Christoph Walter Haudum, BSc.

In Situ Detection of AR-V7 and AR-V12 in Prostate CTCs using Padlock Probe Technology

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Ao.Univ.-Prof. Dipl.-Ing. Dr.techn. tit. Univ.-Prof. Günther Daum
Institute for Molecular Biology, Graz University of Technology

Univ. Ass. Dipl.-Ing. Amin El-Heliebi, PhD
Institute of Cell Biology, Histology and Embryology, Medical University of Graz

Graz, September 2016

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Ich hab euch lieb

"If you trust in yourself . . . and believe in your dreams . . .
and follow your star . . .
you'll still get beaten by people who spent their time working hard
and learning things and weren't so lazy."

- Terry Pratchett, *The Wee Free Men*

Abstract

Deutsche Zusammenfassung

Prostatakrebs ist die häufigste Krebserkrankung bei Männern in Österreich. Österreich weit sind es 4.800 neue Erkrankungen pro Jahr, wobei die Sterberate bei ca. 24 % der Neuerkrankungen liegt. Obwohl die Ergebnisse der Hormontherapie vielversprechend wirken, sind oder werden viele Patienten im Laufe der Therapie resistent. Die Entwicklung dieser fatalen Resistenz ist auf die Veränderung des Androgen Rezeptors hin zur Androgen Rezeptor Splice Variante 7 (AR-V7) zurückzuführen. Ein zuverlässiger, nicht invasiver Nachweis von AR-V7 wäre ein idealer klinischer Marker zur Anpassung der Therapie.

Eine Detektion von AR-V7 ist über zirkulierende Tumorzellen (circulating tumour cells, CTC) möglich. Der Goldstandard, „Cellsearch®", ist in seiner Empfindlichkeit jedoch sehr beschränkt. Unsere Gruppe arbeitet deshalb mit der CTC Isolierungsmethode „CellCollector®". Diese ist ein mit Antikörper beschichteter Draht, der in die Armvene eingeführt wird und CTCs bindet. Zur Detektion der messenger RNA (mRNA) Transkripte von AR-V7 auf einzelnen CTCs etablierten wir nun eine zuverlässige extrem spezifische Methode (In situ Detektion in Kombination mit der „Padlock Probe" Technologie), welche es uns erlaubt, einzelne mRNA Transkripte visuell darzustellen, diese zu identifizieren, quantifizieren und das Signal zu verstärken. Dadurch soll eine besser abgestimmte Therapie für den Patienten ermöglicht werden, um größtmöglichen Nutzen aus den zur Verfügung stehenden Medikamenten gegen Krebs zu erzielen.

English Abstract

Prostate cancer is the most common cancer in men in Austria. There are 4,800 new cases per year, with a mortality rate of about 24 %. Although the results of hormone therapy seem promising, there are many patients becoming resistant during the therapy. The development of these fatal resistances is due to the change in the androgen receptor to the androgen receptor splice variant 7 (AR - V7). A reliable, non-invasive detection of AR-V7 would be an ideal clinical marker to adapt therapy.

Detection of AR - V7 is possible by investigation of circulating tumor cells (CTC). The gold standard, "Cellsearch®", is very limited in its sensitivity. That is why our group is working with the CTC isolation method "CellCollector®". This is a wire coated with antibodies that is inserted in the brachial vein and binds CTCs. For the detection of messenger RNA (mRNA) transcripts of AR -V7 on the wire we now established a reliable method based on the "Padlock probe" technology, and "rolling circle amplification". This highly specific method enables us to identify individual mRNA transcripts and quantify as well as amplify the signal. This may provide the patient with customised treatment with the highest benefit.

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1 Introduction

1.1 Aim of the Thesis

We aim to detect, visualize and quantify prostate cancer specific transcripts directly in circulating tumor cells (CTCs) captured by the ”Cellcollector” wire of the company GILUPI GMBH. We therby aim to establish a novel and robust method for clinicians to optimize a patients therapy according to the androgen receptor splice variant 7 and variant 12 status. This challenging task will be done by using the ”In Situ Hybridization Method using Padlock Probe Technology” for the androgen receptor wild type as well as the androgen receptor splice variants. At first in prostate cancer cell lines VCaP and LNCaP and later on CTCs of patient. In the end we accomplished a robust method to detect androgen receptor wild type as well as variant 7 on slides and the ”Cellcollector”. Due to the low amount of patients and lack of CTCs bound to the ”Cellcollector” we were not able to detect the androgen receptor status in patients. Also the expression level of androgen receptor variant 12 seems to low to be detectable with the ”In Situ Hybridization Method”.

1.2 Prostate

The prostate gland can only be found in men and is located below the bladder and in front of the rectum. It has the size of a walnut in young men but can grow during lifetime to a much larger volume. This growth is caused by male hormones (androgens) such as testosterone.

1.3 Prostate Cancer

Prostate cancer (PCa) remains the most common malignant cancer in men and the third (10.2 %) most common cause of cancer death in males in Europe after lung and colon cancer (Torre et al., 2015). There are 4,800 new diagnosed cases of PCa each year in Austria. Around 24 % of these patients die as a result of PCa (Torre et al.,

2015). Although the prostate consists of many different cell types, almost all PCa incidents arise from abnormal cell proliferation of glandular cells. Therefore prostate cancer is classified as adenocarcinoma. PCa is mostly diagnosed due to symptoms like urination problems, trouble having or keeping an erection, blood in the urine or loss of bladder or bowel control follows by a transrectal ultrasound or a prostate specific antigen (PSA) test (UK, 2015). The amount of PSA should be, below 4 ng/mL in blood of healthy men (Catalona et al., 1994). Although there are other causes for high PSA levels, the risk of PCa increases with PSA levels. If one of these tests show abnormalities, a prostate biopsy is performed by taking 8 to 18 samples from different parts of the prostate. The normal androgen receptor (AR) pathway (see 1.2 on page 8) involves the uptake of the androgen hormone testosterone by the prostate gland cells, where some testosterone is then reduced to 5α-Dihydrotesosterone (DHT) by the 5α-reductase (see 1.1 on page 2).

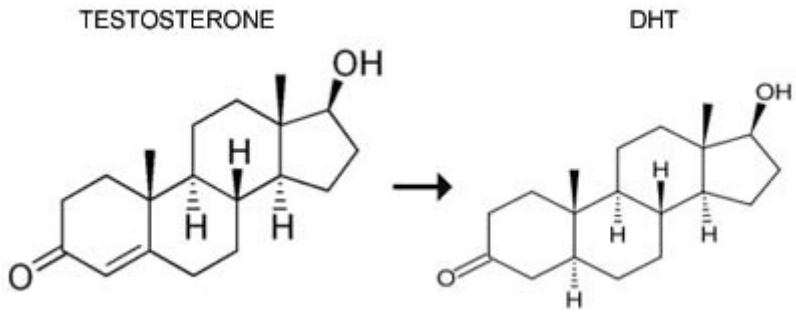

Figure 1.1: Conversion of testosterone to 5α - Dihydrotestosterone by the enzyme 5α-reductase. The two molecules only differ in one double bond in the A ring. The 5α - Dihydrotestosterone is the primary active substance in most cells (Pratt and Toft, 1997).

The expression of 5α - reductase is highly increased in prostate glands compared to normal tissue. Both, testosterone as well as, in a much higher extend, DHT bind to the androgen receptor which is located in the cytoplasm and associated with different heat shock proteins (HSP-90, HSP-70, HSP-56) (Pratt and Toft, 1997). By binding DHT/Testosterone at the ligand binding domain (LBD) a conformational change is induced forming a AF-2 binding surface which has a protein protein interaction site. This AF-2 facilitates intramolecular and intermolecular interaction between the AR-N-terminal domain (NTD) and AR-carboxy-terminal domain (CTD) of another AR molecule leading to a dimerization of two AR molecules. The HSP

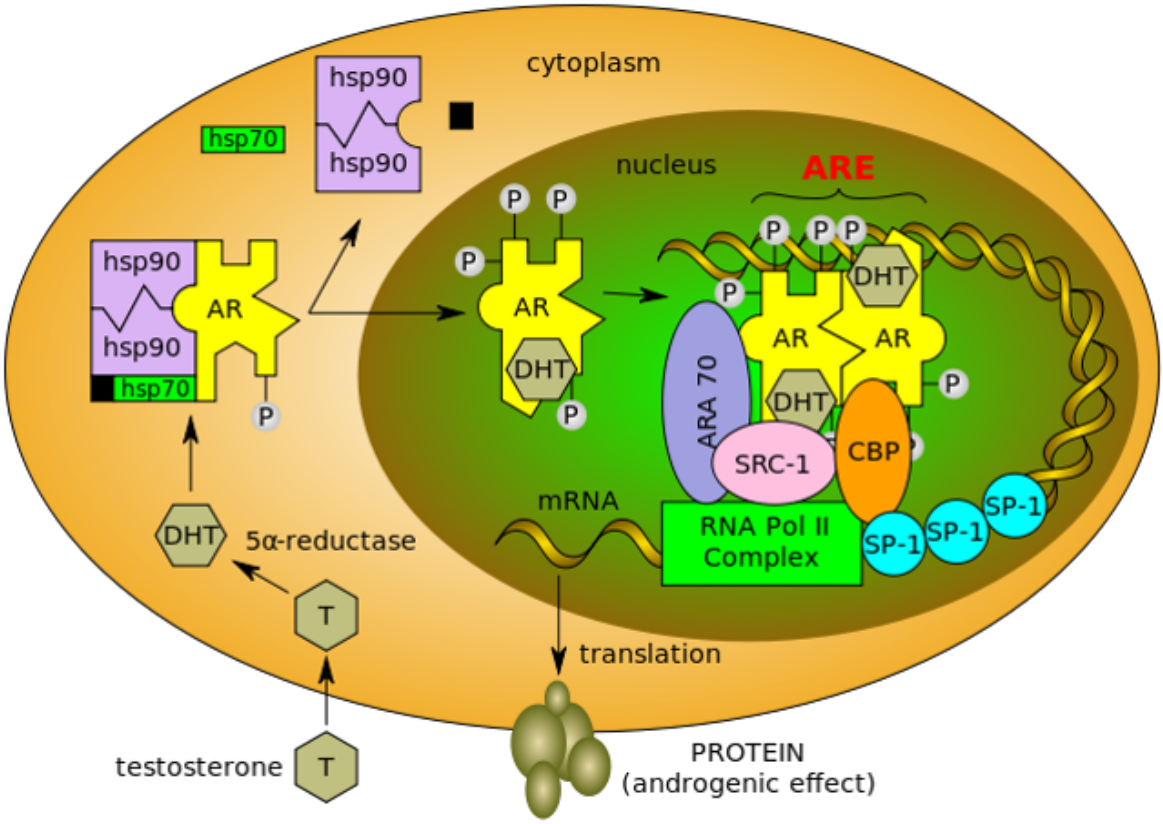

Figure 1.2: Pathway of the androgen receptor. Testosterone is taken up and converted to Dihydrotestestoron by the 5α-reductase. This binds to the androgen receptors (AR) ligand binding site which is still coupled with heat shock proteins (hsp90 and hsp70). The heat shock proteins are then seperated from the androgen receptor allowing translocation into the nucleus where proliferation is induced via binding of AR-associated protein 70 (ARA 70) (Zhou et al., 2002) and ARE. The activation of steroid receptor coactivator-1 (SRC-1) (O ate et al., 1995) and CREB-Binding Protein (CBP) (Ogryzko et al., 1996) triggers histone acylation resulting in more accessible downsteam DNA (O ate et al., 1995). SP-1 is a transcription factor also involved in chromatin remodeling and plays a role in cancer formation (Vizcaíno et al., 2015) (Modified from: Meehan and Sadar (2003)).

detaches from the AR which gives way for coactivators to bind leading to nuclear targeting of the AR-homodimer. In the nucleus AR binds to androgen response elements (AREs) modulate gene expression together with coregulators (Lonergan and Tindall, 2011). In androgen-dependend prostate cancer (ADPCa), AR promotes cell proliferation through regulation of the cell cycle G1/S transition only in the presence of androgen (Comstock and Knudsen, 2007). In contrast, androgen independent prostate cancer (AIPCa), AR is thought to remain active through a variety of potential mechanisms including AR amplification, AR mutation, increased androgen sensitivity, local androgen production and growth factor activation (Heinlein and Chang, 2004). Furthermore Wang et al. (2009) have demonstrated that AIPCa selectively and directly up-regulates M-phase genes. This may explain why maximal androgen blockade that combines AR antagonists with luteinizing hormone-releasing hormone inhibitors (LHRH), which decreases testosterone produced by the testicles, cannot prolong AIPCa patient survival (Group, 2000). Such therapies will only inhibit the ability of androgen-bound AR to promote G1/S transition in ADPCa but cannot prevent unliganded AR from accelerating M-phase transition in AIPCa.

1.3.1 Medical Therapy

If the patient is diagnosed with PCa different actions can be performed. The most obvious is the removal of the whole prostate. This method is a radical but often necessary step to eliminate the dangerous cancer tissue. If this clinical intervention can not be accomplished due to tumour size, overall condition or relapse after prostate removal a medical treatment should be considered.

Treatment involves hormonal therapy with Enzalutamide or Abiraterone targeting the androgen receptor signaling pathways (Fu et al., 2012) (see 1.2 on page 3). This so called first line treatments goal is either decreasing the level of testosterone in the blood (Abiraterone) or to act as an antagonist for the ligand binding site of the AR (Enzalutamide). Enzalutamid, is an orally taken synthetic, non-steroidal pure antiandrogen, which the IUPAC nomenclature 4-(3-(4-Cyano-3-(trifluoromethyl)phenyl)-5,5-dimethyl-4-oxo-2-thioxoimidazolidin-1-yl)-2-fluoro-N-methylbenzamide. It shows a 5 to 8 fold higher binding affinity to AR compared to its precursor drug Bicalutamide. In addition it does not promote translocation into the cell nucleus and therefore prevents binding of DNA and AR to coactivator proteins working not only as an antagonist but also as AR signaling inhibitor (Merseburger et al., 2015). In comparison Abirateron, with its systematic nomenclature (3)-17-(pyridin-3-yl)androsta-5,16-dien-3-ol, is a steroidal antiandrogen, specifically an androgen synthesis inhibitor. It is used in combination with Enzalutamid as a treatment for (metastatic) castration-resistant prostate cancer (mCRPC). Patients who already underwent androgen deprivation therapy (ADT) by surgery and LHRH inhibition are also treated with that compound.

Inhibition of CYP17 activity by Abiraterone thus decreases circulating levels of androgens such as Dehydroepiandrosterone (DHEA), testosterone, and Dihydrotestosterone (DHT) to below 1 ng/dL which is below the detection limit (Neidle, 2013).

1.3.2 Androgen Receptor Variants

The healthy male genome consists of only one copy of the androgen receptor gene at the Xq11-12 locus and it is believed to be the most important gene in the formation of prostate cancer (Ruizeveld de Winter et al., 1994)(Brown et al., 1989) . This locus consists of 9 main exons and 5 cryptic exons (CE1-CE5) (see 1.3 A on page 6). To increase diversity, cells are able to produce more than one variant of the gene by a regulatory process called alternative splicing (see 1.4.2). This event occurs in the nucleus and allows combination of existing exons to create the different splice variants of a certain gene. Splicing is a normal process, not limited to cancer cells. 95 % of all human genes undergo splicing in a tissue-specific, developmental, or signal transduction dependent manner and allows the human genome to use its around 25.000 genes to encode for $>$ 90.000 proteins (Wang et al., 2014). The androgen receptor produces a wide variety of different splice variants (see figure: 1.3 on page 6 B) with enormous range of functions . Starting from the Androgen receptor full length (AR-FL), to the 45 kDa AR45 splice variant, which is mainly expressed in the heart and to a lower extend in skeleton muscle, uterus, prostate, breast, and lung (Lu and Luo, 2013). Latter does not activate the androgen response element (ARE) but acts as a suppressor of AR-FL (Lu and Luo, 2013). The androgen receptor exons encode for different functional domains within the translated protein. Exon 1 is responsible for the N-Terminal domain (NTD) of the protein that consists of 2 transactivation regions (TAU1 and TAU5) which are needed for AR activation and allows AR homodimer formation. The DNA binding Domain (DBD) which is transcribed in Exon 2 and 3, has the nuclear localization signal (NLS) within the C-terminal end (CTE). Together with the hinge region, exon 4, it interacts with importin proteins allowing for translocation trough the nuclear pore complex. The last functional element, transcribed in exon 5-8, is the ligand binding domain (LBD). Lack of this domain leads to constitutively activation of the AR. Our work mainly focused on the splice variant lacking these domain. Namely variant 7 (AR-V7) and variant 12 (AR-V12). The splice variant 7 alone is responsible for the regulation of 117 genes (Lu and Luo, 2013).

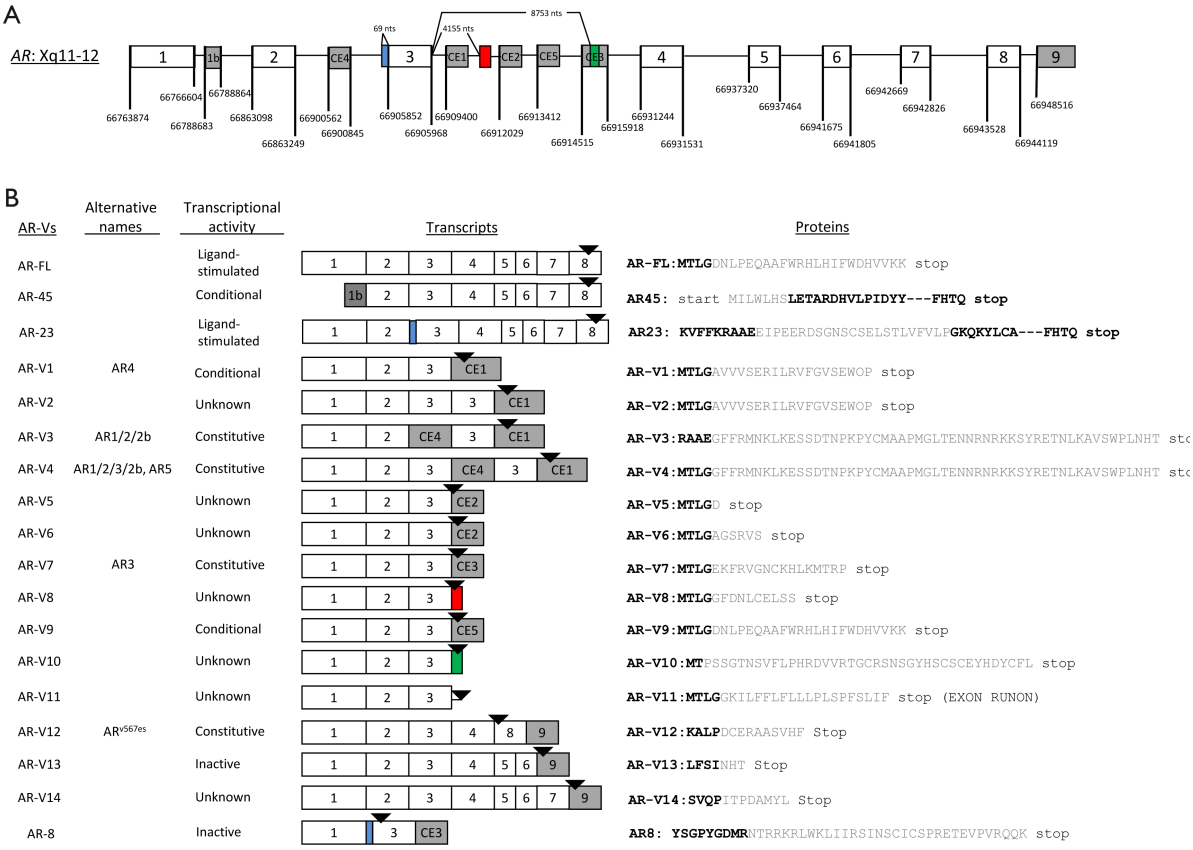

Figure 1.3: (A) The androgen receptor gen locus with its exon and intron structure annotated according to GRCh37/hg19 human genome sequences. (B) A detailed overview of all known AR-Splice variants, their name, specific mRNA sequence and the transcribed protein . Colours are matched with (A) and black arrows indicating stop codons (Modified from: Lu and Luo (2013)).

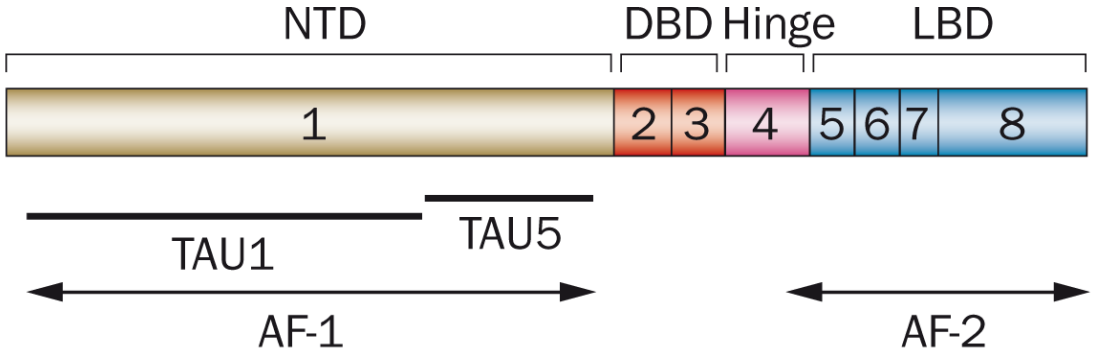

Figure 1.4: The Androgen receptor Full lenght (AR-FL) spliced exon structure mRNA encoding for N-terminal Domain (NTD), the important DNA binding Domain (DBD), a hinge and the Ligand binding domain (LBD)(Modified from: Lu et al. (2015)).

1.3.3 Resistance

Although promising results are obtained with the two drugs Abiraterone and Enzalutamid (described in 1.3.1 on page 4), patients develop a high rate of resistance to these AR-targeting drugs (Armstrong and Carducci, 2006). This resistance can be attributed to an increase of androgen receptor splice variants, especially AR-V7 during treatment (Nakazawa et al., 2015). Because of the missing LBD of AR-V7 the main mechanism behind Abiraterone and Enzalutamid (often called first line treatment), ligand reduction or antagonist binding to the LBD, show no effect. Therefore the AR-V7 translocates into the nucleus inducing proliferation (see figure: 1.2 on page 3). If the patient develops such a resistance the therapy should be changed from the first line treatment to the second line treatment which is mostly a taxane therapy (Antonarakis et al., 2015). This chemotherapeutic drug disruptes the microtubuli leading to mitotic arrest (Schiff and Horwitz, 1980). It also inhibits the cytoplasmic-to-nuclear trafficking and therefore preventing the AR from reaching its final destination (Antonarakis et al., 2015). A recent publication by (Antonarakis et al., 2015) demonstrated the detection of AR-V7 messenger RNA (mRNA) in circulating tumor cells (CTCs) based on a quantitative reverse-transcriptase–polymerase-chain-reaction (qRT-PCR) assay. The authors have shown that detection of AR-V7 mRNA correlates with resistance to anti-hormonal therapies. AR-V7 positive patients treated with the new taxane chemotherapy showed a longer progression free time than AR-V7 positive patient treated with Enzalutamide or Abiraterone (see figure: 1.5 on page 8). Therefore AR-V7 status is a good clinical marker to determine the best therapy for a PCa patient.

1.4 Splicing

There are many essential steps needed for an eukaryotic gene to become translated into protein. One of these steps is RNA splicing. mRNA is generated by exclusion of introns (intragenic region) from a pre-mRNA derived from a eukaryotic gene. The remaining exons are linked together forming the mature mRNA. Splicing is not limited to mRNA which is translated to proteins, it also occurs in other species like tRNA or rRNA (William Roy and Gilbert, 2006).

1.4.1 Splicosomal Splicing

This mechanism occurs either during or shortly after transcription using a molecular machinery called spliceosome, which is a complex of small nuclear ribonucleoproteins

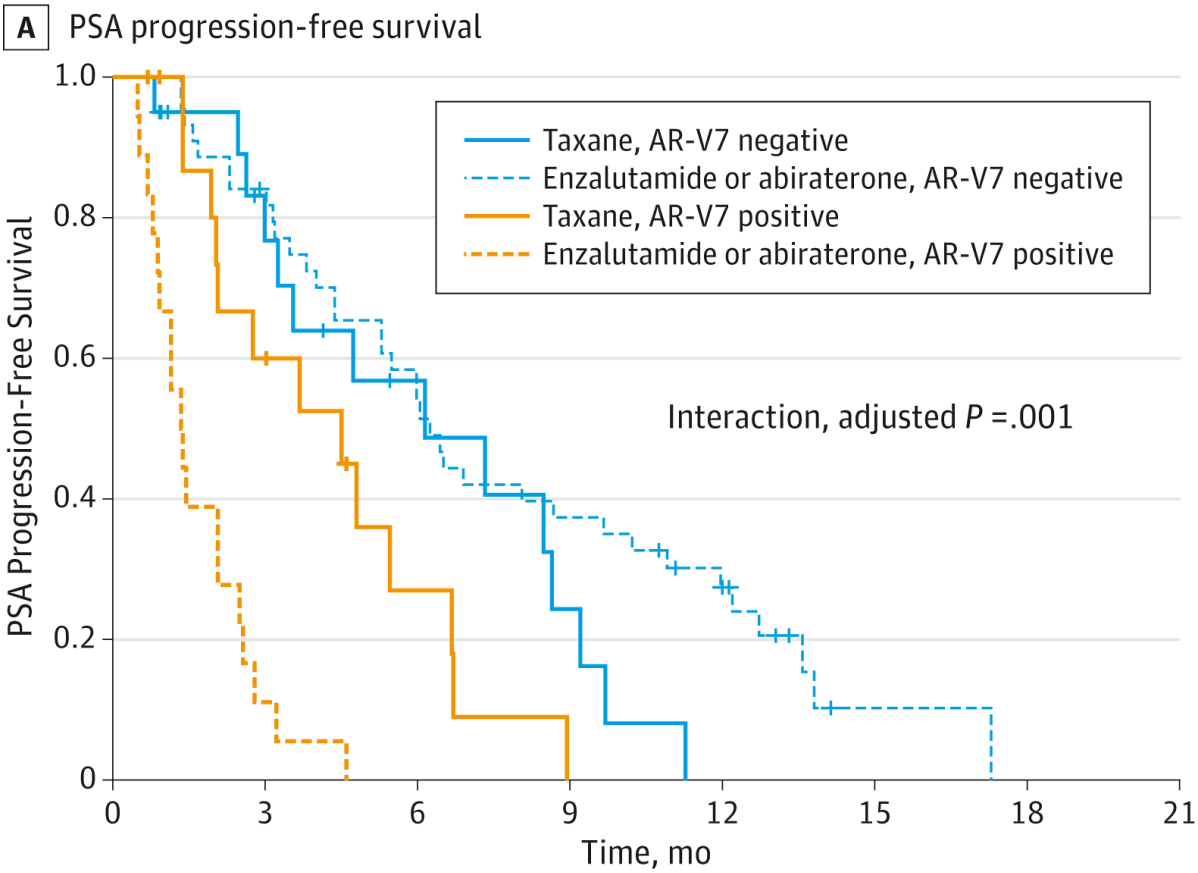

Figure 1.5: The AR-V7 status and the used pharmaceutical compound have a big impact on the PSA Progression Free Survival. AR-V7 positive patients treated with Taxane show a nearly doubled PSA Progression free time in comparison to patients treated with Enzalutamid. Patients with AR-V7 negative status have a shorter progression free survival when treated with Taxane compared to Enzalutamide or Abiraterone (Antonarakis et al., 2015)(Modified from: Antonarakis et al. (2015)).

(snRNPs), most important ones are U1, U2, U4, U5 and U6 (Will and Lührmann, 2011). Also self splicing introns or ribozymes capable of catalyzing their own excision from their parental RNA molecule are known (Zaher and Unrau, 2007). The introns removed by the splicosome need specific motifs which are detected (see figure: 1.6 on page 9).

The intron require, a donor site (5′ end of the intron), a branch site (near the 3′ end of the intron) and an acceptor site (3′ end of the intron) for splicing (Patel and Steitz, 2003). The splice donor site includes an highly conserved sequence GU at the 5′ end of the intron (see figure:1.6 marked with 4), within a larger, less conserved area. The splice acceptor site at the 3′ end of the intron terminates the intron with an again highly conserved AG (marked with 1) sequence (Patel and Steitz, 2003). 5′-upstream from the AG there is a pyrimidines (U and C) rich region, also called polypyrimidine tract (marked with 2). Further upstream from this polypyrimidine tract is the branchpoint, including an A (marked with 3) involved in the formation of the lariat loop, which later during joining of two exons, will be released (Cheng and Menees, 2011).

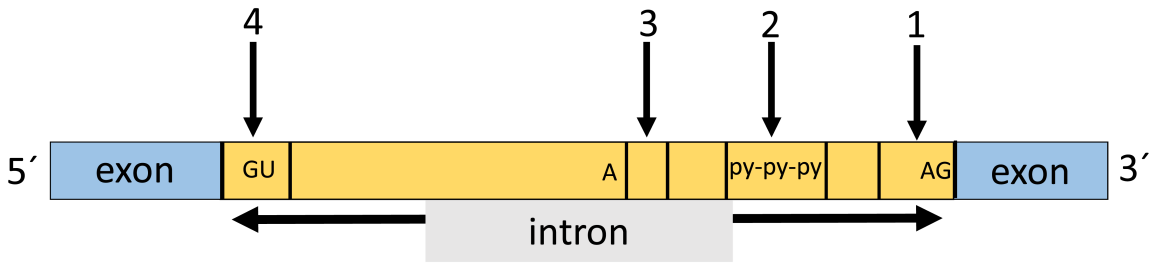

Figure 1.6: The motifs, 5′ GU (4) branchpoint A (3), the polypyrimidine region (2) and the 3′ AG (1) are needed for a splicing. Mutation can lead to cryptic splice sites but also to unspliced introns which would otherwise only affect a single amino acid. (Modified from: Taggart et al. (2012)).

Spliceosomal splicing and self-splicing can be split into a two-step biochemical process, both transesterification reactions between RNA nucleotides. The 2′-OH of a branchpoint A nucleotide within the intron, performs a nucleophilic attack on the first nucleotide of the intron at the 5′ splice site, forming the lariat intermediate (Fica et al., 2013). Afterwards, the 3′OH of the released 5′ exon performs a nucleophilic attack at the very last nucleotide of the intron at the 3′ splice site, which then joins the exons and releasing the intron lariat (Fica et al., 2013).

1.4.2 Alternative Splicing

To increase the amount of proteins encoded by the 25,000 genes, evolution came up with alternative splicing (also called differential splicing). This allows the organism to include or exclude exons which leads to a altered protein isoform with a different amino acid sequence and often also different biological functions (Black, 2003). Splicing is regulated by trans-acting proteins (activators and repressors) and corresponding cis-acting regulatory sites (enhancers and silencers) on the pre-mRNA (Matlin et al., 2005). Splicing silencers, e.g. polypyrimidine tract binding protein (PTB) or heterogeneous nuclear ribonucleoproteins (hnRNPs) decrease, and enhancers e.g. members of the SR protein family, increase the probability that a nearby site will be used as a splice junction (Matlin et al., 2005).

Five basic modes of alternative splicing are generally recognized (as reviewed in: Sammeth et al. (2008) and Black (2003)):

- Exon skipping or cassette exon - most common mode in mammalian pre-mRNAs. One or more exon may be spliced out of the pre-mRNA transcript or retained.
- Mutually exclusive exons - One of two exons is retained in mRNAs after splicing, but not both.
- Alternative donor site - An alternative 5′ splice junction (donor site) is used.
- Alternative acceptor site - An alternative 3′ splice junction (acceptor site) is used.
- Intron retention - A sequence is spliced out as an intron or simply retained. The remaing sequence is not intron flanked which is the difference between exon skipping.

1.5 Circulating Tumor cells - CTCs

A cancerous tumor contains millions or even billions of cells accumulating genetic mutations causing growth, division, and invasion of the local tissue in which they are embedded. Not all of these cells remain bound to the primary tumor but rather detach from it and are swept away by the lymphatic system or bloodstream (Williams, 2013). These cells are so called cirulating-tumor-cells (CTCs). Although the primary tumour consists of a lot of different genetically heterogeneous cells, this CTCs can give precious information about its genetic composition without a dangerous autopsy and therefore are a perfect marker for monitoring patients disease status (Paterlini-Brechot and Benali, 2007). It is estimated that among the cells that have detached from the primary tumor, only 0.01% can form metastases (Zhe et al., 2011). With a

frequency of 1 - 10 CTCs per ml blood, compared to 5x 10^9 erythrocytes per ml blood, CTCs are tremendously outnumbered in the blood (Miller et al., 2010).

1.5.1 Cellsearch

Therefore detection of these rare cells is challenging. The goldstandard for detecting CTCs is the so called „Cellsearch®" method and uses 7.5 mL blood. This approach is based on iron nano particles coated with antibodies against EpCAM. CTCs highly express EpCAM which leads to an CTC enrichment. "Cellsearch®" is the only FDA approved routine method for collecting CTCs. Its disadvantage is the low amount of blood which can be used and therefore its capacity to collect CTCs from patients is limited.

1.5.2 Cellcollector

Another method, for isolating circulating tumor cells, is the so called "Cellcollector™" from the company GILUPI GmbH (see figure: 1.7 on page 12). This is a medical wire which is inserted into the brachial arm vein using a conventional cannula. The tip of the wire is coated with and anti-EpCAM antibody which binds CTCS from the blood stream. The wire stays in the vein for 30 minutes allowing $>$ 1.5 L of blood to pass it (Alix-Panabieres and Pantel, 2013). This tremendously increases the volume compared to the "Cellsearch" method and therefore the probability of detecting CTCs. It has been shown in prostate cancer patients with either a localised (PCa-l) or metastatic (PCa-m) tumor that the Cellcollector can detect CTCs with a probability of 77.5 % (55 patients/71 patients) (PCa-l: 55.2 % [16/29]; PCa-m: 88.1 % [37/42]). The cancer patient positive for CTCs using "Cellsearch®" were only 42.2 % (30/71) in detecting CTCs (PCa-l: 17.2 % [5/29]; PCa-m: 61.9 % [26/42]) (Theil et al., 2014). Therefore "Cellcollector™" increases the probability to find CTCs compared to the "Cellcearch" method. There are currently two other version of the "Cellcollector™" wire on the market. The second version is not yet approved for human use and is a thrilled wire. The thrilling increases the surface and boosts the amount of CTCs binding to the wire (see figure:1.8).

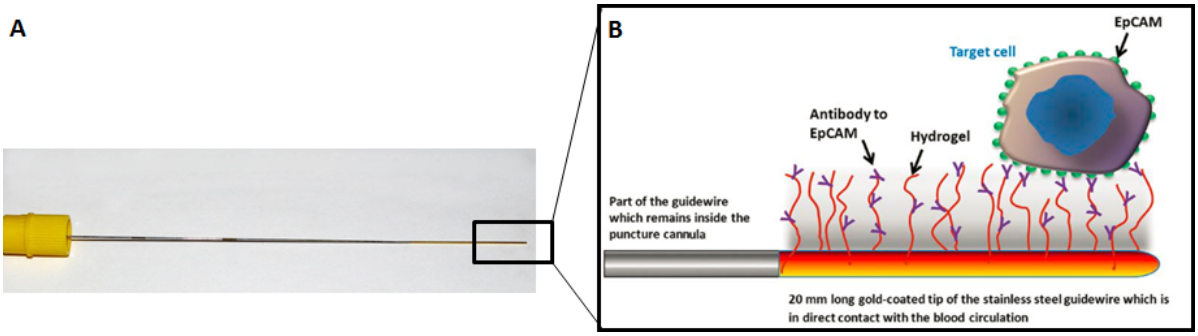

Figure 1.7: A) The "Cellcollector™" wire from the company GILUPI GmbH. It is inserted into the brachial arm vein, whereas the golden tip needs to be inside the blood stream. (B) The golden tip of the wire is coated with Anti-EpCAM antibodies which bind CTCs (Modified from: Saucedo-Zeni et al. (2012)).

1.6 In Situ Hybridisation using Padlock Probe Technology

Nevertheless, the mRNA of CTCs in former studies were mainly investigated by an indirect qRT-PCR approach which precludes any further cell based analysis (Strati et al. (2011), Antonarakis et al. (2015)). On the one side there exist several cell based assays to isolate CTCs in prostate cancer, but on the other side mRNA assays of these isolated cells remain challenging. To combine a cell based approach with mRNA analysis, we established a novel method based on padlock probe technology. This padlock probe approach can differentiate between mRNA transcripts which are just different in one single nucleotide mutation and is based on padlock probing and rolling-circle amplification (Larsson et al., 2004). This procedure allows highly specific and sensitive recognition of nucleic acid sequences with efficient local signal amplification (see figure: 1.9 on page 14) (Larsson et al., 2004). The "In Situ" is a multi-step process were mRNA of interest, targeted by a specific primer, is reverse transcribed to cDNA. If the cDNA is complimentary to the cDNA binding sequence of a padlock probe, consisting out of detection probe-, cDNA binding sequence and linker sequences, it binds, gets ligated and forms a circular DNA molecule. After ligation the circular DNA molecule is amplified and can be visualised by a detection oligonucleotide linked with a fluorophore.

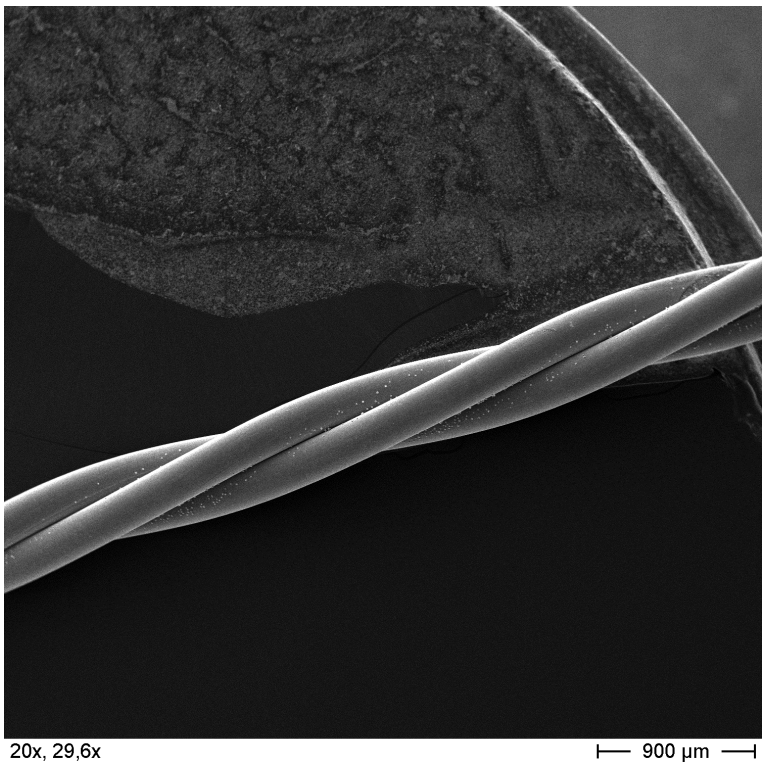

Figure 1.8: An electron-microscopy image of the thrilled "Cellcollector" wire. The dots represent attached cell culture cells. This Image was produced by Gerd Leitinger, Elisabeth Pritz and Shukun Chen of the Institute of Cell Biology, Histology and Embryology Medical University of Graz.

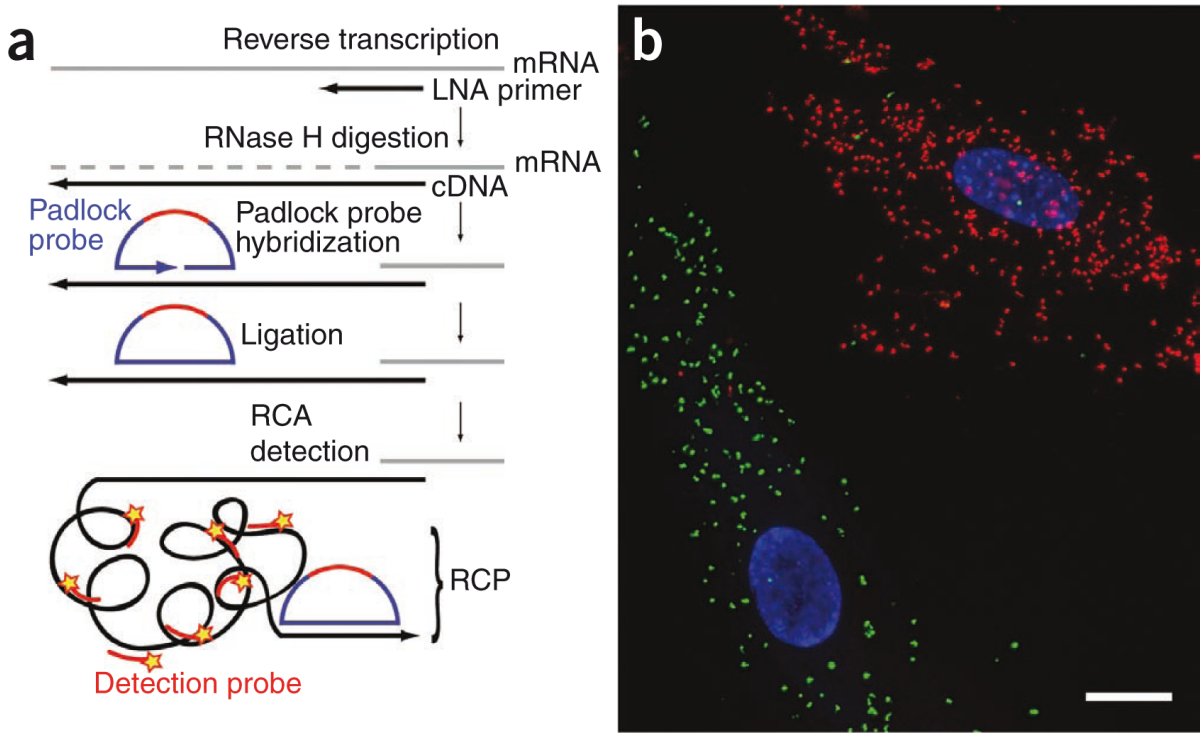

Figure 1.9: Summary of the Padlock probe technology. a) Reverse transcription of mRNA into cDNA, followed by digestion of the mRNA by RNase H and ligation of the Padlock probe to the cDNA. A "rolling circle amplification" (RCA) follows producing large amount of rolling circle products (RCPs). These can be targeted with fluorescent detection probes (shown in red with a star). b) Detection of 2 different transcripts using the Padlock probe technology . Red signals correspond to human beta actin, green signals mouse beta actin. Scale bar = 20 μm (Modified from: Larsson et al. (2010)).

2 Method and Results

As this thesis is a technical approach, no statistical analysis could be performed. The presented results should only be interpreted as a trial and further measurements need to be done to statistically prove them. Images were taken with the Zeiss Observer.Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany) with a X10, X20 or X40 objective and visualised with the software with ZEN 2012 black software (Carl Zeiss, Version 8.1). Maximum intensity projection of Z-stacks was performed and combined in one layer with the ZEN 2 2014 blue software (Carl Zeiss) (Siwetz et al., 2016). Contrast and brightness of each image were adjusted for better visualization again with the software ZEN 2. To exclude false positive signals each fluorescent channel was checked. A detection of a signal in multiple channels is a sign for a false positive finding.

2.1 Signal Counting using Cellprofiler

The high amount of cells as well as signals did not allow for manual counting. Therefore we designed a pipeline consisting of different modules in the CellProfiler Analyst software to analyse the images (Carpenter et al., 2006). Every image set (for example all images of CH0012, or W0027) where analysed with specific settings in the pipeline. First the signals of two images were counted by hand and the pipeline settings adapted until the results fit the hand counted ones. This pipeline was then applied to the other images set pictures. This should limit the human error. The modules used were

- Crop - Only used in wire images to choose the part of the picture which is in focus
- IdentifyPrimaryObjects - To identify nuclei, "In Situ" Signals and the area outside all cells
- IdentifySecondaryObjects - To set the cell borders to be 100 pixels away from the nuclei
- IdentifyTertiaryObjects - To determine the space between nuclei and cell boarder which is labelled as cytoplasm

- EnhanceOrSuppressFeatures - This module allows suppressing unspecific, dark signals, and enhance wanted ones
- ConvertObjectsToImage - Converts the area outside the cell into one image
- ImageMath - To invert the area outside the cell from black to white which can later be identified as one big object
- RelateObjects - To relate signals to one specific cell or the outside of cells
- DisplayDataOnImage - Displays data, for example the number of signals per cell on the image
- OverlayOutlines - Displays the signals and the cell boarder on the image
- SaveImages - This module saves the image (see 2.30 on page 63) with displayed data as well as signals
- ExportToSpreadsheet - Saves the amount of signals, location and other parameters of each cell into a txt file

2.2 In Situ hybridisation using padlock probe technology

As already described in section: 1.6 on page 12 we used the ”In Situ hybridisation method using padlock probe technology” (in short “In Situ”) for our approach to visualise splice variants of the androgen receptor on mRNA level. This method involves many steps and a deep knowledge of the precise mRNA sequence of interest is needed. Otherwise unspecific and therefore false positive results will occur. All experimental settings are listed in the appendix together with a list of all primers and padlocks for all targets.

The ”In Situ” can be separated into four main parts (see figure: 1.9 on page 14, left side):

- Reverse Transcription
- Ligation
- Rolling circle amplification
- Detection probe hybridisation

Prior to the start of the ”In Situ” it is important to determine which mRNA should be detected. In our case this was primary: AR-FL, AR-V7, AR-V12, PSA and B-Actin as a control. Based on the fluorescence microscope, 4 fluorescence colors can be used simultaneously: DAPI, FITC, CY3 and Cy5.

2.2.1 In Situ - Probe Design

The design of the oligonucleotides is a critical step in the planning of the "In Situ" and was already described by Weibrecht et al. (2013). In the first step it is important to know the exact sequence of the target mRNA. This is needed to design a primer for the reverse transcription which transcribes the mRNA to a more stable cDNA (figure 1.9, left side). On this molecule the padlock probes can bind and the amplification can be performed. The binding sites for the primers have to be specific for the mRNA of interest otherwise false positive cDNA transcripts can occur. In case of incomplete cDNA synthesis, the padlock has no site to bind which results in no signals.

Primer Design

The first primers (internal labeling: Go034 and AR-FL_ LNA_ 1) we used were for the padlock already designed by Kiflemariam et al. (2014) (external labeling: Pd_AR, internal labeling: Go035 and plp_ AR-FL1) to detect AR-FL. The software CLC Main Workbench was used for calculation the optimal primer position. It should not be more than 20 nt 5' downstream to the binding site of the padlock probe Go035. The primer itself with the sequence

C+CA+TC+TG+GT+CG+TCCACGTGTAAGTT

is 25 bases long and spans from the bases position 2182 to 2206 of the AR-FL. For optimal amplification, primers should be between 20 and 30 bases long. Larsson et al. (2010) showed that the amount of signals could not be increased by changing the primer length from 25 bases to 30 bases . The six "+" symbols after every second base stand for the base being a locked nucleic acids (LNAs). This bases highly improve mismatch discrimination (You et al., 2006). The amount of LNAs used per primer, with a plateau at seven LNAs (Johansson, 2009), increases its specificity but also the costs. 5 LNA bases at every second base seemed most reasonable according to the data of Johansson (2009) and was also used by Larsson et al. (2010).

With this method we designed also primers for the AR-V7 and AR-V12 (see table 2.1 on page 20). The primer for AR-V7 (internal labeling: Go037 and AR-V7_ LNA_ 1) which is 23 bases long binds at the cryptic Exon 3 (CE3) (see figure: 1.3 on page 6) and allows reverse transcription of the Exon 3-CE3 site where the padlock probe for AR-V7 (internal labeling: Go038 and plp_AR-V7) then binds. The primer for detecting

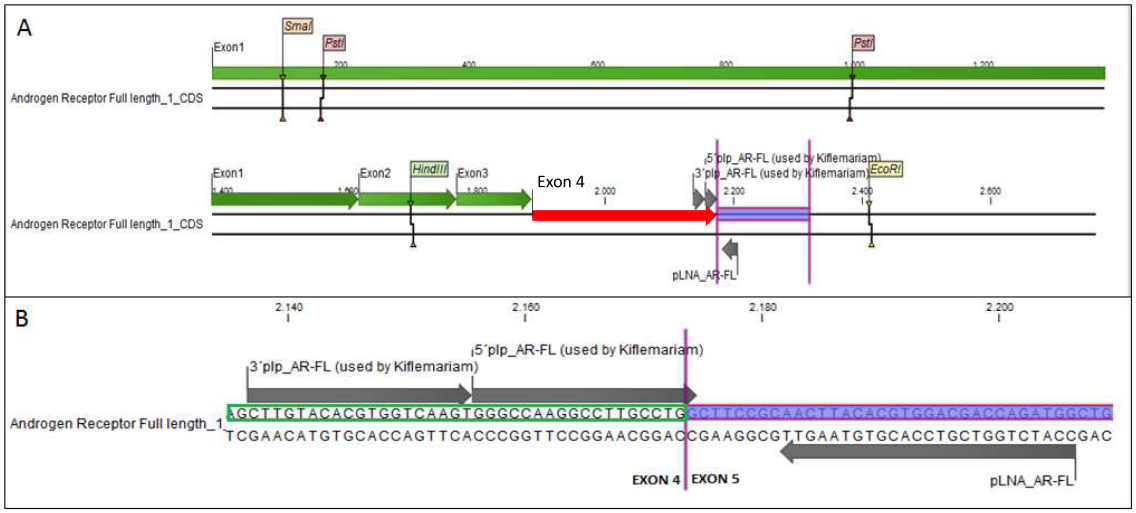

Figure 2.1: Edited screenshot of the CLC-Workbench software. (A) Sequence of the Androgen Receptor Full-Length (AR-FL) was used. The green bars indicating Exon 1-3. The violet bar represent Exon 5 where the specific LNA-primer will bind (grey arrow labeled with pLNA_ AR-FL). The region between the green Exon 3 and the violet Exon 5 is Exon 4 (red arrow). Beginning from the LNA-primer in Exon 5, the mRNA gets reverse transcribed toward the 3' end Exon 3. When the specific padlock probe binding region in Exon 4 reverse transcribed to cDNA, both arms of the padlock probe will bind. Therefore the closer this sequence is to the primer, the higher is the probability of a being reverse transcribed and accessible by the padlock probe. If there is no Exon 4 - Exon 5 site, because of different splicing, the padlock probe cannot bind and no "In Situ" signals will be detectable. (B) A zoom into the very same Exon 4 - Exon 5 site of the AR-FL (Exon 4 marked in green and Exon 5 in violet) Both arms of the padlock probe (5' plp_ AR-FL and 3' plp_ AR-FL) bind to the cDNA.

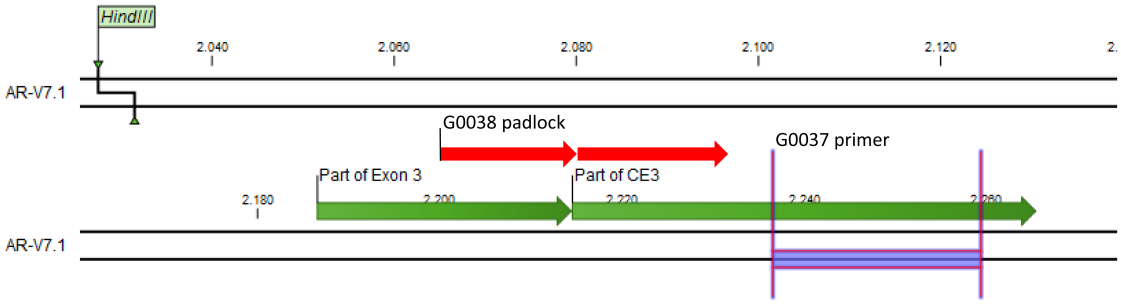

Figure 2.2: The figure shows the Exon 3 and CE3 site. For visualisation there are only the part of the Exon near the Exon junction marked as green arrows. Exon 3 would extend further 5‘ and CE3 more into 3‘direction. Red arrows indicating both cDNA binding sequences of the padlock probe Go038 and there corresponding binding sites on the cDNA. 7 nucleotides 3‘-downstream of that sequence is the binding region for Go037 primer located. Being near the Go038 allows for a higher number of successfully transcribed padlock probe binding regions.

AR-V12 is located near the Exon 8 - Exon 9 site. This site on the mRNA is specific for this splice variant making it the perfect target.

After determination of the optimal region, primers and padlocks were designed according the CLC-Workbench score (see figure: 2.3 and figure: 2.4 on page 20 and 21). On a scale from 0 to 100 with 100 being the best. Our experience with primers showed that scores greater 30 are preferred but even scores from 10-15 work efficient. The sequence also have to be blasted (see: http://blast.ncbi.nlm.nih.gov/Blast.cgi) to eliminate the possibility of false positive target sequences. Selected oligos were then checked for hairpin and self- or heterodimer formation using OligoAnalyzer 3.1 from the company IDT
(see: http://eu.idtdna.com/calc/analyzer).

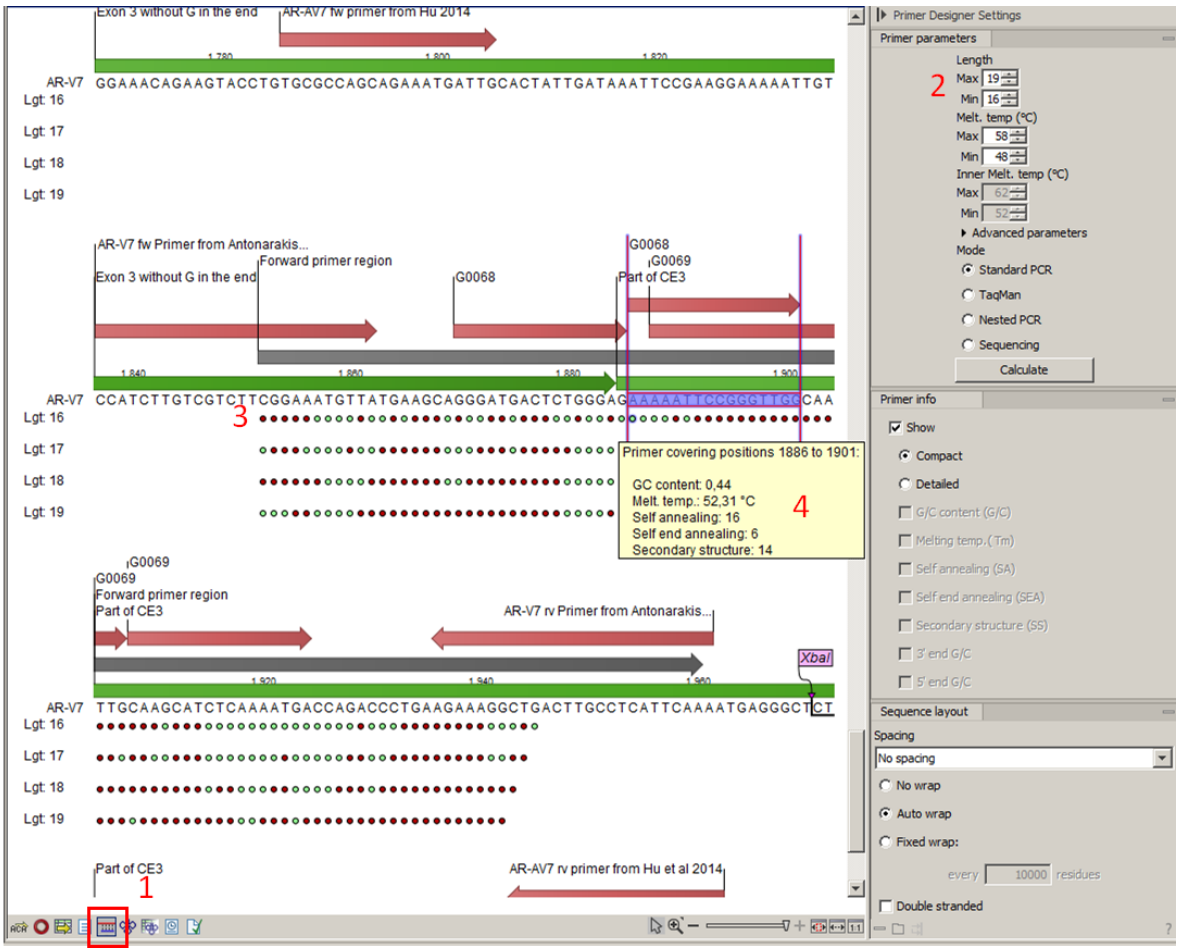

Figure 2.3: The loaded sequence of AR-V7 was used as template to design a padlock (Go068). The button "Show Primer designer" (1) opens a new toolbox (2) which allows to customize the length of the primer or arms of the padlock. The "Primer designer lines" (3) show if a primer at that specific position and length is optimal (green) or not good (red) in the sense of GC-content, melting temperature and other parameters. All parameters at the specific position can be seen with mouseover (4).

Table 2.1: The designed primers used for detection of different splice variants as well as PSA

Internal Labeling	Name	Primer Sequence
Go034	AR-FL_LNA_1	C+CA+TC+TG+GT+CG+TC CACGTGTAAGTT
Go037	AR-V7_LNA_1	T+CT+GG+TC+AT+TT+TGA GATGCTTGC
Go039	AR-V12_LNA_1	G+AT+TA+GC+AG+GT+CA AAAGTG
Go041	PSA_LNA_1	G+AG+GT+CCA+CAC+ACT +GAAGTTT

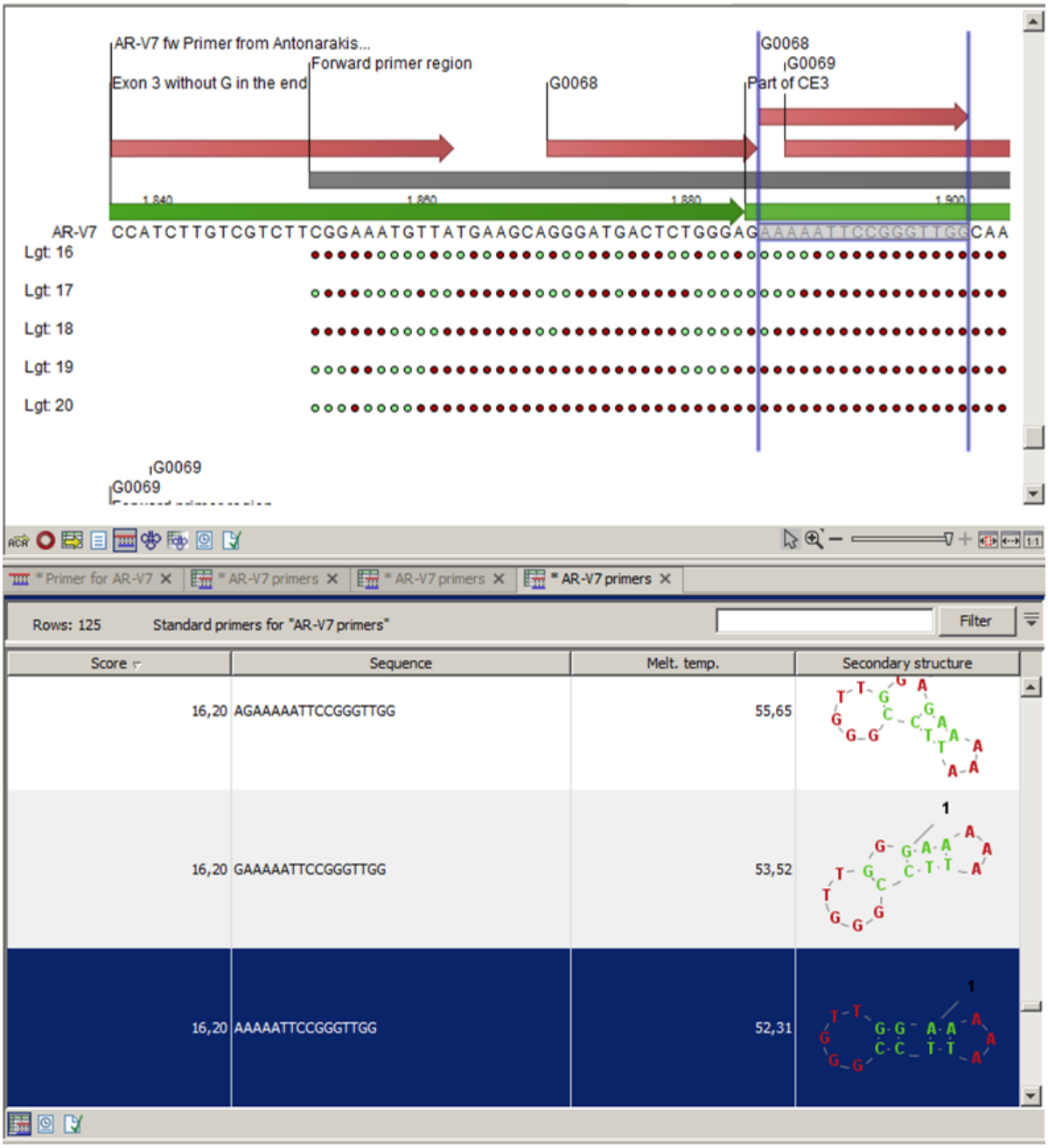

Figure 2.4: By marking a specific nucleotide sequence and pressing the "Calculate" button in the "Primer Parameters" toolbox, all primers in that region are validated and the secondary structure determined. The higher the "Score" the better the primer, being greater 30 is optimal and scores down to 10 reasonable.

Padlock Design

After the mRNA region of interests was successfully reverse transcribed into cDNA and the remaining mRNA digested RNase H, the padlock probe can be added. This linear 70 bases to 90 bases long oligonucleotide consists of different functional regions (see figure: 2.5 on page 22). By binding the cDNA with both (3′ and 5′) arms next to each other, a loop is formed. The last base (3′ end) of the padlock is important for the specifity. This base has to be basepair correctly in order for the ligase, in the following step, to close the nick. The length of each arm can vary between 15 bases and 25 bases being 25 bases the optimum, because of higher specificity and binding strength. The base composition of the cDNA often doesnt̀ allow longer padlock arms at the needed cDNA site because of formation of inhibitory secondary structures of the padlock. Each or at least on cDNA binding arm is followed by a linker sequence. This sequence consists of 4 - 10 random bases (N) minimizing the bending stress caused in the oligonucleotide by loop formation. The final important region is the "Detection probe binding site". One out of a pool of three, Lin16 (seq: CCTCAATGCTGCTGCTGTACTAC), Lin33 (seq: CCTCAATGCACATGTTTGGCTCC) and B2DO (seq: AGTAGCCGTGACTATCGACT), can be used. This 20 to 25 nt long detection oligonucleotide coupled with a fluorophor at the 5´-end will bind their complementary sequence in the backbone of the padlock probe. By using these backbones in combination with different dyes (Fluorescein (FITC), Cyanine (Cy5, Cy3)) detection of up to three targets in one sample is possible.

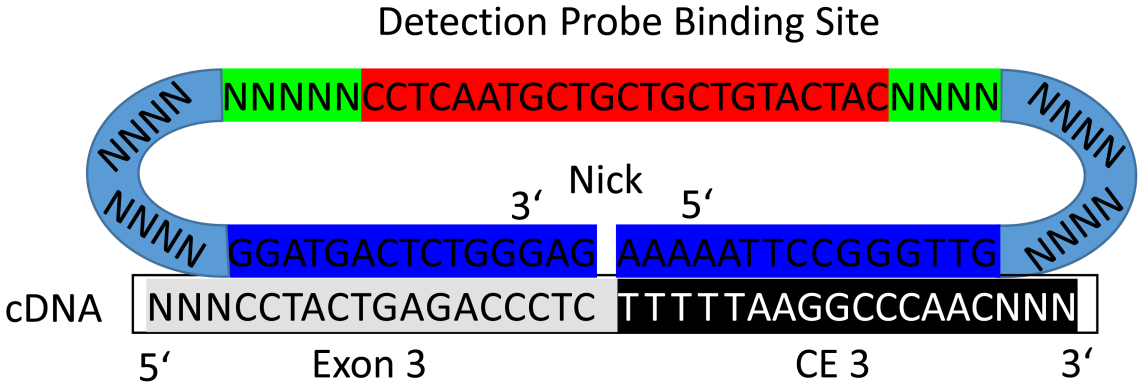

Figure 2.5: The padlock binds with the two ends (also called padlock-arms in dark blue) to the complementary cDNA. To form a loop it is necessary that the padlock has a flexible linker sequence (N, light blue) which flanks (N, green) the detection oligo binding sequence (red).

2.3 "In Situ" on Slides

2.3.1 Comparison of AR-FL1 and AR-FL2 Padlock Probe and Detection of AR-V7 and AR-V12

The first step of establishing a new "In Situ" protocol was to use the available probes (see table 2.1 on page 20 and 2.2 on page 23) on different cell lines to verify their specificity. The cell lines used were prostate cancer cell lines VCaP, LNCaP as they are positive for androgen receptor and as a negative control the colon cancer cell line SW620. Three 50 μl spots were attached to each slide and in each of the three spots different padlocks were used according to figure: 2.6 on page 24.

Table 2.2: The designed primers used for detection of different splice variants as well as PSA. Blue indicating the cDNA binding site, padlock binding sequence with the backbone Lin33 are marked in red, Lin16 in gray and B2_DO in green.

Internal Labeling	Primer Sequence
Go035 plp_AR-FL1	5 ′ - GGGCCAAGGCCTTGCCTGG CCTCAATGCACATGTTTGGCTCCTAAAGTCGGAAGTACTACTCTCTCTTGTACACGTGGTCAAGT
Go036 plp_AR-FL2	5′-GGGCCAAGGCCTTGCCTGGTTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGCTTGTACACGTGGTCAAGT
Go038 plp_AR-V7	5 ′ - AAAAATTCCGGGTTGTTCCTTTTACGA CCTCAATGCTGCTGCTGTACTACTCTTCGGATGACTCTGGGAG
Go040 plp_AR-V12	5′-AAAAATTCCGGGTTGTCCTAGTAATC AGTAGCCGTGACTATCGACTGGTTCAAAGAGAGAGCTGCATCAG
Go042 plp_PSA_1	5′-ACCAGAGGAGTTCTTGTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGCTGGGGCAGCATTGA
Go043 plp_PSA_2	5′-GACCCCAAAGAAACTTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGACCAGAGGAGTTCTT

IN SITU - RT-Step

In the first step secure seals from the company Sigma-Aldrich are mounted on the slides (see figure:2.6 on page 24). This secure seal can hold up to 50 μl of solution. The chambers of the secure seals were then filled with 50 μl of 1x DEPC-PBS-Tween and incubated for 5 minutes to rehydrate the dehydrated cells. The solution was sucked

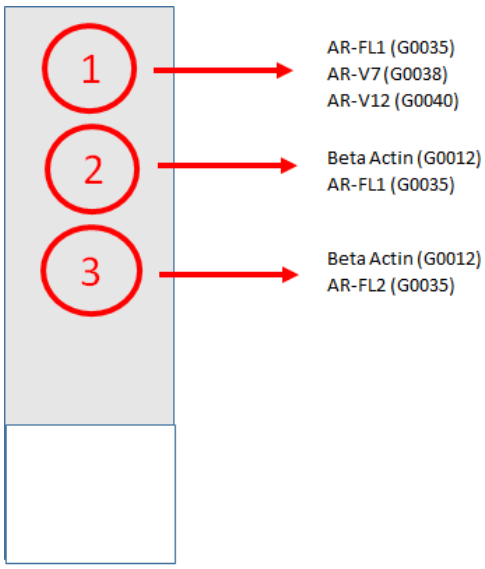

Figure 2.6: Each red circle represents one experimental setting on every slide. The experiment in the first (1) circle was to detect the Androgen Rezeptor Full length 1 padlock (AR-FL1) as well as the splice Variants 7 (AR-V7) and 12 (AR-V12) on slides. It was also used to determine the specificity of not only AR-FL1 but also of the newly designed padlocks and primers for AR-V7 and AR-V12. The second (2) and third (3) experimental setting was to compare the efficiency of the two AR-FL padlock probes.

off and refiled with 0.1 M HCL-DEPC-H2O for 2 minutes to permeabilize the cell walls. The chamber was then washed 2 times with 1x DEPC-PBS-Tween for 5 minutes. Then the master mix for the reverse transcription step was added (see table 2.3 on page 25). The secure seals were then closed with a small piece of PCR tape to avoid evaporation. The slides were incubated in a humid chamber filled with DEPC-H2O and incubated for 3 hours at 45 °C or ON at RT.

The chambers were then filled with 3 % Formaldehyde in DEPC-PBS at RT for 10 minutes for fixation and washed afterwards 2 times with DEPC-PBS-Tween for 2 minutes.

IN SITU - Ligation Step

After mRNA is reverse transcribed to cDNA and the remaining mRNA strand digested, the ligation step takes place. As described in figure 1.9 left side on page 14, if the linear padlock probes perfectly binds the cDNA with both ends, it forms a loop which is then ligated.

Table 2.3: A calculation of the used reagents. The final concentration means is the concentration per 50 μl experiment. Because only 45 μl is needed for the 50 μl secure seal, the mastermix is calculated for 50 μl to have enough in case of pipetting mistakes. The G numbers are unique for every probe. G0001 is the LNA primer for detection of Beta Actin. G0034/G0037/G0039 for Androgen Receptor Full length/Variant 7/ Variant 12 respectively.

Master Mix folds:			1	3	6
In situ RT	stock	final	MIXx1 (LNAs) [μl]	SPOT 1 [μl]	Spot 2+3 [μl]
TranscriptMe RT (pipet last)	200 U/μl	20 U/μl	5	15	30
RT buffer	10X	1X	5	15	30
Rnase Inhibitor (pipet last)	40 U/μl	1 U/μl	1.25	3.75	7.5
pACTB (G0001)	10 μM	1 μM	5	0	30
AR-FL_LNA_1 (G0034)	10 μM	1 μM	5	15	30
AR-V7_LNA_1 (G0037)	10 μM	1 μM	5	15	0
AR-V12_LNA_1 (G0039)	10 μM	1 μM	5	15	0
dNTP	10 mM	0.5 mM	2.5	7.5	15
BSA	20 μg/μl	0.2 μg/μl	0.5	1.5	3
DEPC H2O			15.75	62.25	154.5
Final Volume			50	150	300

Figure 2.7: One secure seal applied to a superfrost slide. The secure seal chambers are available with different reaction volume capacities starting from 35 μl up to 620 μl. For our approach we use 50 μl chambers. The chambers can be filled with a pipette through one of two small holes. During pipetting, bubble formation should be avoinded.

After the RT-step 50 μl of the ligation mastermix (see table 2.4 on page 27) was added in each chamber of the secure seal. The chamber was then again sealed and incubated for 30 min at 37 °C and then 45 min at 45 °C.

Table 2.4: The G numbers G0035/G0036/G0038/G0040/G0012 are the padlock probes for Androgen Receptor Full length primer 1/ Full length primer 2/ Variant 7/ Variant 12/ Beta Actin. Dependent on the experimental setting (figure 5), not every padlock probe is inserted into every chamber.

Master Mix folds:				3	3	3
LIGATION of padlocks	stock	final	1x [μl]	SPOT 1 [μl]	SPOT 2 [μl]	SPOT 3 [μl]
Ampligase (pipet last)	5 U/μl	0.5 U/μl	5	15	15	15
AMP buffer	10X	1X	5	15	15	15
Rnase H (pipet last)	5 U/μl	0.4 U/μl	4	12	12	12
BSA	20 μg/μl	0.2 μg/μl	0.5	1.5	1.5	1.5
KCl	1 M	0.05 M	2.5	7.5	7.5	7.5
Formamide	1	0.2	10	30	30	30
plp_AR-FL1 (G0035)	10 μM	0.1 μM	0.5	1.5	1.5	0
plp_AR-FL2(G0036)	10 μM	0.1 μM	0.5	0	0	1.5
plp_AR-V7 (G0038)	10 μM	0.1 μM	0.5	1.5	0	0
plp_AR-V12 (G0040)	10 μM	0.1 μM	0.5	1.5	0	0
PdACT1cDNA (G0012)	10 μM	0.1 μM	0.5	0	1.5	1.5
DEPC H2O			20.5	64.5	66	66
Volume			50	150	150	150

After the 2 incubation steps, were the chambers washed with 2xSSC-Tween at 37°C for 5 min and then washed again 2 times with DEPC-PBS-Tween.

IN SITU – RCA Step

In the Ligation step, the padlock is circularised and can be forwarded to the rolling circle amplification (RCA). In the RCA step the padlocks are amplified at lieast thousandfold and thereby allow detection by detection oligos. The RCA mastermix (see table 2.5 on page 28) was added to each chamber and the chambers were sealed again to avoid evaporation. The slides were then incubated in a humid chamber at

RT over night or 37°C for 3 hours. The chambers were washed again 2 times with DEPC-PBS-Tween for 5 min the next day.

Table 2.5: The mastermix for the rolling circle amplification is the same for every chamber. The polymerase only amplifies the ligated padlock probes from the mastermix table 2.4.

Master Mix folds:			9
RCA	final	1x [μl]	SPOT1+2+3 [μl]
Φ29 polymerase (pipet last)	1 U/μl	5	45
Φ29 buffer	1x	5	45
dNTP	0.25 mM	1.25	11.25
BSA	0.2 μg/μl	0.5	4.5
Glycerol	0.05	5	45
DEPC H2O		33.25	299.25
Volume [μl]	50	50	450

IN SITU - Detection Probe Hybridisation Step

To visualize the rolling circle products (RCP), from the RCA step we added detection probes. These probes bind to the detection probe binding sequence of the amplified padlock probe. Each target has a different padlock with a different detection oligo sequence. Therefore, limited to the amount of channels in the microscope, we are able to visualize different targets at once. After RCA the detection probe hybridization mastermix (see table 2.6 on page 29) was added to the chambers and incubated for 30 min at 37 °C. To minimise bleaching, the slides were protected from light. After the 30 min the slides were 2 times washed with DEPC-PBS-Tween and then incubated with DAPI (100 [μg/ml]) for 5 minutes at RT to stain the nucleus. It is again 2 times washed and dehydrated with a Ethanol series of 70 %, 85 % and 97 % 2 min each. The slides were then dried and mounted with Slowfade Gold Antifade to minimize bleaching.

Table 2.6: To visualise the amplified rolling circle products fluorescence labelled oligos were used. In this case Cy3, Cy5 and FITCS labelled oligos were used. Whereas the androgen receptor Full length 1 and 2 use the Lin33 backbone and the fluorophore Cy5. Androgen receptor variant 7 and Beta-Actin uses the Lin16 with the Cy3 fluorophore and androgen splice variant 12 B2-DO with a FITC fluorophore.

Master Mix folds:				3	6
Detection probe hybridisation	stock	final	1 x [μl]	SPOT 1 [μl]	Spot 2+3 [μl]
2xHyb buffer	2x	1x	25	75	150
Do001 Lin16_Cy3	10 μM	0.1 μM	0.5	1.5	3
Do004 Lin33_Cy5	10 μM	0.1 μM	0.5	1.5	3
Do008 B2_DO_FITC	10 μM	0.1 μM	0.5	1.5	0
DEPC H2O			23.5	70.5	144
Volume		50	50	150	300

Result - Spot 1 – Detection of Androgen Receptor Full length, Variant 7 and 12

We were able to detect AR-FL as well as Variant 7 in the cell lines LNCaP and VCaP. VCaP cell lines (see figure: 2.8 on page 30) showed a higher overall amount of signals compared to LNCaP (see figure: 2.9 on page 31). The negative control showed no detectable signals (see figure: 2.10 on page 32). Variant 12 could not be detected probably because of the low abundance of the AR-V12 mRNA in both cell lines, confirmed by RT-qPCR (see figure: 2.11 on page 33).

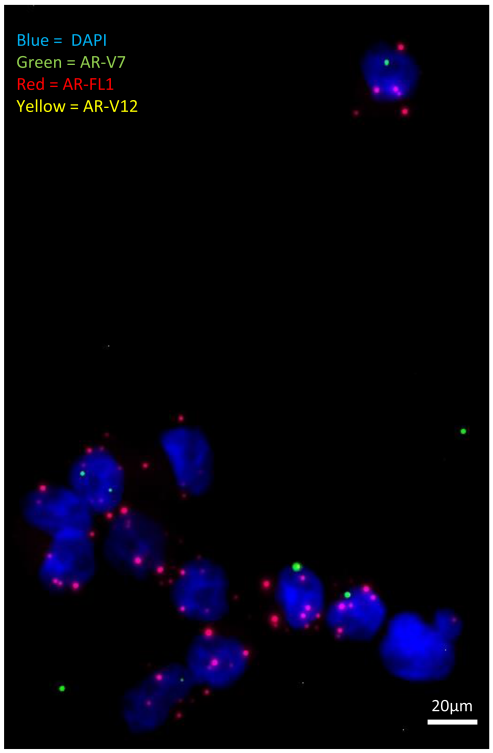

Figure 2.8: The VCaP cells show a high amount of AR-FL (red dots) as well as the splice variant 7 AR-V7 (green dots). No splice variant 12 AR-V12(yellow dots) could be detected.

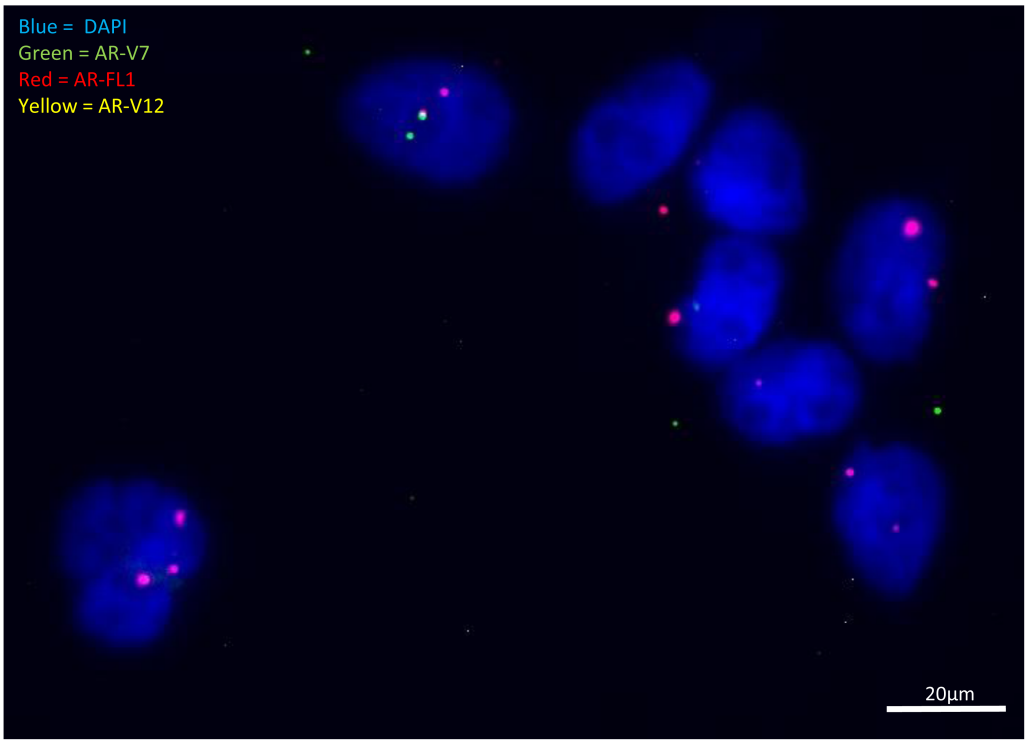

Figure 2.9: LNCaP cell line. The cells show a low amount of AR-FL (red dots) compared to the VCaP cell lines as well as only a few splice variant 7 AR-V7 (green dots) signals. No splice variant 12 (yellow dots) could be found.

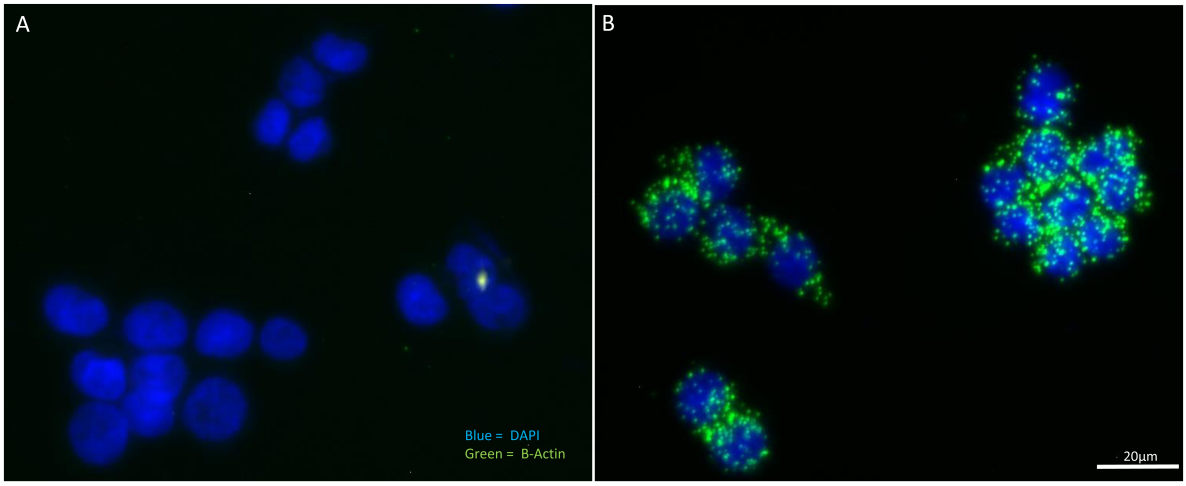

Figure 2.10: A shows the negative control cell line SW620 with the in "In Situ" targeting AR-FL, AR-V7 and PSA. No signal was detected. B shows the same cell line targeting B-Actin (green) and AR-FL (red, no signal detectable).

Although the amount of variant 7 signals per cell is low with around 0.5 dots, we were able to detect them. The level of variant signals in each cell line correlates with the RT-qPCR results (see figure: 2.11). The negative control shows the advantage of the "In Situ" method, which is its selectivity. Also the primers designed for the full length as well as the variants seem to be highly specific. The expression of Variant 12, also confirmed with RT-qPCR, seems to be too low to be detected with the "In Situ" approach. In future experiments we used the software Cellprofiler to count the amount of signals per cell to get an unprejudiced result. Targeting one mRNA of interest not only with one but with different padlock probes and LNA primers could increase sensitivity which is still one of the disadvantages of the method.

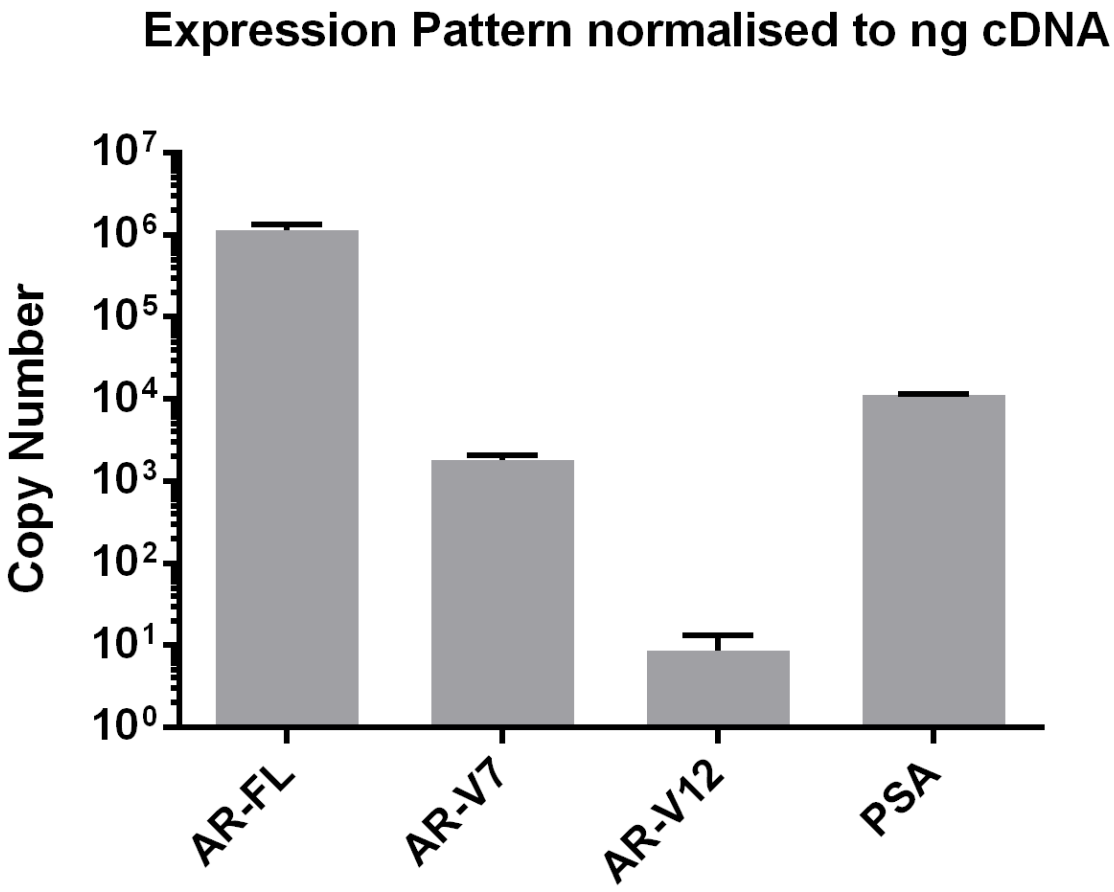

Figure 2.11: The expression of the four targets quantified by RT-qPCR and normalised to the ng cDNA used. AR-FL togehter with PSA showed the highest expression. AR-V12 in contrast is low expressed.

Result - Spot 2 and 3 - Comparison of 2 different padlocks for AR-FL

The results show that both padlocks (AR-FL1 and AR-FL2) worked satisfyingly, with AR-FL1 showing, semi-quantitatively, slightly more signals (see figure: 2.12 on page 34). B-Actin is highly expressed in all cell lines and both spots.

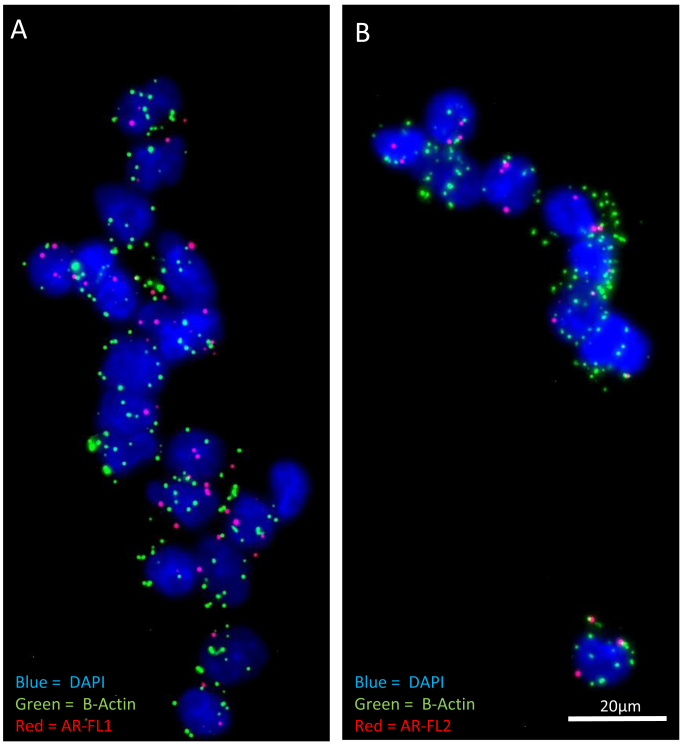

Figure 2.12: Both, spot 2 with the AR-FL1 (A) as well as spot 3 with AR-FL2 (B) padlock probe showed a, sufficient amount of signals in VCaP.

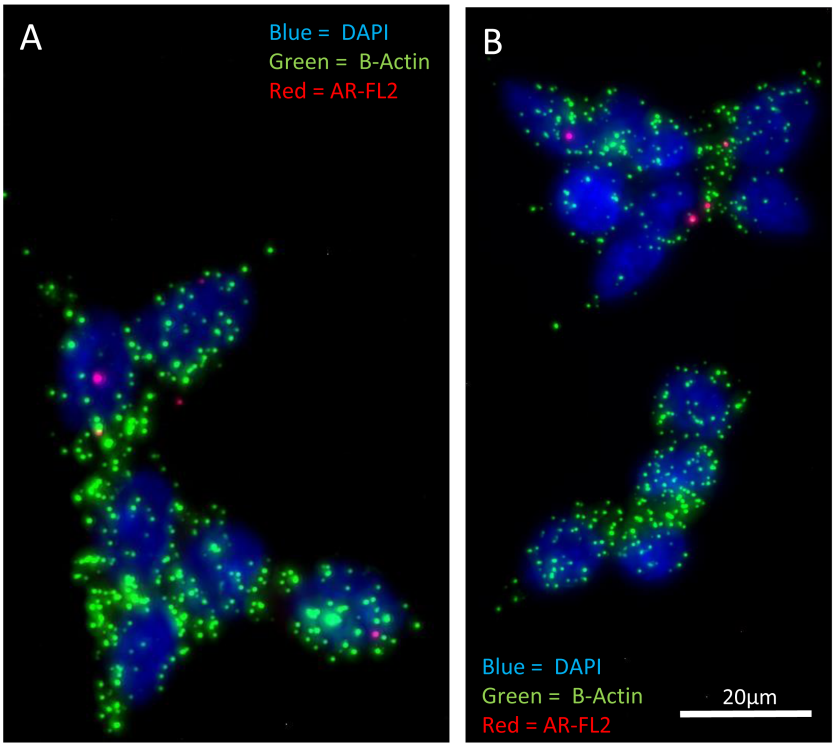

Figure 2.13: Both, spot 2 with the AR-FL1 (A) as well as spot 3 with AR-FL2 (B) padlock probe showed a good amount of signals in LNCaP cell line cells.

2.3.2 Comparison of Cyanine-based Dyes and ATTO Dyes

Cyanine-based dyes (Cy-dyes) are normally used as fluorophore in the detection probe for visualising of rolling circle products. Cy-dyes are prone to bleaching and therefore it is essential during microscopy to use mounting media. For the experiments using cellcollector we needed dyes which are, to a certain extend, resistant to bleaching. The mounting of a 3D-wire with the mounting media Slowfade Gold Antifade, which is also used on slides is challenging because the it accumulated on the bottom side of the wire. This can causing reflections. The bleaching resistant dyes are so called ATTO-dyes (namely ATTO 550 as a equivalent to the Cy3 dye, ATTO488 as FTIC and ATTO 647N as CY5) from the company BIOMERS. They showed an increased resistance to bleaching compared to normal Cy-dyes (Sigma-aldrich, 2015). To determine if the usage of ATTO-dyes compared to Cy-dyes shows similar results we performed an experiment where we tried to detect AR-FL1 (G0034 LNA and G0035 Padlock) and B-Actin (G0001 LNA and G0012 padlock probe) as a control. Also two different PSA

padlocks (G0041 LNA, and G0042 and G0043 padlock probe) were used to investigate binding affinity. Both settings were performed with ATTO as well as Cy-dyes in the negative control SW620, and the AR positive cell lines LNCaP and VCaP .

Table 2.7: Target expression of the different cell lines used throughout the project (Tai et al. (2011) Wen et al. (2014) Uhlen et al. (2015) Mertz et al. (2007) Korenchuk et al. (2000) Hu et al. (2014) Watson et al. (2010)).

Cell Line	Expression Profile			
	Androgen Receptor Full-length (AR-FL)	Androgen Receptor Variant-7 (AR-V7)	Androgen Receptor Variant-12 (AR-V12)	Prostate Specific Antigen (PSA)
VCaP	YES	YES	YES (low)	YES
LNCaP	YES	YES	YES (low)	YES
SW620	NO	NO	NO	NO
PC-3	NO	NO	NO	NO

SPOT 1: Using Cy-dyes labeled oligos to detect AR-FL1, PSA1 and B-Actin
SPOT 2: Using Cy-dyes labeled oligos to detect AR-FL1, PSA2 and B-Actin
SPOT 3: Using ATTO-dyes labeled oligos to detect AR-FL1, PSA1, B-Actin

Results - Comparison of Cyanine-based Dyes and ATTO Dyes

The comparison between the dyes showed an roughly 2 fold decreased amount of signals in LNCaP and VCaP when using ATTO-Dyes. No unspecific signals could be found in SW620 cells and semiquantitative analysis showed no relevant change between PSA1 and PSA2 padlocks.

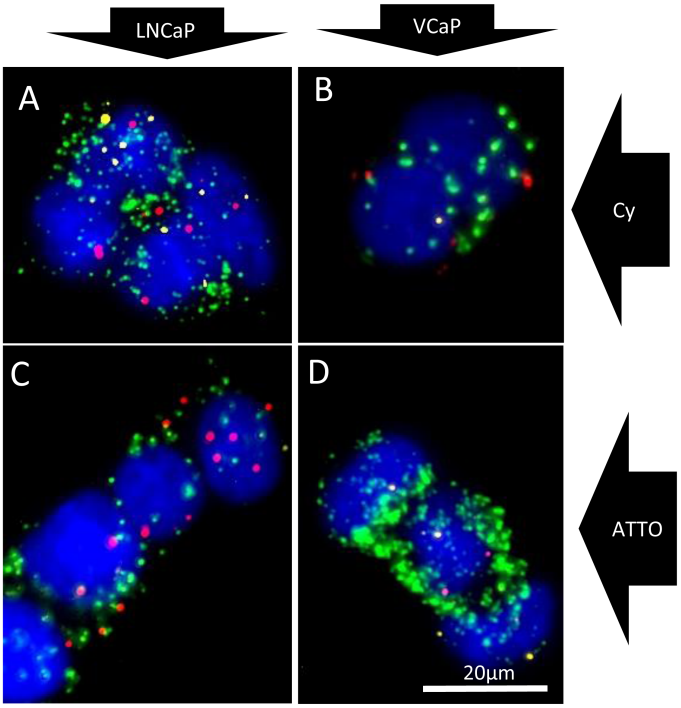

Figure 2.14: The result of the comparison between Cy-Dyes (A and B) and ATTO-Dyes (C and D) in LNCaP (A and C) and VCaP (B and D). High expression of B-Actin in all four images but with less signals per cell when using ATTO-Dyes.

2.3.3 Test of new AR-V7 Probes and Random priming

New "In Situ" primers for the target AR-V7 were designed. These primers are located on the 3´-Untranslated region (UTR) of the mRNA and are for the primers namely:

Table 2.8: As an overview the labeling of new AR-V7 primers. Internal number was used in Graz and External number by the cooperation partners in Stockholm.

Primers		
Internal Number	External Number	Name
G0061	S02794	RV_AR-V7_4
G0062	S02795	RV_AR-V7_3
G0063	S02796	RV_AR-V7_2
G0064	S02797	RV_AR-V7_1
G0070	S02561	pAR-V7_CE3_3'LNA

Table 2.9: As an overview the labeling of new designed AR-V7 padlock probes. Internal number was used in Graz and External number by the cooperation partners in Stockholm.

Padlock Probes		
Internal Number	External Number	Name
G0052	S02786	plp_AR-V7_3
G0053	S02787	plp_AR-V7_4
G0054	S02788	plp_AR-V7_5
G0055	S02789	plp_AR-V7_6
G0056	S02790	plp_AR-V7_7
G0057	S02791	plp_AR-V7_8
G0058	S02792	plp_AR-V7_9
G0059	S02793	plp_AR-V7_10
G0068	S02200	AR-V7_B2DO_2
G0069	S02553	AR-V7_B2DO_3′

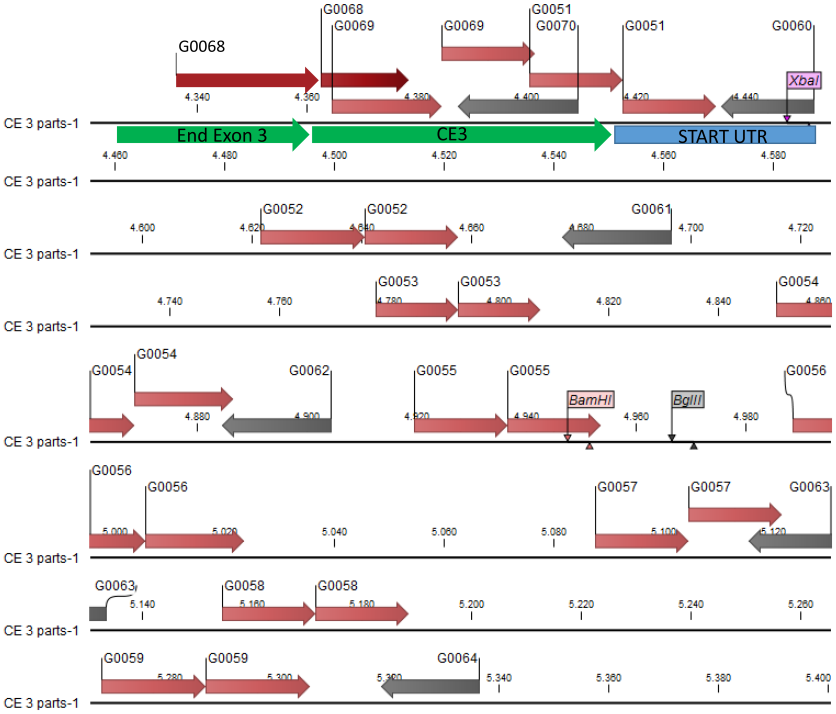

Figure 2.15: The CLC-Workbench layout of the primer as well as padlock location on the 3´-UTR. Only one arm of the padlock G0068 (dark red) binds to the CE3 the other arm on the last exon of AR-V7 to increase specificity. G0038 shares the same location (not shown) as G0068 but the padlock arm of CE3 is one base shorter. Red arrows represent an arm of one padlock, grey arrows primers used. Green arrow indicating the end of Exon 3 and and green bar start of the CE3.

In addition to these probes, random priming was also performed (G0004). The combination of padlock G0051 to G0059 was called "AR-V7 Cocktail". The influence of the new primers together with random priming on AR-V7 positive cell line VCaP as well as on two negative cell lines Sw620 and PC3 is investigated by tested each cell line with 4 spots:

Table 2.10: Layout, similar to the layout from figure: 2.6 on page 24, of the experiment to determine the influence on the new AR-V7 padlocks with there primers.

Target	Padlock Probes			
	Spot 1	Spot 2	Spot 3	Spot 4
AR-V7	G0038, G0051-G0058	G0068, G0051-G0058	G0069, G0051-G0058	G0038, G0068, G0069, G0051-G0058
B-Actin	G0013	G0013	G0013	G0013

Result - Test of new AR-V7 probes and Random priming

The AR-V7 padlock probe cocktail in combination with the AR-V7 padlocks probes G0038, G0068 and G0069 showed an increased amount of AR-V7 signals. VCaP cells on slide CH0014 and CH0015 (see figure: 2.35 on page: 68) showed 3.4 signals per cell and 3.2 signals per cell. With random priming the new AR-V7 padlock probes we could increase the amount of AR-V7 signals per cell to 9.89 for the AR-V7 padlock cocktail (Padlock cocktail consisting out of AR-V7 padlock G0051 - G0059) and 1.74 AR-V7 signals per cell for AR-V7 padlock G0068 and G0069 (tested on slide CH0016 and CH0017). The negative control (SW620, slide CH0018 and CH0019) showed 0.67 signals per cell for the AR-V7 padlock cocktail and 0.22 AR-V7 signals per cell for AR-V7 padlock probe G0068 and G0069 and 58.11 B-Actin signals per cell. Second negative control PC3 (CH0020 and CH0021) showed similar results with 0.32 and 0.17 AR-V7 signals and 66.38 B-Actin signals per cell. The amount of B-Actin signals decreased compared spot 1 or spot 2 and spot 3 or spot 4. Because spot 1 and spot 2 showed no decreased B-Actin signals, G0069 padlock could cause this decreased detection (see figure: 2.16 on page 40.

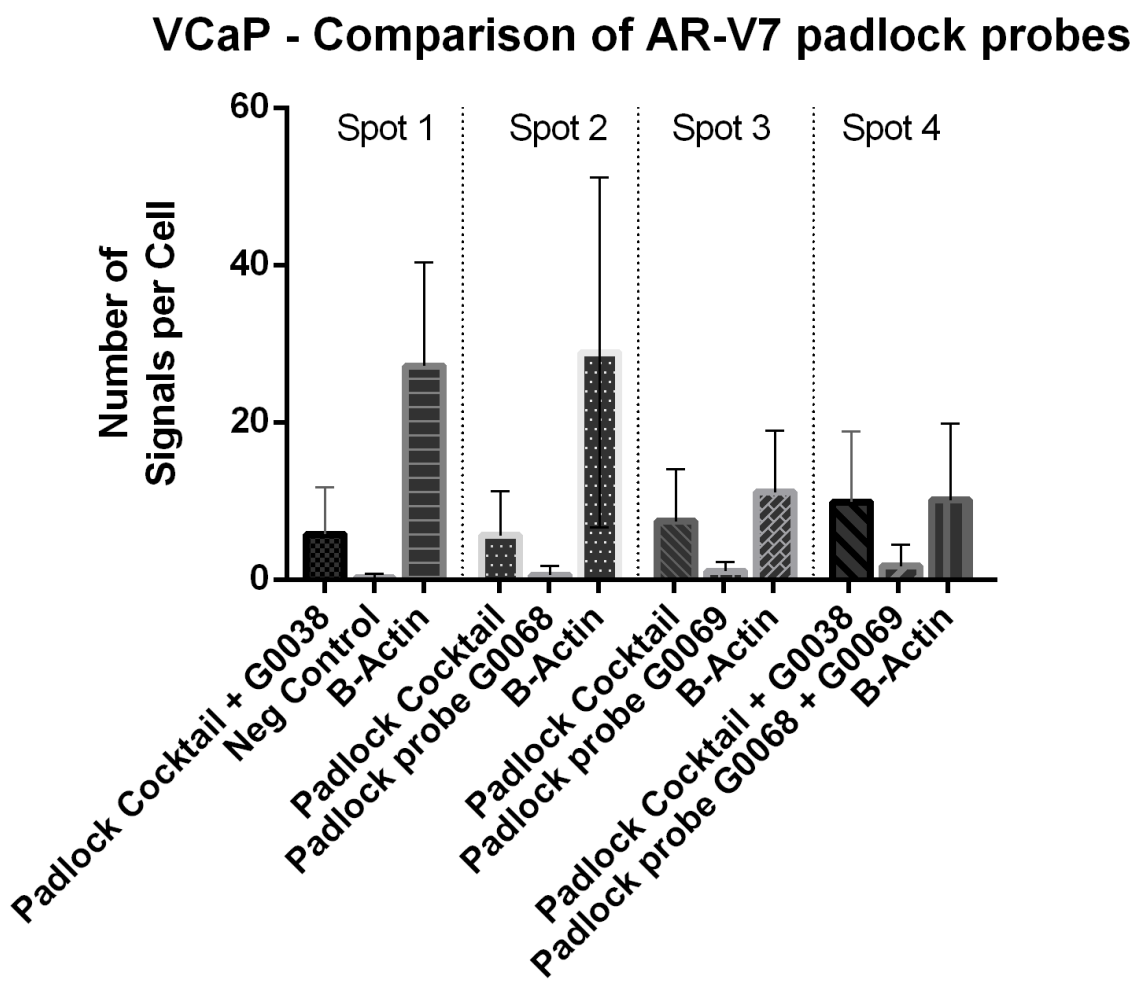

Figure 2.16: Using padlock probe cocktail together with G0038 showed the highest amount of signals. A combination of the padlocks G0068 and G0069 increased the amount compared to the usage of only one padlock probe G0069 or G0069. All four settings were performed on the same VCaP cell line slide with the same mastermix only differing in the primers and padlocks probes used.

2.4 Treatment of Cells

As described before (see section: 1.3.3 on page 7), resistance to the first line treatment Enzalutamid or Abiraterone occurs in a high rate of prostate cancer patients. To evaluate the effect of the drug Enzalutamid (formerly MDV3100 and available from the company Xtandi) on the mRNA expression levels of AR-FL as well as AR-V7, AR-V12 and PSA, we planned a treatment experiment for VCaP cells.

2.4.1 Treatment Experiment 1

Therefore VCaP cells were grown in 6-well plates, starved for 24 hours using 2 ml RPMI + charcoal stripped serum (5 %) and incubated at 37 °C in the brood chamber. The solutions in the 6 wells were then removed using suction pump and the wells were washed using 2 ml of Hank's Balanced Salt Solution (HBSS) afterwards. 3 wells were treated with 2 ml RPMI + CSS (5 %) + MDV3100 (5 μM) for 24 hours and 3 wells with 2 ml RPMI + CSS (5 %) + Vehicle (10 % Dimethylsulfoxid) also for 24 hours as a control (see figure: 2.17 on page 41). Two small experimental errors occurred during the test. Normally the corresponding media, in this case DMEM, should have been used and the amount of vehicle should be the same as the the drug. The first error happened because of a wrong protocol and the second error because of incorrect labelling of the Dimethylsulfoxid (DMSO) tube.

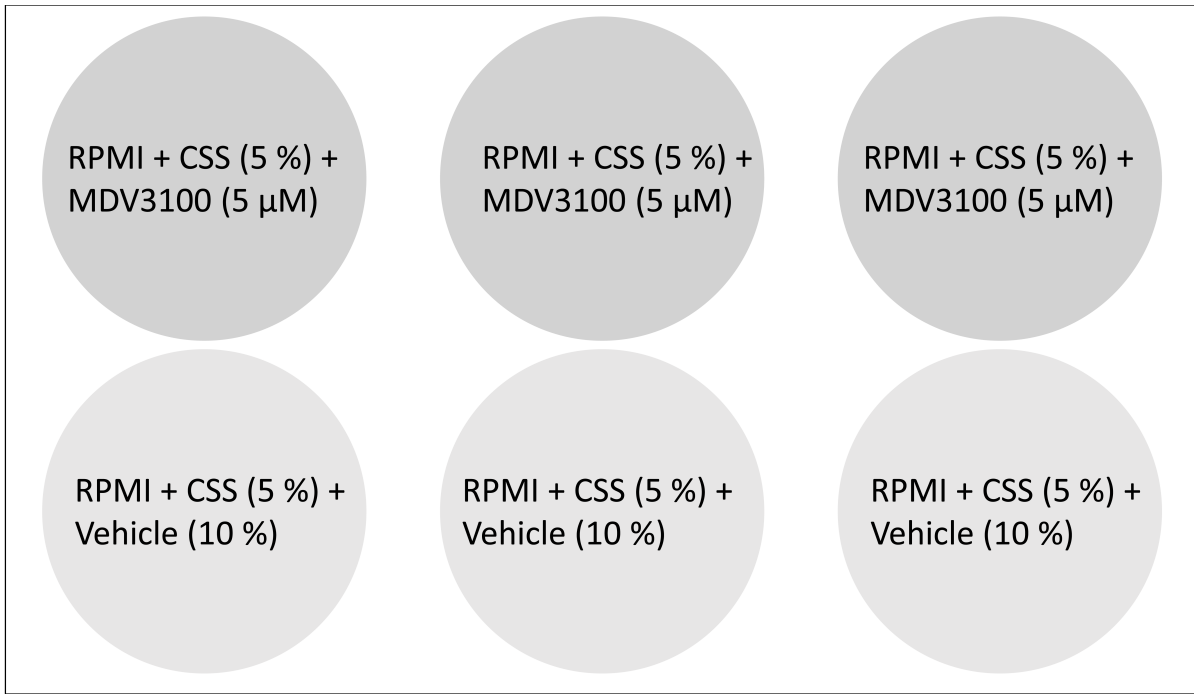

Figure 2.17: Well Layout for the Treatment Experiment. All 6 wells were treated with RPMI + CSS (5 %). In 3 wells (dark grey) MDV3100 (5 μM) and in the other 3 wells (light grey) Dimethylsulfoxid (10 %) as a vehicle control were added.

RNA Extraction and cDNA Syntheses of Treated Cells

The 3 wells with treated cells as well as the 3 wells with control cells were pooled and the RNA was extracted using the "peqGOLD Total RNA Kit" from the company peqLab. During the experiment 400 μl Ethanol for lysis and 2 times 30 μl sterile RNAse-free dH_2O for elution was used. The concentration was determined with the PeqLab "NanoDrop ND-1000".

For the upcoming cDNA synthesis 500 ng RNA should be used. Because of the low amount of RNA (see table 2.11) both elutions of VCap + MDV as well as VCap + control were pooled. The maximal RNA volume used for cDNA synthesis is 15 μl, therefore was the volume reduced from 58 μl to 3 μl using the Speedvac. The reduction was performed at low temperature for 30 min. The walls of the tube were then washed with 12 μl RNAse-free dH_2O which, together with the already enriched RNA (3 μl), was then used for the cDNA synthesis using the GrandScript cDNA Synthesis Kit (Protocol, v1.0, Kit:A103b) from the company TATAABiocenter. We also investigated the expression of two Breast tissue RNA samples, namely Breast RNA MDA 231 and Breast RNA Sum159. 1 μl RNA sample together with 14 μl RNAse-free dH_2O was used for cDNA synthesis. The reverse transcribed cDNA is then diluted to 1:10 with RNAse-free dH_2O and stored at -20 °C.

RT-qPCR of Treated Cells

The cDNA from the experiment: 2.4.1 was then used to perform a RT-qPCR to determine the exact amount of AR-FL, AR-V7, AR-V12 as well as PSA in VCaP+MDV, VCaP+Control as well as for the two additional samples (Breast-MDA 231 and Breast-SUM 159) (see figure: 2.18 on page 43 for the RT-qPCR setting). For the standard curve were primers for AR-FL and a cDNA sequence (namely Go044) with 4 increasing concentrations (2.115 x 10^{-7} ng/ml, 2.115 x 10^{-5} ng/ml, 2.115 x 10^{3} ng/ml, -0,2115 ng/ml which are 301 AR-FL copies, 3,010 AR-FL copies, 30,100 AR-FL copies and 301,000 AR-FL copies respectively) were used.

Results - RT-qPCR of Treated Cells Experiment 1

The amount of copies per transcript were calculated using a standard curve. This standard curve (see figure: 2.19 on page 43) was generated using 4 different amounts of known AR-FL (301 AR-FL copies , 3,010 AR-FL copies, 30,100 AR-FL copies and 301.000 AR-FL copies). This equation was then used for determination of the

Table 2.11: The RNA concentration as well as the purity of the probes. All samples, 30 μl each, showed a good overall quality.

Sample ID	Conc. [ng/μl]	A260 /A280	A260 /A230	Total Amount [ng/30μl]
RNAse free water	0.11	1.72	0.09	-
VCaP + MDV 1	8.08	2.05	1.09	242.40
VCaP + MDV 2	2.67	2.08	0.51	80.10
VCaP + Control 1	16.29	2.12	0.90	488.70
VCaP + Control 2	5.05	2.00	0.64	151.50
RNAse free water	-0.09	-0.56	-0.05	-
RNAse free water	-0.10	0.31	0.02	-
RNAse free water	0.44	0.86	-1.12	-
SUM 159 RNA	2508.22	2.06	2.18	72,246.60
MDA 231 RNA	1471.04	2.07	2.20	44,131.20
RNAse free water	0.30	-2.30	0.47	-

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 AR-FL VCaP+MDV	Unk-1 AR-FL VCaP+MDV	Unk-1 AR-FL VCaP+MDV	Unk-2 AR-FL VCaP-Cont	Unk-2 AR-FL VCaP-Cont	Unk-2 AR-FL VCaP-Cont	Unk-3 AR-FL Breast-MDA	Unk-3 AR-FL Breast-MDA	Unk-3 AR-FL Breast-MDA	Unk-4 AR-FL Breast-SUM	Unk-4 AR-FL Breast-SUM	Unk-4 AR-FL Breast-SUM
B	Unk-5 AR-V7 VCaP+MDV	Unk-5 AR-V7 VCaP+MDV	Unk-5 AR-V7 VCaP+MDV	Unk-6 AR-V7 VCaP-Cont	Unk-6 AR-V7 VCaP-Cont	Unk-6 AR-V7 VCaP-Cont	Unk-7 AR-V7 Breast-MDA	Unk-7 AR-V7 Breast-MDA	Unk-7 AR-V7 Breast-MDA	Unk-8 AR-V7 Breast-SUM	Unk-8 AR-V7 Breast-SUM	Unk-8 AR-V7 Breast-SUM
C	Unk-9 AR-V12 VCaP+MDV	Unk-9 AR-V12 VCaP+MDV	Unk-9 AR-V12 VCaP+MDV	Unk-10 AR-V12 VCaP-Cont	Unk-10 AR-V12 VCaP-Cont	Unk-10 AR-V12 VCaP-Cont	Unk-11 AR-V12 Breast-MDA	Unk-11 AR-V12 Breast-MDA	Unk-11 AR-V12 Breast-MDA	Unk-12 AR-V12 Breast-SUM	Unk-12 AR-V12 Breast-SUM	Unk-12 AR-V12 Breast-SUM
D	Unk-13 PSA VCaP+MDV	Unk-13 PSA VCaP+MDV	Unk-13 PSA VCaP+MDV	Unk-14 PSA VCaP-Cont	Unk-14 PSA VCaP-Cont	Unk-14 PSA VCaP-Cont	Unk-15 PSA Breast-MDA	Unk-15 PSA Breast-MDA	Unk-15 PSA Breast-MDA	Unk-16 PSA Breast-SUM	Unk-16 PSA Breast-SUM	Unk-16 PSA Breast-SUM
E	Unk-17 GAPDH VCaP+MDV	Unk-17 GAPDH VCaP+MDV	Unk-17 GAPDH VCaP+MDV	Unk-18 GAPDH VCaP-Cont	Unk-18 GAPDH VCaP-Cont	Unk-18 GAPDH VCaP-Cont	Unk-19 GAPDH Breast-MDA	Unk-19 GAPDH Breast-MDA	Unk-19 GAPDH Breast-MDA	Unk-20 GAPDH Breast-SUM	Unk-20 GAPDH Breast-SUM	Unk-20 GAPDH Breast-SUM
F	Std-1 AR-FL G0044	Std-2 AR-FL G0044	Std-3 AR-FL G0044	Std-4 AR-FL G0044	Std-5 AR-FL G0044			NTC-1 AR-FL	NTC-2 AR-V7	NTC-3 AR-V12	NTC-4 PSA	NTC-5 GAPDH
G	Std-1 AR-FL G0044	Std-2 AR-FL G0044	Std-3 AR-FL G0044	Std-4 AR-FL G0044	Std-5 AR-FL G0044			NTC-1 AR-FL	NTC-2 AR-V7	NTC-3 AR-V12	NTC-4 PSA	NTC-5 GAPDH
H	Std-1 AR-FL G0044	Std-2 AR-FL G0044	Std-3 AR-FL G0044	Std-4 AR-FL G0044	Std-5 AR-FL G0044			NTC-1 AR-FL	NTC-2 AR-V7	NTC-3 AR-V12	NTC-4 PSA	NTC-5 GAPDH

Figure 2.18: The setting for the RT-qPCR. In blue the AR-FL, AR-V7, AR-V12 and PSA in VCaP+MDV, VCaP+Control as well as for the two additional samples (Breast-MDA 231 and Breast-SUM 159) in triplets. For the standard curve, green, were increasing concentrations of G0044, the androgen receptor full length cDNA, in triplets used. Non template controls, in yellow, were also performed in triplets.

exact amount of transcripts of the targets AR-FL, AR-V7, AR-V12 and PSA. For a comparison between each sample a normalisation for the total cDNA used per RT-qPCR reaction must be considered. Therefore we calculate the concentration of the RNA used for generating cDNA. The volume for this step is 20 μl and the reverse transcribed cDNA was diluted 1:10. From that solution 2 μl were used for RT-qPCR. The equation for calculating total ng amount is:

$$\frac{(ngRNAusedforRT) * 2}{20 * 10}$$

After normalisation the expression shows a different pattern compared to the non normalised results (see figure: 2.20 on page 45). The expression of AR-FL increased by 40.0 % in VCaP treated cells compared to VCaP untreated whereas the expression of AR-V7 increased by 144.9 %. Although the expression of AR-V12 was really low it increased by 113.8 % PSA showed also an increased by 86.7 %. This observation demonstrate that cells have to adopt to the changed environment.

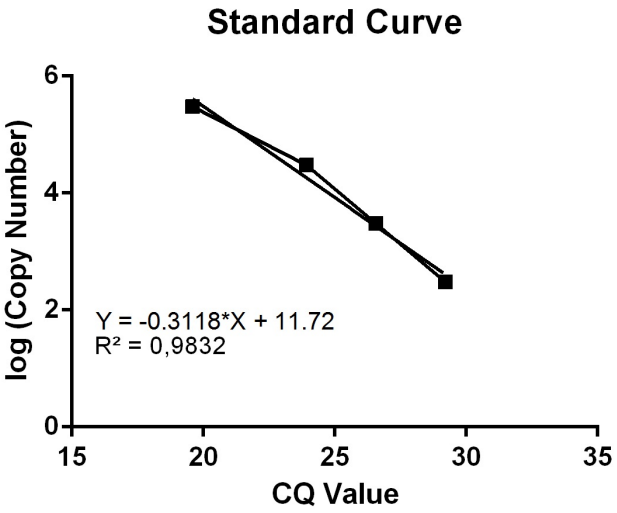

Figure 2.19: The standard curve obtained by four AR-FL samples with an increasing known amount of units.

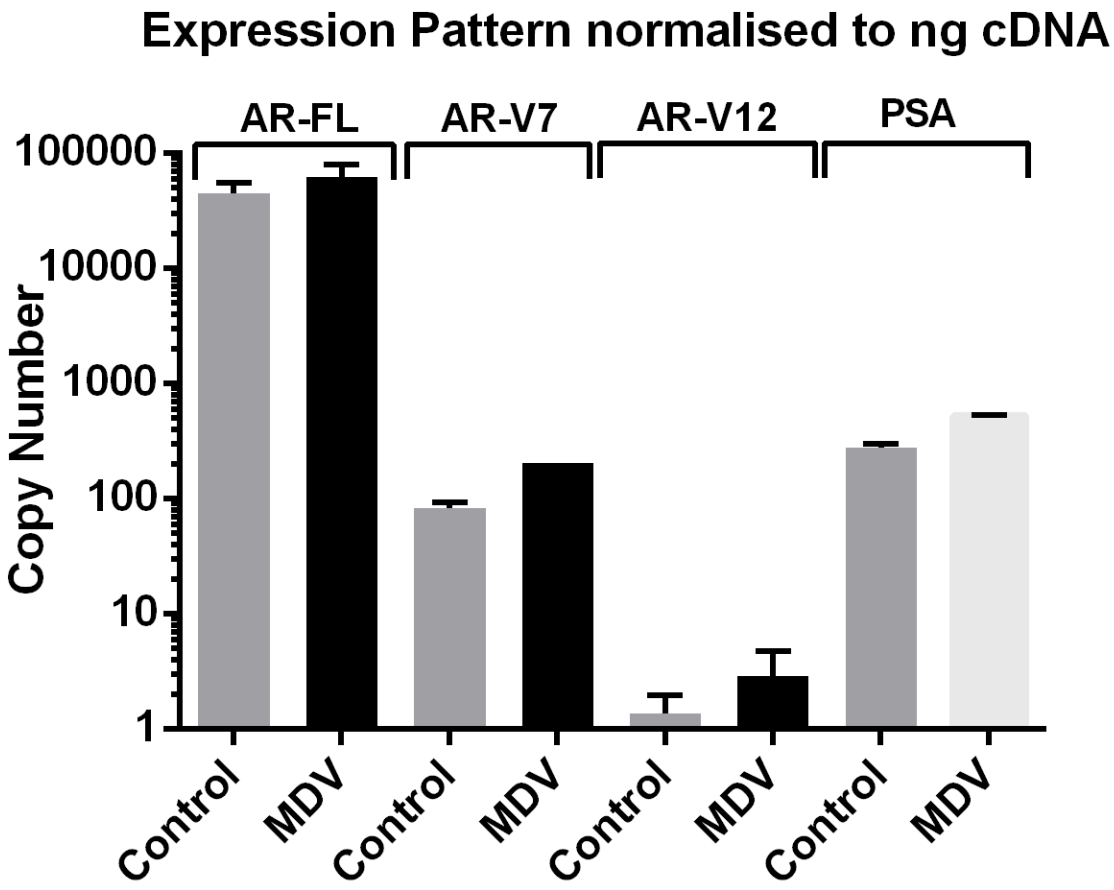

Figure 2.20: The graph shows the total mRNA expression normalised for cDNA used and detected by RT-qPCR calculated by using the standard curve from figure: 2.19. The expression of AR-FL increased by 40.0 % in VCaP cells treated with the drug MDV compared to VCaP untreated whereas the expression of AR-V7 increased by 144.9 %. Although the expression of AR-V12 was really low, it increased by 113.8 % PSA increased by 86.7 %. This shows the need of the cells to adapt to the changed environment.

2.4.2 Treatment Experiment 2

The experimental setting was performed the same way as in experiment 1 (see section: 2.4.1). Only the amount of DMSO used for the control during treatment of the cells was reduced to, as originally intended, 5 %.

RNA Extraction and cDNA Syntheses of Treated Cells

The 3 wells with treated cells as well as the 3 wells with control cells were pooled and the RNA was extracted using the "peqGOLD Total RNA Kit" from the company peqLab. During the experiment 400 μl Ethanol was used for lysis and 2 times 30 μl sterile RNAse-free dH_2O for elution. The concentration was determined with a "NanoDrop ND-1000". The concentration of the VCaP+MDV RNA was 155.43 ng/μl and 164.66 ng/μl and 153 ng/μl respectively for VCaP+Control. Therefore 6.25 μl of the combined VCaP+MDV RNA and 6.52 μl of the VCaP+Control was used for the RT-qPCR. The total RNA amount was for VCaP+MDV 9.60 μg and 9.18 μg for VCaP+Control

RT-qPCR of Treated Cells

To examine the total amount of expression we also analysed the expression of Glyceraldehyde 3-phosphate dehydrogenase(GAPDH). As a control unstarved and untreated VCaP cells cultivated in Dulbeccos modified Eagles medium (DMEM) and Fetal bovine serum (FBS), in contrast to VCaP treated and VCaP untreated cells which were accidental cultivated with RPMI, were analysed. VCaP cells from experiment: 2.4.2) and FBS treated VCaP cells were analysed with RT-qPCR to determine the exact amount of AR-FL, AR-V7, AR-V12 as well as PSA (see figure: 2.21 on page 46). For the standard curve, primers for AR-FL and a syntetic cDNA sequence (namely G0044, from IDT DNA) of known increasing concentration were used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Unk-1** AR-FL VCaP+MDV	**Unk-1** AR-FL VCaP+MDV	**Unk-1** AR-FL VCaP+MDV	**Unk-2** AR-FL VCaP-Cont	**Unk-2** AR-FL VCaP-Cont	**Unk-2** AR-FL VCaP-Cont	**Unk-3** AR-FL VCap-FBS	**Unk** AR-FL VCap-FBS	**Unk** AR-FL VCap-FBS			
B	**Unk-5** AR-V7 VCaP+MDV	**Unk-5** AR-V7 VCaP+MDV	**Unk-5** AR-V7 VCaP+MDV	**Unk-6** AR-V7 VCaP-Cont	**Unk-6** AR-V7 VCaP-Cont	**Unk-6** AR-V7 VCaP-Cont	**Unk** AR-V7 VCap-FBS	**Unk** AR-V7 VCap-FBS	**Unk** AR-V7 VCap-FBS			
C	**Unk-9** AR-V12 VCaP+MDV	**Unk-9** AR-V12 VCaP+MDV	**Unk-9** AR-V12 VCaP+MDV	**Unk-10** AR-V12 VCaP-Cont	**Unk-10** AR-V12 VCaP-Cont	**Unk-10** AR-V12 VCaP-Cont	**Unk** AR-V12 VCap-FBS	**Unk** AR-V12 VCap-FBS	**Unk** AR-V12 VCap-FBS			
D	**Unk-13** PSA VCaP+MDV	**Unk-13** PSA VCaP+MDV	**Unk-13** PSA VCaP+MDV	**Unk-14** PSA VCaP-Cont	**Unk-14** PSA VCaP-Cont	**Unk-14** PSA VCaP-Cont	**Unk** PSA VCap-FBS	**Unk** PSA VCap-FBS	**Unk** PSA VCap-FBS			
E	**Unk-17** GAPDH VCaP+MDV	**Unk-17** GAPDH VCaP+MDV	**Unk-17** GAPDH VCaP+MDV	**Unk-18** GAPDH VCaP-Cont	**Unk-18** GAPDH VCaP-Cont	**Unk-18** GAPDH VCaP-Cont	**Unk** GAPDH VCap-FBS	**Unk** GAPDH VCap-FBS	**Unk** GAPDH VCap-FBS			
F	**Std-1** AR-FL G0044	**Std-2** AR-FL G0044	**Std-3** AR-FL G0044	**Std-4** AR-FL G0044	**Std-5** AR-FL G0044			**NTC-1** AR-FL	**NTC-2** AR-V7	**NTC-3** AR-V12	**NTC-4** PSA	**NTC-5** GAPDH
G	**Std-1** AR-FL G0044	**Std-2** AR-FL G0044	**Std-3** AR-FL G0044	**Std-4** AR-FL G0044	**Std-5** AR-FL G0044			**NTC-1** AR-FL	**NTC-2** AR-V7	**NTC-3** AR-V12	**NTC-4** PSA	**NTC-5** GAPDH
H	**Std-1** AR-FL G0044	**Std-2** AR-FL G0044	**Std-3** AR-FL G0044	**Std-4** AR-FL G0044	**Std-5** AR-FL G0044			**NTC-1** AR-FL	**NTC-2** AR-V7	**NTC-3** AR-V12	**NTC-4** PSA	**NTC-5** GAPDH

Figure 2.21: The setting for the RT-qPCR. In blue the AR-FL, AR-V7, AR-V12 and PSA in VCaP+MDV, VCaP+Control as well as for VCaP treated with FBS. For the standard curve, green, were increasing concentrations of G0044, the androgen receptor full length cDNA, in triplets used. No template controls, in yellow, were also performed in triplets.

Results - RT-qPCR of Treated Cells Experiment 2

The standard curve (R^2=0.9912) was again calculated using primers for AR-FL and 5 known concentrations of a synthetic DNA sequence identical to the AR-FL sequence (301 AR-FL copies , 3,010 AR-FL copies, 30,100 AR-FL copies and 301.000 AR-FL copies per well). Although the results verify the increased production of AR-FL, AR-V7, AR-V12 and PSA in VCaP+MDVS compared to VCaP-Control (see figure: 2.22 on page 48) it increases in a much lower extent compared to experiment 1 (see figure: 2.20 on page 45).

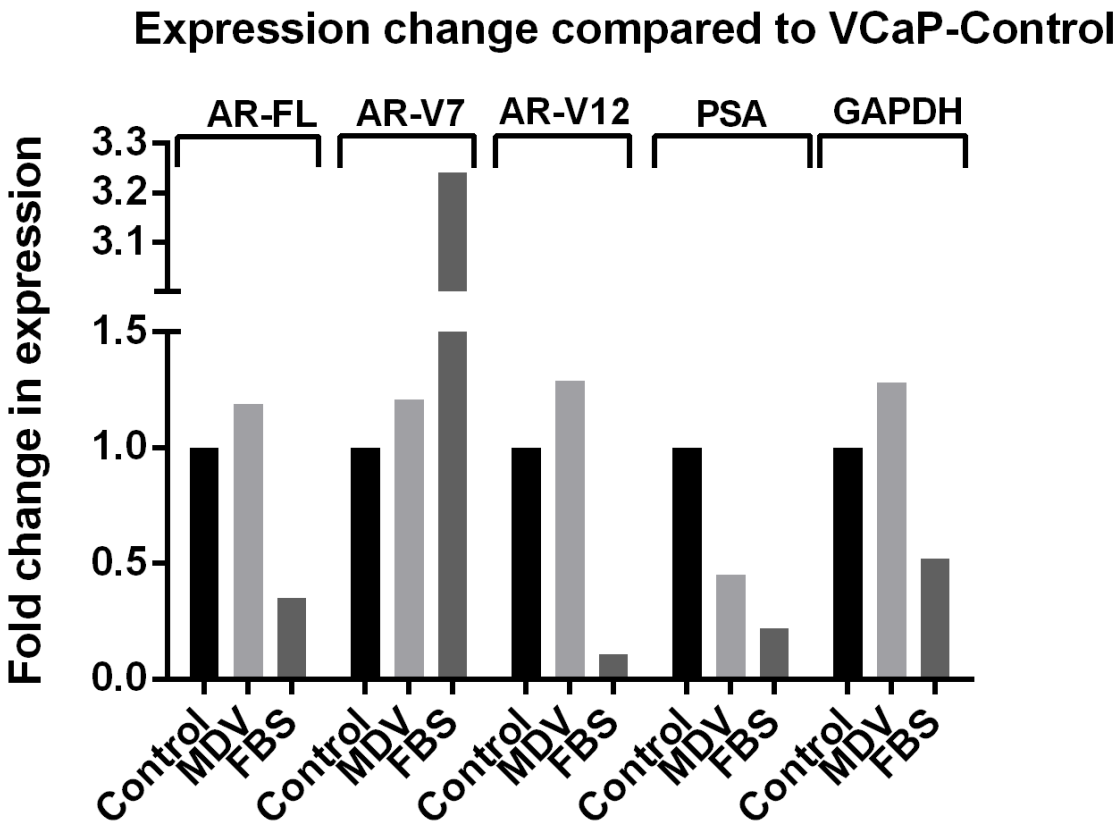

Figure 2.22: The graph shows the total mRNA expression detected by RT-qPCR calculated by using the standard curve from figure: 2.19. The expression is normalised to used cDNA amount as well as im comparison to the untreated VCaP-control. The expression of AR-FL slightly increased by 19.0 % in VCaP+MDV cells compared to VCaP-Control whereas the expression of AR-V7 increased by 21.0 %. Although the expression of AR-V12 was low it increased by 29.0 % PSA decreased by 55.0 %. In VCaP-FBS cells the expression of AR-FL was decreased by 75.0 % relative to VCaP-Control whereas the expression of AR-V7 increased by 324.0 %. Why the expression of AR-V7 was increased that much is yet unclear and should be further investigated. The expression of AR-V12 was down to 11.0 % of the VCaP-Cont expression and PSA decreased by 78.0 %. GAPDH, as a control for expression, was increased in VCaP+MDV an decreased in VCaP+FBS.

2.5 "In Situ" on Cellcollector

2.5.1 Seeding Cells on Wire

The next step after establishing the "In Situ" on Slide was to perform the reactions directly on cells attached to the "Cellcollector" (see 1.5.2 on page 11). For this experiments cells (VCaP) needed to be seeded on the wire. First cells were splitted. The cells were then counted and diluted in PBS-BSA to $2*10^6$ cells/ml in 50 μl. The wire was then put into a glass pipette with the functional part of the wire being in the narrow end of the pipette. The narrow end was then filled with the cell suspension and the wire was, under continually turning with 10-15 RPM, incubated for 30 minutes. The wire was then washed in PBS and cells were fixed with 3.7 % formaldehyde in an 1.5 ml reaction tube for 15 minutes and washed again for 2 minutes in PBS. The wire could now underwent an alcohol dehydration and be stored at -20 °C /-80 °C or an "In Situ" could be started.

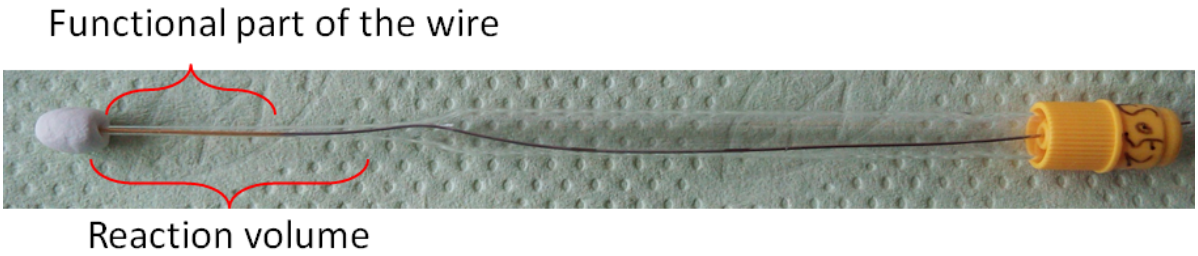

Figure 2.23: The setting for "In Situ" on wire. The "patafix" rubber inhibits the reaction volume (25 - 35 μl) to evaporate during the RT, Ligation, RCA and Detection probe hybridisation steps.

2.5.2 W0002 - KRas & B-Actin

The first experiment on wire (W0002) was performed on SW620 cells to detect B-Actin (G0001 LNA and G0012 padlock probe) as well was KRas (G0002 LNA and G007 Padlock probe) point mutation G12V. Although few cells were on the wire the "In Situ" worked and both, B-Actin and KRas transcripts were detectable on the wire and the control slides.

2.5.3 W0003 - Patient Sample

Wire W0003 was a disposable wire from the Transcan project of Prof. Sedlmayr and his PhD student Shukun Chen from the institute of cell biology, histology and

Table 2.12: Overview of all wire experiments.

Wire Number	Aim	Result
W0002	Detection of KRas and B-Actin on wire	Signals detectable See Page: 49
W0003	Detection of AR-V12, B-Actin and PSA on Antibody stained wire	No signals See Page: 49
W0004 and W0005	Detect AR-Fl, AR-V7 and PSA on wire	No signals See Page: 51
W0006 and W0007	Influence of the DAPI staining on the "In Situ"	No influence and high amount of signals See Page: 51
W0008 and W0009	Detection of AR-FL, AR-V7 and PSA	Signals detectable See Page: 52
W0010	Perform the RCA step at 37°C for 3 hours to increase signal brightness	High background fluorescence See Page: 53
W0011	Background reduction by changing the fixation media	No impact on background See Page: 54
W0012	Background reduction by using another mounting media	Significant reduction of background fluorescences See Page: 55
W0013	Increase signals by treatment of the wire with MDV3100	Increased AR-V7 to AR-FL ratio See Page: 58
W0001	Influence of long -20°C storage of the already seeded wire	Signals still detectable See Page: 59
W0014	Influence of blood on the "In Situ" on wire	Inhibitory effect See Page: 59
W0015	Influence of blood on the "in Situ" on thrilled wire	No results See Page: 60
W0016 and W0017	Compare probes from the cooperation parterns in Stockholm with ones from our lab on Wire	Low amount of cells on the wire See Page: 60
W0018 - W0021	Comparison of probes on twisted and normal wire	Twisted showed a lower amount of cells See Page: 66
W0022, W0025, W0027	Double enzyme concentration to increase amount of signals	Increase of signal amount See Page: 67
W0028	Influence of double enzymes and BSA concentration increased on "In Situ"	Slight increase See Page: 69
W0030 - W0033	Control for the first patient sample	Only the wire without blood showed signals See Page:70

embryology of the Medical University of Graz. The ethical approval (EK-Number: 25-240 ex 12/13) was allowed the usage of the wire for our approach to test the "In Situ" on patient samples. The wire W0003 was already antibody stained. For determination if this Ab-staining influences the "In Situ" we tested for AR-V12 (G0039 LNA and G0040 padlock probe), B-Actin (G0001 LNA and G0012 padlock probe) and PSA (G0041 LNA and G0042 padlock probe). There were no detectable "In Situ" signals from the patient derived wire. This can be explained due to the fact that the wire was already pre stained and treated with reagents for antibody staining which probably inhibiting future "In Situ" reactions.

2.5.4 W0004 & W0005 - AR-FL, AR-V7 & PSA2

Detection of the targets AR-FL, AR-V7 and PSA was the aim of this experiment. The wire W0004 was stored at RT for 2 months and then seeded with VCaP cells for 20 min. "In Situ" was performed. The new wire W0005 (LOT PR19022014-001) was seeded with LNCaP cells. The formaldehyde fixation step after the seeding was accidentally not performed. Both wires were tested for AR-FL1(G0034 LNA and G0035 padlock probe), AR-V7(G0037 LNA and G0038 padlock probe) and PSA (G0041 LNA and G0042 padlock probe). The forgotten formaldehyde fixation after the seeding of the cells on the wire reduced the amount of cells dramatically. Cells which are not fixed on the wire probably lyse during the "In Situ" protocol which led to no cells detectable andtherefore no signals.

2.5.5 W0006 & W0007 - AR-FL, AR-V7 & PSA2 - DAPI Staining First

To investigate the influence of a DAPI staining step ahead of the "In Situ" procedure we compared DAPI staining before and after the "In Situ" method. The wire W0006 was treated the same way as wire W0005 using ATTO dyes to prevent bleaching. DAPI staining as well as analysing the number of cells on the wire with the laser capturing microscope was performed prior to the "In Situ". The wire W0006 ran dry during ligation step which led to high amount of crosstalk and unspecific signals. This experiment was repeated with VCaP cells on wire W0007. The wire W0006 experiment, similar to W0004 and W0005, did not show any cells on the wire as the necessary fixation step was not performed. The cells were lysed during the HCl step and only fragments of the cells remained on the wire. From wire W0007 on formaldehyde fixation was performed as described in chapter: 2.5.1. The wire W0006 ran dry during ligation step which led to high amount of unspecific signals. W0007 showed high amount of cells on the wire as well as a high amount of AR-FL1 signals

and a few AR-V7 and PSA signals (see figure: 2.24 on page 52). The result correlate with the RT-qPCR.

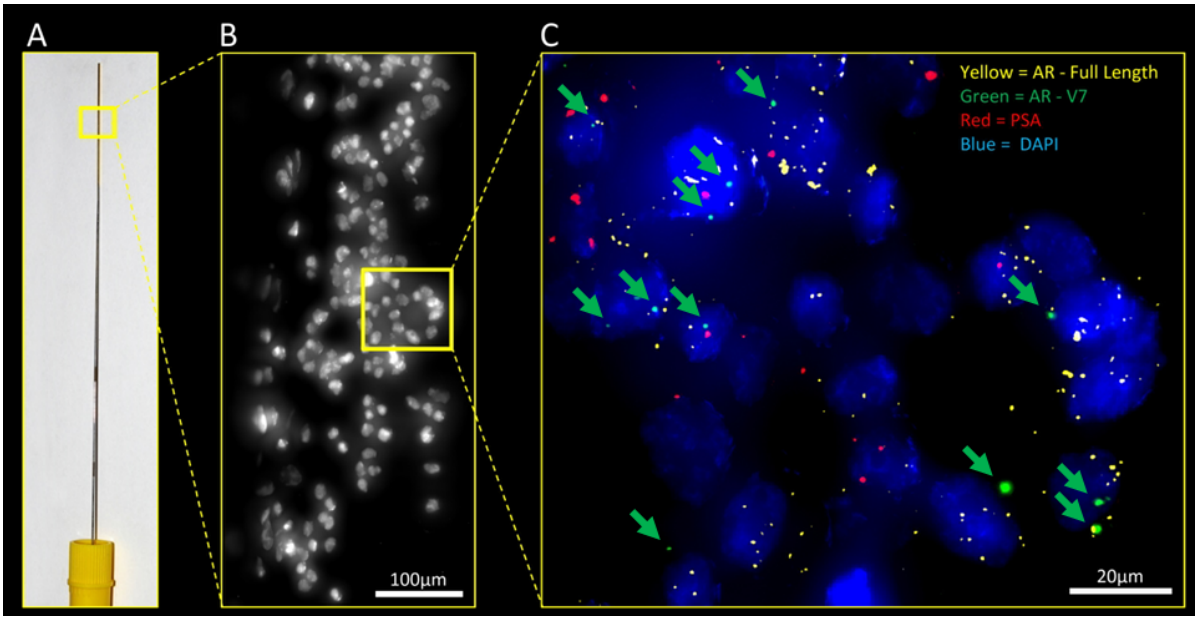

Figure 2.24: (A) Schematic overview of the W0007 wire. (B) Captured VCaP cell (PCa cell line) on the cellcollector. (C)Detected mRNA transcripts by "In Situ Padlock Probe Technology" visualized by fluorescent probes. Each color represents different mRNA transcripts originating from Androgen Receptor Full Length (AR-FL, yellow spots), Androgen Receptor Splice Variant 7 (AR-V7, green spots - arrows) as well as Prostate-Specific Antigen (PSA, red spots). The cell nuclei were stained in blue with DAPI. (Patent application number: 62253907)

2.5.6 W0008 & W0009 - AR-FL, AR-V7 & PSA2 - W0007 confirmation

The aim of W0008 and W0009 was to confirm the very good results from W0007. The cell number used for seeding was very low, resulting in an low amount of cells on the wire. Also trypsination during cell harvesting was done for three minutes. Seeding was, to due the low cell amount, performed in a glass pipette and not as usual in a 1.5 ml Eppendorf tube. Furthermore during cell harvest, cells were trypsinized for only three minutes to minimize a possible chemical damage to expressed EpCam on the surface.We hypothesised that this to allow the cells to be more accessible to the wires EpCam antibodies. This procedure had a rather negative then positive influence on the cell accessibility to the wire leading to a low amount of cells bound. Therefore longer trypsination should be performed. The setting for W0009 was changed to 15 minutes trypsination as well as RCA ON (16.5 hours). Signals of all three targets on

the control slide could be detected. Also all targets could be seen on the wire W0009, although with less and smaller signals.

2.5.7 W0010 - AR-FL, PSA1 & B-Actin - RCA at 37 °C

To increase the number as well as the brightness of the signals the RCA was performed for 3 hours and not ON at 37 °C with LNCaP. Although the control slide showed a high number of B-Actin signals as well as AR-FL1 and PSA1 signals (see figure: 2.25 (A)), the signals on the wire were obscured by unspecific fluorescence (see figure: 2.25 (B) on page 53). The high background signal only occures with "In Situ" on the wire and eliminates the possability to quantify signals. Interestingly this background signal can only be seen at the cell boarder (see figure: 2.26 (B) on page 54)

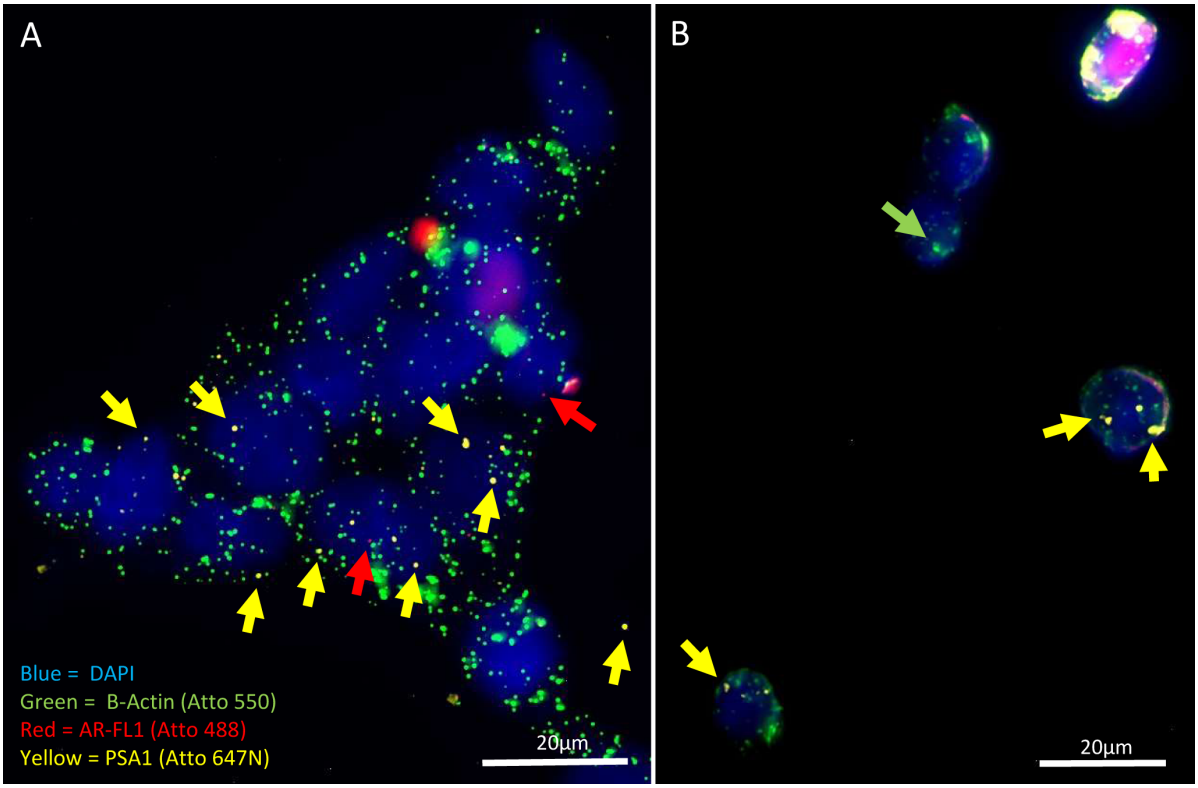

Figure 2.25: (A) Shows the W0010 slide with a high amount of B-Actin signals as well as a few AR-FL1 and PSA1 signals. Nearly no background occur. The right image (B) The same reaction mix was applied to the cells attached to the wire. High amount of background which overlaps with the "In Situ" signals. Arrows highlighting signals in the corresponding colors. Generally big, unspecific signals occur.

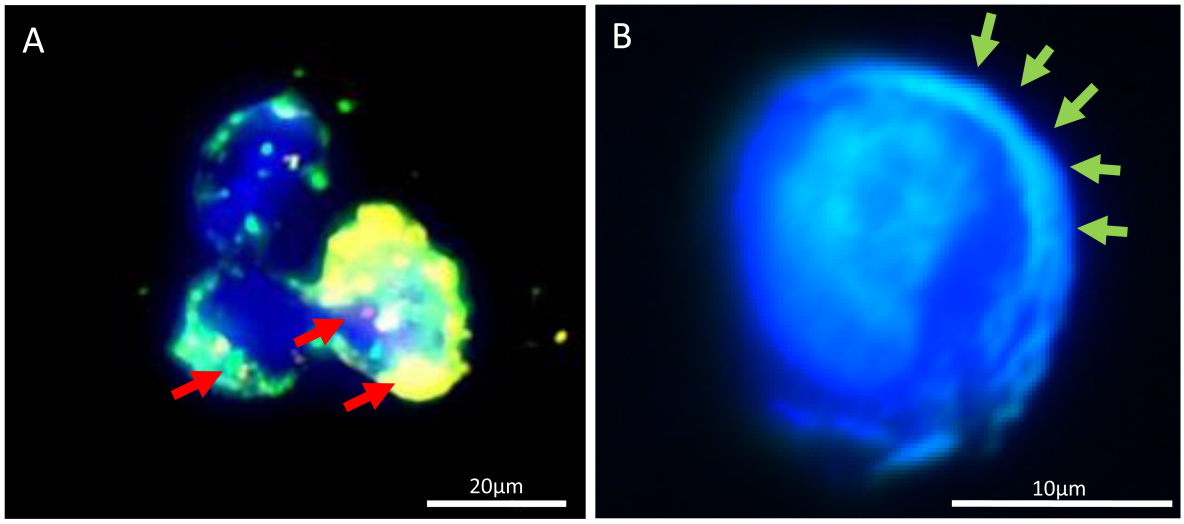

Figure 2.26: (A) Cells on the W0010 wire with a very high background signal. Quantification of AR-FL (red arrow) signals can only be performed partially. (B) In this representative image only the DAPI channel is shown. This channel should only detect blue fluorescence but the right cell boarder (green arrow) shows a greenish fluorescence staining.

2.5.8 W0011 - AR-FL, PSA1 & B-Actin - Reduction of Background Fluorescence

Because of the high background fluorescence (see figure: 2.26) in W0010 the next step was to find a way to reduce the autofluorescence. For that purpose we tested 4 % Paraformaldehyde instead of 3.7 % Formaldehyde. 1 l of 4 % Paraformaldehyde was prepared by heating 800 ml 1X PBS-DEPC to approximately 60 °C without boiling. 40 g Paraformaldehyde powder was then added under stirring and 1 N NaOH was used to set the pH to 6.9. 200 ml 1X PBS-DEPC was added and the pH again adjusted by using HCl. The solution was separated into aliquots and stored at -20 °C. The RCA was performed 1.5 hours at 37 °C. Unfurtunatelly this had no positive effect on the background fluorescence (see figure: 2.27 on page: 55)

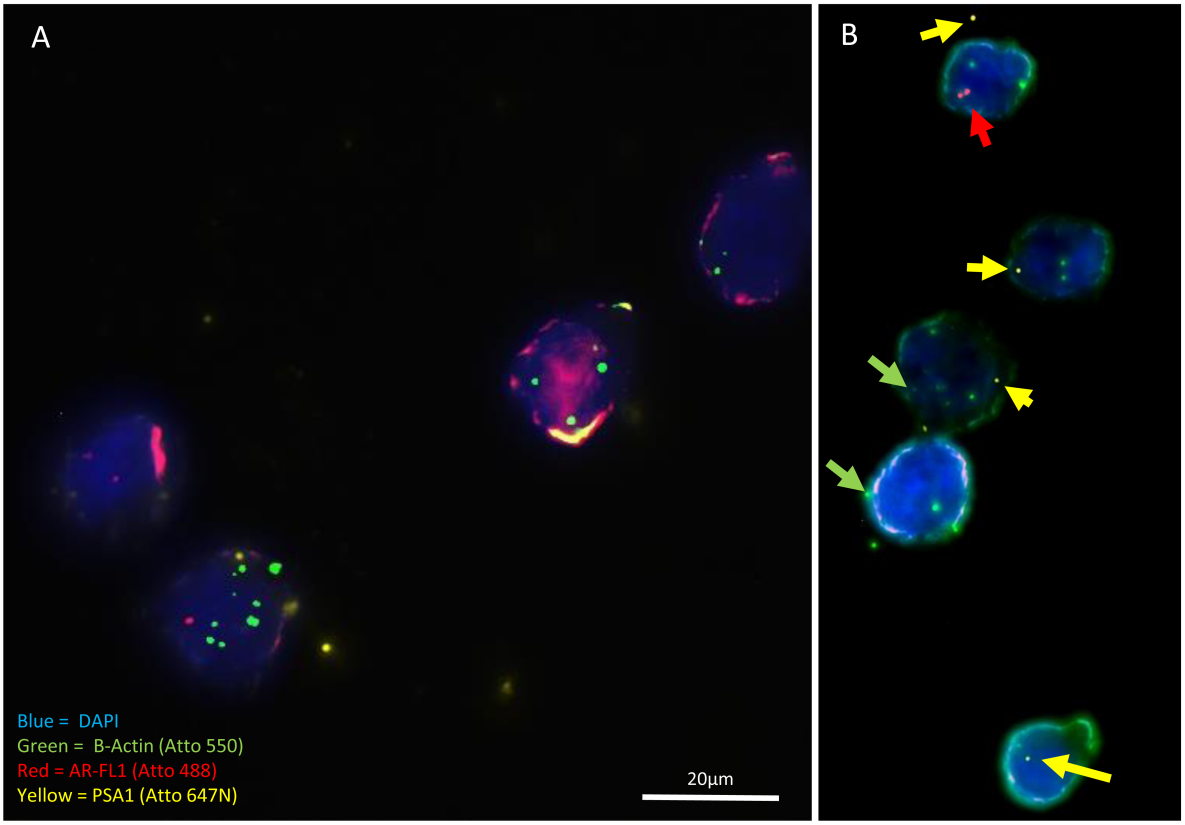

Figure 2.27: (A) VCaP cells attached to the W0011 wire which was fixed using 4%- Paraformaldehyde, with, again, a very high background fluorescence. The software Zen was used to lower the intensity of the channel fluorescence to reduce the background. This decreases also the amount of detectable B-Actin and AR-FL signals. (B) Shows again the high background fluorescence in W0011. Arrows representing AR-FL (red), B-Actin (green) and PSA (yellow) signals.

2.5.9 W0012 - AR-FL, AR-V7 & PSA2 - Mounting Media

To detect the influence of the mounting media on background fluorescence and dyes, we seeded the wire W0012 with VCaP cells and cut it in two pieces. One was incubated with Cy-dyes and one with ATTO-dyes. First we tested the mounting media Slowfade Gold Antifade reagent. This is a liquid mounting media normally used for slides. Because of the three dimensional structure of the wire the mounting media accumulated on the bottom side of the wire. This causes reflection and makes it impossible to detect signals. Therefore we changed the mounting media to Prolong Gold Antifade (Thermo Fisher Scientific, Waltham, MA,USA) reagent which hardens over time. The functional part was cut into 2 pieces and in one part the detection was done using Cy-dyes and one part ATTO-dyes. 30 μl mounting media was applied on

the wire in the tip of a glass pipette for one minute. The wire was then, protected from light, put into a Eppendorf tube and installed on a overhead rotator for two hours. We were able to detect AR-FL as well as AR-V7 signals in the Cy-dyed and ATTO-dyed part of the wire. The number of signals on the wire was slightly decreased compared to the control slide. The new mounting media significantly decreased the background fluorescence (see figure: 2.28). The signals showed some reflections in the different Z-stack levels (see figure: 2.29) which was probably caused by the three dimensional structure of the metal wire in combination with the mounting media. The reflections produced some artefacts if using ”orthogonal projection”. This method combines the brightest signal of each Z-stage into one layer. For follow up experiments the wire was coated with the mounting media Prolong gold. This coating was performed after the last step of the ”In Situ” protocol.

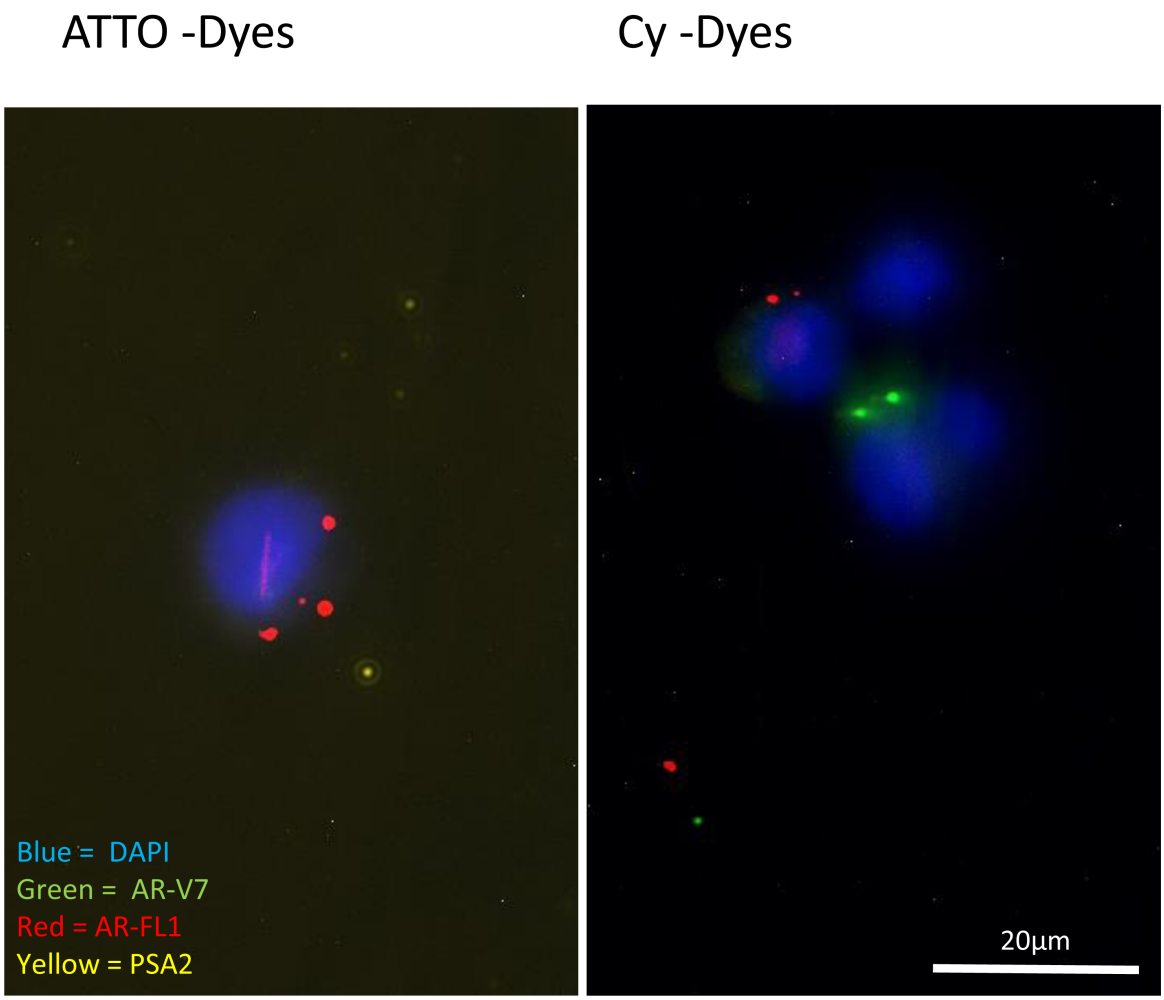

Figure 2.28: Both parts of the wire showed no background fluorescence but the number of cells on the wire was low. (A) ATTO-dyes were used on this part of the wire. AR-V7 was detectable but very low, in both slide and wire. (B) Shows the signals with Cy-dyes used. The control slide as well as the (B) part of the wire contained AR-V7 signals.

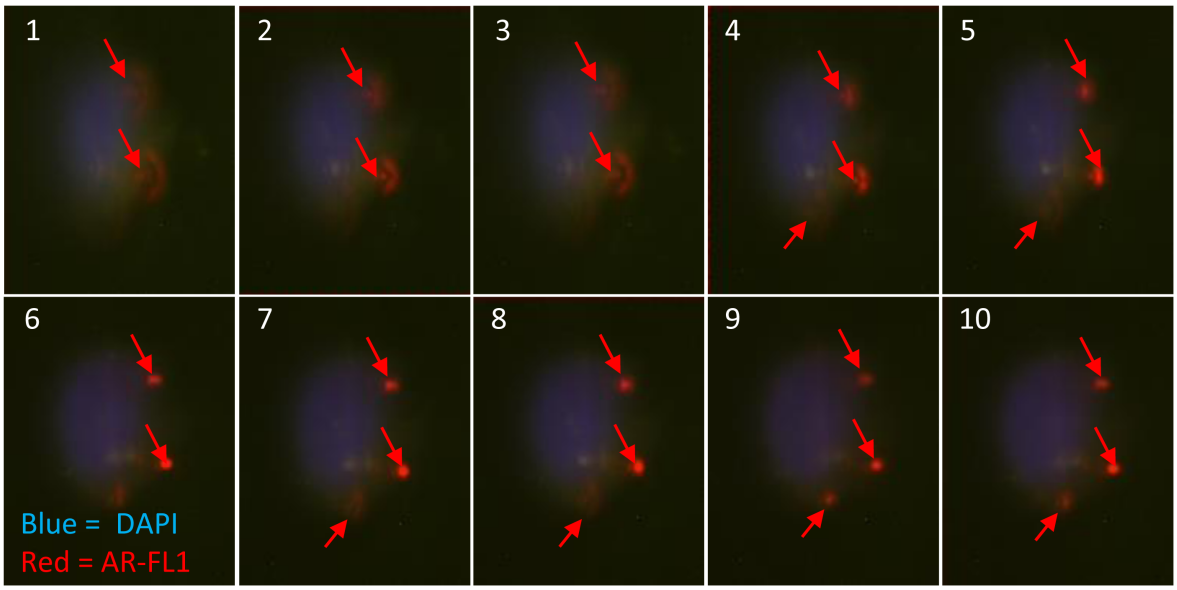

Figure 2.29: Number representing the corresponding Z-stage level of the image. The x-axis moving reflection (indicated by red arrows) on the Z-stage level leads to false results.

2.5.10 W0013 - AR-FL, AR-V7 & PSA1 - Treatment of Cells

With the wire W0013 we wanted to see if treatment and incubation with MDV3100, could also increase the amount of detectable AR-V7 signals on cells already bound to the wire. VCaP cells were seeded on the wire for 30 min (see section: 2.5.1 on page 49) and the wire was then cut into one 7 cm long head piece with the golden functional part of the wire being included. A T25 flask was filled with 5 ml of cell cultivation media (in the case of VCaP DMEM high glucose media with 1 % Penicillin/Streptomycin and 10 % fetal calf serum). The wire was then put into the T25 flask (T25) and incubated for 2 days at 37 °C. The T25 was then washed with PBS and the cells starved for 24 hours using 5 ml of DMEM with 5 % Charcoal Stripped fetal calb serum. Afterwards the media was removed, the T25 was washed again with 5 ml PBS and treated with 5 ml of DMEM with 5 % Charcoal Stripped fetal calb serum and 0.5 % MDV for 24 hours. The wire was then fixed with 3.7 % formaldehyde and "In Situ" was performed ON. After the "In Situ" the wire was shorty incubated with Prolong Gold in a glass pipette. Caused by the treatment, only a low amount (2-10) of cells were detectable on the wire. No background fluorescence was detectable and the ratio of AR-V7 to AR-Fl was approximately 1.5 fold higher compared to the untreated wire. This finding confirms the result of the RT-qPCR with treated VCaP cells (see 2.4.1 on page: 41.

2.5.11 W0001 - B-Actin & KRAS - Storage of Seeded Wires

For clinical sampling, storage of the wire which has already bound patients CTCs would be beneficial. Therefore we tested if a the wire W0001, which was seeded with SW620 cell line and stored at -20 °C for 175 days still show the KRas point mutation G12V after a "In Situ". Our experiment demonstrated that KRas point mutation G12V was still detectable by "In Situ" after long time storage. We could show that it is possible to see KRAS point mutation G12V in SW620 cell line after long time of storage.

2.5.12 W0014 - AR-FL, AR-V7 & PSA1 - Spiked in Blood

The influence of whole blood on the "In Situ" on the wire was tested by adding 75 μl of a solution containing 2x 10^6 cells/ml VCaP cells into 1.5 ml of whole blood of a healthy male donor in a 2 ml Eppendorf tube. The cap of the tube was penetrated using a thick needle and the wire was then put carefully, without touching the golden, fictionalised part, through that hole. The tube was sealed using parafilm and shaken overhead on the rotator for 30 minutes.

- Wires were carefully (30 sec) washed with DEPC-PBS
- Fixed with Aceton for 10 minutes
- Let dry for 10 min
- Frozen for 30 min at -20 °C
- Thawed and washed with DEPC-PBS for 5 min
- Formaldehyde fixed for 15 minutes
- Forwared to "In Situ"

Prolong gold was used as mounting media for 30 seconds.

For detection of AR-FL1 was primer G0034 LNA and G0035 padlock probe used, for AR-V7 primer G0037 LNA and G0038 padlock probe and for PSA the LNA primer G0041 and G0042 padlock was used.

The wire incubated with VCaP cells spiked in blood showed a low amount of cells as well as low number of signals. This low number of signals could be a result of inhibition effects of blood compounds on the "In Situ". Therefore increased amounts of enzymes as well as BSA should be tested.

2.5.13 W0015 - AR-FL, AR-V7 & B-Actin - Thrilled Wire Spiked in Blood

To determine the influence of blood on the ”In Situ” results on a thrilled wire we seeded VCaP cells on the wire W0015 in whole blood with the same method and in the same Eppendorf tube as W0014. The ”In Situ” was performed using same primers and padlocks for AR-Fl and AR-V7. For B-Actin LNA primer G0041 and the padlock G0012 were used. G0012 as well as AR-V7s padlock G0038 share accidentally the same Lin16 backbone. Therefore no differentiation between these 2 signals was possible. After the first Prolong Gold incubation for 3 seconds the background was due to unequally distributed mounting media high. Therefore we removed the mounting media with a alcohol series and performed another prolong gold incubation for 30 seconds. This decreased the amount of background fluorescence. Only few spots with high background fluorescence remained. This could have been caused by uneven distribution of the mounting media on the wire. No ”In Situ” signals were detectable.

2.5.14 W0016 & W0017 - AR-FL, AR-V7 & PSA - Comparison of Probes

The following experiments were performed at the Science for Life Laboratory in Sweden. The padlocks as well as the primers were sent from Graz. To test if these still work we performed an ”In Situ” using our padlocks and primers and the reagents and enzymes from their lab. The control slide CH0012 with VCaP and CH0013 with LNCaP were used with 3 spots each (see table: 2.13 on page 62). Spot 1 is the control experiment for W0016 and W0017. Spot 2 and 3 compares the swedish primers (spot 3)(LNA primer AR-FL LNA STHLM S0007 and padlock plp AR-FL STHLM S02682 as well as for AR-V7 LNA primer AR-V7 LNA STHLM (S02204 and padlock plp AR-V7 STHLM S02200) with the primers from Graz (spot 2)(LNA G0037 and G0034 as well as padlocks G0038 and G0035) with Cy-dyes. This experiment showed that the padlocks from Stockholm should be used because the overall amount of signals was higher as well as unspecific signals outside of cells were less.

Unfortunately the wire W0016 showed no bound cells and the twisted W0017 wire was stored longer then six months at -80 °C which could have a negative influence on the binding ability of the wire. Nevertheless, the ”In Situ” on W0017 worked but with a very low amount of AR-FL signals. No PSA nor AR-V7 could be detected with a lot of unspecific AR-FL signals randomly distributed on the wire without a cell near the signal. This unspecific signals may be a result of bound but lysed cells. The amount of signals were counted using a cellprofiler pipeline (see figure:

2.30 on page 63). This pipeline was also adapted to count signals on the wire. The comparison between the different primers from Stockholm (AR-V7 primer S02204 and the corresponding AR-V7 padlock probe S02200) and Graz (AR-V7 primer G0037 and AR-V7 padlock probe G0038) were tested on the CH0012 slide (see table: 2.13 on page 62 for an overview). On slide CH0014 a new AR-V7 set (AR-V7 primer S02561 and AR-V7 padlock probe S02553) was tested. The target sequence of the primer is shifted toward the 3' direction of the CE3. The new AR-V7 set used in CH0014 (N=504) showed a difference between the and CH0012 (spot 3, Cy-dyes) with the primer AR-V7 (S02204) and padlock (S02200)(N=37). Also CH0014 (N=504) and CH0012 (spot 2, Cy-dyes)(N=47) with the AR-V7 primers (G0037) and AR-V7 padlock probe (G0038) from Graz showed a change in expression. This significant increase did not occur in CH0012 (spot 1, ATTO-dyes) (N=30), although the mean signal amount was lower and the same AR-V7 primer and padlock were used (see graph 2.32 on page 65).

The expression of AR-FL primers and padlocks differ between CH0014 (S0007 and S02682) and CH0012 (G0034 and G0035)(spot 2, Cy-dyes) as well as between CH0014 (S0007 and S02682) and CH0012 (S0007 and S02682)(spot 3, Cy-dyes). Therefore the probes from spot 3 should be used in all further experiments. The signals outside the cells were nearly similar in CH0012 with AR-V7 set in CH0012 (G0034 and G0035)(spot 2, Cy-dyes) being the only one increased (see figure:2.31 on page: 64). Also the low amount of countable pictures the number of N was small ranging from 1 to 3. The amount of signals outside the cells on slide CH0014 was very low. This could be caused by the fact that the density of cells was around 10 times higher (CH0012:CH0014 = 15.8:168 Cells) therefore less space for unspecific signals is available. The unspecific AR-FL signals should be similar to CH0012 (spot 3) because the same probes where used but is much lower (9.8 % compared to 18.2 %).

Table 2.13: Layout, similar to the layout from figure: 2.6 on page 24 only with 3 spots, of the experiment to determine the influence on the new AR-V7 padlocks with there primers.

Slide	Spot	Aim	Primers and Padlocks	Dyes
CH0012 and Ch0013	1	Control for W0016 and W0017	Graz: AR-FL, AR-V7, PSA (G0034, G0037, G0041 and G0035, G0038, G0042)	ATTO-Dyes
CH0012 and Ch0013	2	Comparison of GRAZ and STOCK-HOLM Primers and Padlocks	Graz: AR-FL, AR-V7 (G0034, G0037 and G0035, G0038)	CY-Dyes
CH0012 and Ch0013	3	Comparison of GRAZ and STOCK-HOLM primers and padlocks	Stockholm: AR-FL, AR-V7 (S0007, S02204 and S02682, S02200)	CY-Dyes
CH0014	1	Test different AR-V7 primer and padlock	Stockholm: AR-FL, AR-V7, B-Actin (S0007, S02561, S02203 and S02682, S02553, S02003)	CY-Dyes

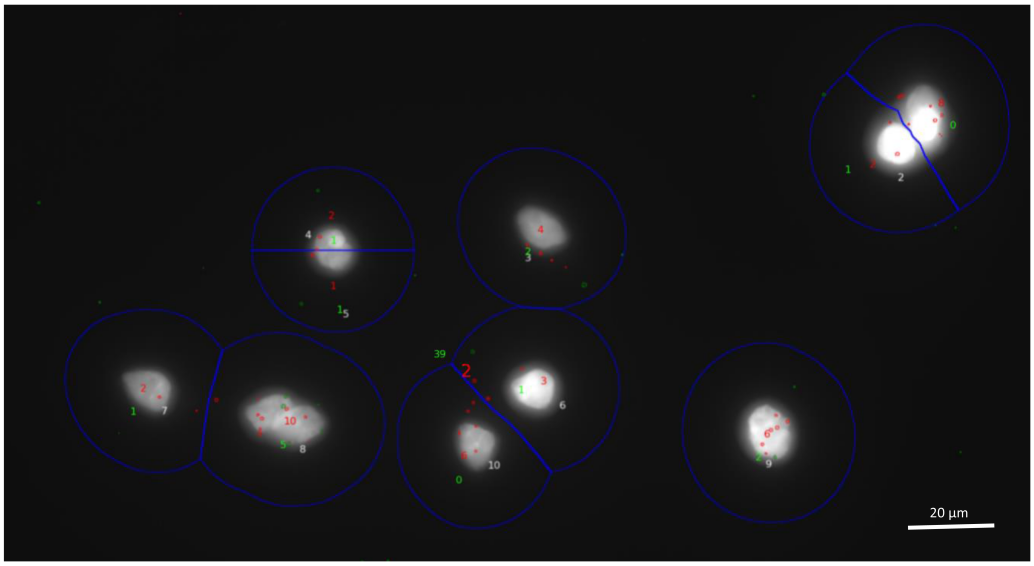

Figure 2.30: Section of the output image of the cellprofiler pipeline of CH0012 (spot2). Cell boarders are marked in blue. White number inside the cell indicates its object number. Red or green dots as well as the red and green number indicating the amount of AR-FL and AR-V7 signals per cell. Numbers outside cells represent the AR-FL and AR-V7 signals outside cells.

The signals counted by cellprofiler were used for the calculation of the signals outside (see figure: 2.31 on page 64) or inside (see figure: 2.32 on page 65) the cells.

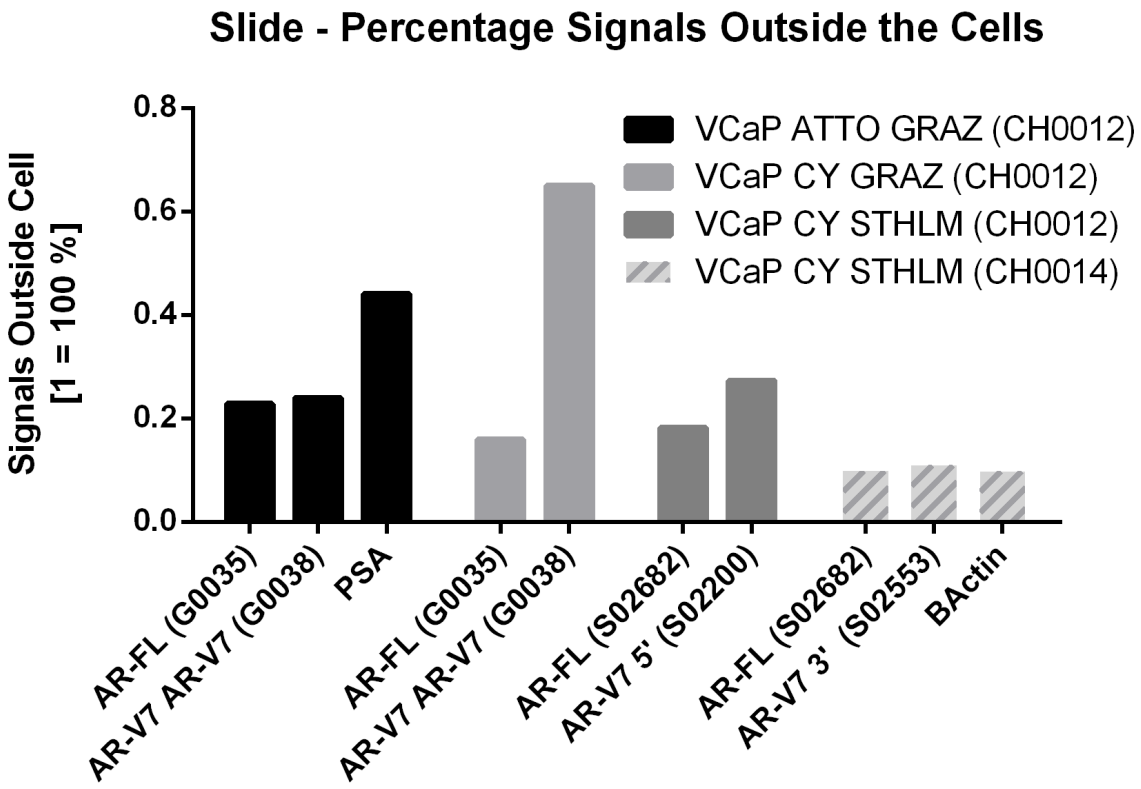

Figure 2.31: Percentage of signals outside the cells dependent on the probes and dyes used. 1 = 100 % of signals outside the cell.

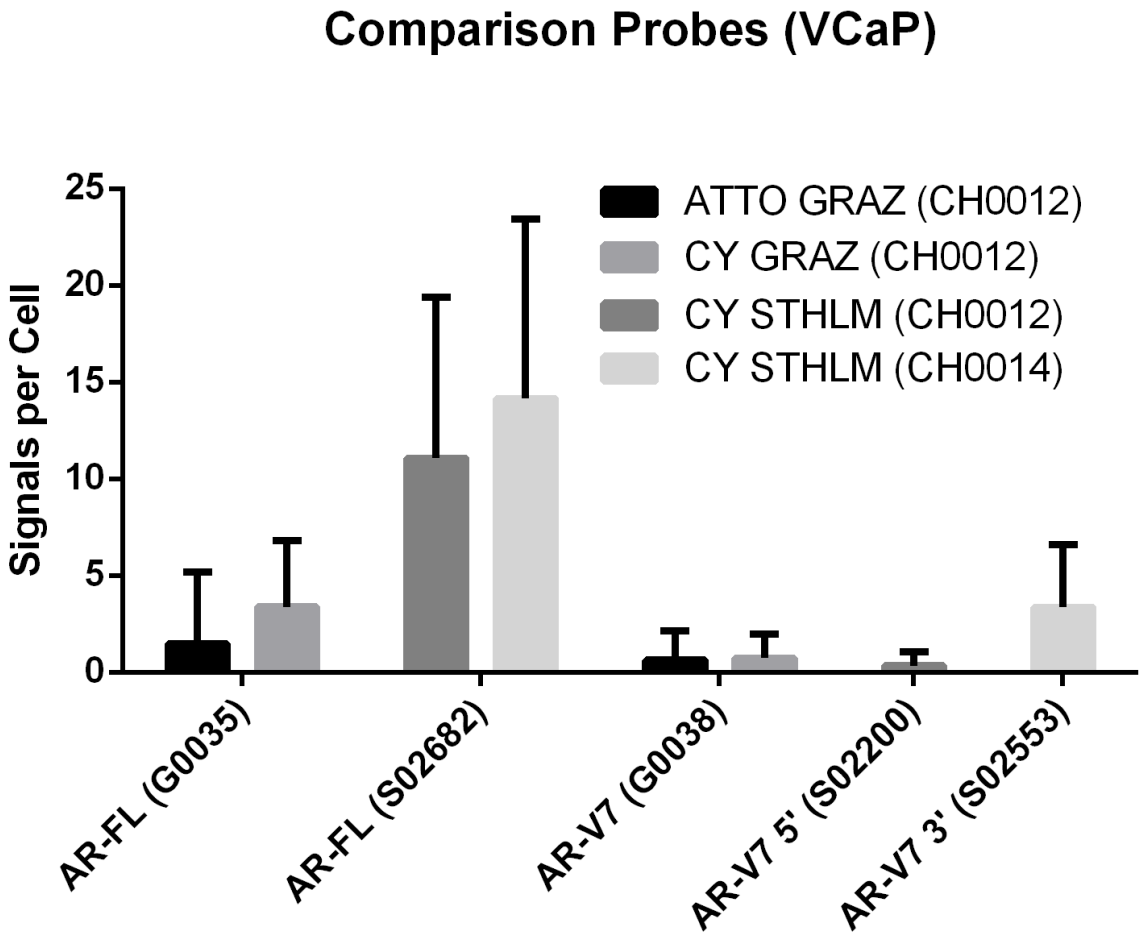

Figure 2.32: Comparison of different AR-FL and AR-V7 padlocks and ATTO and Cy dyes. A small difference between AR-V7 (G0038) from Graz and AR-V7 3′ (S02553) and as well as between AR-V7 5′ (S02200) and AR-V7 3′ (S02553) from Stockholm was detectable. There was no difference between AR-V7 (G0038) (ATTO) and AR-V7 3′ (S02553). The expression of AR-FL (G0035) (ATTO) and AR-FL (G0035)(CY) was not increased between each other but compared to AR-FL (S02682). Because of limitations in Graph pad and a very high amount of cells, only 256 random data points out of 504 were taken for visualisation of VCaP CY STHLM (CH0014) in this graph.

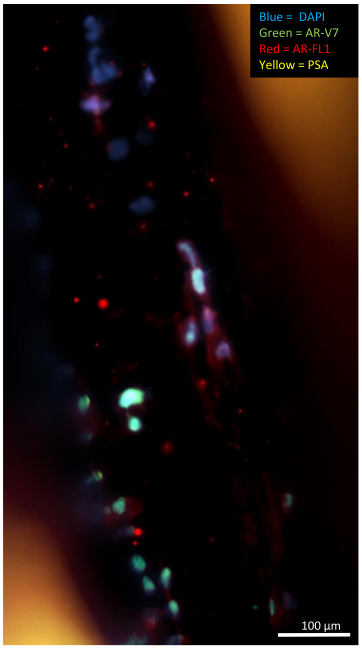

Figure 2.33: The drilled wire W0017 with a high amount of cells but a high background fluorescence. Also a high amount of AR-FL signals are unspecific. Quantification was not feasible.

2.5.15 W0018 - W0021 - AR-FL, AR-V7 & B-Actin - Wires in Blood with Stockholm Probes

To validate the efficiency of the "In Situ" on the wire with the probes from Stockholm we performed this experiment. Also the software Cellprofiler was used for the first time to count signals on wires. The slide CH0014 as well as all 4 wires (W0018 and W0020 flat, W0019 and W0021 thrilled wires) used the Stockholm primers AR-FL(S0007 and S02682), AR-V7 (S02561 and S02553) and B-Actin(S02203 and S02003). Wire W0020 and W0021 were incubated in blood as described in "W0015 - AR-FL, AR-V7 & B-Actin - Thrilled Wire Spiked in Blood". The wires W0020 and W0021 which were incubated in blood showed a low amount of cells as well as a high number of unspecific background signals which could have been caused by blood compounds. These wires could not be analysed with cellprofiler. The amount of signals on the W0018 and W0019 was relative to the control slide CH0014 (see figure: 2.34) lower but detectable and also the amount of cells with a ratio of W0018 to W0019 to CH0014 of 21.33 cells to 13.80 cells to 168.00 cells, differ. The number of detectable signals on the normal wire was 3-10 times higher compared to the drilled one (see figure: 2.34).

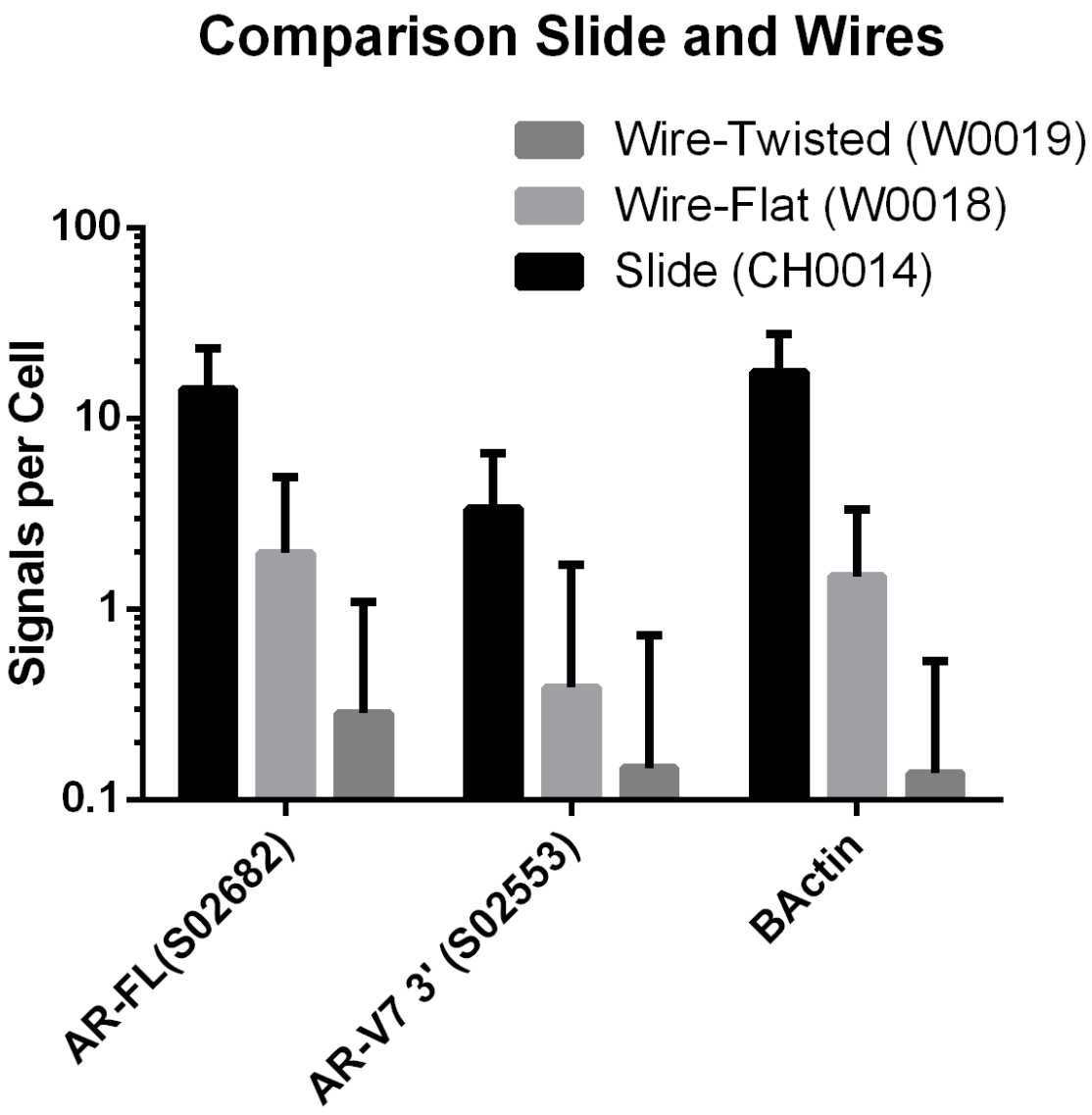

Figure 2.34: The wires showed a change in signal amount compared to the slide. Also the number of signals per cell was reduced on the twisted wire compared to the flat wire.

2.5.16 W0022, W0025, W0027 - AR-FL, AR-V7 & B-Actin - Blood and Double Enzymes

This experiment should show the effect of enzymes concentration on the amount of signals on wires, wires incubated in blood and slides. The reagent concentrations of

the reverse transcriptase (Transcribe ME), RNAse inhibitor, Ampligase, RNASE H and Φ29 polymerase were doubled. We already showed that the "In Situ" is not as efficient on the wire as on slides. Also wires incubated in blood showed no signals. This could be due to PCR inhibitors in the blood as well as lesser access to enzymes or other reagents of the mastermix on the wire. We identified the concentration of the enzymes and BSA as the limitation factor. Slide CH0015 was seeded with VCaP and used as control. W0022 (flat) and W0025 (thrilled) were used and incubated with VCaP cells seeded in whole blood whereas W0027 (flat) was incubated with cells seeded in PBS-BSA. Fixation time was extended to 20 minutes to increase the amount of cells on the wire. The experiment showed that double enzyme only on wires but not on slides increase the amount of signals. B-Actin signals but not AR-FL or AR-V7 signals on slides increase by doubling enzyme concentration (CH0014 and CH0015 see figure: 2.35).

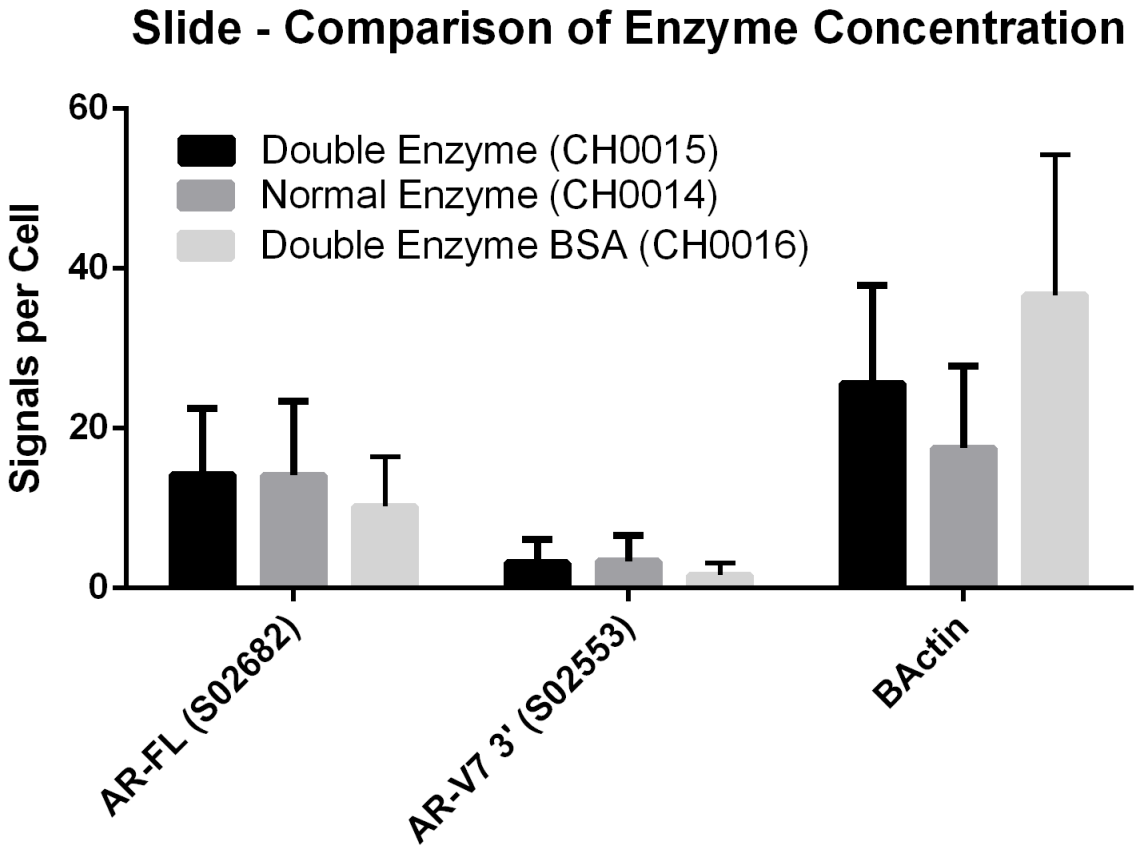

Figure 2.35: Only B-Actin showed a higher (25.6 vs 17.5) amount of signals per cell using double enzyme. AR-FL as well as AR-V7 showed similar result (Double enzyme vs normal enzyme, 14.18 vs 14.15 for AR-FL and 3.15 vs 3.36 for AR-V7). Treatment with double enzyme and BSA concentration even decreased the number of AR-FL signals on slides.

The amount of signals was significantly increased for all three targets on wires (see figure: 2.36) when enzyme concentration is doubled.

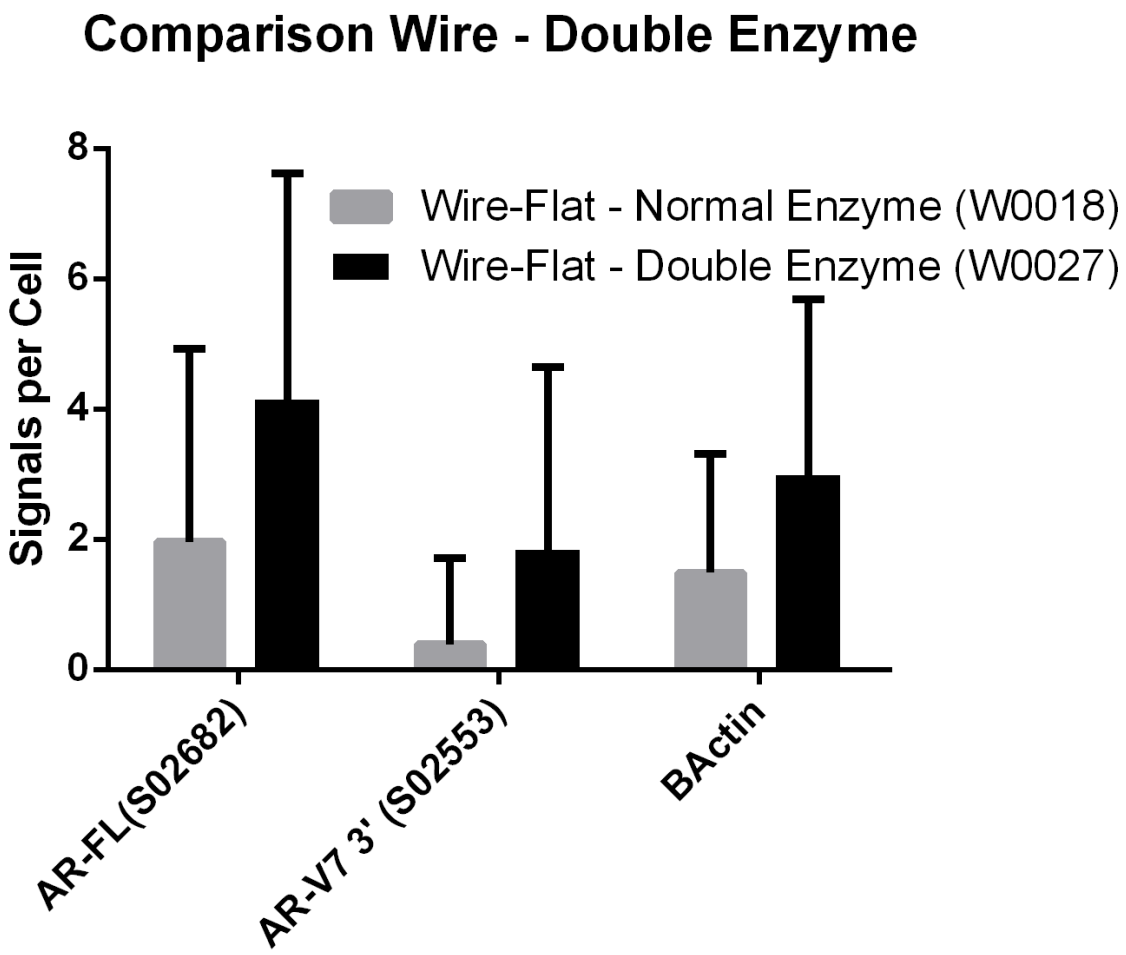

Figure 2.36: Doubling the enzyme concentrations in all "In Site" steps leads to a increased number of signals per cell on the Wire.

2.5.17 W0028 - AR-FL, AR-V7 & B-Actin - Double Enzymes and BSA

To validate if BSA concentration has an effect on the amount of signals on the wire and on slide we repeated the experiment from wires W0022, W0025 and W0027. BSA concentration was doubled in every step. The wire used was W0028 (flat) and slide

CH0016 as control. The change of BSA concentration showed a slightly increase of AR-V7 signals (1.84 to 2.59 signals per cell) on W0028 compared to double enzyme with normal BSA concentration W0027, but B-Actin was decreased (2.98 to 1.31 signals per cell). The results on slide showed a different picture. Here was the amount of AR-FL decreased compared to CH0014 (normal enzyme concentration) and CH0015 (double enzyme) and B-Actin was increased (see figure: 2.35 on page 68). The signals outside of cells where increased in the wire and slides with doubled BSA compared to normal BSA in every target except W0028 AR-V7 (W0028:W0027 AR-FL/AR-V7/BActin = 0.48:0.14/0.24:0.60/0.25:0.08 and CH0016:CH0015 = 0.14:0.08/0.26:0.07/0.24:0.08).

2.5.18 W0030 - W0033 and Patient Wire CB2-G - AR-FL, AR-V7 & B-Actin - Double Enzymes, BSA and Random Priming

The patient wire (CB2-G) was incubated in the patient arm vein for 30 min and afterwards acetone fixed. Double enzymes BSA as well as random primers were used. W0030 and W0032 (both with cells spiked into blood) and W0031 and W0033 (cells spiked into PBS-BSA) and CH0022 with 3 spots were used as control. CH0022 spot 2 and W0032 and W0033 were post fixated after RCA with 3 % formaldehyde to test if more cells can be found on the wires. CH0022 spot 3 was used as a negative control without reverse transcriptase. No cells were attached on wire W0030, W0032 and W0033. Unfortunately we were not able to detect a CTC on the patient sample wire CB2-G. To test if a possible RCP detachment from the wire can be inhibited we performed a post fixation step after the RCA. This detachment could happen because the product of the RCA is not fixed to the wire. A comparison of CH0022 spot 1 and 2 showed a decreased amount of signals when performing a post fixation step after the RCA. Also higher amount of unspecific signals outside cells (Spot 1 : Spot 2 AR-FL: 10.6 % : 20.0 % AR-V7: 22.1 % : 47.1 %) could be detected.

3 Conclusion

Establishing of an robust and reproducible assay for detection of AR-FL and AR-V7 as well as PSA worked perfectly. The random priming in combination with the AR-V7 padlock "Cocktail" consisting of padlock Go051 - Go059 together with the AR-V7 padlock showed a high amount of AR-V7 signals (9.9 dots per cell) and AR-V7 padlocks Go068 and Go069 together resulted in 1.7 dots per cell. So 11.6 AR-V7 signals per cell on slide could be detected with our primer set (see 2.16 on page: 40). Although lower numbers of AR-V7 signals on the wire were observed, compared with slide, we were able to increase the amount of AR-V7 "Cocktail" signals to 44.9 % of the slide AR-V7 "cocktail" signals per cell. The amount of unspecific signals (Wire:Slide = 24.6 %: 22.1 %) did not change. In the 3 patient samples analysed, no CTC was detected on the wire. This finding was unexpected and needs to be further analysed. Furthermore, AR-V12 exhibit a 2 fold lower expression than AR-V7 (see figure: 2.22) and was therefore not detectable via the "In Situ" approach. The RT-qPCR results are a good measurement for validating our "In Situ" results. The higher AR-FL expression compared to AR-V7 can be seen in both, RT-qPCR and the "In Situ" experiments. Especially the low AR-V12 expression detected by RT-qPCR showed us the limits of the "In Situ" method.

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Appendix

Protocol for Splitting Cells into new T25 Flasks

Splitting of cells (1:5)

Cell line:

- VCAP (Prostate cancer cell line)
- SW620 (Colorectal Adenocarcinoma)

Split Ratio: 1:4 to 1:12 recommended (every 2-3days)
Media: VCAP: DMEM high glucose and PenStrep 1 % Vol and FCS 10% Vol (DMEM complete)
SW620: RPMI and PenStrep 1 % Vol and FCS 10% Vol (RPMI complete)

- Warm up Media complete, Trypsin-EDTA (1,5 ml) and HBSS washing buffer in the 37°C water bath for ~15min
- Remove old media from the 75ml culture flasks by the suction pump
- Wash 1 x with 5 ml HBSS, and remove solution
- Add 1,5 ml Trypsin-EDTA, incubate for 3-5 min at 37°C until the cells lose their adhesion to the dish surface (check by microscope)
- Add 5 ml of Media complete, pipette up and down several times and transfer to a sterile 50 ml centrifuge tube (=Deactivation step)
- Centrifuge for 5 min, at 1200 rpm
- Remove supernatant by suction pump and resuspend the pellet in 10 ml Media complete

- Prepare 5x 25ml culture flasks with 5 ml Media complete (for 1:5 splitting)
- Add to each prepared culture flask 1 ml of cell suspension and incubate in the 37°C brood chamber (5% CO_2)

Protocol for Storing Cells at - 80 °C

Storing the cells into N_2:

Cell line: VCap Cell Line

- Prior to storing the cells should be confluent and had fresh media overnight
- Warm up DMEM complete (~15ml), Trypsin-EDTA (5ml) and HBSS washing buffer (~15ml) in the 37°C water bath for ~15min
- Remove old media from the 25 ml culture flasks by the suction pump
- Wash 1 x with 5ml HBSS, and remove solution
- Add 1,5 ml Trypsin
- Incubate for 3-4 min until the cells lose their adhesion to the dish surface (check by microscope)
- Add ~5ml of DMEM complete media, pipette up and down several times and transfer to a sterile 50ml centrifuge tube
- Centrifuge for 5min, at 1200 rpm
- Remove supernatant by suction pump and resuspend the pellet in 3ml RPMI complete supplemented with 10% DMSO (sterile filtered)
- Prepare 2 x cryovials and add 1,5ml of the cell suspension
- Slowly freeze the cells with Mr. Frosty (-1°C/min) in -80°C Freezer >4h
- Store in liquid N_2

Protocol for Seeding Cells on the Wire

Prepare Cells for “In Situ mutation detection”

- Wash humid chamber with EtOH and let it dry (in sterile laminar flow hood) and fill it with 100 ml DEPC - H^2O
- Wash 18 Super frost plus slides in EtOH for 10 minutes and evaporate for 30 minutes (in sterile laminar flow hood) or until dry
- Count cells (according to Neubauer chamber)
- Put the sterile Super frost plus slides into the humid chamber (should be a bit of space between each slide)
- Split cell culture and transfer one part into a new 15 ml flask and dilute it to $3*10^4$ Cells per ml.
- Transfer ~$1,5*10^4$ cells (total cell number) onto each slide in a total volume of 500 µl
- Close the humid chamber and carefully (!) transfer it into the incubator
- 37°C o/n
- briefly wash/ rinse them in PBS
- fix them in 3.7% formaldehyde buffered in PBS, pH 7.4 for 15 min at RT
- wash in twice PBS, pH 7.4
- dehydrate in brief EtOH series (70%->85%->abs), 1 min/ step
- let dry
- store slides at -20°C (for short term use) or -80°C for long-term storage

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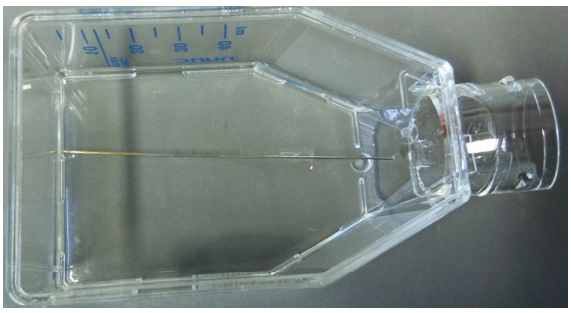
Cell Cultivation on Wire

- Seed cells for 30 minutes see seeding protocol
- Fill 5 ml cell cultivation media into T25 flask
- Cut wire to 7 cm

- Put the wire without the yellow plug inside the T25 flask so that the golden part of the wire is inside the media. The other end of the wire should lay on the rise of the opening of the T25 flask.



- The wire inside the T25 Flask is then put into the incubator for 2 days.
- Additional Information:
  For an “In Situ” on Wire, cut the first 7,5 cm of a 230 mm Pasteur pipette with a wire cutter and plug with rubber.

Treatment of VCaP with Enzalutamid in 6 Well plate

Preparation of aliquots for CSS (charcoal-stripped, steroid-depleted serum)

Use Media according to the cell line used!

Start volume 50 ml →

- 15 x 2ml → in 2ml tubes 4 stored at -20 °C
- 3x (47,5 ml Media + 2,5ml CSS) stored at 4 °C
- Remaining 12 ml CSS stored at 4 °C

Preparing stock solution for Enzalutamide (MDV3100)

- MW = 464.44
- 10mg = 2,153 x 10-5 Mol
- Solubility in DMSO 90 mg/ml
- 10mg + 21,5313 ml DMSO = 1mM Stock Solution
- Split in to aliquots -4 10x 2ml
- 5 aliquots were stored at -20 °C and the remaining at RT

Preparation of drug containing media

5 µl from the Stock Solution (1mM) + 995 µl Medium = 1ml Medium with 5 µM MDV3100 end concentration

Preparation for enzalutamide treatment replacing the media with Media +CSS (5%)

- Cell line: 6 wells of VCaP (Prostate cancer cell line)

Warm up Media complete and PBS washing buffer in the 37°C water bath for -15min
Remove old media from the 6 well culture plate by the suction pump Wash 1 x with 2 ml PBS, and remove solution

Starvation step:
Add 2 ml of Media + CSS (5%) media
incubate in the 37°C brood chamber (5% CO2) for 24h or 48h

MDV Drug treatment

Replacing the media with Media + CSS (5%) + MDV (5 µM) for drug treatment

- Cell line 1: 3 wells of VCaP (Prostate cancer cell line)

Replacing the media with Media +CSS (5%) + DMSO (5 %) for negative control

- Cell line 1: 3 wells of VCaP (Prostate cancer cell line)

For each cell line:

Warm up Media complete and PBS washing buffer in the 37°C water bath for 15min
Remove old media from the 6 well culture plate by the suction pump Wash 1 x with 2 ml HBSS, and remove solution
Add 2 ml of Medi a+ CSS (5%)+ DMSO (5µM) media to the negative control wells Add 2 ml of Media + CSS (5%)+MDV(5µM) for drug treatment
incubate in the 37°C brood chamber (5% CO2) for 24h

Material

Media:

- Dulbecco's Modified Eagle Medium – High Glucose
  (Gibco, Thermo Fisher Scientific, Waltham,MA,USA
- RPMI 1640 (Gibco, Thermo Fisher Scientific)
- Charcoal Stripped Fetal Bovine Serum (Gibco, Thermo Fisher Scientific)
- Fetal Bovine Serum (Gibco, Thermo Fisher Scientific)

Buffers for "In Situ" Protocol

DEPC-PBS-Tween:

Use DEPC milliq H_2O to make a PBS 10x Stock of pH 7.2
20 ml DEPC-PBS (10x) + 180 ml DEPC-H_2O + 100 µl Tween 20 = 200ml DEPC-PBS Tween

0.1M HCl-DEPC-H2O:

Use DEPC milliq H_2O

200 ml H_2O (always water first!) + 1.66ml HCl (37%) = 0.1 M HCl-DPC-H_2O

3 % Formaldehyde in DEPC-PBS

200 ml (3 % Formaldehyde in DEPC-PBS) = 16.22ml (37% Formaldehyde) + 183.78 ml DEPC-PBS-H_2O

KCl [1M]

M [KCl] = 74.5513 g/mol
200ml (KCl 1M)= 14.91 g KCl + 200ml DEPC-H2O

20X SSC

A 20X stock solution consists of 3 M sodium chloride and 300 mM trisodium citrate (adjusted to pH 7.0 with HCl)
175,3 g NaCl
88,2 g Sodium citrate tribasic dehydrate +~800 ml DEPC-H_2O
pH: 7.0 with HCl
Fill up to 1 L and autoclave

2x Hybridisation Buffer

=2xHyb buffer is 20 % Formamide in 4 x SSC
Prepare 80 ml of 4xSSC = 16 ml 20xSSX + 64 ml DEPC-H2O
Prepare 2xHyb buffer = 80 ml 4xSSC + 20 ml Formamide
Protect from light

2x SCC – 0.05 %Tween buffer

=20 ml 20xSCC + 180 ml DEPC-H2O + 100 µl Tween 20

Reagents for "In Situ" Protocol

- Transcriptme Reverse Transcriptase (DNA-Gdansk, Gdansk,Poland),
- RiboLock RNase inhibitor (Thermo Fisher Scientific)
- dNTPs (Thermo Fisher Scientific)
- BSA (NEB, Ipswich, MA, USA)
- RT Reaction Buffer (DNA-Gdansk)
- Ampligase (Epicentre, Illumina,Madison, WI, USA),
- RNase H (Thermo Fisher Scientific)
- phi29 DNA polymerase (Thermo Fisher Scientific)
- phi29 buffer (Thermo Fisher Scientific)
- Primer for reverse transcription (Exiqon Vedbaek, Denmark)
    - Stock conc.: 10 µM [µmol/l]
- Padlock Probes (Integrated DNA Technologies, Coralville, IA, USA)
    - Stock conc.: 10 µM [µmol/l]
- Detection Probes (Ordered from Biomers, Ulm, Germany)
    - Stock conc.: 10 µM [µmol/l]

Labels as well as the Sequences of all Primers and Padlocks used

Label Graz	Label Extern	Name	5´to 3´
		Kras1 (paper Grundberg)	T+GT+AT+CG+TC+AA+GG+CACTCTT
G0003		Kras2 (paper Grundberg)	C+CT+CT+AT+TG+TT+GG+ATCATATTCGTC
G0002	S00122	p_LNA_k-ras_2	T+AT+TC+GT+CC+AC+AA+AATGATTCTGAA
G0001	S00321	pLNAACTB_61	C+GG+GC+GG+CG+GATCGGCAAAG
G0004		Random Primer	random 10mer (ordered from IDT, PAGE purified, machine-mixed option chosen)
G0034		AR-FL_LNA_1	C+CA+TC+TG+GT+CG+TCCACGTGTAAGTT
G0037		AR-V7_LNA_1	T+CT+GG+TC+AT+TT+TGAGATGCTTGC
G0039		AR-V12_LNA_1	G+AT+TA+GC+AG+GT+CAAAGTG
G0041		PSA_LNA_1	G+AG +GT+C CA+C AC+A CT+G AAG TTT
G0060	S02772	AR-V7_LNA_2	T+CT+AG+AG+CC+CT+CATTTT
G0061	S02794	RV_AR-V7_4	AAGCCACATTACAGGAAACA
G0062	S02795	RV_AR-V7_3	ACAAATAAAGATGGCCACAG
G0063	S02796	RV_AR-V7_2	AAGGCTAGATGTAAGAGG
G0064	S02797	RV_AR-V7_1	GACTCCACTTCTCCACTA
G0067	S02204	pAR-V7_CE3_LNA	+GG+TC+TG+GT+CATTTTGAGATGC
G0070	S02561	pAR-V7_CE3_3'LNA	=+GG+GT+CT+GGTCATTTTGAGATGC
G0072	S00007	pLNA_AR	C+CA+TC+TG+GT+CG+TCCACGTGTAAGTT
	Padlock Probes		
Label Graz	**Label Extern**	**Name**	**5´to 3´**
G0005	P4616	plp_KRAS_ mut_c12_ pos1_TGT	GTGGCGTAGGCAAGATTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGTGGTAGTTGGAGCTT
G0006	P4613	plp_KRAS_wt_c12_pos1_GGT	GTGGCGTAGGCAAGATCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGTGGTAGTTGGAGCTG
G0007	P4611	plp_KRAS_ mut_c12_ pos2_GTT	TGGCGTAGGCAAGAGTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGGGTAGTTGGAGCTGT
G0008		Kras_wt_pos2_c12_pos 2 (WT2 Paper_grundberg 2013)	TGGCGTAGGCAAGAGTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGGGTAGTTGGAGCTGG
G0009		PP-KRAS-G12D® (DP-2) (Paper_grundberg 2013)	TGGCGTAGGCAAGAGTTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGGGTAGTTGGAGCTGA
G0010	S00088	Pd_KRAS_wt_c13_pos2_GGC	CGTAGGCAAGAGTGCTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGAGTTGGAGCTGGTGG
G0011	P4618	plp_KRAS_ mut_c13_pos2_GAC	CGTAGGCAAGAGTGCTTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGAGTTGGAGCTGGTGA
G0012	S01394	PdACT1cDNA	AGCCTCGCCTTTGCCTTCCTTTTACGACCTCAATGCTGCTGCTGTACTACTCTTCGCCCCGCGAGCACAG
G0013		PP-ACTB-II® (DP-2) (Paper_grundberg 2013)	AGCCTCGCCTTTGCCTTCCTTTTACGACCTCAATGCACATGTTTGGCTCCTCTTCGCCCCGCGAGCACAG
		experimental AR-V7 PLP	GAAAAATTCCGGGTTTTCCTTTTACGACCTCAATGCTGCTGCTGTACTACTCTTCGGGATGACTCTGGGA
		experimental PSA	CCCACTGCATCAGGATTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGGGGTCCTCACAGCTG
G0035		plp_AR-FL1	PHOS-GGGCCAAGGCCTTGCCTGGCCTCAATGCACATGTTTGGCTCCTAAAGTCGGAAGTACTACTCTCTCTTGTACACGTGGTCAAGT
G0036		plp_AR-FL2	PHOS-GGGCCAAGGCCTTGCCTGGTTCTAGATCCCTCAATGCACATGTTTGGCTCCGGTTCAAGCTTGTACACGTGGTCAAGT
G0038		plp_AR-V7	PHOS-AAAAATTCCGGGTTGTTCCTTTTACGAACCTCAATGCTGCTGCTGTACTACTCTTCGGATGACTCTGGGAG
G0040		plp_AR-V12	PHOS-CCTTGCCTGATTGCGTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGAGAGAGCTGCATCAG
G0042		plp_PSA_1	PHOS-ACCAGAGGAGTTCTTGTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGCTGGGGCAGCATTGA
G0043		plp_PSA_2	PHOS-GACCCCAAAGAAACTTCCTAGTAATCAGTAGCCGTGACTATCGACTGGTTCAAAGACCAGAGGAGTTCTT
G0051	S02785	plp_AR-V7_2	GGCTGACTTGCCTCATTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCGGCTATCACCAGACCCTGAAGAAA
G0052	S02786	plp_AR-V7_3	GGTTACCACTCATGTAGAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCGGCTATCGGGCTGTAGAAGTAATAGT
G0053	S02787	plp_AR-V7_4	TGCTTTTCGTGGTGTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCGTCTATCTCTGGCTCAGTCGCT
G0054	S02788	plp_AR-V7_5	TGTCTGTCTGAGGTTCCTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCGACTATCTTGGACAAGAAGCAACTG
G0055	S02789	plp_AR-V7_6	CTAGCCTTCTGGATCCCAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCCGCTATCTATTTCCCCCTTAGGTT
G0056	S02790	plp_AR-V7_7	AGCTGATCCACACAGAAGTTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCATCTATCACTGAGCTGAAGGTAGT
G0057	S02791	plp_AR-V7_8	GTCCGACTTTCCCTCTTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCCTCTATCCACACTGAGAGACTACA
G0058	S02792	plp_AR-V7_9	TGCTTCTCTTCATCAGTAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCCACTATCTGCTCTCTCACATCACA
G0059	S02793	plp_AR-V7_10	CCATAGCTTCCATATTGACAACCTCAATGCTGCTGCTGTACTACAATGCGTCTATTTAGTGGAGCCAGCTATCATCTTGTTCTCTCTCTGCT
G0068	S02200	AR-V7_B2DO_2	/5Phos/AAAAATTCCGGGTTGGAAAAGTAGCCGTGACTATCGACTAAAAGTAGCCGTGACTATCGACTGGGATGACTCTGGGAG
G0069	S02553	AR-V7_B2DO_3'	/5Phos/CAAGCATCTCAAAATGAAAAAGTAGCCGTGACTATCGACTAAAAGTAGCCGTGACTATCGACTAAATTCCGGGTTGGCAATTG
G0071	S02682	_IS_AR_FL	PHOS-GGGCCAAGGCCTTGCCTGGAGTCGAAGTACTACTCTCTAAAAGTCGAAGTACTACTCTCTCTTGTACACGTGGTCAAGT
	Detection probes		
Label Graz	**Label Extern**	**Name**	**5´to 3´**
D0001	S00103	Lin16_Cy3_SA	CCTCAATGCTGCTGCTGTACTAC
D0002	S00861	Lin16_TR	CCTCAATGCTGCTGCTGTACTAC
D0003	S00086	B2_DO_Cy3	AGTAGCCGTGACTATCGACT
D0004	S00077	Lin33_Cy5	CCTCAATGCACATGTTTGGCTCC
D0005		DP3-FITC (lin16)	FAM - CCTCAATGCTGCTGCTGTACTAC
D0006		Lin33_CY3	CY3-CCTCAATGCACATGTTTGGCTCC
D0007		B2_DO_CY5	CY5-AGTAGCCGTGACTATCGACT
D0008		B2_DO_FITC	FAM-AGTAGCCGTGACTATCGACT
D0009		Lin33_FITC	FAM-CCTCAATGCACATGTTTGGCTCC
D0010		Lin16_ATTO 550 (~CY3)	Atto 550- CCTCAATGCTGCTGCTGTACTAC
D0011		Lin33_ATTO 488 (~FITC)	Atto 488- CCTCAATGCACATGTTTGGCTCC
D0012		B2_DO_ATTO 647N (~CY5)	Atto 647N-AGTAGCCGTGACTATCGACT
	PCR-Primer		
Label Graz	**Label Extern**	**Name**	**5´to 3´**
P0001		AR-FL-f	CAGCCTATTGCGAGAGAGCTG
P0002		AR-FL-r	GAAAGGATCTTGGGCACTTGC
P0003		AR-V7-f	CCATCTTGTCGTCTTCGGAAATGTTATGAAGC
P0004		AR-V7-r	TTTGAATGAGGCAAGTCAGCCTTTCT
P0005		AR-V12-f	CCAAGGCCTTGCCTGATTGC
P0006		AR-V12-r	TTGGGCACTTGCACAGAGAT
P0007		KLK3-f	AACCAGAGGAGTTCTTGACC
P0008		KLK3-R	AGCACACAGCATGAACTTG
G0044		AR-FL_Plus Strand_+6bp up and do	TCCGTGCAGCCTATTGCGAGAGAGCTGCATCAGTTCACTTTTGACCTGCTAATCAAGTCACACATGGTGAGCGTGGACTTTCCGGAAATGATGGCAGAGATCAT CTCTGTGCAAGTGCCCAAGATCCTTTCTGGGAA

Experiment W0002

Date 30.07.2015
Wire W0002

EtOH series 70%, 85% & 100% for 2 minutes

Rehydrate ~300 µl 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 0 0,8

In situ RT		stock	final	1	MM - 1 x SW620
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
pACTB (G0001)		**10µM**	1µM	5	4
pKRAS (G0002)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,4 µg/µl	0,5	0,4
DEPC H_20				25,75	20,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

Put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C̊

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,6

LIGATION of padlocks	Padlock Backbone	stock	final	1x	MM - 1 x SW620
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	3,00
AMP buffer		10X	1X	5,00	3,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	2,40
BSA		20 µg/µl	0.4 µg/µl	0,5	0,30
KCl		1 M	0.05 M	2,5	1,50
Formamide		100%	20%	10	6,00
G0007_plp_KRAS_ mut_c12_ pos2_GTT	Lin33	10 µM	0.1 µM	0,5	0,30
G0012_PdACT1cDNA	Lin16	10 µM	0.1 µM	0,5	0,30
DEPC H_20				22,00	13,20
Volume			**50**	**50,00**	**30,00**
each sample (1x)					**50**

Incubate at 37 C̊ for 30 minutes and 45 C̊ for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C̊ for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,6

RCA		stock	final	1	MM-1
Φ29 polymerase		10 U/µl	1 U/µl	5	3
Φ29 buffer		10x	1x	5	3
dNTP		10 mM	0,25 mM	1,25	0,75
BSA		20 µg/µl	0,4 µg/µl	0,5	0,3
Glycerol		50%	5%	5	3
DEPC H_20				33,25	19,95
Volume [µl]			**50**	**50**	30
each sample (1x)					50

Add MM and Incubate pipette ON at RT
2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

Detection probe hybridisation		stock	final	1	Placenta
2xHyb buffer		2x	1x	25	50
D0001_Lin16_Cy3	Cy3	10 µM	0.1 µM	0,5	1
D0004_Lin33_Cy5	Cy5	10 µM	0.1 µM	0,5	1
DEPC H_20				24	48
Volume			**50**	**50**	100
each sample (1x)					50

From now on protect slides from light
37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Experiment CH0001 - CH0003

Cell line / Tissue LNCaP, VCaP 3 Secure seals per slide
Neg. Controll SW620 3 Secure seals per slide

3 Spots per Slide Spot1: AR-FL1, AR-V7, AR-V12
Spot2: B-ACTIN, AR-FL1
Spot3: B-ACTIN, AR-FL2

Date 12.08.2015

EtOH series 70%, 85% & 100% for 2 minutes

MOUNT 3 SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 3 6

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1	Spot 2+3
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	15	30
RT buffer		10X	1X	5	15	30
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	3,75	7,5
pACTB (G0001)		**10µM**	1µM	5	0	30
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	15	30
AR-V7_LNA_1 (G0037)		**10µM**	1µM	5	15	0
AR-V12_LNA_1 (G0039)		**10µM**	1µM	5	15	0
dNTP		10 mM	0,5 mM	2,5	7,5	15
BSA		20 µg/µl	0,2 µg/µl	0,5	1,5	3
DEPC H_2O				15,75	62,25	154,5
Final Volume			**50**	**50**	**150**	**300**
each sample (1x)					**50**	**50**

Apply tapes (PCR plate seals), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3 3 3

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT1	SPOT 2	SPOT 3
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	15,00	15,00	15,00
AMP buffer		10X	1X	5,00	15,00	15,00	15,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	12,00	12,00	12,00
BSA		20 µg/µl	0,2 µg/µl	0,5	1,50	1,50	1,50
KCl		1 M	0.05 M	2,5	7,50	7,50	7,50
Formamide		100%	20%	10	30,00	30,00	30,00
plp_AR-FL1 (G0035)	Lin33	10 µM	0.1 µM	0,5	1,50	1,50	0,00
plp_AR-FL2(G0036)	Lin33	10 µM	0.1 µM	0,5	0,00	0,00	1,50
plp_AR-V7 (G0038)	Lin16	10 µM	0.1 µM	0,5	1,50	0,00	0,00
plp_AR-V12 (G0040)	B2_DO	10 µM	0.1 µM	0,5	1,50	0,00	0,00
PrIACT1cDNA (G0012)	Lin16	10 µM	0.1 µM	0,5	0,00	1,50	1,50
DEPC H_2O				20,50	64,50	66,00	66,00
Volume			**50**	**50,00**	**150,00**	**150,00**	**150,00**
each sample (1x)					**50**	**50**	**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 9

RCA		stock	final	1	SPOT1+2+3
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	45
Φ29 buffer		10x	1x	5	45
dNTP		10 mM	0,25 mM	1,25	11,25
BSA		20 µg/µl	0,2 µg/µl	0,5	4,5
Glycerol		50%	5%	5	45
DEPC H_2O				33,25	299,25
Volume [µl]			**50**	**50**	450
each sample (1x)					50

Apply tapes
Incubate slides ON at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3 6

Detection probe hybridisation		stock	final	1	SPOT 1	Spot 2+3
2xHyb buffer		2x	1x	25	75	150
D0001_Lin16_Cy3	**Cy3**	10 µM	0.1 µM	0,5	1,5	3
D0004_Lin33_Cy5	Cy5	10 µM	0.1 µM	0,5	1,5	3
D0008_B2_DO_FITC	**FITC**	10 µM	0.1 µM	0,5	1,5	0
DEPC H_2O				23,5	70,5	144
Volume			**50**	**50**	150	300
each sample (1x)					50	50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl Prolong Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment W0003

Protocol received from Amin

Wire WIRE: W003
(Already AB-Stained patient sample wire very few cells!)

Date 03.09.2015

EtOH series 70%, 85% & 100% for 2 minutes

Rehydrate ~300µl 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 0,8

In situ RT		stock	final	MIXx1(LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
pACTB (G0001)		**10µM**	1µM	5	4
AR-V12_LNA_1(G0039)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_20				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-V12(G0040)	B2_DO	10 µM	0.1 µM	0,5	0,40
plp_PSA_1(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
PdACT1cDNA (G0012)	Lin16	10 µM	0.1 µM	0,5	0,40
DEPC H_20				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_20				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette at ON at RT

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0003_B2_DO_Cy3	Cy3	10 µM	0.1 µM	0,5	0,4
		10 µM	0.1 µM	0,5	0
D0005_DP3-FITC (lin16)	FITC	10 µM	0.1 µM	0,5	0,4
DEPC H_20				23,5	19,2
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Experiment CH0004 - CH0006

Protocol received from Amin

Cell line / Tissue	LNCaP, Vcap 3 Secure seals per slide	
Neg. Controll	sw620 3 Secure seals per slide	
3 Spots per Slide	Spot1:	B_ACTIN, AR-FL1, PSA1
	Spot2:	B_ACTIN, AR-FL1, PSA2
	Spot3:	B_ACTIN, AR-FL1, PSA1+ATTO Dyes
Date	07.09.2015	

EtOH series 70%, 85% & 100% for 2 minutes

MOUNT 3 SECURE SEALS (35 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 9,5

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	3,5	33,25
RT buffer		10X	1X	3,5	33,25
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	0,875	8,31
pACTB (G0001)		**10µM**	1µM	3,5	33,25
AR-FL_LNA_1 (G0034)		**10µM**	1µM	3,5	33,25
PSA_LNA_1 (G0041)		**10µM**	1µM	3,5	33,25
dNTP		10 mM	0,5 mM	1,75	16,63
BSA		20 µg/µl	0,2 µg/µl	0,35	3,33
DEPC H_20				14,53	137,99
Final Volume			**35**	**35**	**332,5**
each sample (1x)					**35**

Apply tapes (PCR plate seals), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C˚

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3,1 3,1 3,1

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT1	SPOT 2	SPOT 3
Ampligase (pipet last)		5 U/µl	0.5 U/µl	3,50	10,85	10,85	10,85
AMP buffer		10X	1X	3,50	10,85	10,85	10,85
Rnase H (pipet last)		5 U/µl	0.4 U/µl	2,8	8,68	8,68	8,68
BSA		20 µg/µl	0,2 µg/µl	0,35	1,09	1,09	1,09
KCl		1 M	0.05 M	1,75	5,43	5,43	5,43
Formamide		100%	20%	7	21,70	21,70	21,70
plp_AR-FL1 (G0035)	Lin33	10 µM	0.1 µM	0,35	1,09	1,09	1,09
plp_PSA1(G0042)	B2_DO	10 µM	0.1 µM	0,35	1,09	0,00	1,09
plp_PSA2(G0043)	B2_DO	10 µM	0.1 µM	0,35	0,00	1,09	0,00
PrEACT1cDNA (G0012)	Lin16	10 µM	0.1 µM	0,35	1,09	1,09	1,09
DEPC H_20				14,70	46,66	46,66	46,66
Volume			**35**	**35,00**	**108,50**	**108,50**	**108,50**
each sample (1x)					**35**	**35**	**35**

Incubate at 37 C˚ for 30 minutes and 45 C˚ for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C˚ for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 9,2

RCA		stock	final	1	SPOT1+2+3
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	3,5	32,2
Φ29 buffer		10x	1x	3,5	32,2
dNTP		10 mM	0,25 mM	0,875	8,05
BSA		20 µg/µl	0,2 µg/µl	0,35	3,22
Glycerol		50%	5%	3,5	32,2
DEPC H_20				23,275	214,13
Volume [µl]			**35**	**35**	322
each sample (1x)					35

Apply tapes
Incubate slides at ON at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 6,4 3,2

Detection probe hybridisation			stock	final	1	SPOT 1+2	Spot 3
2xHyb buffer			2x	1x	17,5	112	56
D0010_Lin16_ATTO 550	Cy3		10 µM	0.1 µM	0,35	0	1,12
D0011_Lin33_ATTO 488	FITC		10 µM	0.1 µM	0,35	0	1,12
D00012_B2_DO_ATTO	CY5		10 µM	0.1 µM	0,35	0	1,12
D0003_B2_DO	Cy3		10 µM	0.1 µM	0,35	2,24	0
D0004_Lin33_Cy5	Cy5		10 µM	0.1 µM	0,35	2,24	0
D0005_DP3-FITC	FITC		10 µM	0.1 µM	0,35	2,24	0
DEPC H_20					15,4	105,28	52,64
Volume				**35**	**35**	224	112
each sample (1x)						35	35

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment W0004

Protocol received from Amin

Date	12.09.2015
Wire	W0004
	AR-FL1, AR-V7, PSA,
	NO B-ACTIN because of high signal cross talk
Cell line	VCaP (seeding without ethanol dehydration step afterword)

Rehydrate ~300µl 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 0,8

In situ RT		stock	final	MIXx1(LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	4
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_20				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,40
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,40
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
DEPC H_20				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_20				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette ON at RT

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	0,4
D0011_Lin33_ATTO 488	**Cy5**	10 µM	0.1 µM	0,5	0,4
D00012_B2_DO_ATTO	**FITC**	10 µM	0.1 µM	0,5	0,4
DEPC H_20				23,5	18,8
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Slowfade Gold Antifade 30µl in pipette

Experiment W0005

Protocol received from Amin

Date	15.09.2015
Wire	W0005 AR-FL1, AR-V7, PSA, NO B-ACTIN because of high signal cross talk
Cell line	LNCaP (seeding without ethanol dehydration step afterword)

Rehydrate ~300µl 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 0,8

In situ RT		stock	final	M(x1(LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	4
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_2O				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,40
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,40
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
DEPC H_2O				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_2O				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette at 37 for 3 hour or ON at RT
2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	0,4
D0011_Lin33_ATTO 488	Cy5	10 µM	0.1 µM	0,5	0,4
D00012_B2_DO_ATTO	FITC	10 µM	0.1 µM	0,5	0,4
DEPC H_2O				23,5	18,8
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Wire: Slowfade Gold Antifade 30µl in pipette
Slide: Prolong Gold Antifade

Experiment Wooo6

Protocol received from Amin

Date	29.09.2015
Wire	W0006
	AR-FL1, AR-V7, PSA,
	NO B-ACTIN because of high signal cross talk
Cell line	LNCaP (seeding without ethanol dehydration step afterwards)

Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 0,8

In situ RT		stock	final	MIX x1 (LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	4
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_20				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,40
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,40
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
DEPC H_20				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_20				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette ON at RT
2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	0,4
D0011_Lin33_ATTO 488	**FITC**	10 µM	0.1 µM	0,5	0,4
D00012_B2_DO_ATTO	**CY5**	10 µM	0.1 µM	0,5	0,4
DEPC H_20				23,5	18,8
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Slowfade Gold Antifade 30µl in pipette

Experiment W0007

Date	06.10.2015
Wire	W0007
	AR-FL1, AR-V7, PSA,
	NO B-ACTIN because of high signal cross talk
Cell line	LNCaP (seeding without ethanol dehydration step afterwards)

(Cells are already fixed with Formaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 0,8

In situ RT		stock	final	MIXx1(LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	4
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_20				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,40
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,40
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
DEPC H_20				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_20				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette ON at RT

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	0,4
D0011_Lin33_ATTO 488	Fitc	10 µM	0.1 µM	0,5	0,4
D00012_B2_DO_ATTO	Cy5	10 µM	0.1 µM	0,5	0,4
DEPC H_20				23,5	18,8
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)

Experiment Wooo8

Protocol received from Amin

Date	27.10.2015
Wire	W0008 AR-FL1, AR-V7, PSA,
Cell line	VCaP (seeding without ethanol dehydration step afterwards)

(Cells are already fixed with Formaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 0,8

In situ RT		stock	final	MIXx1(LNAs)	Wire
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	4
RT buffer		10X	1X	5	4
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	4
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	4
PSA_LNA_1(G0041)		**10µM**	1µM	5	4
dNTP		10 mM	0,5 mM	2,5	2
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
DEPC H_20				20,75	16,6
Final Volume			**50**	**50**	**40**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2,5 hrs at 45 C̊

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	4,00
AMP buffer		10X	1X	5,00	4,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	3,20
BSA		20 µg/µl	0,2 µg/µl	0,5	0,40
KCl		1 M	0.05 M	2,5	2,00
Formamide		100%	20%	10	8,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,40
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,40
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,40
DEPC H_20				21,50	17,20
Volume			**50**	**50,00**	**40,00**
each sample (1x)					**50**

Incubate at 37 C̊ for 30 minutes and 45 C̊ for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C̊ for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 0,8

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	4
Φ29 buffer		10x	1x	5	4
dNTP		10 mM	0,25 mM	1,25	1
BSA		20 µg/µl	0,2 µg/µl	0,5	0,4
Glycerol		50%	5%	5	4
DEPC H_20				33,25	26,6
Volume [µl]			**50**	**50**	40
each sample (1x)					50

Add MM and Incubate pipette ON at RT

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 0,8

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	20
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	0,4
D0011_Lin33_ATTO 488	**Cy5**	10 µM	0.1 µM	0,5	0,4
D00012_B2_DO_ATTO	**FITC**	10 µM	0.1 µM	0,5	0,4
DEPC H_20				23,5	18,8
Volume			**50**	**50**	40
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the Wire (5-10min)

Experiment W0009

Protocol received from Amin

Date	11.11.2015
Wire	W0009
Slides	CH0007
	AR-FL1, AR-V7, PSA,
Cell line	VCaP

(Cells are already fixed with Formaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Wire:Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
Wire:2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 1,8

In situ RT		stock	final	MIXx1(LNAs)	Wire and slide
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	9
RT buffer		10X	1X	5	9
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	2,25
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	9
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	9
PSA_LNA_1(G0041)		**10µM**	1µM	5	9
dNTP		10 mM	0,5 mM	2,5	4,5
BSA		20 µg/µl	0,2 µg/µl	0,5	0,9
DEPC H_20				20,75	37,35
Final Volume			**50**	**50**	**90**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2,5 hrs at 45 C̊

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS) in eppi
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire and Slide
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	10,00
AMP buffer		10X	1X	5,00	10,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	8,00
BSA		20 µg/µl	0,2 µg/µl	0,5	1,00
KCl		1 M	0.05 M	2,5	5,00
Formamide		100%	20%	10	20,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	1,00
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	1,00
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	1,00
DEPC H_20				21,50	43,00
Volume			**50**	**50,00**	**100,00**
each sample (1x)					**50**

Incubate at 37 C̊ for 30 minutes and 45 C̊ for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C̊ for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 2

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	10
Φ29 buffer		10x	1x	5	10
dNTP		10 mM	0,25 mM	1,25	2,5
BSA		20 µg/µl	0,2 µg/µl	0,5	1
Glycerol		50%	5%	5	10
DEPC H_20				33,25	66,5
Volume [µl]			**50**	**50**	100
each sample (1x)					50

Add MM and Incubate pipette ON at RT

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	50
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	1
D0011_Lin33_ATTO 488	**FITC**	10 µM	0.1 µM	0,5	1
D00012_B2_DO_ATTO	**Cy5**	10 µM	0.1 µM	0,5	1
DEPC H_20				23,5	47
Volume			**50**	**50**	100
each sample (1x)					50

From now on protect slides/wire from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Slides:Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
Slides: 2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Slide: Coverglass and antifade
Wire: Slowfade

Experiment W0010

Date		16.11.2015
Wire	W0010	
Slides	CH0008	
	AR-FL1, PSA, B-ACTIN	
Cell line	LNCaP	

(Cells are already fixed with Paraformaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Wire:Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
Wire:2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 2

In situ RT		stock	final	MIXx1(LNAs)	Wire and slide
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	10
RT buffer		10X	1X	5	10
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	2,5
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	10
pACTB (G0001)		**10µM**	1µM	5	10
PSA_LNA_1(G0041)		**10µM**	1µM	5	10
dNTP		10 mM	0,5 mM	2,5	5
BSA		20 µg/µl	0,2 µg/µl	0,5	1
DEPC H_20				20,75	41,5
Final Volume			**50**	**50**	**100**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2,5 hrs at 45 C̊

Fixation 3,7% formaldehyde in DEPC-PBS at RT for 5 minutes
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire and Slide
Ampligase (pipet last)		20 U/µl	0.5 U/µl	5,00	10,00
AMP buffer		10X	1X	5,00	10,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	8,00
BSA		20 µg/µl	0.2 µg/µl	0,5	1,00
KCl		1 M	0.05 M	2,5	5,00
Formamide		100%	20%	10	20,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	1,00
PdACT1cDNA (G0012)	lin16	10 µM	0.1 µM	0,5	1,00
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	1,00
DEPC H_20				21,50	43,00
Volume			**50**	**50,00**	**100,00**
each sample (1x)					**50**

Incubate at 37 C̊ for 30 minutes and 45 C̊ for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C̊ for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 2

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	10
Φ29 buffer		10x	1x	5	10
dNTP		10 mM	0,25 mM	1,25	2,5
BSA		20 µg/µl	0,2 µg/µl	0,5	1
Glycerol		50%	5%	5	10
DEPC H_20				33,25	66,5
Volume [µl]			**50**	**50**	100
each sample (1x)					50

Add MM and Incubate pipette at 37 for 3 hour

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	50
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	1
D0011_Lin33_ATTO 488	**FITC**	10 µM	0.1 µM	0,5	1
D00012_B2_DO_ATTO	**Cy5**	10 µM	0.1 µM	0,5	1
DEPC H_20				23,5	47
Volume			**50**	**50**	100
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each
Slides:Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
Slides: 2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Slide: Coverglass and antifade

Experiment W0011

Date		19.11.2015
Wire	W0011	
Slides	CH0009	
	AR-FL1, PSA, B-ACTIN	
Cell line	LNCaP	

(Cells are already fixed with Paraformaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 2

In situ RT		stock	final	MIXx1(LNAs)	Wire and slide
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	10
RT buffer		10X	1X	5	10
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	2,5
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	10
pACTB (G0001)		**10µM**	1µM	5	10
PSA_LNA_1(G0041)		**10µM**	1µM	5	10
dNTP		10 mM	0,5 mM	2,5	5
BSA		20 µg/µl	0,2 µg/µl	0,5	1
DEPC H_20				20,75	41,5
Final Volume			**50**	**50**	**100**
each sample (1x)					**50**

put the Wire into a glas pipette until the golden end of the wire nearly reaches the (small) end of the pipette
Add appropriate volume from the small end make sure no bubbles form, put wire/pipette construct into a preheated humid chamber and incubate for 2,5 hrs at 45 C̊

Fixation 4% Paraformaldehyde in DEPC-PBS at RT for 5 minutes
2x washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

LIGATION of padlocks	Padlock Backbone	stock	final	1x	Wire and Slide
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	10,00
AMP buffer		10X	1X	5,00	10,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	8,00
BSA		20 µg/µl	0,2 µg/µl	0,5	1,00
KCl		1 M	0.05 M	2,5	5,00
Formamide		100%	20%	10	20,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	1,00
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	1,00
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	1,00
DEPC H_20				21,50	43,00
Volume			**50**	**50,00**	**100,00**
each sample (1x)					**50**

Incubate at 37 C̊ for 30 minutes and 45 C̊ for 45 minutes in pipette
Wash 1x with 2xSSC-Tween at 37 C̊ for 5 minutes in eppi
Wash 2x DEPC-PBS-Tween in eppi

Master Mix folds: 2

RCA		stock	final	1	Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	10
Φ29 buffer		10x	1x	5	10
dNTP		10 mM	0,25 mM	1,25	2,5
BSA		20 µg/µl	0,2 µg/µl	0,5	1
Glycerol		50%	5%	5	10
DEPC H_20				33,25	66,5
Volume [µl]			**50**	**50**	100
each sample (1x)					50

Add MM and Incubate pipette at 37 for 3 hour

2 washes 1xDEPC-PBS-Tween for 2 min each in eppi

Master Mix folds: 2

Detection probe hybridisation		stock	final	1	Wire
2xHyb buffer		2x	1x	25	50
D0010_Lin16_ATTO 550	Cy3	10 µM	0.1 µM	0,5	1
D0011_Lin33_ATTO 488	**FITC**	10 µM	0.1 µM	0,5	1
D00012_B2_DO_ATTO	**Cy5**	10 µM	0.1 µM	0,5	1
DEPC H_20				23,5	47
Volume			**50**	**50**	100
each sample (1x)					50

From now on protect slides from light

37°C 30' in pipette
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Slide: Coverglass and antifade

Experiment W0013

Cell line / Tissue

W0013 VCaP	AR,V7,PSA
W001 Sw620 Frozen Wire	BACTIN, KRAS

Date 20.01.2016

(Cells are already fixed with Paraformaldehyde - 15 min)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
1,5 ml in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 1 1

In situ RT		stock	final	MIXx1(LNAs)	VCaP	Sw620
TranscriptMe RT (pipet last)		200 U/µl	5 U/µl	5	5	5
RT buffer		10X	1X	5	5	5
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1,25	1,25
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	5	0
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	5	0
pACTB (G0001)		**10µM**	1µM	5	0	5
pKRAS (G0002)		**10µM**	1µM	5	0	5
PSA_LNA_1(G0041)		**10µM**	1µM	5	5	0
dNTP		10 mM	0,5 mM	2,5	2,5	2,5
BSA		20 µg/µl	0,2 µg/µl	0,5	0,5	0,5
DEPC H_2O				10,75	20,75	25,75
Final Volume			**50**	**50**	**50**	**50**
each sample (1x)					**50**	**50**

Apply rubber to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 2,5 hrs at 45 ℃

Fixation 3% Formaldehyde in DEPC-PBS at RT for 15 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 1 1

LIGATION of padlocks	Padlock Backbone	stock	final	1x	VCaP	Sw620
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	5,00	5,00
AMP buffer		10X	1X	5,00	5,00	5,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	4,00	4,00
BSA		20 µg/µl	0,2 µg/µl	0,5	0,50	0,50
KCl		1 M	0.05 M	2,5	2,50	2,50
Formamide		100%	20%	10	10,00	10,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,50	0,00
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,50	0,00
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,50	0,00
G0007_plp_KRAS_ mut_c12_ pos2_GTT	Lin33	10 µM	0.1 µM	0,5	0,00	0,50
G0012_PdACT1cDNA	Lin16	10 µM	0.1 µM	0,5	0,00	0,50
DEPC H_2O				20,50	21,50	22,00
Volume			**50**	**50,00**	**50,00**	**50,00**
each sample (1x)					**50**	**50**

Incubate at 37 ℃ for 30 minutes and 45 ℃ for 45 minutes
Wash 1x with 2xSSC-Tween at 37 ℃ for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 2 0

RCA		stock	final	1	MM-1	MM-2
Φ29 polymerase		10 U/µl	1 U/µl	5	10	0
Φ29 buffer		10x	1x	5	10	0
dNTP		10 mM	0,25 mM	1,25	2,5	0
BSA		20 µg/µl	0,2 µg/µl	0,5	1	0
Glycerol		50%	5%	5	10	0
DEPC H_2O				33,25	66,5	0
Volume [µl]			**50**	**50**	100	0
each sample (1x)					50	#DIV/0!

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 2

Detection probe hybridisation			stock	final	1	Atto	
2xHyb buffer			2x	1x	25	50	
D0010_Lin16_ATTO 550	**Cy3**		10 µM	0.1 µM	0,5	1	
D0011_Lin33_ATTO 488	**FITC**		10 µM	0.1 µM	0,5	1	
D00012_B2_DO_ATTO	**Cy5**		10 µM	0.1 µM	0,5	1	
DEPC H_2O					23,5	47	
Volume				**50**	**50**	100	
each sample (1x)						50	

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
cut the tip of the 230 mm and fill it with Slowfade Antifade mounting media. Put the wire into the tip and twist slowly. Remove the wire and let it dry
24x55 mm coverslip to slides

Experiment W0012

Cell line / Tissue W0012
Vcap Wire and Slide

Date 15.12.2015
(Cells are already fixed with Paraformaldehyde)
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
300 µl in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 3

In situ RT		stock	final	MIXx1(LNAs)	MM - Placenta 5
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	15
RT buffer		10X	1X	5	15
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	3,75
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	15
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	15
PSA_LNA_1(G0041)		**10µM**	1µM	5	15
dNTP		10 mM	0,5 mM	2,5	7,5
BSA		20 µg/µl	0,2 µg/µl	0,5	1,5
DEPC H_20				20,75	62,25
Final Volume			**50**	**50**	**150**
each sample (1x)					**50**

Apply tapes (PCR plate seals), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 2,5 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3

LIGATION of padlocks	Padlock Backbone	stock	final	1x	MM Placenta
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	15,00
AMP buffer		10X	1X	5,00	15,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	12,00
BSA		20 µg/µl	0,2 µg/µl	0,5	1,50
KCl		1 M	0.05 M	2,5	7,50
Formamide		100%	20%	10	30,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	1,50
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	1,50
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	1,50
DEPC H_20				21,50	64,50
Volume			**50**	**50,00**	**150,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 3

RCA		stock	final	1	MM-1
Φ29 polymerase		10 U/µl	1 U/µl	5	15
Φ29 buffer		10x	1x	5	15
dNTP		10 mM	0,25 mM	1,25	3,75
BSA		20 µg/µl	0,2 µg/µl	0,5	1,5
Glycerol		50%	5%	5	15
DEPC H_20				33,25	99,75
Volume [µl]			**50**	**50**	150
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each
Cut Wire in 2 Pieces

Master Mix folds: 6 2

Detection probe hybridisation		stock	final	1	Atto	Cy
2xHyb buffer		2x	1x	25	150	50
D0001_Lin16_Cy3	**Cy3**	10 µM	0.1 µM	0,5	0	1
D0009_Lin33_FITC	**FITC**	10 µM	0.1 µM	0,5	0	1
D0007_B2_DO_CY5	**CY5**	10 µM	0.1 µM	0,5	0	1
D0010_Lin16_ATTO_550	**Cy3**	10 µM	0.1 µM	0,5	3	0
D0011_Lin33_ATTO_488	**FITC**	10 µM	0.1 µM	0,5	3	0
D00012_B2_DO_ATTO	**Cy5**	10 µM	0.1 µM	0,5	3	0
DEPC H_2O				23,5	141	47
Volume			**50**	**50**	300	100
each sample (1x)					50	50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Wire and Slides were Mount with ~ 50-60µl Prolong Gold

Experiment W0014 and W0015

Cell line / Tissue	W0015 Vcap Wire - blood	AR,V7,PSA
	W0014 Twisted Wire Vcap - blood	AR,V7,Bactin
	CH0011 Vcap Slide	AR,V7,Bactin

Date 25.01.2016

Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
Fix cells with formaldehyde 15 min
Wash 1xDEPC-PBS-Tween in Eppi @ RT for 5 minutes
1,5 ml in Eppi 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each
Check with Microscope if cells are on the wire*

Master Mix folds: 1 1 2

In situ RT		stock	final	MIXx1(LNAs)	VCaP-blood	Twisted Wire + slide
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	5	10
RT buffer		10X	1X	5	5	10
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	1,25	2,5
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	5	10
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	5	10
pACTB (G0001)		**10µM**	1µM	5	0	10
PSA_LNA_1(G0041)		**10µM**	1µM	5	5	0
dNTP		10 mM	0,5 mM	2,5	2,5	5
BSA		20 µg/µl	0,2 µg/µl	0,5	0,5	1
DEPC H_20				15,75	20,75	41,5
Final Volume			**50**	**50**	**50**	**100**
each sample (1x)					**50**	**50**

Apply rubber to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 2,5 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 15 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 1 2

LIGATION of padlocks	Padlock Backbone	stock	final	1x	VCaP-blood	Twisted Wire + slide
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	5,00	10,00
AMP buffer		10X	1X	5,00	5,00	10,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	4,00	8,00
BSA		20 µg/µl	0,2 µg/µl	0,5	0,50	1,00
KCl		1 M	0.05 M	2,5	2,50	5,00
Formamide		100%	20%	10	10,00	20,00
plp_AR-FL1(G0035)	Lin33	10 µM	0.1 µM	0,5	0,50	1,00
plp_AR-V7(G0038)	lin16	10 µM	0.1 µM	0,5	0,50	1,00
plp_PSA_2(G0042)	B2_DO	10 µM	0.1 µM	0,5	0,50	0,00
G0012_PdACT1cDNA	Lin16	10 µM	0.1 µM	0,5	0,00	1,00
DEPC H_20				21,00	21,50	42,00
Volume			**50**	**50,00**	**50,00**	**100,00**
each sample (1x)					**50**	**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 3

RCA		stock	final	1	MM-1
Φ29 polymerase		10 U/µl	1 U/µl	5	15
Φ29 buffer		10x	1x	5	15
dNTP		10 mM	0,25 mM	1,25	3,75
BSA		20 µg/µl	0,2 µg/µl	0,5	1,5
Glycerol		50%	5%	5	15
DEPC H_20				33,25	99,75
Volume [µl]			**50**	**50**	150
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3

Detection probe hybridisation			stock	final	1	Atto
2xHyb buffer			2x	1x	25	75
D0010_Lin16_ATTO 550	**Cy3**		10 µM	0.1 µM	0,5	1,5
D0011_Lin33_ATTO 488	**FITC**		10 µM	0.1 µM	0,5	1,5
D00012_B2_DO_ATTO	**Cy5**		10 µM	0.1 µM	0,5	1,5
DEPC H_20					23,5	70,5
Volume				**50**	**50**	150
each sample (1x)						50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each

EtOH series (2' in 70,85 and 97%)
Cut the tip of the 230 mm and fill it with Slowfade gold mounting media
Put the wire into the tip and incubate and twist for 30 sec. Put wire in an eppi, seal with parafilm and let shake overhead on the rotator for at least 1h.
Remove the wire and let it dry
Add coverslip onto the slide

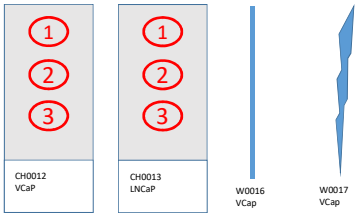
Experiment W0016 and W0017

Protocol received from Amin

Cell line / Tissue — LNCaP, Vcap 3 Secure seals per slide

		Targets	Probes From	Dyes
3 Spots per Slide	Spot1:	AR-FL, AR-V7, PSA	GRAZ	ATTO
	Spot2:	AR-FL, AR-V7	GRAZ	CY
	Spot3:	AR-FL, AR-V7	STHLM	CY
	WIRE Normal (DC01) + Twisted (DC02)	AR-FL, AR-V7, PSA	GRAZ	ATTO

Date 10.02.2016



Wires are charged with VCaP cells and direclty formaldehyde fixed
EtOH series 70%, 85% & 100% for 2 minutes

MOUNT 3 SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Comment: Twisted was accidently stored at -80°C for >6month

Master Mix folds:

				1	4	2,2	2,2
In situ RT		**stock**	**final**	**MIXx1(LNAs)**	**SPOT 1 + both Wires**	**SPOT 2**	**SPOT 3**
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	20,00	11	11
RT buffer		10X	1X	5	20,00	11	11
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	5,00	2,75	2,75
AR-FL_LNA_1 (G0034)		**10µM**	1µM	5	20,00	11	0
AR-V7_LNA_1(G0037)		**10µM**	1µM	5	20,00	11	0
PSA_LNA_1 (G0041)		**10µM**	1µM	5	20,00	0	0
AR-FL_LNA_STHLM (S0007)		**10µM**	1µM	5	0,00	0	11
AR-V7_LNA_STHLM (S02204)		**10µM**	1µM	5	0,00	0	11
dNTP		10 mM	0,5 mM	2,5	10,00	5,5	5,5
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00	1,1	1,1
DEPC H_20				10,75	83,00	56,65	56,65
Final Volume			**50**	**50**	**200**	**110**	**110**
each sample (1x)					**50**	**50**	**50**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3 hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds:

					4	2,2	2,2
LIGATION of padlocks	**Padlock Backbone**	**stock**	**final**	**1x**	**SPOT 1 + both Wires**	**SPOT 2**	**SPOT 3**
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	20,00	11,00	11,00
AMP buffer		10X	1X	5,00	20,00	11,00	11,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	16,00	8,80	8,80
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00	1,10	1,10
KCl		1 M	0.05 M	2,5	10,00	5,50	5,50
Formamide		100%	20%	10	40,00	22,00	22,00
plp_AR-FL1 (G0035)	Lin33	10 µM	0.1 µM	0,5	2,00	1,10	0,00
plp_AR-V7 (G0038)	lin16	10 µM	0.1 µM	0,5	2,00	1,10	0,00
plp_PSA_2 (G0042)	B2_DO	10 µM	0.1 µM	0,5	2,00	0,00	0,00
plp_AR-FL_STHLM (S02682)	Allel 2	10 µM	0.1 µM	0,5	0,00	0,00	1,10
plp_AR-V7_STHLM (S02200 = 5')	B2_DO	10 µM	0.1 µM	0,5	0,00	0,00	1,10
DEPC H_20				20,50	86,00	48,40	48,40
Volume			**50**	**50,00**	**200,00**	**110,00**	**110,00**
each sample (1x)					**50**	**50**	**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds:

				8	0
RCA		**stock**	**final**	**1**	**All Spots + Wire**	**-**
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	40	0
Φ29 buffer		10x	1x	5	40	0
dNTP		10 mM	0,25 mM	1,25	10	0
BSA		20 µg/µl	0,2 µg/µl	0,5	4	0
Glycerol		50%	5%	5	40	0
DEPC H_20				33,25	266	0
Volume [µl]			**50**	**50**	400	0
each sample (1x)					50	#DIV/0!

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds:						4	2,2	2,2
Detection probe hybridisation			**stock**	**final**	**1**	**SPOT 1 + both Wires**	**SPOT 2**	**SPOT 3**
2xHyb buffer			2x	1x	17,5	70	38,5	38,5
D0010_Lin16_ATTO 550	Cy3		10 µM	0.1 µM	0,5	2	0	0
D0011_Lin33_ATTO 488	FITC		10 µM	0.1 µM	0,5	2	0	0
D0012_B2_DO_ATTO 647N	CY5		10 µM	0.1 µM	0,5	2	0	0
S00079_Allel2_Cy5	Cy5		10 µM	0.1 µM	0,5	0	1,1	0
S00086_B2_DO_Cy3	Cy3		10 µM	0.1 µM	0,5	0	1,1	0
D0001	Lin16_Cy3_SA		10 µM	0.1 µM	0,5	0	0	1,1
D0004	Lin33_Cy5		10 µM	0.1 µM	0,5	0	0	1,1
DEPC H_2O					29	124	69,3	69,3
Volume				**50**	**50**	200	110	110
each sample (1x)						50	50	50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

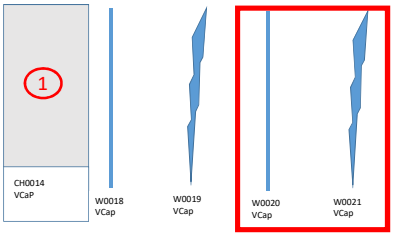
Experiment W0018 - W0021

Protocol received from Amin

Cell line / Tissue Vcap Cells

3 Spots per Slide	Spot1:	Targets	Probes From	Dyes
		AR-FL, AR-V7, B-Actin	Schweden	CY

WIRE Normal (DC01, W0018) + Twisted (DC02, AR-FL, AR-V7, b-Actin, Without Blood Schweden Cy
WIRE Normal (DC01, W0020) + Twisted (DC02, AR-FL, AR-V7, b-Actin, With Blood Schweden Cy
Date 10.02.2016



Wires are charged with VCaP cells and direclty formaldehyde fixed
EtOH series 70%, 85% & 100% for 2 minutes

MOUNT 1 SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Comment: Twisted was accidently stored at -80°C for >6month

Master Mix folds: 1 4

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1 + both Wires
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	20,00
RT buffer		10X	1X	5	20,00
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	5,00
BActin (S02203)		**10µM**	1µM	5	20,00
AR-FL_LNA_STHLM (S0007)		**10µM**	1µM	5	20,00
AR-V7_LNA_STHLM (S02561)		**10µM**	1µM	5	20,00
dNTP		10 mM	0,5 mM	2,5	10,00
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00
DEPC H_2O				20,75	83,00
Final Volume			**50**	**50**	**200**
each sample (1x)					**50**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 4

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT 1 + both Wires
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	20,00
AMP buffer		10X	1X	5,00	20,00
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	16,00
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00
KCl		1 M	0.05 M	2,5	10,00
Formamide		100%	20%	10	40,00
BACTIN (S02003)	Lin16	10 µM	0.1 µM	0.5	2,00
plp_AR-FL_STHLM (S02682)	Allel 2	10 µM	0.1 µM	0.5	2,00
plp_AR-V7_STHLM (S02553 = 3')	B2_DO	10 µM	0.1 µM	0.5	2,00
DEPC H_2O				21,50	86,00
Volume			**50**	**50,00**	**200,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 4

RCA		stock	final	1	All Spots + Wire
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	20
Φ29 buffer		10x	1x	5	20
dNTP		10 mM	0,25 mM	1,25	5
BSA		20 µg/µl	0,2 µg/µl	0,5	2
Glycerol		50%	5%	5	20
DEPC H_2O				33,25	133
Volume [µl]			**50**	**50**	200
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 4

Detection probe hybridisation		stock	final	1	SPOT 1 + both Wires
2xHyb buffer		2x	1x	17,5	70
SO1271_Alexa4750	Cy7	10 µM	0.1 µM	0,5	2
S00079_Allel2_Cy5	Cy5	10 µM	0.1 µM	0,5	2
S00086_B2_DO_Cy3	Cy3	10 µM	0.1 µM	0,5	2
DEPC H_2O				31	124
Volume			**50**	**50**	200
each sample (1x)					50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment W0022, W0025, W0027

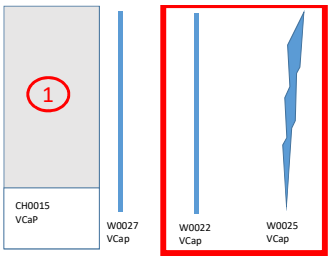
Protocol received from Amin

Cell line / Tissue: Vcap Cells

3 Spots per Slide	Spot1:	Targets	Probes From	Dyes
		AR-FL, AR-V7, B-Actin	Schweden	CY

Double Enzymes

WIRE Normal (DC01, W0022) + Twisted (DC02, \	AR-FL, AR-V7, b-Actin, With Blood		Schweden	Cy
WIRE Normal (DC01, W0027) + Slide	AR-FL, AR-V7, b-Actin, Without Blood		Schweden	Cy
Date		29.02.2016		



Wires are charged with VCaP cells and direclty formaldehyde fixed
EtOH series 70%, 85% & 100% for 2 minutes

MOUNT 1 SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 4

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1 + Wires
TranscriptMe RT (pipet last)		200 U/µl	40 U/µl	10	40,00
RT buffer		10X	1X	5	20,00
Rnase Inhibitor (pipet last)		40 U/µl	2 U/µl	2,5	10,00
BActin (S02203)		**10µM**	1µM	5	20,00
AR-FL_LNA_STHLM (S0007)		**10µM**	1µM	5	20,00
AR-V7_LNA_STHLM (S02561)		**10µM**	1µM	5	20,00
dNTP		10 mM	0,5 mM	2,5	10,00
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00
DEPC H_20				14,50	58,00
Final Volume			**50**	**50**	**200**
each sample (1x)					**50**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 20 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 4

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT 1 + both Wires
Ampligase (pipet last)		5 U/µl	1 U/µl	10,00	40,00
AMP buffer		10X	1X	5,00	20,00
Rnase H (pipet last)		5 U/µl	0.8 U/µl	8	32,00
BSA		20 µg/µl	0,2 µg/µl	0,5	2,00
KCl		1 M	0.05 M	2,5	10,00
Formamide		100%	20%	10	40,00
BACTIN (S02003)	Lin16	10 µM	0.1 µM	0,5	2,00
plp_AR-FL_STHLM (S02682)	Allel 2	10 µM	0.1 µM	0,5	2,00
plp_AR-V7_STHLM (S02553 = 3')	B2_DO	10 µM	0.1 µM	0,5	2,00
DEPC H_20				12,50	50,00
Volume			**50**	**50,00**	**200,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 4

RCA		stock	final	1	All Spots + Wire
Φ29 polymerase (pipet last)		10 U/µl	2 U/µl	10	40
Φ29 buffer		10x	1x	5	20
dNTP		10 mM	0,25 mM	1,25	5
BSA		20 µg/µl	0,2 µg/µl	0,5	2
Glycerol		50%	5%	5	20
DEPC H_20				28,25	113
Volume [µl]			**50**	**50**	200
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 4

Detection probe hybridisation		stock	final	1	SPOT 1 + both Wires
2xHyb buffer		2x	1x	17,5	70
SO1271_Alexa4750 Lin16	Cy7	10 µM	0.1 µM	0,5	2
S00079_Allel2_Cy5	Cy5	10 µM	0.1 µM	0,5	2
S00086_B2_DO_Cy3	Cy3	10 µM	0.1 µM	0,5	2
DEPC H_20				31	124
Volume			**50**	**50**	200
each sample (1x)					50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment W0024 and W0028

Protocol received from Amin

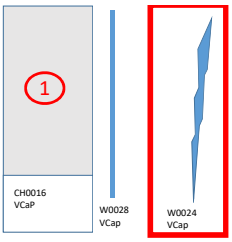
Cell line / Tissue — Vcap Cells and LNCaP

		Targets	Probes From	Dyes
3 Spots per Slide	Spot1:	AR-FL, AR-V7, B-Actin	Schweden	CY

Double Enzymes

WIRE Normal (DC01, W0028)+slide	AR-FL, AR-V7, b-Actin, Without Blood	Schweden	Cy
WIRE Twisted (DC02, W0024)	AR-FL, AR-V7, b-Actin, With Blood	Schweden	Cy
Date	03.03.2016		

W0024 needs to be rehydrated in DEPC-PBS-Tween for 5 min and then Formaldehyde fixed for 15 min



MOUNT 1 SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 3

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1 + Wires
TranscriptMe RT (pipet last)		200 U/µl	40 U/µl	10	30,00
RT buffer		10X	1X	5	15,00
Rnase Inhibitor (pipet last)		40 U/µl	2 U/µl	2,5	7,50
BActin (S02203)		**10µM**	1µM	5	15,00
AR-FL_LNA_STHLM (S0007)		**10µM**	1µM	5	15,00
AR-V7_LNA_STHLM (S02561)		**10µM**	1µM	5	15,00
dNTP		10 mM	0,5 mM	2,5	7,50
BSA		20 µg/µl	0,8 µg/µl	1	3,00
DEPC H_20				14,00	42,00
Final Volume			**50**	**50**	**150**
each sample (1x)					**50**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 20 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT 1 + both Wires
Ampligase (pipet last)		5 U/µl	1 U/µl	10,00	30,00
AMP buffer		10X	1X	5,00	15,00
Rnase H (pipet last)		5 U/µl	0.8 U/µl	8	24,00
BSA		20 µg/µl	0,8 µg/µl	1	3,00
KCl		1 M	0.05 M	2,5	7,50
Formamide		100%	20%	10	30,00
BACTIN (S02003)	Lin16	10 µM	0.1 µM	0.5	1,50
plp_AR-FL_STHLM (S02682)	Allel 2	10 µM	0.1 µM	0.5	1,50
plp_AR-V7_STHLM (S02553 = 3')	B2_DO	10 µM	0.1 µM	0.5	1,50
DEPC H_20				12,00	36,00
Volume			**50**	**50,00**	**150,00**
each sample (1x)					**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 3

RCA		stock	final	1	All Spots + Wire
Φ29 polymerase (pipet last)		10 U/µl	2 U/µl	10	30
Φ29 buffer		10x	1x	5	15
dNTP		10 mM	0,25 mM	1,25	3,75
BSA		20 µg/µl	0,8 µg/µl	1	3
Glycerol		50%	5%	5	15
DEPC H_20				27,75	83,25
Volume [µl]			**50**	**50**	150
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds:

Detection probe hybridisation		stock	final	1
2xHyb buffer		2x	1x	17,5
SO1271_Alexa4750 Lin16	Cy7	10 µM	0.1 µM	0,5
S00079_Allel2_Cy5	Cy5	10 µM	0.1 µM	0,5
S00086_B2_DO_Cy3	Cy3	10 µM	0.1 µM	0,5
DEPC H_20				31
Volume			**50**	**50**
each sample (1x)				

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

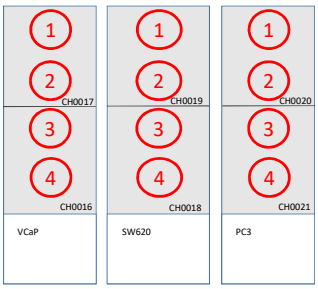
EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip

Experiment CH0016 - CH0021

Cell line / Tissue: Vcap Cells

4 Spots per Slide		Probes From	Dyes
	Spot1:	GRAZ	CY
	Spot2:	GRAZ	CY
	Spot3:	GRAZ	CY
	Spot4:	GRAZ	CY

Date: 02.04.2016



MOUNT SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 13 0 0 0

In situ RT		stock	final	MIXx1(LNAs)	All SPOTS	SPOT 2	SPOT 3	SPOT 4
TranscriptMe RT (pipet last)		200 U/µl	20 U/µl	5	65,00	0,00	0	0
RT buffer		10X	1X	5	65,00	0,00	0	0
Rnase Inhibitor (pipet last)		40 U/µl	1 U/µl	1,25	16,25	0,00	0	0
pLNAACTB_61 (G0001)		**100µM**	1µM	0,5	6,50	0,00	0	0
pAR-V7_CE3_3'LNA (G0070)		**10µM**	1µM	5	65,00	0,00	0	0
RandomPrimer (G0004)		**10µM**	1µM	5	65,00	0,00	0	0
RV_AR-V7_4 (G0061)		**10µM**	1µM	5	65,00	0,00	0	0
RV_AR-V7_3 (G0062)		**10µM**	1µM	5	65,00	0,00	0	0
RV_AR-V7_2 (G0063)		**10µM**	1µM	5	65,00	0,00	0	0
RV_AR-V7_1 (G0064)		**10µM**	1µM	5	65,00	0,00	0	0
dNTP		**10µM**	0,5 mM	2,5	32,50	0,00	0	0
BSA		20 µg/µl	0,2 µg/µl	0,5	6,50	0,00	0	0
DEPC H_20				5,25	68,25	0,00	0	0
Final Volume			**50**	**50**	**650**	**0**	**0**	**0**
each sample (1x)					**50**	**#DIV/0!**	**#DIV/0!**	**#DIV/0!**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3,5 3,5 3,5 3,5

LIGATION of padlocks	Padlock Backbone	stock	final	1x	SPOT 1	SPOT 2	Spot 3	SPOT 4
Ampligase (pipet last)		5 U/µl	0.5 U/µl	5,00	17,50	17,50	17,50	17,50
AMP buffer		10X	1X	5,00	17,50	17,50	17,50	17,50
Rnase H (pipet last)		5 U/µl	0.4 U/µl	4	14,00	14,00	14,00	14,00
BSA		20 µg/µl	0.2 µg/µl	0,5	1,75	1,75	1,75	1,75
KCl		1 M	0.05 M	2,5	8,75	8,75	8,75	8,75
Formamide		100%	20%	10	35,00	35,00	35,00	35,00
plp_AR-V7_2 (G0051)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_3 (G0052)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_4 (G0053)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_5 (G0054)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_6 (G0055)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_7 (G0056)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_8 (G0057)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_9 (G0058)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7_10 (G0059)	Lin16	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
plp_AR-V7 (G0038)	Lin16	10 µM	0.1 µM	0,5	1,75	0,00	0,00	1,75
AR-V7_B2DO_2 (G0068)	B2_DO	10 µM	0.1 µM	0,5	0,00	1,75	0,00	1,75
AR-V7_B2DO_3' (G0069)	B2_DO	10 µM	0.1 µM	0,5	0,00	0,00	1,75	1,75
PP-ACTB-IIa (DP-2) G0013)	Lin33	10 µM	0.1 µM	0,5	1,75	1,75	1,75	1,75
DEPC H_20				16,50	61,25	61,25	61,25	57,75
Volume			**50**	**50,00**	**175,00**	**175,00**	**175,00**	**175,00**
each sample (1x)					**50**	**50**	**50**	**50**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 13

RCA		stock	final	1	All Spots
Φ29 polymerase (pipet last)		10 U/µl	1 U/µl	5	65
Φ29 buffer		10x	1x	5	65
dNTP		10 mM	0,25 mM	1,25	16,25
BSA		20 µg/µl	0,2 µg/µl	0,5	6,5
Glycerol		50%	5%	5	65
DEPC H_20				33,25	432,25
Volume [µl]			**50**	**50**	650
each sample (1x)					50

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 12

Detection probe hybridisation			stock	final	1	SPOT 1-4
2xHyb buffer			2x	1x	17,5	210
D0001 Lin16		Cy3	10 µM	0.1 µM	0,5	6
D0004 Lin33		Cy5	10 µM	0.1 µM	0,5	6
D0008_B2_DO		FITC	10 µM	0.1 µM	0,5	6
DEPC H_20					31	372
Volume				**50**	**50**	600
each sample (1x)						50

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment W0030 - W0033 and Patient Wire CB2-G

Cell line / Tissue: Vcap Cells

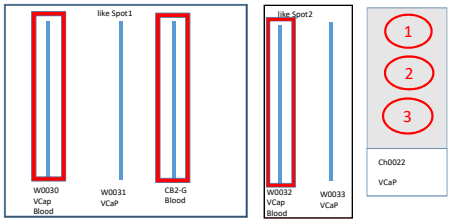
3 Spots per Slide

	All Spots: double enzyme and double Bsa	Targets	Probes From	Dyes
Spot1 + W0030 + W0031 + CB2-G:	positiv	AR-FL, AR-V7, PSA	GRAZ	CY
Spot2 + W0032 + W0033:	positiv post fixation	AR-FL, AR-V7, PSA	GRAZ	CY
Spot3:	negativ	AR-FL, AR-V7, PSA	GRAZ	CY
	WIRE Normal (DC01)	AR-FL, AR-V7, PSA	GRAZ	CY

Date: 0704.2016

Wires are charged with VCaP cells and direclty formaldehyde fixed

Double Enzyme



MOUNT SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes

Master Mix folds: 1 | 7,3 | 1,4

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1 +2 + Wires (30,31,32,33,CB2-G)	spot3
TranscriptMe RT (pipet last)		200 U/µl	40 U/µl	7	51,10	0,00
RT buffer		10X	1X	3,5	25,55	4,90
Rnase Inhibitor (pipet last)		40 U/µl	2 U/µl	0,875	6,39	1,23
pAR-V7_CE3_3'LNA (G0070)		**10µM**	1µM	3,5	25,55	4,90
RandomPrimer (G0004)		**100 µM**	1µM	0,35	2,56	0,49
RV_AR-V7_4 (G0061)		**10µM**	1µM	3,5	25,55	4,90
RV_AR-V7_3 (G0062)		**10µM**	1µM	3,5	25,55	4,90
RV_AR-V7_2 (G0063)		**10µM**	1µM	3,5	25,55	4,90
RV_AR-V7_1 (G0064)		**10µM**	1µM	3,5	25,55	4,90
AR-FL_LNA_1 (G0034)		**100 µM**	1µM	0,35	2,56	0,49
PSA_LNA_1(G0041)		**100 µM**	1µM	0,35	2,56	0,49
dNTP		**10µM**	0,5 mM	1,75	12,78	2,45
BSA		20 µg/µl	0,4 µg/µl	0,7	5,11	0,98
DEPC H_2O				2,62	19,16	13,48
Final Volume			**35**	**35**	**255,5**	**49**
each sample (1x)					**35**	**35**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 8,6

LIGATION of padlocks	Padlock Backbone	stock	final	1x	All Wires and Spots
Ampligase (pipet last)		5 U/µl	1 U/µl	7,00	60,20
AMP buffer		10x	1X	3,50	30,10
Rnase H (pipet last)		5 U/µl	0.8 U/µl	5,6	48,16
BSA		20 µg/µl	0.4 µg/µl	0,7	6,02
KCl		1 M	0.05 M	1,75	15,05
Formamide		100%	20%	7	60,20
plp_AR-V7_2 (G0051)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_3 (G0052)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_4 (G0053)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_5 (G0054)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_6 (G0055)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_7 (G0056)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_8 (G0057)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_9 (G0058)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7_10 (G0059)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-V7 (G0038)	Lin16	10 µM	0.1 µM	0,35	3,01
plp_AR-FL1 (G0035)	Lin33	10 µM	0.1 µM	0,35	3,01
plp_PSA_1 (G0042)	B2DO	10 µM	0.1 µM	0,35	3,01
DEPC H_2O				5,25	45,15
Volume			**35**	**35,00**	**301,00**
each sample (1x)					**35**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 8,6

RCA		stock	final	1	All Spots
Φ29 polymerase (pipet last)		10 U/µl	2 U/µl	7	60,2
Φ29 buffer		10x	1x	3,5	30,1
dNTP		10 mM	0,25 mM	0,875	7,525
BSA		20 µg/µl	0,4 µg/µl	0,7	6,02
Glycerol		50%	5%	3,5	30,1
DEPC H_2O				19,425	167,055
Volume [µl]			**35**	**35**	301
each sample (1x)					35

Apply tapes
Incubate slides at 37°C for 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each
For Spot 2 und W00032,33
Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 8,5

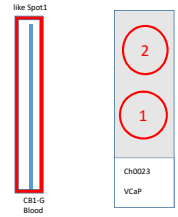
Detection probe hybridisation			stock	final	1	SPOT 1-4
2xHyb buffer			2x	1x	17,5	148,75
D0001 Lin16		Cy3	10 µM	0.1 µM	0,35	2,975
D0004 Lin33		Cy5	10 µM	0.1 µM	0,35	2,975
D0008_B2_DO		FITC	10 µM	0.1 µM	0,35	2,975
DEPC H_2O					16,45	139,825
Volume				**35**	**35**	297,5
each sample (1x)						35

From now on protect slides from light
Apply tapes
37°C 30'
2 washes 1xDEPC-PBS-Tween for 2 min each
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish

Experiment CB1-G



Cell line / Tissue — Vcap Cells

3 Spots per Slide

	All Spots: double enzyme and double Bsa	Targets	Probes From	Dyes
Spot1 + CB2-G:	positiv	AR-FL, AR-V7, PSA	GRAZ	CY
Spot2:	negativ	AR-FL, AR-V7, PSA	GRAZ	CY
	WIRE Normal (DC01)	AR-FL, AR-V7, PSA	GRAZ	CY

Date — 0704.2016

Only wire need to be direclty formaldehyde fixed, Slide allready fixed
Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
Double Enzyme

2 washes 1xDEPC-PBS-Tween for 2 min each

MOUNT SECURE SEALS (50 µl) per slide
Marc the area of cells at the bottom of the glass slide by scratching
Rehydrate 50µl (or 100µl for 100µl seals) 1xDEPC-PBS-Tween @ RT for 5 minutes
50µl (or 100µl for 100µl seals) 0,1M HCl-DEPC-H2O RT for 5 minutes
2x washes 1xDEPC-PBS-Tween RT 5 minutes
Incubate with DAPI (final dilution 1:5000 in DEPC-PBS) for 5 minutes at RT
2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 1 2,4 1,4

In situ RT		stock	final	MIXx1(LNAs)	SPOT 1 + Wire (CB1-G)	spot2
TranscriptMe RT (pipet last)		200 U/µl	40 U/µl	7	16,80	0,00
RT buffer		10X	1X	3,5	8,40	4,90
Rnase Inhibitor (pipet last)		40 U/µl	2 U/µl	0,875	2,10	1,23
pAR-V7_CE3_3'LNA (G0070)		**10µM**	1µM	3,5	8,40	4,90
RandomPrimer (G0004)		**100 µM**	1µM	0,35	0,84	0,49
RV_AR-V7_4 (G0061)		**10µM**	1µM	3,5	8,40	4,90
RV_AR-V7_3 (G0062)		**10µM**	1µM	3,5	8,40	4,90
RV_AR-V7_2 (G0063)		**10µM**	1µM	3,5	8,40	4,90
RV_AR-V7_1 (G0064)		**10µM**	1µM	3,5	8,40	4,90
AR-FL_LNA_1 (G0034)		**100 µM**	1µM	0,35	0,84	0,49
PSA_LNA_1(G0041)		**100 µM**	1µM	0,35	0,84	0,49
dNTP		**10µM**	0,5 mM	1,75	4,20	2,45
BSA		20 µg/µl	0,4 µg/µl	0,7	1,68	0,98
DEPC H_2O				2,62	6,30	13,48
Final Volume			**35**	**35**	**84**	**49**
each sample (1x)					**35**	**35**

Apply tapes (PCR plate seals for slides and rubber for the wires), to avoid evaporation
Add appropriate volume, place slides in a humid chamber and incubate for 3hrs at 45 C

Fixation 3% Formaldehyde in DEPC-PBS at RT for 10 minutes (eg.: for 1ml of 3% Formaldehyde= 81µl of 37% Formaldehyde + 919µl DEPC-PBS)
2x washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3,7

LIGATION of padlocks	Padlock Backbone	stock	final	1x	All Wires and Spots
Ampligase (pipet last)		5 U/µl	1 U/µl	7,00	25,90
AMP buffer		10X	1X	3,50	12,95
Rnase H (pipet last)		5 U/µl	0.8 U/µl	5,6	20,72
BSA		20 µg/µl	0,4 µg/µl	0,7	2,59
KCl		1 M	0.05 M	1,75	6,48
Formamide		100%	20%	7	25,90
plp_AR-V7_2 (G0051)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_3 (G0052)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_4 (G0053)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_5 (G0054)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_6 (G0055)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_7 (G0056)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_8 (G0057)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_9 (G0058)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7_10 (G0059)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-V7 (G0038)	Lin16	10 µM	0.1 µM	0,35	1,30
plp_AR-FL1 (G0035)	Lin33	10 µM	0.1 µM	0,35	1,30
plp_PSA_1 (G0042)	B2DO	10 µM	0.1 µM	0,35	1,30
DEPC H_2O				5,25	19,43
Volume			**35**	**35,00**	**129,50**
each sample (1x)					**35**

Incubate at 37 C for 30 minutes and 45 C for 45 minutes
Wash 1x with 2xSSC-Tween at 37 C for 5 minutes
Wash 2x DEPC-PBS-Tween

Master Mix folds: 3,7

RCA		stock	final	1	All Spots
Φ29 polymerase (pipet last)		10 U/µl	2 U/µl	7	25,9
Φ29 buffer		10x	1x	3,5	12,95
dNTP		10 mM	0,25 mM	0,875	3,2375
BSA		20 µg/µl	0,4 µg/µl	0,7	2,59
Glycerol		50%	5%	3,5	12,95
DEPC H_2O				19,425	71,8725
Volume [µl]			**35**	**35**	129,5
each sample (1x)					35

Apply tapes
Incubate slides at 37°C 3 hours

2 washes 1xDEPC-PBS-Tween for 2 min each

Master Mix folds: 3,7

Detection probe hybridisation			stock	final	1	Slide + Wire
2xHyb buffer			2x	1x	17,5	64,75
D0001 Lin16		Cy3	10 µM	0.1 µM	0,35	1,295
D0004 Lin33		Cy5	10 µM	0.1 µM	0,35	1,295
D0008_B2_DO		FITC	10 µM	0.1 µM	0,35	1,295
DEPC H_2O					16,45	60,865
Volume				**35**	**35**	129,5
each sample (1x)						35

From now on protect slides from light
Apply tapes
37°C 30'

ensure that the position of cells is marked, then remove the seal

EtOH series (2' in 70,85 and 97%)
Air dry the slides (5-10min)
Mount with ~ 50-60µl slow fade Gold Antifdade reagent
24x55 mm coverslip
Seal the cover slip with nail polish