Technology Development in the Field of Ligand Binding Assays

Comparison between ELISA and other methods

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Abstract

In this project, given to us by Mercodia AB, research in the field of immunoassays is done in order to investigate if there are methods that are better than the conventional ELISA. ELISA is known to have some issues, such as "The Hook effect", many washing steps and cross-reactivity with the antibodies used in the assay. Therefore the need of other methods has arised.

The result of the research showed that there are a huge number of methods that measure specific biomarkers. In this report 17 different techniques are presented. These techniques are: Mass Spectrometry (MS), Chemiluminescence Immunoassay (CLIA), AlphaLISA, Lateral Flow Immunoassay (LFIA), Microfluidics-based Immunoassays, Paper Based Immunoassays, Biosensors and Apatasensors, Immuno-PCR, Proximity Ligand Assay (PLA), Proximity Extension Assays (PEA), Meso-scale discovery (MSD), Multiplex Assay, Digital Bioassay, Bioluminescence Resonance Emission Transfer (BRET), Homogeneous Time Resolved Fluorescence (HTRF) and NanoBiT. Each of the listed methods are compared according to several parameters such as specificity, sensitivity, measure range, sample volume, degree of automation, runtime and cost for each analyzed sample.

The methods that showed an upward trend were: AlphaLISA, BRET, Biosensors, CLIA, Digital ELISA, methods using gold nanoparticles (AuNPs), HTRF, Immuno-PCR, Lateral Flow, MSD, Microfluidics, Multiplex methods, NanoBiT, paper-based, PEA, Simoa and Single molecule detection. The methods that showed a downward trend are: ELISA, mass spectrometry with immunoassay and PLA.

The conclusion is that methods that use multiplexing, are digital, use paper based immunoassay methods or that use microfluidics have a great potential in the future field of immunoassays.
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1 Explanation of words

**Analyte:** A substance in a sample that you want to analyze.

**Antibody:** A protein, an immunoglobulin, produced by the immune system as a response to an external unknown substance, known as an antigen.

**Antigen:** A molecule that can provoke an immune reaction. The molecule can be a chemical substance, protein or a carbohydrate. If an immune reaction occurs antibodies will form.

**Aptamer:** A synthetic antibody in the form of DNA or RNA.

**Autoantibodies:** Antibodies produced by the immune system that are specific for one or more proteins from the own body. They react with the body’s own tissue and cause autoimmune diseases.

**Beta-cells:** Cells in the pancreas that produce insulin and c-peptide and releases it to the rest of the body.

**Cross-reactivity:** The phenomenon of interference caused by molecules that are similar in structure as the target molecule which will create unwanted reactions. For example when an antigen binds to an antibody with similar structure that was not the initial target.

**Dynamic range:** The ratio between the largest and the smallest value that can be detected.

**Epitope:** The part of an antigen to which the antibody binds.

**Exogenous insulin secretion:** The origin of insulin is from a source outside of the body and is being inserted, commonly through an injection or a pump.

**Heterogeneous assay:** An assay that has multiple steps needed for the assay to be complete. This increases the risk of errors.

**Homogeneous assay:** An assay that is performed without any washing or separations steps, this minimizes the risk of errors that comes with multiple steps.

**Hyperglycemia:** The blood sugar is at higher levels than normal.

**Hypoglycemia:** The blood sugar is at lower levels than normal.

**Immunogenicity:** A substance’s ability to provoke an immune reaction.

**Insulin resistance:** The cells response to insulin are weakened.
**Isotopically labeled:** Labelling technique in which you replace specific atoms in a reactant with their isotope.

**Linear range (or range of linearity):** A limited linear span of a sigmoid curve during measurements that is crucial to be within to get an accurate result.

**Matrix effect:** The effect or noise caused by all the components in the sample apart from the target component.

**Monoclonal antibodies:** Antibodies that are made from identical immune cells and are considered clones from one unique parent. These antibodies bind to the same epitope.

**Multiplex assay:** Assay that can be able to measure several analytes in one run.

**Point-of-care-testing (POCT):** The principle of the healthcare products and the service of healthcare to be delivered to the patient at the time of care.

**Polyclonal antibodies:** Antibodies that can originate from different plasma cells and can bind to multiple epitopes.

**Synthetic antibodies:** An antibody-like molecule that has an affinity to a specific target but does not derive from an animal.
2 Aim

The purpose of this project is to find out if there are methods or technologies in the CRO and pharma industry that are currently beating ELISA off the immunoassay throne. The aim is to compare the technologies that we find against ELISA and determine the pros and cons. We also want to find out which technologies that are the most used when it comes to metabolic diseases. With this, we hope to determine which methods that have an upward or downward trend. The trend analysis could hopefully be of use for Mercodia AB in their development of detection technologies. We are also going to make an ethical analysis of the project, to conclude what ethical impact it could have on the society.

3 Delimitations

The delimitations we have decided to follow in our project are to compare only those methods used in clinical trials, mostly for metabolic diseases. Mainly, we will look at the biomarkers: insulin, glucagon, proinsulin, c-peptide and drug analogues for GLP-1 and insulin. However, we will not exclude methods that are primarily used for other biomarkers. Also, we will delimitate to discover only those methods used for human testing.

4 Background

In order to better understand our project, the reader might need some background information. In this section we will highlight important parts that are needed, to fully understand the rest of the project. This section will cover: Mercodia AB, metabolic diseases, ligand binding assays, ELISA (enzyme-linked immunosorbent assay), Clinical Research Organization (CRO) and pharma industry, clinical trials and our specific biomarkers.

4.1 Mercodia AB

Mercodia was founded in 1991 in Uppsala (Mercodia 2020a). They are a company that focuses on developing and manufacturing ELISA kits for detecting different biomarkers involved in metabolic disorders. Their products include ELISA kits for detecting biomarkers, such as c-peptide, glucagon, glucagon-like peptide-1 (GLP-1), insulin and proinsulin.
4.2 CRO and the pharma industry

CRO stands for contract or clinical research organizations, they contribute management services such as preclinical as well as clinical research and data management for pharma and biotechnological industries (Reist et al. 2013). Mercordia AB works with bioanalysis for pharma and CRO drug development. The pharma industry refers to the industry that develops and produces drugs.

4.3 Clinical trials

The clinical trials are health-related studies where a new analytical method, candidate drug or a new technology is being tested, evaluated and possibly approved. In a biomedical clinical trial, there are four phases. In the three first phases, the testing group increases for every approved phase. In the last phase, the size of the group does not change but the time of testing increases (WHO 2020).

4.4 Metabolic diseases

Metabolic diseases are caused by abnormal metabolic processes. This abnormality may be acquired as a result of a disease, inherited irregular enzyme activity or caused by a trauma or failure of a significant organ. Metabolism, which is the process where food is transformed to energy, is carried out by enzymatic proteins in several metabolic pathways. These biochemical reactions will be disrupted if a metabolic disease occurs (Britannica Academic 2018). A typical example of a metabolic disease is type II diabetes mellitus, that is caused by insulin resistance.

4.5 Ligand binding assay

Ligand binding assay (LBA) is an umbrella term for any assay where a ligand binds to a receptor, this includes immunoassays which utilize antibodies as ligands. LBA is a common technique within the CRO industry since most therapeutics and drugs are based on binding activity. The main purpose of LBA is to support and validate preclinical and clinical studies by determination of immunogenicity with biomarkers. It is also used to discover potential drug candidates (Sailstad et al. 2013). It is important that the LBA is robust in order to handle long-term clinical studies. Robust in the sense that the assay should not be affected by variations such as different analytes, equipment, lab environment and analysts (Tsou et al. 2014). Traditional LBA methods are CLIA (chemiluminescence immunoassay), RIA (radio immunoassay) and ELISA (enzyme-linked immunosorbent assay) (Sailstad et al. 2013).
4.6 Biomarkers

4.6.1 Proinsulin

Proinsulin is a precursor peptide to both insulin and c-peptide. It is formed within the lumen of the beta cells when the N-terminal of preproinsulin is cleaved (Sims et al. 2019). Proinsulin is cleaved into insulin and c-peptide in the golgi apparatus of the beta cells (ScienceDirect 2020). In proinsulin, the sequences for the two subunits, insulin A and B, are connected with the sequence that forms c-peptide (Vincent et al. 2013), see figure 1. According to Vincent et al. (2013), the peptide of proinsulin will undergo some other posttranslational modifications before the cleavage like cross-linking and folding. Proinsulin is sometimes used as a biomarker for diabetes (Wild 2013). In some immunoassays for insulin, cross-reactivity with proinsulin is a problem since the two peptides are quite similar according to Wild (2013). Some more specific immunoassays have the ability to measure both proinsulin and insulin to be able to discover abnormalities in the pancreatic beta cells.

![Figure 1: The illustration shows how the proinsulin peptide is structured. Illustration by Björn Ancker Persson.](image)

4.6.2 Insulin

The function of insulin in the human body is to regulate the glucose levels in the blood. In the case of hyperglycemia, more insulin will be produced by the beta cells which reduces the levels of glucose in the blood by inhibiting the glucose secretion from the liver (Shen et al. 2019). Hypoglycemia however, causes less insulin to be produced and instead glucagon will stimulate the liver to break down more glycogen into glucose, raising the glucose levels in the blood again. Type II diabetes mellitus makes the endogenous insulin, insulin produced by the body, less effective when reducing the glucose levels in the blood. This leads to chronic hyperglycemia (Shen et al. 2019). Patients that suffer from this disorder are often treated with daily injections of exogenous insulin. Insulin is a good biomarker for...
the detection and prediction of diabetes since any abnormalities in the insulin production can be a sign of the disorder.

4.6.3 C-peptide

C-peptide origins from proinsulin (Igano et al. 1980). It is a commonly used biomarker for detection of diabetes type I or II, since it is produced in the same extent as insulin and thus a good indicator of how much insulin is being produced in the body (Gresch et al. 2017; Jones & Hattersley 2013). If an individual with type I diabetes shows low levels of c-peptide, this could indicate that the breakdown of beta-cells are lacking or are incomplete (Williams et al. 2019).

4.6.4 Glucagon

Glucagon is a peptide hormone released by the pancreas (Unger & Cherrington 2012). It is released to avoid low blood glucose levels in the blood by stimulating the liver to break down glycogen into glucose. Unger & Cherrington (2012) proposed that an excess of glucagon is a more essential characteristic of diabetes than a deficiency in insulin.

The glucagon gene produces several different peptides; glucagon, oxyntomodulin, GLP-1 (Glucagon-Like-Peptide-1) and glicentin (Bak et al. 2014). All the peptides are formed through differential processing of proglucagon and their amino acid sequences overlap. Glucagon is the shortest peptide and both glicentin and oxyntomodulin contain the full glucagon amino acid sequence, see figure 2. The remaining parts of the proglucagon give rise to peptide fragments. Since all the peptides have similar amino acid sequences it is difficult to design immunoassays that can differentiate between all of them.
4.6.5 Drug analogs

Drug analogs are variations of naturally occurring substances. The reason to modify these molecules can vary but for insulin it can be categorized as short-acting and long acting. The short-acting one starts to act immediately after it has been injected and long-acting takes time before it starts acting and has a uniform activity. This is very useful because treatments for diabetes can be more flexible (Hirsch 2005).

Other important analogs, that also is involved with blood sugar and diabetes, are those of GLP-1. This is a hormone that promotes the secretion of insulin which makes it interesting for treating people with low levels of blood sugar. The main reason for developing a new version of this hormone is the short half-life it has in the plasma (Gupta 2013).

4.7 ELISA

ELISA (enzymatic-linked immunosorbent assay) is a method that uses the affinity between antigens and antibodies in order to detect biomarkers. Antigens in our case are the listed biomarkers above. The antigen is adsorbed (attached) to a plate and a protein, carbohydrate or detergent, not interacting with the antibody, is added in order to block non-specific interactions. An enzyme is linked to an antibody which will form a complex that adsorb to the antigen. This is later detected (Voller et al. 1978). Voller et al. (1978) also mentions that ELISA can be used in different forms such as: direct, indirect, competitive and sandwich, see figure 3. All of these methods
require an antigen and antibody reacting, resulting in a signal which is detected.

**Direct ELISA:** The sample with antigen is coated to a 96-well plate. An antibody conjugated to an enzyme is then added to the wells with antigen. The antigen and antibody binds to each other and a substrate specific to the enzyme conjugated to the antibody is added. If a reaction occurs a colorimetric change is observed (Voller et al. 1978).

**Indirect ELISA:** The sample with antigen is coated to a 96-well plate. A primary antibody, without an enzyme conjugate, binds to the antigen. A secondary antibody, with an enzyme conjugate, is added which binds to the primary antibody. A substrate specific to the enzyme is added which will give a colorimetric change when it reacts with the enzyme and will indicate the concentration of antibodies that have reacted with the antigen coated to the plate. Washing is done between each step (Voller et al. 1978).

**Sandwich ELISA:** A capture antibody is attached to the bottom of a well. Antigen from a sample is added and captured by the capture antibody. A specific primary antibody “A” is attached to the antigen. A specific secondary antibody “B” which is conjugated with an enzyme is added and binds to the primary antibody. A substrate, specific to the enzyme conjugated to antibody ”B”, is added and the colorimetric reaction that occurs is detected. Washing is done between each step (Voller et al. 1978).

**Competitive ELISA:** Here an antigen ”A” is coated on the bottom of a well. The test sample, which is thought to contain an inhibitor antigen ”B”, is mixed with the primary antibody. ”B” antigen-antibody complexes are formed but leaves some antibodies free. The ”B” antigen-antibody mixture with free primary antibodies are coated onto the plate. The free antibodies binds to the coated antigen ”A” on the bottom of the well. The ”B” antigen-antibody complexes do not bind to the plate and are washed off. A secondary antibody conjugated to an enzyme is added and binds the primary antibody that is bound to the antigen ”A”. A substrate specific for the enzyme is added which will react and make a signal that can be detected. The more antigen in the sample, the weaker the signal will be. This is because the primary antibody coupled with antigen ”B” is washed off. Washing is done between each step (Voller et al. 1978).
4.7.1 General problems with ELISA

ELISA is in general a very stable and reliable immunoassay. However, as for all assays there are potential problems, particularly with the reagents you use. For example, cross-reactivity between monoclonal antibodies and other non-related proteins can occur giving false results. The batch-to-batch quality can also differ which will not guarantee a consistency when using antibodies (Ali et al. 2019). Batch-to-batch refers to the quality of the antibodies of different batches. An example of this is, as researcher Lars Hellman (personal communication) pointed out; if you have ten goats that you use to get a large quantity of polyclonal antibodies and you take some blood from each and extract the antibodies and pool them together, you will have a stable antibody source for many years. However, when you run out of the pooled antibodies you need to redo the antibody production and this will not guarantee the same quality of the antibodies and the test therefore needs to be re-validated (researcher Lars Hellman, personal communication).

Another problem with ELISA is that the range of linearity in your measurement is very limited, since the measurement is usually done in a spectrophotometer. This can make it necessary for scientists to make a serial dilution of their samples in order to end up within the linear range of the measurement, since the measurement is usually made with OD (optical density). If the sample is not within the linear range, it could suffer from saturation and will not show an accurate reliable value (researcher Lars Hellman, personal communication). This phenomenon is known as...
the Hook Effect. Falsely high concentrations might occur when the immunoglobulins are multispecific and bind to multiple antigens (Hoofnagle & Wener 2009). This can be a source of error in further analysis. A possible solution for this is to use other detection methods than UV-VIS light. Fluorescence for instance is an alternative with a broader linear range.

An additional problem with ELISA is that it does not give any information about the size of the antigen. This can be problematic since you can not check the size of your antigen and therefore you need to have an extra control that the right antigen has been bound. Which means that if something else other than your target antigen happens to bind to your antibody you may get a false positive result (Hoofnagle & Wener 2009). In order to avoid this, it is important to use other methods as control together with ELISA, to check the quality of the sample. Examples of such methods can be western blot or mass spectrometry.

ELISA is not the best choice when detecting multiple targets at the same time. For many years ELISA has been used for protein detection and the method has a wide range of antibody pairs. This is because only one analyte is measured in each well. In addition, if ELISA is used for measuring several antigens it requires a large volume of samples which is a problem.

Considering the problems mentioned above, ELISA might not always be the best method to use and the need for new methods arises.

5 Method

The group divided the specific biomarkers from Mercodia AB, among themselves, to do further research. The biomarkers are listed in the background. A table with the methods and technologies found was constructed. The table contained: the specific biomarker, the method, the source and whether another group member could confirm the relevance of the method. The next step was to decide which methods that were worth looking into further and whether the method was relevant for the specific biomarkers. The methods were then classified into specific groups and each group member was in charge of doing more research for several methods and the articles that already had been found on the subject. During this part of the search the group members wrote down important facts that were going to be in the report and reflected over whether the method still seemed interesting for the project. After this, another round of pruning of methods was made, the remaining technologies
were inserted into the report.

To be able to compare our methods we have considered some different parameters. These include:

- Specificity
- Sensitivity
- Measure range
- Sample volume
- Degree of automation
- Runtime
- Required equipment
- Cost for each analyzed sample

These terms were given to us by Mercodia AB, but we also looked at other, related, parameters. For a detailed explanation of these terms see appendix B. Two trend analysis were also made, in order to determine the trends for each method, see section 8. To be able to visualize the trends, diagrams were made with data from PubMed. The query: ["Method") AND (immunoassay OR "ligand binding assay")] was inserted in the search, where "Method" was exchanged to the name of the method. For each method a trend graphs were made. In these graphs both an upward trend, see figures 21-36 appendix D, and a downward trend, see figures 38-40 appendix E, was noted.

Methods that we have decided not to include in our report are:

- MSIA-HR/AM(Mass spectrometric immunoassay with high resolution and Accurate Mass detection)
- LOCI (Luminescent oxygen channeling assay)
- TRFIA (Time-resolved fluorescence immunoassay)
- MEKC (Micellar electrokinetic chromatography)
- Liquid phase radiobinding assay
- RIA (Radioimmunoassay)
• ID-MS (Isotope dilution mass spectrometry)

This is because these methods were considered to be irrelevant for this project, since a lot of the mentioned method were found in old articles. We had to delimitate our project and therefore these methods were cut.

Reach-outs to experts and researchers in the immunoassay field were also made and several important inputs were collected.

6 Traditional methods

In this section we will present two other methods than ELISA, that have been used for a long time and that we consider as traditional. These methods are CLIA (chemiluminescence immunoassay) and MS (mass spectrometry). Beyond the technical background, this section will also cover the advantages and disadvantages of the methods.

6.1 CLIA: Chemiluminescent immunoassay

The chemiluminescent immunoassay or CLIA, is commonly used for measurement of different biomarkers in clinical studies, often insulin (Carslake et al. 2017). It is a combination between chemiluminescence technique and immunochemical reactions (Vo-Dinh 2003). An immunochemical reaction is the specific reaction between an antigen and antibody. Chemiluminescence is a technique that due to a chemical reaction generates electromagnetic radiation that is emitted and light is produced. The technique uses labeled antibodies to prove and measure the existence of a specific biomarker, like insulin, in a sample. If the biomarker is present, the chemiluminescent signal produced will correlate to the concentration of the biomarker in the sample (Shen et al. 2019). The chemical reaction occurs between the labeled antibody and other molecules added which produces light signals that are measured.

According to the company Creative Diagnostics, there are several ways to produce the light emission by labeling the antibodies in different ways. Depending on which label is used, different reactions occur which yield different intensities of the chemiluminescent signal. Some methods use enzymes as labels, to react with molecules and produce light signals. Since they use enzymes, these methods are similar to ELISA. Other methods use different types of molecules to label the antibodies.
The chemiluminescent technique is applied in many available methods that use the technique in different ways. Assays using this technique is still developing and expanding (Vo-Dinh 2003). One example of such a method is the microfluidic chemiluminescence immunoassay which is described further in 7.2.2.

In general, methods based on this technique have a high signal intensity, a reduced incubation time and a wide dynamic range (Shen et al. 2019). However, with advantages comes disadvantages. According to the article by Shen et al., it has a relatively high cost compared to other methods in the area.

6.2 MS: Mass Spectrometry

A common alternative to immunoassays when measuring biomarkers is mass spectrometry. Mass spectrometry is a technique that has the ability to identify and quantify compounds based on the analysis of the chemical structure. In this section, some mass spectrometry strategies will be discussed.

A difficulty when analysing blood plasma with mass spectrometry is the amount of different proteins in the sample (Holst & Wewer Albrechtsen 2019). To make MS techniques effective, other proteins must be removed apart from your target protein, while still recovering enough of the analyte to adequately quantify it. Many different methods used in conjunction with MS help mitigate this problem but they often have a limiting factor. To make the mass spectrometry specific, the sample could be enriched using antibodies that are specific for the biomarker. This could however cause cross-reactivity problems, similar to those of immunoassays.

6.2.1 LC-MS and LC-MS/MS

In recent years liquid chromatography coupled with mass spectrometry, LC-MS, has evolved to become an important tool for bioanalysis. Conventional ligand binding assays (LBA) are significant in the development of monoclonal antibodies as well as recombinant drugs, since the technique has high sensitivity and high capacity. The limitations regarding specificity and lack of giving structural information makes LC-MS more advantageous. LC-MS has higher specificity, is capable of analyzing multiple analytes simultaneously and has short runtime (Kang et al. 2020). The liquid chromatography part of LC-MS separates the compounds and the mass spectrometer generates a ratio between mass and charge that can be used to identify the structure and concentration in the sample. Before the mass spectrometer does its detection, the analyte from the liquid chromatography
needs to pass through an interface, such as electrospray ionization, that transfers the mobile liquid phase to the mass spectrometer unit (Pacific BioLabs 2020).

LC-MS has two strategies for protein analysis; bottom-up analysis and top-down intact analysis. The first one, bottom-up, digests the protein into pieces and is analysing surrogate peptides (internal standards) by using LC-MS/MS. These surrogate peptides are unique and should be resistant to modification. This approach is very sensitive when analysing large proteins (Kang et al. 2020). LC-MS/MS uses a tandem mass spectrometer which means that it uses at least two analyzers. For instance, in the first the mass is selected by a mass analyzer followed by characterisation in a second mass analyzer. This approach is more sensitive than LC-MS (Karolinska Institutet 2020). The second strategy of LC-MS is top-down, which means that fully intact proteins are analysed in the mass spectrometer (Nedelkov et al. 2018).

6.2.2 Silicon-nanoparticle-assisted MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has a high sensitivity, accurate quantification and high throughput when analysing biomarkers in body fluids (Wang et al. 2019). According to an article by the company Creative Proteomics, the principle of MALDI-TOF MS is that a soft ionization occurs (Creative proteomics 2020). When the MALDI-laser hits a matrix of small molecules it will sublimate them into gas-phase without fragmenting them, see figure 4. The article further explains that since MALDI does not decompose the sample molecules in the matrix, it is suitable for analysing biomolecules. Since the ions are given kinetic energy from the laser, they will “fly” over a free region (with no magnetic or electric field) until they hit a detector. The detector will use the time it took for ions to reach it to calculate the mass. The concept is called Time-Of-Flight (TOF).

According to Creative Proteomics the principle is that, ions with the same M/Z (mass to charge ratio) will hit the detector at the same time. Lighter ions will have a shorter time of flight until they hit the detector. However, Creative Proteomics points out that molecules of low abundance and low molecular weight are still difficult to quantify. The typical molecules used in the MALDI matrix give off ions that interfere at low mass regions. Wang et al. (2019) used a matrix of silicon nanoparticles joined together with antibodies to increase the signal of insulin. They claim this resulted in a very sensitive and accurate MS method that
was also simple, time-saving and cost-effective.

Figure 4: Schematic figure of the silicone-nanoparticle assisted MALDI-TOF MS adapted from (Wang et al. 2019), made by Tanya Al-Khafaf with the program Autodesk Sketchbook. 1) The silicone nanoparticles will bind to the antibodies. 2) The bound complex is added to the sample containing insulin. The insulin will bind to the antibodies which are specific for insulin. 3) The sample is added to a MALDI plate or a target plate. 4) The plate will be hit by a laser beam, the MALDI laser will sublimate the molecules into gas-phase and give them a kinetic energy so that they will "fly" over a region free from magnetic and electric fields. 5) The molecules will then hit a detector which uses the time of flight for identification.

6.2.3 Solid phase extraction HPLC-HRMS

Researchers have developed an MS method using protein precipitation and cation-exchange solid phase extraction (Thomas et al. 2020). The benefits are lower cost and shorter runtime. Using high resolution mass spectrometry (HRMS) gives a very good qualitative and quantitative result. Thomas et al. (2020) also multiplexed measurements of both c-peptide, insulin and its analogs. Furthermore, the method had better linear range in moderate biomarker levels than antibody-based sample preparation. Limitations were matrix effects during ionization and degradation of insulin in the presence of hemoglobin. The method lacks sensitivity because the sample is not enriched enough to use a nano-scale liquid chromatog-
raphy system. Therefore, it cannot cover insulin fasting states in human samples (Thomas et al. 2020).

7 Alternative methods in the field of immunoassays

In this section, some alternative methods in the field of immunoassays will be presented together with their advantages and disadvantages. The methods that will be mentioned below are not considered as traditional by us. For most methods, examples considering the biomarkers of interest will be presented. However, some methods will have other biomarkers than the biomarkers of interest or none at all. We consider all of the methods mentioned as relevant for the project.

7.1 AlphaLISA

This method is developed and distributed by Perkin Elmer and it is based on Luminescent Oxygen Channeling Assay or LOCI. The technique is based on the proximity between an acceptor bead and a donor bead, see figure 5. The acceptor bead is covered with antibodies for the analyte you want to find. When the analyte binds to the acceptor bead, another antibody which is biotinylated, binds to another part of your analyte. This complex is then able to recruit the streptavidin-coated donor beads. Biotin and streptavidin have a very high affinity to each other.

To get a signal from this complex the donor bead is excited by a laser with a wavelength of 680 nm. The LOCI technology is used in the next step since the donor bead will release a lot of singlet oxygen molecules upon being excited. Singlet oxygen molecules are $O_2$ molecules which have an excited electron which makes them reactive. When these come in contact with the acceptor bead, it emits a sharp peak around 615 nm which is proportional to the concentration of the analyte. If the donor bead is not part of a complex the singlet oxygen molecules will revert back to normal oxygen after about 200 nm. This ensures that no signal is produced for unbound donor beads i.e no false positives (PerkinElmer 2020).

AlphaLISA is a homogenous technology which means it does not require any washing at all which makes it easier to perform. The technology is versatile in the way that the acceptor and donor beads are adaptable. In the article by Tak For Yu et al. (2015), they integrated AlphaLISA on a microfluidic chip. It is a promising
technology with a lot of advantages, but there are some drawbacks when it comes to handling and special equipment. The donor beads are sensitive to light so you cannot work with them in a normal room. To excite the donor bead you need an instrument with laser excitation which can be expensive.

![Figure 5: Figure showing the AlphaLISA reaction. a) Streptavidin-coated donor bead absorbs the energy from a 680 nm laser. b) Excited oxygen molecules are released. c) Biotinylated antibody binds to the donor bead and the analyte. d) Analyte e) Acceptor bead covered in antibodies binds to the analyte and emits a signal when it comes in contact with the excited oxygen. Illustration by Björn Ancker Persson. Adapted from (PerkinElmer 2020).](image)

### 7.2 Microfluidics

One technique that offers a fast POCT (point-of-care-testing, a quick diagnostic or analytical test), and LOC (Lab-On-Chip) device and can be applied using different measurement methods is microfluidics. Lab-On-Chip is a commonly used phrase that describes having a laboratory device in a chip-format. Microfluidics is the knowledge of fluids in smaller scales. It is often in the micro- or nanometer scale (Rapp 2017). Two important aspects of microfluidic flows are the higher effects of surface tension and the lower or negligible effect of gravitational forces (Rapp 2017). In the following sections, we will present some approaches that use microfluidics systems coupled with several methods to detect and measure one or several of the biomarkers of interest.

#### 7.2.1 Microfluidic-based capillary electrophoresis immunoassay (CEIA)

During the past 10 years, microfluidic immunoassay has gained popularity. In the field of metabolic disorders, especially diabetes, microfluidic devices are becoming more frequently used. A microfluidic device or a microfluidic-based test can
use different labels and properties of the sample components to be detected and measured. One of the microfluidic-based tests is the capillary electrophoresis immunoassay (CEIA) to monitor glucagon secretion (Shackman et al. 2012). This approach made it possible to perform experiments on the cellular environment which may not be possible with other analytical methods. Experiments on a cellular environment are important due to the detection of glucagon secretion from the pancreas. CEIA is especially powerful when it is microfluidic-based because it allows fast on line separations. In addition, the use of a microfluidic system in mixing reagents decreases sample dilutions, making the tests more sensitive and more accurate. Glass microfluidic devices with 6 µm deep channels are fabricated with a bigger islet chamber to hold islet batches.

### 7.2.2 Microfluidic-based chemiluminescence immunoassay (CLIA)

One further technique based on microfluidics was introduced in a study from 2016 (Yao et al. 2016). The study was about developing a microfluidic-based chemiluminescence immunoassay (CLIA) to detect and measure insulin from samples. The test was also fully-automated. An integrated microfluidic chip with dimensions of 38 mm by 40 mm was fabricated. The chip had five microvalves and one micromixer where insulin reacted with antibodies and chemiluminescence signals were measured. The whole measurement took less than 10 min to complete. The main idea of this approach was to combine CLIA with a microfluidic detection system to get over some limitations, such as expensive supplies, long time consumption, and special laboratory skills. Monoclonal mouse antibodies, insulin antigens, and super-paramagnetic microparticles (strong magnetic particles) were used to capture the target insulin from the sample. All of this was fully automated in the microfluidic chip. Each reagent was loaded onto its corresponding chamber. This decreased the runtime for the same test from 60 min to 10 min. An important factor for developing the sensitivity of the assay was the flow rate of injecting the solution that was added directly before the chemiluminescence microparticles were emitted. If the flow rate would increase, the intensity peak of the chemiluminescence signal would be higher, indicating a higher sensitivity. The light intensity peak is proportional to the insulin level in the tested sample. Higher flow rate puts a higher static pressure on the chip, which can lead to leakage.
7.2.3 Microsphere-based microfluidic device

One more approach using a microfluidic system in our field of interest was reported in 2017 (Cohen et al. 2017). A microsphere-based rapid microfluidic device for quantification of insulin and some insulin analogs was introduced in this study. This approach is somewhat different from the others because it allows continuous detection of insulin levels, so called near real-time monitoring. Which also allows determination of the pharmacokinetic characteristics of insulin and its analogs. This is a benefit for diabetes patients because the rates of absorption of insulin and its analogs differ from patient to patient. Such an application could make it easier to avoid overdose of insulin analogs (Cohen et al. 2017). The biomarkers in focus were fast-acting insulin aspart, fast-acting insulin lispro, and RHI (regular human insulin). Microsphere-based assays are used in many multiplexed assays such as in Luminex platforms (Cohen et al. 2017).

According to the same article, detecting changes in insulin concentration with 30 seconds of interaction, the approach was a near real-time detection device. This occurred in the mixing region 2 of the device, see figure 6. First, the analyte (in this case insulin) streamed in from inlet 1. Streptavidin coated antibody conjugated microspheres streamed in from inlet 2 and met the analyte, see a in figure 6. The analytes and the microspheres were mixed in mixing region 1 to bind together. Fluorescent labeled detection antibodies streamed in from inlet 3 and together with the formed complexes streamed into mixing region 2 where they bound together and the signal was measured, see b and c in figure 6. The measurement was referenced by Abbott Architect insulin assay for measuring insulin in human blood and serum by chemiluminescence microparticle technology. Also, this approach required no washing steps saving much time which made it suitable as a LOC- device (Lab-On-Chip). The concentration was measured by detecting the fluorescent signal accumulation in the microsphere sensor. The signal accumulation was caused by insulin-antibodies complexes captured by the sensor in the very last region of the device.
7.2.4 Microfluidic chip for multi-sample ELISA

Another group of researchers developed a microfluidic chip for multi-sample ELISA (Dai et al. 2019). The chip also came with a semi-automated immunoassay instrument. This instrument was responsible for fluidic delivery and colorimetric detection. The microfluidic ELISA chip was made of three layers, where different reactions occurred. The assay in total, including the colorimetric detection, took 35 min. This approach was applied for detection of human IL-6 (interleukin-6).

7.2.5 Ella

Ella is an automated microfluidic analyzer that uses the sandwich immunoassay format. This technique can be single or multi-analyte microfluidic immunoassay (Dysinger et al. 2017). ProteinSimple, a platform division of Bio-Techne, is the company that has developed the Ella instrument and according to them this is the next-generation ELISA (ProteinSimple 2020).

ProteinSimple has developed an immunoassay platform called SimplePlex, which is loaded with samples and then inserted in the Ella instrument. The analysis will then run automatically, requiring no manually washing steps. This system can be used instead to quantitate four analytes from sixteen samples or a single analyte from 72 individual samples at the same time (Aldo et al. 2016). The analysis is done within an hour and takes place in a single disposable microfluidic plate. The loaded sample will run through a microfluidic channel and the protein of interest
will be bound to the capture antibody, while unbound analytes will be washed away. To be able to detect the binding, a detection antibody with a fluorescent label is added; a step that is also automated. Every plate is factory-calibrated and will generate a result based on its calibrated standard curve (ProteinSimple 2020). This method has many advantages compared to conventional ELISA; it requires a small sample volume, faster reaction-rate, easier since it is automated and also increases cost efficiency (Aldo et al. 2016).

### 7.3 LFIA: Lateral Flow Immunoassay

A lateral flow immunoassay (LFIA) is an immunoassay that uses the biochemical interaction of antigen-antibody or in other cases, the probe DNA-target DNA hybridization (Bahadır & Sezgintürk 2016). The standard LFIA tests are often small, chip-like devices that require small amounts of samples. Samples can be of blood or serum. Once the sample enters the sample pad, it migrates through the chip and makes its first stop at the conjugate pad, see figure 7. The conjugate pad gets rehydrated by the sample and the conjugate antibodies bind to their matched antigens from the sample. The complexes continue to migrate through the chip until they reach the test line where other antibodies catch the first generated complexes if matched. The caught complexes that bind to the antibodies stay on the chip and this causes the test line to change color. This indicates a positive result. The next stop for the sample is the control line. The principle is the same as earlier steps, only now it indicates that the test ran correctly. An LFIA test can contain several test lines after each other, which is called multiplex, but usually, there is only one control line.

Besides the detailed explanation of the standard LFIA tests, Bahadır & Sezgintürk (2016) also talked about some advantages and disadvantages of the technology. Some of the most important advantages are: short runtime, easy to operate and no need for trained personnel. It is also cheap and portable which makes it a very good technology for countries with limited healthcare resources, as effective point-of-care tests (POCTs). Some of the drawbacks with this technology are the low signal intensity and that the LFIA tests are qualitative and sometimes semi-quantitative, measuring the approximate concentration with the color intensity of the test line. Since the amount of the labeled capture antibodies on both the test lines and the control line are proportional to the amount of labeled reaction, the intensity signal can be improved by increasing the sensitivity of the test. One more advantage for the LFIA is a large variety of possible colored detector reagents that can be used. They
can, for example, be AuNPs, carbon nanotubes, magnetic particles (MPs), quantum dots (QDs), enzymes, or colored latex beads. This variety can be used to optimize the system and select the highest specificity and sensitivity (Bahadır & Sezgintürk 2016).

Figure 7: A standard LFIA chip. The waste and other sample components migrate through the whole chip reaching the last part of the chip called the absorbent pad. Illustration by Reneh Kostines, based on figure 1 from Pfützner et al. (2017) with modification.

7.3.1 Glass fiber sheet-based LFIA

In 2012, Oyama et al. (2012) introduced a glass fiber sheet-based electroosmotic LFIA for POCT to measure insulin and CRP (C-reactive protein) as two different model analytes (Oyama et al. 2012). CRP is a biomarker that indicates an infection caused by bacteria in the body. Electroosmosis is the principle of the motion of a liquid under the influence of an electrical field. The ratio of the bound and free labeled antibodies is measured. The test created then used the advantage of the negatively charged glass surface due to its capability of generating an electroosmotic flow. The analyte concentration was measured by coupling antibody-immobilized microbeads to the analyte (antigen). One of the main advantages of this approach is the low cost, also the easy-to-use procedure (Oyama et al. 2012).

7.3.2 LF-based POCT for proinsulin detection

In 2017, Pfützner et al. (2017) used a semiquantitative lateral flow-based POCT (point-of-care-test) to measure elevated levels of intact proinsulin as a biomarker
indicating a prediction of type II diabetes in a study stretched over 5-7 years (Pfützner et al. 2017). The test showed 87.5% sensitivity and 100% specificity compared to ELISA. A ready to use small chip-like device was introduced. The chip contained a sample pad where the blood sample was loaded. It also contained a cell filter pad where big components were filtered out, a detection pad with suitable antibodies for intact proinsulin where the intact proinsulin from the sample should bind in, see figure 7.

### 7.3.3 LFIAs for other biomarkers

For other biomarkers related to diabetes and its complications, LFIA is named many times. One of these biomarkers is glycated hemoglobin A1c (HbA1c) which is a key biomarker for showing the progress of type II diabetes. An approach combining AuNPs (gold nanoparticles) and sandwich assay with monoclonal anti-HbA1c antibodies, IgG1, making an LF-based immunosensor developed in purpose of both increasing sensitivity and selectivity but also decreasing the price and the need of professional lab skills (Ang et al. 2016). This test required 45 min to perform, but the time was shortened to 20 min by diluting the samples. Not only HbA1c but also the detection of cardiac troponin I (cTnI), as a biomarker for indication of one or more heart damages, by LFIA was discussed (Lou et al. 2019). In this approach, integration between nanospheres in the detection pad of the LFIA test chip increased the sensitivity of the test. The fluorescent signals recognized by binding antibodies were amplified using nanoprobes.

### 7.3.4 Multiplexed LFIAs

Under many years, LFIA was developed to become more multiplex. Yet, no relevant studies of multiplexed LFIAs were done in our field of interest in respect of biomarkers. Outside our field of interest, LFIA showed some great forward steps in multiplexity. A review of three different but common designs of multiplexed LFIAs was written by Huang et al. (2020) for a number of biomarkers. The three designs are based on different strategies of biomarker detection (Huang et al. 2020). The first strategy is building the strip with multiple test lines, where each test line has a specific antibody for a specific biomarker, see figure 7 for an example. The second strategy which requires more advanced fabrication is a multi-channel structured assay with multiple test strips. The third strategy is designing an LFIA with a single test line as described before but having multiple receptors on it. Where each receptor is specialized for a specific biomarker. Each
strategy of these will be combined with a signal detection method if the test is quantitative. The number of different combinations is huge due to the availability of many detection methods.

7.4 Paper Based Immunoassays

Several of the mentioned technologies in this report including chemiluminescence, ELISA, biosensors, microfluidic as well as lateral flow, can be applied using paper based systems. Paper based assays are convenient since they are very cost effective, easy to use and easy to transport for use in the field. Most modern immunoassays require large and expensive instruments which are operated with technical difficulty thus making them hard to apply in developing countries. Researchers in these areas therefore tend to use the cheaper alternative, such as paper based immunoassays (Liu et al. 2018b).

This is not only necessary in developing countries since immunoassays are frequently used in hospitals and central labs. Paper based immunoassays are useful due to the fact that they are cost effective, rapid and have on-site performance. The paper based systems usually contain antibodies as a detection element, paper as substrate and a reporter as signaling element. Some traditional paper types used in this approach are cellulose paper, nitrocellulose membrane and glass fiber paper. A newer and more novel type of paper is pseudopaper in which you can adjust the pore size, making it suitable for LFIA. Furthermore, filter paper is often used to fabricate microfluidic chips, used as substrate in immunoassays and as adsorption pads in lateral flow immunoassays.

7.5 Biosensors and Aptamers

The use of biosensors has become more and more popular throughout the years, a vast number of both biosensors and aptasensors have made their appearance on the market. Our definition of a biosensor is a technology that translates a biochemical signal to a readable signal, for example an electrical signal, which is then analyzed.

Some biosensors use aptamers (oligonucleotides) as the part that has specificity and high affinity towards a target molecule. Aptamers have been used in numerous ways, not only in the analyzing of samples but also in treatment by delivering the needed drug to the right cell. Aptamers are also more rigid than antibodies, since aptamers can withstand some heat, acidic environments and salt concentrations while anti-
bodies denature in the wrong conditions. When using aptamers in a sandwich set-up the aptamer will not be so affected after the capture of the target molecule so it should be possible to reuse the aptamer (Toh et al. 2015). Aptamers are smaller than antibodies, they are also cheaper and can be modified more easily. Instead of having the enzyme-linked immunosorbent assay, you will get the enzyme-linked apta-sorbent assay (ELASA). The methods are the same as the ones for ELISA (direct, indirect, sandwich and competitive) (Toh et al. 2015).

A biosensor that uses aptamerer is known as an aptasensor. A common aptasensor is the one that uses an electrochemical signal as the readout signal. The electrochemical signal can come from the difference in voltage from a reaction. Aptasensors have shown high selectivity towards some selected biomarkers. Indicating that the use of adapted biosensors can be used to detect other biomarkers as well (Hanif et al. 2019).

A typical example where biosensors are used, is in diagnostics and monitoring of diabetes. By measuring insulin levels, it is possible to diagnose diabetes since insulin is crucial for the glucose metabolism. In recent years, biosensors have become a great tool for monitoring glucose in the blood. Today a number of different sensors exist. They are, among some, in the form of: electrochemical, enzymatic, non-enzymatic, optical and non-invasive (Sabu et al. 2019). The use of biosensors can be applied to more than just biomarkers for diabetes. Here we will discuss some of the different biosensors and aptasensors available and both note the positive and negative sides of them.

7.5.1 Biosensors: the set-up and multiple different variations of the specific method

The first ever detection of insulin with biosensors was made with a ruthenium modified film in an environment of pH 2 (Singh & Krishnan 2018). The same biosensor was tested in a more neutral pH to mimic the human body. This biosensor was later developed to handle nanomolar concentrations. The development from the first biosensor has now come a long way.

The general set-up for the use of a biosensor requires an analyte of a biorecognition element (the antibody), the transducer and a signal processor that analyzes the result. A transducer is a way to measure your analyte with for example an electrochemical, piezoelectric or optical measurement. The sample needs to bind to the bioreceptor, and when it does, a biological reaction occurs. This is trans-
lated from a biochemical signal to an electronic signal. The last step is for the signal processor to process the signal and give an answer of what analyte it is and its concentration, see figure 8 (Singh & Krishnan 2018).

![Diagram of biosensor workflow](image)

Figure 8: The illustration explains the workflow of a biosensor. First, the analyte needs to react and bind to the antibody. This biochemical signal is translated to an electronic signal by the transducer. The transducer can be magnetic, thermometric, electrochemical, piezochemical and optical. The output signal from this is then processed in a signal processor, which interprets it and gives a final answer of what is in your sample. Illustration by Johanna Cederblad, adapted from (Singh & Krishnan 2018).

The combination of biomolecules and transducers makes it possible to develop analyzing tools for a vast number of applications. The upside of biosensors is that they can have a high selectivity to the target, have a varied range of detection and to be sensitive in its analysis. The possibility of a biosensor to be portable, opens up for point-of-care testing and treatment. A problem with the biosensors that were addressed in the article *Electrochemical and Surface Plasmon Insulin Assays on Clinical Samples* is that the non-specific binding of molecules present in the sample occurs on the free sensor surface (Singh & Krishnan 2018). This will affect the limit of detection and make it less sensitive. This can be prevented if specific blockers are used that physically blocks unwanted binding, like detergents. Now the biosensor can “focus” on the binding of the wanted analyte and the analysis of this. If there is no background noise, the possibility of analyzing complex samples as blood is made possible (Singh & Krishnan 2018).

### 7.5.2 Antibody aptamer immunoarray chip utilizing magnetic nanoparticles and fluorescent QD labels

In the article *Magnetite-Quantum Dot Immunoarray for Plasmon-Coupled-Fluorescence Imaging of Blood Insulin and Glycated Hemoglobin*, a biosensor was
used to detect insulin or glycated hemoglobin (HbA1c). In the study an SPR (surface plasmon resonance) gold microarray chip was used together with antibodies and aptamers. A step to prevent unwanted binding was also made. The antibody was attached to the aptamer for the specific biomarker. When the right binding occurred, a signal was made that indicated the level of insulin or HbA1c in the sample, see figure 9. The signal was captured by a CCD camera (charge-coupled device) and the intensity was noted before and after the electrostatic adsorption of the magnetic nanoparticles present in the biosensor. The difference in the two values was taken in order to determine the number of molecules that were present. Non-specific binding can occur in these sorts of assays if residue of the samples are left on the biosensor (Singh et al. 2017).

Figure 9: The illustration is presenting a workflow of how a biosensor with gold particles and aptamer could be set-up. A) Here the biosensor plate has gold nanoparticles as part of the detection set-up. The gold nanoparticles are connected with a linking component (here mercaptopropionic acid) that is attached to a capture component (here polyamidoamine dendrimer). B) Blocking agent, antibody and the aptamer with magnetic nanoparticles is added one by one to the gold nanoparticle set-up. C) After the components have been added a detection of a possible reaction is made. Illustration by Johanna Cederblad, adapted from (Singh et al. 2017).

7.5.3 AuNP-biosensor

AuNP-biosensor (gold nanoparticle) is an immunosensing method that can be used to detect c-peptide with a label-free electrochemiluminescent (ECL) signal. In this assay the electrode had an indium tin oxide glass as its conductive element. The glass had gold nanoparticles (AuNP) with hydrolysed trimethoxysilane as linker. The AuNP-biosensor was used to detect c-peptide, which is a by-product when insulin is secreted. That is why it is interesting to measure the amount in the body to possibly detect diabetes. One issue of constructing an immunosensor is to immobilize the antigen or antibody. That is why gold nanoparticles are used since they have properties that make the binding more efficient. This method was successful regarding sensitivity, low-cost and simplicity (Liu et al. 2018a).
7.5.4 Sandwich-type electrochemical immunoassay

In an article by (Sun et al. 2019) the use of a Sandwich-type electrochemical immunoassay is done. It uses a developed biosensor based on gold nanoparticle-modified MoS2 nanosheets as well as the hybridization chain reaction, to detect insulin.

7.6 Immuno-PCR

Immuno-PCR is described as the combination of an ordinary immunoassay and a PCR. One of the first formats of this technology had a DNA-antibody conjugate as a streptavidin-protein bridge as the detection antibody with its DNA reporter. There are however different versions of this; one concept has metal ions attached at the bottom of a well and are bridged to a conjugate, see figure 10. No matter the set-up, the first step is always to amplify the oligonucleotide, the aptamer (Chang et al. 2016).

The immuno-PCR is used to visualize the antigen-antibody interaction. The method allows visualization of samples with low concentrations. The method can be used in the same way as ELISA by formats such as: direct, indirect, sandwich and competitive. The principle is the same but the main difference is that instead of having an antibody reacting with an antigen, there is a conjugate between an antibody and an oligonucleotide (Ryazantsev et al. 2016). The conjugate is the connection between the immunoreaction and the amplification of the DNA, see figure 10. The detection can be made through a real-time PCR. However, the attachment of the oligonucleotide to the antibody is difficult. This step can be both costly and time-consuming (Expedeon 2020). It was one of the major down-sides noted in the article, Immuno-PCR: achievements and perspectives, that an immuno-PCR assay can take up to anything between 26 h to 2 days to perform (Ryazantsev et al. 2016). This is partly because of the numerous difficult steps that demand skills and knowledge in the handling of the different components in the assay.

According to Chang et al. (2016), a common method to detect a specific biomarker is to use gel electrophoresis. A downside to the immuno-PCR is the step when the amplified DNA is transferred from the PCR-tube to the gel, that is when the cross-contamination can occur. The agarose gel is also less sensitive than other methods. The advantages of immuno-PCR is that it is often very sensitive in detecting molecules in samples, it is also good regarding reproducibility and the method is also flexible. The disadvantage with this technique is that it has multiple steps that
require the user to have knowledge in the handling of both PCR and ELISA. Another problem is that excessive oligonucleotides may disturb the detection in the sample (Chang et al. 2016).

The conjugate opens for a more diverse use of the antibodies because the oligonucleotide will be the element of specificity. Because of the fusion between the PCR with its sensitivity, and ELISA with its flexibility, the immuno-PCR can be used to detect a vast number of molecules (Ryazantsev et al. 2016).

Why the immuno-PCR is so versatile is because it is able to detect protein antigens and antibodies that are corresponding to those antigens. Immuno-PCR requires a linkage-molecule between the detection antibody and the DNA tag molecule. The sample size of the sample that one wishes to analyze does not have to be that great. This is because the sample is being amplified in a PCR step before being fused together with the conjugate of antibody and DNA tag. Sample type is also versatile, with everything from blood to cell culture (Malou & Raoult 2011). Some of the immunoassays use gold in order to amplify the signal and to make the assay more sensitive. This is also the case with immuno-PCR (Chang et al. 2016).

One aspect that is difficult to interpret is the sensitivity, it is defined as the signal or concentration. Comparing different measurements as electrochemical or spectral will therefore be difficult to determine (Dahiya & Mehta 2019). The NP-I-PCR (nanoparticle immuno-polymerase chain reaction) has a lot of applications and the development of this could lead to a use of it in point-of-care testing. This method can also be applicable when detecting biomarkers for various diseases such as type II diabetes mellitus. There are however toxicity issues and the impact of nanomaterials on human health that needs to be addressed. Dahiya and Mehta (2019) noted that more research is needed before any extended use of the nanoparticle method can be made in diagnostics.
7.7 PEA: Proximity Extension Assay

In proximity extension assay (PEA), the target proteins are bound to pairs of oligonucleotide-conjugated antibodies. The oligonucleotides are extended with the help of DNA polymerase using each other (the two oligonucleotides, that are conjugated to antibodies) as templates, see figure 11. This will create a reporter strand. This strand can be quantified by a real-time PCR (Landegren et al. 2018).
7.8 PLA: Proximity Ligation Assay

In proximity ligation assay (PLA), the target protein is captured by an antibody. Oligonucleotide-conjugated antibodies are also binding to the target protein. In conclusion the target protein is attached to a capture antibody and oligonucleotide-conjugated antibodies. Excessive components are washed away. The oligonucleotides are now in close proximity to each other, due to the binding of antibodies to the target protein and are joined by ligation, see figure 12. The ligation of the oligonucleotide will lead to an amplification of the DNA strand, this amplification is quantifiable by a real-time PCR (Landegren et al. 2018). A problem that might arise is that the oligonucleotides will merge together and become an undetectable mess. To avoid this, reporter tags can be attached to the circular oligonucleotide. This will give a wider range of detection and to distinguish individual signals. Since the signal from each individual oligonucleotide is known, digital analysis is possible (Koos et al. 2014).

Both PEA and PLA can be quantified by DNA sequencing or real-time PCR.

![Proximity Ligation Assay (PLA) requires a pair of PLA probes to both bind to the same target protein. After this the two oligonucleotides that are complementary are ligated. This makes it possible to use a real-time PCR to detect the target protein in the analyte. Illustration made by Johanna Cederblad, adapted from (Cane et al. 2017).](image)

7.9 MSD: Meso-Scale Discovery

When we reached out to experts for advice on which methods that are relevant for the detection of our specific biomarkers, a researcher at Uppsala University advised us to look in the direction of meso-scale discovery (MSD) (José Caballero-Corbalan, personal communication).
While ELISA often uses colorimetric reactions (detection of change in color using absorbance), meso-scale discovery uses electrochemiluminescence (ECL, a luminescent signal caused by a redox reaction) to detect the occurrence of a specific component or biomarker in a sample, see figure 13. When performing a meso-scale discovery sandwich-assay, the capture antibody used will be attached to an electrode at the bottom of a well. The secondary antibody will be linked to a ruthenium metal ion. If a reaction has occurred and the secondary antibody has bound to the antigen, the electrode in the bottom of the well and the ruthenium metal ion will be close enough so that a redox reaction can occur. This reaction can be detected on a camera (CCD, charge-coupled-device). The same idea can be used for direct and indirect MSD-assays. The principle is that the ruthenium metal ion needs to be close enough to the electrode at the bottom of the well, for the reaction to be detected (Pacific BioLabs 2020).

In the article *A novel high-sensitivity electrochemiluminescence (ECL) sandwich immunoassay for the specific quantitative measurement of plasma glucagon*, MSD showed promising results when detecting glucagon with a sandwich assay with affinity-optimized monoclonal antibodies (Sloan et al. 2012). The assay proved to be robust and had a satisfying sensitivity and a broad range of detection.

MSD can be used in multiplex analysis. Since the capture antibody is attached to a specific place in the well which makes it possible to attach several capture antibodies onto the same plate. A camera can then detect from where in the well the redox-reaction occurred (Pacific BioLabs 2020).
Figure 13: A comparative illustration of the difference between a direct MSD and a direct ELISA. The direct MSD requires an electrode and a metal ion in order to detect the antigen in the analyte. The direct ELISA relies on a colorimetric change, when a substrate is added that will react with the antigen. Illustration by Johanna Cederblad, adapted from (Pacific BioLabs 2020).

7.10 Multiplex assays: the possibility of analyzing several biomarkers in the same run

In diagnostics today it is important to be able to measure many protein biomarkers simultaneously since it will provide better clinical information, which ELISA is unable to do. Today there are two classes of multiplex immunoassay that have different methods and protocols; planar and suspension microarray (Lee et al. 2018).

The more we learn about diseases and the human body, the more we realize that detection of multiple biomarkers in the same sample can be of more value. Here, the multiplex assays will be addressed and the principle behind some of the methods that uses this approach.

7.10.1 Multiplexed immunoassay using hydrogel microparticles

Suspension microarray is more favorable since it can offer solution-phase reaction kinetics when planar only offers solid-phase. It also has a higher precision when measuring a large amount of particles (Lee et al. 2018). Luminex technology is established and uses suspension arrays (R&D Systems 2020).

Luminex has shown a promising performance. This has led to the development of new suspension arrays in order to achieve multiplex arrays with high capacity, specificity, sensitivity, increased dynamic range and better detection time. One of these new approaches uses barcoded hydrogel microparticles. The code allows
a signal from every particle of hydrogel to be assigned to the corresponding target in a large mixture of particles (Lee et al. 2019). The hydrogel is a promising material because of its properties; it can intensify the binding kinetics and enhance the loading capacity in regards to capturing antibodies (Lee et al. 2019).

### 7.10.2 Au-NP multiplexed colorimetric immunoassay platform

One multiplex assay is a multiplexed colorimetric immunoassay platform that was used in order to facilitate the detection of cardiovascular disease (CVD), detecting CRP (c-reactive protein) and IL-6 (interleukin 6). In an article by Dong et al. (2020), the multiplex method that utilizes gold nanoparticles, was used in order to make the method less time-consuming, inexpensive and simpler to handle. The gold particles makes the binding of the antigen easier. During the use of the colorimetric immunoassay, milk was added onto the antibody microarray in order to prevent unwanted binding. All the signal and detection probes were added to the array, see figure 14. Then the signal was detected under a microscope. The detection probe will show a signal if the antibody, the antigen and the probe have been successfully linked to each other. No cross reaction was noted in the study, even after several interfering proteins like myoglobin or human serum albumin was added. No cross reaction occurred between the CRP and IL-6 antibodies as well. An observation by the authors was that if more detection probes would be used, then the signal from the microarray will be bigger and will give a clearer signal. The sensitivity of this multiplex assay was similar as the sensitivity of an ELISA (Dong et al. 2020).

![Figure 14](image.png)

Figure 14: Shows a schematic representation of the workflow of a multiplex assay that detects several antibodies at the same time. A) The antibodies are attached to the plate. B) A blocking agent is added to prevent unwanted binding, like BSA (Bovine Serum Albumin). The antigen is added after the blocking agent. C) Detection probes with AuNP (gold nanoparticles) bind to the antibodies on the plate. D) An amplification step is made so the signal will be greater. E) The signal is detected and analyzed. Illustration by Johanna Cederblad, adapted from (Dong et al. 2020).
7.10.3 IMMray Microarray Technology

A microarray has antibodies attached to the array-tray. When the sample is loaded onto the microarray, the correct analyte will attach itself to the correct antibody. The microarray format makes it possible to detect over 400 antibodies. A fluorescence scanner is used to scan the microarray in order to detect whether an analyte and antibody have reacted. The intensity of the signal determines the concentration of the analyte (IMMray® 2020). This microarray technology has been used to determine pancreatic ductal adenocarcinoma (PDAC), which is a signature biomarker that can be used to determine early-stage PDAC (Mellby et al. 2018).

7.11 Digital bioassays

Digital bioassays are a development of conventional bioassays which are performed in tubes or in microtiter plates. In a digital assay, the sample is divided into microreactors. This allows loading most of the sections with 0 or 1 target molecules. This is a binary system; a property that defines a digital bioassay. In this method only the positive signal is measured, instead of quantifying the intensity of the total amount of signals from tubes that traditional methods do, see figure 15. An appropriate mathematical model is then used for further quantification. A digital bioassay has the potential of being connected to existing traditional techniques that are used in laboratories today. This could reduce the workload, cost and difficulties that come with conventional methods (Zhang & Noji 2017).

There are some challenges to achieve single-molecule detection for proteins since they can not be amplified like nucleotides. A digital counting method was first used for fluorescent enzymes that transmit a signal naturally. Since ELISA uses enzyme-conjugated antibodies to target proteins, a combination with digital counting is possible. It has been done in detection of prostate-specific antigen (PSA) without substantial modification in the ligand-receptor affinity. This makes the transition from conventional ELISA to digital ELISA possible and quite easy. Since the technical obstacle is low, the development has accelerated in recent years and more than ten biomarkers in addition to PSA have been detected with this technique. Those biomarkers are Clostridium difficile toxins, Tau, HIV p24 antigen, dengue virus IgG and IgM and cytokines (Zhang & Noji 2017).
7.11.1 Single molecule digital detection

7.11.1.1 Digital HoNon-ELISA

Digital ELISA has potential and is a very powerful method. However, the technique requires several washing steps and an enzyme for signal amplification. In an article by Akama et al. (2019) a science group is proposing a new digital immunoassay called “Digital HoNon-ELISA” (digital homogeneous non-enzymatic immunosorbent assay). In this method, target antigens are reacting with magnetic nanoparticles coated with antibodies, that are magnetically dragged into reactors of a femtoliter-size. Antigens that have reacted with the particles are then captured by antibodies in the reactors. This is done via molecular tethers and is streamlined by magnetic force. Those particles that have bound correctly will show a characteristic Brownian motion, which differs from nonspecific binding. The concentration of the target antigen is directly related to the particles that are tethered. Using this technique no washing steps are required.

The method is comparable with digital ELISA regarding sensitivity and comparable with homogeneous assay regarding simplicity. The research group suggests this technique as a potential for future diagnostic use (Akama et al. 2019).

7.11.1.2 Simoa - Digital ELISA technology

Simoa technology is a digital method to measure proteins. This method lowers the detection limit and is based on the principle of single-molecule analysis. This type of measurement is naturally digital, where each molecule generates a signal that is countable, see figure 15 (Quanterix 2019). By using this technology it is possible to detect inflammation, cancer, neurology related diseases, biomarkers related to cardiovascular health, metabolism and infectious diseases (Wilson et al. 2016).

Quanterix is a company that uses Simoa technology that is based on arrays with reaction chambers of femtoliter-size that have the possibility of detecting single enzyme molecules. Here the volume of the array is 2 billion times smaller than ELISA, which will generate a faster fluorescent product. If a protein with a label is present the fluorescent product will be observable (Quanterix 2019).

According to the same company, the Simoa is the next-generation technology for biomarker detection. The company also claims that this technique is 1000 times more sensitive than ELISA and is able to detect thousands of single-protein
molecules at the same time.

![Diagram of analog vs digital measurements]

Figure 15: An illustration of analog measurements compared to digital. In the analog, traditional measurement, the intensity of the total amount of signals is measured; the intensity increases as the concentration of ligand-binding increases. In digital methods, for instance Simoa, the measurements are independent of intensity and only measure the positive signal; a signal or no signal readout. Illustration made by Lina Löfström based on (Quanterix 2019).

7.11.1.3 Single molecule counting using magnetic microparticles

Single molecule counting (SMC) technology is similar to ELISA but differs in the analysis mechanism. The method has very high sensitivity and can measure down to the femtogram/mL, which makes it suitable for low abundant biomarkers (Hwang et al. 2019). Other sandwich based immunoassays with similar sensitivity have a higher cost.

The basic concept of the technology is to attach magnetic (Merck-Millipore 2020) microparticles to antibodies that bind to the biomarker. They can then be separated from the plate and flow past a detector that is able to detect single molecules. The molecules are fluorescently labeled and give off flashes of light as they pass a laser in the detector. At the upper end of the detection range, the signal is more analog, and total detection can be measured against a standard curve. It has a broad dynamic range because of the low background and high peak signal. It can be adapted to several different biomarkers depending on which antibodies are used.
7.12 BRET: Bioluminescence Resonance Emission Transfer

BRET describes the energy transfer between a bioluminescent protein to a fluorescent protein (Shigeto et al. 2015). Bioluminescence gets its energy from a chemical to transmit light. Fluorescence absorbs the energy from light or photons and will give off a light with a longer wavelength i.e lower energy (Wood 2007). When these proteins are close enough the fluorescent one can use light from the bioluminescent one to produce light with a different wavelength, see figure 16.

In the study by Shigeto (Shigeto et al. 2015) they fused two different parts of a modified insulin receptor (αCT and L1) to a bioluminescent protein and a fluorescent protein to measure insulin concentration. They explain that the bioluminescent part, which in this case is a protein called Nluc, must be within a certain distance of the fluorescent part, called YPet (a yellow fluorescent protein), to get a signal. In order for Nluc and YPet to be close the presence of insulin is needed since they both have an affinity for insulin but not for each other. The study also showed great specificity since no signal was measured when insulin-like compounds were used. They show that the bioluminescent protein emits light at 445 nm and the fluorescent part gives a signal of 528 nm which causes a shift in the wavelength versus intensity curve. It was determined that this shift was noticed at a concentration of 0.8 µM which compared to other methods is quite high. It does have the advantage of being homogenous which makes it simple to perform.
7.13 HTRF: Homogeneous Time Resolved Fluorescence

Homogeneous Time Resolved Fluorescence (HTRF), is an assay that combines fluorescence resonance energy transfer (FRET) technology, with time-resolved measurement (Ding et al. 2018). The method uses two types of antibodies, to be able to detect a specific protein in a sample. Both of the antibodies are specific for the protein of interest. The two kinds of antibodies both coupled with a fluorophore, one antibody is called the donor and the other the acceptor (Cisbio 2020).

When the sample with the protein and the fluorophore antibodies are poured into the wells of the plate, the antibodies will bind to the protein. If the two fluorophores are in close enough proximity to each other, the donor will send a fluorescent signal to the acceptor, see figure 17. This transfers the energy and sends a fluorescent acceptor signal. The acceptor signal is detected and measured by a reader (Cisbio 2020). The measured signal increases with increasing quantity of protein in the sample.

HTRF is a very cost effective and rapid technology for detecting several kinds of proteins. The sample volume needed is low and since the assay is homogenous, the whole procedure is easy and in no need of any separation or washing steps since the
Today, HTRF assays for several biomarkers are available. The insulin HTRF assay has shown a cost effective and rapid detection with a very low cross-reactivity to proinsulin but otherwise with a good specificity towards insulin (Farino et al. 2016). On the other hand, the glucagon HTRF assay available has low specificity towards glucagon since there is a high risk of cross-reactivity against glucagon-like proteins like glicentin and oxyntomodulin (Wewer Albrechtsen et al. 2016). The specificity problem for the glucagon HTRF assay does not relate directly to the HTRF technique itself but can be improved with more specific antibodies against glucagon.

HTRF assays are preferred when you want to use a minimal sample volume. The HTRF technology also has the ability to measure several samples simultaneously, good precision in a short detection time and accuracy with both high and low concentrations of a biomarker in a sample (Einhorn & Krapfenbauer 2015).

![Figure 17: An illustration of the complex between the donor, analyte and acceptor. When light hits the fluorophore on the donor antibody, it sends a signal to the fluorophore on the acceptor antibody. This signal is measured. Illustration by Ella Schleimann-Jensen, based on (Cisbio 2020).](image)

### 7.14 NanoBiT (NanoLuc)

NanoBiT is developed from NanoLuc technology; NanoLuc is an engineered luciferase enzyme. A luciferase enzyme is an enzyme that emits a specific type of light. The main advantage of this technology is studying the dynamics of proteins which is relevant for protein secretion such as insulin secretion and others. The NanoBiT system consists of two subunits, large BiT (LgBiT 18kDa) and small BiT (SmbiT 11aa). When the two subunits come together, they form an enzyme that emits light.
light emission occurs when the substrate of the enzyme is added. The BiT subunits can be conjugated to target proteins of interest. The read-out signal after the protein fusion is luminescence (Hwang et al. 2020).

In the research field of diabetes, it is important to be able to identify post-translational modification (PTM), especially in type I diabetes since the disease is not fully understood yet. Therefore, cell-based studies are relevant. One of the most studied processes is cellular protein phosphorylation. This process can be monitored by several analytical methods such as ELISA, MS, and western blot. The methods named can require many washing steps or they can be tedious while detecting the level of native target proteins (Hwang et al. 2020).

Promega Biotech AB has developed the technique Lumit™ Immunoassays which is based on the NanoBiT technology (Promega 2020). The principle is the same since, like NanoBiT, it uses the SmBiT and LgBiT as well as chemically labeled antibodies and in the presence of the analyte when the subunits SmBiT and LgBiT form an active enzyme complex a luminescent signal is transmitted, see figure 18. The process requires continuous addition of antibodies and reagent to the sample and detection of the luminescent signal, with a total process time of 70 minutes. The enzyme formation occurs only if the labeled antibodies come approximately close. The Lumit™ assay can quantify insulin and glucagon as metabolic targets.

Figure 18: Illustration of NanoBiT technology mechanism.
8 Trend analysis for technologies in the field of immunoassays

During our search we gathered 116 significant articles. This was in order to get an overview of the methods that are used in the field of immunoassays. From this data an overview was made on all the different parameters that were relevant for the methods. The data from this can be seen in table 1-5.

Searches were also made in PubMed for the methods together with the specific biomarkers in order to see how often the biomarker was detected with the help of the current method, see figure 19. The same search was made in Google Scholar, see figure 20. A delimitator was that only the hits from the last ten years was included in the search. It was crucial that the searches were made according to the same criteria, therefore the searches in both PubMed and Google Scholar were done as: [(the method) AND (ligand binding assay OR immunoassay) AND (the biomarker [MeSH terms])]. Where “the method” and “the biomarker” was changed to the relevant search-word. By doing this we could be sure that the result from the searches were collected in a similar fashion, see table 6 and 7. For the methods with abbreviations, the searches were made a little different. Both the abbreviation and the full name were included.

A further search of how many times the methods appeared in just a simple search in both PubMed and Google Scholar was made, see table 8. If a method had an abbreviation, the search was made with both the abbreviation and the full name.

The trends were identified by observing how many times a method or technology was mentioned together with the specific biomarker, and from this the methods with the most recent articles where selected, see figure 20 and 19.

To be able to visualize the trends, graphs were made with data from PubMed. The query: [“the method”) AND (immunoassay OR ”ligand binding assay”)] was inserted in the search, where ”the method” was exchanged to the name of the method. For each method a trend graph was made. See graphs in appendix D-E. The graphs made the visualization of the trends for the different methods more easy. The methods that showed an upward trend were: AlphaLISA, BRET, Biosensors, CLIA, Digital ELISA, methods using gold nanoparticles (AuNPs), HTRF, Immuno-PCR, Lateral Flow, MSD, Microfluidics, Multiplex methods, NanoBiT, Paper-Based, PEA, Simoa and Single Molecule Counting, see appendix D. The methods that showed a
downward trend were: ELISA, Mass Spectrometry with immunoassay and PLA, see appendix E.

Figure 19: The figure shows the distribution of how often a method is used for each of the different biomarkers according to searches in PubMed.
Figure 20: The figure shows the distribution of how often a method is used for each of the different biomarkers according to searches in Google-Scholar.
9 Discussion

This project, given to us by Mercodia AB, was a major task. We feel that given more time we could have dug deeper in the question whether there is a method more used than ELISA. However, we have found some interesting trends and observations in our research, which we will discuss below.

As noted earlier antibodies are dependent on the batch-to-batch quality. Therefore, another option to antibodies is synthetic antibodies. A form of synthetic antibodies are aptamers. Since aptamers can be synthesized chemically, the batch-to-batch variation is no longer a problem. Aptamers do not need to be produced in animals either. It also reduces the cost of production and the time that is needed to make them. However, they are not as specific as antibodies and the production procedure is more complicated than producing antibodies in animals.

Some methods that use aptamers are: immuno-PCR, PLA, PEA and some biosensors. However, these methods use antibodies as well. The goal in the future should be to develop methods that only use synthetic antibodies. A clear advantage of not using antibodies is that it is not necessary to use animals in your research. Read more about this in our ethical section (appendix A).

Some problems associated with immunoassays, including ELISA and especially when using human samples, are for example interference of autoantibodies, anti-reagent antibodies, Hook effect and lack of concordance. There are numerous washing steps required to prevent this. A possible way to avoid cross-reactivity is to not use antibodies in the first place. Another method that does not use antibodies is mass spectrometry, this is therefore one of the main reasons why mass spectrometry sometimes is preferred over immunoassays (Hoofnagle & Wener 2009). To avoid cross-reactivity, microfluidic techniques can also be used. An example of this is the Ella instrument, which run an immunoassay (Simple Plex) that uses microfluidics. It separates each target in its own channel decreasing the risk of cross-reactivity (Cao et al. 2015).

As mentioned earlier in this report, ELISA often has a colorimetric change as detection. The colorimetric detection is rather unspecific. This requires ELISA to have another method, such as size measurement methods, that can confirm that the right antibodies and antigens have reacted. ELISA is often coupled with optical density measurement in order to give a more specific result. This however opens up for a new problem, namely the requirement of the measuring to be within the linear range.
of the measuring curve. The measuring curve can be thought of as a sigmoid curve where the linear range is the “straight line” in the middle of this curve. If this demand is not met, no valuable detection can be made, because the sample is saturated.

Some methods use other detection systems than colorimetric change and optical density, such as fluorescence, chemiluminescence, magnetic beads, electric volt change or light intensity. An example of these methods is CLIA, as one of the most traditional. However, other efficient methods developed such as microfluidics coupled to CLIA, LFIA combined with fluorescence inductor, HTRF, AlphaLISA, AuNP biosensor, MSD, IMMray microarray, NanoBiT, BRET and digital immunoassays such as Simoa. We believe that the way the measurement is made can affect the range of the measurement. We conclude this by analysing the tables for the biomarkers c-peptide, glucagon and insulin, see table 1, 2 and 4. The mentioned methods have a larger range than ELISA, except for microfluidics and lateral flow when detecting insulin, in table 4. For the methods detecting proinsulin, no range was found to compare with ELISA, see table 3.

In the same tables as mentioned above (table 1-4) we can also see that the LOD values (limit of detection values, see appendix B for detailed explanation) differ between all the methods in respect to the biomarkers. One method that significantly differs from the others is BRET which has a LOD value of 800 000 [pM]. This can be due to the fact that BRET does not use antibodies to detect insulin, unlike the other methods, which we believe affect the sensitivity negatively. The biosensor AuNP had the best LOD value (0.0142 ng/mL) regarding the detection of c-peptide. The other two methods, AlphaLISA and ELISA were also quite low, see table 1. MSD was the best method for detection of glucagon with 0.49 pg/mL, see table 2. ELISA had the better value when it came to detecting proinsulin with 1.7 pmol/L, see table 3. However, proinsulin did not have that many values to compare with. Regarding the detection of insulin, the Ella instrument had the best value of 0.006 pM, see table 4.

In the figures 19 and 20 the distribution of how often a method occurred with one of the biomarkers is shown. The searches were made in both PubMed and Google Scholar in order to compare the occurrence of the methods, the search was restricted to the last ten years. The biomarker insulin was more frequently used together with the different methods than the other biomarkers.

We realise that the way the searches where made will give way to some errors and “false hits” will be included. We notice that the searches made in PubMed seem to
be more strict and could be regarded as "more accurate" than the searches made in Google scholar. However, Google scholar had more inclusive hits which would make it possible to find more diverse articles. The search in Google scholar is more inclusive because it includes all hits that are found if a method has several words in the name. For example "paper-based assay" will include hits that are not focused on the method "paper-based" but also articles that discuss similar set-ups. Therefore, these searches can be seen as more of a guideline to how the methods are being used and not an absolute fact. The data is shown in the tables 6, 7 and 8.

We wanted to visualize the trends in the different methods and for these searches we only used PubMed. We had concluded earlier that the searches made in PubMed were more strict and should therefore show a more accurate result. An interesting thing that we noticed during this search, is that ELISA had a notable downward trend since 2010, according to the queries made in PubMed, see figure 38 in appendix E. ELISA is however still used greatly in the field of immunoassay and ligand binding assay, but according to the searches made in PubMed ELISA has decreased from approximately 8000 hits in 2010 to approximately 3000 hits in 2019. Some other methods that showed an upward trend were: Lateral Flow Immunoassay (see figure 29), Meso-Scale Discovery (see figure 30), Paper-Based Immunoassay (see figure 34) and Chemiluminescence Immunoassay (see figure 24). However, all methods that showed an upward trend are included in appendix D. The methods that showed a downward trend are included in appendix E.

How does the future look in the field of immunoassays? When talking to researchers from Uppsala University as well as when reading recently published articles, a possible development in the field of immunoassay is the ability to multiplex the detection of biomarkers. The efficiency of analyzing several biomarkers in one run could be a faster way of detecting important biomarkers and making healthcare more efficient, affordable and informative in diagnostics. Possible methods that could use multiplex are MSD, microfluidics systems, IMMray, lateral flow based systems, Simoa, MS and AuNP.

A big challenge in protein measurement is that important biomarkers could be present in low amounts in the sample. This is an issue in conventional immunoassays like ELISA, chemiluminescence and electrochemiluminescence because they lack enough sensitive and specific measurement. Methods that are able to handle this are digital bioassays, for example Simoa which is a digital ELISA. The Simoa does not require a large amount of sample volume, can detect biomarkers of low concentrations and counts all positive signals in the sample, see figure 15. This technique is a
highly sensitive detection method and will most likely be a more established method in future bioassays.

Another group of methods that we consider to have a future in immunoassay are biosensors, in its many forms. Biosensors can use both antibodies and aptamers and have various forms of detection. One version of a biosensor is in the combination with immuno-PCR. The immuno-PCR can be combined with gold nanoparticles for example, and have been used to detect viral antigens and bacterial antigens (Malou & Raoult 2011). The nanoparticle immuno-PCR has also been shown to be able to detect levels of femtogram per millilitre (fg/mL) concentrations of for example different kinds of viral agents (Dahiya & Mehta 2019). The low limit of detection makes us consider the biosensors to be of importance when very high sensitivity is required.

Another possible idea for the future is to make healthcare more accessible for countries that do not have a developed healthcare system. Instead of investing in expensive machines that might require specially trained personnel, like mass spectrometry, a more simple approach could be used such as a paper based analysis method. This can be combined with several immuno techniques, but a promising combination is with a lateral flow system. Here all you need is some sort of paper that will allow you to run your sample fast, through a device that is easy to read and use. A further use can be to connect the assay to your smartphone. This is an example of how the approach point-of-care testing is used. The accessible and cheap paper-based methods can also be valuable for research projects that stretch over many years.

Since the method is based on paper, the set-up should be easy to transport and also to produce in various different environments. If the paper has a sample that is contagious, a possible way to discard it can be to burn the paper. This would simplify the handling of the device and possible contagious waste, allowing various people with different medical or laboratory backgrounds to use the analysis method. In case of major disease outbreaks, like the COVID-19 pandemic, using such applications would be a benefit regarding the handling of contagious waste and the low test-price.
9.1 Conclusion

In conclusion, there are several methods and techniques that can be used instead of ELISA in order to detect specific biomarkers. In this project we list a great number of such methods that have many advantages regarding given parameters. When it comes to upward trends there are some methods that seem promising. Multiplex immunoassays, digital bioassays, microfluidics-based assays as well as immunoassays that can be used in point-of-care testing are having great potential in future perspectives. Better automatization, higher sensitivity and lower sample cost are the common denominators in the methods that we consider to have an upward trend.

A goal for healthcare should be to make testing affordable and easy to access, but still with a high specificity and sensitivity. This should be the future in the immunoassay-field.
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11 Statement of Contribution

All of the group members cooperated in writing the report and choosing the arrangement of it. Every section had one or two members as main contributors and the other members contributed with feedback and inputs. All of the group members cooperated in writing the discussion section, the abstract section, the explanation of words section and also in gathering the data presented in table 1-8. All of the group members cooperated in creating the figures 21-40 and cooperated in modifying the report after feedback.

A. Häggströms main responsibility in the background was the mercodia and glucagon section, including figure 2. He wrote parts of the mass spectrometry section in the technology description, together with T. Al-Khafaf and L. Löfström. He also wrote the single molecule counting part of the digital bioassay section. In the ethical analysis, he was responsible for the section on research subjects in clinical trials.

B. Ancker Perssons main responsibility in the background was the drug analog section. He was responsible for the AlphaLISA and BRET parts in the technology description section. He created figure 1, 5 and 16 and figure texts for 5 and 16. He also made the “Description of parameters” part. In collaboration with J. Cederblad, T. Al-Khafaf and E. Schleimann-Jensen, he created table 1-8.

E. Schleimann-Jensen had the main responsibility for the background sections about proinsulin and insulin. Also, she was responsible for writing the delimitations part and the methods sections about CLIA and HTRF. She also created figure 17 and wrote a figure text for it. In cooperation with B. Ancker Persson, J. Cederblad and T. Al-Khafaf, she created tables 1-8 and figures 19-20 and wrote table- and figure texts for them. Together with J. Cederblad, she wrote the trend analysis.

J. Cederblad had the main responsibility of the background section of ELISA, c-peptide and aim. She also wrote a major part in the method of the project part and was in charge, together with T. Al-Khafaf, to conduct interviews with third parties and to incorporate them into the report. She was also responsible for writing the immuno-PCR, MSD, PEA, PLA and aptamer sections and parts of the biosensor section. She created illustration 3, 8, 9, 10, 11, 12, 13 and 14 and wrote figure texts for each. Together with B. Ancker Persson, E. Schleimann-Jensen and T. Al-Khafaf, she created table 1-8 and figures 19-20 and wrote table- and figure texts for them. She also wrote the trend analysis together with E. Schleimann-Jensen and L. Löfström.
L. Löfström had the main responsibility of the background section of metabolic diseases. She also wrote the part about digital bioassays including HoNon-ELISA and Simoa, as well as figure 15. Other sections she wrote were the Ella section, the biosensor section together with J. Cederblad, as well as the MS section together with T. Al-Khafaf and A. Häggström. L. Löfström also had responsibility for the ethical analysis about antibody production. She also made a template of the report in LaTeX. L. Löfström also helped J. Cederblad and E. Schleimann-Jensen with the trend analysis.

R. Kostines had the main responsibility for the section about clinical trials, the methods sections about microfluidics and LFIA and finally the ethical analysis about insulin and its abuse. She cooperated in writing the method section about NanoBiT technology with T. Al-Khafaf. She also created figures 6, 7 and 18 and wrote figure texts for them.

T. Al-Khafaf had the main responsibility for the subsections ligand binding assays and CRO and pharma in the background. Together with J. Cederblad, she wrote parts of the section general problems with ELISA. She wrote paper based immunoassays. She also wrote the MS section together with A. Häggström and L. Löfström as well as the NanoBiT technology together with R. Kostines. She made figure 4. In cooperation with B. Ancker Persson, J. Cederblad and E. Schleimann-Jensen, she created tables 1-8 and figures 19-20. Together with J. Cederblad, she conducted the interviews with the third parties.
References


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Appendices

Appendix A  Ethical analysis

A.1 Producing antibodies

Even though synthetic antibodies are available, antibodies from animals are still in use (Dura 2020). According to European Commission, close to one million animals are used for the purpose of antibody production (Gray et al. 2016). It occurs even though other technologies that do not require animals are available. This was something that we noticed during our research of this project. Regarding animal testing there are often ethical discussions; do the animals suffer, is it ethical to use animals in research and so on. Antibodies are very useful and sometimes necessary for doing research, offering great sensitivity and specificity. In order to produce antibodies the use of animals is often required. Polyclonal antibodies are generated by the immune system of an animal, by injecting antigens that trigger an immune response where antibodies are produced. The animal is sometimes injected up to three times, to produce a higher volume of antibodies against a specific antigen (Creative Diagnostics 2020). This procedure could be harmful and could cause a lot of pain for the animal. In some extreme cases too much blood is obtained from the animal when collecting the antibodies which could lead to the animal being killed (AnimalResearch 2014).

To be allowed to use animals in antibody production, a strong scientific justification is always required. According to EU Directive 2010/63 protection of animals should always be done and if there are other valid alternatives they must be used (Official Journal of the European Union 2010). An example is a method called ‘phage-display’ that uses phages to generate antibodies. Using aptamers is also an alternative. Why the transition from animal derived antibodies to non-animal derived antibodies is so slow seems to depend on several factors. The availability of non-animal derived affinity reagents do not exist in the same amount since the majority still uses animal immunisation. Also, a lack of education and knowledge about the quality as well as the validity of the synthetic antibodies exists.

Is it ethically right to use animals for this purpose when there are other alternatives? There are regulations that say that alternative methods should be used but still one million animals are used every year, why? Mercodia AB, for example, uses antibodies from animals in their ELISA-kits in order to have high sensitivity in their
A conflict of whether to use antibodies in the detection and diagnosis of diabetes, weights heavier than the suffering of the animals. If Mercodia AB would not use these antibodies, then possibly thousands of people would go undiagnosed and untreated. Is the suffering of animals less important than human lives? Regarding animal trials the suffering of the animal should be weighed against the utility. If using animals in antibody productions leads to a new treatment that could save a huge number of people from a disease, then it could be worth it. However, animal welfare needs to be of high priority in this ethical discussion.

A.2 Insulin and its abuse

In Sweden, insulin abuse in sport is classified as hormone doping (Rosén 2018). Insulin abuse in elite sports and by bodybuilders has theoretical benefits. The theory behind abusing insulin in sports is that the insulin prevents protein degradation. In addition, insulin combined with high carbohydrate supply can stimulate the glucose flow to cells. This is used mainly for two purposes. The first purpose is to achieve faster recovery after an intense physical effort or exertion. The second purpose is to improve the performance before a competition (Rosén 2018). Both purposes are illegal in Sweden. In addition, improving the performance before a competition favors the abuser and creates an unfair competition. Therefore, insulin abuse in sport is a subject that requires further ethical analysis in order to prevent cheating in competitions.

The main problem with insulin doping is that it is undetectable (Holt & Sönksen 2008; Rosén 2018). No commercial available methods are fast and accurate enough for detecting exogenous insulin. This is based on the fact that fast-acting, which is often used in insulin abuse, exogenous insulin has a half-life of 4-10 minute and vanishes from blood within 4 h (Holt & Sönksen 2008). Since insulin abuse is illegal and considered ethically wrong, improving a fast and accurate detection test is a necessity.

An accurate detection method will limit the possibility for people using insulin for doping. If the people misusing insulin know that it will be discovered, they might refrain from such behaviour. This will possibly lead to insulin reaching the people who needs it.

The same insulin that is considered essential for survival in both type I diabetes and insulin-requiring type II diabetes patients could kill a person (Gundgurthi et al.
An overdose of insulin, also called insulin poisoning, will rapidly lead to hypoglycemic coma (Gundgurthi et al. 2012). If hypoglycemia is not diagnosed and treated in time, it will lead to death. The treatment is always adjusted based on the insulin concentration in the blood. Therefore, finding an accurate and especially fast quantitative method to detect exogenous insulin is necessary.

A less common misuse of insulin is that it can be used as a murder weapon. Performing an insulin measurement in a postmortem investigation is a challenge due to many reasons such as the impact of chemical instability (Labay et al. 2016; Skowronek 2018). Over many years, researchers have tried to validate and develop a method with enough accuracy to detect exogenous insulin in postmortem investigations (Labay et al. 2016). Yet, a method with enough accuracy does not exist. This could mean that a person can commit a murder with an insulin overdose without the person being charged for the crime. It is significant and can be considered our ethical duty to be able to discover the cause of death of a victim in order to achieve justice. Therefore an accurate and precise method to detect exogenous insulin can be of high value.

### A.3 Research subjects in clinical trials

Clinical trials always come with a certain amount of risk, but they also have potential benefits. When deciding whether or not to start a clinical trial it is important to weigh the risks and benefits against each other. It is also important to consider that sometimes it is not people benefiting from the study who are taking the risks. From an ethical perspective, research subjects from certain socioeconomic backgrounds might run the risk of being exploited in clinical trials. One cause of research exploit is poverty, especially situations where researchers from rich countries conduct clinical trials on individuals from poor countries (Schroeder et al. 2019).

In countries with unequal healthcare, being a research subject might be more beneficial to some individuals than others. If a person does not have health insurance, participating in a clinical trial could be a way of getting access to advanced healthcare. There are indications that participants have better overall health outcomes than individuals of the same group who did not participate (Noah 2003). This is probably unrelated to the specific study and indicates that there is a general benefit of participating. It seems somewhat unfair that some groups need to take risks to get adequate health care. Another aspect is that participants in many clinical trials do not represent the distribution of the disease as a whole (Noah 2003). Some mi-
norities are underrepresented in clinical research, which could affect medical research on those groups negatively. It is important for researchers to be aware of this and work harder on including them in their clinical trials.

In Sweden test subjects give different reasons for being part of a clinical trial. One reason is to contribute to research that might lead to better health for other people (Kallenberg 2017). It could be considered unfair that people willing to help others take an disproportional burden of the risks involved in drug development. Another reason for being part of a clinical trial is financial compensation. Student newspaper Ergo in Uppsala interviewed research subjects who had been students when participating in a clinical trial (Ergo 2015). One of them had received 50 000 SEK and another 20 000 SEK, which is a lot of money for a student. They reported getting very unpleasant and unexpected side effects and only one of them had done it more than once. Nevertheless, some of them would consider doing it again or recommend it to a friend. Indicating that some people are willing to take the risk of participating in clinical trials and to be exposed to inconvenient situations. All of this in order to get some financial compensation, which would increase the risk of exploitation. Can we consider this to be ethically right? To use individuals in an exposed situation to participate in studies that could harm them. Can this be considered as ethically justified? Where can we draw the line of what is possible to defend ethically? Is it to justify that new drugs and methods can be released to the market, that will help sick individuals?

Before a study can start, it needs to be approved by the Swedish Ethical Review Authority (Sternudd 2019). The benefits of the study must be proportional to the risks involved. If the study involves a drug it also needs to be approved by the Swedish Medical Products Agency.

Clinical trials are a necessary evil, in order for new research to reach the public we need someone to test it. However, it is important to remember the person behind the test and the possible side effects of participating. Are all clinical trials worth the damage they might cause?


Appendix B  Explanation of parameters

Specificity refers to the ability of the assay to only detect the molecule of choice. This is an important parameter since having poor specificity will result in many false positive results. Antibodies are often used due to them being highly specific to their target. Even if they can be highly specific, cross-reaction with similar molecules can occur. This is especially true for drug analogs which can have only a single amino acid changed.

In addition to being able to detect small changes in the target we also want our method to be able to detect small concentrations of the analyte, this is referred to as sensitivity. This parameter determines the lowest concentration of analyte that can be detected. To get this information we have looked for the terms LOD and LOQ which are terms related to sensitivity. LOD is Limit Of Detection and LOQ is Limit of Quantification, both of these are given in mol/L(molar) or g/ml. The sensitivity requirement is different depending on which biomarker is being tested. Glucagon is a low abundant peptide and requires a method that is able to measure down to 1pmol/L (3.5 pg/mL) (Holst & Wewer Albrechtsen 2019). To get the measure range we look at the highest amount of analyte that can be detected in a sample to get an interval in which the method is effective.

Runtime is simply the amount of time it takes to complete the analysis. This time does not include any preparation of the sample. The sample volume refers to the volume of analyte carrier (blood, serum, plasma) that is required for each test. If a method uses for example multiple wells, this refers to the amount in each well and not the total amount. The sample cost is the cost for each sample in the method and can also be thought of as cost/well, since each well contains a sample.

All the equipment around the methods used will also be of interest. If the method uses equipment already present in most labs or require new specialised equipment can greatly influence the price. Another parameter to consider is to what degree the method can be automated. This can be influenced by the method itself but also the compatibility with other technologies.
### Appendix C  Tables from the research

#### Table 1: Table of the different methods that detected c-peptide and the parameters relevant for those methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>LLOQ [pg/mL]</th>
<th>LOD [pg/mL]</th>
<th>Sample Volume [µL]</th>
<th>Specificity</th>
<th>Precision [%]</th>
<th>Range [pg/mL]</th>
<th>Cost per sample [kr]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlphaLISA</td>
<td>49.5</td>
<td>23.5</td>
<td>1-10</td>
<td>-</td>
<td>Intra: 3.9-4.8</td>
<td>23.5 - 30 000</td>
<td>~26</td>
<td>(PerkinElmer 2012b)</td>
</tr>
<tr>
<td>AuNP immunosensor</td>
<td>-</td>
<td>14.2</td>
<td>10</td>
<td>-</td>
<td></td>
<td>Intra: 3.0-6.8</td>
<td>-</td>
<td>(Liu et al. 2018a)</td>
</tr>
<tr>
<td>ELISA</td>
<td>-</td>
<td>75.5</td>
<td>25</td>
<td>-</td>
<td></td>
<td>302 - 12 000</td>
<td>38.44 /well</td>
<td>(Mercodia AB 2020b)</td>
</tr>
</tbody>
</table>

#### Table 2: Table of the different methods that detected glucagon and the parameters relevant for those methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>LLOQ [pg/mL]</th>
<th>LOD [pg/mL]</th>
<th>Sample Volume [µL]</th>
<th>Specificity</th>
<th>Precision [%]</th>
<th>Range [pg/mL]</th>
<th>Cost per sample [kr]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlphaLISA</td>
<td>60</td>
<td>20</td>
<td>1-10</td>
<td>-</td>
<td>Intra: 12</td>
<td>20-30 000</td>
<td>-</td>
<td>(PerkinElmer 2012a)</td>
</tr>
<tr>
<td>ELISA</td>
<td>0.95</td>
<td>3.49</td>
<td>25</td>
<td>4-10 oxyntomodulin</td>
<td>Intra: 8-15</td>
<td>1.5-45</td>
<td>76.125</td>
<td>(Mercodia AB 2020b)</td>
</tr>
<tr>
<td>IA-LC-MS/MS</td>
<td>272</td>
<td>-</td>
<td>600</td>
<td>-</td>
<td>Intra: 6.8-22.5</td>
<td>76-108</td>
<td>-</td>
<td>(Lee et al. 2016)</td>
</tr>
<tr>
<td>MSD</td>
<td>0.49</td>
<td>-</td>
<td>0.05 - 0.5 pmol/well</td>
<td>-</td>
<td>76-89</td>
<td>-</td>
<td>-</td>
<td>(Stein et al. 2012)</td>
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<tr>
<td>Microfluidics device with CEIA</td>
<td>-</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Shackman et al. 2012)</td>
</tr>
<tr>
<td>Single molecule counting</td>
<td>-</td>
<td>-</td>
<td>&gt; 0.5 oxyntomodulin</td>
<td>Intra: 11</td>
<td>450</td>
<td>15-1000</td>
<td>A little</td>
<td>(Horned et al. 2017)</td>
</tr>
<tr>
<td>UHPLC-MS/MS</td>
<td>75</td>
<td>1100</td>
<td>-</td>
<td>&lt; 20</td>
<td>&gt; 25</td>
<td>15-1000</td>
<td>-</td>
<td>(Horned et al. 2017)</td>
</tr>
<tr>
<td>UHPLC-MS/MS/GLP-1</td>
<td>25</td>
<td>-</td>
<td>0.5</td>
<td>&gt; 25</td>
<td>Width 100</td>
<td>-</td>
<td>-</td>
<td>(Horned et al. 2017)</td>
</tr>
<tr>
<td>Solid phase extraction HPLC-HRMS</td>
<td>-</td>
<td>300</td>
<td>14</td>
<td>112</td>
<td>17-348</td>
<td>-</td>
<td>-</td>
<td>(Mercodia et al. 2017)</td>
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</tbody>
</table>

#### Table 3: Table showing the methods that detected proinsulin and the parameters relevant for those methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD [pmol/L]</th>
<th>Sample Volume [µL]</th>
<th>Precision [%]</th>
<th>Range [pmol/L]</th>
<th>Cost per sample [kr]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Flow Assay</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Pflützer et al. 2017)</td>
</tr>
<tr>
<td>ELISA</td>
<td>1.7</td>
<td>50</td>
<td>Intra: 2.5-3.2</td>
<td>Inter: 5.0-6.1</td>
<td>3.3-130</td>
<td>~43</td>
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</table>
Table 4: Table of the different methods that detected insulin and the parameters relevant for those methods.

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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td>AlphaLISA</td>
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<td>4.63</td>
<td>4-10</td>
<td>97-100</td>
<td></td>
<td></td>
<td>4.63-2748.8</td>
<td>-</td>
<td>Automation</td>
<td>(PerkinElmer 2011)</td>
</tr>
<tr>
<td>Antibody Aptamer</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Singh et al. 2017)</td>
</tr>
<tr>
<td>Immunoarray chip</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Sugito et al. 2015)</td>
</tr>
<tr>
<td>CLIA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Yao et al. 2018)</td>
</tr>
<tr>
<td>Ella instrument</td>
<td>0.040</td>
<td>0.86</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Almost all automated</td>
<td>(ProteinSimple 2020)</td>
</tr>
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<td>HTRF</td>
<td>61</td>
<td>29.6</td>
<td>5-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10-10 000</td>
<td>Automation</td>
<td>-</td>
<td>(Aslanoglou et al. 2018)</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Farrino et al. 2016)</td>
</tr>
<tr>
<td>Microfluidics (CLIA)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Einhorn &amp; Krapfenbauer 2015)</td>
</tr>
<tr>
<td>NanoBiT cell-based</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Hwang et al. 2020)</td>
</tr>
<tr>
<td>Biosensors (general)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Singh &amp; Krishnan 2018)</td>
</tr>
<tr>
<td>Hydrogel microparticle-based assay</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Cohen et al. 2017)</td>
</tr>
<tr>
<td>IMMray microarray</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Larsson et al. 2017)</td>
</tr>
<tr>
<td>PEA</td>
<td>-</td>
<td>0.093</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Wang et al. 2019)</td>
</tr>
<tr>
<td>PLA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Wilson 2018)</td>
</tr>
<tr>
<td>Simoa</td>
<td>-</td>
<td>0.0073</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0073-10 pg/mL</td>
<td>-</td>
<td>-</td>
<td>(Wilson 2018)</td>
</tr>
<tr>
<td>Digital biosensor</td>
<td>-</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.001-10 pg/mL</td>
<td>-</td>
<td>-</td>
<td>(Wilson 2018)</td>
</tr>
</tbody>
</table>

Table 5: Table of the different methods that detected other biomarkers than the ones mentioned and the parameters relevant for those methods.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NanoBiT cell-based</td>
<td>General</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Hwang et al. 2020)</td>
</tr>
<tr>
<td>Biosensors (general)</td>
<td>General</td>
<td>1 ng-25 ng/mL</td>
<td>1.2 pg-50 ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Singh &amp; Krishnan 2018)</td>
</tr>
<tr>
<td>Hydrogel microparticle-based</td>
<td>Placental growth factor</td>
<td>-</td>
<td>17.5-3000 pg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Cohen et al. 2017)</td>
</tr>
<tr>
<td>IMMray microarray</td>
<td>General</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Larsson et al. 2017)</td>
</tr>
<tr>
<td>PEA</td>
<td>Brain natriuretic peptide</td>
<td>-</td>
<td>2000 pg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20000 pg/mL</td>
<td>-</td>
<td>-</td>
<td>(Arrigo et al. 2018)</td>
</tr>
<tr>
<td>PLA</td>
<td>Clenbuterol and ractopamine</td>
<td>0.01 ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.01-30 ng/mL</td>
<td>-</td>
<td>-</td>
<td>(Cheng et al. 2012)</td>
</tr>
<tr>
<td>Simoa</td>
<td>General</td>
<td>0.0073 pg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0073-10 pg/mL</td>
<td>-</td>
<td>-</td>
<td>(Wilson 2018)</td>
</tr>
<tr>
<td>Digital biosensor</td>
<td>General</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Wilson 2018)</td>
</tr>
<tr>
<td>Microfluidics aptasensor</td>
<td>General</td>
<td>-</td>
<td>0.001 ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.001-10 ng/mL</td>
<td>-</td>
<td>-</td>
<td>(Balik-Maghsoodi et al. 2020)</td>
</tr>
</tbody>
</table>
Table 6: Table over the searches made in PubMed of the methods from the literature study and how many times the biomarkers (insulin, proinsulin, c-peptide and glucagon) was mentioned together with that method. The searches were made as: [(the method) AND ((ligand binding assay) OR (immunoassay)) AND (the biomarker)]. “The method” and “the biomarker” was changed to the relevant search word.

<table>
<thead>
<tr>
<th>Method</th>
<th>Insulin</th>
<th>Proinsulin</th>
<th>C-peptide</th>
<th>Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA-LC-MS/MS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>16</td>
<td>17</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Top-down MS</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LC-IM-MS</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<td>UHPLC-MS/MS</td>
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<td>1</td>
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<td>ID-LC/MS</td>
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<tr>
<td>MSIA</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MSIA-HR/AM</td>
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<td>1</td>
<td>0</td>
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<tr>
<td>Bottom-up MS</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td>Nanoparticle MS</td>
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<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>Isotope-Dilution assays MS</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Biosensor (general)</td>
<td>26</td>
<td>27</td>
<td>2</td>
<td>5</td>
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<tr>
<td>Antibody Aptamer Immunoarray chip</td>
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<td>AuNP immunosensor</td>
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<td>Electrochemical aptasensor</td>
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<td>2</td>
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<td>Lateral Flow immunoassay (LFIA)</td>
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<td>AlphaLISA</td>
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<td>0</td>
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<td>Nanobit cell-based</td>
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<td>Single Molecule Counting Using Magnetic Microparticles</td>
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<td>Digital Bioassay</td>
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<td>Simoa</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ella instrument</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IMMray microarray</td>
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<td>22</td>
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<td>1</td>
</tr>
<tr>
<td>PLA (Proximity Ligation Assay)</td>
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<td>0</td>
</tr>
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<td>PEA (Proximity Extension Assay)</td>
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<td>CLIA</td>
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<td>11</td>
<td>5</td>
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<td>ELISA</td>
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Table 7: Table over the searches made in Google Scholar of the methods from the literature study and how many times the biomarkers (insulin, proinsulin, c-peptide and glucagon) was mentioned together with that method. The searches were made as: [(the method) AND ((ligand binding assay) OR (immunoassay)) AND (the biomarker)]. "The method" and "the biomarker" was changed to the relevant search word.

<table>
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<tr>
<th>Method</th>
<th>Insulin</th>
<th>Proinsulin</th>
<th>C-peptide</th>
<th>Glucagon</th>
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<td>LC-MS/MS</td>
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<tr>
<td>Top-down MS</td>
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<td>5</td>
<td>28</td>
<td>57</td>
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<tr>
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<td>0</td>
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<td>3</td>
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<td>49</td>
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<td>1</td>
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<td>50</td>
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<td>PEA (proximity extension assay)</td>
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Table 8: Table showing the number of hits of the different methods in both PubMed and Google Scholar.

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Appendix D  Trend analysis - upward trends

Figure 21: The graph shows the trend for AlphaLISA in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 22: The graph shows the trend for Biosensors in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 23: The graph shows the trend for Bioluminescence Resonance Energy Transfer (BRET) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 24: The graph shows the trend for Chemiluminescence immunoassay (CLIA) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 25: The graph shows the trend for Digital ELISA in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 26: The graph shows the trend for gold nanoparticle (AuNP) technology in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 27: The graph shows the trend for Homogeneous Time Resolved Fluorescence (HTRF) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 28: The graph shows the trend for Immuno-PCR in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 29: The graph shows the trend for Lateral Flow in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 30: The graph shows the trend for Meso-Scale Discovery (MSD) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 31: The graph shows the trend for Microfluidics methods in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 32: The graph shows the trend of multiplexing in the field of immunoassay and ligand binding assay. The data was collected from PubMed.
Figure 33: The graph shows the trend for NanoBiT in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 34: The graph shows the trend for Paper-based immunoassay in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 35: The graph shows the trend for Proximity Extension assay (PEA) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 36: The graph shows the trend for the Simoa technology in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 37: The graph shows the trend for Single Molecule Counting in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Appendix E  Trend analysis - downward trends

Figure 38: The graph shows the trend for enzyme-linked immunosorbent assay (ELISA) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.

Figure 39: The graph shows the trend for Mass Spectrometry (MS) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.
Figure 40: The graph shows the trend for Proximity Ligation assay (PLA) in the field of immunoassay and ligand binding assay. Data was collected from PubMed.