pLIVE® *In Vivo* Expression and Reporter Vectors

Protocol for MIR 5420, 5520, 5620, 5320

SDS and Certificate of Analysis available at mirusbio.com/literature

Introduction

The pLIVE® vector (*L*iver *I*n *V*ivo *E*xpression) is designed for high level, prolonged expression of transgenes in the mouse liver, often delivered by hydrodynamic teil vein injection (HDI). This vector utilizes a chimeric promoter composed of the mouse alpha fetoprotein enhancer II and the minimal mouse albumin promoter. Two introns have been engineered into the vector to increase the expression of the delivered transgene.

pLIVE®-*lac*Z (encoding β-galactosidase) and pLIVE®-SEAP (encoding *S*ecreted *E*mbryonic *A*lkaline *P*hosphatase) were created for use as positive controls. Expression of *lacZ* can be monitored in the liver using either classical X-gal staining of liver sections or quantitative β-galactosidase assays of liver lysates. Expression of the SEAP gene from pLIVE®-SEAP can be easily monitored using a quantitative assay of mouse serum.

Specification

Concentration	Each vector DNA is supplied at 1 mg/ml in TE buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA)
Storage	Store vector DNA at -20°C. Prior to use, thaw at room temperature, vortex and spin down.
Product Guarantee	Guaranteed for 1 year from the date of purchase, when properly stored and handled.

Materials

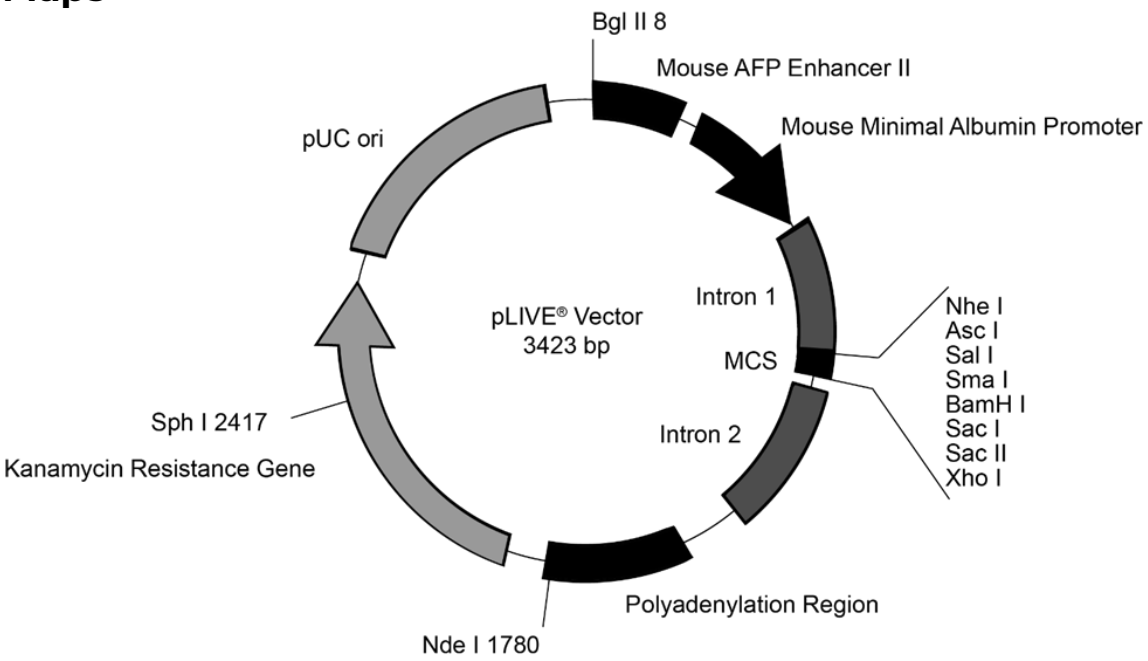
The pLIVE® Vectors are supplied in *one* of the following formats:

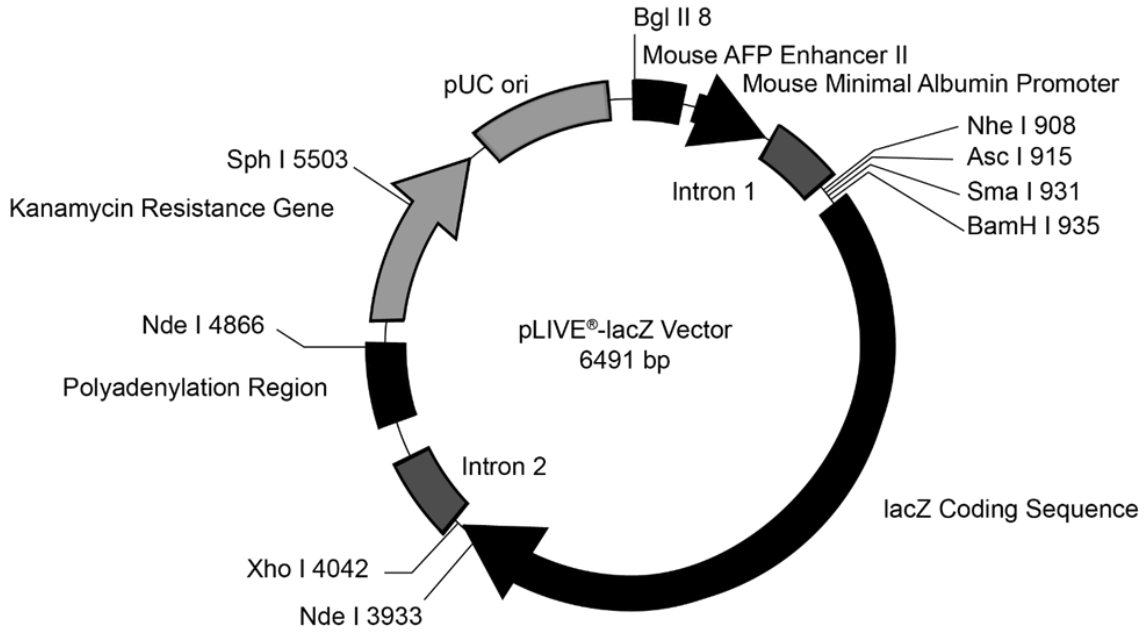
Product Name	Product No.	Quantity	Component(s)
pLIVE® Vector	MIR 5420	20 µg	pLIVE® Vector
pLIVE® Vector/*lacZ* Control Vector Kit	MIR 5520	20 µg each	pLIVE® and pLIVE®-*lacZ* Vectors
pLIVE® Vector/SEAP Control Vector Kit	MIR 5620	20 µg each	pLIVE® and pLIVE®-SEAP Vectors
pLIVE® Vector Complete System (All 3 Vectors)	MIR 5320	20 µg each	pLIVE®, pLIVE®-*lacZ* and pLIVE®-SEAP Vectors

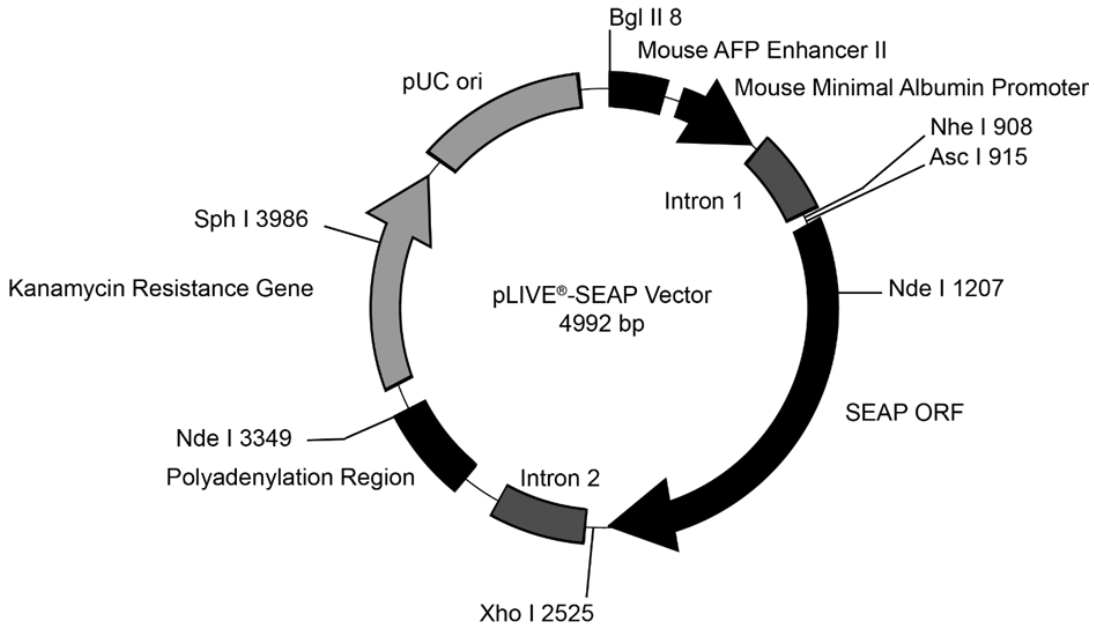
DISCLAIMER
Small-animal research is regulated by federal laws and regulations. Extensive information on this topic is provided by the NIH Office for Protection from Research Risks (**http://www.hhs.gov/ohrp/**). This kit does not confer any approval from regulatory agencies to conduct animal research. Follow all applicable laws and regulations pertaining to the care and use of animals in research. All personnel who handle animals should be properly trained. Familiarity with performing tail vein injections in your particular mouse species will greatly facilitate this procedure.

For Research Use Only

Vector Maps


Bgl II 8
Mouse AFP Enhancer II
Mouse Minimal Albumin Promoter
pUC ori
Intron 1
pLIVE® Vector
3423 bp
MCS
Nhe I
Asc I
Sal I
Sma I
BamH I
Sac I
Sac II
Xho I
Sph I 2417
Kanamycin Resistance Gene
Intron 2
Polyadenylation Region
Nde I 1780


Bgl II 8
Mouse AFP Enhancer II
Mouse Minimal Albumin Promoter
pUC ori
Nhe I 908
Asc I 915
Sma I 931
BamH I 935
Sph I 5503
Kanamycin Resistance Gene
Intron 1
Nde I 4866
pLIVE®-lacZ Vector
6491 bp
Polyadenylation Region
Intron 2
lacZ Coding Sequence
Xho I 4042
Nde I 3933



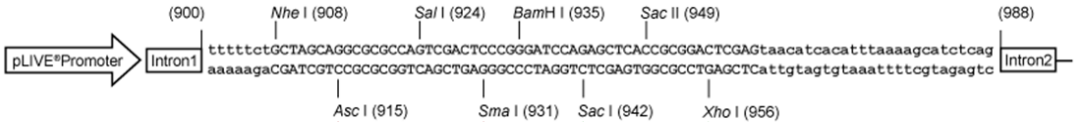
Feature	Vector (bp position)		
	pLIVE®	**pLIVE®-*lacZ***	**pLIVE®-SEAP**
Vector Size (bp)	3423	6509	4992
Mouse Alpha Fetoprotein Enhancer II	8 – 226	8 – 226	8 – 226
Mouse Minimal Albumin Promoter	269 – 586	269 – 586	269 – 586
Intron 1	602 – 900	602 – 900	602 – 900
MCS (*Nhe*I/ *Asc*I/ *Sal*I/ *Sma*I/ *Bam*HI/ *Sac*I/ *Sac*II/ *Xho*I)*	908 – 961	n/a	n/a
E. coli lacZ Coding Sequence	n/a	978 – 4034	n/a
Human Placental Secreted Alkaline Phosphatase (SEAP) ORF	n/a	n/a	933 – 2489
Intron 2	988 – 1329	4074 – 4415	2557 – 2898
Polyadenylation Region	1472 – 1805	4558 – 4891	3041 – 3374
Kanamycin Resistance Gene	1888 – 2683	4974 – 5768	3457 – 4251
pUC Origin	2746 – 3334	5832 – 6420	4315 – 4903

*MCS (Multiple Cloning Sites) restriction enzyme sites can be used for the insertion of the gene of interest

Cloning the gene of interest (GOI) into the pLIVE® Vector

Clone the GOI into the MCS (Figure 1.) of the pLIVE® Vector using standard molecular biology techniques. For detailed information on cloning, see Current Protocols in Molecular Biology[1] or Molecular Cloning: A Laboratory Manual[2].

Figure 1. DNA Sequence of the pLIVE® Vector MCS



A. Compatible Restriction Enzymes

Many of the overhangs produced by the restriction enzymes in this MCS are compatible with the overhangs produced by other restriction enzymes.

Restriction Site	Compatible with overhangs produced by
*Nhe*I	*Avr*II, *Spe*I, *Sty*I (C/CTAGG) and *Xba*I
*Asc*I	*Afl*III (A/CGCGT) and *Mlu*I
*Sal*I	*Psp*XI and *Xho*I
*Sma*I produces a blunt end	any other blunt-ended fragment
*Xma*I also cuts at the SmaI recognition sequence and produces a 5' overhang	*Age*I, *Bsa*WI, *Bsp*EI, *Bsr*FI, *Ngo*MIV and *Sgr*AI
*Bam*HI	*Bcl*I and *Bgl*II
*Sac*II	*Bsi*EI
*Xho*I	*Sal*I

B. General Cloning Architecture

When cloning the GOI fragment into the MCS of the pLIVE® Vector, be certain to include a stop codon at the 3' end of the GOI open reading frame and design the sequence around the ATG start codon (underlined) such that it matches the Kozak translational consensus sequence: **(G/A)**NNATG**G**[3,4]. This consensus sequence will promote the optimal translation efficiency of the GOI mRNA.

1. Due to the presence of intron 2 downstream of the MCS, cloning a GOI such that the stop codon is more than 50 bp upstream of the 5' end of intron 2 could induce nonsense mediated decay (NMD) of the GOI mRNA[5,6] and reduce expression. In order to avoid NMD, clone the GOI open reading frame into the MCS such that the 3' end of the ORF is within 50 bp of the 5' end of intron 2 (bp 988). If the GOI 3' end restriction site is engineered immediately downstream of the GOI stop codon, the fragment can be cloned into the *Sac*I, *Sac*II or *Xho*I sites and the stop codon will be within 50 bp of the 5' end of intron 2.

2. The 5' end of the GOI ORF can be cloned using any of the restriction sites in the MCS.

C. Confirm Sequence

If the GOI fragment was generated by polymerase chain reaction (PCR) before cloning into the pLIVE® Vector, it is important to sequence the entire cloned GOI fragment to verify that no detrimental mutations were introduced during PCR amplification.

Preparation of Plasmid DNA for Delivery

Once the correct clone has been identified and verified, amplify the vector DNA in a suitable *E. coli* strain such as DH5α or DH10b using 30 µg/ml kanamycin sulfate in the culture media for plasmid selection. Use a plasmid purification system that results in vector DNA with endotoxin (lipopolysaccharides, pyrogens) levels that are <30 EU/mg of DNA which are suitable for *in vivo* delivery. If the pLIVE®-*lacZ* and pLIVE®-SEAP Reporter Vectors are going to be used for animal studies, they will have to be amplified in *E. coli* as well using the same procedure.

If the plasmid DNA is contaminated with endotoxin, the MiraCLEAN™ Endotoxin Removal Kit (MIR 5900 or MIR 5910) can be used to easily remove the unwanted endotoxin.

Hydrodynamic Tail Vein Injections

As a leader in the development of the highly efficient hydrodynamic tail vein injection procedure for the delivery of nucleic acids, Mirus Bio uses and recommends this technique for the delivery of nucleic acids to the liver. Optimal expression from pLIVE® Vectors generally occurs 3-5 days post delivery.

Deliver the pLIVE® Vector DNA (pLIVE®-GOI, pLIVE®-*lacZ*, or pLIVE®-SEAP) to the mouse liver using the hydrodynamic tail vein injection procedure developed by Mirus Bio (See Related Products Section). Normally, 10-50 µg of plasmid DNA per mouse is delivered using this technique and the amount injected will ultimately depend on the level of expression necessary for the experiment. The hydrodynamic tail vein injection procedure is explained in detail in the *Trans*IT®-QR (MIR 5240) and *Trans*IT®-EE (MIR 5340) Hydrodynamic Delivery Solution protocols (https://www.mirusbio.com/literature).

The pLIVE® Vectors can be used in conjunction with any delivery technique that will deliver the vector DNA to the mouse liver efficiently.

The ***TransIT®-QR Hydrodynamic Delivery Solution (MIR 5240)*** is optimized for efficient delivery of naked nucleic acids, including the pLIVE® Vectors, to the liver, with the additional benefit that the injected mice demonstrate quick recovery post-injection compared to animals injected using normal saline as the delivery solution.

The **TransIT®-EE Hydrodynamic Delivery Solution (MIR 5340)** is formulated to promote enhanced expression from plasmid expression vectors such as the pLIVE® Vectors after delivery to the liver using hydrodynamic tail vein injections. The expression level obtained using the *Trans*IT®-EE Delivery Solution is approximately 2-3 fold greater than the level of expression obtained after delivery of DNA using the *Trans*IT®-QR Delivery Solution.

Detection of Gene Expression After Delivery

A. Detection of *lacZ* Gene Expression from the pLIVE®-*lacZ* Vector in the Liver

Liver *lacZ* expression from the pLIVE®-*lacZ* Vector can be detected using two different methods. Liver tissue sections can be stained with X-gal, turning the β-galactosidase positive (*lacZ* expressing) cells blue. This assay is useful when a measure of vector delivery efficiency is important or a visual readout of delivery is desired.

LacZ expression can also be quantified in liver lysates using a chemiluminescent assay, such as the Galacto-Light™ Kit (Applied Biosystems). As a general recommendation, livers should be harvested and lysed in approximately 4 ml of lysis buffer. The liver lysates should then be diluted 100-500 fold before being assayed to ensure accurate quantification.

B. Detection of SEAP Gene Expression from the pLIVE®-SEAP Vector in the Liver

SEAP expression from the pLIVE®-SEAP Vector can be detected in the serum of mice using a quantitative chemiluminescent assay, such as the Phospha-Light™ Kit (Applied Biosystems). Serum samples may require approximately 80-300 fold dilution depending on the amount of Vector DNA delivered and the expression level of SEAP obtained.

Troubleshooting Guide

POOR EXPRESSION OF THE GENE OF INTEREST (GOI)	
Problem	**Solution**
Poor delivery of the pLIVE® Vector	Poor delivery will result in low levels of expression from a pLIVE® Vector. If using hydrodynamic tail vein injection (HDI), be certain to inject the full volume of the nucleic acids solution rapidly (4-7 seconds) with steady pressure into the tail vein. For more details on hydrodynamic delivery, consult the *Trans*IT®-QR Hydrodynamic Delivery Solution protocol (https://www.mirusbio.com/literature) or contact Mirus Bio technical support. Use pLIVE®-*lacZ* or pLIVE®-SEAP Reporter Vectors to verify that the delivery technique is working.
Poor translational context surrounding the ATG start codon of the GOI	The best translation efficiency in mammalian cells is usually obtained when the sequence surrounding the ATG start codon of the gene of interest has the following sequence: **(G/A)**NNATG**G**[3,4].
Suboptimal location of the GOI stop codon in the pLIVE® Vector MCS	If the stop codon of the GOI open reading frame is more than 50 bp upstream of the 5' end of intron 2 (bp 988), nonsense mediated decay (NMD) could be induced in the cell, leading to a decreased level of the GOI mRNA in the cell[5,6]. Mirus has observed a 2-3 fold decrease in the level of luciferase expression when the luciferase stop codon is more than 50 bp upstream of the start of intron 2.
Suboptimal amount of pLIVE® Vector DNA delivered	Using the pLIVE®-*lacZ* or pLIVE®-SEAP Vectors, scientists at Mirus Bio routinely deliver 10 µg of vector DNA per mouse and observe robust expression of the reporter genes using the hydrodynamic tail vein injection procedure, optimize the mass of DNA delivered accordingly.
Protein product expressed from the delivered transgene induces an immune response in the mouse	If the transgene protein product is immunogenic in mice, it will induce an immune response which will ultimately lead to the clearance of the liver cells expressing the transgene. An immune response in the mice will usually be indicated by a rapid decrease in the level of expression from the delivered pLIVE® Vector 10-14 days post-delivery. Harvest the mouse livers at earlier timepoints post-injection and re-assay for expression. Alternatively, use C57Bl/6 mice which tend to be less immune-responsive to closely related human genes.
DIFFICULTY WORKING WITH THE pLIVE® VECTORS	
Problem	**Solution**
No transformants obtained with a pLIVE® Vector	The various pLIVE® Vectors contain a kanamycin selectable marker. Be certain to propagate the *E. coli* cells harboring the pLIVE® Vectors in liquid and solid media containing 30 µg/ml kanamycin. DO NOT USE ampicillin.
Poor plasmid yields from plasmid preparations	The vector backbone of the pLIVE® Vectors is derived from pUC, a high copy number plasmid. When purifying plasmid DNA using Qiagen's Maxiprep Kit, use 100 ml of an LB + 30 µg/ml kanamycin liquid culture.

References

1. Ausubel, F.M. *et al.* (2005) *Current Protocols in Molecular Biology* John Wiley & Sons, Inc. Hoboken, New Jersey.
2. Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Press, New York.
3. Kozak, M. (1987) *Nuc. Acids Res.* 15:8125-8148.
4. Kozak, M. (1991) *J. Cell Biol.* 115:887-903.
5. Beyers, P.H. (2002) *J. Clin. Invest.* 109:3-6.
6. Baker, K.E. and R. Parker (2004) *Curr. Opin. Cell Biol.* 16:293-299.