Monitoring adenoviral gene therapy

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Background:

- Gene therapies utilize both integration and non-integrating viral vectors.
- Adenoviral vectors are non-integrating vectors that form episomes within host's cells and are used in gene therapy for stable expression of therapeutic gene(s). Adenoviral vectors (Ad) are widely used in vaccines and gene therapy including oncolytic therapies. One of the commonly used Ad vectors is based on wild-type adenovirus type C5, (Ad5), widely present in the environment. The Ad5 vector has been engineered to be replication-deficient, conditionally replicating or replicating vectors for killing cancer cells, called oncolytic viral therapies.
- Humans develop immunity against wildtype Ad5 and are naturally shed from the body through secretions without any harm to the host. Thus, any potential therapy with these Ad5 vectors needs monitoring of the therapy for shedding through bodily secretions.
- There is also a hypothetical chance that replication-deficient Ad5 vectors administered to a patient can recombine with other wild type adenoviruses and helper viruses present in the patient and become replication competent adenoviruses (RCA).
- This needs sensitive assays that can determine shed vector particles and any potential RCA. We have developed assays for detecting shedding by viral copy numbers (VCN) and for RCA in clinical trial samples.

Materials & Methods:

- Wild type Ad5 reference DNA from WHO (NIBSC, UK) and the reference sequence from NCBI were obtained for assay development.
- Probe based-qPCR assays were designed on conserved sequences within the Ad5 genome that target RCA and VCN as shown in figure 1. The assays were designed for both singleplex and duplex assays. Duplex assays include a human reference assay for a single copy gene (REF) from human genome to be used as internal control and quantification of viral load.
- Sample matrices used for spike-in experiments were blood, plasma and nasal swab transport medium.
- gBlocks, synthetic DNA, for the target sequences that encode for both the RCA and VCN were used for the qPCR assay development. gBlocks, primers and probes were sourced from IDT, Belgium.
- qPCR assays were performed on light cycler LC480 (Roche) and threshold calling with the high confidence settings in the software (Roche).
- Regression analysis of the linearity was done with Excel.

Results:

- Specificity:

1. *In silico* verification: Primer specificity to target amplicon sequences, both RCA and VCN, was verified by using primer BLAST (NCBI) followed by primer heterodimerization (both target and reference assays) analysis. The analysis showed that the primers and probes are specific for their intended targets on both adenoviral and human reference assays.
2. *In silico* PCR was performed to verify if the RCA and VCN assays amplify human genome. This analysis showed that adenoviral assays do not amplify human genomic sequences.
3. qPCR verification: We tested the primers for RCA and VCN with human genomic DNA as template and human reference assays against both adenoviral gBlock templates in separate qPCR runs. Both the primer-probe sets were specific and did not amplify unintended targets, as shown in figure 3. These results showed that the designed assays were highly specific.

- Sensitivity:

Having ascertained the specificity, we next analysed the sensitivity of these assays by using a dilution series of respective gBlocks. Separate qPCRs were performed for each of the RCA and VCN assays. Linearity was analysed by regression analysis of the log transformed values of concentration against their respective Cp values. The results are shown below in figure 2 A-H and both the assays meet the specifications.

Conclusion:

We have developed qPCR assays that sensitively detect any shedding from Ad5 vectors and determine if the shed vectors are replication competent and infectious or not. These assays are essential in any clinical trial to monitor shedding and to ensure appropriate safety of Ad5 gene therapy including oncolytic therapies.

Future Considerations:

- We are testing the sensitivity of duplex assay by combining either the RCA or VCN assays with REF assay.
- Infectivity assays to detect the RCA by TCID50 assay is being developed to complement the molecular assays.
- To further qualify these assays with wide variety of sample matrices in the background for different oncolytic and gene therapy applications.
- We are developing an unbiased sequencing and analysis pipeline based on Oxford Nanopore sequencing to precisely identify the recombined Ad5 (RCA+) sequence, if present.

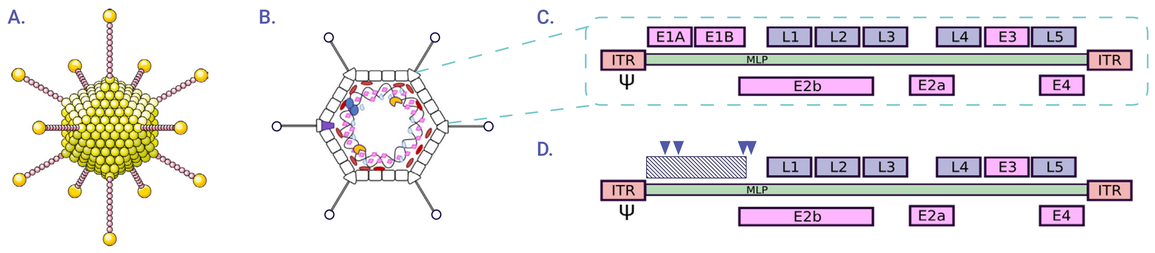

Figure 1:
A. Schematic visualisation of the wild type Adenovirus C5 (Ad5).
B. Artistic impression of inside the adenoviral capsid that contains the double stranded DNA and associated proteins necessary for the integrity of the adenoviral capsid.
C. The linear representation of the wild type Ad5 genome is shown with all essential genetic elements.
D. E1 deleted replication defective Ad5 is depicted and the arrow heads point to the regions where the RCA and VCN assays are targeted. Adapted from references 1 & 2.

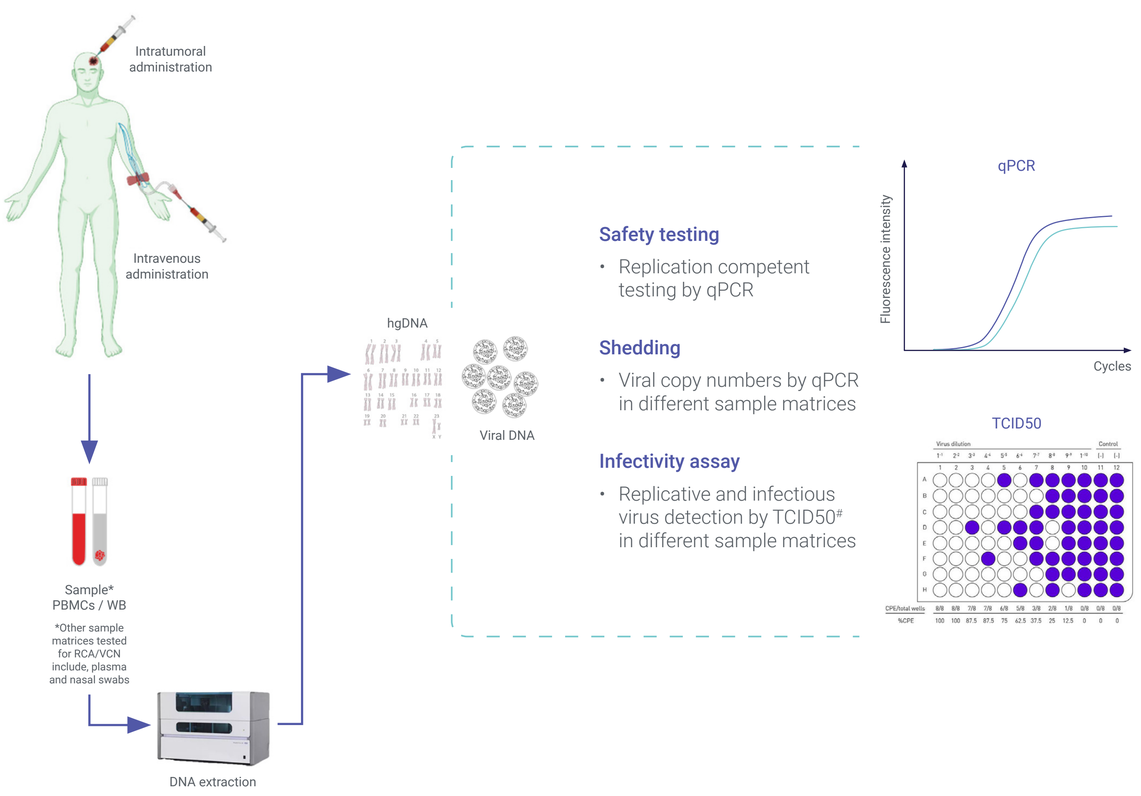

Figure 3 : A schematic representation of clinical sample testing workflow for safety by replication competent virus testing and shedding by viral load using qPCR followed by infectivity assays to verify the replication competency. PBMC/WB is used as an example sample matrix. #TCID50 – Tissue Culture Infectious Dose 50%

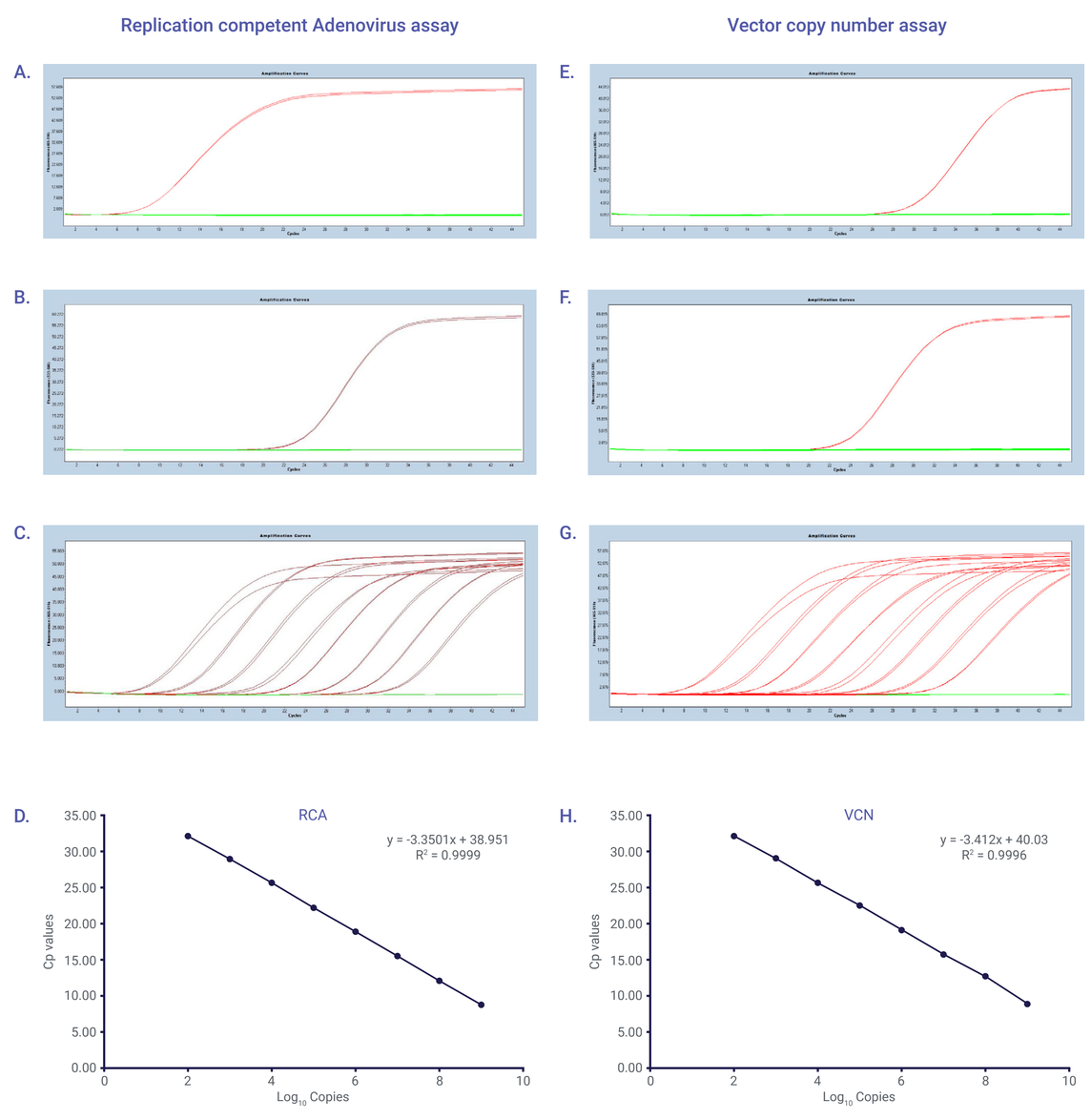

Figure 2: Results of qPCR for both RCA (A – D) and VCN (E – H) assays that includes specificity and sensitivity testing. REF assays verified against gBlocks for RCA (A.) and VCN (E.). The reference assay did not amplify adenoviral targets. The positive control wells for RCA or VCN with respective assays alone were positive. RCA (B.) and VCN (F.) assays verified against human genomic DNA. The RCA or VCN assays did not amplify genomic DNA. The positive control wells for reference assay were positive. The linearity of the RCA (D.) and VCN (G.) assays were tested with a gBlock dilution series. The amplification plots and the regression analysis of the Cp values against log transformed concentration of copies for both the assays show that it is efficient and linear. This shows that the assays are sensitive enough to further qualify for clinical testing.

Key References
1. https://en.wikipedia.org/wiki/Oncolytic_adenovirus#/media/ 2. https://doi.org/10.3390/v4050847 3. doi:10.3390/v2081681 4. doi: 10.3389/fmicb.2016.01503
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