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On the Expression of the Gi-coupled ADP-Receptor P2RY12 in Human Platelets

T H E S I S

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By

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1 Introduction

1.1 Role of thrombocytes in atherosclerotic and atherothrombotic diseases

Platelets play a crucial role in the maintenance of a normal homeostasis. Disturbances in this system can lead to pathological thrombus formation and vascular occlusion, contributing to the development of unstable angina, myocardial infarction and stroke. Whether platelets contribute significantly to restenosis after percutaneus coronary interventions in humans is a matter of debate.

In flowing blood, the endothelial cell monolayer provides an anti-thrombotic surface by separating blood from the subendothelial matrix proteins and by secretion of antithrombotic factors, e.g. prostacyclin (PGI2) and nitric oxide (NO), which inhibit platelet aggregation. Atherosclerotic damages or vascular intima injuries impair the anti-thrombotic properties of the endothelium and thus platelet activation is promoted. Circulating platelets may adhere to the site of injury which is mediated by *von Willebrand factor* (vWF) and involves the glycoproteins GPIa/IIa and GPVI. Concomitantly adherent platelets become activated by several agonists, e.g. collagen and thrombin. The platelets release their dense granules containing amongst other mediators the nucleotide ADP. This process contributes to the activation of additional circulating platelets in the vicinity of the site of injury by stimulation of ADP-receptors on thrombocytes.

Simultaneously, the platelets de novo synthesize and release the platelet activator and vasoconstrictor thromboxane A2 (TXA2), which is the major end product of arachidonic acid metabolism in platelets. The enzymes responsible for the production of this mediator are cyclooxygenase 1 (COX1; PGHS1) and thromboxane synthetase. Thus, both the ADP-release and the TXA2-realease constitute self-amplifying pathways of thrombocytes. Both pathways are targeted clinically. TXA2-production is suppressed by irreversible aspirin-mediated inhibition of COX-1 activity. A subset of platelet ADP-receptors, namely P2RY12 is irreversibly inhibited by thienopyridine compounds (ticlopidine, clopidogrel and prasugrel).

When not inhibited, all these pathways result in activation of GPIIb/IIIa which leads to binding of fibrinogen and vWF. Thus, multivalent adhesive proteins like fibrinogen

form complexes with GPIIb/IIIa* causing cross-bridge formation between adjacent platelets. This common final pathway of platelet aggregation is also a target of antiplatelet therapy by antibodies (like abciximab) or peptides (like tirofiban or eptifibatide), which impair the cross-bridge formation. GPIIb/IIIa-inhibitors strategy constitutes the most powerful antiplatelet therapy available at present, since it targets the common final step of platelet aggregation (Lefkovits et al. 1995)

1.2 Antiplatelet-therapy in primary and secondary prevention

Antagonizing platelets in the therapy of vascular diseases is of major importance especially during early steps of thrombus formation. Several randomized trials have proven the overall importance and benefits of antiplatelet therapy for a wide range of vascular diseases. To exemplify this, subsequently some of the landmark trials with ASA and Clopidogrel are briefly summarized.

1.2.1 ISIS-2 study

The ISIS-2 study was a randomized trial carried out between 1985 and 1987 in 17187 patients with acute myocardial infarction (ISIS-2-collaborative-group 1988). Intravenous streptokinase (1.5 MU in 1 hour) and oral aspirin (162 mg daily for 1 month) versus matching placebos were tested in a two x two factorial design. The study demonstrated an impressing risk reduction for vascular death versus placebo: 35 days after the index infarction, the absolute risk reduction amounted to 2.5% and 2.8% for aspirin and streptokinase, respectively. Moreover, the drugs acted synergistically and the combined treatment caused an absolute risk reduction of 5.2% at 35 days. Thus, the number of patients needed to treat to prevent one vascular death was 40 for aspirin, 38 for streptokinase and 19 for the combination of both drugs. The study was large enough to show a consistent effect of both compounds given alone or in combination in several subgroups on a hard endpoint, namely mortality, in patients with acute STEMI.

ISIS-2 and its pilot study were the first trials performed on the acute effects of antiplatelet therapy in myocardial infarction. In contrast, long-term effects of antiplatelet agents in secondary prevention of coronary artery disease were already known at the time when ISIS-2 was published. The effect sizes of the acute treatments were largely sustained after one year of follow-up, with an absolute risk

reduction of vascular death of 5.5% for the combined treatment (NNT: 18.2). For some patients (6213) a follow-up was done till 1997 (Baigent et al. 1998). The follow-up analysis showed that the early survival advantages produced by fibrinolytic therapy and a month of aspirin therapy started in acute myocardial infarction is maintained for at least 10 years.

1.2.2 CAPRIE trial

The CAPRIE trial published in 1996 was a randomised, blinded trial designed to assess the relative efficacy of clopidogrel (75 mg once daily) versus aspirin (325 mg once daily) in reducing the risk of a composite outcome cluster of ischemic stroke, myocardial infarction, or vascular death (CAPRIE-steering-committee 1996). Another major endpoint was the safety of the two compounds since the predecessor of clopidogrel, ticlopidine exhibited a high rate for hematological complications such as neutropenia. This hematological complication caused death in some patients e.g. by septic thrombosis of a recently implanted stent. The frequency of an agranulocytosis after ticlopidine administration was estimated to be in the order of 1% per 100 patient years (Janzon et al. 1990).

The population studied comprised patients with manifest atherosclerotic vascular disease (recent ischemic stroke, recent myocardial infarction or symptomatic peripheral arterial disease). A total of 19,185 patients, with more than 6,300 patients in each of the clinical subgroups, were recruited over 3 years, with a mean follow-up of 1.9 years. This trial showed that long-term administration of clopidogrel to patients with atherosclerotic vascular disease was only slightly more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death. The overall safety profile of clopidogrel was at least as good as that of medium-dose aspirin. The study demonstrated that clopidogrel unlike ticlopidine was not associated with a detectable risk for agranulocytosis.

1.2.3 CURE trial

The CURE trial published in 2001 was carried out to evaluate the efficacy and safety of the antiplatelet agent clopidogrel when given concomitantly with aspirin in patients with acute coronary syndromes without ST-segment elevation (Mehta & Yusuf 2000; Mehta et al. 2001). The trial randomized 12,562 patients within 24 hours after the

onset of symptoms of unstable angina to clopidogrel or placebo for 3 to 12 months. All patients received aspirin. The antiplatelet agent clopidogrel had beneficial effects in patients with acute coronary syndromes without ST-segment elevation. However, the risk of major bleeding increased among patients treated with clopidogrel (Yusuf et al. 2001). The absolute risk reduction for death from cardiovascular causes with combined treatment with clopidogrel was 2.1% (dual-platelet therapy; AR: 9.3% versus ASA; AR: 11.4%). The NNT was 48.

However, the combined use of both compounds was associated with higher rates of bleeding. The absolute risk increase with the use of clopidogrel amounted to 1.0%. The NNH was 100.

1.2.4 COMMIT trial

The COMMIT trial (clopidogrel and metoprolol in Myocardial Infarction Trial) reported on 45,852 patients with a diagnosis of established or suspected myocardial infarction – evidenced by either ST change or newly developed left bundle branch block in combination with typical symptoms (COMMIT-trial-collaborative-group 2005). Within 24 hours after onset of symptom patients were randomized to receive either clopidogrel 75 mg/day or placebo in addition to aspirin 162 mg/day. The duration of treatment with clopidogrel or placebo was for the duration of the hospital stay (mean: 16 days). The primary end point was a composite of death, re-infarction, or stroke at hospital discharge. The study showed a significant absolute risk reduction of 0.9% in the clopidogrel group within 28 days (relative risk reduction 9%; NNT 111 for 28 days of treatment). Mortality was also significantly reduced (ARR: 0.6%; RRR: 7%), as was reinfarction (RRR: 13%). No excess bleeding with clopidogrel was detected. This proved the additive effect of clopidogrel with aspirin in the treatment of acute myocardial infarction in a setting without reperfusion therapy as the study was performed in China were such treatment options are not routinely available.

1.2.5 TIMI-28

The question of whether the addition of clopidogrel to standard thrombolytic care with the various thrombolytic drugs, heparin and aspirin improves the prognosis of patients with acute STEMI was addressed in the TIMI-28 Trial which was again a double-blind, randomized, placebo controlled trial (Sabatine et al. 2005a). The study

included 3,491 STEMI-patients treated with standard thrombolytic therapy, aspirin and heparin randomized in 1:1 manner to receive either clopidogrel 300-mg loading dose followed by 75 mg/day for 30 days or to receive placebo.

The results showed that the risk of a composite clinical endpoint (death, reinfarction, reintervention stroke) was reduced by clopidogrel treatment. The absolute risk reduction for the composite endpoint amounted to 2.5% (NNT 40).The difference was largely driven by a reduction of reinfarction in patients treated with clopidogrel. The risk for death was not reduced by the treatment with clopidogrel (4.4 and 4.5% within 28 days in clopidogrel-treated and placebo-treated patients, respectively).

1.2.6 PCI-TIMI-28

A subgroup-analysis of patients who underwent percutaneus coronary intervention in this trial additionally showed a significantly reduced incidence of cardiovascular death, strokes or myocardial ischemia for these patients when pre-treated with clopidogrel (Sabatine et al. 2005b).

The results of these studies were considered in the development of the current guidelines for the treatment of patients after acute coronary syndromes and coronary interventions with stent placement. In the following paragraphs, the current recommendations of the European guidelines as of 2007/2008 are briefly summarized.

1.2.7 European Society of Cardiology Guidelines on antiplatelet therapy

Patients with elective coronary interventions

Clopidogrel is recommended in addition to ASA in patients in whom a percutaneous coronary intervention is planned. In this case, it is recommended to give a loading dose of at least 300 mg. 600 mg loading dose can achieve a more rapid and stronger inhibition of platelet aggregation can be achieved. Given the variability of drug response to clopidogrel (see below) the appropriate dose of clopidogrel is still subject of debate at present. After placement of a bare-mental stent (BMS), it is recommended to continue with a daily dose of 75 mg of clopidogrel for 3-4 weeks and for a minimum of 12 months after placement of a drug-eluting stent (DES).

Acute coronary syndromes

Antiplatelet agents are an essential component of the treatment of acute coronary syndromes (ACS). In numerous clinical trials ASA, clopidogrel and glycoprotein IIb/IIIa inhibitors have been shown to reduce the incidence of ischemic events.

Clopidogrel in patients with ST-elevation myocardial infarction

In a dose of 75 mg/day, clopidogrel is as effective as aspirin in preventing ischemic events in patients with history of recent MI, with similar safety profile. Greater effects are obtained by combining clopidogrel and aspirin. In the COMMIT trial, the routine addition of clopidogrel to aspirin therapy for 4 weeks in patients with acute MI resulted in proportional reduction in death, re-infarction, or stroke.

Clopidogrel in patients with non-ST-elevation acute coronary syndromes

Like aspirin, clopidogrel has an established role in NSTE-ACS, based to a large degree on the results of the CURE trial. The CURE trial showed that patients who received a combination of aspirin and clopidogrel in loading dose of 300 mg followed by 75 mg/day for 3-12 months profited. Cardiovascular deaths, non-fatal MI, or stroke occurred significantly less.

Thus, both the American Heart Association and the European Association of Cardiology recommend routine use of ASA and clopidogrel as soon as possible after onset of symptoms of acute myocardial infarction (Bassand et al. 2007; Antman et al. 2008; Van de Werf et al. 2008). Considering the long-term effect of clopidogrel in patients after a non-ST-segment acute coronary syndrome in the CURE trial and taking into account the current recommendation for non-STEMI patients, treatment duration of 12 months is recommended whether or not a stent has been placed, although patients with drug-eluting stents might need a longer duration of treatment according to European guidelines. ASA should be given lifelong. In cases where patients cannot tolerate ASA, clopidogrel is recommended.

1.3 Brief characterization of antiplatelet drugs

1.3.1 ASA (acetylsalicic acid)

Aspirin is a salicylate drug which is readily absorbed from the stomach and upper small intestine. The cyclooxygenases hydrolyze acetylsalicic acid to yield salicylic

acid and an acetic acid residue. The acetic moiety acetylates serine residues within both isoforms causing an irreversible inactivation of COX-1 and a markedly reduced and altered catalytic activity of the COX-2 isoform. The inactivated COX-1, the COX-form present in platelets, cannot catalyze the oxygenation of arachidonic acid to prostaglandin G2 and its subsequent transformation to PGH2. Thus, the formation of TXA2 – which is important for vasoconstriction and platelet aggregation – is blocked by treatment with aspirin.

Aspirin blocks the function of platelets exposed to it for their remaining lifetime (about 7 to 10 days). Aspirin achieves its maximal effect within 15 to 30 minutes. A single dose of 100 mg almost completely suppresses platelet TXA2-synthesis in patients. Daily administration of even lower doses exerts a cumulative effect and results in complete inhibition of TXA2-synthesis from endogenous arachidonic acid.

ASA is the most frequently used antiplatelet agent. It is a very cost effective drug in particular in the secondary and primary prevention of atherosclerotic diseases. Its use is impaired by an increased bleeding risk and by an idiosyncratic reaction which usually presents as pseudoallergic clinical diseases (urticaria, angioedema, salicylate asthma). However, most patients tolerate the drug very well.

1.3.2 Thienopyridines

Both, ticlopidine and clopidogrel are ADP-receptor antagonists which block the ADP-induced pathway of platelet activation by specific inhibition of the P2RY12 ADP-receptor. Ticlopidine has been investigated in the setting of NSTEMI-ACS in only one study, which showed a significant risk reduction of death and MI (Janzon et al. 1990). However, the use of ticlopidine has declined due to its serious side effects, in particular the risk of neutropenia as well as of gastrointestinal side affects and rashes. As a result, it has been replaced with clopidogrel over time albeit a head-to-head comparison of both compounds in terms of efficacy has never been carried out.

Clopidogrel is a pro-drug. The active metabolite blocks an adenosine diphosphate (ADP) receptor on platelet cell membranes. The specific subtype of ADP receptor that clopidogrel irreversibly inhibits is P2RY12 (formerly termed P2TAC) and is important in platelet aggregation and the subsequent cross-linking of platelets by activated GPIIb/IIIa and fibrin.

A dose of 75 mg clopidogrel daily begins to weakly inhibit ADP-induced platelet aggregation within hours after the first dose but requires three to seven days to achieve maximal inhibition of platelet aggregation. The time to maximal inhibition can be markedly shortened by administration of a loading dose of 300 mg of clopidogrel (Müller et al Heart 2001). This shortens the time to peak-effect to 6 to 12 hours. Increasing the loading dose to 600 mg achieves full platelet effect of clopidogrel after two to three hours.

1.3.3 GPIIb/IIIa antagonists

Thrombin and collagen are potent platelet agonist that cause ADP- and serotonin-release and activate GP IIb/IIIa fibrinogen receptors on the platelet surface, all activating the “final common pathway” of platelet aggregation. Since several aggregation pathways converge to this final step, it is understandable that inhibition of this step is most efficacious in terms of antagonizing platelet aggregation.

At present, there are three GP IIb/IIIa antagonist approved for clinical use in Germany.

- Abciximab
- Eptifibatide
- Tirofiban

The GP IIb/IIIa inhibitors, in particular abciximab and epitifibatide have shown benefits in clinical outcome of patients with NSTEMI and unstable angina who were not pretreated with clopidogrel, and patients with positive troponin NSTEMI who have been treated with Clopidogrel. Patients with STEMI can also be treated with GP IIb/IIIa inhibitors, preferably with abciximab. These patients have also been shown benefit within the first 30 days after percutaneus coronary intervention.

1.3.4 Comparison

Taken together, clopidogrel and aspirin are moderately active antiplatelet drugs, which in combination act in an additive manner. This so called dual platelet therapy is a mainstay in the treatment of acute coronary syndromes and after coronary interventions. The combination of both drugs is recommended for at least 12 months after acute coronary syndromes (regardless of whether an intervention was

performed or not) and after placement of drug-eluting stents. After placement of bare metal stents in an elective situation the recommendation is to use dual platelet therapy for 1 to 3 months (until the stent is fully covered by antithrombotic endothelium).

Clopidogrel and aspirin are irreversible inhibitors of platelet aggregation. However, since clopidogrel activity is dependent on the formation of an active metabolite generated in low amounts only, clopidogrel is administered in a loading dose. In contrast, ASA is in itself the active antiplatelet principle and its action is almost instantaneous after injection and rapid after oral administration. Frequently practiced administration of more than 200 mg of ASA in acute infarction patients is unsubstantiated. A comparison on the outcome in GUSTO I and GUSTO II patients revealed that 162 mg of chewable ASA is as effective as a dose of 325 mg ASA in prevention of cardiovascular effects (Berger et al. 2008).

The assessment of the overall safety of clopidogrel versus ASA showed a lower risk for hemorrhagic complications for clopidogrel (CAPRIE-TRIAL).

The GP IIb/IIIa inhibitors have proven their importance in the therapy of patients with ACS in whom an intervention is performed, in particular those with a high thrombus load. Their usage is declining due to high costs and also improvements in the techniques employed in percutaneous coronary interventions. The administration of the GP IIb/IIIa inhibitors is presently only by intravenous injection since all oral GP IIb/IIIa inhibitors failed in clinical trials for complex reasons.

1.4 ADP as platelet agonist and the P2RY12-Receptor

1.4.1 General

ADP was the first low-molecular weight compound that was identified as a platelet activator. It is stored within dense granules of the platelets. With use of different analogues of ADP and a detailed analysis of the different physiological reactions emerging after receptor stimulation at various agonist concentrations it became clear that there are different receptors for ADP present on platelets (Macfarlane et al. 1983) (Colman 1990; Mills 1996). Cell surface receptors which bind purinergic nucleotides are assigned to the P2-subgroup. On platelets, there are three different receptors of the P2-class: one ionotropic receptor (P2X1) and two metabotropic

receptors (P2Y1 and P2TAC = P2RY12). The characteristics of the different receptor subtypes are given in Table 1.

Of the P2-receptors on platelets the Gi-coupled ADP-receptor P2RY12 plays a central role in platelet activation and is the receptor targeted by the active metabolites of thienopyridine compounds. Its activation contributes to the completion of the platelet aggregation after ADP-stimulation and plays a crucial role in amplification of the aggregation induced by other platelet agonists.

Property	P2RY12 P2TAC	P2Y1 $P2T_{plc}$	P2X1
Adenosine diphosphosphate(ADP) an agonist	Yes	Yes	Yes
2-Methylthio-ADP an agonist	Yes	Yes	?
α,ß-MeATP an agonist	No	No	Yes
Antagonized by adenosine triphosphate	Yes	Yes	No
Antagonized by ARL 66096	<1μM	>10μM	No
Mobilization of intracellular calcium stores	No	Yes	?
Rapid calcium influx	?	?	Yes
Inhibition of adenyl cyclise	Yes	No	No
Stimulation of inositol trisphosphate formation	No	Yes	No
Mediator of shape change	No	Yes	No
Essential for ADP-induced aggregation	Yes	Yes	No
Antagonized by A3P5PS,A3P5P, or A2P5P	No	Yes	?
Chromosomal localization	3q25-28	3q25	17p13
Number of amino acids	342	373	399
Predicted Molecular weight [kD]	39.4	42.1	45.0
Number of known transcript variants	2	1	1

Table 1: Properties of the three known ADP-receptors subtypes in human platelets

Activation of P2RY12 by ADP activates a Gαi-protein and releases βγ-subunits which couple to independent downstream signalling cascades. The activated Gαi-subunit decreases cAMP-levels by inhibition of adenylyl cyclase. Subsequently the activity of cAMP-dependent protein kinase (PKA) is decreased. Diminished PKA-activity results in reduced phosphorylation levels of several target proteins amongst which the vasodilatator-stimulated phosphoprotein (VASP) is the most well-known one (Li et al. 2003). The diminished protein phosphorylation of PKA-targets is associated with

activation of GPIIb/IIIa and subsequent platelet aggregation. The βγ-subunits, particularly in non-smoking individuals, activate the C-class protein kinases, phosphoinositol 3-kinase and phosphotyrosine kinases, which are also linked to GPIIb/IIIa activation and platelet dense granule secretion (Cavallari et al. 2007).

1.4.2 Identification and cloning of the gene

Despite several attempts in the past, the P2RY12 has only recently been characterised on the molecular level. One group used a specific approach to clone the receptor from rat platelet cDNA whereas two groups deorphanized a G-protein coupled receptor (GPCR) with a previously unknown ligand.

Hollopeter et al followed a specific approach to clone P2RY12 from a rat platelet cDNA-library (Hollopeter et al. 2001). A sensitive electrophysiological system was used to get a read-out of G_i-linked responses in Xenopus oocytes which were modified by artificial expression of Kir3.1 and Kir3.4. The group used the knowledge that upon activation, several G_i-coupled receptors release Gβγ-subunits from the heterotrimeric G proteins. The released βγ-subunits activate inwardly rectifying K+-channels and thus evoke hyperpolarizing potassium currents.

After functional screening of the platelet-derived cDNA library by oocyte-injection of the corresponding cRNA the authors demonstrated the presence of an ADP-receptor which upon activation evoked potassium currents. After analyses of fractions of the cDNA-library, finally a single cDNA-clone was obtained. After injection of the cRNA into the modified oocytes, an activity was detectable which fulfilled all the characteristics predicted for the P2RY12 $G_{\alpha i}$-coupled receptor. The receptor evoked potassium currents upon stimulation with ADP and 2-MeSADP. 2-MeSADP was by two orders of magnitude more potent than ADP which is in accordance with the observations on P2RY12 in platelets. The response to ADP was entirely abolished by pertussis-toxin treatment. Adenosine did not evoke any significant responses. A human P2RY12 homologue was isolated from human platelet library and similar results were obtained after injection of the derived cRNA into oocytes. The implication that the cloned GPCR was indeed the P2TAC searched for was further substantiated by genetic analysis of a patient with a specific defect in P2TAC-function which detected a frame-shift deletion in one of the patient's alleles. The details of the genetic analyses are described in section 1.4.5.1, page 18.

Another approach which resulted in the molecular identification of P2RY12 was carried out by Zhang et al., 2001 #8. In this work, it was demonstrated that the SP1999 orphan GPCR is a G_i-coupled receptor that is potently activated by ADP. It also showed similarities between the characteristics of SP1999 and P2TAC receptors (subsequently termed P2RY12) in platelets.

SP 1999 was a cDNA-clone of an orphan human GPCR highly expressed in human spinal cord and brain. To identify the ligand of SP 1999, the cDNA was cotransfected together with a mixture of plasmids encoding chimeric Gαi/Gαq and other chimeric Gαq-proteins into CHO-DHFR-cells. Regardless of the G-protein to which the receptor couples under native conditions, it will activate Gαq-dependent pathways through transmission via the chimeric protein since all chimeras contain a functional Gq-domain activated upon receptor stimulation. The Gαq-chimera-approach allows for the detection of increases of calcium concentrations after stimulation of a GPCR. The activation of the Gαq-dependent pathway was monitored by determination of intracellular calcium concentrations by Fluo-3 fluorescence.

Fractionated rat spinal cord extracts were assayed for their activity to evoke elevation of calcium concentrations in cells transfected with expression plasmids for SP 1999 and the aforementioned chimeric Gα-subunits. After a series of chromatographic steps, a narrowed fraction was obtained that activated the receptor. Finally, ADP was identified in this fraction as activating ligand by mass spectroscopy. Subsequently, it was found that the receptor recognizing ADP coupled exclusively to Gq-chimeras which contained functional domains of Gαi-proteins. In a stable CHO-cell line expressing SP 1999, the receptor evoked the suppression of forskolin-induced cAMP accumulation in a manner dependent on the concentration of ADP. Pharmacological characterization of the ligand activation profile with several nucleotides revealed that 2-MeSADP was more potent than ADP with EC_{50}-values differing by ~1.5 orders of magnitude. More than 30 other nucleosides and nucleotides exhibited no activity. The authors concluded that SP 1999 is a good candidate for the P2TAC-receptor.

In a subsequent work it was determined whether SP1999 indeed encodes the P2RY12-receptor (Foster et al. 2001). SP1999-null mice were generated by targeted gene disruption (knock-out). These mice displayed prolonged bleeding time and their platelets aggregated poorly in response to ADP and displayed reduced sensitivity to

thrombin and collagen. It was shown that SP 1999 is essential for a maximal and irreversible aggregation response to ADP, but not for shape change of the platelets. Results also showed that Gi-coupled pathways are not generally disturbed in platelets isolated from knock-out mice: Epinephrine responses mediated by Gi-coupled adrenergic α2-receptors were unaffected by disruption of SP 1999. In addition and in contrast to wildtype mice, clopidogrel had no effect on aggregation in SP1999-null mice. Thus, the clone previously designated SP 1999 contained a functional P2RY12-receptor (P2TAC).

Takasaki et al. identified the P2RY12 through molecular cloning of a GPCR with unknown function (Takasaki et al. 2001). Using the TBLASTN algorithm, dbEST and the genomic database were queried with the amino acid sequence of a leukotriene receptor (CysLTR2). A genomic draft-sequence derived from chromosome 3 was predicted to contain a novel GPCR coding sequence homologous to CysLTR2 and termed HORK3. Primers were constructed from the sequence of the GPCR and cDNA was amplified from human brain RNA. The coding sequence was cloned into an expression vector and stably transfected into a rat glioma cell line. The sub-line responded to ADP and 2-MeSADP with an inhibition of forskolin-stimulated cAMP-accumulation, thus showing that HORK3 is a Gαi-coupled receptor with ADP as ligand.

These three different strategies identified the very same coding sequence and the current essential knowledge about the molecular biology of P2RY12 is summarized in the following paragraphs.

1.4.3 The genomic structure and transcript variants of human P2RY12

The P2RY12-gene is located on chromosome 3q.28 (Figure 1 A). At present two transcript variants of the P2RY12-gene have been characterized, namely NM_022788 and NM_176876. Both transcripts encode the same protein since the entire coding sequence is contained within the 3'-located exon which is shared by both variants. The variants differ however in their exon-composition in the 5' untranslated region of the mRNA (Figure 1 B and Figure 2).

The transcript variant 1 (NM_022788) consists of three exons. The exon situated in the most 5' position is unique for variant 1 whereas the second exon is partially shared with exon 1 of variant 2 (NM_176876). The latter variant consists of two

exons only and its first exon is slightly longer than exon 2 of variant 1. Thus, the variants are due to different promoter usage and subsequent alternate splicing.

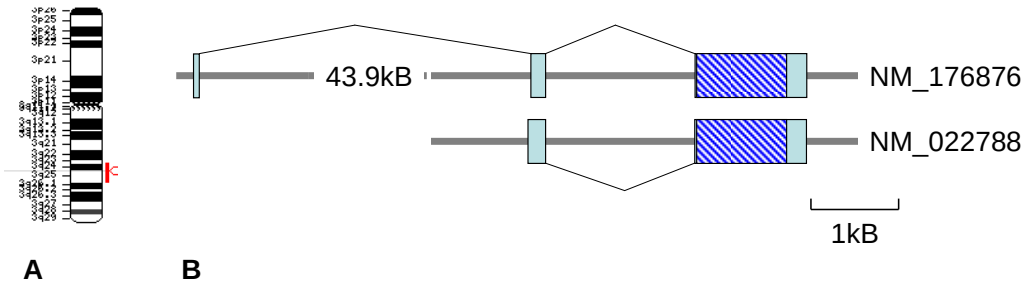

Figure 1: Chromosomal localization (A) and exon-intron-structure of the P2RY12 gene and schematic depiction of the two transcript variants known. Exons are shown as light blue boxes and the coding sequence is shown as hatched blue box. The large intron 1 of the NM_176876 is not drawn to scale.

The detailed alignment of the variants is given in Figure 2. At present it is unknown whether the entries NM_022788 and NM_176876 are complete, in particular with respect to the 5'- ends of the cDNA. The coding sequence of P2RY12 is entirely contained within the large 3'-exon and comprises 342 codons.

```
            1                                                   50
NM_022788   ATCACAATCA GAAGACAGGA GCTGCAGAAC AGAACACTTT CTCATGTCCA
NM_176876   .......... .......... ..       TG AAGCCCTCTT TTTCTCTCCT

           51                                                  100
NM_022788   GGGTCAGATT ACAAGAGCAC TCAAGACTTT ACTGACGAAA ACTCAGGAAA
NM_176876   TCTATTTCTC TCTAGAGCAC TCAAGACTTT ACTGACGAAA ACTCAGGAAA

          101                                                  150
NM_022788   TCCTCTATCA CAAAGAGGTT TGGCAACTAA ACTAAGACAT TAAAAGGAAA
NM_176876   TCCTCTATCA CAAAGAGGTT TGGCAACTAA ACTAAGACAT TAAAAGGAAA

          151                                                  200
NM_022788   ATACCAGATG CCACTCTGCA GGTTGCAATA ACTACTACTT ACTGGATACA
NM_176876   ATACCAGATG CCACTCTGCA GGTTGCAATA ACTACTACTT ACTGGATACA

          201                                                  250
NM_022788   TTCAAACCCT CCAGAATCAA CAGTTATCAG GTAACCAACA AGAAATGCAA
NM_176876   TTCAAACCCT CCAGAATCAA CAGTTATCAG GTAACCAACA AGAAATGCAA

          251                                                  300
NM_022788   GCCGTCGACA ACCTCACCTC TGCGCCTGGT AACACCAGTC TGTGCACCAG
NM_176876   GCCGTCGACA ACCTCACCTC TGCGCCTGGT AACACCAGTC TGTGCACCAG

          301                                                  350
NM_022788   AGACTACAAA ATCACCCAGG TCCTCTTCCC ACTGCTCTAC ACTGTCCTGT
NM_176876   AGACTACAAA ATCACCCAGG TCCTCTTCCC ACTGCTCTAC ACTGTCCTGT

          351                                                  400
NM_022788   TTTTTGTTGG ACTTATCACA AATGGCCTGG CGATGAGGAT TTTCTTTCAA
NM_176876   TTTTTGTTGG ACTTATCACA AATGGCCTGG CGATGAGGAT TTTCTTTCAA

          401                                                  450
NM_022788   ATCCGGAGTA AATCAAACTT TATTATTTTT CTTAAGAACA CAGTCATTTC
NM_176876   ATCCGGAGTA AATCAAACTT TATTATTTTT CTTAAGAACA CAGTCATTTC

          451                                                  500
NM_022788   TGATCTTCTC ATGATTCTGA CTTTTCCATT CAAAATTCTT AGTGATGCCA
NM_176876   TGATCTTCTC ATGATTCTGA CTTTTCCATT CAAAATTCTT AGTGATGCCA

          501                                                  550
NM_022788   AACTGGGAAC AGGACCACTG AGAACTTTTG TGTGTCAAGT TACCTCCGTC
NM_176876   AACTGGGAAC AGGACCACTG AGAACTTTTG TGTGTCAAGT TACCTCCGTC

          551                                                  600
NM_022788   ATATTTTATT TCACAATGTA TATCAGTATT TCATTCCTGG GACTGATAAC
NM_176876   ATATTTTATT TCACAATGTA TATCAGTATT TCATTCCTGG GACTGATAAC

          601                                                  650
NM_022788   TATCGATCGC TACCAGAAGA CCACCAGGCC ATTTAAAACA TCCAACCCCA
NM_176876   TATCGATCGC TACCAGAAGA CCACCAGGCC ATTTAAAACA TCCAACCCCA

          651                                                  700
NM_022788   AAAATCTCTT GGGGGCTAAG ATTCTCTCTG TTGTCATCTG GGCATTCATG
NM_176876   AAAATCTCTT GGGGGCTAAG ATTCTCTCTG TTGTCATCTG GGCATTCATG

          701                                                  750
NM_022788   TTCTTACTCT CTTTGCCTAA CATGATTCTG ACCAACAGGC AGCCGAGAGA
NM_176876   TTCTTACTCT CTTTGCCTAA CATGATTCTG ACCAACAGGC AGCCGAGAGA

          751                                                  800
NM_022788   CAAGAATGTG AAGAAATGCT CTTTCCTTAA ATCAGAGTTC GGTCTAGTCT
NM_176876   CAAGAATGTG AAGAAATGCT CTTTCCTTAA ATCAGAGTTC GGTCTAGTCT

          801                                                  850
NM_022788   GGCATGAAAT AGTAAATTAC ATCTGTCAAG TCATTTTCTG GATTAATTTC
NM_176876   GGCATGAAAT AGTAAATTAC ATCTGTCAAG TCATTTTCTG GATTAATTTC
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           851                                                 900
NM_022788  TTAATTGTTA TTGTATGTTA TACACTCATT ACAAAAGAAC TGTACCGGTC
NM_176876  TTAATTGTTA TTGTATGTTA TACACTCATT ACAAAAGAAC TGTACCGGTC

           901                                                 950
NM_022788  ATACGTAAGA ACGAGGGGTG TAGGTAAAGT CCCCAGGAAA AAGGTGAACG
NM_176876  ATACGTAAGA ACGAGGGGTG TAGGTAAAGT CCCCAGGAAA AAGGTGAACG

           951                                                1000
NM_022788  TCAAAGTTTT CATTATCATT GCTGTATTCT TTATTTGTTT TGTTCCTTTC
NM_176876  TCAAAGTTTT CATTATCATT GCTGTATTCT TTATTTGTTT TGTTCCTTTC

           1001                                               1050
NM_022788  CATTTTGCCC GAATTCCTTA CACCCTGAGC CAAACCCGGG ATGTCTTTGA
NM_176876  CATTTTGCCC GAATTCCTTA CACCCTGAGC CAAACCCGGG ATGTCTTTGA

           1051                                               1100
NM_022788  CTGCACTGCT GAAAATACTC TGTTCTATGT GAAAGAGAGC ACTCTGTGGT
NM_176876  CTGCACTGCT GAAAATACTC TGTTCTATGT GAAAGAGAGC ACTCTGTGGT

           1101                                               1150
NM_022788  TAACTTCCTT AAATGCATGC CTGGATCCGT TCATCTATTT TTTCCTTTGC
NM_176876  TAACTTCCTT AAATGCATGC CTGGATCCGT TCATCTATTT TTTCCTTTGC

           1151                                               1200
NM_022788  AAGTCCTTCA GAAATTCCTT GATAAGTATG CTGAAGTGCC CCAATTCTGC
NM_176876  AAGTCCTTCA GAAATTCCTT GATAAGTATG CTGAAGTGCC CCAATTCTGC

           1201                                               1250
NM_022788  AACATCTCTG TCCCAGGACA ATAGGAAAAA AGAACAGGAT GGTGGTGACC
NM_176876  AACATCTCTG TCCCAGGACA ATAGGAAAAA AGAACAGGAT GGTGGTGACC

           1251                                               1300
NM_022788  CAAATGAAGA GACTCCAATG TAAACAAATT AACTAAGGAA ATATTTCAAT
NM_176876  CAAATGAAGA GACTCCAATG TAAACAAATT AACTAAGGAA ATATTTCAAT

           1301                                               1350
NM_022788  CTCTTTGTGT TCAGAACTCG TTAAAGCAAA GCGCTAAGTA AAAATATTAA
NM_176876  CTCTTTGTGT TCAGAACTCG TTAAAGCAAA GCGCTAAGTA AAAATATTAA

           1351                                               1400
NM_022788  CTGACGAAGA AGCAACTAAG TTAATAATAA TGACTCTAAA GAAACAGAAG
NM_176876  CTGACGAAGA AGCAACTAAG TTAATAATAA TGACTCTAAA GAAACAGAAG

           1401                                               1450
NM_022788  ATTACAAAAG CAATTTTCAT TTACCTTTCC AGTATGAAAA GCTATCTTAA
NM_176876  ATTACAAAAG CAATTTTCAT TTACCTTTCC AGTATGAAAA GCTATCTTAA

           1451                                               1500
NM_022788  AATATAGAAA ACTAATCTAA ACTGTAGCTG TATTAGCAGC AAAACAAACG
NM_176876  AATATAGAAA ACTAATCTAA ACTGTAGCTG TATTAGCAGC AAAACAAACG

           1501
NM_022788  AC
NM_176876  AC
```

Figure 2: Alignment of the two transcript variants of P2RY12.
The unique sequence of transcript variant 1 (NM022788) is indicated in light blue. Indicated in light yellow is the unique sequence of the transcript variant 2 (NM 176876) stemming from the first exon of variant 2. The common nucleotides of the 2 variants stemming from the exon 2 of TV1 and the exon 1 of TV 2 are indicated with a grey background. The last exon of both variants is indicated in purple with the start and stop codon indicated by green and red background, respectively.

1.4.4 Protein characteristics

The molecular weight calculated from the amino acid composition is 39.4 kD with a markedly alkaline predicted isoelectric point of 9.60 due to the high number of basic amino acids.

The hydrophobicity profile of the amino acids is given in Figure 3.

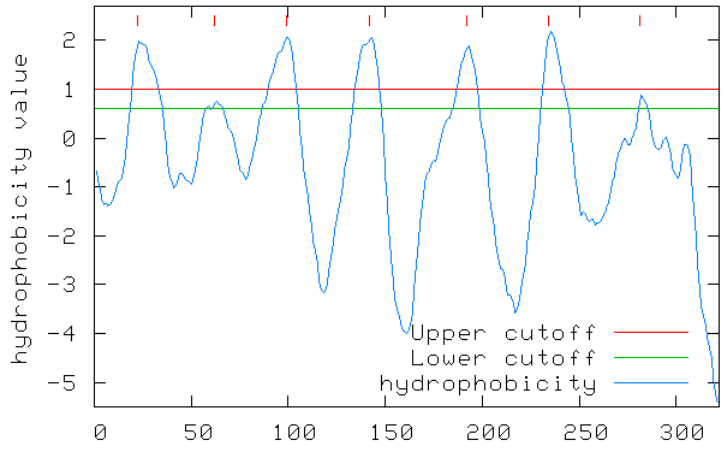


Figure 3: Hydrophobicity profile of the P2RY12-receptor.
Seven transmembrane domains are predicted from the amino acid composition of P2RY12. The transmembrane-domains 2 and 7 have a weaker score than the other hydrophobic a-helices. The hydrophobicity scores were calculated with Toppred (Insitute Pasteur, http://mobyle.pasteur.fr/).

The most likely model predicts the structure of the receptor shown in Figure 4. The N-terminus is predicted to face the extracellular surface whereas the C-terminus is located intracellularily.

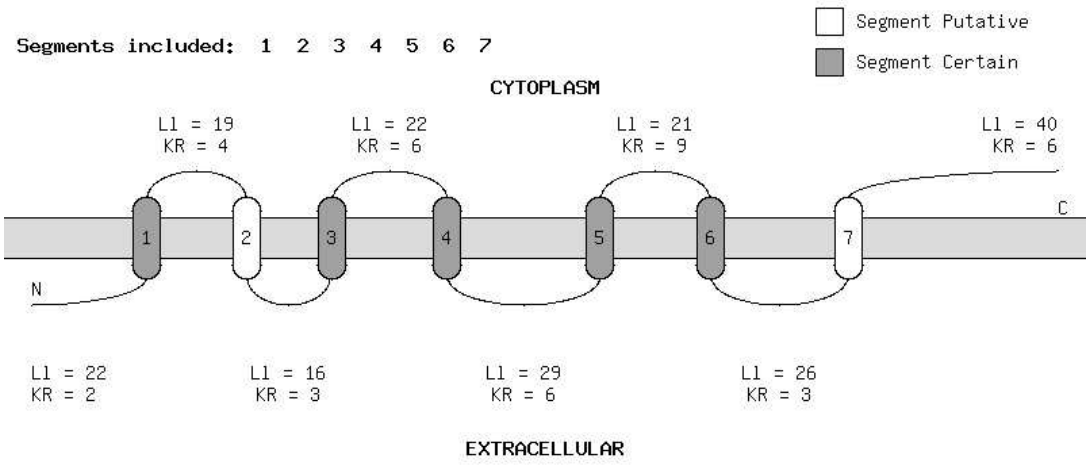


Figure 4: Topology prediction for the P2RY12 protein.
The model with the highest likelihood is shown and demonstrates an N-terminal extracellular tail of 22 amino acids and an intracellular carboxy-terminus of 40 amino acids. Ll and KR denote the total number of amino acids and the number of lysine and arginine residues in the loop, respectively. Topology prediction was performed with the Toppred-program.

The protein is predicted to contain 10 cysteine residues which are considered important for the action of thienopyridine compounds (see below). A detailed topology model has been proposed by Savi's group and is depicted in a modified version in figure 5 (Savi et al. 2006).

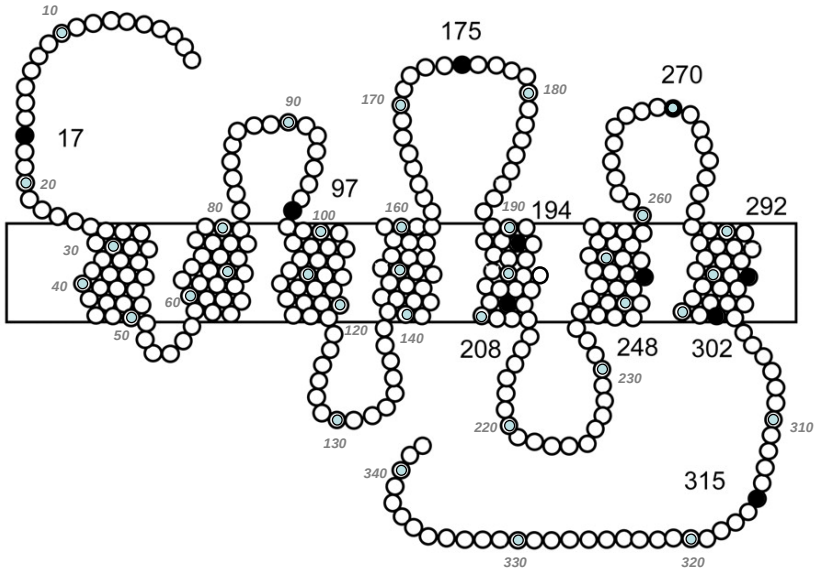


Figure 5: Topology prediction of the P2RY12 receptor.
Each amino acid shown is shown as a circle. Every tenth amino acid is indicated with an additional blue circle and a corresponding small number indicating the position. Cysteine residues are shown by black circles and the position is given by bold numbering. Modified from (Savi et al. 2006).

1.4.5 Genetic variants of P2RY12

1.4.5.1 Disease causing mutations

The first patients with a specific inherited defect of P2YTAC-signalling were described before molecular cloning of the receptor (Cattaneo et al. 1992; Nurden et al. 1995). The patients suffered from mildly to moderately severe bleeding disorders. Platelets from the patients exhibited markedly impaired ADP-dependent platelet aggregation and greatly reduced ADP-binding capacity. Moreover, platelets from both patients lacked the ability to inhibit the PGE1-evoked rise in cAMP-levels in response to ADP. In contrast, the P2Y1-dependent shape change and calcium mobilization were not affected, indicating that the patients had a selective defect in the Gi-linked platelet ADP-receptor.

Hollopeter's work looked for the molecular cause of the defect in the patient described by Nurden et al (1995). and demonstrated that the patient had a deletion of

two basepairs causing a frameshift in the coding sequence of one P2RY12-allele (Nurden et al. 1995; Hollopeter et al. 2001). Surprisingly, the other allele did not show any abnormalities within the subsequent coding sequence. A dominant negative effect of the mutated allele was considered but ruled out: Coinjection of the mutant and the wildtype cRNA into modified Xenopus-oocytes did not abolish the activity of the wildtype-allele. Moreover, the daughter of the patient who inherited the frameshift-allele from her father exhibited an intermediate phenotype with a less pronounced diminution of receptor-density and receptor-activity again arguing against a dominant negative effect of the frameshift-allele. RT-PCR and sequencing of the patient's platelet RNA demonstrated markedly reduced levels of P2RY12-transcripts and an exclusive expression of the mutated allele. Taken together, it is likely that the patient has an additional mutation in the other allele which affects transcription or splicing but apparently is not located within the coding sequence. The reason for the overall markedly lower RNA-expression level is unknown. If the mutated frameshift allele is spliced according to the regular splice-signals of P2RY12, nonsense-mediated decay should not be effective: The entire coding sequence is contained within the last exon and thus conventional nonsense-mediated mRNA decay (NMD) is considered to be not effective since it mandates the presence of a premature stop-codon upstream of an exon-exon boundary. After publication of the genomic sequence of P2RY12, the group of Cattaneo analyzed their own patient first described in 1992 and found compound heterozygosity for two missense mutations on different alleles, namely Arg256Gln and Arg265Trp (Cattaneo et al. 1992; Cattaneo et al. 2003).

1.4.5.2 Polymorphisms

Recently, several groups investigated whether P2RY12-polymorphisms affect platelet responses or are associated with artherothrombotic diseases.

The first study within this context studied the genomic variations in healthy volunteers and their impact on platelet aggregation. The genomic region analyzed was restricted to the exons and the intron of the shorter transcript (NM_176876). The analyzed region comprised 3068 bp and was amplified as a single amplicon. Five frequent variants were detected, four of which were in complete linkage disequilibrium forming the more frequent haplotype H1 and the minor haplotype H2 (i-139T; i-744C; i-ins801A; CDS52T). The fifth sequence variation (C34T) was not coupled to the other

polymorphisms. Neither of the polymorphisms is predicted to alter the amino acid sequence.

Rs number	Trivial SNP-Name	Amino Acid	Region	Allele frequency of the minor allele
rs10935838	i-C139T	–	Intron	T:13.8%
rs2046934	i-T744C	–	Intron	C:13.8%
rs62925561	i-ins801A	–	Intron	Insertion of an A: 13.8%
rs6809699	C34T	Asn/Asn	Exon 2	T:27.5%
rs6785930	G52T	Gly/Gly	Exon 2	T:13.8%

Table 2: Single nucleotide polymorphisms within the genomic region of the P2RY12 gene. The H1/H2 haplotypes are defined by the i-C139T, i-T744C, i-ins801A andG52T polymorphisms. The rs-numbers of the respective variants were identified by SNP-blast. Modified from Fontana et al. (2003a).

The group studied whether the haplotypes are associated with differences in platelet responsiveness to ADP. As compared to the more frequent H1-haplotype the minor H2-haplotype was associated with an increased maximum platelet aggregation upon stimulation with submaximal concentrations of ADP.

The same group investigated in a case-control study whether the identified haplotypes are associated with differences in the frequency of peripheral arterial disease. The study published in 2003 comprised 184 male patients less than 70 years of age and 330 age-matched control subjects with no cardiovascular history and free from peripheral arterial disease. The H2-haplotype was more frequent in patients with peripheral arterial disease than in control subjects. The association remained significant even after adjustment for other risk factors such as diabetes mellitus, smoking, hypertension, hypercholesterolemia and other selected receptor gene polymorphisms (Fontana et al. 2003).

However, the impact of the aforementioned polymorphisms on platelet reactivity has not been uniformly observed. In a publication from 2005 the impact of 4 polymorphisms in P2RY12 and of one variation in P2Y1 on ADP-stimulated fibrinogen binding in samples from 200 white patients from northern Europe was investigated. Whereas none of the variations in the sequence of P2RY12 was associated with differences in platelet response, the investigated silent polymorphism (rs701265; NM_002563.2:c.786A>G; NP_002554.1:p.262Val>Val) in P2Y1 was associated with differences in fibrinogen binding in particular at low concentrations of

ADP: Platelets from subjects homozygous for the rarer variant exhibited a higher ADP-stimulated binding as compared to platelets from subjects homozygous for the wildtype allele. Platelets from heterozygous volunteers showed an intermediate phenotype at a low concentration of ADP (Hetherington et al. 2005).

A further study carried out by Staritz et al. (Int J Cardiol, 2008) addressed the influence of the H2 haplotype on clopidogrel responsiveness. A comparison was carried out with patients who underwent PCI and stent implantation. One day after the intervention, blood was drawn and genetic analyses were carried out. Included were patients with coronary artery disease who received ASA and clopidogrel but not glycoprotein IIb-IIIa receptor inhibitors. Additional healthy blood donors who denied taking antiplatelet medication were also included. Platelet aggregation was measured with impedance aggregometry. The H2 allele was associated with enhanced platelet aggregation in clopidogrel-*naïve* subjects. The homozygote H2 haplotype was associated with an impairment of clopidogrel effect.

Cavallari et al. (2007) demonstrated the association with H2-haplotype and presence of significant coronary artery disease (CAD), particularly in non-smoking individuals.

1.5 Aims of the present study

The aims of this study were:

- To identify the mRNA-variants of P2RY12 present in human platelets.
- To clone and sequence the cDNA of P2RY12 from human platelets.
- To establish a stable cell line expressing a HA-tagged P2RY12-receptor.
- To investigate the specificity and reactivity of available antibodies against P2RY12 and to characterize the suitability of the different antibodies for quantitation of the receptor.
- To establish conditions for platelet preparation and subsequent enrichment of membrane proteins containing the receptor.

The cell line should aid in studying the glycosylation of the receptor and to generate a positive control for experiments addressing the expression of P2RY12 in human platelets. The characterization of transcript variants is important for the correct identification of P2RY12 gene promoters and to further understand the restricted expression pattern of the mRNA and protein.

2 Material and Methods

Beginning with the isolation of total RNA from human thrombocytes, cDNA was synthesized through reverse transcription of the previously isolated total RNA. The cDNA was then amplified through polymerase chain reaction (PCR). Frequently, the DNA-products were modified by mutagenesis and ligated with a suitable vector after ensuring optimal conditions. This strategy was chosen after failure of a previous cloning and overexpression strategy (see discussion).

To ensure accuracy, all transformed DNA products were sequenced in both directions. Afterwards, subsequent experiments were carried out in cell culture, through transient and finally through stabile transfection of in HEK-293 cell line. Detection of proteins was then carried out by immunofluorescence in cell lines and by Western blots in cell lines and human platelets, leukocytes and erythrocytes.

2.1 Materials

2.1.1 Platelets and cDNAs

RNA samples were derived from human thrombocytes of healthy human donors with help of RNeasy mini kits. The corresponding cDNAs were generated from the isolated total RNA by reverse transcription into cDNA with P2RY12- specific primers.

2.1.2 Cell line

The 293T cell line used is a subline of the HEK-293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted. The line is a highly transfectable derivative of the original cell line which was established from human primary embryonal kidney cells transformed by adenovirus type 5 (Graham et al. 1977; Pear et al. 1993).

2.1.3 Vectors and Bacterial Strains

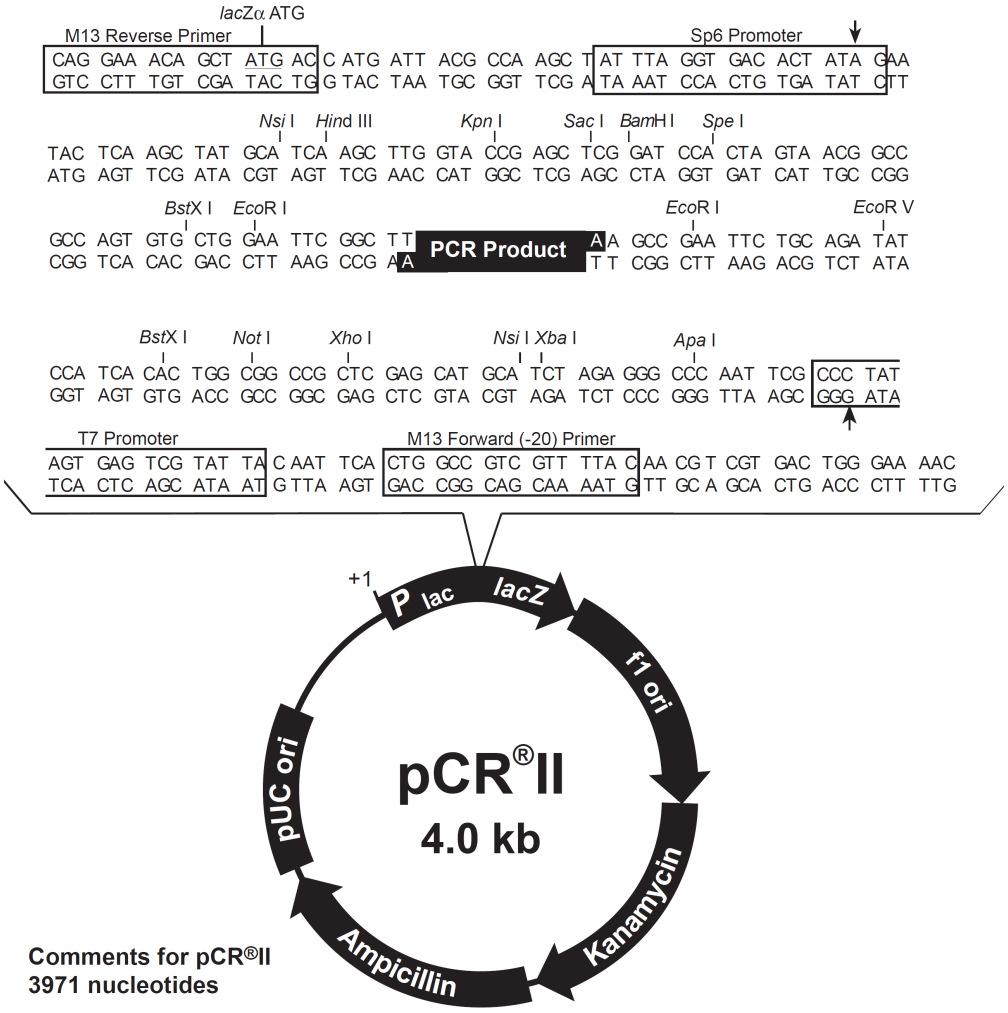
In the present study, two vectors were used: PCRII and pcDNA3.1-Hygro(-). The PCRII vector was used to clone the fragments of the P2Y12-cDNA which were then reassembled in the mammalian expression vector pcDNA 3.1 for both transient and stable expression of P2Y12 in HEK293-cells

2.1.3.1 pCRII-Vector

The vector consists of 3,971 nucleotides. Its multiple cloning site contains 16 restriction cutting sites e.g EcoR1, EcoRV, Xba I etc. The vector also contains ampicillin and kanamycin resistance genes. It is suitable for blue-white screening enabling identification of clones with inserted DNA-fragments.

Screening is based on the disruption of so called α-complementation. Inside the gene encoding the lacZα-fragment lays the multiple cloning site (MCS) of the vector where the PCR product can be cloned into. The lacZ α fragment codes for the N-terminal α-fragment of β-galactosidase. When the α-fragment is expressed together with the C-terminal ω-fragment, this leads to the formation of functionally active β-galactosidase. The ω-fragment is encoded by a gene encoded in the bacterial chromosome or episomes present in the bacteria. The enzyme physiologically splits galactose into lactose and glucose. However, functional β-galactosidase is also capable to break down the sugar X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the medium, which causes a color change from colorless to blue. For this reason, X-Gal is called a chromogenic substrate.

Since the multiple cloning is contained within the gene for the α-fragment, cloning of DNA into the MCS will disrupt the gene of the α-fragment and thus abolish the α-complementation. Thus, clones with inserts in the MCS will usually be whitish, whereas clones with an uninterrupted MCS will stain blue in the presence of X-Gal. Thus, white colonies are likely to contain inserts in the plasmids, whereas this is unlikely for blue colonies.



Comments for pCR®II
3971 nucleotides

*Lac*Zα gene: bases 1-587
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
T7 promoter: bases 404-423
M13 (-20) Forward priming site: bases 431-446
f1 origin: bases 588-1025
Kanamycin resistance ORF: bases 1359-2153
Ampicillin resistance ORF: bases 2171-3031
pUC origin: bases 3176-3849

Figure 6 Depiction of pCR II vector with the multiple cloning site (MCS) and other functional features. (TA cloning-kit from Invitrogen)

The pUC origin is important for the replication of the vector within bacterial cells. Then we also have the primer binding sites for M13 forward and reverse which are used for sequencing of the insert, since their respective positions flank the MCS.

The vector is particularly suitable for the cloning of cDNA: The vector is purchased in linearized form with single 3'T-overhangs, which are generated by a proprietary

technique of the producing company. Taq-polymerases frequently add an additional A to the most 3' base incorporated into the newly synthesized DNA-strand complementary to the template strand. This is termed terminal transferase activity and is common to Taq-polymerases lacking 3'-5'-proof reading activity. The added A-residues are complementary to the T-overhangs on both strands of the vector. The overhangs thus facilitate cloning of the PCR-products by Watson-Crick pairing of the ends and by prevention of re-ligation, since both stands of the vector have T-overhangs that can't be easily joined by ligase unless an insert is present.

2.1.3.2 pcDNA3.1-Hygro(-)

The pcDNA 3.1-Hygro(-) vector contains 5,427 bp. It is designed for both stable and transient expression in mammalian cells. The vector contains a cytomegalovirus immediate early promoter for expression of the recombined protein, followed by a T7-promoter/priming site which allows for in vitro transcription and also for sequencing. It also contains a multiple cloning site; in our case we used the (-) orientation of the pcDNA3.1-Hygro vector. Examples of the restriction sites used were NheI, EcoRV, this allowed for insertion of DNA fragments. The vector properties are depicted in Figure 7.

The bovine growth hormone polyadenylation signal is located downstream of the insert and eases for efficient termination of transcription and also for polyadenylation of the mRNA if the respective signals are lacking in the cDNA-insert.

An SV40 early promoter and origin allow efficient high level expression of neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen, repectively. The hygromycin resistance gene allows for selection of transfected mammalian cells and to produce stabile cell lines. An ampicillin resistance gene is used for the selection in transfected E. coli.

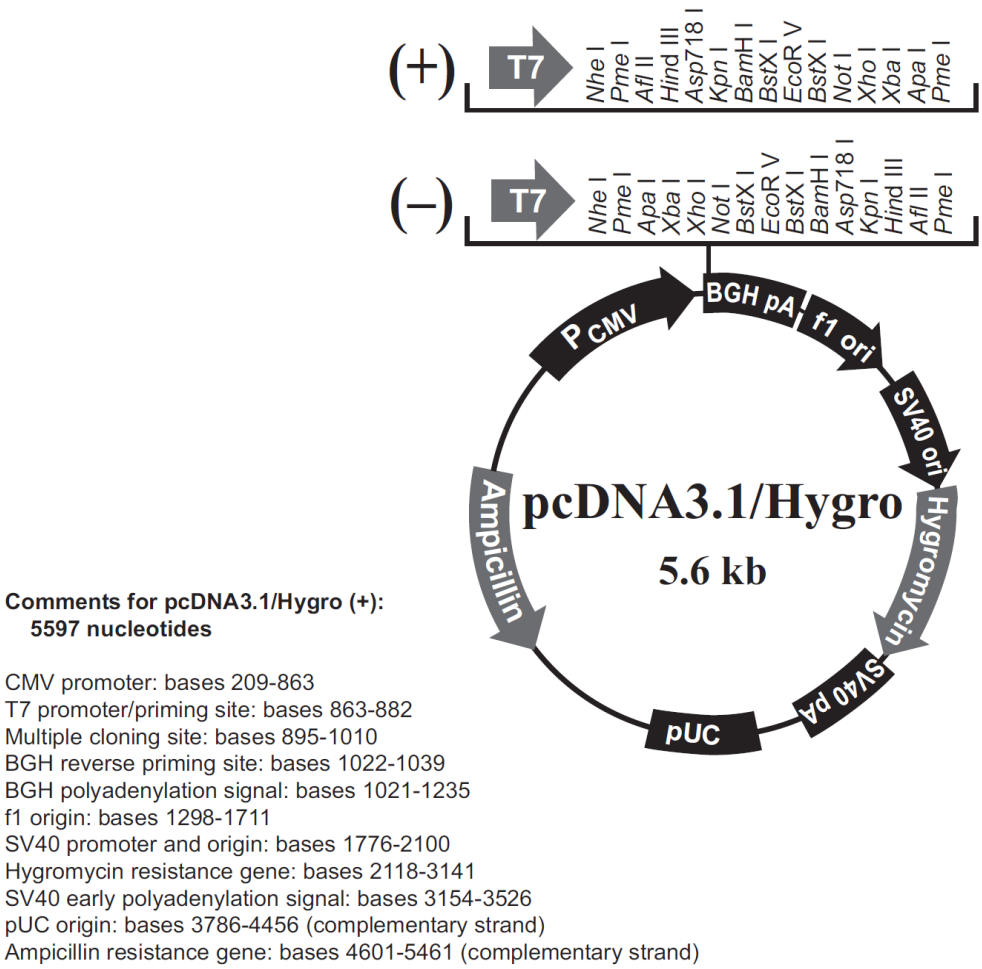


Figure 7: Scheme on the features of the expression vector pcDNA3.1-Hygro(-) (Invitrogen).

2.1.3.3 **Top** 10 F'-cells

The Top 10 F'- cells are a strain of E. coli bacteria, which is able to successfully depict transformed bacteria clones through blue-white screening with help of its genotype (Φ80lacZΔM15).

2.2 Methods

2.2.1.1 Preparation of thrombocytes, erythrocytes and lymphocytes

Platelets were prepared from freshly drawn human blood anticoagulated with an appropriate amount of buffered citrate. The samples were spun in a centrifuge at 200 g for 15 min at room temperature (RT). This low-speed centrifugation yields three phases: the bottom phase consisting largely of erythrocytes and granulocytes, then

follows a whitish interphase of lymphocytes. The uppermost, turbid phase is termed platelet-rich plasma (PRP).

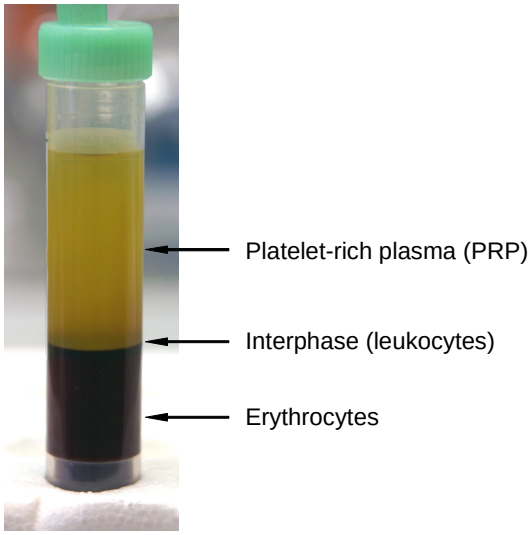


Figure 8: Preparation of platelet-rich plasma from citrate-anticoagulated human blood by low-speed-centrifugation at 200 x g.

The PRP was transferred to a new tube, leaving the whitish interphase intact to avoid spill-over of leukocytes or erythrocytes to the platelet-enriched fraction. The PRP was then centrifuged at 3200 g at RT to pellet the thrombocytes. The supernatant (platelet-poor plasma) was discarded leaving the thrombocytes on the bottom of the tube. In some experiments, the platelets were suspended in a 9:1-mixture of PBS/citrate and an aliquot analyzed in a phase contrast microscope to test the purity of the platelet preparation. The resulting cell suspension was almost devoid of contaminating leukocytes and contained only a small number of erythrocytes.

Erythrocytes and lymphocytes were prepared by Ficoll-density separation. EDTA-anticoagulated blood was mixed with 1 x PBS in a 1:1 ratio. The blood-PBS mixture was carefully layered over the similar amount of Ficoll-Paque^{TM} PLUS solution (Amersham Biosciences). Phases were separated by centrifugation at 400 x g for 30 minutes at room temperature.

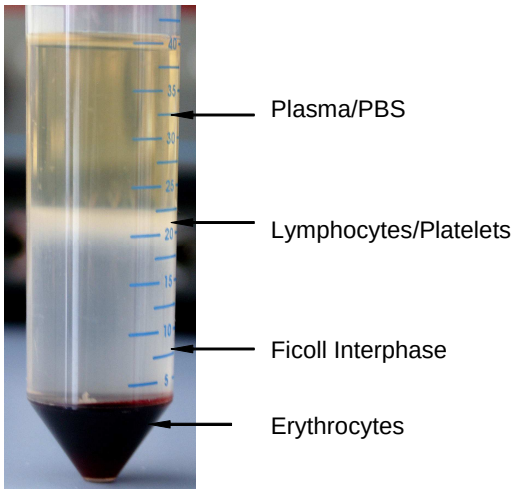


Figure 9: Separation of different particulate blood components by ficoll-density gradient centrifugation.

The lymphocyte-platelet fraction was further separated into lymphocytes and platelets by a low speed centrifugation. To this end the white interphase was resuspended in the PBS/Plasma mixture in a new tube and subsequently subjected to centrifugation at 150 g.

2.2.2 Isolation of total RNA from thrombocytes

The extraction of RNA is always tricky because of the RNAses. The thrombocytes were rapidly lysed in a buffer with guanidinum-isothiocyanate and β-mercaptoethanol in order to deactivate RNAses. After lysis, the RNA is bound to a silica-based matrix and subsequently cleaned by different wash steps and finally eluted from the column. Eluted RNA was immediately stored on ice and split into aliquots stored at -80℃ if it was not used immediately.

1,200 µl of RLT-buffer were added to the isolated thrombocytes and mixed by vigorous vortexing. The lysate was transferred to a shredder spin column placed in a collecting tube and spun for 2 min at 13,200 rpm to homogenize the sample.

One volume of 70 % ethanol was added to the homogenized lysate and the sample was again thoroughly mixed. Then 700 µl of the sample was added to a mini column which had been placed in a 2 ml collection tube. The tube with the inserted column was then spun for 15 s at 13,200 rpm at room temperature. The remaining of the sample was also loaded onto the column after discarding of the flow-through. The

column used could contain a total volume of only 700 µl so similar steps were repeated with the same column.

Then 700 µl of RW1-buffer was added to the RNeasy column and the column was spun for 15 s at 8,000 x g. The flow-through was again discarded. The RNeasy mini-column was then transferred into a new collecting tube. 500 µl RPE-buffer was added and the column spun at 8,000 x g for 15 s and the flow-through discarded subsequently.

This step was repeated with a centrifugation-time of 2 min. The RNeasy mini-column was then transferred into a new collecting tube to prevent contamination. To ensure that the membrane was free from RPE-buffer, the column was centrifuged at the same conditions for 1 min without addition of any buffer.

The dried column was then transferred into a sterile 1.5 ml Eppendorf-vial. 50 µl of RNAse-free water was added directly to the matrix of the column. The RNA was eluted by centrifugation at 13,200 rpm for 1 min.

For the photometric detection of the RNA-content and purity determination, 5 µl of the sample was transferred to a new tube. RNAsin was added to the remaining sample to protect the RNA from degradation from several RNAse isoforms (0.8 µl RNAsin per 45 µl sample) and thereafter, the sample was immediately split into aliquots and shock-frozen in liquid nitrogen. Human brain cDNA was used from a preparation already present in the lab.

2.2.3 Photometry

The concentration and purity of the RNA was determined by spectrophotometry at 260 nm, 280 nm and 320 nm after dilution of the 5 µl aliquot to 120 µl with Aqua ad injectabilia (AAI). Readings were done in a Smart spec 3000® spectrophotometer (Eppendorf, Germany). First, a blank reading was done by filling a quartz semi-micro-cuvette with 120 µl of AAI. Subsequently, the cuvette was emptied and the diluted sample was filled into the cuvette and read in the photometer. The concentration was estimated assuming an optical density at 260 nm (OD_{260}) of 1 per 40 µg x ml^{-1} x cm^{-1}. Purity was assessed by calculating the OD_{260}/OD_{280}-ratio. The ratios were > 1.7 in all samples analyzed.

2.2.4 cDNA-synthesis from total platelet RNA

In reverse transcription polymerase chain reaction (RT-PCR) , the RNA strand is first reversely transcribed into its DNA complement (complementary DNA, or cDNA) using a reverse transcriptase, and the resulting cDNA is amplified by PCR.

For the RT-PCR, we used a two-step method with the Superscript III first strand synthesis system (Invitrogen).

For a 20 µl reaction, ~50 ng of total RNA was added. The reaction was setup by preparing two different reaction pre-mixes: One contained the RNA, the random-primers and the dNTPs whereas the other pre-mix contained the enzyme, the buffer and the other constituents of the reaction.

Composition pre-mix 1

Random priming	Amount in µl
RNA	8 µl (aprox. 50 to 100 ng)
Random primer mix	1 (125 ng/µl)
dNTP mix	1 (5 mM each)

Composition of pre-mix 2

Component	Amount in µl
10 x Puffer Rt	4
25 mM $MgCl_2$	8
0.1 M DTT	4
RNAse out	2
Superscript III RT	2

We heated pre-mix 1 to 65℃ for 5 min in PCR-cycler to denature the RNA and thereafter quickly chilled the reaction on ice. We then collected the contents of the tube by brief centrifugation and added 10 µl of the cDNA-synthesis-mix. Subsequently, the reaction was incubated at 25℃ to allow for annealing of the primers to the RNA. The cDNA-synthesis was then performed at 50℃ for 50 min. The reverse transcriptase was inactivated by incubation at 85℃ for 5 min. Then, 1 µl

RNAseH was added and the reaction incubated at 37℃ for 10 min to remove interfering remaining RNA-molecules. The cDNA was then used as a template for amplification in PCR. Unused cDNA was stored at -20℃ until further use.

2.2.5 Polymerase Chain Reaction (PCR)

The purpose of a PCR is the amplification of DNA, which was acquired in our case through the reverse transcription. A PCR consists of many cycles which include denaturation, annealing and extension.

The denaturation step induces separation of the DNA into single strands; this is carried out at high temperatures in our case at 94 ℃ for 20 s. Primer annealing to the single-stranded DNA-template is performed at a lower temperature in our case e.g. 58℃ for 30 s. The extension or elongation is depen dent on the Taq-polymerase used. In our case we used a temperature of 68℃ for 2 min. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs in 5' to 3' direction. This is repeated for a total of 35 cycles and results in an exponential increase of DNA. The overall reaction is started by a prolonged initial denaturation (e.g. for 2 min). Subsequently, the 35 cycles of amplification follow. The reaction is ended by a final elongation step for 5 to 7 min at the elongation temperature before the sample is cooled down to 4℃ until further use. A typical reaction setup using the XL-PCR Kit from Roche is given below.

PC reaction set-up

Reagent	Amount [µl]
3.3 x buffer	7.60
dNTP-blend	2.00
Fw-Primer [10mM]	0.63
Rv-Primer [10mM]	0.63
MgOAc	1.00
Polymerase	0.50
AAI	7.64
cDNA	5.0

Cycling conditions

Step	Temperature [℃]	Step-duration	Repetition
Initial denaturation	94	2 min	1 x
Denaturation	94	30 s	35 x
Annealing	58	30 s	
Elongation	68	2 min	
Final elongation	68	5 min	1 x
Cool-down	4	∞	1 x

After completion of the reaction, a small quantity of the PCR-product was analyzed by agarose gel electrophoresis to ensure that the product was correct by band sizing and also specific, in order to proceed with subsequent cloning.

The cloning strategy finally used is explained in detail in the results section. We used an approach of three overlapping fragments of coding cDNA-sequence and reassembled these fragment to a full length clone. This is the final approach used. We started with an attempt of expressing a full length clone stemming from a single reversely transcribed cDNA . Attempts to obtain overexpression in Hek- cells of the untagged receptor were negative. Retrospectively, the failure is more likely attribute to the failure of the commercial available antibodies. Thus, a step-wise approach to generate the tagged receptor was planned.

Fragment	Amplicon Size (bp)	Primer (5′ → 3′)
F1	437	Y12_nat_F1_Fw CATTCAAACCCTCCAGAATCAw Y12_nat_F1_Rv TAAATGGCCTGGTGGTCTTC
F2	620	Y12_nat_F2_Fw CCTCCGTCATATTTTATTTCACAA Y12_nat_F2_Rv TCTGAAGGACTTGCAAAGGAA
F3	249	Y12_nat_F3_Fw GAGCACTCTGTGGTTAACTTCC Y12_KpI_F3_Rv AAGGTACCGCTTTGCTTTAACGAGT

Table 3: Three overlapping fragment, which were reassembled to a full length clone of P2Y12 with their sizes and primer combinations.

Three primer-combinations were used for the detection of the splice variants of P2Y12 in platelets and human brain. The strategy for the determination of the splice variants and the expression on RNA-level is depicted in Figure 10.

Transcript-Variants detected	Calculated Amplicon Size (bp)	Primers (5'→3')
Long, NM_022788.3	240	P2Y12_L_F GAC AGG AGC TGC AGA ACA GA P2Y12_SL_R GGC TTG CAT TTC TTG TTG GT
Short, NM_176876.1	225	P2Y12_S_F TGA AGC CCT CTT TTT CTC TCC P2Y12_SL_R GGC TTG CAT TTC TTG TTG GT
Both variants	236	P2Y12_co_F GAG CAC TCA AGA CTT TAC TGA CGA P2Y12_co_R CTG GTG CAC AGA CTG GTG TT

Table 4: Three Transcript-variants with their calculated amplicon sizes and various primers

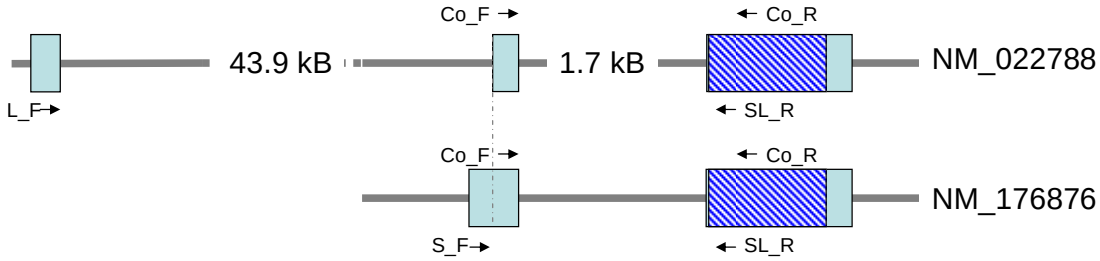


Figure 10: Depiction of the strategy used for the detection of the known splice variants of the P2RY12 gene.
The longer (NM_022788) and short (NM_176876) can be discriminated by unique sequences in their 5'UTR. The primer L_F and S_F bind to these unique sequences present in the cDNA. The reverse primer (SL_R) binds to a site present in both variants. The primer set Co_F/Co_R allows for the detection of the overall expression of P2RY12 RNA since both primer binding sites are shared by the transcript variants.

2.2.6 Addition of A-ends to PCR Product.

To increase efficiency when cloning and also to enable a simple ligation of PCR-product and vector, A-ends are added to blunt ends of PCR product. A-ends were added to the PCR-product by means of the terminal transferase activity of Taq-polymerase.

The reaction mix is as follows

Component	Amount
PCR-Product	10.0 µl
dATP (2.5mM)	2.5 µl
Buffer Y (10 x)	2.5 µl
Taq-polymerase	1.0 µl

AAI	9.0 µl

The reaction was performed in a thermocycler for 1 hour at 72℃. The resulting mixture containing the DNA with unpaired A-overhangs was mixed with 1 µl glycogen and 300 µl of absolute ethanol and kept for 20 min at -20℃ and then centrifuged for 10 min at 14000 rpm. The supernatant was removed and the pellet and left to dry. The pellet was finally dissolved in 10 µl of AAI.

2.2.7 Analytical and preparative DNA-electrophoresis

Gel electrophoresis of nucleic acids and proteins is driven by the electromotive force (EMF) that moves the molecules through the gel matrix for e.g. agarose. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass. Smaller molecules move faster through the gel insuring a separation of the DNA-fragments according to their sizes.

Agarose gel for DNA analysis

Mostly used was a 1% agarose gel-matrix with tris-hydroxymethyl-aminomethane-borate-EDTA (TBE) as a buffer-system.

For a small 1%-gel, 0.5 g agarose was thoroughly suspended with 50 ml of 1 x TBE buffer by a magnetic stir bar. The resulting suspension was heated in a microwave oven at 800 W untill it boiled. This procedure was repeated three times, until the solution was clear and free of foam. The solution was allowed to cool down to about 50℃ before adding 2 µl of ethidium-bromide to the solution. During this time the gel chamber and combs were prepared.

The solution was then poured into the chamber, the comb was inserted and the gel left to harden. Subsequently, the comb was removed and the hardened gel was transferred into the electrophoresis chamber filled with 1 x TBE-solution, ensuring that the gel was entirely covered by the buffer. Samples were then loaded into the slots formed by the comb during gel hardening. To ensure proper loading, the samples were mixed with 6 x DNA-loading buffer which contained glycerol, xylene cyanol and bromphenol-blue. The glycerol increases the density of the samples enabling the DNA-molecules sink into the slots nicely. The two dyes allow for

visualisation of the progress of the electrophoresis. At least one DNA-sizing standard was loaded onto the gel to determine DNA-fragment sizes.

After electrophoresis the gel was transferred to an electronic gel documentation system (CHEMIGenius2, bio-imaging system from Syngene). The gel was illuminated with short wave UV-light by a transilluminator. Images were captured by a computer-linked CCD-camera equipped with a filter specific for the orange-red fluorescence of ethidium bromide. The first lane mostly contained a DNA- ladder for sizing, and the other lanes contained the DNA fragments that were to be analyzed. The molecules in the gel were visualized. The distance a band travels is approximately inversely proportional to the logarithm of the size of the molecule.

2.2.8 DNA extraction after electrophoretic separation

The DNA-extraction from agarose gel was done after running a gel electrophoresis using mostly in 0.7% gels. The bands were visualized with UV-light only briefly to avoid UV-induced damage to the nucleic acid. The band with the correct size was cut out of the gel using a scalpel taking care to cut out as little agarose as possible. The gel piece was then weighed and the DNA was extracted using the QIAquick extraction kit (Qiagen), according to the protocol of the manufacturer.

After the gel piece containing the DNA had been weighed, three volumes QG-buffer were added to one volume of DNA-containing agarose slice. Then the sample was transferred to a thermoblock (Eppendorf) and incubated at 50℃ with constant shaking (550 rpm) for 10 min. During this step the gel dissolved completely. The colour of the lysis-reagent was yellow indicating that the pH was optimal for binding of the DNA to the subsequently used matrix (≤7.5). Subsequently one gel volume of isopropanol was added and the preparation was thoroughly mixed. The solution was then transferred to the QIAquick spin-tube with a silica matrix and then spun at 14, 000 rpm for 1 min at room temperature. The flow-through was discarded. When the volume of the dissolved agarose/DNA-mix exceeded 750 µl the remnant of the sample was transferred into the same QIAspin-tube and spun again at 14,000 rpm for 1 min (The QIAspin-tube could only contain a total volume of 750 µl). Then 400 µl of QG-buffer were added onto the column and again spun at 14,000 rpm for 1 min. Again the flow-through was discarded. Then we added 750 µl of PE-buffer to the column which was subjected to centrifugation at room temperature for 1 min at

14,000 rpm. Again the flow-through was discarded. The empty column was again centrifuged for 30 s at 14,000 rpm, ensuring that the DNA was free from buffer. The column was then transferred to a sterile vial and 50 µl of EB-buffer was loaded directly onto the matrix and then incubated for 1 min at room temperature. Afterwards we centrifuged at 12,000 rpm for 1 min at room temperature and obtained the eluate.

2.2.9 Ligation of PCR-Product

Ligation is a method used to covalently link DNA fragments by newly formed phosphodiester bonds with the help of a DNA-ligase. We used the TA-cloning Kit following the protocol of the manufacturer. The ligase was always added as last compound after all other contents had been thoroughly vortexed. After addition of the ligase, the reaction mixture was only gently mixed by pipetting up and down 5 times.

Reaction mix

Component	Amount
A-PCR-product	4 µl
TA-Cloning vector	1 µl
10 x ligation buffer	1 µl
T4-Ligase	1 µl
AAI	3 µl

The incubation was performed overnight in a thermal incubator at 14 ℃.

To remove salts, the product was precipitated with:

Component	Amount
Glycogen	1,5 µl
3 M Na-Acetat	0.3 µl
Absolute Ethanol	300 µl

The reaction was vortexed and then incubated at -20℃ for at least 30 min.

Subsequent to precipitation, the ligation products were spun down at 14,000 rpm for 10 min. The supernatant was discarded, the pellet washed in 200 µl of 80% ethanol and the pellet dissolved in 10 µl AAI after removal of the ethanol with subsequent drying.

2.2.10 Transformation of competent bacterial cells

As a first step, we prepared kanamycin blue-white plates by spreading of 50 µl of X-gal and 50 µl of IPTG on kanamycin-containing selection plates.

For transformation, 5 µl of desalted ligated DNA was added to 25 µl of top-10-F´ chemically competent cells. The mixture was then carefully stirred with the pipette tip and left on ice for 30 min. Meanwhile, a thermoblock was set to 42℃. After 30 min on ice, the mix was put into the preheated thermoblock for exactly 1 min to cause a heat-shock of the bacteria. Thereafter 300 µl of SOC-medium pre-warmed to 37 ℃ was added to the bacterial suspension and incubated in a thermoblock at 37 ℃ with constant swirling at 300 rpm for one hour (h).

The transformation mix was then spread onto the kanamycin plates prepared for blue-white-screening with a Drigalski spatula and incubated overnight at 37℃. In general, two different amounts of the suspension were spread on two plates to achieve well isolated colonies.

After overnight incubation at 37℃, cream-white well isolated colonies were picked and transferred by means of a sterile pipette tip into 5 to 10 ml LB medium supplemented with the appropriate antibiotics for selection. To generate mini-preps, the suspension was incubated overnight at 37℃ with constant agitation to aerate the broth.

2.2.11 Plasmid-DNA preparation from E. coli bacteria in mini format

The principle of plasmid preparation can be described as follows: the bacterial pellet is re-suspended in a buffer. Subsequently, an alkaline buffer containing sodium dodecyl sulphate is added, causing cell lysis. The lysate is then neutralized by an acidic buffer containing a high concentration of potassium and acetic acid. Under these conditions the proteins and chromosomal DNA of the bacteria are trapped in a precipitate containing a large amount of potassium dodecyl sulphate. In contrast, plasmids remain soluble. After separation of the solid from the liquid phase, the DNA-molecules are bound to the silica-matrix of a plasmid preperation column. Finally washing steps are carried out, to free the DNA-molecules from contaminants and the DNA-molecules are eluted.

We followed the standard protocol for plasmid DNA-preparation with Nucleospin Plasmid kits (Macherey-Nagel). In general, we used 5 - 10 ml of saturated E. coli culture after overnight incubation in LB-medium containing the appropriate antibiotic. The cell suspension was spun for 10 min at 4000 rpm at 4℃. We discarded the supernatant leaving the bacterial pellets in the tube. To the bacterial pellet, 250 µl of buffer A1 was added to re-suspend the cells. The suspension was vigorously vortexed till it was free from clumps. The cell suspension was then transferred into sterile 1.5 ml Eppendorf-tubes. 250 µl of buffer A2 was added and the vial inverted 6 - 8 times and left to incubate for 5 min at room temperature. The mixture became clear and viscous indicating cell lysis. Then 300 µl of A3 buffer was added and inverted 6 - 8 times in order to neutralize the lysate. Then it was spun for 10 min at 13,200 rpm for 10 min at room temperature in order to separate the forming precipitate from the lysate containing the DNA. The supernatant was carefully separated from the white-yellowish pellet with a pipette tip and transferred to the NucleoSpin plasmid column in a 2 ml collecting tube. This was then centrifuged for 1 min at 13,200 rpm at room temperature. The flow-through was discarded. 500 µl pre-warmed AW buffer was applied to the column and again spun at 13,200 rpm for 1 min at room temperature. The flow-through was discarded.

The NucleoSpin column was placed in a 2 ml collecting tube and 600 µl of buffer A4 was added and spun at 13,200 rpm for 1 min at room temperature. The flow-through was discarded.To dry the silica matrix, the column was re-inserted into a 2 ml collecting tube and centrifuged at 13,200 rpm for 2 min. The column was then transferred into a sterile 1.5 ml vial and then eluted with 50 µl of buffer AE which was added directly on the matrix and incubated for 1 min, spun for 1 min at 13,200 rpm at room temperature.

2.2.12 Maxi-format plasmid preparation

The maxi-format plasmid preparation resembles the mini-plasmid preparation described above with the difference that through this method, we are able to obtain a higher amount of plasmid-DNA.

The maxi plasmid preparation was carried out using the Qiagen HiSpeed plasmid purification kit following the protocol of the manufacturer.

An overnight culture in 200 ml LB-medium was obtained after inoculation with a small amount of pre-culture and subsequent incubation for 15 hours at 37℃ and shaking at 180 rpm. The bacterial suspension was then transferred into 50 ml blue cap tubes which were then spun for 15 min at 4,000 rpm and 4°C. The supernatant was discarded and the tubes were inverted to remove traces of remaining medium.

The pellets were resuspended completely by vortexing and pipetting up and down in 10 ml buffer P1. Then 10 ml buffer P2 was added to the suspension and the suspension mixed by inverting 4-6 times and incubated for 5 min. The lysate was viscous indicating that lysis had taken place. During this time the QIAfilter maxi cartridge was prepared and put into a receiving container. Then 10 ml of pre-chilled buffer P3 were added and the reaction again mixed gently. The suspension was cloudy due to precipitation of potassium-dodecyl-sulphate and proteins. Then the lysate was transferred into QIAfilter maxi cartridges and incubated for 10 min at room temperature. During this time the matrixes HiSpeed maxi tips were equilibrated with 10 ml buffer QBT by allowing the buffer flow through by gravity flow. The cap of the QIAfilter cartridge outlet nozzle was removed. Then the plunger inserted into the cartridge and the cell lysate was filtered into the equilibrated HiSpeed tip. The lysate was allowed to enter the resin by gravity flow.

The Qiagen tip was then washed with 2 x 30 ml QC buffer and the DNA eluted with 15 ml of QF buffer and the solution transferred into a sterile 50 ml-tube. The DNA was precipitated by addition of 10.5 ml isopropanol and incubation for 5 min at room temperature.

During the incubation, the plunger from a 30 ml syringe was removed and the QIA-precipitator was attached to the outlet nozzle. The outlet of the QIA-precipitator was placed in a waste bottle and then the eluate/isopropanol mixture was placed into the syringe and the plunger was reinserted. The mixture was then filtered through using constant pressure on the plunger.

The QIA precipitator was removed from the syringe and thereafter the plunger was withdrawn from the syringe. The QIA-precipitator was then re-attached to the syringe and afterwards the plunger was reinserted. Then air was pressed through the filter to dry the membrane. The outlet nozzle was also cleaned with absorbent paper to prevent carry-over of ethanol.

Then the plunger of a 5 ml syringe was pulled out, the QIA-precipitator was attached to the small syringe and placed over a sterile 1.5 ml Eppendorf tube. One ml of buffer TE was transferred into the syringe. The plunger was inserted and the solution pressed through using constant pressure.

The QIA-precipitator was removed and afterwards the plunger. Then the QIA-precipitator was re-attached. The eluate was placed again into the syringe and pressed into the same 1.5 ml Eppendorf tube. Ten µl of the eluate were added to 140 µl of AAI and the DNA-concentration and purity were determined by photometry. The rest of the eluate was stored at 4℃.

2.2.13 Restriction endonuclease digestion.

In order to be able to ligate different DNA fragments, it was important to be able to construct the ends of these fragments to fit into the vector. This was done using restriction endonucleases. These are enzymes that are able to recognize specific base sequences in a DNA-double helix and in both strands of the helix; they cleave phosphodiester bonds producing the needed ends (sticky or blunt ends). In addition restriction enzyme digestion was used for analytical purposes to verify e.g. correct cloning of a DNA-fragment into a vector.

A typical reaction is exemplified in the following table:

AAI	20.5 µl
10 x Reaction buffer[1]	2.5 µl
Restriction enzyme	1.0 µl
DNA (up to 2 µg)	1.0 µl
Sum	25.0 µl

[1]Reaction buffer specific for the enzyme used

This mix was vortexed and incubated normally for an hour or longer at a temperature given by the manufacturer and specific for the restriction enzyme used. The reaction temperature usually reflects the natural living conditions of the bacterium from which the restriction enzyme was originally isolated.

Some rules were applied to calculate the reaction mixtures. The proportion of the restriction enzyme of the total volume did not exceed 10% to avoid a too high concentration of glycerol stemming from the storage buffer of the enzyme. This is

important to avoid either impairment of activity or star-activity (increasingly non-specific cleavage of the DNA by relaxation of the recognition site detection). The buffer used for digestion was in general the optimal buffer recommended by the manufacturer. Again, deviation from the recommended buffer-type can cause either impairment of activity or star-activity. In some cases, we added a small amount of bovine serum albumin to the reaction mixture to stabilize the enzyme as suggested by the manufacturer of the enzyme. The amount of water in the mix was then reduced accordingly.

These considerations were particularly important for double digestions. Usually it was attempted to find a buffer-type in which both enzymes are sufficiently active. If no star-activity is caused by the non-optimal buffer-system, the reduced activity of an enzyme was compensated for by increasing the enzyme amount. In cases where star-activity was not likely preventable in a simultaneous double digestion, the DNA was digested in a sequential approach with intermittent cleaning of the product by ethanol precipitation.

2.2.14 Sequencing

As we built expression constructs for P2Y12, high quality sequencing was mandatory and all intermediate constructs used for assembly of the cDNA and the final construct were extensively verified.

Sequencing was performed by a modified didesoxy-chain-termination technique the principle of which has been first described by Sanger. The technique uses the chain termination caused by dideoxy-nucleotides when these are incorporated as terminal base in a newly synthesized DNA-strand. For each of the four bases the reaction mix contains a respective dideoxy-nucleotide labelled with a specific fluorochrome. The reaction contains regular deoxy-nucleotides in stochiometric excess so that the chain termination by a dideoxy nucleotide is occurring stochastically. Thus DNA-molecules of different lengths are synthesized by polymerase-driven extension of a primer hybridized to the DNA to be analysed. Each new molecule can contain only one fluorescent dye (at the last base added). When this termination reaction occurs is determined by the ratio of dideoxy- and regular deoxy-nucleotides and the amount of DNA added to the mixture. At the end of the reaction, which was performed as cycle sequencing with Taq-polymerase, the reaction mix contained a blend of differently

long DNA-molecules each labelled with a specific fluorochrome at its terminal base. These molecules were then subjected to an electrophoretic size separation and the terminal label of the DNA detected by its specific fluorescent signal evoked by laser excitation.

To ensure a full length reading of a clone, we used at least two primers per clone, a forward and a reverse primer. When working with the cloned fragments – used for assembly of the entire cDNA of P2Y12 – usually two primers binding within the vector upstream and downstream of the multiple cloning site were used (PCR-II-down and M13-Rev). At later stages of cDNA-assembly, additional internal primers were used.

Reaction-Mix for Sequencing

Plasmid DNA	1.5 µl
Primer (10 µM)	1.5 µl
Sequence mix	2.0 µl
Buffer	8.0 µl
AAI	7.0 µl

Temperature conditions for sequencing cycle

Temperature	Duration	Repetition
96℃	10 s	25 X
50℃	5 s	
60°C	4 min	

The resulting products were precipitated by addition of 300 µl absolute ethanol and 80 µl 3M NaAc to remove unincorporated primers and free nucleotides. The samples were incubated at -20℃ for an hour. Subsequently the reaction products were centrifuged at 14,000 rpm for 10 min. The supernatant was removed and the tube left to dry. Usually no precipitate was visible at this step. The tube was then labelled with a code and transferred to the department of molecular pathology for electrophoresis in a capillary sequencer.

The resulting sequencing data was visualized with the freeware program FinchTV. The entire reading was inspected for accurate base-calling and errors corrected.

Ambiguous sites were left uncorrected. Then the sequence was aligned with the reference sequence of P2Y12. Ambiguous readings were corrected by means of the reverse reaction or by repeating of the sequencing with a modified amount of DNA-input or an additional primer.

2.2.15 Cell culture

2.2.15.1 Coating of cell culture bottles with collagen

To ensure a better adherence of the HEK293T cells, the surface of the cell culture bottles were coated with collagen.

The cooled collagen solution was diluted with sterile ice-cold 1x PBS solution 1:100 (45.5 ml 1x PBS and 500 µl collagen) and completely mixed. Then 5 ml of this mixture was put into a small cell culture (75 cm^2) bottle and then shaken till the bottom was entirely coated with the mix. The amount of mixture is dependent on the size of the bottle. With the help of a 10 ml pipette the rest of the mixture was removed and put into the next bottle until all needed bottles were coated. The rest was then put back into a falcon and stored for further use at 4℃.

2.2.15.2 Passaging of cells

Depending on the experiments the cells were spread into different culture plates with different sizes and amounts of wells.

The medium was first removed and then trypsin was added to the plate in different amounts depending on the size. The plates were shaken and the trypsin was removed. Thereafter a small amount of fresh trypsin sufficient to form a thin layer on the cellular surface was added to the culture and left on the cells for a few minutes. The culture vessel was shaken and rocked to release the cells. Then full medium was added to stop the function of the trypsin by inhibition of α1-antitrypsin stemming from the fetal calf serum within the medium. The suspension was then transferred to a falcon and the cells were carefully suspended by pipetting up and down. Then the suspension was centrifuged for 5 min at 100 x g. The supernatant was discarded and the cell pellets were resuspended in an appropriate amount of medium. Then equal amounts were transferred to the collagen coated wells. After addition of the suspension the culture vials were shaken so that the cells were equally spread on the surface.

2.2.15.3 Transfection of HEK 293T

Transfection is a method used to introduce foreign DNA into a eukaryotic cell. There are different methods of transfecting cells. FuGENE Transfection Reagent (Roche)

was used for the experiments. In contrast to several other liposomal transfer techniques, FuGENE liposomes are non-toxic and do not need to be washed away from the transfected cells after addition to the culture. Moreover, the reagent is working efficiently in more confluent monolayers.

FuGENE HD Transfection

Prior to the experiments, different ratios of FuGENE reagent and DNA were tested to optimize the future experiments. In our experiments optimal transfection was done using 6 µl of DNA (0.1 to 3.0 µg) and 8 µl of FuGENE reagent in a total volume of 100 µl (92 µl of Opti-MEM I reduced serum medium) .

The transfection was carried out as follows:

6 µl of DNA was diluted in 92 µl of OPTI-MEM I Medium. To this mix 8 µl of FuGENE was added directly ensuring that there was no contact with the plastic surface of the tube. The tube containing the transfection complex was mixed by tapping. Afterwards the mix was left to incubate for 15 min at room temperature to allow for formation of DNA-containing liposomes.

The culture dishes containing the cells to be transfected were removed from the incubator. The removal of the growth medium was not necessary. The transfection complex was added in drop-wise manner onto the surface of the medium and the culture vessel swirled carefully after addition of the final drop. The cells were then incubated at 37℃ and 5 % CO_2 for at least 24 - 48 h before harvest to detect P2Y12-expression.

2.2.15.4 Preparation of stable cell lines

The expression vector pcDNA3.1-Hygro(-) encodes a resistance gene protecting cells from the action of hygromycin B an aminoglycoside acting as inhibitor of translocation in eukaryotes. Forty-eight hours after transfection selection with hygromycin B was started at a concentration of 200 µg/mL. This concentration was shown previously in our laboratory to effectively kill the parental HEK293T cell line. The cell culture medium was changed twice a week. Approximately 2 to 3 weeks later, cellular clusters of living cells were visualized by microscopy and the corresponding areas marked with a stamping objective. The cells were then lifted from the culture dish by gently scraping and pipetting up with a 100 µl pipette. The

resultant suspension was spilt in halves and seeded to new 24 well cell culture clusters. Of the resulting two wells containing the quasi-clonal cells, one well was analysed by immunofluorescence and the other kept for propagation of the line. In general it took 4 to 6 weeks to get hold of a confluent 10 cm diameter cell culture dish.

2.2.15.5 Harvesting cells from cell culture

To analyse the expression of P2Y12 in the transfected cells and cell lines, we prepared lysates by detergent extraction with the non-ionic detergent NP40 or RIPA-buffer.

The cells were rinsed with the cell medium in the wells. To release all adherent cells (HEK 293T) we repeated pipetting up and down to enforce mechanical release of the cells from the bottoms of the wells. The harvested cells were then transferred into 10 ml tubes. The cells were pelleted at 100 x g at a temperature of 4 ℃ for 10 min.

The supernatant (medium) was then discarded. 1 ml PBS was added to each pellet and the cells carefully resuspended to remove serum proteins. The cells were then spun down again. This washing step with PBS was repeated once. The supernatant was then discarded and the pellets were resuspended in 250 µl of 1% NP40 with a protease inhibitor cocktail .

The cytosolic and soluble membrane proteins were then separated from insoluble material by centrifugation at 13,200 rpm for 20 min at 4℃. The supernatants were split in aliquots and transferred into vials, shock frozen and stored at -80℃.

In some experiments, enrichment for membrane protein was performed by a modification of the aforementioned procedure. After washing of the cell pellet with 1 x PBS, the cells were resuspended in 1/10 PBS with protease inhibitors and left on ice to swell in this hypotonic milieu for 30 min. Thereafter, the cell membranes were disrupted by 3 freeze-thaw-cycles. Then the suspension was centrifuged at 16,000 x g for 20 min to pellet most of the particulate fraction. The supernatant containing soluble cytosolic proteins was removed and shock frozen in aliquots. Thereafter, the particulate fraction was incubated with 1% NP40 in PBS for 30 min on ice to extract soluble membrane proteins. By these means an enrichment of the membrane proteins was achieved and a higher amount of this fraction of proteins could be loaded within a single lane of the Western blot. In whole cell extracts, the

amount of membrane proteins analyzable was limited due to the high content of cytosolic proteins causing overloading of the lanes.

2.2.15.6 Stimulation of cells with ADP

As a read out of the receptor function the phosphorylation status of the MAP-Kinases ERK1 and ERK2 (p42/p44) were analysed.

One stable cell-line expressing low to moderate amounts of P2RY12 (HA-P2Y12/HEK293-3/I) and the parental, non-transfected HEK293T-line were seeded in 6-well clusters to yield a density of about 60 to 70% after one day of growth. One day after seeding,the cells were starved for 6 hours by replacing the regular growth medium (10% FCS) with serum-reduced medium (0.2% FCS). The starvation phase of the experiment was necessary to reduce the amount of MAP-Kinase phosphorylation. Regular cell culture medium contains too many mitogenic factors and the phosphorylation of the target proteins would be too high to detect the effect exerted by stimulation of P2Y12. Subsequently, the medium was withdrawn and the cells washed with PBS at room temperature and challenged with increasing concentrations of ADP for 5 min. The stimulator solution was then entirely withdrawn and the cellular proteins were harvested by addition of ice-cold RIPA-buffer to the monolayer. Nuclei and other non-dissolved remnants were removed by centrifugation at 14,000 x g for 10 min and the dissolved proteins shock frozen in liquid nitrogen. To investigate the time dependence of activation a time series with a single stimulator concentration was performed. Phosphorylation was investigated by means of phospho-specific antibodies after Western-blotting of the proteins (see below).

2.2.16 Immunofluoresence

For immunoflurescence detection, cells were grown in multiwell dishes on cover-slips. Cells were transfected with a dilution of different amounts of plasmid in a liposomal transfection reagent. At 48 h after transfection, the cells were rinsed twice with PBS and an anti-HA-antibody directly coupled to fluorescein was used to visualize the successfully transfected cells with the settings on an inverted confocal microscope. The cells were left with a 1:200 dilution of the antibody in PBS for 2 h at room temperature. The PBS contained 5% bovine serum albumin and 10% fetal calf serum to block non-specific binding. A mock reaction (transfection procedure in the absence of DNA) served as negative control.

A similar procedure was employed for the detection of the recombinant protein by means of the specific anti-P2Y12 rabbit antiserum. The cells grown on coverslips were fixed for 20 min in 4% buffered formaldehyde. This fixation step was followed by blocking of non-specific binding sites and permeabilisation of the cells by incubation in 10% fetal calf serum, 0.1% Triton-X100 in 1 x PBS. After 1 h, the blocking solution was withdrawn and the solution replaced by a 1:1000 to 1:2000 dilution of the antiserum in blocking solution. After 2 h incubation at room temperature the cover slips were washed for 3 x 2 min in 1 x PBS and subsequently incubated with 1:1000 diluted secondary antibody. The solution additionally contained the DNA-staining dye DRAQ-5 to counterstain cellular nuclei (Cell signalling technologies). We used a highly cross-absorbed Alexa-488-coupled goat anti-rabbit serum (Invitrogen). Thereafter the cover slips were again washed for 3 x 2 minutes in 1 x PBS. After removal of PBS the cover slips were mounted on a slide by means of Paramount aqueous mounting medium.

The fluorescence was detected by means of a confocal microscope with excitation by an Argon-Laser at 488 nm.

2.2.17 Western Blot

The western blot is an analytical technique used to detect specific proteins in a complex protein sample, e.g. tissue extract. It separates proteins in a polyacrylamide matrix by means of electrophoresis, mostly under denaturing and reducing conditions. The separation velocity of a given protein depends on its molecular weight. However proteins with e.g. unusual secondary structures or extensive posttranslational modifications, in particular glycosylation, may not travel in accordance with standard proteins of the same molecular weight.

After completion of the separation, the proteins are then transferred to a membrane (nitrocellulose or polyvinylidenfluoride, PVDF) by electrophoresis. After transfer the proteins are accessible for antibodies to probe for the proteins of interest. The detection was achieved by enhanced chemoluminescence. After incubation with a mono- or polyclonal antibody against the protein of interest the bound primary antibody is detected by a secondary antibody. The latter antibody is directed against a constant part of the primary antibody. The secondary antibody is coupled to an enzyme that catalyses a light-emitting reaction. Most techniques use peroxidase to

catalyse the reactions. The emitted light is detected by exposure of X-ray films or means of an electronic camera.

Electrophoretic separation of proteins

We used the discontinuous SDS buffer system described in its modifications by Laemmli et al. First the separation gel was cast, after the glass plates of the mini-gel chambers (BioRad Mini Protean II,) had been correctly assembled in the casting rack. The reaction mixture of the separating gel was poured between the glass plates and filled ¾ of the volume. The solution was then overlaid with isopropanol to prevent contact of air with the reaction mixture. The oxygen contained in air may prevent the formation of a clear-cut surface of the separating gel by interference with the polymerization process. After completion of the polymerization, the isopropanol was discarded and the surface of the gel was thoroughly rinsed with deionised water. Thereafter, remaining droplets of water were removed with filter paper and the stacking gel mixture was poured into the remaining ¼ of the volume formed by the assembled glass plates. The coomb was inserted into the stacking gel solution. Care was taken to avoid the inclusion of air bubbles since they prevent polymerisation of the stacking gel and cause deformation of the slots. After completion of the polymerisation of the stacking gel, the coomb was removed and the slots were rinsed with deionised water to remove any non-polymerized solution. The electrophoresis apparatus was thereafter assembled and the electrophoresis tanks filled with running buffer.

The samples were prepared by loading of the proteins with SDS, heat-denaturation and reductive cleavage of intra- and intermolecular disulfide-bonds with DTT. To this end, the protein-preparation and the appropriate amount of 6 x Laemmli-sample buffer was mixed together and incubated at 65℃ for at least 10 min. After brief chilling the samples were loaded with a Hamilton-syringe into the slots of the stacking gel. A prestained protein standard served as size marker and was loaded in one slot.

After all samples and the standard had been loaded, the electrophoresis was started by connecting the apparatus to a power-supply set to 60 V until the dye front had migrated through the stacking gel zone. Subsequently, the voltage was increased to 100 to 120 V and the electrophoresis was run at least until the dye front had reached the lower end of the glass-plates.

Blotting

In order to make the proteins accessible to antibody detection, they are transferred from the gel onto a nitrocellulose or polyvinylindenfluoride (PVDF) membrane again by means of electrophoresis. The orientation of the electrical field is changed by 90° so that the proteins travel out of the gel and become stuck on a membrane by largely unknown forces. We used the wet transfer system in which the gel containing the separated proteins is submerged entirely in a buffer containing 20% methanol. Since the proteins are negatively charged due to their loading with SDS, the positive pole is oriented towards membrane.

A sandwich-assembly is made of a sponge, two layers of filter paper, followed by the gel and the membrane. The membrane is covered on the side facing away from the gel by two layers of filter papers and a second sponge. This sandwich is held in place between the electrodes by means of a cassette. It is important is that all materials are thoroughly soaked in blotting buffer and that the system is bubble free since bubbles – in particular those between the gel and the membrane – would cause an insulating effects with the consequence of band-distortion.

Using electro-blotting the proteins are pulled out of the gel and transferred to the PVDF-membrane. We ran the system usually for 1 h at 450 mA. The buffer was prechilled to 4℃ and the entire blotting apparatus was submerged in wet ice to prevent overheating of the system. The uniformity and overall effectiveness of the transfer from gel to the membrane was checked by inspection of the marker bands and staining of the transferred proteins with ponceau S. This azo-dye causes a reversible semi-quantitative staining of proteins. For staining, the membrane was briefly rinsed with water and the submerged in the staining solution for ~5 min. Thereafter, the blot was transferred to a dish containing deionised water and gently rocked to wash away excessive dye. Care needed to be exerted not to destain the proteins too much given the easy reversibility of the staining reaction.

Detection

After staining the membrane was entirely destained by incubation in TBS/T-buffer. Then remaining free protein binding sites on the membrane were blocked by over-night incubation in 5 % fat-free milk-powder or 5% BSA (for phosphor-proteins) dissolved in TBS/T-buffer. The detection was done in 2 steps.

We used different primary antibodies against P2Y12 and against hemagglutinin of human influenza viruses (which served as a tag of recombinant P2Y12). The antibodies were titrated to achieve a good signal-to-noise ratio. In our hands, the only specific antibody detecting the untagged P2Y12 antigen was a non-commercial rabbit-antiserum which was a kind gift of Dr. Pierre Savi, Sanofi-Aventis, France. In contrast, the commercially available antibodies did not yield any specific signal as detected by western-blotting of transfected and non-transfected HEK-cells.

The specific anti-P2Y12 rabbit antiserum was used in a dilution of 1:500 in 5% TBS/T supplemented with 0.2% SDS to reduce non-specific binding of the antibody. The monoclonal α-HA rat antibody 3F10 was purchased from Roche. The antibody was used in a 1:1000 dilution in 2 % Milkbuffer/TbstTween.

The incubation in the dilution of the primary antibody was performed for 4 to 5 hours under constant agitation on a roller mixer. Thereafter, the membranes were washed four times in TBST or TBST with SDS according to the different antibodies used.

Thereafter, the membrane was incubated in a 1:10000 to 1:20000 dilution of the appropriate peroxidase-coupled secondary antibody for one hour on a roller mixer. Then the membranes were washed four times in TBST or TBST with SDS.

In the presented experiments we used ECL plus substrate (GE Healthcare) as detection reagents. The appropriate amount of reagent A and reagent B were mixed after the reagents had been brought to room temperature. The reagent mixture was carefully spread onto the membrane which had been removed from the washing solution and very briefly dried by blotting with a sheet of filter paper. The reagent mix was pipetted onto the membrane in droplets evenly dispersed over the membrane area. The mix was homogenously spread by means of an overhead foil. To this end, the foil was repeatedly lifted and put back onto the membrane and gently squeezed with a soft tissue.

After five min of incubation the membrane was removed from the foil sandwich and put between to fresh transparency foils. The assembly was fixed on one side of an X-ray cassette. The cassette was transferred to a dark room and an appropriately cut piec of X-ray film was stuck to the cassette side facing away from the membrane site. The X-ray film was exposed by rapidly closing of the cassette for an appropriate time to yield non-saturated grey-values of the specific bands. The correct time was

determined experimentally. The X-ray films were manually developed by incubation in a series of photographic baths (developer-bath 1 min; stop-bath 1 min, fixation 5 min; water 10 min). The films were finally rinsed with deionised water and hung to dry.

2.3 Substances, Reagents, Machines

2.3.1 Substances

- 2-Mercaptoethanol: SIGMA Corporation, St. Louis, MO, USA
- 6x loading dye (Loading buffer): Fermentas GmbH, St. Leon-Rot, Germany
- Acrylamid 30%: AppliChem GmbH, Darmstadt, Germany
- Agar: Bacto Agar, Becton, Dickinson and Company, Sparks, USA
- Agarose: Electrophoresis Grade, invitrogen Ltd., Paisley, UK
- Ampicillin: SERVA Electrophoresis GmbH, Heidelberg, Germany
- Aqua ad injectabilia: Baxter Germany GmbH, Unterschleißheim, Germany
- Bacto Yeast Extract, Becton, Dickinson and Company, Sparks USA
- Bovines Serumalbumin: BSA, SIGMA Corporation, St. Louis, MO, USA
- Complete Protease Inhibitor: Roche Applied Science, Indianapolis, USA
- DMEM: Biochrom AG, Berlin, Germany
- DNA-Ladderstandard: GeneRuler 100 bp Plus und 1 kb, Fermentas GmbH, St. Leon-Rot, Germany
- dNTP-Mix, PCR-Reagenzien: Qiagen GmbH, Hilden, Germany
- DRAQ5^{tm}, Biostatus limited, Leicestershire, United Kingdom
- E.Z.N.A. Cycle Pure-Kit: PEQLAB Biotechnologie GmbH, Erlangen, Germany
- ECL: ECL-Plus Western Blotting Detection System, Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA
- Ethanol: J.T. Baker, 7400 AA Deventer, Niederland
- Ethidiumbromid: Fluka Biochemica, Buchs, Swizerland
- Fetal calf serum: FCS, Gibco, invitrogen Ltd., Paisley, UK
- Glukose: Roth Chemie GmbH, Karlsruhe, Germany
- Glycerin: Merck KgaA, Darmstadt, Germany
- Hepes: SIGMA Corporation, St. Louis, MO, USA
- Kanamycin: Gibco, invitrogen Ltd., Paisley, UK

- Methanol: J.T. Baker, 7400 AA Deventer, Netherland
- $MgSO_4$: Merck KgaA, Darmstadt, Germany
- $MnCl_2$: Merck KgaA, Darmstadt, Germany
- NaCl: J.T. Baker, 7400 AA Deventer, Netherland
- Nonidet P 40: SIGMA Corporation St. Louis, MO, USA
- NucleoSpin Plasmid-Kit: MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany
- Penicillin: Gibco, invitrogen Ltd., Paisley, UK
- Ponceau S: SIGMA Corporation, St. Louis, MO, USA
- QIAquick Gel Extraction-Kit, Qiagen GmbH, Hilden, Germany
- QIAshredder-Kit, Qiagen GmbH, Hilden, Germany
- Restrictionsendonucleasen: New England Biolabs GmbH, Frankfurt am Main, Germany
- RNeasy Mini-Kit: Qiagen GmbH, Hilden, Germany
- SDS, SIGMA Corporation, St. Louis, MO, USA
    - Small DNA Agarose, Biozym Scientific GmbH, Hessisch Oldendorf, Germany
- Super Script lll First Strand Synthesis System for RT-PCR: invitrogen Ltd., Paisley, UK
- Taq-Polymerase, PEQLAB Biotechnologie GmbH, Erlangen, Germany
- TEMED: Bio-Rad, Hercules, CA, USA
- Transfectionsreagents: Fugene 6 Transfection Reagent, Roche Applied Science, Indianapolis, USA
- Tris Base: Trisma base, SIGMA Corporation St. Louis, MO, USA
- Tris-HCl: SIGMA Corporation, St. Louis, MO, USA
- Trypsin: Trypsin-EDTA 0,5%, Gibco BRL Life Technologies, Eggenstein, Germany
- Trypton: Bacto Tryptone, Becton, Dickinson and Company, Sparks, USA
- TWEEN 20: SIGMA Corporation, St. Louis, MO, USA

2.3.2 Solutions

Solutions	Preparation	End concentration
Agar Medium	7.5 g agar suspended in 500 ml LB-medium, autoclaved	1.5 mg/ml
Ampicillin	40 mg ampicillin dissolved in 8 ml Water	50 µg/ml
Blot buffer	10x solution: 60.5 g Tris, 288 glycin dissolved in 2 l. For 2 l 1x- buffer: 200 ml solution, 1,400 ml water, 400 ml MetOH	25 mM Tris 192 mM Glycin
Ethidiumbromide	10 mg dissolved in 1 ml water, diluted 1:10 with water	1 mg/ml
Kanamycin	120 mg kanamycin dissolved in 4 ml water	30 µg/ml
Laemmli	6x solution: 2,4 g SDS, 20 mg bromphenolblue, 2,4 ml 0,5 M Tris-HCl pH 6,8, 12 g glycerol, filled up with water to a total volume of 2 ml, 1,86 g DTT	
LB-Medium (low salt)	10 g trypton, 5 g yeastextrakt, 5 g NaCl with demineralised water ,total volume of 1 l and autoclaved, pH 7,4	
Loading dye 6x	18.9 g glycerin, 25 mg bromphenolblue und 30 mg xylene cyanol, filled up with water,total volume 50ml	
RIPA	Total volume of 500 ml: 5 g nonidet P40, 5 g NaDOC, 0,5 g SDS, 870 mg NaH_2PO_4, 4,38 g NaCl, 1050 g NaF; pH- of 7,5 using NaOH 7,5, 1 tablet complete protease-inhibitor for 50 ml	1% NP40, 1% NaDOC, 0,1% SDS, 10 mM NaH_2PO_4, 150 mM NaCl, 50 mM NaF
SOC Medium	20 g trypton, 5 g yeast extrakt, 0,5 g NaCl, 10 ml 250 mM KCl filled up with 900ml demineralised water ,pH 7. Autoclaved. Cooled down and 10 ml sterile 1 M $MgCl_2$ solution was added plus 20 ml sterile 1 M Glucose solution.	
TBE-Puffer	108 g Tris Base, 55 g boracid und 40 ml 0,5 M EDTA, pH 8,0 filles up with demineralised	

Solutions	Preparation	End concentration
	water to 1 l . Diluted 1:10.	
TBS/T	For 10x TBS solution: 242.2 g Tris, 175.32 g NaCl, with 37% HCl . pH 7.5 . TBST Addition of 0.2% v/v Tween	1 M Tris, 1,5 M NaCl
Western-Blot-stacking gel	5,6 ml water, 1,7 ml acrylamide 30%, 2.5 ml Tris pH 8,8, 0,1 ml 10% SDS, 0,1 ml 10% APS, 0,01 ml TEMED. for 4 small gels.	
Western-Blot separating gel 10%	4,8 ml water, 2,5 ml acrylamid 40%, 2,5 ml Tris pH 8,8, 0,1 ml 10% SDS, 0,1 ml 10% APS, 0,004 ml TEMED; for 2 small gels	
Western-Blot-seperating gel 12%	4,3 ml Water, 3,0 ml acrylamid 40%, 2,5 ml Tris pH 8,8, 0,1 ml 10% SDS, 0,1 ml 10% APS, 0,004 ml TEMED; for 2 small gels	

2.3.3 Equipment

- Scale: OHAUS Precision Advanced, OHAUS Corp., Florham, NJ, USA
- CHEMI Genius², Bio Imaging System, Syngene, Cambridge, United Kingdom
- Electrophoresis
  - Hölzel E 452, H. Hölzel GmbH, Wörth, Germany
  - Sub-Cell GT, Biorad Laboratories, Hercules, CA, USA
  - Maxi Cell Primo und Mini Cell Primo Electrophoresis Gel System, E-C Apparatus Corporation, Holbrook, N.Y., USA
  - Power Pac Basic Power Supply, Bio-Rad Laboratories GmbH, Münich, Germany
- Precision scale: Sartorius Genius, Sartorius Machatronik AG, Göttingen, Germany
- Confocal microscope: Zeiss Axiovert 200 M, Carl Zeiss Microimaging GmbH, Göttingen, Germany
- Thermomixer comfort, Eppendorf, Hamburg, Germany
- Incubators:
  - Heraeus B5050E, Kendro Laboratory Products, Langenselbold, Germany
  - Heraeus HeraCell 240, Kendro Laboratory Products, Langenselbold, Germany

  - Julabo WS 60, Julabo Labortechnik GmbH, Seelbach, Germany
- Cuvette: Precision cuvette made from quarz glass, Hellma GmbH, Müllheim, Germany
- Microliter injection: Hamilton Bonaduz AG, Bonaduz, Schweiz
- Multiply-PCR-Ke, SARSTEDT AG & Co., Nümbrecht, Germany
- PCR-Cycler:
  - ABI Prism 7900 H Sequence Detection System, Applied Biosystems, Foster City, CA, USA
  - Applied Biosystems GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA
  - Hybaid PCR-Sprint, Thermo Fisher Scientific Inc., Waltham, MA, USA
- Photometer: Smart Spec 3000, Bio-Rad Laboratories GmbH, Munich, Germany
- Pipette: Eppendorf, Hamburg, Germany
- Biosphere Filter Tips, 10 µl, 100 µl und 1000 µl, SARSTEDT AG & Co., Nümbrecht, Germany
- Brand Accu-Jet, Brand GmbH, Wertheim, Germany
- 1,5 ml- reaction tubes: Sarstedt Safe-Seal, SARSTEDT AG & Co., Nümbrecht, Germany
- Milli-Q Plus, Ultra Pure Water System, Millipore GmbH, Eschborn, Germany
- Rollincubator: Fröbel Labortechnik CAT RM 5, Lindau, Germany
- Sterile workbench: class 2, Hera Safe, Typ HS 12, Kendro Laboratory Products, Langenselbold, Germany
- 1,5 ml-Vials: Greiner Bio-One GmbH, Frickenhausen, Germany
- Scientific C25 Incubator Shaker, New Brunswick, Edison New York, USA
- Poly-Q-Vial, Perkin-Elmer Life and Analytical Sciences Boston, MA, USA
- Beckman Centrifuge Tubes Polyallomer, Beckman Coulter Inc. Palo Alto, CA, USA
- Vortex-Mixer: Boskamp Mixomat, Boskamp GmbH, Hersel, Germany
- Western-Blot-Accesories: Bio-Rad, Hercules, CA, USA
- Western-Blot-Membrane: Hybond-ECL, Amersham ,Biosciences, Piscataway, NJ, USA
- Western-Blot-Proteinstandard: Bio-Rad Precision Plus Protein Western C, Bio-Rad, Hercules, CA, USA

- Western-Blot-power source: EC 250-90, E-C Apparatus Corporation, Holbrook, NY, USA
- Cell culture-Medium: Dulbecco's MEM 1x, Biochrom AG, Berlin, Germany
- Cell culture dish 10 cm, 15 cm, Cellculturebottles 75 cm^2 : SARSTEDT AG & Co., Nümbrecht, Germany
- Cell culture dish 6-well, 12-well, 96-well: Greiner Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany
- Cell Scraper, SARSTEDT AG & Co., Nümbrecht, Germany
- Cell counter: CASY, innovatis AG, Reutlingen, Germany
- Centifuge:
  - Beckman Coulter J-6B, Beckman Coulter Inc., Fullerton, CA, USA
  - Beckman Coulter L-90 K Ultra centrifuge, Beckman Coulter Inc., Fullerton, CA, USA
  - Eppendorf Centrifuge 5415 D, Eppendorf, Hamburg, Germany
  - Eppendorf Centrifuge 5415 R, Eppendorf, Hamburg, Germany
  - Eppendorf Centrifuge 5810 R, Eppendorf, Hamburg, Germany
  - Hettich Universal 30 RF, Hettich Centrifuge, Tuttlingen, Germany

3 Results

3.1 Cloning of P2RY12 with and without an N-terminal HA-tag

At present two splice variants of the human P2RY12 are known which encode the same protein. The two known splice variants contain 2 and 3 exons, respectively. The differences between the splice variants affect the 5' UTR and thus leave the amino acid chain of the protein unaltered. The coding part of the mRNA comprises 1043 bases.

3.1.1 Strategy

An inspection of the coding P2RY12 cDNA-sequence revealed the presence of a single BamH1-site located in the 3' part of the CDS suitable for cloning. Subsequently, the cDNA was inspected for the possibility to introduce an additional restriction site in the middle 5' part of the cDNA by silent mutagenesis. A suitable site was found for the blunt-end cutting enzyme EcoRV at position 367 of the CDS.

First, primers were designed to amplify the coding sequence in its native form (Table 5). The strategy chosen was to amplify the sequence in three overlapping parts to get hold of sufficient clones without mutations introduced by the PCR. During this first round of PCRs, only the 3' sequence of the fragment 3 encoding the C-terminal amino acids was modified to introduce a KpnI-site 59 bp downstream of the stop codon.

Subsequently, two of the fragments (fragments 1 and 2) were subjected to a mutagenesis by PCR to introduce the EcoRV-site by silent mutagenesis. Moreover, the 5' end of fragment 1 was modified in two different ways: either to abrogate the native ATG and introduce a Nhe1-restriction site for the addition of an HA-tag or by adding a Nhe1-restriction site for directional cloning leaving the native ATG intact.

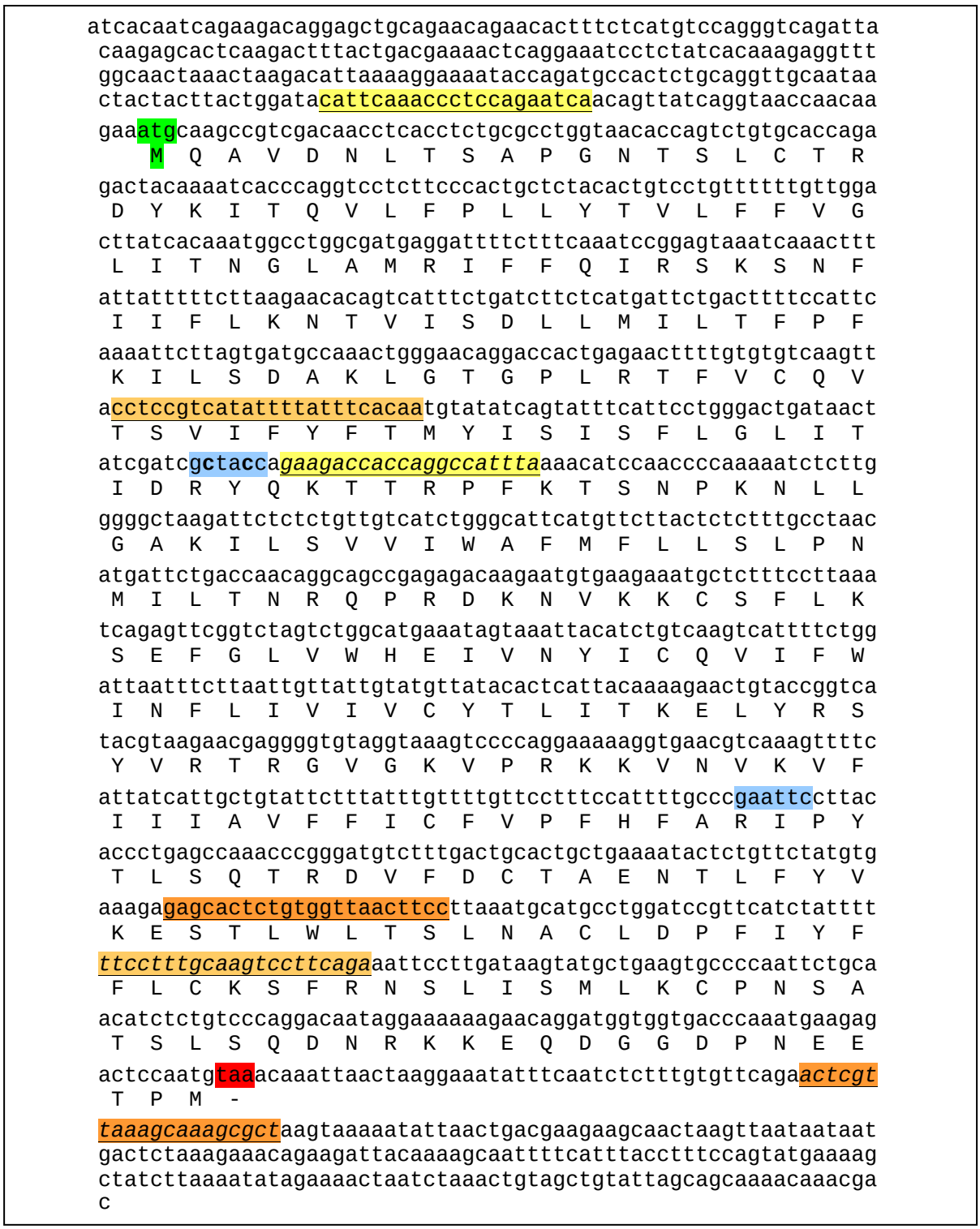


Figure 11: cDNA-sequence of the P2RY12 cDNA (reference NM_022788.3) and cloning strategy. The start and stop codons are indicated by green and red shading, respectively and the amino acid sequence is indicated. The primer binding sites for the forward and reverse primers are indicated by underlining. The reverse primer binding sites are in italics. The three different PCR-primer sets are indicated by yellow to orange shading. The native BamH1-restriction site and the site to yield an EcoRV-site after silent mutagenesis are shown in blue. Bases to be subjected to silent mutagenesis are bold and underlined. The F1 forward primer binds to both P2RY12 splice variants known.

3.1.2 Amplification of the P2RY12 native fragments 1, 2 and 3

Amplification was performed as a touchdown PCR with thrombocyte cDNA. The RNA isolation and reverse transcription were described on page 32.

The expected amplicon sizes of the three first PCRs and primers are depicted in Table 5 .

Fragment	Calculated Amplicon Size (bp)	Primers (5' → 3')
F1	437	Y12_nat_F1_Fw CATTCAAACCCTCCAGAATCA Y12_nat_F1_Rv TAAATGGCCTGGTGGTCTTC
F2	620	Y12_nat_F2_Fw CCTCCGTCATATTTTATTTCACAA Y12_nat_F2_Rv TCTGAAGGACTTGCAAAGGAA
F3	249	Y12_nat_F3_Fw GAGCACTCTGTGGTTAACTTCC Y12_KpnI_F3_Rv AAGGTACCGCTTTGCTTTAACGAGT

Table 5: Primers used in the first round of PCRs

The expected fragments were successfully amplified from human thrombocyte cDNA as depicted in Figure 12. The sizes of the amplicons were in good accordance with the expected sizes. The PCR-products were subjected to a preparative gel electrophoresis and gel extraction procedure to remove any remaining primers which could interfere with the subsequent mutagenesis PCRs.

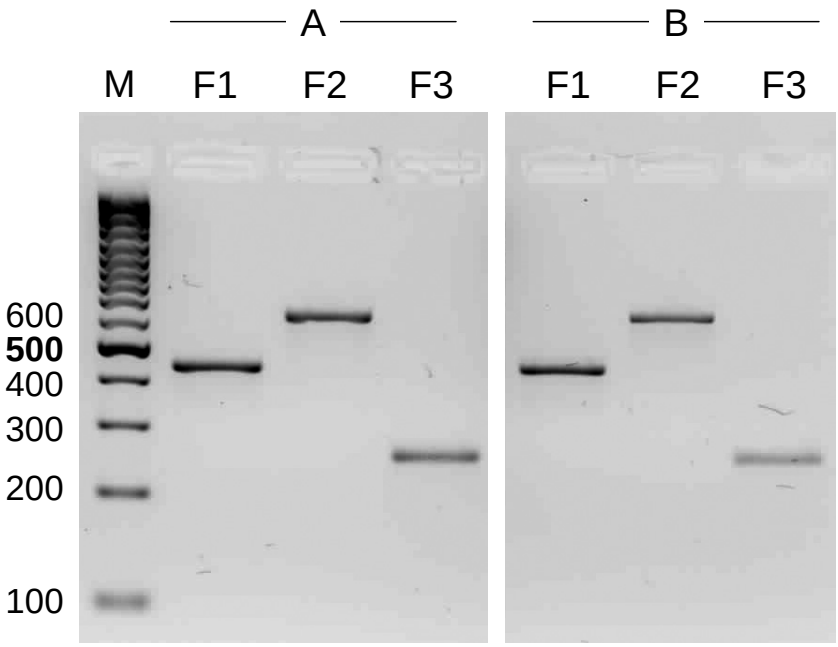


Figure 12: PCR-products of the first sets of PCRs generating three overlapping fragments of the P2RY12 cDNA.
Figure 1A shows an aliquot of the unpurified PCR-products. Figure 1 B shows an aliquot of the very same reaction products after gel extraction of the products from a preparative agarose gel.

3.1.3 Mutagenesis PCR and Cloning

The fragments F1 and F2 were subsequently modified by mutagenesis PCR with the primers given in Table 6.

Fragment	Calculated Amplicon Size (bp)	Primer (5' → 3')
1M1	388	Y12_Muta1 AAAGCTAGCGAAATGCAAGCCGTCGACAACCTC Y12_Muta3 TCTTCTGATATCGATCGATAGTTATCAGTCC
1M2	382	Y12_Muta2 AAAGCTAGCGCCGTCGACAACCTCACC Y12_Muta3 TCTTCTGATATCGATCGATAGTTATCAGTCC
2M	559	Y12_Muta4 CGATCGATATCAGAAGACCACCAGGCCA Y12_nat_F2_Rv TCTGAAGGACTTGCAAAGGAA

Table 6: Primers used in mutagenesis PCRs of the fragments F1 and F2.

Two variants of the F1-amplicon were generated by mutagenesis PCR. In both variants, an EcoRV-site (gatatc) was introduced into the 3' part of the F1-amplicon by

the reverse primer Y12_Muta3. The F1M1 and F1M2 variant differed however in their 5'-region. F1M1 was modified by introducing a Nhe1-restriction site upstream of the endogenous start-ATG for directional cloning. This was done using Y12Muta1 as forward primer. Into the 5'end of 1M2 a Nhe1 restriction site was introduced to enable the in frame addition of a HA-tag and delete the native ATG. This was done with the forward primer Y12_Muta2.

The primer Y12_Muta4 was used to introduce an EcoRV-site into the 5´part of F2 by silent mutagenesis, the 3`part of the F2 was left unchanged. The resulting PCR products were significantly shorter than those amplicons generated in the first round of PCRs. The PCR-products were subjected to an agarose gel electrophoresis to determine their size and clean them by gel extraction. The result of the electrophoresis is shown in Figure 13.

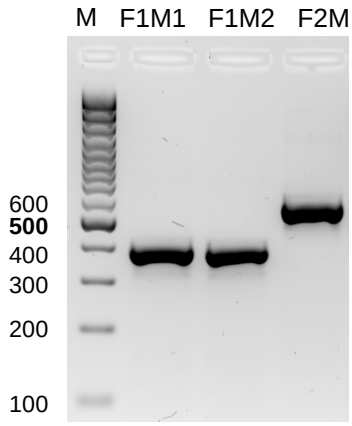


Figure 13: PCR products after mutagenesis-PCR with primers given in table 6.
The observed fragment sizes were in good accordance with those expected form the calculation. On left side is the used 100 bp standard marker.

After purification the products were cloned into the TA-Cloning vector pCRII.

Positive bacterial colonies were identified by blue/white-screening and the plasmids isolated after an overnight mini culture. The isolated plasmids were digested with EcoRI to screen for plasmids with an appropriate size of the insert.

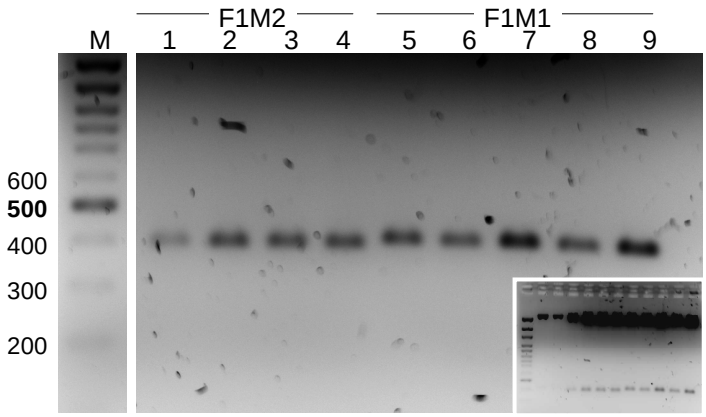


Figure 14: pCRII vectors with F1M2 and F1M1 inserts digested with EcoRI.
Inserts in the pcRII vector can be cut out after cloning due to the presence of two EcoRI-sites flanking the cloning site. The observed fragment sizes were in good accordance with those expected form the calculation. Due to the presence of some additional nucleotides stemming from the plasmid, the fragments were slightly larger than the original PCR-products themselves. The inset at the lower right corner shows the entire gel with the vector backbone. The gel was exposed in the lower part of the gel due to a long run time with subsequent loss of ethidium bromide fluorescence (M=100 bp marker).

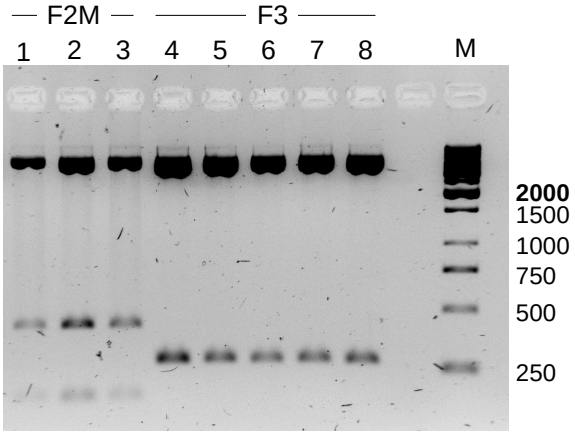


Figure 15: pCRII vectors with F2M and F3 inserts were analysed by digestion with EcoRI.
F2M clones are split into three fragments due to the presence of an internal EcoRI-site. The observed fragment sizes were in good accordance with those expected from the calculation (M=1 kbp marker).

Subsequently, plasmids containing inserts of the correct size and restriction pattern were subjected to sequencing with the primers M13-F and M13-R. Both primers bind within the vector backbone. The results of the sequencing are shown below showing also the restricting sites introduced through mutagenesis, start- and stop-codons and native restriction sites.

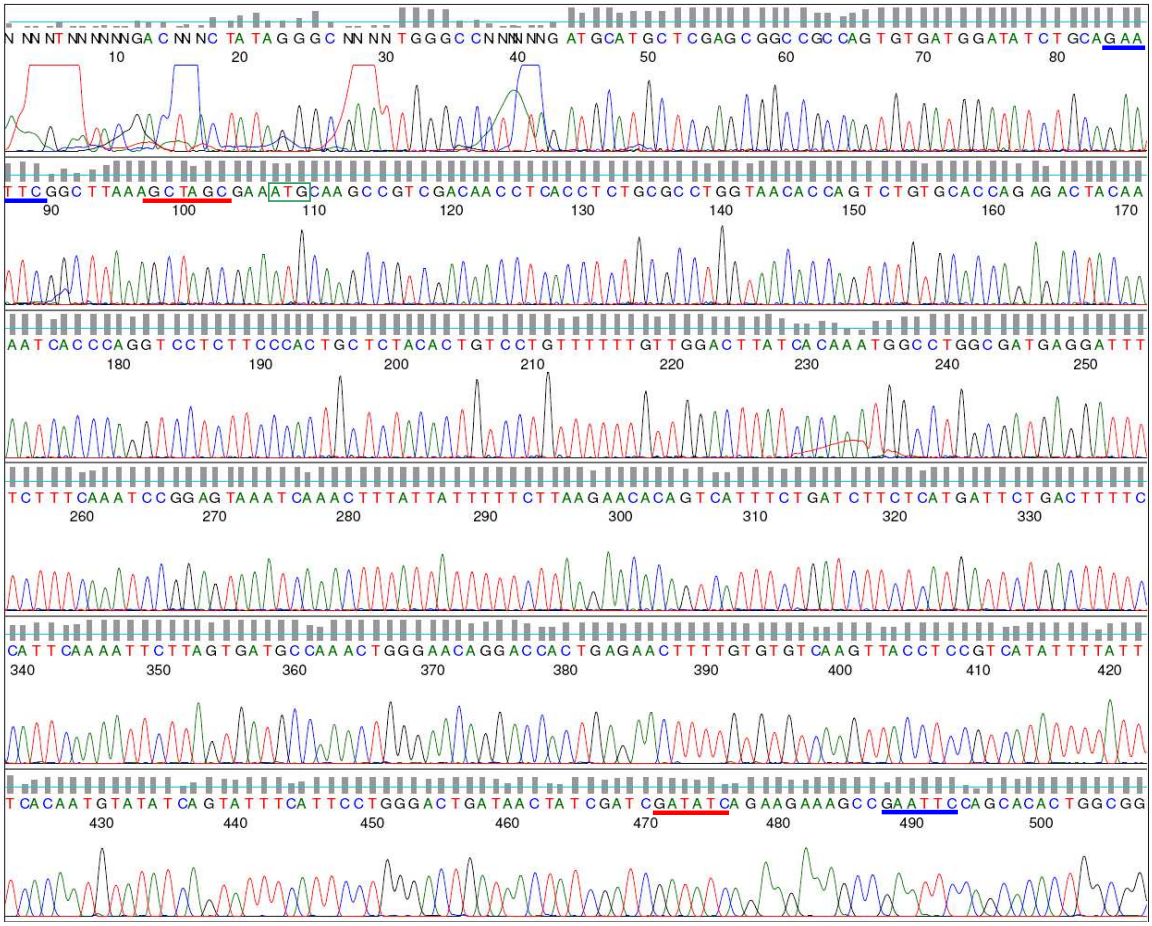


Figure 16: Sequence of fragment F1M1 (clone A) sequenced with Primer M13F.
The start codon is boxed in green. The Nhe1-site (gctagc) and the EcoRV-site (gatatc) induced by mutagenesis are underlined in red. The EcoRI-sites underlined in blue are at the left and right borders of the cloned sequence. The nucleic acid sequence matched completely with the corresponding part of the reference sequence apart from those sites, where the mutations were introduced.

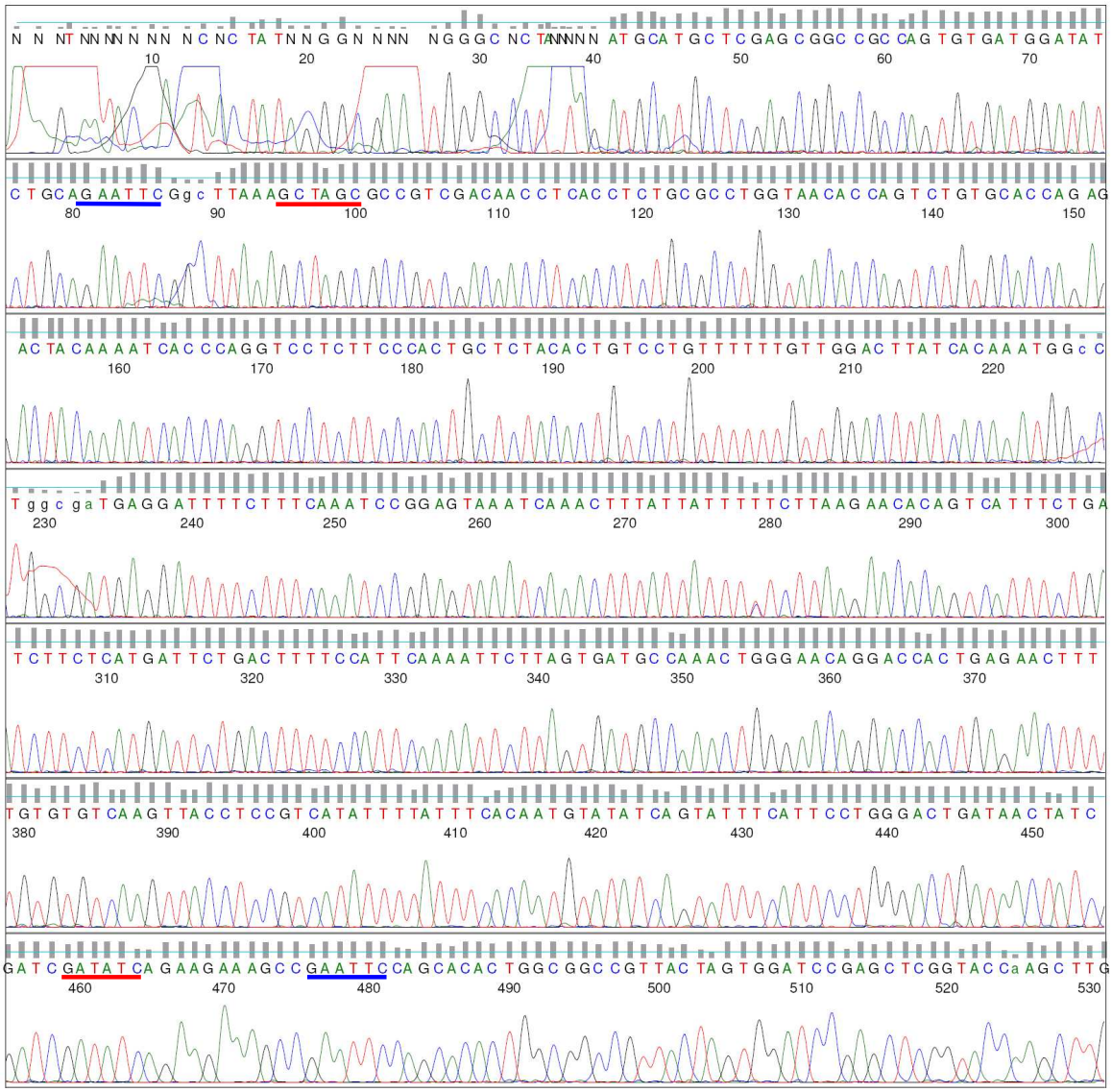


Figure 17: Sequencing of F1M2 (clone nn) in pCRII carried out with the M13F-primer.
Underlined in blue is the EcoRI-site upstream of the cloning site. Subsequently follows the NheI-site (gctagc, red underlining) that abrogates the native ATG and introduces a site for the addition of an HA-tag. Adjacent to the downstream cloning site the EcoRV-site (gatatca) introduced through silent mutagenesis is indicated by red underlining. The downstream EcoRI-site flanking the cloning site is given by blue underlining.

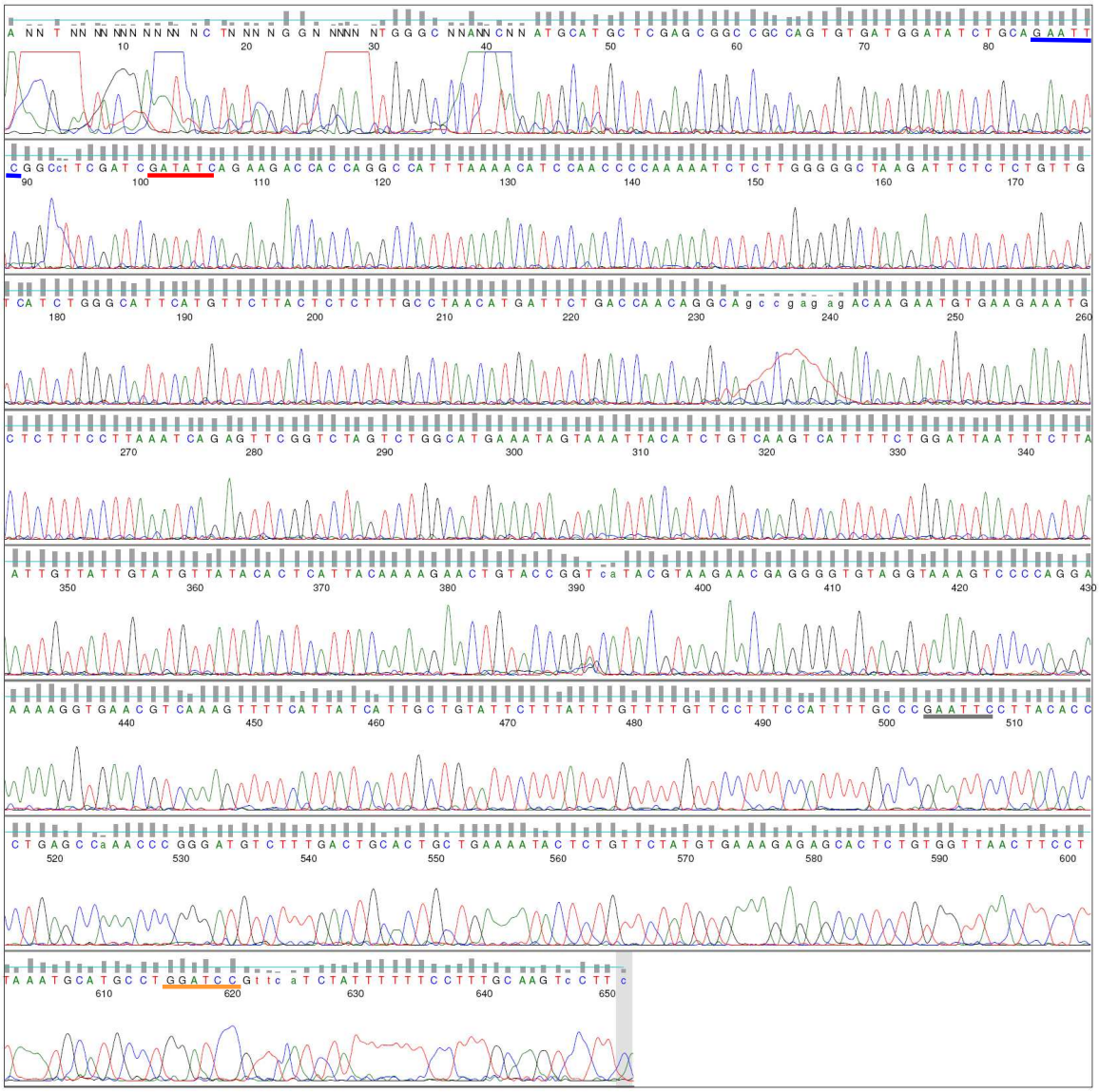


Figure 18: Sequencing of fragment F2M carried out with M13F-primer.
Underlined in blue is the EcoRI site upstream of the border of the insert. The EcoRV-site introduced through silent mutagenesis is indicated in red, underlined in grey the internal EcoRI-site and followed by the native BamHI-site (ggatcc; orange).

Since the sequencing quality of the 3' part of the cloned F2M fragment was limited, the insert was additionally sequenced with the primer M13R to ensure correctness of the nucleotide sequence.

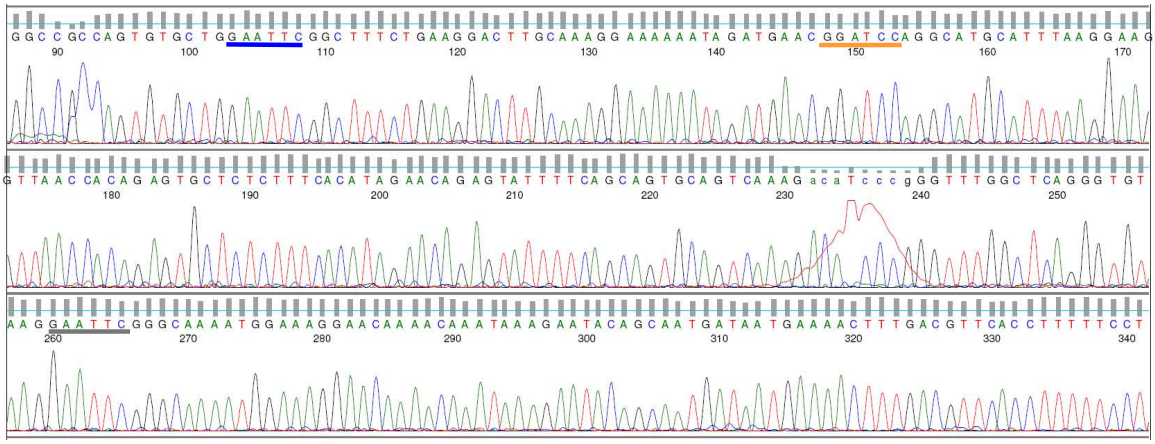


Figure 19: Sequencing of F2M carried out with M13R primer.
The EcoR1 site downstream of the border of the inserted fragment underlined in blue (ggaatt). The native restriction sites for BamH1 (ggatccc; underlined in orange) and EcoR1 (ggaatt; grey underlining) are indicated.

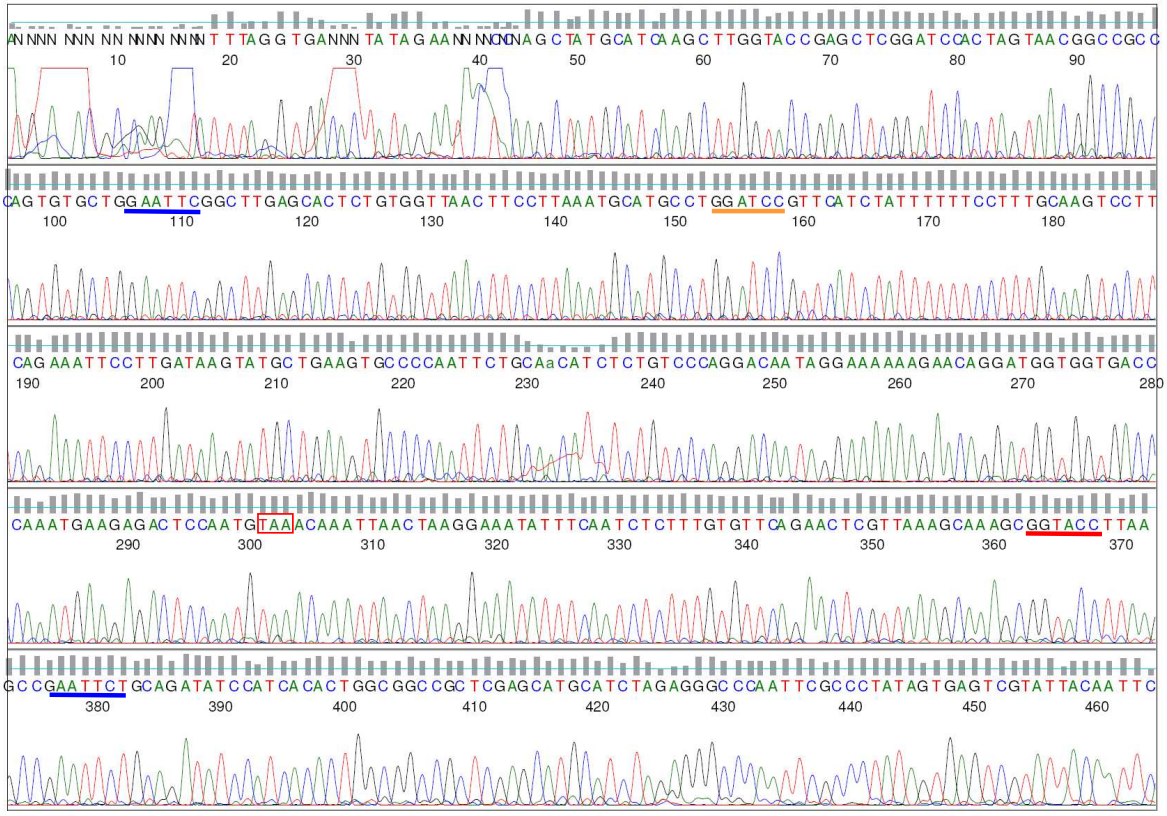


Figure 20: Sequencing of the fragment F3 carried out with the M13R-primer.
The EcoRI sites flanking the insert are underlined blue. Again, the BamH1 site is underlined in orange. The stop-codon is boxed in red and the artificially introduced Kpn1-site located ~60 bp downstream of the stop-codon is underlined in red (ggtacc).

3.1.4 Assembly of fragments within target vector pcDNA/Hygro(-) vector

After the fragments were successfully cloned into the PCRII-vector it was important to assemble them into the expression vector, pcDNA3.1/Hygro(-). This was possible by using the introduced restriction sites to cut out the fragments from the modified pCRII vector. Before assembling of the fragments in pcDNA3.1/hygro(-) we increased our plasmid amounts by carrying out each 1 maxi preparation, the final photometric concentrations were as follows. F3 607,7 µg/ml, F2M 332,3 µg/ml, F1M1 1116.7 µg/ml and F1M2 1167,5 µg/ml.

The assembling was carried out in three steps, always introducing one fragment at a time. We started with fragment F3B subsequently F2M was then inserted into the target vector from step one (F3B-pc DNA 3.1/Hygro(-)). Finally, F1M1 and F1M2 were inserted into the target vector from step 2 (F2M-F3-pc DNA 3.1/Hygro(-)).

The different steps were performed according to the following principle and always occurred in the same manner.

Firstly the vector was opened with the correct restriction enzymes sometimes in double digestion and sometimes sequentially depending on the protocol of the manufacturer. Then the insert was cut out of the PCRII vector in a manner that the ends of vector and insert were compatible.

Thus, the pcDNA vector and the sequenced F3 in its vector were sequentially digested with Kpn1 and BamH1. Simultaneous double digestion was impaired by the star activity of BamH1. The Kpn1 was used in an overnight digestion and the nucleic acids subsequently subjected to an ethanol precipitation. The nucleic acids were then re-dissolved, cut with BamH1 and again precipitated to concentrate the nucleic acids.

The digested nucleic acids were separated in a preparative agarosis gel. The bands that represented the pcDNA vector and the insert F3 were excised and extracted from the gel.

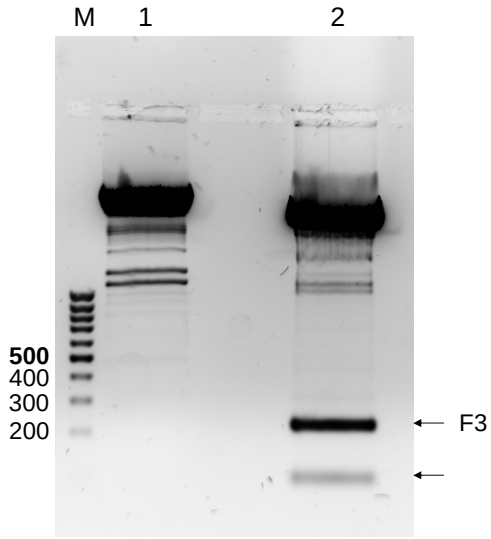


Figure 21: Digestion with Kpn1 and BamH1 of pcDNA3.1/Hygro(-)to open the vector (lane1) and digestion of pCRII plasmid containing F3 in order to extract F3 from the plasmid for the subsequent ligation with the opened pcDNA/Hygro(-) vector.
M1=100bp ladder. Lane 1: KpnI/BamHI digested pcDNA3.1-vector, Lane 2 PCRII/F3 vector cut with Kpn1 and BamH1. The band with a size of approximately 260bp is F3. The lowest band in lane 2 corresponds to a vector fragment due to the presence of an additional KpnI-site within the vector. The star activity of BamH1 could only be partially reduced by precipitation after the first digestion-step.

Afterwards we ligated the insert with the newly opened vector and transformed as described above. After successful transformation, mini preparations were made and a double digestion was carried out with Kpn1 and BamH1.

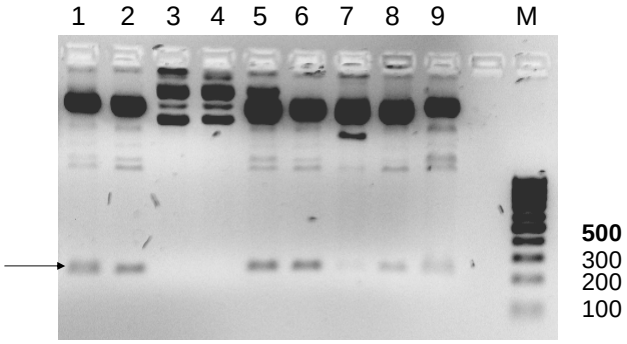


Figure 22: Sequential digestion with Kpn 1 and BamH1 of pcDNA hygro(-) clones to verify recloning of F3 fragment.The clone in lane 1 was used in the further experiments. Slot M shows the 100bp marker.

The vector obtained in step 1 containing F3 was subjected to a double digestion with BamH1 and EcoRV. In parallel the PCRII vector containing F2M was digested with BamH1 and EcoRV to yield F2 in a form which is compatible for directional ligation with the prepared F3-vector. After precipitation we separated using gel electrophoresis and further more we extracted the opened vector and the insert from

the gel using our usual kit. Then carried on with ligation and transformation and after making mini prepations we re-digested small quantities to confirm a successful digestion.

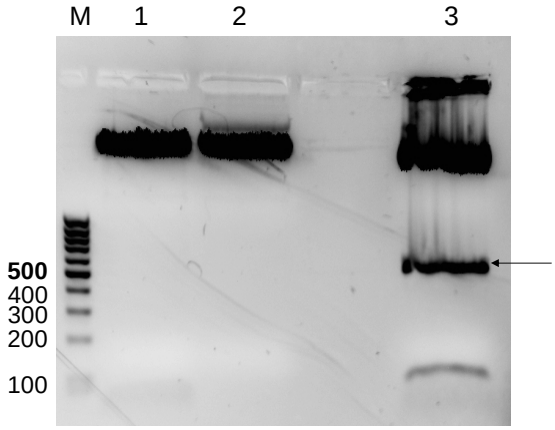


Figure 23: EcoRV and BamH1 digestion to open the F3-pcDNA3.1 vector (lanes 1, 2) and excise the insert form F2M-pCRII (lane 3) for the subsequent ligation.
The arrow indicates the excised part of F2M (~ 520bp). The lowest band in lane 3 is a vector fragment. In lane M is the 100bp ladder.

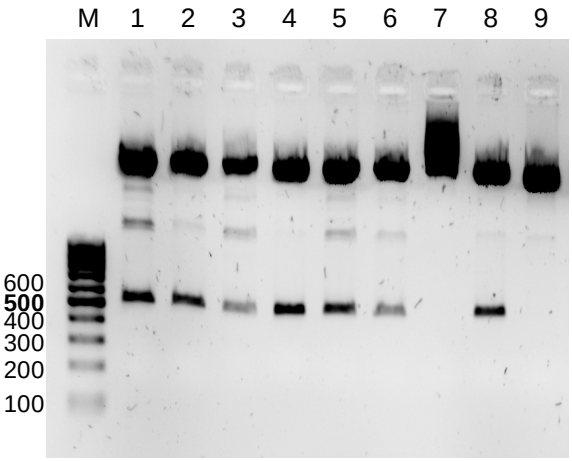


Figure 24: Double digestion with BamH1 and EcoRV of F2M-F3-pcDNA clones to assess whether the cloning of the insert F2M into the F3-pcDNA was successful.
Apart from Clone 7 and 9 all clones contained an F2-fragment of correct size of (~520 bp). Lane M is the 100bp ladder.

Additionally, the clones in lanes 2 and 5 were digested with EcoRV and KpnI to yield the joined insert fragments.

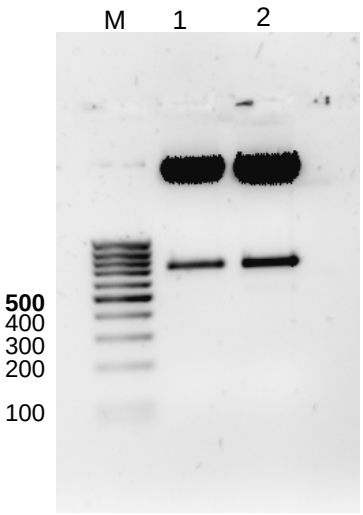


Figure 25: EcoRV and KpN1digestion of F2M-F3-pcDNA plasmids (clones 2 and 5).
Both clones demonstrated the expected size of the joined fragments F2M-F3 (~725 bp). Lane M: 100bp ladder.

Step 3 Re-cloning Fragments F1M1 and F1M2 into F2M-F3pcDNA3.1

The vector from assembly step 2 and the plasmids F1M1-pCRII and F1M2-pCRII respectively were digested with the restriction enzymes Nhe1 and EcoRV. In this manner, the accepting vector F2M-F3pcDNA3.1 was prepared for the ligation and the fragments F1M1 and F1M2 with compatible ends were generated.

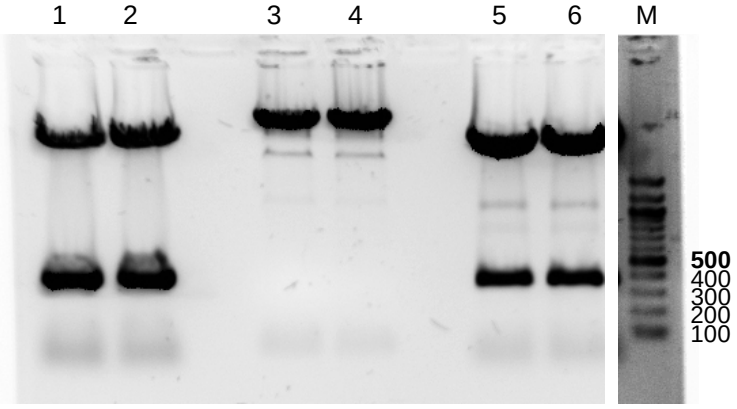


Figure *26*: Double digestion with Nhe1 and EcoRV to cut out the inserts F1M1 (lanes 1 and 2) and insert F1M2 (lanes 5 and 6) and to linearize F2M-F3-cDNA (lanes 3 and 4). Lane M: 100bp ladder.

After transformation and preparation of minis, a confirmation digestion was carried out using EcoRV and Nhe1.

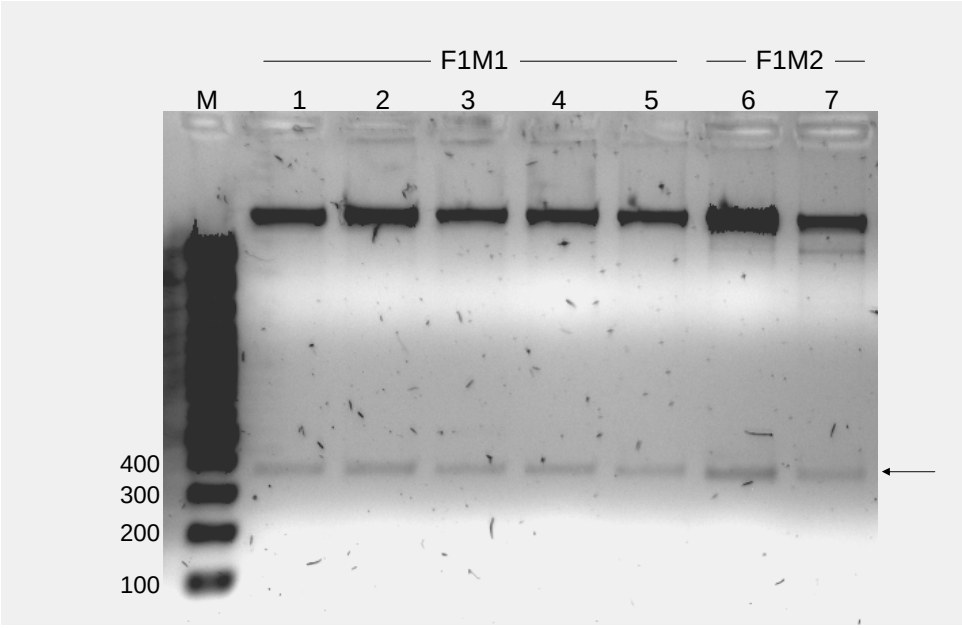


Figure 27: Double digestion with NHe1 and EcoRV of the cloned pcDNA vector with the final fragment F1M1 and with F1M2 in order to confirm the cloned inserts.
Expected were 2 bands, the size of the inserts was <400bp. The arrow at the right side indicates the expected bands. M is the standard 100bp ladder. 1 to 5 are positive cloned pcDNA hygro(-) with F1M1. Slot 6 and 7 are positive cloned pcDNA hygro(-) vector with F1M2.

So at this stage the Vector containing the F1M1 fragment was ready to use while we furthermore continued with the vector containing the F1M2.

Step 3b

Firstly the pcDNA 3.1 hygro(-) vector was cut using Nhe1 and EcoRV in a double digestion step. This was incubated at 37℃ for 3 hours and then placed on a 0.6% agarosis gel and extracted as usual.

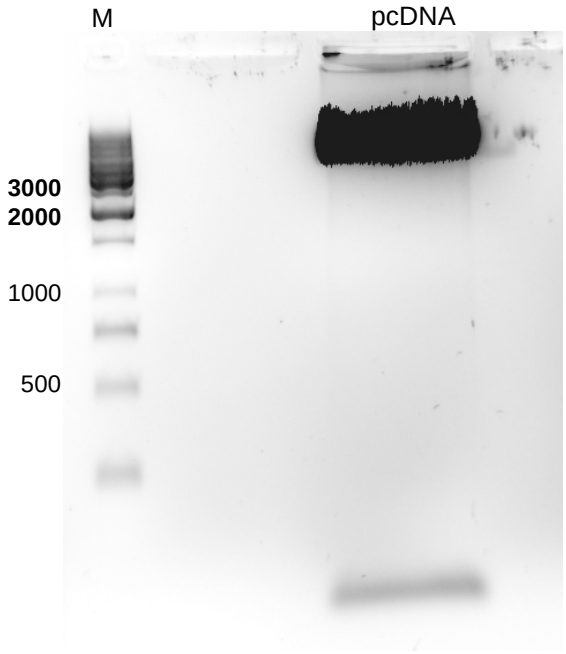


Figure 28: Gel extraction digestion of pcDNA3.1 hygro vector with Nhe1 and EcoRV for subsequent ligation with HA-tag. Digestion yielded the prepared target vector and a small fragment between the Nhe1 and the EcoRV-sites.

```
                    M  G  Y  P  Y  D  V  P  D  Y  A  S  D
HA-tag F 5′-CTAGGAATGGGGTACCCCTACGACGTGCCCGACTACGCTAGCGAT-3′
HA-tag R  3′-   CTTACCCCATGGGGATGCTGCACGGGCTGATGCGATCGCTA-5′
```

Figure 29: The annealed oligo-HA-tag with the opened pcDNA vector.

The transformation was again carried out using the usual protocol. Afterwards the vector with the insert HA-tag was sequenced using segHygUp and SeqHygDown primers. A clone of the tagged vector (A2HA-tag-V) was without faults, so this was used for further cloning.

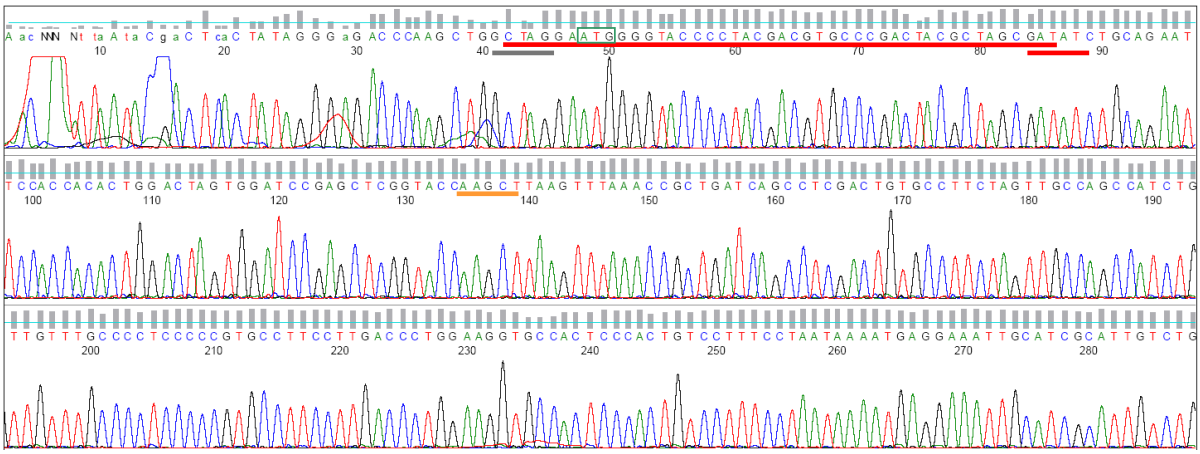


Figure 30: Sequence of pcDNA vector with the accepted insert HA-tag, clone A2HA-tag-V.

Finally the entire insert with the joined fragments F1M2-F2M-F3 was cut out of the pcDNA vector by double digestion with Nhe1 and HindIII. The HA-tagged Vector was also cut with Nhe1 and HindIII. Insert and vector were cleaned by preparative gel electrophoresis.

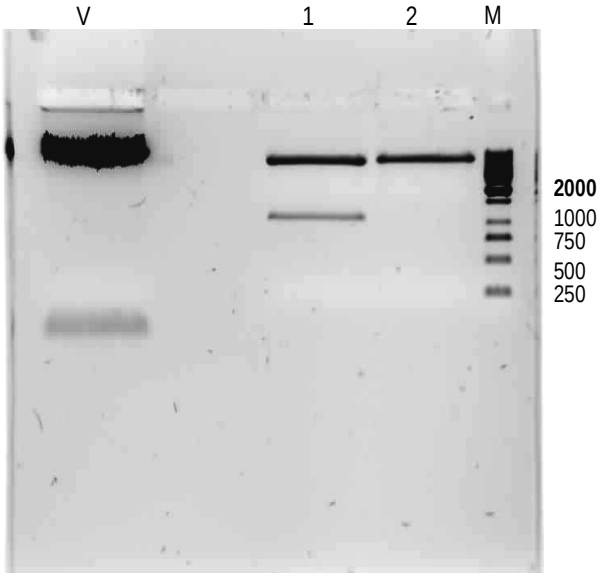


Figure 30: Extraction digestion with Nhe1 and HindIII.
Indicated as V is the opened pcDNA vector.1 shows the PCRII vector (upper band) and the joined fragments F1M2+F2M+F3 have a size of 1231 bp. 2 shows an undigested plasmid vector. M is the 1kb standard marker.

Then a final digestion was carried out to be double of the correct cloning of the Vector containing all fragments without the HA-tag and also the vector containing the HA-tag. The table shows the various digestions carried out to confirm the clones.

Resulting Bands	Restriction Enzymes	Expected sizes in bp
Fragment 3	BamH1/HindIII	249
Fragment 2	EcoRV/BamH1	561
Fragment 1	EcoRV/Nhe1	~ 400
Fragment F1+F2+F3	Nhe1/HindIII	~ 1210

Table 7: Various digestions carried out to confirm the clones

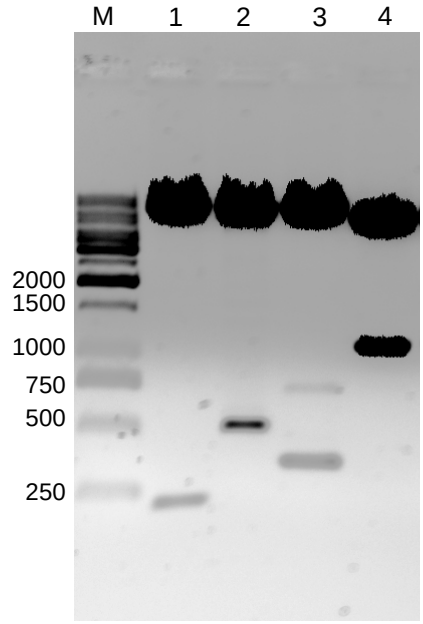


Figure 31: Digestion of HA-tagged clone with all fragments of P2RY12 fragments digested with various enzymes.
The first slot shows the 1kb marker; all upper bands are the vector pcDNA and the lower bands the inserts. Starting from left, M is the 1kb marker, 1 is the expected size of fragment 3, 2 is fragment 2 and then fragment 1 indicated as 3, indicated as 4 we see the expected size of the sum of all 3 fragments.

Sequencing of HA-tagged clone.

The final clone of the HA-tag-P2RY12 was sequenced. With the primers SeqHypUp and SeqHygdown an overlapping sequence of good quality was obtained. To resolve some uncertainties in the sequencing reactions, sequencing was performed with two internal primers, namely Y12_nat_F1_Rv and Y12_nat_F2_Fw. The alignment of the overlapping sequencing reactions is given in figure nn.

```
          1                                                    50
RefCDS    .......... .......... ........   ATGCAAGCCG TCGACAACCT
F1HWdh    ATGGGGTACC CCTACGACGT GCCCGACTAC GCTAGCGCCG TCGACAACCT
 2F1RC    ATGGGGTACC CCTACGACGT GCCCGACTAC GCTAGCGCCG TCGACAACCT
          51                                                  100
RefCDS    CACCTCTGCG CCTGGTAACA CCAGTCTGTG CACCAGAGAC TACAAAATCA
F1HWdh    CACCTCTGCG CCTGGTAACA CCAGTCTGTG CACCAGAGAC TACAAAATCA
 2F1RC    CACCTCTGCG CCTGGTAACA CCAGTCTGTG CACCAGAGAC TACAAAATCA
          101                                                 150
RefCDS    CCCAGGTCCT CTTCCCACTG CTCTACACTG TCCTGTTTTT TGTTGGACTT
F1HWdh    CCCAGGTCCT CTTCCCACTG CTCTACACTG TCCTGTTTTT TGTTGGACTT
 2F1RC    CCCAGGTCCT CTTCCCACTG CTCTACACTG TCCTGTTTTT TGTTGGACTT
          151                                                 200
RefCDS    ATCACAAATG GCCTGGCGAT GAGGATTTTC TTTCAAATCC GGAGTAAATC
F1HWdh    ATCACAAATG GCCTGGCGAT GAGGATTTTC TTTCAAATCC GGAGNNNATC
 2F1RC    ATCACAAATG GCCTGGCGAT GAGGATTTTC TTTCAAATCC GGAGTAAATC
          201                                                 250
RefCDS    AAACTTTATT ATTTTTCTTA AGAACACAGT CATTTCTGAT CTTCTCATGA
F1HWdh    AAACTTTATT ATTTTTCTTA AGAACACAGT CATTTCTGAT CTTCTCATGA
 2F1RC    AAACTTTATT ATTTTTCTTA AGAACACAGT CATTTCTGAT CTTCTCATGA
          251                                                 300
RefCDS    TTCTGACTTT TCCATTCAAA ATTCTTAGTG ATGCCAAACT GGGAACAGGA
F1HWdh    TTCTGACTTT TCCATTCAAA ATTCTTAGTG ATGCCAAACT GGGAACAGGA
 2F1RC    TTCTGACTTT TCCATTCAAA ATTCTTAGTG ATGCCAAACT GGGAACAGGA
          301                                                 350
RefCDS    CCACTGAGAA CTTTTGTGTG TCAAGTTACC TCCGTCATAT TTTATTTCAC
F1HWdh    CCACTGAGAA CTTTTGTGTG TCAAGTTACC TCCGTCATAT TTTATTTCAC
 2F1RC    CCACTGAGAA CTTTTGTGTG TCAAGTTACC TCCGTCATAT TTTATTTCAC
          351                                                 400
RefCDS    AATGTATATC AGTATTTCAT TCCTGGGACT GATAACTATC GATCGCTACC
F1HWdh    AATGTATATC AGTATTTCAT TCCTGGGACT GATAACTATC GATCGATATC
   3F1    .......... .GTATTTCAT TCCTGGGACT GATAACTATC GATCGATATC
 2F1RC    AATGTATATC AGTATTTCAT TCCTGGG... .......... ..........
          401                                                 450
RefCDS    AGAAGACCAC CAGGCCATTT AAAACATCCA ACCCCAAAAA TCTCTTGGGG
F1HWdh    AGAAGACCAC CAGGCCATTT AAAACATCCA ACCCCAAAAA TCTCTTGGGG
   3F1    AGAAGACCAC CAGGCCATTT AAAACATCCA ACCCCAAAAA TCTCTTGGGG
```

```
                451                                                 500
   RefCDS       GCTAAGATTC TCTCTGTTGT CATCTGGGCA TTCATGTTCT TACTCTCTTT
   F1HWdh       GCTAAGATTC TCTCTGTTGT CATCTGGGCA TTCATGTTCT TACTCTCTTT
      3F1       GCTAAGATTC TCTCTGTTGT CATCTGGGCA TTCATGTTCT TACTCTCTTT
                501                                                 550
   RefCDS       GCCTAACATG ATTCTGACCA ACAGGCAGCC GAGAGACAAG AATGTGAAGA
   F1HWdh       GCCTAACATG ATTCTGACCA ACAGGCAGCC GAGAGACAAG AATGTGAAGA
      3F1       GCCTAACATG ATTCTGACCA ACAGGCAGCC GAGAGACAAG AATGTGAAGA
                551                                                 600
   RefCDS       AATGCTCTTT CCTTAAATCA GAGTTCGGTC TAGTCTGGCA TGAAATAGTA
   F1HWdh       AATGCTCTTT CCTTAAATCA GAGTTCGGTC TAGTCTGG.. ..........
      3F1       AATGCTCTTT CCTTAAATCA GAGTTCGGTC TAGTCTGGCA TGAAATAGTA
                601                                                 650
   RefCDS       AATTACATCT GTCAAGTCAT TTTCTGGATT AATTTCTTAA TTGTTATTGT
      3F1       AATTACATCT GTCAAGTCAT TTTCTGGATT AATTTCTTAA TTGTTATTGT
                651                                                 700
   RefCDS       ATGTTATACA CTCATTACAA AAGAACTGTA CCGGTCATAC GTAAGAACGA
      3F1       ATGTTATACA CTCATTACAA AAGAACTGTA CCGGTCATAC GTAAGAACGA
F1HDWdhRC       .......... .......... .......... .....CATAC GTAAGAACGA
                701                                                 750
   RefCDS       GGGGTGTAGG TAAAGTCCCC AGGAAAAAGG TGAACGTCAA AGTTTTCATT
      3F1       GGGGTGTAGG TAAAGTCCCC AGGAAAAAGG TGAACGTCAA AGTTTTCATT
F1HDWdhRC       GGGGTGTAGG TAAAGTCCCC AGGAAAAAGG TGAACGTCAA AGTTTTCATT
                751                                                 800
   RefCDS       ATCATTGCTG TATTCTTTAT TTGTTTTGTT CCTTTCCATT TTGCCCGAAT
      3F1       ATCATTGCTG TATTCTTTAT TTGTTTTGTT CCTTTCCATT TTGCCCGAAT
F1HDWdhRC       ATCATTGCTG TATTCTTTAT TTGTTTTGTT CCTTTCCATT TTGCCCGAAT
                801                                                 850
   RefCDS       TCCTTACACC CTGAGCCAAA CCCGGGATGT CTTTGACTGC ACTGCTGAAA
      3F1       TCCTTACACC CTGAGCCAAA CCCGGGATGT CTTTGACTGC ACTGCTGAAA
F1HDWdhRC       TCCTTACACC CTGAGCCAAA CCCGGGATGT CTTTGACTGC ACTGCTGAAA
                851                                                 900
   RefCDS       ATACTCTGTT CTATGTGAAA GAGAGCACTC TGTGGTTAAC TTCCTTAAAT
      3F1       ATACTCTGTT CTATGTGAAA GAGAGCACTC TGTGGTTAAC TTCCTT....
F1HDWdhRC       ATACTCTGTT CTATGTGAAA GAGAGCACTC TGTGGTTAAC TTCCTTAAAT
                901                                                 950
   RefCDS       GCATGCCTGG ATCCGTTCAT CTATTTTTTC CTTTGCAAGT CCTTCAGAAA
F1HDWdhRC       GCATGCCTGG ATCCGTTCAT CTATTTTTTC CTTTGCAAGT CCTTCAGAAA
                951                                                1000
   RefCDS       TTCCTTGATA AGTATGCTGA AGTGCCCCAA TTCTGCAACA TCTCTGTCCC
F1HDWdhRC       TTCCTTGATA AGTATGCTGA AGTGCCCCAA TTCTGCAACA TCTCTGTCCC
                1001                                               1050
   RefCDS       AGGACAATAG GAAAAAAGAA CAGGATGGTG GTGACCCAAA TGAAGAGACT
F1HDWdhRC       AGGACAATAG GAAAAAAGAA CAGGATGGTG GTGACCCAAA TGAAGAGACT
                1051
   RefCDS       CCAATGTAA
F1HDWdhRC       CCAATGTAA
```

Figure 32: Alignment of the finally obtained vector HA-tag P2RY12 pcDNA/Hygro(-)

Model of P2RY12 without and with HA-tag.

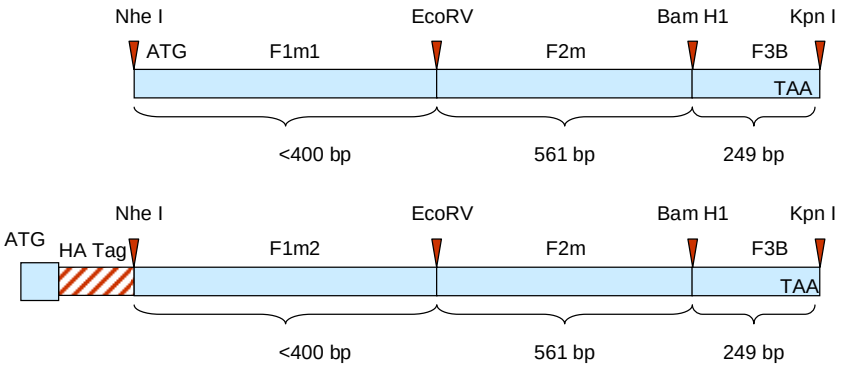


Figure 33: The first upper panel shows the P2RY12 without the HA-tag but with the new EcoRV and Nhe 1 sites introduced by silent mutagenesis.

The lower panel shows the HA-tagged P2RY12 with the start codon abrogated and the restriction sites sown above.

For the subsequent experiments the variant with the HA-tag was used.

3.2 Transcription variants expressed in human platelets and in brain

Currently two transcriptional variants of human P2Y12 mRNA are established. The longer variant (NM_022788.3) comprises three exons whereas the shorter transcript (NM_176876.1) comprises only two exons. These different transcripts are generated by usage of alternative promoters and consequently transcriptional start sites. Three RT-PCRs were performed from cDNA of human platelets and human brain to investigate differential usage of the promoters in these tissues. Two forward primers mapping to unique sequences in the 5'-UTR of either of the transcripts allow for the specific detection of the transcripts: The 5'-primer specific for the longer transcript is located in the first exon, which is not present in the shorter transcript. The transcriptional start of the shorter variant is upstream of the exon-part shared with the longer variant. Thus a primer located 5' to the shared part of the exon allowed the specific detection of the shorter transcript variant.

The products under high-yield PCR-conditions are depicted in Figure 34 A and B.

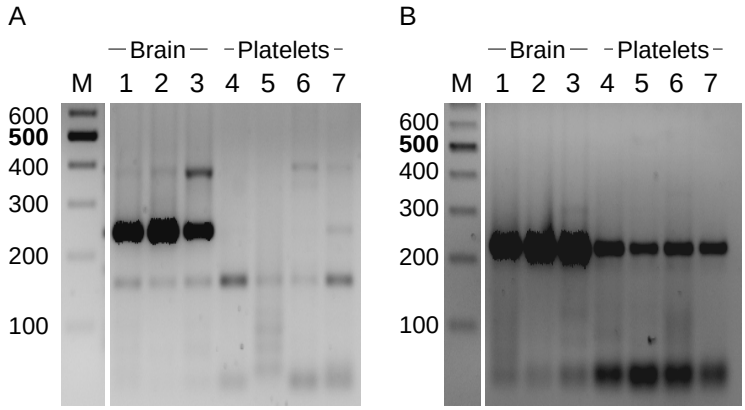


Figure 34: Analysis of the expression of different P2RY12-transcript-variants in platelets and human brain. Primers specific for the longer three-exon variant (A) or specific for the shorter two-exon variant (B) were used in RT-PCRs. Figure A demonstrated the presence of a product of about 240 bp specific for the longer variant only in cDNA prepared from brain (lanes 1 to 3). Lanes 4 to 7 demonstrated the absence of this variant in platelet cDNAs. As depicted in B, the shorter variant was detected in both cDNAs.

As expected, the common primer set amplifying a common part of both RNA-transcripts yielded PCR-products of identical size from platelet and brain cDNA (Figure 35). Notably, the PCR-products from platelets amplified with the common primer set were stronger than the brain-products.

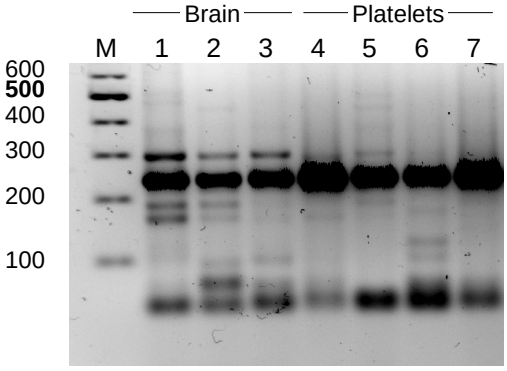


Figure 35: Analysis of P2RY12-expression in brain and platelet mRNA. RT-PCR products were generated from human brain and platelet cDNA with an intron-spanning primer set amplifying both known transcript-variants.

Products of the expected sizes from all three RT-PCR reactions were excised from the gel, cleaned and sequenced. The results were entirely congruent with the reference sequences of the transcript variants (data not shown).

The results demonstrate the expression of the two-exon-variant (NM_176876) in human platelets whereas the longer variant (NM_022788) was not present in human platelets. Both variants were detectable in brain.

3.3 Transient and stable expression of HA-P2Y12 in HEK293 cells

The entirely sequenced HA-P2Y12 cDNA clone in pcDNA3.1/Hygro(-) was transiently transfected in HEK293-cells and the protein expression analysed 48 to 72 h after transfection. Expression was addressed by direct immunofluorescence and Western blotting with an anti-HA monoclonal rat antibody (clone 3F10).

Direct immunofluorescence with anti-HA-antibody coupled to fluoresceine on fixed, permeabilized cells revealed a correct localization of the protein within the outer cellular membrane.

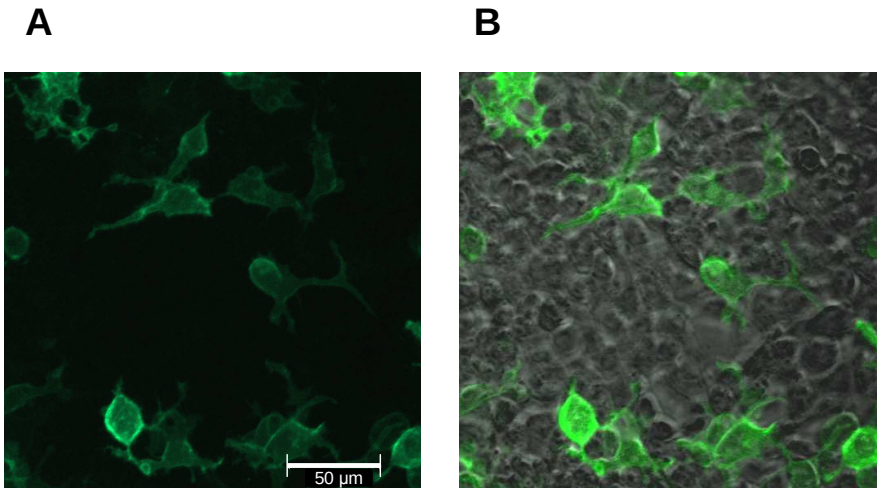


Figure 36: Direct immunofluorescence with anti-HA-antibody (clone3F10) 48 h after transient transfection of HEK293 cells with HA-P2RY12/pcDNA3.1/Hygro(-). Fluorescence (A) and fluorescence-overlay with phase contrast (B). The signal is consistent with a localisation of the expressed protein in the cellular membrane. As is to be expected after transient transfection, expression levels varied between different cells due to the different amounts of plasmid-DNA taken up by the individual cells.

Western blotting after transient transfection in dependence of the amount of DNA added to the transfection mix revealed the occurrence of a broad band with an apparent molecular weight in the range of 50 to 75 kD.

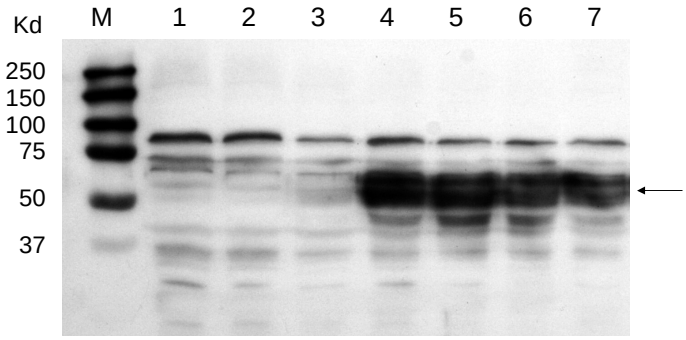


Figure 37: Effect of different DNA-doses on the expression levels of P2Y12 after transient transfection in HEK293 cells. Cells were transfected in 6-well cluster dishes with 0, 0.28, 0.55, 1.1, 2.2, 4.4 and 8.8 µg plasmid-DNA per well (lanes 1 to 7).

The broad migration pattern of the band is suggestive of posttranslational glycosylation of the protein. Since the extent of the glycosylation frequently varies from molecule to molecule proteins with different molecular weights emerge. Moreover, the glycosylation may cause a major difference between the apparent, experimentally determined and the expected molecular weight of a protein. The expected molecular weight of the HA-tagged P2Y12 is 39 kD, whereas the protein size in Western blots was in the range between 50 and 60 kD.

To investigate whether the shift in molecular weight and the broadening of the band is indeed caused by glycosylation, proteins were subjected to digestion with the N-linked-glycopeptide-L-asparagine amidohydrolase (PNGase F). The results are depicted in Figure 38.

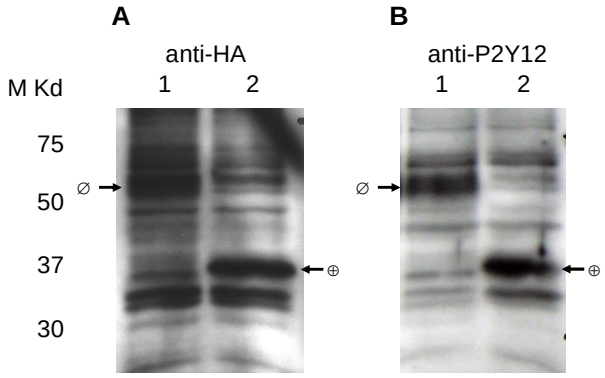


Figure 38: Effect of PNGase F cleavage on the apparent molecular weight and the band pattern of the cell line HA-P2Y12/HEK293-3/I. Western blots were probed with anti-HA-antibody (3F10) detecting the C-terminally located tag (A) or the anti-P2Y12-antiserum (P. Savi) raised in rabbits against an N-terminally located epiotpe (B). Lane 1 untreated membrane proteins; Lane 2 membrane proteins treated with PNGAse F at 37°C for 1 h. Note the shift of the broad band with an apparent molecular weight of above 50 kD (∅) to a band with an apparent molecular weight of ~37 kD (⊕) detected by both antibodies.

It is concluded that the band picked up by both antibodies, namely the monoclonal anti-HA-antibody (C-terminally located tag) and the rabbit antiserum (raised against an N-terminally located epitope of the receptor) is the HA-tagged P2RY12-protein. Since the epitopes detected are located at the outmost C- and N-terminally located regions of the protein, respectively, the protein is likely the desired full length clone. The observed molecular weight after deglycosylation is in good agreement with the expected molecular weight.

3.4 Functionality of the P2Y12-receptor in HA-P2Y12/HEK293-3/I-cells

To address whether the P2Y12-receptor in the stable cell line HA-P2Y12/HEK293-3/I is functionally coupled we took advantage of the fact that P2Y12-stimulation causes activation of the MAP-kinases ERK1/ERK2 (p42/p44) by phosphorylation. To this end, non-transfected HEK293 cells and HA-P2Y12/HEK293-3/I cells were seeded at similar density in 6-well-plates. After one day of growth cells were starved by serum reduction. Subsequently the cells were challenged by the addition of ADP. The results are depicted in Figure 39.

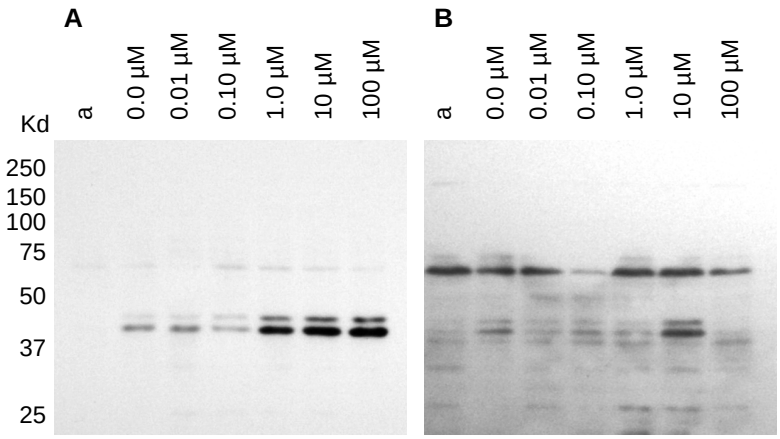


Figure 39: Phosphorylation of MAP-kinases p42/p44 in HA-P2Y12/HEK293-3/I (A) and non-transfected HEK293 cells (B) stimulated for 5 minutes with increasing concentrations of ADP. Lanes a contain proteins harvested directly after withdrawal of the plates from the incubator. The other lanes contain proteins harvested 5 minutes after addition of the indicated concentration of ADP at room temperature. The experiment is representative of three independent experiments.

Increasing concentrations of ADP evoked an increase of the phosphorylation of p42/p44 in the stable cell line HA-P2Y12/HEK293-3/I. The non-transfected mother line (HEK293) does occasionally exhibited a slight increase of p42/p44-phosphorylation under the experimental conditions. However the increase is weaker and unrelated to the ADP-concentration of the experiment.

3.5 Detection of P2Y12 in human platelets

The expression of P2RY12 in platelets and other blood cells was addressed by Western-blotting after fractionation of cellular components by differential centrifugation and detergent extraction.

Erythrocytes and lymphocytes were prepared from EDTA-anticoagulated blood by Ficoll-separation. Platelets were prepared by centrifugation from citrate-anticoagulated platelet-rich plasma.

Lymphocytes and erythrocytes were lysed in icecold water supplemented with protease inhibitors on ice for 20 minutes. Subsequently non-lysed cells and nuclei were removed by low-speed centrifugation (10 min; 500 G; 4 C). This fraction was not further analysed. The supernatant was separated into a particulate and a soluble fraction by ultracentrifugation at 100,000 G for 1 h (4℃). The resulting supernatant contains soluble proteins, largely derived from cellular cytosol. The particulate

fraction contains cellular membranes and big protein complexes e.g. formed by the cellular skeleton. This particulate fraction was extracted with a detergent-containing buffer to solubilise lipophilic proteins by means of their inclusion into micelles. The fraction remaining insoluble was spun down at 20,000 g for 20 min (4℃). The insoluble fraction was analysed separately after solubilisation in SDS-containing loading-buffer.

Since platelets are largely resistant to hypotonic lysis, platelet membranes were broken by freeze-thaw cycles after suspending the platelets in water supplemented with protease-inhibitors. The particulate fraction was separated from the cytosolic fraction by ultracentrifugation at 100,000 g for 1 h (4℃). The particulate fraction was extracted by detergent-containing buffers to solubilise the membrane proteins and separated from insoluble components by centrifugation at 20,000 g for 20 min (4℃).

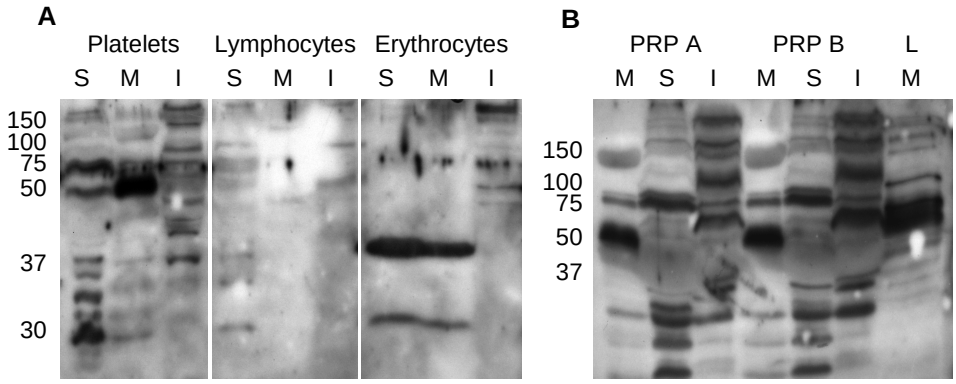


Figure 40: A) Western blot of the soluble(S), detergent-soluble (M) and detergent-insoluble (I) fraction of platelets, lymphocytes and erythrocytes. Proteins obtained by detergent-extraction (1% TX-100) of the particulate fraction (after 20,000 G centrifugation) of platelets exhibited a strong signal at 50 kD. A minor signal observed in the fraction S. No specific signal detected within platelet-proteins insoluble in 1%TX-100. Neither lymphocytes nor erythrocytes exhibit a specific signal in any of the fractions investigated. **B)** Westernblot of the soluble(S), detergent-soluble (M) and detergent-insoluble (I) fraction of platelets after optimization of the centrifugation procedure. Centrifugation of the platelet homogenate at 100,000 g ensures quantitative recovery of P2Y12 in the fraction obtained by detergent-extraction of the particulate fraction. L= Membranes of HA-P2Y12/HEK293-3/I cells loaded for comparison.

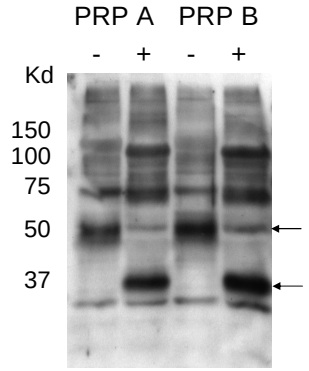


Figure 41: Western-blot of P2RY12 from platelets without and with enzymatic deglycosylation of N-linked saccharides. Platelet membranes of two buffy coats were prepared by freeze-thaw homogenisation with subsequent preparation of a P100-fraction which was extracted with 1% TX-100. Proteins were subjected to digestion with PNGaseF (+) at 37℃ for 1 h. In accordance with findings in the HA-P2Y12/HEK293-3/I line the protein is strongly glycosylated and exhibits a shift in the molecular weight from ~50 kD to ~ 37kD after removal of N-linked sugar moieties.

In conclusion, we demonstrated by Western blotting that the P2RY12 protein is present in platelet but not in other blood cells. The protein has an apparent molecular weight of ~50kD. N-Deglycosylation yielded a protein with an apparent molecular weight of ~37 kD which is in good accordance with the predicted molecular weight of 39 kD. The technique developed to enrich platelet membrane proteins was fairly rapid and quantitative with respect to P2RY12.

4 Discussion

In this work we tried to set up different prerequisites for further studies on the expression, function and pharmacology of the human P2RY12-receptor for ADP.

4.1 Detection of transcriptional variants in platelets

We addressed the expression of P2RY12 variants in human platelets by RT-PCR. The primers were chosen in a way that the known transcriptional variants were selectively amplified. The results demonstrated that platelets express the two-exon variant (NM_176876.1). In contrast, cDNA prepared from human brain contained the shorter NM_176876.1 and the three-exon splice variant (NM_022788.3). The results from the RT-PCR suggest that significant amounts of the P2RY12-RNA in human platelets remain intact at their 5'-end, since the primer specific for the transcriptional variant binds to the outmost 5'-region of the RNA. Thus, future analyses regarding the impact of promoter-polymorphisms on RNA-transcription should focus on the region upstream of the transcriptional start site of NM_176876.1. To our best knowledge, this is the first demonstration that the expression of P2Y12 in platelets is entirely restricted to the two-exon variant. At present it is unknown whether there are additional splice variants of P2Y12-mRNA. After completion of the experimental parts of the study, we readdressed the knowledge about splice variants of P2Y12 in the pertinent databases and no additional variants have been described meanwhile.

4.2 Cloning and Expression in HEK-cells

We chose a stepwise build-up strategy after we encountered problems to identify positive expression after generation of expression constructs in a different system without a tag (data not shown). Retrospectively, the failure to detect positive expression in the former approach was likely entirely due to the failure of two commercially available antisera recognizing two different epitopes (KTTRPFKTSNPKNLLGAK, corresponding to amino acid residues 125-142 of human P2Y12 receptor, Alomone labs and P-14 an affinity purified goat polyclonal antibody raised against an internal epitope with proprietary sequence within an internal region of human P2RY12, Santa Cruz).

Whether this was a problem of only the lots we had in our hands or whether these antibodies are simply not detecting P2Y12 cannot be solved at present. At the time we started the experiments no published data on the apparent molecular weight of mature P2RY12 in human platelets were available. Retrospectively, the specifications of both antibodies are not congruent with our own subsequent data and the Western-blot signals which have been published by other groups (Savi et al. 2006). The problem of the specificity and accuracy of commercial antibodies is well known and is obscuring our knowledge on protein expression in biomedical science (Bodei et al. 2009; Hamdani & van der Velden 2009; Jensen et al. 2009; Michel et al. 2009; Pradidarcheep et al. 2009).

The subsequent built-up strategy involved addition of a HA-tag to the N-terminus of P2Y12 and two additional silent mutagenesis steps to allow for a stepwise reconstruction of the reading frame. This strategy has an additional advantage: In subsequent work (regarding e.g. the role of phosphorylation sites in desensitization of the receptor) parts of the coding sequence can be exchanged by mutagenesis in a part of the coding sequence and subsequent exchange of the cassette. At present it is known that P2Y1 AND P2Y12 rapidly desensitize after agonist stimulation. There is indication for contribution of different mechanisms in the desensitization of these ADP-receptors. However, the exact target sites of the protein kinases involved have not been mapped. Studies addressing the mechanisms responsible are facilitated by the exchange of parts of the coding sequence of the protein by means of the artificially introduced silent restriction sites.

The transient expression yielded a DNA-dose-dependent expression of a glycosylated protein in HEK 293 cells. The glycosylation caused the appearance of a blurred band and a difference between the expected molecular weight (39 kD) and the apparent molecular weight (~ 50 kD). The protein expressed in HEK293-cells was detected with both antibodies, the anti-HA-tag antibody (clone 3F10; rat monoclonal by Roche) and by the polyclonal antiserum raised in rabbits against a C-terminal peptide. The latter antibody was a kind gift of Dr. Pierre Savi (Sanofi Aventis, France). The band was somewhat more condensed in the P2RY12-expressing stable cell line likely due to a more homogenous glycosylation pattern after clonal selection. Immunofluorescence revealed correct localization of the overexpressed tagged

protein on the plasma membrane. The results of a glycosylation-prediction program suggested the presence of two putative N- glycosylation sites (N6 and N13) and thus we attempted to inhibit the glycosylation by incubation with tunicamycin, a compound specifically inhibiting N-glycosylation. Moreover we removed the glycosylation by incubation of membranes with PNGase-F. Both experimental approaches caused a shift of the specific signal from a blurred band above 50 kD to about 37 kD which is in good accordance with the predicted molecular weight of 39.4 kD. This conclusion is supported by the findings of Kumar's group who reported that mutation of two glycosylation sites entirely abrogated the difference between the predicted and the observed molecular weight (Zhong et al. 2004). Indeed, both glycosylation sites appear to be functional in our overexpressing cell line, since abrogation of one of the sites by mutagenesis yielded partially glycosylated proteins with different molecular weights in the aforementioned study.

The glycosylation appears to be essential for signal transduction of the receptor since its abrogation by site-directed mutagenesis abrogated its cAMP-lowering activity despite of essentially unaltered binding of MeSADP and MeSAMP and a correct transport of the protein to the cellular surface (Zhong et al. 2004). The reasons for this phenomenon are not entirely clear at present. Kumar's group proposed that the extracellular portions of the P2Y12 receptor including its amino-terminus and extracellular loops are directly involved in the signal transfer to the cytoplasm. They assume that the activating conformational change triggered or stabilized by ligand binding at the transmembrane domain regions is dependent on intact glycosylation of the receptor. A different view however is, that the altered glycosylation causes an enhanced intrinsic activity of the receptor with subsequent desensitization yielding a ligand-binding uncoupled status of the protein.

4.3 Functional Assays

The signalling evoked by P2Y12-receptors comprises classical Gi-dependent and independent pathways (Soulet et al. 2004). In our cellular model, we used the phosphorylation of MAP-Kinases (p42/p44) as indicator of functional coupling of the receptor. This assay is relatively simple and inexpensive compared to e.g. the determination of cAMP after first stimulating the cells with low concentrations of

forskolin and subsequently inhibiting this response by addition of agonists at the P2Y12 receptor. In CHO cells overexpressing P2Y12, activation of MAP-kinases is entirely abrogated when Gi-proteins are inactivated by pertussis-toxin catalysed ADP-ribosylation. Likewise, ERK-phosphorylation was suppressed by Wortmannin and LY294002 (inhibitors of PI3-kinases). Similarly to the phosphorylation of p42/p44 the phosphorylation of Akt was dependent on functional Gαi-proteins (abrogation by PTX-catalysed ADP-ribosylation).

It is thought that the activation of ERK1/ERK2 is partially mediated by transactivation of the PDGFR given that stimulation of P2RY12 by MeSADP evokes PDGFR-phosphorylation and that inhibition of its kinase-activity by AG1296 reduces the extent of ERK- and AKT-phosphorylation. In contrast, stress fibre formation upon stimulation of CHO-cells overexpressing P2Y12 is not disturbed after PTX-incubation or PI3-kinase inhibition by Wortmannin and is not influenced by inhibition of the PDGFR by AG1296 (Soulet et al. 2004). The involved pathway likely includes activation of RhoA which upon stimulation binds GTP and is redistributed to the cellular membrane. These phenomena are not affected by Gαi-inhibition (PTX), by PI3-Kinase inhibition (Wortmannin) or by PDGFR-inhibition (AG1296). These findings suggest the involvement of pathways independent of Gαi and are compatible with an alternative coupling of the receptor to e.g. Gα12/13 which however has not been formally proven.

With the recent discovery of polymorphisms in the sequence of P2Y12 several studies emerged which yielded inconsistent results regarding the impact of these polymorphisms on platelet aggregation, the propensity to develop cardiovascular diseases and treatment effects of clopidogrel. At present only one study addressed the impact of the polymorphisms on mRNA- and protein expression. However, this study used the antibody supplied by Alomone labs, which – as we have clearly demonstrated – yields a non-specific band in Western-blotting. Moreover, nothing is known about the inter- and intra-individual variability of P2Y12-expression on mRNA- and protein-levels and the correlation of these two variables. Moreover, the relationship between expression levels and either platelet reactivity in laboratory tests or – more importantly – clinical outcomes is at present unknown, too.

The frequent silent polymorphisms within the gene are located within the first intron of the shorter variant (NM_176876.1) and in the coding part of the following exon which contains the entire coding sequence. There are no known polymorphisms altering the amino acid sequence. Only three mutations with alteration of the amino acid sequence causing a very rare bleeding disorder (OMIM *600515) have been described (Hollopeter et al. 2001; Cattaneo et al. 2003).

SNP	Type	Location	Official name	MAF
rs10935838	SNP	intron	NM_176876.1:c.-15+137	0.225 (n=120) [1] 0.138 (n=98)[2]
rs2046934	SNP	intron	NM_176876.1:c.-15+742	0.224 (n=120)[1] 0.138 (n=98)[2]
rs5853517	DIP	intron	NM_176876.1:c.-15+798delA	n.a. [1] 0.138 (n=98)[2]
rs6785930	SNP	exon, cds	NM_176876.1:c.18C>T p.6N>N	0.305 (n=226)[1] 0.275 (n=98)[2]
rs6809699	SNP	exon, cds	NM_176876.1:c.36T>G p.12G>G	0.186 (n=226)[1] 0.138 (n=98)[2]

Table 8: Rs-numbers, location and official nomenclature of the currently known frequent polymorphisms of P2RY12 annotated to the shorter transcript-variant NM_176876. [1]Data from HAP-Map consortium; [2] Data from Fontana and colleagues (Fontana et al. 2003)

This work established two methods to measure the expression of both, the mRNA by means of a qRT-PCR and the proteins after enrichment for platelet membrane proteins. We developed a procedure to enrich the P2Y12 protein in a quantitative manner. This procedure encompasses disruption of the platelets by three freeze-thaw cycles, followed by a high-speed centrifugation (100,000 g) to obtain a particulate fraction and subsequent extraction of this particulate fraction by the non-ionic detergent Triton X-100. The preparation is finalized by another medium speed centrifugation to spin down proteins not solubilised by the detergent. After optimization of the procedure, P2Y12 was solely detectable in the TX-100 extracted fraction whereas neither the supernatant of the first centrifugation step nor the pellet

remaining after TX-100 extraction contained detectable amounts of the protein. Essential to this was the increase in centrifugal force in the separation after the three freeze-thaw cycles.

Current status on variability of clopidogrel and prasugrel response

The last years have seen an enormous amount of publications regarding the impact of genetic variants of drug-metabolizing enzymes and of drug-interactions on variability of drug response towards clopidogrel and more recently prasugrel. There have been various reports about poor responders to treatment with clopidogrel (Snoep et al. 2007) and also a high inter-individual variability the biological response to treatment with clopidogrel by cardiovascular patients. Although P2YR12 receptor has a significant role in normal haemostasis and thrombosis, no genetic study has proven the association of polymorphism of the receptor and different degree of response to therapy with clopidogrel (Giusti et al. 2007). In a study carried out by Angiolillo et al. no association was shown between a specific sequence variation (T744C) and platelet response to clopidogrel among patients with coronary artery disease. Clearly the variability seems to be multifactorial and has partly been explained by its metabolism through the cytochrome P450 system. Until today it is not entirely understood which cytochromes are involved. Several publications show that CYP1A2, 2C9 and 2C19 produce the intermediate metabolite and CYP3 A4, 2B6 and again 2C19 are relevant to produce the active metabolite (Savi et al. 2000; Ingelman-Sundberg et al. 2007; Kim et al. 2008). Worse clinical outcomes appear to be associated with variant alleles of CYP2C19 (Hulot et al. 2006; Brandt et al. 2007). In the work of Simon et al. patients carrying CYP2C19 loss of function alleles had a higher rate of subsequent cardiovascular events (Simon et al. 2009). This was particularly evident for patients undergoing percutaneous coronary interventions. This led to the assumption that inhibition of CYP2C19 activity by drug-interactions e.g. with PP-inhibitors could lead to a reduction in clopidogrel antiplatelet effects. The trials carried out show a discrepancy in results. Some observational studies demonstrated increased cardiovascular risks associated with the combination of a PPI with clopidogrel (Ho et al. 2009; Juurlink et al. 2009). Moreover, an attenuation of *ex vivo* antiplatelet effects of clopidogrel was described, too (Gilard et al. 2008). A

recent observational analysis of data from the TRITON-TIMI 38 clinical trial of clopidogrel found no cardiovascular risk associated with concurrent use of PPI (O'Donoghue et al. 2009). Additionally, concurrent PPI use with clopidogrel was associated with reduced incidence of hospitalizations for gastrointestinal bleeding. (Ray et al. 2010).

The FDA (Food and Drug Administration) as well as the EMA (European Medicines Agency) warned against the reduced efficacy of clopidogrel combined with a PPI. At present, both agencies discourage the combination of omeprazole with clopidogrel. In patients with a gastrointestinal risk profile, concomitant use of a PPI with a reduced interaction potential (e.g. pantoprazol) or a second generation H2-blocker is recommended. This recommendation is based on the finding of reduced plasma concentrations of the active metabolite of clopidogrel after administration of omeprazole (Angiolillo et al. 2011). In contrast, pantoprazole did not affect the biotransformation of the parent compound.

Prasugrel shows a better efficacy than clopidogrel and a reduced drug-drug interaction as compared to clopidogrel (Wiviott et al. 2007; Small et al. 2008). However, this was associated with an increased risk for major bleeding, including fatal bleeding particularly in elderly patients. Overall mortality did not significantly differ between treatment with clopidogrel or prasugrel.

All these findings already play an important role in the therapy of cardiovascular diseases. Future findings will be very important to reduce the risk of drug interactions and unwanted side effects of antiplatelet therapy.

5 Summary

P2RY12 is an important platelet receptor for ADP and an important drug target. Little is known about the inter- and intra-individual variability of the receptor on both, mRNA- and protein levels. Moreover, the tertiary and quaternary structure of the intact receptor is only partially known at present.

The aims of this study were: 1. to characterize the transcript variants expressed in human platelets; 2. to establish a method for the quantitation of the protein in human platelets; 3. to characterize the specificity of commercial antibodies against P2Y12; 4. to establish a cell line with stable overexpression of a functional P2Y12-receptor.

Methods and Results: As shown by RT-PCR, human platelets only expressed the shorter transcript variant of P2Y12, whereas in brain RNA both the longer and shorter transcript were detectable. After optimizing the procedure developed for the preparation of platelet membrane proteins (comprising differential centrifugation and detergent-extraction), P2Y12 was entirely contained in one fraction. Under denaturing reducing conditions, the protein migrated as a single broad band with an apparent molecular weight of ~ 50 kD. After deglycosylation with PNGase F the protein shifted to a condensed band with an apparent molecular weight of ~37 kD which is in good accordance with the predicted weight (39 kD). An HA-tagged P2Y12 receptor was transiently and stably expressed in HEK 293 cells, which had similar characteristics as the protein detected in platelets. The overexpression was used to analyse the specificity of two commercial antibodies which both yielded no correct signal (Anti-P2RY12, APR-012 from Alomone labs and P2RY12, P-14 from Santa Cruz). The only antibody tested which correctly identified both, the native and the overexpressed HA-tagged protein was a non-commercial antibody (gift of Dr. P. Savi). The functionality of the overexpressing was proven by concentration-dependent ADP-induced phosphorylation of the MAP-kinases p42/p44.

Discussion: Human platelets express only the shorter of the two known RNA transcript variants (NM_176876). Future studies on differences in expression levels should firstly address the promoter of the shorter transcript variant. We were able to develop a fairly rapid method for the quantitation of P2RY12 mRNA and protein from

human platelets enabling future studies on differences in the mRNA and protein expression in dependence on genetic or pathophysiological factors.

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7 List of abbreviations

µg	Microgram
µl	Microliter
2-MeSADP	2-Methyl-Thio-ADP
AAI	Aqua ad injectabilia
ACS	acute coronary syndrome
ADP	Adenosine diphosphate
ARR	Absolute risk reduction
ASA	Acetylsalicylic acid, aspirin
BMS	Bare-mental stentAbsolute risk reduction
Bp	Base pair
CAD	Coronary artery disease
cDNA	Complementary DNA
CDS	Coding sequence
Cm	Centimeter
CO_2	Carbondioxide
COX1/COX-2	Cyclooxygenase 1 and 2
dATP	2´-Deoxyadenosine-5´Triphosphate
DES	drug-eluting stent
dNTPs	Desoxynucleosidtriphosphate
DTT	Dithiothreitol
E. coli	Escherichia coli
EMF	Electromotive force
G	Gram
GPIIb/IIIa	Glycoprotein IIb/IIIa
HEK-293 cell line	Human embryonic kidney 293 cells
IPTG	Isopropyl-[beta]-D-thiogalactopyranosid
kD	kilo Dalton
LB medium	Luria broth medium
MCS	multiple cloning site
$MgCl_2$	Magnesium chloride
MgOAC	Magnesium acetate
MI	Myocardial Infarction
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	messenger RNA

Na-Ac	Sodium acetate
ng	Nanogram
nm	Nanometer
NNH	Number needed to harm
NNT	Number needed to treat
NO	Nitric Oxide
NP40	Nonyl phenoxypolyethoxylethanol
NSTE-ACS	Non-ST-Elevation Acute-Coronary Syndrom
NSTEMI	Non-ST-Elevation Myocardial Infarction
PBS	Phosphate buffered saline
PCI	Percutaneus coronary intervention
PCR	Polymerase chain reaction
PGI2	Prostacyclin
PRP	Platelet-rich plasma
PVDF	Polyvinylidenfluoride
PVDF-membrane	Polyvinylidene fluoride membrane
RNA	Ribonucleic acid
RNAse	Ribocuclease
Rpm	Revolutions per minute
RRR	Relative risk reduction
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
S	Seconds
SDS buffer	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STEMI	ST-elevation myocardial infarction
TBE	TRIS-borate-EDTA
TBS/T-buffer	TRIS-buffered saline-Tween buffer
TRIS	Tris-hydroxymethyl-aminomethane
TXA2	Thromboxan A2
VASP	Vasodilatator-stimulated phosphoprotein
vWF	von Willebrand factor
W	Watt
X-Gal	Bromo-chloro-indolyl-galactopyranoside

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