The use of the Yeast Two Hybrid system to detect CKS2 dimerization and interactions with CDK1 and CDK2

Thesis submitted for Master’s degree in Pharmacy

Line Therese Myhrstad
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Arton and Marianne; I cherish our friendship and many laughs.

Sincere thanks to my family and especially my parents for their continuous support and interest in what I do. Finally, I would like to thank Christian for making my days so bright.

Line Therese Myhrstad,
Oslo, November 2011
### LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FOA</td>
<td>5-fluoroorotic acid</td>
</tr>
<tr>
<td>3-AT</td>
<td>3-amino-1,2,4-triazol</td>
</tr>
<tr>
<td>AD</td>
<td>activating domain</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>BD</td>
<td>binding domain (also termed DBD)</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>BP</td>
<td>basepair</td>
</tr>
<tr>
<td>CAK</td>
<td>CDK-activating kinase</td>
</tr>
<tr>
<td>CDC25</td>
<td>cell division cycle 25</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDK1</td>
<td>cyclin-dependent protein kinase 1 (alias: CDC2)</td>
</tr>
<tr>
<td>CDK1 AF</td>
<td>double mutated CDK1 (T14A, Y15F)</td>
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<tr>
<td>CDK2</td>
<td>cyclin-dependent protein kinase 2</td>
</tr>
<tr>
<td>CDK2 AF</td>
<td>double mutated CDK2 (T14A, Y15F)</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>cyclin-dependent kinase inhibitor 1A (alias: p21)</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CKS2</td>
<td>CDC28 protein kinase regulatory subunit 2</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>GAL4</td>
<td>galactose-gene activating transcription factor</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Broth medium</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine</td>
</tr>
<tr>
<td>MYT1</td>
<td>protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCBI</td>
<td>national center for biotechnology information</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rY2H</td>
<td>reverse yeast two-hybrid</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>T14</td>
<td>threonine 14</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature of primer</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor protein 53</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequences</td>
</tr>
<tr>
<td>Urac</td>
<td>uracil</td>
</tr>
<tr>
<td>WEE1</td>
<td>protein kinase</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>Y15</td>
<td>tyrosine 15</td>
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ABSTRACT

Elevated expression of CKS2 protein has been detected in various types of cancer. Emerging evidence suggests that CKS2 affects cell cycle regulation through interactions with CDK1 and CDK2, and discovery of compounds that inhibit this interaction may be used to develop a novel cancer medicine. The aim of this thesis was to establish the yeast two hybrid (Y2H) system for detection of CKS2 interaction with CDK1, CDK2 and itself, using the URA3 reporter gene, as the system can be reversed and used to screen for compounds that dissociate the interactions. The HIS3 reporter gene was also used. The former two interactions have been detected with this reporter in a previous Y2H study. Versions of CDK1 and CDK2 that mimick the dephosphorylated, active state of the proteins were used. These proteins contained substitutions at amino acid 14 and 15, and are referred to as CDK1 AF and CDK2 AF. Primers were designed for use in cDNA amplification of CKS2, CDK1 AF and CDK2 AF by PCR. Each gene sequence was fused into the pENTR™/D-TOPO® vector, generating entry clones by a topoisomerase based cloning methodology. The gene sequences were transferred to the yeast expression vectors pDEST™32 and pDEST™22 in a site-specific recombination reaction. These vectors contained the CEN6/ARS4 sequence for replication which maintained low expression levels of the fusion protein. The expression vector constructs were verified through sequencing, and combinations of the constructs were transformed into S. cerevisiae MaV203. The CKS2 interactions were measured by plating onto specific media. Transcription of the HIS3 reporter gene was activated in the S. cerevisiae MaV203 cells when the interactions between CKS2 and the CDKs were investigated, providing further support to previous findings. Transcription of the URA3 reporter gene was, however, not activated, probably because the strength of the interactions was too low to be detected through URA3. An attempt to activate the URA3 reporter by using high copy-number plasmids from a different system did not show activation of URA3 either. The CKS2-CKS2 interaction was not detected either by use of the HIS3 or the URA3 reporter gene. Further work has to be performed in order to counteract the obstacles of URA3 activation.
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INTRODUCTION

Continuity of life depends on cells to proliferate, and the series of events allowing this is collectively called the cell cycle (1). The major components responsible of progression through the cell cycle are the cyclin-dependent kinases (CDKs). As the name indicates, cyclin binding is the primary determinant for their activity. Amongst other proteins shown to participate in the regulation of CDK activity, is the CDC28 protein kinase regulatory subunit 2 (CKS2) (2). The protein is assumed to affect the cell cycle progression through interactions with the CDKs, but its exact physiological role is yet to be determined (3;4). CKS2 proteins are also reported to interact with each other, forming a dimer (5;6). Upregulation of the CKS2 gene have been detected by analyses of cell material from patients with various types of cancer, in search for genes involved in progression of the disease (7-14). Tumors where high levels of this protein have been detected, include breast, cervical, prostate, gastric, bladder and hepatocellular carcinomas (8-12;14). A common feature associated with these high-level CKS2 tumors are poor response upon treatment and low survival probability of the patients. A novel therapeutic strategy may therefore be to inhibit the CKS2 binding to the CDKs.

The yeast two-hybrid technology (Y2H) is the most widely used method to identify an interaction between two proteins (15). The method is based on the properties of the yeast GAL4 protein, which consists of two separable domains responsible for DNA-binding and transcriptional activation, respectively. By fusing two proteins to each of these domains, one can detect if the proteins interact by activation of one or more reporter genes, causing a phenotypic change of the recipient cell. An added advantage is that once the interaction is demonstrated in the Y2H system, the interaction can be screened against compounds that dissociate the undesirable interaction by reversing the original system (reverse Y2H) (16). The first step towards development of such a screen is confirmation of the interaction in the original system.

The interaction of CKS2 to CDK1 and CDK2 was recently identified in our laboratory by activation of the HIS3 reporter gene in the Y2H system (17). cDNA of the genes were cloned into a high copy-number vector system, utilizing a high expression level of the proteins. The HIS3 reporter gene is considered to be the most sensitive (18), and its use was therefore ideal in the establishment of the Y2H method. The use of this reporter gene in a screen for compounds that dissociate protein bindings is however less advantageous, as disruption of binding will cause lack
of growth. Negative selection assays where disruption of reporter gene activation yields viable cells are more favorable, since yeast cells with no reporter activity can be selected from the plate for further analysis (16). One such reporter gene allowing negative selection is URA3. Identification of interactions through this reporter would enable the system to be reversed in search for inhibitors of the interactions (16).

The aim of this thesis was to establish the Y2H system using the URA3 reporter gene, to explore whether the interactions between CKS2 and CDKs and between two CKS2 proteins could be detected in this system. It was also of interest to see whether previous studies showing the interactions could be confirmed, as the findings are not well established in the literature. Although detection of CKS2 dimers may not have any therapeutic potential at present, a method to inhibit this interaction could provide more insight into the function of CKS2. Furthermore, the HIS3 reporter gene was used to increase the stringency of the analyses.

The work included fusion of the CKS2 and CDK proteins to each domain of the GAL4 protein, using a recombinational cloning technique (19). Co-transformation of the fusion proteins in yeast, following growth on specific media was conducted to test for reporter activity of URA3 and HIS3. The expression vectors that were used utilized low expression levels of the fusion proteins. An attempt was also made to activate the URA3 reporter by using high copy-number plasmids. The physiological regulation of the CDKs includes inhibitory phosphorylations on threonine 14 and threonine 15 (20;21). To prevent the inactivation of the CDKs; mutated cDNA sequences of the CDKs simulating the dephosphorylated, active state were used.
1 BIOLOGICAL BACKGROUND

1.1 The cell cycle
The continuity of life depends on the ability of cells to reproduce (1;22). The series of events allowing duplication of the cells content and division into two daughter cells is collectively called the cell cycle. The cycle consists of four distinct phases separated in time, referred to as G\textsubscript{1} (gap), S (synthesis), G\textsubscript{2} (gap) and M (mitosis), where G\textsubscript{1}/S/G\textsubscript{2} corresponds to interphase. Figure 1.1 is an illustration of the different phases of the cell cycle. During all of interphase, the cell duplicates its content and grows in mass. DNA is replicated in the S phase. In M phase, nuclear division (mitosis) followed by cytoplasmic division (cytokinesis) occur. There are also further subdivisions of the phases in mitosis; prophase, prometaphase, metaphase, anaphase and telophase. After mitosis, cells again enter G\textsubscript{1}, and repeat the cycle. Cells in G\textsubscript{1} can also enter a specialized resting state called G\textsubscript{0}. It may stay in this state forever or for a short period of time (23). Cells in G\textsubscript{0} can reenter the cycle if it receives the appropriate signals.

![Cell Cycle Diagram](image)

**Figure 1.1 The eukaryotic cell cycle.** The cell cycle is comprised of four successive phases. G\textsubscript{1} and G\textsubscript{2} are gap phases where the cell grows in mass. S phase stands for synthesis, indicating the replication of DNA. In M phase, mitosis and cytokinesis occur. The cell can also enter a specialized resting state known as G\textsubscript{0} (23).

1.2 The cell cycle control system
To ensure that each daughter cell is genetically identical to its mother cell, the onset and progression of each phase are under strict control (1;24). Serine/threonine protein kinases termed cyclin-dependent kinases (CDKs) are major components of this control system. These proteins are
activated at specific points of the cell cycle and make the decision to proceed, pause or exit the cell cycle.

As the name indicates; the activity of CDKs are dependent on forming a complex with cyclins (1). Cyclins are regulatory protein subunits whose levels oscillate during the different phases of the cycle. The concentration of CDKs in the cell are constant, and the cyclins are in this way regulating kinase activity in a timely manner (25). When activated, the CDKs induce downstream processes by phosphorylating selected proteins, triggering the progress of the cell cycle if the internal and external environment is appropriate (25).

1.2.1 The CDKs and their cyclin binding partners
Four main CDKs; CDK1, CDK2, CDK4 and CDK6, and ten cyclins that belong to four different classes (the A, B, D and E-type cyclins) are involved in the cell cycle of human cells (23). In mammalian cells, cyclin complexes with CDK2, CDK4 and CDK6 have been implicated in the regulation of events during interphase, while CDK1 (also known as cell division control protein 2, cdc2) controls the initiation of mitosis (26). According to the classical model (figure 1.2), progression trough G1 phase is dependent on the activation of CDK4 and CDK6 by cyclin D. In late G1 phase, cyclin E associates with CDK2 which regulates progression into S phase and initiation of DNA replication (23). When the cell has entered S phase, a complex between cyclin A and CDK2 is formed and required throughout replication. In late G2 and early M phase, the CDK1/cyclin A complex promotes entry into M. Mitosis is further regulated by cyclin B in complex with CDK1.
The classical model of cell cycle regulation in the eukaryotic cell cycle. Cyclin complexes with CDK2, CDK4 and CDK6 have been implicated in the regulation of events during interphase, while CDK1 controls the initiation of mitosis. Compensatory mechanisms are however identified among the CDKs, and also among the different cyclins. Both positive and negative phosphorylations of the CDKs are also necessary for full activation of the complexes as described in section 1.3.2 (figure modified from (23)).

Experiments with knockout mice have however revealed diverse compensatory mechanisms between the functions of the different CDK/cyclin complexes, challenging the classical model of cell cycle regulation (27-29). CDK1 has emerged as the master regulator of human cell cycle regulation, able to drive the progression through the different phases of the cell cycle alone, in complex with cyclins (27;29). Studies have shown that CDK1 compensates for depletion of CDK2, as CDK2−/− mice are viable, but sterile. Deletion of CDK1 on the other hand, leads to early embryonic lethality. Similar studies were performed for CDK4 and CDK6, and also these have shown to play compensatory roles (30). Increasing evidence is therefore rejecting the theory that the CDKs and cyclins serve strictly determined phase-specific functions.

1.2.2 Regulation of CDK activity
The rise and fall of cyclin concentration is the primary determinant for CDK activity (1). Other mechanisms are however also important for fine-tuning CDK activity during the different phases of the cycle. These include both positive and negative regulatory phosphorylation of the CDKs (21), and the accumulation of CDK inhibitory proteins (23). To fully activate CDK1 and CDK2, phosphorylation at threonine 161 and 160, respectively, is required. This action is performed by a separate kinase, the CDK-activating kinase, CAK (25). Inhibitory phosphorylation on threonin 14
and tyrosin 15 by the kinases WEE1 and MYT1 leads to the inactivation of CDK1 and CDK2 (21;31;32), whereas the dual-specificity phosphatase CDC25 counteracts this inactivation (33).

Another important level of CDK regulation is specific inhibitors of CDK/cyclin complexes, called CDK inhibitors (CKI) (23;23;34). Two such families of CKI exist; the INK4 family and the Cip/Kip family. The INK4 family includes CDKN2A, CDKN2B, CDKN2C and CDKN2D, which inactivate CDK4 and CDK6 by forming stable complexes with the CDK enzyme, inhibiting binding with cyclin D (35). The activity of CDK1 and CDK2 are opposed by the second family; the Cip/Kip family (23). This family consists of CDKN1A, CDKN1B and CDKN1C.

1.2.3 Checkpoints and mechanisms of cell cycle arrest

The events of the cell cycle are at certain defined checkpoints monitored for abnormalities (23;36). DNA damage checkpoints are positioned in each of the gap phases before the cell enters S phase (G1/S checkpoint) and after DNA replication (G2/M checkpoint). There also appear to be DNA damage checkpoints during S and M phases and a spindle checkpoint (23), but these will not be discussed in this paper.

Before the cell is committed to S phase, the DNA of the cell is closely examined for possible defects. If the DNA is found to be damaged, the progress through G1 will be delayed while mechanisms attempting to repair the damage are mobilized. The cell may also enter the resting state of G0 or even commit programmed cell death (apoptosis) if the damage is too severe and repair is not possible. This is referred to as the G1/S checkpoint of the cell cycle and prevents the replication of damaged DNA. The arrest is induced by the tumor suppressor gene TP53 (37). In its activated form, TP53 acts as a transcription factor that enhances the rate of transcription of genes that carry out effects arresting the cell cycle. For example, the transcription of the CDK-inhibitor CDKN1A is stimulated by TP53, which will inhibit the CDK2/cyclin E complex and thereby the initiation of replication. TP53 is also a major mediator for apoptosis (37).

The mechanisms of the G2/M phase checkpoint does not allow the cell to enter mitosis if replication is incomplete or if DNA has damage to it (1;23). Although TP53 may play a role in this checkpoint, it is not dependent on it as the main mechanism for preventing entry into the M phase is activation of the ataxia telangiectasia mutated (ATM) kinase (23). ATM is responsible
for inhibiting the activity of CDC25, with the net results of also maintaining the CDK1/cyclin B complex in its inhibited form.

1.2.4 Alteration of cell cycle regulators and cancer
Defects in genes regulating the cell cycle are hallmarks of cancer cells (23). The main regulator of cell cycle arrest and apoptosis, TP53, has been subject to intense studies as it has become clear that it is the most frequently mutated gene in human cancer (37). Defects in other regulating molecules such as CDK inhibitors (CDKN2A, CDKN2B, CDKN2C, CDKN2D and CDKN1A) has also been implicated in tumor formation (24). The uncontrolled cell division and proliferation caused by such mutations, promotes tumor growth where the mutations are passed to their daughter cells (1). The cancerous cells can expand to surrounding tissue and dislodge from the tumor. These can further enter the blood circulation or lymphatic vessels and form secondary tumors in other organs (metastasis).

1.3 CKS proteins
In addition to the CDK-regulatory pathways described above, yet another group of proteins have shown to interact with the CDKs; the CDC28-protein kinase regulatory subunit (CKS proteins) (2). The detection of the human CKS proteins is derived from the identification of suc1 in fission yeast as a suppressor of mutations of the gene coding for CDK (38). Homologs in budding yeast (CKS1), frogs (Xe-p9) and humans (CKS1 and CKS2) have since then been identified (39-41). The human CKS proteins, CKS1 and CKS2, have 81% identical gene sequence (41). Research with knockout mice suggests that these proteins share one or more functions as the depletion of either one have shown to not impact viability (42;43). CKS1/− mice are abnormally smaller than the wildtype, but has an otherwise normal phenotype (43). Mice nullozygous for CKS2 are associated with both male and female sterility (42). The sterility is thought to be due to an arrest of the germ cells in meiosis (42). On the other hand, depletion of both genes led to embryonic lethality (2). Collectively, these findings support the theory that the proteins are essential components of the cell cycle, although their precise function remains to be elucidated.

A few studies have been performed to reveal the CKS proteins role in controlling CDK function. Overall, it appears that the CKS proteins do not act as inhibitors or activators of CDKs in the classic sense, but rather seem to modulate substrate choice or the extent of phosphorylation (4). It is now well recognized that the CKS proteins interact with CDKs by forming ternary complexes
containing a CDK, a cyclin, and a CKS protein (2;3;44;45). Egan and Solomon (3) showed that binding of CKS2 to CDK1 was stimulated in the presence of cyclin B. The binding was however highly dependent on the CDK1 phosphorylation at threonine 161, as binding was reduced with about 90% in the absence of this phosphorylation. This association with CKS2 seemed to activate the dephosphorylation needed for passage through the G2/M checkpoint. They also investigated the binding of CKS2 to the CDK1/cyclin B complex with mutations in the gene sequence of the inhibitory phosphorylation sites, changing threonine 14 to alanine (T14A) and tyrosine 15 to phenylalanine (Y15F). Experiments where the mutations were tested individually (T14A and Y15F), or in combination (AF), showed that the dephosphorylated sites are only important for activation of CDKs, and not for binding of CKS2 (3). Interactions between CKS2 and CDK2 have also been detected (3;45;46). In which cyclin complex this occurs, is uncertain. The CKS2 binding to CDK1 and CDK2 has also been identified by the Y2H technique, using the HIS3 reporter gene (17).

The CKS proteins have also been linked to induction of transcription of certain genes, such as cyclin A, cyclin B and CDK1 (2). They also appear to be essential in some destruction processes. Cyclin A, cyclin B, and CDKN1B have been shown to be degraded by the help of CKS proteins (43;47;48). Studies with Xenopus egg extracts suggests that the CKS proteins are required for optimal phosphorylation of certain CDK regulators; CDC25, CDC27; MYT1 and WEE1 (40;46).

Characterization of the crystal structure of CKS2 has revealed that the protein can possess three different forms; as monomers, dimers or hexamers (5;6;49). The monomeric form is composed of four anti-parallel β strands and two α helices. The CKS proteins dimerize via an exchange of β strands between two monomers, a rather unusual interaction between proteins (4;5). Three strand-exchanged dimers can in turn form the ring-structured hexamer form (6). Bourne et al. (5) predicted trough molecular modelling that the CKS proteins would be unable to bind to a CDK in the form of a dimer because it was sterically hindered. Pines (50) suggests the possibility that there is an equilibrium of the monomer and dimer form in the cell, where only the monomer form is able to bind to the CDKs (figure 1.3) (5).
Figure 1.3  CKS2 protein interactions. The CKS2 protein is thought to be in equilibrium between the forms of monomers and dimers (three dimer molecules may also constitute the hexamer form, but this is not displayed in the figure). Only the monomer form is thought to bind to CDKs, where binding to CDK1 and CDK2 have been detected. Which cyclin CDK2 is in complex with when CKS2 binds is uncertain.

1.3.1 CKS2 and cancer
Despite their elusive physiological role, tumour profiling has revealed that both CKS1 and CKS2 are frequently elevated in a variety of human cancers (7-14). Furthermore, some studies associate especially CKS2 with aggressive disease and poor survival rates of the patients. In a study where gene expression profiling were used to predict clinical outcome of breast cancer, overexpression of CKS2 was associated with low survival (14). Lyng et al. (11) also identified the CKS2 gene as one amongst other genes associated with metastatic phenotypes of uterine cervical cancer, where high levels were associated with poor survival. To characterize the CKS proteins contribution to prostate tumorigenesis, a study performed by Lan et al. (10) revealed that elevated expression of CKS2 in prostate tumor cells protects the cell from apoptosis. Other carcinomas where elevated CKS2 is detected includes, gastric, bladder, nasopharyngeal, lymphoid and hepatocellular carcinoma (7-9;12;13).

Although the link between elevated CKS2 proteins and their role in cancer development is yet to be determined, the studies above do indicate that CKS2 might be a contributing factor in cancerous cell proliferation. Since evidence points towards CKS2 exhibiting effects through binding with CDK1 and CDK2, a potential therapeutic strategy could therefore be inhibition of this interaction. Yeast two-hybrid (Y2H) technology is a method to detect an interaction between two proteins (51). An added advantage with this system is that it can be reversed (rY2H) and used
for identification of compounds inhibiting the interaction (52). The interactions of CKS2 with CDK1 and CDK2 have been shown to be robust enough to give expression of the \textit{HIS3} reporter in a Y2H system with high expression levels of the fusion proteins (17). In this thesis, the interactions of CKS2 with both CDK1 and CDK2 by expression of the \textit{URA3} and \textit{HIS3} reporters were investigated, in a system that yielded low expression levels of the fusion proteins. The interaction between two CKS2 molecules forming a dimer was also investigated. Figure 1.3 displays the CKS2 interactions this project assessed.
2 METHODICAL BACKGROUND

2.1 The yeast two-hybrid system
All proteins interact with other molecules, and the biological properties of a protein depend on this physical interaction with other molecules (1). Signal transduction, enzymatic reactions, metabolic pathways and regulation are some examples of essential biological processes that require selective interactions between proteins (15;53). Many experimental techniques have been developed to study protein-protein interactions (54). Phage display, co-immunoprecipitation, cross-linking and the yeast two-hybrid (Y2H) system are some examples. The most frequently used method today is the Y2H system (15), a method originally developed by Fields and Song in 1989. It is an in vivo technique that takes advantage of the properties of the galactose-gene activating transcription factor (GAL4) of the yeast Saccharomyces cerevisiae (S. cerevisiae) (51). The GAL4 protein consists of two separable domains; the N-terminal domain and the C-terminal domain. The N-terminal domain, also called DNA binding domain (BD or DBD), recognizes and binds to specific sequences in the DNA upstream of a promoter. These sequences are termed upstream activation sequences, or UAS. The C-terminal domain (activating domain, AD) stimulates transcription by binding of RNA polymerase. By fusing a protein to each of these domains, one can detect if two proteins interact by transformation into S. cerevisiae. Provided the two proteins that are fused to the two separable domains interact, the reconstituted GAL4 protein activates transcription of one or more reporter genes that enable a color reaction or growth on specific media. The protein X that is fused to the BD of GAL4 is termed the ‘bait’, whilst the second protein, Y, fused to the AD of GAL4 is termed the ‘prey’ (Fig. 2.1) (51;55).
Figure 2.1 The classical yeast two hybrid system. A protein of interest is fused to the DNA-binding domain (DBD), and this construct is called bait. Another protein is fused to the transcriptional activation domain (AD) and is called prey. The bait and prey is co-transformed into the yeast *S. cerevisiae*. The bait binds to the UAS of the promoter and if an interaction occurs between the bait and prey, AD is recruited and a functional transcription factor is reconstituted. This will lead to the recruitment of RNA polymerase and subsequent transcription of a reporter gene, generating a phenotypic signal (figure modified from (56)).

Interactions between proteins in the Y2H system are scored by testing for expression of reporter genes. Fields and Song introduced the bait and prey into an *S. cerevisiae* strain harboring the *lacZ* reporter gene fused to the *GAL1* promotor (51). Neither the bait nor the prey was able to activate the reporter gene when expressed isolated from each other. However, when they were co-expressed, transcription of *lacZ* resulted in production of the enzyme beta-galactosidase, detected by the formation of blue yeast colonies on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (51). This colorimetric reaction with the *lacZ* gene is still used, but other commonly used reporter genes in Y2H nowadays are auxotrophic growth markers (52;57). Examples of such are the *HIS3, ADE2, LEU2* and *URA3* genes, which transcribe enzymes that participate in the synthesis of specific nutrients. Activation is therefore detected by growth on minimal media lacking the nutrients the enzymes provide.

### 2.1.1 The HIS3 reporter gene

An interaction between two proteins can be detected by activation of the *HIS3* reporter gene (57). This reporter is expressed from a *GAL1* promotor, and activation leads to the transcription of the enzyme imidazole glycerol phosphate dehydratase. This enzyme is involved in the histidine biosynthesis pathway. Detection of interaction by the use of this reporter is therefore growth on
medium lacking this amino acid (figure 2.2). The HIS3 reporter is considered to be the most sensitive, but also the least selective, and is reported to be leaky in most yeast strains (18). This can possibly generate a large number of false positives. To adjust the stringency of the reporter, a histidine analogue, 3-amino-1,2,4-triazol (3-AT) is added in the media. 3-AT is a competitive inhibitor of the HIS3 reporter gene product and is usually required to reduce the level of background growth (58).

![Diagram](image)

**Figure 2.2 Representation of activation of the HIS3 and URA3 reporter gene.** The transformed yeast are plated onto medium that selects for the presence of both plasmids (synthetic complete -leu-trp), and are then screened for HIS3 and URA3 activity.

(i) Detection of interaction by the use of the HIS3 reporter is growth on medium lacking histidine.

(ii) The URA3 gene product (ODCase) is essential for uracil biosynthesis, and the cells will be able to grow on medium lacking uracil where an interaction occurs. ODCase also catalyze the transformation of 5-FOA into a toxic compound, and subsequently the transformed yeast strain will die in 5-FOA-containing media (figure modified from (56)).

### 2.1.2 The URA3 reporter gene

Activation of the URA3 reporter will produce orotidine-5-monophosphate decarboxylase (ODCase), an enzyme involved in the biosynthesis of the base uracil (53;57;59). Detection strategy is similar to that of the HIS3 reporter; growth on medium where uracil is omitted (figure 2.2). An added advantage with the URA3 reporter is that it also allows negative selection (59). In
addition to participate in the formation of uracil, the ODCase also catalyzes the conversion of 5-fluoro-orotic acid (5-FOA) into the toxic compound 5-fluorouracil, causing cell death. If an interaction causes activation of the URA3 reporter, the transformed yeast strain will subsequently die in 5-FOA-containing media. That attribute has also proven to be highly convenient for use in screens that search for inhibitors of an interaction (16;56). If an interaction is disrupted on a 5-FOA containing media, it will yield viable yeast cells that can be selected from the plate. By analyzing these further it can be determined how the interaction was inhibited. The positive growth also indicates that the compound is not toxic for the cell, thereby raising its potential for possible therapeutic use. A protein interaction disrupted with the conventional reporter genes such as HIS3 are detected by lack of growth (56). To isolate the cells in which the interaction is abolished requires replica plating of the yeast on general maintenance media, in addition to media that test for reporter activity. Those yeast cells showing no reporter gene activity must then be recovered from the general maintenance plate, which makes it more time consuming.

The URA3 reporter is expressed from a modified SPO13 promoter (16). This promoter has a strong upstream repressing sequence that tightly controls transcription, and only strong protein interactions induce this gene sufficiently to allow growth on plates lacking uracil (16;60).

### 2.1.3 Advantages and limitations with the Y2H system

The Y2H system offers a number of advantages over other technologies to detect protein interactions (56-58). The Y2H system is often more sensitive than many in vitro techniques, and may therefore be more suited for detection of weak or transient interactions (56). Proteins expressed using bacterial cells or in vitro systems often lack key post-translational modifications that could be important for some protein interactions. There is also a possibility that the proteins may not be stable or fold correctly in the buffer conditions used, thereby making detection difficult. The Y2H technique is also considered to be less time-consuming and more inexpensive compared to the more classical methodologies (58).

Choice of reporter gene has a great impact on the outcome of the Y2H analysis as some reporter genes are inherently more sensitive than others (57). Altering expression levels of bait and prey proteins may increase sensitivity, and the traditionally used vectors in the Y2H utilize the 2μ origin of replication which maintain plasmids at high copy-number (15-30 copies per cell) (57;61). In some cases however, high levels of the proteins are toxic to the cells, thereby inhibiting growth.
causing the interaction to escape detection (62). The abnormally high expression level of bait and prey by the use of high copy-number vectors may also force an interaction to happen generating a false positive (58). The use of centromere-based low copy-number vectors with reduced expression levels could provide a solution for the toxicity (57;61). These vectors contain the CEN6/ARS4 sequence for replication which maintains the vector at 1-4 copies per cell. A study performed by Durefee et al. (61) have also shown that low copy-number vectors may provide stronger interaction signals despite of the much lower expression. Another approach that has been shown to reduce toxicity is the use of plasmids with modified promotors driving low level expression of the fusion proteins (56).

Unfortunately the most selective reporters tend to be the least sensitive (57). As increased expression levels not always correlate with increased detection; altering the number of GAL4 binding sites in the promoter of the reporter gene can increase sensitivity directly.

The use of a highly sensitive reporter gene tends to generate large numbers of false positives (57). Some fusion proteins may also themselves transcriptionally activate the reporter genes, leading to false results as there is in fact no interaction present (62). Another reason that may lead to false interactions can arise as the system relies on proteins localizing in the nucleus of the yeast, which may not correspond to their natural cellular environment (58). The different environment yeast and mammalian cells possess can also contribute to a false interaction, even though they share many similarities as both are eukaryotes (58;63). Proteins known to be ‘sticky’ or that do not fold correctly in the system can also display a false interaction (58).

For the same reason as false positive can occur because the system relies on proteins localizing in the nucleus of the yeast cell, false negatives can appear (56). Although many current Y2H vectors encode signals to localize non-nuclear proteins into the nucleus, many proteins can carry stronger signals for localization other places in the cell or contain strongly hydrophobic domains (such as membrane proteins). The proteins may also adopt a different conformation when expressed as fusions (62). This may result in the true interaction is inhibited. Finally, transient interactions, or interactions dependent on for example phosphorylation of one of the proteins before an interaction can occur, may also escape detection (58). A solution to this problem may be the use of modified proteins where the needed phosphorylation is present, retaining the affinity for the other protein (58).
In general, to reduce artificially occurring interactions more than one reporter gene should be used (56). The reporter genes should also possess different promoter structures. Swapping the two domains for the two fusion proteins can also eliminate some false interactions (62). To increase the stringency even further, a detected interaction should be confirmed with alternative techniques (54;58).

The popularity of this method has led to numerous modifications. The technique has been adapted for systems detecting protein-nucleic acid interactions (the yeast one-hybrid system) and protein-RNA interactions (the yeast three-hybrid system) (56;64). New plasmids and strains for the use in the traditional Y2H system are continuously being developed, and the system is also extended for use in other organisms than yeast cells such as bacterial and mammal cells (56;65).

Other than its use for identification of interacting proteins, the system can also be reversed (rY2H) to screen small molecule libraries for potential inhibitors of a given protein interaction, as described above (16;58).

2.1.4 Steps involved in Y2H analysis
Different molecular biological methods must be performed to carry out the Y2H analysis. Site-specific recombinational cloning provide an efficient methodology for constructing the fusion plasmids (19;66), but other cloning techniques are also available. With the technique, entry clones are first constructed by fusing amplified cDNA of the gene sequences into a specific vector, generating entry clones. The gene sequences are further transferred into yeast expression vectors in a site-specific recombination reaction, generating bait and prey. Co-transformation of the bait and prey constructs into yeast are then performed, following a test for reporter activity on specific media. These steps are presented in figure 2.3.
Figure 2.3 Overview of the different steps in protein interaction analyses by the use of the Y2H technique. Amplification of the genes by PCR must initially be performed. Generation of entry clones, followed by a transfer of the gene sequences into expression vectors by a site-specific recombination reaction generates bait and prey constructs. These are transformed into yeast, followed by test of reporter gene activity by growth on selective plates.

2.2 Amplification of genes by Polymerase Chain Reaction

Similar to the replication process that occurs in human cells, a given nucleotide sequence can be amplified in vitro by the polymerase chain reaction (PCR) (1;67). If the aim of the PCR is to later introduce the amplified products into bacteria or yeast, the nucleotide sequence of human genes (mRNA) must be converted to complementary DNA (cDNA). This conversion is important because in humans, the mRNA produced after replication must facilitate different modification processes in order to be translated into a protein (1). Bacteria and yeast are however not able to perform these modifications when a mammalian RNA transcript is introduced in the cell. The main difference between cDNA to both RNA and DNA is that cDNA contains only the coding sequence for the selected gene. Converting the mRNA of the gene sequence to cDNA is therefore crucial when bacterial and yeast cells are used to express mammalian gene sequences. An enzyme called reverse transcriptase is used to synthesize cDNA from mRNA (1). cDNA is then used as template in the PCR.

A typical amplification reaction includes target DNA, a thermostable DNA polymerase, a forward and reverse primer, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium (68). This reaction mix is placed in a thermal cycler, an instrument that subjects the reaction to a series
of different temperatures for set amounts of time. The cycles in PCR consists of three steps; denaturation of the template, annealing of the primers to the single-stranded target sequences(s), and extension of the annealed primers by a DNA polymerase (67). The GC content is the primary determinant for which temperature double-stranded DNA denaturate. The higher the GC concentration, the higher temperature is required to separate the two DNA strands. Lengths of the DNA molecule determine the time needed for complete separation; the longer the DNA molecule, the longer time is needed. Typical temperatures are 93-95 °C (67).

The temperature is reduced to approximately 40-60 °C in the annealing step. As DNA polymerase can only add a nucleotide onto a preexisting 3'-OH group, a primer to which it can add the first nucleotide is therefore needed. In this step the forward and reverse primers associate with the denatured DNA, flanking it to allow a DNA polymerase to initiate synthesis of a complimentary strand (1). The temperatures which are the optimal for the primers is critical for the overall efficiency of amplification (67). There are several strategies in how to calculate this temperature, but none of them are accurate for all primer lengths and sequences. Experimental determination of the optimal temperature through a gradient PCR prior to the main PCR will yield the best results. However, if this is not possible it is recommended to use temperatures 3-5 degrees below the lowest calculated melting temperature (T_m) of the pair of primers used (67).

In the third step, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. This temperature is in the range of 68–72 °C for most thermostable DNA polymerases. Every cycle doubles the amount of DNA synthesized in the previous cycle, and the newly synthesized strands serve as templates for the next cycle (1;67). To yield sufficient DNA amplification, 20-30 cycles is usually required (68).

### 2.2.1 Primer design

A vital step in the overall success of a PCR experiment is the design of suitable primers (69). The aim is to obtain a balance between specificity and efficiency of amplification. Specificity is the frequency with which a mispriming event occurs, giving undesirable products. Efficiency is defined as giving a high amplification yield (69). To achieve this, some rules must be followed in the design of the primers (67-69). Typical primers are 18-30 nucleotides in length having 40-60 % GC content. The 3’ end should be G or C, and the calculated T_m should not differ by more than 5 °C between the forward and reverse primers (67). Complementarity between the primer pair
must also be avoided, as this can lead to synthesis and amplification of primer dimers. This reaction can compete for the DNA polymerase and the other components in the reaction mix, suppressing amplification of the target DNA (67). Computer programs are available that aid in the primer design (70).

2.3 Plasmid vectors
Plasmids are independent DNA molecules separated from a cell’s chromosomal DNA (1). These extrachromosomal DNAs occur naturally in bacteria, yeast and some higher eukaryotic cells, but are not essential for viability. They rather contain genes that are advantageous to the host, for example genes encoding resistances to toxic substances such as antibiotics (1;71). Plasmids have evolved a variety of mechanisms to partition plasmid molecules accurately to their daughter cells, and to maintain a stable copy-number in their host (72). The copy number of a plasmid is defined as the average number of plasmids per bacterial/yeast cell, or per chromosome under normal growth conditions. The plasmids contain a replication origin, which enable them to be self-replicating, independent of the cells chromosome. The fact that it is easier to manipulate, copy and purify recombinant DNA when it is maintained in a vector, separate from the bacterial chromosome, has led to their popularity for use in gene cloning (1;72). A fragment of DNA, called an insert, may be ligated into the plasmids and in this way be used to carry and transfer a gene into a recipient cell, such as a bacteria or a yeast cell. This introduction of plasmid DNA into a recipient cell is called transformation (72).

2.3.1 Selectable properties of plasmid vectors
Only a fraction of the host cells will acquire the plasmid after a transformation (73). Separating cells that carry the insert DNA from the majority of non-recombinants is required to ensure that only the correct product is obtained. The use of genes coding for a specific selectable property such as antibiotic resistance is common (72). Growing transformed bacteria on medium containing the antibiotic the vector has a resistance gene against, will allow growth only of bacterial colonies carrying the plasmid. This enables cells that have taken up the recombinant DNA to be easily identified. Another selection technique which is used to get rid of unwanted plasmid-bearing cells is the use of the lethal ccdB-gene (73;74). The ccdB gene is purified from the E. coli miniF plasmid. This plasmid encodes two proteins, CcdB and CcdA, where Ccd stands for control of cell death. The interplay of these two proteins promotes a stable maintenance of plasmids by killing daughter cells that have not inherited a miniF plasmid at cell division (75;76). The CcdB protein is
a potent cytotoxin, whereas the CcdA protein acts as an antidote preventing the actions of CcdB (77). This lethal effect of the *ccdB* gene makes it an efficient tool for positive selection in recombination reactions.

### 2.4 Recombinational cloning

Cloning genes into vectors has traditionally relied on restriction enzyme digestion and ligation (67). In recent years, an alternative cloning methodology that takes advantage of site-specific recombination has been developed (19). The principle behind this technique is based upon the enzymatic mechanisms where the bacterial virus, bacteriophage λ, recombines its own DNA into the host’s chromosome. This action is performed by a set of three different enzymes; Integrase (Int), Excisionase (Xis) and the *E. coli* Integration Host Factor (IHF). These enzymes recognizes and binds to specific attachment (*att*) sites on the hosts’ DNA, makes an incision and inserts the phages own DNA (78).

As the recombination occurs at specific *att* sites, construction of vectors containing two such *att* sites each will cause a site-specific recombination between the *att* sites from one vector to another by performing a reaction with the two vectors and the Int, Xis and IHF enzymes (78). Because the transfer of DNA segments in the recombination reaction does not rely on a replicative step, alterations to the nucleotide sequence are not expected (78).

#### 2.4.1 Construction of entry clones

The pENTR™/D-TOPO® vector containing the attachment sites *attL1* and *attL2* can be used to produce entry clones (figure 2.4) (79). A blunt end PCR product is inserted between these *att* sites by a topoisomerase based cloning method. The vector is linear and has a topoisomerase enzyme covalently bound to each 3’ end. It carries a kanamycin resistance gene that allows selection of plasmid in *E. coli*, and it replicates in *E. coli* from the pUC ori.
Figure 2.4 Features of the pENTR™/D-TOPO® vector (79). A topoisomerase enzyme is bound to each 3’ end, allowing the gene of interest to be ligated between the two att sites. The kanamycin resistance gene allows selection in E. coli.

Topoisomerase I enzyme is a vaccinia virus that specifically binds to double-stranded DNA at CCCTT sites on the 5’ end, cleaving the phosphodiester backbone in one strand (78;80;81). A covalent bond between the 5’ phosphate of the incised strand and a tyrosyl residue (Tyr-274) of topoisomerase will be formed. An overhang with the bases GTGG, will basepair with 3’- CACC blunt-end PCR products. The PCR product will be fused to the vector, flanked by the att-sites (figure 2.5).

Figure 2.5 Topoisomerase based cloning. Topoisomerase is covalently bound at 3’ blunt CCCTT ends on the vector, making it linear. CACC blunt end PCR products will basepair with a GTGG overhang at the 5’ end, fusing the PCR product into the vector (79).
The plasmid vector and gene insert must be present at an appropriate molar ratio in the reaction, in order to obtain ‘correct’ clones (72). If the plasmid vector is present in a too high concentration, the reaction may generate an undesirable number of empty plasmids. If too low, the reaction may yield an excess of linear and circular homo-and heteropolymers of varying sizes, compositions and orientation. For this reason, the orientation of the insert must be validated by restriction analysis (72).

### 2.4.2 Construction of expression vectors

By performing a recombination reaction between the entry clone and a destination vector, an expression vector can be generated (figure 2.6). An enzyme mix with the three enzymes described above; Int, Xis and IHF (LR clonase II mix) will facilitate the transfer of the gene from the entry clone to the destination vector. The reaction is highly specific; the attL1 will recombine with attR1, and not attR2, maintaining the orientation of the sequence during the reaction (19).

![Figure 2.6 Recombination reaction generating expression constructs.](image)

As described in section 2.4, the segment between two att sites is ‘switched’ in a recombination reaction. Using a destination vector that contains the ccdB gene between its att sites, will replace the ccdB gene with the gene of interest when a recombination occurs with an entry clone. Theoretically only the successful recombinant clones will grow. Transforming the vectors into \textit{E. coli} lacking the miniF plasmid (\textit{F}−) will cause death of cells that has taken up the by-product molecules retaining the ccdB gene, or unreacted vectors carrying the gene. The two recombination sites, attR1 and attR2, flanking the ccdB gene are converted to attB sites following the recombination reaction.

pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22 (figure 2.7) are destination vectors from Invitrogen that can be used in a recombination reaction to generate expression vectors (66). pDEST\textsuperscript{TM}32 carries the GAL4
DNA binding domain forming the bait, while pDEST\textsuperscript{TM}22 generate the prey (carries the GAL4 AD).

Figure 2.7 Features of CEN-based Y2H destination vectors (66). pDEST\textsuperscript{TM}32 contains the GAL4 DNA binding domain (DBD), while pDEST\textsuperscript{TM}22 carries the activation domain (AD) coding sequence (66). Both vectors contain the ARS/CEN origin that permits the maintenance of the plasmid in low copy. Both are also designed with the same ADH1 promotor for expression of the two hybrids.

The bait plasmid carries a gentamicin resistance gene (G\textsubscript{m}R) for maintenance in \textit{E. coli}, and the \textit{LEU2} gene for selection in yeast. The prey plasmid on the other hand contains the ampicillin resistance gene for selection in \textit{E. coli}, and the \textit{TRP1} nutritional marker for selection in yeast. The constitutive promotor \textit{ADH1} is featured in both vectors to drive medium-level expression of the fusion proteins in yeast, while the pUC origin permits high-copy replication and maintenance in \textit{E. coli}. Both vectors are also equipped with the \textit{ARS4/CEN6} sequence for low-copy number replication. The attachments sites, \textit{attR1} and \textit{attR2}, is the bacteriophage \textit{λ}-derived sequences that allow directional recombinational cloning. In between these sites both vectors contain the chloramphenicol and \textit{ccdB}-gene that permits negative selection.

2.5 Yeast strain and transformation
The yeast strain used in an Y2H experiment must carry mutations on the genes the bait and prey vectors are markers for (57). Thus, a vector carrying the \textit{TRP1} yeast marker must be paired with a yeast strain that carries a \textit{trp1} mutation. It is also important that the markers are not used as reporter genes, because the same gene cannot be used to select for both the presence of the plasmid and the interaction.

A simple and efficient method for introducing plasmid DNA into yeast is the lithium acetate method (82). Yeast cells grown on rich medium are harvested and treated with lithium acetate
(LiAc), polyethylene glycol (PEG) and single stranded carrier DNA (ss DNA) to induce uptake of the plasmid. Plasmid-bearing yeast cells are selected by growth on synthetic complete (SC) minimal medium lacking the nutritional marker the plasmid is coding for.

### 2.6 Purification of plasmid DNA

Many techniques to purify plasmids from bacteria have been developed (72). The alkaline lysis method is a simple and effective method where a strong anionic detergent at high pH destructs the cell wall of the bacterium (83). Chromosomal DNA and proteins denaturates, whereas the strands of closed circular plasmids are protected against denaturation because they do not break, and stay topologically intertwined. The denaturated chromosomal DNA, proteins and broken cell wall of the bacterium are precipitated from the solution during lysis. The plasmid is recovered from the supernatant. This procedure can be done in different scales dependent on the need of the experiment. Mini-preparations yields between 100 ng to 5 µg of DNA, depending on the copy number of plasmid. Midi-preparations are useful when larger amount of DNA is needed, as it can yield as much as 50 µg (72).

### 2.7 Restriction nucleases

Restriction nucleases are enzymes naturally occurring in bacteria for protection against viral infection, by degrading incoming DNA (1). Each nuclease recognizes a specific DNA sequence, and cut the double helix into fragments of strictly defined sizes. The bacterium’s own DNA is protected from this cleavage by modification of these sequences. Different restriction nucleases have different sequence specificities known as restriction sites, and a large number of restriction nucleases have been isolated from prokaryotic organisms and used in research (1). One application is verification of insertion of genes into vectors after a cloning reaction. The cut fragments can further be analyzed by separation with gel electrophoresis (72).

### 2.8 Gel electrophoresis

Gel electrophoresis is a technique to separate, purify and identify DNA fragments of different sizes (1;72). For DNA in the size range 300 to 10 000 bp, the most effective separation is done by the use of agarose gel (1). Agarose is a linear polymer composed of alternating residues of D- and L-galactose, forming a lattice the fragments must migrate through (72). Samples of DNA are loaded in wells at one end of the gel, and a voltage is applied across the gel. A buffer solution
covering the gel is leading the electricity. Because DNA is negatively charged, the fragments will migrate toward the positive electrode. Smaller fragments more easily migrate through the gel, as they do not get impeded by the agarose matrix (1). Other factors will also determine the rate of migration, such as the applied voltage, agarose concentration and the conformation of DNA. The conformation can be either superhelical circular, nicked circular and linear (84). Which conformation that migrate the fastest can vary greatly, depending on the present conditions; such as concentration and type of agarose, the ionic strength of the buffer and the electric field. Because of this, the best way to distinguish between the different conformational forms of DNA is to include a sample of untreated circular DNA in the gel, and a sample of the same DNA that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one place (72).

Before samples are loaded into the gel, a loading buffer is added. The purpose of these buffers is to increase the density of the samples, assuring that the DNA sinks evenly in the well. The buffer will also add a color to the samples, simplifying the loading process. The dye also migrates through the gel in the same direction as DNA in predictable rates, to monitor the progress of the electrophoresis. For example, the dye bromophenol blue will migrate at a rate equivalent to 300 bp DNA in 0.5-1.4 % agarose gels (72). In order for the DNA to be visible, the gel is stained with a dye, such as GelRed, which fluoresces under ultraviolet light when it is bound to DNA (1). If necessary, these bands of DNA can be excised from the gel and used for a variety of purposes (72).
3 METHODS

3.1 Experimental outline

The flowchart in figure 3.1 illustrates the major steps in the experimental work of the thesis:

Step 1:
Design primers for PCR and amplify the genes of interest by PCR

Step 2:
Generate entry clone by topoisomerase based cloning

Step 3:
Generate bait and prey plasmids by LR recombination reaction
Step 4:
Transform bait and prey into *S. cerevisiae* MaV203

![Diagram of transformation process]

Step 5:
Test bait and prey transformants on selective plates:
The Yeast Two-Hybrid Analysis

![Select plates diagram]

Figure 3.1 Illustration of the experimental work. Primers were designed for use in a PCR reaction to amplify cDNA of *CDK2, CDK1 AF* and *CDK2 AF* (step 1). The PCR products were fused into an *att*-site-containing entry vector by a topoisomerase based cloning method (step 2). The gene was then transferred from the entry vector into two different destination vectors (pDEST*TM*32 and pDEST*TM*22) by a site-specific recombination reaction between the *att* sites in step 3, generating expression constructs for bait and prey. These expression constructs were introduced into *S. cerevisiae* (step 4), and analyzed for interaction by plating onto selection plates (step 5).
3.2 Primer design and PCR (step 1)

PCR were used to amplify cDNA of the genes CKS2, CDK1 and CDK2. The gene sequences were retrieved from the department of cell biology’s strain collection, present in pGBK-vectors transformed into XL10-Gold ultracompetent E. coli cells. CDK1 and CDK2 contained two mutations; amino acid 14 was changed from threonine to alanine, and amino acid 15 was replaced with phenylalanine instead of tyrosine. The purpose of these substitutions was to mimic the dephosphorylated, active state of the CDKs. CDK1 and CDK2 with these substitutions will from now on be referred to as CDK1 AF and CDK2 AF.

The nucleotide sequences obtained from internet resources of the National Center for Biotechnology Information, NCBI (85) (appendix 16), were used in the design of forward and reverse primers for each gene. Primer lengths were between 19 and 27 nucleotides, and all 3’ends were a G or a C. The melting temperature \( (T_m) \) and GC-content were calculated by using the program OligoCalc (version 3.26) (70), where the \( T_m \) was in the range from 52.8 – 55.4 °C, while the GC content varied from 33-63 % for the six primers. An addition of 4 nucleotides, CACC, to the 5’ end of the forward primers were added to allow directional cloning in the pENTR™/D-TOPO® vector for creation of the entry clones (step 2 in figure 3.1). The nucleotide sequences for the designed primers are shown in appendix 1 (table 1), and they were purchased from Eurogentec S.A in Belgium.

Before PCR was conducted, plasmid DNA (pGBK-gene) was purified from the E. coli cells by alkaline lysis of overnight cultures, in a process described in appendix 6 (miniprep). The procedure for making overnight cultures is described in appendix 5.

3.2.1 Gradient PCR

A gradient PCR was performed in order to experimentally determine the optimal primer annealing temperature. Using the gradient function of the Mastercycler®, a temperature gradient programmed between 45 °C to 65.6 °C were built up across the heating block, as the calculated temperature was in the range from 52.8 – 55.4 °C for the six primers. Reaction mixtures and cycling conditions are described in appendix 2a. Following the PCR, the samples were further subjected to a 1.5 % agarose gel for gel electrophoresis (appendix 3). The gel was stained with GelRed, and visualized and photographed under a UV transilluminator after electrophoresis.
3.2.2 Preparative PCR and agarose gel purification
A preparative PCR was executed under the same setup and amounts as in the gradient PCR, but with the optimal primer annealing temperature. Each PCR reaction had a total reaction volume of 100 μl, and the reaction mixes and cycling parameters are described in appendix 2b.

Following the PCR, the samples were size fractioned on a 1.5 % agarose gel, according to the procedure described in appendix 3. Four teeth of the comb were taped up to form one large narrow well, and three such taped up sections were made, one for each gene. As CDK1 AF and CDK2 AF are of similar size (894 and 897 bp respectively), these genes were loaded at either side of CKS2, to avoid contamination into each other’s wells. After immersing the agarose gel in GelRed after the electrophoresis, the separated DNA fragments were visualized by UV detection and excised with a scalpel. The gel pieces were further purified using the Wizard® SV Gel and PCR Clean-Up System from Promega, as described in appendix 4.

3.3 Construction of entry clones (step 2)
The gel purified PCR products were cloned into the entry vector, pENTR™/D-TOPO®, by topoisomerase-based molecular cloning (step 2 in figure 3.1). The pENTR™ Directional TOPO® Cloning Kits from Invitrogen was used for this purpose (appendix 10), where the blunt-end CACC of the PCR product base paired with the overhang sequence, GTGG, in the pENTR™/D-TOPO® vector. The reaction mixtures were transformed into One Shot chemically competent E. coli cells, and plated on Luria Bertani (LB) agar containing kanamycin to select for plasmid-bearing E. coli clones. The plates were incubated overnight at 37 °C.

3.3.1 Restriction enzyme analysis of entry clones
Restriction enzyme analysis was performed on randomly selected positive transformants from the cloning reaction to confirm the presence of the genes in the pENTR™/D-TOPO® vector. Transformants were cultured overnight in LB medium containing kanamycin (appendix 5), whereafter a glycerol stock were made (appendix 8), and the plasmid DNA was isolated by alkaline lysis (appendix 6).

Restriction reactions containing the plasmid DNA to be analyzed, the restriction enzymes NotI HF and Ascl, and an enzyme buffer mix were set up (appendix 14). After 2-4 hours of digestion, the
samples were further subjected to gel electrophoresis (appendix 3). The cut DNA fragments were visualized and photographed using GelRed under UV illumination.

3.4 Construction of bait and prey plasmids (step 3)

CKS2, CDK1 AF, and CDK2 AF were transferred from the entry vector into two different destination vectors (pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22) by a site-specific recombination reaction between the \textit{att} sites in step 3, generating expression constructs for bait and prey. The vector generating the bait was pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22 produced the preys, and maps for these vectors are described in appendix 12. The three genes were cloned both as bait and prey to investigate the interactions fused to both domains of the GAL4 protein in the subsequent Y2H analyses in step 5 (figure 3.1). Six recombination reactions were therefore set up (appendix 11). The enzymes that facilitate the recombination; Int, Xis and IHF (LR clonase II enzyme mix) were mixed with equal molar ratio of miniprep entry clone DNA and the destination vectors, and incubated at 25 °C overnight. The following morning proteinase K solution was added to terminate the enzyme activity. Aliquots from each reaction mixture were transformed into TOP 10 One Shot chemically competent \textit{E. coli}, and plated on gentamicin (pDEST\textsuperscript{TM}32 vector) and ampicillin (pDEST\textsuperscript{TM}22 vector) plates, and incubated at 37 °C overnight.

Propagation of the destination vectors pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22 was performed in XL 10-Gold\textsuperscript{®} Ultracompetent \textit{E. coli} cells, which strain is resistant to \textit{ccdB} effects. The technique is described in appendix 9.

3.4.1 Restriction enzyme analysis of expression constructs

Restriction analysis was also performed on positive expression constructs following the recombination reaction, to confirm the presence of the genes in the destination vectors. In the recombination reaction, the chloramphenicol and \textit{ccdB} gene are replaced by the genes of interest. The chloramphenicol and \textit{ccdB} gene together constituted 1589 basepairs (bp), whilst 240 bp for CKS2, 894 bp for CDK1 AF and 897 bp for CDK2 AF were fused into the destination vectors instead. The vector generating the bait, pDEST\textsuperscript{TM}32, constituted 12266 bp. pDEST\textsuperscript{TM}22 (generate preys) had a size of 8930 bp.
Simple mathematical calculations where 1598 bp (the chloramphenicol and \textit{ccdB} gene) was subtracted from the destination vector size and the inserted gene size was added, gave expression constructs of following sizes represented in the table 3.1.

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>Size (bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bait</td>
<td></td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}32/CKS2</td>
<td>10917</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}32/CDK1 AF</td>
<td>11571</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}32/CDK2 AF</td>
<td>11574</td>
</tr>
<tr>
<td>Prey</td>
<td></td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}22/CKS2</td>
<td>7581</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}22/CDK1 AF</td>
<td>8235</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}22/CDK2 AF</td>
<td>8238</td>
</tr>
</tbody>
</table>

* For all correct recombinant clones the chloramphenicol and \textit{ccdB} gene were cut out (1598 bp), whereas the desired gene was inserted. The gene sizes are described above. A simple mathematical calculation gave the expression constructs the following sizes represented in bold.

Overnight cultures containing LB medium with appropriate antibiotics (appendix 5) were made in order to make glycerol stocks for storage (appendix 8). The plasmid DNA was purified from the \textit{E. coli} cells using the alkaline lysis (miniprep) method described in appendix 6, and digested with \textit{SwaI} enzyme for 2-4 hours as described in appendix 15. The enzyme digested fragments were analyzed under a UV transilluminator after GelRed staining, following gel electrophoresis.

### 3.4.2 Sequencing

To ascertain the presence and correct orientation of the insert into the destination vectors, the clones that appeared correct in the restriction enzyme analyses were subjected to nucleotide sequencing by GATC Biotech in Germany. Appropriate primers were designed and included in the shipment to GATC Biotech. These primers are described in appendix 1, table 2. The nucleotide sequences received in return from GATC Biotech, were compared with the correct nucleotide sequences of the \textit{CKS2}, \textit{CDK1} and \textit{CDK2} genes (appendix 16), by using the internet based alignment tool; Basic Local Alignment Search Tool (BLAST) provided by NCBI (86). The results of the BLAST searches are shown in appendix 17.

### 3.5 Transformation in \textit{S. cerevisiae} MaV203 (step 4)

In order to transform the bait and prey constructs into \textit{S. cerevisiae} MaV203, it was necessary to first make a working plate with the yeast. This was done by transferring a small amount from the
80 °C glycerol stock in 0.8 % sodium chloride (appendix 18). Dilutions of this suspension were plated onto agar plates containing a blend of yeast extract, peptone, adenine and dextrose (YPAD), by the receipt described in appendix 21. Adenine was added because MaV203 has a mutation in the ade2 gene that accumulates a red pigment that slows growth (57). Addition of adenine delays this accumulation, resulting in enhanced growth rate. The genotype of S. cerevisiae MaV203 is described in appendix 22. The plates were incubated at 30 °C for 3-5 days depending on growth.

In this thesis, interactions between CKS2 with both CDK1 and CDK2, and the interaction between two CKS2 molecules forming a dimer was investigated. The interactions were tested with the proteins fused to both domains of the GAL4 protein. The combinations of bait and prey constructs transformed into S. cerevisiae MaV203 are described in table 3.2. A modified lithium acetate method was used (appendix 19).

### Table 3.2 Transformation of experimental interactions.

<table>
<thead>
<tr>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Experimental interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEXP™32/CKS2</td>
<td>pEXP™22/CDK1 AF</td>
<td>CKS2 and CDK1 AF</td>
</tr>
<tr>
<td>pEXP™32/CDK1 AF</td>
<td>pEXP™22/CKS2</td>
<td></td>
</tr>
<tr>
<td>pEXP™32/CKS2</td>
<td>pEXP™22/CDK2 AF</td>
<td>CKS2 and CDK2 AF</td>
</tr>
<tr>
<td>pEXP™32/CDK2 AF</td>
<td>pEXP™22/CKS2</td>
<td></td>
</tr>
<tr>
<td>pEXP™32/CKS2</td>
<td>pEXP™22/CKS2</td>
<td>Dimerization of CKS2</td>
</tr>
</tbody>
</table>

S. cerevisiae MaV203 contains the auxothropic mutations that are complemented by the bait and prey vectors (leu2 and trp1). The cells were therefore plated on solid SC (-leu-trp) medium (appendix 21) following transformation. The plates were incubated at 30 °C for 3-5 days depending on growth.

Five control interactions were used in the Y2H analyses; these were therefore also transformed into S. cerevisiae MaV203 with the same technique. Three of the controls were based on the interaction of Krev1 with RalGDS (the Ral guanine nucleotide dissociator stimulator protein) (87;88). The interaction between the Krev1 and the wild-type of RalGDS (RalGDS-wt) is known to be a strong positive interaction in the Y2H system. As a weak positive interaction control, the interaction between Krev1 and the mutant1 of RalGDS (RalGDS-m1) were used. An interaction with Krev1 with a second mutant of RalGDS (RalGDS-m2) served as a negative control. In addition to these strong-weak-absent controls, to test that neither the bait nor the prey was able to
autoactivate the promoter on its own, each bait and prey were transformed into the \textit{S. cerevisiae} with its corresponding empty bait and prey destination vector (negative activation controls). The five control interactions and their purpose are described in table 3.3.

<table>
<thead>
<tr>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEXP\textsuperscript{TM}32/Krev1</td>
<td>pEXP\textsuperscript{TM}22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}32/Krev1</td>
<td>pEXP\textsuperscript{TM}22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>pEXP\textsuperscript{TM}32/Krev1</td>
<td>pEXP\textsuperscript{TM}22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>Bait plasmid</td>
<td>pDEST\textsuperscript{TM}22</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>pDEST\textsuperscript{TM}32</td>
<td>Prey plasmid</td>
<td>Negative activation control</td>
</tr>
</tbody>
</table>

As an additional experiment, transformation of the genes present in high-copy number vectors (pGBK and pGAD) was also conducted. The same technique and \textit{S. cerevisiae} strain were used. This experiment was performed to give an indication of whether non-detectable interactions could be detected when the proteins were expressed at high levels, compared to the low-level vectors (pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22). pGBK function as a bait fusion vector, while the pGAD is the prey vector. The combinations of bait and prey constructs transformed into \textit{S. cerevisiae} MaV203 are described in table 3.4.

<table>
<thead>
<tr>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Experimental interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBK/CKS2</td>
<td>pGAD/CDK1 AF</td>
<td>CKS2 and CDK1 AF</td>
</tr>
<tr>
<td>pGBK/CDK1 AF</td>
<td>pGAD/CKS2</td>
<td></td>
</tr>
<tr>
<td>pGBK/CKS2</td>
<td>pGAD/CDK2 AF</td>
<td>CKS2 and CDK2 AF</td>
</tr>
<tr>
<td>pGBK/CDK2 AF</td>
<td>pGAD/CKS2</td>
<td></td>
</tr>
</tbody>
</table>

### 3.6 \textit{S. cerevisiae} two-hybrid analysis (step 5)

As step five in figure 3.1 shows, the experimental interactions are scored by plating the bait and prey transformants onto selective plates. The transformation and Y2H analyses (step 4 and 5) were repeated once, and a total of fourteen transformants were conducted from each interaction (seven transformants in each analysis).
Overnight cultures with the transformed *S. cerevisiae* cells were made (appendix 20). The yeast cells were harvested the following day by centrifugation, and after two washing procedures with sodium chloride, optical density were measured at 600 nm (OD$_{600}$). The suspensions were adjusted to OD ~ 1 with sodium chloride, and $10^{-1}$, $10^{-2}$, and $10^{-3}$ serial dilutions were prepared. 10 μl aliquots of these four dilutions were spotted onto agar selection plates with a marked-up grid, as illustrated in figure 3.2. The OD ~ 1 concentration was spotted in the leftmost square of the lane, and the $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilutions were applied in the remaining squares to the right. The OD adjustments ensured that approximately equal numbers of cells were plated on each plate, thereby facilitating comparison of growth between plates.

**Figure 3.2 Illustration of petri dish with a marked up grid.** Four different dilutions of transformed *S. cerevisiae* suspensions are spotted from the left to the right. Six samples can fit into the plate.

### 3.6.1 Control interactions

Five out of the six lanes on the plate were controls. In lane 1, the strong positive interaction between the Krev1 and the wild-type of RalGDS (RalGDS-wt) was placed. The weak positive interaction control between Krev1 and the mutant1 of RalGDS (RalGDS-m1) was spotted in lane 2. A negative interaction control by the Krev1 interactions with a second mutant of RalGDS (RalGDS-m2) was applied in lane 3. The negative activation controls to test that neither the bait nor the prey was able to autoactivate the promoter, were applied in lane 4 and 5. In the very last lane (# 6), the experimental interaction was spotted. The five control-interactions and their purpose are described in table 3.3.

### 3.6.2 Selection plates

Four different synthetic complete (SC) selection plates were used in the Y2H analysis, and all four were lacking the amino acids leucine and tryptophan. Omitting these verified that both bait and
prey were present in the yeast cell, as the bait transcribe an enzyme in the leucine biosynthesis pathway, and the prey will synthesize an enzyme in the tryptophan pathway. If only one of the expression constructs were present, the cell should not be able to grow. The first plate SC (-leu-trp) was the control plate, merely indicating that the cells contained both bait and prey plasmids. The SC (-leu-trp-his+10 mM 3-AT) scores for interactions using the HIS3 reporter gene, and should only grow if bait and prey interacts, thereby able themselves to produce histidine. 3-AT was added as a supplement to prevent false positives. The SC (-leu-trp-ura) plate scores for interactions using the URA3 reporter gene. As with the HIS3 plates; cells should only grow if bait and prey interacts, that means they were able themselves to produce uracil. The SC (-leu-trp+0.2 % 5-FOA) was also a test for activation of the URA3 reporter, but will yield opposite phenotypes of the first URA3 test. Because 5-FOA was added, cells where the URA3 reporter is activated will not survive. In contrast, if no interaction occurs the cell will survive on the 5-FOA containing plate. The selection plates and their purpose are summarized in table 3.5.

<table>
<thead>
<tr>
<th>Selection plate</th>
<th>Added supplement(s)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc -leu-trp</td>
<td>20 µg/ml uracil 125 µg/ml L-histidine</td>
<td>Transformation control</td>
</tr>
<tr>
<td>Sc -leu-trp-his + 10 mM 3-AT</td>
<td>20 µg/ml uracil 10 mM 3-AT</td>
<td>HIS3 activation (positive selection)</td>
</tr>
<tr>
<td>Sc -leu-trp-ura</td>
<td>125 µg/ml L-histidine</td>
<td>URA3 activation (positive selection)</td>
</tr>
<tr>
<td>Sc -leu-trp + 5-FOA</td>
<td>20 µg/ml uracil 125 µg/ml L-histidine 0.2 % 5-FOA</td>
<td>URA3 activation (negative selection)</td>
</tr>
</tbody>
</table>

### 3.6.3 Testing the interactions and interpretation of results

Although only one single transformant could be tested on the plates since it included five controls, a second plate made from the same preparation as the first plate for the respective plates tested six more transformants. Not including controls on the second plate was justified because the SC agar with the appropriate supplements was mixed simultaneously in the same tube upon preparation (appendix 21). As a total volume of 25 ml constituted one petri dish, the agar and supplements were mixed in a tube to a final volume of 50 ml. The content was carefully shaken and split between two plates. Provided the container was indeed mixed well, ascertained that these
two plates were equal. No controls were therefore spotted on the second plate, as this plate was only used for six additional transformants of the experimental interactions.

The plates were incubated at 30 °C for 3 days, whereafter the phenotypes of the cells were compared to the expected results provided by Invitrogen (66) in the figure 3.3:

**Figure 3.3 Expected results for the controls of the Y2H analysis.** Control 1-3 is the strong, weak and absent control interaction provided by interactions between the Krev1 and different mutants of RalGDS. Control 4 and 5 are negative activation controls to test that neither the bait nor the prey was able to autoactivate the promoter. Figure modified from the manual from Invitrogen (66).

3.6.4 High copy number vectors
The same selection plates were used in the analyses for when the genes were present in the high-copy number vectors (pGBK and pGAD), but only the three first controls were included, excluding the auto activation controls. The marked up grid drawn on the selection plates in this experiment fitted seven samples on each plate, and not six as in the Y2H analyses above. Only the interactions with CKS2 to the CDK1 AF and CDK2 AF (in both domains) were conducted and only one transformant of each were tested.
4 RESULTS

4.1 PCR optimization and cDNA amplification

The gradient PCR was performed to experimentally determine the optimum primer annealing temperatures for the primers. Figure 4.1 shows that amplification product was achieved in the range of 45 °C and 45.3 °C for CKS2, whereas for both CDK1 AF and CDK2 AF amplification product appeared at 45 °C. At temperatures higher than this, no amplification product was visible. The best annealing temperature was therefore 45 °C for all the primers.

![Gradient PCR with temperatures in the range from 45 °C to 65 °C. M: 2 log DNA ladder. Lane 2-13: CKS2. Lane 14-25: CDK1 AF. Lane 26-37: CDK2 AF.](image)

Figure 4.1 1.5 % agarose gel of cDNA of CKS2, CDK1 AF and CDK2 AF. Gradient PCR with temperatures in the range from 45 °C to 65 °C. M: 2 log DNA ladder. Lane 2-13: CKS2. Lane 14-25: CDK1 AF. Lane 26-37: CDK2 AF.

For all three genes, the preparative PCR was set to a uniform temperature of 45 °C in the annealing phase. The PCR products analyzed by agarose gel electrophoresis (figure 4.2) showed a clear product band for all the three genes corresponding with the correct sizes (240 bp for CKS2, 894 bp for CDK1 AF and 897 bp for CDK2 AF). The bands marked in red were excised from the gel and purified. The purified products were further used in the cloning reaction to make entry clones.
Figure 4.2 1.5 % agarose gel of PCR amplification products of CKS2, CDK1 AF and CDK2 AF. Amplification performed under optimized temperature conditions. The positions and sizes (bp) of marker DNA fragments (lane M) are indicated at the left. The marked bands were excised out of the gel with a scalpel, and purified.

4.2 Generation of entry clones
Plasmid DNA from randomly selected transformants from the cloning reaction generating the entry clones were digested with the restriction enzymes NotI HF and Ascl. Gel electrophoresis was performed to separate the cut fragments, and the results are shown in figures 4.3, 4.4 and 4.5 for the three genes. An uncut and cut sample of each selected transformant was placed next to each other on the gel. The bands yielded by the two samples were used for comparison, where the uncut plasmid DNA serves as a control to determine whether the other plasmid had been cut or not. The lane that contained the digested sample was expected to yield two bands; one for the gene, and the other for the empty pENTR™/D-TOPO® vector. As described in section 2.8, different conformational forms of DNA migrate at different rates through the agarose gel (84). The samples of uncut circular entry clones were seen around 2000 bp. The cut empty pENTR™/D-TOPO® vector is linear and the band can be visualized close to 3000 bp.

4.2.1 Entry clone containing the CKS2 gene
Lane 1, 3, 5 and 7 in figure 4.3 contained uncut circular plasmid DNA of the CKS2 entry clone. Lane 2, 4, 6 and 8 are plasmid DNA of the double digested CKS2 entry clone, into 240 bp and 2580 bp fragments. The two bands seen on the gel for the samples in lanes 4, 6 and 8 are consistent with these sizes, indicating that the CKS2 gene was present in the pENTR™/D-TOPO® vector. The bands for the CKS2 gene showed very weak bands. This was most likely due to the bigger size of the cut vector than the gene. Due to the weak CKS2 band, the sample in lane two
might also be correct even if the band for CKS2 was not visible. This was not investigated any further since the three other lanes had a visible CKS2 band. The clone in lane 6 was selected for the further experiments.

![Figure 4.3 1.5 % agarose gel of double digested CKS2 entry clone.](image)

**Figure 4.3 1.5 % agarose gel of double digested CKS2 entry clone.**
The leftmost lane (M) shows the DNA marker 2-log ladder. The next lane (G) is gel-purified PCR product of the CKS2-gene. The following two and two lanes contains plasmid DNA from the same sample. The leftmost lane of the two is uncut plasmid DNA of a CKS2 entry clone, expected to yield one band. The rightmost lane of the two is the same plasmid DNA double digested with NotI HF and AscI, and is expected to yield two bands. The samples that appeared correct are marked with a red circle.

**4.2.2 Entry clone containing the CDK1 AF gene**
Figure 4.4 shows the results for digested entry clones containing the CDK1 AF gene. Lane 1, 3, 5 and 7 are uncut circular plasmid DNA, while lane 2, 4, 6 and 8 contained plasmid DNA cut with the two restriction enzymes into 894 bp and 2580 bp fragments. The samples in lanes 4 and 8 contained two bands which was consistent with the sizes of empty entry vector and the CDK1 AF gene. The clone in lane 4 was selected for the further experiments.
4.2.3 Entry clone containing the CDK2 AF gene

Uncut circular plasmid DNA of the CDK2 AF entry clone was applied in lane 1, 3, 5, 7 and 9 (figure 4.5). The cut fragments in lane 2, 4, 6, 8 and 10 contained plasmid DNA cut with the restriction enzymes into 897 bp and 2580 bp fragments. Only the sample in lane 2 contained two bands which were consistent with these sizes. This clone was therefore used in the further experiments.

Figure 4.4 0.8 % agarose gel of double digested CDK1 AF entry clone.
The leftmost lane (M) shows the DNA marker 2-log ladder, and the following lane (G) is gel-purified PCR product of the CDK1 AF-gene. Only the samples in lane 4 and 8 appear to be correct (marked in red).

Figure 4.5 0.8 % agarose gel of digested CDK2 AF entry clone. The leftmost lane, marked M, shows the DNA marker (2-log ladder) and the following lane is gel-purified PCR product of the CDK2 AF-gene (marked G). Only the clone in lane 2 contained two bands which were consistent with the sizes of empty vector and the CDK2 AF-gene.
4.3 Generation of bait and prey expression constructs
Following the recombination reaction between the entry clones and destination vectors, pDEST\textsuperscript{TM}32 and pDEST\textsuperscript{TM}22, the expression constructs were tested for the presence of gene inserts by digestion with SwaI. The resulting DNA fragments were separated according to size by electrophoresis, and the results are shown in the figures 4.6, 4.7 and 4.8. Panel A in the figures shows the gene cloned as bait, while panel B is the gene cloned as prey. Empty destination vectors were also cut with SwaI to serve as a linear control, to compare the size of empty vectors to vectors with the inserted gene. These were placed in the second lane from the left in the gel (marked V), and showed a band consistent with their sizes of 12 266 bp for pDEST\textsuperscript{TM}32 (panels A) and 8930 bp for pDEST\textsuperscript{TM}22 (panels B) on all the gels. The samples marked with a red circle in the figures were selected for sequencing by GATC Biotech, Germany.

4.3.1 CKS2 cloned as bait and prey
Figure 4.6, panel A shows five transformants from the recombination reaction where CKS2 was cloned into pDEST\textsuperscript{TM}32. A successful bait construct containing CKS2 should have a size of 10 917 bp. The only transformant showing a band of this size was the one in lane 2. This clone was selected for sequencing. Eight transformants from the reaction where CKS2 was cloned into pDEST\textsuperscript{TM}22 to generate prey constructs are shown in fig. 4.6.B. A correctly transformed construct should have a size of 7581 bp. All the eight transformants appeared to be consistent with this size. The clone in the first lane was selected for sequencing.

![Figure 4.6 Gelectrophoresis of linearized bait and prey.](image)

0.8 % agarose gel. The leftmost lane shows the DNA marker (2-log ladder), and the next lane is the linear control (pDEST\textsuperscript{TM}32 in panel A, pDEST\textsuperscript{TM}22 in panel B). Only the marked transformant in panel A appeared to be correct. In panel B, all eight transformants appeared to be correct, but only the one marked with the red circle was selected for sequencing.
4.3.2 CDK1 AF cloned as bait and prey
Successful cloning of CDK1 AF as bait (panel A) and as prey (panel B) should yield bands of the sizes 11 571 bp and 8235 bp, respectively. Figure 4.7.A shows that all four transformants was above the 10 000 bp fragment of the DNA marker (2-log ladder), but slightly below the control size of 12 266 bp. All four transformants therefore seemed to be correct. The transformant in lane 1 was selected for verification through sequencing. In panel B, the transformants in lane 1 and 2 appeared to contain a fragment of the right size (8245 bp). The one in lane 2 was selected for sequencing.

Figure 4.7 Gel electrophoresis of linearized bait and prey. The leftmost lane shows the DNA marker (2-log ladder), and the next lane was linear control of pDEST32 (marked V). The transformant in lane 1 in panel A and the transformant in lane 2 in panel B, was selected for sequencing.

4.3.3 CDK2 AF cloned as bait and prey
Correct recombinant products from the cloning of CDK2 AF as bait should yield a fragment of the size 11 574 bp on the agarose gel (panel A, figure 4.8). Since the four tested transformants appeared larger than 10 000 bp, but not as large as the empty vector control (12 266 bp), the analysis indicated that all the four transformants contained the gene. The transformant in lane 1 was sequenced. Similar results can be seen for the cloning of CDK2 AF as prey (panel B, figure 4.8); the band for the empty vector was consistent with its size of 8930 bp, while the eight transformants were somewhat below that size (should give a fragment of 8238 bp). Two transformants were sent to GATC Biotech for sequencing (lane 1 and 2).
Figure 4.8 Gelectrophoresis of linearized bait and prey. The leftmost lane shows the DNA marker (2-log ladder), and the next lane was linear control of pDEST32. The transformant in lane 1 in panel A appeared correct and was selected for sequencing. In panel B, all the transformants appeared to be correct. The two transformants marked in the red circle were sequenced.

4.3.4 Sequencing of the bait and prey constructs
The nucleotide sequences of the transformants marked with a red circle in the figures 4.6, 4.7 and 4.8 received in return by GATC Biotech, Germany, were compared with the correct nucleotide sequences of the CKS2, CDK1 and CDK2 genes (appendix 16). The results of these comparisons are described in appendix 17, and showed that successful cloning of the genes into the two expression vectors was achieved for all the three genes. The sequencing also verifies the presence of the CDK1 and CDK2 mutations on amino acid 14 and 15 (changed from threonine to alanine and tyrosine to phenylalanine, respectively).

4.4 The two-hybrid analysis
The Y2H analyses for the interactions between CKS2 to CDK1 AF and CDK2, and dimerization of two CKS2 molecules are shown in the figures 4.9 to 4.13. The transformation in S. cerevisiae and Y2H analysis was repeated once, and a total of fourteen transformants were conducted from each interaction (seven transformants in each analysis). Only one transformant is shown for each analysis.

The four different selection plates used are described in table 3.5. All four plates were lacking leucine and tryptophan to select for the presence of both plasmids. The first plate (plate A) was
merely a positive transformation control to show that both bait and prey plasmids are present in the cell. The three next plates performed the test for reporter gene activation. In plate B, \( \text{HIS}3 \) reporter activation was tested. 3-AT was added to reduce auto activation. In plate C and D, \( \text{URA}3 \) reporter activation was tested, whereas plate C was the positive growth control, and plate D served as a negative growth control. Comparison of the experimental interactions with the controls were used to evaluate results (activation of \( \text{HIS}3 \) and \( \text{URA}3 \) reporter), and assess the strength of the interaction being tested (strong, weak or no interaction). The controls also verified that the plates were correct, according to the phenotypes these should produce described in figure 3.3.

As the growth of the controls is common in all the Y2H analyses presented they will not be discussed any further under each section. Lane 1 was the strong positive interaction control and showed growth on plates A, B and C as expected, because the proteins Krev1 and RalGDS-wt are known to have a strong interaction in the Y2H system. No growth on plates D was also expected, as 5-FOA prevents cells containing strongly interacting proteins to grow. The weak positive interaction control in lane 2 between Krev1 and RalGDS-m1 showed growth only on plates A, B and D. This was consistent with that a weak interaction will activate the \( \text{HIS}3 \) reporter, but not the \( \text{URA}3 \) reporter in this system. The absent interaction control in lane 3 only showed growth on the control plates (A), and the 5-FOA containing plates (D), which were also consistent with the expected result for this control. Lane 4 and 5 were negative auto activation controls, and no growth should appear with these two controls except on the control plate and 5-FOA plate, indicating that neither of the plasmid constructs was able to activate the \( \text{HIS}3 \) and \( \text{URA}3 \) reporter. Lane 6 at the bottom was the test scoring for the experimental interaction. For all interactions, the second set of plates containing six more transformants shared the same result (data not shown) as the one interaction that is shown. The photographs for the selection plates are edited so that only the marked up grid is shown.

Figure 4.14 shows the Y2H experiment where the cDNA of the genes are transformed into the high-copy number vectors (pGBK and pGAD).

**4.4.1 Interactions between CKS2 and CDK1 AF**

Lane six in figure 4.9 below shows the result obtained when CKS2 was fused as bait and CDK1 AF fused as prey. In figure 4.10 (lane six) the domains were swapped; CDK1 AF as bait whereas CKS2 as prey. Both two-hybrid analyses showed that CKS2 interacted with CDK1 AF by
activating the *HIS3* reporter, regardless of whether CKS2 (figure 4.9) or CDK1 AF (figure 4.10) was cloned as the bait protein. The *URA3* reporter on the other hand, was not activated in any of the two Y2H analyses performed. All fourteen transformants tested for this interaction gave these results. No signs of toxicity are observed as the growth on the control plates (plate A) were not reduced compared to the controls.

**CKS2 as bait, CDK1 AF as prey**

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pEXP™32/CKS2</td>
<td>pDEST™22 vector (empty)</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>5</td>
<td>pDEST™32 (empty)</td>
<td>pEXP™22/CDK1 AF</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>6</td>
<td>pEXP™32/CKS2</td>
<td>pEXP™22/CDK1 AF</td>
<td>Test of interaction, sample #1</td>
</tr>
</tbody>
</table>

Figure 4.9 *S. cerevisiae* Y2H analysis of CKS2 fused as bait and CDK1 AF fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.
**CDK1 AF as bait, CKS2 as prey**

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pEXP™32/CDK1 AF</td>
<td>pDEST™22 vector (empty)</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>5</td>
<td>pDEST™32 (empty)</td>
<td>pEXP™22/CKS2</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>6</td>
<td>pEXP™32/CDK1 AF</td>
<td>pEXP™22/CKS2</td>
<td>Test of interaction, sample #1</td>
</tr>
</tbody>
</table>

![Figure 4.10 S. cerevisiae Y2H analysis of CDK1 AF fused as bait and CKS2 fused as prey.](image)

The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for HIS3 reporter gene activation. Plate C and D: Test for URA3 reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.

**4.4.2 Interactions between CKS2 and CDK2 AF**

The results obtained for the Y2H analyses of CKS2 interactions with CDK2 AF (figure 4.11 and 4.12) displayed similar results as described for CKS2 and CDK1 AF. However, four out of the fourteen transformants tested in the analyses where CDK2 AF fused as bait and CKS2 fused as prey, did not activate the HIS3 reporter. The URA3 reporter was not activated in any of the
analyses. Additionally, no toxicity was shown in these analyses, indicated by the dense growth on the control plate.

**CKS2 as bait, CDK2 AF as prey**

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXPR™32/Krev1</td>
<td>pEXPR™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXPR™32/Krev1</td>
<td>pEXPR™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXPR™32/Krev1</td>
<td>pEXPR™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pEXPR™32/CKS2</td>
<td>pDEST™22 vector (empty)</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>5</td>
<td>pDEST™32 (empty)</td>
<td>pEXPR™22/CDK2 AF</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>6</td>
<td>pEXPR™32/CKS2</td>
<td>pEXPR™22/CDK2 AF</td>
<td>Test of interaction, sample #1</td>
</tr>
</tbody>
</table>

![Figure 4.11](image)

**Figure 4.11 S. cerevisiae Y2H analysis of CKS2 fused as bait and CDK1 AF fused as prey.** The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for **HIS3** reporter gene activation. Plate C and D: Test for **URA3** reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.
CDK2 AF as bait, CKS2 as prey

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pEXP™32/CDK2 AF</td>
<td>pDEST™22 vector (empty)</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>5</td>
<td>pDEST™32 (empty)</td>
<td>pEXP™22/CKS2</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>6</td>
<td>pEXP™32/CDK2 AF</td>
<td>pEXP™22/CKS2</td>
<td>Test of interaction, sample #1</td>
</tr>
</tbody>
</table>

Figure 4.12 S. cerevisiae Y2H analysis of CDK2 AF fused as bait and CKS2 fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for HIS3 reporter gene activation. Plate C and D: Test for URA3 reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.

4.4.3 Dimerization of CKS2

Lane six in figure 4.13 shows the result for the analysis scoring for an interaction between two CKS2 proteins. No interaction was detected with either the HIS3 reporter or the URA3 reporter. All fourteen transformants shared this result.
**CKS2 as bait, CKS2 as prey**

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pEXP™32/CKS2</td>
<td>pDEST™22 vector (empty)</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>5</td>
<td>pDEST™32 (empty)</td>
<td>pEXP™22/CKS2</td>
<td>Negative activation control</td>
</tr>
<tr>
<td>6</td>
<td>pEXP™32/CKS2</td>
<td>pEXP™22/CKS2</td>
<td>Test of interaction, sample #1</td>
</tr>
</tbody>
</table>

Figure 4.13 *S. cerevisiae* Y2H analysis of the interaction between two CKS2 proteins. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for HIS3 reporter gene activation. Plate C and D: Test for URA3 reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.

**4.4.4 Test of CKS2-CDK interaction using high copy-number vectors**

On plate B in figure 4.14, a weak activation of the HIS3 reporter gene was shown for CKS2 interactions to both CDK1 AF and CDK2 AF, tested in both domains. Growth was reduced compared to the controls in lane 1-3, and to the Y2H analyses above. This was best visualized in
the $10^{-2}$ and $10^{-3}$ dilution on the plates, and indicates a mild toxicity. No URA3 activation was detected. The results on plate D indicate severe toxicity during growth in 0.2 % 5-FOA.

<table>
<thead>
<tr>
<th>Lane</th>
<th>LEU2 Plasmid (BAIT)</th>
<th>TRP1 Plasmid (PREY)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-wt</td>
<td>Strong positive interaction control</td>
</tr>
<tr>
<td>2</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m1</td>
<td>Weak positive interaction control</td>
</tr>
<tr>
<td>3</td>
<td>pEXP™32/Krev1</td>
<td>pEXP™22/RalGDS-m2</td>
<td>Negative interaction control</td>
</tr>
<tr>
<td>4</td>
<td>pGBK/CKS2</td>
<td>pGAD/CDK1 AF</td>
<td>Test of interaction</td>
</tr>
<tr>
<td>5</td>
<td>pGBK/CKS2</td>
<td>pGAD/CDK2 AF</td>
<td>Test of interaction</td>
</tr>
<tr>
<td>6</td>
<td>pGBK/CDK1 AF</td>
<td>pGAD/CKS2</td>
<td>Test of interaction</td>
</tr>
<tr>
<td>7</td>
<td>pGBK/CDK2 AF</td>
<td>pGAD/CKS2</td>
<td>Test of interaction</td>
</tr>
</tbody>
</table>

Figure 4.14 *S. cerevisiae* Y2H analysis of the interaction of CKS2 to CDK1 AF and CDK2 AF present in high copy-number vectors. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for HIS3 reporter gene activation. Plate C and D: Test for URA3 reporter gene activation. The control interactions were spotted in lane 1-3, while the experimental interactions were applied in lane 4-7.
5 DISCUSSION

5.1 CKS2 interactions with CDK1 and CDK2 were detected through the HIS3 reporter gene

The HIS3 reporter gene was activated in both CDK interactions, and it did not matter in which domain CKS2 was present. All fourteen transformants tested for each Y2H analysis shared this result, except when CDK2 AF was fused as bait and CKS2 was fused as prey. In this analysis four out of the fourteen transformants did not activate the reporter. A mild toxicity of CDK2 AF in yeast has however been described (89), and this might be the cause why an interaction was not observed for all transformants in this specific interaction.

Although interactions were detected by the use of the HIS3 reporter gene, the possibility that some of these are spurious cannot be excluded, taken into consideration the leaky character the GAL1 promoter possesses (18;57). On the other hand, because the results were so consistent it is reasonable to believe that this is in fact a true interaction. The interactions are also demonstrated in previous studies (2;3;17;44-46), which strengthen the possibility for the interactions to be true.

5.2 CKS2 interactions with CDK1 and CDK2 were not detected through the URA3 reporter gene

Establishment of the Y2H using the URA3 reporter gene was successful, considering the expected results of the controls where only the strongest protein interaction tested (the Krev1-RalGDS control) activated the reporter. The CKS2-CDK interactions were however not strong enough to be detected through this system. None of the transformants activated the reporter in either selection assays. Taken into account the strong repression of the URA3 reporter gene by the SPO13 promotor (16), failure of activating transcription should therefore not exclude them as true interactors. Combined with the results for HIS3 described above, the findings indicate that the CKS2 interactions with CDK1 and CDK2 are too weak to be detected by the URA3.

An attempt was also made to activate the URA3 reporter by using high copy-number plasmids from a different system. Y2H analysis of S. cerevisiae MaV203 cells with CKS2, CDK1 AF and CDK2 AF present in the 2μ origin of replication vectors (pGAD and pGBK) were performed, but did not show activation of URA3. The result of the experiment indicates that MaV203 cells grow
poorly with this level of fusion protein expression, but the problem needs to be investigated further. It could also have to do with compatibilities between different yeast strains and plasmids.

A prerequisite for the use of Y2H is that the interacting proteins are localized in the nucleus (58). Although it remains unknown where the binding of CKS2 to the CDKs takes place, studies have shown nuclear presence of both CKS2 and the CDKs (8;28;90). The reason for escaping detection should therefore not be derived from this Y2H limitation.

The CDKs used in these experiments mimicked the dephosphorylated, active state as both CDK1 and CDK2 contained two substitutions on amino acid 14 and 15 (20;21). Whether or not this is of any relevance to the binding of CKS2 is somewhat unclear, as CKS2 is shown to bind also to CDKs where only one of these mutations are present (3;17). In addition, Egan and Solomon (3) found that phosphorylation on T161 stimulated binding of CKS2. As Sunnvolls work did detect interactions although lacking this phosphorylation (17), the same CDKs were chosen for use in this project. It is however not certain if the strength to which CKS2 is able to bind is altered because of these modifications on the CDKs. Investigation of the binding with CDKs lacking these should be performed to see if a stronger interaction were provided.

The robustness and low amount of false positives yielded by this reporter are however good arguments to continue the effort obtaining the interactions through this reporter. One approach could be modification of the SPO13 promotor by increasing the number of UAS\textsubscript{GAL} binding sites. The URA3 system used here contains ten such binding sites (appendix 22). Constructing different S. cerevisiae strains with different reporter genes may be another solution. The ADE2 gene also allows negative selection and can thus be used in a reverse screen (57).

5.3 CKS2 dimerization could not be detected by the Y2H system
The interaction between two CKS2 proteins forming a dimer was also assessed with the same Y2H technique, but this interaction was not detected with either the HIS3 or URA3 reporter gene. As the CKS2 dimer could be an inactive form in respect to CDK binding, inhibition of the dimers does not have any therapeutic potential at present. The objective behind the investigation of this interaction was rather to provide more insight into the function of CKS2.

The dimerization of two CKS2 molecules is reported to be an unusual type of binding, so far only seen in two other proteins; the diphtheria toxin and seminal RNase (50). Other unknown
contributing factors needed for dimerization that is not present in the Y2H system, may be the reason why it was left undetected.

5.4 Further prospects
The work carried out as part of this thesis, demonstrates that development of a screen for inhibition of CKS2 interaction can be challenging. It also highlights the value of utilizing multiple reporters. In an effort to counteract the obstacles of URA3 activation, it could be interesting to perform experiments using non-mutated CDKs to investigate if this provides a stronger interaction. Using a system with increased numbers of GAL4 binding sites or other reporter genes could also be useful.
Reference List


(17) Sunnvoll I. Identifying CKS2 protein interactions with CDK1 and CDK2 by the use of yeast two-hybrid technology 2010.


(36) Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science 1989 Nov 3;246(4930):629-34.


(90) SOURCE. The Genetics Department, Stanford University 2011Available from: URL: http://smd.stanford.edu/cgi-bin/source/sourceResult
APPENDICES

1. Primers for polymerase chain reaction (PCR) and sequencing
2. Protocol for PCR of CKS2, CDK1 AF and CDK2 AF genes
   a. Gradient PCR reaction
   b. Preparative PCR reaction
3. Protocol for analysis by gel electrophoresis
4. Protocol for gel purification of PCR products
5. Protocol for preparation of bacterial culture
6. Protocol for plasmid purification, miniprep
7. Protocol for plasmid purification, midiprep
8. Protocol for glycerol stock preparation
9. Protocol for transformation of XL 10-Gold® Ultracompetent E. coli cells
10. Protocol for construction of entry clone: TOPO cloning reaction
11. Protocol for construction of bait and prey plasmids: LR clonase reaction
12. Maps and features of plasmids: pENTR™/D-TOPO® vector, pDEST™32 and pDEST™22
13. Restriction map for pENTR™/D-TOPO® vector-gene, pDEST™32-gene and
   pDEST™22-gene
14. Protocol for restriction analysis of transformants from TOPO cloning reaction
15. Protocol for restriction analysis of transformants from LR clonase reaction
16. Nucleotide sequences of the CKS2, CDK1 and CDK2 genes
17. Sequencing
18. Protocol for preparing S. cerevisiae MaV203 working plate
19. Protocol for transformation in S. cerevisiae MaV203 by the Lithium Acetate/single-
   stranded carrier DNA/polyethylene glycol method (one-step method)
20. Protocol for S. cerevisiae MaV203 two-hybrid analysis
21. Recipes
22. List of materials used
Appendix 1

**Primers for polymerase chain reaction (PCR) and sequencing**

Table 1. Primers for use in PCR amplification of **CKS2**, **CDK1 AF** and **CDK2 AF**:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CKS2</strong></td>
<td>Forward</td>
<td>5’ – CAC CAT GGC CCA CAA GCA G -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ – TCA TTT TTG ATC TTT TGG AAG AGG -3’</td>
</tr>
<tr>
<td><strong>CDK1 AF</strong></td>
<td>Forward</td>
<td>5’- CAC CAT GGA AGA TTA TAC CAA AAT AG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’- CTA CAT CTT CTT AAT CTG ATT GTC C -3’</td>
</tr>
<tr>
<td><strong>CDK2 AF</strong></td>
<td>Forward</td>
<td>5’- CAC CAT GGA GAA CTT CCA AAA GG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’- TCA GAG TCG AAG ATG GGG TAC -3’</td>
</tr>
</tbody>
</table>

Table 2. Primers for use in sequencing of pEXP™32-gene and pEXP™22-gene (GATC Biotech, Germany):

<table>
<thead>
<tr>
<th></th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDEST™32</td>
<td>Forward</td>
<td>5’ – CAC-AGA-TAG-ATT-GGC-TTC-AGT-GGA-G – 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ – AGC-CGA-CAA-CCT-TGA-TTG-GAG –3’</td>
</tr>
<tr>
<td>pDEST™22</td>
<td>Forward</td>
<td>5’ – GCG-TAT-AAC-GCG-TTT-GGA-ATC –3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ – CCG-ACA-ACC-TTG-ATT-GGA-GAC –3’</td>
</tr>
</tbody>
</table>
Appendix 2

**Protocol for PCR of CKS2, CDK1 AF and CDK2 AF genes**

a. **Gradient PCR reaction:**

The protocol *Pfu Ultra* II Fusion HS DNA Polymerase from Stratagene is used.

1) Add the following components in the described order. Mix gently before adding the DNA polymerase.

<table>
<thead>
<tr>
<th>Component</th>
<th>pGBK/CKS2</th>
<th>pGBK/CDK1 AF</th>
<th>pGBK/CDK2 AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10x <em>PfuUltra</em> II reaction buffer</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>dNTP mix (25 mM each dNTP)</td>
<td>2,5</td>
<td>2,5</td>
<td>2,5</td>
</tr>
<tr>
<td>Vector DNA (10 ng)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Primer FW (10 µM)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Primer RW (10 µM)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Pfu Ultra</em> II fusion HS DNA polymerase</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total reaction volume (µl)</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

2) Aliquot the reaction mixes into twelve PCR tubes for each reaction (8 µl in each tube).

3) Carry out PCR using the following amplification cycles:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial step</td>
<td>2 min</td>
<td>95 °C</td>
<td>(initial denaturation)</td>
</tr>
<tr>
<td>30 cycles</td>
<td>20 sec</td>
<td>95 °C</td>
<td>(denaturation)</td>
</tr>
<tr>
<td></td>
<td>55 °C ± 10 °C</td>
<td></td>
<td>(primer annealing)</td>
</tr>
<tr>
<td></td>
<td>20 sec</td>
<td>72 °C</td>
<td>(elongation)</td>
</tr>
<tr>
<td>Final step</td>
<td>10 min</td>
<td>55 °C</td>
<td>(final elongation)</td>
</tr>
</tbody>
</table>

4) Store amplified DNA at 4 °C, or analyze directly on agarose gel.
b. **Preparative PCR reaction:**

1) The second PCR was performed with the same amount of components as described in appendix 2a, but with the following amplification cycle:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial step</td>
<td>2 min</td>
<td>95 °C</td>
<td>(initial denaturation)</td>
</tr>
<tr>
<td>30 cycles</td>
<td>20 sec</td>
<td>95 °C</td>
<td>(denaturation)</td>
</tr>
<tr>
<td></td>
<td>20 sec</td>
<td>45 °C</td>
<td>(primer annealing)</td>
</tr>
<tr>
<td></td>
<td>20 sec</td>
<td>72 °C</td>
<td>(elongation)</td>
</tr>
<tr>
<td>Final step</td>
<td>10 min</td>
<td>55 °C</td>
<td>(final elongation)</td>
</tr>
</tbody>
</table>

2) Store amplified DNA at 4 °C or analyze it directly on agarose gel, followed by gel purification (appendix 4).
Protocol for analysis by gel electrophoresis

1) Add appropriate amount* of agarose powder and 1 x TAE buffer to a 250 ml bottle.
2) Heat the mixture in the microwave oven and boil and swirl the solution until the agarose powder is completely dissolved.
3) Let the mixture cool down and pour it into a gel rack.
4) Apply an appropriate comb for forming the sample wells in the gel**.
5) Allow the gel to set completely at room temperature (25-30 minutes).
6) Remove the comb and transfer the gel to an electrophoresis tank containing enough 1 x TAE buffer to cover the gel.
7) Prepare the DNA ladder and samples for loading using loading buffer (for receipt, see appendix 21). Loading volume is dependent upon the type of comb used (well thickness and length) and thickness of the gel.
8) Load the DNA samples along with a suitable DNA marker to individual wells.
9) Run the gel at 100-150 V until the DNA fragments are clearly separated.
10) Immerse the gel in stain*** and allow to stain for 15-30 minutes.
11) Examine the gel by UV light and photograph the gel (when appropriate, excise the desired gel fragments with a scalpel).

* Determine the amount of agarose powder required to make the desired agarose gel concentration and volume. Example: For a 1% agarose gel, add 1 gram of agarose to 100 ml of 1x TAE buffer.

** For purification of PCR product: Tape up 3-4 teeth of the comb to form one large narrow well.

*** 100 ml dH₂O and 30 μl 10 000xGelRed
Appendix 4

**Protocol for gel purification of PCR products**

*Wizard ® SV Gel and PCR Clean-Up System* from Promega is used for this purpose. Modified from Quick Protocol, following the steps for DNA purification by centrifugation.

**Dissolving the Gel Slice**

1) Following electrophoresis, excise bands from gel while visualizing the bands on a UV transilluminator. Collect the gel fragments into 1.5 ml microcentrifuge tubes.

2) Add 10 μl of membrane binding solution per 10 mg of excised gel. Vortex and incubate at 50-65 °C until the gel slice is completely dissolved.

**Binding of DNA**

3) Insert SV minicolumn into collection tube.

4) Transfer dissolved gel mixture to the minicolumn assembly. Incubate at room temperature for 1 minute.

5) Centrifuge at 14 000 rpm for 1 minute. Discard flowthrough and reinsert minicolumn into collection tube.

**Washing**

6) Add 700 μl of membrane wash solution. Centrifuge at 14 000 rpm for 1 minute. Discard flowthrough and reinsert minicolumn into collection tube.

7) Repeat step 6 with 500 μl of membrane wash solution. Centrifuge at 14 000 rpm for 5 minutes.

8) Empty the collection tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.

**Elution**

9) Transfer minicolumn to a clean 1.5 ml microcentrifuge tube.

10) Add 30 μl of nuclease-free water to the minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 14 000 rpm for 1 minute.

11) Discard minicolumn, and measure DNA concentration.

12) Store DNA at -20 °C.
Appendix 5

Protocol for preparation of bacterial culture

Preparation of *E. coli* overnight culture:
- Transfer a small amount of the appropriate -80 °C glycerol stock of bacterial culture to a flask containing 100 ml LB medium and appropriate antibiotics*.
- Incubate at 37 °C overnight in a shaking water bath.

* Plasmid          | Antibiotic resistance gene | Concentration, µg/ml |
---               |                             |                     |
*pGBKTT7*        | Kanamycin                  | 50                  |
*pGADT7*         | Ampicillin                 | 100                 |
*pENTR™/D-TOPO®* | Kanamycin                  | 50                  |
*pDEST™32*       | Gentamicin                 | 2                   |
*pDEST™22*       | Ampicillin                 | 100                 |
Appendix 6

**Protocol for plasmid purification, miniprep**

Wizard® Plus SV Minipreps DNA Purification System from Promega is used for this purpose. Modified from Quick Protocol, following the steps for centrifugation protocol.

**Production of Cleared Lysate**

1) Pellet 1-10 ml overnight culture of *E. coli* cells containing plasmid at 15 000 rpm for 5 minutes, and remove the supernatant.
2) Thoroughly resuspend pellet with 250 μl cell resuspension solution.
3) Add 250 μl cell lysis solution to each sample; invert 4 times to mix. Incubate until the cell suspension clears (1-5 minutes).
4) Add 10 μl alkaline protease solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
5) Add 350 μl neutralization solution; invert 4 times to mix.
6) Centrifuge at 15 000 rpm for 10 minutes at room temperature.

**Binding of Plasmid DNA**

7) Insert spin column into collection tube.
8) Decant cleared lysate into spin column.
9) Centrifuge at 15 000 rpm for 1 minute at room temperature. Discard flowthrough and reinsert column into collection tube.

**Washing**

10) Add 750 μl wash solution (with ethanol added). Centrifuge at 15 000 rpm for 1 minute. Discard flowthrough and reinsert column into collection tube.
11) Repeat step 10 with 250 μl wash solution.
12) Centrifuge at 15 000 rpm for 2 minutes at room temperature.

**Elution**

13) Transfer spin column to a sterile 1.5 ml microcentrifuge tube, being careful not to transfer any of the column wash solution with the spin column.
14) Add 50 μl of nuclease-free water to the spin column. Centrifuge at 15 000 rpm for 1 minute.
15) Discard column, measure the concentration of DNA and store at -20 °C.
Appendix 7

Protocol for plasmid purification, midiprep

Modified from JETSTAR 2.0 Plasmid Mini/Midi/ Maxi-Protocol, following the steps stated for Midi which is suitable for low copy plasmids (0.2-1 μg DNA/ml LB medium).

Day 1. Preparation of E. coli overnight culture:
- Transfer a small amount of the appropriate -80 °C glycerol stock of bacterial culture to a flask containing 100 ml LB medium and appropriate antibiotics.
- Incubate at 37 °C overnight in a shaking water bath.

Day 2.
1) Column equilibration: add 10 ml of solution E4 to the column. Allow it to empty by gravity flow, do not force out remaining solution.
2) Harvesting bacterial cells: pellet the overnight culture (centrifuge at 4 °C, 8500 rpm for 7 minutes). Remove all traces of LB medium.
3) Cell resuspension: resuspend the pellet with 4 ml of solution E1.
4) Cell lysis: add 4 ml of solution E2. Mix gently by inverting the tube until the lysate appears to be homogenous. Incubate at room temperature for 5 minutes.
5) Neutralization: add 4 ml of solution E3 and mix immediately by multiple inverting. Centrifuge at room temperature, 8500 rpm for 10 minutes.
6) Column loading: transfer the supernatant from step 5 to the equilibrated column, and allow the lysate to run by gravity flow.
7) Column washing: add 10 ml of solution E5, and allow emptying by gravity flow. Repeat step 7.
8) Plasmid elution: switch tube under the column. Add 5 ml of solution E6 to the column and allow to run by gravity flow.
9) Plasmid precipitation: precipitate the DNA with 3.5 ml isopropanol and centrifuge at 4 °C, 8500 rpm for 30 minutes. Discharge the supernatant.
10) Wash the plasmid DNA with 1 ml 70 % ethanol. Centrifuge at 4 °C, 8500 rpm for 5-10 minutes. Remove the ethanol by using a pipette.
11) Air dry the DNA pellet for at least 10 minutes.
12) Redissolve the DNA pellet in 100 μl nuclease-free water. Measure the concentration of DNA and store at -20 °C.

Solutions supplied with kit:

<table>
<thead>
<tr>
<th>Solution E1 (Cell Resuspending)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris</td>
<td>HCl ad pH 8.0</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td></td>
</tr>
</tbody>
</table>

The RNase-containing solution E1 must be stored at 4°C.

<table>
<thead>
<tr>
<th>Solution E2 (Cell Lysis)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM NaOH</td>
<td></td>
</tr>
<tr>
<td>1.0 % SDS (w/v)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution E3 (Neutralization)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 M potassium acetate</td>
<td></td>
</tr>
<tr>
<td>acetic acid ad pH 5.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution E4 (Column Equilibration)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>100 mM sodium acetate</td>
<td></td>
</tr>
<tr>
<td>0.15 % TritonX-100</td>
<td></td>
</tr>
<tr>
<td>acetic acid ad pH 5.0</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution E5 (Column Washing)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>800 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>100 mM sodium acetate</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution E6 (DNA Elution)</th>
<th>Store at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1250 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>100 mM Tris</td>
<td></td>
</tr>
<tr>
<td>HCl ad pH 8.5</td>
<td></td>
</tr>
</tbody>
</table>

RT = room temperature
Appendix 8

Protocol for glycerol stock preparation

Prepare glycerol stocks of *E. coli* and *S. cerevisiae* as follows:

1) Combine equal amounts of overnight culture and 50% glycerol to a 2 ml cryovial.
2) Vortex the vial vigorously to ensure even mixing of the culture and the glycerol.
3) Store at -80 °C.
Appendix 9

Protocol for transformation of XL 10-Gold® Ultracompetent E. coli

Modified protocol provided by Stratagene.

1) Pre-chill a 2 ml tube on ice.
2) Thaw the XL 10 Gold® cells on ice. When thawed, gently mix the cells by pipetting, and transfer 100 μl to the pre-chilled tube.
3) Add 4 μl β-Mercaptoethanol and incubate on ice for 10 minutes, vortexing gently every 2 minutes.
4) Add 0.1 – 50 ng of the experimental DNA, swirl gently and incubate on ice for 30 minutes.
5) Heat-shock the tube at 42 °C for 30 seconds.
6) Incubate the tube on ice for 2 minutes.
7) Add 900 μl preheated (42 °C) LB, and incubate at 37 °C for 1 hour with shaking at 300 rpm.
8) Prepare serial dilutions (10⁻¹ - 10⁻⁵) with LB medium for each sample.
9) Plate 100 μl of each dilution on LB plates containing appropriate antibiotics.
10) Incubate the plates at 37 °C overnight.
Appendix 10

**Protocol for construction of entry clone: TOPO cloning reaction**

Modified protocol provided from Invitrogen™: pENTR™ Directional TOPO® Cloning Kit.

The following equation is used to calculate the amount of PCR product (insert) in the reaction:

\[
\frac{\text{ng of vector} \times \text{lb size of insert}}{\text{lb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}
\]

Where; pENTR™/D-TOPO® = 2580 bp, CKS2 = 240 bp, CDK1 AF: 894 bp, CDK2 AF = 897 bp.

The recommended insert:vector molar ratio is 0.5:1 – 2:1. The ratio 1:1 provided correct result for CKS2 and CDK1 AF, while the correct clone for CDK2 AF was achieved with ratio 0.5:1.

1) Add the following components in the described order, mix gently and incubate for 5 minutes at room temperature.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product (insert)</td>
<td>0.5 – 4 µl</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>add to a final volume of 5 µl</td>
</tr>
<tr>
<td>pENTR™/D-TOPO® vector</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Final volume:</strong></td>
<td><strong>6 µl</strong></td>
</tr>
</tbody>
</table>

2) Place the reaction on ice.

3) Add 2 µl from step 2 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently.

4) Incubate on ice for 30 minutes.

5) Heat-shock the cells for 30 seconds at 42 °C without shaking.

6) Immediately transfer the tubes to ice and add 250 µl of room temperature LB medium.

7) Shake the tube (200 rpm) at 37 °C for 1 hour.

8) Spread 100 µl directly per sample onto a selective plate.

9) Centrifuge the remaining suspension at 15000 rpm for 5 minutes, and discharge the supernatant. Resuspend the cell pellet in 100 µl LB medium, and spread it onto a second plate.

10) Incubate the plates at 37 °C overnight.
Appendix 11

**Protocol for construction of bait and prey plasmids: LR clonase reaction**

Modified protocol provided from Invitrogen™: Gateway® LR Clonase II Enzyme Mix.

1) Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:

- Entry clone (150 ng) 1-7 µl
- Destination vector (150 ng/µl) 1 µl
- TE buffer, pH 8.0 to 8 µl

2) Thaw on ice the LR Clonase II enzyme mix for about 2 minutes. Vortex the LR Clonase II enzyme mix briefly twice (2 seconds each time).

3) To each sample (Step 1, above), add 2 µl of LR Clonase II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.

4) Incubate reactions at 25 °C overnight.

5) Add 1 µl of the proteinase K solution to each sample and vortex briefly. Incubate samples at 37 °C for 10 minutes.

**Transformation**

6) Add 1 µl from each LR-reaction mixture into a vial of TOP 10 One Shot chemically competent *E. coli* and mix gently.

7) Incubate on ice for 30 minutes.

8) Heat-shock the cells for 30 seconds at 42 °C.

9) Add 250 µl of room temperature LB medium.

10) Shake the reaction mix at 300 rpm for 1 hour at 37 °C.

11) Plate the transformation mix on LB plates supplemented with appropriate antibiotics.

12) Incubate overnight at 37 °C.
Appendix 12

Maps and features of plasmids: pENTR™/D-TOPO®, pDEST™32 and pDEST™22

Comments for pENTR™/D-TOPO®
2580 nucleotides

- rmb T2 transcription termination sequence: bases 268-296
- rmb T1 transcription termination sequence: bases 427-470
- M13 forward (-20) priming site: bases 537-552
- attL1: bases 569-968 (c)
- TOPO® recognition site 1: bases 680-684
- Overhang: bases 685-688
- TOPO® recognition site 2: bases 689-693
- attL2: bases 705-804
- T7 Promoter/priming site: bases 821-840 (c)
- M13 reverse priming site: bases 845-861
- Kanamycin resistance gene: bases 974-1783
- pUC origin: bases 1904-2577

(c) = complementary sequence
Comments for pDEST™ 32
12266 nucleotides

ADH1 promoter: bases 103-1557
GAL4 DNA binding domain: bases 1581-2024
attR1 site: bases 2037-2161
Chloramphenicol resistance (CmR) gene: bases 2411-3070
ccdB gene: bases 3411-3718
attR2 site: bases 3757-3831
ADH1 transcription termination region: bases 4119-4276
f1 ori: bases 4603-5058
Leu2 gene: bases 5767-9081
ARS4/CEN6 origin: bases 7589-8107
Gentamicin resistance gene: bases 8452-8985 (c)
pUC origin: bases 9833-10506
Cycloheximide sensitivity (CYH2): bases 11445-11894 (c)
(c) = complementary strand
Comments for pDEST™22
8930 nucleotides

ADH1 promoter: bases 272-1726
Nuclear localization signal (NLS): bases 1734-1754
GAL4 DNA activation domain: bases 1761-2105
attR1 site: bases 2121-2145
Chloramphenicol resistance (Cm¹⁰) gene: bases 2495-3154
ccdB gene: bases 3495-3800
attR2 site: bases 3841-3965
ADH1 transcription termination region: bases 4203-4360
f1 origin: bases 4687-5142
TRP1 gene: bases 5245-5919 (c)
ARS4/CEN6 origin: bases 6455-6972
Ampicillin (bla) resistance gene: bases 7104-7964
pUC origin: bases 8109-8782

(c) = complementary strand
Appendix 13

Restriction map for pENTR™/D-TOPO®-gene, pDEST™32-gene and pDEST™22-gene

pENTR/D-TOPO

pDEST32
Appendix 14

**Protocol for restriction analysis of transformants from TOPO cloning reaction**

1) Set up the restriction enzyme digest as shown below (listed in order of addition):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>-</td>
</tr>
<tr>
<td>10xNEBuffer4</td>
<td>5</td>
</tr>
<tr>
<td>10xBSA</td>
<td>5</td>
</tr>
<tr>
<td>0.5 – 1 µg DNA</td>
<td>-</td>
</tr>
<tr>
<td>NotI HF enzyme</td>
<td>0.5</td>
</tr>
<tr>
<td>AscI enzyme</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>

2) Gently flick the tubes to mix the reaction.
3) Incubate reaction tubes for 2-4 hours at 37 °C.
4) Store at -20 °C after inactivation of the enzymes at 65 °C for 20 minutes, or add loading dye and load samples directly onto agarose gel for electrophoresis.
5) Stain the gel with GelRed and photograph the gel under UV light.
Appendix 15

Protocol for restriction analysis of transformants from LR Clonase reaction

1) Set up the restriction enzyme digest as shown below (listed in order of addition):

- Nuclease-free water
- 10xNEBuffer3
- 10xBSA
- 0.5 – 1 µg DNA
- SwaI enzyme

Total volume 50 µl

2) Gently flick the tubes to mix the reaction.

3) Incubate reaction tubes for 2-4 hours at 25 °C.

4) Store at -20 °C after inactivation of the enzymes at 65 °C for 20 minutes, or add loading dye and load samples directly onto agarose gel for electrophoresis.

5) Stain the gel with GelRed and photograph the gel under UV light.
Appendix 16

**Nucleotide sequences of the CKS2, CDK1 and CDK2 genes**

These nucleotide sequences are obtained from internet resources of the National Center for Biotechnology Information, NCBI.

**CKS2 240 bp**

```
ATGGCCCAACAGCAGATCTACTACTCTGGACAGAAACTACGAGTACCGGCATGTTATGT
TACCCAGAGAATTTCCAAACAGTACCTAAAACACTCTGTGACAGGAGGACCTCCTATCG
TGTCAGAGAGTGTTGCTTACATGATTCATGAGCCAGAACCACATATTCTTTTTAGA
CGACCTCTCTCCAAAAAGATCACACAAAAATGA
```

**CDK1 (variant 1) 894 bp**

```
ATGGGAAGATTATACCAAATAGAGAAATTTGGAGAAGTACCTATGGAGTTGTGTAAGAGGATG
CAAAAATCCAGGTTATTTTCACGTTTCCATTTTGGGACACTGATCTTTCCATGGATCTGAA
ACTGCAATTCGGGAAATTTCTCTTTACCCATGGGATGTTATATATATACATTCCTATGGGA
ATTAGTAAACACTCTGCTGATCAGATCTTGCTCTGATCTCCTCTTTTCATGGGATG
TTATGCAGAGATTTCTGTTGCTTACAGAGACTTTAATCTTTTAGATGTGACAAAG
GAACATTTAAACTGCAGTTATTTTGCCCTTCCAGAGCTTTTTGGAATACCTATCATGGATATATACACATGA
GGTAGTAAACACTCTGCTGATCAGATCTTGCTCTGATCTCCTCTTTTCATGGGATG
TTATGCAGAGATTTCTGTTGCTTACAGAGACTTTAATCTTTTAGATGTGACAAAG
GAACATTTAAACTGCAGTTATTTTGCCCTTCCAGAGCTTTTTGGAATACCTATCATGGATATATACACATGA
GGTAGTAAACACTCTGCTGATCAGATCTTGCTCTGATCTCCTCTTTTCATGGGATG
TTATGCAGAGATTTCTGTTGCTTACAGAGACTTTAATCTTTTAGATGTGACAAAG
GAACATTTAAACTGCAGTTATTTTGCCCTTCCAGAGCTTTTTGGAATACCTATCATGGATATATACACATGA
```

- 82 -
CDK2 (variant 1) 897 bp

ATGGGAGAACTTCAAAAAGGTGAAAAAGACGCACCAGGTGATTGTGTAACAAAGCCAGAAACA
AGTTGACCGGAGAGGGTGCCTTGTTAGGAAATCCGCCCTGGACACTGAGACTGAGGGTGTGCCCAGTG
TGCCCATCGAGATCTCTCTGTCTTAAGGAGCTTAACCTAACTCTAAATTTCTGCAACGAGATCTCAAGGATCCT
CTGCTCTCTGCTGCTCTTCTCTCTCTCTCTCTCTCTCTCTCTGAATTCTACCCACAGATGTGAATCTG
TGACCCTGTGGAACCGCTCTCTGAAATTTTGGGCAATTATTTATCCACAGGTGACATCTG
GAGCTTCAGGCTCTCTTTGTGATGTTGACTCCGCGGCCCTATTTCTGGAGATTGAGTTGACATCTG
CAGCTCTCTCTGCTCTCTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTGCTCTACAGGATTTGACATCTG
CTGATTACAAGGCAGTTCCAAAGGTGGCCCAGCAGATTATTTAGTAAGTTGTAACCCCTCTGGATGA
AGATGGGACCGAGCTTTATCTGCAATAGCTGACTACGACCCCTGAACAGCGGATTTGCGCCTACAGGCCG
CTGGCTCACCCTTTCCAGGATGTTACCAAGCCAGTACCACTCTGCCTCTCTCTGA
Appendix 17

**Sequencing**

A comparison of the nucleotide sequences received in return from GATC Biotech (Germany) with the correct gene sequences were performed using the Basic Local Alignment Search Tool (BLAST) provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Appropriate primers were prepared (appendix 1) and shipped along with the samples. FW is forward primer, RW is reverse primer.

Query: correct gene sequence
Subject: nucleotide sequence received from GATC Biotech, Germany

**Result of BLAST search for CKS2 cloned into pEXP32, FW:**

>lcl|55809
Length=920

Score = 444 bits (240),   Expect = 4e-129   
Identities = 240/240 (100%),   Gaps = 0/240 (0%)   
Strand=Plus/Plus

<table>
<thead>
<tr>
<th>Query</th>
<th>Sbjct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>181</td>
<td>181</td>
</tr>
<tr>
<td>241</td>
<td>241</td>
</tr>
</tbody>
</table>

**Results of BLAST search for CDK1 AF cloned into pEXP32, FW:**

>lcl|16385
Length=1017

Score = 1596 bits (864),   Expect = 0.0   
Identities = 877/885 (99%),   Gaps = 1/885 (0%)   
Strand=Plus/Plus

<table>
<thead>
<tr>
<th>Query</th>
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<td>181</td>
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<tr>
<td>241</td>
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</table>
Results of BLAST search for CDK1 AF cloned into pEXP32, RW:
>lcl|34167
Length=982

Score = 1550 bits (839),  Expect = 0.0
Identities = 856/871 (98%),  Gaps = 0/871 (0%)
Strand=Plus/Minus

Query 24  GAAAATTGGAGAAGGTACCTATGGAGTTGTATAAGGGTAGACACAAAATCTACAGGTC  83
Sbjct 371  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  430

Query 301  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
Sbjct 361  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  490

Query 421  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
Sbjct 491  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  550

Query 601  AAAACCTTTCCATGGAGCTTACCAACTTCCCTGGAGTTTTGTCACAGCTTTTG  660
Sbjct 731  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  790

Query 661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
Sbjct 791  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  850

Query 721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
Sbjct 911  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  910

Query 840  AGTGGTAGCCATGAAAAAAATGGACTAGAAAGTGAAGAGGAAGGGGTTCCTAGTACTGC  884
Sbjct 921  AGTGGTAGCCATGAAAAAAATGGACTAGAAAGTGAAGAGGAAGGGGTTCCTAGTACTGC  862

Query 144  AATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTCTTCAGGA  203
Sbjct 861  AATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTCTTCAGGA  802

Query 204  TGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGGATCTGAA  263
Sbjct 801  TGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGGATCTGAA  740

Query 264  GAAATACTTGGAGTATACCTTCCCTCGTCAACTTGAGATTTGGTACATGGATTCTTCACTTGTTAAGAGTTA  323
Sbjct 741  GAAATACTTGGAGTATACCTTCCCTCGTCAACTTGAGATTTGGTACATGGATTCTTCACTTGTTAAGAGTTA  682

Query 324  TTTATACAAATCTACAGGGGATTTGGTATCTTCACTTGAAGGTTCTTCCACAGAAGA  383
Sbjct 971  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  1015

- 85 -
Results of BLAST search for CDK2 AF cloned into pEXP32, FW: #9

>lcl|37315
Length=1012

Score = 1620 bits (877),  Expect = 0.0
Identities = 884/888 (99%),  Gaps = 0/888 (0%)
Strand=Plus/Plus

Query 1  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA
Sbjct  125   ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCTTCGGAGTTGTGTACAAA

Query 61  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG
Sbjct  185   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAATCCGCCTGGACACTGAG

Query 121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT
Sbjct  245   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT

Query 241  GAATTTC
gcccagggcctagcctttctgccattct
Sbjct  365   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT

Query 301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT
Sbjct  425   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT

- 86 -
Result of BLAST search for CDK2 AF cloned into pEXP32, RW:

>`lcl|39983`

Length=1069

Score = 1637 bits (886), Expect = 0.0
Identities = 893/897 (99%), Gaps = 0/897 (0%)
Strand=Plus/Minus
Results of BLAST search for CDK2 AF cloned into pEXP32, FW: #10

Score = 1572 bits (851), Expect = 0.0
Identities = 879/893 (98%), Gaps = 7/893 (1%)
Strand=Plus/Plus
Results of BLAST search for CDK2 AF cloned into pEXP32, RW:

>lcl|17759
Length=1109

Score = 1640 bits (888), Expect = 0.0
Identities = 894/897 (99%), Gaps = 0/897 (0%)
Strand=Plus/Minus

Query  1   ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
Sbjct  1013  ATGGAGAACTTCCAAAAGGTGGAA

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
Sbjct  953   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  894

Query  121   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
Sbjct  893   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  834

Query  181   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
Sbjct  833   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  774

Query  241   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  300
Sbjct  773   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  594

Query  301   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
Sbjct  593   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  534

Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
Sbjct  653   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  594

Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
Sbjct  593   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  534

Query  481   CATGAGGTGGTGGCCTGGTGACCGGCCTGGCTGGTACCCGAGTTCTCCTGCTGGATGGAGTGGGG  540
Sbjct  533   CATGAGGTGGTGGTGGCCTGGTGACCGGCCTGGCTGGTACCCGAGTTCTCCTGCTGGATGGAGTGGGG

---

- 89 -
Result of BLAST search for **CKS2** cloned into **pEXP22**, FW:

Query: CKS2
Subject: pEXP22-CKSA FW

**>lcl|21587**

Length=905

Score = 444 bits (240), Expect = 4e-129
Identities = 240/240 (100%), Gaps = 0/240 (0%)
Strand=Plus/Plus

Query 1  ATGGCCCAAGCACATCTACTACTCGGACAAGTACTTCGACGAACA

Sbjct 130  ATGGCCCAAGCACATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG 189

Query 61  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA

Sbjct 190  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA 249

Query 121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT

Sbjct 250  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT 309

Query 181  GAGCCAGAACCACATATTCTTCTTCCAGGTAGCCACTACCTTCCTCCAAAAGATCAACAAAAATGA

Sbjct 310  GAGCCAGAACCACATATTCTTCTTCCAGGTAGCCACTACCTTCCTCCAAAAGATCAACAAAAATGA 369

---

**Results of BLAST search for **CDK1** AF cloned into **pEXP22**, FW:**

**>lcl|18249**

Length=934

Score = 1483 bits (803), Expect = 0.0
Identities = 810/815 (99%), Gaps = 0/815 (0%)
Strand=Plus/Plus

Query 1  ATGGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTAAGCTACTTGGAGTTGTGTATAAG

Sbjct 115  ATGGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTAAGCTACTTGGAGTTGTGTATAAG 174

Query 61  GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA

Sbjct 175  GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA 234
Query 121 GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAGGAACTTCGTCAT 180
Sbjct 235 GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAGGAACTTCGTCAT 294
Query 181 CCAATAATGTCAGCTTCTCCAGGATGCTTTAGCCAGGTACCTCAGTATCTTCATCTTT 240
Sbjct 295 CCAATAATGTCAGCTTCTCCAGGATGCTTTAGCCAGGTACCTCAGTATCTTCATCTTT 354
Query 241 GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCCTGGTCAGTACATG 300
Sbjct 355 GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCCTGGTCAGTACATG 414
Query 301 GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC 360
Sbjct 415 GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC 474
Query 361 TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA 420
Sbjct 475 TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA 534
Query 421 ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT 480
Sbjct 535 ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT 594
Query 481 GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCCTGGTCAGTACATG 540
Sbjct 595 GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCCTGGTCAGTACATG 654
Query 541 TACTCAACTCTGAGCTTACATGGGATGTAATCTATGATCC 600
Sbjct 655 TACTCAACTCTGAGCTTACATGGGATGTAATCTATGATCC 714
Query 601 AAACCATTTTCCATGGGAAGCTTACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA 660
Sbjct 715 AAACCATTTTCCATGGGAAGCTTACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA 774
Query 661 GGCACCTCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA 720
Sbjct 775 GGCACCTCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA 834
Query 721 TTTCCCAAATGGAAACCAGGAAGCTTACATGGATATATGTTGAATGTTTCAACTCAATCCGAGCTTTTG 780
Sbjct 835 TTTCCCAAATGGAAACCAGGAAGCTTACATGGATATATGTTGAATGTTTCAACTCAATCCGAGCTTTTG 894
Query 781 TTGGATTTGCTCCTCGAAATGTATACTATGATCC 815
Sbjct 895 TTGGATTTGCTCCTCGAAATGTATACTATGATCC 929

Result of BLAST search for CDK1 AF cloned into pEXP22, RW:
>lcl|44275
Length=975

Score = 1493 bits (808), Expect = 0.0
Identities = 813/818 (99%), Gaps = 0/818 (0%)
Strand=Plus/Minus
Result of BLAST search for CDK2 AF cloned into pEXP22, FW:

`>lcl|51541`

Length=908

Score = 1428 bits (773), Expect = 0.0

Identities = 784/791 (99%), Gaps = 0/791 (0%)

Strand=Plus/Plus

Query 1  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCCGTCACGGAGTTGTGTACAAA  60

Sbjct 118  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCCGTCACGGAGTTGTGTACAAA  177

Query 61  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120

Sbjct 178  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  237

Query 121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180

Sbjct 238  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  297

Query 241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300

Sbjct 358  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  417

Query 241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300

Sbjct 358  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  417

Query 301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360

Sbjct 418  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  477
Result of BLAST search for CDK2 AF cloned into pEXP22, RW:

>lcl|29011
Length=977

Score = 1557 bits (843), Expect = 0.0
Identities = 848/853 (99%), Gaps = 0/853 (0%)
Strand=Plus/Minus

Query 45  CCGAGTTGTGATCAAAGCCAGAAACAGATTGAGGAGGAGTGGTGGCGCTTAAGAAAAT  104
Sbjct 964  CCGAGTTNTNTAAGCCAGAAACAGATTGAGGAGGAGTGGTGGCGCTTAAGAAAAT  905

Query 105  CCCCTGAGCAGATCGAGACGGTGGGCGCCACTGAGATCAGAGAGCTCTCTCTGGCT  164
Sbjct 904  CCCCTGAGCAGATCGAGACGGTGGGCGCCACTGAGATCAGAGAGCTCTCTCTGGCT  845

Query 165  TAAAGAGCTAAACCATCCAATATTTGTCAAGCCTGAGATGCATGCACAGCAGAATAA  224
Sbjct 844  TAAAGAGCTAAACCATCCAATATTTGTCAAGCCTGAGATGCATGCACAGCAGAATAA  785

Query 225  ACTCTACTGCTGTTTTTGAATTTCTCCTGACACAGATCTCAAGAAATCTAGTGCAGCTCCTGCT  284
Sbjct 784  ACTCTACTGCTGTTTTTGAATTTCTCCTGACACAGATCTCAAGAAATCTAGTGCAGCTCCTGCT  725

Query 285  TCTCACTGCGATCTCTCTCCCTCTCCTACAGAGCTATCTGCTCCACAGGCTCCTGCT  344
Sbjct 724  TCTCACTGCGATCTCTCTCCCTCTCCTACAGAGCTATCTGCTCCACAGGCTCCTGCT  665

Query 345  AGCTTTCTGCCATTCTCATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTAT  404
Sbjct 664  AGCTTTCTGCCATTCTCATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTAT  605

Query 405  TAAACAGAGGGGGCAATCAAGCTTAGAGCTTTTGGGACTACGACAGCTTCTGCTGCTGCC  464
Sbjct 604  TAAACAGAGGGGGCAATCAAGCTTAGAGCTTTTGGGACTACGACAGCTTCTGCTGCTGCC  545

Query 465  TGCTCGAAGCTCAACACCCTGAGGTTGACTACCGAGCTTCTGCTGCTGAGAATCCCTCT  524
Sbjct 544  TGCTCGAAGCTCAACACCCTGAGGTTGACTACCGAGCTTCTGCTGCTGAGAATCCCTCT  885
Appendix 18

**Working plate of *S. cerevisiae* MaV203**

1) Use a micropipette-tip to transfer and resuspend an appropriate amount of *S. cerevisiae* MaV203 from the -80 °C glycerol stock in 1000 µl sterile 0.8 % NaCl.

2) Plate 100 µl of serial dilutions (10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\) and 10\(^{-5}\)) onto YPD agar (appendix 21) supplemented with adenine hemi sulfate to a final concentration of 100 µg/ml (YPAD).

3) Let dry before incubation at 30 °C for 3-4 days.

4) The working plates can be stored at 4 °C for 4-6 weeks.
Appendix 19

Protocol for transformation in *S. cerevisiae* MaV203 by the
Lithium Acetate/single-stranded carrier DNA/polyethylene glycol method
(LiAc/SS carrier DNA/PEG)
(one-step method)

1) Mix 100 µl of one-step buffer with 10 µl of sterile 1 M dithiothreitol (DTT).
2) Harvest and resuspend 3 colonies of *S. cerevisiae* MaV203 from the working plate
   (size: 2-3 mm) in this solution by vortexing.
3) Add this mix in a microcentrifuge tube containing ~ 500 ng of each plasmid DNA (the total
   volume of DNA should be up to 10 µl and approximately 1 µg) and 1µl Herring Testes
   Carrier DNA.
4) Vortex gently.
5) Incubate at 45 °C for 30 minutes.
6) Plate the cells on solid SC -leu-trp medium (appendix 21). Use glassbeads to promote even
   spreading of the cells.
7) Incubate the plates at 30 °C for 3-5 days.
Appendix 20

**Protocol for S. cerevisiae MaV203 two-hybrid analysis**

*Day 1. Preparation of S. cerevisiae MaV203 overnight culture:*

Add the following to separate Falcon tubes:
- 2 ml SD-DO medium (-ade -his -leu -trp)
- Adenine hemisulfate to a final concentration of 20 µg/ml
- L-histidine to a final concentration of 125 µg/ml

Transfer one *S. cerevisiae* MaV203 transformant per separate Falcon tube. Incubate at 30 °C overnight in a shaking water bath.

*Day 2.*

1) Centrifuge 1.5 ml from the overnight culture at 16400 rpm for 3 minutes, and discharge the supernatant.
2) Resuspend the cell pellet in 1 ml sterile 0.8 % NaCl. Centrifuge at 16400 rpm for 3 minutes and discharge the supernatant.
3) Repeat step 3.
4) Resuspend the cell pellet in 500 µl sterile 0.8 % NaCl.
5) Dilute 100 µl from step 4 with 900 µl of sterile 0.8 % NaCl, and measure optical density (OD) at 600 nm. Adjust with sterile 0.8 % NaCl to OD ~ 1.0.
6) Prepare 10⁻¹, 10⁻², and 10⁻³ dilutions with sterile 0.8 % NaCl for each sample.
7) Spot 10 µl aliquots of each dilution (OD = 1, 10⁻¹, 10⁻², 10⁻³) onto agar selection plates with a marked-up grid.
8) Let dry, and incubate at 30 °C for 3 days.
Appendix 21

**Recipes**

**Luria-Bertani (LB) Medium, Agar and Plates**

Rich medium for growth of *E. coli*

**LB medium:**
- Bacto tryptone: 10 g
- Yeast extract: 5 g
- NaCl: 10 g
- dH$_2$O to 1000 ml

Sterilize by autoclaving: 121°C, 15 min

**LB agar:**
- Bacto agar: 20 g
- Yeast extract: 5 g
- NaCl: 10 g
- Bacto tryptone: 10 g
- dH$_2$O to 1000 ml

Sterilize by autoclaving: 121°C, 15 min

**LB agar plates:**
Melt LB agar in microwave. Cool to ~55°C, add appropriate antibiotic*. Dispense into sterile 10 cm petri dishes. Store plates when solidified upside down at 4°C.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Antibiotic resistance gene</th>
<th>Concentration, µg/ml</th>
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</thead>
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<tr>
<td>pGBK T7</td>
<td>Kanamycin</td>
<td>50</td>
</tr>
<tr>
<td>pGAD T7</td>
<td>Ampicillin</td>
<td>100</td>
</tr>
<tr>
<td>pENTR™/D-TOPO®</td>
<td>Kanamycin</td>
<td>50</td>
</tr>
<tr>
<td>pDEST™32</td>
<td>Gentamicin</td>
<td>2</td>
</tr>
<tr>
<td>pDEST™22</td>
<td>Ampicillin</td>
<td>100</td>
</tr>
</tbody>
</table>

**YPD Medium, Agar and Plates:**

Rich medium for growth of *S. cerevisiae*

**YPD medium**
- YPD: 25 g
- dH$_2$O to 500 ml

Sterilize by autoclaving: 121°C, 15 min

**YPD agar**
- YPD: 20 g
- dH$_2$O to 300 ml and let it dissolve under stirring
- Adjust pH to 5.9 – 6.2
- Bacto agar: 9 g
- dH$_2$O to 400 ml

Sterilize by autoclaving: 121°C, 15 min
YPAD plates
Melt YPD agar in microwave. Cool to ~55 °C, add adenine sulfate to a final concentration of 100 µg/ml. Dispense into sterile 10 cm petri dishes. Store plates when solidified upside down at 4 °C.

**SC Medium, Agar and Plates:**

Synthetic Complete medium for growth of *S. cerevisiae*.

Synthetic complete consists of a nitrogen base, a carbon source, and a ‘dropout’ solution containing essential amino acids, nucleic acids, trace elements and vitamins. For selection purposes, certain amino acids are omitted (‘dropped out’) from the medium.

**SC medium**
Minimal SD base 10.68 g
DO supplement (-his -leu -trp -ura) 0.24 g
dH₂O to 500 ml
Sterilize by autoclaving: 121 °C, 15 min

**SC agar**
Minimal SD base 10.68 g
DO supplement (-his -leu -trp -ura) 0.24 g
Adjust pH to 5.9 – 6.2
Bacto agar 9 g
dH₂O to 400 ml
Sterilize by autoclaving: 121 °C, 15 min

**SC plates**
Melt SC agar in microwave, Cool to ~55 °C, add appropriate supplements (see table below). Dispense into sterile 10 cm petri dishes. Store plates when solidified upside down at 4 °C.

<table>
<thead>
<tr>
<th>Selection plate</th>
<th>Added supplement(s)</th>
<th>Purpose/assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc -leu-trp</td>
<td>20 µg/ml uracil 125 µg/ml L-histidine</td>
<td>Transformation control</td>
</tr>
<tr>
<td>Sc –leu-trp-his + 10 mM 3-AT</td>
<td>20 µg/ml uracil 10 mM 3-AT</td>
<td>HIS3 activation (positive selection)</td>
</tr>
<tr>
<td>Sc -leu-trp-ura</td>
<td>125 µg/ml L-histidine</td>
<td>URA3 activation (positive selection)</td>
</tr>
<tr>
<td>Sc -leu-trp + 5-FOA*</td>
<td>20 µg/ml uracil 125 µg/ml L-histidine 0.2 % 5-FOA</td>
<td>URA3 activation (negative selection)</td>
</tr>
</tbody>
</table>

* see direction for preparation below
Medium, Agar and Plates for the 5-FOA test:

2xSC medium
Minimal SD base 26.70 g
DO supplement (-his -leu -trp -ura) 0.60 g
dH₂O to 300 ml and let it dissolve under stirring
Adjust pH 5.9 – 6.2
Complete with dH₂O to 400 ml
Sterilize by autoclaving: 121 °C, 15 min

4 % agar:
Bacto agar 16 g
dH₂O to 400 ml
Sterilize by autoclaving: 121 °C, 15 min

5-FOA plates:
Heat 2xSC medium to ~ 55 °C
Add 5-FOA to a final concentration of 0.2 %
Adjust pH~4.5
Combine with equal amount melted 4 % agar (cooled down to ~ 55 °C)
Dispense into sterile 10 cm petri dishes. Store plates when solidified upside down at 4 °C

Other solutions:

0.8 % NaCl
Sodium chloride 4 g
dH₂O to 500 µl

10x loading buffer:
Glycerol, 87 % 500 µl
dH₂O 500 µl
Bromophenol blue 0.03 %*

*add appropriate amount until a deep blue color is achieved

For preparation of 1x loading buffer: dilute 1:10.
## List of materials used

### Materials and reagents used for Y2H

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<thead>
<tr>
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<th>Relevant features</th>
<th>Source</th>
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<tbody>
<tr>
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<td>Bacto agar</td>
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<td>BD</td>
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<tr>
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<td>Clontech</td>
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<tr>
<td>Herring Testes Carrier DNA</td>
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<td>Clontech</td>
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<tr>
<td>Minimal SD base</td>
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<td>Clontech</td>
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<td>5-FOA</td>
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<td>Fermentas</td>
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### Bacterial strains

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<td>TOP10 One Shot</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) Õ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-</td>
<td>Invitrogen</td>
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<td>XL 10-Gold</td>
<td>Tet'Δ(mrcA)183Δ(mrcCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI'Z ΔM15 Tn10 (Tet') Amy Can']</td>
<td>Stratagene</td>
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### Nutritional/selection

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<td>Kan/ TRP1</td>
<td>Clontech</td>
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<td>Kan/ TRP1</td>
<td>Irene Sunnvoll</td>
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<tr>
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<td>pGBKKT7 bearing CDK1AF</td>
<td>Kan/ TRP1</td>
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<tr>
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<td>This work</td>
</tr>
<tr>
<td>pEXP™32/CDK1 AF</td>
<td>pDEST™32 bearing CDK1 AF</td>
<td>Gent/ LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>pEXP™32/CDK2 AF</td>
<td>pDEST™32 bearing CDK2 AF</td>
<td>Gent/ LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>pDEST™22</td>
<td>PpADH1, pUC ori, ARS4/CEN6 ori, attR1, attR2, ccdB gene, Cm(^R); yeast two-hybrid vector bearing the GAL4 AD binding domain</td>
<td>Amp/ TRP1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEXP™22/RalGDS-wt</td>
<td>pDEST™22 bearing RalGDS-wt</td>
<td>Amp/ TRP1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEXP™22/RalGDS-m1</td>
<td>pDEST™22 bearing RalGDS-m1</td>
<td>Amp/ TRP1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEXP™22/RalGDS-m2</td>
<td>pDEST™22 bearing RalGDS-m2</td>
<td>Amp/ TRP1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEXP™22/CKS2</td>
<td>pDEST™22 bearing CKS2</td>
<td>Amp/ TRP1</td>
<td>This work</td>
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<tr>
<td>pEXP™22/CDK1 AF</td>
<td>pDEST™22 bearing CDK1 AF</td>
<td>Amp/ TRP1</td>
<td>This work</td>
</tr>
<tr>
<td>pEXP™22/CDK2 AF</td>
<td>pDEST™22 bearing CDK2 AF</td>
<td>Amp/ TRP1</td>
<td>This work</td>
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<table>
<thead>
<tr>
<th>Restriction enzymes</th>
<th>Recognition site</th>
<th>Buffer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AscI (10 000 U/ml)</td>
<td>5’…GGCGCGCC…3’ 3’…CCGCGGCG…5’</td>
<td>NEBuffer4</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NotI-HF (20 000 U/ml)</td>
<td>5’…GCGGCCGC…3’ 3’…CGCGCGCG…5’</td>
<td>NEBuffer4</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Swal (10 000 U/ml)</td>
<td>5’…ATTTAAAT…3’ 3’…TAAATTTA…5’</td>
<td>NEBuffer3</td>
<td>New England Biolabs</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Other enzymes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfuUltra™ II fusion HS DNA Polymerase</td>
<td>Stratagene</td>
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### Antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock solution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/ml</td>
<td>Bristol-Meyers Squibb</td>
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<tr>
<td>Gentamicin</td>
<td>10 mg/ml</td>
<td>Merck</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 mg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

### Commercial kits

<table>
<thead>
<tr>
<th>Commercial kits</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProQuest&lt;sup&gt;TM&lt;/sup&gt; Two-Hybrid System</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pENTR&lt;sup&gt;TM&lt;/sup&gt; Directional TOPO&lt;sup&gt;®&lt;/sup&gt; Cloning Kits</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Gateway&lt;sup&gt;®&lt;/sup&gt; Technology with Clonase&lt;sup&gt;™&lt;/sup&gt; II</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Wizard&lt;sup&gt;®&lt;/sup&gt; SV Gel and PCR Clean-Up System</td>
<td>Promega</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit Protocol</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>Wizard&lt;sup&gt;®&lt;/sup&gt; Plus SV Minipreps DNA Purification System</td>
<td>Promega</td>
</tr>
<tr>
<td>JetStar (Mid-prep)</td>
<td>Genomed</td>
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### Chemical/Reagent

<table>
<thead>
<tr>
<th>Chemical/Reagent</th>
<th>Concentration</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>2-Log DNA Ladder (marker)</td>
<td>1000 μg/ml</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Adenine</td>
<td></td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Agarose, Type I</td>
<td></td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td></td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>1 M</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>25 mM each dNTP</td>
<td>AB</td>
</tr>
<tr>
<td>GelRed</td>
<td></td>
<td>Biotium</td>
</tr>
<tr>
<td>Glycerol</td>
<td>99 %</td>
<td>Merck</td>
</tr>
<tr>
<td>Histidine</td>
<td></td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Leucine</td>
<td></td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>Uracil</td>
<td></td>
<td>Sigma-Aldrich</td>
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