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作品名稱 **HOST TARGET PROTEINS OF SPIKE PROTEIN OF SARS-COV-2**

得獎獎項 一等獎

國　　家 **Singapore**

就讀學校 **Clementi Town Secondary School**

指導教師 **Lehming-Teo Shi Hui, Rachel**

作者姓名 **LIM AINSLEY**

BRYAN LEE JIA CHENG

N D DURGHADEVE

關鍵詞 **HOST TARGET PROTEINS OF SPIKE PROTEIN OF SARS-COV-2**

作者照片

ABSTRACT

Coronavirus Disease 2019 (COVID-19) is a newly emerged infectious disease caused by the new severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV-2). In less than one year, the virus has spread around the entire world, killing millions of people and disrupting travel and business worldwide. During infection, the virus uses its Spike protein to dock onto the Ace2 protein on the surface of its human host cell. Spike is 1273 amino acids long and only a short fragment of Spike (319-541) is sufficient to bind Ace2. We hypothesized that the remaining protein sequences of Spike might have functions for viral replication beyond the binding of Ace2. We have performed Split-Ubiquitin protein-protein interaction screens to isolate human proteins by their ability to bind to Spike, and we have identified Annexin2A2 and Cytochrome b as novel human protein interaction partners of Spike. Annexin2A2 is involved in both endocytosis and exocytosis, and the protein interaction with Spike might help the virus to enter and exit its host cell. The presence of the mitochondrial Cytochrome b protein inside the cytosol promotes apoptosis, and the protein interaction with Spike could speed up apoptosis of the infected human cell. The Nub cDNA libraries that we have generated also allowed us to screen for synthetic peptides that interact with Spike. We have isolated two synthetic peptides, FL1a and FL7a, derived from the non-coding parts of human mRNAs by their ability to interact with Spike. We found that both FL1a and FL7a interact with the C-terminal half of the Spike protein. We also found that FL7a is able to block the Spike-Spike self-interaction at the C-terminal half of the Spike protein and we think that this could block the reassembly of the Spike protein in the host cell during viral reassembly. We hope that those synthetic peptides could be used as drugs due to their ability to block protein-protein interactions of Spike with human host proteins that are essential for viral replication.

BACKGROUND AND PURPOSE OF RESEARCH

Coronavirus Disease 2019 (COVID-19) is a newly emerged infectious disease caused by the new severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV-2). It originated in the Hubei province in China at the end of December 2019, after which the disease spread rapidly around the world, resulting in over 253,640,693 million confirmed cases and more than 5,104,899 million deaths at the time of writing (World Health Organisation, 2021). The genome of SARS-CoV-2 was made known to the public by 12 January 2020 (World Health Organisation, 2020). Only about 5 months later, many countries all over the world deployed travel restrictions and almost the entire world went into some form of a lockdown (World Health Organisation, 2021). Many lives were changed completely and the world, including Singapore, was left to imagine possibilities in a term coined as the 'new normal' (Ghebreyesus, 2020). Many social activities and events globally have been halted and lives disrupted as people find a way to live with reduced social contact to minimise COVID-19 transmission. Economic activity has slowed down as businesses struggle to earn an income and more people are forced into a quarantine-focused lifestyle (OECD, 2020).

There has been a concerted effort in the direction of SARS-CoV-2 research to better understand its mechanism so that better and more effective treatment can be developed against COVID-19 infection. We wanted to use techniques we learnt to screen proteins interacting with the Spike protein (Spike) of SARS-CoV-2 virus so that we can contribute to the understanding about viral-host entry, its subsequent replication and re-assembly in the host cell. We wanted to focus on Spike (instead of the other proteins found in the viral membrane) as it is the determining factor that decides if the virus infects a human host cell (Huang et al., 2020).

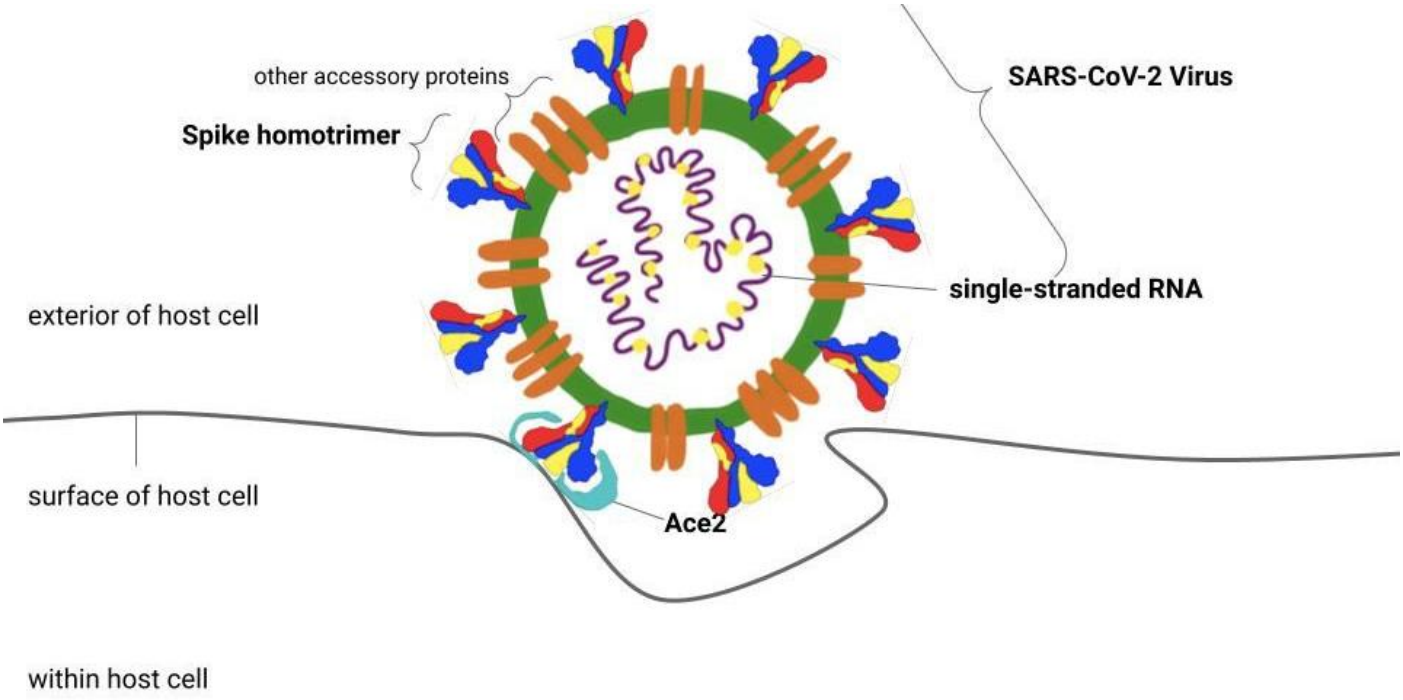

Figure 1: Diagram of a SARS-CoV-2 virus entering the host cell. The SARS-CoV-2 virus is constituted by its membrane protein, envelope protein, nucleocapsid protein, Spike protein, single-stranded RNA and other accessory proteins. To infect a human host cell successfully, the SARS-CoV-2 enters the cell by using its Spike protein to interact with Ace2, fusing with the cell surface membrane to enter the cell.

The SARS-CoV-2 virus is composed of single-stranded RNA encased in a protective envelope containing four main structural proteins, Spike glycoprotein, small envelope glycoprotein, membrane glycoprotein and nucleocapsid protein and several accessory proteins (Klein et al., 2020). The virus infects a human host cell by attaching its Spike protein to Ace2 which is especially found on the surface of the human type II alveolar cells in the respiratory tract (Huang et al., 2020), thereby mediating the fusion of the virus and cell causing the entire virus to enter the host cell via endocytosis (Walls et al., 2020). The human body's response to COVID-19 is mediated by cytokines. The host's immune response is triggered when the virus enters the host cells and innate immune cells like dendritic cells and antigen-presenting cells are involved (Huang et al., 2020). When the virus first infects an individual, the lungs become inflamed and pneumonia, an infection of alveoli in the lungs, occurs (Attaway et al., 2021). Many COVID-19 patients experience mild symptoms or illness

like fever, fatigue, shortness of breath, loss of taste or smell and chest or abdominal pain. About 10%-15% of cases become severe diseases and 5% are critically ill (World Health Organisation, 2020). Patients usually recover after 2 to 6 weeks; however, some people can have symptoms that linger for months or develop conditions that have lasting health complications. Examples of this include damage to heart muscle, heart failure, damage to lung tissue, cognitive impairment and stroke (World Health Organisation, 2020).

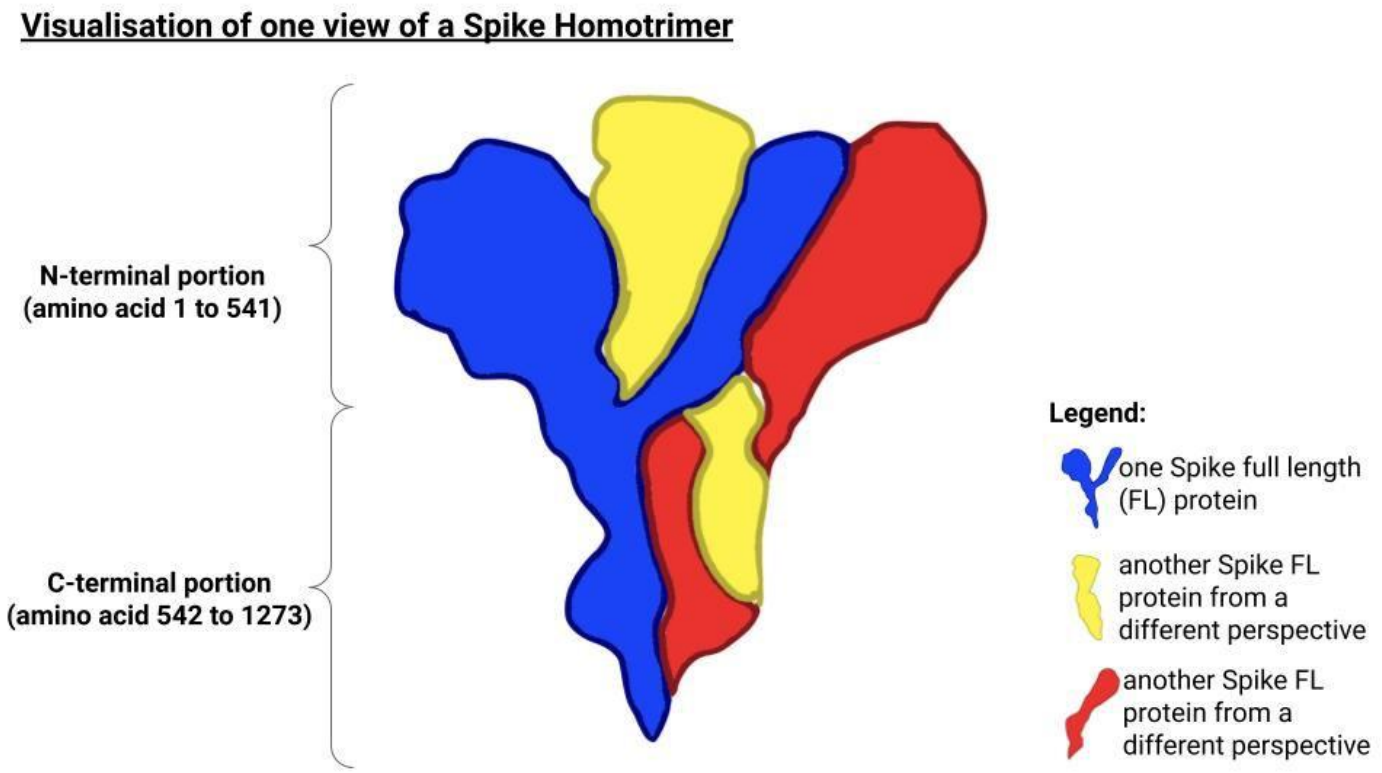

Figure 2: Illustration of Spike Homotrimer and components. A single Spike protein on the surface of the SARS-CoV-2 virus is assembled from three spike individual monomers, forming one Spike homotrimer. Amino acids 1 to 541 represent the N-terminal half of each Spike monomer, while amino acids 542 to 1273 represent the C-terminal half of each Spike monomer.

A virus usually uses its own proteins to target certain host proteins (Flint et al., 2015). The (1) Spike protein, (2) RNA-dependent RNA polymerase (RdRp) (Gao et al., 2020) which helps to make copies of the SARS-CoV-2 RNA genome and (3) the main protease which helps to process viral protein production are important to study because they are important targets for treatment of those infected with SARS-CoV-2 virus (Gil et al., 2020). Studying viral-human host protein interactions is an important way to gain a systems understanding of the molecular mechanism of viral infection (Alguwaizani et al., 2018; Yang et al., 2019). Transmembrane Spike glycoprotein forms homotrimers protruding from the viral surface (Walls et al., 2020). The protruding portion of the Spike homotrimer for SARS-CoV-2 virus has been found to have a higher affinity for human angiotensin converting enzyme 2 (Ace2) (Conceicao et al., 2020) than the SARS-CoV virus that caused the severe acute respiratory syndrome (SARS) in 2003 (Liu et al., 2020). A crystal structure of the receptor-

binding domain (RBD) of the Spike protein of SARS-CoV-2 virus bound to the cell receptor of Ace2 has been determined and published (Lan et al., 2020). In this paper, Spike viral protein amino acids 319 to 541, corresponding to the N-terminal portion of Spike visualised in Figure 2, binds to Ace2 on the host cell. Since amino acids 319 to 541 are sufficient for binding to Ace2 (Lan et al., 2020) and that a full length (FL) Spike protein is 1273 amino acids long (Bangaru et al., 2020), we wonder what other possibilities might amino acid 542 to 1273 hold for Spike. Are the remaining residues there to help form the structure for the Spike protein and its subsequent homotrimer or do they also have a function inside the cell once Spike is internalised into the host cell that would prevent the cell from committing suicide once the virus enters the cell? Split-Ubiquitin (Split-Ub) (Johnsson and Varshavsky, 1994; Laser et al., 2000; Lehming, 2002), which is an alternative Yeast-2-Hybrid (Fields and Song, 1989; Wong et al., 2017) system, would be used to identify host target proteins for the SARS-CoV-2 Spike protein.

OUR HYPOTHESIS

We hypothesize that since Spike is a homotrimer (Huang et al., 2020), it interacts with itself. We also hypothesise that finding human proteins that interact with Spike through a screen would provide insight into viral host entry, its subsequent replication and re-assembly mechanism inside the host cell. In addition, we also hypothesise that synthetic peptides may be able to block interactions between Spike and its human target proteins. These synthetic peptides could be used as drugs to prevent Spike assembly or to prevent interactions between Spike and interacting proteins. These potential drugs can be used, in conjunction with vaccination efforts, to induce a more effective and targeted therapeutic treatment for patients infected with SARS-CoV-2.

PROJECT FLOWCHART

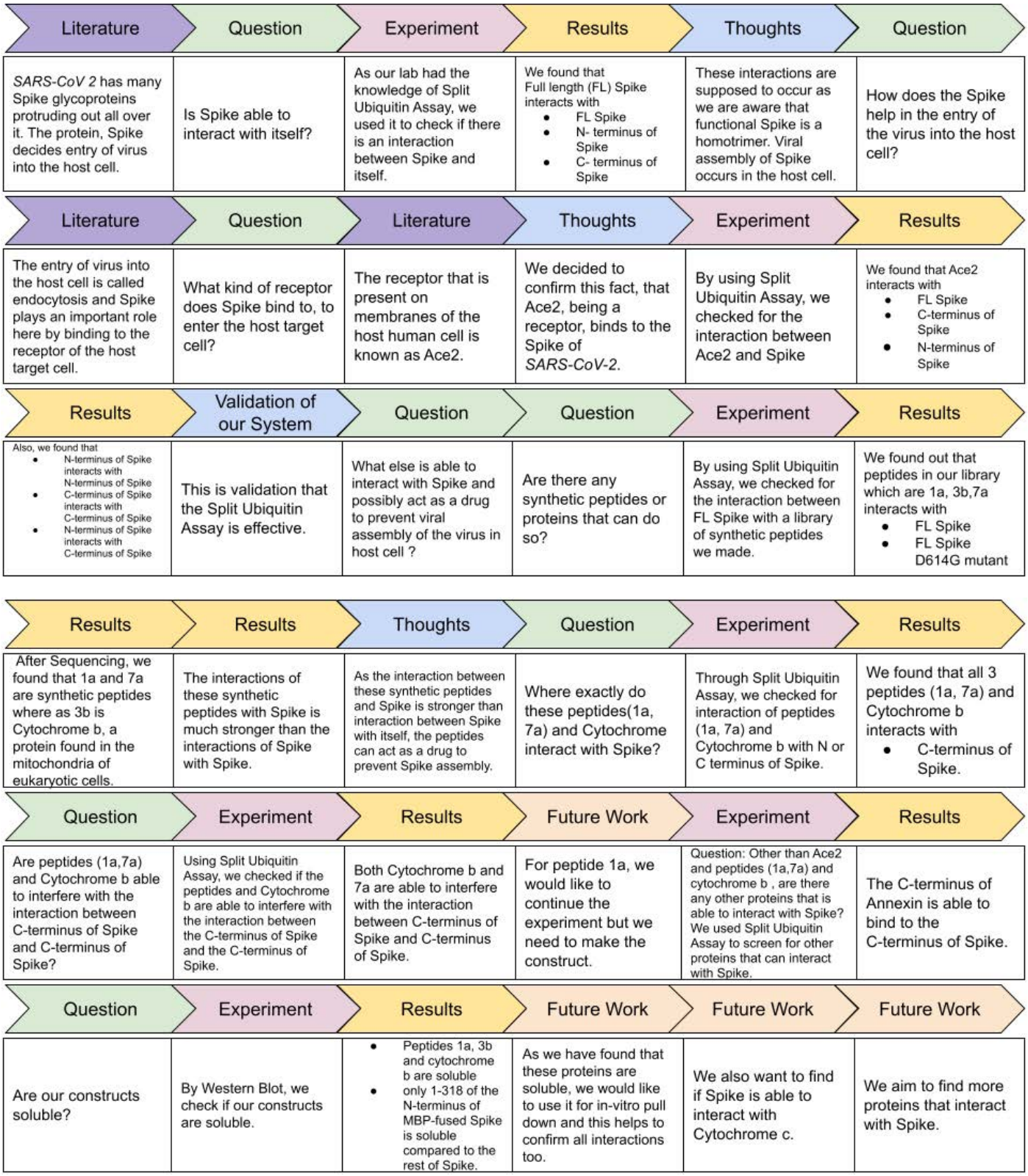

Figure 3: Project flowchart. To summarise the project, we made an overview of our thought processes, methods and results. First, we used literature to expand our knowledge, then came up with questions and performed various experiments in an attempt to answer those questions. We then analysed these results and came up with conclusions, performing additional experiments whenever necessary.

METHODOLOGY

Isolating DNA from *Escherichia coli* (*E. coli*): A single colony of *E. coli* cells is inoculated and left overnight to grow. The grown cells are transferred from black cap tubes into 1.5ml microtubes. The 1.5ml microtubes are centrifuged at full speed for 30 seconds and the supernatant is discarded. 200µl of cell suspension buffer is added and vortexed to resuspend the cells. 200µl cell lysis buffer is added and the tube is inverted 5 times to mix. 200µl of cell neutralisation buffer is added to precipitate the cell debris out. Centrifuge at full speed for 10 minutes to bring down the cell debris and transfer the supernatant containing DNA into a new 1.5mL microtube. 300µl of isopropanol is then used to precipitate the DNA out. Then, the resultant solution is centrifuged to bring the precipitated DNA to the bottom. Next, the DNA pellet is washed with 800µl 70% ethanol. To obtain pure DNA, the tubes are spun in a speed vacuum for drying and the DNA is dissolved in 100µl sterile water to be used for subsequent experiments.

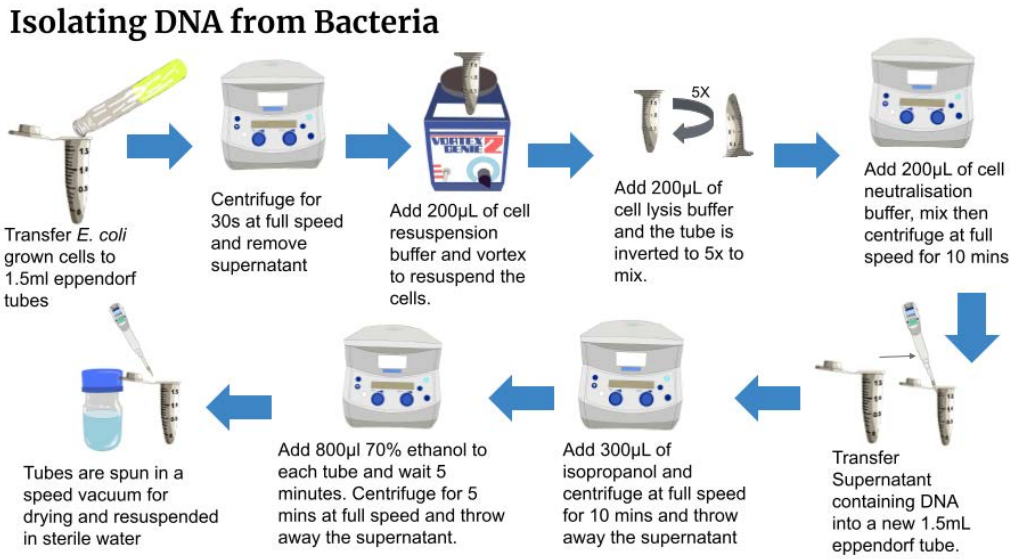

Figure 4: Diagram of isolating DNA from bacteria procedure

Restriction enzyme digest (R.E. digest): R.E. digest is the process that cuts the restriction sites of DNA to check for inserts or conduct genetic modification. 0.2ml tubes are prepared with plasmids from samples. A mix is prepared using restriction enzymes, water and buffer. The mix is pipetted into the plasmid samples and the samples are left for 2 hours in the 37˚C incubator.

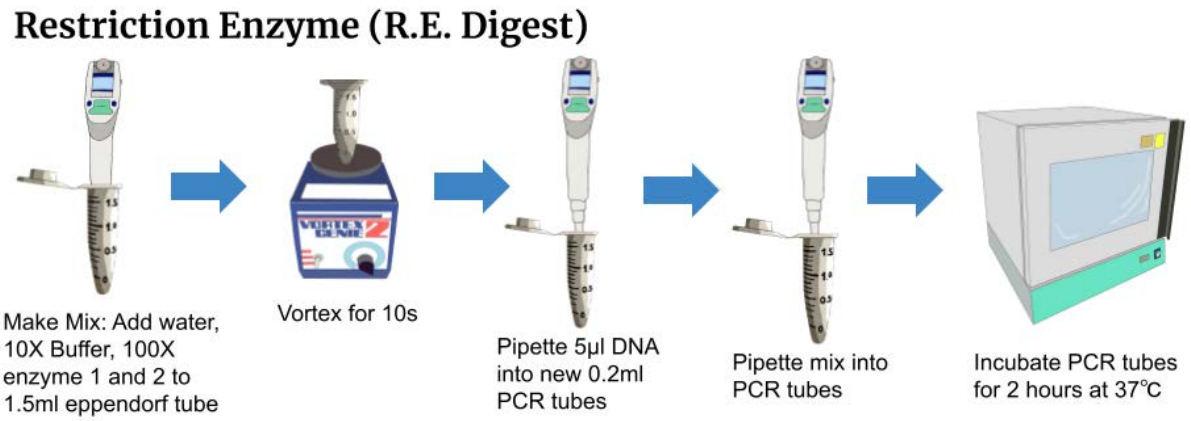

Figure 5: Diagram of R.E. digest procedure

Electrophoresis: Electrophoresis is the process that sorts DNA by size. A 1% agarose gel with 3µl ethidium bromide is made and soaked in TAE buffer. DNA samples were mixed with 1.11µl DNA loading dye, then loaded into the wells of the gel, along with a size marker at the first well of each row. The electrophoresis machine is left to run for 40 minutes at 100V and a current passes through the gel, pulling the DNA samples along with it. The further away the DNA sample was from the starting point, the smaller the size of the DNA. Pictures of these gels were taken to document their results.

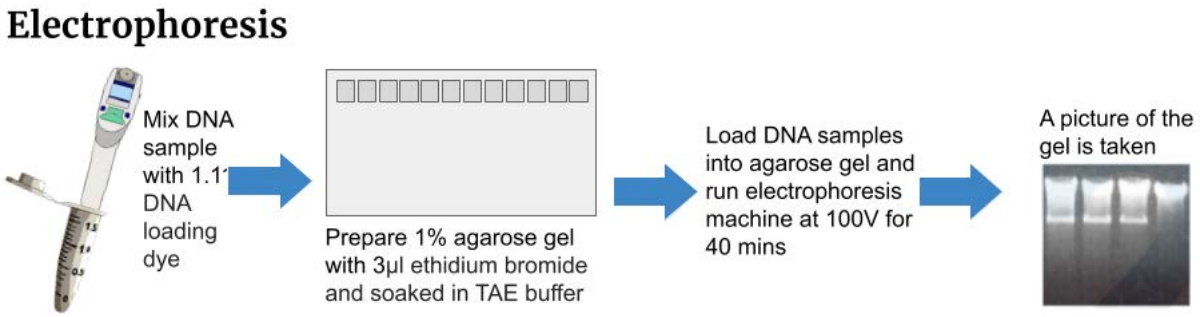

Figure 6: Diagram of electrophoresis procedure

IPTG Induction : After the *E. coli* Inoculation (using BL21 Cells), take black cap tubes out of the incubator when OD reaches 0.8>OD>0.6. Add 50µl of 1M IPTG (Isopropyl β- d-1-thiogalactopyranoside) and wait 4 hours. Next, centrifuge the cells at 8000rpm for 10 mins before discarding the supernatant. Resuspend the pellet in 1ml of PBS buffer, then add DNase and $MgSO_4$. The cells will then be kept at the -80°C freezer for storage.

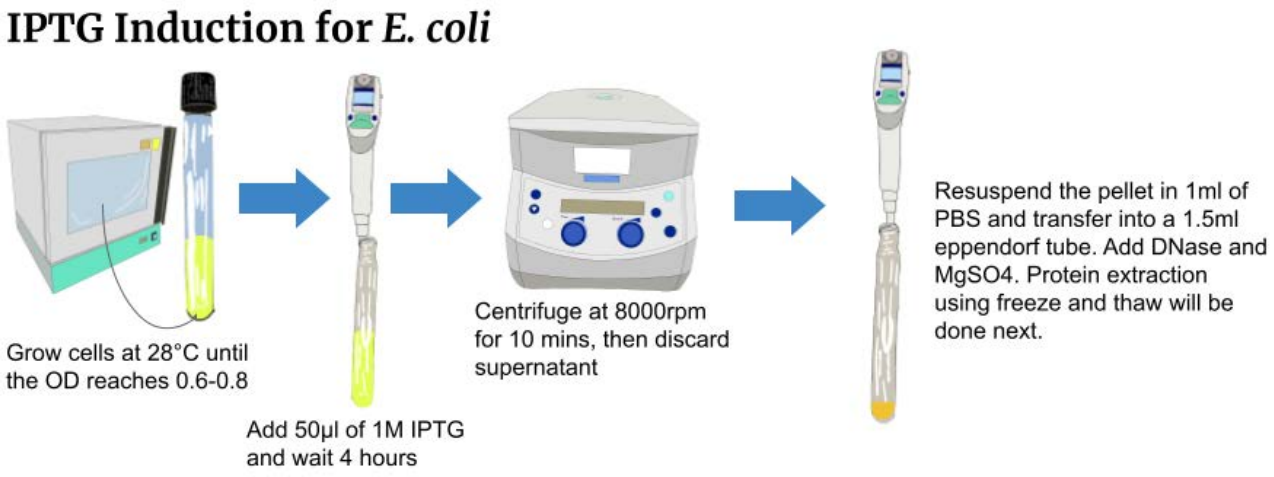

Figure 7: Diagram of IPTG induction for *E. coli* procedure

Protein extraction from *E. coli*: *E. coli* cells are frozen at -80°C and then thawed at 42°C. This is repeated three times. Then, an aliquot of cells will be transferred into 1.5ml microtubes while the remaining will be centrifuged to bring the cell debris down to the bottom of the tube while leaving the extract as the supernatant. The supernatant will be kept for subsequent experiments.

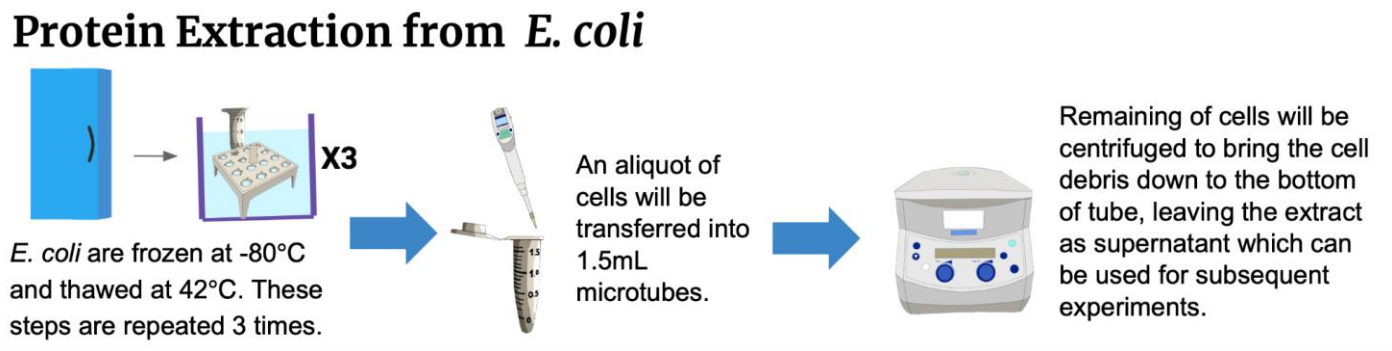

Figure 8: Diagram of protein extraction from *E. coli* procedure

***Saccharomyces cerevisiae (S. cerevisiae)* transformation :** *S. cerevisiae* transformation is the addition of foreign DNA into *S. cerevisiae* cells so that the proteins of interest can be expressed by the cells. 1µl of fish sperm, 2µl of Plasmid DNA, 5µl of competent *S. cerevisiae* cells and 50µl of PEG are mixed together and left in an 28°C incubator for an hour. Then, the samples are placed in a 42˚C water bath for 15 minutes and centrifuged. The supernatant is discarded and resuspended in sterile water to be plated on synthetic deplete (SD) plates. The cells are left to grow in incubators at 28°C.

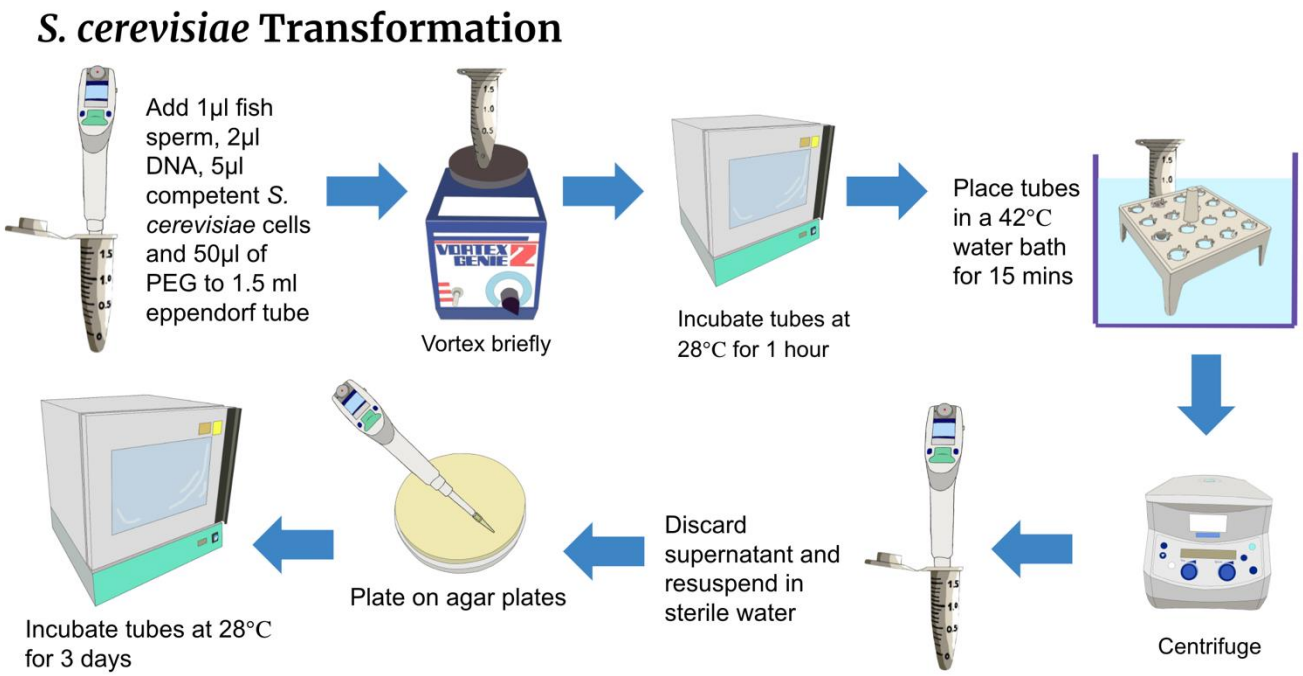

Figure 9: Diagram of *S. cerevisiae* transformation procedure

Mating: Mating mixes together two different *S. cerevisiae* strains to prepare for a split-ubiquitin assay. First, the BY4741 strain cells are taken from a transformation plate using a sterile toothpick and a diagonal slash is made to transfer cells from the toothpick to the YPDA plate used to grow the mated cells. Next, the BY4742 strain cells are taken from another plate and another slash is drawn on top of the previous slash such that an "X" shape is formed. The toothpick is then used to spread out the "X" to mix the two different strains together.

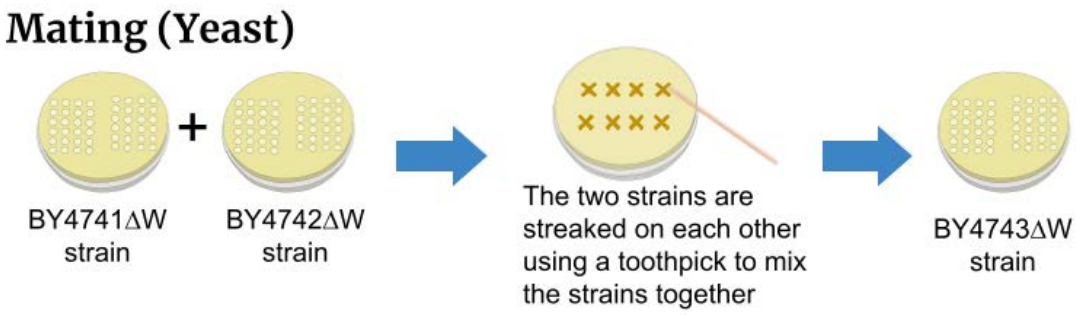

Figure 10: Diagram of mating (Yeast) procedure

Ten-fold serial titration: Titrations are used to carry out split-ubiquitin by growing *S. cerevisiae* cells on plates with fluoroorotic acid (FOA) and plates lacking uracil. This will show us if there is interaction between proteins. The wells of a sterile 96-well plate are filled with 90µl of sterile water. Each well with 3 adjacent wells is mixed with cell samples and dilution is done by taking 10µl of cell-water mixture from the well on the left and mixing the 10µl with the pure water in the adjacent well. This is repeated 3 times. Then, 5µl is taken from each of the 4 wells and loaded onto the different plates neatly using a multi-channel pipette. The cells are left to grow in incubators at 28℃ and pictures are taken at intervals to record result.

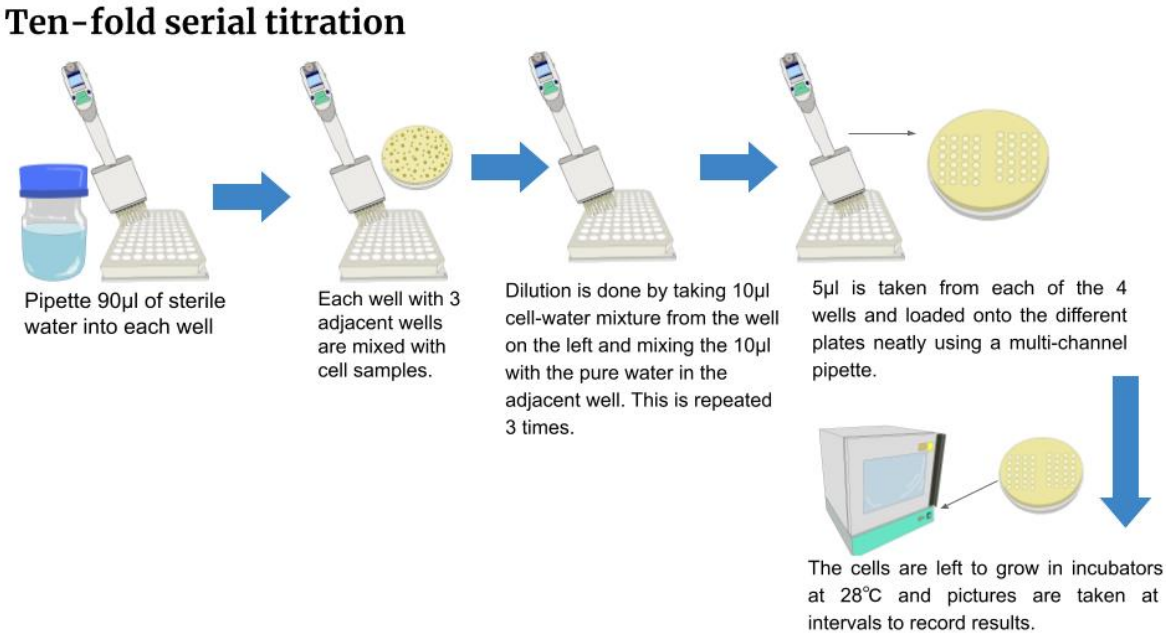

Figure 11: Diagram of ten-fold serial titration procedure

Restreaking: Using a sterile toothpick, pick a yeast colony from the respective plate. Restreak onto a new agar plate lacking the suitable nutritional markers for selection.

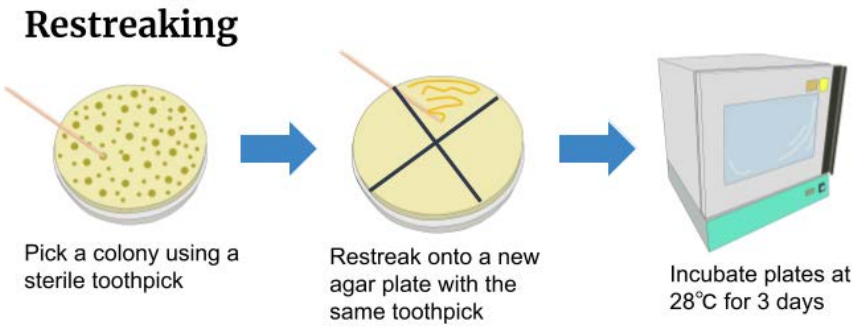

Figure 12: Diagram of restreaking procedure

Ligation: Mix 6µl of cut PCR fragment, 2µl of cut vector, 0.2µl of 100X DNA ligase, 2µl of ligation buffer and 9.8µl of water for each ligation reaction. Flick to mix the tubes and place the tube in a 28℃ incubator for 3 hours.

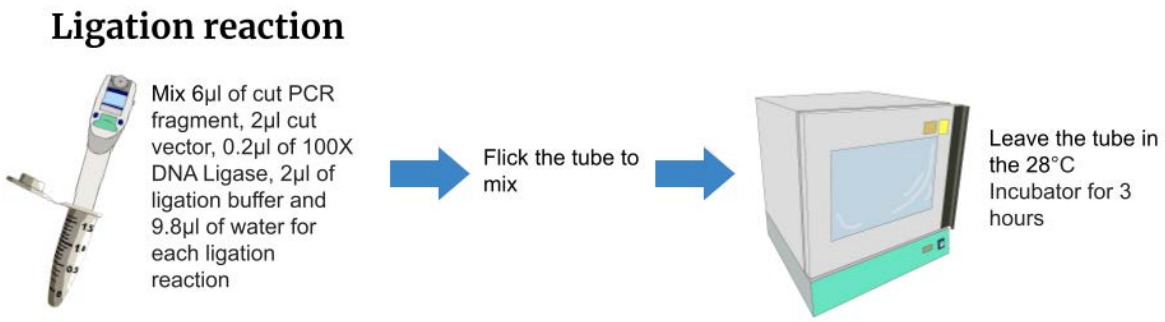

Figure 13: Diagram of ligation reaction procedure

***E. coli* Inoculation**: Fill black cap tubes with 1.665µl of LB media with Ampicillin. Pick a bacteria colony from the LB plate containing Ampicillin using a pipette tip and transfer it into the media. Place the black cap tubes into a 37℃ shaker overnight.

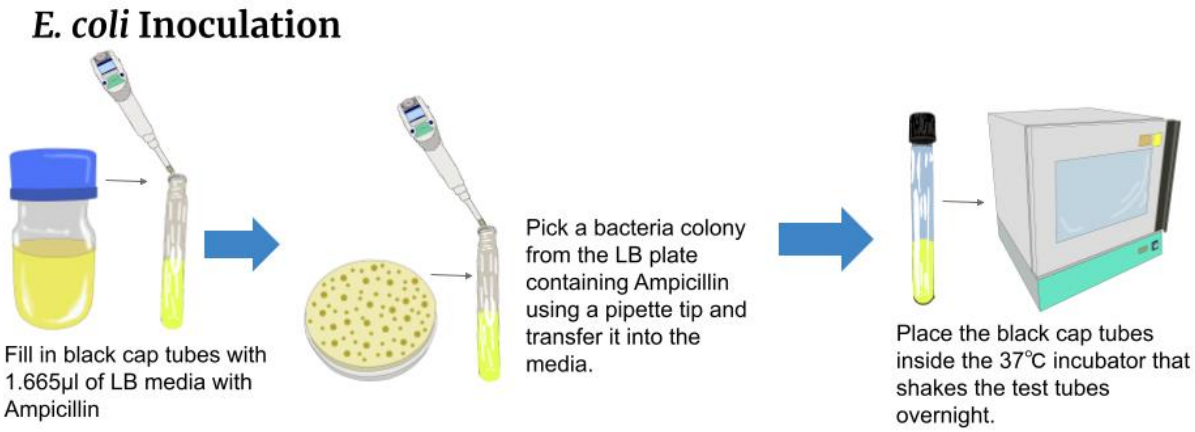

Figure 14: Diagram of *E. coli* inoculation procedure

***S. cerevisiae* Inoculation:** Fill black cap tubes with 10 mL of respective media. Pick a yeast colony from the plate using a tip of the pipette and transfer it into the media.

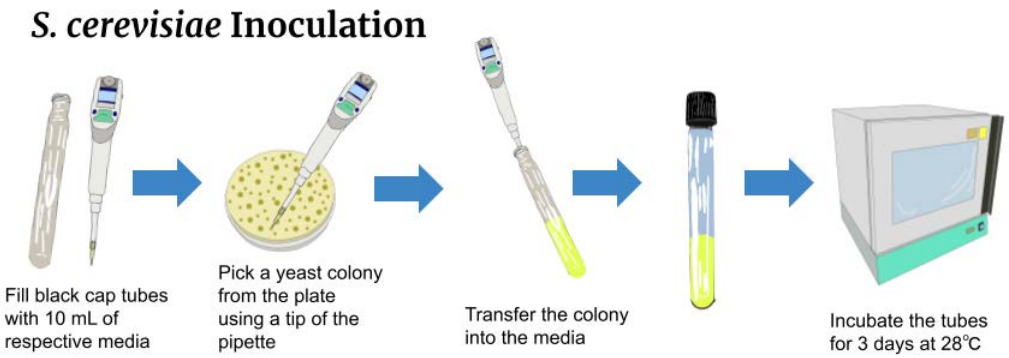

Figure 15: Diagram of *S.cerevisiae* inoculation procedure

Polymerase Chain Reaction (PCR): Mix 0.5µl of each primer, 1µl of template, 10X buffer, 10X dNTPs and $MgSO_4$ into 0.2ml PCR tubes. Run the PCR programme according to these settings: Denaturation for 10 seconds at 98˚C, Annealing for 30 seconds at 50˚C and Extension for 1 min at 68℃.

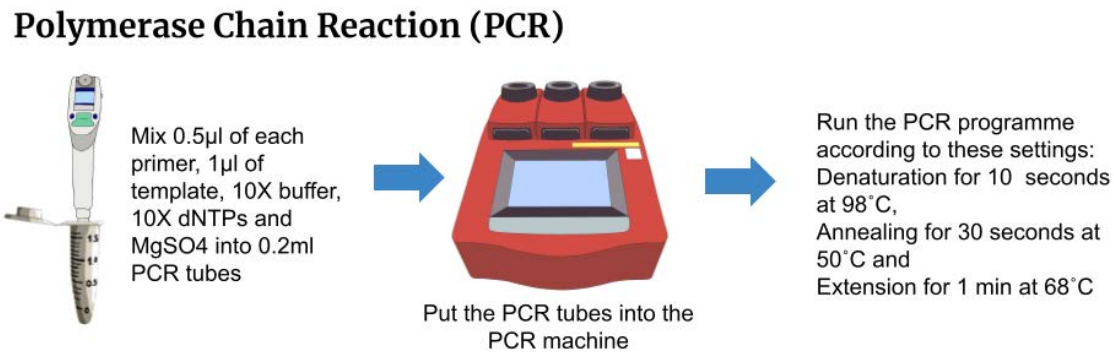

Figure 16: Diagram of PCR procedure

PCR Clean-Up: Add 100µl of DNA Binding Buffer with the PCR mix and transfer mixture into the spin column. The column is then centrifuged for 30s at 6000 rpm and the flow through is discarded. After that, wash the membrane with 500µl of 75% Ethanol and centrifuge the column for 30s at 6000 rpm twice. Then, transfer the spin column to a new 1.5ml Eppendorf tube and bring the tubes to a clean bench. Elute DNA using 80µl of sterile water, put in the warm room for 5 min and centrifuge the tubes for 30s at 6000 rpm.

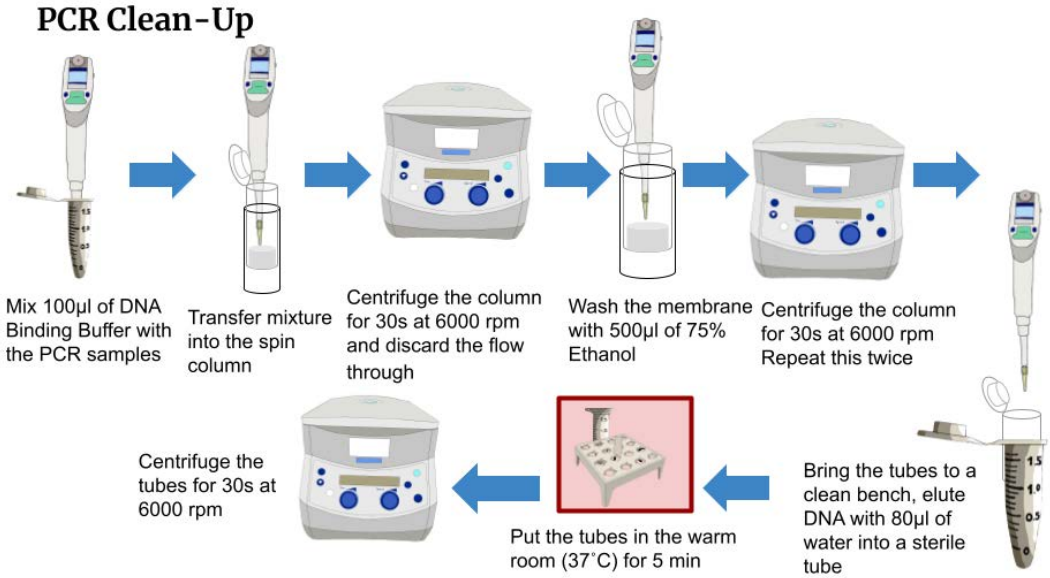

Figure 17: Diagram of PCR clean-up procedure

***E. coli* Transformation (Heat shock):** Take out DH5α *E. coli* competent cells from the -80°C freezer. Pipette 20µl of *E. coli* cells into tubes containing the ligation mixture. Incubate the tubes on ice for 15 minutes. Then, put the tubes in the 42°C water bath for 1 minute for heat shock. Add 80µl of LB Media to each tube and incubate them in the warm room for 1 hour. Afterwards, plate 60µl of the mixture onto a LB plate containing ampicillin.

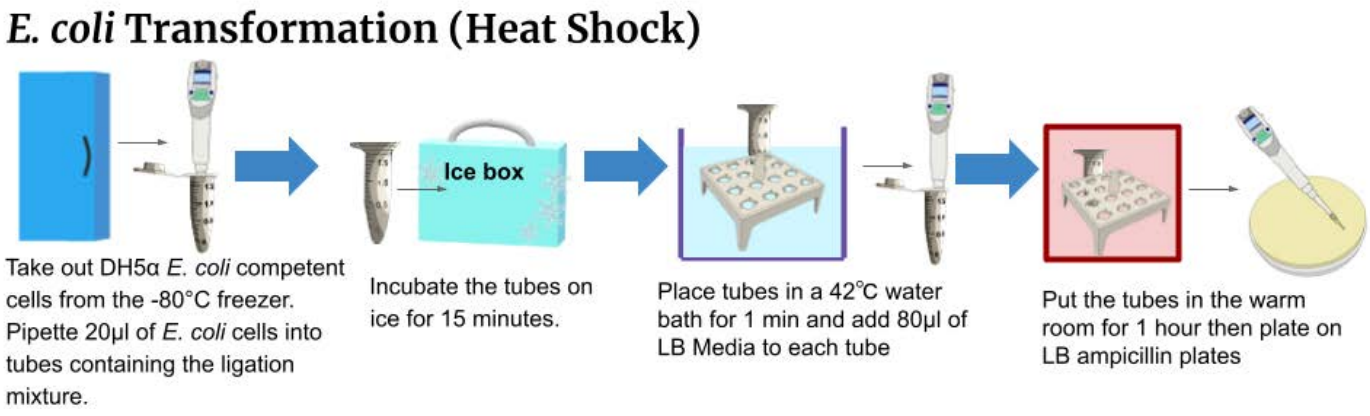

Figure 18: Diagram of *E. coli* transformation (Heat shock) procedure

Preparation for Sequencing: Mix 1µl of primer, 2µl of plasmid DNA, 4.5µl sterile water, 2µl sequencing buffer and 0.5µl Big Dye into a 0.2ml PCR tube. Vortex the tube briefly and centrifuge for 10 seconds. Run the samples in the Thermocycler under the STeP Program. Afterwards, mix 90µl of Sequencing Solution with the samples and transfer into a new Eppendorf tube. Wait 5-10 mins and then centrifuge for 10 mins at 14680 rpm, removing the supernatant afterwards. Add 500µl of 75% Ethanol to the tubes and wait 5 mins, then centrifuge the tubes for 5 mins at 14680 rpm before removing the supernatant again. Put tubes inside the speed vacuum for 15 mins and then send it to the company to carry out the electrophoresis.

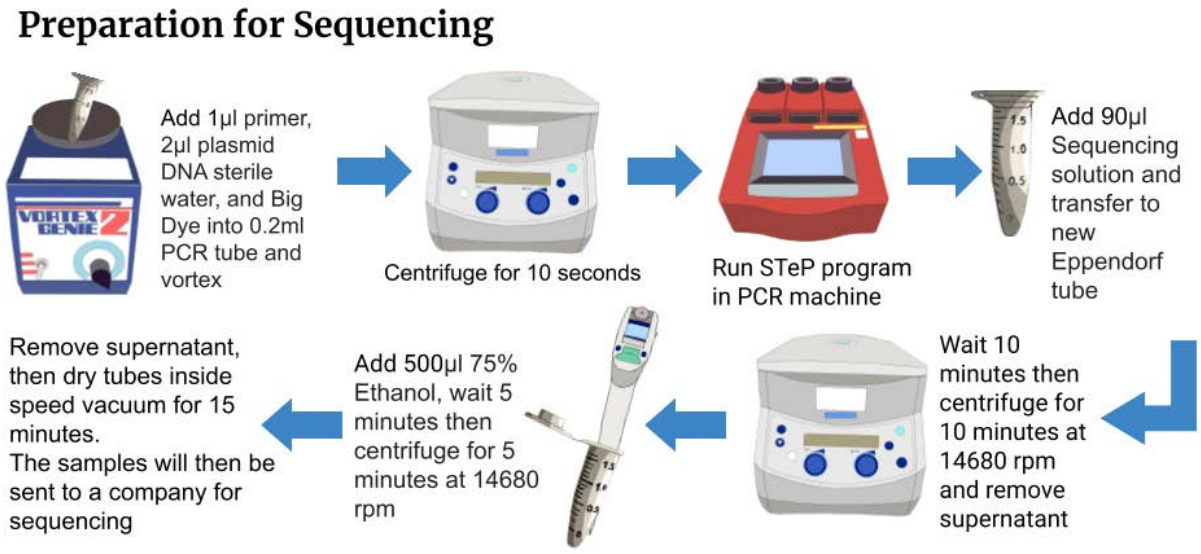

Figure 19: Diagram of preparation for sequencing procedure

Making Competent *S. cerevisiae* Cells: Inoculate *S. cerevisiae* cells in 10ml YPDA media in black cap tube and shake in the 28°C incubator over-night. The next morning, measure the OD until it reaches 1.5. Centrifuged the black cap tubes at 2000 rpm for 5 mins. Use 1ml of sterile water to transfer the cells to a new Eppendorf tube. Centrifuge for 1 min at 7000 rpm and remove the supernatant. Resuspend the cells in 0.1M of Lithium Acetate. Store the cells in the 4˚C fridge.

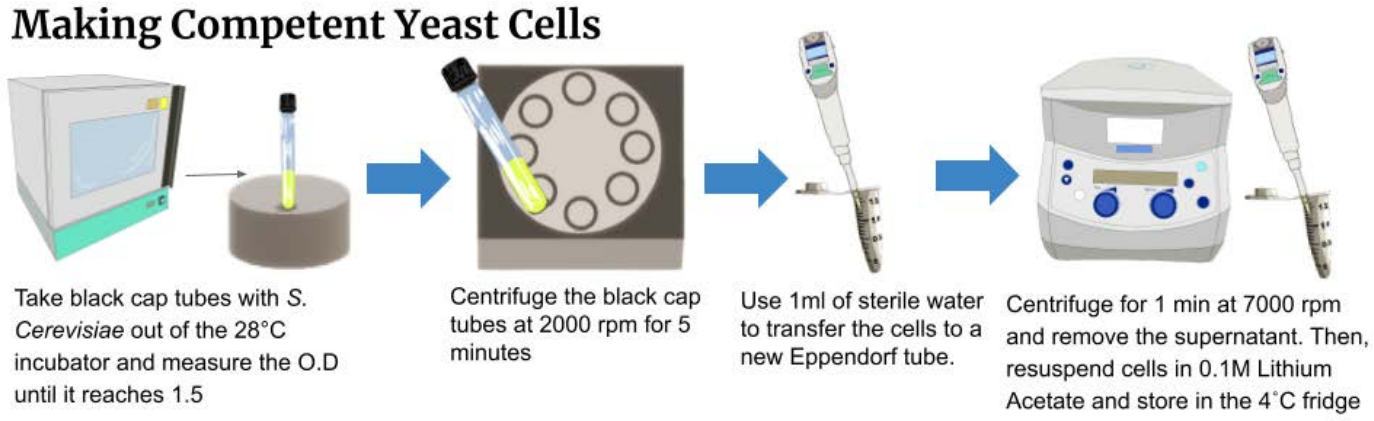

Figure 20: Diagram of making competent yeast cells procedure

***E. coli* Transformation (Electroporation):** Take DH10ß *E. coli* electrocompetent cells out of -80°C freezer. Mix 2µl of plasmid with 28µl of cells in a new Eppendorf tube. Mix and transfer mixture into a cuvette. Put the cuvette inside the electroporation machine (MicroPulser) and apply electric pulse. Mix 300µl of LB media with the cells in the cuvette and transfer the mixture back into the tubes. Put the tubes in the warm room for 1 hour and then plate the cells on a LB plate containing Ampicillin.

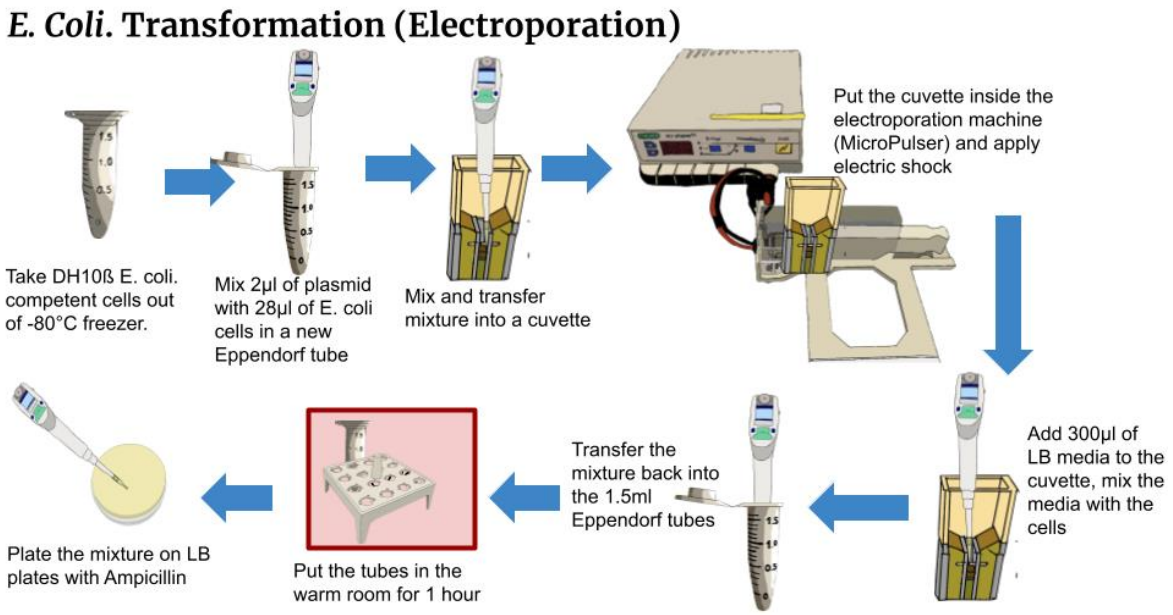

Figure 21: Diagram of *E. coli* transformation (Electroporation) procedure

Western blot: Western blot is used to identify proteins separated by size. The stacking gel and separating gel is made using appropriate amounts of water and 30% Bisacrylamide, pH 6.8 or pH 8.8 Tris-HCl buffer (respectively), 10% SDS, 10% APS solution and TEMED. After the stacking gel has solidified, the samples are loaded into the gel and ran at 100V for 90 minutes. After that, transfer the protein bands onto a nitrocellulose membrane using a TransBlot machine. Next, block the membrane using 25ml of 5% milk. The membrane is then incubated with 0.5µl of primary antibody in 12.5ml of 5% milk in the cold room overnight. Wash the membrane with 30ml of TBST for 10 minutes thrice before adding 0.5µl of secondary antibody into 12.5ml of fresh 5% milk and incubating for 2 hours. Wash the membrane again with 30ml of TBST for 10 minutes three times. Perform enhanced ChemiLuminesence and image the blots using Gel-Imager.

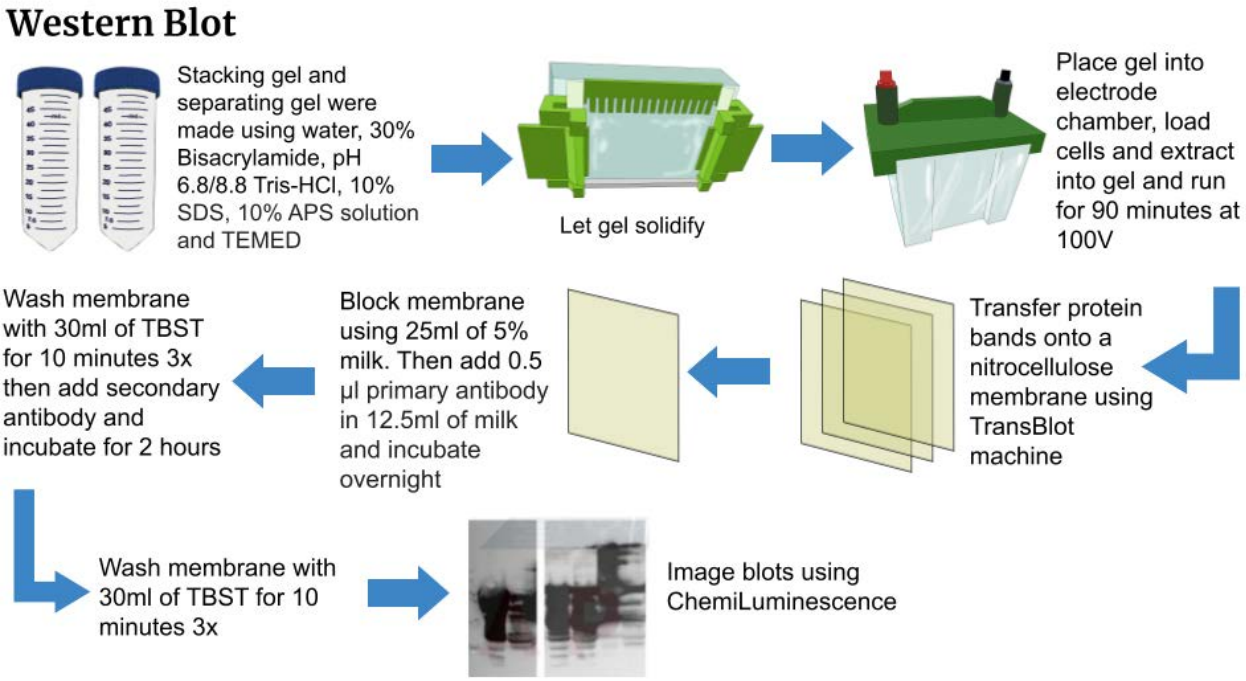

Figure 22: Diagram of western blot procedure

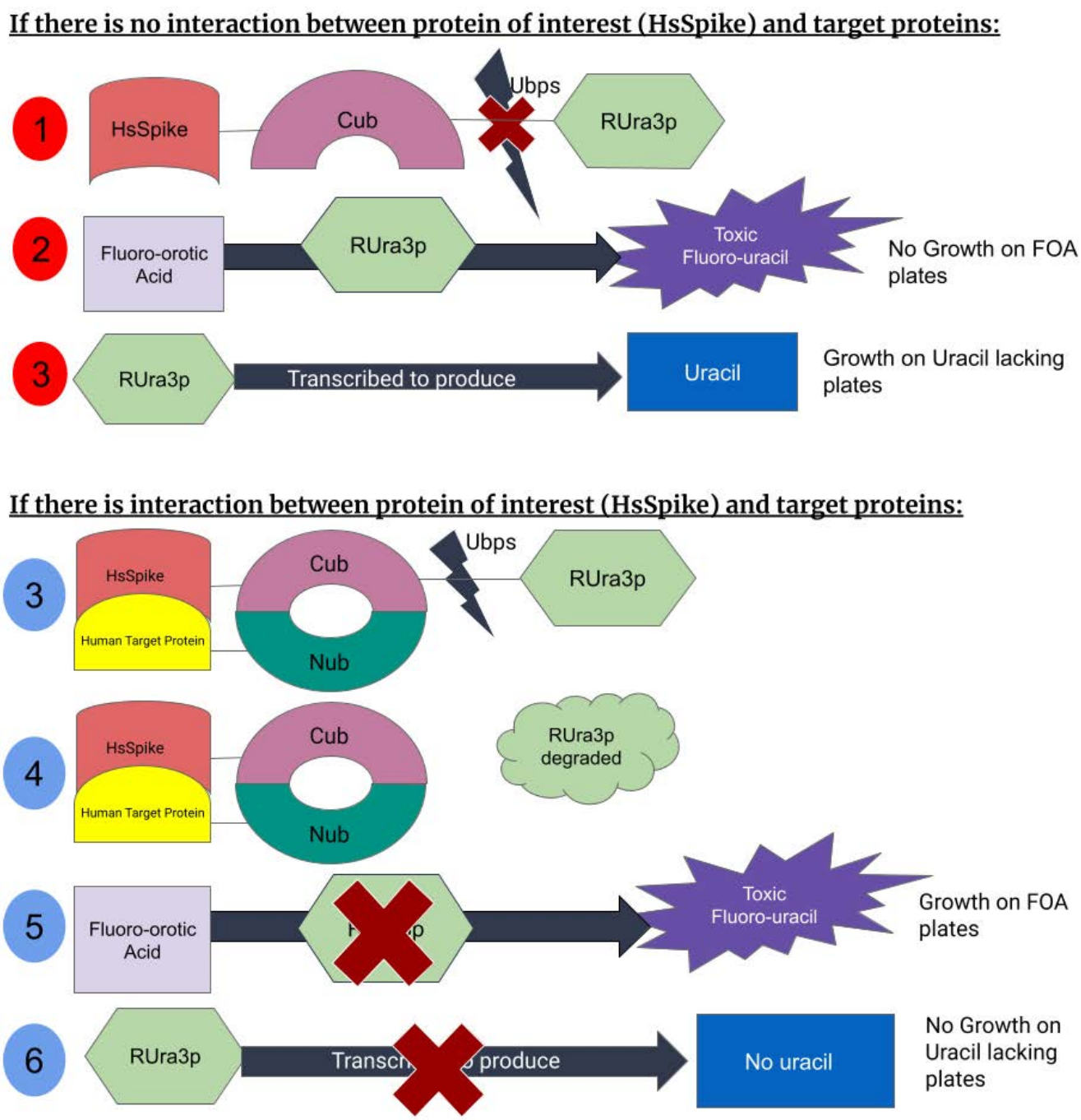

Figure 23: Diagram of Split-Ubiquitin Assay.

1. If there is no interaction between target protein and protein of interest, Ubps does not cleave the fused proteins and RUra3p is still fused to Cub.
2. RUra3p converts harmless fluoroorotic acid into toxic fluorouracil, which kills the cells, thus there is no growth on plates with fluoroorotic acid.
3. Since RUra3p is still fused to Cub, cells can produce their own uracil, thus there is growth on plates lacking uracil.

4. If there is an interaction between protein of interest (HsSpike) and target, the the Nub and Cub ends of Ubiquitin fuse back together, causing the cell to recognise the fused Nub and Cub as Ubiquitin and Ubps will cleave peptide bond between Cub and RUra3p.
5. The RUra3p is then degraded by the N-end Rule, which states that a protein degrades through recognition of the N-terminal residue of proteins, if a protein starts with Arginine (R) it is not stable and will be degraded through the 26s Proteasome pathway.
6. As Ubps cleaved the peptide bond between RUra3p and Cub, RUra3p is no longer fused to Cub, causing it to be degraded by the N-end Rule. This causes the cell to no longer be able to produce Uracil, and thus the cells cannot grow on plates lacking Uracil

RESULTS AND DISCUSSION

Full-length (FL) SARS-CoV-2 Spike protein as well as its N-terminal half (1-541) and its C-terminal half (542-1273) were fused to the N-terminal half of ubiquitin (Nub) and to the C-terminal half of ubiquitin that had been extended by the RUra3 reporter (CubRUra3), respectively. The Spike cDNA that had been used as template for the PCR to generate the Split-Ub fusions had been provided by a commercial company. Figure 24 shows that Spike FL was successfully fused to CubRUra3 for plasmid 4, Spike (1-541) for plasmid 8, and Spike (542-1273) for plasmids 9, 10 and 11. All plasmids were confirmed by DNA sequencing of the inserts.

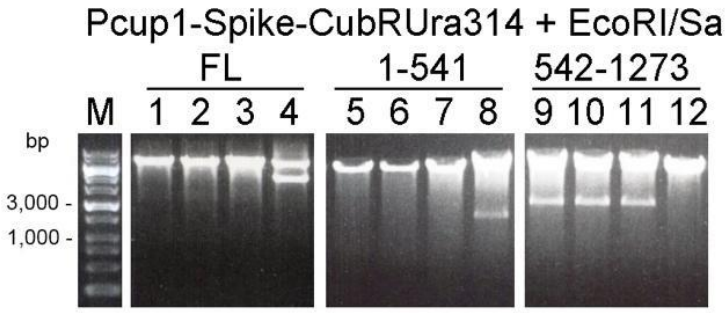

Figure 24: Spike is successfully cloned into the Pcup1-CubRUra314 plasmids. DNAs encoding the respective parts of Spike were amplified by PCR with the forward primer containing an EcoRI site and the reverse primer containing a SalI site. Successful cloning of PCR fragments into the Pcup1-CubRUra314 plasmid was confirmed by cutting the plasmids with EcoRI and SalI.

Figure 25 shows that Spike interacts with itself in the Split-Ub assay, as the co-expression of Nub-Spike under the control of the *ADH1* promoter with Spike-CubRUra3 under the control of the *CUP1* promoter resulted in reduced growth on the plate lacking uracil and resistance to FOA (compare lines 1 and 3 with line 2).

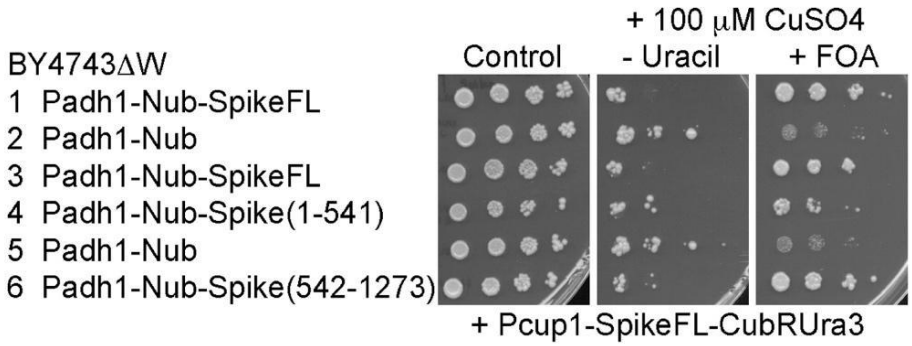

Figure 25: SpikeFL interacts with itself at both the N-terminal and C-terminal halves. *BY4743ΔW* cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days. Protein interaction results in the degradation of RUra3, which means that the cells display reduced growth on the plate lacking uracil and resistance to 5-fluoroorotic acid (FOA).

Next, human Ace2 was fused to Nub and the co-expression with Spike-CubRUra3 resulted in resistance to FOA (Figure 26, line 1), confirming that HsAce2 interacts with Spike. The cDNA derived from human lung cells that was used as a template for the PCR to generate the Nub-HsAce2 fusion had been provided by a commercial company. Surprisingly, the Split-Ub assay revealed not just the well-documented interaction between the N-terminal half of Spike and HsAce2, but it showed that the C-terminal half of Spike interacted with HsAce2 as well (Figure 26, line 1).

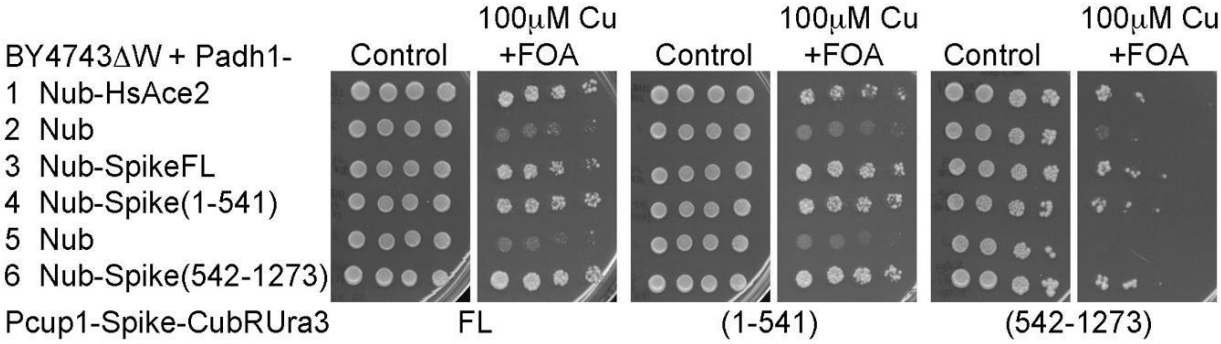

Figure 26: Spike interacts with HsAce2 at both the N-terminal and C-terminal halves. *BY4743ΔW* cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days.

In order to identify proteins that are targeted by Spike inside its human host cell, we fused cDNA libraries derived from human B cells and Lung cells to Nub using a PCR-based strategy (Figure 27) and screened the libraries with Spike-CubRUra3 as a bait.

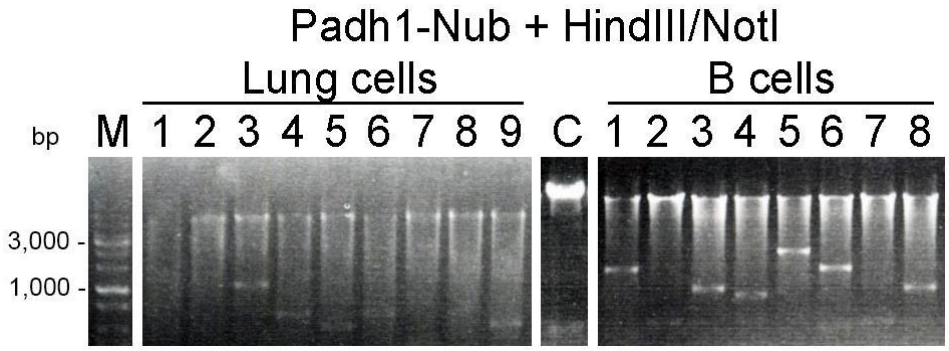

Figure 27: Gel electrophoresis pictures of the constructed Nub cDNA libraries. cDNA libraries derived from human lung and B cells were amplified by PCR with a forward primer that contained a random hexamer downstream of a BglII or BamHI site and a reverse primer that contained a NotI site downstream of an oligodT. Successful cloning of PCR fragments cut with BglII (BamHI)/NotI into the Padh1-Nub plasmid followed by electroporation was confirmed by cutting plasmids of randomly picked colonies with HindIII/NotI (which includes the DNA encoding Nub). The empty Padh1-Nub vector served as control (C). The cDNA libraries consist of about 1 million independent colonies which were washed off 100 plates followed by DNA isolation.

In addition, cDNA libraries derived from mouse tissue culture cells and mouse embryonic stem cells were fused to Nub using the same method and screened with MHVS-CubRUra3 (Mouse Hepatitis Virus Spike protein fused to CubRUra3). Approximately 100,000 primary transformants were plated onto FOA plates and the plasmids from colonies arising on the FOA plates were isolated and tested for plasmid-linkage. Confirmed plasmids were sequenced and revealed two kinds of results. Firstly, the screens had been able to isolate cDNAs from human and mouse mRNAs generating in-frame protein fusions to Nub by their ability to bind to Spike or to MHVS and to confer growth on FOA plates. Secondly, synthetic peptides derived from the non-coding parts of the mRNAs that are unlikely to encode real proteins were also isolated in the screens. While these synthetic peptides do not reveal anything about the biology of the virus, they might have application potential if they are able to interfere with its replication cycle. Figure 28 shows three peptides that had been isolated with full-length Spike. FL3b is a 42-amino acid peptide representing the C-terminus of human Cytochrome b (CytB), while FL1a and FL7a are synthetic peptides derived from the non-coding parts of human mRNAs. CytB is part of the electron transport chain inside mitochondria. Caspase 8 cleaves CytB inside the mitochondria and the C-terminal half of CytB is exported to the cytosol where it triggers apoptosis (Komarov et al., 2008). We hypothesize that the virus could promote apoptosis of the cell that it has just infected by binding to the C-terminus of CytB using its Spike proteins. Figure 28 further shows that the peptides interacted with the original Spike protein as it was found in Wuhan as well as with the D614G mutant protein that arose in Europe and US (Korber et al., 2020). Cells co-expressing the peptides together with Spike-CubRUra3 were completely unable to grow on the plate lacking uracil, indicating that the interactions between the peptides and Spike are stronger than the Spike-Spike interactions, as those cells had just displayed reduced growth on the plate lacking uracil (Figure 25).

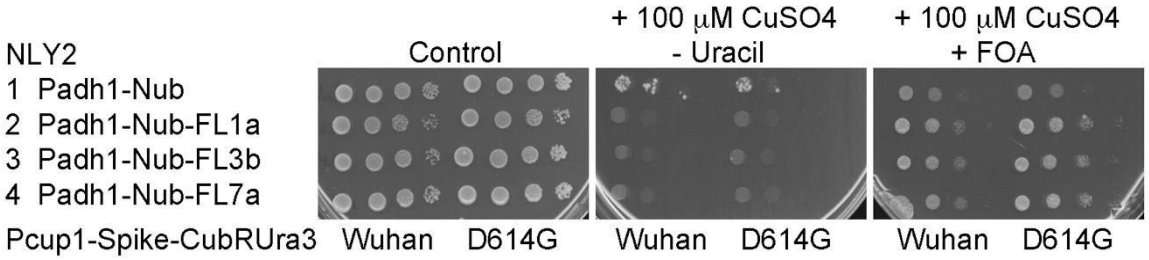

Figure 28: FL3b and synthetic peptides FL1a and FL7a interact with the original Spike and the D614G mutant Spike. *NLY2* cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days. *NLY2* had been used for the screen as it has a higher rate of transformation as compared to *BY4743ΔW*.

Figure 29 shows that all three peptides interacted with the C-terminal half of Spike (542-1273) and not with the N-terminal half of Spike (1-541). The figure further shows that all three peptides interacted with the C-terminus of Spike (930-1273) and not with Spike (542-929), indicating that those interactions are specific.

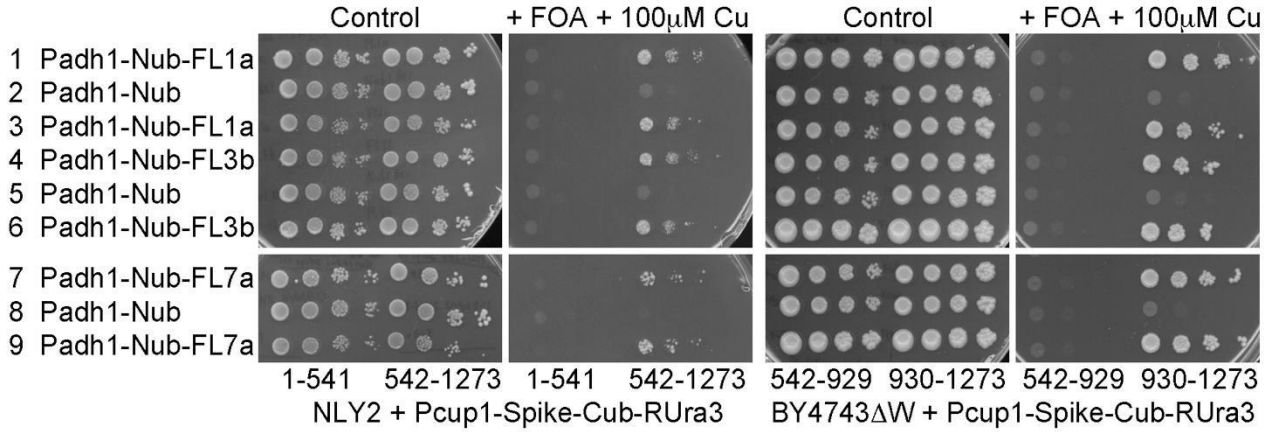

Figure 29: FL3b and synthetic peptides FL1a and FL7a interact with Spike at amino acids 930-1273. Cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days.

Peptides FL3b and FL7a were over-expressed from the *HIS3*-marked multi-copy vector *PactT423* under the control of the strong *ACT1* promoter/terminator cassette. Figure 30 shows that they were able to block the Spike(542-1273)-Spike(542-1273) interaction and to prevent the growth of cells co-expressing Nub-Spike(542-1273) and Spike(542-1273)-CubRUra3 on the FOA plate (compare lines 3 and 4 with the other four lines). We are planning to confirm the competitions *in vitro* and to test peptides FL3b and FL7a for their ability to reduce viral replication in tissue culture cells.

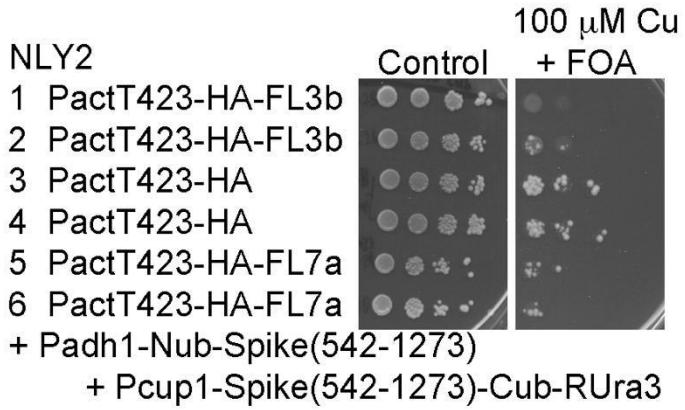

Figure 30: FL7a and FL3b are able to block the Spike(542-1273)-Spike(542-1273) self-interaction. *NLY2* cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days.

A second human protein that we identified as a novel Spike-interacting protein in our screens was Annexin2A2, and Figure 31 shows that cells co-expressing Nub-Annexin2A2 and Spike-CubRUra3 were able to grow on the plate containing FOA. Surprisingly, Annexin2A2 was found to bind both to the N- and the C-terminal halves of Spike. Further deletion analysis, however, indicated that Annexin2A2 bound to the C-terminus of Spike (930-1273) and not to Spike (542-929), suggesting that both interactions of Annexin2A2 with the N- and the C-terminal halves of Spike had been specific. We are planning to confirm these protein interactions *in vitro* and to test if Annexin2A2 is able to block the Spike-Ace2 interaction. Annexin2A2 is involved in both endocytosis and exocytosis (Gerke and Moss, 1997) and we hypothesize that the protein interactions of Spike with Annexin2A2 might help the virus to enter and exit the cell.

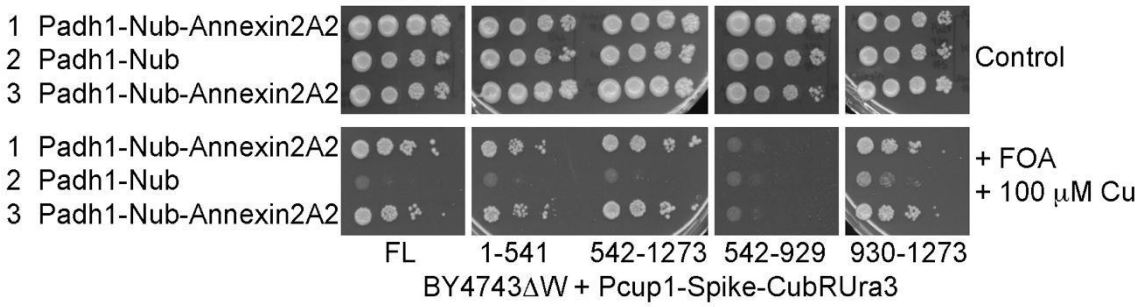

Figure 31: Annexin2A2 interacts with SpikeFL, N-terminal half, C-terminal half and C-terminus of SpikeFL. Cells of the indicated genotype were tenfold serially diluted, spotted onto the depicted plates and incubated at 28°C for six days.

Before we can confirm the protein interactions of Spike with Annexin2A2 and Cytochrome b as well as with the synthetic peptides FL1a and FL7a *in vitro*, we have to express all these proteins *in E. coli* cells and confirm that they are soluble. We fused different portions of Spike to the maltose-binding protein MBP and found that they were soluble when expressed in *E. coli* cells (Figure 32, parts A and B), even though to various degrees. GST fused to FL1a and FL7a was soluble when expressed in *E. coli* cells, while GST fused to the C-terminal 42 residues of Cytochrome b was not well expressed (Figure 32, part C).

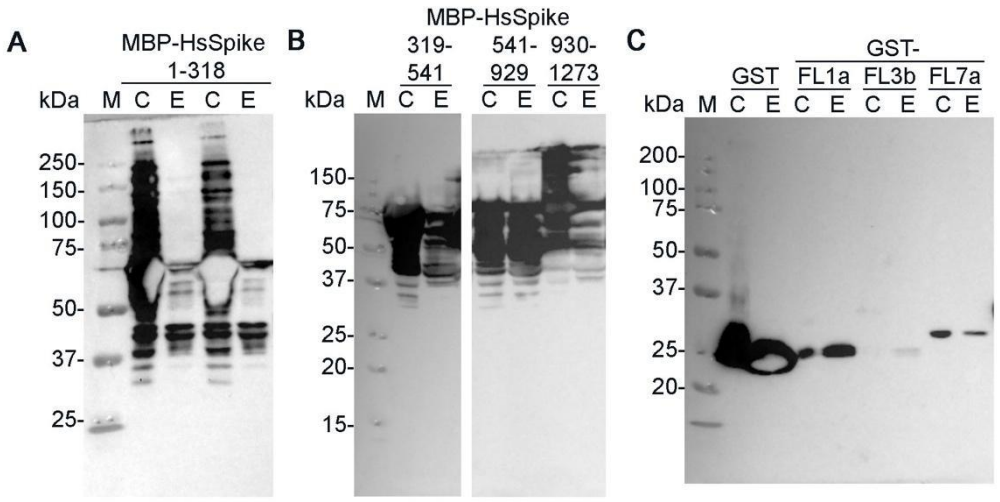

Figure 32: MBP-Spike, GST-FL1a, GST-FL3b and GST-FL7a are soluble. GST-FL1a and GST-7b were soluble when expressed in *E. coli* cells. *E. coli* cells expressing the indicated fusions were broken by freeze/thaw and aliquots were mixed with SDS loading dye before (C = Cells) or after centrifugation (E = Extract). (A) MBP-Spike (1-318) is soluble. (B) MBP-Spike (319-541), MBP-Spike (541-929) and MBP-Spike (930-1273) are soluble. (C) GST-FL1a and GST-FL7a are soluble, while GST-FL3b is not well expressed but soluble.

FUTURE WORK

For future work, we would like to confirm *in vitro* with the help of GST pulldown assays that Spike interacts with the human proteins Annexin2A2 and Cytochrome b as well as with the synthetic peptides FL1a and FL7a. For Annexin2A2, we would like to test if it can block the Spike-Ace2 interaction in the Split-Ubiquitin Assay as both Ace2 and Annexin2A2 bind to the N-terminal half of Spike. During viral infection, the binding of Spike to Ace2 takes place outside the cell, but one could inject Annexin2A2 into the bloodstream in case it is found to block Spike from binding to Ace2. For the synthetic peptide FL1a, we would like to test if it also can block the self-interaction of the C-terminal halves of Spike in the Split-Ubiquitin Assay. We found that Spike interacts with Cytochrome b, whose cleavage by Caspase 8 triggers apoptosis indicating that the virus prevents apoptosis by binding to the C-terminal half of Cytochrome b. Since Cytochrome c is also involved in apoptosis, it might be interesting to find out if Spike interacts with Cytochrome c as well. In order to confirm our hypothesis, we can express Spike in tissue culture cells and test if it can delay or block apoptosis. Finally, we could express the synthetic peptides in tissue culture cells and test if they are able to delay or prevent viral replication. As working with SARS-CoV-2 has to take place in the BSL3 facility to which we do not have access, we could use the mouse virus MHV as a surrogate model.

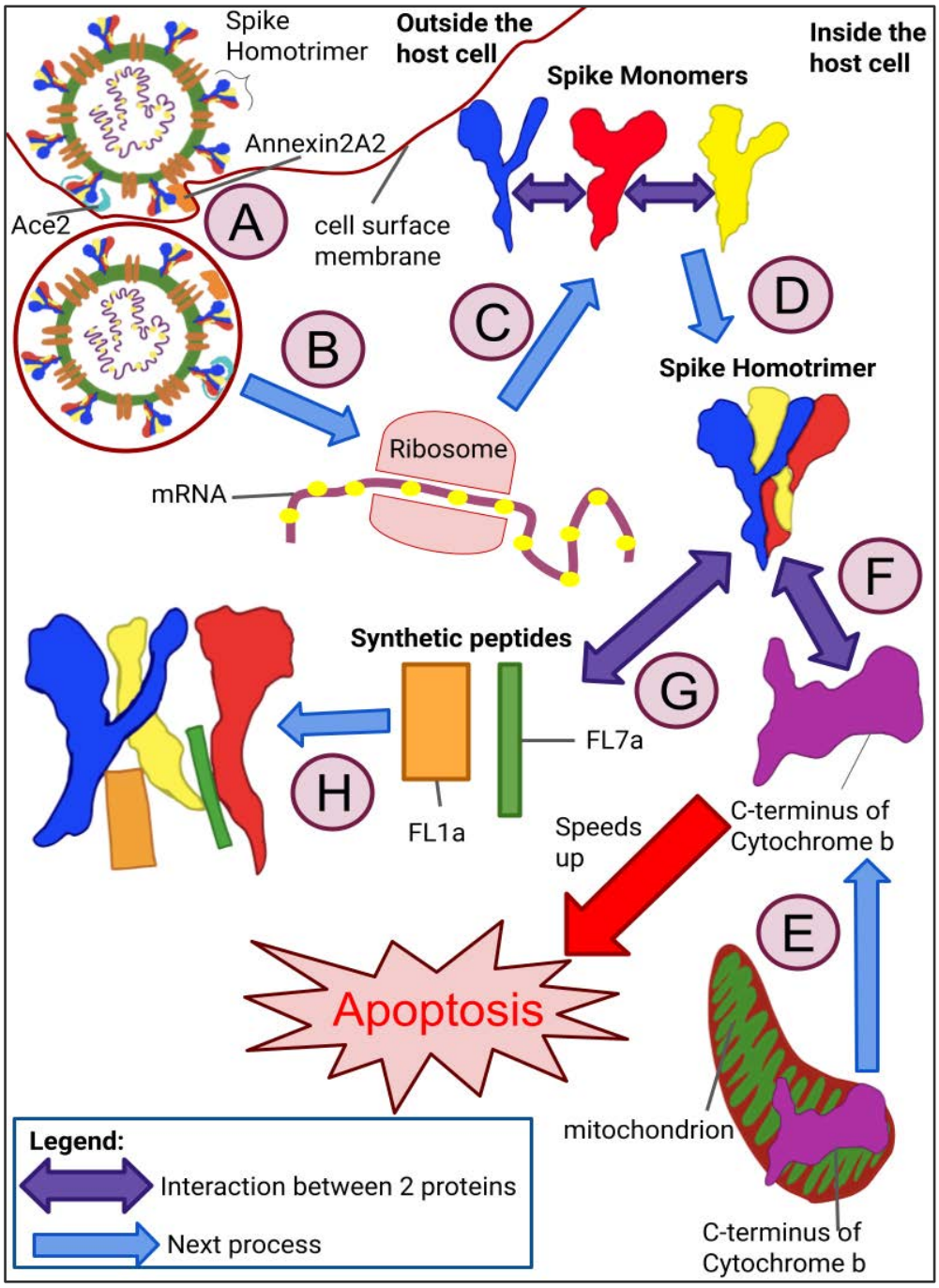

Figure 33: Our Model - A Visualisation of how Spike protein of SARS-CoV-2 aids viral entry, evasion of immune response, viral replication and reassembly and viral exit. (A) As the SARS-CoV-2 nears the cell surface membrane, the Spike glycoprotein of the virus binds to Ace2 on the cell surface membrane. The C-terminus of the Spike protein also binds to the C-terminus of Annexin2A2, helping SARS-CoV-2 enter the cell. **(B)** Once SARS-CoV-2's viral membrane and the cell surface membrane fuses together, the virus enters and uncoats, releasing its single-stranded RNA genome into the cytoplasm. **(C)** The RNA is translated using human ribosomes, eventually producing the Spike glycoprotein monomers. **(D)** The Spike monomers interacts with each other to then assemble to form a Spike homotrimer. **(E)** Alarmed by the entry of the virus, the cell causes caspase 8 cleaves Cytochrome b and its C-terminal half is exported to the cytosol. **(F)** The C-terminus of the Spike homotrimer interacts with the C-terminus of Cytochrome b. This speeds up apoptosis, allowing the cell to die faster so that the likelihood that T cells will detect viral proteins being presented on the cell surface membrane will be lower, allowing the virus to evade the immune response and exit the cells after replication. **(G)** We found 2 synthetic peptides that can bind to the C-terminus of the Spike glycoprotein, FL1a and FL7a. **(H)** FL7a disrupts the interaction between the Spike monomers, preventing a Spike homotrimer from being assembled. We hypothesize that FL1a also does the same.

CONCLUSION AND APPLICATIONS OF PROJECT

We showed that Spike proteins interact with itself, establishing that the Spike protein monomers interact with each other to form the Spike homotrimer (Figure 24). We also found Annexin2A2, a human protein that interacts with Spike (Figure 31). In addition, we identified Cytochrome b, another human protein that interacts with Spike (Figure 28 and 29).

We illustrated a model (Figure 33) that documents our current understanding of how the Spike protein of SARS-CoV-2 aids viral replication after it has used Ace2 (Huang et al., 2020) to dock onto the human cell. The protein interaction of Spike with Annexin2A2 helps to trigger endocytosis and the virus enters the infected cell (Figure 1; Figure 33A). Next, the viral reverse transcriptase makes a DNA copy of the viral RNA and the host RNA polymerase starts viral replication. Alerted by these reactions, Caspase 8 tries to start apoptosis by cleaving CytB (Komarov et al., 2008) and the C-terminal half of CytB is exported from the mitochondria to the cytosol. Spike interacts with the C-terminus of Cytochrome b to speed up the process of apoptosis, reducing the probability that an immune cell can detect the virus through viral proteins presented on the cell surface membrane (Figure 33F). Finally, new Spike proteins assemble and Annexin2A2 aids progeny viruses to exit the cell.

We showed that synthetic peptide FL7a was able to block interactions between Spike and itself (Figure 30; Figure 33H). By extension, the synthetic peptides FL1a and FL7a might be able to block the interactions between Spike and its human target proteins, such as CytB, or itself, preventing the assembly of a Spike homotrimer. These synthetic peptides could then be used as drugs to prevent assembly of a Spike homotrimer or to prevent interactions between Spike and target proteins. These potential drugs can be used, in conjunction with vaccination efforts, to induce a more effective and targeted therapeutic treatment for patients infected with SARS-CoV-2. Vaccines only help human host cells recognise the foreign antigens on the Spike protein. This would induce host cells to produce antibodies that bind to the foreign antigens, stopping them from entering more cells (Centre of Disease Control and Prevention, 2021). When a vaccinated person is infected, although they may not fall sick, they can still transmit the SARS-CoV-2 virus to others (National Centre for Infectious Diseases, 2021). However, if they take drugs containing the synthetic peptides, the rate of assembly of new viruses in host cells could be reduced, thus reducing the number of new viral particles produced and hence, the rate of transmission of COVID-19. To add on, by reducing viral assembly and replication, these drugs may also be used on other infected patients, slowing the progression of COVID-19 to life-threatening stages as the rate of spread to other cells in the body could be reduced.

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【評語】090025

1. The study is a well-study project with good presentation
2. How do those mutant proteins FL-3b or FL7a interacts with spike protein？ Does the cell express those two proteins since those are ncRNAs.
3. The physiological role of Annexin2A2 should be determined.