Application note

Comparison of 2 emulsion-based digital PCR systems: ddPCR from BioRad versus dPCR from Stilla, for reliable determination of AAV genome titre

About Quality Assistance

Quality Assistance is a leading European Contract Research Organisation providing the pharmaceutical industry with all the analytical services required by EMA and FDA regulations for the development and marketing of innovative human medicinal products.

Quality Assistance holds a unique place on the market with all of its laboratories on one site in Belgium, 260 highly-qualified professionals and more than 40 years' expertise at the forefront of analytical sciences.

The company assists its clients from candidate selection, through non-clinical and clinical studies, to marketing authorisation, using our state-of-the-art, product-dedicated expertise in analytical sciences. Quality Assistance designs customised solutions, defines analytical protocols, develops and validates specific new analytical methods and performs characterisation, stability, pharmacokinetics, biomarker and immunogenicity studies as well as batch release testing, in order to evaluate the Quality, Safety and Efficacy of the given drugs.

About OXB

OXB is a quality and innovation-led CDMO in cell and gene therapy with over 25 years of experience in manufacturing. We offer end-to-end capabilities, from plasmid design and optimisation to clinical and commercial GMP manufacturing, accompanied by robust control systems, analytical methods and deep regulatory knowledge.

We have an expertise, among others, across lentivirus, AAV, and adenoviral vectors and have developed our LentiVector® and inAAVate™ platforms.

Introduction

Adeno-associated viruses (AAV) have emerged as a leading vector for gene delivery for treating various diseases due to its safety profile and efficient transduction of numerous target tissues. AAV, like other viral vectors, are complex molecules leading to challenges in their characterisation. OXB is advancing its AAV process development and manufacturing platforms and is becoming an industry leader in AAV manufacturing and control of several serotypes (currently AAV2/5/6/8 and 9). For its part, as a leading actor in cutting-edge analytical services dedicated to innovative medicinal products, Quality Assistance is committed to develop a reliable titration method to assess the safety and efficacy of AAV-based gene therapies from clinical or preclinical stages to drug product QC.

Whether for monitoring the entire manufacturing process (enrichment of the full / empty ratio of AAV particles) or ensuring accurate dosing of the drug product, an exact viral titration of the vector genome copies is critical for rAAVs-based products. Historically, qPCR has been a fast, accurate, and inexpensive method for quantifying viral vectors. Recently, digital PCR has gained popularity, particularly given that it allows for absolute quantification without the need for a reference standard (thus avoiding standard curve bias or the lack of existing standards) and because it offers better performance in terms of accuracy and precision. It is now considered an interesting alternative to the classical qPCR method.

This application note describes how different digital PCR equipment – here two major actors of the emulsion-based digital PCR, the BioRad QX200 and Stilla Naica platforms – can provide a reliable AAV genome titre while evaluating the difference they encompass.

BioRad System

The BioRad QX series is one of the most popular droplet-based digital PCR platforms on the market (Tan *et al.* 2022). The QX200 system stands out by using distinct instruments for sample partitioning, its amplification by PCR, and finally the fluorescence reading of the droplets.

Figure 1: BioRad QX 200 system

The QX200 Droplet Generator is a vacuum-assisted device that ensures the partitioning of the reaction volume using microfluidic cartridges.

After amplification, the droplet fluorescence is read through the QX200 Droplet Reader. This two-colour fluorescence detection system is a flow cytometer-like device allowing for the serial analysis of each single droplet for the positive and negative droplet count and further interpretation through Poisson statistics.

Stilla System

The Naica® system (by Stilla) is a next-generation digital PCR platform; an easy-to-use dPCR platform that harnesses cutting-edge microfluidic technology to integrate the dPCR workflow onto a single consumable chip. The technology, known as Crystal Digital PCR™, is composed of two pieces of equipment; the Geode partitions samples into a large array of thousands of individual droplet crystals (each with its own reaction compartment) before amplifying nucleic acid molecules in each droplet crystal; after amplification, the droplet fluorescence is read through the Prism3. This three-colour fluorescence detection system detects and outputs an image of all droplets in a sample. Finally, Naica Analysis Pro software can be used to quantify the expression of all targets based on positive and negative droplet counts and Poisson statistics.

Figure 2: Stilla Naica system

Comparison of the two digital PCR Systems

Both BioRad and Stilla digital PCR solutions are based on the same principle: sample partitioning in droplets formed by emulsion, followed by PCR amplification and end-point fluorescence readout. Nevertheless, they display some differences such as in the workflow, assay format, etc. (Table 1.)

Table 1 : Comparison of 2 digital PCR systems

Parameter	BioRad	Stilla
List of equipment	QX200 Droplet Generator + QX200 Droplet Reader	Geode (fractioning & PCR) + Prism3 (read out)
Number of droplets	About 20 000 droplet per well	With Ruby chip: up to 17000 droplet per chamber
Throughput	96 samples / run	48 samples / run
Full plate run duration	4 hours	< 3 hours
Detection channels	2*	3*

* *BioRad and Stilla offer other readout instruments providing up to 6 or 7 channels, respectively.*

Material and method

AAV manufacturing process and sample generation

OXB Lyon produced different AAV serotypes (from HEK293 transfected cells) where samples from different manufacturing steps were taken: at the harvest, clarification, capture and polishing steps (Figure 3).

Samples from AAV2 serotype were tested on both digital PCR technologies to assess the potential impacts of the matrix (such as DNA impurities, different buffers, etc.) that evolves through the process. For example, the level of DNA impurity was indicated in Figure 3 at each process step.

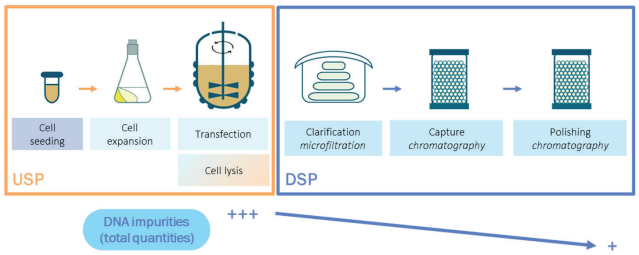


Figure 3: AAV Manufacturing process - USP (Upstream) and DSP (Downstream) steps

DNA extraction protocols

Following scientific publications, OXB Lyon has developed its own DNA extraction protocol and primer/probe selection[1], appropriate for all purification steps and serotypes, while *Quality Assistance* (QA) initially used the BioRad extraction protocol[2].

Given that the objective was to compare the Stilla and BioRad platforms, QA performed an optimisation on the sample preparation recommended by BioRad to harmonise the extraction protocols used at both sites.

In brief, an additional step of 10x predilution in poly(A)+ was added before DNase treatment, as well as a dilution of the sample before thermally-induced capsid lysis. Regarding the preparation for ddPCR, the restriction enzyme digestion step was omitted since no clear difference in titration was observed between samples treated with MspI, SmaI or those which were not treated (data not shown). These observations were already reported by Furuta-Hanawa *et al.* who demonstrated that ddPCR titres were not affected by conformational changes of the AAV genome following RE treatment compared to qPCR titres[3].

The resulting extraction protocol used at OXB or QA involves several common pre-PCR steps that are classically reported for AAV genome titration (Figure 4.):

- The sample is prediluted, reducing the impurity concentration and improving the efficiency of the next DNAse digestion step
- The non-encapsidated DNA is depleted through DNase I digestion
- A second dilution in buffer is performed before the thermally-induced capsid lysis step (10 min at 95°C) allowing to release viral DNA
- The extracted DNA is finally serially diluted to the detection range of the dPCR equipment and processed for partitioning, PCR amplification and fluorescence reading.

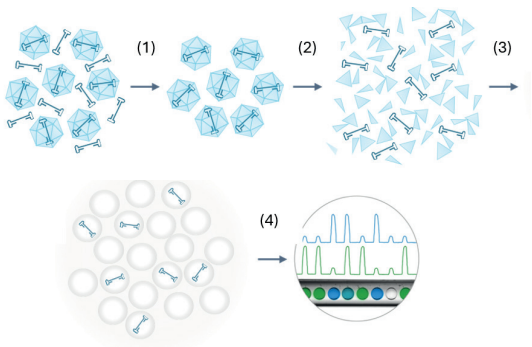
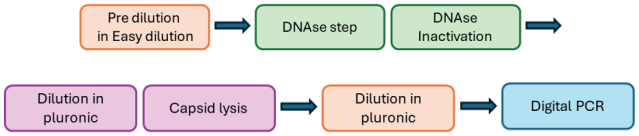


Figure 4: Schematic view of the sample preparation for AAV genome titration, involving [1] non-encapsidated DNA depletion, [2] capsid lysis, [3] Viral genome partitioning and PCR amplification, and [4] droplet fluorescence reading

As described is Figure 5 (A & B), the OXB and QA extraction protocols differ only by:

- The buffer used for dilutions: Easy dilution Buffer for the initial predilution and a Pluronic-based buffer for the subsequent dilutions for the OXB extraction protocol, versus a polyA/ Pluronic-based ("Poly(A)+") buffer used at QA for all the dilution steps
- The absence of a dedicated DNAse inactivation step in QA protocol, considering the DNase inactivation through the thermally induced capsid lysis as sufficient, according to the literature. [4,5,6]

(A) OXB extraction protocol:



(B) QA extraction protocol:

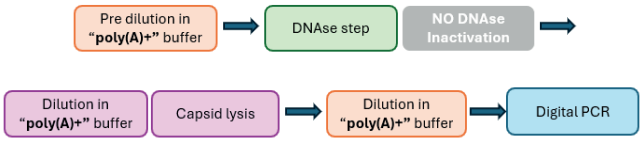


Figure 5: DNA extraction protocols prior to digital PCR (A : OXB & B: QA)

Strategy for inter-site cross test

An inter-site testing was performed in order to compare the two dPCR systems, while assessing the potential variability coming from the two extraction protocols. AAV2 samples at different purification steps (1 harvest, 1 clarified product, 1 capture product, 1 polishing product and 1 commercial purified reference) were selected and extracted simultaneously on both sites, each site using its own extraction protocol. Then, the DNA aliquots were shipped to the other site for PCR analysis.

As soon as received, each site tested "OXB extractions" and "QA extractions" within the same run, by diluting them into the appropriate buffer, and by using its own ITR primer/probe sequences and dPCR systems. The ITR target was chosen since it is applicable to all AAV serotypes and was available on both sites. All extractions were performed as a single determination and two dilutions of each extraction were submitted to a dPCR quantification.

This testing allowed comparison of the performance at different levels:

- For each extraction protocol, comparison of the dPCR systems (OXB/Stilla vs. QA/BioRad, involving dPCR equipment and respective PCR assays)
- For each dPCR system, comparison of the extraction protocols.

The global testing and analysis strategy is summarised in Figure 6.

The titre results are expressed as vg/mL for each condition.

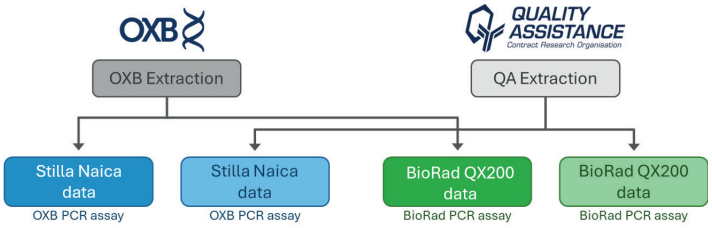


Figure 6: Design of the inter-site testing

Results & Discussion

1D-plot view : separability score and "rain"

The raw data obtained with a dPCR system can be represented through amplitude plots, also known as "1D-plot" if data are explored through only one detection channel. These plots allow to differentiate clusters of positive (containing at least one copy of the target) and negative droplets, depending on the fluorescence of the droplets and the position of a threshold allowing to discriminate both populations. When the amplification within the droplet is partial, a "rain" phenomenon can be observed revealing a heterogeneity in amplification results.

Figure 7 below represents the 1D-plots obtained on both the BioRad and Stilla systems, for two products at different purification stages: a clarified product and a polishing product.

The recorded amplitude of fluorescence appears higher with the Stilla platform (keeping in mind that amplitudes, reported as RFU, rely on the sensor of the instrument and on the PCR assay, including the nature of fluorophore and the concentration of the primers and probe), but both dPCR systems show similar profiles in terms of separability and "rain" for the clarified product (Figure 7.A). Conversely, the data obtained for the polishing product (Figure 7.B) show a higher "rain" amount when extracted with the OXB protocol, independently of the dPCR system (an even slightly higher amount of rain is observed in the data obtained with the Stilla system). These results highlight the impact that the extraction protocol may have on the "rain" phenomena depending on the nature of the sample. Yet, the "rain" profile also depends on the dilution tested and the impact on quantification results is related to the total number of droplets (for both systems, only samples with at least 10 000 droplets where analysed, which corresponds to the MIQE[7] recommendation to ensure a good precision of the results).

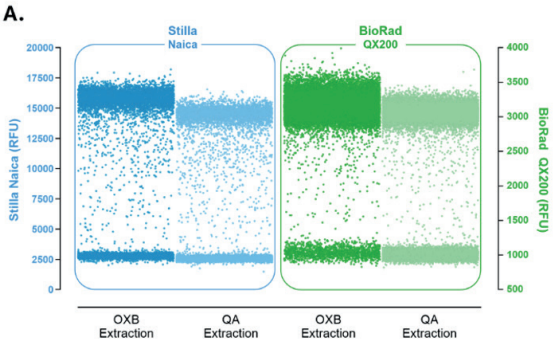


Figure 7: Amplitude-Plots showing fluorescence data obtained with the Stilla platform (blue) or the BioRad platform (green) for the sample prepared with the OXB (dark) or the QA (light) extraction method A. Clarified samples

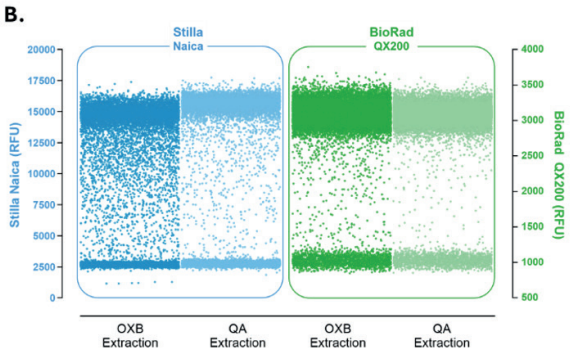


Figure 7: Amplitude-Plots showing fluorescence data obtained with the Stilla platform (blue) or the BioRad platform (green) for the sample prepared with the OXB (dark) or the QA (light) extraction method B. Polishing samples

To assess the segregation of positive and negative droplet clusters, Stilla Crystal Miner software automatically computes a separability score[4]. This score is mainly based on the distance between the positive and negative clusters and on the widths of the two clusters: the greater the distance between clusters, the higher the score; however the wider the clusters, the lower the score. To allow a cross-platform data comparison, this indicator was also manually calculated for BioRad raw data (Figure 8).

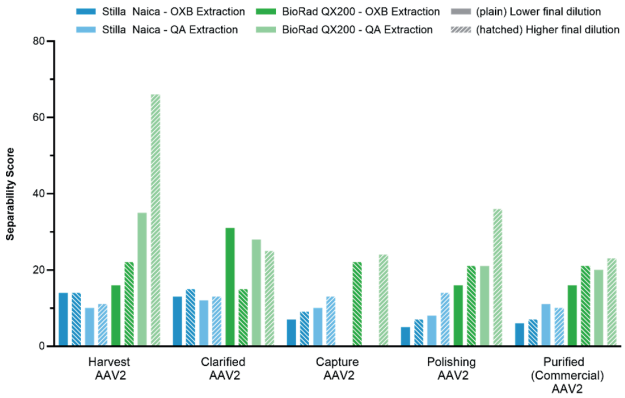


Figure 8: Comparison of the separability scores (absent data – Capture sample: 100% positive droplets, incorrected dilution tested and data unusable)

The separability score might appear to be higher if samples are analysed using the BioRad platform. However, as previously mentioned, the two platforms use different fluorescence detectors and different PCR assays. Moreover, the separability score strongly relies on the threshold value used to separate positive and negative droplets, which can lead to strong variations for dPCR data showing "rain". With non-comparable RFU values and different threshold strategies, separability scoring does not appear to be the most reliable quality metric to compare platform performances. Nevertheless, it remains a useful tool to support assay optimisation during method development on a defined platform.

Quantification of genomic titre

The titre expressed in vg/mL for each condition is represented in Figure 9.

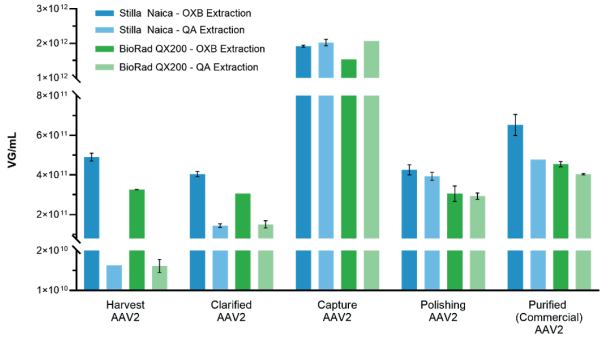


Figure 9: Genome titre quantification - analysis of the extractions and digital PCR systems impacts. Error bar corresponds to the 2 dilutions, when no error bars titer obtained with only one dilution.

When considering each sample from the same extraction independently (● vs. ● ; ● vs. ●), the two dPCR platforms performed similarly with close titre values. It must be noted that, for the sample extracted with the OXB protocol, a systematically higher titre was observed when analysed with the Stilla platform (● vs. ●) while the difference remained lower for samples extracted with the QA extraction protocol (● vs. ●), except for the polishing sample.

When comparing extraction protocols (● vs. ● ; ● vs. ●), the titres obtained from samples extracted with the QA protocol appeared to be systematically lower when samples were in the early phases of the purification process (harvest and clarified), regardless the dPCR platform used. Based on other measurements using the same extraction protocol, it appears that the titres shown here for the harvest sample extracted with the QA protocol are an order of magnitude below the expected value and should therefore be considered as outliers. Nevertheless, this difference related to unpurified samples has been confirmed with further analysis (data not shown). A lower titre does not necessarily mean a less efficient extraction protocol since a DNA depletion step is included and is particularly important in unpurified samples with expected high DNA impurities. A better understanding of this difference in titration between both protocols will require additional investigations.

On the other hand, a reliable titration of viral genomes is important essentially for purified samples since they are the final product to be administered to patients. The results show a very comparable titre for more purified products, whatever the extraction protocol used, thus highlighting that even if the "rain" profile differs (e.g. for polishing sample), it has a limited impact on the titres.

At this stage and considering the number of sources of variability between the two sites, the results for products at advanced stage of purification on the two instruments are very encouraging and show a good robustness.

Conclusion

In summary, this application note reports on the comparison of two different emulsion-based digital PCR systems: the BioRad QX200 and Stilla Naica platforms.

Both systems provided a reliable and comparable AAV genome titre, showing consistent quantification for purified samples (from capture chromatography to polishing), one of the main parameters necessary for product release for clinical trial use.

However, this comparison identified some differences in titres observed on less purified samples (harvest and clarified matrices) possibly related to the protocols used for viral DNA extraction. Additional comparison assays should be performed to further investigate the potential impact of extraction parameters on genome titres.

References

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