UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biotecnologie

ELABORATO DI LAUREA

Experimental Confirmation of Progerin–Small Peptide Interaction Using NanoLuc® Binary Technology Assay in Human Cell Lines to Validate *in Silico*–Designed Progerin Interactors.

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ANNO ACCADEMICO 2024/2025

Abstract

This thesis focuses on exploring a novel therapeutic strategy for Hutchinson-Gilford Progeria Syndrome (HGPS), a rare genetic disorder caused by mutations in the *LAMIN A* (*LMNA*) gene. This leads to the production of Progerin, a truncated form of lamin A. Progerin disrupts the nuclear architecture of affected cells, contributing to accelerated aging. The project proposes a protein-clearance approach by using a modified RING-Bait system to target Progerin for degradation. The research aims to identify Progerin-specific interactors through computational methods and experimental validation.

In silico tools, including AlphaFold, RFdiffusion, ProteinMPNN, HADDOCK, and ClusPro, were used to design peptides that interact with Progerin's C-terminal region. These peptides were further evaluated the NanoBiT protein-fragment complementation system to assess protein-protein interactions. The thesis outlines the methods used to synthesize and optimize the DNA fragments encoding the interacting peptides, including codon optimization, primer design, and plasmid preparation.

The in silico predictions and subsequent experimental assays are anticipated to play a crucial role in the development of *Progerase*, the Uni-Padua-IT iGEM project for this year. Future directions include expanding the peptide library and evaluating the efficacy of the RING-interactor fusion proteins in vivo, providing a promising therapeutic strategy for alleviating the harmful effects of Progerin in Progeria patients.

1. Introduction

1.1 Progeria

In metazoan cells, the nuclear lamina is an intricate protein network composed of polymeric assemblies of lamins, a class of filament proteins. The polymeric association of lamins serves as scaffolding for the nuclear envelope, and plays a key role in chromatin and nuclear pore anchorage. However, like any biological system, it is prone to mutations which can profoundly affect the complex's behaviour.[1]

Hutchinson-Gilford progeria syndrome (HGPS), a rare premature aging disorder, arises from a single dominant *de novo* mutation in the gene encoding lamin A (*LMNA*). This mutation produces an altered form of lamin A that fails to undergo normal post-translational processing: the resulting protein is truncated, retains a farnesyl group, and carries an abnormal C-terminal sequence. In this defective form, it remains predominantly associated with the inner nuclear membrane, rather than integrating into the nuclear lamina, where Lamin A normally localizes. Alterations in protein:protein interactions, such as LMNA-Progerin, may lead to neurodegenerative disorders among other complications.[2]

Progerin overexpression in HGPS directly disrupts the nuclear lamina, leading to alterations in nuclear architecture and function. Affected cells show a misshapen nuclei characterized by: i) blebs and other structural abnormalities; ii) impaired chromatin organization; iii) genomic instability and irregular cell division.

So far, no definitive treatment for Progeria has been commercialized. However, breakthroughs in understanding nuclear morphology and recent advances in HGPS research have highlighted possible therapeutic strategies.

These approaches fall into two main categories: targeting Progerin itself such as Gene Editing, Progerin farnesilation blockers and Mis-splicing Modulation; or function by mitigating harmful downstream effects caused by Progerin. [3]

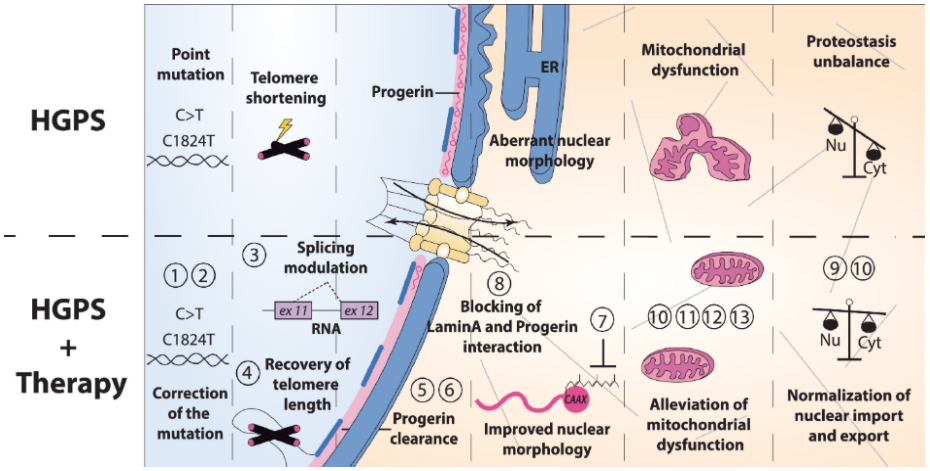


Figure 1.1: Schematic model showing the beneficial effects of different therapeutic approaches on HGPS affected cells.[3]

1.2 PROGERASE

Of the two main categories of possible strategies, the Mutans team (https://mutans.biologia.unipd.it/it/home-2/) a small extra-curricolar team of Synthetic Biology enthusiasts, composed of 16 students including myself, participating in this year's annual iGem competition, chose to follow a protein clearance strategy, focusing on a repurposing of the RING-Bait approach used by L.V.C. Miller *et al.* .[4]

In the RING-Bait system, an aggregating protein fused to an E3 ubiquitin ligase – TRIM 21's RING domain – was recruited onto pathological proteic aggregates, stimulating RING-domain dimerization and consequently inducing proteasomic disposal of the targeted proteins.

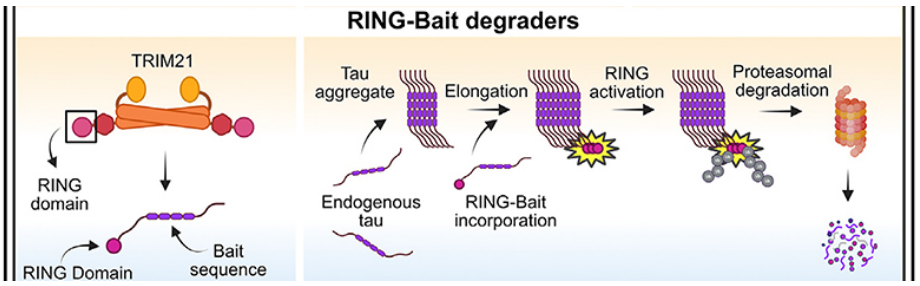


Figure 1.2: Visualization of RING-Bait driven Tau degradation[4]

To adapt the usage of TRIM-21 derived RING domain to a Progerin degradation focused approach, the team had to determine *ex novo* Progerin interactors, forcing RING Domain accumulation on Progerin and subsequent proteasomic degradation.

1.3 *In silico* definition of Progerin-interacting peptides

To fully harness RING domain's E3 ubiquitin ligase activity, a chimeric protein, composed of both (A) a RING domain and (B) a Progerin specific interactor, needed designing. Literature however lacks description of Progerin-specific interacting peptides, posing a strong bottle-neck in the design of our protein depletion model; whereas Addgene Plasmid #233691 was utilized to obtain the RING domain DNA sequence utilized in the RING-Bait system previously described.

We then sought to utilize different on-line softwares to produce a library of *in silico* designed Progerin interactors.For an in depth analysis of the bioinformatics pipeline and results, consult: Andrea D'Amico's and Anna Cenedese's thesis.

However these interacting peptides do not have any experimental validation of *in vivo* association with Progerin, hence we sought for an assay that could confirm and quantify protein-protein interaction in a cellular environment.

Protein-fragment complementation assays (PCAs) make such studies possible, of which the NanoBiT technology, employing fragment complementation of NanoLuc luciferase, stands out for its high sensitivity, wide dynamic range, and straightforward read out.[5]

1.4 NanoLuc® Binary Technology

NanoLuc Binary Technology (NanoBiT®) is a complementation reporter, designed to carry out P:PIS (Protein:Protein Interaction Systems) for quantitative protein interaction dynamics. We chose to follow this path to further validate our synthetic peptides' association with Progerin, hopefully confirming all *in silico* predictions. [6]

The NanoBiT system relies on the enzymatic activity of NanoLuc (Nluc), an engineered luciferase derived from a deep sea luminous shrimp. Structurally speaking, the enzyme is quite small (19kDa), stable, and easy to observe (as it produces bright and substantial luminescence).

Evolving from the Nluc reporter system, NanoBiT was designed to be a binary reporter system having minimal steric burden on the tested proteins, providing nonetheless a high detection sensitivity. The test is specifically designed to allow accurate quantification of protein interactions under physiological conditions (cellular environment).

The system relies on two complementary peptides: a 1.3 kDa peptide (Small BiT) and a 18 kDa polypeptide (Large BiT), that weakly associate to assemble a weaker yet functional luminescence reporter gene.

Qualitative measures have determined both an intrinsic affinity: K_D=190 uM, and association constants k_{on} = 500 M^{-1} s^{-1}; k_{off} = 0.2 s^{-1}. These constants were found to be outside of the ranges typical for protein interactions, providing an accurate indication of interaction dynamics.

Small BiT and Large BiT started as a dissection of the Nluc protein, but further mutation-driven stability and efficiency assays determined the actual sequences behind the complementation reporter. Small BiT is an 11 amino acid long peptide, comparable to other peptide tags used in various procedures. This minimizes structural burden and confirms more reliable results. The Large BiT, composed of 159 amino acids, is the bigger peptide of the two, but is still relatively small in the protein tag family (around ⅔ of GFP's size).

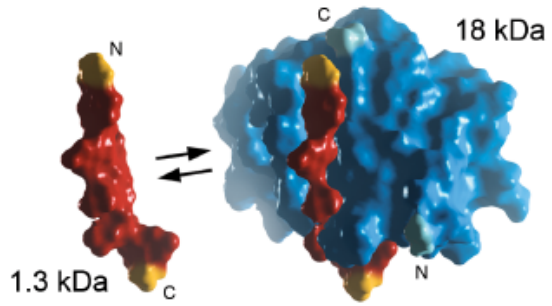


Figure 1.3: Small BiT (in red) is shown both detached (left) and attached (right) from the Large BiT (in blue).[6]

As seen in Figure 1.3, as the SmallBiT and LargeBiT are brought ever so closer together in function of the interacting proteins they are bound to, the system undergoes a structural change due to peptide driven complementation, leading to the expected light signal.

Furthermore, the system is designed to properly function in mammalian cell lines, perfect to exploit possible Progerin-interacting peptides in MRC5 cell cultures (Human fetal lung fibroblasts).

1.5 Aims of the thesis

Once we defined which PPIS assay to carry out, we had to decide which of the different protein fusions was to be designed.

Since Progerin is processed at its C-ter, we opted to utilize it only positioned at the C-ter of the BiT sequence. Furthermore, since the protein GFP probably delocalizes Progerin, we thought that by designing the fusion protein Small BiT - Progerin, our work would shed optimal results.

For these reasons we will be utilizing Vector N198 for SmBiT-Progerin expression.

In the following thesis you will find step-by-step how 10 different inserts i) were designed on Benchling.com, ii) ordered online from TWIST Bioscience® and iii) laboratory procedures necessary to ensure correct cloning into the NanoBiT® Vectors.

2. Method details

2.1 Synthetic gene fragment definition

2.1.1 NanoBit Plasmids

The NanoBiT® PPIS (Protein:Protein Interaction System) is composed of 7 different Vectors. The specific design and added features can be found at: https://www.promega.com/products/protein-interactions/live-cell-protein-interactions/nanobit-ppi-starter-systems/?catNum=N2014&accordion0=0[7]; whilst they are designed as shown on table 2.1.

Name	Base pairs	Promoter	Resistance gene	RS in MCS
N196 pBiT1.1-C [TK/LgBiT] Vector	3865	HSV-TK promoter	Amp	SacI; EcoRI; XhoI; NheI; BglII; XbaI FseI
N197 pBiT2.1-C [TK/SmBiT] Vector	3423	HSV-TK promoter	Amp	SacI; EcoRI; XhoI; NheI; BglII; XbaI FseI
N198 pBiT1.1-N [TK/LgBiT] Vector	3858	HSV-TK promoter	Amp	XhoI; SacI; EcoRI; SgfI; NheI; BglII
N199 pBiT2.1-N [TK/SmBiT] Vector	3417	HSV-TK promoter	Amp	XhoI; SacI; EcoRI; SgfI; NheI; BglII

Table 2.1: schematic summary of NanoBiT ® vectors and features

Altogether the kit “NanoBiT® PPI MCS Starter System” includes:

- 2 × 125µl Nano-Glo® Live Cell Substrate
- 2 × 2.5ml Nano-Glo® LCS Dilution Buffer
- 20µg pBiT1.1-C [TK/LgBiT] Vector
- 20µg pBiT2.1-C [TK/SmBiT] Vector
- 20µg pBiT1.1-N [TK/LgBiT] Vector
- 20µg pBiT2.1-N [TK/SmBiT] Vector
- 20µg SmBiT-PRKACA Control Vector
- 20µg LgBiT-PRKAR2A Control Vector
- 20µg NanoBiT® Negative Control Vector

2.1.2 Synthetic gene fragment preparation and optimization

To exploit maximum assay luminescence, the synthetic DNA (insert) design must follow a series of strict parameters. This is an essential step, for the NanoBiT vector’s MCS is inside a rigid open reading frame that needs to be maintained after cloning in order to express the correct protein fusion between BiT (Large or Small) and the interacting protein.

We start by carrying out a host based codon optimization. We then design primers with specific overhangs for enrichment PCRs, and finally simulate virtual enzyme restrictions and ligations, all carried out on Benchling.com utilizing different tools. Each step is described in the following paragraph.

All information listed below can be found in: NanoBiT® Protein:Protein Interaction System Technical Manual; Instructions for Use of Products; N2014 and N2015.[7]

In order to have a complete assay, test all possible NanoBiT protein fusions as shown on table 2.2, where proteins A and B are the proteins the system will test for *in vivo* interaction.

Protein A	A-LgBiT	Protein B	B-LgBiT
	LgBiT-A		LgBiT-B
	A-SmBiT		B-SmBiT
	SmBiT-A		SmBiT-B

Table 2.2: all necessary protein fusions for a complete NanoBiT ® assay

2.1.2.1 Codon Optimization

Benchling.com, a cloud based Molecular Biology tool, offers an array of different features. In this case their codon optimization tool is essential for a correct retro-translation from the amino acidic sequence of the peptides to their DNA sequence.

Codon optimization is essential when designing synthetic DNA fragments since different organisms have different codon usage, given by different tRNA transcription rates. It is strongly recommended to optimize the codon rate to best match the organism in which the assay will be carried out's codon usage.

2.1.2.2 Primer Pair design

When using a Multiple Cloning Site (MCS) vector, the first step is to flank the synthetic DNA sequence to be cloned with specific primers. Designing

primers with tailored overhangs enables the same DNA fragment to be reused for multiple NanoBiT vector cloning reactions via the MCS.

This approach is essential when testing a DNA fragment in both NanoBiT-N and NanoBiT-C vectors, as their MCS sequences differ. Because cloning relies on restriction digestion and ligation, performing an enrichment PCR with specifically designed primers allows a single DNA fragment to be adapted for different assays.

Benchling.com allows for a simple yet efficient Primer Pair definition. Whilst designing the primers, make sure they have: 25-20pb homology; T_M 55-65°C; T_M that differs no more than ± 1°C between the two; a GC% of 40-60%; a CG clamp at 3'.

The overhangs are to be designed as shown on table 2.3, where NNNNNN are random bases that assure optimal endonuclease enzymatic function.

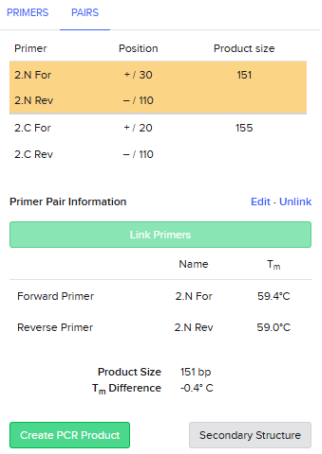
NanoBiT® Vector	Restriction Enzyme	Primer Sequence
pBiT1.1-C [TK/LgBiT] Vector	5´-NNNNNNGAGCTCC(RC GOI)-3´	SacI
	5´-NNNNNNGAATTCCC(RC GOI)-3´	EcoRI
	5´-NNNNNNCTCGAGCC(RC GOI)-3´	XhoI
pBiT2.1-C [TK/SmBiT] Vector	5´-NNNNNNGAGCTCC(RC GOI)-3´	SacI
	5´-NNNNNNGAATTCCC(RC GOI)-3´	EcoRI
	5´-NNNNNNCTCGAGCC(RC GOI)-3´	XhoI
pBiT1.1-N [TK/LgBiT]	5´-NNNNNNCTCGAGCGGT(GOI)-3´	SacI

Vector	5´-NNNNNNGAGCTCAG(GOI)-3´	EcoRI
	5´-NNNNNNGAATTCA(GOI)-3´	XhoI
pBiT2.1-N [TK/SmBiT] Vector	5´-NNNNNNCTCGAGCGGT(GOI)-3´	SacI
	5´-NNNNNNGAGCTCAG(GOI)-3´	EcoRI
	5´-NNNNNNGAATTCA(GOI)-3´	XhoI

Table 2.3: schematic summary of all possible restriction sites in NanoBiT vectors' multiple cloning sites, with consequent primer overhangs needed.

Once the primer pair has been attached to the sequence, Benchling.com's function "Create PCR product" allows visualization of the expected PCR product.

A deep analysis of the output sequence acts as a useful proof-read, allowing for possible corrections.



ATTENTION: at least one restriction site between SacI; EcoRI; XhoI must be used for MCS cloning to maintain a GSSGGGGSGGGGSSG flexible peptide linker inside the fusion protein.
If the cut sites are chosen incorrectly, an in-frame Stop Codon placed downstream of the MCS will inhibit the chimeric protein's correct translation.

Figure 2.1: Graphical User Interface for PCR simulation on Benchling.com

To conclude this phase, simulate a "Restriction and Ligate" cloning on Benchling.com, after virtually digesting both PCR product and the desired vector sequence with the appropriate Restriction Enzymes.
This function allows visualization of the expected cloning into the NanoBiT plasmids, allowing final proof-reading.

2.1.3 Synthetic DNA synthesis and cloning

Since TWIST Bioscience is an iGEM Platinum Partner, each participating team can order up to 20 '000 bp of either DNA fragments of clonal genes.

For each interacting peptide, we ordered two diverse synthetic genes: one for C-large cloning and the other for N-large, since the MCS and requirements are different from vector to vector (as shown on table 2.1).

Further sequence optimization on TWIST's interface was not needed before confirming the order, the sequences being of "Standard synthesis complexity".

2.2 NanoBiT plasmid preparation

2.2.1 Restriction Enzyme Functionality

To address a typical laboratory problem: partially functional enzymes; an endonuclease reaction followed by Cut / UnCut Electrophoresis analysis is essential. This procedure allows for the fine tuning of reaction volumes to be used and as a proof of the endonucleasic's correct functionality.

Note: Set gel parameters according to the DNA bp length.
Note: ensure that the two enzymes used for restriction digestion are compatible with the same reaction buffer.
We utilized New England Biolabs® EcoRI-HF and XhoI, both 100% functional in NEB® CutSmart® Buffer 10X.

Our reactions were set as follows.

Endonuclease Reaction:

Plasmid DNA	200 ng
Reaction Buffer	2 ul Cut Smart 10X
Enzyme 1 - BamHI-HF	1 ul 20k U/ml
Enzyme 2 - XhoI	1 ul 20k U/ml

H_20	to volume
Final Volume	20 ul

Table 2.4: reagents and quantities needed for enzymatic restriction

2.2.2 Plasmid Amplification

Every NanoBiT plasmid is shipped dry with a mass of 2000 ng: proper rehydration and amplification is necessary to perform a complete assay. Any transformation protocol is functional. Follow laboratory protocols if proceeding with another strategy.

2.2.2.1 Plasmid Rehydration

Place the received tubes with dry NanoBiT plasmids in a laboratory centrifuge set at ambient temperature.

1. Spin dry for 1 minute at 14’000 rpm
2. Add 50ul of Tris to every tube
    a. Avoid touching the tubes directly with your pipette tip
    b. The plasmid concentration will be 40 ng/ul
3. Incubate at room temperature for 10 minutes.

2.2.2.2 Plasmid Transformation

If utilizing DH5α use the following protocol:

1. Place the chosen bacterial competent strain on ice. Maintain a sterile environment by working next to a turned on Bunsen burner
2. Once thawed (1-2 minutes) add 60 ng of resuspended plasmid to the tubes.
    a. If resuspended in 50 ul, utilize 1.5 ul.
    b. Do not resuspend DH5alfa competent cells after adding DNA.
3. Maintain the transformation tubes on ice for 30 minutes
4. Heat shock: place at 37°C for 4 minutes; utilize a Water Block
5. Whilst maintaining a sterile environment, add 200 ul of LB (liquid terrain) to each tube.

6. Place in a 37°C set water bath for 45 minutes
7. Whilst maintaining a sterile environment, plate the whole cell culture spreading evenly on the entire plate.
    a. Vectors N196; N197; N198; N199 are to be plated in plates containing LB + AMP as they express Ampicillin Resistance

       Vectors N202; N203; N204 are to be plated in plates containing LB + KAN as they express Neomicin-Kanamicin Resistance
8. incubate O.N at 37°C

2.2.2.3 Inoculum preparation

From every plate, select the 3 better defined single colonies. Utilize the following protocol to start cultures from individual colonies grown on LB agar.
Make sure to work in a sterile environment.

1. Add 3ml of LB+Amp or LB+Kan ([Amp] and [Kan] = 50ug/ul) to each inoculum tube
2. With a 200ul pipet tip, collect a single colony from the agar plates and resuspend it into the inoculum tube
    a. repeat 3 times per plate (3 different colonies per plate)
3. Loosely seal the different tubes, and incubate O.N. at 37°C; 150 RPM.

2.2.2.4 MiniPrep of plasmidic DNA

To extract the amplified plasmid from the liquid cultures left to proliferate O.N., proceed with Invitrogen™ “PureLink™ Quick Plasmid Miniprep Kit”, following the manufactures’ protocol.

2.2.2.5 Plasmid quantification

To correctly quantify the plasmidic DNA obtained via MiniPrep, utilize Thermofisher™ NanoDrop®. Prepare a tube containing Invitrogen® “MiniPrep Elution Buffer”, essential for machine blanking prior to DNA concentration quantification. Follow NanoDrop protocol to measure correctly.

2.2.3 Plasmid Restriction

2.2.3.1 Reaction set up

To proceed with a standard O.N. sticky-end ligation, every plasmid must be processed through an endonucleasic reaction to open its multiple cloning site and allow restriction-derived specific overhangs.

We chose to utilize endonucleases XhoI and EcoRI-HF. To test enzymes' reliability, go to paragraph 2.2.1.

Endonuclease Reaction:

Plasmid DNA	2000 ng
Reaction Buffer	5 ul 10X Buffer
Enzyme 1	2 ul from 20k U/ml
Enzyme 2	2 ul from 20k U/ml
H_20	to volume
Final Volume	50 ul

Table 2.5: reagents and quantities needed for enzymatic restriction

1. Let the reagents thaw on ice
2. Place the correct amount of each reagent on previously signed 1.5ml Eppendorf tubes
    a. add reagents by decreasing volume
    b. fully resuspend the reaction mixture
3. incubate the reaction Eppendorf tubes in a heated Water Bath set at 37°C for 1 hour

a. consider adding 1 ul of each restriction enzyme after 1 hour of enzymatic reaction in order to obtain completely digested linear DNA

b. if adding an extra 1 ul of the endonucleases, let the reaction proceed for 1 additional hour

2.2.3.1 Restriction reaction Purification

Once the reaction has come to an end, proceed by purifying the product via commercial kits. We utilized Quiagen™: “QIAquick® PCR Purification Kit”, following manufacturer’s protocol.

2.2.3.2 Quality and Quantification analysis

To correctly quantify the plasmidic DNA obtained via MiniPrep, utilize NanoDrop®. Prepare a tube containing “MiniPrep Elution Buffer”, essential for machine blanking prior to quantifying DNA concentration values. Follow NanoDrop protocol.

To confirm the correctness of the restriction reaction, prepare a 1% agar Electrophoresis Gel.

3. Results

In the following paragraphs I will discuss the paths I chose to prepare the five Progerin-interacting peptides for a NanoBiT assay. Chapter 3.1 focuses on the *in silico* design, of which sequence maps have been added; whilst chapter 3.2 holds specifics on our plasmid amplification's quality and yield.

3.1 Synthetic construct definition of the Progerin interacting peptides

3.1.1 Codon optimization

Four different peptides were chosen from a small library defined by our Bioinformatics team (information of the bioinformatic pipeline is found on chapter 1.3).

The chosen peptides are:

Name	Aminoacid Sequence
1. 62aa11_1	GLEEAQRRAEEARRQIALANRAGRDQEEAARLQRELE ALEAEIEEAKTG
2. LOGO	APGRGRCRGNPPVCCCPNCPRCDADCTQGGGSGCP ACPCP
3. n80_02	AAGCGRCIGNPPVCCCCNCPECGQDCTQCGGSGCPN CPCP
4. Rank_15	GLPLPELDLPEAMFRGKCAQANGAGASGTTHTAPPPE PREPLSGE

Table 3.1: name and amino acid sequence of the 4 Progerin-interacting peptides we will be testing with a NanoBiT ® assay

After importing each amino acid sequence onto Benchling.com, we proceeded to retro-translate each peptide to its DNA sequence, optimizing the codon usage to best match *Homo sapiens*, for we will be carrying out the NanoBiT assay on MRC5 cell lines.

To prevent the formation of undesired restriction sites, we added EcoRI and XhoI as "Avoided Cut Sites" before carrying out the retro-translation.

3.1.2 Primer Pair design

Once we defined the DNA sequence for our interacting peptides, we found the endonucleasic pair XhoI and EcoRI optimal for our work since we already had both of them in house and they both have complete function with New England Biolabs' restriction buffer CutSmart 10X.

Following Promega: "TECHNICAL MANUAL NanoBiT® Protein:Protein Interaction System; Instructions for Use of Products; N2014 and N2015", we defined the overhangs to add to each primer pair to ensure correct restriction sites and other additional features such as a Stop Codon and Kozak sequence.

C-plasmid cloning

(EcoRI) FOR: CGTCAG**GAATTC**GCCACCATGG - GOI-3'
(XhoI) REV: CGTCAG**CTCGAGCC** (RC GOI)-3´

N-plasmid cloning

(XhoI) FOR: CGTCAG**CTCGAGCGGT** GOI-3'
(EcoRI) REV: CGTCAG**GAATTCTTA** (RC GOI)-3´

Highlighted in **ORANGE**: additional Kozak sequence, helpful to enhance transcription rates in mammalian cells; highlighted in **GREEN**: essential Start Codon; highlighted in **RED**: essential Stop Codon.

Following these constraints, two different primer pairs were designed so that the virtual PCR product obtained using specific primer pairs would be ready to cut DNA sequences.

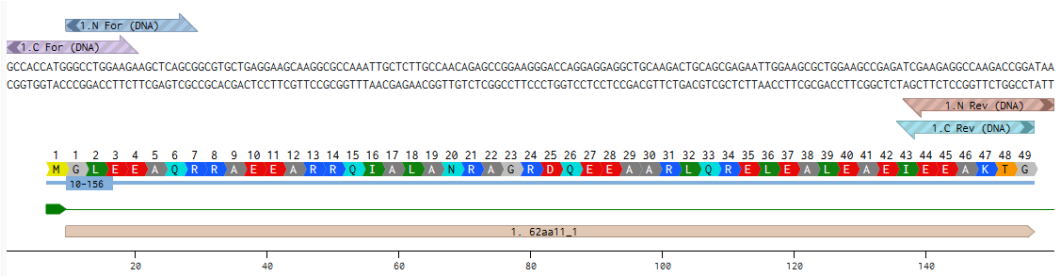


Figure 3.1: peptide 1. 62aa11_1 with primer pairs N and C attached

Since we ordered our synthetic DNA sequences ready to cut and clone into NanoBiT plasmids, we did not need to follow primer design constraints (such as T_M, GC clamp; GC%).

3.1.3 Virtual MCS cloning

To proof-read our primer's design, we first created the virtual PCR product for every primer couple, obtaining both N-ter and C-ter optimized sequences for cloning into NanoBiT vectors through MCS restriction and ligation.

Every PCR product and NanoBiT vector then underwent virtual restriction digestion, producing EcoRI and XhoI specific overhangs.

Finally, utilizing the function "Assembly → Assembly Wizard → Digest and Ligate" we were able to simulate every NanoBiT vector - synthetic Peptide ligation.

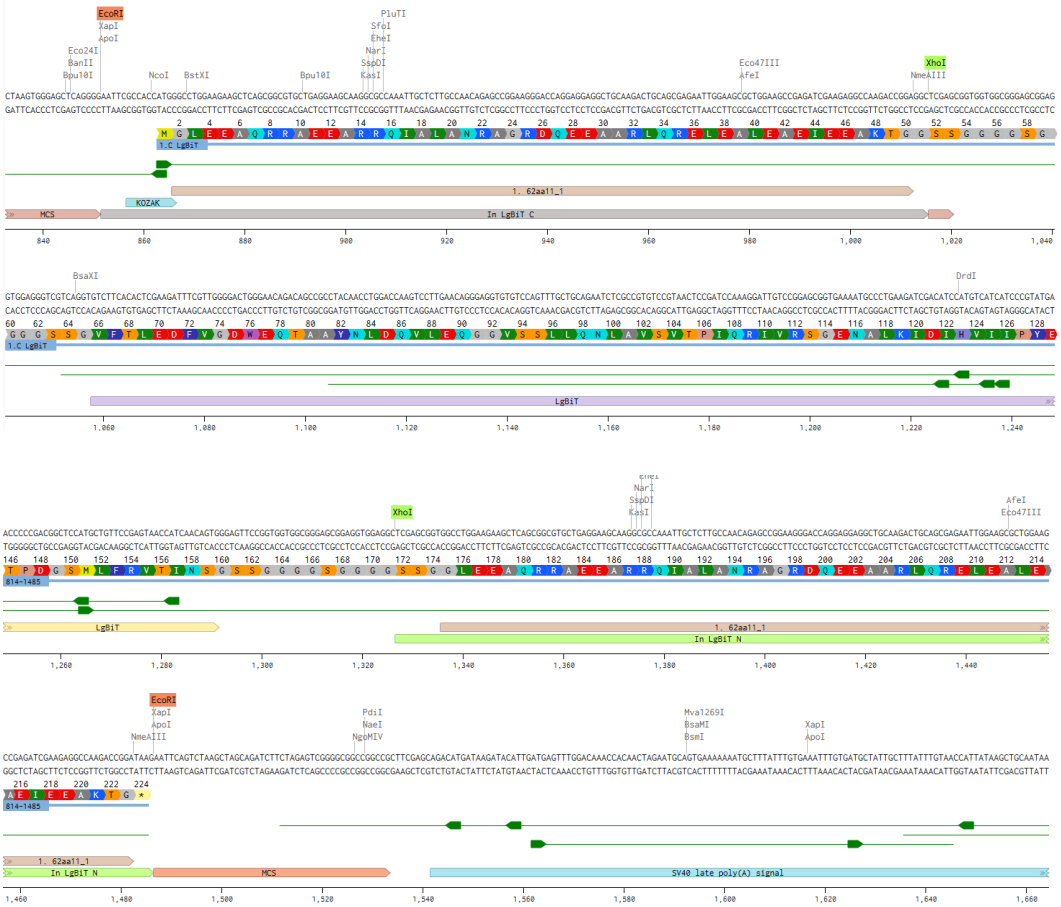


Figure 3.2: virtual cloning of peptide sequence 1.62aa11_1 into LgBiT-C (NanoBiT Vector N196) and LgBiT-N (NanoBiT Vector N198)

After having simulated every restriction-ligation cloning, we had to decide how to proceed with the synthetic DNA order. Firstly we thought to order the precise sequences that would have been cloned directly into the NanoBiT vectors, however, the minimum synthesizable sequence length was 300 bp, and our constructs varied between 100-150 bp.

Hence we choose to order 300pb fragments, selecting extra bases from the 5' and 3' of the plasmid-insert digital assembly simulation.

By doing so, we can still proceed with a digest and ligate type of cloning (since EcoRI and XhoI restriction sites flank the interactors' sequence), but we could also try a different cloning strategy such as a recombination based one, since the 5' and 3' additional bases are identical to the NanoBiT vectors' bases surrounding the MCS.

3.2 Amplification of $N_{lg\ e\ sm}$; C_{lg}; $C^{+}_{N\ e\ C}$

We were gifted with 5 of the 7 different plasmids from the kit: N196; N198; N199; N203; N204. They were all sent already diluted in 2 ul of Nuclease Free H_2O, at a concentration of 50ng/ul. We amplified the different plasmids using *E. coli* strain DH5α.

After Plasmid purification, we obtained 50 ul of plasmid - Elution Buffer solution. NanoDrop quantification read the following:

Vector name	Concentration (ng/ul)	A260/280	A260/230
N196	90	1.93	2.50
N198	150	1.93	2.37
N199	90	1.82	1.95
N203	110	1.91	2.28
N204	110	1.91	2.20

Table 3.2: NanoDrop concentration and purity quantification of different NanoBit amplified plasmids

3.3 Plasmid Restriction

To prepare the NanoBiT vectors for a stick-end based ligation, each plasmid needs to undergo EcoRI-HF and XhoI digestion. We utilized 40 ul of each plasmid's purified amplification solution (MiniPrep eluted solution). After purifying the reaction mixture, the following (*Table 3.3*) concentration values were obtained through NanoDrop analysis:

Vector	Concentration (ng/ul)	A260/80	A260/230
N 196	36	1.78	1.92
N198	75.5	1.84	2.04
N199	42.7	1.81	2.18

Table 3.3: NanoBiT vectors concentration values

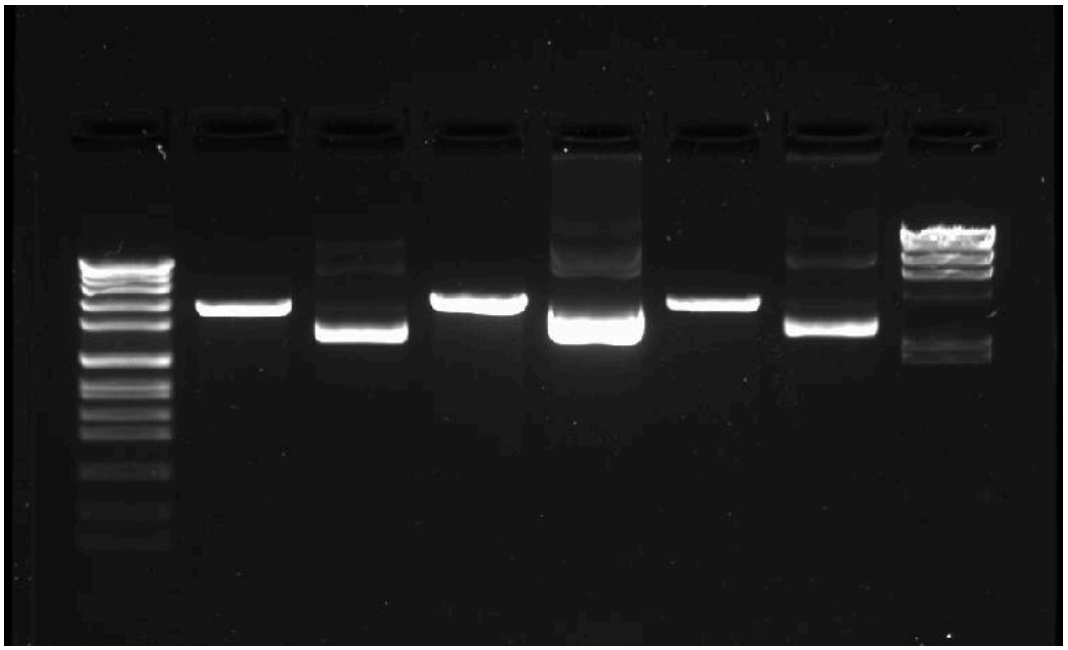


Figure 3.3: electrophoresis result. We loaded 2 ul of enzyme-less restriction product per well. The gel loading was done as follows: well 1: DNA Molecular Weight Marker VII; well 2: N196 Cut; well 3: N196 Not Cut; well 4: N198 Cut; well 5: N198 Not Cut; well 6: N199 Cut; well 7: DNA Molecular Weight Marker 2

Since 150 ng of plasmid will be utilized for each ligation, and we plan on testing 4 different peptides, the amplification's yield is more than enough to proceed with the ligation reaction, us having 45 ul of each vector at concentrations listed above.

4. Discussion

We expect that the results obtained from the NanoBiT assay will shed light on new therapeutic models for Progeria and other Protein-Aggregation Based Syndromes.

4.1 Synthetic DNA and NanoBiT plasmids - Conclusions

After a series of adjustments and optimizations, the sequences we ordered (8) will be essential for the different tests we plan to perform. For each of the Progerin-interacting peptide's amino acidic sequence (reader is referred to Table 3.1) we ordered two different DNA clonal genes from TWIST Bioscience® as the NanoBiT vectors Large-BiT N and Large-BiT C require different bases incorporated at the 5' and 3' of the DNA sequence for the correct expression of the fusion proteins.

Sequence maps of both the primer design (figure 3.1) and the sequences (figure 3.2) for peptide 1. 62aa11_1 can be found on chapter 3.1.

Since we already had Progerin and LMNA *Homo sapiens* optimized sequences in house, we only had to design primers with NanoBiT specific overhangs in order to amplify NanoBiT-compatible DNA fragments. The amplified sequences will be cloned into NanoBiT Vectors N198 and N199.

4.2 Possible NanoBiT assays with our yield

We plan on following with the ligation of each vector and interacting peptide sequence. For this procedure, we will utilize 150 ng of plasmid per reaction, counting 4 different reactions for each plasmid.

The quantity of vectors N196, N198 and N199 are sufficient to allow proper ligation without further plasmid amplification.

On the other hand, plasmids N202 and N203 do not need further manipulation and are ready to be transfected into our MRC5 cell culture utilizing a lipofectamine-based delivery system. Our current yield –4950 ng for both N202 and N203– is sufficient for more than 50 transfection for only 100 ng are consumed per transfection.

4.3 Progerin and LMNA: amplification and assays

As soon as the ordered primer pairs arrive, and both Progerin and LMNA gene are cloned, we will start our NanoBiT assay. We shall first test the control vectors (N203, N204), confirming: i) the functionality of our

reagents and ii) the compatibility of our MRC5 Cell Lines with a NanoBiT assay.

We shall then test LgBiT-LMNA and SmBiT-Progerin. If light signals are confirmed, Progerin is set to function properly in our NanoBiT assays. Assays between SmBiT-Progerin and LgBiT-Interactor will then test for interaction, whilst SmBiT-LMNA on the other hand will test for Progerin-specificity. This is to ensure that our interacting peptides do not target the wild-type LMNA gene. If such constructs were to test positive for interaction, further applications would be useless, since LMNA depletion could destroy the nuclear lamina, harming an already altered nuclear region for HGPS patients.

4.4 Possible implementations

If the assay proves its functionality, we plan on screening a larger number of possible interacting peptides. Furthermore, we could add experimental data by testing all different Progerin protein fusions possible, for in this first screening we will only test Progerin in the C terminal of the fusion protein.

Subsequently, each interactor would be fused to a complementary BiT, allowing a complete NanoBiT assay.

Furthermore, we could opt for a delayed transfection of the Progerin-interacting peptides in order to allow SmBiT-Progerin nuclear localization.

4.5 Future use - Progerase *in vivo*

If an interaction between progerin and any of our constructs is to be recorded, we plan on testing a RING-interactor fusion protein directly on HGPS affected cell lines (Primary HGPS-affected fibroblast cell line cc00366s from Biobanca delle Laminopatie e del Muscolo).
This therapeutic approach, if designed correctly, will provide HGPS patients with a reliable solution by degrading Progerin accumulation in the nucleus.

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