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Develop an automated staining protocol for ProxHCR

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Abstract

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Proximity hybridization chain reaction (ProxHCR) is a novel in situ hybridization technique capable of detecting protein-protein interactions and post-translational modifications, having the potential to be a diagnostic and prognostic tool in clinical practice. As molecular pathology is becoming an integrated part of clinical evaluations, most staining procedures done today are automated. Compared to other similar methods ProxHCR is suitable for automated staining. The aim of this project was therefore to develop an automated staining protocol for ProxHCR, which could be used in an instrument normally performing immunohistochemistry (IHC). During the developing process, several parameters in the current protocol was tested and adjusted to suit a chosen instrument. The result from this project was an automated ProxHCR protocol which was run in an IHC instrument. A small study was also performed to find suitable biomarkers for ProxHCR, indicating the systems' capabilities. The conclusion from this project was that ProxHCR can be run on an existing IHC instrument and that the method can be used to study several relevant biomarkers.

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SAMMANFATTNING

Molekylär patologi är numera klinisk praxis och framför allt immunhistokemi (IHK) används ofta inom diagnostisering av en mängd olika sjukdomar, däribland cancer. Detta har inneburit att en hel del automatiserade instrument som kan genomföra IHK har tagits fram. I IHK lokaliseras proteiner med hjälp av inmärkta antikroppar som binder till specifika antigen. Nyare metoder, såsom ProxHCR, har under de senaste årtiondena upptäckts. Metoder som bland annat möjliggör en mer komplex diagnostik inom sjukvården. Men, för att ProxHCR ska kunna tävla med redan befintliga metoder inom detta område är det gynnsamt att ha ett protokoll som går att köra på ett befintligt IHK instrument.

ProxHCR är en indirekt *in situ* hybridiseringsmetod som kan användas för att detektera protein-protein interaktioner och även post-translationella modifikationer. Dessa interaktioner och modifikationer påverkar och reglerar många funktioner i kroppen och är viktiga element att studera i många sjukdomar. Förändringar i dessa kan användas som biomarkörer för att bevaka patienter samt indikera när en specifik behandling är värdefull. För att demonstrera ProxHCR användbarhet diskuteras i rapporten även några relevanta biomarkörer som kan studeras med hjälp av metoden.

I detta examensprojekt utvecklades och optimerades ett redan befintligt protokoll för ProxHCR för att kunna köras på ett valt automatiserat instrument. Utvecklingsprocessen omfattade att testa och anpassa olika parametrar från det befintliga protokollet till detta instrument. Resultat från detta projekt visade att ProxHCR kan köras på ett IHK instrument även om ytterligare optimering behövs för att uppnå önskvärda resultat.

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ABBREVIATIONS

APC	Adenomatous polyposis coli
CD29	Clusters of differentiation 29
CD38	Clusters of differentiation 38
CD40	Clusters of differentiation 40
CD40L	Clusters of differentiation 40 ligand
CD49d	Clusters of differentiation 49b
CLL	Chronic lymphocytic leukemia
CK1	Casein kinase 1
DAB	3,3' diaminobenzidine
DNA	Deoxyribonucleic acid
DVL1/2	Dishevelled Segment Polarity Protein 1/2
Dsh	Dishevelled
EMT	Mesenchymal transitions
Eya2	EYA Transcriptional Coactivator and Phosphatase 2
GSK3	Glycogen synthase kinase 3
HCC	Hepatocellular carcinoma
HCR	Hybridization chain reaction
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL-17	Interleukin 17
ISH	*In situ* hybridization
LATS1/2	LATS conserved domain 1/2
LMO2	LIM domain only 2
MET	Mesenchymal to epithelial transitions
MM	Multiple myeloma
MOB1A/B	Mps one binder 1A/B
MST1/2	Mammalian Ste20 like kinases 1/2
PLA	Proximity ligation assay
POC	Proof of concept
PPI	Protein-protein interaction
ProxHCR	Proximity hybridization chain reaction
PTM	Post-translational modification
RNA	Ribonucleic acid
SAV1	Protein salvador homolog 1
Six1	Homeobox protein SIX1
TAZ	Transcriptional co-activator with PDZ binding motif
TGF-β	Transforming growth factor beta
VLA-4	Very Late Antigen 4
WNT	Wingless-type MMTV integration site family member
YAP	Yes associated protein

1 INTRODUCTION

Immunostaining is a broad term representing antibody based methods used to detect specific targets such as proteins or gene expressions. These powerful methods enable diagnostics of a broad range of applications and has become even more relevant in later years as the biomarker industry has increased rapidly. Biomarkers are often used as a measurable indicator of disease and does in many cases contribute to fast and precise judgement of a patient's condition. Though, many of the immunostaining methods used today does not have the capacity to make more complex diagnoses.

However, newer and more advanced techniques have in the last decade been developed. One of these methods is the *in situ* detection assay Proximity Hybridization Chain Reaction (ProxHCR) (Koos *et al.* 2015), developed by Olink Bioscience. The ProxHCR method is able to detect protein-protein interactions (PPIs) and even post-translational modifications (PTMs), both being important regulatory elements in the human body. There are already similar methods available such as *in situ* Proximity ligation assay (PLA), commercially available as Duolink®. Whereas most staining systems widely used for routine clinical diagnostics today are automated, PLA have some drawbacks, such as enzyme dependence, making them unfit for automated staining. ProxHCR does not share the disadvantages of PLA and still have the potential for highly specific assays for proteins, PPIs and PTMs. However, the current protocol needs to be further developed and optimized to be suitable for an automated system. There is also a need to find suitable biomarkers for ProxHCR, demonstrating the systems' feasibility and indicating its potential of making the more advanced patients evaluations.

1.1 AIM OF PROJECT

The aim of the degree project was to develop and optimize an automated staining protocol for ProxHCR and to identify suitable assays and applications based on this procedure that can be used in an automated system. The primary goal of the project was to study clinically interesting biomarkers on relevant material and to perform a proof of concept (POC) study demonstrating the feasibility of ProxHCR. Additionally, a survey was done with the aim to study potential protein biomarkers suitable for clinical diagnostics.

2 BACKGROUND

2.1 IMMUNOSTAINING

Immunostaining is being used in histology as well in molecular and cell biology and includes a broad range of antibody based techniques. A prerequisite for these methods to be successful is good antibodies and antibody engineering has helped made immunostaining to one of the most important diagnostic tools today. The procedures include exposing fixed cells or tissue to antibodies which bind to the target of interest. A direct staining is a one-step procedure were

primary antibodies is conjugated with appropriate detection molecule and visualized directly. An indirect staining is a multistep procedure, often in two steps, were conjugated secondary antibodies directed against the primary antibody are used for detection (Schacht & Kern). Detection in tissue is called immunohistochemistry (IHC) whereas detection in cells is referred to as immunocytochemistry (ICC).

In situ hybridization (ISH) is an immunostaining technique which is used to localize specific gene expression in their cellular environment. In ISH, secondary antibodies are labeled with complementary DNA,RNA or modified nucleic acids strand to localize and hybridize to specific DNA or RNA sequence in sections of tissue or cells (Jin & Lloyd 1997). The detection technique can be either fluorescent or chromogenic. In immunofluorescence, antibodies are conjugated with fluorescent dyes called fluorophores. These absorbs light energy of a specific wavelength and emit light of a longer wavelength making it possible to distinguished the emitted fluorescence from the excitation light (Drummen 2012). Chromogenic detection is another detection technique using antibodies conjugated with enzymes that converts soluble substrates into insoluble chromogenic products, giving rise to colors. Horseradish peroxidase (HRP), being one of the most commonly used enzyme, converts 3,3' diaminobenzidine (DAB) into a brown product (Lyon 2012).

2.2 PROXHCR

ProxHCR is an indirect *in situ* hybridization method using the feasibility of both proximity binding and hybridization chain reaction (HCR), enabling effective signal amplification with proximity restriction (Koos *et al.* 2015).

The ProxHCR method is based on two proximity hairpins conjugated on secondary antibodies (referred to as proximity probes) that will bind to primary antibodies, being bound to the target of interest. These kinetically trapped hairpin structures (PH1 and PH2) are kept as monomers, prevented from opening and cross hybridization until an activator oligonucleotide (referred to as activator) is introduced (Koos *et al.* 2015). The activator first binds in the loop of the PH1 displacing the stem, releasing its 3’ end (Figure 2b). If PH1 and PH2 are sufficiently close to each other this 3’ end can hybridize to and invade PH2, leading to the 3’ end of PH2 sticking out (Figure 1c.). This will trigger a cascade of hybridization events, incorporating photophore labeled hairpin oligonucleotides (referred to as HCR oligos) with sticky ends to PH1 and PH2. The first amplification oligonucleotide (H1) will hybridize to the 3’ end of PH2 (Figure 1d). This event will recruit a second amplification oligonucleotide (H2) which will hybridize to its corresponding hairpin structure and recruit another H1 and so forth (Koos & Söderberg 2001) (Figure 1e). The process is driven by the release of potential energy stored in the hairpin structure of the oligonucleotides and will continue until the hairpin species are exhausted. The result is a long, double stranded DNA molecule visible in fluorescence microscope (Koos *et al.* 2015). Likewise, the hairpin oligonucleotides can be labeled with enzymes, allowing for visualization in light microscope.

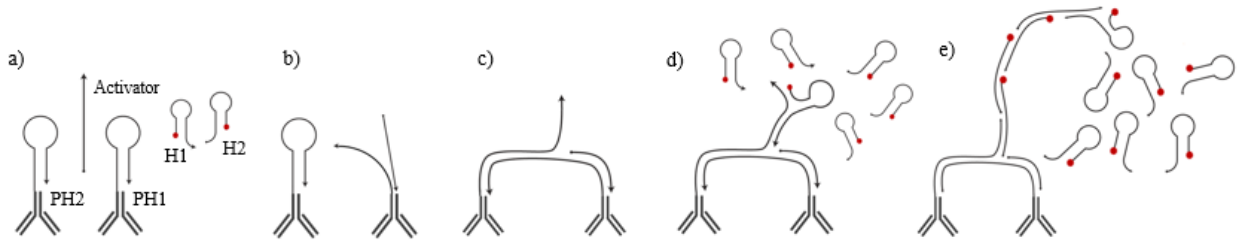

Figure 1. The ProxHCR technique. a) The components of the ProxHCR method b) The activator invades PH1 which in turn c) binds to PH2 and invades it as well. d) H1 will then hybridize to PH2, recruiting H2, which will hybridize to PH1 recruiting a new H1 and so on. e) The amplification will continue until the hairpin is exhausted or the reaction stopped.

2.3 BIOMARKERS

Many of these methods described in 2.1 are widely used in diagnostics and research for the detection, localization, and quantification of specific targets in preserved tissue or cells. For these methods to be valuable, it is essential to have relevant targets to study, such as biomarkers. A biomarker is a measurable biological indicator, such as a molecule or a gene, by which the presence or severity of a pathological or physiological processes can be identified. There are numerous applications for biomarkers, including drug discovery, clinical markers of efficacy and toxicity, diagnostic assay development and many more (Strimbu & Tavel 2010). In clinical practice, biomarkers have been extensively used as an indicator of disease state and today most clinical trials use biomarkers as primary endpoints to assess the efficacy of a therapeutic treatment (Fleming & Powers 2012). For instance, by measuring assayed biomarkers, a patient reaction to a drug can easily be monitored and valuable information concerning the efficacy and safety of the treatment can be attained.

Biomarkers have typically been considered as analytes measured in the blood (Carvajal-Hausdorf *et al.* 2015). Proteins such as prostate specific antigen (Shariat *et al.* 2011) and carcinoembryonic antigen (Okada *et al.* 2004) are good illustrations of this. However, in the last decades more and more *in situ* biomarkers have been discovered. The identification of more localized biomolecules can be of more value than circulating biomarkers since they include more spatial and contextual information (Carvajal-Hausdorf *et al.* 2015). An example of an *in situ* protein biomarkers routinely used in clinical settings is HER2. HER2 is a biomarker frequently amplified in breast cancer, being associated with poor prognosis (Ménard *et al.* 2001 p. 2). For early cancer detection and risk assessment biomarkers can contribute to valuable molecular signatures which can indicate the phenotype of a cell, perhaps demonstrating disease (Hazelton & Luebeck 2011).

2.3.1 Signaling pathways leading to cancer

There are several signaling pathways being associated with cancer where PPIs and PTMs are important signaling nodes and hubs in these networks[14]. The ProxHCR technique could enable clinicians and researchers to use PPIs and PTMs to map critical changes in several signaling pathways leading to cancer. Additionally, immune-oncology is becoming a more common treatment in cancer (Adams *et al.* 2015). These treatments utilize the body's own immune system

to fight cancer and several antibodies that inhibit key functions in tumor development are being discovered. PPIs and PTMs could be used as diagnostic and prognostics biomarkers for immune-oncology, indicating when a particular treatment is suitable. Some of these signaling pathways and proteins involved in cancer development will be discussed below, indicating potential biomarkers for ProxHCR.

Elevated levels of nuclear β catenin promote uncontrolled cell proliferation

The WNT signaling pathway play a crucial role during embryonic development and tissue homeostasis by regulating the level of the β-catenin for signal transduction. B-catenin can be used as a valuable biomarker associated with invasion, metastasis and poor prognosis in cancers such as colorectal and breast cancer (Lin *et al.* 2000, Nazemalhosseini Mojarad *et al.* 2015). There are three WNT signaling pathways discovered which are activated through the binding of a WNT protein ligand to the Frizzled receptor complex, transducing a signal to several intracellular proteins, including cytoplasmic β-catenin, AXIN, APC, CK1, GSK3 and Dsh (Komiya & Habas 2008). These proteins form a destruction complex which, in absent of WNT signaling, degrades cytoplasmic β-catenin. When WNT is activated β-catenin, previous bound to the complex, is no longer degraded and will accumulate in the cytoplasm following nuclear translocation. In the nucleus, β-catenin promote the expression of WNT target genes leading to uncontrolled cell proliferation (MacDonald *et al.* 2009).

β-catenin/E-cadherin is an important PPI in the WNT pathway and the complex is central for epithelial to mesenchymal (EMT) and mesenchymal to epithelial (MET) transitions, taking part in embryo development and tissue fibrosis. They are also key players in cancer development and the initial steps of metastasis (Tian *et al.* 2011). The E-cadherin/ β-catenin interaction is disturbed in many human gastrointestinal cancers indicating the complex's prognostic value in these tumors (Debruyne *et al.* 1999). This PPI is an appropriate model system for ProxHCR and was also used in this project. Another interesting PPI, participating in the regulation of canonical WNT signaling and tumorigenesis, is between LMO2 and DVL1/2. In breast and colorectal cancer cells LMO2 has shown to be co-localized with DVL1/2 in the cytoplasm, an interaction which reduced the activation of β-catenin (Liu *et al.* 2016). This interaction could be used as a prognostic biomarker for breast and colorectal cancer, suitable for the ProxHCR technique. There are several antibodies available for LMO2 and DVL1/2 and the interaction could be studied in breast or colorectal cancer cell lines such as MDA-MB-231 or SW480.

The role of YAP/TEAD interactions in cancer progression and metastasis

The Hippo signaling pathway controls organ size in many species but has also been found in a wide range of human carcinomas, especially in liver cancer (Sebio & Lenz 2015). The heart of the Hippo pathway comprises of a protein-complex including; MST1/2, LATS1/2, SAV1 and MOB1A/B. MST1 and MST2 facilitate LATS1 and LATS2 phosphorylation, which in turn facilitates LATS dependent phosphorylation of the oncoproteins YAP and TAZ. In the activated Hippo pathway, YAP and TAZ are phosphorylated by the protein-complex, retained and degraded in the cytoplasm. When the pathway is inactivated or suppressed YAP and TAZ translocate into the nucleus, regulating the activity of various transcription factors, including SMADs and TEADs (Johnson & Halder 2014).

Studies have indicated that increased YAP/TEAD activity play a fundamental role in cancer progression and metastasis (Johnson & Halder 2014). Mao *et al.* (2014) reveled a regulatory

mechanism of YAP2/TEAD4 being involved in Hepatocellular carcinoma (HCC) tumorigenesis, the most common type of liver cancer. Deacetylate of YAP2 protein in HCC cells strengthened the YAP2/TEAD4 association, leading to enhanced YAP2/TEAD4 transcriptional activation which promoted cell growth in HCC. In a similar study by Gua *et al*. (2015), the authors showed that the dephosphorylation of YAP1 promoted the nuclear accumulation of YAP1, upregulating the YAP1/TEAD2 association, increasing YAP1/ TEAD2 transcriptional activation and cell invasion in HCC cells. YAP/ TEAD interactions could be suitable biomarkers for ProxHCR, helping to evaluate the state of liver cancer. There are several antibodies available against YAPs and TEADs and these interactions are best studied in human liver carcinoma tissue or in human liver cancer cell, such as HepG2 cells.

Targeting the switch from suppressive to promoting effector in TGF-β signaling
The transforming growth factor beta (TGF-β) signaling pathway is involved in a wide range of cellular processes including cell growth, cell differentiation, cellular homeostasis and apoptosis as well as in hormonal and immune responses (Nagaraj & Datta 2010). TGF-β signaling pathways have a tumor suppressor effect in normal and early carcinoma cells something that is often lost as tumors develop and progress. TGF-β signaling then switches to promote cancer progression, invasion, and tumor metastasis (Lebrun 2012). A critical mediator of the switch in TGF-signaling is Six1 (Micalizzi *et al.* 2010). Six1 is predominantly involved in embryonic development where it interacts with Eya proteins (Eya1-4) (Farabaugh *et al.* 2012). Independently, both proteins have been shown to be overexpressed in multiple cancer types such as breast, ovarian and glioblastomas (Blevins *et al.* 2015). In addition, elevated levels of both Six1 and Eya2 correlated with shorter time to relapse and metastasis as well as reduced survival rates (Farabaugh *et al.* 2012). This suggest that the Six1-Eya interaction is critical to promote tumor progression and could perhaps be used as a prognostic biomarker suitable for ProxHCR. There are antibodies towards both Six1 and Eya2 and the interaction can be studied in human breast cancer tissue or in mammary carcinoma cells, such as MCF7 cells.

2.3.2 Cell surface proteins

Cell surface proteins mediate cell-cell communication and respond to external stimuli such as the occurrence of pathogens or chemical signals. The cell surface proteins describe the cells phenotypic and functional characteristics which can be used distinguishing normal and diseased cells, such as cancer cells (Várady *et al.* 2013). These diagnostic and prognostics markers are suitable targets for the ProxHCR technique and some of these markers and their use will be discussed further below.

CD38-CD49d
CD38 and CD49d are both independent prognostic risk markers in chronic lymphocytic leukemia (CLL), associated with very poor diagnosis (Brachtl *et al.* 2014). CLL is a type of cancer of the white blood cells and is the most common type of leukemia in adults. CD38 and CD49d mediate interactions between the chronic lymphocytic leukemia cells and their microenvironment in secondary lymphoid tissues and bone marrow, promoting CLL cell survival and proliferation (Dal-Bo *et al.* 2009). CD38 and CD49d are physical association in macromolecular multiprotein complex, indicated by their common co-expression (Zucchetto *et al.* 2012). It has been showed that CD49d and CD38 co-expression is significantly high in progressive stages of CLL and suggestions are made both CD38 and CD49d expressions should be considered as prognostic

markers for CLL patients and should be used to determine patients' therapy and to evaluate disease prognosis. The CD38/CD49d interaction could be a suitable biomarker to study with ProxHCR, using it to evaluate a CLL patents prognosis and treatment. There are several antibodies towards CD38 and CD49d and the interaction could be studies in the Burkitt's lymphoma cell line Raji or in the myeloma like cell line RPMI-8226.

VLA-4

The integrin dimer VLA-4 is composed by CD49d (alpha 4) and CD29 (beta 1). Many cells with hematopoietic origin express VLA-4, which mediate key functions in the cellular immune response, regulating leukocyte tethering, rolling, binding, and transmigration of the vascular wall at inflammatory sites(Chigaev & Sklar 2012 p. 4). Leukemic cells and solid tumors, expressing VLA-4 can use its adhesive functions, giving it a progressive role in the metastatic spread. In multiple myeloma (MM) cells and the surrounding stroma, increased VLA-4 expression has been observed and its activation has been associated with MM pathogenesis(Soodgupta *et al.* 2016). ProxHCR could be used to study its expression as available antibodies exist towards both CD49d and CD29. Several MM Cell lines are also available to study the VLA-4 expression.

CD40-CD40L

The interaction between CD40 and CD40 ligand (CD40L) is one of the most important receptor ligand communications which occurs during the T-dependent immune response(Elgueta *et al.* 2009). The interaction can give valuable information concerning prognosis in various of cancers. In breast cancer patients, expression levels of CD40/CD40L on B-cells and T-cells has shown to be higher compared to those in control groups. There has also been shown to be a positive correlation between pathological grades and lymph node metastasis of breast cancer and the expression levels of CD40 and CD40L (Pan *et al.* 2013). Additionally, co-expression of CD40 and its ligand *in vitro* increased the proliferation, motility and invasion of immortalized human epithelial cells suggesting that neoplastic growth uses the CD40/CD40L pathway independent of the immune-system (Baxendale *et al.* 2005). In a study from Kim *et al.* (2015) the CD40-CD40L interaction was reported to be involved in Th17 development. The direct CD40-CD40L interaction between breast cancer cells and activated T-cells showed to increased TGF-β production, inducing the production of IL-17 which in turn enhanced the proliferation of the cancer cells. ProxHCR could be used to study the CD40/CD40L interaction and provide valuable information concerning tumor growth and spreading. Additionally, since several cancer treatments, aiming to inhibit this interaction, are becoming available ProxHCR can be used to validate when these treatments would be effective.

2.4 AUTOMATED STAINING

Molecular pathology is becoming an integral part of clinical practice. The use of biomarkers to identify disease status and conditions, potentially influencing treatment, has led to an increasing demand for IHC in clinical diagnostics. As an example, IHC is often used to detect HER2 positive metastatic breast cancer patients. These patient are suitable for Trastuzumab treatment, an antibody which will bind to the HER2 receptor, slowing down cell proliferation (Perez *et al.*). This and similar applications of methods such as IHC have led to a request for effectiveness and standardization regarding the use of these methods. This have future led to automation in this field. In clinical settings, automated staining is the new standard and to be able to compete with

other staining methods it is important to have a functioning method running on an automated instrument. There are several vendors on the market, such as Ventana and Dako, selling instruments with a variety of features suitable for diverse needs. When the first instrument was released it had limited capability, but today an instrument can manage everything from slide labeling, baking, deparaffinization, antigen retrieval, staining, cover slipping to digital image analysis. There is no perfect system, as all has weaknesses and strengths, depending on their intended use (Myers 2004). For a clinic, the slide capacity might be of most importance, being able to run as many tests as possible during a day. As for a researcher, reagent and protocol flexibility might be of more value.

3 METHODS AND MATERIAL

3.1 THE PROXHCR PROTOCOL

The ProxHCR protocol for HaCaT and SkBr3 cells, being described in 3.1.1 and 3.1.2, was the basis of the developing process. They were used as a starting point in the development and by testing various parameters, mimicking an automated system, the current protocol could be optimized towards an automated staining platform.

3.1.1 HaCaT cells

The HaCaT cell line is an immortalized human keratinocyte cell line which was first described by Boukamp *et al*. (1988). These cells were the first spontaneously transformed human epithelial cell line from adult skin which maintained full epidermal differentiation capacity and has since then been widely used in research. HaCaT cells was used to study the interaction between β-catenin/E-cadherin which is found in the cell-cell junctions in the epithelial barrier.

HaCaT cells were permeabilized with 0.2% triton in PBS for 5 min and then washed 3x5min in PBS. Cells were then blocked using Duolink® blocking solution and incubated in humidity chamber for 1 hour at +37°C. Blocking solution is tapped off and slides were incubated with primary antibody against β-catenin (1:100, rabbit, Santa Cruz) and E-cadherin (1:100, mouse, bd biosciences) mixed in Duolink® antibody diluent, overnight at 4 °C. The slides were then washed 3x5 min in TBS-T under gentle agitation. Proximity probes (1:10) directed against rabbit IgG and mouse IgG were mixed in Duolink® antibody diluent and added to samples. The slides were incubated in humidity chamber for 1 hour at 37°C. The slides were then washed 1x5 min in PBS and 2x5 min in HCR buffer under gentle agitation. The activator was mixed in HCR buffer to a concentration of 20 nM and added to corresponding samples. The slides were then incubated in humidity chamber for 30 min at 37°C. Slides were then washed 2x3 min in HCR buffer under gentle agitation. Amplification solution was prepared by mixing two HCR oligos to a concentration of 40nM in HCR buffer. The amplification solution is added to corresponding sample and the slides were incubated in a humidity chamber for minimum of 1h at 37°C. The slides were washed 2x5 min in HCR buffer under gentle agitation and then rinsed twice in water. Coverslips were mounted on the slides using mounting medium with DAPI and placed to rest in a dark space for 5 minutes. The samples were then analyzed in fluorescence microscope.

3.1.2 SkBr3 cells

The SkBr3 cell line is a human breast cancer cell line which overexpresses the HER2 gene product. The cell line was established in 1970 being derived from a 43-year-old Caucasian female with malignant adenocarcinoma of the breast (Fogh *et al.* 1977). In this protocol, the ProxHCR technique allowed for dual recognition of the single protein expression of HER2.

SkBr3 cells were permeabilized with 0.2% triton in PBS for 5 min and then washed 3x5min in PBS. Cells were then blocked using Duolink® blocking solution and incubated in humidity chamber for 1 hour at +37°C. Blocking solution was tapped off and slides were incubated with two primary antibodies against HER2 (1:50, goat, Santa Cruz biotechnology and 1:500, mouse, Chemi Con), mixed in Duolink® antibody diluent, overnight at 4 °C. The slides were then washed 3x5 min in TBS-T under gentle agitation. Proximity probes (1:10) directed against goat

IgG and mouse IgG were mixed in Duolink® antibody diluent and added to samples. The slides were incubated in humidity chamber for 1 hour at 37°C. The slides were then washed 1x5 min in PBS and 2x5 min in HCR buffer under gentle agitation. The activator was mixed in HCR buffer to a concentration of 20 nM and added to corresponding samples. The slides were then incubated in humidity chamber for 30 min at 37°C. Slides were then washed 2x3 min in HCR buffer under gentle agitation. Amplification solution was prepared by mixing two HCR oligos to a concentration of 40nM in HCR buffer. The amplification solution was added to corresponding sample and the slides were incubated in a humidity chamber for minimum of 1h at 37°C. The slides were washed 2x5 min in HCR buffer under gentle agitation and then rinsed twice in water. Coverslips were mounted on the slides using mounting medium with DAPI and placed to rest in a dark space for 5 minutes. The samples were then analyzed in fluorescence microscope.

3.2 THE LAB VISION™ AUTOSTAINER 360 2D

To be able to design and optimize the ProxHCR protocol towards an automated platform the choice of an appropriate instrument is important. A list containing several automated staining machines with their different key features was created (Table 1). This list was then used to narrow down the choice of machine by comparing and evaluating the distinctive features. Features such as flexibility and user friendliness was among the most important things to consider. These are essential since the protocol used in the instrument needs to be programmable without having specialized knowledge. Parameters need to be adjustable and easy to change. This was true for the instruments having open systems. In an open system, the user can choose reagents from any company and the protocols are adjustable. The selected instrument was the Lab Vision™ Autostainer 360 2D from Thermo Fischer scientific. When the instrument was decided, there were several parameters in the manual protocol which needed to be addressed to suit the environment in the machine.

Table 1. Key features of automated IHC instruments

	BioCare	Ventana	Leica		Dako		Thermo Fisher
	Intellipath	Benchmark Ultra	Bond III	Bond Max	Omnis	Autostainer Link 48	Lab Vision™ Autostainer 360-2D
Slide capacity	50	30	30	30	60	48	36
Reagent Application	Rinsed probe	Disposable dispenser	Rinsed probe	Rinsed probe	Rinsed probe	Rinsed probe	Rinsed probe
Regent mixing method	Open slide	Liquid coverslip	Covertiles	Covertiles	Dynamic capillary	Open slide	Open slide
Dispensing range	100-600µl	100µl	50-1000µl	50-1000µl	-	100-600µl	100-600 µl
Temperature range	Ambienet	Ambient	5-35°C	5-35°C	32°C	Ambient	Ambient
Accept other regents	All types	Primary ab	Primary ab	Primary ab	Primary ab	All types	All types
Programming capacity	Open	Closed	Closed	Closed	Closed	Open	Open
Space	Benchtop	Floor	Floor	Benchtop	Floor	Benchtop	Benchtop
Automated steps							
Dewaxing	NO	YES	YES	YES	YES	Separate module	Separate module
Onboard heat	NO	YES	YES	YES	YES	Separate module	Separate module
Counterstaining	YES	YES	YES	YES	YES	YES	YES
Coverslip	NO	NO	NO	NO	NO	NO	9

The ProxHCR protocol done manually has many different buffers and an automated instrument only has two buffer inlets. Consequently, the buffers in the automated protocol needs to be reduced to two. Also, in the manual protocol the washing is done in a jar or directly on the slide under agitation whereas the instrument washes the sample from above by spraying out buffer directly on the slide**.** The Autostainer 360-2D has an ambient working temperature while in the

manual ProxHCR protocol all the incubations are done in humidity chamber at +37°C. Therefore, all incubation times needs to be adjusted to room temperature (RT). Furthermore, the HCR wash buffer does not include any surfactant, making it hard to distribute the reagents on the slide after washing. Additives need to be incorporated in the buffer to reduce this problem. Additionally, since the protocol will take several hours pre-mixed solutions, loaded in instrument, need to manage storage in RT during these hours. Also, if selling this method as a product it would be feasible to have some of the reagents in ready-to-use (RTU) concentrations. Therefore, a storage buffer will be developed, helping to keep pre-mixed solutions stable.

3.3 BUFFERS AND WASHING

The SkBr3 cells were treated as described in 3.1.2 and the ProxHCR assay preformed as described in 3.1.2 but changing the washing with HCR buffer to HCR-T (HCR buffer with 0.01% tween). The PBS wash was replaced with a HCR-T wash.

3.4 ROOM TEMPERATURE

3.4.1 Testing all incubations in RT

The HaCaT cells were treated as described in 3.1.1 and the ProxHCR assay preformed as described in 3.1.1 but leaving one slide out in RT for every incubation. The washing was done using only HCR-T.

3.4.2 Optimal incubation time for activator in RT

The HaCaT cells were treated as described in 3.1.1 and the ProxHCR assay preformed as described in 3.1.1 but having four different incubation times for the activator, 0.5, 1, 1.5 and 2h. The washing was done using only HCR-T.

3.5 RTU CONCENTRATIONS

3.5.1 First evaluation of RTU concentrations

RTU solution of the activator was prepared by mixing it to a concentration of 20nM in HCR buffer. Two RTU solutions were mixed for the HCR oligos. One solution with the HCR oligos together in a 1x dilution (40nM) using HCR buffer and one solution where the HCR oligos were mixed separately in a 2x solution (80nM) using HCR buffer. All RTU solutions where stored in -20°C for 1 day. Skrb3 cells were treated as described in 3.1.2 and the ProxHCR assay preformed as in 3.1.2 using both RTU solutions and freshly made solutions of activator and HCR oligos.

3.5.2 Storage buffer for RTU concentrations

One RTU solutions with HCR oligos was prepared by mixing them in HCR buffer, containing 0.25% tween, to a concentration of 40nM. One RTU solutions with activator was prepared by mixing it in HCR buffer, containing 0.25% tween, to a concentration of 20nM. The solutions are stored in -20°C in 4 and 1 day, respectively. HaCaT cells were treated as described in 3.1.1 and the ProxHCR assay preformed as described in 3.1.1, using both RTU solutions (containing tween) and freshly made solutions of activator and HCR oligos. The washing was done using only HCR-T.

One RTU solutions with HCR oligos was prepared by mixing them in HCR buffer, containing 0.1% bovine serum albumin (BSA), to a concentration of 40nM. One RTU solutions with activator was prepared by mixing it in HCR buffer, containing 0.1 % BSA, to a concentration of 20nM. HaCaT cells were treated as described in 3.1.1 and the ProxHCR assay preformed as described in 3.1.1, using both RTU solutions (containing BSA) and freshly made solutions of activator and HCR oligos. The washing was done using only HCR-T.

3.5.3 Stability study

RTU solutions of activator and HCR oligos was prepared by using the storage buffer tested in 4.5.2, HCR buffer containing 0.25% tween and 1% BSA. These were frozen and stored in -20°C and tested after 2 and 5 weeks. Additional evaluations will be done after 3 and 6 months storage. HaCaT cells were treated as described in 3.1.1 and the ProxHCR assay preformed as described in 3.1.1, using both RTU solutions (containing tween and BSA) and freshly made solutions of activator and HCR oligos. The washing was done using only HCR-T.

3.6 THE COMPLETE AUTOMATED PROTOCOL

All reagents (primary antibodies, blocking solution, proximity probes, activator and HCR oligos) were pre-mixed and left in RT during the whole experiment. RTU solutions of activator (20nM) and HCR oligos (40nM) were mixed in HCR buffer containing both 0.25% tween and 0.1% BSA. The HaCaT cells were permeabilized as described in 3.1.1. The blocking and primary antibodies were incubated in RT for 1h each. The ProxHCR assay was then preformed as described in 3.1.1, but with all incubations in RT and a pro-longed incubation time for activator to 2h. The washing was done using only HCR-T. Both RTU solutions (containing tween and BSA) and fresh solutions of activator and HCR oligos was used during the experiment.

3.6.1 Test in Autostainer 360 2D

The first test on the instrument included two slides of HaCaT cells, studying only positive signal. One slide was surrounded with a hydrophibic fatpen whereas the other one was left without, evaluating if surface tension was enough to hold the reagent on slide. The instrument was run according to protocol in Table 2.

Table 2. The automated protocol tested in Autostainer 360-2D.

	Wash	**Blocking**	**Wash**	**Primary Ab**	**Wash**		**Proximity pr.**	**Wash**		**Activation**	**Wash**		**Amplification**	**Wash**	
Volume		3x 100µl		3x 100µl			3x 100µl			3x 100µl			3x 100µl		
Reagents	2x TBS-T	Duolink blocking	Blow	β-cat/E-cad	4 x TBS-T	Blow	p18R6/M7	4x HCR-T	Blow	Activator old	2x HCR-T	Blow	HCR Oligos texas red	2x HCR-T	H20
Time		1h		1h			1h			2h			1h		

4 RESULTS

4.1 BUFFERS AND WASHING

To solve the problem with distributing the reagents on slide 0.01% Tween 20 was added to HCR wash buffer. In the same experiment, it was also tested to remove the washing with PBS after the proximity probes and to replace this with HCR-T. The results indicated that the changing to only HCR-T washes did not affect the results negatively (Figure 2).

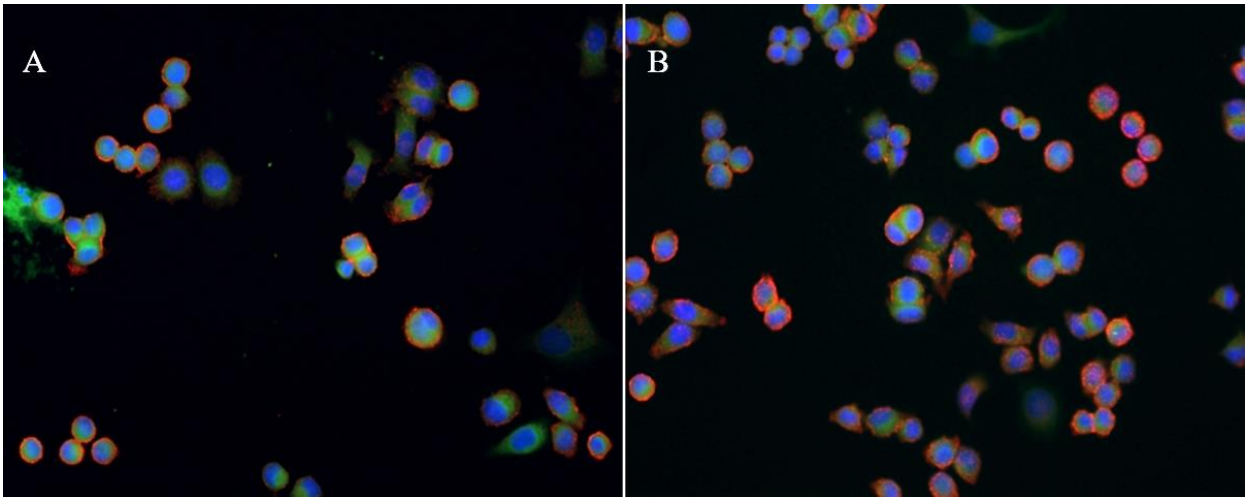

Figure 2. Expression of Her2 on SkBr3 cells surface using two Her2 primary antibodies. Strong staining (shown in red) when using A) HCR wash buffer or B) HCR T wash buffer and no PBS wash.

4.2 ROOM TEMPERATURE

For every incubation, one of the slides were left in RT. For reference, all incubation steps were done in humidity chamber at +37°C. This experiment indicated that the reaction with the activator was mostly affected by incubation in RT (Figure 3).

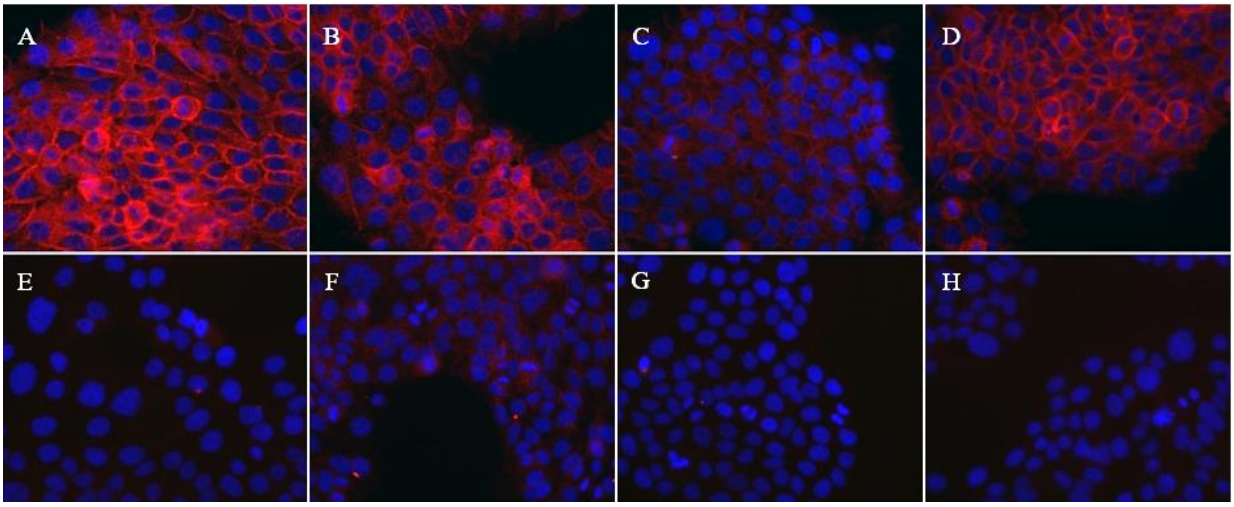

Figure 3. Technical controls for the expression of E-cadherin/β-catenin interaction in HaCaT having A-D) both primary antibodies and E-H) one primary antibody as negative control. Membranous staining (shown in red) was observed when incubating A) all ProxHCR steps in +37°C B) proximity probes in RT D) oligos in RT. No staining was observed for negative controls E-H) or when C) incubating activator in RT.

The next experiment was to test the optimal incubation time for activator. The result indicated that the longest incubation time in RT gave the highest signal (Figure 4).

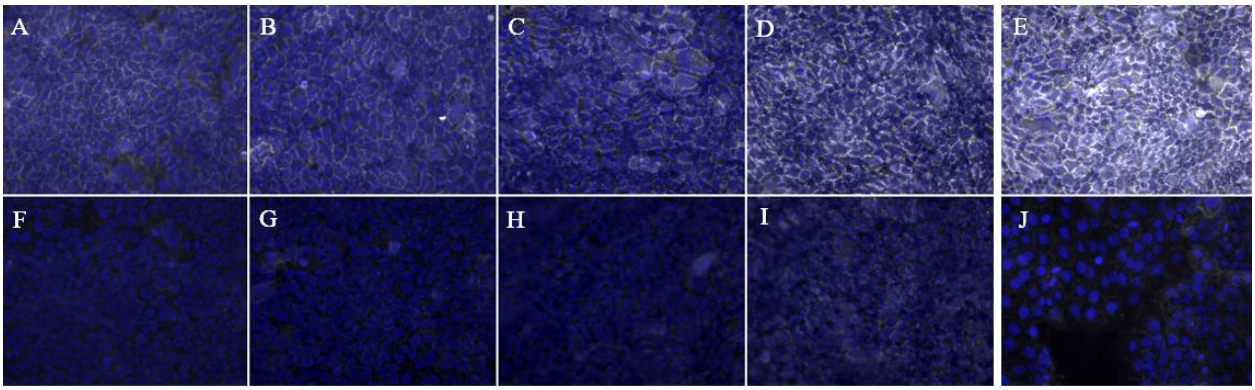

Figure 4. Technical controls for the expression of E cadherin/β catenin interaction in HaCaT having A-E) both primary antibodies and F-J) one primary antibody as negative control. Increasing membranous signal (shown in white) was observed when incubating activator A) 0.5h in RT C B) 1h in RT C) 1.5h in RT D) 2h in RT. E) Reference, running all ProxHCR incubations in +37° using the times described in 3.1.1. F-J) No or little staining was observed for negative controls.

4.3 RTU CONCENTRATIONS

An initial test to store activator and HCR oligos in RTU solutions was prepared by mixing them in HCR buffer and storing them in -20°C for 1 day. This experiment indicated that the activator was capable of storage in RTU concentrations using only HCR buffer (Figure 5B) whereas none of the solutions with the HCR oligos gave any signal (Figure 5C-D). To solve the problem with low or no signal from RTU solutions of HCR oligos it was tested to add tween in the storage buffer. The results indicated that tween did improve the conditions for the HCR oligos in the tube and the signal was now much higher (Figure 5K).

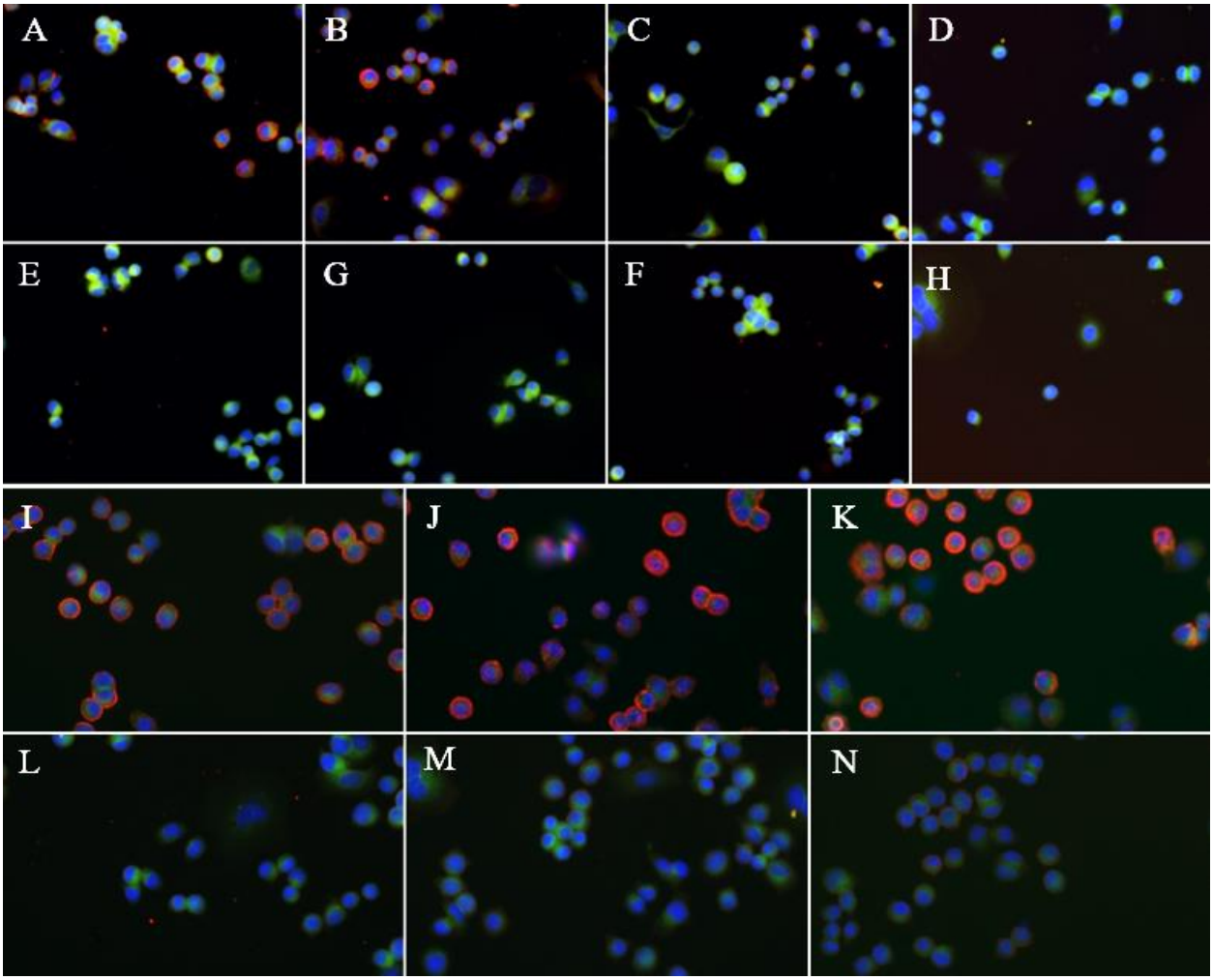

Figure 5. Expression of Her2 on SkBr3 cells surface (shown in red) using A-D) two Her2 antibodies and L-M) one Her2 antibody as negative control. Strong surface staining for A) freshly made reagents (reference) B) activator stored in HCR buffer -20°C. No staining for C) mixed HCR oligos stored in HCR buffer -20°C D) HCR oligos stored separated in HCR buffer in -20°C. Strong staining for I) freshly made reagents (reference) J) activator stored in HCR-T (0.25% tween) and K) mixed HCR oligos stored in HCR-T (0.25% tween). All negative controls E-H) and L-M) shown no or little staining.

It is feasible to have BSA in solutions containing low protein concentrations, making it suitable to include in this storage buffer. It is tested to include BSA in the ProxHCR assay, evaluating if it has a damaging effect on the reaction. Reagents mixed with only HCR buffer or HCR buffer containing 0.1% BSA show similar staining (Figure 6A-B) as well as similar background (Figure 6C-D).

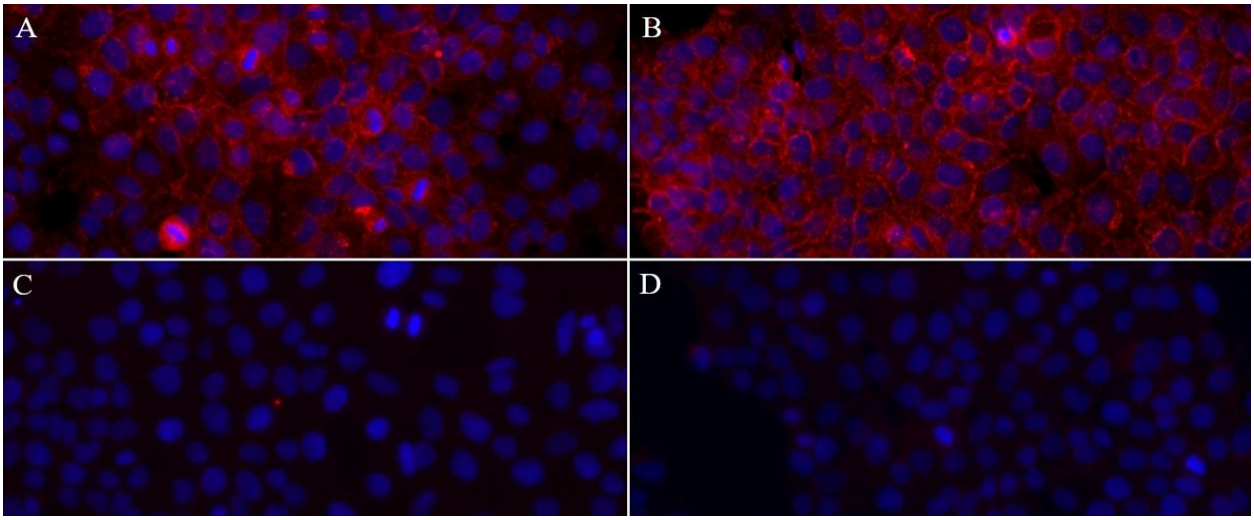

Figure 6. Technical controls for the expression of E cadherin/β catenin interaction in HaCaT having A B) both primary antibodies and C D) one primary antibody. A) reagents mixed with HCR buffer (reference) B) reagents mixed with HCR buffer containing 0.1% BSA.

To further test the storage buffer developed from the previous experiment a small stability study was started, evaluating RTU concentrations of activator and HCR oligos stored in -20°C at four different time points, two of these was assessed during this project (Data not shown).

4.4 RUNNING THE COMPLETE AUTOMATED PROTOCOL

Before running on the Autostainer 360-2D the complete automated protocol was tested, running all steps in RT with pre-mixed solutions to simulate the environment in the instrument. When comparing the pre-mixed solutions to the freshly mixed solutions, the signal from pre-mixed was higher (Figure 7). Overall the result from this experiment was positive, generating signal.

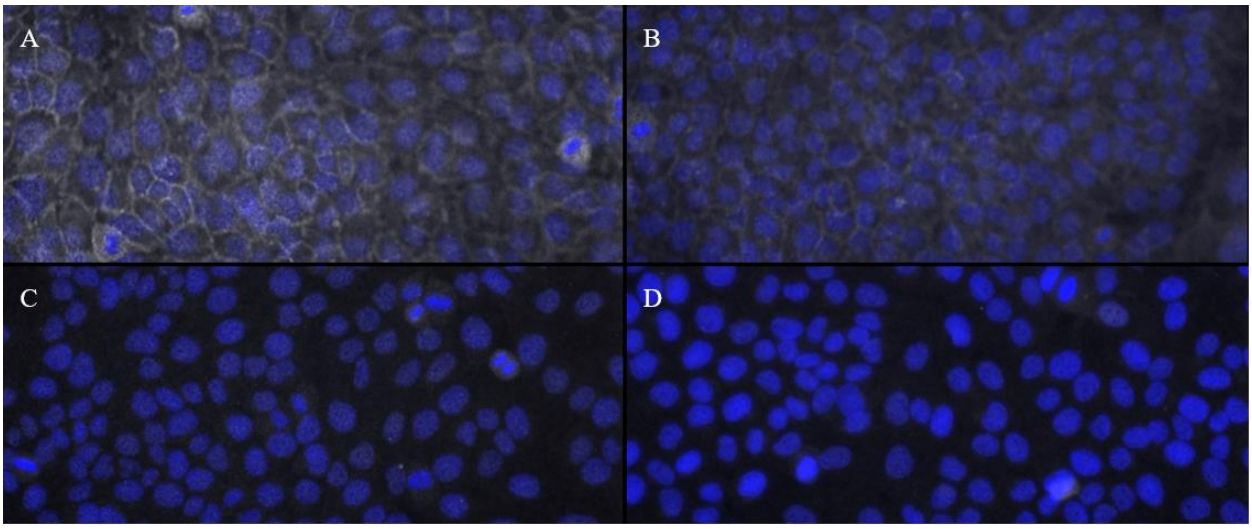

Figure 7. Technical controls for the expression of E-cadherin/β-catenin interaction in HaCaT cells having A-B) both primary antibodies C-D) no primary antibodies. Membranous staining (shown in white) for A) reagents mixed with HCR buffer containing BSA and tween B) reagents mixed with HCR buffer (reference). C-D) negative controls showing no staining

The first run in the Autostainer 360 2D was successful and the instrument handle the protocol well. The results showed positive signal for E cadherin/β catenin interaction (Figure 8).

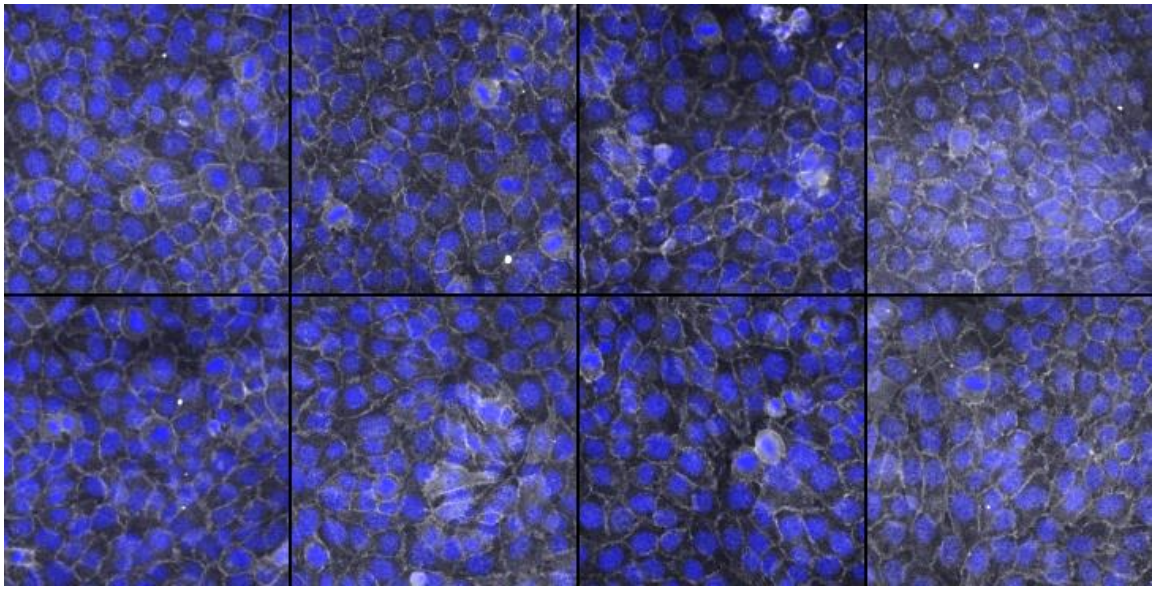

Figure 8. Expression of E cadherin/β catenin interaction in HaCaT cells. Membranous staining (shown in white) for all wells on the slide surrounded with hydrophobic fatpen

5 DISCUSSION AND CONCLUSION

As indicated from the scientific review described in chapter 2.3, discussing possible biomarkers for ProxHCR, the technique has the potential to be a leading diagnostic and prognostic tool for diseases such as cancer. For example, by studying the CD40/CD40L interaction valuable information concerning tumor growth and spreading can be achieved, perhaps even indicating when existing treatments would be effective. This even further demonstrates the need of having a method such as ProxHCR, running on an automated instrument. There were three major challengers which needed to be addressed when adopting the current ProxHCR protocol towards an automated platform. The challenges needed to be addressed included; the composition of HCR wash buffer to facilitate proper washing spreading of reagent, the composition of storage buffer for pre-mixed reagents and room temperature conditions.

To be able to spread reagent on the slide after washing Tween 20 was included in the HCR wash buffer. Tween 20 is a commonly used surfactant in IHC and is added to buffers and reagents, enhancing reagent spreading in both manual and automated processes. Most automated system suggest a concentration of minimum 0.05% tween in their wash buffer. The HCR wash buffer in this project only included 0.01% of tween, this since problems with autofluorescence increased when adding tween. With 0.01% tween, the reagent was able spread properly, giving acceptable autofluorescence. However, alternatives to tween needs to be considered to circumvent this problem.

Since reagent needs to be pre-mixed and left in the instrument for several hours during a run, storage buffer for RTU concentrations needed to be tested. The activator seemed to manage storage in only HCR buffer whereas the HCR oligos were more sensitive. The first experiment having only HCR buffer as storage buffer did not generate any signal for the HCR oligos. Since these oligos are very sticky the problem could be caused by the HCR oligos simply sticking to the tube, resulting in a low concentration of HCR oligos applied on samples. This was solved by adding tween into the storage buffer, making the tube more slippery. Signal was now obtained for both activator and HCR oligos stored in RTU concentrations. Additionally, since diluted protein solutions are susceptible to degradation when stored in concentrations >1mg/ml it is motivated to add “filler” protein, such as BSA in storage buffers. By adding protein to the solution, the loss of antibody due to binding to the tube will be further decreased. The storage buffer comprised of HCR buffer containing 0.25% tween and 1% BSA gave good signal. This storage buffer was further tested in a small stability study were RTU concentrations of activator and HCR oligos were stored in 20°C, to be tested at four different time points. Two of the time points have been evaluated showing satisfactory results. This storage buffer was only tested for activator and HCR oligos but appropriate storage buffers need to be considered for all ProxHCR reagents.

In theory, the ProxHCR reaction should manage to be performed in RT even if the current system is not adapted to this temperature. Previous experiments have also indicated that it is possible to run the present ProxHCR method in RT. First, all ProxHCR incubations were tested in RT separately. This indicated that the incubation with activator was most effected by being

carried out in RT, suggesting that this step need to be prolonged. Different incubation time for the activator was tested, demonstrating that the highest incubation time generated the highest signal. The incubation time for activator was therefore increased in the automated protocol whereas the other incubation times were unchanged. In addition, previous experiment has indicated that prolonging the amplification step in RT will further enhance the signal. This make sense, since more HCR oligos can be incorporated during a longer incubation time. Longer amplification time was tested (data not shown) without giving any conclusive results.

Before running on the instrument, the complete automated protocol needed to be tested. This means testing all the steps in RT, including the incubation of primary antibodies and the blocking. All reagents were also pre-mixed and left in RT during the complete protocol to simulate the environment in the instrument. The results from this experiment indicates that a first test in the Autostainer 360-2D should be performed. The first run in Autostainer was successful, generating a fairly strong signal. However, due to problems with material and reagents there were no time for further tests in the instrument. Several more test in the instrument need to be carried out before the optimization process is completed. Several more model systems need to be evaluated to validate the protocols utility. This project only studied signals from fluorescence whereas most clinics use chromogenic detection systems. It would therefore be favorable to be able to run the automated protocol using a chromogenic detection. Additionally, the project only included cells but as many clinical evaluations are made on tissue this material should be tested in the Autostainer as well. Nevertheless, the result from this project does indicate that it is possible to run ProxHCR on an automated system. This possibility could potentially revolutionize diagnostics, enabling more complex and precise evaluations of disease states leading to more accurate treatments.

6 REFERENCES

Adams, Smothers J, Srinivasan R, Hoos A. 2015. Big opportunities for small molecules in immuno-oncology. Nat Rev Drug Discov 14: 603–622.

Baxendale, Dawson CW, Stewart SE, Mudaliar V, Reynolds G, Gordon J, Murray PG, Young LS, Eliopoulos AG. 2005. Constitutive activation of the CD40 pathway promotes cell transformation and neoplastic growth. Oncogene 24: 7913–7923.

Blevins, Towers CG, Patrick AN, Zhao R, Ford HL. 2015. The SIX1-EYA transcriptional complex as a therapeutic target in cancer. Expert opinion on therapeutic targets 19: 213–225.

Boukamp P, Petrussevska R T, Breitkreutz D, Hornung J, Markham A, Fusenig N E. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. The Journal of Cell Biology 106: 761–771.

Brachtl, Piñón Hofbauer J, Greil R, Hartmann TN. 2014. The pathogenic relevance of the prognostic markers CD38 and CD49d in chronic lymphocytic leukemia. Annals of Hematology 93: 361–374.

Carvajal-Hausdorf, Schalper KA, Neumeister V, Rimm DL. 2015. Quantitative Measurement of Cancer Tissue Biomarkers in the Lab and in the Clinic. Laboratory investigation; a journal of technical methods and pathology 95: 385–396.

Chigaev, Sklar LA. 2012. Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion. Frontiers in Immunology 3: 242.

Dal-Bo, Bertoni F, Forconi F, Zucchetto A, Bomben R, Marasca R, Deaglio S, Laurenti L, Efremov DG, Gaidano G, Del Poeta G, Gattei V. 2009. Intrinsic and extrinsic factors influencing the clinical course of B-cell chronic lymphocytic leukemia: prognostic markers with pathogenetic relevance. Journal of Translational Medicine 7: 76–76.

Debruyne, Vermeulen S, Mareel M. 1999. The role of the E-cadherin/catenin complex in gastrointestinal cancer. Acta Gastro-Enterologica Belgica 62: 393–402.

Drummen. 2012. Fluorescent Probes and Fluorescence (Microscopy) Techniques — Illuminating Biological and Biomedical Research. Molecules 17: 14067–14090.

Elgueta, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunological reviews 229: 10.1111/j.1600-065X.2009.00782.x.

Farabaugh, Micalizzi DS, Jedlicka P, Zhao R, Ford HL. 2012. Eya2 Is Required to Mediate the Pro-Metastatic Functions of Six1 Via the Induction of TGF-β Signaling, Epithelial-Mesenchymal Transition, and Cancer Stem Cell Properties. Oncogene 31: 552–562.

Fleming, Powers JH. 2012. Biomarkers and Surrogate Endpoints In Clinical Trials. Statistics in medicine 31: 2973–2984.

Fogh, Fogh JM, Orfeo T. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. Journal of the National Cancer Institute 59: 221–226.

Guo, Wang X, Liang L. 2015. LATS2-mediated YAP1 phosphorylation is involved in HCC tumorigenesis. International Journal of Clinical and Experimental Pathology 8: 1690–1697.

Hazelton, Luebeck EG. 2011. Biomarker-Based Early Cancer Detection: Is It Achievable? Science translational medicine 3: 109fs9-109fs9.

Jin, Lloyd RV. 1997. In situ hybridization: Methods and applications. Journal of Clinical Laboratory Analysis 11: 2–9.

Johnson, Halder G. 2014. The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. Nature reviews Drug discovery 13: 63–79.

Kim, Kim Y, Bae S, Kong JM, Choi J, Jang M, Choi J, Hong J, Hwang Y, Kang JS, Lee WJ. 2015. Direct Interaction of CD40 on Tumor Cells with CD40L on T Cells Increases the Proliferation of Tumor Cells by Enhancing TGF-β Production and Th17 Differentiation. PLoS ONE 10: e0125742.

Komiya, Habas R. 2008. Wnt signal transduction pathways. Organogenesis 4: 68–75.

Koos, Cane G, Grannas K, Löf L, Arngården L, Heldin J, Clausson C-M, Klaesson A, Hirvonen MK, de Oliveira FMS, Talibov VO, Pham NT, Auer M, Danielson UH, Haybaeck J, Kamali-Moghaddam M, Söderberg O. 2015. Proximity-dependent initiation of hybridization chain reaction. Nature Communications 6: 7294.

Koos, Söderberg O. 2001. Designing and Applying Proximity-Dependent Hybridization Chain Reaction. Current Protocols in Protein Science, doi 10.1002/cpps.9.

Lebrun. 2012. The Dual Role of TGF in Human Cancer: From Tumor Suppression to Cancer Metastasis. International Scholarly Research Notices 2012: e381428.

Lin, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung M-C. 2000. β-Catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. Proceedings of the National Academy of Sciences of the United States of America 97: 4262–4266.

Liu, Huang D, Wang Z, Wu C, Zhang Z, Wang D, Li Z, Zhu T, Yang S, Sun W. 2016. LMO2 attenuates tumor growth by targeting the Wnt signaling pathway in breast and colorectal cancer. Scientific Reports 6: 36050.

Lyon. 2012. Theory and Strategy in Histochemistry: A Guide to the Selection and Understanding of Techniques. Springer Science & Business Media

MacDonald, Tamai K, He X. 2009. Wnt/β-catenin signaling: components, mechanisms, and diseases. Developmental cell 17: 9–26.

Mao, Hu F, Cheng J, Wang P, Xu M, Yuan F, Meng S, Wang Y, Yuan Z, Bi W. 2014. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. Oncogene 33: 1468–1474.

Ménard, Fortis S, Castiglioni F, Agresti R, Balsari A. 2001. HER2 as a Prognostic Factor in Breast Cancer. Oncology 61(suppl 2): 67–72.

Micalizzi, Wang C-A, Farabaugh SM, Schiemann WP, Ford HL. 2010. Homeoprotein Six1 Increases TGF–β Type I Receptor and Converts TGF-β Signaling from Suppressive to Supportive for Tumor Growth. Cancer research 70: 10371–10380.

Myers. 2004. Automated slide stainers for SS, IHC, and ISH: A review of current technologies and commercially available systems. MLO: medical laboratory observer 36: 28–30.

Nagaraj, Datta PK. 2010. Targeting the Transforming Growth Factor-β Signaling Pathway in Human Cancer. Expert opinion on investigational drugs 19: 77–91.

Nazemalhosseini Mojarad, Kashfi SMH, Mirtalebi H, Almasi S, Chaleshi V, Kishani Farahani R, Tarban P, Molaei M, Zali MR, J.K. Kuppen P. 2015. Prognostic Significance of Nuclear β-Catenin Expression in Patients with Colorectal Cancer from Iran. Iranian Red Crescent Medical Journal 17: e22324.

Okada, Nishio W, Sakamoto T, Uchino K, Yuki T, Nakagawa A, Tsubota N. 2004. Prognostic significance of perioperative serum carcinoembryonic antigen in non-small cell lung cancer: analysis of 1,000 consecutive resections for clinical stage I disease. The Annals of Thoracic Surgery 78: 216–221.

Pan, Gong J, Yang C, Feng R, Guo F, Sun Y, Chen H. 2013. Peripheral blood CD40–CD40L expression in human breast cancer. Irish Journal of Medical Science 182: 719–721.

Perez, Cortés J, Gonzalez-Angulo AM, Bartlett JMS. 2014. HER2 testing: Current status and future directions. Cancer Treatment Reviews 40: 276–284.

Schacht, Kern JS. 2015. Basics of Immunohistochemistry. Journal of Investigative Dermatology 135: 1–4.

Sebio, Lenz H-J. 2015. Molecular Pathways: Hippo Signaling, a Critical Tumor Suppressor. Clinical cancer research : an official journal of the American Association for Cancer Research 21: 5002–5007.

Shariat, Semjonow A, Lilja H, Savage C, Vickers AJ, Bjartell A. 2011. Tumor markers in prostate cancer I: blood-based markers. Acta oncologica (Stockholm, Sweden) 50: 61–75.

Soodgupta, Zhou H, Beaino W, Lu L, Rettig M, Snee M, Skeath J, DiPersio JF, Akers WJ, Laforest R, Anderson CJ, Tomasson MH, Shokeen M. 2016. Ex Vivo and In Vivo

Evaluation of Overexpressed VLA-4 in Multiple Myeloma Using LLP2A Imaging Agents. Journal of nuclear medicine : official publication, Society of Nuclear Medicine 57: 640–645.

Strimbu, Tavel JA. 2010. What are Biomarkers? Current opinion in HIV and AIDS 5: 463–466.

Tian, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, Zhao Y, Harris DCH, Zheng G. 2011. E-Cadherin/β-Catenin Complex and the Epithelial Barrier. Journal of Biomedicine and Biotechnology 2011: 567305.

Várady, Cserepes J, Németh A, Szabó E, Sarkadi B. 2013. Cell surface membrane proteins as personalized biomarkers: where we stand and where we are headed. Biomarkers in Medicine 7: 803–819.

Zucchetto, Vaisitti T, Benedetti D, Tissino E, Bertagnolo V, Rossi D, Bomben R, Dal Bo M, Del Principe MI, Gorgone A, Pozzato G, Gaidano G, Del Poeta G, Malavasi F, Deaglio S, Gattei V. 2012. The CD49d/CD29 complex is physically and functionally associated with CD38 in B-cell chronic lymphocytic leukemia cells. Leukemia 26: 1301–1312.