Bachelor Thesis

Optimisation of subcellular fractionation of the lung epithelial cells as exploration of the Hippo signalling pathway.

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A word of gratitude

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List of abbreviations

YAP	Yes associated protein
TAZ	WW domain–containing transcription regulator 1
MLE12	Mouse lung epithelial 12
ESC	Embryotic stem cell
IPF	Idiopathic pulmonary fibrosis
SRC	The proto-oncogene c-Src
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
PVDF	Polyvinylidine Fluoride
DMEM	Dulbecco's Modified Eagle Medium
COPD	Chronic obstructive pulmonary disease
TBST	Tris-buffered saline with 0.1% Tween
TF	Transcription factor
TGFB	Transforming growth factor

1 Abstracts

Samenvatting van
Optimalisatie van sub cellulaire fractionering en validatie

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Achtergrond

De ziekte Idiopathische pulmonaire fibrose (IPF) is een ziekte die steeds meer en meer gediagnostiseerd wordt. Helaas is er voor deze ziekte nog geen behandeling beschikbaar. Zelfs een correcte classificatie van deze ziekte staat nog sterk onder discussie aangezien de oorzaak nog niet bekend is. De ziekte heeft een sterke invloed op de alveolaire epitheelcellen waar er een herstructurering en onbehandelbare schade wordt aangericht aan de epitheelcellen waardoor de capaciteit van de long verminderd en de patiënt uiteindelijk zal overleiden aan de exponentiele verspreiding en verzieking van de organen. De Hippo is een signaalweg die in normale omstandigheden een belangrijke rol speelt bij differentiatie en proliferatie van cellen. Bij IPF zal de activiteit van de alveolaire epitheel cellen type II laten wijzigen waardoor deze cellen afgestorven epitheelcellen minder tot niet meer zullen vervangen en het epitheelweefsel gecomprimeerd raakt. De transcriptiefactoren die hierbij een rol spelen zijn YAP & TAZ. Zij zullen samen met onbekende cofactoren transcriptie gaan induceren waardoor transcriptie/ translatie geïnduceerd wordt.

Doel

Het doel van deze studie is om een MS compatibel sub cellulair fractionering protocol te optimaliseren en valideren met westernblotting, vervolgens te gaan kijken hoe de voornaamste fibrotische factor de YAP activiteit beïnvloed van MLE12 cellen.

Methode

De cellen werden uitgepraat en vervolgens gefractioneerd en gevalideerd met de BCA-assay. Na het fractioneren van de cellen werd er ook een full lysate bereid door de cellen in RIPA-buffer te gaan brengen. Vervolgens werd een westernblotting uitgevoerd op een PVDF-membraan en gestained me Lamin B1 en GAPDH.

Tijdens het volgende experiment werden de cellen voor de fractionering uitgehongerd en vervolgens werd TGFB toegevoegd aan de helft en een negatieve controle aan de andere helft. Vervolgens werd na deze behandeling de fractionering uitgevoerd en vervolgens de westernblotting. De primaire antilichamen die gebruikt werden zijn p-smad2, p-YAP & YAP.

Resultaten

De resultaten van de BCA-assay toonden aan dat er geen verschil is tussen cel extractie van cellen die gesuspendeerd of aanhechtend zijn. De resultaten van de westernblotting toonden aan dat de fractionering efficiënt en succesvol werd uitgevoerd. De resultaten van het uithongeringsexperiment toont aan dat wanneer TGFB wordt toegevoegd de hoeveelheid p-YAP toeneemt alsook de heelheid YAP in de nucleus.

Conclusie

Het fractioneringsprotocol werd geoptimaliseerd. Er is geen verschil tussen cel extractie van gesuspendeerde of aanhechtende cellen.
Het induceren van een fibrotische factor als TGFB zal ervoor zorgen dat de hoeveelheid YAP in de nucleus van de cel zal toenemen en dat de hoeveelheid p-YAP in de cel ook verhoogt zal zijn.

Abstract

Optimisation of subcellular fractionation and validation

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Background

IPF or idiopathic pulmonary fibrosis is a disease where the amount of diagnosed cases has been increasing steadily. The cause of this disease is currently unknown and no cure is currently available. The life expectancy of a newly diagnosed patient is 3-10 years. In this disease, many pathways play an important role in the pathogenesis, one of these pathways is the Hippo signalling pathway, where transcription YAP and TAZ influence the ability of the cell to proliferate and differentiate. Studies have already shown that some alteration happens with these factors en lung epithelial cells during IPF causing the lung epithelial cells to change, die and stop proliferating.

purpose

The main goal of this project is to optimize a subcellular fractionation protocol with buffers that are mass spec compatible and validate it using western blotting. When this process was optimised and validated, the activity of YAP was studied with a starvation experiment and later treatment of TGFB

Method

The cells were plated, after which they were fractionated (full lysates using RIPA buffer were prepared as well)and validated using the BCA assay to determine the protein amounts. Next a western blotting on PVDF membrane was performed using Lamin B1(nuclear Stainer) and GAPDH(cytoplasmic Stainer) as primary antibodies on the blot to determine the localisation of the proteins in each fraction.

After the prior experiments (previous paragraph) were analysed and repeated, a second experiment was started where before the commencing of the fractionation, the DMEMF12 medium on each plate was replaced with foetal bovine serum poor medium for 12 hours, after which TGFB was added to the medium with an incubation time of 6 hours, after which the fractionation was initiated and the experiment was performed as previously except working with two conditions: the TGFB treated and the treated with a negative control. After the fractionation, the samples were quantified with the BCA assay after which the westernblotting was performed. The primary antibodies in this case were p-smad2, p-YAP, YAP

Results

The results of the first experiment show how the type of cells that are extracted IE adherent or suspended does not influence the result of the experiment. The results of the westernblotting shows that the isolation of the proteins to each fraction was successful with no contamination of protein that could be visibly seen.

The starvation experiment showed that the treatment with TGFB took effect and that p-YAP could actually be found in the nucleus. The westernblotting from the starvation experiment also showed that TGFB increases YAP activity in the cell and the amount of YAP in the nucleus.

Conclusion

The fractionation protocol was optimised. The TGFB treatment took was administered successfully.
The introduction of TGFB will cause the YAP activity to increase and cause the amount of YAP in the nucleus to increase. P-YAP was found in the nucleus which is unusual but suggested by some papers and requires more research for confirmation.

2 Preface

Lung diseases have been a problem and will remain one for the foreseeable future. Of these diseases, a serious amount is still uncurable and have only limited treatment available (COPD, IPF, etc …). A transplant is an option in some cases but is never a flaw free solution. Complications can arise in many cases and a transplant usually does not have the lifespan of the innate organ of the individual. Among these, IPF is a very important one of these hard to handle diseases and is one of the most common idiopathic interstitial pneumonias. Many studies have shown that the number of cases is rising around the world. One problem is that the diagnoses of IPF have no mandatory registration like cancer. Even with the medicinal technology of our age, the disease is incredibly hard to diagnose Once diagnosed any intervention would be in vein.

One of the primary pathways that have been linked to IPF is the HIPPO pathway. This pathway has mostly been associated with proliferation and differentiation of cells. This pathway inhibits the working of the co-transcriptional factors called YAP and TAZ. These co-factors, when uninhibited, go to the nucleus and combined with other various transcription factors will initiate transcription which in the end will result in proliferation and or differentiation of the cells. Some of YAP and TAZ interaction partners are unknown, however in the context of lung disease, most are not. The discovery of these currently unknown transcription factors is the main goal of the doctoral study performed by Hani Alsafadi from which this bachelor thesis is only a small part. This will be attempted by optimising a subcellular fractionation protocol that is mass spectroscopy compatible.

2.1 Research question/ aim of the project

- The main goal of the project is to optimise the subcellular fractionation protocol which allows separation between the nuclear and cytoplasmic fraction, each fraction must be measurable with Mass spectroscopy.
  - What is the influence of the culturing method suspended vs adherent?
  - How does treatment with TGFB alter YAP activity/localisation

2.2 Problem statement

The disease IPF(idiopathic pulmonary fibrosis is influenced by a signalling pathway called the Hippo pathway. In this pathway two transcription factors named YAP and TAZ play a major role in the proliferation of lung epithelial cells in the lungs. These factors have been noticed to be altered in IPF. It is thought that YAP is phosphorylated in the cytoplasm of the cells and dephosphorylates after which it undergoes translocation to the nucleus to initiate transcription combined with it's cofactors. YAP is viewed as a possible target for medical treatment and has been known to behave differently in the cytoplasm and nucleus of the lung epithelial cells, therefore these transcription factors must be identified, which is the main goal of the overarching study. The goal of this study is to first optimise the subcellular fractionation protocol resulting in two fraction that are mass spectroscopy compatible.

Literature study
3 The lungs

3.1 General structure

The lungs can be seen as a sponge -like air-filled organ situated on both sides of the thorax. However, the position of the heart in the mediastinum in between the lungs results in an uneven in size (right lung is 56% and left lung is 44%). The right lung consists of three lobes (superior, middle, and inferior), while the left lung only consists of two lobes, the right lung contains three. All lobes are separated by fissures, these are the oblique and/or the horizontal fissure. The lungs rest on a series of muscles. Each lobe can be further divided into bronchopulmonary segments (10 in right lung, 9 in left lung). Each of these segments is supplied by one of the segmental bronchi. This modular design of the lung significantly simplifies a surgical procedure on the lung; Instead removing an entire lobe, an individual diseased segment can be removed.(Kotton & Fine, 2008)

The lungs in their entirety are enveloped by a pleural sac that consists of two membranes, these membranes line the pulmonary cavity and adhere to the thoracic wall. The inner pleura or the visceral pleura lines the lungs and is connected to the outer pleura by the pleural fluid. The outer pleura or parietal pleura is connected to the intercostal muscle. When inhaling, the intercostal muscle will cause the lung to expand. When the muscle expands, so does the parietal pleura. Interestingly, there is no solid connection between the partial pleura and the visceral pleura, the tension created by the pleural fluid between these two is sufficient to let the visceral pleura connected to the lung expand with the partial with a force strong enough for a proper respiration of an individual. (Franks et al., 2008)

When inhaled, air that travels into the lung is further guided by the tracheobronchial tree. This tree allows air to travel to each specific section of the lung. Every passage from the trachea to the bronchioles is part of this fascinating structure. The first division that is created in the trachea is the division in between the left and right bronchus each bronchus enters the lung through an entry point called the hilum. The hilum is not only the entry point for the bronchus but for other vessels like blood and nerves as well. The collective of these three “vessels” at the entry point are called the root of the lung. Interestingly, the right bronchus is slightly wider due to its higher capacity. This means that objects entering through the thorax have a higher chance of causing an obstruction in the right lung.(Barkauskas et al., 2017; Franks et al., 2008)

3.2 Lung epithelium

The lung epithelium is most exposed layer of the complex branching system in the lung; therefore, it must be able to provide any kind of necessary protection against outside dust or microorganisms. The epithelium of the lungs can be divided into two regions with two regions: proximal and distal. Each of these regions can be recognised by both their position 411 amongst an axis in the lung and their cellular composition. The distal regions constitute the alveolar spaces and consist of alveolar type I and type II cells while the trachea and main bronchi in the proximal region is build-up of mainly ciliated, basal cells and goblet cells which form the columnar epithelium (Fig. 1). The difference between the proximal and distal region is important to understand. Distal regions consist less of basal cells but of an epithelial surface with an increasing ratio of ciliated epithelial cells. The Bronchiolar and alveolar region are separated by the BADJ (a region that harbours a variant of cells which possesses airway epithelial regenerative potential). The Alveolar epithelium consists mostly of the flat epithelial cells type I which covers most of the surface area, type II cells have the ability to proliferate and differentiate in to the type I cells.(Rackley & Stripp, 2012)

The lung, in a healthy state is remarkably non proliferative. Although the lung cells have a slow rate of mitosis and a prolonged survival, the epithelial cells type II can switch to rapid proliferation and differentiation to epithelial cells type I in the alveolar region which allows replacement of the damaged cells that were exposed to toxins or pathogens. The conducting airway is built up from numerous cells. This includes subsets of basal and non-ciliated columnar cells that have the ability to proliferate into other epithelial cell types like goblet or ciliated cells. (Barkauskas et al., 2017; Gokey et al., 2018)

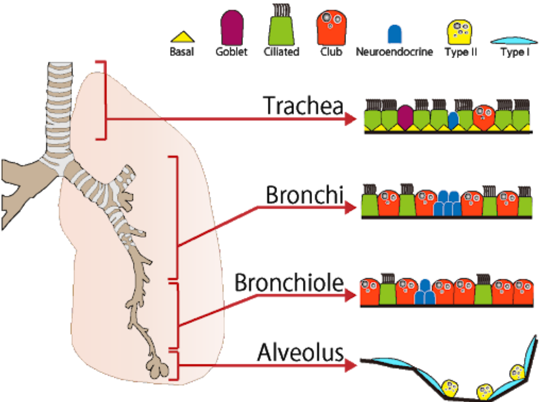


Figure 1: build of epithelium throughout the lung

3.3 Lung diseases

3.3.1 Obstructive vs restrictive diseases

One of the first steps in diagnosing lung diseases is differentiating if the disease is an obstructive or a restrictive disease. Both cases are related with a shortness of breath, it has been known that obstructive diseases are more related with a higher difficulty when exhaling, while restrictive diseases are more related to problems when inhaling. This difference however slight it may seem but is important in diagnosing and estimating the residual capacity.

The obstruction caused by asthma or COPD (chronic obstructive pulmonary disease) occurs due to inflammation and swelling. Because of this, a high amount of volume of air can get trapped behind these restricted airways, drastically decreasing the volume of air available.

Restrictive diseases in contrast to obstructive diseases limit the amount of air that can be inhaled in normal conditions. This mostly occurs due to difficulty's filling the lungs completely by (intrinsic (stiffening of the lung), extrinsic (problem originates from outside of the lung) or neurological factors (neurological disorders that limit the amount of air that could be inhaled). (Hillas, Perlikos, Tsiligianni, & Tzanakis, 2015)

3.3.2 COPD

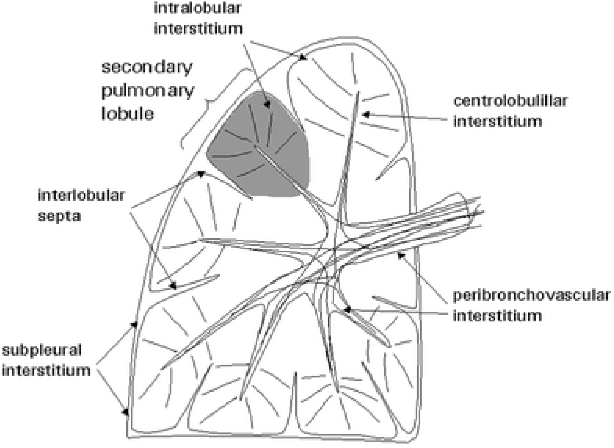
COPD or chronic obstructive pulmonary disease is categorised as an irreversible inflammatory disease with the most renowned symptom known as the difficulty that the patient experiences when exhaling. Other symptoms can include chronic coughing, fatigue, a tightness in the chest and a production of mucous. The most common cause for COPD is smoking (both active and passive) and mostly work-related pollution (factory fumes, etc…) COPD can be a genetic disorder in very rare cases where the problem is caused by a deficiency in alpha-1-antitripsin. COPD proceeds in 4 stages, starting at mild and ending at very severe. In the latest stage, COPD can cause the patient to decease.

4 Idiopathic pulmonary fibrosis (IPF)

4.1 Lung epithelium in IPF

IPF or idiopathic pulmonary fibrosis is a chronic and progressive disease. As of this yet there is no known cure and is in most cases lethal. The symptoms of the disease are irreversible, and the cause is unknown. IPF will in most cases occur with patients that are middle aged or older. The disease is limited to the lung and the radiological pattern that occurs is typical for an interstitial pneumonia. This means that a progressive scarring of fibrosis will occurs in the gas exchange of the lungs (interstitial (where it permanently damages the alveoli).

The different features are usually optimally visible under a low magnification and most notably are the subpleural and parastatal fibrosis (scaring at the alveoli in the distal region)



Honeycombing (figure 2) is another typical pattern that can be seen in IPF. The formation of fibrotic airspaces as a radiological pattern. The space created by this process is not empty but filled with inflammatory cells and mucin. The airspaces are usually lined by bronchial epithelium. There are often areas where the parenchyma(tissue) is less or even not affected. Collagen deposition usually plays an important role during wound healing where it supplies the wound with strength and is broken down after its role is fulfilled in normal conditions.(Martinez et al., 2017; Schwartz, 2018)

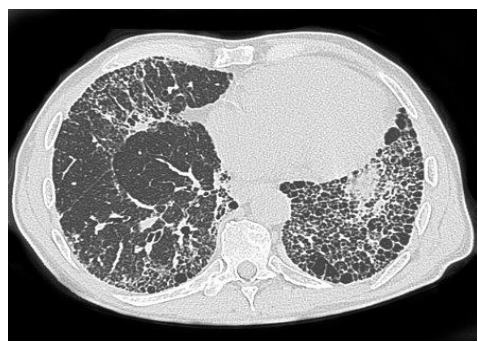


Figure 2: IPF lungs with honeycombing

Interestingly. During IPF distal region epithelial cells start to show immunological markers from cells that originate in the proximal airway epithelial. The reason why these markers originating from cells (like goblet and basal cells) are restricted to different parts of the lung. When fibrotic lesions occur and cause honeycombing that replaces the usual mainly niche consisting of alveolar type I and type II cells. When a genome wide analysis is performed on healthy and fibrotic epithelial cells, there have been some extraordinary results showing dramatic changes in the gene expression by these "intruders" in the interstitial lung epithelium. It is known that mutations in the type II alveolar cells cause interstitial lung diseases (ILD).

IPF results in the remodelling of the lung parenchyma, which results in loss of gas exchangeable regions. The normal niche of alveolar epithelial cell types I and II becomes replaced with lesions containing mucosal cells and other unusual cells.

4.2 Working mechanism of IPF

It is not unusual for lungs to be exposed to injury. Their ability to constantly repair any damaged tissue is truly remarkable. This is possible through a very finetuned mechanism of certain biological processes by the activated mesenchymal cells. However, when repetitive alveolar epithelial injury occurs or the cells become genetically susceptible due to some of the previous mentioned factors, it could lead to an epigenetic (alterations to the DNA brought on by outside factors) reprogramming to the cell which could lead to an excessive activation of mesenchymal and overproduction of profibrotic mediators. This results in fibrosis, but it will take years for any notable symptoms to develop. It has been noted that there have been clear signs of an accelerated cell aging, mostly in alveolar epithelial cells(type2) whose main function is the production of surfactant (has a detergent like working where it lowers the surface tension around the alveoli and inhibits alveolar collapse during respiration). These cells also produce the alveolar type I epithelial cells during lung regeneration. These functions make these cells critical to the normal functioning of the lung.

The working mechanism of this disease is extremely complicated and relies on an exceeding amount of cell signalling pathways, enzymes and fibrotic mediators. This means that it is challenging to develop effective therapy. Most of the current research is focused on the mechanism and attempts to discover possibilities to disable these.(Martinez et al., 2017)

(Yan, Kui, & Ping, 2014)

4.2.1 Pathogenesis

The old assumption and strongly questioned theory about IPF is that it is an inflammatory disease that takes place due to a chronic inflammation that causes inflammatory cells to be recruited and a release of cytokines, which activates the fibroblasts to proliferate and initiate the deposition of the extracellular matrix (ECM). One of the strong reasons why this theory has been questioned is the fact that anti-inflammatory pharmaceuticals have an effectiveness next to none. This proves that the inflammation is not the main pathogenic process that takes place during idiopathic pulmonary fibrosis (IPF).

One of the main characterisations of IPF is the extreme activation of alveolar epithelial cells (type I and II), myofibroblasts, fibroblasts, combined with a high production of extracellular matrix (ECM). All of these previous mentioned will cause the architecture of the lungs to be altered and result in an inability for a proper respiration.

The working mechanism or cause of IPF is to this day still an enigma. Even the classification (genetic or mucociliary) is unknown. However, mounting evidence suggests that the main factors that influence the development are genetic instability, the attrition of telomers due to aging, mitochondrial dysfunction, insufficient autophagy (installed self-devouring of the cell) or epigenetic alterations. It has been observed that the epithelial cells in developing IPF are going into a mode of senescence. This combined with mutations in the genes and alterations in transcriptional outputs supports the fact that the epithelium slowly starts to fail over time.

The hypothesis has been proven that the single nucleotide polymorphism identified as rs35705950 in the MUC5B gene's promoter region poses a high risk in the formation of IPF. The risk is strongly associated with inhalation of wood/metal particle, smoking, infection. Under these previous mentioned and vastly associated factors with IPF, can cause the alveolar epithelial cells type II to become senescent much faster than in normal "healthy" circumstances. These cells will in this case start displaying a senescence associated secretory phenotype (SASP), where the alveolar epithelial cells type II start to release chemokines and proteases, which will induce inflammation (+oxidative stress) and contribute to the accumulation of extracellular matrix caused by fibroblasts in myofibroblast. Eventually this will lead to the alterations in the alveolar architecture. (Gokey et al., 2018; Yan et al., 2014)

(Yan et al., 2014)

4.2.2 Influence of myofibroblasts & fibroblasts on the pathogenesis

Myofibroblasts originating from mesenchymal transition and fibroblasts (activated and translocated fibrocytes) play a major role in the fibro genetic process. Myofibroblasts will deposit the extracellular matrix (ECM) which consist of collagens, proteoglycans and glycoproteins. Due to the overproduction of the ECM caused by the myofibroblasts, more and more cells will become compromised as for the lung architecture, it will be ultimately destroyed by this matrix. This will lead to a disruption of the lung function. The important regulators during this fibrotic process that have been identified are PDGF, TGF-B,TNF-α, endothelin-1 and C-C motif chemokine ligand 2. TGFbeta is regarded to be the most important and strongest pro fibrotic mediator that is proven to cause ECM production by use of the TGF-beta/smads pathway.(Gokey et al., 2018; Schwartz, 2018)

4.3 Mechanosignaling in IPF and the hippo pathway

The centre of mechanosignaling in IPF is the pathological matrix. This matrix causes the fibroblast activation that drives the progression of the fibrosis. This has caused the identification of the mechanisms that stimulate this response from the fibroblasts to the matrix is seen as one of the high priority's during research in this disease.

The role that YAP and TAZ play in this form of signalling is important and play a strong regulatory role during the development of the disease. Both YAP and TAZ are expressed in an over plentiful amount in the spindle shaped fibroblastic cells and there is a strongly nuclear localisation of both YAP and TAZ. TAZ is strongly found in the nucleus of epithelial cells during a stiffening of the extracellular matrix. This localisation will increase the stiffness of the matrix even further and drive a more localisation of YAP and TAZ in the fibroblasts (and nucleus of epithelial cells). The link between matrix stiffness and fibroblast activity has been proven by deliberately introducing a YAP-TAZ siRNA knockdown. This results in a strong downturn in matrix synthesis, most noticeably on pathogenic matrices. (Brodowska et al., 2014)

Fibroblasts that were introduced with a mutated YAP or TAZ (the mutation makes them constantly active) have been even known to overcome a soft matrix and its limitations for a fibrotic reaction.

4.3.1 YAP in IPF

During IPF in epithelial cells, YAP will have an increased nuclear activity while its inhibitor MST1/2 is decreased. Experimental results have also shown that one of YAP's known transcriptional targets called Ajuba was increased. And is verry detectable in epithelial cells present in the lesions formed by IPF. Even more interesting is that the actual lung epithelial cells type II that are fibrotic) rarely show any Nuclear(active) YAP while MST1/2 levels are highly elevated. Epithelial cells type II usually perform maintenance and repair while they also secrete surfactant and perform transepithelial movement. It is very interesting that during IPF their proliferation and differentiation is inhibited combined with the high presence of MST1/2.

(Barry & Camargo, 2013; Gokey et al., 2018)

5 The Hippo pathway

5.1 General

The hippo pathway is most known for regulating the growth, proliferation and differentiation of the cells. However, it's mechanism can be significant in different cell types. When the hippo pathway is promoted, it will activate LATS kinase which will inhibit the activity of transcriptional cofactors named YAP and TAZ. The hippo pathway can be seen as a sensor which takes different mechanical and chemical inputs into account and makes the organoid adjust to the altered condition (organ size, overgrowth of cells). The Hippo pathway was first discovered in the *Drosophila* (fruit fly) when tumour suppression genes in this organism were "knocked out" and an overgrowth occurred as a result. Over time some of these tumour suppressors were linked to the hippo pathway and were named. After some research, a mammalian equivalent was found named YAP and TAZ. Yap and TAZ are responsible for the differentiation, proliferation and apoptosis of the cell. The Hippo pathway inhibits any of this process until it is deactivated through upstream chemical or mechanical cues. After which both YAP and TAZ will initiate the transcription of many proteins that play an important role during differentiation, proliferation or apoptosis.(Liu et al., 2015; Misra & Irvine, 2018)

5.2 Function of the hippo pathway

The Hippo cascade is a growth suppressing signalling pathway that is highly conserved. It was initially discovered in the drosophila where somatic mutations in components of this pathway resulted in the dramatic overgrowth of the influence tissue. The pathway consists of a biochemical phosphorylation cascade that controls the transcriptional outputs. It has been discovered that this same pathway is also found as a conserved size regulator in the mammalian cell. The exact trigger of the hippo pathway is a controversial subject with numerous proposed mechanisms, some of these mechanisms are cell-cell junctions or a G-protein coupled receptor signalling. (Barry & Camargo, 2013)

(Barry & Camargo, 2013)

5.2.1 The core kinase cascade of the hippo pathway

The core of the hippo pathway in mammals is viewed as a kinase cascade in which the Ste20-like kinases 1 and 2 phosphorylate and activate the large tumour suppressor called LATS 1 and 2. The result of this cascade is a restriction of the Yes associated protein or YAP. YAP and TAZ are active, they translocate to the nucleus where they cause the expression of a wide range of genes that play an important role to the proliferation and differentiation of the cell.

The LATS kinases play a key role in the hippo pathway since they are direct regulators of YAP and TAZ. LATS kinases themselves are activated by MLST1/2 by phosphorylation.(Barry & Camargo, 2013; Misra & Irvine, 2018)

5.3 Working of the hippo pathway

The hippo pathway as it is known in mammals is as seen from a classical view a cascade in which the Ste20-like kinases ½ (MST1/2) phosphorylate and activate the large tumour suppressor ½ (LATS1/2) this activation will restrict the activities of the Yes associated protein or known as YAP and the transcriptional coactivator with PDZ binding motive or known as TAZ. TAZ is the mammalian homologue of the drosophila Yorkie. YAP and TAZ will translocate to the nucleus when active where they will bind to the TEAD transcription factor. After the binding, the restriction factors will induce expression of a great collection of protein that will contribute to not only the survival of the cell, but to the proliferation and differentiation as well. The Hippo kinase cascade. (Hansen, Moroishi, & Guan, 2015)

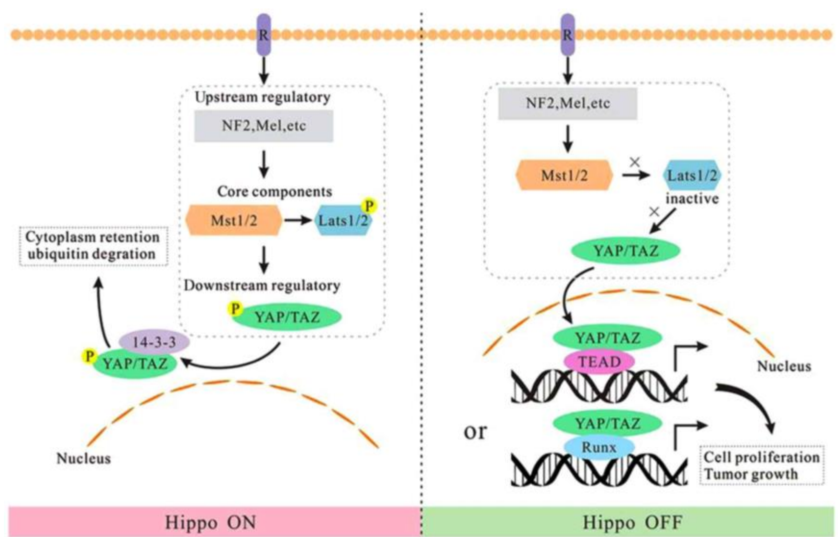


Figure 3:working of the Hippo pathway

5.4 Upstream factors that regulate the hippo pathway

Studies conducted in the last decade have cemented that YAP and TAZ are the major effectors in the hippo pathway. This regulates in a cytoplasmic retention and protein degradation of YAP and TAZ which is phosphorylation induced. This is a response to a myriad of intrinsic and extrinsic signals. These signals modulate in most scenarios' phosphorylation events of the core kinase cascade. This happens through peripheral components of the Hippo pathway. There are several proteins that can directly regulate the YAP localization or transactivation without having an effect on the LATS kinase activity. The crosstalk between the Hippo pathway and the Wingless/ints (Wnt) and some other pathways modulates the activity of YAP and TAZ.

5.4.1 Upstream signal 1: physical cues

There are many coordinated actions of cells during organ growth and development required to adapt to physical restraints or extracellular mechanical cues. The architecture of tissue creates a physical restriction on cell growth and proliferation which eventually will lead to a quiescence or dormant state of the cells in which the proliferation grinds to a hold. A strong example of this is when cell-cell contact in a high cell density creates a growth inhibitory signal that partially is mediated by the Hippo pathway. LATS kinase is as a result of this activated while it notably is inactive at a low cell density. The inactivation of YAP is critically important for cell contact inhibition in a cell culture. During the embryonal development is the inactivation of YAP critical

for the cell contact inhibition. A regulation of the YAP- TEAD transcription program by contact inhibition is crucial for embryonal development as well. The increased number of adherents and tight junction in confluent cells contribute to the activation of LATS and an inactivation of both YAP and TAZ. Furthermore, a decreasing in cell spreading or size is involved with the activation of YAP and TAZ as well since it has been discovered that the rigidity of and stiffness of the extracellular matrix (ECM) regulates YAP and TAZ subcellular localization. This happens through both changes in the geometry of the cell and a cytoskeletal tension. The cells need to be able to attach to a cytoskeletal matrix to be able to survive. This attachment induces a nuclear localization of YAP through the activation of RHO-GTPases or the FAK- Sre-PI3K (g-signalling protein) pathway. A disruption of F-actine is able to block the effect of attachment on YAP phosphorylation and nuclear localization.

Detachment of cells inactivates YAP and TAZ and triggers kinases in a LATS dependent matter. The attachment of the cell thus supplies a mechanical signal to the cell this is the case in culture plates where there is a high stiffness. The activity of YAP and TAZ are also modulated by stretching in an epithelial sheet.(Hansen et al., 2015)

5.4.2 Upstream signal 2: soluble factors and G-protein coupled receptors.

The ability for tissue to grow has a certain requirement of nutrients and hormonal signal via autocrine, paracrine and endocrine mechanisms. The uptake of nutrients is under mediation of growth stimulating signals. There has been a long speculation that it is in fact hormones or growth factors that regulate the hippo pathway, this is in order to maintain the homeostasis. The discovery that certain diffusive molecules like sphingosine-1-phosfate and lysophosphatidic acid (LPA) activate and stabilize YAP and TAZ through their G-protein coupled receptor (GPCR) and LPA receptor. A series of further studies did demonstrate that GPCR’s regulate the Hippo pathway as a universal respond to hormonal cues. Rho-GTPases mediate the working of GPCR on YAP and TAZ. Rho- GTPases are activate by $G\alpha_{12/13}$- and $G\alpha_{q/11}$-coupled GPCRs, and itself inactivates LATS1/2(the mechanism that does this is as of yet unknown but is dependent on F-actin assembly).(Barry & Camargo, 2013)

5.5 Hippo pathway and organ size

The regulatory system behind the organ size has always remained one of the most mysterious questions in biology. Classical experiments that were performed in the 60’s have demonstrated that there was an exquisite mechanism that senses organ size actively regulated by the organism. This theory is supported by an experiment in which multiple spleens of roughly the same size were transplanted from small dogs into recipients that were much bigger than the donors. Impressively the transplanted organs grew to a normal size (considering the girth of the receiver). Similarly, the same results have been observed in a baboon to human liver transplantation where the baboon liver doubled in size after a mere 24 days. These observations suggest that there is a stringent control over the growth and organ size ’memory’. The secreted negative growth regulators or chalones are supposed to serve as mediators of the tissue size. Due to lacking evidence, they have remained a theory. The identification of myostatin a chalone like muscle specific growth regulator is something that as of yet remains unclear.(Barry & Camargo, 2013)

5.6 Hippo pathway in development and disease

5.6.1 Regulation of cell fate decisions during embryogenesis by the Hippo pathway

Both YAP and TAZ protein are expressed during the early embryogenesis. An experiment showed that *Taz* knockout mice can develop into adults without noticeable complications, while mice that have a deletion of *Yap* will result in death of the embryo, this is due to a yolk sac defect. The role that both *Yap/Taz* play during embryogenesis was uncovered with help of Tead4(primary nuclear binding partner of YAP and TAZ). This protein will induce Cdx2, a transcription factor that plays an important role during the development of the trophectoderm. The trophectoderm consist of the outer cells from a blastocyte stage embryo, where Tead4 expression was found in both these outer cells and the inner cells. It became very clear that this Tead4 binding partner was Yap which can be found nuclear in the outer cells and in a more cytoplasmic position within the compacted inner cellular mass. This means that the more outside cells will be prone to proliferate and differentiate while it is the opposite in the more inner cells. A knockout of both Yap and Taz will cause the embryo to die in the morula stadium. The loss of the Yap function in outer cells will result in suppression of *Cdx2* and a loss of *Lats1/2*. The loss of both *Lats1/2*(in the cells has a strong effect on the inner cells of the embryo where it causes an overexpression of *Cdx2*. These experimental results demonstrate how the hippo pathway plays an essential role in the mammalian embryotic development. (Barry & Camargo, 2013)

5.6.2 Yap throughout Embryotic stem cell differentiation

The intriguing note about the hippo pathway is that it appears at a crossroad of both differentiation and reprogramming of cells to a pluripotent state. Yap is found in the nucleus in embryotic stem cells, but the Yap nuclear localisation and protein levels are all but diminished during the embryotic stem cell (ESC) differentiation, this can be linked to the increased Hippo pathway activity (proven by the phosphorylation of yap). Overexpression of YAP will lead to an increased programming of fibroblasts to induced pluripotent stem cells, but a total loss of yap will result in a loss of embryotic stem cells pluripotency.

A possible explanation of the transformation mechanism of fibroblasts to induced pluripotent stem cells (IPS):

Yes-kinase phosphorylation of Yap which causes the induction of a YAP/TEAD2 gene expression program. The now formed TEAD2 binds directly to *Oct3/4*(promoter who is now activated. The Yes-kinase causes an increase in the activity of *Oct3/4* and the *nanog* promoter. This all suggest that the activity of YAP enhances the reprogramming process and that the main function of the Hippo pathway is to serve as a barrier against total pluripotency. (Barry & Camargo, 2013; Liu et al., 2015)

5.6.3 Hippo/ WNT crosstalk in the intestinal cells

The intestine is a fascinating organ to study the behaviour of stem cells in because the intestinal cells renew themselves approximately once every week. This makes it ideal for studying the stem cell dynamics. The main factor that promotes this self-renewing capacity is called the Wnt signalling pathway which instructs the intestinal stem cells or ISC's to self-renew and retain certain components. Beta catenin is the main driving force behind the Wnt signalling pathway. Beta catenin is usually marked for protein degradation by a destruction complex. Wnt ligand in the intestinal crypt instructs the ISC under normal condition to induce the beta catenin transcriptional program. When a component the protein destruction complex is mutated making it unable to degrade beta catenin which causes an accumulation of this protein in the cell and induces a transcriptional program when it enters the nucleus. This causes the cell to form an adenoma(cancer) which is probably derived from an ISC.

There are several pieces of evidence that suggest that the Hippo signalling plays a vital role in the ISC. Yorkie activity is induced in the enterocytes of a drosophila's intestine when a disruption of Hippo upstream components. This can lead to a non-cell-autonomous activation of proliferation through an upregulated unpaired cytokine expression. Yorkie causes the same process when there is tissue damage or disruption of Hippo. This happens specifically in the intestinal stem cells. The role of YAP in the mammalian intestine is as of yet unclear. Overexpression of YAP in mouse tissue has resulted in a loss of differentiation markers and an expansion of the undifferentiated cell population in the intestine of the mouse.(Liu et al., 2015; Piersma, Bank, & Boersema, 2015; Varelas, 2014)

5.7 Mechanotransduction of YAP and TAZ in diseases.

There has been a noticeable association between mechanical signalling and the pathogenesis of a disease. This association was probably caused by changes in both the structural and physical features of the microenvironment of the cell or by defects in the receiving mechanical input of the cell. It is most impressive how many of these defects eventually will affect the cell and its behaviour through a de-regularised YAP and TAZ.(Panciera, Azzolin, Cordenonsi, & Piccolo, 2017)

5.7.1 Mechanotransduction and tissue fibrosis

Tissue fibrosis is caused by many fibrotic diseases like liver cirrhosis, pulmonary, cardiac and kidney fibrosis and systemic sclerosis. This kind of diseases are responsible for at least 33% of the deaths. These diseases have some common traits like the excessive production of Extra cellular matrixes, this process is activated by fibroblasts. This process will resolve in tissue stiffening, cellular dysfunction and organ failure in its final stage. It has been noticed that the YAP and TAZ mechanotransduction plays an important role during the fibrotic process.(Liu et al., 2015; Panciera et al., 2017)

5.7.2 Liver fibrosis

During the process of liver fibrosis, YAP is activated in the hepatic stellate cells. This is as a response to liver damage in vivo or to stiffening of the extracellular matrix ex vivo. The YAP activation by damage to the hepatic stellate cells triggers translocation of YAP to the nucleus and an increase in total YAP activity, which will result in an increase in collagen deposits and smooth muscle actin expression. Both smooth muscle actin and collagen are associated with the production of myofibroblasts. Experiments showed that myofibroblasts have a strong YAP nuclear staining in addition to that it is known that hepatic stellate cell activation could be reversed with a pharmacological inhibition of YAP. This concludes that YAP and TAZ will be essential in the development of a treatment to fibrosis in the liver as they play an important role in the regulation of this process.(Panciera et al., 2017)

5.7.3 YAP and TAZ during cancer development

YAP and TAZ are noticeably activated in human cancers where they induce the malignant properties of the tumour like the unrestricted proliferation or cell survival and the chemoresistance (therapeutic resistance) and metastasis (spread of cancer cells over a new area). A possible explanation of these effects is that activated YAP and TAZ can convert benign neoplastic (neoplastic is tumour like cell growth) cells into cancer stem cells. While both YAP and TAZ activity is widespread in tumours, there have been no signs of a mutation in *yap or taz*(genes) in human cancer cells. The Hippo pathway itself has never been found mutated in any researched cancer. This means that it is mostly mechanical inputs from the microenvironment that causes the YAP and TAZ overactivity in the tumour cells. These inputs include aberrant tissue organisation, accumulation of stromal cells, inflammation, increased compression forces and interstitial pressure, metalloproteinase-mediated ECM remodelling by cancer-associated fibroblasts (CAFs) and overall extra cellular matrix stiffening.

The regulation of YAP and TAZ by the cytoskeleton could be a representative of an interesting target for the design of anti-cancer therapeutics in the future.(Varelas, 2014)

5.7.4 YAP and TAZ as mediators of an inflammatory response

Inflammation is a natural response to injury, where leukocytes and plasma proteins are recruited. During inflammation, a fibro genetic response and extracellular matrix remodelling plays an important role as well. The acute inflammation is integral for the regeneration of damaged tissue and stops after the healing process has been completed. In the context of YAP and TAZ, YAP mediates the regeneration of epithelial tissue after inflammatory damage. The activation of YAP is in this context independent from Hippo/LATS, but it is dependent on SRC-family kinases. The activity of the proto-oncogene c-Src (SRC) works as an inducer on cytoskeletal remodelling. There also is a possibility that inflammation activates YAP and TAZ as well by inducing an increased Mechano-responsiveness in the epithelial cells.

The link between inflammation and YAP and TAZ mechontransduction is most appealing in the study of chronic inflammatory disease which in difference to the acute inflammatory processes are represented by persistent conditions that are characterised by a constant damaging and repair of the tissue what will resolve in a progressive erosion of the tissue. The causes of a chronic infection are as of yet poorly conceptualised and understood.(Varelas, 2014)

Practical work
6 Materials and methods

6.1 Cell culturing and maintenance

All experiment were performed using an immortalized cell-line, MLE12 cells. All MLE12 cells used were cultured in t-75 flasks prior to any experiments. These cells are originally stored at -160°C First cryotube containing the cells was placed on dry ice to keep the temperature at an optimal level. The medium, Dulbecco's Modified Eagle Medium (DMEM F12) supplemented with Foetal bovine serum (FBS) (10%) and placed in the warm water bath at 37°C. Next, the cells were thawed by holding the cryotube in the warm water bath until only a small particle remains. The cryovial is disinfected and placed under the laminar hood. The cells were displaced to a 15ml falcon tube using a 1ml micropipette. Next droplets of the DMEM f12 medium are added to the falcon which transition into a stream until a volume of 10ml is reached. Finally, the cells were moved from the falcon tube to a t-75 culture flask which was swirled and next incubated at 37°C with a carbon dioxide level of 5%.

The medium of the cells is refreshed every 2-3 days until an experiment is started. When the cell line had to be kept but a confluency of 85% was reached when no experiments had to be started, the cells were split. This was done by first heating up trypsin (0,005%), phosphate buffered saline PBS and medium in the warm water bath at 37°C. after all of these solutions were sufficiently heated, the cells were washed with 10ml of PBS for 2min. Next, the PBS was removed, and 2 ml of trypsin was added. The cells were incubated with the trypsin for 5 minutes. After the incubation, the flask was hit on the side with the hand to make sure that all the cells were loosened from the surface of the flask. After this, 8ml of DMEMF12 contain 10% FBS was added to the cells and used to wash the surface of the flask to remove any residual cells. Finally, 500µl of the medium and cells was translocated to a new flask and supplemented with 9,5ml of DMEM F12 containing 10%FBS. In advance of our experiments, two T-75 flasks were created with these steps.

6.2 Cell experiments for subsequent fractionation

The experiment where cells plated on Petri dishes were needed, we commenced by washing the t-75 culture flasks with 10ml of PBS. Next, 2ml of trypsin was added after which the culture flasks were incubated for 5 minutes at 37°C with a CO_2 level of 5%. After 5 min incubation, the culture flasks received three mechanical shocks on the side to remove the adhering cells from the flask. Proceeding, 8ml of DMEM F12 was added to each of the flasks. Next, the flasks were put into an upright position to inhibit re-attachment of the cells to the adhering site. And the entire contents of the flasks were combined in a 50ml falcon tube. Next the cells were counted by bringing 10µl of the suspended cell solution in a 96 well plates well together with 10µl of methylene blue. The cells are then counted using a Neubauer chamber. Next, the cells are plated on 4x10cm petri dishes at a concentration of 150.000 cells/ml. finally the cells are incubated at 37°C with a CO_2 level of 5% until a sufficient confluency is reached.

6.2.1 Experimental design of subcellular fractionation

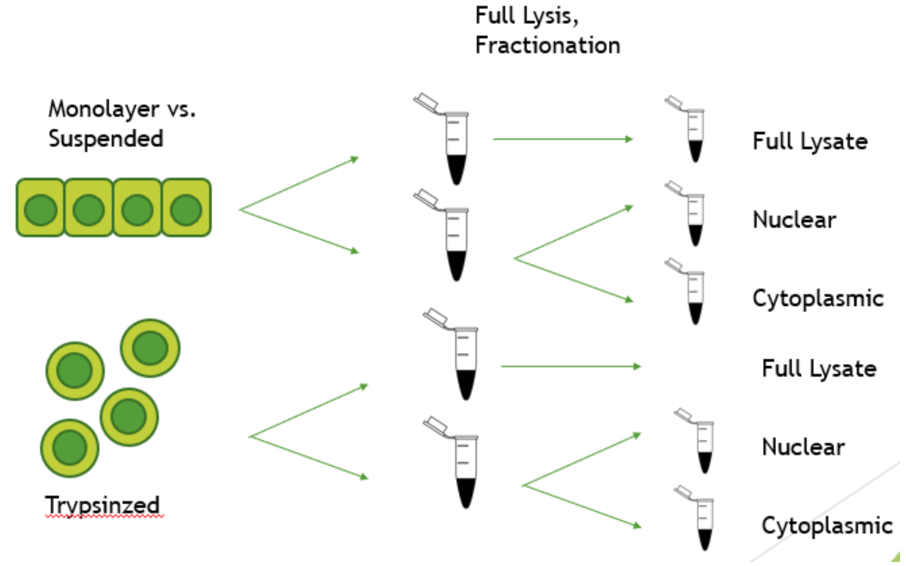


Figure 4:Experimental design of subcellular fractionation

6.3 TGF beta experiment

Prior to the cell extraction of subsequent fractionation, all plates were starved and proceeding, 4 plates were treated with TGFB and 4 with a negative control.

First, the plates were checked under the microscope to ensure sufficient confluency and that the cells are in good health. Next, all the medium was removed using suction and starvation medium was very carefully brought on to the plate. The experiment was proceeded by incubating the cells for 11 hours in the incubator at 37°C with a CO_2 level of 5%. After this incubation time, the starvation medium was removed followed by adding 12ml fresh FBS free medium containing 6 µl of TGF Beta to four plates and 6µl of 4mM HCl in 0,1% BSA in PBS to the other four plates. This treatment was incubated for 6 hours and is stopped by initiation of nuclear fractionation.

6.4 Experimental design of the starvation experiment

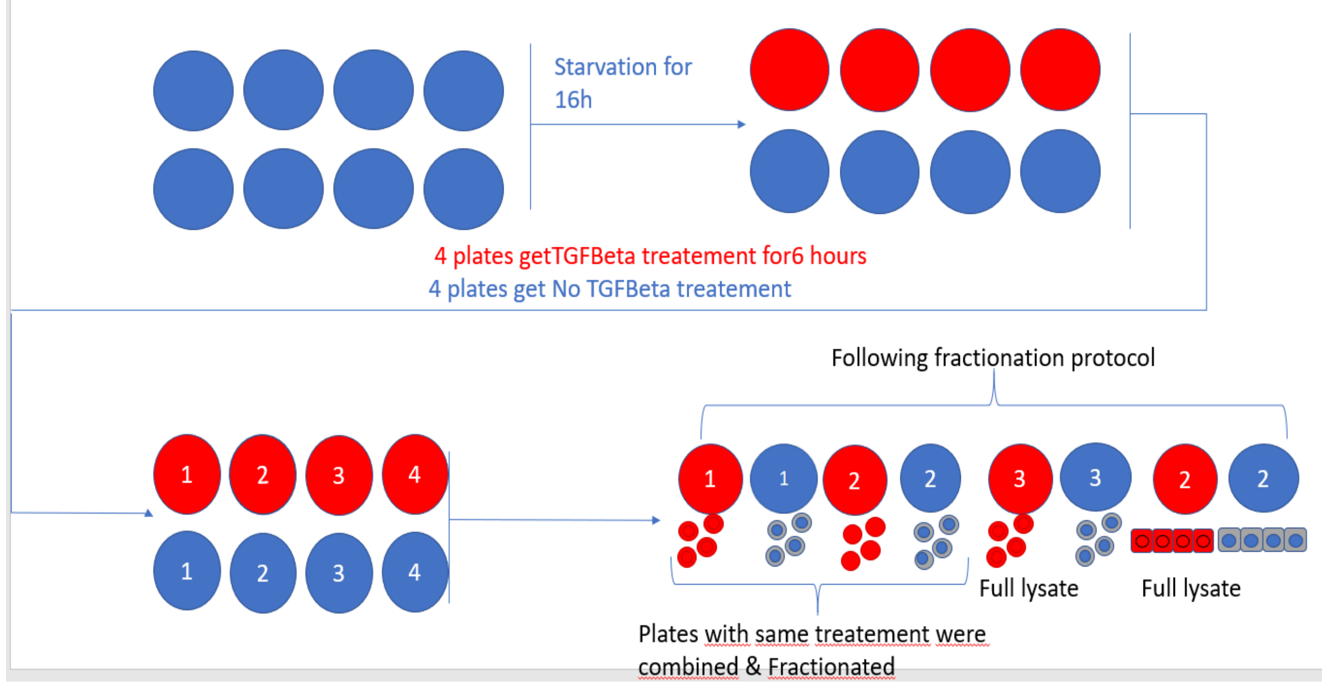


Figure 5:experimental design of the performed starvation experiment

6.5 Fractionation of suspended cells

First, there was ensured that the selection of 2 plates to be fractionated consisted of 1 plate treated with TGF-beta and 1 plate treated with the negative control. Next the medium was removed from the plates, proceeding to this the plates were incubated with PBS for two minutes after which the PBS was removed using suction and 2ml of trypsin was added to each plate. Each plate was incubated with trypsin for 5 minutes at 37°C and a CO_2 level of 5%. Following this, the plates were mechanically shocked on the side to make sure that the cells released from the surface. After this, 8ml of starvation medium was added to the plates. Next, a 10 ml pipet was used to suspend all cells from the plate in the added starvation medium and translocate the medium combined with cells from the plate to a labelled 15ml falcon tube. The falcon tubes were proceeding spun down for 10 minutes at 1000xg after which the pellet was suspended in 10ml PBS and again spun down for 10 minutes at 1000xg. This pellet was resuspended in 1ml of PBS and translocated to a labelled 1,5ml microcentrifuge tube which was next centrifuged for 10 minutes at 1000xg, the formed pellet was resuspended in 300µl of fractionation buffer and next sucked through a hydraulic needle(27g) for six times and incubated on ice for 20 minutes. After this, the supernatant was removed and translocated to a new 1,5ml microcentrifuge tube labelled cytoplasmic fraction. The original sample was resuspended in 200µl of fractionation buffer and spun down for 10minutes at 1000xg after which the supernatant was removed and discarded. The resulting pellet was resuspended in 200µl of MS compatible buffer after which the samples were sonicated for 3 minutes. Finally, the samples were spun down for 20minutes at maximal speed and the resulting supernatant was translocated to a new tube and labelled as nuclear fraction. All samples are stored at -20°C or immediate quantification using the BCA assay.

A full lysate of each condition was prepared by substituting fractionation buffer with RIPA lysis buffer, incubating on ice for 20 minutes and next, spinning down and translocating the supernatant to a new 1,5ml microcentrifuge tube, labelling the tube as full lysate and storing it at -20°C.

Solution	Amount added in 50ml falcon tube
Note: add all solutions to a 50ml falcon in the order as stated below(due to a difference in polarity between Np-40+glycerol and the polar chemicals)	
25mM HEPES	1,25ml
5mM KCl	18,7mg
25mM MgC_{l2}	0,254g
0,05mM EDTA	0,25ml
Add triple distilled water until the tube is half full	
0,1% NP-40	50µl
Shake thrice after the addition of NP-40	
10% glycerol	5ml
Vortex vigorously after the addition of glycerol	
Triple distilled H_2O	Until a total volume of 50ml is reached
Total volume	50ml
Note: this buffer can be stored at "temperature" °C	

Figure 6: recepi for fractionation buffer

Solution	Amount added in 50ml falcon tube
Note: add all chemicals in the order and amount(for total volume of 50ml) as stated below	
25mM tris-HCl (ph 7,5)	1,25ml
300mM NaCl	3,75ml
1mM EDTA	5ml
Triple distilled H_2O	35ml
Add 1M HCl to reach a ph of 7-8 (about 10 drops)	
Triple destilled H_2O	5ml
Total volume	50ml

Figure 7: recepi for ms compatible buffer

6.6 Fractionation of adherent cells

First, there was ensured that the selected plates for fractionation of adherent cells consisted of both TGFB treated a non-treated cell. After which all medium was removed from the plates and 10ml of PBS at a temperature of 4°C was added and removed via suction after 2 minutes. Next 300µl fractionation buffer was added after which the cells were removed from the plate by using a pipet tip to scrape them thoroughly lose from the surface and incubating them at an angle on ice for 15minutes, after which the cells were translocated to a 1,5ml microcentrifuge tube. Each of the samples was pulled through a hydraulic needle (27G) six times and next spun down for 10 minutes at 1000xg. After this, the supernatant was removed and translocated to a new 1,5ml microcentrifuge tube labelled cytoplasmic fraction. The original sample was resuspended in 200µl of fractionation buffer and spun down for 10minutes at 1000xg after which the supernatant was removed and discarded. The resulting pellet was resuspended in 200µl of MS-compatible buffer after which the samples were sonicated for 3 minutes. Finally, the samples were spun down for 20minutes at maximal speed and the resulting supernatant was translocated to a new tube and labelled as nuclear fraction. All samples are stored at -20°C or immediately quantification using the BCA assay.

A full lysate of each condition was prepared by substituting fractionation buffer with RIPA lysis buffer, incubating on ice for 20 minutes and next, spinning down and translocating the supernatant to a new 1,5ml microcentrifuge tube, labelling the tube as full lysate and storing it at -20°C.

6.7 Protein concentration determination (BCA assay)

First, a standard curve of protein was prepared at concentrations: 2000ng/µl,1000ng/µl, 500ng/µl, 250ng/µl, 125ng/µl, 62,5ng/µl, 31,25ng/µl, 0ng/µl. From each of these standards, 10µl was brought on a 96 well plate in two different wells. Next 10µl of each sample was brought on two of the wells as well. From the BCA kit reagent, A & B were taken and combined in an A/B margin of 50/1. From this solution, 200µl was brought in each of the wells containing either sample or standard. This had to be done in as little time as possible, therefore a multiwall micropipette was used. After adding of the BCA reagents, the well plate containing the samples was incubated at 37°C for 30minutes. Finally, the absorbance was measured using the citation 5.

6.8 Western blotting

First, the samples had to be prepared by making a sample solution containing 15µg of samples combined with RIPA lysis buffer to a total volume of 30µl. Next, 10µl of LB loading dye was added to the sample solution. Finally, the sample solution was desaturated for 5 minutes at 95°C on the heat Blok.

The gel was made in a batch of two following the recipe below. (Fig.7). Each gel had a thickness of 1,5mm and a polyacrylamide percentage of 10%. The gel was made by combining all described chemicals in 50ml falcon tubes but withholding the TEMED until the gel was cast. First the resolver gel was cast in the mould (setup of the mould is described in the SOP in appendix 1). Straight after the casting of the resolver gel, propanol was added on top of the resolver gel to remove any bubbles that may have formed. The propanol was removed after hardening of the resolver. Finally, the stacking gel was added on top, and the comb was inserted to provide the wells for the experiment.

Available Casting options	1	1,5	mm

**ENTER DATA HERE

Choose Gel Thickness	1,5	mm
# of gels	2,5	gels
Desired Percentage	10	%

Stacking Gel	Volume (mL)
40% acrylamide (37,5:1)	0,938
dH2O	4,905
0,5 M Tris pH6,8	1,500
10% SDS	0,075
10% APS	0,075
TEMED	0,008
Total Volume	7,500

Resolver Gel	Volume (mL)
40% acrylamide (37,5:1)	5,000
dH2O	10,480
1,5 M Tris pH 8,8	4,000
10% SDS	0,200
10% APS	0,200
TEMED	0,020
2,2,2, Trichloroethanol (TCE)	0,100
Total Volume	20,000

Figure 8: Recipe for stacking gel(left) & resolver gel (right)

After casting the gels, 5µl of the all blue standard ladder was brought on to the first well, after which 39µl of each sample was brought on to one well each. The gel was run at 90v while the samples were passing through the stacking gel and turned up to 130v when they reached the resolver gel. The electrophoresis was stopped when the dye was about 0,3 cm from the bottom of the glass casing.

After the electrophoresis was completed, the gel was removed from the glass casing and transferred to a container with running buffer. Using a stain free gel, allowed for an imaging prior to the transfer to ensure that the electrophoresis had taken place in the expected manner. After Imaging the transfer buffer was prepared. This was done by combining 200ml transfer buffer with1400ml milliQ water and 400ml methanol. After preparation, the buffer was kept cool in the cold room at 4°C (the transfer buffer was often prepared up to 24h before use to ensure that it had cooled properly since the reaction in the dilution is slightly exothermic.)

The PVDF membrane used for the transfer was cut to fit the size of the gel nicely. Right before use, it was activated by submerging it for 1min in methanol.

The sandwich was prepared by removing the transfer buffer from the cold room and pouring it in a case. Every component from the sandwich was properly submerged in transfer buffer before it was assembled and all air pockets that may formed were removed from the sandwich during assembly. The sandwich was formed by first placing the cassette in the case (black side down) and placing the first Ion reservoir in it. The ion reservoir was next covered with two filter papers on which the activated PVDF membrane was placed. The gel was placed on top of the PVDF membrane (face down) and finally covered with two filter papers and the second ion reservoir. Finally, the sandwich was closed, and the cassette was placed in the blotting station (black side of the cassette to black side of the blotting station). The blotting case was filled with transfer buffer and an icepack and placed on ice. The transfer was run at 250mA for 90minutes.

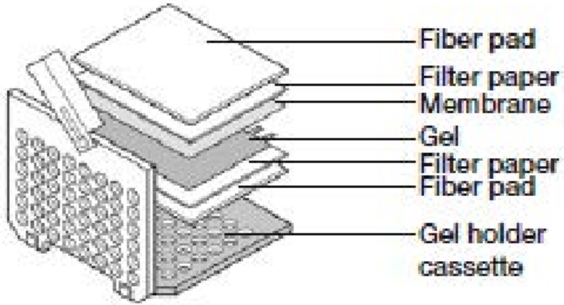


Figure 9:Setup of a westernblotting

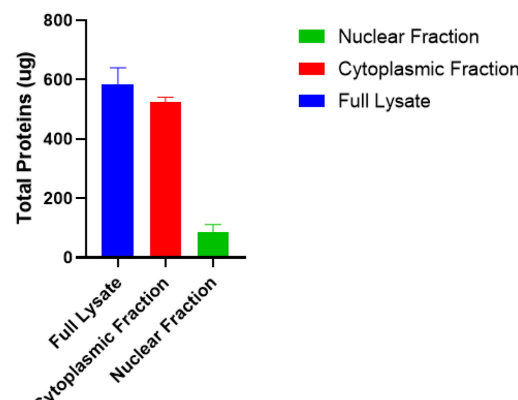
When the transfer was finished, the blot was removed from the sandwich and transferred to a 50ml falcon tube. 5ml of blocking buffer (ROTI) was added and incubated for 1 hour on the roller. When the blocking buffer was finished, the primary antibody could be added (Lamin B1, Alpha tublin, GAPDH, p-YAP, p-SMAD2, YAP) The concentration of these antibody's was either 1:1000 or 1:500.The primary antibody either incubated for 1 hour at room temp or overnight at 4°C. When the incubation was competed, the blot was washed 3x with TBST (Tris-buffered saline with 0.1% Tween) for 10 minutes. The secondary antibody was added after the washing steps in a concentration of 1:5000. The used antibody was Antirabbit IGG. After the 1hour incubation, the blot was washed 3x with TBST for 10 minutes and was then finally ready to be developed and imaged.

7 Results

7.1 The culturing method and its influence on the fractionation

During the experiments, the cells were extracted in two different ways, in case the extraction method would influence the results of the fractionation experiment. The first extraction method was the lifting using trypsin to dissolve the extracellular matrix and lift the cells from their surface. The second method used was a direct addition of fractionation buffer on the plates. There was observed that in some cases (when plated on the 9,5cm petri dishes) a high amount of viscosity was present when either treated with fractionation buffer and manually extracted or when trypsin was added. This viscosity was observed to make the extraction of the cells from the petri dishes much more laborious by the addition of extra washing with DMEM F12 starvation medium, due to the difficulty of removing the formed viscosity. In every case of extracting and pelleting the cells from their plates, extra washing steps with PBS were introduced to ensure that no residual medium was left in the tubes that could contaminate the experiment. During the first experiment, 2ml Eppendorf tubes were used. In these, the pellets were observed to be quite unstable after spinning them down for 10 minutes at 1000xg which has been linked to their relatively flat tip in comparison to their 1,5 ml counterparts which have a much more conical shape. The 1,5ml conical shaped tubes were observed to have a much more stable pellet which allowed an improved removal of supernatant/medium. During the first performed experiment, the samples were frozen overnight. The freezing process mad the samples highly difficult to work with due to hardening and difficulty with dissolving them with use of a 1ml tip and a micropipette.

7.2 Protein amounts in fractionated samples and their correspondence with the full lysate's protein amount



Total proteins of full lysate vs Fractions - imposed

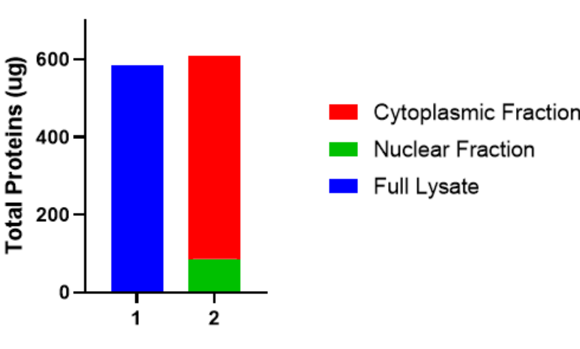


Figure 10:Total proteins full lysate vs fractions

In order to test if the fractionation caused a loss of protein, the concentration of protein in the full lysates, cytoplasmic and nuclear fraction. These results showed that the amount of protein in the fractions when combined is almost equal to the amount of protein in the full lysate.

7.3 Results of western blot test experiments

7.3.1 Failed SDS-page vs successful SDS-page

a. Failed Gel   b. Successful Gel

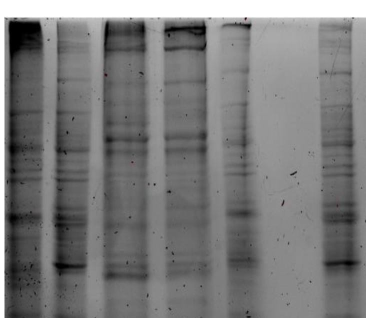


Figure 11: comparison between failed blot from the first test experiment(left) and a successful blot from the second test experiment(right)

The failed gel (a.) showed verry few bands in the experiment while the successful gel(b.). The failed gel has bands that are very slight and shows mostly only traces of the proteins that have been loaded in the gel. The first lane shows some protein present, but this is mostly in the shape of a smear through the gel.

The successful gel contained samples after the protocol was more optimised and some features were adjusted. The protein bands are present in a clear manner while nothing is shown that might suggest any issues during the preparation, loading of the samples or running of the gel that could suggest any issues.

7.4 Validation of subcellular fractionation with western blotting

The results of the second test experiment have shown great improvement over the first experiment. After the imaging of the gel (loading control), the transfer was performed, and the membrane was stained with Lamin B1(anti rabbit igg as secondary) first and GAPDH as the second primary antibody.

Lamin B1 showed bands in both the nuclear fraction of the fractionated plates and in the full lysates as well. No bands were shown in the cytoplasmic fraction after the staining with the antibody. The full lysate of the suspended cells showed some smearing on the most left side of the membrane (full lysate suspended cells). All Lamin B1 bands had a size of 70kda.

GAPDH showed bands in all the cytoplasmic fractions and in the full lysates. Except for the suspended full lysate which showed no band from the cytoplasmic fraction. The GAPDH staining resulted in bands that had a size of about 38kda.

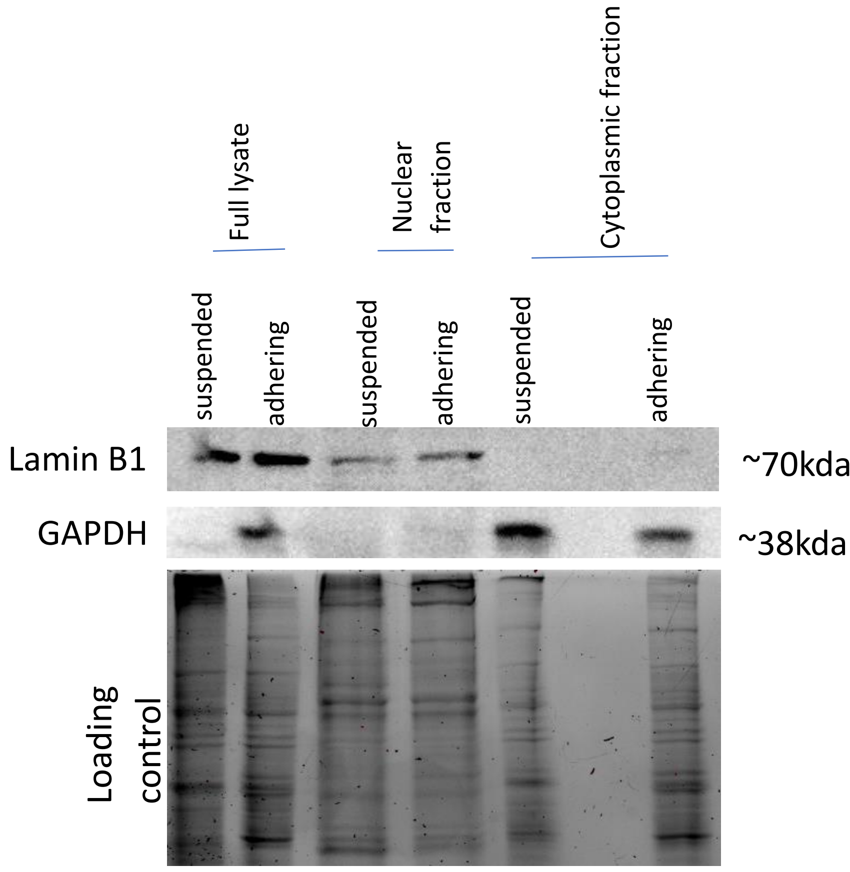


Figure 12:Results of second test experiment

7.4.1 TGF beta signalling is activated in MLE12 cells evident by phosphorylation of smad2

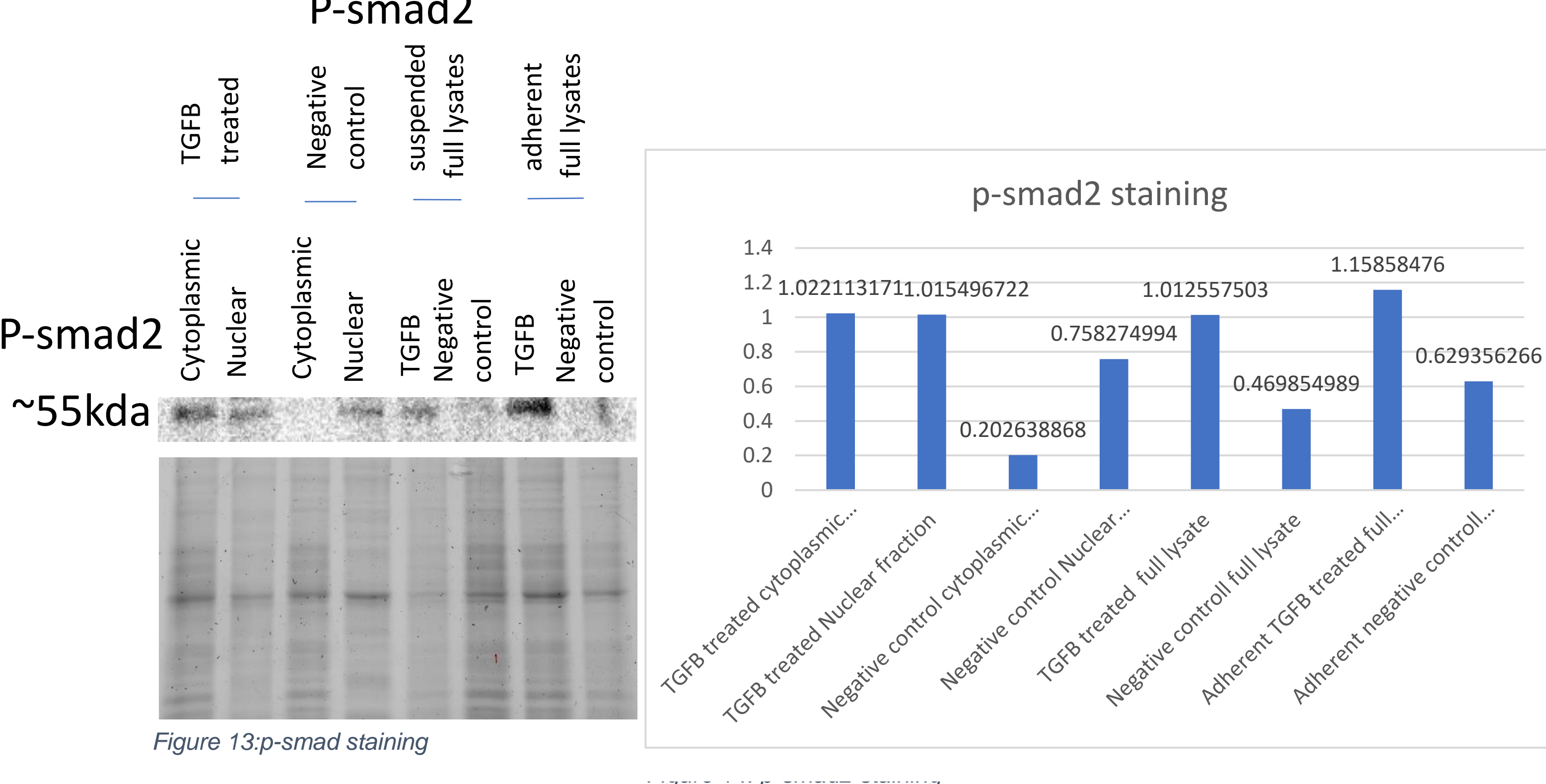


Figure 13:p-smad staining

The p-smad2 staining has interestingly shown an almost equal amount of p-smad in the nuclear and cytoplasmic cells. While in the negatives the amount in the nucleus is still quite high and even higher compared to the full lysates.

7.4.2 Yap activation upon TGF-Beta treatment

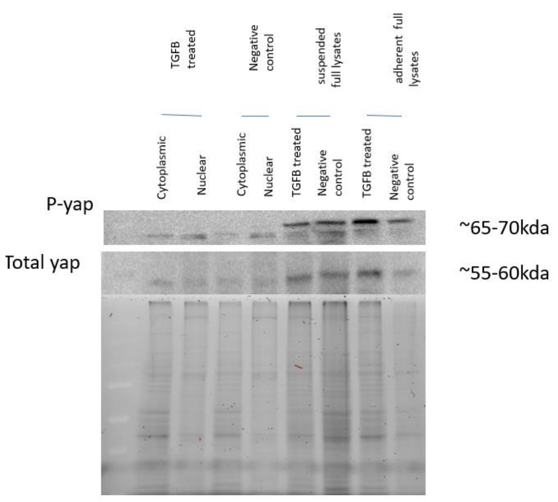


Figure 16:YAP activation results on wb

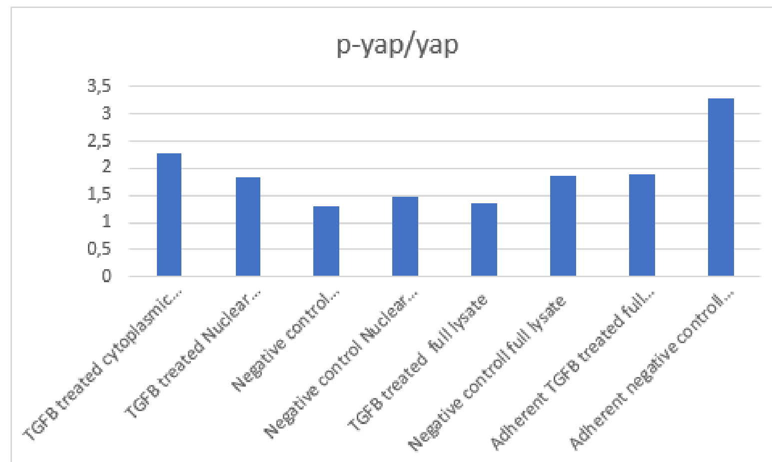


Figure 15: quantified results of p-YAP/YAP quantification

Interestingly, p-YAP was detected in the nuclear fraction in a verry strong amount. The negative control showed a similar result where the amount of p-YAP/YAP in the nucleus is lower than the amount of p-YAP in the cytoplasmic fractions. The blot was quite clear but had some secondary appearing bands that appeared on the blot these bands were combined and added when the graph was assembled.

The graph (fig16) showed the normalised results of what could be seen on the imaged blot. The amount of protein calculated in the nuclear fractions was much higher than in the cytoplasmic fractions, this difference was even more noticeable in the fractions that were treated with TGFB. If the resulted amount is high like in TGFB treated cytoplasmic fraction, then the results show a high hippo pathway activity and thus a high phosphorylation of YAP. This graph show of the fractions however is difficult to interpret because the protein margin is disrupted in the fractions and therefore hard to interpret.

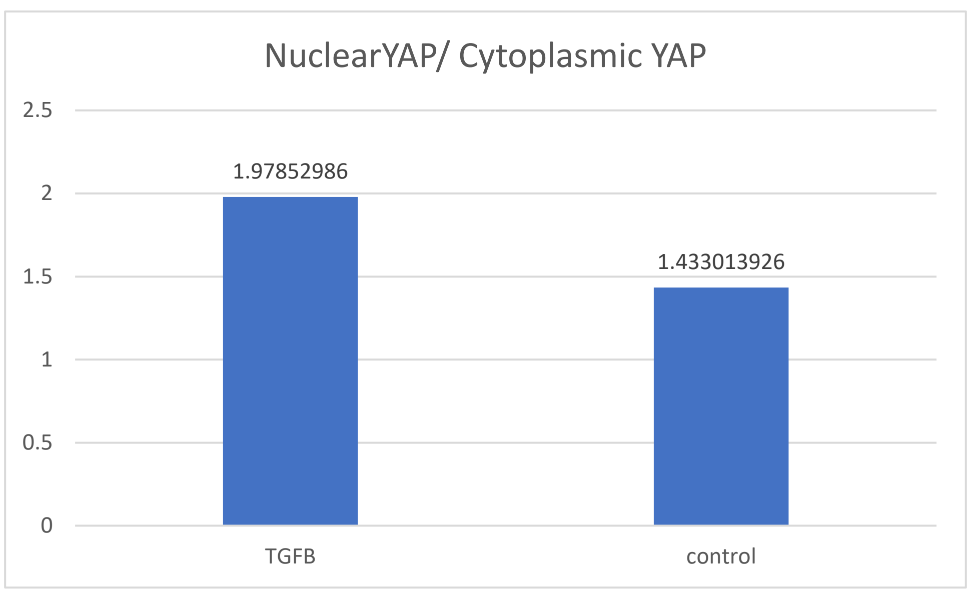


Figure 17:graph of nuclear YAP/Cytoplasmic YAP

The nuclear/cytoplasmic YAP margin showed the margin of YAP in the nuclear fraction compared to the amount of YAP in the cytoplasmic fraction. A high value results in a high amount of nuclear YAP. The amount of YAP present in the TGFB treated nucleus is much higher then the amount of YAP in the nucleus of the negative control.

8 Discussion

While performing the different experiments (data not shown), it was shown that some adjustments had to be made to the protocol. Extra washing steps during the fractionation had to be introduced due to “contamination” caused by improper washing that caused medium to stay present after the first washing. The use of less conical shaped Eppendorf tubes also caused the pellets that were created during fractionation to be much softer which caused them to become easily sucked up when the supernatant was removed.

During the TGFB starvation experiment (data not shown), there was a high amount of difficulty when plating the cells on 9,5cm petri dishes that had a surface that made attachment for the cells very difficult. The cells had to grow as confluent as possible to prohibit loosening of the cells when starving or when treating them. This problem still isn’t solved to this day but should be solved by using plates that are either collagen coated or plates that have a surface that is more attachable for the MLE12 cells. This in combination with letting the cells grow to the highest confluency possible while keeping the cells in growing mode (might influence the hippo pathway and localisation of YAP and TAZ if the growth cycle of the cells is to become stumped.

The results of the BCA assay (figure 9-10) shows that the amount of protein found in the fractions when combined, is the same amount that can be found in the full lysates. This showed that the protocol after the adjustments that were made E.I(using different microcentrifuge tubes and extra washing steps in the fractionation protocol) were sufficient to optimize the subcellular fractionation to a near optimal point.

The staining of Lamin B1(figure13) during the second experiment showed all nuclear proteins that were present on the blot. The slight distortion of the band in comparison of the gel that isn’t distorted suggest that this abnormality originates from the presence of an air bubble during the transfer of the protein. The proteins stained with Lamin B1 are the nuclear proteins and the fact that these proteins are exclusive to the nuclear fraction and the full lysates shows that the fractionation was performed in a proper manner if this is the same for the cytoplasmic (GAPDH) staining.

The GAPDH staining(figure13) resulted in bands formed in the cytoplasmic fraction of each samples and the full lysates. However, one of the full lysates shows that the cytoplasmic fraction is not present, this is expected not to originate from an insufficient fractionation but from problems during the transfer. The combination of the results from the Lamin B1 and GAPDH staining concludes that the fractionation protocol was sufficiently optimised. The results from both the staining and the BCA assay also show that there is no real noticeable difference between cells that were suspended when they were fractionated or cells that were adherent when they were fractionated. This means that cells in future experiments could be extracted using trypsin without having to worry about any changes in the proteins in the cytoplasmic fraction or the nucleus that might affect the experiment.

The first result of the starvation experiments (p-smad2 staining) shows with the highly raised concentration of smads2 in the blots resulting in the specific bands. The raised concentration of p-smads2 staining is most remarkable in the cytoplasmic, the nucleus has a more then noticeable raise as well but not as strong as the cytoplasmic fraction. The p-YAP/YAP graph shows how p-YAP is present in the nucleus which complies with some research papers indicating that p-YAP is not exclusive to the cytoplasm and dephosphorylates in the nucleus. The experiments however need to be repeated to confirm this data. The full lysates show how the TGFB treatment took effect and how the rigidity of the surface influences the YAP activity. To quantify the fractionated samples, the YAP nucleus/ YAP cytoplasmic fraction was

calculated. The results show that after TGFB treatment the amount of YAP in the nucleus rises a serious amount after treatment.

9 Conclusion

IPF is a disease that has the number of diagnosed cases increasing verry highly in the past few years. The disease is in almost every case only diagnosable in a late stage when the lungs have already taken a serious amount of damage due to the restructuring of the lungs. The disease is while seeming like an inflammation, different. Infection is one of the pathogenic drivers but not the cause, therefore anti-inflammatory's don't have an effect in treating the patient. The disease can be seen as a vicious cycle that increases exponentially over time causing damage to more and more epithelial resulting in the release of the fibrotic inducers (like TGF-B) causing more and more fibrotic cells to become active and start releasing extracellular matrix components which will result in restructuring of the lung inevitably rendering the alveoli useless.

In this disease, unusual activity has been noticed in the hippo pathway of the epithelial cells. Where the Hippo pathway has become highly active resulting in a senescence of the cells, making the epithelial cells type II unable to replace any damage caused by the disease or any other outside factors like smoking or inhaling metal/wooden dust particles. This could be a possible explanation why individuals who have had a high exposure to these factors to have a much more aggressive version of the disease decreasing their survival time after diagnosis by more than half in some cases.

The results of the BCA assay show that the extraction of protein from suspended and adherent cells shows that the state of the "source" cells (adherent or suspended) doesn't influence the results or the efficiency of the subcellular fractionation.

The results of the BCA assays show that the fractionation was performed in an efficient manner with a minimal loss of proteins. The pureness of each of the fraction was then validated with western blotting and staining with GAPDH and Lamin B1. The presence of only 1 kind of staining per fraction(cytoplasmic/nuclear) showed that the separation of the proteins was proper.

TGF-B was added to simulate the fibrotic environment in the cells. The amount of p-YAP/YAP in the full lysates shows how TGFB causes the amount of p-YAP to increase and YAP activity to decrease. The samples are illogical at first glance. This probably is due to the fractions being separated and causing the p-YAP to YAP margin to become disturbed, therefore the amount of Nuclear YAP/cytoplasmic YAP was calculated, actually showing that YAP becomes more nuclear after treatment with TGFB.

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11 Appendix-1ad

Lung Bioengineering and Regeneration Group *Standard Operating Procedure and Risk Assessment*		
TITLE: Immunoblotting – sample preparation, gel electrophoresis, immunoblotting, and developing.		Page 1 of 12
SOP #: LBR-sop-001	REVISION LEVEL:	EFFECTIVE DATE:
AUTHOR(S): Hani N Alsafadi, Margareta Mittendorfer	PRIMARY REVIEWERS (at least two): John Stegmayr, Deniz Bölükbas, Darcy Wagner	

1. **Purpose**
   A detailed description of western-blotting (immunoblotting) procedure from sample preparation to development of protein bands. This protocol contains all information regarding risk assessment for each step or material used in this protocol.

2. **Materials (please list all chemicals, concentrations and location of where and how the chemicals are stored)**

Reagent	Type	Concentration	Product #	Risk	Storage
PhosphoSTOP	Tablets	NA	Fishersci REF 04906837001		4ºC main lab
Protease Inhibitor	Tablets	NA	Fishersci A32953		4ºC main lab
RIPA lysis buffer	Solution	See RIPA preparation SOP	Homemade		4ºC main lab or -20°C if inhibitors are added
2-Mercaptoethanol	Liquid stock	14,2 M	BioRad 1610710		RT, Ventilated cupboard main lab
Loading Buffer	Liquid	See Loading Buffer preparation SOP	BioRad 161-0747 And Homemade		RT, non-ventilated chemical cupboard, main lab
Rotiphorese® Gel 40 (37,5:1) Acrylamide	Liquid	40%	Carl Roth T802.1		4°C main lab
Tris pH 8,8	Solution	1,5 M	Homemade		RT, WB shelf
Tris pH6,8	Solution	0,5 M	Homemade		RT, WB shelf
Sodium dodecyl sulfate (SDS)	Solution	10%	Homemade		RT, WB shelf
Ammonium persulfate (APS)	Solution	10%	Homemade		4ºC main lab
2,2,2-Trichloroethanol (TCE)	Liquid	≥99%	Sigma T54801		RT, Locked ventilated cupboard main lab

N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Liquid	~99%	Sigma T9281		4ºC main lab
2-propanol	Solution	≥99,8%	Fishersi 603117000		RT, Chemicals cabinet main lab
10X Tris/Glycine Buffer	Solution	250 mM Tris, 1.92 M glycine, pH 8.3	Biorad 161-0734, also homemade	NA	RT, WB shelf
Methanol	Liquid	≥99,8%	20837.320		RT, Chemicals cabinet main lab
10X Tris buffered saline (TBS)	Solution	10X	170-6435		RT, WB shelf
Tween-20	Liquid (very viscous)	1,1 kg/L	28829.296	NA	RT, Ventilated cupboard main lab
Non-fat dry milk	Powder	NA	Bioard 170-6404	NA	RT, WB shelf

3. Procedure

(Note: Make sure that you ask for an introduction before the first time you use this protocol to make sure you know where everything is)

1. Sample Preparation:

1.1. Add phosphatase and protease inhibitors to RIPA buffer; add one tablet each of protease inhibitors and phosphatase inhibitors to 10 mL of RIPA buffer. This mix can be stored at -20°C.

1.2. Lyse your sample depending on your sample type; here are some suggestions:

Type of experiment	Lysis method
Cell Culture	- Add lysis buffer to your stopped experiment; shake on orbital shaker for 15 minutes on ice. - Scrape cells using a cell scraper or pipette tip. - Collect lysate and centrifuge at 15000g for 15 minutes at 4°C; collect supernatant.
Lung Tissue / Lung slices	- If not lysed fresh, tissue is usually stored frozen. - Thus, add lysis buffer and let tissue thaw inside lysis buffer. - Homogenize tissue using a tissue-lyzer or an equivalent device. - Collect homogenized sample and centrifuge at 15000g for 15 minutes at 4°C; collect supernatant (soluble fraction) and save pellet for future analysis (insoluble fraction).
If you need all subcellular fractions (RNA, DNA, protein) from the same sample, you can use the TRIzol reagent. Consult Hani/Darcy for more details.	

1.3. Measure protein concentration using an appropriate method; here are some suggestions:

Note that all of these methods are well described by their manufacturers. You can find manufacturer protocols in the protocol folder above Hani's desk.		
Method name	When to use	Brief procedure

BCA assay	Most common, use when proteins are lysed with a mild protocol and you expect a good yield.	- Use 10 uL of each sample or standard in each well of a 96well plate. (for samples: you can use a diluted amount as you do not need to waste much sample for protein measurement) - Mix reagents A and B in a 50:1 ratio to make the working solution - Add 200ul of working solution to each well. - Incubate at 37C for 30 minutes. - Measure absorbance at 562nm; generate standard curve and calculate protein conc.
Bradford Assay	Use for high yields of proteins.	- Use 10 uL of each sample or standard in each well of a 96 well plate. (for samples: you can use a diluted amount as you do not need to waste much sample for protein measurement) - Add 250ul of Coomassie Blue. - Color change should occur immediately, but shake gently for 10 minutes at RT. - Measure absorbance at 595nm
RC DC Kit	Use this kit when your sample contains high concentrations of reducing agents	- A detailed protocol can be found in the labs' protocols folder.
CBQCA Protein Quantification Kit	Use this kit when your protein yield is expected to be very low.	- Use manufacturer's protocol

1.4. Calculate the volumes from each samples to choose the same amount of proteins in each well (a.e. 5, 10, or 15ug proteins / well), the choice of the protein amount is dependent on your target proteins; a.e. use higher amounts if you need to analyze a low-expressed protein and vice versa.

1.5. Make sure to dilute all your samples so they all have the same volume and concentration.

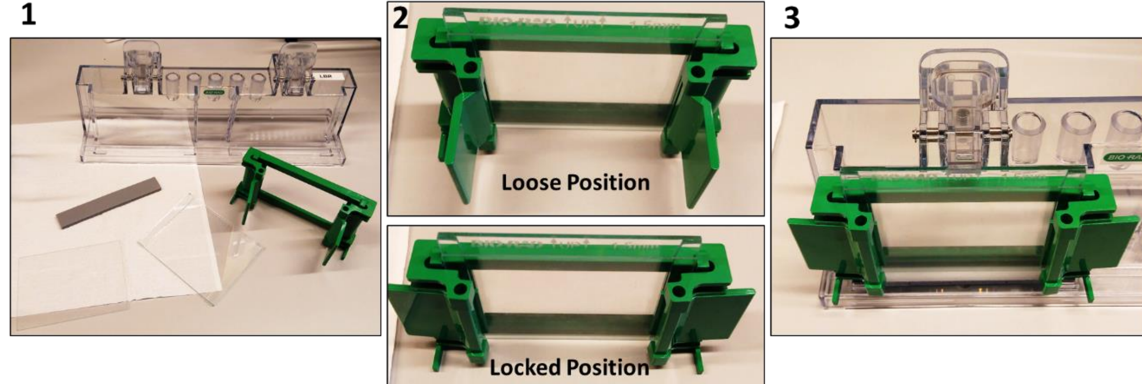
1.6. Add Loading buffer to your samples at the recommended ratio (a.e. 1:3, loading buffer: Sample)

(Note: Use loading buffer containing (2-mercaptoethanol or DTT) if you require reducing conditions for your electrophoresis. Otherwise, use it without a reducing agent. Most of the time we run with reducing conditions. BUT Turn on your brain when you are making these decisions.)

1.7. Incubate samples containing Loading buffer at **95°C for 5 minutes**.

2. Gel-Casting:

2.1. Set up the casting devices as shown in image:



2.2. You need to decide the percentage of your gel according to your target proteins. (a.e. higher percentage for smaller proteins and vice versa) and the size and number of wells for each gel.

2.3. Use excel sheet (SDS-PAGE-gel _calculator.xlsx) to calculate the volumes needed for each solution. (See image). Input numbers in the red box and calculations will be automatically made. (be cautious when working with Acrylamide; the monomer is quite toxic, pipette acrylamide and TEMED in a ventilated hood)

**ENTER DATA HERE

Choose Gel Thickness	1	mm
# of gels	4,5	gels
Desired Percentage	10	%

Stacking Gel	Volume (mL)		Resolver Gel	Volume (mL)
40% acrylamide (37,5:1)	1,125		40% acrylamide (37,5:1)	6,000
dH2O	5,886		dH2O	12,576
0,5 M Tris pH6,8	1,800		1,5 M Tris pH 8,8	4,800
10% SDS	0,090		10% SDS	0,240
10% APS	0,090		10% APS	0,240
TEMED	0,009		TEMED	0,024
Total Volume	9,000		TCE	0,120
			Total Volume	24,000

2.4. Prepare both the stacking and resolver gels (WITHOUT TEMED). Only add TEMED just before casting the gels. The addition of TEMED will immediately initiate polymerization of the gels.

2.5. Add TEMED to resolver gel only and fill in the casting device till 1cm bellow the glass edge.

2.6. Add 1 mL of 2-propanol to get rid of any bubbles and to smoothen out the surface of the resolver gel.

2.7. Let the resolver gel solidify for **15 minutes**.

2.8. Discard all of the propanol from the top by inversion carefully.

2.9. Add TEMED to stacking gel and fill in until the top of the casting device and immediately insert the combs of your choice.

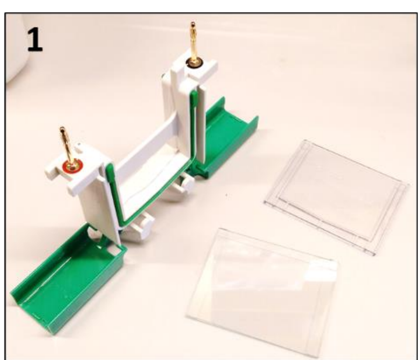
(Note: The choice of comb depends on the number of samples to be run and the volume of these samples and thickness of gel (see table below). Do not forget to count at least one well for ladder)

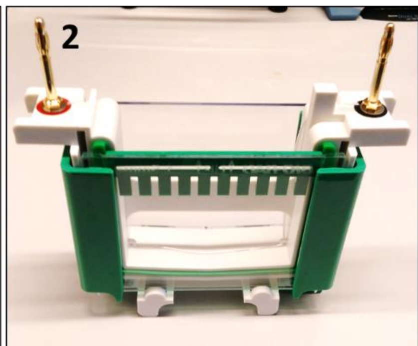
# wells	Well Width	1.0 mm gel	1.5 mm gel
10	5.08mm	44 ul	66 ul
15	3.35mm	26 ul	40 ul

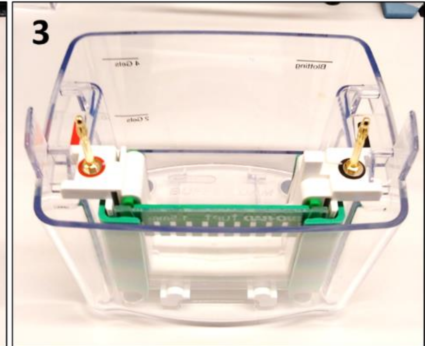
* These values are obtained from www.bio-rad.com
(https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10007296D.pdf)

2.10. Allow the stacking gel to solidify for another **15 minutes** and gels are ready.

3. Gel Electrophoresis:
    3.1. Assemble the electrophoresis chambers as shown in the image bellow:







3.1.1. Once you have assembled the inner part (image 2) fill it with 1X running buffer to ensure there is no leakage. If there is any leakage repeat this step until there is no leakage.

3.1.2. Place the inner part inside the main chamber as shown in image 3.

3.1.3. Once the inner chamber is filled slowly remove the combs in an upward direction. Make sure that the wells are immediately filled with running buffer.

3.2. Load the protein ladder and samples in each well as necessary:
There are several types of ladders that can be used such as (2 color ladder, which is visible on the membrane) and unstained ladder that is visualized by the stain-free technology. We use the latter, but it is important to select according to your needs.

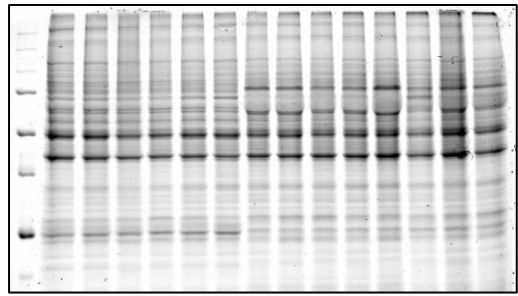
3.3. Close the chamber and start running with a constant voltage of **90V** until the samples pass through the stacking gel.

3.4. Once samples reach the line between stacking and resolver gels, you can increase the voltage up to **130-140V**. At this point the samples will look like a straight line. Leaving the gel running at 90V is ok, however, it will take a long time to run through the whole gel.

3.5. Stop electrophoresis once the samples have reached the bottom the gel within the last 0,5 cm. Make sure that your samples never run outside of the gel. If that happens the gel is no longer trustworthy.

4. Imaging of the stain free gel:
Imaging of the stain free gels and the developed blots is done using the ChemiDoc Touch. At the moment, we use the device that belongs to the group of Anja Meissner which is located in D12.

    4.1. Carefully open the casting device. Be careful not to harm the gel.
    4.2. Spray some dH2O on the UV tray of the ChemiDoc.
    4.3. Place the gel at the center of the tray in the desired direction
    4.4. Choose the appropriate function on the ChemiDoc and image your gel. For the first time, you need to activate your gel for **45 seconds** with UV light prior to imaging. However, if you are re-imaging the gel to change the exposure or any other parameter, you no longer need to activate again. Excessive exposure of UV light could be harmful to the gel. Here is an example of an obtained image.



5. Immunoblotting – Transfer of proteins:
   Transfer has to be done in cold conditions, so bring a basket of ice.
   5.1. Prepare transfer buffer. Add 100mL of 10X Tris/Glycine buffer + 700mL $H2O$ + 200mL methanol. Use cold water to make this if possible. Keep cold while preparing other things.
   5.2. Membrane Choice and preparation:
   You can either use nitrocellulose membranes or PVDF membranes depending on the target proteins you have and the intended use (If you intend to strip several times, then use PVDF: note that this is only a suggestion based on my experience ~Hani).
   Membranes also come with different pore size, 0.2 or 0.4 um. Bigger pore size is recommended for bigger proteins.
   - If you select nitrocellulose membrane, you can proceed to the next step with no prior preparations.
   - If you use PVDF, you need to activate it in Methanol for **30 second to 1 minute**. Discard methanol and incubate membrane in transfer buffer.
   5.3. Prepare the transfer sandwich:
      5.3.1. Place the main chamber in ice and make sure that it is leveled horizontally.
      5.3.2. Make sure that you have the correct order and correct direction for transfer:
      If you use the transfer sandwich cassette with the black side on the bottom. First place an ion reservoir, filter paper, gel, membrane, filter paper, ion reservoir and close the sandwich and place it in the electrophoresis chamber with the black side of the cassette facing the black side of the inner transfer block.
      Place transfer block in the correct orientation; match the correct electrode to the correct side (color coded)
      5.3.3. Place an ice-pack inside the main chamber and fill the whole chamber with transfer buffer till mark.
      5.3.4. You can run the transfer at either a constant voltage or a constant current. I prefer using constant current as this will reduce the amount of heating. **(250mA for 90 minutes).**
      Otherwise, you can use constant voltage **(100V for 60 minutes)** but be aware that extra heat may cause loss of proteins.

   5.4. You may also use semi-dry transfer, which is much faster. However, you may lose a lot of the proteins in the semi-dry transfer. You can use this if your protein of interest is located at the middle of the gel, a.e. 50 – 100 kDa in a 10% gel. Otherwise wet transfer is preferred.
6. Blocking:

6.1. Once the transfer has completed remove the membrane from the transfer sandwich and place it in a 50mL falcon tube or alternatively use a flat surface container. If you use 50 mL falcon, use the roller mixer for all incubation steps. Otherwise, use an orbital shaker for flat containers.

6.2. Incubate blot for **1 hour** with blocking buffer at **room temperature**. Blocking buffer could be: 5% milk, 1-5% BSA, RotiBlock or any other block you prefer.

7. Primary Antibody:

7.1. After blocking add your primary antibody diluted in blocking buffer to your membrane. Note that there is no need for a washing step between blocking and primary antibody incubation.

7.2. Incubate primary antibody for **1 hour** at **room temperature**, or **overnight at 4°C** while rotating.
(Note: Use the dilution for antibody as recommended by the supplier; if no information is given in the datasheet, use a 1:1000 dilution for the first time and optimize accordingly)

7.3. Wash membrane with 5-10 ml 1X TBS-T (0,05%Tween-20 in 1X TBS) **3 times** for **10 minutes** each time.

8. Secondary Antibody:

8.1. Make sure you choose the correct secondary antibody. Dilute your antibody in the same buffer you used for the primary antibody.
(Note: Use the dilution for antibody as recommended by the supplier; if no information is given in the datasheet, use a 1:3000 dilution for the first time and optimize accordingly)

8.2. Incubate for **1 hour** at **RT**, or **4°C overnight**.

8.3. Wash in TBS-T **3 times** for **10 minutes** each time.

9. Developing:

There are a lot of variations in developing your blot depending on the target protein and the quality of the antibody, etc. Here are steps for the general method that needs to be optimized for your specific targets. In here, we use Clarity ECL reagent from BioRad. Talk to Hani about what options we have and what you should use.

9.1. Place your blot in a flat container (a.e. a clean pipette tips cover could suffice)

9.2. Mix developing reagents (A and B) in 1:1 ratio.

9.3. Incubate blot with developing mix while shaking for **3 minutes**

4. Waste Removal Considerations

- Waste containing 2-Mercaptoethanol needs to be disposed only in sealed bags or containers. 2-Mercaptoethanol is a reducing agent and hazardous if not reacted. **Can be disposed in the autoclaveable waste.**
- Waste from Running and transfer buffers can be drained in the sink.
- Casted Gel waste must be disposed of in the blue waste boxes.
- Blocking buffers / Wash buffers can be drained in the sink

5. Identify the hazards

Biological		
☒ Blood/Bodily Fluid	☒ Tissue	☒ Virus/Disease
Chemical – Note: Refer to the label and Material Safety Data Sheet (MSDS) for the classification and management of all chemicals		
☒ Non-hazardous chemicals	☒ Hazardous chemicals (refer to a completed hazardous chemical risk assessment)	
Name of chemical(s): 2-Mercaptoethanol, SDS, Acrylamide, TEMED, isopropanol, methanol In case of spillage:		
Radiation		
☐ No	☒ Yes (refer to a completed radiation risk assessment)	
Fire		
☒ No	☐ Yes (refer to a completed fire risk assessment)	
Environment/Waste		
☐ No	☒ Yes (refer to a completed environmental/waste risk assessment)	
Equipment		
☒ No	☐ Yes (refer to a completed equipment risk assessment)	

6. **Assess the level of risk**

The risk of this protocol is low if all chemicals are handled as instructed. All personnel must wear protective clothing, and gloves while performing this procedure. Specific instructions for risk handling are written within the protocol. Those substances for which the handling poses serious health hazards need to be handled in the fume hood or under other ventilation.