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Development and evaluation of an immunoassay for Keratin 5/19 complex in lung cancer

Master's thesis in Biotechnology

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Gothenburg, Sweden 2015

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Abstract

14 antibody combinations were evaluated as candidates for a keratin 5/19 enzyme immunoassay. By enzyme immune assay and western blot, 1A12+Ks19.1 was determined as the superior combination. The most successful test assay was the two-step enzyme immunoassay with 2 hours incubation time for the biotinylated antibody and 1 hour incubation for the HRP-conjugated antibody.

The experiments used to evaluate antibody combinations also indicates a structural difference between keratin 5/19 in pleura liquid compared to serum and that K5/19 complex containing the N-terminal is likely to be present in serum in higher extent than complex with intact C-terminal. The western blot also revealed cross-reaction of G-2 antibody with keratin 8, although G-2 is stated specific for keratin 5 by its manufacturer. [1]

A randomized lung cancer study including 110 lung cancer plasma samples, 52 serum samples of benign lung disease and 52 plasma samples from healthy blood donors was performed. The study was carried out by analyzing the samples in the constructed 1A12+Ks19.1 test and CYFRA21-1. The constructed test was not able to discriminate between histological classes of lung cancer in the lung cancer study.

The ability of the test to detect early stage lung cancer varied among the different histological classes. Adenocarcinoma and large cell carcinoma samples detected by the 1A12+Ks19.1 test were progressed cancer, stage III or stage IV. For small cell lung cancer 3 of the 13 early stage lung cancer were detected, indicating the test is thus not suitable for detecting early stages of these histological classes. For Squamous cell carcinoma in lung early stages are detected in larger extent than stage III and IV for K5/19 (1A12+Ks19.1) as a separate test, but primarily in combination with CYFRA21-1 where 9 of 13 early stage samples were detected. From this study one can recommend to perform further studies on early stage SCC in lung to determine the relevance of CYFRA21-1 in combination with 1A12 as diagnostic tool.

Key words: Enzyme immunoassay, ELISA, Keratin 5/19, K5/19, lung cancer

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1 Introduction

Lung cancer is one of the most common cancer forms in the world and has a poor survival rate. [2] The majority of the new cases are patients with progressed cancer (stage III or IV) and 5-year survival rate is less than 15 % for adults. The low survival rate is due to the lack of diagnostic methods for early stage cancer and methods for treating the more progressed cases [3]. However, when the lung cancer is caught in an early stage the survival rate reaches 60-90% depending on the tumor size. [4] Depending on the histological type, the cancer treatment will differ. A new diagnostic tool of lung cancer with ability to early diagnose lung cancer and to discriminate between different lung cancer forms, would be of great value. [5]

A widely used immunoassay used in diagnosing cancer is the CYFRA21-1, which detects the total amount of keratin 19. However, the CYFRA21-1 is not effective in detecting early stage lung cancer and therefore a complement to this test would be desirable. [6]

A possible complement to CYFRA21-1 would be to detect the keratin complex K5/19 which is expressed in squamous cell carcinoma but not in small cell nor adenocarcinoma. If levels of K5/19 in tissue and serum correlates, a K5/19 would possibly enable discrimination between different lung cancers. This would aid significantly in the diagnosis and treatment plan of patients. In addition, since K19 is mainly expressed in poorly differentiated squamous cell carcinoma, possibly making the test discriminate between different stages of lung cancer and enable an early detection. [7]

1.1 Aim

The aim of this project is to design a sensitive enzyme immunoassay (EIA) for detection of keratin 5/19 fragments found in sera from patients suffering from lung cancer. The assay will be evaluated as a single marker and in combination with assays measuring total keratin 19 (CYFRA 21-1) for the ability to discriminate between benign and malignant disease and to aid in the differential diagnosis between different histological forms of lung cancer.

1.2 Limitations

In this study the developed keratin 5/19 serum test will only be evaluated as a marker for squamous lung cancer. Other possible applications will not be evaluated. Neither the 3D-structure of the keratin 5/19 complex nor its epitopes where antibodies used in the project will be investigated.

No effort in developing a keratin 5 test will be done in this project although such a test might be successful in detecting squamous cell carcinoma.

1.3 Background

The most common cause of all lung cancers is smoking, but the correlation is substantially higher for squamous carcinoma and small cell carcinoma. Smoking is be responsible for approximately 80% of all male lung cancer cases and 50% of the female cases. On the other hand, a country with relatively high prevalence of lung cancer is China, a land with less than 4 % adult smokers. The cause of this is thought to be the indoor air pollution, coal-fueled stoves and from cooking fume. Other possible causes of lung cancer are asbestos, arsenic, ionizing radiation and combustion gases. [2]

Worldwide lung and bronchus cancer are the cancer form responsible for the highest number of new causes as well as deaths caused by cancer for men. Among women it is the fourth most frequently diagnosed cancer form every year and has second highest death rate. [2] Lung cancer is diagnosed by medical history, x-ray computed tomography, examination, sputum cytology test, blood sampling, lung x-ray, biopsy and bronchoscopy among other methods. [4] Nevertheless, the low survival rate is due to the lack of prognosis methods for early stage cancer and methods for treating the more progressed cases [3]. Another factor interfering with the ability for diagnosis are the unspecific symptoms of early stage lung cancer. [4]

Lung cancer can be divided into two major subclasses, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 80% of all lung cancer cases are the NSCLC, which can be further divided into the histological classes adenocarcinoma, squamous cell carcinoma and large cell lung cancer Whereas the SCLC and NCLC respond differently to therapy, all NSCLC responds fairly similar to treatment and due to this the main interest is in discriminate between SCLC and NSCLC. [5]

1.4 Keratins

Like all intermediate filaments, keratins is made up by three regions; the N-terminal region, the C-terminal region and the α-helical domain. The α-helical domain is a highly conserved region of the keratin and is further divided into the sub regions: 1A, 1B, 2A and 2B by the linkers L1, L2 and L3 (see Figure 1).The N-terminal and C-terminal on the other hand are the two regions that varies between different keratins [8][9] There are two types of keratins, type I (acidic) and type II (neutral/basic). Keratins filaments consists of an equal mixture of type I and type II keratins. Together, the type I and type II keratin will form a heterodimer and these will in pair form the essential tetramer. The tetramers will then stack together in to a rope-like form that is flexible and resilient to mechanical strength. [8] [10]

Keratin act as an anchor by linking neighboring cells together via desmosomes and linking cells with the basal lamina in the extracellular matrix via hemidesmosomes. In order for the cells to be able to maintain their functions in proliferation and migration plasticity and flexibility is required properties of the keratin filaments. Although keratins have important roles in organelle transport, stress response and tissue differentiation fairly little is known about the substructure of the keratin molecule and the N- and C-terminal in particular. [8]

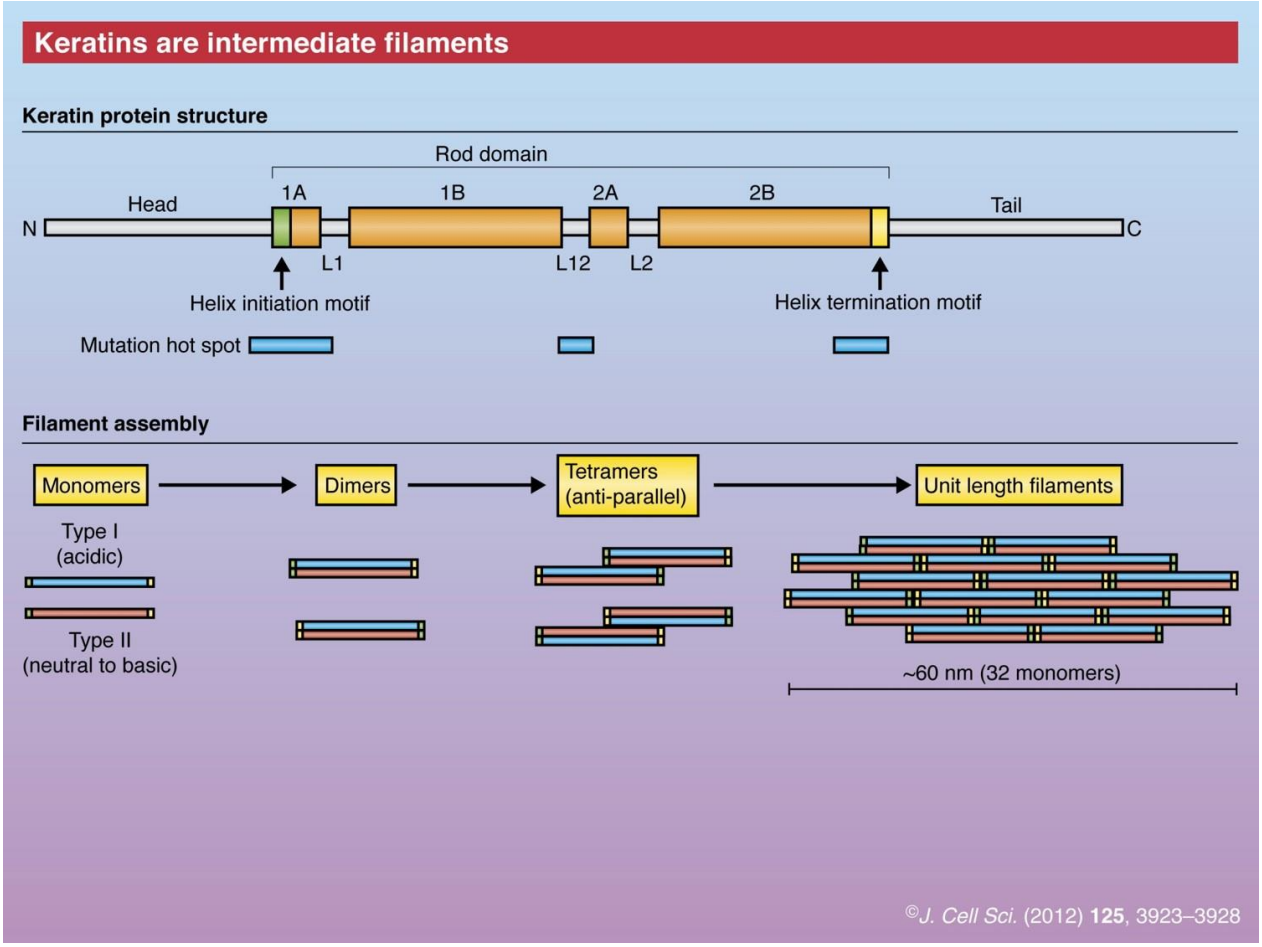

Figure 1. The structure and assembly of keratins. [8]

There are 54 different keratins that all have different expression profiles for different cell types. The keratins can also be divided into three functional groups; simple keratins, barrier keratins and structural keratins. Simple keratins are found in embryonic and one-layered epithelia, which comprises liver, pancreas, colon and glandular cells. The barrier keratins are expressed in the multilayer stratified squamous epithelia such as the skin epidermis and the structural keratins will be found in hair and nail. [8]

1.4.1 Keratin 5/19

Keratins function as markers for several different cancers. When comes to lung cancer, the pattern of keratin expression varies among histological types. No matter of the origin, squamous lung cancer will have a high expression of K5, K14, K17, K6 and K16. One has also observed expression of K8, K18 and K19 in the less differentiated squamous cell carcinomas. When it comes to lung cancer neither adenocarcinoma nor small cell carcinoma tissue express K5/19. [7] [11]

1.5 Enzyme linked immunosorbent assay (ELISA)

One of the most commonly used immunoassays are the enzyme linked immunosorbent assay (ELISA) test. The outline of the method is to immobilize an antibody onto a solid material, typically a microtiter plate. The antibody, often referred to as the catcher antibody, will trap the

antigen onto the solid phase. A second antibody, known as the tracer, conjugated with reporter enzyme will then bind to the antigen. The reporter enzyme enables quantification of the antigen and due to the enzymes ability to catalyze the same reaction multiple times, it will also amplify the reaction. When adding a substrate, the enzyme will convert the substrate into a signal in form of color, chemiluminescence or fluorescence. The most commonly used reporter enzymes is the horseradish peroxidase (HRP) enzyme, see *Conjugation of Horseradish peroxidase (HRP)*, and when used together with the colorimetric substrate 3,3',5,5'-Tetramethylbenzidine (TMB), it converts the TMB in to a blue product which can be quantified by measuring the absorbance at 620 nm. By adding acid the conversion stops and the product turns yellow. [12] [13]

In a variant of the ELISA, the enzyme immunoassay (EIA), streptavidin is immobilized onto a solid phase and the catcher antibody is biotinylated. The assay is typically carried out by first adding the sample containing the antigen on the microtiter plate. The antibodies are then added either in a one-step method or two-step method. In the one-step both antibodies are added together while in the two step the catcher antibody is first added and incubated with the sample alone, followed by a wash step, before the secondary antibody is added. For example of this, see page 63. [12] [14].

1.5.1 Chemical conjugation of antibodies

There are different approaches for chemical conjugation of the antibodies in immunoassays. Below are two conjugation method for catcher and tracer antibodies described.

1.5.1.1 Biotinylation

Biotin is a B vitamin, with a strong avidity for streptavidin and avidin, making the biotinylation suitable as conjugation method for immunoassays with streptavidin/avidin. The small size of biotin also minimizes the risk for it to effect the protein's function when used for conjugation. [14] [10] Since more than one biotin molecule can be conjugated to each protein, the signal in the immunoassay can be augmented and the detection of the protein will be more sensitive. The conjugation can be carried out via enzymatic or chemical methods, where chemical is the most commonly used and easy to customize. Many biotinylation reagents are therefore available on the market but all contain the biotin (blue), the spacer arm and the reactive moiety (red) as seen in Figure 2, where NHS-LC-Biotin is illustrated. The reactive moiety will cross-link the biotin to amines of the protein of interest. The length of spacer arm varies among different biotinylation reagents and will affect the biotin's availability for avidin/streptavidin. The solubility of the reagent will depend on the reactive moiety, the spacer arm or a combination of both. [15]

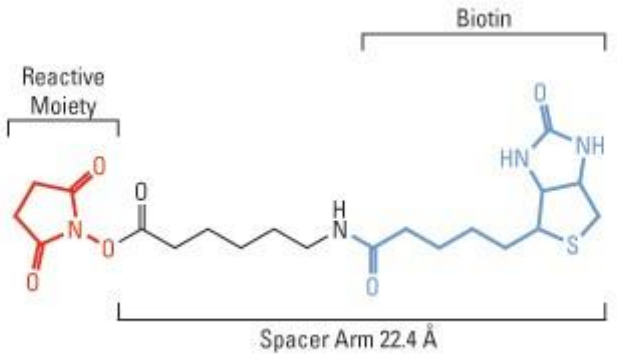

Figure 2. Composition of the biotinylation reagent NHS-LC-Biotin. [15]

1.5.1.2 Conjugation of Horseradish peroxidase (HRP)

As mentioned above, HRP is one of the most common enzymes used for labelling the tracer antibody. HRP is a glycoprotein with an approximate length of 300 amino acids. The protein has very few reactive groups but can be activated by using the periodate method. By using sodium periodate ($NaIO_4$) in acidic conditions (Figure 3), $NaIO_4$ will oxidize the eight chains of carbohydrates on the surface of HRP, resulting in aldehyde groups. Under conditions with pH >9, the aldehyde groups can react with the amine groups of the antibody forming an intermediate. The linkage between the amine groups of the antibody and the aldehyde groups of the glycoprotein is called a Schiff base. However, this molecule will be rather unstable and needs to be reduced in order to make the reaction irreversible. $NaBH_4$ is a frequently used reducing agent suitable for this purpose. The reaction is light sensitive and should be carried out in the dark. [16] [17]

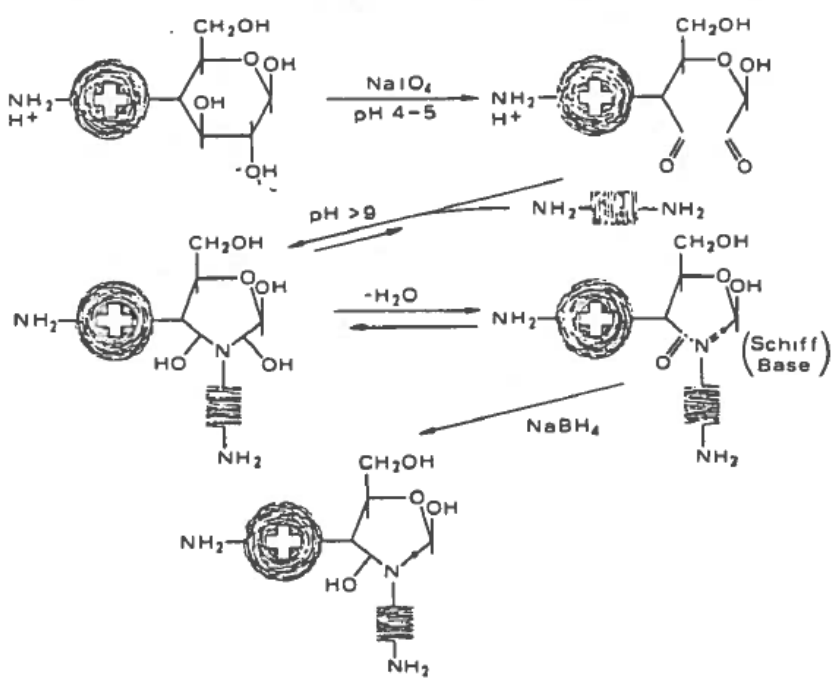

Figure 3. Conjugating HRP to a protein. The HRP protein is activated by $NaIO_4$. When reacting with an antibody, an unstable intermediate is formed. NaBH4 reduces the molecule to a more stable form where the antibody and HRP are covalently bound. [16]

1.6 Antibodies

Antibodies are divided into two major classes, monoclonal and polyclonal antibodies. The polyclonal antibodies are produced by many different cells while monoclonal are produced by one single cell. The monoclonal antibodies are produced in vitro by one single animal cell line, which are called hybridomas. The cell producing the monoclonal antibodies are a fusion between a cancer cell line and B cells from an animal donor. The production of the monoclonal antibody is induced by presenting the B cells to antigen fragment by macrophages and other antigen-presenting cells. The monoclonal antibodies produced by the hybridomas will have the same features as the donor B cells. Polyclonal antibodies on the other hand are produced by injecting an animal with an antigen and collecting the serum from the animal. Many different B cell clones in the animal will produce polyclonal antibodies for different epitopes of the injected antigen. The polyclonal antibodies will be a population of different antibodies contributed by different B cell clones. While the monoclonal antibodies will be epitope specific, the polyclonal antibodies will be antigen specific. [12]

1.6.1 Structure

The Y-shaped molecule is the base for all naturally occurring antibodies. The Y structure consists of two pair of polypeptide chains, a light chain (L) and a heavy chain (H) linked together by disulphide bonds (**Fel! Hittar inte referenskälla.**). The members of each pair are identical. The constant region of the antibody is located at the base of the Y-structure and the variable region at the end of the arms. Within a species the constant region is rather fixed but will deviate between different species. The variable region consists of approximately 110-130 amino acids. The variable regions are typically the binding sites of the antibody and changes in

the amino acid structure of the variable region thus causes changes in epitope recognition. The antibody normally react with an epitope of minimum three amino acids. [12]

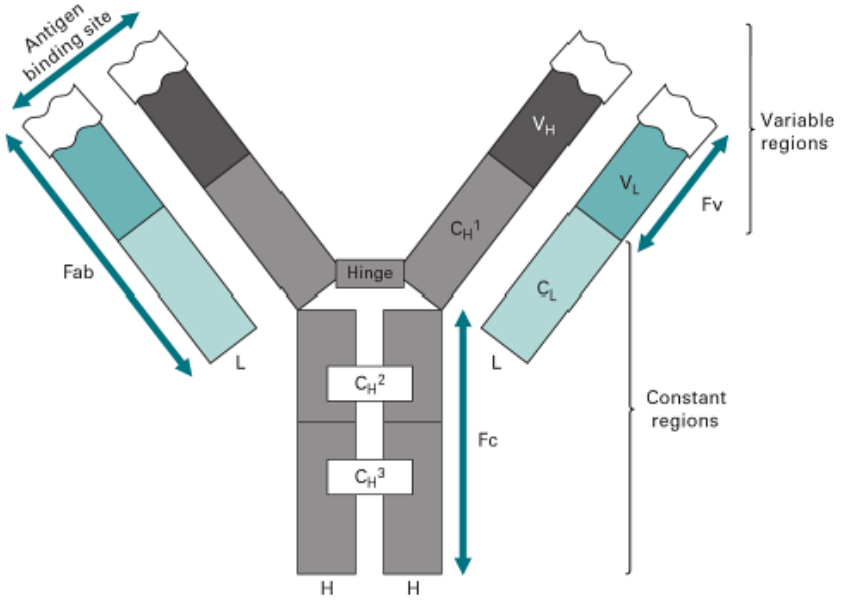

Figure 4. Structure of Immunoglobulin G the most common antibody structure. L and Hare the marking the light chain and the heavy chain respectively, Fc the constant fragment and C_L, C_H, V_L and V_H the constant and the variable regions of respectively chain. [12]

The antibody molecule, also called immunoglobulins (Ig), are further divided into five subclasses IgG, IgA, IgM, IgE and IgD (see Figure 3) Out of these five, only IgG and IgM are suitable for immunochemistry. IgG, the most common structure, is featured by its Y-shape and is also the base for all the other IgGs. The IgG can be further divided into four subclasses IgG1, IgG-2a, IgG-2b and IgG3. It is robust, can be labelled without losing affinity for the antigen and can easily be produced both as a monoclonal and polyclonal antibody. IgM on the other hand, tend to be more unstable and harder to label thus making IgG a more prominent choice for assay development. [12]

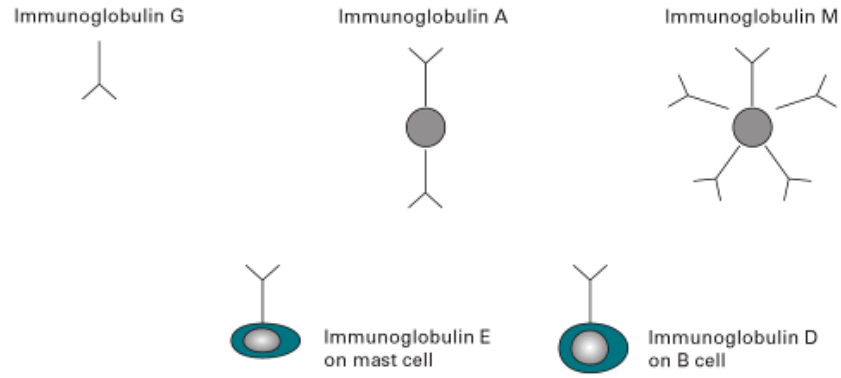

Figure 5. The five major classes of the antibody molecule. [12]

2 Materials and methods

The materials used in the project will be described under each method section. For materials not stated otherwise they were provided by Fujirebio Diagnostics AB. All programs used for enzyme immune assay is found at page 63.

2.1.1 Antibodies used in the project

Eight different antibodies against keratin 5 and two against keratin 19 was used in the project. Two of the K5 antibodies, 1A12 and AE14, used were received in two batches which were both used in the project. The information of each of them are listed in Table 1. Note that specificity refers to binding specific to one and only one protein. All antibodies were commercially available. [1] [18] [19] [20]

Table 1. General information about the antibodies used in the project. ID refers to supplier's ID-number.

Antibody	Isotype	ID	Specificity/other comment	Concentration (mg/ml)	Buffer	Supplier
RCK103	IgG1	Sc-32721	Reacts with several keratins, K5 among others	0.2	PBS buffer with <0.1% sodium azide and 0.1 % gelatin.	Santa Cruz Biotechnolgy
AE14 (batch 1)	IgG2b	sc-80606	K5. [21]	0.2	PBS buffer with <0.1% sodium azide and 0.1 % gelatin.	Santa Cruz Biotechnolgy
3E2F1	IgG1	Sc-81702	Raised against a recombinant protein correspond. to amino acids 316-491	0.3	PBS buffer with <0.1% sodium azide and 0.1 % gelatin.	Santa Cruz Biotechnolgy
1E1	IgG1	NBP1-47432	Immungoen: recombinant fragment of K5 expressed in E.cloi	Unpurified	Ascites, 0.03 % Sodium Azide	Novus Biologicals
1A12 (batch 1)	IgG2a	H00003852-M08	Immunogen: full length recombinant K5 protein	0.6	PBS buffer pH 7.4	Novus Biologicals
PAb K5	Protein A purified	H00003852-D0IP	Immunogen: full length recombinant K5 protein	0.897	PBS buffer pH 7.4	Novus Biologicals
1A12 (batch 2)	IgG2a	H00003852-M08	Immunogen: full length recombinant K5 protein	0.659	PBS buffer pH 7.4	Novus Biologicals
G-2	IgG2a	Sc-377431	Specific for the epitope between amino acids 13-47 on K5.	0.2	PBS buffer with <0.1% sodium azide and 0.1 % gelatin.	Santa Cruz Biotechnology
XM26	IgG1	Sc-58732	Raised against a recombinant protein correspond. to 103 amino acids of the C-terminal region of K5	Unknown	250 µl of culture supernatant	Santa Cruz Biotechnology
AE14 (batch 2)	IgG2b	Special ordered	K5	1.85 mg/ml	PBS buffer pH 7.4	Santa Cruz Biotechnology
Ks8.7	IgG1	61038	K8	0.05	0.09 % sodium azide, 0.5% BSA in PBS buffer, pH 7.4	Progen
Ks19.1	IgG2a		K19	2.5	NaCl	Fujirebio Diagnostigs AB
BM19.21	IgG		K19	0.042	HRP-labelled	Fujirebio Diagnostigs AB

2.1.2 Recombinant proteins

Table 2 contains information about the recombinant proteins used in the project.

Table 2. Information of the recombinant proteins used in the project. [1] [18]

Recombinant protein	Length	Concentration (mg/ml)	Buffer	Supplier
K5	Full length	0.29	100 mM NaH_2PO_4, 10mM Tris-HCl, 8 M Urea, 1000 mM imidazole.	AH Diagnostics
K8	Full length	1	30 mM Tris-HCl, pH 8, 9.5 M Urea, 2 mM DTT, 2 mM EDTA, 10 mM methylam-monium chloride	Progen
K19	Full length	1	30 mM Tris-HCl, pH 8, 9.5 M Urea, 2 mM DTT, 2 mM EDTA, 10 mM methylam-monium chloride	Progen

2.2 Multiple Sequence Alignment

Multiple sequence alignment (MSA) is a method for arranging three or more sequences in order to identify homologous regions among the sequences. The purpose of the alignment is to obtain and compare information about structure, function or evolutionary distance between the sequences. [22]

2.2.1 ClustalW2

The ClustalW2 is a MSA using successive pairwise alignment, which mean it will first pairwise align the two first sequences. This alignment will be fixed and then the next sequence with the already paired sequences. [22][23] The EMBL-EBI multiple sequence alignment tool ClustalW2 was used to obtain a sequence alignment of K5, K7 and K8. [23] Alignment was performed with K5 K7 and K8 (sequences page 56-57).

2.3 Purification of RCK103, AE14 (batch1), 3E2F1, 1E1, G-2 and XM26 with Protein G affinity chromatography

Protein G affinity chromatography was carried out in the same way for RCK103, AE14 (batch1), 3E2F1, 1E1, G-2 and XM26:

- The antibody was 1:4 diluted with PBSx1, 0.05% NaOH buffer, pH 7.3.
- The protein G purification on Hitrap 1 mL column was equilibrated with 5 column volumes PBSx1, 0.05% NaOH buffer, pH 7.3 (binding buffer).
- Sample with antibody applied to the column
- Column was washed with 5 column volumes PBS buffer.
- To elute the protein 5 column volumes 0.1M Citrat – NaOH buffer pH 2.53 was applied onto column. Fractions of 0.5 mL were collected when application started. 1.5 µl 5M NaOH was added to all fractions.
- Absorbance was measured on the fractions by Nanodrop2000. 0.1M Citrat – NaOH buffer pH 2.53 was used as reference.

- Fractions containing the antibody was selected for concentration with Amicon Ultracel 4 ml. The tube was centrifuged 10 minutes 4000 rpm. The absorbance was measured on Nanodrop2000C with 0.1M Citrat – NaOH buffer pH 2.53 as reference.
- Column was washed with 3 column volumes 0.1M Citrat – NaOH buffer pH 2.01 and elute was collected in fractions of 0.5 mL. Absorbance was measured with Nanodrop2000 in the fractions. No absorbance was observed in any sample and one could consider the antibody to have been eluted in earlier fractions. After re-equilibration with binding buffer, 4 mL of 20%Ethanol (v/v) was applied onto the column for storage.

2.4 Conjugation of antibodies

Biotinylation and HRP-conjugation was performed on the antibodies as described below. All keratin 5 antibodies were conjugated with biotin and antibodies against keratin 19 was conjugated with horseradish peroxidase (HRP).

2.4.1 Biotinylation of antibodies RCK103, AE14 3E2F1, 1E1, 1A12, PAbK5, G-2 and XM26

Biotinylation was carried out for antibodies RCK103, AE14 (two different batches), 3E2F1, 1A12 (two different batches), 1E1, PAb –K5, XM26 and G-2 during five different occasions. Materials, preparations of buffers and reaction proportions are found in Supplementary page 58-59. For each occasion, different amount of BNHS was weighed in. The reaction proportion was different for each of the total ten biotinylation reactions, see page 59-60. Concentration of antibodies was determined via absorbance measurement by Nanodrop 2000c. To all antibody solutions was carbonate buffer, pH 8.5, added to 1/10 of the final volume. The Biotin-N-hydroxysuccinimide ester (BHNS) in DMSO was prepared. For conjugation were concentrations of antibodies used as described in Table 42 and a 10 times molar redundancy of the BHNS for respectively antibody concentration. The reactants were mixed by vortex according to the proportions inTable 42, page 60. The reaction was incubated at room temperature for 2-2.5 hour. In order to exchange buffer of the biotinylated antibody a NAP-5 column (GE Healthcare) was used. The operation was carried out according to Operation protocol NAP-5 (page 58). 50 mM Tris-HCl was used as buffer. Concentration was measured on the Nanodrop 2000c with 50 mM Tris-HCl as reference. For stability, BSA was added to a final concentration of 5 mg/ml. The biotinylated antibodies were hereafter stored at +4 °C.

2.4.2 Horseradish peroxidase (HRP) conjugation of Ks19.1

Materials, preparations of buffers and reaction proportions are found at page 60-61. HRP was weighed out and dissolved in H_2O to a final concentration of 10 mg/mL. The HRP solution was mixed with 0.05M acetate buffer pH4.0 to the final concentration of 0.0083 M acetate. 0.2 M Sodium periodate ($NaIO_4$) solution was prepared right before use by dissolving 71 mg of $NaIO_4$ in 1.65 mL H_2O. The HRP solution was mixed with the $NaIO_4$ solution to a final concentration of 0.028 M $NaIO_4$. This was performed by mixing 1 part of $NaIO_4$ with 6 parts of HRP solution. The mixture was incubated 15 minutes in dark at room temperature.
The MAb solution was adjusted to 0.1 M carbonate with the 1 M carbonate buffer pH 9.2By mistake, 0.222 mL 0.1 M carbonate buffer was added instead of 1 M carbonate buffer. To

correct this 0.222 mL 1 M carbonate buffer was added. The new MAb concentration was calculated. The HRP solution was applied to a foil covered, equilibrated NAP column according to Operation protocol NAP-5 (page 58). The HRP solution was eluted by 0.1 M carbonate buffer pH 9.2 according to the protocol of the column. The volume elute was estimated to 1.2 mL, giving the solution a concentration of 4 mg/ml HRP. The MAb solution was mixed with the HRP solution in a ratio of 2:1 (weight:weight). The reaction was incubated 2 hours at room temperature in dark. 0.1 M $NaBH_4$ was prepared and was added to the MAb-HRP solution to a final concentration of 2.5 mM NaBH4.The solution was incubated in room temperature the dark for 1 hour.

140 µL 3 M HAc was added drop wise until pH 5 – 6 was obtained. The pH was measured with Litmus paper. The sample was applied onto the equilibrated Sephacryl S200-HR column and gel filtered with ÄKTA purifier. The MAb was eluted with 0.15 M NaCl. Fractions was collected in collection tubes and absorbance was monitored at 280 nm and 403 nm. Following run parameters were used:

- Fraction size: 0.7 mL
- Flow rate: 5 cm/h.
- Number of fractions: 60
- Run time: 16 hours

The peak starts in fraction 10 and ends approximately in fraction 20. The highest absorbance was measured in the fraction around 40 mL. Fractions 12-18 was selected for pooling. The earlier eluting fractions were avoided since they may contain high molecular weight aggregates and the latter fractions could possibly dilute the sample due to its low concentrations and due to presence of unlabeled antibody. The concentration of the HRP-antibody pool was determined by the Nanodrop 2000C (see results). From the concentration was total amount (mg) and yield (antibody weight) calculated.50 µl BSA was added from a stock solution of 100 mg/ml to a final concentration of 1 mg BSA/ml HRP-conjugated Ks19.1. The HRP conjugated MAb was diluted 1:1 with Surmodics stabilizer by adding 4.95 mL Surmodics Stabilizer. The HRP conjugated MAb was filtered through a 0.22 µm filter. The HRP conjugated antibody was stored in +4 °C.

2.5 Keratin extraction from CaSki cell line

Keratin from Caski was extracted to use as antigen and possible calibrator for the enzyme immunoassay. All materials used and solution preparations used in the experiment are presented in Supplementary page 61-63.

2.5.1 Extraction

2.5.1.1 Extraction of cytoskeleton

Three tubes with cell pellets were thawed at room temperature. 8 mL cytoskeleton buffer with Triton X-100 was added to each of the pellets. The pellets were incubated in the buffer for 30 minutes. As soon as the cells had detached from the tube walls, the solution was transferred to preweighed tubes (Tube 1: 6.7130 g, Tube 2: 6.6658 g, Tube 3: 6.7186 g). The tubes were put on ice bath.

The tubes were shaken 2 times and vortexed 3 seconds on speed 6.5, so the content spun up. Tubes were centrifuged 10 minutes, 4000 rpm, +4 °C. DNAse was thawed on ice. 400 µl DNAse was mixed in 31.6 mL of cold cytoskeleton buffer. The supernatant was discarded. 8 ml DNAse in cytoskeleton buffer was added to each tube and was incubated for 20 minutes, counted from adding to the first tube. The tubes were vortexed 3 seconds on speed 6.5 with even intervals (3 times in total) during the incubation. Tubes were centrifuged 10 minutes, 4000 rpm, +4 °C. Supernatant was discarded and each pellet was dissolved in 4.5 mL cytoskeleton buffer/tube. The three tubes were pooled in tube 1. The tube was centrifuged 10 minutes, 4000 rpm, +4 °C. Supernatant was discarded and the last supernatant was removed with a Pasteur pipette. The tube was kept on ice. The tube was weighed and the pellet weight was calculated to 0.1742 g. The cytoskeleton was dissolved in 1.5 mL cold EDTA in phosphate buffer. The tube was kept at -20 °C over night.

2.5.1.2 Extraction of intermediate filaments

The tube with cytoskeleton was thawed on ice. The cytoskeleton was centrifuged 10 min, 2000 rpm, 4 °C. The supernatant was discarded. Extraction of cytoskeleton was performed by adding 0.5 mL 9.5 M Urea in EDTA/phosphate buffer, followed by vortex and 12 minutes incubation. The tube was vortexed and additional 0.5 mL 9.5 M Urea in EDTA/phosphate buffer was added, followed by vortex and 18 minutes incubation. The tube was centrifuged 10 min, 2000 rpm, 4 °C. Supernatant was transferred to a fresh tube. Concentration was measured by Nanondrop 2000c. 9.5 M Urea in EDTA/phosphate buffer was used as reference.

To precipitate the intermediate filaments was 7 mL of 95% ethanol added to the 4.2 mg of intermediate filaments. The tube was centrifuged 10 min, 2000 rpm, 4 °C. Supernatant was carefully discarded. The tube containing the pellet was gently washed with purified water. The water was removed with a Pasteur pipette. The pellet was dried by letting the tube stand in room temperature overnight loosely covered with aluminum foil.

2.5.2 SDS-treatment of extracted keratin

2.5.2.1 SDS-treatment of extracted keratin step 1

0.5 ml 2 % SDS with 10 mM EDTA in 50 mM Na2HPO4, pH 7.8 was added to the dried keratin pellet. The pellet was dissolved by vortexing. The solution was transferred to a clean tube. The tube which contained the pellet was cleaned with 0.5 ml SDS buffer and this solution was transferred to the clean tube. The sample was heated 10 minutes at 90 °C. The tube was centrifuged 10 min, 2000 rpm. Supernatant was carefully transferred to a clean tube. The 1 ml, 4 mg/mL extracted protein was stored at room temperature. 10 µl was saved for DNA-content test on agarose gel electrophoresis.

2.5.2.2 SDS-treatment of extracted keratin step 2

The 1 ml keratin from SDS-treatment of extracted keratin step 1 was diluted 1:10 with 1 % PBS-BSA Tween20. The sample was incubated at 50 °C during 4 hours. The SDS-treated keratin, 400 µg/mL was hereafter stored in +4°C.

2.6 Evaluation of antigen from CaSki

To evaluate the quality of the extracted keratin, a DNA content test and a test immunoassay with the antigen coated onto a microtiter plate was performed.

2.6.1 DNA-content test of SDS-treated antigen with agarose gel electrophoresis

To ensure the keratin solution did not contain high concentration of DNA and that the DNAse treatment was successful, the keratin solution was run on agarose gel electrophoresis.

Material: SDS-treated keratin from SDS-treatment of extracted keratin step 1, approximately 4 mg/ml
Agarose
TAE buffer
5X DNA Loading dye, Bio-Rad
Low DNA mass ladder (diluted 1:4 with loading dye Table 3), Bio-rad

Keratin: 2 µl keratin was mixed with 1 µl loading dye. 45 g of agarose was dissolved in 45 mL TAE buffer. The solution was heated to 50 °C and poured into a gel chamber with combs. The combs were removed when the gel had solidified. The gel was covered with TAE buffer. 2 µl ladder and 2 µl keratin sample was applied to well 1 and 2 respectively. This gives an approximately amount of 5.5 µg keratin added to the gel. For amount of added ladder, see the table below. The gel was run for 20 min with starting values 85 mV and 45 mA.

Table 3. Low DNA Mass Ladder.

Fragment size (base pairs)	2 µl ladder
2000	100 ng
1200	60 ng
800	40 ng
400	20 ng
200	10 ng
100	5 ng

2.6.2 Coating of antigen onto Maxisorp plate

The purpose was to evaluate the antigen function before usage in K5/19 test development. This quality control was only performed to minimize the number of source of errors if the assay would not work.

Material: SDS-treated keratin from SDS-treatment of extracted keratin step 1, approximately 4 mg/ml
Sodium bicarbonate ($NaHCO_3$)
Sodium hydroxide (NaOH) 5M
Purified water
Maxisorp microtiter plate
Self-adhesive plastic film
1 % PBS-BSA 0.1% Tween 20 buffer
Wash buffer (according to page 65)

2.6.2.1 Preparation of solutions

2.6.2.1.1 $NaHCO_3$ buffer 0.1 M, pH 8.5

0.84 g $NaHCO_3$ was dissolved in 100 mL H_2O. pH was adjusted to 9.2 with 6M NaOH. The buffer was filtered through a 0.22 µm filter.
SDS-treated keratin was diluted in carbonate buffer to 4 µg/ml. 100 µl of keratin solution was added to each well in total 7 strips of microtiter plate. The plate was covered with self-adhesive plastic film and incubated in +4°C over night.

The plate was washed 3 times in strip mode with wash buffer. 100 µl 1 % PBS-BSA 0.1% Tween 20 was added to each well to prevent non-specific binding. The plate was incubated 1 hour at room temperature. The plate was washed 3 times in strip mode with wash buffer. 100 µl 1 % PBS-BSA 0.1% Tween 20 was added to each well. The plate was covered with self-adhesive plastic film and stored at +4°C until usage.

2.6.3 Evaluation of antigen coated Maxisorp plate with two-step enzyme immunoassay

Material: Maxisorp plate with coated antigen
Biotinylated antibody 1E1, 233 µg/mL
Biotin antibody from CYFRA-kit (CB) 1.25 µg/mL
HRP-conjugated Polyclonal Rabbit Antimouse 1.3 g/L
TMB, Fujirebio Diagnostics AB
1 % PBS-BSA 0.1% Tween 20 buffer
Wash buffer (according to page 65)

Biotinylated antibody 1E1 was diluted in 1 % PBS-BSA 0.1% Tween 20 buffer to the final concentrations 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL and 0.0625 µg/mL.
Biotinylated antibody CB was diluted in 1 % PBS-BSA 0.1% Tween 20 buffer to the final concentrations 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL and 0.0625 µg/mL.
HRP-conjugated antibody was diluted to 2 µg/mL.

The plate was washed 3 times with wash buffer in strip mode. 100 µl biotinylated antibody/buffer was added according to Table 4. The plate was incubated 60 minutes on shaker. The plate was washed 3 times with wash buffer in strip mode. 100 µl HRP-conjugated antibody was added to each well. The plate was incubated 60 minutes on shaker. The plate was washed 6 times with wash buffer in strip mode. 100 µl TMB was added to each well and was incubated 30 minutes with immediate absorbance measurement at 620 nm as followed.

Table 4. Addition of primary antibody solutions. A-H denotes the wells on each strip. Buffer = 1 % PBS-BSA 0.1% Tween 20 buffer, was added as negative control.

	Strip 1	Strip 2	Strip 3	Strip 4
A	1E1 2 µg/mL	1E1 0.125 µg/mL	CB 0.25 µg/mL	Buffer
B	1E1 2 µg/mL	1E1 0.125 µg/mL	CB 0.25 µg/mL	Buffer
C	1E1 1 µg/mL	1E1 0.0625 µg/mL	CB 0.125 µg/mL	Buffer
D	1E1 1 µg/mL	1E1 0.0625 µg/mL	CB 0.125 µg/mL	Buffer
E	1E1 0.5 µg/mL	CB 1 µg/mL	CB 0.0625 µg/mL	Buffer
F	1E1 0.5 µg/mL	CB 1 µg/mL	CB 0.0625 µg/mL	Buffer
G	1E1 0.25 µg/mL	CB 0.5 µg/mL	CB 0.0625 µg/mL	Buffer
H	1E1 0.25 µg/mL	CB 0.5 µg/mL	CB 0.0625 µg/mL	Buffer

2.7 K5/19 Calibrators

The keratin from CaSki was used for making calibrators to the test. The keratin was diluted in Calibrator A (CalA) from CYFRA21-1 (page 65) to the concentrations of keratin seen inTable 5. The Concentration of K19 was determined in CYFRA21-1 (protocol page 65). These calibrators will hereafter be referred to as Calibrators K5/19.

Table 5. Concentration of Calibrators.

Name	Concentration keratin (µg/mL)	Concentration K19 (ng/mL)
C1	0	0
C2	0.1	0.474
C3	0.5	2.236
C4	0.75	3.491
C5	1	4.908
C6	1.5	7.717
C7	2	9.877
C8	4	21.085
C9	6	32.056

2.8 Evaluation of biotinylated antibodies

The biotinylated antibodies were evaluated to control if biotinylation was successful and to determine optimal antibody concentration for test.

2.8.1 RCK103, AE14 (batch 1), 3E2F1, 1E1, 1A12 (batch1), PAbK5

Materials: Biotinylated antibodies RCK103, AE14 (batch 1), 3E2F1, 1E1, PAb K5 and 1A12 (batch 1)
HRP-conjugated Polyclonal Swine Anti-Rabbit
HRP-conjugated Polyclonal Rabbit Anti-Mouse
1% PBS-BSA, 0.1% Tween20 buffer
TMB
Microtiter plate with streptavidin
Wash buffer (according to page 65)

The biotinylated antibodies were diluted to 2µg/mL, 1 µg/mL, 0.5 µg/mL and 0.25 µg/mL.The microtiter plate was washed 1 time in strip mode with wash buffer. 100 µl biotinylated antibody and 1% PBS-BSA, 0.1% Tween20 buffer was added according to Table 6. The plate was incubated 60 minutes on shaker in room temperature. The plate was washed 3 times with wash buffer in strip mode.

Table 6. Addition of biotinylated antibodies. A-H denotes the wells on each strip. 2, 1, 0.5 and 0.25 refers to antibody concentration in µg/mL. Buffer is 1% PBS-BSA, 0.1% Tween20 buffer. Well G7 and H7 was left empty.

	Strip 1 RCK103 **(µg/mL)**	Strip 2 AE14 **(µg/mL)**	Strip 3 3E2F1 **(µg/mL)**	Strip 4 1E1 **(µg/mL)**	Strip 5 1A12 **(µg/mL)**	Strip 6 PAbK5 **(µg/mL)**	Strip 7 Buffer
A	2	2	2	2	2	2	Buffer
B	2	2	2	2	2	2	Buffer
C	1	1	1	1	1	1	Buffer
D	1	1	1	1	1	1	Buffer
E	0.5	0.5	0.5	0.5	0.5	0.5	Buffer
F	0.5	0.5	0.5	0.5	0.5	0.5	Buffer
G	0.25	0.25	0.25	0.25	0.25	0.25	
H	0.25	0.25	0.25	0.25	0.25	0.25	

100 µl HRP-conjugated antibody was added to each well (Table 7). The plate was incubated for 45 minutes in room temperature on shaker. The pate was washed 6 times with wash buffer in strip mode. 100 µl TMB solution was added to each well and incubated for 30 minutes in room temperature on shaker.

Table 7. Addition of HRP-conjugated antibodies. A-H denotes the wells on each strip. Well G7 and H7 was left empty.

	Strip 1 (RCK103)	Strip 2 (AE14)	Strip 3 (3E2F1)	Strip 4 (1E1)	Strip 5 (1A12)	Strip 6 (pAb K5)	Strip 7 Buffer
A	Anti-mouse	Anti-mouse	Anti-mouse	Anti-mouse	Anti-mouse	Anti-Rabbit	Anti-mouse
B							
C							
D							
E							Anti-Rabbit
F							
G							
H							

2.8.2 1A12 (bach 2)

Materials: Biotinylated 1A12 (batch 2)
HRP-conjugated Ks19.1
Calibrators K5/19
TMB
Microtiter plate with streptavidin
Stop solution: 0.12 M hydrochloric acid.
Biotin dilution buffer (page 66)
Wash buffer (according to page 65)

Antibodies were diluted with biotin dilution buffer to following concentrations:
1A12: 2 µg/mL, 0.5 µg/mL, 0.125 µg/mL.
Ks19.1: 1 µg/mL
The assay was run according to Program C. Sample and biotinylated antibodies were added according to Table 8 and Table 9 respectively.

Table 8. Addition of calibrators (samples) to microtiter plate. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3
A	C1	C1	C1
B	C1	C1	C1
C	C3	C3	C3
D	C3	C3	C3
E	C5	C5	C5
F	C5	C5	C5
G	C7	C7	C7
H	C7	C7	C7

Table 9. Addition of biotinylated antibody solutions. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3
A-H	2 µg/mL	0.5 µg/mL	µg/mL

2.8.3 G-2, XM26 and 1A12 (batch 2)

Materials: Biotinylated antibodies G-2, XM26, 1A12 (batch 2)
HRP-conjugated Ks19.1
Calibrators K5/19
Microtiter plate with streptavidin
TMB
Stop Solution: 0.12 M hydrochloric acid.
Biotin dilution buffer (page 66)
Wash buffer (according to page 65

Antibodies were diluted with biotin dilution buffer to following concentrations:
G-2: 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL
XM26: 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.125 µg/mL
1A12: 0.5 µg/mL, 0.125 µg/mL
Ks19.1: 1 µg/mL

The assay was carried out according to Program C. Addition of calibrators (samples) and biotinylated antibody solutions was carried out according to Table 10 and Table 11 respectively.

Table 10. Addition of sample. A-H denotes the wells on each strip.

	Strip 1-10
A	C1
B	C1
C	C3
D	C3
E	C5
F	C5
G	C8
H	C8

Table 11. Addition of biotinylated antibody solutions. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8	Strip 9	Strip 10
A-H	G-2 1 µg/mL	G-2 0.5 µg/mL	G-2 0.25 µg/mL	G-2 0.125 µg/mL	XM26 1 µg/mL	XM26 0.5 µg/mL	XM26 0.25 µg/mL	XM26 0.125 µg/mL	1A12 0.5 µg/mL	1A12 0.125 µg/mL

2.8.4 AE14 (batch 2)

Materials: Biotinylated AE14 (batch 2)
HRP-conjugated Ks19.1
Microtiter plate with streptavidin
TMB
Biotin dilution buffer (page 66)
Wash buffer (according to page 65)
Stop solution: 0.12 M hydrochloric acid.
Calibrators K5/19

The assay was carried out according to Program C. Addition of calibrators (samples) and biotinylated antibody solutions was carried out according to Table 12 and Table 13 respectively.

Table 12. Addition of samples. A-H denotes the wells on each strip.

	Strip 1-4
A	C1
B	C1
C	C3
D	C3
E	C5
F	C5
G	C8
H	C8

Table 13. Addition of biotinylated antibodies. A-H denotes the wells on each strip.

	Strip1	Strip 2	Strip 3	Strip 4
A- H	1.0 µg/ml	0.5 µg/ml	0.25 µg/ml	0.125

2.9 Evaluation of antibody combinations and assay format

2.9.1 Test of Ks19.1 with anti K5 against serum and pleura samples

Materials: Biotinylated antibodies RCK103, AE14 (batch 1), 3E2F1, 1E1, PAb K5 and 1A12 (batch 1)
HRP-conjugated Ks19.1
Biotin dilution buffer (page 666)
CalA and CalF CYFRA21-1 (page 65)
Calibrator C5, K5/19
Pleura sample ID: TB43
Microtiter plate with streptavidin
TMB
Serum samples from lung cancer patients according to Table 14.
Serum sample from healthy blood donor M2010-184
Wash buffer (according to page 65)

Serum samples was chosen from patients with non-small cellular lung cancer according to recorded CYFRA21-1 values. Due to low volumes the samples were pooled according to the table below. As negative control (background noise sample) serum from a healthy blood donor was chosen. The pleura sample from lung cancer patient was chosen based on its high value in CYFRA21-1 (not shown).

Table 14. Lung cancer pools used in the experiment.

Pool 1		
Sample ID (FDAB)	**Diagnosis**	**CYFRA21-1 value**
71	Non small lung cancer	16.3
81		22.6
83		21.6
Pool 2		
Sample ID (FDAB)	**Diagnosis**	**CYFRA value**
63	Non small lung cancer	3.1
63		3.1
78		3.4
79		3.1
75		2.0

The antibodies were mixed with biotin dilution buffer to the following antibody solutions:
RCK103+Ks19.1: 0.5 µg/mL RCK103, 1 µg/mL Ks19.1
AE14+Ks19.1: 0.125 µg/mL AE14, 1 µg/mL Ks19.1
3E2F1+Ks19.1: 0.125 µg/mL 3E2F1, 1 µg/mL Ks19.1
1E1+Ks19.1: 0.5 µg/mL 1E1, 1 µg/mL Ks19.1
1A12+Ks19.1: 0.5 µg/mL 1A12, 1 µg/mL Ks19.1
PAbK5+Ks19.1: 0.5 µg/mL PAbK5, 1 µg/mL Ks19.1

Pleura sample were diluted in three different concentrations (1:1=P43.1, 1:5=P34.2, 1:10, P43.3) with CalA from CYFRA21-1. The assay was run according to Program A. Addition of samples was carried out according to Table 15. Antibody solutions were added according to Table 16.

Table 15. Scheme for addition of samples. A-H denotes the wells on each strip. 1 and 2 refers to pool 1 and 2, 184 the healthy blood donor, C5 the calibrator, P43.1-P43.3 the pleura samples and CalF is the calibrator F from CYFRA21-1.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8
A	1	1	1	1	1	P43.1	1	P43.1
B	1	1	1	1	1	P43.1	1	P43.1
C	2	2	2	2	2	P43.2	2	P43.2
D	2	2	2	2	2	P43.2	2	P43.2
E	184	184	184	184	184	P43.3	184	P43.3
F	184	184	184	184	184	P43.3	184	P43.3
G	C5	C5	C5	C5	C5	CalF	C5	CalF
H	C5	C5	C5	C5	C5	CalF	C5	CalF

Table 16. Addition of antibody solutions.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8
A-H	RCK103	AE14	3E2F1	PAbK5	1E1	1E1	1A12	1A12

2.9.2 Test of BM19.21 with anti K5 against serum and pleura samples

Materials:
Biotinylated antibodies RCK103, AE14 (batch 1), 3E2F1, 1E1, PAb K5 and 1A12 (batch 1)
HRP-conjugated BM19.21
Pleura sample, ID: TB43
Biotin dilution buffer (page 66)
CalA and CalF CYFRA21-1, (page 65)
Calibrator 5, K5/19
Microtiter plate with streptavidin
TMB
Serum samples from lung cancer patients according to Table 17
Serum sample from healthy blood donor
Wash buffer (according to page 65)

Serum samples was chosen from patients with non-small cellular lung cancer according to recorded CYFRA21-1 values. Due to low volumes the samples were pooled according to the table below. As negative control (background noise sample) serum from a healthy blood donor was chosen. The pleura sample from lung cancer patient was chosen based on its high value in CYFRA21-1 (not shown).

Table 17. Lung cancer pools used in the experiment.

Pool 3		
Sample ID (FDAB)	**Diagnosis**	**CYFRA21-1 value**
96	Non small lung cancer	10.5
99		10.1
85		8
Pool 4		
Sample ID (FDAB	**Diagnosis**	**CYFRA21-1 value**
67	Non small lung cancer	5.5
68		5.9
92		6.5
72		4.2

Pleura sample were diluted in three different concentrations (1:1=P43.1, 1:5=P34.2, 1:10, P43.3) with CalA from CYFRA. The antibodies were mixed with biotin dilution buffer to the following antibody solutions:

RCK103+BM19.21: 0.5 µg/mL RCK103, 1 µg/mL BM19.21
AE14+ BM19.21: 0.125 µg/mL AE14, 1 µg/mL BM19.21
3E2F1+ BM19.21: 0.125 µg/mL 3E2F1, 1 µg/mL BM19.21
1E1+ BM19.21: 0.5 µg/mL 1E1, 1 µg/mL BM19.21
1A12+ BM19.21: 0.5 µg/mL 1A12, 1 µg/mL BM19.21
PAbK5+ BM19.21: 0.5 µg/mL PAbK5, 1 µg/mL BM19.21

The assay was run according to Program A. Addition of samples was carried out according to Table 18. Antibody solutions were added according to Table 19.

Table 18. Sample addition to microtiter plate. A-H denotes the wells on each strip. 1-4 refers to pool 1-4, 186 the healthy blood donor, C5 the calibrator, P43.1-P43.3 the pleura samples and CalA and CalF are the calibrators from CYFRA21-1.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8	Strip 9
A	3	3	3	3	3	P43.3	CalF	C+	1
B	3	3	3	3	3	P43.3	CalF	C+	1
C	4	4	4	4	4	P43.2	3	P43.3	2
D	4	4	4	4	4	P43.2	3	P43.3	2
E	186	186	186	186	186	P43.1	4	P43.2	CalA
F	186	186	186	186	186	P43.1	4	P43.2	CalA
G	C5	C5	C5	C5	C5	CalA	186	P43.1	CalF
H	C5	C5	C5	C5	C5	CalA	186	P43.1	CalF

Table 19. Addition of antibody solutions is presented in the table. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8	Strip 9
A	RCK103	AE14	3E2F1	PAbK5	1E1	1E1	1E1	1A12	1A12
B									
C							1A12		
D									
E									
F									
G									
H									

2.9.3 Comparison between one-step and two-step ELISA

A comparison between the two assay formats were made to distinguish if any of them was beneficial. The incubation time for the antibody solution in the one step was increased to two hours which was compared with previous results for one-step with 1 hour incubation time (Program A and Program B respectively). The two-step assay was carried out according to Program C.

Material:
Biotinylated antibody 1A12 (batch 1)
HRP-conjugated antibody Ks19.1
Cal A and CalF, CYFRA21-1 (page 65)
TMB
Calibrator C5 (1.0 µg/mL)
Microtiter plate with streptavidin
Biotin dilution buffer (page 66)
Pleura samples TB43 diluted 1:10 (P43.3)
Serum pool 2, 3 and 4 (Table 144 and Table 17)

Serum from lung cancer patients ID: 1145-04 (LC1), 1146-04 (LC2)
Serum from healthy blood donor ID: M2010-186
Stop Solution: 0.12 M hydrochloric acid.
Wash buffer (according to page 65)

The antibodies were diluted with biotin dilution buffer to the following antibody solutions:
One-step: 1A12+Ks19.1: 1 µg/ml 1A12, 1 µg/ml Ks19.1

Two-step: 1A12. 1 µg/ml 1A12
Ks19.1: 1 µg/ml Ks19.1

The two step assay was run according to Program C and the one-step assay according to Program B. Table 20 displays the sample addition.

Table 20. Scheme for addition of samples for the comparison between one-step and two-step assay. A-H denotes the wells on each strip. LC1 and LC2 denotes lung cancer samples, 2, 3 and denotes the serum pools from non-small lung cancer patients, P43.3 the diluted pleura sample. C5, CalA and Calf are calibrators from K5/19 and CYFRA21.1.

	Strip 1	Strip 2
A	LC1	P43.3
B	LC2	P43.3
C	2	C5
D	3	C5
E	3	CalA
F	4	CalA
G	186	CalF
H	186	CalF

2.9.4 Endometrial cancer samples G-2, XM26, 1A12

Materials: Biotinylated antibodies G-2, XM26 and 1A12
HRP-conjugated Ks19.1
TMB
Stop solution: 0.12 M hydrochloric acid.
Microtiter plates with streptavidin
Biotin dilution buffer (page 66)
Calibrators K5/19
Serum samples from endometrial cancer patients (Table 44)
Wash buffer (according to page 65)

Antibodies were diluted with biotin dilution buffer to following concentrations:

G-2:0.5 µg/ml, XM26: 0.25 µg/ml, 1A12. 0.125 µg/ml and Ks19.1 to 1 µg/ml.

Three assays were carried out (according to Program C) in parallel with the same sample distribution on microtiter plate (

Table 21). Plate 1 was incubated with G-2 as biotinylated antibody, plate 2 with XM26 and plate 3 with 1A12.

Table 21. Addition of samples to microtiter plate. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5
A	C1	C9	En3	En11	En19
B	C1	C9	En4	En12	En20
C	C2	B181	En5	En13	En21
D	C2	B181	En6	En14	En22
E	C3	B186	En7	En15	En23
F	C3	B186	En8	En16	En24
G	C5	En1	En9	En17	En25
H	C5	En2	En10	En18	En26

2.10 Western Blot

In order to determine the specificity of G-2, 1A12 and AE14 for keratin 5 was a western blot performed. The antibodies were tested against keratin 5, keratin 8 and keratin 19. As positive controls for keratin 19 and 8 controls were Ks19.1 and Ks8.7 used respectively.

Day 1

Blocking solution was prepared by dissolving 20 g of dried milk in 400 mL of wash buffer. The antigens to be used in the western blot were diluted according to Table 22. + marks the higher concentration and – the lower concentration. Not that K5 only needed to be diluted for the lower concentration.

Table 22. Dilution of antigens. The "d" in front of the antigen denotes dilution.

dK19+	dK19-	dK8+	dK8-	dK5-
10 µl K19 1mg/ml	2.5 µl K19 1mg/ml	10 µl K19 1mg/ml	2.5 µl K19 1mg/ml	8.6 µl K19 1mg/ml
15 µl 6M Urea	22.5 µl 6M Urea	15 µl 6M Urea	22.5 µl 6M Urea	16.4 µl 6M Urea

Samples were prepared according to the proportions in Table 23. The amount refers to the amount of the prepared sample aimed to be loaded on the gel.

Table 23. Sample preparation. The amount of the antigen refers to the amount of the sample aimed to be loaded on the gel.

K19+, 200 ng	K19-, 50 ng	K8+, 200 ng	K8-, 50 ng	K5+, 200 ng	K5-, 50 ng
5 µl DTT	5 µl DTT	5 µl DTT	5 µl DTT	2 µl DTT	5 µl DTT
25 µl Laemmli sample buffer	25 µl Laemmli sample buffer	25 µl Laemmli sample buffer	25 µl Laemmli sample buffer	10 µl Laemmli sample buffer	25 µl Laemmli sample buffer
25 µl dK19+	25 µl dK19-	25 µl dK8+	25 µl dK8-	10 µl K5 0.29 mg/ml	25 µl dK5-

45 µl H_2O	45 µl H_2O	45 µl H_2O	45 µl H_2O	18 µl H_2O	45 µl H_2O

Due to miscalculation were the above samples not appropriate to use and the samples were therefore diluted 1:5 according to:

20 µl sample
20 µl Laemmli sample buffer
60 µl H_2O

The samples were denatured for 10 minutes in 70°C. The amount of antigen added to gel was calculated based on adding 10 µl of sample in each well. The new diluted samples are referred to as sample K5-, K5+, K8-, K8+, K19- and K19+ from now on.

The chambers were cleaned. The combs were removed from the gels and the total three gels were put into the chambers according to manufacturer's instructions. Buffer was poured into the chambers by first filling the inner chamber and then the outer. The wells were cleaned with running buffer.
Samples were loaded according to Table 24. Well 8 was left empty. L marks the ladder. 3 µl of ladder was applied and 10 µl of the other samples to respectively well. Gels were ran for 15 minutes with starting values 252mA, 360 mV.

Table 24. Sample loading on gel. Well 8 was left empty.

Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Sample	K19+	K19-	K8+	K8-	K5+	K5-	L		K19+	K19-	K8+	K8-	K5+	K5-	L

A The Trans-Blot® Turbo™ bottom pad was put in the Trans-Blot® Turbo™ box with the membrane side up. The gel was gently removed from the. The gel was put on top on the nitrocellulose membrane and the top pad was put on top of the gel. Air bubble was removed by a roller. This was carried out in the same way for all three gels. The Trans-Blot® Turbo™ boxes were put into the Trans-Blot® Turbo™ Transfer System and 2.5 A and 25 V was applied for 3 minutes. The membranes were taken out and cut in half along lane 8 resulting in membranes with the following samples:

K19+ K19- K8+ K8- K5+ K5- L

Each membrane was transferred to a plastic trays with the protein side up. The membrane was covered in blocking solution. Membrane 1-4 was blocked overnight and Membrane 3-6 was blocked over two nights.

Day 2

Antibody AE14, 1A12, G-2 and Ks19.1 was diluted to 1 µg/ml (total 10 ml) in blocking solution. The antibodies were incubated 45 minutes in room temperature. Blocking solution was discarded. Each membrane was moved to a petri dish and the antibody solutions were poured onto the membranes according to:

Membrane 1	AE14
Membrane 2	1A12
Membrane 3	G-2
Membrane 4	Ks19.1

The membranes were incubated for 1.5 hours on shaker in room temperature. The antibody solutions were discarded and the membrane was rinsed two timed with wash buffer. The membranes were moved back to their plastic tray and covered with wash buffer. The membranes were washed 15 minutes on shaker in room temperature. The washing was repeated two times with rinsing the membrane two times in between each wash.

During the washing procedure four tubes with 10 ml of 0.02 µg/ml Polyclonal Rabbit Anti-mouse HRP-labelled antibody (secondary antibody) and 1 µl Precision Protein™ Strep-Tactin-HRP conjugate in blocking solution were prepared. The antibody was incubated in dark for 45 minutes. The wash buffer was discarded and the membranes were moved to clean petri dishes. Each membrane was covered with the secondary antibody solution. The membranes were incubated for 1.5 hours on shaker in room temperature in dark.

The antibody solutions were discarded and the membrane was rinsed two timed with wash buffer. The membranes were moved back to their plastic tray and covered with wash buffer. The membrane was washed 15 minutes on shaker in room temperature. The washing was repeated two times with rinsing the membrane two times in between each wash.

Detection solution was prepared according to Bio-Rad instructions. Each membrane was completely covered with the solution but only so much that could be held by surface tension. The membranes were left for incubation in 5 minutes. Excess solution was removed by letting the edge of the membrane touch a piece of tissue. The membrane was move to the ChemiDOC™ Touch Imaging System. The Chemiluminscence application was used to expose the membranes during 300, 100 and 30 seconds. The best pictures for each membrane was selected and is shown in Figure 13.

Day 3

Antibody AE14 and Ks8.7 was diluted to 2 respectively 1 µg/ml (total 10 ml) in blocking solution. The antibodies were incubated 45 minutes in room temperature. Blocking solution was discarded. Each membrane was moved to a petri dish and the antibody solutions were poured onto the membranes according to:

Membrane 5	AE14
Membrane 6	Ks8.7

The rest of the blotting was carried out in the manner as during day 2. The best pictures for each membrane was selected and is shown in Figure 13.

2.11 Preparation of antibody solutions for Drift test and Lung cancer Study

Antibody solutions was prepared by mixing the antibodies in biotin dilution buffer with magnetic stirrer for 1 hour in autoclaved glass bottles (Table 25).

Table 25. Concentration of reagents.

	Biotinylated 1A12 (batch 1)	HRP-conjugated Ks19.1	Biotinylated 1A12 (batch 1)
Concentration	0.125 µg/ml	1 µg/ml	0.5 µg/ml

2.12 Assay robustness to wash modes (drift over the plate test)

In enzyme immunoassays one can either wash the microtiter plate in strip mode wash or plate mode wash. With strip mode wash each all wash cycles will be completed on one strip before the washer moves on to the next strips. For example, with six wash cycles the wash manifold will start washing strip 1 six times and aspirate the strip wells completely dry before it moves on to the next strip. For plate mode wash on the other hand, the washer will wash strip 1 one time, leaving the wells with liquid, and then go on to strip two and so on until all strips have been washed one time. After washing each strip one time it starts over with strip 1 again, aspirating and dispensing, repeating the procedure until all strips have been washed six times. Strip mode was used for all enzyme immune assays except the lung cancer study. Before the lung cancer study drift test with strip mode and plate mode was performed in order to evaluate the robustness of the enzyme immunoassay.

2.12.1 Strip mode

Material: HRP-conjugated Ks19.1, 1 µg/mL.
Biotinylated 1A12 (batch2), 0.125 µg/mL
Serum from helathy blood donors, ID: M2010-165, M2010-166
(Referred to as 165 and 166 in Table 26Table 26)
TMB
Microtiter plate with streptavidin
Stop solution: 0.12 M hydrochloric acid.
Wash buffer (according to page 6565)

The assay was performed according to Program C. Scheme for sample addition can be seen in Table 27.

Table 26. Scheme for addition of serum samples on microtiter plate. Only strip 1, 6 and 10 was used for the experiment. A-H denotes the wells on each strip.

	Strip 1	Strip 6	Strip 10
A	165	166	165
B	165	166	165
C	166	165	166
D	166	165	166
E	165	166	165
F	165	166	165
G	166	165	166
H	166	165	166

2.12.2 Plate mode

Material: HRP-conjugated Ks19.1, 1 µg/mL.
Biotinylated 1A12 (batch2), 0.125 µg/mL
Serum bladder cancer patients, sample ID 96
Serum from healthy blood donor ID: M2010-178 (Referred to as 178)
Calibrators K5/19
TMB
Microtiter plate with streptavidin
Stop solution: 0.12 M hydrochloric acid.
Wash buffer (according to page 5)

The assay was performed according to Program D. Scheme for sample addition can be seen in Table 27.

Table 27. Scheme for addition of samples on microtiter plate. Only strip 1, 4, 7 and 10 were used. A-H denotes the wells on each strip.

	Strip 1	Strip 4	Strip 7	Strip10
A	C3	96	178	C8
B	C3	96	178	C8
C	C8	C3	96	178
D	C8	C3	96	178
E	178	C8	C3	96
F	178	C8	C3	96
G	96	178	C8	C3
H	96	178	C8	C3

2.13 Lung cancer study

Materials: HRP-conjugated Ks19.1, 1 µg/mL.
Biotinylated 1A12 (batch2), 0.125 µg/mL
Biotinylated 1A12 (batch2), 0.5 µg/mL
Plasma samples from healthy blood donor
Serum samples from patients with benign lung disease (page 68)
Lung cancer plasma samples (page 68-73)
Calibrators K5/19
Microtiter plate with streptavidin
TMB
Stop solution: 0.12 M hydrochloric acid.
Wash buffer (according to page 65)

During the study, no other information than healthy, benign or cancer were known about the samples. Samples were given run ID for the study, see page 688-73. Each run ID was paired with the sample information. 0.125 µg/mL of 1A12 was used for plate 1 and 2 and 0.5 µg/mL for plate 3 and 4. The assays were carried out according to Program D. Samples were added according to Table 48-Table 55. Samples were run in single replicate.

3 Results and discussion

Results and discussion of these are presented in this section.

3.1 Multiple Sequence Alignment

The multiple alignment was performed in order to determine the homology between the protein sequences of keratin 5, 7 and 8. These keratins are often expressed in complex with keratin 19 as well. [9] If the antibody dedicated for detecting keratin 5 detects keratin 7 or 8 in addition, it would result in a less specific signal. The result from multiple alignment of K5, K7 and K8 is presented in Figure 6. The sequences are presented with keratin 5 as the upper, keratin 7 in the middle and keratin 8 as the lower. As expected, the three type II keratins are highly conserved in the rod domain and variable in head and tail domains. Thus, there is a high risk of cross-reaction for antibodies with binding site on the rod domain. Amino acids marked in bold green are unique K5 regions of minimum three amino acid. Most regions are located at the head or tail domain and marks linear epitopes where antibodies specific for K5 could bind without risk for cross-reacting with keratin 7 or 8. The yellow marked region on K5 correspond to the binding site of G-2. [1] Possible sub-epitopes on K7 and K8 of the G-2 binding site are marked with yellow. Several possible sub-epitopes for G-2 are available on both keratin 7 and 8 and the high homology between the sequences implies risk for cross-reaction of other antibodies as well. Therefore, it is of great importance to test the specificity of the antibodies used in the final keratin 5/19 immunoassay.

```
1    MSRQSSVSFRSGGSRSFSTASAITPSVSRTSFTSVSRSGGGGGGGFGRVSLAGACGVGGYGSRSLYNLGGSKRISISTSG  80
1    MSIHF-------------------------------------------------------------------SSPVFTSR  13
1    MSIRVT------------------------------------------------------------------QKSYKVSTSG  16

81   GSFRNRFGA-----GAGGGYGFGGGAGSGFG---FGGGAGGGFGLGGGAGFGGGFGGPGFPVCPPGGIQEVTVNQSLLTP  152
14   SAAFSGRGAQVRLSSARPGG-LGSSSLYGLGASRPRVAVRSAYG--GPVG-AG--------------IREVTINQSLLAP  75
17   PRAFSSRSY-----TSGPGSRISSSSFSRVGSSNFRGGLGGGYG--GASGMGG--------------ITAVTVNQSLLSP  75

153  LNLQIDPSIQRVRTEEREQIKTLNNKFASFIDKVRFLEQQNKVLDTKWTLLQEQGTKTVRQN-LEPLFEQYINNLRRQLD  231
76   LRLDADPSLQRVRQEESEQIKTLNNKFASFIDKVRFLEQQNKLLETKWTLLQEQ--KSAKSSRLPDIFEAQIAGLRGQLE  153
76   LVLEVDPNIQAVRTQEKEQIKTLNNKFASFIDKVRFLEQQNKMLETKWSLLQQQ--KTARSN-MDNMFESYINNLRRQLE  152

232  SIVGERGRLDSELRNMQDLVEDFKNKYEDEINKRTTAENEFVMLKKDVDAAYMNKVELEAKVDALMDEINFMKMFFDAEL  311
154  ALQVDGGRLEAELRSMQDVVEDFKNKYEDEINHRTAAENEFVVLKKDVDAAYMSKVELEAKVDALNDEINFLRTLNETEL  233
153  TLGQEKLKLEAELGNMQGLVEDFKNKYEDEINKRTEMENEFVLIKKDVDEAYMNKVELESRLEGLTDEINFLRQLYEEEI  232

312  SQMQTHVSDTSVVLSMDNNRNLDLDSIIAEVKAQYEEIANRSRTEAESWYQTKYEELQQTAGRHGDDLRNTKHEISEMNR  391
234  TELQSQISDTSVVLSMDNSRSLDLDGIIAEVKAQYEEMAKCSRAEAEAWYQTKFETLQAQAGKHGDDLRNTRNEISEMNR  313
233  RELQSQISDTSVVLSMDNSRSLDMDSIIAEVKAQYEDIANRSRAEAESMYQIKYEELQSLAGKHGDDLRRTKTEISEMNR  312

392  MIQRLRAEIDNVKKQCANLQNAIADAEQRGELALKDARNKLAELEEALQKAKQDMARLLREYQELMNTKLALDVEIATYR  471
314  AIQRLQAEIDNIKNQRAKLEAAIAEAEERGELALKDARAKQEELEAALQRGKQDMARQLREYQELMSVKLALDIEIATYR  393
313  NISRLQAEIEGLKGQRASLEAAIADAEQRGELAIKDANAKLSELEAALQRAKQDMARQLREYQELMNVKLALDIEIATYR  392

472  KLLEGEECRLSGEGVGPVNISVVTSSVSSGYGSG--SGYGGGLG-GGLGGGLGGGLAGGSSGSYYSSSSGGVGLGGGLSV  548
394  KLLEGEESRLAGDGVGAVNISVMNST------GGSSSGGGGIGLTLGGTMGSNALSFSSSAGPGLL-KAY----------  455
393  KLLEGEESRLES---GMQNMSIHTKT-TSGYAGGLSSAYG-GLTSPGLSYSLGSSFGSGAGSSSFSRTS----------  456

549  GGSGFSASSGRGLGVGFGSGGGSSSSVKFVSTTSSSRKSFKS  590
456  --------SIRTASA-------SRRSARD-------------  469
457  --------SSRAVVV-------KKIETRDGKLVSESSDVLPK  483
```

Figure 6. Multiple sequence alignment of K5, K7 and K8 by ClustalW2. K5 is the upper, K7 in the middle and K8 the lower sequence. Amino acids marked in bold green are unique K5 regions of minimum three amino acid. The yellow marked region on K5 correspond to the binding site of G-2. [1] Possible sub-epitopes on K7 and K8 of this binding site are marked with yellow.

3.2 Purification of antibodies with Protein G affinity chromatography

The results from the absorbance measurement by Nanodrop 2000C on the antibodies purified with Protein G affinity chromatography are seen in Table 28.

Table 28. Results from Protein G chromatography purification of antibodies RCK103, AE14, 3E2F1, 1E1, G-2 and XM26.

Antibody	Concentration (mg/ml)	Volume µl
RCK103	0.5 mg/ml	70
AE14 (batch 1)	1.16	80
3E2F1	2.09	80
1E1	4.6	80
G-2	1.0 mg/ml	180
XM26	1.9 mg/ml	180

3.3 Conjugation of antibodies

Results from biotinylation and HRP-conjugation are found in this section.

3.3.1 Biotinylation of RCK103, AE14, 3E2F1, 1E1, 1A12, PAb K5, G-2 and XM26

The results from biotinylation of RCK103, AE14, 3E2F1, 1E1, 1A12, PAb K5, G-2 and XM26 can be seen in Table 29.. Note that all volumes were estimated and therefore are weights and yields approximate. Because of this RCK103 gets a higher output than input weight and thus a yield exceeding 100 %. Yields were varying from 17-106 for the different antibodies even though the same type of column was used. The variations is most likely due to that very low amount were used in the experiments.

Table 29. Concentration and amount of biotinylated antibodies.

Antibody	Concentration(µg/ml)	Final weight (µg)	Start weight (µg)	Yield
RCK103	69	36	34	106 %
AE14 (batch 1)	30	15	93	17 %
3E2F1	50	30	167	18 %
1E1	233	117	327	36 %
1A12 (batch 1)	143	71.5	102	70 %
PAb K5	75.6	38	81	47 %
1A12 (batch 2)	138.5	69.3	125.2	55 %
G-2	233.7	116.9	160	73 %
XM26	252.2	126.1	285	44.2 %
AE14 (batch 2)	297	163.4	222	74 %

3.3.2 Horseradish peroxidase (HRP) conjugation of Ks19.1

The chromatogram from the gel filtration on ÄKTA purifier is seen in Figure 7. Absorbance monitored at 280 nm (blue) and 403 nm (red). The highest (blue) peak starts in fraction 10 (37 mL) and ends approximately in fraction 20 and corresponds to the HRP-conjugated antibody.

The highest absorbance was measured in the fraction around 40 mL. Fractions 12-18 was selected for pooling. The earlier fractions were avoided due to risk for can containing aggregates and the latter fractions could possibly dilute the sample due to its low concentrations. Results from concentration measurement, volume and yield of the pool can be seen in Table 30.

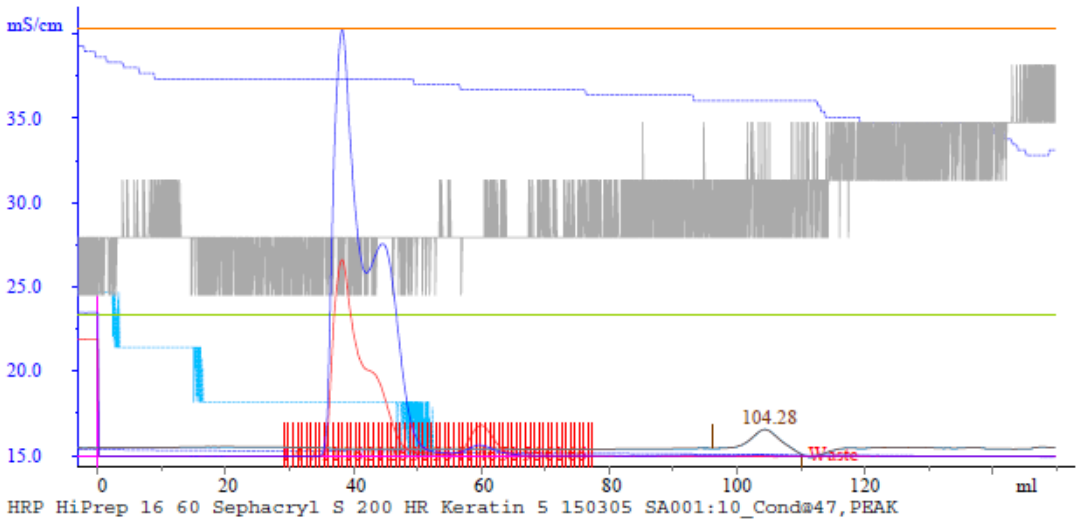

Figure 7. Chromatogram from HRP-conjuation of antibody Ks19.1 on ÄKTA purifier with Sephracryl S 200. Absorbance monitored at 280 nm (blue) and 403 nm (red). Vertical red lines denotes the fractions. The highest (blue) peak starts in fraction 10 and ends approximately in fraction 20 and corresponds to the HRP-conjugated antibody. The peak at 403 nm (red) corresponds to the free HRP.

Table 30. Results from the HRP-conjugation of Ks19.1. Volume and Concentration HRP-Ks19.1 pool corresponds to measurements right after pooling. Total weight and yield were calculated from the two previous values. Final volume and concentration refers to after the addition of BSA and Surmodics.

Volume HRP-Ks19.1 pool	Concentration HRP-Ks19.1 pool	Total weight	Yield	Final volume	Final concentration HRP-Ks19.1
4.9 mL	0.4367 mg/ml	2.14 mg	41.4 %	9.8 mL	0.128 mg/mL

3.4 Evaluation of antigen from CaSki

3.4.1 DNA-content test of SDS-treated antigen with agarose gel electrophoresis

The picture from the agarose gel is shown in Figure 8. Well 1 contains the ladder and well 2 the keratin sample. Two bands are marked in the figure. The weak, upper band is larger than 2000 base pairs (largest band in ladder is 2000 base pairs). Between 800 and 400 base pairs is a "schmere" band occurring. The schmere band is probably many small fragments. The weakest band can barely be seen, thus containing maximum 10 ng of DNA. This is approximately 0.2 % of DNA, which can be considered as negligible. The schmere band is maximum 20 ng, 0.4 % which also is negligible. The concentration of DNA can therefore be considered as low and the

antigen should not be affected by the DNA content. No uncleaved DNA is in the start of lane, meaning the DNAse treatment was effective.

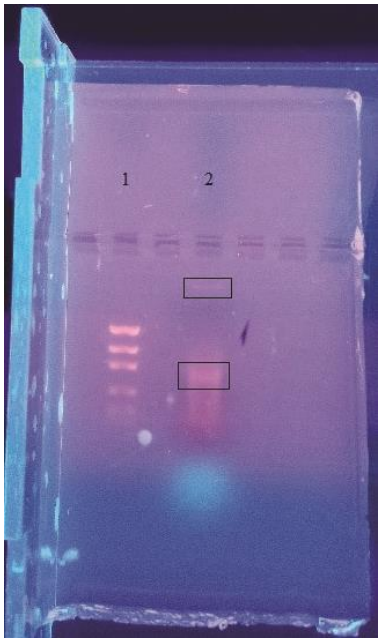

Figure 8. DNA-content test of SDS-treated antigen. Well 1 contains the ladder and well 2 the keratin sample. Boxes marks band in the keratin sample.

3.4.2 Evaluation of coated Maxisorp plate

Results from absorbance measurement of the two-step enzyme immunoassay are seen in Table 31. The addition of antibodies can be seen in Table 4 on page 23. The background noise is the absorbance measured in strip 4, which only contained buffer. A pattern of dose response for increasing antibody concentration is observed for strip 1-3. In general was absorbance higher for the keratin 19 antibody (CB) compared to the keratin 5 antibody, indicating a higher content of keratin 19 than keratin 5. Although, it is clear that the extracted keratin contained both keratin 5 and keratin 19. Nevertheless, one cannot know which other proteins the keratin solution contains

Table 31. Results from absorbance measurement of the two-step enzyme immunoassay. A-H denotes the wells on each strip. The background noise is the absorbance measured in strip 4. A pattern of dose response for increasing antibody concentration is observed for strip 1-3. Strip 1 and wells A2-D2 contained biotinylated antibody against keratin 5 whereas and well E2-H2 and strip 3 contained the antibody towards keratin 19.

	Strip 1	Strip 2	Strip 3	Strip 4
A	2.823	0.497	3.376	0.279
B	2.831	0.476	3.292	0.277
C	1.785	0.378	3.024	0.268
D	1.814	0.381	3.089	0.270
E	1.130	3.652	2.472	0.264
F	1.099	3.640	2.702	0.271
G	0.783	3.586	2.780	0.314
H	0.783	3.666	2.746	0.304

3.5 Evaluation of biotinylated antibodies

Results from evalution of biotinylated antibodies and determination of their optimal concentrations is presented in this sectio.

3.5.1.1 RCK103, AE14 (batch 1), 3E2F1, 1E1, 1A12 (batch 1) and PAbK5

As seen in Figure 9, for RCK 103 is higher absorbance correlated with higher antibody concentration, which is an expected result. There is almost no difference between 2 µg/ml and 1 µg/ml, which probably is due to biotin saturation of the microtiter plate. On the other, the absorption is reaching its limits at what could be detected. Since 1 µg/ml gets high absorption compared to lower concentration and there is no need to obtain higher absorbance, this concentration of RCK103 should be optimal. However, limited amounts of the antibodies were available for the project and therefore was 0.5 µg/ml used in the latter experiments.

For all the other antibodies the absorbance was decreasing with increasing antibody concentration. This could be due to biotin saturation at very low concentration or due to conformational changes making the epitope unavailable for Ks19.1. Because of this, a rather low concentration of the five antibodies were selected for future experiments. 0.125 µg/mL was chosen as concentration for AE14 and 3E2F1, due to the low amount available. For the remaining three antibodies was 0.5 µg/mL used for latter experiments.

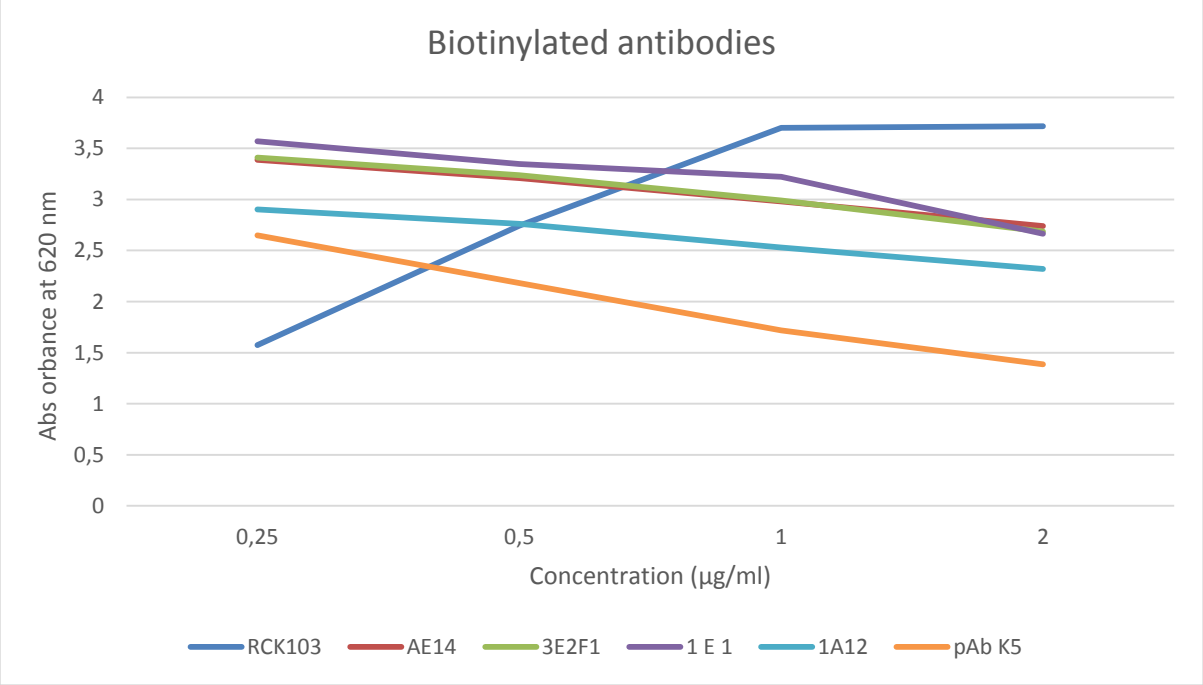

Figure 9. Test of biotinylation of antibodies.

Figure 10 illustrates the microtiter plate from the experiment. Clear colorimetric differences can been seen between the strips. Strip 1 (containing RCK103) goes from medium blue to dark blue

whereas strip 2-6 (containing remaining antibodies), where the latter are more intense bordering black. The last strip, with buffer colored light blue is background noise.

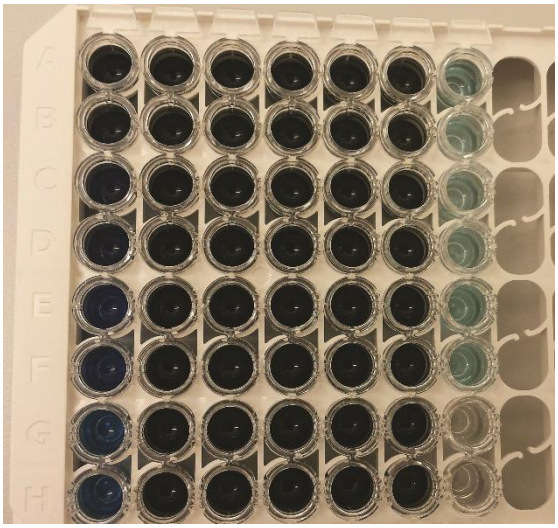

Figure 10. Picture of the microtiter plate.

3.5.1.2 1A12 (batch 2)

Results from the biotinylation test is shown in Table 32. Addition of antibody solutions is described on page 25. The biotinylated antibody is able to detect the samples and thus, biotinylation was successful. Since results from the different concentrations were similar, it was decided to retest 0.125 µg/ml and also test 0.5 µg/ml when evaluating the biotinylation of G-2 and XM26 in order to determine the optimal antibody concentration. .

Table 32. Results from biotinylation test of 1A12. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3
A	0.156	0.210	0.252
B	0.162	0.214	0.240
C	0.278	0.303	0.373
D	0.278	0.295	0.334
E	0.398	0.385	0.386
F	0.393	0.390	0.434
G	0.632	0.560	0.554
H	0.596	0.519	0.553

3.5.1.3 G-2, XM26 and 1A12 (batch 2)

Results from biotinylation test of G-2, XM26 and 1A12 (batch 2) is seen in Table 33. Addition of samples and antibody solutions are described on page 26. For all antibody concentrations is higher keratin concentration correlated with higher absorbance. Increasing antibody concentration gave a decreasing absorption, indicating a low antibody concentration would be desirable to use in latter experiments. The following concentrations were selected for latter experiments: G: 0.5 µg/mL; XM26: 0.25 µg/mL and 1A12: 0.125 µg/mL.

Table 33. Biotinylation test results. A-H denotes the wells on each strip.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8	Strip 9	Strip 10
A	0.099	0.106	0.118	0.125	0.093	0.113	0.138	0.154	0.180	0.210
B	0.096	0.104	0.124	0.126	0.091	0.106	0.141	0.150	0.172	0.202
C	0.173	0.157	0.168	0.169	0.126	0.146	0.176	0.193	0.217	0.256
D	0.154	0.154	0.162	0.169	0.124	0.156	0.166	0.191	0.221	0.244
E	0.221	0.228	0.230	0.230	0.171	0.198	0.221	0.245	0.292	0.322
F	0.224	0.224	0.221	0.233	0.165	0.195	0.215	0.241	0.290	0.319
G	0.908	0.891	0.845	0.808	0.591	0.651	0.748	0.802	0.959	0.977
H	0.887	0.875	0.831	0.767	0.559	0.650	0.803	0.792	0.876	0.935

3.5.1.4 AE14 (Batch 2)

Results from Biotinylation test of AE14 is presented below. Largest variations between the antibody concentrations is seen for low calibrator concentrations (C1 and C3). C1 does not contain any keratin and is thereby a measurement of the background noise. Since as low background noise as possible is desirable the concentration corresponding to lowest value on C1 was selected for latter experiments (0.5 µg/mL).

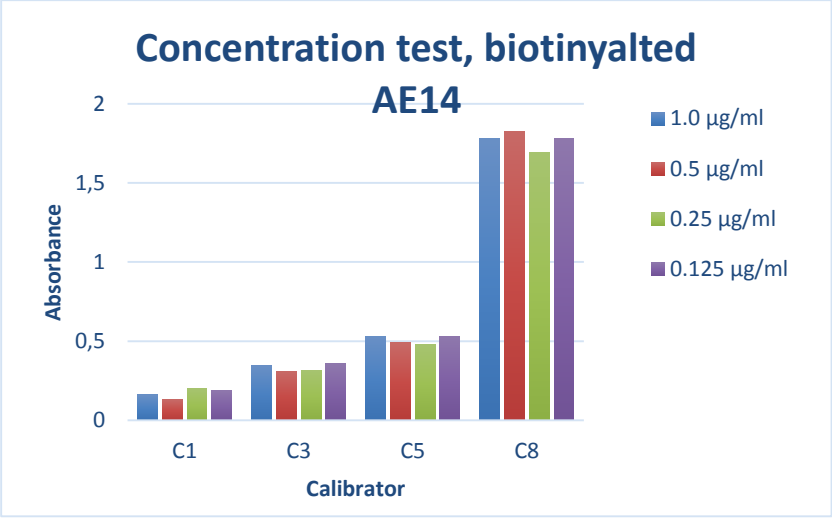

Figure 11. Biotinylation test of AE14.

3.6 Evaluation of antibody combinations

This section presents the experiments performed to determine the optimal antibody combination and assay procedure.

3.6.1 Test of Ks19.1 with K5 antibodies against serum and pleura samples

Results from Ks19.1 in combination with different antibodies against K5 are presented in Table 34. Compared to healthy blood donors, both lung cancer samples are have an enhanced absorbance in the test. However one should keep in mind that histological classes of these samples were unknown and therefore one cannot draw to much conclusions from this. The pleura sample had been chosen based on its high CYFRA21-1 value, which measure the K19

concentration. One would assume that a K5/19 would be correlate to some extent with the CYFRA21-1 value, which would have given an absorbance between 2 and 3. Nonetheless, absorbance of the pleura samples are under 0.2. The pleura sample was diluted 1:10, 1:5 and 1:1, which is not reflected in the absorbance measurement. The C5 calibrator however is detected, implying a structural difference between the keratin complex from CaSki cells and from the pleura samples. Strip 7, containing 1A12, obtains higher absorbance in comparison with strip 1-5. This indicated that 1A12 would be a superior K5 antibody in the test and is was decided to proceed with it as long as no contradictive results were obtained in the corresponding assay with BM19.21.

Table 34. Results from Ks19.1 with K5 antibodies against serum and pleura samples. A-H denotes the wells on each strip. Strip 1: RCK103, Strip 2: AE14, Strip 3: 3E2F1, Strip 4: PAbK5, Strip 5+6: 1E1, Strip7+8: 1A12. Strip 7 (1A12) obtains higher absorbance in comparison with strip 1-5.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8
A	0.087	0.079	0.089	0.086	0.095	0.086	0.152	0.191
B	0.095	0.089	0.097	0.085	0.089	0.084	0.163	0.171
C	0.091	0.100	0.084	0.084	0.081	0.061	0.143	0.084
D	0.090	0.097	0.089	0.089	0.083	0.061	0.120	0.082
E	0.045	0.042	0.042	0.042	0.041	0.053	0.080	0.065
F	0.045	0.042	0.043	0.044	0.043	0.055	0.077	0.060
G	1.300	1.083	1.237	1.158	1.069	0.270	1.368	0.373
H	1.184	1.111	1.183	1.091	1.098	0.265	1.457	0.357

3.6.2 Test of BM19.21 with K5 antibodies against serum and pleura samples

Compared to the assay with Ks19.1, the BM19.21 detects pool 1 equivalent with the Ks19.1 assay although pool 2 received a worse absorbance in the BM19.21 assay (Table 355). Pool 3 and 4 (not tested in Ks19.1) gave equivalent results with blood donor, thus not detected in the test. Pleura samples obtained the high absorbance expected, which was not obtained in the Ks19.1 assay. However, the C5 calibrators is not detected. This confirmed the theory of structural differences of K5/19 in pleura compared to CaSki cells and how this structural difference affects the affinity of the antibodies to their epitopes can only be speculated on. Nevertheless, the keratin from CaSki was intended to be used as calibrator in the final test, Ks19.1 was superior to BM19.21 and chosen to proceed with together with 1A12.

Table 35. Results from BM19.21 with K5 antibodies against serum and pleura samples. A-H denotes the wells on each strip. Strip 1: RCK103, Strip 2: AE14, Strip 3: 3E2F1, Strip 4: PAbK5, Strip 5 and 6, well A7 and B7: 1E1, Well C-H7, Strip 8-9: 1A12.

	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6	Strip 7	Strip 8	Strip 9
A	0.046	0.046	0.047	0.047	0.047	1.180	0.119	0.219	0.133
B	0.049	0.046	0.048	0.045	0.045	1.216	0.119	0.212	0.131
C	0.041	0.041	0.041	0.040	0.039	2.276	0.051	1.146	0.051
D	0.041	0.039	0.040	0.041	0.040	2.177	0.045	1.166	0.052
E	0.036	0.037	0.036	0.039	0.037	3.665	0.041	2.244	0.037
F	0.039	0.037	0.035	0.037	0.038	3.709	0.046	2.340	0.038
G	0.190	0.195	0.196	0.195	0.190	0.040	0.037	3.712	0.190
H	0.180	0.188	0.195	0.191	0.190	0.038	0.037	3.771	0.130

3.6.3 Comparison between one-step and two-step ELISA

Compared to the 2-ste assay, the one-step detected all samples higher, including CalF from CYFRA21-1 (Table 36). However, CalF is not supposed to contain high amount of K5/19, which indicates higher rate of unspecific binding in the one-step. Due to this, some of the enhanced samples might be falsely positive in the 1 step. Since the two-step assay still detected the highest samples well and received low absorbance for the healthy blood donor, the two-step seemed as the superior assay format. For the two-step assay, pool 2 is detected by approximately the same absorbance as for the one step. Pool 4, LC1 and LC2, C5 and CalF is detected by the test. The results were also compared with the previous enzyme immunoassays (page 44-45). For the one-step, the increased incubation time increased absorbance for the samples run in the Ks19.1 with K5 antibodies against serum and pleura samples. Due to this, one did not try 1 hour incubation time for biotinylated antibody in two-step. However, no further optimizations of the two-step test was performed neither in procedure nor reagents except antibody combinations and the two-step assay according to this experiment was used for the future experiments. Pool 3 and 4, which previously only been tested in BM19.21 assay, obtained enhanced values for at least pool 4, which was not detected by BM19.21 and confirming the choice of Ks19.1 as antibody against K19. Aside from the antibody selection the results may imply a structural difference between serum and pleura samples. Based on these two antibodies ability to detect samples from serum, pleura and keratin from CaSki one could assume that the K5/19 complex in serum and CaSki is more alike compared to pleura. The ability of 1A12+Ks19.1 to detect the two other lung cancer samples also makes the combination possible in a K5/19-test.

Table 36 Results from the two-step and one-step assays. A-H denotes the wells on each strip.

	two-step assay		one-step assay	
	Strip 1	**Strip 2**	**Strip 1**	**Strip 2**
A	3.453	0.211	3.633	0.174
B	0.380	0.200	1.007	0.170
C	0.189	1.452	0.315	1.849
D	0.193	1.387	0.212	1.651
E	0.189	0.158	0.209	0.138
F	0.302	0.191	0.652	0.145
G	0.135	0.671	0.163	1.000
H	0.135	0.561	0.171	0.902

3.6.4 Enzyme immunoassay with endometrial cancer

Results from the endometrial cancer samples are presented as concentration values in Figure 12. All values (concentration) below C1 (zero calibrator) were set to 0 and above C9 were set to 35 (highest calibrator 35 µg/L). All absorbance values are found in Table 45, page 68. The cutoff was set to 3 µg/L based on the cutoff set for the lung cancer study.

G-2 detected 11 endometrial cancer samples and no blood donors. XM26 detected 5 endometrial samples and 1A12 detected 11 endometrial samples and one of the blood donors. XM26 could not detect as many samples as the other two antibodies and had a higher background noise. For the G-2 test were two samples out of range (higher absorbance than the highest calibrator) and both 1A12 and G-2 received many positive samples, but not for XM26. From this experiment

one could assume that there is a higher amount of K5/19 that contain the N-terminal than C-terminal part of the protein. Due to detecting only half of less than half of the samples G-2 and 1A12 detected, XM26 was excluded as a possible candidate for the K5/19 test. G-2 and 1A12 results are not correlating, thus not detecting the same complex. This could either be due to levels of K5/19 containing the N-terminal and rod domain or only the rod domain. Cross-reaction between one of the antibodies and another keratin could also be causing of difference.

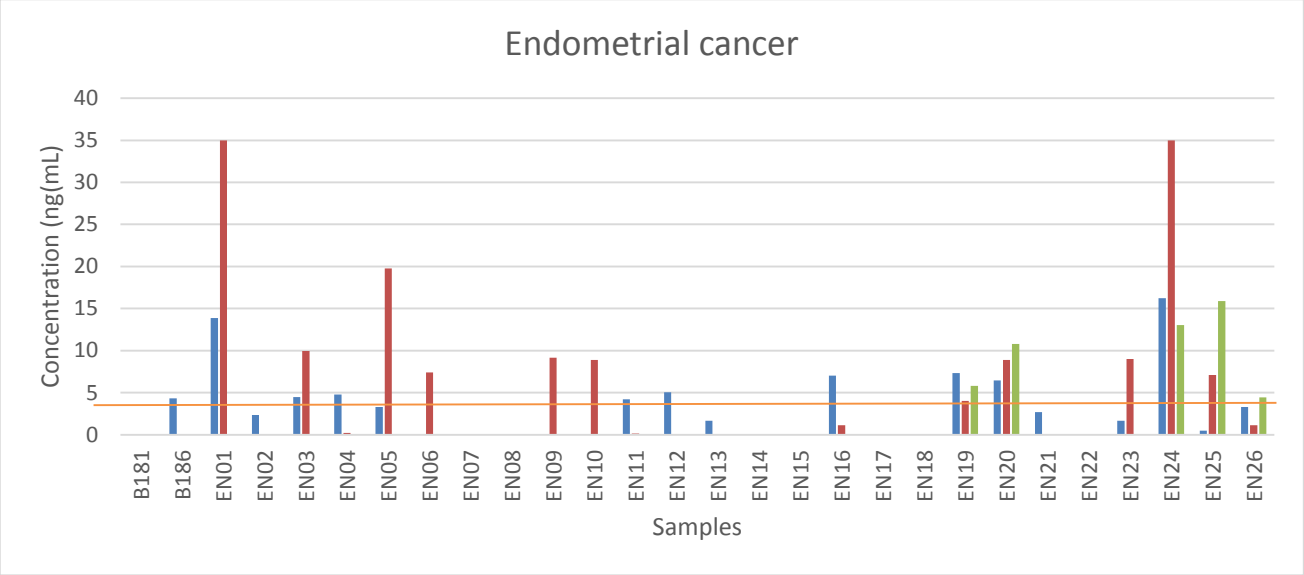

Figure 12. Results from the endometrial cancer presented with concentration values. The orange line at 3 ng/mL denotes the cut off limit. Blue: 1A12, Red: G-2, Green: XM26.

3.7 Western Blot

The western blot was performed to determine the specificity of AE14, 1A12 and G-2 for keratin 5. The antibodies was tested against keratin 5, 8 and 19 and Ks19.1 and Ks8.7 was used as positive controls for keratin 19 and keratin 8 respectively. In Figure 13, one can see the results from both day 2 and 3 of the Western Blot run. The positive controls Ks8.7 and Ks19.1 (membrane 6 and 4) obtain the expected results, only reacting with K8 and K19 respectively. G-2, which according to the manufacturer [1] should be specific for K5, reacted with both K5 and K8. Although the K5 band are much more intense than K8, which could mean that is G-2 reacts in larger extent with K5 compared to K8. The G-2 antibody reacts against epitope 13-47 on the head of the K5 molecule. As shown in the multiple alignment (Figure 6) parts of this epitope may be located on several possible sites of the K8 molecule. Taken together with the EIA results of endometrial cancer samples and western blot data this implies cross-reactivity and G-2 antibody is thus not suitable for a K5 or K5/19 test. 1A12 on the other displays specificity for K5 and does not cross-react with K8, implying 1A12 is a suitable antibody for immunoassays involving K5. AE14 did not bind to any of the proteins neither at day 2 nor day 3 seven though its manufacturer states K5 specificity. [1] This could be due to not optimized primary antibody concentration or that the antibody can no longer bind to the antigen due to structural variation between the native and recombinant protein or the reduction with DTT. DTT was used to reduce the samples, which results in breakage of sulfide bonds. This could have affected the results for K5 samples in the western blot since K5 is thought to have sulphide bridges making a cross-like

3D-structure. Since the K5 binding sites are not known for antibodies AE14 and 1A12, one cannot say if the reducing affected the binding of these. However, due to the contingency of the reactivity of AE14, only 1A12 was selected to proceed with in the lung cancer study. One should also keep in mind that K7 was not used in the western blot and therefore, one could not say anything about an eventual cross-reactivity with K7 for the antibodies.

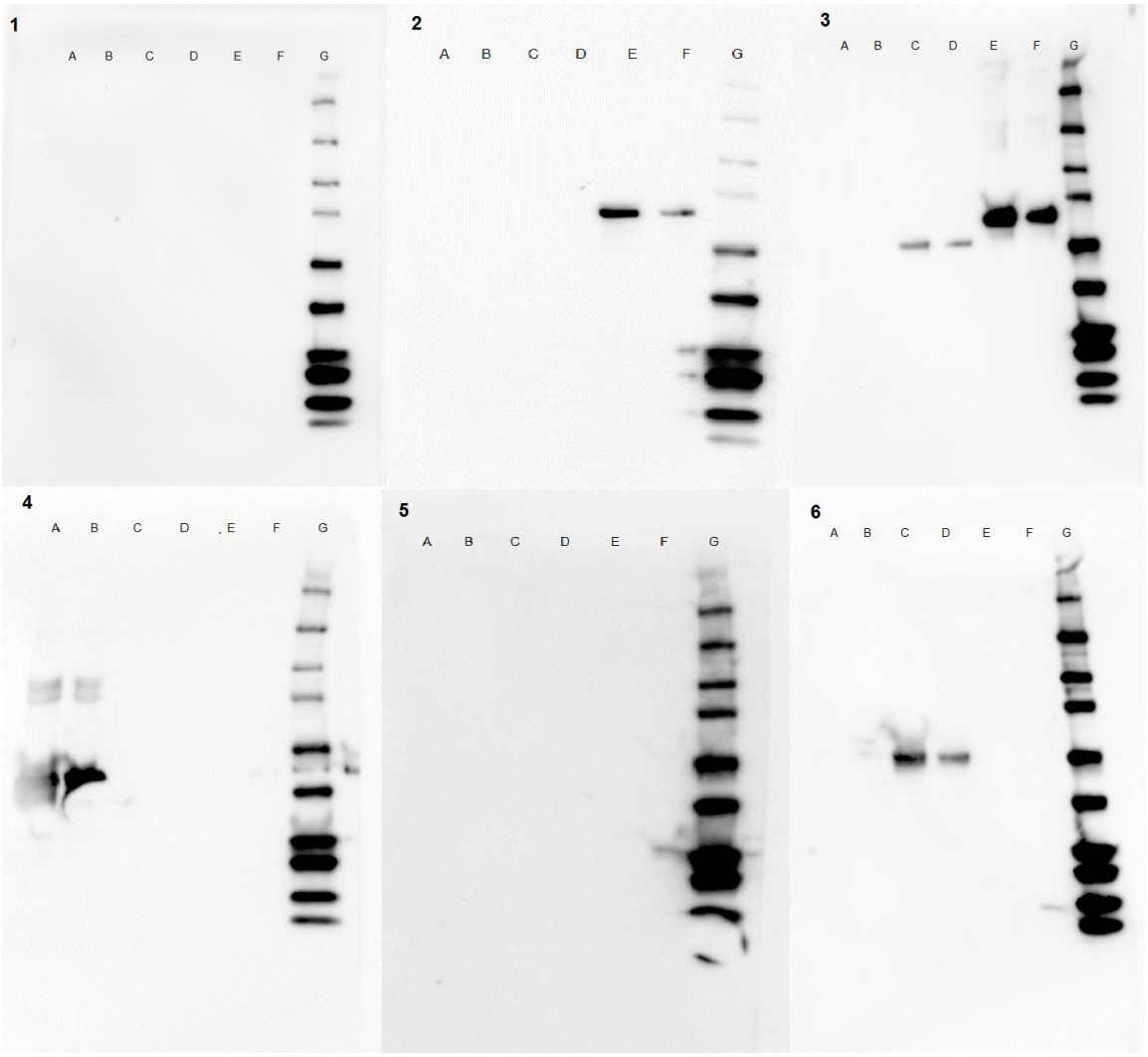

Figure 13. Results from Western Blot. The six membranes are shown from left to right upper row: AE14 day 2, 1A12, G-2. From left to right lower row: Ks19.1, AE14 day 3, Ks8.7. Lane A=K19+, B=K19-, C=K8+, D= K8-, E=K5+, F=K5- and G=Ladder (reference). Membrane 1 and 5 are not containing any protein bands, meaning AE14 have not bound to any of the recombinant proteins for neither of the two antibody concentrations. Membrane 2 (1A12) shows clear bands at both K5+ and K5-, meaning 1A12 is specific for K5 and not cross-reacting with K8. Membrane 3, incubated with G-2 show reactivity for both K5 and K8, even though it is more intense for K5. Ks19.1 (membrane 4), only shows reactivity for K19 as expected. However, protein bands at higher kDA are shown for both lane A and B, indicating a dimerization. Membrane 6, incubated with Ks8.7 only has reactivity for K8.

3.8 Robustness testing of the EIA Wash step (Drift test)

The absorbance is hard to compare between the tests since the samples were not used. However, serum samples from healthy blood donors should generate approximately the same absorbance.

3.8.1 Strip process wash mode

From results of the strip mode drift test below one can note that although the 1A12+Ks19.1 test is rather stable within a certain strip, there are as much as twice the absorbance generated for strip 10 as strip 1 for the same sample. The 1A12+Ks19.1 is thus unstable in strip mode and should not be carried out in strip mode.

Table 37. Results from Strip mode drift test. A-H denotes the wells on each strip.

	Strip 1	Strip 6	Strip10
A	0.124	0.188	0.253
B	0.126	0.194	0.254
C	0.131	0.194	0.252
D	0.127	0.175	0.238
E	0.128	0.185	0.251
F	0.128	0.181	0.245
G	0.154	0.193	0.251
H	0.149	0.195	0.271

3.8.2 Plate process wash mode

Compared with the strip mode drift test, the plate mode washing gives uniform results within samples independent of position. No pattern between higher/lower absorbance can be seen between or among strips even though the absorbance is not exactly the same for all measurements on the same sample. The plate mode can thus be considered as much more reliable than strip mode and was therefore used in the Lung cancer study.

Table 38. Results from Plate mode drift test. A-H denotes the wells on each strip.

	Strip 1	Strip 4	Strip 7	Strip10
A	0.194	0.106	0.098	3.482
B	0.184	0.106	0.089	3.217
C	3.246	0.192	0.099	0.093
D	3.221	0.176	0.098	0.107
E	0.089	3.043	0.308	0.131
F	0.090	3.241	0.190	0.153
G	0.128	0.093	3.252	0.217
H	0.183	0.100	3.304	0.223

3.9 Lung cancer study

Results from the lung cancer study are presented in this section (for exact values and sample information, page 68-82). When analyzing the results, no pattern between result and position on strip or plate could be observed, implying the randomization was successful. The calibrators used in the project were found to be unstable and despite making fresh calibrators for each day it is impossible to predict how the instability might have affected the results. Even though the

absorbance of each sample might be true one should not rely too much on the estimated concentrations. If one intends to further develop the test, other calibrators should be used. One could test to exchange the matrix the extracted keratin was diluted in or simply to find entirely different calibrators.

During the study different concentrations of biotinylated antibodies were used for plate 1 and 2 than 3 and 4. The samples were also ran in single replicate and these two factors brought uncertainty to results. However, all lung cancer samples were rerun together with some of the low and some of the enhanced samples from healthy blood donor and benign (not shown). These results were in line with the lung cancer study in the project, making the results more trustworthy.

The limits were set to get as high percentage of true positive without getting too low number of negative healthy samples. The proportion of positive diseased samples is referred to as the sensitivity of the test and the proportion of negative control samples as the specificity. Cut off limits for the study were determined to 2.5 ng/mL for CYFRA21-1 and 3.0 ng/mL for 1A12+Ks19.1. The cutoffs give a specificity of 92.3 % true negative when benign is used as control and 97 % when both benign and healthy are used as control in the 1A12+Ks19.1 test. For a combined test these cutoff limit corresponds to 90.4 % specificity with benign as control and 92.3% with benign and healthy as control. However, these cut off limits might be revised if one optimizes the test.

Firstly results were compared between 1A12+Ks19.1 and CYFRA21-1. For blood donors results correlated fairly (not shown). Only 3 of the samples were positive (1 in 1A12+Ks19.1 and 2 in CYFRA21-1) given the cutoffs limits. For benign lung disease 1 sample was positive in CYFRA and 4 in 1A12+Ks19.1 (data not shown). None of positive samples were however positive in both tests.

For lung cancer samples positive samples were seen for all histological classes. For the largest sample groups, adenocarcinoma and squamous cell carcinoma, were approximately the same number of samples positive in 1A12+Ks19.1 meaning the test is not able to discriminate between histological classes (Figure 14-17). The results were not correlating well with CYFRA either (not shown).

If one study the stages detected within each histological class, the results differ between the histological classes. Adenocarcinoma and large cell carcinoma samples detected by the 1A12+Ks19.1 were progressed cancer, stage III or stage IV (Figure 14 and 15). The test are thus not suitable for detecting early stage adenocarcinoma or large cell lung cancer. Note though that only 6 samples of large cell carcinoma was evaluated in the study and therefore are further conclusions not possible to draw.

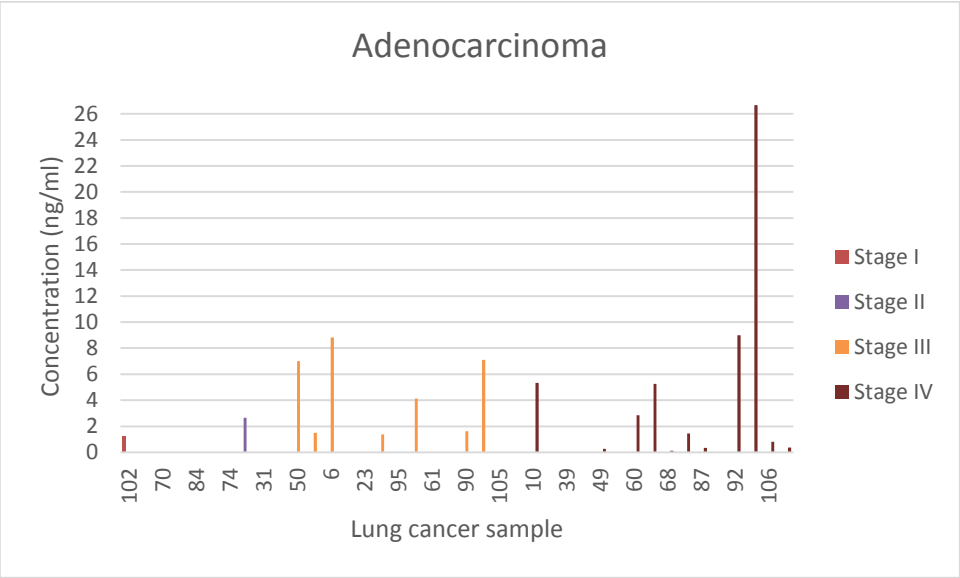

Figure 14. Results from EIA 1A12+Ks19.1against Adenocarcinoma. Samples are denoted according to stage. Of all 40 samples are 8 detected by the test. All 8 samples are from patient with progressed cancer (stage III or Stage IV).

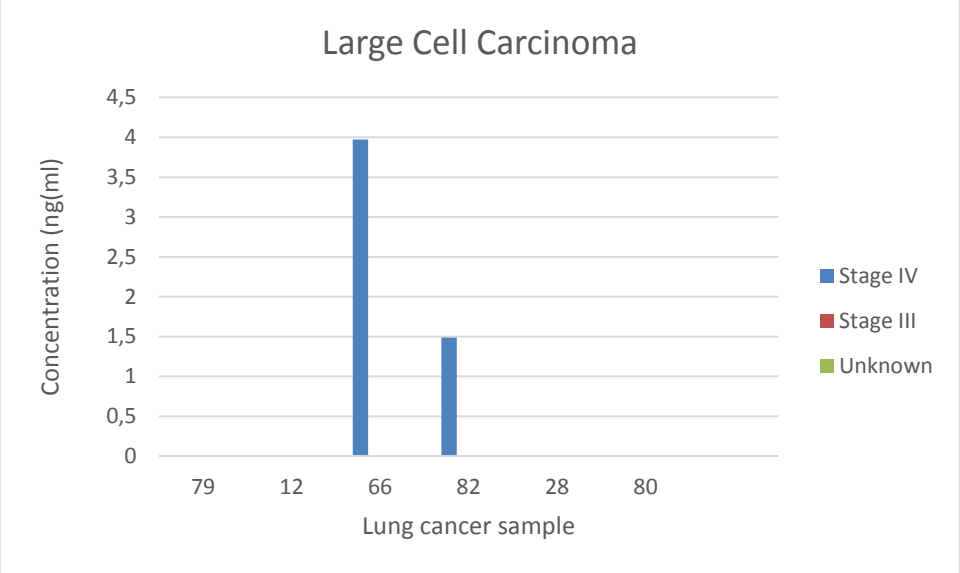

Figure 15. Results from EIA 1A12+Ks19.1against Large Cell carcinoma. Samples are denoted according to stage. Of all 6 samples are 1 (Stage IV) detected by the test. None of the samples with unknown stage or stage III were detected.

For small cell are 5 of 24 samples detected by the 1A12+Ks19.1 test and of these were 3 limited stage and 2 were extensive. Of the 24 samples were 13 early stage. With 3 of 13 detected, this corresponds to 23% sensitivity. Due to few samples used in the study, one cannot draw any conclusion from the small cell lung cancer results. The results are not too promising but further testing should be performed to evaluate the usefulness of the test for small cell lung cancer of early stage.

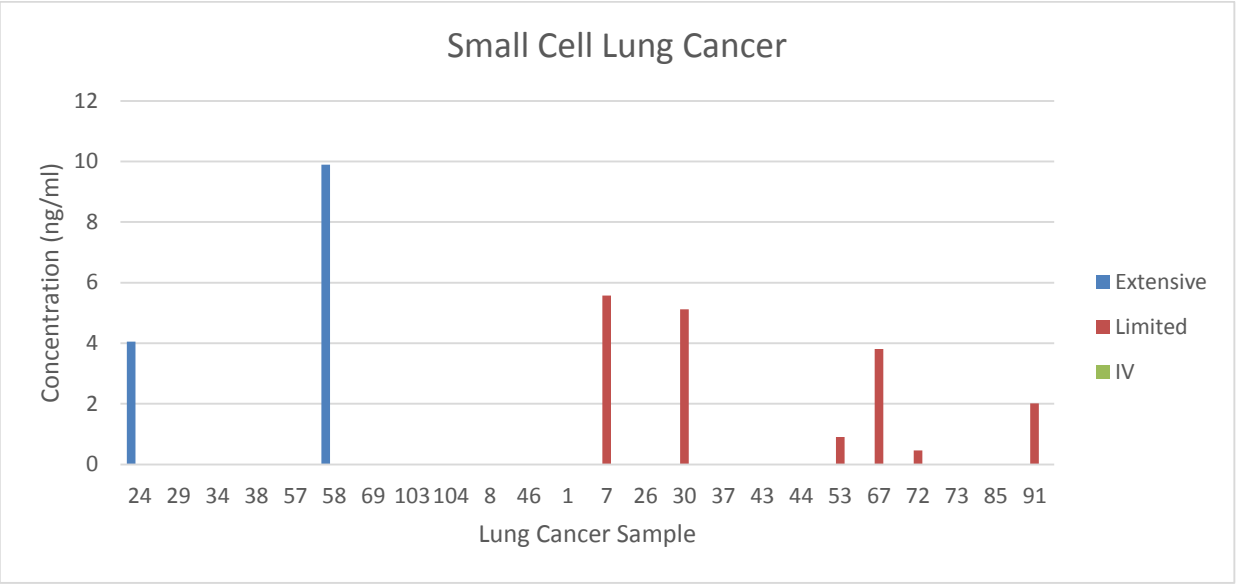

Figure 16. Results from Small Cell lung cancer in 1A12+Ks19.1. Samples are denoted according to stage. Of the 24 samples are 5 detected by the test. Of the detected samples were 3 denoted limited stage and the other two extensive.

Even though the Keratin 5/19 test was not able to discriminate between histological classes the results for detecting early stages of squamous cell carcinoma (SCC) were more promising. For SCC were 9 of 40 samples detected (Figure 17). Of these were 9 positive samples were 6 early stage. Since there only 13 early stage SCC samples were available, these 6 positive corresponds to detecting 46 % of the early stage SCC samples and thus having a 46 % sensitivity. These results implies the possibility of the test to be a useful tool in detecting early stage SCC in lung. Nevertheless, the combination of the developed test together with CYFRA21-1 gives even more interesting results. Figure 18 presents results from the combination of 1A12+Ks19.1 with CYFRA21-1 for early stage SCC samples. As an alone marker a Keratin 5/19 assay (1A12+Ks19.1) detects 6 out of 13 early stage SCC and CYFRA21-1 5 out of 13. However when assays are combined they detects 9 of 13 samples, giving a 69 percent sensitivity (true positive of all diseased) given the cut off limits described above. From these results one can draw the conclusions that there is a possibility for developed test, both separately but in combination with CYFRA21-1 above all, to function as a tool in the diagnosis of early stage SCC in lung. Even though these results are promising one should keep in mind they are based on 13 early stage samples. Therefore one should perform further studies with a greater quantity of early stage SCC lung cancer samples in order to be able to draw conclusions whether or not 1A12+Ks19.1 could be a complement for CYFRA21-1 in detecting early stage SCC lung cancer.

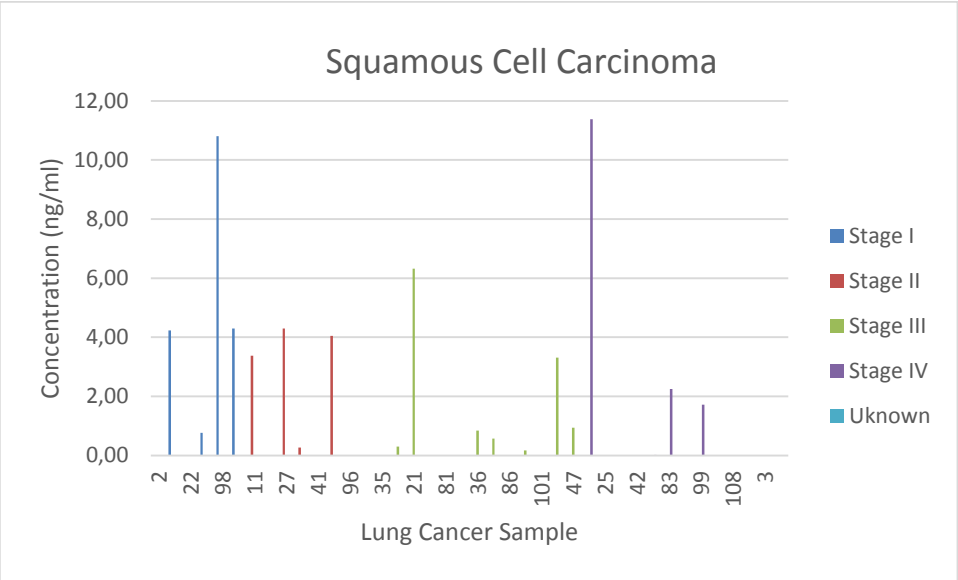

Figure 17. Results from EIA 1A12+Ks19.1against Squamous Cell carcinoma. Samples are denoted according to stage. Of all 40 samples are 9 detected by the test. Of early stages (I and II) were 6 of 13 samples detected. Neither of the samples with unknown stage were detected.

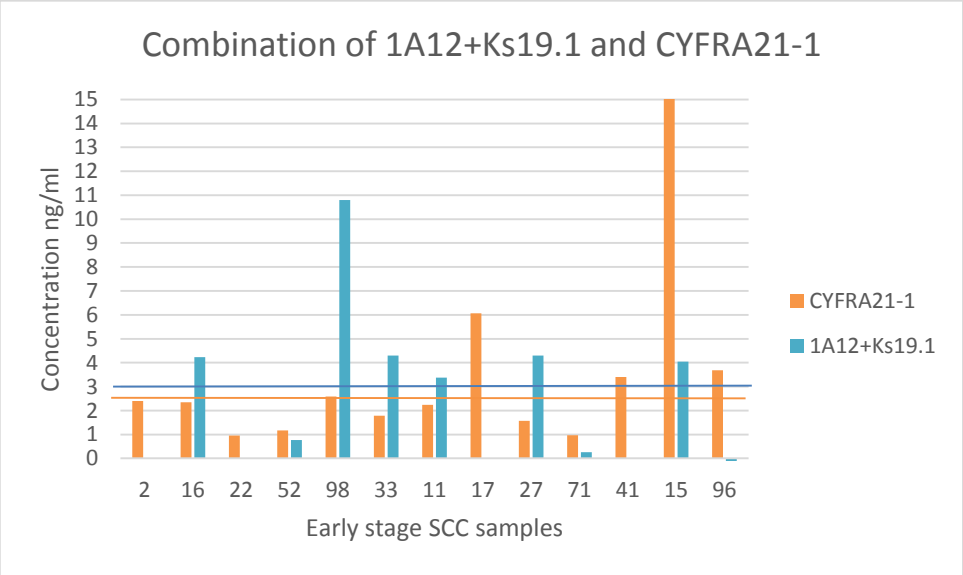

Figure 18. Combination of CYFRA21-1 and 1A12+Ks19.1 for early stage SCC lung cancer. The blue and orange lines denotes cutoff limits for 1A12+Ks191. and CYFRA21-1 respectively.

4 Conclusions

Of all 14 antibody combinations, 1A12+Ks19.1 was determined as the superior one. Assay procedure was chosen to a two-step assay with 2 hours incubation time for the biotinylated antibody and 1 hour incubation for the HRP-conjugated antibody.

The experiments used to evaluate antibody combinations also indicates a structural difference between keratin 5/19 in pleura liquid compared to serum. From the experiments one can also conclude that K5/19 complex containing the N-terminal is likely to be present in serum in higher extent than complex with intact C-terminal. The western blot also revealed cross-reaction of G-2 antibody with keratin 8, although G-2 is stated specific for keratin 5 by its manufacturer. [1]

The extracted solubilized keratin from CaSki cells is not stable in the CYFRA21-1 calibrator matrix, a phosphate buffered salt solution containing bovine serum albumin. One should exchange matrix for the calibrators if one intends to use the keratin from CaSki as calibrator for a K5/19 test or use another protein as calibrator.

A randomized lung cancer study including 110 lung cancer plasma samples, 52 serum samples of benign lung disease and 52 plasma samples from healthy blood donors was performed. The study was carried out by analyzing the samples in the constructed 1A12+Ks19.1 test and CYFRA21-1. The constructed test was not able to discriminate between histological classes of lung cancer in the lung cancer study.

The constructed keratin 5/ keratin 19 (K5/19) test detected the early stages among the different histological classes in various extent. Adenocarcinoma and large cell carcinoma samples detected by the 1A12+Ks19.1 test were progressed cancer, stage III or stage IV, the test is thus not suitable for detecting early stages of these classes. Five of twenty four Small Cell Lung Cancer (SCLC) were detected and 3 of 13 early stage cancers (limited disease) were detected. The low number of SCLC samples in the study, nonetheless, makes it impossible to draw any conclusions of the results.

For squamous cell carcinoma in lung early stages are detected in larger extent than stage III and IV for K5/19 (1A12+Ks19.1) as a separate test but primarily in combination with CYFRA21-1, where 9 of 13 early stage samples were detected. From this study one can recommend to perform further studies on early stage SCC in lung to determine the relevance of CYFRA21-1 in combination with K5/19 as diagnostic tool.

5 Acknowledgements

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7

8 Supplementary

8.1 Protein sequences

All protein sequences was collected from interfile.org. [24]

8.1.1 Keratin 5

Below one finds the protein sequence of keratin 5. Table 39 lists the locations of the different subunits on the keratin molecule.

```
>NP_000415.2|K5
MSRQSSVSFRSGGSRSFSTASAITPSVSRTSFTSVSRSGGGGGGGFGRVS
LAGACGVGGYGSRSLYNLGGSKRISISTSGGSFRNRFGAGAGGGYGFGGG
AGSGFGFGGGAGGGFGLGGGAGFGGGFGGPGFPVCPPGGIQEVTVNQSLL
TPLNLQIDPSIQRVRTEEREQIKTLNNKFASFIDKVRFLEQQNKVLDTKW
TLLQEQGTKTVRQNLEPLFEQYINNLRRQLDSIVGERGRLDSELRNMQDL
VEDFKNKYEDEINKRTTAENEFVMLKKDVDAAYMNKVELEAKVDALMDEI
NFMKMFFDAELSQMQTHVSDTSVVLSMDNNRNLDLDSIIAEVKAQYEEIA
NRSRTEAESWYQTKYEELQQTAGRHGDDLRNTKHEISEMNRMIQRLRAEI
DNVKKQCANLQNAIADAEQRGELALKDARNKLAELEEALQKAKQDMARLL
REYQELMNTKLALDVEIATYRKLLEGEECRLSGEGVGPVNISVVTSSVSS
GYGSGSGYGGGLGGGLGGGLGGGLAGGSSGSYYSSSSGGVGLGGGLSVGG
SGFSASSGRGLGVGFGSGGGSSSSVKFVSTTSSSRKSFKS
```

Table 39. Domain distribution of the K5 protein. [24]

Domain Name	Probable Starting Amino Acid Position	Probable Ending Amino Acid Position
Head	1	168
1A	169	203
L1	204	215
1B	216	316
L12	317	333
2A	334	352
L2	353	360
2B	361	481
Tail	482	590

8.1.2 Keratin 7

Below one finds the protein sequence of keratin 7.

```
>NP_005547.3|K7
MSIHFSSPVFTSRSAAFSGRGAQVRLSSARPGGLGSSSLYGLGASRPRVA
VRSAYGGPVGAGIREVTINQSLLAPLRLDADPSLQRVRQEESEQIKTLNN
KFASFIDKVRFLEQQNKLLETKWTLLQEQKSAKSSRLPDIFEAQIAGLRG
QLEALQVDGGRLEAELRSMQDVVEDFKNKYEDEINHRTAAENEFVVLKKD
VDAAYMSKVELEAKVDALNDEINFLRTLNETELTELQSQISDTSVVLSMD
NSRSLDLDGIIAEVKAQYEEMAKCSRAEAEAWYQTKFETLQAQAGKHGDD
LRNTRNEISEMNRAIQRLQAEIDNIKNQRAKLEAAIAEAEERGELALKDA
RAKQEELEAALQRGKQDMARQLREYQELMSVKLALDIEIATYRKLLEGEE
SRLAGDGVGAVNISVMNSTGGSSSGGGIGLTLGGTMGSNALSFSSSAGPG
```

LLKAYSIRTASASRRSARD

8.1.3 Keratin 8

Protein sequence of keratin 8 is shown below.>NP_002264.1|K8

MSIRVTQKSYKVSTSGPRAFSSRSYTSGPGSRISSSSFSRVGSSNFRGGL
GGGYGGASGMGGITAVTVNQSLLSPLVLEVDPNIQAVRTQEKEQIKTLNN
KFASFIDKVRFLEQQNKMLETKWSLLQQQKTARSNMDNMFESYINNLRRQ
LETLGQEKLKLEAELGNMQGLVEDFKNKYEDEINKRTEMENEFVLIKKDV
DEAYMNKVELESRLEGLTDEINFLRQLYEEEIRELQSQISDTSVVLSMDN
SRSLDMDSIIAEVKAQYEDIANRSRAEAESMYQIKYEELQSLAGKHGDDL
RRTKTEISEMNRNISRLQAEIEGLKGQRASLEAAIADAEQRGELAIKDAN
AKLSELEAALQRAKQDMARQLREYQELMNVKLALDIEIATYRKLLEGEES
RLESGMQNMSIHTKTTSGYAGGLSSAYGGLTSPGLSYSLGSSFGSGAGSS
SFSRTSSSRAVVVKKIETRDGKLVSESSDVLPK

8.2 Protocols and recipes

8.2.1 Operation protocol NAP-5

Operation protocol of GE Healthcare illustra NAP-5 column is found below. [25]
Note: Prior to commencing, ensure that the NAP-5 Columns have equilibrated to room temperature (20–25°C).

8.2.1.1 1. Column Preparation

a. Remove the top and bottom caps from the NAP-5 Column and allow the excess liquid to flow through the column. Support the column over a suitable waste receptacle to catch buffer flow through.
Note: Not allowing the excess liquid to fully flow through the column will result in slow flow rates.

8.2.1.2 2. Column Equilibration

a. Equilibrate the column with 10 ml of Buffer 1 (50mM Tris-HCl, pH 7.75).
Note: This volume corresponds to 3 complete refills of the column. Any nuclease-free buffer is suitable, including water or Tris/EDTA (TE); both are available from GE Healthcare. It is important to use the same buffer in both the Column Equilibration and the Elution steps.
b. Allow the Buffer 1 to completely enter the gel bed by gravity flow. Do not apply positive pressure.

8.2.1.3 3. Sample Application

a. Add the sample to the column in a maximum volume of 0,5 ml. Allow the sample to enter the gel bed completely.

Note: If the sample volume is less than 0,5 ml, and you wish to elute your sample using the minimum volume of Buffer 1 (see Buffer volume guide, Table 40), it is important not to adjust the sample volume to 0,5 ml with Buffer 1 at this point.

Table 40. Buffer Volume guide.

Column type	Sample volume (ml)	Volume of Buffer 1 for Column Equilibration step	Volume of Buffer 1 for Elution step(ml)
NAP-5	0,1	0,4	0,5
	0,25	0,25	0,7
	0,5 (max vol)	0	1,0

Note: Concentration of the DNA sample should be less than 1 mg/ml, as higher concentrations tend to reduce resolution and give lower yields due to increased viscosity.

b. Where a sample volume of less than 0,5 ml has been loaded onto the column, load additional Buffer 1 onto the column such that the combined volume of sample and buffer added equals 0,5 ml. Allow the Buffer 1 to enter the gel bed completely.

8.2.1.4 4. Elution

a. Place an appropriate size collection tube under the column.b. Elute the purified sample with an appropriate volume of Buffer 1 (Table 15).

Note: If you require a concentrated sample, collect 0,1 ml fractions as the sample elutes off the column. The concentration of the different fractions can be quantified using a spectrophotometer or by loading 5 µl of each fraction onto an analytical gel (agarose or acrylamide).

c. Store the purified sample at -20°C.

8.2.2 Conjugation of antibodies

8.2.2.1 Biotinylation of antibodies

Materials:

Monoclonal antibodies RCK103, AE14 (batch 1), 3E2F1, 1E1, 1A12 (batch 1), 1A12 (batch 2), G-2, XM26, AE14 (batch 2)
Polyclonal antibody PAbK5
Biotin N-hydroxysuccinimide ester (BHNS)
Dimethyl sulfoxide (DMSO)
Sodium bicarbonate ($NaHCO_3$)
Elution buffer Tris-HCl, 50 mM
6 M Sodium hydroxide (NaOH), 6 M
Sodium chloride (NaCl), 0.15 M
Bovine serum albumin (BSA), 100 mg/ml
NAP-5 column

8.2.2.1.1 Preparation of solutions

1 M $NaHCO_3$ buffer, pH 8.5

8.4 g $NaHCO_3$ was dissolved in 100 mL H_2O. pH was adjusted to 8.5 with 6M NaOH. The buffer was filtered through a 0.22 µm filter.

The general reaction proportions used are presented in Table 41 and actual proportions for each reaction are seen in Table 42.

Table 41. Reaction proportions of the biotinylation reaction.

Reaction proportions			
Molar concentration Antibody	Volume Antibody	Molar concentration BNHS	Volume 1M Carbonate Buffer
A µM	B µl	10×A µM	B/9 µl

Table 42. Proportions of each biotinylation reaction.

Antibody	Amount BNHS (mg)	Molar concentration Antibody (µM)	Desired molar concentration BNHS	Molar concentration BNHS (mM)	Volume Antibody (µl)	Volume BNHS (µl)	Volume 1M Carbonate Buffer (µl)
RCK103	6	3.025	30.625	1.32	70	1.6	7.8
AE14 (batch 1)	6	7.25	72.5	1.32	80	4.4	8.9
3E2F1	6	12.5	125	1.32	80	7.6	8.9
1E1	5	29.19	291.9	1.1	80	21.2	8.9
1A12 (batch 1)	5	3.77	37.7	1.1	170	5.8	18.9
PAb K5	5	5.61	56.1	1.1	90	10	10
1A12 (batch 2)	5.2	4.12	41.2	1.14	190	6.8	21.1
G-2	5.7	6.25	62.5	1.254	160	8	17.8
XM26	5.7	11.875	118.75	1.254	150	14.2	16.7
AE14 (batch 2)	6	11.56	115.6	1.32	120	10.5	13.3

8.2.2.2 Horseradish peroxidase (HRP) conjugation of Ks19.1

Materials: Monoclonal antibody, Ks19.1
Horseradish peroxidase (HRP) enzyme
StabilZyme HRP, Surmodics
Sodium chloride (NaCl), 0.15 M
Sodium bicarbonate ($NaHCO_3$)
Sodium hydroxide (NaOH), 6M
Acetate buffer, 0.05 M, pH 4.0
Acetic acid (HAc)
Sodium periodate ($NaIO_4$)
Sodium borohydride ($NaBH_4$)
Bovine serum albumin (BSA), 100 mg/ml
Sephadex NAP-10, NAP-5 column
Sephacryl S200-HR column, 43.3 mL

8.2.2.3 Preparation of solutions

8.2.2.3.1 $NaHCO_3$ buffer 0.1 M, pH 9.2

0.84 g $NaHCO_3$ was dissolved in 100 mL H_2O. pH was adjusted to 9.2 with 6M NaOH. The buffer was filtered through a 0.22 µm filter.

8.2.2.3.2 $NaHCO_3$ buffer 1 M, pH 9.2

8.4 g $NaHCO_3$ was dissolved in 100 mL H_2O. pH was adjusted to 9.2 with 6M NaOH. The buffer was filtered through a 0.22 µm filter.

8.2.2.3.3 3 M HAc

17.16 mL HAc was dissolved in 82.84 mL H_2O.

8.2.2.4 Conjugation

The Sephacryl S200-HR column was washed with 300 ml of 0.15 M NaCl (Elution buffer) with 0.5 ml/h.
Reaction proportions:
Amount MAb for conjugation: 5.168 mg, 2.584 mg/ml
Volume Mab for conjugation: 2 mL

Amount of MAb: 5.168 mg
Amount of HRP: 5.186/2 =2.584 mg
Volume HRP solution for conjugation: 2.584mg /4 mg/ml = 0.646
Total volume: 3.09 mL

Volume MAb-HRP solution: 3.09 mL

$$\text{Volume } NaBH_4 = \frac{Volume\ MAb{-}HRP\ solution}{39} = \frac{3.09}{39} = 0.079\ mL$$

Total volume: 3.88 mL

8.2.3 Keratin extraction from CaSki cell line

Materials:
- CaSki cell pellets (freezed)
- Tris (hydroxymethyl) aminomethane (TRIS)
- Sodium chloride (NaCl)
- Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$)
- DNAse (freeze dried)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Sodium hydroxide (NaOH) 5 M
- Hydrogen chloride 3 M
- Hydrogen chloride 6 M
- Magnesium sulphate heptahydrate ($MgSO_4$ x $7H_2O$)
- Disodium phosphate dihydrate (Na_2HPO_4 x $2H_2O$)
- Ethylenediaminetetraacetic acid (EDTA)
- Sodium dodecyl sulphate (SDS)
- Ethanol 95%

Urea $CO(NH_2)_2$
Purified water
1 % PBS-BSA 0.1% Tween20 buffer

8.2.3.1 Buffer preparation

8.2.3.1.1 Cytoskeleton buffer pH 7.6

TRIS	1.21 g/L	10 mM
$MgSO_4$ x $7H_2O$	1.2324 g/L	5 mM
NaCl	8.18 g/L	140 mM
Purified water	1 L	

The TRIS, $MgSO_4$ x $7H_2O$ and NaCl was dissolved in 950 mL purified water. The solution was mixed with magnetic stirrer for 10 minutes. pH was adjusted to 7.6 with 3 M HCl. The volume was adjusted with purified H_2O to 1 L. The solution was filtered through a 0.2 µm filter. The buffer was stored at room temperature.

8.2.3.1.2 Cytoskeleton buffer with 0.5 % Triton X-100

1 ml of Triton X-100 was added to 200 mL Cytoskeleton buffer pH 7.6. The solution was mixed with magnetic stirrer for 20 minutes. The solution was filtered through a 0.2 µm filter. The buffer was stored in room temperature.

8.2.3.1.3 DNAse 10 mg/ml

4 mg of DNAse was dissolved in 0.4 mL of purified water. The solution was stored in freezer.

8.2.3.1.4 EDTA 10 mM in NaOH solution

11.7 mL of 5M NaOH was added to 200 mL purified water, to the final concentration of 0.277 M NaOH. 1.49 g EDTA was dissolved in the NaOH solution with magnetic stirrer.

8.2.3.1.5 Phosphate buffer 0.013 M pH 7.5

Na_2HPO_4 x $2H_2O$	2.08 g
KH_2PO_4	0.23 g
Purified water	

The components were dissolved in 500 mL purified water by stirring 10 minutes with a magnetic stirrer.

8.2.3.1.6 EDTA in phosphate buffer pH 7.5

210 mL of phosphate buffer was added to the EDTA in NaOH solution.

8.2.3.1.7 Urea 9.5 M in EDTA/phosphate buffer pH 7.5

57 g of Urea was dissolved in 50 ml EDTA in phosphate buffer with magnetic stirring. The solution was diluted with 100 mL phosphate buffer.

8.2.3.1.8 2 % SDS with 10 mM EDTA in 50 mM Na_2HPO_4, pH 7.8

Na_2HPO_4 x $2H_2O$	8.9 g/L
EDTA	3.72 g/L
SDS	20.0 g/L
Purified water	1 L

500 mL of buffer was prepared by dissolving EDTA, SDS and Na_2HPO_4 x $2H_2O$ in 500 mL of purified water. pH was adjusted with 5M NaOH to 7.5. The buffer was stored in room temperature.

8.2.4 Western Blot

Materials: Recombinant K5 0.29 mg/ml, 100 mM NaH_2PO_4, 10mM Tris-HCl, 8 M Urea, 1000 mM imidazole. N-terminal His-tag. Source: E.coli, Species: Human Lotnr: 25G0913W, AH Diagnostics.
Recombinant K19 freeze dried, diluted with water to the final concentration of 1 mg/ml Keratin 19, 30 mM Tris-HCl, pH 8, 9.5 M Urea, 2 mM DTT, 2 mM EDTA, 10 mM methylammonium chloride, Progen
Recombinant K8 freeze dried, diluted with water to the final concentration of 1 mg/ml Keratin 19, 30 mM Tris-HCl, pH 8, 9.5 M Urea, 2 mM DTT, 2 mM EDTA, 10 mM methylammonium chloride, Progen
Dithiothretol (DTT), 1 M
6M Urea in PBS
2X Laemmli sample buffer
Mini-Protean® TGX Stain-Free™ Precast gel, 15 wells, Bio-Rad
Ladder: Precision Plus Protein™ WesternC™, Bio-Rad
Running buffer: 25 mM Tris, 192 mM Glycine, 0.1 % (w/v) SDS buffer, pH 8.3
Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Pack including filter, nitrocellulose membrane and buffer ready to use, Bio-Rad
Trans-Blot® Turbo™ Transfer System, Bio-Rad
Wash Buffer: TBS Tween 0.1% Buffer
Nonfat dry milk powder, Blotting grade, Bio-Rad
Antibody Ks19.1, 2.5 mg/ml
Antibody AE14, biotinylated, 297 µg/ml
Antibody AE14, unlabeled, 1.85 mg/ml
Antibody 1A12, biotinylated, 138.5 µg/ml
Antibody G-2, biotinylated, 233.7 µg/ml
Antibody K8, Ks8.7, Mouse monoclonal, IgG1, Cat. No 61038, Lot 706180, Progen, 50µg/ml (unlabeled)
HRP-conjugated antibody polyclonal Rabbit Antimouse Progen
Precision Protein™ Strep-Tactin-HRP conjugate, Bio-Rad
Detection solution: Clarity™ Western ECL Prime substrate
ChemiDOC™ Touch Imaging System, Bio-Rad

8.2.5 Enzyme immunoassay programs

8.2.5.1 Program A

- The microtiter plate was prewashed in strip mode wash by washing each strip one time with “wash buffer”.
- 50 µl sample was added to each well.

- 100 µl antibody solution was added to each well. The microtiter plate was incubated on shaker in room temperature for 1 hour.
- The microtiter plate was washed 6 times in strip mode with “wash buffer”.
- 100 µl TMB was added to each well. The microtiter plate was incubated for 30 minutes.
- The absorbance was measured at 620 nm by micro plate reader.

8.2.5.2 Program B

- The microtiter plate was prewashed in strip mode wash by washing each strip one time with “wash buffer”.
- 25 µl sample was added to each well.
- 100 µl antibody solution was added to each well. The microtiter plate was incubated on shaker in room temperature for 2 hours.
- The microtiter plate was washed 6 times in strip mode with “wash buffer”.
- 100 µl TMB was added to each well. The microtiter plate was incubated for 30 minutes.
- 100 µl Stop solution was added to each well
- The absorbance was measured at 4500 nm by micro plate reader.

8.2.5.3 Program C

- The microplate was prewashed in strip mode wash by washing each strip one time with “wash buffer”.
- 25 µl sample was added to each well.
- 100 µl of biotinylated antibody was added to each well. The microtiter plate was incubated on shaker in room temperature for 2 hours.
- The microtiter plate was washed 3 times in strip mode with “wash buffer”.
- 100 µl of HRP-conjugated antibody was added to each well. The microtiter plate was incubated on shaker in room temperature for 1 hour.
- The microtiter plate was washed 6 times in strip mode with “wash buffer”.
- 100 µl TMB was added to each well. The microtiter plate was incubated for 30 minutes.
- 100 µl Stop solution was added to each well
- The absorbance was measured at 450 nm by micro plate reader.

8.2.5.4 Program D

- The microplate was prewashed in strip mode wash by washing each strip one time with “wash buffer”.
- 25 µl sample was added to each well.
- 100 µl of biotinylated antibody was added to each well. The micro plate was incubated on shaker in room temperature for 2 hours.
- The micro plate was washed 3 times in plate mode with “wash buffer”.
- 100 µl of HRP-conjugated antibody was added to each well. The micro plate was incubated on shaker in room temperature for 1 hour.
- The micro plate was washed 6 times in plate mode with “wash buffer”.
- 100 µl TMB was added to each well. The micro plate was incubated for 30 minutes.
- 100 µl Stop solution was added to each well
- The absorbance was measured at 450 nm by micro plate reader.

8.2.6 CYRFA21-1 Reagents and protocol

A selection of the instructions for use and information about reagents is presented in this section. Only content of components used in the K5/19 test is described. Information of remaining components of the CYFRA21-1 test can be found on http://www.fdab.com/world_home/products/manual_kits/eia_kits/cyfra_21_1.html. [6]

8.2.6.1 Reagents

CalA: Phosphate buffered salt solution containing bovine serum albumin, an inert yellow dye, and a non-azide antimicrobial preservative.

CalF: The lyophilized calibrator contain 52.0 ng/ml CYFRA 21-1 antigen in a phosphate buffered salt solution containing bovine serum albumin, an inert yellow dye, and a nonazide antimicrobial preservative

Wash Buffer: A Tris-HCl buffered salt solution with Tween 20. Contains Germall II as preservative. To be diluted with distilled or deionized water 25 times before use.

8.2.6.2 Assay procedure

Perform each determination in duplicate for calibrators, controls and unknown samples. A calibration curve should be run with each assay. All reagents and samples must be brought to room temperature (20-25 °C) before use.

1. Start to prepare CYFRA 21-1 Calibrators, Controls 1 & 2, Wash Solution and Antibody Solution. It is important to use clean containers. Follow the instructions carefully.

2. Transfer the required number of microplate strips to a strip frame. (Immediately return the remaining strips to the aluminum pouch containing a desiccant and reseal carefully). Wash each strip once with the Wash Solution. Do not wash more strips than can be handled within 30 min.

3. Mix samples by gentle inversion. Do not Vortex for more than 1 second. Pipette 50 µL of the CYFRA 21-1 Calibrators (CAL A, B, C, D, E, and F), Controls 1 & 2 and unknown specimens (unknowns-Unk).

4. Add 100 µL of Antibody Solution to each well using a 100 µL 8-channel precision pipette (or a 100 µL precision pipette). Do not allow the pipette tip to touch the surface of the liquid in order to avoid carry-over.

5. Incubate the plate for 1 hour (± 5 min) at room temperature (20-25°C) with constant shaking of the plate using a microplate shaker.

6. After the incubation aspirate and wash each strip 6 times.

7. Add 100 µL of TMB HRP-Substrate to each well using the same procedure as in item 4. The TMB HRP-Substrate should be added to the wells as quickly as possible and the time between addition to the first and last well should not exceed 5 min.

8. Incubate for 30 min (± 5 min) at room temperature with constant shaking. Avoid exposure to direct sunlight.

9. Immediately read the absorbance at 620 nm in a microplate spectrophotometer

8.2.7 Recipe Biotin Dilution Buffer

The biotin dilution buffer used in the project the following components:

Tris	12.12 g
NaCl	58.4 g
EDTA	7.48 g
2-Methylisothiazolone	0.2165 g
Pluronic F68	20 mL
Patent Blue	0.0288 g
γ–globulins	0.2031 g
BSA	59.58 g
HBR-2	2.5 mL

8.3 Results and sample information

8.3.1 Evaluation of biotinylated antibodies

Results from absorbance measurement of evaluation of biotinylated antibodies RCK103, AE14 (batch1), 3E2F1, 1E1, 1A12 (batch1), PAbK5 are displayed in Table 43.

Table 43. Results from Biotinylation test of RCK103, AE14, 3E2F1, 1E1, 1A12, PAbK5. A-H denotes the wells on each strip.

	Strip 1 (RCK103)	Strip 2 (AE14)	Strip 3 (3E2F1)	Strip 4 (1E1)	Strip 5 (1A12)	Strip 6 (PAb K5)	Strip 7 Buffer
A	3.730	2.725	2.657	2.640	2.287	1.366	0.181
B	3.704	2.751	2.713	2.687	2.352	1.407	0.148
C	3.643	2.955	2.956	3.016	2.528	1.690	0.138
D	3.761	3.005	3.023	3.060	2.528	1.746	0.148
E	2.762	3.242	2.261	3.387	2.795	2.183	0.190
F	2.726	3.176	3.214	3.305	2.727	2.182	0.180
G	1.593	3.393	3.407	3.541	2.898	2.637	
H	1.557	3.381	3.412	3.599	2.908	2.663	

8.3.2 Enzyme immunoassay with endometrial cancer samples

Information about endometrial cancer are presented in Table 44. The results from the absorbance measurement is presented in Table 45.

Table 44. Serum samples from endometrial cancer run. He4 is a marker for ovarian cancer [20].

Sample-ID	He4 values
En1	208.9
En2	546.7
En3	222.1

En4	252.3
En5	383.7
En6	170.3
En7	137.4
En8	1800
En9	722.6
En10	131.1
En11	138.3
En12	203
En13	205.5
En14	158.4
En15	174.9
En16	241.4
En17	1016
En18	437.1
En19	279.6
En20	152.9
En21	448.2
En22	593.6
En23	172.2
En24	196.8
En25	393.1
En26	575.4

*Table 45. Results from Endometrial cancer study. * marks samples masked during concentration calculation.*

Sample	1A12 absorbance	G-2 absorbance	XM26 absorbance
C1	0,113	0,138	0,152
C2	0,114	0,143	0,157
C3	0.112*	0,148	0,158
C5	0,128	0,152	0,16
C9	0,231	0,297	0,239
B181	0,1035	0,134	0,126
B186	0,1255	0,137	0,132
EN01	0,16	0,389	0,141
EN02	0,119	0,132	0,135
EN03	0,126	0,168	0,14
EN04	0,127	0,14	0,13
EN05	0,122	0,217	0,128
EN06	0,106	0,159	0,123
EN07	0,101	0,122	0,13
EN08	0,101	0,122	0,124
EN09	0,113	0,165	0,149
EN10	0,111	0,164	0,132

EN11	0,125	0,139	0,145
EN12	0,128	0,124	0,135
EN13	0,117	0,13	0,134
EN14	0,109	0,121	0,122
EN15	0,108	0,123	0,114
EN16	0,135	0,147	0,145
EN17	0,113	0,132	0,146
EN18	0,112	0,13	0,141
EN19	0,136	0,15	0,162
EN20	0,133	0,164	0,174
EN21	0,12	0,131	0,151
EN22	0,108	0,132	0,146
EN23	0,117	0,134	0,143
EN24	0,169	1,366	0,18
EN25	0,114	0,158	0,188
EN26	0,122	0,147	0,159

8.3.3 Lung cancer study

Below samples used in the lung cancer study is presented and distribution on microtiter plate during the experiment. BD denotes healthy blood donor, BL benign lung disease and LC lung cancer. No sample information about blood donors is provided.

Table 46. Sample information of lung cancer samples used in the lung cancer study.

Sample code (during run)	Plate	Primary Diagnosis	Subtype	Overall Clinical Stage	Tobacco History
LC1	2	Lung Cancer	Small cell	Limited Stage	Previous Use
LC2	2	Lung Cancer	Squamous Cell Carcinoma	I	Previous Use
LC3	2	Lung Cancer	Squamous Cell Carcinoma	Unknown (Reviewed)	Current Cigarette
LC4	2	Lung Cancer	Adenocarcinoma	III-B	Previous Use
LC5	2	Lung Cancer	Squamous Cell Carcinoma	IV	Previous Use
LC6	2	Lung Cancer	Adenocarcinoma	III-A	Previous Use
LC7	2	Lung Cancer	Small cell	Limited Stage	Previous Use
LC8	2	Lung Cancer	Small cell	IV	Previous Use
LC9	2	Lung Cancer	Adenocarcinoma/Bronchioloalveolar	II	Previous Use
LC10	2	Lung Cancer	Adenocarcinoma	IV	Never Used

LC11	2	Lung Cancer	Squamous Cell Carcinoma	II	Previous Use
LC12	2	Lung Cancer	Large Cell	IV	Previous Use
LC13	2	Lung Cancer	Adenocarcinoma	III-A	Previous Use
LC14	2	Lung Cancer	Adenocarcinoma	IV	Current Cigarette
LC15	2	Lung Cancer	Squamous Cell Carcinoma	IIB	Previous Use
LC16	2	Lung Cancer	Squamous Cell Carcinoma	I	Previous Use
LC17	2	Lung Cancer	Squamous Cell Carcinoma	II	Current Cigarette
LC18	2	Lung Cancer	Squamous Cell Carcinoma	III-B	Current Cigarette
LC19	2	Lung Cancer	Adenocarcinoma	I-B	Current Cigarette
LC20	2	Lung Cancer	Squamous Cell Carcinoma	III	Previous Use
LC21	2	Lung Cancer	Squamous Cell Carcinoma	IIIA	Current Cigarette
LC22	2	Lung Cancer	Squamous Cell Carcinoma	I	Previous Use
LC23	2	Lung Cancer	Adenocarcinoma	III-A	Current Cigarette
LC24	1	Lung Cancer	Small cell	Extensive Stage	Current Cigarette
LC25	1	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC26	1	Lung Cancer	Small cell	Limited Stage	Previous Use
LC27	1	Lung Cancer	Squamous Cell Carcinoma	II	Previous Use
LC28	1	Lung Cancer	Large Cell	Unknown (Reviewed)	Previous Use
LC29	1	Lung Cancer	Small cell	Extensive Stage	Current Cigarette
LC30	1	Lung Cancer	Small cell	Limited Stage	Current Cigarette
LC31	1	Lung Cancer	Adenocarcinoma	II/III	Current Cigarette
LC32	1	Lung Cancer	Squamous Cell Carcinoma	Unknown (Reviewed)	Previous Use
LC33	1	Lung Cancer	Squamous Cell Carcinoma	I-B	Previous Use
LC34	1	Lung Cancer	Small cell	Extensive Stage	Previous Use

LC35	1	Lung Cancer	Squamous Cell Carcinoma	III	Current Cigarette
LC36	1	Lung Cancer	Squamous Cell Carcinoma	III-B	Previous Use
LC37	1	Lung Cancer	Small cell	Limited Stage	Current Cigarette
LC38	1	Lung Cancer	Small cell	Extensive Stage	Current Cigarette
LC39	1	Lung Cancer	Adenocarcinoma	IV	Current Cigarette
LC40	1	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC41	1	Lung Cancer	Squamous Cell Carcinoma	II-A	Previous Use
LC42	1	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC43	1	Lung Cancer	Small cell	Limited Stage	Previous Use
LC44	1	Lung Cancer	Small cell	Limited Stage	Previous Use
LC45	1	Lung Cancer	Squamous Cell Carcinoma	III-A	Previous Use
LC46	1	Lung Cancer	Small cell	IV	Current Cigarette
LC47	3	Lung Cancer	Squamous Cell Carcinoma	IIIB/IV	Previous Use
LC48	3	Lung Cancer	Adenocarcinoma	IV	Never Used
LC49	3	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC50	3	Lung Cancer	Adenocarcinoma	III	Previous Use
LC51	3	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC52	3	Lung Cancer	Squamous Cell Carcinoma	I	Previous Use
LC53	3	Lung Cancer	Small cell	Limited Stage	Previous Use
LC54	3	Lung Cancer	Adenocarcinoma	II-B	Current Cigarette
LC55	3	Lung Cancer	Squamous Cell Carcinoma	III	Current Cigarette
LC56	3	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC57	3	Lung Cancer	Small cell	Extensive Stage	Current Cigarette
LC58	3	Lung Cancer	Small cell	Extensive Stage	Previous Use
LC59	3	Lung Cancer	Squamous Cell Carcinoma	III-B (Wet)	Previous Use
LC60	3	Lung Cancer	Adenocarcinoma	IV	Current Cigarette
LC61	3	Lung Cancer	Adenocarcinoma	III-B	Previous Use
LC62	3	Lung Cancer	Adenocarcinoma	III-B (Wet)	Previous Use

LC63	3	Lung Cancer	Squamous Cell Carcinoma	III-B	Previous Use
LC64	3	Lung Cancer	Adenocarcinoma	I-A	Previous Use
LC65	3	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC66	3	Lung Cancer	Large Cell	IV	Previous Use
LC67	3	Lung Cancer	Small cell	Limited Stage	Current Cigarette
LC68	3	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC69	3	Lung Cancer	Small cell	Extensive Stage	Previous Use
LC70	3	Lung Cancer	Adenocarcinoma	I-A	Previous Use
LC71	3	Lung Cancer	Squamous Cell Carcinoma	IIA	Previous Use
LC72	3	Lung Cancer	Small cell	Limited Stage	Previous Use
LC73	3	Lung Cancer	Small cell	Limited Stage	Current Cigarette
LC74	3	Lung Cancer	Adenocarcinoma	II	Previous Use
LC75	3	Lung Cancer	Adenocarcinoma	IV	Never Used
LC76	3	Lung Cancer	Adenocarcinoma	I-A	Never Used
LC77	3	Lung Cancer	Adenocarcinoma	III-A	Previous Use
LC78	3	Lung Cancer	Adenocarcinoma	III-B	Previous Use
LC79	4	Lung Cancer	Large Cell	III-B	Previous Use
LC80	4	Lung Cancer	Large Cell		Current Cigarette
LC81	4	Lung Cancer	Squamous Cell Carcinoma	III-A	Previous Use
LC82	4	Lung Cancer	Large Cell	IV	Current Cigarette
LC83	4	Lung Cancer	Squamous Cell Carcinoma	IV	Previous Use
LC84	4	Lung Cancer	Adenocarcinoma/Bronchioloalveolar	I-A	Never Used
LC85	4	Lung Cancer	Small cell	Limited Stage	Previous Use
LC86	4	Lung Cancer	Squamous Cell Carcinoma	III-B	Previous Use
LC87	4	Lung Cancer	Adenocarcinoma	IV	Current Cigarette
LC88	4	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC89	4	Lung Cancer	Squamous Cell Carcinoma	IV	Previous Use
LC90	4	Lung Cancer	Adenocarcinoma	III-B	Current Cigarette
LC91	4	Lung Cancer	Small cell	Limited Stage	Current Cigarette
LC92	4	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC93	4	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC94	4	Lung Cancer	Adenocarcinoma	III-B	Previous Use

LC95	4	Lung Cancer	Adenocarcinoma	III-A	Current Cigarette
LC96	4	Lung Cancer	Squamous Cell Carcinoma	IIB	Previous Use
LC97	4	Lung Cancer	Squamous Cell Carcinoma	III-B	Current Cigarette
LC98	4	Lung Cancer	Squamous Cell Carcinoma	I	Current Cigarette
LC99	4	Lung Cancer	Squamous Cell Carcinoma	IV	Previous Use
LC100	4	Lung Cancer	Adenocarcinoma	III	Previous Use
LC101	4	Lung Cancer	Squamous Cell Carcinoma	III-B	Current Cigarette
LC102	4	Lung Cancer	Adenocarcinoma	I	Previous Use
LC103	4	Lung Cancer	Small cell	Extensive Stage	Previous Use
LC104	4	Lung Cancer	Small cell	Extensive Stage	Previous Use
LC105	4	Lung Cancer	Adenocarcinoma	III-B	Previous Use
LC106	4	Lung Cancer	Adenocarcinoma	IV	Previous Use
LC107	4	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC108	4	Lung Cancer	Squamous Cell Carcinoma	IV	Current Cigarette
LC109	4	Lung Cancer	Squamous Cell Carcinoma	IV	Previous Use
LC110	4	Lung Cancer	Adenocarcinoma	IV	Current Cigarette

Table 47. Sample information of benign lung disease used in the Lung cancer study.

Sample code	Diagnos
BL1	Asthma
BL2	Asthma
BL3	Asthma
BL4	Asthma
BL5	COPD
BL6	COPD and Asthma
BL7	Asthma
BL8	Asthma
BL9	COPD
BL10	Asthma
BL11	COPD
BL12	COPD and Asthma
BL13	Asthma

BL14	COPD
BL15	Asthma
BL16	Asthma
BL17	Asthma
BL18	Asthma
BL19	COPD and Asthma
BL20	COPD
BL21	Asthma
BL22	Asthma
BL23	Asthma
BL24	Asthma
BL25	COPD
BL26	COPD and Asthma
BL27	Asthma
BL28	Asthma
BL29	Asthma
BL30	Asthma
BL31	COPD
BL32	Asthma
BL33	Asthma
BL34	Asthma
BL35	Asthma
BL36	Asthma
BL37	COPD
BL38	COPD
BL39	COPD
BL40	COPD
BL41	COPD
BL42	COPD
BL43	Asthma
BL44	COPD
BL45	COPD
BL46	Asthma
BL47	COPD
BL48	Asthma
BL49	Asthma
BL50	Asthma
BL51	Asthma
BL52	Asthma

8.3.3.1 Plate 1

Below can the calibrators and samples used on plate 1 be seen along with their respectively values.

Table 48. Calibrators from Plate 1 in the Lung cancer study. Concentration refers to concentration determined from CYFRA21-1 and back calculated concentration is the measured concentration. Co01=Blood donor serum control, Co02= Bladder cancer serum control.

Sample	Wells	Concentration	Back calculated concentration	Absorbance
C1	A1	0	-0,448	0,116
	B1		0,448	0,118
C3	C1	2,236	1,447	0,121
	D1		2,779	0,129
C5	E1	4,908	4,685	0,15
	F1		5,125	0,156
C6	G1	7,717	7,221	0,181
	H1		12,599	0,186
C7	A2	9,877	6,568	0,175
	B2		7,376	0,182
C8	C2	21,085	21,318	0,326
	D2		20,853	0,317
C9	E2	32,056	31,852	0,514
	F2		32,26	0,521
Co01	G2	-		
Co02	H2	-		

Table 49. Samples from lung cancer study plate 1 are shown. Wells refers to positon on the plate where the number denotes the strip and the letter position within the strip (A-H). Negative values were set to 0. BD = blood donor, BL = Benign Lung disease and LC =Lung cancer. Values marked green are those considered as positive.

Sample	Wells	Absorbance 1A12	Concentration 1A12
BD639	F3	0,088	0
BD640	G3	0,092	0
BD641	E4	0,099	0
BD642	F4	0,09	0
BD643	D6	0,092	0
BD644	C6	0,1	0
BD645	G7	0,104	0
BD646	H7	0,118	0,448
BD647	A5	0,094	0

BD648	B5	0,088	0
BD649	E8	0,118	0,448
BD650	F8	0,09	0
BL1	A3	0,086	0
BL10	F5	0,087	0
BL13	G6	0,133	3,223
BL14	H6	0,095	0
BL17	A7	0,107	0
BL18	B7	0,084	0
BL2	B3	0,084	0
BL21	C8	0,129	2,779
BL22	D8	0,093	0
BL5	C4	0,099	0
BL6	D4	0,09	0
BL9	E5	0,087	0
LC24	C3	0,142	4,051
LC25	D3	0,1	0
LC26	E3	0,097	0
LC27	H3	0,145	4,297
LC28	A4	0,101	0
LC29	B4	0,09	0
LC30	G4	0,156	5,125
LC31	H4	0,11	0
LC32	C5	0,099	0
LC33	D5	0,145	4,297
LC34	G5	0,106	0
LC35	H5	0,107	0
LC36	A6	0,119	0,842
LC37	B6	0,111	0
LC38	E6	0,117	0
LC39	F6	0,096	0
LC40	C7	0,101	0
LC41	D7	0,094	0
LC42	E7	0,09	0
LC43	F7	0,097	0
LC44	A8	0,094	0
LC45	B8	0,092	0
LC46	G8	0,101	0

8.3.3.2 Plate 2

Below can the calibrators and samples used on plate 2 be seen along with their respectively values.

Table 50. Calibrators from Plate 2 in the Lung cancer study. Concentration refers to concentration determined from CYFRA21-1 and back calculated concentration is the measured concentration. Co01=Blood donor serum control, Co02= Bladder cancer serum control.

Sample	Concentration	Wells	Back calculated concentration	Absorbance
C1	0	A1	2,33	0,113
		B1	Out of range	0,105
C3	2,236	C1	2,498	0,114
		D1	1,89	0,111
C5	4,908	E1	5,074	0,14
		F1	4,741	0,136
C6	7,717	G1	7,015	0,163
		H1	8,497	0,178
C7	9,877	A2	9,676	0,189
		B2	10,072	0,193
C8	21,085	C2	21,281	0,372
		D2	20,889	0,365
C9	32,056	E2	32,675	0,562
		F2	31,437	0,542
Co01		G2	Out of range	0,094
Co02		H2	Out of range	0,103

Table 51. Samples from lung cancer study plate 2 are shown. Wells refers to positon on the plate where the number denotes the strip and the letter position within the strip (A-H). Negative values were set to 0. BD = blood donor, BL = Benign Lung disease and LC =Lung cancer. Values marked green are those considered as positive.

Sample	Wells	Absorbance 1A12	Concentration 1A12
BD651	F3	0,097	0
BD652	G3	0,092	0
BD653	E4	0,083	0
BD654	F4	0,085	0
BD656	A5	0,099	0
BD657	B5	0,083	0
BD658	C6	0,084	0
BD659	D6	0,083	0
BD661	H7	0,086	0
BD660	G7	0,082	0
BD662	E8	0,084	0
BD663	F8	0,103	0

BL11	E5	0,081	0
BL12	F5	0,081	0
BL15	G6	0,083	0
BL16	H6	0,091	0
BL19	A7	0,095	0
BL20	B7	0,089	0
BL23	C8	0,085	0
BL24	D8	0,087	0
BL3	A3	0,108	0
BL4	B3	0,092	0
BL7	C4	0,086	0
BL8	D4	0,082	0
LC1	C3	0,097	0
LC10	D5	0,143	5,322
LC11	G5	0,121	3,373
LC12	H5	0,106	0
LC13	A6	0,097	0
LC14	B6	0,09	0
LC15	E6	0,128	4,049
LC16	F6	0,13	4,228
LC17	C7	0,109	0
LC18	D7	0,1	0
LC19	E7	0,102	0
LC2	D3	0,103	0
LC20	F7	0,104	0
LC21	A8	0,155	6,32
LC22	B8	0,098	0
LC23	G8	0,108	0
LC3	E3	0,088	0
LC4	H3	0,129	4,139
LC5	A4	0,208	11,382
LC6	B4	0,181	8,823
LC7	G4	0,146	5,57
LC8	H4	0,094	0
LC9	C5	0,115	2,649

8.3.3.3 Plate 3

Below can the calibrators and samples used on plate 3 be seen along with their respectively values.

Table 52. Calibrators from Plate 3 in the Lung cancer study. Concentration refers to concentration determined from CYFRA21-1 and back calculated concentration is the measured concentration. Co01=Blood donor serum control, Co02= Bladder cancer serum control.

Sample	Concentration	Wells	Back calculated concentration	Absorbance
C1	0	A1	-0,07	0,117
		B1	0,07	0,122
C3	2,236	C1	2,22	0,196
		D1	2,252	0,197
C5	4,908	E1	5,419	0,276
		F1	4,44	0,256
C6	7,717	G1	8,056	0,345
		H1	7,323	0,319
C7	9,877	A2	9,699	0,423
		B2	10,056	0,441
C8	21,085	C2	19,801	0,899
		D2	22,309	1,029
C9	32,056	E2	31,126	1,559
		F2	32,986	1,677
Co01		G2	-0,911	0,087
Co02		H2	-0,348	0,107

Table 53. Samples from lung cancer study Plate 3 are shown. Wells refers to positon on the plate where the number denotes the strip and the letter position within the strip (A-H). Negative values were set to 0. BD = blood donor, BL = Benign Lung disease and LC =Lung cancer. Values marked green are those considered as positive.

Sample	Wells	Absorbance 1A12	Concentration 1A12
BD668	E5	0,097	0
BD669	F5	0,093	0
BD664	A3	0,121	0,042
BD665	B3	0,098	0
BD666	C4	0,157	1,054
BD667	D4	0,186	1,909
BD670	B6	0,103	0
BD671	C6	0,147	0,769
BD672	C7	1,102	23,624
BD673	G8	0,132	0,348
BD674	H8	0,14	0,572
BD675	D9	0,102	0
BD676	E9	0,12	0,014
BD677	A10	0,142	0,629
BL25	E3	0,083	0

BL26	F3	0,097	0
BL27	F4	0,094	0
BL28	G4	0,101	0
BL29	A5	0,212	2,745
BL30	B5	0,183	1,818
BL31	E6	0,693	15,414
BL32	F6	2,428	>34
BL33	G7	0,105	0
BL34	H7	0,306	6,853
BL35	C8	0,179	1,697
BL36	D8	0,108	0
BL37	H9	0,228	3,31
BL38	D10	0,148	0,798
LC47	C3	0,153	0,94
LC48	D3	0,113	0
LC49	G3	0,129	0,265
LC50	H3	0,31	7,007
LC51	A4	0,113	0
LC52	B4	0,147	0,769
LC53	E4	0,152	0,911
LC54	H4	0,11	0
LC55	C5	0,13	0,293
LC56	D5	0,12	0,014
LC57	G5	0,118	0
LC58	H5	0,433	9,897
LC59	A6	0,228	3,31
LC60	D6	0,215	2,847
LC61	G6	0,091	0
LC62	H6	0,095	0
LC63	A7	0,14	0,572
LC64	B7	0,107	0
LC65	D7	0,273	5,263
LC66	E7	0,245	3,97
LC67	F7	0,241	3,808
LC68	A8	0,124	0,125
LC69	B8	0,104	0
LC70	E8	0,1	0
LC71	F8	0,129	0,265
LC72	A9	0,136	0,46
LC73	B9	0,096	0
LC74	C9	0,12	0,014

LC75	F9	0,171	1,46
LC76	G9	0,091	0
LC77	B10	0,168	1,372
LC78	C10	0,107	0

8.3.3.4 Plate 4

Below can the calibrators and samples used on plate 4 be seen along with their respectively values.

Table 54. Calibrators from Plate 4 in the Lung cancer study. Concentration refers to concentration determined from CYFRA21-1 and back calculated concentration is the measured concentration. Co01=Blood donor serum control, Co02= Bladder cancer serum control.

Sample	Concentration	Wells	Back calculated concentration	Absorbance
C1	0	A1	0,098	0,12
		B1	-0,098	0,111
C3	2,236	C1	2,477	0,216
		D1	2,014	0,201
C5	4,908	E1	5,654	0,279
		F1	4,158	0,254
C6	7,717	G1	7,919	0,343
		H1	7,496	0,326
C7	9,877	A2	9,643	0,434
		B2	10,112	0,461
C8	21,085	C2	20,971	1,04
		D2	21,199	1,052
C9	32,056	E2	31,626	1,623
		F2	32,486	1,671
Co01	-	G2	-0,578	0,089
Co02	-	H2	-0,141	0.109

Table 55. Samples from lung cancer study plate 4 are shown. Wells refers to positon on the plate where the number denotes the strip and the letter position within the strip (A-H). Negative values were set to 0. BD = blood donor, BL = Benign Lung disease and LC =Lung cancer. Values marked green are those considered as positive.

Sample	Wells	Absorbance 1A12	Concentration 1A12
BD682	E5	0,121	0,119
BD683	F5	0,159	0,961
BD406	D9	0,104	0
BD407	E9	0,136	0,446
BD408	A10	0,124	0,184
BD678	A3	0,1	0
BD679	B3	0,106	0

BD680	C4	0,201	2,014
BD681	D4	0,126	0,228
BD684	B6	0,089	0
BD685	C6	0,101	0
BD686	C7	0,192	1,767
BD687	G8	0,109	0
BD688	H8	0,096	0
BL39	E3	0,126	0,228
BL40	F3	0,095	0
BL41	F4	0,091	0
BL42	G4	0,118	0,054
BL43	A5	0,098	0
BL44	B5	0,095	0
BL45	E6	0,085	0
BL46	F6	0,117	0,033
BL47	G7	0,213	2,378
BL48	H7	0,099	0
BL49	C8	0,105	0
BL50	D8	0,107	0
BL51	H9	0,09	0
BL52	D10	0,086	0
LC100	A8	0,182	1,51
LC101	B8	0,107	0
LC102	E8	0,171	1,241
LC103	F8	0,107	0
LC104	A9	0,113	0
LC105	B9	0,096	0
LC106	C9	0,152	0,802
LC107	F9	0,092	0
LC108	G9	0,094	0
LC109	B10	0,087	0
LC110	C10	0,132	0,359
LC79	C3	0,11	0
LC80	D3	0,1	0
LC81	G3	0,088	0
LC82	H3	0,181	1,485
LC83	A4	0,209	2,251
LC84	B4	0,157	0,915
LC85	E4	0,089	0
LC86	H4	0,098	0
LC87	C5	0,131	0,337

LC88	D5	0,094	0
LC89	G5	0,112	0
LC90	H5	0,186	1,611
LC91	A6	0,201	2,014
LC92	D6	0,103	0
LC93	G6	1,348	26,671
LC94	H6	0,313	7,111
LC95	A7	0,11	0
LC96	B7	0,102	0
LC97	D7	0,123	0,163
LC98	E7	0,5	10,799
LC99	F7	0,19	1,714