



# HIGH THROUGHPUT AAV VIRAL TITERING USING NANOPLATE-BASED DIGITAL PCR

Kelly Geosits, Associate Scientist, Viral Vector R&D  
Timothy Dougherty MS, Scientist, Analytical Development  
Arielle Kilner MS, Sr. Scientist, Analytical Development  
Khan Umaer, PhD, Research Scientist, Viral Vector R&D  
Brian Tomkowicz PhD, Sr. Director, Viral Vector R&D  
David Colter PhD, Sr. Director, Analytical Development  
**Center for Breakthrough Medicines**

TECHNICAL APPLICATION  
NOTE

## SUMMARY

Determining the physical titer of viral vectors has typically been accomplished using digital droplet PCR (ddPCR). Droplet-based methods, however, are challenged by matrix effects and long analysis times (up to 7 hours). With advanced nanoplate-based digital PCR technology (dPCR) from QIAGEN, it is possible to achieve accurate titers in 2-hour run times using simple workflows, even for challenging samples. The multiplexing capabilities of the QIAcuity Digital PCR System allow quantification of more targets using fewer runs, accelerating time to market. Digital PCR can also be performed on a more diverse set of sample matrices, enabling titering at more stages of AAV production to ensure product quality, safety, and efficacy.

## INTRODUCTION

Viral vectors are effective vehicles for delivering genetic material into cells and widely used in gene therapies, gene-modified cell therapies and certain vaccines. Vectors based on adeno-associated viruses (AAVs) are preferred for gene therapies due to their low immunogenicity, non-pathogenicity and ability to readily enter multiple cell types.<sup>1</sup>

AAVs used in drug products must be fully characterized during process development and manufacturing to ensure product quality, safety and efficacy. Physical viral titer - the concentration of viral particles containing viral genomes - is an important critical quality attribute (CQA) to enable informed decision-making in trial design and regulatory data submissions.

Physical titer can be calculated using polymerase chain reaction (PCR)-based methods such as quantitative PCR (qPCR) or digital droplet PCR (ddPCR). Historically, ddPCR

is the preferred method for determining AAV titers over qPCR because it is more precise and repeatable and does not require the use of a standard reference curve. However, droplet-based methods can be challenged by matrix effects and long analysis times. It can be difficult to implement ddPCR methods across all phases of AAV development and manufacturing, particularly early ones when resources are scarce. With limited data, companies run the risk of developing processes that fail during clinical and commercial scale, resulting in time and resources to correct.

The microfluidic nanoplate technology leveraged in the QIAcuity Digital PCR System from QIAGEN overcomes the challenges with inconsistent droplet generation, complex workflows, and slow droplet readouts.



**Figure 1. QIAcuity Digital PCR System, the three available QIAGEN Nanoplates (24-well with 8,500 and 26,000 partitions and 96-well with 8,500 partitions) and the special seal used with the plates.**

In this technical application note, nanoplate-based dPCR is compared to ddPCR analyses for different types of AAV samples to demonstrate that the QIAcuity is a successful platform for AAV titering. The results show that the nanoplate-based dPCR system rapidly delivers accurate titer values, providing more data across more sample types, faster, and with higher throughput to drive towards first-time right process development.

## THE SPECIFICS OF NANOPLATE-BASED dPCR

Absolute quantification is achieved by dividing a bulk qPCR-like reaction mixture into numerous partitions



enabling individual reactions and then measuring the endpoint fluorescence of each partition to determine the presence or absence of the target. Random distribution across all available partitions results in some having no copy of the target molecule, some containing one copy and some containing more than one copy.

Each partition undergoes PCR amplification to the endpoint, then the plate is imaged. Partitions with and without amplified products are individually counted and analyzed for the presence or absence of a fluorescent signal. Statistical methods (e.g., Poisson distribution) are used to calculate the absolute concentration of the target based on the number of positive and negative partitions.

By randomly distributing molecules into partitions (8,500 or 26,000 per well), dPCR minimizes the effects of competing targets and enhances the sensitivity of detection. It offers significant advantages when quantifying rare targets in complex backgrounds, analyzing low-titer samples and detecting small-fold changes. Essentially, it is able to find a metal needle in a haystack using a magnet vs. combing through by hand.

Furthermore, because of absolute quantification, dPCR does not require a standard curve or reference sample to determine the genomic copy quantity. Eliminating the reliance on standard curves reduces error, improves precision, frees up space for more samples, saves time, and ultimately saves money.

## **ADVANTAGES OF THE QIACUITY dPCR SYSTEM**

---

The QIAcuity system integrates reaction partitioning, thermal cycling and imaging into a single fully automated instrument. It is also compatible with ddPCR detection chemistries and similar workflows should those methods be needed. In addition, because all partitions in a well are read simultaneously, the time-to-result is reduced compared to ddPCR.

User-friendly software allows importation or creation of plate templates that can be saved and later edited. Sealing of the Nanoplates, meanwhile, eliminates the risk of contamination and sample aerosolization. The 96-plate system also has a wide dynamic range, with a lower limit of detection of 1.4 copies per microliter and a maximum of 14,000 copies per microliter.

With the QIAcuity 8, eight different users can have eight different plates set up, loaded, and ready to run. It is also possible to create all necessary serial dilution plates the day prior and freeze them in a -20°C freezer overnight. The 8-plate capacity allows up to 1,248 samples to be analyzed in a single workday using a 96-well nanoplate. The multiplexing capabilities of the system, meanwhile, allow quantification of up to 5 target molecules (against a non-template control) in a given assay, saving time and reagents. Two separate thermocyclers can run simultaneously and non-dependent upon the tray – the instrument will cycle whichever plate is scheduled to run next, regardless of the tray position).

## **THE QIACUITY WORKFLOW FOR AAV TITERING**

---

The nanoplate-based QIAcuity dPCR System provides a qPCR-like workflow - sample preparation includes the transfer of diluted samples and the addition of master mix, probes and primers to the 96- or 24-well nanoplates. The system then automates a fully integrated dPCR workflow—partitioning, thermocycling and imaging, delivering results in about two hours.

There are five main steps to the process ([See Figure 2](#)). **Step one** is sample preparation. Depending on their purity (whether Bulk, purified or polished AAV) and titer, AAV samples are pre-diluted with QIAGEN's pre-dilution buffer (described in Figure 2) and then capsid lysed. Lysis is performed in a thermo-cycler at 95°C for 10 minutes, then at 4°C for holding. **Step two** involves serial dilution, which is performed using the pre-dilution buffer in a 96-well plate with automatic repeater pipette



mixing 10-times with 90% of the total volume, changing pipette tips between each dilution. Automated pipetting is recommended to ensure consistency.

In **step three**, the Master Mix containing the primers and probe is prepared and loaded into a new 96-well pre-PCR plate. The samples are then added and appropriately mixed using the automatic pipettor. The plate is then centrifuged for 1 minute. It is essential at this stage that sufficient mixing be achieved without the formation of any bubbles.

**Step four** involves transfer of the mixes from the pre-PCR plate into the QIAcuity Nanoplate with tips at a 45-degree angle down the side of the wells to ensure no air-bubbles. QIAGEN's seal is then placed on the plate and rolled with a specific roller to ensure all wells and partitions are sealed. The seal is very important since it prevents cross-contamination and evaporation of samples while ensuring proper partitioning.

In **step five**, the Nanoplate is loaded into the QIAcuity and the run parameters are created including priming, thermocycling temperature and duration, probe channels, exposure and gain. The plate template can be as detailed as necessary and should indicate the well samples and non-template controls.

During the run, the partition chambers are loaded with the reaction mixture automatically. A roller then compresses the bottom seal to individually seal each partition, and individual copies of target DNA are distributed throughout the partitions. Thermocycling is then performed. The target DNA doubles with each cycle, and partitions containing the target DNA fluoresce. The nanoplate is then imaged to count the number of positive/fluorescent and negative/no-color partitions. During analysis, oversaturated wells are excluded in the statistical breakdown.

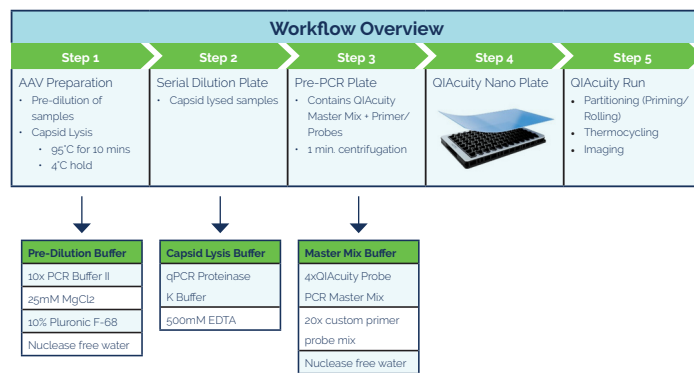
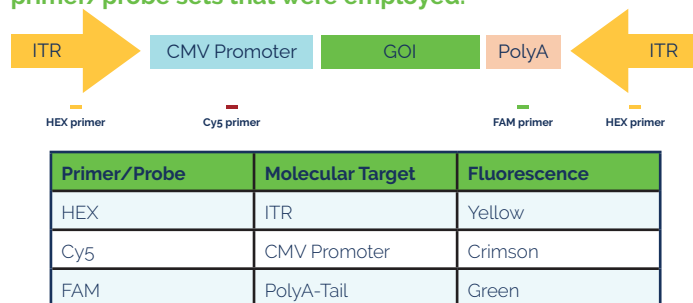


Figure 2: QIAcuity AAV Titering Workflow

## COMPARISON OF dPCR AND ddPCR

Since ddPCR is widely used for viral titering, it is important to demonstrate that dPCR provides robust and reliable results that are comparable to or better than those obtained using ddPCR. Two runs were performed using AAV2 and AAV8 bulk harvest, affinity-purified and polished samples. Assay 1 compared the multiplexing capabilities of dPCR vs. ddPCR using polished AAV2. Titers determined using Bio-Rad's QX ONE Droplet Digital PCR System are compared to those obtained using the QIAcuity Digital PCR System employing dPCR and ddPCR cycling. Assay 2 compared the multiplexing capabilities of dPCR vs. ddPCR for a diverse set of sample matrices using the QIAcuity Digital PCR System and the QX ONE.

Figure 3. General structure of the target AAV vectors for which titers were determined in the current assays and the primer/probe sets that were employed.





## MATERIALS AND METHODS

### Assay 1: Comparison of the multiplexing capabilities of dPCR vs. ddPCR using polished AAV2

Five total analyses were performed using a polished AAV sample. The reactions sets included three singleplexes (ITR-2 HEX, CMV-Cy5, PolyA-FAM) and two duplexes (PolyA-FAM and CMV-Cy5).

**Table 1. Serial dilution scheme for Assay 1**

Steps	Dilution Scheme		
1	Sample Dilution	1:100	
2	Capsid Lysis	1:2	(1:200)
3	Serial Dilution Plate	14	(1:800)
			(1:3200)
			(1.128e+4)
			(1.512e+4)
			(1.205e+5)
			(1.819e+5)
4	Pre-PCR Plate/Final Dilution Scheme	15	(1.328e+6)
			(1.4000)
			(1.160e+4)
			(1.640e+4)
			(1.256e+5)
			(1.103e+6)
			(1.410e+6)
			(1.164e+7)

The same serial dilution plate was used to create the two plates used for dPCR (Plate 1) and ddPCR (Plate 2) cycling. The dilution scheme can be seen in [Table 1](#), and the cycling conditions can be seen in [Figure 4](#). In order to compare dPCR to ddPCR as closely as possible, the dPCR cycling conditions were those outlined in the QIAcuity rAAV technical note<sup>2</sup>, while the ddPCR conditions were designed to mimic those employed in Bio-Rad's QX ONE Droplet Digital PCR System<sup>3</sup>. Because the dPCR does not form droplets, however, the 25°C steps were removed.

During sample preparation, to conserve the amount of AAV used, two (1:10) dilutions were performed in step

Number of Cycles	dPCR		rAAV	
	Temp. (C)	Duration	Temp. (C)	Duration
1x	95	2min	95	2min
40x	95	15sec	95	15sec
	60	30sec	60	30sec
1x	95	2min	-	-

**Figure 4. Cycling conditions used in Assay 1 for dPCR and ddPCR**

one. For capsid lysis, a 1:2 dilution was performed prior to thermo-cycling. Seven sequential 1:4 serial dilutions were then performed in the 96-well serial dilution plate. Because the QIAGEN Master Mix is viscous, an overall excess volume (calculated for 130 wells) was used to ensure no bubbles were introduced and that the 1:5 dilution was achieved in each well.

When the 20 µl samples were transferred from the Pre-PCR plate to the QIAcuity Nanoplate, no dilution was performed. In each well of the pre-PCR plate, 5 µl was left to reduce the risk of introducing air bubbles. A non-template control was essential for setting the threshold during analysis.

### Assay 2: Comparison of the multiplexing capabilities of dPCR vs. ddPCR using different AAV samples

Bulk harvest, capture-affinity-purified and polished (eluate from anion exchange chromatography) AAV 8 samples were used in this assay. The reaction sets included the singleplexes CMV-Cy5 (for all samples) and PolyA-FAM (polished sample only) and the duplexes CMV-Cy5 and PolyA-FAM (polished sample only). Analyses were performed using the QIAcuity Digital PCR System and Bio-Rad's QX ONE Droplet Digital PCR System with the QX ONE cycling parameters.

The bulk harvest samples were prediluted at 1:10, then diluted at 1:2 for capsid lysis. Serial dilution was performed at 1:4 and the dilution for the pre-PCR plate was 1:5. All of the dilutions were the same for the purified and polished samples except the predilution, which was 1:100.

## RESULTS AND DISCUSSION

### Assay 1: Comparison of the multiplexing capabilities of dPCR vs. ddPCR using polished AAV2

An overview of the 96-well nanoplate design used for comparison of the dPCR and ddPCR analyses of polished AAV 2 samples is shown in Figure 5. Analyses were run in triplicate for each primer/probe combination.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ITR-2 HEX			CMV-Cy5			PolyA-FAM			CMV-Cy5 + PolyA-FAM		
B												
C												
D												
E												
F												
G	Non Template Control (NTC)											
H	Non Template Control (NTC)											

Figure 5. 96-well Nanoplate overview for Assay 1

For all of the analyses, both rAAV Cycling and QX ONE Cycling on the QIAcuity Eight provided similar results to the those obtained via ddPCR using the QX ONE Droplet Digital PCR System, as can be seen in Table 2.

Instru.	Method	Singleplex			Duplex	
		PolyA-FAM	ITR-2 HEX	CMV-Cy5	PolyA-FAM	CMV-Cy5
Qx One	ddPCR	1.06E+12	1.07E+12	1.02E+12	1.02E+12	1.10E+12
QIAcuity	rAAV Cycling	1.16E+12	8.29E+11	8.38E+11	1.05E+12	1.04E+12
	QxOne Cycling	1.23E+12	9.28E+11	1.25E+12	1.06E+12	9.99E+11

Table 2. Analysis results for rAAV and QX ONE cycling on the QIAcuity Eight compared to results (copies/ $\mu$ l) obtained using the QX ONE Droplet Digital PCR System for three singleplexes and two duplexes of a polished AAV2 sample

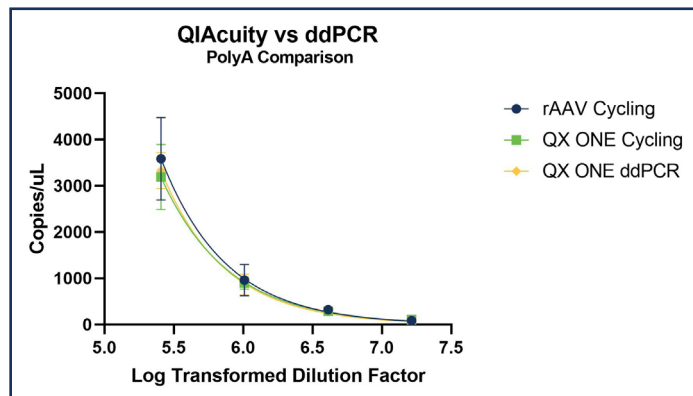


Figure 6. PolyA-FAM Singleplex Analysis results for a polished AAV2 sample on the QIAcuity Eight using rAAV and QX ONE Cycling

The results for the PolyA-Fam singleplex and duplex assays are shown in Figures 6 and 7, respectively. The ddPCR data obtained using the QX ONE system are shown in yellow. In all cases, the average rAAV (navy) and QX ONE Cycling (green) results obtained using the QIAcuity Eight align well with the actual ddPCR results obtained using the QX One system.

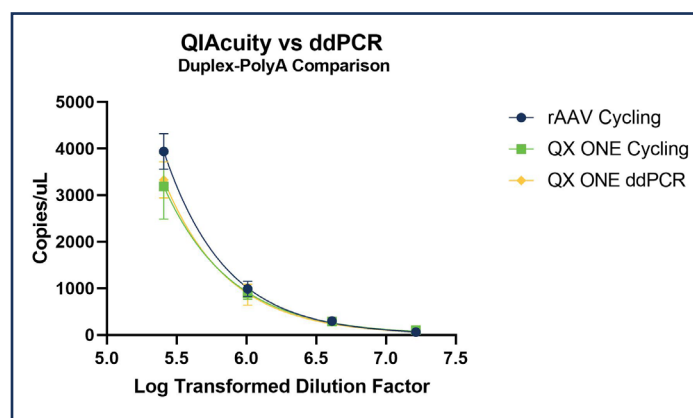
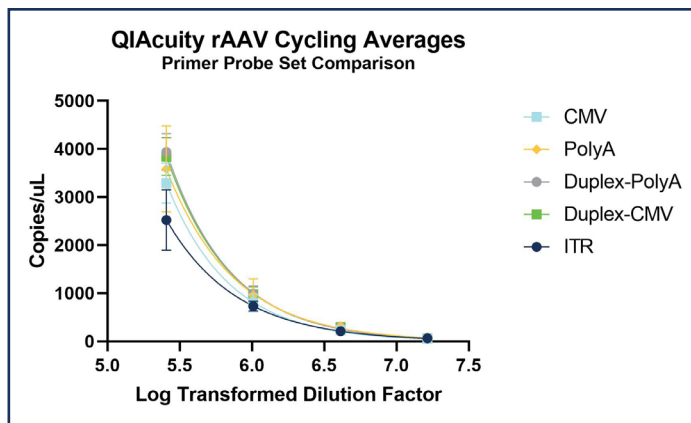
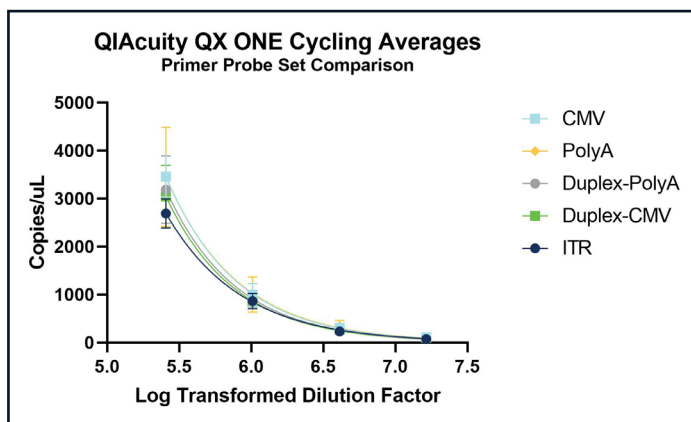


Figure 7. PolyA-FAM Duplex Analysis results for a polished AAV2 sample on the QIAcuity Eight using rAAV and QxOne Cycling

**Figure 8** presents a comparison of the results for all of the singleplexes and duplexes obtained under the two different cycling conditions (dPCR and ddPCR) using the QIAcuity Eight. This data clearly show that the QIAcuity Eight serves as a successful platform to AAV titering whether using rAAV or QX ONE cycling conditions.



**Figure 8. Comparison of results for different singleplexes and duplexes of a polished AAV2 sample obtained using AAV and QxOne Cycling conditions on the QIAcuity Eight**

**Assay 2: Comparison of the multiplexing capabilities of dPCR vs. ddPCR using AAV8 samples in different matrices**

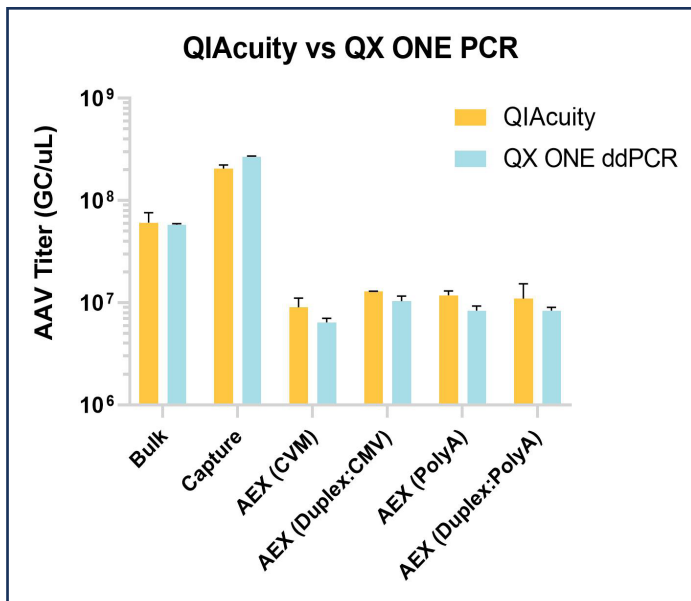
An overview of the 96-well Nanoplate design used for analysis of AAV8 samples in different matrices (bulk harvest, purified and polished) is shown in Figure 9. In this study, the samples were analyzed on the QIAcuity Eight dPCR and QX ONE ddPCR systems using relevant cycling conditions.

	1	2	3	4	5	6	7	8	9	10
	CMV-Cys						PolyA -FAM	PolyA -FAM Rep.	Multi-plex	Mul-ti-plex Rep
A	Bulk	Bulk Rep.	AEX	AEX Rep.	Cap-ture	Cap-ture Rep.	AEX	AEX Rep.	AEX	AEX Rep.
B										
C										
D										
E										
F										
G										
H	Non Template Control (NTC)									

**Figure 9. 96-well nanoplate overview for Assay 2.**

The results are shown in **Figure 10**. It can be clearly seen that dPCR analysis using the QIAcuity Eight provided comparable results to ddPCR analysis using the QX ONE for all of the AAV8 samples, regardless of the sample matrix and the reaction set.





**Figure 10. Comparison of results for different AAV8 samples obtained using the QIAcuity Eight dPCR and QX ONE ddPCR systems. The bulk and affinity results are for CMV-Cy5 singleplexes.**

## CONCLUSIONS

The studies presented in this technical note clearly show that dPCR analysis using QIAGEN's QIAcuity Digital PCR System provides comparable precision and sensitivity to those obtained using Bio-Rad's QX ONE Droplet Digital PCR System, but with shorter run times (~ 2 hours vs. ~ 7 hours) and comparable multiplexing capabilities for improved speed and throughput.

Digital PCR using the QIAcuity also accommodates a comparable diversity of sample matrices, thus enabling titering at more stages of AAV production. In addition, because it leverages individual PCR reactions rather than analysis of bulk droplets, it is possible to find rare targets (<10–20 copies per reaction). Furthermore, the QIAcuity comes in three models (One, Four and Eight) designed to support different workloads and allows multiple independent users to run multiple assays simultaneously.

In summary, the QIAcuity system saves both time and money and can displace qPCR, ddPCR and existing dPCR systems as the method of choice for AAV titering.

## REFERENCES

1. Naso, M., Tomkowicz, B., Perry, W., Strohl, W. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs*. 31, 317-334 (2017).
2. Hui-wen Liu, Emmitt Tucker, Laima Antanaviciute, A'Drian Pineda, Mike Valliere and Patrick Starremans, "Optimized in-process recombinant adeno-associated virus (rAAV) vector genome titer protocol using the QIAcuity Digital PCR System," QIAGEN Technical Note, March 2022. <https://www.qiagen.com/us/resources/resourcedetail?id=e918c957-bc6e-46f2-bb91-bf67dce88ca7&lang=en>
3. Bio-Rad QX ONE Software Standard Edition User Guide v1.2 page 46





## ABOUT THE CENTER FOR BREAKTHROUGH MEDICINES

CBM is a cell and gene therapy contract development and manufacturing organization (CDMO) based in the heart of Philadelphia's Cellicon Valley. CBM offers pre-clinical through commercial manufacturing capabilities including process development, plasmid DNA, viral vector manufacturing, cell banking, cell processing, and a full suite of complimentary testing and analytical capabilities. Through a single-source, end-to-end solution, CBM accelerates time to market without compromising quality.

Co-locating manufacturing, process development and analytical services prevents delays and handling errors. CBM's aim was to create one campus, one building, one manufacturing site. Our purpose built 700,000 sq. ft. manufacturing center is future-proofed in terms of infrastructure within and around the site. The current facility sits on over 1 million sq. ft. of space, allowing for future expansion to match the growing demand of the cell and gene therapy industry. Internally, the suites have been designed so that complementary services and labs are adjacent or nearby, to ensure we can accelerate time to market without compromising quality.

More specifically, the testing and analytical services occupies approximately 200,000 sq. ft. across four buildings along with a stand-alone safety testing laboratory segregated from GMP operations on one campus. In addition to massive analytical development capabilities, the campus has advanced analytics at the forefront of the operation.



Scan or click the QR code to learn more about our testing and analytical services.