Plasminogen Activator Inhibitor-2

A Spontaneously Polymerising Serpin in a Preeclampsia Perspective

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Preface and Acknowledgements

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Abstact

Approximately 2-5% of all pregnancies are affected by preeclampsia, the cause of which remains unknown. Patients with established preeclampsia have decreased Plasminogen Activator Inhibitor-2 (PAI-2) plasma levels, indicating that PAI-2 might have a role in the development of preeclampsia, or that the decrease in PAI-2 levels might be a result of preeclampsia. PAI-2 has the ability to spontaneously form polymers, which could be relevant in a preeclampsia perspective. Thus, the possible role of PAI-2 in the etiology of preeclampsia needs to be investigated in full.

Therefore, the aim of this study was to develop experimental methods, models and tools to aid in the investigation of PAI-2 and its role in preeclampsia. These methods aimed to evolve on existing methods to induce polymerisation of PAI-2 and models to visualise the degree of polymer formation in samples. In addition, the aim herein was to generate and characterise Monoclonal Antibodies (mAbs), with the intent to establish an ELISA with the purpose of quantifying of the levels of PAI-2 in plasma samples from pregnant individuals.

In vitro models for polymerisation of PAI-2 were established. The polymerisation was induced chemically and thermally, using exposure to Guanidine Hydrochloride (GuHCl) or heat, respectively. These samples were then visualised using SDS-PAGE, native PAGE and western blotting techniques. Mouse mAbs towards PAI-2 were characterised and a new generation of rat mAbs against PAI-2 was generated and partially characterised. An ELISA based assay was established, with a mouse mAb as the capture antibody, and a pAb to detect captured PAI-2 from samples. This assay was used on plasma samples from preeclamptic patients and controls. Moreover, PAI-2 in pregnancy plasma samples was investigated, utilising magnetic DynaBeads^{TM} with a mouse mAb, to capture PAI-2 from the sample. The results of this were illustrated with western blotting techniques. Lastly, anti-PAI-2 mAbs were raised in rats, in an attempt to create high-affinity antibodies with different specificities, which might be applicable in further optimisation of ELISA and for the detection of PAI-2 polymers.

The results from *in vitro* polymerisation of PAI-2, induced chemically and and heat, showed polymerisation in the samples. Furthermore, PAI-2 in plasma samples showed intact, non-polymerised PAI-2 and polymerised PAI-2. The established ELISA for quantification of PAI-2 levels in plasma samples from preeclampsia patients compared with controls, showed detectable amounts of PAI-2. The rat mAbs were able to detect PAI-2, when used for western blotting applications. Furthermore, they could bind both intact and polymerised PAI-2, when PAI-2 samples were bound on an ELISA

plate. However, none of these antibodies seemed to have specificity towards only polymerised PAI-2.

The ELISA based assay established in this study remains to be validated. Additionally, rat mAbs must be characterised in their ability to recognise native, human PAI-2 from plasma samples. Future studies must be designed to investigate if there is a difference in the amount of PAI-2 polymers present in plasma from patients affected by preeclampsia.

Resumé

Omtrent 2-5% af alle graviditeter bliver ramt af præeklampsia hvortil årsagen ikke er kendt. Patienter med etableret præeklampsi har et nedsat koncentrationsniveau af Plasminogen Activator Inhibitor-2 (PAI-2) i plasma, hvilket indikerer at PAI-2 kan spille en rolle i udviklingen af præeklampsi, eller faldet i PAI-2 kan være et resultat af præeklampsi. PAI-2 har evnen til spontant at danne polymerer, hvilket kan være relevant i et præeklampsi perspektiv. Dermed må rollen af PAI-2 i ætiologien af præeklampsi undersøges fuldt ud.

Derfor var formålet med dette studie at udvikle metoder, modeller og værktøjer til at hjælpe undersøgelsen af PAI-2 og dens rolle i præeklampsi. Disse metoder stræbte efter at udvikle på metoder og værktøjer til at inducere polymeriseringen af PAI-2, og modeller til at visualisere graden af polymerdannelse i plasmaprøver fra gravide. Yderligere var formålet her at generere og karakterisere Monoklonale Antistoffer (mAbs), med hensigt på at etablere et ELISA med formål i at kunne kvantificere koncentrationsniveauet af PAI-2 i plasmaprøver fra gravide.

In vitro modeller til polymerisering af PAI-2 blev etableret. Polymerisering blev induceret henholdsvist kemisk og termisk, ved eksposering af PAI-2 prøver til Guanidin Hydrochlorid (GuHCl) eller varme. Disse prøver blev visualiseret ved hjælp af SDS-PAGE, nativ PAGE og western blot teknikker. Muse anti-PAI-2 mAbs blev karakteriseret, og en ny generation rotte anti-PAI-2 mAbs blev udviklet og delvist karakteriseret. Et ELISA baseret assay blev etableret, med et muse mAb som det primære antistof, og et Polyklonalt Antistof (pAb) til detektion af det bundne PAI-2. Dette assay blev brugt til at måle PAI-2 koncentrationsniveau i plasmaprøver fra patienter med præeklampsi og kontroller. Endvidere blev PAI-2 i plasmaprøver fra gravide undersøgt, ved brug af magnetiske DynaBeads^{TM} med et muse mAb til at binde PAI-2 i prøven. Resultat af dette blev illustreret med western blotting teknikker. Endelig blev anti-PAI-2 mAbs produceret i rotter, i et forsøg på at danne antistoffer med høj affinitet og forskellige specificiteter, der formentlig kan være brugbare i yderligere optimering af ELISA og for detektion af polymeriseret PAI-2.

Resultaterne af *in vitro* polymerisering af PAI-2, induceret kemisk eller termisk, gav polymerisering i prøverne. Ydermere, viste PAI-2 fra plasmaprøver både intakt, ikke polymeriseret PAI-2, samt PAI-2-polymerer. Det etablerede ELISA til kvantificering af PAI-2 niveauer i plasmaprøver fra præeklampsi patienter og kontroller, viste målbare koncentrationsniveauer PAI-2. Rotte mAbs kunne detektere PAI-2 ved brug i western blotting. Endvidere kunne de detektere både intakt og polymeris-

eret PAI-2, når PAI-2-prøver var bundet til ELISA plader. Imidlertid fremstod ingen af antistofferne til at have specificitet alene mod polymeriseret PAI-2

Det ELISA baseret assay etableret i dette studie kræver validering. Ydermere bør rotte mAbs videre karakteriseres i deres evne til at genkende nativt, humant PAI-2 fra plasmaprøver. Fremtidige studier bør designes til at undersøge om der findes en forskel i mængden af PAI-2 polymerer i plasma fra patienter påvirket af præeklampsi.

Contents

Abbreviations

BK	Bradykinin
BM	Basement Membrane
BP	Blood Pressure
C1INH	C1-Inhibitor
CAS	Contact Activation System
cDNA	Complementary Deoxyribonucleic Acid
ConA	Concanavalin-A
DM-water	Demineralised Water
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FXI	Factor XI
FXII	Factor XII/Hageman Factor
GuHCl	Guanidine Hydrochloride
HEK	Human Embryonic Kidney Cell
HELLP-syndrome	Hemolysis, Elevated Liver enzymes, and Low Platelet count
HMWK	High-Molecular-Weight Kininogen
HRP	Horse-Raddish Peroxidase
HSA	Human Serum Albumin
IP	Immunoprecipitation
IUGR	Intrauterine Growth Restriction
kDa	Kilodalton
mAb	Monoclonal Antibody
mAbs	Monoclonal Antibodies
mRNA	messenger-Ribonucleic Acid
MW	Molecular Weight
OD	Optical Density
OPD	*o*-phenylenediamine Dihydrochloride
PA	Plasminogen Activator
pAb	Polyclonal Antibody
PAGE	Polyacryalamide Gel Electroforesis
PAI	Plasminogen Activator Inhibitor
PAI-1	Plasminogen Activator Inhibitor-1
PAI-2	Plasminogen Activator Inhibitor-2
PBS	Phosphate-Buffered Saline
PK	Plasma Kallikrein
PMNs	Polymorphonuclear Leukocytes
PPK	Plasma Prekallikrein
PVDF	Polyvinylidene Difluoride

rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
Serpin	Serine Protease Inhibitor
tPA	Tissue-type Plasminogen Activator
uPA	Urokinase Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor

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1 Introduction

Worldwide, approximately 2-5% of all pregnancies are affected by preeclampsia; a pregnancy complication that affects both the pregnant woman and the infant, and can lead to morbidities and mortality for both [1–3].
Preeclampsia is a multisystemic disorder, and the etiology of the disease is not fully understood. It is associated with a high rate of maternal and perinatal morbidity and mortality. It is the pregnancy complication with the highest mortality worldwide, and it is estimated that preeclampsia is the cause of mortality for 76,000 mothers and 50,000 infants every year [2–4].

Pregnancies affected by preeclampsia must be followed closely, as progression of the disease can be catastrophic. In some cases, even a mild case of preeclampsia can quickly develop into severe preeclampsia, characterised by an increase of the already raised blood pressure, and worsening of symptoms. Preeclampsia can also lead to the rare Hemolysis, Elevated Liver enzymes, and Low Platelet count (HELLP-syndrome) or even eclampsia [2–4].

The etiology of preeclampsia is not fully understood [2, 4], and an understanding of the causes of the disease is a prerequisite for the development of safe and effective interventions to ensure survival of both the mother and the infant, and to decrease the risk of poor outcomes for either. PAI-2 has been suggested to play a role in preeclampsia, as levels have been shown to be decreased in patients presenting with the disease [5]. PAI-2 is a spontaneously polymerising Serine Protease Inhibitor (Serpin), but the role of this in preeclampsia has not previously been investigated.

This study seeks to establish methods with the purpose of investigating PAI-2 and its function as a spontaneously polymerising Serpin in a preeclampsia perspective.

2 Background

2.1 Preeclampsia

Preeclampsia is defined by the occurrence of hypertension in previously normotensive women (Blood Pressure (BP) $\geq$ 140 mmHg systolic and/or $\geq$ 90 mmHg diastolic), accompanied with proteinuria, signs of stress on other organs (trombocytopenia, elevated liver enzymes and/or neurological complications, among others) [3, 6], after the 20th week of pregnancy. Previously, proteinuria has been a diagnostic criteria for this disease, however, in Denmark, preeclampsia can be diagnosed even in the absence of proteinuria [3]. Although research into this disease have been ongoing for many years, the pathology has still not been fully elucidated. There are many different theories as to why this disease affects pregnant women [4, 6].

The fact that the pathology of preeclampsia has been difficult to unravel, is likely due the multifactorial development caused by the imbalance of several physiological processes and factors. It is proposed that preeclampsia is due to the complex interactions between the genome of both the mother and the fetus, as well as the influence of external factors [7]. The placenta has been the central focus of the research into the disease, as the removal of it is the only curative intervention for preeclampsia [6, 7]. Impaired placentation caused by the defective establishment of the spiral arteries, which provide the blood flow to the placenta, increases the risk of ischemia and reperfusion damage to the placenta has been a central focus in preeclampsia research [6–8]. Impaired blood flow to the placenta can result in a further underdevelopment of the placenta, oxidative stress, ischemia, infarct, and necrosis in the placental tissue, and fetal malnutrition [6, 7]. However, placental insufficiency can be present in pregnancies not complicated by preeclampsia [5–7, 9]. This highlights the complexity of the disease, as the insufficient placenta previously was thought to be the central pathogenic factor of preeclampsia [6, 10, 11]. However, when the finding of incomplete spiral arteries and insufficient placenta, with infarcts and necrosis, is not a preeclampsia-specific find, there must be some other factors influencing the progression to preeclampsia [6, 7]. Based on this, the progression of preeclampsia is often explained in two stages; the inadequate placental perfusion, resulting in placental insufficiency, often referred to as the root cause of the disease, and the maternal syndrome of preeclampsia, characterised by multisystemic stress and symptoms [4, 12].

One theory in the research of preeclampsia, is the involvement of the fibrinolytic system. The

fibrinolytic system is involved in maintaining the thrombohemorrhagic balance, in the sense that it dissolves fibrin clots formed by coagulation, by complicated interactions between the coagulation system and the fibrinolytic system [13, 14].

During pregnancy, there is a notable decrease in fibrinolytic activity in the blood which returns to normal closely following delivery. This decrease has been shown to be partly due to a selective inhibition of uPA in pregnancy blood samples [15], and was later contributed to the placenta itself rather than from pregnancy plasma [16]. Thus, it is well established that an inhibitor of fibrinolysis is present during pregnancy [15–17].

This Plasminogen Activator Inhibitor (PAI) was first isolated and described from placental extracts in 1985 by Åstedt and colleagues [18]. Herein, the later named PAI-2 was isolated and described as a protein of approximately 47 kDa, which can form 1:1 complexes with uPA and tPA [18]. PAI-2 is produced by the trophoblastic cells of the placenta, and is only found in measurable levels in plasma during pregnancy (up to 200-300 ng/mL) [5, 19–21]). Since its discovery, multiple groups have investigated the role of PAI-2 in the homeostasis of pregnancy. Some of these studies have linked PAI-2 to preeclampsia, since the levels of PAI-2 in plasma samples from preeclamptic pregnancies have been shown to be lower than those observed in healthy pregnancies [9, 22–27]. Therefore, PAI-2 has been suggested as a candidate for an early biomarker to identify pregnancies that will later be affected by preeclampsia [5, 28]. However, there is some level of ambiguity in the results regarding the role of PAI-2 in preeclampsia patients [5, 9, 20, 22–34], indicating a need for methods to gain a deeper understanding of the relationship between PAI-2 and preeclampsia.

To gain a better perspective on PAI-2, the structure and functions of PAI-2 will be elucidated in the following.

2.2 Plasminogen-Activator Inhibitor Type 2 (PAI-2)

2.2.1 The Fibrinolytic System

The fibrinolytic system is responsible for the lysis of fibrin clots, generated by the coagulation system [13]. Plasmin is a serine proteinase responsible for degradation of fibrin clot formation in the blood, to ensure the prevention of clot formations, and for remodelling after an injury. It is generated as a result of the cleavage of plasminogen, which activates the zymogen into the active plasmin. Aside from

being important for the maintenance of vascular homeostasis, plasmin also plays a role in Extracellular Matrix (ECM) remodelling, and wound healing, among others. The activity of plasmin is closely regulated by Plasminogen Activator (PA)s and Plasminogen Activator Inhibitor (PAI)s, and the enzymatic system responsible for this, is often referred to as the fibrinolytic system or the plasminogen activation system [35–37]. An overview of this system is presented in figure 2.1.

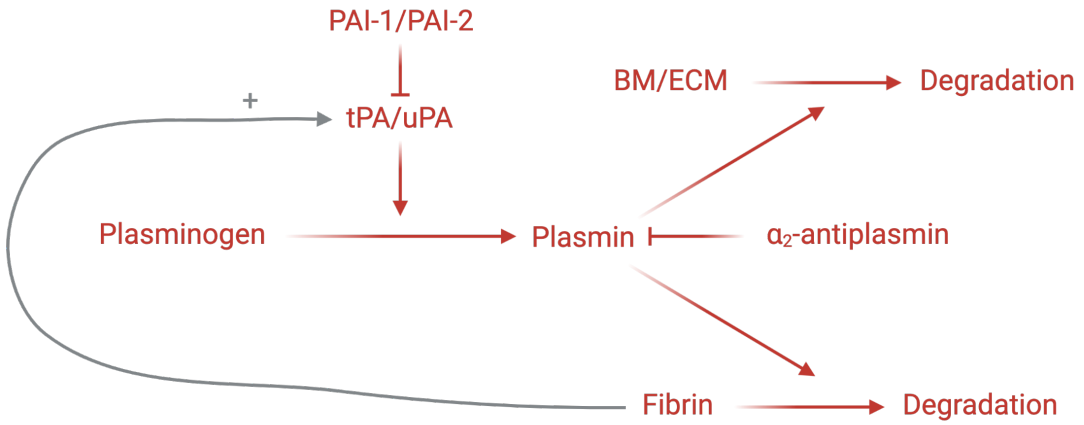


Figure 2.1: Schematic overview of the Fibrinolytic system/Plasminogen Activation System.
The zymogen plasminogen is converted into the active plasmin by tPA, or uPA; alone or in complex with uPAR. This activation can be inhibited by either PAI-1 or PAI-2. When activated, plasmin can degrade fibrin into soluble fibrin degradation products, and is also capable of degradation of BM and ECM components for remodelling.
Abbriviations: Plasminogen Activator Inhibitor-1 (PAI-1), Plasminogen Activator Inhibitor-2 (PAI-2), Tissue-type Plasminogen Activator (tPA), Urokinase Plasminogen Activator (uPA), Basement Membrane (BM), Extracellular Matrix (ECM), Urokinase Plasminogen Activator Receptor (uPAR). Figure modified from [13]; created with BioRender.com.

For plasminogen to be activated into plasmin, it has to be cleaved by either Tissue-type Plasminogen Activator (tPA) or Urokinase Plasminogen Activator (uPA), both of which are serine proteases. tPA is the most prominent PA in plasma. It is secreted by endothelial cells into the vascular system as a single-chain enzyme, and is therefore thought to be the most important PA for inhibition of plasminogen vascularly [38]. It can be converted to its two-chain form by proteolytic cleavage of a peptide bond, facilitated by fibrin. The two-chain form of tPA has a higher enzymatic capacity than the single-chain form, and the activity of tPA is therefore significantly lower in the absence of fibrin [21, 37].

Like tPA, uPA is secreted as a single-chain enzyme. This is considered a pro-enzyme with little to no enzymatic activity that can be cleaved into a two-chain enzyme with a higher activity, catalysed by fibrin or other proteinases [37]. Hereby, there is a positive feedback loop involved in both tPA

and uPA facilitated plasminogen activation [21, 36, 37]. Specific to uPA, function is often linked to the membrane bound Urokinase Plasminogen Activator Receptor (uPAR), causing uPA dependent plasminogen activation to be concentrated near cell surfaces [36, 37].

There are two main Plasminogen Activator Inhibitor (PAI)s, PAI-1 and PAI-2, both of which belong to the Serine Protease Inhibitor (Serpin) family. PAI-1 is predominantly secreted from endothelial cells, and is found at relatively high concentrations in plasma. Therefore, PAI-1 is presumably the predominant inhibitor of plasminogen activators intravascularly [38]. The physiological function of PAI-2 has not been fully established [39]. The focus of this study is PAI-2, the form and function of which will be further investigated in the following. A deeper review of PAI-1 is beyond the scope of this study.

2.2.2 The Discovery of PAI-2

As described in section 2.1, a pregnancy associated PAI was first isolated from placental extracts, and described by Åstedt and colleagues in 1985. Herein, it was described as a protein of approximately 47 kDa, which can inhibit uPA and tPA, by the formation of 1:1 inhibitory complexes [18].

The previous year, an inhibitor of fibrinolysis, capable of forming complexes with uPA, was isolated from the monocyte-like lymphoma cell line U-937 [40]. This was later characterised as a PAI, later recognised to be an effective inhibitor of uPA and two-chain tPA, and a less effective inhibitor of one-chain tPA [41]. The PAI from U-937 cells was found to be different than the one isolated earlier from endothelial cells (PAI-1) [42], with regard to Molecular Weight (MW) (52 kDa PAI-1; 47 kDa PAI-2), binding to ConA Sepharose (PAI-1 binds to ConA Sepharose, while PAI-2 does not) and immunogenicity (PAI-2 is not bound by antibodies raised against PAI-1). The newfound PAI was also compared to the one previously isolated from placental extracts [18], and they were found to be identical with regards to structure, functionality, and immunogenicity [41].

Later, both types of PAIs were found to be present in the placenta, and the notable decrease in fibrinolytic activity described during pregnancy was found to be a result of an increased amount of both PAIs. Because of this, the previously named endothelial-type and placental-type PAIs were renamed PAI-1 and PAI-2, respectively [35, 43–45].

2.2.3 PAI-2 - Structure and Function

PAI-2 exists in two well characterised forms, estimated to 47 kDa and 60 kDa, respectively [41, 45, 46], both of which have been shown to be transcribed from a single *pai-2* mRNA stand [47].

Initially, only the 47 kDa PAI-2 was described. However, a molecular heterogeneity of PAI-2 was discovered, corresponding to the location of the molecule, where the 47 kDa form has been shown to remain intracellularly, while the 60 kDa form has been shown to be related to the secretory pathway and is the main PAI-2 form found extracellularly [45, 46]. The heterogeneity has been contributed to a glycosylation of the molecule. This was initially studied by passing a mixture of the two forms over a ConA sepharose column, which binds carbohydrates. Only the 47 kDa form passed freely over the column, while the 60 kDa PAI-2 was retained by the column [45].
Isolated cDNA for PAI-2 predicts a protein of 415 amino acids, with the predicted MW of 46.543, closely corresponding to the 47 kDa observed with gel electrophoresis [35, 45, 48]. With this predicted amino acid sequence, there is three potential N-linked glycosylation sites (asn-75, asn-115, and asn-339), all three of which have been shown to be employed after glycosylation [35, 45, 49]. The 47 kDa and the 60 kDa form have been found to be identical with regards to function and immunology [45, 50].

The molecular structure of PAI-2 is similar to that of other Serpins; it is made up of nine helices, three β-sheets and its reactive centre loop is supported by the primary α-β-sheet. Furthermore, it has a long CD-loop, that connects helices C and D [51–53]. Even though it is close to PAI-1 in function, in structure the closest relative of PAI-2 is chicken ovalbumin [52].

Another characteristic of PAI-2 is the seemingly inefficient secretion. The protein does not have a N-terminal secretion signal that is cleaved after processing in the endoplasmatic reticulum [49]. Instead, the secretion signal of PAI-2 is internal and remains uncleaved after processing [54]. Investigations of the secretion of PAI-2 revealed an inefficient secretion, where most PAI-2 remained in the cytosol, even when the cells performed *de novo* synthesis of the protein [45, 46, 54].

PAI-2 has the the ability to spontaneously form polymers [46, 51, 55]. The formation of polymers occurs when PAI-2 is exposed to denaturing conditions, such as after treatment with Guanidine Hydrochloride (GuHCl) [46], but the polymers also form spontaneously *in vitro* [46, 51, 55]. PAI-2 belongs to the family of Serine Protease Inhibitors, or Serpins for short [41]. One feature of the proteins belonging to this family, is the mobility of their reactive centre loop that is required for their

inhibitory activity, and, as a result of which, most of the Serpins can enter an array of conformations, for example locked-latent states and loop-sheet polymers [51, 55, 56]. It has been confirmed that both the 47 kDa and the 60 kDa form of PAI-2 can form polymers by a loop-sheet mechanism, and that this happens in cells that naturally express PAI-2 at physiological expression levels [55]. Furthermore, it has been shown that intracellular PAI-2 is almost completely on singular form, while the PAI-2 associated with the endoplasmatic reticulum (i.e. in the secretory pathway) largely is polymerised [51, 55]. The unique ability of PAI-2 to change molecular conformations, allows for the spontaneous polymer-formations. This function is modulated by a redox-sensitive molecular switch that changes the formation of PAI-2 between a monomeric form and a polymerogenic conformation that can spontaneously form polymers with nearby polymerogenic PAI-2 molecules [51]. The stable monomeric form exists primarily under reducing conditions (i.e. in the cytoplasm), but under oxidative conditions (i.e. in the secretory pathway and extracellularly), a disulfide bond can be formed, resulting in a conversion from the monomeric form to the polymerogenic form with an open A-β-sheet. The polymerogenic PAI-2 can then spontaneously form loop-sheet polymers with nearby polymerogenic PAI-2 molecules [51, 57].

An illustration of these interconvertible conformations of PAI-2, modified from [51], is provided in figure 2.2.

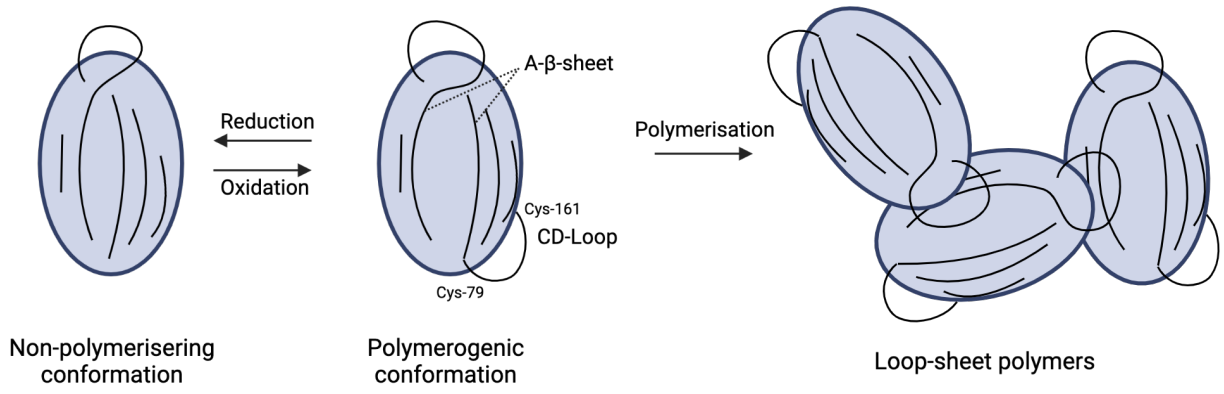


Figure 2.2: Illustration of the three interconvertible conformations of PAI-2.
Non-polymerising conformation: When PAI-2 is in a reducing environment where disulfide bonds cannot be established. Polymerogenic conformation: In oxidative environments, PAI-2 contains a disulfide bond, that opens the A-β-sheet allowing for PAI-2 to readily form loop-sheet polymers with nearby polymerogenic PAI-2 proteins. *Modified from [51]. Created with Biorender.com*

Both the monomeric and the polymerogenic forms are inhibitory active, but when polymerised, PAI-2 becomes inactive [51, 55]. This polymerisation might influence the secretion efficiency of PAI-2, as cells with lower expression rates have a higher secretion rate, while cells with higher expression rates have a lower secretion rate with a higher percentage of polymerised PAI-2 near the secretory pathway [51, 55].

Although the structure of PAI-2 is well described, the physiological function of this Serpin is yet to be determined. Throughout years of research, the ability of PAI-2 to inhibit the PAs, and especially uPA, have been confirmed *in vitro* [41, 46]. However, since the levels of PAI-2 are below detection levels in plasma under normal conditions, and because PAI-1 is readily available in plasma, it is highly unlikely for PAI-2 to have a crucial role in the maintenance of homeostasis under normal physiological conditions [39].

Due to a large amount of produced PAI-2 remaining intracellularly, there has been a lot of focus on finding the intracellular function of PAI-2. One of the proposed functions of PAI-2, is a role in apoptosis [39]. However, the results of studies on the role of PAI-2 in apoptosis are inconsistent [39, 58], as some indicate promotion of apoptosis [59], some indicate suppression of apoptosis [60, 61], and others show no connection [62].

Because PAI-2 has been found in different types of Polymorphonuclear Leukocytes, its role in inflammatory reactions has been investigated. Recently, it was shown that PAI-2 in macrophages inhibits migration, perhaps facilitated by uPA inhibition, *in vitro* [58].

Since the levels of PAI-2 increases dramatically during pregnancy, reaching levels up to 300 ng/mL during the last trimester [21], it has been postulated to play a role for the maintenance of homeostasis during pregnancy. However, except for the before mentioned rise of uPA specific decrease in fibrinolytic activity, this role is yet to be characterised *in vivo* [39].

At the time of writing, no literature describing PAI-2 as a pathogenic factor in any diseases has been located. Correspondingly, there are currently no therapies targeting PAI-2. The current clinical implications of PAI-2 are reviewed in the following.

2.2.4 PAI-2 - Clinical Implications

The tendency of other Serpins to form polymers, has been shown to be involved in pathological processes. For example, a mutation in the gene encoding for α_1-antitrypsin causes the protein to be more prone to form polymers, even under physiological conditions. This results in the accumulation of α_1-antitrypsin in the endoplasmatic reticulum in hepatocytes, which can cause hepatocellular damage, while the circulating α_1-antitrypsin can predispose to emphysema [55, 63–66]. Another example is in the case of hereditary angioedema, where mutations in the *SERPING1* gene encoding for C1-Inhibitor (C1INH) promotes a high rate of polymerisation of C1INH. This results in decreased levels of functional C1INH in plasma, ultimately causing recurrent attacks of angioedema [67–69].

At the time of writing, there is no evidence that the polymerisation of PAI-2 is involved in any pathological conditions [39]. Several studies have investigated PAI-2 in pregnancy, and specifically in preeclampsia. These generally comment on a correlation between decreased levels of plasma PAI-2 in pregnancy, and a higher incidence of preeclampsia [24–26, 70]. No literature, commenting on the polymerisation status of PAI-2 in either healthy or complicated pregnancies is available at the time of writing.

Due to the ability of PAI-2 to readily form polymers, especially during oxidative conditions, it is plausible to think this polymerisation could be involved in the pathogenesis of preeclampsia. As polymerised PAI-2 is not enzymatically active [51, 55], an increased amount of polymerised PAI-2 in a developing pregnancy, might interfere with the development of the placenta. Significantly, preeclampsia is characterised by increased oxidative stress in the placenta [6, 7], and since polymerisation of PAI-2 is dependent on oxidative conditions [51], there is a good chance that polymerisation of PAI-2 occurs in the preeclamptic placenta and/or systemically in the preeclamptic pregnancy. This might be of significance independently, or it could interact with other factors. For instance, it has been shown previously that misfolded/polymerised proteins can activate the contact system [69, 71].

In the following, the contact activation system and the effect of misfolded proteins will be briefly elucidated.

2.3 The Contact Activation System

The Contact Activation System (CAS) is comprised of the intrinsic coagulation pathway, but also the kallikrein-kinin system. The four proteins involved in CAS are Factor XII/Hageman Factor (FXII), Plasma Kallikrein (PK), Factor XI (FXI) and High-Molecular-Weight Kininogen (HMWK) [72, 73]. A brief overview of the factors comprising the CAS, is shown in figure 2.3.

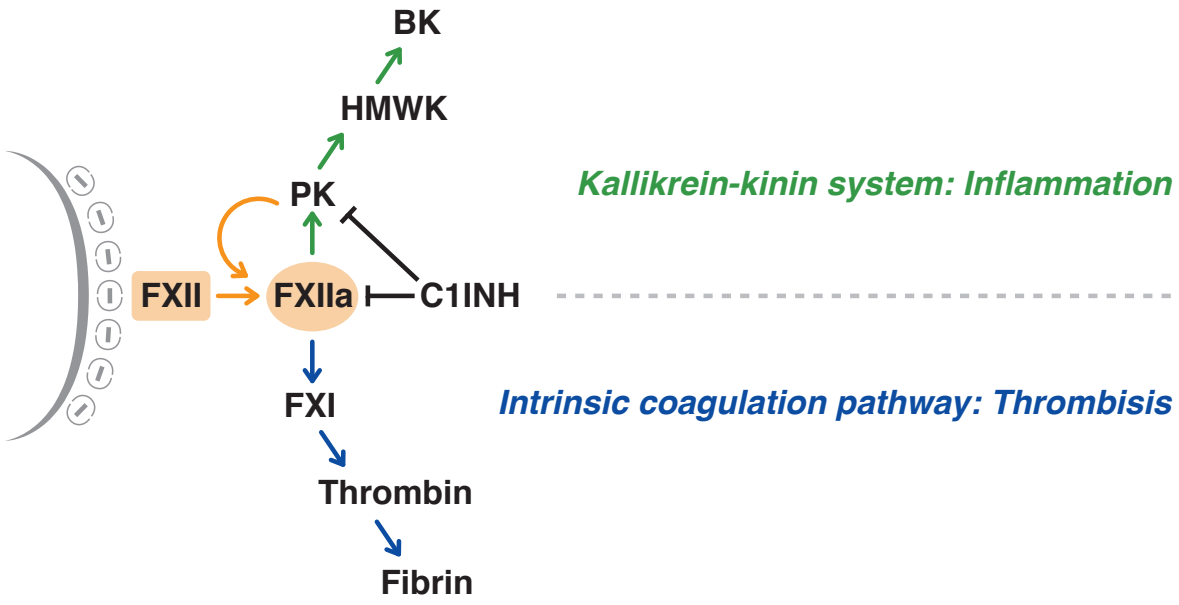


Figure 2.3: Brief overview of the Contact Activation System (CAS).
Exposure to negatively charged surfaces induce activation of Factor XII/Hageman Factor (FXII) to FXIIa. FXIIa can then activate Plasma Kallikrein (PK) that will cleave High-Molecular-Weight Kininogen (HMWK), activating Bradykinin (BK). This is the kallikrien-kinin system. Furthermore, FXIIa can activate Factor XI (FXI), inducing the intrinsic coagulation pathway. *This figure has been modified from [72].*

Contact activation is initiated by the exposure of a negatively charged surface to plasma. FXII will bind this surface, inducing a conformational change, activating FXII into FXIIa. Plasma Prekallikrein (PPK) will subsequently be activated by FXIIa into PK that will in turn cleave HMWK. PK will furthermore induce an amplification loop with activation of additional FXII [73]. Activation of FXII can also lead to the activation of FXI, which in turn would promote coagulation [72], while the activation of HMWK activates Bradykinin (BK), promoting inflammation [72].

One way that PAI-2 polymers could be involved in the pathogenesis of preeclampsia, is by activation of the contact activation system, which in turn affects the fibrinolytic system. Previously, studies

have found that misfolded proteins can activate the CAS and lead to kallikrein formation [71]. It is proposed that this ability of CAS is designed to initiate an immediate inflammatory reaction to the pathologically misfolded proteins, to ensure clearance [71]. An overview of this proposed activation of CAS and its effects on the fibrinolytic system is depicted in figure 2.4.

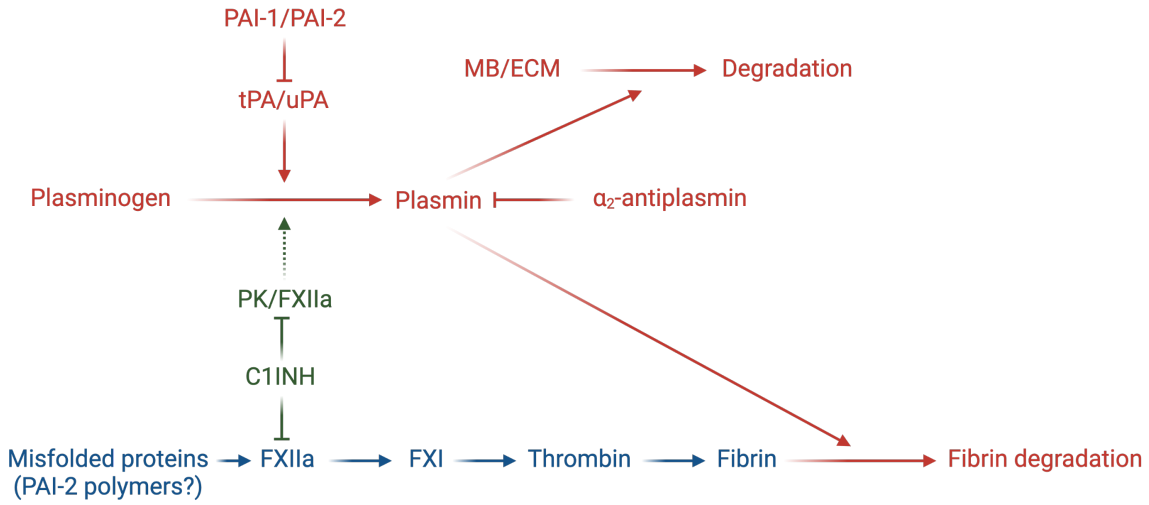


Figure 2.4: Schematic overview of CAS activation and the effect on the fibrinolytic system. Misfolded proteins activate FXII, resulting in activation of thrombin and formation of fibrin. Furthermore, FXIIa and PK can also activate plasminogen into plasmin, with low efficacy. The factors and processes illustrated in red, are those considered a part of the fibrinolytic system. The blue and green factors are all considered part of CAS. The green illustrates the kallikrein-kinin system, while the blue illustrates the intrinsic coagulation pathway. *Abbreviations: Plasminogen Activator Inhibitor-1 (PAI-1), Plasminogen Activator Inhibitor-2 (PAI-2), Tissue-type Plasminogen Activator (tPA), Urokinase Plasminogen Activator (uPA), Basement Membrane (BM), Extracellular Matrix (ECM), Plasma Kallikrein (PK), Factor XII/Hageman Factor (FXII), C1-Inhibitor (C1INH), Factor XI (FXI). This figure is modified from figure 2.1, Created with Biorender.com*

One proposed example of this is the involvement of C1-inhibitor polymers in the initiation of the pathological pathway that induces an angioedema episode in patients suffering from hereditary angioedema. C1-inhibitor polymers have been shown to mediate FXII activation *in vitro*, resulting in activation of the kallikrein-kinin system [69]. Thus, the mutations in C1-inhibitor that results in a protein more inclined to form polymers, not only result in loss-of-function of C1-inhibitor, but may also result in gain-of-function [69]. Currently, no available literature investigates the involvement of CAS activation by PAI-2 polymers in a preeclampsia perspective.

3 Aim of Study

The pathology of preeclampsia is yet to be fully elucidated. As described, there is evidence that PAI-2 levels are affected in cases of preeclampsia. This is mainly seen as a decrease in the levels of PAI-2 antigen, detected using ELISA based assays. This indicates that PAI-2 might have a role in the development of PAI-2, or the decrease in PAI-2 might be a result of development of preeclampsia. Whether PAI-2 is involved in the pathogenic processes that lead to the development of preeclampsia, must be established. Furthermore, the characteristic of PAI-2 to spontaneously form polymers could be relevant in a preeclampsia perspective. Thus, the possible role of PAI-2 in the pathology of preeclampsia needs to be investigated in full.

Based on this, the aim of this study was to establish valid experimental tools and methods for the purpose of investigating PAI-2. These methods aimed to evolve on methods to induce polymerisation of PAI-2 and models to visualise the degree of polymer formation in samples. Furthermore, to generate and characterise Monoclonal Antibodies (mAbs) with the intend to establish an ELISA based assay with the purpose of quantification of the levels of PAI-2 in plasma samples from pregnant individuals.

4 Methods and Materials

4.1 Buffers and Solutions

The buffers and solutions used for experiments and analyses in connection with this study are summarised in table 4.1

PBS	1.45 mM NaH_2PO_4, 6.46 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, in ultrapure water, pH 7.4
SDS-PAGE Electrophoresis Buffer	NuPAGE® MES SDS running buffer (20X) (Novex®, NP0002) diluted into a 1X concentration with DM-water. Prepared in advance and stored at °C
SDS-PAGE sample buffer	NuPAGE® LDS Sample Buffer (4X) (Invitrogen®, NP0007) diluted to a 1X concentration in samples and ultrapure water immediately before boiling
SDS-PAGE reducing sample buffer	NuPAGE® LDS Sample Buffer (4X) (Invitrogen®, NP0007) + 10% β-mercaptoethanol was diluted to a 1X concentration in samples and ultrapure water immediately before boiling, resulting in an end-concentration of 2.5% β-mercaptoethanol in samples
Native PAGE Electrophoresis Buffer	10X TG Buffer (Bio-rad, 161-0734) diluted to a 1X working concentration in ultrapure water. Prepared in advance at stored at 4°C
Wash Buffer	1X PBS with 0.05% Tween® 20 Surfact-Amps® Detergent Solution (Thermofisher, 85113)
Western Blot Blocking Buffer	1X PBS with 0.05% Tween™ 20 Surfact-Amps™ Detergent Solution (Thermofisher, 85113) and 2.5% (w/v) skimmed milk powder (Fluka Analytical, 70166)
Western Blot Developer	0.04% 3-amino-9-ethylcarbazole and 0.015% H_2O_2 in 50 mM sodium acetate buffer, pH 5.0
IP Buffer	1X PBS with 0.01% HSA (Statens Seruminstitut, Copenhagen, Denmark, batch no. 203300)
Elution Buffer	0.5% (w/v) Citric acid (Sigma-Aldrich®, C0759) in ultrapure water (sterile filtrated)

Table 4.1: Table displaying all buffers and working solutions used for present study.

4.2 Acquisition of PAI-2

PAI-2 was supplied by the Institute of Molecular Medicine, University of Southern Denmark. It was expressed in, and purified from, HEK 293 cells (Human Embryonic Kidney Cells) using the Expi293^{TM} Expression System Kit (Gibco^{TM}, A14635). The expression vector PAI-2-Tyr-Pho-Sig plasmid pcDNA 3.4 was used, consisting of the *pai-2* gene with an additional standard signal peptide to ensure secretion of translated PAI-2, as well as an C terminal hexa-His sequence, to allow for purification of the expressed protein.

4.3 Mouse Monoclonal Antibodies

The mouse Monoclonal Antibodies (mAbs) used in this project were all manufactured at the Institute for Molecular Medicine, Southern University of Denmark, from a mouse immunised with recombinant human PAI-2 (e. coli derived) (R&D Biosystems®, 9206-PI-025). These antibodies were denoted 19-26-X, with X indicating the number of the specific clone.

Mouse mAb were purified from culture supernatant, using protein A columns that specifically binds the Fc region of IgG [74]. Prepacked protein A columns were stored at 4°C in a 20% ethanol solution. Prior to purification, the culture supernatants were supplemented with 0.5 mol/L NaCl, and centrifuged on 7000 RCF for 15 minutes. Subsequently, the supernatants were passed through a pre-filter (Satorius, 13400-47-Q) and afterwards a 0.45μm sterile filter (Advantec, C045A047A). The columns were rinsed with a full column volume of PBS, after which the culture supernatant was run over the column. Subsequently, the column was washed with PBS. After this last wash step, the mAbs, now bound to the protein A in the columns, were eluted with Elution Buffer (table 4.1) and the eluate was collected. Prior to further analyses of the antibodies, they were dialysed overnight in Spectra/Por molecularporous membrane tubing (Spectrum Laboratories Inc., lot no. 3318105) against 500-1800 mL/sample PBS.

Prior to the beginning of this project, mouse mAbs were tested for application in western blotting. A combination of the mouse mAb 19-26-2 and 19-26-11 was shown to give excellent resolution of protein bands with little background, thus these have been used for initial analyses of PAI-2 and polymerisation in this project.

4.4 Polymerisation of PAI-2

4.4.1 Chemically Induced Polymerisation of PAI-2

For chemical induction of polymerisation, PAI-2 was diluted to an end-concentration of 100 μg/mL, and aliquoted to Eppendorf tubes, each treated with a concentration of Guanidium Chloride (Sigma #BCBF0939V) ranging between 2M-0M, and incubated at 4°C in an end-over-end rotator for one hour. Following incubation, the samples were dialysed using Spectra/Por molecularporous membrane tubing (Spectrum Laboratories Inc., lot no. 3318105) against 500-1800 mL/sample PBS at 4°C overnight. The samples were retrieved and stored in a -18°C freezer.

4.4.2 Thermally Induced Polymerisation of PAI-2

For heat induced polymerisation, PAI-2 was diluted to a concentration of 100 μg/mL in ultrapure water, and aliquoted to Eppendorf tubes that were then incubated for 10 minutes to one hour at 63°C and 37°C. One sample was moved directly to a -18°C freezer for control. The samples were retrieved and moved to a -18°C freezer after their incubation period.

4.4.3 Characterisations of PAI-2 and Polymerisation

To confirm polymerisation, all samples were evaluated using Sodium Dodecyl Sulfate (SDS)- Polyacryalamide Gel Electroforesis (PAGE) and native PAGE. The gels were subsequently stained or used for western blotting. The procedures are described in the following.

4.4.3.1 SDS-PAGE of PAI-2 Samples

SDS-PAGE samples were diluted 1:4 in MilliQ-water and SDS-PAGE Sample Buffer (table 4.1), also diluted 1:4 in the total solution. For reducing sample conditions, the sample buffer was substituted with SDS-PAGE Reducing Sample Buffer (table 4.1). All samples were heated in a thermomixer at 80°C and 400 rpm for 10 minutes. Samples were then loaded into Bolt™ 4-12% Bis-Tris Plus gels (Invitrogen, #NW04127BOX) inserted into an Invitrogen Novex® Mini-cell electrophoresis chamber, filled with SDS-PAGE Electrophoresis Buffer (table 4.1). For gels bound for Coomassie staining, Novex™ Sharp Unstained Protein Standard (Invitrogen™, LC5801) was loaded into one well for

Molecular Weight (MW) estimation. Similarly, for gels bound for protein transfer to a PVDF membrane for western blotting, Novex™ Sharp Pre-stained Protein Standard (Invitrogen™, LC5800) was used for MW estimation. Electrophoresis was run using a XCell SureLock™ power-supply lid for 40-65 minutes at 180 V.

After electrophoresis, the gel was removed from the cassette, placed in a single-use plastic tray and rinsed briefly in DM-water. For Coomassie staining, SimplyBlue™ SafeStain (Thermofisher, LC6065) was added to cover the gel. The gel with SimplyBlue was microwaved on high for 30-60 seconds (until the solution was nearly boiling), and the gel with the warm SimplyBlue was incubated for approximately one hour. Hereafter, the SimplyBlue was replaced with DM-water, and the gel was left to destain overnight, before pictures were captured.

Some of the SDS-PAGE gels were not stained, but transferred to membranes and used for western blotting (see section 4.4.3.3.)

4.4.3.2 Native PAGE of PAI-2 Samples

Samples were prepared on ice with NativePAGE™ Sample Buffer (4X). Samples were diluted 1:10 in PBS, and sample buffer was diluted to a 1X concentration. All following steps were performed in a cold room. Subsequently to sample preparation, a NativePAGE™ 4-16% Bis-Tris Gel (Invitrogen™, BN1002BOX) was loaded into an Invitrogen™ Novex® Mini-cell Electrophoresis Chamber and the wells were flushed through with Native PAGE Electrophoresis Buffer (table 4.1). The electrophoresis chamber was filled with Native PAGE Electrophoresis Buffer, and the samples were loaded into the wells. After loading, the electrophoresis was run at 200 V for 4-7 hours. Thereafter, the gel was placed in a single-use plastic tray in DM-water. The gel was then either stained, using Pierce™ Silver Stain Kit according to manufacturer's instructions [75], or used for western blotting (see section 4.4.3.3).

4.4.3.3 Western Blotting

Transfer of SDS-PAGE gels was accomplished using the Trans-Blot® Turbo™ Transfer System (Bio-rad) with the Trans-Blot® Turbo™ consumables, using the pre-set setting, Mixed Molecular Weight proteins (7 min, 1.3 A constant; up to 25 V).

Native PAGE gels were transferred using the same system and consumables on a user-defined setting (30 min, 25 V constant; up to 2.4 A).

After protein transfer, all membranes were treated the same; they were transferred to a single-use plastic tray, which was filled with Western Blot Blocking Buffer (table 4.1) and incubated in a cold room with agitation overnight.

Following blocking, the two mouse mAbs 19-26-2 and 19-26-11 were added to the trays at a concentration of 2 μg/mL in Wash Buffer (table 4.1), and incubated with agitation for one hour at room temperature. The membranes were rinsed thoroughly in wash buffer and left to wash for five minutes. A secondary rabbit-anti-mouse antibody (Sigma-Aldrich, product# A9044), diluted 1:10.000 in wash buffer, was added to the membranes which was left to incubate for 30 minutes with agitation at room temperature. Following incubation, the membranes were rinsed thoroughly in wash buffer, left to wash in wash buffer while the developer was prepared, and rinsed in briefly DM-water before developing. To develop the protein bands on the membrane, a Western Blot Developer (table 4.1) was prepared and added to the membrane. The membrane was closely observed and the reaction was stopped after protein bands appeared with a satisfying intensity without excessive background.

4.5 ELISA

An illustration of the ELISA established in this study can be seen in figure 4.1.

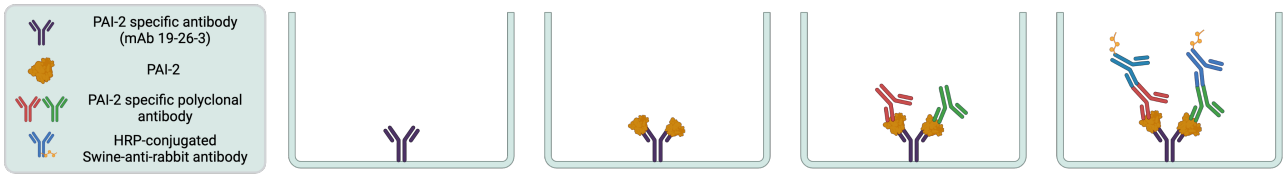


Figure 4.1: Illustration of the ELISA assay established in this study.
ELISA plates were coated with mouse mAb 19-26-3 overnight. When samples were added, mAb 19-26-3 captures PAI-2 present in the sample. A polyclonal rabbit anti-human-PAI-2 antibody (antibodies-online.com) detect PAI-2 captured by mAb 19-26-3. Lastly, a HRP conjugated swine-anti-rabbit antibody was added, and detects the Fc portion of the polyclonal antibody. A substrate of HRP is added (TMB-Ultra), providing a chemiluminescent signal that can be quantified with an ELISA plate reader. *Abbreviations: Plasminogen Activator Inhibitor-2 (PAI-2), Horse-Raddish Peroxidase (HRP), Enzyme-linked Immunosorbent Assay (ELISA), Monoclonal Antibody (mAb). Figure created with BioRender.com.*

MaxiSorp plates (Nunc^{TM} Maxisorp^{TM}, Roskilde Denmark) were coated with 5 μg/mL mAb 19-26-3 diluted in 1X PBS overnight stored at 4°C. All following incubation steps were performed with constant agitation at room temperature. The plates were washed thrice in Wash Buffer (table 4.1), and incubated for one hour in wash buffer. Subsequently, plasma was added 1:10, diluted in wash

buffer with 50 mM EDTA. Plates with plasma samples were incubated for one hour, after which the plates were washed thrice. The pAb (antibodies-online.com, ABIN349627) was diluted to a concentration of 2 μg/mL in wash buffer, added to the plates and incubated for one hour. The plates were washed thrice, and HRP-conjugated swine-anti-rabbit (Dako A/S, P0217) was added to the plates, diluted 1:1000 in wash buffer. After incubation for 30 minutes, the plates were washed thrice and developed for 10 minutes with TMB Ultra (Thermo Scientific^{TM} 1-Step^{TM} Ultra TMB-ELISA Substrate Solution, 34029). The reaction was stopped with 100μL 200 mM H_2SO_4 and the plates were read at 450 nm.

This ELISA was used to quantify the concentration of PAI-2 in plasma samples from 10 preeclampsia patients and 10 healthy, matched controls. These samples were obtained from an ongoing PhD project, where larger cohorts of preeclampsia patients and control plasma samples are currently being collected. All samples were collected at the third trimester of pregnancy, prior to the initiation of preeclampsia treatment. Control samples were collected in a comparable gestation week. A standard curve was established using a serial dilution of PAI-2 purified from expression in HEKs, with a known starting concentration of 1 μg/mL. All samples and the standards were added in technical duplicates. PAI-2 concentrations were interpolated using the standard curve constructed and the software Softmax Pro® (see appendix A for standard curve).

4.6 Immunoprecipitation

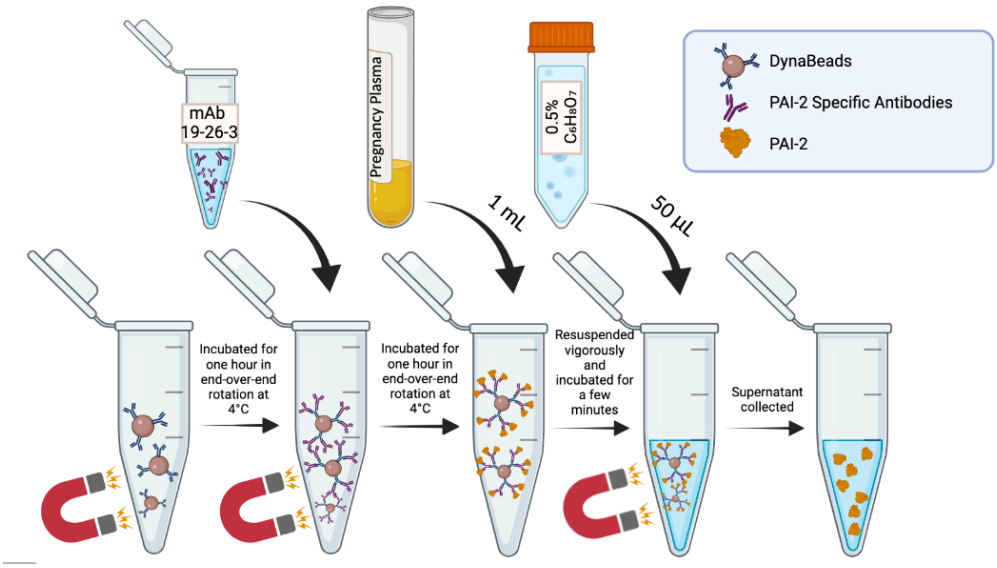


Figure 4.2: Brief summery of the method of immunoprecipitation of PAI-2 from pregnancy plasma. DynaBeads™ were added to Eppendorf tubes placed in a magnetic rack, and the beads were washed thrice. Hereafter, mAb 19-26-3 was added to the beads and the mixture was incubated for one hour in an end-over-end rotator at 4°C. Subsequently, the beads were washed thrice, and pregnancy plasma was added. The tubes were incubated for one hour in an end-over-end rotator at 4°C. The beads were again washed thrice. Elution was performed by adding 0.5% citric acid and resuspending vigorously. The mix was incubated briefly, after which the tubes were placed back on the magnet and the supernatant was collected and used for further analyses. Refer to the legend in the top right corner of the figure for symbol description. *Abbreviations: Monoclonal Antibody (mAb), Plasminogen Activator Inhibitor-2 (PAI-2). Created with BioRender.com.*

Figure 4.2 briefly summarises the methods used to immunoprecipitate PAI-2 from pregnancy plasma. A detailed description is given in the following.

DynaBeads™ M-280 Sheep Anti-Mouse IgG (Invitrogen™, 11202D) were washed thrice with IP Buffer (table 4.1). Antibodies were added in a 2 μg/mL concentration in IP buffer (table 4.1) and a volume of 1 mL to the beads, which were then incubated for one hour at 4°C in an end-over-end rotator. After the incubation period, the beads were washed thrice with IP buffer. Subsequently, samples were added to the beads. Samples of HEK expressed PAI-2 (used as positive controls) were diluted to a concentration of 5 μg/mL or 200 ng/mL in IP buffer, while plasma samples from men were diluted 2:1 in in IP buffer, for negative controls. Pregnancy plasma was added as samples diluted 2:1 in IP buffer.

The beads with the samples added were then placed in an end-over-end rotator at 4 °C for one hour.

Subsequently, the beads were washed thrice in IP buffer. After the last wash, the beads were carefully sucked dry of buffer, and 50 μL of 0.5 % citric acid (elution buffer, see section 4.1) was added to the beads. This mixture was carefully mixed by pipetting up and down until it appeared homogeneous. The beads were left off the magnet for approximately 2-3 minutes, after which they were placed back in the magnetic separation rack for 2-3 minutes, until the supernatant was clear and could be collected for further analysis.

The collected supernatants from immunoprecipitations were visualised using western blotting techniques. SDS-PAGE was performed as described in section 4.4.3.1, except the samples were diluted only in LDS Sample Buffer (4X) (Invitrogen®, NP0007) 4:1. Additionally, for reducing sample conditions, an end concentration of 100 mM of DTT was added to the samples. After electrophoresis, the gels were blotted onto a PVDF membrane using the Bio-rad TransBlot Turbo system and consumables, on the pre-set setting Mixed Molecular Weight.

Native PAGE was performed essentially as described in section 4.4.3.2, except the samples were diluted only in NativePAGE™ Sample buffer 4:1. The running buffer was exchanged with Native PAGE™ Running Buffer (20X) (Invitrogen®, BN2001) diluted into a 1X concentration in ultrapure water, and electrophoresis was run for approximately two hours on 200 V.

After protein transfer, all membranes were treated the same; they were transferred to a single-use plastic tray, which was filled with Wash Buffer (table 4.1), and incubated for one hour at room temperature with agitation. All subsequent incubation steps were performed at room temperature with constant agitation. After incubation, the membrane was incubated in pAb (Abcam, 137588). Prior to application, this pAb had been diluted to a concentration of 100 μg/mL and biotin-conjugated as described in 4.9. This pAb stock was diluted 1:750 and added to the membrane. The membrane with the pAb dilution was incubated for one hour. The membrane was rinsed thrice and washed for approximately five minutes in wash buffer, while HRP-conjugated streptavidin (GenScript, M00091) was diluted 1:3000 in wash buffer. This solution was added to the membrane that was then incubated for 30 minutes, and then rinsed thrice and washed for approximately five minutes. Lastly, the Western Blot Developer (table 4.1) was prepared, and the membrane was quickly rinsed in DM-water, before the developer was added. The membrane was developed with constant agitation for 3-8 minutes, until bands appeared.

4.7 Rat Monoclonal Antibodies

2 rats (Sprague Dawley^{TM}) were immunised with intact, non-polymerised, and polymerised HEK expressed PAI-2, respectively. Immunisations were performed by a laboratory technician, and were given subcutaneously twice with a 14 day interval, using 2x 50 μg of their respective antigen, coupled with diphteria toxoid via the end cysteine, adsorbed to $AI(OH)_3$ and mixed in a 1:1 ratio with Freud's incomplete adjuvant. Three days prior to the fusion, the rats were boosted intravenously with 50 μg antigen supplemented with epinephrine. An overview of the immunisation and methods can be seen in figure 4.3.

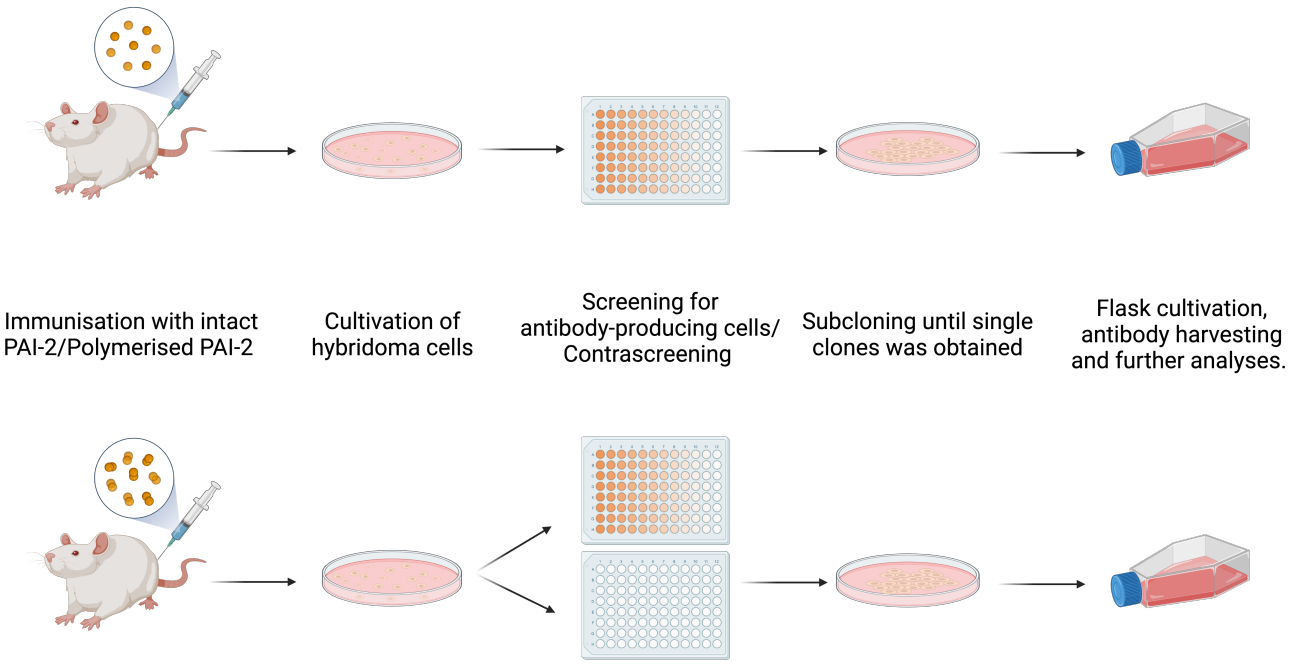


Figure 4.3: Brief overview of the production of monoclonal antibodies with hybridomas.
Two rats were immunised, one with intact PAI-2; another with polymerised PAI-2. Cultivation of hybridoma cells gave cell culture supernatant, which was used to screen for antibody-producing cells. Hybridomas from the rat immunised with polymerised PAI-2, were contra-screened for antibodies specific to polymerised PAI-2 but not intact PAI-2. Positive wells were subcloned until a single clone was obtained and cultivated in a flask for antibody production that was later harvested and used in further analyses. *Created with BioRender.com.*

Immunised rats were sacrificed, the spleens were removed, and fusions were performed essentially as described by Köhler and Milstein in 1975 [76]. However, the myeloma cell line SP2/0-AG14 was used as a fusion partner. Cells were seeded in Nunc^{TM} MicroWell^{TM} 96-Well, Nunclon Delta-Treated, Flat-Bottomed Microplate plates (Thermo Fisher, 191093) throughout the entire process.

Cells from the immunisation with intact, non-polymerised PAI-2 were screened for antibody pro-

duction by coating MaxiSorp plates (Nunc™ Maxisorp™, Roskilde Denmark) with 0.05 μg/mL PAI-2 in PBS overnight at 4°C. Cells from the immunisation with polymerised PAI-2, were contra-screened against both polymerised PAI-2, as well as intact PAI-2: these plates were coated with 0.5 μg/mL antigen. The antigens were diluted to the specified concentration in PBS.

Plates were coated overnight, prior to analyses. All following incubation steps were performed at room temperature with constant agitation. The plates were washed thrice and left to block in wash buffer for one hour. Hereafter, 100 μL of culture supernatants from the fusion plates was added to the wells and incubated for one hour. The plates were washed thrice in wash buffer. HRP-conjugated rabbit anti-rat IgG (Sigma-Aldrich, A9542) was diluted 1:2000 in wash buffer, added to the wells, and left to incubate for one hour. The plates were washed and developed using *o*-phenylenediamine Dihydrochloride (OPD). This was added to the plates that were then incubated in the dark for 15 minutes and the reaction was stopped using 50μL 2M H_2SO_4.

Positive wells were cloned onto new culture plates, using the limited dilution method until single clones were achieved. Positive single clones were transferred to T75 flasks Nunc™ EasYFlask™ Cell Culture Flasks (Thermo Fisher™, 156499) and grown in sterile medium (RPMI 1640 Medium with UltraGlutamine™ (Lonza, BE12-702F/U1) supplemented with 10% fetal bovine serum (Biowest®, S1810-500), Sodium Pyruvate 100X (Gibco™, 11360070), diluted to 1X concentration and 40μL Gentamicin Sulfate (Biowest®, L0012-100). When confluency was obtained, culture supernatant was collected for antibody purification, whilst aliquots of cells were collected and frozen. Each clone was denoted with a number in series. The hybridomas from immunisation with intact PAI-2 was denoted 21-11-X, while clones from the polymerised PAI-2 immunisation was denoted 21-12-X, with X indicating the number of the specific clone.

Rat mAbs were purified from collected cell culture supernatants using a HiTrap Protein L column (Cytiva, 29007877) on an ÄKTA™ system.

4.7.1 Evaluation of Rat Monoclonal Antibodies

4.7.1.1 Western blot

Two zoom gels were prepared by removing all wells except for one from Bolt™ 4-12% Bis-Tris Plus gels (Invitrogen, #NW04127BOX), one gel was prepared with a PAI-2 sample non-reduced, and the other with reducing sample conditions. Samples were prepared by diluting a pre-dilution of PAI-2

with a concentration of 100 μg/mL in Ultrapure water and LDS Sample Buffer (4X) (Invitrogen®, NP0007) to a total dilution of 1:10. For reduced sample conditions, the sample buffer was replaced with Reducing Sample Buffer (table 4.1). 500 μL was loaded onto the zoom gels and Novex™ Sharp Pre-stained Protein Standard (Invitrogen$_{TM}$, LC5800) was loaded in the remaining well. The Novex™ Mini-cell electrophoresis chamber was filled with SDS-PAGE Electrophoresis Buffer (table 4.1) and electrophoresis was run using a XCell SureLock™ power-supply lid for 40-65 minutes at 180 V. The gels were removed from the cassette and transferred into single-use plastic trays and rinsed briefly in DM-water. The proteins were transferred from the gels onto a PVDF membrane using the Bio-rad TransBlot Turbo system and consumables, on the pre-set setting Mixed Molecular Weight. The membranes were cut into strips and blocked overnight in Western Blot Blocking Buffer (table 4.1). One cell culture supernatants was added to one non-reduced and one reduced strip, and the strips were incubated in the supernatants for one hour at room temperature. They were briefly washed in Wash Buffer (table 4.1), after which HRP-conjugated rabbit anti-rat IgG (Sigma-Aldrich, A9542) was diluted 1:2000 in wash buffer and added to the strips. The strips were incubated for 30 minutes and briefly rinsed before they were developed using Western Blot Developer (table 4.1).

4.7.1.2 ELISA

MaxiSorp plates (Nunc™ Maxisorp™, Roskilde Denmark) were coated overnight at 4°C, with a serial dilution of either intact, non-polymerised or polymerised PAI-2, starting at 0.2 μg/mL, diluted in PBS. The plates were washed thrice and incubated for one hour in Wash Buffer (table 4.1). Culture supernatants were added to the plates undiluted and the plates were incubated for one hour at room temperature with constant agitation. The plates were washed thrice, after which HRP-conjugated rabbit anti-rat IgG (Sigma-Aldrich, A9542) was diluted 1:2000 in wash buffer and added to the plates. The plates were incubated for 30 minutes at room temperature with constant agitation. Subsequently, the plates were washed thrice and developed for 10 minutes with TMB Ultra (Thermo Scientific™ 1-Step™ Ultra TMB-ELISA Substrate Solution, 34029). The reaction was stopped with 100 μL 200 mM H_2SO_4 and the plates were read at 450 nm.

4.7.1.3 Immunoprecipitation

Immunoprecipitation was performed essentially as described in section 4.6, with the exception that the DynaBeads used were Invitrogen™ DynaBeads™ Sheep Anti-Rat IgG (Invitrogen™, 11035). 100 μL of DynaBeads was added to each Eppendorf tube and washed thrice in IP buffer supplemented with 2mM EDTA. 13 cell culture supernatants were added non-diluted in pools of two and three. The beads were incubated with culture supernatants for one hour in a cold room in an end-over-end rotator. The beads were washed thrice, and the sample (either a prepared sample of HEK expressed PAI-2 diluted to 5 μg/mL or 200 ng/mL in IP buffer, or plasma samples diluted 2:1 in IP buffer) was added. The beads with the samples were incubated for one hour in a cold room in an end-over-end rotator. The beads were then washed thrice, and all the supernatants was carefully removed. Elution was performed by adding 50 μL Elution Buffer (table 4.1) to each tube. The mix of beads and elution buffer was resuspended vigorously and was incubated for a few minutes, before the Eppendorf tubes were placed back on the magnet. The tubes were left on the magnet for approximately two minutes, until the solution was clear, before the supernatant was collected for further analyses. The collected eluates were analysed exactly as described in section 4.6.

4.8 Polyclonal Antibodies

Two polyclonal antibodies were purchased and used throughout this study. One was biotin-conjugated and used for western blotting (Abcam, 137588) and another was used in the establishment of the ELISA (antibodies-online.com, ABIN349627).

4.9 Biotinylation of Antibodies

Biotinylated antibodies used in this study were biotinylated by adding 200 μg biotin ((+)-Biotin N-Hydroxysuccinimide Ester, Sigma-Aldrich, H1759) to an appropriate amount of antibody. This was incubated in a cold room for two hours, and dialysed overnight in Spectra/Por molecularporous membrane tubing (Spectrum Laboratories Inc., lot no. 3318105) against 500-1800 mL/sample PBS.

4.10 Data analysis

ELISA plates were read using the software Softmax Pro®. Dose-response curves were constructed using Microsoft Excel (version 16.16.27) for MacOS Catalina using the OD values obtained at 450 nm. Samples and standards were analysed in technical duplicates. The standard curve was constructed by performing a two-fold serial dilution of HEK expressed PAI-2, and using the OD values to construct an 11-point standard curve with a four-parameter logistic fit model generated by the software Softmax Pro®. From this, the concentration of PAI-2 was interpolated. The obtained values were then analysed, described and illustrated with GraphPad Prism 8 software (version 8.4.3) for MacOS Catalina.

5 Results

5.1 Acquisition of PAI-2

PAI-2 was expressed and purified from HEKs. The purification of PAI-2 produced a stock of PAI-2 with an end-concentration of 1.3 mg/mL protein in HBS. This PAI-2 was compared to PAI-2 from bacterial expression from R&D Biosystems (9206-PI-025) (figure 5.1A). The two PAI-2s from different expression systems were compared on a Coomassie stained SDS-PAGE gel, displayed in figure 5.1A.

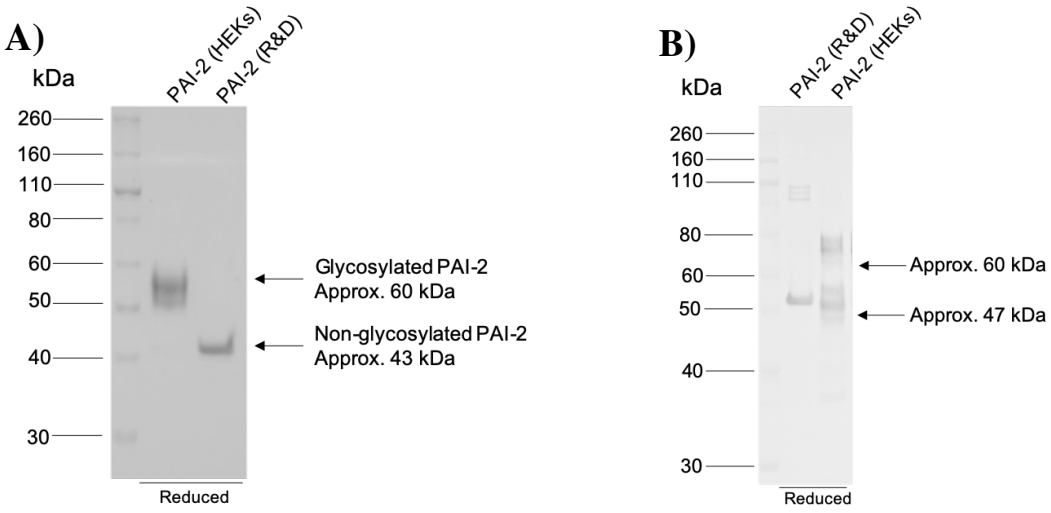


Figure 5.1: Comparison of PAI-2 expressed in different expression systems.
A) PAI-2 (HEKs) and PAI-2 (R&D) was visualised in reduced state with a Coomassie stained SDS-PAGE.
B) PAI-2 (HEKs) and PAI-2 (R&D) was analysed using western blotting techniques. Samples were analysed in reducing sample conditions. Mouse mAb 19-26-2 and 19-26-11 was used for western blotting, with rabbit-anti-mouse IgG for detection. Samples labelled R&D were bacterially expressed PAI-2 purchased from R&D Biosystems®(9206-PI-025), whilst samples labelled (HEKs) were PAI-2 expressed in and purified from HEKs. *Abbriviations: Plasminogen Activator Inhibitor-2 (PAI-2), Human Embryonic Kidney Cell (HEK), Kilodalton (kDa).*

Here, it was evident that there was a difference between the two proteins with regard to size, as the lane with PAI-2 from HEKs showed a protein band at approximately 60 kDa, while the lane with the PAI-2 from a bacterial expression system showed a protein band at approximately 43 kDa. As described in section 2.2.3, PAI-2 exists in two different forms that differ in glycosylation. Glycosylation is a post-transcriptional modification to some proteins. Glycosylation in mammalian and bacterial expression system varies, and a mammalian expression system offers glycosylation closely responding to the one of the native protein [77]. Thus, the difference in the two PAI-2s displayed, is likely a result of the glycosylation of PAI-2 that happens in HEKs, while the PAI-2 expressed in a bacterial expression

system is non-glycosylated.

The western blot in 5.1B confirmed that the protein expressed and purified from HEKs share immunological properties with the purchased PAI-2 protein. Moreover, it further illustrated the size difference between mammalian and bacterial expressed PAI-2, due to glycosylation, as the lane with PAI-2 from bacterial expression (R&D Biosystems) showed no bands at 60 kDa, whilst bands this size can be found in lanes with PAI-2 from HEKs (PAI-2 (HEKs) 1 and 2).

5.2 Polymerisation of PAI-2

Previously, it has been shown that Plasminogen Activator Inhibitor-2 (PAI-2) is a spontaneously polymerising Serpin [41, 46, 55]. Denaturing conditions, such as treatment with the chemical agent GuHCl, have been shown to induce the polymerisation [55]. To test this, PAI-2 samples were subjected to varying concentrations of GuHCl, ranging from 0.25M to 2M. The state of these samples was then visualised using SDS-PAGE and western blotting techniques. The results of this can be seen in figure 5.2.

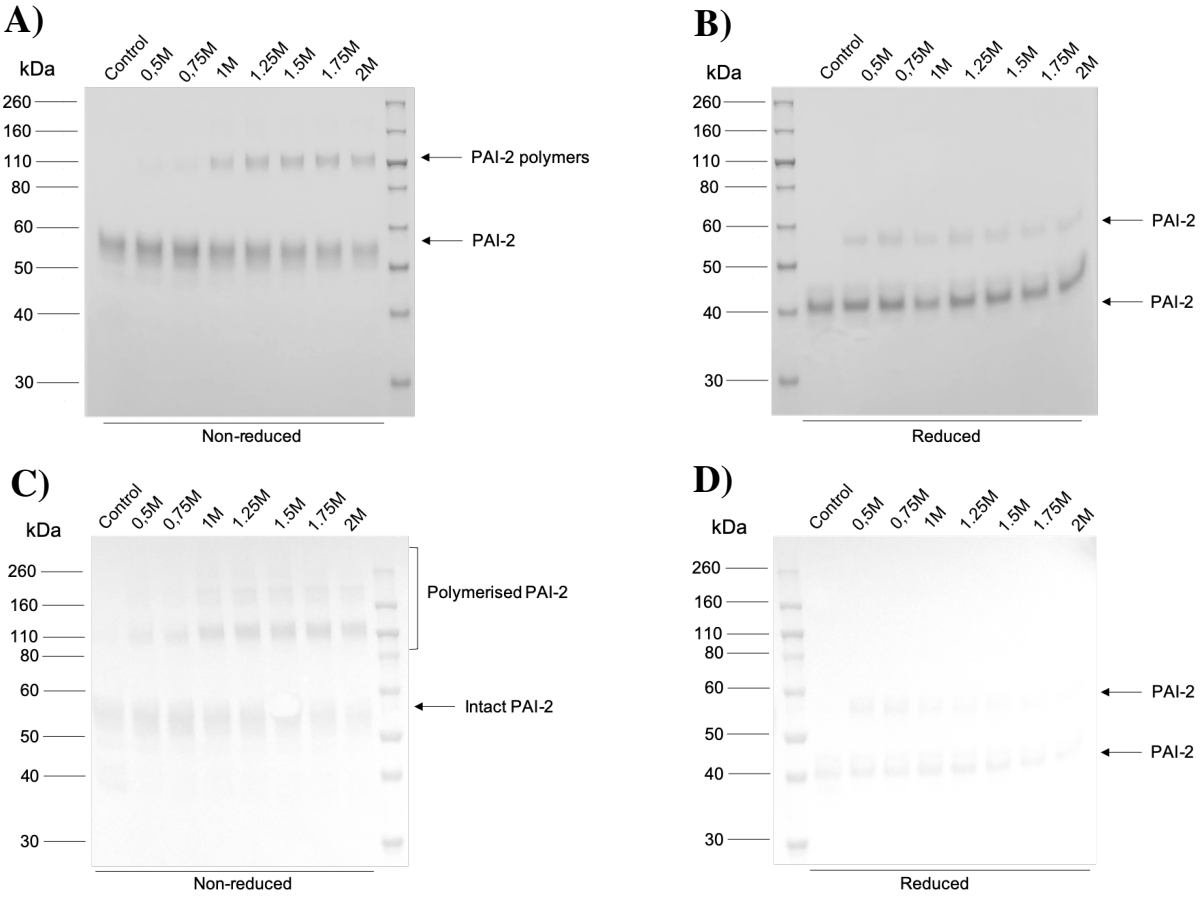


Figure 5.2: SDS-PAGE and Western Blot of samples polymerised with GuHCl at varying concentrations. Plasminogen Activator Inhibitor-2 (PAI-2) was polymerised using varying concentrations of Guanidine Hydrochloride (GuHCl). A control was treated similarly to test samples, however, GuHCl was not added. The samples were then visualised both reduced (**B** and **D**) and non-reduced (**A** and **C**) with a Coomassie stained SDS-PAGE gel (**A** and **B**) and western blotting (**C** and **D**). The western blot was developed using mouse mAb 19-26-2 and 19-26-11, detected with a HRP-conjugated rabbit-anti-mouse IgG antibody.

Evidently, all concentrations $\geq$1M GuHCl caused polymer formation of PAI-2 samples. This can be seen in both the SDS-PAGE gel as well as on the western blot in figure 5.2 as bands of MW of approximately 110 kDa. This was confirmed by the observation that these bands disappear when the samples were treated with reducing sample buffer (figure 5.2B and D). Furthermore, bands of a smaller size (corresponding to approximately 40 kDa) was observed on the blots showed here, and in figure 5.4.

From this point, non-polymerised PAI-2 is described as intact PAI-2.

In an attempt to further visualise the polymerisation of PAI-2, the samples polymerised using GuHCl was run on native PAGE. The gels were stained using silver staining or analysed using western blotting techniques. The results of this is presented in figure 5.3.

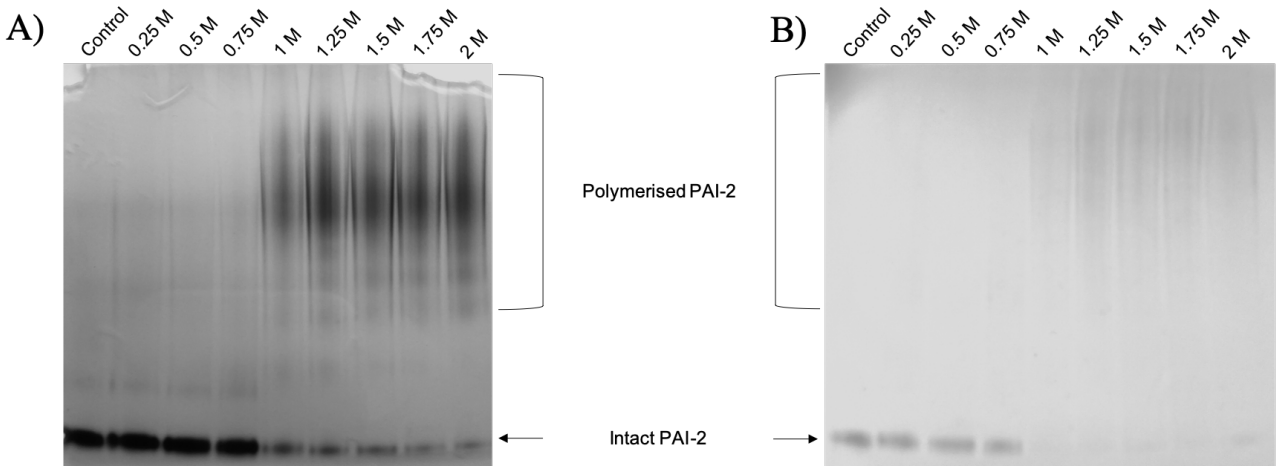
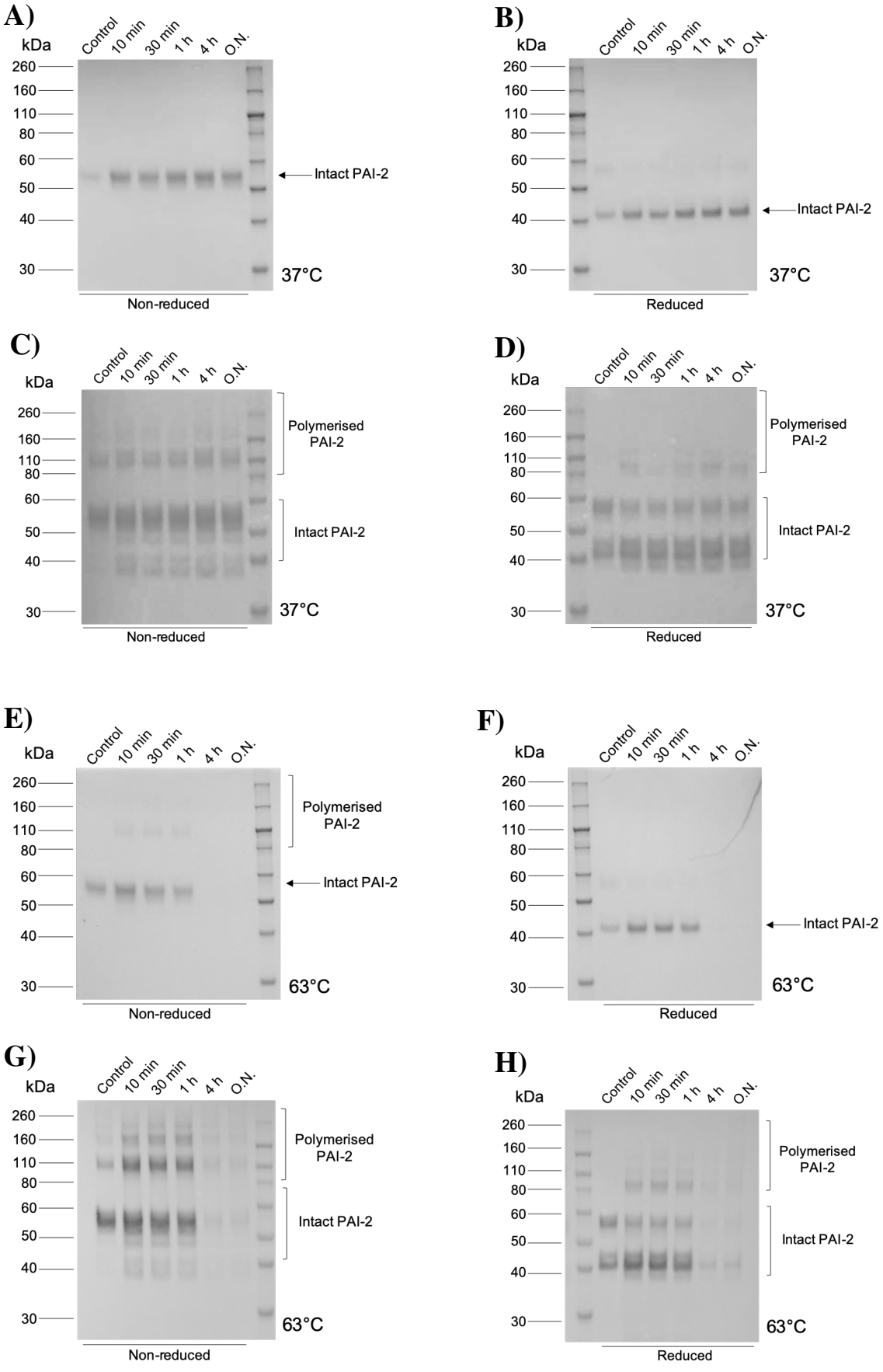


Figure 5.3: Silver stain (A) and western blot (B) native page of GuHCl polymerised PAI-2.
Samples of polymerised Plasminogen Activator Inhibitor-2 (PAI-2), polymerised with GuHCl at varying concentrations, were run on native PAGE. One gel was stained with silver stain, the other blotted onto a membrane and used for western blotting. The blot was developed with mAb 19-26-2 and 19-26-11, detected with a HRP-conjugated rabbit-anti-mouse IgG antibody.

The results of the native PAGE confirmed the observations from the SDS-PAGE, as it shows significant polymer formation in samples treated with GuHCl concentrations of 1M and above, and little to no polymer formation corresponding with the control sample in samples treated with 0-0.75M GuHCl.

Overall, these results indicated that PAI-2 has a tendency to form polymers under denaturing conditions. To further study the tendency of PAI-2 to form polymers, samples of HEK expressed PAI-2 were exposed to 37°C and 63°C for different time intervals.


A)
kDa
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10 min
30 min
1 h
4 h
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Intact PAI-2
37°C
Non-reduced
B)
kDa
Control
10 min
30 min
1 h
4 h
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Intact PAI-2
37°C
Reduced
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Control
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Polymerised PAI-2
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Polymerised PAI-2
Intact PAI-2
63°C
Reduced

Figure 5.4

Figure 5.4: SDS-PAGE and Western Blot of samples polymerised with heat in different time intervals. Plasminogen Activator Inhibitor-2 (PAI-2) samples were exposed to 37°C and 63°C for 10 minutes, 30 minutes, one hour, four hours and overnight. A control sample was continuously stored at 4°C. The samples were prepared for SDS-PAGE with or without 2.5% mercaptoethanol for reducing conditions. After electrophoresis, gels were either stained with Coomassie staining (**A**, **B**, **E** & **F**), or used for western blotting (**C**, **D**, **G** & **H**). Blots were developed using mouse mAb 19-26-2 and 19-26-11.

The results of this experiment are shown in figure 5.4. It was evident when looking at the western blots displayed in the figure (5.4C, D, G & H) that a polymerisation of PAI-2 occurred after just 10 minutes in either temperature. This was seen as protein bands at MW >60 kDa. It is important to note that the control also displays some degree of polymerisation. However, polymerisation was induced to a higher degree when exposing PAI-2 samples to 37°C or 63°C for just 10 minutes. In figure 5.4E, F, G & H, results from exposure to 63°C is displayed. In these, little to no bands appeared after four hours of heat exposure. This was not seen in the samples exposed to 37°C, where protein was apparent in all samples (5.4A, B, C & D). As mentioned previously, PAI-2 polymerisation is dependent on a stable disulfide bond. However, in the blots displayed in figure 5.4D and H, bands of 110 kDa and above (corresponding to polymer formation) still appeared.

In summary, polymerisation seemed to occur in PAI-2 samples exposed to both 37°C and 63°C after 10 minutes. To further study the polymerisation after exposure to heat, native PAGE was performed and gels were silver stained or used for western blotting. The results of this are shown in figure 5.5.

The results displayed in 5.5 supported the ones obtained from SDS-PAGE, as the same pattern was observed. All samples display some amount of polymerisation.Additionally, it seemed that samples exposed to 63°C mostly had polymers of a larger size, seen as streaks higher in the gel.

In summation, these results suggested that polymerisation of PAI-2 can be achieved using both a chemical denaturant, GuHCl, as well as denaturing heat at 63°C. They also suggested that polymerisation occurs spontaneously in 37°C, but also under control conditions at 4°C.

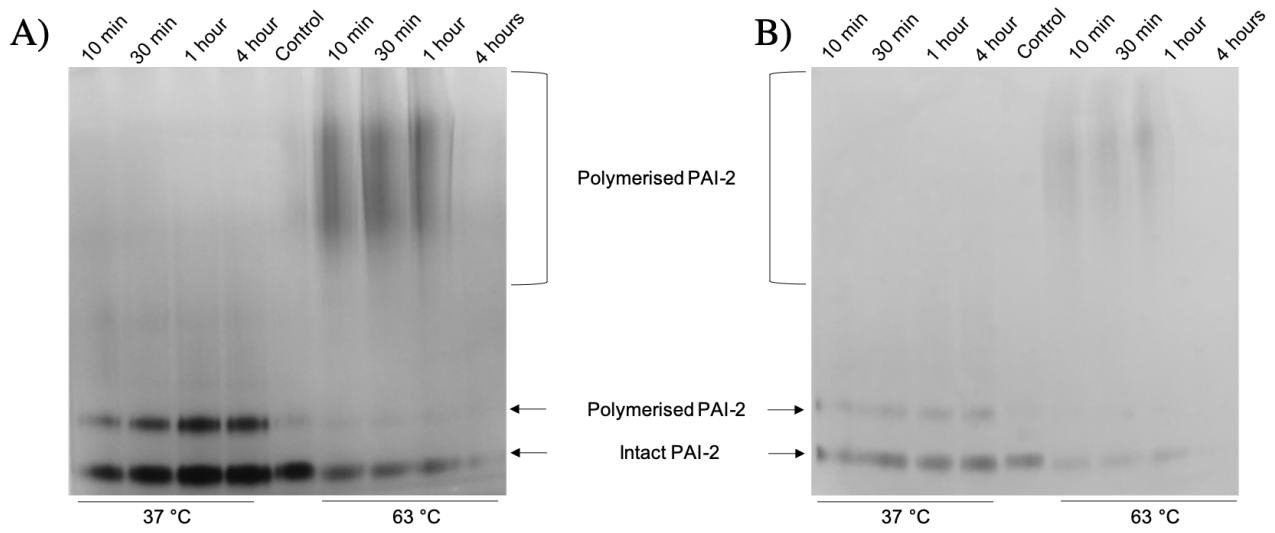

Figure 5.5: Silver stain (A) and western blot (B) of heat polymerised PAI-2 samples.
Plasminogen Activator Inhibitor-2 (PAI-2) samples were exposed to 37°C and 63°C for 10 minutes, 30 minutes, one hour, four hours and overnight. A control was continuously stored at 4°C. Samples were prepared for native PAGE, and after electrophoresis, one gel was visualised with SilverStain **(A)**, while another was visualised with western blotting techniques **(B)**. Mouse mAb 19-26-2 and 19-26-11 was used for western blotting, and detected with a HRP-conjugated rabbit-anti-mouse IgG antibody.

5.3 An ELISA for Quantifying PAI-2 in Plasma

One objective of this study was to establish an Enzyme-linked Immunosorbent Assay (ELISA) that could measure the PAI-2 levels in pregnancy plasma. After in-depth characterisation of mouse mAbs in western blotting and Immunoprecipitation (IP) (see section 5.4), mouse mAb clone 19-26-3 was chosen for application in ELISA. This was based on the ability of this mAb to recognise native, human PAI-2 from plasma samples from pregnant individuals, thereby making it a valid candidate as a capture antibody in this assay. When pairing mouse mAb 19-26-3 as a capture antibody with the pAb (antibodies-online.com, ABIN349627), an ELISA setup was established.

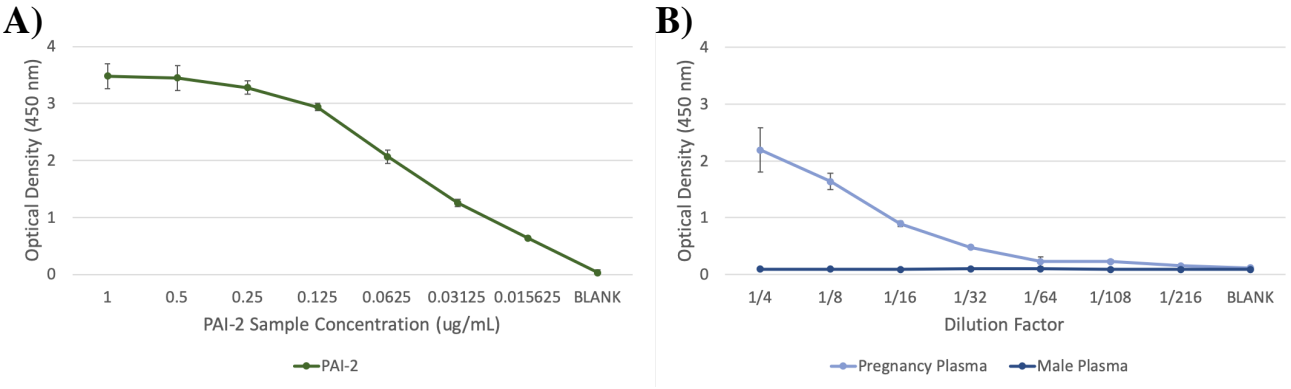

Figure 5.6: Dose-response curves obtained when analysing different samples on the established ELISA. **A)** Dose-response curve of the ability of this ELISA to detect PAI-2 in a prepared sample with a known concentration. Constructed by plotting the mean of OD values obtained by measuring a prepared HEK expressed PAI-2 sample with a know concentration on the ELISA with 5 μg/mL mAb 19-26-3 as a capture antibody, and 2 μg/mL pAb (antibodies-online.com, ABIN349627) as a detection antibody. **B)** Dose-response curves of the ability of this ELISA to measure PAI-2 in plasma samples. Constructed by plotting the means of OD values obtained when analysing a serial dilution of pregnancy plasma on this assay. The male plasma curve is the OD values for a dilution of a negative control (plasma sample from a man). All curves are fitted with their respective standard deviations of the mean.

Figure 5.6 shows the dose-response curves of this ELISA obtained, when a prepared dilution of HEK expressed was analysed (figure 5.6A), and when diluted plasma samples from a pregnant invidual and a negative control was analysed (figure 5.6B). The graph in 5.6A showed that the ELISA setup in this study was able to detect PAI-2 in a prepared control PAI-2 sample, while the graphs in 5.6 suggested that the assay could detect PAI-2 in pregnancy plasma and discern PAI-2 from other components of plasma, as no signal was detected in plasma samples from a man.

The ELISA was now established and was used in an experiment to quantify the levels of PAI-2 in plasma samples from preeclamptic patients (*n=10*) and a group of healthy controls (*n=10*). All samples was analysed in technical duplicates. The results of this are illustrated in figure 5.7 and figure 5.8.

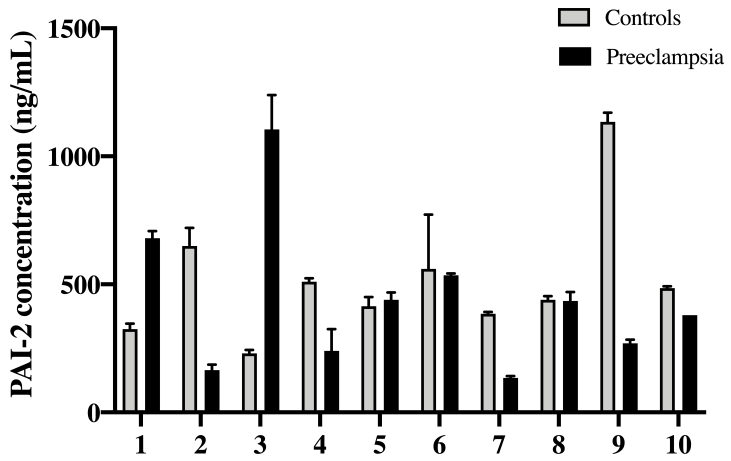

Figure 5.7: Column Chart illustration the levels of PAI-2 measured ELISA.
Preeclampsia (*n=10*) and control (*n=10*) samples were quantified in technical duplicates using the ELISA. The values were calculated using the standard curves displayed in appendix A. The patients and matched controls are divided into groups, numbered on the x-axis. Each sample is fitted with their respective standard deviation from the mean.

Figure 5.7 represents a column chart of the results obtained from analysing the preeclampsia and control samples. Each column represents the mean of two repeats and are fitted with the standard deviation of the mean. This column chart thus depicts the mean values obtained on the ELISA when analysing patient samples. It shows that it was possible to measure PAI-2 in patient samples with this established ELISA, however, this bar chart did not indicate a clear tendency of one of the groups having generally higher plasma concentrations of PAI-2.

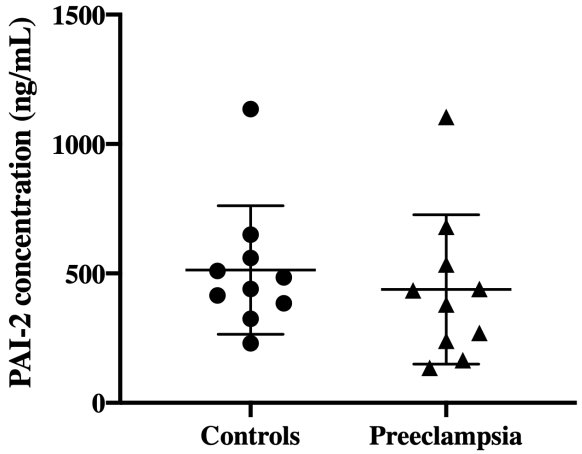

Figure 5.8: Scatterplot of mean values of measurements of samples obtained from ELISA.
Preeclampsia (*n=10*) and control (*n=10*) samples were quantified using the ELISA. Concentrations were interpolated from the standard curve (see appendix A), and values was plotted: dots illustrate the means of the two technical duplicated for each of the control samples, while triangle represents the means of the two repeats for the each of the preeclampsia samples. Standard deviations for the means of the two groups are illustrated on the scatter plot.

Figure 5.8 represents a scatter plot of means of values for the two sample groups, preeclampsia and control. Taken together with figure 5.7, figure 5.8 likewise illustrated that this ELISA setup could measure PAI-2 levels in plasma of patient samples.

The results from the ELISAs performed in this study gave a range of PAI-2 concentration of 155-1134 ng/mL; mean 423.8 ng/mL for control samples, and a range of 153-1107 ng/mL; mean 376.25 ng/mL for preeclampsia samples. When looking at figure 5.7, no tendency of one of the groups having a generally higher or lower level of PAI-2 was observed and taken together with figure 5.8, it seemed there was no clear tendency, even if the mean of the preeclampsia group was a little lower than that of the control group.

In summation, the ELISA established within this project could detect PAI-2 from pregnancy plasma. However, the results obtained did not clearly represent a pattern of PAI-2 levels being decreased in one of the groups compared to the other.

5.4 Immunoprecipitation of PAI-2 from Pregnancy Plasma

To illustrate the presence of PAI-2 in pregnancy plasma, immunoprecipitation was performed. Using DynaBeads^{TM}, PAI-2 was precipitated from plasma as well as controls. In figure 5.9, the DynaBeads were tested with pools of mouse mAbs against prepared samples of PAI-2. From these results it

was evident that HEK expressed PAI-2 could be precipitated using mouse mAbs in combination with DynaBeads, as PAI-2 bands appeared in almost all lanes on the blot. The lane with the pool of antibodies 19-26-22 and -23 showed only a faint protein band, and the bands at the lane of 19-26-7 and -15 were also vague compared to the rest. Therefore, these two pools were excluded from further analyses.

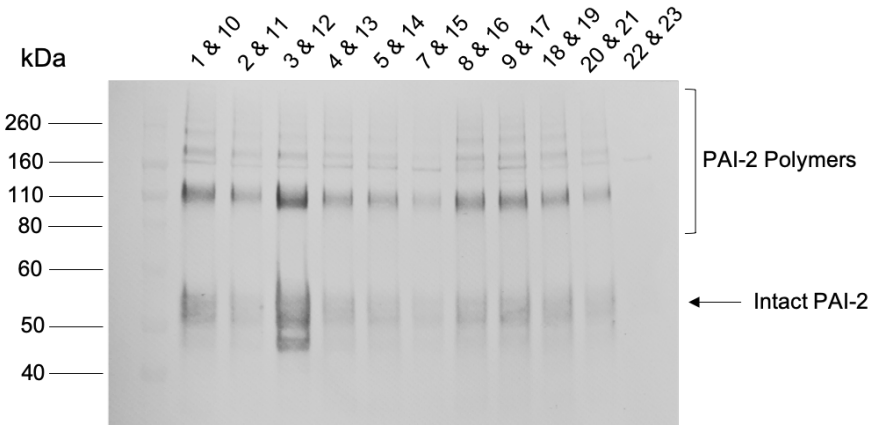

Figure 5.9: Western blot of immunoprecipitated HEK expressed PAI-2.
Samples of 5μg/mL PAI-2 were immunoprecipitated using pools of mouse mAbs (19-26-X) at a concentration of 2μg/mL, with DynaBeads. After precipitation, samples were analysed with SDS-PAGE, subsequently transferred to a membrane and used for western blotting, developed with biotin-conjugated pAb (Abcam, 137588).

After it was confirmed that the mouse antibody pools were capable of precipitating PAI-2 from a prepared sample using DynaBeads, precipitation of PAI-2 from pregnancy plasma was attempted in a similar fashion. The results from this immunoprecipitation can be seen in figure 5.10. Here, it was evident that precipitation of PAI-2 from pregnancy plasma had been successful, mainly for the pool with mAbs 19-26-3 and 19-26-12, as this lane showed strong protein bands closely corresponding to those of the control. Weak bands could also be seen in the lane of the 19-26-1 and 19-26-10 pool. These four antibodies were therefore used in further analyses, while the rest were excluded.

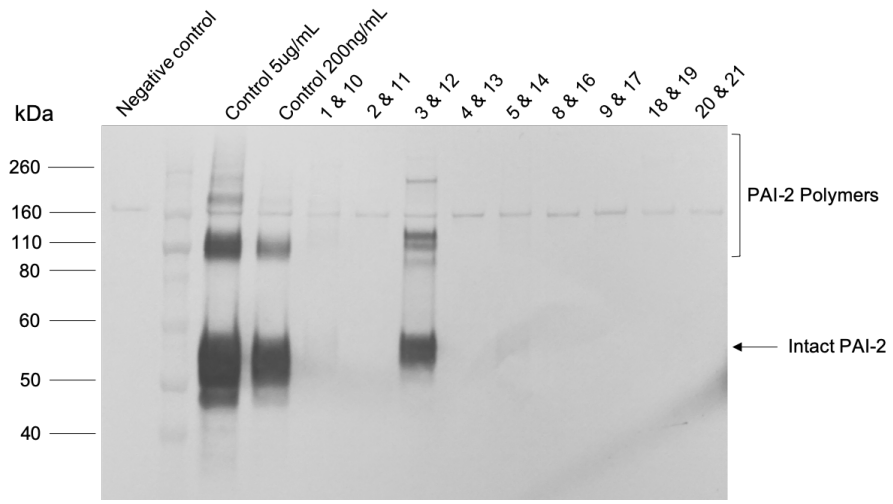

Figure 5.10: Western blot of precipitates from immunoprecipitation of PAI-2 from pregnancy plasma. DynaBeads were bound with pools of mouse mAbs in a concentration of 2 μg/mL, after which controls and samples were added. Positive controls were prepared samples of HEK expressed PAI-2 at the given concentrations; the negative control was a similar precipitation attempted on male plasma. All controls were precipitated with a pool of mouse mAbs 19-26-3 & 19-26-12. After precipitation, precipitates were loaded on a SDS-PAGE gel that were subsequently transferred to a membrane and used for western blotting. The blot was developed with biotin-conjugated pAb (Abcam, 137588).

Because it was now evident that precipitation of PAI-2 from pregnancy plasma could be achieved with available mouse mAbs, it was relevant to investigate which of the antibodies was working to precipitate PAI-2 from pregnancy plasma. Therefore, DynaBeads were coated with single mouse mAbs at a concentration of 2 μg/mL and subsequently used for precipitation of pregnancy plasma.

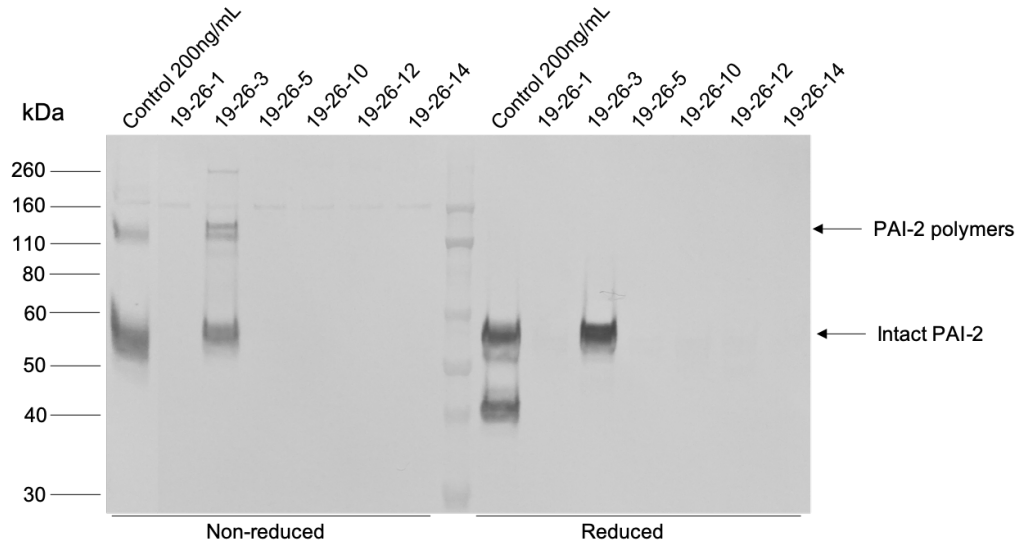

Figure 5.11: Western blot of precipitation of PAI-2 from pregnancy plasma with single mouse mAbs. DynaBeads were bound with single mouse mAbs in a concentration of 2 μg/mL and samples were added. The control was HEK expressed PAI-2 diluted to a concentration of 200 ng/mL in IP buffer, precipitated with a pool of mouse mAbs 19-26-3 & 19-26-12. After precipitation, precipitates were run on an SDS-PAGE gel, with or without the addition of 2.5% mercaptoethanol for reducing sample conditions, subsequently transferred to a membrane used for western blotting. The blot was developed with a biotin-conjugated pAb (Abcam, 137588).

From figure 5.11, it was evident that the precipitation of PAI-2 from pregnancy plasma only succeed when using mouse mAb 19-26-3. This was a significant find for the present study, as it shows that mAb 19-26-3 worked as an antibody towards naturally occurring, human, placental PAI-2. Therefore, this antibody was used in other parts of the present study.

In summation, mAb 19-26-3 has characteristics that enables recognition of naturally occurring human PAI-2 from pregnancy plasma, thus enabling precipitation of PAI-2 from patient samples.

5.4.1 Precipitation of PAI-2 from Preeclampsia Patient Plasma Samples

One hypothesis of this study was that PAI-2 is polymerised to a higher degree in preeclampsia patients than in their healthy counterparts. Samples were obtained from preeclampsia patients and matched controls. To illustrate the state of PAI-2 in plasma from patients with preeclampsia, immunoprecipitation was performed. The results of this immunoprecipitation are illustrated in figure 5.12.

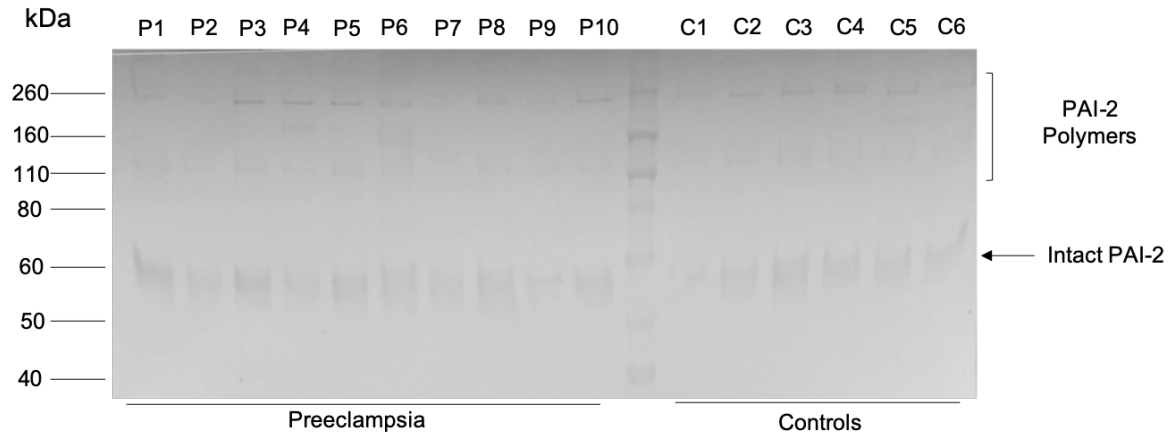

Figure 5.12: Western blot of immunoprecipitated preeclamspsia samples and healthy matched controls. DynaBeads^{TM} were bound with mouse mAb 19-26-3. These were used to precipitate PAI-2 from preeclampsia plasma samples from 10 different patients (P1-P10) and plasma samples from 6 healthy, pregnant, matched controls (C1-C6). After precipitation, samples were run on SDS-PAGE and subsequently transferred for western blotting. The blot was developed with a biotin-conjugated polyclonal antibody (Abcam, 137588).

The western blot displayed in figure 5.12 showed that all plasma samples have both intact and polymerised PAI-2 present, regardless of preeclampsia.

To further visualise the polymerisation of PAI-2 in preeclamptic plasma samples, native page of the eluates was performed. The result of this can be seen in figure 5.13. Samples used in this analysis are denoted correspondingly to the samples in 5.12. The results of this native PAGE western blot analysis supported those obtained from the SDS-PAGE western blot: All patients, regardless of whether they were affected by preeclampsia, had both intact and polymerised PAI-2 that was precipitated with Immunoprecipitation with DynaBeads bound with mouse mAb 19-26-3 and observed with western blotting techniques. Some samples gave bands with lower intensity, but all samples showed both polymers and intact PAI-2. These two analyses (figure 5.12 and figure 5.13) did not display a clear pattern with regards to quantity of PAI-2 or the proportion of PAI-2 polymers to intact PAI-2 in preeclamptic patients and their healthy counterparts.

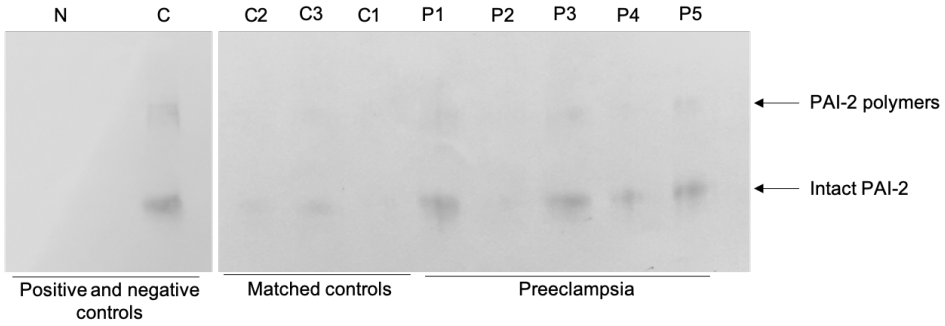

Figure 5.13: Western blot of a native PAGE of immunoprecipitated preeclamspsia samples and controls. Eluates from immunoprecipitation, performed with DynaBeads bound with mAb 19-26-3, of PAI-2 from preeclamptic patients (P1-P5) and healthy, pregnant, controls (C1-C3), were run on a native PAGE gel, subsequently transferred to a membrane for western blotting. For controls, immunoprecipitation was performed on plasma samples from one male (N) and one healthy, pregnant control (C). The western blot was developed with a biotin-conjugated polyclonal antibody (Abcam, 137588).

In parallel with the development of the ELISA, and experiments to visualise the naturally occurring PAI-2 in pregnancy plasma, a new panel of Monoclonal Antibodies was produced by immunisation of rats with HEK expressed PAI-2. The purpose of this was mainly to achieve a new generation of mAbs using glycosylated PAI-2 as the immunogen. These antibodies can be used in combination with above-characterised mouse mAbs, and have some other specificities which can prove useful, in particular for applications with PAI-2-polymer specific aims.

5.5 Generation and Characterisation of Rat Monoclonal Antibodies

Fusion and cloning provided 19 stable antibody producing clones from the rat immunised with polymerised PAI-2 (21-12-X). These were characterised western blotting techniques (figure 5.14).

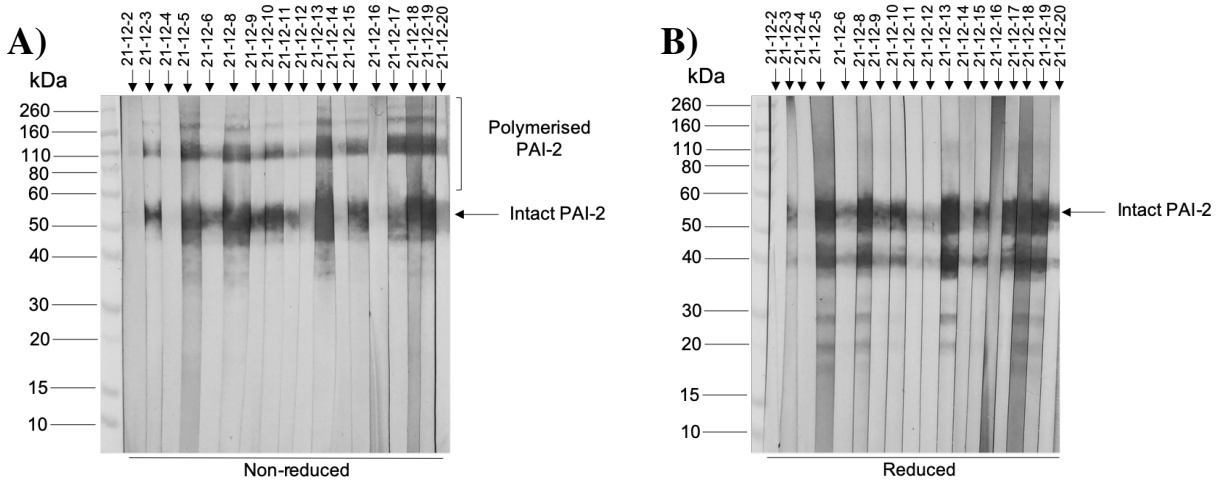

Figure 5.14: Western blots of zoom gels loaded with PAI-2 and blotted with rat mAbs.
Two zoom gels were loaded with HEK expressed PAI-2 sample with (**B**) or without (**A**) the addition of 2.5% mercaptoethanol, and was after SDS-PAGE blotted onto a membrane. The membranes were cut into strips, and cell culture supernatant from each antibody producing clone was added to one reduced and one non-reduced strip. A rabbit-anti-rat antibody was used as a secondary antibody.

The results of this showed that most of the clones achieved from immunisation produce antibodies that could detect PAI-2 with western blotting techniques. However, antibodies 21-12-2, -4, -14, and -16 detected little to no PAI-2 on the strips, while antibodies 21-12-6, -13, -18 and -19 detected PAI-2 on the strips, but with a high background. The remainder of the antibodies (21-12-3, -5, -8, -9, -10, -11, -15, -17, -20) all bound PAI-2 on the membrane, both the intact PAI-2 with MW at approximately 60 kDa as well as the polymers with MW approximately 110-260 kDa.

In correspondence with testing the abilities of the produced rat mAbs on western blots, a subset of the produced antibodies were tested on an ELISA, the results of which are displayed in figure 5.15. The results of this ELISA indicated a similar affinity of the antibodies to both intact, non-polymerised PAI-2 and polymerised PAI-2.

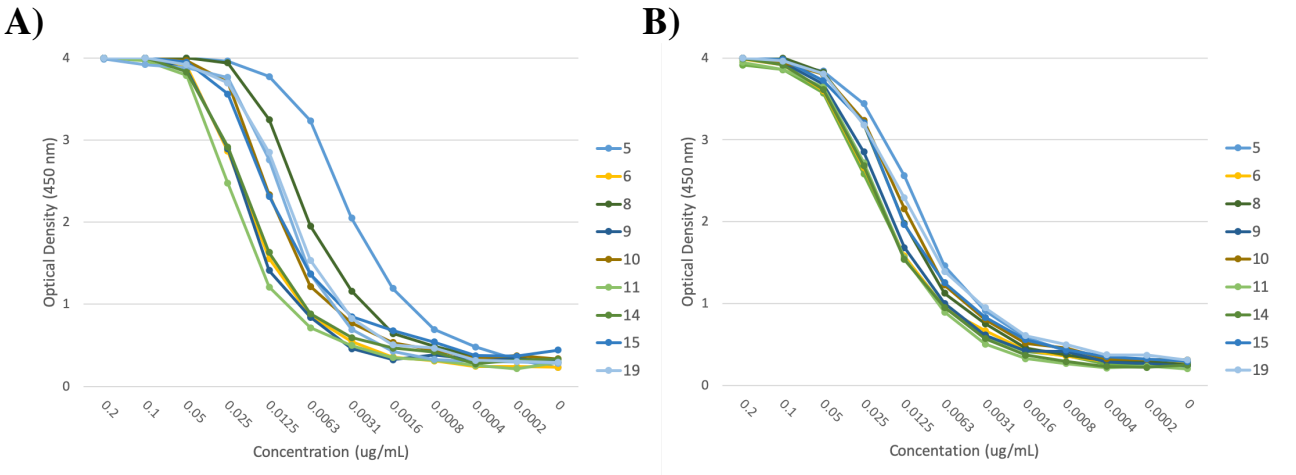

Figure 5.15: Dose-response curves of rat mAbs for intact PAI-2 (A) and polymerised PAI-2 (B). Nunc MaxiSorp^{TM} ELISA plates were coated with a serial dilution of 0.2 μg/mL PAI-2 (**A**)) or PAI-2 polymerised with 1.5M GuHCl (**B**)) overnight. Rat culture supernatants 21-12-X were added to the plates, and HRP-cojugated rabbit-anti-rat antibody was used as a secondary antibody. The plates were read at 450 nm on a plate reader, and the results were plotted into the graphs shown, with the concentration of the coated PAI-2, polymerised or not, on the X-axis.

In summation, the rat mAbs produced for this study detected both intact PAI-2 and polymerised PAI-2 in both western blotting techniques and on ELISAs.

To further analyse the abilities of the produced rat mAbs, immunoprecipitation was attempted with DynaBeads^{TM}. The results of this are displayed in figure 5.16. It was evident that all pools of antibodies could precipitate HEK expressed PAI-2 from a prepared sample diluted in IP buffer to a concentration of 5μg/mL, as all lanes showed protein bands corresponding to PAI-2.

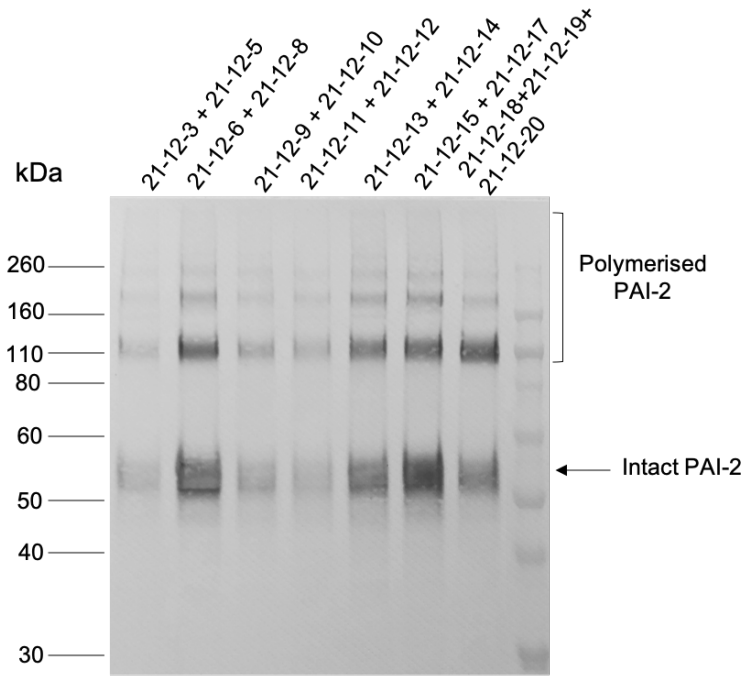

Figure 5.16: Immunoprecipitation of PAI-2 samples using rat monoclonal antibodies.
Pools of cell culture supernatants from antibody producing clones was used with DynaBeads^{TM} to precipitate PAI-2 samples with a concentration of 5 μg/mL. Subsequently, the precipitates were run non-reduced on an SDS-PAGE gel, which was blotted onto a membrane, subsequently developed using a biotin-conjugated polyclonal antibody (Abcam, 137588).

To test if rat mAbs could also precipitate PAI-2 from pregnancy plasma, the same experiment was repeated, however, the prepared PAI-2 sample was exchanged with pregnancy plasma samples. The results of this immunoprecipitation are depicted in figure 5.17.

On this blot, there was no visible protein bands, expect from in the control lane. This suggested that the rat mAbs produced in this study cannot be used with DynaBeads to precipitate PAI-2 from pregnancy plasma.

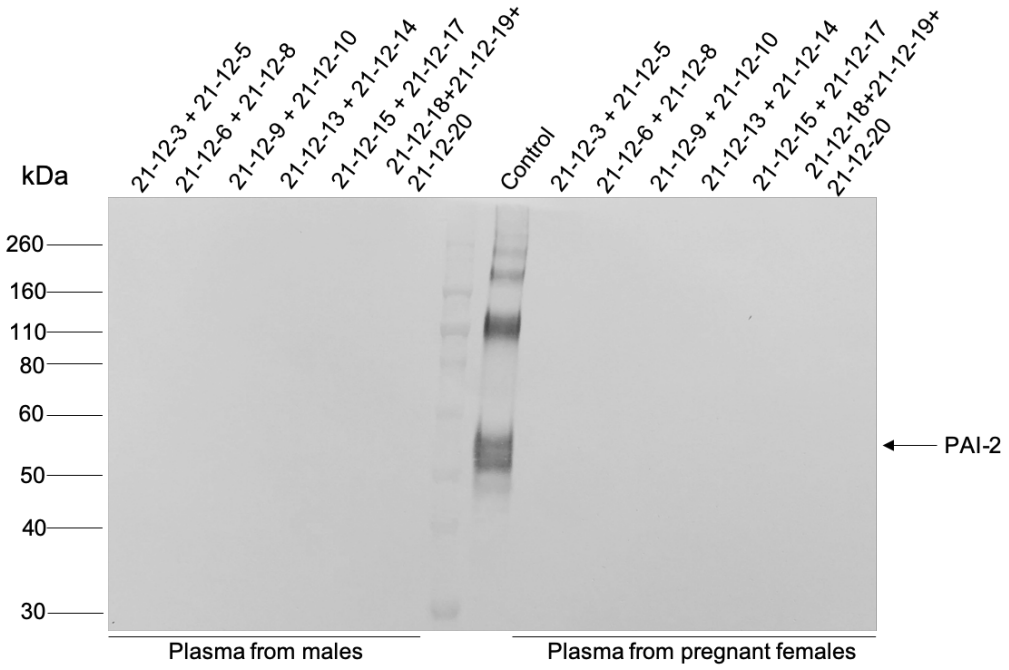

Figure 5.17: Immunoprecipitation of pregnancy plasma using rat monoclonal antibodies.
Pools of cell culture supernatants from antibody producing clones was used with DynaBeads^{TM} to precipitate PAI-2 from pregnancy plasma samples. Subsequently, the precipitates were analysed on SDS-PAGE, which was blotted onto a membrane. The control was a precipitate from figure 5.16. This membrane was then developed using a biotin-conjugated polyclonal antibody (Abcam, 137588).

To summarise, the produced rat monoclonal antibodies detected PAI-2 using western blotting techniques, and could also detect PAI-2 when this was coated on an ELISA plate even in low concentrations. With regards to immunoprecipitation, when binding the antibodies to DynaBeads, precipitation was possible when precipitating HEK expressed PAI-2 of a known dilution in IP buffer, but not when attempting to precipitate PAI-2 from pregnancy plasma.

6 Discussion

The aim of this study was to establish a toolbox and provide the necessary tools and methods for future research to investigate PAI-2 and its role as a spontaneously polymerising Serpin in preeclampsia. Firstly, a model to induce polymerisation in PAI-2 samples was established using an addition of $\geq$1M GuHCl to PAI-2 samples, as well as exposure of PAI-2 samples to 63°C. Moreover, an ELISA based assay was assembled with the goal of being able to quantify the concentration of PAI-2 in pregnancy plasma samples. An attempt to visualise the state of PAI-2 in pregnancy plasma samples was furthermore carried out with immunoprecipitation using magnetic DynaBeads.

In parallel with the above-mentioned methods, rat mAbs were produced and partially characterised, in an attempt to create high-affinity antibodies against PAI-2, as well as an attempt to create antibodies specific for PAI-2 polymers. The end-goal of this was to create a novel generation of mAbs, created by immunisations with HEK expressed PAI-2, as opposed to the mouse mAbs used in this study, which was created by immunisation with bacterially expressed PAI-2. The aim of this was to create high-affinity antibodies applicable for uses targeting native, human PAI-2. Furthermore, by immunisation with polymerised PAI-2, the aim was to create mAbs for PAI-2-polymer specific applications. Lastly, mAbs created by immunisations with HEK expressed PAI-2 could be a valid candidate for the replacement of the pAb used in the ELISA based assay established in this study.

6.1 PAI-2 polymerisation

To be able to establish methods to investigate PAI-2 polymerisation, there was a need to establish a method of inducing PAI-2 polymerisation.

This was accomplished by exposing PAI-2 to denaturing conditions. In 1993, Mikus and colleagues found that the addition of 1.3M GuHCl to PAI-2 induced almost total polymerisation of a PAI-2 sample [46]. Furthermore, other studies have shown that the exposure of other Serpins to heat can induce polymerisation [68]. Based on this, samples of PAI-2 were diluted to a concentration of 100 μg/mL and exposed to different conditions; 7 samples had different concentrations of GuHCl added, while 10 samples were incubated at either 37°C or 63°C, respectively, for different time intervals. As expected, polymerisation was induced. In samples with GuHCl, all samples showed some degree of polymerisation, but concentrations $\geq$1M induced a high level of polymerisation in the samples, when analysed on SDS-PAGE (figure 5.2), while native PAGE analysis indicated almost total poly-

merisation of the samples (figure 5.3). The results from heat polymerisations were very similar. All samples showed some level of polymerisation (figure 5.4), even in those exposed to 37°C, but native PAGE analyses suggested almost total polymerisation of the samples left at 63°C for 10-60 minutes (figure 5.5). Polymerisation in samples exposed to 37°C was expected, as studies have shown that polymerisation of PAI-2 occurs spontaneously even at physiological conditions [46, 55, 78].

One unusual result obtained with the polymerisations of PAI-2 performed in this study, was the fact that polymerised PAI-2 was still present in the western blots of polymerisations with 37°C and 63°C (figure 5.4D and H). It is well established that a disulfide bond is required for the polymerogenic conformation that allows PAI-2 to form polymers with nearby polymerogenic PAI-2 molecules [51]. The reducing agents β-mercaptoethanol and DTT used in this study, reduce this disulfide bond by donating an electron and accepting a sulfide. For this reaction to be successful, an excess of the reducing agent is required, as each disulfide bond requires its own reducing molecule for complete reduction of all disulfide bonds in the sample [79].

One explanation that polymerised PAI-2 was still seen in figure 5.4D and H might be that β- mercaptoethanol was not added in a high enough concentration to reduce all disulfide bridges. This corresponds with the fact that most polymerised PAI-2 had been reduced, and only some prevailed in the sample. However, the same amount of sample and β-mercaptoethanol was added to the samples displayed in figure 5.2D and all polymers appear to have been reduced on this blot. Thus, further investigations must examine if it is possible to reduce all polymerised PAI-2 in the samples displayed in 5.4D and H, for example with a titrating approach, using different concentrations of β-mercaptoethanol or DTT.

Moreover, PAI-2 protein in samples appeared largely degraded after four hours at 63°C. This was evident in both SDS-PAGE analysis as well as on native PAGE. Coomassie staining of the gels (figure 5.4E and F) suggested degradation of nearly the entire sample, while the western blots (G and H) showed that some PAI-2 still prevailed in the samples. The thermostability of PAI-2 has not been thoroughly studied. One study by Mikus and colleagues investigated the thermostability of PAI-2, and their results showed that after exposing PAI-2 to 60°C for two hours, nearly 40% of PAI-2 antigen remains in the sample [46]. This is in good concurrence with the results obtained in the present study, as some of the PAI-2 sample remained as bands in samples incubated for four hours - overnight in 63°C on the blots, while most of the sample appeared to be degraded.

It is notable that the polymerisation-inducing methods used in this study, are also considered to have a denaturing effect on proteins. Thus, prolonged exposure to either method is likely to induce degradation of the samples, which was also seen in this study, especially when HEK expressed PAI-2 was exposed to 63°C for four hours and overnight.

In addition to the results obtained regarding the polymerisation, one unexpected observation was made when comparing the non-reduced and the reduced HEK expressed PAI-2 samples. When looking at figure 5.2, it was evident that a second band appeared just above 40 kDa, when reducing the sample, while the protein band observed near 60 kDa in the non-reduced sample, lessened in intensity. The same observations applied to similar analyses (figure 5.4). The reason for this apparent drop in molecular weight is currently unknown, but might be due to the presence of non-intact PAI-2 in the protein expressed by HEKs. No available studies comments on similar changes in MW, only a reduction of the 47 kDa form to a 43 kDa have been observed previously in reduced samples or from cellular extracts [51, 55]. No fragments indicating cleavage of PAI-2 were observed on either Coomassie stained gels or western blots (see appendix B). The hypothesis that the drop observed in this study was a result of a fault of the HEKs in expression of PAI-2, was supported by the fact that these observations were not reproducible when reducing PAI-2 precipitated from pregnancy plasma samples (figure 5.11), where only the PAI-2 polymers appeared reduced in the reduced sample. However, the reason for this change in MW in the HEK expressed PAI-2 must be characterised in future studies.

In summary, different methods for inducing polymerisation was established in this study. Polymerisation occured not only spontaneously in PAI-2 samples at physiological temperatures (37°C), but was induced to a higher degree by denaturing conditions, with the addition of GuHCl in concentrations of 1-2M or by exposure to 63°C for 10-60 minutes. Altogether, the results obtained in this study regarding polymerisation in PAI-2 samples largely corresponded with the results provided by earlier research.

6.2 Establishment of an ELISA

An ELISA based assay was established with the objective of being able to quantify PAI-2 concentrations in pregnancy plasma samples. The ELISA was of the sandwich type, meaning that a capture antibody coats the wells to capture antigen from the samples, and a detection antibody is placed on

top to detect the antigen bound by the capture antibody.
In the assay established in this study, mouse Monoclonal Antibody 19-26-3 was used as a capture antibody to capture PAI-2 from a sample, then detected by a Polyclonal Antibody. This setup provided dose-response curves (figure 5.6A) indicating that PAI-2 was detectable at concentrations lower than those expected in pregnancy plasma samples (up to 200-300 ng/mL) [5, 19–21]). Furthermore, the dose-response curves portrayed in figure 5.6B suggested that the ELISA could distinguish PAI-2 in plasma samples from other components of plasma. Thus, the basic setup for an ELISA capable of quantifying the PAI-2 levels in pregnancy plasma samples has been developed in this study. However, it is important to note that this assay is still under development and remains to be validated.

After the ELISA based PAI-2 assay was established, it was used to measure the levels of PAI-2 in samples from preeclampsia patients and controls. The results of this reflected no clear pattern, however, the mean of PAI-2 concentration seemed to be slightly depressed in the preeclampsia group, compared with the control group (figure 5.8). The currently available research primarily suggests that a difference can be seen when comparing the plasma levels of PAI-2 in preeclampsia patients and controls [23, 26, 29, 33, 80], while a few suggest no significant differences [30, 81]. Differences in approach and sample sizes might explain this difference. In this present study, only 10 patient samples were included in each group, and there might be a need to include a larger sample size to obtain a more pronounced difference between the two groups. Along with further validation of the ELISA established in this study, a larger sample size and a validated assay will provide a better tool for investigations of the PAI-2 levels in preeclampsic patients and controls.

Some of the previous literature separate preeclamptic patients into either groups of severity, or into groups with or without established IUGR. The results of this generally suggest that preeclamptic patients with IUGR have decreased levels of PAI-2 in plasma samples, while preeclampsia without IUGR have levels less decreased or not significantly different PAI-2 levels, compared with controls [9, 24, 26, 27, 29–31]. Because IUGR have been suggested to be a result of decreased placental function, and because PAI-2 levels have been shown to correlate with the presence of IUGR, there is a good consensus in the literature that PAI-2 is a good candidate for a biomarker to reflect placental dysfunction [20, 24, 27, 29, 30, 32, 33, 80–82]. The samples analysed in this study have not been categorised based on the presence of IUGR, but future studies might consider including this to obtain more specific results regarding the influence of PAI-2 on preeclampsia and IUGR.

Samples analysed in this study were all obtained in the third trimester of pregnancy. Previous research has suggested that the time of blood sampling matters, as studies that investigates PAI-2 levels at different time points only found significant differences at their latest sample point [22, 26]. However, one of these studies obtained significant differences of PAI-2 levels in the second trimester [22], while the other only obtained significance late in the third trimester [26]. The studies discussed here and above in this section have a widespread sampling time, between 24-42 weeks of gestation, and most of these resulted in a significant difference between PAI-2 levels in preeclampsia patients and controls [9, 20, 25–27, 29, 33, 80, 82, 83].

Though different groups have found a correlation between PAI-2 levels and the prevalence of preeclampsia, decreased PAI-2 levels must precede the emergence of preeclampsia, if PAI-2 is to be used as a biomarker to predict preeclampsia. Few groups have studied this, with varying results [5, 27, 34]. Halligan and colleagues studied a group of a total of 36 pregnancies, of which 4 later developed preeclampsia. Their results indicated no difference in PAI-2 levels between the two groups, thus indicating no predictive potential of PAI-2 in preeclampsia [27]. Contrarily, Clausen and colleagues found a positive association between PAI-2 levels early in pregnancy and the later presentation of preeclampsia. However, in contrast to all other studies cited in this section, their association of PAI-2 with preeclampsia came with an increased level of PAI-2 in plasma of the group destined to develop preeclampsia [34]. Akolekar and colleagues concluded that the decreased levels of PAI-2 in preeclampsic patients does not precede the clinical onset of preeclampsia, as they found no statistically significant difference in PAI-2 levels between pregnancies that was later affected by preeclampsia [5].

On an important note, the levels of PAI-2 measured in pregnancy plasma samples in this study, gave a range of 155-1134 ng/mL; mean 423.8 ng/mL PAI-2 for control samples, and a range of 153-1107 ng/mL; mean 376.25 ng/mL PAI-2 for preeclampsia samples. Some of the values obtained were larger than those expected in pregnancy plasma samples (up to 200-300 ng/mL) [5, 19–21]). The likely reason of this, was that the standard curve (see appendix A) was constructed using the purified HEK expressed PAI-2 sample. This sample had a concentration calculated to 1.3 mg/mL from measurements on a NanoDrop Spectrophotometer [Data not shown]. However, this sample might contain impurities, and these are included in the calculation of the concentration, thus providing a higher total concentration of the sample than the actual concentration of PAI-2 in the sample. This might skew

the measurements obtained using the ELISA, giving larger quantities of PAI-2 than actually present in the plasma sample. This uncertainty must be addressed in the further development of the assay.

In summation, the results obtained in the present study did not suggest a clear pattern of PAI-2 levels in preeclamptic patients and controls. There is a general consensus in the available literature that PAI-2 levels are decreased in patients presenting with preeclampsia. Moreover, there is a general agreement that PAI-2 seems to be an indicator of placental health, with decreased levels indicating placental insufficiency, and not specific to preeclampsia. Whether or not PAI-2 is a valid candidate for a biomarker to predict the emergence of preeclampsia is not as well agreed upon, and more research is needed before conclusions can be drawn.

6.3 The State of PAI-2 in Pregnancy Plasma

An effort to visualise the polymerisation state of PAI-2 was made in this study. This was done by using mouse mAb 19-26-3 bound to magnetic DynaBeads. No corresponding investigations of polymerisation of PAI-2 in plasma samples from pregnant individuals have been identified in the available literature. The results obtained in present study suggested that PAI-2 was available in pregnancy plasma in both an intact, non-polymerised state at approximately 60 kDa, as well as in polymerised forms at different sizes.

In 1988, Booth and colleagues used zymography with SDS-PAGE to show that PAI-2 circulates in pregnancy plasma in two forms, a fast form of 70 kDa and a slow form of 130 kDa [84]. The results obtained in this present study were similar, however with bands of approximately 60 kDa and with polymerisation at different sizes $\geq$110 kDa. In the study by Booth and colleagues, they hypothesise that the slow form of PAI-2 was due to protein aggregations or interactions with other proteins. However, in present study, it was confirmed that the PAI-2 available in pregnancy plasma samples at sizes of 110 kDa and above, was indeed a result of the polymerisation of PAI-2. Since these protein bands are not present under reducing conditions (figure 5.11), they must be dependent on a disulfide bond, thus providing evidence that it is indeed polymers of PAI-2, as this polymerisation is dependent on a disulfide bond [51].

After precipitation of PAI-2 had proved successful from pregnancy plasma samples, immunoprecipitation was performed with the same method on plasma samples from preeclamptic patients as well as from healthy controls. This was done to investigate if there was any difference in the

polymerisation status of PAI-2 in plasma from preeclampsia patients. The results suggested that all samples had PAI-2 present in both the intact form as well as in polymerised forms with bands $\geq$110 kDa. There was no clear pattern as to whether preeclamptic patients had more or less polymerised PAI-2 than their healthy counterparts. These samples were also analysed using native PAGE methods. This gave similar results, showing that all analysed samples, regardless of preeclampsia, had both intact PAI-2 as well as PAI-2-polymers. One study using zymography to detect active PAI-2 in pregnancy samples from preeclampsia patients and controls was available [20], and similarly to the study mentioned above [84], they found PAI-2 in pregnancy plasma samples in a fast form of 70 kDa and a slow form of 130 kDa [20]. When investigating these two forms in healthy pregnancies and preeclampsia afflicted pregnancies, their results suggested that patients afflicted by preeclampsia had reduced levels of PAI-2, both the 70 kDa and the 130 kDa form, with the 130 kDa form being the most reduced [20]. Nonetheless, it must be noted that their methods rely on the activity of PAI-2, as PAI-2 must react with uPA to be detected [84, 85]. This is important, as PAI-2 polymers have later been reported to be inactive [51, 55], thus should not give a corresponding signal. Therefore, it is not clear whether the observed slow form of 130 kDa is due to polymerisation or other protein interactions. They also measured the amount of PAI-2 in the plasma samples and observed a notable decrease of PAI-2 concentration in preeclampsia samples. Therefore, the reduced levels of PAI-2 seen on their SDS-PAGE were likely due to an overall decrease of PAI-2 concentration in the samples [20].

Nevertheless, the results obtained in this study regarding the polymerisation status of PAI-2 in pregnancy plasma suggested that PAI-2 was available in both an intact form as well as in polymers of different sizes. Future research should focus on investigating the relationship between these two forms, and determine if polymerised PAI-2 is increased in preeclamptic patients, relative to the total PAI-2 concentration in plasma. Furthermore, the end-goal should be determining if PAI-2 polymers play a role in the pathogenesis of preeclampsia.

6.4 Production of Rat Monoclonal Antibodies

Simultaneously to the development of the ELISA, and with the experiments to visualise PAI-2 from plasma samples, a novel group of mAbs were produced. The mouse mAbs used in this study, were produced by immunising a mouse with the *E. coli* expressed PAI-2 (R&D Biosystems®, 9206-PI-025). The rat mAbs produced in present study were generated by immunising a rat with the HEK

expressed PAI-2. The difference herein lies mainly on the post-translational modifications that the cells can perform with expression. Thus, PAI-2 expressed in a mammalian systems (i.e. in HEKs) should be glycosylated correctly, and therefore be closer corresponding to the native, human PAI-2 expressed in the placenta of a pregnant individual, than the PAI-2 expressed in an eukaryotic system (i.e. in *E. coli*) [77].

The antibodies were produced by immunising two rats, one with intact, non-polymerised PAI-2, while a second rat was immunised with PAI-2 polymerised by 1.5M GuHCl. Hybridomas were obtained and cultured, and hybridomas from the first rat were screened for antibodies with affinity for intact, non-polymerised PAI-2, and those from the second rat were contra-screened, with the purpose of identifying clones with a higher affinity for PAI-2-polymers. Positive clones were selected and subcloned until single clones were obtained and moved to flasks.

The purpose of the novel generation of mAbs was mainly to achieve high-affinity anti-PAI-2 antibodies, with capabilities allowing for antigen-recognition of the native, human PAI-2. Furthermore, with the immunisation with polymerised PAI-2, generation of PAI-2-polymer specific antibodies was attempted.

The antibodies obtained from this process were evaluated based on their performance in several applications; western blotting, ELISA and immunoprecipitation. Most of the produced antibodies were able to bind and give clear protein bands corresponding to PAI-2, both in the intact, non-polymerised form, as well as the polymerised form, with little to no background, when used for western blotting (figure 5.14). This indicated that the antibodies produced herein could bind PAI-2 specifically with little to no non-specific binding to the PVDF membranes used for western blotting or impurities in the loaded samples.

Similarly, mAbs were tested in their ability to bind PAI-2 and polymerised PAI-2. ELISA plates were coated with a serial dilution of 0.2 μg/mL of either intact PAI-2 or polymerised PAI-2. The results indicated that antibodies that bind polymerised PAI-2 also bind intact, non-polymerised PAI-2 with similar affinity. Furthermore, low background indicated little to no non-specific binding to the ELISA wells or impurities in the samples (figure 5.15).

One of the objectives of this study was to obtain PAI-2 polymer specific mAbs that could be used to establish a PAI-2 polymer specific assay. This was attempted by immunising one of the rats with polymerised PAI-2. Initial contra-screenings gave no indication of PAI-2-polymer specific antibodies

[Data not shown].

There could be different reasons as to why mAbs specific to polymerisation of PAI-2 were not obtained in this study. As mentioned earlier, the conformations of PAI-2; non-polymerising conformation and polymerogenic conformation that can form the loop-sheet polymers, are fully inconvertible depending on the milieu PAI-2 is present in. Even though the oxidative conditions in the tissue and blood [86] of the rat should allow for the polymerogenic conformation and the polymers of PAI-2 to remain, it is difficult to accurately predict what happens to the polymerised PAI-2 once it enters the rat. If the polymers are dissociated after injection into the rat, only intact PAI-2 will give rise to antibodies.

The rat mAbs produced in this study were also tested in their ability to precipitate PAI-2 from pregnancy plasma. Results using mouse mAbs showed that this was possible using DynaBeads^{TM} M-280 Sheep Anti-Mouse IgG (Invitrogen^{TM}, 11202D) in combination with mouse mAb 19-26-3. In light of this, Invitrogen^{TM} DynaBeads^{TM} Sheep Anti-Rat IgG (Invitrogen^{TM}, 11035) were purchased for use in conjunction with rat mAbs. Similarly to analyses with mouse mAbs, rat mAbs was first tested in the ability to precipitate HEK expressed PAI-2 from a prepared sample with a known concentration, in pools of 2-3 culture supernatants. This analysis suggested that all rat mAb pools were capable of precipitation of HEK expressed PAI-2 from a prepared sample (figure 5.16). However, when testing the same mAb pools on pregnancy plasma samples, no PAI-2 appeared to be precipitated (figure 5.17). There are several possible reasons for the inability of the produced antibodies to perform immunoprecipitation of PAI-2 from pregnancy plasma when used in combination with Sheep Anti-Rat IgG DynaBeads^{TM}.

One explanation could be that the antibodies produced in the present study simply do not recognise native PAI-2 present in human pregnancy plasma. The antibodies have been produced by immunisation with a HEK expressed recombinant human PAI-2, expressed with a standard signal peptide to ensure secretion, and a C-terminal hexa-His sequence, to enable purification. These are both present in the protein after purification from HEK culture supernatant. They are therefore also present in the PAI-2 samples used for immunisations of the rats. Thus, there is a chance that antibodies were produced with specificity towards either of these elements which are not present on the native PAI-2 in human pregnancy plasma. This could explain why the application of the produced antibodies in immunoprecipitation with pregnancy plasma fails, as the native, human PAI-2 would not have the epitope the applied antibodies recognises. It would therefore not be captured and precipitated. However,

when testing the antibodies on ELISA on an irrelevant antigen, also carrying the C-terminal hexa-His sequence bound to the MaxiSorp plates, the antibodies did not bind and provide a signal at 450 nm [Data not shown], thus indicating that this is not the case. Furthermore, it seems unlikely that out of the total of 13 antibodies applied, all antibodies have specificity for an epitope not occurring on the human, native, placental PAI-2 protein.

Another possible reason that the immunoprecipitation of PAI-2 from pregnancy plasma failed, could be that the chosen DynaBeads are not applicable for protein precipitation from plasma samples. DynaBeads^{TM} M-280 Sheep Anti-Mouse IgG used for immunoprecipitation with mouse mAbs are specifically marketed for use to precipitate target proteins from a solution (appendix C). Contrarily, DynaBeads^{TM} Sheep Anti-Rat IgG used for immunoprecipitation with rat mAbs are only marketed for the use of isolation cells from a heterogeneous cell sample (appendix D). Furthermore, literature using the anti-rat DynaBeads only contain studies for cell isolation, including the citation provided by ThermoFisher^{TM}. Thus, the reason for the inability to immunoprecipitate PAI-2 from pregnancy plasma with the novel rat mAbs in this study, might be due to a technical issue with the applied DynaBeads, and not due to an inability of the mAbs to bind native, human PAI-2.

Why these DynaBeads were not applicable for immunoprecipitation of a target protein is not clear, but whether this is the cause for the failure of the immunoprecipitation of PAI-2 from pregnancy plasma, needs to be fully investigated.

6.5 Contact Activation - Implications in Preeclampsia

As described in section 2.3, polymerised proteins have previously been found to activate CAS [71]. This is interesting, when investigating PAI-2 in a preeclampsia perspective. PAI-2 has previously been found to form polymers spontaneously *in vitro*, even at physiological expression levels in physiological conditions [46]. Therefore, this study sought to investigate the state of PAI-2 in pregnancy plasma. The results suggested that all the analysed pregnancy plasma samples had an amount of polymerised PAI-2.

As described in section 2.3, a mutation in the gene encoding for C1INH results in a phenotype more inclined to form polymers [68]. C1INH polymers have been shown to mediate FXII activation *in vitro*, resulting in activation of the kallikrein-kinin system [69]. Thus, the mutations in C1INH that results in a protein more inclined to form polymers, not only result in loss-of-function, but may also

result in gain of function [69]. The hypothesis in this present study was that a similar mechanism plays a role in preeclampsia. Since PAI-2 polymers have been shown to be present in pregnancy plasma samples in the current study (figure 5.12 and 5.13), and previously [20, 84], and because protein-polymers have previously been found to do so [71], PAI-2 polymers could initiate CAS activation. This might cause an amplification of fibrin-formation (figure 2.4), causing an imbalance between the fibrinolytic system and the coagulation system, and/or cause an increased amount of Bradykinin (the kallikrein-kinin system, figure 2.3). Both of these outcomes could possibly play a role in the development of preeclampsia. As preeclampsia is characterised by endothelial dysfunction as well as pro-inflammatory, pro-coagulant, and pro-fibrinolytic processes [87], it is not unlikely that the formation of PAI-2 polymers might contribute to the development of preeclampsia.

7 Future Perspectives

In this study, an ELISA was established, with the ability to to quantify PAI-2 levels in pregnancy plasma. However, this assay must be validated for use in future experiments. Several analyses and different methods are needed to validate an ELISA, and the assay must be tested in robustness, preciseness, and limits of detection, among others. Once the assay is validated and fully developed, it is a tool for use in further experiments to analyse plasma samples from pregnancies with or without preeclampsia.

Another aspect of the ELISA is the possibility of replacing the detection antibody. In this study, a pAb was used for detection of PAI-2 captured by the mouse mAb 19-26-3. However, the accessibility of a pAb will always be limited, and it can therefore be favourable to replace the pAb used in this assay. Future studies should therefore investigate if any of the rat mAbs produced in this study can be used in such applications. Furthermore, the interest of future studies can be to refine the assay established in this study in a way to detect only polymerised PAI-2 from pregnancy samples, to determine if plasma samples from preeclampsia patients have a higher level of PAI-2 polymers, compared with healthy controls, and if this plays a part in the pathology of preeclampsia.

In continuation of this, a method to visualise the state of PAI-2 was developed in this study. This was done using magnetic DynaBeads for immunoprecipitation of PAI-2 from pregnancy samples, and analysing the results using SDS-PAGE and native PAGE. However, to be able to determine if there is a difference between the levels of intact and polymerised PAI-2 between preeclamptic and control samples, there is a need to find quantifiable methods to evaluate the results of these analyses.

Immunoprecipitation of PAI-2 from pregnancy plasma with DynaBeads and the novel rat mAbs was not successful in this study. The reason for this might be that the rat mAbs simply do not recognise native PAI-2. However, there is a chance that it might be due to a technical issue and the fact that the Sheep Anti-Rat IgG DynaBeads are not applicable for the precipitation of proteins from plasma samples. This needs to be fully investigated before any conclusions can be drawn. This investigation can be executed with several different methods. One method is to use established rat mAbs that have been tested in their ability to recognise an antigen present in plasma samples. If this is successful, the beads must be applicable for such a purpose, and the inability to precipitate PAI-2 from pregnancy plasma in this study, must be due to the antibodies not recognising native PAI-2 from pregnancy plasma samples. However, this is a rather difficult experiment to conduct, as a new rat mAb, already

confirmed to work on native proteins in plasma samples, must be procured. Furthermore, an antibody that can detect immunoprecipitated protein on a western blot must also be obtained, to confirm immunoprecipitation. Another way would be to test the ability of the antibodies to bind native PAI-2, for example by using established immunoprecipitation methods with the rat mAbs produced herein.

In conclusion, a model for PAI-2 polymerisation was established in this study. Polymerisation can be performed by exposure of PAI-2 samples to either 63°C or $\geq$1M GuHCl. This polymerisation model can be used in further research to deeper investigate the relationship between PAI-2 polymerisation and preeclampsia.

An ELISA was established to quantify PAI-2 levels in pregnancy plasma. This assay is capable of distinguishing PAI-2 from other components of plasma, as evident by the low backgrounds obtained when analysing male plasma samples. However, this assay has not been validated herein, and there is a need for further investigation of the assay, before it ready for use in preeclampsia research.

The state of PAI-2 was visualised using immunoprecipitation. This means that a model has now been established for use in future research to investigate if preeclampsia patients present with a higher level of PAI-2 in their plasma. However, the method of western blotting used in this study is somewhat qualitative and could be prone to observer bias, and methods for quantitatively measuring the ratio between PAI-2 and polymerised PAI-2, and even the ratio between polymerised PAI-2 in preeclampsia samples and healthy controls, must be established for further research.

Lastly, an effort was made in this study to produce novel rat monoclonal anti-PAI-2-polymer antibodies. The results shown herein suggested that this was not successful. In future attempts, a new immunisation should be performed, preferably using stable PAI-2 polymers that will not dissociate when entering the host. Nonetheless, rat monoclonal anti-PAI-2 antibodies were produced in this study. They proved to be useful in both western blotting and ELISA methods, where they were able to detect both intact PAI-2, as well as polymerised PAI-2. Futhermore, immunoprecipitation of native PAI-2 from pregnancy plasma samples with DynaBeads proved ineffective. Further research must determine if this is due to the antibodies not recognising the native PAI-2, or if it is due to a technical issue with the DynaBeads not being applicable for such purposes.

PAI-2-polymers could play a role in the development of preeclampsia. Currently, no available literature investigates the involvement of CAS in preeclampsia. The tools developed in this study are meant to aid in such future investigations.

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A Standard Curve

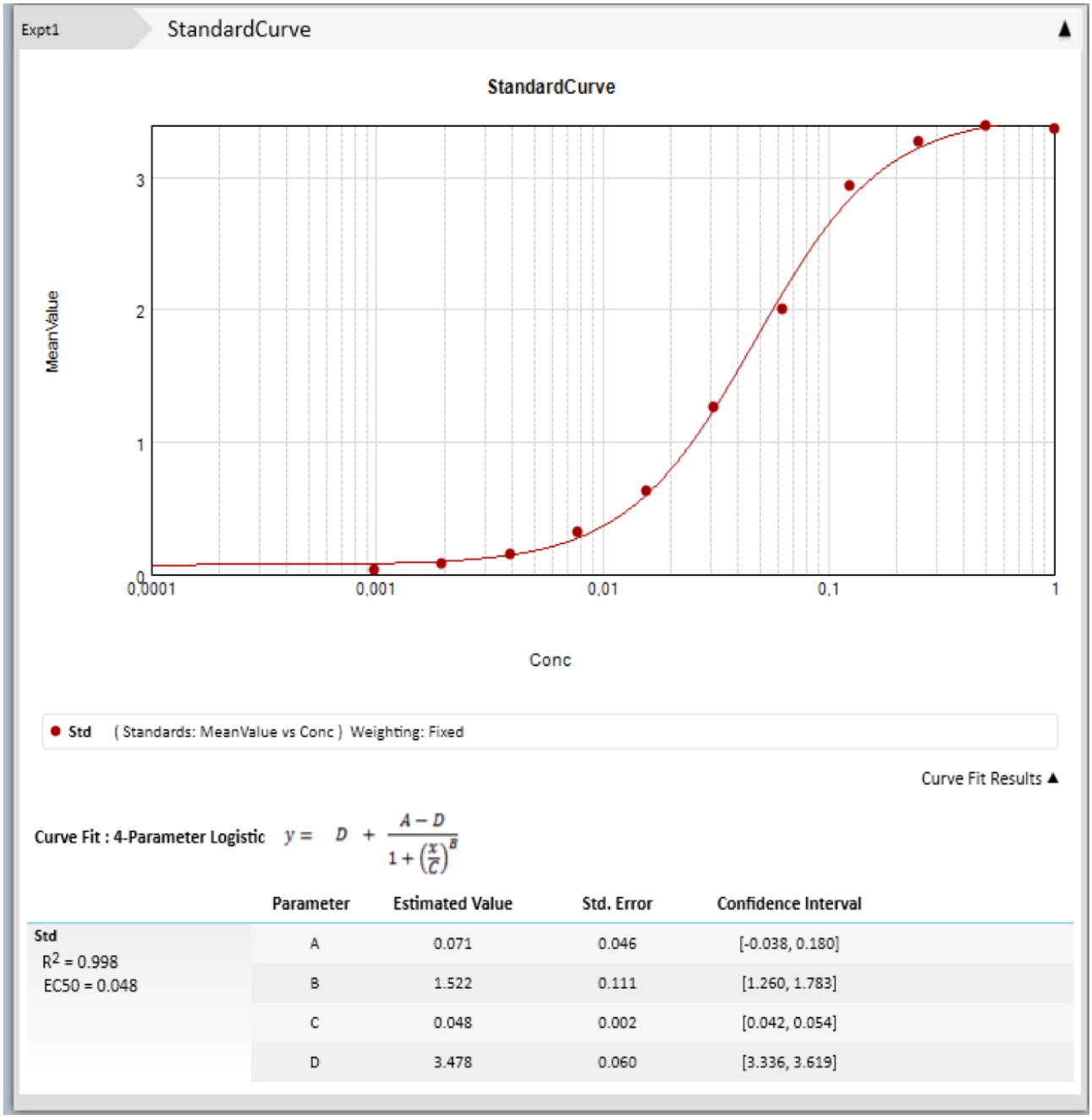

Figure A.1: Standard curve used to interpolate PAI-2 concentration of pregnancy plasma.
This standard curve was generated using OD values obtained from a serial dilution of HEK cell expressed PAI-2 in a 2-fold serial dilution starting at 1 μg/mL. The standard curve was constructed by the software Softmax Pro®with a four-parameter logistic fit model.

B Uncropped SDS-PAGE and Western Blots

Gels and blot were displayed in this study in cropped versions. However, to show that no cleaved fragment were observed, in continuation of the discussion in section 6.1, the figures in question are displayed here uncropped.

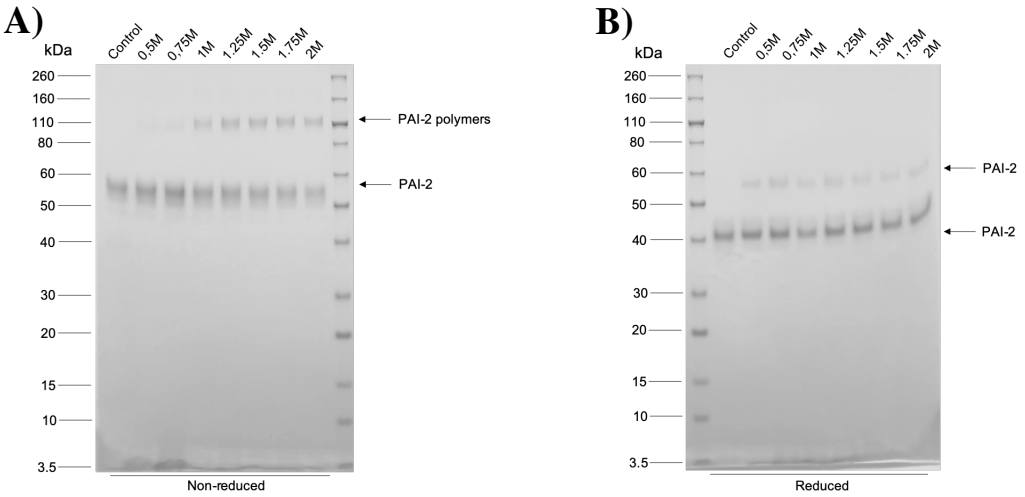

Figure B.1: Figure 5.2A&B in an uncropped version.

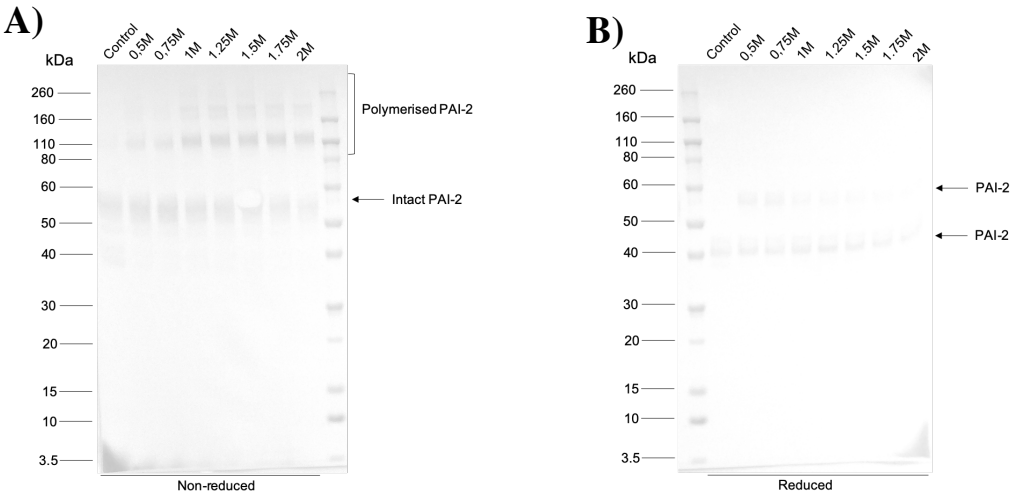

Figure B.2: Figure 5.2C&D in an uncropped version.

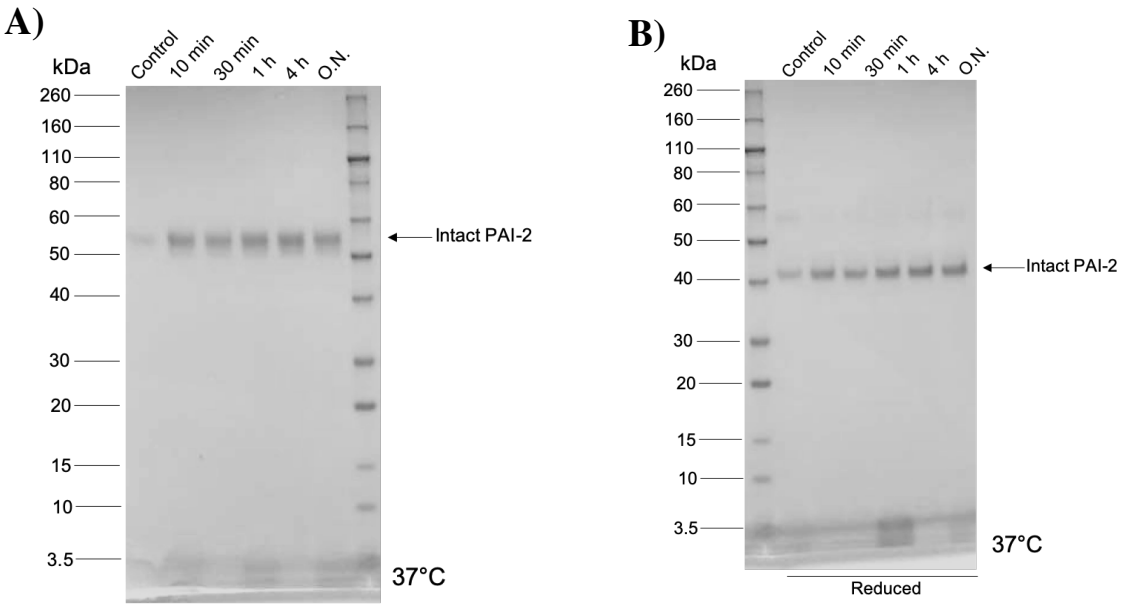

Figure B.3: Figure 5.4A&B in an uncropped version.

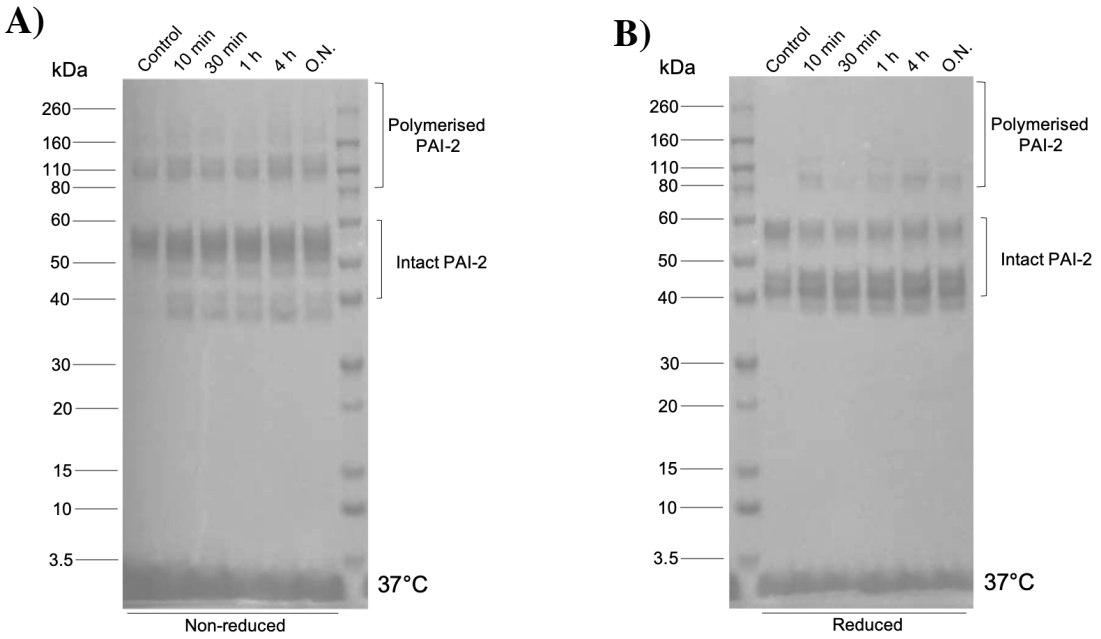

Figure B.4: Figure 5.4C&D in an uncropped version.

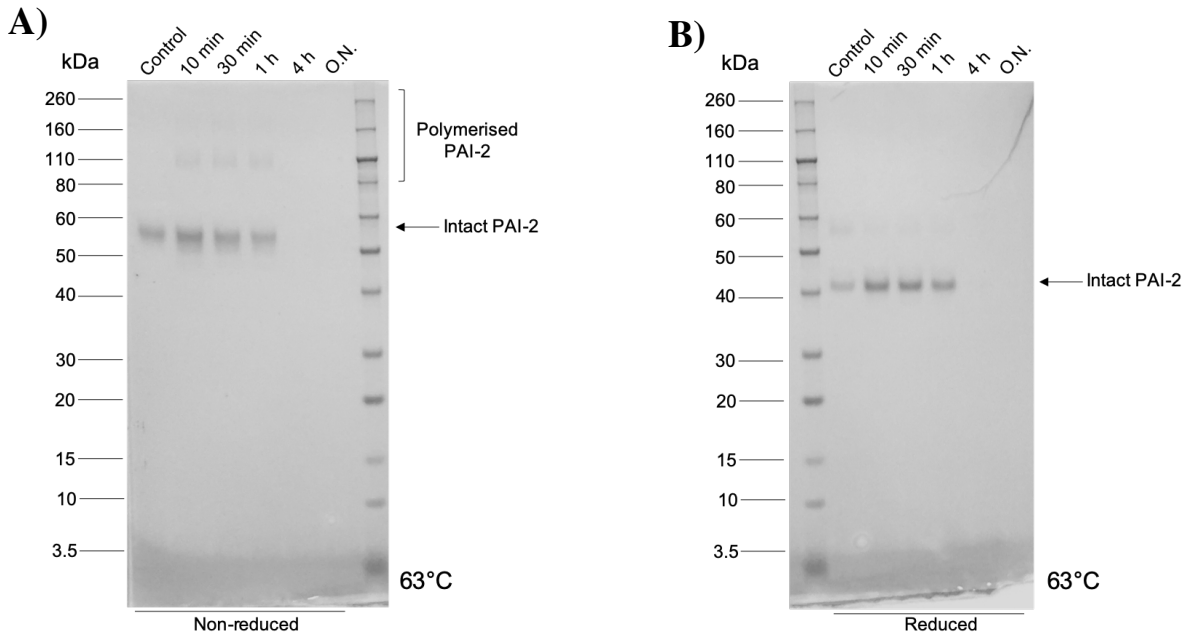

Figure B.5: Figure 5.4E&F in an uncropped version.

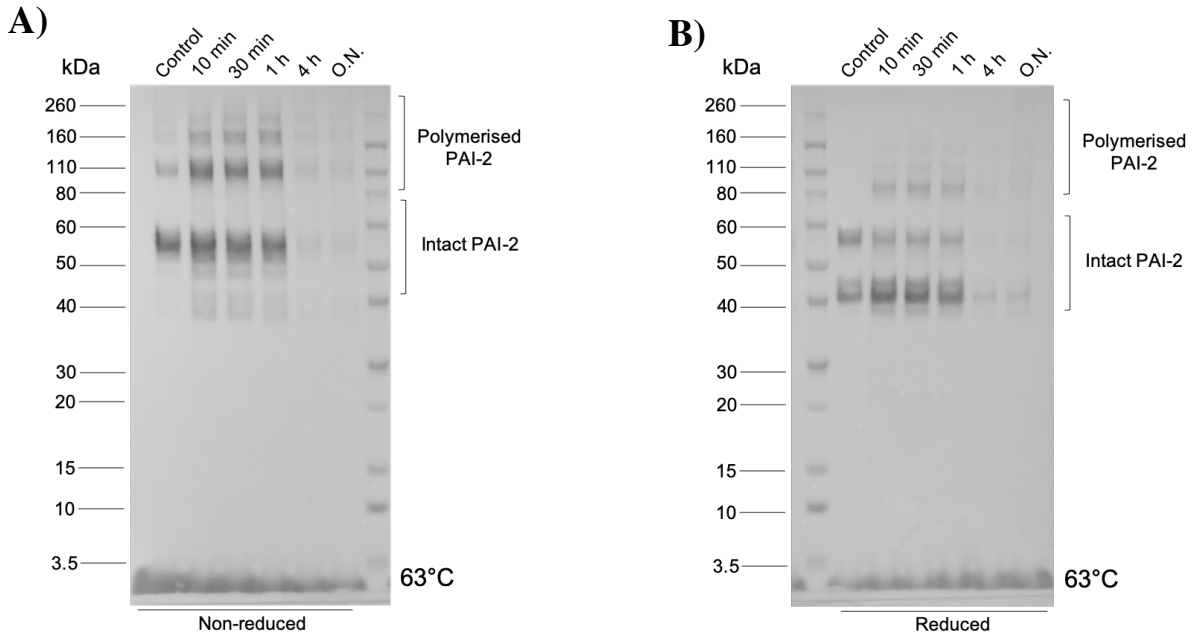

Figure B.6: Figure 5.4G&H in an uncropped version.

C Sheep Anti-Mouse IgG Dynabeads

novex
by life technologies

Dynabeads® M-280 Sheep anti-Mouse IgG
Catalog nos. 11201D, 11202D

Dynabeads® M-280 Sheep anti-Rabbit IgG
Catalog nos. 11203D, 11204D

Store at 2°C to 8°C

Rev. Date: May 2012 (Rev. 000)

Product Contents

Product contents	Cat. no.	Volume
Dynabeads® M-280 Sheep anti-Mouse IgG	11201D	2 mL
	11202D	10 mL
Dynabeads® M-280 Sheep anti-Rabbit IgG	11203D	2 mL
	11204D	10 mL

Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG both contains 6–7 × 10^8 beads/mL (~10 mg/mL) in phosphate buffered saline (PBS) pH 7.4 with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide.
Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Product Description

Note that this manual describes the protocols for two separate products: Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG. The protocols and handling steps are the same; the only difference being different primary antibody targets:

- Dynabeads® Sheep anti-mouse IgG bind defined antigens via a mouse primary antibody.
- Dynabeads® Sheep anti-Rabbit IgG bind defined antigens via a rabbit primary antibody.

Dynabeads® M-280 Sheep anti-Mouse IgG and Dynabeads® M-280 Sheep anti-Rabbit IgG are designed as a solid support for simple and efficient binding of immunoglobulins (Ig) or other target molecules (see fig. 1).

The size of the beads (2.8 µm) makes them particularly suitable for isolation of antibodies (Ab) and their target proteins. The beads can also be used for cell isolation, but visit www.lifetechnologies.com/dynabeads to view our full range of cell isolation products.

The beads with primary antibody may be added directly to the sample containing your target antibody/antigen. The beads bind to the target during a short incubation, then the bead-bound target is separated by a magnet (direct technique). Alternatively, the primary antibody is allowed to bind to the target in suspension prior to adding the beads (see fig. 1).

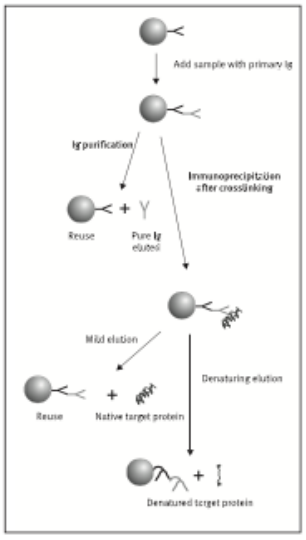

Figure 1: Overview of method

Required Materials

- Magnet (DynaMag™ portfolio). See www.lifetechnologies.com/magnets for recommendations.
- Mixer allowing tilting and rotation of tubes (e.g. HulaMixer® Sample Mixer).
- Primary mouse antibody for Dynabeads® M-280 Sheep anti-Mouse IgG.
- Primary rabbit antibody for Dynabeads® M-280 Sheep anti-Rabbit IgG.

Recommended Buffers

- Washing Buffer: Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4.
  Note: BSA can be replaced by human serum albumin (HSA) or fetal calf serum (FCS). EDTA can be replaced by sodium citrate.
- Elution Buffer: 0.1 M citrate, pH 2-3.

General Guidelines

- Visit www.lifetechnologies.com/samplepreparation for recommended sample preparation procedures.
- Use a mixer that provides tilting and rotation of the tubes to ensure that the beads do not settle in the tube.
- These products should not be used with the MPC™-1 magnet (Cat. no. 12001D).
- Avoid air bubbles (foaming) during pipetting.
- Never use less than recommended volume of beads.
- Carefully follow the recommended volumes and incubation times.

Direct vs. Indirect Technique

- *Use the indirect technique when:* The concentration of antibody is low, the antibody-antigen affinity is weak, the binding kinetics is slow or the direct technique gives unsatisfactory purity.
- *Use the direct technique when:* The affinity of the primary antibody is high, there are high numbers of target antigens, or to make a larger stock preparation of primary coated beads (will generally have the same shelf life as stated on the beads vial).

Cross-linking prior to Immunoprecipitation

Immunoprecipitation (IP) is done by either adding the primary coated beads directly to a new sample containing the target protein, or by first covalently cross-linking the primary Ig to the antibody on the beads. Bound Ig will be co-eluted along with the target using different elution methods (e.g. for SDS-PAGE followed by Western blotting or autoradiography). For other applications (e.g. protein purification or amino acid sequencing) where co-elution of the Ig is not desired, the primary Ig should be cross-linked to the antibody on the beads prior to IP. If the Ig-coated beads are to be re-used, cross-linking is necessary.

Target Protein Elution

One of the major advantages of using Dynabeads® products in protein/Ig isolation is the possibility to elute in small volumes. Low pH (2.8–3.5), change in ionic strength, affinity elution, electrophoresis, polarity reducing agents, deforming eluents can be applied, or even boiling the bead-target complex in SDS-PAGE application buffer for direct characterization of protein on SDS-PAGE. The method of choice depends on the Ig's affinity for the specific target protein, stability of target protein and downstream applications and detection methods. Most proteins will be eluted at pH 3.1. Some protein functionality might be lost under these conditions. If maintaining functionality of the target protein is important, try milder elution conditions, such as high salt (e.g. 2 M NaCl) or step-wise elution reducing pH from 6 down to 3. This is also recommended if the bead-bound ligand must remain functional to allow re-use of the beads.

Figure C.1: Datasheet and instructions for DynaBeads™ M-280 Sheep Anti-Mouse IgG (Invitrogen™, 11202D). Page 1. *Sourced from Thermofisher.com*

Protocol

Wash the Beads

See "Couple Beads with Target Ig" to determine the bead volume.

1. Resuspend the beads in the vial (i.e. vortex for >30 sec, or tilt and rotate for 5 min).
2. Transfer the desired volume of beads to a tube.
3. Add the same volume of Washing Buffer, or at least 1 mL, and resuspend.
4. Place the tube in a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed beads in the same volume of Washing Buffer as the initial volume of beads (step 2).

Couple Beads with Target Ig

- This protocol is based on 50 µL Dynabeads® Sheep anti-Mouse IgG or Dynabeads® Sheep anti-Rabbit IgG, but is directly scalable. It is not recommended to work with lower volumes. When working with larger volumes, scale up all volumes accordingly.
- Use 0.4–4 µg Ig/50 µL beads. Optimize for your application.

6. Add the sample containing ~0.4–4 µg target-Ig (optimize) to 50 µL of pre-washed and resuspended beads.
7. Incubate with gentle tilting and rotation for 30 min or up to 24 hours at 2°C to 8°C.
8. Place the test tube on the magnet for 2 min and pipet off the supernatant.
9. Remove the test tube from the magnet; add 1 mL Washing Buffer and resuspend.
10. Repeat steps 4 and 5 twice.
11. Place the tube on the magnet and remove the supernatant.

Elute Isolated Ig

- Elute the isolated Ig off the beads using 0.1 M citrate (pH 23) to lower the pH. Most Ig will be eluted off at pH 3.1, but the degree of acidity required will depend on the specific Ig.
- This protocol is based on the 50 µL beads from the "Couple the Beads with Target Ig" section. If using higher volumes, scale up accordingly.

1. Add 50 µL 0.1 M citrate to the Ig-coupled beads.
2. Mix well by tilting and rotation for 2 min.
3. Place the test tube on a magnJet for 2 min and transfer the supernatant containing the purified Ig to a new tube.
4. Add another 50 µL 0.1 M citrate to the bead fraction to elute any remaining Ig.
5. Mix well by tilting and rotation for 2 min.
6. Place the test tube on a magnet for 2 min, pipet off the eluate and pool the two supernatants containing pure Ig.

The Ig-eluted beads may be re-used at least five times. For re-use after elution, the beads should immediately be brought to neutral pH using a Na-phosphate buffer. For storage, the beads should be resuspended in Washing Buffer.

Cross-linking Ig to the Beads

If you want to avoid co-elution of the antibody, cross-link your antibody to the beads before continuing with e.g. IP. We recommend using the cross-linking reagent BS3.For further information and procedure, visit: www.lifetechnologies.com/crosslinking.

Antigen-Binding to Ig-Coated Beads

Use approximately 25 µg target antigen/mL beads to assure an excess of antigen. Dilute with PBS or 0.1 M phosphate buffer (pH 7–8), if necessary.

1. Add 25 µg target antigen/mL Ig-coupled beads.
2. Mix well by tilting and rotation for 1 hour. (Incubation time can be reduced to as low as 10 min, if the protein concentration is high).
3. Place the tube on the magnet for 2 min and pipet off the supernatant.
4. Remove the test tube from the magnet; add 1 mL Washing Buffer and resuspend.
5. Repeat steps 3–4 twice.

See "General Guidelines" for more information on elution of the target protein.

Description of Materials

Dynabeads® M-280 Sheep anti-Mouse IgG are uniform, superparamagnetic, polystyrene beads with affinity purified sheep anti-mouse IgG covalently bound to the bead surface. The antibody binds both heavy and light chains of mouse IgG1, IgG2a and IgG2b. They may have low reactivity towards mouse IgG3 and IgM. Human cross reactivity is minimal.

Dynabeads® M-280 Sheep anti-Rabbit IgG are uniform, superparamagnetic, polystyrene beads with affinity purified sheep anti-rabbit IgG covalently bound to the bead surface. The antibody binds all rabbit IgG subclasses.

Related Products

Product	Cat. no.
DynaMag™-2	12321D
DynaMag™-5	12303D
DynaMag™-15	12301D
Dynabeads® Protein A	10001D
Dynabeads® Protein G	10003D
HulaMixer® Sample Mixer	15920D

REF on labels is the symbol for catalog number.

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Manufactured by Life Technologies AS, Norway. Life Technologies AS complies with the Quality System Standards ISO 9001:2008 and ISO 13485:2003.

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SPEC-07657

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com

www.lifetechnologies.com

life technologies™

Figure C.1: Datasheet and instructions for DynaBeads™ M-280 Sheep Anti-Mouse IgG (Invitrogen™, 11202D). Page 2 *Sourced from Thermofisher.com*

D Sheep Anti-Rat IgG Dynabeads

invitrogen
by life technologies

Dynabeads® Sheep anti-Rat IgG

Catalog no. 11035 **Store at 2°C to 8°C**

Rev. Date: May 2012 (Rev. 004)

Product Contents

Product contents	Volume
Dynabeads® Sheep anti-Rat IgG	5 mL

Maximum product capacity
MNC*: ~2 × 10^9 cells
Whole blood/buffy coat: ~200 mL

*Note: If using the product for negative isolation of multiple cell types simultaneously, the bead volume used is higher, thus giving a lower product capacity (see Table 1 and 2).

Dynabeads® Sheep anti-Rat IgG contains 4 × 10^8 Dynabeads®/mL in phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide as a preservative.
Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Product Description

Dynabeads® Sheep anti-Rat IgG in combination with primary rat IgG antibodies are ideal for depletion or positive isolation of cells from different species (e.g. mouse, human), depending on the specificity of the primary antibody. Cells can be directly isolated from any sample such as whole blood, bone marrow, MNC suspensions or tissue digests.

The primary rat IgG antibodies are either added to the cell sample (indirect technique) or pre-coated onto the beads (direct technique) prior to cell isolation Dynabeads® are then mixed with the cell sample in a tube. The Dynabeads® bind to the target cells during a short incubation, and then the bead-bound cells are separated by a magnet.
Positive isolation – Discard the supernatant and use the bead-bound cells for downstream applications (e.g. isolation of proteins, nucleic acids (NA) or cell culture).

Note: For positive isolation of cells for downstream cellular applications or for use in flow cytometry, bead-free cells are required. For these applications, use Dynabeads® FlowComp Flexi (to obtain bead-free cells). The kit contains biotinylation and release reagents. Standard biotinylated antibodies will not give release with that kit.
Depletion/negative isolation – Discard the bead-bound cells and use the remaining bead-free and untouched cells for any application. Different rat IgG antibodies can be used to deplete several cell types simultaneously (negative isolation) to obtain untouched cells.

Required Materials

- Magnet (DynaMag™ portfolio). See www.lifetechnologies.com/magnets for recommendations.
- Mixer allowing tilting and rotation of tubes (e.g. HulaMixer® Sample Mixer).
- Isolation Buffer: Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) supplemented with 0.1% BSA and 2 mM EDTA, pH 7.4.
  Note: BSA can be replaced by human serum albumin (HSA) or fetal calf serum (FCS). EDTA can be replaced by sodium citrate.
- Rat IgG antibodies.

General Guidelines

- Visit www.lifetechnologies.com/samplepreparation for recommended sample preparation procedures.
- The choice of primary antibody is the most important factor for successful cell isolation. Note that some antibodies may show reduced antigen-binding efficiency when coated onto beads, even though the antibody shows good results in other immunological assays.
- To avoid non-specific binding of cells (e.g. monocytes, B cells), add aggregated IgG to block Fc-receptors prior to adding the primary antibodies.
- Wash cells prior to adding rat IgG antibodies or Dynabeads® to remove density gradient media (e.g. Ficoll) or soluble factors in serum (e.g. antibodies or cell surface antigens), which can interfere with the cell isolation protocol.
- Use a mixer that provides tilting and rotation of the tubes to ensure that Dynabeads® do not settle in the tube.
- This product should not be used with the MPC™-1 magnet (Cat. no. 12001D).
- Avoid air bubbles (foaming) during pipetting.
- Never use less than recommended volume of Dynabeads®.
- Carefully follow the recommended pipetting volumes and incubation times.
- Keep all buffers cold.

Indirect versus Direct Technique
Use the indirect technique when: A cocktail of rat monoclonal antibodies is used to deplete several cell types simultaneously (use MNC as a starting sample to remove erythrocytes, platelets and granulocytes), very high depletion efficiency is required, the affinities of rat antibodies are low, the cells express low number of target antigens or the direct technique gives unsatisfactory purity.
Use the direct technique when: The affinity of the primary antibody is high, the cells express a high number of target antigens or to make a larger stock preparation of primary coated Dynabeads® (will generally have the same shelf life as stated on the Dynabeads® vial).

Protocol

Wash Dynabeads®

See Table 1 and 2 for volume recommendations.
1. Resuspend the Dynabeads® in the vial (i.e. vortex for >30 sec, or tilt and rotate for 5 min).
2. Transfer the desired volume of Dynabeads® to a tube.
3. Add the same volume of Isolation Buffer, or at least 1 mL, and resuspend.
4. Place the tube in a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and resuspend the washed Dynabeads® in the same volume of Isolation Buffer as the initial volume of Dynabeads® (step 2).

Prepare Cells

Cells can be directly isolated from any samples such as whole blood, bone marrow, MNC suspensions, or tissue digests. See "General Guidelines" for sample preparation.
- Prepare a MNC suspension according to "General Guidelines". Resuspend the cells at 1 × 10^7 cells/mL in Isolation Buffer.
- See "General Guidelines" for recommendation of when to use the direct vs. indirect cell isolation technique.
- This protocol is based on 1 × 10^7 MNC or 1 mL whole blood, but is directly scalable from 1 × 10^7 to 4 × 10^8 cells or 1–40 mL whole blood. When working with lower volumes than 1 × 10^7 cells or 1 mL blood, use the same volumes as for 1 × 10^7 cells or 1 mL blood. When working with larger volumes, scale up all volumes accordingly, as shown in Table 1 and 2.
  Note: When doing negative isolation it is recommended to isolate the cells from a prepared MNC sample rather than from whole blood to remove erythrocytes, platelets and granulocytes.

Isolate Cells – Indirect Technique (labeling cells with rat IgG antibodies)

Use approximately 10 µg of primary antibody (rat IgG) per 10^7 target cells. Titrate the primary antibody to optimize the amount used.
1. Add ~10 µg primary antibody to 1 mL cell suspension and mix (titrate the antibody amount for your use).
2. Incubate for 10 min at 2°C to 8°C.
3. Wash the cells by adding 2 mL Isolation Buffer and centrifuge at 350 × g for 8 min. Discard the supernatant.
4. Resuspend the cells in Isolation Buffer back to 1 × 10^7 MNC/mL (or 1 mL for blood).
5. For positive isolation or depletion of one cell type – add 25 µL pre-washed and resuspended Dynabeads®. For negative isolation (removal of multiple cell types simultaneously) add 100 µL Dynabeads®.

For research use only. Not for human or animal therapeutic or diagnostic use.

Figure D.1: Datasheet and instructions for Invitrogen™ DynaBeads™ Sheep Anti-Rat IgG (Invitrogen™, 11035). Page 1. *Sourced from Thermofisher.com*

6. Incubate for 20 min (positive isolation) or 30 min (depletion/negative isolation) at 2°C to 8°C with gentle tilting and rotation.
7. *Optional:* Add 1 mL Isolation Buffer to limit trapping of unbound cells.
8. Place the tube in a magnet for 2 min.
9. *Depletion/negative isolation:* Transfer the supernatant containing the unbound cells to a fresh tube for further experiments.
   or
   Positive isolation: While the tube is still in the magnet, carefully remove and discard the supernatant.
10. Remove the tube from the magnet and add 1 mL Isolation Buffer, pipet 2–3 times (or vortex 2–3 seconds) and place the tube in a magnet for 2 min.
11. Repeat steps 10–11 at least twice to wash the cells. These steps are critical to obtain a high purity of isolated cells.
12. Resuspend the cell pellet in preferred cell medium.

Table 1: Volumes for indirect cell isolation.

Step	Step description	Volumes per 1×10^7 MNC	Volumes per 2×10^8 MNC
	Recommended tube	5–7 mL tubes	50 mL tubes
	Recommended magnet	DynaMag™-5	DynaMag™-50
1	Primary rat IgG antibody	~10 µg	~200 µg
1	Cell volume (MNC/blood)	1 mL	20 mL
3*	Wash cells (Isolation Buffer)	~2 mL	~40 mL
4	Resuspend cells	1 mL	20 mL
5**(*)	Add Dynabeads® (positive isolation/depletion) Add Dynabeads® (negative isolation)	25 µL 100 µL	500 µL 2 mL
7*	Increase volume (Isolation Buffer)	~1 mL	~ 20 mL
10-12*	**For positive isolation only:** Wash the cells (Isolation Buffer)	3 × ~1 mL	3 × 20 mL

* Adjust the Isolation Buffer volumes to fit to the tube you are using.

** If very high depletion-efficiency is required or you are depleting many cells simultaneously, increase/optimize the amount of Dynabeads®.

*** When incubating, tilt and rotate the vial so the cells and beads are kept in the bottom of the tube. Do not perform end-over-end mixing if the volume is small relative to the tube size.

Isolate Cells – Direct Technique (antibody-coating of Dynabeads®)

Use 0.5–1.5 µg of primary rat IgG antibody per 25 µL (1×10^7) Dynabeads®.
Titrate the primary antibody to optimize the amount used.

1. Transfer 25 µL pre-washed and resuspended Dynabeads® to a tube.
2. Add ~1 µg antibodies (titrate the antibody amount for your use).
3. Incubate for ≥30 minutes at 2°C to 8°C with gentle tilting and rotation.
4. Place the tube in a magnet for 1 min and discard the supernatant.
5. Remove the tube from the magnet and add 2 mL Isolation Buffer.
6. Repeat step 4–5 once to remove excess of antibodies.
7. Place the tube in the magnet for 1 min, discard the supernatant, remove the tube from the magnet and resuspend the Dynabeads® in 1 mL Isolation Buffer.
8. Add the beads to 1 mL cell sample (1×10^7 cells) and resuspend.
9. Incubate for 20 min (positive isolation) or 30 min (depletion) at 2°C to 8°C with gentle tilting and rotation.
10. *Optional:* Add 1 mL Isolation Buffer to limit trapping of unbound cells.
11. Place the tube in a magnet for 2 min.
12. *Depletion:* Transfer the supernatant containing the unbound cells to a fresh tube for further experiments.
    or
    Positive isolation: While the tube is still in the magnet, carefully remove and discard the supernatant.
13. Remove the tube from the magnet and add 1 mL Isolation Buffer, pipet 2–3 times (or vortex 2–3 seconds) and place the tube in a magnet for 2 min.
14. Repeat steps 13–14 at least twice to wash the cells. These steps are critical to obtain a high purity of isolated cells.
15. Resuspend the cell pellet in preferred cell medium.

Table 2: Volumes for direct cell isolation.

Step	Step description	Volumes per 1×10^7 MNC	Volumes per 2×10^8 MNC
	Recommended tube	5–7 mL tubes	50 mL tubes
	Recommended magnet	DynaMag™-5	DynaMag™-50
1	Dynabeads®	~25 µL	~500 µL
2	Primary rat IgG antibody	~1 µg	~20 µg
5–6	Wash Dynabeads® (Isolation Buffer)	2 × ~2 mL	2 × ~40 mL
7	Resuspend Dynabeads® (Isolation Buffer)	1 mL	20 mL
8	Cell volume	1 mL	20 mL
10*	**Optional:** Increase volume (Isolation Buffer)	~1 mL	~8 mL
13–14*	**For positive isolation only:** Wash the cells (Isolation Buffer)	3 × ~1 mL	3 × 20 mL

* Adjust the Isolation Buffer volumes to fit to the tube you are using.

** If very high depletion-efficiency is required or you are depleting many cells simultaneously, increase/optimize the amount of Dynabeads®.

*** When incubating, tilt and rotate the vial so the cells and beads are kept in the bottom of the tube. Do not perform end-over-end mixing if the volume is small relative to the tube size.

Description of Materials

Dynabeads® Sheep anti-Rat IgG are uniform, superparamagnetic polystyrene beads (4.5 µm diameter) coated with polyclonal sheep anti-rat IgG antibodies.
Cross-reactivity to mouse antibodies is high and cross-reactivity to human antibodies is minimal.

Related Products

Product	Cat. no.
DynaMag™-5	12303D
DynaMag™-15	12301D
DynaMag™-50	12302D
HulaMixer® Sample Mixer	15920D
Dynabeads® FlowComp™ Flexi	11061D

REF on labels is the symbol for catalog number.

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SPEC-05657

For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com

www.lifetechnologies.com

life technologies™

Figure D.1: Datasheet and instructions for Invitrogen™ DynaBeads™ Sheep Anti-Rat IgG (Invitrogen™, 11035). Page 2. *Sourced from Thermofisher.com*