Optimization of PCR protocols used for genotyping transgenic mice
&
Evaluation of a method for co-detecting mRNA and protein

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Abstract

The aim of the current study was divided into two separate goals, (i) optimization of a number of PCR-based protocols employed for genotyping transgenic mouse lines and (ii) evaluating a protocol for co-detection of mRNA and its correlated protein in the mouse midbrain. The optimization was performed on PCR protocols for genotyping the following transgenic mouse lines; Dat-Cre, Vglut2-Lox, Vglut2-Cre and Vmat2-Lox. Also, two different polymerases were evaluated parallel to each other – KAPA and Maxima Hot Start. One of the main findings from the PCR optimizations were that for the Vglut2-Lox protocol. By decreasing the annealing temp and increasing the MgCl₂ the bands appeared brighter.

For the second part of the project, in-situ hybridization (ISH) was used to detect the mRNA expression with a `non-radioactive in situ hybridization´ protocol, using digoxigenin or fluorescein labelled riboprobes (mRNA probes). To detect the correlated protein a basic immunohistochemistry (IHC) protocol with the use of primary and secondary antibodies was implemented. The combined protocol was tested with Nd6 and Grp markers. Before testing to combined the protocols the ISH protocol was performed alone with riboprobes for Girk2, Lpl and Fst. The combined protocol detected mRNA and protein for both the control marker Th and the Nd6 marker.

In conclusions, the optimized PCR protocols were optimal when used with the Maxima Hot Start polymerase and the new combined ISH and IHC protocol worked for markers Th and Nd6.

Keywords

Ventral tegmental area; Substantia nigra; Parkinson’s disease; transgenic mice; Cre-Lox recombination
Introduction

The brain is the focus of a variety of studies, everything from understanding the human mind to understanding the physiological mechanisms behind diseases. The study of volitional behaviour and voluntary movement are of especially great interest today. The areas in the midbrain have been linked to disorders such as schizophrenia and addiction, but also degenerative diseases such as Parkinson’s disease (PD) and Alzheimer’s disease.

The midbrain is a part of the brainstem and located between hypothalamus – a part of the forebrain, pons, a part of the brainstem and the hindbrain. The most relevant parts of the midbrain, in studies of volitional behaviour and voluntary movement are the ventral tegmental area (VTA) and the substantia nigra (SN), or more specific the substantia nigra pars compacta (SNc) (Björklund et al., 2007). The SN and SNc is located lateral to the VTA, which is located on the “floor” of the midbrain. The SNc is associated with PD (Patt et al., 1991; Yamada et al., 1991).

PD is a very complex degenerative disease where neurons are damaged and go through apoptosis, which causes atrophy and shrinkage of the brain tissue. The pathological signs of PD are the loss of neurons that transmit dopamine (DA) from the SNc, leading to impairment of the nigrostriatal pathway (d'Anglemont de Tassigny et al., 2015; Roberson et al., 1989). The loss of neurons is caused by aggregations, Lewy bodies, of misfolded protein found within the neurons. The mechanism behind PD however is still somewhat unknown, although genetic predispositions and environmental factors are believed to play big roles in the grand scheme of things. The most observed protein found in Lewy bodies is misfolded alpha-synuclein, which is caused by a mutation in the SNCA gene. The alpha-synuclein is therefore a promising biomarker for early onset PD and its progression (Kang et al., 2013; Skogseth et al., 2015). Commonly known symptoms for PD is loss of motor control, which makes it self-known through tremors, stiffness and postural imbalance. Early warning signs can also be observed, often of a non-motor type, e.g. sleep disorder or psychiatric symptoms (Khoo et al., 2013). In addition to the usual symptoms it is important to be aware of the neuropsychiatric symptoms, such as depression and apathy. Many PD patients can feel like they are a burden to the people around them, family and friends.
Together with the confusion and loss of control, which are major stress factors, this can sometimes lead to suicidal thoughts (Hiro et al., 2015). There is no treatment available today that can stop or regress PD. The most effective treatments today are symptomatic relief treatments, such as levodopa (L-DOPA) and deep brain stimulation (DBS). The long-term effects are still undesirable.

The ability to perform voluntary movements and volitional behaviour involves the four major dopaminergic (DAergic) pathways. The DAergic neurons starts out in the midbrain and later connects to different parts throughout the brain. Only three of the pathways were relevant to this project. The mesolimbic pathway induces both reward- and aversion-like signals by transmitting DA from the VTA to the nucleus accumbens (NAc) (Ilango et al., 2014). Closely associated with the mesolimbic reward pathway, as it is also known, is the mesocortical pathway. The DAergic neurons starts out from the VTA and are located almost side-to-side, with the mesolimbic DAergic neurones branching off first (Ferreira et al., 2008). The mesocortial pathway transmits DA to the prefrontal cortex, which is believed to be the centrum for cognitive control, motivation and emotional response (Fuster et al., 2000; Goldman-Rakic et al., 1996). Pathological changes to these pathways are highly linked to disorders such as addiction, ADHD and schizophrenia (Perez-Costas et al., 2012). The DAergic neurons of the last pathway, the nigrostriatal pathway, starts out in the SN and connects to the caudate nucleus and putamen, both located in the dorsal stratum (Salvatore et al., 2012). Here the DAergic neurons latches onto GABAergic neurons in the dorsal stratum, which is a part of the basal ganglia motor circuit that produce movement. GABA is an inhibitory neurotransmitter also known as gamma-Aminobutyric acid (Ikemoto et al., 2015; Morita et al., 2013).

To comprehend these elaborate networks of neurons, the science community have dissected the brain and mapped out proteins that are intimately linked to the different neurotransmitters or the transmitters themselves through use of different methods. The methods commonly used for this kind of research are ISH and different transgenic mouse models. With ISH the gene expression is localized, but before that the correlated protein function is observing by artificially altering the genome. To alter the gene of interest, the DNA sequence is cloned and a mutation is introduced, in the end
effecting the gene expression of this specific gene. A vector containing this altered DNA sequence is inserted into embryonic stem cell that are later screened for the positive clones, which will be injected into mouse embryos that are implanted into host mothers. The mutated offsprings are then bred to achieve the desired transgenic mouse model (Beglopoulos et al., 2004; Thomas et al., 1987). The effects of the altered gene can be observed with optogenetics and behaviour analysis (Pupe et al., 2015). This was just a brief description and there are many different systems to obtain transgenic mouse lines and models.

One of the systems for developing transgenic mouse lines is the Cre-Lox recombination. Developed during the 80-90th, the Cre-Lox recombination system enables conditional knock-out mice, restricting the mutation to either a specific cell type or tissue. The Cre recombinase, found in P1 bacteriophage, and its ability to carry out site specific recombination of Lox sites. Genes flanked by Lox sites can either be removed completely, deletion, or inverted by the Cre enzyme (Nagy 2000; Sternberg et al., 1981).

When studying voluntary movements and volitional behaviour, using the method described, there are a few proteins that are linked to different neurotransmitters, or are transmitters. Again focusing on the VTA and SN structure of the midbrain, and the DAergic neurons, GABAergic neurons and also subpopulations of glutamatergic neurons (Dobi et al., 2010; Nair-Roberts et al., 2008). Vesicular glutamate transporter 2 (VGLUT2) mediates the uptake of glutamate in synaptic vesicles, enabling the transportation of the neurotransmitter to the cliff between the axonal terminal and dendrite. The gene encoding VGLUT2 is therefore used as a marker for glutamatergic neurons (Herzog et al., 2006; Nordenankar et al., 2015; Takamori et al., 2001). Tyrosine hydroxylase (TH) is an enzyme that works as a catalyst when producing L-DOPA from tyrosine, and L-DOPA is in turn a precursor for DA (Kaufman 1995). This makes TH a marker for DA, and subsequently a marker for the VTA due to the structure having high production of DA (Daubner et al., 2011; Swanson 1982). A little more general marker for DA is the DA transporter (DAT), which carries the DA molecule across the plasma membrane of nerve terminals effectively completing the neurotransmission (Chen et al., 2000). An even more general marker is the vesicular
monoamine transporter 2 (VMAT2) and like previous markers it can be used to detect DA, but it also detects noradrenaline, adrenaline and other monoamine molecules (Liu et al., 1997; Lohr et al., 2014).

A recent gene-screening have identified a few new possible markers for the VTA and SN areas. Among these were the neuronal differentiation-6 (ND6), gastrin-releasing peptide (GRP), G-protein-gated, inwardly rectifying potassium channel (GIRK2), Follistatin (FST) and Lipoprotein lipase (LPL). Gene expression from Grp, Lpl, Nd6 and Fst was restricted to different parts of the VTA, while Girk2 mRNA was detected in both the VTA and SNc, more laterally than medially but not limited (Viereckel et al., 2016).

The project was divided into two different methods and goals. The first aim of the project was to optimize the PCR protocol for transgenic mouse lines Dat-Cre, Vglut2-Lox, Vglut2-Cre and Vmat2-Lox. The protocols have already been implemented in earlier studies and are routine, therefore this is not an optimization from scratch, where e.g. primers are designed. In addition to the optimization, two different polymerase were evaluated – KAPA and Maxima Hot Start. The second aim was to combine an ISH protocol with an IHC protocol to co-detect mRNA and the correlated protein, performed with markers for Nd6 and Grp. To get to know the method a double ISH was first performed with newly synthetized riboprobes for genes Girk2, Fst and Lpl. All markers used were recently identified as possible markers for the VTA and SN areas (Viereckel et al., 2016).

To optimize and validate the routine methods and protocols used in a lab is a crucial part of maintaining a good laboratory practice. This should be done at a regularly basis, but also when the need arises. The lab where this project took place were having problems with theses specific protocols and therefore wanted them optimized and validated.
Methods

Animals
The animals used during this project were provided by the Department of Comparative Physiology at EBC. Uppsala animal ethics committee approved the department’s application, the reference numbers are as followed – C156/14, C158/15 and C138/15. The housing of all animals was according to both Swedish regulation and EU legislation.

The optimization of PCR protocols for the transgenic mouse lines were performed on biopsies from test subjects in ongoing studies, so the number and transgene genotype varied from trial to trial. For the second project only tissue from a C57BL/6 mouse was used.

DNA extraction
The genotype of each animal was confirmed with biopsies from the ears, taken at the same time as the weaning of the mice at 3-4 weeks of age. With a lysis buffer diluted to a 1X solution (prepared in 10X solution: 250 mM NaOH, 2 mM EDTA) the DNA was extracted while incubated on a heat-shaker at 96°C for 30-40 min. To neutralize the lysis buffer, a neutralization buffer diluted to a 1X solution (prepared in 10X solution: 400 mM Tris-HCl pH 8.0) was later added. The ratio between the buffers was 1:1 and the extracted DNA sample had an average concentration of 100 ng/μL. The extractions buffers were stored on the benches, next to the heat-shaker.

PCR genotyping
All master mixes, or original reagent recipes were pipetted in the following order: water, MgCl₂ together or separately with the reaction buffer, dNTPs, primers (forward and reverse) and last the polymerase was added. All the reagents concentrations were calculated for X1 PCR reaction and then multiplied with the numbers of samples. Prepared on ice 24 μL were pipetted in each PCR tube together with 1 μL DNA sample, adding up to a total reaction volume at 25 μL. For all the recipes the water volume was adjusted accordingly to maintain the total reaction volume at 25 μL.
Original reagent recipe for the Dat-Cre PCR was pipetted in the following order and concentration when using KAPA Taq (KAPA Biosystems, #KE1000): 1.5 mM MgCl$_2$ at 1X (KAPA Biosystems, #KB1003), 0.2 mM dNTPs, 0.04 µM fw and rev primer (Dat-Cre fw: 5´-AGG AGT GAT GAG GTT CGC AGG A-3´ [Tm 60.3°C], rev: 5´-ACC GAC CAT GAA GCA TGT TTA G-3´ [Tm 58.4°C]) and 0.03 units/µL polymerase KAPA Taq. The same concentration dNTPs were used when using Maxima Hot Start Taq DNA Polymerase (Thermo Scientific, #EP0602) for the Dat-Cre PCR: 2.0 mM MgCl$_2$, 1X Hot Start PCR 10X buffer (Thermo Scientific #EP0602), 0.2 µM fw and rev primer and 0.036 units/µL polymerase Maxima Hot Start. Original PCR protocol initialization temp at 95°C for 4 min, denaturation temp at 95°C for 30 s, annealing temp at 55°C for 30 s, elongation temp at 72°C for 40 s, final elongation temp at 72°C for 6 min and an extra step at temp 20°C for 20s. Cycles of the denaturation, annealing and elongation were repeated 30 times.

The original reagent recipe for the Vglut2-Lox PCR differed in primer and polymerase concentration when using the KAPA polymerase: 0.2 µM fw and rev primer (Vglut2-Lox fw: 5´-CAG GCA AAA TCT GTC CAC CT-3´ [Tm 57.3°C], rev: 5´-AGG GTA GGC CAA AAG CAA TC-3´ [Tm 57.3°C]). Same Maxima Hot Start recipe, but of course using the Vglut2-Lox primers. The original PCR protocol started with the same initializations and denaturation step as the Dat-Cre PCR, but had a higher annealing temp at 58°C for 30 s. The elongation step was again the same as for the Dat-Cre PCR, but the Vglut2-Lox had a shorter final elongation step at 5 min and an extra step at temp 25°C for 20s. The same cycle repetition was used.

For the Vglut2-Cre PCR the original reagent recipe with KAPA only differed with the polymerase concentration used, 0.02 units/µL, and the primers (Vglut2-Cre fw: 5´-TTG CAT CGC ATT GTC TGA GTA G-3´ [Tm 58.4°C], rev: 5´-TTC CCA CAC AGG ATA CAG ACT CC-3´ [Tm 60.6°C]). No changes to the Maxima Hot Start recipe, but of course now using the Vglut2-Cre primers. The original PCR protocol had the same initialization step as before, but the denaturation temp at 94°C for 20 s and the same annealing step as the Vglut2-Lox PCR protocol. A shorter elongation step at 1 min and final elongation temp at 72°C for 10 min. The cycles of denaturation, annealing and elongation were repeated 32 times.
For *Vmat2-Lox* original reagent recipe when using KAPA a higher concentration primers where used, 0.4 µM fw and rev primer (*Vmat2-Lox* fw: 5´-GAC TCA GGG CAG CAC AAA TCT CC-3´ [Tm 64.2°C], rev: 5´-GAA ACA TGA AGG ACA ACT GGG ACC C-3´ [Tm 62.7°C]). Also a higher polymerase concentration, 0.03 units/µL polymerase. Same Maxima Hot Start recipe, but again using the higher concentration of the *Vmat2-Lox* primers at 0.4 µM. The original PCR protocol differed completely from the other protocols. Initialization temp at 94°C for 4 min, denaturation temp at 94°C for 1 min, annealing temp at 62°C for 1 min, elongation temp at 72°C for 1 min – this initial cycle was just performed one time. The duration of the steps were cut in half to 30 s and then repeated 29 times. Final elongation temp at 72°C for 3 min.

The dNTPs reagent used had a concentration of 10 mM and all primers were bought from Eurofins Genomics, custom made for each transgenic mouse line. The primers were diluted 1:10 from stock to an end-concentration at 10 µM. The PCR machines used in this project were both S1000™ Thermal Cycler (Bio-Rad).

**Detection of PCR products**

Agarose gels were used with GelRed™ nucleic acid stain (Biotium) to detect the PCR product, and with a 100 bp ladder to confirm the different sizes of the bands. The *Vglut2-Cre* PCR product were larger in size (~700 bp) than the other transgenic mouse lines (~50-120 bp), so a 1 kb ladder should be used instead of the 100 bp ladder. For transgenic Cre mice the products could either run through a 1 % agarose gel for 30-40 min at 130-140 V, or a 2 % agarose gel for 50-60 min at 100 V. There is only a small difference in size between the heterozygote bands in the Lox transgenic mice line, so an agarose gel with a higher density was used to separate the 2 bands. So to properly separate the heterozygote bands for the transgenic Lox mice a 2 % agarose gel had to be used as descried. All gels were photographed with C200 (Azure Biosystems) gel imaging workstation and accompanying cSeries Capture Software. The photographs were later processed with ImageJ software.
Co-detecting mRNA and protein

Harvesting and fixation of brain tissue from the C57BL/6 mouse was done beforehand by authorized personnel and according to the lab's routine for fresh frozen tissues. All brain tissue used in this project were harvested after the mark of adulthood at 10 weeks. The brain tissue was sliced with a cryostat CM1950 (Leica) in sections of 16 µm and placed on superfrost plus® (Thermo Scientific) slides. The placement of tissue section on the slides were done according to routine, in series of 8 slides where one slide contained 10-12 tissue sections. One brain was divided onto approximately 40-48 slides and set to air dry briefly before being stored in a -20C freezer.

ISH protocol

The ISH was based on `non-radioactive in situ hybridization´ protocol found in the Viereckel (2016) article. Riboprobes (mRNA probes) labelled with digoxingenin (DIG) or fluorescein were already synthesised and aliquoted for each gene – Th, Grp, Nd6, Girk2, Fst and Lpl (Viereckel et al., 2016).

Slides with tissue sections were hybridized for 16-18 h at +65C coated with 1 µg/mL denaturated riboprobe: DIG and/or fluorescein-labelled. DIG riboprobe pared with horseradish peroxidase (HRP)-conjugated anti-DIG antibody (Roche 1120773910) at 1:1000 emits a red fluorescent signal when revealed with TSA™ Kit (Perkin Elmer) using Cyanine 3 (Invitrogen, excitation/emission maxima 554-568 nm) tyramide at 1:150. Fluorescent detected the tissue sections when incubated with HRP conjugated anti-fluorescein antibody (Roche 11426346910) at 1:1000. Also revealed with TSA™ Kit, but using Biotin-tyramide at 1:75 and Neutravidin Oregon Green conjugate at 1:500, emitting a green fluorescent signal.

Double ISH protocol

If two genes were to be detected on the same tissue, double ISH, both types of riboprobes were needed. The fluorescein-labelled riboprobe was first detected with its primary antibody. The HRP activity was stopped with 0.1 M glycine and then 3 % H2O2 before detecting the DIG riboprobe. The background structures were stained last
with DAPI diluted at 1:50 000 in PBST and then the cover slip was mounted with fluoromount.

**Combined protocol**

When combining the ISH and IHC protocols, the ISH was performed according to the "non-radioactive in situ hybridization" protocol, up to but not including the inhibition of HRP with glycine and H₂O₂. This was because the IHC protocol starts off with an inhibition with permeabilization solution (BL) (5% donkey serum, 0.1% TX-100, 0.05% Tween in PBS). Slides were washed in PBS and then incubate overnight at +4°C with primary antibody diluted in BL.

Proteins detected with IHC were GRP, ND6 and TH as control. Primary antibody were as followed: rabbit (rb)-Grp (abcam ab22623) and rb-Nd6 (abcam ab85824) at 1:1000, mouse (m)-Th (Millipore #MAB318) at 1:400. Wash 3x 15 min in PBS and incubate secondary antibody at 1:1000 for 1.5 h in room temperature. Secondary antibodies were as followed: donkey (dk)-anti-rb conjugated with fluorescent Alexa Fluor 488 (Invitrogen, excitation/emission maxima 490-525 nm) and dk-anti-m conjugated with Cyanine 3. Wash 3x 15 min in PBS before mounting cover slip with fluoromount and set to dry overnight.

**Fluorescent microscopy**

The slides was photographed with Leica DM5500 B (Leica Microsystems CMS) microscope and the accompanying software Leica Application Suite Advanced Fluorescence (2.3.5 build 5379, Leica Microsystems CMS). Figures were made with GIMP (GNU Image Manipulation Program). The book *The mouse brain in stereotaxic coordinates* (Franklin and Paxinos, 4th edition 2013) and Allen Brain Atlas¹ (website) was used to navigate in the brain tissues.

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Results

First aim: Optimization of PCR protocols

The first aim of the project was to optimize the protocols for the transgenic mouse lines, while at the same time evaluate the two polymerases – KAPA and Maxima hs. The protocols were performed in order to establish the genotype of the specific transgenic mouse line, but for the four protocols in this project – Dat-Cre, Vglut2-Lox, Vglut2-Cre and Vmat2-Lox – there had been recurring problems with consistency.

Starting out with the original temperatures and concentrations, the optimizations were performed according to general ‘troubleshooting’ guides provided by the polymerase manufactures. If the bands were not up to the usual standard with clear and bright bands, but instead were diffuse, the wrong size or even absent, a re-run was performed with a suitable modification.

Dat-Cre

We started out by testing the original PCR protocol with the different polymerases to see which yielded the clearest bands. After a few trials we concluded that the bands were significantly clearer with the Maxima hs polymerase and at a lower concentration (fig. 1, C). Not the same could be observed with the KAPA polymerase, we the bands often were diffuse and weak (fig. 1, A). No other modifications were deemed needed beyond the use of Maxima hs at a lower concentration.

‘Shadow bands’ or false positives were observed frequently just above were the positive bands should have been located (fig. 1, B). By running the agarose gel on a lower voltage and for a longer time helped reduce these false positives. Because of this residue, after a few weeks a new and more extensive cleaning schedule was implemented.
Figure 1. PCR products from Dot-Cre (80-90 bp) animals, all on 1% agarose gels with a 100 bp ladder. Original PCR protocol performed with polymerase KAPA on gel A and gel B, but with different samples – to illustrate the inconsistency of the protocol and the shadow bands seen on gel B. PCR products on gel C were performed with Maxima hs polymerase, but the enzyme has been titrated down from 0.036 units/µL to 0.03 units/µL. The numbers seen in the pictures under each sample is the animals tag number.

Vglut2-Lox

We observed the same results when evaluating the polymerases for the Vglut2-Lox PCR. That the polymerase Maxima hs yielded brighter and clearer bands (fig. 2), while the KAPA polymerase generated a low to almost no amplification (fig. 2). This was a recurrence with the KAPA polymerase, also observed with transgenic mouse line Dot-Cre (fig. 1, A) and Vmat2-Lox (fig. 5).

To obtain more defined heterozygous bands further modification was performed. We found that reducing the annealing temp from +58C to +57C helped yield a brighter band (fig. 3, A). Increasing the MgCl₂ concentration had the same effect (fig. 3, B). By increasing the concentration from 2.0 mM to 2.5 mM the band appeared brighter. The polymerase concentration was also evaluated and we reduce the polymerase concentration two times. First from the original 0.036 units/µL to 0.03 units/µL and then to 0.02 units/µL, which caused the larger heterozygous band to disappear (fig. 3).
Figure 2. PCR products from Vglut2-Lox (50-90 bp) animals, all on 2 % agarose gels with a 100 bp ladder. Comparing the polymerases KAPA (left) and Maxima hs (right) on the gel. The concentrations used were 0.03 units/µL for KAPA and 0.036 units/µL for Maxima hs. The numbers seen in the pictures under each sample is the animals tag number.

Figure 3. PCR products from Vglut2-Lox (50-90 bp) animals, all on 2 % agarose gels with ladder for 100 bp. Testing different annealing temp (A) and MgCl₂ concentration (B) on the same samples, with Maxima hs polymerase. The polymerase had been titrated down from the original recipes 0.036 units/µL to 0.02 units/µL – no other alterations were performed to the original PCR protocol. The water volume was adjusted accordingly, so the total volume remained 25 µL. The numbers seen in the pictures under each sample is the animals tag number.

Vglut2-Cre

We used Maxima hs polymerase (fig. 4, A), due to the perceived irregularity of the KAPA polymerase when used with the other PCR protocols. When the Maxima hs yielded satisfactory bands it was deemed not necessary to test the KAPA polymerase.

Furthermore, the polymerase concentration was decreased from 0.036 units/µL to 0.03 units/µL (fig. 4, B), and still yielded detectible bands. Because the bands already
were detectible with the original protocol, no further modification was performed beyond the polymerase. Because the positive control did not work the first trial, a new one and the old ones were tested (fig. 4, A). The old controls did not yield any bands and were discarded, and the new control was used from here on.

Figure 4. PCR products from Vglut2-Cre (~700 bp) animals on 2 % agarose gel (A) and 1 % agarose gel (B), with a 100 bp ladder. Both geles were performed with polymerase Maxima hs. On gel A the original recipe was used and all old controls (2350, 2352, 2353, 2355, 2356), plus one new positive control (6783), were tested. For PCR products seen on gel B, the polymerase have been titrated down from 0.036 units/µL to 0.03 units/µL. The numbers seen in the pictures under each sample is the animals tag number.

Vmat2-lox
It was once again considered valuable to test the protocol with the KAPA polymerase, due to a faulty run with Maxima hs that did not yield any bands. We found that the KAPA polymerase yielded diffuse bands, which made it hard to make out the larger
band of the heterozygous band (fig. 5, A). When using Maxima hs again it was easier to distinguish the heterozygous band (fig. 5, B).

![Figure 5](image)

**Figure 5.** PCR products from *Vmat2-Lox* (100-120 bp) animals, all on 2% agarose gels with ladder for 100 bp. Original PCR protocol with the KAPA polymerase on gel A. Switching to Maxima hs polymerase on gel B, no alterations to the PCR protocol or recipe. The numbers seen in the pictures under each sample is the animals tag number.

**Second aim: ISH and IHC protocols**

The second aim was to try to combine the ISH protocol with a routine IHC protocol. To confirm that the tissue sections were in level with the midbrain, we used the Th-marker that detects the VTA and SN structures. All protocols were performed on tissue sections from a C57BL/6 mouse and a total of three protocols were performed; double ISH, ISH combined with IHC, and IHC alone.

**Only newly synthesized riboprobes for *Girk2* observed**

To get to know the method we performed a double ISH protocol with gene combinations *Girk2/Th, Fst/Th* and *Lpl/Th*. This allowed practice and confirmation of the newly synthesised riboprobes for genes *Girk2, Fst* and *Lpl*. We observed mRNA expression from the control gene *Th* in the VTA and SNc (fig. 6, B), confirming that
the sections were in level with the midbrain area. Of the newly synthesised riboprobes, Grik2 mRNA expression was observed giving a weak signal at 20x magnification (fig. 6, A). However, no mRNA expression from genes Fst or Lpl were detected on their respective slide and therefore no pictures were taken.

Figure 6. Genes Girk2 (A) and Th (B) detected with double ISH on coronal cross-sectioned midbrain tissue. DIG-labelled mRNA probe for Girk2 was detected with HRP-conjugated anti-DIG antibody. Emitting a red fluorescent signal when revealed with TSA™ Kit using Cyanine 3. Fluorescein labelled mRNA probe for Th gene was detected with HRP-conjugated anti-fluorescein. Emitting a green signal when revealed with TSA™ Kit using biotin-tyramide and a following incubation with Neutravidin Oregon Green. The Co-location of mRNA and protein is illustrated with an overlay that have been color balance treated (C).

The combined protocol worked to a certain extent

We obtained the following results when combining the ISH protocol with the IHC protocol. The combined protocol was performed with markers Nd6, Grp and control marker Th.

Control marker Th was detected with both ISH and IHC (fig. 7). The mRNA expression (fig. 7, A) and the corresponding protein (fig. 7, B) was observed in the VTA and SNC structures. This confirms that the tissue sections are in midbrain level.
Figure 7. Combined ISH and IHC protocol on coronal cross-sectioned midbrain tissue. ISH detected the Th gene (A) green with HRP conjugated anti-fluorescein antibody revealed with TSA™ Kit, using biotin-tyramide and a following incubation with Neutravidin Oregon Green. IHC detected the TH protein (B) with secondary donkey-anti-mouse antibody with conjugate Cyanine 3 emitting a red signal. Overview at 20x magnification.

We found that the combined protocol also worked for the Nd6 marker, observing the mRNA expression (fig. 8, A) and its protein (fig. 8, B). The mRNA expression of Nd6 was detected both intracellular and intranuclear in the pyramidal (Py) cells of hippocampus. However, the protein was only observed intranuclear in the same Py cells. This co-location is illustrated with yellow colour in the overlay (fig. 8, C).
Figure 8. Combined ISH and IHC protocol on coronal cross-sectioned midbrain tissue. ISH detected the \textit{Nd6} gene (A) with HRP conjugated anti-DIG antibody, emitting a red signal when revealed with TSA\textsuperscript{TM} Kit using Cyanine 3. IHC detected the ND6 protein (B) with secondary donkey-anti-rabbit antibody with conjugate Alexa fluor 488 emitting a green signal. The Co-location of mRNA and protein is illustrated with an overlay that have been color balance treated (C).

For the Grp marker we only observed the mRNA expression, in the area near the hippocampus (fig. 9, A and B). No fluorescent signal was detected for the correspondent GRP protein (fig. 9, C).

Because we did not observe any GRP protein with the combined protocol the IHC was performed again, but this time on its own. To see if it was the combination of the two protocols that caused the antibodies not to work or if they were compromised in some other way.
Figure 9. Combined ISH and IHC protocol on coronal cross-sectioned midbrain tissue. ISH detected the Grp gene (A) with HRP conjugated anti-DIG antibody, emitting a red signal when revealed with TSA™ Kit using Cyanine 3. A closer image of the Grp gene in cells stained with ISH (B). IHC detected the GRP protein (C) with secondary donkey-anti-rabbit antibody with conjugate Alexa fluor 488 emitting a green signal.

Successfully performed IHC protocol
We observed the Th control again in the VTA and SNc structures (fig. 10), once again confirming the level of the sections. The signal was weaker this time at 20x magnification, in comparison with the overview from the combined protocol (fig. 7, B).
Figure 10. IHC detected the ND6 protein with secondary donkey-anti-mouse antibody conjugated with Cyanine 3 emitting a red signal. The ND6 protein is first localized with primary mouse antibody. Overview at 20x magnification.

The IHC protocol successfully detected the Nd6 markers protein again. We observed the ND6 protein intranuclear in the Py cells of hippocampus (fig. 11). The protein was also detected intranuclear in the cells in the VTA of the midbrain (fig. 12).

Figure 11. IHC detected the ND6 protein with secondary donkey-anti-rabbit antibody conjugated with Alexa fluor 488 emitting a green signal. The ND6 protein is first localized with primary rabbit antibody. Overview of the Py cells of hippocampus (A1) and a close up on the cells (A2) in that same area.
Figure 12. IHC detected the ND6 protein with secondary donkey-anti-rabbit antibody conjugated with Alexa fluor 488 emitting a green signal. The ND6 protein is first localized with primary rabbit antibody. Overview of the VTA at 20x magnification (A1) and a close up at 40x (A2) in that same area.

The GRP protein was detected with the IHC protocol (fig. 13, A1) and we observed a weak signal from two structures in the midbrain. Both in the red nucleus (RN) (fig. 13, A2 and A3) and in the nucleus of Darkschewitsch (Dk) (fig. 13, A1).

Figure 13. IHC detected the GRP protein green with secondary donkey-anti-rabbit antibody conjugated with Alexa fluor 488 emitting a green signal. The GRP protein is first localized with primary rabbit antibody. Overview of the whole tissue at 5x (A1), close up at 20x (A2) and at 40x (A3).
Discussion

The first part of the project was to optimize the PCR protocols for transgenic mouse lines Dat-Cre, Vglut2-Lox, Vglut2-Cre and Vmat2-Lox. Two different polymerases were also evaluated, KAPA and Maxima hs, to see which was most suitable for all the protocols.

No alterations were needed for the original Vglut2-Cre and Dat-Cre PCR protocols, the different temperatures and concentrations yielded satisfactory results with a clear positive or negative band (fig. 1 and 4). There was however a recurring problem when running the Dat-Cre PCR. Frequently `shadow bands´, a slightly larger band, could be seen just above were the positive bands should have been located (fig. 1, B). This could lead to the bands being interpreted as positives, false positives, which could result in that the wrong genotype being used in very specific projects and this would inevitable then compromise the obtained data. First we thought the `shadow bands´ were a residual product of the KAPA polymerase. But the bands were also present when using polymerase Maxima hs, although not as regular and this is also why the Maxima hs polymerase was preferred.

If the unsatisfactory bands was not a residual product from the polymerase, we suspected that maybe the extraction buffers could have been contaminated, so a new cleaning schedule was therefore implemented and the extraction buffers were replaced more often. Before the new schedule the extraction buffers (10X stocks and 1X dilutions) were stored on the benches near the heat-shaker where the extraction took place. After the implemented schedule, the 10X stocks were aliquoted and stored in the -20C freezer until use, and then diluted to a 1X solution just before use. The new schedule also covered weekly cleaning of the different working areas; cleaning the benches with chlorine, UV treat the PCR hood, wipe down the PCR machines, clean the gel electrophoresis tanks and change the TAE buffer. Unfortunately, the `shadow bands´ did not disappear after the new implemented cleaning schedule.

With this information and earlier findings the next step were to explore the transgenic combination; Dat-Cre and Vglut2-Lox. Because when looking back at the samples that have had these `shadow bands´, it was exclusively this transgenic combination. This is only so far and in this specific optimization. Anyhow, maybe the primers for Dat-
*Cre* are able to amplify some other gene and needs to be re-designed so they are more specific. Just briefly researching other studies were *Dat-Cre* mice are used there is a number of different primers that could be tested (Parlato *et al.*, 2006; Vuong H.E. *et al.*, 2015). Just keep in mind that these primers design are most likely based on how the gene targeting was performed. However, letting the bands run longer on the gel, on a lower voltage, helped to distinguish true positive bands from these false positive `shadows bands`.

The *Vglut2-Lox* protocol underwent the most modifications: Comparison of polymerase, polymerase concentration correction and a modified annealing temp as well as MgCl$_2$ concentration. The *Vglut2-Lox* PCR protocol was optimized by decreasing the annealing temp to 57°C and increasing the MgCl$_2$ to 2.5 mM/µL, yielding brighter bands.

When comparing the polymerases a clear winner was crowned – Maxima hs. Although, both polymerases worked when running the *Vglut2-Cre* PCR, Maxima hs was preferred for all other transgenic mouse lines. The KAPA polymerase have also doubled in price since it was sold to another company, making it less cost-effective, and therefore the optimizations was focused on the use of Maxima polymerase. It was also found that a lower polymerase concentration could be used in all PCR protocols and still yield detectible bands. Using a lower concentration means using a smaller volume and this will in the end save the lab money.

Originally the *Vmat2-Lox* PCR protocol were also planned to be optimized and all the optimizations would be validated, but due to a limited time frame this was not possible.

For the second part of the project – confirming newly synthetized riboprobes and testing a combined ISH and IHC protocol. The DA specific TH marker detected the VTA and SN structures for all protocols performed; double ISH (fig. 6), combined protocol (fig. 7), and IHC alone (fig. 10). This visualization of the VTA and SNC confirms that we are in level with the midbrain. It also means that the protocols should in theory work for the other markers. However, the *Fst* and *Lpl* were not detected with the double ISH protocol. This could indicate that something was wrong with the new synthesized riboprobes, but most likely a higher concentration is needed for a stronger
signal. This theory is strengthened by the weak signal of Girk2 that was detected, only visual at 20x magnification (fig. 6, A). Thus, there’s a possibility that Fst and Lpl also had a signal, but that it was weaker than the Girk2 and was therefore overlooked. The Girk2 gene was detected in the VTA and SNc which is consistent with the observations made in the recent gene-screening (Viereckel et al., 2016) and earlier studies (Reyes et al., 2012).

The results obtained when testing the combined ISH and IHC protocol was overall hopeful. The gene expression and correspondent protein for the Nd6 marker was detected with the combined protocol, except not in the midbrain area. The co-location was observed intranuclear in the Py cells of hippocampus, which do comply with references pictures from Allen Brain Atlas (website) but not with what we were looking for. The Grp gene expression was detected, but not the marker for the corresponding protein. This was unfortunate and to exclude the possibility that the antibodies were somehow compromised the IHC protocol was performed alone.

With the IHC protocol the ND6 protein was detected again in the Py cells of the hippocampus, but also in the VTA area and again verifying the findings from the gene-screening (Viereckel et al., 2016). The GRP protein was detected this time around and observed in the RN and Dk in the midbrain, freeing the antibodies from suspicion. The detection is backed by similar observations made in reference pictures from Allen Brain Atlas (website). One could argue that there would have been detection in the VTA, consistent with the gene-screenings observations (Viereckel et al., 2016), if the tissue section had not been totally butchered in that specific area.

These genes were used because of the observations found in the gene-screening (Viereckel et al., 2016), but also because of the potential usage as markers when diagnosing neurodegenerative diseases such as PD (Chung et al., 2005; Duda et al., 2016).

In summary, three of four PCR protocols were optimized and the Vglut2-Lox protocol underwent the most modifications. Furthermore, for all the PCR protocols a lower concentration polymerase was considered sufficient to ensure that the bands were stable and after evaluating the two polymerases Maxima Hot Start was considered to be the most cost effective and stable. Although there was no time to
validate the modifications properly, the new protocols were produced by gradually changing the original protocol and was therefore performed several times. For the second part of the project the main finding was that the combined protocol worked to a certain extent, but there is room to test other concentrations for the antibodies and incubation times. Again, there was a too limited timeframe to run more test.
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References


