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Degree Final Project

Lama2 lentiviral payload
Directing a treatment for
muscular dystrophy

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ABSTRACT

In this degree final project will be summarized the results from one of the experimental procedures performed in the Translational Synthetic Biology Department in the PRBB-UPF, a research group which has a project focused on finding a treatment for merosin-deficient congenital muscular dystrophy (MCD1A) disease, a variation of congenital muscular dystrophy (CMD) which may be caused by mutations in LAMA2 gene. These mutations may provoke a deficiency or a functional reduction of laminin $\alpha 2$ subunit, the heavy chain of Laminin-211 molecule, also known as merosin, a cell-adhesion molecule that is highly expressed in the basement membrane on skeletal muscle and protects the muscle fiber from damage because of the constant stress of muscle contractions.

We aim to explore a curative treatment for Lama2 patients using animal models to explore its efficacy.

To achieve this purpose, we will improve the expression of mice Lama2 gene in bone marrow cells by using lentiviral vectors to treat muscular dystrophy.

To carry out this process we cloned Lama2 gene into lentiviral vector for this way infect mammal cells with lentivirus and, by extracting RNA, DNA and proteins from those cells, check the lentiviral titer with quantitative PCR assay to analyse its infective capacity.

The results of the project have shown that despite the huge length Lama2 gene it's possible to clone into lentiviral vectors and then by lentivirus infection, transfer the transgene to mammalian cells so that it produces functional proteins in a ratio that can be used to treat the disease.

Keywords: MCD1A, muscular dystrophy, LAMA2 gene, Laminin-211, laminin $\alpha 2$ subunit, curative treatment, animal models, bone marrow, lentiviral vectors, lentiviral titer, quantitative PCR, mice.

INTRODUCTION

Muscular dystrophies are a group of inherited **myogenic disorders**¹ characterized by the increasing weakening and breakdown of skeletal muscles over time.

There are nine major forms of muscular dystrophy [1,2] depending in which type of muscle is affected, the age of onset, the rate of progression and the different symptoms and associated pathologies that can develop.

Of all those types we focused in a subtype of congenital muscular dystrophy (CMD) called merosin-deficient congenital muscular dystrophy [2] (MDC1A) that is present since birth, affects both males and females and is the commonest form of congenital dystrophy in the European population, about 30 – 50 %. Most cases of CMD are from this subtype.

MDC1A causes muscular weakness since birth or during the first months of life and continue by developing severe contractures that can produce joint problems and deformities. The problems that cause this disease may only affect skeletal muscle or can be accompanied within brain effects such as **lissencephaly**² and **hydrocephalus**³ and other organ systems. Severe **hypotonia**⁴ [2], progressive muscle weakness and wasting, joint contractures and consequent impeded motion characterize this incurable disorder, which causes great difficulty in daily life and often leads to premature death.

Several forms of the CMD are caused by defects in proteins thought to have some relationship to the **dystrophin-glycoprotein complex**⁵ [3] and to the connections between muscle cells and their surrounding cellular structure.

One of the ways to develop this disease is by mutations in the LAMA2 gene, found in the 6th chromosome. This may provoke a deficiency or a functional reduction of laminin α 2 subunit, the heavy chain of Laminin-211 molecule (**Figure 1.A**), also known as merosin [3].

Laminin-211 or merosin is a cell-adhesion molecule that is highly expressed in the basement membrane [3] on skeletal muscle and protects the muscle fiber from damage because of the constant stress of muscle contractions. Is composed of three chains: α 2, a β 1 and a γ 1 thus forming an heterotrimeric molecule[2]. This laminin's distribution includes the brain apart from muscle fibres. In muscle, it binds to α -dystroglycan receptor[3] and α 7 β 1 integrin[3] via the LG domain, and via the other end it binds to the extracellular matrix (**Figure 1.B**).

The importance of laminin-211 in skeletal muscle is evident from MDC1A, in which absence of the α 2 chain of laminin-211 leads to skeletal muscle dysfunction.

¹ **Myogenic disorders:** dysfunction that affects muscular tissues.

² **Lissencephaly:** is rare brain disorder where the whole or parts of the surface of the brain appear smooth.

³ **Hydrocephalus:** accumulation of cerebrospinal fluid within the brain.

⁴ **Hypotonia:** is a state of low muscle tone, often involving reduced muscle strength.

⁵ **Dystrophin-glycoprotein complex:** provides the linkage between the cytoskeleton and the extracellular matrix from skeletal muscle fibers.

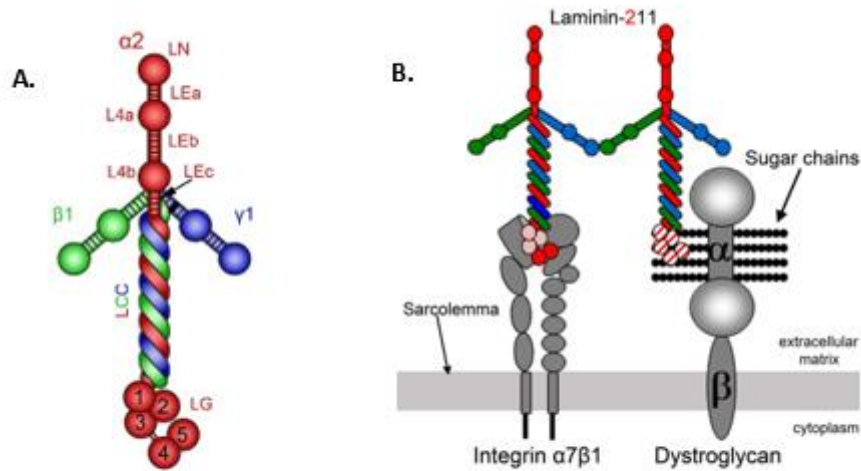


Figure 1 - Schematic representation of laminin-211 structure and its receptors. (A). As it is a heterotrimeric molecule it has 3 main domains: laminin $\alpha 2$ chain is depicted in red, $\beta 1$ in green and $\gamma 1$ in blue. Laminin $\alpha 2$ is the heavy chain and consist of a N-terminal globular domain (LN) followed by 3 tandem rod domains of epidermal growth factor (LEa,LEb,LEc) that separate the LN, L4a and L4b globular domains between them. Then it has the laminin coiled coil (LCC) domain tangled with the LCC domains of the other two chains, $\beta 1$ and $\gamma 1$; and finally, at the C-terminal some laminin globular domains (LG). **(B).** Heavy chain of Laminin-211, the $\alpha 2$ subunit, binds to Integrin $\alpha 7\beta 1$ and to dystroglycan by the laminin globular domains (LG) found in the C-terminal.

Skeletal muscle main function is to move bones. Also, those muscles are in charge to transmit force and resist the stress that muscle contractions entails thanks to the protein chains attaching to the cytoskeleton of the muscle fiber to the surrounding extracellular matrix (**Figure 2**). One of the major components of the extracellular matrix is Laminin-211 that when suffers any mutation results in a defect in the linkage between the muscle fiber and the basement membrane leading the muscle to support too much stress and start consuming and losing strength till it develops the disease.

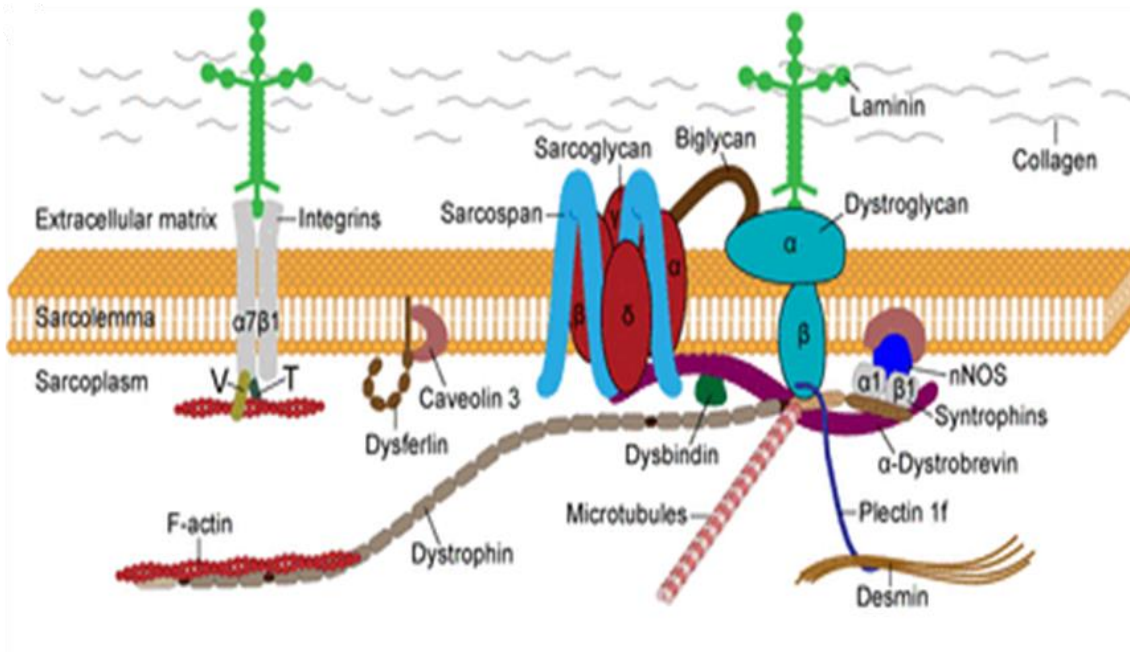


Figure 2 – Overview of Laminin-211 in basement membrane. As it is shown in the image, Laminin 211 is attached in both $\alpha7\beta1$ integrin and dystroglycane. In the case of $\alpha7\beta1$ integrin it acts as a receptor for Laminin 211 in skeletal muscle and promotes the activation of PI3-K/Akt and Ras/Raf/MEK/Erk cell survival pathways a part from others. Then, dystroglycane is a major receptor of Laminin 211 in skeletal muscle which is necessary for binding in the surrounding basement membrane.

Nowadays there is still not a cure for muscular dystrophy but there are many labs working on it. Our lab is one of them. We're doing assays with mice with laminin $\alpha2$ chain deficiency which are reliable models for studies of disease mechanisms and potential therapeutic approaches.

There are some techniques that are being tested to treat this type of disease. Some of those techniques are the use of **CRISPR-Cas9** [4]⁶ technique that allows to do genome editing to correct disease causing splice-site mutations in a mouse model with MCD1A by using an homology-directed-repair-independent mechanism [5].

Another technique used is bone marrow transplant [6] in which has been seen that the injection of bone marrow from healthy mice lead to an increase in lifespan by producing an increase in muscle tone and strength.

Also, there are some assays that tried, using lentiviral vectors [7], to do full length dystrophin gene therapy.

We aim to explore a curative treatment for Lama2 patients using animal models to explore its efficacy.

⁶ **CRISPR-Cas9**: relatively new tool for gene editing.

OBJECTIVES

The aim of our project is to improve the expression of mouse Lama2 gene in bone marrow cells by using lentiviral vectors to treat muscular dystrophy. Is a combination between bone marrow transplant treatment, that will improve the expression of Lama2 protein, Laminin-211, in skeletal muscle fibers and the use of lentiviral vectors deliver Lama2 which slightly exceed the lentiviral packaging limit.

To achieve this purpose, we will start by cloning Lama2 gene in lentiviral transfer vector pSico.

After the cloning we will proceed to produce lentivirus containing the Lama2 gene and infecting cells to obtain modified gDNA, RNA and proteins which will be used to analyse the lentivirus titer by quantitative PCR (qPCR) to see its infective capacity.

MATERIALS AND METHODS

The complete protocols that we used for the experimental procedure are fully explained in the ANNEX A.

Generation of plasmid constructs

To create a construct that contains our Lama2 gene, which is a large gen since it's almost 9,5 kb, we chose pCCL plasmid which is a commonly used plasmid for lentiviral assays. Also, we wanted to clone green fluorescent particle (GFP) into pCCL to use it as a control.

But, as the trial of cloning both Lama2 and GFP gene into pCCL did not work, we proceed to perform the cloning into pSico plasmid, also a commonly used plasmid in lentivirus trials.

All the process that involves the cloning assay into pCCL plasmid will be described in the annex (**ANNEX B**) and only the cloning process in pSico will be explained here.

The pSico plasmid that we have in our lab was already containing GFP (figure on **ANNEX C.c**) so we directly proceed to the cloning of Lama2 in pSico. The plasmid was shortened by removing GFP and other non-relevant parts and a multicloning site (MCS) was added forming a plasmid which we named pSico-MCS (figure on **ANNEX C.b**) that will be used for the cloning assay.

For the cloning of Lama2 gene inside a pSico backbone⁷, first of all a stop codon was needed to add at the end of Lama2 gene in its original plasmid pENTRY, a widely used vector for cloning. This was done by a **site directed mutagenesis**⁸.

Once the stop codon was correctly added, we proceeded to digest both pSico-MCS backbone and pENTRY containing Lama2-stop with *Ascl* and *NotI* restriction enzymes. After the digestion we treated the digested pSico to rSAP enzyme [8] which is a phosphatase that dephosphorylates the 3' and 5' end. This procedure makes easier to join backbone and insert because only one of the fragments has the phosphorylated ends and this promotes the ligation.

When we had both digestions, we purify the samples by using PCR purification kit which would take off all the possible reagents from the digested sample that could interfere with the ligation or simply are not relevant.

Then, when the sample contains only the fragment of interest, we measure the amount of DNA by using nanodrop machine which gives DNA concentration in ng/ μ L.

Next step was the ligation between the Lama2 gene insert and pSico backbone.

For this procedure is necessary to put proportional amounts of each part in order to make an optimal ligation. Also, the amount of each fragment will vary depending on the length of the fragment. In this case as our Lama2 insert has 9,5 kb and pSico backbone 7,5 kb so, we add a 1:1 proportion of the samples, as they have quite similar length.

To know how many μ L were necessary from each sample, we used a digital converter with which knowing the amount of DNA of the sample and the length of the fragment can calculate the amount of DNA needed, which we will multiply by the concentration of our sample (obtained before with the nanodrop) and this way, obtain the amount that needs to be added from each part.

The ligation was done with T4 Ligase [9] and letting it 3 hours at 16°C. Once the ligation was completed we proceeded to transform the resultant plasmid into Stbl3 competent cells [10], which are *E.Coli* competent bacteria that will allow us to know if the cloning had work or not.

We plated the transformation sample into a **Carbenicillin**⁹ antibiotic agar plate and let overnight at 37 °C to promote colony growth.

Next day colonies had grown so we made a random choosing of some of those colonies and prepare culture media to make them grow in suspension. We let them shaking at 37°C overnight and next day we proceed to do plasmid extraction by Miniprep Kit.

⁷ **Backbone:** is the part of the plasmid that includes the replication origin, the antibiotic resistant marker and a polilinker or multicloning site (MCS).

⁸ **Site directed mutagenesis:** is a molecular biology method that is used to make specific and intentional changes to the DNA sequence.

⁹ **Carbenicillin:** is a bactericidal antibiotic belonging to the carboxypenicillin subgroup of the penicillins. Is the analogue of ampicillin and more resistant to degradation.

Finally, to see if the insert had correctly inserted in the backbone, we performed a comprovative digestion with BspQI.

Lentivirus production and cell infection

For lentiviral production, second generation packaging system [11,12] was used. This packaging system consists in 3 plasmid types: the lentiviral transfer plasmid encoding our Lama2 insert, the packaging plasmid that encode components of the viral capsid (psPAX2) and the envelope plasmid encoding the wrap (pMD2.G). Those plasmids were co-transfected into 3.8×10^6 **Lenti-X 293T cells**¹⁰ [12] in concentrations of 1,3 pmol of psPAX2, 0,72 pmol of pMD2.G and for both transgenes, pSico Wild type (WT) that has GFP and pSico with Lama2 1,64 pmol.

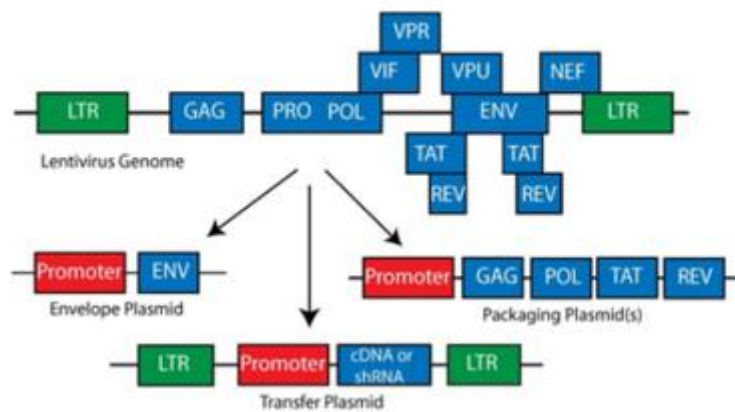


Figure 3 – Second Generation Lentiviral Plasmids. This system contains a single packaging plasmid encoding the following genes: **Gag**¹¹, **Pol**¹², **Rev**¹³ and **Tat**¹⁴. The transfer plasmid is the one containing the **LTR's**¹⁵ and **psi packaging signal**¹⁶. The envelope protein (**Env**) is encoded on a third plasmid.

¹⁰ **Lenti-X 293T cells:** are a subclone of the transformed human embryonic kidney cell line, HEK 293T, which are highly transfectable and support high levels of viral protein expression.

¹¹ **Gag gene:** produces structural proteins for the virus.

¹² **Pol gene:** produces DNA polymerase enzyme.

¹³ **Rev gene:** produces a transactivating protein that is essential for the regulation of lentiviral protein expression.

¹⁴ **Tat gene:** encodes for Tat protein which is a regulatory protein that drastically enhances the efficiency of viral transcription.

¹⁵ **LTR's:** Long terminal repeats. Are identical sequences of DNA that repeat hundreds or thousands of times that are used by viruses to insert their genetic material into the host genomes.

¹⁶ **Psi packaging signal:** is involved in regulating the essential process of packaging the retroviral RNA genome into the viral capsid during replication.

A mixture of the plasmids was done and after, added to a plate containing the Lenti-X 293T cells. We incubate the plates for 3-4 days at 37°C. Day 2, 3 and 4 post transfection, viruses were harvested.

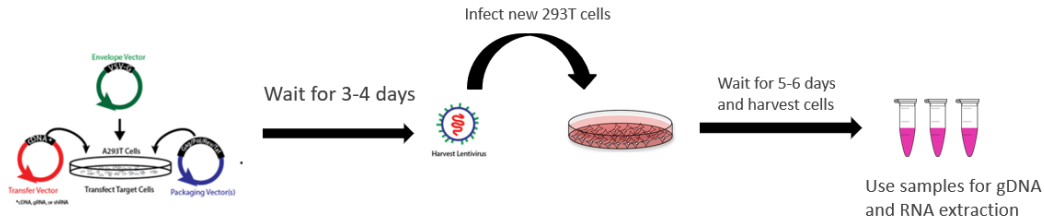


Figure 4 - Lentivirus production process. Scheme of the lentivirus production process since the viral particle formation to cell infection to obtain the modified genetic material and the subsequent obtaining of samples for gDNA, RNA and protein extraction.

After harvesting those viruses, they are added to a new plate containing healthy HEK 293T cells¹⁷ [13] (no Lenti-X 293T cells this time) to infect them and this way, transfer the DNA of interest into them.

As these viruses are all replication incompetent, infected cells will not produce virus and will be only genetically modified as we wanted. These modified cells will be used to extract gDNA, RNA and proteins to lentiviral titer analysis.

gDNA, RNA and protein extraction

Once the lentivirus had infected the cells and those have the inserted transgene; aliquots were done to this way extract gDNA, RNA and protein by then titration can be analysed.

For gDNA extraction we followed the steps stipulated by the kit that we used, DNeasy Blood and Tissue Kit of house QIAGEN.

To perform RNA extraction, we mostly followed the protocol of the kit we used, RNeasy from QIAGEN but, for the steps of Phase separation, RNA precipitation, RNA solubilization and the first steps of sample clean-up we used a different protocol (**ANNEX A.k**).

For the rest of the RNA cleaning we followed the RNasey protocol.

¹⁷ **HEK 293T cells:** are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture that are widely used in cell biology.

Finally, to extract protein, we centrifuged 1 mL of cell suspension and washed the pellet with PBS.

Once the pellet was clean of any medium, we added Radioimmunoprecipitation assay buffer (RIPA buffer), a lysis buffer that releases the proteins from cells.

Lentiviral titration by qPCR

For lentiviral titration by the use of RNA and gDNA we opted for using qPCR instead the other techniques such as ELISA, by measuring the p24 antigen concentration or by flow cytometry (FACS), that counts florescent particles.

In one hand, p24 concentration measures both functional and non-functional vector particles so this assay does not work for us because we only want to detect functional particles. In the other hand FACS do only detect fluorescent particles so it could not detect Lama2 particles since they do not have GFP.

To perform this assay, we used plates of 384 wells which we filled with 5 μ L of the sample we wanted to analyse and 5 μ L of the mastermix sample of SYBRGreen mastermix kit from ThermoFisher house, with the pertinent oligos added.

Once the plate was filled, we seal it with MicroAmp Optical Adhesive Film, also from Thermo Fisher house, to avoid the evaporation of the sample and to make easier for the qPCR machine to read the results of each well. After that we proceed to run the qPCR.

We used the standard cycling mode (default in the program) that consists in 50°C during 2 minutes for activation, 95°C during 2 minutes for the amplification, 95°C for 15 second as denaturation and 60°C for 1 minute for the annealing and extension. The las two steps were repeated x40 cycles.

Once the run has ended the machine creates a .txt document with all the results.

The values that the document provides are the number of cycles or Ct value that the qPCR has done to amplify the amount of DNA we loaded, the standard deviation of those Ct values and other values that for now are not relevant for the titer analysis.

Each sample has its Ct value and as we plated each sample for triplicate, we need to do the average of those Ct values and calculate its standard deviation, even if the document provides it, this way is more accurate.

The following calculations to obtain the transduction units per mL (TU/mL) to analyse lentiviral titer are explained in the results section.

Western blotting

Before starting with the Western Blot, a Bradford assay was done to know protein concentration of our samples.

Once we knew the protein concentration of the samples we extracted from infected cells, we performed a Western Blot to be sure that the protein of our construct, Lama2, was expressing.

We run Bis-Tris Gel 4-12%, a special gel for Western blot assays, for 3 hours in which each well we used, was containing a total mixture of 25 μ L of our sample, **β -mercaptoetanol**¹⁸ and LDS Sample Buffer.

For the transfer we used nitrocellulose membrane and we let it overnight at 4°C.

Next morning, we stained the membrane with **Ponceau S solution**¹⁹ to observe the bands that had transferred from the gel and this way cut the sections in which we intuit that there are the proteins of interest.

Once cut, we began to block the membrane with milk (5%) + TBST 1x solution for 1h at room temp. Then we pass to incubate with the primary antibody, also overnight at 4°C. After that, we washed the membranes 3 times with TBST buffer 1x (10'', 5' and 5') and began the incubation with the secondary antibody for 1 hour at room temp. For primary antibody we used rabbit antibody for Lama2 and mouse antibody for GAPDH and for the second incubation we used anti-rabbit for both membranes.

When the incubation with the secondary antibody was done, we washed again the membrane with TBST buffer 1x (10'', 5' and 5') and proceed to reveal the results by using a chemiluminescent developer and developing sheets in dark room.

¹⁸ **β -mercaptoetanol**: chemical compound used to denature proteins.

¹⁹ **Ponceau S solution**: chemical red coloured dye used to a rapid reversible detection of proteins.

RESULTS

1. Cloning of Lama2 in lentiviral vector

To check if Lama2 gene had correctly inserted inside pSico backbone, a comprovative digestion was performed. The aim of this digestion was that by comparing the results of the real digestion with a digital model generated by **Benchling**²⁰, we could know if the bands that appear in gel electrophoresis in which we loaded the digested samples, were the fragments we expected.

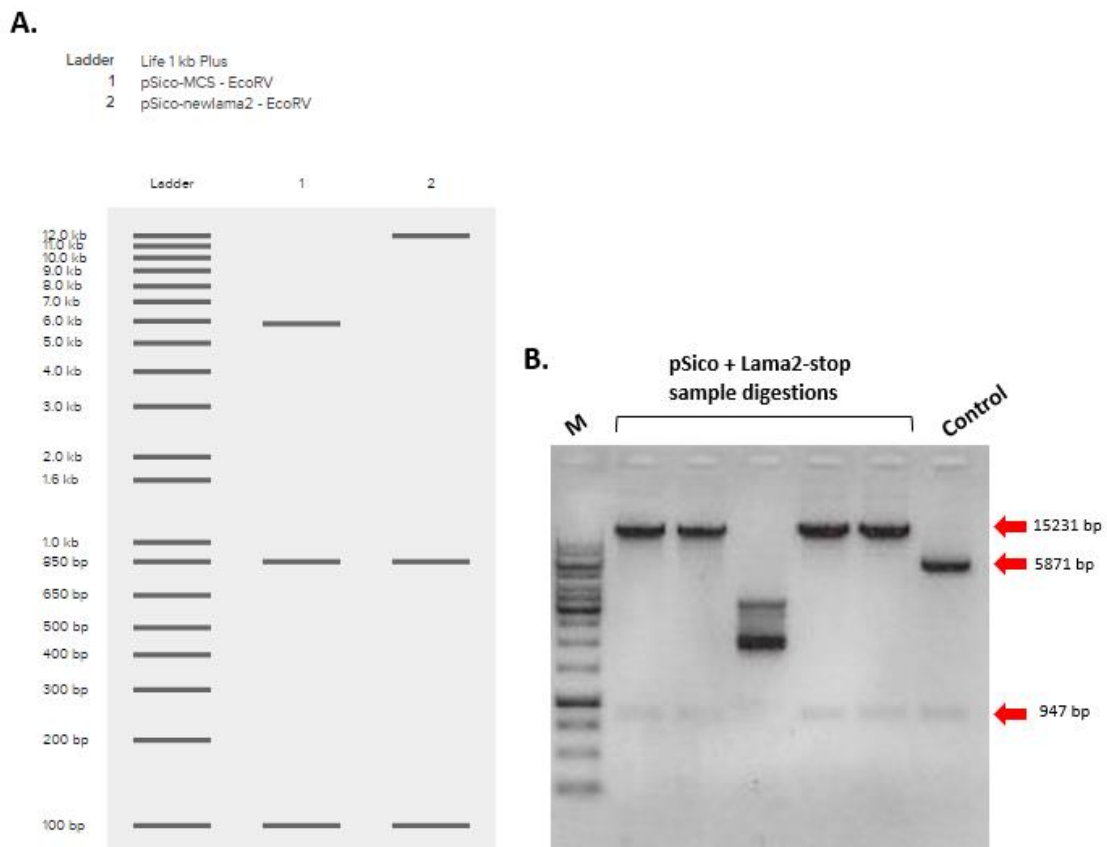


Figure 5 - Comprovative digestion of pSico + Lama2-stop with EcoRV. (A) Digital digestion representation of what should appear when we digest the plasmids. In number 1 there is the digestion of pSico - MCS as control and in number 2 the digestion of pSico + Lama2-stop, both with EcoRV. **(B)** The real digestion. First, we have the marker, then the 5 samples of pSico + Lama2-stop digested with EcoRV and finally pSico - MCS as digestion control.

We can observe that for pSico samples (**Figure 5.A**) the bands that appear concord with the digital digestion (**Figure 5.B**) except for the 3rd sample that we don't know what can be. Also, the bands that appear for the control sample are correct.

²⁰ **Benchling**: is a cloud-based software platform for biology researchers.

In the digital digestion (**Figure 5.A**) is shown that a band of approximately 100 bp should appear for both samples but since the marker we use starts at 250 bp is difficult to see bands with such a little molecular weight.

Also, as a more reliable method, we send to sequence a couple of samples of pSico + Lama2-stop Miniprep, randomly chosen. For both samples the results where favourable but it was showed that one had more coverage that the other.

As the results for pSico were good, we decided to use the sample of pSico + Lama2-stop that had more coverage in sequencing to perform new lentivirus so, we send to sequence the whole Lama2-stop gene to check if it was correct and there were no random mutations.

2. Lentiviral production and titration

To analyse Lama2 lentiviral titer we first perform a qPCR with samples of cDNA done from RNA extracted from cells which were infected with pSico alone (WT) and pSico with Lama2 (the clone we achieved to create) to see the expression of Lama2 through the RNA.

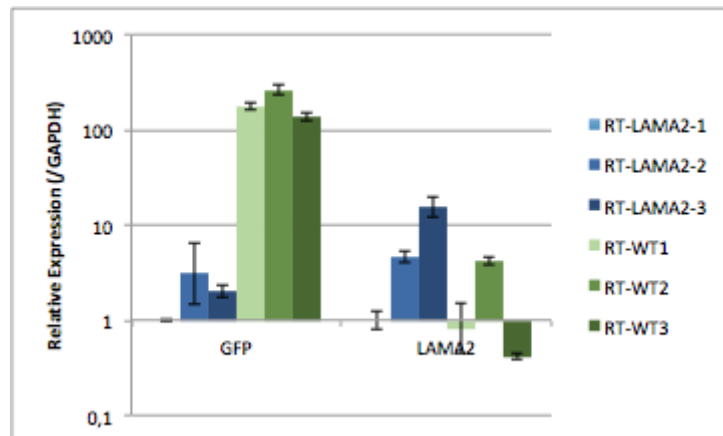


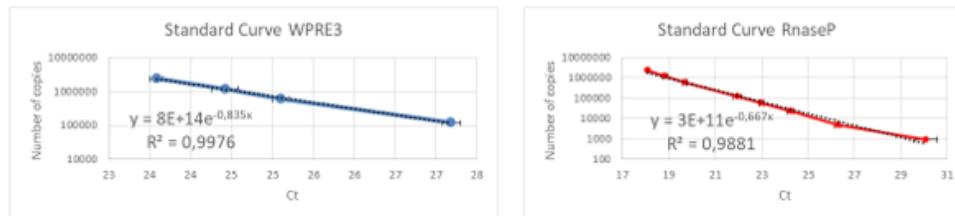
Figure 6 – Results from qPCR in which we were analysing RNA to see its expression in front GFP, used as control. Samples in blue are RNA extracted from infected cells with Lama2 plasmid and the green ones are from cells infected with GFP plasmid that we called WT as pSico has already GFP on it. On the left we can find the relative expression of those samples by using a GFP probe and on the right the same samples but with Lama2 probes and both regarding to GAPDH as housekeeping gene.

In **Figure 6** is represented the relative expression of Lama2 and GFP for probes of those genes regard to GAPDH as housekeeping gene. We can appreciate that for GFP probe, induction of GFP

samples are clear but for Lama2 probe, only RT-Lama2-3 sample express a significant level which means some induction.

Then we analysed gDNA samples. For this assay we loaded dilutions of a control gDNA to create pattern curves for each probe, WPRE3 and RnaseP, to this way then be able to calculate the number of copies per genome that infected cell had. Also, we loaded the gDNA samples extracted from the different infected cells with which we will be able to analyse the lentiviral titer.

A.



B.

	WPRE			RnaseP			Nº copias/Genom e	TU/ml	TU/ml Mean	SD
	Ct Mean	SD	Nº copias	Ct Mean	SD	Nº copias				
GFP-1:2000-5ng	26,39	0,38	255706	21,31	0,09	192464	2,66	425150115	436244591	15689958
GFP-1:2000-5ng	26,42	0,05	249439	21,42	0,15	178434	2,80	447339066		
GFP-1:2000-10ng	26,38	0,26	257036	21,82	0,28	136415	3,77	602951486	445743296	222325955
GFP-1:2000-10ng	26,90	0,06	166885	21,37	0,01	185084	1,80	288535105		
Lama 1:10-5ng	24,70	0,14	1036356	20,23	0,06	396353	5,23	4183568	4297079	160528
Lama 1:10-5ng	24,70	0,04	1037790	20,31	0,02	376472	5,51	4410589		
Lama 1:10-10ng	25,65	0,07	472240	21,30	0,62	193131	4,89	3912294	3727442	261420
Lama 1:10-10ng	25,78	0,03	422443	21,32	0,15	190795	4,43	3542590		

C.

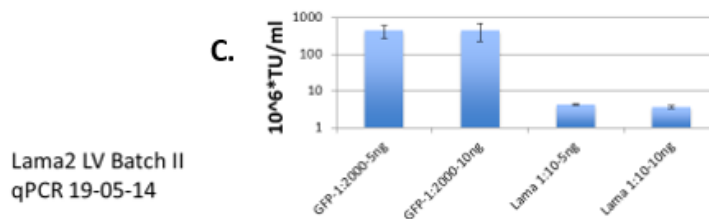


Figure 7 – Results from qPCR in which we were analysing gDNA. (A) Pattern curves done with a control sample of gDNA for probes of WPRE3 and RnaseP. (B) Table which contains the Ct mean for each sample dilution, the Standard deviation and the number of copies for both probes. At the end we can see the number of copies per genome, the transfected units per mL (TU/mL), the mean of TU/mL and its standard deviation. (C) The value of TU/mL of GFP and Lama2 for each sample dilution.

The aim of analysing gDNA samples extracted from infected cells was to obtain the transfected units per mL value (TU/mL) which is necessary to determine the lentiviral titer [14].

First of all, we designed the pattern curves by analysing different dilutions of a gDNA from HEK 293T cells as a control. As we know the number of DNA copies that were in these control samples, we made a relation between these copy numbers and the Ct value that the qPCR

analysed from the control samples thus creating a formula, shown in **Figure 7.A**, that allows to obtain the number of DNA copies of the gDNA samples we were analysing to obtain lentiviral titer.

To obtain the TU/mL value we calculated the number of copies that our analysed samples had, thanks to the formula of the pattern curves, and we made a relation between the number of copies from WPRE3 probe and RnaseP probe, obtaining this way the number of copies per genome. HEK 293T cells are triploid so as the numbers of copies per genome that appear are quite similar means that there is almost one insertion per chromosome. Finally, to calculate the TU/mL value or titer value we multiplied the number of cells at transduction x [Number of copies per genome/mL of lentiviral stock used at transduction]. Those values can be observed in **Figure 7.B**. and the difference between GFP and Lama2 titer in **Figure 7.C**.

3. Western blot assay

As we did not see too much Lama2 expression we decided to perform some Western Blots this way see if actually Lama2 was expressing. We performed two trials.

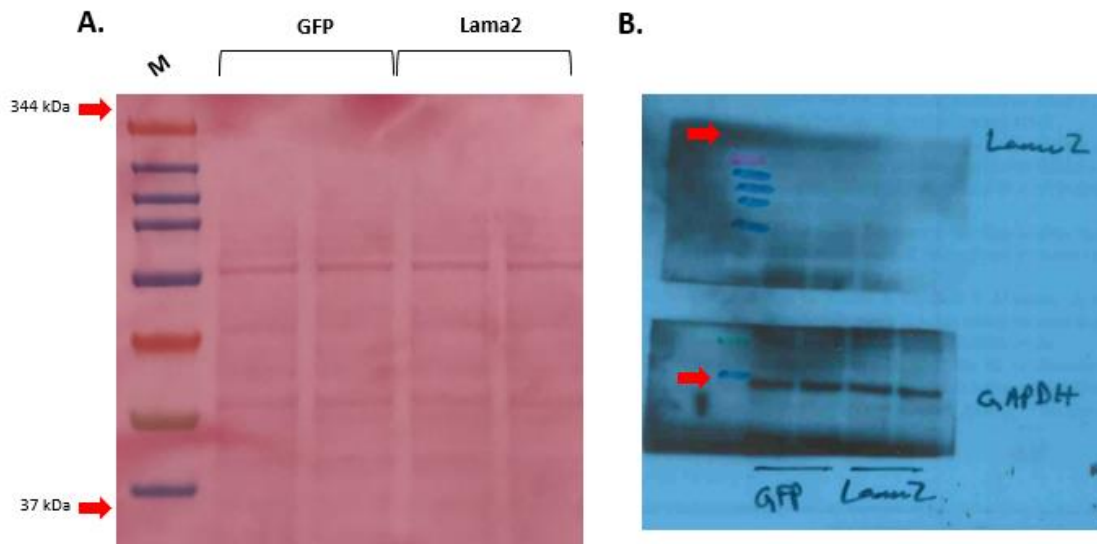


Figure 8 – Western blot assay first trial with 50 ug of protein loading. (A) Nitrocellulose membrane stained with Ponceau S dye. First, we have the marker, then the two samples of GFP and following them the two samples of Lama2. **(B)** Developing sheet with the revealed bands by chemiluminescent developer. Bands are in the same position as in image A.

GAPDH was detected well and loading seems adequate, but no Lama2 seen (**Figure 8.B**). This can be due to lack of expression from transfer plasmid, lack of viral particle production, poor transfer to membrane (which is confirmed) or maybe lack of detection from antibodies, among

other possibilities. After those results, we decided to repeat the western blot by loading more proteins and transferring with the traditional wet RioRad system overnight.

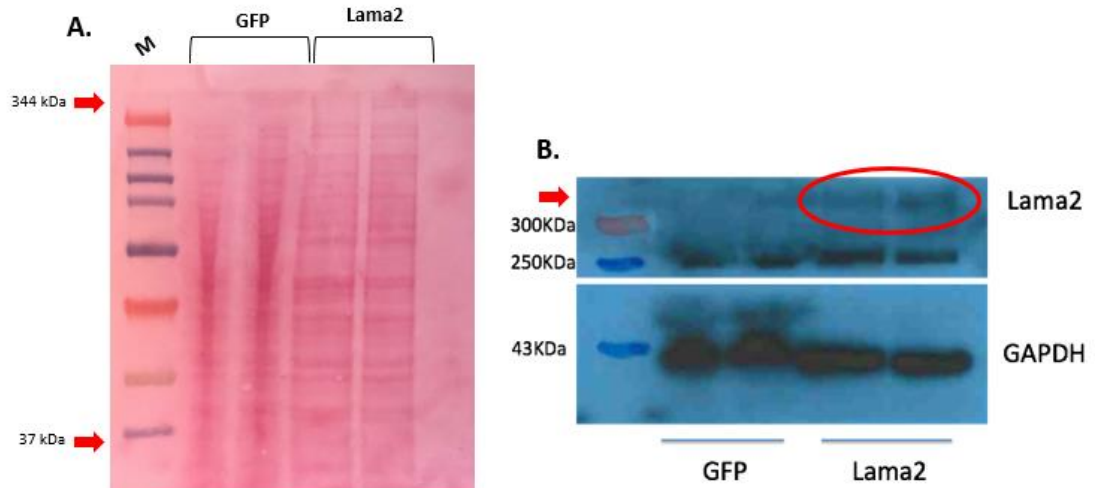


Figure 9 - Western blot assay first trial with 100 ug of protein loading. (A) Nitrocellulose membrane stained with Ponceau S dye. First, we have the marker, then the two samples of GFP and following them the two samples of Lama2. **(B)** Developing sheet with the revealed bands by chemiluminescent developer. Bands are in the same position as in image A. The circled zone shows Lama2 expression, band at 344 kDa.

After primary and secondary incubation, a faint band compatible with Lama2 was seen mostly in Lama2 samples (**Figure 9.B**). This result would need further confirmation, as running and transfer of GFP samples was a bit weird and could affect the results but at least this time seems that we can say that Lama2 is expressing.

DISCUSSION

1. Cloning of Lama2 in pSico lentiviral vector

Cloning Lama2 into pSico backbone was relatively easy compared to the cloning into pCCL, which finally did not work.

As pSico has only one cutting site either for *Ascl* and *NotI* we did not have any problems to insert Lama2 gene which was also cut with those enzymes.

To verify that the insertion was correct done a comproivative digestion was performed and as it can be seen in **Figure 5.B**, the bands that appear for pSico + Lama2-stop samples, one at 15 kb and other at 950 bp approximately, concord if we take a look to the digital digestion represented in **Figure 5.A**. Same happens for the control sample which means that the enzyme worked well.

For the third sample of pSico + Lama2-stop present in **Figure 5.B**, an unexpected band appears that does not agree with any of the expected bands, we have no explanation of what can be.

2. Lentiviral titration by qPCR

For the qPCR in which we were analysing the RNA the results showed that GFP is easy to induce at least in HEK 293T cells but Lama2 does not have that much capacity but seems that at least is possible.

Then, in the analysis of gDNA as it showed in the graphic from **Figure 7.C** there is more titration for GFP than Lama2 in a ratio of x100 times which makes sense due the length of the insert, but this also means that the assay in mammal cells HEK 293T there is production of Lama2 protein so it could also work for bone marrow cells for mice assay.

3. Western blot to check protein expression

The aim of the western blot assay was detecting any expression of Lama2 protein to this way ensure that the cloning has worked and, no less important, that actually the gene is expressing. We expected 2 types of bands: one corresponding to the housekeeping gene GAPDH with a molecular weight of 37 kDa which should appear in all the samples and another at 344 kDa corresponding to Lama2 protein which should appear only in Lama2 samples. We have compared the expression between the membrane stained with Ponceau S solution and with a chemiluminescent developer which prints the results in a developing sheet revealed in a dark room.

For the first assay that was performed, in **Figure 8.A**, image from the membrane stained with Ponceau S dye, none of the bands of interest can be appreciated but only GAPDH in the chemiluminescent developer, **Figure 8.B**.

For the second assay for the Ponceau S staining, present in **Figure 9.A**, a faded band can be assumed above the 300 kDa marker band which could be Lama2, but apparently, there is no appearance of GAPDH band. Then when we developed the membrane with the chemiluminescent reagent, in **Figure 9.B**, we can observe bands in all samples around 40 kDa which is GAPDH and other two bands in Lama2 samples that are above 300kDa that is a sign of Lama2 protein expression.

CONCLUSION

We finally achieved to clone Lama2 gene in a lentiviral vector, even if was not the one that we wanted to use at first, but finally pSico worked very well for all the assay.

Also, that we could clone a full functional large gene, which is quite difficult because of random mutations and loose of functionality, by using a lentiviral vector was not a great difficulty.

Lentiviral vectors have a limited transgene carrying capacity and this is why as the size of the payload increases the lentiviral titer is reduced. Despite this, we could achieve an acceptable expression of Lama2 that allows us to start the assay with mice and see if the bone marrow treatment with this lentiviral titer is optimal and could really work or still need improvement and optimization.

As future directions, besides the assay with mice, will begin to try to clone human LAMA2 gene into the pCCL plasmid.

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ANNEX

A. PROTOCOLS

In the following pages there are explained all the protocols that were used to perform all the experiments described in the project.

a. Protocol for plasmid digestion with restriction enzymes

We use NEB restriction enzymes [15] which use different types of buffer depending for which efficiency we want to obtain with each enzyme.

STEPS:

- Set up reaction as follows:

COMPONENT	50 μ l REACTION
DNA	1 μ g
10X CutSmart Buffer	5 μ l (1X)
Restriction enzyme	1.0 μ l (or 10 units)
Nuclease-free Water	to 50 μ l

- Incubate at 37°C for 1 hour, inactivate at the optimal temperature for the enzyme used for 20 minutes.

b. Protocol to dephosphorylate 5' and 3' ends of DNA either RNA

To perform this reaction, we used an enzyme called Shrimp Alkaline Phosphatase (rSAP), a heat labile alkaline phosphatase purified from a recombinant source that allows to remove 5' and 3' phosphates from DNA, RNA and dNTPs. Usually we used this enzyme after restriction enzyme digestions.

STEPS:

- Add to the digested sample 2,5 μ l of rSAP.
- Incubate at 37°C for 30 minutes.
- Inactivate the enzyme at 65°C 5 minutes.



c. Protocol for ligation with ligase T4

STEPS:

- Mix in a nuclease-free eppendorf the following reagents:

Reagent	1x
10X buffer	2 μ L
T4 ligase	1 μ L
Vector	Variable
Insert	Variable
Nuclease free Water	Up to 20 μ L

- Depending on the fragments used and its previous treatments incubate the samples as convenient between the following options.
- 16°C overnight.
- 37°C 10 minutes.
- Room temp (~25°C) for 2 hours.

d. Protocol for transformation to Stbl3 competent cells

We used One Shot® Stbl3™ Chemically Competent *E. coli* from ThermoFisher house that are designed especially for cloning direct repeats found in lentiviral expression vectors.

STEPS:

- Thaw, on ice, One Shot™ Stbl3™ chemically competent cells and transfer 25 μ L to a nuclease-free eppendorf previously cooled in ice.
- Add 5 μ L of the DNA (10 pg to 100 ng) sample into the cooled eppendorf mix gently without vortexing.
- Incubate the mixture on ice for 30 minutes.
- Heat-shock the cells for 45 seconds at 42°C without shaking.
- After heat-shock place the sample on ice for 2 minutes.
- Add 250 μ L of pre-warmed S.O.C. medium to the cells.
- Let them for 1 hour at 37°C shaking incubator at 225 rpm.
- Spread 25–100 μ L (50 μ L recommended) from transformation sample on a pre-warmed selective plate.
- Store the remaining transformation mix at 4°C. Additional cells may be plated out the next day, if desired.
- Invert the selective plate(s) and incubate at 37°C overnight.
- Select colonies and analyse by plasmid isolation, PCR, or sequencing.



e. Protocol for colony PCR [16]

From a plate in which colonies have grown, follow the next steps:

- Add 3 μL of Nuclease-free Water in an eppendorf (we usually use strips of 200 μL eppendorf).
- Prepare a plate with the same antibiotic as the one with the colonies and draw a grid to classify the copied colonies and number the parcels.
- Once drawn, proceed to chop colonies. Touch softly a colony, then touch the plate with the grid and finally soak the tip inside the strip of eppendorf filled with water.
- After the colonies have been chopped proceed to prepare the PCR. We usually use Standard Taq. DNA Polymerase Kit.
- Prepare the following mastermix. Multiply the amount of reagents depending of the number of colonies. Prepare in ice:

Reagent	For 12,5 μL
10x Standard Taq. (Mg-Free) Reaction Buffer	1,25 μL
3mM MgCl ₂	1,5 μL
10mM dNTPs	0,25 μL
10uM Forward Primer	0,25 μL
10uM Reverse Primer	0,25 μL
Taq. DNA Polymerase	0,0625 μL
Nuclease-free water	5,9375 μL
Template DNA	3 μL (Already in the Eppendorf)
TOTAL	9,5 Mastermix + 3 sample

- Run the PCR:

STEP	TEMP	TIME
Initial Denaturation	95°C	30 seconds
30 Cycles	95°C	15-30 seconds
	45-68°C	15-60 seconds
	68°C	1 minute/kb
Final Extension	68°C	5 minutes
Hold	4-10°C	

- Once the PCR has finished run an agarose gel at the proper agarose percentage to see the results.



f. Protocol for end repair [17]

STEPS:

- Add the following components to a sterile nuclease-free tube:

Reagents	µL
NEBNext Ultra II End Prep Enzyme Mix	3 µl
NEBNext Ultra II End Prep Reaction Buffer	7 µl
Fragmented DNA	50 µl
Total Volume	60 µl

- Gently pipette the entire volume up and down at least 10 times to mix thoroughly.
- Perform a quick spin to collect all liquid from the sides of the tube.
- Then use thermocycler machine to perform the following process:

TEMP	TIME
20°C	30 minutes
65°C	30 minutes
4°C	Infinite

g. Protocol for lentiviral production

This is a summary of the real lentiviral production protocol from Addgene. To get all the information check the full protocol of Addgene – Lentiviral Production.

This protocol can be used to produce lentivirus from a lentiviral vector transfected into Lenti-X 293T cells using a polyethylenimine (PEI) transfection protocol. This procedure can be modified for alternative packaging cell lines or transfection reagents. Once produced, lentivirus can be used for a variety of downstream applications such as stable-cell line generation.

Workflow Timeline:

Day 0	Seed 293T packaging cells
Day 1 (pm)	Transfect packaging cells
Day 2 (am)	18 hours post transfection. Remove media, replace with fresh media



Day 3-4 (am)	Harvest virus

STEPS:

- Seed 293T packaging cells at 3.8×10^6 cells per plate in DMEM complete in 10 cm tissue culture plates.
- Incubate the cells at 37 °C, 5% CO₂ for ~20 hours.
- Gently aspirate media, add 10 mL fresh DMEM complete containing 25 μM chloroquine diphosphate and incubate ~5 hours.
- For 10 mL of DMEM complete, add 10 μL of 25 mM chloroquine diphosphate.
- Prepare a mixture of the 3 transfection plasmids:

Reagents	Amount per 10 cm dish*
psPAX2	1.3 pmol
pMD2.G	0.72 pmol
Transfer Plasmid*	1.64 pmol
OptiPro SFM to total volume	500 μL

*Plasmid concentrations and ratios should be optimized for each transfer plasmid.

Pro-Tip Endotoxins can inhibit transfection; therefore, plasmid DNA purification should include an endotoxin removal step. For high quality plasmid DNA, the plasmid should also be propagated in an endonuclease negative E. coli strain such as NEB stable.

- Dilute the above 500 μL mixture into 500 μL PEI-OptiPro SFM with enough PEI such that the ratio of μg DNA:μg PEI is 1:3 (1000 μL total per 10 cm dish).
 - Gently add the diluted PEI to the diluted DNA. Add the diluted PEI dropwise while gently flicking the diluted DNA tube. Incubate the mixture 15-20 min at room temperature.
 - Carefully transfer the transfection mix to the Lenti-X 293T packaging cells. Add the transfection mix dropwise being careful not to dislodge the cells.
 - Incubate the cells for 18 hours, or until the following morning.
 - The following morning, carefully aspirate the media. Replace the media with 15 mL of DMEM complete.
 - Incubate the cells.
- Virus can be harvested at 48, 72, and 96 hours post transfection in individual harvests or a combined harvest where all the individual harvests are pooled. If pooling harvests,

transfer the harvested media to a polypropylene storage tube and store at 4 °C between harvest.

- Centrifuge the viral supernatant at ~500 g for 5 minutes to pellet any packaging cells that were collected during harvesting.
- Filter supernatant through a 0.45 µm PES filter.
- The viral supernatant can be stored at 4 °C for several hours but should be aliquoted and snap frozen in liquid nitrogen and stored at -80 °C as soon as possible to avoid loss of titer.

h. Protocol for Quantitative PCR

Quantitative PCR or real-time PCR is a technique used to amplify and simultaneously quantify the DNA of a given sample in absolute terms.

The agents that are used for this type of technique are the same as for a normal PCR but in addition a substance marked with a fluorophore is added which will allow to measure the amount of copies that are produced due to the fluorescence sensors of the qPCR machine that measure the light that occurs every time a copy is made.

To test samples for qPCR, a 384 wells plate is used. The sample distribution has to be planned before the sample loading so as not to make mistakes when placing reagents.

This is an example of qPCR planning:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1					WPRE 1	
2	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5					WPRE 2	
3	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10					WPRE 3	
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1					Amp	
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5					Rnasel	
6	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10					Lama2	
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1					GFP	
8	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5					Samples	
9	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10					Ctrl	
10	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20	H20						
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
12	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10						
13	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100						
14	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000						
15	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000						
16	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05	1E+05						
17																												
18	Concentrations are from - to +.																											

Figure 10 - Example of qPCR planification. The colours mark the different probes that will be used as a target to amplify the DNA. The numbers that appear within each plot correspond to the different sample concentrations that will be analysed.

As in each experiment there must always be a control sample analysis to be able to make a pattern curve and the typical deviation, these samples are those of the grid with the thick edge.



Once planned the 384 well plate will dilutions are done and then can start filling the plate.

The qPCR reagents are already mixed in the kit that we use, so only mix it with the sample.

In each well, 5 μL of sample and 5 μL of qPCR mastermix are placed. To go faster when filling the wells, an 8-channel multipipet is used with which you should be careful not to make mistakes as the samples have no colour.

Once all the necessary wells are filled, the plate is placed on the machine specialized in qPCR process and the program is activated, where the wells are arranged and what they contain.

Once the program is available, the wells will start the analysis and the machine will begin to perform the PCR.

Once finished, a .txt file will be created with the results, named as marked on the machine's program. This file contains information about the number of copies made for each sample, the typical deviation and certain aspects but they are not needed for the analysis we carry out.

i. Protocol for Bradford assay (BPA)

Always do samples and blanks in triplicates

MATERIALS:

- BPA Reagent
- Samples9
- 6 well White Wall plates

STEPS:

- Prepare 1X BPA reagent by diluting 5X stock in deionized water.
- Add 100 μL of 1X BPA reagent into each well.
- Use 1 μL of protein samples to test the volume of protein to add to each well by eyeballing the colour change (if colour change is too faint, use 2 μL).
- Measure absorbance at 595 nm.
- Calculate the concentration of the protein with the equations below (obtained from a standard curve).



j. Protocol for Western Blot assay [18]

MATERIALS

- Samples
- NuPAGE® LDS Sample Buffer (4X) (Invitrogen)
- NuPAGE® Reducing Agent (10X) (Invitrogen)
- NuPAGE® MOPS SDS Running Buffer (20X) (Invitrogen)
- NuPAGE® Bis-Tris Gels (Invitrogen)
- XCell SureLock® Mini-Cell gel running tank (Invitrogen)

STEPS:

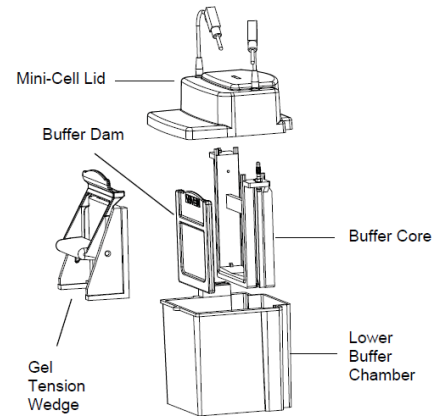
Sample preparation – Cell Lysate

- After washing cell pellet with PBS, completely remove PBS.
- To lyse cells, add 40ul of Protein Lysis Buffer (PLB) to cell pellet (for 12-well plates). Pipette up and down to completely resuspend pellet.
- Incubate on ice for 10 mins. Flick the bottom of the tubes occasionally to ensure that lysate is homogenous.
- Spin at maximum speed for 10mins at 4 degrees.
- Transfer supernatant to a fresh Eppendorf tube.
- Prepare sample buffer – combine 800ul of 4x sample buffer and 200ul of 10x reducing agent for a final concentration of 4x sample buffer and 2x reducing agent.
- Measure the protein concentration with BPA (refer to BPA protocol)
- Prepare samples for loading by calculating the amount of proteins needed per well, dilute it in PLB, and add 4x sample buffer to a 1x final concentration in an Eppendorf tube.
- Heat the tubes for 5 mins at 95 degrees.
- Spin down at max speed for 1 min.

Set up Gel Tank

- Prepare 1X running buffer by diluting 20X NuPAGE MOPS SDS Running Buffer with milliQ water.
- Remove the white tape near the bottom of the plate.

- Pull out the comb in one fluid motion to expose the wells (take care not to collapse the wells).
- Place the plates on either side of the buffer core with the short place facing inside.
- Place the buffer core into the lower buffer chamber.
- Place the gel tension wedge into the tank and lock it in place.
- Fill the upper chamber with enough 1X running buffer to completely cover the sample wells. ENSURE that it is not leaking.
- Fill the lower chamber with enough running buffer to cover at least 1/3 of the tank. (Higher voltage – More buffer)



Load samples

- Load samples in an order similar to the below:

Ladder	1	2	3	4	5	6	7	8	9	10	11	12	Ladder
--------	---	---	---	---	---	---	---	---	---	----	----	----	--------

- Connect the tank to the power supply and run the gel at 150V until the dye front passes the stacking layer and run at 180V until the dye front is at the bottom of the gel.
- Proceed to western blot, Coomassie staining etc.

Electrotransfer

After the gel has run, proceed to transfer the proteins to a nitrocellulose membrane.

- Cut a piece of membrane from the stock roll suitable with the gel dimensions.
- Wet the sponge and filter paper in transfer buffer, and wet the nitrocellulose membrane in methanol.
- Separate glass plates and retrieve the gel.
- Create a transfer sandwich as follows: Sponge, 2 filter papers, Bis-Tris Gel, nitrocellulose membrane, 2 filter papers and sponge.
- *Tip: Ensure there are no air bubbles between the gel and PVDF membrane, and squeeze out extra liquid.
- Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4°C.
- Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer.
- Place electrodes on top of the sandwich, ensuring that the membrane is between the gel and a positive electrode.
- Transfer for 90 minutes or for a better transfer, overnight at 4°C chamber.



Blocking, antibody incubation and revelation

The membrane can be stained with Ponceau S dye to a rapid reversible detection of proteins. Then proceed to:

- Block the membrane with 5% skim milk in TBST* for 1 hour.
- Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4°C on a shaker.
- Wash the membrane with TBST for 5 minutes. Do this 3 times.
- Add secondary antibody in 5% skim milk in TBST, and incubate for 1 hour.
- Wash the membrane with TBST for 5 minutes. Do this 3 times
- Prepare a chemiluminescent mixture and add to the membrane.
- Visualize the result in the dark room.

k. Protocol for RNA extraction

Those are the steps we followed for RNA extraction which are different from the kit protocol.

STEPS:

Prepare RNA samples

- Remove growth media from culture dish
- Add 1mL of Quiazol reagent directly to the cells in the culture dish per 10 cm² of culture dish surface area.
- Lyse the cells directly in the culture dish by pipetting the cells up and down several times.
- Lysate can be stored at -80°C.
- Incubate the homogenized sample for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
- Transfer the samples to bench top to defrost.
- After the samples are completely thawed, centrifuge at 12000 g, 4°C for 10 minutes.
- Transfer the supernatant to clean pre-labelled tube.

Phase separation

- Add 200 µL BCP per 1mL of Quiazol. Vortex continuously for 30 seconds. Incubate at room temperature for 10 minutes.
- Centrifuge at 12000 g, 4°C for 15 minutes.



- Transfer two times of 200 μ L of the aqueous phase to a clean pre labelled 1,5 mL tube. This step is critical for not to get any contamination from edge or the lower phase, in case of contamination, vortex and centrifuge for 10 minutes.

RNA precipitation

- Add 500 μ L of isopropanol to each tube. Invert the tube 2-3 times. Incubate at room temp for 10 minutes.
- Centrifuge at 12000 g, 4°C for 10 minutes. Pour the supernatant.
- Add 1mL of ethanol 75%. Vortex to resuspend the RNA pellet.
- Centrifuge at 12000 g, 4°C for 5 minutes. Pour the supernatant.

RNA solubilization

- Spin again at 12000 g, 4°C for 5 minutes. Remove the remaining liquid by a P200 tip.
- Dissolve the RNA pellet in 100 μ L of MiliQ water.

RNA clean-up

- Make a solution of 3,5 mL RLT buffer + 2,5 mL of 100% ethanol. Add 600 μ L of this solution to each RNA sample tubes. Load sample in to a Qiagen RNasey column. Continue with the kit protocol.

B. CLONING OF LAMA2 AND GFP INTO PCCL LENTIVIRAL VECTOR

In this section cloning of Lama2 and GFP genes into pCCL and also pSico lentiviral plasmids will be explained due both assays were done in parallel.

Cloning of Lama2 and GFP were done at the same time.

We started the experiment by pretending to clone Lama2 and GFP gene inside a pCCL vector that we renamed pCCL Clinically Relevant (pCCLcr) as was modified for this project.

The first cloning's were done by digesting pCCLcr with Ascl and NotI, same restriction enzymes that Lama2 and GFP were cut.

Electrophoresis gel of the assembly between GFP and pCCLcr both digested with Ascl/NotI:

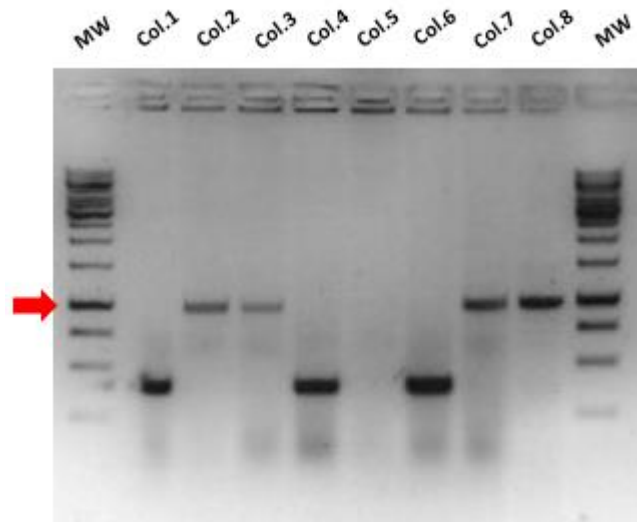


Figure 11 – Results from Colony PCR of assembly between GFP and pCCLcr. The first and the last well contains MW, the samples between them are the ones resultant from the colony PCR.

In the image we can see that in samples 2,3,7 and 8 there's a band around 1 kb that means that GFP has inserted in the plasmid.

To see if indeed GFP was correctly inserted in pCCLcr a comproivative digestion was done. We chose BspQI as restriction enzyme.

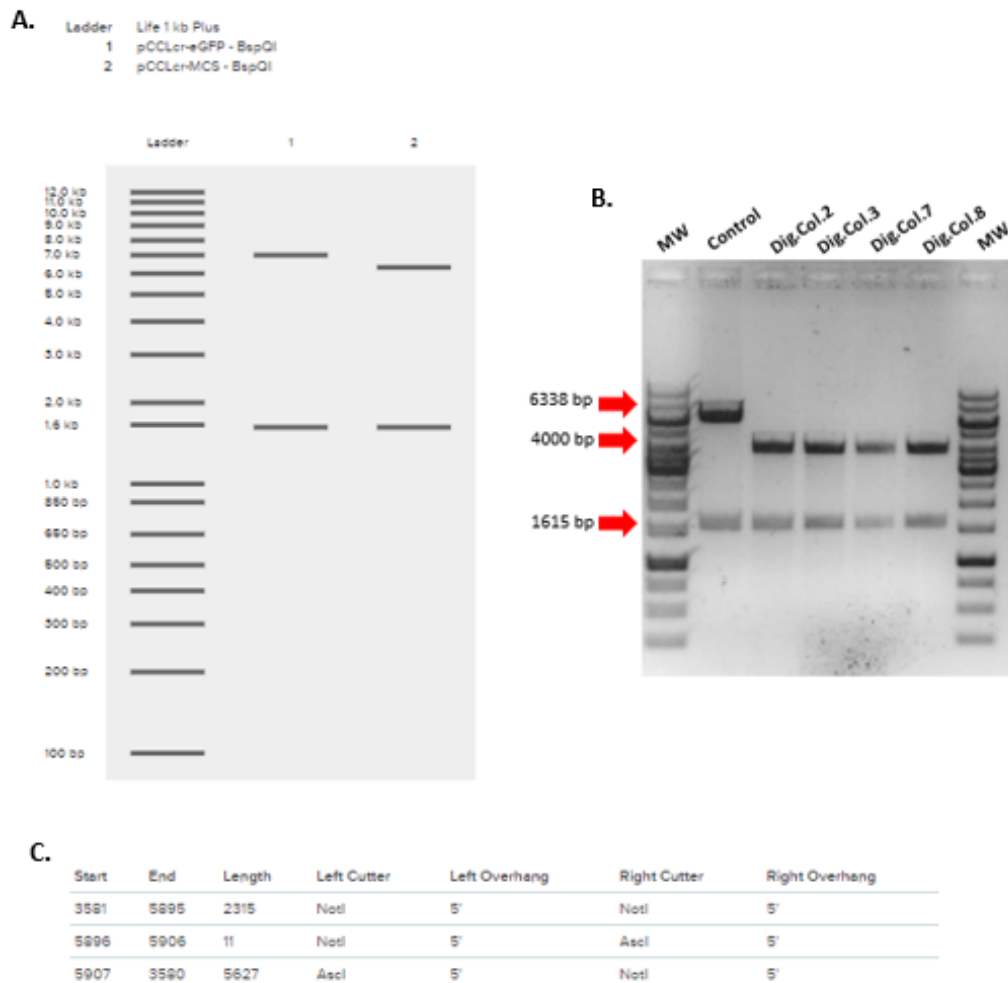


Figure 12 - Comproivative digestion of pCCLcr-GFP and control plasmid with BspQI. (A) Digital digestion representation of pCCLcr-MCS as control and samples of the assembly between pCCLcr and GFP, both digested with BspQI. (B) The real digestion. First and last well there are MW and then the control digestion followed by the samples of pCCL-GFP. (C) pCCL-MCS fragments when digested with Ascl and NotI.

As it is shown in **Figure 12.B** the first and the last band are MW, then second band is pCCLcr-MCS digested with BspQI, loaded as a digestion control, and the bands are the expected, one at 1615 and the other at 6338 bp. The other samples are pCCLcr-GFP digested with BspQI and the bands that appear are not the ones that we expected, as the digital digestion showed. The bands that supposed to appear were one at 1615 bp and the other at 7061 bp but it appears at around 4 kb.

We supposed that what happened is that in the digestion of pCCLcr-MCS with NotI and Ascl (**Figure 12.C**), the fragments from NotI to NotI and the other fragment cut by Ascl and NotI that has 11 bp, couldn't attach to the insert.

When the GFP fragment attaches to the remaining backbone from the digestion of *Ascl* and *NotI* makes a plasmid that has about 6kb. That's why when we cut the resultant plasmid with *BspQI* the result is the band at 4 kb.

We started using pSico vector in parallel with pCCLcr as it is also functional with lentivirus and because we were searching alternatives as we see that cloning into pCCLcr was failing.

Then we continue by doing the following assays:

- pCCLcr cut with *Ascl* + GFP
- pSico cut with *NotI* and *Ascl* + new Lama2-stop

After doing minipreps of the colonies, a comprovative digestion was made. This time with *EcoRV* for pSico-new Lama2-stop and *EcoRI* for pCCLcr-GFP.

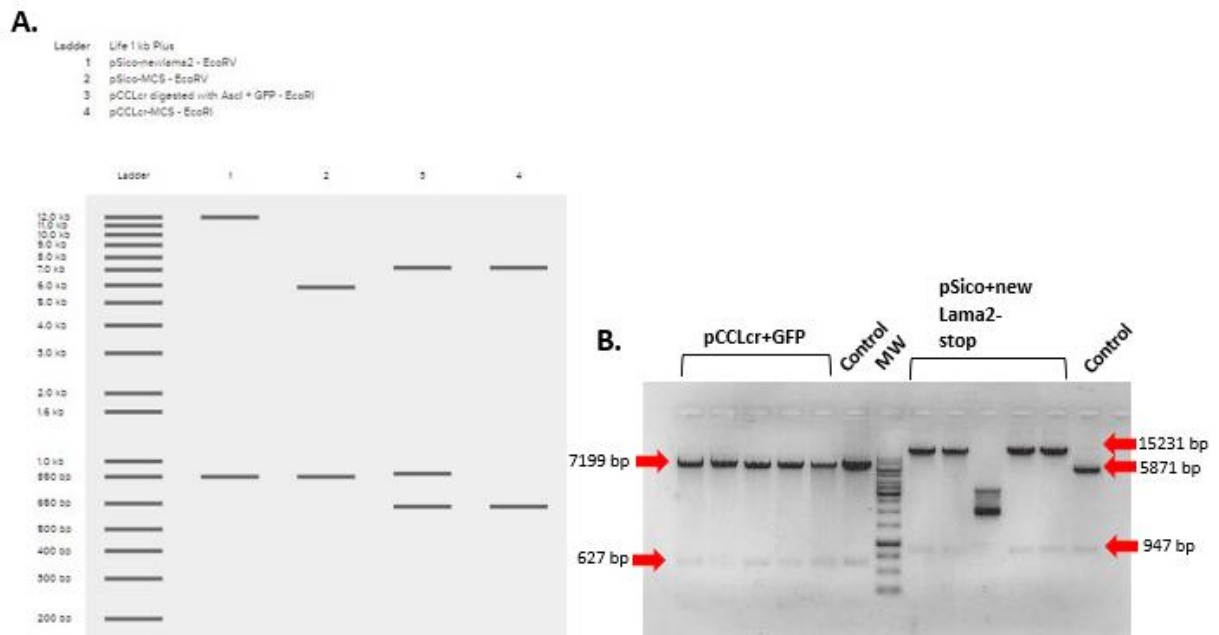


Figure 13 - Comprovative digestion of pSico + new Lama2-stop with EcoRV and pCCLcr + GFP EcoRI. (A) Digital digestion representation of what should appear when we digest the plasmids. In number 1 there is the digestion of pSico + new Lama2-stop with EcoRV, in number 2 the digestion of pSico-MCS with EcoRV as control, in number 3 the digestion of pCCLcr + GFP digested with EcoRI and finally in number 4 pCCLcr-MCS digested with EcoRI as control. **(B)** The real digestion. First, we have the 5 samples of pCCLcr + GFP digested with EcoRI followed by its control, then the MW and finally the 5 samples of pSico + new Lama2-stop digested with EcoRV followed by its control.

For pCCLcr + GFP seems that GFP has not inserted inside pCCLcr because the band that should appear at 950 bp is not visible. For pSico we can see that the bands are the ones

expected except for the 10th sample that we don't know what it can be. Also, both controls are correct.

We sent sequence one random sample of pCCLcr + GFP to look why the band of 950 bp do not appear in digested samples and also a couple of pSico + new Lama2-stop to verify that it has inserted inside the plasmid.

Sequencing results were: for the sample of pCCLcr + GFP the backbone was empty, no GFP was inserted, which concords with the comprovative digestion. Then for the samples of pSico + new Lama2-stop both had Lama2 inserted but one had more coverage than the other.

As the results for pSico were good, we decided to use the sample of pSico + new Lama2-stop that had more coverage in sequencing to perform new lentivirus so, we send to sequence the whole new Lama2-stop gene to check if it was correct and there were no random mutations.

Even if we had Lama2 inserted in pSico we keep trying the cloning in pCCLcr that was the principal objective. So, the next cloning we performed was:

- pCCLcr cut only with Ascl with new Lama2-stop.
- pCCLcr cut with NotI/Ascl with new Lama2-stop.

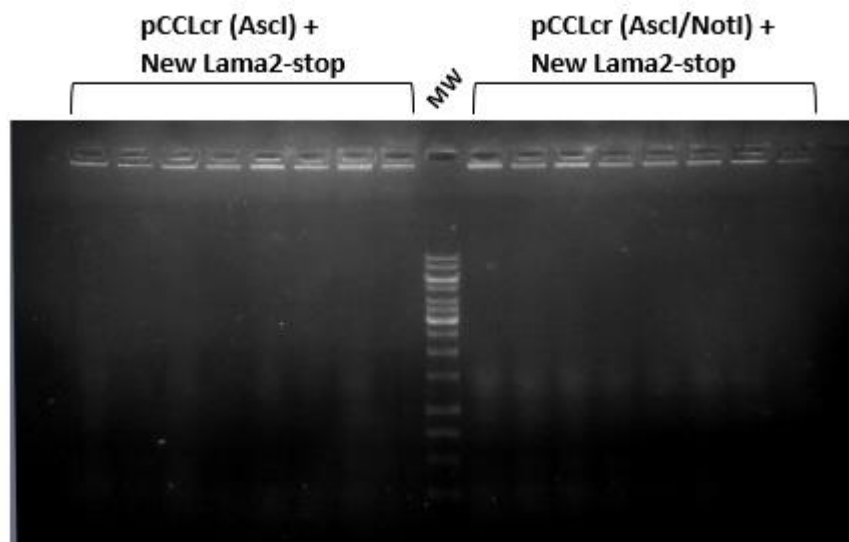


Figure 14 – Colony PCR of pCCLcr (Ascl) + New Lama2-stop and pCCLcr (Ascl/NotI) + New Lama2-stop samples.



At the left we have the 8 samples corresponding to pCCLcr (Ascl) - New Lama2-stop, then the MW and finally the 8 samples from pCCLcr (Ascl/NotI) + New Lama2-stop. Apparently, nothing appears. Only the MW is visible.

As the results weren't visible, we decided to run some samples in an e-gel to perhaps have some new images.

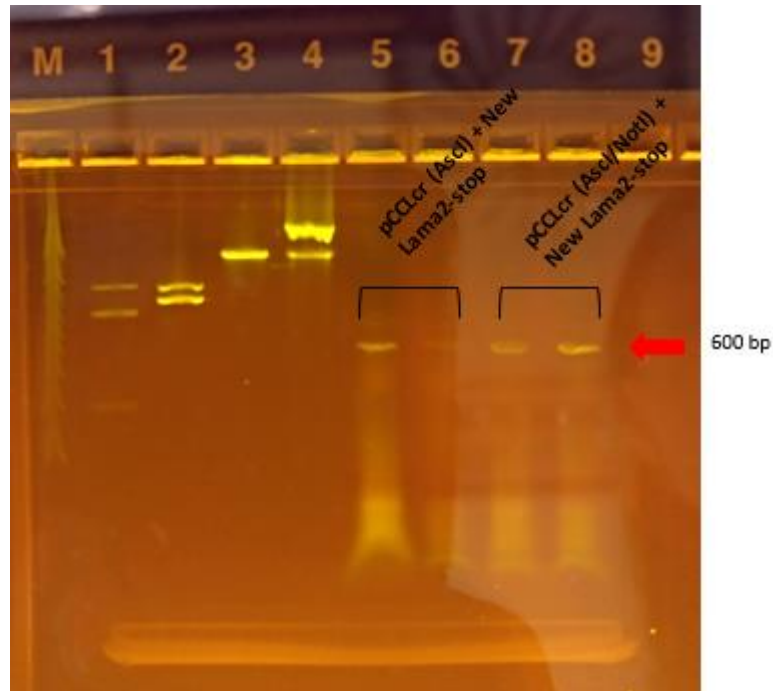


Figure 15 – Colony PCR ran again in an e-Gel at 1%. We loaded two samples of pCCLcr (Ascl) + New Lama2-stop and 2 pCCLcr (Ascl/NotI) + New Lama2-stop.

Seems that there is some chance that the cloning of Lama2 have been successful as there are some bands at around 600 bp (we compared the length of the bands with a partner that knows the length of its bands because of the bad running of the MW).

As those were the results, we decided to repeat the colony PCR by taking one colony of pSico (Ascl/NotI) + New Lama2-stop as a control to be sure that the amplification was correct and 4 new colonies of each plate of pCCLcr + New Lama2-stop to see if this time, we have better results.

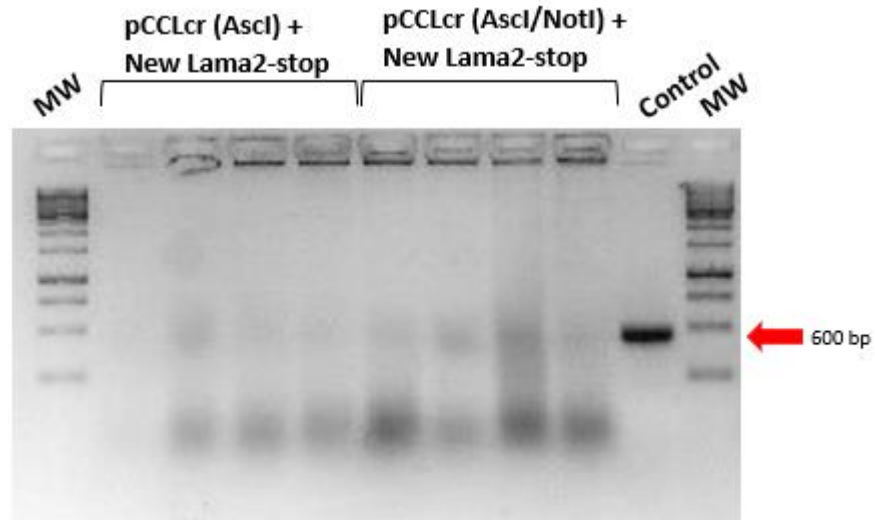
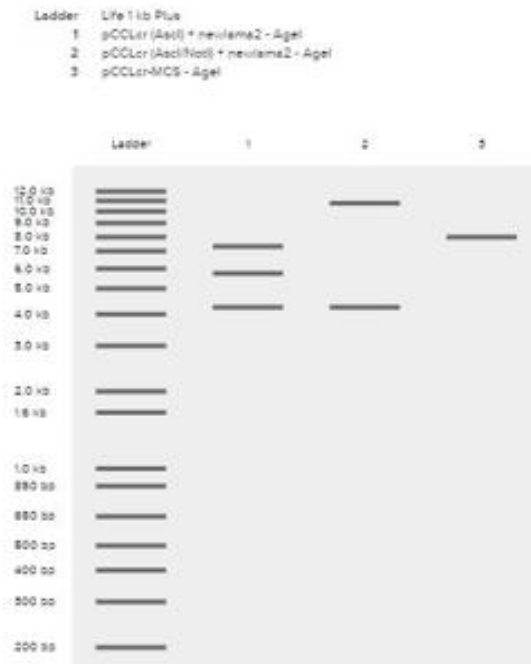


Figure 16 – Repetition of Colony PCR from the plates of pCCLcr + New Lama2-stop (backbone digested with Ascl and asci/NotI) and pSico + New Lama2-stop as control.

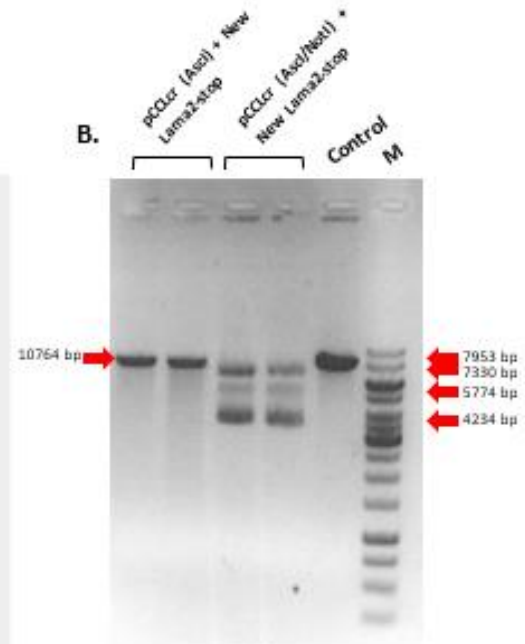
We can't see much again but we can distinguish some light bands at 600 bp that more or less are at the same line of pSico positive control Lama2 gene that means that there are chances that it has integrated in some plasmids. Also, we can appreciate that at the bottom of the gel there are some marks that could be primer dimers that means that not much DNA has been amplified, this could explain the light bands.

Also, from the colony PCR that apparently did not appear anything in the gel we prepare 4 tubs to make cell culture, 2 for pCCLcr (Ascl) + new Lama2-stop and 2 more for pCCLcr (Ascl/NotI) + new Lama2-stop to digest them and try to observe if the new Lama2-stop has entered the plasmid.

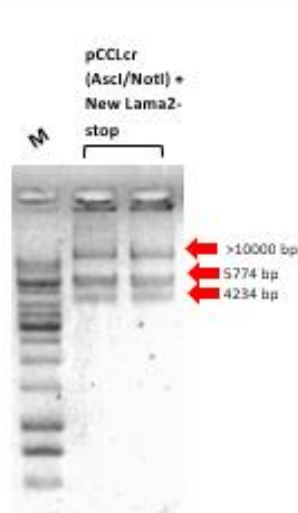
A.



B.



C.



D.

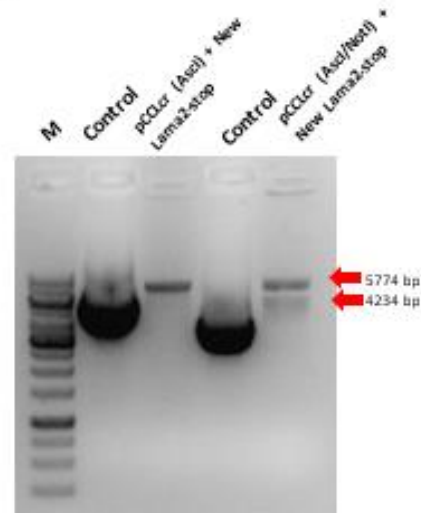


Figure 17 - Comproivative digestions of minipreps of pCCLcr (Ascl) + new Lama2-stop and pCCLcr (Ascl/NotI) + new Lama2-stop done with Agel. (A) Digital digestion representation of what should appear when we digested the plasmids. In number 1 there is the digestion of pCCLcr (Ascl) + new Lama2-stop, in number 2 the digestion of pCCLcr (Ascl/NotI) + new Lama2-stop and in number 3 the digestion of pCCLcr-MCS as a control. If new lama2-stop hasn't inserted in pCCLcr (Ascl/NotI) we won't see any band. (B) The real digestion. First, we have the two samples of pCCL (Ascl) + new Lama2-stop, next the two samples of pCCLcr (Ascl/NotI) + new Lama2-stop, then the control sample and finally the MW. (C) Digested samples of pCCLcr (Ascl/NotI) + new Lama2-stop that we have run a day after.

The bands concord with the digital digestion representation (**Figure 17.A**) of the digested samples seem switched so, as we do not know if the samples were wrong labelled, we send to sequence one sample of pCCL (Ascl) + new Lama2-stop and one sample from



pCCL (Ascl/NotI) + new Lama2-stop to see if there was lama and also if the samples were switched.

Sequencing showed that both backbones were empty and pCCL + new Lama2-stop sample digested with Ascl and NotI was also lacking the NotI-NotI part.

It seems strange because when we run the digestion in an e-gel there was a sample that showed three bands which means that the new Lama2-stop had inserted, due this it has not. To prove what is in the digestion we run an agarose gel to do a gel extraction and send to sequence to see what was in this strange sample.

We run again the samples but a day after and as the **Figure 17.C** shows seems that the bands are upper than the first time, that might be because of the recircularization of the fragment. We waited till next day to run again the samples and this time with control samples which was a pCCLcr-MCS empty backbone.

As can be observed in **Figure 17.D** this time the samples seem to be again in the position, we first time saw them but in well 4 (pCCL (Ascl/NotI) + new Lama2-stop) a band is missing.

So, we finally decided not to cut the gel and generate a new cloning by using pCCL cut with Ascl and new Lama2-stop cut with Ascl and NotI by doing blunt ends on both fragments and then ligate.

From the plate we seeded containing the blunt ended ligation from pCCLcr + new Lama2-stop, a few colonies have grown. Enough to do a colony PCR (16 samples).

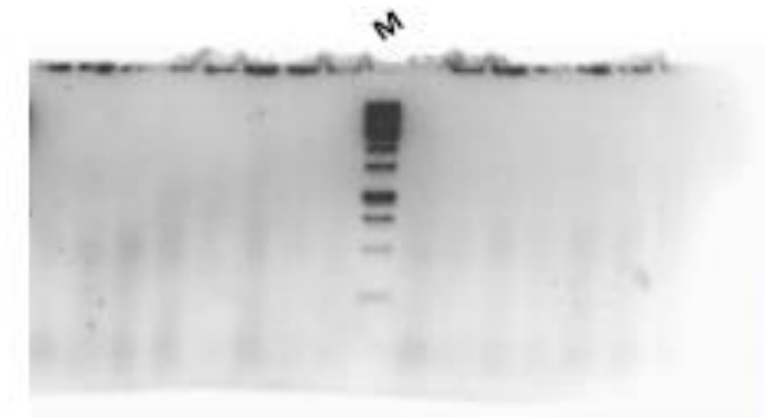


Figure 18 - Colony PCR samples from blunt ended ligation of pCCLcr (Ascl) + new Lama2-stop.

As we can appreciate any well shows the expected 650 bp band so we repeated the colony PCR.

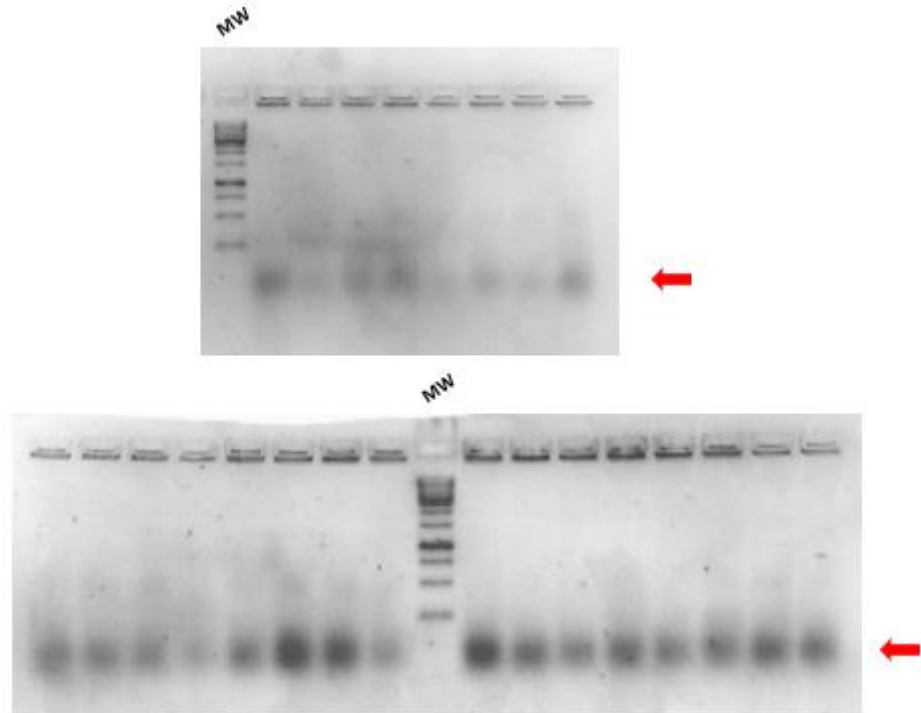


Figure 19 - Repetition of colony PCR samples from blunt ended ligation of pCCLcr (Ascl) + new Lama2-stop.

The first image are the first 8 samples and the second image the rest of them. Again, seems that the expected band (650 bp) does not appear.

As the cloning of GFP was not working, new oligos were asked (put them) to produce more GFP.

We proceed to ligate the new GFP digested with Ascl with the sample of pCCL digested also with Ascl and with rSAP.

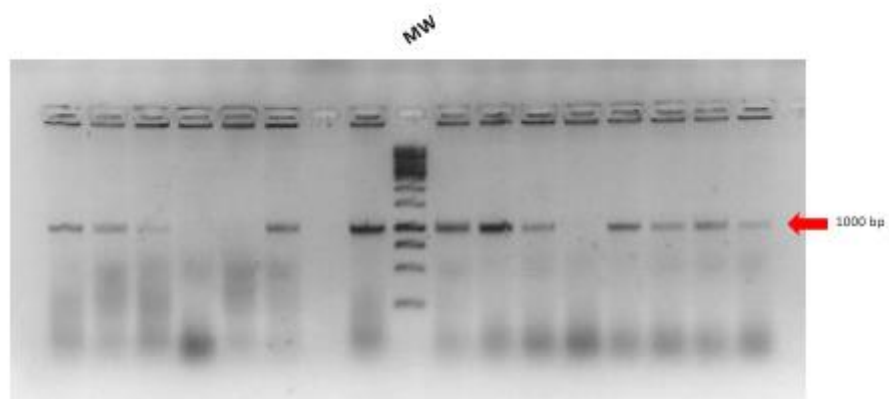


Figure 20 - Results of colony PCR from pCCLcr (Ascl) + GFP.



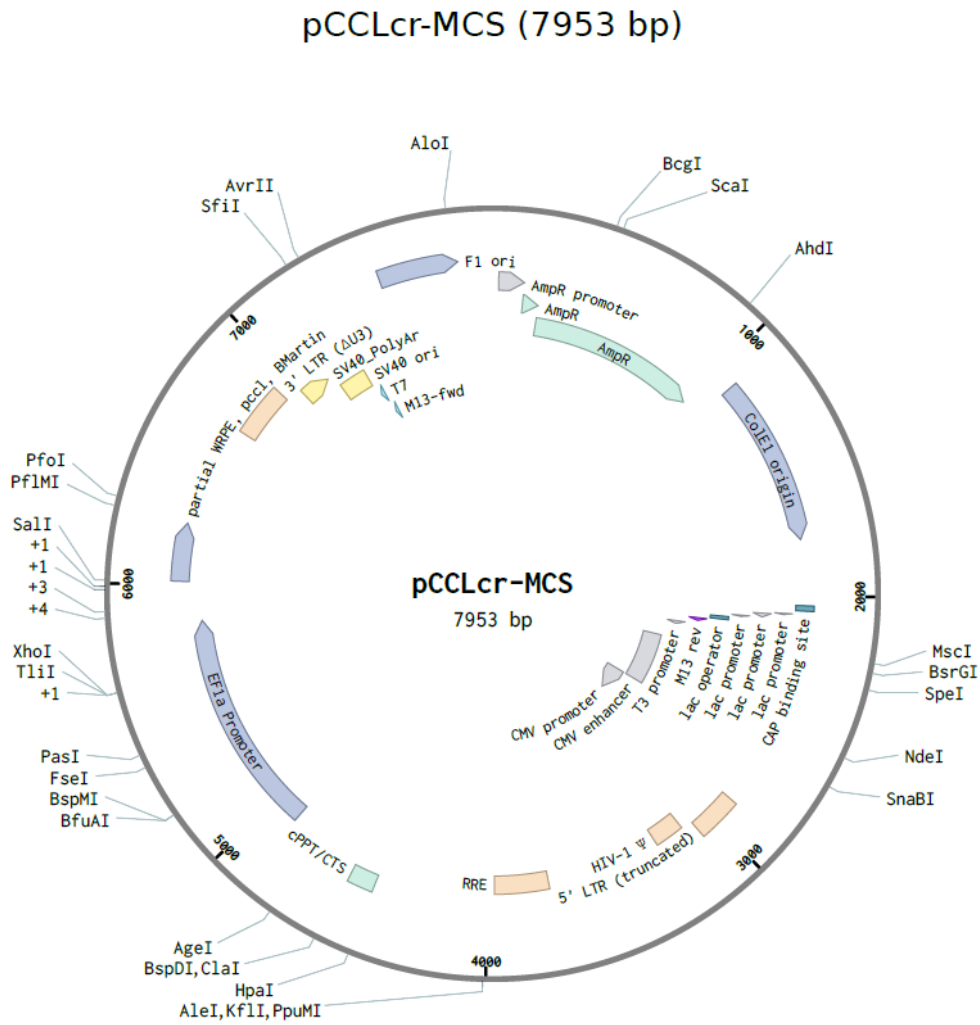
All the samples are from the same plates (except from well 9 that is the MW). The expected band is, more or less of 1 kb, so we can appreciate that in most samples the band appears. In well 7 there is nothing because the eppendorf was wrong closed and the sample evaporated. We took colonies from sample 8 and 10 to make cell culture and do miniprep next day and send to sequence.

Finally, both colonies that we send to sequence, nº8 and 10, were empty.

As the trial of cloning GFP in pCCLcr did not work as well as Lama2, we continued to the next step, lentivirus production, by using pSico cloned plasmids.

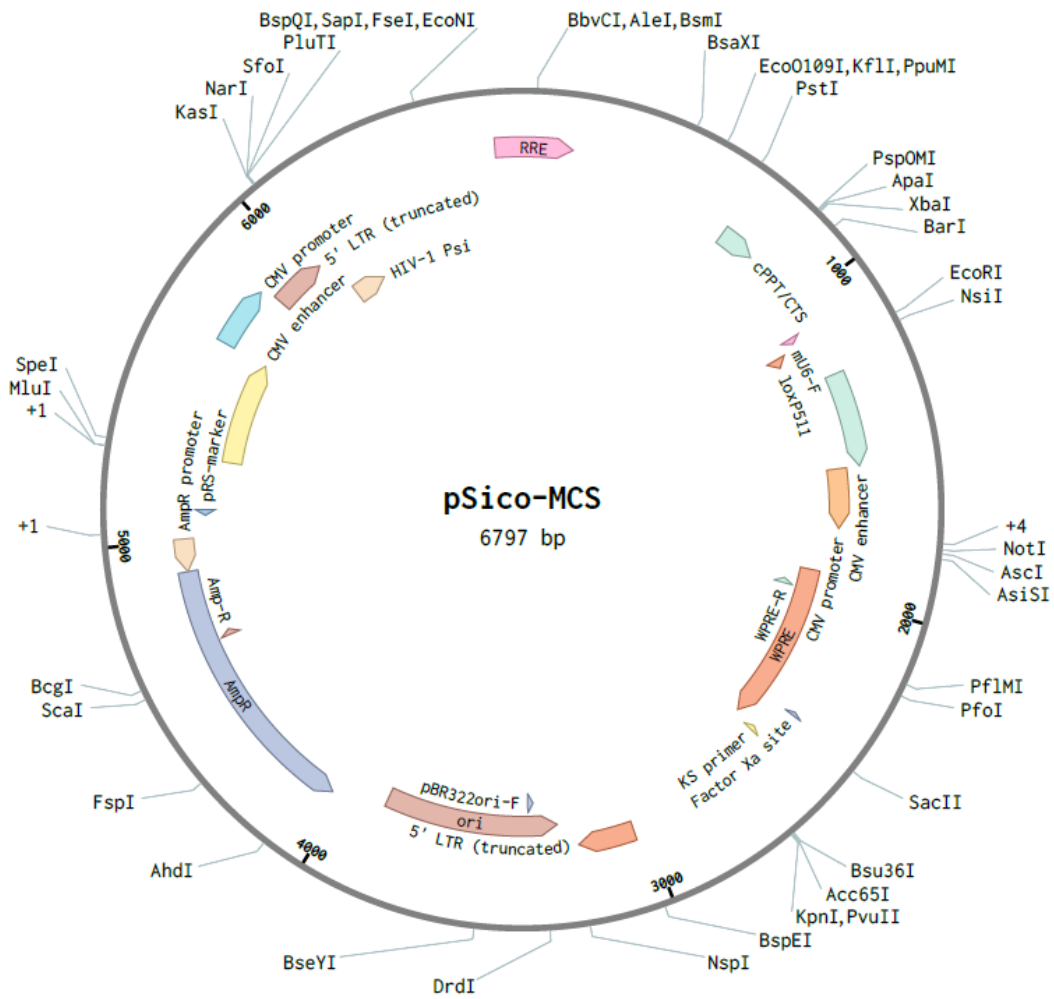
C. PLASMIDS USED FOR THE CLONING ASSAY

a. pCCLcr-MCS plasmid



b. pSico-MCS plasmid

pSico-MCS (6797 bp)



c. pSico plasmid (contains GFP)

