CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Development of anti-AAV assay for detection of pre-existing AAV immunity

John Chappell & Zhe Liu

The high natural prevalence of AAV wild-type viruses has resulted in a high frequency of capsid-directed humoral immunity in human populations and many animal models, which may interfere with the effectiveness of AAV-based gene therapies if not identified and managed properly. This case study describes the development of an anti-AAV antibody assay for use in preclinical studies that involve cynomolgus monkeys. This will allow the ability to screen for pre-existing AAV immunity and identify potential low-responding individuals to increase the likelihood of successful transduction and to maximize the utility of the preclinical investigations.

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AAV & RECOMBINANT VECTORS

AAV belongs to the parvovirus family and is a small non-enveloped virus with an icosahedral capsid and a single-stranded DNA genome. To date, 13 serotypes have been identified with different tissue tropisms, which can transduce a wide variety of dividing and nondividing cells in mammalian hosts. As the name indicates, AAV is replication-deficient and dependent on helper viruses such as adenovirus or herpes simplex virus to replicate and complete its life-cycle. The virus infects humans but does not cause any known disease.

Easily modified into vector delivery systems, recombinant adeno associated virus (AAV) has become a popular and promising tool for gene therapy. Advantages include minimal vector-related toxicities and long-term gene expression—up to several years. Approved products are already on the market using different AAV vectors to treat genetic diseases of the skeletal muscle, retina, and central nervous system, with many more in preclinical and clinical development.

Many humans and animal models have already been exposed to AAV and developed capsid-directed humoral immunity.

This creates a challenge for AAV-based gene therapies since pre-existing neutralizing antibodies (NAbs) to the wild-type virus can block vector transduction and gene delivery and activated immune memory may destroy the transduced cells. Treatment-emergent or boosted anti-drug antibodies (ADAs) can result in failure of AAV re-administration and loss of efficacy over time.

CASE STUDY: DETECTING PRE-EXISTING AAV IMMUNITY IN CYNOMOLGUS MONKEYS

The goal of the current study was to develop an assay to detect antibodies to AAV in serum to support a preclinical trial of an AAV-based gene therapy in cynomolgus monkeys (cyno). Using this assay, monkey colonies can be screened for pre-existing AAV immunity, and negative or low-responding animals selected for the trial, to maximize the likelihood of successful transduction. For this purpose, the assay must be quick, simple, sensitive, and robust.

A ligand-binding assay for total antibodies (TAbs) was selected over cell-based assays for NAbs. The presence of ADA, regardless of neutralizing activity, is a biomarker of previous exposure to the virus, and even non-neutralizing ADA could alter biodistribution and accelerate clearance and therefore reduce the biological activity of the vector.

We selected the Gyrolab® immunoassay platform primarily due to its automation, high throughput, and flexible assay design. The small sample volume is particularly well suited to preclinical animal work.

ABOUT GYROLAB TECHNOLOGY

The core of the Gyrolab technology is the compact disc (CD), which is the equivalent of a microtiter plate for an ELISA. The Gyrolab Bioaffy™ CD is made up of highly reproducible microfluidic structures and operates at a nanoliter scale. The microstructure has a defined volume and is fully automated, to avoid human error.

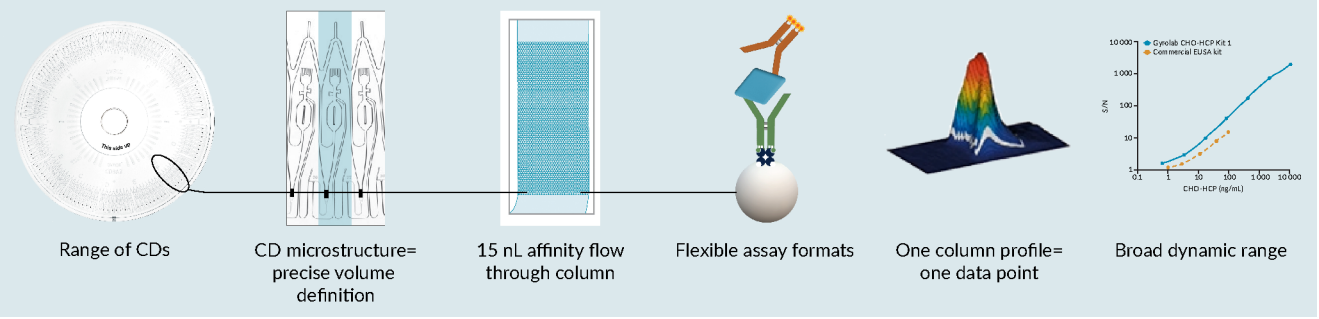
A combination of centrifugal force and capillary action drives movement through the microstructure and into an affinity column below. The 15 nL affinity flow-through column contains streptavidin beads and is the basis for the immunoassay (Figure 1). Various assay formats can be used, including sandwich assays.

Using a fluorophore-detection antibody results in a laser-induced fluorescent peak. There is effectively one column profile per microstructure, which avoids assay drift or other plate or CD effects because everything is processed at the same time. The resulting viewer profile allows interpretation of the binding happening within the column.

Characteristics of the automated Gyrolab immunoassay platform include:

FIGURE 1

Key features of the Gyrolab platform.



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- Fully automated, with minimal hands-on time and fewer errors resulting from manual operations;
- Nanoliter scale, saving both sample volume and reagent volume;
- Uses laser-induced fluorescence allowing a broad dynamic range;
- An open platform with excellent flexibility. Ready-to-use kits or custom-developed assays can be provided, to support applications from discovery to clinical.

Gyrolab assay protocols—pre-qualified methods using commercial reagents—speed up assay development and provide high-quality data.

BRIDGING ASSAY DEVELOPMENT

The project was initiated with a bridging assay, in which virus capsid is biotinylated as capture and Alexa Fluor labeled as detection. Materials and methods are summarized in Table 1.

Briefly, we labeled AAV2 empty capsid in-house with Biotin and Alexa Fluor™ 647 separately. Monoclonal anti-AAV2 antibody was used as a positive control. Conjugated checkerboards performed and both conjugates at one in six dilution were selected for good signal-to-noise ratio and minimizing virus used.

Bioaffy™ 1000 CD was used initially, before changing to the 4000 CD for increased sensitivity. Together with the highest PMT setting of 25%, this gave the optimal response.

Using pooled serum from low-responding cyno, we titrated the positive control antibody at different serum levels. Matrix interference was minor and MRD one in four (25%) was selected.

Serum from 25 cyno individuals was screened in three runs. Results were very consistent, and several high-responding animals were identified, proving the utility of the assay. The same animals were identified as outliers in each run, and all gave very similar cut points (Figure 2 & Table 2).

However, the bridging assay often gave a high coefficient of variability (CV) from a single sample well. CVs higher than 20% were highlighted in red in Table 2. They mostly happened in samples with low signal responses; therefore, for the cut point calculation, high CV samples were not excluded, as to do so would skew the result.

BRIDGING ASSAY CHARACTERIZATION & OPTIMIZATION

Assay specificity was confirmed from capsid depletion of the positive control signal in a dose-dependent manner. A Gyrolab slow spin method further improved assay signal and sensitivity.

With a high prevalence of pre-existing antibodies, it was decided to use capsid inhibition to generate pseudo-negative samples for the cut point. A 1 in 40 dilutions was used for sample inhibition. Signals were well depleted apart from one animal, which likely had too high ADA. Sensitivity was approximately 150 ng/mL using cut point from the pseudo-negative samples.

Again, there were some high CVs, mostly in samples with a low signal response. The slow spin and extra mixing did not fully resolve the issue of high CVs, resulting in the need to test an alternative assay.

TABLE 1

Materials & methods.

AAV2 empty capsids 1013 PP/mL (SIRION BIOTECH GmbH)
In-house Biotin and Alexa Flour™ 647 capsid labeling
Positive control: anti-AAV2 (intact particle) monoclonal antibody, A20 (PROGEN)
Rexxip™ F for all samples and reagent dilution
Checkerboards with both conjugates at one in six dilution (c. 1012 PP/ML nominal) (Figure 2)
Bioaffy™ 1000 CD, PMT=25%

FIGURE 2

Bridging assay profiles for individual animals in runs 5–7.

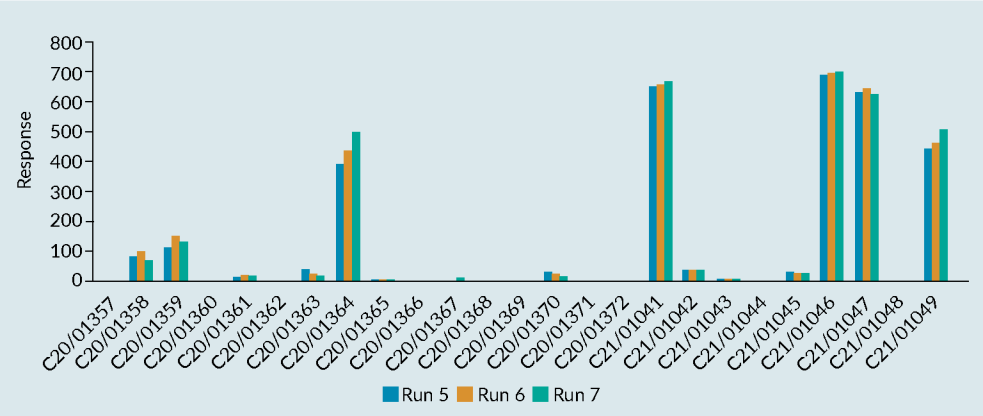


TABLE 2

Bridging assay results for runs 5–7.

Sample ID	Run 5		Run 6		Run 7	
	Mean	%CV	Mean	%CV	Mean	%CV
C20/01357	2.200	42.6	2.302	6.0	5.070	29.5
C20/01358	86.486*	14.1	102.447*	6.1	76.592*	1.8
C20/01359	115.964*	7.0	152.503*	12.3	136.123*	5.9
C20/01360	3.169	37.4	2.595	14.5	5.014	13.9
C20/01361	18.161	14.2	19.719	5.1	20.213	3.3
C20/01362	2.176	224.0	2.227	51.2	3.860	26.2
C20/01363	42.258	28.9	27.917	1.3	26.806	20.3
C20/01364	400.437*	9.4	446.359*	0.4	507.687*	1.4
C20/01365	10.021	14.1	9.673	6.4	9.480	6.4
C20/01366	0.757	28.9	1.485	64.7	2.129	55.1
C20/01367	2.193	11.4	1.648	4.4	13.745	91.0
C20/01368	5.243	13.5	4.739	1.3	5.409	11.7
C20/01369	1.885	7.5	2.670	18.5	2.204	16.7
C20/01370	33.604	7.6	28.0048	6.2	20.951	0.8
C20/01371	2.969	17.7	2.745	25.0	6.131	11.8
C20/01372	2.861	1.4	2.962	3.4	4.422	6.8
C21/01041	665.382*	1.0	671.207*	1.1	685.726*	1.8
C21/01042	42.937	11.7	41.725	5.8	44.956	7.5
C21/01043	13.465	22.2	12.873	3.2	13.264	1.7
C21/01044	2.392	14.5	1.190	12.4	3.576	68.8
C21/01045	35.752	8.4	32.695	5.0	32.229	11.5
C21/01046	706.104*	1.0	715.975*	1.1	719.144*	0.7
C21/01047	647.474*	1.1	656.058*	2.4	641.373*	2.9
C21/01048	2.154	9.2	1.667	1,6	1.821	96.7
C21/01049	454.426*	4.3	472.272*	3.5	521.615*	1.6
Mean	12.455		11.049		12.294	
SD	15.222		13.058		12.218	
Cut point	59.5		51.4		50.0	

*Outlier

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GENERIC ASSAY DEVELOPMENT

Next, a generic assay was developed, with the ability to easily screen ADA to different AAV serotypes without capsid labeling. The Biotin pan anti-AAV capture antibody was used, which binds all AAV serotypes except AAV9.

The anti-AAV2 positive control antibody from the bridging assay was tested using anti-mouse IgG detection and Bioaffy™ 1000 CD. The results showed that the generic format worked well and the overlap of the matrix curve with the buffer curve indicated that cyno serum does not interfere with mouse detection (Figure 3).

With no cyno anti-AAV positive control antibody available, the cyno serum from the bridging assay was used to develop the method. For the detection, three commercial anti-monkey antibodies were tested. However, the negative control serum showed a higher instrument response than the positive sample. Another experiment with different serum samples confirmed the issue. This was caused by nonspecific binding of the capture antibody to the cyno serum components, which was subsequently picked up by the anti-monkey IgG detection.

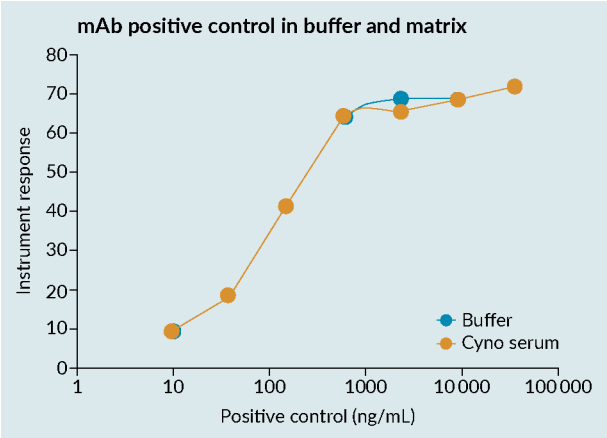
GENERIC ASSAY CHARACTERIZATION & OPTIMIZATION

To overcome the problem of high signals for negative samples, blocking using anti-camelid antibodies or unlabeled camelid capture, or even sample extraction was considered. However, this would add additional steps to the process and be unlikely to work with such a large background.

Instead, two measurements were run in parallel for each sample. In addition to the anti-capsid measurement, a second measurement was made in which the capsid was removed, so that only the non-specific signal was measured. By subtracting the nonspecific signal from the total measurement, a specific anti-capsid signal was obtained.

FIGURE 3

mAb positive control in buffer and matrix.



After the background signal was subtracted, the negative control serum responses were very low, while positive control responses remained high.

The following parameters were selected for further experiments:

- Capsid 1 in 5 dilution
- MRD 1 in 4
- Detection antibody 25 nM
- PMT 5%.

Serum from the same 25 cyno individuals used earlier was analyzed with the generic assay, with a similar profile to the bridging assay (Figure 4). There was good inter-assay consistency, and the generic assay did not suffer from the high CVs seen with the bridging assay.

With the pre-existing antibodies, the cut point resulting from individuals was too high to safely determine the negative animals. Using the variation of negative control response, the cut point was more conservative.

For future experiments, a cut point control (CPC) will be used for this assay at a positive control dilution of 1 in 2000. The CPC will give a more consistent result than the negative control and will be run alongside the samples.

Samples with results at or above CPC will be deemed positive, and below, negative.

To verify the first generic assay, a second serotype (AAV8) was analyzed (Figure 5). Cyno are natural hosts of AAV8 and, similar to AAV2, there was a spread of responses across individuals. Some respond to a single serotype, whereas others respond to both or neither.

Matrix positive control titration showed good intra- and inter-assay consistency. Similar to the anti-AAV2 assay, a CPC at the positive control dilution of 1 in 2000 could be used to define positive and negative samples.

Testing for specificity revealed that AAV8 inhibits the AAV8 ADA response and to a lesser extent AAV2 inhibits this response as well, illustrating anti-AAV antibodies' cross-reactivity to different serotypes. Further research will be needed to assess the cross-reactivity of the anti-AAV8 antibodies.

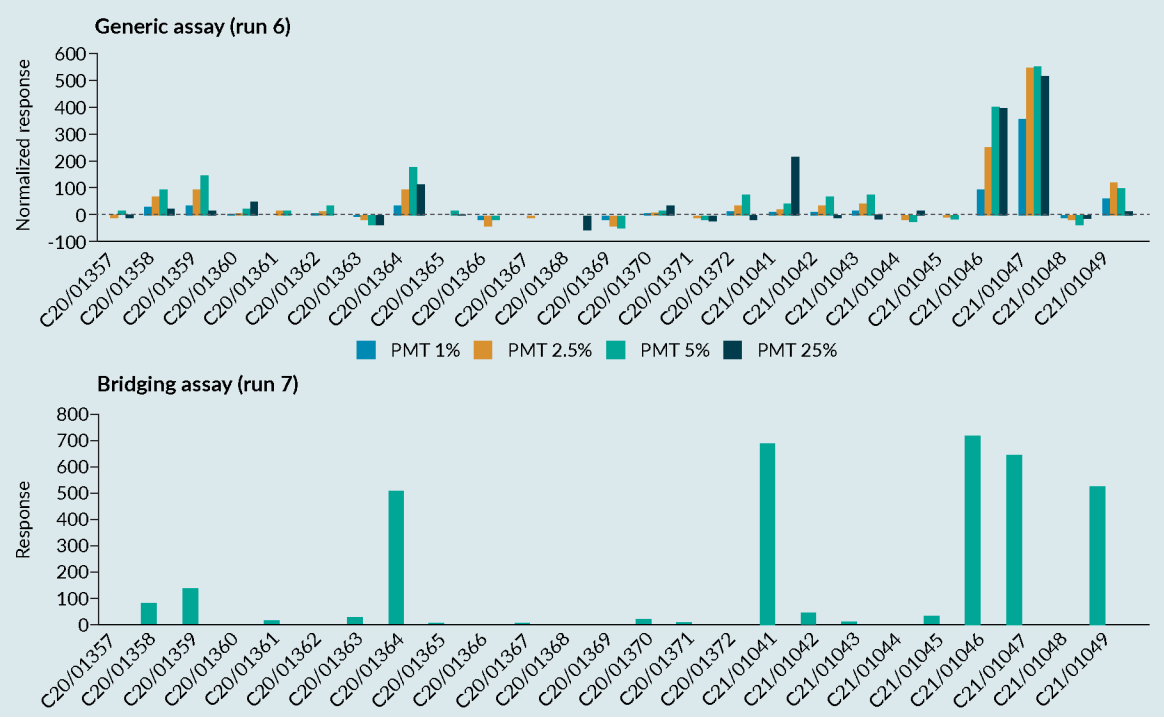
CONCLUSION

The bridging anti-AAV2 assay had a sensitivity of approximately 150 ng/mL and was deemed specific and day-to-day reproducible. There were issues with poor replicate % CV, albeit mainly in low responders.

The generic anti-AAV assay removed the issue of high CVs seen with the bridging assay. Non-specific reactivity between serum and capture reagent was resolved by subtracting the signal of a capsid unloaded column. The assay was deemed specific, and day-to-day reproducible. Without a characterized cyno-positive control, it was not possible to report sensitivity. However, the matrix-screening profile and capsid inhibition results correlated well with the bridging assay, suggesting a sensitive generic assay that is fit for purpose. The generic assay format has been tested and confirmed on both AAV2 and AAV8 stereotypes.

FIGURE 4

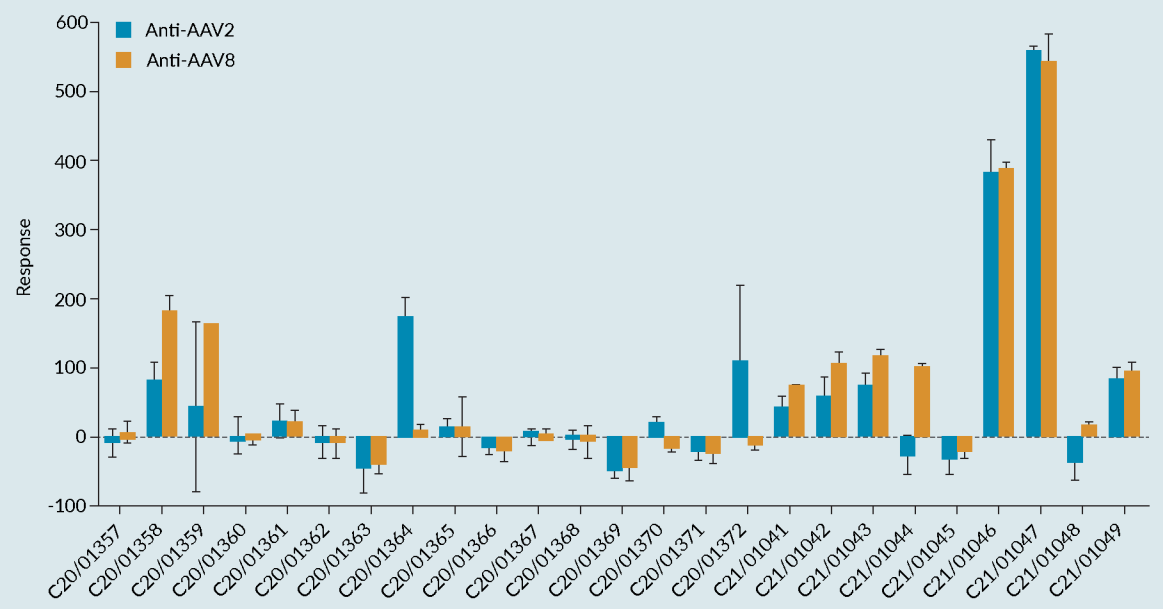
Comparison of generic and bridging assay matrix profiles.



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FIGURE 5

Responses for anti-AAV2 versus anti-AAV8.



ASK THE AUTHORS





Here, **Zhe Liu,** Staff Scientist, Labcorp Early Development, and **John Chappell,** Director of Scientific Support, EMEA and Asia Pacific, Gyros Protein Technologies, answer your questions about anti-AAV assays.

Q For the bridging assay format, there's a requirement to specifically label the capsid. Can you comment on the process for labeling, and did you encounter any difficulties?

ZL: We used a commercial kit for labeling. Gyros Protein Technologies were able to share some relevant data with us, so we had very few difficulties.

JC: We had a labeling procedure based on internal work, so we were able to recommend a labeling protocol based on the capsid concentration.

Q Why did Labcorp select Gyrolab for the work?

ZL: For this particular project, we selected Gyrolab primarily due to the automation. The Gyrolab also provided a quick turnaround time, and small sample and capsid volumes so you can better optimize the assay with the reagent available. We also found good matrix tolerance because of the flow-through system, allowing shorter assay incubation.

Q How do you add a small volume of sample?

JC: The Gyrolab Bioaffy CDs use nanoliter volumes of sample, but you need to load excess sample into a microtiter plate that you place in the instrument. For example, for the 200 nL compact disc (CD), you need to add approximately 4 µL of sample into the microtiter plate. Then the instrument itself transfers the sample from the microtiter plate well to the Gyrolab Bioaffy CD. As it adds sample to the Gyrolab Bioaffy CD, the volume is defined within the Gyrolab Bioaffy CD, and excess sample moves into the overflow part of the microstructure. The volume is completely controlled within the Gyrolab Bioaffy CD itself. The Gyrolab Software will tell you when you set your run-up how much volume you need to add to your plate.

Q Can we analyze crude lysate from upstream with your assay for full capsid detection in AAV production?

JC: Yes, this assay can be used with lysate. The presentation today was based on anti-adeno associated virus (AAV) in serum samples, but we also have an assay kit for measuring AAVX or AAV9 titer. The AAVX titer kit can measure all serotypes apart from AAV9 and is specifically designed to support the measurement of AAV titer in upstream and downstream processing. Depending on the amount of cell debris, you may need to carry out another treatment of the sample, but ultimately, you can measure samples from upstream processing.

Q Can you comment on the capability of the generic assay format for clinical assays?

ZL: The generic assay can be used for clinical analysis, and we are currently planning such an application.

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Q Is there a correlation between TAb and NAb titer?

ZL: For this project, we haven't analyzed our final matrix sample in a neutralizing antibodies (NAb) assay because we appropriate it for screening the total. However, there is plenty of evidence in the literature to show the high concordance between the ligand-binding assay total antibodies (TAb) and NAb for multiple serotypes in both non-human primate and human samples.

Q What did you use for the negative control serum?

ZL: We screened cyno individuals from in-house and commercial sources and selected those that were negative for signal response, Ig depletion, and capsid depletion. We made a natural pool from those individuals.

Q When you see positive samples from one serotype, do you observe that they will cross-react with other serotypes?

ZL: Yes, we did see anti-drug antibodies (ADA) cross-reactivity between the AAV2 and AAV8, the two serotypes we tested. Some animals are positive in both serotypes, especially very high responders. This is understandable because of the substantial capsid structure homology.

Q In the AAV titer kit, is there a calibrator included?

JC: In general, we do not supply a standard with the kit. The kit is designed for customers to use with their specific standard. However, if the customer needs to source a standard, we can make a recommendation.

Q Can you discern whether antibodies are neutralizing via the TAb assay only, or does one also need to perform a transduction inhibition assay?

ZL: It depends on what you need. The NAb is a proportion of the TAb. If the animal has a very high response in the total assay, it is more likely to be positive in the NAb assay.

Q When using the generic assay format, is there a requirement to use the specific customer vector or would general serotype control be sufficient?

ZL: The ease of using specific customer vectors is one advantage of this generic assay format. A new capsid can be easily plugged in as you don't need a label or any other preparation.

The use of a general serotype control may not be ideal and could be a compromised approach because the gene therapy vector and the commercial vector are not exactly the same. They may have different antigen epitopes and different manufacturing conditions, so if possible, we should use the same vector in the assay as in the real clinical and preclinical studies, so the ADA detected will be more relevant and complete, and no bridging activity is needed.

Q The background phenomenon with samples is interesting. What do you think is the cause of that and are there any potential solutions?

ZL: We consider that the background comes from the nonspecific binding of this pan anti-AAV capture antibody to the cyno serum component—most likely the serum Ig. We considered different blocking approaches, but these did not look very promising, so we decided to use a background subtraction, which worked pretty well.

BIOGRAPHIES

ZHE LIU is a Staff Scientist at Labcorp Early Development. Zhe received her PhD in neurobiology from King's College London in 2002. She joined Labcorp Early Development at the Huntingdon site as a Staff Scientist in April 2020. Zhe is a subject matter expert for immunoassay and performs development and validation work. She has extensive laboratory experience in cell-based assay, immunoassay, and flow cytometry in both academia and industry. In particular, she has been working in the CRO sector for 15 years with substantial experience in assay development, validation, and sample analysis of ADA, PK, biomarkers, and potency.

JOHN CHAPPELL is Director of Scientific Support, EMEA and Asia Pacific at Gyros Protein Technologies AB. John has approximately 25 years of experience in the Contract Research industry supporting both preclinical and clinical drug development. He has specialized in supporting biological compounds from an analytical perspective e.g., PK, immunogenicity, and biomarker analysis. He is particularly interested in validation requirements and ensuring that the data generated will be acceptable to the regulatory authorities. He now leads the Application Support and Service teams for Gyros Protein Technologies.

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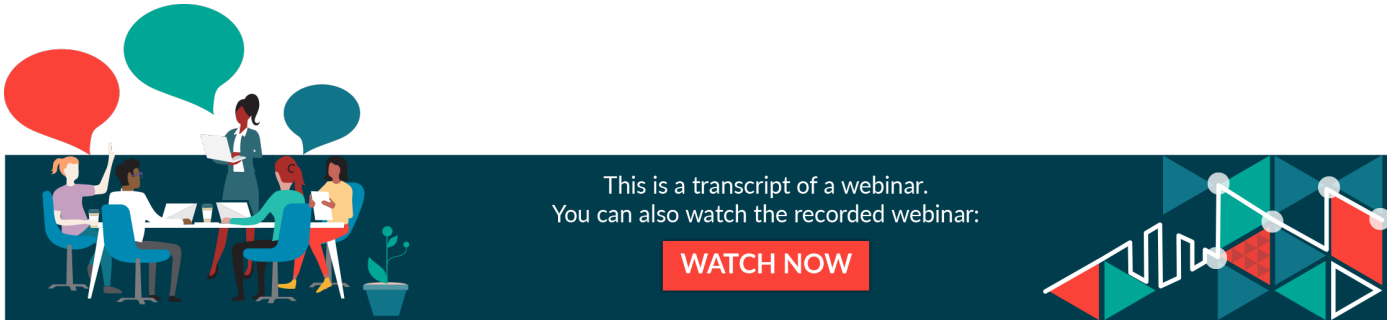
GYROS PROTEIN Technologies

AUTHORSHIP & CONFLICT OF INTEREST

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