DIPLOMARBEIT

COORDINATED EXPRESSION OF THE ANTIBODY FRAGMENT 3H6 FAB CHAINS IN THE YEAST *PICHIA PASTORIS*

Characterisation of Fab producing clones by expression of light chain and heavy chain genes under control of different constitutive promoters

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Abstract

Since the last years there is an increasing demand for monoclonal antibodies. For some applications not the whole antibody is required, but fragments like Fab (antigen binding fragment) or ScFv (Single chain variable domain fragment) are even preferred. This is because of their properties like easy penetration into the target tissues or causing fewer side effects because of lower immunogenicity. Yeasts, like *Pichia pastoris,* have shown to be potential host organisms for the production of Fabs. They combine the advantages of a microbial host, with the abilities of eukaryotics to perform posttranslational modifications. However, the yields in yeast are far from optimal. Therefore different strategies are developed to improve the heterologous protein production.

In the present study the Fab of the antibody 3H6 was expressed in *P. pastoris.* The chosen strategy was to express the light (LC) and heavy chain (HC) genes under control of different constitutive promoters from separate expression vectors. The strong promoters P_{GAP} (glyceraldehyde 3-phosphate dehydrogenase promoter for the LC gene) and P_{TEF} (translation elongation factor 1-α promoter for the HC gene) were used. The productivity of the constructed clones and the quality of the produced protein had to be determined by analysis of the culture supernatant containing the secreted Fab. Analysis by quantitative RT-PCR (Real time polymerase chain reaction) should shed light on factors influencing the productivity like amounts of the transcript and the gene copy number of the Fab chain expression cassettes.

An increase of the Fab yield could not be obtained by expression of the HC gene regulated by the growth-associated TEF-promoter and the LC gene driven by the GAP-promoter. Generally, a higher transcript level resulted in a higher amount of the expressed polypeptide chain, while P_{GAP} showed a higher transcriptional strength than P_{TEF}. A good correlation of the ratio gene copies of the LC per HC to productivity could be observed. A higher amount of LC alone did not increase the amount of intact Fab, but resulted in secretion of LC to the culture supernatant, where it was partly degraded. Therefore it can be concluded that a balanced HC:LC ratio is advantageous for higher yields of Fab in *P. pastoris*, as was also described for other expression systems.

Zusammenfassung:

Seit den letzten Jahren ist die Nachfrage nach monoklonalen Antikörpern gestiegen. Für manche Anwendungen wird nicht der ganze Antikörper benötigt, sondern Fragmente wie Fab (das antigenbindende Fragment) oder scFv (das einkettige variable Domänen Fragment) werden bevorzugt. Gründe dafür sind Eigenschaften wie ihr Vermögen in das Zielgewebe einzudringen oder weniger Nebenwirkungen aufgrund ihrer geringeren Immunogenität. Hefen, wie *Pichia pastoris,* haben sich als potentielle Wirtsorganismen erwiesen. Sie vereinigen in sich die Vorteile eines mikrobiellen Wirtes mit den Fähigkeiten von Eukaryoten, posttranslationale Modifikationen durchzuführen. Jedoch sind die Ausbeuten in Hefen bei weitem nicht im optimalen Bereich. Daher werden neue Strategien entwickelt, um die Produktion heterologer Proteine zu verbessern.

In dieser Arbeit wurde Fab des Antikörpers 3H6 in *P. pastoris* exprimiert. Die gewählte Strategie war, die Gene der leichten (LC) und schweren Kette (HC) unter Kontrolle von verschiedenen, konstitutiven Promotoren zu exprimieren. Die starken Promotoren P_{GAP} (Glyeraldehyd 3-phosphat-Dehydrogenase-Promotor für das LC-Gen) und P_{TEF} (Translations-Elongationsfaktor1-α-Promotor für das HC-Gen) wurden verwendet. Die Produktivität der konstruierten Klone und die Qualität des produzierten Proteins sollten, mittels Analyse des Fab-enthaltenden Kulturüberstandes, bestimmt werden. Die Analyse durch quantitative RT-PCR (Real time polymerase chain reaction) sollte Aufschluss geben über die Faktoren, die die Produktivität beeinflussen, wie Transkriptmengen und Genkopienzahl der Expressionskassetten der Fab-ketten.

Eine Steigerung des Ertrages an Fab konnte nicht erreicht werden durch die Expression des HC-Gens unter Regulation des wachstumsassoziierten TEF-Promotors und des LC-Gens unter Kontrolle des GAP-Promotors. Allgemein resultierte eine höhere Genkopienzahl in größeren Mengen der exprimierten Polypeptidkette, wobei P_{GAP} eine höhere Promotorstärke aufwies als P_{TEF}. Es konnte eine gute Korrelation zwischen dem Verhältnis der Genkopienzahl der LC zur HC und der Produktivität beobachtet werden. Eine höhere LC-Menge alleine, führte nicht zu einem Anstieg der Menge an intaktem Fab, sondern resultierte in der Sekretion von leichter Kette in den Kulturüberstand, wo diese teilweise abgebaut wurde. Das lässt darauf schließen, dass ein ausgeglichenes Verhältnis von HC zu LC vorteilhaft für höhere Ausbeuten an Fab in *P. pastoris* ist, wie es auch für anderen Expressionssysteme beschrieben wurde.

Aim of the study

The antibody fragment 3H6 Fab (antigen binding fragment) was expressed in the yeast *Pichia pastoris*. The aim of this study was to determine whether the chosen expression strategy results in an increase of the Fab yield or not. Furthermore factors, influencing the productivity, should be described. Based on the heterodimeric structure of antibodies, the strategy was to express the light and heavy chain genes of the Fab from separate vectors, under expression control of different constitutive promoters. As the amount of heavy chain is probably a bottleneck in the production of Fab, the HC gene was driven by the growth associated and strong TEF-promoter. For the characterisation of the constructed clones, the protein amount and quality and also influencing factors like the gene copy number and transcript levels were determined.

1 Introduction

1.1 Antibodies

Antibodies are heterodimeric proteins which specifically recognise foreign molecules, the so called antigens. They play an important role in the immune response and are produced by the B-lymphocytes when the host is invaded by antigens. Their specificity and the fact that antibodies against almost any antigen can be created made them an interesting tool for medical and scientific research. Antibodies are also called immunoglobulins and 5 different classes are known: IgG, IgM, IgA, IgD and IgE. IgG are the most abundant form of antibodies in the blood.

1.1.1 Structure of Immunoglobulin G

Immunoglobulin G consists of two complexes of two identical heavy chains (HC) and two identical light chains (LC). The LC and HC are both divided into variable and constant regions. The light chain is assembled of one constant (C_L) and one variable region (V_L), while the heavy chain consists three constant regions (C_H1, C_H2, C_H3) and one variable region (V_H). The CDR's (complementary determining regions) of the variable regions differ a lot in their amino sequences and are characteristic for each antibody. The V_H and V_L together form the unique antigen-recognition site that assigns the antigen-specificity.

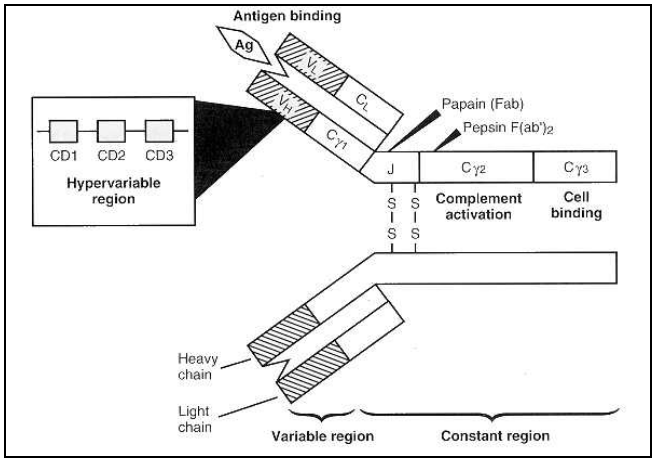

Figure 1: Schematic structure of an antibody (taken from: Samuel Baron: "Medical Microbiology" 1996 Fourth Edition)

1.1.2 Antibody fragments

As reviewed by Joosten et al. 2003, for most applications only the antigen-binding site of a certain antibody is required. In some cases it is even preferred e.g. when they are used in vaccines because they are better tolerated and thus cause less undesired side effects. Several fragments can be produced by different treatment of the whole antibodies. The use of enzymes yields in Fab (antigen binding fragment), scFab (single chain antigen binding fragment), Fv (variable domain fragment) and scFv (single chain variabledomain fragments) (see Fig. 2).

1.1.2.1 IgG Fab

The Fab is composed of the total light chain (V_L and C_L) and the variable region (V_H) as well as the first constant region (C_H1) of the heavy chain (see Fig. 2), which are connected by at least one disulfide bond.

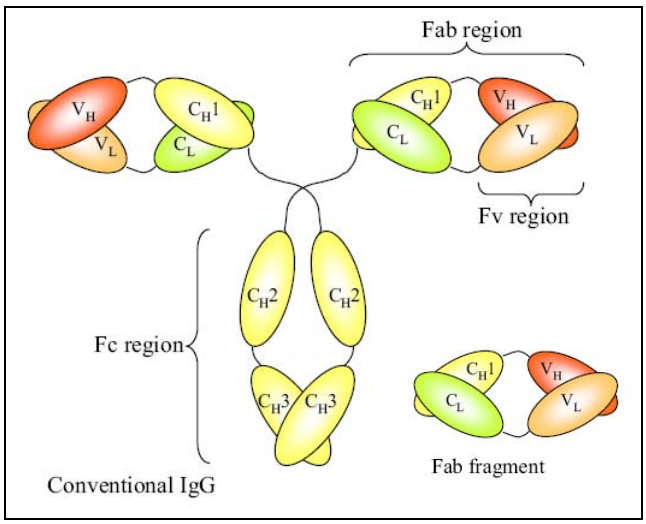

Figure 2: Schematic structure of an IgG full length antibody and a Fab fragment (taken from Joosten et al. 2003)

1.1.2.2 Advantages of Fab

The Fab has several advantages in comparison to whole antibodies as well as to smaller fragments. In most cases Fabs have a better binding affinity than the scFvs and the great advantage over smaller antibodyfragments is their long-time stability, which makes long time storage possible. On the other hand Fabs show lower immunogenicity than the whole antibody, which means fewer side effects when they are used in vaccines. For this application also the fact that they accumulate more rapid at the target tissue is an important and advantageous characteristic (Takahashi et al. 2000, Hust et al. 2007).

1.1.2.3 3H6-Fab

The antibody 3H6 is an anti-idiotypic antibody-fragment directed against the human monoclonal antibody 2F5, which is broadly neutralising HIV-1 (human immunodeficiency virus). The 3H6-Fab fragment seems to be a promising candidate for the production of vaccines against HIV-1 (Gach et al. 2007).

1.2 Fab-production

1.2.1 Enzymatic production: papain digestion

Papain digestion of a whole IgG leads to 2 Fab fragments and one Fc fragment. It cleaves antibodies above the hinge region (see Papain restriction site in Figure 1) and thus destroys the bonds joining the C_H1 to the other constant regions of the heavy chain. The disulfide bond between the light and the heavy chain persists.

1.2.2 Biotechnological production by different expression systems

Another approach to Fab production is their recombinant expression in cultivated cells. Both prokaryotic organisms (like *Escherichia coli*) and eukaryotic organisms (lower eukaryotes like yeasts or filamentous fungi and higher ones like plant, insect or mammalian cells) have been used so far as host systems for the production of Fabs. By insertion of expression vectors containing the sequences of the light and heavy chains into the host cell, the antigen binding fragment can be produced directly, instead of producing the whole immunoglobulins followed by an enzymatic treatment. The decision, which system to chose, depends on factors like the type of antibody to be expressed and the required amount and purity of the product (reviewed by Verma et al. 1998).

1.3 Advantages and disadvantages of some common expression systems for the production of antibodies and fragments thereof

As antibodies are a growing class of biotherapeutics, and large amounts are required, the demand for appropriate, highly productive expression systems increases. The properties of three different important, commonly used expression systems are shortly described in the following:

- **Mammalian cells**

Mammalian cells are the “natural” hosts of antibodies. An argument for mammalian systems is their capacity to properly recognise signals for the synthesis, processing, secretion or posttranslational modifications of eukaryotic proteins (reviewed by Verma et al. 1998). These extensive control mechanisms prevent incorrectly folded proteins to proceed along the secretory pathway (Schoonjans 2000). Additionally high antibody or Fab titers/cell can be obtained with mammalian cell cultures. Nevertheless it has to be considered that working with mammalian cell cultures is time-consuming and cost intensive (reviewed by Verma et al. 1998). Furthermore there are different glycosylation mechanisms in different mammalian hosts, which may interfere with the intended application. Additionally for the use of Fabs for medical applications, the product has to be purified from virus particles.

- ***E. coli***

E. coli is a commonly used host organism for the production of immunoglobulin fragments, which is characterized by its fast growth, easy handling, inexpensive media and the fact that only small amounts of DNA are required for transformation (reviewed by Verma et al. 1998 and Joosten et al. 2003). For many applications of antibody engineering the very well characterized bacterial expression system is used, for example for the production of scFv. An essential advantage is that high titers of Fabs can be obtained when using *E. coli* as a host organism (Andersen and Reilly 2004). However a great disadvantage is that the Fab is usually expressed in the cytoplasm where the reducing conditions often lead to the formation of insoluble inclusion bodies, which makes the recovery of the product rather complex. Another critical point is that bacteria are due to their prokaryotic nature not able to perform certain essential posttranslational modifications (reviewed by Verma et al. 1998 and Daly and Hearn 2005). A further problem with bacterial expression systems is the production of endotoxins, which have to be removed carefully, if the recombinant protein is used for medical applications.

- **Yeasts and fungi**

The great advantage of yeasts is that they combine the properties of micro-organisms with the capabilities of eukaryotes like glycosylation, disulphide bridge formation and advanced protein folding pathways. Yeasts and fungi can be grown on cheap mineral media to high cell densities. Additionally they are able to secrete the product as a soluble protein into the culture supernatant which makes a convenient recovery possible (Gasser and Mattanovich 2007, Joosten et al. 2003, Verma et al. 1998). However yeasts are inable to perform amidation and certain types of phosphorylation and glycosylation and the product titers/cell are generally low.

For the production of Fab, for example the yeast *P. pastoris* has shown to be a suitable host (Ning et al. 2005, Gasser et al.2006, Dragosits et al. 2009, Gach et al. 2007, Lange et al. 2001, Takahashi et al. 2000). Also filamentous fungi like *Trichoderma reesei* (Nyyssönen et al. 1993) and *Aspergillus niger* (Ward et al. 2003) showed to be potential hosts for the production of whole antibodies and fragments thereof. A reason to prefer yeast to fungi as an expression host is that fungi produce a lot of proteases, which can have a negative effect on the yields of the heterologous protein (Frenken et al.1998).

The choice of the suitable expression system depends on several factors like the product to be expressed. In view of the easier purification of the product, expression hosts that secrete the recombinant protein into the culture medium are generally preferred (Frenken et al. 1998). On the whole, it can be noted that mammalian cell cultures are a more appropriate host system for the production of full length human antibodies while for the expression of antibody-fragments micro-organisms seem to be the system of choice (reviewed in Chadd and Chamow 2001).

1.4 The methylotrophic yeast *Pichia pastoris* as an expression system

P. pastoris is a methylotrophic budding yeast which belongs to the family of Saccharomycetaceae. For biotechnological applications it was first used for the production of single cell proteins (SCPs) on methanol containing media (Phillips Petroleum Company) in the 1970s.
Nowadays it is a popular and widely used expression organism for a variety of proteins for several reasons like easy genetic manipulation, its fast growth to high cell densities, the production of heterologous proteins in high levels and their secretion to the culture supernatant. As an eukaryotic system it is able to perform posttranslational modifications and allows correct folding of most eukaryotic proteins. The foreign DNA is integrated into the *P. pastoris*-genome via homologous recombination and can be expressed under inducible promoters (like the alcohol oxidase promoter (AOX) → which is induced by methanol) as well as constitutive promoters like the glyceraldehyde 3-phosphate dehydrogenase promoter (GAP) reviewed by Cereghino and Cregg 2000; Macauley-Patrick et al. 2005.

1.4.1 Integration via homologous recombination

Integration of the expression vectors into the genome leads to a stable expression system. If the *P. pastoris* vector to be inserted is cleaved within a sequence homologous to a sequence in the host genome, it is integrated by homologous recombination. This means that (linearised) plasmids are integrated to the chosen genomic locus by a single reciprocal crossover. Thus the gene can be targeted to a distinct locus (Orr-Weaver et al. 1981; Cereghino and Cregg 2000; Rosenfeld et al 1996).

1.4.2 Secretion of the heterologous protein: The secretion-signal alphafactor-leader from *Saccharomyces cerevisiae*

In *P. pastoris* different secretion signals are known: The expressed protein can be secreted by a native secretion signal, or by other signal sequences from either the alphafactor-leader of *S. cerevisiae*, the acid phosphatase or the invertase sequence. The most commonly used secretion signal is the alpha mating factor prepro leader of *S. cerevisiae.* This signal sequence comprises of a pre-peptide consisting of a 19 amino-acid-sequence which targets the nascent polypeptide to the ER, followed by the pro-peptide, a 66 amino-acid sequence. Upon the transport along the secretory pathway, the sequence is processed in three steps as reviewed in detail by Cereghino and Cregg 2000 and Daly and Hearn 2005.

1.4.3 Expression vectors

Yeast expression vectors are usually designed as *E. coli* / yeast shuttle vectors containing a bacterial origin of replication for plasmid maintenance and amplification in *E. coli* as well as selection markers (for auxotrophic selection or selection due to antibiotic resistance) for both species. Further essential parts of such vectors are a promoter regulating the expression of the gene of interest, as well as a transcription-terminator, a secretion signal if necessary and a multiple cloning site (MCS) for the insertion of the gene. There is a variety of *P. pastoris* expression vectors with different properties for various applications available. The basic vectors used in this work are shortly described in the following:

- **pGAPZαA**

The pGAPZαA vector is a *P. pastoris* expression vector for constitutive high level expression of recombinant proteins commercially available by Invitrogen Inc., Carlsbad, CA. The resistance marker is Zeocin.

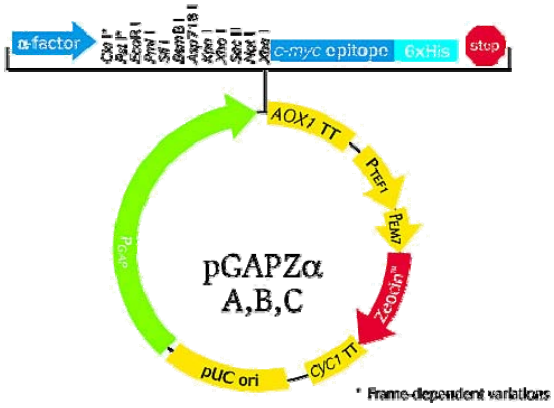

Fig. 3: Vectormap of the vector pGAPZαA (www.invitrogen.com)

- **pPUZZLE**

The pPUZZLE vector is a *P. pastoris* expression vector which has been designed in house (Stadlmayr et al. submitted 2010). The vector is constructed to be integrated in the *P. pastoris* genome via homologous recombination. It can be integrated into different loci of the genome by varying the homologous region parts (here: 3' AOXtt-region). The promoters used in this work were either the glyceraldehyde-3-phosphate dehydrogenase (GAP) or the translation elongation factor 1 α (TEF1). As selection markers a Zeocin cassette or the Kanamycin/G418 (KanMX)-cassette were utilized.

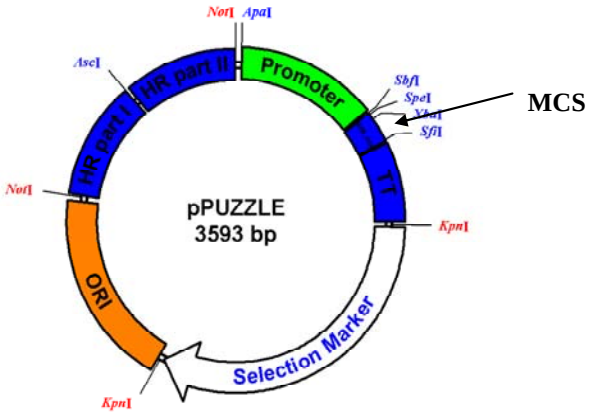

Fig. 4: Vectormap of the basic *P. pastoris* expression vector pPUZZLE

1.4.4 Promoters

There is a set of promoters for the production of heterologous proteins in *P. pastoris*. Three of them are described here in the following:

- **AOX-promoters (alcohol oxidase promoters)**

The AOX-promoters (AOX1-promoter and AOX2-promoter, Tschopp et al.; 1987a) are methanol-inducible promoters regulating the alcohol oxidase genes *AOX1* and *AOX2*. The principle of this system is based on the fact that enzymes essential for the methanol utilization pathway are only activated, if methanol is present. The *AOX1*-promoter is the most widely used when *P. pastoris* expression system is cultivated on methanol. However there are also some disadvantages of this well-established expression strategy like the costs and the hazard when working with large volumes of methanol. The hazardous work with the flammable methanol can be overcome with a constitutive promoter. Additionally high concentrations of methanol may also cause stress to the cell. The cell viability is decreased (Hohenblum et al 2004) and also the degradation of the recombinant protein by intracellular proteases as well as cell lyses can be a significant problem (Jahic et al 2003). For these reasons constitutive promoters are an interesting alternative.

- **P_{GAP} (glyceraldehyde 3-phosphate dehydrogenase promoter)**

The enzyme glyceraldehyde 3-phosphate dehydrogenase plays an important role in the glycolysis, where it catalyzes the reaction from glyceraldehyde-3-phosphate (GAP) to 1,3-bis-phosphoglycerate (1,3-BPG). Its promoter was first isolated by Waterham et al. in 1997. This is a strong constitutive promoter which is most often used with glucose or glycerol as carbon source. (Zhang, A.-L. et al. 2009, Cos et al. 2006)

- **P_{TEF} (translation elongation factor 1-α promoter)**

The translation elongation factor 1-α is a key factor in the transport mechanism of amino acyl transfer tRNAs to the ribosome. In *S. cerevisiae* there is a long use of its promoter (as for example described in Mumberg et al. 1994). In *P. pastoris* the gene and the promoter were first isolated by Ahn et al. in 2007. They suggest the P_{TEF} as a promising promoter for the constitutive expression in *P. pastoris* as it showed high levels of expression resulting in the same ore even higher product levels than with P_{GAP}. These two promoters are different in expression because the TEF-promoter is associated to cell-growth.

1.4.5 Selection markers

Generally selection markers are designed for dominant selection (such as antibiotic resistance genes) or they rely on the complementation of a genetic defect (auxotrophic markers).

- Antibiotic selection

There are a few selectable marker genes for *P. pastoris.* In this work the two ones mainly used were the *Sh ble* gene originating from the bacteria *Streptoalloteichus hindustans*, which confers resistance to the bleomycin-related drug Zeocin (Zeo^R) (Cereghino and Cregg 2000;

Macauley-Patrick et al. 2005) and the bacterial gene Tn903kan^r (Kan^R), conferring resistance to the antibiotic G418 (geneticin) (Lin-Cereghino et al 2008). Both are used as expression cassettes for the selection in *E. coli* as well as in yeast.

1.5 Strategies for Fab-production in microbial systems

1.5.1 Fab-production in yeast

The observation that yeast is a potential expression system for antibodies and Fab was first described by Horwitz et al. 1988. They showed that Fabs produced by *S. cerevisiae* had the same binding activity as the Fabs prepared by the enzymatic cleavage of the whole antibody by papain. However the product titer they obtained was very low.

As several heterologous proteins have been successfully expressed in *P. pastoris*, it was also used for the production of Fab as shown by Takahashi et al. 2000 and Lange et al. 2001. In these studies the Fab chains were expressed under the control of the inducible promoter AOX. Gasser and co-workers studied the Fab-production (2F5) in *P. pastoris* by the constitutive promoter P_{GAP} (2006). Based on the promising results, the working group expressed another antibody (with P_{AOX}) (Gach, J. et al. 2007). The Fab-chains of the anti-idiotypic antibody 3H6 gene were constitutively expressed on one vector under control of the same regulatory units. In the present work the 3H6-Fab should be produced by *P. pastoris* by coordinated expression of the light and heavy chain gene by two different constitutive promoters on two separate vectors.

1.5.2 Inducible versus constitutive protein-expression

Expression under control of a constitutive promoter means that the heterologous protein is continuously expressed at rather constant levels. A strong constitutive promoter in *P. pastoris* is the GAP promoter, which allows a gene expression that is going along with the generation of biomass, independent from an inducing substance. This is in contrast to the AOX promoter system, which only expresses the recombinant protein in the presence of the inducer methanol during the induction phase when glucose is not present. When *P. pastoris* is grown to high cell densities, the heterologous protein is accumulated during cell growth continuously. At the end of the cell growth phase the secreted protein can be recovered. For these reasons the constitutive system might be more suitable for large scale production, however, it is limited to proteins that are non-toxic to the cells (Wu et al 2003).

1.5.3 Coordinated expression of Fab light and heavy chain genes from separate vectors

For the coordinated expression of Fab, either the gene sequences of both chains are combined on one single vector, or the genes are on two separate expression vectors.

In *E. coli* there is a different initial situation than in yeast. Generally, one single vector is used with one promoter expressing a bicistronic mRNA. This means the light chain and heavy chain genes are expressed in one expression cassette as an operon. A comparison of this standard expression system for *E. coli* to the expression of the two chains in two separate expression cassettes, each with its own promoter, was done by Corisdeo and Wang (2004). Leonard et al. (2006) showed that the expression of the LC and HC from two separate vectors in *E. coli* (with mutant ColE1 replication origins) lead to similar expression levels as the expression from one single vector. They suggest that co-expression of the Fab-chains from separate plasmids makes it possible to combine every light chain with every heavy chain and therefore creating the best combination that would be possible. In mammalian cells antibody(fragment)-chain genes are generally expressed from two separate vectors, but also one single vector systems are available (Persic et al. 1997, Norderhaug et al. 1997, Schlatter 2005, Li 2007 , Sanna 1999). For example Schatz and co-workers (2003) expressed a chimeric Fab in CHO-cells with the LC and HC gene under control of the CMV-promoter (Cytomegalovirus) from two separate vectors. 3H6 Fab was expressed in CHO-cells by Kunert et al. 2008 with the light and heavy chain genes on separate vectors. They compared its expression in the CHO mammalian system to that in the *P. pastoris* yeast system. In *P. pastoris* the expression of the Fab was accomplished on one single vector. In yeast Fab chains are normally expressed from one single vector with the HC and LC genes in two separate expression cassettes, driven by two separate promoters (Takahashi et al. 2000, Lange et al. 2001, Gasser et al.2006, Gach et al. 2007). To our knowledge for the production of Fab in yeast, the light and heavy chain genes have not been expressed from two separate vectors so far. In the present work two separate plasmids, one with the light chain gene and the other with the heavy chain gene, were used.

The LC and HC genes can be regulated by the same promoter or by the use of different promoters. Two other important aspects that have to be considered, especially when separate vectors are used, are the gene copy number and the resulting ratio of the heavy chain to the light chain.

During the present work, these parameters (additionally to the determination of product amount and quality) were tested, to characterise clones which express the light and heavy chain genes of the Fab under control of different constitutive promoters.

2 Materials and Methods

2.1 The strains

- yeast: *Pichia pastoris* X-33 (Invitrogen, Carlsbad USA)
- bacteria: *Escherichia coli* Top10 (Invitrogen, Carlsbad USA)

2.2 The vectors

- **Light chain vector: pGAPZαA_(3H6)LC**

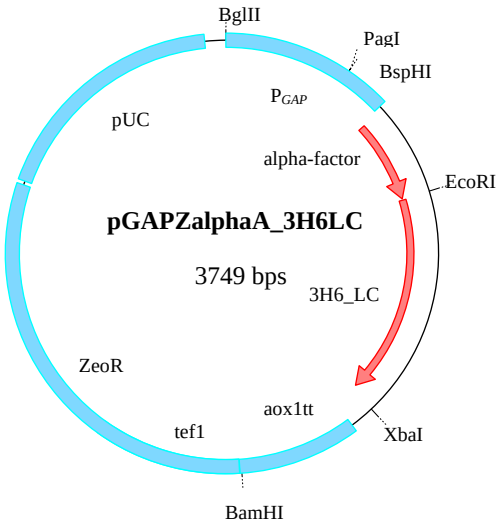

Fig. 5: Vector map of the LC-vector pGAPZalphaA_(3H6)LC (Gach et al. 2007)

- **pPZGAP_(3H6)HC**

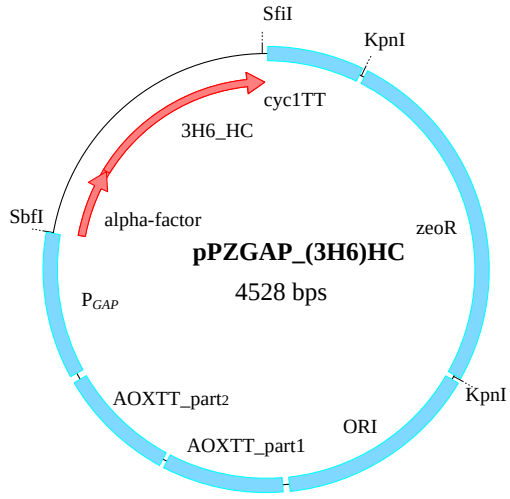

Fig. 6: Vector map of the vector pPZGAP_(3H6)HC (constructed by K. Benakovitsch)

- **Heavy chain vector (TEF-promoter): pPKTEF_(3H6)HC**

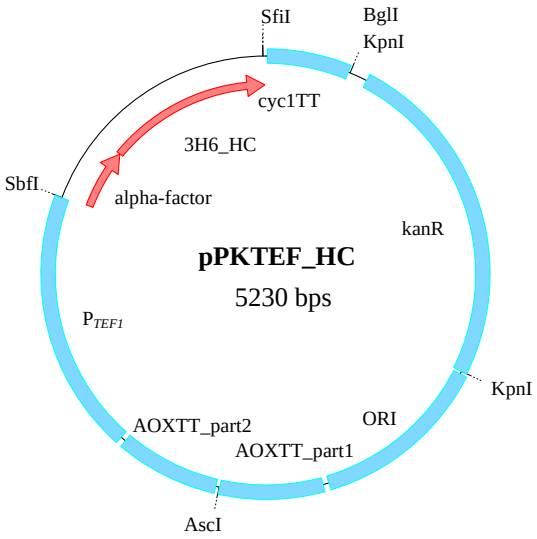

Fig. 7: Vector map of the HC- vector pPKTEF_(3H6)HC (constructed by K. Benakovitsch)

- **Heavy chain vector (GAP-promoter): pPKGAP_(3H6)HC**

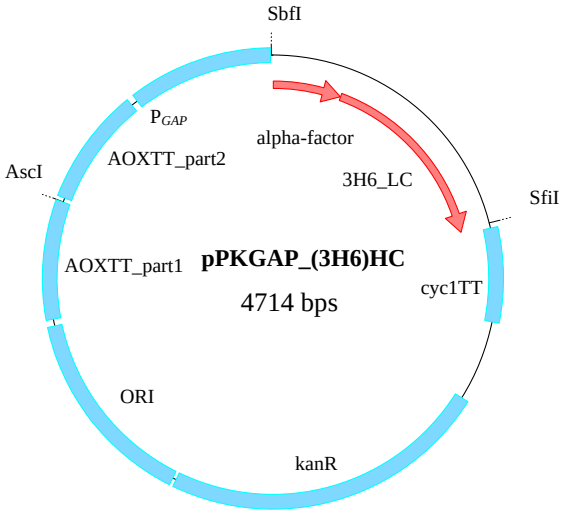

Fig. 8: Vector map of the HC- vector pPKGAP_(3H6)HC (constructed by K. Benakovitsch)

- **Vector production clone: pGAPZ αA_(3H6)Fab**

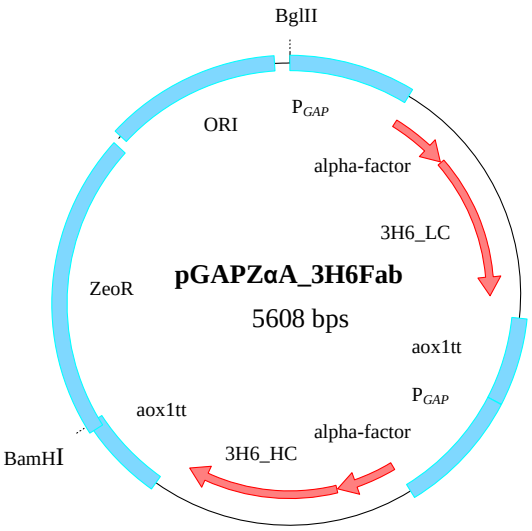

Fig. 9: Vector map of the vector pGAPZαA _(3H6)Fab (Gach et al. 2007, Gasser et al. 2006)

2.3 Media

All media and solutions have been heat-sterilized by autoclaving at 121 °C and 2 bar for 20 min, if not stated otherwise.

2.3.1 Media for bacteria cultivation

LB medium/ LB-agar:

10 g soy peptone (HY QUEST)
5 g yeast extract
5 g NaCl
ddH2O up to 1000 mL
pH was adjusted to 7.4 with 0.8 M NaOH

LB medium containing 1.5 % agar- agar (20 g for 1000 mL) was retained at 52 °C prior to pouring out into plates.

Antibiotic selection marker:

Kanamycin final concentration 100 µg/mL (Kanamycin stock solution: 100mg/mL Kanamycin (2000 x), Roth Karlsruhe Germany), sterile filtered

or Zeocin final concentration 25 µg/mL (Zeocin stock solution: 100mg/mL Zeocin (4000x), (Invivogen, San Diego USA)

SOC-medium:

2 g soy peptone (HY QUEST)
0.5 g yeast extract
0.58 g NaCl
0.19 g KCl
0.203 g $MgCl_2.6H_20$
0.396 g glucose-monohydrate
0.246 g $MgSO_4.7\ H_20$
ddH_20 up to 100 mL

2.3.2 Media for yeast cultivation

YPD-medium/-agar:

10 g yeast extract
20 g of soy peptone
20 g agar-agar if required
900 mL ddH_20

after sterilization: addition of 100 mL separately sterilized 10 x glucose

BMDY-medium:

10 g yeast extract
20 g soy peptone
700 mL ddH20
100 mL 1M phosphate buffer pH=6.0
100 mL 10xYNB
2mL 500x Biotin
100 mL of separately sterilized 10 x glucose

Stock solutions

10 x glucose (20%): 220 g D(+)-glucose monohydrate / 1000 mL
10 x glycerol: 100 mL (126 g) glycerol water free / 1000 mL
10 x YNB (13.4 %): 134 g yeast nitrogen base / 1000 mL, the solution was heated until YNB was completely dissolved, then sterilised by filtration Millex-GP-filter, stored at 4°C, dark
500 x biotin (0.02 %): 20 mg d-biotin / 100 mL, sterilisation by filtration Millex-GP filter storage at 4 °C

Antibiotic selection markers:
Zeocin (Invivogen, San Diego USA) stock solution 100 mg / mL (4000x),
final concentration 25 µg /mL
Geneticin (G418) (Invivogen, San Diego USA) stock solution 50 mg / mL (100x),
final concentration 500 µg / mL

2.4 Strain Conservation by Cryo Culture

For short periods, cells can be stored by repeated passages on appropriate agar plates at 4°C, however, for long time storage and preservation of the produced strains cryo culture conservation is required.

- **Procedure:**

5 mL of LB medium (for bacteria, with selection marker) or YPD medium respectively (for yeasts, without selection marker) were inoculated with a single colony from a plate and grown overnight at 37°C with shaking (bacteria) or 28 °C (yeast) respectively. Aliquots of the overnight cultures were transferred into cryo tubes (Nunc) and mixed carefully with sterile water-free glycerol up to a concentration of approximately 20% for the bacteria and 30% for yeast culture. The mixtures were immediately frozen at – 80 °C. To bring cryo-preserved strains in culture again, cultures were taken from the frozen surface with a sterile inoculation loop and used to inoculate either liquid media or plates.

2.5 Cloning procedures

2.5.1 Restriction digest of DNA

Restriction endonucleases are bacterial enzymes which are used by bacteria as a tool against viral infections by destroying (cleaving) the bacteriophagal DNA. There are four types of restriction endonucleases of which only the type II (and sometimes type I) ones are used in the laboratory for cloning procedures. The classification is considering their specificity and cofactors, the subunit composition and the position when they cleave. The type II enzymes cut at defined specific sequences of the DNA within or close to their recognition sites. About 250 different sequence specificities have been discovered so far. Most of the enzymes recognize symmetric sites because they bind as homodimers and cut within these

sequences, a few bind as heterodimers and recognize asymmetric DNA sequences. The cleavage leaves a 3'-hydroxyl terminus on one side of the cut and a 5'-phosphate on the other. Depending on the used enzyme there are either resulting sticky ends, which can easy be ligated by using inserts with sticky ends with the complementary sequence, or blunt ends. For a restriction digest the restriction enzymes are chosen that cut within a specific, desired sequence in the DNA. The procedure is normally performed in a specific buffer for about two hours at the temperature optimum of the particular enzyme. For a wide range of restriction enzymes this means 37°C. In some cases double digestion (two compatible enzymes used in one preparation) are performed.

- **Procedure:**

Table 1: Components and volumes of a preparation for a restriction digest:

100 µL preparation	
10 µL	10x buffer (suitable for the used restriction enzyme)
10 µL	10x BSA (if required for the used restriction enzyme)
100 ng to 5 µg	DNA in solution (10mM Tris/Cl pH 8)
2-5 µL	restriction enzyme
Volume up to 100 µL	ddH_20 (if necessary)

The components (as described in Table 1) were mixed in a 1.5 mL tube and incubated for about 2h at 37°C (or at the temperature the restriction enzyme required). After incubation an aliquot (5µL) was analysed on an agarosegel by gel electrophoresis as control. For cloning purposes the DNA-fragment of correct size was purified by gel extraction (using the mi-Gel Extraction Kit, metabion) (see 2.5.3)

2.5.2 Separation of DNA Fragments by Agarose Gel Electrophoresis

The separation of DNA-fragments by electrophoresis is based on the migration in an electric field which is applied. The DNA is migrating towards the positive anode due to the negative charge of the phosphate-groups along the backbone. On their way the fragments are retarded by the pores of the agarose gel-matrix. Agarose is a linear uncharged carbohydrate polymer which is extracted from seaweed. After melting and solidifying, it builds a porous gel. Larger molecules move more slowly through the gel than smaller fragments. Depending on the size of the DNA the agarose-concentration in the gel can be varied (normally 1-3% agarose, the smaller the fragments the higher the agarose-concentration). The migration distance of the DNA is mainly depending on the size and conformation of the nucleic acid, the agarose-concentration and the applied voltage. Before the application the samples are mixed with a loading buffer which contains three main components: A high density solution

that allows the DNA-solution to sink to the bottom of the well, a tracking dye which migrates in the same direction as the DNA and a chelating agent that stops enzymatic reactions by complexing divalent cations. Visualisation of the DNA is obtained through ethidium-bromide (3, 8-diamino-6-ethyl-5-phenyl-phenanthridium-bromide). This is a dye that intercalates between the stacked bases of double-stranded DNA. It is added to the agarose-gel and the running-buffer and gives an orange fluorescence while it is illuminated with UV-light (260-360 nm) by a transilluminator, if it is intercalated in the DNA. To estimate the DNA-fragment size, the obtained bands are compared with the ones from DNA-standards. These DNA size markers consist of a number of bands of known size. The agarose-gel electrophoresis can also be performed as a preparative gel electrophoresis to purify a certain DNA-fragment from a mixture of digest products or for the purification of a PCR-product. After the electrophoresis the DNA can be extracted from the gel-matrix, for example with gel-extraction kits (Molecular Biomethods Handbook 1998, publisher: Humana press, chapter 3: D.R. Smith: „Gel electrophoresis of DNA“).

- **Material**

- ethidiumbromide stock: 10 mg/mL (Roth, Karlsruhe Germany)
- 50 x TAE buffer: 242 g Tris base
  57.1 mL glacial acetic acid
  0.5 M EDTA pH 8.0
  ddH_2O up to 1000 mL
- 6 x loading buffer (Fermentas, St. Leon-Rot Germany)
- 1 % agarose gel: 1 g / 100 mL agarose
  2 mL / 100 mL 50 x TAE buffer
  4 µL / 100 mL ethidiumbromide stock

2.5.3 Gel extraction

As a next step the desired DNA-fragment had to be extracted from the agarose-gel. The gel-extraction technique with kits proceeds in several steps: After identifying and cutting out of the desired band, the DNA is separated from the gel by melting the agarose-matrix. The DNA-solution is transferred to a special spin column, which contains a silica-gel-membrane that specifically binds the DNA. Chaotropic salts assure the inactivation of nucleases and enhance also the DNA-binding-step. After the binding the DNA is washed by specialized wash buffers. During this process, impurities like primers, nucleotides, enzymes, salts and remains of agarose and ethidium bromide are removed. The terminal step is the elution of the DNA with a small volume of a low-salt buffer e.g. Tris/Cl (10mM, pH8).

- **Material:**

see mi-Gel Extraction Kit (cat #: mi-GE100), metabion, Martinsried, Germany

- **Procedure** (according to the supplier's instructions)

The solution was transferred to an agarose gel and gel electrophoresis was performed (2h, 100V). The desired DNA-band was cut and put into a 1.5 mL micro-centrifuge tube. 300 µL gel extraction buffer was added and incubation at 65°C for about 10 min was done in order to melt the agarose. The process was supported by inverting the tube for several times. After melting of the gel, the solution was added onto the spin column which was placed on a collection tube and after short incubation (1min), it was centrifuged at 13,000 rpm for 1min. The flow-through was discarded and the spin column replaced to the collection tube. 750 µL column wash buffer was added and centrifugation followed (13,000 rpm, 1min). After the washing step, the flow-through was discarded and another centrifugation step was performed (13,000 rpm, 1min). The spin column was placed on a fresh 1.5 mL micro-centrifuge tube and 10-50 µL of 10 mM Tris/Cl (pH 8) was added to the spin column. After 2 min of incubation the DNA was eluted by centrifugation (13,000 rpm, 2 min)

2.5.4 Dephosphorylation of the vector

To prevent religation of the vector, it was dephosphorylated (the phosphate group is enzymatically cleaved from the 5'-end). Thus the probability of a recirculation of the plasmid is minimized, because a phoshodiester bond can only be built between a 5'-phosphate group and a 3'-hydroxyl group.

- **Procedure:**

Table 2: Components and volumes of a preparation for the dephosphorylation of a vector.

60 µL preparation	
3 µL	CIP (alkaline phosphatase, calf intestinal) (1 U/µL)
6 µL	10x CIP reaction buffer (restriction endonuclease reaction buffer 3, NEB Massachusetts USA)
6 µL	10x BSA
45 µL	vector solution

The reagents were mixed as shown in table 2 and incubated at 37°C for 30 min.

2.5.5 Ligation of the vector and the insert

Restriction fragments with compatible (complementary) ends can be recombined with the enzyme DNA-ligase. DNA-ligases are enzymes which form phosphodiester-bonds between a

5'-phosphate-group and a 3'-hydroxy-group of the open ends of double-stranded DNA-restriction fragments. In vivo they repair breaks of single-strands. The ligase mainly used for DNA-recombination originates from bacteriophage T4. The reaction of this ligase needs ATP as a co-factor.

- **Procedure:**

Table 3: Components and volumes of a preparation for the ligation of vector and insert:

15 µL preparation	
1 µL	vector solution (50 to 500 ng of vector DNA solution in 10 mM Tris/Cl pH 8)
7 µL	insert solution
1.5 µL	10x T4 ligation buffer
1 µL	T4 DNA ligase
up to 15 µL (--> 4.5 µL)	ddH_2O

The reaction mix (see Table 3) was incubated at room temperature for 20 min. (As negative control a ligation was done using ddH_2O instead of the insert).

2.5.6 Purification with the mi-PCR-purification-Kit (metabion)

The PCR-reaction or other DNA-solutions are mixed with the DNA-binding-buffer, which enhances the selective binding of the double-stranded DNA to the silica-gel-membrane in the spin column. Additional components like remaining polymerase, buffer, primers or dNTP are removed from the DNA by washing steps with the Column wash buffer. The solutions are passed through the column by centrifugation. Finally the DNA is eluted with Tris/Cl (10 mM, pH 8).

- **Material:**

see mi-PCR Purification Kit (cat #: mi-PP200), Metabion, Martinsried, Germany

- **Procedure:**

500 µL binding buffer were transferred to the spin column and the DNA-solution was added. After 1 min of incubation it was centrifuged for 1 min at 13,000 rpm. The flow-through was discarded and a washing step was performed. After addition of 750 µL column wash buffer and centrifugation for 1 min at 13,000 rpm, another washing and centrifugation step followed. The spin column was put on a fresh 1.5 micro-centrifuge tube and 20 µL Tris/Cl (10 mM, pH 8) were given onto the column. After 1 min incubation the DNA was eluted by centrifugation (1 min at 13,000 rpm).

2.6 Transformation of the prepared vectors into the bacteria

2.6.1 Transformation in *E. coli*

The DNA has to be introduced into the cells either by chemical treatment ($CaCl_2$) or electroporation. Here the commonly used electroporation-method has been performed. The linearised vectors were transformed into electrocompetent *E. coli.*

2.6.1.1 Electroporation:

Electroporation is a highly efficient method of transformation for *E. coli.* The cell suspension and the DNA to be inserted are mixed and exposed to a brief and intense electrical pulse. As a consequence of the electrical pulse the conductivity of the cell membrane drastically increases and therefore pores are built, which make the membrane permeable for DNA. Thus results up to 10^9-10^{10} transformants/µg DNA can be reached (Dower 1988, Jahnson 2007).

- **Material:**

SOC-media
LB-agar
(see media 3.2)

- **Procedure:**

Electro-competent *E.coli* Top10 stored at -80°C were slowly thawn on ice. 80 µL of the cells were mixed with 20 µL of the purified vector-solution and then transferred into a chilled electroporation cuvette (4 mm).
The electroporation was performed under following conditions on a BTX ECM 630 Electrocell Manipulator:
Voltage: 2500 V
Capacitance: 25 µF
Resistance: 1000 Ω
After the pulsing, the cells were taken up in 900 µL 37°C tempered SOC-Media and incubated for 1h at 37°C to give them the possibility to regenerate. Then the transformed cells were plated in volumes of 50 µL and 200 µL on LB-agar containing the appropriate antibiotic (Kanamycin: 50 µg/mL or Zeocin 25 µg/mL). The plates were incubated at 37°C for 24h. From the grown transparent colonies six were streaked out on fresh LB-plates containing the selection marker.

2.7 Preparation and purification of the plasmids

2.7.1 Plasmid preparation with mi-plasmid miniprep kit (metabion)

The preparation of circular plasmid DNA from bacteria can be easily done by alkaline lysis. This method was developed by Birnboim and Doly (1979) and is based on the difference of plasmid and chromosomal DNA in size and conformation. Therefore the plasmid DNA consistent of small supercoiled molecules can selectively be extracted from the chromosomal DNA (larger, linear molecules). The lysis with an alkaline detergent effects the denaturation of proteins and nucleic acids. After alkaline lysis of the cells DNA-binding buffer is added. The potassium acetate neutralizes the solution while the plasmid DNA is refolded and stays in solution the chromosomal DNA and proteins are not able to correctly refold and precipitate. The mix is transferred to a spin column which contains an anion-exchange resin that binds the solved plasmid DNA while the chromosomal DNA passes through. The bound plasmid DNA is washed with Column wash buffer and can then be eluted with Tris/Cl (10mM, pH8) (Birnboim and Doly 1979).

- **Material:**

see mi-Plasmid Mini Prep Kit (cat #: mi-PM200), Metabion, Martinsried, Germany

- **Procedure:**

Precultures of the clones picked from the plate were prepared. 2 mL LB media + antibiotics were inoculated by picking the clones from transformed bacteria with sterile toothpicks. The cells were grown overnight at 37°C under shaking conditions. The cells were collected by centrifugation at 13,000 rpm for 1 min and the supernatant was discarded. 250 µL resuspension buffer were added and the cells were resuspended by vortexing. After addition of 250 µL cell lysis buffer, the suspension was mixed by inversion (5 times) and incubated for 2 min. 350 µL DNA binding buffer were added and the solution was inverted again and incubated for another 2 min. After centrifugation for 10 min at 13,000 rpm, the supernatant was transferred into a spin column placed on a collection tube. The solution was spun for 1 min at 13,000 rpm after 1 min of incubation. The flow-through was discarded and 600 µL column wash buffer were added followed by another centrifugation step (1 min, 13,000 rpm). The spin column was placed on a fresh 1.5 mL micro-centrifuge tube, and DNA was eluted by addition of 50 µL Tris/Cl (10mM, pH8) and centrifugation after 1 min of incubation (2 min, 13,000 rpm). As a control the prepared plasmids were digested with restriction enzymes. One preparation was performed with a restriction enzyme that should give one cut in the insert to linearise the plasmid (here: AscI) and one preparation with a double cutter enzyme

(here: Hind III). The digested plasmid was then analysed by separation of the resulting fragments by agarose gel electrophoresis.

2.8 Transformation of the amplified vectors into the yeast *P. pastoris*

The transformation into the yeast cells was also done by electroporation. The principle of the procedure for yeasts is nearly the same as that described for the *E.coli* in 3.11. The differences in cell preparation and electroporation parameters are described below. The plasmid was linearised (either in the promoter region or in the AOX-TT region) to enable correct homologous recombination into the yeast genomic DNA.

2.8.1 Preparation of electro-competent yeast cells

- **Material:**

YPD-media
1 mM sterile HEPES buffer
1 M sterile sorbitol

- **Procedure:**

An aliquot of the respective *P. pastoris* cryo-culture (-80°C) was the inoculation material for the starter culture in 10 mL YPD-media (28°C, shake flask culture, overnight). The main culture was prepared by transferring 50 µL from the preculture in 100 mL YPD-Media and incubated (28°C, shake flask culture, overnight) to an OD_{600} about 1. The cell cultures with OD_{600} ~ 1 were harvested by centrifugation (5 min, 3,500 rpm in 50 mL tubes). The pellet was resuspended in 1 mL 1mM HEPES (ice-cold) and transferred into 2 mL tubes (cooled). Cells were washed by centrifugation (4°C, 1 min, 3,000 rpm). Further another 4 washing steps with 1mL 1mM HEPES followed (centrifugation: 4°C, 1 min, 3,000 rpm). 1 mL 1M sorbitol (cooled) was added and after resuspension of the pellet, cells were centrifuged (4°C, 5 min, 3,000 rpm). The supernatant was discarded by pipetting. Cells were taken up in 200 µL 1M sorbitol, and cooled on ice until the electroporation.

2.8.2 Electroporation

- **Material:**

1 M sterile ice-cold sorbitol
YPD – agar (+ required antibiotic as selection pressure)

electrocompetent cells (*P. pastoris* X-33)
vector solution

- **Procedure:**

80 µL of competent *P. pastoris* cells were mixed with 20 µL of linearised plasmid in a micro-centrifuge tube stored on ice for about 5 min. As negative control one preparation was performed with 20 µL 10mM TrisCl instead of plasmid. After the incubation the suspension was transferred to pre-cooled 2 mm electroporation cuvettes.

Electroporation was performed under following conditions:
Voltage: 1500 V
Capacitance: 25 µF
Resistance: 400 Ω
Transformed cells were taken up in 1 mL pre-cooled 1M sorbitol and transferred into a 50 mL tube to be incubated at 30 °C for 1h for regeneration.

2.8.3 Regeneration, plating and antibiotic selection

When using G418 as selection marker, 4 mL YPD-media were added to the GAP-clone cultures after the regeneration and the transformed *P. pastoris* cells were kept at 28°C under shaking conditions for 2h. After that volumes of 200 and 50 µL were plated on YPD-agar containing G418 (500 µg/mL) and additionally Zeocin (25 µg/mL) as selection pressure. The plates were incubated at 30°C for 48h. After that 16 clones were streaked out fractionated on fresh YPD-agar plus G418 and Zeocin (conc. as above) and incubated for 48h at 30°C. For Zeocin as selection marker, directly after the regeneration-time volumes of 200 µL as well as 50µL were plated on YPD-agar + Zeocin (25µg/mL) (as selection pressure) and incubated at 30°C for 48h. Afterwards 16 clones were streaked fractionated on YPD-agar + antibiotic for selection pressure (Zeocin 25µg/mL). Incubation was performed for 48 h at 30°C. The clones were again freshly streaked on a masterplate (YPD-agar + Zeocin (25µg/mL)) and incubated for 48 h at 30°C.

2.9 Screening of the selected yeast clones

The constructed *P. pastoris* clones were cultivated to examine Fab production. A screening is performed in order to identify clones which give an outstandingly high protein expression

(Penneli and Eldin 1998). The procedure was done in 24-deep-well plates as described by Boettner et al. 2002 using a culture volume of 2 mL.

- **Material:**

YPD-medium
BMDY-medium
24 deep well microplates (UniPlate® 10000, 24 Well, 10 mL, Whatman)
50% glucose

- **Procedure:**

For a pre-culture, clones were picked from a freshly streaked selective plate (YPD+ Zeocin and/or G418) and dispended in 5 mL YPD in 50 mL Falcon tubes overnight at 28°C. After determination of the optical density at 600 nm, the main culture was prepared. The calculated volume of the pre-culture for a starting OD_{600} was inoculated in 2 mL BMDY-media/well in 24 deep well microplates and incubation was performed for 48h at room temperature and vigorous shaking. Feeding with 0.5% glucose was done every 12h.

Harvesting of the cells and determination of the yeast wet mass (YWM):
After 48 h of cultivation the wet biomass was determined. Empty tubes were weighed and cultures were transferred into these tubes. Cells were harvested by centrifugation (2min at 13,000rpm) and the culture supernatant (~ 1.5 mL) was transferred into fresh tubes. For determination of yeast wet biomass pellets have been weighed. Pellets were stored at -20°C. The culture supernatant was immediately used for analysis or stored at -20°C.

2.10 Analysis of the culture supernatant containing the recombinant proteins

2.10.1 SDS-PAGE:

The sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) is a method to separate proteins by their molecular size. The basic principle is the migration of charged molecules in the electric field. To overcome the influences because of different charges and structures, the samples are treated with the detergent SDS. First the samples have to be heated under denaturing and reducing conditions. The proteins are coated with the SDS-molecules to unfold the proteins and give all proteins a negative charge. The net charge is then proportional to the length of the poly-peptide chain. The prepared samples are loaded to

a polyarcylamide-gel and an electric field is applied in which the proteins migrate to the positive electrode. The migration distance gives information about the size of the denatured proteins. After the electrophoresis the gel can either be stained directly (comassie blue, silver staining) or the separated proteins can be transferred to a nitrocellulose-membrane and probed with protein-specific antibodies (Western blot).

- **Material:**
  - NUPAGE 12% Bis-Tris-Gel, 1mm, 15 well, (Invitrogen, Carlsbad USA)
  - 20 x MOPS NuPAGE SDS Running buffer, (Invitrogen, Carlsbad USA)
  - ddH_2O
  - NuPAGE LDS 4x sample buffer, (Invitrogen, Carlsbad USA)
  - 100 mM DTT (1,4-Dithio-DL-threitol Dithiothreitol), used for reducing conditions
  - Page Ruler Prestained Protein ladder (Fermentas, Massachusetts USA)
  - MagicMark™ XP Western Protein Standard (20-220 kDa) (if Western blotting was performed)

- **Procedure:**

Preparation of the samples:

Non reducing conditions:

Table 4: Components and volumes of a preparation for a non-reducing SDS-PAGE:

60 µL preparation	
15 µL	NuPAGE LDS 4x sample buffer, Invitrogen
45 µL	culture supernatant

Reducing conditions:

Table 5: Components and volumes of a preparation for a reducing SDS-PAGE:

60 µL preparation	
15 µL	NuPAGE LDS 4x sample buffer, Invitrogen
6 µL	DTT 100 mM
39 µL	culture supernatant

The components (see above) were mixed, kept at 95°C under shaking conditions (800 rpm, 10 min) and spun down. After short vortexing 15 µL of the sample or 5-7 µL marker were put on the gel which was placed in the electrophoresis chamber (Novex) filled with 800 mL running buffer. The gel electrophoresis was performed at 200 V for 45-50 min until the dye-front was on the end of the gel.

2.10.2 Silver staining

The silver staining is a very sensitive colorimetric method for protein visualisation. It takes advantage of the reduction of cationic silver in the form of silver nitrate in the stain reagent to

metallic silveriones and their binding to functional protein-groups. The Ag^+ ions are complexed by certain groups of the proteins in the gel matrix especially by carboxyl- and sulfhydrylgroups. This procedure leads to a black-brown colour on the places of the gel where the protein-bands are located. Formaldehyde and glutaraldehyde function as enhancers by crosslinking proteins to the polyacrylamide-gel (Jahnson 2007).

- **Material:**

- Fixing solution:	50 % (v/v) ethanol
	10 % (v/v) acetic acid
- Incubation solution:	30 % (v/v) ethanol
	0.83 M sodium acetate
	13 mM sodium thiosulfate
	0.25 % glutaraldehyde (freshly added before use)
- Staining solution:	6 mM $AgNO_3$
	0.02 % formaldehyde (freshly added before use)
- Development solution:	0.25 M Na_2CO_3
- Stop solution:	50 mM EDTA

- **Procedure:**

The gel was transferred into the fixing solution for 1h under gentle shaking at room temperature. After 30 min of shaking in 25 mL incubation solution, the gel was rinsed with distilled water 3 times for 10 min each. 25 mL of the staining-solution was added and the gel incubated for 20 min (gentle shaking) to allow the silver ions to bind to cysteine residues of the separated proteins on the gel. After washing with distilled water for 10 min, the gel was incubated in the development solution until the desired intensity of the respective bands has been reached. (The silver ions were then reduced to molecular metallic silver in the alkaline, formaldehyde-containing containment of the development solution). The reaction was stopped with 25 mL of stop solution. The gel was scanned.

2.10.3 Western blotting

Western blotting means the electrophoretic transfer of proteins from the polyacrylamide gel onto a nitrocellulose membrane after the SDS-PAGE. As Towbin et al (1979) described, with this method the original pattern of the separated proteins on the gel can be replicated onto a membrane on which they are immobilized. The proteins adsorb to the nitrocellulose due to hydrophobic interactions as well as different charges, while salts, smaller molecules, RNA and DNA are not retained. The immobilized proteins are then incubated with an antibody

specific to the target protein. Before this step the blocking solution (usually skimmed milk powder or BSA) is added to prevent unspecific binding. The antibody-protein-complex is detected by a second, enzyme-labelled antibody which is directed against the first antibody. Alternatively, the first antibody can be directly labelled with the enzyme.
In this work the reporter enzyme was either alkaline phosphatase or horseradish peroxidase, in conjunction with a chemiluminescent agent which led to a luminescent reaction. The visualisation can either be done by a colorimetric or chemiluminescent signal. The emerging chemiluminescence was measured by the CCD-camera (charged coupled device) of a luminograph (lumi-imager), which gives a signal that is proportional to the measured luminescence. The intensity (number of photon fluxes) was given in Boehringer Light Units (BLU) (Towbin et al. 1979; Manaresi et al. 1999).

- **Material:**

- Transfer buffer 1x:

per 200 mL (for one Novex electrophoresis chamber):

10 mL NuPAGE Transfer buffer (20x) (Invitrogen, Carlsbad USA)

20 mL MeOH (final concentration: 10%)

up to 200 mL ddH_2O

- Whatman paper
- sponges
- Nitrocellulose membrane (Transfer-Medium Pure Nitrocellulose Membrane, 0.2µm, #162-0112, BioRad)
- Washing buffer: PBST ((1x phosphate buffered saline (PBS) pH 7.4; 0.1% Tween 20)

per litre:

1.15 g $Na_2HPO_4.2H_2O$

0.2 g KH_2PO_4

0.2 g KCl

8.0 g NaCl

1 mL Tween20

- Blocking buffer: PBST + 1 % skimmed milk powder
- Antibody buffer: PBST + 1% BSA (bovine serum albumin)
- Antibodies: Anti-Human IgG (Fab specific)−Peroxidase antibody produced in goat (Sigma-Aldrich A0293 gahFab-HRP)
- Anti-C_H1(3H6)-domain-antibody, produced in mouse, polyclonal (made in house)
- Anti–mouse-IgG –HRP (Sigma-Aldrich # K6078)
- standard for quantification: hFab Std (Bethyl P80-115, Lot.:P80-115-6)

- horse radish peroxidase chemiluminescence kit (chemiluminescent peroxidase substrate
  CPS160-1KT 038K1063, Sigma-Aldrich)
- Lumi Imager (Roche)

- **Procedure:**

After the SDS-PAGE , the gel was transferred into the transfer-buffer and the front was discarded. The blot was assembled in following order: cover 1, 2 sponges, 1 whatman paper, the gel, the nitrocellulose membrane, 1 whatman paper, 2 sponges, cover 2. These components were placed into the Novex blotting chamber, transfer buffer was added on top, in the outer spaces of the chamber sterile water was added. After attaching the lid, the blotting process was performed under the following conditions: 50 V, 1h. The blot was transferred into the blocking solution and incubated for at least 1h or overnight.

Staining of the blot:

- Fab:

The goat anti human Fab-HRP was diluted 1: 20,000 in PBST + 1% skimmed milk powder and the blot was incubated for one hour at gentle shaking. 3 washing steps followed with PBST for 10 min each. The detection was performed with a HRP chemiluminescence kit. 2 volumes of buffer reagent were mixed with 1 volume of the second reagent.1.5 mL detection solution were added to the gel, which was placed in a plastic foil and stored light proof until measurement. The bands were visualised by chemiluminescence with the Lumi Imager (Roche)

- Heavy chain:

The anti-C_H1 (3H6)-domain-antibody was diluted 1:1,000 in PBST + 1% BSA for incubation of the blot for 1h. After washing 3 times in PBST the blot was incubated for 1h with the antibody anti mouse-HRP (diluted 1:1,000) in PBST + 1% skimmed milk powder. 3 washing steps followed with PBST for 10 min each. The detection was performed as described above.

2.10.4 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a method to determine the concentration of specific molecules in a sample. It is based on the specificity of antibodies and the possibility to attach proteins to polystyrol-surfaces of microtiter plates. In this work a “sandwich”-ELISA was performed. Therefore the first (capture-) antibody is bound to the solid

phase by incubation. After this coating procedure, the sample (here: culture supernatant) is added. During an incubation step the target-protein binds specifically to the immobilized first antibody. Every incubation step is followed by a washing step to remove unbound reagents. In order to be able to detect the bound product, a second specific and enzyme-labelled (here: alkaline-phosphatase) antibody is added. For the detection, the incubation with an enzyme substrate is performed (here: p-nitrophenylphosphate which is converted to the yellow p-nitrophenol). The enzymatic reaction leads to a colorimetric reaction product which is soluble in contrast to the Western blot, where it is precipitated. The signal-intensity is measured by a spectrophotometer. For the quantification a dilution series of a standard (product of known concentration) is measured as well on each microtiter plate, and concentration of the samples are calculated from a calibration curve of this standard (enzyme activity per specific antibody content).

- **Material:**

Solutions contained per litre:

▪ Coating buffer: PBS, pH 7.4 (see Western blotting, 2.10.3)

▪ Dilution buffer: PBST (PBS, pH 7.4, 0.1% Tween20 ((see Western blotting, 2.10.3))+ 2% BSA)

20 g BSA

▪ Washing buffer PBST (PBS, pH 7.4, 0.1% Tween20), see Western blotting 2.10.3

▪ Detection buffer: 0.1M $NaHCO_3$ buffer, pH 9.6 -9.8

8.4 g $NaHCO_3$

4.0 g Na_2CO_3

▪ Maxisorp 96-well plates – Nunc 442404

▪ Tween 20 - Sigma P7949

▪ ANTI-HUMAN IgG (Fab specific) - Sigma I5260

▪ ANTI-HUMAN Kappa Light Chains (bound)-Alkaline Phosphatase - Sigma A3813

▪ Serum Albumin, bovine – Sigma A7906

▪ Phosphatase substrate pNPP – Sigma S0942

▪ standard: hFab Std (Bethyl P80-115, Lot.:P80-115-6)

- **Procedure:**

Pre-coating of 96-well plates:

A 1:1,000 dilution of ANTI-HUMAN IgG (Fab specific) in coating buffer was prepared. 100µL/well were pipetted into the plates which were incubated with gently shaking at room temperature over night.

Preparation of the samples:

The culture supernatant was diluted in PBST + 1% BSA in several steps to the final concentration of about 40-100 µg/mL to be in the range of the Fab-standard (the dilution rates ranged between 1:200 and 1:500). The pre-coated 96 well-plates were 3 times washed with PBST to remove unbound antibody. The plates were beated on a piece of tissue to get rid of the remaining liquid. Next step was the application of 140 µL of dilution buffer in each well of row A to G of the plate. In row H the samples were applicated (280 µL/well). H1: dilution buffer (blank), H2 and H3: Fab-standard (200 ng/µL, diluted in dilution buffer) H4-12: the culture supernatant of the chosen clones. A dilution series was reached by taking 140 µL from row H and transferring it into row G which meant a 1:2 dilution. After mixing by pipetting up and down, 140µL were transferred to the next row. This step was continued until the last row (A). The last 140 µL in row A were discarded, so that 140 µL were in each well. Incubation was performed on a shaker for 1h at room temperature. The plates were washed as described above. The second antibody (Anti human kappa light chain bound alkaline phosphatase) was diluted 1:1,000 in dilution buffer and then applied to the wells (100 µL/well). Another hour of incubation on the shaker was performed.

Detection:

The plates were washed as described above. A detection solution was freshly prepared by solving 2 pellets of phosphatase substrate in 11 mL of detection buffer for one plate (= 10 mg/mL pNPP). The plates were developed until the standard series showed a good colour gradient (5-10 min). The absorption of the highest concentration of the Fab standard should be 2.0 ± 0.5. The plates were measured at 405nm (reference wavelength 620nm) in the microtiterplate photometer (TECAN Sunrise™). Data evaluation was performed by using a calibration curve (hFab-standard) and 4-parameter analysis.

2.11 Preparation of yeast RNA, cDNA and DNA

2.11.1 Isolation of RNA

The isolation of RNA with the TRI-Reagent after disruption of the cells combines phenol and guanidine-thiocyanate in one solution. The cell components are dissolved without harming the RNA, because of inactivation of the RNases. The method is based on the work of Chomczynski and Sacchi (1987) and the principle that total RNA stays in the aquatic phase under acidic conditions while the DNA and proteins are precipitated and accumulated in the interphase or organic phase respectively. The separation into the phases was done by

addition of chloroform. Isopropanol is added to precipitate the RNA that is subsequently washed with ethanol and solubilised in DEPC treated water (= water treated with Diethyl-pyrocarbonate to inactivate RNase). The RNA-Isolation was performed in a RNase-free zone to avoid RNA-degradation.

- **Material:**
    - YPD
    - BMD
    - TRI Reagent (SIGMA; T-9424)
    - Chloroform
    - Isopropanol
    - 75% Ethanol in DEPC(diethylpyrocarbonate)-treated water
    - Phenol
    - 0.5 % SDS solution or DEPC-treated water
    - acid washed glass beads (0.5 mm)
    - RNase free tips, tubes
    - UV cuvette

- **Procedure:**

The culture for the RNA-Isolation was grown in a 3-step-mode. For the pre-culture 5 mL YPD-media were inoculated with the picked colonies from the selective agar-plates and grown over night under shaking conditions at 28°C (in a 50 mL Falcon tube). The OD_{600} was determined and the required volume for an OD of 0.1 was calculated and pipetted in 10 mL YPD in a shake flask. The 1st main culture was grown overnight at 28°C and shaking. To fix the cells in the exponential growth phase a 2nd main culture was prepared, which was grown for 6 h. After determination of the OD_{600} the required volume for an OD of 1 after 6h was calculated and was transferred into 50 mL BMD- media (in a 500 mL shake flask). A volume of the cultures (8 mL) was cooled on ice. In a 50 mL falcon tube the ethanol/phenol mix was prepared: ½ volume of ethanol (4mL) and 5% (final concentration) phenol (200 µL) were mixed and cooled on ice. 50 mL falcon tubes and 2 mL microcentrifuge tubes were pre-cooled on ice. 4 mL of the ethanol/phenol mix were mixed with 8 mL of the culture in the pre-cooled 50 mL tubes and shaked well to reach a good stirring.

The suspension was portioned in the 2mL tubes and cells were spun down by centrifugation at 4°C at full speed for 2 min. The supernatant was discarded (→phenol waste) and the pellets were directly used for isolation or stored at -80°C.

- Yeast-cell-disruption:

The cell pellets were combined with 1mL Tri-Reagent and 500 µL mini-glass-beads in a sealed tube. The cells were disrupted using a cell homogenisator (FastPrep®, Thermo Scientific, Waltham USA) two times for 20 sec each at 6.5 m/s. 200 µL chloroform were added, shaked vigorously and incubated 5-10 min at room temperature. Then the cells were spun down by centrifugation at 4°C at 13,000 rpm for 15 min. This led to a separation into three phases: on the bottom a pink one which was the organic phase, in the middle a white phase containing the DNA, and on top a transparent, aqueous phase, containing the RNA. The RNA-phase was carefully taken up and transferred in fresh RNase-free tube. 500 µL isopropanol were added and mixed well. After 5-10 min of incubation at room temperature, centrifugation at 4°C, full speed for 10 min was performed. The supernatant was discarded and the pellet washed with 1 mL ethanol and centrifugation at 4°C at 13,000 rpm for 5 min. The supernatant was discarded and the RNA-pellet was air-dried for 5-10 min. The pellet was taken up in 50 µL DEPC-water and dissolution was supported by treatment at 60°C at gentle shaking for 20 min.

- DNase-treatment:

The DNase-treatment was performed with the DNA-*free*™ -kit (Ambion, cat # AM1906, Applied Biosystems, Foster City USA). To the RNA-solution 5 µL 10x DNase-buffer and 1 µL rDNase 1 were added and components were carefully mixed before incubation at 37°C for 20-30 min. The reaction was stopped by addition of 5 µL DNase inactivation reagent and incubated at room temperature for 2 min followed by centrifugation at 10,000 g for 1.5 min. The supernatant containing the RNA was transferred in a fresh RNase-free tube. The RNA-content was determined by measuring on a ND-1000 nanodrop-photometer (Thermo scientific) (wavelength 260 nm). To assess the purity of the isolated RNA, the ratio 260/260nm was determined as well, to show possible (large amounts of) protein contaminations, since proteins tend to absorb at 280nm.

2.11.2 cDNA-synthesis: Reverse transcription

For the use in analysis (q-RT-PCR) cDNA (= complementary DNA) was needed, therefore the RNA was reverse transcribed. The SuperScript™ III First-Strand synthesis kit (Invitrogen, Carlsbad USA) was used. cDNA is a DNA reverse transcribed from RNA and the responsible enzyme is therefore called reverse transcriptase (RT). The enzyme used in this work (Superscript III RT) origins from the retrovirus Moloney Murine Leukemia Virus (M-MLV-reverse transcriptase, isolated from *E. coli*) and is engineered in terms of stability.

As a starting point the RT needs a short double-stranded sequence at the 3´end of the messenger-RNA. Using oligo (dT) (a short complementary synthetic oligonucleotide) as

priming method, the 3'-poly(A)tail is hybridized, which can be found in eukaryotic mRNAs. A RNase –Inhibitor (RNase out) is added to prevent enzymatic degradation of the RNA. After addition of dNTPs (deoxy-nucleotide triphosphates), magnesium ions and RT under neutral conditions, a complementary DNA on the mRNA is synthesized. A digestion with RNase H after the cDNA-synthesis improves the sensitivity of the following PCR as it digests RNA on the cDNA:RNAhybrid.

- **Material:**

SuperScript™ III First-Strand synthesis kit (cat # 18080-051, Invitrogen, Carlsbad USA) containing following components that have been used:

- Oligo(dT)12-18 (0.5 µg/µL)
- 10X RT buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]
- 25 mM $MgCl_2$
- 0.1 M DTT
- 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
- SuperScript. II RT (50 units/µL)
- RNaseOUT [Recombinant Ribonuclease Inhibitor (40 units/µL)]
- *E. coli* RNase H (2 units/µL)
- DEPC-treated water

- **Procedure:**

The required volume for an amount of 2.5 µg RNA was calculated and the RNA diluted in DEPC-water. To 11 µL of this solution 1 µL oligo dT 18 and 1µL dNTPs were added. The mixed components were incubated for 5 min at 65°C and then cooled on ice and spun down.

Following components were added:
2 µL 10x RT buffer
4 µL 25 mM $MgCl_2$
2 µL 0.1 M DTT
1 µL RNaseOUT (40 U/µL)
1 µL SuperScript™ III RT (200 U/µL)

Then the mix was incubated at 50°C for 50 min, and inactivated at 85°C for 5 min afterwards The tubes were placed on ice and spun down. 1 µL RNase H was added and incubated at 37°C for 20 min. The cDNA-solution was purified with the mi-PCR-purification kit (Metabion) (see 2.5.6) and the DNA was taken up in 50 µL Tris/Cl (10 mM, pH8).

2.11.3 Isolation of DNA (DNeasy Blood & Tissue kit)

The DNA-Isolation with DNeasy Blood & Tissue kit (cat # 69504, QIAGEN, Hilden Germany) is based on the use of a guanidinium-detergent (guanidinium-chloride) after enzymatic lysis (lyticase) of the yeast cells in a sorbitol-buffer. The spheroplasts are treated with proteinase K to re-move proteins and subsequently treated with the guanidinium-detergent, which destroys the RNA and assures a selective preparation of the genomic DNA. The purification of the DNA is provided by a silica-based column system. The DNA is eluted in Tris/Cl (10mM, pH8) (Ligozzi and Fontana 2003).

- **Material:**

see DNeasy Blood & Tissue kit (cat # 69504, QIAGEN, Hilden Germany)
additionally:
Ethanol (96–100%)
Lysis buffer (1 M sorbitol; 100 mM sodium EDTA; 14 mM β-mercaptoethanol)
Lyticase (Sigma-Aldrich St. Louis Missouri USA #4025-50KU, yeast-lysing enzyme, 3,400 Units, → diluted 1:10 in lysis buffer)

- **Procedure:**

DNA-Isolation was performed with the DNeasy Blood & Tissue Kit (QIAGEN). An overnight culture was prepared. 5 mL YPD were inoculated with a clone picked from a selective plate and incubated at 28°C and shaking. The cells of 2 mL culture were spun down by centri-fugation (10 min, 7,500 rpm), the supernatant was discarded and 600 µL lysis buffer were added to the cells as well as 100 µL of lyticase (340 Units) and the mixture was incubated for 45 min at 30°C and shaking (600 rpm). After centrifugation for 10 min at 300x g, the supernatant was discarded, buffer ATL was added and the pellet was resuspended by vortexing. Addition of 20 µL proteinase K was followed by another vortex step and incubation at 56°C for 45 min. 4 µL RNase A was added and the reaction was performed by incubation at 37°C for 1h. The mix was vortexed for 15 sec and 200 µL buffer AL. After vortexing 200µL ethanol were added and the solution was transferred to a column on a collection tube. After a short incubation the DNA was bound to the column by centrifugation at 8,000 rpm for 1 min. The flow-through was discarded and the column was placed on a fresh collection tube. 500 µL buffer AW1 were pipetted onto the column and centrifugation was performed as above. The same procedure was done with buffer AW2 followed by centrifugation at full speed for 3 min. The flow-through was discarded and the column was placed on a fresh 1.5 mL micro-centrifuge tube. The DNA was eluted with 200 µL Tris/Cl (pH 8, 10 mM) in two steps (100 µL each). The concentration was determined by measuring the OD_{260} with the nanodrop photometer (ND-1000 Nanodrop-photometer, Thermo Scientific).

An aliquot (5µL) was loaded on an agarose gel and gel electrophoresis was performed to check the DNA quality. The DNA-solution was used for the real time reverse PCR to determine the gene copy number.

2.12 DNA amplification by polymerase chain reaction (PCR)

2.12.1 Qualitative PCR

DNA can easily be amplified by the polymerase chain reaction. It is a very sensitive method so that smallest amounts of DNA can be copied multiplicative in short time periods. For this process several components are needed: The target DNA (template) to be amplified, the nucleotides (the dNTPs), the DNA-polymerase II (which provides the elongation of the nucleotides to longer molecule strains), the primers (small pieces of DNA of known sequence that determine the start and stop position of the amplification process on the matrix-strand). The technique was published in 1987 by Mullis and Faloons and is based on three cylces which are repeated.

The cycles and temperatures following a standard protocol are as follows:

The first cycle is the denaturation-step: A mix of the components described above in a suitable buffer is initially heated to ~ 95°C (in this work 94°C) to denature the double-stranded DNA-template in order to make it accessible for the primers. During the following annealing-step the temperature is lowered (to e.g. 55°C) to allow the primers to anneal to the matrix-strain on the position with the complementary sequence. After this hybridization the polymerase can start to synthesise the complementary primer-labelled sequence of the template-DNA in 5´→3' direction. To reach a high yield the optimal working temperature of the polymerase of 72°C is set during this elongation-step. Unspecific binding shall also be minimized at this temperature. These three cycles are repeated again and again. This leads, due to the exponential amplification, to approximately 1 million of molecules after about 20 cycles, from only one specific DNA-fragment. The polymerase mostly used for PCR is a thermostable Taq-polymerase isolated from the thermophilic bacteria *Thermus aquaticus (*Saiki et al. 1988). Primers are synthetically produced oligo nucleotides, which are complementary to the 3´ - or the 5´- end of the template, and can additionally bear restriction sites for subsequent cloning strategies.

2.12.2 Quantitative real time polymerase chain reaction (q-RT_PCR)

The q-RT-PCR is a widely used method for quantification of mRNA levels because of its high sensitivity and specificity, good reproducibility and wide dynamic quantification range (M. Tevfik Dorak "Real time PCR", 2006 chapter 3 by M. W. Pfaffl). The real time PCR gives the possibility to detect and measure the PCR products as they are accumulating because they are bound by a fluorescence dye. The product is measured in the exponential phase of the amplification, when it is most efficient and least affected by reaction-limiting conditions (Ginzinger 2002; Walker 2002). A fluorescence signal threshold (cycle threshold = Ct) is determined at which point a comparison of all the samples becomes possible (Ginzinger 2002). The fewer cycles are necessary to exceed the defined Ct, the greater is the number of target sequence in the sample (Higuchi 1993). In this work, the SYBRgreen-detection system was used. The fluorescent dye Sybr green I (excitation max 494 nm, emission max 521 nm, Jahnson "Gentechnische Methoden", 4.Auflage Spek-trum) binds to double stranded DNA of all sorts. During the polymerase chain reaction the target sequence is amplified and the fluorescence is increasing with every cycle. As the increase of the intensity of fluorescence is proportional to the amount of built double-stranded PCR-product the amplification can be observed in real time. The amounts were determined by relative quantification with external standards. To balance variations the data were normalised by parallel determination of the reference gene actin.

- **Reference gene actin *ACT1*:**

The data have to be normalised by a reference gene, to overcome variations caused by differences in different amounts of initial sample, sample preparation (pipetting, etc..), RNA/DNA-extraction, Reverse transcription (cDNA-synthesis), and variations between different qRT-PCR-runs and therefore resulting efficiency differences (Roche Technical note No. LC 15/2002 "Selection of Housekeeping genes", Neuvians, 2005). A gene sequence had to be chosen that was contained in all samples and as far as possible constant in expression and unaffected by experimental conditions (Heid 1996, Neuvians 2005). The housekeeping gene β-actin was used as a reference gene for normalisation. Beta-actin is a cell-structure element found in all eukaryotes. It encodes a ubiquitous cytoskeleton protein (Bustin 2000). Similar to the target gene a standard curve was generated for the actin gene. For each target gene measure point, an actin-value was determined. The data were normalised by dividing the amount of the target gene by the amount of the reference gene actin (Roche Technical note No. LC 15/2002 "Selection of Housekeeping genes").

- **Material:**

Rotor-Gene 6000™ (Corbett Lifesciences, Australia)

2x SensiMix Plus SYBR (QT-605-02/05)

HQ-water

0.1 mL PCR tubes 4 strips GX-18-3001-002 (genXpress)

- **Standards for the calibration curve:**

The PCR-product of the sample-sequence from plasmid DNA was used as calibration standard. The primers were designed with appropriate software to eliminate complications such as primer-dimers and secondary-structures (Sigma Aldrich Sybr green quantitative rt-pcr kit, technical bulletin).

- **Primer-design:**

The designed primers were chosen under consideration of following aspects (as far as possible):

  - The range of the melting temperature (T_m) (between 58-60°C)
  - The length of the primers (19-24 bps)
  - The length of the built PCR-amplicon (100-200 bps)
  - The content of the bases guanine and cytosine (about 45-55%)
  - Status: standard desalting purified

(Quantitative RT-PCR Protocol SYBR Green I, Schnable lab, Iowa state university, USA)

- **Primer for the qRT-PCR:**

Table 6: QRT-PCR primer: sequences and characteristics

primer	sequence	length (bp)	Tm (°C)	GC-content (%)
HC fw	5’ GC ACA CCT TCC CGG CTG 3’	17	61	70.6
HC rv	5’ GAT TCA CGT TGC AGA TGT AGG 3’	21	59	38.1
LC fw	5' CAA AGT ACA GTG GAA GGT GGA TAA 3'	24	62	41.7
LC rv	5' CTT TGT GTT TCT CGT AGT CTG C 3'	22	60	45.5
actin fw	5’ CCT GAG GCT TTG TTC CAC CCA TCT 3’	24	61	54.2
actin rv	5’ GGA ACA TAG TAG TAC CAC CGG ACA TAA CGA 3’	30	61	46.7

primer-concentration used in PCR: 10 pmol

- **The sequences: (utilized primer sequences are underlined)**

- 3H6_HC-sequence:

ATGAATTCGTCCAACTGCAGCAGTCTGGACCTGAACTAGTGAAGACTGGGGCTT
CAGTGAAGATTTCCTGCAAGGCTTCTGGTTACTCATTCACTGATTACTTCATGCA
CTGGGTCAAGCAGAGCCATGGAAAGAGCCTTGACTGGATTGGATATATTAATTG
TTACACTGGTGCTACTAACTACAGCCAGAAATTCAAGGGCAAGGCCACATTTACT
GTAGACACATCCTCCAACACAGCCTACATGCAGTTCAACAGCCTGACATCTGAG
GACTCTGCGGTCTATTACTGTGCAAGAACGTCAATTGGGTACGGTAGTAGCCCC
CCCTTTCCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAGCCTCCACC
AAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGG
CACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGG
TGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGT**GCACACCTTCCCGGCTGTC**
CTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAG
CAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACA
CCAAGGTGGACAAGAAAGTTGA

- 3H6_LC-sequence:

ATGGAATTCGAAACAACTGTGACCCAGTCTCCAGCATCCCTGTCCATGTCTATAG
GAGAAAAAGTCACCATCAGATGCATAACCAGCACTGATATTGATGATGATATGAA
CTGGTATCAGAGAAGCCAGGGGAACCTCCTAGGCTCCTTATTTCAGATGGCAAT
ACTCTTCGTCCTGGAGTCCCGTCCCGATTCTCCAGCAGTGGCTATGGTACAGAT
TTCGTTTTTACAATTGAAAACATGCTCTCAGAAGATGTTGCAGATTACTACTGTTT
GCAAAGTGATAACTTGCCGTACACGTTCGGAGGGGGGACCAACCTGGAAATAAA
ACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTT
GAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAG
GC**CAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGG**
AGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCAC
CCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAA
GTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGA
GTGTTAG

- Actin (1085 bp):

ATGTGTAAGGCCGGATACGCCGGAGACGACGCCCCACACACAGTGTTCCCATC
GGTCGTAGGTAGACCAAGACACCAAGGTGTCATGGTCGGTATGGGTCAAAAGG
ACTCCTTCGTCGGTGACGAGGCTCAATCCAAGAGAGGTATCTTGACCTTGAGAT
ACCCAATCGAGCACGGTATCGTCACTAACTGGGACGATATGGAAAAGATCTGGC
ACCACACCTTCTACAACGAGTTGCGTCTGGCCCCAGAAGAGCACCCAGTTCTTT
TGACTGAGGCTCCAATGAACCCAAAGTCCAACAGAGAGAAGATGACCCAAATCA
TGTTCGAGACTTTCAACGTTCCAGCCTTCTACGTTTCTATTCAGGCCGTTTTGTC
CCTGTACGCTTCCGGTAGAACCACTGGTATCGTTTTGGACTCTGGTGACGGTGT
TACCCACGTTGTCCCAATTTATGCCGGTTTCTCCTTACCACACGCTATTTTGCGT
ATCGACTTGGCCGGTAGAGATTTGACCGACTACTTGATGAAGATCTTGTCTGAG
CGTGGTTACACTTTTTCTACCTCTGCTGAGAGAGAAATCGTCCGTGACATCAAGG
AGAAGCTTTGTTACGTTGCTCTTGACTTTGACCAGGAATTGCAAACTTCTTCTCAA
TCTTCATCCATTGAGAAGTCTTACGAGTTGCCAGATGGCCAAGTTATCACTATCG
GTAACGAGAGATTCAGAGCT**CCTGAGGCTTTGTTCCACCCATCTGTACTTGGCC**
TTGAGGCTTCTGGTATCGACCAAACCACTTACAACTCCATCATGAAGTGTGATG
TTGATGTTCGTAAGGAACTCTACAGTAACATCGTTATGTCCGGTGGTACTACTA
TGTTCCCAGGTATTGCTGAGCGTATGCAAAAGGAGCTTACTGCCTTGGCTCCAT
CTTCGATGAAGGTCAAGATTTCTGCTCCACCAGAAAGAAAGTACTCCGTATGGAT
CGGTGGTTCTATCCTCGCTTCTTTGGGTACTTTCCAACAAATGTGGATCTCAAAG
CAAGAGTACGACGAATCTGGACCATCCATTGTGCACCTCAAGTGTTTCTAAGT

- **Design of the standard:**

The heavy chain sequence was amplified from the plasmid pPKTEF_HC by PCR, the light chain sequence from pPKTEF_LC (sequences (3H6_HC, 3H6_LC) see above). Primer see Table 6.

- **Actin- standard:**

The template was amplified from *P. pastoris* X-33 genomic DNA by PCR. Sequence: see above, primer: see Table 6.

- **PCR-Conditions:**

Hotstart at 94°C was performed. The polymerase requires this temperature for its activation and hotstart as well as touchdown PCR reduces unspecific PCR-product. During touchdown PCR, the annealing temperature is decreased in each step from a higher starting point down some degrees below the primers melting point.

Table 7: Components and volumes of a preparation for PCR of the calibration standard (LC and HC)

50 µL preparation	
5 µL	10x buffer (Biotools)
5 µL	$MgCl_2$ (Biotools)
1 µL	10mM dNTPs
1 µL	fw primer
1 µL	rv primer
34 µL	ddH_2O
1 µL	Taq-polymerase (Biotools)
2 µL	template (plasmid DNA)

Table 8: Components and volumes of a preparation for the PCR for the calibration-standard (actin):

50µL preparation	
5µL	10xbuffer
4µL	Mg^{2+}
1.5 µL	dNTPs (10mM)
1.5 µL	fw primer
1.5 µL	rv primer
1µL	Taq-polymerase (Biotools)
30.5µL	ddH2O
5µL	template (genomic DNA)

The PCR was performed under following conditions:

Table 9: Conditions of the PCR for the production of the qRT-PCR standards (LC, HC):

step	temperature	time	cycles
1	94°C	5 min	
2	94°C	15 sec	
3	60°C	15 sec	
4	72°C	15 sec	
5	go to step 2		25 x
6	72°C	10 min	
7	20°C	1 sec	
8	End		

Table 10: Conditions of the PCR for the production of the actin-standard:

	touchdown PCR touchdown -1°C/cycle, 10cycles		
step	temperature	time	cycles
1	94°C	5min	
2	94°C	30sec	
3	60°C	30sec	T-1°C/cyle
4	72°C	20sec	
5	Go to 2		10x
6	94°C	30sec	
7	50°C	30sec	
8	72°C	30sec	
9	Go to 6		15x
10	72°C	10min	
11	10°C	1min	
12	END		

The PCR-product was purified by gel-purification (mi-Gel Extraction Kit, metabion, see 2.5.3). The purified DNA was taken up in Tris/Cl 10 mM pH8 and the concentration (ng/µL) was determined by measuring OD_{260nm} on the ND-1000 Nanodrop-photometer, Thermo Scientific.

- **Calculation of the standards:**

Based on the concentration and the length of the fragment, the copies/µL for the standards were calculated.

1 bp ~ 650 Da

fragment length (bp)* 650= MW g/mol

Avogadro-constant (*NA* =6,022 * 10^{23} 1/mol) * MW g/mol = copies/g

(copies/g : 10^9) * conc (ng/µL) = copies/µL

The PCR-product was diluted to a concentration of 1*10^9 copies/µL.

This was the stock solution and a dilution series was produced (1:10 dilutions) to final concentrations of 10^8, 10^7,10^6, 10^5, 10^4, 10^3 copies/µL

- **qRT-PCR Samples:**

Either the amount of 2.5 µg reverse transcribed RNA (cDNA) or 4 ng genomic DNA were used as template.

- **qRT-PCR: The procedure:**

The metallic rack was put on ice and the tubes were placed on it to pre-cool them.

The mastermix was prepared:

Table 11: Components and volumes of a mastermix- preparation for the qRT-PCR:

one reaction (10 µL preparation → 9 µL mastermix)	
0.25 µL	primer fw (10 pM)
0.25 µL	primer rv (10 pM)
5 µL	reaction buffer (2x SensiMix Plus SYBR)
3.5 µL	HQ-water (= high quality)

The final reaction volume was 10 µL. 9 µL of the master mix were pipetted into the tubes.
1 µL of the diluted sample or the calibration standard respectively was added. Each sample was measured in quadruple, each standard concentration was determined in double. As negative controls two samples containing HQ-treated water instead of template were prepared. The tubes were closed and placed into the rotor of the Rotor Gene™ PCR-machine. The continous spinning of the tubes (400 rpm) ensures that the samples stay at the base of the tube, reduces air-bubbles, condensation and variation.

- **qRT-PCR: The conditions:**

Table 12: Conditions of the qRT-PCR:

step	temperature	time	cycles
hotstart	95°C	10 min	1
denaturation	95°C	15 sec	45
primer-annealing	60°C	20 sec	45
elongation	72°C	15 sec	45

The acquisition of the signal was detected at the 60°C step during each cycle.

- **Melting curve acquisition:**

range: 65-99°C

- rising by 0.5°C each step
- wait for 0 sec on first step
- wait for 2 sec on each step afterward

- **Melting curve:**

SYBR-Green binds every kind of double stranded DNA in a sequence-independent way (Lekanne Deprez 2001). As a control for specificity, a melting curve was generated after PCR-amplification for each PCR-product. The fluorescence was plotted as a function of temperature. By increasing the temperature above the melting temperature (Tm) and simultaneous measuring of the fluorescence, a graph can be achieved. The nucleotide composition is an important factor for the Tm, therefore the specific signal from the built target product can be identified. The characteristic melting peak of the amplicon can be distinguished from unspecific products, which have a peak at a lower temperature (Bustin 2000). A correct curve comprises of only one clearly separated peak. Additional peaks for one product are an indication for primer dimers or unspecific PCR-product (artefacts).

- **Analysis:**

The raw data were analysed with Rotor-Gene^{TM} 6000 system software version 1.7 (Build75) and exported to MS Excel for further analysis (calculation of copy number). A relative quantification method with an external standard-curve was used. The results were given as a ratio of the calculated gene copies of the target gene to the ones of the reference gene (the housekeeping gene actin). Standard curves were used to obtain the concentration of the target and the reference gene. This method corrects for factors influencing the q-RT-PCR. It balances variations that would adulterate the results. This could be variations relating to the initial amount of sample as well as nucleic acid recovery. The starting amount of sample is mainly affected by variations in cDNA-synthesis efficiency. Degradation because of the instability of the RNA and the quality of the RNA/DNA are also critical points. Furthermore variations due to preparation errors like loading or pipetting errors have an influence on the results (Roche, Technical note No. LC 13/2001). The software created a standard curve of the fluorescence signals from the sample standards as well as from the reference standards. A threshold was defined at a point in which the signal generated from a sample was significantly greater than the background fluorescence. The cycle threshold (Ct) was the number of PCR-cycles required to exceed this threshold in fluorescence intensity (Gin-zinger 2002). Based on the determined standard equation (fluorescent threshold values Ct correlate linearly with relative DNA copy numbers) and the defined Ct, the copies are calculated by the soft-ware (Heid 1996). The standard curve and the separate curves of the quadruple determination were checked and outliers were not used in calculation. The ratio of target gene copies to reference gene copies was calculated with Microsoft Excel and is shown in the diagrams (see 3.4.1).

2.13 Quantitative Southern blot

2.13.1 Preparations

2.13.1.1 DNA-Isolation (QIAGEN genomic tips)

Isolated genomic DNA was used as sample. The DNA was isolated with QIAGEN genomic tips 100G. The isolation and purification of genomic DNA is provided by anion-exchange. First the cells are lysed by enzymatical treatment (lyticase) in SCED-buffer, which denatures impurities like proteins. Under the given pH-and low-salt-concentration conditions the DNA is bound to the anion-exchange resin in the spin-column (based on the contrarious charges of the DNA-backbone and the DEAE = DiEthylAminoEthane groups on the resin-surface). The DNA remains on the column over a wide range of salt concentrations while undesired substances as RNA, protein, carbohydrates and small metabolites are removed by washing steps with medium-salt buffers. The DNA is finally eluted in Tris/Cl (10mM, pH8).

- **Material:**

QIAGEN genomic tips 100 G containing following solutions:

- QBT: 43.83 g NaCl, 10.46 g MOPS (free acid) dissolved in 800 mL distilled water, pH 7.0 (adjusted with NaOH) addition of 150 mL pure isopropanol and 15 mL 10% Triton X-100 solution → volume adjusted to 1 liter with distilled water.
- QC: 58.44 g NaCl, 10.46 g MOPS (free acid) dissolved in 800 mL distilled water, pH 7.0 (adjusted with NaOH) addition of 150 mL pure isopropanol,
  → volume adjusted to 1 liter with distilled water.
- QF: 73.05 g NaCl and 6.06 g Tris base dissolved in 800 mL distilled water,
  pH 8.5 (adjusted with HCl), addition of 150 mL pure isopropanol, addition of 150 mL pure isopropanol, → volume adjusted to 1 liter with distilled water.
    - YPD-media
    - ddH_2O
    - SCED-buffer (final conc.: 1M sorbitol, 10 mM sodium-citrate pH= 7.5, 10 mM EDTA, 10 mM DTT) freshly prepared
    - lyticase (10,000 U/mL)
    - 1% SDS in water
    - 5 M potassium acetate (pH 8.9)

- **Procedure:**

An overnight culture was prepared with 20 mL YPD-media and the chosen clones (picked with a sterile tooth pick) (30°C, under shaking conditions). The cells were spun down by centrifugation (4,200x g, 10 min), washed with 5 mL ddH_2O and again centrifuged under same conditions. Next step was the resuspension of the cells in 4 mL SCED-buffer and addition of 50 µL lyticase followed by incubation at 37°C for about 50 min.2 mL 1% SDS in water was added, gently mixed and stored on ice for 5 min. After addition of 1.5 mL 5 M potassium acetate the mix was vortexed and centrifuged for 5-10 min at 4°C. The culture supernatant was transferred into fresh tubes and vortexed. The column (100 G) was placed on a collection tube and equilibrated with 4 mL of QBT-buffer. The culture supernatant was transferred to the column. After flow-through by gravity flow the column was washed twice with 7.5 mL of buffer QC (flow-through by gravity). The DNA was eluted with 5 mL pre-warmed (50°C) QF-buffer collected in a fresh 50 mL falcon tube. 3.5 mL isopropanol (room temperature) were added to precipitate the DNA and the procedure was supported by inverting the tube 10 to 20 times. The suspension was portioned to 2 mL tubes and centrifuged at 4°C for 20 min at 10,000 x g. The supernatant was discarded and the pellet was washed with 2 mL of 70% ethanol, vortexed and centrifuged at full speed for 10 min. After careful removal of the supernatant the pellet was air-dried for 10 min and resuspended in overall 650 µL Tris/Cl (10mM, pH 8) per sample. The resuspension was realised by shaking at 55°C for 1h at 600 rpm. The DNA was quantified by photometry on the nanodrop-photometer and by determination using the Hoechst-dye 33258.

2.13.1.2 Quantification of the isolated DNA with HOECHST 33258

The quantification of the total DNA is carried out with the fluorimetric dye bisbenzimidazol (Hoechst 33258). Bisbenzimidazol becomes attached to DNA without reacting with it. After isolation of the DNA via QIAGEN genomic-tip G100, the sample is quantified by fluorescence measurement: The reaction is excited at 365nm and emits light at 458nm where the measurement is taking place. The fluorescence of Hoechst 33258 increases in the presence of DNA, binding specifically and quantitatively. It is hardly affected by common laboratory reagents (Paul and Myers 1982). The DNA content of the samples is determined according to a calf-thymus DNA standard curve.

- Material:

- HITACHI F-2000 Spectrofluorometer
- Fluorescence quartz cuvette
- Fluorometric dye Hoechst 33258 (bisbenzimidazolderivate) (10 mg/mL)
- Calf thymus DNA (stock solution 200 µg/mL, D 3664 SIGMA) as standard solution

- Dilution buffer: 1xTNE (Tris, NaCl, EDTA):
  10x TNE: 1M Tris, 10 mM EDTA, 1M NaCl, pH 7.4 adjusted with HCl
  the buffer was used as 1xTNE (1:10 dilution)
- Fluorescence reagent (freshly prepared before use): 100 mL dilution buffer and 10 µL of Hoechst 33258

- Standard:

The stock-solution of the calf-thymus DNA was diluted in HQ-water to a dilution-series of 1.25; 2.5; 5; 10 µg/mL.

- Samples:

The DNA-samples were diluted 1:10 in HQ-water in order to fit the calibration range.

- **Measurement:**

First the standards were measured in ascending order beginning with a 0 µg/mL reference point by measuring the fluorescence reagent without calf thymus DNA. 1.9 mL fluorescence reagent were provided in a fluorescence quartz cuvette and 100 µL of the DNA-standard or diluted DNA-sample respectively, were added and mixed with the reagent by pipetting. The cuvette was placed in the bracket and measuring of fluorescence measuring (excitation 365 nm, emission 460 nm) was started. Based on the before generated standard curve and the sample dilution the spectrofluorometer-software was calculating the concentration of the given sample in ng/mL.

2.13.2 Quantitative Southern Blot (alkaline transfer)- The procedure

The Southern Blot is named after E. M. Southern who invented this method at Edinburgh University in the 1970ies. It is a DNA-blotting technique to specifically locate particular DNA-sequences from complex mixtures, such as a single gene within an entire genome. It comprises the separation of DNA-restriction fragments according to their size by agarose-gel electrophoresis and the sub-sequent transfer of the bands onto a nylon membrane followed by a hybridization-process with a specific probe and finally a detection step. After the gel ectrophoresis, the gel undergoes an alkaline treatment to denature the DNA to get single strands. The transfer of the DNA to the membrane is provided only by capillary interactions, from the paper soaking the buffer and the DNA-fragments are covalently bound by the nylon membrane. A neutralizing step is following the blotting procedure and the band-pattern is fixed on the membrane by heat treatment. The next step is to hybridize the blot with a specific DIG-labelled single-stranded DNA-probe which binds the complementary target DNA sequence immobilized on the membrane. The detection was performed with the nonradio-

active DIG-system (= Digoxygenin: a steroid extracted from plants (*Digitalis purpurea and D. lanata*)). An AP (alkaline phosphatase) conjugate of an anti-DIG-antibody was directed against the DIG-labelled probe. The DIG was incorporated to the probe-sequence by PCR with DIG-dUTPs. The enzyme (alkaline phosphatase) activates the chemiluminescent substrate CDP-star (NEB) and during the enzyme reaction a light signal is produced. The labelled bands were visualised by measuring the light signal with the Lumi Imager (Boehringer Mannheim).

- **CDP-Star:**

CDP [Disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tri-cyclo [3.3.1.13,7]decan}-4-yl) phenyl phosphate] is a sensitive and fast chemiluminescent substrate for alkaline phosphatise. The principle of the CDP-reaction: The dephosphorylation of CDP-Star by the enzyme alkaline phosphatase leads to the production of the metastable dioxetane phenolate anion. This intermediate decomposes and emits light (h*ν) at 466 nm.

- **Material:**

- Alkali transfer buffer (0.4M NaOH, 1M NaCl)
- Neutralising buffer (0.5M Tris*HCl pH 7.2, 1M NaCl)
- 20xSSC stock solution (3M NaCl, 0.3M NaAc*3H_2O)

- 2xSSC, 0.1% SDS	20xSSC	50mL
	20% SDS	2.5mL
	ddH_2O	447.5mL
- 0.5xSSC, 0.1% SDS	20x SSC	12.5mL
	20% SDS	2.5mL
	ddH_2O	485mL
- Washing buffer:	Add 0.3% Tween20 to Maleic acid buffer, not autoclaved	
- Maleic acid buffer	maleic acid (Disodiumsalt – Dihydrate)	0.1 M
	NaCl	0.15 M
	pH adjusted with HCl, sterilized by autoclaving	

- 10x Blocking Reagent stock solution

  10 g Blocking Reagent were added to 100 mL Maleic acid buffer.

  It was placed on a stir plate and heated to 60°C for approximately 1hour.

- Blocking buffer: Blocking Reagent stock solution was diluted in Maleic acid buffer (1:10)
- High SDS concentration hybridization buffer 500mL

100% formamide	250mL
20x SSC	124.5mL
1 M sodium-phosphate, pH 7.0	25 mL

10 % blocking solution	100 mL
10 % N-lauroylsarcosine	5 mL

The solution was poured onto 35 g SDS and heated while stirring to dissolve the SDS.

- N-lauroylsarcosine stock solution (10% in ddH2O, stirred for 1 hour, filtered through a 0.2 – 0.45 μm membrane)
- Sodium-Phosphate-buffer: 1M pH 7.0

1 M NaH_2PO_4	34.5g / 250mL
1 M Na_2HPO_4 (*$12H_2O$)	89.5g / 250mL

NaH_2PO_4 was provided and the pH was adjusted by adding Na_2HPO_4

- Detection buffer (100mM Tris*HCl pH 9.5, 100mM NaCl)
- Substrate: CDP-Star (Tropix) (NEB)

- **Procedure:**

The required amount of plasmid for a solution containing one copy of the target gene was calculated: The fragment length of the LC standard was 665 bp and of the HC standard 725 bp. The size of the *P. pastoris* genome is approximately 10^7 bp which meant 100%. Accordingly were the standard-fragments LC: $6.65*10^{-3}$ and HC: $7.25*10^{-3}$ % of the genome size. The whole used amount of standard-plasmid in the Southern blot should be 300 ng. The corresponding amount of the target was 0.01995 ng for the light chain and 0.02175 ng for the heavy chain. The LC-fragment were 17.7% of the whole plasmid, the heavy chain fragment was 15.4%.

The required amount was calculated:

Light chain: (0.01995ng*100%)/17.7%= 0.1271 ng = 112 pg

Heavy chain: (0.02175ng*100%)/15.4%= 0.14123 ng = 141 pg

For the restriction digest the standard solutions with the theoretical gene copies 0.5, 1, 2, 3, 4, 6 and 10 were made by using the respective volume of plasmid.

Following components were used for one restriction digest preparation:

Light chain:

Table 13: Components and volumes of a preparation for the SB light chain standard:

15 µL preparation	
1 µL	buffer 2 (NEB)
1 µL	10xBSA
1 µL	EcoRI
1 µL	XbaI
x µl	plasmid DNA
up to 15µL	ddH_2O

Heavy chain:

Table 14: Components and volumes of a preparation for the SB heavy chain standard:

15 µL preparation	
1 µL	buffer 3 (NEB)
1 µL	EcoRI
1 µL	BglI
x µl	plasmid DNA
up to 15µL	ddH_2O

These components were mixed with the DNA (either LC or HC) (the volume was according to the copies the certain standard should have → see gene copies mentioned above). The restriction digest was performed at 37°C over night.

- **Probes:**

The probes were amplified by PCR from plasmid DNA.

Table 15: Components and volumes of a preparation for the SB-probe

50 µL preparation	
5 µL	10x buffer (Biotools)
5 µL	$MgCl_2$ (Biotools)
1 µL	10mM dNTPs
1 µL	fw primer
1 µL	rv primer
35 µL	dH_2O
1 µL	Taq-polymerase (Biotools)
1 µL	template

- **Templates:**

HC: PPKTEF_(3H6) HC, LC: PPKGAP_(3H6)LC

- **Southern-blot primer:**

Table 16: Southern blot-primer: Sequences and characteristics:

primer	sequence	length (bp)	Tm (°C)	GC-content (%)
HC fw	5' GGC TTC TGG TTA CTC ATT CAC 3'	21	59	47.6
HC rv	5' GAT TCA CGT TGC AGA TGT AGG 3'	21	59	38.1
LC fw	5' AGG AGA AAA AGT CAC CAT CAG 3'	21	57	47.6
LC rv	5' CTT TGT GTT TCT CGT AGT CTG C 3'	22	60	45.5

concentration used in PCR: 10 pmol

- **The probe-production:**

The probes were amplified by PCR from plasmid DNA under following conditions:

Table 17: PCR-conditions for the production of the SB-probes

	PCR-Program touchdown		
step	**temperature**	**time**	**cycles**
1	94°C	5 min	
2	94°C	30 sec	
3	57°C	30 sec	
4	-1°C per cycle		
5	72°C	1 min 30 sec	
6	go to 2		**8 x**
7	94°C	30 sec	
8	50°C	30 sec	
9	72°C	1 min 30 sec	
	go to 7		**25x**
10	20°C	1 sec	
11	End		

An aliquot of the generated PCR-product was controlled by electrophoresis. The remaining solution (42 µL) was applied to a preparative agarose gel and separated by electrophoresis for 1h at 100 V. The bands (HC: 725 bp, LC: 665 bp) were cut out and the DNA was purified by gel extraction (gel extraction kit metabion) (see 2.5.3). The purified product was taken up in 30 µL of Tris/Cl 10mM, pH8.

- **Labelling of the probes:**

The probes were labelled with the Digoxygenin-system (DIG). A PCR was performed to incorporate Digoxygenin -11-dUTP by Taq DNA polymerase (PCR DIG Probe Synthesis Mix features a 1:2 DIG-11-dUTP:dTTP ratio). The DIG-mix was used instead of dNTPs. The primer and conditions were the same as above.

Table 18: Components and volumes of a preparation for the PCR for the DIG-labelling of the probes.

50 µL preparation	
5 µL	10x buffer (Biotools)
5 µL	$MgCl_2$ (Biotools)
5 µL	DIG-mix
1 µL	fw primer
1 µL	rv primer
30 µL	HQ-water
1 µL	Taq-polymerase (Biotools)
2 µL	template

As a control step an aliquot of the DNA was determined by agarose gel electrophoresis. The remaining labelled probes (HC, LC 2x 42µL each) were purified by gel extraction (gel extraction kit metabion) (for 1h at 100 V see 2.5.3) and taken up in 2x 50 µL each in Tris/Cl 10mM pH8. The clean probes were quantified by nanodrop-photometer measurement and then stored at -20°C until use.

- **Samples:**

The isolated DNA (genomic tips 100 G) was used for the Southern blot after quantitation (Nanodrop, Hoechst 33258). The samples were diluted to 30 ng/µL. 300 ng were chosen as the amount suitable for the Southern blot, therefore 10 µL of the 30 ng/µL solution were used.

For the restriction digest following compounds were added to the 10 µL of the sample-solution:

Light chain:

Table 19: Components and volumes of a preparation for the restriction digest of the SB-samples

15 µL preparation	
1 µL	buffer 2 (NEB)
1 µL	10x BSA
1 µL	EcoRI
1 µL	XbaI
x µL	plasmid DNA
up to 15µL	ddH_2O

Heavy chain:

Table 20: Components and volumes of a preparation for the restriction digest of the SB-samples

15 µL preparation	
1 µL	buffer 3 (NEB)
1 µL	EcoRI
1 µL	BglI
x µL	plasmid DNA
up to 15µL	ddH_2O

The components were mixed with the DNA (either LC or HC). The volumes were the same as for the standards (see above). The preparations were stored at 37°C over night to ensure a complete digest of the DNA.

2.13.2.1 Separation of DNA with agarose gel electrophoresis

The digested samples and standards (15 µL) were mixed with 2.5 µL Loading dye (6x MBI Fermentas) and applied to a 0.7% agarose gel. The DNA fragments were separated by agarose gel electrophoresis (0.7 % agarose) over night at 30 V. Additionally two markers were applied: 5 µL MassRuler™DNA Ladder Mix, ready to use (Fermentas) and 7 µL DNA-molecular weight marker VII DIG-labelled 0.01 µg/µL (Roche). A photo of the gel was taken and the genomic DNA appeared as a smear. The gel was incubated in the alkaline transfer buffer by shaking at room temperature for 30 min.

2.13.2.2 Blotting procedure

6 Whatman papers, the nylon membrane (Nytron SPC 0.45µm Nylon Transfer Membrane, What-man) and about 10 cm Kleenex paper (all cut in size of the gel) were prepared. The blots were assembled in following order (from bottom to top) in an electrophoresis chamber:

- wick made of Whatman paper (sopped in alkaline transfer buffer)
- 3 Whatman papers
- the gel (upside-down)
- the nylon membrane
- 3 Whatman papers
- ~10 cm Kleenex papers
- Bottle of water for weight and pressure

The blotting was performed by upward capillary transfer. After 5h of blotting at room temperature the slots of the gel were marked on the membrane. The membrane was incubated in neutralizing buffer by shaking for 10 minutes at room temperature. To fix the bands on the membrane, the blot was baked placed on a Whatman paper in a suitable oven for 30 min at 120 °C and stored in a box until it was detected.

2.13.2.3 Hybridization and Detection

- Pre-hybridization and Hybridization:

The high SDS buffer was thawn and tempered to 60°C to solve it. The blot was incubated in 40 mL of the prepared buffer while rotating for 1 hour at 42°C in the hybridization-oven. For the hybridization-procedure the DIG labelled probes were heated for 5-10 min at 99°C and were put immediately on ice to avoid re-hybridization of single stranded probe. The blot was incubated in high SDS buffer enriched with 24 µL of the DIG-labelled probe (the probe-amount should be in the range of 5-25 ng/mL) at 42°C over night in the hybridization oven under shaking conditions.

- Washing procedure and detection of the Southern Blot:

The blot was washed twice in 2xSSC, 0.1% SDS for 5 minutes at room temperature under shaking conditions. Next step was to wash the blot with 0.5x SSC, 0.1% SDS twice in the hybridisation oven at 68°C for 5 minutes. The blot was rinsed in a special washing buffer (DIG-System) for 1 minute at room temperature and then blocked in 40 mL blocking buffer for 30 minutes. In another 40 mL of blocking buffer 4 µL Anti-DIG-Fab-AP antibody (dilution 1:10,000) were added and the blot was incubated by shaking for 30 minutes. The antibody solution was discarded and the blot was washed twice in 40 mL washing buffer (DIG-System) for 15 minutes. Incubation in detection buffer for 2 minutes was performed. 400 µL substrate CDP-Star was diluted in 40 mL detection buffer (1:100) and the blot was incubated for 5 minutes always covering the solution with tin foil to keep it dark. The blot was weld in a plastic bag and the bands visualised by chemiluminescence with the Lumi-Imager (Roche).

3 Experiments and Results

3.1 The cloning

3.1.1 Preparation of the vectors

Table 21: Short names of the constructed vectors

name	short name	insert	antibiotic resistance	enzyme (linearisation)
pGAPZαA_(3H6)LC	pGAPZ αA_LC	3H6 light chain	Zeocin	PagI or BspHI
pPUZZLEKanTEF_(3H6)_HC	pPKTEF_HC	3H6 Fd-fragment [+)]	Kanamycin/G418	AscI
pPUZZLEKanGAP_(3H6)_HC	pPKGAP_HC	3H6 Fd-fragment	Kanamycin/G418	AscI

[+)] Fd-fragment: $C_H1 + V_H$

3.1.2 The vectors

In this work the biotechnological production of antibody fragments (Fab) in the yeast *P. pastoris* was in the focus. The heavy and light chain of the chimeric human/mouse anti-idiotypic antibody 3H6 Fab (first expressed in *P. pastoris* by Gach et al., 2007) have been co-expressed from separate vectors, driven by 2 different constitutive promoters (LC: P_{GAP}, HC: either P_{GAP} or P_{TEF}).

The construction of the vectors and the used promoters are described in the following.

An overview of the cloning steps is given in Fig. 10. The sequences of the inserts, which have been integrated into the genome by homologous recombination, are shown in Materials and Methods (see 2.12.2).

3.1.2.1 Overview: Construction of the vectors

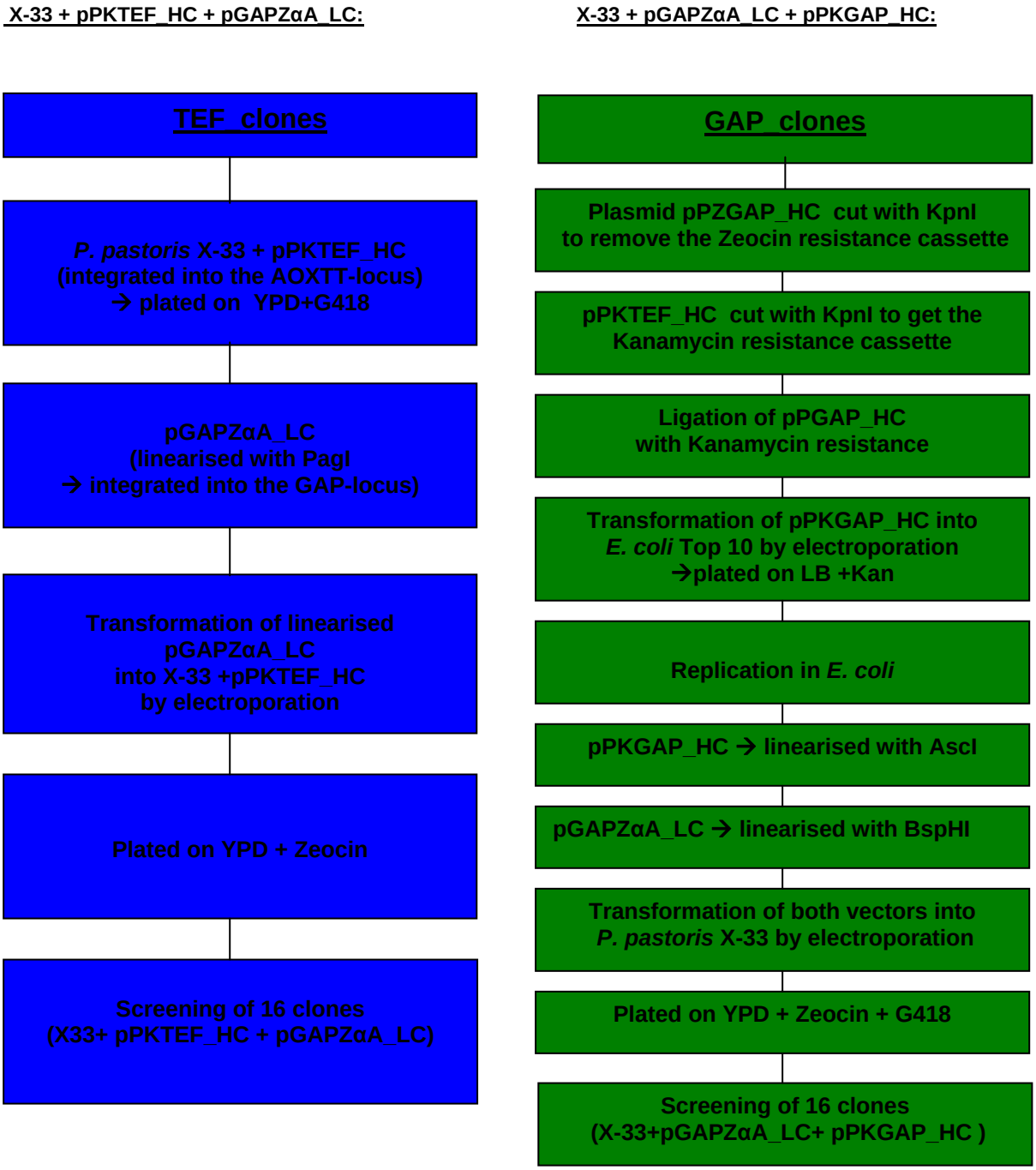

Fig. 10: Overview of the cloning strategy of the expression vectors

3.1.2.2 The construction of the vectors

- **Vector: pGAPZαA_LC**

The 3H6_Light chain was inserted into pGAPZαA as described in Dragosits et al. 2009.

- **Vector for the HC (TEF-promoter) pPKTEF_HC**

For the TEF-clones the vector pPKTEF_HC-vector was used (3H6-heavy chain inserted into the pPKTEF-vector by restriction with Sbf1 and Sfi1). The pPUZZLE vector is described in Stadlmayr et al. 2010.

- **Vector for the HC (GAP-promoter) pPKGAP_HC**

The vector for the GAP-clones was constructed by exchange of the resistance gene. The Zeocin-cassette was removed with KpnI and the Kanamycin resistance cassette was integrated into the vector pPGAPHC (3H6-HC inserted with Sbf1, Sfi1).

- **Vector : pGAPZαA_Fab (=PK, production clone)**

As a control a construct with the commercial available vector pGAPZαA was used in which the 3H6-Fab sequence has been integrated (Baumann et al. 2007; Dragosits et al. 2009).
Here the expression cassette of the light chain and heavy chain were combined on one plasmid as concatemers each under control of the GAP promoter. This construct was analysed in parallel as a positive control as well as to find out if there are differences in comparison to the other constructs with the light and heavy chain expression-casettes placed on two different plasmids.

3.1.3 Construction of *P. pastoris* expression strains

3.1.3.1 GAP-clones

The completed vector pPKGAP_HC was transformed into *E. coli* Top 10 to replicate it. This was done by electroporation. The selection of the transformed clones was done by plating on LB + Kanamycin (final concentration: 50 µg/mL). The two GAP-vectors were integrated into the *P. pastoris* X-33 genome by homologous recombination. The vector pPKGAP_HC was linearised with AscI to enable the integration into the AOXTT-locus, the linearisation of the light chain vector (pGAPZαA_LC) was done by BspHI to integrate it into the GAP-promoter-locus. After the electroporation process positive transformants have been selected by plating on YPD+ Zeocin and G418. After incubation, 16 clones growing under selection pressure of the 2 antibiotics were selected for testing. An aliquot of these clones was cryo-cultivated for long-time storage.

3.1.3.2 TEF-clones

An aliquot of the cryo-cultivated *P. pastoris* X-33 + vector pPKTEF_HC (integrated into the AOXTT-locus) was plated on YPD + G418. The light chain vector pGAPZαA_LC was integrated to the *P. pastoris* X-33 strain that was already carrying the heavy chain vector, by homologous recombination after linearisation with PagI to integrate it into the GAP-locus. The transformation procedure was done by electroporation. The selection of the transformed clones was done by plating on YPD+ Zeocin. After incubation also 16 clones were selected for testing. An aliquot of these clones was cryo-cultivated for long-time storage.

3.2 The screening

3.2.1 Expression

The selected clones (16 GAP-clones, 16 TEF-clones, 3 x production-clone) were screened in 2 mL BM-media/well in deep well plates at room temperature and under shaking conditions for 48h. (Detailed information about the screening-conditions, pre-culture, glucose-feed, etc. can be found in Materials and Methods, see 2.9). After the incubation, the culture-supernatant was recovered by centrifugation and then used for analysis of the produced protein. The remaining supernatant was stored at -20°C. The yeast wet mass (YWM) was determined by weighing the pellets (which have been separated from the supernatant by centrifugation) of the cultures.

Following clones have been analysed:

Table 22: Description of the clones chosen for analysis and their short names used in the following

clone	short name
X-33+ pPKTEF_HC(AscI) + pGAPZαA_LC (Pag1)	TEF 2-15
X-33+ pGAPZαA_LC (BspHI)+ pPK_GAP_HC(AscI)	GAP 1-16
pGAPZαA_3H6_Fab	PK 1-3

3.3 Determination of the protein level and productivity

P. pastoris is known as an expression system that has the ability to secrete the expressed protein into the culture supernatant as a quite pure product (Lange et al. 2001). Thus the culture supernatant can be directly used without further purification, for the analysis of the protein level.

3.3.1 Determination of the productivity

First of all, the amount of the recombinant protein was determined. ELISA was used to check if the product (Fab) was built and to identify potential differences in the amounts of produced antibody fragments between the constructs and the individual clones. As samples, the diluted supernatants of the cultures were used after 48 hours of incubation in deep well plates at room temperature (for details see Materials and Methods 2.10.4). To overcome differences in product titers due to different final cell densities in-between the separate clones, the results were related to the yeast wet mass (YWM). This means the concentration of built product related to the concentration of the biomass (YWM) after the incubation was calculated ([mg/L product per g/L YWM] after 48h of incubation). This calculated product yield ($y_{P/X}$) was in the following named P (productivity). For the quantification hIgGFab was used as standard. The sandwich-ELISA works after the following principle: After coating with a Fab-specific ahIgG the plates were incubated with the diluted sample, so the built antibody fragments, that are able to bind the pre-coated antibody, can bind. In this experiment this means the intact heterodimeric Fab, the free (unassembled) light chain monomers, light chain dimers and possible degraded light chain fragments. Therefore the signals given by the ELISA are the result of the sum of these antibody fragments. The plates with the bound fragments have been incubated with an alkaline-phosphatase labelled antibody directed against the LC and read at 405 nm. 14 of the TEF-clones (due to the loss of sample 1 and 16), 16 GAP-clones and 3 production clones have been measured in triplicate or duplicate determination.

The productivity P was calculated as follows:

$$P = \frac{\text{average product concentration (mg/L)}}{\text{concentration of yeast wet mass (g/L)}} \quad \text{after 48 hours of incubation}$$

- **ELISA-results of the TEF clones**

Table 23: Yeast wet biomass of the TEF-clones determined after 48h of incubation

	YWM after 48h of 2mL in g	YWM after 48h (g/ L)
TEF 2	0.1482	74.1
TEF 3	0.1489	74.45
TEF 4	0.1576	78.8
TEF 5	0.1497	74.85
TEF 6	0.1523	76.15
TEF 7	0.1498	74.9
TEF 8	0.1644	82.2
TEF 9	0.1491	74.55
TEF 10	0.1595	79.75
TEF 11	0.1451	72.55
TEF 12	0.1446	72.3
TEF 13	0.1868	93.4
TEF 14	0.1545	77.25
TEF 15	0.1534	76.7

Table 24: Determined product-concentration of the TEF-clones (mg/L) per concentration of yeast wet biomass (g/L) after 48h of incubation, pc= product concentration, SEM= standard error of the mean

	1st value	2nd value	3rd value	4th value	5th value	6th value			
	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	average conc.	SEM	P
TEF 2	20.05	21.5	-	-	-	-	**20.77**	0.01	**0.28**
TEF 3	17.94	26.29	-	-	-	-	**22.11**	0.06	**0.3**
TEF 4	45.33	40.58	33.03	37.9	45.33	41.99	**40.69**	0.02	**0.52**
TEF 5	17.99	21.02	17.94	16.56	17.25	15.37	**17.69**	0.01	**0.24**
TEF 6	20.75	24.39	-	-	-	-	**22.57**	0.02	**0.3**
TEF 7	21.42	24.58	-	-	-	-	**23**	0.02	**0.31**
TEF 8	34.73	34.94	26.41	27.44	22.28	20.55	**27.73**	0.03	**0.34**
TEF 9	16	22.31	17.6	17.89	19.32	19.48	**18.77**	0.01	**0.25**
TEF 10	51.43	52.45	53.89	36.66	62.92	54.71	**52.01**	0.04	**0.65**
TEF 11	19.43	24.37	-	-	-	-	**21.9**	0.03	**0.3**
TEF 12	14.5	18.7	14.42	22.28	14.2	15.52	**16.61**	0.02	**0.23**
TEF 13	16.8	23	18.17	-	24.31	24.14	**21.28**	0.02	**0.23**
TEF 14	43.4	47.97	44.49	34.29	47.58	41.88	**43.27**	0.03	**0.56**
TEF 15	16.49	21.08	-	22.04	20.56	18.3	**19.7**	0.01	**0.26**

- **ELISA-results of the GAP_clones**

Table 25: Yeast wet biomass of the GAP-clones determined after 48h of incubation

	YWM after 48h of 2mL in g	YWM after 48h (g/ L)
GAP1	0.1437	71.85
GAP2	0.1467	73.35
GAP3	0.1467	73.35
GAP4	0.1547	77.35
GAP5	0.1768	88.4
GAP6	0.1447	72.35
GAP7	0.1673	83.65
GAP8	0.1497	74.85
GAP9	0.1504	75.2
GAP10	0.1441	72.05
GAP11	0.185	92.5
GAP12	0.1731	86.55
GAP13	0.1784	89.2
GAP14	0.1779	88.95
GAP15	0.1567	78.35
GAP16	0.1607	80.35

Table 26: Determined product-concentration of the GAP-clones (mg/L) per concentration yeast wet biomass (g/L) after 48h of incubation

	1st value	2nd value	3rd value
	pc (mg/L)	pc (mg/L)	pc (mg/L)
GAP1	-	-	-
GAP2	4.37	6.05	5.57
GAP3	15.6	19.14	17.18
GAP4	-	-	
GAP5	-	-	-
GAP6	20.58	20.02	20.81
GAP7	13.33	15.06	13.44
GAP8	22.81	27.93	-
GAP9	16.03	15.66	-
GAP10	-	-	-
GAP11	51.75	59.09	-
GAP12	14.74	18.47	-
GAP13	22.78	33.87	-
GAP14	45.12	74	-
GAP15	-	-	-
GAP16	25.73	51.74	-

not within the standards --> not used in calculation

- **ELISA-results of the PK clone**

PK 1-3 are three cultivations of the same clone

Table 27: Yeast wet biomass of the production-clone determined after 48h of incubation

	YWM after 48h of 2mL in g	YWM after 48h (g/ L)
PK1	0.1885	94.25
PK2	0.1492	74.6
PK3	0.16	80

Table 28: Determined product-concentration of the production-clone (mg/L) per concentration of the yeast wet biomass (g/L) after 48h of incubation

	1st value	2nd value	3rd value	4th value	5th value	6th value			
	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	pc (mg/L)	average conc.	SEM	P
PK1	22.45	26.15	22.54	18.77			**22.48**	0.02	**0.24**
PK2	23.6	-	25.17	21.35			**23.38**	0.01	**0.31**
PK3	46.76	-	21.71	21.45	23.85	23.22	**22.56**	0.01	**0.28**

not within the standards --> not used in calculation

- **ELISA-results: summary of all clones**

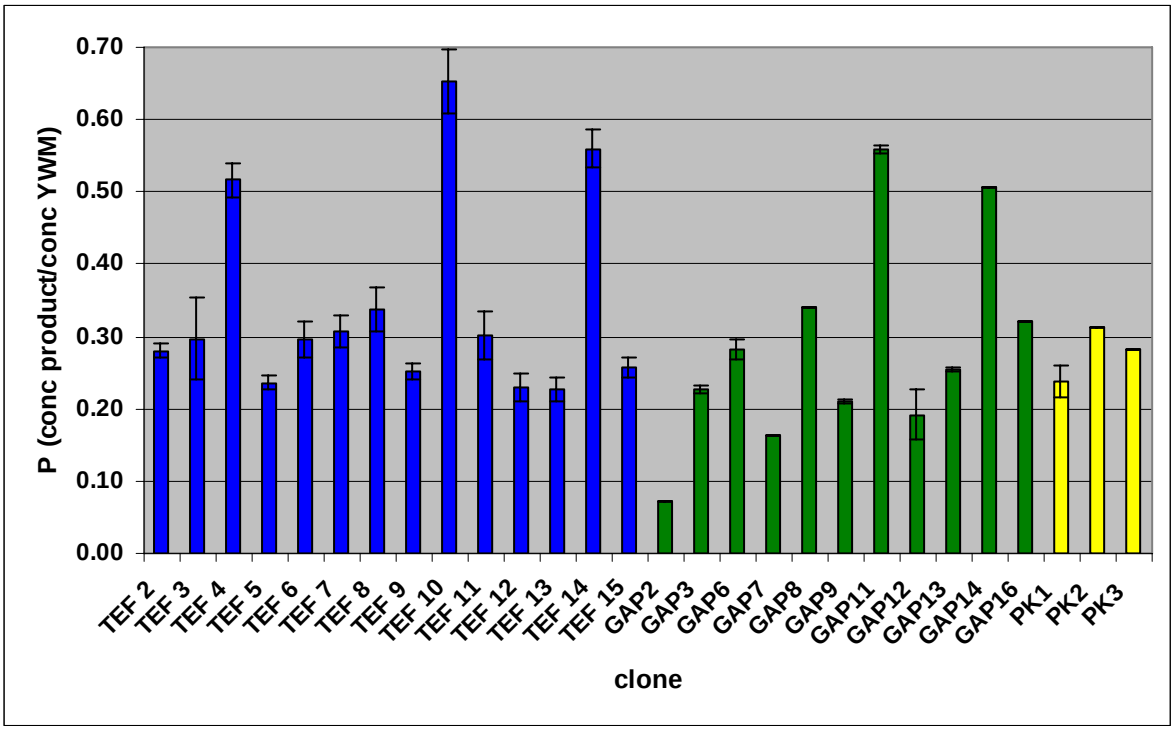

Fig. 11: Determined productivity (P) (product concentration/ concentration of the yeast wet mass).
The standard errors of the mean (SEM) are shown as error bars.

The clones GAP 1, 4, 5, 10 and 15 gave unexpectedly no signal and were therefore excluded for the rest of the study (with the exception of GAP 5). The signals for the other GAP-clones were not uniform, but most of them seemed to be in a mean production range. Two clones (GAP 11 and 14) gave noticeable higher, and GAP 2 noticeable less amounts of product.

The production clone showed production rates of a middle range of about 0.2-0.3 mg per g YWM after 48 hours. Most of the clones with the heavy chain under the TEF-promoter showed a productivity (product/ yeast wet biomass/48 hours) similar to the one of the production clone. The resulting product amounts of all TEF clones together compared to the ones of all GAP-clones showed no significant difference in the statistical analysis (Students t-test: P= 0.3418 >0.05). Interestingly, three clones (TEF 4, 10 and 14) seemed to produce almost two times higher amounts of product than the other TEF-constructs. This difference was also statistically analysed by Student´s t-test and the result shows that the TEF-high producers (TEF 4, 10 and 14) did differ significantly from the TEF normal producers (P= 0.0123 <0.05). In Fig. 12 it can be seen that the mean value of the product amounts of the three high producing TEF-clones is about a 2-fold higher in comparison to the mean values calculated from the amounts of all GAP clones together of the normal producing TEF clones and the production clone.

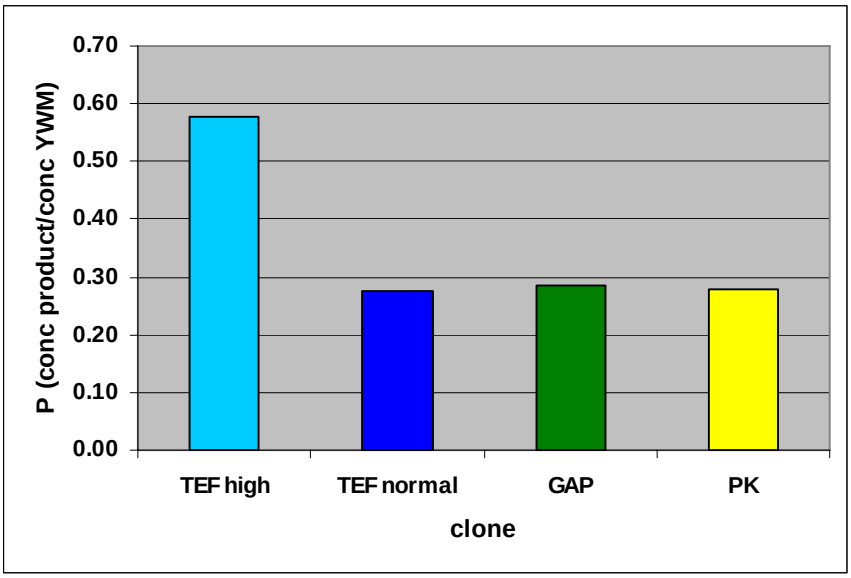

Fig. 12: Mean values of the determined productivity (product concentration/ concentration of the yeast wet mass) after 48 hours of incubation.

In order to investigate the hypothesis that the higher signals are due to the strength of the TEF-promoter, further analyses had to be done.

3.3.2 Determination of the product quality

As a next step visualisation of the proteins on agarose gels was performed to determine the quality of the produced Fab. The separation of the proteins in the culture supernatant by their molecular weight was performed by SDS-PAGE. The undiluted culture supernatants of the selected clones were taken as samples. The samples for the silverstaining have not been reduced but shortly heated before the electrophoresis (to cause denaturation) to reduce the background and ensure a consistent running performance. The samples for the Western

blots have been reduced by DTT (dithiothreitol) and heat treatment. Detailed handling can be found in Materials and Methods (2.10.1-2.10.3). After the electrophoretic separation of the proteins, the gels have been silver stained to identify the fragments by their size (molecular weight).

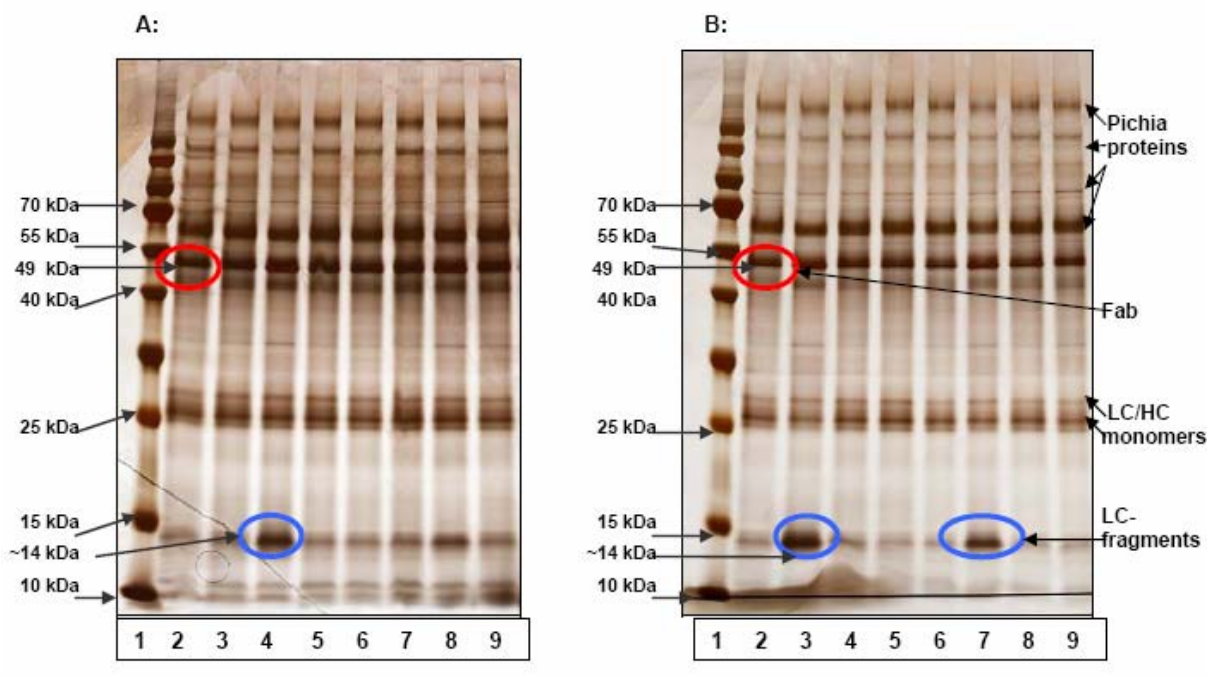

Fig. 13: A: Gel1 (TEF-clones 2-8 and PK1) silver stained after SDS-PAGE under non reducing conditions. B: Gel2 (TEF-clones 9-15) silver stained after SDS-PAGE under non reducing conditions.

Table 29: Sample order (marker = Fermentas Page Ruler Prestained Protein ladder)

lane	gel1	gel2
1	marker	marker
2	TEF2	TEF 9
3	TEF 3	TEF 10
4	TEF 4	TEF 11
5	TEF 5	TEF 12
6	TEF 6	TEF 13
7	TEF 7	TEF 14
8	TEF 8	TEF15
9	PK1	PK8

At ~49 kDa a band representing the intact Fab appeared (red labelling). The Fab band had quite the same intensity for all TEF-clones. The numerous bands with a higher molecular weight were native proteins expressed by *P. pastoris*. The bands around 25 kDa were probably HC and LC monomers. Another interesting band was visible at ~14kDa (blue labelling) which showed an outstanding intensity for the clones TEF 4, 10 and 14. This was probably a matter of fragmented LC in these samples. These three clones were exactly the ones that gave a higher signal in the ELISA.

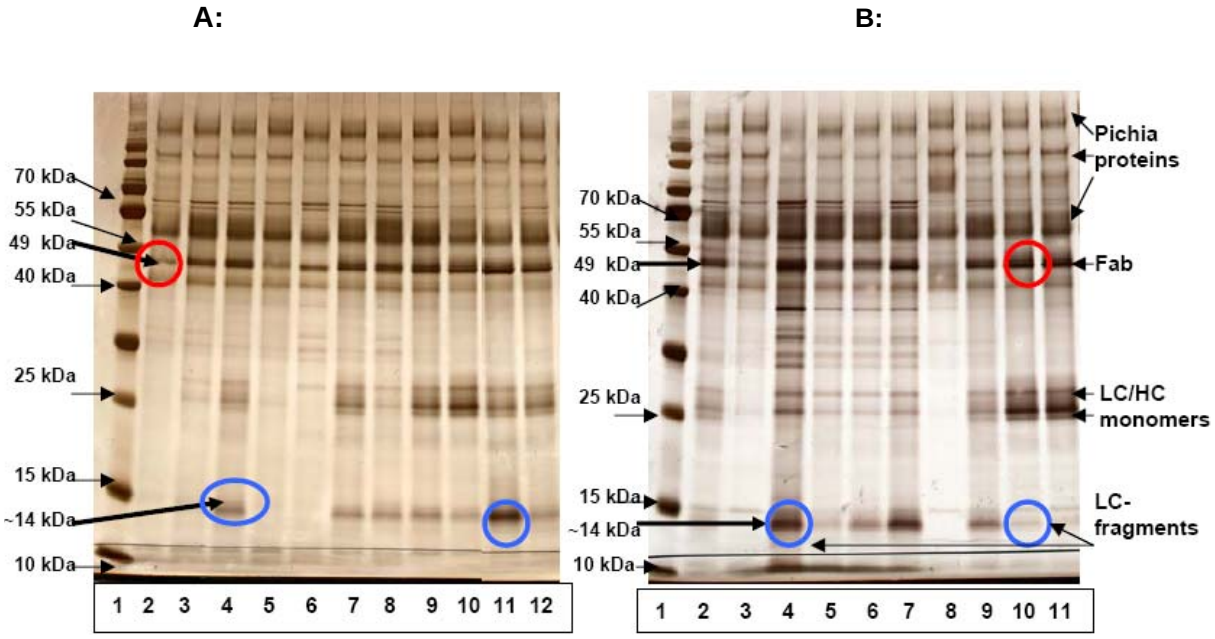

Fig. 14: A: Gel3 (GAP-clones 1-8 and PK1; TEF 4 and 15) silver stained after SDS-PAGE under non reducing conditions. B: Gel4 (GAP clones 9-16 and PK2 and PK3) silver stained after SDS-PAGE under non reducing conditions.

Table 30: Sample order (marker: Fermentas Page Ruler Prestained Protein ladder)

lane	gel 3	gel 4
1	Marker	marker
2	GAP 1	GAP 9
3	GAP 2	GAP 10
4	GAP 3	GAP 11
5	GAP 4	GAP 12
6	GAP 5	GAP 13
7	GAP 6	GAP 14
8	GAP 7	GAP 15
9	GAP 8	GAP 16
10	PK 1	PK 3
11	TEF 4	PK 2
12	TEF 15	-

Fig. 14 A shows the same band pattern as appeared in Fig 13. Noticeable is that the band at 14 kDa of most of the GAP-clones, as well as from the production clone, was very low in intensity. The three TEF clones that have given two times higher product amounts in the ELISA (TEF 4, 10 and 14) showed a band at about 14 kDa, which is considerably more intense than the band from the clones with average yields (see figure 13). About the same intensity of this band can be observed for the GAP-clones 11 and 14 (see figure 14 B), which were also the ones with the highest productivity in the ELISA. Based on the knowledge that unassembled heavy chain from 3H6 Fab (like from several other Fabs) is not secreted into the supernatant (Gach et al. 2007), it can be concluded that this band derives from free light

chain. As the protein parts were rather small, the free remaining light chain seems to be degraded after release into the culture supernatant. The fact that these clones (TEF 4, 10, 14 and GAP 11 and 14) gave such an intense band in comparison to the other clones, but not a stronger band corresponding to the intact Fab (assembled light and heavy chain), probably indicates, that these clones have produced more light chain. For the production clone the intensity of the band representing the Fab, was in the range of the intensities from the TEF-clones. As the silver staining method is not really quantitative, this theory had to be proved by quantitative Western blotting.

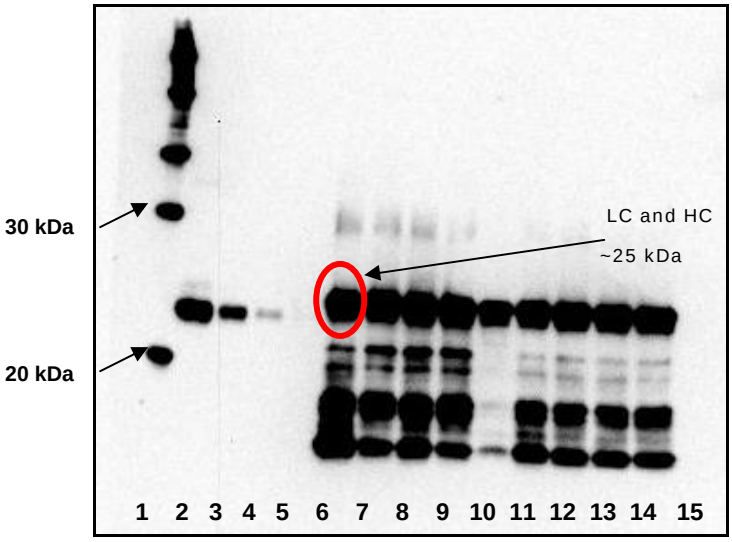

Fig. 15: Blot 1 stained with goat_antihuman_Fab labelled with horse radish peroxidase after SDS-PAGE under reducing conditions.

Table 31: Sample order blot1 (Fab stained)

lane	sample
1	Marker (Magic NuPAGE)
2	hFab Std 20 µg/mL
3	hFab Std 15 µg/mL
4	hFab Std 10 µg/mL
5	hFab Std 5 µg/mL
6	TEF4
7	TEF5
8	TEF8
9	TEF9
10	GAP2
11	GAP3
12	PK1
13	PK2
14	PK3
15	Prest. Prot. Lad.

The principle of the detection for all Western blots was chemiluminescence. Under reducing conditions a band at about 25 kDa (red labelling) is visible. The same pattern of bands was identifiable as with the silver staining, although differences between the clones were hard to define because the blot was over-stained. The second blot (Fig. 16) with the remaining clones allowed a more accurate interpretation.

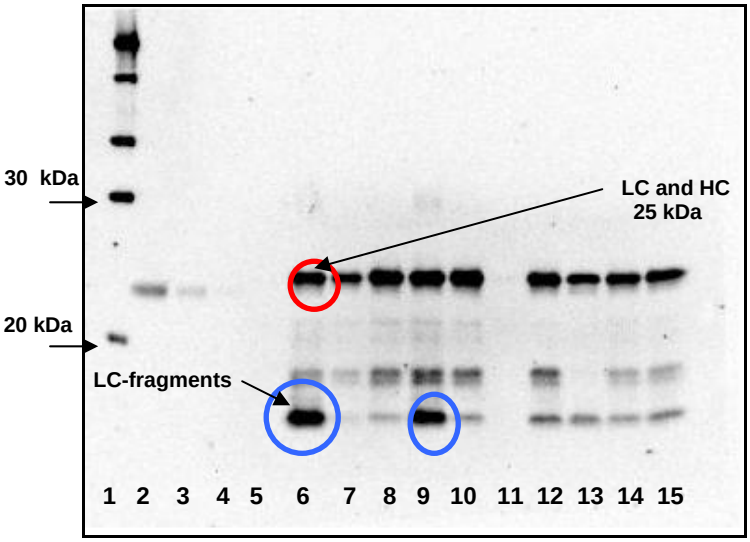

Fig. 16: Blot 2 stained with goat antihuman Fab labelled with horse radish peroxidase after SDS-PAGE under reducing conditions

Table 32: Sample order blot 2 (Fab stained)

lane	sample
1	Marker (Magic NuPAGE)
2	hFab Std 20 µg/mL
3	hFab Std 15 µg/mL
4	hFab Std 10 µg/mL
5	hFab Std 5 µg/mL
6	TEF10
7	TEF12
8	TEF13
9	TEF14
10	TEF15
11	GAP5
12	GAP6
13	GAP7
14	PK4
15	PK5

A band appeared under reducing conditions at approximately 25 kDa, representing the LC and HC. The TEF-clones 10, 13, 14 and 15 and GAP 6 showed quite the same intensity of Fab band, but they differed clearly in the LC-fragment band at ~14 kDa. The Fab bands for TEF 12 and GAP 7 were in the range of the ones from the production clone. The clone GAP 5, from which no signal was obtained in the ELISA, seemed actually to secrete and probably also to express no product and was therefore a kind of negative control. The distinct LC-fragment band for the clones TEF 10 and 14, which was indicated by silver staining was confirmed by the Western blot. The antibody ahFab binds not only the intact protein (the whole assembled Fab), but also the light or heavy chain alone as well as light chain dimers and fragments thereof. As the Fab-bands were a lot more intense than the Fab-standard of highest concentration, quantification was not possible. The next step was to perform a Western blot with a mouse-anti-C_H1-antibody, which only binds the heavy-chain and therefore gives more detailed information about the amount of correctly assembled Fab-fragment, because only assembled heavy chain is assumed to be transported out of the cell into the culture supernatant. Moreover, it was performed to assure that the fragments are derived from LC.

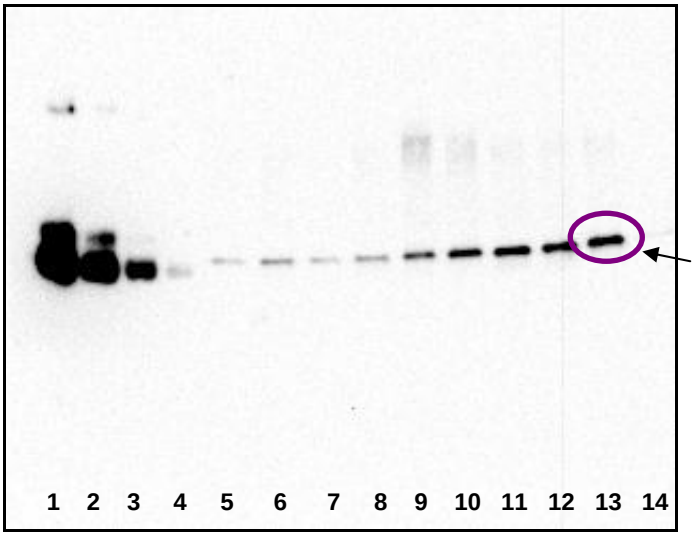

Fig. 17: Blot 3 stained with mouse anti human 3H6-HC.
2nd antibody: anti mouse labelled with horseradish peroxidase after SDS-PAGE under reducing conditions

Table 33: Sample order blot 3 (aHC stained)

lane	sample
1	hFab Std 20 µg/mL
2	hFab Std 15 µg/mL
3	hFab Std 10 µg/mL
4	hFab Std 5 µg/mL
5	TEF4
6	TEF5
7	TEF8
8	TEF9
9	GAP2
10	GAP3
11	PK1
12	PK2
13	PK3
14	TEF10

The band at about 25 kDa is the bound HC which complies with the reduced Fab (violet labelling).

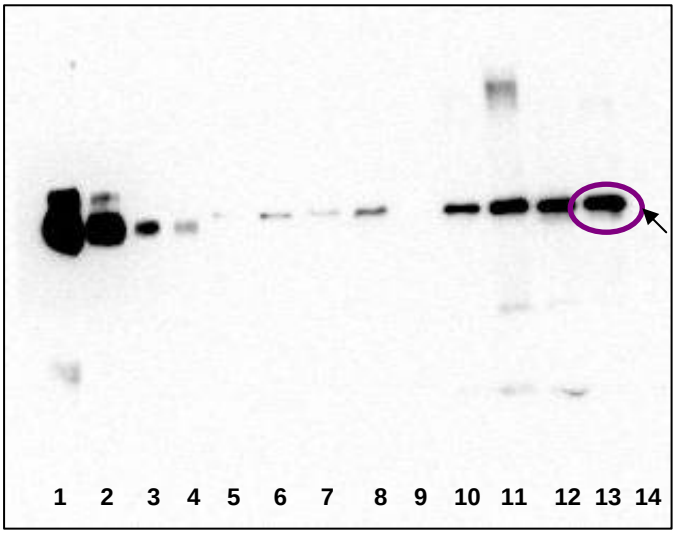

Fig. 18: Blot 4 stained with mouse anti human 3H6-HC.
2nd antibody: anti mouse labelled with horseradish peroxidase after SDS-PAGE under reducing conditions.

Table 34: Sample order blot 4 (aHC stained)

lane	sample
1	hFab Std 20 µg/mL
2	hFab Std 15 µg/mL
3	hFab Std 10 µg/mL
4	hFab Std 5 µg/mL
5	TEF12
6	TEF13
7	TEF14
8	TEF15
9	GAP5
10	GAP6
11	GAP7
12	PK4
13	PK5
14	Prest. Prot. Lad..

Here again the clear visible band at ~25 kDa is the bound HC (violet labelling). To get information about the real amount of Fab the clones produced, a Western blot with mouse-anti-C_H1(3H6)-domain-antibody was performed. It was remarkable that there is only one band on the blot, which is on the one hand a sign for the great specificity of the anti heavy chain antibody and on the other hand degradation of the heavy chain can be excluded. This means that no HC-fragments were built. The GAP/GAP constructs (except for clone 5, which gave

also no ELISA signal) seemed to produce higher amounts of correctly assembled Fab-amounts compared favourably with those of the control-clone PK. However, the clones with the heavy chain under control of the TEF-promoter gave significantly lower yields of HC**.** The clones that showed higher signals in the ELISA did not give any outstandingly higher product amount.

- **Quantification of the HC**

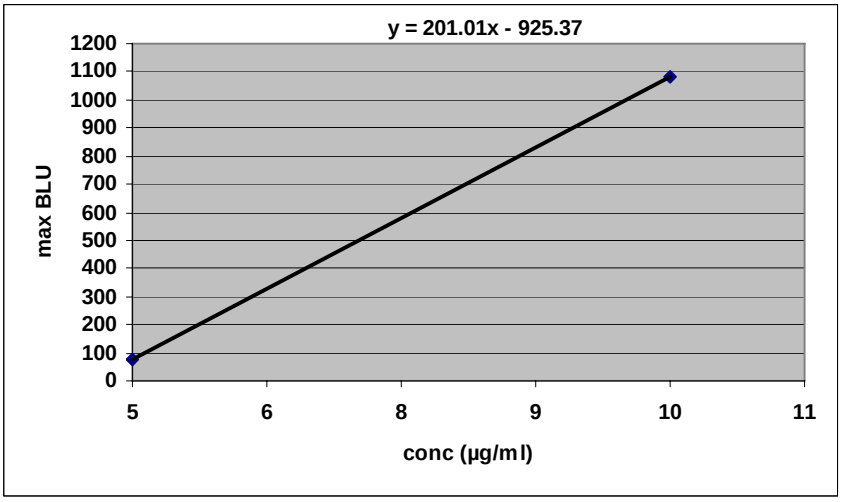

Fig 19: Calibration curve aHC-Westernblot #1

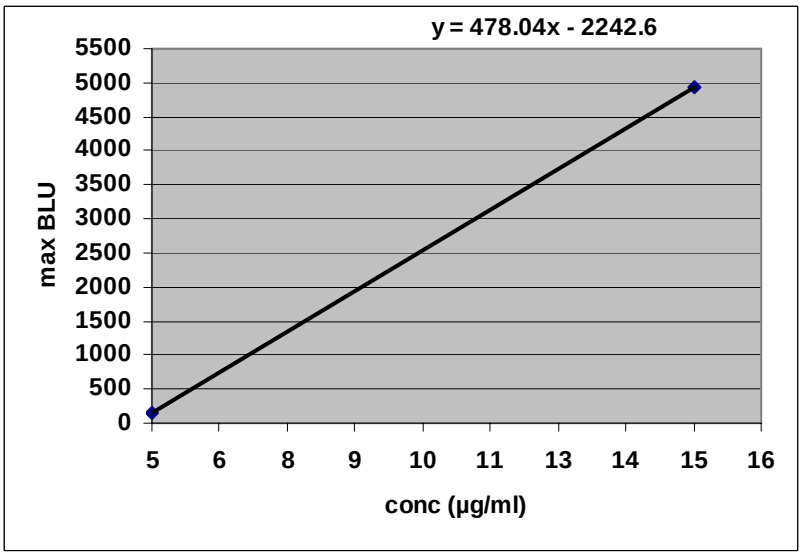

Fig 20: Calibration curve aHC-Westernblot #2

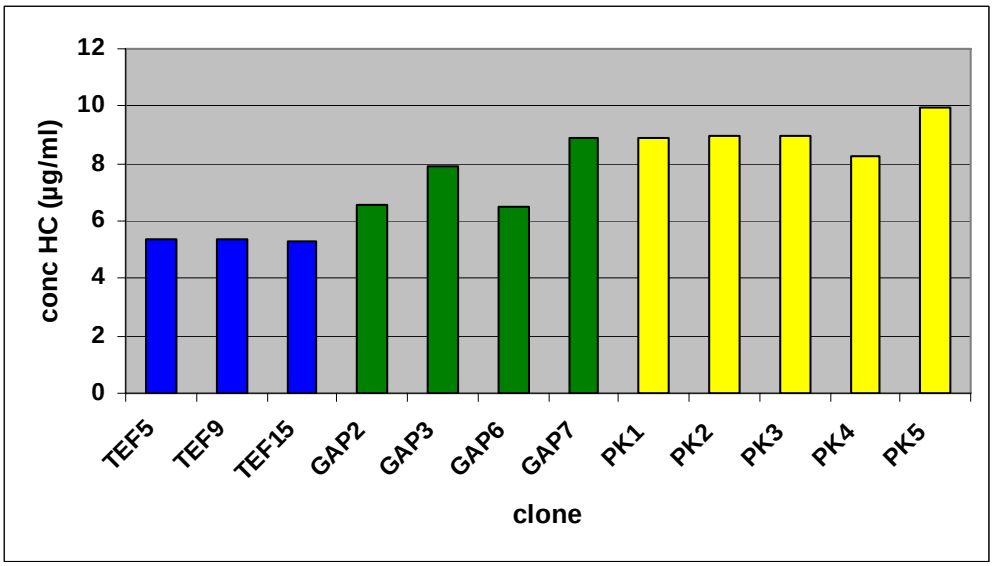

Fig 21: HC-concentration determined by the quantitative aHC-Western blot

As the standard bands of the higher concentrations were oversaturated, the standard curves were only calculated from two standards. Therefore the absolute values are not exact, but in this case only the comparison of the values of the different constructs was essential. The quantification of the HC-Western blot showed that the HC-amount and therefore the amount of produced Fab showed that all TEF- clones gave less amounts compared to the GAP-clones and the production clone. The clones TEF 5, 9 and 15 were in the range of the GAP and PK-clones, while all the other TEF-clones had considerably less amounts, which could not be calculated because they showed band intensities lower than the standard of the lowest concentration. However it can be noted that the clones TEF 4, 8, 10, 12, 13 and 14 produced less HC than 5 µg/mL. A remarkable observation that can be seen in Fig. 17 was, that the clone that gave the highest signal in the ELISA (TEF 10), showed one of the lowest amount of HC and thus secreted Fab. Therefore it can be assumed that the higher ELISA signal was caused by LC and fragments thereof.

As the previous analysis concentrated on the protein level, the following tests should shed light on transcriptional events and their effects on Fab expression. The first method in this respect was the quantitative real time PCR.

3.4 Determination of the transcript level

3.4.1 Quantitative Real time PCR

In the following the transcript level was analysed by quantitative Real-time PCR. The results are given as relative amounts. This means the calculated gene copies/µL of the sample are related to the expression of the reference gene actin in each sample. The gene copies/µL were first calculated from external standards (of actin and of the samples) respectively. (Detailed information about the RNA- and DNA-isolation, transcription into cDNA and the performed qRT-PCR can be found in Materials and Methods 2.11. and 2.12.2). Detailed data-tables are shown in the appendix.

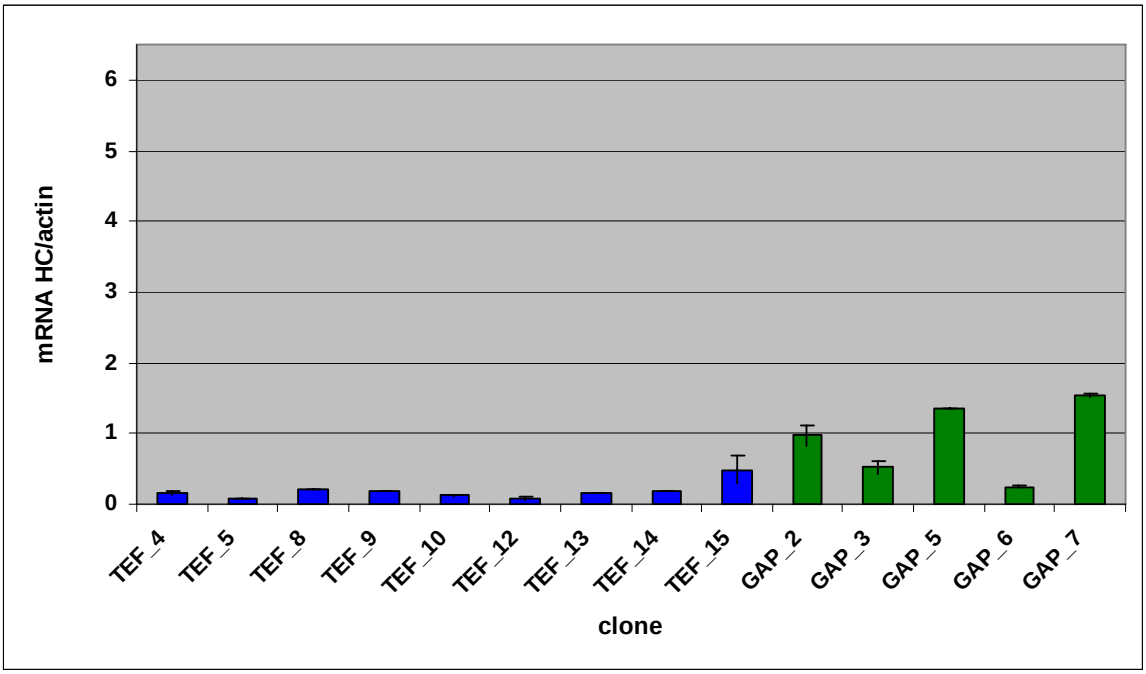

Fig. 22: Calculated ratio of the transcript from the heavy chain gene (HC) per calculated transcript (calculated copies/µL) of the reference gene actin. The standard error of the mean value is shown as error bars.

The GAP/GAP clones had more HC-transcript-copies than the GAP/TEF-constructs, which is consistent with the amounts of Fab detected by the aHC-Western blot. This is another argument for the theory that Fab production depends predominantly on the HC amount. The PK-clone gave no signal for the heavy chain in the qRT-PCR. This could be due to steric problems of these samples. The HC and LC genes in PK were placed one after another on one vector which could have caused problems with accessibility to the HC gene.

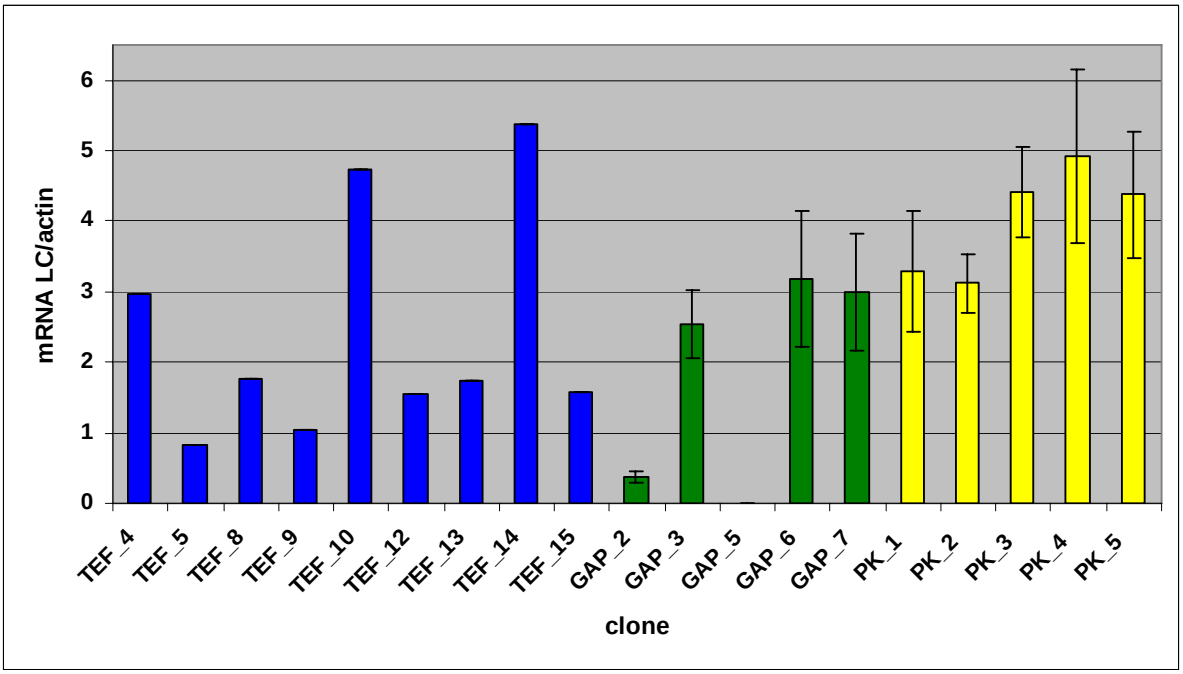

Fig. 23: Calculated ratio of the transcript from the light chain gene (LC) per calculated transcript (calculated copies/µL) of the reference gene actin. The standard error of the mean value is shown as error bars.

Regarding the LC transcript level, the GAP/TEF constructs showed the same pattern as in the ELISA. Outstanding amounts of LC-transcript levels were observed for the same three clones: TEF 4, 10 and 14. The GAP clones gave quite consistent values (except 2, which gave a lower signal, which goes along with the ELISA-result) and the production clone provided a constant transcript-level. All the TEF clones showed a higher amount of LC-transcript as HC-transcript. This holds also true for GAP 3, 6 and 7 but not for the clones GAP 2 and 5.

3.5 Determination of the Gene copy number

3.5.1 Determination of the gene copy number by q-RT-PCR

To find out if the higher amounts of Fab/LC of some clones are due to gene copy number effects, the gene copies of the expression cassettes integrated into the genomic DNA had to be determined.

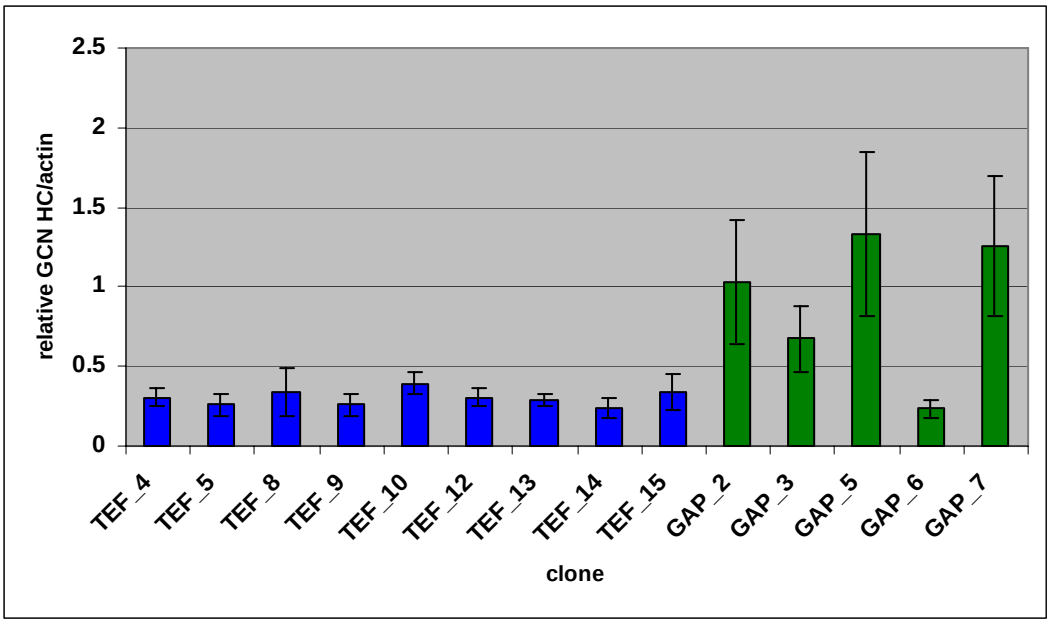

Fig. 24: Calculated concentration of gene-copies (calc. copies/µL) of the heavy chain gene (HC) per calculated concentration of gene-copies (calc. copies/µL) of the reference gene actin.
The standard error of the mean value is shown as error bars.

The genomic results represented almost the same pattern as the mRNA run. The GAP-clones varied in their gene copy numbers. If Fig. 22 and 24 are compared, it can be seen that the GAP-clones with higher gene copy numbers gave also a higher amount of transcript. The variation within the TEF-clones may be a matter of measurement inaccuracy which appears if the gene copy number is in such a low concentration range. The statistic analysis using the Student´s t-test (2-tailed, different variances) showed that the difference between the HC gene copy numbers of the TEF-clones and the ones of the GAP-clones is significant, but not highly significant. ($P = 0.0411 < 0.05$).

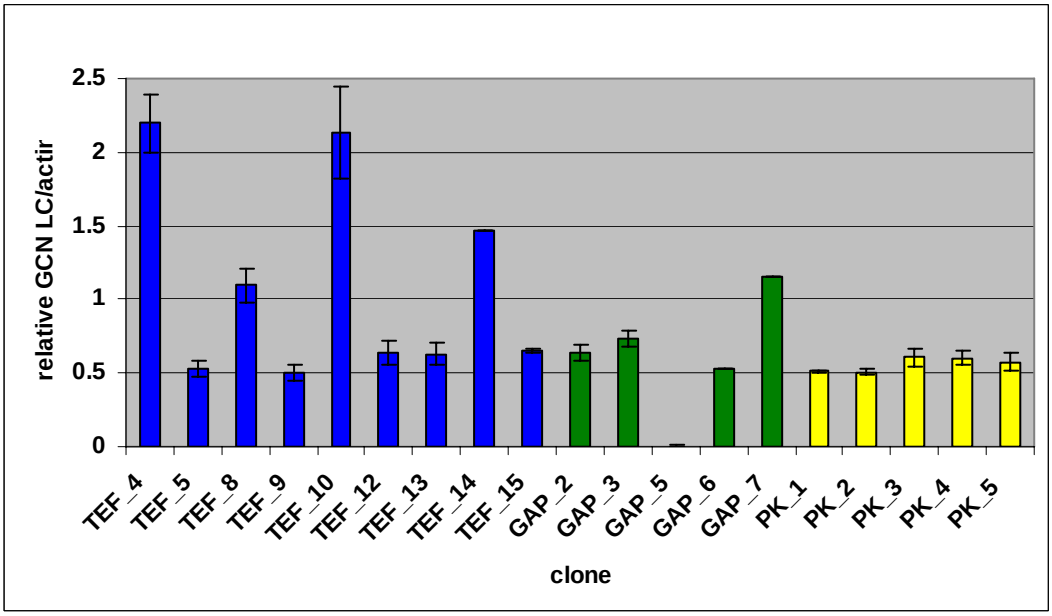

Fig. 25: Calculated concentration of gene copies (calc. copies/µL) of the light chain gene (LC) per calculated concentration of gene copies (calc. copies/µL) of the reference gene actin. The standard error of the mean value is shown as error bars.

The values that were observed in the determination of the LC transcript (see Fig. 23) reflected overall the determined gene copies of the LC, which means that the amount of transcript depends on the GCN. This underlined once more previous results, the LC-gene copy number for the clones TEF 4, 10 and 14 showed again about twice the amount compared to the other clones. A statistical analysis (Student's t-test, two tailed, different variances) showed the significance of the difference of the LC-copy number comparing the TEF high producers (TEF 4, 10 and 14) to the TEF normal producers (TEF 5, 8, 9, 12, 13 and 15) (P= 0.0206 <0.05). The TEF and the GAP clones do not differ significantly in their copies of the LC gene. (P= 0.1273 >0.05)

3.5.2 Determination of the gene copy number by quantitative Southern Blot

To quantify the absolute gene copy number some clones were selected for the next analyses. Another method to determine the gene copy number is the quantitative Southern blot. This technique is also very specific because of the use of a specific probe. The isolated DNA of the selected clones was used as samples. The standards for the quantification were plasmid solution with the adequate sequence and defined theoretical number of gene copies. (Details about the procedure are shown in the part Materials and Methods.) The detection was done by the DIG-labelling system and measurement of the emerging chemiluminescence. The results are given in absolute amount of the gene copies, as calculated from the standard curve.

3.5.2.1 Quantification of the gene copy number of the light chain

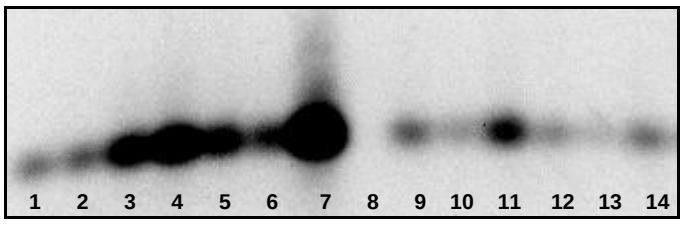

Fig 26: Southern blot labelled with light chain probe measured by chemiluminescence after two min exposure time

Table 35: Sample order of the Southern blot light chain gene, (std = standard)

lane	samples_Southern blot (LC)
1	LC_std_(plasmid)_0.5 gene copies
2	LC_ std _(plasmid)_1 gene copy
3	LC_ std _(plasmid)_2 gene copies
4	LC_ std _(plasmid)_3 gene copies
5	LC_ std _(plasmid)_4 gene copies
6	LC_ std _(plasmid)_6 gene copies
7	LC_ std _(plasmid)_10 gene copies
8	-
9	GAP3
10	GAP6
11	TEF4
12	TEF10
13	TEF13
14	PK2

Table 36: Data of the standards quantitative Southern blot light chain

standards	
mass	gene copies
0.08028	0.5
0.10394	1
0.41477	2
2.2741	10

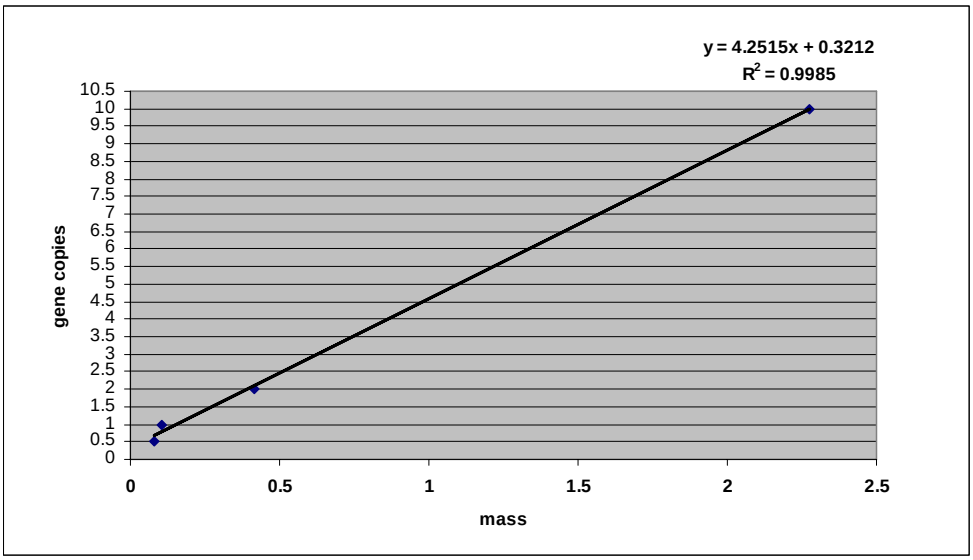

Fig. 27: Standard curve quantitative Southern blot light chain

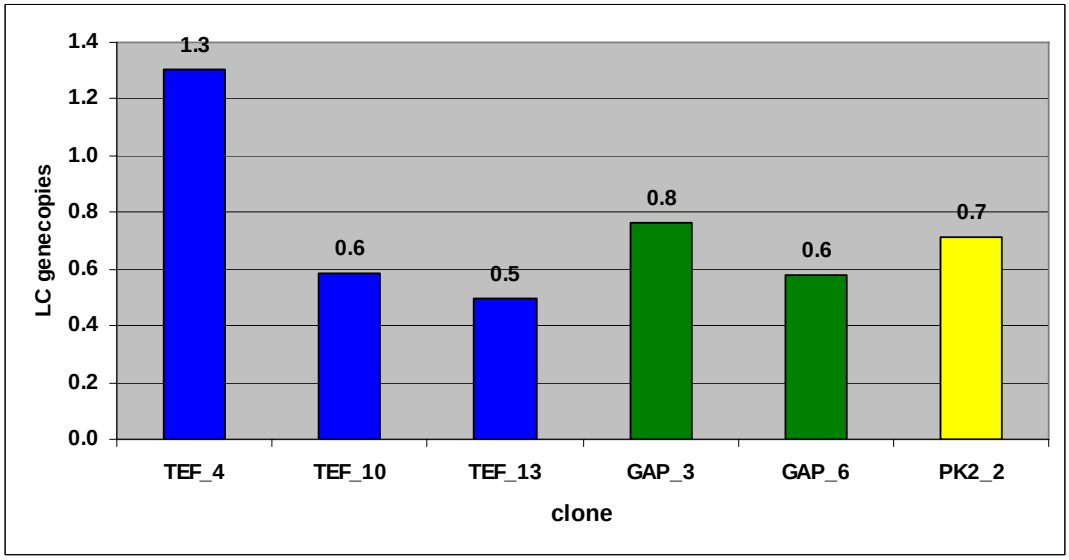

Fig 28: Quantitative Southern blot: Calculated copies of the light chain gene

The Southern blot showed only one band, which stands for a specific binding of the probe on the immobilised sample. The LC-bands of GAP 3, 6, TEF 13 and PK 2 gave the expected intensities. The high producer TEF 4 had about the twofold intensity in comparison to the other clones, which confirms the previous observations. The band of clone TEF 10 was less intense than it was expected, which could be due to a blotting effect (like an air bubble,etc.).

3.5.2.2 Quantification of the gene copy number of the heavy chain

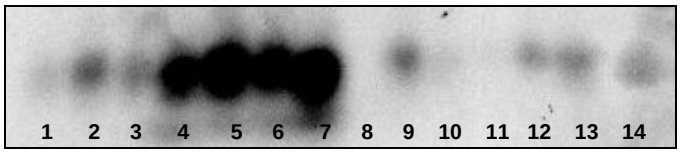

Fig. 29: Southern blot labelled with heavy chain probe measured by chemiluminescence after two min exposure time

Table 37: Sample order of the Southern blot heavy chain gene, (std= standard)

lane	samples_Southern blot (HC)
1	HC_std_(plasmid)_0.5 gene copies
2	HC_std_(plasmid) 1 gene copy
3	HC_std_(plasmid)_2 gene copies
4	HC_std_(plasmid)_3 gene copies
5	HC_std_(plasmid)_4 gene copies
6	HC_std_(plasmid)_6 gene copies
7	HC_std_(plasmid)_10 gene copies
8	-
9	GAP3
10	GAP6
11	TEF4
12	TEF10
13	TEF13
14	PK2

Table 38: Data of the standards quantitative Southern blot heavy chain

standards	
mass	gene copies
0.04244	0.5
0.24594	2
1.7667	10

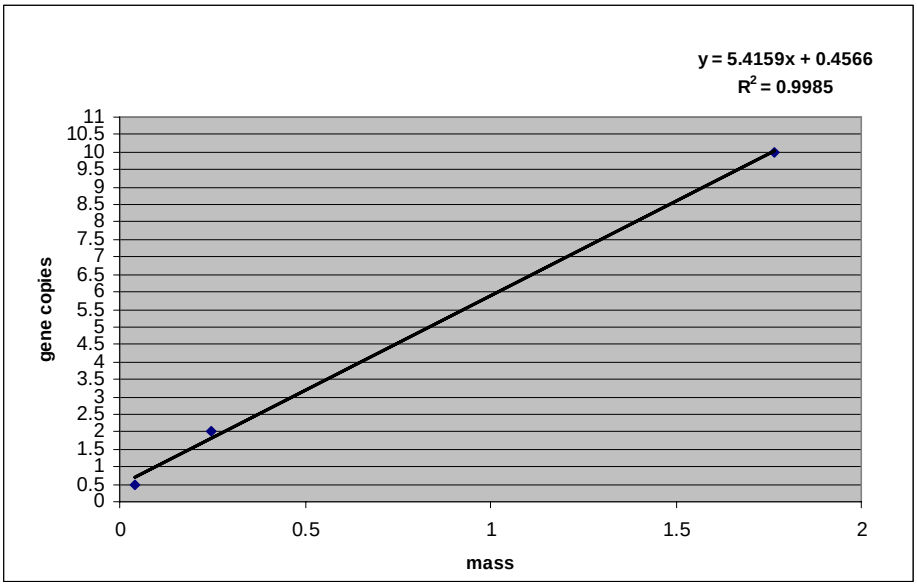

Fig. 30: Standard curve quantitative Southern blot heavy chain

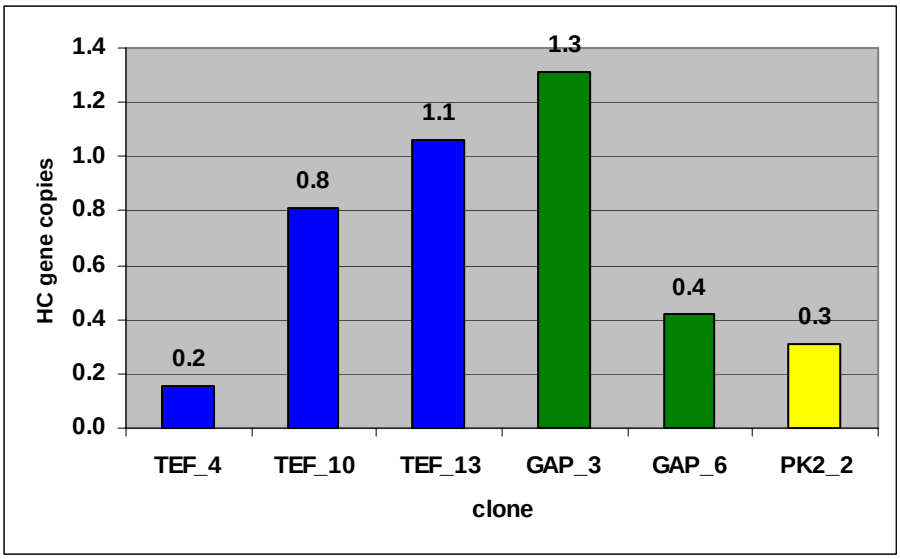

Fig. 31: Quantitative Southern blot: Calculated copies of the heavy chain gene

Noticeable was the significant lower amount of heavy chain gene copies of the high producer TEF 4. A high gene copy number was observed for the clones GAP 3, TEF10 and 13. The production clone and GAP 6 showed a rather low GCN. As the bands on the HC-blot were not very clear and the standards gave no clear grading according to the gene copies, these results should not be over-interpreted.

4 Discussion

4.1 Antibody-fragment production in the *P. pastoris* expression system

Antibodies and fragments thereof are important tools in medical research and application. The demand will probably increase in the next decades. Therefore there is a need for high level expression systems for Fab fragments. The yeast *Pichia pastoris* turned out to be a potential candidate as a host system (Pennel and Eldin 1998; Cereghino and Cregg 1999; Gasser et al. 2006). As antibody molecules and Fabs are hetero-dimeric proteins composed of a heavy and a light chain, their assembly suggests for a co-expression of the heavy chain and light chain from separate vectors (Leonard et al. 2006).

In the present work the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene P_{GAP} (Waterham et al. 1997) and the promoter of the translation elongation factor gene P_{TEF1} (Ahn et al. 2007) were used. Clones with the heavy chain expressed under control of P_{TEF1} should be characterised and the influence of this promoter in comparison to the GAP-promoter in terms of product titers determined. A “reference”-construct (named production clone, short name: PK) with both chain sequences on one vector and under control of P_{GAP} was analysed the same way as the constructs of interest. The analysis of the protein-level was done by detecting the protein (3H6-Fab) secreted in the culture supernatant and determining of the product titers and quality. The methods used for these applications were ELISA and SDS-PAGE followed by silverstaining and Western blot. In order to analyse some influencing parameters on the product titers and quality, apart from the promoter strength, like the gene copy number and the HC:LC ratio, quantitative real time-PCR and Southern blot were performed.

4.2 Product amount and quality

The analysis by ELISA of the culture supernatant after 48h of incubation showed that three of the nine constructs with the HC under control of the TEF promoter seemed to be high producers, as they gave about double amounts of product in comparison to the other clones. The yields of the GAP-clones were in the range of the PK-clones (both having the LC and HC under control of P_{GAP}). This would suggest for no difference of product titers by expression of the chains from two separate vectors in comparison to the expression from one

single vector. This would mean that the locus of integration has no influence on the productivity.

The analysis by silverstained SDS-PAGE was in good agreement with the ELISA-results that these three TEF-clones produce a slightly higher amount of Fab. In contrast to the ELISA, the production clone PK gave quite the same results as the TEF-clones. Another important observation was that within the TEF-constructs the band corresponding to the intact Fab showed quite the same intensity, and the potential high producers had an additional intense band for a considerably smaller fragment. The aFab-stained Western blot experiments reflect these results. These lead to the conclusion that the higher product titers have been due to the increased production of light chain in these three clones and not due to a higher Fab production. A Western blot stained with aHC-antibody (see Figures 17, 18 and 21 in 3.3.2) verified this conclusion. The effect, described in previous studies (Gach et al. 2007), that, apart from heterodimers, only unassembled light chain of the 3H6 Fab is secreted in the culture supernatant by *P. pastoris* under control of the AOX-promoter and that heavy chain remains in the cell, holds also true for constitutive expression. This observation has also been made with other Fabs in *P. pastoris* (Gasser et al. 2006) as well as with whole IgGs in mammalian cells (Li et al. 2007). A second conclusion that could be made from the analyses performed so far was the instability of the secreted free 3H6 light chain. The unassembled light chain seems to be (partly) degraded into smaller fragments of about 14 kDa, probably by proteases in the culture supernatant. This is for example different to studies made with the antibody Fab 2F5, which seems to be quite stable when it is expressed in *P. pastoris* (Gasser et al. 2006). The GAP-constructs as well as the production clone gave higher amounts of produced intact assembled Fab as the TEF-clones. This supports the assumption of a gene copy number effect concerning the light chain as can be seen in Figure 25 in 3.5.1 and Figure 26 and 28 in 3.5.2.1. Furthermore it can be presumed that the TEF-promoter is not stronger than the GAP-pomoter as far as HC-expression levels are concerned (see Figure 22 in 3.4.1). The aHC blots display that the production clone gives the highest amounts of intact Fab. Hence no increase of Fab production could be observed by co-expression of heavy and light chain under separate expression control.

4.3 Expression of the Fab HC gene under control of the TEF-promoter

Antibodies as well as Fabs are heterodimeric structures. The two polypeptide chains are separately expressed and transported into the ER, where the folding and the assembly are taking place. Due to developmental reasons, (in mammalian cells) the HC is synthesized before the LC is expressed. In pre B-cells built HC-homodimers remain in the ER because of

strong interactions with BiP (immunoglobulin binding protein) until the heterodimeric structure can be completed because of expressed LC and subsequently be secreted as antibody (-fragment) (Schlatter et al. 2005). LC is known to form also homodimers but in contrast to the HC-dimers they can generally be secreted into the culture supernatant. Thus in the assembly-step there seems to be some kind of competition between LC and HC for the LC binding sites (Schlatter et al. 2005).

Therefore the idea in the present work to obtain a higher yield of Fab, was to express the HC gene under control of a promoter that is growth-associated and would show higher expression rates in early stages of cell growth. As a consequence the accumulation of HC could lead to a higher amount of Fab in comparison to LC-homodimers. In the present work the constitutive promoter TEF1 (translation elongation factor 1 α) was chosen because it shows a higher activity in the exponential than in the stationary phase as it is described by Ahn et al. 2007. The authors also reported that expression of a heterologous gene under control of P_{TEF1} yielded in protein levels in the range of the ones obtained from P_{GAP} or even higher. Additionally in previous works of our working group about novel *P. pastoris* promoters for heterologous protein production, it was also found that from P_{TEF1} higher expression activity was obtained in early phases of growth, followed by a decrease to a constant level (Stadlmayr et al. submitted 2010). In this work the cells were grown on glucose and the reporter proteins eGFP (green fluorescent protein) over a batch or fed batch cultivation respectively.

However, the expected effect of a higher Fab yield due to higher concentrations of HC could not be observed in the present work. As transcript analysis shows, there were no higher expression rates of the HC gene for the constructs with the TEF-promoter in comparison to those with the GAP-promoter, which is known to be less growth associated. In comparison to the above mentioned studies on P_{TEF1} this indicates, that different genes under the same promoter can lead to quite different levels of expression. Admittedly the GCN has also to be considered in this case, as most of the GAP clones had all a higher number of HC expression cassettes than the TEF-clones. GAP 6 that had a GCN in the range of the TEF-constructs, showed also similar amounts of transcript like the TEF-clones (see Fig. 22 in 3.4.1. and 24 in 3.5.1).

4.4 Co-expression of the LC and HC gene from two separate vectors

The production of recombinant Fab can either be performed by expression of the HC and LC gene from one single vector or from two separate vectors. A comparison of these two strategies was described in *E. coli* by Leonard et al. 2006. They compared the expression from

both genes on one vector driven by one promoter with the HC in front of the LC with the expression from each Fab chain from two separate vectors. They reported that the amount of Fab obtained from the co-expression of the polypeptide chains from separate vectors was comparable to the one from one single vector.

In the present work the co-expression of the Fab chains from two separate vectors was compared to the expression from one single vector with each gene driven by a separate promoter. The results from the aHC-Westernblot showed, that the Fab yield could not be increased by the use of two different expression vectors. The clones with the LC and HC expressed from one single vector showed higher productivity than the two vector constructs with the TEF promoter driving the HC as well as the one with GAP promoter as HC expression control (except for one GAP-clone). This is not surprising, as the production clone is a selected clone, while the GAP and TEF-clones are randomly picked clones.

During the work it became evident that the HC to LC ratio is one essential parameter when the Fab chains are expressed from two separate vectors, as will be discussed later in 4.6.

4.5 Gene copy number (GCN) can be an important factor for protein production

The gene copy number (GCN) is one of the critical points influencing expression levels. In literature different statements about the influence of the GCN on protein production can be found. This suggests that it is dependent on factors like the chosen expression system or the protein to be produced. One attempt to increase product titers was the systematic increase of the GCN which was performed in lots of studies. For example Morlino et al. (1999) observed an increased production of heterologous proteins (glucoamylase from yeast and mammalian interleukin-1β) by inducible amplification of the GCN in the yeast *Kluyveromyces lactis.* Furthermore in 2001 Vassileva and colleagues described a direct correlation between the GCN and the produced amounts of HBsAg (Hepatitis B surface antigen) in *P. pastoris.* They obtained an enhanced HBsAg expression from the clones with increased copy numbers. On the other hand, Hohenblum et al. (2004) showed that the increase of the GCN correlates only to a certain limit with higher yields of the protein trypsinogen (human). They suggest that other limitations of the entire expression pathway counteract the positive effect of higher GCN beyond a certain level of copies. In their work this increasing effect on the product yield was only observed from 1 to 2 copies, while a GCN of 3 or more copies had even a negative influence (decrease of cell viability and cellular proteins were released into the culture supernatant in higher amounts). Reisinger and co-workers (2008) concluded that the GCN can only have a positive effect on the product yield if the gene can be transcribed at efficient levels. To our knowledge, the determination of the GCN for Fab in yeast was not done so far,

so this was one reason to perform it in the present work. It was observed that a higher gene copy number of the Fab chains (obtained by random multiple integration) leads in most clones to a higher transcript signal. Higher gene copy numbers resulted also in higher product titers (= total amount of protein), but not in a higher productivity in terms of higher amounts of intact Fab. This might be due to the unfavourable HC:LC ratio and the lower GCN for the HC. Additionally it has to be mentioned that other influencing factors like the correct folding, assembly and secretion of the antibody fragment are important factors beside the GCN, to reach higher yields of the desired product.

4.5.1 Ratio transcript/GCN: gene copy number effect

The results show that the transcript of the heavy chain for the TEF-constructs are all quite at the same low level (see Fig. 22 in 3.4.1). The GCN of these samples gave the same result as Fig. 24 in 3.5.1 shows. This is due to the fact that the TEF-clones all origin from one initial clone containing only one copy of the HC-vector. Another effect that appeared was that most of the GAP-clones showed a higher amount of heavy chain gene copies, which resulted also in higher amounts of the respective transcript. The "high producers" of the TEF-clones showed a higher transcript amount of LC but not of HC (compare Fig. 22 and 24 in 3.4.1). This indicates again that the clones with higher signal in the ELISA produce only more light chain instead of correctly, assembled Fab. The three clones with the outstanding results on the protein level are only high producers as far as it concerns the light chain. The production clone shows similar high amounts of light chain transcript as the high producing TEF-constructs, but is significantly lower in the genomic DNA results. However one has to be aware that the production clone is one specific selected high producing clone, while the other clones were randomly picked. The determination of transcriptional activity by correlating the transcript copies to the Fab chain gene copies in the genomic DNA should shed light on the assumed copy number effect.

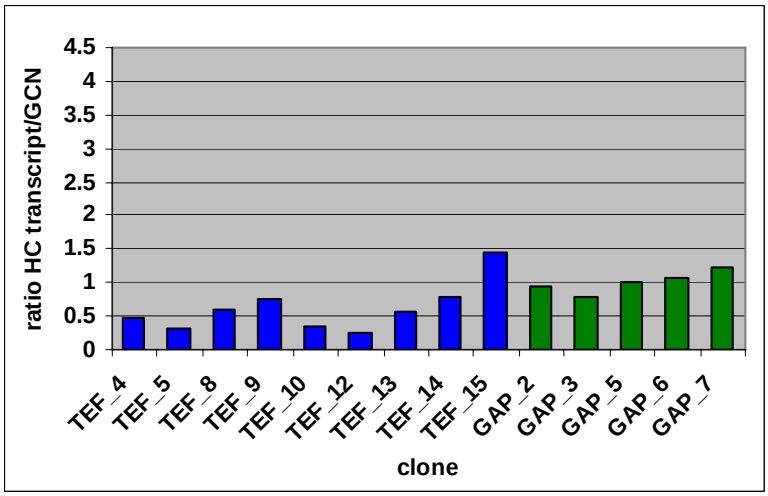

Fig.32: Ratio transcript/ gene copies of the heavy chain gene

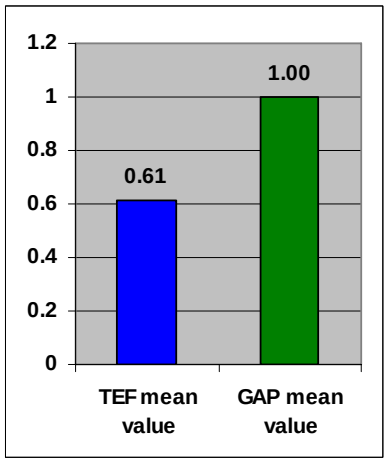

Fig. 33: Ratio transcript/GCN of the HC-gene: Comparison of the mean values of the TEF- to the GAP-clones

For nearly all TEF-clones the gene copy number of the heavy chain was lower than the gene copies of the light chain. The ratio HC transcript/GCN was a bit higher for the GAP-clones. Regarding the mean values, the TEF-constructs produced less transcript related to their gene copy numbers. The difference between the mean value of the TEF-clones to the one of the GAP clone is statistically significant, as the analysis by the Student´s t-test (2-tailed, diff. variances) showed (P= 0.0183 < 0.05). The GAP-promoter seems to have a higher transcriptional strength, which has also been observed by Stadlmayr et al. (submitted 2010).

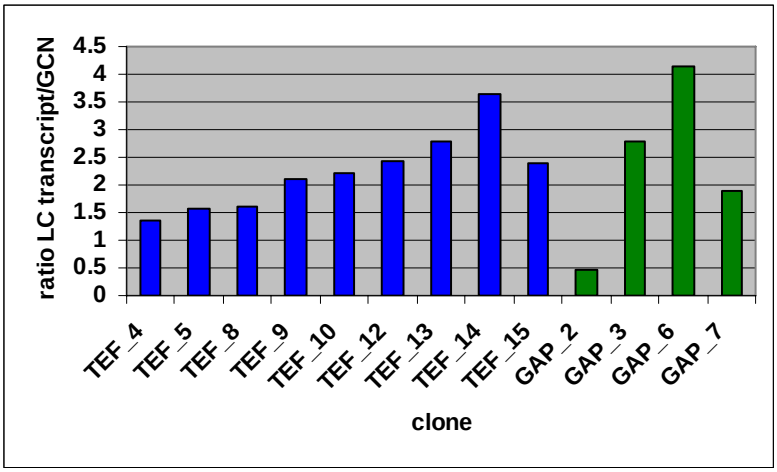

Fig. 34: Ratio transcript/gene copies of the light chain gene

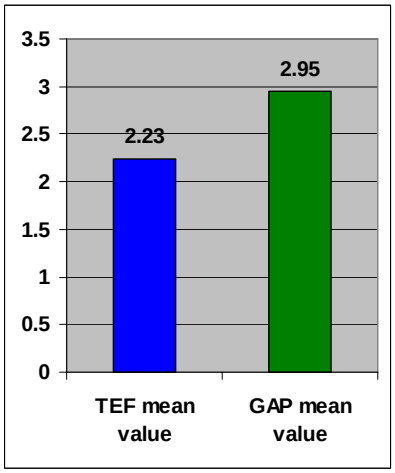

Fig. 35: Ratio transcript/GCN of the LC-gene: Comparison of the mean values of the TEF- to the GAP-clones (without GAP 2)

The ratio of the LC transcript per GCN, is in agreement with the previous analyses and confirm a gene copy number effect for the light chain of the high producers. As can be seen in Fig. 34, more than one copy of the light chain gene has been integrated to the genome of those clones. In the calculation of the mean values, the outlier GAP 2 was not considered. The LC ratio transcript per GCN for the TEF clones was approximately in the range of the one from the GAP clones (mean value). This is plausible as in both constructs the LC gene was under expression control of the GAP-promoter. The statistical analysis by the Student´s t-test confirmed that the difference between the mean values was not significant (P= 0.3944 > 0.05).

For validation of previous results and to get information about absolute gene copy numbers, Southern blot was performed as concluding method. The HC-bands were very weak, which made a confidential interpretation hard, but it showed again the lower copy number in comparison to the light chain gene. The light chain Southern blot gave for one (TEF 4) of the two tested high producing clones a significant higher signal than for the other clones.

The HC:LC of 0.12 (0.16:1.31) ratio clarifies the light chain dominance of this clone even more:

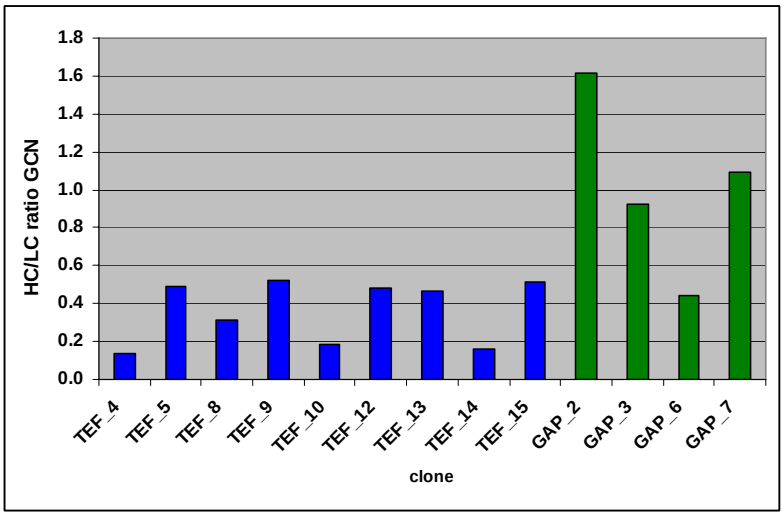

Fig. 36: Ratio gene copies heavy chain gene per gene copies light chain gene (qRT-PCR)

4.5.2 Correlation between the produced protein amounts and the LC:HC GCN

Regarding the productivity P (concentration of the product in mg/L per concentration of the yeast wet mass in g/L after 48h) resulting from the ELISA in comparison to the ratio of calculated LC:HC copies from the qRT-PCR, the correlation between them for each clone became evident (see Fig. 40). This is plausible and can be explained with reagard to the protein-fragments that are determined by this ELISA, which are the whole assembled Fab as well as LC-homodimers and free LC.

Table 39: Number of the clones in the diagram
(in ascending order of the q-RT-PCR-values)

clone number	clone
1	GAP_2
2	GAP_7
3	GAP_3
4	TEF_15
5	TEF_9
6	TEF_5
7	TEF_12
8	TEF_13
9	TEF_8
10	GAP_6
11	TEF_14
12	TEF_4
13	TEF_10

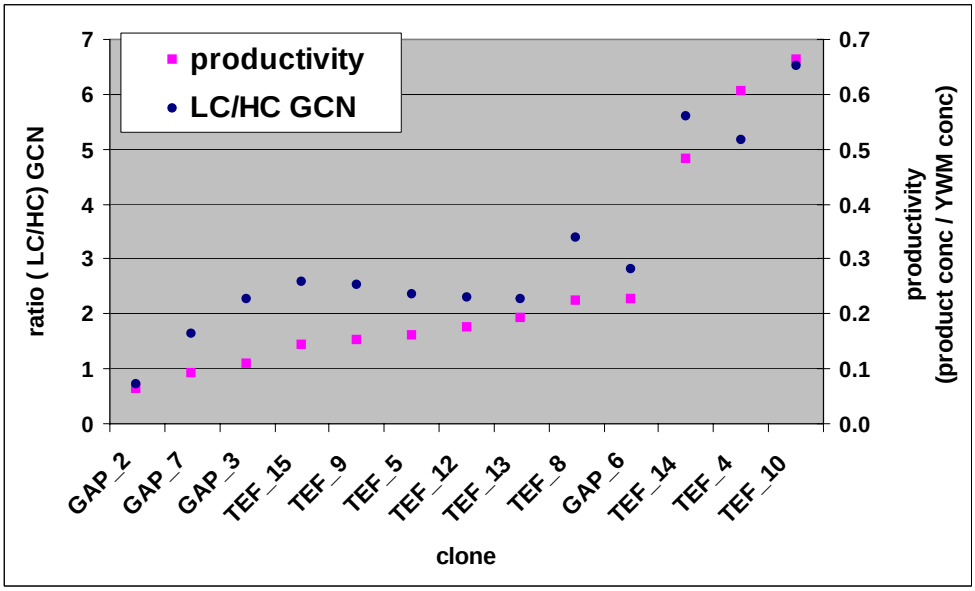

Fig. 37: Correlation of the productivity (in concentration of the product in mg/L per concentration of the yeast wet mass in g/L after 48h) to the ratio calculated gene copies light chain /calculated gene copies heavy chain

4.6 Influence of the HC:LC- ratio on the Fab expression

A critical point in antibody as well as Fab production is the assembly of the antibody (fragment) in the endoplasmatic reticulum (ER). One bottle neck for the correct assembly of the chains in *P. pastoris* seems to be the amount of heavy chain in relation to the amount of light chain (the expression ratio HC:LC). This has also been observed in previous studies for Fabs in other expression systems (Leonard et al. 2006), as well as for whole IgG antibodies (Li et al. 2007). As antibodies have a hetero-dimeric structure, an equivalent molar ratio of light and heavy chain polypeptides was attempted in most studies concentrating on production of recombinant antibodies and fragments thereof. Li and co-workers suggest an intracellular HC:LC molar ratio about 1 in HEK 293T cells (Human Embryonic Kidney Cells) and 0.58 in CHO-K1 cells (Chinese Hamster Ovary cells) (both transiently transfected) to reach an optimal result for the IgG yield in the culture supernatant.

In the present work also a quite balanced HC:LC ratio seems to be advantageous for high yields of 3H6-Fab in the supernatant. A significantly higher amount of light chain gives no higher yields of Fab, but leads to secretion of LC-monomers and dimers thus causing impurities. A similar effect has been observed for Fab' in the periplasm of *E. coli* by Humphreys et al. (2002). They reported that a slightly higher amount of LC over HC is permitted to a certain degree, but a significantly excess of LC is inhibiting the production of high yields of Fab'.

By altering the HC and LC gene ratio, the optimal ratio can be determined. This strategy was for example used by Schlatter et al. 2005 in mammalian cells (CHO). In their work the optimal HC:LC ratio on the gene level for transiently transfected cells was determined, which reached the highest yields of a whole monoclonal antibody and was then compared to the HC:LC polypeptide ratio. Additionally they determined the HC:LC ratios of stably transfected cells with a HC:LC gene ratio of 1:1, for the transiently transfected cells the optimal HC:LC gene ratio was 1.5.

4.7 Conclusion and further perspectives

The results demonstrate that the 3H6 Fab production in *P. pastoris* by co-expression of the HC and the LC from separate vectors under the control of different constitutive promoters lead to Fab formation and secretion. The productivity could not be increased by the expression of the HC under control of the TEF promoter. Furthermore the co-expression of the Fab-chains from separate vectors had no positive effect on the Fab-yields.

The HC:LC ratio is an important point for the recombinant Fab production, the optimum seems to be a quite balanced ratio. The higher gene copy number of the LC gene that some clones showed, led to an increase of the amount of LC transcript and protein, but not of intact Fab. A reason for this result could be that no higher amounts of HC transcript were available due to a low GCN of the HC gene. An attempt to overcome this problem could be the directed amplification of the GCN. One strategy in *P. pastoris* was recently described by Marx et al. 2009. They amplified the gene copies of human serum albumin and human superoxide dismutase by integration into the ribosomal locus. They report to get reliably higher copy numbers by selection on increasing concentrations of the antibiotic Zeocin. This method appears to be also a promising strategy for 3H6-Fab by alternate, separate amplification of the LC and the HC gene copy number as the present work showed that efficient co-expression from separate vectors of this antibody fragment in *P. pastoris* is possible.

On the whole, it can be concluded that the production of high yields of Fab depends on a lot of influencing factors. Beside the critical points analysed in the present work, stability of the mRNA and the expressed Fab, folding, assembly (disulphide bridge formation), glycosylation and secretion play also an important role. The demand for antibody fragments will definitely increase in the future due to their properties like easy penetration into tissues invaded by antigens and at the same time shorter retention time in non-target tissues, their lower immunogenicity and therefore less side effects.

P. pastoris has shown to be a potential host for large scale production. The challenge for the next years will be the optimization of this expression system for Fab production by strategies like choice of the right promoter, co-expression of helper-factors, codon optimization, balancing of the HC:LC ratio or the amplification of the GCN. The publication of the whole genome sequence (9.43 Mbp) of two different *P. pastoris* strains in 2009 (De Schutter et al., Mattanovich et al.) will facilitate these studies and will open up new possibilities for working with this yeast as an expression system for antibodies and fragments thereof.

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6 Appendix

Table 40: QRT-PCR data HC transcript: Calculated copies of the heavy chain transcript and the corresponding values of the reference gene actin

Clone	aver. calc copies_ HC_transcript	aver. calc copies_ actin_transcript	calc conc HC/actin 1st value	calc conc HC/actin 2nd value	aver. HC/actin transcript
TEF_4	3341530.2	23961378.7	0.18	0.11	**0.15**
TEF_5	426380.3	5511225.3	0.08	-	**0.08**
TEF_8	1670794.7	8184279.5	0.20	-	**0.20**
TEF_9	5645453.2	28523600.1	0.20	-	**0.20**
TEF_10	768021	6036208.8	0.15	0.11	**0.13**
TEF_12	341816.3	4606610.1	0.11	0.06	**0.08**
TEF_13	1179876.2	7217797.7	0.16	0.16	**0.16**
TEF_14	1398313.7	7736379.2	0.20	0.17	**0.19**
TEF_15	687848.1	3255380.1	0.21	0.77	**0.49**
GAP_2	4473110.8	4652742.5	1.19	0.77	**0.98**
GAP_3	2730828.8	5401926.4	0.66	0.38	**0.52**
GAP_5	10188631.5	7589356.4	1.34	-	**1.34**
GAP_6	943976.4	3781243.9	0.24	0.26	**0.25**
GAP_7	10941590.5	7139317.1	1.50	1.57	**1.54**
PK_1	320.8	4254896.5	0.00	0.00	**0.00**
PK_2	4658.4	4354871.1	0.00	0.00	**0.00**
PK_3	1178.6	5325998.8	0.00	0.00	**0.00**
PK_4	577.9	4862028	0.00	0.00	**0.00**
PK_5	3101.3	8072184.2	0.00	0.00	**0.00**

Table 41: QRT-PCR data HC GCN: calculated gene copies of the heavy chain gene copies and the corresponding values of the reference gene actin

clone	aver. calc copies_HC	aver. calc copies actin	calc cop. HC/actin 1st value	calc cop . HC/actin 2nd value	aver . HC/actin
TEF_4	75279.2	219545.3	0.36	0.38	**0.37**
TEF_5	11491.9	48146.5	0.33	0.19	**0.26**
TEF_8	45707.3	157940.3	0.49	0.19	**0.34**
TEF_9	25710.6	103236.9	0.33	0.19	**0.26**
TEF_10	21042.6	42844.7	0.32	0.66	**0.49**
TEF_12	41408.5	80029.3	0.36	0.67	**0.52**
TEF_13	23256.8	47771	0.33	0.65	**0.49**
TEF_14	20132.8	39531.1	0.30	0.71	**0.51**
TEF_15	38137.8	125981.5	0.45	0.22	**0.34**
GAP_2	69810.5	67696.4	1.42	0.65	**1.03**
GAP_3	27236.6	40379.3	0.88	0.47	**0.67**
GAP_5	73124	54776	1.85	0.82	**1.33**
GAP_6	27292.9	116049.2	0.29	0.18	**0.24**
GAP_7	45218.7	35952.1	1.69	0.82	**1.26**
PK_1	140.1	211156.2	0.00	0.00	**0.00**
PK_2	173	20132.1	0.01	0.01	**0.01**
PK_3	175	74414.3	0.00	0.00	**0.00**
PK_4	102.4	70190.8	0.00	0.00	**0.00**
PK_5	96.6	64799.9	0.00	0.00	**0.00**

Table 42: QRT-PCR data LC transcript: Calculated copies of the light chain transcript and the corresponding values of the reference gene actin

clone	aver. calc copies_ LC_transcript	aver. calc copies_ actin_transcript	calc conc LC/actin 1st value	calc conc LC/actin 2nd value	aver. LC/actin transcript
TEF_4	53272714.9	17942421	2.97	-	**2.97**
TEF_5	4540443.9	5511225.3	0.82	-	**0.82**
TEF_8	14363676.6	8184279.5	1.76	-	**1.76**
TEF_9	29792944.6	28523600.1	1.04	-	**1.04**
TEF_10	23966915.4	5049135.9	4.75	-	**4.75**
TEF_12	4888481.8	3164775.5	1.54	-	**1.54**
TEF_13	9421035.4	5409035.1	1.74	-	**1.74**
TEF_14	29158288.7	5422932.9	5.38	-	**5.38**
TEF_15	5132074	3255380.1	1.58	-	**1.58**
GAP_2	1343916.2	3751091.2	0.29	0.45	**0.37**
GAP_3	9963094.1	4061609.2	2.05	3.03	**2.54**
GAP_5	7264.2	5888889.6	0.00	0.00	**0.00**
GAP_6	8426069.2	2921537.6	2.21	4.15	**3.18**
GAP_7	16929918.7	6084028.5	2.18	3.83	**3.00**
PK_1	11126210.4	3619174.7	2.43	4.14	**3.29**
PK_2	12027713.5	3963593.9	2.71	3.54	**3.13**
PK_3	18313470.7	4253647.3	3.77	5.06	**4.42**
PK_4	16358622.3	3564822.8	3.69	6.14	**4.91**
PK_5	26118970.6	6337804.8	3.48	5.27	**4.38**

Table 43: QRT-PCR data LC GCN: Calculated copies of the light chain gene (LC) and the corresponding values of the reference gene actin

clone	aver. calc copies_LC	aver. calc copies actin	calc cop. LC/actin 1st value	calc cop. LC/actin 2nd value	Aver . LC/actin
TEF_4	472906.2	222559.6	2	2.39	**2.19**
TEF_5	26095.1	48146.5	0.47	0.58	**0.53**
TEF_8	166356.5	157940.3	1.21	0.98	**1.09**
TEF_9	52389.9	103236.9	0.44	0.55	**0.5**
TEF_10	104369	48173.3	1.82	2.45	**2.13**
TEF_12	57117	88389.2	0.55	0.72	**0.64**
TEF_13	33635.5	53028.8	0.55	0.7	**0.63**
TEF_14	71351.6	48477.8	1.47	1.47	**1.47**
TEF_15	83121.7	125981.5	0.64	0.67	**0.66**
GAP_2	38841.6	61391.1	0.7	0.58	**0.64**
GAP_3	28436.1	38694.7	0.68	0.79	**0.73**
GAP_5	311.7	46297.4	0.01	0.01	**0.01**
GAP_6	53616.8	116049.2	13.17	0.53	**0.53**
GAP_7	80707.1	61734.5	1.15	2.51	**1.15**
PK_1	96856.6	187540.9	0.51	0.52	**0.52**
PK_2	9577.4	18803.3	0.49	0.53	**0.51**
PK_3	39386.3	65969.2	0.67	0.54	**0.6**
PK_4	38029.4	63908.7	0.65	0.55	**0.6**
PK_5	34471.7	60230.6	0.64	0.52	**0.58**

not within the standards --> not used in calculation

Table 44: Ratio transcript HC per gene copy number HC:

clone	transcript HC/actin	GCN HC/actin	ratio transcript/GCN HC
TEF_4	0.15	0.36	**0.41**
TEF_5	0.08	0.33	**0.23**
TEF_8	0.20	0.49	**0.42**
TEF_9	0.20	0.33	**0.61**
TEF_10	0.13	0.32	**0.41**
TEF_12	0.08	0.36	**0.23**
TEF_13	0.16	0.33	**0.50**
TEF_14	0.19	0.30	**0.61**
TEF_15	0.49	0.45	**1.08**
GAP_2	0.98	1.03	**0.95**
GAP_3	0.52	0.67	**0.77**
GAP_5	1.34	1.33	**1.01**
GAP_6	0.25	0.24	**1.06**
GAP_7	1.54	1.26	**1.22**

Table 45: Ratio transcript LC per gene copy number LC:

clone	transcript LC/actin	GCN LC/actin	ratio transcript/GCN LC
TEF_4	2.97	2.19	**1.35**
TEF_5	0.82	0.53	**1.56**
TEF_8	1.76	1.09	**1.60**
TEF_9	1.04	0.50	**2.10**
TEF_10	4.75	2.13	**2.23**
TEF_12	1.54	0.64	**2.42**
TEF_13	1.74	0.63	**2.78**
TEF_14	5.38	1.47	**3.65**
TEF_15	1.58	0.66	**2.41**
GAP_2	0.37	0.64	**0.58**
GAP_3	2.54	0.73	**3.47**
GAP_5	0.00	0.01	**0.00**
GAP_6	3.18	0.53	**5.98**
GAP_7	3.00	1.15	**2.60**