Development of a triplex one-step RT-ddPCR method as a quantitative potency assay for the vMiX™ platform

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OBJECTIVE

To develop a triplex one-step reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) method to quantify the extent of ataxin 2 (*ATXN2*) mRNA knockdown (KD) by AVB-205. This approach can be applied across the vMiX™ gene silencing platform for rapid gene silencing potency assay development.

INTRODUCTION

- AVB-205 is an investigational gene therapy product encoding a vMiX™ gene silencing microRNA (miRNA) that targets and degrades *ATXN2* mRNA transcripts (**Figure 1**).

Figure 1: AVB-205 miRNA payload design

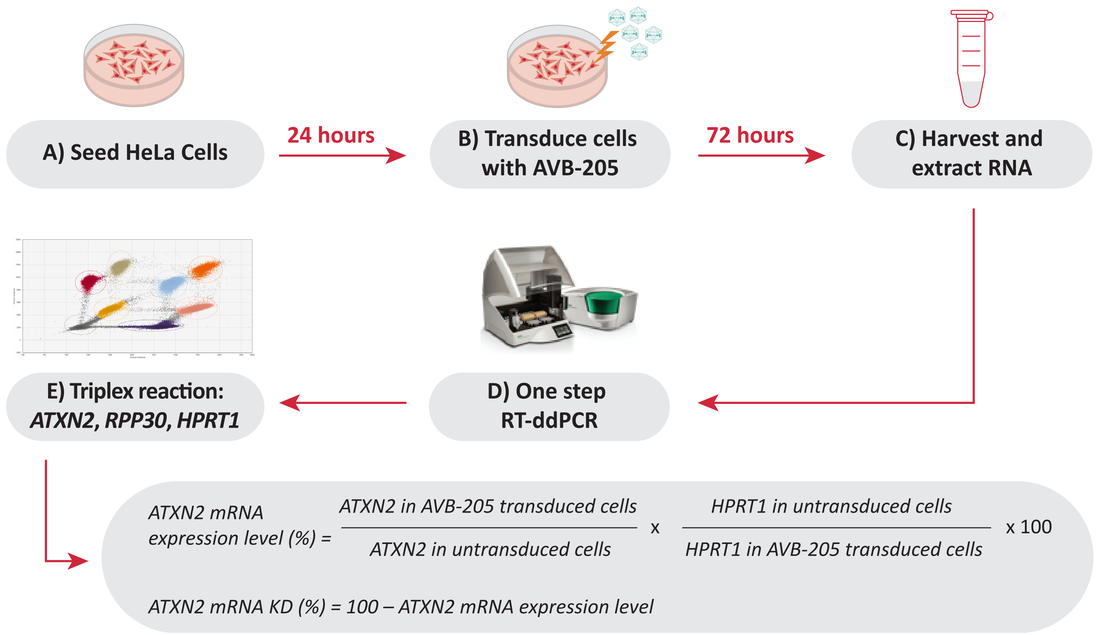
AVB-205 is composed of a recombinant adeno-associated virus serotype 9 (AAV9) vector containing DNA encoding an miRNA construct targeting *ATXN2* mRNA, under control of the ubiquitous chicken β-actin promoter (CB7). The position of inverted terminal repeats (ITRs) and miRNA optimizes miRNA processing, and the single-stranded genome optimizes packaging and manufacturability.

- *ATXN2* mRNA degradation significantly slows disease progression in TAR DNA binding protein 43 (TDP-43)-overexpressing mouse models of amyotrophic lateral sclerosis (ALS), so has emerged as a potential therapeutic strategy.[1]
- Here, we describe the development of a triplex one-step RT-ddPCR *in vitro* potency assay that can quantify the KD of endogenous *ATXN2* mRNA caused by the transduction of AVB-205.
- For such gene silencing approaches, measuring the KD of endogenous genes in relation to multiple housekeeping genes (HKGs) in a single reaction can be used to assess mechanism of action, and potency for routine testing of batch release and stability.

METHODS

- The RT-ddPCR method was developed using the following steps (**Figure 2**):
  - Determine total RNA load, linearity, and sensitivity of RT-ddPCR multiplexing
  - Evaluation of HKG stability in response to an AAV9 infection and *ATXN2* KD
  - Determination of dose-response curve to assess linearity and range
  - Evaluation of assay precision

Figure 2: Method for measuring *ATXN2* knockdown (%) in cells treated with AVB-205



A) HeLa cells are seeded at 8 x 10^4 cells/well in 500 µL of complete media then incubated at 37°C for 24 hours. **B)** Cells are transduced with AVB-205 at selected multiplicites of infection (MOIs) in the linear range of the dose response curve. **C)** Cells are further incubated at 37°C for 72 hours before automated RNA extraction using the Qiacube by Qiagen. **D)** RNA samples are loaded at a concentration of 10 ng/well in the one-step ddPCR Biorad machine. **E)** The triplex gene clusters are selected and analyzed, and the KD (%) is calculated from the formula above.

CONCLUSIONS

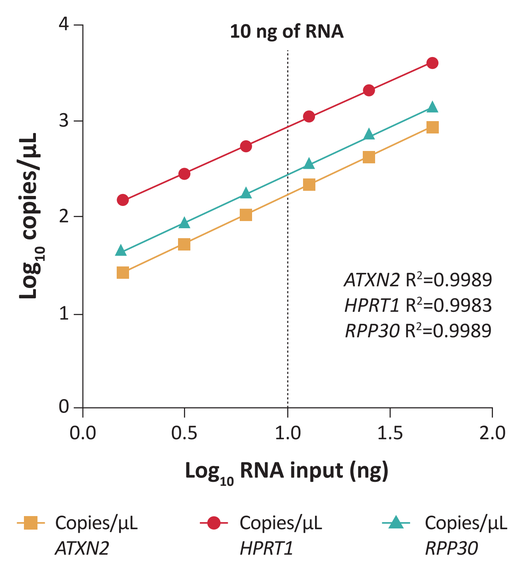
- **This one-step RT-ddPCR *in vitro* potency assay shows excellent sensitivity and repeatability, working in triplex to detect two HKGs alongside the gene of interest and confidently reporting knockdown of *ATXN2*.**
- **The one-step RT-ddPCR method provides a platform approach for vMiX™ potency assay development.**

RESULTS

Determination of total RNA load, linearity and sensitivity

- To determine total RNA load in the linear range of all three target genes, a multiplexing experiment was performed where 10 ng of RNA was identified to be an optimum RNA load (**Figure 3**).
- RNA load was assayed in both simplex and multiplex (data not shown) to ensure the multiplex reaction had no impact on gene expression quantification.
- All three genes demonstrated high sensitivity and good precision between replicates with a linear detection range of 1.56–50 ng of RNA input.

Figure 3: *ATXN2*, *HPRT1* and *RPP30* multiplex of total RNA load and linearity

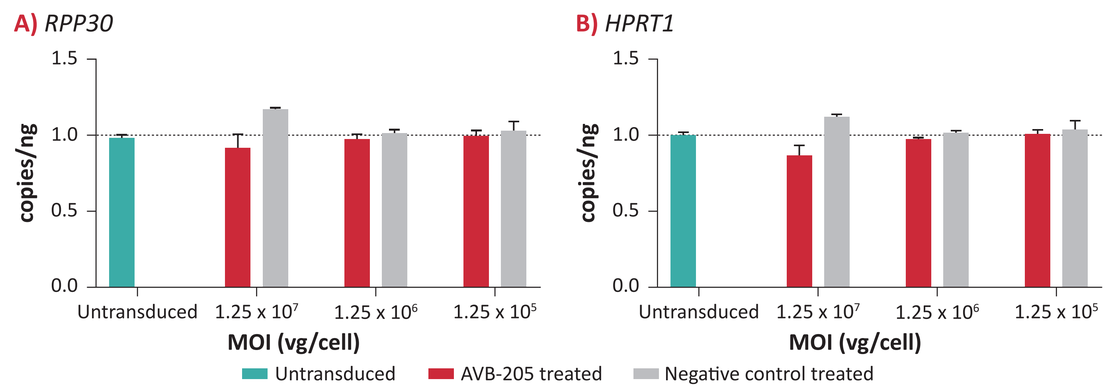


Log_{10}-log_{10} transformation indicated linearity and high sensitivity of triplex one-step RT-ddPCR for all three genes.

Assessing the stability of chosen HKGs across a range of MOIs

- HeLa cells were transduced with either AVB-205 or a negative control with non-targeting mRNA across a 3 log range.
- Both HKG expressions were stable across the range of MOIs regardless of AAV9 infection and subsequent *ATXN2* mRNA KD (**Figure 4**).
- A ratio of *RPP30:HPRT1* expression was used as an assay control to monitor variability in cell culture within the assay.

Figure 4: HKG stability in AVB-205-treated HeLa cells



A) The gene expression profile of *RPP30* (copies/ng) and of **B)** *HPRT1* was stable in HeLa samples treated with AVB-205 (red) against the negative control with non-targeting mRNA (in grey) across three MOIs and the untransduced sample (in teal). All samples were normalized to untransduced samples. *RPP30* and *HPRT1* gene expression was stable.

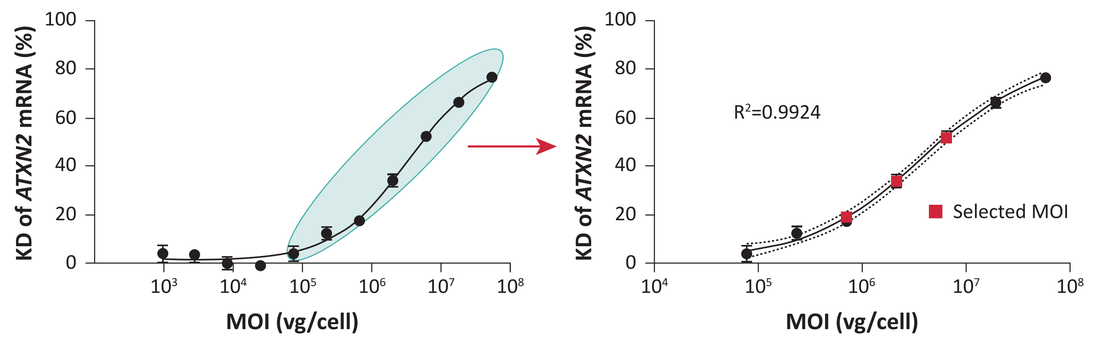
Dose response curve of *ATXN2* mRNA percentage knockdown

- Three MOIs were selected for testing *ATXN2* mRNA percentage KD routinely: 6.95 x 10^5, 2.06 x 10^6 and 6.25 x 10^6 (**Figure 5**).

Inter-assay precision

- The assay was tested three times between two operators evaluate inter-assay precision. All values were below a 15% coefficient of variation (CV; **Table 1**).

Figure 5: Dose response curve of *ATXN2* mRNA percentage knockdown



A) HeLa cells were transduced with MOIs covering a 6-log range. MOIs between 5.6 x 10^7 and 9.48 x 10^3 were fitted with a four-parameter logistic sigmoidal curve. **B)** The three MOIs highlighted in red were selected for further assay development.

Table 1: Inter-assay precision knockdown of *ATXN2*

MOI	Run 1, Operator 1 KD (%)	Run 2, Operator 2 KD (%)	Run 3, Operator 1 KD (%)	Average KD (%)	CV (%)
6.25 x 10^6	53.3	50.5	58.7	54.2	7.1
6.95 x 10^5	20.3	21.5	23.7	21.9	10.6

Inter-assay precision shown by KD (%) achieved at two MOIs (6.25 x 10^6 and 6.95 x 10^5) across three runs by two operators. The CV (%) was calculated using independent triplicate values from each run at the respective MOIs. CV values less than 15% indicate high precision of the assay.

REFERENCES: Becker LA, *et al.* Nature 2017;544:367–71.

ABBREVIATIONS: **AAV9:** adeno-associated virus serotype 9; **ALS:** amyotrophic lateral sclerosis; ***ATXN2*:** ataxin 2; **AVB-205:** AAV9-miR-ATXN2; **CB7:** chicken β-actin promoter; **CV:** coefficient of variation; **HKG:** housekeeping gene; ***HPRT1*:** hypoxanthine-guanine phosphoribosyltransferase; **ITR:** inverted terminal repeat; **KD:** knockdown; **miRNA:** microRNA; **MOI:** multiplicity of infection; **mRNA:** messenger RNA; **RNA:** ribonucleic acid; ***RPP30*:** ribonuclease P protein subunit p30; **RT-ddPCR:** reverse transcription droplet digital polymerase chain reaction; **TDP-43:** TAR DNA binding protein 43; **vg:** viral genome.

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