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Optimization of Enzyme-Linked Immunosorbent Assay (ELISA) Format Utilizing SpyTag/SpyCatcher- Interaction To Capture Mammalian Cell-Expressed Protein Antigens

Metropolia University of Applied Sciences

Bachelor of Laboratory Services

Laboratory Science

Bachelor's Thesis

7 December 2025

Abstract

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Title:	Optimization of Enzyme-Linked Immunosorbent Assay (ELISA) Format Utilizing SpyTag/SpyCatcher- Interaction To Capture Mammalian Cell-Expressed Protein Antigens
Number of Pages:	36 pages + 7 appendices
Date:	7 December 2025
Degree:	Bachelor of Laboratory Services
Degree Program:	Laboratory Science
Supervisors:	Jussi Hepojoki, Docent in Virology, University of Helsinki Tomas Strandin, Docent in Virology, University of Helsinki Tiina Soininen, Senior Lecturer, Metropolia

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The aim of this thesis was to optimize an ELISA (enzyme-linked immunosorbent assay) format that utilizes SpyTag/SpyCatcher interaction to immobilize recombinant protein antigens expressed in mammalian cells. This approach offers an alternative to conventional antibody-based immobilization and enables the use of unpurified recombinant antigens without the need for target-specific capture antibodies. HEK293T cells were employed for protein expression, and optimal incubation time was determined by observing the ratio between target antigen and total protein.

ELISA optimization focused on testing variables for coating microplates with SpyCatcher and blocking nonspecific binding sites. The experiments compared reagents and materials, incubation times and temperatures, SpyCatcher concentrations, and antigen lysate dilution ratios. Antibody specificity and background signal levels were evaluated to distinguish analyte-specific signal from nonspecific signal.

The best results were achieved when wells were coated with 2 µg/mL SpyCatcher in carbonate–bicarbonate buffer at +37 °C for 1.5 hours, followed by blocking with milk powder. These conditions minimized background and enhanced specific signal detection. The developed method provides a practical tool for identifying pathogen antigens and studying antibody responses, particularly in cases where validated antibodies are unavailable.

Keywords: SpyTag-SpyCatcher interaction, ELISA assay, sandwich ELISA, antigen recognition, serum antibodies

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Tiivistelmä

Tekijä:	Sari-Anna Lämsä
Otsikko:	Entsyymivälitteisen immunosorbenttimääritys (ELISA) -formaatin optimointi hyödyntäen SpyTag-SpyCatcher-interaktiota nisäkässoluissa tuotettujen proteiiniantigeenien kiinnittämiseen
Sivumäärä:	36 sivua + 7 liitettä
Aika:	7.12.2025
Tutkinto:	Laboratorioanalyytikko (AMK)
Tutkinto-ohjelma:	Laboratorioanalyytikko
Ohjaajat:	Dosentti Jussi Hepojoki Dosentti Tomas Strandin Lehtori, Tiina Soininen

Tämän opinnäytetyön tavoitteena oli optimoida ELISA (entsyymivälitteinen immunosorbenttimääritys) -formaatti, joka hyödyntää SpyTag/SpyCatcher-vuorovaikutusta nisäkässoluissa tuotettujen rekombinanttiproteiiniantigeenien immobilisoimiseksi. Tämä lähestymistapa tarjoaa vaihtoehdon perinteiselle vasta-ainepohjaiselle immobilisoinnille ja mahdollistaa uusien, puhdistamattomien antigeenien tunnistamisen ilman validoituja vasta-aineita. HEK293T-soluja käytettiin proteiinien ilmentämiseen, ja optimaalinen inkubointiaika määritettiin tarkastelemalla kohdeantigeenin ja kokonaisproteiinin välistä suhdetta.

ELISA-optimoinnissa keskityttiin testaamaan muuttujia mikrolevyjen päällystämisessä SpyCatcherilla sekä epäspesifisten sitoutumiskohtien blokkaamiseen. Kokeissa verrattiin käytettyjä reagensseja ja materiaaleja, inkubointiaikoja ja -lämpötiloja, SpyCatcher-pitoisuuksia sekä antigeenilysaattien laimennussuhteita. Käytettyjen vasta-aineiden spesifisyyttä ja taustasignaalin tasoa arvioitiin, jotta voitiin erottaa analyyttispesifinen signaali epäspesifisestä sitoutumisesta.

Parhaat tulokset saavutettiin, kun kaivot päällystettiin karbonaatti–bikarbonaattipuskurilla, jossa on 2 µg/ml SpyCatcheriä, inkuboimalla levyjä +37 °C:ssa 1,5 tunnin ajan ja blokkaamalla kaivot maitojauheella. Nämä olosuhteet vähensivät taustasignaalia ja paransivat spesifisen signaalin havaitsemista. Kehitetty menetelmä tarjoaa käytännöllisen työkalun patogeeniantigeenien tunnistamiseen ja vasta-ainereaktioiden tutkimiseen erityisesti tilanteissa, joissa validoituja vasta-aineita ei ole saatavilla.

Avainsanat: SpyTag/SpyCatcher-interaktio, ELISA määritys, Sandwich ELISA, antigeenitunnistus, seerumi vasta-aineet

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1 Introduction

The detection of proteins is a central challenge in molecular biology, biomedicine and immuno-sciences where antibodies are crucial elements, especially in diagnostic applications. Many widely used laboratory techniques, such as immunoblotting and enzyme-linked immunosorbent assay (ELISA), depend on the availability of target-specific antibodies and purified antigens. The lack of validated antibodies poses a significant limitation to research, particularly in cases involving proteins that undergo rapid or frequent mutations, such as virus-derived antigens. In emerging pathogen research, there is a rapid need for tools to identify pathogen-exposed or infected individuals. (Forthal, 2014).

This thesis addresses that need by developing a flexible and reproducible ELISA platform for studying antibody response against previously uncharacterized antigens based on the SpyTag–SpyCatcher interaction. The method enables immobilization of SpyTag-bearing target proteins directly onto ELISA plate wells coated with SpyCatcher, bypassing the need of capture antibodies or prior antigen purification process. The SpyTag sequence spontaneously and irreversibly binds to the SpyCatcher with high specificity, thereby theoretically enabling the assay set up. Combining the SpyTag/SpyCatcher-interaction with sandwich ELISA technique offers the potential to develop universal method indicating and quantifying different proteins without prior purifying (Baba Ahmadi et al., 2020). This approach expands the applicability of ELISA to contexts where conventional tools are not yet available. SpyTag/SpyCatcher-based immobilization of novel pathogen-related antigens enables the identification of antibodies specific to these proteins. This, in turn, facilitates the analysis of patient samples for immunological markers indicative of infection by the corresponding pathogen.

2 Background

2.1 ELISA: Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) is a widely applied analytical technique for detecting and quantifying antigens, antibodies, and other proteins in biological samples. The method relies on specific molecular recognition via interaction between target molecules and their binding partners. In its basic form, the assay involves coating microplate well surfaces with either an antibody or antigen, to immobilize the target molecule (Table 1). (Alhajj et al., 2023; Gibbs et al., n.d.).

Table 1. Most common ELISA methods and their basic functions adapted from Alhajj et al., 2023.

ELISA method	Immobilizer	Target	Binding
Direct ELISA	Antigen	Antibodies in the sample	Enzyme-conjugated primary antibody binds directly to the immobilized target antigen.
Indirect ELISA	Antigen	Antigens or antibodies in the sample	Primary antibody binds to the immobilized target antigen and enzyme-conjugated secondary antibody binds to primary antibody.
Sandwich ELISA	Antibody	Antigens in the sample	Target antigen is captured between an immobilizing antibody and an enzyme-conjugated detection antibody.
Competitive ELISA	Antigen or antibody	Antibodies in the sample	Sample antibodies compete with labelled antibodies for the same binding sites.

In sandwich ELISA, target antigen is captured between antibody layers. The ELISA microplate wells are coated with immobilizing antibodies that capture the target antigen present in sample, binding it to the well surface. An enzyme-conjugated detection antibody binds directly or indirectly through primary antibody to the immobilized target (Figure 1). Detection of the analyte is accomplished by adding a substrate that reacts with the enzyme, producing a measurable colorimetric or fluorometric signal. (Anon., 2024; Gibbs et al., n.d.). The presence of the analyte can be determined qualitatively just by comparing color change to controls, whereas its concentration is quantified by measuring the intensity of the detected signal using a spectrophotometer and comparing it to a formed standard curve. (Horlock, 2025).

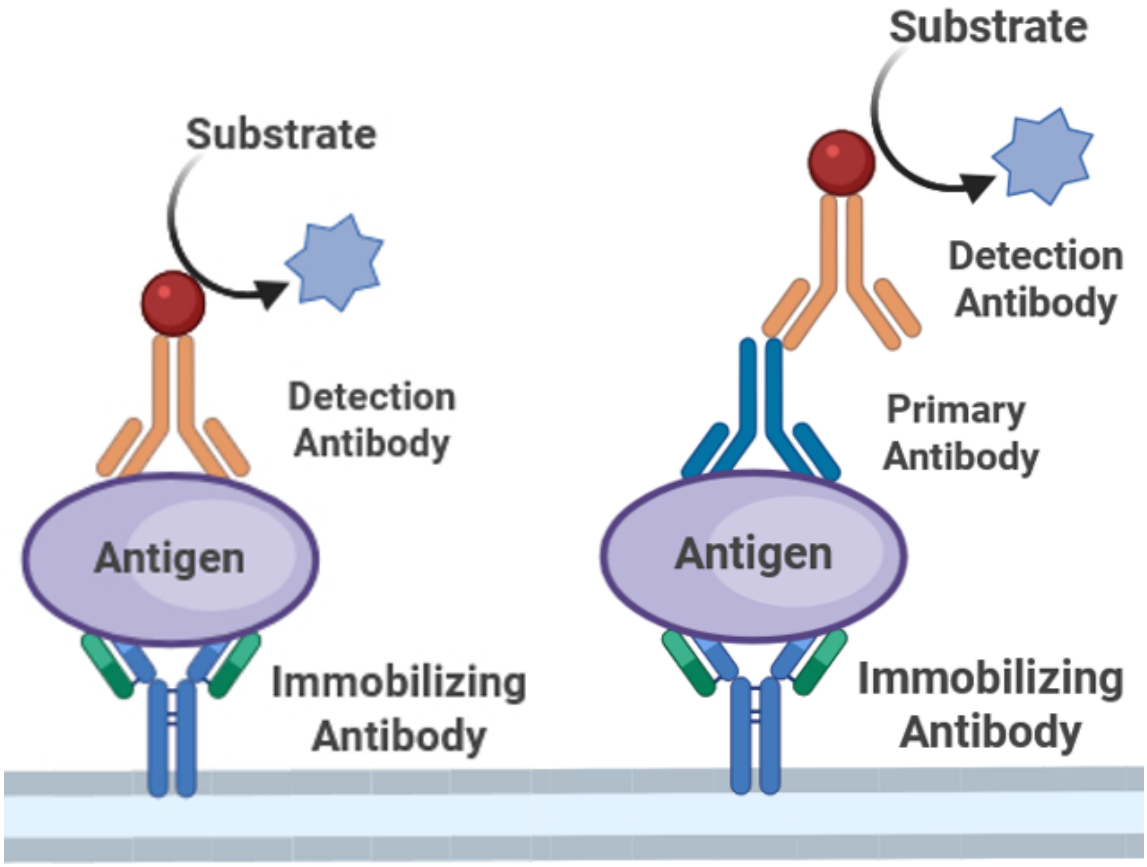

Figure 1. Principle of sandwich ELISA. An immobilizing antibody captures the target antigen. An enzyme conjugated detection antibody binds directly (left) or indirectly through primary antibody (right) to the immobilized target antigen. Upon addition of a suitable substrate, the enzyme catalyzes a reaction that generates a measurable product. (Anon., 2024; Gibbs et al., n.d.) (Image created with BioRender)

Different types of ELISA applications are widely used due to their sensitivity, selectivity, rapidity, efficiency, and easiness to handle without complex instruments (Sakamoto et al., 2017). Despite its wide range of benefits, ELISA's multi-step nature and reliance on antigen-antibody interactions make it susceptible to technical challenges that can compromise assay accuracy and reliability. (Jiang et al., 2021). Two of the most frequently reported issues are low signal (weak or absent analyte detection) and high background (non-specific signal that hide actual analyte measurement). Non-specific binding is a principal factor causing high background in ELISA. It occurs when analytes unintentionally adhere to the microplate surface or to non-target molecules in the sample matrix. This phenomenon is aggravated by incomplete blocking of unoccupied plate sites, poor antibody specificity, or the presence of cross-reactive agents. Other factors such as suboptimal incubation conditions, contaminated reagents or improper pipetting technique and inadequate washing add up to background signal level. (Drummond, n.d.; Anon., n.d.).

The issues with high background and low signal can be minimized in many ways. When washing wells between steps the volume of washing buffer in wells should always surpass the area covered with the reagent that is being washed off. In addition, tapping the plate or using absorbent tissue ensures removal of residual wash buffer, preventing analyte dilution or carryover of subsequent reagents. ELISA relies on enzymatic reaction; therefore, buffers and reagents should be free of substances that inhibit enzyme activity or interfere with antigen-antibody binding. Optimal conditions such as incubation time and temperature for each step can help reduce background and increase specific signal. Use of positive and negative controls is important to distinguish the level of the background from analyte signal and setting up robust ELISA procedures. (Drummond, n.d.; Anon., n.d.)

2.2 SpyTag and SpyCatcher

The SpyTag/SpyCatcher system originates from the fibronectin-binding protein, FbaB, found in *Streptococcus pyogenes*. It contains an immunoglobulin-like collagen adhesion domain, CnaB2, that forms an internal isopeptide bond (Reddington & Howarth, 2015; Lo et al., 2015). Mark Howarth's research group discovered that this domain could be split into two separate components that still spontaneously reconstitute this isopeptide bond when brought together (Figure 2). The interaction between these components is highly specific and does not readily occur with unrelated proteins. (Reddington & Howarth, 2015; Zakeri et al., 2012).

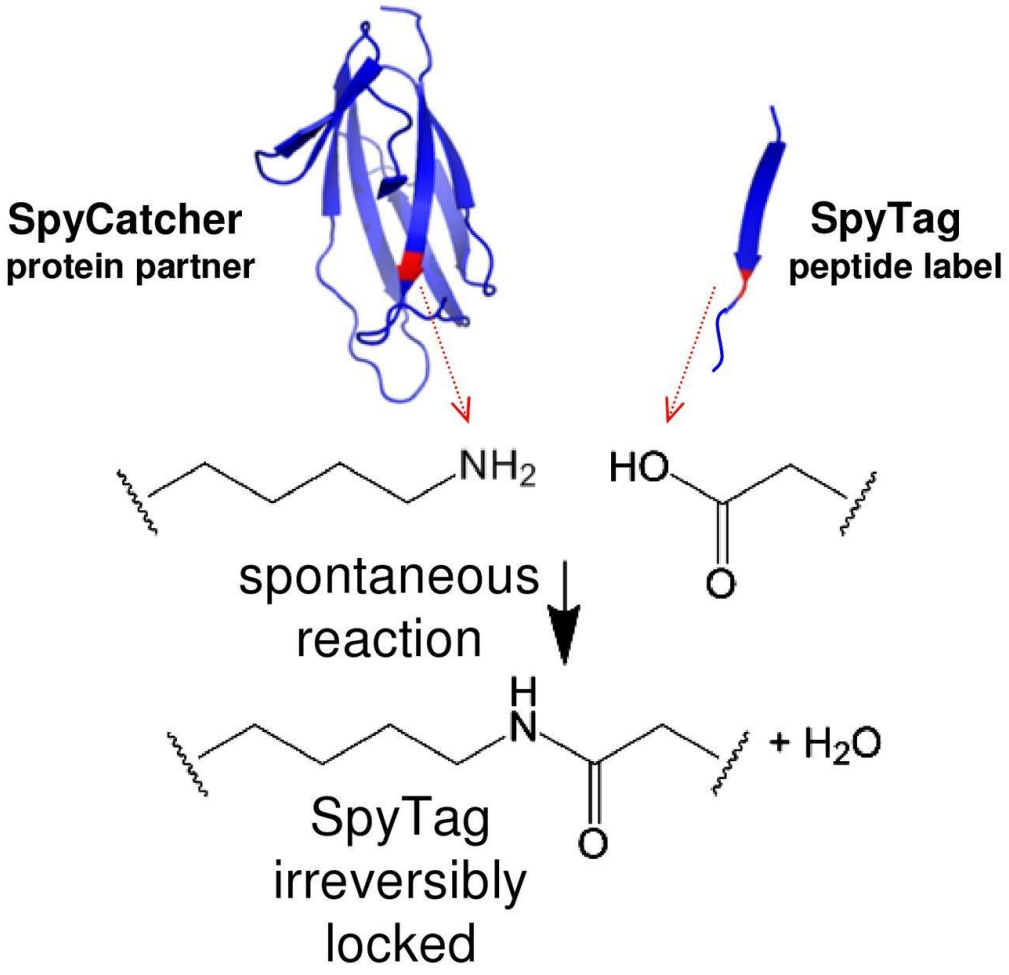

Figure 2. The SpyTag–SpyCatcher interaction involves a spontaneous covalent reaction between the ε-amino group of lysine (K31) in the SpyCatcher and the side-chain carboxyl group of aspartic acid (D117) in the SpyTag peptide, resulting in the formation of an isopeptide bond. (Hatlem et al., 2019; Zakeri et al., 2012). Image brought from Zakeri et al., 2012.

Splitting the CnaB2 domain produces unfolded 13 amino acid peptide residue, called SpyTag, and complementary 116 amino acid protein fragment, called SpyCatcher. (Reddington & Howarth, 2015). The covalent bond forms spontaneously between the ε-amine of lysine K31 (SpyCatcher) and the side chain carboxyl of aspartic acid D117 (SpyTag). (Hatlem et al., 2019; Reddington & Howarth, 2015). This bond between amino acids is classified as an isopeptide bond—a second-class peptide bond—because it is formed between amino acid side chains, rather than along the main polypeptide chain. (Hatlem et al., 2019). Structural studies have revealed that 10 of the 13 amino acids in SpyTag residue make direct contact with SpyCatcher. (Lo et al., 2015).

The original SpyTag/SpyCatcher system was first introduced in 2012, providing a specific, covalent and irreversible protein ligation method with high efficiency. Unlike most protein–protein interactions that require strictly controlled physical and chemical conditions, this “wild-type” SpyTag/SpyCatcher system worked under substrate excess conditions at 25°C–37°C and pH 5–8 without the need for cofactors or additional reagents. Reaction was approved to work in all buffers tested by Mark Howarths’ research group. (Zakeri et al., 2012; Cai et al., 2025) Since then, the system has been refined to enhance performance, reduce constraints and broaden its applications. Second generation (SpyTag002/SpyCatcher002) introduced sequence adjustments at the N- and C-termini of SpyTag to improve binding affinity, and a GAMVTT mutation in SpyCatcher to prevent unwanted self-polymerization. The modifications increased the reaction rate more than tenfold compared to the original and enabled functionality across much wider pH and temperature range. The third and latest generation (SpyTag003/SpyCatcher003) introduced further mutations that accelerated kinetics and eliminated self-reaction via enhanced surface polarity augmentation and loop stabilization. With a reaction rate approximately 400 times greater than the wild type, this variant enables rapid cell surface protein labelling, restoration of adhesion and migration functions, and remains compatible with earlier versions. (Cai et al., 2025). The key differences between generations are summarized in Table 2.

Table 2. Overview of SpyTag/SpyCatcher systems with their basic features and main modifications adapted from Cai et al., 2025.

Version	Changes	Reaction efficiency	Features and applications
Original	Basic system without mutations	$1.4 \times 10^3\ M^{-1}\ s^{-1}$	Protein ligation
SpyTag002 / SpyCatcher002	N-terminal VPT-sequence optimization C-terminus fine tuning Prevention of self-polymerisation through GAMVTT-mutation	$2.0 \times 10^4\ M^{-1}\ s^{-1}$ (12× original)	Live-cell imaging pH range 4–9 and temperature range 4–40 °C
SpyTag003 / SpyCatcher003	T112H-mutation + RG-dipeptide in N terminal (positive charge) Loop stabilization (A89P) + 4 charge mutations (e.g., T91E)	$5.5 \times 10^5\ M^{-1}\ s^{-1}$ (≈ 400× original)	Quick cell surface protein labelling Restore the ability of cell adhesion and migration Compatible with earlier versions

Fusion of proteins with either SpyTag or SpyCatcher typically function well, when they are positioned either the N- or C- terminus of the target protein (Reddington & Howarth, 2015). As Figure 3 demonstrates, these recombinant proteins are produced through gene expression in procaryotic or eucaryotic cell cultures. This process involves construction of recombinant plasmids that contain the relevant gene sequences next to each followed by their introduction to production cells via transformation or transfection (Martin & Zuk, 2025). Within cells, the transcription and translation machinery expresses these gene sequences by synthesizing recombinant protein from the introduced DNA. (Figure 3).

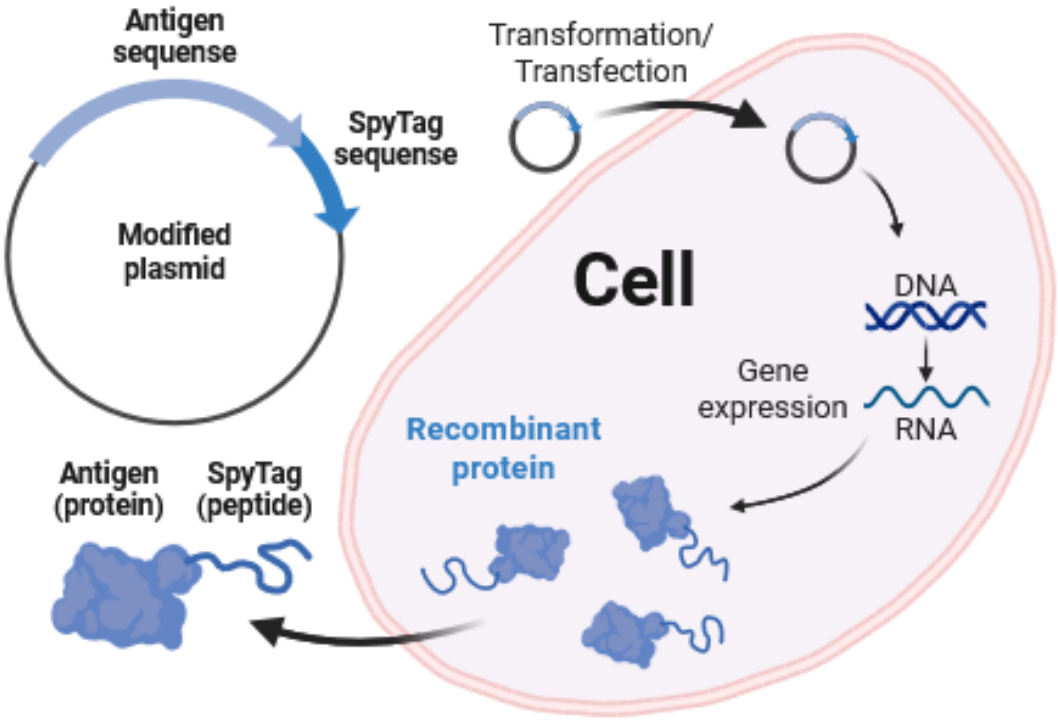

Figure 3. Production of recombinant protein with SpyTag using a modified plasmid as gene vector. After transformation or transfection, the plasmid is expressed by cellular machinery through transcription of DNA into RNA and translation into protein. (Image created with BioRender)

Procaryotes are often favored for recombinant protein production due to their cost effectiveness. They can be grown in simpler medias and exhibit faster growth rates compared to eucaryotic cells. However, procaryotes are limited in their ability to produce large and complex proteins, since they lack cellular machinery required for post transitional modifications. Regardless of cell type during expression, downstream processing (e.g., purification) inevitably results in losses in overall protein yield. The extent of protein loss increases proportionally with the number of steps in purification process. (Puetz & Wurm, 2019).

The adaptability to work in large range of different physical and chemical conditions makes the SpyTag–SpyCatcher system highly suitable for diverse applications outside of cellular environments (Zakeri et al., 2012). Over the years SpyTag/SpyCatcher system has been utilized in many different functions such as localizing bacterial proteins vaccine optimization, immunoregulatory functions and engineering biomaterials via bioconjugation. (Lo et al., 2015; Hatlem et al., 2019; Toledo-Garcia et al., 2025). The modified version of sandwich ELISA takes

advantage of SpyTag/SpyCatcher system as an immobilizer**.** Unlike in the conventional sandwich ELISA (Section 2.1, Figure 1, page 3), where the well surface is coated with antibody, this format coats the wells with the SpyCatcher protein. The target antigen - recombinant protein including a SpyTag sequence - binds spontaneously and specifically to the SpyCatcher protein (Baba Ahmadi et al., 2020). Primary antibody binds to the immobilized antigen. An enzyme-conjugated detection antibody is then added, which binds to the previously added primary antibody. For example, antigens from patient serum sample binds to immobilized antigen from unfamiliar pathogen, followed by detection antigen specific for human-derived antibodies. As a result, the target antigen becomes sandwiched between the SpyTag/SpyCatcher system and antibodies (Figure 4).

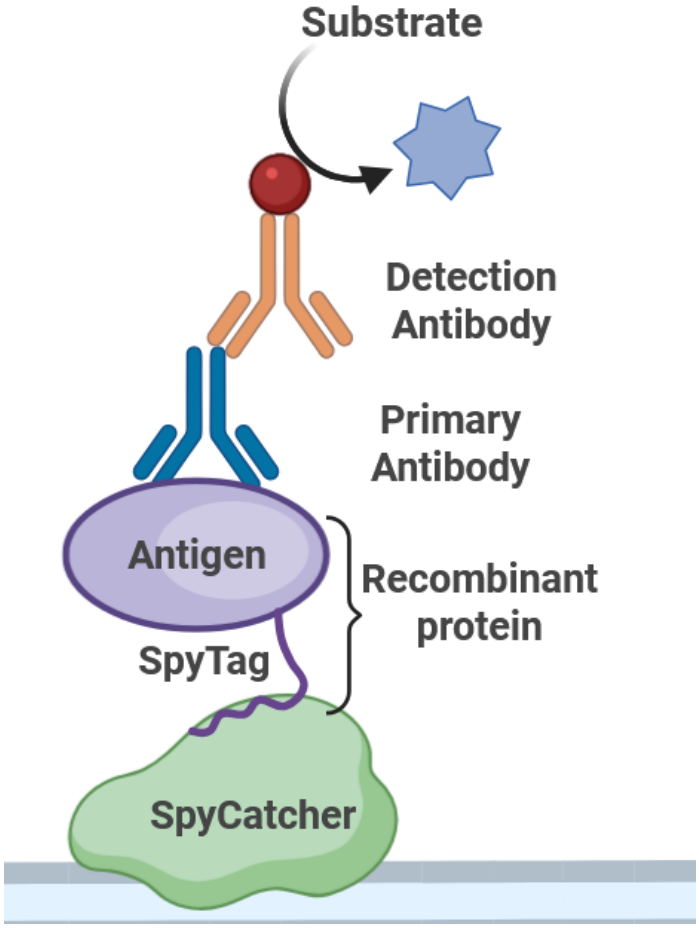

Figure 4. Principle of the modified sandwich ELISA utilizing SpyCatcher-SpyTag interaction. ELISA microplate wells are coated with the SpyCatcher protein to which the SpyTag peptide fused with the target antigen binds to. The Primary antibody will bind to the target antigen while the enzyme conjugated detection antibody binds to the primary antibody. Upon addition of a suitable substrate, the enzyme catalyses a reaction that generates a coloured product. (Image created with BioRender)

Structurally the assay resembles typical sandwich ELISA, but the coating step deviates from the conventional implementation of the method. The advantages of this method are that there is no need for purification of the produced recombinant protein nor to have target specific immobilizing capture antigens. Any protein of interest can be fused with SpyTag, as shown in Figure 3 (Section 2.1, page 8), and immobilized to the ELISA wells surface with SpyCatcher.

Despite many advantages, there are several limitations and practical challenges that should be considered when using SpyTag/SpyCatcher. Because system is derived from bacterial protein, it might cause an immune response that compromises assay specificity, reduce therapeutic effectiveness, or affect its usage with vaccines. (Hatlem et al., 2019). The efficiency of bond formation depends critically on the steric accessibility and relative orientation of the reactive residues meaning that SpyTag peptide and SpyCatcher protein must be brought into proximity for reaction to occur. Dense coating or poor insertion of SpyTag or SpyCatcher in fusion proteins may fully or partially cover the reactive sites and restrict interaction between SpyTag and SpyCatcher. Fusion of SpyTag/SpyCatcher to a target protein can disrupt the target's native folding, solubility and stability, potentially promoting misfolding or aggregation and thereby impairing both the function of target protein and the reaction between SpyTag and SpyCatcher. (Krajcovicova et al., 2025).

3 Materials and methods

Experiments conducted for this thesis were divided into two main parts. The first part of the thesis focuses on studying SpyTag003-bearing recombinant protein expression in mammalian cells, with the aim of identifying the ideal collection time of cell lysates following transient transfection. The second part of thesis focuses on optimization of SpyTag/SpyCatcher-based ELISA protocol coating and blocking conditions to produce ready-to-use SpyTag–SpyCatcher–based ELISA plates. The resulting protocols were compiled for use by the Helsinki University laboratory personnel, either as provided or adapted for specific experimental needs.

3.1 Protein production

HEK 293T cells (human embryonic kidney cells) were used for protein expression for their high transfectability and rapid growth. Mammalian cells were used to ensure the antigens are folded in proper structure. The cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 200 mM L-glutamine. Cells were transfected with plasmids encoding recombinant viral proteins fused with a SpyTag sequence in C-terminus. Plasmid constructs can be seen in Figure 5. Plasmids were already created, purified and sequence-verified before the start of this thesis.

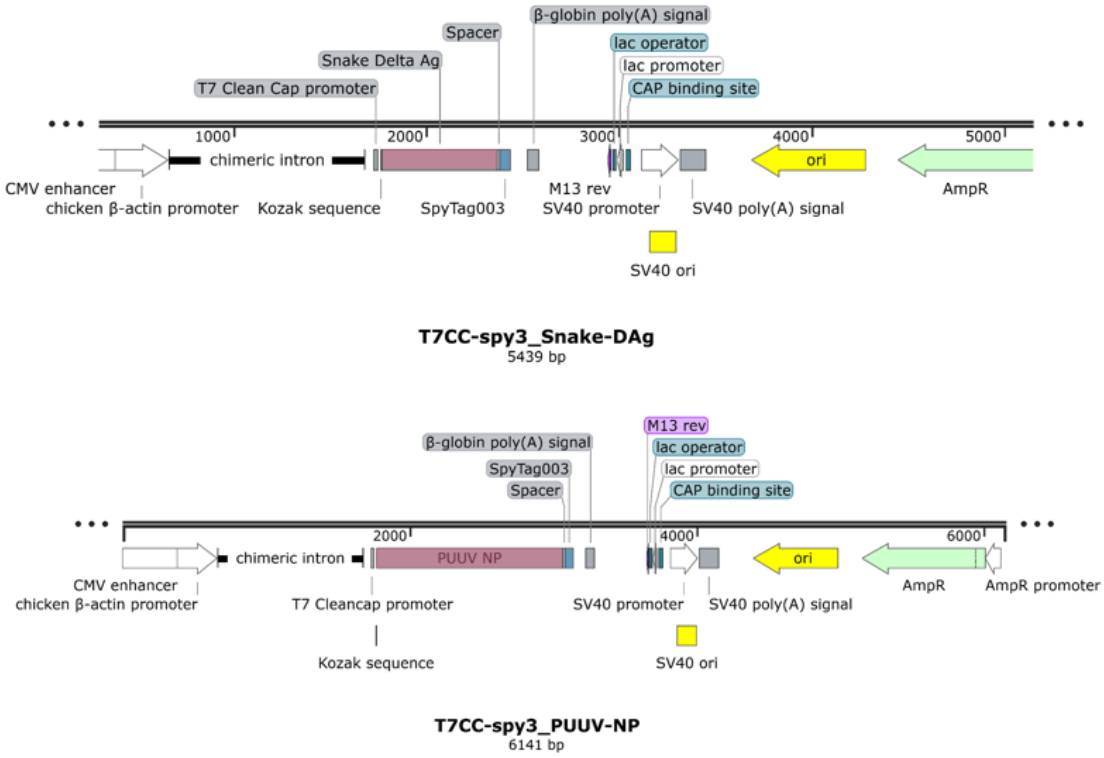

Figure 5. Plasmid constructs used in transfections (Snake Delta virus antigen (top), Puumala nucleocapsid protein (bottom)). The target antigen sequence is marked with red color and the SpyTag003 sequence with blue color. Sequences are connected with spacer, that ensures the SpyTag peptide will not be sterically hindered from interacting with SpyCatcher. T7CC refers to the T7 clean cap promoter.

The expressed constructs included two SpyTag-fused proteins (antigens) of different sizes: Puumala virus nucleocapsid protein (PUUV NP) and a Snake Delta antigen (Snake DAg). The transfection protocol is provided in Appendix 1. Used transfection reagent: DOPE:DOTPA (1:0,5) (Ojha et al., 2025).

Expression of target antigens was tested between 1–4 days of incubation. Following incubation, cells were harvested from 6-well plate surfaces by first removing media and then detaching with PBS. Cells were washed three times by taking turns of centrifuging cells for 3 minutes with 500 g and resuspending cells to PBS. Finally, cells were pelleted by centrifuging and were divided into two handling schemes: (1) lysed immediately and stored as lysates, and (2) frozen as cell pellets and lysed after thawing prior to analysis. Samples were frozen and stored at - 20 °C. The lysis was performed using RIPA buffer supplemented with protease inhibitor cocktail (PIC: Sigma Aldrich, REF:11873580001) (ingredients of RIPA and volume of the lysis buffer can be seen in appendix 2).

Total protein concentration was determined with Thermo Scientific Pierce™ BCA protein assay kit (Ref: 23227) using microplate procedure. Presence of the recombinant target proteins in lysates was confirmed by Western blotting (method in appendix 3). Used primary antibodies: rabbit anti-Delta (Hetzel et al., 2019) and rabbit anti-PUUV (Strandin et al., 2020), used secondary antibody: LI-COR Biosciences, IRDye 800CW-conjugated donkey anti-rabbit IgG (LicorBio, n.d.). The blotting membrane was imaged using Odyssey imaging system with 700 nm and 800 nm detection channels and Image Studio. The experiment was repeated incubating cells for 2-4 days.

3.2 Optimization of ELISA format

Coating and blocking were optimized using lysates of the two recombinant proteins (antigens) produced above. Materials and reagents were selected based on their general common usage in ELISA protocols and laboratory availability. Reagents and materials compared in this thesis are listed in Table 3.

Table 3. List of materials that were compared during SpyTag/SpyCatcher based ELISA format optimization.

Plate materials	Thermo Scientific 96F Maxisorp, straight W/O lid, LOT:183810 Thermo Scientific 96F Polysorp, straight W/O lid, LOT: 183610
Coating buffers	Carbonate-bicarbonate buffer (pH 9,6) Medico, Lot 253416 PBS buffer (0.14 M NaCl, 0,0027 M KCl, 0,010 Phosphate buffer pH 7.4), Medicago, LOT 301405
Blockers	BSA (Bovines serum albumin) Fraction V, Cas: 9048-46-8 Milk powder (Valio, skimmed milk powder)

The initial coating test was performed between Polysorp and Maxisorp plates using SpyCatcher003 (Kerafast Ink, Cat EOX003, Lot: 2023-03-0) coated to ELISA wells with concentration from 0.5 to 1.5 µg/mL in carbonate-bicarbonate buffer and blocking wells with 3% BSA for one hour at 37 °C. Antigen-containing lysates were diluted from 1:100 to 1:150. Coating conditions were further evaluated at room temperature and +37 °C and compared with overnight coating at +4 °C, using SpyCatcher concentrations ranging from 0.5 to 1.5 µg/mL and diluting lysate to 1:100 to assess the effects of coating incubation temperature. Initial experiments compared two different coating buffers (Carbonate-bicarbonate and PBS) and 2 different blockers either individually or in combination (3% BSA, 3% milk powder and 1.5% BSA-1.5% milk powder mixture). Blocking incubation was assessed at room temperature for 1-2 h and +37 °C for 0.5–2 h, monitoring effects on signal intensity.

Signal specificity and source experiments were performed, further testing blockers (3 % BSA and 3 % Milk powder) and coating buffers (carbonate-bicarbonate and PBS) using antigen dilution series or SpyCatcher concentration series with incubation times determined during initial testing. Antigen dilution

series (from 1:25 to 1:800) were tested using both blockers in two different concentrations (1 % and 3 %) with 2 µg/mL of SpyCatcher in tested buffers. SpyCatcher concentration series (from 0 to 5 µg/mL) were tested with both buffers using either of the tested blockers and diluting antigen to 1:150. To assess unspecific serum antibody binding, signal intensity of wells with antigen-containing samples were compared to wells with samples lacking the specific antigen. Signal intensities of wells coated with SpyCatcher were compared to wells without SpyCatcher to evaluate background signals arising from lysate components, blocker and coater.

Throughout the process, coated and blocked wells followed SpyTag/SpyCatcher the ELISA protocol presented in appendix 4. The experiment's signal was generated using rabbit polyclonal serums (containing primary antibodies, specific for Puumala (Strandin et al., 2020) or Delta virus (Hetzel et al., 2019) antigens). Polyclonal Swine Anti-Rabbit HRP conjugated antibody (Dako Denmark A/S, LOT 0069121) was used as the secondary antibody. Used substrate was 1-Step™ Ultra TMB-ELISA (Thermo Scientific, LOT: AF4606131), and enzymatic reaction was stopped with 0.5M sulphuric acid (Chem-Lab NV, Batch Nr: 30.3051406).

Desired SpyCatcher concentration was prepared by diluting SpyCatcher stock into coating buffer, blockers were prepared dissolving blocker reagents into the PBST solution, and all other solutions were prepared using a blocker diluted to 1:3. Used volume for blocker was 100 µl per well, volume for coating, antigens, antibodies, substrate and stopper was 50 µl per well. Wells were washed three times with PBST between steps with the wash volume at least 50 µl greater than the volume of reagent being washed off. Plates were emptied thoroughly between washes by tapping the plate on an absorbent surface. Absorbance readings were acquired with a HIDEX plate reader at 450 nm.

4 Results

4.1 Protein production

Transfection and expression of plasmids encoding target antigens - Puumala nucleocapsid protein (PUUV NP) fused with SpyTag and snake delta antigen (Snake DAg) fused with SpyTag - were performed in HEK293T cell cultures in two experiments. In the first experiment, cells were harvested on days 1–4 of incubation, whereas in the second experiment on days 2–4 of incubation. At each time point, the collected cells were processed according to two handling schemes: immediate lysis followed by freezing (Lysate), and freezing cells as pellets, which were subsequently lysed after thawing prior to analysis (Pellet). All samples were lysed with equal volume of 100 µl lysis buffer (Appendix 2), and protein concentration was measured from each sample.

Among the samples from the first transfection, the highest total protein concentrations were observed in antigen samples incubated for 2 or 3 days (Figure 6). In general, samples frozen as cell pellets had higher total protein content than samples frozen as lysates. Samples collected on day 4 showed a decrease in protein levels across all samples. At this time point, the cells in the 6-well plates had detached completely from the surface of the well.

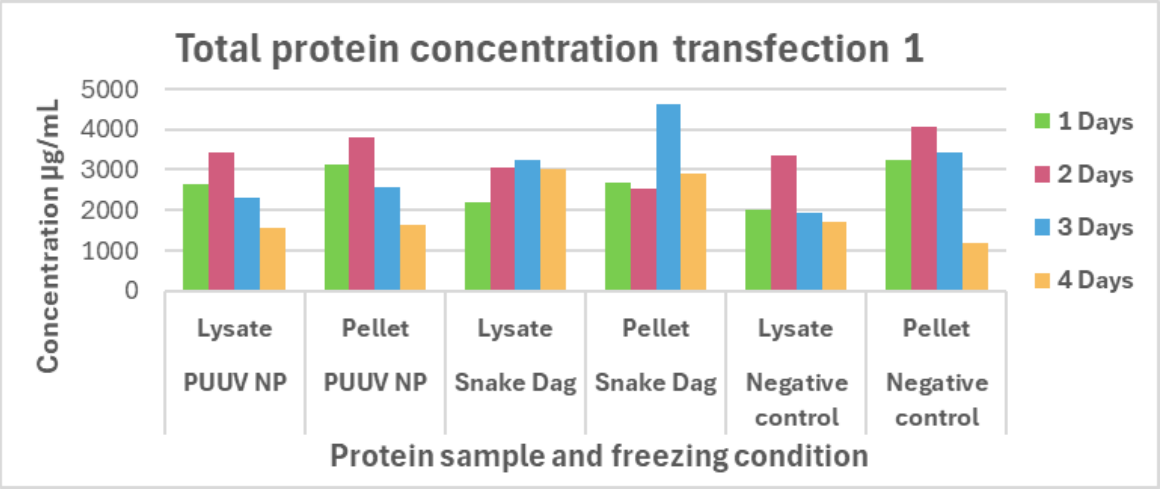

Figure 6. Total protein concentrations(µg/mL) of PUUV NP and Snake DAg antigen samples from the first transfection. Samples were collected after 1–4 days of incubation and stored in the freezer either as antigen lysates (Lysate) or intact cell pellets (Pellet) that were lysed after thawing prior to analysis.

The antigen samples from second transfection exhibited generally lower total protein content compared to samples from the first transfection (Figure 7). Unlike in the previous experiment, most day-4 samples retained relatively high protein content, and the cells remained attached to the well surface at the time of harvesting. Although no clear trend was observed between the two handling schemes, samples from both transfections suggest that freezing as cell pellets may preserve protein more effectively.

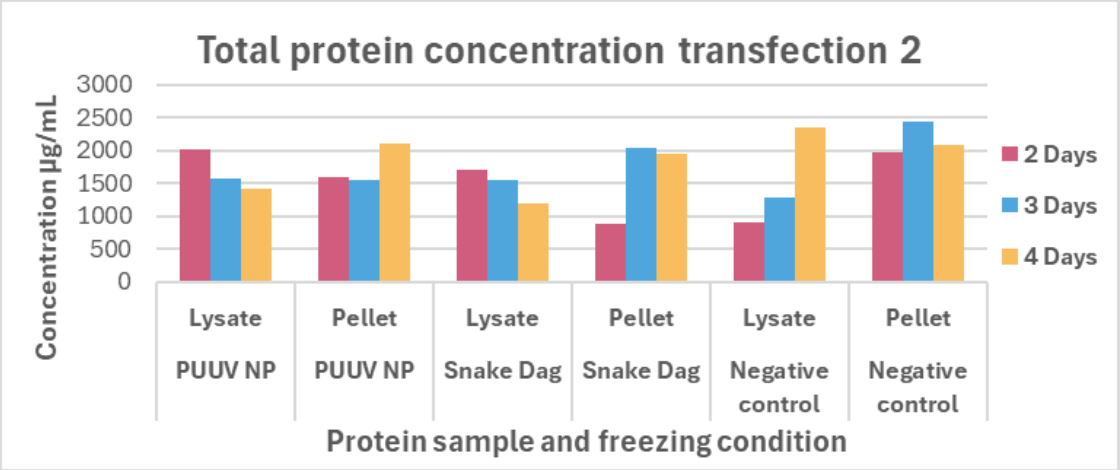

Figure 7. Concentrations of samples collected from second transfection. Samples were frozen as lysate and cell pellets. Samples were collected from 2-4 days of incubation.

Antigen samples obtained from the second transfection were subjected to further evaluation to determine relative amount of target protein in total protein content. This was achieved by combining quantitative western blot analysis with protein concentration measurements: target antigen band intensities were divided with corresponding mass of protein pipetted to the well. Target protein bands from Western blot membranes were scanned and quantified with both 700 and 800 nm detection channels. The pellet samples from day 2 (well 5) showed no detectable signal and were therefore excluded from the analysis (Figure 8). Snake DAg western blot bands on the membrane appeared smudged, and therefore the intensity values were less reliable than those obtained from PUUV NP samples. The target antigen-to-total protein ratio was interpreted as an approximate indicator for progression of relative abundancy of target antigen rather than as precise quantitative values. Total protein pipetted to each well and measured band intensities of samples can be seen in Table 4.

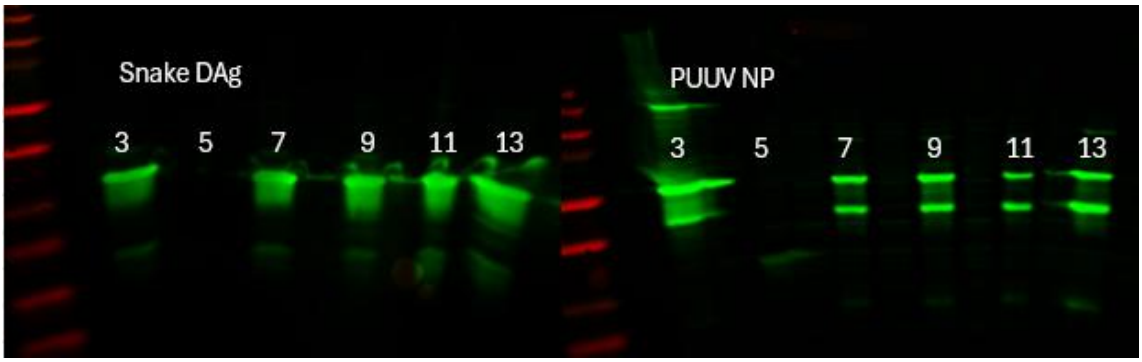

Figure 8. Western blot images from second transfection. Samples in both Snake DAg and PUUV NP membranes listed from left to right: 3 - day 2 lysate, 5 - day 2 pellet, 7 - day 3 lysate, 9 - day 3 pellet, 11 - day 4 lysate and 13 - day 4 pellet.

Table 4. List of Snake DAg and PUUV NP western blot samples with the amount of protein pipetted to each well and intensities of target antigen band at 700 nm and 800 nm.

Well	**Snake DAg**		**µg of total protein in well**	**IR 700**	**IR 800**
3	2 Days	Lysate	4.75	5780	1320000
7	3 Days	Lysate	4.34	5780	1160000
9		Pellet	5.70	9720	1100000
11	4 Days	Lysate	3.36	4910	1140000
13		Pellet	5.44	5850	1470000
Well	**PUUV NP**		**µg of total protein in well**	**IR 700**	**IR 800**
3	2 Days	Lysate	5.65	3320	536000
7	3 Days	Lysate	4.40	2500	481000
9		Pellet	4.36	2660	506000
11	4 Days	Lysate	3.98	1450	239000
13		Pellet	5.87	2580	527000

As illustrated in Figure 9, the progression of relative amount of target antigen slightly differs between Snake DAg and PUUV NP. All the graphs representing PUUV NP antigen samples showed that the relative amount of target antigen increased or remained high until day 3, and then clearly decreased on day 4. Whereas most graphs representing Snake DAg antigen samples showed that the protein ratio remained high still on day 4. Overall, the highest target-to-total protein ratio was observed in antigen samples incubated for three days and samples frozen as cell pellets. By day 3, the wells were already overgrown, and cell death became evident on day 4. Taken together, these observations suggest that a three-day incubation period is optimal for recombinant protein production in 6-well plates, as it balances cell viability with a favorable target protein-to-total protein ratio.

Figure 9. Pole chart of relative target protein-to-total protein levels of the samples from transfection 2. y-axis represents signal intensity divided with the corresponding protein mass loaded to the well.

4.2 Coating and blocking ELISA wells

4.2.1 Initial testing

In this experiment, two different ELISA plate materials—Polysorp and Maxisorp—were evaluated. Plates were coated overnight at +4 °C using carbonate–bicarbonate buffer containing 0.5–2 µg/mL of catcher protein, while antigen lysates were diluted at a 1:100–1:150 ratio 1:3 diluted blocking buffer (3% BSA, 1 h at +37 °C).

Visual inspection of the wells after signal development revealed a clear difference between the two plate materials. Maxisorp plates produced brightly colored reactions in wells containing lysate samples, while blank wells showed only minimal background coloration. In contrast, all Polysorp wells remained clear or displayed only faint coloration (Figure 10). Quantitative measurement of absorbance at 450 nm confirmed these observations: signals from Polysorp wells were nearly identical to those of blank controls while Maxisorp plates provided strong signals in antigen-containing wells that were easily distinguishable from blank wells (Figure11).

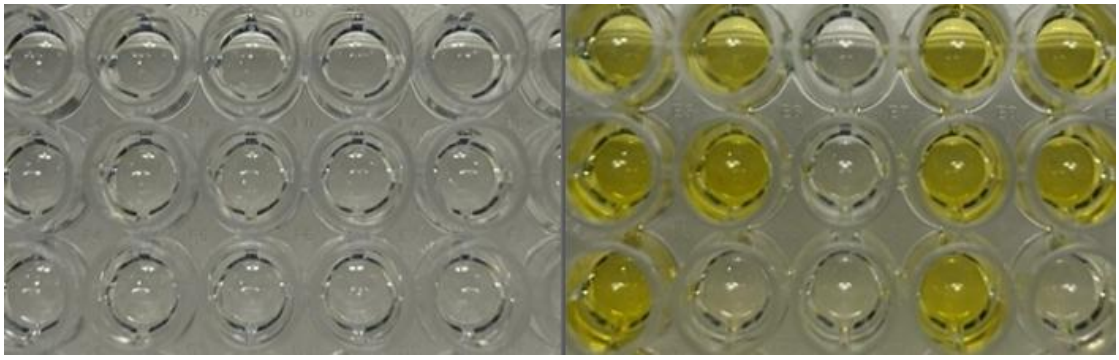

Figure 10. Difference in ELISA reaction color change between two different plate materials coated using the carbonate-bicarbonate buffer including 0.5-2 µg/mL of catcher overnight at + 4 °C. Polysorp (left) and Maxisorp (right).

When evaluating signals in Maxisorp plates coated with different SpyCatcher concentrations, the Snake DAg antigen lysate signal decreased as the coating concentration was lowered (Figure 11). In contrast, signals from the PUUV NP samples did not follow this trend and remained at similar levels regardless of the

SpyCatcher concentration. When comparing different antigen dilutions, signals remained more consistent for both antigen lysates. These findings suggest that the amount of SpyCatcher present in the well is the limiting factor for signal generation. Based on these observations, it was concluded that Maxisorp is a more suitable material for ELISA assays than Polysorp. All subsequent tests were conducted using Maxisorp plates.

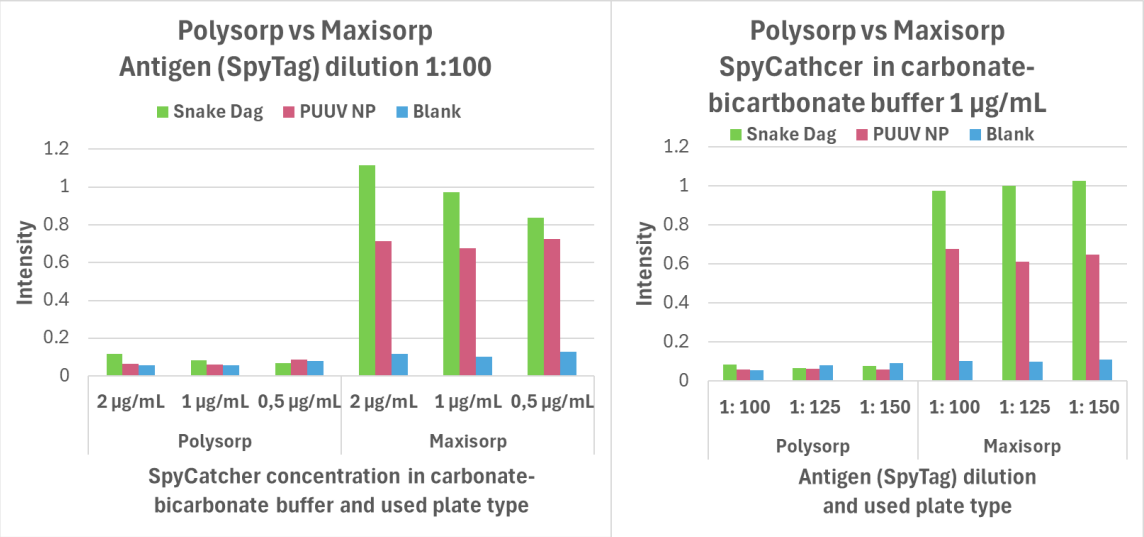

Figure 11. Pole chart of intensities measured from Polysorp plates and Maxisorp plates using the carbonate-bicarbonate coating buffer coated overnight at +4 °C. The left chart includes series of SpyCatcher concentrations with 2, 1 and 0.5 µg/mL using 1:100 diluted antigen. The chart on the right includes antigen dilution series between 1:100 -1:150 using a catcher concentration of 1 µg/mL in the coating buffer. All wells were blocked with 3 % BSA at +37 °C for 1 h.

Coating incubation conditions were assessed overnight at +4 °C, 2 hours at room temperature, and 1 or 1.5 hours at +37 °C. Plates were coated using a carbonate–bicarbonate buffer containing 0.5–1.5 µg/mL SpyCatcher, blocking wells with 3 % BSA at +37 °C for 1 hour and diluting antigen to 1:100.

As illustrated in Figure 12, the Snake DAg lysate samples signal intensities correlated with the SpyCatcher concentrations in all conditions except room temperature. The highest signals received from the SpyCatcher concentration of 1.5 µg/mL remained similar across + 4 and +37 °C conditions, but the signal declined more rapidly at elevated temperatures as the SpyCatcher concentration decreased. Incubating wells at room temperature formed the poorest signal.

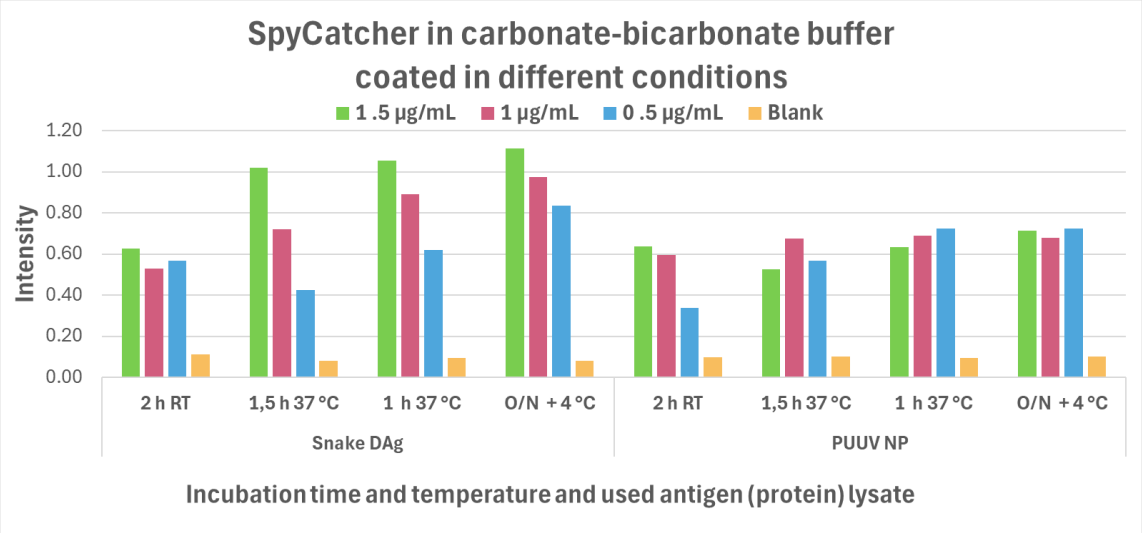

Figure 12. Bar chart of intensity of the Snake DAg and PUUV NP antigen samples incubated in different conditions (overnight at + 4°C, 2 h at RT, 1 h and 1.5 h at + 37°C) and different SpyCatcher concentrations in the carbonate-bicarbonate coating buffer (0.5-1.5 µg/mL) and antigen diluted to 1:100. Wells were blocked using 3 % BSA at +37 °C for 1 h.

In contrast, all the PUUV NP samples showed generally lower signal compared to the Snake DAg samples and did not exhibit a consistent response to changes in SpyCatcher concentration. According to Snake DAg results, overnight incubation at +4 °C yields highest signal with all SpyCatcher concentrations and was selected to be used in following experiments.

Blocking conditions and blocker reagents were compared using a SpyCatcher concentration of 1 µg/mL in either carbonate-bicarbonate or PBS coating buffer coated overnight at + 4 °C. Three blocking strategies were tested: 3% BSA, 3% milk powder, and a 1:1 mixture of both (1.5% BSA and 1.5% milk powder). Blocking was performed at room temperature for 1 or 2 hours, and at +37 °C for 0.5–2 hours.

As shown in Figure 13, the differences between the two coating buffers were minimal for both antigen samples. The Snake DAg samples show very little variation between different blocking reagents, but the BSA intensities fluctuate more than the intensities of wells that had milk powder or combined blocker reagent. Signal intensity did not vary significantly between incubation times at +37 °C or room temperature.

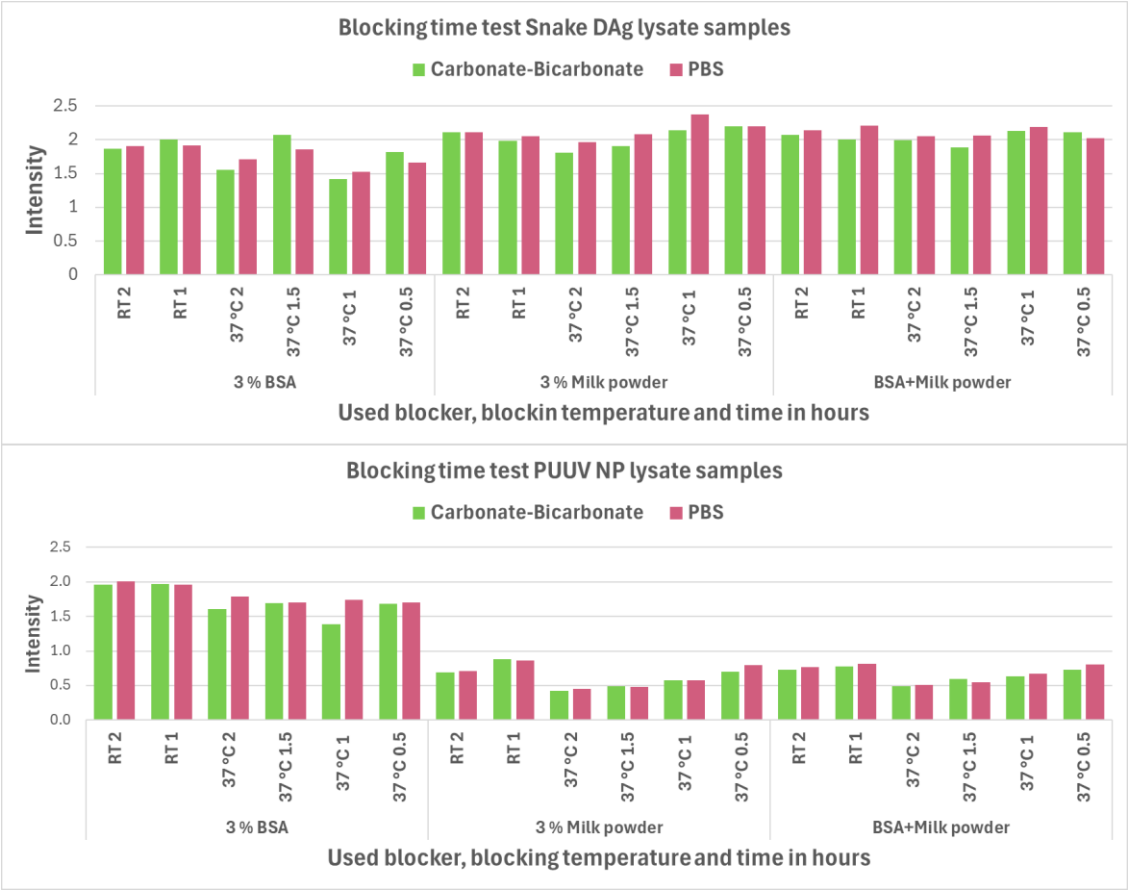

Figure 13. Signal using 1µg/mL SpyCatcher (coated overnight at +4 °C) on 2 different coating buffers (Green: Carbonate-Bicarbonate, Red: PBS) blocked with 3 different blockers (left to right: 3% BSA, 3% Milk powder, 1.5%BSA with 1.5% milk powder) in room temperature for 1 and 2 hours and at + 37°C for 0.5-2 h.

Room temperature produced consistently stronger signals than + 37 °C with the PUUV NP lysate samples. Notably, the PUUV NP samples blocked with BSA exhibited higher signals compared to those blocked with milk powder or the BSA–milk mixture. These milk containing samples showed slight decreasing trend over time when blocked at + 37 °C. The Signal of PUUV NP lysates decreased significantly when the blocker included milk powder. Overall, results obtained with milk powder were nearly identical to those with the 1:1 mixture of the tested blocking reagents. It was decided to continue using 1- hour incubation at 37 °C as blocking condition in the following experiments.

All measured results analyzed in this section can be found in Appendix 5.

4.2.2 Analyzing source of signal

To further analyze differences between BSA and milk powder as blocking reagents, antigen lysates were serially diluted from 1:25 to 1:800 using 2 µg/mL of SpyCatcher in the PBS coating buffer (coated overnight at +4 °C) with the blocking time of 1 hour at 37 °C. BSA and milk powder were tested at concentrations of 1% and 3 % in PBST. The used antibody serums were tested for their specificity by adding negative control samples by switching used antigen.

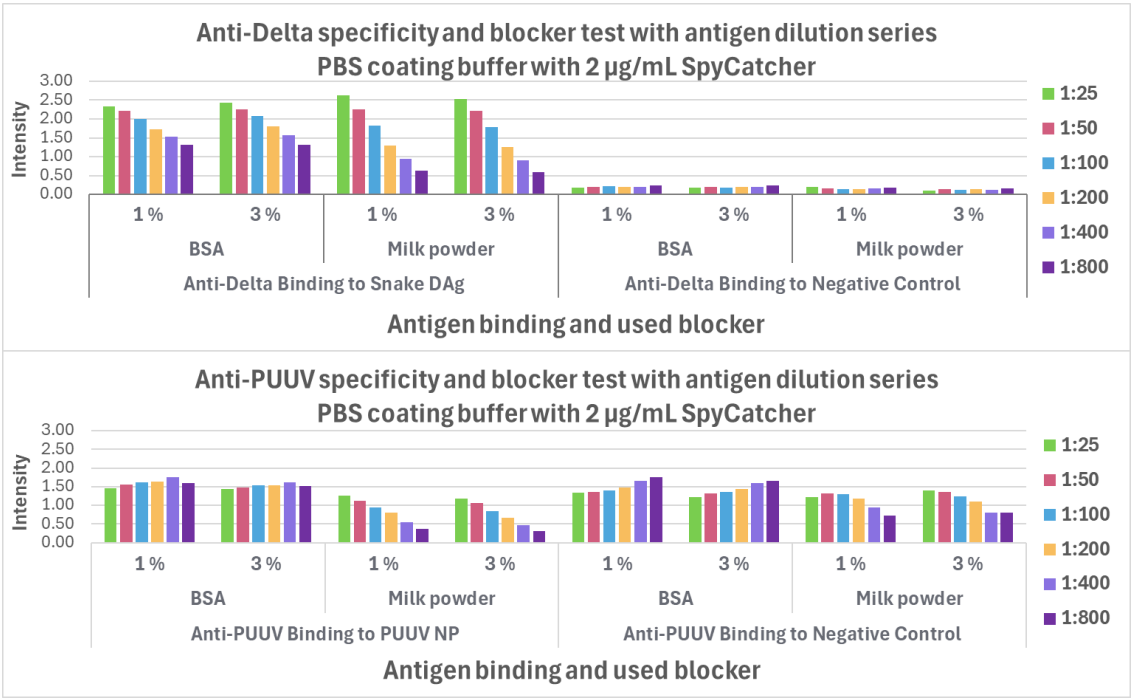

Figure 14. Specificity of the anti-Delta (top) and anti-PUUV (bottom) serum antibodies across antigen dilution series. Signal intensities using the PBS coating buffer with 2 µg/mL SpyCatcher (incubated overnight at +4°C) and antigen dilutions ranging from 1:25 to 1:800. Blocking was performed with 1% and 3% BSA or milk powder in PBST at 37 °C for 1 hour.

As shown in Figure 14, signal intensities produced with the anti-Delta serum remained at the level of blank sample values from previous experiments when the Snake DAg antigen was absent. This indicates that the serum is specific to its corresponding antigen. When analyzing the anti-Delta serum with the Snake DAg antigen, a clear dilution-dependent trend was observed with both BSA and milk powder. Among the lowest dilution ratio (1:25), wells blocked with milk powder produced higher signal than wells blocked with BSA, but the signal declined more rapidly as the dilution ratio increased.

In the case of the anti-PUUV serum with its corresponding antigen (PUUV NP), milk powder produced signal trend that aligns with serial dilution, whereas BSA did not. However anti-PUUV expresses a clear signal with negative control samples indicating that anti-PUUV is not specific for the PUUV NP antigen.

These results suggest that milk powder may be more effective than BSA in certain cases, whereas BSA tends to provide stronger signal as the dilution ratio is above 1:50. In both Snake DAg and PUUV NP samples, no noticeable differences were observed between the 1% and 3% concentrations of the blocking reagent.

To investigate the origin of signal, a SpyCatcher concentration series ranging from 0.3 to 5 µg/mL was prepared using either carbonate–bicarbonate or PBS coating buffer (coated overnight at +4 °C). Wells were blocked with 3% BSA at +37 °C for 1 hour, and antigen lysates were diluted at a 1:150 ratio. Control wells were included to assess nonspecific binding and generation of background signal. The negative control wells (containing opposite antigen intended for serum) and the blank wells (without any antigens) were coated with or without SpyCatcher to determine unspecific binding of either antibody serum. The background signal control wells contained antigen lysate without SpyCatcher to evaluate whether lysate components bind directly to the well surface.

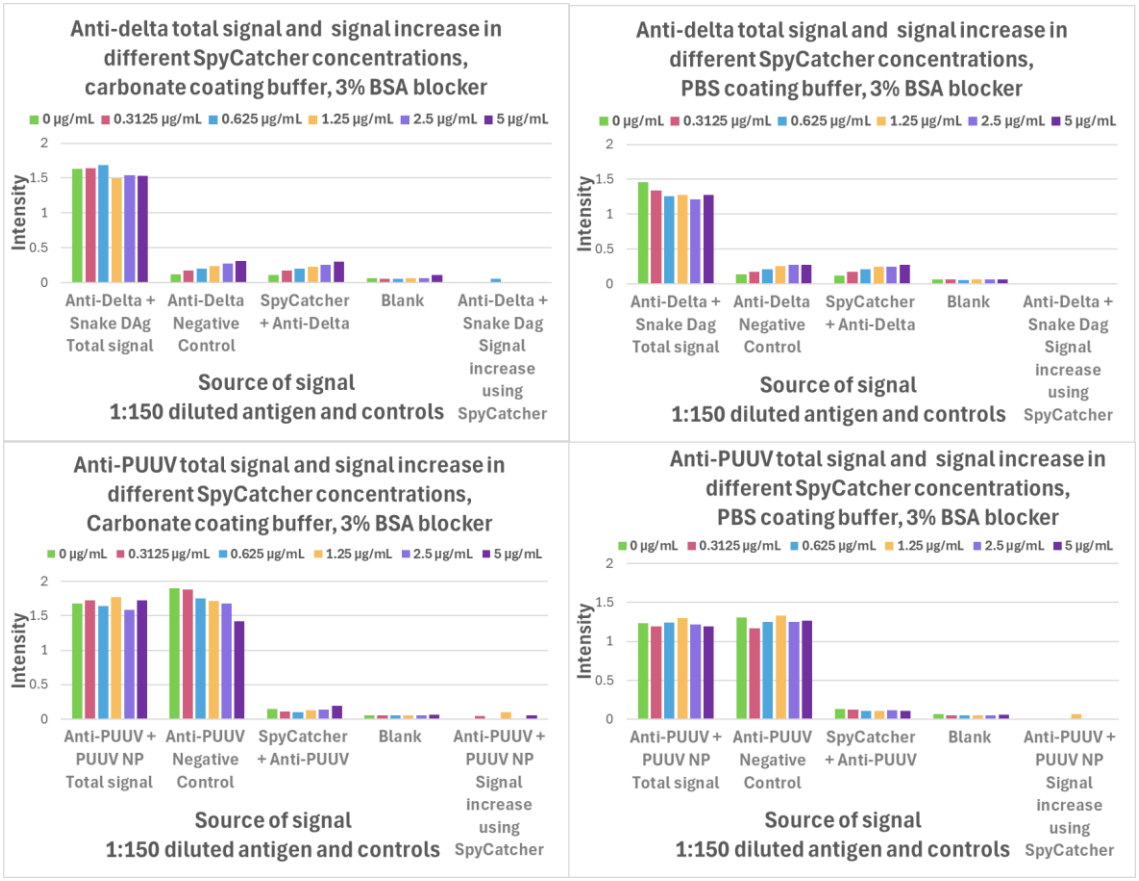

Figure 15. Bar charts of signals with different catcher concentrations (0–5 µg/mL), coated overnight at +4 C. Antigen lysates (top: Snake DAg, bottom: PUUV NP) were diluted 1:150 and tested using the carbonate–bicarbonate buffer (left) or PBS buffer (right). Wells were blocked with 3% BSA at +37 °C for 1 hour. Controls included wells without antigen or SpyCatcher to assess nonspecific binding and the background signal. Signal increase represents the difference between the total signal and the background signal in wells containing SpyCatcher.

The results seen in Figure 15 show that anti-PUUV serum antibody produced similar signal intensities in both the PUUV NP lysate samples and the negative controls, further confirming previous indications that the serum is not specific for the PUUV NP antigen. Similarly, the anti-Delta serum showed no significant signal in the negative controls, further confirming previous indications its specificity for the Snake DAg.

Wells containing SpyCatcher and serum antibody (without antigen) provided signal slightly above levels of blank wells without SpyCatcher, suggesting that neither serum antibody binds to the coating or blocking buffer components. The wells coated without SpyCatcher (0 µg/mL; green bars in Figure 15) produced

signal intensities similar to or higher than those of wells coated with SpyCatcher, indicating that lysate components bind directly to the well surface. This background signal appears to mask any analyte-specific signal, as the addition of SpyCatcher did not enhance signal intensity throughout the tested SpyCatcher concentrations. The experiment was repeated several times with consistent result: SpyCatcher failed to increase the signal above the background level.

To investigate the cause of the high background signal, the coating protocol was modified. Wells were coated with a SpyCatcher concentration series in carbonate–bicarbonate buffer, but instead of overnight incubation at +4 °C, coating was performed at +37 °C for 1.5 hours. Wells were then blocked with 3% BSA at +37 °C for 1 hour, and antigen lysates or negative controls were diluted at a 1:150 ratio. The same signal evaluation controls as in the previous experiment were included.

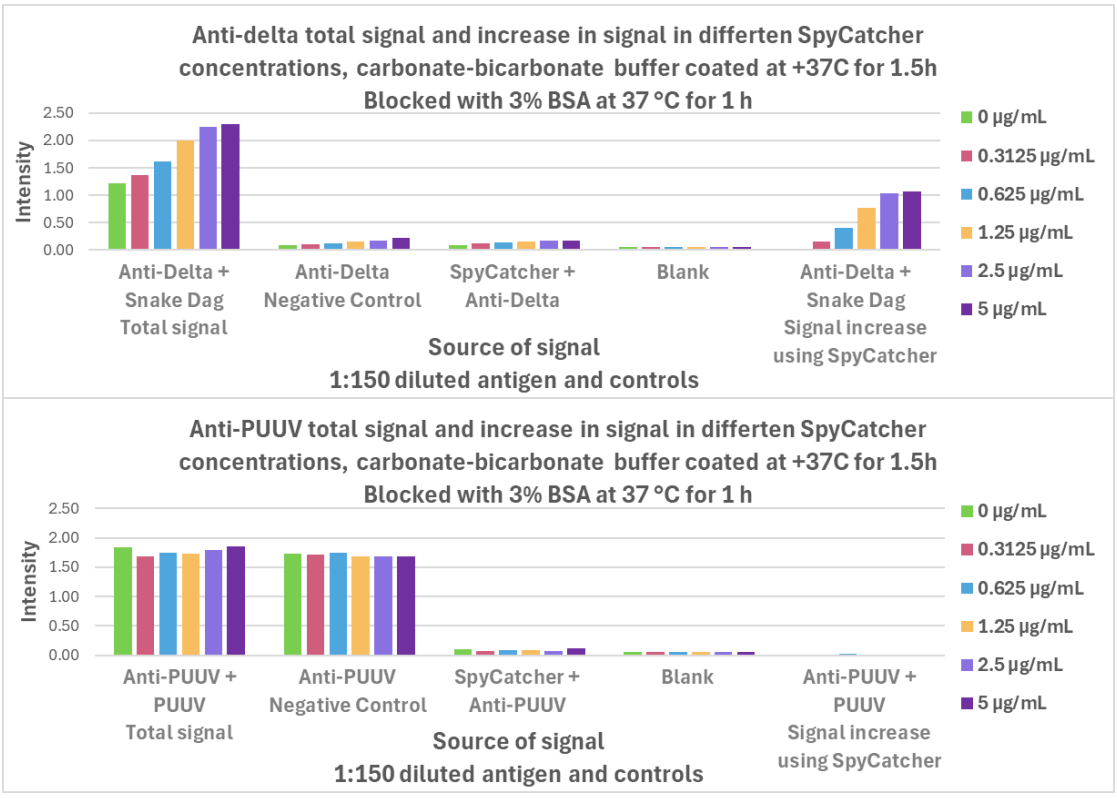

Figure 16. Signal intensities and specificity of serum antibodies (top: anti-delta, bottom: anti-PUUV) across the SpyCatcher concentrations (0–5 µg/mL) in the carbonate-bicarbonate buffer, coated at +37 °C for 1.5 hours. Wells were blocked with 3% BSA at +37 °C for 1 h using 1:150 diluted antigen lysates. Signal increase reflects the difference between the total signal and the background signal in wells containing SpyCatcher

As illustrated in Figure 16, even though clear signals were still observed in wells without SpyCatcher, the background did not fully mask the analyte-specific signal. Incubating wells at + 37 °C for 1.5 hours led to a measurable increase in signal as SpyCatcher was present and formed a trend along with increasing SpyCatcher concentration. Signals observed from wells without SpyCatcher indicate that some of the antigen lysate components still bind to the coating or blocking components.

From Figure 17 can be seen how signal intensities from different SpyCatcher concentrations formed a logarithmic curve, reaching maximum intensity approximately at 2.5 µg/mL. Based on formed curve in Figure 17, concentration of 2 µg/mL of SpyCatcher in coating buffer was assessed to be sufficient to generate a reliable analyte-specific signal.

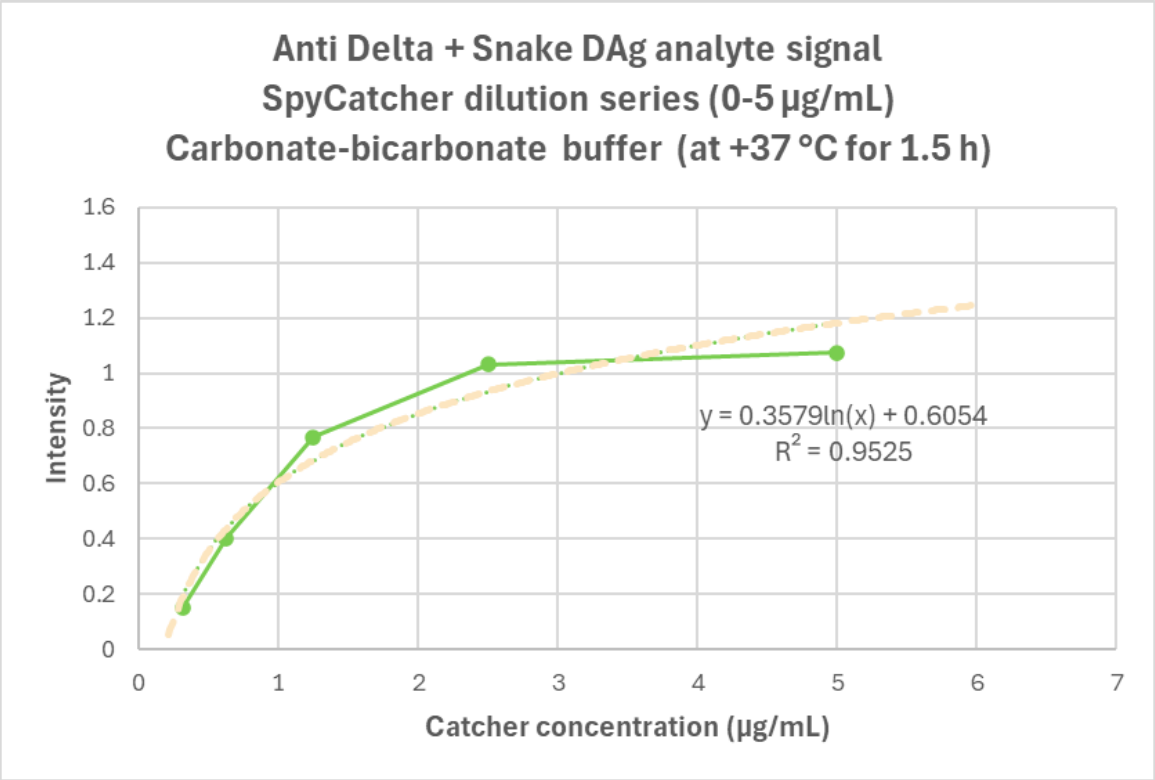

Figure 17. Snake DAg analyte signal intensities of with SpyCatcher concentrations incubated at +37 °C for 1.5 hours. Wells were blocked with 3% BSA at +37 °C for 1 h using 1:150 diluted antigen lysates. The background signal decreased from the total signal values.

Both carbonate-bicarbonate and PBS coating buffer were tested on antigen serial dilutions again with a SpyCatcher concentration of 2 µg/mL This time, wells were coated at +37 °C for 1.5 hours, as previous test suggested. Wells were blocked with either 3% BSA or 3% milk powder at +37 °C for 1 hour. Negative control and blank wells were included to assess nonspecific binding. Background controls contained an antigen lysate without SpyCatcher to evaluate whether the lysate components bind directly to the well surface and produce background signal.

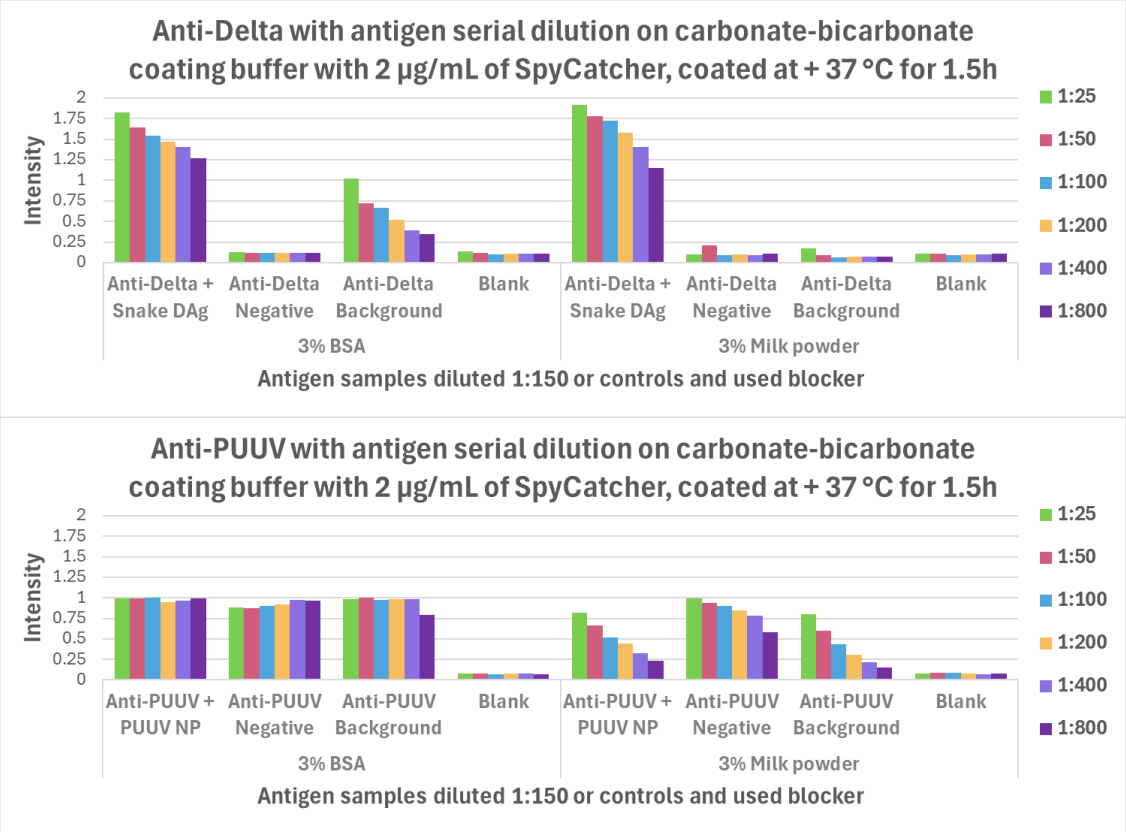

Figure 18. Specificity of the anti-Delta (top) and anti-PUUV (bottom) antibodies across antigen dilution series. Signal intensities measured using the carbonate-bicarbonate coating buffer with 2 µg/mL SpyCatcher (incubated at +37°C for 1.5 hour) and antigen dilutions ranging from 1:25 to 1:800. Blocking was performed with 3% BSA or milk powder in PBST at 37 °C for 1 hour.

As shown in Figure 18, carbonate–bicarbonate coated wells reveal that the specific anti-Delta antibody combined with the Snake DAg antigen produces clear signals that align with the antigen dilution series. The negative anti-Delta controls present elevated signal when BSA is used, but not when milk powder is used, indicating that the background signal is caused by antigen binding to BSA. For the non-specific anti-PUUV antibody, milk powder did not eliminate background

signal, but it did allow the PUUV NP antigen signal to follow the dilution trend. This indicates that the PUUV NP antigen components still bind to wells without the use of SpyTag-SpyCatcher-interaction. Based on observations from Figure 18, milk powder appears to be a more suitable blocking reagent due to its tendency to reduce the background signal.

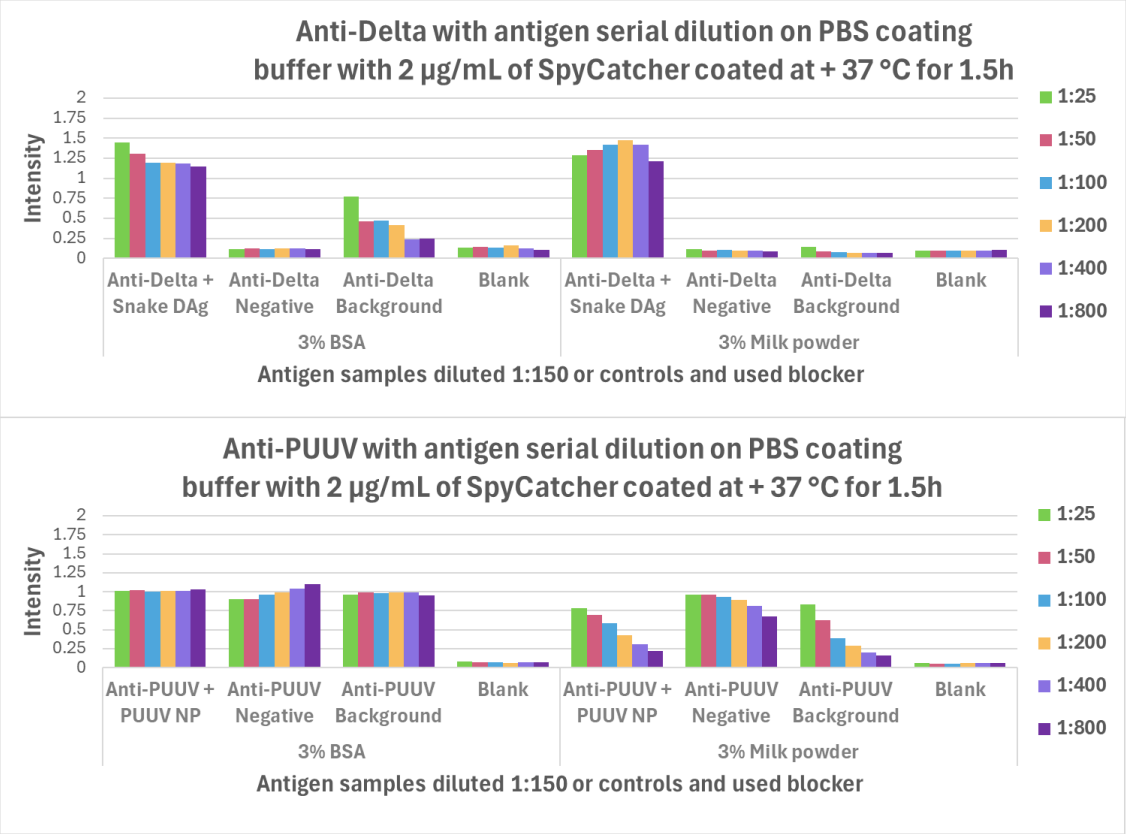

Figure 19. Specificity of the anti-Delta (top) and anti-PUUV (under) antibodies across the antigen dilution series. Signal intensities using the PBS coating buffer with 2 µg/mL SpyCatcher (incubated at +37°C for 1.5 hour) and antigen dilutions ranging from 1:25 to 1:800. Blocking was performed with 3% BSA or milk powder in PBST at 37 °C for 1 hour.

PBS-coated wells (Figure 19) showed similar signals from both blockers in comparison with the carbonate-bicarbonate coating buffer: The milk powder blocked wells exhibited no signal in the anti-Delta antibody background signal controls and enabled the anti-PUUV antibody to follow the PUUV NP antigen dilution series trend. However, the PBS coated wells blocked with milk powder exhibited a systematic increase of the anti-Delta signal up to the dilution of 1:200, after which it reached the same signal intensity with carbonate-bicarbonate

coated wells, and began to follow decreasing dilution trend. PBS coated well signals present generally slightly lower signal than carbonate-bicarbonate coated wells. In both buffer conditions, the specific anti-Delta signal at the 1:800 dilution would sufficiently be strong enough to allow analyte quantification, provided that known standards were included

Subtracting the signal of background wells (0 µg/mL SpyCatcher in coater) from the total signal reveals the analyte-specific signal illustrated in Figure 20.

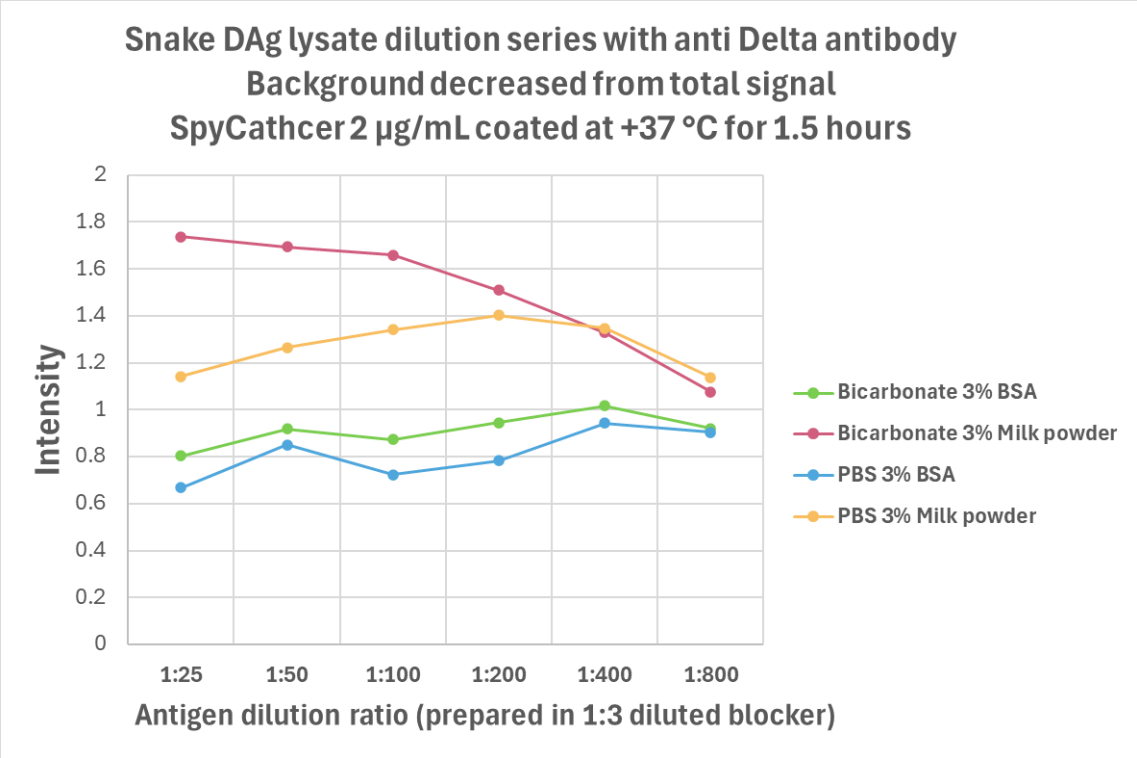

Figure 20. Actual Snake DAg analyte signal with the specific anti-Delta rabbit serum calculated by subtracting the background signal from the total signal. The wells were coated with 2 µg/mL SpyCatcher at +37 °C for 1.5 hours and blocked with 3% BSA or milk powder. Antigen dilutions ranged from 1:25 to 1:800.

The wells blocked with BSA (green and blue lines) reach intensities close to 1, but do not form a trend consistent with the antigen dilution series. In contrast, the PBS-coated wells blocked with milk powder (orange line) start with a lower signal that systematically increases up to the dilution of 1:200 and then declines. Wells coated with carbonate–bicarbonate buffer and blocked with milk powder (red line) display a clear curve that corresponds to the antigen dilution series. These wells show the highest overall signal intensities, although at the largest dilutions (1:400

and 1:800), the signal levels cross with those of the PBS-coated, milk powder-blocked wells. On the basis of these observations, the optimal combination is concluded to be carbonate–bicarbonate coating buffer with milk powder as the blocking reagent.

All measured results analyzed in this section can be found in Appendix 6.

5 Discussion

The cells used in the first transfection were grown beyond optimal confluency for transfection, exceeding 100%. Therefore, the used volume of culture medium during transfection should have been greater to achieve intended cell concentration in the media. After transfection, cells began detaching from the surface of the flasks by day 3 and were completely detached and floating in the medium by day 4 indicating cell death. In contrast, the second transfection was performed with 90% confluent cells, which remained viable for a longer period on 6-well plates. As a result, protein concentration declined earlier in the first transfection compared to the second and cell death began sooner. Although the Western blot bands from Snake DAg antigen lysates were unclear, the experiment was able to demostrate that a 3-day incubation maintained a high target antigen-to-total protein ratio, and the cells remained viable throughout the incubation period.

Signal source experiments revealed a critical difference between two coating methods. While overnight coating at + 4°C is typically the standard procedure for preparing ELISA wells, it produced a different outcome compared to coating at +37 °C for 1.5 hours. Tests showed that when SpyCatcher was applied using the overnight method, the signal originated primarily from nonspecific binding of the antigen lysate to the BSA blocker. Signal intensities remained high across all wells coated with SpyCatcher, without showing a consistent trend in relation to increasing SpyCatcher concentrations. When ELISA microplates were coated at 37 °C for 1.5 hours, the background signal was significantly reduced, and a clear

trend in signal increase was observed with the increasing SpyCatcher concentrations.

When the coating was first tested in section 4.2.1, the SpyCatcher concentrations used were very close to one another, with only a few concentration points, and the experiments lacked proper controls to measure the signal source. As a result, this difference could not be observed initially. It is unfortunate that the initial coating tests did not include proper background signal controls, as such data would have been valuable from the start. This shortage led to additional laboratory tests and repetitions limiting the time available to evaluate other ELISA steps. The reasons for the observed difference between coating methods remain unknown. There is a possibility that the overnight coating method affected SpyCatcher, altering its capability of interacting with SpyTag or covered the reactive sites of SpyCatcher. Other reasons could be that the method enhanced background signal so much it simply covered any analyte signal received from SpyTag-SpyCatcher interaction. Future experiments are required to clarify the underlying causes of this difference between coating incubation methods.

As for the blocking results, when antigen was added to the wells without SpyCatcher, a high signal was detected when BSA was used as the blocker, but not when milk powder was used. The antibodies used during this thesis were used in the blank wells without lysate and produced no signal, confirming that antibodies did not bind to coating or blocking components. This indicates that a high background signal is derived from the lysate components that bind to BSA. A mixture of BSA and milk powder produced results similar to wells blocked only with milk powder, suggesting that the presence of milk powder reduced the unspecific signal derived from BSA. The outcomes were nearly identical with both 1% and 3% blocker concentrations, suggesting that the amount of blocker did not affect the results. Further experiments are required to determine whether changing the blocking method could reduce the BSA-derived signal, and to assess how prolonging the blocking time influences background and analyte signals.

Despite observed differences and lack of background controls in the beginning, the Tag–Catcher interaction was shown to work on sandwich-like ELISA format. The method was proven capable of antigen detection even in extremely low antigen concentrations.

6 Conclusion

The experiments demonstrated that the transfection method is functional and a 3-day incubation period can be applied to produce recombinant SpyTag-proteins. Furthermore, the SpyCatcher–SpyTag interaction was shown to be suitable for use in a sandwich-like ELISA platform, and clear signals were obtained from samples diluted up to a 1:800. SpyCatcher concentration of 2 µg/mL was determined sufficient to ensure reliable detection in all used dilutions. It was determined that coating incubation at +37 °C for 1.5 hours is superior to overnight incubation at +4 °C, as it provides lower background signal allowing detection of analyte signal and formation of signal trend that correlates with different SpyCatcher concentrations. When milk powder was used as a blocker, background signal reduced to the level of signal from blank wells. Differences in signal intensity between tested buffers were slim, but carbonate-bicarbonate buffer worked more consistently compared to PBS coating buffer throughout the experiments. The final optimized procedure for coating and blocking based on results of this thesis is presented in Appendix 7.

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Appendix 1: Transfection method

Cell transfection:

Reagents and tools:

- Transfection stock (200 µg/ml)
- Transfection reagent
- Media (Dulbeccos Modified Eagles' medium, high glucose +10% FBI +1% penstrep +1% L-glut)
- OptiMEM
- Trypsin EDTA
- 6-well plates

Method uses cells grown in T75 bottles and transfected cells are cultivated in 6-well plates

1. **Prepare transfection mixes**
   mix each solution thoroughly

Transfection mix 1:	Transfection mix 2:
Mix is prepared for different sample types, positive control and negative control separately	Transfection reagent is mixed with OptiMEM. Same mix ratio is used for all samples (9:1 (reagent: plasmid) ratio)
For one reaction in 6 well plate: 12,5 µl transfection stock 487,5 µl optiMEM (total volume 500 µl) Negative control: use 500 µl of OptiMEM	For one reaction in 6 well plate: 22,5 µl transfection reagent (200 ng/µl) 477,5 µl optiMEM (total volume 500 µl)

2. **Combine transfection mixes:**
   1. Mix transfection mixes with 1:1 ratio by pipetting up and down 10-15 times.
   2. Incubate tubes in RT for 10-15 minutes.

Cell suspension		
Confluency	Media addition	Suspension volume
Use cell T75 bottles with minimum of 80% confluency	Media addition in T75 bottles: 35 ml * confluency index - V trypsin *(35 ml * 0,8 – 2 ml= 26 ml)*	Use 4 ml of prepared media for each sample.

3. **Prepare cell suspension:**
   1. Detach cells from T75 bottles with trypsin and add media.
   2. Mix carefully pipetting up and down.
4. **Combine cell suspension and transfection mix**
   1. Pipette 1 ml transfection mixture and 4 ml cell suspension and mix carefully, trying not to disrupt the cells, by pipetting up and down.
   2. Incubate mixes in RT for 10-15 minutes. Pour samples to 6-well plates (5 ml/well)
   3. incubate cells in 37°C for 3 days.

Collect cells and wash the media off with PBS. Centrifuge cells 500 g for 3 minutes and discard the supernatant.

Store cell pellets at -18 °C

Appendix 2: Lysis buffer

RIPA buffer:

50 mM Tris (pH 8)

150 mM NaCl

1% Tx-100

0.1 % SDS

0.5 % Sodium Desoxycholate

+ milliQ water to decided volume

+ protease inhibitor cocktail

Protein inhibitor cocktail:

Sigma Aldrich, cOmplete, EDTA-free, REF:11873580001, LOT: 8442300

1 tablet to 50 µl volume

Volume of lysis buffer for one 6-well plate well: 200 µl

Appendix 3: Western blotting

Western blotting method

Reagents and tools:

- Laemmli buffer
- DTT (dithiothreitol)
- Running and transfer buffers
- Nitrocellulose membrane
- Whatman paper
- TBS (Tris-buffered saline)
- TBST (Tris-buffered saline + Tween 20)
- 3 % milk powder in TBST (blocker)
- Primary antibody (1:2500)
- Secondary antigen (1:10 000)

Gel electrophoresis	Transfer to membrane
For 10 µl of protein lysate, add 3 µl Laemmli buffer and 1µl DTT	Soak Whatman papers and sponges in transfer buffer for 15 minutes before assembling
SDS running buffer	Transfer buffer
Run for 35 mA (1 gel) Run for 40 min with 70 mA (2 gels)	Run for 1 h with 400 mA

Gel electrophoresis:

1. Prepare electrophoresis sample combining protein sample with Laemmli buffer and DTT
2. Incubate mixture at 85-90 °C for 5 min (max 10 min)
3. Assemble gels to the running chamber and fill with running buffer.
4. Pipette a volume of prepared mixture so that it contains 5-10 µg of protein to SDS-gel wells.
5. Pipette 2,5 µl of marker in one well.
6. Run SDS electrophoresis for 35 mA (1 gel) or for 40 min with 70 mA (2 gels)

Transfer to a membrane:

1. Cut nitrocellulose membrane and Whatman papers into desired size to cover electrophoresis gel then soak Whatman papers and sponges in running buffer
2. Assemble the blotting sandwich in transfer buffer in following order starting from the negative charge side: sponge, 2-3 Whatman papers, SDS gel from electrophoresis, nitrocellulose membrane, 2-3 Whatman papers, sponge. Avoid forming bubbles between layers.
3. Insert the sandwich into the running chamber and fill with transfer buffer
4. Run for 1 h with 400 mA

Blocking	Primary antibody	Secondary antibody (IR)
3 % Milk powder in TBS	Dilute in 1:2500 ratio to 3 % milk powder in TBST	Dilute in 1:10 000 to 3 % milk powder in TBST
Volume needed for 1 gel: 10 ml	Volume needed for 1 gel: 10 ml	Volume needed for 1 gel: 10 ml
Incubation at room temperature for 1 h	Incubation at + 4 °C overnight	Incubation at room temperature for 45 min

Blocking and antibody probing

1. Wash nitrocellulose membranes with TBS 3 times for 5 minutes each time in shaker
2. Block the unspecific sites in nitrocellulose membranes with milk powder blocker for 1h at RT
3. Wash the membranes with TBS 3 times for 5 minutes each time in shaker
4. Incubate the membranes with primary antibody at + 4 °C overnight
5. Wash the membranes with TBST 3 times for 5 minutes each time in shaker
6. Incubate membranes with secondary antibody at RT for 45 minutes
7. Wash the membranes with TBST 3 times for 5 minutes each time in shaker
8. Scan the nitrocellulose membranes with Odyssey and Image studio

Appendix 4: ELISA method

SpyTag-SpyCatcher based ELISA

Reagents:

- Protein including SpyTag
- Blocking buffer diluted in 1:3 ratio
- PBST (for washing)
- Primary antibody (serum)
- Secondary antibody (anti-serum HRP)
- TMB-substrate solution
- Stop solution: 0,5 M H_2SO_4 (sulphuric acid)

Lysing stored cell pellet	Protein (antigen) with SpyTag
Melt the stored cell pellets with SpyTag protein	Dilute protein lysate in suitable ratio
Lyse stored cell pellets with RIPA+PIC	Volume antigen lysate in well: 50 µl
Use 200 µl volume of lysis buffer to cells from one well 6 well plate	Incubate antigen-Tag lysate RT 1,5-2 h

SpyTag-SpyCatcher interaction:

1. Dilute SpyTag protein lysates in desired ratio in 1:3 diluted blocking buffer
2. Pipette 50 µl SpyTag dilution to wells (avoid solution getting to walls of the wells)
3. Cover wells with sticker and incubate ELISA plates at RT for 1,5-2 hours.
4. Wash wells 3 times with PBST

   Tap wells dry between washes

Primary antibody (serum):	Secondary antibody (α- serum HRP):
Dilute serum: 1:1000 in 1:3 diluted blocker	Dilute antibody: 1:5000 in 1:3 diluted blocker
Volume of serum antibody in well: 50 µl	Volume of HRP antibody in well: 50 µl
Incubate antibody at RT for 1,5 hours	Incubate antibody at RT for 45 minutes

ELISA reaction:

1. Dilute primary antibody in 1:3 blocking buffer in 1:1000 ratio
2. Pipette 50 µl of antibody solution to wells
3. Cover wells with sticker and incubate plates in RT for 1,5-2 hours
4. Wash wells with TBST 3 times

   Tap wells dry between washes

5. Dilute secondary antibody (Anti-Serum HRP) in 1:3 blocking buffer to 1:5000 ratio and pipette 50 µl of solution to wells
6. Wash wells with TBST 3 times

   Tap wells dry between washes

7. Add 50 µl TMB substrate to each well (blue color should start to form almost immediately)
8. Incubate wells in RT for 15 minutes **covered from light**
9. Add 50 µl of stop solution (0,5M sulphuric acid) and read the absorbance at 450 nm

   Read the plates as soon as possible

Appendix 5: ELISA test result tables section 4.2.1

Initial testing between compared materials

1: 100 antigen dilution					
Snake DAg			**PUUV NP**		
Material	SpyCatcher concentration	Intensity	Material	SpyCatcher concentration	Intensity
Polysorp	2 µg/mL	0,12	**Polysorp**	2 µg/mL	0,06
	1 µg/mL	0,08		1 µg/mL	0,06
	0,5 µg/mL	0,07		0,5 µg/mL	0,09
Maxisorp	2 µg/mL	1,12	**Maxisorp**	2 µg/mL	0,72
	1 µg/mL	0,97		1 µg/mL	0,68
	0,5 µg/mL	0,84		0,5 µg/mL	0,73
SpyCatcher concentration in coater 1 µg/mL					
Snake DAg			**PUUV NP**		
Material	Antigen dilution	Intensity	Material	Antigen dilution	Intensity
Polysorp	1: 100	0,08	**Polysorp**	1: 100	0,06
	1: 125	0,07		1: 125	0,06
	1: 150	0,08		1: 150	0,06
Maxisorp	1: 100	0,97	**Maxisorp**	1: 100	0,68
	1: 125	1,00		1: 125	0,61
	1: 150	1,02		1: 150	0,65

Table 1. Intensities measured from Polysorp plates and Maxisorp plates coated overnight at +4 °C using carbonate-bicarbonate coating buffer. Wells were blocked using 3 % BSA at +37 °C fort 1 h.

Initial coating incubation condition test results

Antigen	**Condition**	**1 .5 µg/mL**	**1 µg/mL**	**0 .5 µg/mL**	**Blank**
Snake DAg	2 h RT	0.63	0.53	0.57	0.11
	1.5 h 37 °C	1.02	0.72	0.43	0.08
	1 h 37 °C	1.06	0.89	0.62	0.09
	O/N + 4 °C	1.12	0.97	0.84	0.08
PUUV NP	2 h RT	0.64	0.59	0.34	0.10
	1.5 h 37 °C	0.53	0.68	0.57	0.10
	1 h 37 °C	0.64	0.69	0.72	0.09
	O/N + 4 °C	0.72	0.68	0.73	0.10

Table 2. Intensities from initial coating condition tests from testing. Wells were blocked using 3 % BSA at +37 °C for 1 h.

Initial coating buffer comparison in different incubation conditions

Snake DAg				PUUV NP			
Blocker	**Condition**	**Carbonate-bicarbonate buffer**	**PBS buffer**	**Blokcer**	**Condition**	**Carbonate-bicarbonate buffer**	**PBS buffer**
3 % BSA	RT 2	1.87	1.90	3 % BSA	RT 2	1.96	2.01
	RT 1	2.01	1.91		RT 1	1.97	1.96
	37 °C 2	1.56	1.71		37 °C 2	1.60	1.79
	37 °C 1.5	2.07	1.86		37 °C 1.5	1.70	1.71
	37 °C 1	1.42	1.53		37 °C 1	1.39	1.74
	37 °C 0.5	1.82	1.66		37 °C 0.5	1.68	1.70
3 % Milk powder	RT 2	2.11	2.11	3 % Milk powder	RT 2	0.69	0.71
	RT 1	1.98	2.06		RT 1	0.88	0.87
	37 °C 2	1.81	1.96		37 °C 2	0.43	0.45
	37 °C 1.5	1.91	2.08		37 °C 1.5	0.49	0.48
	37 °C 1	2.14	2.38		37 °C 1	0.57	0.58
	37 °C 0.5	2.20	2.20		37 °C 0.5	0.70	0.79
BSA + Milk powder	RT 2	2.07	2.14	BSA + Milk powder	RT 2	0.73	0.77
	RT 1	2.01	2.21		RT 1	0.77	0.81
	37 °C 2	2.00	2.05		37 °C 2	0.49	0.51
	37 °C 1.5	1.89	2.06		37 °C 1.5	0.59	0.55
	37 °C 1	2.13	2.19		37 °C 1	0.63	0.67
	37 °C 0.5	2.12	2.02		37 °C 0.5	0.73	0.81

Table 3. Intensities with two different coating buffers with 1µg/mL of SpyCatcher (coated overnight at +4 °C). Wells were blocked with different blocker reagents for various times at room temperature or +37 °C.

Appendix 6: ELISA test result tables section 4.2.2

Blocker tests with background signal

Antigen	Binding	Blocker		1:25	1:50	1:100	1:200	1:400	1:800
Snake DAg	Anti-Delta Binding to Snake DAg	BSA	1 %	2.34	2.22	2.01	1.72	1.54	1.31
			3 %	2.43	2.26	2.08	1.80	1.57	1.32
		Milk powder	1 %	2.63	2.25	1.82	1.30	0.95	0.64
			3 %	2.52	2.21	1.79	1.27	0.89	0.58
	Anti-Delta Binding to Negative Control	BSA	1 %	0.17	0.19	0.22	0.19	0.20	0.23
			3 %	0.17	0.21	0.18	0.20	0.21	0.24
		Milk powder	1 %	0.20	0.15	0.15	0.15	0.16	0.19
			3 %	0.11	0.13	0.13	0.14	0.13	0.16
PUUV NP	Anti-PUUV Binding to PUUV NP	BSA	1 %	1.46	1.55	1.61	1.64	1.76	1.59
			3 %	1.44	1.48	1.53	1.54	1.62	1.52
		Milk powder	1 %	1.25	1.12	0.94	0.81	0.56	0.37
			3 %	1.18	1.06	0.84	0.67	0.47	0.31
	Anti-PUUV Binding to Negative Control	BSA	1 %	1.34	1.35	1.40	1.48	1.65	1.76
			3 %	1.22	1.33	1.36	1.43	1.59	1.66
		Milk powder	1 %	1.21	1.31	1.30	1.17	0.94	0.73
			3 %	1.40	1.36	1.24	1.09	0.81	0.79

Table 1. Signal intensities using the PBS coating buffer with 2 µg/mL SpyCatcher (incubated overnight at +4°C). Antigen lysates were diluted ranging from 1:25 to 1:800. Wells were blocked either with 1% or 3% BSA or milk powder in PBST at 37 °C for 1 hour.

Coating tests with background signal controls (coated overnight at + 4 °C) using SpyCatcher concentration series

Carbonate-bicarbonate		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Total signal	Anti-Delta + Snake DAg	1.63	1.64	1.68	1.49	1.54	1.53
	Anti-Delta Negative Control	0.12	0.17	0.20	0.23	0.27	0.31
	SpyCatcher + Anti-Delta	0.11	0.17	0.20	0.23	0.25	0.30
	Blank	0.06	0.05	0.05	0.06	0.06	0.11
Total signal	Anti-PUUV + PUUV	1.68	1.72	1.65	1.77	1.58	1.73
	Anti-PUUV negative control	1.90	1.89	1.75	1.71	1.68	1.42
	SpyCatcher + Anti-PUUV	0.14	0.11	0.10	0.13	0.14	0.20
	Blank	0.06	0.05	0.05	0.05	0.05	0.06
Carbonate-bicarbonate analyte signal		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Signal increase using SpyCatcher	Anti-Delta + Snake DAg	0.00	0.01	0.05	-0.14	-0.09	-0.10
	Anti-Delta negative control	0.00	0.05	0.08	0.11	0.16	0.19
	SpyCatcher + anti-Delta	0.00	0.06	0.09	0.12	0.14	0.19
	Blank	0.00	-0.01	-0.01	0.00	0.00	0.05
Signal increase using SpyCatcher	Anti-PUUV + PUUV	0.00	0.05	-0.03	0.10	-0.09	0.05
	Anti-PUUV negative control	0.00	-0.02	-0.15	-0.19	-0.23	-0.48
	SpyCatcher + Anti-PUUV	0.00	-0.04	-0.05	-0.02	-0.01	0.05
	Blank	0.00	-0.01	-0.01	-0.01	0.00	0.01
PBS		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Total signal	Anti-Delta + Snake DAg	1.46	1.34	1.25	1.28	1.21	1.27
	Anti-Delta negative control	0.13	0.17	0.20	0.25	0.27	0.28
	SpyCatcher + anti-Delta	0.12	0.18	0.21	0.24	0.24	0.27
	Blank	0.06	0.06	0.06	0.06	0.06	0.06
Total signal	Anti-PUUV + PUUV	1.24	1.19	1.24	1.30	1.22	1.20
	Anti-PUUV negative control	1.30	1.17	1.25	1.33	1.25	1.27
	SpyCatcher + Anti-PUUV	0.13	0.12	0.11	0.11	0.12	0.11
	Blank	0.07	0.05	0.05	0.05	0.05	0.06
PBS analyte signal		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Signal increase using SpyCatcher	Anti-Delta + Snake DAg	0.00	-0.12	-0.21	-0.18	-0.26	-0.19
	Anti-Delta negative control	0.00	0.04	0.07	0.12	0.14	0.15
	SpyCatcher + anti-Delta	0.00	0.06	0.09	0.13	0.13	0.15
	Blank	0.00	0.00	0.00	0.00	0.00	0.01
Signal increase using SpyCatcher	Anti-PUUV + PUUV	0.00	-0.04	0.00	0.06	-0.02	-0.04
	Anti-PUUV negative control	0.00	-0.14	-0.06	0.03	-0.05	-0.03
	SpyCatcher + Anti-PUUV	0.00	-0.01	-0.03	-0.03	-0.02	0.37
	Blank	0.00	-0.02	-0.02	-0.02	-0.02	-0.01

Table 2. Signals with different catcher concentrations (0–5 µg/mL), coated overnight at +4 C. Wells were blocked with 3% BSA at +37 °C for 1 hour. Signal increase represents the difference between the total signal and the background signal in wells containing SpyCatcher

Coating tests with background signal (coated at +37 °C for 1.5 hours)

Carbonate-bicarbonate		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Total signal	Anti-Delta + Snake DAg	1.46	1.34	1.25	1.28	1.21	1.27
	Anti-Delta negative control	0.13	0.17	0.20	0.25	0.27	0.28
	SpyCatcher + anti-Delta	0.12	0.18	0.21	0.24	0.24	0.27
	Blank	0.06	0.06	0.06	0.06	0.06	0.06
Total signal	Anti-PUUV + PUUV	1.24	1.19	1.24	1.30	1.22	1.20
	Anti-PUUV negative control	1.30	1.17	1.25	1.33	1.25	1.27
	SpyCatcher + Anti-PUUV	0.13	0.12	0.11	0.11	0.12	0.11
	Blank	0.07	0.05	0.05	0.05	0.05	0.06
Carbonate-bicarbonate analyte signal		0 µg/mL	0.3125 µg/mL	0.625 µg/mL	1.25 µg/mL	2.5 µg/mL	5 µg/mL
Signal increase using SpyCatcher	Anti-Delta + Snake DAg	0.00	-0.12	-0.21	-0.18	-0.26	-0.19
	Anti-Delta negative control	0.00	0.04	0.07	0.12	0.14	0.15
	SpyCatcher + anti-Delta	0.00	0.06	0.09	0.13	0.13	0.15
	Blank	0.00	0.00	0.00	0.00	0.00	0.01
Signal increase using SpyCatcher	Anti-PUUV + PUUV	0.00	-0.04	0.00	0.06	-0.02	-0.04
	Anti-PUUV negative control	0.00	-0.14	-0.06	0.03	-0.05	-0.03
	SpyCatcher + Anti-PUUV	0.00	-0.01	-0.03	-0.03	-0.02	0.37
	Blank	0.00	-0.02	-0.02	-0.02	-0.02	-0.01

Table 3. SpyCatcher concentrations (0–5 µg/mL) in the carbonate-bicarbonate buffer, coated at +4 °C for 1.5 hours. Blocked with 3% BSA at +37 °C for 1 hour using 1:150 diluted antigen lysates. Signal increase reflects the difference between the total signal and the background signal in wells containing SpyCatcher

Coating tests with background signal controls (coated at +37 °C for 1.5 hours) using SpyCatcher concentration series

Carbonate-bicarbonate			1:25	1:50	1:100	1:200	1:400	1:800
Anti-Delta	3 % BSA	Anti-Delta + Snake DAg	1.83	1.64	1.54	1.47	1.41	1.27
		Anti-Delta Negative	0.13	0.12	0.12	0.12	0.12	0.12
		Anti-Delta Background	1.02	0.72	0.67	0.52	0.39	0.35
		Blank	0.14	0.12	0.11	0.11	0.11	0.11
	3 % Milk powder	Anti-Delta + Snake DAg	1.92	1.78	1.73	1.58	1.40	1.15
		Anti-Delta Negative	0.10	0.21	0.09	0.10	0.09	0.11
		AntiDelta Background	0.18	0.09	0.07	0.07	0.07	0.08
		Blank	0.11	0.11	0.10	0.10	0.10	0.11
Carbonate-bicarbonate analyte signal			**1:25**	**1:50**	**1:100**	**1:200**	**1:400**	**1:800**
Anti-PUUV	3 % BSA	Anti-PUUV + PUUV NP	0.99	0.99	1.00	0.95	0.96	0.99
		Anti-PUUV Negative	0.88	0.87	0.90	0.92	0.97	0.97
		Anti-PUUV Background	0.99	1.00	0.97	0.98	0.98	0.79
		Blank	0.08	0.08	0.07	0.08	0.08	0.07
	3 % Milk powder	Anti-PUUV + PUUV NP	0.82	0.66	0.51	0.44	0.32	0.23
		Anti-PUUV Negative	0.99	0.94	0.90	0.85	0.78	0.58
		Anti-PUUV Background	0.80	0.60	0.43	0.31	0.22	0.15
		Blank	0.08	0.08	0.09	0.07	0.07	0.07
PBS			**1:25**	**1:50**	**1:100**	**1:200**	**1:400**	**1:800**
Anti-Delta	3 % BSA	Anti-Delta + Snake DAg	1.44	1.31	1.19	1.20	1.18	1.15
		Anti-Delta Negative	0.12	0.12	0.12	0.12	0.12	0.11
		Anti-Delta Background	0.78	0.46	0.47	0.41	0.24	0.24
		Blank	0.14	0.15	0.13	0.16	0.12	0.11
	3 % Milk powder	Anti-Delta + Snake DAg	1.28	1.35	1.42	1.47	1.41	1.21
		Anti-Delta Negative	0.12	0.10	0.10	0.10	0.10	0.09
		AntiDelta Background	0.14	0.09	0.07	0.07	0.07	0.07
		Blank	0.10	0.10	0.10	0.10	0.10	0.10
PBS analyte signal			**1:25**	**1:50**	**1:100**	**1:200**	**1:400**	**1:800**
Anti-PUUV	3 % BSA	Anti-PUUV + PUUV NP	1.01	1.02	1.00	1.02	1.01	1.03
		Anti-PUUV Negative	0.90	0.90	0.96	0.99	1.04	1.10
		Anti-PUUV Background	0.96	0.99	0.98	0.99	1.00	0.95
		Blank	0.08	0.07	0.07	0.07	0.07	0.07
	3 % Milk powder	Anti-PUUV + PUUV NP	0.78	0.70	0.59	0.43	0.31	0.22
		Anti-PUUV Negative	0.96	0.97	0.93	0.90	0.81	0.68
		Anti-PUUV Background	0.83	0.63	0.39	0.29	0.21	0.16
		Blank	0.06	0.06	0.06	0.06	0.06	0.06

Table 4. Signal intensities measured using either carbonate-bicarbonate or PBS coating buffer with 2 µg/mL SpyCatcher (incubated at +37°C for 1.5 hour) and antigen dilutions ranging from 1:25 to 1:800. Blocking was performed with 3% BSA or milk powder in PBST at 37 °C for 1 hour

Appendix 7: Created method for coating and blocking

Preparing plates for SpyTag-SpyCatcher based ELISA:

Tools and reagents:

- Maxisorp plates
- SpyCatcher
- PBS coating buffer (Phosphate-buffered saline)
- PBST for washing (PBS with 0,05% (v/v) Tween 20)
- Blocker with 1 % of milk powder in PBST

Spy Catcher coating buffer	Blocker buffer:
SpyCatcher 2 µg/ml in PBS	1% Milk powder in PBST
Volume of coater in well: 50 µl	Volume of blocker in well: at least 100 µl
Incubate wells 1,5h at +37 °C	Incubate blocker for 1h at 37 °C

Coating wells:

1. Prepare coating buffer with 2 µg/ml of SpyCatcher in PBS.
2. Coat Maxisorp plate wells with 50 µl of coating buffer.
   Pipette buffer straight to the bottom of the well avoiding walls of the wells.
   Shake wells gently and make sure the coater is spread evenly in the bottom of all wells
3. Cover the wells with sticker and incubate wells 1,5h at +37°C.
4. Wash wells with PBST 3 times
   Tap wells dry between washes

Blocking wells:

1. Prepare blocking buffer using 1% of milk powder in PBST
2. Block wells. Use at least 100 µl of blocking buffer in each well.
   Make sure that the amount of the blocker covers all the area coated with SpyCatcher.
3. Incubate wells at 37 °C for 1 hour.
4. Wash wells with PBST 3 times
   Tap wells dry between washes

Plates can be used in ELISA experiments right after blocking