CHALMERS
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Development of a recombinant CHO cell line

Anti-Müllerian Hormone (AMH)

Master's thesis in Biotechnology

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Gothenburg, Sweden 2017

MASTER'S THESIS 2017

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Abstract

Anti-Müllerian hormone (AMH) is biological marker for the ovarian reserve, independent of the menstrual cycle. The AMH serum level can be measured with immunoassay in order to predict premature ovarian failure, chance for success of in vitro fertilization and aid in the diagnosis of polycystic ovary syndrome (PCOS).

The aim of the thesis was to develop a CHO cell line with overexpression of recombinant human AMH. Characteristics of the produced AMH were investigated in order to evaluate suitability for use in calibrators for an immunoassay. CHO cells are well established safe hosts for production of recombinant protein and have numerous of advantages such as capacity for efficient post-translational modifications and easy adaptation to growth in serum free medium. The experiments were held out with two different CHO cell lines and two different vectors, one with codon optimized AMH and one with the native human AMH sequence. Two different transfection methods were used, chemically based and electroporation followed by two different selection systems.

The results of the thesis show that a stable recombinant CHO cell line could be established for AMH by transfection of a vector containing the native AMH sequence. The results also indicated that the C-terminal purification tag in the expression vector altered expression. Selection with G418 enabled establishment of clones with stable expression. The produced AMH had desirable stability and properties like native AMH present in serum and plasma. This indicates that the produced AMH could be suitable for use in calibrators for immunoassay measuring native human AMH.

Keywords: Anti-Müllerian hormone, AMH, CHO cells, Cell line development, Immunoassay

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1. Introduction

Diagnostic methods play a major role in patient management and treatment, since better diagnostics enable better patient care. The diagnostic tests have to be sensitive, specific, fast and cost effective in order to fulfill the economic and scientific requirements. Since many years one important part of diagnostics is immunoassays measuring a certain biomarker in body fluids such as blood or urine. Biomarkers are indicators of a biological state and measurement of them provide information about a condition of interest (Buszewski and Schubert 2011, Florea and Anand 2012). Generous effort has been devoted to searching for biological marker substances which are specifically linked to a disease or a diseased state. Today various biomarkers for a number of diseases and biological states have been identified but there is still a big need of more specific biomarkers and to develop more tests available for more systems (Mendelsohn, Mortimer and Peeters 1998).

This thesis focuses on a fertility biomarker, anti-Müllerian hormone (AMH), an antigen expressed by the ovarian granulosa cells with important auto- and paracrine regulator functions in follicle development (Pearson et al. 2016). AMH serum level has emerged as an exceptional biomarker for the ovarian reserve that can be used for prediction of premature ovarian failure, prediction of the chance for success of in vitro fertilization and aid in the diagnosis of polycystic ovary syndrome (PCOS) (Gassner and Jung 2014). One big advantage with AMH compared to other clinically available fertility biomarkers such as follicle stimulating hormone (FSH) or inhibin B is that the level is constant through the menstrual cycle (Pearson et al. 2016). This means that the blood test could be taken any day without affecting the result.

One essential part of immunoassays and their detection of biomarkers are the calibrators. Calibrators contain known amounts of the biomarker that should be detected in the test. Important properties of a calibrator are that it should be stable and have properties like native AMH present in serum and plasma. When developing a test for an immunoassay is a big amount of the antigen needed and one way to produce this amount is by over expression in Chinese hamster ovary (CHO) cells. CHO cells is a frequently used system for production of recombinant antigens because of their adaptability and ease of genetic manipulation, their capacity for proper protein folding and post translational modification (Kim et al. 2012, Jayapal et al. 2007).

It is essential that both the produced recombinant antigen and the native antigen are recognized by the antibodies in the immunoassay. Earlier attempts have been made to over-express AMH in CHO cells at the Fujirebio Diagnostics AB (FDAB), but unsuccessfully. The attempts were carried out with two different vectors and chemical transfection. Both of the vectors have C-terminal tags for purification and one theory for

the unsuccessful transfections is that the C-terminals tags are too big and cause problem with folding or hinder translation and thereby protein secretion or expression. The starting point for this project was to introduce a stop codon before these C-terminals and verify the theory.

1.1. Aim

The goal of the project is to develop a CHO cell line with overexpression of recombinant anti Müllerian hormone (rAMH). The characteristics of the produced AMH should be evaluated for its suitability as a component in calibrators for an automated immunoassay.

1.2. Limitations

The project is limited to protein expression and will not include protein purification because of time limitation.

One other limitation of the project is the use of CHO cells as expression host without trying other mammalian cell lines. This is since CHO cells are the most commonly used mammalian host for production of recombinant proteins and is thereby the most promising alternative.

1.3. Outline of the thesis

The thesis is divided into six different chapters. Chapter 1 describes the background of the thesis, aim and limitations.

Chapter 2 contains necessary theoretical background about in vitro diagnostics (IVD), recombinant proteins, anti-Müllerian hormone (AMH), mammalian cell lines and the important steps in CHO cell line development.

Chapter 3 is describing the materials and method procedures used during the project. The chapter is guiding the way from construct design and initial work with CHO cells to transfection, development of stable clones and evaluation of the produced protein.

Chapter 4 presents the obtained results related to the method procedure and in Chapter 5 are the results discussed. Finally chapter 6 contains the conclusions drawn from the thesis.

2. Theory

In this chapter the theoretical background of the project is presented. Initially the background of immunoassays in diagnostics, recombinant proteins and mammalian cell lines is described. Following the background there is an overall description of the strategy for developing a stable CHO cell line.

2.1. In vitro diagnostics (IVD)

In vitro diagnostics (IVD) is an important category of medical devices. IVDs are, by definition of the Food and Drug Administration (FDA), "… reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body." A shorter and simplified definition is stated by *Wayne R. Patterson* "IVDs are laboratory instruments, test kits, or reagent systems".

2.1.1. Immunoassays

Immunoassays are frequently used analytical methods with the purpose to detect and quantify the presence of a specific analyte in a sample. Various methods for immunoassays exist but the general principle is that detection appears through specific interaction between antibody (Ab) and antigen (Ag). Immunoassays have various applications and one of them is to detect disease biomarkers, which enable to develop correct diagnosis, to follow the progression of a disease and to design a course of therapy (Van Emon 2011). Acquired reagents for an immunoassay are antibodies, analyte (antigen), calibrators, sample matrices, labels, substrates and control samples. The analyte used in calibrators and controls is normally a recombinant form of the native analyte. Important properties of such recombinant antigen are that it mimic the native antigen, is stable and show linearity over a pre-determined concentration *(Cox et al.* 2014).

Today both manual and automated formats of immunoassays are used, even though the use of automated systems has increased dramatically in almost all areas of clinical laboratory. An automated immunoassay system minimizes human activity in performing the task. Advantages with the automated system are improved test formats, improved reproducibility and greater within-laboratory uniformity (Bock 2000).

Enzyme immunoassay (EIA) is a method used to detect specific antigens or antibodies in a sample and is well known in IVD. The method relies on antibodies and enzymes, the antibodies detect specific antigens through affinity interactions and the enzyme that is conjugated to the antibodies catalyzes a chemical reaction by addition of substrate and result in a color switch (Kurstak 1986). Figure 1 shows a schematic picture of one arrangement of a sandwich EIA. The optical density of the samples can be recalculated

to concentration of the specific antigen, since the degradation of the enzyme substrate is proportional to the concentration of the antigen or antibody concentration in the sample (Voller, Bidwell and Bartlett 1976).

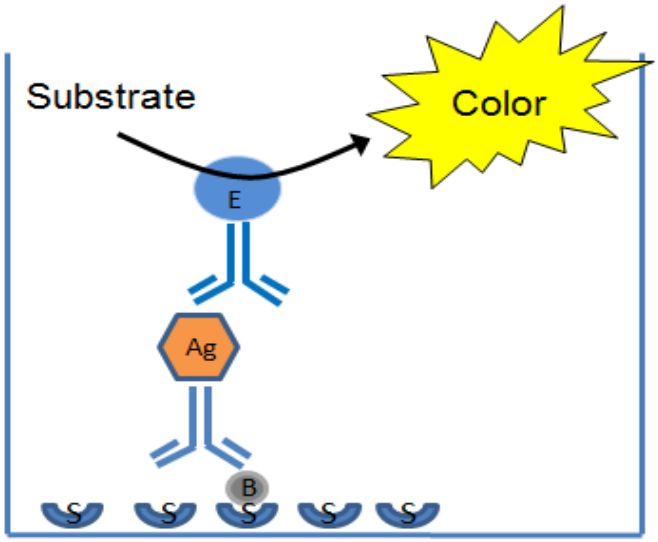

Figure 1. Schematic description of a sandwich EIA with a streptavidin (S) coated well, a biotinylated (B) catcher antibody and an enzyme (E) conjugated tracer antibody.

The most frequently used enzymes in immunoassays are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). HRP is an oxidoreductase that can be used with various hydrogen donors to reduce hydrogen peroxide, which can generate colored, fluorescent or luminescent derivatives, depending on the substrate (Cox et al. 2014). Biotin is a small water-soluble vitamin with very high affinity for the egg white protein avidin. Streptavidin is the bacterial form of avidin, which unlike avidin has a neutral isoelectric point and does not contain carbohydrates (Weeks, Kricka and Wild 2013). These properties are favorable in assay systems, resulting in lower nonspecific binding and hence greater sensitivity. The strength and the speed of binding between streptavidin and biotin can be used for linkage of molecules and generation of signal. One application of streptavidin and biotin in immunoassays is generic capture systems, where streptavidin is coated on the solid phase and the catcher antibody is biotinylated. The generic capture system reduces the need for new coating methods and facilitates usage of antibodies with high affinities for the analyte but weak coating properties (Weeks et al. 2013).

Chemiluminescent enzyme immunoassay (CLEIA) is a variant of EIA, where the tracer antibody is labeled with an enzyme detectable with luminescent substrates. The approach increases in popularity because of its competitive detectability and sensitivity (Roda et al. 2004).

2.2. Recombinant Proteins

Production of recombinant proteins is a challenging task that plays an essential role in the drug discovery process and IVD (Bollin, Dechavanne and Chevalet 2011). The first therapeutic protein from a recombinant mammalian cell line was approved on market 1986 and was human tissue plasminogen factor, tPA. This approval triggered the use of mammalian cells to express recombinant proteins in industry and 2004 were about 60-70% of all recombinant protein pharmaceuticals produced in mammalian cells (Wurm 2004).

2.2.2. Anti-Müllerian Hormone

Anti-Müllerian Hormone (AMH), also known as Müllerian Inhibiting Substance (MIS) is a glycoprotein homodimer consisting of two 72 kDa monomers linked by disulfide bridges (Kumar *et al*. 2010). It is a differentiation factor that belongs to the transforming growth factor -β (TGF-β) family. A common characteristic for the TGF-β family is that they are synthesized as large precursors with a short signaling sequence followed by the pre-hormone that forms the homodimer. Before secretion the mature protein is glycosylated and forms a 144 kDa homodimer of the 560-amino acid preproprotein. The 24 first amino acids are cleaved of from the preproprotein during synthesis, resulting in the propeptide (proAMH), which is an inactive form of AMH that does not interact with AMH receptor (Pankhurst and McLennan 2013). The 72 kDa monomer subunits contain a N-terminal domain, also called pro-region, and a C-terminal domain, also called mature-region. The N-terminal domain is believed to be required in order to enhancing the activity of the C-terminal domain and thereby reach the full bioactivity of AMH, this in contrast to other TGF-β family members *(Kumar et al.* 2010). Between 5 and 20 % of the secreted AMH is enzymatically cleaved at a specific site (between amino acids 451 and 452, where amino acid 1 is the start coding methionine) to form two polypeptides of 58 kDa proregion (AMH_N) and 12 kDa mature region (AMH_C), where the receptor activating component is AMH_C. The two parts of the molecule remain in a noncovalent complex ($AMH_{N,C}$) (Kumar *et al*. 2010, Pankhurst and McLennan 2013). An illustration of the cleaved AMH monomer is shown in figure 2.

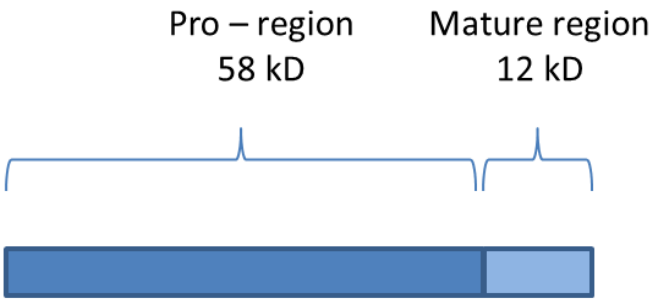

Figure2. Monomer of human AMH with a N-terminal pro region and a C-terminal mature region.

AMH perform various physiological functions but is limited to expression in genitals. The level of AMH in blood varies between sex and age, highest level is found in male embryos and prepubescent boys where it plays a crucial role in male sexual differentiation (Kumar et al. 2010). AMH in males is secreted by the sertoli cells of the testis and induces regression of the Müllerian ducts during embryonic development. AMH is then continued to be produced in testicles until puberty when it starts to decrease, and the sex dimorphic pattern is lost (Kumar *et al.* 2010). In females AMH is expressed in low levels after birth, when menstrual cycle begins the level of AMH is slowly decreasing until menopause where it is undetectable (Gassner and Jung 2014). In females AMH is secreted by granulosa cells in ovary and has an important role in the ovarian folliculogenesis by inhibition of primordial follicle recruitment and oocyte maturation (Gassner and Jung 2014). Figure 3 shows the acting of AMH in ovary. AMH is acting by inhibiting the response of larger follicles to Follicle Stimulating Hormone (FSH)-induced growth and selection, which gives that the AMH level in bloodstream is proportionate to the size of the primordial follicle pool (Pearson *et al*. 2016, Visser *et al*. 2006). Since the AMH level in male decreases at puberty, young men and women will have about the same levels of AMH, which are about 10% of those in young boys (Pankhurst and McLennan 2013).

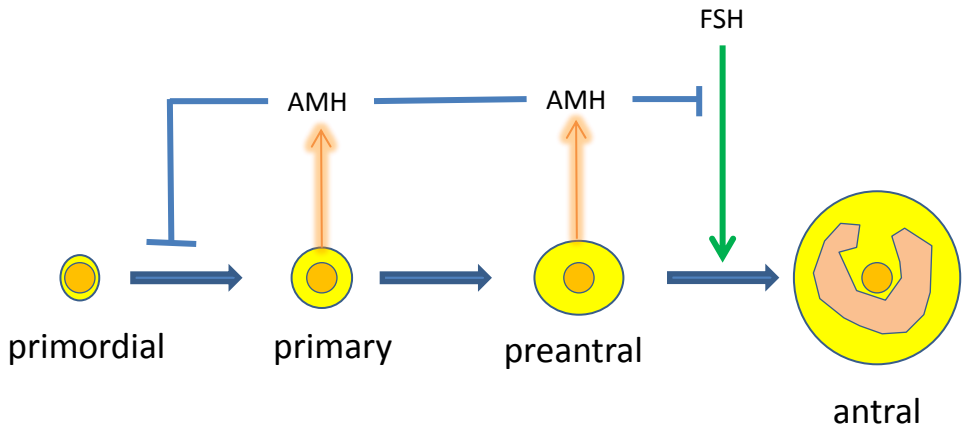

Figure 3. Model of the action of AMH in ovary and its expression in growing preantral follicels. AMH is inhibiting initial follicle recruitment and FSH stimulated growth of preantral follicels.

There are various proposed clinical applications of measuring serum AMH in humans. Due to its expression particularly by small primordial follicles AMH is classified as a direct serum marker of the ovarian reserve and a fertility marker (Gassner and Jung 2014). This has resulted in that the main field for AMH measurement is presently seen in ovarian function such as the assessment of the ovarian reserve, for ovarian pathophysiology, aid in the diagnosis of polysteric ovarian syndrome (PCOS) and for optimization of in vitro fertilization treatments (Gassner and Jung 2014, Visser et al. 2006, Pearson et al. 2016). Measurement of AMH for these applications is advantageously since it is independent of gonadotropin and thereby remains constant trough the menstrual cycle, which make it a unique marker (Kumar *et al.* 2010, Pearson *et al.* 2016). Other applications are diagnosis of intersex disorders in children (Lee *et al*.

2003), precocious puberty and the delayed onset of puberty, cryptorchidism, anorchidism and evaluation of male gondal function (Teixeira *et al*. 2001) and monitoring of granulosa cell cancer patients (Long et al. 2000).

2.2.2.1. AMH Immunoassays

The wide clinical application area has emerged the interest of developing immunoassays for AMH detection. The challenge is to develop assays with accurate AMH measurements, since there have been problems with the reliability of existing manual AMH assays (Gassner and Jung 2014). It is of high importance to have a robust and reproducible assay with a stable AMH sample in order to give the correct analyze of patient samples. It is critical that the sources of variability in AMH assays are identified and understood. Moreover clear recommendations for storage and proper assay conditions for AMH samples are needed (Rustamov et al. 2012).

The first reported AMH immunoassay was developed by Hudson *et al.* (1990) and did use a pair of monoclonal antibodies directed towards epitopes in the AMH_N pro region of human recombinant AMH (rhAMH) (Kumar *et al.* 2010). Nonetheless this assay gave altered results (Lee *et al.* 1996). The alterations were believed to be caused by storage and freeze thaw instability. Another assay developed by Long *et al*. 200 also used a pair of monoclonal antibodies, but one directed towards epitopes in the AMH_N pro region and the other one towards AMH_C mature region. Further, a third assay was developed by Al-Qahtani *et al*. 2005 that uses a monoclonal antibody pair directed to epitopes in the AMH_N pro region.

During this project two monoclonal antibodies that bind to two different epitopes in AMH were used. The tracer antibody bind to an epitope in the C-terminal region and the catcher antibody binds to the N-terminal region.

2.3. Mammalian cell lines

Mammalian cell technology is currently in focus for production of recombinant proteins in biopharma and IVD industry. A number of different cell lines have been used to produce recombinant proteins, among these mouse myeloma (NS0) cells, baby hamster kidney (BHK) cells, human embryo kidney (HEK-293) cells and Chinese hamster ovary (CHO) cells (Jayapal et al. 2007). Despite the availability of cell lines are CHO cells most commonly used and nearly 70% of all recombinant therapeutic proteins are produced in CHO cells (Jayapal *et al.* 2007).

2.3.1 Chinese hamster ovary (CHO) cells

CHO cell line is widely used as an expression system to produce recombinant proteins. The popularity of CHO cells has various explanations; the most important factors are their adaptability and their ease of genetic manipulation. Also an explanation is that it may be easier to obtain market approval for recombinant protein expressed in CHO cells since they have been proved as safe hosts for a long time. Another reason is that

the low specific productivity (q) can be overcome by gene amplification in CHO cells by using dihydrofolate reductase (DHFR) or G418 as selection markers (Kim et al. 2012). The low q is normally a disadvantage with using mammalian cells for production of proteins. Further, CHO cells are capable of post-translational modifications such as glycosylation and can relative easily be adapted to serum-free (SF) medium, which is an attractive property for large scale culture (Kim *et al.* 2012, Jayapal *et al.* 2007).

2.4 Serum free cell culture

One of the most common used serum in cell culturing is fetal bovine serum (FBS). Serum is an essential supplement in cell culture medium supporting cell growth and proliferation and contain a complex mixture of hormones, growth factors, cytokines, fatty acids, vitamins, carbohydrates and nitrogens (Brunner et al. 2010). But the use of serum comes with various disadvantages, ethical, scientific and economical (Do 2003, Brunner et al. 2010, Rodrigues et al. 2013). Ethical in terms of the harvesting and collection of the serum, that is obtained from various species but often prepared from blood extracted from cow fetuses, a process that could be painful and cause suffering (Do 2003). Scientific since serum is largely undefined, which result in serious lot to lot variations that make it hard to control culture conditions and thereby causing variability in protein expression. Moreover the serum may contain contaminants such as fungi, bacteria, mycoplasma and prions (Brunner *et al.* 2010, Do 2003). Furthermore the high protein content can complicate product purification (Rodrigues *et al.* 2013). Economical because of the high cost of high quality serum and because of the costly process when screen each lot of serum to identify components (Do 2003).

The major disadvantages with serum as a supplement in culture media have led to development of alternative serum free culture supplement. As earlier explained can various benefits be earned by serum free cell culturing, but cell adaptation to serum free is a time consuming step of the process development (Rodrigues *et al*. 2013). Mammalian cell lines can be adapted to the serum free alternatives through gradual decrease of serum concentrations in the medium or by direct adaptation. During the adaptation it is of high importance to measure cell growth rate and viability in order to determine successful adaptation or not (Rodrigues *et al.* 2013, Costa *et al.* 2010).

2.5. Strategy for recombinant mammalian cell line development

The strategy for a recombinant cell line development includes some essential steps, illustrated in figure 4.

Figure 4. Overall flow chart for a common strategy for production of recombinant protein in cultivated mammalian cells used in various studies (Jayapal *et al.* 2007, Kim, Kim and Lee 2012, Dalton and Barton 2014).

2.5.1. Transfection

The first step of developing a recombinant CHO cell line is the introduction of product DNA into the nucleolus of the cells for integration in the chromosomes (Jayapal et al. 2007). The introduction of the DNA of a protein of interest (POI), if successful, makes the cells to produce the protein. The transfection involves several steps such as design of the expression vector and choice of suitable transfection method. The ideal method should show a high efficiency, be easy to use and repeat and have low cell toxicity. The different methods for transfection can be categorized into biologically, chemically and physically mediated methods(Kim and Eberwine 2010).

2.5.1.1. Transient transfection versus Stable transfection

Transient gene expression (TGE) is a frequently used technique for rapid and cost effective production of recombinant proteins (Bollin et al. 2011). Transiently transfected genetic material is not integrated to the host genome and is only expressed for a limited time (Kim and Eberwine 2010). TGE have made it possible to produce high quantities (milligram to gram) of recombinant proteins just some days or weeks after initiation of the process (Bollin et al. 2011).

In stable transfections, genomic material is integrated to the host genome and sustain the expression even after replication (Kim and Eberwine 2010). Establishment of stable transfectants is far more time consuming. However it is generating cells with continuous expression of the product for a longer period and is therefore most commonly used for clinical and commercial procedures (Costa *et al*. 2010). Stable transfection have numerous advantages such as it give a reliable and consistent yield and quality of the recombinant protein (Dalton and Barton 2014).

The different integration sites of the product DNA into the genome in transient and stable transfection also affects which factor that is most important for the productivity. For transient transfection is the transfection efficiency of highest importance and for stable transfection is the frequency of DNA integration into the chromosome of higher importance (Costa et al. 2010). The productivity of both of the methods are strongly correlated with the strength of the promoter driving the expression of the gene of interest (Costa *et al.* 2010).

2.5.1.1. Expression vector

There are three main features that correspond with an ideal vector for recombinant protein production: 1) the expression should be independent of the site of integration in the genome, 2) the expression should correlate with the number of transgene copies that have been integrated to the genome and 3) the expression efficacy should be preserved over time (Blaas *et al.* 2009). These features result in that most of the mammalian vectors are plasmids.

The ability of a plasmid to express genes in mammalian cells requires a cassette containing a promoter and an enhancer (Costa *et al.* 2010). Promoters are upstream elements needed to drive the expression of the recombinant gene, while enhancers are elements that intensify transcription. When using mammalian cells for expression of recombinant proteins it is common to use a strong viral promoter/enhancer or a promoter/enhancer combination known to be particularly active to a certain host cell. The most widespread promoters are strong viral promoters from cytommegalovirus (CMV) and simian virus 40 (SV40) (Dalton and Barton 2014).

Furthermore cis-acting elements that stabilize and enhance translation are needed in the expression vector (Dalton and Barton 2014). These elements can be placed both within and outside the coding sequence. A number of different elements can be used including polyadenylation signals and the Kozak sequence. The Kozak sequence is the consensus sequence for surrounding the initiator codon and play a role in translation initiation (Dalton and Barton 2014, Costa et al. 2010). Downstream of the ORF is a polyadenylation signal derived from SV40 or bovine growth hormones commonly found that is important for the efficiency of transcriptional termination and 3' processing (Dalton and Barton 2014). Also the inclusion of introns effect the transfection efficiency, one or more introns between the promoter and the coding sequence can result in a more efficient cytoplasmic transport (Dalton and Barton 2014). Linearization of the plasmid could also be a way to improve the efficiency of stable transfection (Costa et al. 2010).

Generation of a cell line with stable expression requires a selectable marker, which could be expressed in the expression vector. It should be driven from a weak promoter

in order to increase the chance to obtain a high level of producer cells (Costa et al. 2010).

2.5.2. Selection methods

After DNA integration by transfection only stable transfectants survive and are selected while the cells with unsuccessful integration are killed. The selection is performed by subjecting the cells for culture conditions that only cells expressing the marker gene from the vector can stand.

A common method to establish a CHO cell line with high specific productivity levels is to use the methotrexate (MTX) induced DHFR amplification of recombinant genes (Grillari *et al*. 2001, Dalton and Barton 2014). DHFR is a small enzyme involved in nucleotide metabolism and catalyzes conversion of folic acid to tetrahydrofolate (THF). THF is a cofactor for one-carbon moieties that is needed for synthesis of thymidine, glycine and purine (Jayapal *et al.* 2007). DHFR deficient CHO cell lines (dhfr-) are thereby lacking the ability to produce thymidine, glycine and purine and are used in the DHFR gene amplification system. Introduction of heterologous genes into these cells can be accomplished by co-transfection with a functional copy of the DHFR gene, and thereby make the transfected cell able to synthesize the nutrients (Jayapal *et al.* 2007). Clone selection can then be performed by growing cells in media that lack the nutrients. When the cells are cultured in high levels of MTX, an inhibitor of DHFR, the transfected cells will fight against the decrease of the DHFR level and multiply its copies of the DHFR gene and the gene of interest, resulting in amplification. The cells that have been successfully transfected contain the DHFR gene and will survive.

Another alternative is selection with a drug selection marker. One of the most common selection agents for mammalian cells is geneticin (G418), an aminoglycoside antibiotic that inhibits the elongation step in polypeptide synthesis, both in eukaryotic and prokaryotic cells by disrupting ribosome function (Becker and Cooper 2012). The cells that have G418 resistance carry a neomycin resistance gene (neo) on the expression vector (Dalton and Barton 2014). The optimal concentration of G418 for selection has to be determined for each cell line and is normally done by a kill curve assay.

2.5.3. Screening/Cloning

After the selection and recovery step the cells held are under selection pressure for some further time in order to isolate a pool of heterogeneous high expressing cells. These cells are heterogeneous since they have different integration sites of the protein DNA, various copy numbers and a range of production rates. The screening step is performed in order to isolate individual clones and thereby a monoclonal cell population with the highest possible productivity and growth rate (Jayapal *et al.* 2007). This is commonly performed with limiting dilution technique in multi well plates.

2.5.4. Expansion

After the screening process, when promising clones with high production rate have been successfully isolated, the cultivation of the chosen clones is expanded. The clones are expanded by several passages, the growth is evaluated and the process is optimized (Jayapal *et al.* 2007). The methods that should be used for the expansion depends on each cell line.

Scale up of adherent cells is limited by surface-attachment requirements, which make the surface to volume ratio an important parameter when choosing culture system. Adherent cells can be adapted to various culture systems such as roller bottles, microcarriers suspended in bioreactor and multilayer flasks (Chu and Robinson 2001, Dalton and Barton 2014, Wurm 2004).

2.5.5. Cell banking

The final step of the cell line development is cell banking. The cell line with best evaluation results is chosen and vials are frozen for future use. This is the clone that will be used in commercial manufacturing (Jayapal *et al.* 2007).

3. Methods and materials

In this chapter the methods and the materials used during the project are explained. The methods are presented in chronological order, beginning with methods used at initiation of the project.

3.1. Construct design

As a starting point for the vector design, two plasmids were chosen: pD18-AMHFc that is a derivate of pCDNA 3 and pcMV6-AMH, TrueORF® from Origene. Little is known about the pD18-AMHFc vector but the AMH sequence is codon optimized for CHO cells and contain a C-terminal Fc-tag. The pcMV6-AMH vector seen in figure 3 contains the native AMH sequence and has a C-terminal Myc-tag. These vectors have earlier been used for transfection in CHO cells but without success. The hypothesis was that the C-terminal tags were too big and resulted in incorrect folding or somehow prevented expression or secretion. The idea was to get rid of the C-terminal tags in order to get a shorter sequence. Introduction of a stop codon after the AMH sequence by site directed mutagenesis could be a way to eliminate these tags and obtain successful expression and recognition.

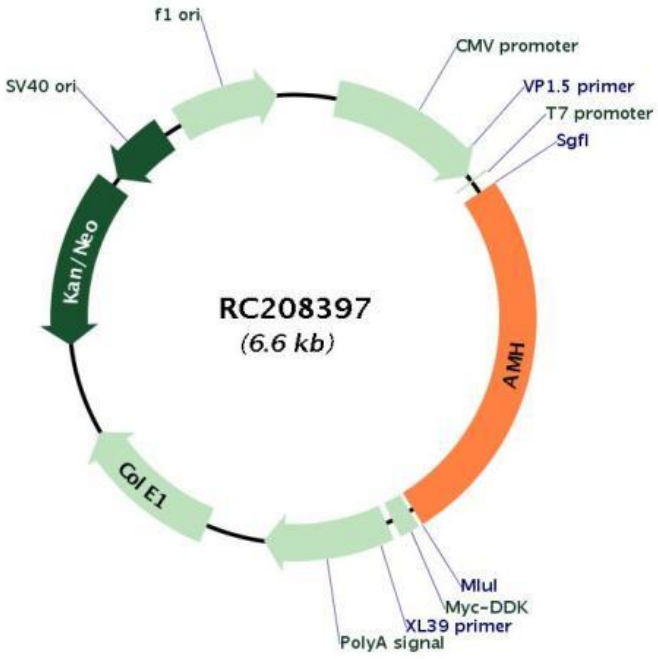

Figure 5.Plasmid map for thepcMV6-AMHvector (Origene Plasmid map RC208397).

3.1.1. Site directed mutagenesis

Site directed mutagenesis was performed with the use of QuickChange II Site-Directed Mutagenesis Kit from Agilent Technologies. Mutagenic primers were designed by help of the web-based QuickChange Primer Design Program available at Agilents webpage. The stop codon was introduced by changing the nucleotides one by one, as point mutations. Two different stop codons were used, TAG (proposed by the program) and

TGA (most common stop codon in CHO cells), resulting in two different mutagenesis experiments for each vector, see table 1.A total of eight designed mutagenesis primers were needed for the mutations and were ordered from Integrated DNA Technologies. These primers are listed in Appendix A.

Table 1.The different mutations performed by site directed mutagenesis

Short name	*Vector*	*Mutation*
Mut 1	pD18-AMHFc	From GAT (Asp) to TAG (STOP)
Mut 2	pD18-AMHFc	From GAT (Asp) to TGA (STOP)
Mut 3	pcMV6-AMH	From ACG (Thr) to TAG (STOP)
Mut 4	pcMV6-AMH	From ACG(Thr) to TGA (STOP)

The site directed mutagenesis was performed as explained in the included protocolwith the QuickChange II Site-Directed Mutagenesis Kit from Agilent Technologies. The cycling of the reactions was performed with a T100™ Thermal Cycler from Bio Rad Technologies. The transforming reactions with pD18-AMHFc were plated on agar plates containing 100 µg/ml of ampicillin and transforming reactions with pcMV6-AMH were plated on LB agar plates containing 50 µg/ml of kanamycin. The agar plates were incubated over night at 37 °C in an INCU-Line digital mini incubator from VWR collection.

Five colonies from each mutation were freshly streaked on new selective LB agar plates and inoculated overnight in liquid culture containing LB and 50 µg/ml ampicillin or 25 µg/ml kanamycin. The cultures were placed in an Innova 4000 incubator shaker from New Brunswick Scientific at 37 °C and 250 rpm overnight.

The cells from the overnight culture were harvested and the plasmid was purified with Plasmid DNA purification kit from Machery-Nagel and performed as explained in the Nucleospin® Plasmid protocol included in the kit.

Concentrations of the purified plasmids were measured with a NanoDrop 2000c spectrophotometer from Thermo Scientific. The plasmids were diluted to a concentration of 50 – 100 ng/µl and sequenced at Eurofins Genomics. The sequencing primers can be seen in Appendix A. The FASTA files from the sequencing were analyzed with the nucleic tools CLUSTALW, CLUSTALWPROF and REVCOMP available on the web based program Biology Work Bench (version 3.2).

3.2. Initial work with CHO cells

The two different CHO cell lines used during the project are presented in table 3 together with information of some culturing conditions. Both cell lines were supplied by American Type Culture Collection, ATCC. The CHO K1 cell line was used together

with the pcMV6-AMH vector and the CHO dhfr- cell line was used together with the pD18-AMH vector. The standard culturing medium is the same for both cell lines, Iscove's modified Dulbecco's medium (IMDM) with 1% Dulbecco's Modified Eagle Medium (DMEM) and 1 x Gibco® HT Supplement containing sodium hypoxanthine (10 mM) and thymidine (1.6 mM) all from Thermo Fisher Scientific.

Cell line	*Growth type*	*Standard Medium*	*Amplification system*	*Selection Medium*
CHO-K1	Adhesion	IMDM, 1% DMEM supplement 1xHT, 10%FBS	G418	IMDM, 1% DMEM-supplement 1xHT, 5% FBS, G418
CHO dhfr-	Adhesion	IMDM, 1% DMEM supplement 1xHT, 10%FBS	DHFR-MTX	IMDM, 1% DMEM -supplement, 5 %FBS, increasing amount of MTX

Table 2.CHO cell lines used during the project together with culturing information.

The cell lines were thawed and suspended in standard medium in T75 tissue culture flask and were incubated in 37 °C, 8 % CO_2, passaged every third to fourth day. All laboratory procedures involving cells were performed in a laminar flow cabinet.

3.2.1. Antibiotic kill curve

First experiment

A kill curve for G418 was performed by seeding CHO-K1 cells at a density of 1.6 x 10^5 cells/ml in four 6-well tissue culture plates. The cells were cultured overnight in standard medium, 5 ml in each well. Increasing amounts of G418 were added to duplicate wells (0 - 1000 µg/ml), see appendix A. Media was replaced every second to third day for a week and the cultures were examined every day for signs of toxicity.

Second experiment

A second kill curve for G418 was performed with lower initial concentration of cells, this time CHO-K1 cells were plated in a 6-well tissue culture plate at a density of 0.5 x 10^5 cells/ml. The cells were directly cultured in media containing increasing amounts of G418 (0 - 600 µg/ml), see appendix A. Media was replaced every 2 - 3 days for a week and the cultures were examined every day for signs of toxicity.

3.2.2. Adaptation to growth in serum-free media

The serum free media used for the adaptation was the EX-CELL® CHO DHFR⁻ Medium from Sigma-Aldrich, complemented with 11 ml/l GlutaMAX™ and 1 X HT

supplement. The adaptation procedure was performed as recommended in the included product information that came together with the medium and as Rodrigues *et al.* 2013 described for direct adaptation to serum free medium. CHO dhfr- cells were cultured in different ratios of standard medium and EX-CELL® CHO DHFR⁻ medium, starting with 1:1 following by 1:3, 1:7 and finally 100 % of EX-CELL® CHO DHFR⁻medium. The cells were considered as adapted when the concentration had reached 1×10^6 cells/ml, with at least 90 % cell viability.

The cells were diluted to a density of 2×10^5 cells/ml in a T75 tissue culture flask with 50 ml medium and incubated at 37 °C, 8 % CO_2. For each new ratio the viability was checked by addition of trypan blue exclusion 1:2 and the concentration was calculated by use of a Bürker chamber.

3.3. Transfection

An initial step for all of the transfection methods was to prepare and purify the plasmid DNA from endotoxins. For this was the Qiagen plasmid mini kit used and performed as stated in the included protocol "Plasmid or cosmid DNA purification". One of the successfully sequenced colonies for each mutation from the site directed mutagenesis step was picked from the LB agar plates and placed into 14 ml sterile falcon tubes with 3 ml liquid LB and appropriate antibiotic (50 µg/ml ampicillin for pD18 and 25 µg/ml for pcMV6).The tubes were incubated over day in an Innova 4000 incubator shaker from New Brunswick Scientific at 37 °C and 300 rpm. At the end of the day the cultures were diluted 1:500 with fresh LB medium.

The diluted cultures and the original cultures were incubated overnight under the same conditions as the incubation over day. The diluted cultures were harvested and purified using the plasmid mini kit and the undiluted cultures were added 15% glycerol and saved as freezing cultures. In the final step of the protocol the purified precipitated plasmid pellets were dissolved in 30 µl of 10mM Tris-HCL, pH 8,5. The concentrations were determined by measurements with a NanoDrop 2000c spectrophotometer from Thermo Scientific. This purification procedure was repeated various times in order to get enough amounts of the purified plasmids for the transfections stated in table 3.

Transient and stable expression after transfection was measured with EIA and CLEIA as described in sections 3.4.1 and 3.4.2.

Table 3. Transfections performed during the project.

Experiment Nr	*Cell line*	*Passage*	*Plasmid*	*Transfection method*
1	CHO dhfr$^-$	P4	Mut 1	Chemically
1	CHO dhfr$^-$	P4	Mut 2	Chemically
2	CHO dhfr$^-$	P1	Mut 1	Chemically
2	CHO dhfr$^-$	P1	Mut 2	Chemically
2	CHO K1	P1	Mut 4	Chemically
3	CHO K1	P5	Mut 3	Chemically
4	CHO K1	P8	Mut 4	Electroporation
4	CHO K1	P8	Mut 4 -linearized	Electroporation

3.3.1. Chemical-based transfection

Chemical-based transfection was performed with X-tremeGENE HP DNA Transfection reagent from Roche, a multi-component reagent that forms a complex with DNA and transports the complex into the host cells. The procedure was performed as described in the protocol included with the reagent.

One day before the transfection cells were cultivated in a 6-well tissue plate at various densities between 2 –3.5 x 10^5cells/ml (2 ml/well). On the day of transfection the wells with a monolayer of cells with 70 – 90 % confluence were chosen for transfection. The medium in the wells was removed and replaced with fresh medium. At the first transfection four different ratios (1:1, 2:1, 3:1 and 4:1) of microliter X-tremeGENE HP DNA Transfection reagent to microgram DNA were used. For the later transfection experiments just two different ratios were used (2:1 and 4:1). The incubation time of the transfection reagent and the DNA complex was 15 min for the first transfection and 25 min for the later transfections. After transfection the plates were incubated for 48 - 96 hours in 37 °C and 8 % CO_2. The medium was evaluated of transient protein expression. The transfected cells were diluted 1:4 and moved to T75 tissue flasks with 50 ml selection medium, in order to investigate stable expression.

3.3.2. Transfection by electroporation

The electroporation was performed with two different articles as guidelines for the experimental procedure (Lin, Lee and Cheung 2009) and (Longo *et al.* 2013). Electroporation was performed using both circular and linear DNA.

Linearization was performed with two different restriction enzymes, DraII and ApaLI, from New England Biolabs. The reactions were prepared in PCR tubes, two tubes of each reaction, 50 µg purified plasmid DNA, 250 U enzyme, 1 X cutsmart buffer and MQ water was mixed in each tube. The DNA was digested in the PCR incubator, T100™ Thermal Cycler from Bio Rad, at 37 °C for 150 minutes. The result was evaluated by electrophoresis. Linearized DNA was extracted from the agarose gel using QIAquick® Gel Extraction kit from Qiagen.

Both linear and circular DNA were prepared by combining 65 µg of purified vector DNA and Hanks balanced salt solutions (HBSS) to a final volume of 0.2 ml in sterilized tubes. The solutions were incubated in room temperature while preparing cells.

The cells were loosened with trypsin and suspended in 10 ml standard medium. The cells were counted and centrifuged in 340 x g for 3 minutes, the supernatant was discarded. The pellet was washed with HBSS two times cientrifuged at 340xg for 3 minutes and was dissolved with HBSS to a concentration of 20 x 10^6 cells/ml. The cell solution (0.2 ml) was mixed with the pre prepared vector DNA and then transferred to 2 mm electroporation cuvettes from Bio-Rad. The cuvettes were capped and incubated on ice for 10 minutes. The cells were electroporated with two pulses of 400 V, 375 µF, kept in the electroporation chamber for 2 minutes followed by incubation on ice for 10 minutes. Fresh medium was added to the cuvettes and mixed gently. The electroporated cells were transferred to a centrifuge tube and diluted with 10 ml fresh medium, 2 ml was transferred to a well in a 6 well plate and the rest was transferred to T25 culture flask and adjusted to a volume of 10 ml with standard medium. The plates and flasks were incubated in 37 °C and 8 % CO_2 for 72 hours.

After three days the medium was saved for evaluation of transient protein expression. The medium was replaced with fresh medium containing 0,7 mg/ml G418 for initiation of selection of successfully transfected cells and stable expression.

3.4. Evaluation of protein expression

After transfection the protein expression was evaluated with various methods in order to quantify the amount of the produced AMH in different stages, such as transient and stable expression and later on to determine the best expressing clone.

3.4.1. Enzyme immunoassay – EIA

EIA was performed in order to quantify the amount of the produced AMH in the growth medium of the transfected cells. It was used both to measure transient and stable expression and later on to measure the expression levels of individual clones. A one step test was used which means that both of the antibodies and the sample were added at the same time.

96-well streptavidin coated plates were prewashed in a Hydroflex washer from Tecan and 50 µl medium together with 100 µl of specific antibodies for the antigen (biotinylated catcher antibody A (2 µg/ml) and HRP tagged tracer antibody B (2 µg/ml) were added to the wells. The plate was incubated one hour in room temperature on a DELFIA® plateshake from PerkinElmer at 150 rpm. After the incubation the plate was washed 6 times using strip-mode program and 100 µl of TMB was immediately added and incubated on the shaker for 30 more minutes. The plate was read both before (at

620 nm) and after addition of stop solution (at 450 nm). The plate was briefly shaken before the read with a VMax Microplate Reader from Molecular Devices.

3.4.2. Automated CLEIA

Automated CLEIA runs were performed as a complement to the EIA runs. This method was most frequently used in the end of the project.

3.5. Establishment of stable clones

After a number of selection cycles, when the cells reached relatively high AMH expression level and had a good growth rate screening/cloning experiments were initiated by limiting dilution. Two separate cloning procedures were performed during the project. The first cloning experiment was on CHO K1 cells chemically transfected with mut 4 (transfection number 2) and the second cloning experiment was on CHO K1 transfected by electroporation with linearized mut 4 (transfection number 3).

First experiment

The first cloning experiment was performed with the CHO K1 cell line transfected by X-tremeGENE HP DNA transfection reagent with pCMV6-AMH with codon optimized introduced stop codon (Mut4).

The cells were loosened with trypsin and counted in a Bürker chamber in order to estimate the concentration of the cells. The cells were diluted to contain 3.33 cells/ml and 33.3 cells/ml in 125 ml standard medium which corresponds to 1 cell/well and 10 cells/well in four 96 well tissue culture plates with 300 µl in each well. Four plates four each concentration were seeded with 300 µl medium in each well. After five days of incubation the wells containing cells from one distinct clone were marked with a dot at the lid of the plate. First screening by EIA for highest expressing clones was performed when the wells contained about 100 cells/well, which was approximately six days after screen initiation. 200 µl medium from each marked well were harvested and saved in sterile tubes and were replaced with fresh standard medium. The EIA was pursued as explained in section 3.4.1.

For the plates with the higher concentration (10 cells/well) all wells were tested with EIA in order to identify the best expressing wells. The two wells with the highest expression on each plate were saved and cultivated in wells in a 6-well plate and the two highest expressing of those were cloned once again. This time the cells were diluted to a concentration of 0.67 cells/ml in 125 ml medium and distributed at 300 µl/well in four 96-well plates. After five days of incubation were the wells containing cells from just one single clone marked with a dot and the media of these wells were analyzed with EIA.

After the first screen, when the cells covered 50 % of the surface area of the well the best expressing clones were transferred to one well in 6-well plates with 8 ml fresh

medium. To avoid cross-contamination between the clones, one clone was cultured on each plate. The 96-well plates were saved as a backup with freshly added medium. When the cells covered about two thirds of the well the cells were loosened with trypsin and cultured in three wells on the 6-well plate. The old medium from the first well were divided equally between the three wells and fresh media was added to a volume of 8 ml in each well.

When cells in the three wells reached 70 % confluence, freeze-cultures were prepared (five cryo tubes for each clone). The culture medium was removed and the cells were loosened with trypsin, suspended in media and transferred to a 15 ml centrifuge tube. The cells were centrifuged at 2000 x g for 8 minutes, the supernatant was discarded and the cell pellet was suspended in cold FBS with 10 % DMSO, 1,8ml/ampoule. The ampoules were placed in the fridge for 30 minutes and then transferred to -70 °C for 1- 3 days, finally the ampoules were placed in a -170 °C nitrogen freezer. 6 ml of the old media was transferred to each of the three wells once again and 2 ml of fresh media was added in each well, 5 days after the freezing, when the medium was yellowish, the medium was saved for a second screening.

Second experiment
The second cloning experiment was performed on the CHO K1 cell line electroporated with linear mut 4. This experiment followed the same procedure as the first experiment, with one exception: the cells were directly diluted to a concentration of 0.67 cells/ml in 125 ml medium and distributed at 300 µl/well in four 96-well plates.

3.6. Characterization of the produced AMH

This section describes the methods used for characterization of the produced AMH.

3.6.1. Western blot

Western blot was performed in order to confirm that the detected protein in the immunoassays had the expected size of AMH and to compare the produced AMH to a commercially available recombinant AMH. Two Western blots were performed, one with the tracer antibody and one for the catching antibody in order to see how the antibodies recognizes AMH. A Trans-Blott Turbo Blotting system from Bio Rad was used during this experiment.

Both non-reduced and reduced samples were analyzed with western blot. The samples are reduced in order to break disulfide bridges and to allow random coil conformation, which enable better separation by size in protein electrophoresis. Reduced samples are frequently used for Western blot. Some antibodies can't detect reduced proteins and therefore non-reduced samples were also tested.

Day one
Reduced samples were prepared by mixing medium with laemmli buffer x4, MQ-water and for the reduced samples DTT. Different concentrations were prepared by altering the medium and MQ-water volume. Mini protean® TGX stain free gels from Bio-Rad were used and 10 µl of sample was loaded in each well containing 1 – 50 ng of AMH. All of the samples were prepared in PCR tubes and was denaturized by incubation in the PCR incubator, T100™ Thermal Cycler from Bio Rad, at 70 °C for 70 minutes. Precision plus protein westernC™ standard from Bio Rad was used as protein marker. The gel electrophoresis was performed with Mini-Protean Tetra cell from Bio Rad and was mounted and used as described in the belonging set up and basic operations instructions. Two identical gels were run, one for tracer antibody A and one for catching antibody B. The gels were run for 45 minutes at 200 V and 1 X TGS was used as running buffer.

The gel was placed in a transfer-blot turbo transfer pack from Bio-Rad and the trans-blot turbo blotting system was used for transfer of proteins from gel to membrane. The membrane was transferred to a tray and cleaned with washer buffer, TBST (0.2 % Tween20), and incubated over night with 15 ml blocker (5 % blotting-grade blocker from Bio-Rad.

Day two
Blocker solution with 1 µg/ml of primary antibody was prepared and incubated in RT for 30 minutes under stirring, one solution for each antibody. One of the membranes was then incubated with antibody A blocker solution and the other on with antibody B on a shaker for one hour. The membranes were washed 4 times manually and 4 times on the shaker for 5 minutes with washer buffer. Meanwhile a secondary antibody blocker solution was prepared with 0.65 µg/ml polyclonal rabbit anti-mouse imunglobin-HRP from Dako, and 1.5 µl precision protein streptactin-HRP conjugate. The solution was incubated at RT for 30 minutes under stirring. The secondary blocker was added, 15 ml to each tray with membrane and was incubated on the shaker, in room temperature (RT) for one hour. The membranes were washed once again in the same way as previously. For detection Amersham™ ECL™ prime Western blotting detection reagent from GE Healthcare was used. The detection solution was prepared by mixing 700 µl of solution A and 700 µl of solution B directly before use for each membrane and pipetted over the membrane, followed by incubation for 5 minutes. The signal was detected with Chemidoc™ Touch imaging system from Bio Rad by chemiluminescence.

3.6.2. Stability
The section describes the procedures for the stability tests.

Stability in different matrixes
A stability study was initiated when the first clone had been frozen and once again confluent in three 6-hole wells, as explained in section 3.5. The antigen was spiked in

six different matrixes in a concentration of 10 ng/ml, the different matrixes are stated in table 4.

Table 4. Matrixes tested for stability

Matrix number	*Description*	*Concentration of AMH (ng/ml)*
1.	Serum A	10
2.	Serum A + 10 mM EDTA	10
3.	Serum B	10
4.	Serum B + 10 mM EDTA	10
5.	Phosphate buffer with BSA	10
6.	Tris buffer with BSA	10

The solutions of matrixes and antigen were prepared in 50 ml falcon tubes in a volume of 10 ml, stirred and aliquoted to 25 sterile 0,5 ml tubes with screw cap, 400 µl in each tube. The tubes were placed in boxes containing five tubes of each solution. These boxes were stored in different temperatures, one in – 70 °C as a reference, one in 4 °C, one in RT, one at 25 °C and the last one at 37 °C. The concentration of the AMH was measured weekly in order to determine the stability in each matrix.

Different BSA lots

Three different BSA lots were used in matrix six. Tubes containing matrixes from different BSA lots were put in boxes and stored in different temperatures as described above. The stability was checked weekly.

Freeze-thaw stability

A second stability test was performed in order to determine the effect of freeze thawing. 1 ml samples of medium from two different clones were stored at – 70 °C and one sample of each medium was stored at – 4 °C as reference. The samples were frozen and thawed at RT five times as stated in table 5 and the AMH concentration was measured both in the references and the freeze thawed samples.

Table 5. Setup for the freeze-thaw stability

Process	***Time (h)***
Freeze 1	12
Thaw 1	1
Freeze 2	1
Thaw 2	1
Freeze 3	12
Thaw 3	1
Freeze 4	1
Thaw 4	1
Freeze 5	1
Thaw 5	1

3.6.3. Linearity

To establish the linear rang of the produced AMH eleven concentration levels were analyzed, see table 6.

Table 6.Concentration levels investigated for establishment of the linearity.

11 concentration levels	Expected values (ng/ml)
1. Calibration matrix (L)	0
2. 0,9L + 0,1H	1,72
3. 0,8L + 0,2H	3,44
4. 0,7L + 0,3H	5,16
5. 0,6L + 0,4H	6,88
6. 0,5L + 0,5H	8,6
7. 0,4L + 0,6H	10,32
8. 0,3L + 0,7H	12,04
9. 0,2L + 0,8H	13,76
10. 0,1L + 0,9H	15,48
11. Medium containing AMH (H)	17,2

The expected values were calculated with equation 1.

$$Expected\ concentration\ = (C_H \cdot V_H + C_L \cdot V_L)/(V_L + V_H) \qquad (1)$$

Where C_L is the concentration in the calibrator matrix, V_L is the volume of the calibrator matrix, C_H is the concentration of the medium and V_H is the volume of the medium.

The expected values were compared with the measured values and the recovery was calculated with equation 2.

$$\%\ Recovery = \frac{Measured\ value}{Expected\ value} \cdot 100 \qquad (2)$$

3.7. Expansion

The best expressing clones were cultured in a T125 tissue flask with 100 ml medium. The AMH concentration was measured at some different time points. To evaluate the highest possible AMH production rate, the cells were cultured until cell death.

One of the clones was cultivated in four T125 tissue flasks with 100 ml medium, two with standard medium and two with DMEM. The cells from these flasks were then transferred to roller flasks with 300 ml DMEM medium and incubated 3 days at 37°C 8% CO_2, allowing the cells to attach on the surface. The roller flasks were placed in a roller incubator and expression was measured weekly.

4. Results

This chapter presents the obtained results from the project. Figure 4 shows a simplified scheme of the connections between the different steps and what they have contributed to in the project.

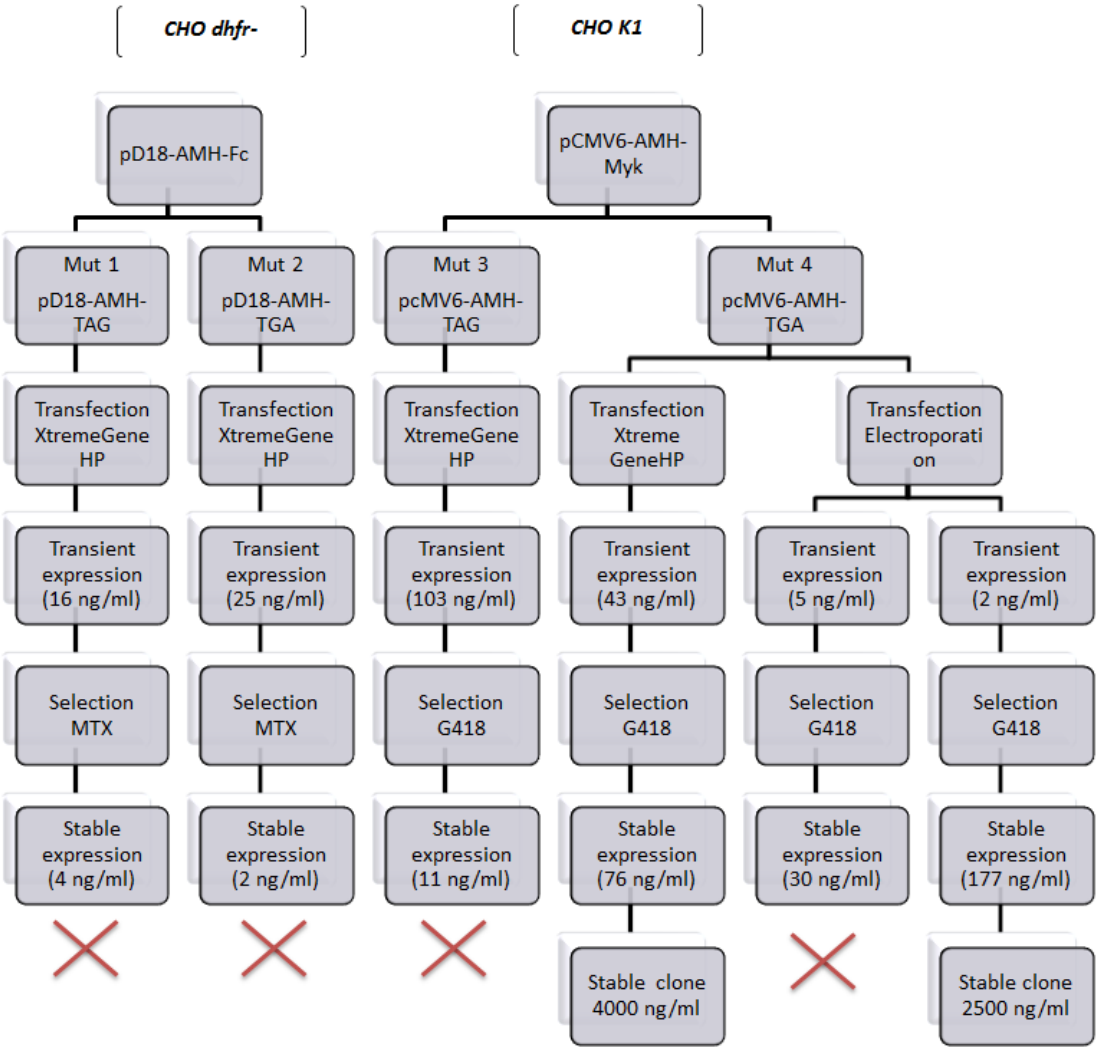

Figure 6. Simplified schedule over important results and their connection with each other's. Cross means that the experimental procedure ended at that step.

4.1. Construct Design

This section presents the results related to the construct design.

4.1.1. Site directed mutagenesis

The concentrations of the purified plasmids varied between 600 and 1000 ng/µl, exact concentrations are presented in Appendix B.

All of the four different mutations were confirmed by sequencing, one of the successful colonies for each mutation was used in the upcoming experiments.

4.2. Initial work with CHO cells

This section presents results from the methods related to the initial work with the two CHO cell lines.

4.2.1. Antibiotic kill curve

From the kill curves for G418 the following antibiotic doses could be determined for CHO-K1, see table 7. Low dose corresponds to the lowest dose with toxic response, optimal dose correspond to the dose with total cell death after seven days and high dose corresponds to total cell death after three days.

Table 7.Determined antibiotic doses from the G418 kill curves for the CHO-K1 cell line.

Dose	***Concentration (µg/ml)***
Low dose	400
Optimal dose	700
High dose	>1000

4.2.2. Adaptation to grow in serum free media

The result for the adaptation of the CHO dhfr- cell line to grow in serum free media is presented in table 8. The cells did adapt well until culturing in 1.25 % serum, but 0 % serum resulted in total cell death.

Table 8. Result of the attempt for adaptation to serum free media.

Ratio (standard medium:EXCELL)	***FBS (%)***	***Adaptation time (days)***
1:1	5	4
1:3	2,5	4
1:7	1,25	4
0:1	0	Cell death

4.3. Transfection

This section describes the results from the transfection procedures. The measured concentrations of the purified plasmid DNA varied between 1000 – 1400 ng/µl.

4.3.1 Linearization

Figure 7 shows a photo of the results for the gelelectrophoresis with the linearized smaples. The DNA digested with DraII shows two bands with sizes similar to the circular undigested vector DNA.

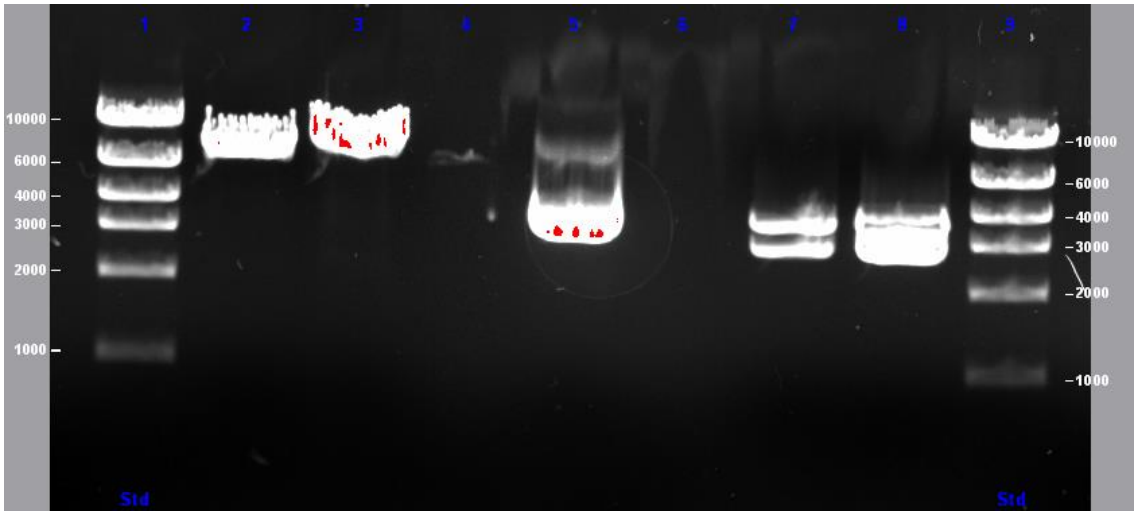

Figure 7. Electrophoresis of mut 4 digested with ApaLI and DraII. From left to right: High ladder, ApaLI, ApaLI, negative control (circular mut 4), DraII, DraII and finally high mass ladder.

4.3.2 Transient and stable transfection

Note that the stable expressions were measured on media from T75 culturing flasks containing 50 ml medium unlike the transient expressions that were measured on media from wells containing 2 ml medium. This results in that the first stable transfection seems lower than the transient expression.

Chemical based transfection

Transfection number 1 did not give any transient expression of AMH. Specific results are stated in Appendix B.

Table 9 shows the transient and stable expression of AMH for transfection number 2. The transient expressions were measured 2 days after transfection, the highest transient expression was measured for mut 4 4:1 with 39.7 ng/ml. Transient expression for mut 1, 2 and 4 2:1 were higher than calibration curve and no exact values are thereby available.

After 7 days selection and three passages mutation 4 2:1 showed the highest expressing with 18.1 ng/ml while the others varied between 1-3.1 ng/ml. After 14 days of selection and five passages was mut4 2:1 still the one with highest expression, 76,5 ng/ml. The others varied between 1.8-12.7 ng/ml. Mut 1 and 2 had a protracted morphology and were growing in "islands" with much lower confluence than mut 4.

Based on the low expression and lack of increased expression by selection the work with mut 1 and 2 were canceled. Mut 4 2:1 was chosen as candidate for further work.

Table 9.Transient and stable expression levels for transfection number 2.The ratios2:1 and 4:1 stand for the proportions of microliter X-tremeGENE HP DNA Transfection reagent to microgram DNA. The concentration was calculated by subtracting with the contribution of the medium.

Sample	***Transientexpression (ng/ml)***	***Stable expression (ng/ml) 7 days***	***Stable expression (ng/ml) 14 days***

Mut 1 2:1	>10	1.8	1.8
Mut 1 4:1	12.7	1.5	1.8
Mut 2 2:1	>10	1	1.5
Mut 2 4:1	21.9	1	1.3
Mut 4 2:1	>10	18.1	76.5
Mut 4 4:1	39.7	3.1	12.7

Table 10 shows the transient and stable expression of AMH for transfection number 3. The transient expressions were measured four days after transfection, mut 3 2:1 and mut 3 4:1 expressed about 100 ng/ml.

After seven days of selection and two passages mut 3 2:1 was expressing 11.2 ng/ml and mut 3 4:1 only 2 ng/ml. After 11 days of transfection the expression levels were more close to each other again and about 11 ng/ml. Since mut 4 and 3 only differ by the stop codon and that the stable expression levels didn't vary noticeable between them was further work with mut 3 canceled.

Table 10.Transient and stable expression levels for transfection number 3. The ratios 2:1 and 4:1 stand for the proportions of microliter X-tremeGENE HP DNA Transfection reagent to microgram DNA. The concentration was calculated by subtracting with the contribution of the medium.

Sample	*Transient expression (ng/ml)*	*Stable expression (ng/ml) 7 days*	*Stable expression (ng/ml) 11 days*
Mut 3 2:1	100	11.2	11.5
Mut 3 4:1	110	2	10.7

Electroporation

Table 11 shows the transient and stable expression level of AMH for transfection number 4. The transient expressions were measured two days after transfection, with expression levels of 1.36 ng/ml for the cells transfected with linear DNA and 5.07 ng/ml for the cells transfected with circular DNA. The confluence of cells was very low, about 20 %. After four days of selection and one passage the cells were about 40% confluent and the cells transfected with circular DNA did still express the highest level, 7 ng/ml. After 15 days of selection and four passages the cells were about 100 % confluent and now the cells transfected with linear DNA was highest expressing, 177.5 ng/ml. Since the cells transfected with linear DNA showed such high stable transfection it was chosen as candidate for further work. Further work with mut 4 EC was canceled.

Table 11.Transient and stable expression levels for transfection number 4. The concentration was calculated by subtracting with the contribution of the medium. EL stands for electroporation linear and EC stands electroporation circular. The negative control is medium from untransfected cells.

Sample	*Transient expression (ng/ml)*	*Stable expression (ng/ml) 4 days*	*Stable expression (ng/ml) 15 days*
Mut 4 EL	1.36	2.2	177.5

Mut 4 EC	5.07	7	70.1

4.4. Evaluation of protein expression

The results from the methods of evaluation of protein expression are divided between the transfection, section 4.3 and the establishment of stable clones, section 4.5.

4.5. Establishment of stable clones

This section states the results from the cloning experiments.

First experiment

The first cloning experiment was initiated with the best stable expressing cells from transfection number 2 after 10 days selection. As can be seen in table 9 mut 4 2:1 isthe best expressing with a concentration of 18.1 ng/ml AMH.

At the first screening the expression levels varied from 0.05 – 1.1 ng/ml after regulation with the negative control. The two best expressing clones were expressing 1.1 and 0.99 ng/ml and kept cultivated. The best clone of the second screening expressed about 1000 ng/ml.

For the plates seeded with 10 cells/well did the expression levels vary between 0.1 to 3.8 ng/ml after regulation with the negative control for the first screening. The two best expressing wells were expressing 3.8 and 2.75 ng/ml and were re cloned.

At the first screening after recloning did the expression levels vary from 0.1 to 17.3 ng/ml after regulation with the negative control. The two best expressing clones were expressing 17,3 and 17,0 ng/ml and kept cultivated. At the second screen the best clone expressed about 1100 ng/ml.

Second experiment

The second cloning experiment was initiated with the mut 4 linear electroporated cells from transfection number four when expressing 177.5 ng/ml, with 15 days of selection.

At the first screen did the expression levels varied from 1.5 to 3.8 ng/ml after regulation with the negative control. The two best expressing clones were expressing 3.8 and 2.3 ng/ml and kept cultivated. At the second screen the best clone expressed about 700 ng/ml.

4.6. Characterization

In this section are the results from the characterization presented.

4.6.1 Western blot

Figure 8 shows a picture of the western blot results for the detection antibody A. This antibody have an epitope in the N-terminal region (pro-region)of the AMH, which should show bands in the size of about 120 kDa for non-reduced samples and 60 kDa for reduced samples if truncated into mature- and pro-region.

No signal is shown the negative control, which means that the standard medium do not contain high levels of AMH itself. The non-reduced samples show similar signals for both produced and reference AMH, but the bands for the produced AMH are slightly bigger, about 140 kDa while the bands for the commercially available AMH are about 120 kD. The signals from the reduced samples differ between produced and reference AMH, the produced AMH seems to be harder to reduce than the commercially available AMH. The size of the bands for the reduced commercially available AMH are of expected size, about 60 kDa.

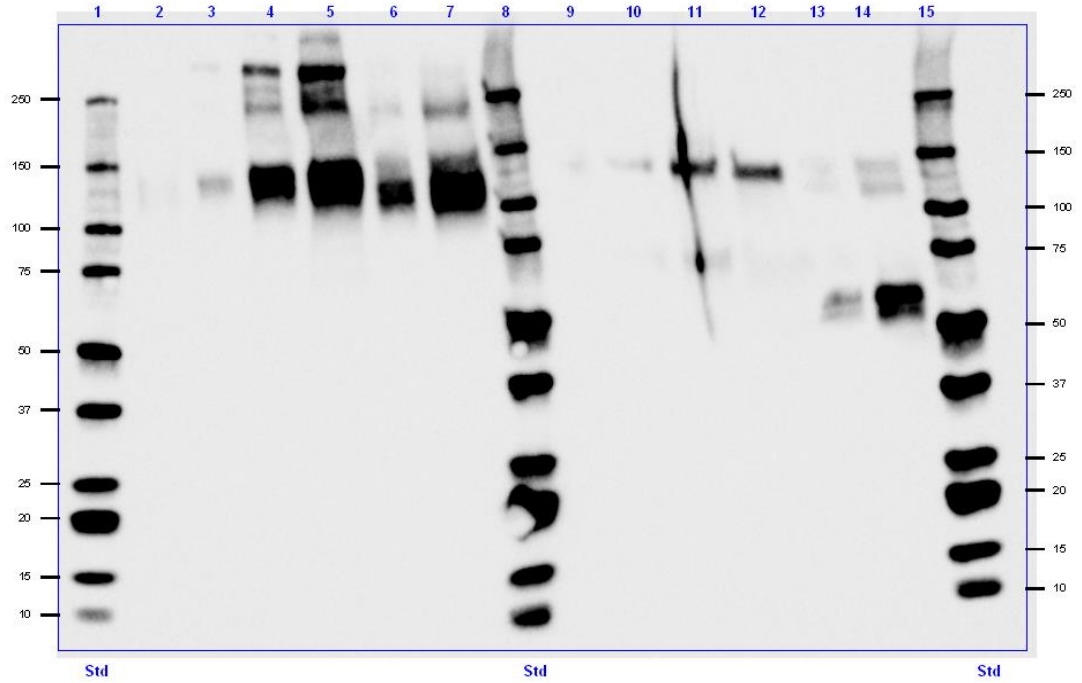

Figure 8. Western blot of AMH tracer antibody with epitope in the c-terminal region of AMH. The wells 2 - 7 are non-reduced samples and well 9 – 14 are reduced samples. Well 1,8 and 15 contain the marker, precision plus protein Western C standards, well 2 and 9 contain standard medium (negative control), wells 3, 4, 5, 10, 11 and 12 contain medium from one of the stable clones with increasing amount of AMH 1, 10 and 50 ng, wells 6, 7, 13 and 14 contain 10 and 50 ng of reference AMH, commercially available.

Figure 9 shows a picture of the western blot results for the capturing antibody B. This antibody have an epitope in the C-terminal region (mature-region) of the AMH, which should show bands in the size of about 24 kDa for non-reduced samples and 12 kDa for reduced samples if truncated into mature- and pro-region.

No signal is shown for the negative control, which means that the standard medium do not contain high levels of AMH itself. The catcher antibody show weaker signal than

the tracer antibody, which indicate that a higher antibody concentration should be used for optimizing the blot. The commercially available AMH is not detected in its non-reduced form at 50 ng, this indicate that the catcher antibody could have a conformation dependent epiotope that is disturbed by the truncation. Also in this blot is it shown that the reduction of the produced AMH was incomplete.

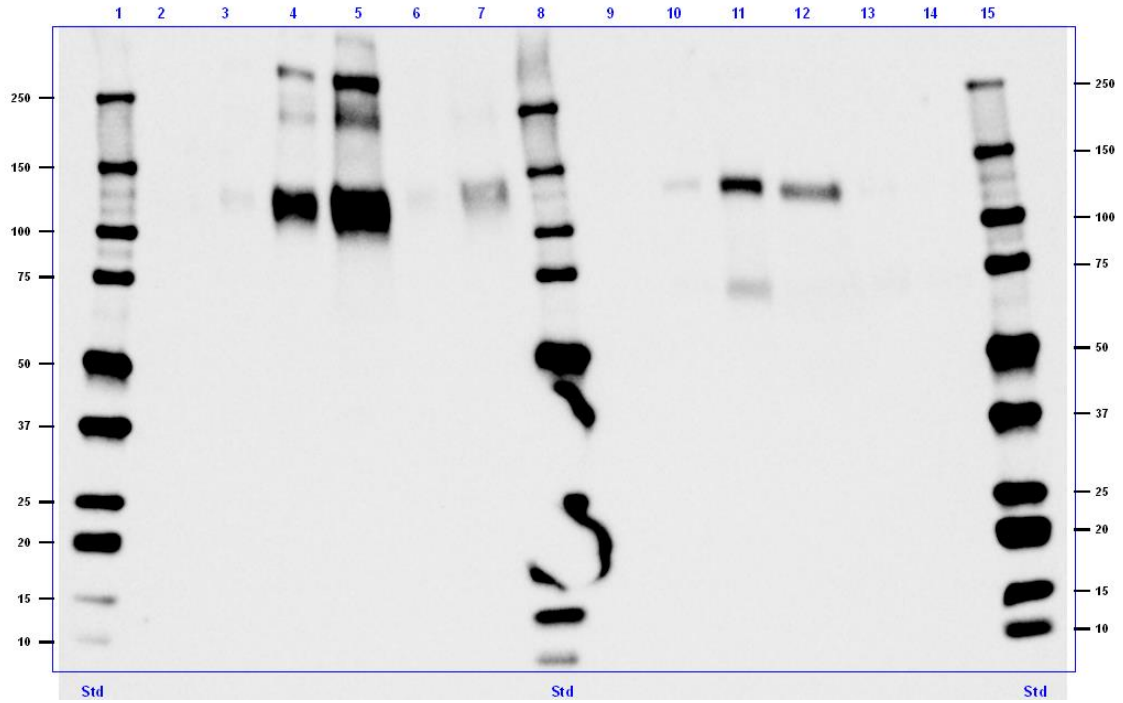

Figure 9. Western blot of AMH catcher antibody with epitope in the N-terminal region of AMH. The wells 2 - 7 are non-reduced samples and well 9 – 14 are reduced samples. Well 1,8 and 15 contain the marker, precision plus protein Western C standards, well 2 and 9 contain standard medium (negative control), wells 3, 4, 5, 10, 11 and 12 contain medium from one of the stable clones with increasing amount of AMH 1, 10 and 50 ng, wells 6, 7, 13 and 14 contain 10 and 50 ng of reference AMH, commercially available.

4.6.2. Stability

This section present the result for the stability studies performed during the project.

Stability in different matrixes

In table 12 and 13 are the result for the most promising buffer based and the most promising serum based matrix presented. Result for the other matrixes can be found in Appendix B. The first two weeks was measured with EIA and The second two weeks with the automated imunnoassy.

Table 12 shows the stability results for matrix number 6, a trisbuffer based matrix with BSA. The reference are the sample stored in – 70 °C. The low recovery for week one is caused by the high reference that probably was not equilibrated resulting in the false high value. The recovery in 37 °C decrease to about 95 % during the first weeks, but then stabilizes and remains relatively constant.

Table 12.Stability of produced AMH in matrix 6, a Tris buffer based matrix containing BSA. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 6	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	11.2		10.8		10.9		11.2	
4 °C	10.5	94.4	10.9	101.1	10.8	99.5	11.2	100.0
RT	10.6	94.9	10.8	100.0	10.8	99.5	11.1	98.7
25 °C	10.6	94.6	10.8	100.4	10.9	100.0	10.9	97.3
37 °C	10.5	94.0	10.3	96.2	10.3	94.9	10.6	94.6

The results four week stability study for matrix 4, a serum based matrix with EDTA is seen in table 13. The reference are the sample stored in – 70 °C. The low recovery for week one is caused by the high reference that probably was not equilibrated resulting in the false high value. Just as in the buffer based matrix above the recovery in 37 °C decreases the first week, but then stabilizes and remain constant. The measured concentrations are higher than 10 ng/ml, this because of that the serum itself contain human AMH.

Table 13.Stability of produced AMH in matrix 4, a serum based matrix with EDTA. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 4	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	18.4		16.6		13,4		13.2	
4 °C	16.8	90.8	15.9	95.7	13,3	99.3	13.1	99.6
RT	15.9	85.9	15.6	93.9	13,2	98.9	12.9	98.1
25 °C	16.5	89.3	15.7	94.7	13.2	98.9	13.0	98.9
37 °C	14.8	80.0	13.6	81.8	10.9	81.4	10.8	81.8

Stability in different BSA lots

Results of the stability study with different lots of BSA in matrix number six is presented in table 14. No lot to lot variation is seen and the result corresponds well with the earlier stability study in matrix 6 above. The second week the samples stored at 4 °C was used as reference.

Table 14. Stability results for AMH spiked the Tris buffer based matrix (matrix nr 6) containing three different lots of BSA.

		Week 1		*Week 2*	
Lot nr	**Temp**	**Conc. (ng/ml)**	**Recovery (%)**	**Conc. (ng/ml)2**	**Recovery (%)3**
Lot 117	Ref	4.7			
Lot 117	2 - 8 °C	4.75	101.1	5.2	
Lot 117	25 °C	4.75	101.1	5.1	98.1
Lot 117	37 °C	4.5	95.7	4.9	94.2
Lot 116	Ref	4.8			
Lot 116	2 - 8 °C	4.85	101.0	5.3	
Lot 116	25 °C	4.8	100.0	5.25	99.1
Lot 116	37 °C	4.6	95.8	4.95	93.4
Lot 114	Ref	4.9			
Lot 114	2 - 8 °C	4.85	99.0	5.2	
Lot 114	25 °C	4.95	101.0	5.2	100.0
Lot 114	37 °C	4.6	93.9	5.1	98.1

Freeze thaw stability

The result for the freeze thawing is presented in table 15. The concentration of produced AMH was decreased after five cycles of freeze thawing of medium from clone D10 and increased for clone D5. This experiment shows untrustworthy results since the AMH level can't increase, but probably the margin of error is within this 10 %.

Table 15. Stability results for five times of freeze-thawing on AMH produced by two different clones.

Clone	***Concentration (ng/ml)***	***(%)***
D10 ref	3810	
D10	3420	90
D5 ref	1915	
D5	2145	112

4.6.3. Linearity

The result of the linearity study is presented in table 16 and shows a good recovery between expected and observed concentrations.

Table16. Results for the linearity study.

Sample	***Expected Conc. (ng/ml)***	***Observed Conc. (ng/ml)***	***Recovery (%)***
1.Calibration matrix (L)	0	0	100
2. 0,9L + 0,1H	1.72	1.9	110
3. 0,8L + 0,2H	3.44	3.5	102
4. 0,7L + 0,3H	5.16	5.3	103

5. 0,6L + 0,4H	6.88	6.9	100
6. 0,5L + 0,5H	8.6	8.8	102
7. 0,4L + 0,6H	10.32	10.4	101
8. 0,3L + 0,7H	12.04	12.4	103
9. 0,2L + 0,8H	13,76	13,9	101
10. 0,1L + 0,9H	15,48	15,8	102
11. Medium containing AMH (H)	17,2	17,2	100

Figure 10 shows a plot of the observed concentration (y) vs. Expected concentration (x). The linear curve in the plot shows that the produced AMH is dilutable and have the same properties as the AMH used in the calibrators today.

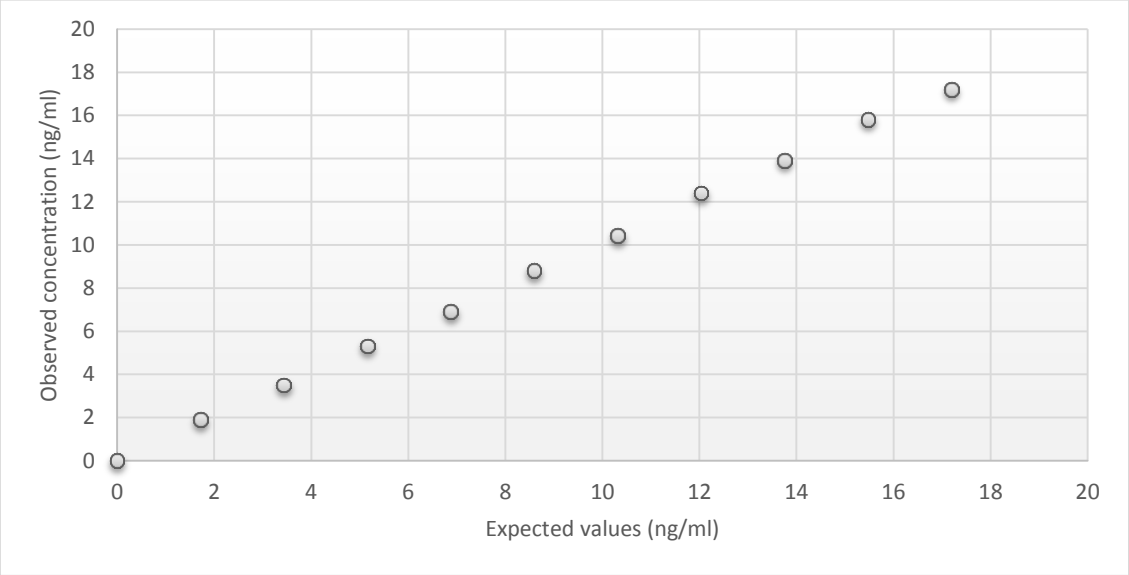

Figure 10. Linearity plot with expected concentration vs. observed concentration.

4.7. Expansion

In this section the results from the expansions are presented.

The result for the different clones cultured in T175 tissue flasks with 100 ml standard medium differed between 2000 – 4000 ng/ml. The highest expressing clone expressed 4000 ng/ml after 12 days.

The weekly measured concentration levels in the roller flasks are presented in figure 12. The cells cultured in standard medium before culture in roller flask with DMEM express higher levels than the cells adapted to DMEM before culture in roller flask. Week 3 was 10 ml HT added to the DMEM roller, which seems to have accelerated the cell growth and expression.

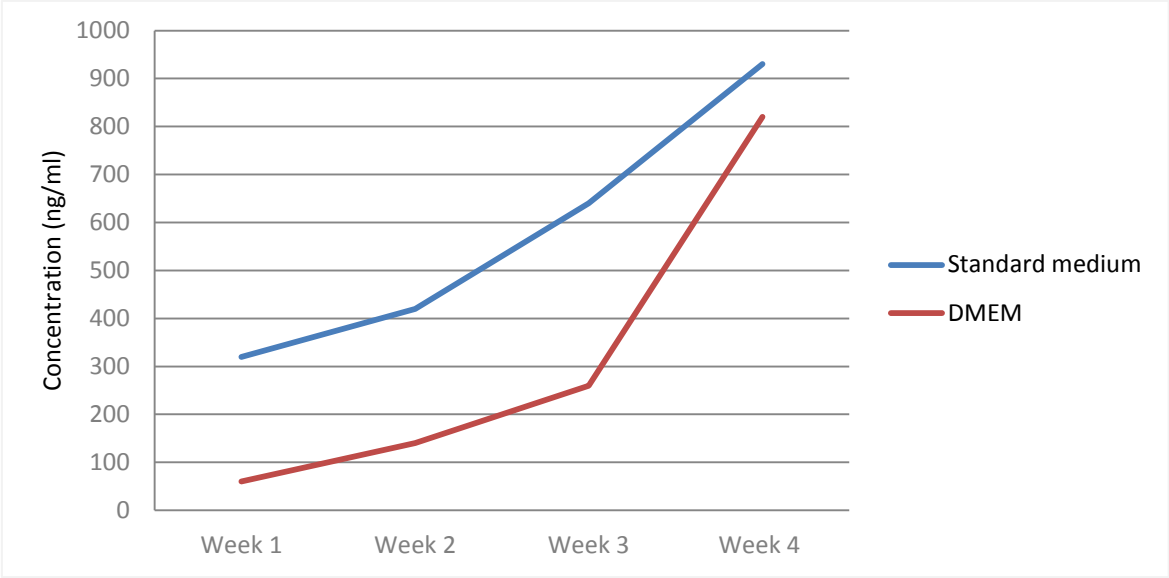

Figure 11. Expression from cells in roller flasks measured weekly. Standard medium is the roller that was inoculated with cells cultivated in standard medium and DMEM is the roller flask inoculated with cells adapted to growth in DMEM.

5. Discussion

This chapter contains the discussion of obtained results.

Construct design
Successful mutations could be confirmed by sequencing of the DNA in the colonies, which means that stop codons were introduced between the end of the AMH sequence and the beginning of the C-terminal tag.

Initial work with CHO cells
The CHO cells were successfully cultured and a G418 kill curve for the CHO K1 cell line could be established. The second antibiotic kill curve establishment was performed with a lower concentration since the first experiment had 100 % confluent cells, which made it hard to decide the optimal concentration of G418. With the second experiment was the optimal and low dose easily determined.

The adaption procedure to serum free media with the CHO dhfr- cell line was successful until cultured with 1.25 % serum but 0 % serum resulted in total cell death. A possible explanation for this is that the serum contains essential attachment factors such as fibronectin and laminin, making it impossible for adherent cells to survive without serum (Rodrigues *et al.* 2013, Brunner *et al.* 2010). One way to work through this problem could be to first adapt the adherent cells to grow in suspension, even though it is a time consuming and cost intensive procedure, often associated with loss of productivity and quality (Zahn *et al.* 2007). A less time consuming alternative but probably more costly would be to by a commercially available CHO dhfr- cell line already adapted to grow in suspension.

Transfection
The linearization results from the gel electrophoresis in figure 4 indicate that the restriction enzyme DraII had two different restriction sites in the vector, which resulted in two fragments and unsuccessful linearization. Digestion with restriction enzyme ApaLI resulted in just one band at about 7kb and seems to have digested the construct at just one restriction site, resulting in successful linearization.

The chemical based transfection worked better for transfection number 2 than for transfection number 1, which indicates that the longer incubation time for the DNA and reagent before addition to cells could be favorable for the cell lines, just as lower passage numbers.

Surprisingly the stable expression for cells selected with MTX never reached as high levels as the ones selected with G418.This could possibly be explained by to short adaptation period between each MTX dose increase (Chun *et al.* 2002). Another

explanation could be that the AMH sequence in vector pD18 wasn't successfully integrated in the host chromosomes or some problem with the translation, even if that AMH sequence is codon optimized for CHO cells. One way to test the theory with badly integrated sequence would be to transfect CHO dhfr- cells with pD18 carrying the native AMH-sequence. Such attempt was made by amplification of the AMH sequence from pcMV6 and digestion of the ends of the fragment with restriction enzymes. The AMH sequence in pD18 was cut and the AMH sequence from pcMV6 was ligated into the pD18 vector. If successful amplification with MTX and establishment of stable expression it was probably the codon optimized AMH sequence that caused the problem, if not the problem was probably due to the amplification method.

The electroporation is a rough method and the cells needed longer recovery before initiation of selection. When the cells had recovered the transient expression levels were comparable with the ones from the chemically based transfections. The transient expression was higher in the cells transfected with circular DNA but the stable expression was higher for the cells transfected with linear DNA. It was not surprising that the linearized DNA incorporated better to the chromosomes of the host genome since that process is most likely to occur during replication of nucleus as linear DNA fragments can be randomly copied into the host chromosome (Stuchbury and Münch 2010).

The different stop codons did not seem to affect the expression of AMH. Mut 4 with the codon optimized stop codon was chosen for establishment of stable clones because the transfection was performed earlier in the project than for mut 3. Either one of them would be just as suitable.

Overall are the successful transfections a confirmation on that the C-terminal tags earlier caused the problem with the expression and identification of AMH and that the problem could be solved by introduction of stop codon by site directed mutagenesis.

Establishment of stable clones

The first cloning experiment was seeded in higher concentration than the second experiment because of the concern that the cells were dependent of each other during selection and that no single cells would survive if seeded in lower concentration. This was shown not to be the case since single colonies from the plates with 1 cell/well did survive and expressed AMH. This resulted in that the second experiment was carried out with 0.67 cells/ml, which is the standard concentration for cloning at the company. The obtained stable clones were all transfected with the same vector and thereby the same AMH sequence and no major differences in the expression were seen in the second screening. Thereby the choice of clone for stability testing of produced AMH only was taken considering how far in the expansion procedure the clone was.

Western blot

The Western blots show a clear difference between the produced AMH and the commercially available AMH used as reference. The AMH produced in this project is more resistant to reduction than the reference AMH. It also appears like the AMH haven't been cleaved into the 58 kDa proregion (AMH_N) and the 12 kDa mature region (AMH_C), but sitting together as proAMH. That the produced AMH is in the form of proAMH could be an explanation for its resistance for reduction, since more cysteines are available to form disulfide bridges between the monomers. The reference AMH couldn't be detected with the capturing antibody B when reduced. These factors could maybe explain the excellent stability of the produced AMH.

Stability of AMH protein

The stability of the produced AMH was evaluated with some different stability tests. The first was a four week stability test in different matrixes, the result showed clear differences between the matrixes and indicated that the Trisbuffer based matrix with BSA was the choice of matrix for potential us as calibrators, with almost 95 % expression left after four weeks in 37 °C and that matrix number 4 with serum B and EDTA was the best choice for potential use as a serum control. The high recovery in the calibrator matrix also indicates that liquid calibrators instead of lyophilized may be possible, depending of the requirements.

The BSA lot did not show any lot to lot variation after two weeks at 37 °C. This indicates that the BSA does not affect the stability of the AMH.

The freeze thawing stability test showed that the produced AMH was pretty resistant for the effect of freezing and thawing.

Dilution Linearity

The dilution linearity plot in graph 5 shows the produced AMH has a good linearity between 0 and 17 ng/ml, which is an important property for antigens used in calibrators for immunoassays. The result indicates the produced AMH could be suitable for use in calibrators.

Expansion of stable clones

The expansion study in T175 culture flasks shows that it is possible to reach relatively high concentrations in this culturing system. Upscaling is easily done by culturing the cells in more T175 flasks or by the use of multilayered T flasks with more surface area for the cells to attach and thereby probably also a higher production.

Expansion was also tried in roller flasks with standard medium for production, DMEM with 5 % FBS. The results indicate that the cells have not grown well, since the production of AMH was very low compared to the level in the T175 flasks.

Nevertheless weekly increasing AMH concentration indicates that the cells are still proliferating. In order to draw any further conclusions of roller flasks as culture system for the cell line the experiment should be repeated.

The clone dedicated for the final production should be recloned in order to validate a monoclonal cell population. Properties of the cell line should be characterized such as the growth and stability of the productivity.

6. Conclusion

In this chapter the conclusions from the project are summed up.

The aim of the present research was to develop a CHO cell line with over-expression of recombinant anti Müllerian hormone (rAMH) and to evaluate important characteristics of the produced AMH in order to determine its suitability for use in calibrators for an immunoassay measuring AMH level in serum samples.

The study shows that recombinant AMH could be successfully produced in CHO K1 cells and that establishment of stable expression clones could be performed by amplification with G418. The hypothesis about the C-terminal-tags causing problem was true and that the introduction of stop codon eliminated the earlier problems with expression in CHO cells. The vector with the native AMH sequence tended to give better expressions and the G418 selection system was shown to be favorable over MTX in the establishment of stable clones within a tight time line. The stability and linearity of the produced AMH is promising and indicate that the AMH could be suitable in calibrators for immunoassay test. The success of CHO cell line development for production of recombinant proteins is clearly supported by the current findings.

Further studies need to be carried out in order to characterize the final cell line that is dedicated for the final production. Moreover it is recommended that further expansion experiments in roller flasks should be carried out before choice of production culture system. Greater efforts are needed to enable the production in a serum free cell culture.

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Appendix A

This appendix contains additional information regarding the method of the project. Data is presented in the same order as mentioned in the report.

Site directed mutagenesis

Table 17 shows the mutagenesis primers used for introduction of stop codons. Each mutation required two mutagenesis primers, one forward (fw) and on reverse (rev). In total were eight different mutagenesis primers used.

Table 17. Mutagenesis primers for introduction of stop codons before C-terminal tags.

Primer name	***Mutation***	***Primer sequence***	***Tm***
Rev pD18AMHFc-TAG	Mut 1	5'-gatcccttgtcgtcgtcctaacgacaaccacactcag-3'	78.9 °C
Fw pD18AMHFc-TAG	Mut 1	5'-ctgagtgtggttgtcgttaggacgacgacaagggatc-3'	78.9 °C
Rev pD18AMHFc-TGA	Mut 2	5'-cggatcccttgtcgtcgtctcaacgacaaccacactcagta-3'	78.7 °C
Fw pD18AMHFc-TGA	Mut 2	5'-tactgagtgtggttgtcgttgagacgacgacaagggatccg-3'	78.7 °C
Rev pcMV6-AMH-TAG	Mut 3	5'-cggccgcgtacgctaccggcagccacac-3'	81.0 °C
Fw pcMV6-AMH-TAG	Mut 3	5'-gtgtggctgccggtagcgtacgcggccg-3'	81.0 °C
Rev pcMV6-AMH-TGA	Mut 4	5'-gcggccgcgtacgtcaccggcagccacac-3'	78.9 °C
Fw pcMV6-AMH-TGA	Mut 4	5'-gtgtggctgccggtgacgtacgcggccgc-3'	78.9 °C

Sequencing primers used for sequencing of the colonies are stated in table 18.Three different primers for each vector were needed for complete sequencing of the AMH sequences.

Table 18. Sequencing primers for sequencing of colonies after site directed mutagenesis.

Primer name	*Mutation*	*Primer sequence*
Rev XL39	Mut 3& Mut 4	5' attaggacaaggctggtggg3’
Rev AMH nt1146-1166	Mut 1 & Mut 2	5’tcgaagagtggctgccga3’

Antibiotic kill curve

Table 19 shows the used concentration of G418 for the first experiment for establishment of an antibiotic kill curve.

Table 19. Concentrations used during the first experiment for establishment of an antibiotic kill curve for G418.

Volume of stock solution added (µl) G418 (50mg/ml)	*Final concentration (µg/ml)*	*Total volume (ml)*
0	0	10
10	50	10
20	100	10
40	200	10
60	300	10
80	400	10
100	500	10
120	600	10
140	700	10
160	800	10
180	900	10
200	1000	10

Table 20 state the concentrations used for the second experiment for establishment of an antibiotic kill curve for G418.

Table 20. Concentrations used during the second experiment for establishment of an antibiotic kill curve for G418.

Volume of stock solution added (µl) G418 (50mg/ml)	*Final concentration (µg/ml)*	*Total volume (ml)*
0	0	5
20	200	5
30	300	5
40	400	5
50	500	5
60	600	5

Appendix B

This appendix contains additional information regarding the result of the project. Data is presented in the same order as mentioned in the report.

Site directed mutagenesis

The concentrations of the purified plasmids were measured with a NanoDrop 2000c spectrophotometer from Thermo Scientific. Plasmids were purified from five colonies for each mutation and the concentrations of these are presented in table X.

Table 21. Measured concentration of purified plasmids from colonies after site directed mutagenesis.

Sample	***Concentration (ng/µl)***	***260/280***
Mut 1:1	843,1	1,91
Mut 1:2	901,0	1,89
Mut 1:3	833,3	1,90
Mut 1:4	679,6	1,89
Mut 1:5	808,9	1,92
Mut 2:1	673,7	1,92
Mut 2:2	848,8	1,91
Mut 2:3	701,5	1,92
Mut 2:4	610,7	1,87
Mut 2:5	754,4	1,91
Mut 3:1	981,1	1,90
Mut 3:2	677,7	1,92
Mut 3:3	886,7	1,90
Mut 3:4	581,2	1,88
Mut 3:5	686,2	1,91
Mut 4:1	1000,3	1,90
Mut 4:2	958,9	1,90
Mut 4:3	936,1	1,90
Mut 4:4	932,0	1,90
Mut 4:5	763,5	1,93

Transfection

Transfection number 1

In table 22 is the level of transient AMH expression two days after transfection for transfection number 1 stated. The cells were about 90% confluent and were cultured in one well each in a 6-well tissue culture plate with 2 ml standard medium. These values are measured with EIA.

Table 22. Transient expression levels measured on the medium two days after transfection. 1:1, 2:1, 3:1 and 4:1 stand for the ratio of microliter X-tremeGENE HP DNA Transfection reagent to microgram DNA. The negative control is medium from untransfected cells. The actual concentration is the concentration after regulation with the negative control.

Sample	*Volume (ml)*	*Mean conc. (ng/ml)*	*Actual conc. (ng/ml)*
Mut 1 1:1	2	6,269	0,412
Mut 1 2:1	2	5,818	-0,039
Mut 1 3:1	2	6,52	0,663
Mut 1 4:1	2	5,344	-0,513
Mut 2 1:1	2	5,658	-0,199
Mut 2 2:1	2	5,722	-0,135
Mut 2 3:1	2	5,992	0,135
Mut 2 4:1	2	5,651	-0,206
Negative Control		5,857	

Stability

Stability results for matrix 1, 2, 3 and 5 are presented in tables 23 - 26. The first two weeks are measured with EIA and the last two weeks are measured with the automated system.

Table 23 presents the stability results for AMH spiked in matrix 1, serum A.

Table 23. Stability of produced AMH in matrix 1, Serum A. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 1	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	15,6		13,5		12,7		13,0	
4 °C	13,9	88,7%	13,5	100,0 %	12,1	95,3%	11,8	91,1%
RT	11,8	75,4%	7,0	52,0%	2,9	22,4%	1,0	7,7%
25 °C	9,5	60,8%	3,1	23,1%	0,6	4,3%	0,2	1,5%
37 °C	0,9	5,6%	-0,1	-0,9%	0,0	0,0%	0,0	0,0%

Table 24 presents the stability results for AMH spiked in matrix 2, Serum A with 10 mM EDTA. EDTA improved the stability.

Table 24. Stability of produced AMH in matrix 2, Serum A with EDTA. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 2	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	15,8		13,6		12,3		12,0	
4 °C	11,6	73,4%	13,6	100,2%	12,0	98,0%	11,6	96,7%
RT	13,7	86,8%	13,4	98,2%	11,4	92,7%	11,6	96,3%
25 °C	13,7	86,6%	13,5	98,9%	11,7	95,5%	12,7	105,4 %
37 °C	11,6	73,8%	10,2	74,5%	8,4	68,6%	7,4	61,3%

Table 25 presents the stability results for AMH spiked in matrix 3, serum B.

Table 25. Stability of produced AMH in matrix 3, Serum B. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 3	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	18,2		14,9		12,5		13,4	
4 °C	15,1	83,0%	15,2	102,6%	12,4	98,8%	12,5	93,3%
RT	15,1	83,3%	14,8	99,5%	12,4	99,2%	12,2	91,0%
25 °C	15,4	84,8%	14,8	99,8%	12,5	100,0%	12,3	91,8%
37 °C	15,1	83,0%	12,6	85,1%	11,3	90,4%	9,8	73,1%

Table 26 presents the results for AMH spiked in matrix 5, a phosphate based buffer with BSA.

Table 26. Stability of produced AMH in matrix 3, a phosphate buffer with BSA. Rec is short for recovery and is the remaining AMH level compared to the reference value, ref.

Nr 5	*Week 1*		*Week 2*		*Week 3*		*Week 4*	
Temp	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)	Conc. (ng/ml)	Rec. (%)
Ref	Range?		27,7		29,4		28,9	
4 °C	Range?		29,0	104,4%	29,2	99,2%	29,3	101,6%
RT	Range?		28,6	103,2%	28,4	96,4%	28,6	99,1%
25 °C	Range?		29,0	104,5%	27,7	94,1%	28,4	98,4%
37 °C	27,6		28,6	103,3%	25,1	85,2%	25,9	89,6%