Assembling a Comprehensive Potency Assay Matrix for Late-Stage Manufacturing of AAV Viral Vectors

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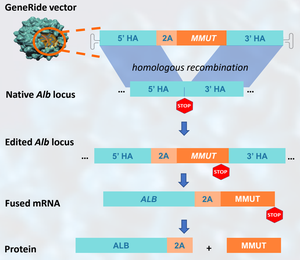
Abstract

Understanding of commercial gene therapy products has accelerated rapidly in the last few years, resulting in stringent specifications to maintain a consistent level of potency through process development changes. Viral vectors have a particularly complex mechanism of action and several steps must occur in series for a therapeutic benefit to be observed in patients. As a result, it is critical to demonstrate that various stages in the biological process occur successfully when developing a potency package. SUNRISE is a Phase I/II clinical trial which is exploring the use of hLB-001 to potentially treat pediatric patients with methylmalonic acidemia characterized by methylmalonyl-CoA mutase gene (*Mmut*) mutations. hLB-001 uses GeneRide technology and incorporates homology arms in the transgene design to precisely integrate a corrected *Mmut* gene into the albumin locus of targeted cells through homologous recombination. Selective advantage then promotes the proliferation of edited cells that express the functional protein. Here, a combination of mRNA expression and enzymatic activity assays are used to show comparable levels of potency when both genomic integration and functional activity are required to effectively treat the disease. The assays were initially assessed by performing linearity and inter/intra-assay precision experiments, and then were further evaluated using different batches of drug substance and heat-treated samples to demonstrate alignment of the two analytical methods in measuring vector potency.

GeneRide®, a Nuclease-free Promoterless AAV Genome-Editing Technology

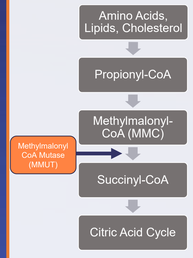
GeneRide is a novel AAV-based genome-editing technology that is designed to leverage homologous recombination to insert a therapeutic transgene into a specific locus of the host genome [1]. GeneRide is designed to work as follows.

For liver-directed targets, the transgene is integrated into the albumin locus (*Alb*). The integrated transgene (*e.g.* methylmalonyl-CoA mutase; *Mmut*), which does not have a promoter, can thus "hitch a ride" on the highly active endogenous albumin promoter, which is designed to result in high transgene expression selectively in hepatocytes. The transgene is precisely inserted in frame between the penultimate and the stop codons of albumin and utilizes a P2A peptide sequence that allows for polycistronic protein expression. This results in the production of two separate proteins: a C-terminal 2A-tagged albumin (ALB-2A) and the therapeutic transgene (*e.g.* MMUT). The percentage of modified *albumin* allele in liver can be directly determined by a qPCR-based assay, and ALB-2A levels in circulation can be quantified by ELISA. Levels of genomic integration in liver and transgene expression as well as ALB-2A in circulation linearly correlate with each other, making ALB-2A a universal circulating biomarker to monitor GeneRide-mediated genome editing in the liver.



LB-001: Methylmalonic Acidemia (MMA)

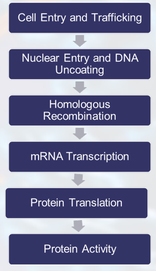
MMUT Metabolic Pathway



Methylmalonic acidemia, or MMA, is a rare and life-threatening genetic disorder, reported to affect 1 in 50,000 newborns in the US. Isolated MMA is primarily caused by mutations in the *Mmut* gene, resulting in an inability to properly process certain fats and proteins. The current standard of care for MMA is patient management and includes severely restrictive, low-protein, high-calorie diet, often delivered through a feeding tube. Even so, patients with MMA can experience significant morbidity and mortality.

GeneRide technology is used in the SUNRISE clinical trial to introduce a corrected *Mmut* gene into targeted cells. Once functional MMUT enzymes are produced in the body, they convert the methylmalonyl-CoA substrate into succinyl-CoA, potentially providing therapeutic effects to the patient. Because sufficient levels of mRNA expression and enzyme functionality are essential in providing a therapeutic effect, two separate analytical methods to measure both steps were developed.

GeneRide Mechanism



Fused mRNA Expression

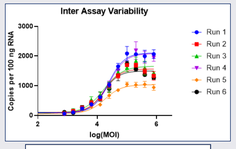
- This assay measures the amount of MMUT fused mRNA that is expressed as a result of site-specific integration of the transgene into the genome.
- Data from a 12-point multiplicity of infection (MOI) curve is fit to a four-parameter logistic fit. The EC50 from the fitting is used to calculate **relative potency** compared to a reference standard.

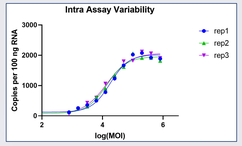
Fused mRNA Assay Workflow

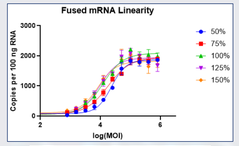
Albumin Locus and Primer/Probe Design

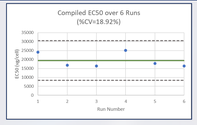
The fused RNA primer/probe set span exons 12 through 14, and will only recognize spliced, fused MMUT RNA and not endogenous or episomal forms

- Experiments were conducted to assess the inter- and intra-assay variability as well as the measurement linearity of the fused mRNA method:

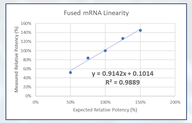








Best-fit values	rep1	rep2	rep3	AVERAGE	%CV
Bottom	121.7	74.93	44.79	80.47	39.32%
Top	2021	1955	2072	2016	2.38%
HillSlope	1.430	1.320	1.122	1.291	9.87%
LogEC50	4.253	4.138	4.144	4.178	1.26%
EC50	**17906**	**13740**	**13932**	**15193**	**12.64%**
R squared	0.9872	0.9866	0.9837	0.9858	0.16%

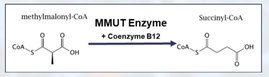


- EC50 %CV values were <20%, indicating that the assay showed good precision. The method also showed strong linearity in relative potency measurements.

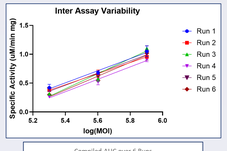
Enzymatic Activity

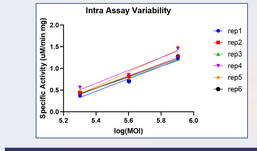
- The amount of MMUT activity as a result of treatment with the sample vector is measured by performing a controlled enzymatic reaction with transduced cells.
  - A knockout cell line is used to measure enzymatic activity without the presence of endogenous MMUT.
- Three MOIs are transduced in triplicate to calculate an Area Under the Curve (AUC) value used to measure **quantitative potency**.
  - The quantitative values can be compared to a reference standard to calculate a relative potency value.

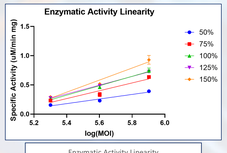
Enzymatic Activity Assay Workflow

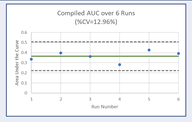


- Experiments were conducted to assess the inter- and intra-assay variability as well as the measurement linearity of the enzymatic activity method:

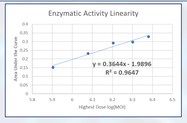








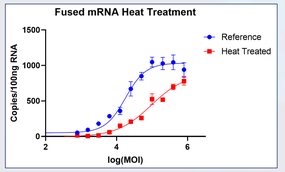
Best-fit values	rep1	rep2	rep3	rep4	rep5	rep6	AVERAGE	%CV
Slope	1.439	1.379	1.274	1.483	1.683	1.365	1.437	8.88%
R^2	0.9801	0.9990	0.9731	0.9601	0.9528	0.9527	0.9696	1.71%
AUC	**0.4504**	**0.5005**	**0.4698**	**0.5613**	**0.5353**	**0.4753**	**0.4988**	**7.76%**

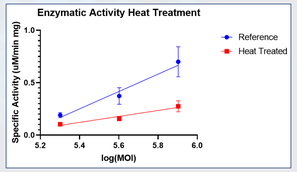


- AUC %CV values were <15%, indicating that the assay showed excellent precision. The method showed moderate linearity in quantitative potency measurements.

Challenging Methods with Heat Treated Sample

- Reference sample was exposed to elevated temperatures to determine if both assays can detect a decrease in potency.





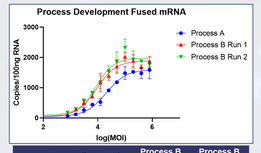
Best-fit values	Reference	Heat Treated
Bottom	51.76	-5.506
Top	1034	886.6
HillSlope	1.526	0.9019
LogEC50	4.252	4.978
EC50	17865	95060
R squared	0.968	0.9738
Relative Potency (%)	**100**	**19**

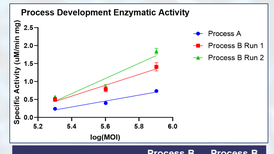
Best-fit values	Reference	Heat Treated
Slope	0.8277	0.2844
R squared	0.8733	0.8347
AUC	**0.2463**	**0.1043**
Relative Potency (%)	**100**	**42**

- Both assays were able to detect a loss of potency in the heat-treated sample demonstrating that they were stability indicating.

Comparing Manufacturing Processes

- The initial manufacturing Process A was optimized by the process development team to yield Process B.
- Drug substance from the two manufacturing processes were run on both assays to compare potency.





	Process A	Process B Run 1	Process B Run 2
Bottom	45.33	49.93	94.99
Top	1602	1686	1983
HillSlope	1.164	1.236	1.331
LogEC50	4.308	3.959	3.942
EC50	20324	9099	8750
R squared	0.9609	0.9504	0.9252
Relative Potency (%)	**100**	**223**	**233**

	Process A	Process B Run 1	Process B Run 2
Slope	0.8245	1.510	2.109
R squared	0.9384	0.9247	0.8920
AUC	**0.2668**	**0.5240**	**0.6192**
Relative Potency (%)	**100**	**196**	**232**

- Material produced with Process B was observed to be more potent than with Process A.
- The two Process B runs demonstrated similar levels of potency, highlighting consistency of the new manufacturing process.

Conclusions

- Precision and linearity parameters were assessed for fused mRNA expression and enzymatic activity assays; both methods were determined to be acceptable for routine use of potency measurements.
- Both methods demonstrated good correlation when measuring samples with differing levels of potency and were also capable of detecting a loss in product stability.
- Future work will focus on developing an ELISA based method to measure MMUT protein translation.
- An assay matrix of fused mRNA expression, protein expression, and enzymatic activity can be used to measure the last three steps in the GeneRide mechanism of action.

References

[1] Barzel A. et al. Nature (2015)