaction cannot necessarily be replaced with an analysis using multiple 2-plex combinations.
- The integrity calculation is robust over a broad dynamic range. Extensive dilutions to obtain very small λ values are not needed.
- AAV genome stability can be assessed via dPCR leading to important insights on the impact of storage and processing procedures.

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