PXL TECH

Professional Bachelor Agro and Biotechnology

Biotechnology

OPTIMIZATION OF A GENOTYPING PROTOCOL FOR PINK1 KNOCKOUT MICE AND CRE MICE

Eline Celis

Promotoren:

Sebastian Haesler — NERF/Imec
Evelyne Wirix — PXL

nerf
Empowered by
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Preface

To finish my education Agro- and Biotechnology at PXL I had to do an internship. During this internship I had to work on a project which would be the subject of my bachelor thesis. This project consisted of the optimization of genotyping protocols for both PINK1 knockout mice and cre mice. I did my internship in the lab of Sebastian Haesler at NERF which is empowered by Imec, KULeuven and VIB.

By doing this internship I experienced how it was to work as if I had a real job. I learned to communicate with people in Dutch and in English and to be independent. It was essential to plan everything because the project lasted for 4 months and I had to take other people into account. It was not that easy but I managed it very well. I was able to perform the techniques I learned the past three years, by myself. For my literature study, I had to find articles. I had to be critical about them and extract the right information.

I had a good connection with all the people at NERF. There were no conflicts or difficulties. Overall is was a very good experience which taught me a lot. I am sure that I am ready to start a real job now.

First of all I would like to thank Sebastian Haesler for being my promotor and evaluating me. I could always ask for help and it was no problem for him to immediately help me. If I needed something, for example new primers, it was no problem for him that I ordered them. I would also like to thank José Esquivelzeta. He was a huge help to me, I could ask him everything and he helped me right away. He also took me under his wing and involved me in some activities with the team outside the office. Evelyne Wirix was my school promotor and she guided me through the whole process. I am very grateful for that. If I had questions, she always answered them and it was no problem for her at all.

Besides these three people, I would also like to thank the whole NERF team because they were really good to me. Last but not least I thank the teachers of PXL who taught me everything I needed to know to write this thesis, to do the experiments, to make the presentation, to defend my thesis and to take the next step in my life which is finding an interesting job.

Abstract

Every year there are 1500 new cases of Parkinson's disease (PD) in Flanders. This is the primary form of parkinsonism and the main symptoms are tremor, rigidity and bradykinesia. PD is progressive and some patients suffer more than others. The disease is a consequence of the loss of dopaminergic neurons in the substantia nigra. Due to the lack of dopamine, the nigrostriatal pathway changes which causes hyperactivity of the globus pallidus and subthalamic nucleus. This overactivity results in the hypoactivity of the frontal cortex. This makes it difficult for a person to plan movements so patients move slow. To diagnose PD is quite difficult for the symptoms could also represent other diseases. Usually a neurologist does some examinations and then prescribes a medicine for PD. If this works the person has PD, if not it could be another condition. Techniques that are used often are CAT scan, DaT-SPECT scan, MRI and an EEG.

PD still can't be cured but there are some treatments that alleviate the symptoms and make the patient's life easier. Examples of these treatments are medication, DBS-STN, pallidotomy and thalamotomy which are surgeries to remove some parts of the brain, kinesiotherapy, ergo therapy and speech therapy.

Researchers are still not sure what the actual cause of PD is. Most of them think it's a combination of gene mutations and environmental factors. Also Lewy bodies and oxidative stress could trigger it. One of the genes in which a mutation can cause PD is the PINK1 gene. It codes for PTEN induced putative kinase 1 and plays a role in the pathway that gets rid of damaged mitochondria.

Research is necessary to better understand the mechanism of the brain and hence the mechanisms of Parkinson's disease. At NERF, PINK1 knockout mice are used for this research. To create such knockout animals, labs use the cre-lox system that modulates the genomic DNA through homologous recombination. Before mice can be used for experiments the researchers have to be sure about the genotype. There are different techniques for genotyping like RFLP, sequence analysis, micro array,… In this case PCR is used to determine whether or not an animal contains the complete PINK1 gene and the cre gene. The protocol needs to work optimally to be sure that the results are right. An optimization is performed to determine which parameters need to change in a provided protocol to make sure that the results are clear.

First of all the provided protocols were tested to check if the results were clear. This was not the case in both the PINK1 and cre protocol. A touchdown PCR was executed with the PINK1 samples to check whether the primers worked. They worked but the results were rather confusing. Afterwards the right annealing temperature, primer concentration and DNA volume were determined. The optimal annealing temperature was 56,1°C, the right primer concentration was 0,125 µM and the right amount of DNA in a PCR sample of 40 µl was 0,5 µl.

On the cre samples only the optimization was performed not a touchdown because the primers worked. The protocol was used before but the results weren't clear enough. Because it was used before, it wasn't in the plan to determine the right annealing temperature but the results of the different primer concentrations showed a lot of nonspecific binding. So the annealing temperature was determined afterwards and this was 51°C. The right primer concentration is 0,25 µM. The right amount of DNA to be added is 0,25 µl.

The results of the optimized protocols are good. The bands are very clear so the researchers can be sure about the genotype of the animals used in their experiments.

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List of abbreviations

3D	3 dimensional
AGE	advanced glycation end products
bp	base pairs
CAT	computerized axial tomography
cDNA	copy deoxyribonucleic acid
cre	cyclization recombination
dATP	deoxyadenosine triphosphate
Dat-SPECT	dopamine transporter single photon emission computed tomography
DBS-STN	deep brain stimulation in subthalamic nucleus
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleotide triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
ES	embryonic stem cells
GABA	gamma aminobutyric acid
GPe	external segment of the globus pallidus
GPi	internal segment of the globus pallidus
kb	kilo base
kDa	kilo Dalton
KO	knockout
L	liquid
loxP	locus of crossing over of bacteriophage P1
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction

PD	Parkinson's disease
pH	potentia Hydrogenii
PTEN	induced putative kinase 1
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
S	solid
SDS	sodium dodecyl sulphate
SOP	standard operating procedure
STN	subthalamic nucleus
TAE	tris-acetate-EDTA
Taq	*Thermus aquaticus*
TD-PCR	touchdown polymerase chain reaction
tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
WT	wildtype

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease that in most cases develops at a later age. The main symptoms are disturbance of the motoric skills and tremor. The condition is progressive and really decreases the quality of life. Every year there are 1500 new cases of PD in Flanders, Belgium. This is mainly due to the aging of the population [1] [2].

Because of the increase of PD patients and the fact that the real cause is still unknown, research is necessary to better understand the functioning of the brain. Once the several brain circuits are fully understood, an evolution towards a cure for Parkinson's disease is possible. Nowadays there is only treatment available to alleviate the symptoms.

To be able to perform this research there is need of a model organism for the experiments. In this case Parkinson's research mice are used. These mice need to suffer from PD and they are created by labs that make transgenic and knockout mice. To create such a knockout mouse there is need of a technique that deletes a gene or at least a part of it. This technique is called the cre-lox system which consists of the enzyme cre recombinase and loxP sites. Only when mice have the gene for cre recombinase the cre-lox system can be used to modulate the mouse's genome. The genotyping of the cre mice is necessary to find out if the animals contain this gene. The PINK1 mice are used in experiments regarding olfaction and novelty detection. One of the first symptoms of Parkinson's is the loss of olfaction and the inability to detect new things. The researchers train the mice with novelty tasks, this means exposing them to familiar and new odours. Afterwards they cut and stain the mouse's brain to check the loss of dopaminergic neurons. They want to know if this really corresponds with the loss of olfaction and novelty detection. Before the animals can be used for the experiments it is necessary to be sure of their genotype otherwise the tests will be useless. PCR is one of the techniques that can be used for genotyping. For the results to be reliable the protocols need to work optimally. There is a need for a standard operating procedure (SOP) that is established by doing experiments to find out which parameters need to be changed in the provided protocol to make sure the PCR is optimal.

2. Literature study

2.1. Parkinson's disease

2.1.1. General

Parkinson's disease is a kind of parkinsonism. This term represents several neurodegenerative diseases that have similar symptoms as Parkinson's and are divided into primary parkinsonism and secondary parkinsonism.
Primary parkinsonism represents PD itself. The condition is also called the idiopathic form which means that there is no known cause for it.
Secondary parkinsonism represents the diseases that do have a known cause and have similar symptoms as PD [3].

2.1.2. Symptoms

The pattern of symptoms is different for every patient. Some suffer more than others. According to Hoehn and Yahr there are five phases in Parkinson's disease:

- Phase one: This phase includes mainly tremor of one limb and there are changes in posture, mobility and facial expression. The symptoms occur only at one side of the body. They are mild and inconvenient.
- Phase two: In this phase the symptoms are visible on both sides of the body. Because of this the posture and the way a patient walks will change completely.
- Phase three: In this stage the balance will be disturbed. The patient can still function independently although movements will be slower.
- Phase four: The symptoms get more severe. The limbs will get more rigid and mobility will get difficult. The patient can't fully function on his own and will need help every day. Although most symptoms get worse at this stage, the tremor could become less than in the previous phases.
- Phase five: This is the most severe phase. The patient will be completely disabled and will need constant care because they won't be able to walk anymore.

There are a lot of symptoms that can occur during the patient's life. The first symptoms are mainly constipation, loss of olfaction, constant desire to move the legs, sleep disorders and mood swings. Everyone links Parkinson's disease to disturbance of the motoric skills. There are a lot of symptoms regarding the locomotion but the most known is the tremor of the limbs. Besides this there is also rigidity, bradykinesia, pain in the muscles, … The condition affects the nervous system and that's why some people have trouble controlling things. They might have problems with their bladder, augmented sweating and a lot more. There aren't only physical symptoms as some patients undergo behavioural changes [4] [5] .

There are two forms of Parkinson's disease. The early-onset and late-onset form. There is not much difference between these two regarding the symptoms but patients with the early-onset form suffer more from bradykinesia and rigidity. The main symptom of people with the late-onset form is gait disturbance. The younger the patients, the slower the progression of the symptoms. So for people with late-onset PD the progression will be more severe [6].

2.1.3. Consequences

As mentioned before PD is a neurodegenerative disease which means it induces the death of important brain cells, called neurons. These nerve cells are specialized in receiving and passing on information. They consist of an axon and dendrites at both sides as you can see in figure 1. The information is received through the synapse that connects the neuron to another cell (this can also be a neuron). The incoming neurotransmitters produced by the neurons, generate an electric charge that's divided all over the cell body. When this charge reaches the limit, the neuron will start firing to pass on the information to other connected cells [7].

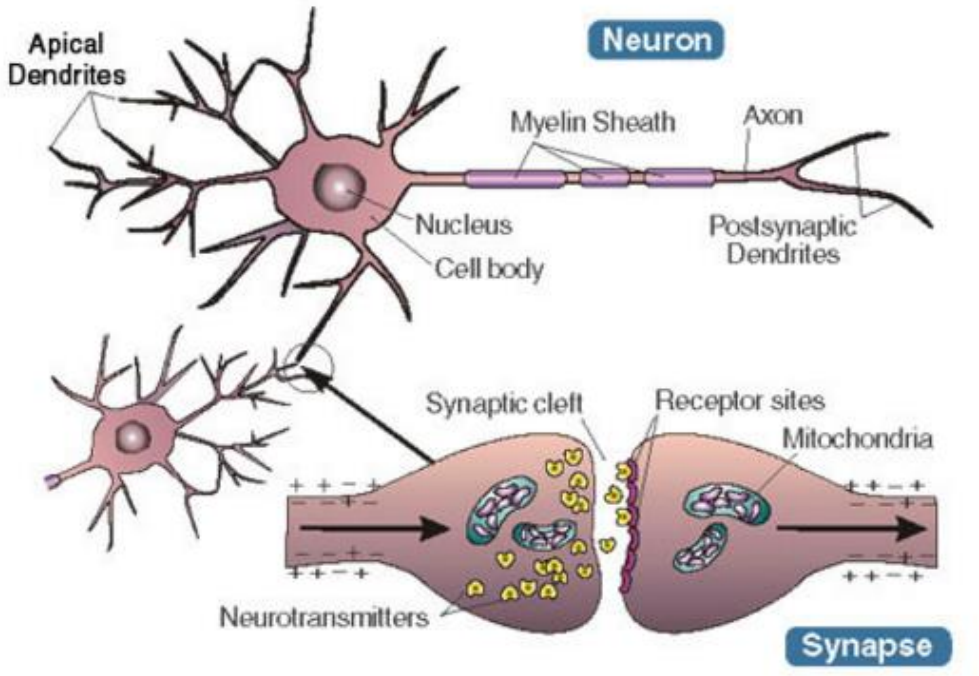

Figure 1: Neuron and synapse [8]

PD specifically targets the neurons that produce dopamine. Dopamine is a neurotransmitter that is active in several brain circuits. One of these circuits is the nigrostriatal pathway. This one coordinates the motoric activities and consists of the substantia nigra and the dorsal part of the striatum (caudate nucleus and putamen) (see figure 2) [9] [10]. The subthalamic nucleus and globus pallidus are areas between the striatum and the substantia nigra [11].

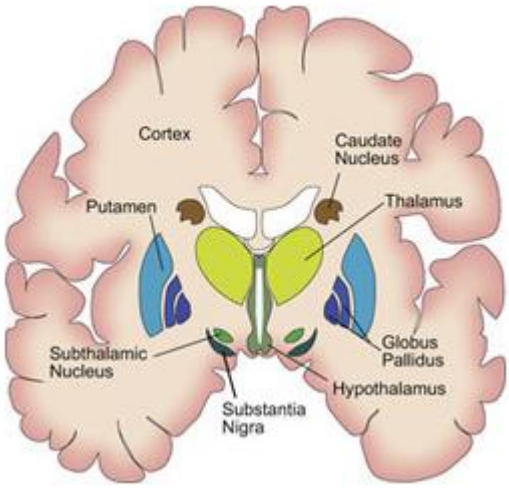

Figure 2: Important components of the nigrostriatal pathway [12]

Patients will lose dopaminergic neurons in the substantia nigra [13]. The name refers to the dark colour of these neurons. They produce a large amount of neuromelanin which is a colour pigment. The substantia nigra consists of two parts. One is the pars compacta and the other one is called the pars reticula. The dopaminergic neurons that get affected by PD are found in the pars compacta [14]. In figure 3 you can see the substantia nigra of a healthy person (top) and a PD patient (bottom).

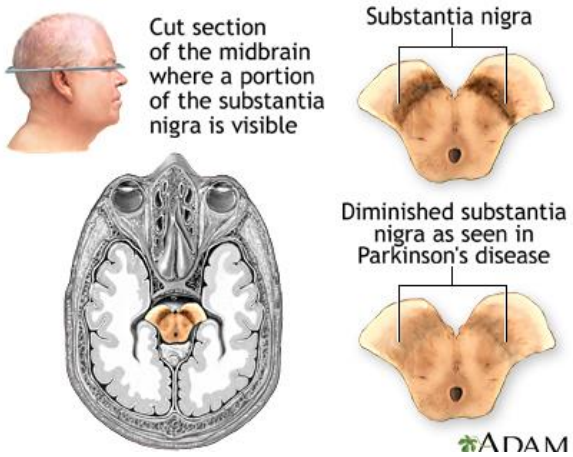

Figure 3: Substantia nigra in healthy person and patient [15]

The amount of dopaminergic cells in the substantia nigra decreases thus the level of dopamine decreases which results in less motoric movement because of the loss of neurotransmitter to pass on information. Because the substantia nigra becomes less active, there are other parts in the brain that become more active, namely the internal segment of the globus pallidus (GPi) and the subthalamic nucleus (STN). The dopaminergic neurons in the substantia nigra project to the caudate nucleus and putamen in the striatum. Dopamine can have an inhibitory effect or an excitatory effect. The striatum contains GABAergic cells which produce γ-aminobutyric acid (GABA). This neurotransmitter has an inhibitory effect. The inhibitory projections from the striatum to the external part of the globus pallidus (GPe) will increase which makes this part less active. A smaller amount of inhibitory projections from the striatum will go to the internal segment (GPi) which makes it more active than in the normal situation. The GPe also contains GABAergic neurons and because it's less active there will be less inhibitory neurotransmitters projected to the STN. This results in overactivity. The STN contains neurons that produce the excitatory glutamate. And

because it's more active there are increased excitatory projections to the GPi. This part is really active which results in an excess of inhibitory projections from GABAergic neurons to the thalamus. This part becomes less active because of the inhibition. It projects less excitatory neurotransmitters to the frontal cortex which results in a decreased activity in that part. This hypoactivity makes the planning of movements difficult and that's why PD patients are slower (see figure 4) [12] [16].

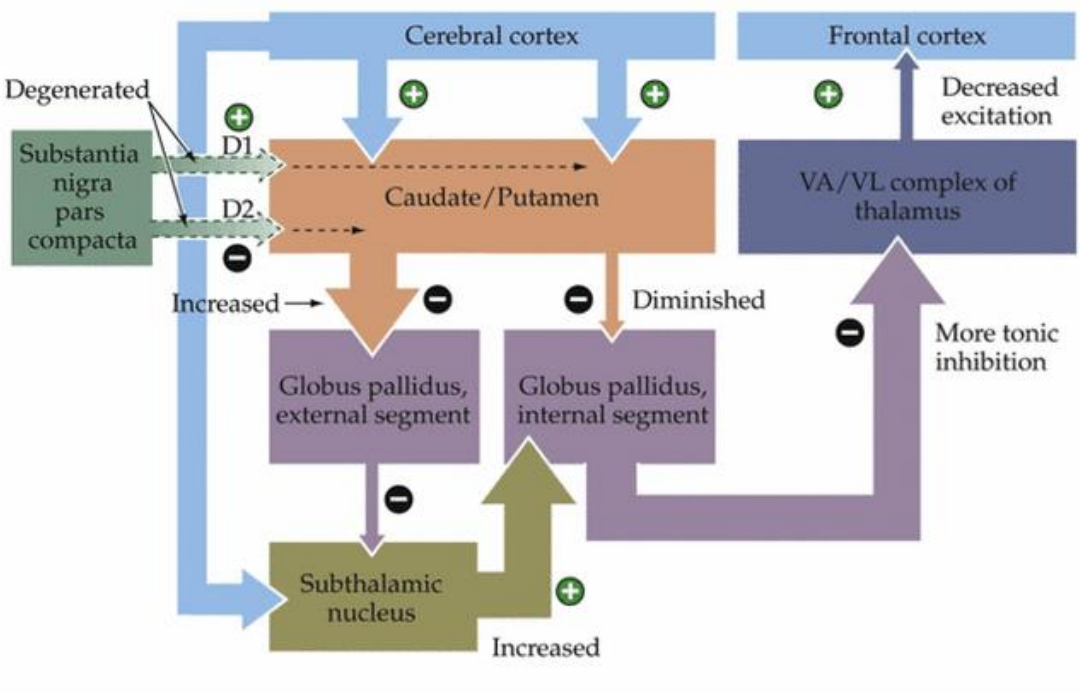

Figure 4: Dopamine circuit in PD patient [12]

2.1.4. Diagnosis

Diagnosing Parkinson's disease is not easy as there are other conditions with the same symptoms. When the doctor suspects PD, he will sent the patient to a specialist. This neurologist will talk about the person's history regarding illnesses and medication. Afterwards he will let the patient do some exercises to check the functioning of the brain. An example of such a test is following an object with the eyes. Besides the brain function, he will also test reflexes, muscle strength, coordination and muscle tension. The specialist checks this by making the patient do some movements like stretching an arm. If the patient has some other complaints, the neurologist will test the memory, concentration, … There will also be a general clinical check-up.
To conclude that a person has PD, he has to suffer from rigidity and at least two of the following symptoms:

- slowness,
- tremor,
- gait disturbance,
- balance disturbance [17].

The neurologist can then prescribe a medicine for PD. If patients react well, the diagnosis will be PD. If they don't react to the medication, there are some additional tests that can be executed to make a right diagnosis [18].

A Dopamine Transporter Single Photon Emission Computerised Tomography scan or DaT-SPECT scan is used to check the amount of living dopaminergic neurons. Before going under a scanner the patient will be injected with a radioactive tracer which will be taken up by the dopaminergic neurons. The computer will then show a 3D (3 dimensional) image of the brain (see figure 5) [18] [19].

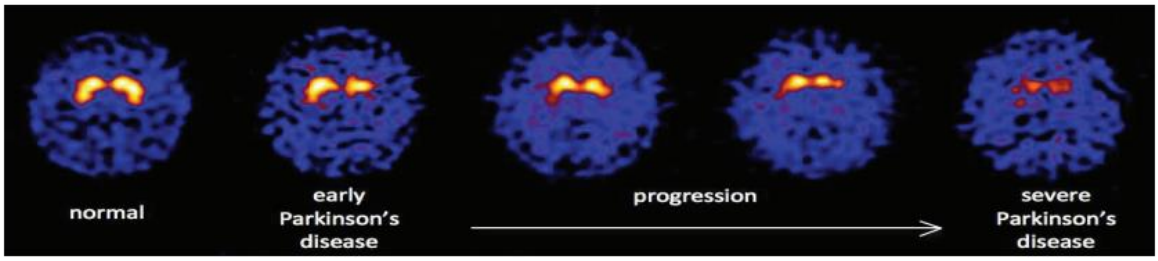

Figure 5: DaT-SPECT scan of healthy person and patient + progression [20]

A CAT scan or Computerised Axial Tomography will be performed to exclude other diseases like a brain tumour or a stroke. Parkinson's disease itself cannot be detected by this kind of scanning. A CAT scan makes several cross section images of the brain by X-rays [18] [21]. Also an MRI scan (Magnetic Resonance Imaging scan) and an EEG (Electro Encephalogram) are used for this purpose. The MRI scanner makes cross section images of the brain by using a magnetic field and radio waves [18].

2.1.5. Treatment

There is still no medication to cure Parkinson's disease completely. This is mainly due to the fact that the actual cause is still unknown. However, there are some treatments that make the symptoms bearable.

Medication

Because of the decrease in dopamine levels, patients can take dopaminergic medication. An example is Levodopa. This medicine is used the most by the people who suffer from PD. It is in the form of pills and when it's swallowed, it will be absorbed by blood and travel to the brain. Here, the medicine is converted to dopamine that will be stored in the neurons until it is needed for movement. In most cases Levodopa comes with another medicine called Carbidopa. This is an enhancer that makes it possible to take a lower dose of Levodopa to reduce the side effects. The combination can be bought as extended release capsules. These capsules maintain the concentrations of the medication for a longer period of time. After four to five hours the concentration will start decreasing. Besides the capsules there is also the option of a dopamine intestinal infusion pump (DUOPA™). This pump will provide the right dose of medication for sixteen hours. There are a couple of side effects of Levodopa like nausea, vomiting, light-headedness, loss of appetite, lower blood pressure, confusion and dyskinesia. Dyskinesia means the sudden involuntary movements of the arms, legs, face, …. [22]

Another type of medication are dopamine agonists. These are molecules that mimic the dopamine that is normally produced in the brain while Levodopa is converted to dopamine and stored in the neurons. Examples are pramipexole, ropinirole, rotigotine and apomorphine. Some side effects are confusion, hallucinations, sleepiness, dyskinesia, compulsive behaviour and swelling of the ankles [23].

Medicines for PD can interact with certain kinds of food like protein rich food, vitamins, herbal supplements, …. [23]

Deep brain stimulation of the subthalamic nucleus (DBS-STN)

For deep brain stimulation two electrodes will be implanted in the brain (one on the left and one on the right). First of all a metal ring will be placed around the patient's head and fixed to the skull. The specialist will make a brain scan to make sure that the procedure will be very precisely executed. The patient will be anesthetised and the specialist will make a hole in the skull. Through the hole the micro electrodes will be inserted to measure the activity of the nerve cells. That way the specialist takes the electrode to the subthalamic nucleus. To make sure that the electrode is placed in the right position, the patient will no longer be anesthetised and will have to do some exercises. During these exercises an electric current will be sent to the brain to reduce the symptoms. Once the right place for the electrode is determined, it will be attached to the hole definitively. This procedure will be repeated to implant the second electrode.

The definitive stimulator which will send electric impulses to the brain is implanted in the chest. A wire will connect the stimulator in the chest and the electrode in the head (see figure 6).

There are some negative aspects about this treatment. Stimulation can induce psychological or psychiatric symptoms such as euphoria, depression, confusion, … These symptoms are temporarily in most cases. There is always a slight chance of a stroke. The stimulator in the chest works with batteries. After approximately three years the batteries are empty and a new operation is necessary [24].

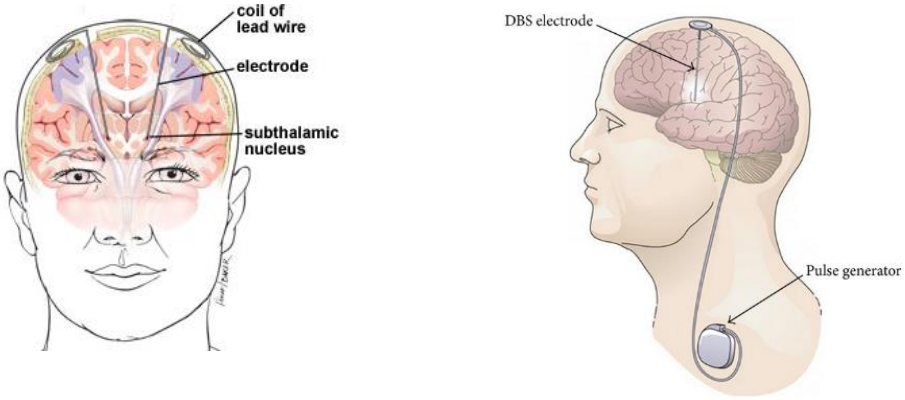

Figure 6: DBS-STN [25] [26]

Pallidotomy and thalamotomy

As mentioned before some parts of the brain become overactive, namely the globus pallidus and the subthalamic nucleus. Pallidotomy and thalamotomy are surgeries to damage the overactive parts. The surgeons use radio-frequency energy to destroy a small part. Pallidotomy reduces the tremor, bradykinesia, rigidity and balance disturbance. Thalamotomy only eliminates tremor, so this type of surgery is not chosen very often as a treatment. Patients really have to think about this option because the surgeries are irreversible [27] [28].

Kinesiotherapy, ergo therapy, speech therapy

Kinesiotherapy is a very important element during the life of a PD patient. By doing the right exercises the patients can train their muscles for strength, muscle activity and coordination.

Because of the slower movements of the muscles, eating and speaking will also get difficult. Speech therapy will improve articulation, communication, swallowing, chewing, …
Ergo therapy will make a patient's life easier. The therapist gives advise on how to adjust the house to the difficult movements and how to do daily life activities in a more easy way [29].

2.1.6. Cause of the disease

The actual cause of PD is still unknown but researchers think it's a combination of several factors [30].

Genetics

Researchers don't fully understand the genetics of PD yet. They know that there are mutations which are responsible for the development of the disease. These mutations may occur in the following genes: SNCA, PARK2, PARK7, LRRK2 and PINK1. Inheritance of Parkinson's disease is possible but it's very rare and the mechanism of inheritance is not fully understood yet [31]. Many researchers think that it's a combination of mutations in the genes and the environment [32].

- SNCA gene: alpha-synuclein
    - Function: It's mainly found in the presynaptic terminals of neurons and is considered to play a role in the supply of presynaptic vesicles. These vesicles contain the neurotransmitters used to pass on signals from one neuron to the other [33].
    - In case of mutations: All of the mutations result in a gain of function, which means that the activity of the protein is enhanced. Studies of animal models with an overexpression of α-synuclein showed an increase of intracellular reactive oxygen species, decreased proteasome activity, stress of the endoplasmic reticulum and mitochondrial dysfunction which eventually induced cell death. *Drosophila* models with overexpression showed degeneration of the dopaminergic neurons, inclusions that looked like Lewy bodies and motor deficits [32].
    - Form of PD: autosomal dominant early onset [32]

- LRRK2 gene: dardarin or leucine-rich repeat kinase 2
    - Function: This protein is a kinase and probably plays a role in passing on signals, making up the cytoskeleton and protein-protein interactions [34].
    - In case of mutations: It's a kinase and cell models have shown that a mutation in the LRRK2 gene results in ubiquitinated inclusions and induces death of neurons. The clinical features of PD caused by a mutation in LRRK2 depend on the kind of mutation. Some of them cause Lewy bodies, others only cause loss of cells in the substantia nigra [32].

- PARK2 gene: parkin protein.
    - Function: This is a E3 ubiquitine ligase and it tags unneeded proteins with ubiquitin. These tagged proteins will be recognized by proteasomes and will be degraded. According to researchers parkin plays a role in the maintenance of mitochondria and makes sure that the organelles are broken down when they're not working properly [35].
    - In case of mutations: A mutation in this gene results mainly in loss of nigral neurons and loss of function. Mice wherein the PARK2 gene was knocked out, displayed

mitochondrial dysfunction and oxidative stress. Overexpression of the parkin protein had a protective effect [32].
    - Form of PD: autosomal recessive early onset

- PARK7 gene: DJ-1 (protein deglycase).
    - Function:This protein is a protein deglycase which removes the sugar groups from a protein and in this way prevents the formation of advanced glycation end products (AGE) which are dangerous for our health [36]. It also plays a role in the protection against oxidative stress.
    - In case of mutations: Just like the parkin knockout mice, the PARK7 knockouts displayed a dysfunction of the mitochondria and oxidative stress [32].
    - Form of PD: autosomal recessive early onset

- PINK1 gene: Just like the PARK7 gene it codes for a protein that plays a role in the protection against oxidative stress. This will be discussed into detail in chapter 2.2 PINK1 gene.

Environment

There are a couple of environmental factors that can increase the risk of developing PD. These factors can be toxins like carbon monoxide, carbon disulphide, manganese, pesticides and herbicides which are generated by farming, traffic and industrial pollution. Also viruses could influence the development of Parkinson's disease [30] [31].

Proteins

Researchers found that the brain of PD patients contains Lewy bodies (see figure 7) [31]. These structures are also present in the brains of people with Lewy body dementia, Alzheimer's and Gaucher disease [37].They are clumps of accumulated proteins that are formed in the brain cells. The main protein that these bodies contain is alpha-synuclein. They still don't know what causes the formation of these Lewy bodies and which consequences it has. More research is needed to fully understand it but there are already some theories. It could be that the cell can't function properly because of the large structure that can't be broken down. Another theory is that the accumulation of such a high level of proteins could be harmful and eventually induce cell death [31]. Recently there was some new evidence that the Lewy bodies could have a protective function. So there is still no conclusion about the function and the consequences of these structures. A lot more research is needed [37].

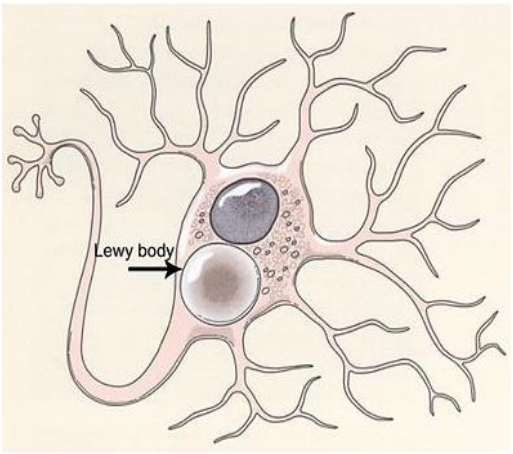

Figure 7: Lewy body in neuron [31]

Oxidative stress

Oxidative stress is also one of the things that may cause the death of dopaminergic neurons. Mitochondria are the organelles that supply the energy for proper functioning of the cell. Free radicals are formed during the oxidation cycle. These are molecules that lack an electron and react with other molecules to replace the missing electron. If the mechanisms to get rid of these free radicals don't work properly there will be oxidative stress. This stress damages all kinds of molecules

like DNA, molecules in the cell membrane and so on. This phenomenon was found in patients of PD. Also the environmental toxins can cause an abnormal formation of free radicals [31] [30].

2.2. PINK1 gene

2.2.1. Location

In figure 8 you can see that the PINK1 gene is located on the short arm of chromosome one at position 36.12 (1p36.12) [38]. The gene counts eight exons. These are the parts that are coding for the protein induced putative kinase 1 (PTEN) [39].

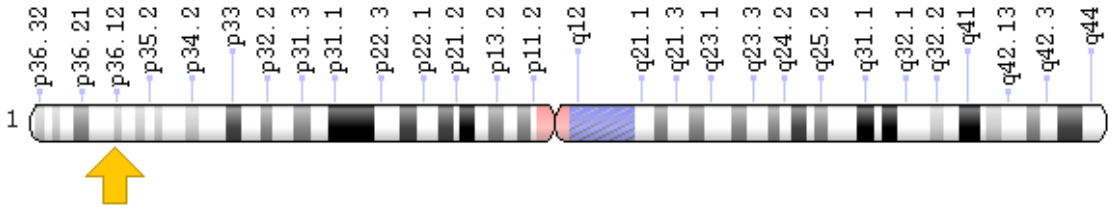

Figure 8: Chromosomal location of PINK1 gene [40]

2.2.2. Mutations

There are a lot of mutations in the PINK1 gene that can cause Parkinson's disease. Most of these result in the autosomal recessive early onset form of PD [38]. The mutation that is introduced in the PINK1 mice that need to be genotyped is the deletion of exon 2 and 3 (see figure 9). This one too results in the early onset form [41].

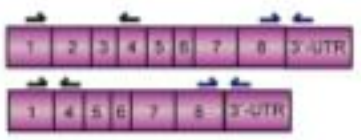

Figure 9: Schematic representation of exon 2 and 3 deletion [41]

2.2.3. PTEN induced putative kinase 1

2.2.3.1. Properties

As mentioned before, PTEN stands for induced putative kinase 1. Another name that's commonly used is serine/threonine-protein kinase. This protein is produced in the mitochondria of every cell but at higher levels in the skeletal muscles, testis and heart. After production, PTEN is transported to the outer membrane of the mitochondria. There are two fragments of the protein, namely one of 55 kDa and one of 48 kDa. The fragments are formed by the proteolytic cleavage after the production of the whole protein. They are transported to the cytosol of the cell while the intact form stays in the outer membrane of the mitochondria. The protein has a protein kinase domain, which means that it can phosphorylate other proteins [42] [43].

2.2.3.2. Function

The main function of PTEN is to make sure that the parkin protein is translocated to damaged mitochondria. The mechanism of this process is not fully understood yet but some research has been done to understand most parts.

PTEN is situated in the outer membrane of mitochondria. In case of an excess of this protein it will translocate to the inner membrane where it is degraded by a proteasome. For this translocation there needs to be a membrane potential. But when a mitochondrion is damaged, the potential will

change. The excess of PTEN will not be able to translocate to the inner membrane anymore and will stay in the outer membrane. The protein will then autophosphorylate its serotonin residues. This phosphorylation will induce the translocation of the parkin protein to the outer membrane of the damaged mitochondrion. As mentioned before, parkin works as an enzyme that catalyses the ubiquitylation of the proteins in the mitochondrial outer membrane (see 2.1.6 Cause of the disease). In healthy mitochondria the ubiquitin ligase activity of the parkin protein is suppressed. Ubiquitylation means that ubiquitin proteins are going to bind to the damaged mitochondrion. A proteasome will then degrade the mitochondrial proteins in the membrane through autophagy (see figure 10).

So PTEN is a protein that helps to get rid of the damaged mitochondria. Once this protein doesn't function properly the damaged organelles will continue living and consequently decrease the functioning of the muscles. The working of the PTEN/parkin interaction in neurons itself are not yet known [41].

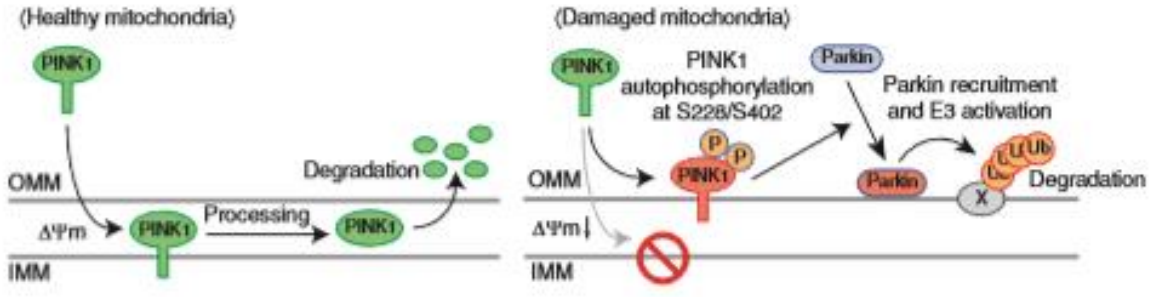

Figure 10: Schematic representation of the function of PTEN [41]

2.3. Knockout mice

Knockout mice are used in the lab to perform research on genetic disorders and functions of particular genes. In these mice a gene of interest is inactivated. This can be done by replacing or deleting a complete gene, by disrupting the gene with an artificial DNA insert or by deleting exons (coding regions) [44].

In figure 11 you can see how knockout mice are created. First of all embryonic stem cells (ES) are taken from a mouse, for example a black mouse. The mutation is introduced in these cells and this can be done by two different methods which will be explained later. The altered ES (one normal chromosome + one mutant chromosome) are injected in an embryo that is in the blastocyst stage. These embryos are from another mouse, for example a brown mouse. The blastocyst with the altered ES is then implanted in a surrogate female mouse. In case of the figure it is a white one. When the mother gives birth, the pups will have two different colours, namely brown and black because the blastocyst contained ES from the black mouse and the brown one. Mice with two different fur colours are called chimeras. After a few weeks a male and a female chimera will mate. But the gonads of both mice have to be formed out of the ES of the black mouse (these cells contain the mutant gene). After giving birth, 50% of the offspring will be heterozygous for the mutant gene. 25% will have two normal genes and 25% will have two mutant genes so these last ones are the knockout mice.

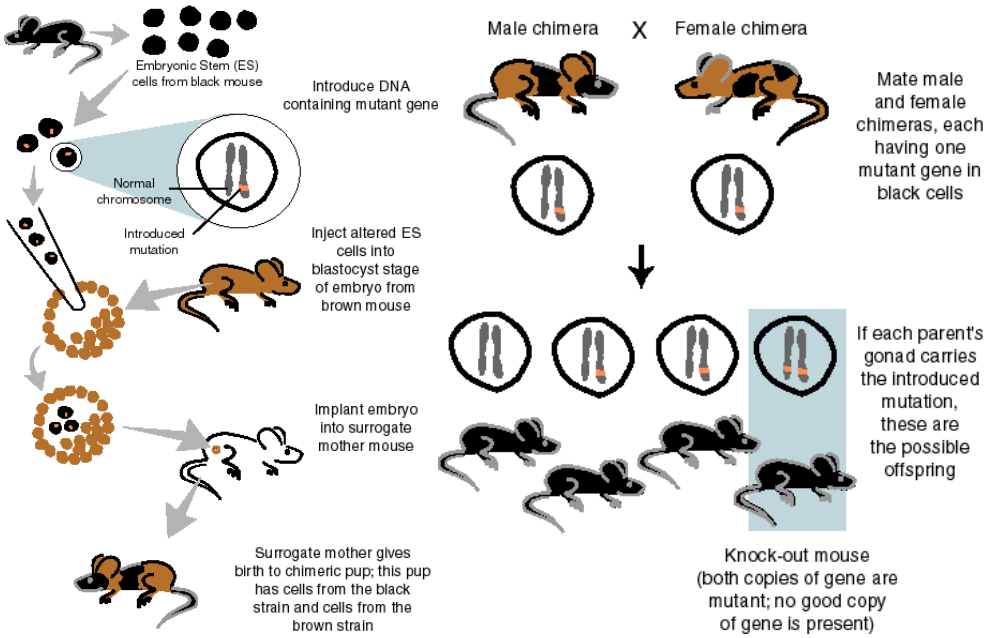

Figure 11: Engineering knockout mice [45]

2.3.1. Gene targeting

As mentioned before, there are two different techniques for introducing an artificial DNA fragment to knock out a gene. One of the methods is gene targeting.

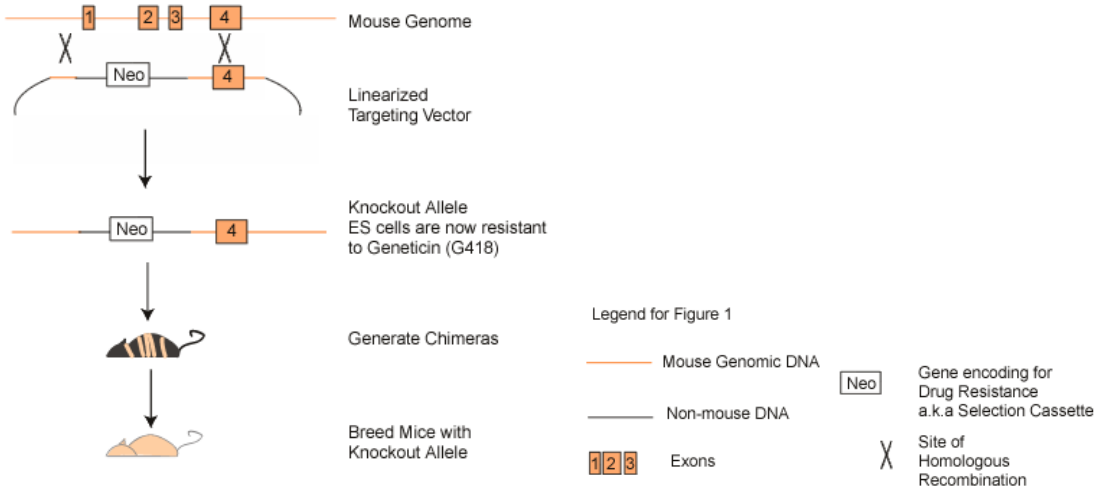

Figure 12: Schematic representation of gene targeting [46]

In figure 12 you can see that exon one, two and three are deleted. This can be done by using a vector. For gene targeting this vector contains a marker. In this case it is a gene that codes for drug resistance. The DNA sequences on both sides next to the marker are homologous to the DNA sequences flanking the first three exons. The vector will be injected in embryonic stem cells and through homologous recombination it will be inserted in the genomic DNA of the mice, replacing the first three exons. Homologous recombination is the exchange of sequences between two similar DNA molecules. The ES are inserted in a blastocyst which is implanted in a surrogate mouse. The chimeras will mate and 25% of the offspring will be knockout mice [44] [46].

2.3.2. Gene trapping

The second method is called gene trapping. This technique also includes a vector which is not the same as the one used for gene targeting. It contains a splice acceptor, a DNA sequence coding for a reporter protein and a poly-A tail. Unlike the gene targeting, gene trapping is completely random. There is no gene that is targeted. The vector will be inserted into the genomic DNA at a random place. The splice acceptor will make sure that there will be no normal splicing after the first exon. Instead the sequence following exon one will be transcribed. The poly-A tail stops the transcription early to produce a truncated protein. This protein consists of a part of the original protein and the reporter protein (see figure 13) [47].

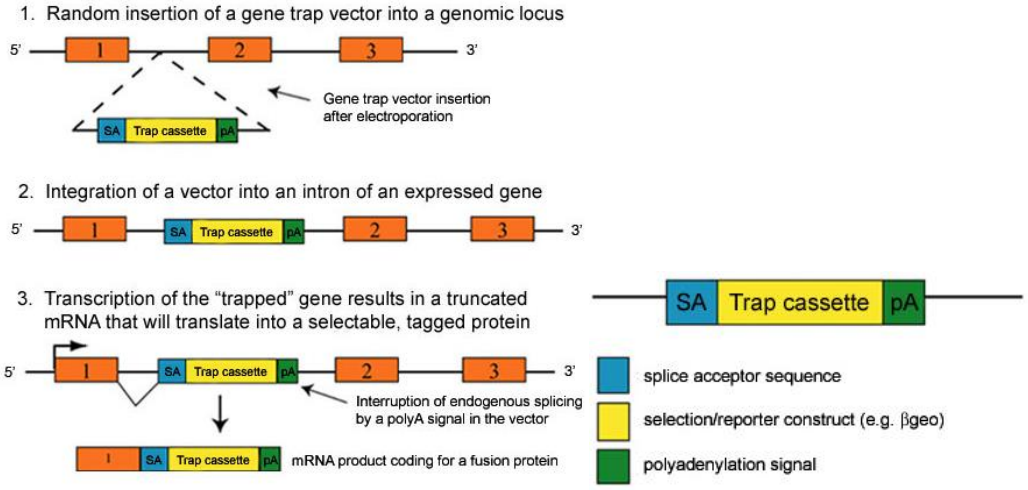

Figure 13: Schematic representation of gene trapping [47]

2.4. Cre recombinase

Cre recombinase is an enzyme that is derived from the P1 bacteriophage and catalyses recombination of targeted DNA sequences . It belongs to the family of the site-specific recombinases. These enzymes are used to edit the genomic DNA [48] [49].

Cre stands for cyclization recombination for the recombination mechanism occurs in a cyclic way. The enzyme is used in combination with loxP sites which forms the cre-lox system. According to the orientation of these sites, DNA sequences can be translocated or a targeted gene can be inversed or deleted (see figure 14) [50].

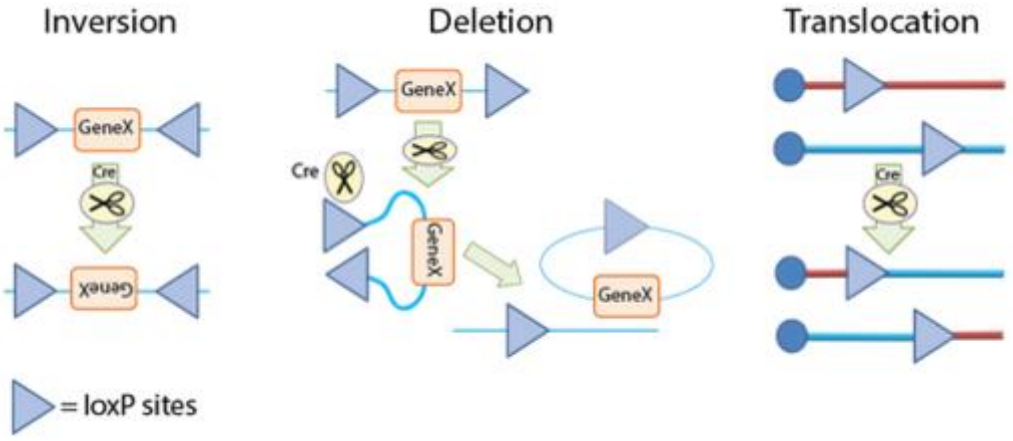

Figure 14: Three different orientations of the loxP site and their functions [51]

The loxP sequences consist of 34 base pairs. In the middle is a spacer region of eight base pairs and at the flanks are two palindromic sequences. In figures the loxP sites are indicated by a triangle (see figure 15) To delete a specific sequence the loxP sites need to be in the same direction [52].

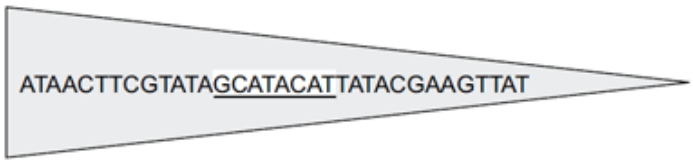

Figure 15: Schematic representation of a loxP site [52]

The cre recombinase doesn't need any cofactors to fulfil its function which makes it a really desired enzyme to create knockouts. The amino domain interacts with the phosphate backbone of DNA strands. The carboxyl domain contains the active site of the enzyme and participates in interactions with DNA. When bound to a loxP site it will form a dimer. Each monomeric subunit of the dimer is bound to one side of the loxP site (see figure 16). Another dimer is bound to the other loxP site. These two dimers will then form a tetramer with in the middle a hydrophobic cavity where the strand exchange occurs (see figure 17). The cre recombinase will excise the DNA strands to form a circular fragment of the DNA with the targeted gene that is now cut off the genomic DNA. One of the loxP sites will remain in the genomic DNA of the chromosome [50].

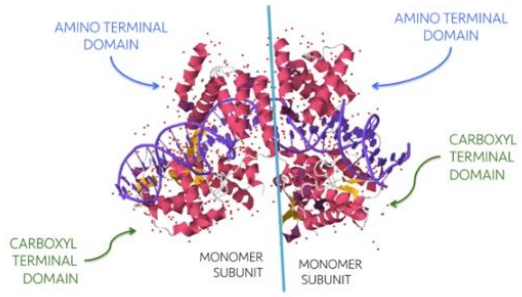

Figure 16: Cre recombinase dimer [50]

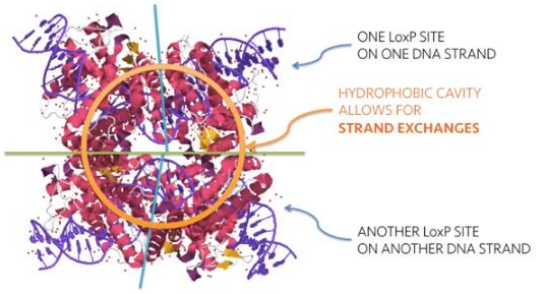

Figure 17: Cre recombinase tetramer [50]

2.5. PINK1 knockout mice

The PINK1 knockout mice that need to be genotyped are generated by the gene targeting method. Cre-lox recombination was used to delete the targeted exons.

Figure 18 represents the method used to create the PINK1 knockouts. The black arrows represent the vector. This one contains a 5' homology arm which is the sequence homologous to a part of exon one and intron one and a 3' homology arm which is homologous to exon four, five, six, a part of exon seven and the introns in between. In the midde of the vector is a floxed homology arm. This one consists of a marker (neomycin) and exon two and three floxed by two loxP sites in the same direction. The vector was inserted into embryonic stem cells by electroporation. The mice with the vector were neomycin resistant and the succes of the homologous recombination was double checked with Southern blotting and a restriction reaction with ScaI and HindIII (insertion of the vector gives a third restriction site for HindIII). The ES that were altered succesfully, were injected into a blastocyst which was implanted in a surrogate mouse. The offspring were chimeras and these were bred to get mice with the artificial DNA fragment in both chromosomes. These were then crossed with mice who had the cre gene. The offspring of these ones have both the artificial DNA fragment and the cre gene. Cre recombinase encoded by the cre gene spliced the artifical DNA fragment at the loxP sites to delete exon two and three [41].

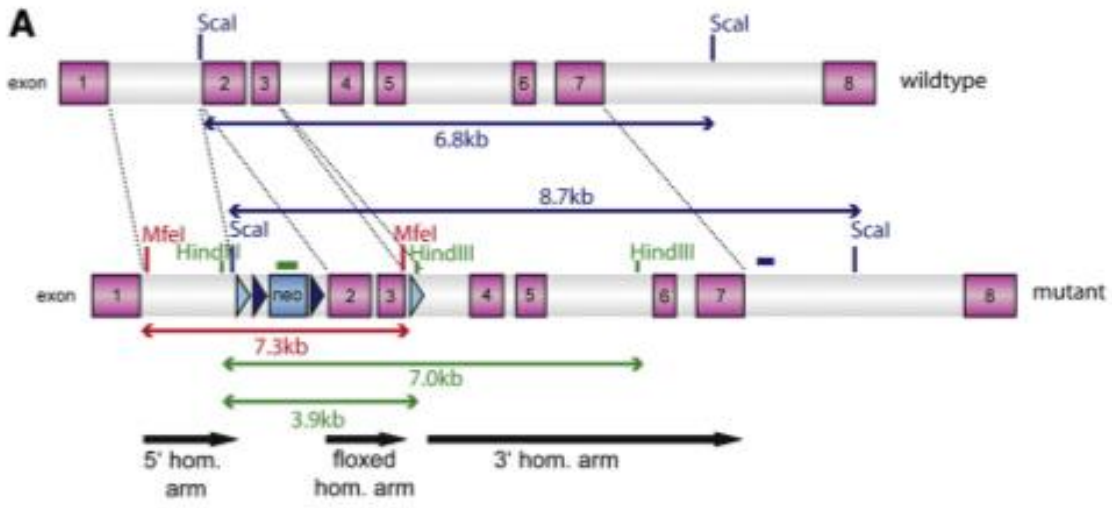

Figure 18: Schematic representation of the exon 2 and 3 deletion [41]

2.6. Genotyping

Genotyping is a technique used to determine the genetic make-up of organisms by examining the DNA sequence. The genotype is the collection of genes found on the chromosomes but this is now called the genome in most cases. Genotype is now mainly used to indicate a part of the total amount of genes [53] [54]. Genotyping can be done by several different methods. In this essay only the most common ones are explained:

- RFLP stands for restriction fragment length polymorphism. In this method DNA molecules are cut into smaller fragments by restriction enzymes. Every enzyme recognizes its specific short sequence of minimum four base pairs and cuts only in this site. The cutting occurs by hydroxylation of the phosphodiester bonds. A mutation in one of the molecules at the

restriction site will display a different pattern of bands after gel electrophoresis. By comparing the patterns with a pattern of a negative or positive control, one knows in which sample the mutation is present [55].

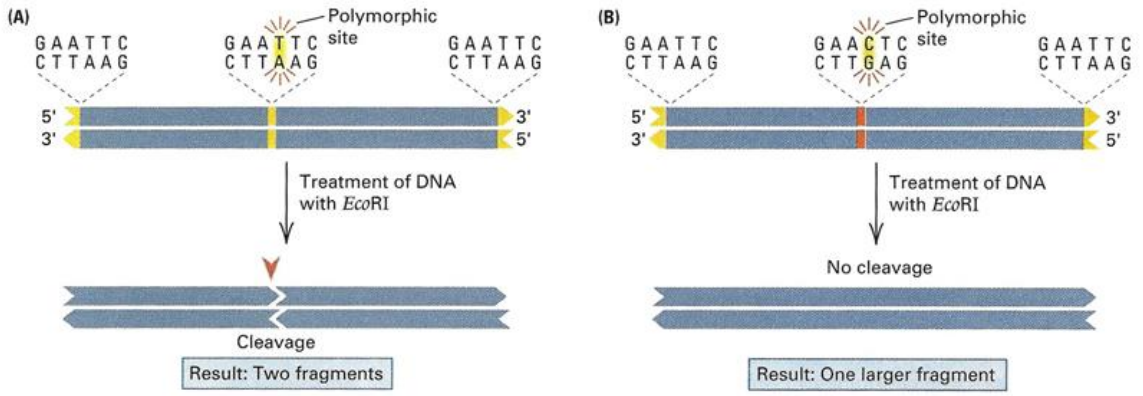

Figure 19: Principal of RFLP [56]

In figure 19 you can see that GAATTC is the sequence that is recognised by restriction enzyme EcoRI. In (A) there is no change in the sequence, so EcoRI will cleave the DNA which results in two fragments which means two bands on a gel after electrophoresis. In (B) you can see there is a mutation in the restriction site which means that there will be no cleavage. EcoRI will no longer recognize this site. This results in just one larger band after gel electrophoresis [55].

- Sequence analysis is determining the order of nucleotides of a DNA molecule. Like the RFLP a reaction with several components is needed. This reaction resembles a PCR reaction but there are some differences. Only one primer is used and besides dNTP's the mastermix also contains ddNTP's (dideoxynucleotides) with a fluorescent label. When these ddNTP's are added to the DNA fragment by a polymerase the elongation will stop. The 3' end will contain a hydroxyl group on which nucleotides can't attach. So the result after a reaction is an amount of DNA molecules with different lengths. These fragments will be separated by capillary electrophoresis. The shortest fragment will come out first and the largest one last. The output is a chromatogram which shows the sequence (see figure 20) [55].

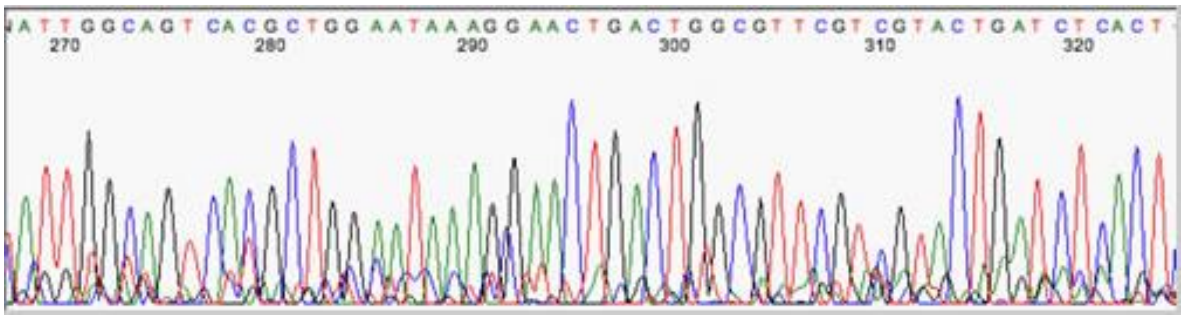

Figure 20: Chromatogram [57]

- A technique that's used more often this time is the DNA microarray. With this array a lot of genes can be examined at the same time. It consists of spots and each spot contains a sequence of a particular gene. mRNA (messenger RNA) is extracted from the human tissue and is transformed to cDNA (copy DNA) by reverse transcriptase. This cDNA is labelled with fluorescent proteins and added to the microarray. The cDNA will hybridize with the DNA

attached to the surface of the spots. If the fluorescence is intense there is a lot of gene expression. No fluorescence in a particular spot which contains the DNA sequence of a gene means that there is no gene expression [58] [59].

- PCR is a polymerase chain reaction which can be used for several applications including genotyping. Primers can be designed to bind to a specific sequence that needs to be studied. To check whether there is expression of a particular gene, the primers have to bind to the sequence just before and just after the gene sequence or on the gene itself. A PCR reaction will amplify DNA fragments which are determined by the primers. The reaction will be checked by gel electrophoresis. The gel will display bands of DNA fragments [55]. PCR was used for the genotyping of the PINK1 mice for it is fast, cheap and not difficult to execute. Two mastermixes need to be prepared, one for the wildtypes and one for the knockouts. The primers for the wildtype mastermix are 5'- gacagtacttgcctagcgtaggtag-3', the forward primer and 5'-cagacacgcgcttggttttctgtgt-3', the reverse primer. The forward primer will bind to a sequence in intron 1 and the reverse primer will bind to a sequence in exon 2. After the PCR the products should show a band of 781 bp (base pairs) on the gel when the mouse is a wildtype. The forward primer of the knockout mastermix is the same as the one used for the wildtype mastermix. The reverse primer is 5'-cttgccctgggtgaatggtgaca-3' and will bind to a sequence in intron 3. When the mice are knockouts, the samples should display a band of 736 bp on the gel [41]. Imagine you have two DNA samples of the PINK1 mice that need to be tested. One sample shows a band after PCR with the wildtype mastermix but none with the knockout mastermix. The conclusion is that this mouse is a wildtype. If the other sample shows a band after PCR with the knockout mix and none with the wildtype mix , then this sample came from a knockout mouse.

2.7. Touchdown PCR

Touchdown PCR is a type of PCR where the starting annealing temperature is a couple of degrees higher than the melting temperature of the primers. Every cycle the temperature will decrease gradually until the calculated annealing temperature is reached. Afterwards the amplification will continue at this annealing temperature. TD-PCR is in most cases used to check if the primers work and it consists of two phases:

- Phase 1: In phase one the starting annealing temperature will be approximately 10°C higher than the melting temperature of the primers. This one will decrease in the next 10-15 cycles. During this phase the binding of the primers to the DNA will be very specific but the yield will be lower.
- Phase 2: In the second phase there are 20-25 cycles at the calculated annealing temperature. Because of the lower temperature the primers will bind more easily which increases the yield of the reaction.

During TD-PCR the yield increases while the specificity of the primer annealing is conserved [55].

2.8. PCR optimization

The purpose of a PCR optimization is to get a product yield that is large enough but still conserve the specificity of the primer binding. The important things that can be optimized are the annealing temperature, the primer concentration and the DNA concentration. These have the most impact

on the PCR reaction. After this the concentration of Mg^{2+}, additives and dNTP's can also be changed to create a better working protocol.

The annealing temperature is very important for a PCR. Normally it is 5°C lower than the melting temperature of the primers. As mentioned before the higher the annealing temperature, the higher the specificity. The lower the temperature, the bigger the yield but then there is the risk of nonspecific binding. To check which one is the best annealing temperature the machine needs to use a different temperature in each row of samples. This can be performed by changing the setups of the PCR machine. Afterwards the reaction will be checked by gel electrophoresis.

The second thing that can be optimized is the primer concentration. A concentration that is too low results in a smaller yield because there are not enough primers that can bind to the DNA molecules. If the concentration is too high there is a chance of nonspecific binding and the primers can also bind to each other forming primer dimers. The primer concentration can be determined by preparing different reactions with different concentrations. This will also be checked by gel electrophoresis.

Also the DNA concentration can be optimized. If there is too little DNA in the reaction there will not be a lot of product. However, this can be fixed by increasing the amount of cycles but then the risk of smear on the gel, after electrophoresis, needs to be taken in account. Too much DNA can cause nonspecific bands after gel electrophoresis. Like the primer concentration this can be optimized by preparing different reactions with different concentrations of DNA [55].

3. Methodology

3.1. Flowchart

The flowchart shows all the steps that needed to be executed for the optimization of the genotyping protocols. The starting point were samples of DNA that were extracted from the mice's tails. This phenol chloroform extraction was done at the beginning of all the experiments. The end point is a standard operating procedure for the genotyping of PINK1 mice and cre mice.

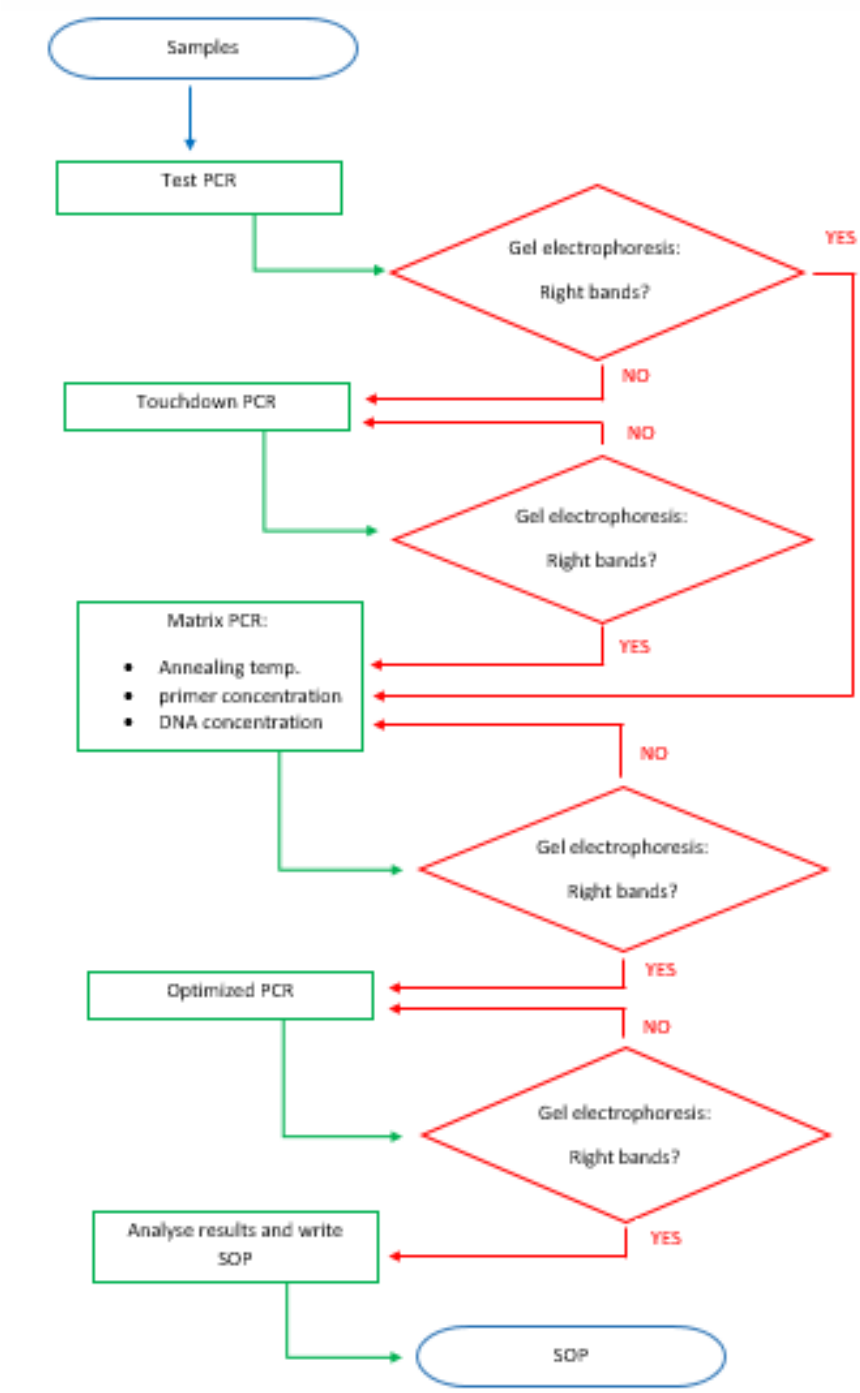

Figure 21: Flowchart of experiments

3.2. Identification and origin of samples

PINK1 samples

Table 1: PINK1 samples [60]

Sample	Identification	Origin
Irun	PINK1 -/-	Tail from PINK1 knockout mouse
Bevel	PINK1 -/-	Tail from PINK1 knockout mouse
Huesca	PINK1 -/-	Tail from PINK1 knockout mouse
Jaca	PINK1 -/-	Tail from PINK1 knockout mouse
Genova	PINK1 +/+	Tail from PINK wildtype mouse
Antwerp	PINK1 -/-	Tail from PINK1 knockout mouse

The names of the mice represent cities in different countries and were given by the lab that created the mice.

Cre samples

Table 2: Cre samples

Sample	Identification	Origin
12/	?	Tail from cre mouse
13L	?	Tail from cre mouse
14R	?	Tail from cre mouse
Founder	Positive for cre	Tail from cre mouse

A founder is a positive control. So this mouse contained the cre gene. The names of the mice were given by the people who take care of them.

3.3. DNA extraction

3.3.1. Samples

Table 3: Samples to use

PINK1 Samples	Cre Samples
Irun	12/
Bevel	13L
Huesca	14R
Jaca	
Genova	
Antwerp	

The DNA of the founder was already available.

3.3.2. Materials

List of used hardware

Table 4: Hardware for DNA extraction

Hardware	Brand	Purpose
Micropipette (1-1000 µl)	Eppendorf	Pipette small volumes of liquid
Thermomixer	Eppendorf	Lysis at 55°C overnight
Fume hood	Vinitex	Avoid dispersion of harmful compounds
Pipetboy	Integra biosciences	Pipette volumes with a volumetric pipet
Centrifuge	Eppendorf	Pelleting of DNA
Micropipette (2-200 µl)	Eppendorf	Pipette small volumes of liquid
Freezer (-20°C)	Liebherr	Store samples
Analytical balance	Satorius	Weighing
Magnetic stirrer	VWR	Stirring to dissolve compound
Heating plate	VWR	Heating to dissolve compound
pH metre	InoLab	Measure the pH

List of glassware and disposables

Table 5: Glassware and disposables for DNA extraction

Hardware	Purpose
Pipette tips	Pipette small volumes of liquid
Gloves	Avoid contamination
Holder for microtubes	To hold the microtubes
Tissue	Clean
1,5ml-microtubes	Container for liquids
Waste beaker	Container for all the waste
Small beaker	Container for liquids (1 for sterile H_2O, 1 for phenol chloroform)
Permanent marker	Label the microtubes
Schott bottles (3 of 200 ml and 3 of 500 ml)	Container for liquids
Spoon	Take small amount of compound
Plastic weighing bowl	Container for compounds
Volumetric pipette (10 ml and 25 ml)	Pipette volumes of liquid

List of reagents and solutions

Table 6: Reagents and solutions for DNA extraction

Name	Batch and brand	S/L	Composition /conc.	H-sentences	P-sentences	Purpose	Waste disposal
Demi H_2O	/	L	/	/	/	Solvent	Sink
Lysis buffer		L	1M Tris-HCl 0,5M EDTA 20% SDS 5M NaCl Demi H_2O			Lysis of tails	WIVA barrel
- Tris base	SLBG2651V Sigma	S	/	/	/	Buffer, stabilize DNA	WIVA barrel
- EDTA	SLBD6046V Sigma	S	/	H319	P305+P351+ P338	Chelation of DNase cofactors	WIVA barrel
- SDS	098K0067V Sigma	S	/	H228,H302+H332, H315, H318, H335, H412	P210, P261, P273, P280, P305+P351+ P338	Break down cell membrane, denaturation of proteins	WIVA barrel
- NaCl	SZBD0700V Sigma	S	/	/	/	Stabilize DNA	WIVA barrel
Proteinase K	1601/002 Macherey-Nagel	L	/	H315, H319, H334, H335	P261, P280, P284, P304 +P340+P312, P337+P313, P342+P311	Denaturation of proteins	WIVA barrel

Name	Batch and brand	S/L	Composition /conc.	H-sentences	P-sentences	Purpose	Waste disposal
Phenol chloroform	BCBP3226V Sigma	L		H301, H312+H332, H314, H336, H341, H351, H361d, H372, H411	P201, P261, P301+P310+ P330, P303+ P361+P353, P304+P340+ P310, P305+ P351+P338	Remove proteins and organic compounds	Solvent waste barrel
Ethanol	58465PRB Sigma	L	70%	H225, H319	P210, P280, P305+P351+ P338, P337 +P313, P403+P235	Remove salts, disinfect	Sink
Isopropanol	49296APV Sigma	L		H225, H319, H336	P210, P261, P305+P351+ P338	Precipitation of DNA	Sink
HCl	AGVF2544V Sigma	L		H290, H314, H335	P261, P280, P305+P351+ P338, P310	Adjusting pH	Acid waste barrel
NaOH pellets	APTR5879B Sigma	S		H290-H314	P280, P303+ P361+P353, P304+P340+ P310, P305+ P351+P338	Dissolve EDTA by adjusting pH	WIVA barrel

3.3.3. Method

Prepare lysis buffer

Always wear gloves and a mask for protection!

To make two Schott bottles of 500 ml of lysis buffer:

- Clean balance
- Place weighing bowl on balance and press 'tare'
- Weigh 60,57 g of Tris base to make 1M Tris base solution
- Add to a Schott bottle with 400 ml demi water
- Stir with magnetic stirrer to dissolve (if it doesn't dissolve very well: heat it a little bit)
- Check if pH is not higher than 8.5, otherwise add HCl to adjust 
- Add demi water until 500 ml
- Repeat previous steps, except for the last two, for:
    - 20 g of SDS in 100 ml demi water to make 20% SDS solution 
    - 29,21 g of NaCl in 100 ml demi water to make 5M NaCl solution
    - 14,612 g of EDTA in 100 ml demi water to make 0,5 M EDTA solution (if this is not dissolving well: add concentrated NaOH pellets until EDTA is dissolved) 
- If solutions are prepared, add the following together in a Schott bottle of 500 ml
    - 50 ml 1M Tris base solution
    - 5 ml 0,5M EDTA solution
    - 5 ml 20% SDS solution
    - 20 ml 5M NaCl solution
- Add demi H_2O until 500 ml
- Label and store in cupboard with solutions
- Clean working space

Cell lysis

Always wear gloves to avoid contamination!

DAY 1

- Mix 1% proteinase K in lysis buffer (60 µl in 6 ml lysis buffer) 
- Carefully open the tubes without touching the inside of the cap
- Add 500 µl lysis buffer (+ prot. K) to each tube
- Lyse overnight at 55°C in the thermomixer (300 rpm)

DAY 2

Work under the fume hood and wear gloves to avoid contamination and for protection!

- Pipette (volumetric pipette + pipetboy) phenol chloroform in a small beaker

- Add 500 µl phenol chloroform to each tube
- Close tubes and cover with paper (to avoid spilling) and mix by turning up and down with your hand for ±10 times
- Centrifuge for 5 min. at full power (13 000 rpm)
- If the two layers are not very clear: repeat previous step
- Label new microtubes
- Clean the table with 70% ethanol and add 700 µl isopropanol to new tubes 
- After centrifugation, take as much supernatant as possible without disturbing interphase layer and add to tubes with isopropanol
- Mix tubes by hand and check if the DNA is precipitated
- Centrifuge for 10 min. at full power (13 000 rpm)
- Carefully decant supernatant in waste beaker (do not disturb pellet)
- Remove last amount of fluid by patting the tubes on an absorbing tissue
- Add 500 µl 70% ethanol to the DNA pellet and tap the tube to make the pellet flow
- Centrifuge for 5-10 min. at full power (13 000 rpm)
- Remove supernatant with 200 µl pipette without disturbing the pellet
- Leave tubes open, cover them with tissue, to dry for 5 min.
- Add 50 µl of sterile H_2O
- Put them in the thermomixer and leave them there for 20 min. at 55°C
- Tap the tubes to mix and store at -20°C
- Clean working space

3.4. PCR

3.4.1. Samples

Table 7: Samples to use

PINK1 Samples	Cre Samples
Irun	12/
Bevel	13L
Huesca	14R
Jaca	Founder
Genova	
Antwerp	

3.4.2. Materials

List of used hardware

Table 8: Hardware for PCR

Hardware	Brand	Purpose
Micropipette (1-1000 µl)	Eppendorf	Pipette small volumes of liquid
Micropipette (1-100 µl)	Eppendorf	Pipette small volumes of liquid
Micropipette (1-10 µl)	Eppendorf	Pipette small volumes of liquid
Thermocycler	Bio-rad	PCR reaction
Vortex	VWR	Mix
Mini centrifuge	VWR	Remove bubbles and liquid on the side of the tube

List of glassware and disposables

Table 9: Glassware and disposables for PCR

Hardware	Purpose
Pipette tips	Pipette small volumes of liquid
Gloves	Avoid contamination
Holder for microtubes	To hold the microtubes
Tissue	Clean
1,5ml-microtubes	Container for liquids
Permanent marker	Label the tubes
PCR tubes	Container for liquids
Box with ice	Keep the reagent cold

List of reagents and solutions

Table 10: Reagents and solutions for PCR

Name	Batch and brand	S/L	Composition/ conc.	H-sentences	P-sentences	Purpose	Waste disposal
10x Key buffer	13I2 VWR	L	Tris-HCl $(NH_4)_2SO_4$ 15 mM $MgCl_2$ 1% Tween®20	/	/	High yield, Optimal conditions for PCR	WIVA barrel
10x Extra buffer	13F21 VWR	L	Tris-HCl KCl 15 mM $MgCl_2$ 1% Triton X-100	/	/	High specificity, Optimal conditions for PCR	WIVA barrel
$MgCl_2$	14A27 VWR	L	25 mM	/	/	Cofactor for Taq polymerase	WIVA barrel
Taq polymerase	14E01 VWR	L	5U/µl	/	/	Elongating the DNA	WIVA barrel
dNTP mix	13D22 VWR	L	10 mM dATP 10 mM dGTP 10 mM dCTP 10 mM dTTP	/	/	Building blocks of DNA strand	WIVA barrel
Forward primer	IdtDNA	L	10 µM	/	/	Starting point for Taq	WIVA barrel
- Intron 1 (PINK1)	198190605						
- sh001 (cre)	166741143						

Name	Batch and brand	S/L	Composition/ conc.	H-sentences	P-sentences	Purpose	Waste disposal
Reverse primer	IdtDNA	L	10 µM	/	/	Starting point for Taq	WIVA barrel
- Exon2 (PINK1)	198190606						
- Intron 3 (PINK1)	198190607						
- sh002 (cre)	166741144						
Sterile H_2O	/	L	/	/	/	Solvent	Sink
Ethanol	58465PRB Sigma	L	70%	H225, H319	P210, P280, P305+P351+ P338, P337 +P313, P403+P235	Disinfection	Sink

3.4.3. Method

Wear gloves to avoid contamination!

- Clean working surface with ethanol 
- Get the reagents out of the -20°C freezer and thaw them on ice
- Add following reagents to a labelled microtube in the same order as below (see tables 11, 12 and 14):
    - Sterile H_2O
    - 10x Key buffer
    - 10x Extra buffer
    - $MgCl_2$
    - dNTP mix
    - Reverse primer
    - Forward primer
    - Taq polymerase
- Keep on ice the whole time and pipette up and down after adding a reagent
- Vortex
- Add 39 µl of mastermix to each labelled PCR tube
- Add 1 µl of DNA sample in the right tube and pipette up and down
- Leave one PCR tube as a negative control: add 1 µl of sterile H_2O to this one
- Close tubes and spin in the mini centrifuge for ± 10 sec.
- Place tubes in thermocycler
- Make sure that they're closed well
- Choose the right programme and start the reaction

Mastermix for PINK1 wildtype

Table 11: Mastermix for PINK1 wildtype [60]

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	30,75	246
10x Extra buffer	10x	5	40
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	1	8
Primer exon 2	10 µM	1	8
Taq polymerase	5U/µl	0,25	2
Total		39	312
DNA		1	

Mastermix for PINK 1 knockout

Table 12: Mastermix for PINK1 knockout [60]

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	30,75	246
10x Extra buffer	10x	5	40
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	1	8
Primer intron 3	10 µM	1	8
Taq polymerase	5U/µl	0,25	2
Total		39	312
DNA		1	

PCR programme for PINK1

Table 13: PCR programme for PINK1 mice [60]

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min.	1
Denaturation **Annealing** **Elongation**	95°C 60°C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	7 min.	1
Conserving	4°C	∞	

Mastermix for cre

Table 14: Mastermix for cre

Reagents	Stock concentration	Volume/sample (µl)	Volume/6 samples (µl)
Sterile H_2O	/	7,5	45
10x Extra buffer	10x	1,25	7,5
$MgCl_2$	25 mM	1	6
dNTP mix	10 mM	0,5	3
Primer sh001	10 µM	1	6
Primer sh002	10 µM	1	6
Taq polymerase	5U/µl	0,25	1,5
Total		12,5	75
DNA		0,5	

PCR programme for cre

Table 15: PCR programme for cre

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min.	1
Denaturation **Annealing** **Elongation**	95°C 55°C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	1 min.	1
Conservation	4°C	∞	

3.5. Touchdown PCR

See materials and method in chapter 3.4 PCR. A touchdown PCR was only performed for the samples of the PINK1 mice.

Mastermix for PINK1 wildtype

Table 16: Mastermix for PINK1 wildtype (touchdown PCR)

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	30,75	246
10x Extra buffer	10x	2,5	20
10x Key buffer	10x	2,5	20
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	1	8
Primer exon 2	10 µM	1	8
Taq polymerase	5U/µl	0,25	2
Total		39	312
DNA		1	

Mastermix for PINK1 knockout

Table 17: Mastermix for PINK1 knockout (touchdown PCR)

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	30,75	246
10x Extra buffer	10x	2,5	20
10x Key buffer	10x	2,5	20
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	1	8
Primer intron 3	10 µM	1	8
Taq polymerase	5U/µl	0,25	2
Total		39	312
DNA		1	

Touchdown PCR programme for PINK1

Table 18: PCR programme for PINK1 mice

Step	Temperature	Time	Cycles
Initial denatruration	95°C	2 min.	1
Denaturation **Annealing** **Elongation**	95°C 68°C (-0,5°C/cycle) 72°C	1 min. 1 min. 1 min.	30x
Denaturation **Annealing** **Elongation**	95°C 53,5°C 72°C	30 sec. 30 sec 1 min.	10x
Finale elongation	72°C	5 min	1
Conservation	4°C	∞	

3.6. PCR optimization

3.6.1. Annealing temperature

See materials and method in chapter 3.4 PCR.

Mastermix for PINK1 wildtype

Table 19: Mastermix for PINK1 wildtype (optimization annealing temperature)

Reagents	Stock concentration	Volume/sample (µl)	Volume/32 samples (µl)
Sterile H_2O	/	30,75	984
10x Extra buffer	10x	2,5	80
10x Key buffer	10x	2,5	80
dNTP mix	10 mM	1	32
Primer intron 1	10 µM	1	32
Primer exon 2	10 µM	1	32
Taq polymerase	5U/µl	0,25	8
Total		39	1248
DNA		1	

Mastermix for PINK1 knockout

Table 20: Mastermix for PINK1 knockout (optimization annealing temperature)

Reagents	Stock concentration	Volume/sample (µl)	Volume/32 samples (µl)
Sterile H_2O	/	30,75	984
10x Extra buffer	10x	2,5	80
10x Key buffer	10x	2,5	80
dNTP mix	10 mM	1	32
Primer intron 1	10 µM	1	32
Primer intron 3	10 µM	1	32
Taq polymerase	5U/µl	0,25	8
Total		39	1248
DNA		1	

PCR programme for PINK1

Table 21: PCR programme for PINK1 mice (optimization annealing temperature)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min.	1
Denaturation **Annealing** **Elongation**	95°C Row A: 58 °C Row B: 57,7 °C Row C: 57 °C Row D: 56,1 °C Row E: 55 °C Row F: 54,1 °C Row G: 53,4 °C Row H: 53 °C 72°C	30 sec. 1 min. 1 min.	30
Final elongation	72°C	5 min.	1
Conservation	4°C	∞	

Mastermix for cre

Table 22: Mastermix for cre (optimization annealing temperature)

Reagents	Stock concentration	Volume/sample (µl)	Volume/6 samples (µl)
Sterile H_2O	/	7,5	45
10x Extra buffer	10x	1,25	7,5
$MgCl_2$	25 mM	1	6
dNTP mix	10 mM	0,5	3
Primer sh001	10 µM	1	6
Primer sh002	10 µM	1	6
Taq polymerase	5U/µl	0,25	1,5
Total		12,5	75
DNA		0,5	

PCR programme for cre

Table 23: PCR programme for cre mice (optimization annealing temperature)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min.	1
Denaturation **Annealing** **Elongation**	95°C Row A: 55 °C Row B: 54,7 °C Row C: 54,2 °C Row D: 53,5 °C Row E: 52,6 °C Row F: 51,9 °C Row G: 51,3 °C Row H: 51 °C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	1 min.	1
Conservation	4°C	∞	

3.6.2. Primer concentration

See materials and method in chapter 3.4 PCR.

Concentrations to test

Table 24: Primer concentrations to test

PINK1 samples	Cre samples
0,5 µM	0,5 µM
0,38 µM	0,38 µM
0,25 µM	0,25 µM
0,125 µM	0,125 µM
0,1 µM	
0,075 µM	

For the cre only 4 concentrations were tested because the test PCR showed very light bands. So the concentration can't be too low.

Mastermix for PINK1 wildtype

6 different mastermixes need to be prepared:

Table 25: Mastermix for PINK1 wildtype (optimization primer concentration)

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
10x Extra buffer	10x	2,5	25
10x Key buffer	10x	2,5	25
dNTP mix	10 mM	1	10
Taq polymerase	5U/µl	0,25	2,5
Sterile H_2O	/		
1	/	28,75	287,5
2	/	29,75	297,5
3	/	30,75	307,5
4	/	31,75	317,5
5	/	31,95	319,5
6	/	32,15	321,5
Primer intron 1	10 µM		
Primer exon 2	10 µM		
1	10 µM	2 (0,5 µM)	20
2	10 µM	1,5 (0,38 µM)	15
3	10 µM	1 (0,25 µM)	10
4	10 µM	0,5 (0,125 µM)	5
5	10 µM	0,4 (0,1 µM)	4
6	10 µM	0,3 (0,075 µM)	3
Total		39	390
DNA		1	

Mastermix for PINK1 knockout

6 different mastermixes need to be prepared:

Table 26: Mastermix for PINK1 knockout (optimization primer concentration)

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
10x Extra buffer	10x	2,5	25
10x Key buffer	10x	2,5	25
dNTP mix	10 mM	1	10
Taq polymerase	5U/µl	0,25	2,5
Sterile H_2O	/		
1	/	28,75	287,5
2	/	29,75	297,5
3	/	30,75	307,5
4	/	31,75	317,5
5	/	31,95	319,5
6	/	32,15	321,5
Primer intron 1	10 µM		
Primer intron 3	10 µM		
1	10 µM	2 (0,5 µM)	20

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
2	10 µM	1,5 (0,38 µM)	15
3	10 µM	1 (0,25 µM)	10
4	10 µM	0,5 (0,125 µM)	5
5	10 µM	0,4 (0,1 µM)	4
6	10 µM	0,3 (0,075 µM)	3
Total		39	390
DNA		1	

PCR programme for PINK1

Table 27: PCR programme for PINK1 mice (optimization primer concentration)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min.	1
Denaturation **Annealing** **Elongation**	95°C 56,1°C 72°C	30 sec. 1 min. 1 min.	30
Final elongation	72°C	5 min.	1
Conservation	4°C	∞	

Mastermix for cre

4 different mastermixes need to be prepared:

Table 28: Mastermix for cre (optimization primer concentration)

Reagents	Stock concentration	Volume/sample (µl)	Volume/6 samples (µl)
10x Extra buffer	10x	1,25	7,5
$MgCl_2$	25 mM	1	6
dNTP mix	10 mM	0,5	3
Taq polymerase	5U/µl	0,25	1,5
Sterile H_2O	/		
1	/	5,5	33
2	/	6,5	39
3	/	7,5	45
4	/	8,5	51
Primer sh001	10 µM		
Primer sh002	10 µM		
1	10 µM	2 (0,5 µM)	12
2	10 µM	1,5 (0,38 µM)	9
3	10 µM	1 (0,25 µM)	6
4	10 µM	0,5 (0,125 µM)	3
Total		12,5	75
DNA		0,5	

PCR programme for cre

Table 29 PCR programme for cre mice (optimization primer concentration)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min.	1
Denaturation **Annealing** **Elongation**	95°C 55°C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	1 min.	1
Conservation	4°C	∞	

3.6.3. DNA volume

See materials and method in chapter 3.4. PCR.

Amounts of DNA to test:

Table 30: DNA volumes to test

PINK1 samples	Cre samples
1 µl	1 µl
0,85 µl	0,85 µl
0,7 µl	0,7 µl
0,55 µl	0,5 µl
0,4 µl	0,4 µl
0,25 µl	0,25 µl

Table 31: DNA concentrations of PINK1 samples

Amount of DNA (µl)	Concentration Irun (ng/µl)	Conc. Bevel (ng/µl)	Conc. Huesca (ng/µl)	Conc. Jaca (ng/µl)	Conc. Genova (ng/µl)	Conc. Antwerp (ng/µl)
1	24,0	22,4	9,02	25,1	26,1	11,1
0,85	20,4	19,0	7,67	21,4	22,2	9,42
0,7	16,8	15,7	6,32	17,6	18,3	7,75
0,55	13,2	12,3	4,96	13,8	14,4	6,09
0,4	9,60	8,95	3,61	10,1	10,5	4,43
0,25	6,00	5,59	2,26	6,28	6,53	2,77

The concentrations of the cre DNA are not known because normally they don't check it with the Nanodrop®. There is no Nanodrop® available in the lab. Access to a lab of Imec itself is needed to use this device. So they just dilute the extracted DNA in 50 µl of sterile water.

Mastermix for PINK1 wildtype

6 different mastermixes need to be prepared:

Table 32: Mastermix for PINK1 wildtype (optimization DNA volume)

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
10x Extra buffer	10x	2,5	25
10x Key buffer	10x	2,5	25
dNTP mix	10 mM	1	10
Primer intron 1	10 µM	0,5	5
Primer exon 2	10 µM	0,5	5
Taq polymerase	5U/µl	0,25	2,5
Sterile H_2O	/		
1	/	31,75	317,5
2	/	31,9	319
3	/	32,05	320,5
4	/	32,2	322
5	/	32,35	323,5
6	/	32,5	325
DNA	See table 31		
1		1	
2		0,85	
3		0,70	
4		0,55	
5		0,40	
6		0,25	

Mastermix for PINK1 knockout

6 different mastermixes need to be prepared:

Table 33: Mastermix for PINK1 knockout (optimization DNA volume)

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
10x Extra buffer	10x	2,5	25
10x Key buffer	10x	2,5	25
dNTP mix	10 mM	1	10
Primer intron 1	10 µM	0,5	5
Primer intron 3	10 µM	0,5	5
Taq polymerase	5U/µl	0,25	2,5
Sterile H_2O	/		
1	/	31,75	317,5
2	/	31,9	319
3	/	32,05	320,5
4	/	32,2	322
5	/	32,35	323,5
6	/	32,5	325
DNA	See table 31		
1		1	
2		0,85	

Reagents	Stock concentration	Volume/sample (µl)	Volume/10 samples (µl)
3		0,70	
4		0,55	
5		0,40	
6		0,25	

PCR programme for PINK1

Table 34: PCR programme for PINK1 mice (optimization DNA volume)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min.	1
Denaturation **Annealing** **Elongation**	95°C 56,1°C 72°C	30 sec. 1 min. 1 min.	30
Final elongation	72°C	5 min.	1
Conservation	4°C	∞	

Mastermix for cre

6 different mastermixes need to be prepared:

Table 35: Mastermix for cre (optimization DNA volume)

Reagents	Stock concentration	Volume/sample (µl)	Volume/6 samples (µl)
10x Extra buffer	10x	1,25	7,5
$MgCl_2$	25 mM	1	6
dNTP mix	10 mM	0,5	3
Primer intron 1	10 µM	1	6
Primer exon 2	10 µM	1	6
Taq polymerase	5U/µl	0,25	1,5
Sterile H_2O	/		
1	/	7	42
2	/	7,15	42,9
3	/	7,3	43,8
4	/	7,5	45
5	/	7,6	45,6
6	/	7,75	46,5
DNA			
1	/	1	
2	/	0,85	
3	/	0,70	
4	/	0,50	
5	/	0,40	
6	/	0,25	

PCR programme for cre

Table 36: PCR programme for cre mice (optimization DNA volume)

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min.	1
Denaturation **Annealing** **Elongation**	95°C 51°C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	1 min.	1
Conservation	4°C	∞	

3.7. Gel electrophoresis

3.7.1. Samples

Table 37: Samples to use

PINK1 Samples	Cre Samples
Irun	12/
Bevel	13L
Huesca	14R
Jaca	Founder
Genova	
Antwerp	

3.7.2. Materials

List of used hardware

Table 38: Hardware for gel electrophoresis

Hardware	Brand	Purpose
Micropipette (1-10 µl)	Eppendorf	Pipette small volumes of liquid
Electrophoresis tank	CBS scientific	Electrophoresis
Combs	CBS scientific	Create wells
Source of voltage	VWR	Migration of DNA molecules
UV transilluminator		Visualize DNA
Analytical balance	Satorius	Weighing

List of glassware and disposables

Table 39: Glassware and disposables for gel electrophoresis

Hardware	Purpose
Pipette tips	Pipette small volumes of liquid
Gloves	Avoid contamination
Holder for microtubes	To hold the microtubes
Tissue	Clean
1,5ml-microtubes	Container for liquids
Schott bottle	Container for liquids
Spoon	Take small amount of compound
Weighing bowl	Container for compounds

List of reagents and compounds

Table 40: Reagents and solutions for gel electrophoresis

Name	Batch and brand	S/L	Composition/conc.	H-sentences	P-sentences	Purpose	Waste disposal
Gelred®	15G0611 Biotum	L	/	H301, H312+H332	P262, P338, P280, P201, P312	Visualisation of DNA	WIVA barrel
1 x TAE Buffer		L	Tris-base Glacial acetic acid EDTA Demi H_2O				
- Tris-base	SLBG2651V Sigma	S	/	/	/	Maintain right pH for conductivity and DNA stabilisation	WIVA barrel
- EDTA	SLBD6046V Sigma	S	/	H319	P305+P351+ P338	Chelation of DNase cofactors	WIVA barrel
- Glacial acetic acid	SZBA3300V Sigma	S	/	H226, H314	P280, P305+ P351+P338, P310	Maintain right pH for conductivity and DNA stabilisation	Acid waste barrel
BlueJuice	1697755 Invitrogen	L	/	/	/	Running dye	WIVA barrel
Agarose	BCBK7498V Sigma	S	/	/	/	To make a gel	WIVA barrel
1kb+ ladder	10.1 Prime	L	/	/	/	To check the length of the amplicon	WIVA barrel

3.7.3. Method

Prepare 2 L 10x TAE buffer

Always wear gloves and a mask for protection!

- Clean balance
- Place weighing bowl on balance and press 'tare'
- Weigh:
    - 96,8 g Tris base
    - 7,4 g EDTA 
- Add to Schott bottle with some demi water
- Stir with magnetic stirrer to dissolve (if it doesn't dissolve very well: heat it a little bit)
- Add 22,8 ml of acetic acid 
- Stir with magnetic stirrer to mix
- Add demi water till 2L
- Label bottle and store in cupboard with solutions
- Clean working space

Prepare 1% gel

Wear gloves to avoid contamination and for protection!

- Weigh 2 grams of agarose
- Add the agarose to 200 ml of 1x TAE-buffer in a Schott bottle
- Put in the microwave to solve the agarose
- Cool until you can hold it in your hand
- Add 40 µl of Gelred® to the solution and pipette up and down 
- Pour the solution into the gel template in the electrophoresis tank
- Let it harden
- While the gel is hardening, add 1 µl BlueJuice to every sample and pipette up and down
- When the gel is hard, pour 1x TAE buffer into the tank until the gel is under and the wells are filled
- Load 9 µl of 1kb+ marker
- Load 8 µl of each sample
- Close the tank and connect it with the voltage source
- Run the gel for 2,5 hours on 90 V
- After running, place the gel in the UV transilluminator
- Switch on the UV light and start the software on the computer to take a picture

- Before taking the gel out, switch off the light
- Throw the gel in the WIVA barrel
- Clean working space

3.8. UV spectrometry

3.8.1. Samples

Table 41: Samples to test

PINK1 Samples
Irun
Bevel
Huesca
Jaca
Genova
Antwerp

3.8.2. Materials

List of used hardware

Table 42: Hardware for UV spectrometry

Hardware	Brand	Purpose
Micropipette (1-10 µl)	Eppendorf	Pipette small volumes of liquid
Nanodrop®	Nanodrop®	Check concentration and purity of samples
Computer	Dell	For Nanodrop® software
Vortex	VWR	Mixing

List of glassware and disposables

Table 43: Glassware and disposables for UV spectrometry

Hardware	Purpose
Pipette tips	Pipette small volumes of liquid
Gloves	Avoid contamination
Holder for microtubes	To hold the microtubes
Tissue	Clean

List of reagents and solutions

Table 44: Reagents and solutions for UV spectrometry

Name	Batch and brand	S/L	Composition /conc.	H-sentences	P-sentences	Purpose	Waste disposal
Sterile H_2O	/	L	/	H301, H312+H332	P262, P338, P280, P201, P312	Visualisation of DNA	Sink
Ethanol	58465PRB Sigma	L	70%	H225, H319	P210, P280, P305+P351+ P338, P337 +P313, P403+P235	Disinfect	Sink

3.8.3. Method

Wear gloves to avoid contamination!

- Switch on the computer and software
- Carefully clean the capillary with a tissue and ethanol
- Pipette 1 µl of sterile H_2O on the capillary
- Close the lever and press 'Blank'
- Vortex the first sample before pipetting 1 µl on the capillary
- Press 'Measure'
- Save the data
- Repeat this for the other samples
- Clean the lens with ethanol and switch off the computer

4. Results + discussion

4.1. PINK1 protocol

4.1.1. Provided protocol

First of all the PCR mastermix and programme that were provided by the lab that created the PINK1 knockouts, needed to be tested. The result of this experiment is a gel that should show the right bands. The wildtypes should show a band of 781 base pairs and the knockouts one of 736 base pairs.

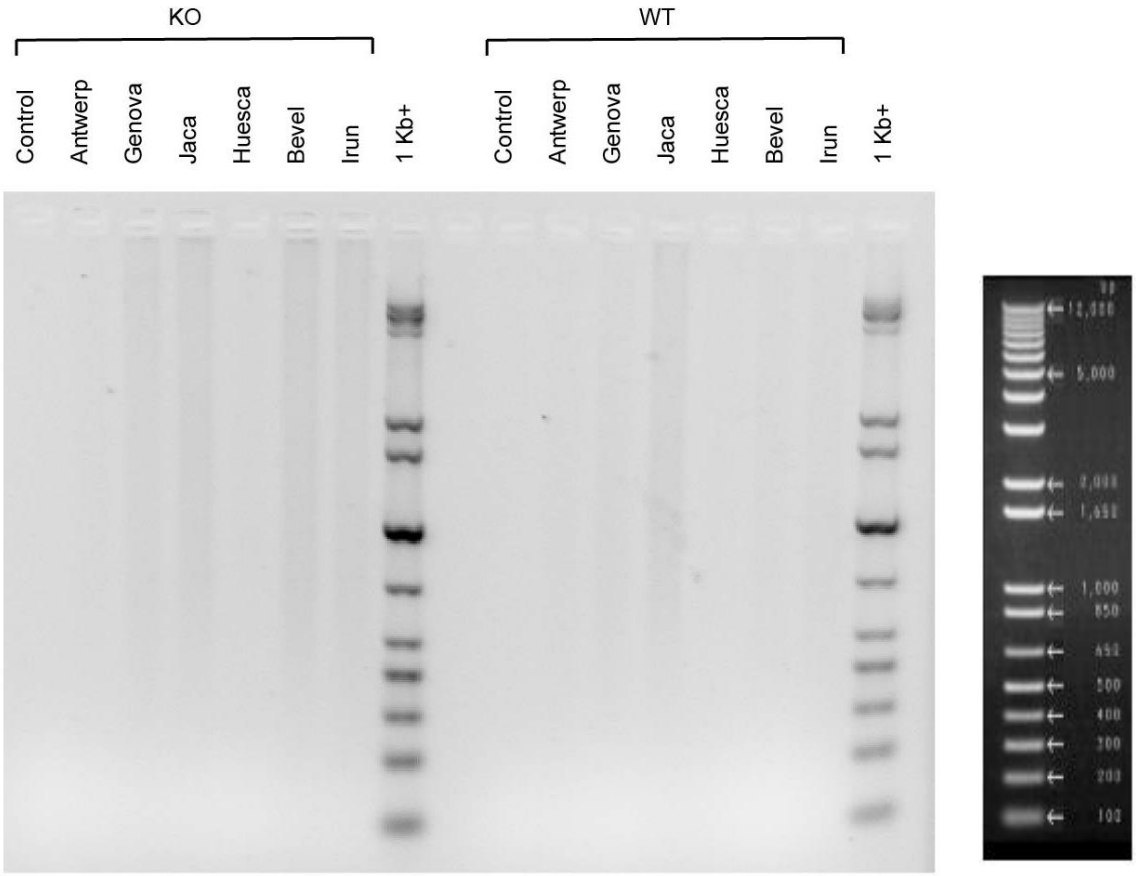

Figure 22: Electropherogram provided protocol

Results

The ladders are well separated except for the bands of 850 and 1000 base pairs. There is smear of DNA in almost every lane except for the control. None of the samples show a band (see figure 22).

Discussion

There was no amplification of DNA which could have been caused by the following reasons:

- Mastermix:
    - No primers: when the primers were delivered they needed to be diluted in sterile water. During this step the primers probably weren't mixed well enough, they were still at the bottom of the tube. The pipetting of the primers occurs just under the surface of the working solution, so there were probably no primers in the pipette.

    - Taq polymerase: When this enzyme is left out of the freezer for too long it will stop functioning properly. Someone could have taken it out and forgotten to put it back at -20°C.
- PCR programme:
    - Denaturation step: this step lasted for 5 min. at 95°C. When Taq polymerase is exposed to such a high temperature for a long time, it will start degrading.
    - Annealing temperature: the annealing temperature is 60°C. This is too high because the melting temperatures of the primers are 58,1°C (primer intron 1), 62°C (primer exon 2) and 61,8°C (primer intron 3). Because of the high annealing temperature the primers didn't bind well to the sequence of interest.

The concentration of the gel was 1,5%. This is a high concentration for such large amplicons (781 bp and 736 bp). It is better to use a concentration of 1% in the next experiments so the DNA fragments experience less resistance.

The results of the control samples are correct for they display no bands. There was no contamination during this experiment.

Adjustments

- Remix the primer solutions
- Use a new tube of Taq polymerase
- Change the time of the denaturation step to 2 min.
- Annealing temperature was optimized later
- Change the concentration of the gel from 1,5% to 1% because of the large amplicons
- Add Key buffer to increase the specificity of the PCR reaction

Next experiment

Since there was no DNA amplification, the activity of the primers was tested by a touchdown PCR.

4.1.2. Touchdown PCR

Because of the results of the test PCR, a touchdown PCR was performed to check the primer activity.

First attempt

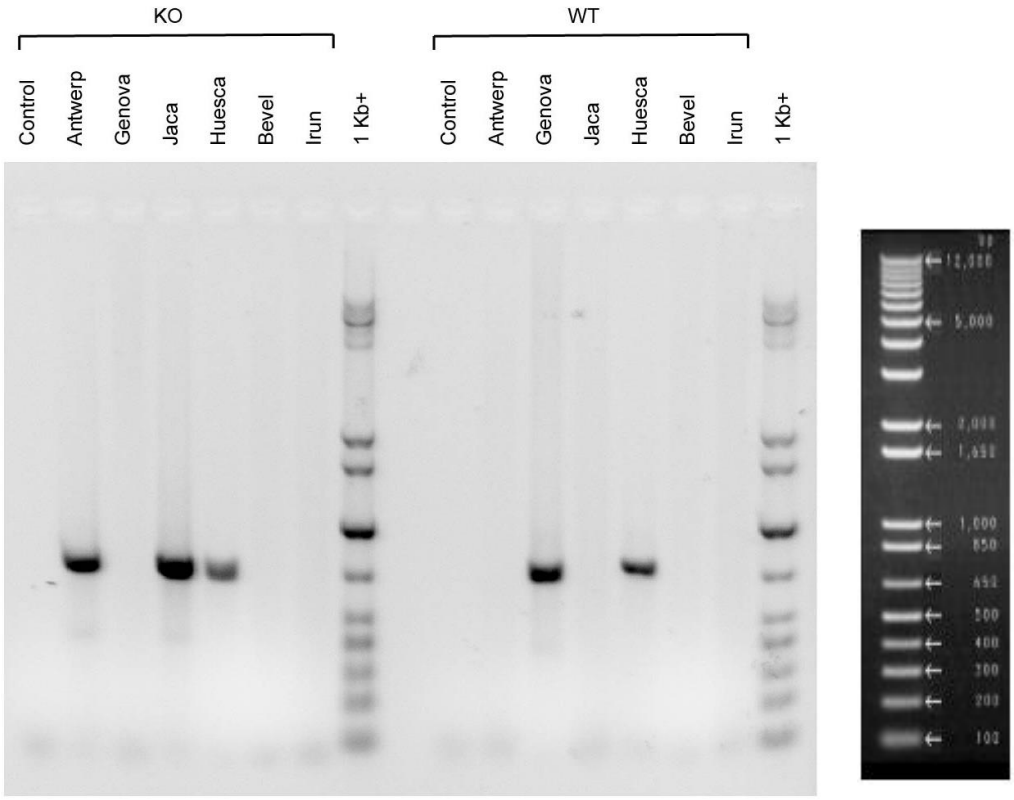

Figure 23: Electropherogram TD-PCR first attempt

Results

The ladders are separated except for the bands of 850 and 1000 base pairs (see figure 23). There is smear of DNA in almost every lane except for the control. The samples Irun and Bevel don't show a band. Huesca shows a band of the right height at the wildtype and knockout side. Jaca and Antwerp show a band of ±736 base pairs at the knockout side. Genova shows a band of ±781 bp at the wildtype side. At the bottom of every lane are small amounts of primer dimer.

Discussion

The results are not as expected:

- The DNA of the Irun sample didn't get amplified because it shows no band at the wildtype side neither at the knockout side. It should have displayed a band of 736 base pairs at the knockout side. The problem could have been the amount of DNA that was added to the mastermix, it could have been too small.
- Bevel should have shown a band of 736 bp at the knockout side. There is no band at both sides. This could have been because of the same reason as for the Irun sample, too little DNA.

- Huesca should show a band at the knockout side but instead it also displays one at the wildtype side. This is not according to the expectations. The band at the wildtype side could be a contamination or the mouse must be heterozygous for the knocked out PINK1 gene because another clean pipette tip was used for every sample.
- Jaca displays a band of the right height (736 bp) at the knockout side. This is in accordance with the identification of the samples in table 1.
- Genova shows a band of ±781 base pairs at the wildtype side as it should because this mouse is a wildtype for the PINK1 gene.
- There is a band of the right height in the lane of Antwerp at the knockout side. This is correct since Antwerp is a knockout mouse.

The controls show no band which means that no contamination occurred during this experiment.

Adjustments

None

Next experiment

Since the results were not as expected and there is no clear reason for it, a second touchdown PCR was executed.

Second attempt

The results of the first attempt were confusing and that is why a second touchdown PCR was executed.

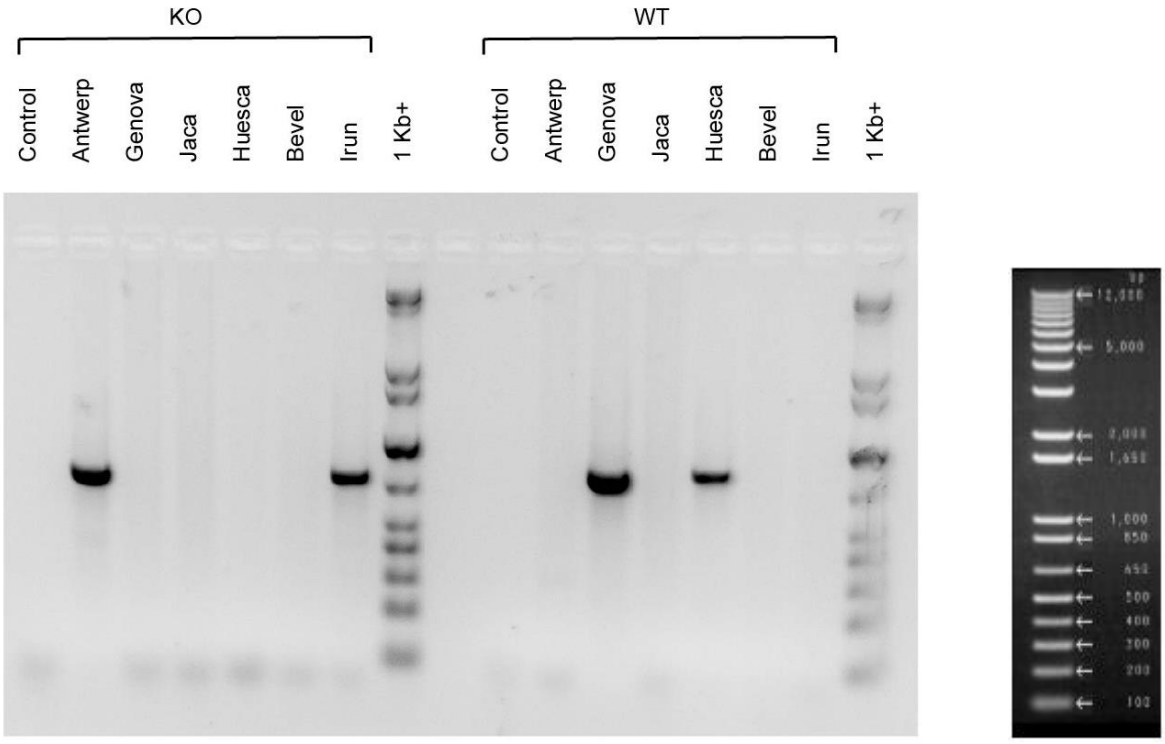

Figure 24: Electropherogram TD-PCR second attempt

Results

The ladders are well separated apart from the bands of 850 and 1000 base pairs (see figure 24). There is a smear of DNA in almost every lane except for the controls. Irun displays a band of ±736 base pairs at the knockout side. Bevel doesn't show a band at the knockout side nor at the wildtype side. Huesca shows a band of the right height at the wildtype side. Jaca shows no band at both sides. Genova shows a band of ±781 bp at the wildtype side and Antwerp shows a band of ±736 base pairs at the knockout side. At the bottom of every lane are small amounts of primer dimer.

Discussion

The results are again not as expected:

- After this second attempt Irun shows a band of the right height at the knockout side. This is correct as Irun is a knockout mice.
- The DNA of Bevel again didn't get amplified for it shows no band. The cause could still be the too small amount of DNA that was added to the mastermix
- Huesca should show a band at the knockout side but instead it now only displays one of the right height at the wildtype side. There is no clear reason for why this sample didn't get amplified in the knockout mix.
- Jaca didn't work in this attempt. It shows no band at the knockout side nor at the wildtype side. There is no obvious reason for this since this sample got amplified in the first attempt.
- Genova shows the correct band of ±781 bp at the wildtype side.
- Antwerp is a knockout so it shows a band of the right height at the knockout side as expected.

There was no contamination since the controls show no bands.

The reason why some samples didn't work and some did could be the DNA. If the DNA wasn't pure enough after the extraction, the contaminations could have inhibited the PCR reactions.

These touchdown PCR's were performed to check the activity of the primers. There were bands of the right height visible on the gel which means that some DNA was amplified so the primers work.

Adjustments

None

Next experiment

Since the problem could have been the DNA, the purity was checked by a Nanodrop® analysis.

4.1.3. Nanodrop®

The purity and concentration of the DNA was analysed to check for contiminations.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample
1	Irun PINK1	Westburg	9-3-2016 12:11	960,2	ng/µl	19,204	10,152	1,89	2,26	DNA
2	Antwerp PINK1	Westburg	9-3-2016 12:17	443,1	ng/µl	8,861	4,698	1,89	2,23	DNA
3	Genova PINK1	Westburg	9-3-2016 12:18	1045,5	ng/µl	20,910	11,018	1,90	2,30	DNA
4	Jaca PINK1	Westburg	9-3-2016 12:19	1005,4	ng/µl	20,107	10,880	1,85	2,24	DNA
5	Huesca PINK1	Westburg	9-3-2016 12:20	360,9	ng/µl	7,218	4,021	1,80	2,08	DNA
6	Bevel PINK1	Westburg	9-3-2016 12:21	994,7	ng/µl	19,894	10,431	1,91	2,29	DNA

Figure 25: Results of Nanodrop® analysis

Results

Figure 25 shows the results of the Nanodrop® analysis. The ratio 260/280 is around 1,8 for all samples. The ratio 260/230 is above 2 for every DNA sample.

Discussion

There are no proteins left in the samples since the ratio 260/280 is around 1,8. There are no organic compounds in the samples for the ratio 260/230 was above 2. The DNA concentrations are high enough to perform a well working PCR reaction. All DNA samples are pure so this could not have been the reason for why some of them didn't work.

Adjustments

None

Next experiment

The DNA was not the problem that caused the unexpected results after the touchdown PCR's. It's difficult to find a clear reason for this outcome so the next experiment was the optimization of the annealing temperature. This one wasn't changed yet so this could have still been the problem.

4.1.4. Optimization

4.1.4.1. Annealing temperature

The primers work, the DNA is pure and the concentration is high enough so the optimization could start. The first thing that was optimized for both the knockout and wildtype mastermix was the annealing temperature because it was too high. A PCR with the optimal annealing temperature should show clear correct bands, as little primer dimer as possible and no nonspecific bands.

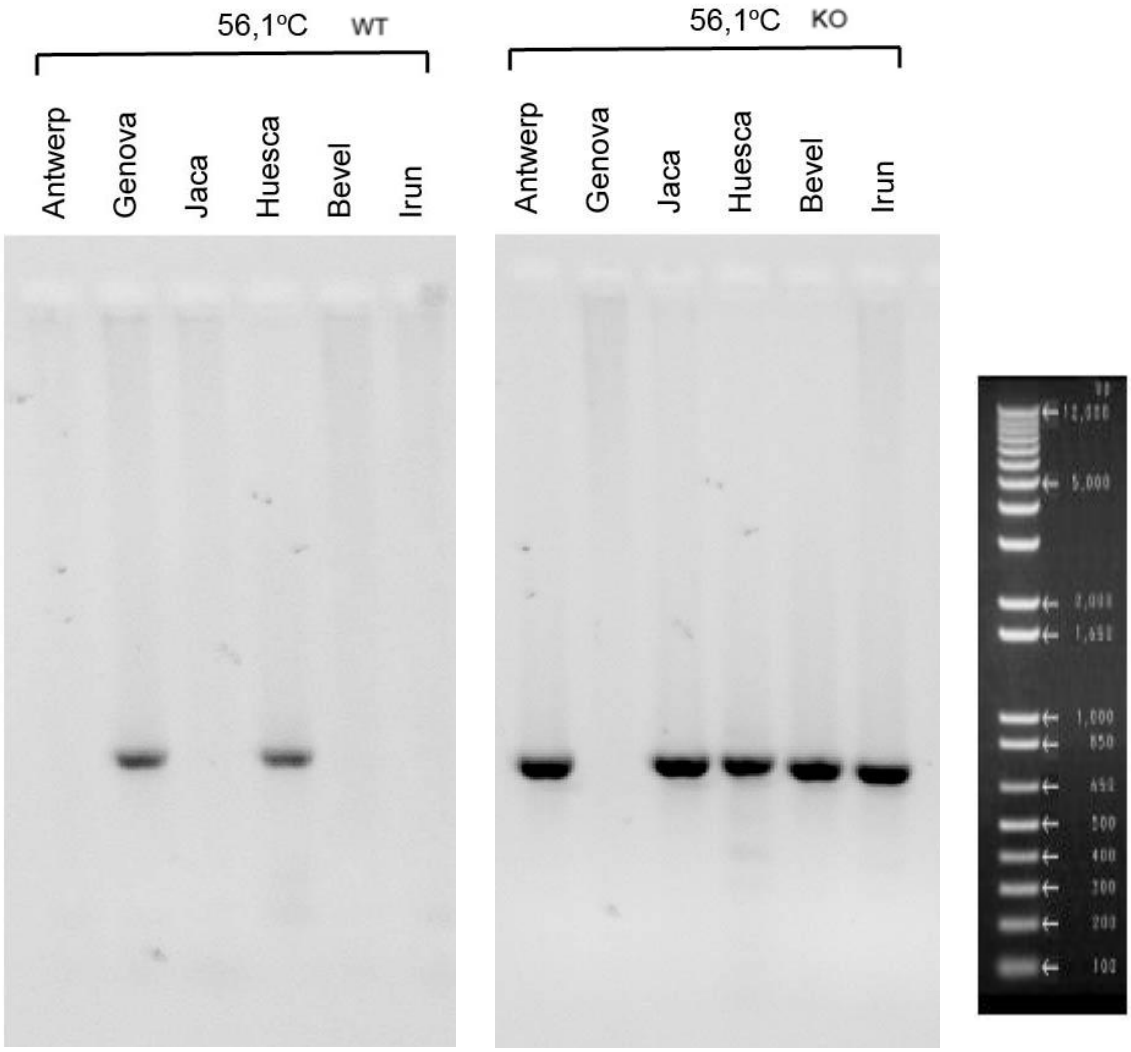

Figure 26: Electropherogram optimization annealing temperature WT (left) and KO (right)

Results

The electropherogram in figure 26 shows the results of the optimization of the annealing temperature for both the knockout and wildtype mastermix. The ladder is shown on figures 35, 36,38 and 39 in the attachments pages 86-88. The bands are well separated except for again the bands of 850 and 1000 bp. The ones at the top are not visible. Irun, Bevel, Jaca and Antwerp show a band of the right height at the knockout side. Genova displays one of ±781 base pairs at the wildtype side. Huesca again shows two bands of the same height. One at the knockout side and one at the wildtype side. There is just a little smear of DNA visible in every lane and there is no primer dimer at the bottom.

Discussion

All the samples show the right bands expect for Huesca which displays one at the wildtype side and one at the knockout side. According to the identification of the samples (table 1) it should show one band of 736 base pairs at the knockout side. The mouse could be heterozygous for the knocked out PINK gene.

56,1°C is the optimal temperature as it shows better results than the other ones for both mastermixes. The other temperatures display bands that are too light, more primer dimer or a darker smear of DNA (see attachments pages 86-88).

There was no control in this experiment. The PCR machine contains 8 rows and 6 columns for the samples. 6 samples needed to be tested and the annealing temperature could only be adjusted per row so there was no place for a control.

Adjustments

The annealing temperature was changed from 60°C to 56,1°C.

Next experiment

The next step of the optimization is determining the optimal primer concentration. This has to be done with an annealing temperature of 56,1°C.

4.1.4.2. Primer concentration

The second step in establishing the SOP for the genotyping of the PINK1 mice was the optimization of the primer concentration.

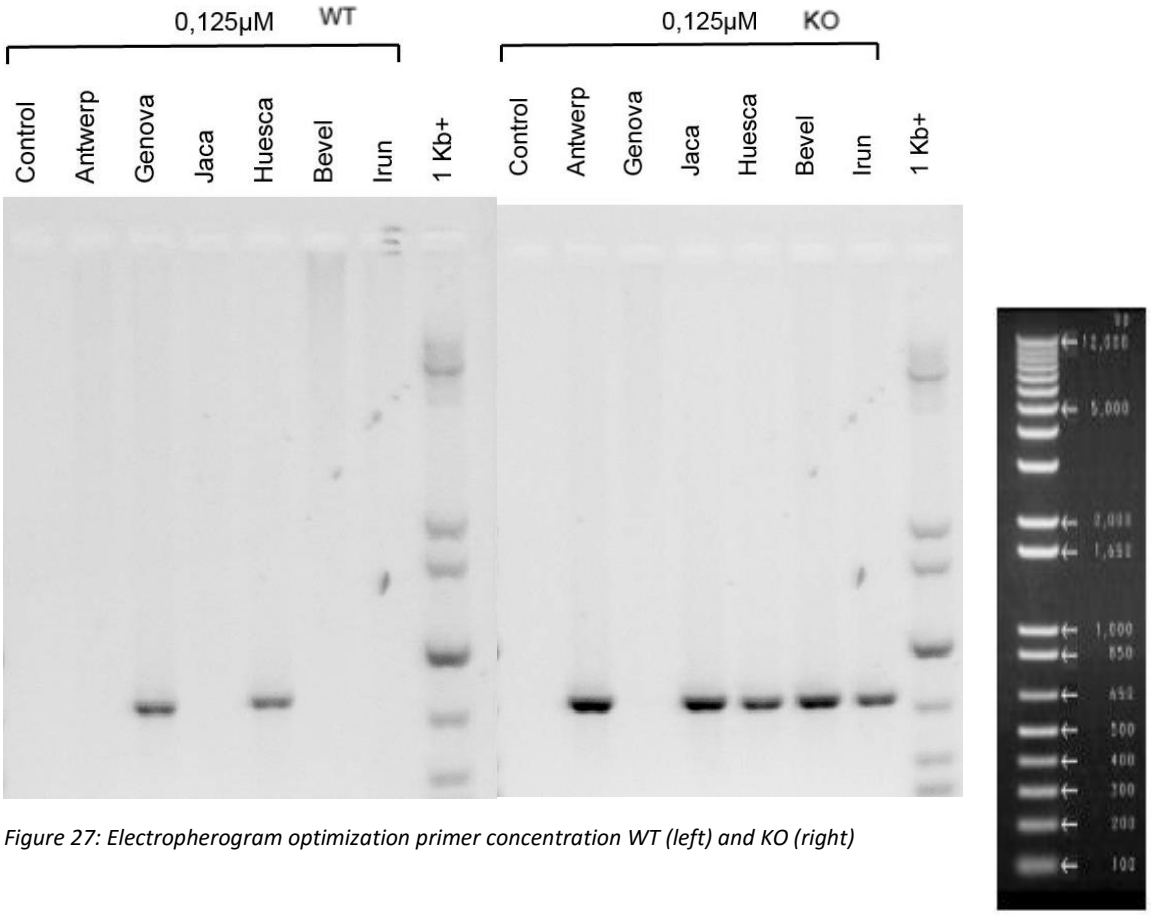

Figure 27: Electropherogram optimization primer concentration WT (left) and KO (right)

Results

The ladder is separated except for the bands on 850 and 1000 base pairs. The bands at the top are not visible. There is just a light smear of DNA in every lane. The bands that are displayed are clear and at the right height. Irun, Bevel, Jaca and Antwerp show one at the knockout side. Genova shows a band at the wildtype side and Huesca again at both sides.

Discussion

All samples show the correct bands at the right height. Huesca displays two bands again. It's getting clear that this mouse is heterozygous. It can't be a contamination for the samples were kept separate all the time and for every sample a different clean pipette tip was used.

The optimal primer concentration is 0,125 µM. The bands are clear enough. 0,25 µM also shows good results but it is better to use less to save more primer solution. The results of the other concentrations are not optimal (see attachments pages 89-92):

- 0,075 µM: The knockout mastermix shows no bands and the ones of the wildtype mix are too light.
- 0,38 µM and 0,55 µM: These concentrations show good results for the wildtype mix but the knockout mix display some nonspecific bands. The reason for this could be the large amount of primers.
- 0,1 µM: The bands for this concentration are light especially for the wildtype mastermix.

Adjustments

The primer concentration was changed from 0,25 µM to 0,125 µM.

Next experiment

The next experiment was the determination of the optimal DNA volume that needs to be added to the mastermix to get good results.

4.1.4.3. DNA volume

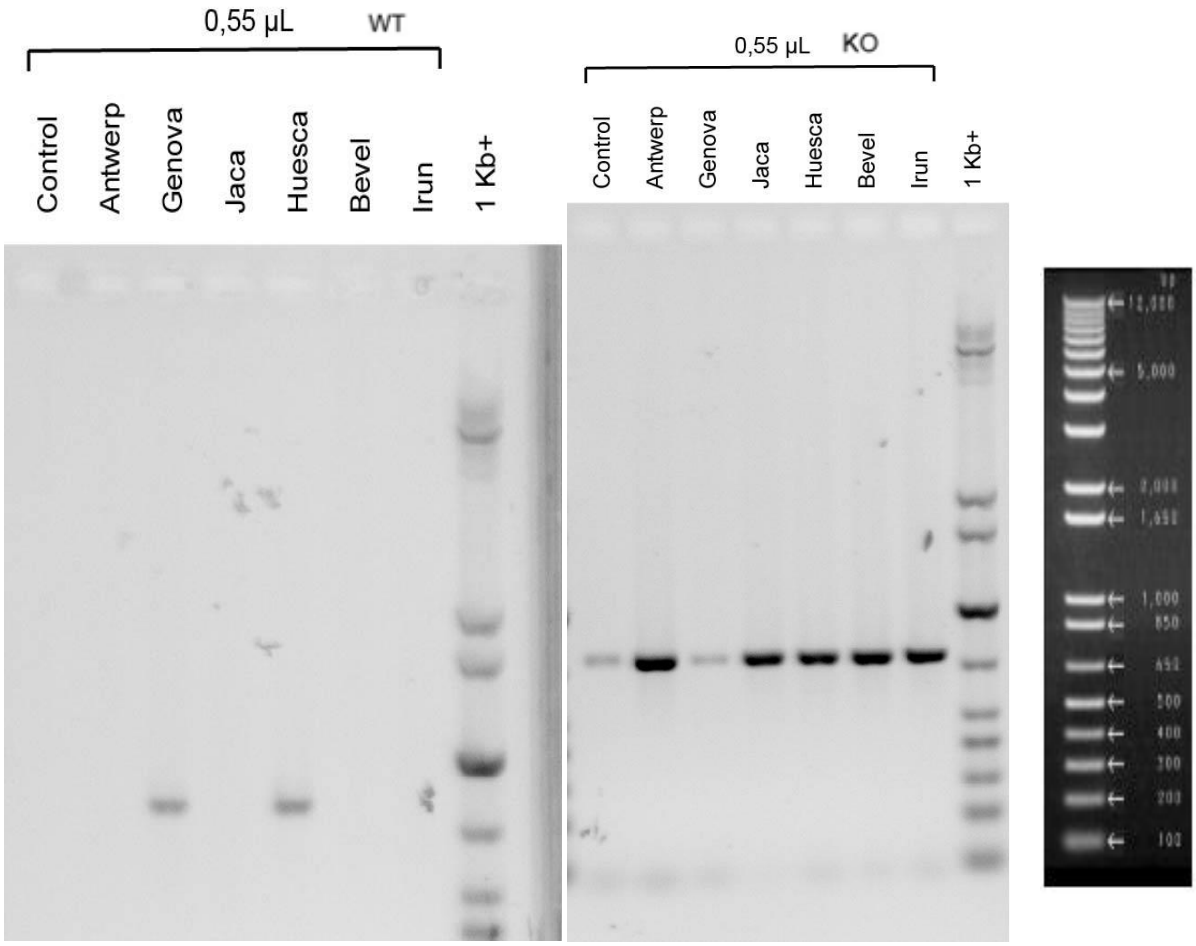

Figure 28: Electropherogram optimization DNA volume WT (left) and KO (right)

Results

The bands of 850 and 1000 base pairs of the ladder are overlapping. The bands at the top are not clear. At the knockout side, there is a smear of DNA in almost every lane. The amount of primer dimer is small. Irun, Bevel, Jaca and Antwerp show clear bands at the right height on the knockout side. Genova displays one at the wildtype side and also a very light one at the knockout side. Huesca shows one band at the right height on the knockout side and one on the wildtype side. The control at the knockout side displays one light band of the same length as the other fragments.

Discussion

Irun, Bevel, Jaca and Antwerp show the correct bands at the knockout side because these mice are knockouts.

Genova displays a band at the wildtype side of ±781 bp which is correct since this mouse is a wildtype for the PINK1 gene. But the sample also shows a very light band at the knockout side. This is a contamination.
The control on the knockout side shows a very light band which means that there is some contamination.

During the loading of the gel the sample and loading buffer came out of the pipette too early and then float in the buffer. Some of the Antwerp sample could have landed in the wells next to it (control and Genova). This experiment didn't need to be repeated since this results were good for the one that has to use the mice.

The optimal DNA volume is 0,55 µl. 0,85 µl and 0,7 µl also display good results but it is better to save more sample thus use less for the mastermix. The results of the other volumes are not as expected (see attachments pages 93-96):

- 1 µl and 0,4 µl: The wildtype mastermix for these two volumes doesn't show bands. The PCR didn't work. The reason for this could be that an important component of the mastermix was forgotten. For every volume a different mastermix was prepared. The knockout mastermix displayed correct clear bands although there is some contamination in the lane of the control and Genova. This could have occurred while loading the sample into the well.
- 0,25 µl: The bands on this electropherogram are too light.

Adjustments

The DNA volume was changed from 1 µl to 0,5 µl. 0,55 µl was rounded off to 0,5 µl for this volume is easier to pipette with the pipettes available in the lab.

Next experiment

The next step is to check if the optimized protocol works and shows correct and clear results.

4.1.5. Optimized protocol

After the optimization of the genotyping protocol for the PINK1 mice, the optimized SOP needed to be tested. Table 45, 46 and 47 represent the optimized mastermixes and PCR programme.

See materials and method of chapter 3.4 PCR.

Mastermix PINK1 wildtype

Table 45: Optimized mastermix for PINK1 wildtype

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	31,25	250
10x Extra buffer	10x	2,5	20
10x Key buffer	10x	2,5	20
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	0,5	8
Primer exon 2	10 µM	0,5	8
Taq polymerase	5U/µl	0,25	2
DNA		0,5	

Mastermix PINK1 knockout

Table 46: Optimized mastermix for PINK1 knockout

Reagents	Stock concentration	Volume/sample (µl)	Volume/8 samples (µl)
Sterile H_2O	/	31,25	250
10x Extra buffer	10x	2,5	20
10x Key buffer	10x	2,5	20
dNTP mix	10 mM	1	8
Primer intron 1	10 µM	0,5	8
Primer intron 3	10 µM	0,5	8
Taq polymerase	5U/µl	0,25	2
DNA		0,5	

PCR programme PINK1

Table 47: Optimized PCR programme for PINK1 mice

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min.	1
Denaturation **Annealing** **Elongation**	95°C 56,1°C 72°C	30 sec. 1 min. 1 min.	30
Final elongation	72°C	5 min.	1
Conservation	4°C	∞	

Result

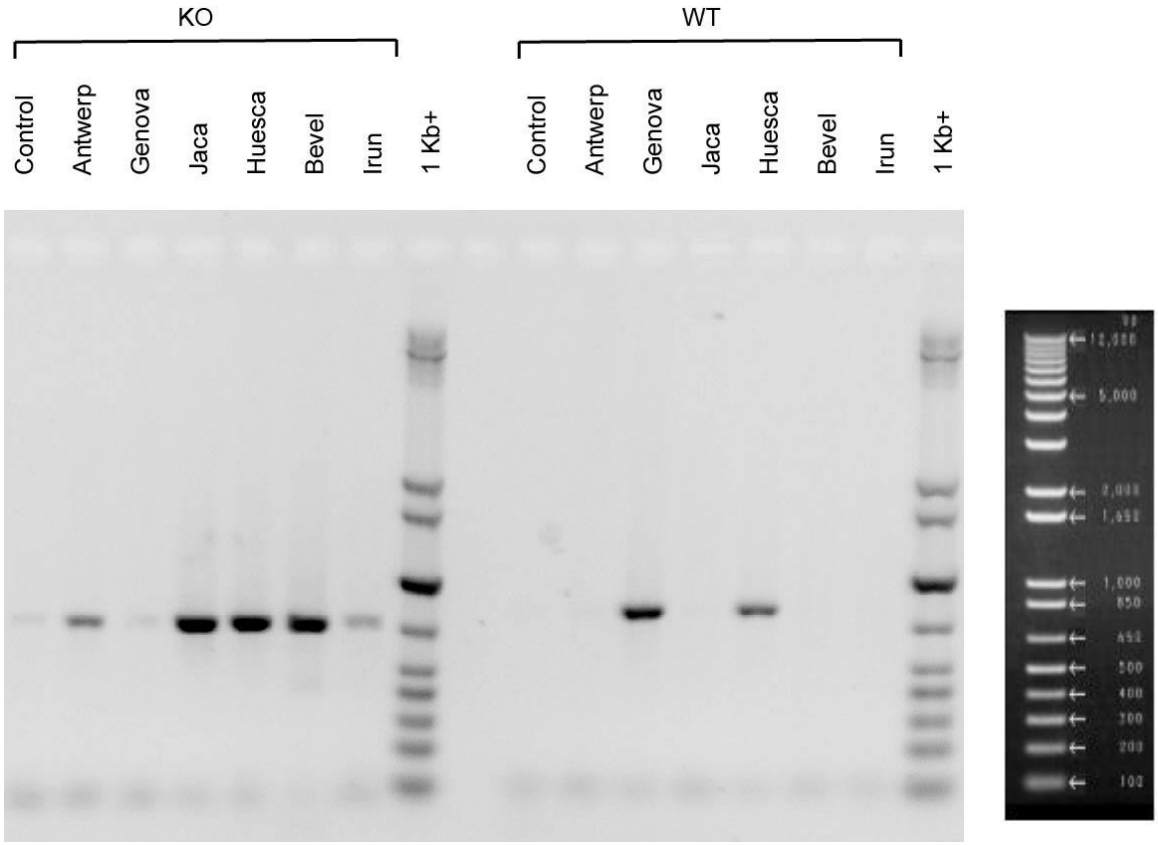

Figure 29: Electropherogram optimized PINK1 protocol

Results

The ladder in figure 29 is well separated apart from the bands of 850 and 1000 bp which are overlapping. The bands at the top are not clear. In every lane there is a light smear of DNA. There is a small amount of primer dimer at the bottom of the lanes. Irun and Antwerp show a light band of ±736 bp at the knockout side. Jaca and Bevel show very clear bands at the same side. Huesca displays a band at the right height on the knockout side and one on the wildtype side. Genova shows a clear band at the wildtype side and a very light one at the knockout side. The lane of the control at the wildtype side is empty. The one at the knockout side shows a very light band.

Discussion

Irun, Bevel, Jaca and Antwerp show the correct bands at the knockout side because these mice are knockouts. The bands are clear except for the ones of Irun and Antwerp. These are light but still visible. Huesca is probably heterozygous so this one too displays the correct bands. Genova shows a correct band of ± 781 base pairs on the wildtype side and a very light one at the knockout side. This is a contamination that probably occurred during the loading of the Antwerp sample into the well. This also could have caused the contamination (very light band) found in the lane of the control.

The control on the wildtype side is clear of bands which means that there was no contamination during the experiment with the wildtype mastermix. Despite the contaminations the experiment

wasn't repeated since the results were good for the person who's going to use the mice for further experiments.

4.2. Cre protocol

4.2.1. Provided protocol

There was a protocol available in the lab for the genotyping of the cre mice. This protocol first needed to be tested to check which parameters could be optimized to get clearer results.

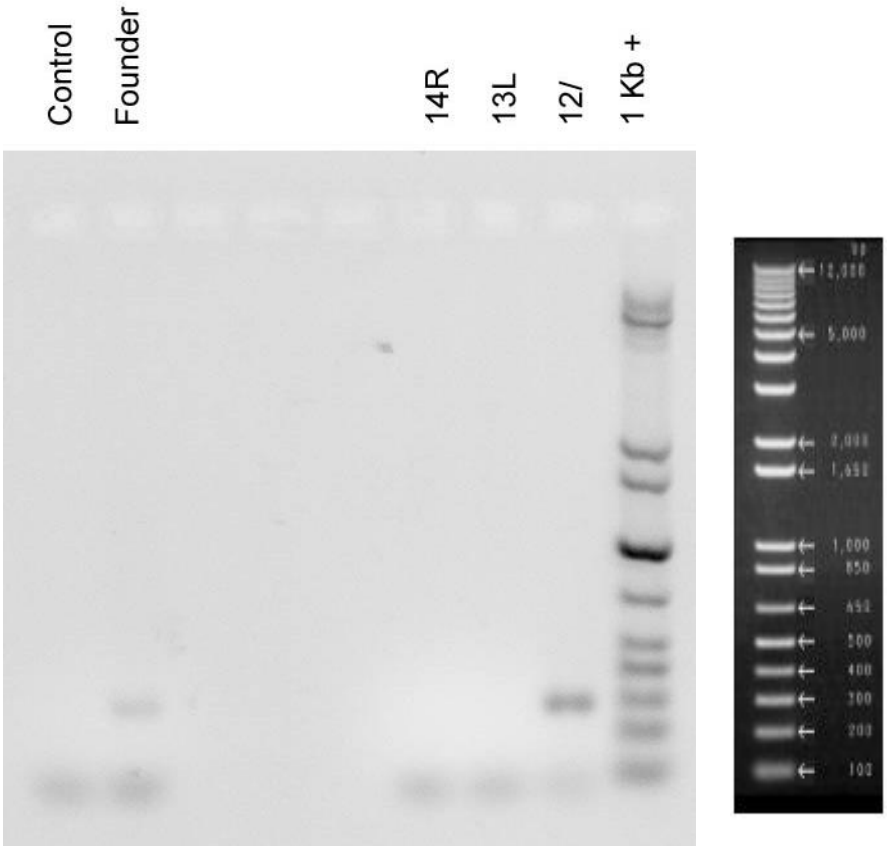

Figure 30: Electropherogram provided protocol for cre

Results

Figure 30 shows the results of the test PCR for the genotyping of the cre mice. The bands of 850 and 1000 base pairs of the ladder are overlapping and the ones at the top are not clearly visible. At the bottom of the lanes with the samples is a small amount of primer dimer. The founder shows a very light band that is at a height of 200-300 base pairs. 12/ displays a band of the same height that is more visible. 13L and 14F show no bands. The same goes for the control.

Discussion

The founder shows a band at the height of 200 – 300 bp. This was expected since the sample is a positive control. The only sample that displays a band at the same height is 12/ which means that

this sample is positive for the cre gene. 13L and 14F don't show a band, these samples are negative for the gene.
There was no contamination for the control doesn't show a band.

The displayed bands are very light and not clearly visible. There is need for optimization of the protocol.

Adjustments

None

Next experiment

The next step is the optimization of the genotyping protocol.

4.2.2. Optimization

4.2.2.1. Primer concentration

The first thing that was optimized is the primer concentration. Since the protocol that was used in the lab worked there was no need for the optimization of the annealing temperature.

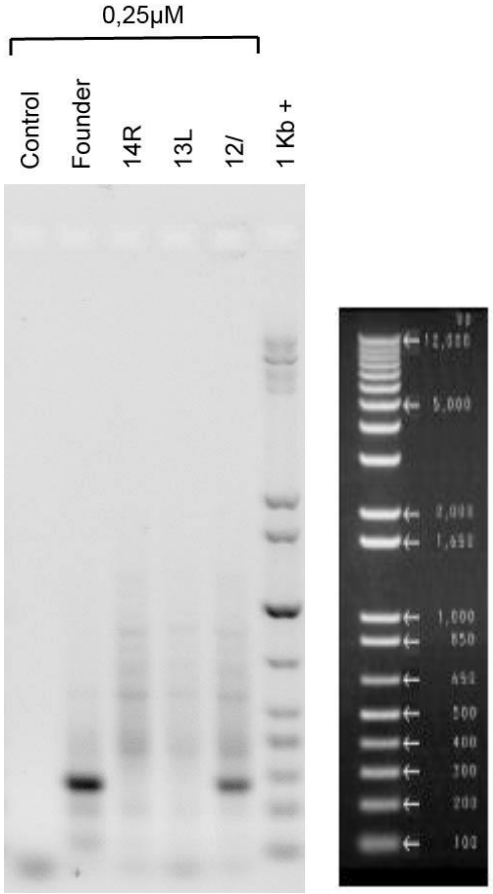

Figure 31: Electropherogram optimization primer concentration cre

Results

The electropherogram in figure 31 displays the results of a primer concentration of 0,25 µM. The bands of the ladder are separated apart from the ones of 850 and 1000 base pairs. The bands at the top of the ladder are not clearly visible. The two samples that show a band at a height of 200 – 300 are the founder and 12/. The one of 12/ is less clear. 13L and 14R show no clear bands but there are some light bands that can be distinguished from the DNA smear. There are also some light bands at a different height in the lane of the founder and 12/. The control shows no band, only a small amount of primer dimer at the bottom.

Discussion

The founder and 12/ show a clear correct band but there are also some light nonspecific bands. 13L and 14R are negative for the cre gene so they don't show a clear band at the height of 200 – 300 base pairs. In these lanes are also some light nonspecific bands. These bands are formed when the primers bind to other sequences than the ones that are complementary to the sequence of the primers. This nonspecific binding can disappear by adjusting the annealing temperature and by addition of Key buffer which increases the specificity of the PCR reaction.

The control shows no band which means that there was no contamination.

The optimal primer concentration is 0,25 µM which is the one that was already used in the protocol, so this parameter doesn't need to be changed. The results of the other temperatures are not good (see attachments page 97):

- 0,5 µM: The bands are thick so it is better to use less primer solution.
- 0,38 µM: The amount of primer dimer is very small but there are a lot of nonspecific bands.
- 0,125 µM: The nonspecific bands of this concentration are more clear than the ones on the electropherogram of 0,25 µM (figure 31).

Adjustments

Addition of Key buffer.

Next experiment

Because there was a lot of nonspecific binding, the next step was determining the optimal annealing temperature.

4.2.2.2. Annealing temperature

To prevent the nonspecific binding, the annealing temperature needed to be optimized.

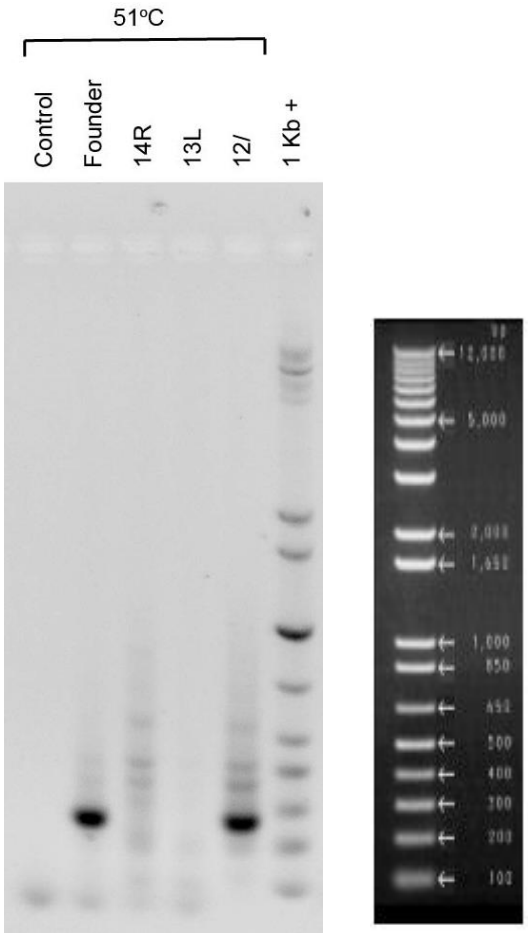

Figure 32: Electropherogram optimization annealing temperature cre

Results

Figure 32 shows the result of the adjustment of the annealing temperature. The ladder is separated except for the bands of 850 and 1000 bp. The bands at the top are also not very clear. The founder and 12/ display one clear band at a height of 200 – 300 base pairs. In the lane of 12/ are nonspecific bands. 13L shows no bands. The same goes for the control. In the lane of 14R are nonspecific bands. At the bottom of every lane is a small amount of primer dimer.

Discussion

The founder and 12/ show the correct clear band but in the lane of 12/ are some light nonspecific bands. 13L doesn't display a band which is correct since this mouse is negative for the cre gene. 14R is also negative so it doesn't show a clear band at 200 – 300 base pairs but there are nonspecific bands visible. Now the annealing temperature is adjusted the nonspecific binding could also be a consequence of too much added DNA.

51°C is the optimal annealing temperatures as the other ones show more nonspecific bands and more smear of DNA (see attachments pages 98-99).

The control doesn't show bands so no contamination occurred during this experiment.

Adjustments

Change annealing temperature from 55°C to 51°C.

Next experiment

The nonspecific bands could also be a consequence of the excess of DNA that was added to the mastermix. The next step is the adjustment of the DNA volume.

4.2.2.3. DNA volume

To prevent the formation of nonspecific bands, the DNA volume needs to be optimized.

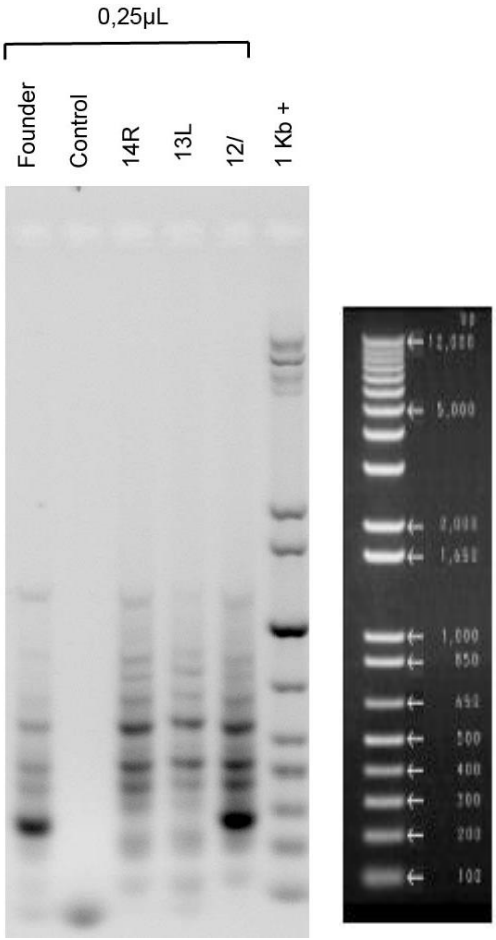

Figure 33: Electropherogram optimization DNA volume cre

Results

The ladder in figure 33 is separated apart from again the bands of 850 and 1000 base pairs. And it is also a bit stretched out. There are a lot of nonspecific bands in the lane of every sample. The founder and 12/ also display a clear band at the height of 200 – 300 bp. The control shows no band and there is some primer dimer at the bottom of this lane.

Discussion

The founder and 12/ show one clear band at the correct height. There are also a lot of nonspecific bands in these lanes. So the DNA volume isn't the cause of the nonspecificity of the primers. 13L and 14R also display a lot of nonspecific bands. The problem could be the excess of $MgCl_2$. According to the protocol an extra amount of this component needs to be added to the mastermix although it's already present in the buffers.

All the volumes that were tested, show a lot of nonspecific bands (see attachments page 100). 0,25 µl is the lowest volume that still displays clear visible bands at the right height.

The control shows no bands so there was no contamination.

Adjustments

Change DNA volume from 0,5 µl tot 0,25 µl.

Next experiment

The optimal values for the most important parameters were determined, so the next step was to test the optimized protocol. Because the excess of $MgCl_2$ could have caused the formation of the nonspecific bands, it wasn't added to the mastermix of the optimized PCR.

4.2.3. Optimized protocol

Table 48 and 49 represent the optimized mastermix and PCR programme.

See materials and method in chapter 3.4 PCR.

Mastermix cre

Table 48: Optimized mastermix for cre

Reagents	Stock concentration	Volume/sample (µl)	Volume/6 samples (µl)
Sterile H_2O	/	7,4	44,4
10x Extra buffer	10x	1,3	7,8
10x Key buffer	10x	1,3	7,8
dNTP mix	10 mM	0,5	3
Primer sh 001	10 µM	1	6
Primer sh 002	10 µM	1	6
Taq polymerase	5U/µl	0,25	1,5
Total		12,75	
DNA		0,25	

PCR programme cre

Table 49: Optimized PCR programme for cre mice

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1 min.	1
Denaturation **Annealing** **Elongation**	95°C 51°C 72°C	30 sec. 30 sec. 1 min.	30
Final elongation	72°C	1 min.	1
Conservation	4°C	∞	

Result

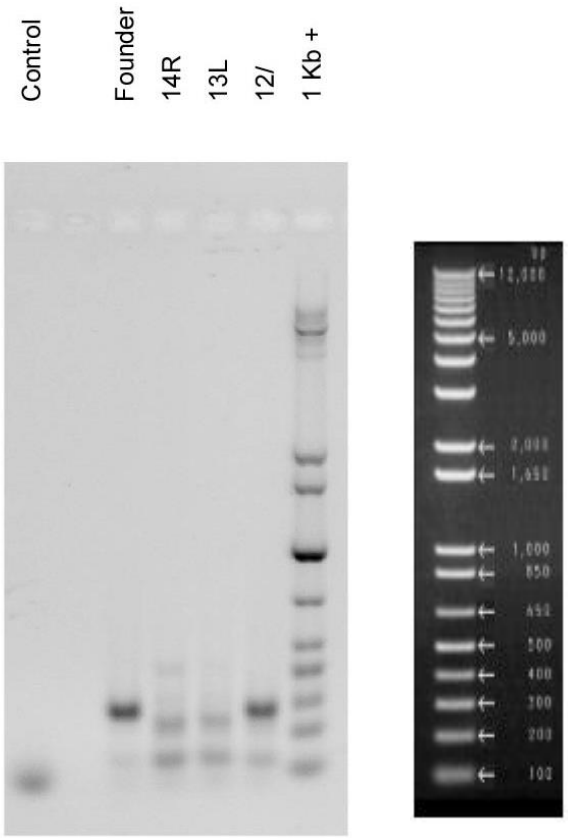

Figure 34: Electropherogram optimized cre protocol

Results

Figure 34 shows the result of the optimized cre protocol. The ladder is well separated apart from the bands of 850 and 1000 base pairs. The ones at the top are not clearly visible. The control shows no band, only some primer dimer at the bottom of the lane. The founder displays one clear band at a height of 200 – 300 bp and one band that is very light at a lower height. 12/ also shows one clear band at the same height as the founder and one lighter band below. 13L and 14R show the same pattern of light bands. There is just a light smear of DNA in each lane and there is no primer dimer at the bottom of the lanes with the samples.

Discussion

The founder shows the correct band since this sample is positive for the cre gene. The same goes for 12/ but in both lanes there is a light nonspecific band. 13L and 14R show the same nonspecific bands. Normally they should display no bands since these samples are negative for the cre gene. Because there is still a lot of nonspecific binding after the changes in the protocol the problem is probably the pair of primers. They weren't designed well, therefore they bind to sequences that are not completely complementary to the primers' sequences. Despite the nonspecific bands, it is still clear which mice are positive for the cre gene and which ones are not.

The control shows no band which means that no contamination occurred during this experiment.

5. Conclusion

The paramaters that needed to be changed in the PINK1 protocol were the annealing temperature, the primer concentration and the amount of DNA. 56,1°C is the optimal annealing temperature. The right primer concentration is 0,125 µM and the amount of DNA that needs to be added to get a good and clear result is 0,5 µl. Key buffer was added to the mastermix to increase the specificity of the primers. Besides the mastermix the programme was also adjusted. The initial denaturation step now lasts for 2 minutes instead of 5.

The changes in the mastermix for the genotyping of the cre mice are the addition of 0,25 µl of DNA instead of 0,5 µl, the addition of Key buffer and the extra $MgCl_2$ that is left out. The primer concentration remains the same (0,25 µM). In the programme only the annealing temperature is changed from 55°C to 51°C.

Both protocols show good and clear results and can be used for the genotyping of the PINK1 and cre mice. Because of the optimization, the results are now reliable so researchers will be able to start experiments with the right mice. The mice with deviating results won't be used in experiments and will be killed.

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Attachments

Results of optimization of the annealing temperature PINK1 (wildtype)

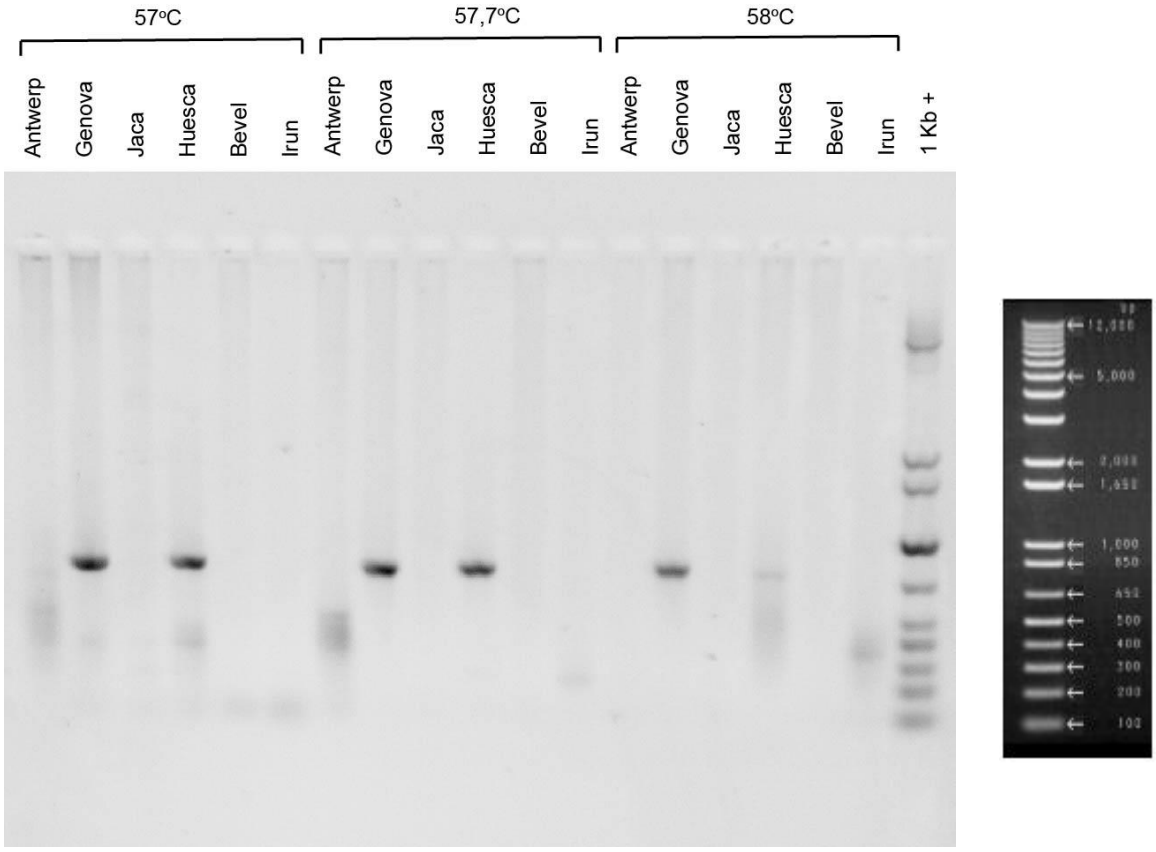

Figure 35: Electropherogram optimization of annealing temperature for PINK1 wildtype (1)

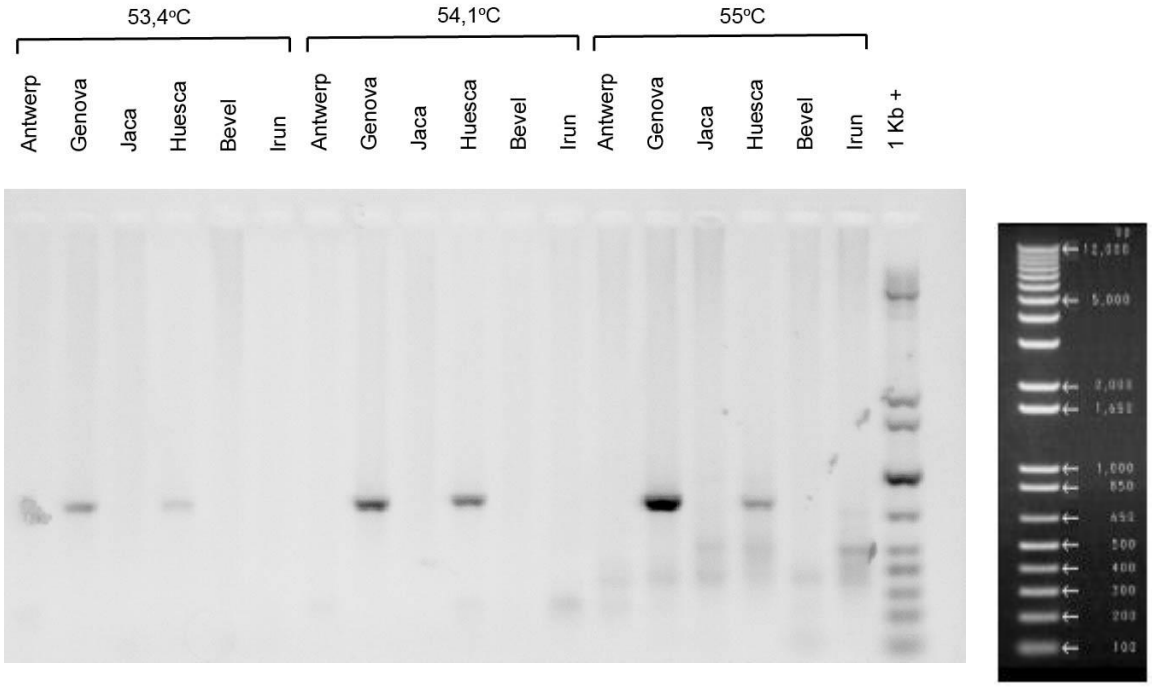

Figure 36:Electropherogram optimization of annealing temperature for PINK1 wildtype (2)

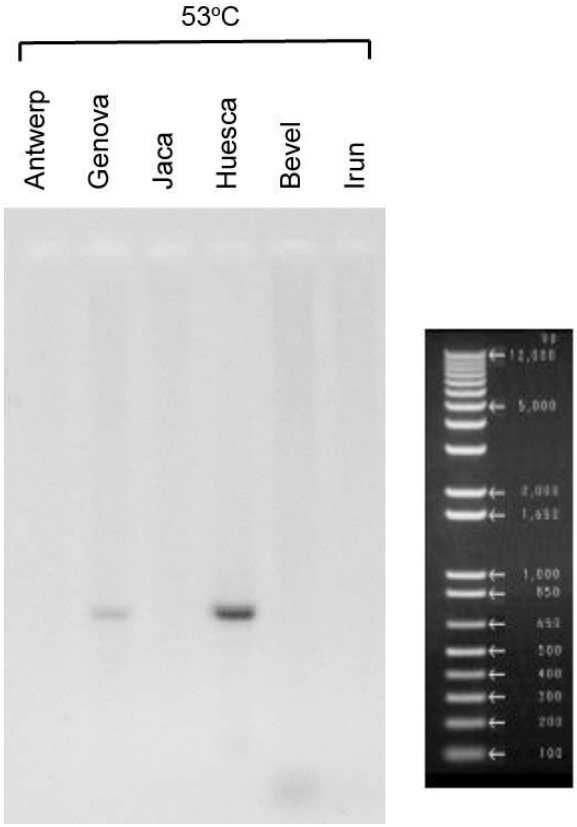

Figure 37: Electropherogram optimization of annealing temperature for PINK1 wildtype (3)

Results of optimization of the annealing temperature PINK1 (knockout)

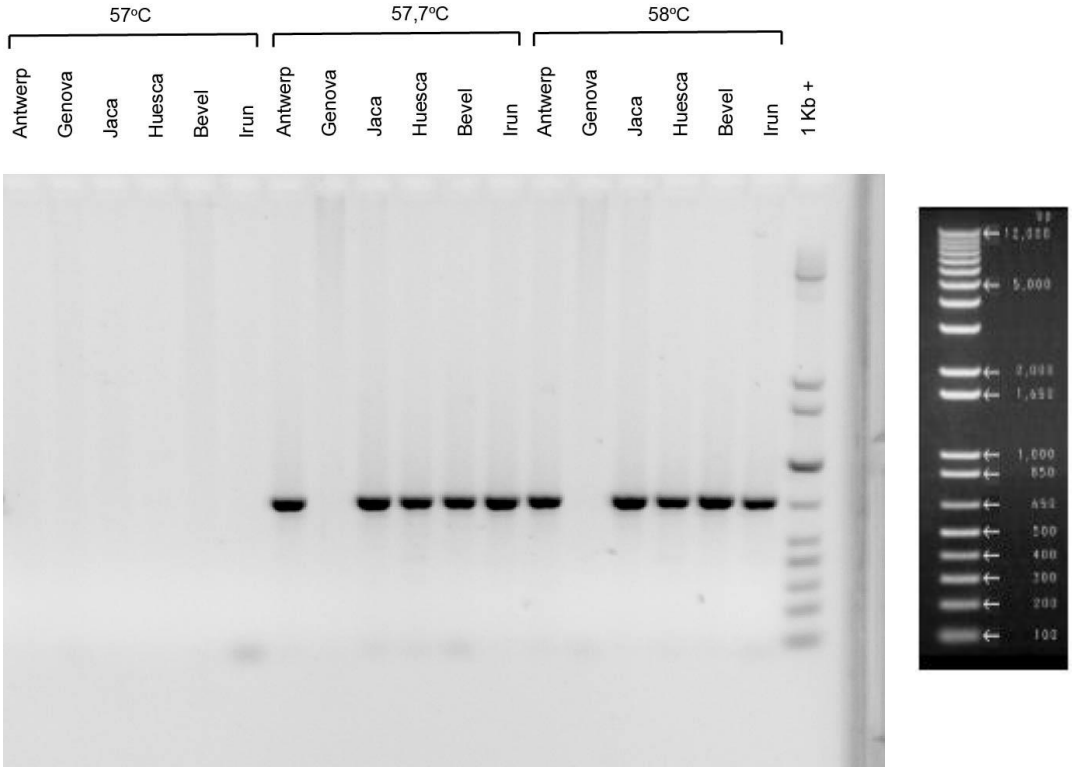

Figure 38: Electropherogram optimization of annealing temperature for PINK1 knockout (1)

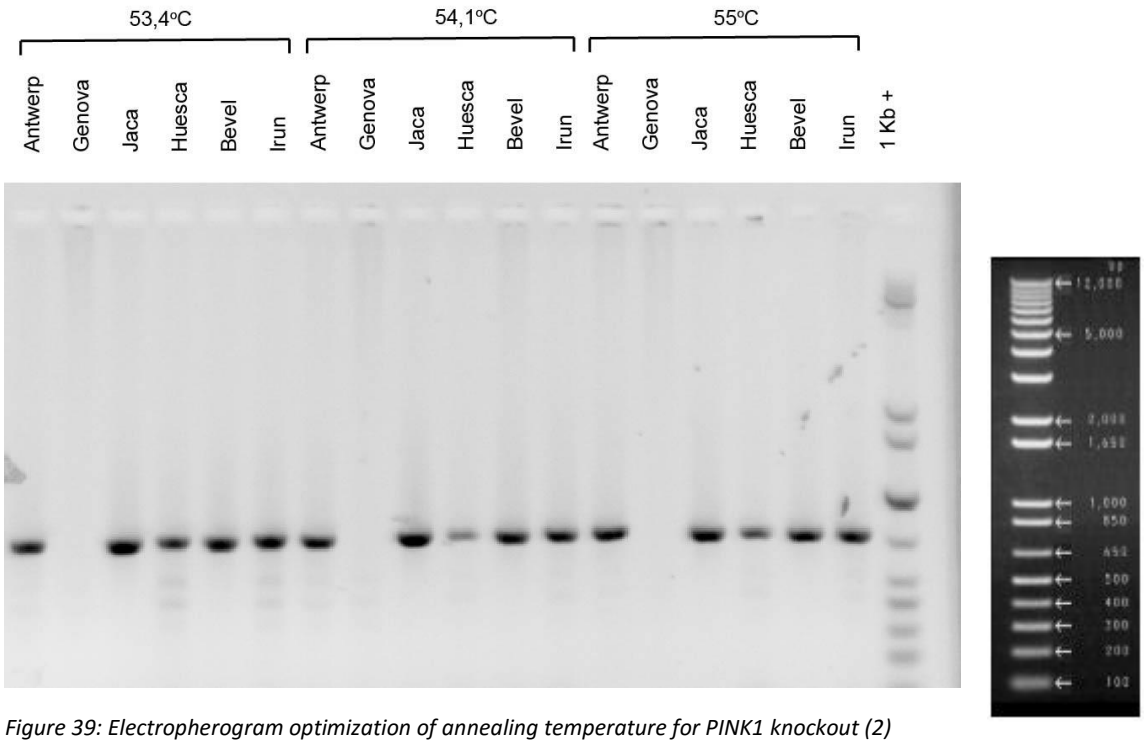

Figure 39: Electropherogram optimization of annealing temperature for PINK1 knockout (2)

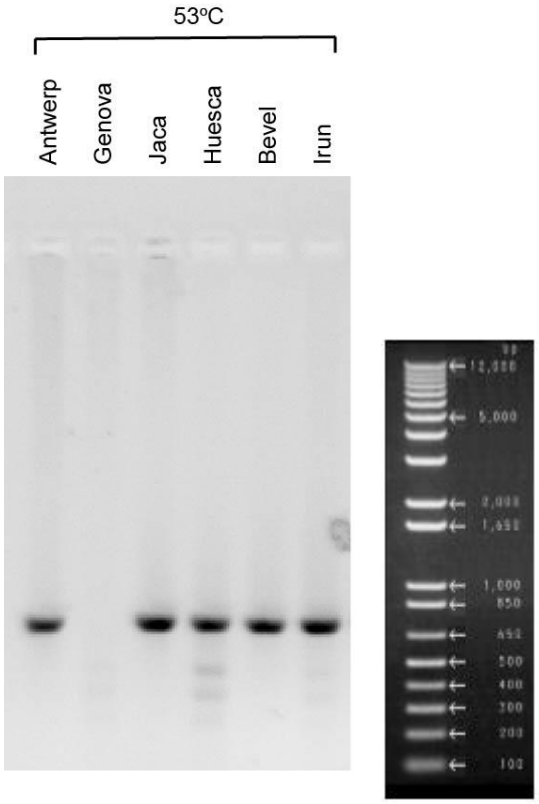

Figure 40: Electropherogram optimization of annealing temperature for PINK1 knockout (3)

Results of optimization of the primer concentration PINK1 (wildtype)

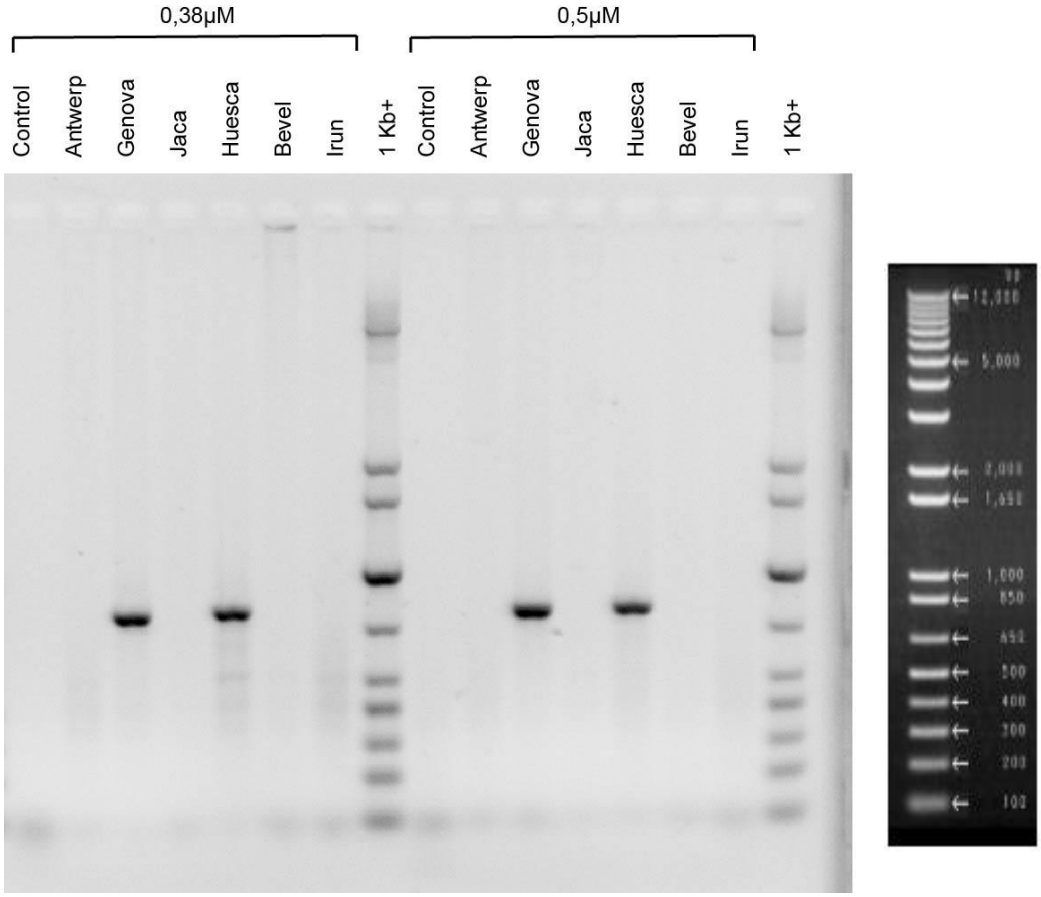

Figure 41:Electropherogram optimization of primer concentration for PINK1 wildtype (1)

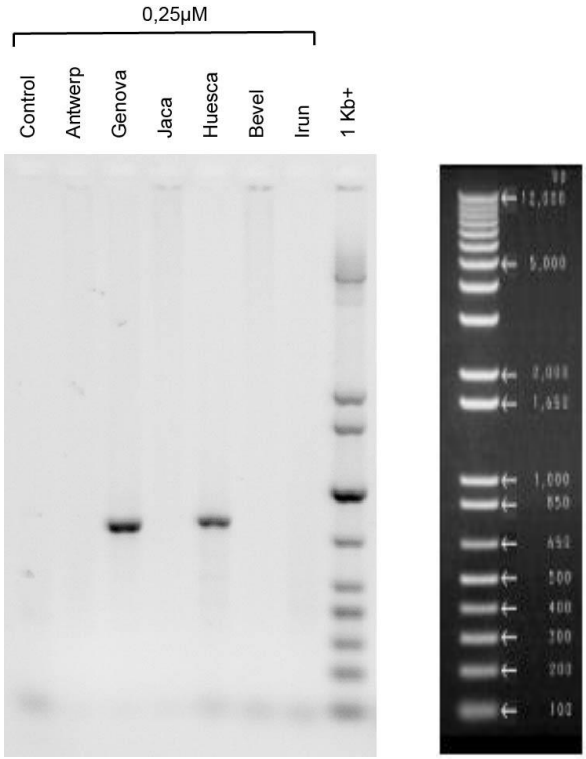

Figure 42: Electropherogram optimization of primer concentration for PINK1 wildtype (2)

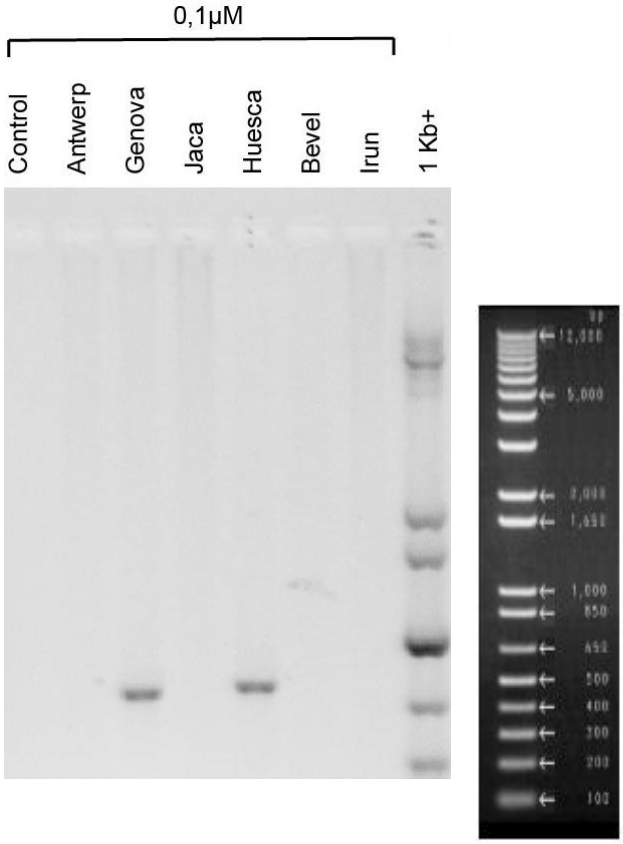

Figure 43: Electropherogram optimization of primer concentration for PINK1 wildtype (3)

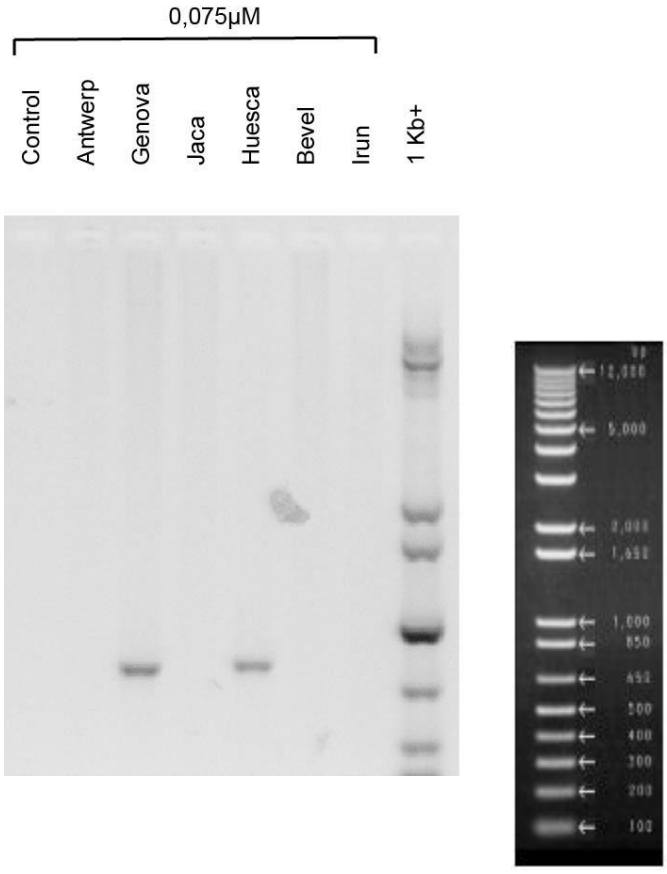

Figure 44: Electropherogram optimization of primer concentration for PINK1 wildtype (4)

Results of optimization of the primer concentration PINK1 (knockout)

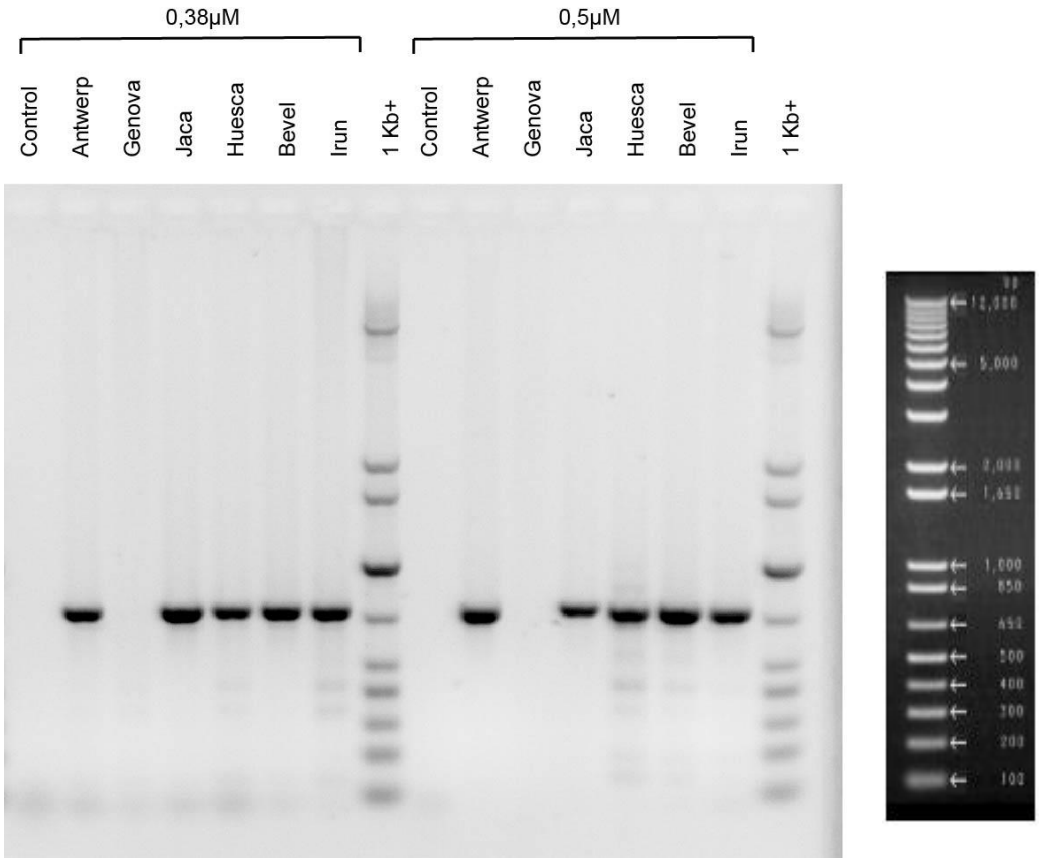

Figure 45: Electropherogram optimization of primer concentration for PINK1 knockout (1)

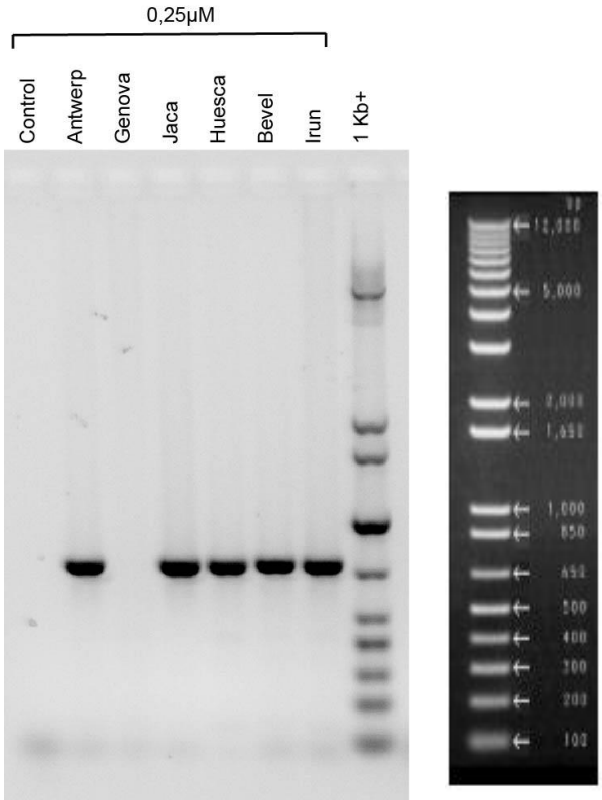

Figure 46: Electropherogram optimization of primer concentration for PINK1 knockout (2)

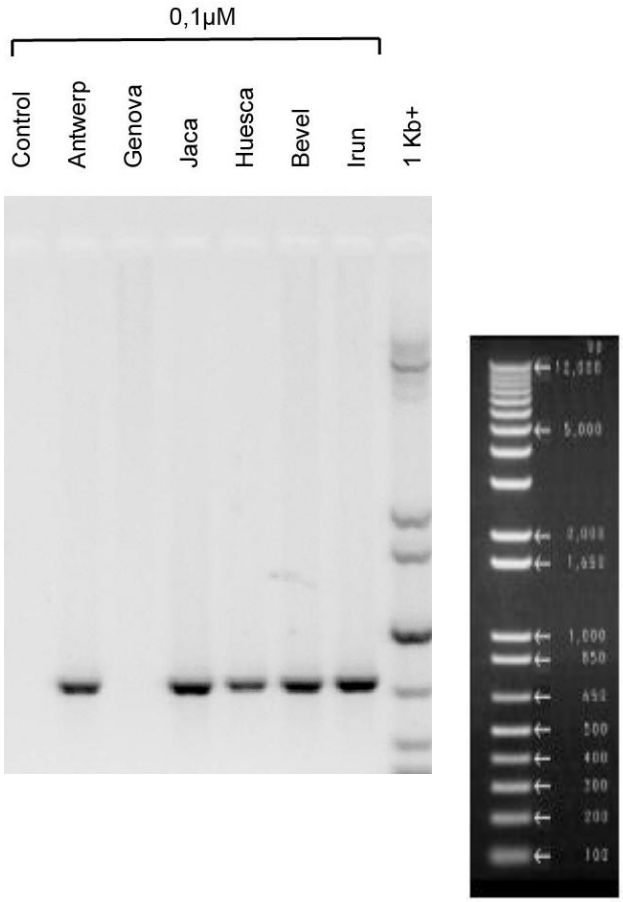

Figure 47: Electropherogram optimization of primer concentration for PINK1 knockout (3)

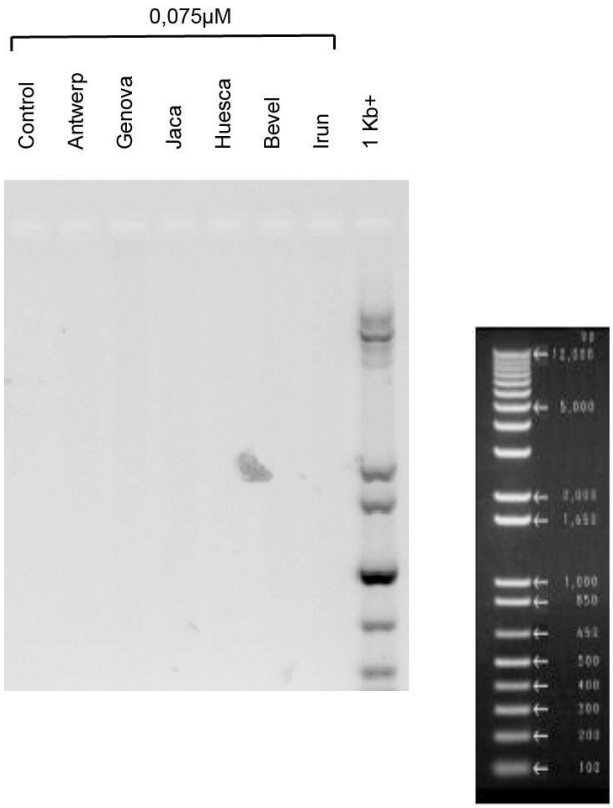

Figure 48: Electropherogram optimization of primer concentration for PINK1 knockout (4)

Results of optimization of the DNA volume PINK1 (wildtype)

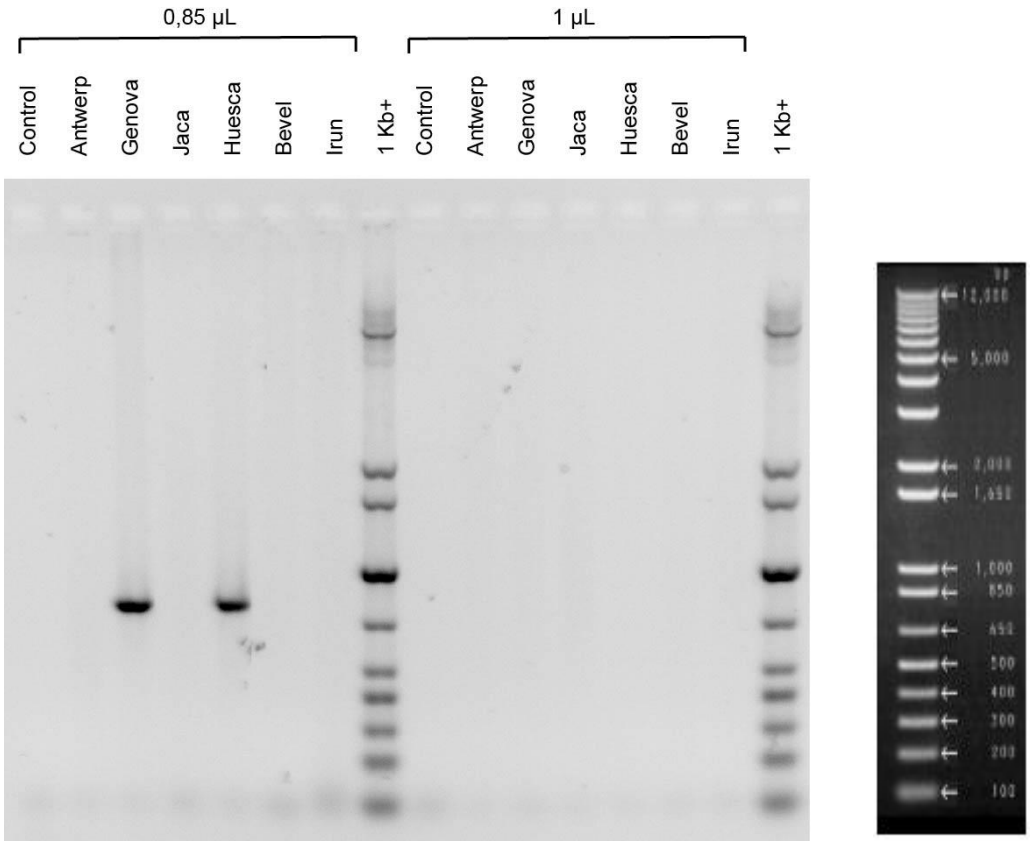

Figure 49: Electropherogram optimization of DNA volume for PINK1 wildtype (1)

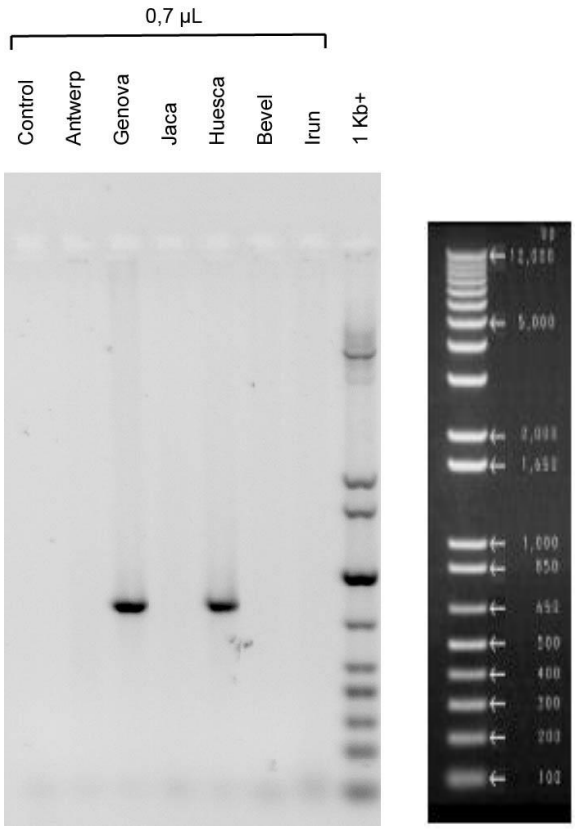

Figure 50: Electropherogram optimization of DNA volume for PINK1 wildtype (2)

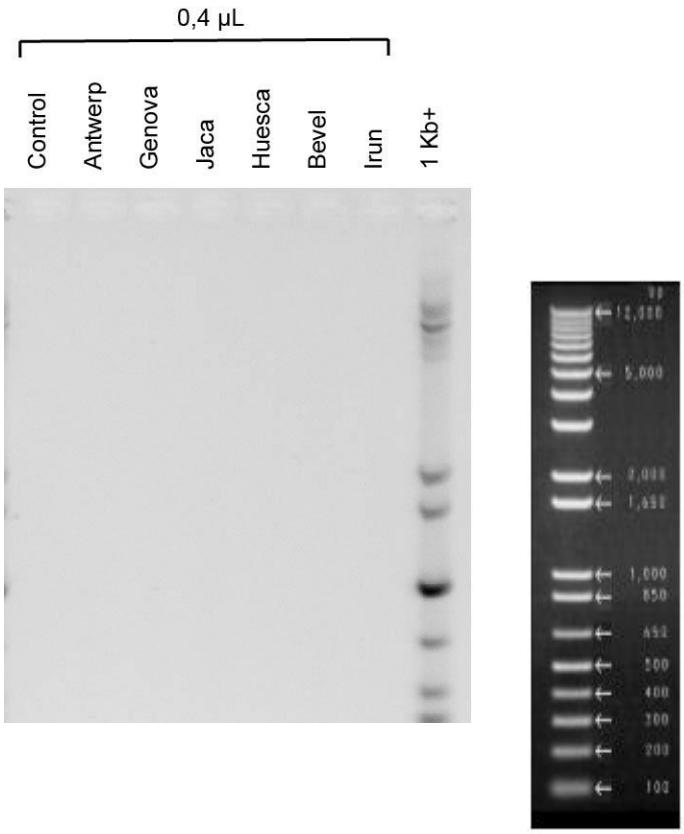

Figure 51: Electropherogram optimization of DNA volume for PINK1 wildtype (3)

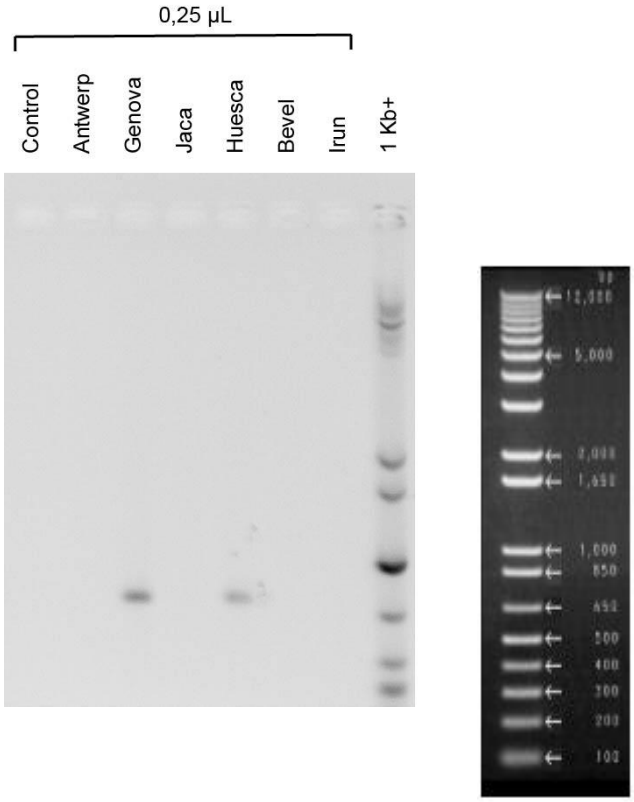

Figure 52: Electropherogram optimization of DNA volume for PINK1 wildtype (4)

Results of optimization of the DNA volume PINK1 (knockout)

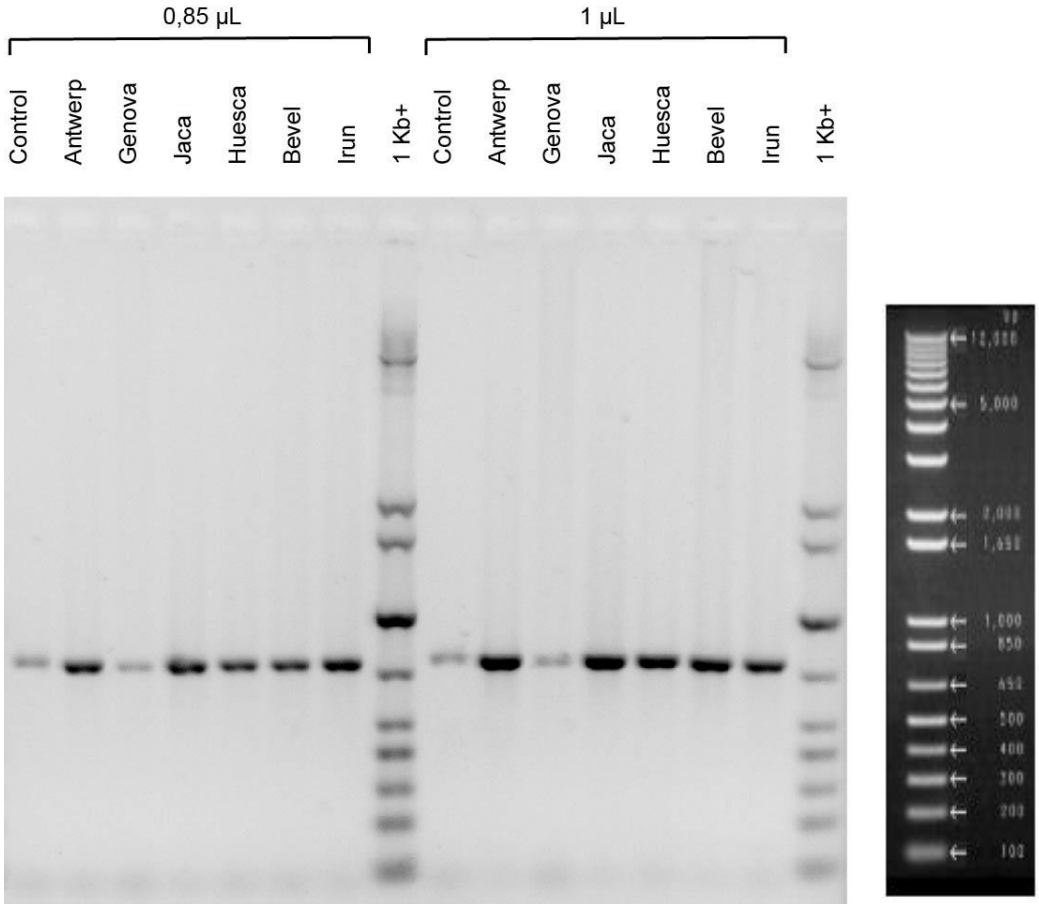

Figure 53: Electropherogram optimization of DNA volume for PINK1 knockout (1)

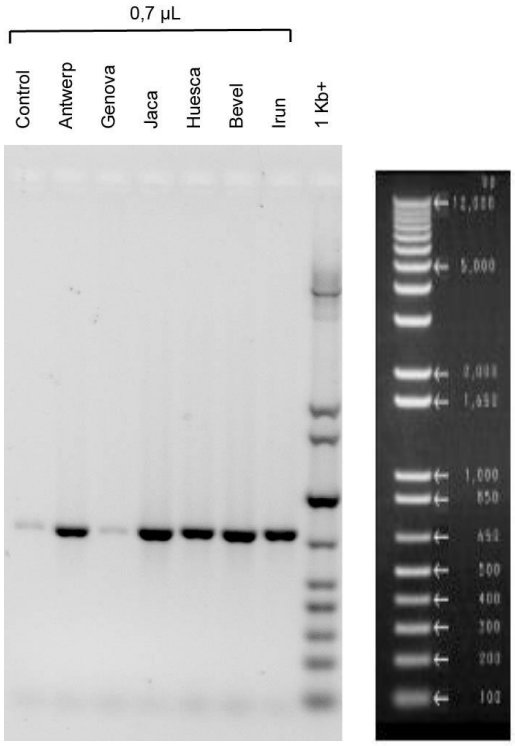

Figure 54: Electropherogram optimization of DNA volume for PINK1 knockout (2)

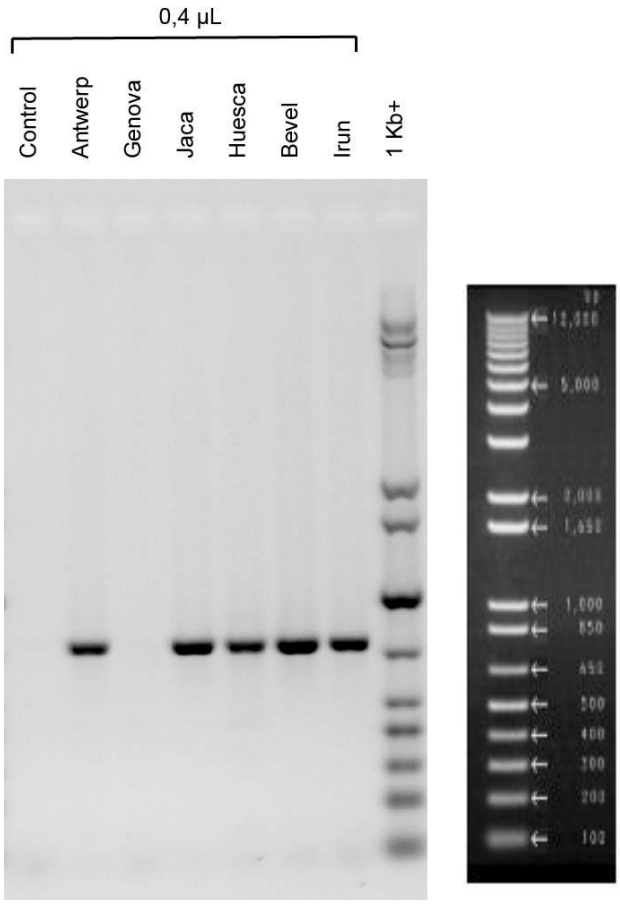

Figure 55: Electropherogram optimization of DNA volume for PINK1 knockout (3)

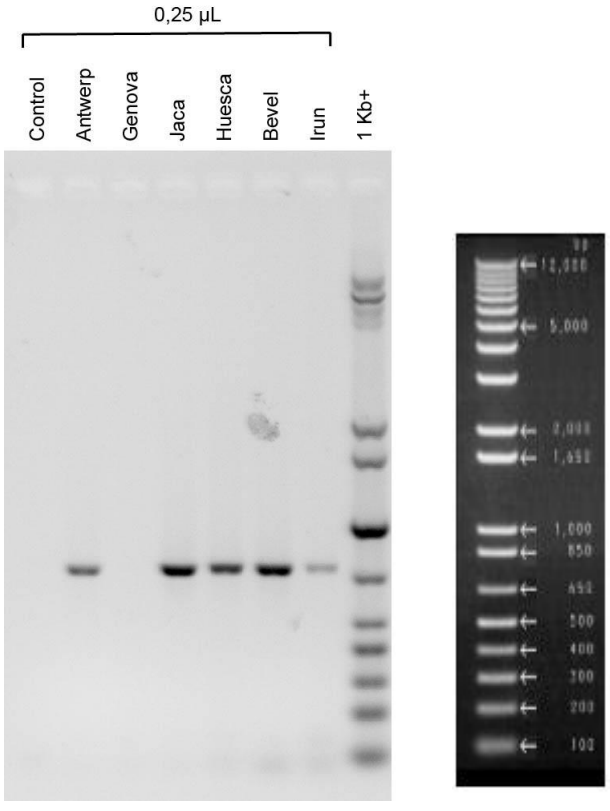

Figure 56: Electropherogram optimization of DNA volume for PINK1 knockout (4)

Results of optimization of the primer concentration cre

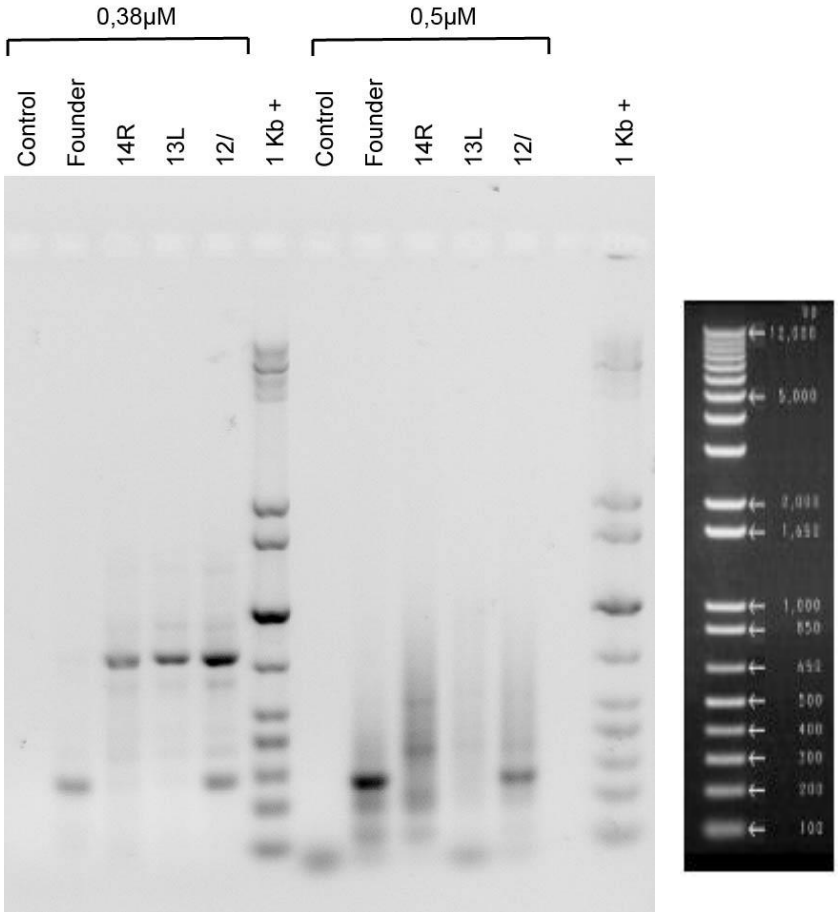

Figure 57: Electropherogram optimization of primer concentration for cre (1)

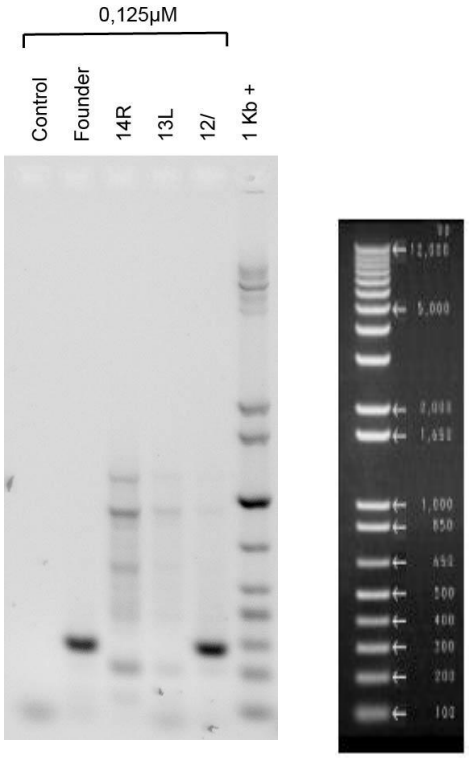

Figure 58: Electropherogram optimization of primer concentration for cre (2)

Results of annealing temperature cre

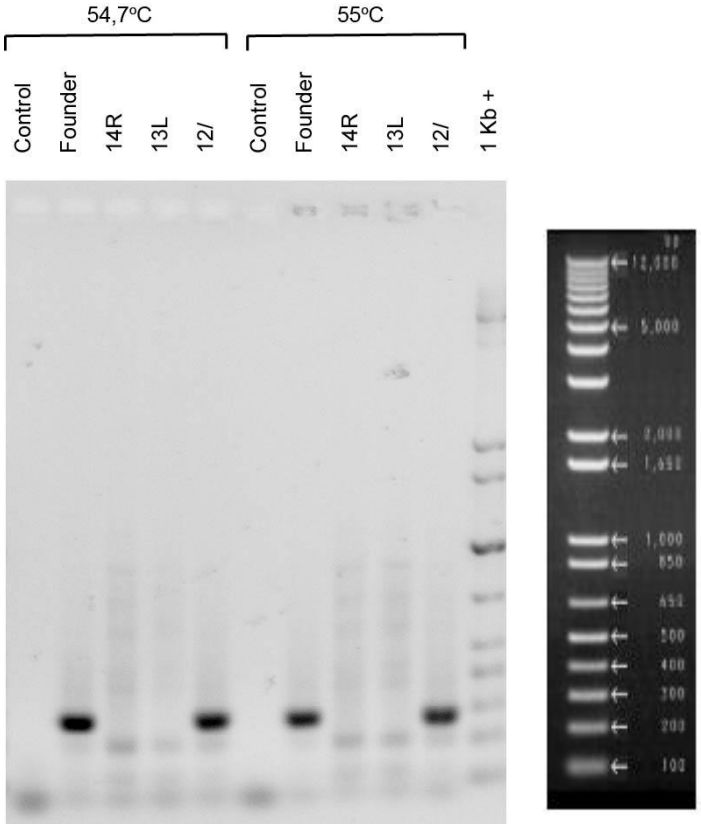

Figure 59: Electropherogram optimization of annealing temperature for cre (1)

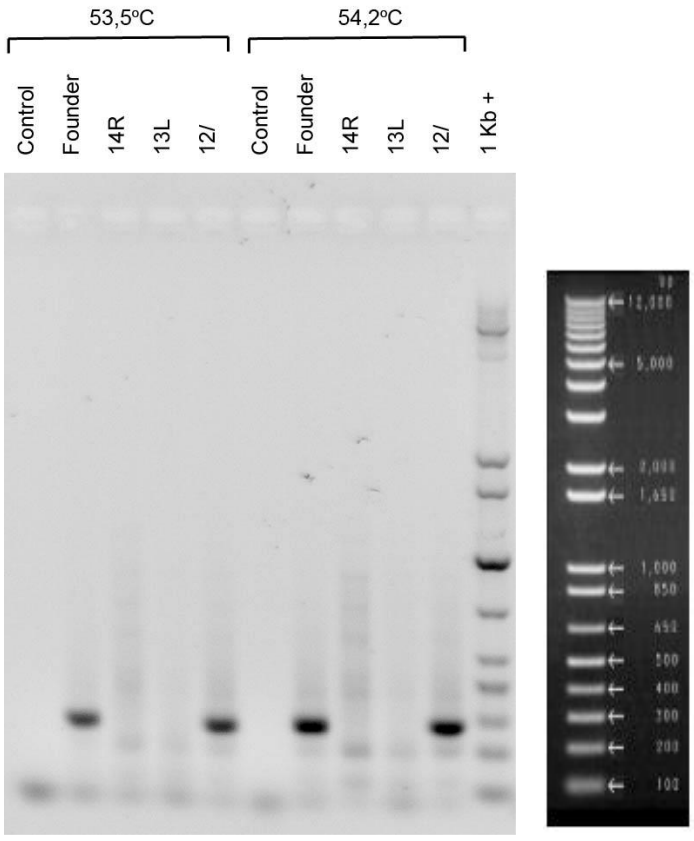

Figure 60: Electropherogram optimization of annealing temperature for cre (2)

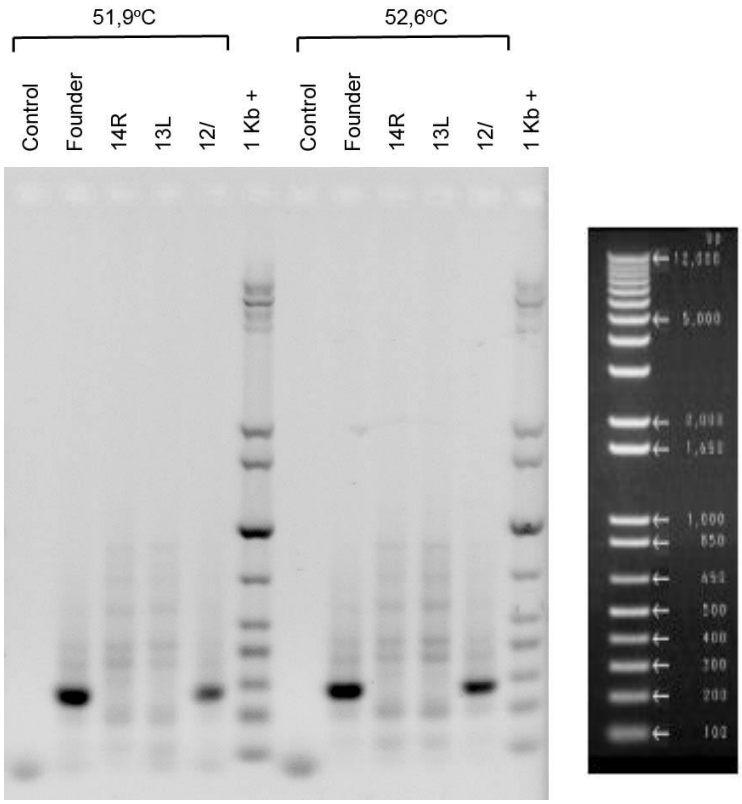

Figure 61: Electropherogram optimization of annealing temperature for cre (3)

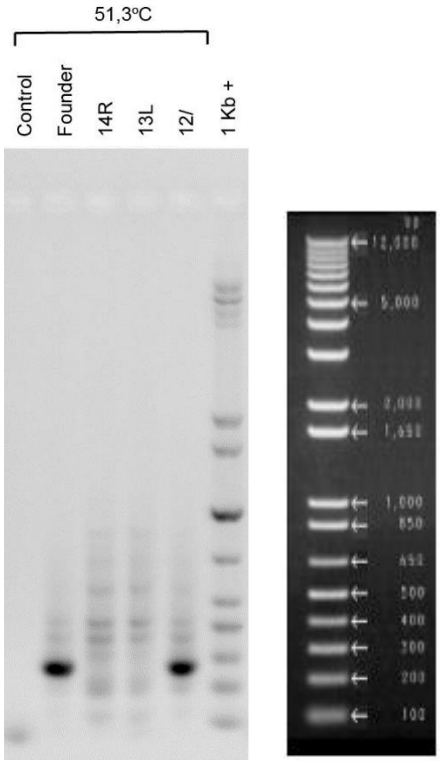

Figure 62: Electropherogram optimization of annealing temperature for cre (4)

Results of optimization of the DNA volume cre

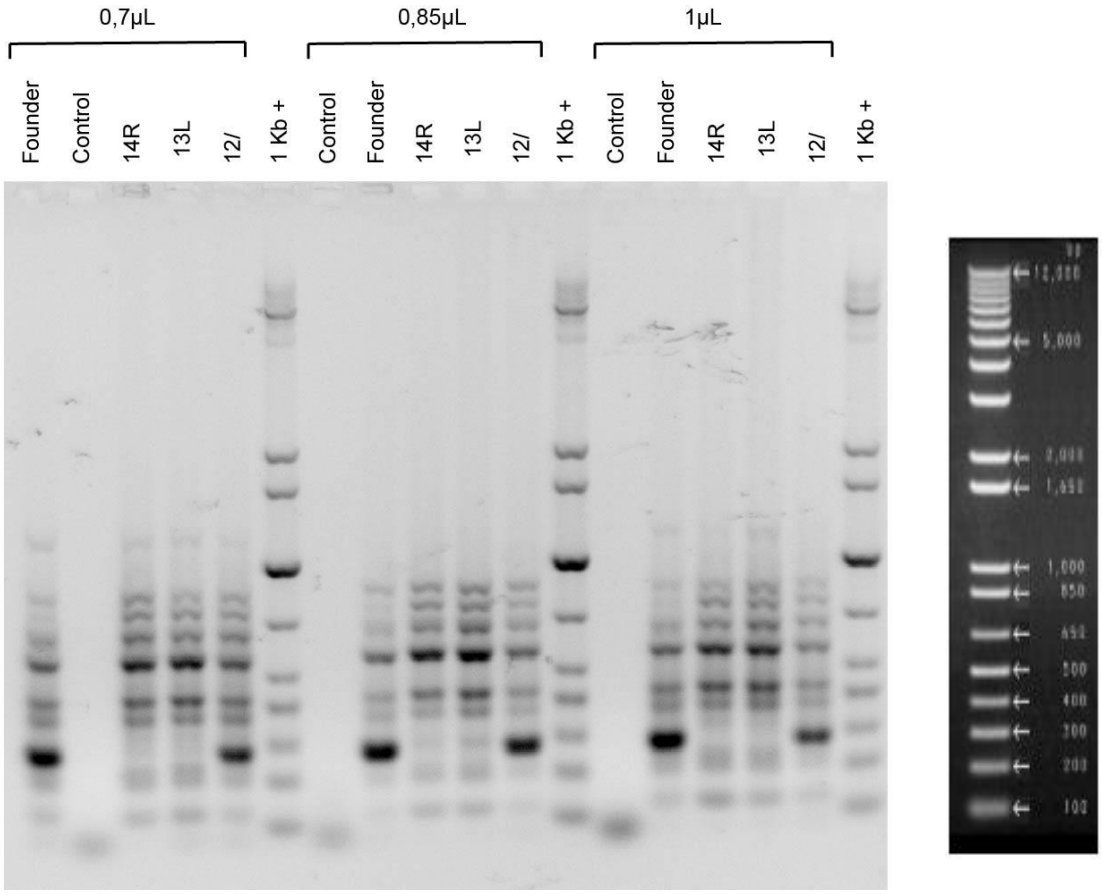

Figure 63: Electropherogram optimization of DNA volume for cre (1)

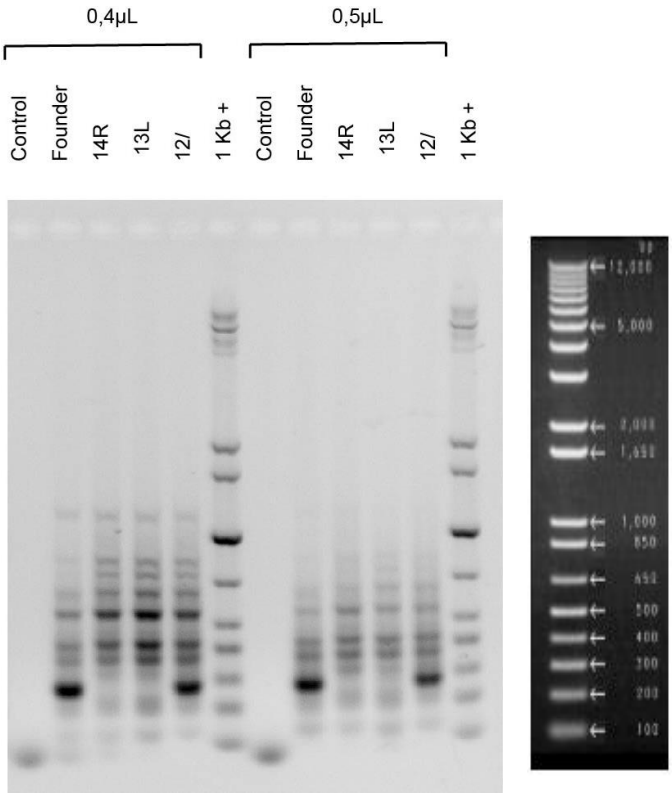

Figure 64: Electropherogram optimization of DNA volume for cre (2)