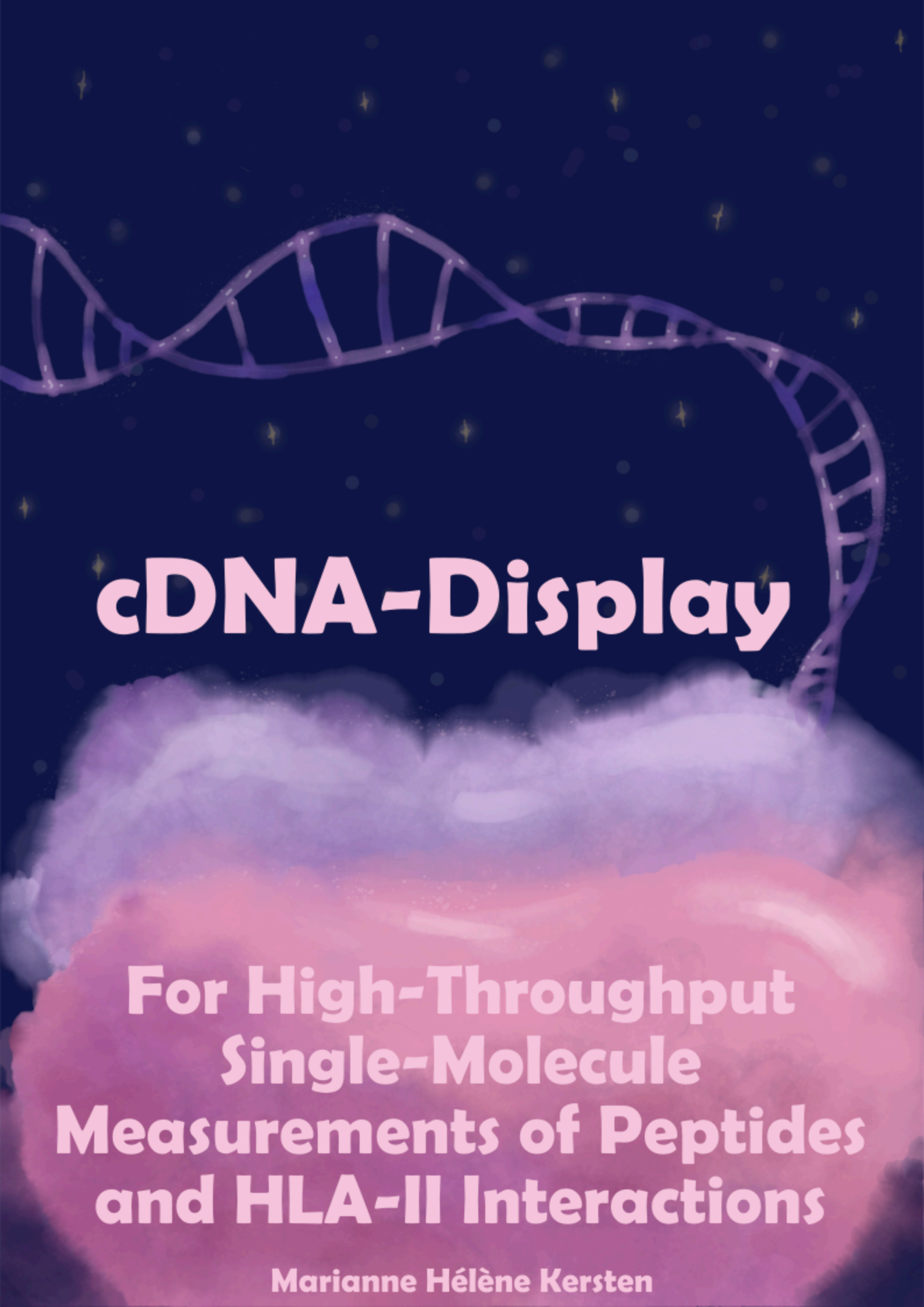




cDNA-Display

**For High-Throughput
Single-Molecule
Measurements of Peptides
and HLA-II Interactions**

Marianne Hélène Kersten

cDNA-DISPLAY FOR HIGH-THROUGHPUT SINGLE-MOLECULE MEASUREMENTS OF PEPTIDES AND HLA-II INTERACTIONS

Master End Project Thesis By

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ABSTRACT

Major histocompatibility complex (MHC) class II molecules play a crucial role within the immune system. They bind and present various peptides on the cell surfaces to T-cells, which activate the immune response upon recognition. Failure to recognize pathogens results in an insufficient immune response, but incorrectly recognizing self-antigens can cause an autoimmune response. Although T-cells ultimately determine the immune response to peptides, MHC-II determines which peptides are presented, thus which peptides can be recognized. Therefore, studying the binding kinetics between MHC-II and peptides is crucial for understanding the immune response. However, studying the binding characteristics of MHC-II and peptides is challenging due to the infinite number of possible peptides and the thousands of MHC-II polymorphisms.

Single-molecule Parallel Analysis for Rapid eXploration of Sequence space (SPARXS) could address this challenge as it combines single molecule FRET (smFRET) experiments with Illumina sequencing to measure binding kinetics at high-throughput. Extending this technique to peptide libraries would enable high-throughput measurements of MHC-II peptide binding. SPARXS would be able to obtain high-throughput kinetic data of these interactions through a smFRET assay. To be able to use this data however, it is dependent on robust genotype-phenotype coupling. This ensures that the kinetic data extracted is matched with its corresponding DNA-sequence, which is extracted through Illumina sequencing. Robust genotype-phenotype coupling is possible when using cDNA-display, which links a peptide to its encoding DNA through a puromycin linker. Building on this principle, this thesis has established the groundwork for a cDNA-display designed, which is compatible with SPARXS to enable high-throughput characterization of peptide-MHC-II interactions.

1

INTRODUCTION

1.1. GENERAL BACKGROUND ON ANTIGEN PRESENTATION

Major histocompatibility complexes (MHCs) are crucial proteins within the adaptive immune system, responsible for the presentation of antigens to T-cells. T-cells have to distinguish between self and non-self, while also recognizing aberrated self antigens that might signal cellular malfunction or mutations. Recognition of these antigens by the T-cell receptors (TCRs) will result in the activation of an immune response. Through this mechanism, TCRs ultimately determine the immune response. However, TCRs can only do so in the context of MHC as they probe the peptide-MHC complex, rather than the peptide by itself. Therefore, the binding kinetics of MHC and a given peptide can greatly affect the immune response to said peptide, with grave consequences. Failure to recognize pathogens hinders the immune response, whereas incorrectly recognizing self as pathogenic leads to autoimmune diseases such as type 1 diabetes.

MHC can be found on the membrane of all nucleated cells, as well as on professional antigen presenting cells (APCs). However, the distribution of MHC across cell types depends on the structure and function of the specific class. MHC class I, encoded in humans by the highly polymorphic human leukocyte antigen (HLA) loci HLA-A, HLA-B, and HLA-C, is expressed on all nucleated cells, where it mainly presents endogenous proteins to CD8⁺ T cells (Hewitt, 2003). In healthy, non-infected cells, none of the peptides derived from endogenous proteins should be recognized by the TCR, whereas peptides with mutations or of foreign origin, such as viruses, are likely to cause an immune response. In contrast, class II proteins (HLA-DR, DQ, DP) are expressed only on professional APCs, which include dendritic cells, B cells, macrophages and thymic epithelial cells (Pishesha et al., 2022). MHC class II, is specialized in presenting exogenous peptides to CD4⁺ T-cells (Figure 2)(Rock et al., 2016). These peptides have been captured into the cell through various processes and are subsequently processed in endolysosomal compartments within the cell, before being presented (Roche & Furuta, 2015; Rossjohn et al., 2015).

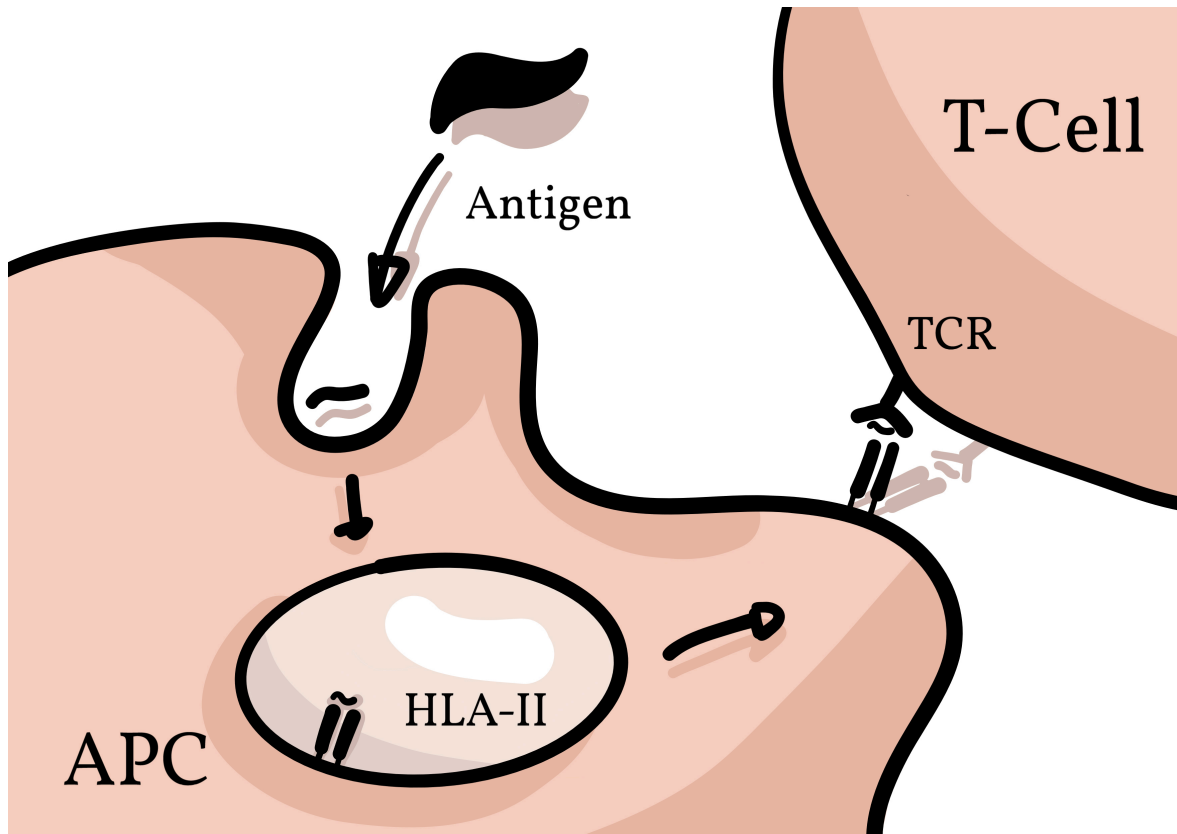


Figure 2: Overview of antigen presentation by HLA-II. Antigens are taken up into the cell, processed into peptides and transported to the antigen processing compartment. Here they bind to HLA-II, which is transported to the outer cell membrane, where it presents the antigen to T-cells. The TCR of the T-cell can recognize the antigen and initiate the immuneresponse.

The functional difference between the two classes is reflected in their structure. Although both classes share great similarities, as they both present peptides, key distinction can be made between them. Both are heterodimers, but are composed of different chains. MHC-I consists of a heavy α -chain combined with a β 2-microglobulin chain. The peptide binding groove is formed solely by the α -chain. In contrast, MHC-II is composed of an α and a β -chain, both of which contribute to the formation of the peptide binding groove (Rossjohn et al., 2015). These features affect the architecture of the peptide binding groove, which results in distinct peptide-binding properties, which will be discussed in more detail later.

So far, the discussion has focused on classical MHC proteins. In addition to the classical MHC molecules there exist non-classical molecules. These non-classical MHC molecules share similarities in structure, but can differ greatly in function. Only a few non-classical proteins of class I present peptides, in a similar fashion to the classical ones. Most non-classical proteins either present non-peptide antigens, including phospholipids, lipids, fatty acids and vitamin B metabolites or they do not bind an antigen at all. Instead they can act as a cofactor for binding of classical MHC to TCRs or in the loading of classical MHC proteins. The latter is also the case for the non-classical class II proteins (Halenius et al., 2014).

As mentioned previously, HLA class II molecules present exogenous proteins captured by the cell. Additionally, the peptides derived from these proteins can originate from other somatic cells within the organism. If they are then presented and recognized by the TCRs, they elicit an immune response to the cell of origin. This is why class II molecules in particular are associated with autoimmunity. Moreover, alleles of this highly polymorphic gene have shown to correlate with higher risks for specific autoimmune diseases. Despite their central role in the immune-system and autoimmunity, the peptide interactions of class II molecules remain less well-characterized than those of class I. Therefore, this work aims to further the understanding of these interactions. The focus will be mainly on the classical MHC class II molecules, with additional attention to the relevant non-classical proteins that modulate their function.

1.2. THE ANTIGEN PROCESSING COMPARTMENT

It is within the antigen processing compartment where the majority of antigens are loaded onto the HLA class II molecules. The compartment is a specialized micro environment with unique properties that cannot be found in the cytoplasm. The compartment is complex and is typically a late endosomal or lysosomal structure with complex internal membrane structures including intraluminal vesicles, which form multi-vesicular bodies. Considering the unique properties of these bodies is required to understand peptide binding kinetics and its implications.

The typical pH of these bodies is acidic, with an pH between 4.5-5.5. The importance of this pH is highlighted by the numerous studies that show greatly reduced binding kinetics of peptide to HLA-II at higher pH (Sette et al., 1992). Secondly, proteins have to enter one of the antigen processing pathways in order to enter the compartment and possibly be presented, thus favoring the presentation of non-self antigens which leads to self-tolerance. Finally, the HLA class II molecules are on the membrane facing inwards. There are multiple accessory proteins which play a role in the regulation of peptide binding, with the most important ones, invariant chain, HLA-DM, HLA-DO being membrane proteins as well. This allows for the control of HLA-II activity in co-localization by membrane micro domains. Altogether, the compartment can be viewed as a carefully controlled microenvironment in which pH, membrane organization, and accessory proteins converge to regulate antigen loading. The starting point of this regulated pathway is the association of MHC-II with the invariant chain (Ii), which ensures that peptide binding occurs only under the appropriate conditions (Roche & Furuta, 2015).

1.2.1. REMOVAL OF THE INVARIANT CHAIN (Ii)

The Ii is a type II non-polymorphic membrane protein which associates with MHC-II during biosynthesis within the endoplasmic reticulum (ER). Ii binds into and blocks the peptide binding groove, thus effectively preventing the premature binding of peptides to MHC-II. Furthermore, it stabilizes the MHC-II complex by occupying the peptide binding groove. (Busch et al., 1996; Landsverk et al., 2009; Stumptner-Cuvelette & Benaroch, 2002).

Within the antigen processing compartment, the invariant chain has to be removed, to allow for the binding of antigens to HLA-II (Figure 3). In order to achieve this, the Ii is cleaved into fragments. The cytosolic fragment, of approximately 15 amino acids is Class II-associated invariant chain peptide (CLIP) (Landsverk et al., 2009; Stumptner-Cuvelette & Benaroch, 2002). All other fragments of Ii but CLIP dissociate from the HLA-DR complex. CLIP remains bound within the peptide binding groove, where it still blocks antigens from binding. Different alleles of HLA-II have different affinities for CLIP (Sanderson & Trowsdale, 1995). It sometimes dissociates spontaneously, but often requires mediation by HLA-DM in order to remove the peptide (Figure 3). Notably, alleles with lower affinities of CLIP are associated with a higher risk for autoimmunity (Busch et al., 1996; Honey et al., 2004; Ito et al., 2018).

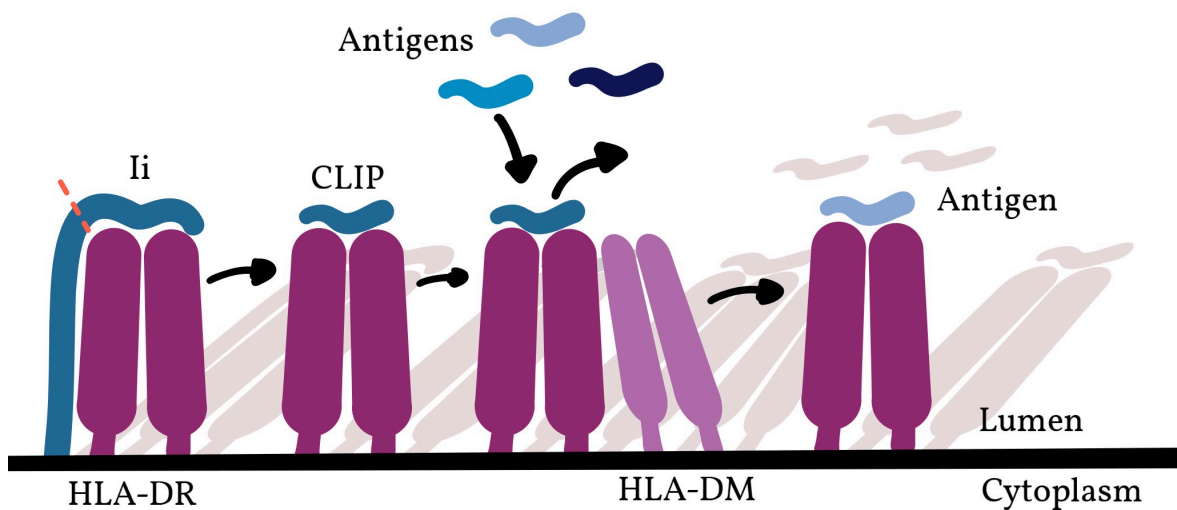


Figure 3: Overview of HLA-DM mediated peptide exchange within the antigen processing compartment. HLA-DR is bound to the invariant chain (Ii), which is then cleaved and degraded into Class II-associated invariant chain peptide (CLIP). HLA-DM catalyzes the removal of CLIP and subsequent peptide exchange. Upon stable binding of an antigen, HLA-DM disassociates. HLA-DR is transported to the cell membrane, where it can present the antigen.

1.2.2. HLA-DM MEDIATED PEPTIDE EXCHANGE

HLA-DM is a non-classical HLA-class II protein, which unlike its classical counterpart, cannot bind peptides, due to a shallower and negatively charged groove (Sanderson & Trowsdale, 1995). It is a molecular chaperone and its function is to induce the removal of CLIP that even at low affinities can take hours to dissociate from the complex (Schulze & Wucherpennig, 2012). Furthermore, after the removal of CLIP, it further catalyzes the selection process of peptide binding (Figure 3). It is thought that HLA-DM stabilizes a conformational intermediated of HLA-DR with rapid binding kinetics, thus allowing for the quick exchange of peptides, until a stable binder is found (Pos et al., 2012).

1.2.3. HLA-DO CONTROLS HLA-DM ACTIVITY

HLA-DO is another non-classical HLA-class II protein, which is rather similar in structure to classical HLA-DR. It can bind the active binding site of HLA-DM, as it is similar in structure, although it is not capable of binding peptides. It is thought that by binding to HLA-DM, HLA-DO down-regulates the activity of HLA-DM. Through its effects on HLA-DM, HLA-DO is thought to edit the peptide binding repertoire of HLA-II, as it either favors HLA-DM edited or non-edited peptides (Álvaro-Benito & Freund, 2020). By lowering the ability of HLA-DM to edit the peptidome, the number of self-restricted peptides is limited. Self-peptides, besides CLIP, are less efficiently presented. Therefore, it is thought that the presence of HLA-DO promotes self-tolerance (Guce et al., 2012).

The binding kinetics of peptides to HLA-II are thus depended on several factors. Ii, HLA-DM and HLA-DO regulate when, where and how fast peptide exchange occurs. This is further regulated by the compartmentalization, which creates a microenvironment, with unique properties including a lower pH the specific membrane properties and the employment of an antigen processing pathway. Together this promotes efficient, but controlled peptide exchange, promoting self-tolerance while protecting the ability to instigate an immune response.

1.3. STRUCTURE OF HLA-DR MOLECULES

Although the microenvironment created in the cell is thus of great importance for efficient peptide exchange, it is ultimately HLA-DR itself that determines whether a peptide can be stably bound. Thus an understanding of the structure of HLA class II molecules is required in order to be able to study the binding kinetics of the peptides and HLA-II and their immunological consequences. This can be better understood by first understanding the basic structure of HLA-II. They are membrane proteins, as they have to present the antigen to the outside of the cell. Furthermore the HLA class II molecules are dimers, consisting of an α - and a β -chain. Each chain contains a cytoplasmic tail, a transmembrane segment and two extracellular domains. The membrane-proximal extracellular domains $\alpha 2$ and $\beta 2$ are immunoglobulin-like in structure. The $\alpha 1$ and $\beta 1$ domains are positioned above and together form the peptide binding groove (Stern, 2016).

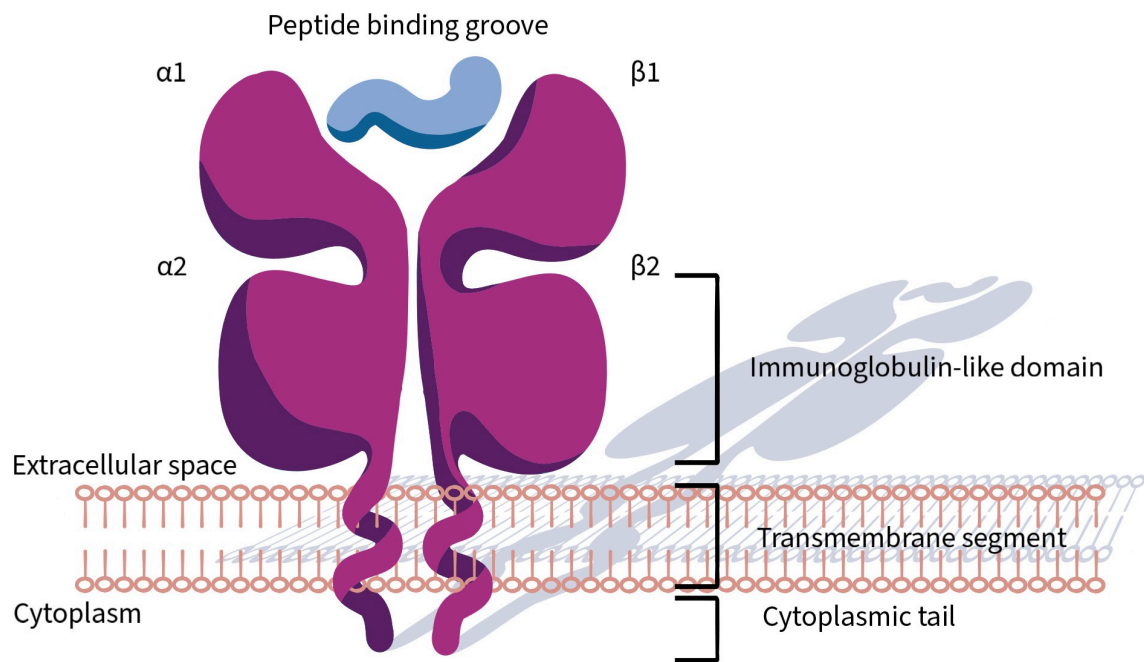


Figure 4: Structure of HLA-DR. The structure consists of an α - and β -chain, with domains as indicated. The $\alpha1$ and $\beta1$ domains together form the peptide binding groove, where in red the peptide is depicted.

1.3.1. GENETIC POLYMORPHISM

The genes of HLA-DR are generally the most expressed HLA-II variants. The gene is located on chromosome 6. The α -chain is encoded by one gene whereas four variants of the β -chain, (DRB1, DRB3, DRB4 and DRB5) are located on the same gene. As of June 2025, few polymorphisms of the α -chain have been found, in contrast with 2499 polymorphisms of the DRB1 gene. Additionally, over a hundred polymorphisms have been found for each of the DRB3, DRB4 and DRB5 genes have been found. The polymorphisms are a likely result of pathogenic selection driven evolution, which results in more resistant immune systems (Barker et al., 2023; Boegel et al., 2018). Although expression levels differ across cell types, DRB1 is generally the most expressed β -chain across all professional APCs. It plays a dominant role in the determination of the peptidome presented to T-cells. However, the other genes are however not unimportant. Evidence has shown that DRB3, DRB4 and DRB5 bind a distinct peptidome that is complementary to the one presented by DRB1. This complementary nature likely arose from the selective pressure of pathogens and has subsequently been conserved across individuals, as the alleles are located in close vicinity on the same chromosome (Faner et al., 2009; Kaabinejadian et al., 2022).

1.3.2. THE PEPTIDE BINDING GROOVE

The peptide binding groove constitutes the most functionally critical region of the protein, as it directly determines the binding and presentation peptides. It is within the groove that most polymorphisms are found, which alter the peptide binding repertoire of the molecule.

The peptide binding groove is created by both the α - and β -chain. Each chain contributes a β -sheet, which forms the floor of the groove, as well as an α -helix, which creates the sides of the groove (Figure 5A). The HLA class II groove is open at both the N-terminal and C-terminal end. This allows peptides of variable length to bind. Despite the absence of such restraints the typical length of a bound peptide is between 13 to 25 amino acids long (Stern, 2016).

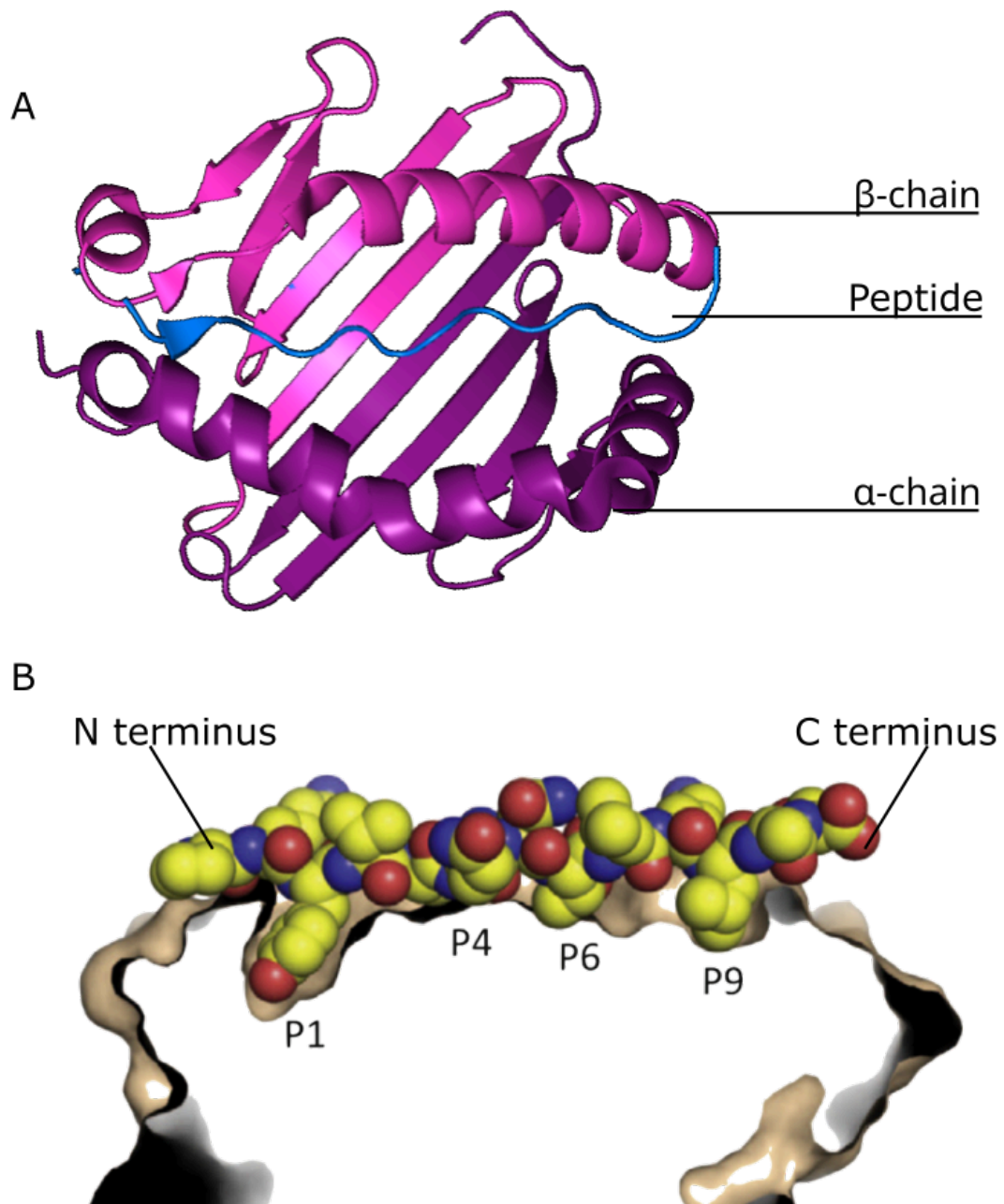


Figure 5: Structure of peptide binding groove. **A)** Topview of peptide binding groove, which consists of a β -sheet at the bottom of the groove, created by both the α - and β -chain. Structure from (Chen et al., 2023). Both chains contribute an α -helix that form the sides of the binding groove. In the binding groove a peptide (blue) bound. **B)** Sideview of peptide binding groove, outlining the main binding pockets: P1, P4, P6, P9 Adapted from (Stern, 2016).

Peptides bind in an extended conformation along the groove, with only approximately 12 residues in direct contact with the HLA molecule. Stretched out, the peptide takes a polyproline type II conformation, with a series of hydrogen bonds forming between the groove and the peptide backbone. Although the hydrogen bonds are formed across the peptide, the N-terminal hydrogen bonds are seemingly more important than those in the shallower C-terminal region (McFarland & Beeson, 2002). Most side chains of the peptide are oriented outwards, facilitating the recognition by TCRs, while some protrude into the pockets within the groove. These interactions help anchor the peptide but only indirectly affect TCR interactions (Rossjohn et al., 2015; Stern, 2016).

1.3.3. BINDING POCKETS

The groove can be subdivided into a series of nine pockets, termed P1-P9, with the N-terminus of the peptide at the P1 pocket side. The side chains anchor themselves into the P1, P4, P6 and P9 pockets (Figure 5B). They are composed of both the residues of the α - and β -chain, except P4, which is entirely formed by the β -chain. These pockets differ across the different alleles of HLA class II, directly affecting their peptide binding repertoire (Jones et al., 2006; Stern, 2016). Arguably the most important pocket is the P1, pocket which is also the deepest. This pocket anchors the peptide into the groove. Generally it contains either a glycine or valine residue in the β -chain at position 86. Incorporation of valine results in a shallow P1 pocket, that can only accommodate small hydrophobic acids. Substituting it for glycine creates a larger pocket, that can thus accommodate bulkier hydrophobic acids and even aromatic residues (Kaabinejadian et al., 2022).

P9 pocket is also protruding into the groove, completely burying the side chain of the residue at this position. However, it is slightly less deep than the P1 pocket. Together, P1 and P9 are the most important determinants for the binding of peptides. The P4 and P6 pockets are much shallower. Amino acids bound into these pockets are still partially exposed and contribute to T-cell recognition. The P3, P7 and P10 pockets are very shallow and also referred to as shelves, rather than pockets. They contribute to the overall binding interaction, but to a much lesser extend than the aforementioned pockets. The side chains that do not interact with HLA-II at all are P2, P5 and P8. These project away from the binding groove and are major sites for interaction with TCRs (Jones et al., 2006; Kaabinejadian et al., 2022; Stern, 2016).

1.3.4. HLA-DM CATALYZED PEPTIDE SELECTION OF HLA-DR

As aforementioned, HLA-DM plays a central role in the regulating peptide–MHC-II interactions by catalyzing the exchange of bound peptides. It promotes the removal of CLIP, catalyzes peptide exchange and promotes the binding of high-affinity peptides (Pos et al., 2012). Understanding this mechanism is crucial for elucidating how antigen presentation is shaped at the molecular level. Here, the structural model of peptide exchange as catalyzed by HLA-DM will be outlined.

After the degradation of Ii, HLA-DR is left bound to CLIP. A model for HLA-DM mediated peptide selection as proposed by (Pos et al., 2012), suggests that the P1 pocket is the most important for peptide exchange. This pocket is initially stabilized by the α W43 residue, which is oriented into the binding pocket and stabilizes the residues in its vicinity. The peptide exchange then starts with the dissociation from the P1 pocket. This causes α W43 to rotate outwards (Figure 6A). This rotation induces a conformational change in the vicinity of the P1 pocket. The flip creates a wider pocket and leads to the loss of stabilizing interactions with the β sheet at the floor of the groove and notably the DR α 52 to 55 strand. These changes combined allow for the formation of an extended helix, which spans from DR α 46 to 77 (Figure 6B). Simultaneous to the rotation outwards of α W43, three key residues are repositioned into the P1 pocket. First of all α F51 is moved (Figure 6A), thanks to the newly obtained helical conformation of the α 52 to 55 strand. There it stabilizes the hydrophobic pocket. Secondly, β F89 interacts with α F51, due to the repositioning of the DR β 86-91 segmenting, which further stabilizes the now empty pocket. Finally α E55 forms a water-mediated hydrogen bond with β N82, which is an essential residue for stable peptide binding. (Pos et al., 2012)

As α W43 is now oriented outwards, it is available for interaction with the α N125 residue of HLA-DM. Both residues have shown to be essential for the activity of HLA-DM, as mutation of either residue leads to greatly reduced or even complete loss of activity. It is thought that by the binding of HLA-DM to HLA-DR, through interaction with α W43 and others, it stabilizes HLA-DR into a peptide receptive conformation. This is supported by the notion that when bound, the P4, P6 and P9 pockets are available for binding of peptides (Anders et al., 2010). These pockets are shallower as compared to the P1 pocket, thus allowing for faster associating and disassociation kinetics. Incoming peptides first bind into these pockets. High-affinity peptides then compete with the side-residues of HLA-DR itself for binding in the P1 pocket. If it outcompetes the sidechains, it occupies the P1 pocket, which leads to the reversal of the conformational changes as described above and long-lived, stable binding (Pishesha et al., 2022; Pos et al., 2012).

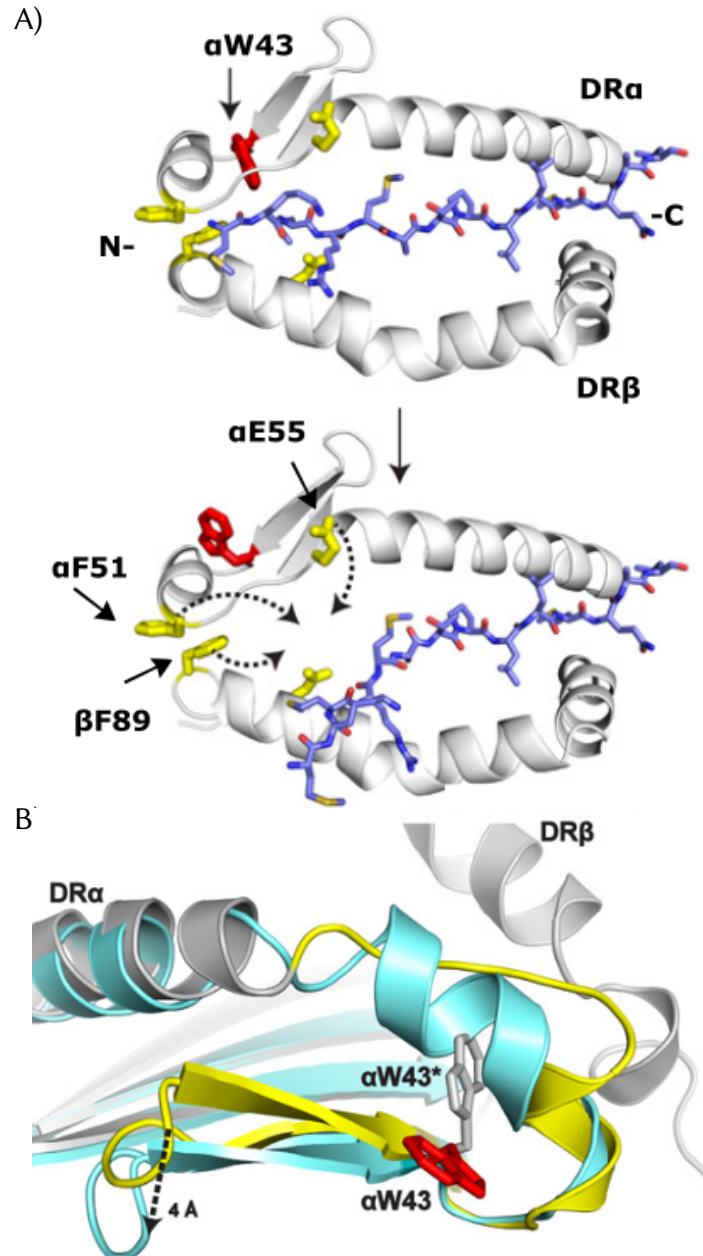


Figure 6: Structural changes in HLA-DR upon dissociation of CLIP peptide. **A)** αW43 (red) is oriented into the P1 pocket of the binding groove when the CLIP peptide. CLIP dissociates from the P1 pocket, causing αW43 to rotate outwards. This causes αF51, βF89 and αE55 to rotate inwards and stabilize the P1 pocket. **B)** In grey, the structure of HLA-DR bound to haemagglutinin₃₀₆₋₃₁₈ peptide. In yellow, the area, which undergoes a conformational change upon release of the peptide, and binding to HLA-DM is highlighted. In cyan, these changes are shown. In red, the new position after rotation outwards is shown for αW43. Adapted from (Pos et al., 2012)

1.4. EXPLORING HLA-II-PEPTIDE KINETICS

Extensive research into the peptide binding repertoire of HLA-II molecules, has revealed key amino acid motifs for given HLA allotypes. Nonetheless, binding kinetics for many HLA-II-peptide pairs remain poorly characterized. This knowledge gap stems from the high diversity present in both epitopes and HLA-II molecules. As a result, scientists rely on predictive algorithms to estimate binding kinetics. Although valuable, these inevitably contain a degree of uncertainty. To either increase the training data for such predictive algorithms, or to bypass their limitations, one has to rely on experimental procedures.

1.4.1. HIGH-THROUGHPUT PEPTIDOME DETERMINATION

A commonly used method for the identification of the peptidome is the usage of mass spectrometry. This method achieves high throughput measurements of the peptidome. The general procedure is outlined in Figure 7. It does this by using the cell lysate of various origins, including whole organs, cultured cells or biopsies. The HLA complex is purified by immunoaffinity from the lysate. Then the peptides are separated from the MHC molecules, which can be done through various methods, such as acid elution. The HLA-II peptide mixture is then used for liquid chromatography tandem mass spectrometry (LC-MS/MS). Using a high performance liquid chromatography system, the peptides are separated from the HLA-II molecules and analyzed using the tandem mass spectrometry to determine the compositions of the peptides (Bozzacco et al., 2011; Purcell et al., 2019). The advantage of this technique is the high throughput of peptides. However, it does not analyze the binding kinetics of the peptides and HLA-II molecules. For this other techniques are available.

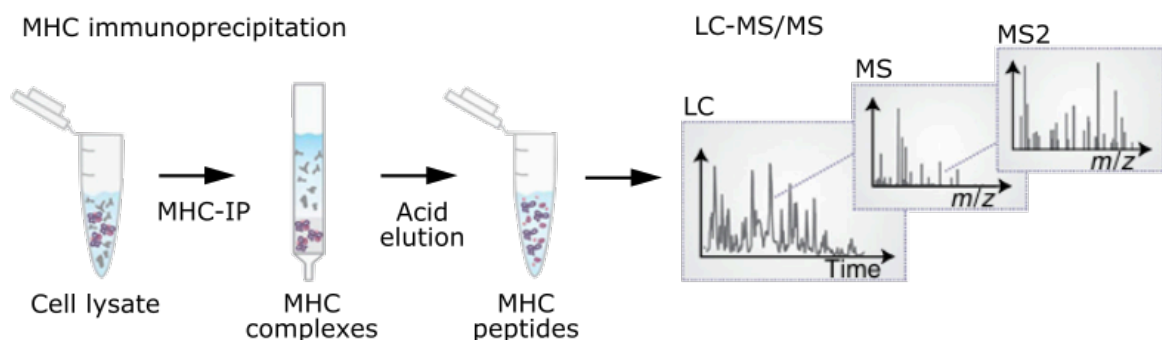


Figure 7: Overview of workflow of mass spectrometry for determining the peptidome. Cells are lysated and MHC complexes are immunoprecipitated (MHC-IP). Using acid elution, peptides are dissociated from the MHC complex and separated using liquid chromatography, which is followed by tandem mass spectrometry (LC-MS/MS). Adapted from (Purcell et al., 2019)

1.4.2. LOW-THROUGHPUT KINETICS

Commonly employed methods include fluorescence polarization and surface plasmon resonance. Fluorescent polarization relies, as the name suggests, on polarized light excitation of a fluorophore. This fluorophore can be attached to a small peptide of interest. Due

to Brownian motion, the peptide rotates rapidly, resulting in depolarized emission from the fluorophore. Upon binding to a larger molecule, such as HLA-II, the rotational speed decreases, resulting in more polarized emission. This technique has high sensitivity and allows for realtime in solution measurement of peptide binding and dissociation.

A label free method is surface plasmon resonance, which is also highly sensitive. For this method an incident light is directed at a thin metal sheet, under a specific angle. At a particular angle the electrons in the sheet become excited and travel parallel through the metal sheet. This causes an intensity drop in the reflected light, which can be detected. The angle at which the intensity drop is observed is related to the reflective index of the material. Ligands can be adsorbed onto the surface. When an analyte binds to the ligand, it causes a small, localized change in the refractive index. This causes an angular shift of the detected intensity drop. Therefore, this highly sensitive method is able to detect in real time binding events.

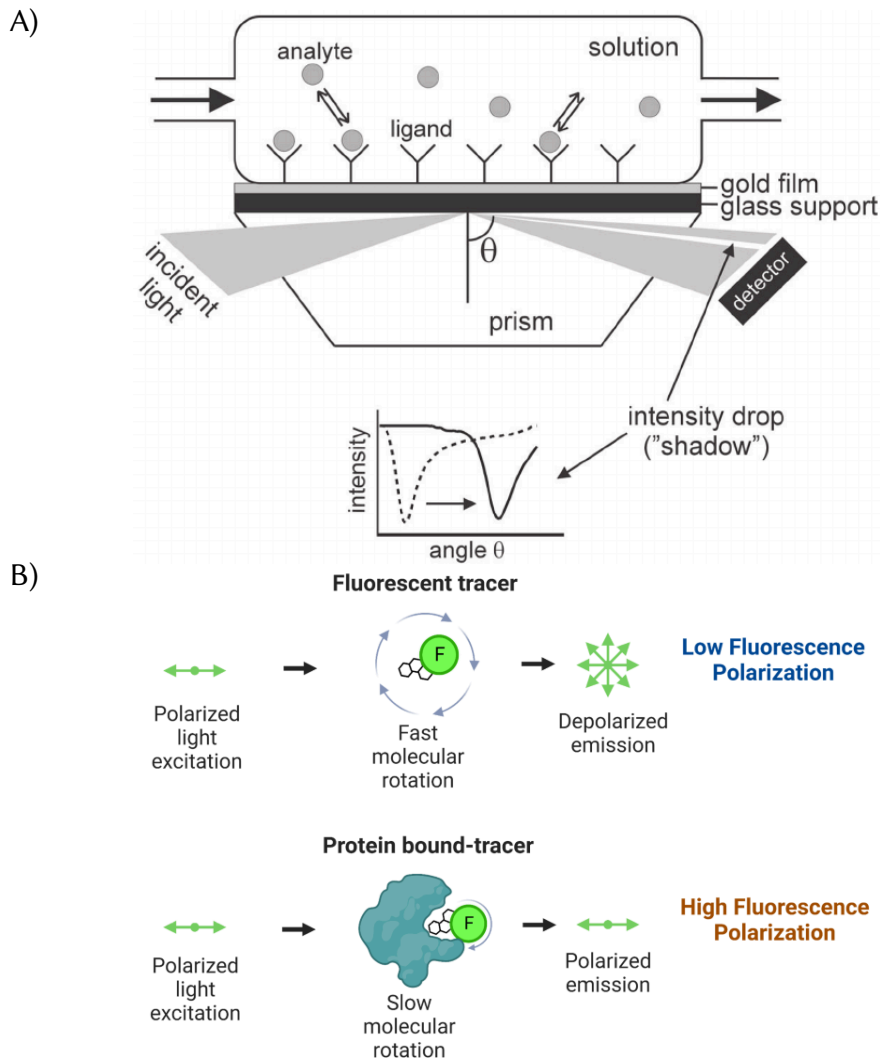


Figure 8: **A)** Schematic overview of surface plasmon resonance. From: (Hahnefeld et al., 2004). **B)** Schematic overview of underlying principle of fluorescence polarization measurements. From: (BPS Bioscience, 2025).

Although these methods both have high sensitivity and allow for real time measurements, they are both bulk measurements, averaging over all peptides and HLA-II molecules. As such, neither method is able to resolve distinct heterogeneous states. However, different conformational states of HLA-II can cause differences in binding kinetics that are obscured in bulk measurements. Furthermore, transient binding of peptides into the groove cannot be observed. To be able to observe this, a single molecule assay is required. This can be done using single molecule fluorescence resonance energy transfer microscopy (smFRET).

1.5. SINGLE MOLECULE FLUORESCENCE RESONANCE ENERGY TRANSFER (SMFRET) MICROSCOPY

The spatial resolution of conventional widefield microscopy techniques is fundamentally limited by the Rayleigh criterion. This results in a spatial resolution limit of approximately 200nm for most microscopes in biological research. Although this is sufficient for the localizations of molecules, interactions within and between proteins remain unobserved, as they occur at a much smaller scale. To overcome this limitation, Fluorescence (or Förster) resonance energy transfer (FRET) can be employed. FRET is a technique that detects the energy transfer between two chromophores in close proximity. This energy transfer requires extremely close proximity of approximately 1-10nm, thus greatly improving the resolution of the interactions between the molecules. (Sasmal et al., 2016)

The high spatial resolution of FRET relies on its underlying properties. When a molecule absorbs a photon, its energy increases, resulting in one or more electrons of the molecule to be excited to a higher energy level. Similarly, the electron can fall back to a lower energy state, and release a photon, which results in fluorescence. The emitted light has a lower energy and thus a longer wavelength, resulting in a different color than the absorbed light.

FRET occurs when an electron of a donor falls back to a lower energy state, but does not emit a photon. Instead the energy is transferred through non-radiative dipole-dipole coupling to an acceptor fluorophore, which is excited. The excited fluorophore returns to a lower state, emitting a photon of lower energy and longer wavelength (Sasmal et al., 2016). This is illustrated in the Jablonski diagram in Figure 9.

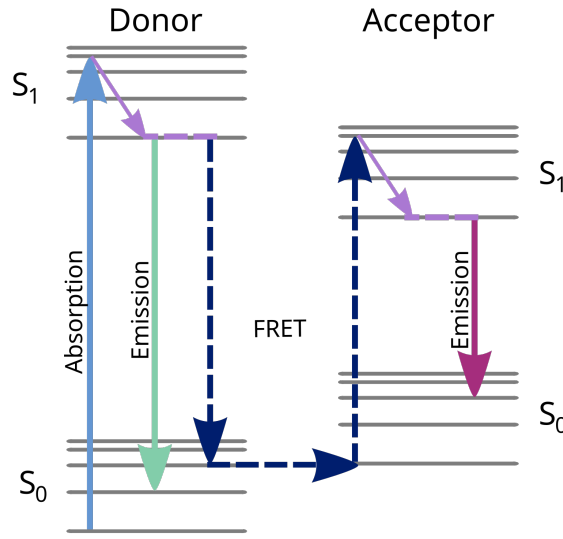


Figure 9: Jablonski diagram of fluorescence and FRET, purple lines denote relaxation.

$$E_{\text{FRET}} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \quad \text{Eq. 1}$$

The measured FRET efficiency (E_{FRET}) can be calculated using Eq. 1, where r is the distance between the acceptor and the donor and R_0 is the distance at which the FRET efficiency is 50%. Because the FRET efficiency is inversely proportional to the sixth power of the radius, even small changes in distance greatly effect the FRET efficiency. This strong distance dependency allows for resolving distances at the nanometer scale. This is useful for determining intramolecular interactions. Typically this is done using either confocal microscopy or total internal reflection fluorescence (TIRF) (Sasmal et al., 2016). In this work, TIRF is used as it has a higher signal to noise ratio and uses lower laser power.

1.6. TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF) MICROSCOPY

TIRF microscopy is widely used for single molecule experiments and particularly well suited for observing near surface events. TIRF relies on an optical phenomenon that occurs at the interface between a solid glass and a transparent liquid. When an excitation light is totally internally reflected within the glass, an electromagnetic field is generated. This field, also known as an evanescent wave is generated in the liquid and has the same frequency as the excitation light. The intensity of the wave decays exponentially, typically penetrating only to an approximate 200nm into the sample. As a result, TIRF only illuminates fluorophores near the surface, providing a high signal-to-noise ratio (Fish, 2009).

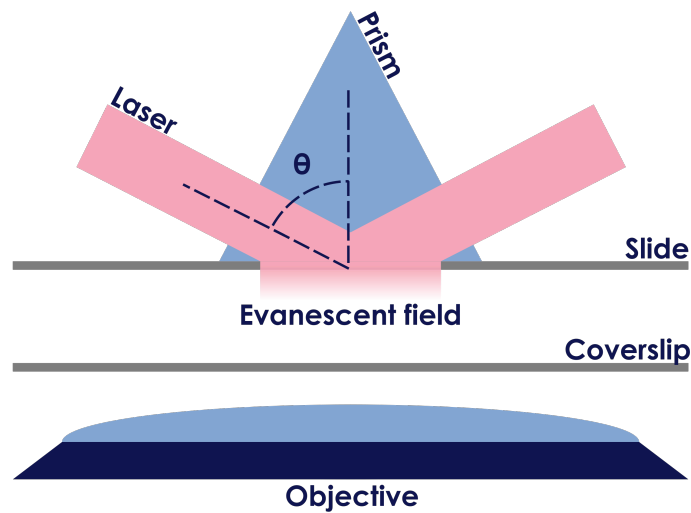


Figure 10: A slide is placed with the coverslip facing down. On top a laser is passes through a prism, at an angle to create an evanescent field. The objective placed underneath the slide captures the fluorescent signal of the molecules. Adapted from:(MARTIN-FERNANDEZ et al., 2013)

1.7. SINGLE-MOLECULE PARALLEL ANALYSIS FOR RAPID EXPLORATION OF SEQUENCE SPACE (SPARXS)

smFRET using TIRF microscopy is able to show heterogeneity in binding kinetics. Furthermore, it is able to discern transient binding states. However, like surface plasmon resonance and fluorescence polarization, it remains limited by low-throughput. Each assay typically measures the interactions between a single HLA molecule and a single peptide. This is in stark contrast to the almost endless peptide sequence space, which HLA-II can possibly encounter *in vivo*. As a result, none of these techniques can uncover broader trends independently in the peptide binding repertoire of HLA-II molecules. Therefore, adjustments to enable high-throughput measurements using smFRET are essential.

A method that achieves high-throughput while maintaining single molecule resolution is Single-molecule Parallel Analysis for Rapid eXploration of Sequence space (SPARXS). SPARXS combines smFRET with Illumina sequencing to achieve high-throughput (Severins et al., 2024). It accomplishes this by immobilizing DNA or DNA labeled samples on the surface of an Illumina flowcell. These samples are fluorescently labeled. Using a fluorescent probe, the binding between the probe and the immobilized samples can be observed with smFRET measurements, which provide kinetic data for the immobilized samples.

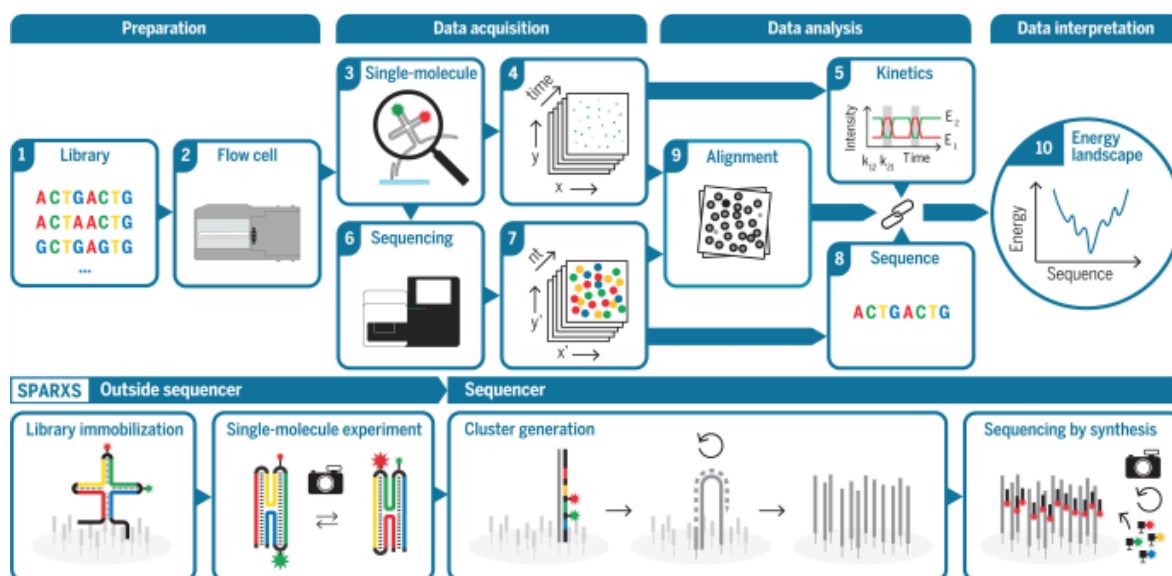


Figure 11: Overview of SPARXS workflow. Adapted from: (Severins et al., 2024).

The underlying principles are similar to a conventional smFRET experiment. However, the usage of an Illumina flowcell is the critical innovation that enables high-throughput. The immobilized DNA or DNA-labeled sample can be sequenced after smFRET measurements. Because each molecule retains their spatial coordinates, the sequencing data and the kinetic data can be aligned. Therefore, the kinetic data of an individual molecule can be directly matched with its sequence. The full procedures are outlined in Figure 11.

This allows for the introduction of diversity within the immobilized sample, by constructing a DNA-library. The samples can be analyzed simultaneously using the same binder with smFRET. Afterwards, using the sequencing data, they can be identified. This allows for high-throughput single molecule measurements which have previously been reported to analyze over 400,000 molecules (Severins et al., 2024). Using this analysis, an energy landscape of the sequence space can be created. Therefore, this method can provide both single-molecule resolution and high-throughput.

1.8. HLA-II-PEPTIDE MEASUREMENTS USING SPARXS

The implementation of peptide HLA-II measurement into SPARXS is non-trivial. The most important feature required for SPARXS is that each peptide can be identified through either a DNA or RNA mediated immobilization and identification. As a peptide consists of amino acids, encoded by mRNA a straightforward method of encoding the peptide is phenotype-genotype coupling. Various display methods exist, which are able to link the peptide to its template RNA or DNA.

1.8.1. PHAGE DISPLAY

A commonly employed display method, suitable for high-throughput measurements is phage display. Phage display uses bacteriophages to display peptides and proteins of interest on the surface. This is done by fusion of the protein of interest with capsid proteins

of the bacteriophage. The fusion is achieved by incorporating the protein sequence into the DNA of the phage. Using a large gene library, a highly diverse population of phage particles displaying different peptides can be generated (Smith & Petrenko, 1997).

A typical phage display experiment involves the immobilization of a target of interest, such as HLA-II. Phages, displaying high-affinity binders will bind to the target on the surface, thus becoming immobilized as well (Figure 12). Weak binders are washed off, leaving only the strong binders. These can then be amplified and undergo multiple rounds of selection to allow for evolution by introducing small mutations. Furthermore, increasing the stringency each round, will further isolate high-affinity binders (Smith & Petrenko, 1997).

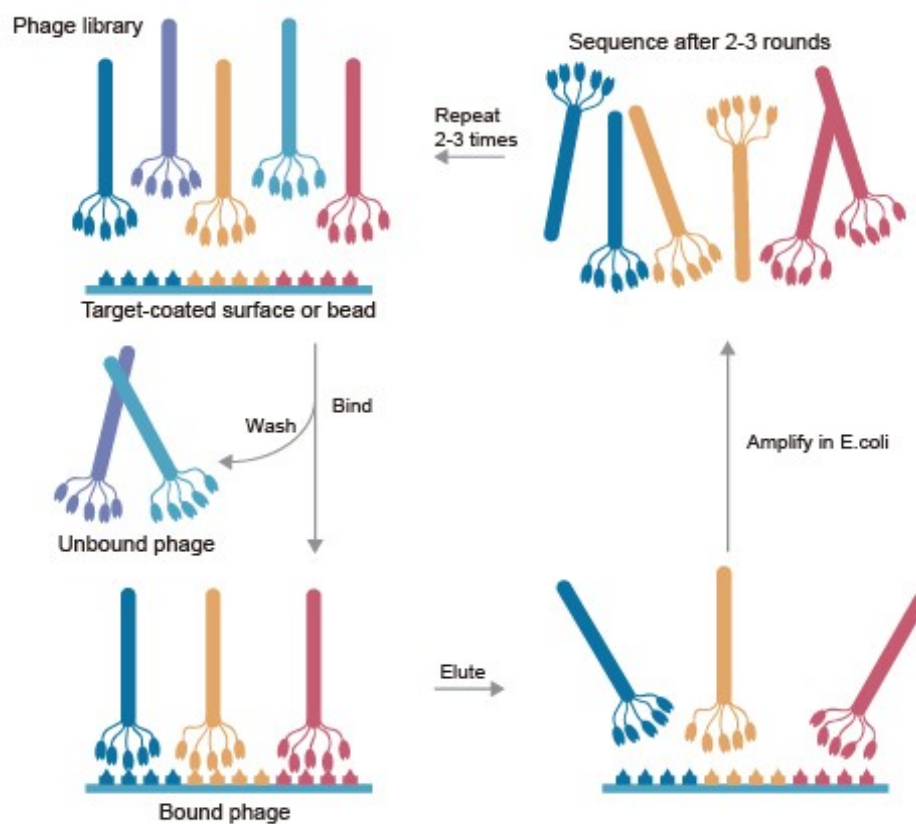


Figure 12: Procedure of phage display. A library of protein displaying phages is generated. High-affinity binders bind to the surface immobilized targets. Unbound phages are washed off. The high-affinity binders are eluted and amplified for sequencing or for additional selection. Adapted from (BiologicsCorp, 2025).

1.8.2. CELL FREE DISPLAY

A display method that is compatible with SPARXS is thus needs to either contain a DNA or RNA sequence that is immobilized on the surface. There are several methods available that satisfy this requirement. One of these methods is ribosome-display. For the implementation of this method, a DNA library is created, which is fused to a spacer sequence. The library is transcribed into RNA products and subsequently translated. The spacer sequence lacks a stopcodon, causing the ribosome to stall. The spacer sequence remains

bound to the tRNA. This creates a complex of the ribosome, mRNA and protein, which is able to fold as it protrudes out of the ribosome. The biggest limitation of this method is the instability of the complex. Although the effects can be mitigated, it does require working at low temperatures and using a high concentration of magnesium ions (Li et al., 2018).

An alternative method is RNA display or mRNA display. An RNA sequence is linked to a puromycin linker. is a amino-nucleoside antibiotic that is produced by the *Streptomyces alboniger*. It consists of a nucleoside covalently linked to an amino acid. This configuration mimics the 3' end of aminoacylated tRNAs, which deliver amino acids to ribosomes for the elongation of peptides. Puromycin can enter the A-side of the ribosome and bind to the growing peptide in similar manner to amino acids delivered by tRNAs. However, puromycin does not facilitate further elongation, thus prematurely halting the translation (Aviner, 2020). These properties are utilized for the creation of mRNA display. During in vitro translation of the peptide, the puromycin will be incorporated into the peptide. Then the elongation of the peptide will be halted, and the peptide conjugated to mRNA via a puromycin (Aviner, 2020).

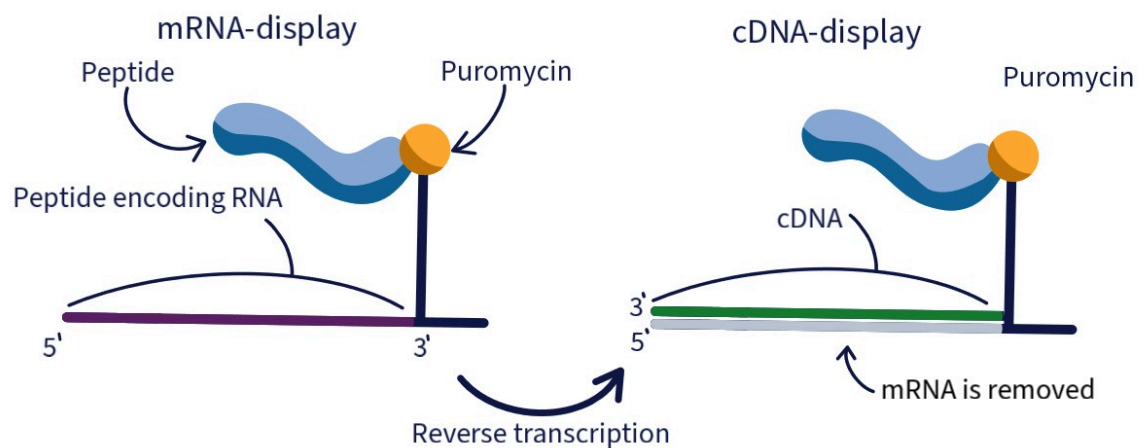


Figure 13: mRNA and cDNA display, they share close similarities, but cDNA display involves additional reverse transcription to convert the RNA into DNA and achieve higher stability.

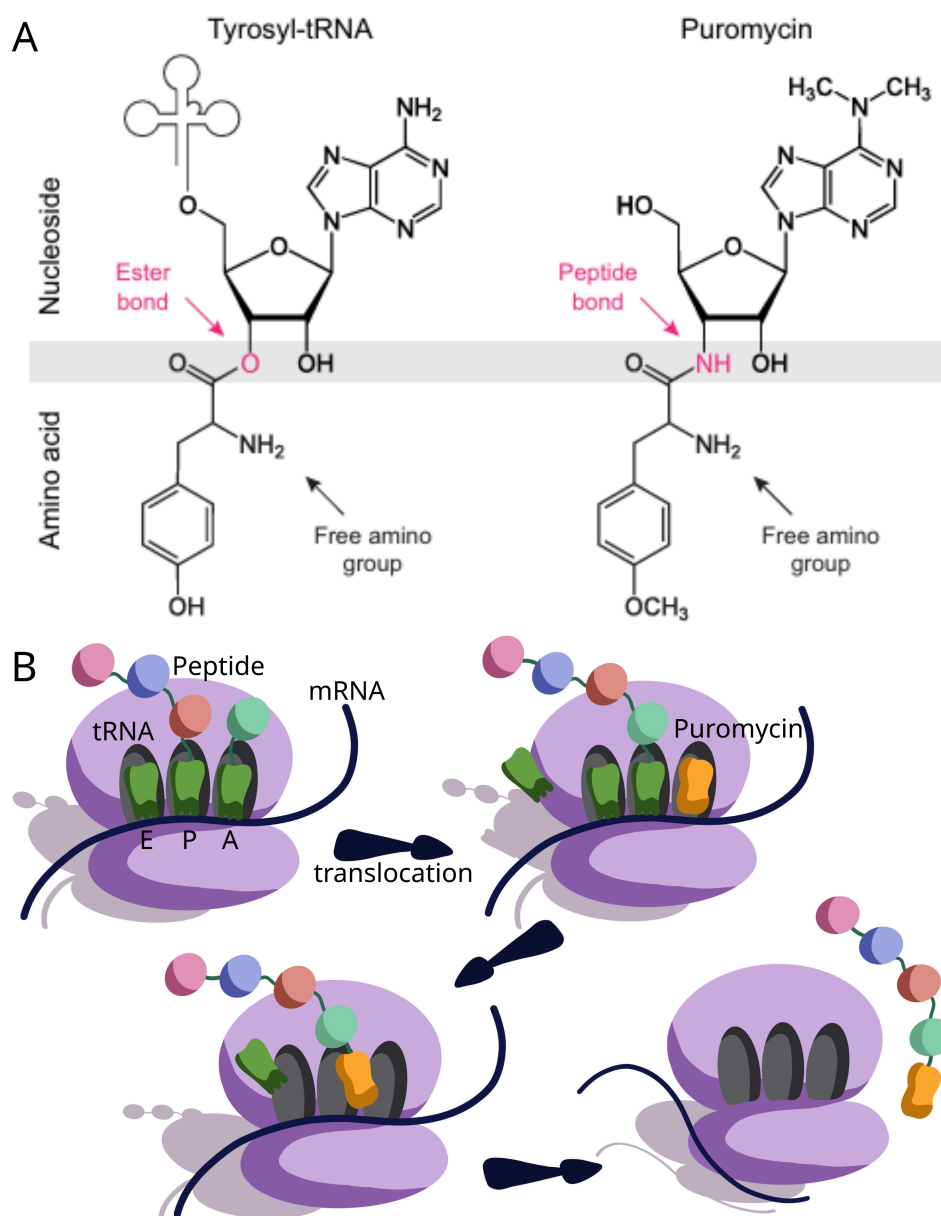


Figure 14: **A)** The chemical structure of Tyrosyl-tRNA and Puromycin. **B)** Mechanism of incorporation of puromycin. Aminoacyl-tRNA (aa-tRNA) enters the A-site of the ribosome. The P-site is occupied by the peptidyl-tRNA. The nascent chain is transferred to the A-site aa-tRNA, thus elongating the peptide. This is followed by translocation, which leaves the A-site free. Puromycin enters the A site, and the nascent chain is transferred to the puromycin, which leads to the termination of peptide elongation and ribosome disassembly. Adapted from: (Aviner, 2020)

1.8.3. cDNA DISPLAY

mRNA display could possibly be used for the implementation of SPARXS, however it can be further improved. RNA is relatively unstable and can be rapidly degraded due to RNase contamination. Therefore, the usage of DNA would be preferred. This can be achieved through reverse transcription of the mRNA construct. By degrading the RNA, cDNA display is obtained, which is more stable and can endure higher temperatures.

cDNA display can be compatible with SPARXS as it satisfies important requirements. It cleverly is able to link the phenotype directly to its genotype, thanks to the usage of a puromycin linker. Furthermore, the DNA, can be immobilized on an illumina sequencing chip on which a smFRET assay can be performed Figure 15. This retains the spatial information of the single molecules, which lets the sequencing information be correlated to its corresponding single molecule kinetic data. Finally, cDNA display will have to be fluorescently labeled in order to obtain smFRET measurements. As the FRET distance should be taken into account, the peptide itself should be labeled, which could be done at the N-terminus.

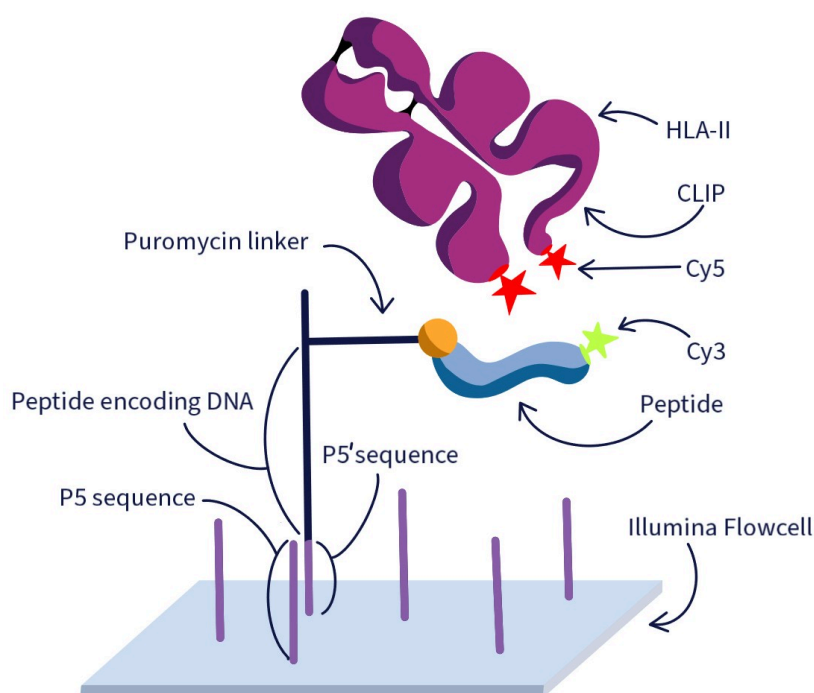


Figure 15: Schematic of smFRET experiment with cDNA display immobilized on an Illumina flowcell. cDNA-display is immobilized through incorporation of a P5 sequence, which ligates to its complement on the surface. HLA-II is in solution and labeled with Cy5. The peptide in the cDNA construct is labeled with Cy3. Upon binding, the dyes are in close proximity and FRET can be observed.

Overall, SPARXS has the ability to uncover the kinetic landscape of the peptidome of HLA-DR molecules. However, its application to measuring the binding kinetics of peptides to HLA-II molecules has not yet been established. In order to do this, cDNA display has to be developed as it shows the most potential in compatibility with SPARXS. Therefore this body of work aims to develop a cDNA display construct, which is fully compatible with the SPARXS assay.

2

METHODS

2.1. OVERVIEW OF WORKFLOW

The workflow is depicted in Figure 16, Figure 17 & Figure 18. It starts with a DNA template as described previously. This template is used to obtain RNA strands using *in vitro* transcription. Then the puromycin linker is prepared by conjugating the puromycin segment with the biotin segment through a click-chemistry reaction. The RNA strand is then ligated to the puromycin linker by hybridization to the pairing region of the DNA. This is followed by *in vitro* translation, which starts at the translation start site of the DNA strand. The ribosome stalls near the 3' end of the DNA-puromycin linker, due to the lack of a stop codon. Due to this, the puromycin is in close proximity and will be incorporated into the peptide chain, effectively conjugating the linker and peptide. Upon incorporation, the ribosome dissociates. The peptide sequence includes a 6-his tag, which is utilized for purification using Ni-NTA magnetic beads. This is followed by reverse transcription and treatment with RNase H, resulting in cDNA display. This is preferable over RNA-display as it is more stable and easier to work with. Utilizing the biotin included in the linker, the cDNA-display construct is purified using streptavidin magnetic beads. Cleavage of deoxyinosine, using Endonuclease V (Endo V) frees the construct from the biotin. The product is purified one final time using an oligo-nuclease purification column. Please note that the overview as depicted is a general overview and has gone through slight alterations in order to increase the yield and lower the risk of RNA degradation, changes will be detailed later.

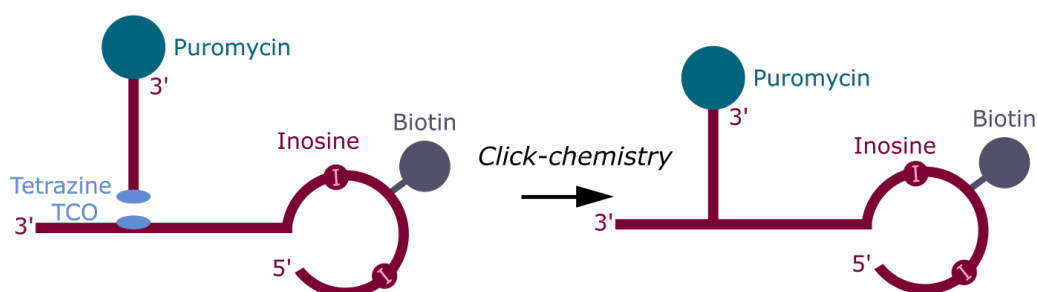


Figure 16: The Puromycin linker consists of two segments: the puromycin and the biotin segment. These are conjugated through a click-chemistry reaction with TCO and tetrazine.

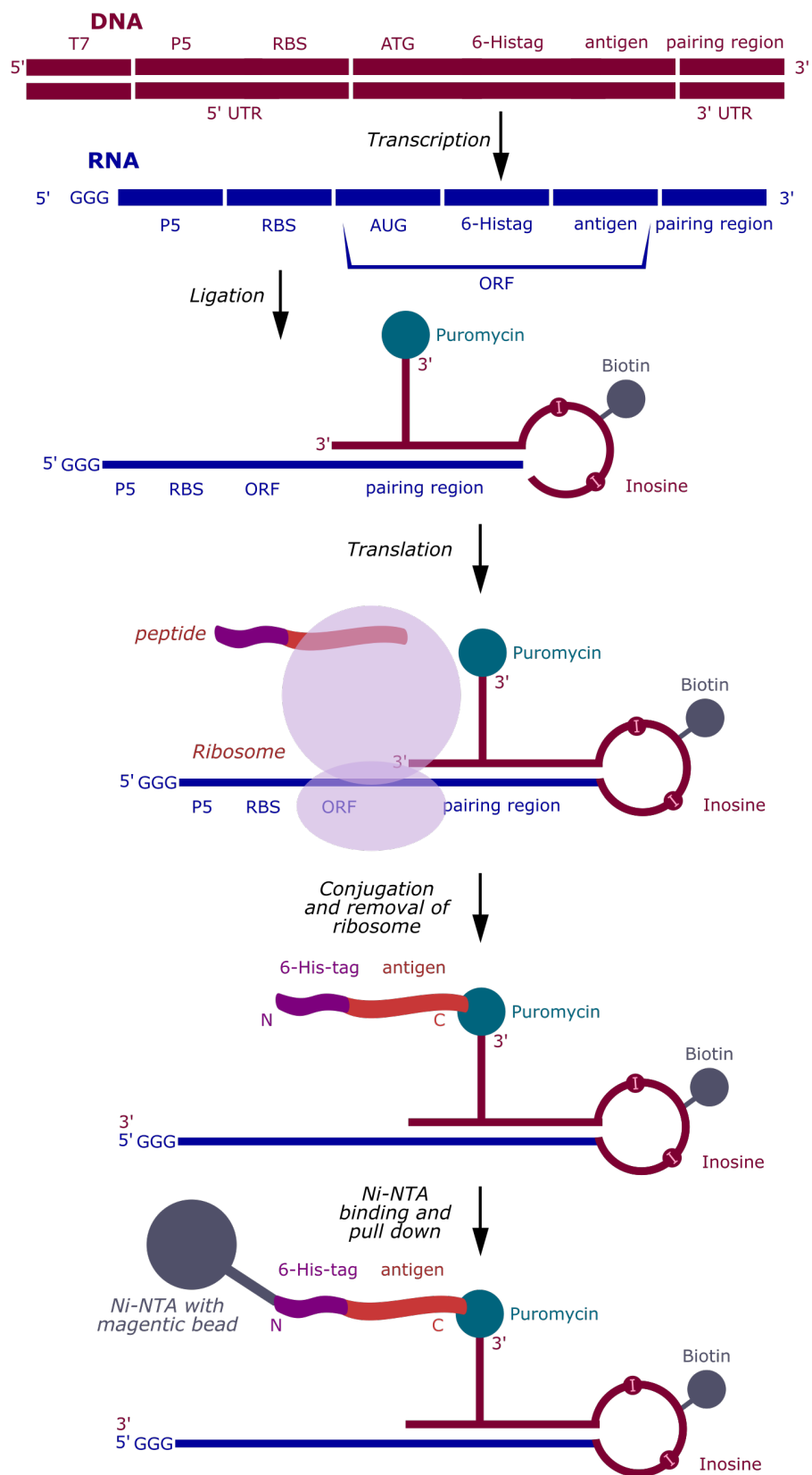


Figure 17: Detailed overview of the procedures for obtaining cDNA display, part 1

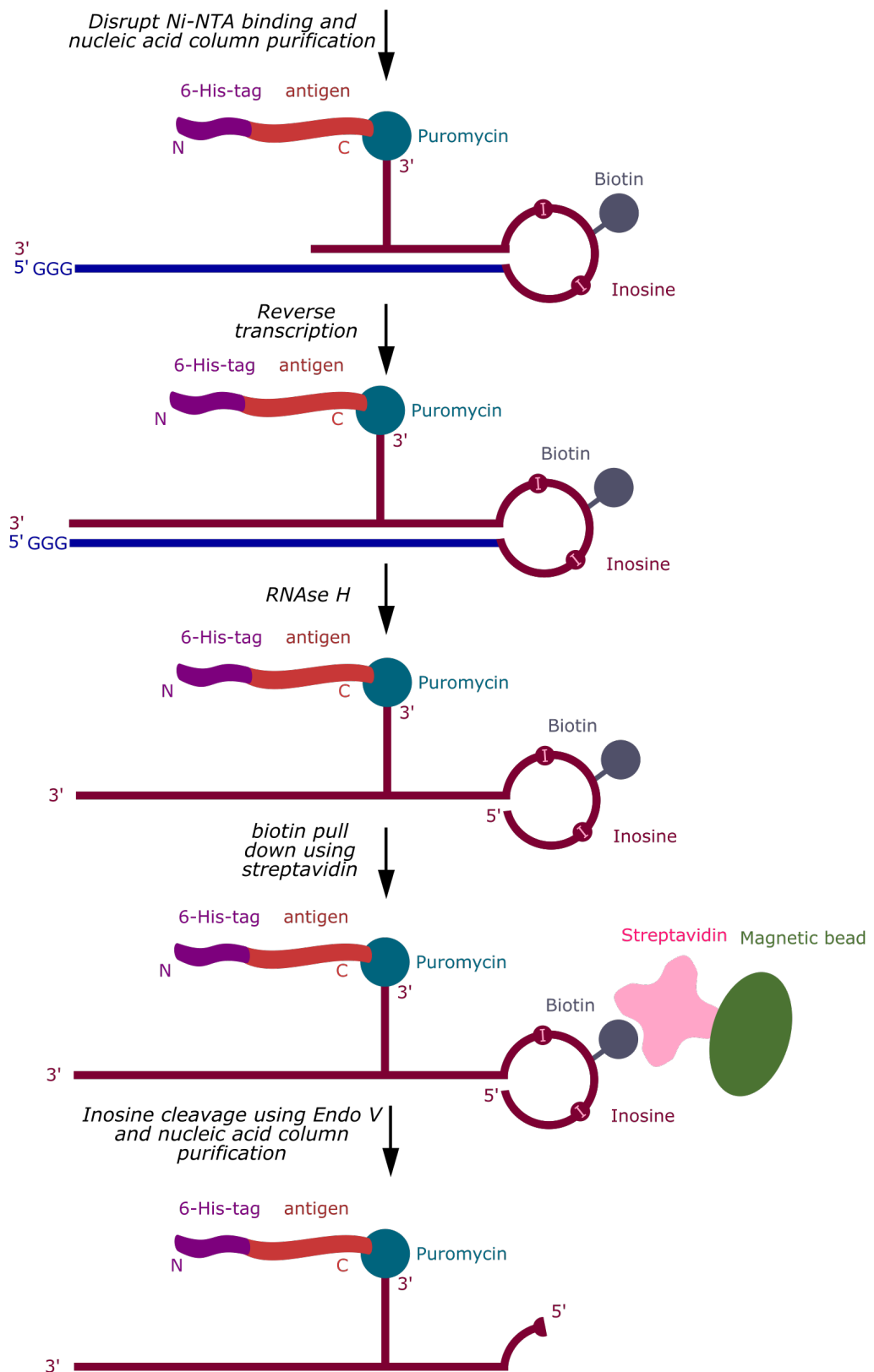


Figure 18: Detailed overview of the procedures for obtaining cDNA display, part 2

2.2. EXPERIMENTAL PROCEDURES

2.2.1. PCR OF DNA TEMPLATES

Assemble the following components in a PCR tube. Use the following primers: **Forward primer:**

GgaaaaaatATGCATCATCACCACCACCAACACCTACAGAAAGATTATAGGGCTTAC-TACACCTTCCAAGGGACGGGGGGCGGCGGGGAAA

Reverse primer:

TTTCCCCGCCGCCCGCCGTC

Reagent	Volume (μL)	Amount
Forward primer (10 μM)	5	50 pmol
Reverse primer (10 μM)	5	50 pmol
Q5 Hot Start High-Fidelity 2X Master Mix	10	
Total	20	

Table 1: Reagents, volumes, and amounts for reaction setup

Second forward primer:

GCGAATTAATACGACTCACTATAGGgAATGATACGGCGACCACCGAGATCTACACAAG-GAGgaaaaaatATGCATCATCACCACCACCAC

Put it in the thermocycler using the time and temperature settings as shown in Table 2. Use the following second forward primer.

Second forward primer:

GCGAATTAATACGACTCACTATAGGgAATGATACGGCGACCACCGAGATCTACACAAG-GAGgaaaaaatATGCATCATCACCACCACCAC

Temperature	Time	Cycle
98 °C	30 sec	35x
98 °C	10 sec	
65 °C	15 sec	
72 °C	20 sec	
12 °C	Hold	

Table 2: Temperature and time settings of Thermocycler for PCR

Use the QIAquick PCR purification kit (QIAGEN) to purify the PCR product and elute the product in 30uL of TDW.

Perform a second round of PCR by combining the following components in a PCR reaction tube.

Reagent	Volume (μL)	Amount
Template: Column purified 94 bp products (diluted to 10 ng/μL)	5	50 ng
Second forward primer (10 μM)	2.5	25 pmol
Reverse primer (10 μM)	2.5	25 pmol
TDW	up to 25	
Q5 Hot Start High-Fidelity 2X Master Mix	25	
Total	up to 35	

Table 3: Reagents, volumes, and amounts for PCR setup

Place the tube in the thermocycler using the settings as shown in Table 2. Then purify the product again using the QIAquick PCR purification kit (QIAGEN). Elute using 30 μL of TDW

2.2.2. HIGH YIELD *IN VITRO* TRANSCRIPTION

Combine the following components according to the protocol of TranscriptAid T7 High Yield Transcription Kit (Catalog Number K0441, Pub. No. MAN0012652)

Reagents	Volume (μL)	Amount
T7 templates	6	6–9 pmol
5X buffer	4	-
ATP	2	-
UTP	2	-
GTP	2	-
CTP	2	-
Enzyme mix	2	-
Total	20	-

Table 4: Temperature and time settings of thermocycle for PCR

Incubate at 37°C for 4 hours, then add 1 μL of DNase I and incubate for another 15 minutes at 37°C. Purify the product using the Oligo clean and concentrator kit (Zymo D4060/D461). Follow instructions provided and elute into 10 μL TDW. Proceed to the next step or store at –20°C.

2.2.3. GEL PURIFICATION

Prepare a 10% denaturing polyacrylamide stock solution.

Reagent	Amount	Final concentration
Urea	420 g	7 M
Acrylamide/Bis-acrylamide (19:1), 30% solution	333 mL	10%
10× TBE solution	100 mL	1×
TDW	up to 1 L	-
Total	1 L	-

Table 5: Composition fo 10% denaturing polyacrylamide stock solution

Prepare a 10% denaturing gel of 1.5mm thickness add together:

Reagent	Amount	Final concentration
10% denaturing polyacrylamide stock solution	45 mL	10%
20% ammonium persulfate (APS) solution	200 µL	
Ultrapure TEMED	20 µL	
Total	45.22 mL	

Table 6: Composition of 10% denaturing PAGE gel

Pre-run the gel at 370V for 50 min, using 0.5x TBE as running buffer and start preparing samples and ladders. Prepare the samples for gelloading by adding 10uL of RNA loading dye to 10uL of concentrated RNA sample. Prepare the Low range ssRNA ladder (NEB: N0364S), by adding 1uL of marker to 9 µL TDW and 10uL RNA loading dye

Prepare the RNA century plus marker (Thermofisher: AM7145), by adding 0.5 uL of marker to 9.5 µL TDW and 10 µL RNA loading dye. After preparing the samples, incubate them at 70 °C for 5 minutes. Wash the wells before loading the samples. To avoid cross-contamination between samples, ensure there are two lanes in between samples. Run the gel 370V for 2.5h, check progression of the gel using Gel electrophoresis tables

% Acrylamide	Xylene Cyanol (nucleotides)	Bromophenol Blue (nucleotides)
5	130	35
6	106	26
8	75	19
10	55	12
20	28	8

Table 7: Gel electrophoresis from (Thermo Fisher Scientific, 2023)

Prepare a metaltray, with 0.5x TBE buffer and 10uL of SYBR™ Gold Nucleic Acid Gel Stain (Thermofisher: S11494). Once the run is finished, disassemble the gel set-up and place the gel in the metal tray. Incubate for 5 minutes. Transfer the gel to a blue light converter and illuminate with a UV light from below. Be careful and wear proper eye-protection.

Typically, neighboring wells will be penetrated as well. Based on the Low range ssRNA ladder and the RNA century plus marker the size of the RNA at the ghostband can be determined. This serves as a check whether the ghostband is the desired RNA. If this is the case, a razor can be used to cut out the desired RNA band and transfer it to a gel breaker tube. Centrifuge the tube at 20 000 g for 1 minute to completely break the gel. Add 500 μ L of 0.3 M NaCl solution and Incubate at 4°C and 1500 rpm overnight (at least 16 h).

Transfer the suspension with debris to Spin-X® Centrifuge Tube Filters. Centrifuge the tubes at 14000 x g at 4°C for 5 minutes and transfer the eluate to new micro centrifuge tubes. Add 1 mL of 100% ethanol and 50 μ L of 3 M NaOAc. Additionally, 1 μ L Glycoblue co-precipitator can be added for easier visualization of the product, which is especially helpful when handling small amounts of sample. Store at –80 °C for at least 1 h.

After incubation, centrifuge the tube at 17 000 g at 4 °C for 20 minutes. Discard the supernatant and wash the pellet twice using 1mL of 75% EtOH. Centrifuge the tube at 17 000 x g, 4 °C for 5 minutes between washes. After the second wash, spin down the tube and carefully completely remove the residual ethanol. Air-dry for 3 minutes to completely remove the ethanol and dissolve the pellet in 11 μ L TDW. Proceed to the next step or store at –20°C.

2.2.4. PREPARATION OF PUROMYCIN LINKER

Incubate overnight in the dark at 25°C:

Reagent	Volume (μ L)	Amount
Puromycin segments (1 mM)	2	
Biotin segment (1 mM)	2	
TDW	6	
Total	10	

Table 8: Reagents and volumes for ligation

Then perform gel purification of the segments as described previously.

2.2.5. LIGATION OF mRNA TO PUROMYCIN-LINKER

Prepare the following components to anneal mRNA template to the puromycinlinker. Then anneal them using the thermocycler, using the settings as shown in Table 10.

Reagent	Volume (μL)	Concentration (or Amount)
Gel purified mRNA (Diluted to 10 μM)	5	50 pmol 3'-OH RNA
Puromycin linker (20.3 μM)	2.5	50 pmol 5'-P DNA
10X T4 RNA ligase buffer (NEB, B0216)	1.5	1X bf
10 mM ATP	3	1 mM ATP
TDW	3	up to 15 μL
Total	15	

Table 9: Reagents, volumes, and concentrations for ligation setup

Temperature	Time	Cycle
90 °C	1 min	x32
$\Delta T = -2\text{ °C}$	1 min	
25 °C	Hold	

Table 10: Temperature and time settings of Thermocycler for annealing of puromycin linker to RNA

Add the following components to the annealed reaction:

Reagent	Volume (μL)	Concentration
10X T4 RNA ligase buffer (NEB, B0216)	1.5	1X bf
TDW	0.5	
50% PEG8000 (NEB, B1004)	12	20% PEG
T4 RNA ligase 1, high concentration (3X) (NEB, M0437M)	1	
Total	30 (15 + 15)	

Table 11: Reagents, volumes, and concentrations for RNA ligation mix

Then incubate it in the Thermocycler at 25°C for 2 h. After incubation, perform Oligo clean and concentrator (Zymo D4060, D461) and elute the samples in 10 μL of TDW. Proceed to the next step or store at -20°C.

2.2.6. IN VITRO TRANSLATION WITH PUREFLEX 2.0

Incubate 4 μL (20pmol) of mRNA-Puromycinlinker at 65°C for 5 minutes. Immediately place the tube on ice after incubation for at least 1 minute. Thaw solution I from the PUREflex 2.0 (GeneFrontier, PF201-EX) kit at RT for 1 minute. Thaw solution II and III on ice. Tap the solutions and spin down to achieve a uniform distribution and assemble.

Reagent	Volume (μL)	Concentration (or Amount)
mRNA–PuroLinker (5 μM)	4	20 pmol of mRNA–PuroLinker up to total 20 μL
TDW	3	
Solution I	10	
Solution II	1	
Solution III	2	40 pmol Ribosomes
Total	20	

Table 12: Reagents, volumes, and concentrations for mRNA–PuroLinker reaction

Incubate at 37 °C for 3 hours, then add 20 μL of 2x high salt buffer (65mM MgCl₂, 750mM KCl). Incubate for an additional hour at 37 °C, then store at –20°C or proceed to the next step.

2.2.7. Ni-NTA PURIFICATION

Reagent	Volume (μL)	Amount
In vitro translated product (mRNA-display)	40	1x PBS w/0.2% Tween-20
TDW	60	
2× PBS w/ 0.2% Tween-20	100	
Total	200	

Table 13: Dilution of in vitro translated mRNA-display product

Prepare the following buffers:

Equilibration buffer (EQ buffer): 1xPBS w/ 0.1% Tween 20

Reagent	Volume	Amount
2× PBS	1 mL	1x PBS
10% Tween-20	20 μL	0.1% Tween 20
TDW	980 μL	Up to 2 mL
Total	2 mL	

Table 14: Composition of equilibration buffer

Wash buffer: 1X PBS w/ 20 mM Imidazole & 0.1% Tween 20

Reagent	Volume	Concentration
2× PBS	1 mL	1× PBS
10% Tween-20	20 µL	0.1% Tween-20
0.5 M Imidazole pH 8	80 µL	20 mM imidazole
TDW	900 µL	up to 2 mL
Total	2 mL	

Table 15: Composition of wash buffer

Elution buffer: 1X PBS w/ 200 mM Imidazole & 0.1% Tween 20

Reagent	Volume	Concentration
2× PBS	1 mL	1× PBS
10% Tween-20	20 µL	0.1% Tween-20
0.5 M Imidazole pH 8	0.8 mL	20 mM imidazole
TDW	180 µL	up to 2 mL
Total	2 mL	

Table 16: Composition of Elution buffer

Transfer 100µL of Pierce Ni-NTA magnetic agarose beads (Thermo Scientific, 78605) to an e-tube. Wash the beads three times by pipetting with 200 µL EQ buffer, while placing the beads on a magnetic stand in between washes and discarding supernatant. After the last wash, add 200 µL of mRNA-display samples to the beads and incubate in a Thermomixer at 25°C at 1000 rpm for 30 minutes. After incubation, place the tube on a magnetic stand for 1 minute and discard the supernatant. Wash the beads three times with the wash buffer, placing the tube on the magnetic stand and discarding the supernatant. Add 50 µL of elution buffer to the beads, mix the suspension and incubate for 1 minute. Place the tube on the magnetic stand for 1 minute and transfer the eluate fraction to a new e-tube. Add once again 50 µL and repeat. Perform Oligo Clean & Concentrator using the instruction provided with the kit. Elute with 12 µL TDW and proceed to the next step or store at –20 °C.

2.2.8. REVERSE TRANSCRIPTION AND RNASE H TREATMENT

Use reagents from the Superscript IV kit (Thermofisher) Add 1 µL of 0.5 M dNTP mix to 12 µL of Ni-NTA purified mRNA-display. Incubate at 65°C for 5 minutes. Then place on ice for at least 1 minute. Add the following components:

Reagent	Volume (μL)	Concentration
5× SSIV bf	4	1× bf
100 mM DTT	1	5 mM DTT
SUPERase In (20 U/μL)	1	1 U/μL
SuperScript IV Reverse Transcriptase (200 U/μL)	1	10 U/μL
Total	20 (13 + 7)	

Table 17: Reagents, volumes, and concentrations for reverse transcription

Incubate at 55°C for 30 minutes. Then heat-inactivate by incubating at 80°C for 10 minutes. Lower the temperature to 37°C, then add 1μL of RNase H (NEB, M0297) and incubate at 37°C for 20 min. Then heat-inactivate at 65°C for 20 minutes. Store at –20°C or proceed to the next step.

2.2.9. BIOTIN PULL-DOWN AND ENDONUCLEASE V TREATMENT

2x binding and wash (B&W) buffer: 2 M NaCl, 20 mM Tris-HCl pH 8, 1 mM EDTA, 0.2% Tween 20

Reagent	Volume	Concentration
5 M NaCl	4 mL	2 M NaCl
1 M Tris-HCl pH 8	0.2 mL	20 mM Tris
0.5 M EDTA, pH 8	20 μL	1 mM EDTA
10% Tween-20	0.2 mL	0.2% Tween-20
TDW	5.58 mL	up to 5 mL
Total	10 mL	

Table 18: Composition of Binding and Wash (B&W) buffer

To 21 μL of unpurified cDNA-display, add 79 μL of TDW. Then add 100 μL of 2x B&W buffer. Transfer 100μL to Streptavidin magnetic beads (ThermoFisher, Dynabeads MyOne Streptavidin C1) to an e-tube. Wash the beads three times using 200 μL of 1x B&W buffer, while placing the beads on a magnetic stand in between washes and discarding supernatant. After the final wash, add 200 μL of sample to the beads and incubate at 25°C, 1000 rpm for 30 min. Place the tube on the magnetic stand and wash the beads again three times using 1x B&W buffer. Discard the supernatant after the final wash. Wash the beads once, using 1X NEBuffer 4 w/ 0.1% Tween 20. Add 98 μL of 1X NEBuffer 4 w/ 0.1% Tween 20 and resuspend the beads. Then add 2 μL of Endonuclease V (NEB, M0305) to the beads and incubate at 37°C, 1000 rpm for 1 h. Centrifuge the beads at RT, 1,000 x g for 1 min and place on a magnetic stand. Transfer the supernatant and heat-inactivate at 65°C for 20 min. Then perform Oligo Clean and concentrator, by following provided instructions of the kit and elute into 11 μL of TCW. Store at –20 °C

2.2.10. TEV PROTEIN PURIFICATION

Lysis & Equilibration & Wash buffer: 200 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM DTT

Reagent	Volume	Final concentration
5 M NaCl	8 mL	200 mM NaCl
1 M Tris-HCl pH 8	10 mL	50 mM Tris
1 M DTT	200 μ L	1 mM DTT
TDW	182 mL	up to 200 mL
Total	200 mL	

Table 19: Composition of Lysis, equilibration and wash buffer

MBP Trap elution buffer: 200 mM NaCl, 50 mM Tris-HCl pH 8, 11.7 mM Maltose, 1 mM DTT

Reagent	Volume	Final concentration
5 M NaCl	2 mL	200 mM NaCl
1 M Tris-HCl pH 8	2.5 mL	50 mM Tris
20% Maltose solution (=584 mM) (Sigma, 63423)	1 mL	11.7 mM Maltose
1 M DTT	50 μ L	1 mM DTT
TDW	44.5 mL	up to 50 mL
Total	50 mL	

Table 20: Composition of MBP Trap elution buffer

Strep-Tactin XT Equilibration & Wash buffer: 200 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM DTT

Reagent	Volume	Final concentration
5 M NaCl	4 mL	200 mM NaCl
1 M Tris-HCl pH 8	5 mL	50 mM Tris
1 M DTT	100 μ L	1 mM DTT
TDW	91 mL	up to 100 mL
Total	100 mL	

Table 21: Composition of Strep-Tactin XT Equilibration & Wash buffer

Strep-Tactin XT Elution buffer: 200 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM Biotin, 1 mM DTT

Reagent	Volume	Final concentration
5 M NaCl	1.6 mL	200 mM NaCl
1 M Tris-HCl pH 8	2 mL	50 mM Tris
1 M NaOH	2 mL	
Biotin (ibalifescience)	490 mg	50 mM Biotin
1 M DTT	40 μ L	1 mM DTT
TDW	34 mL	up to 40 mL
Total	40 mL	

Table 22: Composition of Streptactin XT elution buffer

1 M Triethanolamine (TEAM), pH 8

Reagent	Volume	Final concentration
7.46 M (>99%) Triethanolamine (TEAM) (Sigma, 90297)	7.5 mL	1 M TEAM
1 M HCl (Sigma, H9892)	17.5 mL	
TDW	31 mL (up to 56 mL)	
Total	56 mL	Final pH 8

Table 23: Composition of 1 M Triethanolamine (TEAM)

Storage buffer: 200 mM NaCl, 50 mM TEAM pH 8, 1 mM DTT

Reagent	Volume	Final concentration
5 M NaCl	2 mL	200 mM NaCl
1 M TEAM pH 8	2.5 mL	50 mM TEAN
1 M DTT	50 μ L	1 mM DTT
TDW	45.5 mL	Up to 50 mL
Total	50 mL	

Table 24: Composition of storage buffer

Mini-seeding: Add 10 μ L of 20% Glycerol bacterial stock into 20 mL of LB with 50 ng/ μ L Kanamycin and incubate it overnight at 37°C, 200 rpm.

Inoculation and induction: Inoculate the total 20mL of culture to 2 L of LB with 50 ng/ μ L Kanamycin and incubate it at 37°C, 200 rpm until an optical density value of approximately 0.60 has been obtained, this typically takes several hours. Then cold shock the culture for 30 minutes in ice-cold water to inhibit the further growth. Then add 1 ml of 1 M IPTG to 2L of culture and incubate at 18°C, 180 rpm overnight.

Harvest: Centrifuge the culture at 4000xg, 4°C for 30 min and resuspend the pellet using 25 mL PBS per 1L culture. Centrifuge at 300xg, 4°C for 15 min. Discard the PBS and store

the pellet at -80°C .

Cell lysis: Resuspend the cell pellets using 50 mL of lysis buffer and sonicate the suspension using the following settings: 2 sec ON, 8 seconds OFF with a total ON time of 2 minutes (60 cycles) and amplitude of 40%.

centrifugation and filtration: Centrifuge the sonicated suspension at $39,810 \times g$, 4°C for at least 30 min and store the pellet at 4°C . Filtrate the supernatant using a $0.45 \mu\text{m}$ bottle-top filter and a vacuum pump

HiTrap MBPTrap HP 5ml: Using a flowrate of 5 mL/min equilibrate the column connected to a peristaltic pump using 50 mL of Lysis & EQ & Wash buffer. Using a flowrate of 2 mL/min for loading, load 50mL of filtrated supernatant. Then wash with 50 mL of Lysis & EQ & Wash buffer with a flow rate of 5 mL /min. Finally elute using 30 mL of MBPTrap elution buffer with a flowrate of 5 mL/min and store the eluate at 4°C .

Strep-Tactin XT 4Flow high capacity cartridge, 5 ml: Using a flowrate of 2 mL/min equilibrate the column using 30 mL of STXT EQ & Wash buffer. Using a flowrate of 1 mL/min for loading, load 30mL of filtrated supernatant. Then wash with 50 mL of STXT EQ & Wash buffer with a flow rate of 2 mL /min. Finally elute using 30 mL of STXT Elution buffer with a flowrate of 2 mL/min.

Concentration: Centrifuge at $3,220 \times g$, 4°C for 15 min 30 mL of sample in two Amicon Ultra-15 Centrifugal Filter Units, concentrating the protein to an approximate 1.5 mL per unit. Repeat, to concentrate to approximately 0.25 mL per unit.

Buffer exchange: Use two Zeba desalting columns (0.5 mL) to completely remove biotin and storage buffer, using the concentrated protein as input. Dilute the buffer exchanged protein to 2 mg/ml using the storage buffer. Then further dilute to 1 mg/mL with 100% Glycerol. Aliquot the protein to 1ml per e-tube. Snap freeze the proteins using liquid nitrogen and store at -80°C .

3

RESULTS

3.1. TYPE 1 DIABETES AS MODEL SYSTEM

Type 1 diabetes (T1D) is a disorder, which causes the destruction of insulin producing pancreatic beta cells, leading to an absolute insulin deficiency. The most common form is diabetes type 1a, which is characterized by the autoimmune-mediated destruction of the beta cells. Approximately 10-15% of all diabetes cases are T1D (Sticht et al., 2021). The more prevalent type of diabetes is type 2 diabetes (T2D). It is characterized by insulin resistance in target organs and the failure of pancreatic beta-cells, which leads to a relative insulin deficiency (Chatterjee et al., 2017).

It is thought that in T1D, the destruction is caused by environmental triggers in people with a genetic predisposition (Maahs et al., 2010). Many genes have been found to either confer susceptibility to or resistance against T1D. Most of these genes function within the immune system. The most associated gene are those of HLA-class II. In particular, DR3 and DR4 haplotypes are associated with T1D. Furthermore, primary markers have been identified off which 90-95% of young children with T1D carry either or of the haplotypes: DRB1*03 with DQA1*0501-DQB1*0201 and DRB1*0401 with DQA1*0301-DQB1*0301. However, the general population has only a 5% risk of getting T1D when they carry either of these markers (Mehers & Gillespie, 2008).

3.1.1. ISLET-SPECIFIC GLUCOSE-6-PHOSPHATASE CATALYTIC SUBUNIT-RELATED PROTEIN (IGRP)

A major auto-antigen for T1D has been identified as the Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). This protein, as the name suggests is found in the islet of Langerhans in the pancreas, where insulin is produced. Four minimal epitopes, two for HLA-DR3 and two for HLA-DR4 have been shown to induce a T-cell response in vitro as shown in Table 25 (Yang et al., 2006).

Additionally it is worth noting that the HLA-DRB1*0301 contains a valine at position β 86, which accommodates small hydrophobic side chains, in this case a leucine. The HLA-DRB1*0401 construct contains a glycine at β 86. Therefore it is able to also

accommodate larger hydrophobic side chains, in this case phenylalanine and isoleucine. Therefore, the IGRP epitopes are consistent with general trends in peptide binding preferences. (Kaabinejadian et al., 2022; Yang et al., 2006)

Peptide	HLA	Minimal epitope	Sequence
A	HLA-DRB1*0301	IGRP ₁₃₋₂₅	QHLQKDYRAYYT <u>F</u>
B	HLA-DRB1*0301	IGRP ₂₂₆₋₂₃₈	RVLNIDLLWSV <u>P</u> I
C	HLA-DRB1*0401	IGRP ₂₃₋₃₅	YTFLNFMSNV <u>G</u> DP
D	HLA-DRB1*0401	IGRP ₂₄₇₋₂₅₉	DW <u>I</u> HI <u>D</u> TT <u>P</u> FA <u>G</u> L

Table 25: Identified epitopes by (Yang et al., 2006). Underlined residues are the P1, P4, P6 and P9 anchor residues

3.2. PRELIMINARY smFRET OF HLA-II PEPTIDE BINDING

SPARXS requires a working smFRET experiment in order to be able to obtain any kinetic data. Therefore, before developing a display method with SPARXS a preliminary experiment has to be performed. This will confirm whether it is possible to observe binding kinetics between HLA-II and peptides using smFRET as it is crucial for SPARXS.

For this experiment, an HLA-DR molecule is immobilized on a slide. For this HLA-II has been purified and stabilized using a modified human IgG1 Fc domain Figure 19. It contains a knob-and-hole mutation and two disulfide bridges as adopted from (Serra et al., 2019). The N-terminus of the β -chain is elongated with a CLIP peptide, which binds into the binding groove. Both the N-termini of the α - and β -chain are labeled with a Cy5 dye. The immobilization scheme is shown in Figure 20. As a probe, a N-terminus labeled synthetic peptide is used. The peptide can only be observed when it is near the surface or bound into the peptide binding groove of the immobilized HLA-II molecule.

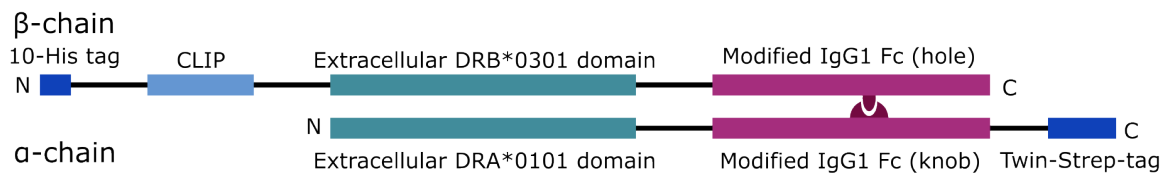


Figure 19: Structural design of the HLA-DR protein. The modified IgG1 Fc domain provides stability of the construct. CLIP, is attached via a linker to the N-terminal end of the β -chain, which allows it to occupy the peptide binding groove and further stabilize the protein. The 6-Histidine tag and Twin-Strep-tag are used for purification of the construct. Labeling of N-termini is non-specific, thus leading to both being labeled.

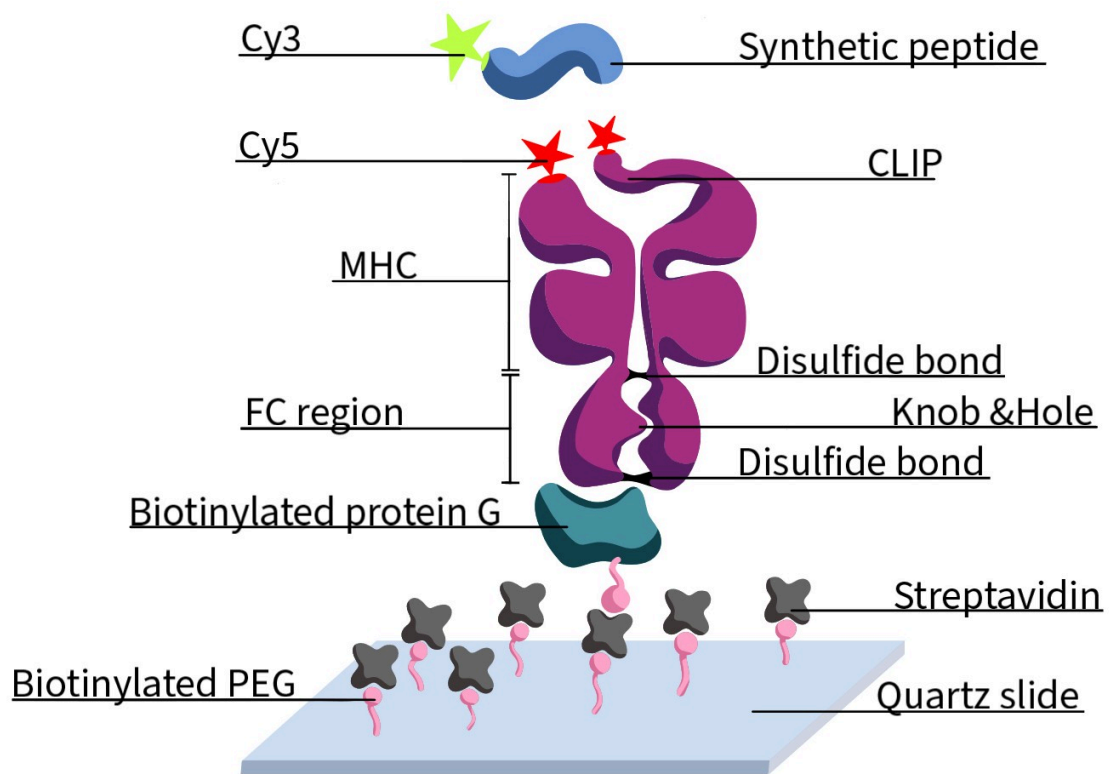


Figure 20: A quartz slide, is treated with biotinylated PEG. Streptavidin binds to biotin and biotinylated protein G. Protein G binds to the Fc domain of the MHC construct, thus immobilizing it on the surface. The N-termini of MHC-II are labeled with Cy5. A FRET signal can be observed when CLIP is displaced and Cy3-labeled synthetic peptide binds into the groove.

In order for the synthetic peptide to bind, the CLIP peptide has to be displaced out of the peptide binding groove. As described earlier, CLIP is likely initially displaced from the P1 pocket, and then dissociates from the rest of the peptide binding groove. Similarly, The incoming peptide likely binds into the shallower part of the groove with the C-terminal end before binding into the P1 pocket as shown in Figure 21. The fluorescent signal of the Cy3 peptide can be observed when the peptide binds into the binding groove. However, FRET requires extremely close proximity between both the acceptor and donor. Thus, no FRET is observed when the synthetic peptide only binds into the more shallow binding groove. Only when the synthetic peptide binds into the P1 pocket can FRET be observed.

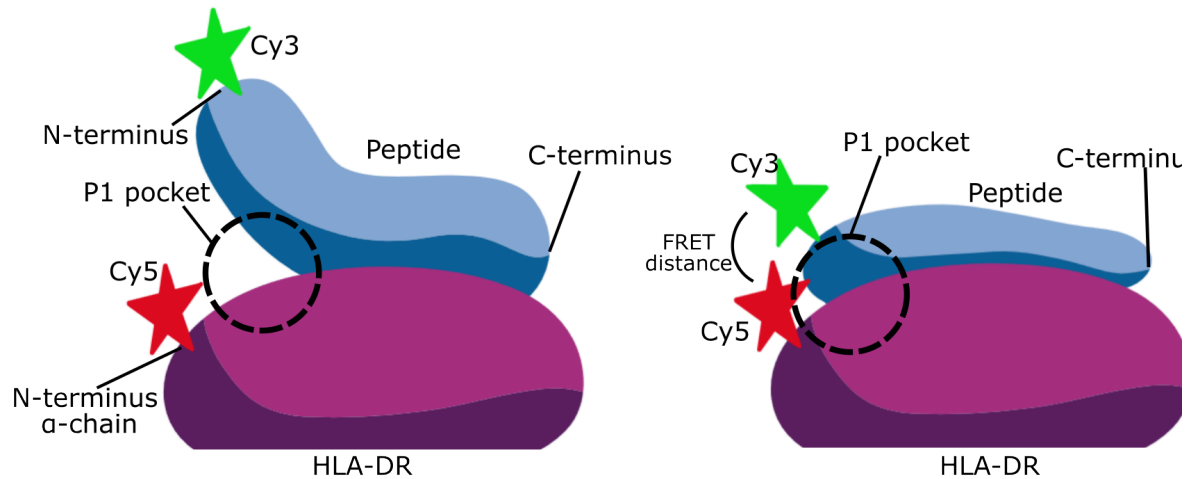


Figure 21: Schematic diagram of hypothesized mechanism. On the left, the peptide is bound in the binding groove, except the N-terminus. The P1 pocket is empty. The Cy3-dye can be detected, but is not within FRET distance of the Cy5-dye, thus no FRET is observed. Then the N-terminal end binds into the P1 pocket, bringing the Cy3-dye within FRET distance of the Cy5 dye. This results in FRET signal.

For the measurement, first the red laser is used to be able to localize the exact position of HLA-II on the surface. Then the green laser is turned on to illuminate the Cy3 dye. Traces are then extracted from the location of the HLA-II molecules. Two example traces are shown in Figure 22. Here the attempt to binding of the synthetic peptide can be observed as the green fluorescent signal of the Cy3 dye becomes visible (Figure 22A box2). Furthermore, clear FRET events can be observed (Figure 22 box, 1, 3, 4). In box 4 in Figure 22B an event is more clearly observed, where the green signal increases, as the synthetic peptide first binds in the shallower part of the groove. Then it binds into the P1 pocket, and is within FRET distance. This results in an increase in red signal and decrease in green signal.

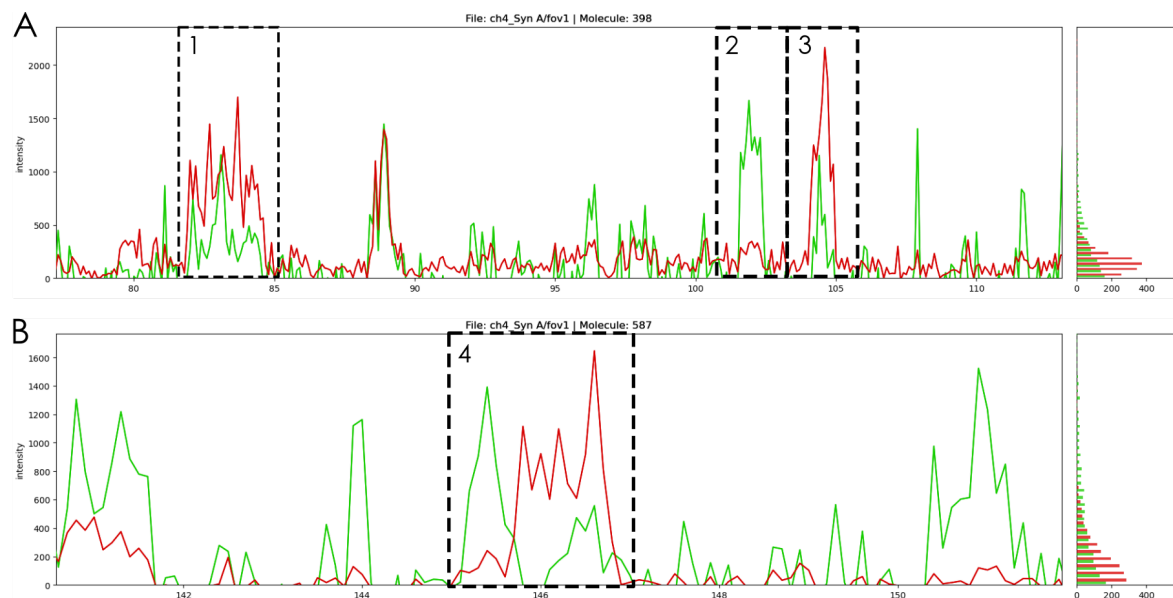


Figure 22: FRET traces of cy5 labeled HLA-II (red) and Cy3 labeled synthetic peptide (green)

The goal is to perform high-throughput smFRET measurements between HLA-II and peptides. This would allow for the rapid determination of peptidomes, but more importantly would also provide binding kinetics of the peptides. This could further enhance the understanding of the binding mechanism of the peptide.

3.3. cDNA DISPLAY DESIGN

The preliminary experiment, using immobilized HLA-II molecules showed FRET, which indicates binding events. Although further optimization for such assays is required, it also indicates that it is possible to observe binding events between HLA and synthetic peptides. Therefore, it can be further developed. However, key components will have to be adjusted.

First of all, the peptide will have to be labeled with a DNA sequence. For this, a cDNA construct will have to be developed and employed rather than using synthetic peptides. As SPARXS relies on spatial information, the cDNA display construct will have to be immobilized. This means that the HLA-II construct will have to be in solution in order to perform this assay (Figure 23). The implementation of such a cDNA display construct, relies on the covalent linking between peptide and DNA. This is achieved by the puromycin linker, which is therefore a critical element for a SPARXS-compatible construct.

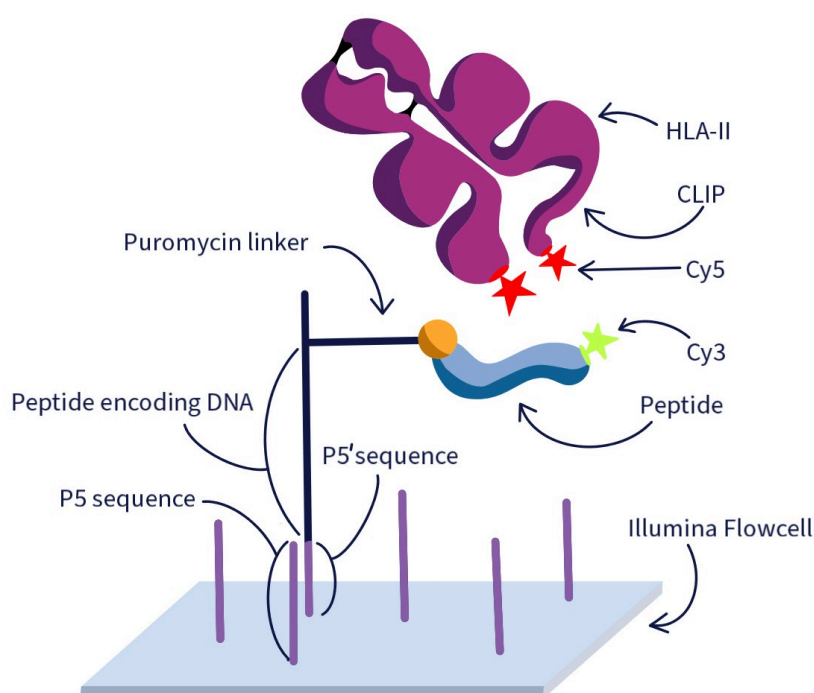


Figure 23: cDNA display is immobilized on the surface of an Illumina flowcell, through the hybridization of its P5' sequence to the P5 sequence on the flowcell. Following the P5 sequence, is the peptide encoding DNA sequence, linked to the puromycin linker. The peptide, which is also linked to the puromycin linker, is labeled with Cy3. HLA-II is in solution and labeled with Cy5. Upon binding of HLA-II to the peptide, FRET can be observed.

3.3.1. DESIGN OF THE PUROMYCIN LINKER

Thus, the key feature that enables the linkage between the peptide and its genetic code is the usage of puromycin. It is the most important component of the linker. During in vitro translation, the puromycin is incorporated into the peptide by the ribosomes, thus effectively conjugating the peptide to the other parts of the construct, most importantly its encoding nucleotide sequence.

However, puromycin is not the only element of the puromycin-linker. The linker is designed in two parts, the puromycin-segment and the biotin segment. These are shown together in Figure 24. The puromycin segment contains additional key elements, besides puromycin at the 3' end of the construct. First of all, there is the FAM dye. Although it is not necessary for the functioning of the linker, it has been used throughout the experiments to be able to visualize the segment and to ensure the quality and functioning of the construct. In between the 5' end and puromycin are two C18 spacers and a poly-A sequence. These have been included to ensure sufficient distance between the RNA strand and the puromycin itself. This allows for adequate spacing between the ribosome bound to the RNA so it can incorporate the puromycin.

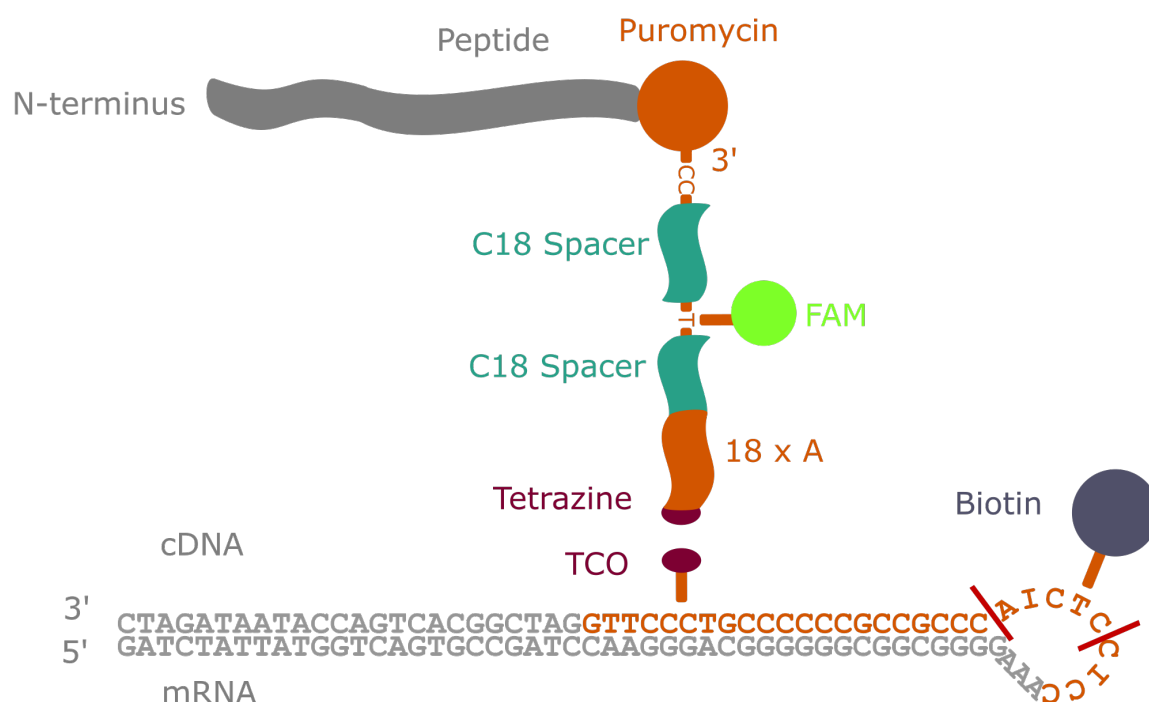


Figure 24: Puromycin-linker design consisting of two segments. The puromycin segment consists of the puromycin, connected to a tetrazine via two C18 spacers and a oligo-dA sequence. In between the C18 spacers, is the FAM dye. The biotin segment is connected to TCO through a DNA linker. Biotin can be cleaved off by endonuclease V cleavage of the second DNA phosphodiester backbone 3' of deoxyinosine, which is indicated with an I.

The biotin segment, as the name suggests, contains biotin, which is used for purification with streptavidin. The biotin can be removed from the construct through cleavage of the DNA strand. This is possible due to the incorporation of deoxyinosine on both sides of the biotin. Deoxyinosine is an altered base, which can be incorporated into DNA strands.

Endonuclease V (Endo V) is an enzyme, which specifically cleaves the second DNA phosphodiester backbone 3' of deoxyinosine. Finally, the rest of the biotin segment contains a ligation region. This region contains a DNA sequence, which allows for the hybridization of a complementary RNA sequence, which is shown ligated in Figure 24.

Finally both segments will have to be conjugated together. This is possible due to the incorporation of a tetrazine at the 3' end of the puromycin segment. A few bases from the 3' end of the biotin segment, TCO is incorporated. Through a click-chemistry reaction, it can be conjugated to the tetrazine as shown in Figure 25.

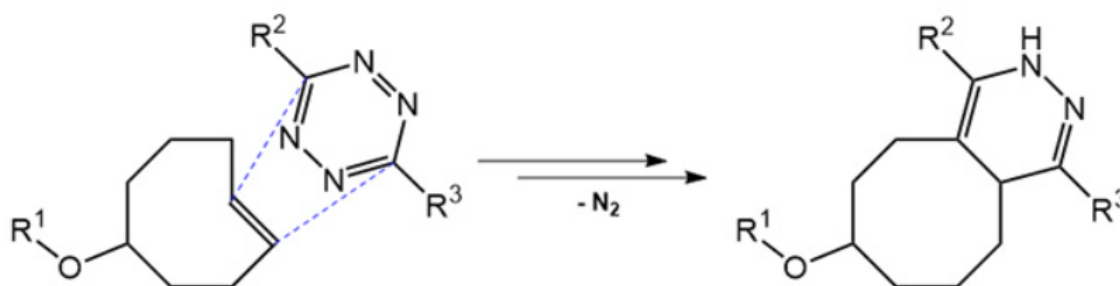


Figure 25: TCO-tetrazine click chemistry reaction. Source: (GmbH, 2016)

The click-chemistry reaction was performed at two different incubation temperatures to test, whether the efficiency could be increased by altering the temperature. Therefore, the reaction was performed at room temperature (25°C) and 37 °C, to test whether a higher efficiency of conjugation could be obtained. The results are shown in Figure 26. No significant difference in efficiency could be found from the gel. Therefore, the decision was made to combine the samples together. The final concentration of this is shown in Table 26.

sample	Concentration (ng/μL)	MW (kDA)	Concentration (μM)
Puromycin linker	322.54	15.87	20.32

Table 26: Measurements of concentrations of RNA products using nanodrop. Total volume = 11μL

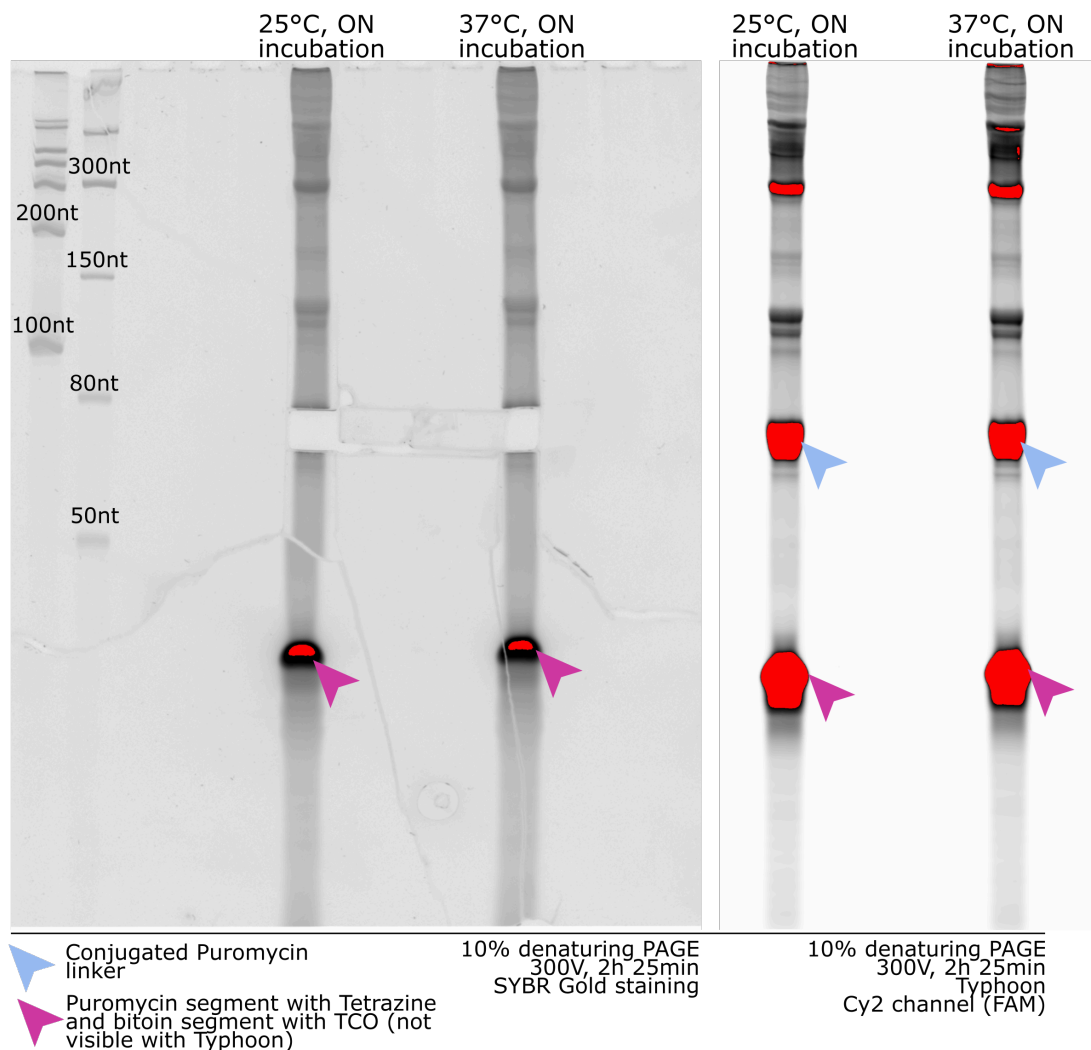


Figure 26: Gel of puromycin linker conjugation.

3.3.2. SEQUENCE DESIGN FOR cDNA DISPLAY

The puromycin-linker on its own is not sufficient to obtain cDNA display. For this, it has to be combined with an RNA template, which is transcribed into DNA after *in vitro* translation of the peptide. Obtaining the RNA template, begins with transcription of a DNA template. The sequence in this template has to be carefully designed.

The DNA sequence used for preparing the cDNA display is shown in Figure 27. It contains several key elements. The T7 promoter is used to start *in vitro* transcription by the T7 RNA-polymerase. This is followed by the 5' UTR, which contains a P5 sequence. This sequence can be used in Illumina sequencing for binding and immobilization on the sequencing slide. Although this procedure is outside the scope of this project, the P5 sequence can still be used for the immobilization of the construct for low-throughput smFRET experiments. Additionally, the 5' UTR contains a ribosomal binding site sequence, for prokaryotic *in vitro* translation and a spacer.

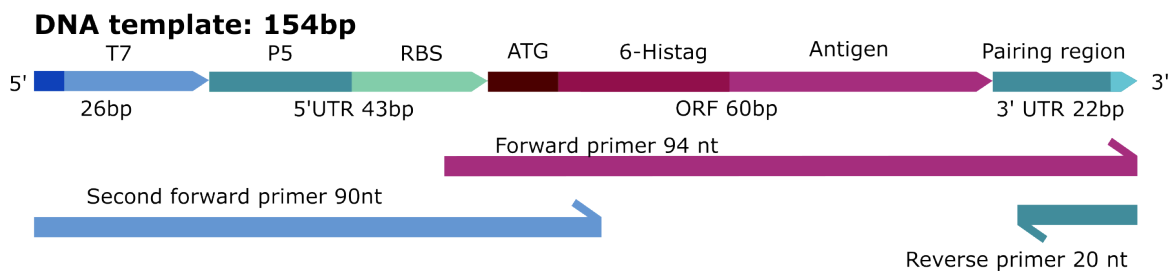


Figure 27: Overview of the elements within the DNA sequence of the different constructs. The antigen sequence is dependent on the chosen antigen (A, B, C and D) as shown in Table 25. Below, the respective forward and reverse primers for the first round of PCR are shown. Together they form a 94 bp product. The second round of PCR is done with the 94 bp product and the second forward primer.

The next region is the open reading frame (ORF), which includes all translated regions. This starts with the start codon and a 6-histidine region for purification of the translated product. The start codon codes for methionine, but as a prokaryotic in vitro translation system is used, this is an N-formylmethionine. This is followed by the epitope sequence, which is the peptide of interest. In this work, the aforementioned IGRP peptides (A, B, C and D) as shown in Table 25 are used. It is important to note that the ORF contains a start codon, but does not include a stop codon. This is to ensure that the ribosome stalls on the RNA, and can incorporate the puromycin. As described earlier, this will terminate translation and conjugate the peptide to the puromycin linker. Finally, the ORF is followed by the 3' UTR, which contains a pairing region. This region contains a complementary sequence to the DNA sequence in the puromycin linker, which allows for the hybridization of RNA to the linker.

3.4. PCR OF cDNA CONSTRUCTS

Producing cDNA thus starts with a DNA template. This template has to be replicated, to obtain a sufficient amount of cDNA display. For this two rounds of PCR were used. For the first round, two primers were used. Together these formed a 94 bp product which is purified and used for the second round of PCR. The second round of PCR, which includes an additional forward primer, results in a 154 bp product.

In Figure 28 the results are shown. Based on these results it can be concluded that both rounds of PCR were successful. Both the first and second round show bands of the expected size. Furthermore, the second round has a higher intensity, indicating that the product was amplified.

DNA template	concentration (ng/ μ L)	260/280	260/230
Construct A	111.60	1.78	2.15
Construct B	145.53	1.82	2.11
Construct C	103.31	1.86	2.20
Construct D	118.74	1.85	2.23

Table 27: Measurements of concentrations of DNA products using nanodrop. Total volume = 30 μ L

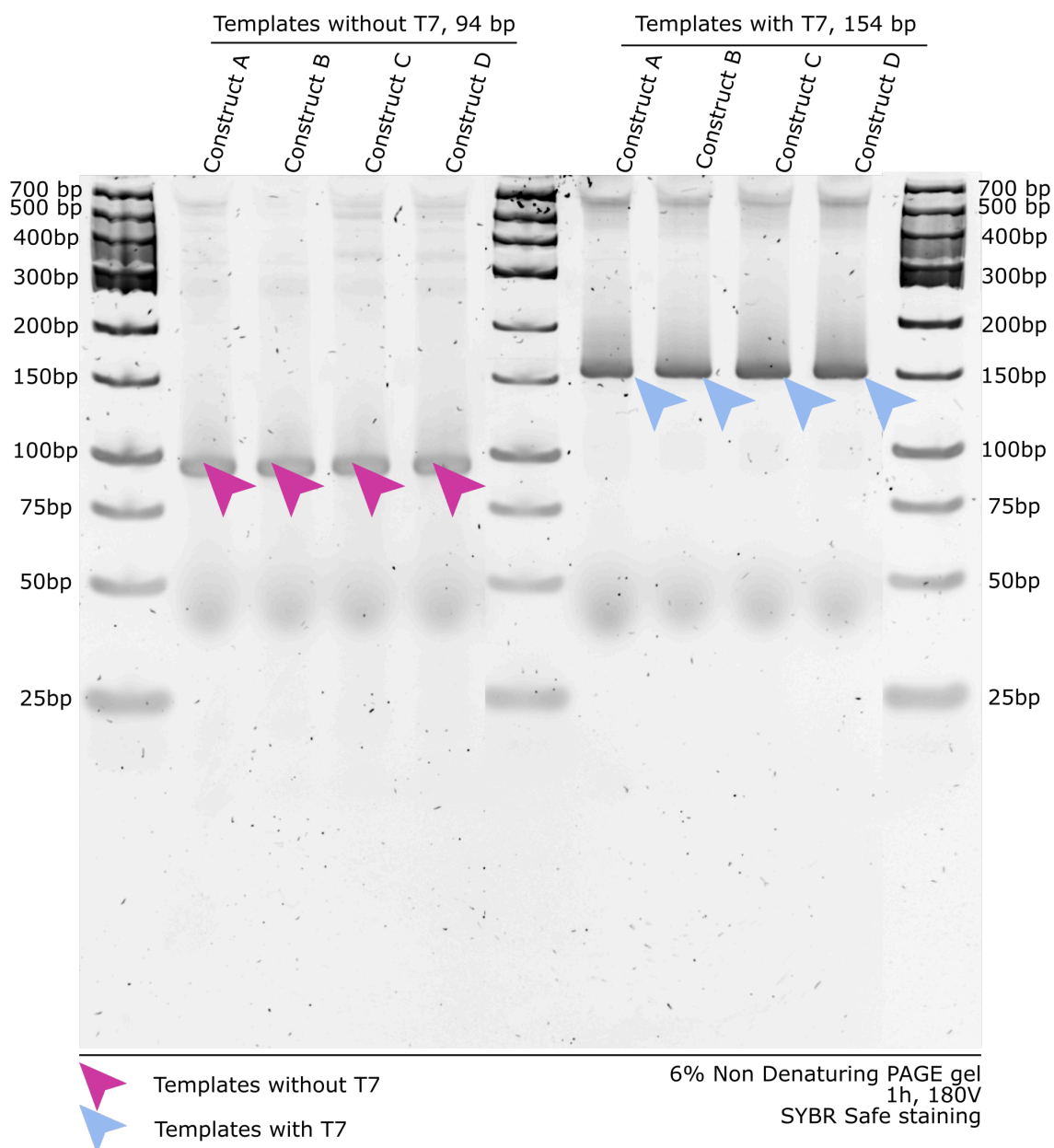


Figure 28: Results of first and second round of PCR

3.5. *IN VITRO* TRANSCRIPTION AND RNA PURIFICATION

After performing PCR, high yield *in vitro* transcription results in RNA (Figure 29). The RNA product is then purified using an Oligo Clean and Concentrator kit and all of the product is loaded onto the gel. As the T7 promoter is not transcribed, the total construct length is 131 nt Figure 27.

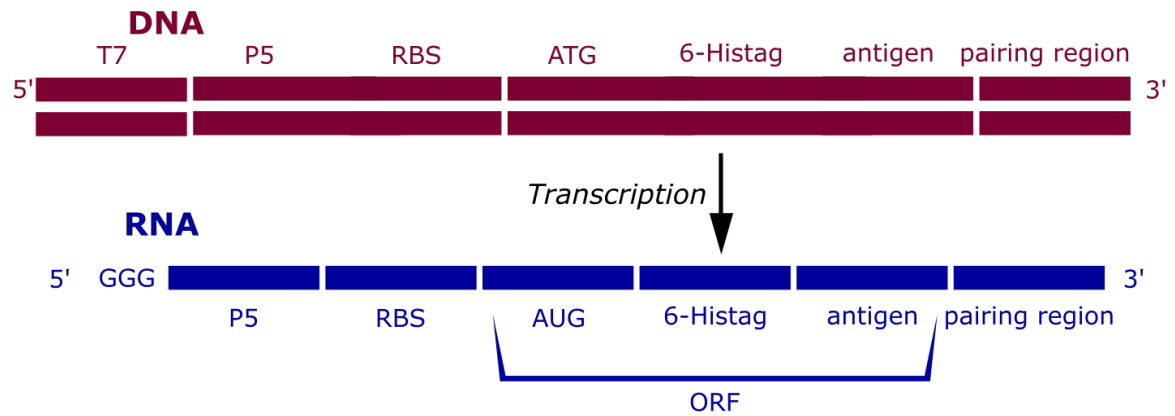


Figure 29: Overview of procedures so far. After PCR, the DNA is transcribed and purified.

The results are shown in Figure 30. Bright squares are visible and indicated with arrows. Here, the gel has been cut for gel purification of the product. The empty wells, next to the loaded lanes show penetration of RNA product. The size of these bands corresponds to the expected size of 131 nt.

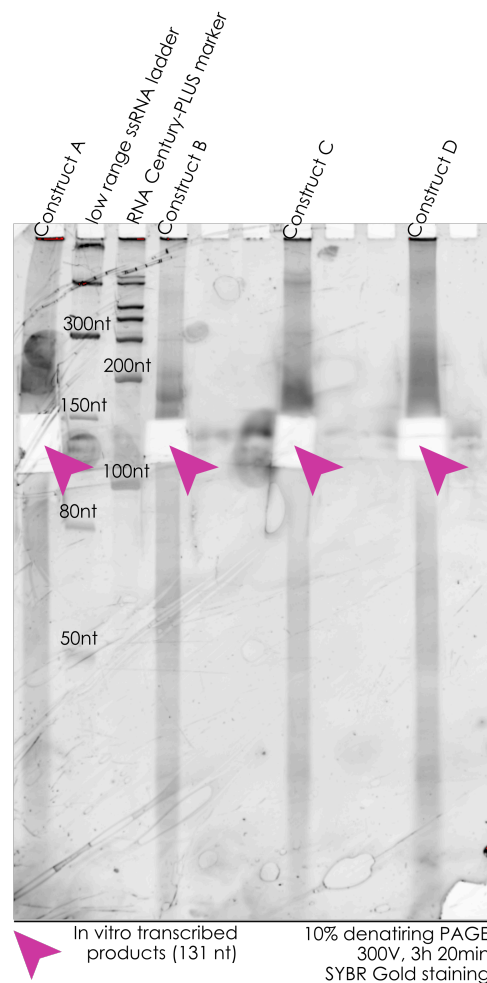


Figure 30: Gel image after removal of mRNA products.

After performing gel purification, the RNA product is diluted into 11 μL of TDW. From this, 1 μL is used to measure the concentration using the NanoDrop. The results of these measurements are shown in Table 28. The 260/280 ratio is to determine whether there is protein contamination. A 260/280 value of approximately 2 is an indication for pure RNA, although the value also depends on the composition of the RNA as different bases have different absorption spectra. The 260/230 ratio is used to detect other non-protein contaminants, such as glycogen, residual phenol or guanidine. Typical values of pure RNA are approximately between 2 and 2.2.

samples	concentration (ng/ μL)	260/280	260/230	nt	MW (kDa)	μM
Construct A	330.67	2.02	2.17	131	42.14	7.85
Construct B	366.30	2.01	2.26	131	42.14	8.69
Construct C	226.82	1.93	2.12	131	42.14	5,38
Construct D	589.32	2.09	2.20	131	42.14	13.98

Table 28: Measurements of concentrations of RNA products using nanodrop. Total volume = 11 μL

3.6. LIGATION OF THE PUROMYCIN-LINKER TO RNA TEMPLATES

Now that both the RNA template and the puromycin linker have been obtained, they have to be ligated together (Figure 31). In Figure 32 the results of the ligation reaction are shown. There are clearly two bands visible, one corresponding to RNA ligated to puromycin and one corresponding to non-ligated puromycin product.

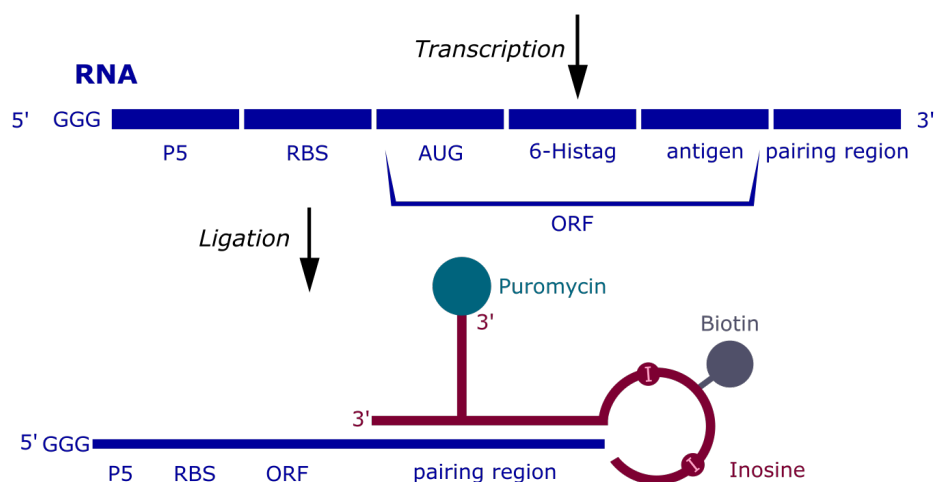


Figure 31: Ligation of puromycin linker after transcription. The pairing region is complementary to the DNA sequence in the puromycin linker.

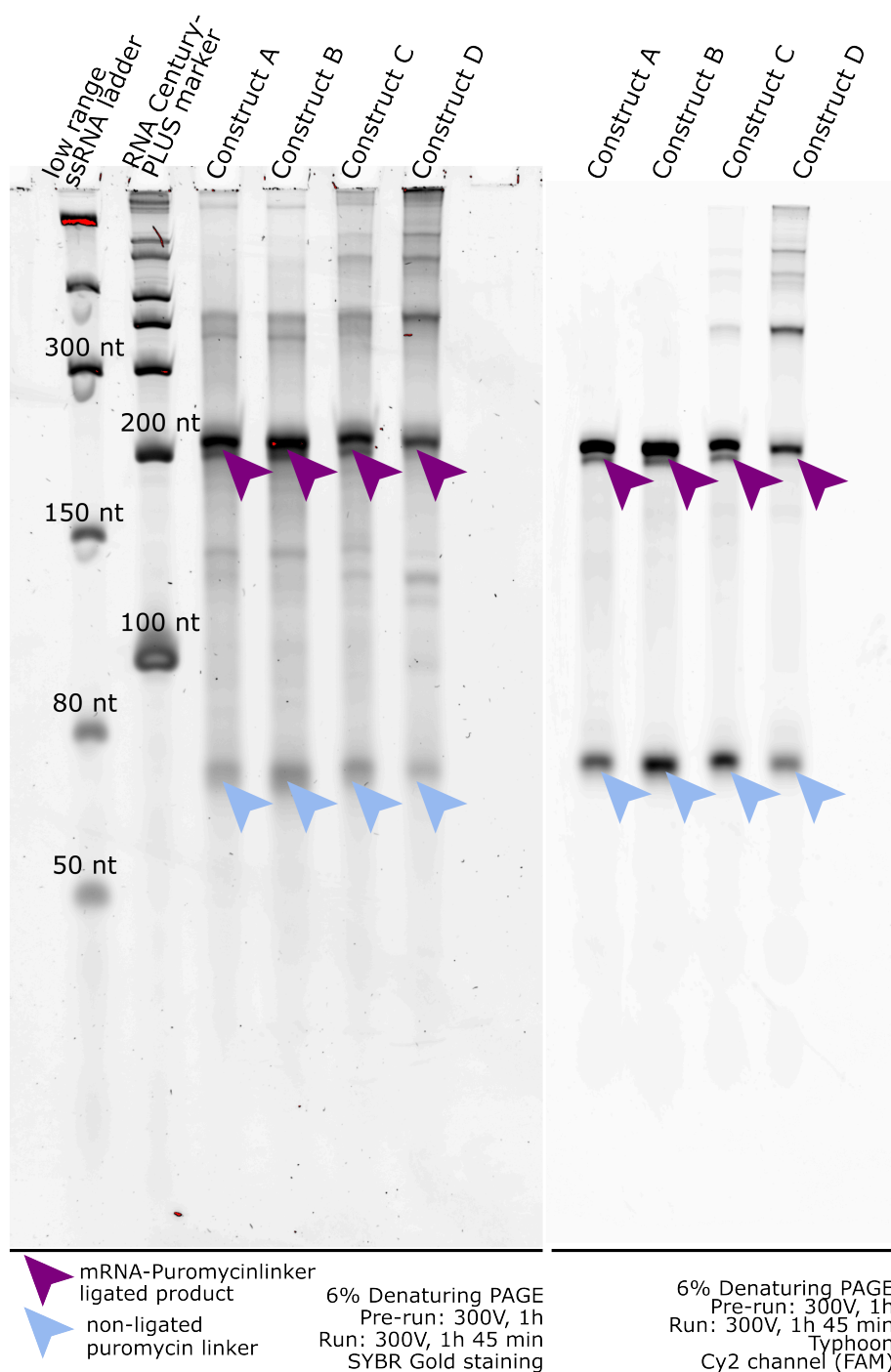


Figure 32: gels

3.7. OBTAINING cDNA DISPLAY

With the ligation of RNA to the puromycin-linker, all elements are present to be able to obtain cDNA display as outlined in Figure 33. The RNA can now be translated, creating a peptide, which can be conjugated to the puromycin linker. If this is carried out successfully, mRNA-display is obtained. This can be seen in Figure 34A & Figure 35A, where the first lane (checkpoint #1) shows both the RNA-puromycin construct as well as the mRNA-display.

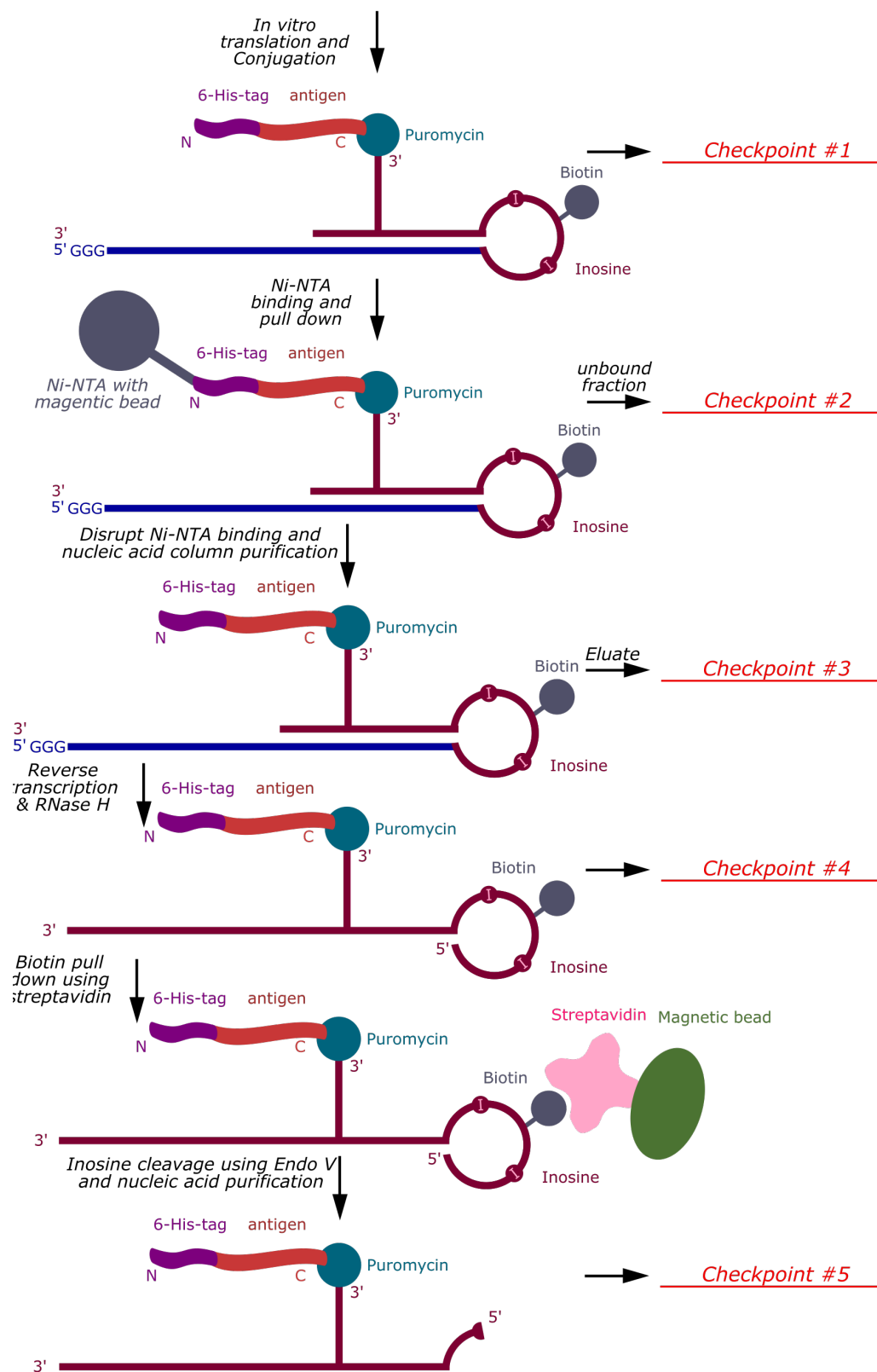


Figure 33: Overview of the procedures including checkpoint as shown in Figure 34 & Figure 35

Both mRNA-display and cDNA display contain very little peptide (<30 a.a.). Therefore, the construct is not suitable for staining with Coomassie Instant Blue staining. Thus, bands shown at a similar height to the construct correspond to other proteins originating from the in vitro translation kit.

mRNA-display by itself could be useful, however, cDNA is much more stable. Therefore, the mRNA-display will have to be further processed. In order to do this, it is first purified using Ni-NTA magnetic beads. The unbound and bound fractions are shown at checkpoint #2 and #3 in Figure 34 & Figure 35 as well. Ni-NTA magnetic beads bind to the 6-histidine tag of the peptide. Therefore, only unbound peptides and mRNA-display constructs are bound to the beads and subsequently purified.

The purified mRNA-display can be used to obtain cDNA-display. This is done through reverse transcription, followed by treatment with RNase H. This slightly reduces the size of the product. This shift can be seen in Figure 34A & Figure 35A. Furthermore, the RNaseH enzyme is visible by InstantBlue staining in lane Checkpoint #4.

After reverse transcription and degradation of the RNA, the product has to again be purified before usage. This is done using streptavidin magnetic beads, which bind to the biotin of the puromycin-linker. Then, after washing the beads, the cDNA-display can be cleaved off using Endo V, which cleaves two bases downstream of deoxyinosine. The results are shown in the final lanes of the gels (Checkpoint #5) in Figure 34A and Figure 35A. Endo V is visualized as well, thanks to the InstantBlue staining.

There are a few important details that have to be taken into account when drawing conclusions based on these gels. First of all, caution has to be taken when considering the efficiency of the different steps. The FAM dye, which is visualized using the Cy2 channel, is constantly exposed to light, due to the required experimental procedures. This can cause photo-bleaching of the FAM dye and result in a fainter signal. Therefore, the efficiencies as calculated and shown in Table 29 are likely to be an underestimation. Despite the possible underestimation, the reverse transcription efficiencies of the samples are all approximately 60%, except for Construct A.

	Construct A	Construct B	Construct C	Construct D
Reverse transcription efficiency	0.45	0.61	0.61	0.60

Table 29: efficiencies calculated from gel images as shown in Figure 34& Figure 35

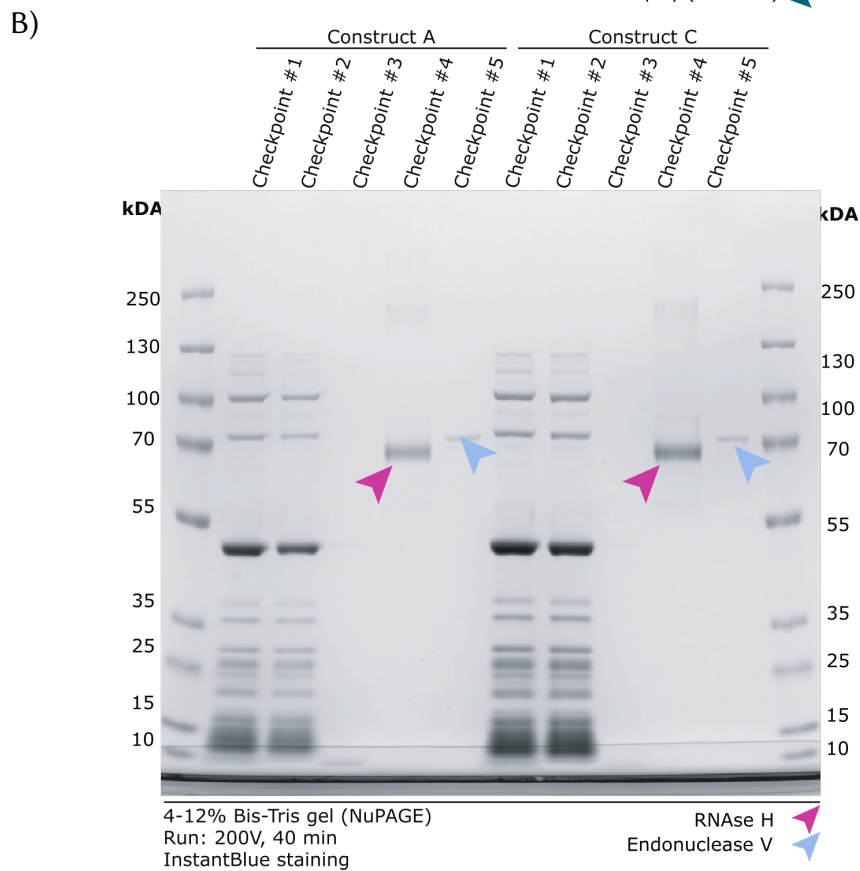
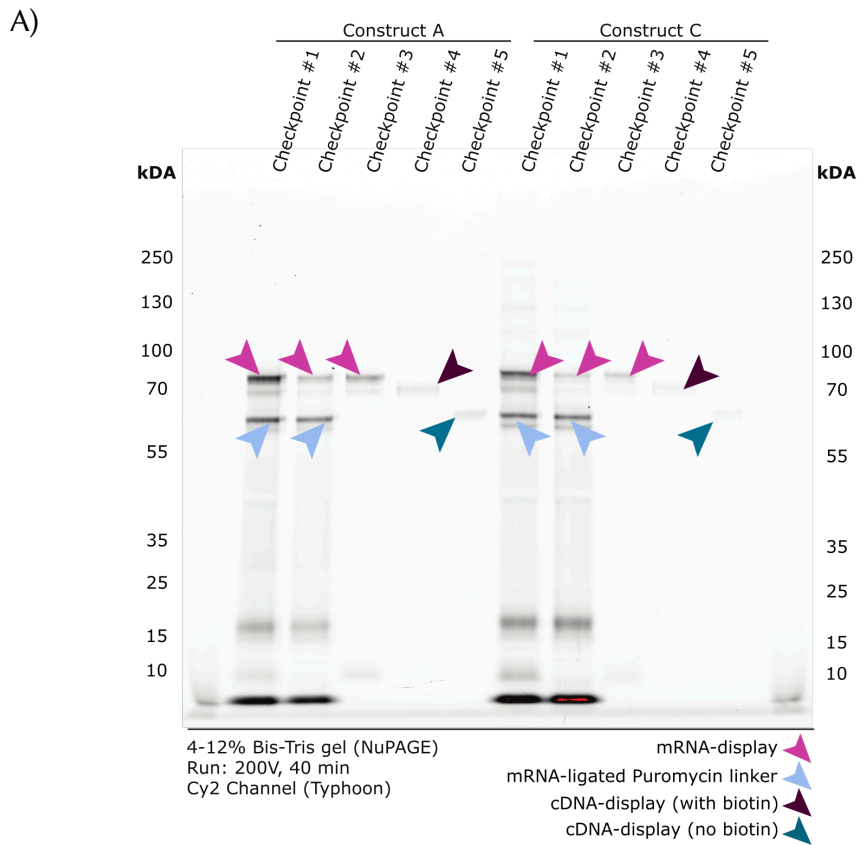
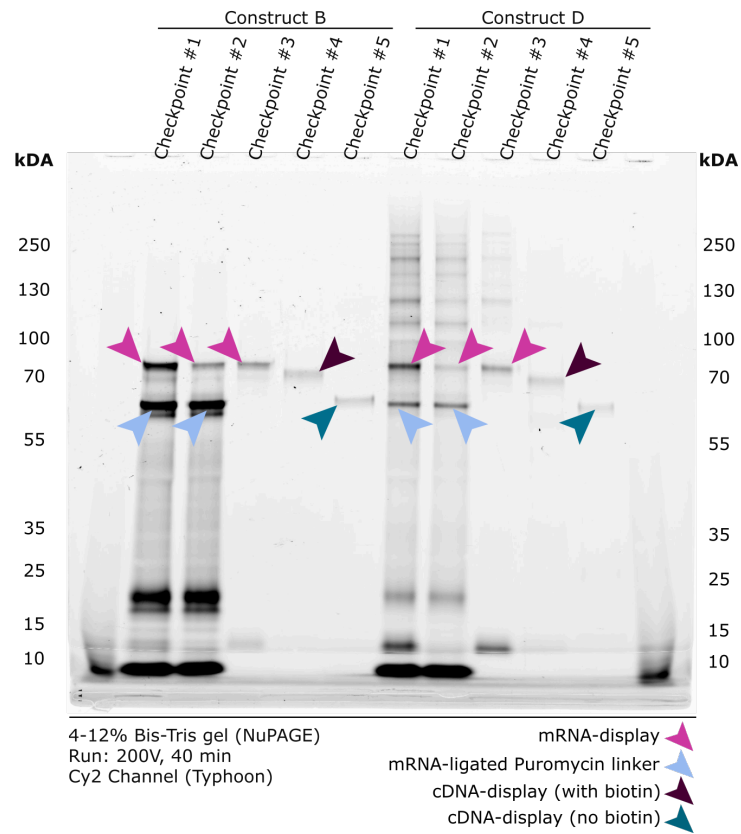


Figure 34: Images of the gel with the 5 checkpoints loaded. Gels visualized with **A)** Typhoon Cy2 channel and **B)** Coomassie InstantBlue

A)



B)

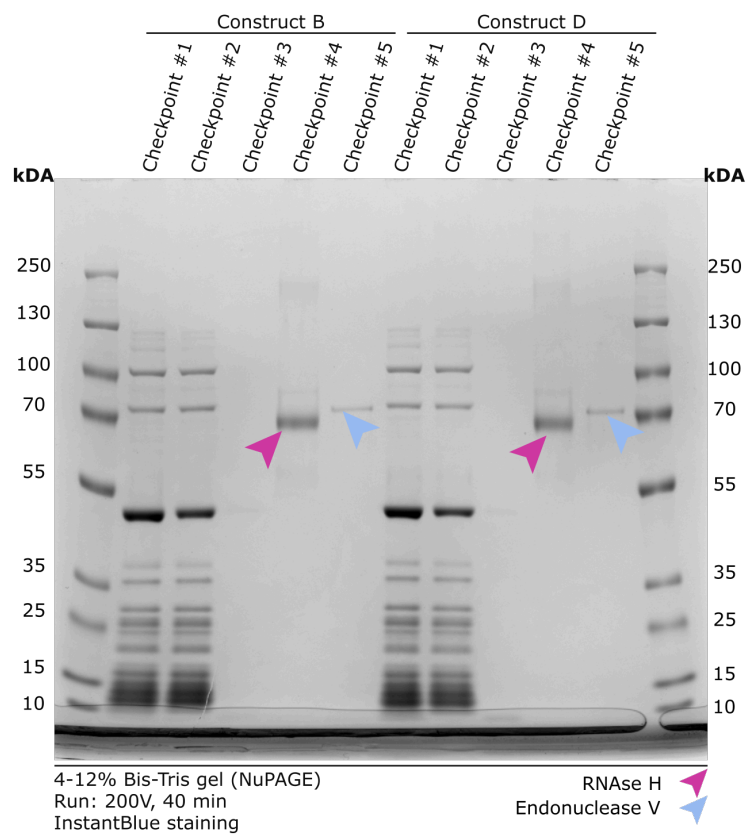


Figure 35: Images of the gel with the 5 checkpoints loaded. Gels visualized with **A)** Typhoon Cy2 channel and **B)** Coomassie InstantBlue

3.8. LABELING cDNA-DISPLAY FOR smFRET

The cDNA-display as produced and purified could now in theory be used for single molecule experiments using TIRF microscopy and a fluorescent probe. However, only the FAM dye is available as a fluorophore, which can be used as a FRET donor (Howell et al., 2002). However, its position is relatively distant from the peptide. If HLA-II is labeled at the N-terminus, as in the preliminary experiment, the distance between the fluorophores becomes too long for efficient FRET. Therefore, the construct will have to be labeled at an alternative location, ideally on the peptide itself.

However, with the construct design as presented, this is not feasible. Therefore, alterations have to be made to the ORF of the DNA template. Through these alterations, the peptide itself can be modified, such that it can be labeled. This can be done by introducing a TEV cleavage site between the peptide and the 6-Histidine tag, resulting in construct-T as shown in Figure 36.

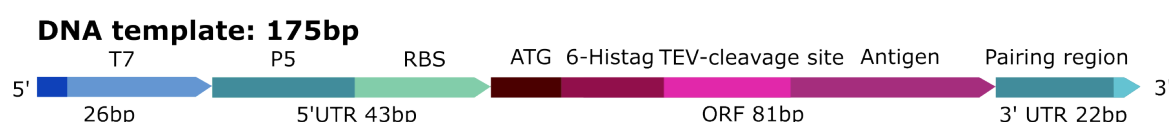


Figure 36: Template of Construct-T, which includes a TEV-cleavage site.

Cleaving the construct will result in a different N-terminus of the peptide. Without cleavage, the N-terminus is the startcodon, which encodes for methionine. However, as a prokaryotic in vitro translation system is used, this is an N-Formylmethionine, which is difficult to label due to the formyl group. Cleavage, on the other hand, results in a free amine. Using imidazole-1-sulfonyl azide (I-1-SA), diazo transfer can be performed, which results in an azide at the N-terminus. (Schoffelen et al., 2011) Using click-chemistry, the construct can then be labeled with Cy3- or Cy5-DBCO as shown in Figure 37.

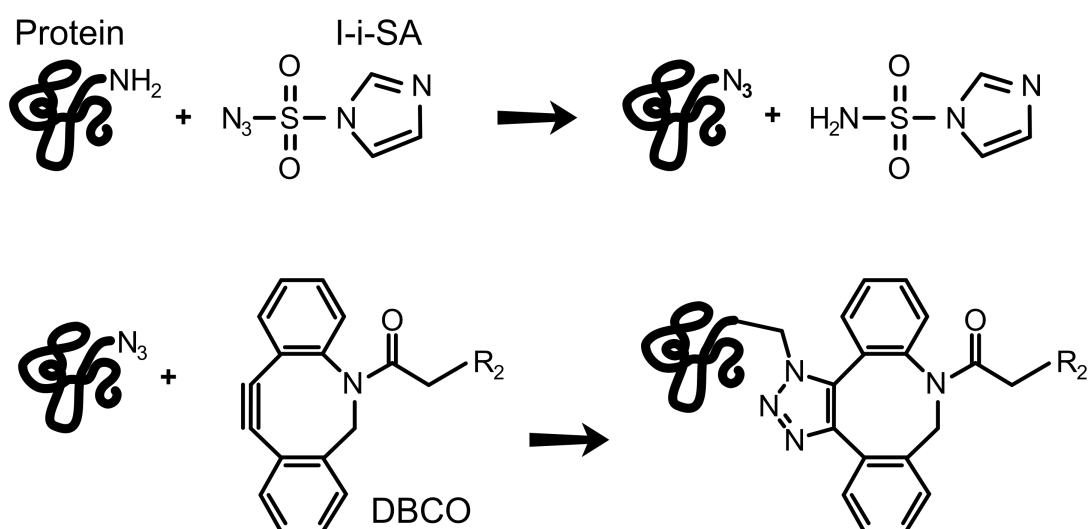


Figure 37: Diazo transfer followed by DBCO click-chemistry. Adapted from: (Schoffelen et al., 2011; Zuccaro, 2023)

Usage of TEV cleavage provides an additional benefit. It ensures that only the epitope sequence is part of the final construct and the 6-histidine tag is cleaved off. Therefore, it can be ensured that any FRET signal observed in experiments is due to the epitope sequence of interest and not due to the binding of the histidine tag in the groove.

If this labeling method is not efficient enough, an alternative labeling method is available, due to the design of the TEV cleavage site. The sequence of this site is designed as follows: ENLYFQC, which will be cleaved between Q and C. Consequently, the peptide contains an additional cysteine, providing a thiol group, which can be specifically labeled using a maleimide-fluorophore conjugate. (Fili & Toseland, 2014) However, the major drawback of this design is its restriction to cysteine free peptides, as the labeling of these could interfere with the binding to HLA-II. The peptides used so far do not contain any cysteine, but it could be problematic when expanding the number of peptides tested during a SPARXS experiment.

3.9. PURIFICATION OF TEV PROTEASE

In order to perform TEV cleavage, a TEV protease is required. Thus, it has to be purified. The TEV protease construct contains an MBP-tag at the N-terminus and a 2xStrep tag at the C-terminus, allowing for two step purification. In Figure 38, the results of the first purification using the MBPTrap HP 5 mLcolumn can be seen. Although some of the product is washed off, a large amount of purified protein remains in the elution. However, other protein bands are visible as well, thus requiring a second round of purification. This is done using the Strep-Tactin XT High capacity 5 mL column. From this purification, little product is lost, but the additional protein bands have disappeared. Thus indicating a pure product of TEV proteases, which can be used for the cleavage of the peptide.

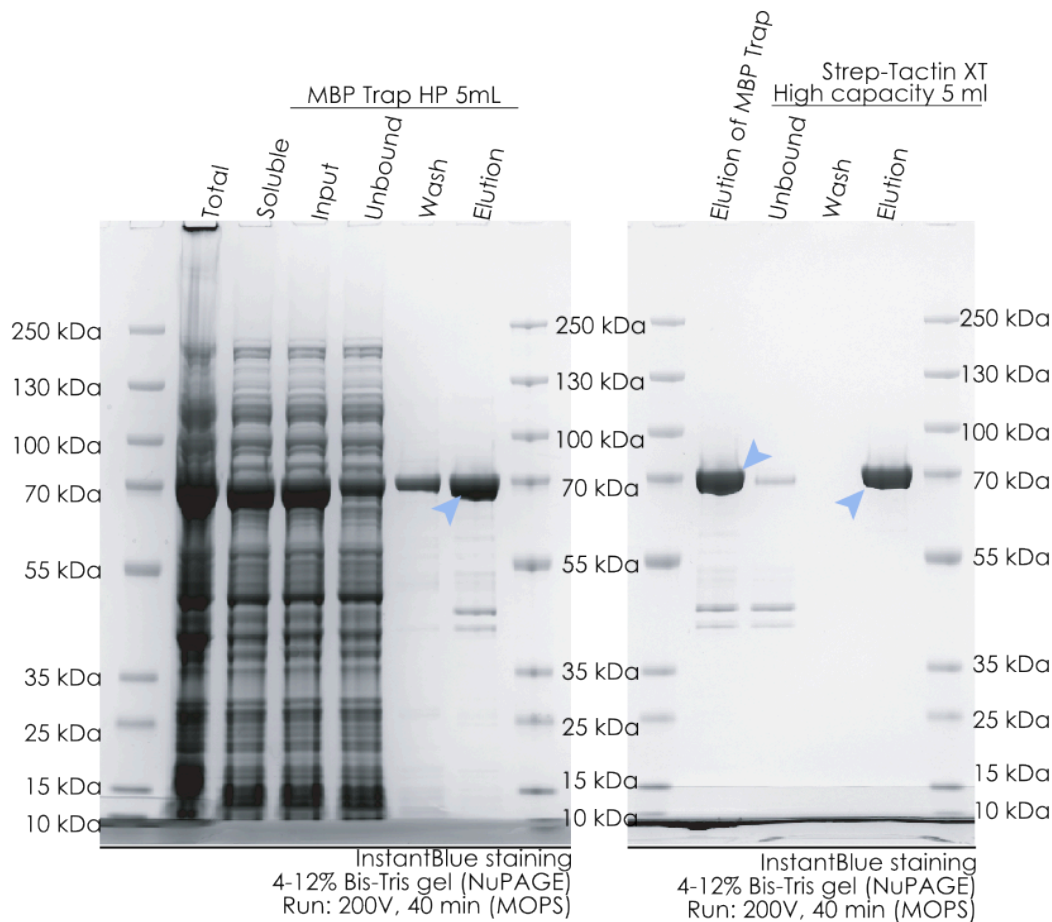


Figure 38: Gel images of intermediate steps of TEV purifications.

3.10. PCR OF CONSTRUCT-T

As the TEV purification was successful, the new construct with a TEV cleavage site could be produced. The procedure for performing PCR is similar to previous PCR steps of Constructs A-D. However, the modified DNA template now contains a TEV cleavage site as shown in Figure 36. This results in slightly larger products, which can be seen in Figure 39 as well. Here, the bands with and without T7 promoter are visible at 106bp and 175bp. No significant difference in purity or efficiency was observed in the purification of the PCR products.

Sample name	ng/ μ L	260/280	260/230
Construct A-T	120.52	1.81	2.20
Construct B-T	95.38	1.81	2.20
Construct C-T	104.56	1.82	2.28
Construct D-T	109.75	1.83	2.02

Table 30: Measurements of concentrations of DNA products using nanodrop. Total volume = 11 μ L

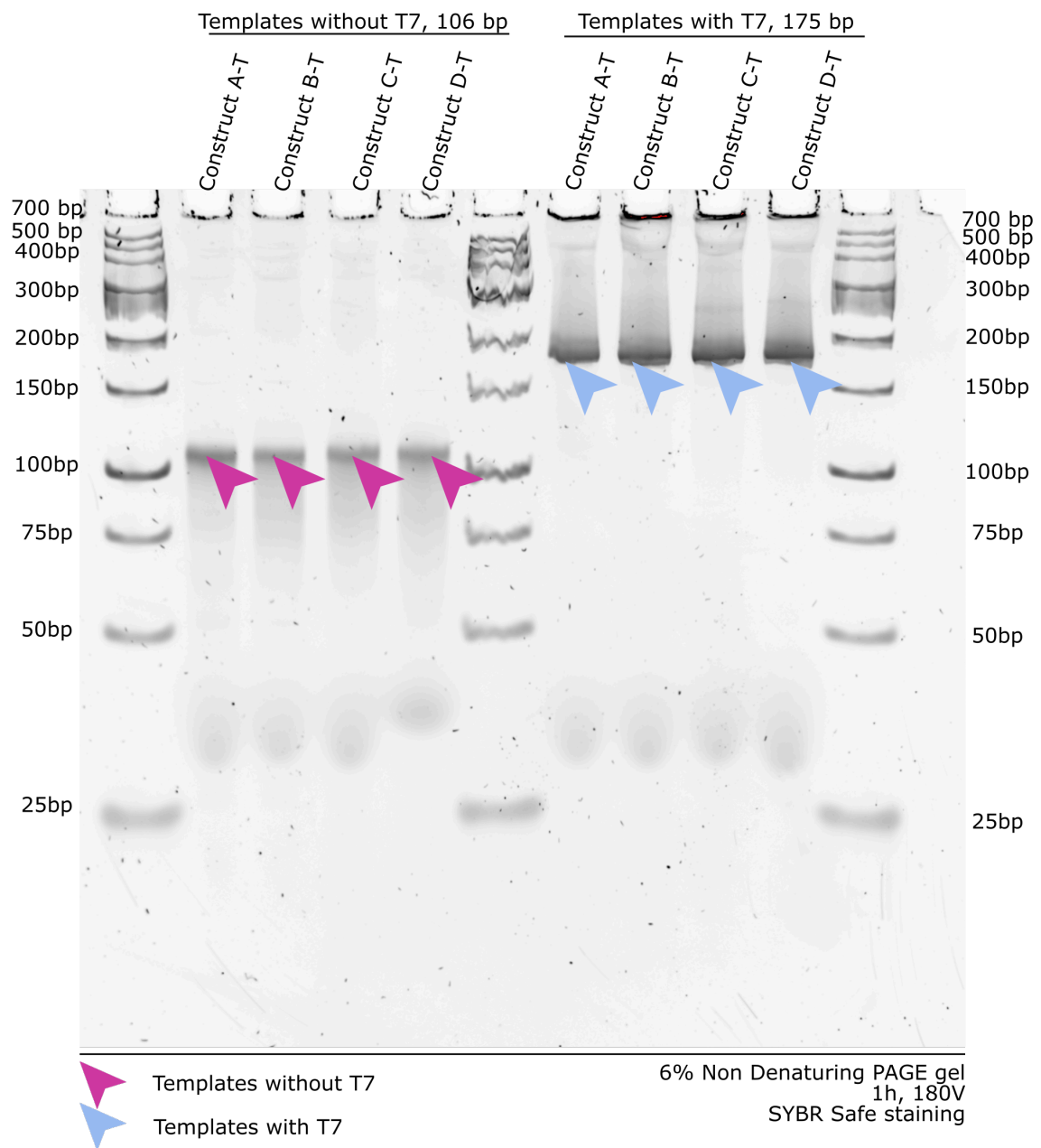


Figure 39: DNA templates: Construct-T without T7: 106 bp, Construct-T with T7: 175 bp

3.11. *IN VITRO* TRANSCRIPTION AND RNA PURIFICATION

The RNA was purified as shown previously for the previous Constructs A-D. At a band height of 152 nt, the gel was cut for purification. The results of the purification are shown in Table 31, which show a similar yield compared to the Constructs A-D shown in Table 28

Sample name	ng/ μ L	260/280	260/230	nts	MW (kDA)	μ M
Construct A-T	382.14	2.24	2.43	149	47.91	7.97
Construct B-T	315.42	2.12	2.49	149	47.91	6.58
Construct C-T	414.39	2.15	2.50	149	47.91	8.65
Construct D-T	514.11	2.13	2.39	149	47.91	10.73

Table 31: Measurements of concentrations of RNA products using nanodrop. Total volume = 11 μ L

3.12. LIGATION OF mRNA AND PUROMYCIN-LINKER

The newly purified puromycin linker then had to be conjugated to the RNA templates and purified. The results of the ligation are shown in Figure 40. This was done twice in order to obtain enough product to be able to optimize the yield for in vitro transcription, reverse transcription and the remaining purification steps.

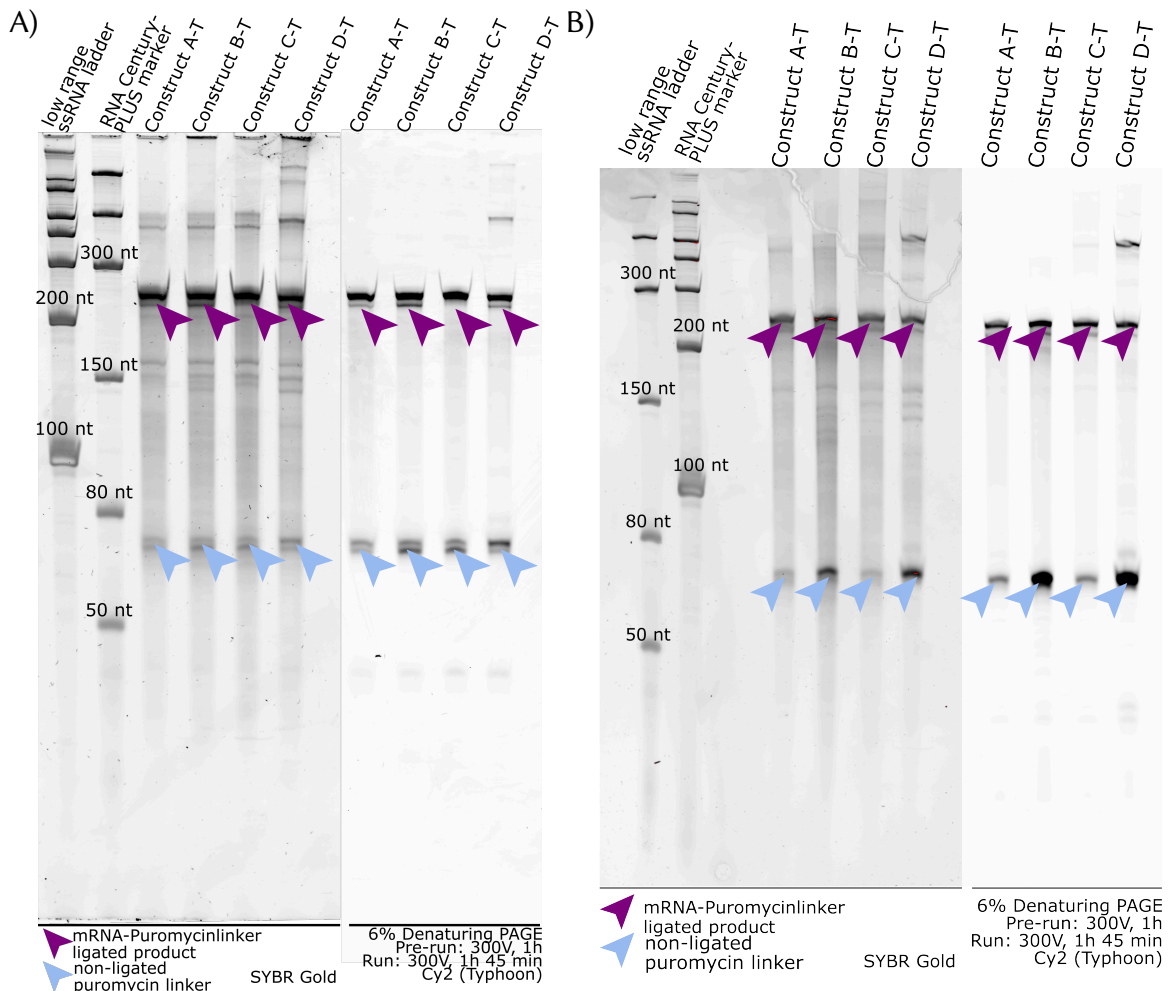


Figure 40: Gel images of ligation of mRNA to the puromycin linker. Results are shown for both purifications

3.13. CONSTRUCT C-T AND D-T: YIELD TO LOW TO BE DETECTABLE

Ligation of mRNA to the puromycin linker was successful, thus *in vitro* translation, Ni-NTA purification, reverse transcription and biotin pull-down were performed. However, the Ni-NTA purification yield was low. Furthermore, the overall yield of subsequent reverse transcription and biotin pull down was low as well, resulting in an undetectable amount of final cDNA construct. Intermediate checkpoints in Figure 41 correspond to the checkpoints shown in Figure 42.

	Construct C-T	Construct D-T
mRNA display	1	1
mRNA-Puromycin linker	1.89	0.50
Overall <i>in vitro</i> translation and peptide conjugation efficiency	35%	67%
Ni-unbound	0.07	0.26
Ni-purified	0.18	0.14
fraction purified	19%	9%

Table 32: Efficiencies calculated from gel, shown relative to mRNA-display of each construct

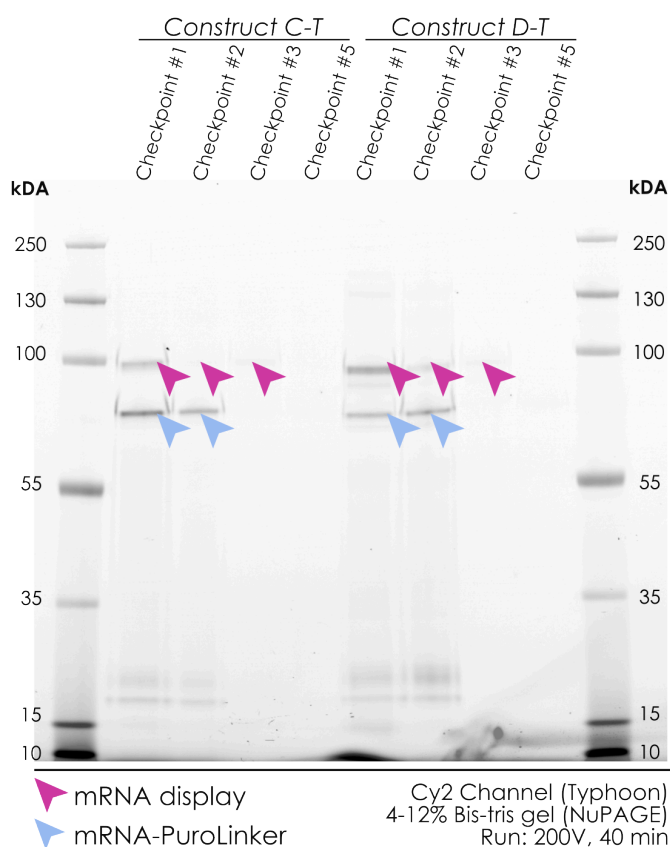


Figure 41: Gel image of intermediate steps for obtaining cDNA display. The yield of cDNA-display was too low to be detected.

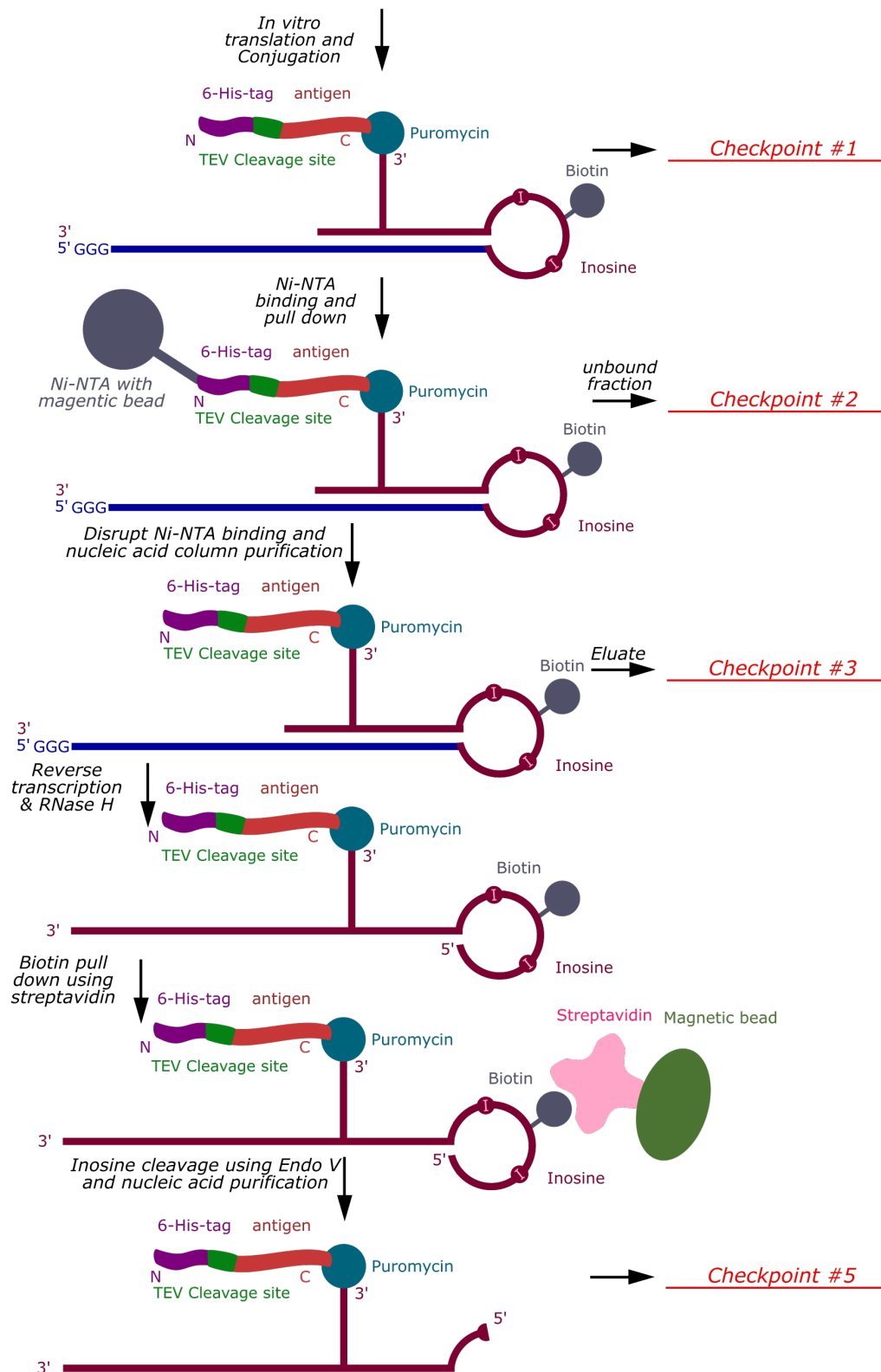


Figure 42: Overview of the procedures including checkpoint as shown in Figure 34 & Figure 35

3.14. CONSTRUCT A-T AND B-T SUCCESSFUL NI-NTA PURIFICATION, DESPITE LOW *IN VITRO* TRANSLATION YIELD OF CONSTRUCT B-T

As the Ni-NTA purification seemed to be a limiting step in the purification of the product, the protocol was performed for Construct A-T and B-T as shown in Figure 43. The *in vitro* translation showed varying efficiency. Despite this, the purification efficiency for both samples is approximately 48% and 41%, which is an improved yield as compared to previous purifications.

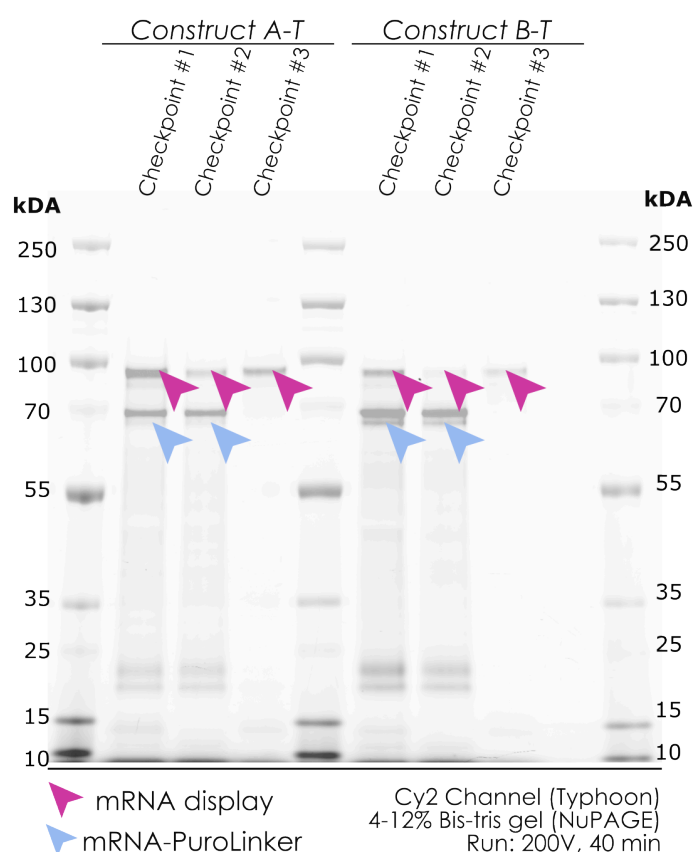


Figure 43: Results of Ni-NTA purification, checkpoints correspond to checkpoints shown in Figure 42

	Construct A-T	Construct B-T
mRNA display	1	1
mRNA-Puromycin linker	0.76	3.85
Overall <i>in vitro</i> translation and peptide conjugation efficiency	87%	21%
Ni-unbound	0.26	0.20
Ni-purified	0.46	0.39
fraction purified	48%	41%

Table 33: Efficiencies calculated from gel, shown relative to mRNA-display of each construct

3.15. DETECTABLE BUT LOW YIELD OF THE PURIFIED cDNA CONSTRUCT

The *in vitro* translation and Ni-NTA purification were repeated for Construct C-T and D-T and for all Constructs (A-T, B-T, C-T and D-T) reverse transcription and biotin pull-down were performed. The results are shown in Figure 44. The yield of the final cDNA construct was extremely low, but detectable, thus requiring optimization to be able to obtain high enough concentrations for smFRET assays.

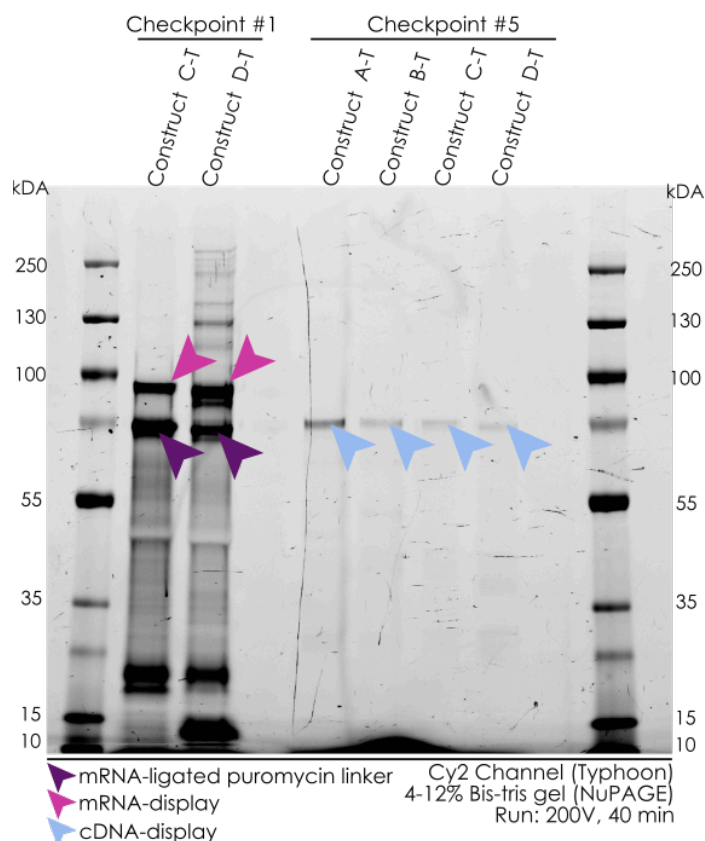


Figure 44: Results of *in vitro* translation and conjugation to the puromycin linker (checkpoint #1) and the final cDNA construct (checkpoint #5)

Sample	Construct A-T	Construct B-T	Construct C-T	Construct D-T
mRNA-display	-	-	1	1.55
puromycin linker	-	-	2.93	0.83
Overall <i>in vitro</i> translation and peptide conjugation efficiency	87%	21%	25%	65%
cDNA display	0.16	0.05	0.03	0.03

Table 34: Efficiencies calculated from Figure 44, shown relative to mRNA-display input of Construct C-T. IVT and ligation efficiency of Construct A-T and B-T are calculated from Figure 43

3.16. TRIAL YIELD OPTIMIZATION RESULTS IN INDETECTABLE YIELD

As the yield was too low for smFRET experiments, the protocol was altered in order to improve to yield. First the in vitro translation was altered. The input of puromycin ligated to RNA was halved, resulting in a theoretical maximum of 10pmol. The amount of ribosomes was kept the same at 40 pmol, thus altering the ratio from 1:2 to 1:4. The ribosomal translation and conjugation are single turn-over reactions, because the ribosomes have to stall before conjugating the peptide to the puromycin. Increasing the number of ribosomes per mRNA-puromycinlinker is thought to increase the yield relative to the input of the RNA-puromycinlinker construct.

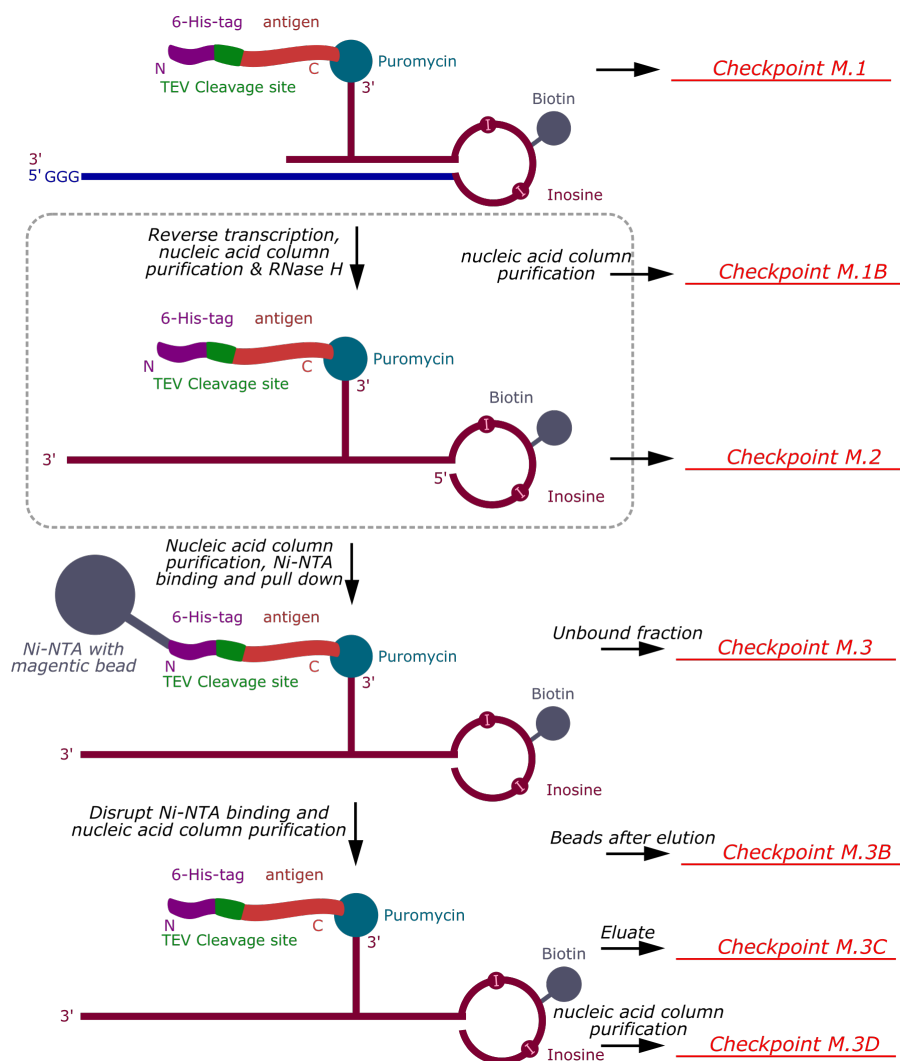


Figure 45: Newly modified protocol, with modified checkpoints. In the gray box, the procedures (Reverse transcription and RNase H treatment) that have been performed before Ni-NTA purification.

The second modification was changing the order of Ni-NTA purification and reverse transcription (Figure 45). In the current working protocol, first Ni-NTA purification is

performed before reverse transcription. In the modified protocol, first reverse transcription is performed to obtain cDNA display, before purification with Ni-NTA magnetic beads. This reduces the time spent handling RNA, thus reducing the risk of (partial) RNA degradation and could potentially increase the yield.

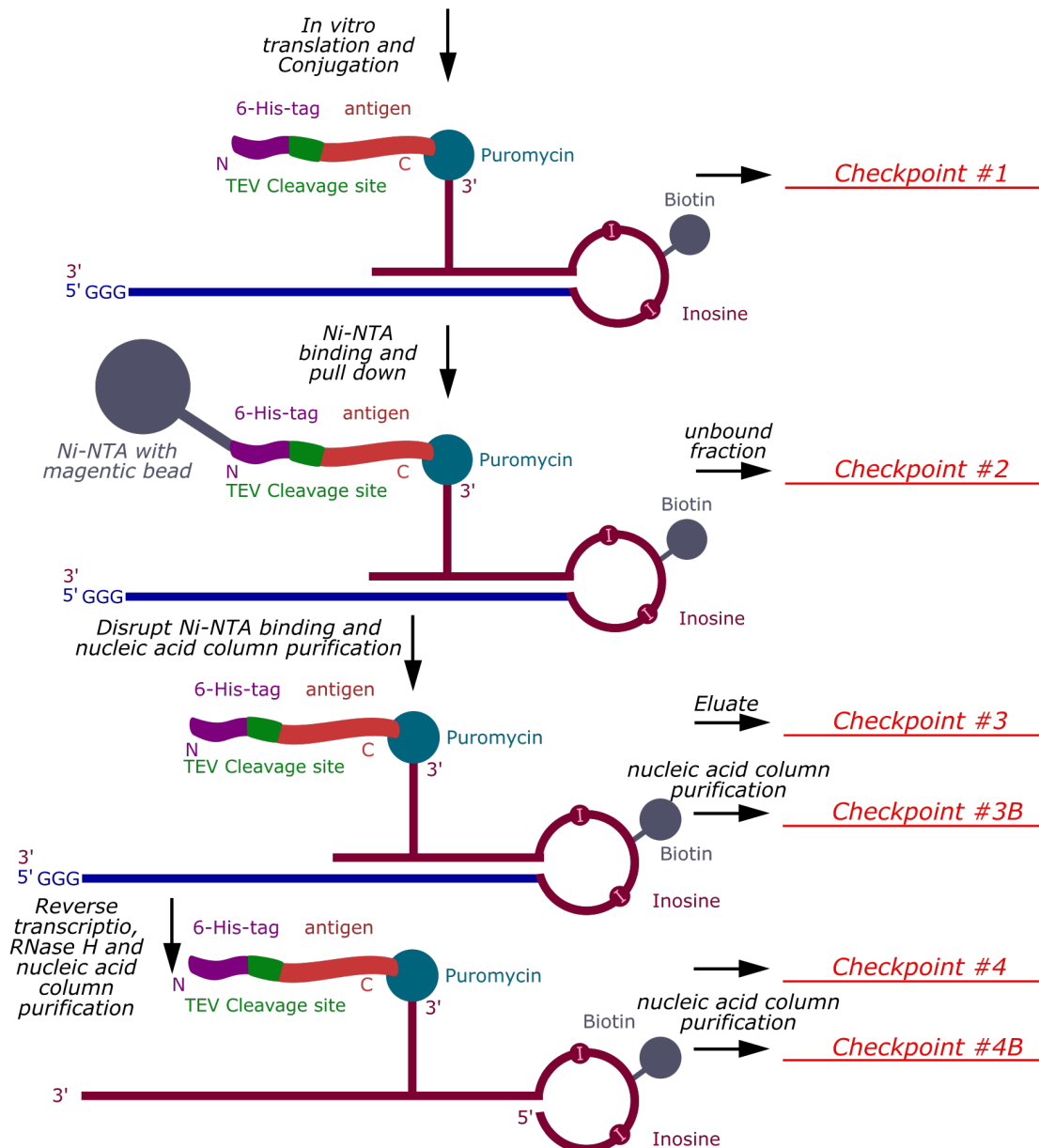


Figure 46: Additional checkpoints (#3B and #4B) which have also been loaded onto the gel

The yield optimization was done using Construct A, which is devoid of a TEV cleavage site. In Figure 47 the results for the modified protocol are shown next to the unmodified control. Both were performed with a 1:4 ratio of ribosomes to mRNA-puromycinlinker. However, both methods showed no yield at all, which indicates RNase contamination as neither the puromycin linker with mRNA, nor the mRNA-display are visible.

RNase contamination can have many sources. A likely source is contamination during the handling of the samples. Insufficient cleaning of contaminated surfaces could easily lead to contamination and subsequent degradation of the samples. Therefore, the experiment was repeated in order to rule out this possibility. The results are shown in Figure 48. Once again, no yield from the in vitro translation and subsequent procedures was observed, once again indicating RNA contamination.

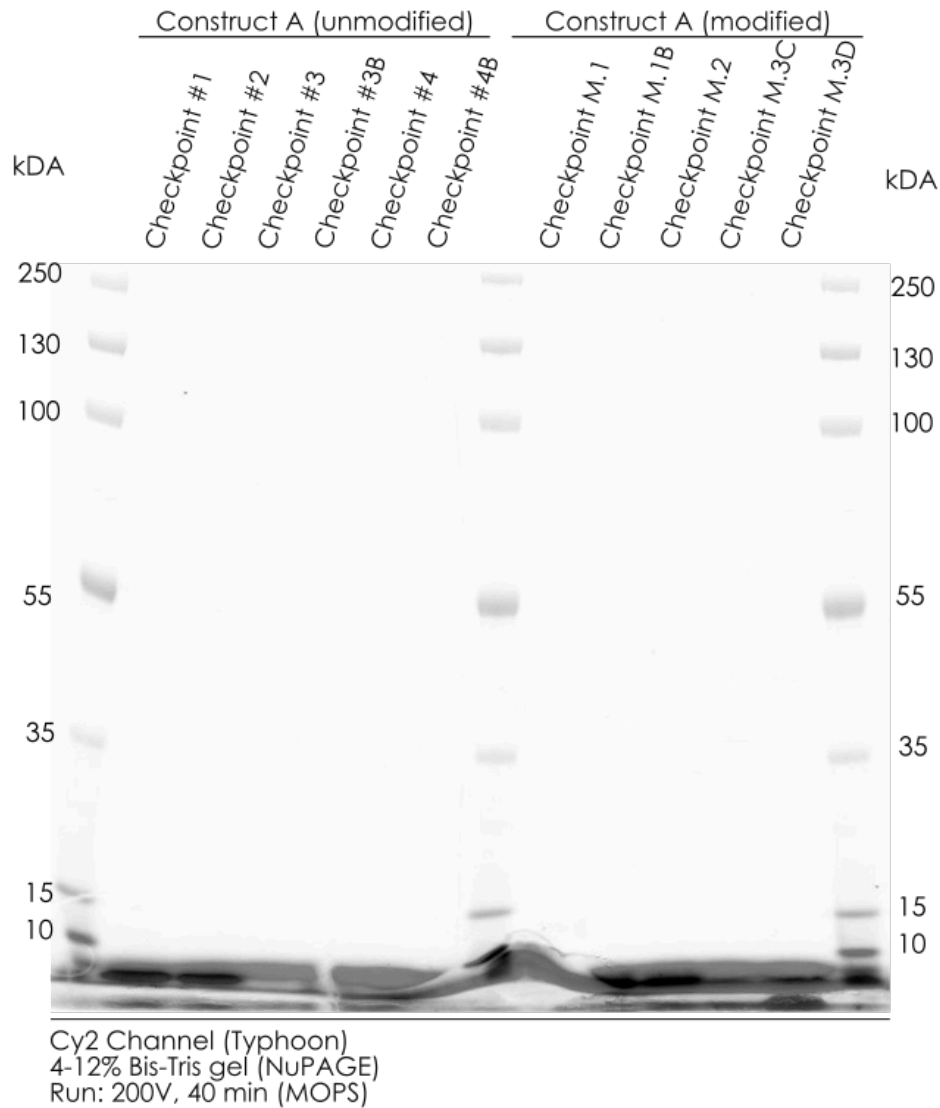


Figure 47: Results of yield optimization, neither the unmodified, nor the modified protocol result in cDNA-display.

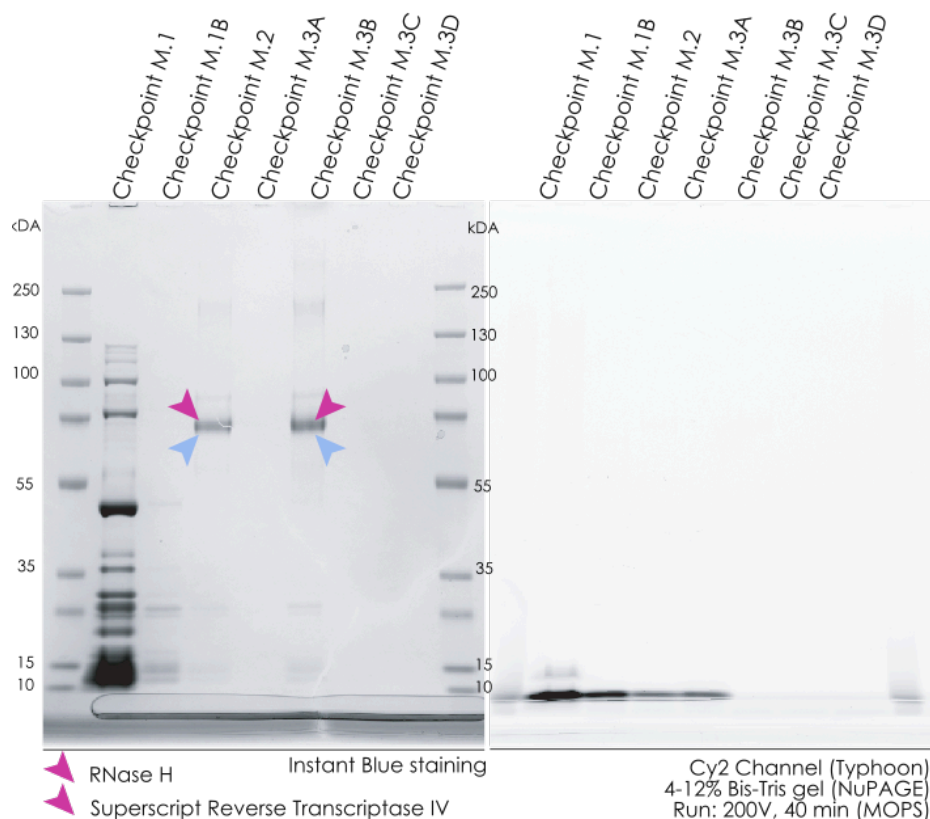


Figure 48: Results of yield optimization, neither the unmodified, nor the modified protocol result in cDNA-display.

3.17. INVESTIGATING SOURCES OF RNA DEGRADATION

From these results it seems clear that contamination occurs during in vitro translation and puromycin conjugation or during gel running. This leaves several sources for contamination.

1. Contamination during handling of the samples. This is unlikely as it would have occurred continuously, while no such problems occurred during previous experiments. Furthermore, intensive cleaning is performed after each contaminated experiment in order to eliminate this possibility.
2. The sample is contaminated during storage before performing in vitro translation.
3. The construct is degraded during the 95 °C incubation before loading onto the gel. This was not problematic when performing in vitro translation initially, but could have become problematic due to reduced stability of the RNA.
4. The FAM dye has bleached, thus resulting in undetectable constructs. The constant handling of samples under the light could have photo bleached the FAM dye, thus making it impossible to detect in the gel. However, at the bottom of the gel, a strong signal remains visible.

The aforementioned points can be addressed by including a control, where a sample of the input for in vitro translation, the mRNA-ligated puromycin linker, is taken and loaded onto the gel with the in vitro translated samples. By using a different construct, Construct-T instead of Construct A, the question can be answered whether storage conditions have caused the degradation of RNA.

5. The high initial temperature of 65 °C before performing in vitro translation degrades the RNA.

The incubation is performed remove secondary structures from the RNA. Although this was not problematic when performing in vitro translation initially, it might become problematic when combined with traces of Mg contamination, which could lead to higher activity of RNases and could subsequently cause the degradation of the RNA.

To address this, the incubation at 65 °C step could be removed before performing in vitro translation. Although this could lower the efficiency of the reaction, it might also prevent degradation of the RNA.

6. The high-salt buffer, which is added for the conjugation of the peptide, is contaminated with RNase

This could be a possible explanation, as contamination of this buffer would indeed lead to degradation of both the mRNA-display as well as the mRNA-puromycin construct. This is because conjugation takes place in the same reaction tube as the in vitro translation.

Whether this is the cause of RNase contamination, can easily be addressed, by taking a sample of the in vitro translated product before puromycin conjugation through addition of the high salt buffer.

7. The in vitro translation system is contaminated (confirmed)

It would be expected to receive non-contaminated product from the company. However, the first time RNA degradation was observed in the gels occurred right after switching between batches from the company. Therefore, it could be possible that the company has provided a contaminated batch although other sources of contamination are still possible.

3.17.1. RNA DEGRADATION OCCURS WITHOUT 65 DEGREE INCUBATION, BEFORE ADDITION OF HIGH-SALT BUFFER

The aforementioned alterations were implemented into the protocol. The results are shown in Figure 50. All intermediate samples were loaded onto the gel as outlined in Figure 49. As can be seen, the input control is clearly present, thus indicating that the 95 °C incubation before loading the gel does not cause the degradation of RNA. Furthermore, it indicates that the FAM dye is not bleached and the constructs aren't degraded during storage. However, the other lanes are empty, thus indicating that degradation occurs before puromycin conjugation, during in vitro translation. *In vitro* translation solely involves the combining of the sample with TDW, RNase inhibitor and solutions as provided in the

in vitro translation kit of PUREfrex as provided by GeneFrontier. Therefore, the in vitro translation kit is likely contaminated.

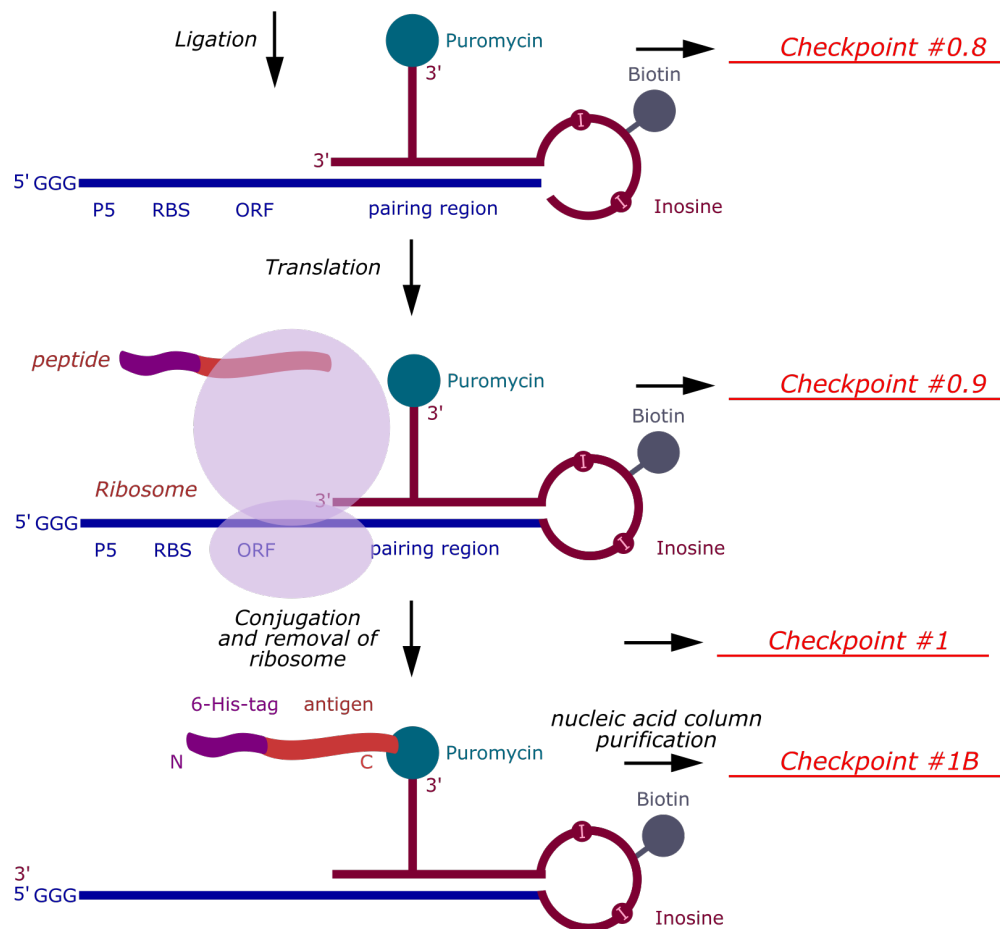


Figure 49: Overview of additional checkpoints

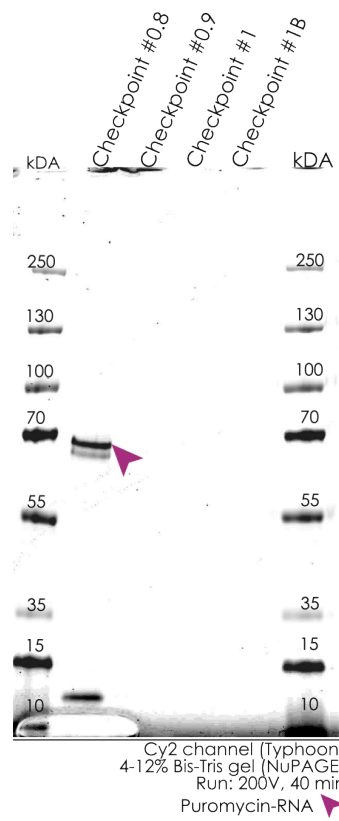


Figure 50: Results of yield optimization, neither the previous applied method, nor the new function

4

DISCUSSION & CONCLUSION

4.1. HLA-II PEPTIDE BINDING

Earlier in this thesis, the peptide binding mechanism has been discussed. A hypothesis based on the crystal structures of HLA-DR and HLA-DM is that the N-terminal end of CLIP is initially displaced from the P1 pocket, before completely dissociating (Pos et al., 2012). Furthermore, HLA-DM stabilizes the HLA-DR construct and facilitates the movement of side residues of HLA-DR into the P1 pocket and P2 site. This results in an only partially accessible groove, only allowing the peptide to bind into the shallower pockets of the groove. Only when the peptide outcompetes the side chain residues of HLA-DR, can it occupy the P1 pocket and stably bind in the entire groove. Thus, both the binding and dissociation are sequential in the presence of HLA-DM. In our data, we observed similar behavior. First, co-localization of the peptide with HLA-II is observed, before FRET occurs, which indicates the binding of the peptide into the P1 pocket.

The question remains, whether these sequential steps as observed in the smFRET data have the same underlying mechanism as proposed (Pos et al., 2012). If this were the case, one would still expect that the P1 pocket is at least partially blocked by the HLA-DR side chains when no peptide is bound, thus preventing the immediate occupation of the P1 pocket. This is difficult to verify, as to date no crystal structures of empty HLA-DR have been resolved. Furthermore, prolonged peptide vacancy increases the chance of HLA-DR adopting an alternative inactive conformation. (Rupp et al., 2011) A computational model has shown that the P1 to P4 pockets seem indeed to be occupied by residues of the HLA-DR molecule (Painter et al., 2008). However, they hypothesize that this conformation could actually be the inactive state of the HLA-DR molecule, where the molecule is non-receptive to peptides.

A logical next step would be purification of HLA-DM and observe whether the same sequential binding process is observed. HLA-DM is a molecular chaperone, which would likely reduce the binding and dissociation times of the peptides. However, in the preliminary experiment, dwell times were already relatively short. Even shorter dwell times would increase the difficulty of the data analysis, as the low and high FRET states would

be harder to classify. The short dwell-time is likely caused by the close proximity of the CLIP peptide, which remains bound to the N-terminus of the β -chain.

Another important consideration has to be made when implementing HLA-DM into the assay as shown in Figure 51. First of all, similar to HLA-DR, it is a membrane protein, this aids in the localization of both proteins within the antigen processing compartment. Furthermore, the micro domains present within the membrane of the antigen processing compartment help to concentrate HLA-DM and HLA-DR. In order to observe the catalytic activity of HLA-DM, it might be required to either use high concentrations of HLA-DM, aid in its localization to HLA-DR or increase the association affinity of the complex.

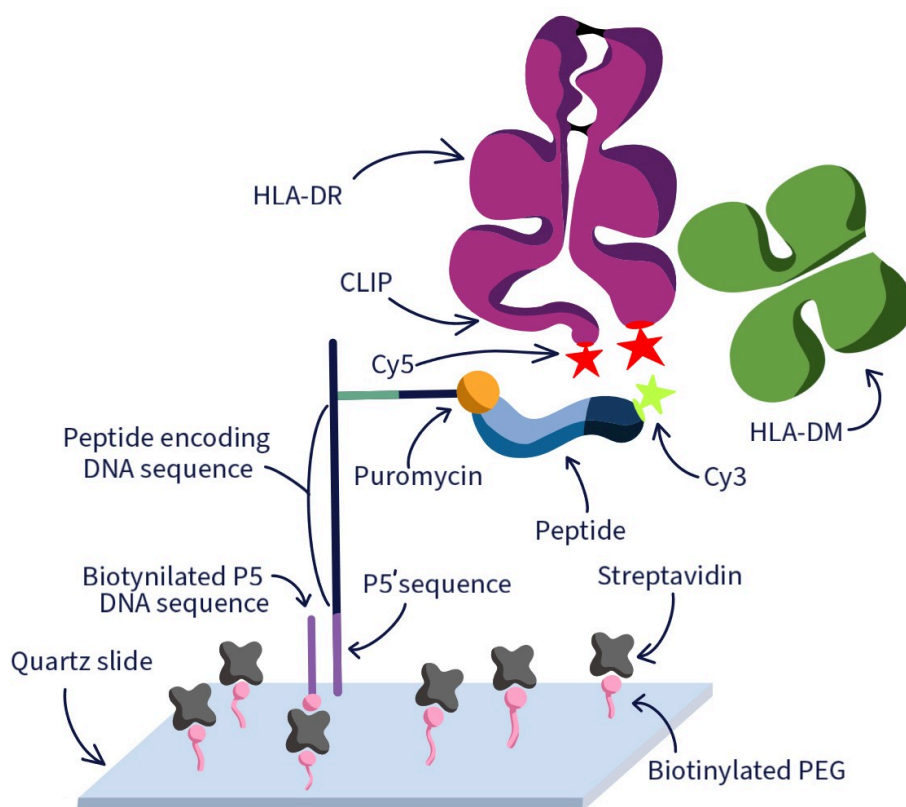


Figure 51: Implementation of HLA-DM into the assay. HLA-DM interacts near the P1-pocket of HLA-DR and acts as a molecular chaperone.

4.2. FUTURE OUTLOOK ON SPARXS

This research has shown that cDNA-display can be generated and purified with relevant qualities, which allows for its adaptation for smFRET experiments. This is an important first step in implementing a peptide-HLA-II assay for SPARXS. However, several additional steps have to be taken in order to be able to perform a high-throughput peptide-HLA-II assay.

4.2.1. TEV-CLEAVAGE AND cDNA DISPLAY LABELING

The cDNA-display molecule will have to be labeled. At the N-terminus of the construct is an N-formylmethionine, which is the first amino acid in a bacterial *in vitro* translation system. Labeling of the N-terminus is difficult due to this formyl group. Therefore it has to be removed. This can be done by first incubating the construct with the previously purified TEV enzyme Figure 52. This enzyme cleaves the peptide at the incorporated TEV-cleavage site, which then exposes a free amine that can be used for labeling. Using, Imidazole-1-sulfonyl azide, diazo transfer can be performed creating an azide group, which can be further labeled using a click-chemistry reaction to conjugate either Cy3-DBCO or Cy5-DBCO (Schoffelen et al., 2011). After labeling, the construct will have to be purified once more, which could be done using nucleic acid column purification.

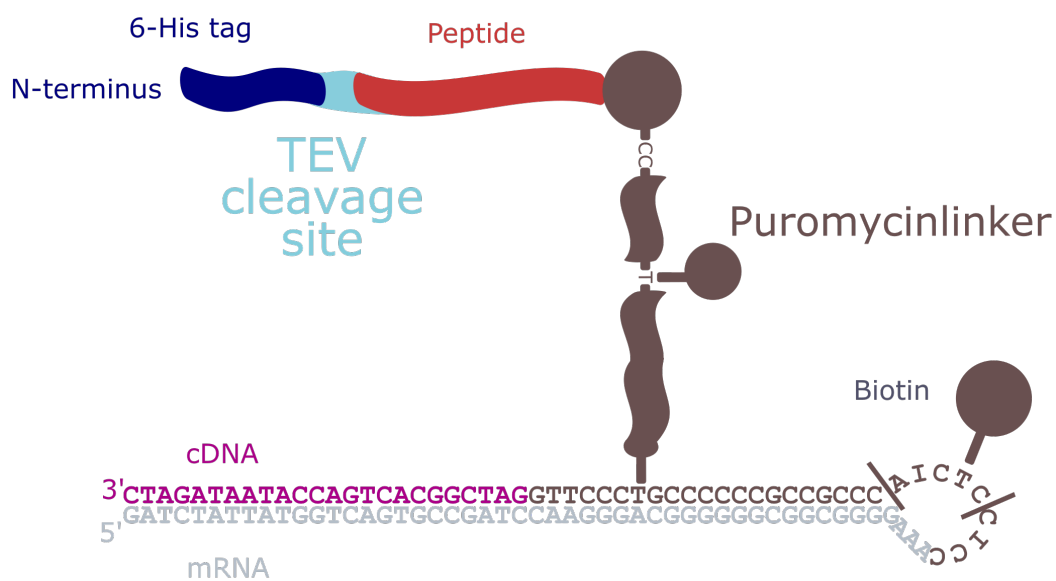


Figure 52: Detailed depiction of the cDNA construct with TEV cleavage site in the peptide. mRNA is removed after reverse transcription, but is included in the figure. Furthermore, when using biotin for the purification of the construct, it will be cleaved off as well.

4.2.2. FURTHER VERIFICATION OF cDNA DISPLAY

Immobilizing the cDNA-display construct is key for studying the interactions between peptide and HLA-II when using smFRET. This can be achieved by utilizing the P5' sequence in the cDNA-display construct. The surface of the quartz slide can be biotin-PEGylated, to which streptavidin can be bound. To the streptavidin, a biotinylated DNA strand can be bound. This DNA strand should contain the complementary P5 region. Then, it will be possible to hybridize the DNA of the cDNA-display to the biotinylated DNA. Through this, the cDNA-display construct can be immobilized on the surface. Successful immobilization is dependent on the generation of cDNA in the cDNA construct. Only if the complete RNA template is reverse transcribed is the cDNA P5 sequence obtained. Therefore, a smFRET assay, with immobilized cDNA-display, can also be used to verify whether reverse transcription was successful.

In order to test whether the construct is immobilized on the surface, either the construct itself has to be fluorescently labeled, or a suitable probe has to be designed. Successful labeling depends on the efficiency of TEV cleavage, diazo transfer and subsequent click-chemistry. If the yield of these reactions remains insufficient, a probe can be useful for testing both immobilization and reverse transcription. Multiple probes can be designed for the construct. A possible probe is a fluorescently labeled oligo-dT sequence of 18 nucleotides (Figure 53). As the puromycin segment contains an oligo-dA sequence of 18 nucleotides. This provides a long region for hybridization of the DNA and thus a suitable probe, as it has strong binding kinetics.

Additionally, if TEV-cleavage and subsequent labeling is not performed, the 6 His-tag of the peptide could be utilized. In the production of cDNA display, it has already been shown that the peptide is generated and conjugated as the construct is purified using Ni-NTA magnetic beads. These beads bind to the 6-histag at the N-terminus of the peptide, thus only peptides with the transcribed tag can be purified through this method. Furthermore, a fluorescently labeled Histag antibody could be used as a probe for the construct (Figure 53). If these antibodies are observed, it would be an even stronger indication that the peptide is indeed a functional element of the cDNA display construct and would indicate the successful immobilization.

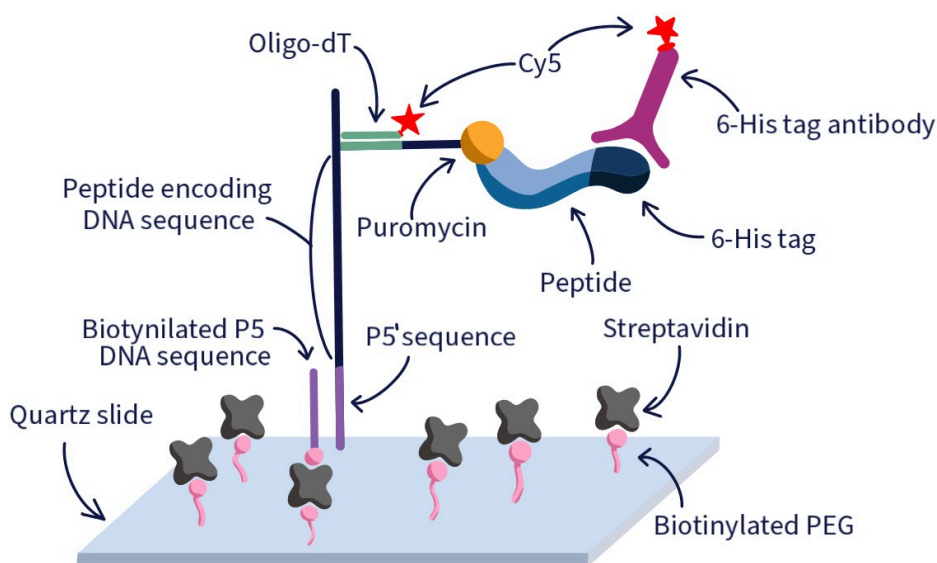


Figure 53: Single molecule localization assay, using either a Poly-dT or 6-His Tag antibody as a probe for the cDNA display construct. Alternatively, Cy5 labeled HLA-DR could be used as a probe as well

Naturally it follows that one could use HLA-II proteins in solution as a probe of the cDNA-display molecules immobilized on the slide surface. This would show whether cDNA-display can be used to test peptide affinities against HLA-II proteins. Ideally, this would be done using smFRET, where both the cDNA-display construct and the HLA-II protein are labeled to be able to obtain a FRET signal, which would be stronger evidence for binding than the localization of HLA-II molecules on the surface. Additionally, it could confirm whether the puromycin linker interferes with peptide binding or not. The peptide

sequences as shown in Table 35 can be used as a positive control. Additionally, it has been shown previously that these epitopes of HLA-DRB1*0301 do not bind to HLA-DRB1*0401 and vice versa, thus also providing a negative control. (Yang et al., 2006)

Additionally, more kinetic data can be extracted from the smFRET experiment, such as association and dissociation constants. These remain unavailable to date, with only estimations from binding affinity models. Observed constants could be compared to these estimations and thus further test and improve their reliability.

HLA	Minimal epitope	Sequence
HLA-DRB1*0301	IGRP _{13–25}	QH <u>L</u> QK <u>D</u> YR <u>A</u> Y <u>T</u> F
HLA-DRB1*0301	IGRP _{226–238}	RV <u>L</u> N <u>I</u> D <u>L</u> L <u>W</u> S <u>V</u> P <u>I</u>
HLA-DRB1*0401	IGRP _{23–35}	Y <u>T</u> <u>F</u> L <u>N</u> <u>F</u> M <u>S</u> N <u>V</u> G <u>D</u> P
HLA-DRB1*0401	IGRP _{247–259}	D <u>W</u> <u>I</u> H <u>I</u> D <u>T</u> T <u>P</u> F <u>A</u> G <u>L</u>

Table 35: Identified epitopes used for preparing cDNA-display (Yang et al., 2006). Underlined amino acids correspond to the identified anchor residues for the P1, P4, P6 and P9 pocket.

4.2.3. SOLUBILITY OF HLA-II

However, before this experiment can be performed, further challenges have to be addressed. Ideally, smFRET will be carried out using immobilized cDNA-display and HLA-II in solution as described earlier. However, currently it has been challenging to obtain soluble HLA-II that is compatible with smFRET experiments. One of the main limitations is the precipitation of the construct at acidic pH. This is problematic, as an extensive body of research has shown that peptide binding occurs endogenously in human cells at approximately pH 4.5. Moreover, almost no binding is observed at pH >7.0 (Roche & Furuta, 2015).

A possibility to overcome this limitation is to adjust the construct such that it remains soluble at acidic pH. However, achieving this is no simple feat and would require a great deal of time, research and resources. An alternative is to adjust the assay such that binding events between peptide and HLA-II can be observed at neutral pH. This could possibly be achieved by using J10-1, which is a small molecule which acts as a catalyst for peptide exchange. Similar to HLA-DM, it acts on the P1 pocket side of the HLA-DR construct, near the N-terminus of the peptide. Furthermore, it has been shown to circumvent the pH dependency. (Call et al., 2009) Therefore, it could be used in combination with the current HLA-DR construct at neutral pH.

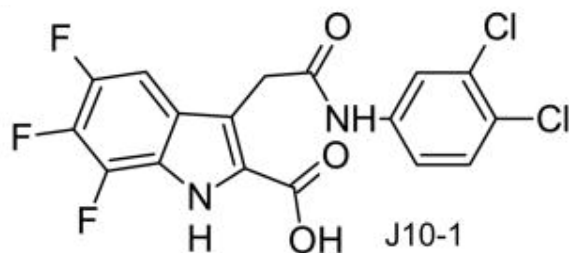


Figure 54: Structure of J10-1 (Call et al., 2009)

4.2.4. ILLUMINA MiSEQ

Once an smFRET assay using both cDNA display and HLA-II in solution has been established, it is still not compatible with SPARXS. For this, the assay will have to be performed on an Illumina sequencing chip, which contains a different surface compared with the biotin-PEG surface of the quartz slide. Therefore, new non-specific binding moieties on the surface could be observed. However, the exact composition of the flowcells is not publicly known, thus only experimental research will be able to show whether cDNA-display is compatible.

Once the smFRET measurements have shown to be successful, the cDNA-display will have to be sequenced using the Illumina MiSeq flowcell. This will pose its own challenges, as either the cDNA display will have to be sequenced with its peptide attached, or the peptide has to be removed. It could be removed from the puromycin construct by treatment with a protease, if it interferes with the sequencing. However, more likely, the puromycin-linker will simply be displaced during amplification. This starts with the DNA-strand, which is immobilized using the P5 sequence of the construct. Then the complementary strand is synthesized, after which the original strand, in this case the cDNA-display construct is detached and amplification continues.

Additionally, it has to be noted that Illumina sequencing requires the sequence to contain both a P5' and P7 region. In the most recent construct, the P5' region has been included, but not the P7 region. The latter could be incorporated using two methods. Either, the P7 region is placed within the ORF, which would ensure its incorporation into the DNA sequence. However, this would result in the P7 region being translated and incorporated into the peptide of the cDNA-display construct. This is undesirable, as it could possibly bind into the HLA-II cleft, instead of the peptide of interest, thus resulting in a false positive result. Alternatively, the region could be included in the puromycin-linker region. Then, when the construct is immobilized using the P5 sequence, the P7 sequence will be sequenced by the Illumina MiSeq as shown in Figure 55.

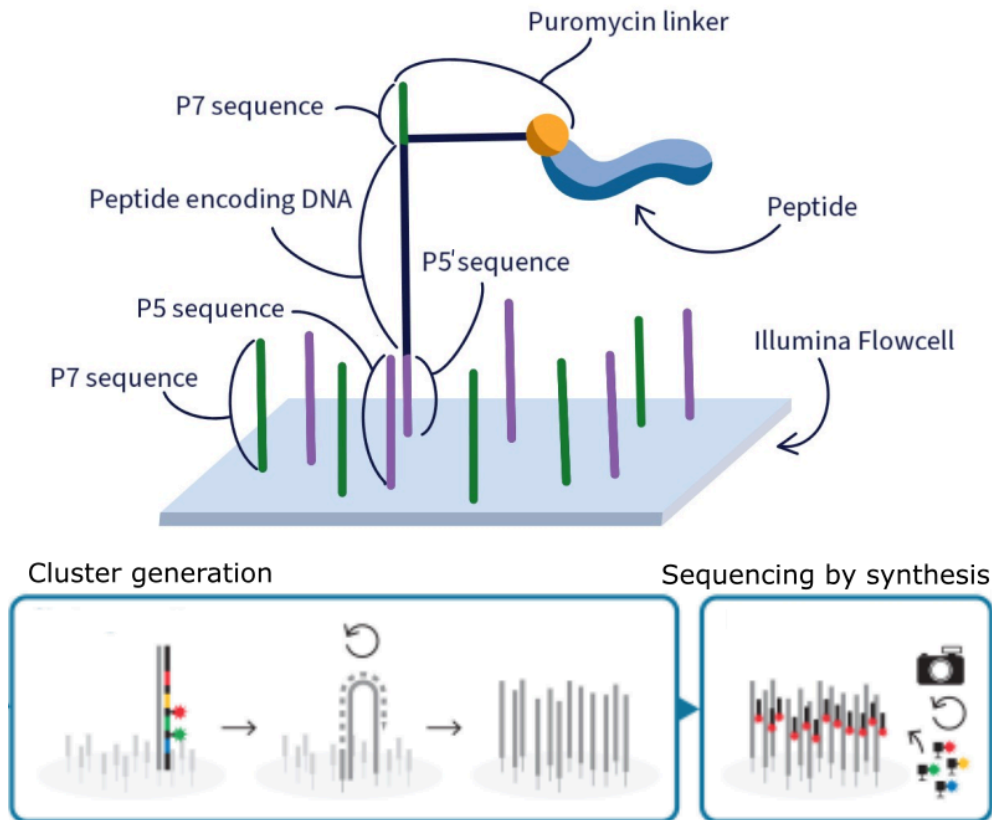


Figure 55: cDNA-display construct with a P7 sequence. The P5 sequence on the Illumina flowcell is elongated during clustering. After elongation, the cDNA-display construct is washed off. The strand folds over and hybridizes to the P7 sequence on the flow cell. From here clustering continues. Adapted from (Severins et al., 2024)

4.2.5. PEPTIDE LIBRARY CONSTRUCTION

Finally, the goal of implementing SPARXS is to achieve high-throughput kinetic measurements of peptides. In order to do this, a peptide library will have to be carefully constructed. The length of the peptides generated in the cDNA display construct so far is 13 amino acids, although both longer and shorter peptides could be generated. If one were to change each single position one would have 20^{13} possible peptides, which is impossible to all be tested using SPARXS. Therefore, careful consideration on which peptides to include have to be made, for which the following strategies could be taken into account.

Varying of anchor residues

It is known that the main determinants for binding between peptide and MHC are the anchor residues. Although the other amino acids play a minor role as well. However, it would greatly reduce the number of peptides tested if one were to vary only the anchor residues. Considering all four binding pockets, this would result in $20^4 = 160.000$ peptide sequence variations. Just varying the residue in the P1 pocket, would result in only 20 different peptide sequences to test, further reducing the complexity. However, the binding positions are relative to the HLA molecule. Varying a residue and observing binding could indicate two distinct scenarios. The first scenario is that the changed residues binds into its predesigned pocket, which results in stable binding. However, another scenario could

be that the binding positions simply shift, where one of the other side chains binds into a pocket.

Amino acid variations based on properties

To further reduce the number of possible variations, the properties of the amino acids themselves have to be taken into account. Altering amino acids with similar properties is less likely to affect the binding kinetics between the peptide and HLA-II molecules. Therefore, the number of amino acids tested could be greatly reduced if they are grouped based on properties, such as charge, polarity, hydrophobicity and size. If one were to group the amino acids into groups of two, and vary only the 4 anchor residues, this would already result in only $10^4 = 10.000$ possible sequences.

Selection using computational affinity prediction

An alternative method for selecting peptides is to make use of binding prediction software such as NetMHCIIpan 4.1 (Reynisson et al., 2020). This tool can predict the binding affinity of peptides to HLA-II molecules. This could be used to perform pre-selection rounds of peptides, as peptides with extremely low expected binding affinity, are more likely to not bind to HLA-DR. During a smFRET assay, this would result in no observed binding. Therefore the experiment would not be able to provide any kinetic data on these peptides as the kinetics are simply not observed. Excluding these peptides, would reduce the number of peptides to be tested and provide more valuable data per experiment.

Selecting physiologically relevant epitopes

One has to consider the relevance of performing a SPARXS experiment. Considering IGRP and its relation to type 1 diabetes, the main goal is to identify binding kinetics of possible epitopes that could be responsible for causing an autoimmune response. An autoimmune reaction targets a peptide within the human body. Therefore, the epitope and thus the peptide tested should be expressed in the human body. In the case of T1D, one could narrow it down further and only consider epitopes specific to insulin producing β -cells in the pancreatic islets of Langerhans and also include common polymorphisms and mutations.

4.3. CONCLUSION

Taken together, this work establishes the groundwork for SPARXS-compatible cDNA-display to study the interactions between HLA-II and peptides. This would probe the kinetic landscape of peptide-HLA-II binding. The design and adaptations outlined in this thesis demonstrate that cDNA-display could be engineered to support the immobilization, labeling and smFRET-based detection required for SPARXS. By adapting the aforementioned modifications, the cDNA-display construct can be improved and successfully used for SPARXS. With this optimization, this technique has the potential to rapidly test the peptidome of HLA-II molecules. Moreover, it can provide high-throughput kinetic data on peptide-HLA-II interactions. These data could help further resolve fundamental questions regarding peptide loading and dissociation mechanisms.

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APPENDIX

CLARIFICATION OF NOMENCLATURE OF HLA GENES AND ALLELES

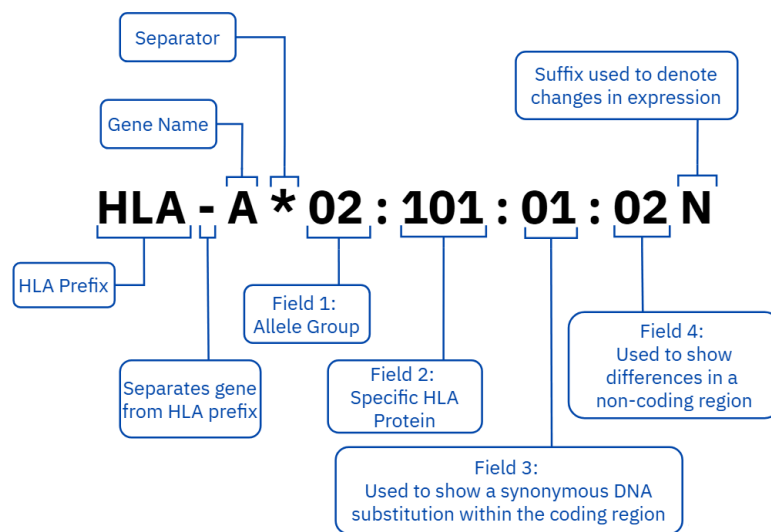


Figure 56: Nomenclature convention of HLA alleles. (Marsh et al., 2010)