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TRABAJO FIN DE GRADO
BIOTECNOLOGÍA

Production of influenza-antigen fusion proteins utilizing recombinant protein technologies.

Álvaro Garcés Cardona

Director/a:

Minna Hankaniemi

Niila Saarinen

TAMPEREEN YLIOPISTO - UNIVERSITY OF TAMPERE, FINLAND

Ponente:

Milagros Medina

Facultad de Ciencias
Universidad Zaragoza

UNIVERSITY OF TAMPERE

INDEX

1. Abstract

Human influenza viruses are respiratory pathogens that transmits by respiratory droplets and by contact and cause about 250.000 deaths each year. Nowadays, vaccination is the most effective method to prevent human influenza diseases. Safer and cheaper than the usual vaccines, Virus-like particles (VLPs) are multiprotein structures that imitate the native viruses but lack the viral genome, so they are not infective. To avoid the annual renewal of these vaccines due to the constant change of these viruses, in this thesis we look for antigens encoded by conserved sequences of the virus, with the aim of developing an universal human Influenza A vaccine candidate that would be valid for several years, or even for a lifetime.

For that, in this thesis we optimize the production of two antigens, M2e and H1F. We search for the best strain, temperature, optical density to induce the protein production and the amount of IPTG inductor, calculating the amount of protein produced under these conditions. The results obtained indicate that the optimum conditions for the production of M2e are the Star strain, 25ºC, an optical density of 0.6 and 1 mM IPTG to obtain a yield of 34 mg/L. To produce H1F, the optimum conditions are the Star strain, 18ºC, an optical density of IPTG of 0.8 and 1mM IPTG to obtain a yield of 7 mg/L. Once the process of production has been optimized, the first candidates for VLP vaccines can be formulated, which will start the whole process of clinical trials.

Los virus de la gripe humana son patógenos respiratorios que se transmiten mediante gotitas respiratorias y por contacto, causando alrededor de 250.000 muertes cada año. Hoy en día, la vacunación es el método más eficaz para prevenir la gripe. Más seguro y más barato que estas vacunas normales, los Virus-like particles (VLPs) son estructuras multiproteicas que imitan la forma nativa de los virus a excepción del genoma vírico, por lo que no son infecciosos. Para evitar la renovación anual de estas vacunas debido al constante cambio de estos virus, en este trabajo buscamos antígenos codificados por secuencias conservadas del virus, con el objetivo de desarrollar una vacuna contra el virus de la gripe A humana universal que se podría utilizar por años, o incluso para toda la vida.

Para ello, en este trabajo realizamos la optimización de la producción de dos antígenos, M2e y H1F. Buscaremos la mejor cepa, temperatura, densidad óptica a la que inducir la producción y cantidad de inductor IPTG, calculando la cantidad de proteína producida en esas condiciones. Los resultados obtenidos indican que las condiciones óptimas para la producción de M2e son la cepa Star, 25ºC, una densidad óptica de 0.6 y 1 mM de IPTG para obtener un rendimiento de 34 mg/L. Para la producción de H1F, las condiciones óptimas son la cepa Star, 18ºC, una densidad óptica de 0.8 y 1mM de IPTG para obtener un rendimiento de 7 mg/L. Una vez optimizado este proceso de producción, se podrán formular los primeros candidatos a vacunas VLP, los cuales comenzarán todo el proceso de ensayos clínicos.

2. Aims for the project

The essence of the project is to create a vaccine using antigens of the non-variable region of the influenza virus and Virus like particles. For that, there are two independent parts:

1) Producing VLP with SpyTag using Insect Cells.
2) Producing SpyCatcher with the antigens using recombinant bacteria. We used two antigens, SpyCatcher-M2e and SpyCatcher-HF1.

After both of them are produced, SpyTag and SpyCatcher will bind and, as result, we will have the VLP with the antigen we want.

This thesis will only be focus in the second point of the project, producing the SpyCatcher with the antigen, more exactly making the purification and production optimization protocol to produce the protein.

The **specific objectives** of the project are to find out the optimal purification conditions for the antigens:

- The most efficient cell line for the production of the antigens

- The optimal cell culture conditions for the production of the antigens: optimal culturing temperature and OD_{600}.

- The optimal induction protocol

- The estimate amount of protein produced with the optimal conditions.

3. Background

3.1 SpyTag and SpyCatcher:

SpyTag is a short peptide that forms covalent bonds with its protein partner SpyCatcher, which is used to create 'unbreakable' peptide tags.

In Biotechnology, this can be used to stabilize enzymes. If you fuse SpyTag and SpyCatcher to the termini of a protein, it leads to a spontaneous cyclisation, forming a SpyRing (1). After that, if you heat the enzyme, for example, at 100 °C and then you re-test it at room temperature, the SpyRing enzyme still has most of its initial activity. In **Figure 1**, we can see as the protein of interest is fused with an N-terminal SpyTag and C-terminal SpyCatcher, which spontaneously lock together.

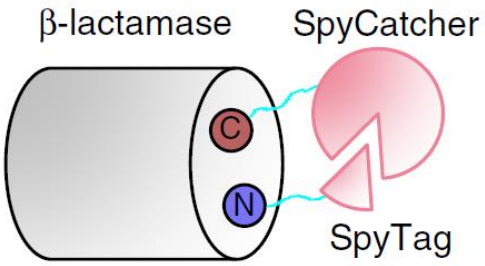

Figure 1. *SpyRing generation: the protein of interest (β-lactamase in this example) is genetically fused with an N-terminal SpyTag (blue) and C-terminal SpyCatcher (red), which lock together*. (2)

Another use in this field is the vaccine optimization (3). The SpyTag is covalently linked to a dendritic cell-targeting antibody to the optimal location. At the same time, SpyCatcher is fused to an antigen (for example, albumin). Then, they are conjugated to create a full vaccine, with both the targeting unit and the antigen. In conclusion, SpyTag/SpyCatcher has now established itself as an improved route to covalent protein conjugation.

3.2 M2e and H1F based universal vaccines

Influenza A viruses can be divided into several subtypes depending on the antigenic differences in their main surface glycoproteins. There are two main glycoproteins in the membrane of the virus (**Figure 2**). Influenza hemagglutinin (HA) is the major influenza virus envelope glycoprotein (4) and it is the primary target of the humoral response during infection/vaccination, using neutralizing antibodies (nAbs) against them. When HA0 (the initial protein) is proteolytically cleaved, it produces HA1, the head domain which is used by the virus to attach itself to its host cell, and HA2, which comprises most of the stem domain of the protein. The other main glycoprotein is neuraminidase (NA).

Both HA and NA are under continuous and rapid evolution. For that reason, vaccines with antigens from these proteins have to be renewed every year (5). On the other hand, to make a long-lasting, universal vaccine against influenza, it can be used the HA2, as it is 90% conserved for H1 and H3 influenza subtypes (6).

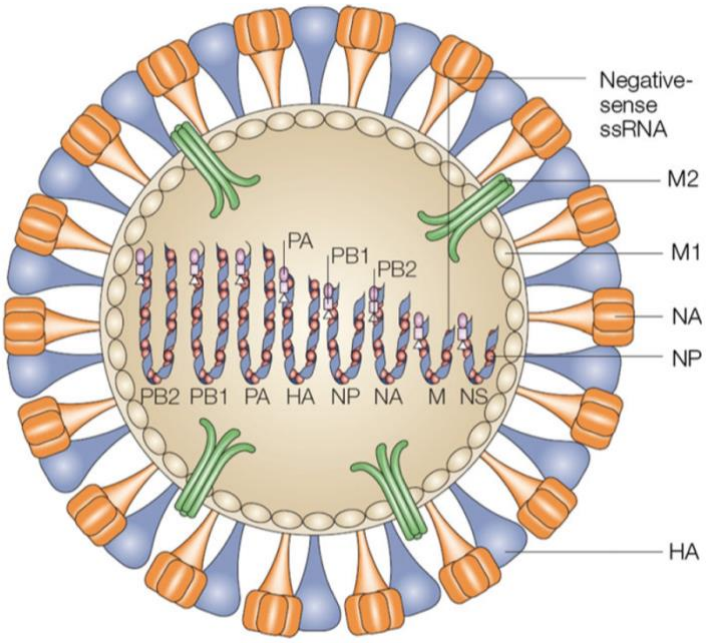

Figure 2. *This is a representation of influenza A virus, which has a lipid envelope (derived from the host cell membrane). We can see three envelope proteins (HA, NA and M2). The ribonucleoprotein complex comprises a viral RNA segment associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1 and PB2). We also can see the matrix protein 1 (M1) and matrix protein 2 (M2, an ion channel protein)* (7).

Matrix-2 protein (M2) is a type III integral membrane protein that forms a proton-selective ion channel, which is activated at acidic pH (8). It is vital for the virus, as during virus entry, via receptor-mediated endocytosis, M2 transport protons across the virus membrane reducing the pH of the virion interior, which is necessary as the low pH facilitates protein-protein dissociation between the matrix protein and the ribonucleoprotein (9). In addition, during infection, M2 ion channel increases the pH of the trans-Golgi network, which helps to prevent premature acid activation of newly synthesized HA and the consequent inactivation of progeny virus. The whole M2 protein has 97 amino acids, but we only use the first 23 amino acids in the N-terminal of M2 (referred to as `M2e´from now on).

H1F is the HA ministem construct derived from the HA protein of an H1 strain influenza virus (10). This construct contains the conserved stem parts of the HA protein without disulfide bonds.
F in the name means the Foldon domain that is added to the C-terminus (bottom) of the protein to promote trimerization.

M2e is the first of the influenza antigen that we are going to produce in *E.coli.* First, we have to order it from GenScript. For that, we provided them with a gene insert that contained SpyCatcher genetically fused to the N-terminus of the M2e peptide. With that, they will synthetize this gene and then subclone it into a pET-11b plasmid, which we will use for our protein production.

H1F is the second one we are going to produce in *E.coli*. We design the plasmid doing the same thing for both of them.

3.3 Plasmids

The antigen coding genes have been cloned into pET11b plasmids (**ANNEX**). In the pET11b plasmids, there are some components of vital importance for successful expression of the target proteins including ampicillin resistance gene, lac operon, his-tag, ori and rop.

Ampicillin resistance gene: This is necessary because it will be our selection marker, so every time that we make a culture with ampicillin, we will be sure that most of the bacteria that grows there will contain our plasmid, and, therefore, our gene, so they will be able to product the protein. Ampicillin is an antibiotic that kills bacteria, so only our bacteria that contains the plasmid with the resistance gene and other bacteria that have developed resistance because of a mutation (a minority) will be able to grow in its presence.

Lac operon: It is used for the gene regulation. Glucose inhibits the lac operator, so if we add it we will make sure that our protein will not be produced, what can be useful if we don't want our protein to be produced all the time. That is because it allows the repressor lacl to bind the lac operator. On the contrary, IPTG (a lactose analogs) induces the protein production as it binds the repressor protein lacl and produce a conformational change that decrease its affinity for the lactose operator. This way, when we add IPTG, we will be allowing our bacteria to produce the protein at the exact moment we want.

6x His: It is the His-tag, which will make easier the purification of the protein. If we use an Inmobilized Metal Anion Resin (IMAC) , our protein will bind it strongly. Meanwhile, the other proteins will not bind so we will be able to separate them easily.

Ori: The origin replication in bacteria will allow to replicate the plasmid once it is inside of the bacteria.

Rop: is a small protein responsible for keeping the copy number of the pET plasmid. The only difference between both plasmid is the antigen we are fusing to SpyCatcher (M2e or H1F).

4. Material and methods

4.1 Theory and instruments relevant to the study

- **Centrifugation:** It is a process used to separate components of a mixture by spinning them at very high speeds. This causes that the more dense particles of the mixture migrate away from the axis of rotation faster than the lighter ones. At the end, we usually have two parts, the pellet (solid) and the supernatant (liquid). According to what you are going to do, you can adjust the speed, time and temperature of the procedure. (Sorvall LYNX 4000 Centrifuge, Thermo Scientific, Italy).

- **Electrophoresis SDS-PAGE:** Electrophoresis is the separation of macromolecules in an electric field. In this case, we use a polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) as a way to denature the proteins. This can be used to estimate relative molecular mass and the amount of protein we have in every sample. SDS is a detergent that, with the help of Mercaptoethanol (to break disulfide bonds), disrupts the tertiary structure of proteins, so every protein is linear. In addition, SDS also gives a uniform negative charge, so the charge of the protein is proportional to its molecular weight and it will not be differential migration based on charge, only its size.

- **Optical density measurement:** optical density was followed at 600 nm Wavelength.
  This measurement, compared to a blank (usually a buffer or water), gives an estimate of the concentration of the cells in the sample. That way, you can identify the growth phase of the population of bacteria.

  At the beginning, when the bacteria are in the lag phase, the OD_{600} measurement is nearly 0.
  In the log phase, reproduction accelerates and the bacteria population increase, which corresponds to 0.5-0.6 OD_{600}.
  Beyond these values, the bacteria will stabilize during the stationary phase, and then it will decline during the death phase. This can be seen in the **Figure 3.** (BioPhotometer Eppendorf AG, Hamburg).

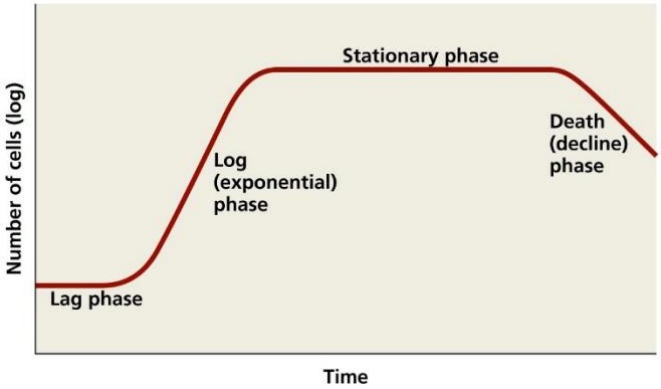

Figure 3. *Four-phase pattern of bacteria population growth. It express the number of cells during the time between the lag phase and death.*

- **Bacteria cell homogenization:** bacterial cells were lyzed using a high pressure homogenizer (Emulsiflex C3 avestin, Canada). High-pressure homogenization is an efficient means of cellular disruption, whereby a sample is subjected to ultra-high pressures as it is forced through a narrow orifice. As the sample exits the orifice, stress forces such as cavitation and shear are generated, lysing the cell.

- **Liquid phase chromatography:** Liquid chromatography is a technique that can be used to separate molecules based on their differential interactions with a chromatography matrix. There are different types of chromatographies, but in this study we used IMAC affinity chromatography, which is based on the ability of immobilized metal anions to bind to histidine amino acids. Using this system, molecules of interest are separated by differential interaction with a chromatography matrix via an affinity tag. With a gradient of ligand, salt or pH, we can elute the protein achieving a better purification. (Ge healthcare, äkta p100. **Figure 4**).

Figure 4 . *ÄKTA Protein Purification System used to make the protein purification.*

- **UV-VIS measurement:** UV-VIS spectophotometry is a way to measure protein, DNA and RNA. We used Nanodrop instruments, that only need 1-2µL of sample. (NanoDrop 2000 ja NanoDrop One. Thermo Fisher Scientific, Wilmington, DE, USA)

- **Bacterial transformation:** Transformation is a process of horizontal gene transfer by which some bacteria take up foreign genetic material (naked DNA) from the environment. Bacteria can be modified to make them more likely to take up foreign genetical material under different conditions, such as heat shock. Such bacteria are referred to as competent bacteria (11).

We are going to test the protein production in three different strains:

- OverExpress™ C43(DE3) pLysS Chemically Competent Cells
- OverExpress™ C41(DE3) Chemically Competent Cells
- BL21 Star™ (DE3)pLysS One Shot™ Chemically Competent E. coli

C41 and C43 have been modified to withstand production of proteins that would be toxic to most bacteria.

All three have in common that are chemically competent cells designed for applications that require high-level expression of non-toxic recombinant proteins from high copy number. They

are descended from the E. coli B strain. This is because of their key genetic markers and their inducibility of protein expression.

4.2 Production and purification of SpyCatcher-M2e and SpyCatcher-H1F

In this protein production and purification, we optimized four different conditions following the order of the **Figure 5.**

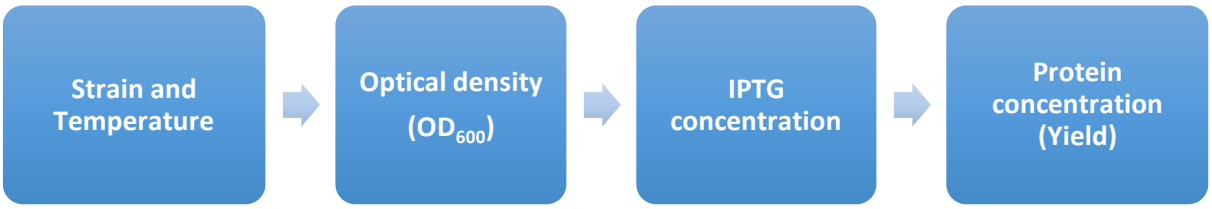

Figure 5. *Order of conditions to optimize of our protein optimization.*

The protocol is the same for each condition. Although the steps are explained in the next pages, in a few words the process we followed four times would be the one shown in the **Figure 6.** In addition, only to calculate the yield we need to calculate the protein concentration using the AKTA purification system.

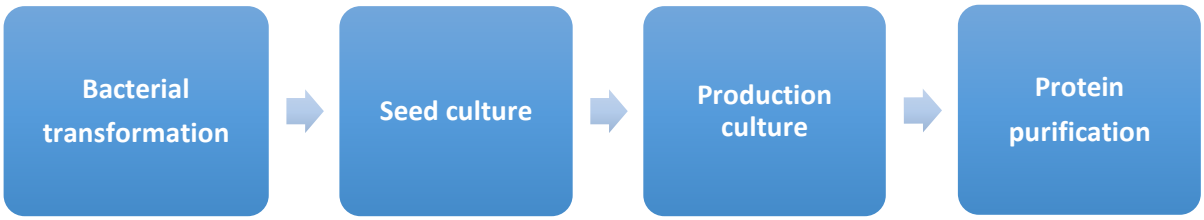

Figure 6. *Process followed to optimize each condition.*

The parameters of the conditions tested were:

- Strain: C41, C43 and Star
- Temperature: 18ºC, 25ºC and 37ºC.
- Optical density: 0.20, 0.40, 0.60, 0.80 and 1.00
- IPTG concentration: 0.01 mM, 0.1 mM, 1 mM and 10 mM.

4.3 Temperature/strain optimizacion

The first thing to do was to insert our construct SpyCatcher-M2e into three different bacteria strains (Star, C43 and C41). That way, we could choose the best one for our construct regarding the protein production. As I already said, this took us four days.

We started doing a bacterial transformation. We mixed the construct with three different bacterial strain (Star, C41 and C43). It was important to maintain the bacteria always in ice, as they are quite sensitive after being frozen. We left it there 30 min so the construct could attach to the bacteria's membrane.

Then, we made a 'Heat Shock' (to insert the plasmid) at 42°C for 30 seconds to help the inserts to penetrate the bacteria, as they had a shuttle plasmid with the necessary genes to incorporate the plasmid pFastBacDual. We had to put it again on ice quickly.

After that, we added 500 µl of SOC, (that had to be at body temperature for a better use), which function was to help the bacteria grow, and we took it to the shaking incubator at 37 °C for 1 hour. Then, we plated the bacteria into two plaques with different concentration (20 µl and 100 µl), which had agar and ampicillin (our selection marker). We left it overnight.

Next day, the bacteria had grown and they were ready to make the seed culture. We plated two different amounts of bacteria for each strain, so now we had to choose the best one to take a colony.

We can see the results in the **Figures 7, 8 and 9**.

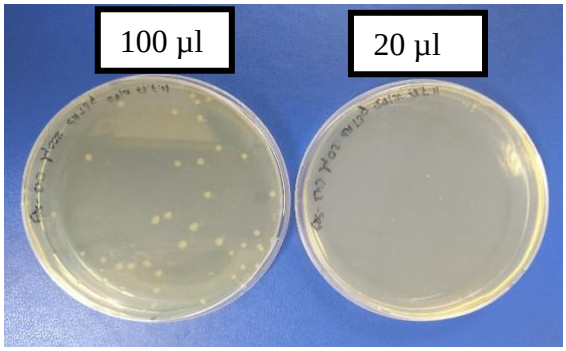

***Figure 7**. In this picture we have two plates with the strain C43 with 100 µl and 20 µl of bacterial culture. Each white spot represents one colony.*

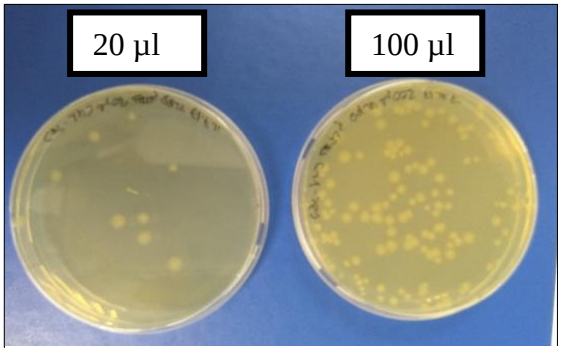

***Figure 8.** In this picture, we have two plates with the strain C41 with 100 µl and 20 µl of bacterial culture. Each white spot represents one colony.*

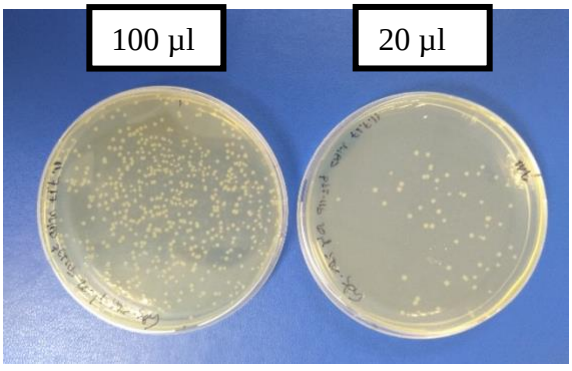

***Figure 9.** In this picture, we have two plates with the strain Star with 100 µl and 20 µl of bacterial culture. Each white spot represents one colony.*

In the **Figure 7**, we have the C43 strain. In this case, in the 20 µl we do not have colonies, so we take the colony from the 100 µl plate.
In the **Figure 8**, we have the C41 strain. In this case, it is easier to take a colony from the 20 µl plate.
In the **Figure 9**, we have the Star strain. In this case, it is also easier to take a colony from the 20 µl plate.

We transferred six colonies (two of every plate) to a six different 50 ml Nunc tube (which have 5 ml of LB medium (it contains NaCl, tryptone and yeast extract), 5 µl of ampicillin (100 µg/ml) and 0.07 ml of glucose(0.5 % w/v)). We took two colonies of every plate to make duplicates, just in case one of them would not work.
The glucose inhibits the lactose operator, so our protein can not be produced and the bacteria can concentrate on multiplying. Now, we left it overnight at 37 °C and shacking in 200 rpm.

Next day, we started pelleting the seed cultures (4,000 g, 10 mins) and resuspending them into the same amount of fresh LB w/o antibiotics, 5 ml. We did this to get rid of the beta lactamase. Then, we inoculated 30 ml LB medium with ampicillin (200 µg/ml, so we added 60 µl) and glucose (0.5 %, so we add 42 µl) in a Erlenmeyer.
After that, we left them grow with vigorous shaking (around 200 rpm) until OD_{600} was between 0.3-0.6. Then, we added the necessary volume of IPTG (induces the lactose operator and the protein expression as well) until 1mM concentration. As IPTG was 1 M, we had to add 30 µl.
We divided the 30 mL production culture into three 10 mL cultures. Now, we could incubate the divided cultures overnight in different incubators in a way that each construct was expressed in 18 °C, 25 °C and 37°C. That way, we also knew which temperature is best for the production.

Last day, we only had to collect the cells by centrifugation (4,000 g, 10 mins), discard the supernatant and make the purification optimization for the his-tagged proteins.
Now that we had the pellet, we resuspended it in EasyLyse solution and we lyzed the bacteria according to the manufacturer´s instructions.

Then, we incubated it 5 min at room temperature, waiting until the solution had broken the bacteria and we pelleted the cellular debris by centrifugation (2 min, 4,000 rpm) and we took a sample of each pellet (we will use it later in a SDS-PAGE). Then, we transferred the supernatant fluids to a clean tube.
Near to the end, we used a resin that binds the protein, so we can separate them. This resin was `2-iminobiotin sepharose 6 fast flow´ (Affiland, Belgium).

We added 100 µl of this resin to the supernatant and we let the resin bind to the proteins 2 hours on agitation.
After that time, we centrifuged the suspension at 20,000 g for 5 min and we separated the supernatant from the resin. This supernatant was equivalent to a flow through sample, which we also used in the SDS-PAGE.
Finally, we added the loading buffer (Laemmli), to the resin to the supernatant and to the pellet, we boiled them for 10 min and we run the gel.

After we imaged the gel, we could compare the different cell lines properly and chose the one with the most target protein in the resin sample.

Now that we knew which strains was the best, we had to test the other conditions (OD_{600}, amount of IPTG and the purification process). We needed many bacteria with our plasmid, so if we want to skip the bacterial transformation (day one in our protocol), we can make a glycerol stock. The advantages of this stock is that we can have a big amount of it frozen, so it will last a long time. As it is made from a single colony, variation due to the production being done in different clones is not present.

To make a glycerol stock from an LB plate we picked a single colony from a plate and put it in 50µl LB. Then we spread it to a new plate with the appropriate antibiotics and incubated it overnight at 37 ºC. Once the new plate was covered by the bacteria, it was betached by using 1-2 ml freezing buffer (15% glycerol and 0.9% NaCl). The bacterial suspension was aliquoted and stored them at -80 ºC.
Now, every time we needed bacteria, we could defrost one of the aliquots and spreaded everything into a new plate with ampicillin.
Making the glycerol stock did not save us a day of work, because after we had put the contain of the tube into a new plate we had to wait one day for the bacteria to grow, before we made the seed culture. However, it did save us work and time, as we did not have to make the bacterial transformation every time (what takes approximately 2-3 hours).

4.4 OD_{600} optimization

This time, we produced the protein again, but this time we used the best strain and temperature to produce it, Star at 25 ° C for SpyCatcher-M2e and Star at 18 ºC for SpyCatcher H1F. We repeated the process but with different OD_{600}.
First we cultured the best cell line at best temperature (same way as we did before) and, when we had one large culture, we measured the OD_{600} and added the IPTG when the OD was at 0.200, 0.400, 0.600, 0.800 and 1.00.

Every time we took 10 ml of the large culture to a separate 50 ml Falcon tube and then, we induced it with 1 mM IPTG. As this time we needed 5 samples of 10 ml each, we had to make a larger production culture, for example of 100 ml. We took the 5 ml of the seed culture and mixed it with 95 ml of LB, so we had a 1:20 dilution. The amount of ampicillin and glucose changed, so we used 200 µl of ampicillin and 140 µl of glucose.
We had to put 50 ml in 5 tubes of 10 ml and other 50 ml to spare, just in case.
Next day, when the IPTG had made effect and the protein had started to be produced, we made the purification and a similar gel as with the temperature/strain selection.

4.5 IPTG concentration optimization

Now that we knew the optimal OD_{600}, we could optimize the IPTG concentration. To do so, we had to repeat the process once more at optimal strain, temperature and at optimal OD_{600}. Then, we could divide the culture to 4 culture tubes of 10 ml and induced them with 0.01 mM, 0.1 mM, 1 mM and 10 mM final concentration of IPTG.

As this time we needed 4 samples of 10 ml each, we had to make a larger production culture, for example of 50 ml.
We took the 5 ml of the seed culture and mixed it with 45 ml of LB, so we had a 1:10 dilution. We added ampicillin (100 µg/ml) and glucose (0.5 % w/v) to have the proper final concentrations. Now we had 50 ml to put in 4 tubes of 10 ml and other 10 ml to spare, again just in case.
Then, after the purification of the protein, we made another SDS-PAGE gel.

4.6 IMAC purification optimization

With this purification, we were able to estimate the concentration of protein we can produce following the determined conditions.

First, we took one of the 100 µl aliquot from the glycerol stock of SpyCatcher-M2e or SpyCatcher-H1F and we made the seed culture (with LB, ampicillin and glucose).
Next day, we made a 500 ml protein production culture with the already optimized production conditions (Strain: Star; Temperature: 25 °C). Then, we waited until OD is 0.6 and we added IPTG until a final concentration of 1 mM.

Next day, we divided the culture solution into 2 tubes and we centrifuged it them to remove the LB medium, 15 min at 12,000 rpm. Then, we resuspended the pellets to binding buffers and we lysed them. The binding buffer we used contained 500 mM NaCl, 50 mM NaHPO4 (pH=7.5) and 20 mM Imidazole.

We lyzed the bacteria as before, using the high pressure homogenizer. Then, we centrifuged again and we took a sample of the insoluble part and another one from the soluble part (what we would use in the SDS-PAGE gel).
After that, we used the ÄKTA protein purification system.

Now, with the graph given by the program UNICORN, we saw that our eluted SpyCatcher-M2e had a peak in the tube B2. For that reason, we made a new SDS-PAGE electrophoresis with the tubes A9, B1, B2, B3, B4, B5, B7, B10 and C3 to see how much protein we had there and with the soluble and insoluble part, the flow-through and the wash.

5. Results and discussion

5.1 Strain and temperature

In the Pellet, we should see many bands, corresponding to all the proteins that the strains were producing (including our target protein).

In the Flow through, we should see all the proteins except ours, as it had been binding to the resin and the others had not. Sometimes we could see our protein in this sample because it did not bind perfectly, what could be either because the affinity was not 100% or because we did not have enough resin to bind all the proteins.

In the Resin, we only should have seen one band corresponding to our protein, as it is the one that had the His-Tag and, consequently, binds the resin. However, other proteins have the amino acid histidine, so they can bind the resin (in minimal amounts) and we can see unspecific bindings.

The results for SpyCatcher-M2e are shown in the **Figure 10.**

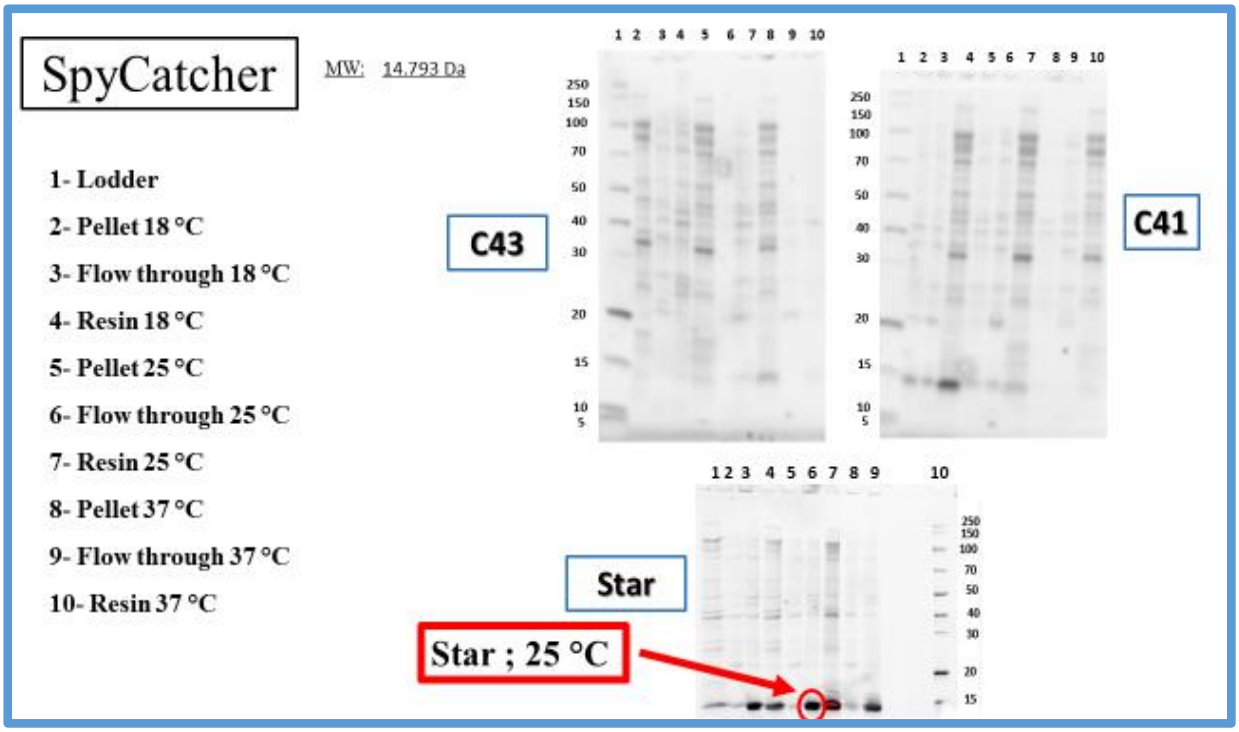

Figure 10. Results of the strain and temperature optimization of the antigen SpyCatcher-M2e. *One SDS-PAGE for each strain (C43, C41 and Star) with the lodder and the pellet, flow through and resin samples at three diferent temperatures, 18ºC, 25 ºC and 37 ºC. Best conditions are indicated by red color.*

In C43 we could see our protein (almost in the 15kDa band) in the three pellets, but just a little, what means that the strain does not produce a lot of protein. In the resin of 25 °C, we can see something, buy it is not as strong as in Star.

In C41, we could see a lot of produced protein at 37 °C, but we were not able to see as much in the resin, and part of it remained in the flow-through.

In Star cell line, we could see a lot of produced protein in the three different temperatures, and almost everything in the resin. As it was hard to know which one is better, the 25 °C spot seemed darker and we did not have a lot in the flow through, so it was a good one. We chose this one for continuing the optimization procedure. In addition, we could see a lot of protein in the three pellets. Although the band of the 37 °C pellet was bigger, it may was because the bacteria produced other proteins of similar weight at that temperature, so it was not necessarily, because at that temperature the strain produced more protein. If we look the pellets, it was bigger the one in the 25 °C band, so that was the amount of protein that binds to the resin.

We made the purification with PBS buffer and that it was not very specific as we can see many bands in the resins of other proteins that are not our protein.

The results for SpyCatcher-H1F are shown in the **Figure 11**.

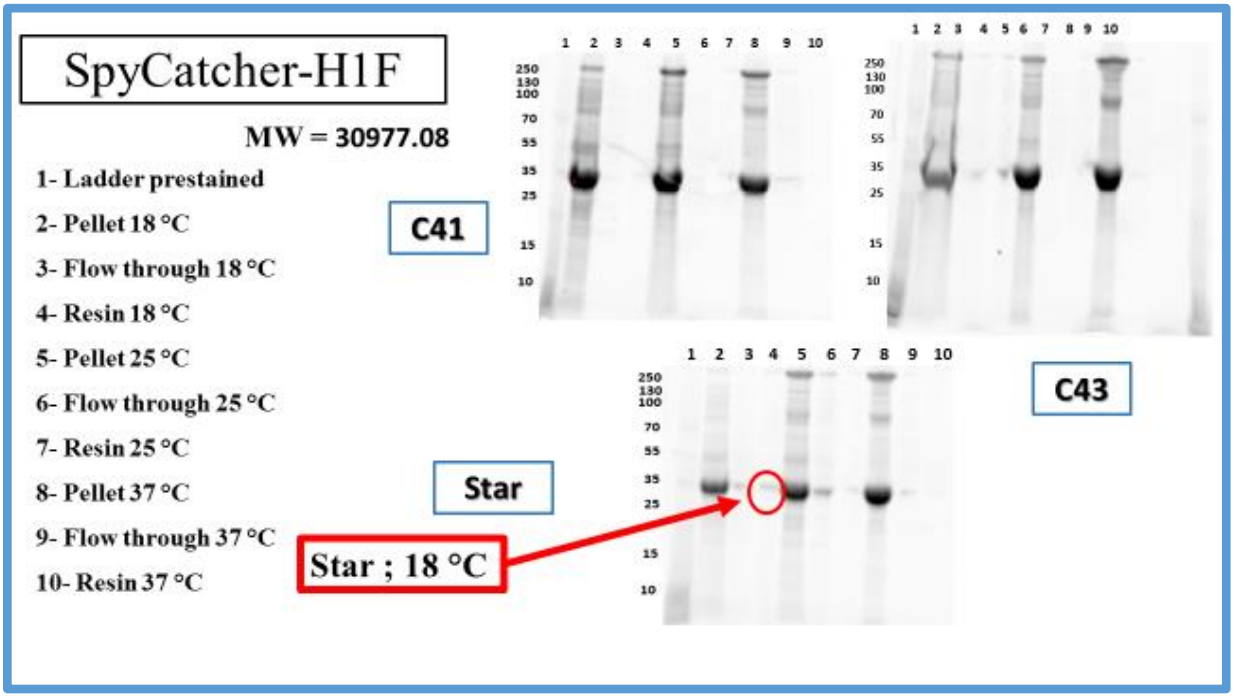

Figure 11. Results of the strain and temperature optimization of the antigen SpyCatcher-H1F. *One SDS-PAGE for each strain (C43, C41 and Star) with the lodder and the pellet, flow through and resin samples at three diferent temperatures, 18ºC, 25 ºC and 37 ºC. Best conditions are indicated by red color.*

In C41, we did not see any bands, so we discarded that one to produce our protein.

In C43 we could see a small band in the resin at 18 °C.

In Star, we could see bands in all the resins, so we chose that strain. Consequently, as we had a darker band in the resin at 18 °C, we chose this one.

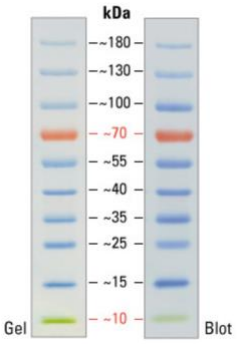

Figure 12. *Prestained Ladder used to optimize the strain and temperature of the antigen SpyCatcher-H1F.*

This time we tried a prestained Ladder, which we can see in the **Figure 12**. It was not as good as the unstained with this kind of gels, so after taking the picture we could not differentiate the different bands of the Ladder perfectly. However, after running the gel, it was clear enough so we could know in which part of the gels are the 25 KDa and 35 KDa bands and compare it with the image.

5.2 Optical density

The results for SpyCatcher-M2e are shown in the **Figure 13.**

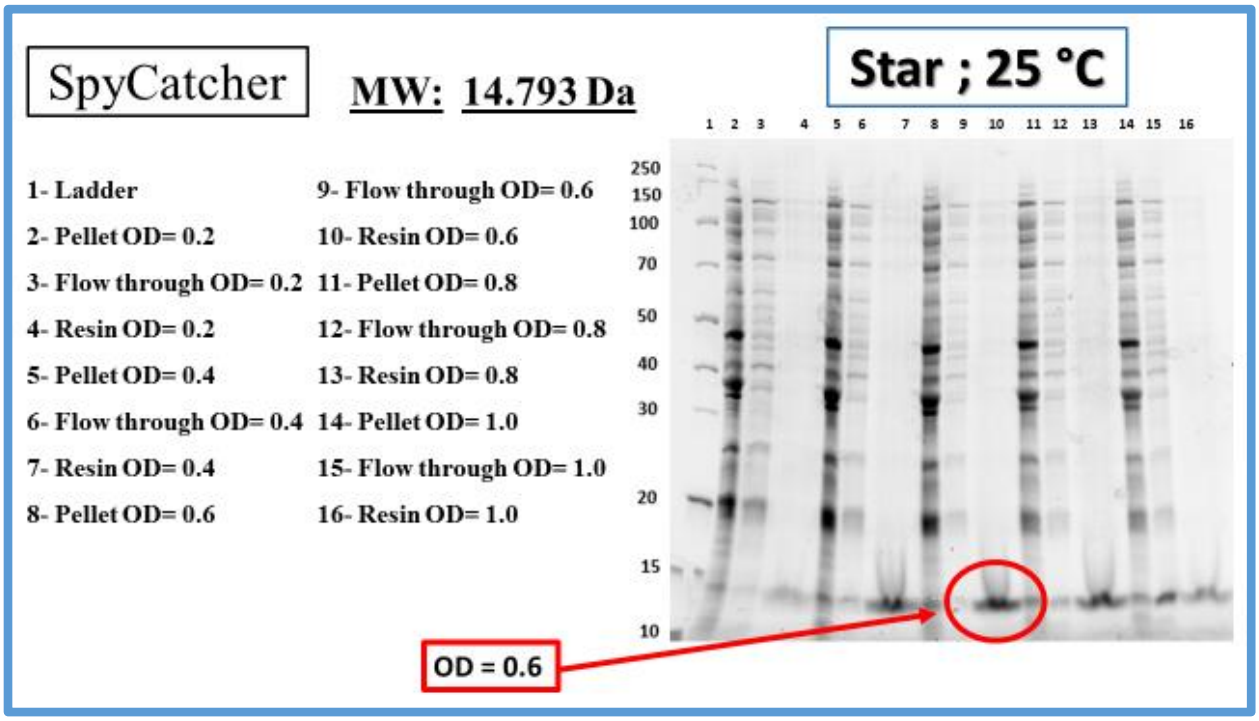

Figure 13. Results of optical density optimization of the antigen SpyCatcher-M2e. *In this figure we can see a SDS-PAGE for Star at 25 ºC with the lodder and the pellet, flow through and resin samples at five diferent optical densities: 0.2, 0.4, 0.6, 0.8 and 1 . Best condition is indicated by red color.*

We saw that we had a lot of protein when OD=0.4, 0.6 and 0.8. However, the best of those three seemed to be the one with OD=0.6.

In the next condition optimization, as we knew because of our experiment results, the best conditions we used for the antigen SpyCatcher-M2e are the strain star at 25 °C and a OD = 0.6 at the moment that we added IPTG.

The results for SpyCatcher-H1F are shown in the **Figure 14**.

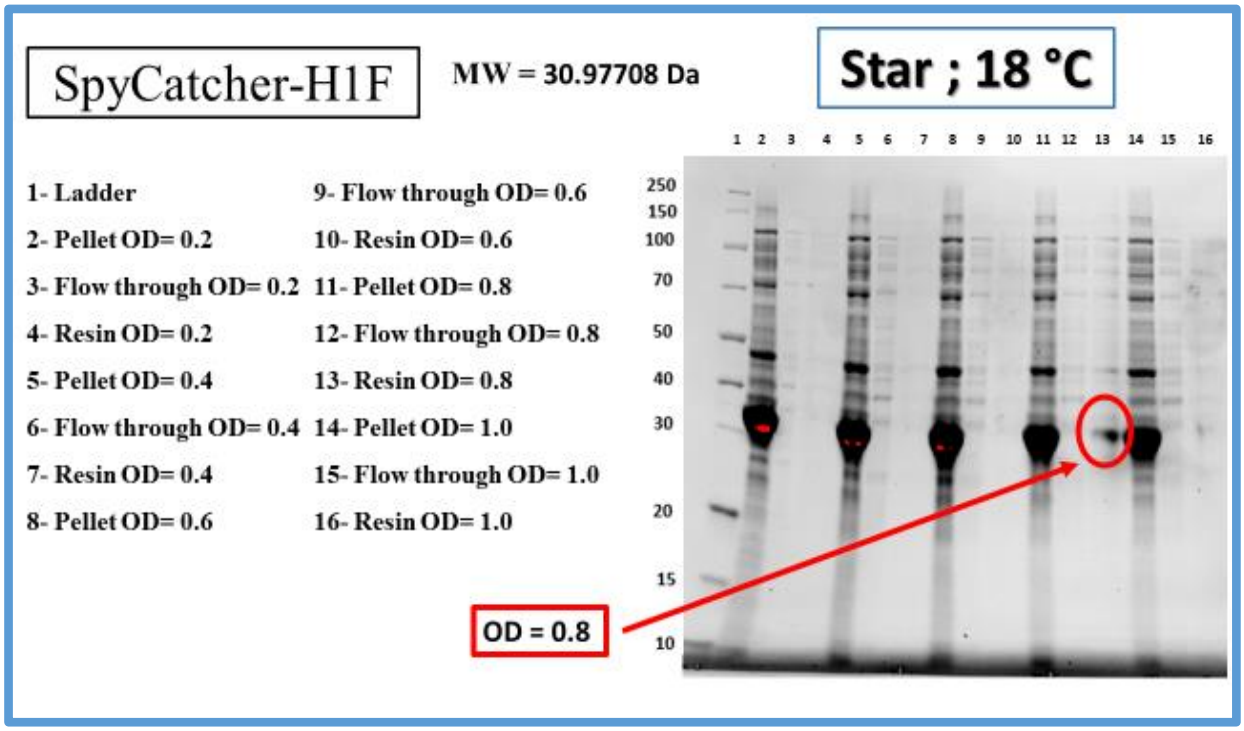

Figure 14. Results of optical density optimization of the antigen SpyCatcher-H1F. *In this figure we can see a SDS-PAGE for Star at 18 ºC with the lodder and the pellet, flow through and resin samples at five diferent optical densities: 0.2, 0.4, 0.6, 0.8 and 1 . Best condition is indicated by red color.*

We only saw a band in the resin when the OD=0.8, so we chose this one for our next step of the optimization.

5.3 IPTG concentration

The results for SpyCatcher-M2e are shown in the **Figure 15**.

It was clear that the stronger and darker band was the one in the resin with 1 mM of IPTG, so we chose that concentration to continue the optimization of SpyCatcher-M2e.

The results for SpyCatcher-H1F are shown in the **Figure 16**.

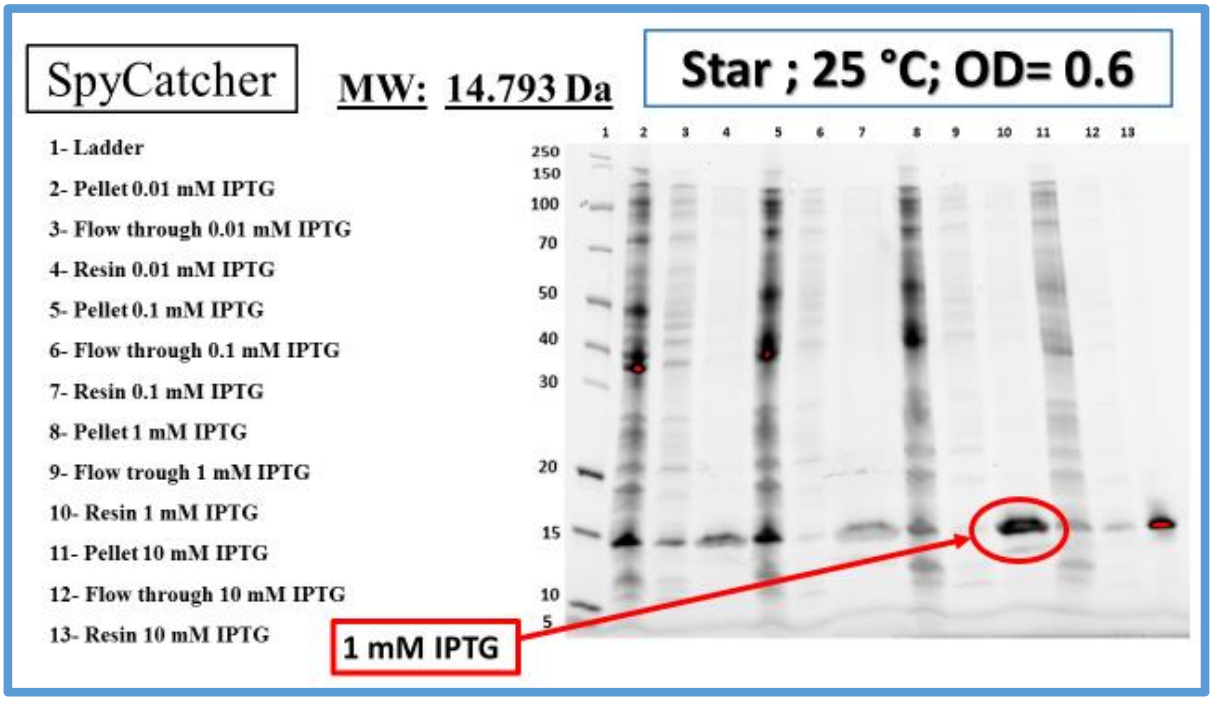

Figure 15. Results of IPTG concentration optimization of the antigen SpyCatcher-M2e. In this figure we can see a SDS-PAGE for Star at 25 ºC and an optical density of 0.6 with the lodder and the pellet, flow through and resin samples at four diferent concentrations of the inductor IPTG: 0.01 mM, 0.1 mM, 1 mM, and 10 mM . Best condition is indicated by red color.

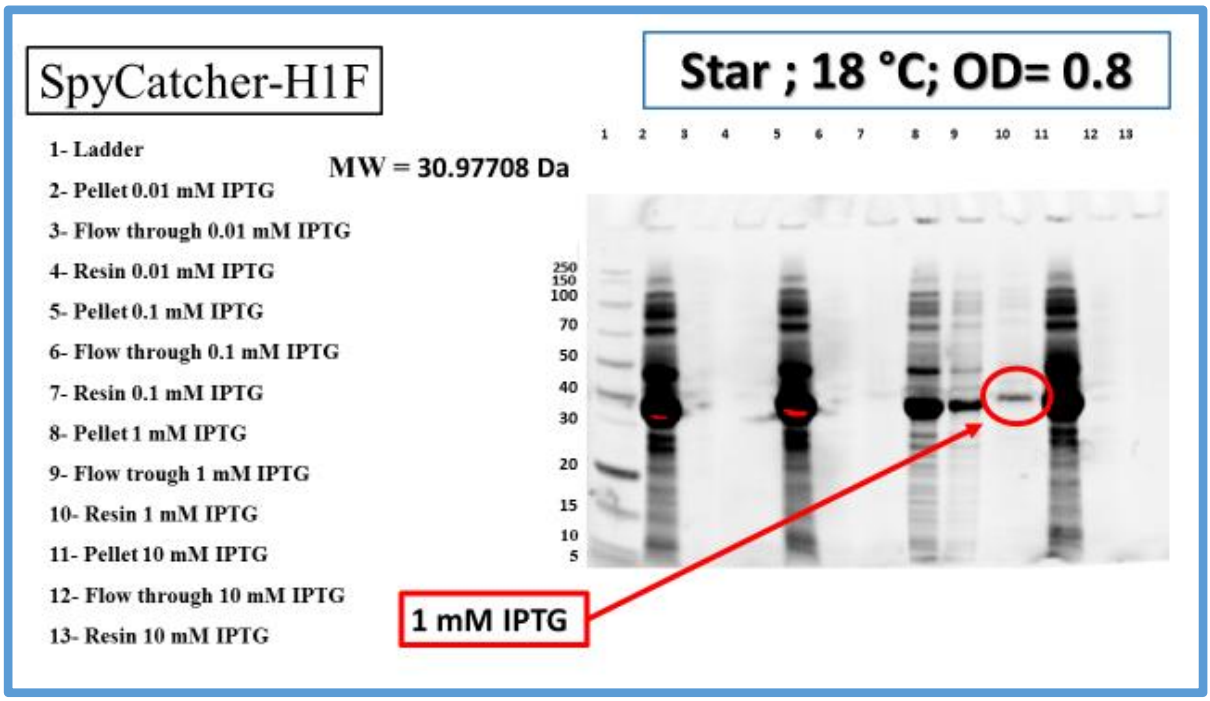

Figure 16. Results of IPTG concentration optimization of the antigen SpyCatcher-H1F. *In this figure we can see a SDS-PAGE for Star at 18 ºC and an optical density of 0.8 with the lodder and the pellet, flow through and resin samples at four diferent concentrations of the inductor IPTG: 0.01 mM, 0.1 mM, 1 mM, and 10 mM . Best condition is indicated by red color.*

As we can see, the clearer and darker band was in the resin with 1 Mm of IPTG, so we chose this parameter for the next step of the purification of SpyCatcher-H1F.

5.4 IMAC purification

For SpyCatcher-M2e we obtained the results shown in the **Figure 17**.

Now, we were sure that we had a lot of protein in the tubes B1, B2 and B3, so we mixed them in one tube and took a measure of the absorbance with a spectrometer. In addition, we also did the same with the tubes B7, B8, B9 and B10 because despite we shouldn't have had a lot of protein there, in the gel we saw there is something, so we have to check if it is important.

We used NanoDrop to measure the absorbance at 280 nm of our samples. The absorbance at 280 nanometers of the tubes B1-B3 was 2.35 and the same absorbance with the tubes B7-B10 was 0.345.

Now, with the extinction coefficients (calculated using http://web.expasy.org/protparam/ and the amino acids sequence) we calculated the estimated concentration using c=A/(ε *b).

Extinction coefficient (ε) is 20,065 M^{-1} cm^{-1}, at 280 nm measured in water.

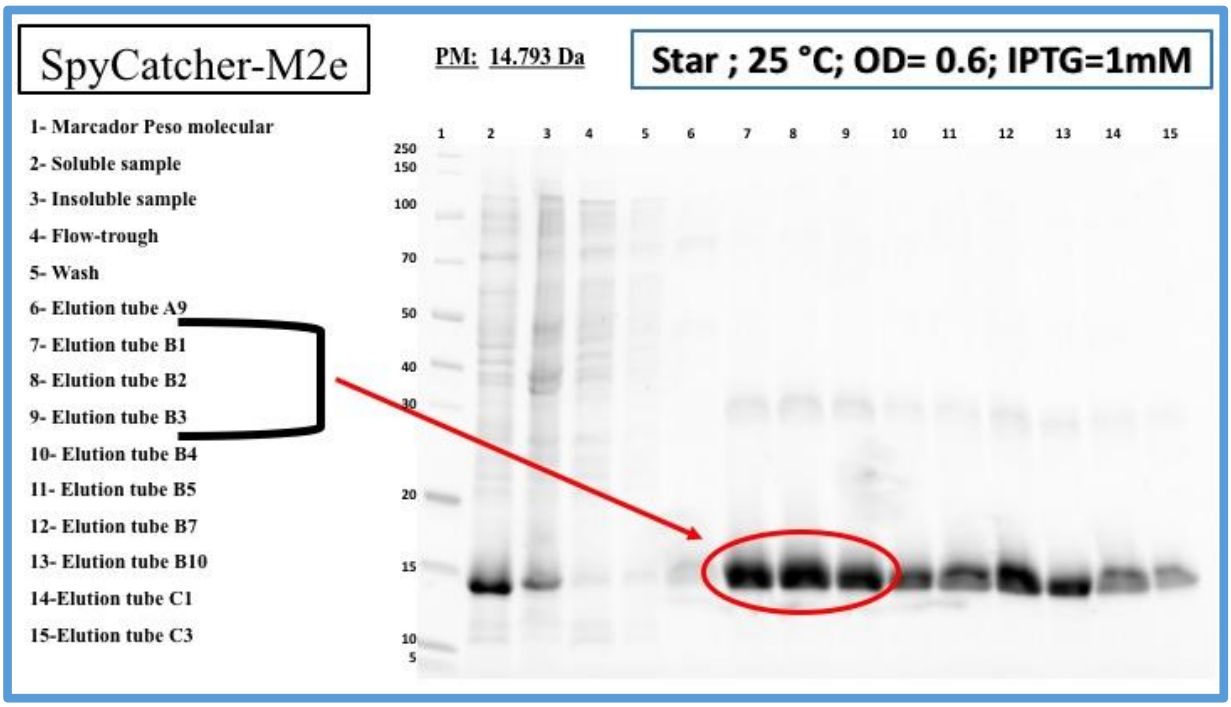

Figure 17. A SDS page with the results of the AKTA protein purification system for the antigen SpyCatcher-M2e. *In this gel it was used the soluble sample, insoluble samble, flow-trough, wash and the different elutions of the antigen SpyCatcher-M2e obtained in the purification. Elution tubes with higher amount of protein are indicated by red color.*

It was assumed that all Cys residues are reduced. `b´ is the length of the pathway light travels in the UV-spectrophotometer in centimeters. The standard value of 1 cm is used here (because NanoDrop automatically gives the absorbance readings according to that).

Table 1. Absorbance and concentration of the NanoDrop pooled fractions B1-B3 and B7-B10.

NanoDrop pooled fractions	A_{280}	A_{260}/A_{280}	Concentration (µmol/ml)
B1-B3	2.35	0.57	0.118
B7-B10	0.345	0.72	0.0286

*An A260/280 reading of 0.57 or 0.72 means that the sample is 100 % protein.

Concentration (µg/mL):
B1-B3: 1,745 µg/mL
B7-B10: 423 µg/mL

We chose the B1-B3 samples. As we had 10 ml, we have 17 mg. We produced this using 500 mL of the protein production culture, so in 1 L we can produce 34 mg.

Now that we knew how much protein we were able to produce, we can start optimizing other antigens. This data is important to compare all the antigens before we start producing the vaccine.

Also, with other graph given by UNICORN, we saw that our eluted SpyCatcher-H1F had a peak in the tube C3. We did another SDS-PAGE electrophoresis with the tubes A9-C9 to see how much protein we had there.

For SpyCatcher-H1F we obtained the results shown in the **Figure 18.**

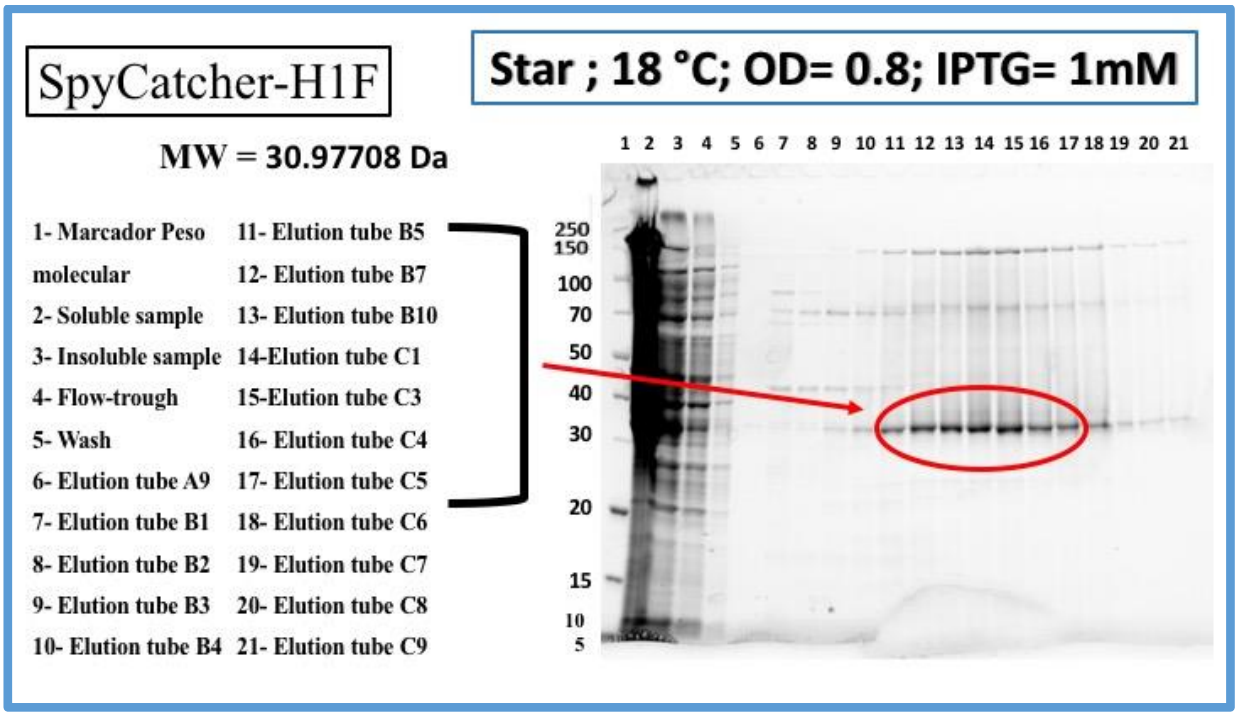

Figure 18. A SDS page with the results of the AKTA protein purification system for the antigen SpyCatcher-H1F. *In this gel it was used the soluble sample, insoluble samble, flow-trough, wash and the different elutions of the antigen SpyCatcher-H1F obtained in the purification. Elution tubes with higher amount of protein are indicated by red color.*

Now that we knew we had a considerably amount of protein between the elution tubes B5- C5, we did the same thing as with the SpyCatcher-M2e.

We mixed those tubes and we used NanoDrop again to measure the absorbance at 280 nm of our samples. The absorbance at 280 nanometers was 0.398.

Now, using c=A/(ε *b), we can estimate at 40 mL of 0.35 mg/mL 90% pure protein.

The extinction coefficients (calculated using http://web.expasy.org/protparam/ and the amino acids sequence) is 32.890 at 280 nm measured in water.

Table 2. Absorbance and concentration of the NanoDrop pooled fractions B5-C5.

NanoDrop pooled fractions	A_{280}	A_{260}/A_{280}	Concentration (mol/l)
B5-C5	0.398	0.66	$1.21 * 10^{-5}$

Concentration (µg/mL):

B5-C5: 350 µg/mL

As we had 10 ml, we have 3.5 mg/mL. We produced this using 500 mL of the protein production culture, so in 1 L we can produce 7 mg/mL.

The results obtained during the protein production and purification can be sum up in the next two tables:

1) The optimal conditions to produce the antigen SpyCatcher-M2e in bacteria are:

Table 3. Optimal conditions to produce the antigen SpyCatcher-M2e in bacteria.

Strain	Temperature	OD	IPTG	Yield
Star	**25 ˚C**	**0.6**	**1 Mm**	**34 mg/L**

2) The optimal conditions to produce the antigen SpyCatcher-H1F in bacteria are:

Table 4. Optimal conditions to produce the antigen SpyCatcher-H1F in bacteria.

Strain	Temperature	OD	IPTG	Yield
Star	**18 ˚C**	**0.8**	**1 Mm**	**7 mg/L**

After the results, we can conclude that the protocol used in the protein production fits our two antigens.

However, some details can be improved for the following procedures:

- For the production culture, we had used a 50 ml Nunc tube. It was good, but it would had been better to use a bigger tube, or a membrane rather than the cap. That way, the bacteria could had grown better, as they had more oxygen. However, as we started the first two experiments using the cap, we continued using it because if we change the parameters and we give more oxygen to the bacteria on the next experiments, we could not compare them with the previous ones.

- Use of imidazole. All the proteins have histidine, so they can bind a little to the his tag resin despite they don't have the his-tag. For that reason, we can use Imidazol. In the binding buffer, the imidazole will bind the resin strongly, and only will be replaced for something that binds the resin with a stronger bind, so our protein with the his-tag will bind but the other proteins with some histidine will not.

- When we made the testing conditions for the different temperatures, we put the samples at 18 °C, 25 °C and 37 °C. However, the tubes came from an incubator at 37 °C, so despite we put

them in incubators at 18 °C and 25 °C, maybe it took them like an hour to achieve that temperature. This could be a problem because the protein production can be finished in around 3 or 4 hours, so the first hour of the production was at 37 °C. Next time, to avoid this, after adding IPTG and before we take the tubes to the respective incubators, we should put them in cold water just for a few minutes, so the temperature would decrease and it would be nearer to 18 °C and 25 °C. At the contrary, the tubes at 37 °C do not have these problems.

6. Conclusions

1) The first conclusion is that both antigens SpyCatcher with M2e and with H1F can be produced in bacteria, obtaining satisfactory amount of proteins at the end of the process.
2) There are a lot of conditions we need to optimize to get the best amount of produced protein. It requires a lot of time of work, but once to have optimized it, this new conditions will save you even more time as you produce a higher amount of protein every time you do it.
3) The protocol used to produce and purify the protein works and should be used in future production, as we can see a higher levels of protein and mostly our target protein, as we have been able to remove others proteins.
4) Comparing SpyCatcher-M2e and SpyCatcher-H1F, we produced much more amount of SpyCatcher-M2e. We could think that antigen is the best one. However, despite we produce more of that, maybe SpyCatcher-H1F produce a better response in the clinic trials in order to produce the vaccine, so for now, we can not assure which one is better.
5) The whole optimization procedure could be streamlined. For instance, more parameters could be tested at once and bacteria could be grown in smaller volumes, e.g. on well plates.

In conclusion, now we have two antigens that could work to produce the VLP vaccine, but there is a long way until there. In 2018, the first vaccine candidates will be formulated and the conjugation reactions will be evaluated. Also, it will take place the first pre-clinical test in mice. In 2019, the best performing vaccine candidates will be further enhanced and there will be more animal trials.
Finally, between 2019 and 2021, clinical trials will be started and the suitability of the vaccine platform will be evaluated in other applications (another infectious disease, immunomodulation).

1) La primera conclusión es que tanto el antígeno SpyCatcher (M2e y H1F) pueden ser producidos en bacterias, obteniendo una cantidad de proteína satisfactoria al final del proceso.
2) Hay muchas condiciones que necesitamos optimizar para conseguir la mejor cantidad de proteína producida. Esto requiere mucho de tiempo de trabajo, pero una vez que se han

optimizado, estas nuevas condiciones ahorrarán incluso más tiempo al producir una mayor cantidad de proteína cada vez que se lleva a cabo la producción.

3) El protocolo usado para producir y purificar la proteína funciona y debería ser usado en futuras producciones ya que podemos ver altos niveles de proteína y mayormente nuestra proteína objetivo, ya que hemos conseguido eliminar las demás proteínas.
4) Comparando SpyCaycher-M2e y SpyCatcher-H1F, produjimos mucha más cantidad con SpyCatcher-M2e. Podríamos pensar que el antígeno es el mejor. Sin embargo, a pesar de que hemos producido más, SpyCatcher podría producir una mejor respuesta en los ensayos clínicos al producir la vacuna. Por lo tanto, por ahora no podemos asegurar cuál de los dos es mejor.
5) Todo el proceso de optimización podría ser optimizado. Por ejemplo se podrían probar más parámetros a la vez y se podrían usar volúmenes más pequeños para que crezcan las bacterias.

En conclusión, ahora tenemos dos antígenos que podrían funcionar al producir la vacuna VLP, pero aun queda un largo camino hasta ahí. En 2018, se formularán los primeros candidatos a vacunas y las reacciones de conjugación se evaluarán. Además, también se llevarán a cabo las pruebas pre-clínicas en ratón.

En 2019, las vacunas que mejor hayan funcionado seguirán adelante y habrá más ensayos en animales.

Al final, entre 2019 y 2021, comenzarán los ensayos clínicos y la plataforma de la vacuna será evaluada en otras aplicaciones (como otra enfermedad infecciosa, inmunomodulación).

7- References

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8- ANNEX

The plasmids used to produce the Spycatcher-M2e and Spycatcher-H1f are:

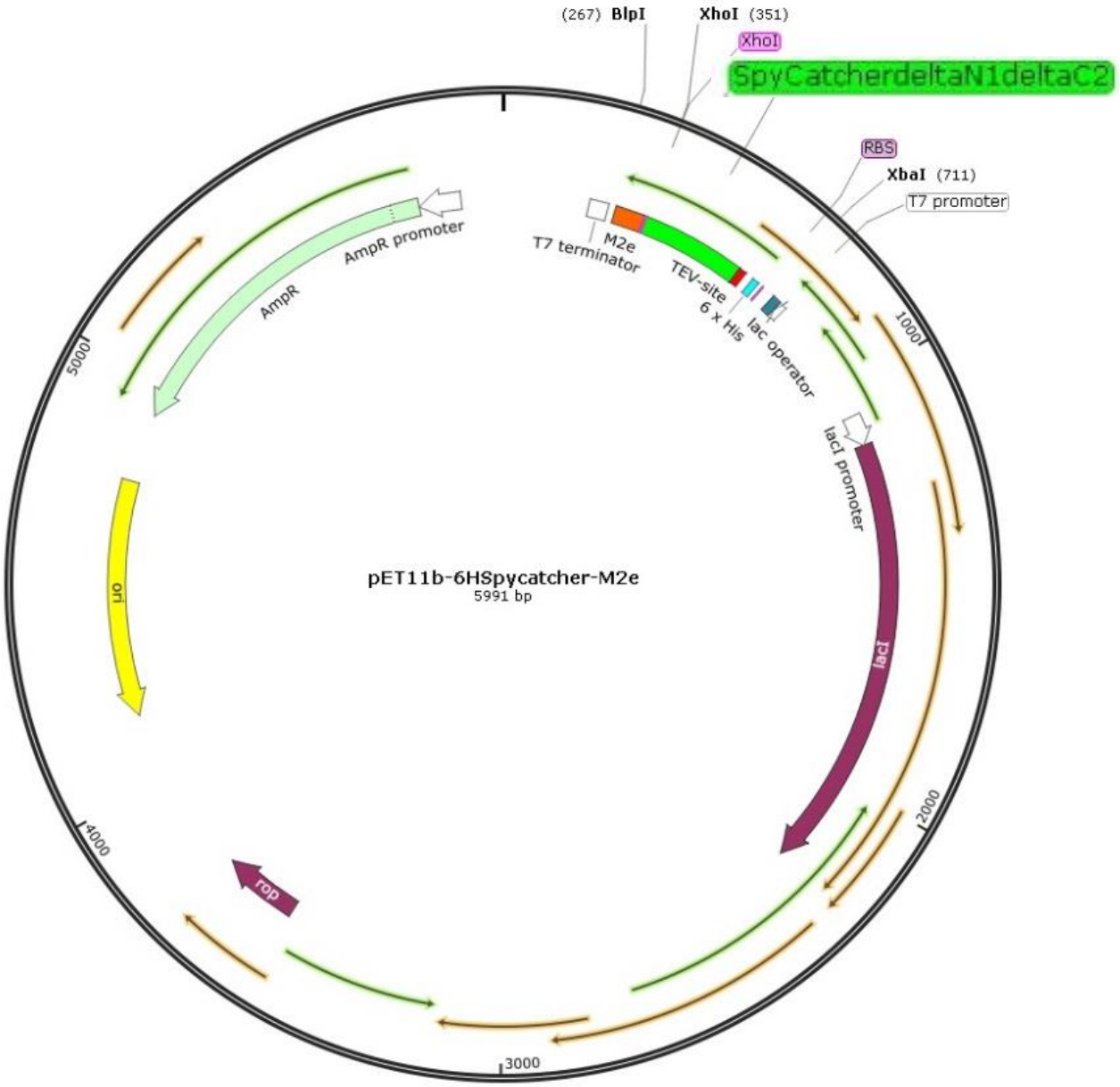

Figure 18. Plasmid pET11b-6HSpycatcher-M2e used to produce SpyCatcher-M2e. *It contains the ampicilin resistence gene, lac operon, 6x His, Ori and Rop.*

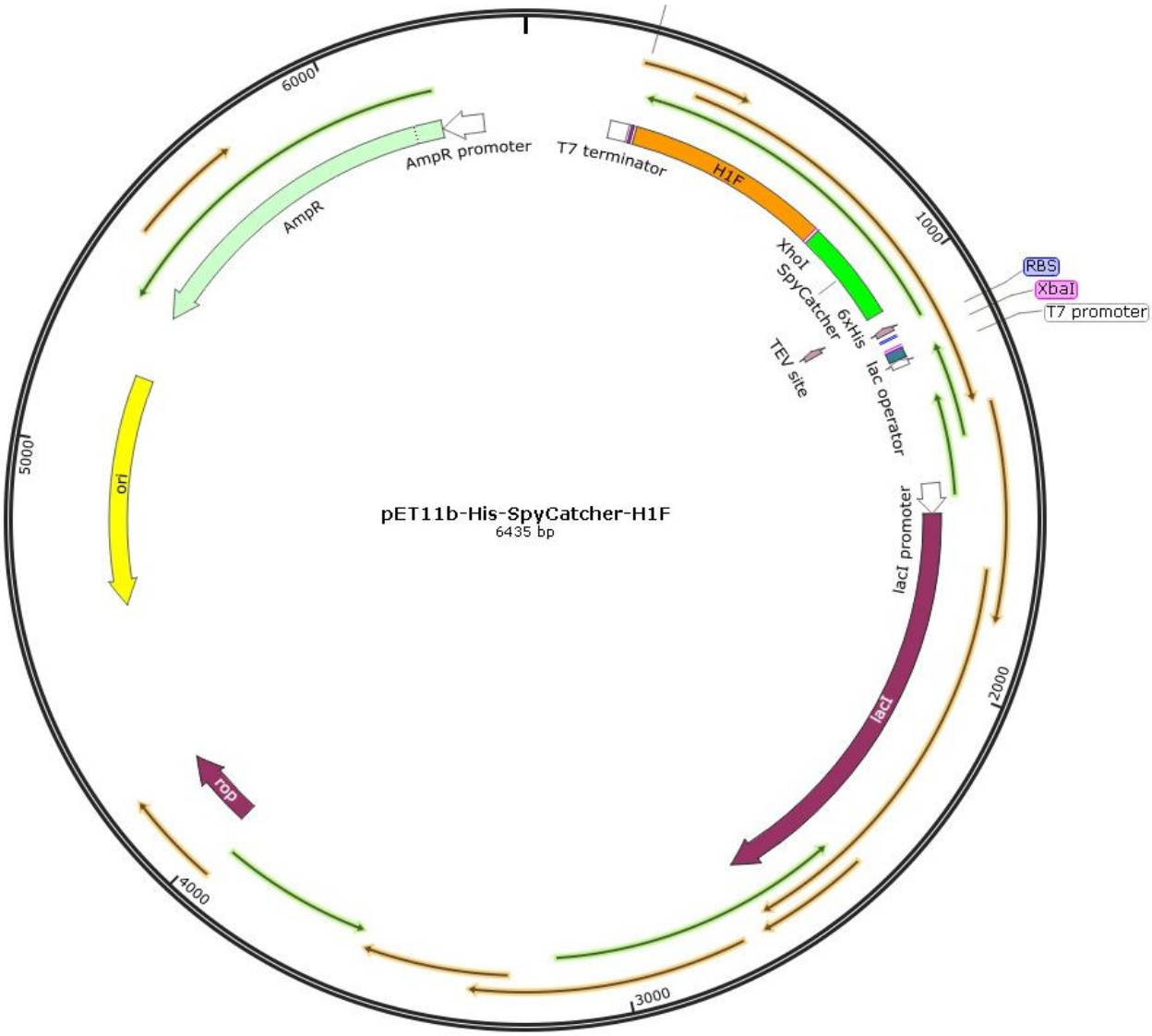

Figure 29. Plasmid pET11b-His-Spycatcher-H1F used to produce SpyCatcher-H1F. *It contains the ampicilin resistence gene, lac operon, 6x His, Ori and Rop.*