INNOVATOR INSIGHT

Current technological trends & advancements in vector purification

Elisa Manzotti speaks to Ying Cai, Nathalie Clement, Chantelle Gaskin, Matt Roach & Ashish Saksule

YING CAI is the Sr. Director of Process Development at Ultragenyx Pharmaceutical. She heads AAV downstream process development and formulation development functions, also a CMC lead of AAV clinical programs. Prior to joining Ultragenyx, Ying worked at Sanofi, Biogen, Merck, and a few CDMOs. Ying has over 20 years' experience in the development, validation, manufacturing and commercialization of different modalities including AAV, plasmid DNA, oligonucleotides, antibodies, antibody conjugates, and fusion proteins. Ying holds a Ph.D. in Chemical Engineering from the University of Arkansas at Fayetteville and a B.S. in Biochemical Engineering from Zhejiang University in China.

NATHALIE CLEMENT has more than 25 years of experience in the field of Gene Therapy, with a strong expertise in viral vectors, specifically adeno-associated vectors, in the academic and industry settings. Her research focus has strongly been focused on optimizing processes to support large-scale production of high quality rAAV stocks and their implementation into the GMP settings. During her thesis work at the University Libre of Brussels, Belgium, she developed new recombinant viruses derived from the parvovirus Minute Virus of Mice (MVM) for cancer-selective gene therapy treatments. She then joined Dr. Michael Linden's laboratory at Mount Sinai School of Medicine, New York, where she developed novel recombinant AAV vectors and directed the AAV Vector Core. She next joined the Powell Gene Therapy Center in 2008 as the Associate Director to supervise AAV production and

testing at research, preclinical and clinical grades. She led the Process and Development Group and the Quality Control group responsible for the production and release of all AAV pre-clinical and clinical lots. During her time at UF she oversaw manufacturing, release and stability campaigns of more than 7 AAV INDs from start to finish, including CMC preparations and interactions with FDA. More recently, she spent several months at Resilience, Alachua, Florida, as the Director of Process and Development of the Viral Vaccines and Gene Therapy franchises. IN that role she oversaw viral vaccine and AAV production scales up to 200L in suspension format and in the icellis 500 platform for adherent platforms of a variety of viruses and AAV vectors. Currently Nathalie is taking a break before starting a new adventure in 2022.

CHANTELLE GASKIN is a Field Applications Scientist, specializing in protein and viral vector purification and downstream process development. She held leadership positions at Applied Genetic Technology Corporation and Brammer Bio, prior to joining the Thermo Fisher Scientific Bioproduction Division in 2020. With over 10 years of experience in gene therapy, Chantelle has accumulated comprehensive knowledge of standard industry practices and regulatory standards, applying this knowledge to advance development of therapies for a variety of indications including ocular, CNS and systemic disease.Chantelle holds a Master's degree in Chemistry from University of Florida and a Bachelor's in Chemistry from Smith College.

MATT ROACH leads the AAV Process Development group at Precision BioSciences, which is focused on designing and implementing new strategies for the production and purification of adeno-associated virus. Matt completed his Bachelor's degree in Biological Sciences at North Carolina State University and his Master's degree in Microbiology and Cell Science at the University of Florida. Prior to Precision, Matt spent time at Pfizer working on the purification of AAV and the Biomanufacturing Training and Education Center training industry professionals on downstream bioprocessing operations.

ASHISH SAKSULE is the Cell and Gene Therapy process development lead and technical expert on bioprocessing platforms for viral vectors (Lentivirus and Adeno-associated virus vector) and non-viral vectors with more than 7 years of experience. Ashish has graduate degree in Chemical Engineering from Michigan Tech University, and Biotechnology graduate degree from Harvard University. His experience spans research & drug development, clinical stage and CRO/CMO settings. Ashish is currently working at Takeda within Global Gene Therapy and have previously worked at MilliporeSigma and Miltenyi Biotec.

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Q Can you sum up the key current technological trends and advancements in AAV vector downstream processing?

YC: There are three key trends regarding AAV gene therapy. First, we want the enrichment for full AAV particles to be as high as possible. This is not only done by removing empty capsids, but also partially filled AAV, which is quite challenging. Secondly, there is a rising regulatory bar for the control of adventitious agents including viral clearance and inactivation. The third trend is manufacturing cost reduction from the clinical phase to commercial. Manufacturing cost consideration is becoming more important. We have seen high cost per dose, especially for AAV and cell therapies. Moving forward, we not only need to improve product quality, but we also need to reduce manufacturing cost per dose. Our ultimate goal is to make these drugs affordable to all patients.

MR: Somewhat unsurprisingly, we are all still working towards improved recovery and purity. There have been innovative revelations on the separation of empty and full capsids that have added to this potential solution. There has been a move to continue reducing the number of purification steps whilst also maintaining sufficient purity, especially around the harvest and capture purification steps.

NC: One substantial advancement over the past five years is the CaptureSelect™ column, or affinity capture column, specifically for AAV8 and 9. Having worked on AAV9 for more than 10 years, this was a huge change in the field. We have seen it widely implemented in downstream processes in the industry.

Another new trend is the enrichment in full capsids. There has also been an effort to develop new reagents to better remove DNA and RNA residuals. Instead of or in addition to benzonase, there are current efforts to remove some DNA species that may be more resistant.

AS: A newer trend I have seen is regarding novel variants and new serotypes. Generation and screening of libraries for AAV variants has emerged as a powerful method for identifying novel capsids. Novel capsids are emerging with numerous advancements in the construct design, and we have multiple synthetic capsid variants that can outperform their natural counterparts. These include new liver-tropic serotypes such as AAV-DJ or AAV-DJ/8, muscle-tropic AAV9MYO, or even the newer AAV7m8.

For downstream processing of this novel capsid, we are still using traditional methods, which were developed for the proteins and monoclonal antibody (mAB) space. There is a key technological need to focus on the newer novel serotypes. There are tools that are being developed specifically for AAV such as CaptureSelect™ AAVX. There are also new key players emerging who can provide custom AAV serotype-specific affinity ligands, as well as newer formats of chromatography media such as monolith or membrane adsorbers formats, which can deliver higher performance as compared to traditional resin formats.

The separation of empty and full creates a mandate for chromatography suppliers to explore new surface chemistries and methods with the goal of achieving adequate separation for all the serotypes. Until then, many of us are still relying on traditional methods such as ultracentrifugation.

Lastly, the application of fast and high-resolution analytical tools is important. Confirmation of all the results with the techniques that we work with for weeks to months is not a problem. Relying on them for day-to-day guidance, especially within process development where decisions need to be made on the spot, is a burden. That is where high-resolution and quick analytical technologies will be necessary.

CG: From the vendor side of things, I personally am looking at the column-free systems on the horizon. One example is essentially a liquid-liquid phase separation approach, based on a hydrophobic affinity reagent binding to the target molecule in the crude harvest phase. This is combined with tangential flow filtration to produce purified material. Another example is a single-use flow-path system using a chromatography resin in a recirculation flow path. The different process buffers are connected and are allowed to circulate in the flow-path along with the crude material. If they are applied at the correct time, then the purified material is eluted in a separate vessel.

Q How are current solutions helping to address the challenge of empty/full capsid separation?

MR: This is an exciting topic that has made significant progress in the last few years. Companies are moving towards designing platforms for AAV. It has become more apparent just how different various AAV capsids are from each other. Additionally, you need to account for the differences in production systems, heterogeneity of viral proteins, and heterogeneity of packaging, which can be a challenge.

The good news is that many groups are tackling this. We have seen an increase in the number of resin and column manufacturers providing specific solutions to empty/full capsid separation. Four years ago, vendors had no specific solutions, only general recommendations and examples of model proteins, like BSA being separated with an anion-exchange resin. We have seen a large increase in the number of vendors approaching us personally with initial methods that have been tested for AAV. It is pretty promising.

There is still a large space to be explored regarding additives. We are seeing that start to develop, and it is promising that people are willing to share that information.

CG: From a regulatory perspective, people are finding they want to get ahead of the bar being raised, as there are not many regulatory guidelines yet. Like Matt said, it is very interesting how people are willing to share methods. A handful of papers and posters published in 2021 have tackled the subject. I have seen the use of divalent salts and other additives to modulate the retention times between the two species, so that you could get baseline separation and even proceed to step gradients in some cases. We have made some advancements, but it is still challenging.

NC: I would like to emphasize the challenge of separating full and empty capsids. The innate nature of these capsids is that the isoelectric point (pI) is so close and requires a specific method. This is why chromatography methods have been slow in becoming efficient,

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though we have seen progress here. Successful separation may end up being very product and platform specific. We will be able to develop standard protocols, but we need to keep in mind that each product will be different. The percentage of empty in a harvest is affected by the AAV construct itself: the length of the genome and the sequence of the genome. It is also affected by the type of helper being used, such as a *rep/cap* helper or adenovirus.

AS: We still need more technological advancements in platforms that can be applied for multiple serotypes. Right now, it is time-consuming, and we need to develop a process individually for each serotype. If we are working with 10 different targets, a lot of hours and resources are spent developing a process for the individual serotypes.

There are technical difficulties and challenges existing specifically around elution. For example, the close similarity of elution conditions leaves the separation vulnerable. The variation in temperature, buffer formulation or lot-to-lot differences among the buffers, the chromatography media, or the AAV feed material itself, can add a lot of challenges. Even small variations can compromise the separation and recovery of AAV.

Empty capsids are reported to have some beneficial effects, under certain conditions, based on their immunological similarity. Empty capsids can act as an effective decoy to reduce the neutralization of AAV vectors by pre-existing antibodies, thus increasing the target tissue transduction following systemic administration. We need to find out exactly how much percent empty and full AAV are beneficial, and whether we should focus on removing all the empty particles. We must balance both sides of the separation, and this will be useful for systemic administration.

YC: Empty/full separation is based on small differences in the pI. Recently, in the October issue of *Cell and Gene Therapy Insights*, my team published a paper using capillary isoelectric focusing to explore this. We demonstrate that measured pH is different from calculated pI; there is also a heterogenous species of different charge profile ranking between 6.2 to 7.0, which is very different from the theoretical pI being reported: 5.9 to 6.3.

This is caused by several factors including the capsid post translational modifications (PTM), not just the length and sequence of the genome. Certain PTMs can shift the charge profile drastically. This is also highlighted during this forced degradation study, where the shifting of the charge profile is visible. The heterogeneity of charge profiles are observed in different products, as well as the same product of different origin (clone, serotype, or different upstream conditions).

Right now, it is more of an art than a science, as we do not understand all the root causes contributing to this charge profile heterogeneity. Mixed mode chromatography will become interesting to apply to this field. We are also exploring gradient separation, and how can we apply it to the industry, but the challenge from the GMP environment is whether it is possible for it to become single use.

Q If you were starting a new process development initiative today, would you recommend utilizing ultracentrifugation or would you bank on chromatography techniques or other new technologies to enrich full capsids?

MR: We have been through this at Precision, and our choice was to go with chromatography. We have devoted effort on the process development side, which has been no small feat. As you get to the later stages of a clinical trial, you think more about comparability. When you are transitioning from phase I/II to phase III, you are going to modify your initial production process. If this is a chromatography method, which it more than likely is for commercial production, it is better to start early. You are likely going to get a worst-case scenario for your percent full at the beginning, but it is ideal to build it early and then improve upon it. That being said, you must hit certain metrics. I would not recommend doing that if you are 10 to 20% full, for example. As you approach higher than 40% full, you are probably in a good state to switch over to chromatography.

Q What are the chief implications of residual testing, for example in terms of cost and time, and what are the keys to optimizing this aspect?

NC: Measuring residuals, whether DNA, protein, or product or process derived, has become a very hot topic over the past few years. In my opinion, there are two reasons. The technology has advanced tremendously, so all the testing has become more sensitive and more accurate, for example ddPCR, next generation sequencing, and RNA sequencing. In parallel to the technology improvements in the assay itself, the clinical doses have dramatically increased, mostly because of the type of indication treated. With higher doses in the clinic, there comes a higher burden of residuals, and therefore a need to better determine the amount and the type of residuals. We have seen toxicity in humans during the course of several trials, furthering the importance of measuring residuals.

One of the keys to success in residual testing is to determine the type of residuals you are going to face in your own platform with your own product. Separate the ones that are very common to every single product and platform like host cell DNA and host cell protein. CMOs have invested in the field, and they are going to be able to offer assays that have been, to some extent at least, validated and standardized.

Spend more time looking at what is going to be specific to your product. If you are using a specific helper virus, for example baculovirus or HSV, you are going to require different types of assays. Keep in mind that developing specific assays for your product could be more time consuming and therefore more expensive, so take this task on early. You also need a well-defined clinical dosing regimen early on. It is sometimes difficult to think about the clinic if you are just about to start screening candidates. However, the importance of your residuals will be impacted by the dose you are going to choose, the route of administration, and also the localization of your administration. Being in the eye, the liver, or being systemic will present very different impacts.

There is still a significant need for improving the technologies, mostly for accuracy and consistency across various products. It is becoming more important to know that the techniques developed are validated across multiple Investigational New Drugs (INDs), so you can have a

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basis for comparability between a product that may show some toxicity or immune reaction in patients and another product that would not. Moving the field towards sharing and standardizing more is critical for everyone, especially in terms of residuals.

AS: There are a lot of guidelines available for residual testing. For example, with cell-substrate DNA, we want to have less than 10 nanograms per dosage with a median DNA size of 200 bp or lower. From a process development point of view, these guidelines need to be addressed by establishing process optimization strategies when the residual host cell DNA is present as a nuclease-sensitive process-related impurity. One of the challenges is that there is residual nuclease-resistant host-cell DNA that has been packaged within the AAV capsid.

For process optimization, based on the close similarity with the desired vector product, it is difficult to eliminate the AAV package host cell DNA impurities by regular vector purification methods. The separation of AAV particles based on density or by gradient ultracentrifugation can remove the AAV package nucleic acid impurity, as they can differ significantly in length from the vector genome based on different densities of the respective particles. In addition to chromatography, gradient ultracentrifugation has been shown to improve this to four to five-fold. This again can represent a scalability challenge when we move into large clinical programs.

CG: One of the more critical aspects of assay development is getting hold of representative reference standards, which the downstream process development team is usually responsible for. It can pose a bit of a problem because before a process is finalized, you must start assay development; it must be developed concurrently with the process development. This means the process that you use to make any reference standards for assay development might not be your final process.

It can be helpful for a sponsor company to establish your formulation buffer early on during the process development. This allows you to use a standard platform column chromatography, or even an affinity chromatography step, then buffer exchange your material into your final formulation buffer, to serve as a surrogate for your reference standard in the interim.

YC: We should always push for improving methods and the manufacturing process. We are always being asked for the residual specifications from a safety perspective as early as possible, but we are reluctant to set the specifications based on very early data. It is equally important to demonstrate impurity clearance as early as possible, by designing scale-down studies to analyze impurities that could introduce certain safety concerns with higher doses. Overall, the keys are demonstrating testing, improving methods, and also demonstrating the process capability for downstream operations as early as possible.

MR: To echo what other panelists have said, an interesting way to approach this is to prioritize testing for final material studies, supply and animal studies, initial animal studies, then compare to confirmation runs as the process improves as a check. These tests, especially if they are outsourced, can get quite expensive. Designing specific Design of Experiments (DOEs) carefully around key steps like the harvest process and in various buffer conditions for possible chromatography steps is important.

Q Is there any trend of companies being more open with sharing critical quality attributes (CQAs) and residuals information, to better understand how products are affecting patients?

CG: In industry, there is a sense of keeping CQAs and other material information close to the chest. However, there is a very slow-moving trend towards being more open with data on reduction of host cell protein and other types of residuals.

NC: Being able to see data, especially on residuals, would be critical to the field. I do agree with Chantelle that there is a trend there, but we are far from being where we should be. I hope that the FDA will push towards sharing this information, because this is exactly how we are going to understand the role of residuals and their toxicity, if any, in a human body.

Turning to adventitious agent inactivation, removal, and viral clearance – what is the current state of the art?

YC: Currently, we inactivate and remove adventitious agents through more traditional approaches. For example, inactivation is typically done through heat, detergent, or lower pH. The removal process typically uses different chromatography modes, including affinity-based modes to find the protein, and different anion exchange steps during separation.

With AAV, we need to be careful when selecting viral filters. Viral clearance is dependent on the manufacturing platform being used. In the early clinical stages, it is possible to get away with not executing viral clearance, especially if you have a low-risk manufacturing process. This is a small part of the control strategy, and you can still test your raw materials, cell bank, starting material, or seed bank. If we have a high-risk process using adenovirus or helper virus, then removal needs to be demonstrated with a viral clearance study, as well as inactivation. If you do not have a key inactivation step, then it can be difficult to add during a later clinical phase.

Due to the rising regulatory bar, I recommend thinking about what the risks of your process are. Also, justify the choice of your model virus. For example, AAV is relatively small, so consider the smallest model virus you want to use. We are not currently using very small viruses; we are not trying to use MNV yet, although we have tried SV40, which has been quite successful.

MR: Having worked on later stage projects, this should be dealt with earlier rather than later. If you do not have something like detergent or an inactivation step built in, it can be quite disruptive to the process to add later. Otherwise, the general steps that people are going through – affinity purification and anion exchange – will help in providing the appropriate log removal values. There may be slight modifications, like low pH holds, that can be added to achieve sufficient viral clearance. It is important to rely on the quality organization within companies as well and have robust raw and starting material qualification.

NC: Focusing on testing your raw material and your cell banks early on is critical. Viral clearance, as Ying said, is not required for phase I or II, so it may not prevent you moving to the clinic, but it is still something to consider. An issue I faced myself is when you are using a virus as a helper, like HSV in my case, testing the raw material, your HSV stock, is a challenge

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in itself because you may get false positives. This makes the development of your adventitious assay a little more complicated.

CG: I have seen the introduction of older technologies like viral reduction filters specifically designed for the removal of larger viruses. This has been adopted in some processes and has worked really well with high recovery.

Q Chantelle, regarding a previous answer: did you observe high aggregation levels in your process intermediates, and what did you include to reduce or remove aggregation? Could you find a good purification solution?

CG: There are certainly some serotypes that have more of a tendency to aggregate, such as AAV2. Some other novel capsids might have some aggregation problems depending on the buffer background. I have worked with a few processes where aggregation was alleviated by adding different excipients throughout the process. Intermediates can suffer aggregation because sometimes you need to include longer hold times between unit operations.

If you have an unstable intermediate product, I recommend looking into either non-ionic detergents or potentially different amino acids in a small-scale screening study. Including a stability study early on during process development allows you to get an idea of what your stability really is.

Q What issues can a lack of serotype-specific technologies present to process development, and what solutions are available?

AS: Unfortunately, due to current lack of serotype specific technology, the approaches that we are using are still traditional methods such as cesium chloride gradient or iodixanol gradient ultracentrifugation combined with filtration technologies. One benefit of this is that we can distinguish our serotypes based on the physical characteristics versus chemical characteristics.

It is easy to develop a process based on the physical characteristics of AAV, because regardless of the serotypes and the capsid differences, we still see similar physical characteristics. Due to this, we can utilize many filtration-based technologies, making process development easier. We can adapt the process based on the physical characteristics of the viruses. On the other hand, if we consider chemical properties, we see multiple differences. As an industry, we need to continuously work on bringing new technologies that can address multiple serotypes and novel variants.

YC: For AAV we have some choices for accommodating different serotypes. We need more publications regarding the fundamental mechanism, regarding which part of the serotype the peptides are binding to associated with the resin. There is continual work to do around developing the technology and working closely with resin manufacturers and vendors.

This includes possible work on the isolation of certain peptides or antibodies which have more specificity to the serotype the company is using.

NC: If you are lucky enough that your serotype or capsid variant works well on any of the current tools, specifically the affinity resins that are available, such as AVB or CaptureSelect™ resins, there is no problem with using the same method for each serotype you have. The exception is the possibility for cross contamination that needs to be assessed once you are in the clinical environment.

MR: We have taken the approach where we have a whole platform, then we test a given serotype as it comes through. We may have to modify a portion of the platform, but the rest will ideally stay intact. However, something like empty/full separation may have to be modified significantly. The various serotypes we have tested to date fall in a few buckets depending on their homology. They may need slightly different buffer conditions, or slightly different load conditions. These options for manufacturing make things easier when novel serotypes come through.

CG: Putting the time in to design a high-throughput screening experiment usually gets overlooked. Often, people want to brute force through small-scale experiments using 1 or 5 ml columns. Static mode small-scale screening tools can be useful in this case, to give good data early on in your process run. I would encourage people not to shy away from doing something like that.

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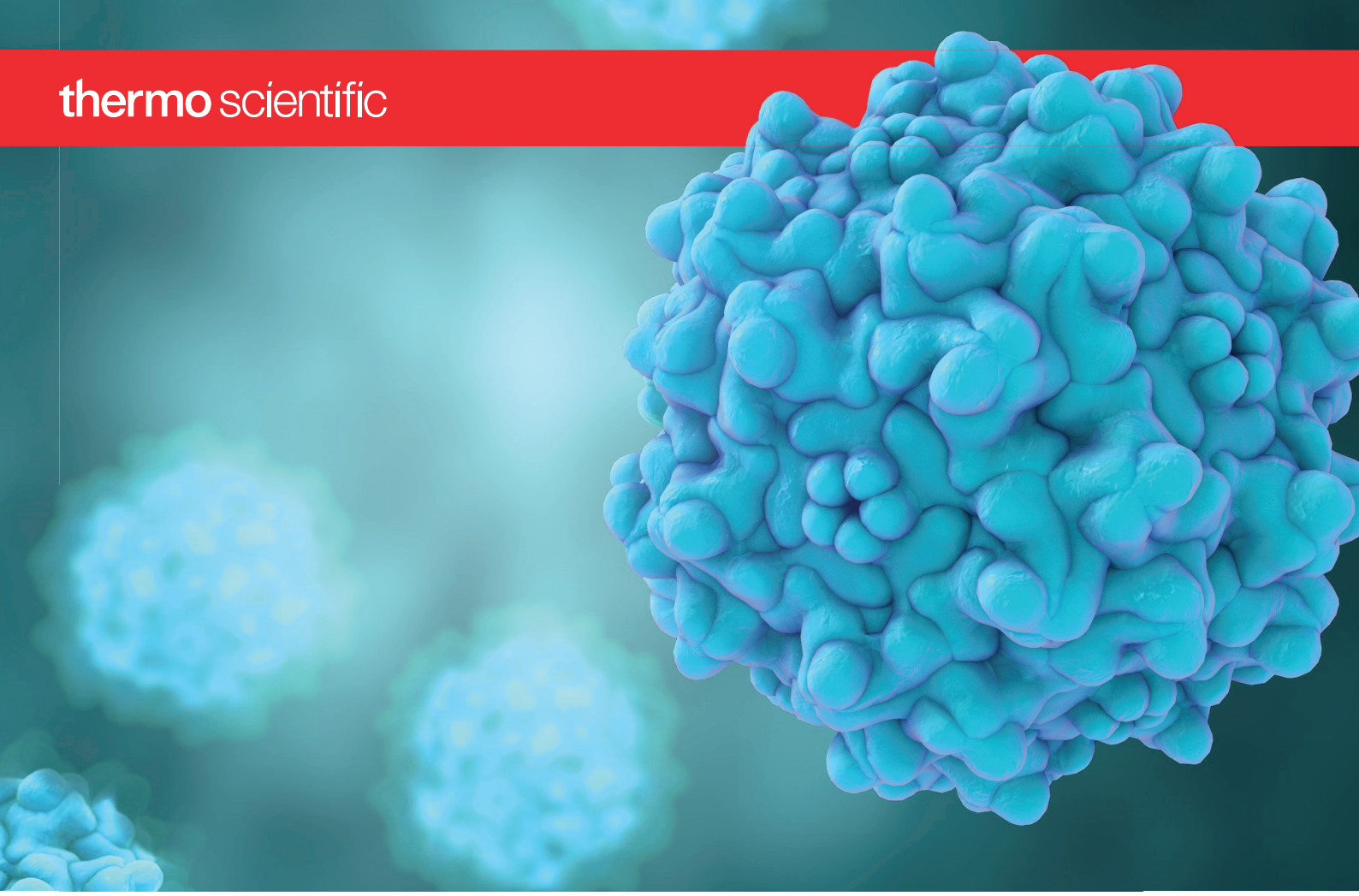

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