Determination of neutralizing antibodies against AAV in human serum using *iLite*® AAV Packaging and *iLite*® AAV Responsive Reporter Assay Ready Cells

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

Currently, the AAV product development and approval processes have multiple challenges that require the development of new tools (1). Among these challenges, the presence of neutralizing antibodies (NAbs) against AAV in patients is one of the most important features to determine before treating patients, as pre-existing immunity can block AAV transduction even at lower titers. The prevalence of anti-AAV antibodies can be as high as 60% of the population for some serotypes (2) and broad cross-reactivity among the different serotypes has been reported (3,4).

The combinational use of the *iLite*® AAV Packaging and *iLite*® AAV Responsive Reporter Assay Ready Cells represent a functional, robust, and agile methodology to detect neutralizing antibodies against AAV of different serotypes in samples containing human serum. The presence of NAbs blocks the transduction of the AAVs produced by the AAV packaging cells, decreasing stimulation of Firefly luciferase production in the AAV responsive reporter cells. Thus, this assay can be used for detection of pre-existing immunity against AAV and to test new approximations to overcome the neutralization.

Principle of the assay

The *iLite*® AAV Packaging Assay Ready Cells are engineered cells capable of producing AAV vectors of a unique serotype e.g. 2, 6, 5, 8 or 9. The *iLite*® AAV Responsive Reporter Assay Ready Cells incorporate a reporter-gene promoter construct that responds to the transduction of the AAV produced in the *iLite* AAV packaging cells. The luciferase signal can be measured in a luminometer following the addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the transduction of the aforementioned AAV in the sample. The presence of neutralizing antibodies reduces the AAV transduction, resulting in a decreased stimulation of Firefly luciferase production. Thus, the Firefly luciferase signal is inversely proportional to the amount of neutralizing activity against the AAV vector (Fig 1). Therefore, the combination of both *iLite* Assay Ready Cells can be utilized for detection of the activity of neutralizing antibodies against recombinant AAV vectors. *iLite*® AAV Responsive Reporter Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter. The constitutive expression of RL allows control of ex. serum matrix effects.

This application note contains the information to perform the neutralizing assay where detection of neutralizing antibodies in samples containing human serum can be performed with human normal immunoglobulin pool as reference and with fixed ratio of *iLite*® AAV Responsive Reporter Assay Ready Cells and *iLite*® AAV Packaging Assay Ready Cells producing AAV of serotype 2, 5, 6, 8 or 9 (from now on AAV2/5/6/8/9).

Specimen collection

The combination of *iLite*® AAV2/5/6/8/9 Packaging Assay Ready Cells and *iLite*® AAV Responsive Reporter Assay Ready Cells can be utilized for detection of the activity of neutralizing antibodies against recombinant AAV vectors in test samples containing human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® AAV Responsive Reporter Assay Ready Cells	Svar Life Science	BM6100
iLite® AAV2 Packaging Assay Ready Cells	Svar Life Science	BM6002
iLite® AAV5 Packaging Assay Ready Cells	Svar Life Science	BM6005
iLite® AAV6 Packaging Assay Ready Cells	Svar Life Science	BM6006
iLite® AAV8 Packaging Assay Ready Cells	Svar Life Science	BM6008
iLite® AAV9 Packaging Assay Ready Cells	Svar Life Science	BM6009
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin)	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Kiovig (Intravenous immunoglobulins = IVIG)	Baxter	NA
IgG-depleted serum	Innovative Research	IIGGDS100ML
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6055680
Microplate Luminometer with appropriate reading software – no filter on the luminometer	Contact Svar Life Science for list of recommended suppliers	NA
Incubator, 37 °C with 5% CO_2	NA	NA
Water bath, 37 °C	NA	NA
Single-channel and multi-channel pipettes with polypropylene disposable tips	NA	NA
Polypropylene tubes or plates for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

The *iLite*® AAV Responsive Reporter Assay Ready Cells together with *iLite*® AAV2/5/6/8/9 Packaging Assay Ready Cells are added to diluted samples containing human serum. Assay sample dilution is to be established for each individual application and after relevant validation – here a final human serum concentration of 2 % for BM6002 (AAV2) and 10 % for all the other packaging cells (AAV5/6/8/9) is used. Intravenous immunoglobulins are here used as reference. The matrix of the reference ought to be similar to the matrix of the tested samples.

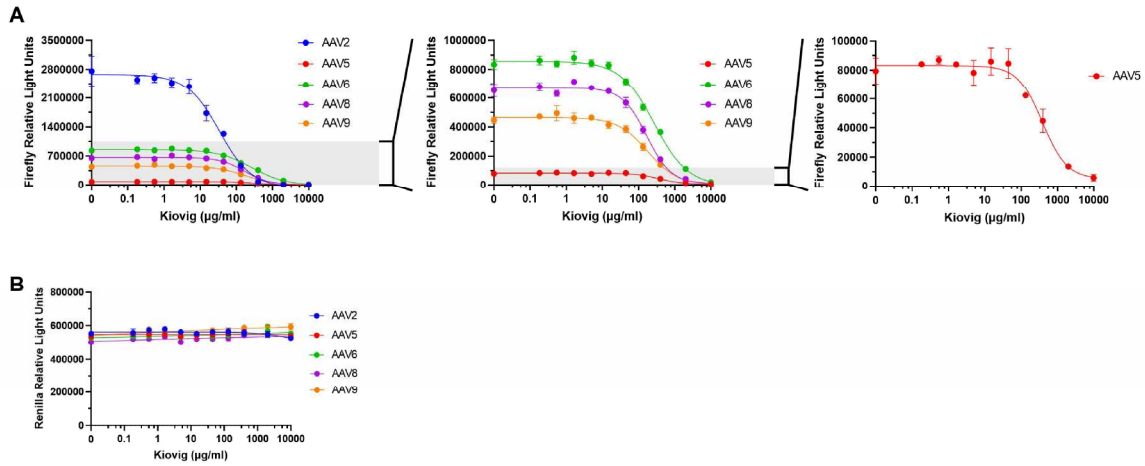

Figure 1. (**A**) IVIG blocking the transduction of the AAVs produced by the packaging cells, decreasing stimulation of Firefly luciferase production in the AAV responsive reporter cells. (**B**) Cell functionality shown by Renilla Luciferase was not affected by IVIG. Values are shown as mean of triplicate ± SD.

Reference	IVIG Suggested **in assay** concentrations, µg/ml
A	10 000
B	2 000
C	400
D	133
E	44
F	15
G	4.9
H	1.7
I	0.55
J	0.18
K	0

Table 1. Suggested reference **in assay concentrations** for IVIG.

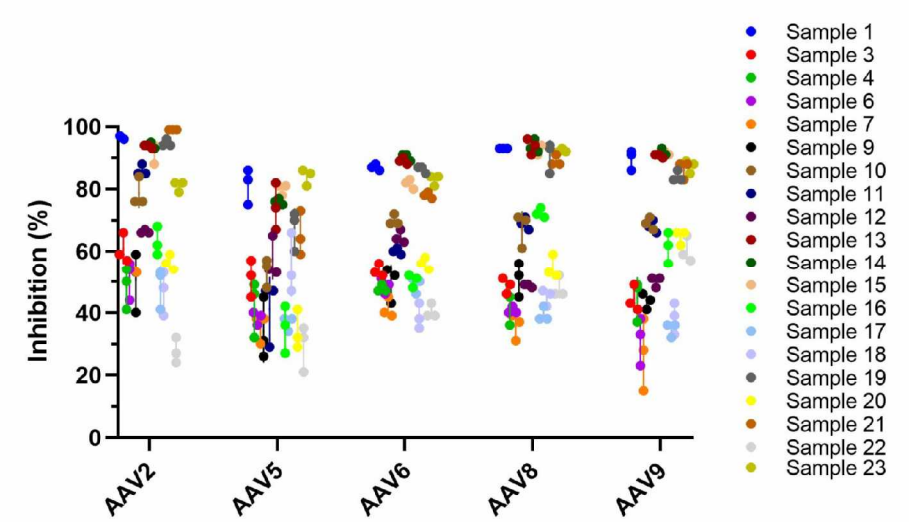

Figure 2. Example of the determination of the presence of neutralizing antibodies on individual samples containing human serum (final concentration 2% for AAV2 and final 10% for AAV5/6/8/9). Values are shown as triplicate values of percentage of inhibition (± SD). Technical note "Calculation of neutralization capacity as percentage of inhibition" describes the analyses of samples containing human serum expressing neutralization capacity as % inhibition using IVIG as reference.

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Dilute human serum test samples in diluent.
3. Perform serial dilution of IVIG in diluent with the same serum concentration as the diluted samples, using IgG-depleted serum or AAV-NAb negative sera.
4. Add 40 µL of IVIG references, controls, and diluted test samples to assigned wells (final concentration will be half of the solution concentration).
5. Thaw the vials of *iLite*® AAV2/5/6/8/9 Packaging Assay Ready Cells and *iLite*® AAV Responsive Reporter Assay Ready Cells in a 37 °C water bath with gentle agitation. The cell suspensions are mixed very carefully ten times with a pipette in order to ensure a homogeneous distribution of cells.
6. Dilute the AAV responsive reporter cells and the AAV packaging cells producing AAV of serotypes 2, 5, 6, 8 or 9 as shown in the Table 2.

Serotype	Media (ml)	Packaging cells **BM600x** Cells (µl)	Reporter cells **BM6100** Cells (µl)
AAV2	5.5	250	250
AAV5	5.65	100	250
AAV6	5.5	250	250
AAV8	5.5	250	250
AAV9	5.5	250	250

Table 2. Recommended dilutions of the AAV responsive reporter cells and the AAV packaging cells producing AAV of serotype 2, 5, 6, 8 or 9.

7. Add 40 µL of diluted cells to each well.
8. Place the lid on the plate, mix and incubate for 18 hours at 37 °C with 5% CO_2.

Adding substrate solutions

9. Equilibrate the plate and the substrate solution to room temperature.
10. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes of incubation at room temperature read in a luminometer.
11. If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes of incubation at room temperature read in a luminometer.

Detection of neutralizing antibodies in samples containing human serum using IVIG as reference

11. Cut-point for neutralization assay is to be established for each individual application and after relevant validation. The technical note “Calculation of neutralization capacity as percentage of inhibition” describe analyse of samples containing human serum expressing neutralization capacity as % inhibition using human normal immunoglobulin pool as reference.

Considerations to be taken into account

The optimal sample dilution and the optimal number of AAV Packaging cells for a neutralization assay is to be established for each individual application and after relevant validation. Each user should consider which are the optimal conditions for them, and the ratios between *iLite*® AAV Packaging Assay Ready Cells and *iLite*® AAV Responsive Reporter Assay Ready Cells shown here are examples only.

- A ratio with a high number of packaging cells will produce a large amount of AAV vectors, reducing the potential neutralizing capacity of some test samples, but it will promote a larger difference in test samples with high NAb titer.
- A ratio with a low number of packaging cells will produce a low amount of AAV vectors, resulting in a fast clearance even in the presence of test samples containing a low amount of NAbs, which will appear positive for NAbs in the same level as other test samples with higher amounts of Nabs.

Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the supplier's/ manufacturer's instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed in accordance with EU directive (2009/41/EC) as Class 2 Genetically Modified Microorganism.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

Propriety Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these patents.

Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

References

1. Gupta V, Lourenço SP, Hidalgo IJ. Development of Gene Therapy Vectors: Remaining Challenges. J Pharm Sci. 2020;

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3. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, et al. Prevalence of Serum IgG and Neutralizing Factors Against Adeno-Associated Virus (AAV) Types 1, 2, 5, 6, 8, and 9 in the Healthy Population: Implications for Gene Therapy Using AAV Vectors. Hum Gene Ther. 2010;21(6):704–12.

4. Aronson SJ, Veron P, Collaud F, Hubert A, Delahais V, Honnet G, et al. Prevalence and Relevance of Pre-Existing Anti-Adeno-Associated Virus Immunity in the Context of Gene Therapy for Crigler–Najjar Syndrome. Hum Gene Ther. 2019;30(10):1297–305.

QUICK GUIDE

Determination of Neutralizing Antibodies against AAV in samples containing human serum using *iLite* AAV Packaging and *iLite* AAV Responsive Reporter Assay Ready Cells

1 Sample and controls dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage.**
- Perform a dilution of the test samples.
- Perform a serial dilution of IVIG, ensuring serum matrix consistency between reference inhibitor solutions, control solutions, and sample solutions using IgG-depleted serum or AAV-NAb negative sera.
- Add 40 µL of the test samples, IVIG references, and controls to assigned wells.

2 Cells dilution

- Thaw the vials of *iLite* AAV Packaging and *iLite* AAV Responsive Reporter cells in a 37°C water bath. Mix the cell suspensions with a pipette in order to ensure a uniform solution.
- Dilute *iLite* AAV Responsive Reporter and *iLite* AAV Packaging cells in accordance with Table 2 - total volume of 6 mL.
- Add 40 µL of diluted cells to each well.

3 Incubation 18 h

- Incubate at 37 °C with 5% CO_2 for 18 hours.

4 Read plate

- Equilibrate the plate to room temperature.
- Prepare the **Firefly luciferase** substrate according to the manufacturer's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.
- If appropriate, prepare the **Renilla luciferase** substrate according to the manufacturer's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.