Developing a Potency Assay for AAV-based Genome Editing Vectors

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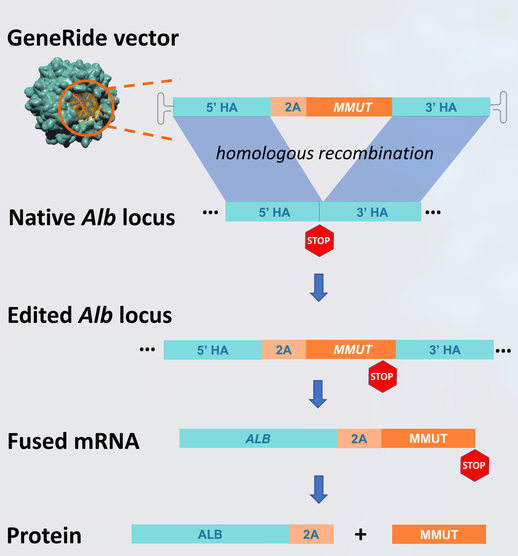
1 GeneRide™, a Nuclease-free Promoterless AAV Genome-Editing Technology

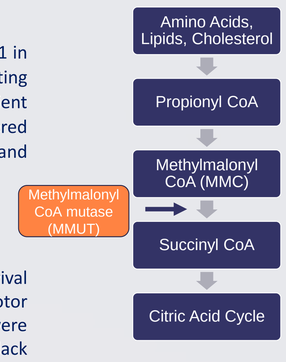
GeneRide is a novel AAV-based genome-editing technology that leverages homologous recombination to insert a therapeutic transgene into a specific locus of the host genome [1]. For liver-directed targets, the transgene is integrated into the albumin locus (*Alb*).

The integrated transgene (*e.g.* methylmalonyl CoA mutase; *Mmut*) can thus "hitch a ride" on the highly active endogenous albumin promoter, which results 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.



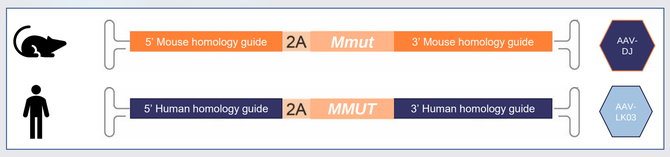
2 LB-001: Methylmalonic Acidemia (MMA)

Methylmalonic acidemia, or MMA, is a rare and life-threatening genetic disorder, affecting 1 in 50,000 newborns. Isolated MMA is primarily caused by mutations in the *MMUT* gene, resulting in an inability to properly process certain fats and proteins. There is no cure for MMA: patient management includes severely restrictive, low-protein, high-calorie diet, often delivered through a feeding tube. Even so, patients with MMA experience significant morbidity and mortality, and the prognosis for long-term survival is poor.



MMA Mouse Model

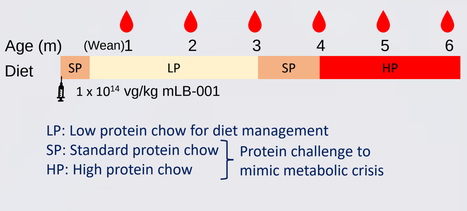
Complete systemic knockout of *Mmut* results in severe neonatal lethality. To improve survival the *Mmut* transgene was reintroduced, driven by the muscle creatine kinase (MCK) promotor for muscle-specific expression. These transgenic *Mut*$^{-/-}$*/MCK-Mut*$^{+}$ mice (MMA mice) were employed as a model for MMA. These mice survive the neonatal period, but because they lack hepatic Mmut activity, they recapitulate many of the clinical characteristics observed in severe MMA patients, including hepatorenal pathology, abnormal growth/development, and increased mortality [2]. Heterozygous *Mut*$^{+/-}$*/MCK-Mut*$^{(+\text{ or }-)}$ mice display normal growth, body weight and lifespan. They serve as housing companions of the MMA mice and are used to determine baseline genomic integration rate.



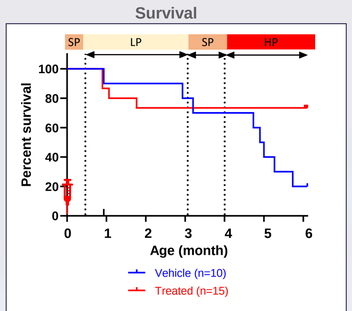
3 mLB-001 Efficacy in Neonatal Mice

Objective and Study Design: A GeneRide vector encoding mouse Mmut (mLB-001) was dosed IV via temporal vein at birth to neonatal MMA mice to evaluate therapeutic efficacy. In order to stabilize MMA mice to allow for observation (untreated animals perish soon after weaning on standard chow) and to mimic clinical management of MMA patients, a low protein diet was introduced prior to weaning.

Once stabilized, a challenge in the form of increased protein diet was applied to MMA mice to mimic a metabolic crisis. The metabolic stress induced by increased protein intake and flux into the metabolic pathway allows for the assessment of therapeutic intervention in the context of a serious medical crisis.



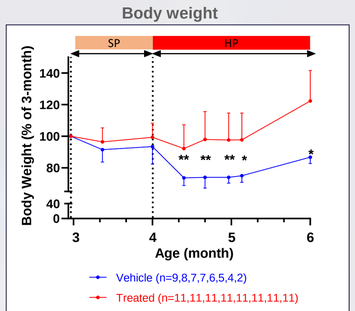
Survival



- **mLB-001 treated mice showed significantly improved survival compared to vehicle controls following high protein challenge**

p<0.05, log-rank test

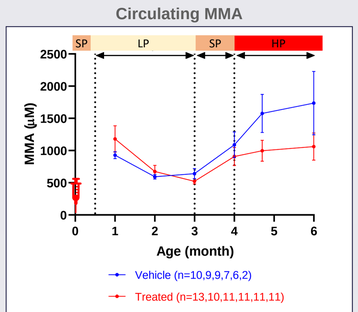
Body weight



- **mLB-001 treated mice showed significantly improved body weight compared to vehicle controls following high protein challenge**

* p<0.05, ** p<0.01, Student t-test comparing vehicle and mLB-001 treated animals at indicated timepoints

Circulating MMA



- **mLB-001 treated mice showed a trend of decreased MMA levels compared to vehicle controls following high protein challenge**

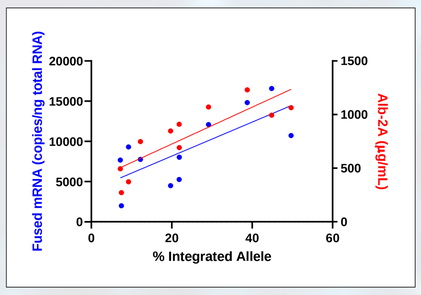
Survival: Health and mortality checks were conducted on all mice, daily. mLB-001 treated mice showed significantly improved survival compared to vehicle controls during the protein challenge phase of the study.

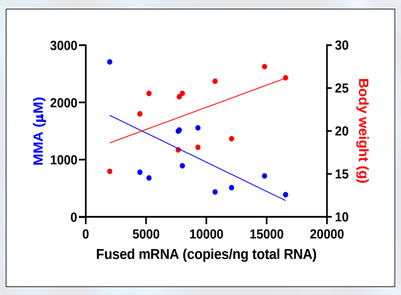
Body Weight: Body weights were measured using scales (precision 0.01 to 0.1 g) approximately bimonthly.

Circulating MMA: Circulating MMA was quantified approximately monthly using LC-MS/MS. mLB-001 treated mice showed a trend of decreased MMA compared to vehicle group during the protein challenge phase of the study.

4 Correlation of Disease Biomarker, Circulating Biomarker, Fused mRNA, and Genomic DNA Integration of *Mmut*

Objective and Study Design: Terminal liver tissue analysis was performed on MMA mice treated with mLB-001 at 6 months post dosing. The variation in survival advantage of edited hepatocytes provided a unique opportunity to correlate genomic DNA integration rate, fused Mmut mRNA levels, circulating ALB-2A protein production, circulating MMA biomarker, and body weight.





With increasing genomic DNA integration of *Mmut*, both higher levels of fused Mmut mRNA and circulating ALB-2A protein are observed. Additionally, with increasing levels of Mmut fused mRNA, a decrease in the levels of circulating methylmalonic acid (MMA) is observed, correlating with an increase in body weight.

Fused mRNA: quantified from frozen liver tissue extracts using an RT-ddPCR assay

Circulating ALB-2A: quantified approximately monthly from mouse plasma using an enzyme-linked immunosorbent assay (ELISA).

Genomic DNA (gDNA) Integration: analyzed terminally from mouse liver samples using an LR-qPCR assay. Genomic DNA was extracted from frozen liver tissues and targeted genomic DNA integration was analyzed by long range PCR amplification followed by qPCR quantification. Integration per allele was calculated from the standard curve with synthesized double-stranded DNA which was used as an integration standard.

Circulating MMA: quantified from diluted whole blood using LC-MS/MS.

5 Development of Human MMUT Fused mRNA Assay

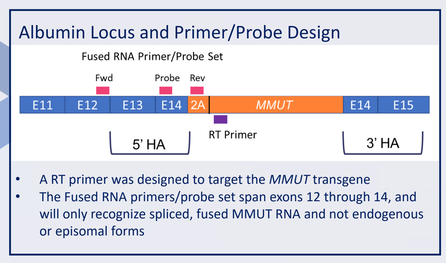
Assay Overview

Transduction of liver cells with hLB-001 → Harvest cells and isolation of RNA → Reverse Transcription → ddPCR quantification of MMUT → Determination of Relative Potency

The goal of the *in vitro* potency assay is to recapitulate the *in vivo* system for screening potential gene therapy candidates.

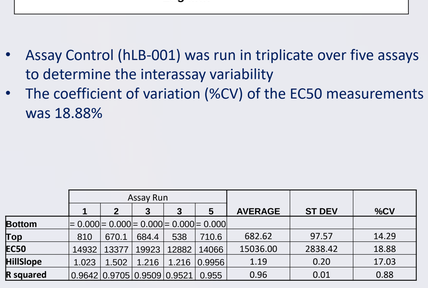
Challenges to developing an *in vitro* potency assay for GeneRide vectors:

- Mouse and human products cannot be directly compared for potency
- A selective advantage is observed with mLB-001 treatment *in vivo*, due to proliferation of corrected hepatocytes. This is not observed *in vitro*.
- Homologous recombination occurs at a low frequency
- High MOIs required for transduction result in high levels of episomal DNA, and may result in cryptic expression of MMUT



- A RT primer was designed to target the *MMUT* transgene
- The Fused RNA primers/probe set span exons 12 through 14, and will only recognize spliced, fused MMUT RNA and not endogenous or episomal forms

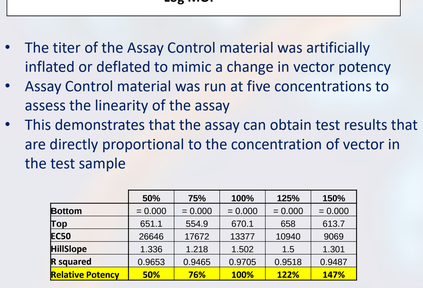
Interassay Variability of Assay Control



- Assay Control (hLB-001) was run in triplicate over five assays to determine the interassay variability
- The coefficient of variation (%CV) of the EC50 measurements was 18.88%

	Assay Run					AVERAGE	ST DEV	%CV
	1	2	3	3	5			
Bottom	= 0.000	= 0.000	= 0.000	= 0.000	= 0.000			
Top	810	670.1	684.4	538	710.6	682.62	97.57	14.29
EC50	14932	13377	19923	12882	14066	15036.00	2838.42	18.88
HillSlope	1.023	1.502	1.216	1.216	0.9956	1.19	0.20	17.03
R squared	0.9642	0.9705	0.9509	0.9521	0.955	0.96	0.01	0.88

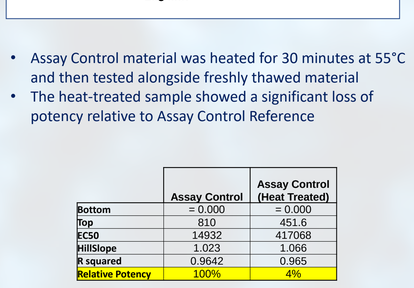
Linearity of Assay Control



- The titer of the Assay Control material was artificially inflated or deflated to mimic a change in vector potency
- Assay Control material was run at five concentrations to assess the linearity of the assay
- This demonstrates that the assay can obtain test results that are directly proportional to the concentration of vector in the test sample

	50%	75%	100%	125%	150%
Bottom	= 0.000	= 0.000	= 0.000	= 0.000	= 0.000
Top	651.1	554.9	670.1	658	613.7
EC50	26646	17672	13377	10940	9069
HillSlope	1.336	1.218	1.502	1.5	1.301
R squared	0.9653	0.9465	0.9705	0.9518	0.9487
Relative Potency	50%	76%	100%	122%	147%

Assay Control Stability



- Assay Control material was heated for 30 minutes at 55°C and then tested alongside freshly thawed material
- The heat-treated sample showed a significant loss of potency relative to Assay Control Reference

	Assay Control	Assay Control (Heat Treated)
Bottom	= 0.000	= 0.000
Top	810	451.6
EC50	14932	417068
HillSlope	1.023	1.066
R squared	0.9642	0.965
Relative Potency	100%	4%

6 Conclusion

- Following protein challenge, mLB-001 treated MMA mice demonstrated benefits in key MMA endpoints including:
  - significant enhanced animal survival
  - significant protection from loss of body weight
  - protection from increase in circulating MMA level
  - in addition, mLB-001 treated MMA mice appeared to be more active than vehicle treated MMA mice (data not shown)
- In the MMA mouse model, increasing levels of genomic DNA integration of *Mmut* was observed to correlate with higher levels of circulating ALB-2A and fused Mmut mRNA. A decrease in the levels of circulating MMA biomarker, and an increase in body weight were also observed with increasing fused Mmut mRNA levels .
- The initial development of the *in vitro* MMUT Fused mRNA assay establishes proof-of-concept as a qualitative potency assay for the assessment of MMUT mRNA expression.
  - The assay demonstrates good repeatability and can detect a loss of potency
- Future work will focus on the development of a potency assay that can demonstrate biological activity of the MMUT enzyme produced from the integrated *MMUT* DNA
  - Further development will investigate the generation and use of a MMUT null cell line to distinguish endogenous MMUT from integrated, GeneRide delivered *MMUT*

References

[1] Barzel A. et al. Nature (2015) *[2] Chandler RJ. et al. FASEB J (2009)*