



Universidade do Minho

Escola de Engenharia

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**Development of molecular and imaging
tools to study SUMO conjugation in plants**

Dissertação de Mestrado

Mestrado Integrado em Engenharia Biológica

Ramo de Tecnologia Química e Alimentar

Trabalho efetuado sobre orientação da

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Outubro 2024

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ACKNOWLEDGMENTS

Firstly, I would like to thank my parents and my sister for their support during the five years of my degree. I also want to show my gratitude to Professor Alcina Pereira, Dr. Maria Lois and Dr. Anna Solé that guided me on the last step of my academic journey. Then, my special thanks to University of Minho and the Centre of Research in Agricultural Genomics for welcoming with open arms and for making feel always at home. Lastly, my appreciation to the project Erasmus + for funding my internship.

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Desenvolvimento de ferramentas moleculares e de imagem para estudo da conjugação de SUMO em plantas

Sumário

A conjugação do Pequeno Modificador Semelhante à Ubiquitina (SUMO) é um mecanismo molecular presente em todos os eucariotas e fornece um nível extra de controlo dos processos celulares na forma de modificação pós-translacional de proteínas. Esta modificação modula a função das proteínas-alvo às quais o SUMO se liga (também conhecido como SUMOlação), resultando na regulação de múltiplos processos fisiológicos, como as respostas ao stress das plantas. O papel crítico da SUMOlação vegetal é ilustrado pelo seu papel essencial durante as fases iniciais do desenvolvimento das sementes.

O principal objetivo deste projeto é descobrir a regulação de uma enzima importante da maquinaria SUMOlação, a grande subunidade da enzima ativadora SUMO E1 SAE2. Resultados anteriores identificaram que o domínio C-terminal de SAE2 é processado, embora o mecanismo que intervém neste processamento seja desconhecido. Para atingir este objetivo, explorou-se uma abordagem experimental baseada na técnica de Transferência de Energia por Ressonância de Förster (FRET), que permite a realização de estudos de alto rendimento. FRET envolve a transferência de energia entre uma molécula fluorescente dadora que é excitada e emite a energia que irá excitar uma segunda molécula, recetora, que emite a energia que pode ser lida e medida. O delineamento experimental utilizou uma proteína recombinante constituída por um doador e um recetor de proteínas fluorescentes separadas pelo domínio SAE2 que é alvo de uma atividade proteolítica (dador-SAE2-recetor). Consequentemente, se uma protéase clivar esse domínio SAE2, dador e recetor será dividido, e FRET não ocorrerá.

Após vários ensaios, a configuração do ensaio FRET não foi possível, e uma nova estratégia foi desenvolvida baseada em eletroforese em gel não desnaturante. Utilizando este método, foi possível observar a presença de atividade de processamento de SAE2 em extratos proteicos de siliques. Apesar deste resultado positivo observado no gel e do seu potencial, esta técnica deve ser melhorada.

Palavras-chave: SUMO, SUMOlação, FRET, fluorescência.

Development of molecular and imaging tools to study SUMO conjugation in plants

Abstract

The conjugation of Small Ubiquitin-like Modifier (SUMO) is a molecular mechanism present in all eukaryotes and provides an extra level of control of cellular processes in the form of post-translational modification of proteins. This modification modulates the function of the target proteins to which SUMO binds (also known as SUMOylation), resulting in the regulation of multiple physiological processes, such as plant stress responses. The critical role of plant SUMOylation is illustrated by its essential role during the early stages of seed development.

The main goal of this project is to uncover the regulation of a critical enzyme of the SUMOylation machinery, the large subunit of the SUMO E1 activating enzyme SAE2. Previous results identified that SAE2 C-terminal domain is processed, although the mechanism that mediates this processing is unknown. To address this objective, we have explored an experimental approach based in the Förster Resonance Energy Transfer (FRET) technique, which allows performing high-throughput studies. FRET involves the transfer of energy between a donor fluorescent molecule that is excited and emits the energy that will excite a second molecule, acceptor, that emits the energy that can be read and measured. The experimental design used a recombinant protein consisting of a donor and an acceptor fluorescent proteins separated by the SAE2 domain that is targeted by a proteolytic activity (donor-SAE2-acceptor). Consequently, if a protease cleaves that SAE2 domain, donor and acceptor will be split, and FRET will not take place.

After several trials, the set-up of the FRET assay was not possible, and a new strategy was developed based in non-denaturing gel electrophoresis. Using this method, it could be observed the presence of SAE2 processing activity in protein extracts from siliques. Despite this positive result observed in the gel and its potential, this technique requires further improvement.

Key words: SUMO, SUMOylation, FRET, fluorescence.

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Abbreviation list

Recombinant proteins	
C	Clover
R	mRuby2
C:R or C-R	Clover:mRuby2
C+R	Clover and mRuby2 with same molarity
L1	Clover:UFDct:Linker1:mRuby2
L2	Clover:UFDct:Linker1:Linker2:mRuby2
Units	
MW	Molecular weight
C	Concentration

1. State of the art

1.1. Protein post-translational modification

The human genome contains between 20.000 and 25.000 genes; however, it is estimated that the human proteome has over one million proteins. This suggests that genes can encode several proteins using mechanisms that generate different mRNA transcripts from one gene (differential transcription termination, and alternative splicing) and by protein post-translational modifications (PTMs) (Ramazi & Zahiri, 2021) (Figure 1).

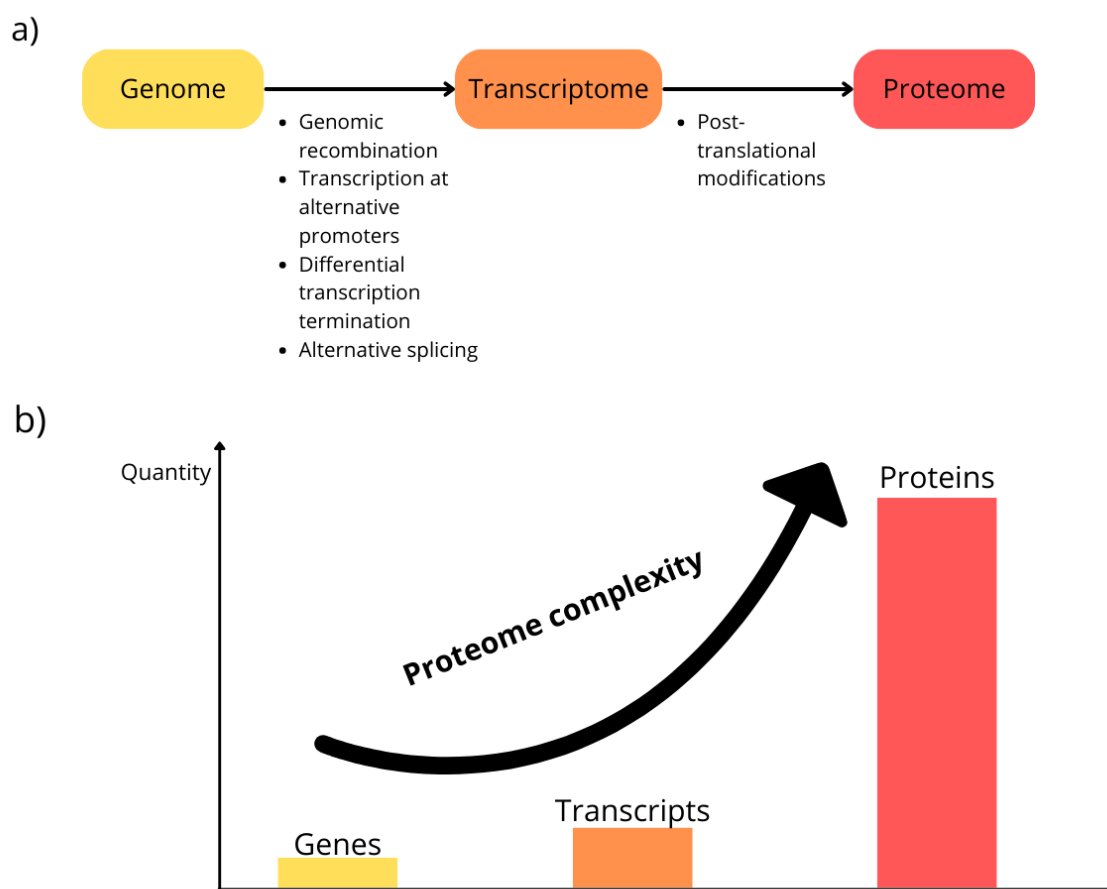


Figure 1. Schematic representations of a) explaining how proteome complexity is originated and b) graph with the relative quantity of genes, transcripts and proteins (Adapted from *(ThermoFisher Scientific, s.d.)*).

PTMs introduce changes in the existing protein structure like breaking or generating covalent bonds on the backbones or amino acid side chains of proteins. These changes allow the modified molecules to take part in multiple biological processes (Zhong, *et al.*, 2020), (Li, *et al.*, 2021) by

regulating their activity, localization, and interaction with other molecules. There are several types of PTMs, as presented in Table 1.

Table 1. Some types of PTMs (Zhong, *et al.*, 2020)

Types of PTMs	Target molecule group
Phosphorylation	Small molecules
Carbamylation	
Methylation	
Short-chain fatty acid-modification	
Ubiquitination	Polypeptides
SUMOylation	
Long-chain fatty acid modification	Lipids
Glycosylation	Carbohydrates
Citrullination	Others
Redox modifications	

This project focus on the study of the regulation of plant SUMOylation through an unknown mechanism consisting of the processing of the large subunit of the SUMO E1 activating enzyme SAE2.

1.2. SUMO conjugation

The small ubiquitin-like modifier, also known as SUMO, is present in all eukaryotes, and, consequently, in plants (Park, *et al.*, 2011). SUMO is a member of the superfamily of ubiquitin-like polypeptides that covalently bind to various target proteins, which promotes diverse changes such as molecular function, cellular localisation or half-life of such proteins (Kurepa, *et al.*, 2003). This process is named SUMOylation.

The SUMO machinery includes several proteins that are involved in SUMOylation and deSUMOylation reactions (Figure 2). This process consists of the formation of a covalent bond between the C-terminal glycine carboxyl group of SUMO and the ϵ -amino group of a specific lysine residue in a target protein (Park, *et al.*, 2011).

SUMOylation occurs by the sequential action of three enzymatic activities: the E1-activating, the E2-conjugating, and the E3-ligase. SUMO proteins are synthesised as immature precursors and processed by a SUMO-specific isopeptidase to expose the di-glycine motif at the C-terminus required to enter the conjugation pathway. The mature SUMO is activated heterodimeric (SAE2/SAE1) E1-activating enzyme, in an ATP-dependent reaction, and then transferred to the catalytic cysteine of the SUMO E2-conjugating enzyme. The E2 is competent to transfer SUMO the target lysine residue of the substrates, although this reaction is accelerated by E3 ligases and, *in vivo*, are often required for efficient SUMOylation (Johnson, 2004).

As for deSUMOylation, this is a process in which specific proteases remove SUMO from its target, breaking the bond between the SUMO-conjugated terminal glycine and the substrate. The SUMO is then released from the target protein and can enter new conjugation cycles. Therefore, proteases are essential for maintaining the balance of SUMO conjugation and deconjugation *in vivo* (Müller, *et al.* 2001 cit in Yates, *et al.* 2016). (Müller, *et al.*, 2001)

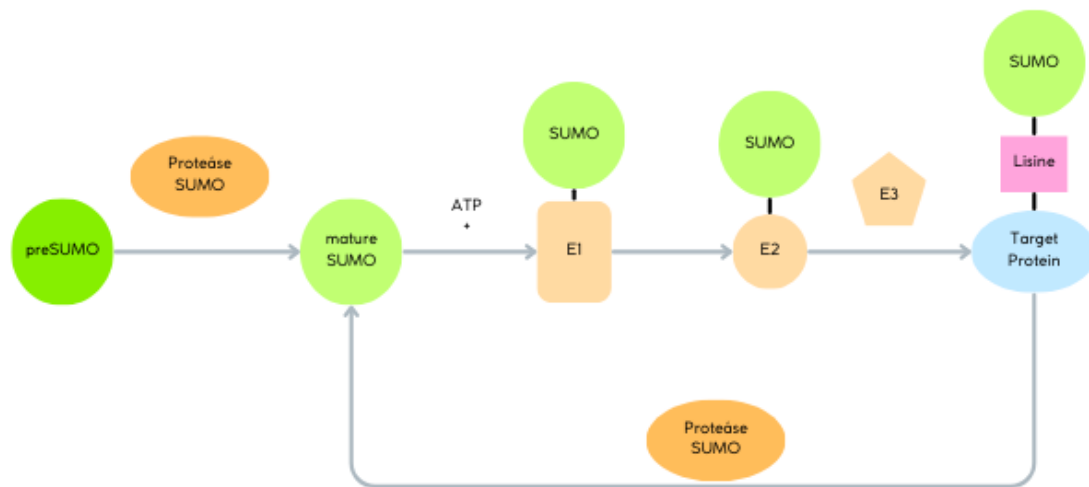


Figure 2. SUMO de- and conjugation process (Adapted from (Yates, *et al.*, 2016)).).

Due to its role in the plant cells and tissues, the SUMO pathway is considered to have an important contribution to defence mechanisms (Chimire, *et al.*, 2021), even though most SUMO studies in plants are based only on *Arabidopsis* as a model. SUMOylation affects several important processes in plants, such as the response to environmental stresses, which can be abiotic (drought, temperature, phosphate deficiency, among others) or biotic (for example, defence against infection by pathogens), regulation of the flowering period, cell growth and development and nitrogen assimilation (Park, *et al.*, 2011). This is due the fact that SUMO targets many different proteins, and, therefore,

SUMOylation and deSUMOylation can affect a large number of processes in plants, such as those shown in Table 2.

Table 2. Examples of processes regulated by deSUMOlation

Function	Process	Reference
Molecular	Post-replicative DNA repair.	(Hoege, <i>et al.</i> , 2002)
	Activation and repression of transcription.	(Ross, <i>et al.</i> , 2002)
Physiological	Regulation of flowering.	(Murtas, <i>et al.</i> , 2003)
	Induction of plant defence responses	(Hanania, <i>et al.</i> , 2002)
	Control mechanism (positive and negative) in different responses to phosphate deficiency.	(Miura, <i>et al.</i> , 2005)
	Regulation of innate immunity.	(Lee, <i>et al.</i> , 2006)
	Salt tolerance.	(Conti, <i>et al.</i> , 2008)
	Stress response and stress adaptation.	(Park, <i>et al.</i> , 2010)

The activating SUMO enzyme (E1) is heterodimeric and has two subunits: SAE1 and SAE2 (Lima & Lois, 2005). The SAE1 is the smaller subunit and has two isoforms, SAE1a and SAE1b, while SAE2 is larger and has four functional domains (adenylation, catalytic cysteine, ubiquitin-fold and C-terminal). Isoforms are proteins that even though they originate from gene duplication events and, although they retain the same molecular function, they differ in their amino acid sequence (Torrens-Fontanals, *et al.*, 2021) and they display functional and/or structural alterations. The heterodimeric E1 is considered to be exclusively in the cell nucleus. This nuclear localization is determined by

molecular determinants present in the C-terminal tail of the SAE2 subunit (Castaño-Miquel, et al., 2013), as shown by the exclusive nuclear localization of the native E1 versus the cytosolic localization of the E1 composed of a SAE2 variant with deletion of the C-terminal domain (SAE2 Δ Ct). The cellular localization of the subunits SAE1 was exclusively dependent on the SAE2 for the subunit SAE1b, while SAE1a seemed to have a wider distribution, suggesting it could exist as monomer. The same analysis was done only with the ubiquitin-fold domain (UFD): when the UFD was fused with the C-terminal (UFD Δ Ct) it was exclusively found in the nucleus. However, in the absence of the C-terminal, the UFD was also present in the cytoplasm. This suggests that the C-terminal integrity is responsible for its localization in the nucleus (Castaño-Miquel, *et al.*, 2013).

Genetic analyses concluded that SAE1a has a crucial role in SUMOylation under stress (heat and drought), and important to maintain SUMOylation homeostasis *in vivo* (Castaño-Miquel, *et al.*, 2013). However, the two isoforms are not fully redundant, contrary to previous research from Catala *et al.*, (2007). Finally, it was speculated that the interaction between SAE1a and SAE2 and between SAE1b and SAE2 confer different stability properties to E1 which can influence the SUMO conjugation (Castaño-Miquel, *et al.*, 2013).

More recently it was demonstrated that SAE2 undergoes processing of the C-terminal in a tissue specific manner, *i.e.*, it is more predominant in seeds (Más, *et al.*, 2020). In this article, the SAE2 processing was assayed in leaf (from rosette) and silique (which contains mostly seeds) protein extracts, and the processing activity was only detected in the presence of siliques extracts both in *Arabidopsis thaliana* Col-0, corroborating initial observation in planta.

1.3. Molecular and imaging tools

In order to study SUMO conjugation in plants we decided to use the FRET technique. The FRET (Förster Resonance Energy Transfer) is a mechanism in which a donor fluorescent molecule is excited and emits a signal. That signal will excite a second molecule (the acceptor) which will emit a signal that can be read and measured. This energy transference is only possible if both the donor and the acceptor are in close contact, and the range of wavelength of excitation and emission of the two molecules overlap. In Figure 3 is presented an example of the application of a FRET-based assay. The protein A is fused to a green fluorescent protein (Clover) and a red fluorescent protein (mRuby2) is fused to a protein B. If the protein A and B interact, Clover and mRuby2 are very close to each other and, after the excitation of Clover, this will emit and excite mRuby2, production a FRET signal.

However, if the interaction does not happen, Clover and mRuby2 are separate, and no FRET signal emitted (Stankovic-Valentin, *et al.*, 2009).

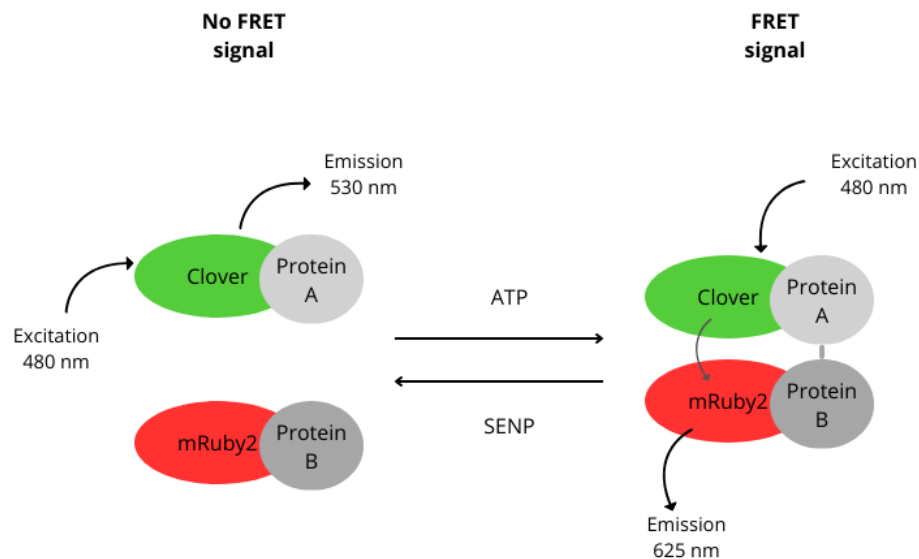


Figure 3. FRET-based assay: des and SUMOylation (Adapted from (Stankovic-Valentin, *et al.*, 2009)).

Therefore, the FRET technique is usually based on the interaction between two proteins, but in this thesis the FRET was used with a different application. In this case, the two fluorophores were fused with a linker that contains the sequence where the protease will cut. According to the experimental design, if the protease cuts, there is no FRET signal, and if it does not cut, the FRET signal is maintained.

Another explored strategy was the nondenaturing polyacrylamide gel electrophoresis of proteins. This method separates and preserves the native structure of proteins, and it is useful to detect the fluorescence of the fluorophores (Walker, 2002). Therefore, it allows to study interactions between proteins while still detecting fluorescence (Safer, 1994). According to Dong, J. *et al.* (2019) it is possible to run a native gel SDS in the reagents and preserve the nondenatured fluorescent protein. Based on this, it was decided to try this different approach and confirm whether the fluorescent protein variants used in this assay were compatible with SDS. In the article the protein used was GFP but in this project it was tested with Clover and mRuby2. (Dong, *et al.*, 2019)

2. Motivation

As mentioned before, in SUMOylation there are three enzymes with important roles: activating (E1), conjugation (E2), and ligation (E3). The E1 has two subunits, SAE1 and SAE2, the latter containing a C-terminal tail. It was believed that E1 to be present solely in the cell's nucleus, however some studies showed that it can also be found in the cytoplasm. Cataño-Miguel, *et al.* (2013), using fluorescent imaging, discovered that full length SAE2 was only detected in the nucleus but if the C-terminal was removed, SAE2 appeared in both the nucleus and cytoplasm. This suggests that the plant can modulate SUMO conjugation by modifying E1 subcellular localization through the processing of SAE2 C-terminal. Despite its importance, the way that this mechanism works is still unknown.

3. Objectives

This dissertation is part of a broader project that aims to uncover the SAE2 C-terminal domain processing enzyme, using FRET, a high through-put assay using *Arabidopsis thaliana* as a model plant organism. In this thesis, we will perform the initial steps to develop this high throughput assay under our experimental conditions, laying the foundation for future research. Our objectives are:

- Setting-up the FRET conditions (this includes producing the purified proteins from plasmids previously produced in the laboratory, quality control of the purified proteins by SDS-PAGE and establishing the optimal protein concentrations for the FRET assay).
- Performing an initial screening of C-terminal domain cleavage by using several protein extracts from different parts of *Arabidopsis* (rosette leaves and siliques).
- Developing an alternative methodology to identify the processed SAE2 treated with the different extracts from *Arabidopsis* tissues.

4. Methodology

All the information of buffers, reagents and medias used in the different experiments are presented in the Appendix A.

4.1. Protein material

The proteins that were used were Clover:mRuby2 (59,45 kDa), Clover (33,34 kDa), mRuby2 (31,76 kDa), L1 (81,13 kDa), L2 (81,74 kDa) and UFDcT (25,62 kDa) and their plasmids constructs are, respectively pET28a::Clover:mRuby2, pET28a::Clover, pET28a::mRuby2, pET28a::Clover-UFDcT(L1)-mRuby2, pET28a::Clover-UFDcT(L2)-mRuby2 and pET-28b(+) rc-AtUBLcT (Appendix B). L1 and L2 sequences have the region of the SAE2 C-terminal (UFDcT) ligated to Clover and the only difference between them is that the first has a linker of 18 base pairs and the second has two linkers of 18 base pairs between the UFDcT region and mRuby2. The processing of the C-terminal domain is probably occurring at a linker sequence right before the C-terminal of SAE2, which contains a protease targeted domain for cutting. Therefore, the rationale for the experiments was to isolate this sequence to see if the protease can cleave this site.

The Clover:mRuby2 does not have the UFDcT region, Clover and mRuby2 are fused together. Finally, all the plasmids have the resistance gene to kanamycin, 35s promoter, His tag and T7-tag.

4.2. Heat shock transformation of *Escherichia coli* cells

The BL21 (DE3) strain was used in this experiment. Around 100 ng of plasmid was added to 50 µL of bacteria on ice and then, incubated 30 min on ice. The heat shock was at 42 °C for 1 min and 15 sec. In a flow hood, 950 µL of LB were added and it incubated for 1 h at 37 °C while shaking. Finally, 100 µL of the samples was plated into a LB agar plates with kanamycin (25 µg/mL) and the plates incubated overnight at 37 °C.

4.3. Induction of protein expression and batch purification (small scale)

The experiment was done in three days: the first day was the bacteria growth, second was the induction and, finally, the third with the lysis and batch purification.

The pre-inoculation was done in 3 mL of 2×YT media with chloramphenicol for 6 h shaking at 37 °C. For that, a colony of BL21 (DE3) carrying the desired plasmid was isolated and used to initiate the bacterial culture (check item 4.2.). In alternative, a glycerol stock of the *E. coli* with the plasmid

can be used. After 6 hours, the inoculation was prepared in 60 mL (2×YT media with chloramphenicol and kanamycin) with the pre-culture diluted 1:50 and incubated overnight shaking at 37 °C.

Then, the final inoculation of 500 mL (2×YT media without antibiotics) was prepared from the overnight culture (1:50), as well as a glycerol stock that was labelled and stored at – 80 °C for future use. The culture was left at 37 °C shaking until it reached an OD (600 nm) between 0,6 and 0,8. Once the desired OD was reached, 1 mL of the sample was saved (T_0) and started the induction by adding IPTG to a final concentration of 0,5 mM and incubated shaking at 30 °C for 4 to 5 h. The OD was measured again, 0,5 mL of the culture was saved (T_1) and the culture was centrifuged for 15 min at 6000×g. The supernatant was discarded, and the pellet was stored at -80 °C until the next step.

For the lysis, the pellet was dissolved in lysis buffer (1:20) and the equipment used was the French press. Before the lysis, 1 mL of the sample was saved. After this step, the sample was centrifuged at 12000 rpm at 4 °C for 1 h. The supernatant was filtered (first 0,45 µm filter and then 0,2 µm filter) and kept on ice.

For the purification, the purification column was washed with water, added 3 mL of IMAC Sepharose beads GE ©. The beads were washed with water, activated with 0,2 M NiCl_2 , washed again with water, washed with equilibrium buffer and, finally, the sample was added. The sample was collected (flow-through) and the column was washed again with equilibrium buffer and the sample collected (wash). The elution buffer was added and collect 3 to 5 elutions of 1 mL (E3-5). The different elutions were quantified by Bradford (check item 4.6.) in order to choose the ones with more protein concentration. After this, the chosen aliquots were pooled together to desalt with PD10 columns (Cytiva ©), filtered with a 0,2 µm filter, and aliquoted and measured by Bradford again and then, frozen in liquid nitrogen and stored at -80 °C.

Finally, the samples saved during this protocol, were analysed in an SDS-PAGE gel (check item 4.7.). The T_0 and T_1 were centrifuged at max speed for 5 min, the supernatant was discarded, and the pellet was resuspended in loading buffer. The lysis sample was centrifuged at max speed for 5 min, the pellet was separated from the supernatant and loading buffer was added in both. For the flow-through and wash sample, the loading buffer was added. Then, the normal procedure of an SDS-PAGE gel electrophoresis was followed.

4.4.FRET assay

Firstly, 500 nM stocks of the proteins of interest were prepared: Clover:mRuby2, Clover, mRuby2, L1, L2 and a mixture of equal parts Clover and mRuby2. Then, 1:2 serial dilutions (250, 125, 63, 31, 16, 8 and 4 nM) were prepared and each dilution of each protein was loaded in a 96 black bottom well plate. The negative control is the mixture of Clover and mRuby2, the positive is Clover:mRuby2 and the blank the buffer used for the protein storage.

After assembling the plate, it was read in a spectrophotometer (SpectraMax® M5 Microplate Reader from Molecular Devices LLC) with an excitation of 480 nm and an emission of 530 nm.

4.5.FPLC fractionation

The equipment used was a FPLC system ÄKTA purifier, GE Healthcare © equipped with S-75 preparative gel filtration column (HiLoad 26/60 Superdex 75 pg, GE Healthcare ©), S-200 preparative gel filtration column (HiLoad 26/60 Superdex 200 pg, GE Healthcare ©) an UV detector and the columns used were Superdex © 75 and 200, both 16/60. Firstly, the column was cleaned with 20 % Et-OH for one to two column volumes. Then, the same procedure with water and, the required buffer. All the three liquids were filtered and degassed beforehand. After the cleaning, the sample was injected to go through the column. The equipment was set-up to fractionate the downstream output.

With the help of the FPLC software, it was possible to visualise a peak that corresponded to the highest concentration of the protein. The fractions that corresponded to the peak or several peaks were collected, quantified by Bradford (check item 4.6.) and ran in an SDS-PAGE gel (check item 4.7.).

4.6.Bradford protein quantification (cuvette)

Firstly, the dye reagent (Bio-Rad © Protein Assasy, ref. 500-0006) was prepared by diluting with deionized water (1:5). Then, six dilutions of Bovine Serum Albumin (BSA) of 1, 0,5, 0,25, 0,125, 0,063 and 0,031 mg/mL were prepared. To 1 mL of diluted dye reagent, 20 µL of the BSA or desired samples were added, incubated minimum for 5 min at room temperature (no more than 1 h) and measured at a spectrophotometer (SpectraMax © M series Multi-Mode Microplate Readers) at 595 nm. With the data of the BSA samples, a standard curve (protein concentration in mg/mL in the x-axis and OD in nm in the y-axis) was constructed and, with the graph's equation (Appendix C) and the DO of the desired samples, it was possible to calculate their protein concentration.

Alternately, if the desired samples were too concentrated, only 1 µL was added to 1 mL of diluted dye reagent and the calculations were corrected by a factor of 20.

4.7. SDS-PAGE gel electrophoresis

The first step was to prepare the gels. The glass cassette and the casting stand (Bio-Rad ©) was assembled with the desired width (1,5 nm). The separation gel (12 % of acrylamide) was prepared and filled in the cassette leaving 2,5 cm free. Around 500 µL of isopropanol was added. After polymerization, the isopropanol layer was removed, and the cassette was filled with 4 % acrylamide stacking solution and the comb was added with the desired number of wells (15). Once polymerized, the electrophoresis module was assembled according to the specifications of the equipment and the samples and the desired protein ladders (pre- or/and un-stained) were loaded. The protein samples to be analysed were previously boiled at 95 °C for 5 to 10 min shaking with loading buffer. To the loading buffer 100 mM of DTT were added for cleaner results. After the loading, the electrophoresis was run until the loading buffer reached the end of the gel.

The first 30 min of the run was done at 110 V and the remaining time was done at 120 V. After that, the gels were stained with Coomassie blue between 15 min and 1 h shaking slowly and then, destained with water. This step can be done overnight in a closed container to avoid contamination.

4.8. Non-denaturing polyacrylamide gel electrophoresis of proteins

This procedure is very similar to the SDS-PAGE gel electrophoresis (check item 4.7.), except that this does not contain SDS, or other reducing agents (like DTT) and the samples were not boiled, therefore, the samples are not denatured. The running gel has 7,5 % of acrylamide, the stacking gel had 3,75 % of acrylamide and the run (at 20 mA for one gel, 40 mA for two) was done at room temperature with cold buffer (4 °C).

4.9. Western Blot

For this procedure, first it was necessary to run an SDS-PAGE gel (check item 4.7.) without staining with Coomassie.

After the protein electrophoresis, the gel(s) were washed two times quickly with water and then washed with freshly made transfer buffer (1×). The nitrocellulose membranes (one per gel) were activated with methanol for 10 seconds, washed with water and then with transfer buffer. For the Whatman papers, two thick papers per gel were used and soaked in transfer buffer. Both the membrane and the Whatman papers were the size of the gel(s). The assemble of the sandwich was done with the following order: Whatman paper(s), membrane, gel and Whatman paper(s). The

electrophoresis was run at 20 to 25 V for 45 min for 1 gel and 1 h for 2 or more and, after the transference, the membrane(s) were washed quickly with water and washed two times for 5 min shaking slowly with TBST buffer. The next step was the blocking for 30 min (10 mL per gel TBST buffer with 3 % milk powder) and the primary antibody solution with TBST and 3 % milk powder was prepared (10 mL per gel). After the blocking, the membrane(s) were washed two times with TBST buffer for 5 min shaking slowly. The buffer was removed, and the primary antibody solution was added and kept at 4 °C overnight shaking slowly. On the next day, the membrane was washed first 15 min and then two times for 5 min with TBST in slow shaking to do the secondary antibody incubation. This step was prepared in the same manner as the first and with the same steps, except for the change from primary to secondary antibody. Lastly, for the revealing solution, 800 µL with equal parts of solution A and B (Amersham™: ECL™ Prime Western Blotting Detection Reagents) was prepared and added to the membrane in a plastic bag. The exposure was done for the necessary time (between 30 s and 10 min).

4.10. Plant tissue production

The *Arabidopsis thaliana* (Col-0) was planted in soil, grown, collected, and preserved at - 80 °C.

The seeds were provided by Dr. Anna Solé from her personal stock from 21/12/23 and the seeds were sown directly to soil (2 to 3 plants per pot in a total of 28 pots) in the greenhouse in long day at 23 °C. The substrate used was a mixture of gramoflor, vermiculite and perlite (3:1:1).

The first collection was done on day 23; all rosette leaves of one plant in each pot was collected, grinded with liquid nitrogen and stored at - 80 °C. On day 28, 34 and 42, the fully developed siliques were collected and stored at – 80 °C.

4.11. Plant protein extraction

For the extraction, the tissue was grinded with liquid nitrogen and 200 µL of plant protein extraction buffer was added to every 100 mg of tissue. The mixture was vortexed until homogenised, the samples were left in the orbital shaking at 4 °C for 30 min and centrifuged at max speed for 20 min at 4 °C. The supernatant was collected and quantified by Bradford (check item 4.6.).

4.12. SAE2 proteolytic processing analysis

In order to prepare the reaction, the freshly protein extracts from *A. thaliana* rosette and siliques (check item 4.11.) were incubated for 1 h at 30 °C with recombinant proteins (Clover:mRuby2, Clover,

mRuby2, L1, L2 and UFDCt) in the presence of the extraction buffer and ATP (1 mM). The mass ratio of protein to plant extract is 1:35.

After the reaction, the samples either were ran in a native or SDS-gel (check item 4.7. and 4.8.). For the native gel, then the gels were read in the FUJIFILM LAS-4000 © (GFP method, 488 nm, blue light, 510DF10 filter, F0,85 Iris) and ChemiDoc © (black tray abd SYPRO Ruby Gel filter: 590/110 nm, UV Trans). With the GFP filter it is possible to detect Clover and with the Ruby filter only mRuby2. The pictures taken from the second equipment and their quantification is presented in Appendix E. For the SDS gel, after the run a Western blot method was followed (check item 4.9.).

5. Results

5.1. Protein production

The plasmids (pET28a::Clover:mRuby2, pET28a::Clover, pET28a::mRuby2, pET28a::Clover-UFDCT(L1)-mRuby2, pET28a::Clover-UFDCT(L2)-mRuby2) were transformed in *E. coli*, induced, extracted, and purified. Then the aliquots were quantified by Bradford, and quality checked by SDS-PAGE.

After the transformation, the *E. coli* grew according to the item 4.3. of the Methodology section. During the purification process, for Clover, L1 and L2, the Sepharose beads were saturated, and the wash flow-through sample had some protein content. In consequence, the flow-through sample was passed twice or even thrice to make sure that the maximum amount of protein was collected. Hence, the sample names use two numbers: the first is the number corresponding to the number of times it passed through the column and, the second, the number of aliquots. As it can be observed in Table 3, a minimum of 5 mg/mL (for Clover and mRuby2) and a maximum of 129 mg/mL (for L2) was obtained from the purifications, which was sufficient to proceed with the subsequent experiments. The Bradford curve with known concentration of BSA that was used for all experiments is presented in the Appendix C.

Table 3. Protein concentration of the final protein aliquots

Protein	Sample	C, mg/mL
Clover:mRuby2	1.1	$34 \pm 0,5$
	1.2	$33 \pm 0,06$
Clover	1.1	$10 \pm 0,04$
	1.2	$8 \pm 0,09$
	2.1	$8 \pm 0,04$
	2.2	$7 \pm 0,1$
	3.1	$6 \pm 0,02$
	3.2	$5 \pm 0,01$
mRuby2	1.1	$5 \pm 0,03$
	2.1	$6 \pm 0,05$
L1	1.1	109 ± 0
	2.1	$77 \pm 0,1$
L2	1.1	$129 \pm 0,2$
	2.1	$56 \pm 0,07$

Eventually, 15 aliquots with a concentration of $0,5 \mu\text{M}$ per protein were prepared and stored at -80°C . Afterwards, an SDS-PAGE was performed as quality control for the purified proteins (Figure 4) with the following samples: T0, T1, supernatant, pellet, flow-through, wash and $0,5 \mu\text{M}$ purified protein. The several samples in the SDS-PAGE inform of how successful the protein purification was.

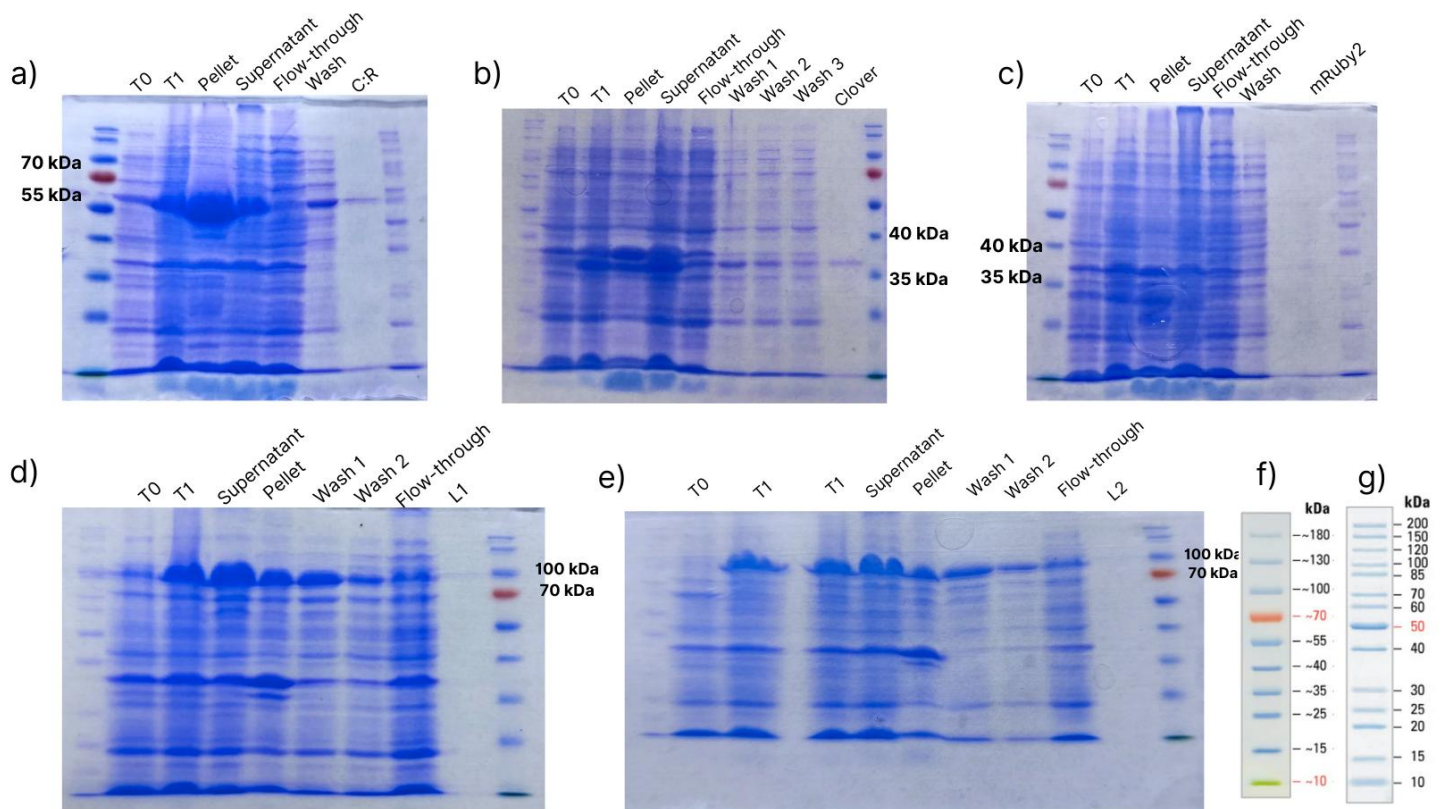


Figure 4. 12 % SDS-PAGE gels of 1,5 mm a) Clover:mRuby2 (C:R), b) Clover, c) m Ruby2, d) L1, e) L2, f) stained ladder sizes, and g) unstained ladder sizes.

The expected bands in The SDS-PAGE gel were between 55 and 70 kDa for Clover:mRuby2; Clover between 40 and 35 kDa; between 35 and 40 kDa for mRuby2; and between 70 and 100 kDa for L1 and L2. As can be observed in Figure 4 a) and b), the bands that correspond to Clover:mRuby2 and Clover were visible, but for mRuby2, L1 and L2 (Figure 4 c), d) and e) respectively) the bands were too faint. Therefore, another gel was run with 10 μ g of each protein (Figure 5).

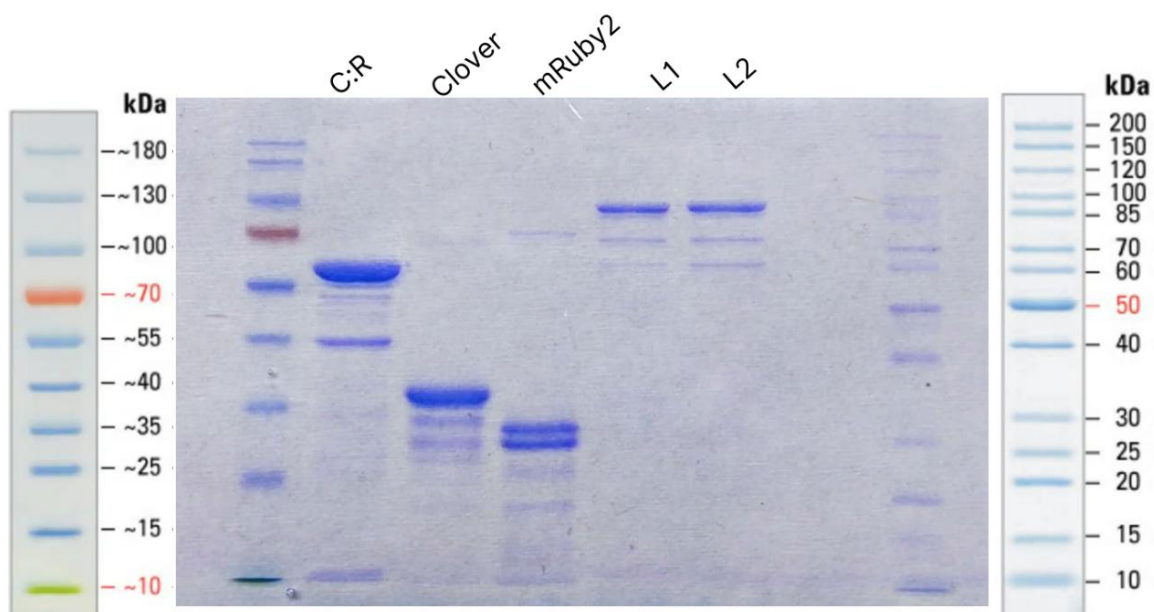


Figure 5. 12 % SDS-PAGE gel of 1,5 mm with all the purified proteins.

From Figure 5, it is possible to visualize a main band with the expected size in most samples; however, for the mRuby2, there are two bands with the same intensity very close in size, and therefore mRuby2 would have to be purified again. In addition, it is also possible to visualize the presence of other unexpected bands in all purified proteins. This can indicate protein degradation since these bands correspond to lower weights. It is also interesting to mention that even though the same weight and molarity per protein was added, the bands have different intensities between proteins; for example, L1 and L2 main bands were significantly less intense than Clover:mRuby2 or Clover main bands. This indicates that the bands that have a higher intensity have a higher protein concentration, which was not expected. Therefore, a new protein quantification was done to compare both quantifications and discard any unforeseen methodological errors during Bradford quantification (Table 4). This time, a minimum of 8 mg/mL (for Clover) and a maximum of 32 mg/mL (for L1) was obtained from the purifications, which was sufficient to proceed with the subsequent experiments.

Table 4. Protein concentration of the final protein aliquots and expected molecular weight

Protein	Sample	C, mg/mL	MW, kDa
Clover:mRuby2	1.1	29 ± 2	59,45
	2.1	26 ± 1	
Clover	1.1	$12 \pm 0,3$	33,34
	1.2	$10 \pm 0,6$	
	2.1	$10 \pm 0,5$	
	2.2	$10 \pm 0,5$	
	3.1	$8 \pm 0,6$	
	3.2	9 ± 2	
mRuby2	1.1	$9 \pm 0,3$	31,76
	2.2	$8 \pm 0,8$	
L1	1.1	32 ± 8	81,74
	1.2	$24 \pm 0,2$	
	2.1	$12 \pm 0,7$	
	2.2	15 ± 2	
L2	1.1	30 ± 3	81,74
	1.2	25 ± 2	
	2.1	13 ± 4	
	2.2	16 ± 6	

There were some differences from the quantification present in table 3 and 4. Clover:mRuby2, L1 and L2 were overestimated and mRuby2 and Clover were underestimated, being the biggest differences for L1 and L2 since the first measurement was over three times the concentration obtained in the second quantification. For Clover:mRuby2, Clover and mRuby2, the differences were over the

margin of error, being the biggest difference for Clover 3.2 and mRuby2 1.1 that it is 1,8 times higher than the original value.

After the plasmid transformation for mRuby2, two cultures were started with two independent colonies. After the centrifugation, the pellet chosen to be purified was the one with a stronger pink colour since it indicated that there was a higher concentration of the fluorescent protein in that culture. The other pellet was stored at -80°C as a backup option. From the purification process two aliquots were obtained and were quantified by Bradford (Table 5), with a very similar concentration. The protein obtained was almost three times more concentrated than in the previous purification (Table 4), which was sufficient for future experiments.

Table 5. Protein concentration of mRuby2 aliquots

Protein	Sample	C, mg/mL
mRuby2	1.1	23 ± 3
	2.1	$23 \pm 0,5$

New aliquots of $500\text{ }\mu\text{M}$ for all proteins were done and an SDS-PAGE with the samples of mRuby2 production was performed to compare the old and new purifications (Figure 6). It was decided to do $500\text{ }\mu\text{M}$ stocks instead of $0,5\text{ }\mu\text{M}$ to ensure band visualization, given that at $0,5\text{ }\mu\text{M}$ the bands were not visible as can be seen in Figure 4.

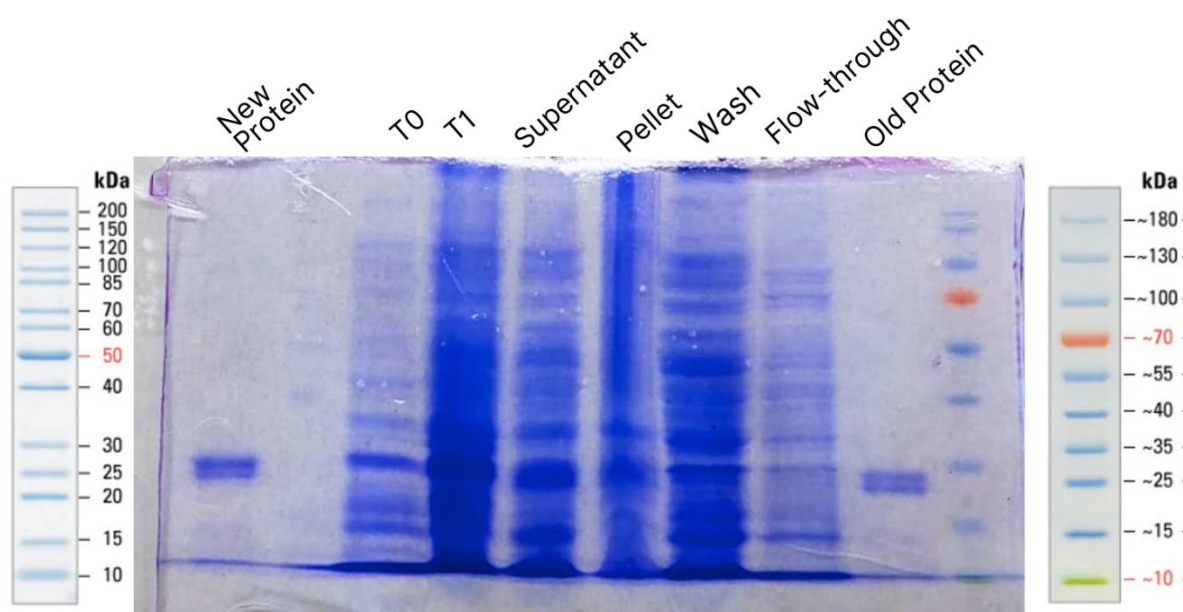


Figure 6. 12 % SDS-PAGE gel of 1,5 mm with purified mRuby2 and protein production samples.

From Figure 6, it was possible to verify that the mRuby2 production was successful and since an expected band between 35 and 25 kDa appeared. In addition, it is possible to compare the new and old protein: both a double band very close, however the lower band in the new mRuby2 is less strong than the upper band. The same did not happen on the previous mRuby2 since both bands had the same intensity. Therefore, it was assumed that the purification was successful and the protein concentration for mRuby2 was correct.

For the next step, even though all proteins had some degradation, it was believed that this should not interfere with the FRET and no additional purification was done at this point.

5.2. Set-up FRET conditions

After the protein production and quantification, the next step was setting up the FRET conditions. The emission and excitation of Clover and mRuby2 are a spectrum (Figure 7), where there is an optimal excitation and emission wavelength for each fluorophore but there is a range of wavelengths that can be used to have FRET.

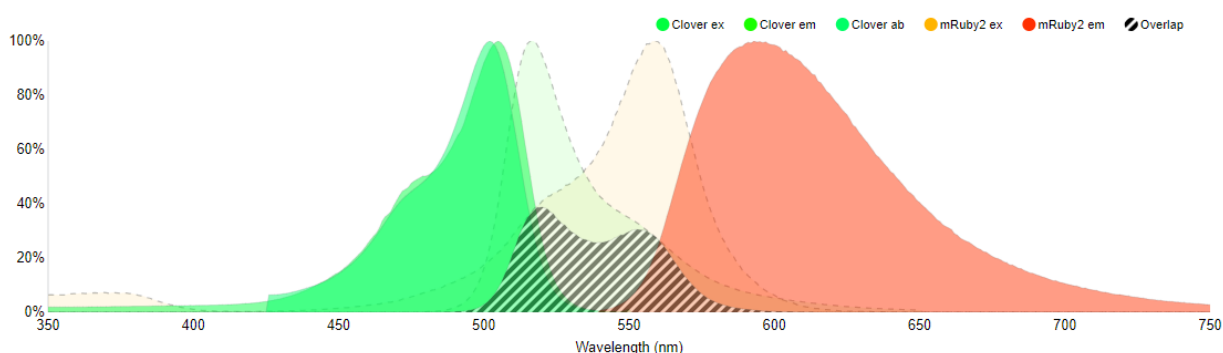


Figure 7. Clover and mRuby2 wavelength spectrum for FRET.

Table 6 illustrates the excitation and emission wavelengths of the fluorescent proteins used, and the conditions for the FRET assay:

Table 6. Clover, mRuby2 and FRET excitation and emission wavelength

	λ excitation, nm	λ emission, nm
Clover	480	530
mRuby2	530	625
FRET	480	625

The experimental design is presented in Figure 8.

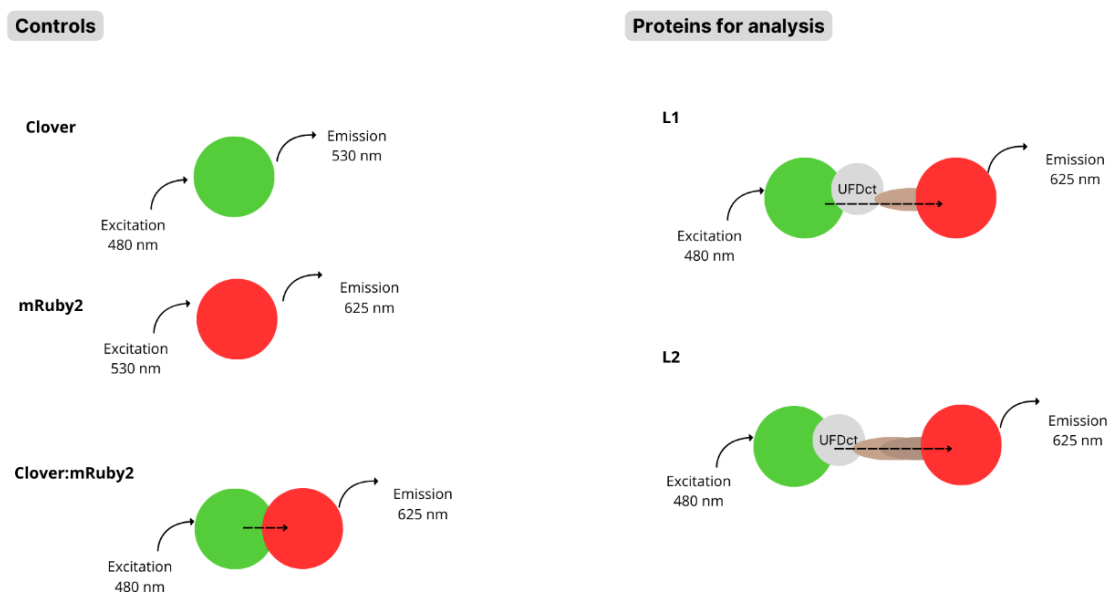
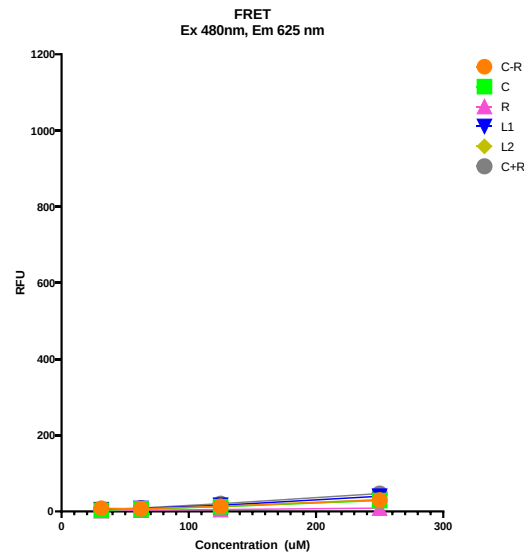


Figure 8. Schematic representation of the experimental design.

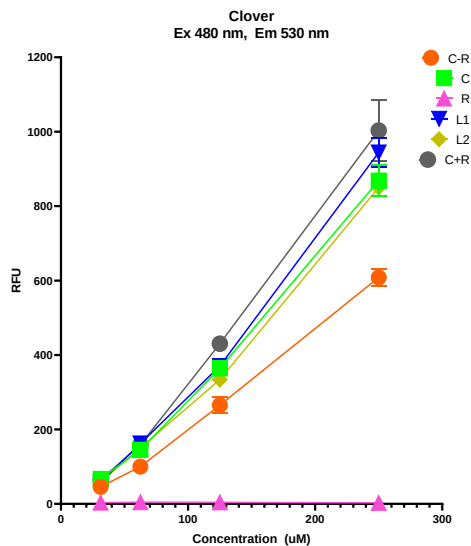
For this experiment Clover:mRuby2 was the positive control for the FRET, Clover+mRuby2 the negative control, and Clover and mRuby2 alone were used to calculate their specific excitation and emission conditions. In the Clover+mRuby2, the molecules were not in close contact because it is not a translational fusion (only Clover and mRuby2 added in equal molarity), and the transfer of energy was not expected to happen. Hence, the only signal that may be expected was by the proteins randomly clashing into each other, which would give a very low signal that could be considered the “basal” signal for the rest of the proteins. Lastly, L1 and L2 were the proteins being tested and it was expected to detect signal from them at FRET conditions.

In the first attempt, the proteins (concentrations of 250, 125, 62,5 and 31,25 μM) were tested for FRET, Clover and mRuby2 conditions. For the data analysis, graphs were constructed with the protein concentration in μM in the x-axis and in the y-axis the relative fluorescent units (RFU) in nm. The value of the buffer and the negative control was subtracted to the signal emitted by all the proteins. The results are presented in Figure 9.

a)



b)



c)

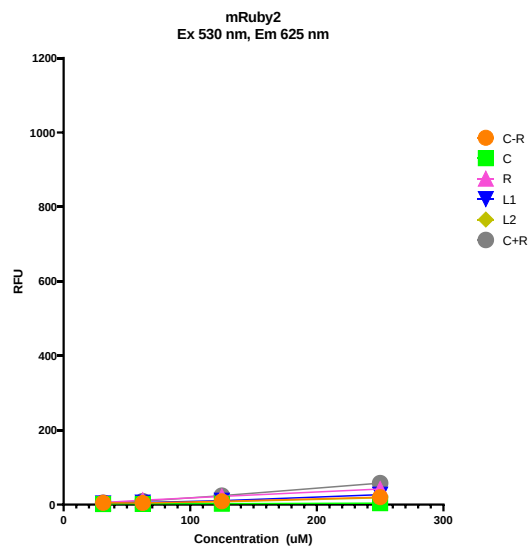


Figure 9. Results with a black bottom plate with buffer extraction of a) FRET, b) Clover excitation and emission conditions, c) mRuby2 excitation and emission conditions. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 - Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 - Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

For Clover conditions (Figure 9 b)), it was expected to detect the highest signal in Clover followed by Clover+mRuby2, Clover:mRuby2, L1 and L2 (which was anticipated to be reduced by half or even more in Clover:mRuby2, L1 and L2 due to possible quenching) and low or no signal of mRuby2, since the signal emission was being read at 530 nm instead of 625 nm. However, Clover was not the highest signal but rather Clover+mRuby2, followed by L1, Clover, L2, Clover:mRuby2 and mRuby2.

Therefore, this experiment suggested that the proteins were not behaving as initially hypothesized, expect for Clover:mRuby and mRuby2.

For mRuby2 conditions (Figure 9 c)), it was expected to have high signal of mRuby2 followed by Clover+mRuby2, Clover:mRuby, L1 and L2, following the same logic as per Clover conditions. In addition, it was expected a low or no signal of Clover, since the excitation was done at 530 nm instead of 480 nm. Once again, the highest signal was from Clover+mRuby2 followed by mRuby2, L1, Clover:mRuby2, L2 and Clover. The signal from Clover+mRuby2 was higher than mRuby2, in particular for 250 μ M concentration, which was not expected but in line with previous results; the remaining proteins behaved as expected.

Regarding the FRET conditions (Figure 9 a)), it was expected the highest signal for Clover:mRuby2, L1 and L2, and very low to no signal of Clover, mRuby2 and Clover+mRuby2 because the excitation was done at 480 nm (excites Clover) and emission at 625 nm (emission for mRuby2). Clover+mRuby2 had, again, the unexpected highest signal, followed by L1, L2, Clover:mRuby2, Clover and mRuby2. To note, that Clover also had a signal that was higher than expected. Together, these results indicate that there was a problem with Clover and Clover+mRuby2 samples.

Due to these results, a new strategy was designed: several emission and excitation values were tested to check if there was a condition what would better fit these proteins and see the results expected. As mentioned before, excitation and emission of a fluorophore are presented in a spectrum (Figure 7), meaning that are an interval of wavelengths where the protein can be excited and can emit signal. Therefore, new emission wavelengths between 595-635 nm were tested, as well as new excitation wavelengths between 470-500 nm, for the FRET. To do so, two different experiments were done: one with B7 Buffer with 0 % and the other with 0,1 % BSA. The main goals of having the presence of BSA in the protein samples is to stabilize the solution and to create a competition between the BSA proteins and the fluorescence proteins with the objective of improving the results by reducing unspecific binding between proteins to avoid Clover and mRuby2 from sticking together in the negative control, reducing the background signal.

The best FRET results obtained for both buffer conditions and all excitations tested were for the emission of 625 nm and 635 nm, which it is logically consistent since mRuby2 peak of emission is at 625 nm. Moreover, the higher the excitation value, the higher RFU was obtained. This could due the fact that the optimal Clover excitation is at 500 nm and, at that wavelength, mRuby2 is also being excited, resulting in higher FRET signals. All the results are presented in Appendix D.

The B7 0 % BSA for 625 nm emission results are presented in Figure 10.

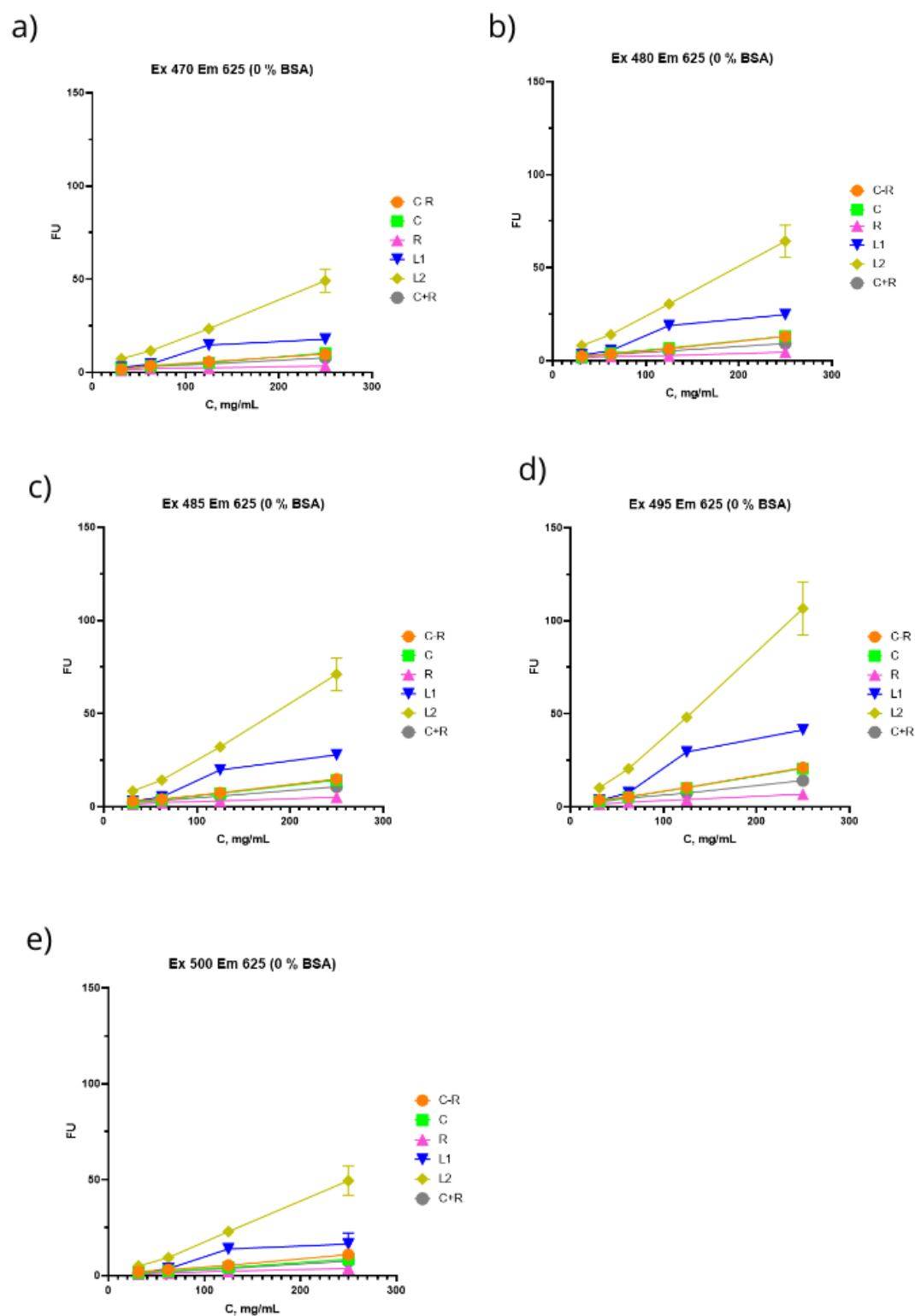


Figure 10. Results with buffer subtraction (B7 0 % BSA, 625 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500.

Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby2 (grey circle).

The B7 0 % BSA for 635 nm results are presented in Figure 11.

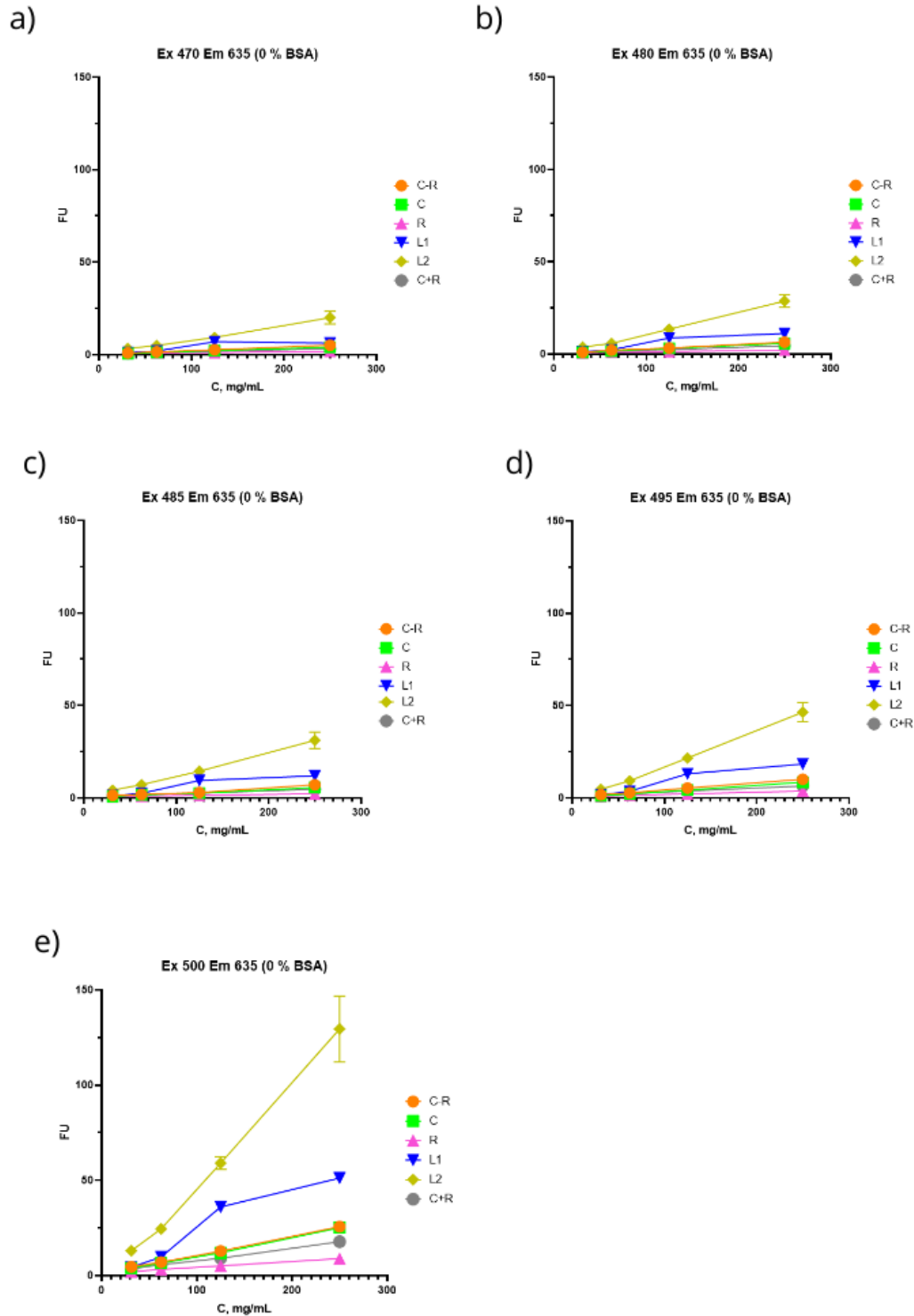


Figure 11. Results with buffer subtraction (B7 0 % BSA, 635 emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2

(orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 - Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 - Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby2 (grey circle).

The B7 0,1 % BSA for 625 nm emission results are presented in Figure 12.

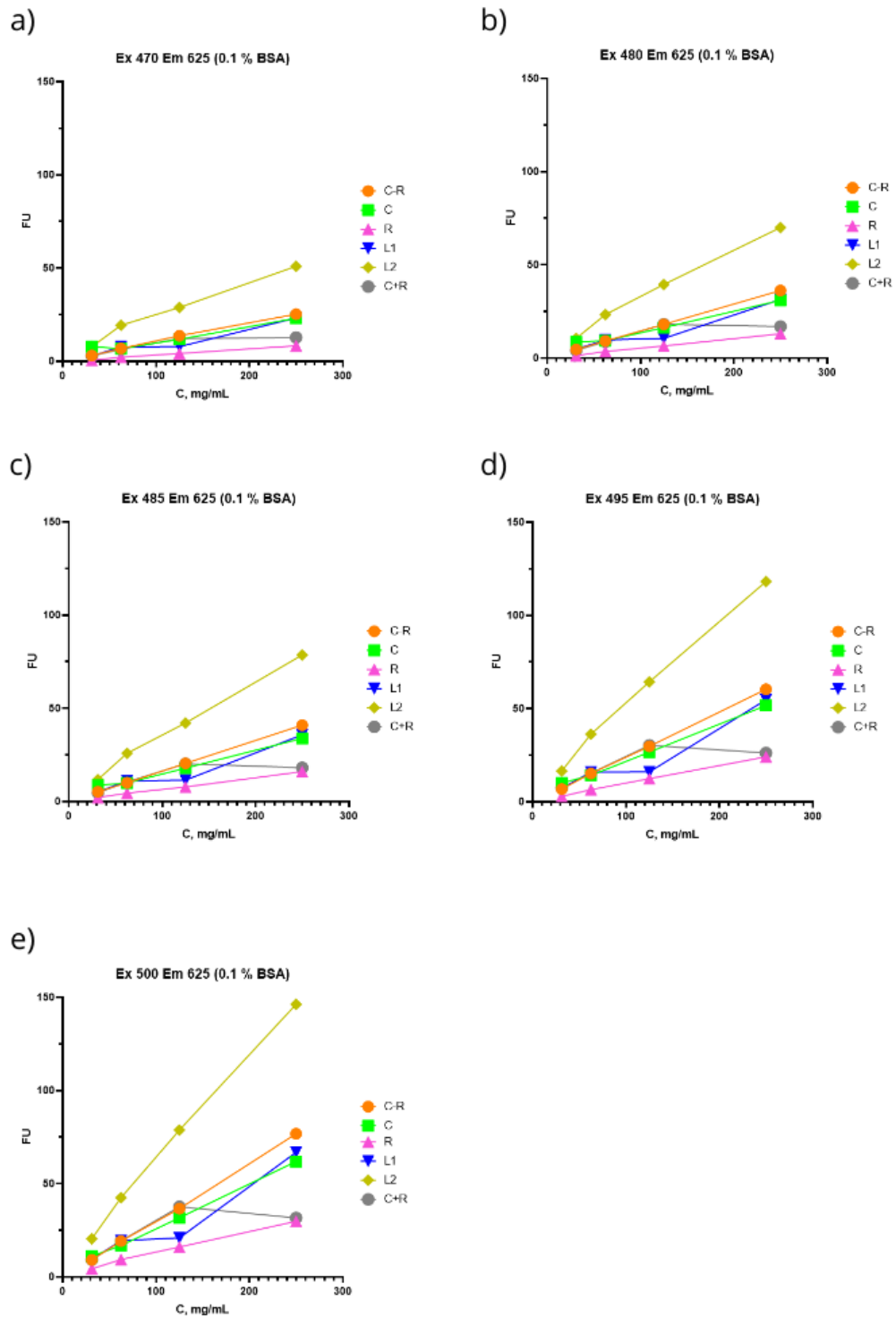


Figure 12. Results with buffer subtraction (B7 0,1 % BSA, 625 emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby2 (grey circle).

The B7 0,1 % BSA for 635 nm emission results are presented in Figure 13.

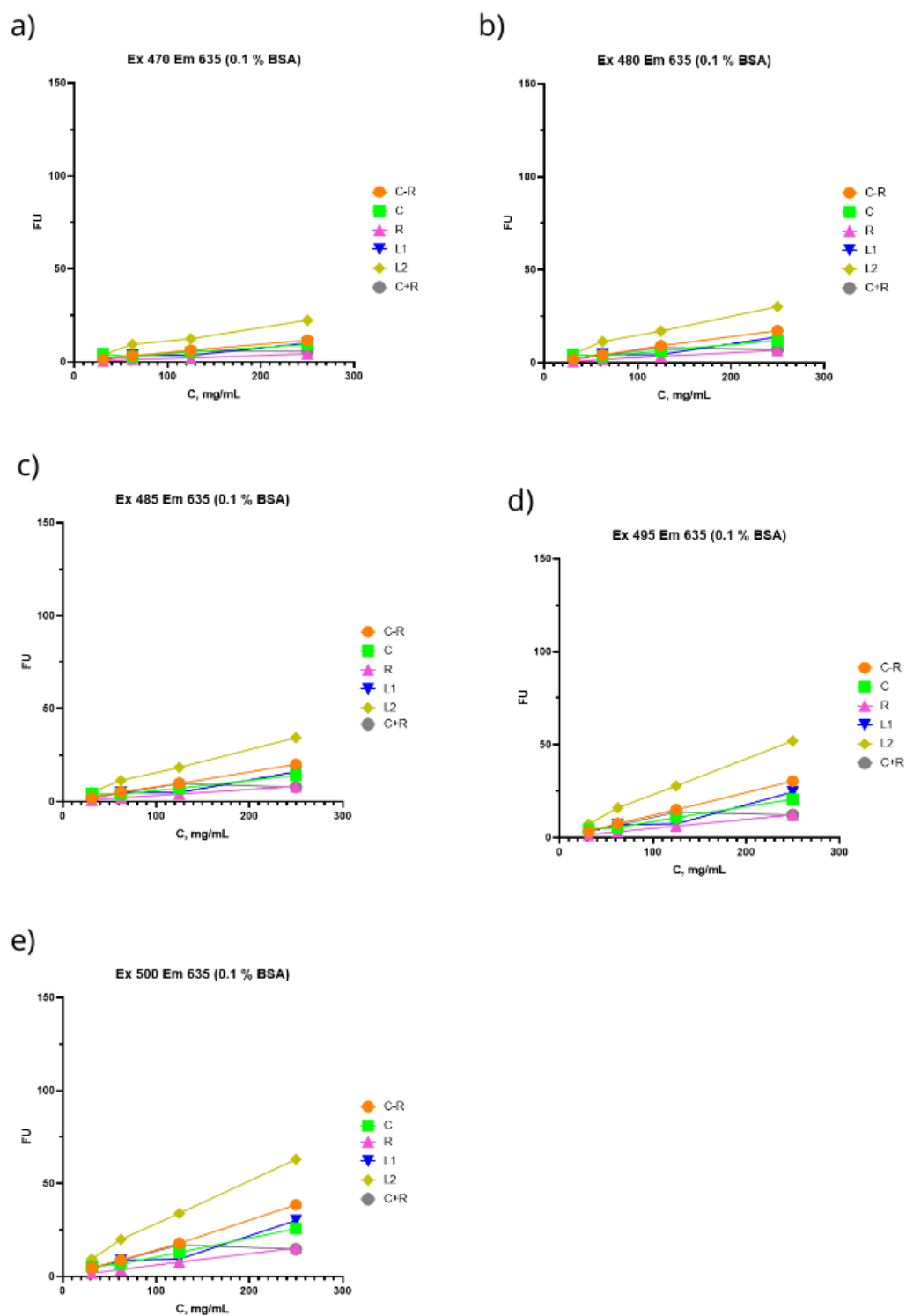


Figure 13. Results with buffer subtraction (B7 0,1 % BSA, 635 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

It is possible to observe that, in Figures 10 to 13, the signal for L2 was higher than all the other proteins in all conditions, especially in the presence of BSA, while the other proteins have a lower signal and closer to each other. It would be expected to have a similar signal between L1, L2 and Clover:mRuby2, which did not happen. Therefore, the experiment was repeated once again and with more concentration points, for both buffer conditions.

This time a higher range of protein concentrations was tested (250, 125, 61,5, 31,25, 15,63 and 7,81 μM) to increase the number of points and evaluate if there was an optimal protein concentration or the signal and the protein concentration were directly proportional. Since in the last experiment the best emissions were 625 and 635 nm, only these two were tested for different excitations.

The B7 0 % BSA for 625 nm emission results are presented in Figure 14.

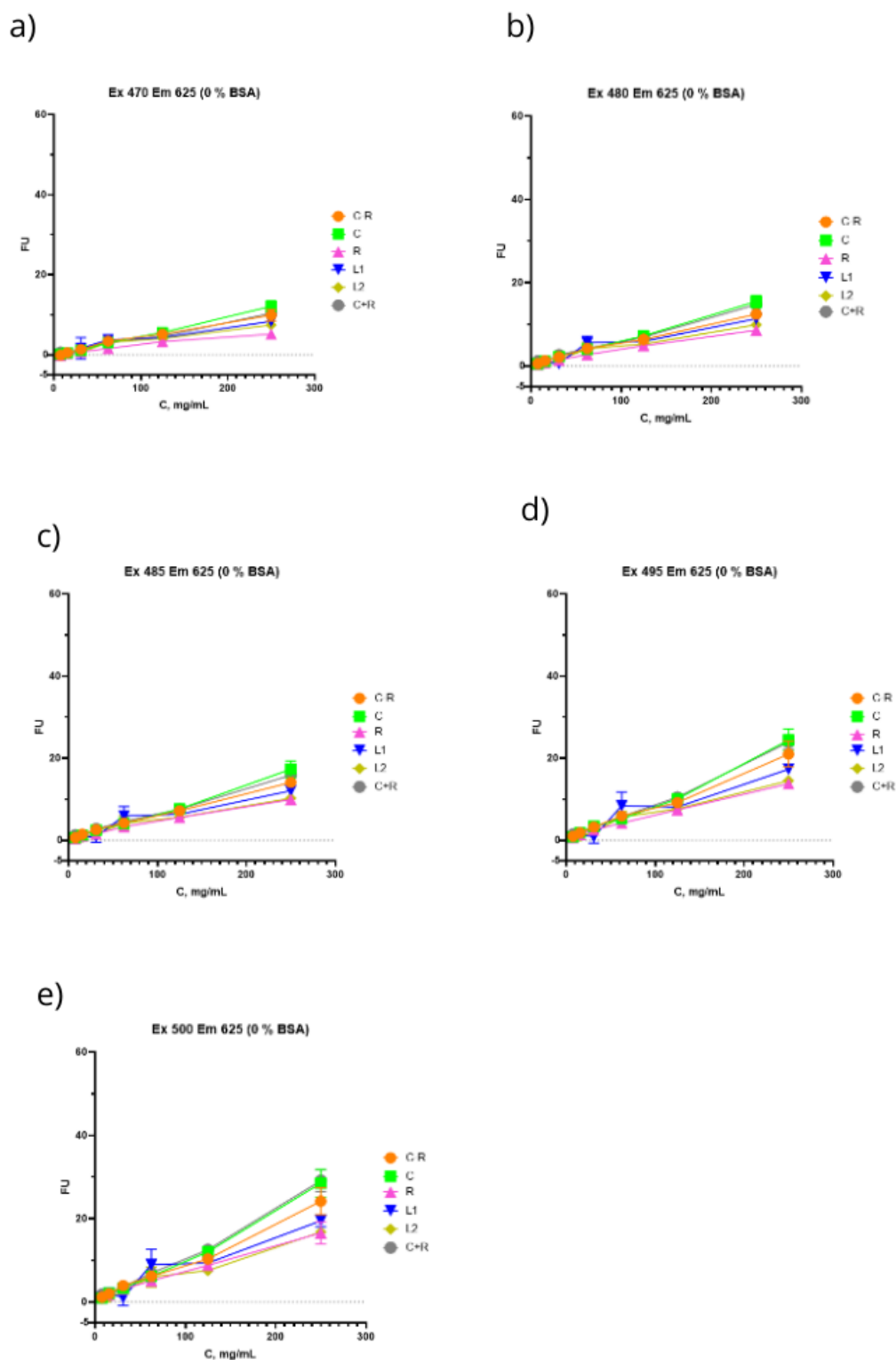


Figure 14. Results with buffer subtraction (B7 0 % BSA, 625 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

The B7 0 % BSA for 635 nm emission results are presented in Figure 15.

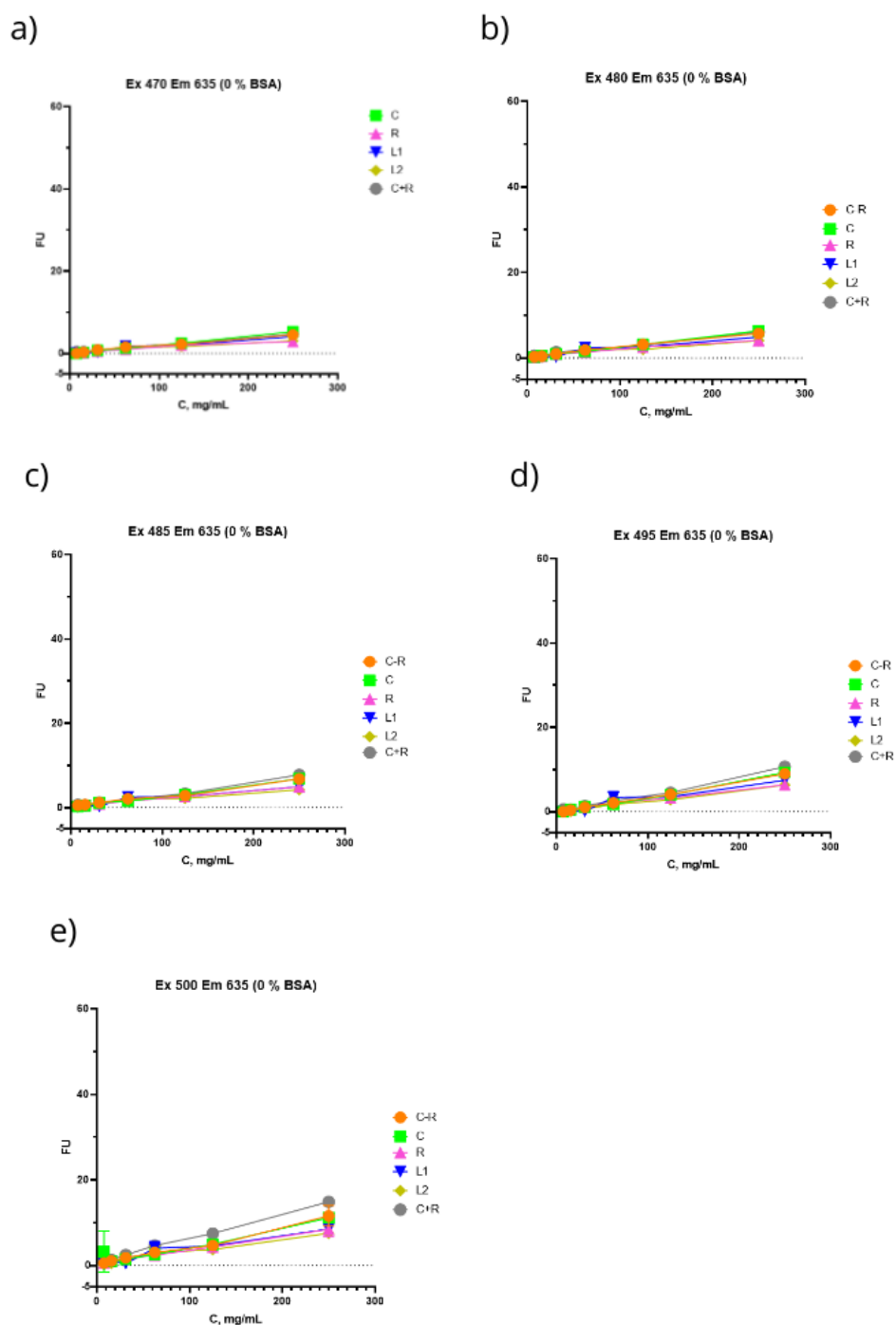


Figure 15. Results with buffer subtraction (B7 0 % BSA, 635 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

The B7 0,1 % BSA for 625 nm emission results are presented in Figure 16.

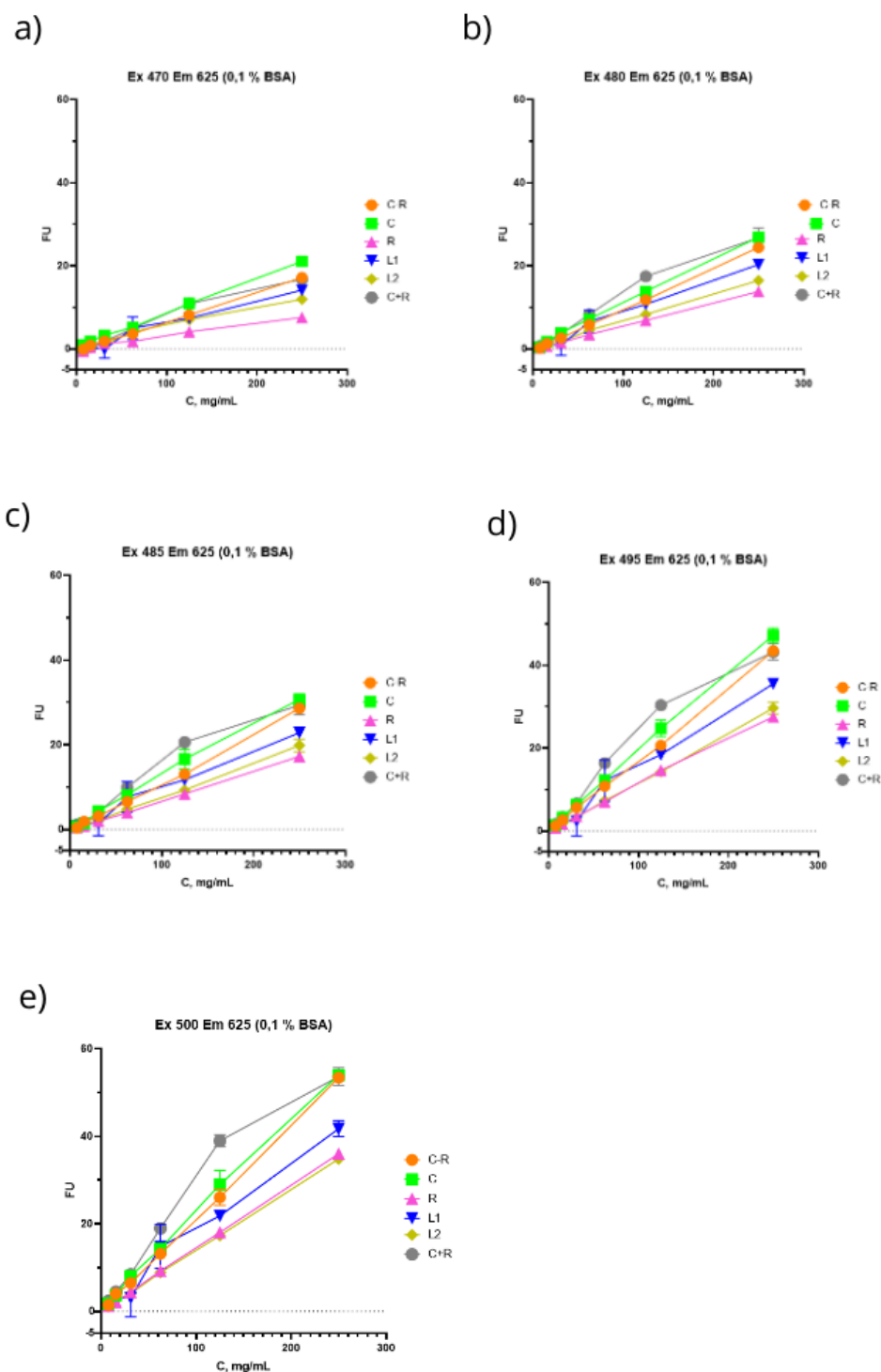


Figure 16. Results with buffer subtraction (B7 0,1 % BSA, 625 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C+R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

Lastly, the B7 0,1 % BSA for 635 nm emission results are presented in Figure 17.

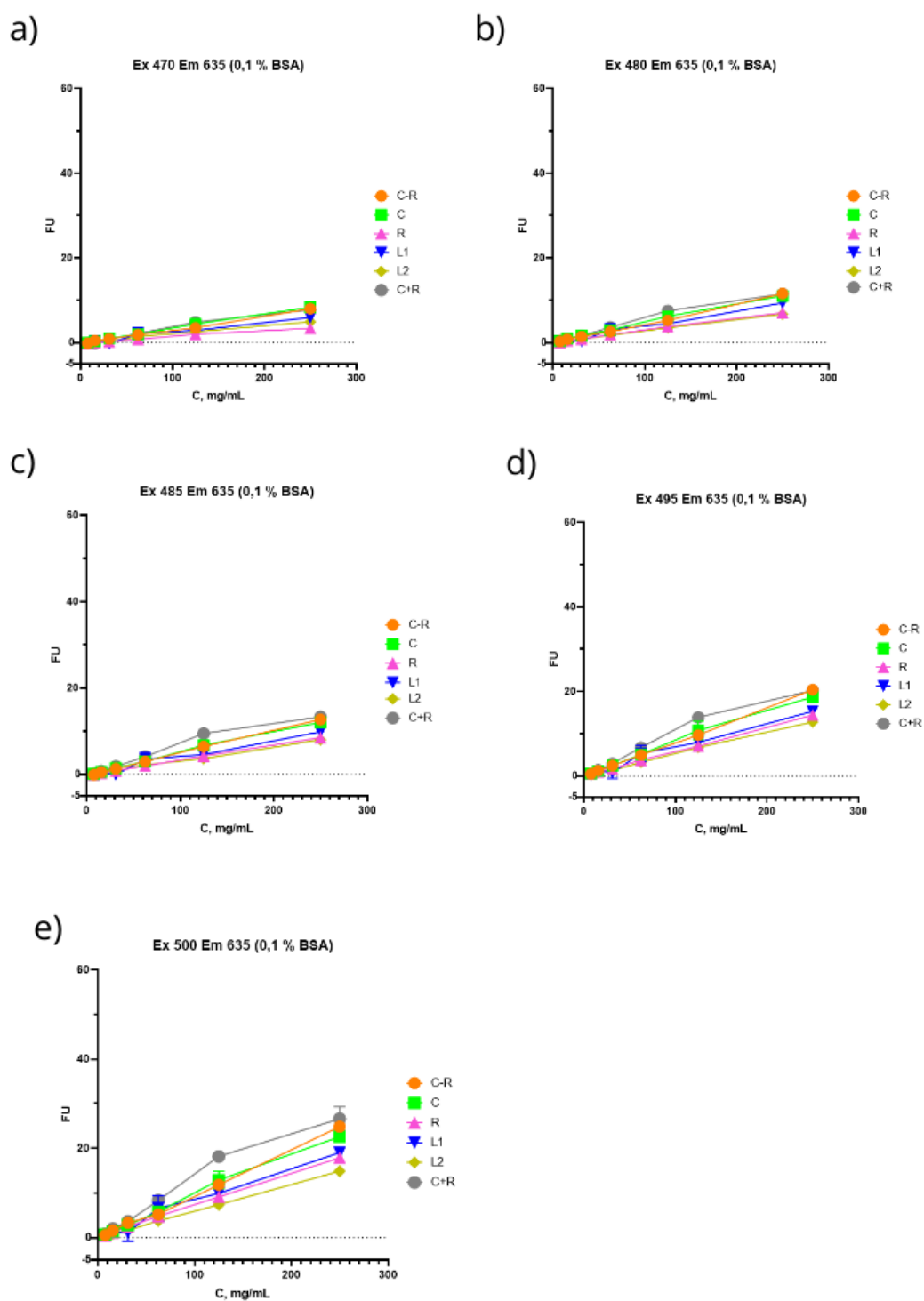


Figure 17. Results with buffer subtraction (B7 0,1 % BSA, 635 nm emission) with a black bottom plate of a) excitation 470 nm, b) excitation 480 nm, c) excitation 485 nm, d) excitation 495 nm, and e) excitation 500. Legend: C-R – Clover:mRuby2 (orange circle), C – Clover (green square), R – mRuby2 (pink triangle), L1 – Clover:UFDct:Linker1:mRuby2 (inverted blue triangle), L2 – Clover:UFDct:Linker1:mRuby2 (yellow diamond), and C+R – Clover + mRuby (grey circle).

In Figures 14 to 17 it is possible to see that Clover, Clover:mRuby2 and Clover+mRuby2 signals are very close to each other, L1 signal is higher than L2 and mRuby2 has the lowest signal. Again, it was expected to have no or very low signal for Clover+mRuby2 and mRuby2 and, also, Clover signal was expected to be lower than Clover:mRuby2, L1 and L2, which was not observed. Finally, similar to the previous experiment, the signal detected was higher in the presence of BSA.

A possible explanation to have such a high signal for Clover is that at 625 and 635 nm this protein can still emit some fluorescence; for mRuby2 something similar may also happen: this protein can be excited between 470 and 500 nm, although at a much less strength, therefore at the 625 and 635 nm mRuby2 may still emit a minimal signal. Clover:mRuby2 was expected to have a higher signal than L1 and L2, although it was not always the case. For Clover+mRuby2, a possible explanation is that Clover and mRuby2 were close enough to interact and emit FRET signal, contrary of what it was believed when this assay was designed.

Taken together, it seems that the FRET assay was impossible to perform using these purified proteins at that moment, due to technical, protein-specific or experimental design related problems. Hence, a new strategy alternative to FRET was created.

5.3.Set-up of alternative method

5.3.1. Selection of the best gel electrophoresis method

After trying to set up the FRET assay with the previously mentioned proteins and not being able to do so, another strategy was defined. This strategy consisted of working with non-denatured proteins in a native gel, which would separate the different proteins by size and isoelectric point. This way, the fluorescent signal could be detected, and we could potentially identify any processing performed by a protease in the SAE2 C-terminal. Since the aim of the project is to find the protease that cuts the UFDCt (region from the SAE2) and the L1 and L2 proteins is UFDCt ligated to Clover with a linker that ligates to mRuby2, mixing a plant extract (which supposedly contains the responsible protease) with L1 and L2 proteins, it should be possible to see the signal of both fluorescence proteins separately. The expected result is schematically represented in Figure 18.

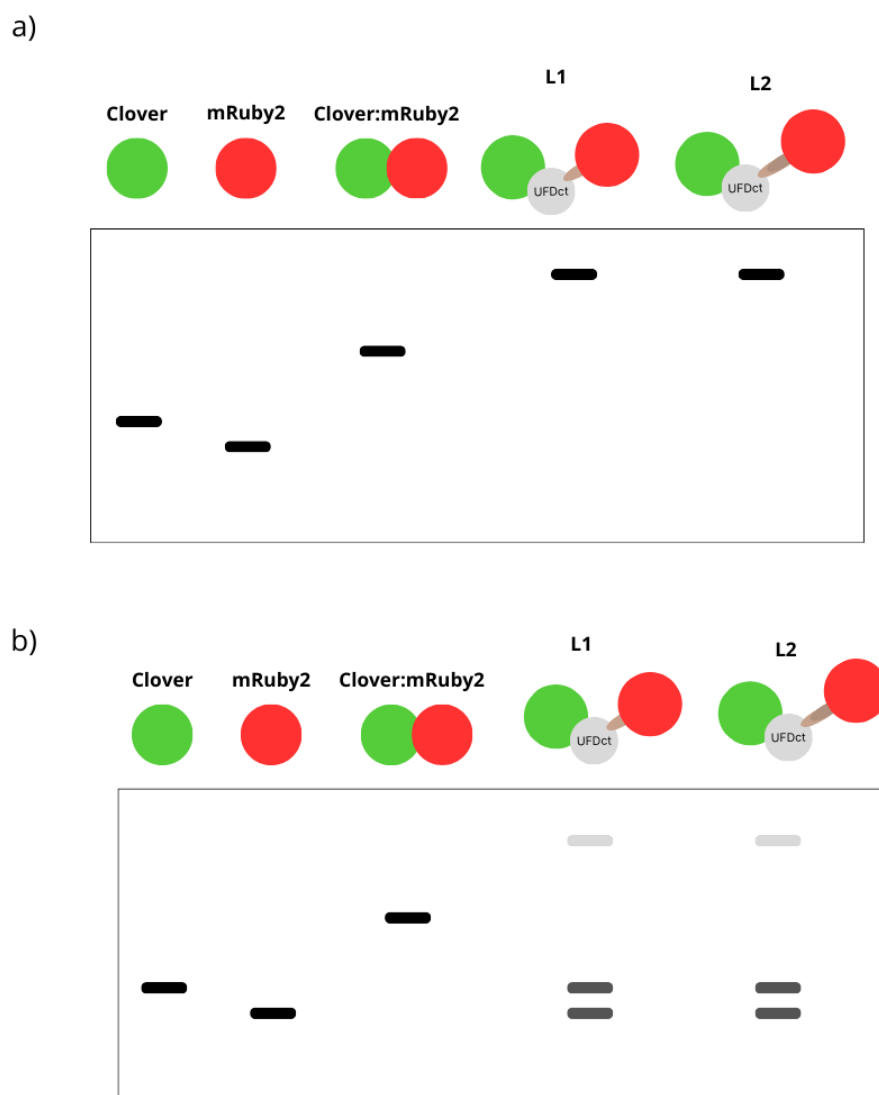


Figure 18. Scheme of expected gel results a) proteins in absence of plant extract b) proteins in presence of siliques extract.

For this experiment, several conditions were tested. Using 500 mM protein stocks and, 1:2 serial dilutions would be tested in native gels, SDS-PAGE gels and in a native gel using buffers and reagents with SDS (check the items 4.7. and 4.8., respectively). In these three different approaches the samples would not be boiled since the objective is to see the behaviour of the samples nondenatured and in cold conditions. The advantage of using the reagents with SDS is that it is not necessary to have two different stocks with reagents for non- and denaturing protein electrophoresis.

After testing the mentioned conditions (Table 7 and Figure 19), the one that lead to the best results, would be the one used for this alternative.

Table 7. Characteristics and information of the gels ran

	Gel composition	Run characteristics
a)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1, L2 and CFP SUMO (kindly provided by a lab member). CFP SUMO was used as a control since it was previously tested in other experiments and has a very strong fluorescent signal. It was useful to check if the assay worked, since it was uncertain that this approach would work for our proteins. - Samples with DTT. - Concentrations tested: 0,5; 0,25; 0,125; 0,63 and 0,31 μM. 2 Native gels with 15 wells ran at 40 mA for 1h51 in the cold room (4 °C). - Loaded 60 μL per well.
c)	SDS-PAGE: 12 % acrylamide Stacking gel: 4 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Concentrations tested: 5; 2,5; 1,25 and 0,625 μM. 2 SDS-PAGE gels with 15 wells ran at 100 V: Gel with L1 and L2 for 3h and the other for 3h28 on ice. - Loaded 30 μL per well.
e)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Buffers used with SDS and no DTT in the samples. - Concentrations tested: 2,5; 1,25 and 0,625 μM. 1 Native gel with 15 wells ran at 20 mA for about 2h on ice. - Loaded 60 μL per well.
g)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover (new), mRuby2, Clover:mRuby2, L1 and L2. - Buffers used with SDS and no DTT in the samples. - Concentrations tested: 2,5; 1,25 and 0,625 μM. 1 Native gel with 15 wells ran at 20 mA for on ice. - Loaded 60 μL per well.

The results of the several runs are presented in Figure 19.

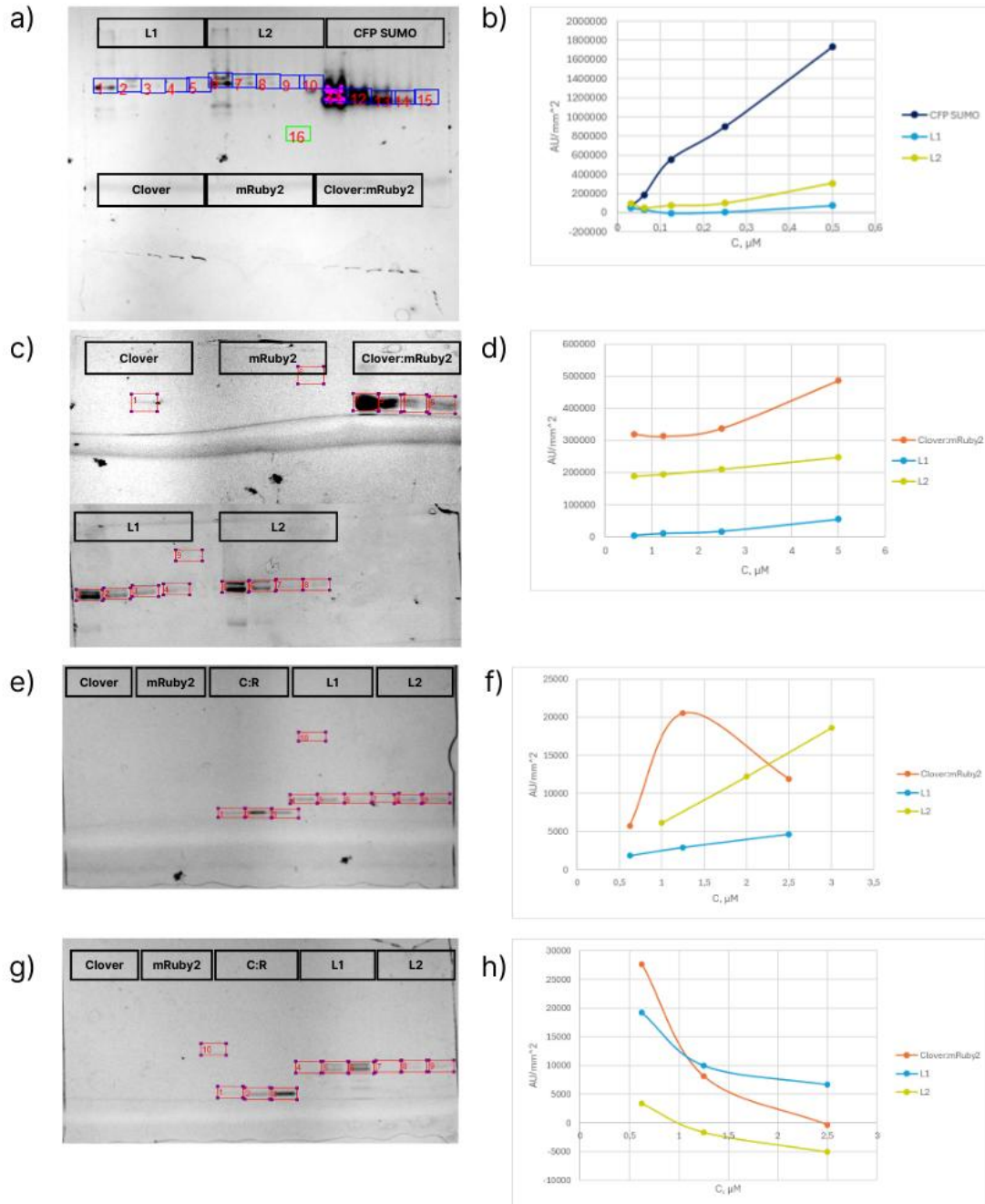


Figure 19. Results a) native gel, b) fluorescent signal quantification of a), c) SDS-PAGE gel and d) fluorescent signal quantification of c), e) native gel with buffers with SDS, f) fluorescent signal quantification of e), g) native gel with buffers with SDS, and h) fluorescent signal quantification of g).

From the results in Figure 19, it is possible to see that all three methods worked since it was possible to obtain bands and their relative size appear correct (L1 and L2 are about the same size and the heaviest, Clover:mRuby is smaller than L1 and L2 and CFP SUMO is smaller than L1 and L2).

As observed in Figure 19 a) it was possible to detect fluorescence for L1, L2 (with a double band) and CFP SUMO, even though it was expected to see also Clover and Clover:mRuby2. The double band in L2 indicates that the proteins had degraded more since the last experiment (Figure 5) that could be due the cycles of frost and defrost of the samples to use them in the experiments. Since the filter used was GFP, mRuby2 was not detected in none of the gels in Figure 19, however in Appendix E it is presented the picture of the gels with the Ruby filter. Additionally, it is possible to see in the graph of Figure 19 b) that the higher the protein concentration, higher the fluorescent signal.

According to Figure 19 c) it was possible to detect Clover:mRuby2, L1 and L2, all with a double band. It was also possible to observe from Figure 19 d) that the higher the protein concentration, higher the fluorescent signal. Finally, all the visible bands are very strong at 5 μ M but still strong and clear at 2,5 μ M, therefore it was determined that from this point on the maximum amount of protein loaded to the gel would be 2,5 μ M in order to not waste sample. Similar results were obtained in

It was possible to, in Figure 19 e), detect Clover:mRuby2, L1 and L2 with no double band, however the quantification (Figure 19 f)) it was possible to see and decrease of signal in the most concentrated Clover:mRuby2 sample. This could have been due to an error of the operator on the preparation of the samples or in the loading order.

Similarly, in Figure 19 g), once again, it was possible to detect Clover:mRuby2, L1 and L2, all with a double band and, for the quantification of all proteins (Figure 19 h)), it was observed that the highest the protein concentration, the lowest signal was detected. Again, this could have been due to an error of the operator on the preparation of the samples or in the loading order.

In conclusion, the worst run was the SDS-PAGE because the samples did not run in a uniform way (Figure 19 c)). The runs using a native gel with or without SDS (Figure 19 a), e) and g)) worked very well, therefore it was concluded that the best assay was using a native gel using buffers and reagents with SDS to avoid maintaining two different buffer stocks and the pictures are the gels and the bands appear to be clearer and a protein concentration of 2,5 μ M.

Additionally, it is possible to see a double band in mRuby2, L1, L2 and Clover:mRuby2 and some protein degradation (Figures 6 and 19 a), c) and g)). Therefore, all proteins were purified with a FPLC as an attempt to get rid of any possible protein degradation.

5.3.2. FPLC protein purification

In order to eliminate the additional bands, an FPLC was performed (check item 4.5.).

The FPLC purified fractions showed that Clover:mRuby2 appeared in one peak that corresponded to six fractions (40-45). Clover with seven fractions (27-33). mRuby2, in turn, appeared in two separate peaks, the first with only three fractions (23-25) and the second with ten fractions (30-39). L1, it was contained in six fractions (42-47) and L2 in six fractions as well (37-42).

After the purification, the fractions of interest were run in SDS-PAGE gels (Figure 20) in order to check the quality of the fractions.

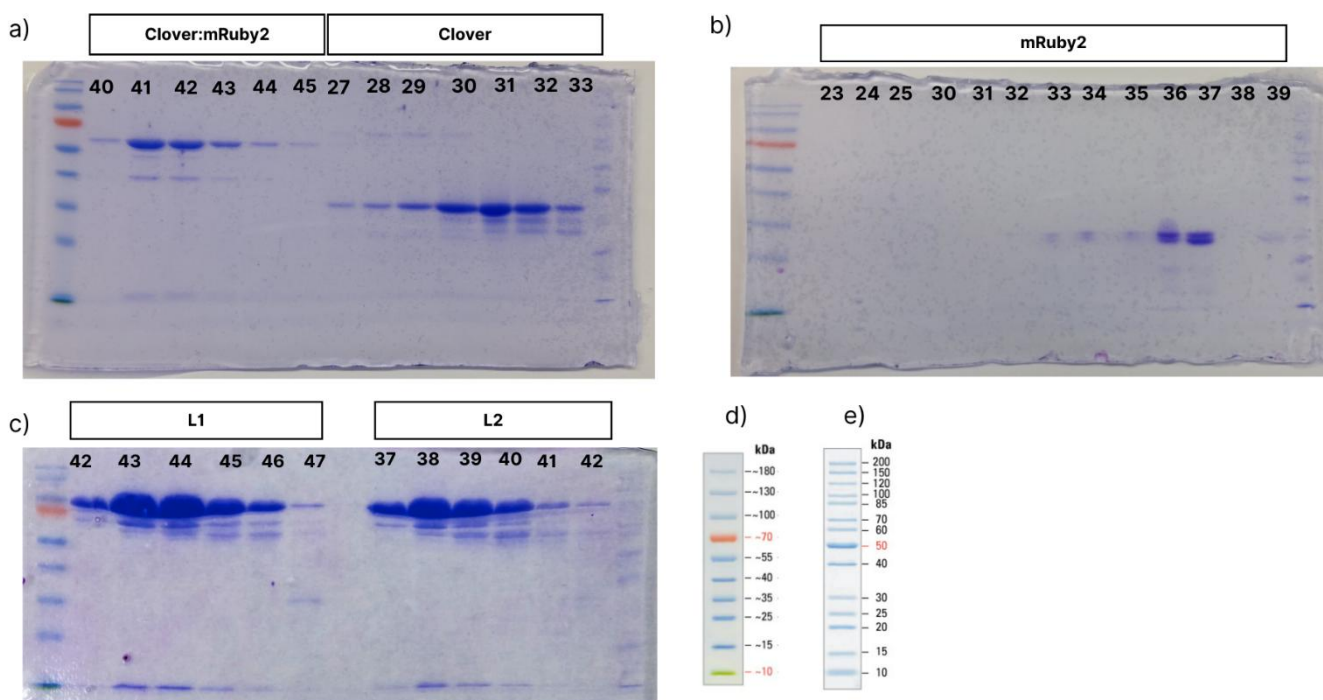


Figure 20. 12 % SDS-PAGE, 1,5 mm a) Clover:mRuby2 and Clover; b) mRuby2; c) L1 and L2, d) stained ladder sizes, and e) unstained ladder sizes.

It is possible to observe that in From Figure 20 a), b) and c) some fractions are stronger than others and there were different degradation bands in the fractions for the same proteins. Therefore, a pre-selection of two potential fractions for each protein was done (Table 8). The reasoning behind that

selection was to choose the fractions that correspond to a stronger band (more protein concentration) but with a smaller number of degradation bands as possible.

Table 8. Fractions to be tested of each protein

Protein	Fractions
Clover	30, 31
mRuby2	34, 37
Clover:mRuby2	40, 41
L1	42, 43
L1	37, 38

These fractions were quantified by Bradford (Table 9). With these, a non-denaturing gel with SDS-Page buffers was run with 2,5 μ M of each sample to select the best fractions with the high protein concentration but less degraded as possible (Figure 21).

Table 9. Bradford quantification of the different fractions of each protein (mg/mL) and standard deviation

Protein	Fraction	C, mg/mL
Clover	30	1,2 $\pm$ 0,01
	31	2,6 $\pm$ 0,02
mRuby2	34	6,8 $\pm$ 0,03
	37	5,9 $\pm$ 0,01
Clover:mRuby2	40	2,1 $\pm$ 0,02
	41	12 $\pm$ 0,1
L1	42	2,92 $\pm$ 0,005
	43	10 $\pm$ 0,04
L2	37	0,740 $\pm$ 0,005
	38	5,6 $\pm$ 0,03

The results are presented in Figure 21.

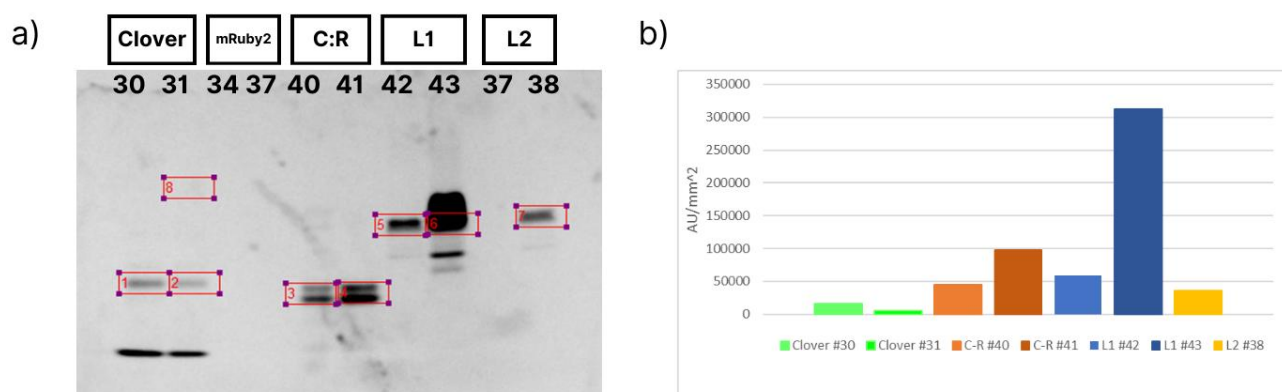


Figure 21. Native gel 1,5 mm, 20 μ L per well of 2,5 μ M of Clover, mRuby2, Clover:mRuby2, L1 and L2, ran for 2h11 at 20 mA a) picture taken with LAS-4000, b) fluorescence quantification of picture a).

From Figure 21 a) and the quantification of b), the fractions chosen to work for this point forward were Clover:mRuby2 #40, Clover #31, L1 #42 and L2 #38. The mRuby2 fractions were not able to be detected with the Ruby filter (Appendix E, Figure 1E g)), therefore the strongest band from Figure 20 which correspond to the fraction #37 was chosen. The reason why mRuby2 at 2,5 μ M was detected with the proper filter previously but not in this gel is unclear. Possible explanations are the calibration of the equipment since its camera was not in good conditions (blurry pictures of the gels) or the preparation of the samples.

5.3.3. Assay validation

After the purification, the next step was running a native gel with the proteins in the presence of the plant extracts: rosette and siliques. Given that the processing of SAE2 occurs differently in rosette and siliques (Más, *et al.*, 2020), we wanted to test whether we can detect this processing using our experimental set-up.

For the plant protein purification of the siliques, it was done in duplicate to ensure there was enough viable sample. Then, after the quantification by Bradford (Table 10), the sample with the highest concentration was chosen.

Table 10. Bradford quantification of plant extracts

	Siliques 1	Siliques 2	Rosette
$C_{average}$, μ g/ μ L	$11 \pm 0,01$	$12 \pm 0,05$	$15 \pm 0,04$

In order to test whether we could observe a processing of the SAE2 C-terminal domain in our experiment set-up in L1 and L2 proteins, a digestion reaction was prepared with 350 µg of freshly prepared protein extracts from rosette and silique Col-0 plants and 10 µg of the produced proteins in the presence of the extraction buffer supplemented with ATP (1 mM); reaction mixtures were incubated during 60 min at 30 °C. After this incubation, the reactions were run in a native gel and documented under GFP light, allowing to detect Clover fluorescence and document any processing of the proteins.

The results of the native gel, the fluorescence quantification and the experimental design are presented in Figure 22. For the rosette extract, it was just tested with L1 and L2 since the objective is to verify the UFDCt cut of L1 and L2 only in the presence of siliques extract (which should contain the target protease).

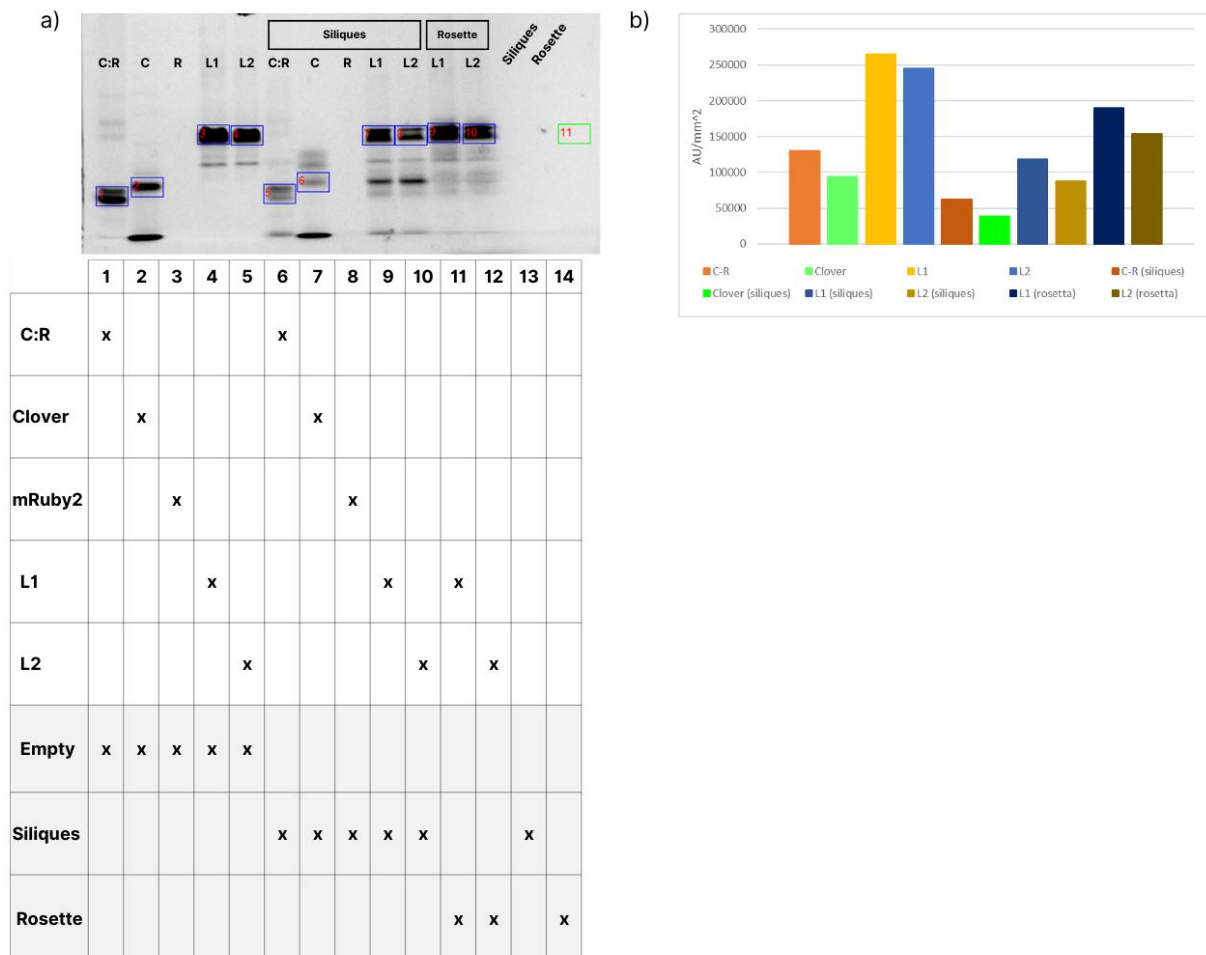


Figure 22. Results a) Native gel 1,5 mm of all proteins with and without plant extracts, b) quantification of a).

As can be observed in Figure 22 a) the L1 and L2 are cleaved in the presence of the proteins extracted from the siliques, since a strong band appeared after the digestions that did not correspond

to the L1 or L1 main bands. Interestingly, these bands were not observed in L1 and L2 under mock reactions but appeared as a weaker signal in the rosette extracts. Additionally, when comparing the fluorescence in L1 and L2 unprocessed bands from siliques and rosette extract (Figure 21 b)), a clear decrease is found at the L1 and L2 from siliques, which correlates with the additional processed band. Although these results cannot prove a differential processing between siliques and rosette extracts, they indicate that the cleavage is stronger in siliques extracts. This could be either due to a higher accumulation of the target protease in siliques extracts, or different types of proteases that can target UFDcT domain in different plant extracts.

However, whether the observed cleaved band in L1 and L2 correspond to Clover or Clover-UFDcT was impossible to discern in this native gel. In fact, the two options would be possible, but with different implications: if it is Clover-UFDcT what was detected, the cleavage was most likely at the UFDcT domain (as expected). Nonetheless, if the band does not contain the UFDcT domain, the cleavage most likely occurred at some unspecific cleavage point in the Clover protein.

Hence, a Western Blot was planned with L1 and L2 proteins and the control, UFDcT from *A. thaliana* (kindly provided by previous lab members) under the same conditions as the previous native gel. This would allow to detect the presence of the UFDcT in the different reactions and discern whether it is found linked to the processed Clover that was observed previously. The primary antibody used was α -T7 (1:5.000), which targets the T7 tag in the UFDcT domain and secondary antibody was α -Rabbit (1:5.000).

The UFD aliquots were quantified by Bradford (Table 11).

Table 11. Bradford quantification of UFDcT stocks

	UFDcT #1	UFDcT #2
C _{average} μ g/ μ L	7 $\pm$ 0,03	7 $\pm$ 0,008

The results and experimental design are presented in the Figure 23.

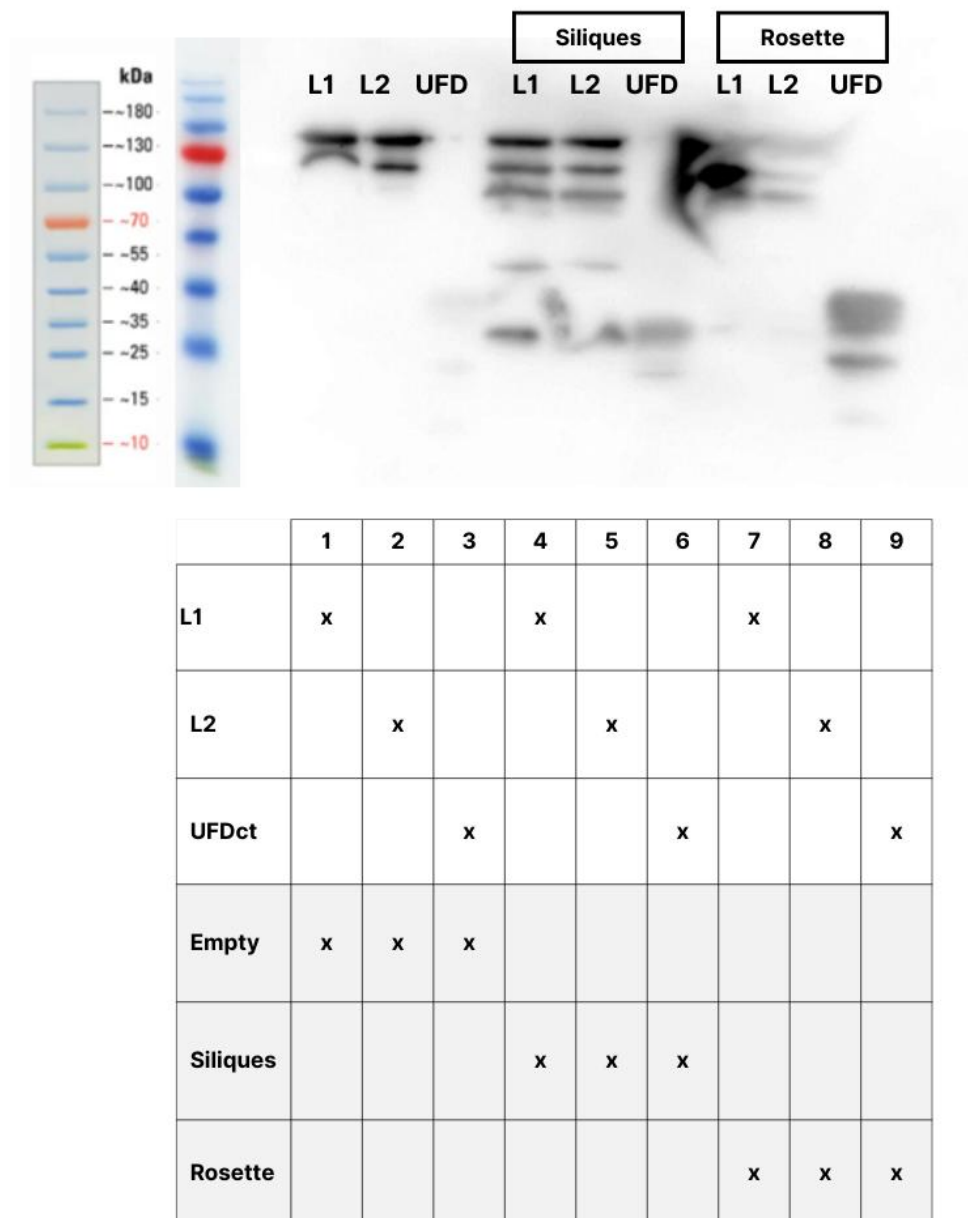


Figure 23. Western Blot membrane taken in IQ800 Amershan © (5 min exposure; α -T7 and α -Rabbit).

The reason to do this western blot was to discern whether the UFDct was linked to Clover or not. So, it is possible to conclude from Figure 23 that, although the transference was not entirely successful (presence of some spots/stains), some bands could be observed clearly in all conditions. The expected sizes of L1 and L2 are 81 kDa and from what is visible, some unexpected bands appear at about 22 and 37 kDa with siliques extract, which does not happen with the rosette extract. This could be unexpected cuttings in the Clover protein, since in the native gel (Figure 22) it is possible to

observe processing of Clover with siliques extract. This was not tried with rosette; therefore, it is not known what happens with rosette extract. Eventually Clover ligated with UFDcT should be around 60 kDa, but we observed the expected band (Clover-UFDcT) at 55 kDa in the presence of siliques and rosette extracts and not in the mock. As mentioned before, the protease that cuts the UFDcT region it is not should be present in rosette, therefore the band that appears in L1 and L2 in the presence of rosette could be another protease that cuts that region in the conditions that were defined. In turn, UFDcT expected size is 25 kDa and, in the mock, the bands are very faint, being hard to state if there were two bands or not. In the presence of both plant extracts however, the UFDcT has two bands, meaning that either the UFDcT was processed, or the sample was degraded. What's more, the UFDcT bands with rosette are stronger than in siliques, even though the same amount of protein was loaded.

A possible solution would be adding Clover and Clover:Ruby as a control in order to see how Clover behaves with plant extracts and use all proteins with siliques and rosette and increase total weight of UFDcT (from 6,5 μ g to 13 μ g). This way, we would be able to see if the unidentified bands are clover being processed and see the UFDcT band more clearly. The antibody used would be the same as the previous experiment.

The protein quantification by Bradford of the plant extracts is presented in Table 12.

Table 12. Bradford quantification of plant extracts

	Siliques	Rosette
$C_{average}$ μ g/ μ L	$8 \pm 0,1$	$16 \pm 0,04$

The results are presented in the Figure 24.

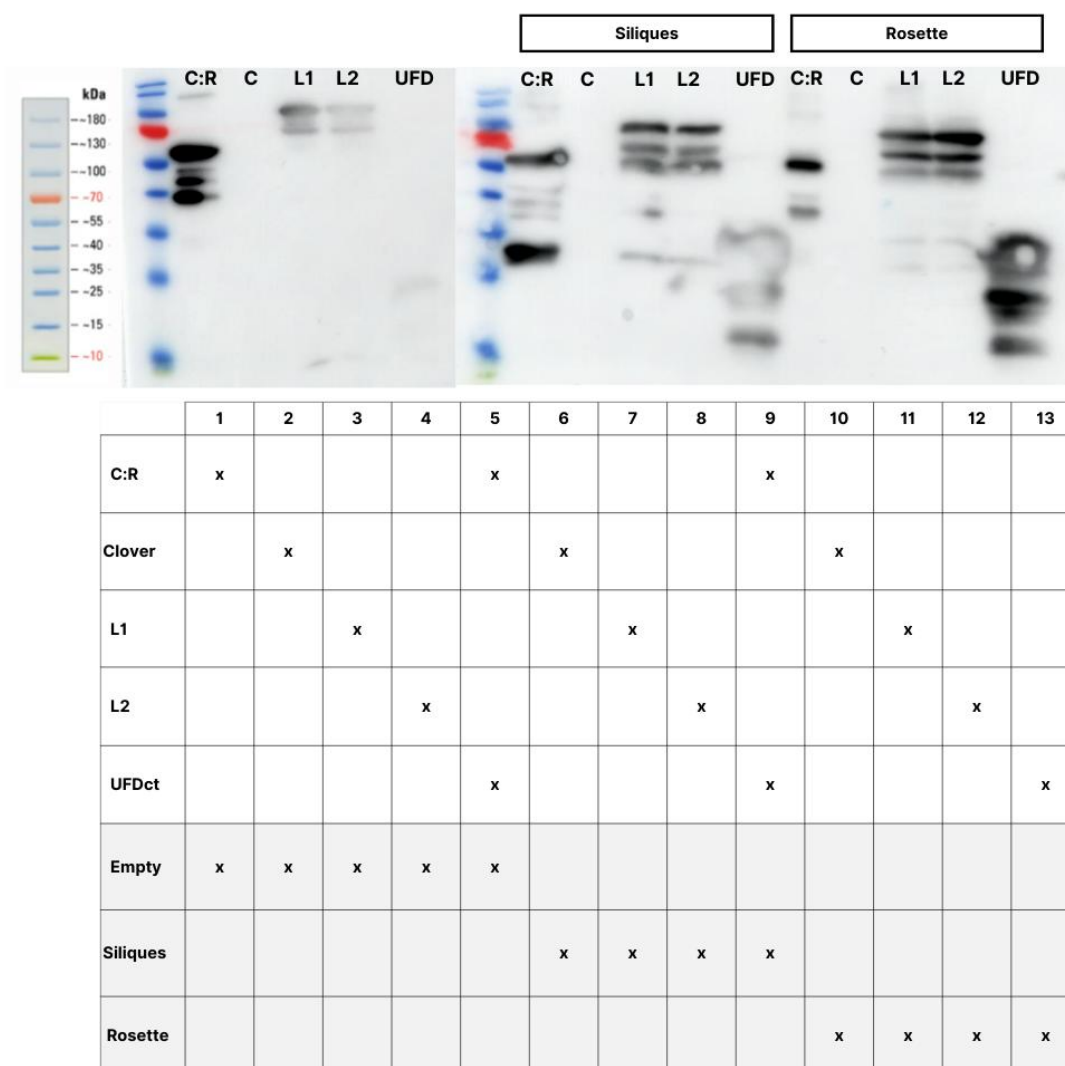


Figure 24. a) Western Blot membrane taken in IQ800 Amershan © (2 min exposure; α -T7 and α -Rabbit).

As is shown in Figure 24, this time around, Clover could not be detected and Clover:mRuby2 was most likely degraded in the mock and possibly processed in the presence of plant extract, since it is possible to see several bands that had different sized compared to the mock and between siliques and rosette. Again, there is a third unknown band at 40 kDa for L1 and L2 for the silique extract and a band at around 50 kDa for L1 and L2 for the rosette extract, which do not correspond to their original expected sizes and that does not appear in the mock. However, even though, this third band in L1 and L2 appear with both plant extracts, this band is stronger with the siliques extract. Lastly, the

UFDcT was processed in the presence of plant extract, although the transference did not allow to see these specific bands very clearly.

In the absence of Clover and the unclear digesting of UFDcT with plant extracts, it was necessary to understand what it is happening. First, the membranes were stained with Coomassie (Figure 25) in order to see the protein bands.

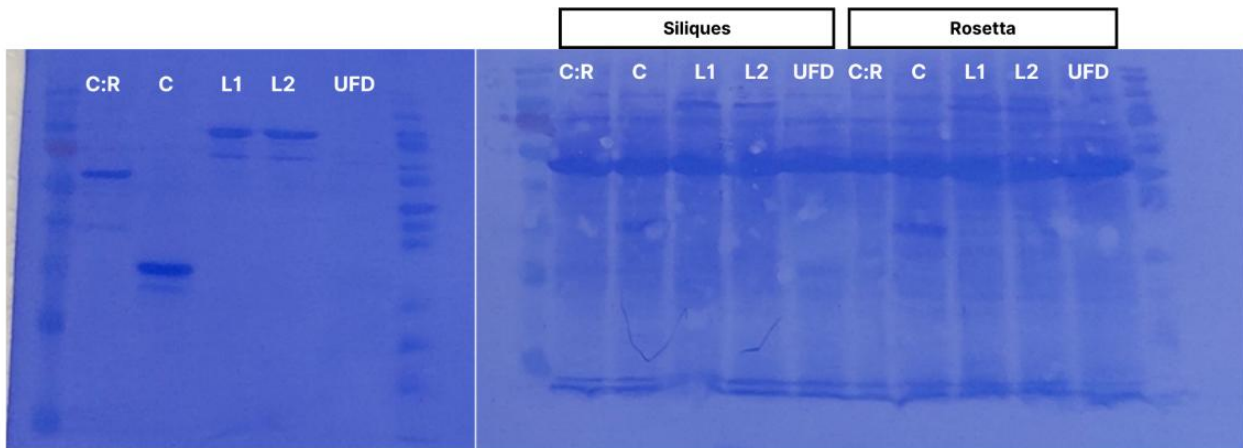


Figure 25. Western Blot membranes stained with coomassie.

It was possible to see Clover, after the staining in both membranes which indicates that the terminal T7 is either being cleaved or not active (Figure 25). Additionally, was impossible to see the UFDcT band in the mock that could indicate a problem with the sample.

After some consideration, another western blot plan was designed to try to improve the results. The reasoning behind this plan was to supervise the protein stability with and without plant extract of the controls and the target protein, testing it with two different plant extracts, one where we expect cleavage (siliques) and the other not (rosette). It was hypothesized that the amount of protein loaded was too high and, to the antibodies to increase antibody specificity, the amount of protein was reduced down to 0,1 and 0,2 μg . In addition, the amount of primary antibody was also reduced from 1:10.000 to 1:20.000 and, since the UFDcT aliquot used seemed to be degraded and not suitable for a positive control, another aliquot was chosen from the lab stock. To do that, several samples were quantified and loaded to an SDS-PAGE gel and ran to ensure their quality (Figure 26).

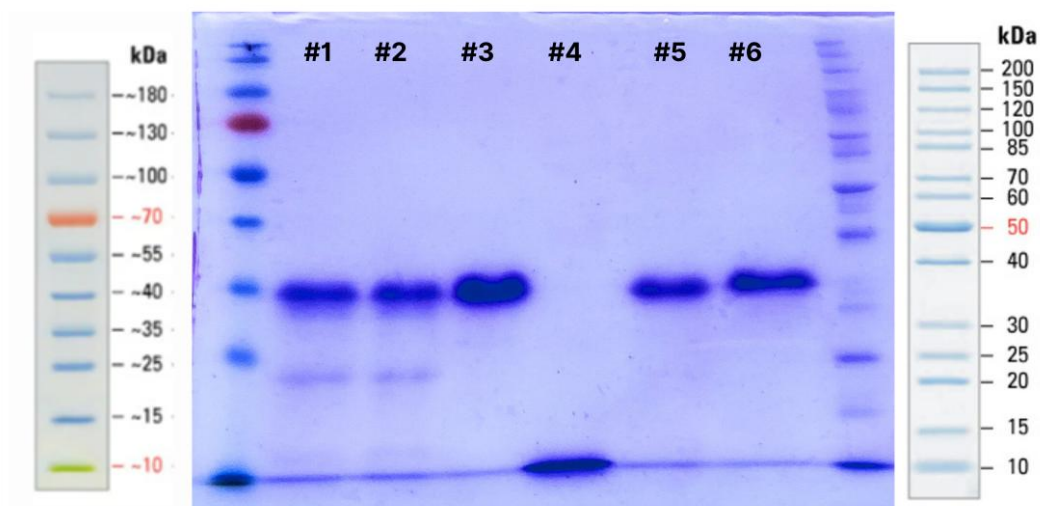


Figure 26. 12 % SDS-PAGE 1,5 mm for six samples of UFDcT (lab stock).

The UFDcT chosen was #6 based on the clarity of the band, the absence of degradation and also because it had the highest volume. With this, it was possible to repeat the western blot as described earlier. Firstly, the plant extracts were prepared and their quantification by Bradford of the plant extracts is presented in Table 13.

Table 13. Bradford quantification of plant extracts

	Siliques	Rosette
C_{average} $\mu\text{g}/\mu\text{L}$	$8 \pm 0,01$	$16 \pm 0,08$

The membranes were first revealed with Amersham™ (ECL™ Prime Western Blotting Detection Reagents) but the bands were not very clear (Appendix F, Figure 1F), therefore it was revealed again with SuperSignal™ West Femto (Figure 27).

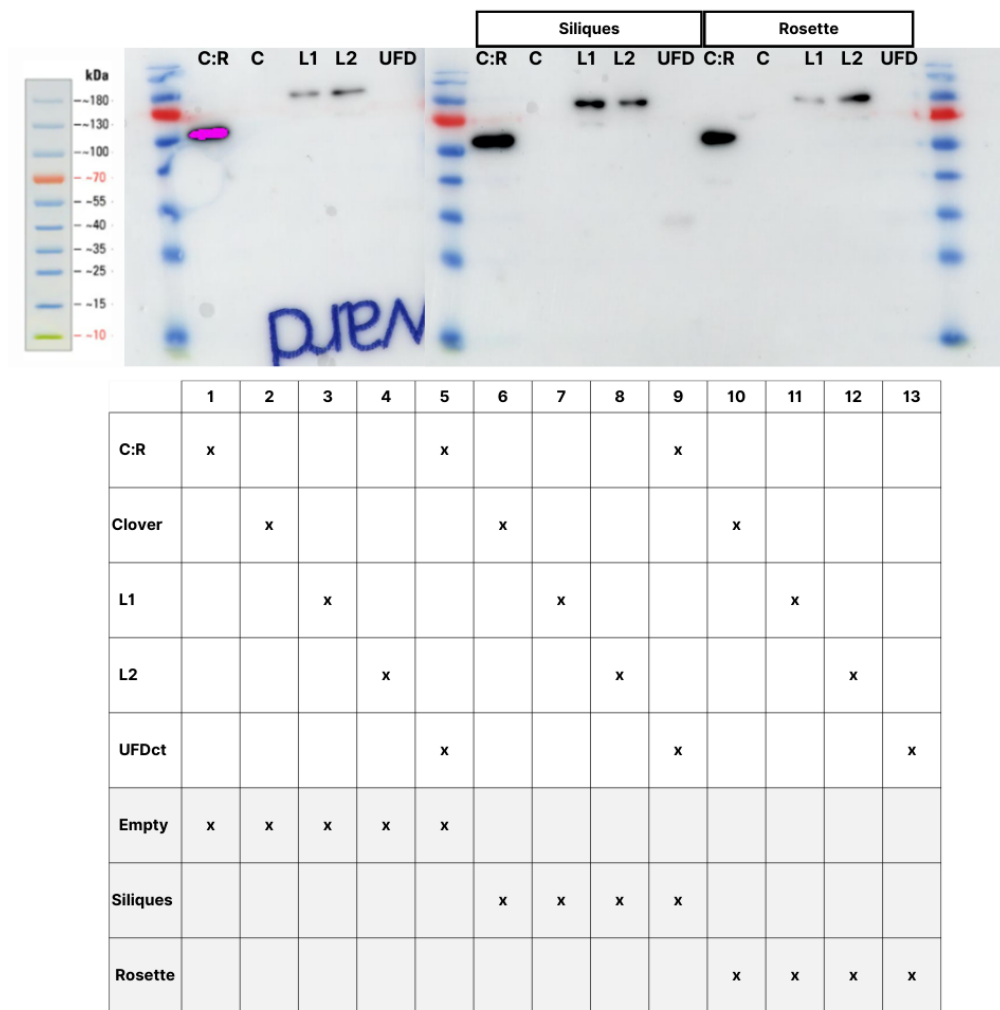


Figure 27. Western Blot membranes taken in IQ800 Amershan © (10 min exposure; α -T7 and α -Rabbit).

In Figure 27 was not possible to see the cut of L1 and L2, neither Clover nor UFDct, but the new antibody concentration adjustments worked since we were able to see the protein bands at their expected size, except UFDct that it was not detected. Also, with the decrease of protein concentration compared with the last experiment, it allowed the antibody to act more specifically only on the target protein, reducing the number of unspecific bands that are visible. To ensure that the fact that it was not detected L1 and L2 cut in siliques and the UFDct in all conditions was due an experimental issue, the experiment was repeated. However, in case the issue was the antibodies used, a second experiment was done using as primary antibody the α -His tag (1:5.000), which targets the His tag, and its secondary antibody is α -Mouse (1:20.000). Since all proteins have a Hist tag, it would be possible to detect all proteins at once.

Another possible explanation for the previous results is that the transfer buffer was damaged and, therefore a new one was made to ensure that it was freshly made. The results are presented in the Figure 28.

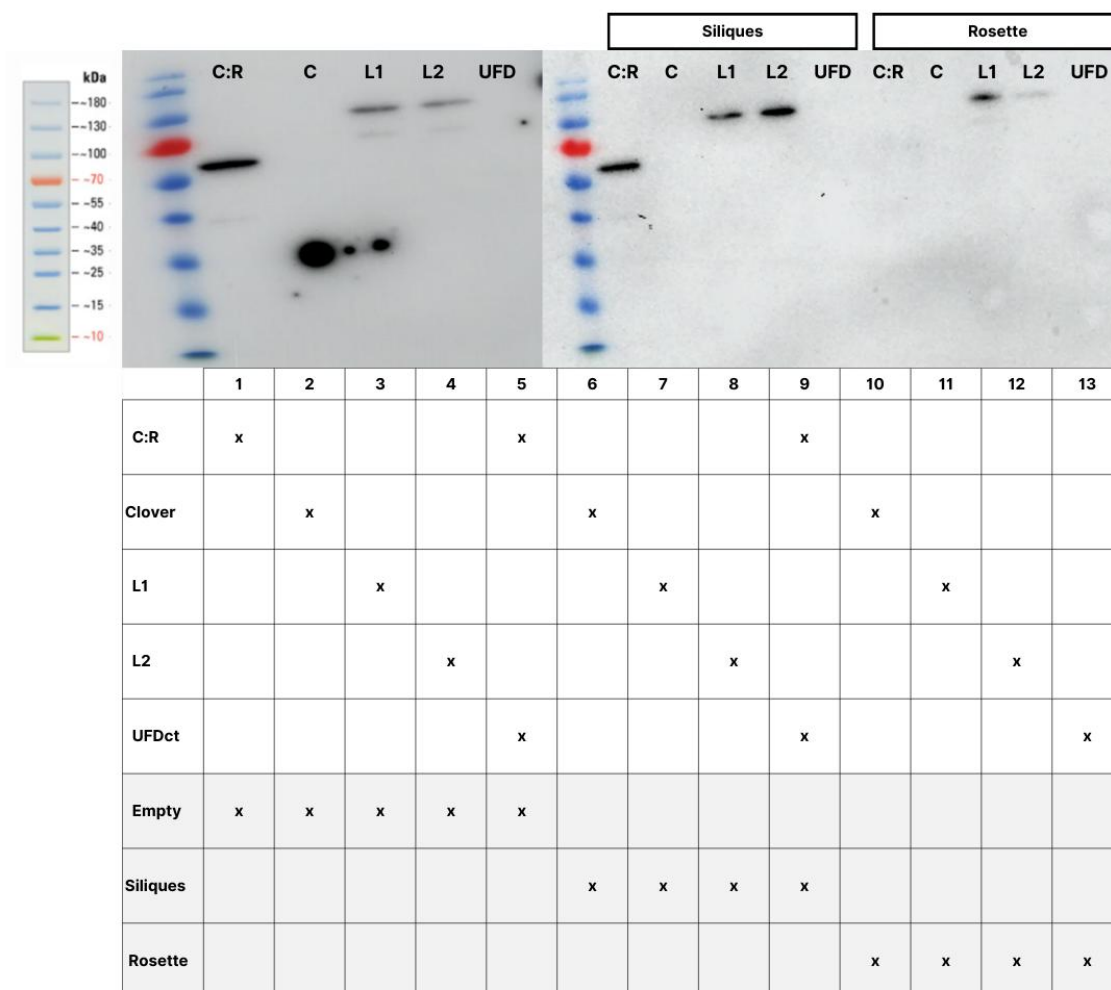


Figure 28. Western Blot membranes using α -T7 taken in IQ800 Amersham © (5 min exposure; α -T7 and α -Rabbit).

The results of the second experiment are presented in the Figure 29.

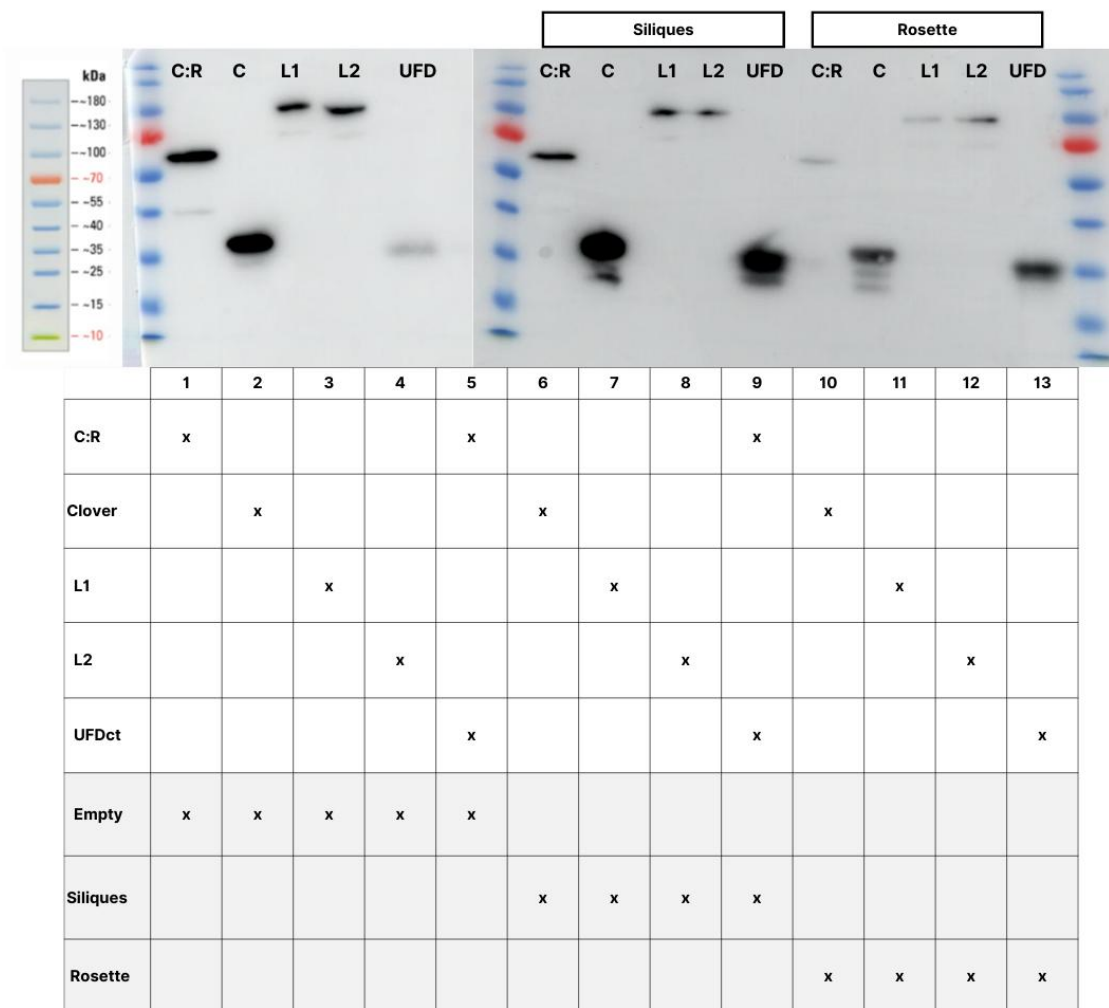


Figure 29. Western Blot membranes using α -His tag taken in IQ800 Amershan © (50 secs exposure; α -His tag and α -Mouse).

The results of the first experiment using anti T7 (Figure 28) were similar to the first attempt (Figure 27), confirming that the problem in band detection was probably not derived from a damaged transfer buffer but rather due to problems with the antibody.

In Figure 29, however, it was possible to detect all proteins at their expected sized with the α -His tag, but it was not possible to see the cut in L1 and L2 with neither siliques nor rosette, however the bands appear fainter with rosette than in siliques, that could be due poor transference of the membrane. In these conditions, it is possible to detect Clover as expected, however Clover is being processed in the presence of the plant extracts since it has several bands that do not appear in the mock. This could be due some unidentified protease cutting Clover. Clover:mRuby2 appear in the

mock and in presence of the plant extracts with no degradation, but it does seem fainter with rosette (possibly due transference). Finally, it was able to detect UFDCt and it was possible to see a cut in the presence of siliques and not in rosette.

In conclusion is that, comparing Figures 28 and 29, Clover T7 is being processed since with the incubation with α -T7 the bands do not appear, but with α -His tag they do. This is an issue because Clover is a control and should behave in the same way in absence and presence of both plant extract. To finish, the new pair of antibodies tested were able to obtain better results than the previous antibodies. It would had been interesting to have tested another antibody that targets the UFDCt (α -SAE2). With this antibody it would be possible to detect only the UFDCt region and if it is being processed or not by the different plant extracts.

6. Discussion

6.1. Protein Production

In the protein production step, it was possible to produce between 8 and 29 mg/mL of the recombinant protein that was more than sufficient for the experiments done during the development of this project, since it allowed to do several one-use protein stocks. However, the mRuby2 production showed an issue since in both protein productions, the purified protein had a double band. Possibly this happened because the bacteria strain was unable to produce the pure mRuby2 and some kind of processing alternative splicing could have happened.

6.2. Set-up FRET conditions

When the proteins were tested in Clover and mRuby2 conditions (excitation at 480 nm and emission at 530, and excitation at 530 nm and emission at 625 nm, respectively) they behaved as expected, except Clover and Clover+mRuby2. However, in FRET conditions (excitation at 480 nm and emission at 530 nm) in the presence or absence of BSA, the proteins did not behave as expected, especially for Clover+mRuby2 and L2. In reality, in every experiment, different results were obtained, which complicated to extract any conclusions: firstly Clover+mRuby2 has too high comparatively with the other, therefore, other conditions were tested with a higher range of excitations and emissions; then, Clover+mRuby2 had a lower signal (close to mRuby2) but L2 had a very high signal in comparison with the other proteins in all conditions and the experiment was repeated; finally, in the last attempt Clover+mRuby2 had again the highest signal in most of the conditions and the mRuby2 signal was close to the other proteins instead of being lower or even close to zero.

Due to the disparity of results obtained in the same conditions and with the same proteins, it was not possible to set-up the FRET conditions. However, the FRET signal detected in the presence of BSA (0,1 %) is higher than in its absence, which could indicate that in the presence of a low concentration of BSA improved the experiment.

6.3. Set-up of alternative method

Since the FRET conditions were not possible to define, another strategy was developed: nondenaturing protein electrophoresis (native gel). In this second approach the conditions were set: native gel with 7,5 % acrylamide and a stacking gel of 3,75 %, ideal protein concentration of 2,5 μ M, ran at room temperature with cold running buffers (4 °C) with SDS and exposed to a GFP filter.

After this and the FPLC purification of the proteins (to improve the experiment discarding unwanted degradation), the following step was validating the assay. To do so, proteins were extracted from plant tissue (rosette and siliques) and incubated with the recombinant proteins to a 35:1 ratio in the presence of ATP. After loading the samples in the native gel, ran the gel and expose it at GFP light, it was possible to detect that only in the presence of siliques L1 and L2 was cut. To ensure that this cut was done by the target protease at the correct site (UFDCt), the digestion was repeated and followed by the western blot protocol. Firstly, with the primary antibody α -T7 and secondary antibody α -Rabbit, it was not possible to detect the cut that corresponds to the expected band size. The protein concentration was doubled in order to test if it was a concentration issue and, once again, the cut was not detected. Then, we realized that the increased in protein amount could cause a loss in the antibody specificity, therefore the protein concentration was reduced to be between 0,1 and 0,2 μ g and the primary antibody was reduced to half. This improved the visualisation of the membrane since no unspecific bands appeared, however, the cut in L1 and L2 in siliques was not visualized even though it was observed in the UFDCt only in the presence of siliques. Finally, in the last trial the antibodies were changed, the primary antibody would be α -His tag and the secondary α -Rabbit, but the cut in L1 and L2 was not detected.

Thus, although we see a cut in L1 and L2 in the native gel analysis, the same was not observed in the western blot, not being able to validate this assay. However, this does not mean that the cut in the UFDCt region in L1 and L2 by the target protease did not happen, it means that with the chosen set-up we were not able to detect it.

6.4.Future prospects

The work developed in this dissertation can be improved in very different ways: changing the method or changing strategy.

In relation to the native gel technique, it could be improved by using other antibodies (α -SAE2 and α -Rabbit) or using other fluorescent proteins. In fact, there is other more efficient fluorescence protein pairs that could work for either the native gel protocol or for the FRET assay. The pairs TagBFD-TagGFP2 and TagGFP2-TagRFP are two examples of high effective FRET pairs that can be used as an alternative to Clover and mRuby2 (Evrogen JSC, 2022).

Another strategy that could improve the FRET assay is measuring the quenching instead of the FRET signal (Jiang, *et al.*, 2019), represented schematically in Figure 30. Quenching of

fluorescence is a process that happens when an excited molecule loses its energy by interacting with other molecules (quenchers) in close proximity, decreasing its fluorescence intensity (Mark, *et al.*, 2011). Even though, quenching is something that can affect the FRET measuring, since some fluorescence is being lost, in this case it is a very interesting approach with the advantage that it can be used in a broader way, *i.e.*, the donor can be a fluorophore or a quencher.



Figure 30. Scheme of the quenching process in FRET assay (Adapted from (Jiang, *et al.*, 2019)).

One of the encountered problems was the protein degradation due to several uses. This can be avoided by using chemically stable fluorescent proteins such as hyperfolder YFP (hYFP) that is a monomeric variant of YFP and large Stokes shift monomeric GFP (LSSmGFP) (Campbell, *et al.*, 2022).

Lastly, an alternative approach to the methods previously described could be the NanoLuc Binary Technology or NanoBiT. NanoLuc is a small luciferase (19 kDa) that can produce bright and sustained luminescence, with high physical stability and tolerance to temperature, pH and urea. This enzyme can be expressed in or outside of cells and its small size allows the fusion of protein tags (Hall, *et al.*, 2012). The NanoLuc is divided in two parts weakly conjugated: a smaller fragment called Small BiT (SmBiT) and a bigger fragment called Large BiT (LgBiT). The luciferase is activated when the fused protein interacts with other proteins, producing bioluminescence (Rozbeh & Forchhammer, 2021). In Figure 31 is presented a scheme of this reaction. Therefore, there are still a lot of strategies

that can be used in order to develop and improve a molecular and imaging tools to study SUMO conjugation in plant.

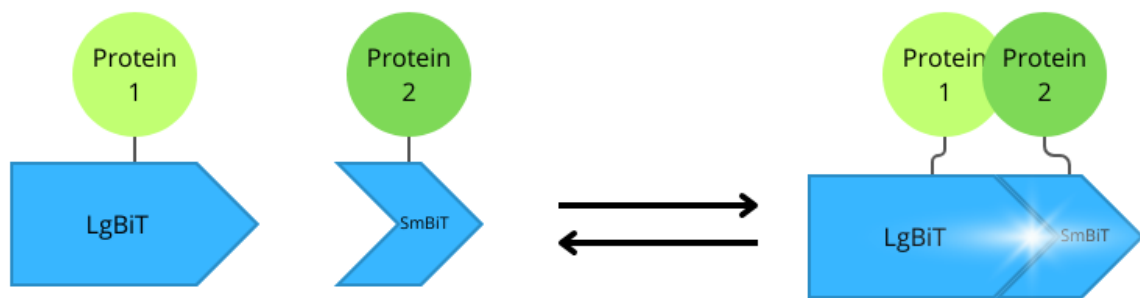


Figure 31. Representation of NanoBiT (Adapted from (Calabretta, *et al.*, 2021)).

7. Conclusion

This thesis was performed with the purpose of establishing a high throughput assay to identify the processing of the SAE2 C terminal domain in *Arabidopsis thaliana*, which is crucial for SUMOylation.

The conclusions that could be obtained were:

- It was possible to produce a suitable amount of the recombinant proteins, even though the purification of mRuby2 was not ideal (presence of double band).
- It was impossible to establish a FRET assay due to unforeseen technical problems with the proteins used.
- The purification of the FPLC was able to remove some degradation of the protein samples, however not all of it, especially proteins that contained double bands that were very close to the desired protein.
- Detecting the proteins with a native gel was successful and would be a promising approach to detect SAE2 processing.
- Processing of the C-terminal domain of SAE2 was documented under silique and rosette extracts, although the differential expression in tissues nor the specificity of the protease could not be confirmed.
- Clover was processed in the presence of plant extracts (siliques and rosette); therefore, it would be ideal to change it for another fluorophore that is not processed in the designed conditions.

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Appendix

A. Buffer composition

The reagents composition used in the different experiments is presented in the Table 1A.

Table 1A. Reagent composition for each experiment

Experiment	Reagent	Composition
Heat shock transformation of <i>Escherichia coli</i> cells	LB agar	For 1L: 5 g Yeast extract 10 g Peptone 10 g NaCl 12 g Agar
	LB broth	For 1L: 5 g Yeast extract 10 g Tryptone 10 g NaCl
Induction of protein expression and batch purification (small scale)	2× YT	For 1L: 10 g Yeast extract 16 g Tryptone 5 g NaCl
	Lysis buffer	20 % (w/v) Sucrose 50 mM Tris-HCl (pH 8) 350 mM NaCl 10 mM MgCl ₂ 1 mM β-mercaptoetanol 0,1 % (v/v) Igepal CA-630 (NP-40) 100 µg/µL DNase 1 µg/mL Lysozyme 1mM PMSF 1 µg/mL Leupeptin 1 µg/mL Pepstatin 20 mM Imidazole
	Equilibrium buffer	50 mM Tris-HCl (pH 8) 350 mM NaCl 20 mM Imidazole 1 mM β-mercaptoetanol
	Elution buffer	50 mM Tris-HCl (pH 8) 350 mM NaCl 300 mM Imidazole 1 mM β-mercaptoetanol
FRET assay	B7 buffer (without BSA)	25 mM Hepes (pH 7,5) 150 mM NaCl
FPLC fractionation	Buffer for FPLC (compatible with Superdex © 75 and 200 16/6 columns)	50 mM Tris-HCl 100 mM NaCl 1 mM β-mercaptoetanol

Table 1A. Reagent composition for each experiment (continuation)

Experiment	Reagent	Composition
SDS-PAGE gel electrophoresis	4x Loading buffer	0,25 M Tris 8 % SDS 0,08 % Bromophenol blue 40 % Glicerol 4 % DTT
	10x Running buffer	1,92 M Glycine 0,25 M Tris 1 % SDS
	12 % acrylamide separation gel (2 gels)	6 mL Acrylamide/ bis 37.5:1, 30% Bio-Rad © (161-0158SP3) 3,74 mL Tris-HCl (4×) 64,2 µL APS 10 % 12,84 µL TEMED 5,18 mL Deionized water
	10 % acrylamide separation gel (2 gels)	5 mL Acrylamide/ bis 37.5:1, 30% Bio-Rad © (161-0158SP3) 3,74 mL Tris-HCl (4×) 64,2 µL APS 10 % 12,84 µL TEMED 6,18 mL Deionized water
	4 % acrylamide stacking gel (2 gels)	1,06 mL Acrylamide/ bis 37.5:1, 30% Bio-Rad © (161-0158SP3) 1,99 mL Tris-HCl/SDS pH 8,8 (4×) 39,9 µL APS 10 % 7,99 µL TEMED 4,95 mL Deionized water
	Coomassie staining solution	0,1 % Brilliant Blue R250 30 % Methanol 5 % Acetic acid
	Destaining solution	40 % Ethanol (70 %) 3 % Acetic acid
Western Blot	Transfer buffer	25 mM Tris 193 mM Glycine To 1 L add 150 mL of Methanol
	10x TBST buffer	2 M Tris 1,5 M NaCl 1 % Tween ® 20 pH 7,6

Table 1A. Reagent composition for each experiment (continuation)

Experiment	Reagent	Composition
Non-denaturing protein gel electrophoresis	5x Sample buffer	0,3 M Tris-HCl (pH 6,8) 0,03 % Bromophenol blue 51 % Glycerol
	Electrophoresis buffer (1L)	3 g Tris 14,4 g glycine pH 8.3
	Acrylamide separation gel (2 gels)	5 mL Acrylamide/ bis 37.5:1, 30% Bio-Rad © (161-0158SP3) 5 mL 1,5 M Tris-HCl pH 8,8 100 µL APS 10 % 10 µL TEMED 9,9 mL Deionized water
	Acrylamide stacking gel (2 gels)	1,5 mL Acrylamide/ bis 37.5:1, 30% Bio-Rad © (161-0158SP3) 3 mL 0,5 M Tris-HCl pH 6,8 100 µL APS 10 % 10 µL TEMED 7,4 mL Deionized water
Plant protein extraction	Plant Protein extraction buffer	50 mM Tris-HCl 150 mM NaCl 0,20 % Triton 5 mM EDTA 1 mM PMSF 1 µg/mL Pepstatin 1 µg/mL Leupeptin 2 mM NEM 10 M Iodoacetamide

B. Plasmids constructs

The plasmid constructs that correspond to Clover:mRuby2 protein is presented in Figure 1B.

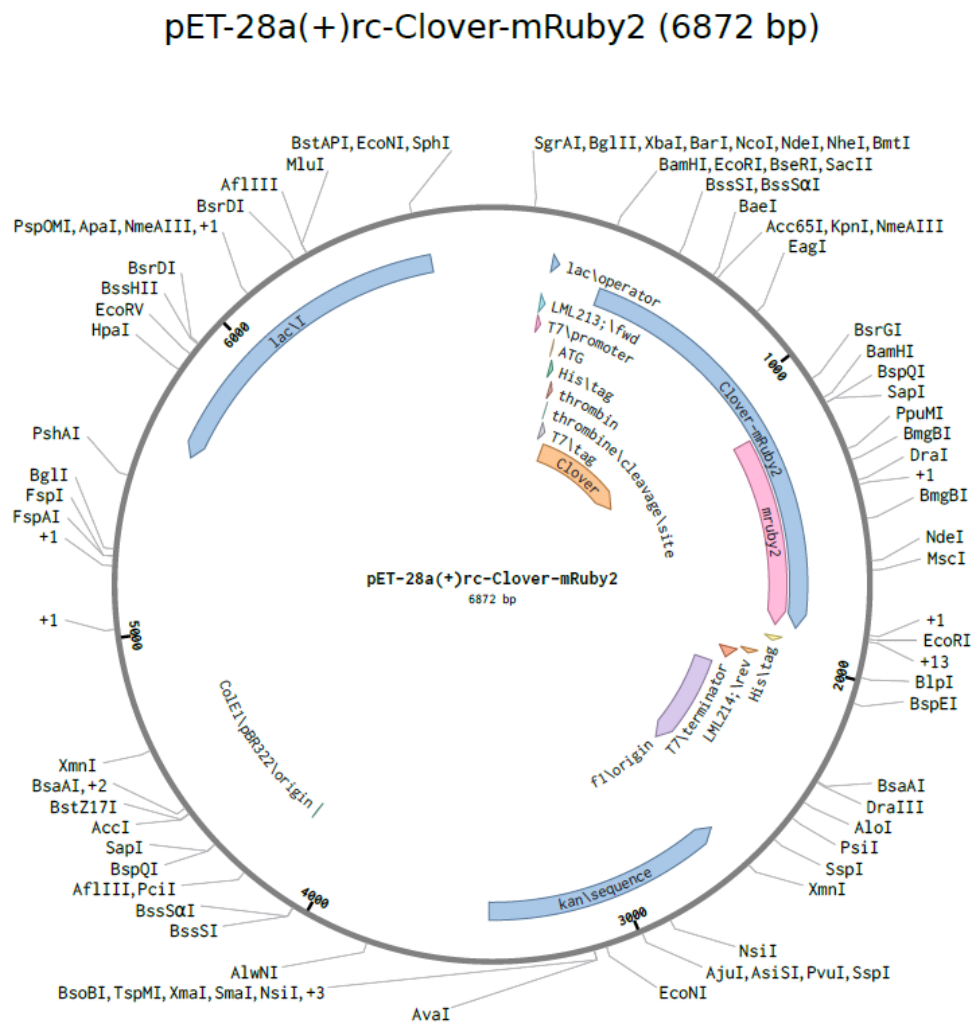


Figure 1B. Protein plasmid construct of Clover:mRuby2.

The plasmid constructs that correspond to Clover protein is presented in Figure 2B.

pET28a(+)_{rc}::Clover (6095 bp)

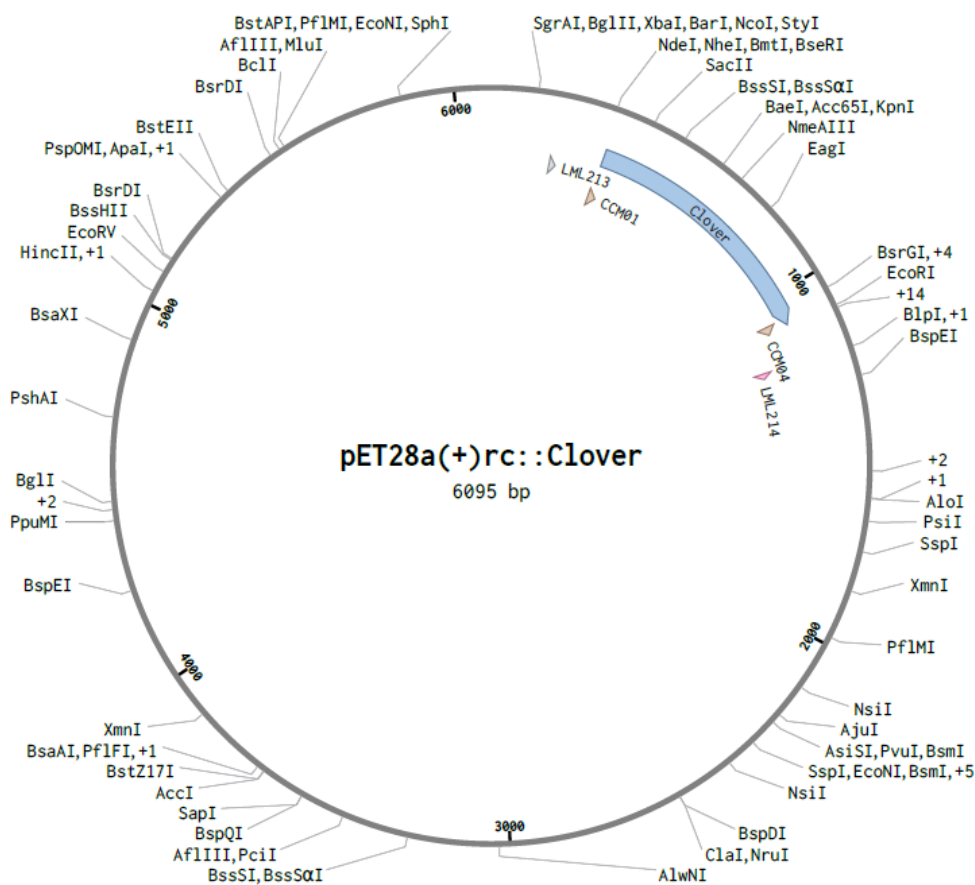


Figure 2B. Protein plasmid construct of Clover.

The plasmid constructs that correspond to mRuby2 protein is presented in Figure 3B.

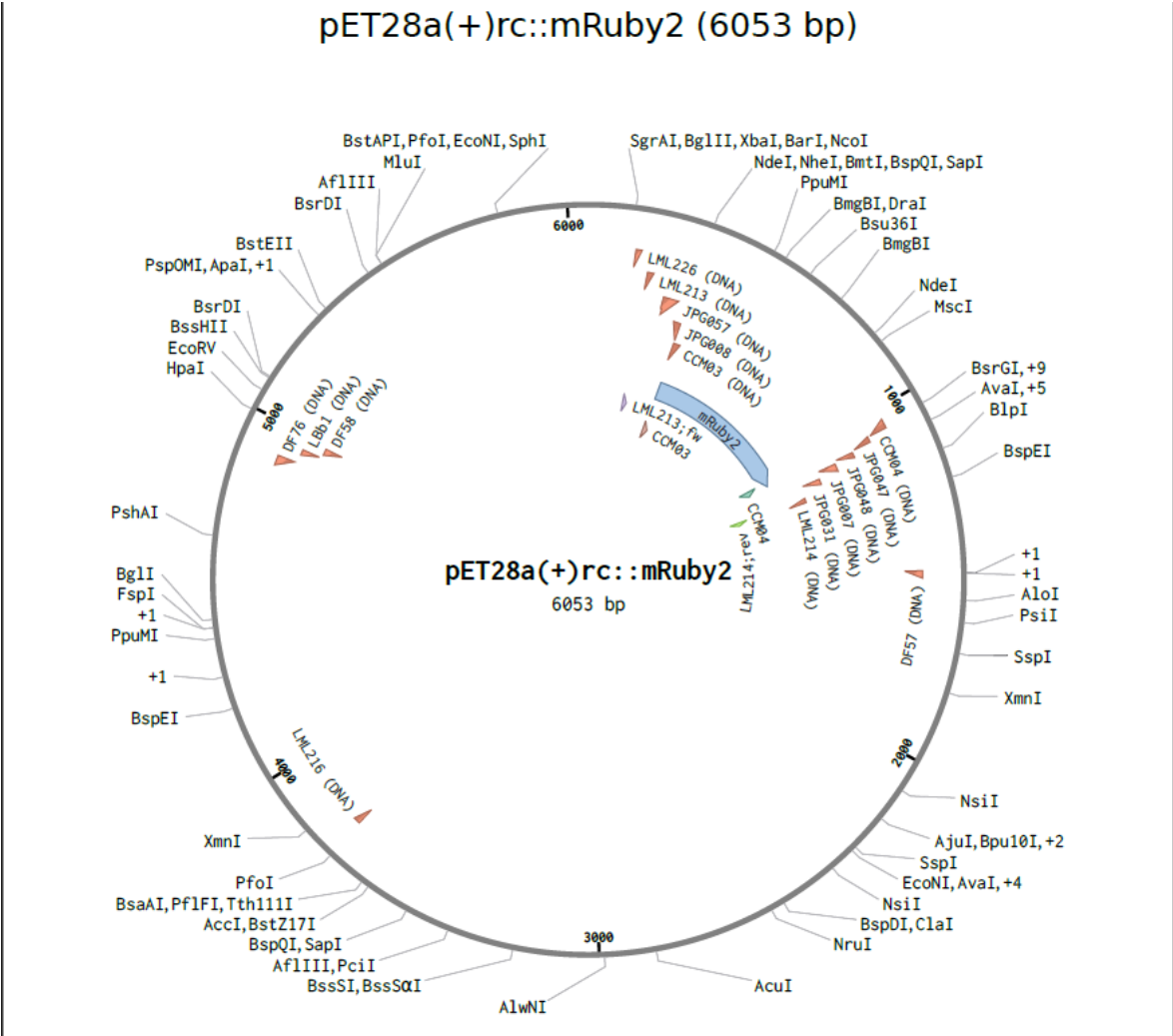


Figure 3B. Protein plasmid construct of mRuby2.

The plasmid constructs that correspond to L1 protein is presented in Figure 4B.

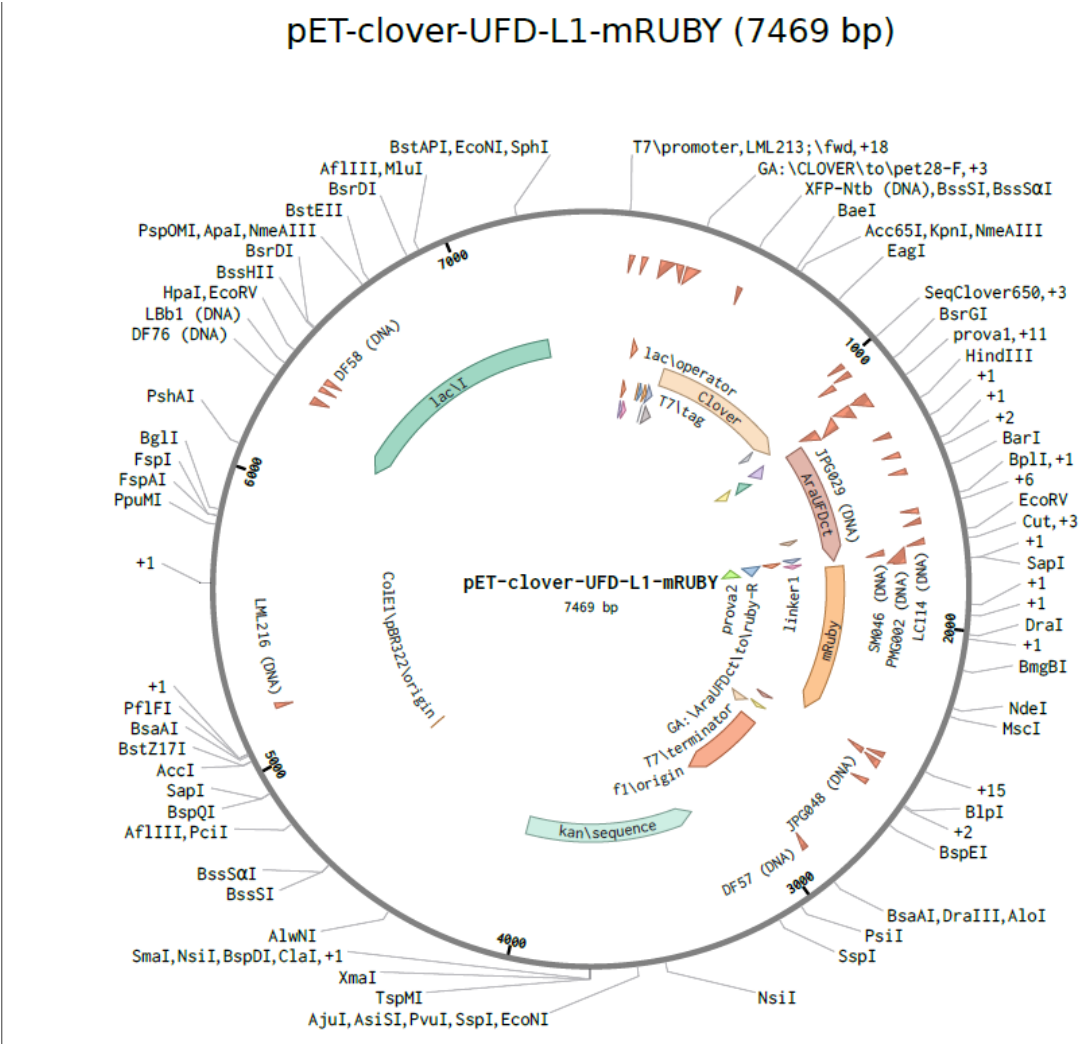


Figure 4B. Protein plasmid construct of L1.

The plasmid constructs that correspond to L2 protein is presented in Figure 5B.

pET28-clover-UFD-L2-mRU (7487 bp)

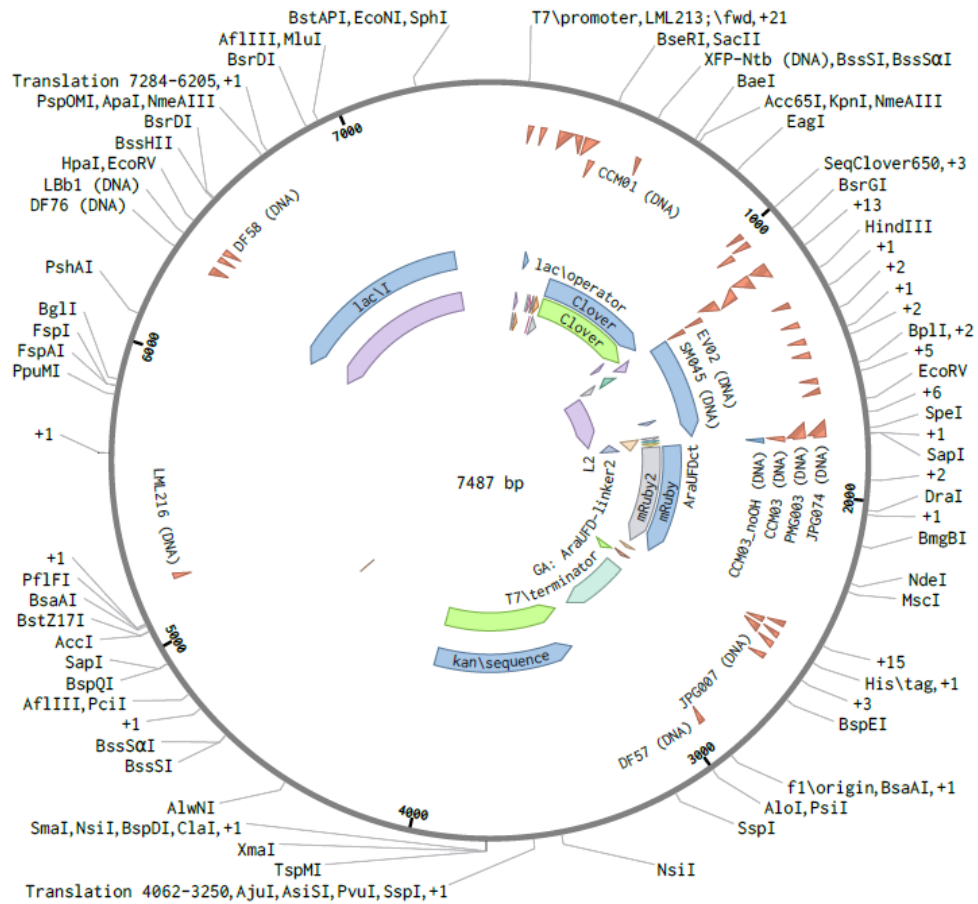


Figure 5B. Protein plasmid construct of L2.

The plasmid constructs that correspond to UFDcT protein is presented in Figure 6B.

pET-28b(+) rc-AtUBLcT (5947 bp)

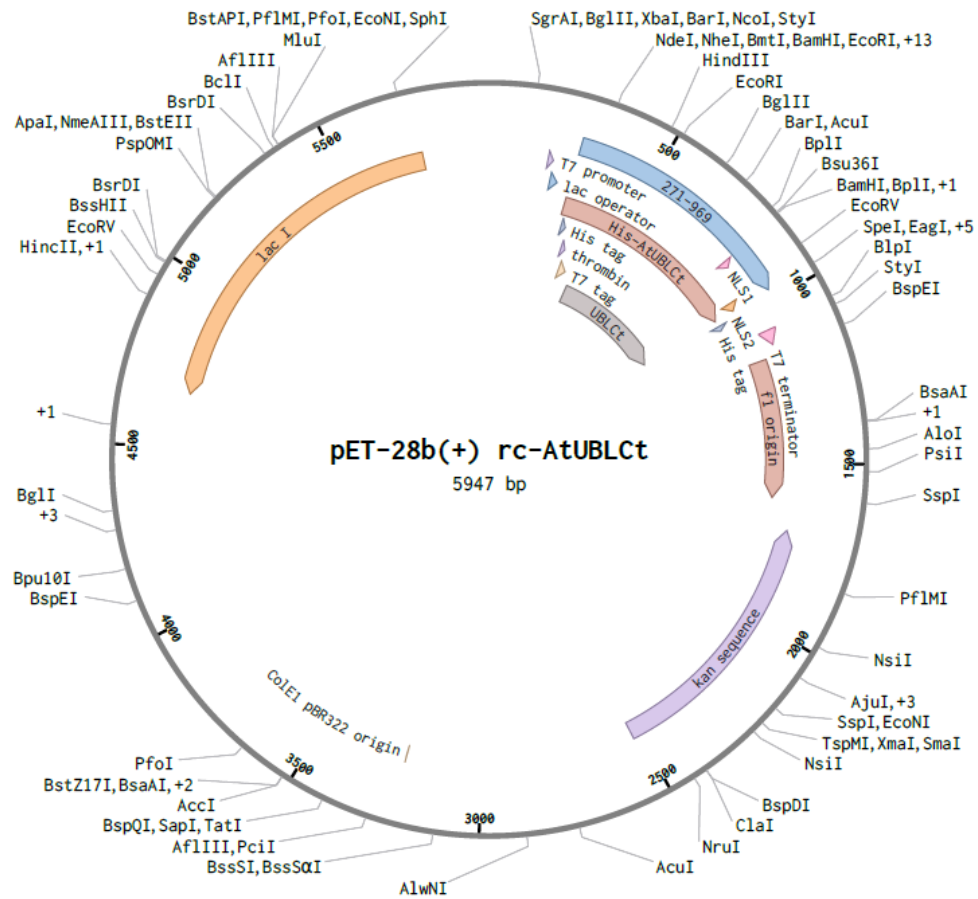


Figure 6B. Protein plasmid construct of UFDcT.

C. Bradford standard curve

The Bradford curve (Figure 1C) used in all experiments is $C, \frac{mg}{ml} = (0,8 \pm 0,9) \times Abs, 595\text{ nm}$.

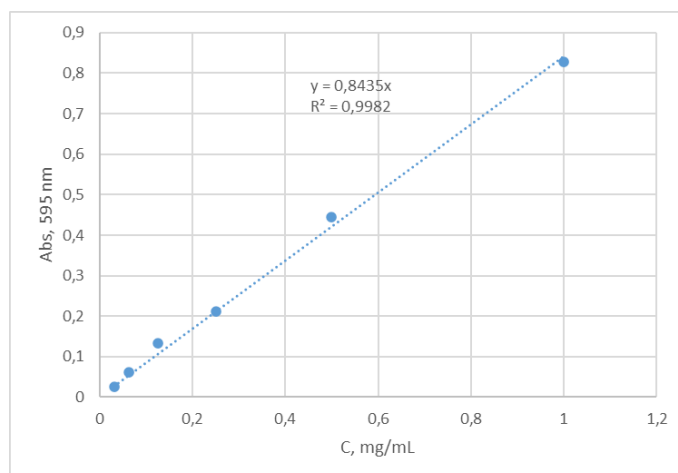


Figure 1C. Bradford curve.

D. FRET Results

The results of the FRET assay for all protein for the excitations between 470 and 500 nm and emission between 595 and 635 for the buffer B7 with 0 % BSA is presented in the Table 1D.

Table 1D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
470	595	C-R	16,7	0,8	10,8	0,8	6,4	0,3	3,0	0,3
		C	27,9	0,7	15,0	0,7	8,2	0,4	4,1	0,4
		R	4,3	0,8	2,8	0,2	3,0	0,2	1,5	0,1
		L1	44,7	0,2	35,1	0,6	10,7	0,7	5,3	0,6
		L2	118,8	13,7	57,2	2,9	27,0	0,2	14,8	0,4
		C+R	16,8	0,5	9,3	0,4	5,6	0,4	3,1	0,6
	600	C-R	18,6	1,5	10,8	0,5	6,6	0,5	3,1	0,7
		C	29,4	0,9	14,8	0,6	7,9	0,4	4,4	0,2
		R	5,2	0,4	3,2	0,3	3,1	0,3	1,7	0,1
		L1	45,1	1,2	34,9	1,0	10,3	0,7	5,4	0,6
		L2	121,4	14,8	58,3	3,3	26,8	0,7	15,0	0,4
		C+R	17,5	0,7	9,4	0,3	5,9	0,1	3,8	0,5
	610	C-R	8,1	1,1	3,8	0,5	2,7	0,2	1,3	0,2
		C	9,1	0,7	5,0	0,3	2,6	0,4	1,3	0,2
		R	2,4	0,3	1,4	0,0	1,4	0,2	0,7	0,1
		L1	15,0	0,2	12,2	0,8	3,7	0,2	2,2	0,2
		L2	40,1	5,7	18,8	1,7	8,4	0,5	5,8	0,2
		C+R	6,3	0,3	3,5	0,1	2,2	0,3	1,3	0,3
	625	C-R	9,8	0,1	5,7	0,3	3,7	0,1	1,7	0,1
		C	10,4	0,2	5,2	0,4	3,3	0,2	1,8	0,3
		R	3,5	0,7	2,4	0,2	2,1	0,1	1,1	0,2
		L1	17,7	0,8	14,7	0,6	4,5	0,5	2,6	0,2
		L2	49,2	6,3	23,4	1,4	11,5	0,5	7,4	0,5
		C+R	7,8	0,8	4,5	0,3	3,2	0,5	1,8	0,3
	635	C-R	5,1	0,5	2,7	0,3	1,5	0,2	0,9	0,4
		C	4,0	0,2	2,2	0,1	1,3	0,3	0,6	0,1
		R	1,8	0,4	1,1	0,2	1,0	0,1	0,6	0,1
		L1	7,5	0,2	7,0	0,5	2,0	0,0	1,1	0,1
		L2	20,0	3,4	9,4	0,6	5,0	0,2	3,4	0,2
		C+R	3,2	0,1	1,8	0,0	1,4	0,1	0,8	0,3

Table 1D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
480	595	C-R	22,5	0,5	11,7	0,7	7,0	0,3	4,0	0,3
		C	35,1	1,2	17,4	0,3	9,6	0,6	5,0	0,5
		R	5,7	0,3	4,0	0,2	2,8	0,2	1,8	0,2
		L1	52,8	0,0	41,0	1,1	11,5	0,3	6,2	0,4
		L2	149,1	19,3	66,4	4,2	30,2	0,5	16,1	0,4
		C+R	20,2	0,4	10,5	0,3	6,9	0,2	3,9	0,7
	600	C-R	23,4	1,3	12,1	0,5	7,2	0,5	3,9	0,2
		C	35,1	0,8	17,8	0,6	10,0	0,9	5,1	0,7
		R	6,9	0,5	4,4	0,7	3,1	0,1	2,1	0,2
		L1	55,7	0,6	41,7	1,0	11,7	0,2	6,4	0,7
		L2	153,5	21,0	70,3	3,2	30,9	1,0	17,0	1,5
		C+R	20,9	0,8	11,4	0,1	7,2	0,4	4,0	0,4
	610	C-R	9,7	0,4	4,9	0,5	3,0	0,4	1,8	0,1
		C	11,1	0,4	5,5	0,3	3,3	0,0	1,7	0,1
		R	3,1	0,3	1,8	0,2	1,6	0,2	0,8	0,2
		L1	20,0	0,7	14,7	0,5	4,4	0,4	2,1	0,2
		L2	52,9	6,3	24,8	1,4	10,9	0,5	6,3	0,2
		C+R	7,5	0,7	3,9	0,2	2,6	0,3	1,4	0,2
	625	C-R	13,0	0,5	6,4	0,4	3,7	0,5	2,4	0,1
		C	13,1	0,8	6,7	0,4	4,0	0,2	2,1	0,2
		R	4,5	0,2	2,7	0,3	2,2	0,0	1,4	0,3
		L1	24,7	0,5	18,8	0,4	5,4	0,2	2,9	0,4
		L2	64,2	8,6	30,5	1,5	14,0	0,1	8,1	0,9
		C+R	9,2	0,1	5,2	0,0	3,3	0,4	2,0	0,4
	635	C-R	6,5	0,6	3,3	0,2	2,2	0,1	1,1	0,2
		C	5,8	0,2	3,1	0,1	1,6	0,2	1,0	0,1
		R	2,2	0,3	1,4	0,1	1,1	0,2	0,6	0,1
		L1	11,1	0,5	8,7	0,2	2,3	0,2	1,2	0,3
		L2	28,7	3,5	13,5	0,3	5,8	0,0	3,8	0,5
		C+R	4,5	0,1	2,4	0,2	1,7	0,2	1,1	0,2

Table 1D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
485	595	C-R	24,0	1,7	11,0	0,5	6,8	0,2	3,8	0,2
		C	36,7	0,3	17,8	0,7	9,3	0,4	5,3	0,1
		R	6,5	0,2	3,7	0,3	2,6	0,3	1,9	0,2
		L1	58,5	0,6	45,0	0,6	11,4	0,8	5,5	0,3
		L2	163,7	22,1	73,0	4,5	31,6	1,8	16,5	0,6
		C+R	19,9	0,5	10,4	0,1	6,2	0,5	3,9	0,9
	600	C-R	26,2	1,4	13,3	0,4	7,4	1,0	5,0	0,5
		C	37,5	0,4	18,1	0,9	9,5	0,5	5,2	0,4
		R	7,6	0,7	4,6	0,2	3,0	0,5	1,8	0,3
		L1	64,5	2,0	47,7	0,8	12,6	0,1	6,4	0,4
		L2	174,0	24,1	78,9	4,2	34,3	0,4	18,8	0,1
		C+R	22,7	0,1	11,5	0,1	6,9	0,2	4,1	0,4
	610	C-R	11,1	1,0	5,4	0,2	3,4	0,8	2,0	0,3
		C	12,0	0,8	6,1	0,3	3,2	0,3	1,9	0,1
		R	3,6	0,3	2,2	0,2	1,5	0,3	0,8	0,1
		L1	22,2	1,4	16,6	0,4	4,4	0,4	2,2	0,1
		L2	58,4	8,2	26,7	1,0	12,2	0,3	6,7	0,2
		C+R	7,7	0,2	4,4	0,1	2,8	0,2	1,7	0,3
	625	C-R	14,9	0,8	7,5	0,2	4,1	0,6	2,9	0,1
		C	14,2	0,6	7,2	0,5	3,8	0,7	2,2	0,3
		R	5,1	0,6	3,1	0,3	2,3	0,3	1,3	0,2
		L1	27,9	0,0	19,8	0,5	5,3	0,1	2,9	0,4
		L2	71,1	8,8	32,1	1,5	14,3	0,2	8,5	0,2
		C+R	10,7	0,4	5,7	0,3	3,4	0,3	2,2	0,1
	635	C-R	7,1	0,6	3,0	0,3	1,9	0,4	1,4	0,2
		C	5,6	0,3	2,6	0,0	1,7	0,1	0,8	0,1
		R	2,2	0,2	1,4	0,3	1,0	0,2	0,7	0,2
		L1	12,0	1,0	9,5	0,4	2,5	0,3	1,2	0,1
		L2	31,2	4,5	14,5	1,1	7,2	0,6	4,2	0,4
		C+R	4,7	0,2	2,6	0,4	1,6	0,3	1,1	0,2

Table 1D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
495	595	C-R	34,2	1,2	16,6	1,3	8,5	0,6	6,1	0,7
		C	52,7	0,6	25,5	0,3	12,3	1,3	7,0	1,1
		R	9,3	0,8	5,4	0,4	3,0	0,2	1,8	0,3
		L1	90,0	0,5	64,5	1,1	15,8	0,7	7,7	1,1
		L2	244,7	32,7	107,9	4,1	43,8	0,5	22,3	0,7
		C+R	30,2	0,8	14,9	0,9	8,9	0,4	4,8	0,6
	600	C-R	38,5	1,2	18,8	1,7	10,1	1,1	7,0	0,5
		C	55,5	1,1	26,0	0,7	13,9	0,7	7,3	0,9
		R	10,3	0,8	5,8	0,3	3,5	0,7	2,2	0,2
		L1	94,3	2,7	68,7	1,3	17,1	0,2	8,1	0,8
		L2	255,5	34,8	115,4	7,2	48,7	0,5	23,1	0,5
		C+R	32,4	1,1	15,9	0,6	9,0	0,2	5,3	0,9
	610	C-R	15,2	0,9	7,2	0,4	3,9	0,2	2,7	0,4
		C	17,3	0,2	8,6	0,5	4,2	0,5	2,7	0,5
		R	4,6	0,4	2,8	0,2	1,8	0,1	0,9	0,2
		L1	33,0	0,5	23,7	0,3	6,2	0,3	2,8	0,5
		L2	87,3	12,7	39,9	2,5	17,0	0,4	8,3	0,1
		C+R	11,8	0,7	5,7	0,3	3,5	0,4	1,9	0,4
	625	C-R	21,0	0,5	10,4	0,2	5,5	0,3	3,6	0,4
		C	20,7	0,4	10,2	0,1	5,3	0,4	2,9	0,3
		R	6,8	0,6	3,9	0,4	2,5	0,2	1,5	0,2
		L1	41,3	1,1	29,5	1,4	7,6	0,3	3,4	0,2
		L2	106,6	14,2	48,1	2,9	20,4	0,7	10,2	0,5
		C+R	14,1	0,7	7,4	0,8	4,7	0,0	2,6	0,6
	635	C-R	10,1	0,7	5,4	0,2	2,8	0,3	1,8	0,2
		C	8,5	0,5	4,2	0,4	2,2	0,1	1,1	0,1
		R	3,7	0,2	2,2	0,1	1,4	0,2	0,9	0,0
		L1	18,3	0,6	13,1	0,7	3,6	0,1	1,7	0,2
		L2	46,5	5,2	21,6	1,2	9,3	0,1	4,7	0,4
		C+R	6,4	0,3	3,8	0,2	2,3	0,2	1,1	0,2

Table 1D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
500	595	C-R	40,3	1,8	19,9	1,3	11,4	0,7	7,1	0,3
		C	63,3	1,2	28,8	0,3	14,6	0,5	8,3	0,7
		R	10,7	1,0	6,3	0,5	3,2	0,2	2,2	0,1
		L1	107,4	1,2	76,2	2,2	19,3	0,9	8,5	1,2
		L2	294,4	39,4	129,7	4,5	53,3	1,2	26,0	1,0
		C+R	36,0	1,4	16,9	0,8	8,9	0,8	5,5	1,2
	600	C-R	45,6	2,7	21,6	1,3	11,4	0,4	7,9	0,6
		C	65,3	1,0	31,3	0,7	15,3	0,4	8,5	0,6
		R	12,3	1,0	7,4	0,4	3,7	0,6	2,4	0,1
		L1	113,9	3,0	82,1	1,5	19,5	0,2	8,9	1,1
		L2	315,3	39,7	138,9	7,7	56,4	1,6	27,0	1,4
		C+R	36,9	0,4	18,4	0,9	10,3	0,5	5,8	1,0
	610	C-R	17,8	0,8	9,0	1,0	4,8	1,1	3,1	0,4
		C	20,7	0,6	9,5	0,7	4,9	0,2	2,9	0,1
		R	5,5	0,7	3,3	0,3	1,9	0,2	0,9	0,2
		L1	39,9	1,4	28,2	0,3	7,3	0,6	3,2	0,3
		L2	105,9	14,9	48,5	3,1	19,3	0,3	9,7	0,5
		C+R	14,1	0,7	6,7	0,8	3,7	0,3	2,3	0,4
	625	C-R	25,3	1,4	12,5	0,2	6,7	0,7	4,2	0,5
		C	24,7	0,2	11,6	0,4	6,1	0,6	3,6	1,0
		R	8,5	0,5	4,7	0,4	2,8	0,4	1,7	0,1
		L1	50,8	1,2	35,6	1,2	9,4	0,6	4,0	0,3
		L2	129,1	17,3	58,7	3,4	24,1	0,5	12,6	0,3
		C+R	17,4	1,1	8,7	0,8	5,3	0,4	3,1	0,3
	635	C-R	10,9	0,4	5,4	0,5	3,1	0,0	2,0	0,2
		C	8,6	0,1	4,2	0,1	2,4	0,5	1,5	0,4
		R	3,7	0,3	2,3	0,3	1,4	0,4	0,8	0,1
		L1	16,4	5,8	13,9	1,1	3,6	0,1	1,6	0,1
		L2	49,4	7,6	23,0	0,1	9,3	0,4	4,9	0,3
		C+R	7,5	0,2	3,7	0,2	2,2	0,4	1,2	0,2

The results for the same conditions but with B7 with 0,1 % BSA is presented in the Table

2D.

Table 2D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
470	595	C-R	43,2	1,0	22,0	1,3	11,4	0,5	4,8	0,5
		C	61,0	2,5	32,3	0,8	17,7	0,5	13,6	0,8
		R	10,0	0,5	5,3	0,1	3,0	0,6	0,7	0,4
		L1	50,4	2,6	17,0	1,2	16,2	0,7	6,6	1,0
		L2	119,5	3,7	66,3	3,1	40,3	0,8	16,4	1,1
		C+R	26,8	2,2	26,6	0,8	14,2	0,8	5,8	0,4
	600	C-R	48,2	2,1	25,7	0,9	12,6	0,2	5,6	0,7
		C	61,2	2,5	32,4	1,1	17,9	0,5	15,6	0,8
		R	12,6	0,3	6,1	0,4	3,5	0,1	1,2	0,2
		L1	53,7	3,3	18,4	3,5	15,9	0,4	7,3	0,4
		L2	125,8	3,4	69,6	5,1	42,2	0,4	17,6	0,5
		C+R	28,9	2,5	28,4	1,2	14,2	0,3	6,4	0,5
	610	C-R	18,5	1,1	9,7	0,6	4,6	0,2	2,1	0,1
		C	18,7	1,4	10,1	0,2	5,4	0,4	6,2	0,3
		R	5,7	0,4	2,9	0,0	1,8	0,2	0,5	0,1
		L1	18,6	0,5	6,1	0,8	5,6	0,1	2,3	0,3
		L2	41,0	1,1	23,3	1,1	14,3	0,7	6,2	0,1
		C+R	9,3	0,7	9,6	0,6	5,2	0,5	2,0	0,3
	625	C-R	25,3	0,1	13,7	1,4	6,8	0,5	3,1	0,0
		C	23,2	0,8	11,8	0,4	6,8	0,5	7,8	0,1
		R	8,3	0,2	4,2	0,2	2,2	0,3	0,6	0,1
		L1	23,2	0,9	7,9	1,6	7,8	0,5	2,8	0,2
		L2	51,0	0,8	28,9	1,9	19,3	0,8	7,8	0,8
		C+R	12,8	0,5	12,2	0,2	6,4	0,5	2,9	0,6
	635	C-R	11,6	0,5	6,3	0,4	3,3	0,1	1,5	0,2
		C	9,4	0,5	5,3	0,2	2,8	0,5	4,2	0,3
		R	4,3	0,1	2,2	0,2	1,4	0,1	0,4	0,1
		L1	10,1	0,5	3,6	0,3	3,6	0,5	1,6	0,3
		L2	22,4	0,6	12,5	0,5	9,4	0,3	3,8	0,2
		C+R	5,7	0,3	5,8	0,2	2,8	0,1	1,4	0,2
		C	25,8	0,9	13,0	0,1	6,8	0,3	5,4	0,6
		R	15,4	1,1	7,8	0,3	3,8	0,4	1,9	0,1
		L1	30,2	1,4	9,4	0,3	8,7	0,2	4,3	0,2
		L2	63,0	2,0	34,0	1,5	20,0	0,6	9,4	0,8
		C+R	14,7	0,7	17,0	1,0	8,7	0,7	4,3	0,2

Table 2D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
480	595	C-R	61,0	2,7	31,0	0,2	15,3	0,1	7,6	0,5
		C	79,8	3,0	42,8	0,6	22,4	0,4	16,5	0,8
		R	17,0	0,6	8,8	0,5	4,5	0,3	1,9	0,2
		L1	69,3	2,6	20,7	1,1	20,3	0,3	9,6	0,7
		L2	160,1	1,1	87,6	0,4	50,0	0,6	22,6	1,1
		C+R	35,4	2,6	36,8	1,2	19,3	0,5	9,1	0,6
	600	C-R	66,7	1,4	34,9	1,1	16,8	1,3	8,1	0,7
		C	83,0	3,6	44,5	0,1	23,2	0,2	17,9	0,6
		R	19,9	1,1	10,2	0,4	5,4	0,6	2,5	0,1
		L1	75,0	2,0	22,0	0,8	21,7	0,3	9,9	0,6
		L2	171,2	3,1	94,5	1,4	54,0	0,6	24,3	1,1
		C+R	38,2	1,3	40,2	1,1	20,8	1,3	9,4	0,4
	610	C-R	26,1	0,3	13,1	0,2	6,7	0,6	3,1	0,0
		C	25,9	0,7	13,8	0,5	7,4	0,3	6,5	0,1
		R	9,1	0,2	4,8	0,3	2,7	0,1	1,2	0,3
		L1	25,2	1,2	7,3	0,5	8,0	0,6	3,3	0,5
		L2	56,9	2,8	31,3	0,6	18,3	0,9	7,8	0,3
		C+R	13,2	0,2	13,8	0,6	7,3	0,2	3,4	0,6
	625	C-R	36,2	1,5	18,1	0,2	9,1	0,4	4,6	0,4
		C	31,0	1,7	16,3	0,6	9,3	0,2	8,7	0,3
		R	13,0	0,8	6,5	0,1	3,5	0,4	1,3	0,1
		L1	31,4	1,0	10,5	0,9	9,7	0,6	4,8	0,3
		L2	70,0	1,4	39,5	1,7	23,3	0,3	10,6	0,9
		C+R	16,9	1,0	18,2	0,7	8,8	0,5	4,1	0,6
	635	C-R	17,3	0,2	9,1	0,2	4,2	0,1	2,1	0,2
		C	12,0	0,2	6,5	0,2	3,6	0,2	4,4	0,2
		R	6,7	0,3	3,5	0,2	1,8	0,1	0,7	0,2
		L1	14,0	0,7	4,5	0,8	4,6	0,2	1,9	0,3
		L2	30,1	1,4	17,0	0,1	11,4	0,3	4,8	0,3
		C+R	7,0	0,3	8,2	0,5	4,1	0,2	1,9	0,1

Table 2D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
485	595	C-R	70,0	3,0	34,1	1,0	17,5	0,4	8,1	0,1
		C	89,0	2,1	46,4	1,0	24,4	0,9	16,7	0,6
		R	20,4	1,6	10,7	0,5	5,9	0,6	2,4	0,1
		L1	77,9	4,2	22,4	0,5	22,4	0,4	11,0	0,4
		L2	177,4	3,6	96,1	2,2	54,6	0,7	25,4	1,0
		C+R	36,7	0,7	40,5	0,5	22,3	1,0	10,3	0,8
	600	C-R	76,6	0,7	37,7	0,9	19,4	0,7	8,7	0,1
		C	94,2	2,5	48,7	0,8	25,5	0,2	18,0	0,6
		R	23,7	1,6	12,0	0,5	6,4	0,2	2,8	0,2
		L1	84,4	3,6	23,9	0,1	24,3	0,6	10,5	0,9
		L2	189,1	0,9	104,3	1,7	57,8	0,8	26,9	0,4
		C+R	40,6	1,2	44,9	2,7	23,1	0,4	11,0	0,3
	610	C-R	32,1	1,5	14,9	0,5	7,8	0,5	3,6	0,3
		C	28,3	0,7	14,6	0,7	7,9	0,4	6,3	0,4
		R	10,9	0,4	5,6	0,2	3,2	0,3	1,1	0,3
		L1	29,2	1,3	9,0	0,5	8,6	0,2	3,9	0,5
		L2	63,8	1,9	33,3	1,0	19,5	0,3	9,2	0,3
		C+R	13,9	1,0	15,7	0,2	8,5	0,7	3,6	0,2
	625	C-R	41,0	0,7	20,5	0,5	10,1	0,4	5,3	0,1
		C	33,9	0,2	17,9	0,4	10,0	0,4	8,7	0,3
		R	16,1	0,8	7,8	0,6	4,5	0,2	2,1	0,5
		L1	35,8	2,2	11,5	0,4	11,1	0,7	5,1	0,3
		L2	78,6	0,4	42,1	1,3	25,9	1,5	11,8	0,2
		C+R	18,1	1,8	20,3	0,5	10,5	0,5	4,7	0,4
	635	C-R	20,1	0,9	9,8	0,6	5,3	0,8	2,0	0,0
		C	14,3	0,1	7,2	0,2	4,0	0,1	4,2	0,2
		R	8,2	0,3	4,1	0,2	2,1	0,2	0,9	0,1
		L1	16,1	1,3	4,9	0,2	4,9	0,1	2,2	0,1
		L2	34,4	1,3	18,4	0,4	11,4	0,6	5,1	0,2
		C+R	7,8	0,6	9,6	0,5	4,6	0,3	2,0	0,1

Table 2D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
495	595	C-R	102,6	0,5	51,1	0,4	25,7	0,7	12,8	0,4
		C	131,9	3,9	69,4	1,6	34,7	0,8	21,5	0,3
		R	31,4	1,4	16,6	0,1	8,7	0,2	4,1	0,3
		L1	117,4	2,9	33,4	1,4	33,3	0,2	15,4	0,4
		L2	263,0	4,0	144,3	3,6	78,7	2,1	36,5	1,5
		C+R	54,7	3,4	62,7	1,4	32,1	1,3	15,7	0,4
	600	C-R	113,6	2,7	55,9	0,7	29,6	0,3	13,9	0,6
		C	140,0	1,8	71,2	1,9	37,7	0,9	22,9	0,8
		R	36,2	0,7	19,0	0,5	10,2	0,5	4,3	0,1
		L1	125,9	4,9	36,2	2,1	36,4	0,2	17,1	0,1
		L2	285,1	5,1	155,0	3,1	83,0	2,0	38,3	2,6
		C+R	59,3	2,5	67,8	1,6	34,5	0,8	16,3	1,1
	610	C-R	47,7	1,2	21,7	0,7	11,5	0,1	5,5	0,1
		C	43,4	0,8	22,6	1,0	11,6	0,2	7,9	0,4
		R	16,6	1,4	8,8	0,1	4,5	0,6	1,9	0,1
		L1	44,4	1,0	12,6	1,0	12,8	0,3	6,3	0,5
		L2	94,5	1,5	50,7	1,7	28,9	0,9	13,0	0,6
		C+R	20,7	0,3	23,9	0,6	12,1	0,4	5,6	0,4
	625	C-R	60,4	1,3	29,8	0,2	15,2	0,5	6,9	0,7
		C	51,7	0,7	26,7	0,2	14,2	0,1	10,1	0,6
		R	24,1	0,4	12,4	0,2	6,5	0,5	3,0	0,5
		L1	54,8	2,2	16,1	0,4	16,0	0,3	7,1	0,4
		L2	118,2	2,4	64,3	1,4	36,3	0,7	16,6	0,9
		C+R	26,2	1,5	30,3	1,1	15,4	0,3	7,5	0,4
	635	C-R	30,4	1,4	15,0	0,6	7,7	0,2	3,3	0,2
		C	20,7	0,6	10,8	0,4	5,5	0,5	4,4	0,2
		R	12,3	0,3	6,3	0,4	3,2	0,2	1,5	0,3
		L1	24,5	1,0	7,4	0,2	6,9	0,6	3,2	0,3
		L2	52,1	1,1	27,8	0,5	16,1	0,5	7,2	0,7
		C+R	12,3	0,6	13,7	0,6	6,6	0,2	3,3	0,3

Table 2D. FRET assay results (Av- average, Dv- standard deviation); color code: red- high, blue- low (continuation)

Ex	Em	Protein	C, μ M							
			250		125		62,5		31,25	
			Av	Dv	Av	Dv	Av	Dv	Av	Dv
500	595	C-R	130,4	4,3	61,9	0,8	31,3	1,7	14,7	1,1
		C	162,0	3,0	85,5	0,9	41,8	0,9	24,6	1,1
		R	40,0	1,8	21,0	0,6	11,2	0,2	5,0	0,6
		L1	146,6	2,9	40,3	1,1	40,1	0,3	19,1	0,2
		L2	322,3	6,6	175,8	3,8	93,6	1,0	45,1	1,7
		C+R	65,6	2,6	76,8	0,9	39,4	0,1	19,8	0,8
	600	C-R	144,4	3,3	69,6	1,2	35,8	1,6	16,4	0,2
		C	169,3	4,7	89,2	0,9	44,7	1,3	25,6	0,6
		R	45,8	1,7	24,4	0,2	12,7	1,5	5,6	0,0
		L1	154,2	7,0	44,6	2,0	43,4	0,7	21,2	0,9
		L2	343,9	5,3	185,3	2,8	101,1	1,2	46,2	2,3
		C+R	71,3	3,6	81,8	1,4	43,8	1,1	20,5	0,3
	610	C-R	59,8	2,9	27,6	2,1	14,2	0,3	6,7	0,0
		C	51,6	1,0	27,8	1,5	14,3	0,5	8,6	0,3
		R	21,2	0,6	11,0	0,3	5,7	0,6	3,0	0,1
		L1	54,3	2,3	15,8	0,9	14,8	0,6	7,7	0,6
		L2	117,0	2,0	63,9	1,3	34,5	1,2	15,8	0,0
		C+R	25,4	0,5	29,9	0,9	15,0	0,2	7,6	0,6
	625	C-R	76,8	0,8	36,6	2,3	19,2	0,4	9,1	0,4
		C	61,9	1,3	31,7	0,6	16,8	0,6	11,2	0,9
		R	29,9	0,8	16,1	0,5	9,3	0,2	4,4	0,7
		L1	66,9	1,6	20,9	0,5	19,4	1,1	9,4	0,6
		L2	146,2	2,0	78,8	0,7	42,5	0,3	20,5	0,2
		C+R	31,7	1,2	37,6	2,0	19,4	0,0	9,3	0,5
	635	C-R	38,6	0,8	17,9	0,8	8,9	0,6	4,8	0,5
		C	25,8	0,9	13,0	0,1	6,8	0,3	5,4	0,6
		R	15,4	1,1	7,8	0,3	3,8	0,4	1,9	0,1
		L1	30,2	1,4	9,4	0,3	8,7	0,2	4,3	0,2
		L2	63,0	2,0	34,0	1,5	20,0	0,6	9,4	0,8
		C+R	14,7	0,7	17,0	1,0	8,7	0,7	4,3	0,2

E. Gels pictures taken with ChemiDoc ©

The gels pictures taken with ChemicDoc © are presented in Figure 1E.

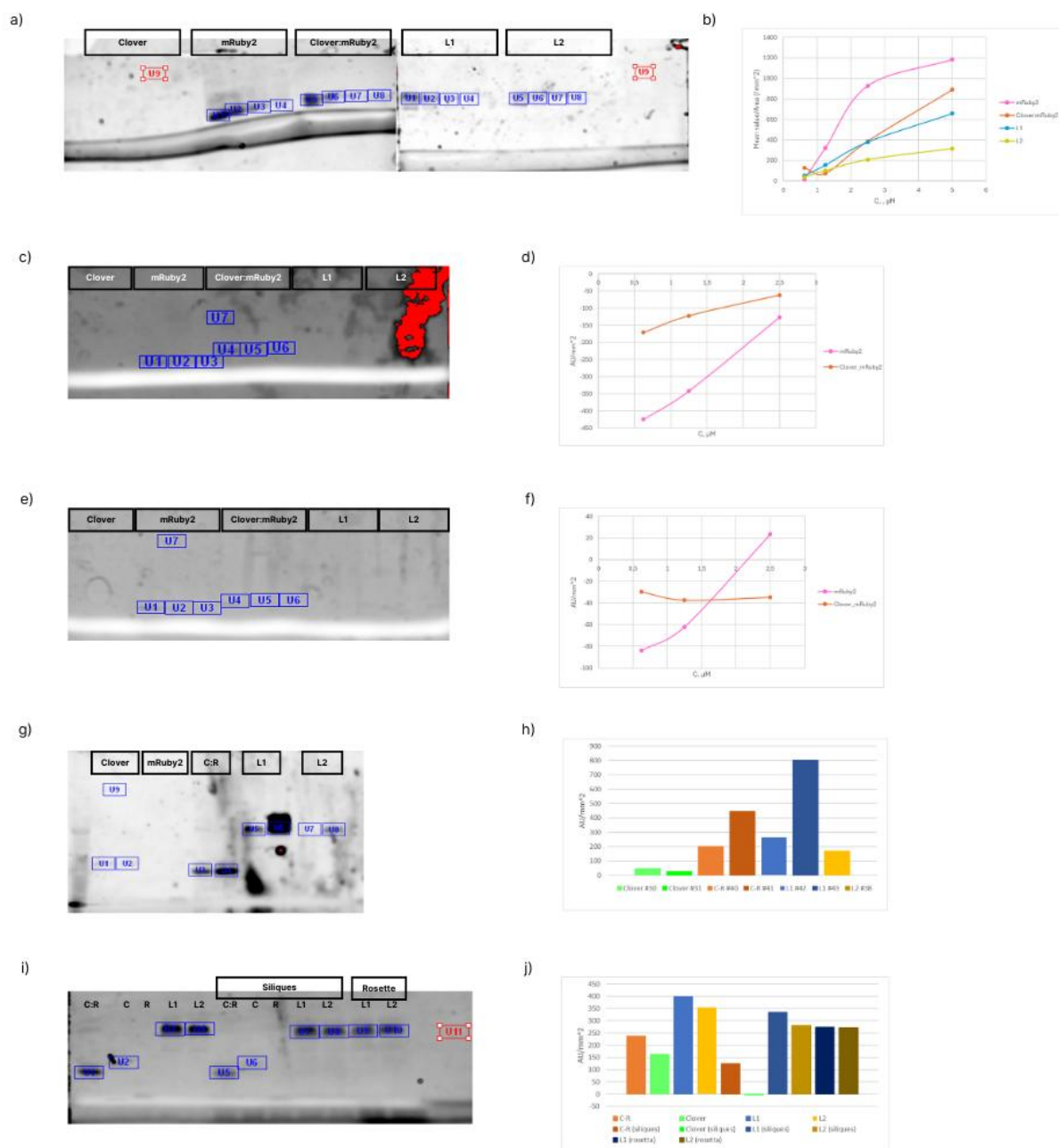


Figure 1E. Results a), c), e), g) and i) pictures of the gel and b), d), f), h) and j) their fluorescent signal quantification, respectively.

The information and characteristics of each gel is presented in Table 1E.

Table 1E. Characteristics and information of the gels ran

	Gel composition	Run characteristics
a)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1, L2 and CFP SUMO. - Samples with DTT - Concentrations tested: 0,5; 0,25; 0,125; 0,63 and 0,31 μM. 2 Native gels with 15 wells ran at 40 mA for 1h51 in the cold room (4 °C). - Loaded 60 μL per well.
c)	SDS-PAGE: 12 % acrylamide Stacking gel: 4 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Concentrations tested: 5; 2,5; 1,25 and 0,625 μM. 2 SDS-PAGE gels with 15 wells ran at 100 V: Gel with L1 and L2 for 3h and the other for 3h28 on ice. - Loaded 30 μL per well.
e)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Buffers used with SDS and no DTT in the samples. - Concentrations tested: 2,5; 1,25 and 0,625 μM. 1 Native gel with 15 wells ran at 20 mA for about 2h on ice. - Loaded 60 μL per well.
g)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Buffers used with SDS and no DTT in the samples. - Concentrations tested: 2,5; 1,25 and 0,625 μM. 1 Native gel with 15 wells ran at 20 mA for on ice. - Loaded 60 μL per well.
i)	Native gel: 7,5 % acrylamide Stacking gel: 3,75 % acrylamide	- Proteins used: Clover, mRuby2, Clover:mRuby2, L1 and L2. - Buffers used with SDS and no DTT in the samples. 1 Native gel with 15 wells ran 36 min at 110 V and 1h07 at 120 V with cold buffer. - Samples were incubated with plant extract for the UFDCt cleavage occur. - Loaded 20 μL per well.

F. Western blot (Revealed with Amersham ©)

The Western Blot membrane with the design and set-up tables 21 and 24 that incubated with α -T7 as primary antibody (1:10 000) and α -Rabbit as secondary (1:20 000) is presented in figure 1F.

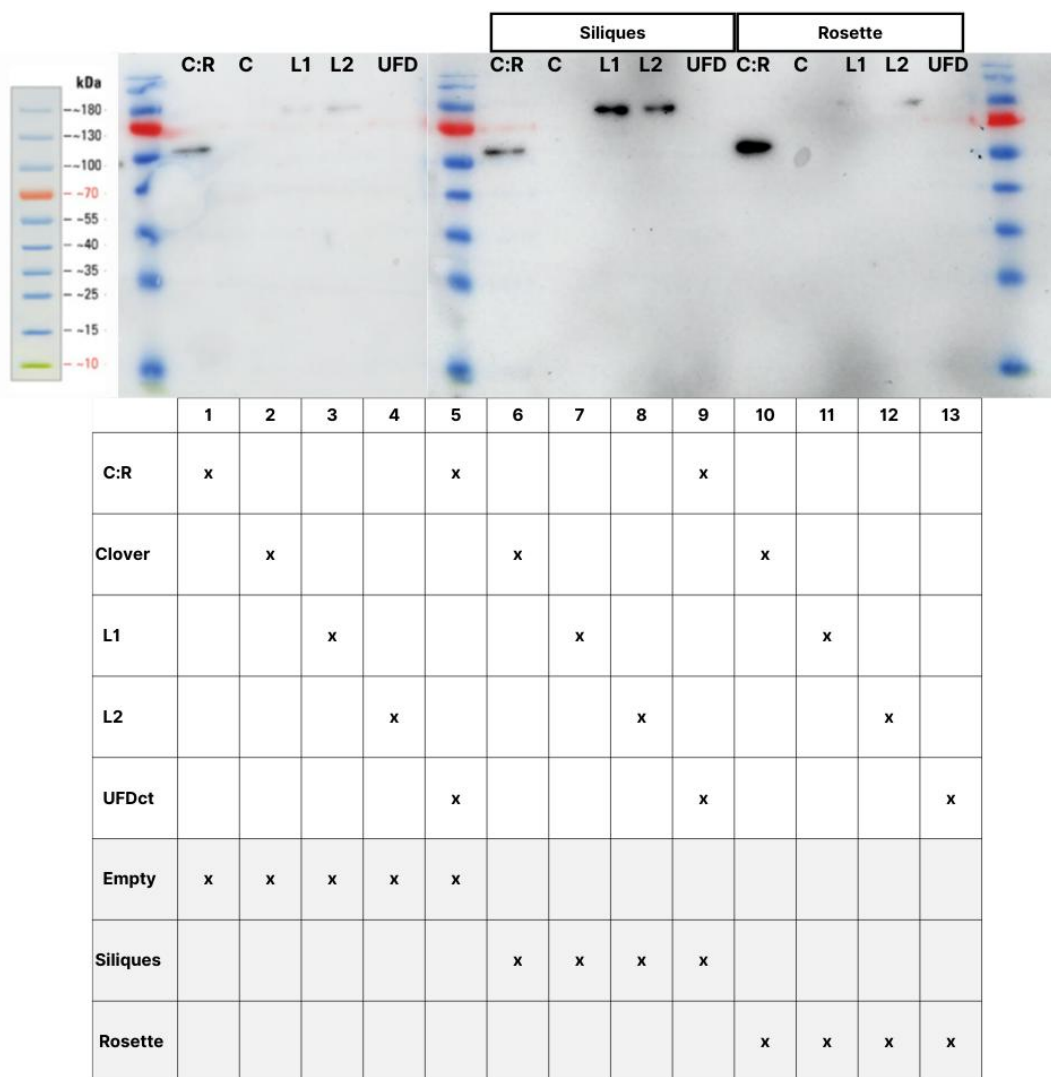


Figure 1F. Western Blot Membrane taken in IQ800 Amersham © (10minutes exposure; α -T7 and α -Rabbit).