UPTEC X 09 007
Examensarbete 30 hp
Februari 2009

Optimization and development of the Padlock probe technique using in situ detection of nucleic acids

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UPTEC X 09 007	**Date of issue 2009-02**

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Title (English)

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Title (Swedish)

Abstract

In this project I have optimized and developed the *in situ* Padlock probe technique for localized RNA and DNA detection. The technique uses a circularizable oligonucleotide to template rolling circle amplification (RCA) product detected with fluorophore-labelled detection probes. The technique is efficient in single-cell analyses for genotyping single nucleotide polymorphisms (SNPs) and will hopefully be a future diagnostic tool improving medical treatment.

Keywords

Padlock probe, Rolling Circle Amplification, localized detection, Single Nucleotide Polymorphisms, Single-cell analyses

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Project name	Sponsors
Language **English**	Security **Secret until 2009-12-31**
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **28**

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Sammanfattning

Alla organismer är uppbyggda av celler som innehåller den information (arvsmassa) som krävs för överlevnad. Eftersom alla individer är olika har det utvecklats tekniker som används för att hitta skillnader i arvsmassan mellan och inom individer. Eftersom många sjukdomar uppstår från en cell, t.ex. cancer, är det viktigt att kunna jämföra sjuka celler mot normala celler inom en individ. Jämförelsen kan leda till att den rätta diagnosen ställs vilket förhoppningsvis kan göra behandlingen mer specifik och kostnadseffektiv.

Skillnaderna i arvsmassan kan vara så små att det bara är en molekyl som skiljs åt i en enda cell. Det krävs då att de tekniker som används kan analysera enskilda molekyler i enskilda celler, ett problem för dagens tekniker.

Viljan att förstå varje enskild cells arvsmassa och därmed dess funktioner har lett till utvecklandet av en ny teknik kallad Padlock probe (hänglåsprob). Padlock probe tekniken är effektiv då den kan hitta skillnader på molekylnivå i arvsmassan på både odlade celler och i vävnadssnitt. Tekniken förlänger arvsmassan med en specifik sekvens lokalt på platsen där skillnaden har hittats. Skillnaderna i arvsmassan kan då efter behandling ses som små fluorescerande signaler med hjälp av ett fluorescensmikroskop.

I detta projekt har jag fortsatt utvecklingen av Padlock probe tekniken med optimeringar av teknikens olika steg i celler och i vävnad med avseende på ökad specificitet och effektivitet.

Examensarbete 30 hp

Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet februari 2009

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1. Introduction

1.1 Background

For many years, scientists have used techniques like Polymerase Chain Reaction, PCR, in their genetic research. PCR is used to amplify an exact fragment of DNA from a starting sample. It is a very fast and highly sensitive technique where only a few molecules of DNA are needed for correct amplification [1]. The analysed samples in PCR are often populations of different cells, e.g. blood, tumours or normal tissue; obtaining only an overall image of all molecules present in the sample. The mix of DNA in the sample will sometimes not provide a good representation of material from the cells you want to analyze and in these cases to fully understand a multicellular organism the detection of DNA will have to be at the base level of single cells.

Single cell analyses are sometimes important for correct diagnosis. Finding differences between cells that would otherwise go undetected could potentially lead to better treatment and understanding of many unknown as well as known diseases in today's society. Single cell analyses can be performed on both cell culture samples as well as on tissue, which is a great advantage for tumour research. Single cell analysis can not only be used for detecting differences in genomes or for expression analysis between cells but can also be used to localize the detection of molecules in the cell.

PCR is also a method highly used in genotyping of individual viruses, bacteria or humans. By genotyping one can find differences, such as polymorphisms, in the genome between different individuals as well as between cells within one individual. Polymorphisms are allelic differences in genes. They can be large or small down to single nucleotide polymorphisms, SNP [2]. Even though SNPs only differ in one nucleotide between alleles they can cause changes in amino acids and therefore produce a protein with different structure and function.

PCR, however, does not, as mentioned before, have the potential of localized detection; it can therefore not find differences between cells in the same sample. Developing new ways of detecting SNPs, using single cell analyses, may lead to better diagnostic tools, improving today's medical care.

The idea of localized detection being a new diagnostic tool in molecular medicine led to the development of a technique called Padlock probe, invented by Mats Nilsson and Ulf Landegren. The Padlock probe technique was originally designed for experiments *in vitro* but was early on believed to be a good candidate for *in situ* experiments [3]. The Padlock probe technique takes the best of PCR and transfers it *in situ* for single cell analysis for detection of both DNA and RNA. Padlock probes can be combined with different amplification methods to analyze DNA, but in contrast to PCR the technique uses a different approach for probing the DNA that results in higher specificity and sensitivity.

In PCR two separate primers are added to the template DNA in order to start amplification. If badly designed primers are used they may be self-complementary or hybridize to each other leaving no or small amounts left for amplification of target DNA. The padlock probe technique has diminished that problem by using a 70 to 90 nucleotides (nt) long oligonucleotide with target-complementary ends as primers. The primer-ends on the padlock probe are designed to hybridize 5' to 3' forming a circle around the target DNA. The fact that it is a linear oligonucleotide prevents hybridization of the primer ends within one probe and those probes that may hybridize to each other will be easily distinguished from the circularized probes and washed away not disturbing the reaction [4]. By designing the primer-ends of the padlock probe, so that two padlock probes differ only in one nucleotide located at the 3'-end, makes this technique excellent in genotyping together with ligase.

1.2 Theory of the in situ Padlock probe technique

Padlock probes can be applied on both cell and tissue samples detecting both DNA and RNA. The procedure of the technique differs slightly between these applications but they all have the main goal of finding differences within or between cells. First of all the cell or tissue sample needs to be fixed to stop all processes in the cell and get a snapshot of the cell's ongoing processes at moment of fixation. The steps of the two procedures can be seen in Figure 1.

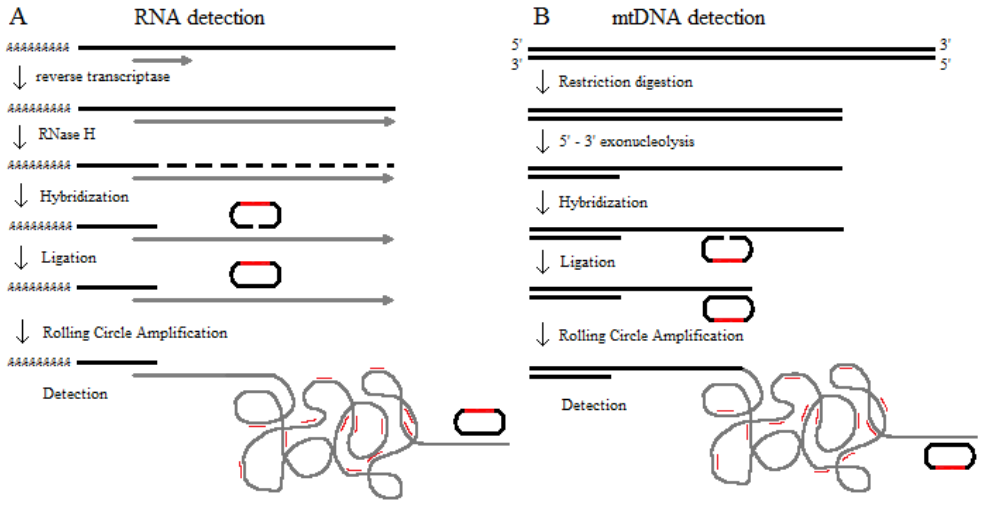

Figure 1. A schematic representation of the reaction steps for RNA and mtDNA detection with padlock probes *in situ*. A. The mRNA is reversly transcribed to cDNA. After RNA degradation by RNase H the padlock probe hybridizes. After ligation of padlock probes a rolling circle product is formed by RCA and detected with fluorophore-labelled detection probes complementary to the RCA product. B. The mtDNA is restriction digested on the 3'-site of the target sequence and made single-stranded by 5'-3' exonucleolysis. The padlock probe is hybridized and ligated to the targets followed by RCA. The RCA product is detected with fluorescent detection probes in the same way as for RNA detection.

1.2.1 Pre-treatment of cells and tissue

To get access to the nucleic acid in the cells, the samples have to undergo a pre-treatment. Treating the cells or tissue with HCl is enough for detecting RNA. However, DNA and in this project mitochondrial DNA, mtDNA, lays within yet two other membranes surrounding the mitochondria and treating the cells with pepsin is therefore necessary for reagents to reach the DNA. Pepsin is a digestive protease active only in very acidic environment; it is therefore used together with HCl in the pre-treatment step.

1.2.2 Getting access to the region of interest

The region of interest within a DNA or RNA molecule needs further treatment in order for the padlock probe to be able to hybridize. For DNA detection the double-stranded DNA is digested with a restriction enzyme just downstream of the target DNA and made single-stranded by 5'-3' exonucleolysis [5]. For RNA detection the mRNA molecule is reversely transcribed to complementary DNA (cDNA) and made single-stranded by RNase H activity. The Padlock probe technique has been shown to work directly on RNA

with ligation of padlock probe by T4 DNA Ligase [6]. However, since the padlock probe is a DNA oligonucleotide, the ligation to the RNA is more unspecific than the ligation to a cDNA molecule [7]. This leads to much slower reaction that requires more enzymes. This is why the way through cDNA is performed.

1.2.3 Hybridization and ligation of padlock probe

The padlock probe hybridizes in an end to tail conformation on the target molecule, with 15 to 20 nt on each end segment. When purchased, the synthesized padlock probe is lacking the phosphate group on the 5'-end. Phosphorylation of the probe prior to hybridization is therefore necessary in order for ligation to occur. Following hybridization to the target DNA a DNA ligase covalently joins the two end segments and closes the padlock probe forming a circle [3].

For most efficient ligation the DNA ligase requires perfect hybridization of the padlock probe arms, which makes the padlock probe technique very suitable for genotyping. When using padlock probes in genotyping experiments the two padlock probes are designed to differ in one base located at the 3' end, as well as to have two different hybridization sites for detection probes encoded in the non-target-complementary region of the probe.

1.2.4 Rolling Circle Amplification, RCA

When detecting targets *in situ* the ligased padlock probe locally amplifies by turning into the amplification template for a DNA polymerase and the target molecule functions as the primer for polymerization. In this way, the target DNA is extended with an RCA product containing hundreds of tandem-repeated copies of the padlock probe [8].

The polymerase mostly used in RCA comes from *Bacillus subtilis* phage Φ29. The Φ29 DNA polymerase has the highest processivity and strand displacement activity among known DNA polymerases as it can synthesize DNA chains greater than 70 000 bp per hour [9].

In DNA detection, and in RNA detection with a padlock hybridization site near the 3'-end of the cDNA, the Φ29 DNA polymerase uses its 3' exonucleolytic activity to remove any protruding 3' end downstream of the padlock probe hybridization site [5]. When the Φ29 DNA polymerase reaches the hybridization site of the padlock probe the target DNA starts functioning as the primer for RCA. If, however, a padlock probe is designed to hybridize further away from the 3'-end of the cDNA molecule than the polymerase has the ability to remove by exonucleolysis, another way of removing the protruding 3'-end is necessary. MutY is an enzyme that removes the adenine from a G:A mismatch leaving an apurinic/apyrimidinic (AP) site (Figure 2).

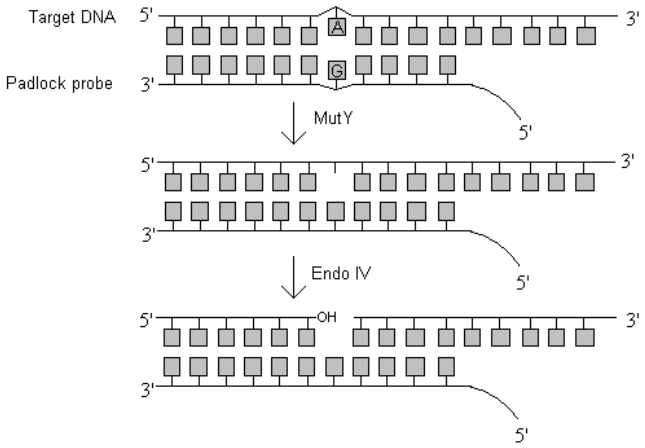

Figure 2. MutY removes the adenine from a mismatched G:A pair forming an AP site. The Endo IV then acts as an AP endonuclease and cleaves the DNA backbone.

The AP endonuclease Endo IV, found in *E. coli*, recognizes the AP site and catalyzes cleavage of the DNA backbone leaving a 5'-deoxyribose and a 3'-hydroxyl end product [10]. The cleavage activity of MutY and Endo IV has been tested on padlock probes ligated to synthetic DNA and mtDNA molecule [Howell In progress]. The mismatch is consciously designed by changing a tymidine base to a guanine base in the padlock probe oligonucleotide. The location of the mismatch must be close to the end of the hybridization site between target DNA and the padlock probe to allow the 3' hydroxyl end to be recognized by the DNA polymerase in order for RCA to start.

1.2.5 Detection of RCA products

The two end segments on the padlock probe are connected by a target-non-complementary segment [11]. This connecting bridge is designed to contain a site where a detection probe can hybridize. The detection probe in turn is tagged with a fluorescent marker making detection easy in a fluorescence microscope. The RCA product, about 70 000 bp after one hour, corresponds to about 1000 copies of a padlock probe meaning that there are 1000 sites where the detection probe can hybridize. The RCA product randomly collapses into a coil of DNA making the detection visible as a single bright object of about 1 µm in diameter [12].

1.3 Problem statement and aim of the project

The padlock probe technique has been in use for several years in numerous applications. Each new modification e.g. new padlock probes, primers, detection probes, cells, tissue etc. as well as changes in a present protocol may require new settings leading to optimization and validation of the technique. The aim of this project was to optimize and develop new methods for present as well as new applications.

1.3.1 Experimental set up

Mitochondria are found in the cytoplasm of eukaryotic cells. They have their own genome that mutates at a very high rate compared to nuclear DNA. Mitochondria are

strictly inherited on the maternal side incorporating hundreds of copies per cell. Because of the high copy number and the mutation rate the cells can be heteroplasmic containing both mutant and wild-type mtDNAs [13]. Because of the heteroplasmic conditions of the human cells the mitochondria makes a good choice for genotyping experiments. The padlock probes for mtDNA detection in human cells were designed to genotype a mutation found in MELAS syndrome [5] (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), whereas the padlock probes for mtDNA detection in mouse tissue were designed to genotype a mutation in the cytochrome b gene in the mitochondrial genome.

For RNA detection a so called housekeeping gene is used that is assumed to be expressed constitutively from cell to cell and sample to sample [14]. In these experiments one of the most widely used housekeeping genes, β-Actin is used. Padlock probes for RNA detection were designed to hybridize at different sites of the cDNA (Appendix 1). For two sets of designed padlock probes a single nucleotide variant, was located at the 3'-end hybridizing either to human or mouse β-Actin.

2. Materials and Methods

2.1 General

For RNA detection H_2O and PBS were DEPC treated for RNase degradation and DEPC H_2O was used in all dilutions. Washes between incubations were performed in Wash buffer 0.1 M Tris-HCl, 0.15 M NaCl and 0.05% Tween 20 for DNA detection and in 0.05% Tween 20 in 1 x PBS for RNA detection. Reactions were incubated either under cover slips (Menzel-Gläser), lifter slips (Erie Scientific Company) or in secure-seal hybridization chambers (Invitrogen). The secure-seals are silicone chambers with one adhesive side which is taped onto the microscope slide for entire protocol. Preincubation in 1 x buffer were performed for all secure-seal steps. All padlock probes, cDNA primers and detection probes can be found in Appendix 1.

2.2 Cell culture and tissue sample preparation

The human fibroblast cell line 143B and the mouse embryonic fibroblast cell line (MEF) were cultured in 1 x Dulbecco's Modified Eagles medium (D-MEM) without phenol red and L-glutamine (11880, Gibco) with addition of 10% fetal bovine serum, 292 µg/ml L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin plus addition of 50 µg/ml uridine for 143B. The human fibroblast cell line 8402 was cultured in 1 x Minimum Essential Medium (MEM) without phenol red and L-glutamine (51200, Gibco) with addition of 10% fetal bovine serum, 292 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 x non-essential amino acids. Prior to experiments the cells were trypsinated in 0.25% trypsin, 10% EDTA in 1 x PBS and seeded on microscopic glass slides (Menzel-Gläser). The glass slides used were Frosted slides for mtDNA detection and Superfrost plus for RNA detection on cell samples. The cells were fixed for DNA detection in 70% EtOH for 20 min in RT and stored in 70% EtOH at 4ºC until use and for RNA detection in 3% formaldehyde in PBS for 30 min in RT followed by washes in 1 x DEPC-PBS and stored if necessary in 70% EtOH in freezer.

Mouse liver tissue samples were sectioned in a cryostat, placed on frosted microscopic glass slides and fixed, for mtDNA detection, in 0.1 M Tris-HCl (pH7.4), 3 mM calcium acetate, 23 mM zinc acetate and 37 mM zinc chloride over night in RT followed with washes in 1 x TBS.

2.3 Phosphorylation of padlock probe

The padlock probes were phosphorylated before ligation in 500 mM Tris-HCl (pH 7.6 at 25ºC), 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1mM EDTA, 1 mM ATP, 2 µM padlock probe and 0.2 U/µl T4 PNK (Fermentas) for 30 min in 37ºC followed by inactivation of the enzyme for 5 min in 65ºC.

2.4 General protocol for mtDNA detection

2.4.1 Pre-treatment of cells and tissue

The cell and tissue samples were pre-treated in 0.01% pepsin (Sigma) in 0.1 M HCl for 80 s (for cells) or 4.5 min (for tissue) in 37ºC followed by washes in 1 x PBS (1 x TBS for tissue).

2.4.2 Restriction enzyme and exonuclease activity

The DNA was cleaved and made single-stranded in 1 x NEB 4 buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 at 25ºC)), 0.2 µg/µl BSA, 0.4 U/µl T7 exo (New England Biolabs) and 0.5 U/µl Msc1 (New England Biolabs) for 30 min (cells) or 60 min (tissue) in 37ºC.

2.4.3 Hybridization and ligation of padlock probe

Ligation with T4 DNA Ligase was performed in 10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM ATP, 0.2 µg/µl BSA, 0.25 M NaCl, 0.1 µM padlock probe and 0.1 U/µl T4 DNA Ligase (Fermentas) for 30 - 45 min in 37ºC.

Ligation with Ampligase was performed in 20 mM Tris-HCl (pH 8.3), 25 mM potassium chloride, 10 mM magnesium chloride, 0.5 mM NAD, 0.01% Triton X-100, 0.2 µg/µl BSA, 0.1 µM padlock probe, 7.5% glycerol, 0.125 M potassium chloride and 0.25 U/µl Ampligase (Epicentre) for 45 - 60 min in 50- 55ºC.

The incubations were followed by a ligation wash in 2 x SSC and 0.05% Tween for 5 min in 37ºC for removal of access padlock probes.

2.4.4 RCA

The RCA was performed in 33 mM Tris-acetate (pH 7.9 at 37ºC), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1% (v/v) Tween 20, 1 mM DTT, 0.25 mM dNTP, 0.2 µg/µl BSA, 5% glycerol and 1 U/µl Φ29 DNA polymerase (Fermentas) for 60 - 150 min in 37ºC.

2.4.5 Detection of RCA products

Hybridization of detection probes was performed in 2 x SSC, 20% formamide and 100 - 250 nM detection probes for 15-80 min in 37ºC. The samples were after washing dehydrated in 70 %, 85% and 99 % ethanol for 3 min each. The nuclei in the cell samples were stained with 100 ng/µl DAPI in Vectashield mounting medium (Vector), under a cover slip, and stored in the dark until analyzed. For tissue samples the nuclei were stained with Hoechst 1:500 in 1 x TBS for 60 min in RT followed by mounting with Vectashield under cover slip.

Signals were detected in an EPI fluorescence microscope with the software AxioVision 4.3 Zeiss. Filters were used to take pictures in specific wavelengths corresponding to each fluorescent marker. Three imaged were collected from each sample using filters for DAPI, Cy3 and/or Cy5. For each experiment exposure time and focus was set manually. The amount of signals was counted with the program Blobfinder [15] written by Carolina Wählby, CBA.

2.5 General protocol for RNA detection

2.5.1 Pre-treatment of cells

The cell samples were pre-treated in 0.1 M HCl for 10 min in RT followed by washes in 0.05 % Tween 20 in 1 x DEPC-PBS.

2.5.2 Reverse transcription of mRNA

The mRNA molecule was reversly transcribed to cDNA in 50 mM Tris-HCl (pH 8.3 at 25ºC), 50 mM potassium chloride, 4 mM magnesium chloride, 10 mM DTT, 0.5 mM dNTP, 0.2 µg/µl BSA, 1 µM cDNA primer, 1 U/µl RNaseIn (Fermentas) and 20 U/µl RevertAid H minus M-MuLV RT (Fermentas) for 3 h to O/N in 37 ºC in secure-seal chamber. After a brief wash in 0.05 % Tween 20 in 1 x DEPC-PBS, the cells were post-fixed in 3% FAH for 30 min in RT.

2.5.3 Hybridization and ligation of padlock probe

The hybridization and ligation of padlock probe was performed as in mtDNA protocol with addition of 1 U/µl RNaseIn and 0.4 U/µl RNAse H. The ampligase incubation temperature was changed to 30 min in 37ºC for optimal temperature of RNAse H to be active, followed by 60 min incubation in 50ºC which results in higher activity for the Ampligase.

2.5.4 MutY cleavage for RNA detection

The MutY reaction was performed in 20 mM Tris-HCl, 30 mM NaCl, 1 mM EDTA, 0.1 M KCl, 1 mM DTT, 0.5 µg/µl BSA, 1 U/µl RNaseIn, 1 µM MutY (USB) and 0.15 U/µl Endo IV (Fermentas) for 45 min in 37ºC.

2.5.5 RCA

The RCA was performed as in mtDNA protocol with addition of 1 U/µl RNaseIn.

2.5.6 Detection of RCA products

The detection of padlock probe was performed as in mtDNA protocol.

2.6 Optimizations

2.6.1 Optimization of cell permeabilization

Optimizing pepsination time for mtDNA detection in cell samples was performed on the cell line 143B. Two experiments were made, the first with incubation time in pepsin for 70, 80, 90 or 100 s and the second for 60, 70 and 80 s. Both experiments were followed by mtDNA protocol for cells including ligation of padlock probe 1 (See appendix) with Ampligase. Detection was made with detection probe 4 (See appendix) followed by staining of cytoplasm with 1:400 WGA488 in 1 x PBS for 45 min in RT and dark.

The same optimization was performed on mouse liver tissue sample A165L but with incubation time in pepsin for 2.5, 3, 3.5 and 4.5 min followed by mtDNA protocol for tissue with ligation of padlock probes 3 and 4 (See appendix) with Ampligase and detection with detection probes 4 and 5 (See appendix).

Optimizing incubation time in HCl for RNA detection was performed on cell line 8402 with exposure of cells to HCl for 0, 10, 20 and 30 min followed by RNA protocol including reverse transcriptase reaction with cDNA primer 1, ligation of padlock probe 6 with T4 DNA Ligase and detection with detection probe 5.

2.6.2 Comparing different reverse transcriptases for *in situ* cDNA synthesis

Comparison between the six different reverse transcriptases (Table 1) was performed on cell line 8402, with RNA protocol, with the final concentration of 20 U/µl of the reverse transcriptases except M-MuLV Reverse Transcriptase (Fermentas) with 2 U/µl. Incubation was done with cDNA primer 1 in each reverse transcriptase's buffer. Ligation of padlock probes 6 and 7 with T4 DNA Ligase followed by MutY cleavage and detection with detection probe 3 and 5.

Table 1|Reverse Transcriptases

1.	RevertAid H Minus M-MuLV Reverse Transcriptase	Fermentas	Minus RNase H
2.	RevertAid M-MuLV Reverse Transcriptase	Fermentas	Active RNase H
3.	M-MuLV Reverse Transcriptase	Fermentas	Active RNase H
4.	M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant	Promega	Minus RNase H
5.	M-MuLV Reverse Transcriptase, RNase H^-	Finnzymes	Minus RNase H
6.	Superscript II Reverse Transcriptase	Invitrogen	Minus RNase H

The Reverse Transcriptases used for *in situ* RNA detection. The table indicates vendor and if the RT has an active RNaseH function or if this property has been inactivated.

2.6.3 Comparing the length of cDNA synthesis with different reverse transcriptases

Comparison in length of synthesized cDNA with the reverse transcriptases 1, 4, 5 and 6 (Table 1) was performed on cell line 8402, with RNA protocol. Incubation was done with corresponding buffer and cDNA primers 1, 2, 3 and 6. Ligation of padlock probe 5 was done with T4 DNA Ligase and detection with detection probe 4 (Appendix 1).

2.6.4 Optimization of LNA containing cDNA primers

Comparing the same cDNA primer in sequence but with different amount of LNA bases incorporated was performed on the cell line 8402 with RNA protocol. Incubation with cDNA primers 6, 7 and 8, ligation of padlock probe 5 with both T4 DNA Ligase and Ampligase and detection with detection probe 4.

2.6.5 Comparing Ampligase to T4 DNA Ligase for RNA detection

Comparing Ampligase to T4 DNA Ligase was performed on co-cultured cell lines 8402 and MEF with RNA protocol. Incubation with cDNA primers 4 and 5, ligation of padlock probes 10 and 11 with both T4 DNA Ligase and Ampligase and detection with detection probes 1 and 2.

2.6.6 Optimization of concentration of detection probe

Optimization of concentration of detection probes was performed on the cell line 143B with mtDNA protocol for cells. Four different experiments were made with two detection probes and either secure-seals or cover slips. Ligation of padlock probe 1 with T4 DNA Ligase followed by detection with 250 nM, 100 nM, 50 nM, 25 nM and 0 nM of detection probes 4 and 5 under cover slips or secure-seals.

2.6.7 Blocking the detection step

Mouse liver tissues A165L and A186.2L were directly after fixation and pepsination, as described above, blocked in 10 mM copper (II) sulphate (Riedel-de Haën) in 50 mM ammonium acetate (pH 5 at RT) for 30 min in RT followed by washes in 1 x TBS. Detection was performed with detection probe 4 and 5. Another experiment was performed with entire mtDNA protocol for tissue on both A165L and A186.2L using padlock probes 3 and 4 with Ampligase and detection with detection probes 4 and 5.

3. Results and Discussion

3.1 Optimization of cell permeabilization

The digestive protease pepsin is used to digest the outer cell membrane as well as the membrane of the mitochondria. In previous experiments pepsin has been weighed and added to HCl in room temperature prior to heating to 37ºC with an optimized pepsination time of 90 s. For time saving, 200 x aliquots of pre-weighed pepsin in solution were made and stored in freezer. The aliquots of pepsin are now added directly to pre-heated HCl leading to higher activity of the enzyme. This suggests that the exposure time should be

shorter to avoid leakage of mtDNA. The results, from first experiment on cells, with pepsination time of 70 s, 80 s, 90 s and 100 s show a signal peak at around 70 s (Table 2).

Table 2 | Presentation of pepsination results on cells

Exp 1				Exp 2		
+					+	+
+	+			+	+	+
+	+	+	+	+	+	+
+	+	+	+	+	+	+
70 s	80 s	90 s	100 s	60 s	70 s	80 s

Classification of the results from the two experiments when optimizing incubation time in pepsin for cells. + represent comparison of the amount of signals obtained for each time point.

However, the amount of signals is low in all samples. Therefore a second experiment was performed with pepsination time of 60 s, 70 s and 80 s samples in order to see more signals and visualize the peak more clearly. The results from the second experiment show fewer signals in the 60 s sample (Table 2). The 70 s and 80 s samples show no visible difference in amount of signals but the higher amount of signals compared to 60 s sample suggests that this is a peak. Even though the 70 s and 80 s samples appear to be equal the pepsination time for pre-weighed frozen pepsin was chosen to 80 s as it corresponds well with the previously used pepsination time.

The results from optimization of pepsination time for tissue show an increase of signals in sample with an incubation time of 4.5 min (Table 3). The first sample of 2.5 min showed fewest signals and surprisingly the third sample of 3.5 min show less amount of signals than in 3 min and 4.5 min sample suggesting something else had gone wrong. It is therefore hard to know if the optimal time is in fact lower than 4.5 min. However, since that sample show more signals than the second sample of 3 min it is used as the pepsination time for the pre-weighed frozen pepsin for tissue.

Table 3 | Presentation of pepsination results for tissue.

			+
	+		+
	+	+	+
+	+	+	+
2.5 min	3 min	3.5 min	4.5 min

Classification of the results from the two experiments when optimizing incubation time in pepsin for tissue. + represent comparison of the amount of signals obtained for each time point.

For RNA detection the cells are pre-treated with HCl to permeabilize the cell membranes. The incubation time of HCl needed optimization to see in what time area the signals increase. The incubation times were 0, 10, 20 and 30 min and the results show an increase at 10 min differing only slightly compared to the other samples (Figure 3). The first sample with no exposure of HCl interestingly shows a very high amount of signals suggesting that the effect of HCl is not crucial for accessing the RNA.

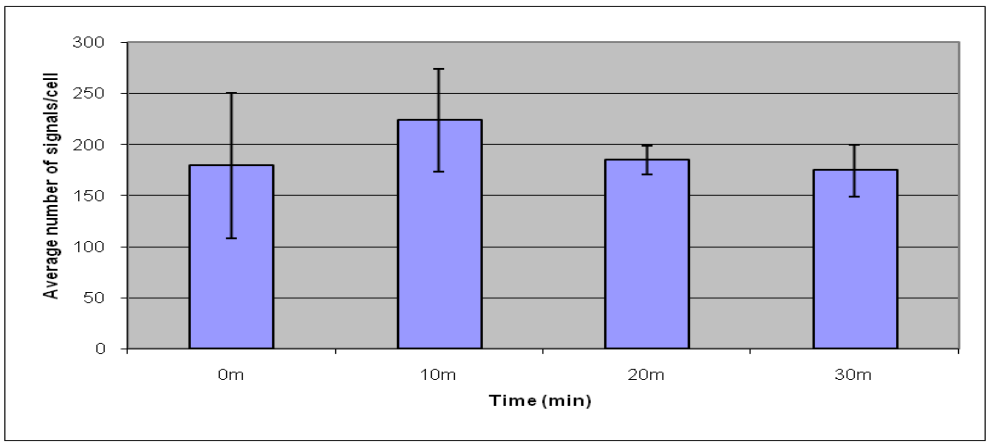

Figure 3. Cells were exposed to HCl for the times indicated. RNA detection in situ was then carried out and number of signals/cell for different HCl exposure times was counted.

3.2 Comparing different reverse transcriptases for in situ cDNA synthesis

The mRNA molecule is reversly transcribed to cDNA by a reverse transcriptase, also known as a RNA dependent DNA polymerase. The reverse transcriptase starts its DNA polymerase activity at the location of a cDNA primer and a cDNA molecule is synthesized. The reverse transcriptases (RT) used in this experiment are all derived from the Moloney Murine Leukemia Virus (M-MuLV). This RT has a natural RNase H activity. RNase H degrades the RNA in a RNA/DNA hybrid molecule. However, if RNase H is present during reverse transcription it can, especially during long incubation time, prevent full-length synthesis of cDNA. To avoid degradation of RNA prior to full-length cDNA synthesis it is common to use reverse transcriptases lacking the RNase H activity and instead degrade the RNA in a separate step.

In this experiment, six reverse transcriptases (Table 1) were compared; four without RNase H activity and two with RNase H activity. Genotyping was performed using human and mouse padlock probe with human cDNA primer. The distance between cDNA primer and padlock probe is only 64 nt which suggests that all RTs should be able to synthesis that length equally well. The amount of mouse signals can be seen as a measure of how specific the T4 DNA Ligase is but it may also show the proof-reading ability of the reverse transcriptase. The previously used RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and the two other RT from Fermentas differ in RNase H activity. Two of the Fermentas RT also differ in structure and catalytic properties, therefore the name RevertAid. The RevertAid M-MuLV RT (Fermentas) shows almost no signals suggesting that RNase H activity degrades the RNA during synthesis (Figure 4).

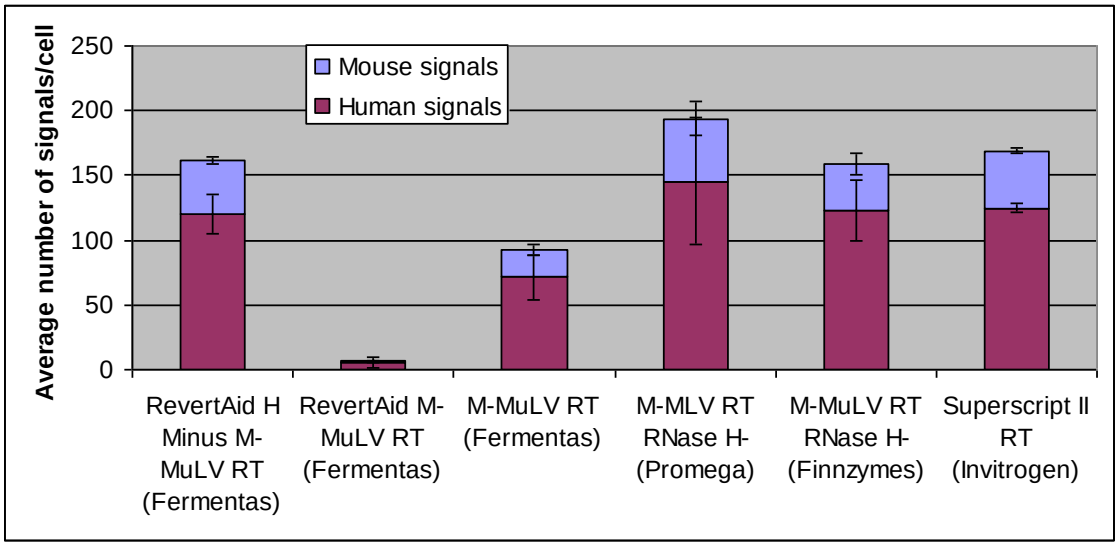

Figure 4. Comparison of different reverse transcriptases for cDNA synthesis on human cell line *in situ*. The mouse padlock probe has sequence similarities to human genome which is why the amount of mouse signals is high.

However, strangely the M-MuLV RT (Fermentas) also with RNase H activity show increased number of signals. This suggests that the structure and catalytic properties that differ between them keep the RNA from degrading, to allow the RT to synthesis a full-length cDNA molecule, even though RNase H is present. The four RT without RNase H activity (Fermentas, Promega, Finnzymes and Invitrogen) appear to be making almost equal amount of signals, between 120 and 150 signals per cell in average with 22-26% wrong signals, with a slightly better result for the RT from Promega. The wrong signals are the mouse padlock probe hybridizing in the human genome because of sequence similarities which explains the high amounts in this experiment. However, to find better proof of best reverse transcriptase, further experiments were performed comparing the length of cDNA synthesis for these four reverse transcriptases.

3.3 Comparing the length of cDNA synthesis with different reverse transcriptases

The reverse transcriptases found to perform best in the previous experiment were compared to find differences in ability to synthesize cDNA molecules. Four cDNA primers were used located at different lengths from a target sequence. The length of the synthesized cDNA fragment corresponds to visible signals in the different cDNA primer samples. If signals are seen in the sample with cDNA primer furthest from the padlock probe the RT is able to make that length of cDNA. The first experiment compared RT from Promega, Finnzymes and Invitrogen (Table 1).

Results show a quick decrease of signals, as the distance between cDNA primer and target site increases, with almost no signals at 500 nt long fragments. An increase was then seen again at more than 1100 nt long fragments (Figure 5). This suggests that the mRNA forms secondary structure making it both difficult for cDNA primers to hybridize at specific sites and for the reverse trancriptase to keep synthesizing if the structure of

mRNA is too rigid. Overall, the RT from Promega gave the highest amount of signals and was therefore compared to the previously used RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas).

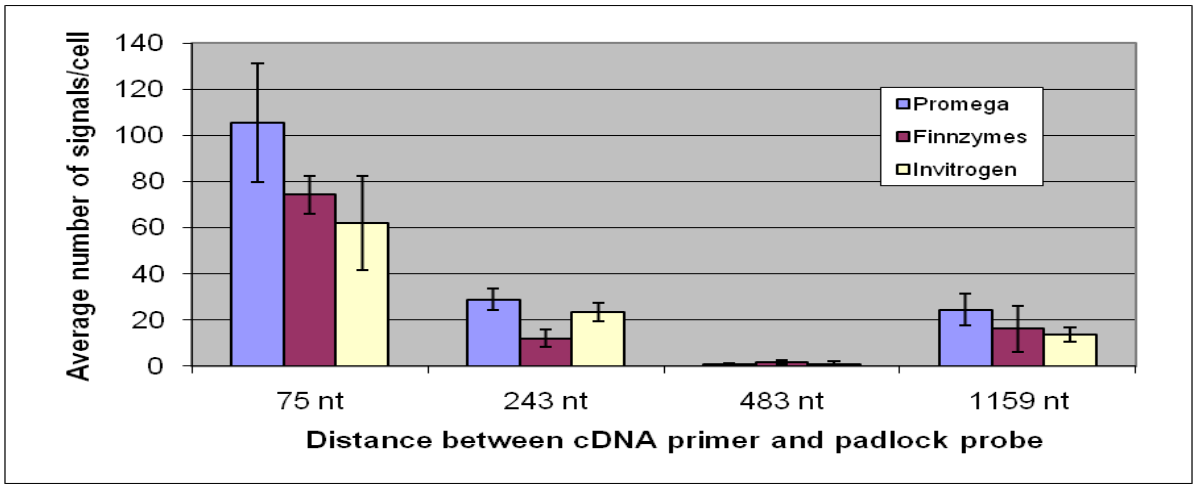

Figure 5. Comparison of length of cDNA synthesis *in situ* for different reverse transcriptases. A decrase in signals is seen where the distance between cDNA primer and padlock probe is about 500 nt.

The results from the second experiment show a similar decrease of signals at about 500 nt distance between cDNA primer and padlock probe, and an increase again at around 1100 nt, suggesting that the mRNAs secondary structure is constant. When comparing the RT from Promega to the previously used RT from Fermentas the results (Figure 6) show more signals at all distances validating that the RevertAid H Minus M-MuLV Reverse Transcriptase from Fermentas already in use is the best cDNA synthesizer.

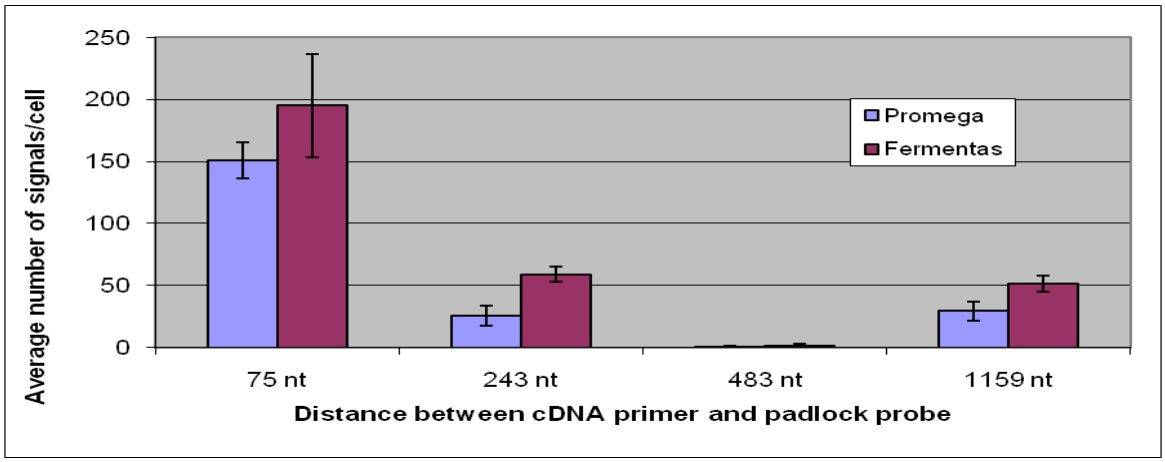

Figure 6. Comparison of length of cDNA synthesis *in situ* for different reverse transcriptases. Similar results as in figure 5, suggesting that the secondary structure of the mRNA molecule is constant. The RT from Fermentas is validated as the best cDNA synthesizer.

3.4 Optimization of LNA containing cDNA primers

For localized detection of RNA it is necessary for the cDNA molecule to stay hybridized to the mRNA during the entire experiment. Since RNase H degrades the RNA in the

RNA/DNA hybrid molecule an anchor is needed to link the two molecules together. One part of the cDNA that can be modified is the primer. If the primer constitutes of bases that RNase H would detect as RNA the degradation will stop leaving the primer hybridized to the mRNA [16]. A novel class of RNA analogs is Locked Nucleic Acid (LNA) (Figure 7) which differs from RNA by having a linking bridge between the 2'-oxygen and the 4'-carbon. This structure makes an oligonucleotide containing LNA more rigid and together with an exceptionally high affinity towards complementary RNA the LNA bases are perfect for use in our cDNA primers [17].

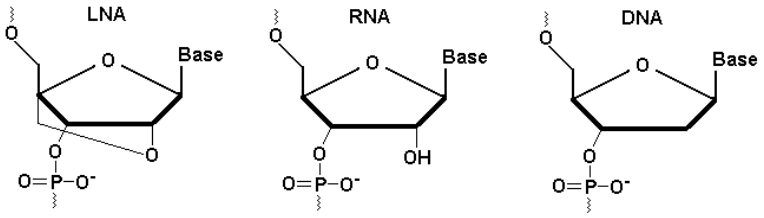

Figure 7. Structure of the sugar-phosphate backbone of LNA, RNA and DNA.

It is necessary to optimize the number of incorporated LNA bases in the cDNA primer. Too many LNA bases form a very rigid primer that could sterically hinder the hybridization and too few LNA bases may not be able to work as an anchor keeping the cDNA molecule hybridized to the mRNA. cDNA primers 6, 7 and 8 were modified by changing every second or every third base incorporating five, seven or nine LNA bases (Appendix 1).

The results show an increase in number of signals for 7 LNA sample (Figure 8). Interestingly though, both T4 DNA Ligase and Ampligase show an equal increase between the 5 LNA and the 7 LNA sample, only to decrease in difference in the 9 LNA sample. The difference shows a large decrease of signals for T4 DNA Ligase suggesting that 9 LNA bases make a cDNA primer too rigid for correct hybridization. If the different cDNA primers were not dependent on which ligase used there would be an equal decrease of signals for Ampligase as seen for T4 DNA Ligase in the 9 LNA samples. However, the results show instead an almost equal amount of signals for 7 LNA and 9 LNA sample for Ampligase. This sudden increase of signals when using Ampligase is explained by the increase of thermal stability of between +1 - 2ºC/LNA that occurs when a LNA monomer is introduced into a cDNA primer [18]. A cDNA primer with more LNA bases is therefore more able to stay hybridized at the higher incubation temperatures used when ligating with Ampligase.

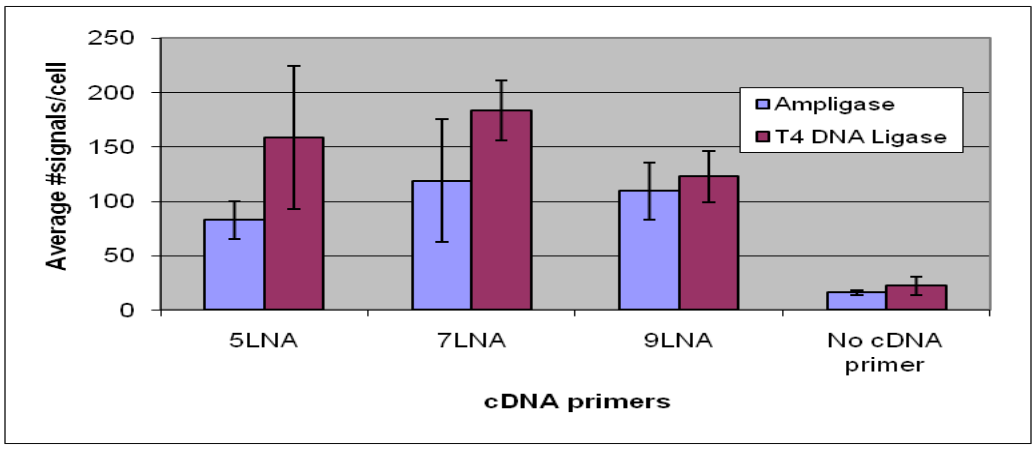

Figure 8. The difference in amount of signals with five, seven or nine incorporated LNA bases in cDNA primer was investigated using either Ampligase or T4 DNA Ligase.

3.5 Comparing Ampligase to T4 DNA Ligase for RNA detection

There are two different kinds of DNA ligases utilizing either ATP or NAD^+ as a cofactor. The ATP-dependent ligases are mostly found in eukaryotes as well as eukaryotic DNA viruses, bacteriophages and archaebacteria whereas the NAD^+ depending ligases are only found in eubacteria [19]. Despite the different requirements all ligases share the same mechanism when they catalyze the repair of the phosphodiester bonds at single- and double- stranded breaks.

Here two different DNA ligases commonly used for ligation of padlock probes are compared. T4 DNA Ligase, which is dependent of ATP, has previously been the ligase of choice in padlock probe experiments because of the large amounts of signals it generates. It has the ability to ligate at incubation temperatures of 37ºC with short incubation time which is another advantage as the morphology of the cells is kept intact. However, we have seen when performing genotyping experiments of SNPs that the T4 DNA Ligase does not have the highest specificity, as some calls from the wrong allele is seen. Ampligase, which is dependent of NAD^+, is a thermostable ligase. It differs from T4 DNA Ligase as it requires higher temperature and longer incubation times. We have seen a higher specificity when using Ampligase but with loss of signals. Therefore Ampligase and T4 DNA Ligase are compared to see if it is possible for Ampligase to achieve as high amounts of signals as T4 DNA Ligase but with better specificity.

A site on the β-Actin gene was found where the sequence between mouse and human differs in one nucleotide. This single nucleotide variant can be looked upon as a model of a SNP. Padlock probes were designed to have the single nucleotide variant at its 3' end leaving it up to the DNA ligase for correct probe ligation. The padlock probes also differ in their detection probe hybridization site, where the human padlock probe gives rise to red Cy3 signals and the mouse padlock probe to green Cy5 signals. Two cDNA primers both hybridizing at the same distance from the target sequence in both genomes were also designed to allow correct cDNA synthesis. The results show more signals when using T4

DNA Ligase compared to Ampligase with a smaller difference in the red human padlock signals (Figure 9).

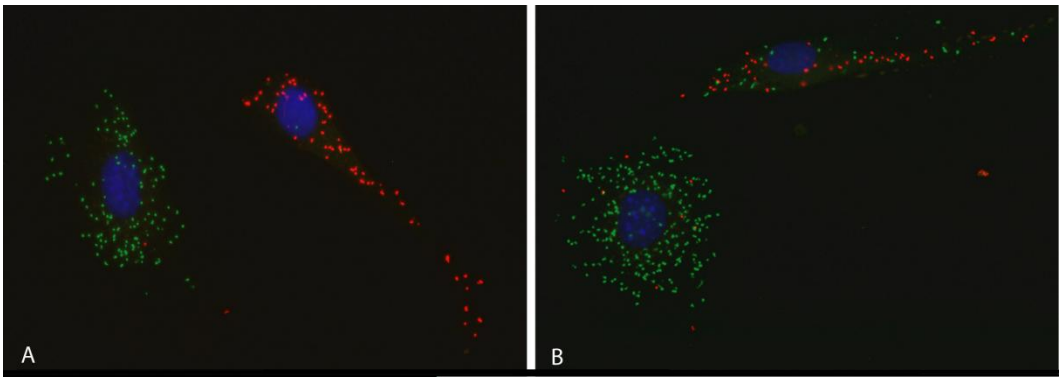

Figure 9. cDNA detection *in situ* on co-cultured human and mouse cell lines with Ampligase (A) and T4 DNA Ligase (B). Nuclei were stained with DAPI (blue) and signals from the two padlock probes are shown in red (human) and green (mouse). A clear difference in specificity can be seen between the ligases.

The reasons for difference in amount of signals are either due to the capacity of the ligase for fast ligation or simply to the different expression patterns seen at moment of fixation. The fact that Ampligase needs a higher incubation temperature may be a reason for lower amount of signals. However, it shows almost no false positives thereby having a very high specificity which is in large contrast to the T4 DNA Ligase. The ligase of choice in following experiments will be depending of the experiment. Ampligase is the best choice for genotyping experiments. However, if experiments with one padlock probe are made where signals are counted and compared depending on other parameters than genotyping of SNPs then T4 DNA Ligase can be chosen in order to see as many signals as possible.

3.6 Optimization of concentration of detection probe

When detecting the signals it is of importance that there is enough detection probes present for full saturation of the entire rolling circle product. However, it is also important to keep the background, from unspecific binding of detection probe, down suggesting that the detection probe concentration should be kept low. In previous experiments 250 nM detection probe has been used. If the concentration could be lowered, not only would it be more cost-effective but may also decrease background which can be a problem especially in tissue samples.

Two different detection probes, labelled with either Cy3 or Cy5, were added to cultured cells subjected to detection of mtDNA in concentrations of 25 nM, 50 nM, 100 nM and 250 nM. Experiments were performed in both secure-seals and under cover slips for both detection probes. The signals detected were kindly analyzed by Carolina Wählby at CBA using custom made program written in Matlab. The program is designed to deal with the most important part of signal analyses; to decide how to define a signal and how to define noise. Noise is pixel values incorrectly believed to be a signal that usually arises from the interference of the detection device. The signal to noise ratio, SNR, is measured by the difference between average values of signal to average value of background divided by the standard deviation of the background. The background is calculated from an intensity

distribution plot (Figure 10) and located where the highest number of pixels has the lowest intensity.

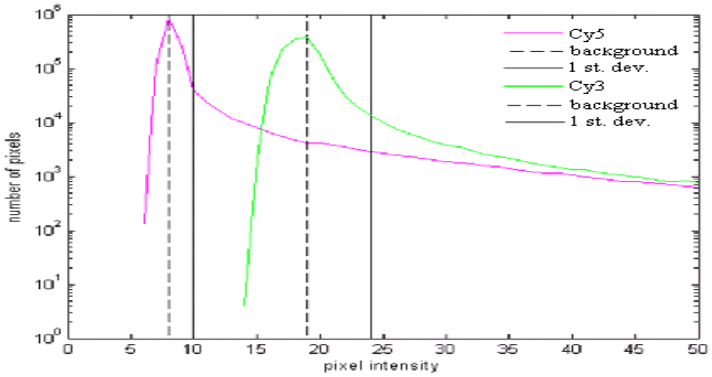

Figure 10. Example of an intensity distribution plot for two detection probes labelled with either Cy5 or Cy3. The background is found where the highest number of pixels has the lowest pixel intensity.

There are different ways in which signals can be defined depending on how many pixels over the background you choose to be a signal. Here three ways of defining a signal was used by Matlab (Figure 11). Definition 1 chooses the average intensity of all pixels over the background plus two standard-deviations of the background. Definition 2 chooses the 90 percentile of the pixels in definition 1 and definition 3 chooses all pixels found by signal enhancement and over a set threshold. Signal enhancement uses a so called Laplace filter searching all pixels. The background is set to zero where no signals are found and intensity is increased at the location of a signal. The enhanced intensity is however given the true intensity in the plot.

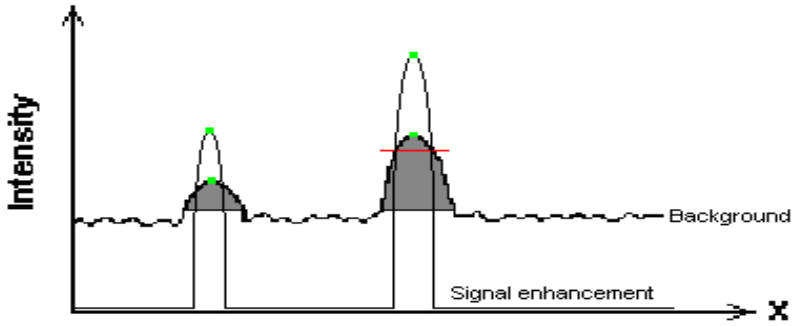

Figure 11. Definitions of a signal. The plot shows intensity of signals if looking in one direction in the image. Definition 1 chooses the average intensity of all pixels over the background plus two standard-deviations of the background (grey area), definition 2 chooses the 90 percentile of the pixels in definition 1 (red line) as the signal intensity and definition 3 chooses the average intensity after signal enhancement and over a set threshold (green dot). Both enhanced and true intensity is shown for each signal.

The number of signals and signal pixels in the images were calculated and plotted against eachother (not shown). The plot shows a linear relationship, meaning that all signals in the different concentrations have the same size. Results are shown in intensity and SNR for the three different definitions (Figure 12).

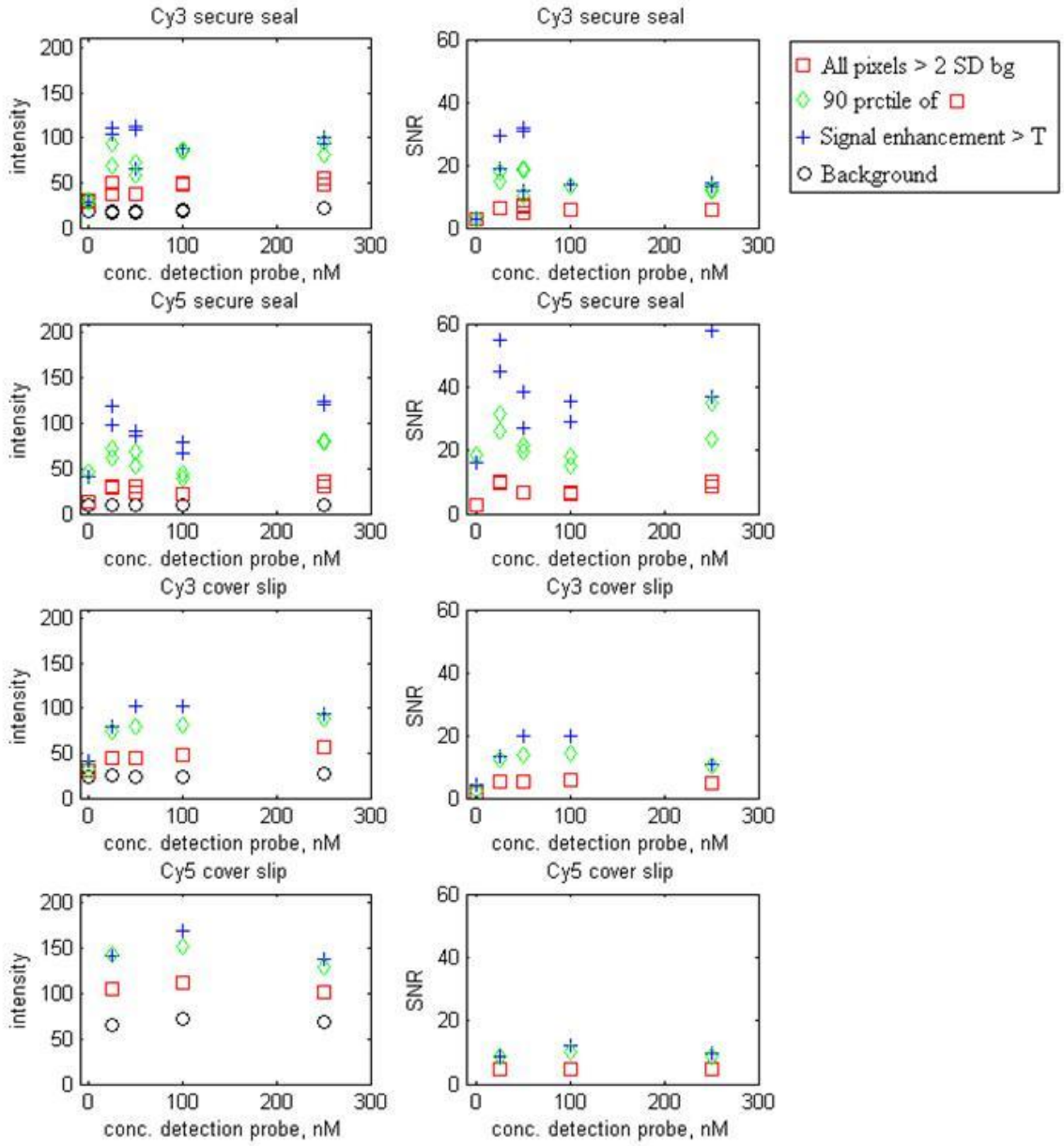

Figure 12. Intensity and SNR for detection probes labelled with Cy3 or Cy5 hybridized in secure-seal or under cover slip. The three definitions of signal were used by Matlab to analyze the images in each sample for intensity and SNR.

Overall, independent on which signal definition used, there seems to be an equal intensity and SNR when comparing the different concentrations meaning that the detection probe concentrations can be decreased for a more cost-effective protocol. A higher SNR is seen in secure-seals sample compared to cover slips. This together with a lower background for secure-seals, as seen in the intensity plot, suggests that the unspecifically bound detection probes are more easily washed off after hybridization in secure-seals. Interestingly though, the background did not decrease at lower detection probe

concentrations, suggesting that the wash after detection probe hybridization removes all unspecifically bound detection probes.

Comparing Cy5 to Cy3 there is a larger difference seen in SNR between secure-seal and cover slip for Cy5 samples. However, the background is much higher for the Cy5 cover slip sample than secure-seal sample, which explains why the SNR is so low in the Cy5 cover slip sample. The background in the Cy5 secure-seal sample is the lowest seen; therefore it has the highest SNR suggesting that using detection probe with Cy5 in a secure-seal chamber gives the best results. The concentration of detection probe was now decreased to 100 nM for a more cost-effective protocol.

3.7 Blocking the detection step

When detecting signals in the microscope, problems like autofluorescence and high background can arise. The detection probes may contribute to increase in background if not properly washed after hybridization and the cells or tissue may have pigments that autofluoresce complicating signal analyses.

One pigment found in many different cells is lipofuscin, an autofluorescent pigment that increases in amount in aging cells [20]. Here blocking of the autofluorescent lipofuscin and the background was attempted by exposing tissue samples to copper (II) sulphate, $CuSO_4$. It has been shown that treatment with $CuSO_4$ can reduce or even eliminate the lipofuscin autofluorescence in tissue [21]. It was also shown by testing either $CuCl_2$ or Na_2SO_4 that the Cu^{2+} is responsible for the blocking.

To test if $CuSO_4$ could be used for reduction of autofluorescence together with our protocol liver tissues from two different mouse individuals were treated with $CuSO_4$. Microscope pictures were taken with the same exposure time for both treated and untreated tissue, setting the time for the blocked tissue first.

Results show, in experiment with blocking directly after fixation, a difference between treated and untreated tissue both before and after detection probe hybridization (Figure 13). It seems like the blocking agent can reduce both autofluorescence and background arising from detection probes.

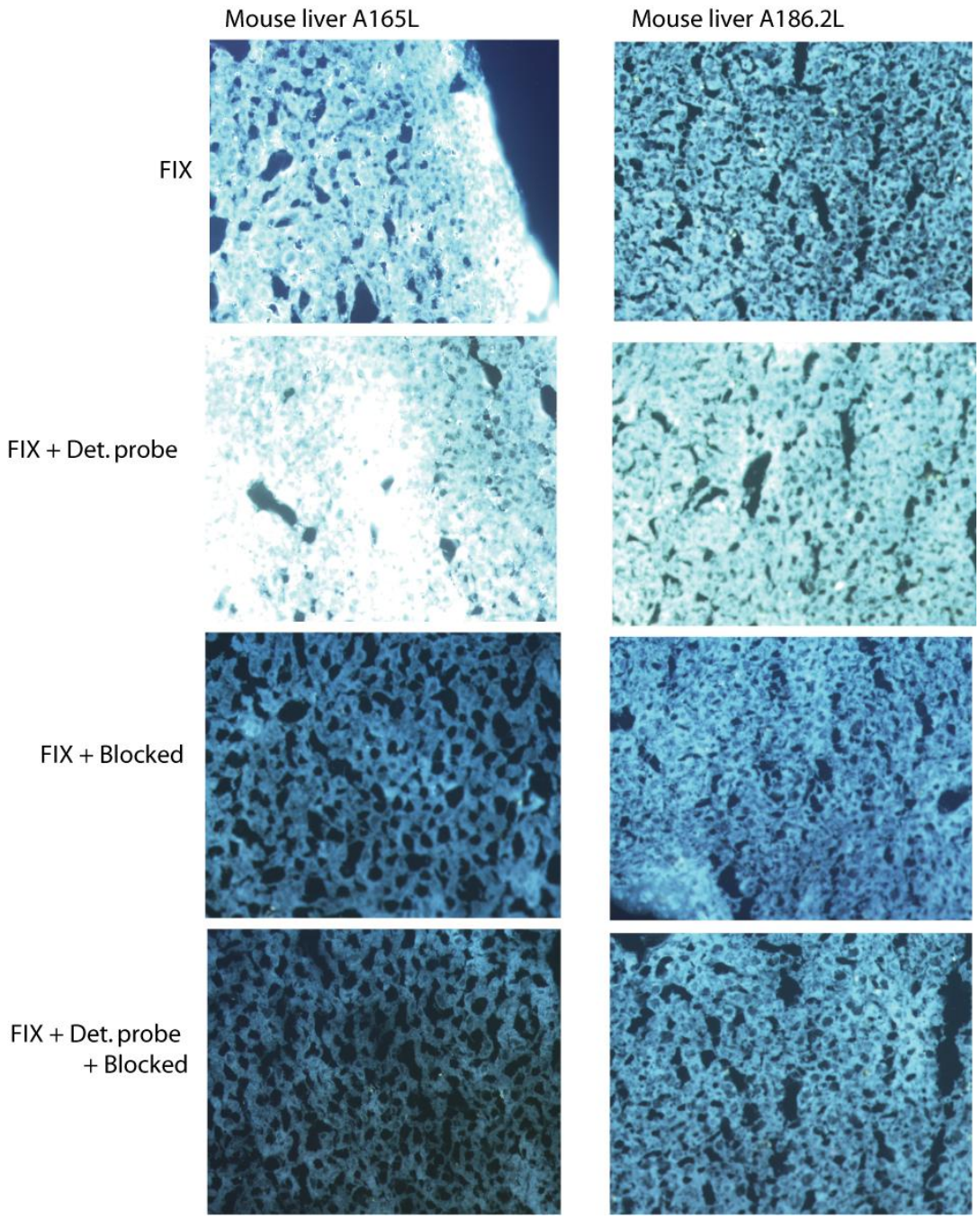

Figure 13. Figure showing blocked and unblocked mouse liver tissue with $CuSO_4$ directly after fixation prior to detection probe hybridization.

The attempt to block the tissue was proceeded by testing with entire protocol to see if the RCA products could withstand the treatment. In the results (Figure 14) a clear difference is seen between treated and untreated tissue. There is also a difference between the different mouse individuals, suggesting that more lipofuscin is accumulated in one of the

tissues compared to the other. Since lipofuscin is an ageing pigment this suggests that the mouse liver tissue A165L is an older individual than A186.2L.

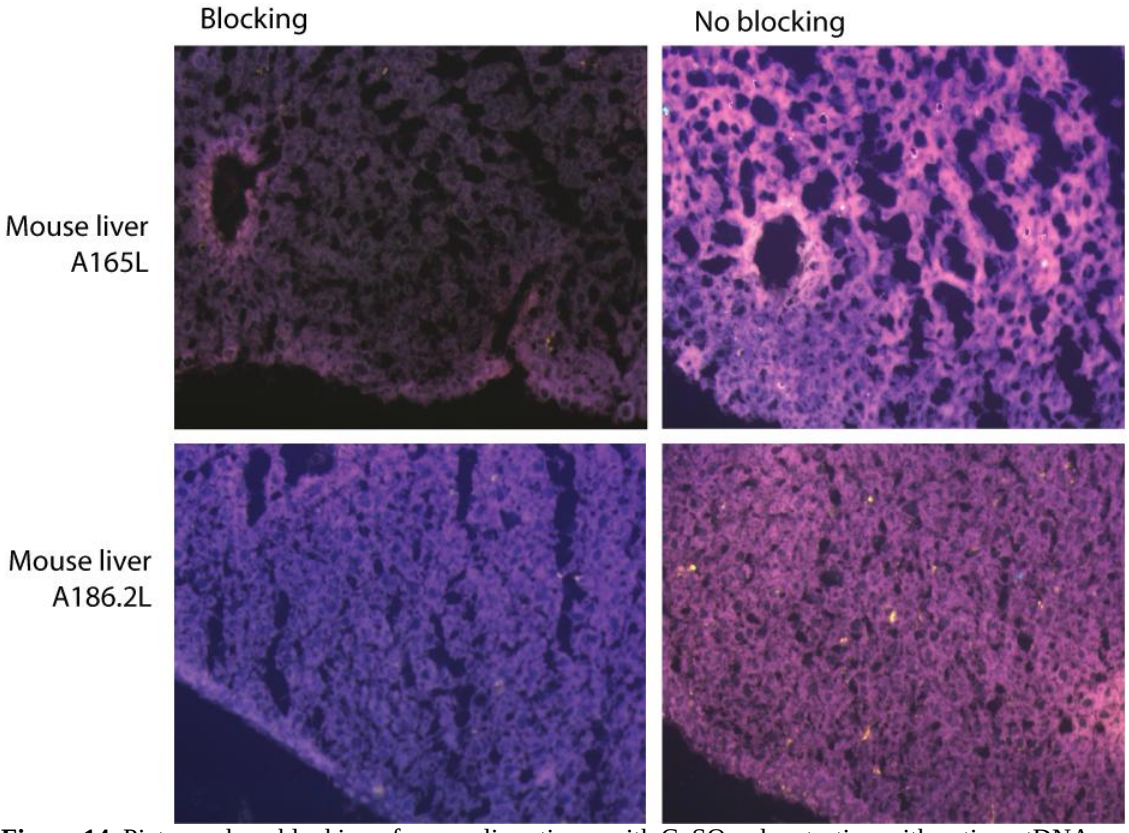

Figure 14. Pictures show blocking of mouse liver tissue with $CuSO_4$ when testing with entire mtDNA protocol. A difference is seen between blocked and unblocked and between the different mouse individuals.

4. Conclusions and future perspectives

Development of techniques for single-cell analyses has become more and more important over the years. The reason being the new knowledge; that looking at single cells is the only way to fully understand an entire multicellular organism. Since many diseases in today's society are originating from one cell e.g. cancer tumours, development of methods for analysing the diseased cells and compare them to the normal cells are very important for correct diagnosis. Once a correct diagnosis is set the treatment can become more specific, easier and more cost-effective.

When studying single cells it is important that the detected molecule is at its right location in the cell. It would otherwise be impossible to be certain of the interactions taken place in the cell since the detected molecule could be lost or move to another cell. Developing methods that can localize the detection of molecules is therefore highly interesting.

Here I have presented optimization for the Padlock probe technique for localized detection of target molecule. The Padlock probe technique uses a circularizable oligonucleotide to template a rolling circle amplification product detected by fluorophore-labelled probes. The method can be used in numerous applications, both on cell cultures and tissues detecting both RNA and DNA.

The rolling circle product is, for localized detection, connected to the molecule of interest either by a covalent link to a mtDNA molecule or via hybridization of the cDNA primer to an mRNA molecule. Development of the technique is crucial for finding nucleic acid differences in cells, such as single nucleotide polymorphisms SNPs. However, the development of different applications needs individual optimization in order to receive the best results possible.

In this project I have optimized and validated the padlock probe technique in different steps, all with the purpose of receiving as many signals with as high specificity as possible.

The RevertAid M-MuLV H Minus Reverse Transcriptase (Fermentas) was compared to five other reverse transcriptases and was validated as the best choice for cDNA synthesis in both specificity as well as cDNA length.

The number of LNA bases in the cDNA primer was optimized showing an increase of signals when using seven LNA bases. However, since cDNA primers with more LNA bases work better in higher temperatures, the cDNA primer with nine LNA bases is interesting for use in applications depending on the use of Ampligase.

Comparison of Ampligase and T4 DNA Ligase show a better specificity with slightly less signals for Ampligase suggesting that Ampligase is more suited for genotyping experiments with SNPs whereas T4 DNA Ligase can continue to be used in experiments with one padlock probe.

The experiment optimizing concentration of detection probes showed an equal intensity and SNR in all different concentrations. Thereby the concentration could be decreased for a more cost-effective protocol. The background did not decrease with lower concentrations; however it is lower overall in secure-seal samples, confirming that the unspecifically bound detection probes are easily washed away.

Blocking with $CuSO_4$ proved to work on lipofuscin when testing only on fixed tissue sample. However, the reduction of detection probe background suggests that it also works well on blocking the surface of the tissue to avoid unspecific detection probe hybridization.

Only a small part of the in situ DNA and RNA detection project by the Padlock probe technique is presented in this report. More experiments need to be performed to validate the results further and to take the Padlock probe technique more easily into future applications.

5. Acknowledgements

I would like to thank my supervisors Chatarina Larsson, Ida Grundberg and Mats Nilsson for giving me the opportunity to do my exam work in their group. Thank you very much for your support and excellent supervision during these weeks. I would also like to thank Carolina Wählby for all help with the signal analyses and my scientific reviewer Anna Asplund for taking the time to read my report. Finally many thanks to everyone in the research groups of Mats Nilsson and Ulf Landegren at the department of genetics and pathology for your friendly support and helpfulness.

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7. Appendix

Appendix 1 | All primers and padlock probes used for β-actin detection.

β-actin 1852 bp (NM_001101.3)
>gi|168480144:c1852-1 Homo sapiens actin, beta (ACTB), mRNA
cDNA sequence
5′-
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATTTTTAAGGTGTGCACTTTTATTCAAC
TGGTCTCAAGTCAGTGTACAGGTAAGCCCTGGCTGCCTCCACCCACTCCCAGGGAGACCAAAAGCCTTCA
TACATCTCAAGTTGGGGGACAAAAAAGGGGGAAGGGGGGGCACGAAGGCTCATCATTCAAAATAAAACAA
AATAAAAAAGTATTAAGGCGAAGATTAAAAAAATTTTGCATTACATAATTTACACGAAAGCAATGCTATC
ACCTCCCCTGTGTGGACTTGGGAGAGGACTGGGCCATTCTCCTTAGAGAGAAGTGGGGTGGCTTTTAGGA
TGGCAAGGGACTTCCTGTAACAACGCATCTCATATTTGGAATGACTATTAAAAAAACAACAATGTGCAAT
CAAAGTCCTCGGCCACATTGTGAACTTTGGGGGATGCTCGCTCCAACCGACTGCTGTCACCTTCACCGTT
CCAGTTTTTAAATCCTGAGTCAAGCCAAAAAAAAAAAAAAAACCAAAACAAAACAAAAAAAACAAATAAA
GCCATGCCAATCTCATCTTGTTTTCTGCGCAAGTTAGGTTTTGTCAAGAAAGGGTGTAACGCAACTAAGT
CATAGTCCGCCTAGAAGCATTTGCGGTGGACGATGGAGGGGCCGGACTCGTCATACTCCTGCTTGCTGAT
CCACATCTGCTGGAAGGTGGACAGCGAGGCCAGGATGGAGCCGCCGATCCACACGGAGTACTTGCGCTCA
GGAGGAGCAATGATCTTGATCTTCATTGTGCTGGGTGCCAGGGCAGTGATCTCCTTCTGCATCCTGTCGG
CAATGCCAGGGTACATGGTGGTGCCGCCAGACAGCACTGTGTTGGCGTACAGGTCTTTGCGGATGTCCAC
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AGCTCTTCTCCAGGGAGGAGCTGGAAGCAGCCGTGGCCATCTCTTGCTCGAAGTCCAGGGCGACGTAGCA
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GCCAGAGGCGTACAGGGATAGCACAGCCTGGATAGCAACGTACATGGCTGGGGTGTTGAAGGTCTCAAAC
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GACGATGCCGTGCTCGATGGGGTACTTCAGGGTGAGGATGCCTCTCTTGCTCTGGGCCTCGTCGCCCACA
TAGGAATCCTTCTGACCCATGCCCACCATCACGCCCTGGTGCCTGGGGCGCCCCACGATGGAGGGGAAGA
CGGCCCGGGGGGCATCGTCGCCCGCGAAGCCGGCCTTGCACATGCCGGAGCCGTTGTCGACGACGAGCGC
GGCGATATCATCATCCATGGTGAGCTGGCGGCGGGTGTGGACGGGCGGCGGATCGGCAAAGGCGAGGCTC
TGTGCTCGCGGGGCGGACGCGGTCTCGGCGGT-3′

Padlock	Name	ID	Length (nt)
8	PdAct2G_90 (Lin33)	P3909	90
6	PdACT2MUTY (Lin33)CC	P2897	70
7	PdAct2A (B2_DO)TC	P3878	70
10	PdACTBhum (B2_DO_27+)	P3975	90
5	pdAct1cDNA (Lin16)	X01735	70

Primer	Name	ID	Length (nt)	Dist. to pd [1, 2, 3, 4, 5] (nt)
1	pLNA30ActcDNA11	P3816	30	69, 64, 64, 1059, 1159
2	pLNAActcDNA4	P3875	30	-, -, -, 383, 483
3	pLNAActcDNA5	P3876	30	-, -, -, 143, 243
4	pLNA_ACTBhum	P3978	25	-, -, -, 113, 213
6	pLNA30ActcDNA7	P3699	30	-, -, -, -, 75
7	P2LNA30ActcDNA7	P3702	30	
8	P3LNA30ActcDNA7	P3814	30	

Appendix 2
The oligonucleotides used in this project

Mitochondrial DNA experiments

			Sequence (5'-3') Probe complementary segment — *Detection probe hybridization site* — Probe complementary segment
Padlock probes			
1. MELAS WT-70	-	Lin16	5' - **CTGCCATCTTAACAA** TTCCTTTTACGA ***CCTCAATGCTGCTGCTGTACTAC*** TCTTC **TGCGATTACCGGGCT** - 3'
2. MELAS MUT-70	-	Lin33	5' - **CTGCCATCTTAACAA** CCTTTCCTACGA ***CCTCAATGCACATGTTTGGCTCC*** TCTTC **TGCGATTACCGGGCC** - 3'
3. Pd15151C	P2719	Lin16	5' - **TTAAGATAAGTAGGT** TTCCTTTTACGA ***CCTCAATGCTGCTGCTGTACTAC*** TCTTC **GGCCCCCAATTCAGG** - 3'
4. Pd15151T	P2720	Lin33	5' - **TTAAGATAAGTAGGT** TTCCTTTTACGA ***CCTCAATGCACATGTTTGGCTCC*** TCTTC **GGCCCCCAATTCAGA** - 3'

cDNA experiments

Padlock probes			
5. PdACT1cDNA	X01735	Lin16	5' - **AGCCTCGCCTTTGCC** TTCCTTTTACGA ***CCTCAATGCTGCTGCTGTACTAC*** TCTTC **GCCCCGCGAGCACAG** - 3'
6. PdACT2MUTY	P2897	Lin33	5' - **CTGTCCACCTTCCAG** CCTTTCCTACGA ***CCTCAATGCACATGTTTGGCTCC*** TCTTC **TCCAGCCTGGCCTCG** - 3'
7. PdAct2A	P3878	B2_DO	5' - **CTGTCCACCTTCCAG** AGAGTGTACCGACCTCAGTA ***AGTAGCCGTGACTATCGACT*** **TCCAGCCTGGCCTCA** - 3'
8. PdAct2G_90	P3909	Lin33	5' - **CTGTCCACCTTCCAGCAGAT** TTCCTCTATGATTACTGACCTA ***CCTCAATGCACATGTTTGGCTCC*** TCTTC **GCGGCTCCAGCCTGGCCTCG** - 3'
9. PdAct2A_90	P3910	Lin16	5' - **CTGTCCACCTTCCAGCAGAT** TTCCTCTATGATTACTGACCTA ***CCTCAATGCTGCTGCTGTACTAC*** TCTTC **GCGGCTCCAGCCTGGCCTCA** - 3'
10. PdACTBhum	P3975	B2_DO_27+	5' - **GCCGGCTTCGCGGGCGACGA** TTCCTCTATGATTACTGACCTA ***TGCGTCTATTTAGTGGAGCC*** TCTTCTTT **ACGGCGCCGGCATGTGCAAG** - 3'
11. PdACTBmus	P3976	Allel2_	5' - **GCCGGCTTCGCGGGCGACGA** TTCCTCTATGATTACTGACCTA ***AGTCGGAAGTACTACTCTCT*** TCTTCTTT **ACGGCGCCGGCATGTGCAAA** - 3'

cDNA primers			**Modifications**
1. pLNA30ActcDNA11	P3816	5'-ACTCGTCATACTCCTGCTTGCTGATCCACA-3'	7 **LNA** bases
2. pLNAActcDNA4	P3875	5'-CACAGCCTGGATAGCAACGTACATGGCTGG-3'	"
3. pLNAActcDNA5	P3876	5'-CTGGGCCTCGTCGCCCACATAGGAATCCTT-3'	"
4. pLNA_ACTBhum	P3978	5'-CTGACCCATGCCCACCATCACGCCC-3'	"
5. pLNA_ACTBmus	P3979	5'-CTGACCCATTCCCACCATCACACCC-3'	"
6. pLNA30ActcDNA7	P3699	5'-ATCATCCATGGTGAGCTGGCGGCGGGTGTG-3'	"
7. p2LNA30ActcDNA7	P3702	5'-ATCATCCATGGTGAGCTGGCGGCGGGTGTG-3'	5 **LNA** bases
8. p3LNA30ActcDNA7	P3814	5'-ATCATCCATGGTGAGCTGGCGGCGGGTGTG-3'	9 **LNA** bases

Detection probes

1. B2_DO_27+_Cy3	L8748	B2_DO_27+	5'-TGCGTCTATTTAGTGGAGCC-3'	5' - Cy3
2. Allel2_+Cy5	X00880	Allel2_	5'-AGTCGGAAGTACTACTCTCT-3'	5' - Cy5
3. B2_DO	X01063	B2_DO	5'-AGTAGCCGTGACTATCGACT-3'	5' - Cy3
4. Lin16-Cy3	L9832	Lin16	5'-CCTCAATGCTGCTGCTGTACTAC-3'	5' - Cy3
5. Lin33_Cy5	L9576	Lin33	5'-CCTCAATGCACATGTTTGGCTCC-3'	5' - Cy5
6. Lin16-Cy5	L9831	Lin16	5'-CCTCAATGCTGCTGCTGTACTAC-3'	5' - Cy5