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Precision-cut kidney slices as a model for acute kidney injury

Targeting mitochondrial dysfunction to improve kidney regeneration after acute kidney injury

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Abstract

Acute kidney injury (AKI) is worldwide a major health concern and the incidence has increased over time. There is, as of now, no medical intervention that significantly impacts AKI. A factor that can cause AKI is ischemia/reperfusion injury (IRI), which is restriction of blood to the kidney followed by restored blood flow. Mitochondrial dysfunction plays a critical role in the progression of renal IRI. Here, we looked at murine precision-cut kidney slices (PCKS) as a model for renal IRI. Furthermore, a compound that theoretically improves mitochondrial functionality, SUL-138, was added during incubation to possibly combat kidney IRI. Finally, a protocol was set up to isolate mitochondria from PCKS. This to specifically study the effects on the mitochondria due to IRI and the possible positive effect of SUL-138. ATP results showed that PCKS's incubated with SUL-138 100µM and 500µM are viable, although not significantly higher than the control samples. Also, different gene markers (Kim-1, NGAL, Il-6, etc.) showed that there is damage induced during incubation in the PCKS's due to IRI. Gene expression of the SUL-138 100µM incubated slices showed no significant protective effect in comparison with control groups. Morphology of the PCKS's showed that SUL-138 100µM had protective effects in comparison with control groups. In the SUL-138 samples, Bax and kim-1 were visually less expressed and Tom-20 was more expressed in comparison with the control groups. Using western blot, and the markers Vinculin, HDAC, Tom-40, Tom-20 and COX4, mitochondria were shown to be isolated from the PCKS's using the protocol. In conclusion, PCKS's incubated with SUL-138 are viable and show morphologic protective effects. Gene expression does not show these protective effects. The used isolation protocol showed that mitochondria can be extracted from the PCKS's and be used for further research.

Contents

Introduction

Acute kidney injury

Acute kidney injury (AKI) is a major global health problem with a relatively high prevalence. Worldwide, 21.6% of hospitalized adults suffer from AKI and for patients admitted to critical care units this number is 30 to 40%(1,2). The incidence of AKI has increased in the last fifteen years and is associated with a significant increase in the length of hospital stay and mortality(2). AKI affects the kidney not only on the short term, but also on the long term. Patients who leave the hospital after an episode of AKI are at persistent risk of adverse outcomes. This includes a 10-fold greater risk of chronic kidney disease (CKD), a 3-fold greater risk of end-stage renal disease (ESRD) and double the risk of premature death(2).

AKI is characterized by the abrupt loss of excretory kidney function over a period of hours to days, and the main pathological phenotype of AKI is tubular damage, including apoptosis(1). Furthermore, AKI can result in the dysregulation of extracellular volume and electrolyte balance, the accumulation of nitrogen metabolism end products (e.g. urea and creatinine) and it can decrease urinary output. There are multiple factors involved in the development of AKI. These include ischemia/reperfusion injury (IRI), cardiovascular surgery, radiocontrast agents and sepsis(1,3). Despite substantial research, there is no medical intervention that significantly impacts AKI, and current therapeutic options are solely symptomatic treatment(3).

Ischemia/reperfusion injury

As stated earlier, IRI is an important and common factor in the pathogenesis of AKI. It is characterized by the restriction of blood supply to the kidney followed by restoration of the blood flow and re-oxygenation(4). Restricted blood flow causes a generalized or localized impairment of oxygen and nutrient delivery to the kidney cells and waste products from the kidney are not removed(5). This causes an imbalance in local tissue oxygen supply and waste product removal. Restoration of blood flow causes re-oxygenation and nutrient delivery to the kidney cells and removal of kidney waste products. However, reperfusion of the kidneys also induces a large production of reactive oxygen species (ROS), which contributes to membrane and cytoskeleton damage. Also, reperfusion leads to cytoplasmic and mitochondrial calcium overloads. The increased mitochondrial calcium content and ROS production causes opening of the mitochondrial transition pore. This results in cell death through different mechanisms, such as apoptosis, necrosis and autophagy(6).

IRI may occur after infarction, sepsis and surgical interventions, e.g. nephrectomy and renal transplantation(7). IRI can activate leukocytes and stimulate the production and release of cytokines and chemokines, which can cause renal tissue damage and stimulate the production of ROS(4). Renal tissue damage can results in a decrease in glomerular filtration rate, an increase in serum creatinine levels, a decrease in urine output or tubular epithelial cell injury. In the case of severe injury, apoptosis and necrosis is induced in the different kidney cells(5,7).

Mitochondrial dysfunction in IRI

Mitochondria play a critical role in the progression of renal IRI. Mitochondrial dysfunction occurs due to a lack of oxygen, which induces apoptosis and production of ROS, thereby contributing to the development and progression of IRI. Mitochondria can also change the metabolic and bioenergetic status of kidney cells, and induce autophagy and inflammation(4,8).

Mitochondria are intracellular organelles mainly tasked with the production of ATP, which is the major cellular energy source. Production of ATP is done via the Oxidative Phosphorylation (OXPHOS) system, which is expressed in the mitochondria, see figure 1(4,8,9).

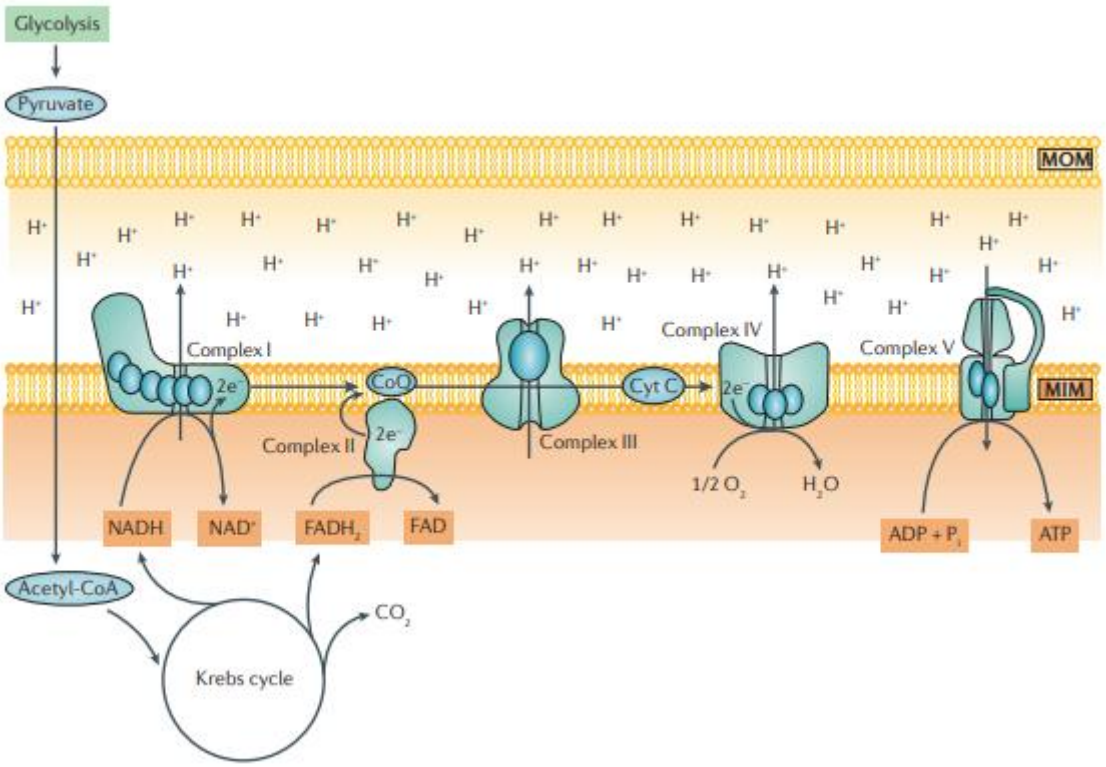

Figure 1. OXPHOS system in mitochondria(10)

The OXPHOS system starts when pyruvate, generated in the cytosol via glycolysis, transfers across the double mitochondrial membrane and enters the matrix. Inside the matrix, pyruvate is converted into the two carbon compound acetyl coenzyme A (acetyl CoA), which is used in the Krebs cycle to produce NADH and $FADH_2$. NADH donates electrons to complex I of the OXPHOS system and $FADH_2$ donates electrons to complex II(8,9). The electrons donated to complex I and II are passed to complex III, via coenzyme Q(11). Cytochrome C passes the electrons from complex III to complex IV, where the electrons are used to produce H_2O(9). The energy that is released through this electron transport is used by complexes I, III and IV to pump protons in the inter membrane space in the mitochondria. This creates an electrochemical gradient, which is used by complex V (ATP synthase) to catalyze the synthesis of ATP from ADP and phosphate(7,8).

During IRI-induced mitochondrial dysfunction, activity of the OXPHOS system is suppressed due to the lack of oxygen. This leads to a reduction in ATP synthesis and concurrently diminishes the activity of cellular energy dependent processes, which contributes to cell death. Reduced ATP production results in an influx of sodium ions that is no longer counteracted by the Na^+-K^+-ATPase, which causes an influx of water and therefore cell swelling, resulting in necrotic cell damage(12).
When oxygen supply is restored (reperfusion phase), mitochondria will be exposed to large amounts of oxygen free radicals. This contributes to the progressive functional deterioration of mitochondria and cells during the reperfusion phase, resulting in apoptosis (12). See figure 2.
The kidney relies on the OXPHOS system to produce the bulk of ATP that is needed for tubular reabsorption, so it is evident that mitochondrial homeostasis is critical for the maintenance of normal renal functioning(1).
Taken together, it is clear that mitochondria play an important role in the pathogenesis of AKI, especially as a cause of renal tubular dysfunction and cell death(1,4,8)

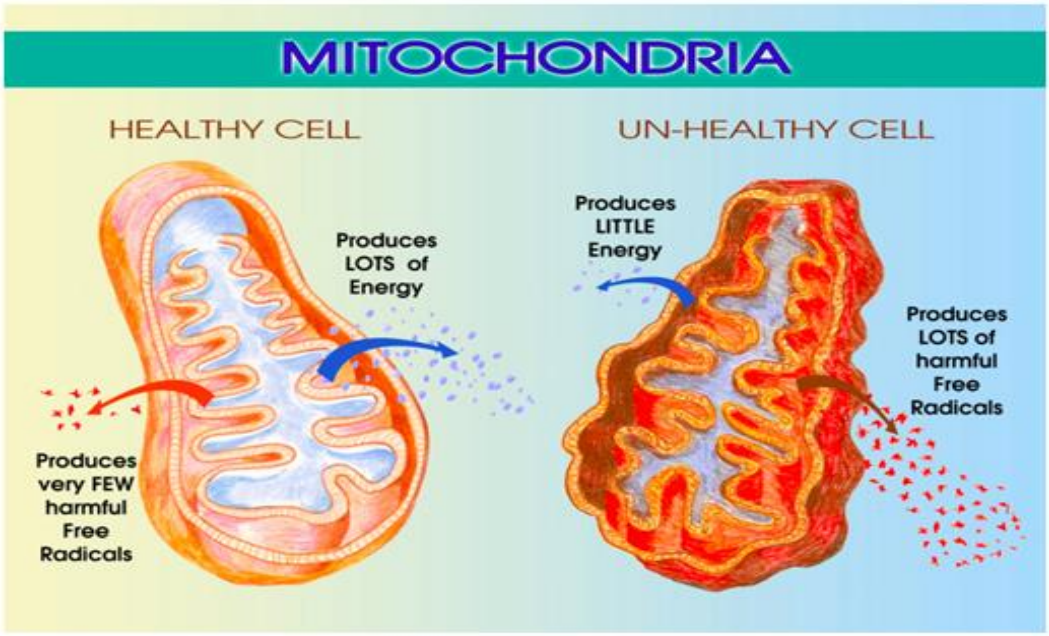

Figure 2. Mitochondria functionality in healthy and un-healthy cells(13)

Kidney slices as a model for AKI due to IRI

A possible model to look at renal IRI is murine and human precision-cut kidney slices (PCKS). When the kidneys are used for slicing, they are harvested and preserved at 4^0C. During this stage, there is no oxygen supply to the tissue. This mimics ischemic stress in the organ. After slicing of the organ, slices are incubated at 37^0C with high oxygen levels. This mimics reperfusion stress(14). Thus, during preparation, PCKS undergo ischemia/reperfusion injury, similar to IRI in kidneys of patients. Viability of PCKS is determined by the ATP content of the slices. The measured ATP levels in PCKS remain fairly constant up to 96 hours(15,16). However, on a morphological level it is observed that the integrity of the slices deteriorates after 48 to 72 hours, which shows that the viability of slices decreases over time and does not correspond with ATP levels(15,16). An explanation for this could be that there is mitochondrial dysfunction in the slices, where ATP levels stay fairly the same, but there is damage in the OXPHOS system.

SUL – 138

Since the current treatment of AKI is supportive, a new treatment that can combat the AKI sounds promising. Improving the functionality of the mitochondria in patients with AKI and PCKS could be such a treatment.
SUL-138, a 6-chromanol derivate, is a compound that seems to be improving the functionality of the mitochondria(12,17). Related compounds have shown to protect the mitochondria in AKI due to IRI and therefore improving the functionality of the mitochondria(12,18).
Preclinical models showed that SUL-138 is able to reach the mitochondria, where it restores electron transport to improve ATP production and reduce the production of ROS, see figure 3(12).

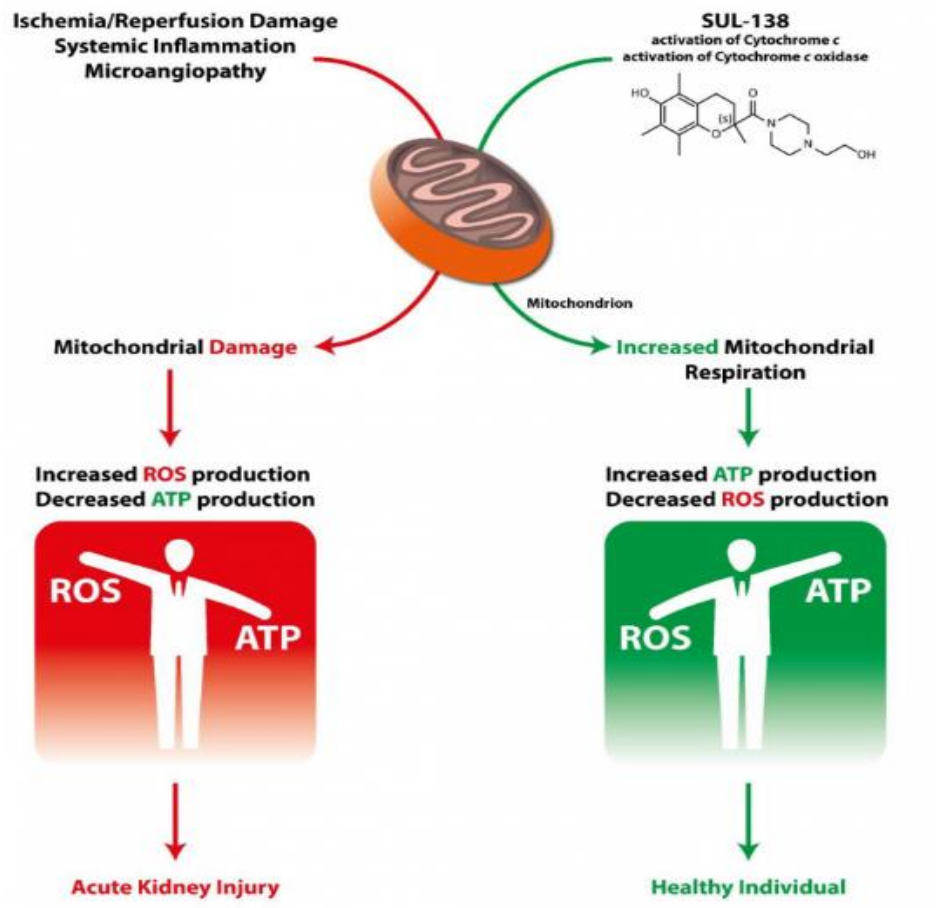

Figure 3. SUL – 138 mechanism of action(17)

Restored electron transport in the mitochondria results in reduced oxidative stress. SUL-138 functions by targeting and helping the mitochondrial complexes III and IV, where it maintains functionality of the enzyme cytochrome c oxidase and is involved in the direct reduction of Cytochrome C, which helps in maintaining the mitochondrial membrane potential(12,17). Therefore, SUL-138 can help to maintain the mitochondrial membrane potential, increase ATP production and reduce ROS production during pathological conditions.
SUL-138 promises to have a significant positive effect on the viability of PCKS, by increasing ATP production and decreasing ROS production. Ultimately, the goal is to use SUL-138 to combat IRI-induced AKI (17).

Mitochondria isolation

Isolation of mitochondria from PCKS is interesting to look at. It is observed that ATP levels of slices stay fairly constant over a period of 96 hours, but morphological research shows that slices deteriorate earlier(15,16).So this could mean that ATP in PCKS is not a complete marker of mitochondrial health and therefore not a good indication of the increase in viability of the slices. In theory, when mitochondria are isolated from the kidney slices, these can be used to determine the viability. Other markers could be determined in the isolated mitochondria. These could indicate damage in PCKS's with 'viable' ATP levels. Also, it can determine if the slices treated with SUL-138 are more viable in comparison with the slices that aren't treated with SUL-138. There are antibodies that could be used in western blot on isolated mitochondria samples to determine if the isolation succeeded. Also, markers for the nuclei and cytosol of the cell could also be used to determine the purity of the isolation.

Materials and Methods

Mice

C57BL/6 mice weighing 14-28g, used for experiments, were obtained from Harlan (Zeist, the Netherlands). Animals were kept in cages with free access to food and water. Mice were anesthetized under 2% isofluorane/O2 (Nicholas Piramal, London, UK), and then sacrificed. The right and left kidneys were retrieved as quickly as possible and placed into ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA). Up until incubation, all further steps were then performed on ice (0-4 ^{0}C). The animal experiments were evaluated and approved by the Animal Ethics Committee of the University of Groningen.

Mouse precision-cut kidney slices

Kidneys were prepared for slicing by removing adipose tissue around the kidney. The whole kidney was then placed into the cylindrical core holder. Precision-cut kidney slices were cut in Krebs-Henseleit buffer (4^{0}C, pH 7.4) using the Krumdieck tissue slicer, according to the protocol by De Graaf et al, 2010. The wet weight of the slices was around 4mg (200-300 µm thickness) and the slices were selected on basis of their appearance, with good slices having an equal thickness, uniform color and smooth edges. The selected slices were transferred into fresh ice cold (4^{0}C) UW and stored on ice until incubation. See figure 4, steps 1 and 2, and appendix A.

Incubation of precision-cut kidney slices

Kidney slices were incubated in 12-well plates containing 1.3 ml William's E medium with GlutaMAX (Life technologies, Carlsbad) supplemented with 10µg/ml ciprofloxacin and 2.7 g/L D-(+)-Glucose solution (Sigma-Aldrich, Saint Louis) per well, see appendix A.
Medium was pre-warmed and gassed with 80% O_2/5% CO_2 before slices were added to the medium. The culture plate was placed on a heating pad to maintain the medium at 37^{0}C while the slices were placed in the medium. Slices were individually transferred to the wells of a culture plate. After transferring the slices, plates were immediately transferred back at 37^{0}C in a gently shaking CO_2 incubator, under continuous supply of 80% O_2/5% CO_2. See figure 4, steps 3 and 4.
Slices were incubated for 0,3,24 and 48 hours, whereby medium was refreshed after 24 hours. Slices were also treated with 100µM (3h, 24h and 48h) and 500µM (24h) SUL-138 compound (Sulfateq B.V.). 100µM DMSO (100%) was used for drug reconstitution and added to control conditions.
After incubation, kidney slices were snap-frozen in liquid nitrogen and kept in -80^{0}C until used for analysis.

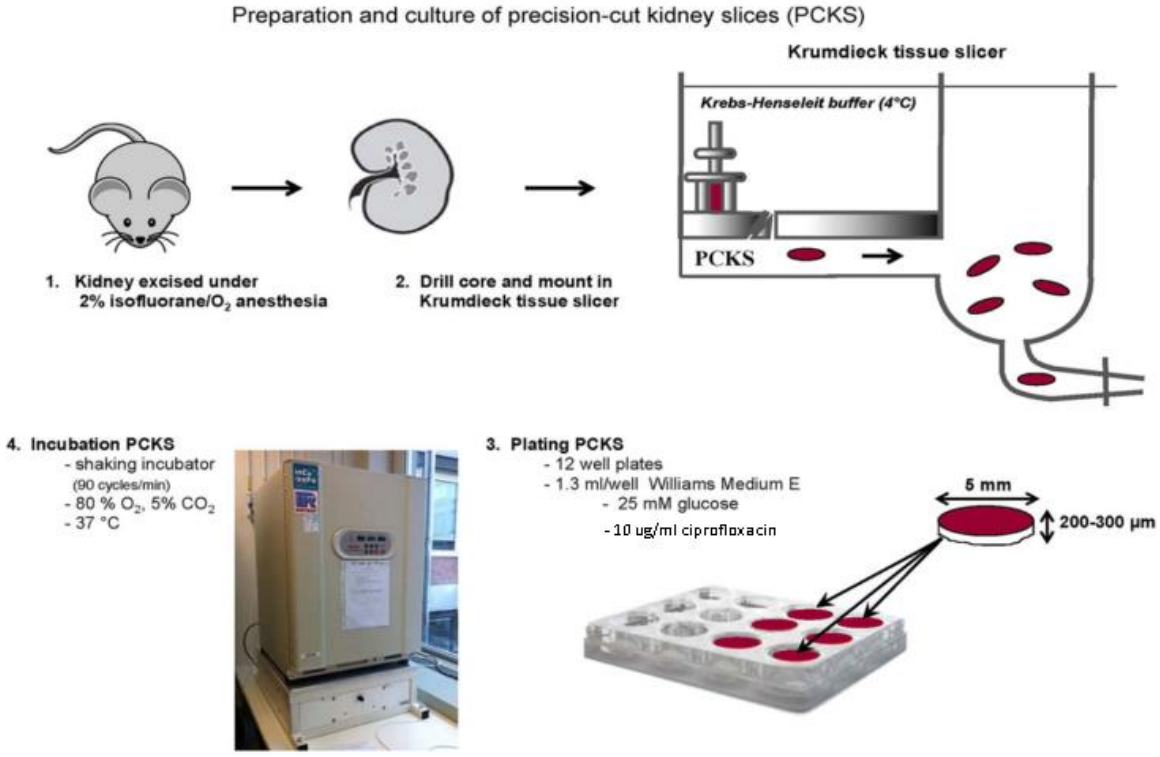

Figure 4. Schematic representatation of the experimental approach for obtaining and culturing mPCKS(15,16)

Viability slices

The general viability of the PCKS was checked by measuring ATP content of the slices, normalized for protein concentrations.

For ATP measurements, single slices were transferred to 1.5ml safe lock vials and a sonication solution (SONOP), containing 70% ethanol (v/v) and 2mM EDTA (pH 10.9), was added. The vials were snap frozen in liquid nitrogen and stored at -80^{0}C until analysis. A Mini-BeadBeater-24 (BioSpec, Bartlesville, OK, USA), was used to homogenize the samples. After homogenization, samples were centrifuged for 5 minutes at 13,000 RPM at 4^{0}C. ATP levels in the supernatant were determined by using the Bioluminescence Assay Kit CLS II (Roche diagnostics, Mannheim, Germany) and accompanying protocol (*protocol)*. Luminescence was measured in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA), see appendix B.

The remaining pellets from the ATP determination were used to determine the protein content of the slices. The pellets were incubated at 37^{0}C to dry overnight. 200µL 5M NaOH was added to the samples and these were incubated for 30 minutes inside a shaking water bath at 37^{0}C. MilliQ water was added to reach a final concentration of 1M NaOH and samples were homogenized. Protein content of the samples was determined using the Lowry method (Bio-Rad DC Protein Assay, Bio-Rad, Munich, Germany). Bovine Serum Albumin (BSA), 3.2 mg/ml, was used for the calibration curve. ATP values in pmol were divided by the total protein content in µg of the PCKS. This was expressed as the ratio ATP/protein, see appendix B.

Gene expression

Three PCKS per treatment group were snap frozen in safe lock vials. RNA was isolated with the FavorPrep tissue total RNA mini kit. Slices were homogenized in FARB buffer with a Mini-Beadbeater-

24 (Biospec. Bartlesville, OK, USA). RNA was isolated by filtering using filter columns. It was quantified on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, Isogen Ijsselstein, the Netherlands), see appendix C.

RNA samples were diluted to 1µg in 10µL. 10µl of cDNA mix was added to every tube to achieve a final volume of 20µl/tube. The cDNA reaction mix contained the following composition/ 1 reaction: 4µl 25mM $MgCl_2$, 2µl 10x RT-buffer, 2µl 10mM dNTP's, 0.5µl Recombinant RNasin ® Ribonuclease Inhibitor, 0.6µl AMV Reverse Transcriptase (High Conc.) and 1µl of Random primers. After this, RNA was amplified to cDNA using the Reverse Transcription System (Promega), see appendix C.

Each sample of cDNA was diluted to 10ng/µl. PCR was performed in a 10-µl reaction volume, for each sample, containing cDNA of samples, SYBR green/Taqman mix and a 6µM working solution containing the forward and reverse primers of the gene of interest and DEPC water. Quantitative real-time polymerase chain reaction (qPCR) was performed with a 7900HT qPCR system (Applied Biosystems) using SYBR Green in duplo. Fold induction of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with GAPDH, a housekeeping gene, see appendix C. See table 1 for the used primer sequences in the qPCR.

Table 1. Forward and reverse primers used in qPCR

Genes	**Forward primer**	**Reverse primer**
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Il-6	TGATGCTGGTGACAACCACGGC	TAAGCCTCCGACTTGTGAAGTGGTA
Il-1β	GCCAAGACAGGTCGCTCAGGG	CCCCCACACGTTGACAGCTAGG
TNFα	CATCTTCTCAAAATTCGAGTGACAA	GAGTAGACAAGGTACAACCC
HMOX1	TGACACCTGAGGTCAAGCAC	CTGATCTGGGGTTTCCCTCG
SFN	GGCCGAACGGTATGAAGACA	GTACTCTTTCACCTCGGGGC
iNOS	AACGGAGAACGTTGGATTTG	CAGCACAAGGGGTTTTCTTC
NOX1	ACCTGCTCATTTTGCAACCGTA	AGAGATCCATCCATGGCCTGTT
SULF2	GCGGCCATAGAGAGAGGAAC	TGATCCAGAGCAAAGCAGGG
Bcl-2	GAACTGGGGGAGGATTGTGG	GCATGCTGGGGCCATATAGT
EIF3C	TGTGCCATCATTGAGCGAGT	GCCTGGTCTTGCTCAGACTT
KIM-1	AAACCAGAGATTCCCACACG	GTCGTGGGTCTTCTTGTAGC
NGAL	CTCAGAACTTGATCCCTGCC	TCCTTGAGGCCCAGAGACTT
Hif-1α	TCAAGTCAGCAACGTGGAAG	TATCGAGGCTGTGTCGACTG

Morphology

Kidney slices were fixated in 4% formalin for 24 hours and stored in 70% ethanol, both at 4^0C, until processing. Slices were embedded in paraffin after dehydration and were cut into approximately 4µm thick cuts. After cutting, the sections were mounted on glass slides and dried overnight at 37^0C. After drying, sections were deparaffinized in xylene and rehydrated in graded alcohol and distilled water. Antigen retrieval was achieved by overnight incubation at 80^0C in 0,1M Tris/HCL buffer (pH 9.0) for Bax, Tom20 and KIM-1 antibodies. Endogenous peroxidase activity was blocked with 0,1% H_2O_2 in PBS for 10 minutes.After that, the sections were stained. Samples were first incubated with a 1:20 avidin and biotin solution (in PBS), before incubation with the first antibody. Primary antibody was detected by sequential incubations with peroxidase labeled appropriate secondary antibodies. Peroxidase activity was visualized using NOVARED (approximately 5-10 min incubation). Sections were

counterstained with hematoxylin for 1 min and embedded in DEPEX medium to asses morphology, see appendix D.

Mitochondria isolation

5x mitochondrial isolation buffer (MIB) and 5x mitochondrial storage buffer (MSB) were prepared. 5x MIB consisted of 1.05M mannitol, 350mM sucrose, 25mM tris, 5mM EDTA, 100ml ddH_2O and the pH was set to 7,5 using HCL or NaOH. 5x MSB consisted of 1.25M sucrose, 5mM ATP, 0,4mM ADP, 25mM succinate, 10mM K_2HPO_4, 5mM DTT, 50mM HEPES, 10ml ddH_2O and the pH was set to 7,5 using HCL. Throughout the procedure, all solutions and tissues were kept on ice (4^0C). 5x MIB and 5x MSB were both diluted to 1x with ddH_2O. For 100mg of tissue, 50µl of 1x MSB was used. Also, 5 ml of 1x MIB-BSA solution was prepared. This solution contained 2 mg/ml BSA. After PCKS were obtained following the protocol stated above, they were added to a 12 wells plate containing 1x MIB on ice. The samples were then washed two times in 1x MIB. Then, the slices were added to Eppendorf tubes containing 10 times the volume (for example for 50mg, 500µl) MIB-BSA, until a weight of 50-100mg was reached. These slices were transferred to a glass tube or eppendorf tube containing MIB-BSA and homogenized. The first isolation was done with the slices transferred to the Eppendorf tubes. These were homogenized using an automated homogenizer with a plastic pestle. The pestle was spinning automatically and inserted in the eppendorf tube containing the samples. The Eppendorf tube was then moved up and down from the spinning pestle ten times. For the second isolation, the homogenizing was done manually with the plastic pestle. Here the pestle was also moved up and down in the tube ten times.
For the third isolation, the samples in the glass tube were used to homogenize. This was done manually, with two glass pestles (2 times up and down with pestle B, 4 times up and down with pestle A). After homogenization, the homogenate was transferred to a 1,5 ml tube and the samples were centrifuged for 5 minutes at 600 x g at 4^OC. Supernatant was transferred to a new 1,5 ml tube and centrifuged at 11000 x g for 10 minutes at 4^0C. Supernatant was then removed and the pellet was resuspended in 10 volumes (example: 50µl pellet volume→500µl 1x MIB) 1x MIB. Homogenate was then centrifuged at 600 x g for 5 minutes at 4^0C. Supernatant was then transferred to a new 1,5 ml tube and centrifuged at 11000 x g for 10 minutes at 4^0C. Pellet was saved for measurements. Then the pellet was resuspended in 1x MSB, 40µl per 100mg tissue. 5µl of this sample was saved for protein measurement. The pellet from an earlier step in the protocol was used as a positive control. The rest of the sample was stored at -80^0C or directly used for measurements. Protein content was measured by dilution in RIPA buffer and the expected protein content of the mitochondria suspension should be approximately 10-25 mg/ml per 100mg tissue. See appendix E. for the full protocol.

Western blot

The mitochondria isolation samples (20µg per sample)were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% running gels and 4% stacking gels. Samples used for the western blot were the isolated mitochondria and samples from earlier steps in the isolation process. The first step of the protocol was used as a positive control and samples from the step before the isolated product were also used. These samples should contain no mitochondrial tissue, only other cell components such as cytosol and nuclei. A molecular weight ladder was added to slot 4 or 5 to separate the samples and to determine the molecular weight of the sample bands. Mitochondria samples were 20µl in total were 4x sample buffer was diluted in the sample and dH_2O. Trans blotting was done using the Biorad Trans-Blot turbo RTA kit. Gels were imaged using the Biorad ChemiDoc imaging system. After trans blotting membranes were imaged. After that, membrane was blocked for 60 minutes in 5% Non-fat dry milk in TBST blocking buffer. Membranes were then cut and incubated in blocking buffer with different antibodies (HDAC1 60kDa, Vinculin 117kDa, Tom-20 20kDa, Tom-40 40kDa, Cox4 17kDa) overnight at 4^0C with continues rotation. Dilution of all antibodies used was 1:500 The next day, imaging was done using the Biorad ChemiDoc Touch imaging system by adding

ECL reagents 1:1 to the membrane. Membrane could be used for a different marker. The membrane was in this case stripped with stripping buffer, see appendix F.

Results and discussion

ATP content

Viability of the control PCKSs and the PCKSs treated with SUL-138 was assessed by ATP concentration. From previous experiments, ATP has shown to increase at the start of the incubation. This is also expected for these samples(15). It is also expected that the viability of the slices remains constant to at least 48 hours(15).And it is expected that the ATP concentration is higher in the SUL-138 samples compared to the control samples, due to the protective nature of the SUL-138 compound. The concentration of SUL-138 used was 100µM, and one 24 hour sample was incubated in a concentration of 500µM. Expectation was that this had a greater effect on the viability of the slice in comparison with the lower concentration SUL-138. See figure 5 for the results.

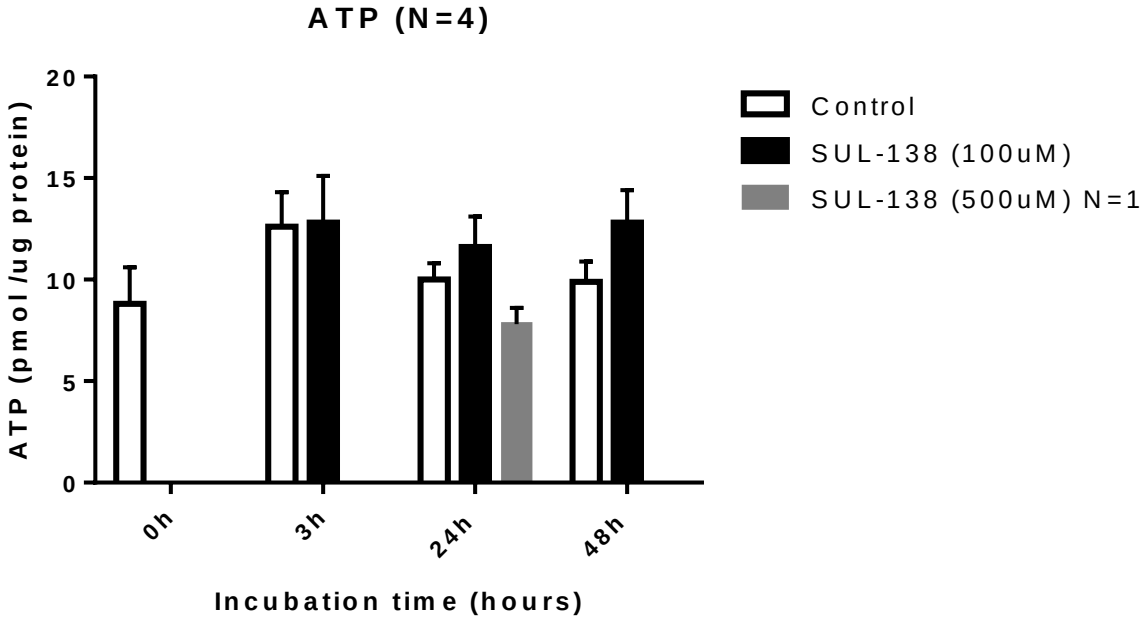

*Figure 5. ATP content in pmol/µg protein in the PCKS treated with SUL-138 and control PCKS during incubation. Data are presented as the mean ± standard error of the mean of 4 independent experiments. Statistical analysis was performed via a Kruskal-Wallis test followed by the Dunn multiple comparison test, compared with the 0h column. *P, 0,05*

Figure 5 shows that ATP levels increase at the start of the incubation, from 8,8 pmol/mg (0 hours) to 12,6 pmol/mg for the control sample and 12,8 pmol/mg for the SUL-138 sample (3 hours; Fig 5). The ATP levels remained relatively stable with a content of 9,9 pmol/mg for the control samples and a content of 12,8 pmol/mg for the SUL-138 samples at 48 hours.
When comparing the SUL-138 and control samples at 3 hours, it is observed that the mean ATP levels from the SUL-138 100µM samples is higher than the control samples. This is not a significant difference. This is also the case between the control and SUL-138 100µM samples at 24 and 48 hours. When looked at the SUL-138 500µM sample at 24 hours, it is observed that the ATP concentration is lower than the other two samples from the 24 hours. It is also lower than the 0h control sample. This difference is not significant, but not following the expectations for this experiment. A possible explanation for this could be that the concentration SUL-138 was too high for the kidney slices. It could be that these concentrations were causing toxicity effects, which could lead to damage in the kidney slices and less viability. This is translated in a low expression of ATP in comparison with viable samples, which is the case.

For future experiments, the concentration SUL-138 could be tested between 100µM-500µM, to see if there is an optimal concentration for the viability of the slices.

Hypothesis genes (1)

Expression of different genes was determined in the control samples and the samples incubated with SUL-138. These genes are a wide range of markers that can indicate damage in the tissue damage or protective effects from the tissue damage. The expression of the genes was determined during incubation and compared with the expression of the same genes in the SUL-138 samples.

IL-6:
Interleukin 6 (IL-6) is demonstrated to be a multifunctional cytokine that regulates numerous biological processes, such as inflammation and immune responses. Under certain circumstances, kidney resident cells such as podocytes, endothelial cells and tubular epithelial cells can secrete IL-6. It was found that in ischemic AKI animal models, IL-6 transcription and signaling are elevated locally and systematically after 60 minutes. (19)
IL-1β:
Interleukin 1β (IL-1β) is part of the IL-1 family of cytokines. This family is important in the regulation of systemic and tissue inflammation, pro and anti-inflammatory factors and signaling pathways. IL-1β mainly contributes to the systemic inflammation. Inflammation of mouse kidneys triggers release of IL-1β in tubular epithelial cells. (20)
TNFα:
Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine, that causes remote organ injury after localized tissue ischemia. TNF-α has been established as an important mediator of renal ischemia reperfusion injury. It is increased as early as 30 minutes after renal ischemia. (21)

The expectation is that IL-6, IL-1β and TNF-α are upregulated during the incubation in comparison with the 0 hour samples. The genes should be highly expressed at the first time point (3h) due to their characteristics. The protective properties of SUL-138 in the samples should show in the expression of these genes in comparison with the control samples. The expression in the SUL-138 samples is expected to be lower than the control samples.(19–21) Results are shown in figure 6.

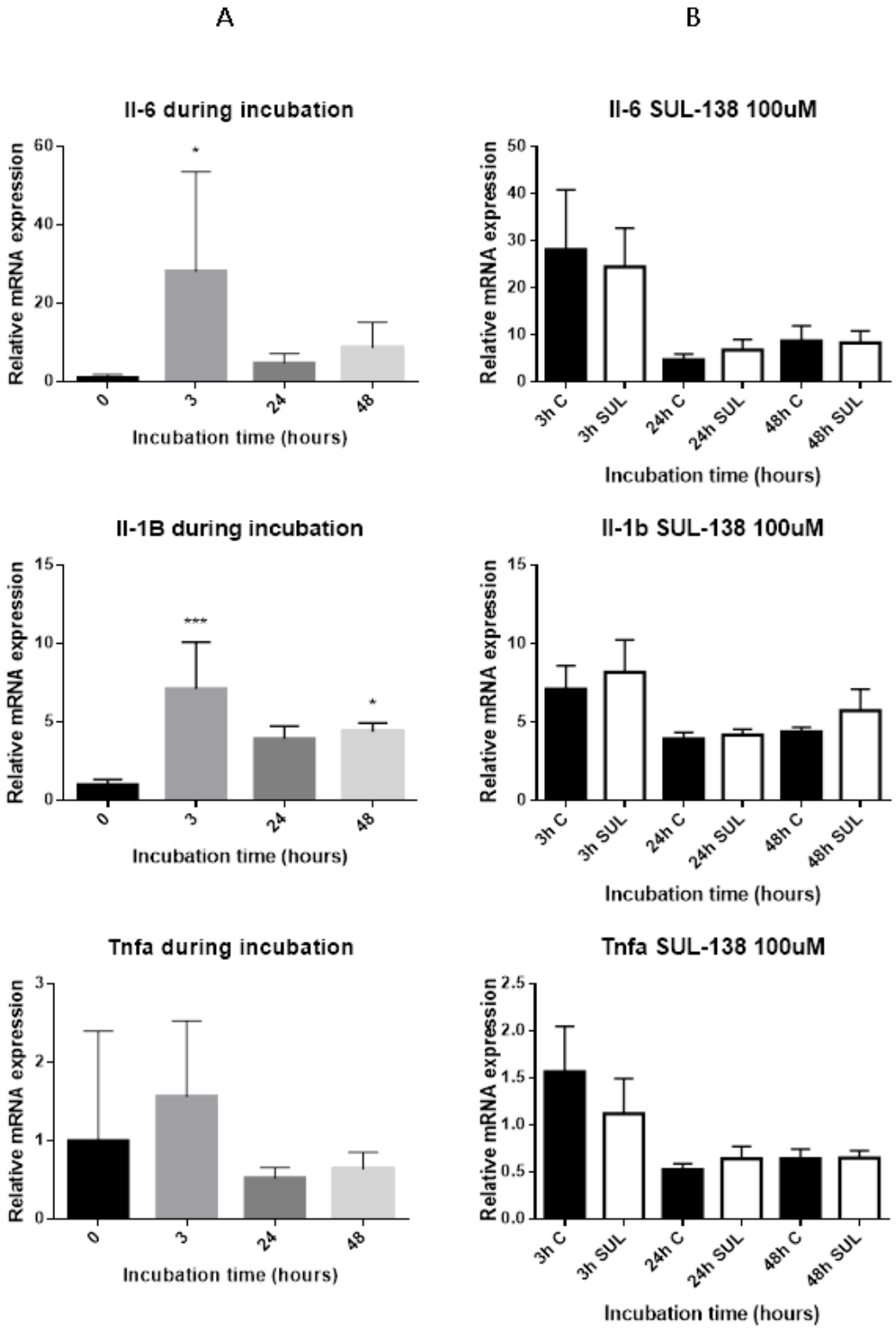

*Figure 6. Relative mRNA expression of Il-6, Il-1ß and Tnfα during incubation of PCKS's (A) and SUL-138 100µM samples (B). Gene expression was studied by qPCR. Relative expression was calculated using the household gene GAPDH (100%). Data are presented as the mean ± standard error of the mean of 4 independent experiments. Statistical analysis on the samples during incubation was performed via a Kruskal- Wallis test followed by the Dunn multiple comparison test, compared with 0 hours. *P,0.05. Statistical analysis on the SUL-138 100µM samples was performed via a Kruskal-Wallis test followed by the Dunn multiple comparison test, were the control samples of each time point were compared with the corresponding SUL-138 samples. *P,0,05*

Figure 6 column A shows a significant upregulation of IL-6 mRNA expression during incubation in the three hour samples in comparison to the 0h samples. The 24 hour and 48 hour samples show no significant upregulation of IL-6 expression. IL-1β samples during incubation show significant upregulation in the 3h and 48h samples compared to the 0h samples, in the 24h sample there is no significant upregulation. Tnfα samples show no significant increase in expression during expression compared to the 0h samples. Expression of IL-6 during incubation is as expected, IL-1β and Tnfα show different patterns. IL-1β upregulation after 48 hours of incubation was not expected. IL-1β is involved in systemic inflammation, which could be a reason that it is involved in early and later inflammatory processes.(20) No significant upregulation of Tnfα could possibly be explained by the nature of Tnfα. It is already significantly upregulated after 30 minutes in kidneys after IRI. Samples after 3,24 and 48 hours could therefore see no significant expression of Tnfα, because the levels have dropped off.(21)
Figure 6 column B shows the relative mRNA expression of the different genes in the control samples and the samples treated with SUL-138 100µM. The results show that there is some difference observed in expression between the control and SUL-138 samples, but not enough to make a significant difference. This could possibly be explained by the amount of samples used per time point. This was four for each time point. With more samples, the differences could become greater and show a significant difference. Also, there is one concentration used of SUL-138 (100µM). It could be that higher or lower concentrations of SUL-138 could have a bigger impact on the samples and show a significant difference. For Tnfα, it is observed that there is no significant up- and/or downregulation during incubation. So the expression stays relatively equal during incubation. This means that there is no big changes in expression, which also is the case in the SUL-138 samples. Due to this, the difference between the control and SUL-138 samples could stay fairly minimal.
Finally, the SUL-138 is expected to improve the health of the mitochondria in the cells of the PCKS's. The health of the mitochondria could have a little effect on the expression of these genes, because of the different pathways and the possible low influence of mitochondria on these pathways. So the mitochondria could be significantly 'healthier' in the SUL-138 samples in comparison with the control samples, but this is not indicated by the different genes tested.

Hypothesis genes (2)

HMOX1:
Heme oxygenase (HMOX1) is an inducible enzyme with potent anti-oxidant, anti-inflammatory, and anti-apoptotic attributes. It is a rapid and protective response due to AKI. It breaks down heme, which is a strong pro-oxidant molecule. It also mediates cytoprotection during AKI through several other pathways. It is shown that HMOX1 induction improves kidney function and survival, whereas chemical inhibition of HMOX1 leads to exacerbation of AKI. (22)
SFN:
Sulforaphane (SFN) is a naturally occurring isothiocyanate, which shows anti-inflammatory and antioxidative effects on cells . SFN activates Nrf2, which in turn upregulates detoxification enzymes which have antioxidant properties. ROS is produced during IRI and these enzymes could neutralize these ROS and play an protective roll in kidney injury. (23)
iNOS:
Nitric oxide synthase (iNOS) increases the expression of NOS proteins. This happens when kidneys experience IRI. NO that is produced by iNOS is expected to be toxic. NO production in the renal proximal tubules due to IRI is mediated by iNOS. (4)
NOX1:
NADPH oxidase 1 (NOX1) is one of the many sources of ROS in biological systems. Kidney damage could upregulate NOX1 and cause more production of ROS. This in turn, could lead to damage in kidney tissue. (24)

Expectation is that the genes described above all show increased expression in the incubation samples in comparison with the 0 hour samples. The incubation samples experience IRI over a longer period of time and should show this in the expression of genes that are related with IRI.
HMOX1 and SFN show protective properties in the kidney cell during AKI. The protective property of SUL-138 should show a higher expression of these genes in the SUL-138 samples in comparison with the control samples. iNOS and NOX1 upregulation indicates NO and ROS production, which are toxic components in the kidney. SUL-138 samples are expected to show a lower expression of these genes in comparison with the control samples. (4,22–24) Results are shown in figure 7.

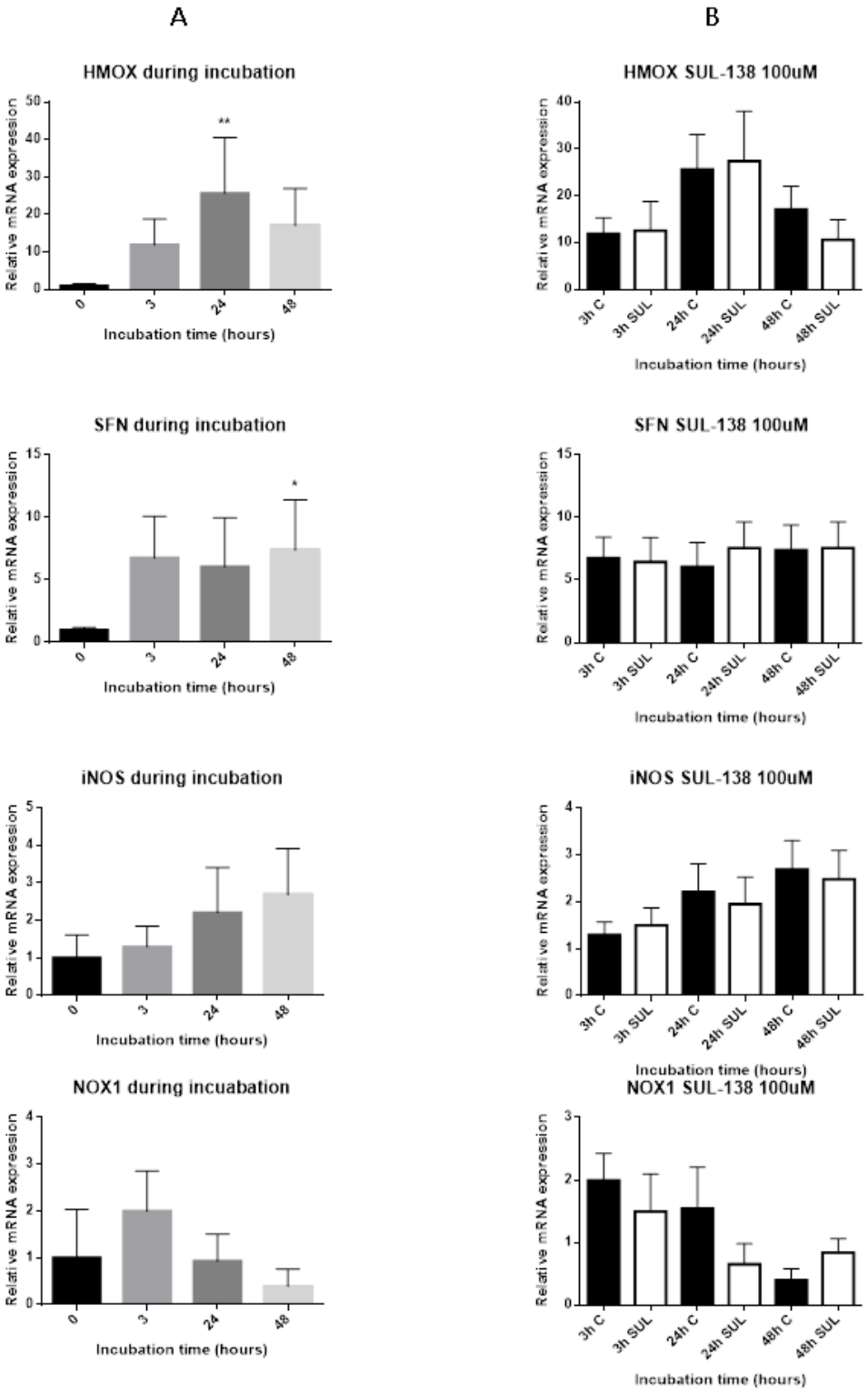

*Figure 7. Relative mRNA expression of HMOX, SFN, iNOS and NOX1 during incubation of PCKS's (A) and SUL-138 100µM samples (B). Gene expression was studied by qPCR. Relative expression was calculated using the household gene GAPDH (100%). Data are presented as the mean ± standard error of the mean of 4 independent experiments. Statistical analysis on the samples during incubation was performed via a Kruskal- Wallis test followed by the Dunn multiple comparison test, compared with 0 hours. *P,0.05. Statistical analysis on the SUL-138 100µM samples was performed via a Kruskal-Wallis test followed by the Dunn multiple comparison test, were the control samples of each time point were compared with the corresponding SUL-138 samples. *P,0,05*

Figure 7 column A shows the expression of HMOX1, SFN, iNOS and NOX1 in the PCKS's during incubation. After 24 hours, HMOX shows a significant upregulation in the samples compared to the 0 hour samples. The 3h and 48h samples show no significant upregulation. SFN shows a significant upregulation after 48 hours and no significant upregulation after 3h and 24h. iNOS and NOX1 show no significant upregulation during incubation compared to the 0h samples. HMOX and SFN are both significantly upregulated as was expected. The protective properties of these genes protect slices from damage due to IRI at different time points. iNOS is upregulated in every time sample, but not significantly. NOX1 is upregulated after 3 hours, not significantly, and even downregulated at 24h and 48h. Expectation was that these markers, due to IRI, would be significantly upregulated. iNOS shows an increasing expression after a longer period of time, so it could be that the slices can protect themselves against the damage for at least 48 hours, but that time points later than that could show significant increased expression of iNOS. NOX even shows downregulation after 24h and 48h. This could also prove that the slices are capable of combating occurred damage in the first 48 hours.(4,24)
Figure 7 column B shows the relative mRNA expression of the different genes in the control samples and the samples treated with SUL-138 100µM. There is no significant difference between the control samples and the corresponding SUL-138 samples for the different genes. HMOX and SFN show higher expression in the SUL-138 samples after 24 hours. The other samples show no difference in expression, except for 48h HMOX, were the expression is lower in the SUL-138 samples compared to the control samples. For iNOS, the expression is higher in the 3h SUL-138 samples compared to the 3h control samples, but lower in the 24h and 48h samples. NOX1 shows lower expression in the 3h and 24h SUL-138 samples in comparison with the control samples, but higher expression in the 48h sample. It appears that there is some protective effect of SUL-138 on the samples, but no significant effect and not at every time point. Possible explanations for this could be as described at the genes IL-6, IL-1β and Tnfα. More samples could be needed, other concentrations of SUL-138 and a possible small effect on the expression of the studied genes. Also, iNOS and NOX1 show no increase or decrease in expression during incubation. The genes stay relatively equal, which could have an effect on the difference in the SUL-138 and control samples.

Hypothesis genes (3)

SULF2:
Sulfatase 2 (SULF2) are highly charged proteins located on the cell surface or in the extracellular matrix. They can modulate the binding and release of signaling molecules. SULF2 is critically involved in the maintenance of the glomerular filtration barrier and therefore the functionality of the glomerulus. (25)
Bcl-2:
B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein that can block cytochrome C release and caspase activation. It resides in the mitochondria and overexpression can block both apoptosis and necrosis. (26)
EIF3C:
Eukaryotic Translation Initiation Factor 3 Subunit C (EIF3C) is a subunit of proteins that regulates global protein translation. Stress to the endoplasmic reticulum, which can be caused by IRI, has influence on EIF3C. It can induce increased translation of selected proteins. (27)

SULF2 and Bcl-2 gene expression is expected to decrease during incubation of the kidney slices compared to the 0 hour sample. Lower expression of these genes indicates damage to the kidney slices, which occurs due to IRI. EIF3C expression is expected to increase in the incubation samples compared to the control samples. Stress to the kidney cells should activate these genes.
For the SUL-138 samples, it is expected that the expression of SULF2 and Bcl-2 is higher in comparison with the control samples and expression of EIF3C is lower compared to the control samples. (25–27) Results are shown in figure 8.

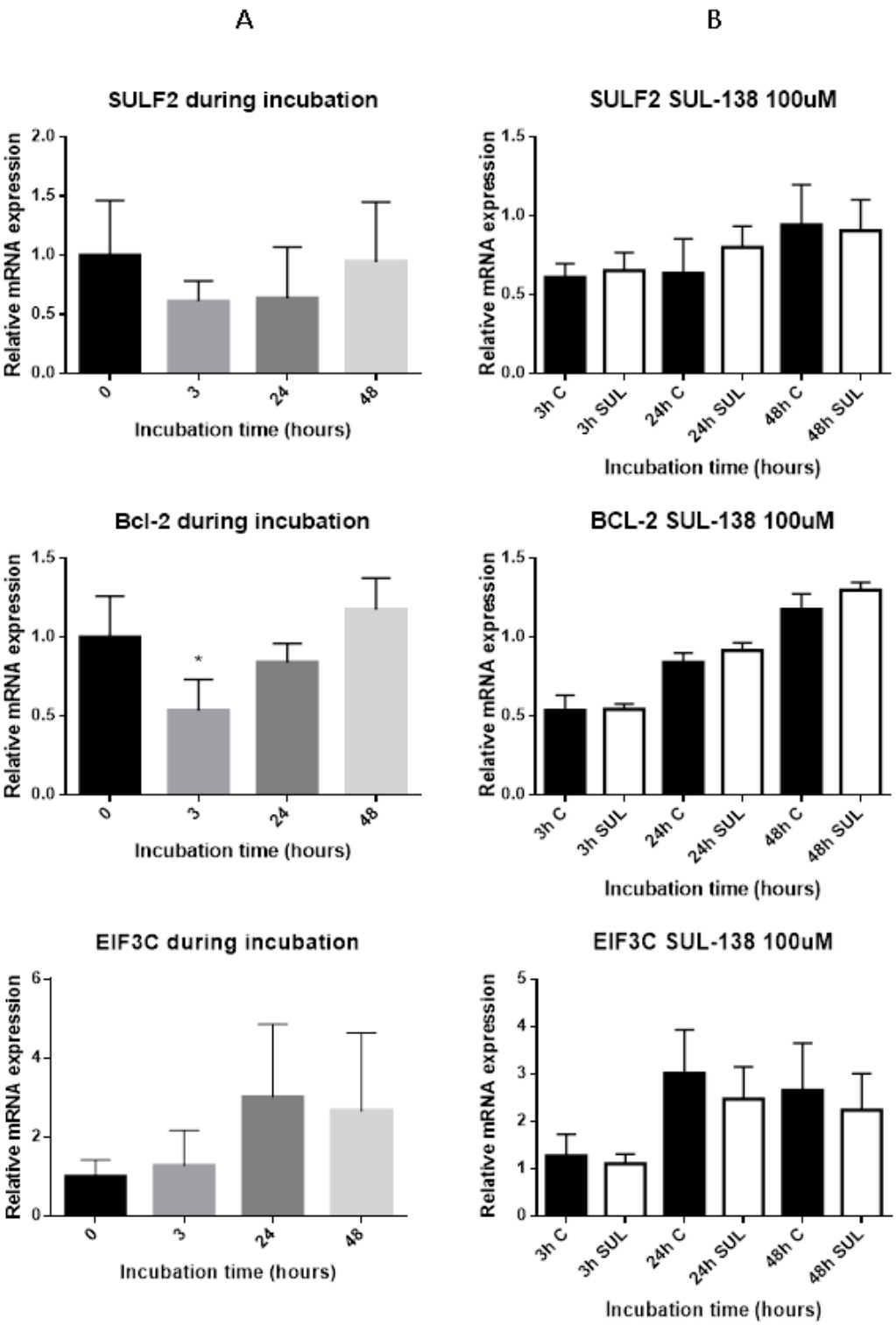

*Figure 8. Relative mRNA expression of SULF2, Bcl-2 and EIF3C during incubation of PCKS's (A) and SUL-138 100µM samples(B). Gene expression was studied by qPCR. Relative expression was calculated using the household gene GAPDH (100%). Data are presented as the mean ± standard error of the mean of 4 independent experiments. Statistical analysis on the samples during incubation was performed via a Kruskal- Wallis test followed by the Dunn multiple comparison test, compared with 0 hours. *P,0.05. Statistical analysis on the SUL-138 100µM samples was performed via a Kruskal-Wallis test followed by the Dunn multiple comparison test, were the control samples of each time point were compared with the corresponding SUL-138 samples. *P,0,05*

Figure 8 column A shows the expression of SULF2, Bcl-2 and EIF3C in the PCKS's during incubation. SULF2 and EIF3C expression shows no significant difference on expression between the different time points and the 0h time point during incubation. Bcl-2 shows a significant decrease in expression after 3 hours of incubation. This was expected. SULF2 was expected to also decrease in expression. This is observed, but is not significant. After 48 hour, the expression is higher in comparison with the 3h and 24h samples. This could indicate that the SULF2 expression due to IRI can be combated by the slices. EIF3C was expected to increase in expression. This is observed, with an increasing expression after a longer period of time. It could be that after the 48h sample, the expression significantly increases.(25,27)
Figure 8 column B shows the relative mRNA expression of the different genes in the control samples and the samples treated with SUL-138 100µM. EIF3C shows lower expression in every SUL-138 sample compared to the corresponding control sample. This is following expectations. It should be mentioned that the difference is not significant.
Compared to the control samples, SULF2 is higher in the 3h and 24h samples and Bcl-2 in the 24h and 48h samples. The 3h Bcl-2 sample shows equal expression and the 48h SULF2 sample shows lower expression compared to the control groups. These differences are not significant. SULF2 is also not significantly decreased during incubation, which could explain the small differences in the SUL-138 samples compared to the control samples. Bcl-2 shows higher expression in the 24h and 48h SUL-138 samples compared to the corresponding control samples, as was expected. The difference is here also not significant. Also here, the not significant differences between the SUL-138 samples and the control samples could be explained as stated earlier. More samples could be needed, other concentrations of SUL-138 and a possible small effect on the expression of the studied genes.

Hypothesis genes (4)

KIM-1:
Kidney injury molecule 1 (KIM-1) is markedly increased in kidney tissue after insults, but is virtually undetectable in healthy kidney tissue. It is qualified by the Food and Drug Administration and European Medicines Agency as a urinary biomarker for kidney damage. It is demonstrated that KIM-1 is an early diagnostic marker, which expression is highly sensitive and specific to kidney injury. KIM-1 levels are positively correlated with the degree of renal injury and are sensitive enough to detect kidney injury caused by 10-min ischemia. (28)
NGAL:
Neutrophil gelatinase – associated lipocalin (NGAL) is a biomarker for AKI, due to renal ischemia. NGAL is produced by injured tubular epithelial cells. It is considered as a marker of acute tubular cell injury. NGAL levels are elevated after 10-min ischemia in kidneys. (29)
Hif-1α:
Hypoxia-inducible factor 1 alpha (HIF-1α) can be activated in all mammalian cells and induce widespread changes in gene expression. Most of these genes are expected to increase the capacity of a cell or tissue when oxygen supply is reduced. This may improve the survival of kidney tissue in the case of IRI. (30)

The expectation is that for these genes the expression is upregulated during incubation in comparison with the 0 hour samples. Kim-1 and NGAL are kidney injury markers and due to IRI in the slices, these genes should be upregulated. HIF-1α is expected to be upregulated to improve cell survival of the kidney slices after IRI. The protective properties of SUL-138 should show in the expression of these samples in comparison with the control samples. The expectation is that the expression of Kim-1 and NGAL is higher in the control samples in comparison with the SUL-138 samples and that the expression of HIF-1α is higher in the SUL-138 samples in comparison with the control samples. (28–30) Results are shown in figure 9.

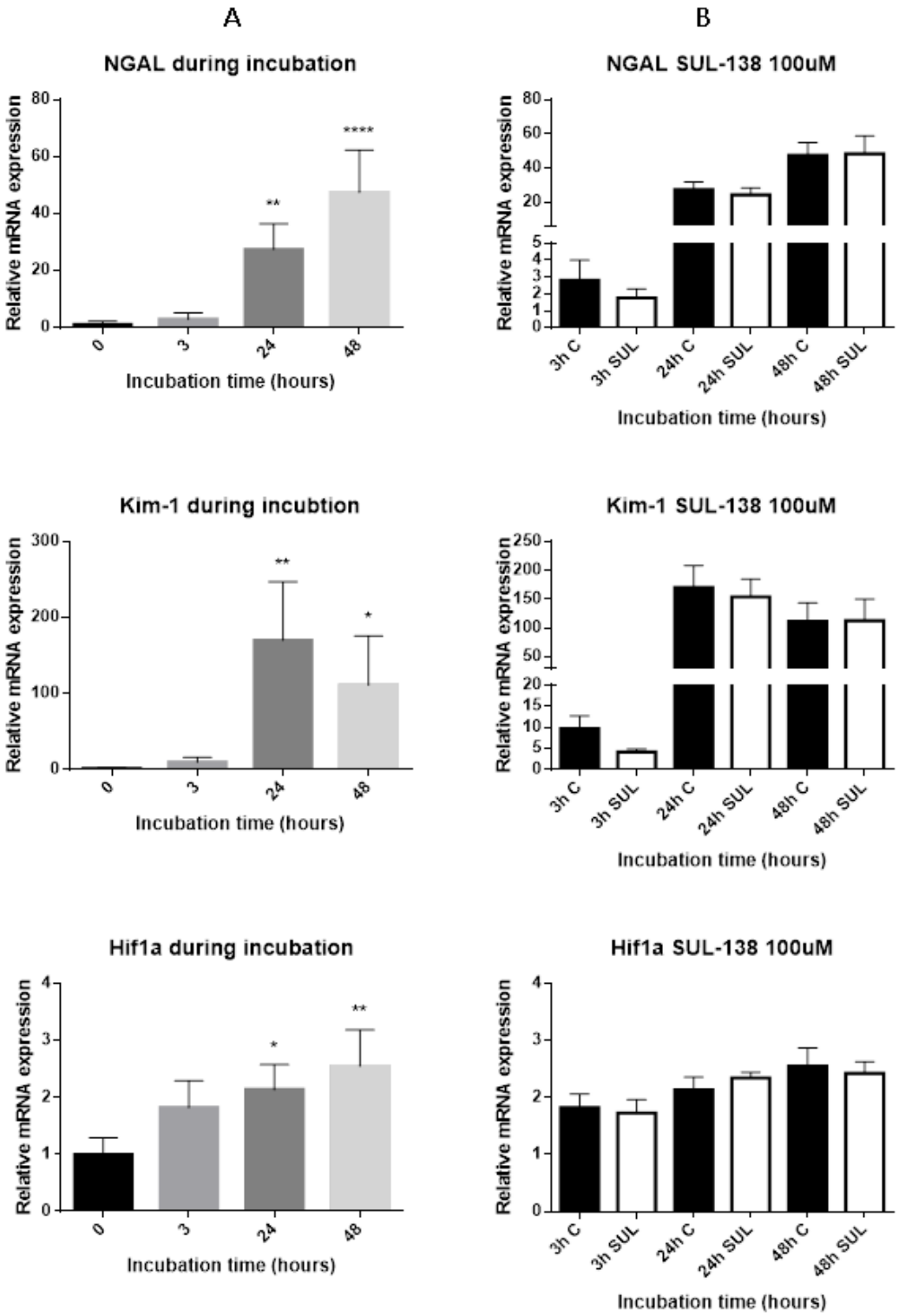

*Figure 9. Relative mRNA expression of NGAL, Kim-1 and Hif1a during incubation of PCKS's (A) and SUL-138 100µM samples (B). Gene expression was studied by qPCR. Relative expression was calculated using the household gene GAPDH (100%). Data are presented as the mean ± standard error of the mean of 4 independent experiments. Statistical analysis on the samples during incubation was performed via a Kruskal- Wallis test followed by the Dunn multiple comparison test, compared with 0 hours. *P,0.05. Statistical analysis on the SUL-138 100µM samples was performed via a Kruskal-Wallis test followed by the Dunn multiple comparison test, were the control samples of each time point were compared with the corresponding SUL-138 samples. *P,0,05*

Figure 9 column A shows the expression of KIM-1, NGAL and Hif-1α in the PCKS's during incubation. All three genes show no significant upregulation after three hours compared to the 0h samples and show a significant upregulation after 24h and 48h. This is following the expectations of the gene expression during incubation beforehand.
Figure 9 column B shows the relative mRNA expression of the different genes in the control samples and the samples treated with SUL-138 100µM. NGAL shows that the SUL-138 samples are lower expressed in comparison with the control samples at 3 hours and 24 hours. At 48 hours, the expression is a little bit higher, but all these compared values are not significant. This is also the case for the Kim-1 samples. Hif-1α shows lower expression in the 3 hour and 48 hour SUL-138 samples compared to the control group and higher expression in the 24 hour samples. These values are also not significant. There is some protective effect observed of SUL-138 in the samples, but not a significant effect. This could be explained as stated earlier. There are some positive differences in expression, but these could be maybe significant with more samples per condition. Which also could make a difference is other concentrations of SUL-138.

Morphology kidney slices

Slices from two mice kidneys (MK52 and MK53) were used for morphology. These samples were collected right after slicing, after 3 hours, after 24 hours and after 48 hours. Control slices were compared with slices incubated with SUL-138. The pictures show morphology of the kidney slices at different magnifications. Different markers were used to stain the samples and indicate the health of the samples.

Staining Bax

Figure 13 and 14 show staining of Bax in PCKS during culture. It also shows examples of a glomerulus, tubular and necrotic damage. Bax is stained red in the picture and the blue staining is hematoxylin, which stains nuclear and cytoplasmic structures. Bax is a pro apoptotic protein. Bax is induced and activated in ischemic kidney tissue. It shows apoptosis and necrotic tubular damage. A higher expression of Bax can also induce mitochondrial fragmentation.(31) High expression indicates therefore cell death and damage to the kidney cells. With the hypothesis that SUL-138 protects the kidney slices, expected was that Bax expression is higher in kidney cells that are not treated with SUL-138 in comparison with cells treated with SUL-138 and that over a longer incubation period, the expression increases.

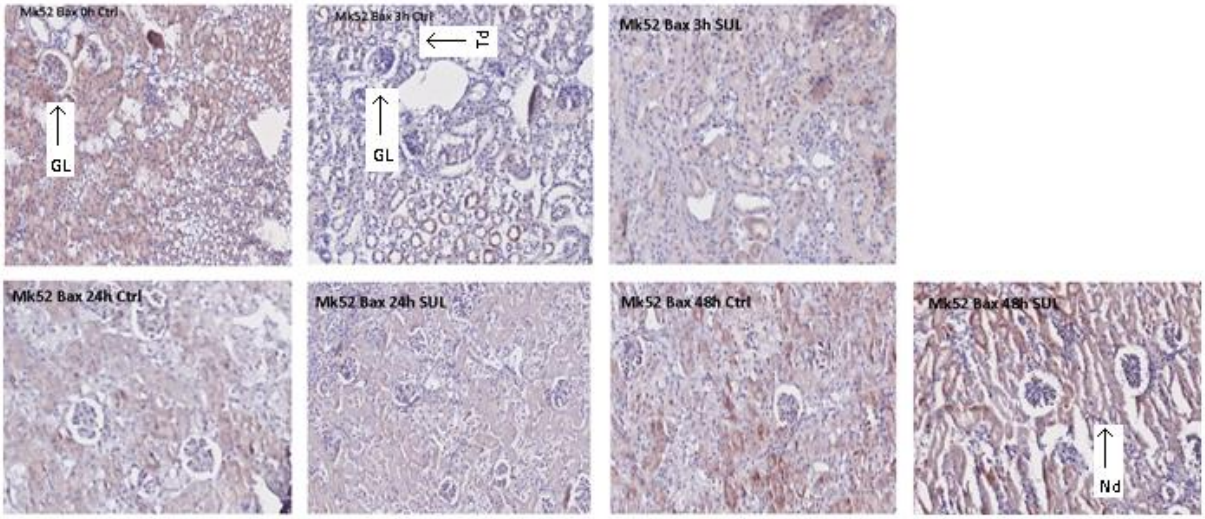

Figure 13. Bax (1:100, diluted in PBS) staining of PCKSs during culture, magnification 10X. Gl: glomerulus, Td: tubules damage and Nd: necrotic damage

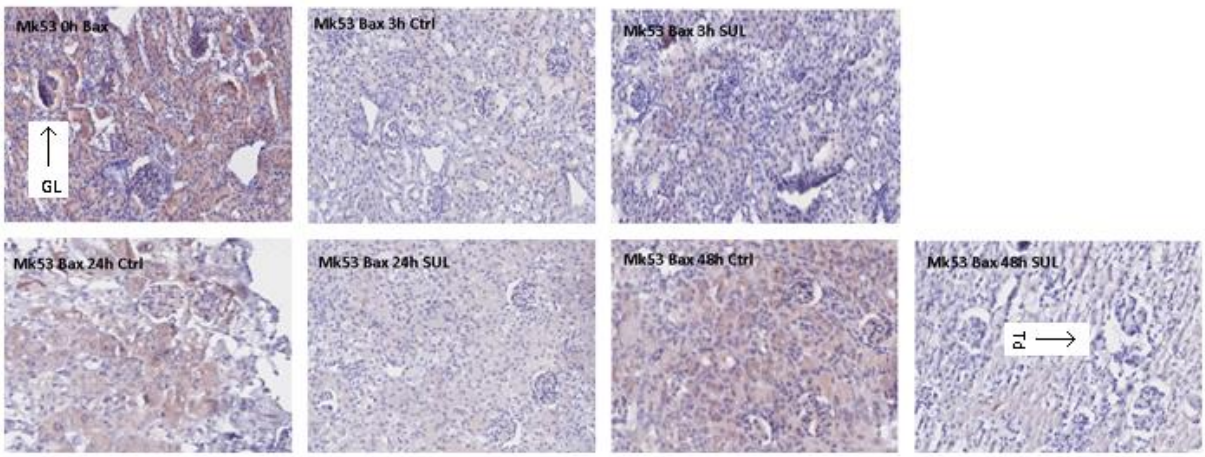

Figure 14. Bax (1:100, diluted in PBS) staining of PCKSs during culture, magnification 10X. Gl: glomerulus and Td: tubules damage

Both mouse kidney slices show strong expression of Bax at 0 hours. At 3 hours, the control and SUL samples of both mouse kidney slices show similar expression of Bax, which is relatively low. At 24 and 48 hours incubation, the MK52 samples show equal expression of Bax in the control and SUL sample. MK53 samples show more expression of Bax in the control sample compared to the SUL sample after 24 hours. This is also the case at 48 hours, but the expression of Bax is higher in comparison with the 24 hour samples.
Bax expression is already upregulated after a short period of ischemia in kidney tissue (around 20 minutes). Other stress factors to the kidney tissue could also cause increased expression of Bax. Reperfusion of the tissue could decrease apoptosis in the kidney tissue and therefore Bax expression. After 0 hours, Bax expression is observed and higher in comparison with the other time samples. The high expression after 0h could be explained by the ischemia injury experienced by the tissue after harvesting of the kidneys from the mice. Also, slicing of the tissue causes extra stress and could lead to apoptosis and necrosis of the tissue. This induces Bax expression. After reperfusion, it is possible that the kidney tissue is able to stop the apoptotic and necrotic process in the cells and thus decrease the expression of Bax. This could explain the lower expression of Bax in the other time samples compared with the 0h time sample.(31,32).
The other MK53 samples show the expected expression of Bax, with more expression after a longer period of time and more in the control samples compared to the SUL samples, with some tubuler damage in the SUL-138 tissue after 48 hour. The MK52 samples show even expression in Bax in both sample types and over the different periods. This can possibly be explained by the tissue used. This is another kidney than the mouse kidney 53, and this could mean that the strength of the tissue is weaker. It could be that the slicing and incubation process caused too much stress for the tissue and induced apoptosis in all tissues, which shows equal Bax expression. The glomeruli in the samples also show damage, observed by shrinkage and the tissue itself shows tubular damage after 3 hours. The tissue after 48 hours incubated with SUL-138 shows a lot of necrotic damage. This indicates that the tissue is damaged and has more damage than healthy tissue.

Staining Tom-20

Figure 15 and 16 show staining of Tom-20 in PCKS during culture. It also shows examples of a glomerulus, tubular and necrotic damage . Tom20 is stained red in the picture and the blue staining is hematoxylin. Tom-20 is a protein that is located on the outer membrane of the mitochondria(33). So this protein indicates mitochondria that are located in the cells. Expected is that slices treated with SUL-138 are healthier in comparison with control slices and due to this, show more mitochondria expression.

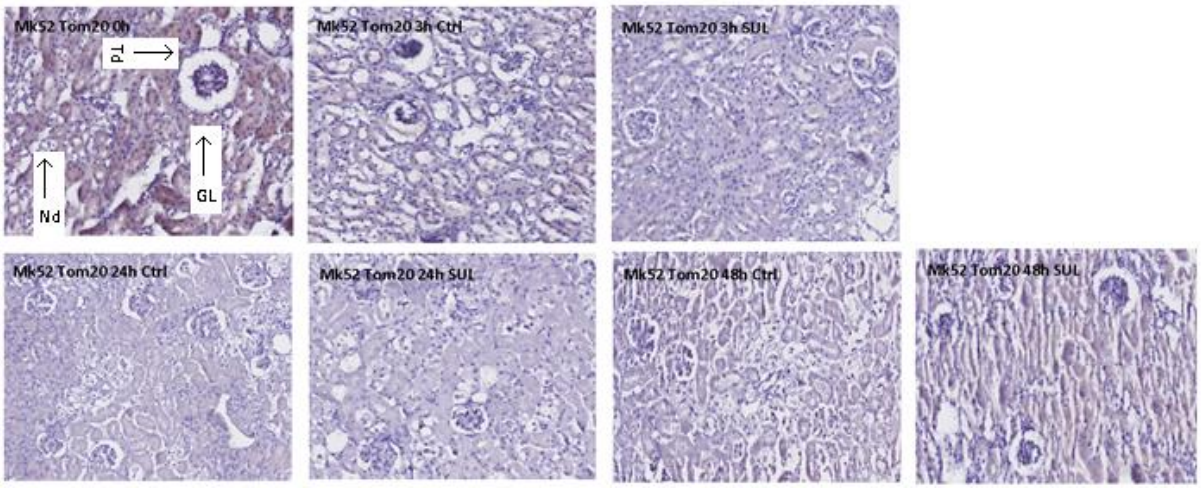

Figure 15. Tom20 (1:100, diluted in PBS) staining of PCKSs during culture, magnification 10X. Gl: glomerulus, Td: tubules damage and Nd: necrotic damage

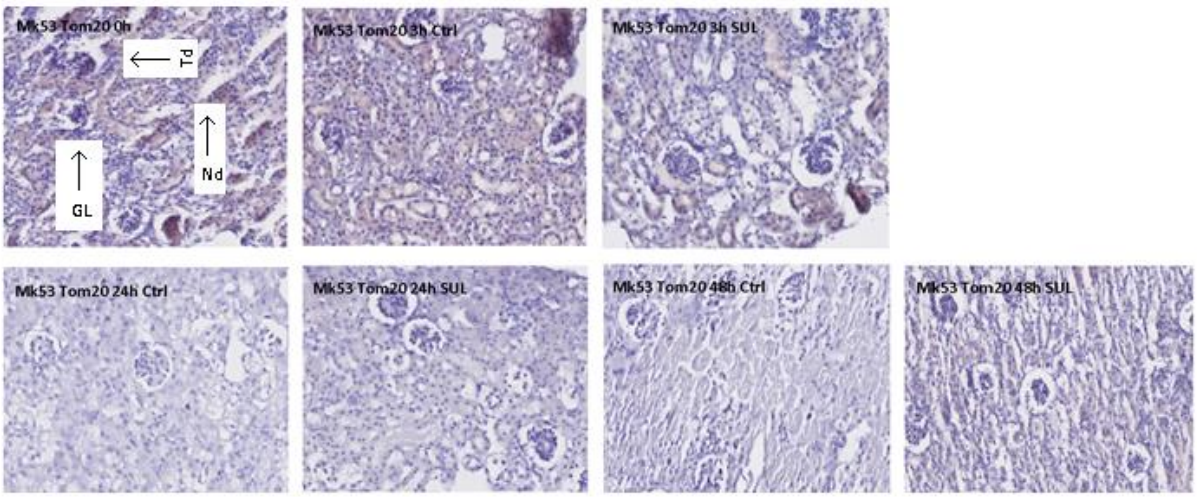

Figure 16. Tom20 (1:100, diluted in PBS) staining of PCKSs during culture, magnification 10X. Gl: glomerulus, Td: tubules damage and Nd: necrotic damage

The 0 hour samples from MK52 and 53 show high Tom20 staining, which indicates a lot of mitochondria in the slices. In both mouse kidneys, the Tom20 expression in both the control and the SUL samples is relatively equal, but less in comparison with the 0 hour. The 24 hour and 48 hour samples show more Tom20 expression in the SUL samples in comparison with the control samples, in both mouse kidneys. This was expected. The glomeruli in both mouse kidney shows damage at every time point. This is indicated by the shrinking of the glomeruli. Also, the tissue is necrotic. So there is tissue damage, which could be caused by the incubation process and the preparation of the staining samples. This damage could have an effect on the mitochondria and could cause a lower expression of mitochondria.

Staining Kim-1

Figure 17 and 18 show staining of Kim-1 in PCKS during culture. It also shows examples of a glomerulus, proximal tubule, necrotic and tubular damage. Kim-1 is stained red in the picture and the blue staining is hematoxylin. Kim-1 is a transmembrane tubular protein, that is undetectable in normal kidneys, but is markedly induced in kidney injury. It is mostly expressed in proximal tubular cells.(28) So expression of Kim-1 indicates kidney injury. Expected is that the expression of Kim-1 is higher in the control samples in comparison to the SUL samples, with increasing expression during the incubation.

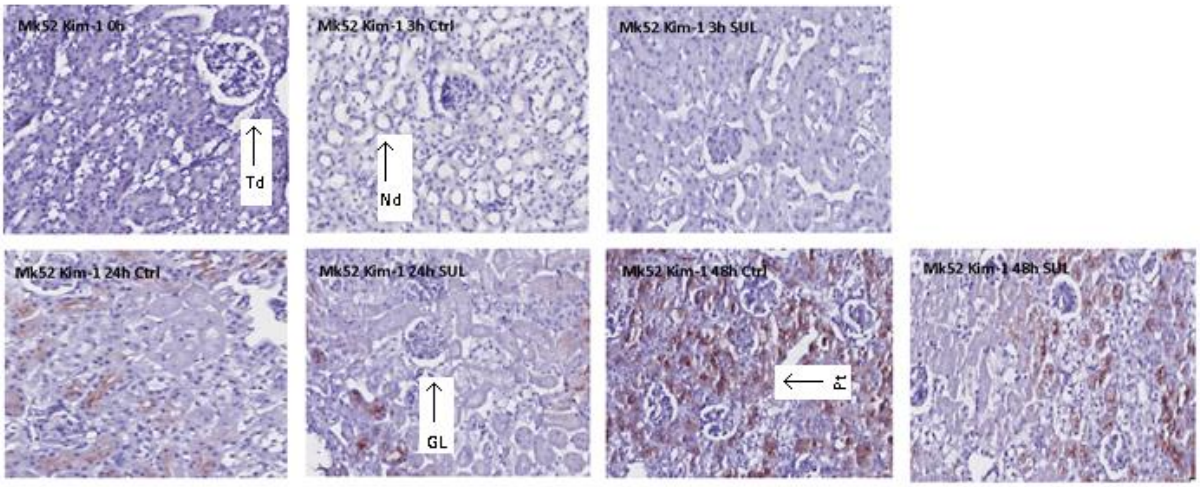

Figure 17. Kim-1 (1:200, diluted in PBS) staining of PCKSs during culture, magnification 10X. Gl: glomerulus, Td: tubules damage, Nd: necrotic damage and Pt:proximal tubules

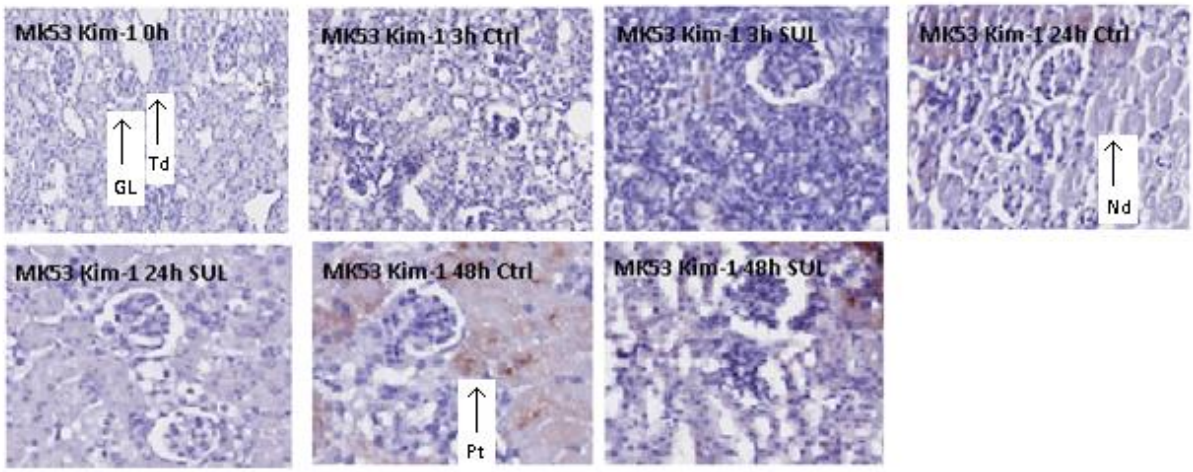

Figure 18. Kim-1 (1:200, diluted in PBS) staining of PCKSs during culture, magnification 10x. Gl: glomerulus, Td: tubules damage, Nd: necrotic damage and Pt:proximal tubules

The 0h samples show no expression of Kim-1. Both Mk 52 and 53 show no expression of Kim-1 after 3 hours in both samples. The 24 hour samples of both kidneys show slight expression of Kim-1 in the control and SUL samples, but compared between those two, there is more expression in the control samples. The expression is observed in the proximal tubules. The 48 hour samples of both kidneys show increased expression of Kim-1 compared to the 24 hour samples. Also, here the expression of Kim-1 is higher in the control samples compared to the SUL samples. This is in agreement with the expectations. There is some necrotic and tubules damage observed, probably due to the cutting and incubation process. This could lead to more expression of Kim-1

Western blot

Mitochondria isolation 1

Mitochondria were isolated from the slices of two mouse kidneys and one mouse liver. During the isolation there are multiple steps were supernatant and pellet are separated. The supernatant is used to continue the isolation and the pellet is normally discarded. For the western blot, the pellet from the last step before mitochondria isolation is used as a control sample. Western blot was used to check if the isolation of mitochondria succeeded and the other cell organelles remained in the control samples. This was done by using antibodies for mitochondria, cytosol and nuclei. Used antibodies to characterize the mitochondria in the samples are: Tom-40 (40 kDa), Tom-20 (20 kDa) and COX 4 (17 kDa)(33–35). To characterize the cytosol, Vinculin (117 kDa) was used(36). For the nucleus, HDAC1 (60 kDa) was used(37). Expectation was that the isolated mitochondria samples showed expression for Tom-20 and Tom-40, but not for HDAC1 and Vinculin. This would indicate that the isolation is pure and only the mitochondria were isolated from the slices. Therefore, expectation was also that the control samples showed no expression for Tom-20 and Tom-40, only for Vinculin and HDAC1. See table 1 for the configuration of the slots and see figures 10.1,10.2,10.3,10.4 and 10.5 for the results of the western blot. For this isolation, a plastic pestle which rotated automatically was used to homogenize the tissue.

Table 1. Configuration samples in slots on western blot isolation 1

Slot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Kidney mitochondria 1	Kidney mitochondria 2	Liver mitochondria	Marker	Control 1	Control 2	Control 3	-	-	-

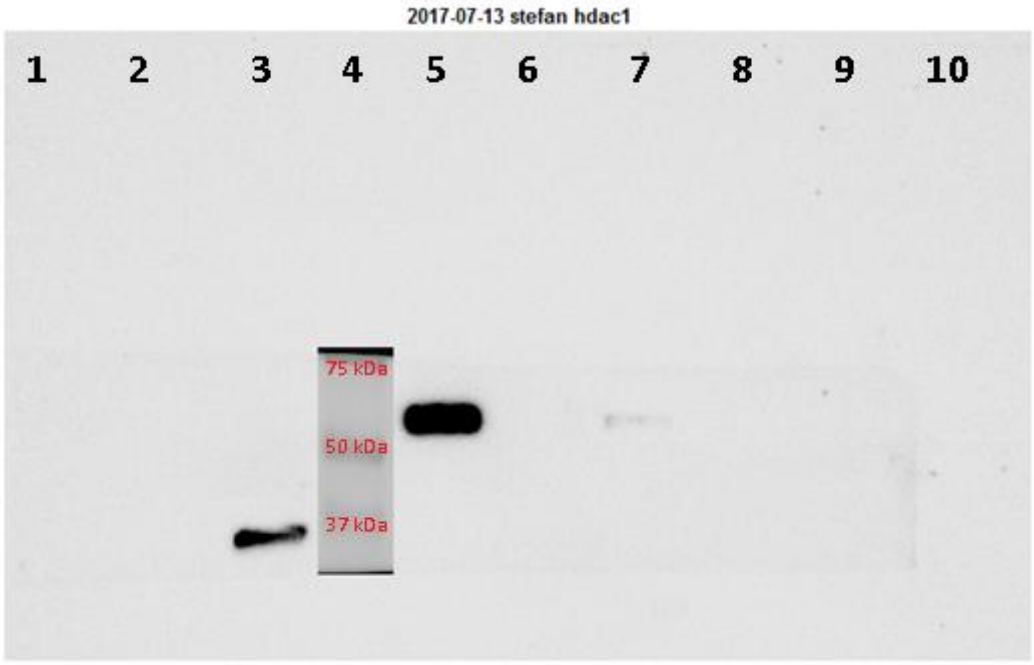

Figure 10.1. Western blot of samples of isolation 1 incubated with HDAC1

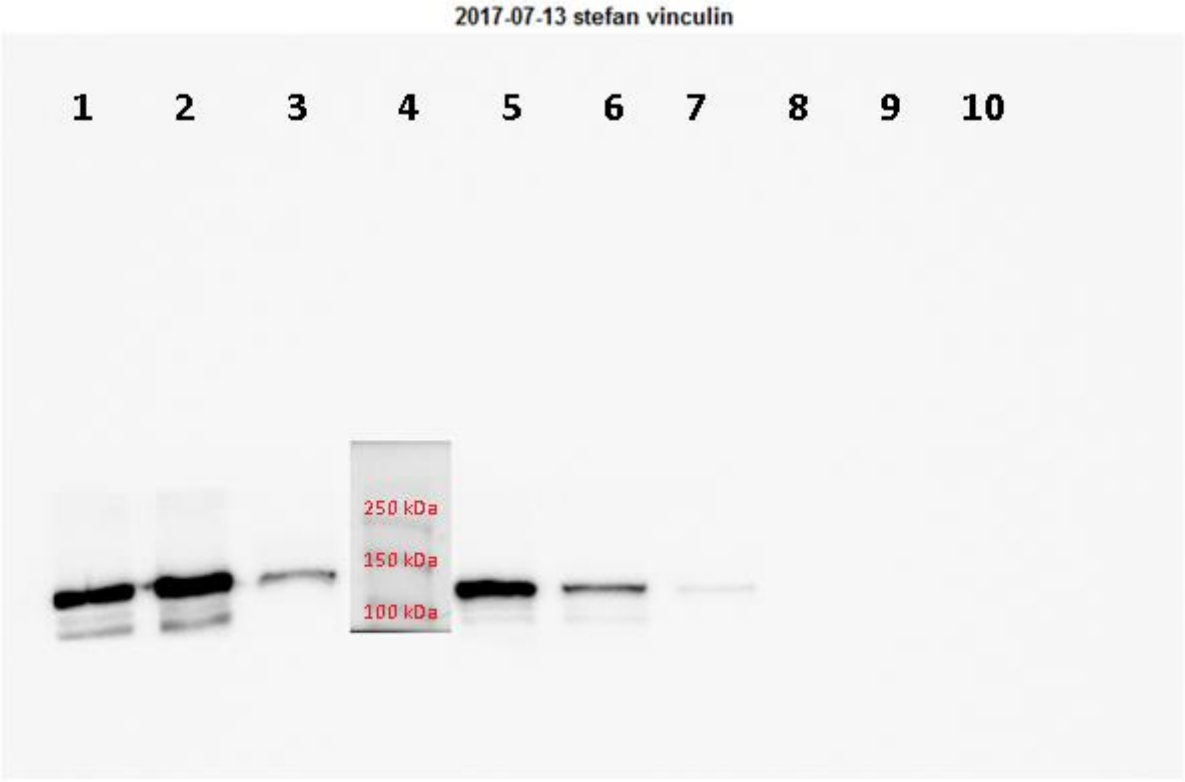

Figure 10.2. Western blot of samples incubated with Vinculin

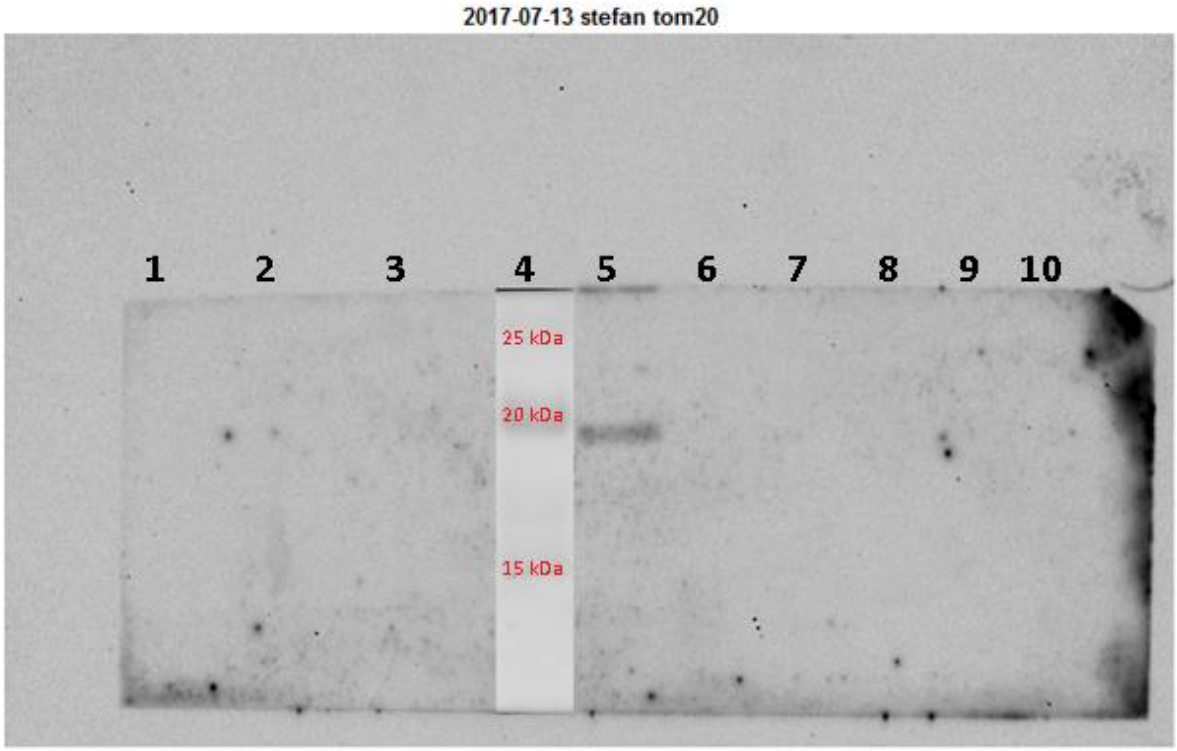

Figure 10.3. Western blot of samples incubated with Tom-20

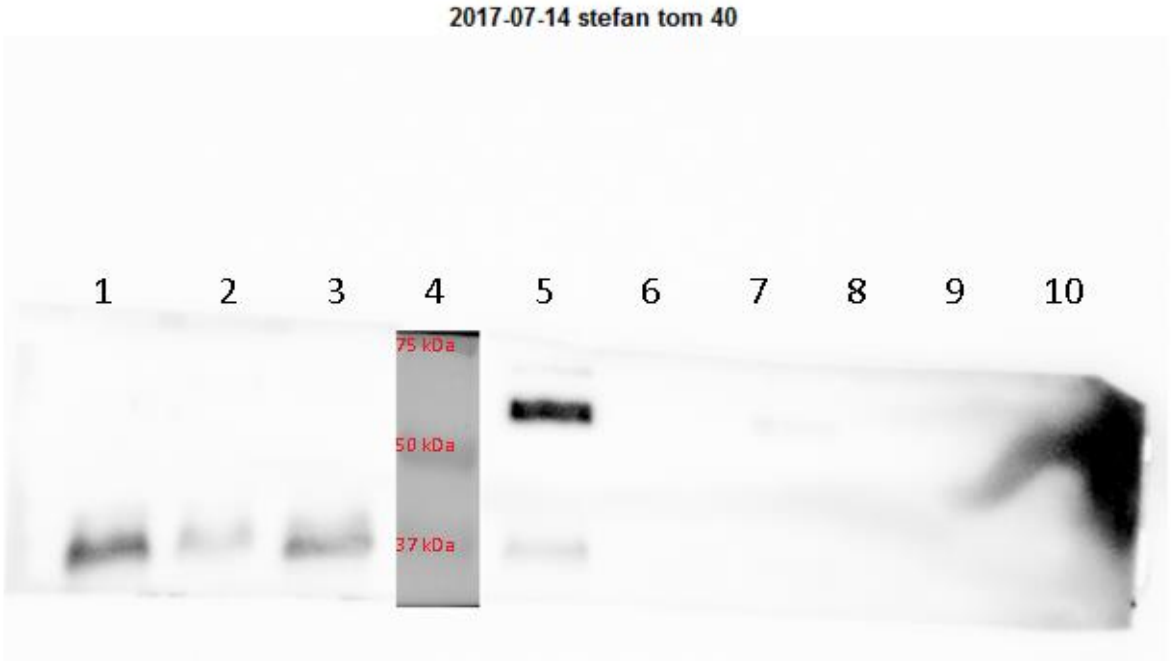

Figure 10.4. Western blot of samples incubated with Tom-40

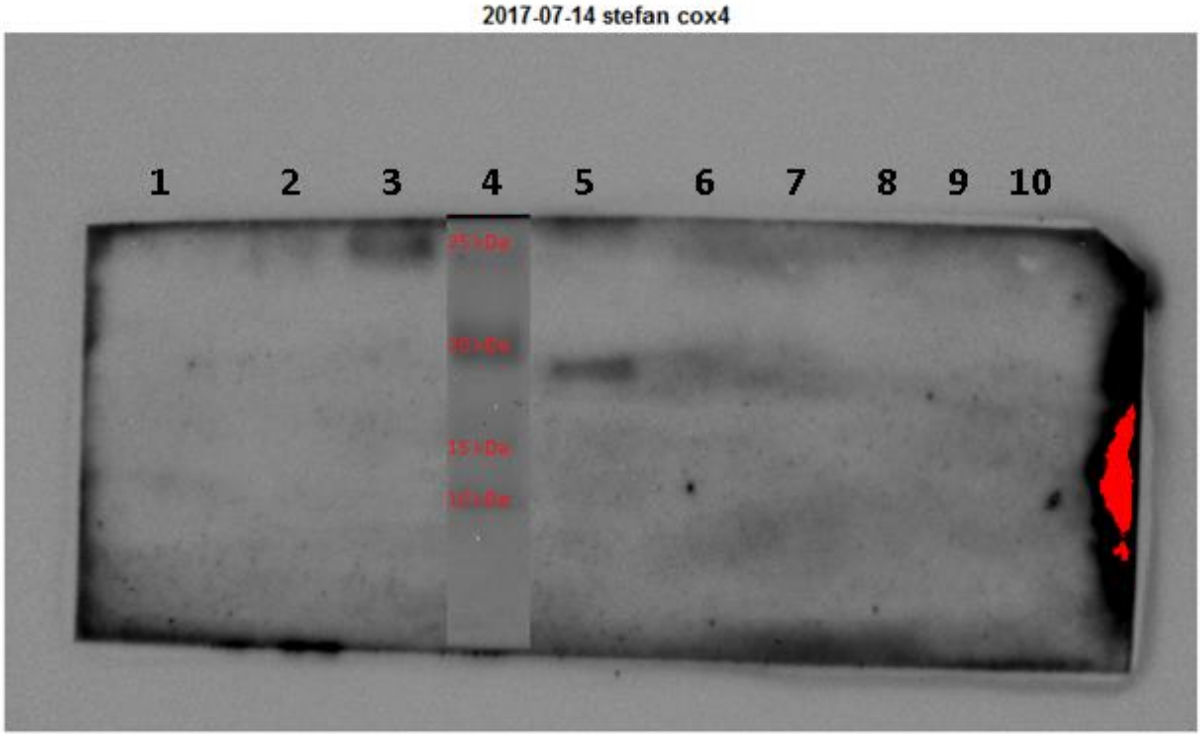

Figure 10.5. Western blot of samples incubated with COX4

HDAC1 has a molecular weight of 60 kDa and is mostly expressed in the nucleus of the kidney and liver cells. Expected was that the mitochondrial isolation samples contained no other cell organelles, such as a nucleus or cytosol. A band around 60 kDa indicates that the sample contains HDAC1 proteins. The clearer the band, the more HDAC1 protein is in the sample. The western blot shows that there are no bands for the mitochondrial samples at 60 kDa and this shows that there are no HDAC1 proteins in the isolated mitochondria samples, as expected. Also, the first control sample shows a thick band at 60 kDa, which indicates that there is HDAC1 protein in the control sample. The

other two control samples show no expression of HDAC1 proteins. A possible explanation for this difference could be that there are multiple steps in the isolation process. It could be that in some samples the HDAC1 proteins are removed before the control sample is extracted. This explains why there is no expression of the HDAC1 proteins in some control samples.(37)
Vinculin has a molecular weight of 117 kDA and is mostly expressed in the cytosol of kidney and liver cells. The expectation was that the isolated mitochondrial samples contained no expression of vinculin proteins. Results show that the first two mitochondrial isolation samples express vinculin proteins. The control samples also show expression of vinculin proteins. A possible explanation for the expression of vinculin proteins in the mitochondria samples could be that there are multiple steps in the extraction process, which increases the chances of not completely isolating the mitochondria. This could lead to bad separation of the mitochondria from the other cell organelles, in this case cytosol proteins. The third isolation sample shows no vinculin protein expression in the isolated mitochondria and shows a faint band in the control sample. The results of these samples was expected. It is possible that with this sample, the isolation steps were performed appropriately. This sample was also liver tissue instead of the other two samples, who were kidney tissue. Possibly there is a difference in extraction of mitochondria from kidney and liver tissue.(36)
Tom-40 (40 kDa), Tom-20 (20 kDa) and COX4 (17 kDa) are all proteins expressed in mitochondria. It was expected that with western blot, the mitochondrial isolation samples showed expression of these three proteins and no expression in the control samples. The results show that there is no expression of Tom-20 and COX4 in the mitochondrial samples. Control sample 1 shows a light expression of Tom-20. A possible explanation could be that the mitochondria were extracted earlier in the process. Also, the antibodies used to determine the expression of the proteins could be not specific enough. This is more likely, because Tom-40 protein is shown to be expressed in the mitochondria isolation samples. This indicates that there is mitochondrial tissues isolated in the samples.(33–35)

The three mitochondrial samples show no expression of hdac1 (nucleus) proteins, two samples show expression of vinculin (cytosol) proteins and all three samples show Tom-40 (mitochondrial) protein expression. Expected was that there was expression of all mitochondrial proteins in the isolated samples and no expression of cytosol and nucleus proteins. Difference in specificity of the antibodies could be an explanation as to why this is not the case. This is also a new isolation protocol for isolation of mitochondrial tissue out of kidney slices, which makes it prone to errors. There are a lot of extraction and centrifuge steps which could cause mistakes in the process. The samples should be kept at 4^0C at all time, which is difficult to do, because samples need to be kept on ice all the time. Also, the extraction should be done as quickly as possible. But before the slices are all extracted from the kidney, weighed, and transported to the centrifuge, there is a lot of time lost. Finally, there was kidney and liver tissue used in this extraction. The difference in tissue could explain the differences in expression of multiple proteins, because this physiologically is different in kidney tissue compared to liver tissue. These are all factors that could influence the extraction in general and are possibly improved by performing the isolation multiple times.

Mitochondria isolation 2

See table 2 for the configuration of the slots and see figures 11.1,11.2,11.3,11.4 and 11.5 for the results of the western blot in the second mitochondria isolation. For this isolation, a plastic pestle was used manually to homogenize the tissue.

Table 2. Configuration samples in slots on western blot isolation 2

Slot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Kidney mitochondria (KM) 1	KM 2	KM 3	KM 4	Marker	Control 1	Control 2	Control 3	Control 4	-

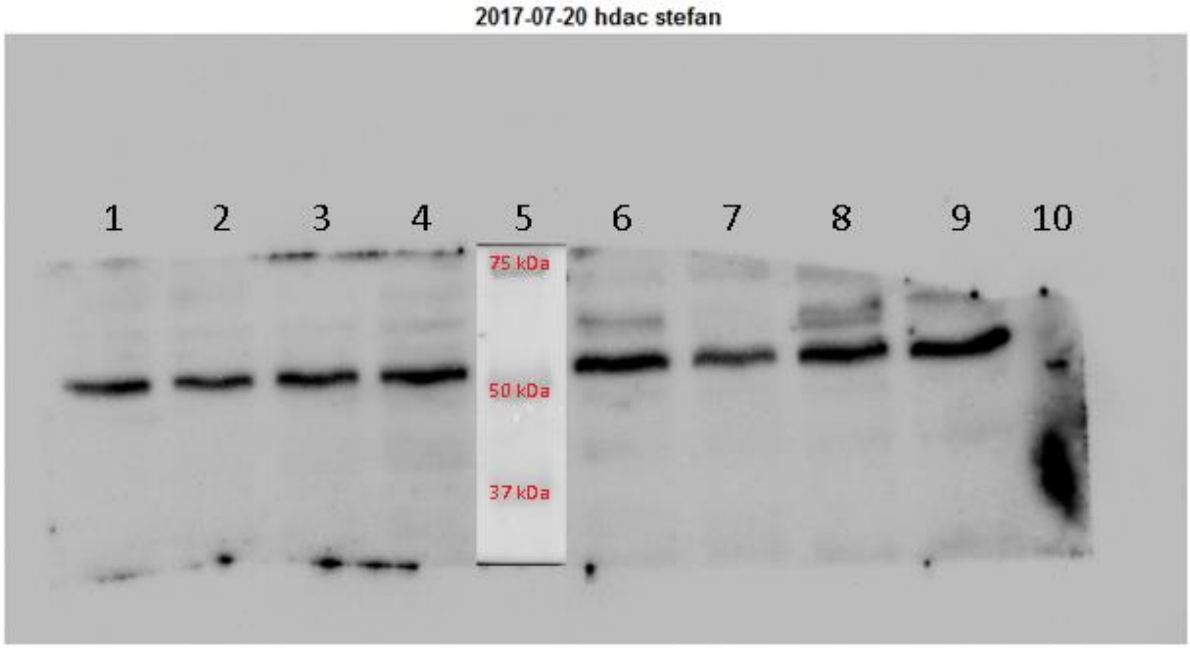

Figure 11.1. Western blot of samples incubated with HDAC1

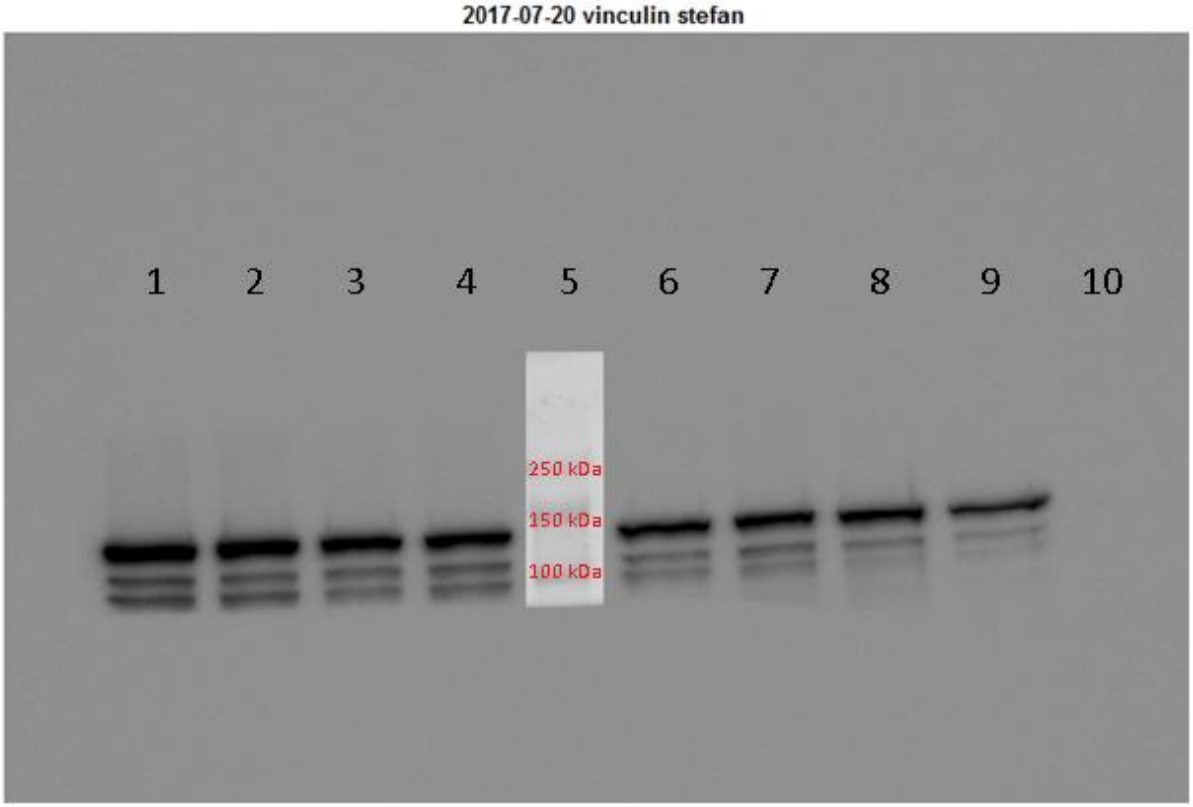

Figure 11.2. Western blot of samples incubated with Vinculin

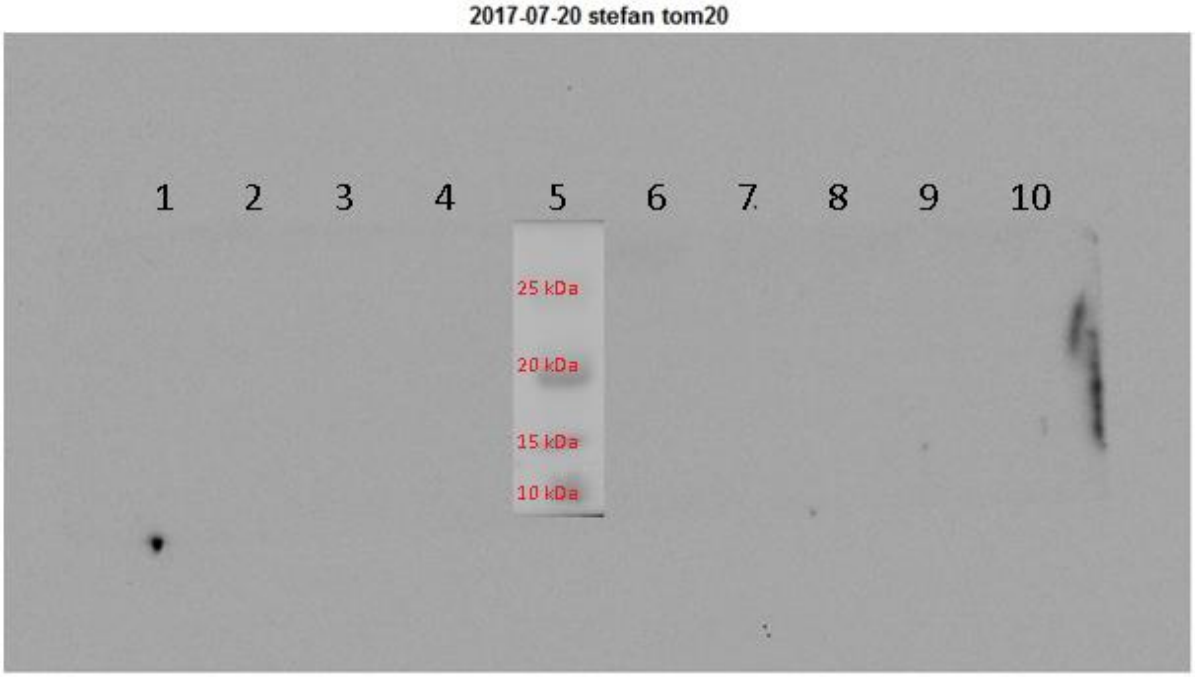

Figure 11.3. Western blot of samples incubated with Tom-20

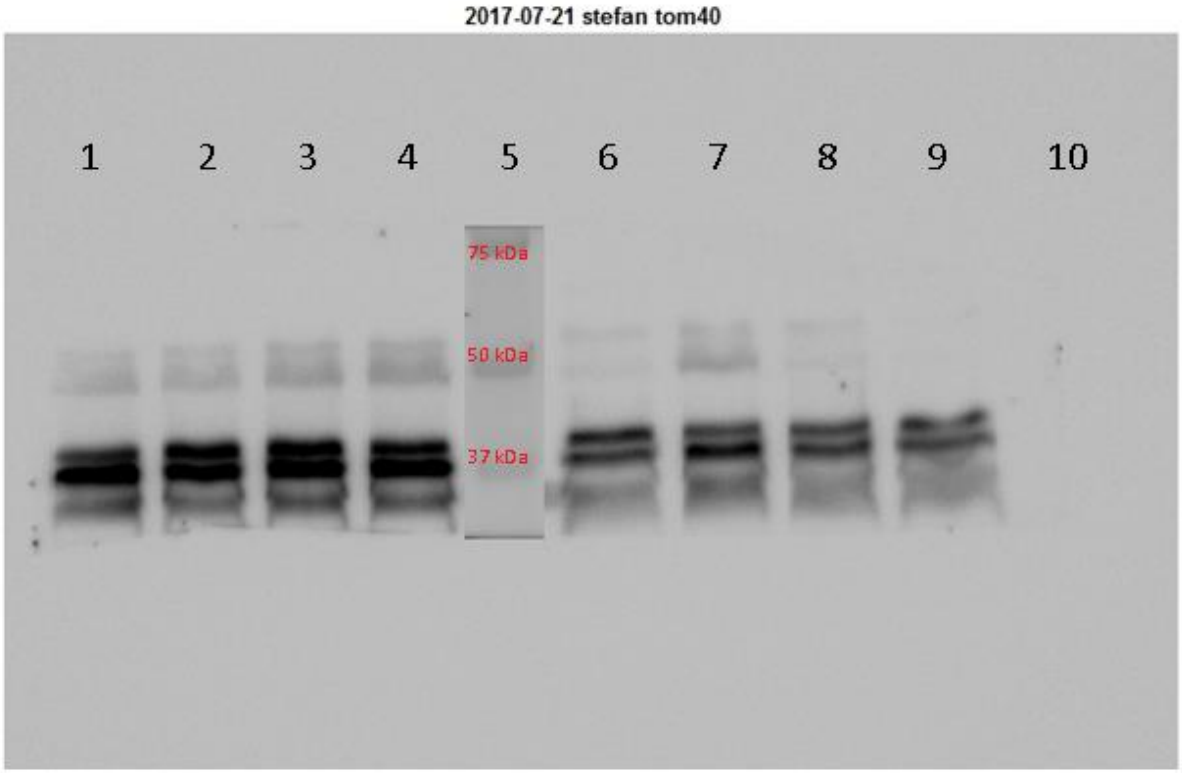

Figure 11.4. Western blot of samples incubated with Tom-40

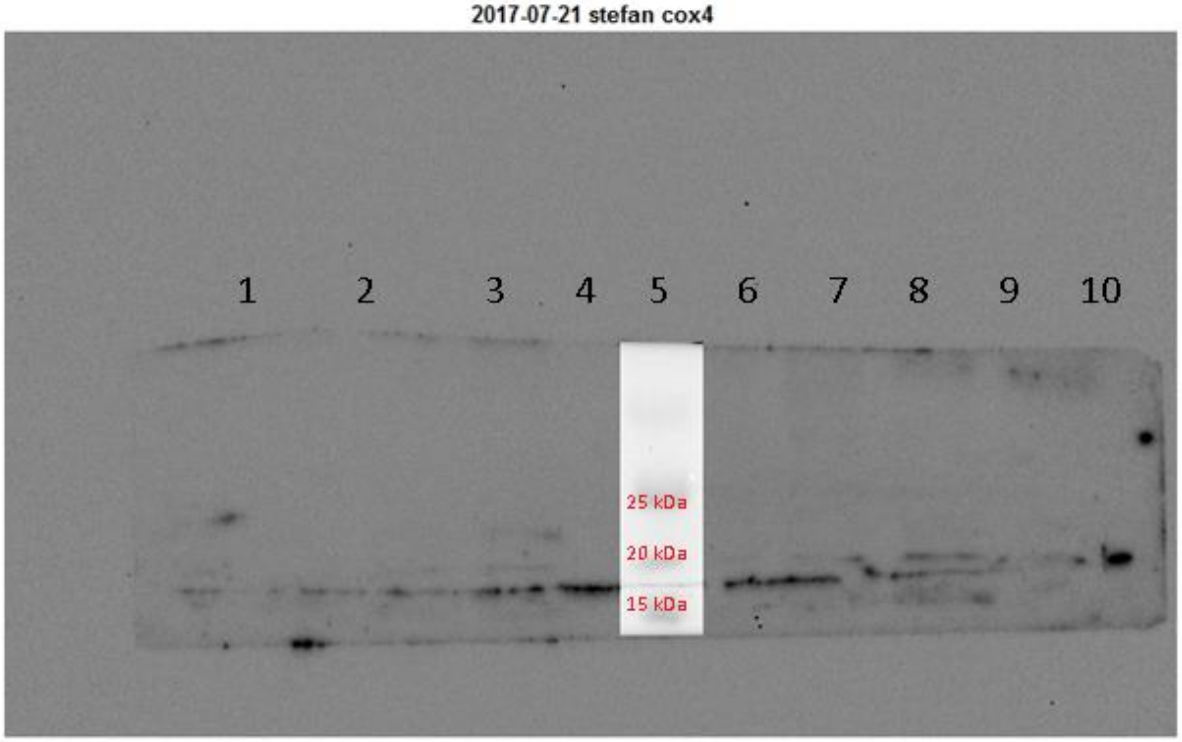

Figure 11.5. Western blot of samples incubated with COX4

HDAC1 protein (60 kDa) shows expression in all mitochondrial samples and all control samples. In comparison with the control samples, there is less expression in the mitochondrial isolation samples. Expected was no expression in the isolation samples. The control samples show that there is some extraction of the HDAC1 proteins, but not completely. An explanation for this could be that the extraction process did not work optimal. This could be due to errors in the isolation process, or that the samples were not at the right temperature (4^0C) for a longer period of time. This could lead to partial extraction of the mitochondria, but not complete.(37)
Vinculin protein (117 kDa) was expected to not be expressed in the mitochondrial samples. The results show that the protein is expressed in the mitochondrial isolation samples and in the control samples. Compared with the control samples, the expression in the mitochondrial samples is more. This shows that there is more Vinculin protein in the mitochondrial samples in comparison with the control samples. It shows that there is some extraction of Vinculin from the mitochondrial samples, but not all Vinculin proteins. A reason for this could be that the performed isolation did not work optimal.(36)
Tom-20 (20 kDa) is not expressed in isolation samples and control samples. Tom-40 (40 kDa) is strongly expressed in all samples and COX4 (17 kDa) is faintly expressed in all samples. Expected was to see these proteins only in the isolated mitochondria samples. Tom-20 protein shows no expression. This can be explained by a not specific enough antibody, as seen in isolation 1, but it could also be that these proteins were extracted in an earlier step of the process. Tom-40 shows expression in the mitochondrial samples and also in the control samples, but less. So the mitochondria are isolated, but the control samples show that not all mitochondrial tissue is isolated. COX4 shows very faint expression. This could also be due to a not so specific antibody, as seen in isolation 1, or extraction of the protein in an earlier step.(33–35)

The results of the western blot show that there is a big difference in antibody expression. Tom-40 showed that mitochondrial tissue was isolated, but COX4 and Tom-20 did not show this. So it difficult to say if the isolation worked, or that there is not enough of mitochondrial tissue to express all antibodies for the proteins. Also, the isolation process could be optimized by performing the isolation as quickly as possible after slicing.

Mitochondria isolation 3

See table 3 for the configuration of the slots and see figures 12.1,12.2,12.3 and 12.4 for the results of the western blot in the third mitochondria isolation. For this isolation, two positive controls were added. These were the samples collected in the first step in the process, after centrifuging. Expected was that the mitochondrial markers were not expressed in the positive controls, and the nucleus and cytosol markers would be expressed strongly. The other samples are as used before, control samples and the isolated mitochondria. Due to bad results in the first two isolations, COX4 was not used as a mitochondrial antibody. For this isolation, two glass pestles were used manually to homogenize the tissue.

Table 3. Configuration samples in slots on western blot isolation 3

Slot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Kidney mitochondria (KM) 1	KM 2	KM 3	Marker	Control 1	Control 2	Control 3	positive control 1	positive control 2	-

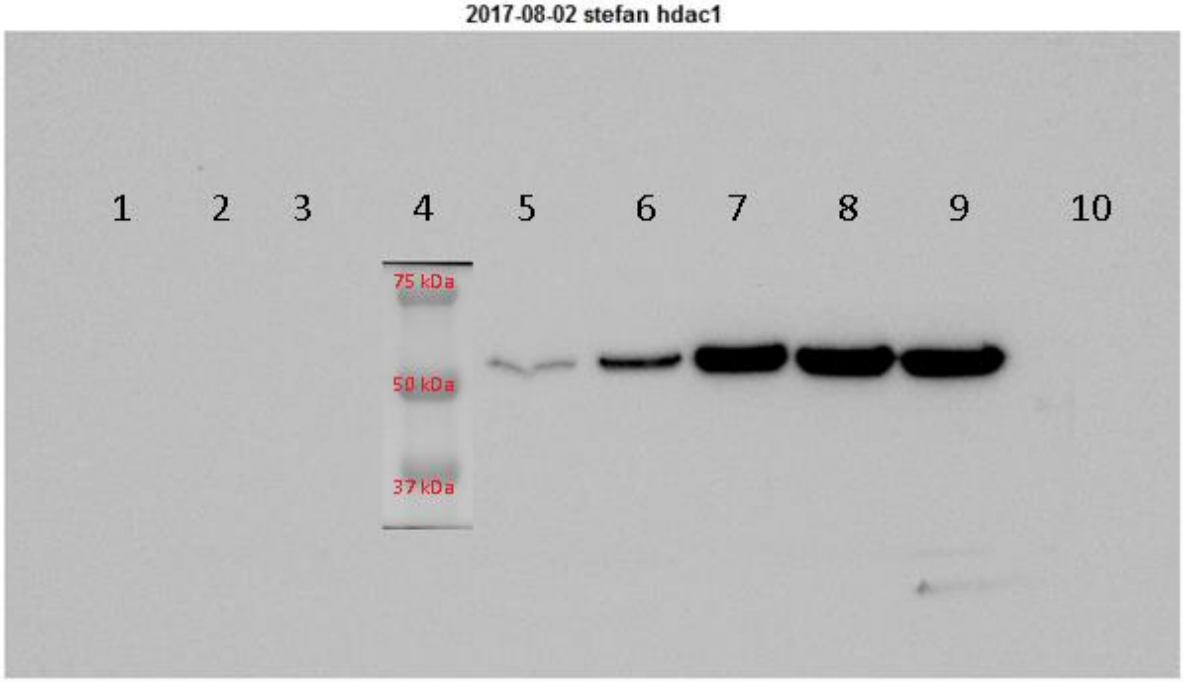

Figure 12.1. Western blot of samples incubated with HDAC1

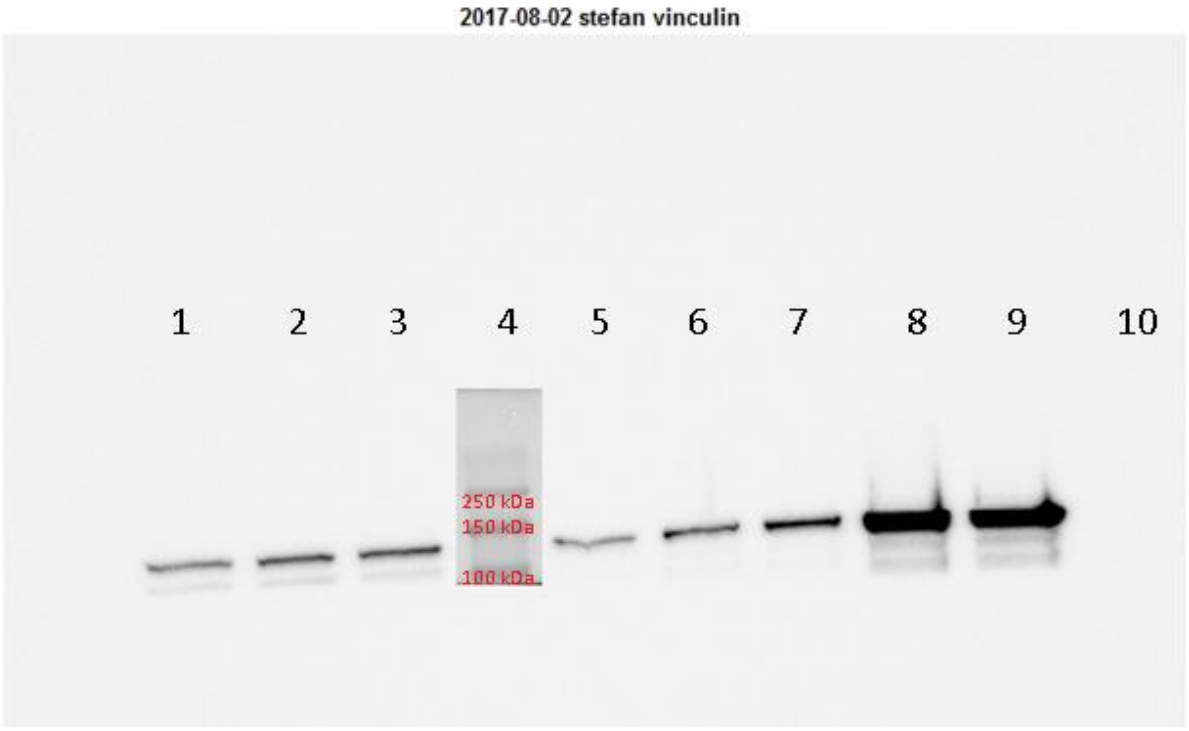

Figure 12.2. Western blot of samples incubated with Vinculin

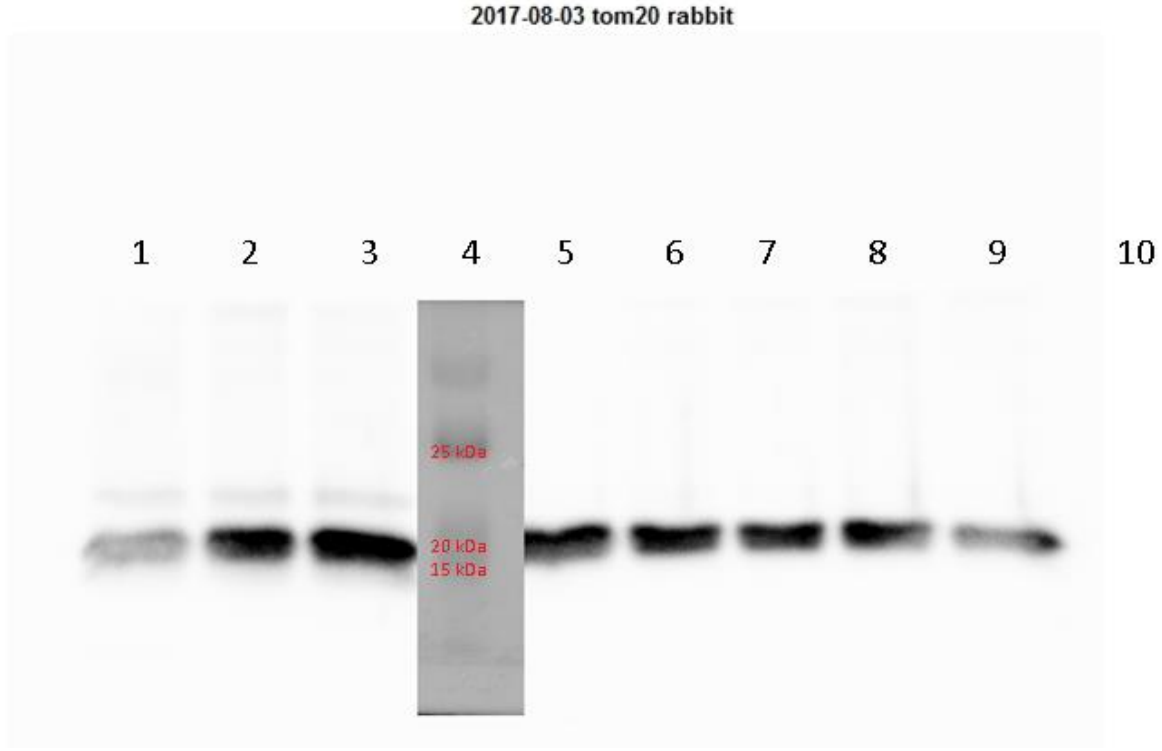

Figure 12.3. Western blot of samples incubated with Tom-20

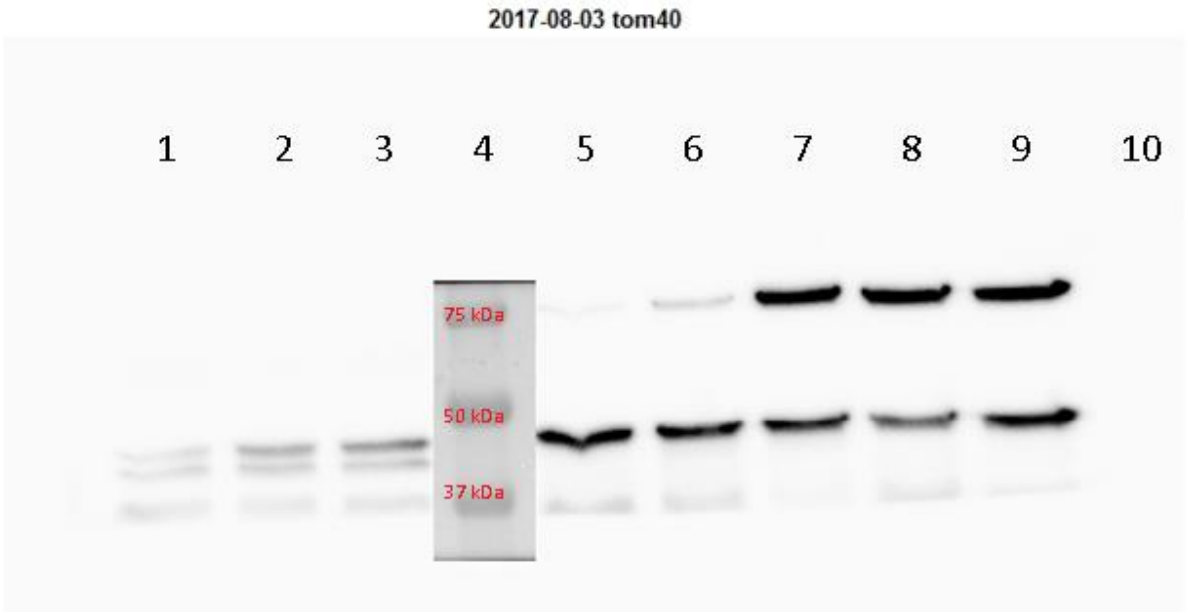

Figure 12.4. Western blot of samples incubated with Tom-40

HDAC1 (60 kDa) western blot shows no expression of the HDAC1 protein in the isolated mitochondria samples, expression in the control samples and stronger expression in the positive controls. This is as expected. Based on these results, the isolated mitochondria contain no nucleus proteins from the kidney cells, because these are all extracted in the earlier steps of the isolation.(37)
Vinculin (117 kDa) shows a strong expression in the positive control samples and a faint expression in the mitochondrial and control samples. Expected was that there would be no expression in the isolated samples. This is not the case, but a lot less in comparison with the positive control samples. This shows that vinculin (cytosol protein) is being extracted from the samples, mostly in the first step. A possible explanation for the protein in the mitochondria samples could be that the first extraction is not optimal. This could cause for some vinculin protein to stay in the samples and not be extracted in the later steps.(36)
Tom-40 and Tom-20 show expression in the isolated mitochondria samples, but also in the positive controls and the other controls. The expression of Tom-20 in the positive controls is lower in comparison with the other controls and the mitochondrial samples. This indicates that some mitochondrial tissue is isolated, but not all tissue. Tom-40 also shows faint expression in mitochondrial isolated samples, but more in the (positive) control samples. So there is some mitochondrial tissue isolated, but there is also mitochondrial tissue that is not obtained due to the isolation process.(33,34)

In general, the isolation shows that it is possible to isolate mitochondrial tissue, which is shown with antibodies specific for mitochondrial tissue. It also shows that other cell tissue, nucleus and cytosol can be separated during the isolation process. This is, however, not optimal. In some samples, the mitochondrial tissue is not completely isolated from the other organelles of the cell. Positive controls show that the isolation steps are working, but not fully. To improve this, the isolation should be done as quickly as possible. Also, all the samples should be treated the same. This includes pipetting steps and keeping the right temperature. Maybe the protocol could be altered (centrifuging steps, buffers) to improve the isolation. Finally, possible improvements of the antibodies could be tried. This can be done by other providers of the same antibodies, or using other antibodies for the same markers.

Conclusions

The goal of this project was to use PCKS's as a model for IRI and to combat this injury by adding the mitochondria protective compound SUL-138 during incubation of the slices. Furthermore, the goal was to set up a protocol for the isolation of mitochondria from the PCKS's.
ATP measurements show that slices incubated with SUL-138 100µM and 500µM are viable for at least 48 hours. There is no significant difference between the control and SUL-138 samples and their accompanying time points. For future experiments, the concentration of SUL-138 could be altered to obtain the concentration that gives the optimal viability of the PCKS's and possibly significant differences between control and SUL-138 groups.

Relative mRNA expression of Il-6, Il-1β, HMOX, SFN, NGAL, Kim-1 and Hif1a is significantly increased during incubation of the PCKS's for at least 48 hours. Relative mRNA expression of Bcl-2 during incubation is significantly decreased and expression of Tnfα, iNOS, NOX1, SULF2 and EIF3C shows no significant increase or decrease during incubation for at least 48 hours. When comparing this relative mRNA expression of the genes at the different time points (3h, 24h and 48h) with the corresponding SUL-138 incubated samples, there are some differences in expression detected. These differences are nevertheless not significant. For future experiments, more samples could be tested to make the differences significant. It could also be tried with other concentrations of SUL-138, possibly the optimal concentration as mentioned earlier in ATP research.

Control PCKS's and slices treated with SUL-138 from two mice kidneys were stained with Bax, Tom-20 and Kim-1. After 48 hours, Bax shows more expression in the control samples compared to the SUL-138 samples, which indicates protective effects of SUL-138. There is also some necrotic and tubular damage observed in the SUL-138 samples after 48 hours and not in the control samples, which indicates damage to the tissue due to SUL-138. Tom-20 expression is higher in the 24h and 48h SUL-138 samples in comparison with the control samples, which indicates protective effects of SUL-138. All these samples show some glomeruli damage and necrotic damage. Kim-1 is more expressed in the 24h and 48h control samples in comparison with the SUL-138 samples, which also shows the protective properties of SUL-138. These samples also show some necrotic damage and glomeruli damage.

Staining and qPCR results show contradicting conclusions. Expression of mRNA showed no significant protective effects of SUL-138, while protein expression showed these protective properties. It could be that the mitochondrial health has little to no influence on the mRNA expression of the tested genes, while it increases expression of the proteins that were stained. Even if mRNA expression is significantly increased, this does not correlate directly with protein expression. So even then, there could be differences in mRNA expression and protein expression in tested tissues. Also, it could be that the incubation of the slices is done in concentration of SUL-138 which is too high or too low. This could affect the differences in mRNA and protein expression. SUL-138 was added every 24 hours, which maybe is not enough to influence the expression. Finally, the incubation time of max 48 hours could influence the expression on protein levels, but on gene expression it could be that there is a longer period of time needed to see these effects happening. So for future experiments, it could be interesting to look at these factors and see if there is less difference between protein expression and mRNA expression relating to the protective effects of SUL-138 in kidney slices.

A protocol to isolate mitochondria from PCKS's was set up and tested. Purity was tested using western blot and the markers Vinculin (117 kDa) for the cytosol, HDAC1 (60 kDa) for the nucleus and Tom-40 (40 kDa), Tom-20 (20 kDa) and COX4 (17 kDa) for the mitochondria were used to characterize these different cell organelles. (Positive) control samples showed that it was possible to isolate mitochondrial tissue from the PCKS's. However, this was not optimal. It showed that, sometimes, not all mitochondrial tissue was isolated or/and contaminated. Improvements could be

applied to optimize the isolation. Time between harvesting of the slices and start of the protocol could be diminished by performing it right after slicing. Each sample should be treated exactly the same. Centrifuging steps and buffers could be altered to get better results. Finally, antibodies used for western blot could be changed to check for more components and better indicate the purity of the isolation.

PCKS's incubated with SUL-138 100µM and 500µM showed to be viable, but not significantly better in comparison with control groups. Different genes showed that there is IRI in the PCKS's during incubation. Incubation with SUL-138 100µM did not show an significantly improved protection in comparison with control groups. Morphology markers showed that SUL-138 100µM had a protective effect on the slices in comparison with control samples. Finally, mitochondria were shown to be (partially) isolated from the PCKS's following the protocol.

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Appendix A.

Title	Slicing and incubation Mouse kidney slice protocol
Lit. Ref	
Lab. Ref	
Date	25/3/2015
Goal	Preparing, slicing and incubating kidney slice
Materials	• **Krumdieck slicer** • **Incubator cabinet with heater and carbogen supply** • **1 ml safelock tubes** • **12 wells plates**
Solutions	• **William E + Glutamax medium** • **Liquid glucose (3ml/500ml Williams E medium)** • **Ciprofloxacin (2.5 ml/500 ml Williams E medium)** • **10x Krebs solution** • **UW solution**

Protocol

1. Turn on the cooler for the Krumdieck slicer and connect the cooler tubes to the slicer. Turn on the flow cabinet and the incubation cabinet.

2. **Preparation Krebs solution:** (for 3 litres) 300 ml 10X KREBS solution + 2.7 L cold ultra pure water + 6.3 g NAHCO3 + 14.85 g Glucose + 7.14 g HEPES. After saturation with carbogen for 20 minutes, adjust pH to 7.4

3. **Preparation culture medium**: 1.3 ml medium (WilliamE+Glucose+cipro) per well. *Note: Medium must be pre-oxygenized in plates at least 30 minutes before incubation with slice*.

4. Pick up the kidneys from OR and store in cold UW.

5. Clean the kidney from excess tissue (fat!) surrounding the kidney with the surgical knife.

6. Put the kidney into the core holder of Krumdieck slicer, and start making slices. The thickness of the slice is measured by weight (between 4 and 5mg/slice).

7. Collect slices and transfer them from KREBS to UW a.s.a.p. Put 1 slice/well for incubation. Use the thermal pad to keep the plate warm. (For SUL-138 incubation, add the SUL stock at this step in each well in the right concentration.

8. After incubation, slices will be collected for ATP (1 slice/1ml SONOP/tube), RNA (3 slices/tube), western blot (3 slices/tube) and histology (3 slices/4%Formalin/well). ATP, WB and RNA samples must be snapfrozen in liquid Nitrogen.

9. **Cleaning the slicer**: Separate the parts of the slicer, put all parts in soap sloution and leave for 5 minutes, wash carefully and transfer to tap water box. Rinse with ethanol and finally with demi water and let them dry out.

10. **Cleaning plates/plastics**: Clean plastic waste can be recycled. Rinse with tap water and dry before recycling (green box).

Remarks	**Always keep tissue material cold and wet**

Example plate layout

Date					**Date**	
Code				0h:		
Experiment	ATP, RNA			3h:		
Store	Fridge -80			24h		
Tissue	Mouse			48h		
Description	PCKS are incubated with SUL-138					
Remark						
			M1	SUL 138 100uM	Add 1.3ul of 100mM stock solution to 1,3 ml medium	
			M2	CTRL	1,3 ml medium with 1,3 ul DMSO	
			M3	Sul 138 500uM	Add 6,5ul of 100mM stock solution to 1,3 ml medium	
0h						

Time	Conditions	Samples			Plate	
0h ATP	*Ctrl*	A1	A2	A3		-
0h RNA	*Ctrl*	R1	R1	R1		-

3h/24h/48h

Time	Condition	Samples			Plate	Medium
3h - ATP	*Ctrl*	MK51 A4	MK51 A5	MK51 A6	**1**	M2
3h - RNA	*Ctrl*	MK51 R2	MK51 R2	MK51 R2		M2
3h - ATP	*SUL-138 100 uM*	MK51 A7	MK51 A8	MK51 A9		M1
3h - RNA	*SUL-138 100 uM*	MK51 R3	MK51 R3	MK51 R3		M1
24h - ATP	*Ctrl*	MK51 A10	MK51 A11	MK51 A12	**2**	M2
24h - RNA	*Ctrl*	MK51 R4	MK51 R4	MK51 R4		M2
						-
						-
24h - ATP	*SUL-138 100 uM*	MK51 A13	MK51 A14	MK51 A15	**3**	M1
24h - RNA	*SUL-138 100 uM*	MK51 R5	MK51 R5	MK51 R5		M1
24h - ATP	*SUL-138 500 µM*	MK51 A16	MK51 A17	MK51 A18		M3
24h - RNA	*SUL-138 500 µM*	MK51 R6	MK51 R6	MK51 R6		M3
48h - ATP	*Ctrl*	MK51 A19	MK51 A20	MK51 A21	**4**	M2
48h - RNA	*Ctrl*	MK51 R7	MK51 R7	MK51 R7		M2
48h - ATP	*SUL-138 100 uM*	MK51 A22	MK51 A23	MK51 A24		M1
48h - RNA	*SUL-138 100 uM*	MK51 R8	MK51 R8	MK51 R8		M1

Appendix B.

Title	ATP determination protocol
Lit. Ref	
Lab. Ref	(Roche ATP assay kit)
Date	10/16/13
Goal	Assay the ATP level in tissue slice
Materials	• **safelock vials** • **minibead-beater** • **Repetitive pipet with 50 µl tip** • **White 96-wells plate** • **Syrnegy HT plate reader**
Solutions	• **SONOP (Sonification Solution), Ethanol (70% v/v) containing 2 mM EDTA (M=372.24 g/mol) with pH=10.9**. *For 500 ml : Dissolve 0.372 g EDTA in ± 100 ml of mQ-water, adjust PH with 5M NaOH to PH=10.9, add 30mL MQ-water and 370 ml Ethanol (96%).* • **100mM Tris-HCl, 2mM EDTA buffer (pH 7.6-8.0)** *For 500 ml: Dissolve 6.0 g Tris (M=121.14) (Tris(hydroxymethyl)amniophen; Merck) and 0.37 g EDTA (Triplex III; M=372.24) in ± 300 ml MQ-water, adjust pH with 6N HCl and fill up to 500 ml total volume with MQ-water* • **ATP Bioluminescence assay kit Roche.** Contents: Luciferase reagent lyophilized (white cap) *(Dissolve lyophilized luciferase in exactly 10.0 ml MQ-water and mix by swinging. Do not vortex)* ATP-standard ± 10 mg lyophilized (red cap) *(Dissolve the ATP-standard from the kit to **exactly** 10 mg/ml (= 16.5mM) with ultra pure water)*
Protocol	
After the incubation put 1 slice in 1 ml SONOP in a safelock vial, 1 cup of minibead and snap frozen in liquid N_2. **1.** Homogenize the sample with minibead-beater for 45 seconds. Keep the samples on ice.	

2. Centrifuge homogenate 5 minutes at 13,000 rpm. Transfer the supernatant into a new tube and keep on ice, the tube with precipitate is dried at 37°C (1 day) or room temperature (3 days) for protein measurement.

3. Make a calibration curve:

Dilution A, B and C are only to prepare the calibration curve, they are not going to be measured. Calibration samples (a,b,c, Cal1-5) should not be stored to reuse

Dilution	Amount (µl)	Tris/EDTA Buffer (µl)	Conc. (M)
A	10µl ATP-standard	***90***	1.65×10^{-3}
B	50µl [A]	450	1.65×10^{-4}
C	50µl [B]	450	1.65×10^{-5}
Cal 1	50µl [C]	450	1.65×10^{-6}
Cal 2	***100µl [1]!***	***400***	*3.30 x 10^{-7}*
Cal 3	50µl [1]!	450	1.65×10^{-7}
Cal *4*	***100µl [3]!***	***400***	*3.30 x 10^{-8}*
Cal 5	50µl [3]!	450	1.65×10^{-8}

4. Pipet 5 µl supernatant + 45 µl Tris/EDTA buffer into white 96-wells plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank1	Blank1	A7	A7	A15	A15	A23	A23	A31	A31	Cal1	Cal1
B	Positive	Positive	A8	A8	A16	A16	A24	A24	A32	A32	Cal2	Cal2
C	A1	A1	A9	A9	A17	A17	A25	A25	A33	A33	Cal3	Cal3
D	A2	A2	A10	A10	A18	A18	A26	A26	A34	A34	Cal4	Cal4
E	A3	A3	A11	A11	A19	A19	A27	A27	A35	A35	Cal5	Cal5
F	A4	A4	A12	A12	A20	A20	A28	A28	A36	A36	Blank 2	Blank 2
G	A5	A5	A13	A13	A21	A21	A29	A29	A37	A37	A39	A39
H	A6	A6	A14	A14	A22	A22	A30	A30	A38	A38		

5. Always include a positive control (stored at –80˚C). Prepare in the same way like the homogenates

6. Pipette 50 µl diluted calibration curve in a white 96-wells plate (at 4˚C)

7. Add to every well 50 µl luciferase (4˚C) by using repetitive pipet with 5mL combi tip (can be attached with 100uL yellow tip)

8. Measure plate after 0, 5 en 10 minutes with the luminometer (follow the SynergyHT protocol)	
Remarks	**a) Important!: The ATP in the slices may be breaking down by present enzyms, therefore store samples at -80°C and keep everything at 4˚C during determination**

Title	Protein estimation protocol
Lit. Ref	Lowry
Lab. Ref	BIO-rad RC DC Protein Assay
Date	17/10/13
Goal	Assay the protein level in tissue slice
Materials	• **BSA stock solution 3.2 (A) and 2.4 (B) mg/mL** • **Water bath with shaking function** • **Minibead beater** • **Transparent flat 96-wells plate** • **Multichannel pipet** • **Protein reader absorbance at wavelength 650nm***
Solutions	• **5M NaOH solution** (20g sodium hydroxide/100mL) • **Reagent A and B of BIO-rad kit.**

Protocol

9. Turn on waterbath
10. Thaw BSA (3.2 and 2.4 mg/ml)
11. Add (to pellet and beads) 200 µl 5M NaOH (20g in 100ml)
12. Incubate 30 min at 37°C (shaking, high speed) inside the waterbath
13. Make the following calibration curve diluted in the buffer at concentrations: 0 - 0.2 - 0.4 - 0.6 - 0.8 - 1.2 mg/ml

.

Dilution	Amount (µl)	1M NAOH (µl)	Conc. (mg/ml)
Calibration curve A (3.2 mg/ml)			
A1	50µl [A]	50	1.6
A2	50µl [A1]	50	0.8
A3	50µl [A2]	50	0.4
A4	50µl [A3]	50	0.2
A5	50µl [A4]	50	0.1
A6		50	0.0

Calibration curve B (3.2 mg/ml)			
B1	30µl [A]	50	1.2
B2	50µl [B1]	50	0.6
B3	50µl [B2]	50	0.3

14. Add 800 µl milliQ water (5x dilution, same as volume in SONOP)
15. Homogenize again with minibeadbeater for 40 seconds
16. Pipette 5 µl of calibration standard or sample in 96 well clear plate.
17. Add 25 µl of reagent A in each well (standards and samples).
(If samples contain detergent than 20 µl of reagent S is added to 1 ml of reagent A)

18. Add 200 µl of reagent B to each well (standards and samples).
19. Keep the plate for 15 minutes at room temperature and measure the absorbance at wavelengths 750 or 650* nm (Stable for 1 hour)

Remarks	**With the Biorad reader, please use 655nm wavelength instead.**

ATP content mouse kidney slices (in pmol/µg)

		Mouse kidney (MK)			
		MK48	MK49	MK51	MK52
0h	Mean	8,9	5,8	9,5	11
	SEM	1,5	1,5	1,8	2,4
3h Ctrl	Mean	10,1	8,5	14,6	17,2
	SEM	1,3	0,7	1,2	3,6
3h SUL-138 (100uM)	Mean	13,6	11,5	7,6	18,3
	SEM	0,8	0,7	1,9	5,6
24h Ctrl	Mean	9,5	6,5	8,6	15,4
	SEM	0,6	0,7	0,7	1,2
24h SUL-138 (100uM)	Mean	10,8	9,2	8,7	17,7
	SEM	0,8	1,3	0,6	3,4
24h SUL-138 (500uM) N=1	Mean			7,8	
	SEM			0,8	
48h Ctrl	Mean	10,5	8,9	7,8	12,4
	SEM	1,3	0,5	0,8	1,4
48h SUL-138 (100uM)	Mean	12,6	10,5	11,5	16,4
	SEM	1,7	0,8	1,3	2,7

Appendix C.

Title	RNA isolation (FavorPrep tissue total RNA mini kit)
Lit. Ref	
Lab. Ref	
Date	
Goal	Isolate RNA from tissue
Materials	• **Minibead beater** • **Centrifuge** • **FavorPrep RNA tissue isolation mini kit (FATRK 001-2)** • **DEPC treated water (keep always under the hood)**
Solutions	• **70% ethanol** – Mix 17.5 ml of (96% to 100%) ethanol with 7.5 ml DEPC water. • **Buffer FARB** – Add 10 µl of Dithiothreitol (DTT) to 1 ml of FARB (prepare fresh). Final concentration of DTT must be 20 mM (stock is 2 M, stored in -20 °C). • **Wash buffer 1** (stable at room temperature). • **Wash buffer 2** (ethanol added – stable at room temperature).

Protocol

20. Prepare and label tubes (1 set collection tubes, 1 set columns, 1 set elution tubes), buffers before start.
21. Keep samples in dry ice; add minibeads and 300 µl of FARB buffer. Homogenize the slices in 300 µl of FARB buffer by minibead beating (45 seconds for liver/kidney samples, 2x45 seconds for intestine samples); be sure the tissue is well homogenized. **Before homogenizing don't let the tissue thaw/melt!!!**
22. Centrifuge the homogenate for 5 min at 13.000 rpm and transfer the supernatant (300 µl) into a clean 1.5 ml tube with 300 µl of 70% ethanol and mix well by pipetting.
23. Transfer 600 µl of the ethanol-mixed sample (including any precipitate) to the FARB Mini column. Centrifuge for 1 min at 10000 rpm and discard the flow-through.
24. Add 500 µl of Wash buffer 1 to the column and centrifuge for 1 min at 10.000 rpm. Discard the flow-through.
25. Add 750 µl of Wash buffer 2 to the column and centrifuge for 1 min at 10.000 rpm. Discard the flow-through.
26. Repeat Step 7.
27. Centrifuge at full speed 14.000 rpm for an additional 3 min to dry the column (this step will avoid the residual liquid to inhibit subsequent enzymatic reaction). Discard the flow-through and collection tube.
28. Transfer the FARB mini column into elution 1.5 ml tube (provided with the kit). Elute the total RNA by applying 40µl of DEPC water onto the center of the FARB mini column. Close the tube gently and wait for 1 minute
29. Centrifuge for 1 min at 13.000 rpm.

30. Collect the flow through and keep samples on ice **31.** Measure the RNA concentration by Synergy HT	
Remarks	**A260 / A 280 = 1.9 to 2.1** **Do not isolate more than 24 samples in one batch** **Using RNAlater, read RNA later protocol.**

Title	cDNA synthesis (Promega kit)
Lit. Ref	
Lab. Ref	
Date	06/07/16
Goal	Synthesis of cDNA from RNA
Materials	• **cDNA tubes** • **Thermal cycler**
Solutions	• **MgCl2 25mM** • **Reverse Transcription 10X Buffer** • **dNTP Mixture, 10mM** • **Recombinant RNasin® Ribonuclease Inhibitor** • **AMV Reverse Transcriptase (High Conc.)** • **Random Primers** • **DEPC treated Water**

Protocol

32. Prepare and label tubes.

33. Calculate the necessary amount of RNA and DEPC water for the reaction (1 µg RNA in 10 µL). If the RNA concentration is lower than 100 ng/µL, do not dilute the RNA sample and pay attention to step **12**).

34. Take out the RNA samples from the freezer. Keep samples on ice.

35. Add the RNA and DEPC water in the reaction tube.

36. Make the cDNA reaction mix with the following composition/ 1 reaction:

- **$MgCl_2$ 25mM** **4 µL**
- **Reverse Transcription 10X Buffer** **2 µL**
- **dNTP Mixture, 10mM** **2 µL**
- **Recombinant RNasin® Ribonuclease Inhibitor** **0,5 µL**
- **AMV Reverse Transcriptase (High Conc.)** **0,6 µL**
- **Random Primers** **1 µL**

37. Add 10 µL of cDNA mix to every tube (final volume 20 µL/tube).

38. Centrifuge the tubes for 1 minute. Make sure there are no air bubbles.

39. Place the reaction tubes in the Thermal cycler machine.

40. Close properly the lid.

41. Select the program "PROMEGA" from "MAIN" (the program includes the following steps:

- **22°C – 10 minutes**
- **42°C – 15 minutes**
- **95°C – 5 minutes**
- **4°C – for ever**

42. After 35 minutes, take out the tubes (the tubes can be left longer in the machine – the program will keep the samples at 4°C indefinetely).

43. Add 80 µL of DEPC water to the samples to a total volume of 100 µL. If your input was less than 1 µg, then dilute accordingly to the final conc of 10 ng/ µL.

44. Place the tubes in the freezer (-20°C).

Remarks	

Dissolving Primers for SybrGreen Real Time PCR

- Dissolve primers in DEPC water to a concentration of 50 µM (according to the manufacturer's recommendations). Prepare the working solution in 1 tube. The working solution should be stored after use at -20°C in the primer's box (label it with the name of the gene and R+F).

Working solution (6 µM primers)	Volumes (µl)
Forward (50 µM)	24
Reverse (50 µM)	24
DEPC water	152
Total volume	200 µl (400 wells)

Setup SYBR Green Real Time PCR

- Use duplicates for each sample (2 wells/sample). Use the template to design your experiment.
- Dilute cDNA samples in DEPC water 5 times, to a final concentration of 2 ng/µl.
- Make mastermix per gene (always make some extra because of pipetting errors):

SYBR Green Mix	volume/reaction (µl)
SYBRgreen mastermix ROCHE	5
Working solution (6 µM)	0,5
Total	5,5

- Use electronic multi dispense pipet to pipet cDNA in 384 well plate (5 µl/well).
- Use electronic multi dispense pipet to pipet **SYBR Green mix** in 384 well plate (5 µl/well).
- End volume is 10 µl/well.
- Put an optical seal on the 384 well plate. Don't touch seal with your hands, always wear gloves. Apply good pressure in the edges to prevent evaporation. Be sure the seal is correctly placed; no sticky ends because this can cause problems when using the robot arm (overnight runs).
- Spin down plate for 2 minutes at 2.000 rpm.

Protocol for Sybr Green Roche

	Temp	**Time**	**Number of cycle**
Enzyme activation	95°C	10 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Melting curve	Increasing temp	According to ViiA7 melting protocol	-

Protocol ViiA7 Real Time PCR

General instructions

Operators: Peter Zwiers

Pytrick Jellema

Always book the ViiA7 machine before use via http://medicalbiology.bookedscheduler.com/

- If you make a reservation mention your name, telephone number and the department project number (190134575**).**
- Book in blocks of 2 hrs.
- Overnight runs are possible to a maximum of 8 plates.

Costs per run (2016) are €25,-.

In both daytime and overnight runs qPCR runs need to be performed using the Robot arm. Instructions on using the robot can be found below.

Take care in removing the edges of the adhesive film properly. Adhesive remains can stick to the robot arm, after which the machine cannot perform the run.

Creating Run File (.eds) using ViiA™ 7 RUO Software

For file set up and analysis, use the ViiA7 analysis PC on the first lab table of the MB-Z lab.

Turn on PC; login with your own P-number and password

Open the ViiA7 software via shortcut on desktop

In Set Up choose Experiment Setup

Complete “Setup” in the “Experiment Menu”

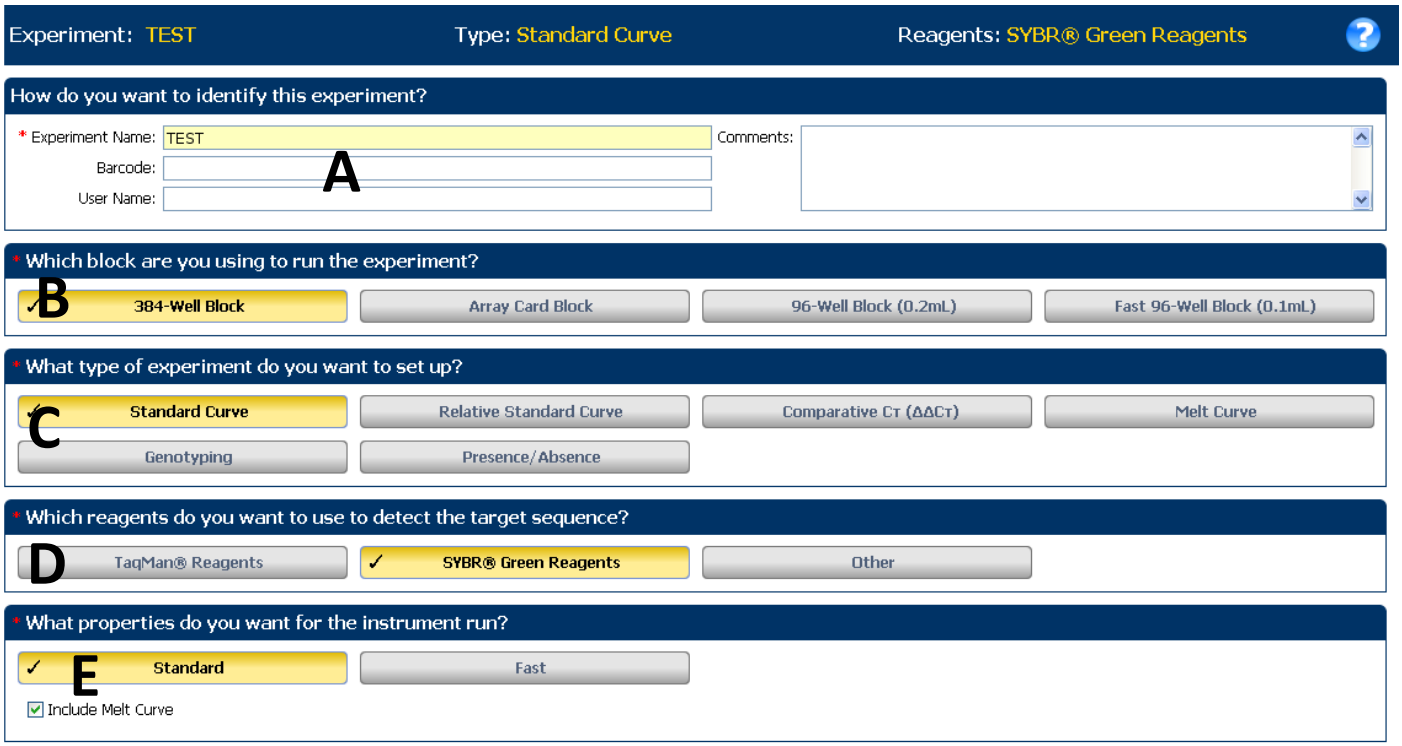
Experiment Properties 

A. Experiment Name (enter an unique file name e.g. 120624 [name exp.] [your name])
Barcode (Preferably use Barcode reader)

Only use capital letters! Be careful: Ø = 0 (zero), O = capital O

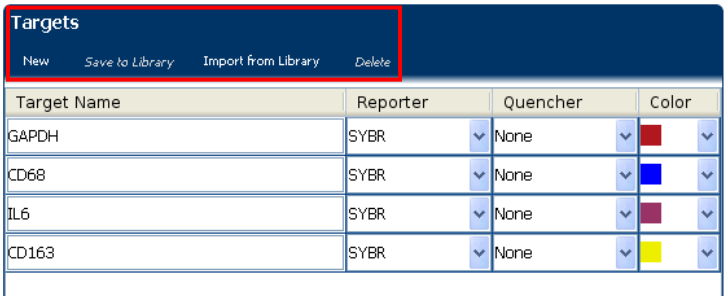
User name (in case we need to contact you)

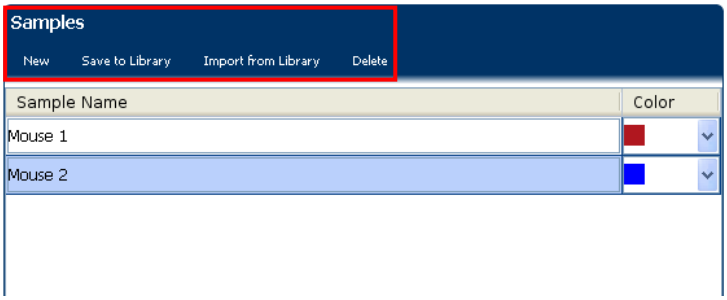
B. Which block are you using to run the experiment : 384-well or Array Card Block (LDA)
C. What type of experiment do you want to set up?
D. Which reagents do you want to use to detect the target sequence?
E. What properties do you want for the instrument run?



Define 

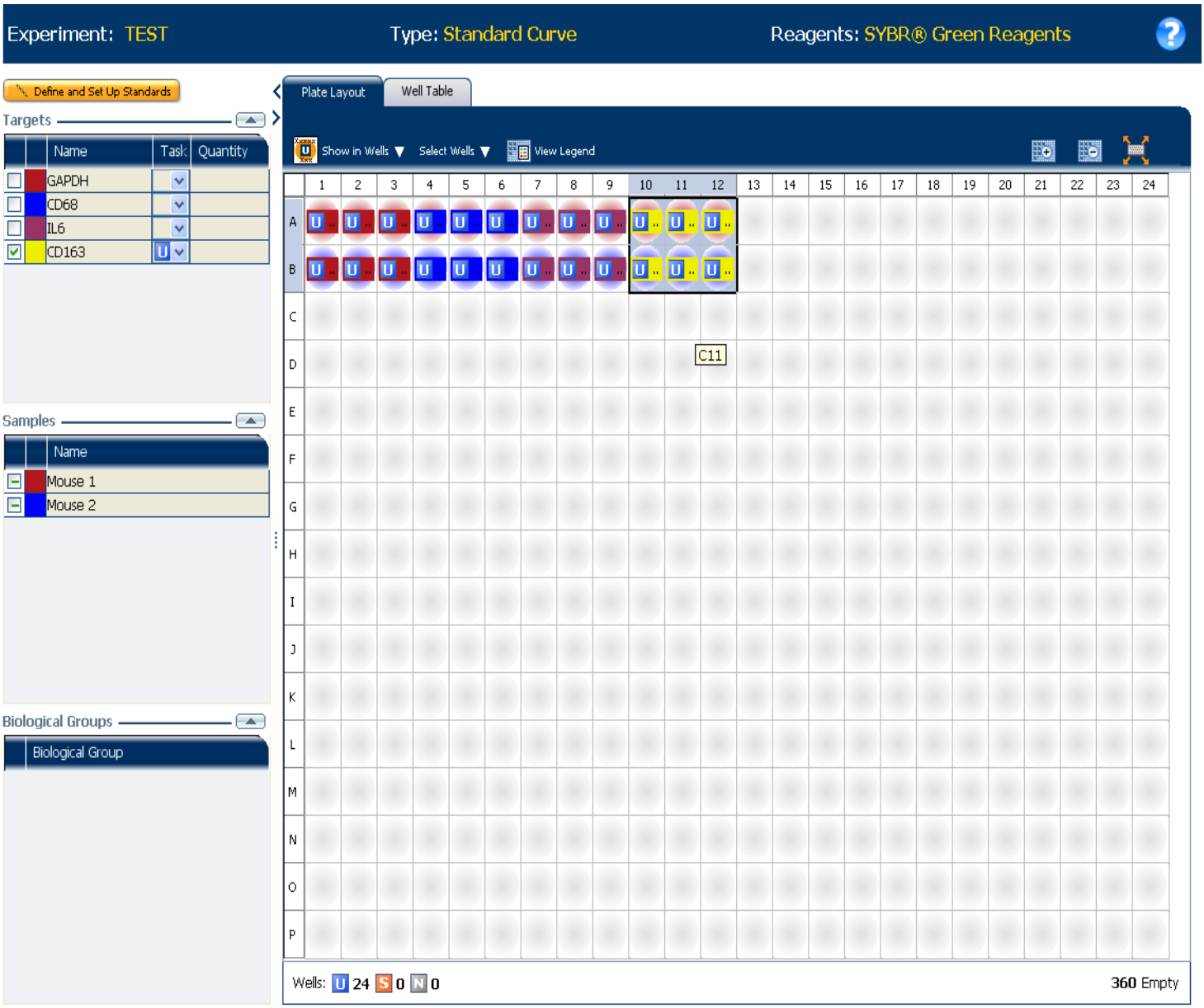
Fill in targets and samples (recommended) New or Import from Library





Assign 

Assign targets and samples by selecting wells and ticking the required checkbox



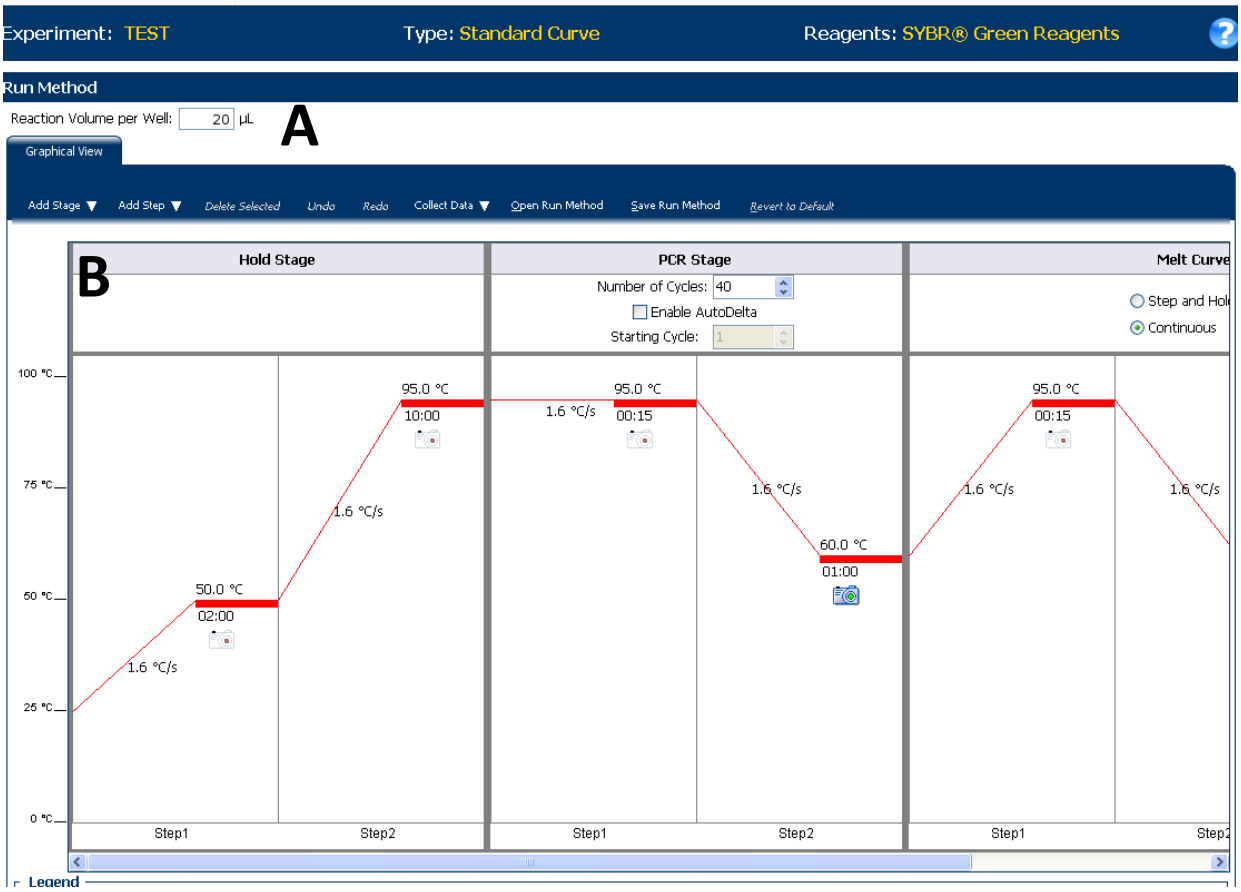
Run Method 

Adjust Run Method to the reagents requirements.

A Reaction volume per well

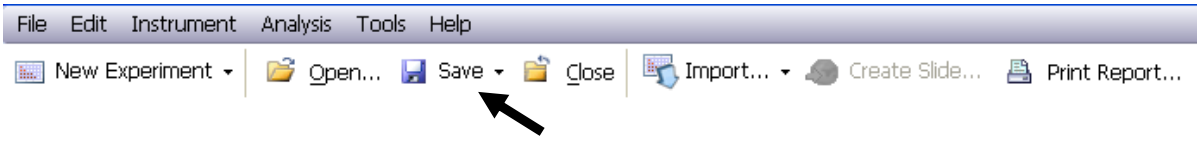
B Review and if necessary edit the run method

Make sure that the thermal profile is appropriate for your reagents.



Pull down the “save” menu and choose save as.

<u>IMPORTANT! File name and Experiment name need to be the same!</u>

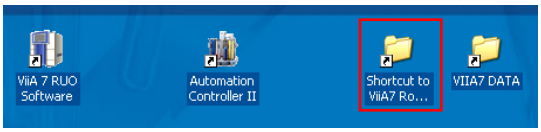


Copy the file to the ViiA7 USB drive (always nearby ViiA7 analysis PC)

Connect the USB drive to the computer connected to the ViiA7.

Copy or drag and drop the files that you want to run from the USB drive to the folder

D:\VIIA7 DATA\ViiA7 Robot Runs (shortcut on desktop).



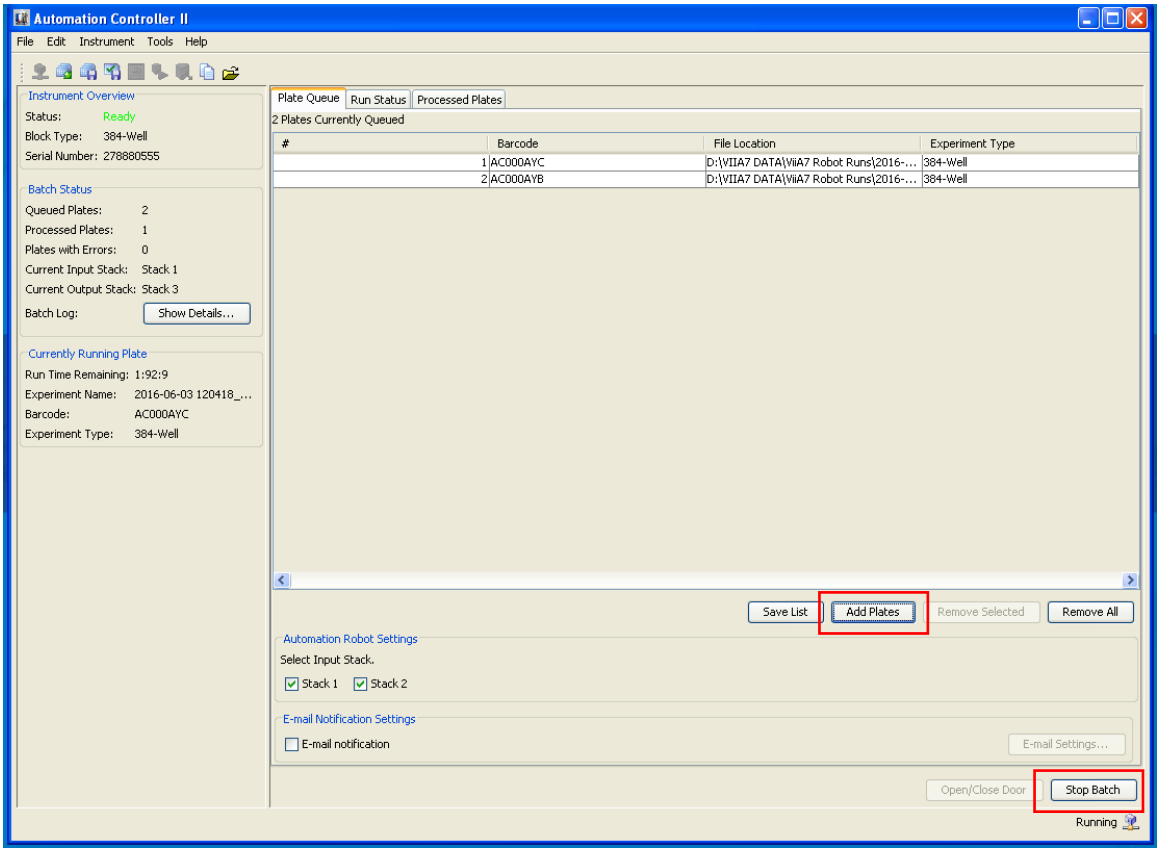
Running the ViiA7 using the Robot arm

Start a new run

Launch Automation Controller II software via shortcut on desktop

ViiA7 software will launch automatically when Automation Controller is launched, this is needed for the Robot arm to function.

IMPORTANT! During a run, NEVER close the ViiA7 software! The robot run will stop and data will not be stored correctly



Click ‘Add plates’, go to desktop, ViiA7 robot runs folder and select the plates you want to add to the Robot run.

Remove finished plates from stack 3 (these are waste, throw in blue bin)

Put your plates in stack 1 in the robot. Make sure the orientation is correct otherwise the barcode cannot be detected.

In Automation Controller II software, click ‘start batch’. (‘start batch’ will now change to ‘stop batch.) After some checks the robot arm will pick up your plate and the run will start.

~5 min after run has started you can touch the screen on the ViiA7 PCR machine, if you select ‘Time view ‘ a screen with the remaining run time will be visible (from the other side of the lab).

When the run is finished, your plate will be put in rack 3.

Copy or drag and drop the files that are run from the folder **D:\VIIA7 DATA\ViiA7 Robot Runs** (shortcut on desktop) to the USB drive.

Analyze you results on the ViiA7 analysis PC on the first lab table of the MB-Z lab.

Add plates to a running batch

If a batch is already running and you want to add your plate to this batch:

In Automation Controller II software: Click ‘Add Plates’, go to desktop, ViiA7 robot runs folder and select the plates you want to add to the Robot run.

Put your plates in stack 1 in the robot. Make sure the orientation is correct otherwise the barcode cannot be detected. After finishing the previous run, the robot will pick up the next plate automatically and continue the run.

When the run is finished, your plate will be in rack 3.

Copy or drag and drop the files that are run from the folder **D:\VIIA7 DATA\ViiA7 Robot Runs** (shortcut on desktop) to the USB drive.

Analyze you results on the ViiA7 analysis PC on the first lab table of the MB-Z lab.

Troubleshooting

My plate is picked up and discarded to rack 3 directly by the Robot arm.

Give each experiment file a unique name, you cannot run 2 plates with the same name, even if the barcode is different.

Solution: Design a new experiment file as described in the protocol above, save under a unique name and start the run again.

My plate is run, but there is no data in the file.

Probably the ViiA7 software was closed during the run, data is not lost, but to retrieve data, ask one of the operators for help

My plate sticks to the Robot arm.

Remove the adhesive film properly; make sure no adhesive is left on the plate. Clean the Robot arm with 70% EtOH.

In multiple plates I see no amplification in 1 specific well.

The well in the PCR block might be dirty, ask one of the operators to clean this well.

The ViiA7 software issues a warning that calibration is expired.

The PCR machine needs calibration every 6 months; tell one of the operators, so calibration can be planned.

I really need more help than provided in this protocol

Go to the Thermo Scientific website and search for “Applied Biosystems ViiA7 Real-Time PCR system Getting Started Guides”. This guide will provide additional information on the possibilities of the ViiA7 PCR system.

Smoke is coming from one or more parts of the machine

Shut the machine down, leave the room and ask for help.

Mean relative mRNA expression biomarkers (N=4)

Biomarkers	Incubation	MK48	MK49	MK51	MK52
Il-6	0h	0,579	0,628	2,2	0,593
	3h Ctrl	7,889	24,904	64,848	15,185
	3h SUL-138 (100uM)	9,625	34,211	42,482	11,69
	24h Ctrl	2,087	6,474	7,152	3,390394
	24h SUL-138 (100uM)	2,703	11,19	9,922	3,659
	48h Ctrl	2,886	13,759	14,901	3,395929
	48h SUL-138 (100uM)	3,273	8,015	15,477	6,438
IL-1β	0h	0,82	1,52	0,851	0,809
	3h Ctrl	3,562	10,746	7,706	6,47
	3h SUL-138 (100uM)	7,86	14,092	4,771	6,06
	24h Ctrl	3,744	2,89	4,768	4,364
	24h SUL-138 (100uM)	3,501	4,774	4,877	3,647
	48h Ctrl	4,449	4,775	3,62	4,772
	48h SUL-138 (100uM)	4,529	3,027	6,2	9,292
TNFα	0h	0,251	0,332	0,323	3,094
	3h Ctrl	0,824	2,837	1,788	0,82
	3h SUL-138 (100uM)	1,462	1,996	0,661	0,372
	24h Ctrl	0,621	0,603	0,541	0,327
	24h SUL-138 (100uM)	0,587	0,825	0,859	0,289
	48h Ctrl	0,945	0,612	0,474	0,529
	48h SUL-138 (100uM)	0,532	0,547	0,668	0,861
HMOX	0h	0,638	1,866	0,818	0,678
	3h Ctrl	18,725	16,784	7,072	4,945
	3h SUL-138 (100uM)	30,419	11,642	5,109	3,215
	24h Ctrl	40,925	35,101	17,654	9,1
	24h SUL-138 (100uM)	31,244	55,751	14,28	8,36
	48h Ctrl	20,599	29,314	11,024	7,459
	48h SUL-138 (100uM)	10,318	22,735	4,979	4,524
NGAL	0h	0,256	0,417	2,708	0,619
	3h Ctrl	0,526	2,457	6,154	2,093
	3h SUL-138 (100uM)	1,011	2,127	3,127	0,783
	24h Ctrl	28,118	22,385	39,794	19,372
	24h SUL-138 (100uM)	20,358	14,955	33,098	28,814
	48h Ctrl	42,804	59,188	59,517	28,545

	48h SUL-138 (100uM)	32,101	51,602	76,578	33,438
Kim-1	0h	0,443	0,691	2,349	0,517
	3h Ctrl	4,697	14,825	15,105	4,043
	3h SUL-138 (100uM)	2,897	5,685	5,197	2,833
	24h Ctrl	204,914	226,984	192,189	56,733
	24h SUL-138 (100uM)	153,158	199,936	195,327	67,744
	48h Ctrl	123,54	195,354	80,506	45,095
	48h SUL-138 (100uM)	71,112	223,533	92,911	62,967
SFN	0h	1,177	1,047	0,73	1,046
	3h Ctrl	5,888	11,491	3,648	5,98
	3h SUL-138 (100uM)	7,961	10,801	1,672	5,365
	24h Ctrl	11,827	4,392	3,178	4,818
	24h SUL-138 (100uM)	10,844	11,318	2,932	5,126
	48h Ctrl	12,58	8,436	3,551	5,031
	48h SUL-138 (100uM)	10,844	11,318	2,932	5,126
SULF2	0h	0,863	1,27	0,414	1,453
	3h Ctrl	0,544	0,732	0,399	0,765
	3h SUL-138 (100uM)	0,767	0,743	0,313	0,791
	24h Ctrl	1,232	0,328	0,303	0,681
	24h SUL-138 (100uM)	0,91	1,026	0,416	0,851
	48h Ctrl	1,666	0,83	0,487	0,786
	48h SUL-138 (100uM)	1,416	0,645	0,551	1,005
Bcl-2	0h	0,779	1,357	0,842	1,023
	3h Ctrl	0,377	0,524	0,814	0,421
	3h SUL-138 (100uM)	0,511	0,633	0,547	0,477
	24h Ctrl	0,976	0,857	0,841	0,683
	24h SUL-138 (100uM)	0,871	1,02	0,967	0,803
	48h Ctrl	1,178	1,442	1,11	0,968
	48h SUL-138 (100uM)	1,171	1,282	1,337	1,4
EIF3C	0h	0,618	1,031	1,563	0,788
	3h Ctrl	0,666	1,107	2,586	0,736
	3h SUL-138 (100uM)	0,967	1,151	1,634	0,665
	24h Ctrl	2,369	5,039	3,848	0,798
	24h SUL-138 (100uM)	1,891	2,438	4,351	1,205
	48h Ctrl	1,769	5,424	2,599	0,838

	48h SUL-138 (100uM)	1,221	4,302	2,513	0,907
iNOS	0h	0,672	0,683	1,916	0,73
	3h Ctrl	0,838	0,985	2,059	1,291
	3h SUL-138 (100uM)	0,91	1,013	2,535	1,516
	24h Ctrl	1,296	2,008	3,958	1,567
	24h SUL-138 (100uM)	1,209	1,237	3,634	1,719
	48h Ctrl	1,587	3,265	4,144	1,747
	48h SUL-138 (100uM)	1,156	2,801	4,02	1,935
NOX1	0h	0,483	2,187	0,33	1
	3h Ctrl	1,727	2,857	0,907	2,484
	3h SUL-138 (100uM)	0,92	3,298	1,009	0,761
	24h Ctrl	0,526	1,594	0,668	3,390394
	24h SUL-138 (100uM)	0,245	0,186	0,588	1,596
	48h Ctrl	0,617	0,147	0,793	0,026
	48h SUL-138 (100uM)	0,348	1,405	0,913	0,69
Hif1a	0h	0,717	1,332	0,786	1,165
	3h Ctrl	1,151	1,929	1,985	2,236
	3h SUL-138 (100uM)	1,706	2,225	1,099	1,878
	24h Ctrl	2,514	1,875	2,51	1,649
	24h SUL-138 (100uM)	2,13	2,288	2,618	2,338
	48h Ctrl	2,821	3,323	2,1	1,964
	48h SUL-138 (100uM)	1,854	2,476	2,816	2,56

Appendix D.

Morphology

Fixation:

1. Add Formalin (4%) to 24 wells-plate.

2. Gently transfer slices using a spatula from incubation plate to Formalin plate, store at 4°C for 24 hours. 3 Slices (condition) per well.

3. After 24 hours, move slices in the same manner to a new 24 wells-plate containing 70% ethanol. Seal this plate with Para film to prevent evaporation and store in the fridge for at least 24 hours. Samples can be stored for longer periods of time but make sure there is enough ethanol in the wells.

Dehydration:

1. Label orange embedding cassettes with a sharp pencil (push hard).

2. Soak biopsy pads in 70% ethanol and place into cassette. Make sure the cassette can still be closed properly.

3. Place 1 condition (3 slices) in each cassette. Slices should be as close to the center as possible, but not overlapping.

4. Cover slices with another soaked biopsy pad (cut in half) and close cassette firmly.

5. Place cassette in a glass beaker filled with 70% ethanol.

6. Take cassettes to the pathology department for dehydration. Once there:

- Put on provided lab coat,
- Put on gloves,
- Enter into the excel sheet on the computer: number of cassettes, colour of cassettes, phone number, name and project number (for payment),
- Put cassettes in bucket labeled “Research Cassettes”, fill with 4% formalin if empty. Make sure to use all spaces; don’t get your own rack. Close lid firmly,
- Throw away gloves in the lab and put labcoat in the bin in the hallway.
- Pick up cassettes (in the stove) the next day.

Materials:

- 70% EtOH
- 4% Formaldehyde (Baker order #4078-9005)
- Biopsy pads (Surgipath 500pcs Leica Biosystems order #01020
- Embedding cassettes (with lid, peach Simport order #493/7)

Development of precision-cut kidney slices for testing toxicity and disposition of platinum anticancer drugs in tubular cells
54 University of Groningen, 2015

Embedding:

1. Turn on the embedding machine at least 2 hours before use. Check whether all paraffin has melted by opening the lid on the top of the machine. Add paraffin if necessary.

2. Turn on cooling part of the machine. Place dehydrated cassettes in the right compartment of the apparatus. Let paraffin melt.

3. Get a cassette and open it. Take out both biopsy pads and put slices on one. Throw away lid and keep labeled part of the cassette.

4. Take a medium sized metal mold from the left compartment and fill with paraffin. Move to non-heated (white) part of the machine and quickly add kidney slices. Place them standing up in the middle of the mold. Make sure to do this fast as all kidney slices should be at the same depth in the solidifying paraffin.

5. Quickly place the labeled lid on top of the mold and add some more paraffin. Move to cold plate and let it cool down for at least 15 minutes.

6. When completely solid remove cassette part + paraffin from mold. This should go smoothly (not forcefully). Check whether all slices are standing upright. If this is not the case, remelt them on the gray area and repeat steps 4-6.

Cutting**:**

0. To use the machine for cutting paraffin blocks, you will first need to get an introduction from one of the technicians.

1. Turn on the small water bath and heating block and set them to heat.

2. Insert knife into the machine. Make sure thickness is set to 4. Do not change this.

3. Clear paraffin of the edges of the cassette part and secure this part to the machine. Make sure the block is straight in relation to the knife.

4. Move the block all the way to the back and put knife right in front of it. Secure knife position and start cutting (fast until you reach the tissue). When all slices are visible in your cuts (3 small pink lines), start cutting slowly.

5. Carefully move good cuts to the water bath and leave them for a few minutes.

6. Mount onto glass slides and place them in the rack vertically (label with pencil!). Let dry.

7. Remove water drops from slides using filter paper.

8. Place slides on heating block for at least 1 hour.

9. Incubate slides at 37°C overnight (use a metal rack). Can be stored here or at room temperature for longer periods of time.

Immunostaining paraffin sections

1. **Removing paraffin**: 15 min xylol I, 10 min xylol II, 5 min alcohol 100%, 3 min alcohol 96%, 3 min alcohol 70%, 3 min demiwater, 3 min PBS.
2. **Antigen retrieval:** Incubate overnight in 0,1 M Tris/HCl buffer pH 9,0 in heater at 80 ^{0}C. Next day cool of at RT for 30 minutes. Wash with PBS (3x)
3. **Block endogenous peroxidases:** 0,1% H_2O_2 in PBS for 10 minutes at RT
4. **Pap samples**
5. **Primary antibody:** 40µl per sample, antibody + 2% BSA + 2% human serum in PBS at RT for 60 minutes.
6. **Wash:** PBS with 0,05% tween-20 , 3x 5 min
7. **Secondary antibody:** secondary antibody + 2% BSA in PBS for 30 minutes at RT
8. **Wash:** PBS, 3x 5 min
9. **Third antibody:** third antibody + 2% BSA in PBS for 30 minutes at RT
10. **Wash:** PBS, 3x 5 min

11. **Staining with Nova Red:** Prepare solution according to the protocol in the box, 40µl per sample. Incubate for x (6-10) minutes, check redness of the slices. Stop reaction in demi-water.
12. **Hematox:** counterstain with hematox for 1 minute and wash in tap water for 5 minutes.
13. **Dehydration:** after washing with tap water: ethanol 96% 20x, ethanol 96% 20x, ethanol 100% 20x and ethanol 100% 20x
14. **Embedding:** embed in depex

Mouse on mouse protocol

Use protocol in black mouse on mouse box

1. **Step 1, 2, 3 and 4:** use immunostaining paraffin sections protocol steps 1,2,3 and 4
2. **Step 6:** Make a avidin and biotin dilution of 1:20 in PBS. Incubate samples first with avidin for 15 min, then with biotin for 15 min. (avidin and biotin in fridge, behind secondary and third antibodies in white box)
3. **Step 10:** Incubate with primary antibody for 1 hour at RT
4. **Step 16:** Use Nova Red staining as described at step 11. from the immunostaining paraffin sections protocol
5. Continue with steps 12, 13 and 14 from the immunostaining paraffin sections protocol

Appendix E.

Isolation of mitochondria from PCKS's

Introduction

This protocol describes the steps needed to isolate mitochondria from fresh kidney tissue. These mitochondria need to be isolated within 1 h after the experiment is finished but can be stored at -80°C once they are isolated.

Protocol

Prepare the following solutions:

5x Mitochondrial Isolation Buffer (MIB):

- 19.128 g Mannitol (1.05 M)
- 11.981 g Sucrose (350 mM)
- 0.303 g Tris (25 mM)
- 0.146 g EDTA (5 mM)
- 100 ml ddH2O
- *Set pH to 7.5 using HCl or NaOH*

5x Mitochondrial Storage Buffer (MSB):

- 4.279 g Sucrose (1.25 M)
- 0.025 g ATP (5 mM)
- 0.002 g ADP (0.4 mM)
- 0.030 g Succinate (25 mM)
- 0.017 g K2HPO4 (10 mM)
- 0.008 g DTT (5 mM)
- 0.119 g HEPES (50 mM)
- 10 ml ddH2O
- *Set pH to 7.5 using HCl*

Albumin solution

- 50 to 250 mg/mL BSA solution. *The Department of Medical Biology uses 220 mg/mL (22%, 110x) BSA solutions.*

Method

Throughout this procedure, make sure all solutions and tissue are kept on ice

Preparation:

- Prepare up to 5 mL of 1x MIB-BSA containing 2 mg/mL of albumin. *Example: For 5 mL, add 200 µL of 50 mg/mL BSA to 4.8 mL of 1x MIB.* BSA is used to remove lipids from the homogenate. Also prepare up to 5 ml 1x MIB without BSA. Use for preparation 5x MIB stored in the fridge.
- Prepare 50 µL of 1x MSB per 100 mg of tissue.

Homogenization and isolation:

1. Use fresh tissue sample (obtained within 1 hour of sacrifice) kept on ice. Do not freeze.
2. Was the sample twice with 2 volumes of 1x MIB in a 12 wells plate
3. Determine the weight of a single PCKS and use 50-100mg tissue per sample.
   Tip: Weigh the empty cups beforehand and write the tare weight on them.
4. Homogenize the sample:
   a. Add MIB-BSA in 10x the volume of the tissue. *Example: For 50 mg, add 500 µL of MIB-BSA*
   b. Transfer the tissue to a glass tissue grinder with two grinders (A and B) and add MIB-BSA
      Tip: Use a 1 mL pipet tip with the point cut off to pipet viscous substances.
   c. First homogenize by moving pestle B up and down two times, followed by pestle A up and down four times.
      Tip: In case of a glass pestle, rinse the pestle with 1 x MIB before continuing with the next sample.
   d. Transfer the homogenate to a 2/1,5 mL tube and centrifuge at 600 x *g* for 5 minutes
   e. Carefully transfer the supernatant into a new 1.5 mL tube and centrifuge at 11000 x *g* for 10 minutes. Save pellet for western blot as positive control.
   f. Remove the supernatant and resuspend the pallet in 10 volumes of 1x MIB. *Example: For a 50 µL pallet volume (about 50µg), add 500 µL of 1 x MIB*
   g. Transfer the homogenate to a 1.5 mL tube and centrifuge at 600 x *g* for 5 minutes
   h. Carefully transfer the supernatant into a new 1.5 mL tube and centrifuge at 11000 x *g* for 10 minutes. Save pellet for protein measurement and western blot.
   i. Resuspend the pallet in 1 x MSB. Use 40 µL per 100mg of starting tissue. Save 5µl per sample for protein measurement.
5. Store the isolated mitochondria in a -80°C freezer or measure protein concentration immediately

Follow-up:

The expected protein concentration of the mitochondria suspension should be approximately 10 – 25 mg/mL for 100mg tissue. For JC-1 experiments, a mitochondria suspension with a protein

concentration of 1 mg/mL is needed as a stock solution. In order to determine the protein concentration, perform a BioRad DC protein assay (based on the Lowry protein assay).

Appendix F.

Western Blot for tissue slices

General Points:

1. Keep everything clean! Wash WB equipment parts after every time you use it, and place them in the drawers (all drawers are specifically marked) once they dried.
2. When 10x buffers (stocks) are finished, please refill.
3. Wash and dry empty bottles.
4. When prepare fresh solution, please put the date on it.
5. Keep Biorad ChemiDoc Touch Imaging System clean (wash the glass tray with dH_2O and 70% EtOH).

Step 1 **Gel preparation**

NOTE:

Gels can be prepared in advance to SDS-PAGE and stored at 4°C up to 7days. Therefor, wrap the gel (incl. comb) in wetted tissues, slide it in a plastic bag (name+date) and place vertically in the fridge.

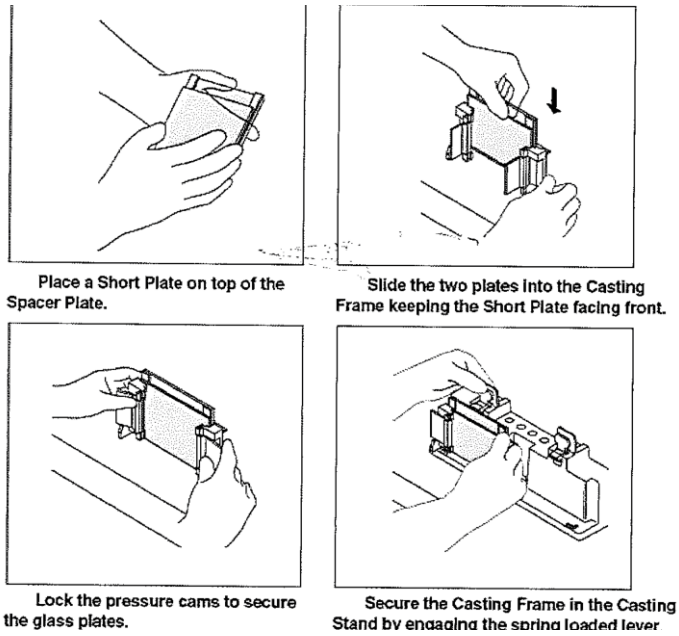
Materials

- Gel casting frames and casting stand
- Short plates, spacer plates and comb
- Isobutanol (**water saturated -> use only upper layer!**) and filter paper strips

Methods:

***** Step 8-16 do not apply when using Biorad Mini-PROTEAN TGX Stain-Free precast gels *****

1. Assemble the casting gel unit:



2. Prepare the running gel *(*percentage depends on type of protein)*:

10% running gel	1	2	3	4	# of gels
40% Acrylamide/Bis (37:1)	2.5	5	7.5	10	mL
1.5M Tris pH 8.8	2.5	5	7.5	10	mL
dH2O	4.8	9.6	14.4	19.2	mL
10% SDS	100	200	300	400	µL
10% APS	100	200	300	400	µL
TCE	50	100	150	200	µL
TEMED (*add in fume hood*)	10	20	30	40	µL
Total volume	*10*	*20*	*30*	*40*	mL

12% running gel	1	2	3	4	# of gels
40% Acrylamide/Bis (37:1)	3	6	9	12	mL
1.5M Tris pH 8.8	2.5	5	7.5	10	mL
dH2O	4.3	8.6	12.9	17.2	mL
10% SDS	100	200	300	400	µL
10% APS	100	200	300	400	µL
TCE	50	100	150	200	µL
TEMED (*add in fume hood*)	10	20	30	40	µL
Total volume	*10*	*20*	*30*	*40*	mL

7.5% running gel	1	2	3	4	# of gels
40% Acrylamide/Bis (37:1)	1.9	3.74	5.61	7.48	mL
1.5M Tris pH 8.8	2.5	5	7.5	10	mL
dH2O	4.8	9.6	14.4	19.2	mL
10% SDS	100	200	300	400	µL
10% APS	100	200	300	400	µL
TCE	50	100	150	200	µL
TEMED (*add in fume hood*)	10	20	30	40	µL
Total volume	*10*	*20*	*30*	*40*	mL

Note: 40% Acrylamide/Bis (37:1) - Add 63mL MQ water per bottle to reconstitute.

3. Pour the liquid gel between the glasses until it reach the green bar of the casting frame. Remove bubbles by filter paper strips.
4. Add few drops of isobutanol on top of the running gel to even the surface.
5. Let the gel polymerize for 20-30min.
6. After the running gel is polymerized, remove the isobutanol (turn over the casting unit above sink), and prepare the stacking gel.

4% stacking gel	1	2	3	4	# of gels
40% Acrylamide/Bis (37:1)	0.25	0.5	0.75	1	mL
0.5M Tris pH 6.8	0.63	1.25	1.88	2.5	mL
dH2O	1.6	3.2	4.8	6.4	mL
10% SDS	25	50	75	100	µL
10% APS	25	50	75	100	µL
TEMED (*add in fume hood*)	2.5	5	7.5	10	µL
Total volume	*2.5*	*5*	*7.5*	*10*	mL

7. Pour the liquid stacking gel till it reaches the top of the short plate and gently insert the comb. Make sure there are no bubbles at the bottom or in between the wells.
8. Let the gel polymerize for 10-15min.
9. Once polymerized, remove the casting frames from the casting stand. Continue with SDS-PAGE or store the gel in the fridge (see instructions on previous page).

Step 2 **SDS PAGE**

Materials:

- SDS PAGE system (power supply, tag, gel cassette sandwiches)
- Biorad Running buffer 1x:

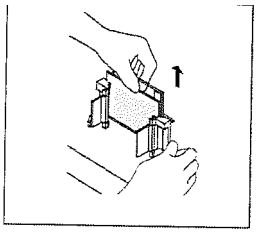
Biorad 1x running buffer (gel electrophoresis buffer)			**Aliquot**
Tris, Glycine, SDS	4°C	10x Tris Glycine SDS buffer	100mL
	RT	dH2O	900mL
** Running buffer can be **re-used 3x**, therefore mark bottle after every use and store at 4°C! **			

- 4x Sample buffer:

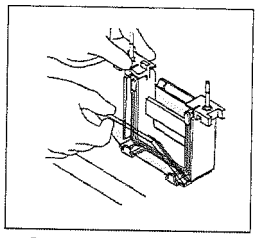
Biorad 4x Laemmli sample buffer (loading buffer)			**Aliquot**
(277.8mM Tris HCl pH 6.8, 44.4% (v/v) glycerol, 4,4% LDS, 0.02% bromophenol blue, 0.2M DTT)	RT (WB cabinet)	4x Laemmli SB	400µL
	RT	1M DTT **Add just before use***	80µL
** Mark DTT-containing SB tubes with preparation date and store at **20°C** (stable for 1w)! **			

Method:

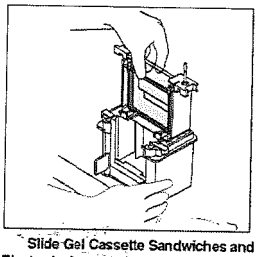
10. Follow the instructions to install the gel cast in the buffer tank:



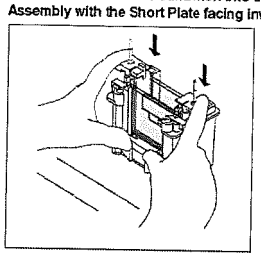
Remove the Gel Cassette Sandwich from the Casting Frame.



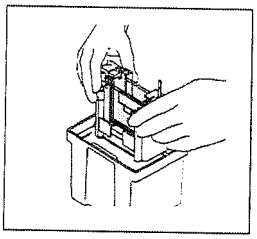
Place Gel Cassette Sandwich into the Electrode Assembly with the Short Plate facing inward.



Slide Gel Cassette Sandwiches and Electrode Assembly into the clamping frame.



Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame.



Lower the Inner Chamber into the Mini Tank.

*** Don't forget to remove and discard the green sticker at the bottom of the precast gels! ***

11. Fill the inner chamber (between the gels) with **running buffer 1x** (stored at 4°C) to detect leakage. Then, fill the tank with buffer until appropriate volume (marked by "2 gels" inscription on tank).
12. Remove the comb and rinse the slots with buffer (using transfer pipet).

13. Take aliquoted samples from -80°C and add the calculated volume of **4x sample buffer**. Vortex and boil the samples at 70°C for 10-15min. Quickly centrifuge before loading.
14. Load the gel with 5µL molecular weight ladder (-20°C) and the calculated volume per sample (**load empty wells** with an average loading volume of **1x sample buffer** for even running).
15. Run the system at 80-100V until the samples pass stacking gel (~30min), increase to run at 120-150V. Wait until the dye reaches the bottom (or lower pink mark leaves the gel), then stop running system.

Step 3: **Western Blotting**

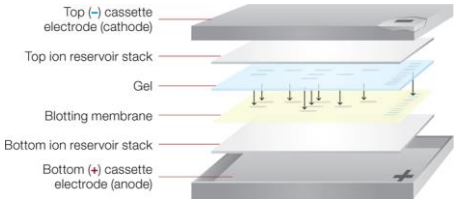
Materials

- Biorad Trans-blot Turbo Transfer System
- Biorad ChemiDoc Imaging system
- Biorad Trans-blot Turbo Transfer RTU (ready to use) Packs (mini format, 0.2µm PVDF, stored at 4°C) **or** Biorad Trans-Blot Turbo RTA (ready-to-assemble) Kit (mini format, mini format, 0.2µm PVDF, stored at 4°C)
- Blot roller

Methods

16. Disassemble the running system, retrieve gels from glass plates and remove stacking gel part using the green plastic gel releaser. Pour the 1x running buffer back in the bottle if it is still re-usable and store at 4°C. Rinse system with dH_2O and clean gel glasses with handsoap.
17. Rinse the gels in MQ water (in a large Petri dish).
18. Quickly **image the gels** to activate proteins with UV (to check equal loading): gently place a gel on the glass tray of the image system, make sure there are no bubbles trapped under it. In the menu choose "gels" → "Stain-free" → "activation 45sec" (intensive or faint bands). Acquired images are automatically saved in the gallery.

19. **A. When using Biorad Trans-blot Turbo RTU packs:** Take 1 Trans-blot turbo transfer pack per gel. Place bottom part of the transfer pack in the tray, roll over to remove the airbubbles. Place the gel on top of the membrane (align the gel straight), cover with the top part from the pack, roll over again. Close the tray and place it in Transfer System (you can fit two trays with 2 mini gels per cassette).



B. When using Biorad Trans-Blot Turbo RTA kit: Soak the PVDF membrane in 100% MeOH until membrane is translucent, then transfer to a gel tray containing 30ml of **1x transfer buffer** (0.5L of 1x transfer buffer =100mL of 5x transfer buffer, 300mL MQ water, 200mL 96% ethanol, store at **4°C**, discard after use) for 2-3min (ensure that membrane is submerged). Wet two transfer stacks (separated by blue sheets) in a gel tray containing 50ml of 1x transfer buffer for 2–3min. Prepare transfer stack: place one wetted stack on bottom of cassette, place wetted membrane above and place gel on top of membrane. If needed, remove air bubbles with blot roller. Finally, place second wetted transfer stack on top of gel (roll the assembled sandwich with the blot roller to expel trapped air bubbles, do NOT pour additional transfer buffer in the cassette). Close the tray and place it in Transfer System.

20. Choose the transfer program based on the amount of gels and the molecular weight of the protein of interest, separately for each tray:
21. After trans-blotting is finished, open the tray and place the membranes in MQ water, discard the rest. Rinse the trays with dH_20, spray them with ethanol and let them dry. Also check the Transfer System machine for the presents of salts and clean with dH_20 and ethanol when needed.
22. **Image the gels** (control for trans-blotting efficiency): "gels" → "stain-free" → "no activation", then discard them.
23. **Image the membranes** (for total protein): gently place membrane on the glass tray of the image system, make sure there are no bubbles trapped under it. In the menu choose "blots" → "Stain-free" → "no activation" (intensive or faint bands). Acquired images are automatically saved in the gallery.

Protocol Name	MW (kD)	Time (min)	2 Mini Gels or 1 Midi Gel	1 Mini Gel
STANDARD SD	Any	30	Up to 1.0A; 25V constant	
1.5 mm GEL	Any	10	2.5A constant, up to 25V	1.3 constant; up to 25V
HIGH MW	>150	10		
LOW MW	<30	5		
MIXED MW	5-150	7		
1 Mini TGX	5-150	3	N/A	2.5A constant; up

Step 3 **Blocking, washing, and incubating**

Materials

- Blocking buffer (5% non-fat dry milk in TBST (or 5% BSA in TBST **exclusively** for phospho-proteins)).

Blocking buffer	Storage	Volume	*10mL*	*25mL*	*50mL*	
5% Non-fat dry milk in TBST	RT*	Non fat dry milk	0.5	1.25	2.5	g
	RT	1xTBST	10	25	50	mL
5% BSA in TBST **ONLY** for P-proteins	4°C (-20°C aliquots)	BSA	0.5	1.25	2.5	g
	RT	1xTBST	10	25	50	mL

*5%Milk-TBST can be kept at 4°C for a week (general use, date on bottle)

- Washing buffers (1x TBS and 1x TBST): can be kept at RT for a week (mark bottle with date).

Methods

24. Rinse membrane with MQ water (3-4 times) before incubation in blocking buffer.
25. Block the membrane at RT for 60 min in the appropriate blocking buffer while rotating (50mL tube = 5mL blocking buffer; 15mL tube = 3mL blocking buffer).
26. Prepare antibodies in the appropriate blocking buffer according to the scheme:

Primary antibody				Incubation in 15mL tube
Rabbit	COL1	1:2000	MW = 129kD	3mL 5% milk-TBST + 1.5µL Ab
	HSP47	1:2000	MW = 47kD	3mL 5% milk-TBST + 1.5µL Ab
	pSmad2	1:1000	MW = 60kD	3mL 5% BSA-TBST + 3µL Ab
	pSmad1	1:1000	MW = 60kD	3mL 5% BSA-TBST + 3µL Ab
Mouse	Smad2/3	1:2500	Smad2 MW = 60kD	3mL 5% milk-TBST + 1.2µL Ab
			Smad3 MW = 52kD	3mL 5% milk-TBST + 1.2µL Ab
	Fibronectin	1:1000	MW = 220kD	3mL 5% milk-TBST + 3µL Ab
	PICP	1:1000	MW = 100kD	3mL 5% milk-TBST + 3µL Ab
	αSMA	1:5000	MW = 42kD	3mL 5% milk-TBST + 0.6µL Ab
	GAPDH	1:5000	MW = 36kD	3mL 5% milk-TBST + 0.6µL Ab
	β-actin	1:5000	MW = 45kD	3mL 5% milk-TBST + 0.6µL Ab

Primary antibodies are stored at 4°C (black box), only COL1 is stored at 20°C (black box)

Secondary antibody			Incubation in 15mL tube
Anti-**rabbit** IgG-HRP (GARPO)	COL1	1:2000	3mL 5% milk-TBST + 1.5µL Ab
	HSP47		
	pSmad1		3mL 5% BSA-TBST + 1.5µL Ab
	pSmad2		
Anti-**mouse** IgG-HRP (RAMPO)	Smad2/3	1:2000	3mL 5% milk-TBST + 1.5µL Ab
	Fibronectin		
	PICP		
	αSMA	1:5000	3mL 5% milk-TBST + 0.6µL Ab
	GAPDH		
	β-actin		

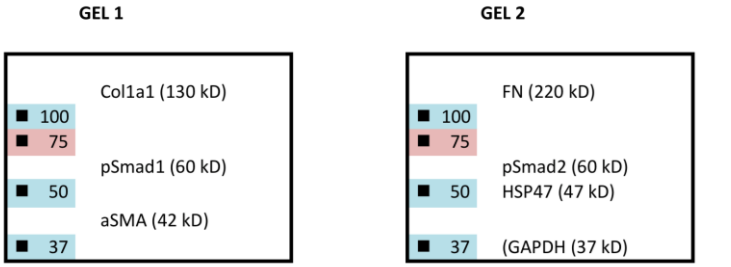
Secondary antibodies are stored at 4°C (white cardboard box)

NOTE:
Prepared antibodies dilutions in blocking buffer can be re-used for 3 days if stored at 4°C. For longer storage keep them frozen at -20°C.

27. *Optional: Ponceau S staining:* Place the membrane in the tube with dye for 5min (rotating). Wash with demi water several times, then image. To de-stain: wash in 0.5M NaF, then in demi water until the pink dye is gone.

	Storage	Recipe	50 mL
Ponceau S (staining solution)	RT	Ponceau S	0.05g
		Glacial acetic acid	2.5mL
		dH2O to	50mL

28. Cut the membrane according to the proteins of interest and the molecular weight ladder (example).



1. Cut between 75 and 100 (COL1)
2. Cut a bit higher or directly at 50 (lower part - aSMA)

1. Cut between 75 and 100 (FN)
2. Cut higher than 50 (but not too high)
3. Cut a bit higher than 37

29. Incubate pieces of membrane with primary antibody at 4°C overnight with continuous rotation.
30. The next day, wash pieces of membrane with **washing buffer (1x TBST)** for 10min, then 5min twice (50mL tube = 5 mL TBST; 15mL tube = 3ml TBST).
31. Incubate pieces of membrane with secondary antibody at RT for 1h.
32. Wash pieces of membrane with **washing buffer (1x TBST)** for 10min, then 5min twice (50mL tube = 5mL TBST; 15mL tube = 3ml TBST).
33. Wash pieces of membrane with **TBS** twice (50mL tube = 15mL TBS; 15mL tube = 7ml TBS) and keep membranes in TBS until the detection.

Step 4 **Chemiluminescence detection**

Materials

- ECL kit (black and white reagents)
- Plastic film
- Biorad ChemiDoc Touch Imaging System

Methods

34. Mix ECL reagents 1:1 (to estimate total amount of the mix: use ~250µL per piece of membrane). For highly abandoned proteins (such as GAPDH) you may use a diluted mix (1:1 in MQ water).
35. Place a piece of membrane on the film, shake off the excess of washing buffer.
36. Drop about 250µL of ECL mix to cover all the area of the piece of membrane, wait for a while, seal with the other plastic film, remove the excess of ECL and air bubbles.

37. Place the film on the glass tray of the Imaging System, choose the right size of the image.
38. Choose “colorimetric” option in “blots” section to detect the ladder.
39. Choose “chemiluminescence” option and detect a band in automatic mode first. After, adjust the exposure time manually if needed (example: first image taken: 120sec; exposure: 1200sec; images: 4). All images are saved in the gallery automatically. Rename the images and copy to USB. Wash the glass tray of Imaging System with dH_2O followed by EtOH 70%.
40. After imaging, wash the membrane in MQ water to remove ECL and dry pieces on filter paper.

Step 6 (additional) **Re-blotting**

Re-blot for the same protein

Methods

41. After imaging, briefly wash membrane in MQ water, then 5-10min in TBST.
42. Transfer the membrane to the primary antibody and incubate at 4°C overnight.
43. Follow further steps according to the protocol.

Re-blot for different protein

Methods

44. After imaging, briefly wash membrane in MQ water.
45. Wash the membrane twice in **stripping buffer** for 10min.

	Storage	Recipe	500 mL	1 L	Unit
Stripping buffer (mild)	RT	Glycine	7,5	15	**g**
Stripping buffer 10 mins x 2 then		SDS	0,5	1	**g**
TBS 10 mins x 2, TBST 5 mins x 2, block		Tween 20	5	10	**mL**
			HCl 25% adjust to pH 2.2		
		Water qs to	0,5	1	**L**

46. Wash the membrane twice in TBS for 10min.
47. Wash the membrane twice in TBST for 10min.
48. Block the membrane in blocking buffer at RT for 1h.
49. Incubate the membrane with the primary antibody at 4°C overnight.
50. Follow further steps according to the protocol.

Attachments

Former homemade lysis buffer formulation:

Lysis buffer	Storage	Recipe	10mL	25mL	50mL	Unit
(30Mm Tris, 150mM NaCl, 1mM EDTA, 0.54% Triton X-100, 1mM Na3VO4, 10mM NaF, 1% SDS)	-20°C	1M Tris HCl pH 7.4	0.3	0.75	1.5	mL
		5M NaCl	0.3	0.75	1.5	mL
		0.5M EDTA pH 8.0	20	50	100	µL
		Triton X-100	0.054	0.135	0.27	gr
		0.5M Na3VO4	20	50	100	µL
		0.5M NaF	0.2	0.5	1	mL
		10% SDS	1	2.5	5	mL
		PhoSphoStop TBS (10x stock)	1	2.5	5	mL
		PIC (protease inh. cocktail)	100	250	500	µL
		Water qs to	10	25	50	mL

Stock solutions for western blot

04/10/14	Storage	Recipe	100 mL	200 mL	250 mL	500 mL	1 L	Unit
1 M Tris HCl pH 7.4	4 °C	Tris base	12,11	24,22	30,275	60,55	121,1	g
		Water	80	160	200	400	800	mL
		25% HCl to pH 7.4 (about 26 mL/250 mL)						
		Water qs to	100	200	250	500	1000	mL
1.5 M Tris HCl pH 8.8	4 °C	Tris base	18,15	36,3	45,375	90,75	181,5	g
		Water	70	140	175	350	700	mL
		25% HCl to pH 8.8 (about 8.5 mL/250 mL)						
		Water qs to	100	200	250	500	1000	mL
0.5 M Tris HCl pH 6.8	4 °C	Tris base	6,06	12,12	15,15	30,3	60,6	g
		Water	80	160	200	400	800	mL
		25% HCl to pH 6.8 (about 15 mL/250 mL)						
		Water qs to	100	200	250	500	1000	mL
10X TBS pH 7.6	4 °C	Tris base	6,06	12,12	15,15	30,3	60,6	g
(10X = 500 mM Tris, 1500 nM NaCl)		NaCl	8,8	17,6	22	44	88	g
		Water	90	180	225	450	900	mL
		25% HCl to pH 7.6 (about 45 mL/1000 mL)						
		Water qs to	100	200	250	500	1000	mL
1X TBS pH 7.6	Room temp	10X TBS	10	20	25	50	100	mL
(1X = 50 mM Tris, 150 nM NaCl)	Fresh dilution	Add water	90	180	225	450	900	mL
1X TBST	Room temp	10X TBS	10	20	25	50	100	mL
(1X = 50 mM Tris, 150 nM NaCl)	Fresh dilution	Tween-20	0,1	0,2	0,25	0,5	1	mL
		Add water	90	180	225	450	900	mL
0.5 M EDTA pH 8.0	RT	Na-EDTA	16,81	33,62	42,025	84,05	168,1	g
		Water	50	100	125	250	500	mL
		5 M NaOH to pH 8.8 (about 14 mL/100 mL)						
		Water qs to	100	200	250	500	1000	mL
10% SDS	RT	SDS	10	20	25	50	100	g
		Water qs to	100	200	250	500	1000	mL
10% APS	4 °C	APS	0,1	0,2	0,25	0,5	1	g
	aliquot	Water qs to	1	2	2,5	5	10	mL
5 M NaCL	RT	NaCl	10	20	25	50	100	g
		Water qs to	100	200	250	500	1000	mL
0.5 M NaF	RT	NaF	2,1	4,2	5,25	10,5	21	g
		Water qs to	1	2	2,5	5	10	mL
0.5 M Na3VO4 pH 10.0	minus 20 °C	Na3VO4	0,092	0,184	0,23	0,46	0,92	g
	aliquot	Water	1	2	2,5	5	10	mL
		HCl or NaOH to pH 10.0 (NaOH first)						
		Boil and adjust pH until colourless and pH stable						
PhosphoSTOP in TBS	4 °C for 1 month minus 20 °C for 6 month	1 tab in 5 mL TBS (1 tablet for 50 mL Lysis buffer)						

Western blot samples

Isolation 1

Western blot	**20 µg per sample**	**20 µl total volume per sample**					
Isolation 1							
Well	*Sample*	*Average concentration (µg/µl)*	*Volume sample (µl)*	*Volume dH2O (µl)*	*Volume 4x sample buffer (µl)*	*Total Volume (µl)*	*Note*
1	MK1	2,49	8,0	7,0	5	20	-
2	MK2	0,18	111,4	-96,4	5	20	No dH2O was added, all of sample was used
3	ML3	2,03	9,9	5,1	5	20	-
5	Step Kg. 1	0,29	68,3	-53,3	5	20	No dH2O was added, all of sample was used
6	Step Kg. 2	0,01	1350,7	-1335,7	5	20	No dH2O was added, all of sample was used
7	Step Lg. 3	0,06	318,8	-303,8	5	20	No dH2O was added, all of sample was used

Isolation 2

Western blot	**20 µg per sample**	**20 µl total volume per sample**					
Isolation 3							
Well	*Sample*	*Average concentration (µg/µl)*	*Volume sample (µl)*	*Volume dH2O (µl)*	*Volume 4x sample buffer (µl)*	*Total Volume (µl)*	*Note*
1	M1	4,92	4,1	10,9	5	20	-
2	M2	4,16	4,8	10,2	5	20	-
3	M3	7,61	2,6	12,4	5	20	-
4	M4	6,49	3,1	11,9	5	20	-
6	Step g. 1	1,75	11,4	3,6	5	20	-
7	Step g. 2	3,38	5,9	9,1	5	20	-
8	Step g. 3	2,95	6,8	8,2	5	20	-
9	Step g. 4	2,39	8,4	6,6	5	20	-

Isolation 3

Western blot	**20 µg per sample**	**20 µl total volume per sample**					
Isolation 4							
Well	*Sample*	*Average concentration (µg/µl)*	*Volume sample (µl)*	*Volume dH2O (µl)*	*Volume 4x sample buffer (µl)*	*Total Volume (µl)*	*Note*
1	M1	9,47	2,1	12,9	5	20	-
2	M2	9,73	2,1	12,9	5	20	-
3	M3	12,86	1,6	13,4	5	20	-
5	step a.1	14,30	1,4	13,6	5	20	-
6	step a.2	13,27	1,5	13,5	5	20	-
7	Step g. 1	11,12	1,8	13,2	5	20	-
8	Step g. 2	8,14	2,5	12,5	5	20	-
9	Step g. 3	7,81	2,6	12,4	5	20	-