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UPTEC X 15 021
Examensarbete 30 hp
Augusti 2015

A yeast three-hybrid (Y3H) screen to evolve BirA biotin ligase variants with histone biotinylation activity

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UPPSALA UNIVERSITET

Degree Project in Molecular Biotechnology

Masters Programme in Molecular Biotechnology Engineering,
Uppsala University School of Engineering

UPTEC X 15 021	**Date of issue 2015-08**

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Title (English)

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Title (Swedish)

Abstract

Histones are heavily modified and marked with epigenetic tags. How these essential proteins together with their modifications are faithfully transmitted through mitotic cell division is still an unanswered question. Such process could be studied using a synthetic modification to tag and trace histone proteins. *In vivo* lysine biotinylation of histones could ideally serve as a synthetic marker. To be able to bind biotin to histones *in vivo*, an enzyme with that capacity would be necessary. Preferably an engineered specific enzyme that only biotinylate histones. BirA is an enzyme that regulates biotinylation in eukaryotic and prokaryotic cells. This project is an attempt to create a BirA mutant through directed evolution, with the capability to biotinylate histones *in vivo*.

Keywords

Yeast three-hybrid, Y3H, BirA, Biotin, directed evolution.

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **36**

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Populärvetenskaplig sammanfattning

Länge var det trott att gener tillsammans med miljö var de enda faktorer som styrde över våra fysiska egenskaper. På senare tid har epigenetiken dock fått allt större utrymme och beskrivs numera som en brygga mellan arv och miljö. Epigenetiska markörer kan styra över vilka, och hur, gener uttrycks i våra celler och påverkar därför också troligen hur vi ser ut och fungerar. Exakt hur och i vilken utsträckning epigenomet ärvs mellan generationer är okänt. Eftersom epigenomet till viss del styr över genomet, är information om hur det fungerar inte bara intressant för forskarna utan också för läkemedelsindustrin och hälsovården.

Detta arbete är ett försök att tillverka ett verktyg som kan användas för att spåra hur en epigenetisk markör ärvs. Verktyget är ett enzym som ska kunna fästa en syntetisk epigenetisk etikett på histoner, ett sorts protein som DNA lindas runt i våra celler och som är en naturlig bärare av olika former av epigenetiska etiketter. Hur en sådan syntetisk flagga ärvs mellan generationer skulle kunna ge ledtrådar till hur epigenetiska markörer ärvs. I detta projekt har ett litet steg på vägen emot ett sådant enzym tagits.

Examensarbete 30 hp
Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet, Augusti 2015

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1 ABBREVIATIONS

AD	Activation domain
DB	DNA binding domain
SDO	Single drop out media
DDO	Double drop out media
QDO	Quadruple drop out media
DDO/X/A	Double drop out media with X-α-Gal and Aureobasidin A
QDO/X/A	Quadruple drop out media with X-α-Gal and Aureobasidin A
Y2H	Yeast two hybrid system
Y3H	Yeast three hybrid system
mSA	Monovalent streptavidin

2 INTRODUCTION

2.1 Background

Biotinylation of proteins has found many applications in biotechnology methods. It is the sub-nanomolar affinity between the small molecule Biotin and the protein streptavidin that is very useful and widely used in research (Weber *et al*., 1989, Wilchek *et al*., 2006). Biotinylation can be used to label proteins in various biotechnology assays, by chemically attaching biotin to a protein *in vitro* (Wilchek and Bayer, 1990). Versions of streptavidin have been developed that bind Biotin with reduced affinity (Lim *et al*., 2013).

BirA is the enzyme that facilitates biotinylation in nature. It is a biotin protein ligase that biotinylates one of the subunits (BCCP) of the enzyme acetyl-CoA carboxylase that is involved in the fatty acid synthesis. BirA transfers biotin onto a specific lysine residue in the BCCP protein. In *Escherichia coli*, this is the only protein that is biotinylated in the cell. Even though other organisms can have a few biotin dependent proteins, the reaction that BirA catalyze is considered highly specific. (Chapman-Smith and Cronan, 1999)

The DNA in an eukaryotic cell is packaged around histones in structures called nucleosomes (Kornberg, 1977). In each nucleosome, eight histones form an octameric protein core around which approximately two turns of DNA are wrapped. The N-teminal tails of the histones are exposed, making modification of those amino acids possible (Luger *et al*., 1997). A large number of posttranslational modifications are known to occur on the histone tails, and in particular on lysine residues. The lysine can undergo several different modifications including mono-,di and trimethylation and acetylation (Bannister and Kouzarides, 2011).

Whether biotinylation of histones is a natural phenomenon in eukaryotic cells has been a disputable subject. Stanley *et al.* (2001) stated that biotin is found covalently bond to all 5 classes of histones in human cells. Subsequent studies have found biotinylated histones in various cell lines (Kothapallia, *et al*. 2005). However Healy *et al.* (2009) critically inspected the methods used to detect biotin attachment on histones and claimed in 2009 that Biotin is not a natural histone modification in living cells. Later studies concluded that biotinylation of human histones are a rare and most likely an off-target or non-enzymatic event (less then 0.001%), and its biological impact in the cells can be questioned (Kuroishi *et al*. 2011). Thus, biotinylation of lysine residues would represent a synthetic and orthogonal means of modifying histone proteins.

Replicating the DNA poses a structural problem onto chromatin – histones have to be completely removed from the DNA molecule for the replication fork to passage through chromatin. At the replication fork, DNA strands are separated and copied into two daughter strands by two DNA polymerases. It is unclear how histones are shuttled and distributed to reassemble chromatin on the two newly formed double stranded DNA molecules behind the replication fork, and in particular how epigenetic markers along the DNA are maintained. Histones are heavily modified and marked with epigenetic tags. How these essential proteins together with their

modifications are faithfully transmitted through mitotic cell division are still an unanswered question (Jasencakova and Groth, 2010). (Probst *et al.*, 2009). Such process could be studied using a synthetic modification to tag and trace histone proteins.

Assuming that biotinylation of histones in fact is extremely rare in eukaryotic cells, *in vivo* lysine biotinylation could ideally serve as a synthetic histone mark. To be able to attach biotin to histones *in vivo*, an enzyme with that capacity would be necessary. Preferably an engineered specific enzyme that only biotinylate histones, or even better a defined site in a single histone. This project is an attempt to create a BirA mutant, with the capability to biotinylate histones *in vivo*.

2.2 Yeast Two-Hybrid system

The Yeast Two-Hybrid System (Y2H) is a method to search for protein-protein interactions in yeast. Chien *et al.* developed the method in 1991 based on earlier studies by Fields and Song (1989). The basic application of the method involves screening a library of proteins or fragments of proteins (pray proteins), originated from a cDNA library, against a protein of interest (bait), see figure 1. The selection procedure reveals hits that can bind the protein of interest. The biological technique has been widely used in research since it appeared 25 years ago (Vidal and Fields, 2014).

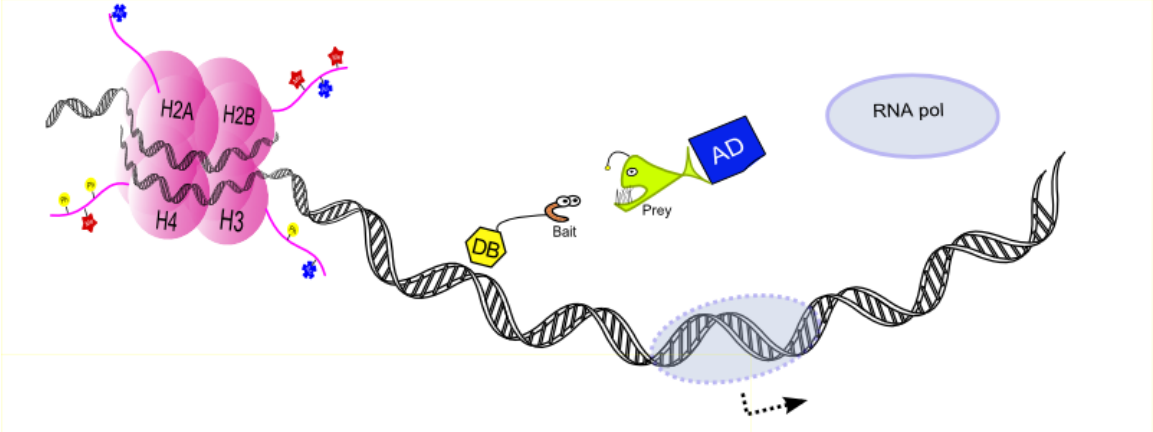


Figure 1. Yeast Two hybrid System. The DNA binding domain (DB) binds the promotor sequence, if Bait and Pray proteins bind to each other, the activation domain (AD; which is a transcription factor) recruit RNA polymerase to the site and transcription is stimulated.

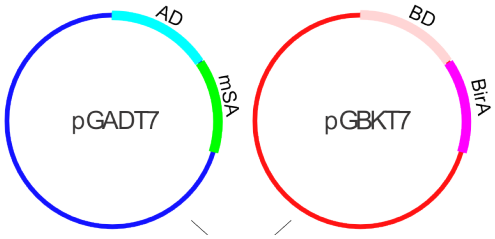
The Matchmaker® Gold Yeast Two-Hybrid System is a GAL4-based Y2H system from Clontech Laboratories. Gal4 is a native transcription factor in yeast, which activate transcription of a gene if galactose is present. The Ptashne Laboratory discovered that Gal4 could be separated into two fragments, one DNA-binding domain and one activating domain. When the two fragments reunite they can form a functional Gal4 again (Keegan *et al.*, 1986). This discovery came to be the fundamental building block in the screening method.

2.2.1 The screening method

The system is based on Gal4-dependent induction of reporter and selection genes. Transcription is highly regulated in eukaryotic cells and requires a transcriptional activation domain (AD) to be stimulated. To recruit the activation domain to the target gene (reporters and selection markers) in the yeast genome, a DNA- binding domain (DB) is required. The pray and bait proteins are fused to respective domain. When the bait protein interacts with one of the pray proteins, the AD domain is brought in contact with the BD domain and transcription of the reporter and selection genes are activated (Brückner *et al.*, 2009).

The pray and bait must be fused to the separated DB and AD domains, respectively. The protein-coding sequence are cloned in frame as a C-terminal fusion to the DB and AD domains in two plasmids, see figure 2. The plasmids are transformed in to two different strains of *Saccharomyces cerevisiae*, the Y2H strain and the Y187 strain. These strains can then be mated to create diploid yeast with both plasmids. It is inside the diploids that protein interactions between the pray and bait can occur (Brückner et al., 2009).

A



B

Plasmid	Type
BD-BirA	Wildtype, negatve control
BD-BirA*	Promiscuous enzyme, positive control
L-BD-BirA 310G	Library plasmid, Bait
L-BD-BirA 310A	Library plasmid, Bait
L-BD-BirA	Library plasmid, Bait
AD-msA	Pray

Figure 2. A; Prey and bait proteins fused to respective domain in two separate plasmids. B; All the final plasmids created before library formation. Three slightly different versions of BirA were used when creating the libraries; L-BD-BirA, L-BD-BirA 310G and L-BD-BirA 310A.

2.2.2 The selection of yeast

Different media are used to select and control the growth of yeast cells. Drop out media, which lack one or several of the nutrients required for normal yeast, are used to select for transformed yeast. In this project two different types of Single drop out (SDO) media were used to select for each plasmid, and Double drop out (DDO) media select for diploids containing both plasmids. DDO/X/A is the media used to select for diploids that have interacting pray and bait proteins. "X" stands for X-α-Gal, which is a substrate that will turn the cells blue if the MEL1 gene is active. "A" stands for Aureobasidin A, which is a very toxic drug to yeast cells unless a resistance gene (AUR1-C) is activated. A list of all the selection and reporter genes can be seen in table 1.

2.2.3 The promotors and selective genes

There are four reporter genes under the control of three different promotors in the Matchmaker® Gold Yeast Two-Hybrid System. A Gal4-DNA-Binding site is engineered into each of the heterologous promotors, G1, G2 and M1. The promotors are unrelated in sequence to each other and the pray and bait proteins must activate transcription on all three sites for a cell to survive on selective DDO/X/A media.

Table 1. A list of the selection genes that are used in the system. A positive hit of mated diploid yeast cells will have all the genes in the table activated.

Gene	Selection/reporter	Strain
AUR1-C	Aureobasidin resistance	Y2H
HIS3	Survival on media without histidine (QDO)	Y2H
ADE2	Survival on media without adenine (QDO)	Y2H
MEL1	Encodes α-galactosidase, turn colonies blue in the presence of its substrate X-α-Gal	Y2H and Y187
Gene	**Selection**	**Plasmid**
LEU2	Survival on media without Leucine (SD–Leu)	pGADT7
TRP1	Survival on media without Tryptophan (SD–Trp)	pGBKT7

2.3 Yeast three-hybrid screen for directed evolution of a histone biotinylation enzyme

In this project, the Matchmaker® Gold Yeast Two-Hybrid System was used to screen through a library of BirA mutants. However, the system was used in a slightly different way than described above, and works through an intermediate enzymatic modification, biotinylation, thus referred to as a Yeast-three hybrid (Y3H) screen. BirA mutants are directed to the DNA, and must biotinylate yeast chromatin before monovalent streptavidin (mSA) can bind to a biotin group somewhere along the DNA. Thus BirA mutant libraries are fused to the DNA-binding domain (BD), to be recruited to the reporter genes. Monovalent streptavidin (mSA) is coupled to the transcription activator domain (AD). Gene activation is achieved when a BirA mutant is able to locally biotinylate a chromatin component that can recruit the mSA-AD fusion, see figure 3.

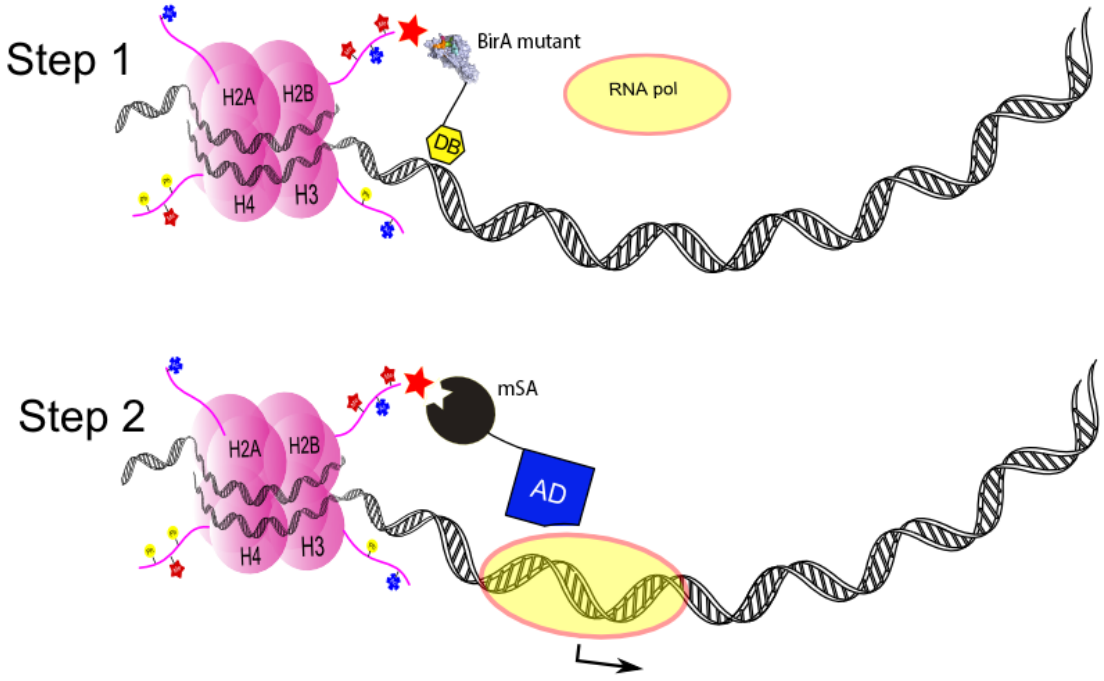


Figure 3. Yeast three-hybrid screen for directed evolution of a histone biotinylation enzyme.

2.4 Different variants of BirA

New variants of the wild type biotin ligase enzyme BirA can be developed with common biotechnology methods. These mutant enzymes could possess the ability to biotinylate a Y3H system to select for positive hits. The ambition is to develop an enzyme that can put biotin groups on one of the lysine tails of histones in nucleosomes. Figure 2B contains a table with all the final plasmids created for the screen.

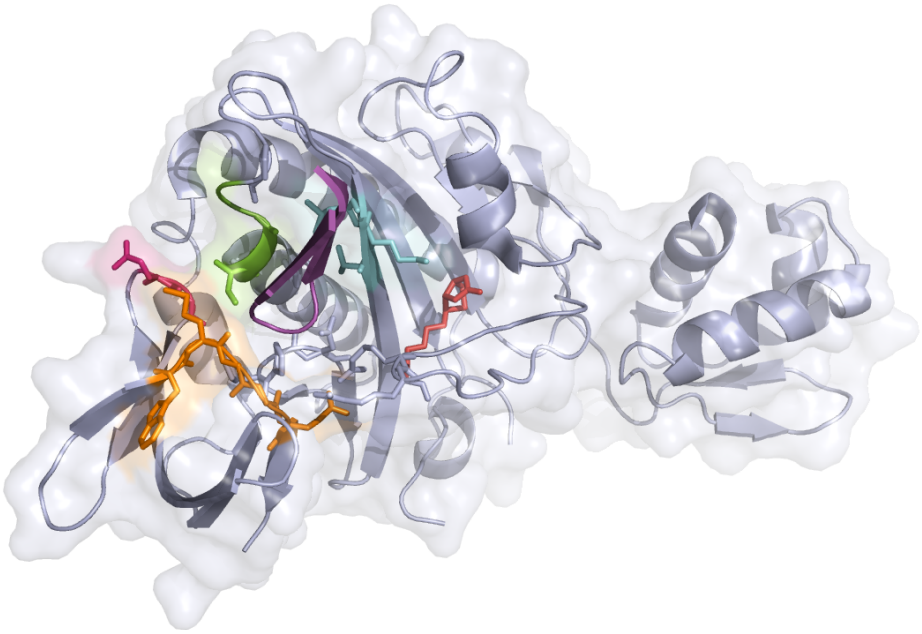
2.4.1 The positive control

In any scientific experiment a positive and negative control are usually required to ensure a reliable result. In earlier studies a promiscuous variant of BirA mutant has been reported. It differs from the native enzyme in one single codon, R118G (Kwon *et al*, 2000). The enzyme has been shown to effectively biotinylate proteins in a promiscuous manner both in *E. coli* and mammalian cells (Choi-Rhee *et al*., 2004; Roux *et al*., 2012). The promiscuous enzyme, BirA*, could act as a positive control in this screen, when fused with the DNA binding domain.

2.4.2 The library design

A library of BirA mutants can be created in a PCR with randomized primers. The sites of randomization were based on rational structural analyses. Randomizing primers can be designed in different ways. A certain type of amino acids can be selected by randomizing a codon position to amino acids with the desired characteristic. The amino acids around the active site of the native enzyme are mostly hydrophilic. By controlled randomization of codons (with bases DVT), identity of randomized amino acids was skewed towards polar and negatively charged amino acids, which were expected to be most suited for creating binding surfaces for the positively charged histone tails. The structure and active site of BirA is taken into account when designing the library.

Five different regions on the enzyme surface were changed with randomizing primers. Each codon was replaced with the codon library containing 8 hydrophilic amino acid (A, C, D, G, N, S, T, Y). As seen in the table in figure 2B, three slightly different variations of the BirA were used as templates when creating the libraries. In two, Met310 was mutated to a small amino acid to widen the substrate binding pocket. All the libraries are listed in figure 4. These libraries can be used separately in individual screens, but in the future, a selection of them can be put together into one library.



Plasmid	Site of Mutation (protein sequence)
L-BD-BirA	144, AAAI
	173, WPN
	189, VEL
	295, QG
	310, WMGG
L-BD-BirA M310G	144, AAAI
	173, WPN
	189, VEL
	295, QG
L-BD-BirA M310A	144, AAAI
	173, WPN
	189, VEL
	295, QG

Figure 4. A view of the native BirA enzyme. Each color represent a position near the active site that undergo library creation. In the table to the right all the different libraries are listed together with the amino acids that is replaced. The number indicating 'site of mutation' is referring to the amino acid sequence.

2.5 Analysing the outcome of the assay

Colonies growing on DDO/X/A selective plates after mating were further selected by patching the colonies to QDO/X/A. Cells that survived on the more selective plate were further analysed by western blot, to visualise biotinylated proteins and by sequencing, to reveal the identity of the mutant enzyme.

3 MATERIAL AND METHODS

3.1 Provided material and equipment

The Y185 Yeast strain and Y2HGold Yeast strains of *Saccharomyces Cerevisiae* were provided in the Matchmaker® Gold Yeast Two-Hybrid System from Clontech Laboratories, as well as the two plasmids, pGADT7 and pGBKT7 (see figure 12 and 13 in supplementaries). The pACYC plasmid with the native BirA sequence was provided by the Protein science Facility at MBB of Karolinska Institutet in Solna (see figure 13 in supplementaries). All PCR amplifications were made with AmpliTaq DNA Polymerase and purified with GeneJET PCR Purification Kit from Thermo Scientific. OD_{600} refers to the optical density of the cell culture at 600 nm.

3.2 General

For all methods except Western Blot, media and chemicals used were either autoclaved or pressed through a sterile filter to prevent contamination. All new sequences created were sent to Eurofins genomics for Sanger sequencing and the result were analysed with CLC sequence viewer before used in further steps. Three different variations of BirA were template for library construction. However only the library based on the native BirA sequence was mated, the BirA 310G and BirA 310A will be analysed later due to time and scale up limitations.

3.3 Plasmid construction

A flowchart of the plasmid construction can be seen in figure 5. The purpose of the figure is to get an overview of the cloning steps.

3.3.1 pGADT7-mSA

A synthetic template sequence was ordered for mSA. The sequence for mSA was fused with the AD in the pGADT7 plasmid. The mSA insertion sequence was synthesized, designed with 15 overlap in ordering to be cloned directly into a linearized pGADT7 vector. pGADT7 was linearized by cutting the single restriction sites, BamHI and EcoRI, with Thermo Scientific™ FastDigest™ restriction enzymes (see vector in Supplementaries). In-Fusion ® HD Cloning Kit was used to fuse the synthesized insertion sequence with the vector.

3.3.2 pGBKT7 -BirA and mutants

The native BirA sequence was inserted in the pGBKT7 plasmid. The pACYC plasmid was used as a template in a PCR with appropriate primers to amplify the BirA sequence. DpnI digestion of the template sequence was done. DpnI is an enzyme that cuts methylated DNA. The PCR primers was designed with a 15bp nucleotide overhang, corresponding to the target site in the pACYC plasmid. The BirA insertion sequence was then inserted downstream of the BirA gene in the vector. pGBKT7 was linearized by cutting the two single restriction sites, EcoRI and smal, with Thermo Scientific™ FastDigest™ restriction enzymes (see vector in Supplementaries). The In-Fusion ® HD Cloning Kit was used to fuse the insertion sequence with the vector. The necessary point mutations to the BirA sequence were created through site-directed mutagenesis (BirA*, BirA310G and BirA310A). The whole BirA-BD was

then reunited with the pGBKT7 backbone when transforming the yeast (see 3.4 Yeast competent cells and transformation).

3.3.3 Modifications to the native BirA sequence by site-directed mutagenesis

All BirA point mutants were engineered from the pGBKT7-BirA plasmid in a PCR with phosphorylated primers. The primers was designed with a flanking region corresponding to the site of mutation. Thermo Scientific™ T4 DNA Ligase was used to ligate the ends of the vectors.

3.3.4 Cloning with In-Fusion ® HD Cloning Kit

The In-Fusion® HD Cloning Kit was used to fuse the pray and bait to respective domain. The method was used to insert the sequence for mSA right after the AD domain in the pGADT7 plasmid. In-Fusion was also used to paste in the BirA sequence downstream of the BD domain in the pGBKT7 plasmid, see figure 5. In-Fusion is an enzyme that fuses a DNA insertion sequence with a linearized vector. The DNA sequence must have a 15 base pair overlapping homology at both ends. 2µl digested vector, 2µl insert and 1 µl In-Fusion® mastermix was prepared on ice. The mixture was then incubated at 50°C for 15 minutes. The reaction mix was placed on ice prior to transformation.

3.3.5 Transformation of E. coli and amplification of plasmids

Plasmids created with the In-Fusion® HD Cloning Kit were transformed with stellar competent cells (*Escherichia coli* HST08 strain) from Clontech Laboratories. 2µl of the In-fusion mixture were added to 50 µl of cell suspension. The mixture was incubated on ice for 30 minutes, then heat-shocked in a 42°C water bath for 55 seconds. The cells were allowed to recover for 1 hour in 37°C with shaking. The cells were plated on LA with appropriate antibiotic (ampicillin [100 µg/ml] or Kanamycin [50 µg/ml]). Plates were incubated in 37 °C over night. 3-5 single colonies from each plate was selected and amplified in an overnight culture in LA media. Extraction and purification of the plasmids were done with the Thermo Scientific GeneJET Plasmid Miniprep Kit. Final plasmids were Sanger sequenced for validation.

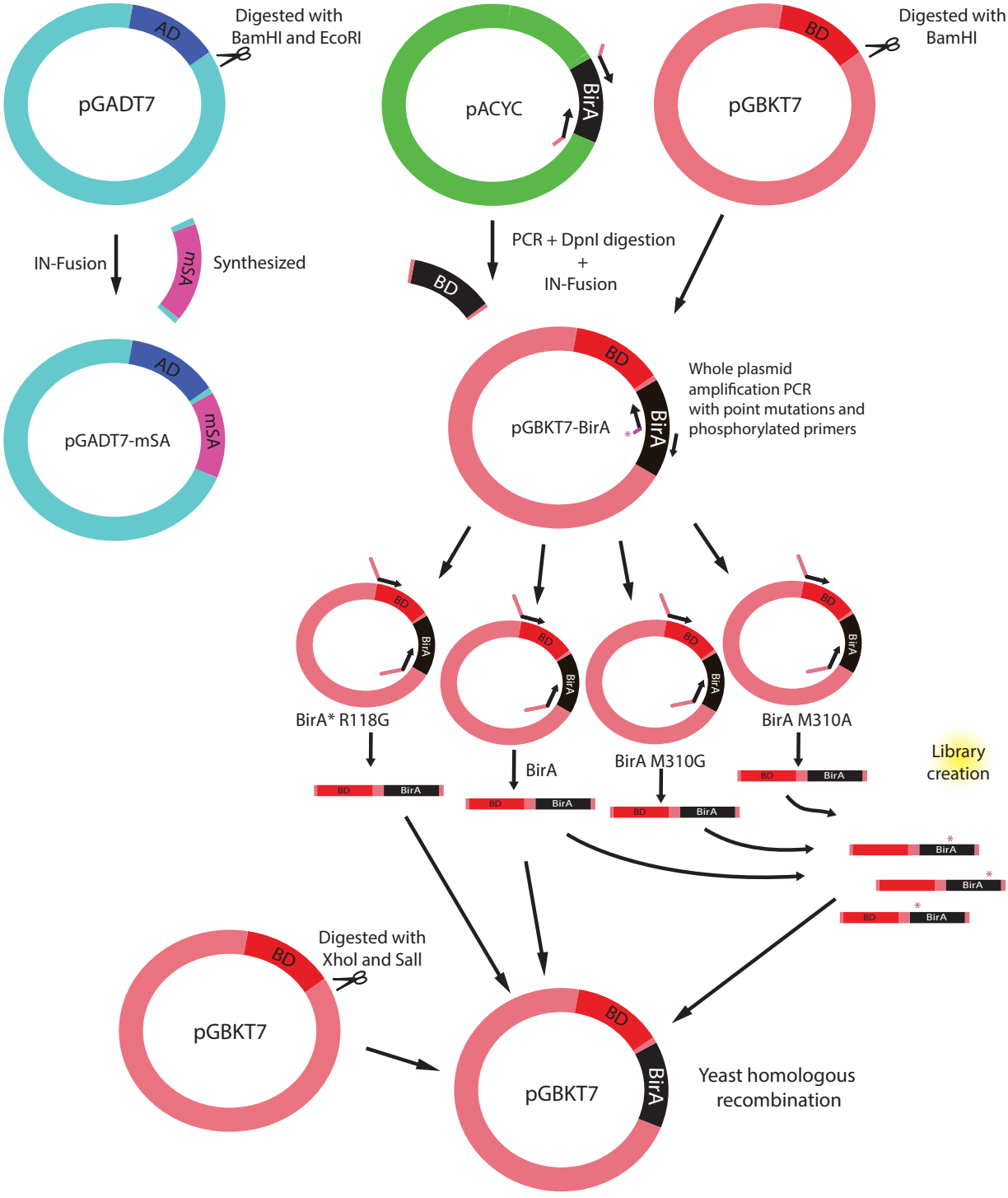


Figure 5. Flowchart of plasmid construction, the starting plasmids can be seen in the supplementaries. As seen in the figure, In-Fusion was used to paste in Bait and Prey (BirA and mSA) to respective domain. After this, point mutations to the BirA sequence were created with whole plasmid amplification PCR and phosphorylated primers. After ligation of the plasmids, the different variants were pasted in the original pGBKT7 plasmid through yeast homologous recombination. BirA, BirA M3110G and BirA M310A were used as templates for the library construction, the library with a BirA template was inserted in the original pGBKT7 plasmid through yeast homologous recombination.

3.3.6 Library creation; Overlap extension PCR with randomizing primers

Overlap extension PCR was used to create the library in two steps. In the first step, two separate PCR reactions were used to create two DNA sequences with an overlapping part, for each library, see figure 6. The two pieces are referred to as left and right fragment. An analytical gel-electrophoresis was used to validate the result. A second gel-electrophoresis loaded with all the remaining material was used to purify the PCR product. The bands corresponding to the correct length of the sequences where cut out from the gel using a scalpel and blue light. The DNA was extracted with the GeneJET Gel Extraction Kit from Thermo Scientific. In the second PCR, the purified left and right fragment and the two outside primers were mixed in one reaction. The two strands with a homology part anneal in the PCR, acting primers to one another.

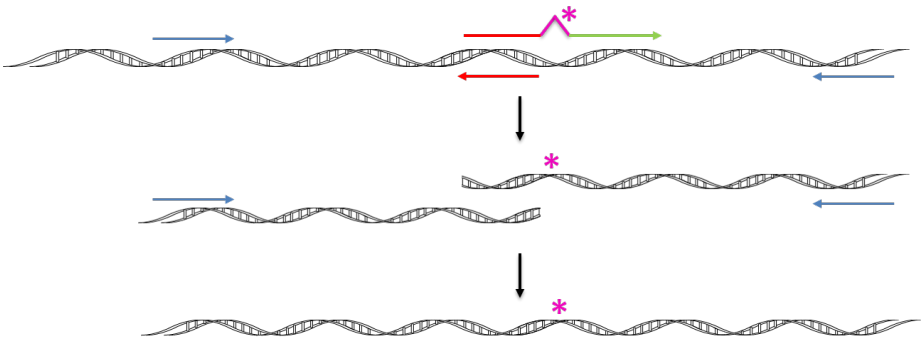


Figure 6. Two PCRs were done to create the libraries with randomising primers.

The final sequence of each library contained the BirA gene with a randomised region at the site of mutation. The final sequence, 1836bp in length, was inserted in the pGBKT7 plasmid by yeast *in vivo* recombination by cotransformatin with a linearized vector. (see section *3.4.4 library scale transformation of yeast competent cells*). PCR was used to create homology on each side of the insertion sequence to the desired site in the plasmid.

3.4 Yeast competent cells and transformation

Two different protocols were used for yeast competent cells and transformation. Both protocols used are based on the LiAc/SS carrier DNA/PEG method (*Gietz and Schiestl, 2007).*

3.4.1 Yeast competent cells for small scale transformations

100ml YPD media was inoculated with an overnight culture of an appropriate yeast strain, to an OD_{600} of about 0.1, and then grown at 30°C with shaking until an OD_{600} of 0.7 was reached. The cells were pelleted by centrifugation of 2000rpm for 5 minutes. The pellet was resuspended in 1 ml of LiOAc mix (1ml TEx10, 1ml 1M LiOAc, 8 ml dH_2O). Freezing media (50% glycerol in LiOAc mix) was added to a final glycerol concentration of 25%. Cells were stored at -70°C prior transformation.

3.4.2 Small scale transformation of competent cells

Transformation of competent cells was performed as follows: The cells were thawed at room temperature. 5µl carrier DNA (Deoxyribonucleic acid sodium salt from salmon testes, 10mg/ml) together with 0.4-1 µg of plasmid were added to 50µl of competent cells. 230 µl of PEG mix (8ml 50% PEG, 1ml 10xTE, 1ml 1M LiOAc) were added to the cells before incubation at 30°C for 30 minutes. The cells were heat-chocked for 12 minutes at 42°C. 100µl of the solution was spread on plates with appropriate drop-out selection carried by the plasmid. Plates were incubated at 30°C until colonies appeared.

3.4.3 Yeast competent cells for library transformations

High transformation efficiency is required when transforming libraries. The protocol used was provided in Yeastmaker™ Yeast Transformation System 2 User Manual from Clontech Laboratories. 50 ml YPDA was inoculated with 5 µl of overnight culture from an appropriate stain. Cells were grown at 30°C with shaking until the culture reached an OD_{600} of 0.15-0.3. Cells were gently pelleted at 700g for 5min and resuspended in 100 ml of YPDA media. The suspension was incubated at 30°C with shaking until an OD_{600} of 0.4-0.5 was reached. Cells were pelleted and washed with 30ml of sterile dH_2O. The pellet was then washed with 1.5 of 1.1x LiOAc mix and finally resuspended in 600µl of 1.1x LiOAc mix. Competent cells were transformed directly.

3.4.4 Library scale transformation of competent cells

The library insertion sequence was transformed into the yeast together with linearized pGBKT7. Yeast cells are efficient for homologous recombination, if a 30-40bp of homology at each end of the insertion sequence are provided (Oldenburg *et al,* 1997). 20µl of carrier DNA was mixed together with 12µl of digested plasmid (0.5µg/µl) and 2.5µg of library insertion. The competent cells were added to the mix. 2.5 ml of PEG mix was added and the suspension was incubated at 30°C for 45 min, turning the tube upside down after half of the time to mix gentle. 160µl of DMSO was added to the tube followed by incubation for 20 min in 42°C. The yeast was pelleted at 700g for 5 minutes and resuspended with 3 ml YPD. Then incubated at 30°C for 90 minutes. The cells were then pelleted and resuspended in 4 ml 0.9% NaCl before plating. The suspension was plated on SDO plates, selective for the library plasmids. From each library, 1:100 dilutions were made and plated so that transformation efficiency could be calculated. Plates were incubated at 30°C for 3 days. The library was then collected with glass beads and stored in 1.2 ml aliquots in the -80°C freezer. Only the 5 first libraries in the table in figure 4 were transformed into yeast cells. The libraries were transformed twice, due to inefficient transformation efficiency in the first round.

3.5 Mating of Yeast Strains

3.5.1 Small scale mating

All the control mating experiments were done at small scale. One colony from each strain, Y2HGold [pGBKT7+bait] and Y187 [pGADT7+pray], was resuspended in 1.2 ml YPDA in a 2ml tube. The tube was incubated with shaking at 30°C for 24h. The cells were plated on SDO–L, SDO-T, DDO-L-T and DDO-L-T/X/A media and incubated in 30°C for 2 days.

3.5.2 Library scale mating

Mating of the yeast strains to form diploids was performed on the following principle; A 50 ml overnight culture with the Y187[pGADT7-pray] strain was grown until it reached OD_{600} 0.8. The cells were then pelleted at 1000g for 5 min. The pellet was resuspended in 4ml SDO media. The Y187[pGADT7-pray] cell suspension was then added to 45ml YPDA media together with 1ml Y2HGold [pGBKT7+bait] thawed library strain in a 2L flask. The flask was incubated at low speed shaking (65rpm) at 30°C for 24h (First library, with inefficient transformation efficiency, was incubated for 60h, over weekend mating). The cells were pelleted (1,000 g for 10 min) and suspended in 7ml YPDA before plated on SDO–L, SDO-T, DDO-L-T and DDO-L-T/X/A media and incubated in 30°C. Positive colonies were patched on QDO/X/A media. Colonies surviving on QDO/X/A media were further analyzed with western blot.

3.6 Control experiments

3.6.1 Tests of the Y2H system

A control experiment was done to test if the mSA and BirA proteins did not produce a false positive result in the Matchmaker® Gold Yeast Two-Hybrid System. Three control vectors were provided with the kit. One contained a pray protein, SV40 large T-antigen, fused to the activation domain (pGADT7-T). The other two containing a positive and negative bait protein, murine p53 and lamin, fused to a DNA- Binding domain (pGBKT7-53, pGBKT7-Lam). The pGBKT7 vectors were transformed into the Y2H strain and the pGADT7 was transformed into Y187 strain. A test mating of the strains and media was done with the tree control vectors described above. The control vectors were transformed and mated at small scale and then plated on selective plates.

To test if the system could be self-activated, leading to false positive results, the following mating experiment was preformed; The BirA-BD, BirA*-BD, BirA320A-BD and BirA310G-BD (Y2H strain) were mated with the mSA-AD (Y187 strain) at small scale.

3.6.2 Tests of biotin levels in the system

Different concentrations of biotin in the media were tested to see if the positive control, the promiscus BirA* enzyme, would become more active and able to provide colonies on DDO/X/A plates. 3 different concentrations were used in the media when preforming small scale mating, and also in the agar plates (10nM, 100nM and 1µM).

Western blot was used to test if different levels of biotin in the media could have an effect on the amount of biotinylated proteins in the cell. Cells cultures were grown with different concentrations of Biotin and the crude yeast cell extract was analysed with western blot. In previous studies, a small addition of biotin (10nM) made a difference of biotinylation in yeast cells (Athavankar and Peterson, 2003). The Yeast Nitrogen Base (without amino acids), used to cultivate the yeast, had a concentration of 8.19nM of biotin. Biotin was added to the following concentrations: 10nM, 100nM and 1µM.

3.7 Western Blot

3.7.1 Cell lysis prior to western blot

The samples for which different levels of biotin were tested, were treated in the following way: Overnight cultures of the yeast were prepared and diluted to OD_{600} 0.1 and then grown again for 4h. The cells were boiled (99°C) for 10min and directly placed on dry ice. Afterwards the cells were tip-sonicated with 2 second intervals for 30 seconds at 20 kHz.

When analyzing the cells from the small scale library, cells were lysed in a different way. 10ml culture was grown to an OD_{600} of 0.68. Cells were pelleted and washed with ice cold 20% TCA and then resuspended in 200µl ice-cold 20% TCA. About ~0.2ml cold, acid-washed glass beads (Sigma, 425-600 micron) was added to the tubes. The tubes were subjected to harsh vortexing in 30 seconds, 2 minutes ice, harsh vortexing, 2 minutes ice. 1ml cold 5% TCA was added and the liquid was transferred to new tubes and incubated on ice 10 minutes. The cells were pelleted at 14000rpm for 10 minutes at 4°C and suspended in 100µl SDS loading buffer. Samples were boiled for 5 minutes. 15µl was loaded per lane in the precast gels.

3.7.2 Running conditions

All western blots were run with the same conditions. The samples were loaded on Mini-PROTEAN TGX precast Gels in a gel-electrophoreses with 1x TGX running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). WB ladder was used (Spectra™ Multicolor Broad Range Protein Ladder ThermoScientific). Running conditions; 200 V constant, 400 mA, 35 min.

3.7.3 Membrane transfer, blocking and antibodies

The proteins from the gel were transferred to a Nitrocellulose membrane (Bio-Rad Torboblot) for 7 minutes. The membrane was rinsed with water. Ponceau S Staining Solution (0.1% Ponceau S (w/v), 5.0% acetic Acid (w/v)) was added to the membrane and incubated for 3 minutes. The membrane was washed with water and

TBST (Tris-buffered saline with Tween(1%)). For the experiment with the yeast cells grown with different biotin concentrations; the membrane was treated in the following manner; Blocking buffer was added (5% dry milk in TBST) to the membrane and then incubated for 1h at room temperature. Detection agent (1:10000 biotin-conjugated antibody Streptavidin-HRP) was then added to 15ml of the blocking buffer and incubated overnight. The membrane was washed with TBST for 5 minutes, three times.

For the screened yeast, fluorescent streptavidin (1:3000 IRDye 680RD Streptavidin) in blocking buffer (Casein blocking buffer, Sigma) was used for the development of the membrane. The IRDye 680RD Streptavidin marker give a florescent signal which is more quantitative than the HRP signal.

3.7.4 Imaging

Images were made with LiCor Odyssey with settings for chemiluminescent measurements when using Streptavidin-HRP antibody. For the IRDye 680RD Streptavidin a wavelength of 700nm and 800nm was used. Images were viewed and analyzed with Imagelab.

4 RESULTS

4.1 Creating the libraries

The library sequences were created with two PCR reactions. The result of the two reactions were analyzed by gel electrophoreses (figure 7A and 7B). The bands on the gel can be compared with the expected sizes of each fragment in table 2. The predicted length of each fragment was collected from the CLC sequence viewer. The left fragment of each library resulted in one strong band in each lane of the gel, see the upper part of Figure 7A. The bands look like the right sizes and no other bands can be seen on the gel. In the lanes with the right (R) sequences, there is one strong band followed by one or two week bands. The strong bands were assumed to be the correct fragment of each library. The weaker bands were carefully cut away before gel extraction and purification.

The full length sequences, in figure 7B, included a 40 bp long homology sequence at each end. The library sequences were merged with the pGBKT7 plasmid inside the yeast cells, relying on the yeasts ability of homology recombination. Only the library based on the native BirA sequence was transformed into yeast cells.

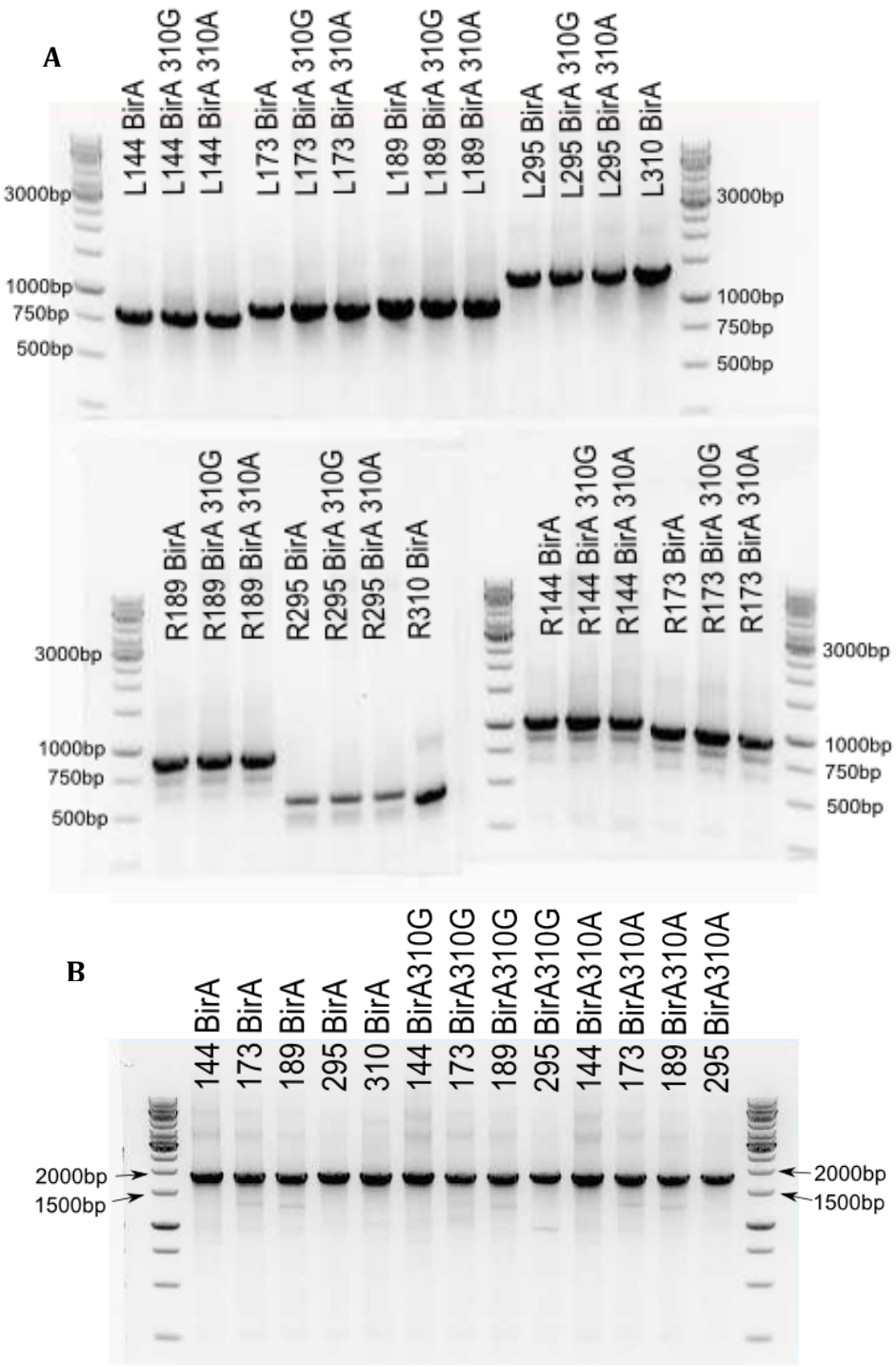


Figure 7. Gel electrophoreses of library construction. A: The result form the first PCR where every library has two sequences, indicated left (L) and right (R). B: The final result of the second PCR. Ladder used was Thermo Scientific GeneRuler 1kb DNA

Table 2. The expected sizes of each fragment from the PCR with randomised primers. R and L fragments have a homology region that varies in length between the libraries.

Sequence	Length	Sequence	Length
R114 BirA R114 BirA 310G R114 BirA 310A	1028bp	L114 BirA L114 BirA 310G L114 BirA 310A	820bp
R173 BirA R173 BirA 310G R173 BirA 310A	949bp	L173 BirA L173 BirA 310G L173 BirA 310A	907bp
R189 BirA R189 BirA 310G R189BirA 310A	894bp	L189 BirA L189 BirA 310G L189BirA 310A	955bp
R295 BirA R295 BirA 310G R295 BirA 310A	580bp	L295 BirA L295 BirA 310G L295 BirA 310A	1273bp
R310 BirA	540bp	L310 BirA	1315bp

4.2 Control experiments

A small scale mating was done with strains holding the control plasmids (pGADT7-T, pGBKT7-53 and pGBKT7-Lam). The purpose with the experiment was to test the strains, media and technique before proceeding with further experiments. The results are presented in the supplementary data, table 4.

A test mating with the strains holding the native BirA gene (fused to the BD domain) as well as the stain with the BirA* mutant was mated with the strain containing the mSA gene (fused to the AD domain). The experiment showed no signs of auto activation or toxicity of the system. However, the promiscuous enzyme BirA* that was planned to be a positive control, showed no colonies on the DDO/X/A plates after mating. The result is presented in the supplementary data, table 5.

4.3 The impact of biotin concentration

Since the positive control failed to work in the first mating experiment, different levels of biotin were tested to see if it would have an effect on the result. Cells were mated in different concentrations of biotin (8.1nM, 10nM, 100nM, 1µM) and then plated on agar plates with the same concentration. No difference in the result was obtained.

Western blots of cells grown in different concentrations of biotin can be seen in figure 8. The aim was to see if the amount of biotinylated protein was related to the amount of biotin in the media. A clear band just under 50 kD is visualized on the blot. The difference in strength of the band is likely to be related to the amount of crude yeast cell extract loaded to the gel (see figure 15 of ponceau stained membrane in the supplementary data).

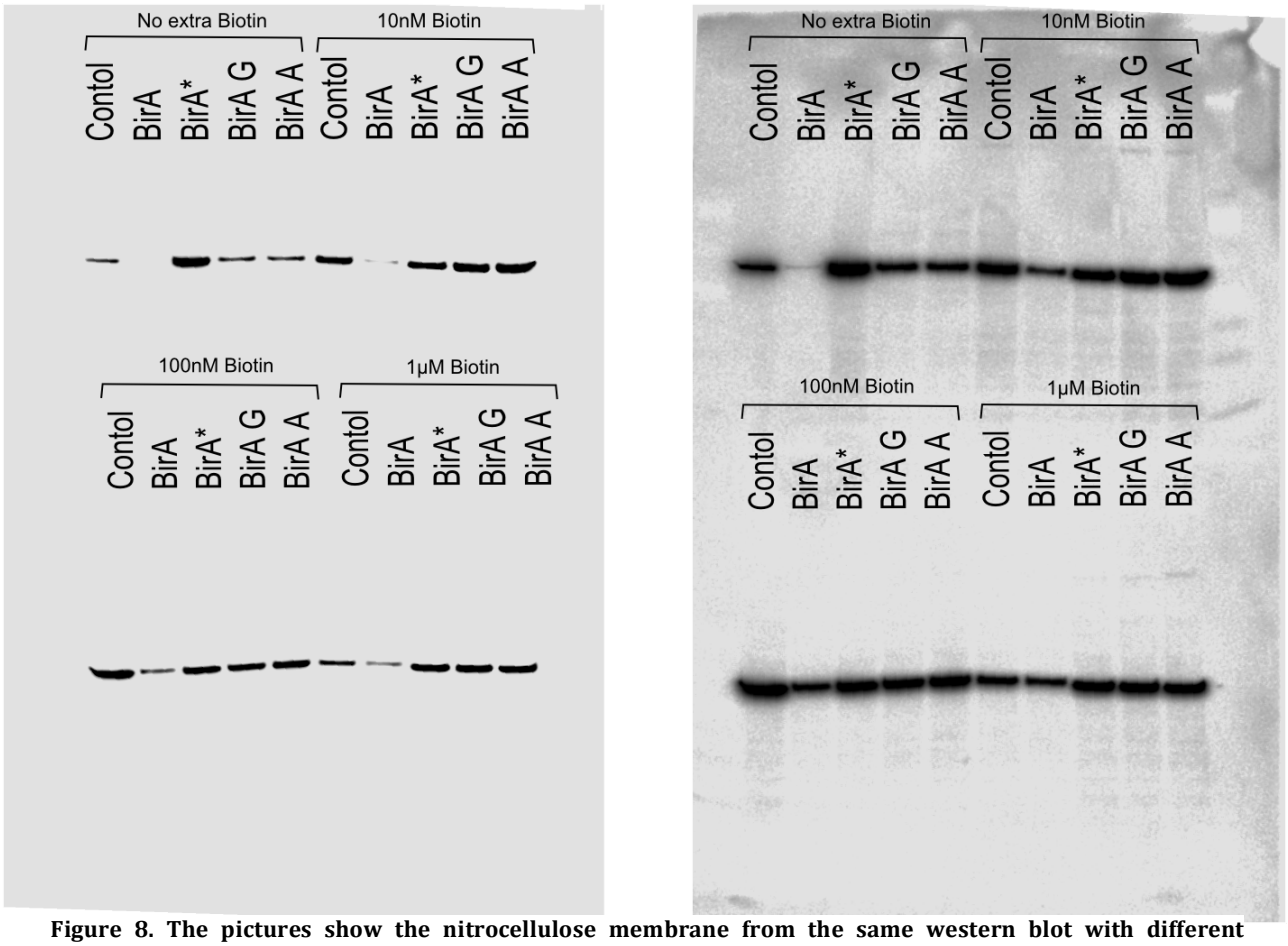


Figure 8. The pictures show the nitrocellulose membrane from the same western blot with different contrast. The cells have been grown with different concentrations of Biotin in the media.

4.4 Result of the transformation efficiency

The yeast transformation efficiency was calculated according to the following equation:

$$Transformation\ efficency = \frac{colonies \times Suspension\ Volume\ (ml)}{Vol.plated\ (ml) \times amount\ DNA(\mu l)} \times dil.factor$$

The first transformation resulted in a "small scale library" with inefficient efficiency of 1600 cfu/µl. The second one resulted in 1.8 ·10^5 cfu/µl. The libraries are of different sizes depending on the amount of codons that are randomized. The largest library have 65536 (4^8) different mutants of BirA with 4 codons affected (see table in figure 4). It is necessary to collect a larger amount of individual colonies than number of mutants to ensure full representation of the library in the screen.

4.5 Mating of the library

After mating the libraries (Y2H strain) with the pray-plasmid (Y182), the number of colonies were counted on each selective media. The results from the mating of the first library (low transformation efficiency) is descried in table 3. For the 310 library plates, colonies appeared on DDO/X/A after 3 days. It took longer time for colonies to appear on the plates with the 189 library. Tiny colonies showed up after 4 days and were normal sized after 5 days. Not all colonies were patched on QDO selective media.

Table 3 Number of colonies on the selective plates after mating

	SDO -Tryp		SDO -Leu		DDO		DDO/X/A	
Library	No. Colonies	Dilution	No. Colonies	Dilution	No. Colonies	Dilution	No. Colonies	Dilution
144	To dense	1:100	116	1:100	≈520	1:10	-	
173	To dense	1:100	234	1:100	≈920	1:10	-	
189	To dense	1:100	270	1:100	≈1400	1:10	112	1:1
295	To Dense	1:100	80	1:100	≈500	1:10	-	
310	To Dense	1:100	152	1:100	≈860	1:10	320	1:1

A mating of the second round of libraries, with better transformation efficiency, was also done. This generated colonies on all the five libraries. Due to time limitations these libraries were not further analysed.

4.6 Patched colonies on QDO selective media

Pictures of patched colonies are viewed in figure 9. The plate to the left shows patched colonies from the 189 library and the plate to the right are patched colonies from the 310 library. It was noted that longer storage time caused more of the blue pigment to diffuse into the agar. The plate with the 310 library were older than the plate with the 189 library when the picture was taken, thus comparison of the colour between them are not feasible. The 29th patch on the plate with the 189 library is more blue compared to the other patches.

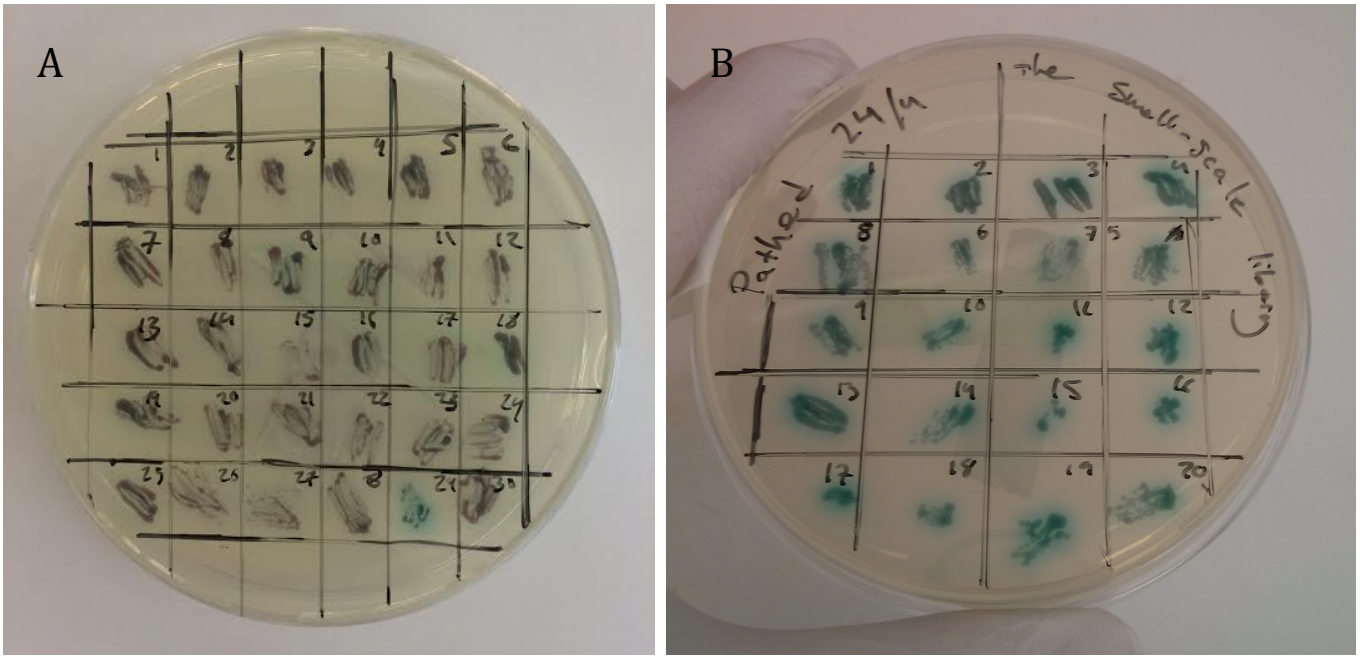


Figure 9. The pictures shows colonies that have been patched with sterile flat toothpicks onto QDO. Picture A shows patched colonies from the 189 library. Picture B shows patched colonies from the 310 library.

4.7 Western blots of positive patched colonies.

A western blot was prepared of the positive hits from the Y3H screen to visualise the biotinylated proteins in each clone. The result of the developed membrane is showed in figure 10 and 11. Comparison to the western blot of haploid yeast cell extract can be made, figure 8. Two bands are visualised, one clear band at 50 kD and one weaker at 35kD. The blots look smeared out which can be explained by background of the florescence label, or maybe promiscuous activity of the BirA mutant. The membrane was washed with stripping buffer to remove florescence label, and subjected to Streptavidin-HRP, the compound used for the previous western blot. The result is presented in figure 11.

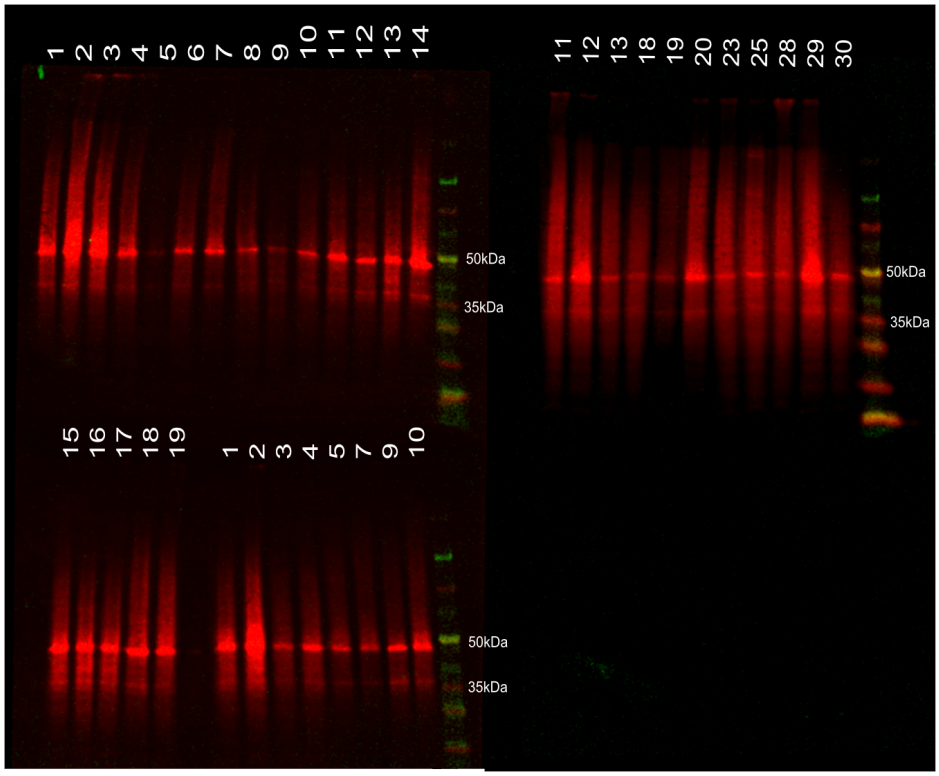


Figure 10. Western blot of BirA mutants developed with IRDye 680RD Streptavidin marker. Two bands are seen more clear than the rest of the smear on the membrane. The clearer band at 50kD is seen here as well as in the western blot in figure 8. The weaker band is around 35kb.

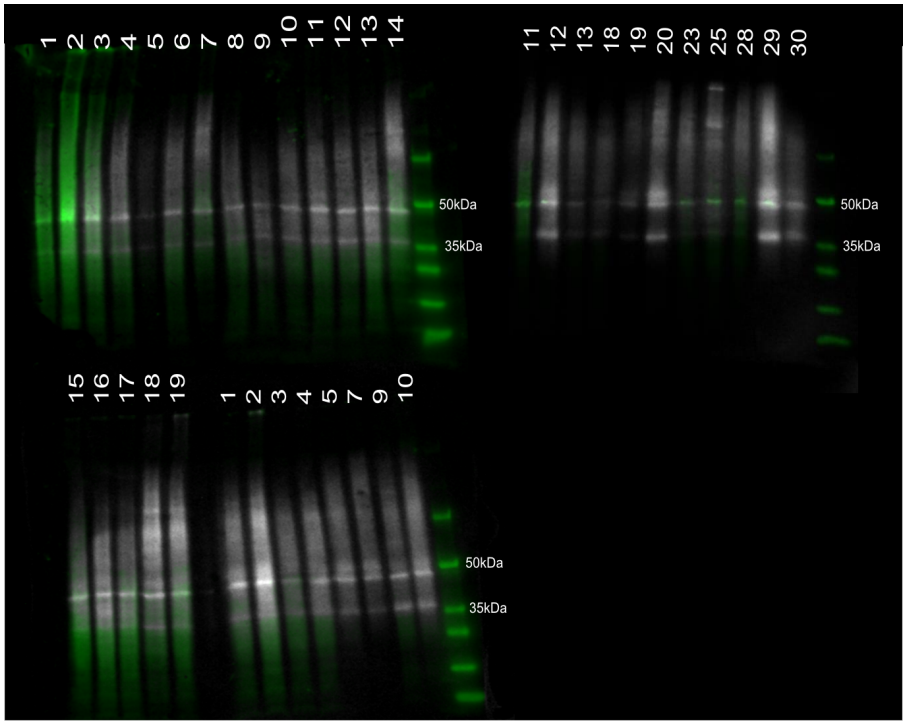


Figure 11. Western blot of BirA mutants developed with Streptavidin-HRP. The membrane from figure 10 have been striped with stipping-buffer to remove the IRDye 680RD Streptavidin marker. The result is not very different from figure 10.

5 DISCUSSION

In the presented project, a Y3H screen was successfully set up and a number of positive hits were identified. From the preliminary results presented in this report, however, no conclusions can be made whether the positive hits represent BirA mutants with direct histone biotinylation activity. Even though the libraries generated positive colonies on selective media, the western blots do not reveal any band at the expected size of histones. It is possible that the BirA mutants created, have acquired promiscuous activity and/or the ability of self biotinylation. Different protocols will thus be tested to extract a cleaner population of histones from yeast to be able to detect histone biotinylation without the background from other proteins.

It should be mentioned that not all the colonies from the DDO/X/A agar plates were patched over to QDO/X/A after mating. The reason was time limitations and the fact that the extended mating time (60h instead of 24h) was predicted to give many duplicates of the same mutants. However the second library that was transformed with higher transformation efficiency, produced colonies on all five libraries. These colonies have not been screened yet (preserved in -80). It is possible that an enzyme with the desired activity have been created but not analysed.

The Western blot bands

On the western blot membranes, both from the haploid and diploid cells, there are one band just under 50 kD. The native Biotin-dependent acetyl-coenzyme which is the enzyme that gets biotinylated of BirA in yeast is about 250kD (Hasslacher *et al*., 1993). In *E. coli* the Biotin-dependent acetyl-coenzyme consist of three independent subunits. One of these subunits, the biotin carboxylase (BC) are running slightly under 50kD. (Cronan and Waldrop 2002) (Broussard *et al*., 2013). It is possible that the yeast 250kD protein is processed proteolytically in similar fragments. Wildtype yeast cell extract from the haploid Y2H stain with control vector (pGBKT7-53) also contains the 50kDA biotinylated protein (see “control” in figure 8), thus it must be an edogenous yeast protein.

One band at slightly over 35kD appears on the blot of the positive hits of the Y3H screen. When comparing to the blot from the Biotin concentration experiment, no band at 35kD is visualised (using a different lysis protocol). BirA from *E. coli* is a 35kD protein (Chapman-Smitha and Cronan, 1999). Mutants of BirA have shown to be able to biotinylate themself before, one example is the promiscuous enzyme BirA* (Choi-Rhee *et al*., 2004). Interestingly, as BirA* did not score positive colonies in our Y3H screens, the newly found variants, if indeed promiscuous, may have a much higher activity than the published and widely used BirA* mutant. Such more active variant would be excellent to replace the BirA* mutant in many assays.

How would a positive hit appear on western blot?
The Y2H system has three distinct promotors that the DNA binding domain recognises. This reduces the risk of getting false positives or biotinylation of an element that is only present at one of the sites in the genome, like a transcription factor. However in the Y3H screen, there is no protection against enzymes with self biotinylating activity. It may be possible to add a degradation tag on the BirA-AD fusion to continuously degrade the protein. In such scenario, only biotinylation of histones or other chromatin proteins would maintain the gene activation.
The most promising BirA mutant would be an enzyme with activity against a specific histone, let us say with a special type of post-translational modification. The question is if this enzyme would be able to activate transcription on three promotor sites. It really is a question of how frequent this specific type of histone is, and if it is present in the three promoter's closest environment. If a mutant BirA would be able to biotinylate a very specific histone on all these sites, is it reasonable to think that the quantity of that type of histone is enough to appear on a western blot membrane? If it is specificity we are looking for, how to detect it in a method that requires quantity? Maybe we should look for a BirA mutant that give us surviving cells on selective media, but with a western blot result that look like the native one. To answer these type of questions, a more extensive literature study together with further experiments are probably needed.

The positive control
The promiscuous enzyme, BirA*, did unfortunately not work as predicted. Studies in *E. coli* have shown that this enzyme has the capacity of self-biotinylation, which in theory would be a very good positive control. Choi-Rhee *et al*., (2004) have published western blot results from crude *E. coli* cell extracts of BirA*. However the *E. coli* culture was grown with a higher concentration of biotin (5 µM biotin). No study has been found that uses expression on BirA* in *Saccharomyces cerevisiae.* It is possible that the BirA* enzyme actually has a very low activity, not sufficient for activating all the selection genes. For future studies, one of our clones could possible work as a positive control after plasmid extraction and sequencing.

Future perspectives
For further troubleshooting it will be beneficial to identify the sequence of the positive clones. Their activity could also be tested in a different system, e.g. overexpression in yeast. The second round of transformed libraries showed more promising positive hits on each of the 5 libraries. If those turn out to be mutants with new activity, there is a possibility to combine the libraries into one, with higher amount of combinations of randomized amino acids. There are also reasons to consider other methods to analyse the positive outcome of the Y3H screen.

6 ACKNOWLEDGEMENTS

First of all, my deepest thanks to my supervisor Simon Elsässer for his supervision, guidance and encouragement during this project. I am grateful to my scientific reviewer Helena Jernberg Wiklund and to my opponents Agnes Svärd and Yonatan Gatica Gustafsson for their useful comments when reviewing this report. I would also like to thank the other members of the Elsässer lab for their helpfulness in the lab and friendly support.

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8 SUPPLEMENTARIES

Table 4. Result of mating of control plasmids.

	SDO - Tryp	SDO -Leu	DDO	DDO/X/A
Lam+ T	Very dense	Very dense	≈300	-
P53+T	Very dense	Very dense	≈200	≈50 Blue colonies

Table 5. Test of Auto activation and toxicity

	SDO -Tryp		SDO -Leu		DDO		DDO/X/A	
	No. Colonies	Dilution	No. Colonies	Dilution	No. Colonies	Dilution	No. Colonies	Dilution
BirA M310A	354	1:100	12	1:100	95	1:1	-	
BirA M310G	332	1:100	27	1:100	120	1:1	-	
BirA*	156	1:100	1	1:100	100	1:1	-	
Lam+T	To Dense	1:100	192	1:100	Dense ≈500 50	1:1 1:10 1:100	-	
P53+T	To Dense	1:100	50	1:100	Dense 63 1	1:1 1:10 1:100	180 blue 2 blue -	1:1 1:10 1:100

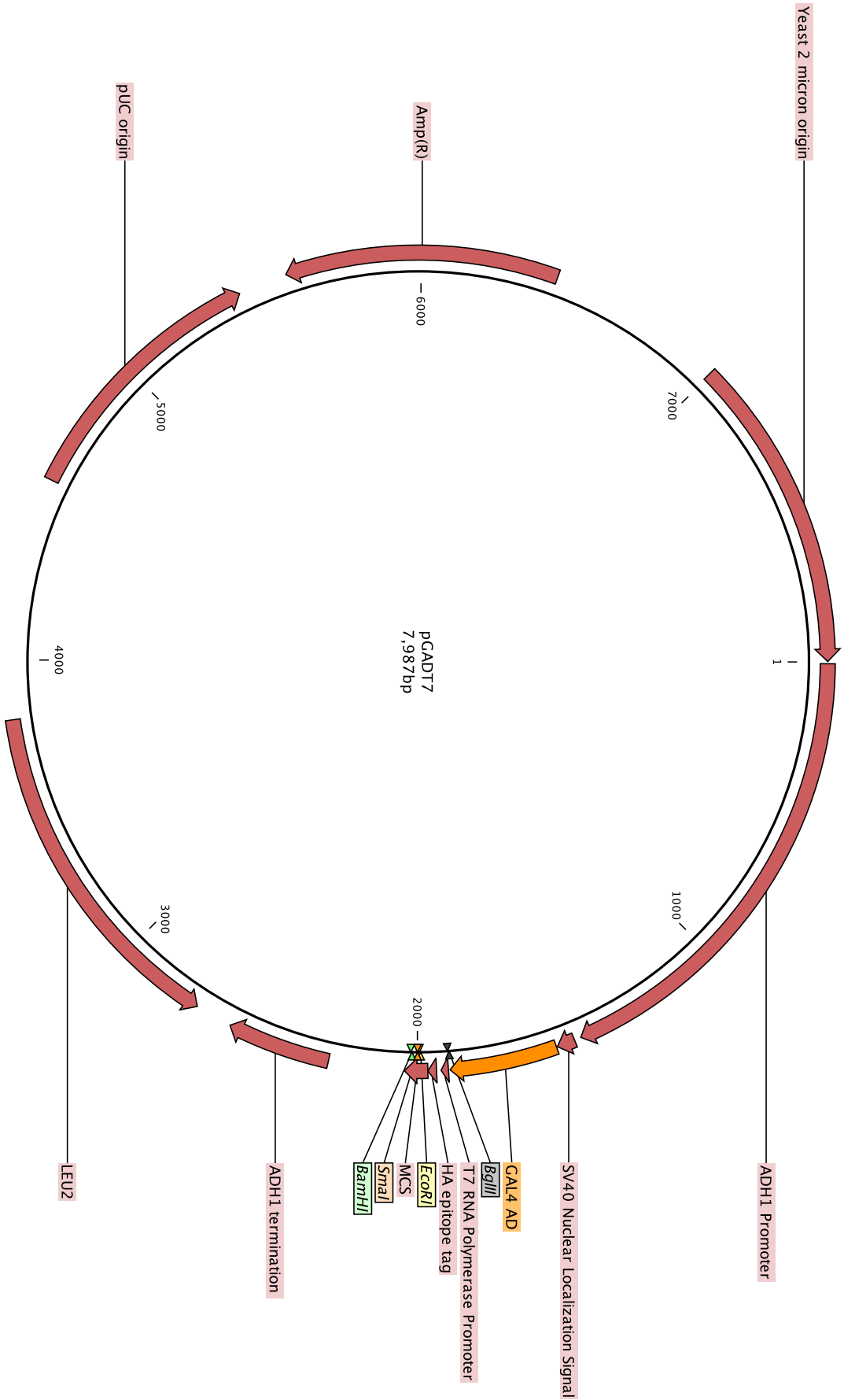

Figure 12. Original pGADT7 plasmid, provided with the Matchmaker® Gold Yeast Two-Hybrid System s from Clontech Laboratories

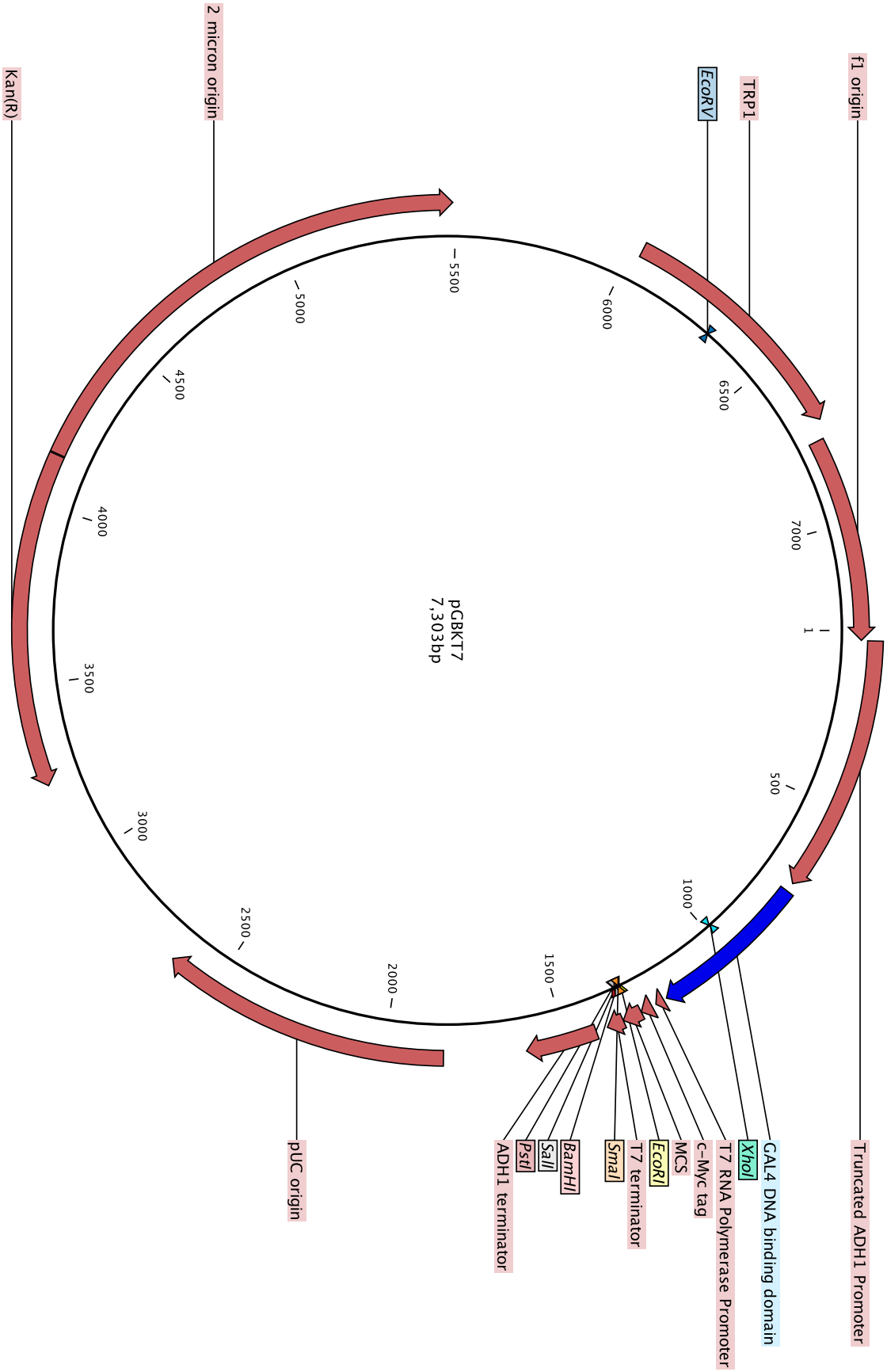

Figure 13. Original pGBKT7 plasmid, provided with the Matchmaker® Gold Yeast Two-Hybrid System from Clontech Laboratories.

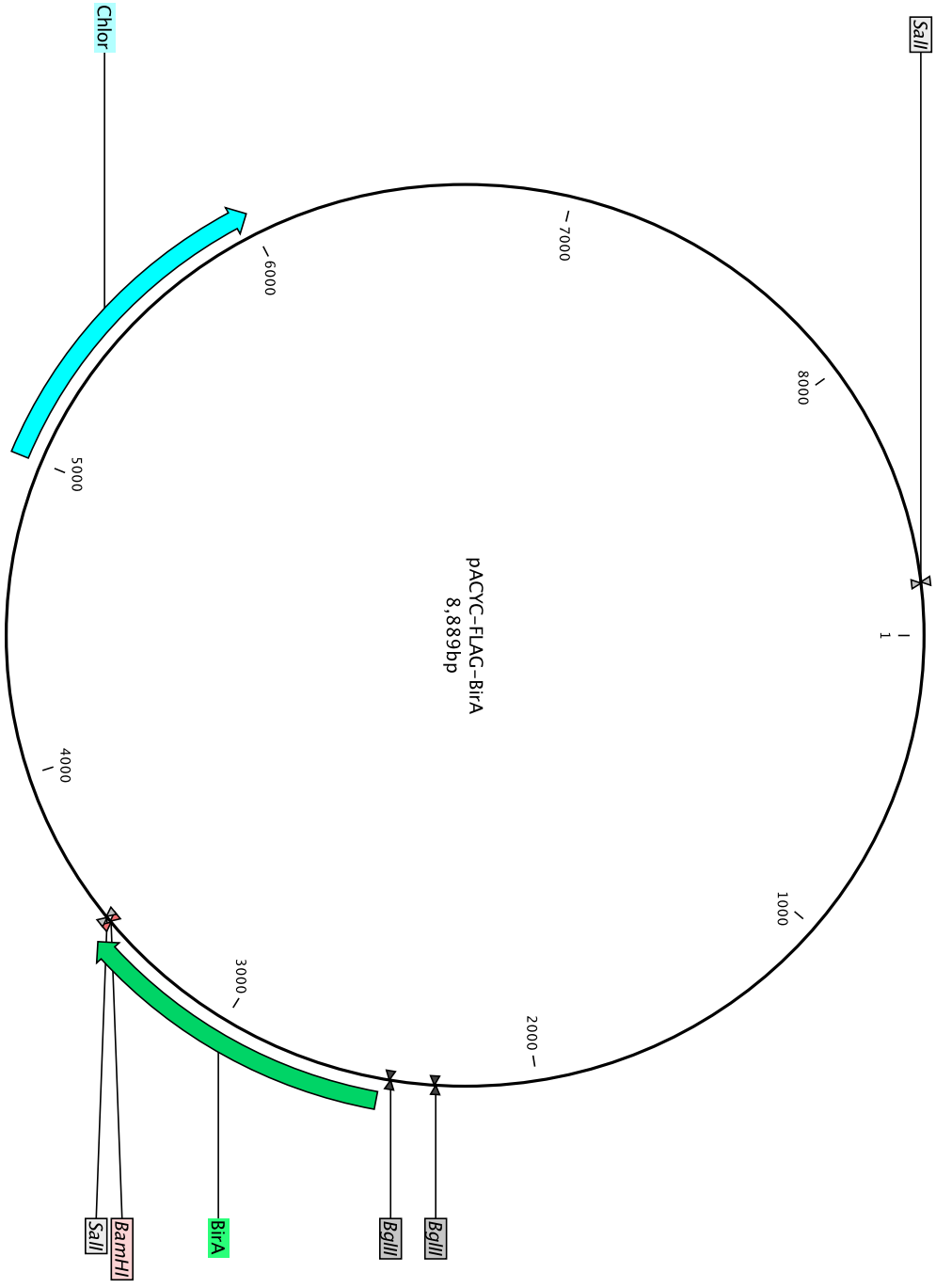

Figure 14. pACYC-BirA plasmid, holding the native BirA sequence.

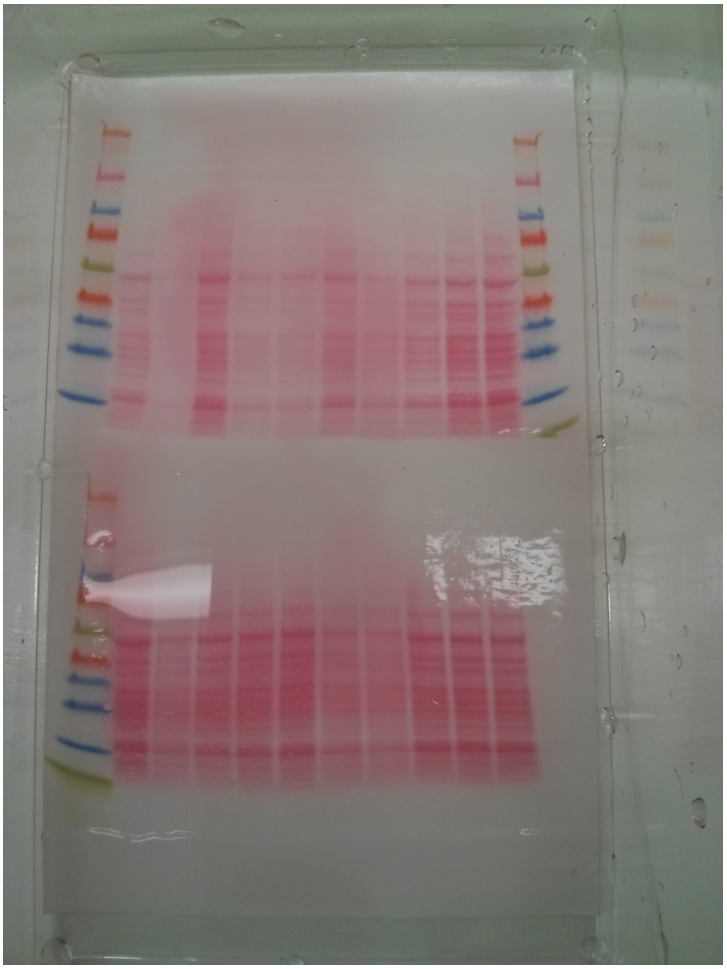

Figure 15. Ponceau S Staining of a Nitrocellulose membrane, same membrane as in figure 8.