Modifiers of AAV-mediated gene expression in implication for serotype-universal neutralizing antibody assay

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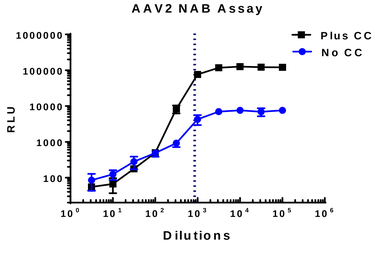
ABSTRACT

AAV-based gene therapy is undergoing major expansion into clinical practice with two treatments currently being granted FDA approval. However, the presence of pre-existing neutralizing antibodies is one of the significant hurdles for the clinical application of AAV vectors that significantly limits the patient population, which might benefit from the treatment. A reliable diagnostic to evaluate the patient's seropositivity is required to ensure the effectiveness of the AAV-mediated therapeutic. Here we describe a simple method for determination of AAV neutralizing antibodies activity based on our original observation that selective inhibitor of AMPK Dorsomorphin, also known as Compound C (CC), makes HEK293 cell highly permissive for infection by ten (10) commonly used AAV serotypes (1, 2, 3, 5, 6, 7, 8, 9, 10, and recently identified Anc80L65). The assay was validated on serum from C57BL6 mice injected intramuscularly with 10^{10} vg/mouse. AAV vectors expressing luciferase are incubated with serial dilutions of tested serum sample, and then added to HEK293 cell pre-treated with CC with at MOI 2000 vg/cell. The expression of luciferase is analyzed 24 or 48 h later in cells by measuring enzyme activity using Bright-Glo luciferase substrate. NAB titer corresponds to the dilution of the test serum sample at which 50% of the luciferase signal is inhibited compared to the "virus only" control. In summary, our findings resulted in the development of easy to set up a universal protocol for analysis of AAV-specific NAB for commonly used serotypes. We planning to further validate this assay on human samples.

The performance of NAB assay in the presence of CC does not change NAB titer.

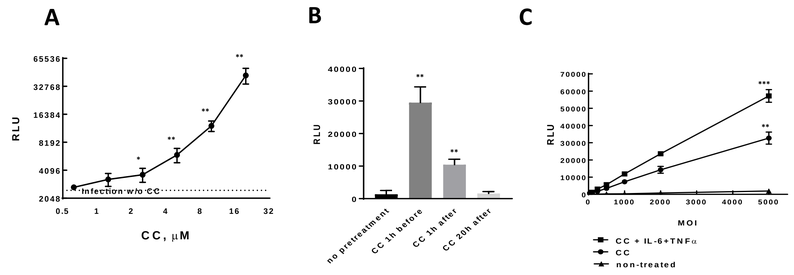


AAV2 NAB titer: In the presence of CC - 832± 24
Without CC - 882 ± 98

The mouse plasma was collected one month after injection of AAV2 and analyzed on the presence of NAB by utilizing HEK293 cell either pre-treated or non-treated with CC. While CC significantly increased the values for luciferase activity, it did not affect NAB titer (as demonstrated overlaid vertical dotted lines corresponded to NAB titers for AAV2 measured in the presence and absence of CC). The observed difference is not statistically significant.

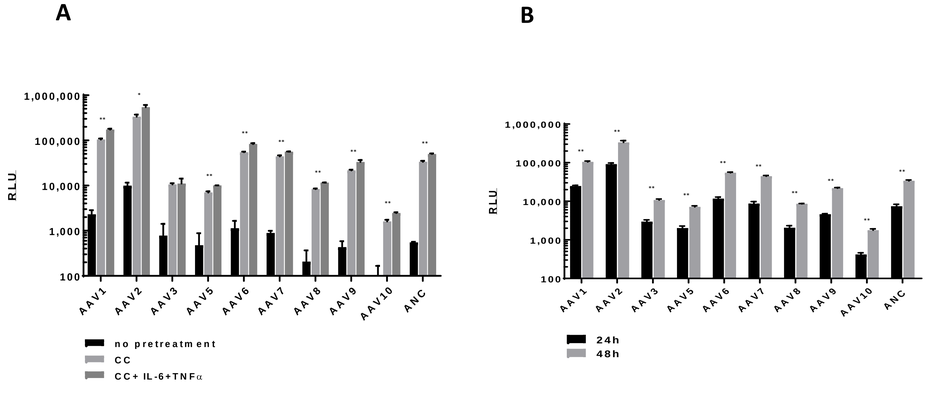
RESULTS

Pre-treatment of HEK293 cell with Compound C (CC) significantly increase the infectivity of AAV8-Luc.



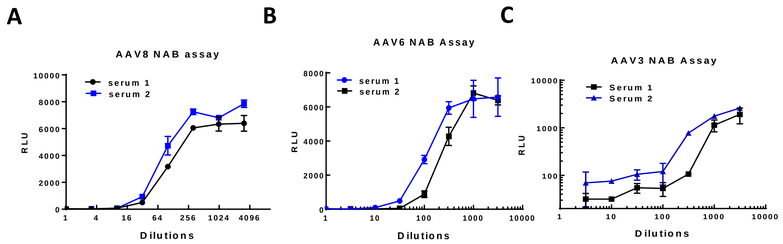
A. HEK293 cell were treated with different concentrations of CC and 1h later infected with AAV8-Luc at MOI=2,000 vg/cell. * p< 0.05 and ** p< 0.01 compared to luciferase activity in untreated cells **B.** 10 μM CC was added to HEK293 cell at different time points during infection with AAV8-Luc. ** p< 0.01 compared to luciferase activity in untreated cells **C.** HEK293 cell were infected with different MOI (100-5,000 vg/cell) of AAV8-Luc in the presence of 10 μM CC or 10 μM CC+20 ng/ml IL-6 + 20 ng/ml TNF-α. Luciferase activity was measured 24 hrs later. ** p< 0.01 for all MOI in the presence of CC compared to infections without pre-treatment of HEK293 cell, *** p<0.01 for MOI in the range 500-5,000 vg/cell in the presence of CC+IL-6+TNF-α compare to the infections in the presence of CC. For MOI =250 vg/cell *p<0.05 compare to mock.

CC enhanced the infectivity of all tested AAV serotypes.



A. HEK293 cell were infected with different AAV-Luc serotypes at MOI 2000 vg/cell in the presence of 10 μM CC, or CC+ IL-6+TNF-α. Luciferase activity was measured 48 hrs later. For all serotypes, luciferase activity was higher in the presence of CC compared to non-treated cells ($p < 0.01$). *p<0.05 and ** $p < 0.01$ for cells infected in the presence of CC+IL6+TNF-α compared to CC only **B.** Comparison of Luciferase activity at 24 hrs and 48 hrs after infection. Cells were infected in the presence of CC as described in A. *p<0.05 and ** $p < 0.01$ for 48 hrs compared to 24 hrs.

Examples of NAB assay for different serotypes performed with HEK293 cells pretreated with CC.



AAV8 NAB titer: Serum #1 - 100.9± 4.0
Serum #2 - 76.8 ± 5.0

AAV6 NAB titer: Serum #1 - 111.5± 10.9
Serum #2 - 239.6 ± 14.1

AAV3 NAB titer: Serum #1 - 907.7± 118
Serum #2 - 746 ± 97.3

For each AAV serotype mice were injected with 10^{10} vg/animal and serum was collected 3 weeks later for NAB assay. **A.** Assay for AAV8 **B.** Assay for AAV6 **C.** Assay for AAV3. NAB titer corresponds to the dilution of the test serum sample at which 50% of the luciferase signal is inhibited compared to the "virus only" control.

Table. Summary of critical steps of the protocol with possible troubleshooting

PROBLEM	SOLUTION
High Variability in readout across triplicate wells	The major source of such variability is unequal number of cells in the different wells. HEK293 cell is readily detached from plate during trypsinization step, but doesn't dissociate easily from each other. Ensure that cells form a single cell suspension at step of plating. In addition, wrapping the plate in aluminum foil during incubation time in CO_2 incubator will help to maintain even temperature across the plate and as result more even growing. Since HEK293 cell detach easily, to prevent the loss of cells during the assay, avoid aspiration of media or plate washing.
Low level of luciferase readout	The aliquot of reporter AAV lost activity or the titer was miscalculated. Take another aliquot or re-titer virus. The quality of the HEK293 cell also very important. Cells should be of low passage and be 50-70 % confluent at the beginning of experiment. To increase the signal for serotypes with low infectivity several approaches can be utilized. 1. Time of incubation can be extended from 24 hrs to 48 hrs. 2. MOI can be increased. However, make sure that luciferase signal is dose dependent and is not saturated. 3. For many serotypes pre-treatment of HEK293 cell with CC together with IL-6 and TNFα will additionally increase the luciferase readout.
The RLU of MAX Luciferase signal is significantly lower than some of dilutions of test sample	FBS used as diluent inhibits AAV infection by itself. Different providers and lot of FBS should be tested on the ability to affect the AAV infectivity.

CONCLUSION

1. **The presented NAB assay based on HEK293 cells pretreated with Compound C is universal and convenient tool to analyze blood samples against variety of AAV serotypes.**
2. **Increased permissiveness of HEK293 cells pretreated with Compound C also can be used for the assessment of AAV infectivity, particularly for hard-to-infect *in vitro* serotypes.**

Acknowledgements

This project was supported by NIH/NIGM 1R01HL131586 and startup funds from the Hormel Institute.