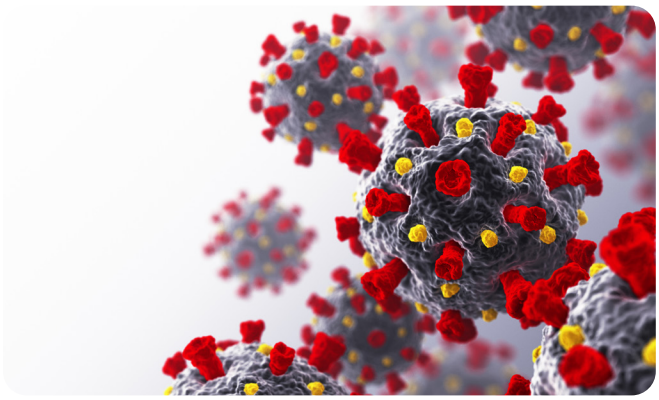
Bioassays used to characterize viral particles for gene therapy discovery and viral vector development

Abstract

The goal of gene therapy is to correct genetic disorders where an absent or mutated protein leads to a debilitating disease. Development of safe, reliable, and efficient gene transfer vectors is one of the keys to successful gene therapy. Two of the most prominent vehicles for carrying gene transfer vectors are the Adeno-Associated Virus (AAV) and Lentiviral Vectors (LVs). In this review, we will highlight examples of different types of assays that can be used in the development and batch testing workflow of viral particles for gene therapy.



Introduction

Lentiviruses (LVs) are RNA based viruses that have a large capacity for stable transgene integration in the genome of target cells. They have been used for ex *vivo* gene therapy, where cells are harvested from a patient, transduced, and re-infused. They have also been used for *in vivo* gene therapy where LVs are directly injected into a patient. Adeno-Associated Viruses (AAVs) are single stranded DNA viruses that have a smaller viral packaging capacity (4 kilobases compared with 8.5 kilobases for LVs), and are considered marginally safer because unlike LVs they are not derived from a pathogenic virus. However, modifications to LVs have been made and the third generation LVs are considered stable and safe (White et al.). To date, while only two AAV-based gene therapies, LUXTURNA® (voretigene neparvovec-rzyl) and ZOLGENSMA® (onasemnogene abeparvovec-xioi) have been FDA approved, many more AAV-based and lentiviral-based gene therapies are currently in clinical trials.

Figure 1 summarizes a general workflow for gene therapy discovery with the key applications covered in this literature review. Good Laboratory Practices (GLP) requires many tests to be performed on gene therapy products, such as AAV particles and lentiviral particles, prior to injecting them into a test subject. In development, rigorous testing must be performed to ensure a high benefit to risk ratio. Similar tests must be performed again on different batches during manufacturing to ensure safety and reliability. These tests include biophysical analysis on the viral particles such as viral titer, vector integrity, and transduction efficiency or genomic integration all of which are required to guarantee a standard amount of genomic material will be administered to the subject. The safety profile includes tests for purity, sterility, replication incompetence, and cytotoxicity, as particle components or contaminants can trigger immune responses. In addition, LVs may inadvertently interfere with normal gene regulation of surrounding genes by altering

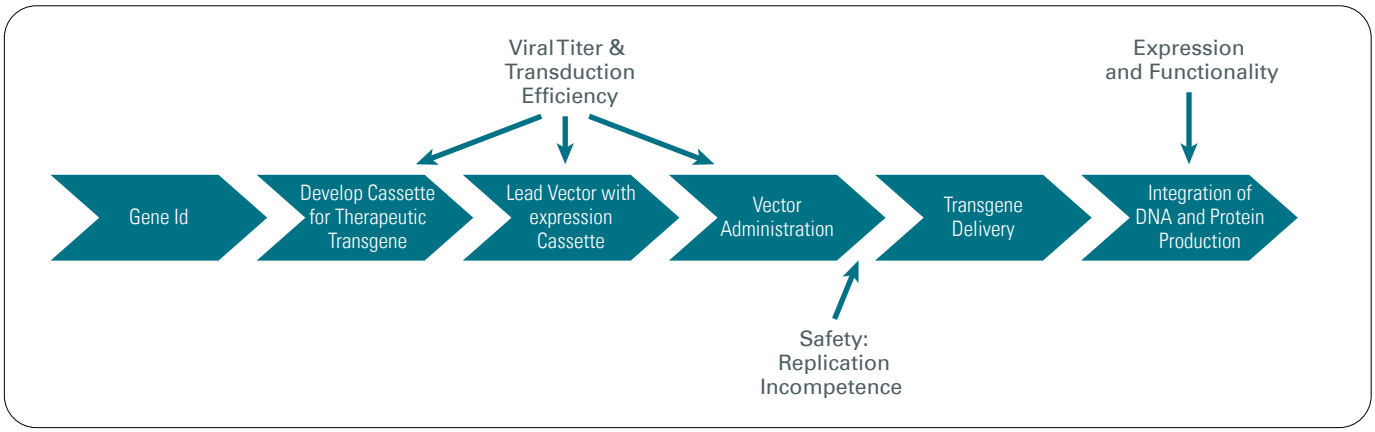
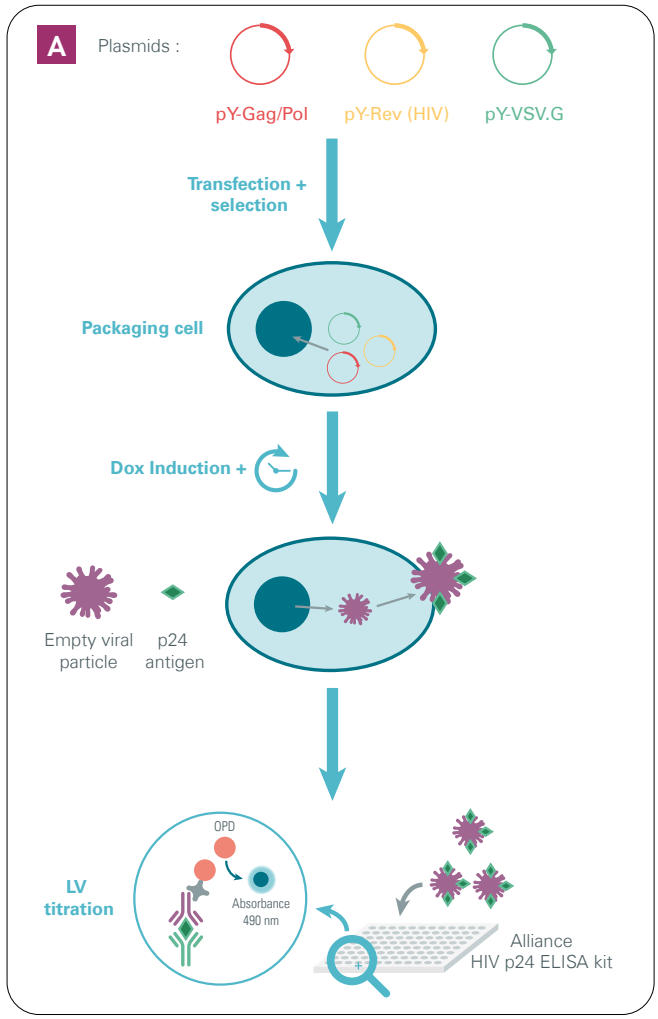


Figure 1: Gene therapy discovery workflow overview of steps for viral vector development and optimization for gene delivery.

transcriptional activity, splicing, or premature termination of transcripts. Therefore, it is important to verify that there are no off-target effects. Finally, the expression and functionality of the transgene is tested to confirm that the protein of interest can be expressed in therapeutic levels and is able to produce a phenotype that is consistent with reversing the genetic disorder (White et al.).

Biophysical analysis: Viral titer

For LVs and AAVs, manufacturing consistently pure batches in high quantities is extremely important prior to *in vivo* therapeutic administration. Milani et al. developed an inducible scalable packaging cell line that produces LVs capable of high yield gene transfer. These LVs are more resistant to inactivation by human sera and lack plasmid DNA contamination. They also modified the LV to enhance its capacity to escape the immune system. Throughout the process of developing the inducible cell line and testing the modified LVs, it was necessary to quantify the LV particles. Since the most common LVs are derived from the Human Immunodeficiency Virus (HIV), they contain the HIV p24 Gag protein. The amount of p24 is therefore directly correlated with the number of LV particles and can be used as a measurement of viral titer. In Milani et al, the amount of p24 was measured (Figure 2) using the Alliance HIV p24 ELISA kit (Revvity). This paper shows the importance of cell line selection for packaging the LVs and how new LVs can be engineered to be more resistant to the immune system.



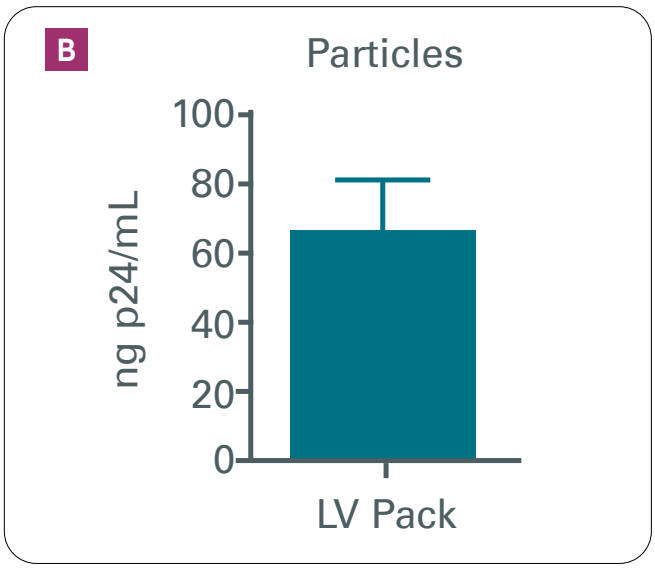


Figure 2: ELISA p24 schematic (Figure 2A). LV physical particle content is determined by measuring the ng/mL of the HIV Gag p24 protein in medium collected from the packaging cell line after induction (Figure 2B).

Transduction efficiency

Transduction efficiency of the viral particles can greatly vary depending on the species and the target tissue. Delivery of gene therapy products across the blood brain barrier has been particularly challenging. Variants of AAV have been engineered that are better at gene delivery across the central nervous system, specifically variants of AAV9. The enhanced CNS tropism of the variant, AAV-PHP.eB, appears to vary greatly in different mouse strains and in other species. Huang . looked for a genetic component to understand these large variations and narrowed it down to the Ly6 gene, which appeared to enhance CNS tropism. They transfected Ly6 family members into various cell lines, transduced the cell lines with either AAV9 or AAV-PHP.eB containing a luciferase gene, and assayed for transduction efficiency using Revvity's britelite™ plus technology. Britelite plus is a homogeneous, ultra-high sensitive luciferase reporter gene assay system for the quantitation of firefly luciferase expression in mammalian cells. The authors report that Ly6a increased transduction in HEK293 cells, greater than 50-fold compared with mock transfected cells (Figure 3).

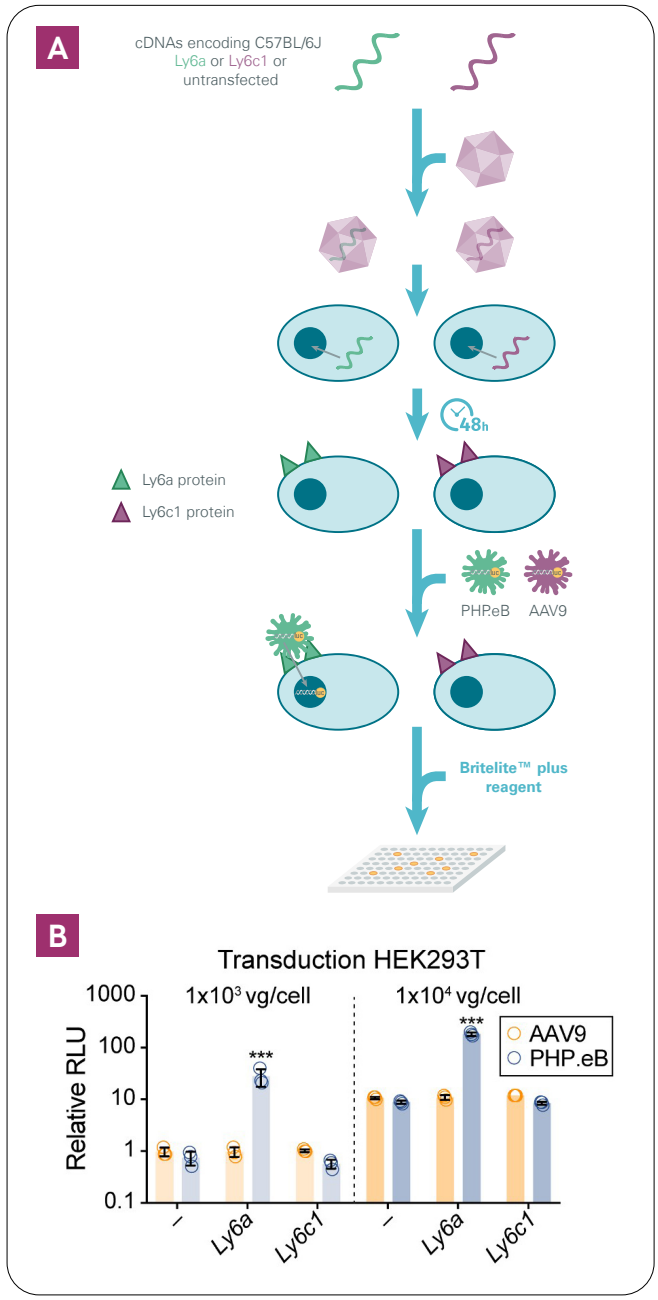


Figure 3: Britelite™ plus schematic (Figure 3A). Ly6a allows for enhanced binding and transduction using AAV-PHP.eB. Using Britelite, luciferase activity was measure and normalized to AAV9 on mock transfected cells. (Figure 3B).

Next, they knocked out the Ly6a and Ly6c1 genes in primary brain cells (BMVECs) and using britelite, they showed a 50% reduction in transduction efficiency (Figure 4a). Finally, they pre-incubated the BMVEC cells with antibodies against Ly6a and saw a dose-dependent decrease in transduction efficiency (Figure 4b), solidifying their theory that Ly6a acts as a receptor for AAV and the interaction with Ly6a increases transduction efficiency. This paper demonstrates that AAVs can be engineered to utilize new transduction mechanisms without necessarily having to rely on interactions with natural receptors.

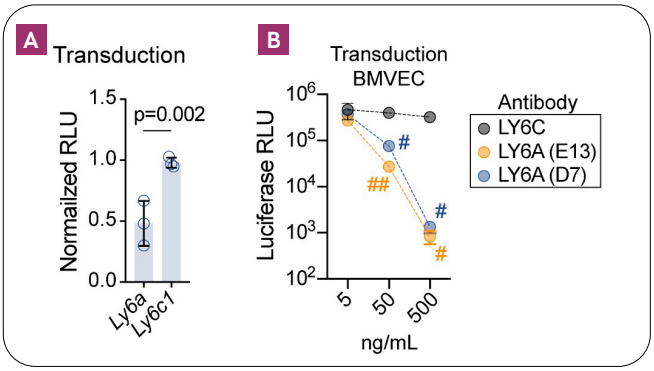


Figure 4: Ly6a allows for enhanced binding and transduction using AAV-PHP.eb. Transduction efficiency in knockout mice. Normalized to cells without sgRNA for knocking out the gene (Figure 4A). AAV. PHP.eB transduction efficiency following preincubation with indicated antibodies (Figure 4B).

Safety: replication incompetence

Aberrantly generated replication competent lentiviruses (RCL) can be a dangerous side effect of administering lentiviral-based gene delivery vectors. RCLs are capable of infecting other cells and creating infectious particles. p24 can be used as a proxy for virus titer and the presence of circulating p24 should be considered evidence of lentivirus replication. Lidonnici et al. explored the safety profiles of ex *vivo* engineering hematopoietic stem cells (HSCs) using LVs and administering the genetically modified cells through a bone marrow transplant in a mouse β-thalassemia disease model. β-Thalassemia is an inherited blood disorder caused by reduced or absent production of hemoglobin beta chains. The low levels of hemoglobin result in a lack of oxygen to various parts of the body and a shortage of red blood cells (anemia). It was one of the first diseases identified as a good candidate for gene therapy (Lidonnici et al.). Lidonnici et al. tested for RCL by taking serum samples from the mice and used the Alliance HIV p24 ELISA kit to measure circulating p24. They showed that all analyzed samples tested negative for p24. The variety of tests outlined in this paper provide important information about the safety and quality profiles of using their lentiviral transduced HSCs for β-thalassemia patients. LentiGlobin, marketed at ZYNTEGLO® in Europe, is in the process of getting approved by the FDA as a one-time gene therapy treatment for β-Thalassemia.

Expression and functionality

The final step in the workflow is to verify that the replacement protein is expressed and functional. This can be achieved using assays that specifically detect the target protein in a sample such as tissue or plasma. Recently, gene therapy has shown promise for a variety of rare neurological disorders. For example, Sanfilippo Syndrome is a rare Lysosomal Storage Disorder (LSD) affecting young children that results in the accumulation of substrates in lysosomes. Specifically, one type of LSDs, MPS IIIA, arises from a deficiency of the sulfamidase enzyme (SGSH), leading to partially degraded heparin sulfate-derived oligosaccharides. Winner et al, selected AAVrh10 to deliver recombinant human SGSH because this serotype is known to better evade the immune system than some of the other common serotypes. It also shows the largest distribution of the transgene when transducing neurons. The mice (either MPS IIIA model or unaffected mice) were injected with either AAVrh10 carrying the replacement SGSH gene or a GFP control. Mice were euthanized at three different time points post-injection (7, 18, and 25 weeks), the brain tissue removed, and frozen for subsequent analysis. The brains were sliced and the four brain slices (L2, L4, R2, and R4) were tested for biodistribution of the protein, with L2 being the injection site. Using a standard recombinant SGSH protein for signal interpolation, the SGSH protein in each slice was quantified by performing a DELFIA® immunoassay (Revvity). DELFIA (Dissociation-Enhanced Lanthanide Fluorescence Immunoassay) is a Time-Resolved Fluorescence (TRF) intensity technology which detects the presence of a compound or biomolecule using lanthanide chelate labeled reagents, separating unbound reagent using wash steps (Figure 5A). They saw that although the injection site, L2, has the highest expression level, they were able to detect lower protein expression in other parts of the brain (Figure 5B). The data in this paper showed the therapeutic potential of the AAVrh10-SGSH serotype, although a multi-injection site may still be necessary for optimal biodistribution of the protein. From this data, they were able

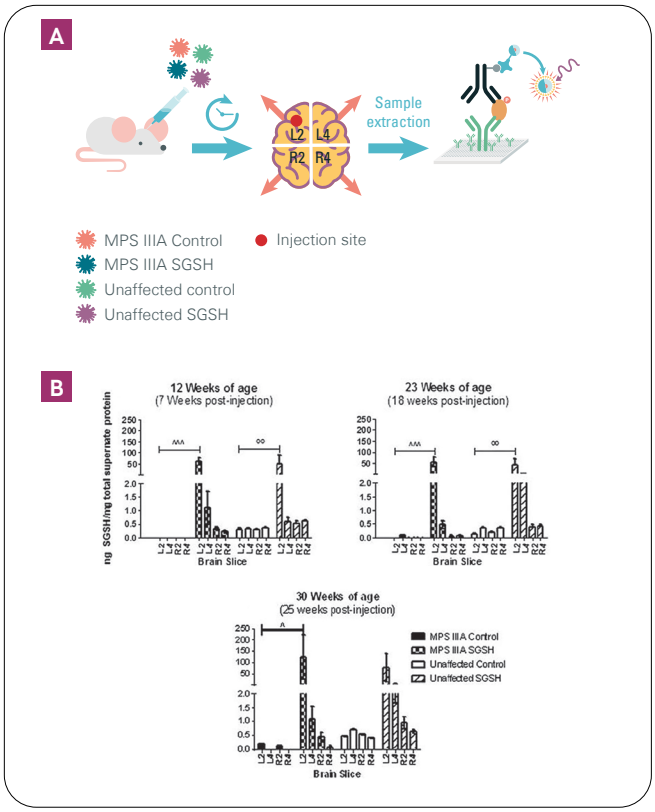
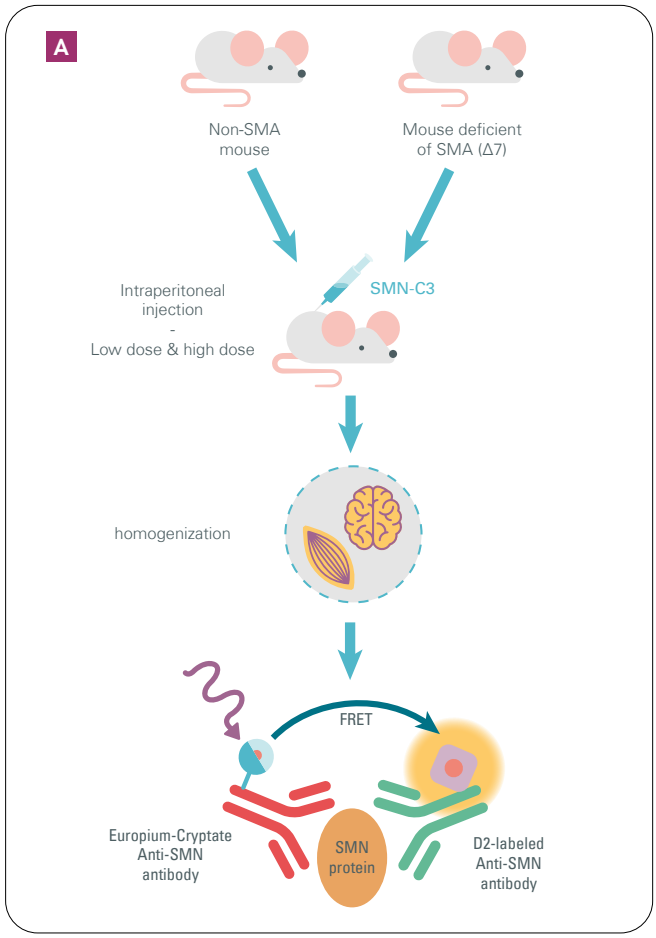


Figure 5: DELFIA schematic for detection of SGSH protein. (Figure 5A). The distribution of sulfamidase after injection at site L2 as measured by DELFIA. SGSH concentration in 12-week old mice, 23-week old mice, or 30-week old mice. MPSIIIA control compared with MPS IIA-SGSH using unaffected control mice compared with unaffected-SGSH mice.

to proceed with a clinical trial using this vector construct. In February of 2020, Lysogene was granted FDA fast track designation for LYS-SAF302 gene therapy in MPS IIIA, which uses AAVrh10 virus to replace the faulty SGSH gene.

SMN1-related proximal Spinal Muscular Atrophy (SMA) is another example of a rare genetic disease where gene therapy has shown promise. It is caused by a deletion or mutation of the Survival Motor Neuron 1 (SMN1) gene. Zolgensma® (formerly, AVXS-101) is an FDA-approved gene therapy treatment that delivers a copy of the SMN1 gene in a self-complementary adeno-associated viral serotype 9, scAAV9 (Rao et al.). Another method to target SMA is to alter the expression of the SMN2 gene. SMN2, which is nearly identical to the SMN1 gene, does not generate adequate levels of the SMN protein due to a splicing defect. Therefore, altering the splicing of the SMN2 gene so that it can produce higher levels of the SMN protein, is another therapeutic strategy. Feng et al. looked at an SMN2 splicing modifier administered to a mouse model with severe SMA. They used HTRF® (Revvity) to quantify the expression levels of SMN protein in brain and quadricep muscles using low or high doses (Figure 6) and compared them to levels in a normal mouse. HTRF (homogeneous time resolved fluorescence), a TR-FRET technology, can be used to assess molecular interactions by coupling antibodies with either a donor (long-lived fluorescence) or an acceptor (short-lived fluorescence) fluorophore. When both antibodies bind the target, energy transfer between the two fluorescent dyes can be measured and is proportional to the concentration of the target protein in the sample. They showed that higher levels of SMN protein correlated with increased survival of the mice. Since one of the attributes of SMA is muscle atrophy, they also looked at blocking myostatin function, a known inhibitor of muscle cell growth and differentiation. For this, they used AAV to administer recombinant follistatin, a known inhibitor of myostatin. The combination therapy showed enhanced muscle growth in the adult diseased mice; promising results which could eventually provide significant benefit for muscle atrophy in SMA patients.



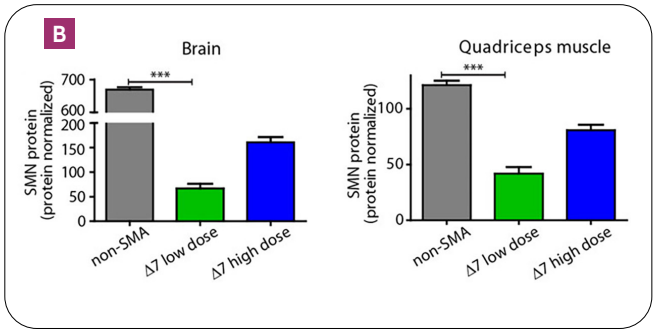


Figure 6: HTRF schematic for detection of SMN protein. Anti-SMN antibodies are coupled with Eu+ cryptate (donor) and d2 (acceptor). (Figure 6A). Quantification of SMN protein in brain and quadricep muscles using HTRF.

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In another paper looking at SMA, Poirier et al. designed a set of pharmacokinetic pre-clinical studies in animals to look at the bioavailability of Risdiplam®, another SMN2 splicing modifier. Using an HTRF assay to quantify SMN1 extracted from brain and muscle tissues of mice, they showed a correlation between the level of SMN protein and a dose response of Risdiplam in both brain and muscle compared to a control.

Conclusion

Here we presented recent examples of a variety of assays that have been used throughout the gene therapy workflow. These assays can be used for analyzing the biophysical properties and safety profiles of viral particles, such as AAV and LVs, during development or manufacturing. We also show examples of both wash and no wash technologies that allow for quantitative measurements of protein expression as a readout of the gene therapy efficacy.

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