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

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Departments of Radiation Biology and Cell Biology

Institute for Cancer Research

The Norwegian Radium Hospital

Oslo University Hospital

Department of Pharmaceutical Biosciences

School of Pharmacy

The Faculty of Mathematics and Natural Sciences

University of Oslo

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

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

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_1 (gap), S (synthesis), G_2 (gap) and M (mitosis), where G_1/S/G_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_1, and repeat the cycle. Cells in G_1 can also enter a specialized resting state called G_0. It may stay in this state forever or for a short period of time (23). Cells in G_0 can reenter the cycle if it receives the appropriate signals.

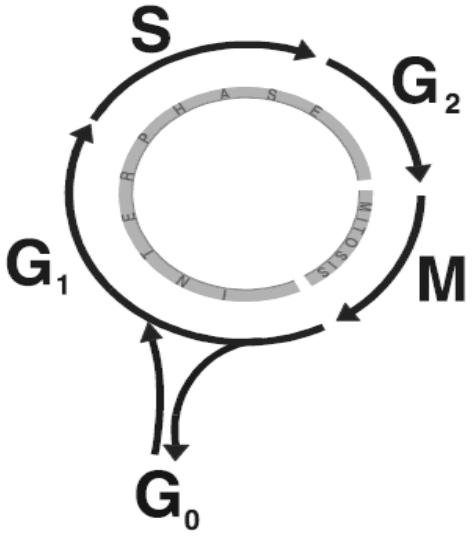


Figure 1.1 The eukaryotic cell cycle. The cell cycle is comprised of four successive phases. G_1 and G_2 are gap phases where the cell grows in mass. S phase stands for synthesis, indicating the replication of DNA. In M phase, mitosis and cytokinesis occur. The cell can also enter a specialized resting state known as G_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 G_1 phase is dependent on the activation of CDK4 and CDK6 by cyclin D. In late G_1 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 G_2 and early M phase, the CDK1/cyclin A complex promotes entry into M. Mitosis is further regulated by cyclin B in complex with CDK1.

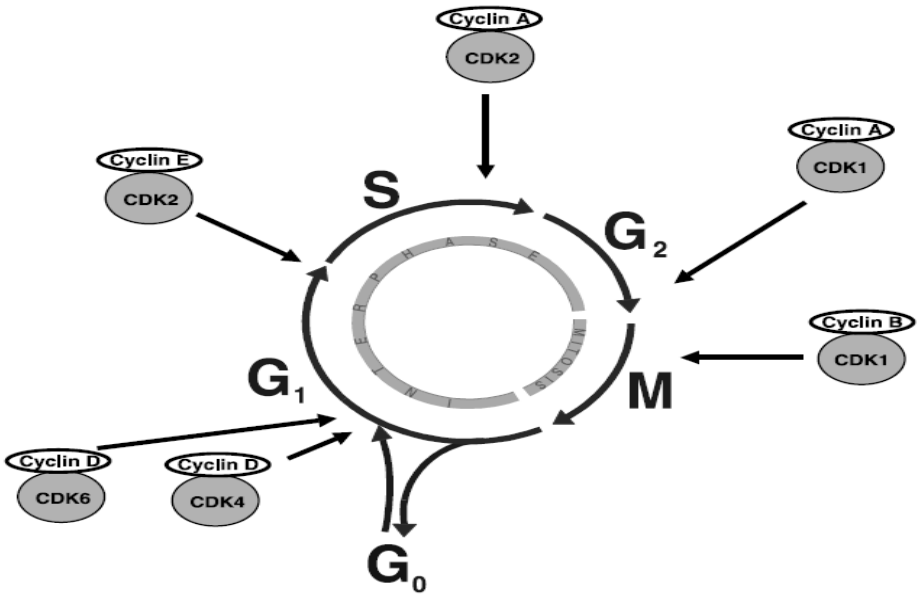


Figure 1.2 The classical model of cell cycle regulation in the eukaryotic cell cycle. Cyclin complexes with CDK2, CDK4 and CDK6 have been implicated in the regulation of events during interphase, while CDK1 controls the initiation of mitosis. Compensatory mechanisms are however identified among the CDKs, 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 (G_1/S checkpoint) and after DNA replication (G_2/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 G_1 will be delayed while mechanisms attempting to repair the damage are mobilized. The cell may also enter the resting state of G_0 or even commit programmed cell death (apoptosis) if the damage is too severe and repair is not possible. This is referred to as the G_1/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 G_2/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 G_2/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).

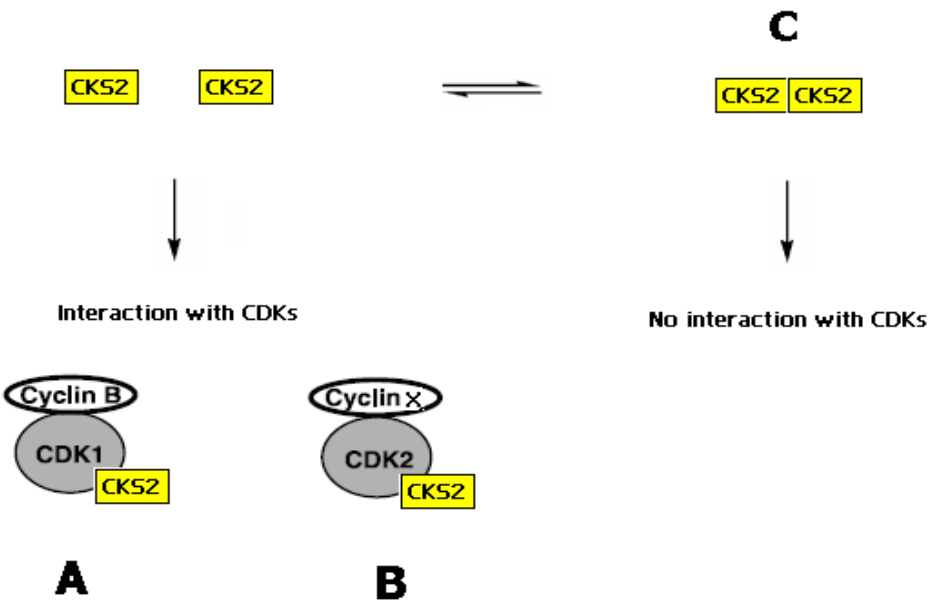


Figure 1.3 CKS2 protein interactions. The CKS2 protein is thought to be in equilibrium between the forms of monomers and dimers (three dimer molecules may also constitute the hexamer form, but this is not displayed in the figure). Only the monomer form is thought to bind to CDKs, where binding to CDK1 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 *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 *URA3* and *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).

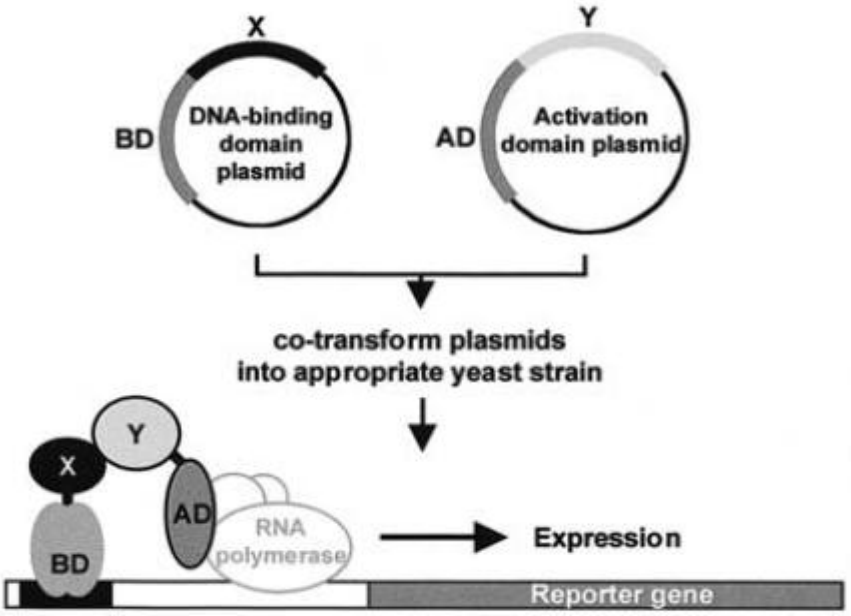


Figure 2.1 The classical yeast two hybrid system. A protein of interest is fused to the DNA-binding domain (DBD), and this construct is called bait. Another protein is fused to the transcriptional activation domain (AD) and is called prey. The bait and prey is co-transformed into the yeast *S. cerevisiae*. 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 auxotropic 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).

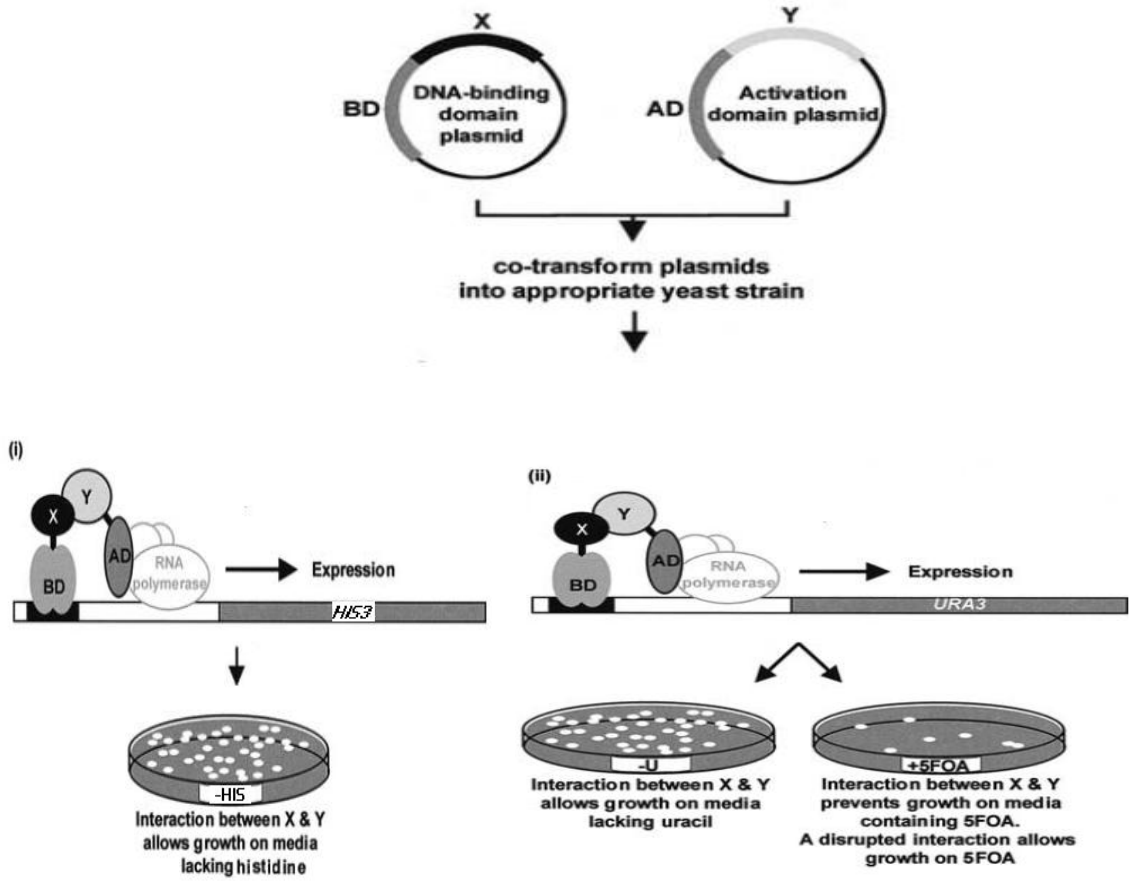


Figure 2.2 Representation of activation of the *HIS3* and *URA3* reporter gene. The transformed yeast are plated onto medium that selects for the presence of both plasmids (synthetic complete -leu-trp), and are then screened for *HIS3* and *URA3* activity.

(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-fluoroorotic 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 phosporylation 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.

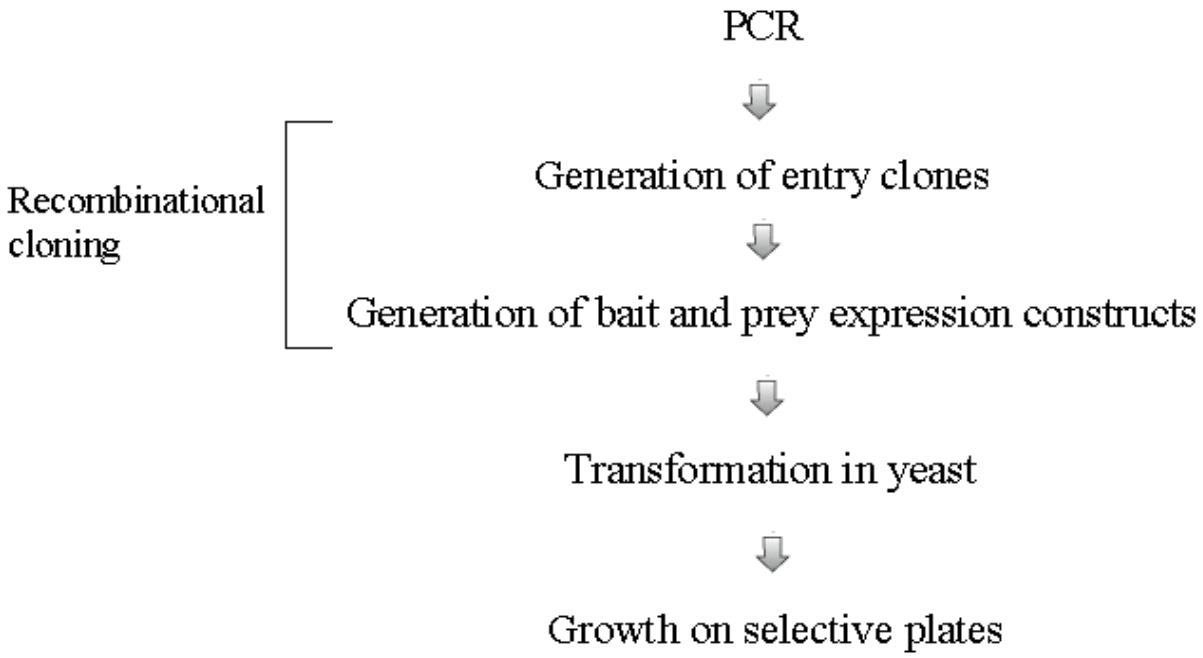


Figure 2.3 Overview of the different steps in protein interaction analyses by the use of the Y2H technique. Amplification of the genes by PCR must initially be performed. Generation of entry clones, followed by a transfer of the gene sequences into expression vectors by a site-specific recombination 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 *att*L1 and *att*L2 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.

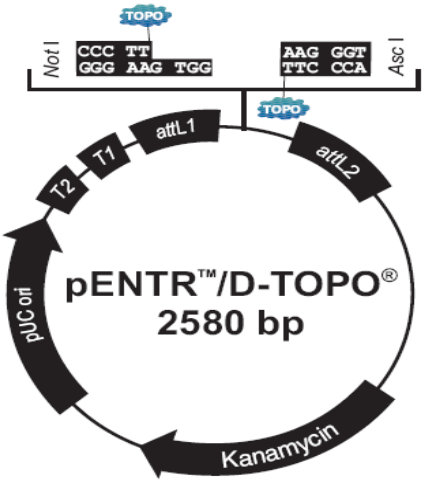


Figure 2.4 Features of the pENTR™/D-TOPO® vector (79). A topoisomerase enzyme is bound to each 3'end, allowing the gene of interest to be ligated between the two *att* sites. The kanamycin resistance gene allows selection in *E. coli*.

Topoisomerase I enzyme is a *vaccinia* virus that specifically bind 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).

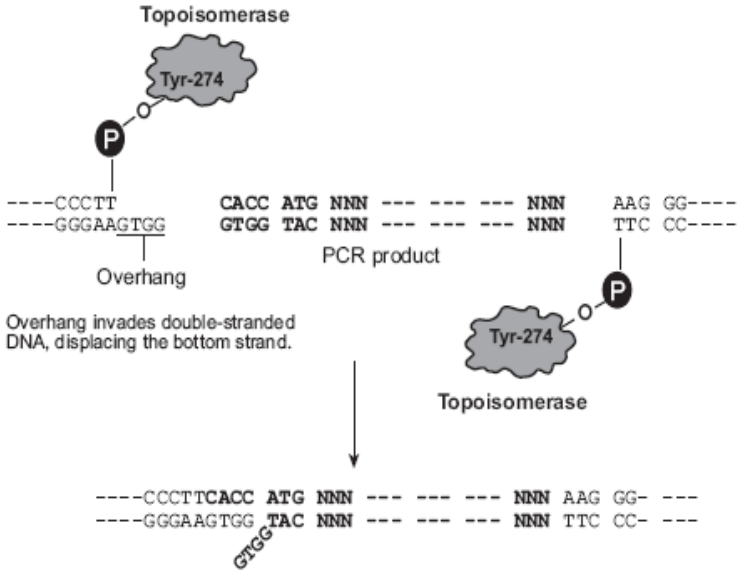


Figure 2.5 Topoisomerase based cloning. Topoisomerase is covalently bound at 3' blunt CCCTT ends on the vector, making it linear. CACC blunt end PCR products will basepair with a GTGG overhang at the 5' end, fusing the PCR product into the vector (79).

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 *att*L1 will recombine with *att*R1, and not *att*R2, maintaining the orientation of the sequence during the reaction (19).

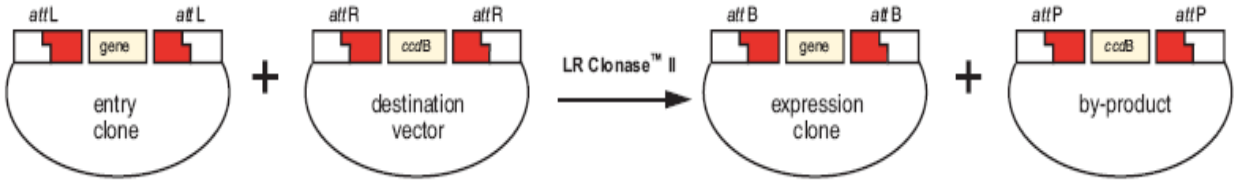


Figure 2.6 Recombination reaction generating expression constructs. The LR clonase II enzyme mix consists of the Int, Xis and IHF enzymes which catalyze the recombination of an entry clone with a destination vector to create an expression clone (66).

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 *E. coli* lacking the miniF plasmid (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, *att*R1 and *att*R2, flanking the *ccdB* gene are converted to *att*B sites following the recombination reaction.

pDEST^{TM}32 and pDEST^{TM}22 (figure 2.7) are destination vectors from Invitrogen that can be used in a recombination reaction to generate expression vectors (66). pDEST^{TM}32 carries the GAL4

DNA binding domain forming the bait, while pDEST^{TM}22 generate the prey (carries the GAL4 AD).

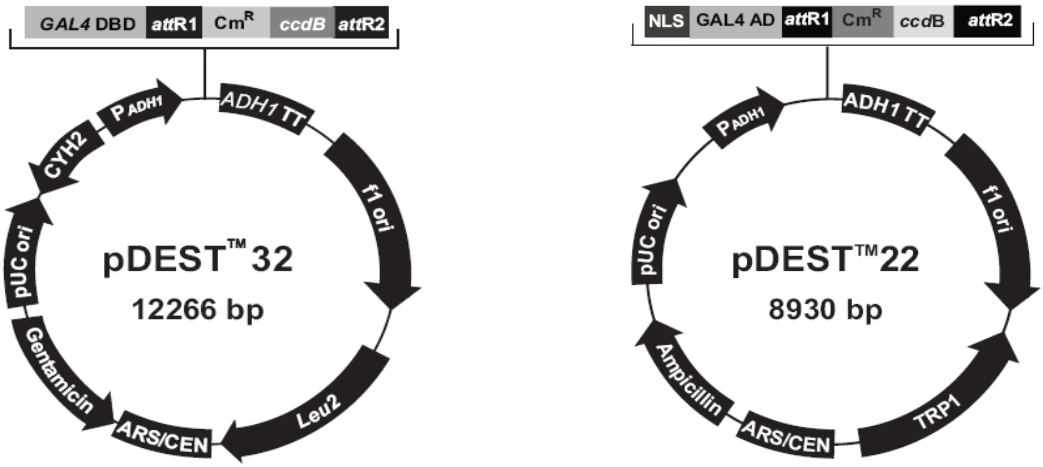


Figure 2.7 Features of *CEN*-based Y2H destination vectors (66). pDEST^{TM}32 contains the GAL4 DNA binding domain (DBD), while pDEST^{TM}22 carries the activation domain (AD) coding sequence (66). Both vectors contain the ARS/CEN origin that permits the maintenance of the plasmid in low copy. Both are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

The bait plasmid carries a gentamicin resistance gene (Gm^R) for maintenance in *E. coli*, and the *LEU2* gene for selection in yeast. The prey plasmid on the other hand contains the ampicillin resistance gene for selection in *E. coli*, and the *TRP1* nutritional marker for selection in yeast. The constitutive promoter *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 *E. coli.* Both vectors are also equipped with the *ARS4/CEN*6 sequence for low-copy number replication. The attachments sites, *att*R1 and *att*R2, is the bacteriophage λ-derived sequences that allow directional recombinational cloning. In between these sites both vectors contain the chloramphenicol and *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 *TRP1* yeast marker must be paired with a yeast strain that carries a *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).

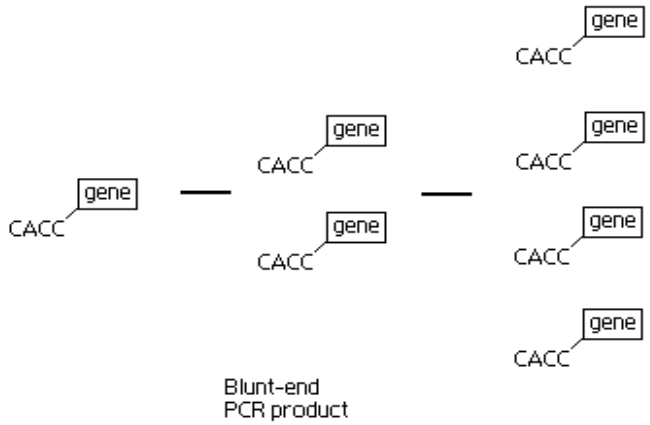
3 METHODS

3.1 Experimental outline

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

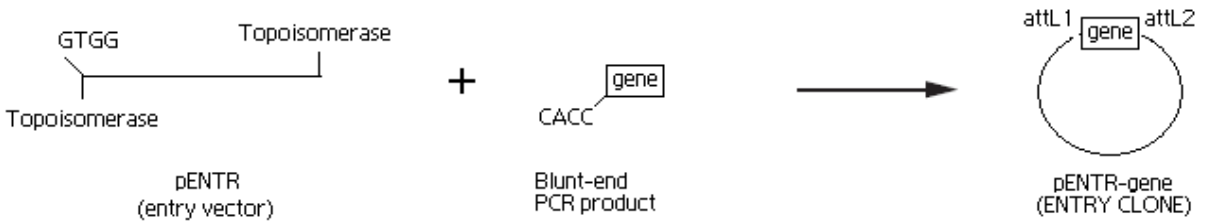
Step 1:

Design primers for PCR and amplify the genes of interest by PCR



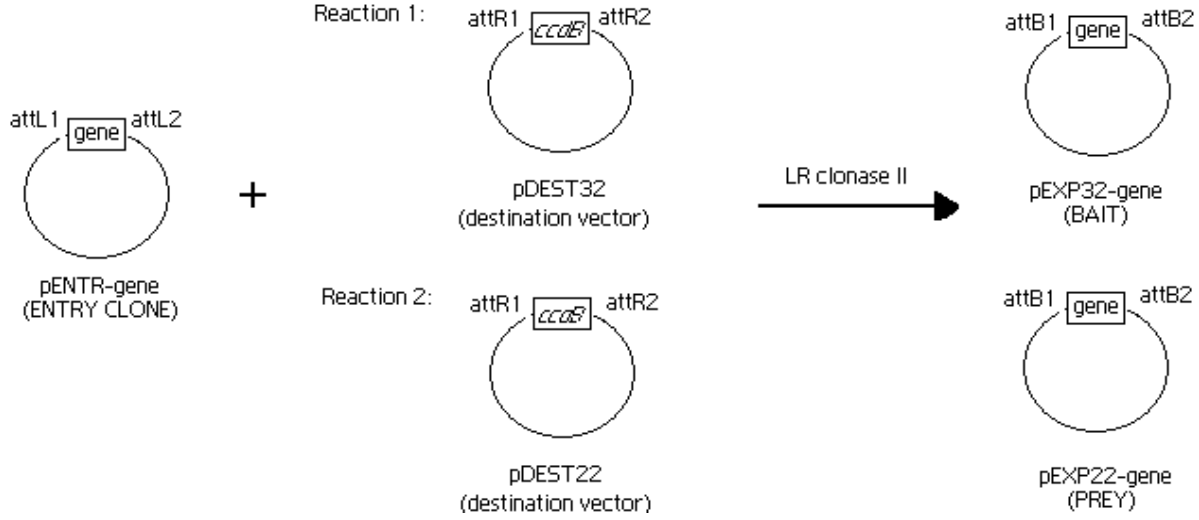
Step 2:

Generate entry clone by topoisomerase based cloning



Step 3:

Generate bait and prey plasmids by LR recombination reaction



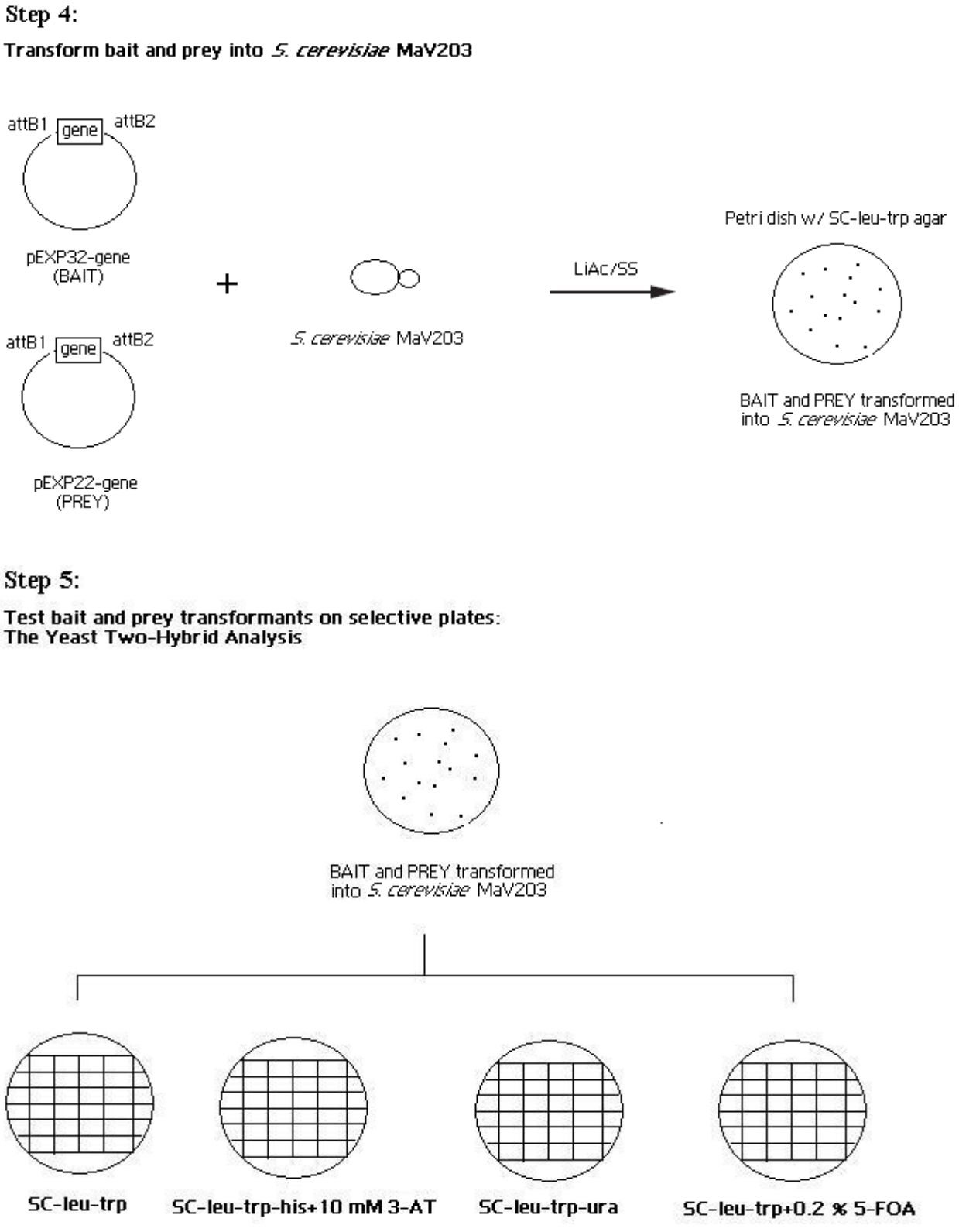


Figure 3.1 Illustration of the experimental work. Primers were designed for use in a PCR reaction to amplify cDNA of *CDK2*, *CDK1 AF* and *CDK2 AF* (step 1). The PCR products were fused into an *att*-site-containing entry vector by a topoisomerase based cloning method (step 2). The gene was then transferred 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' 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 AscI, 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^{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. The vector generating the bait was pDEST^{TM}32 and pDEST^{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 *E. coli,* and plated on gentamicin (pDEST^{TM}32 vector) and ampicillin (pDEST^{TM}22 vector) plates, and incubated at 37 °C overnight.

Propagation of the destination vectors pDEST^{TM}32 and pDEST^{TM}22 was performed in XL 10-Gold® Ultracompetent *E. coli* cells, which strain is resistant to *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 *ccdB* gene are replaced by the genes of interest. The chloramphenicol and *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^{TM}32, constituted 12266 bp. pDEST^{TM}22 (generate preys) had a size of 8930 bp.

Simple mathematical calculations where 1598 bp (the chloramphenicol and *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 3.1 Size of bait and prey plasmids.

Expression construct		Size (bp)*
Bait	pEXP^{TM}32/CKS2	**10917**
	pEXP^{TM}32/CDK1 AF	**11571**
	pEXP^{TM}32/CDK2 AF	**11574**
Prey	pEXP^{TM}22/CKS2	**7581**
	pEXP^{TM}22/CDK1 AF	**8235**
	pEXP^{TM}22/CDK2 AF	**8238**

***** For all correct recombinant clones the chloramphenicol and *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 *E. coli* cells using the alkaline lysis (miniprep) method described in appendix 6, and digested with 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 *CKS2*, *CDK1* and *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 S. cerevisiae MaV203 (step 4)

In order to transform the bait and prey constructs into *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.

LEU2 Plasmid (BAIT)	TRP1 Plasmid (PREY)	Experimental interaction
pEXP™32/CKS2	pEXP™22/CDK1 AF	CKS2 and CDK1 AF
pEXP™32/CDK1 AF	pEXP™22/CKS2	
pEXP™32/CKS2	pEXP™22/CDK2 AF	CKS2 and CDK2 AF
pEXP™32/CDK2 AF	pEXP™22/CKS2	
pEXP™32/CKS2	pEXP™22/CKS2	Dimerization of CKS2

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 *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 3.3 Transformation of control interactions.

LEU2 Plasmid (BAIT)	TRP1 Plasmid (PREY)	Purpose
pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
Bait plasmid	pDEST™22	Negative activation control
pDEST™32	Prey plasmid	Negative activation control

As an additional experiment, transformation of the genes present in high-copy number vectors (pGBK and pGAD) was also conducted. The same technique and *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^{TM}32 and pDEST^{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 *S. cerevisiae* MaV203 are described in table 3.4.

Table 3.4 Transformation of the genes present in high copy-number vectors.

LEU2 Plasmid (BAIT)	TRP1 Plasmid (PREY)	Experimental interaction
pGBK/CKS2	pGAD/CDK1 AF	CKS2 and CDK1 AF
pGBK/CDK1 AF	pGAD/CKS2	
pGBK/CKS2	pGAD/CDK2 AF	CKS2 and CDK2 AF
pGBK/CDK2 AF	pGAD/CKS2	

3.6 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.

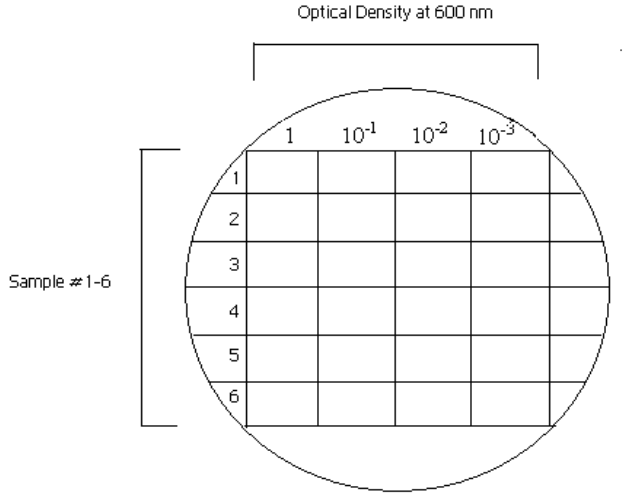


Figure 3.2 Illustration of petri dish with a marked up grid. Four different dilutions of transformed *S. cerevisiae* suspensions are spotted from the left to the right. Six samples can fit into the plate.

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 3.5 The selections plates and their purposes.

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

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:

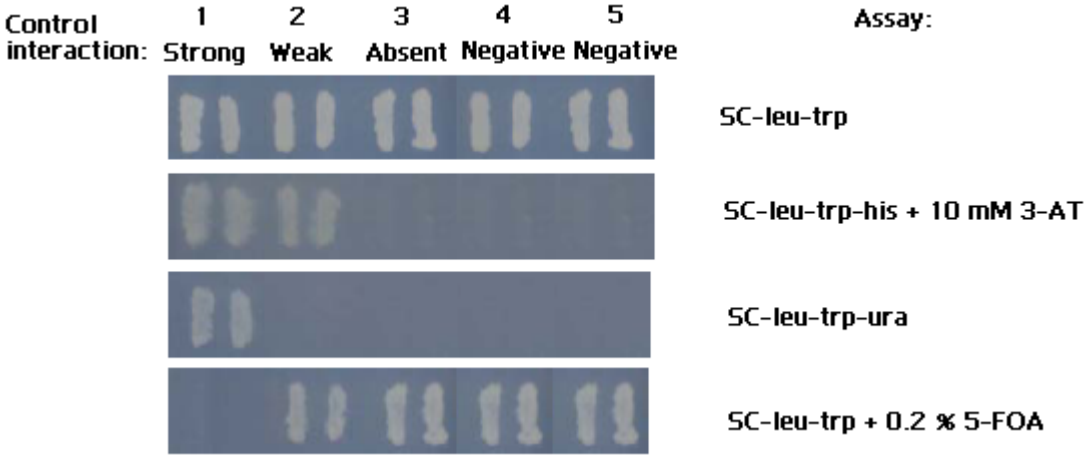


Figure 3.3 Expected results for the controls of theY2H analysis. Control 1-3 is the strong, weak and absent control interaction provided by interactions between the Krev1 and different mutants of RalGDS. Control 4 and 5 are negative activation controls to test that neither the bait nor the prey was able 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 optimalization and cDNA amplification

The gradient PCR was performed to experimentally determine the optimum primer annealing temperatures for the primers. Figure 4.1 show 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.

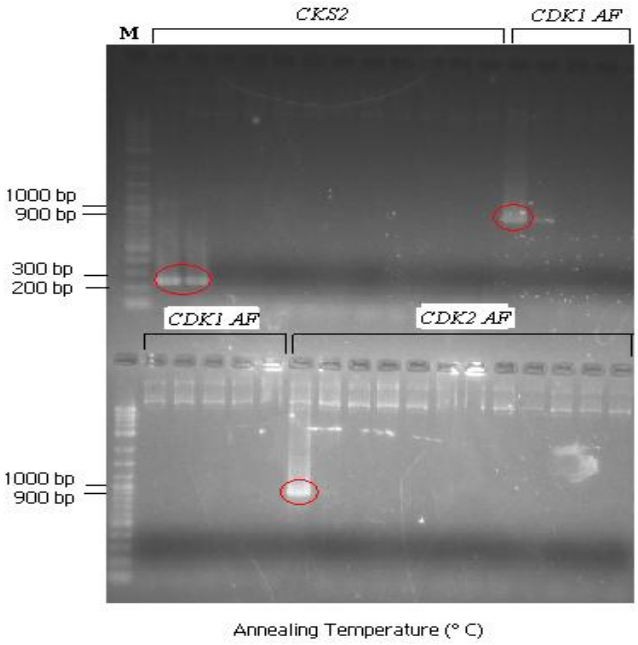


Figure 4.1 1.5 % agarose gel of cDNA of *CKS2, CDK1 AF* and *CDK2 AF*. Gradient PCR with temperatures in the range from 45 °C to 65 °C. M: 2 log DNA ladder. Lane 2-13: *CKS2*. Lane 14-25: *CDK1 AF*. Lane 26-37: *CDK2 AF*.

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.

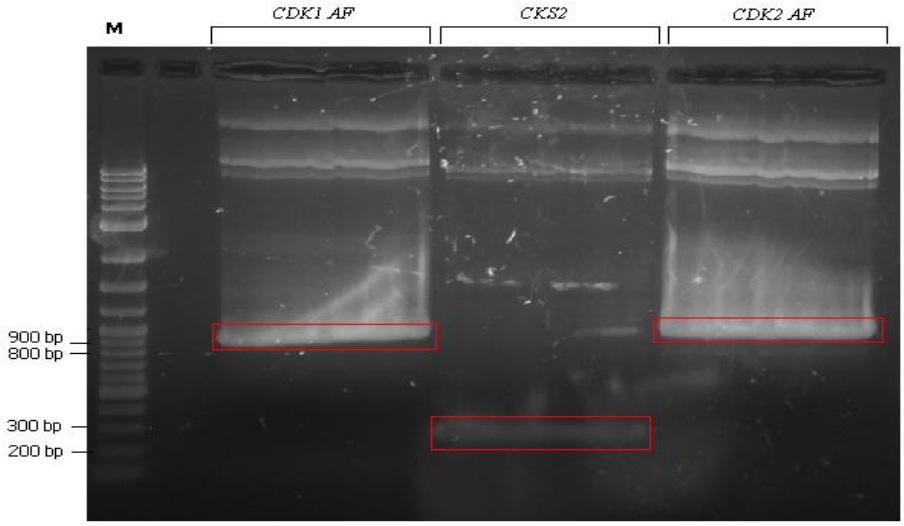


Figure 4.2 1.5 % agarose gel of PCR amplification products of *CKS2, CDK1 AF* and *CDK2 AF*. Amplification performed under optimized temperature conditions. The positions and sizes (bp) of marker DNA fragments (lane M) are indicated at the left. The marked bands were excised out of the gel with a scalpel, 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 AscI. 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.

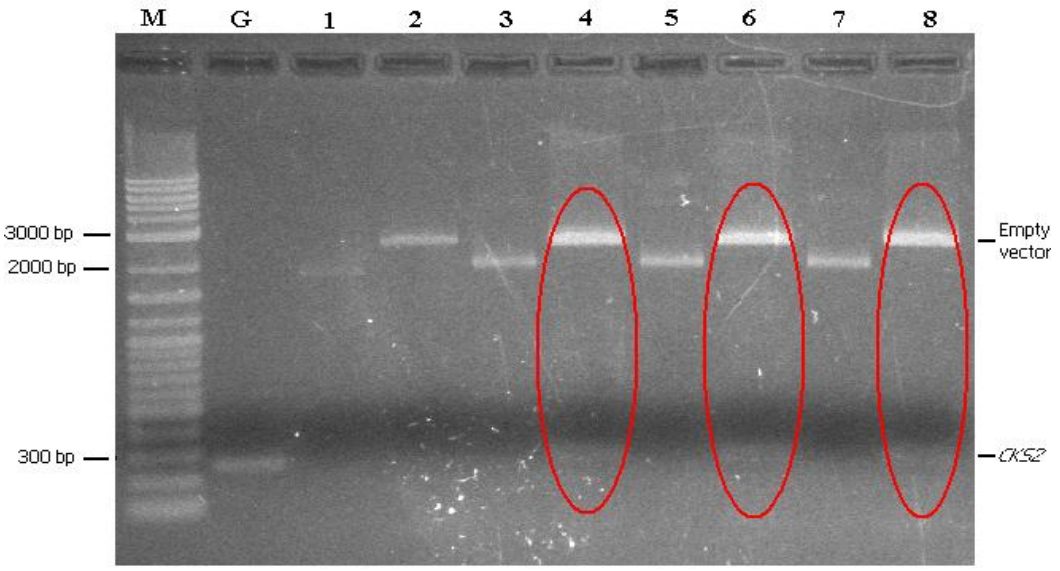


Figure 4.3 1.5 % agarose gel of double digested *CKS2* entry clone.
The leftmost lane (M) shows the DNA marker 2-log ladder. The next lane (G) is gel-purified PCR product of the *CKS2*-gene. The following two and two lanes contains plasmid DNA from the same sample. The leftmost lane of the two is uncut 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.

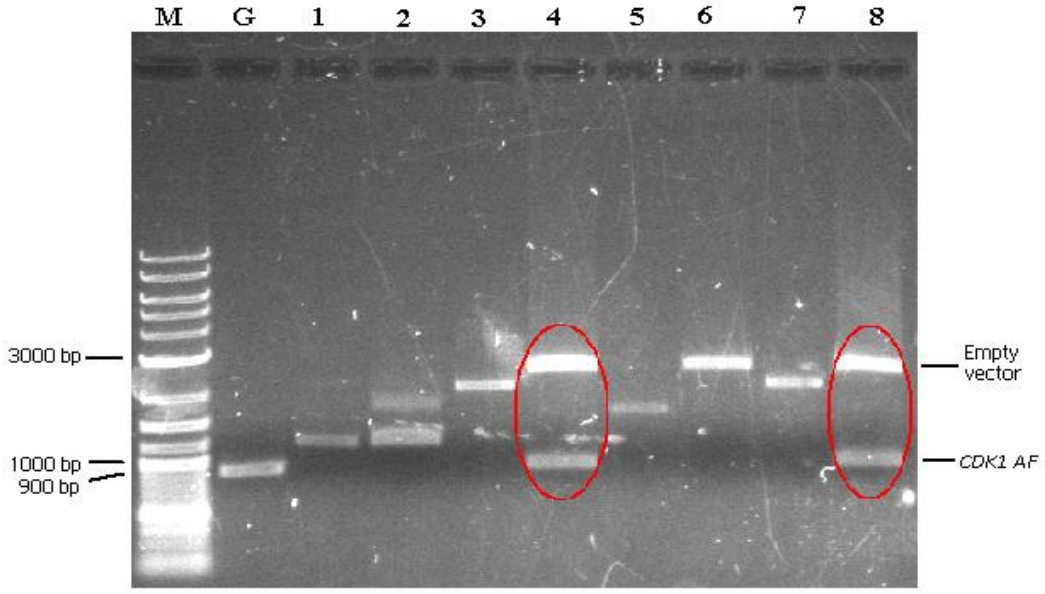


Figure 4.4 0.8 % agarose gel of double digested *CDK1 AF* entry clone.
The leftmost lane (M) shows the DNA marker 2-log ladder, and the following lane (G) is gel-purified PCR product of the *CDK1 AF*-gene. Only the samples in lane 4 and 8 appear to be correct (marked in red).

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.

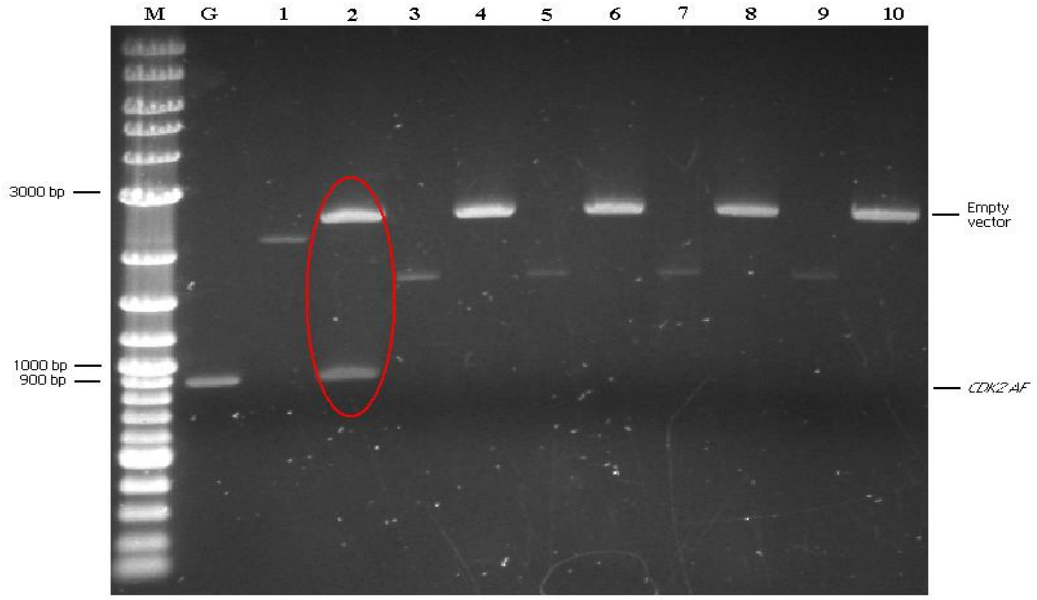


Figure 4.5 0.8 % agarose gel of digested *CDK2 AF* entry clone. The leftmost lane, marked M, shows the DNA marker (2-log ladder) and the following lane is gel-purified PCR product of the *CDK2 AF*-gene (marked G). Only the clone in lane 2 contained two bands which were consistent with the sizes of empty 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^{TM}32 and pDEST^{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^{TM}32 (panels A) and 8930 bp for pDEST^{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^{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^{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.

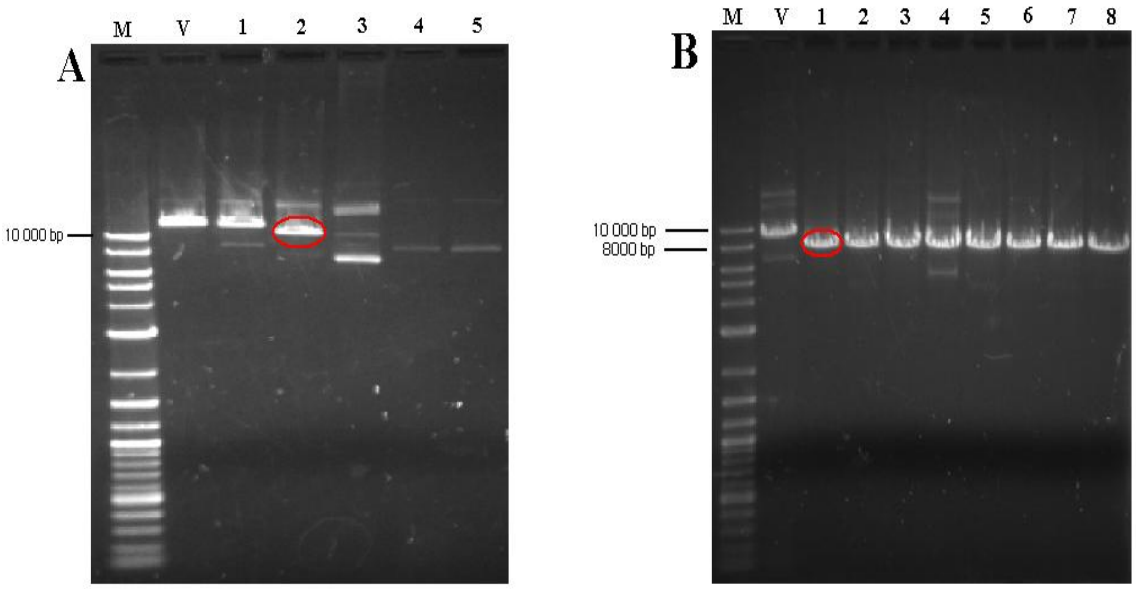


Figure 4.6 Gelelectrophoresis of linearized bait and prey. 0.8 % agarose gel. The leftmost lane shows the DNA marker (2-log ladder), and the next lane is the linear control (pDEST^{TM}32 in panel A, pDEST^{TM}22 in panel B). Only the marked transformant in panel A appeared to be correct. In panel B, 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.

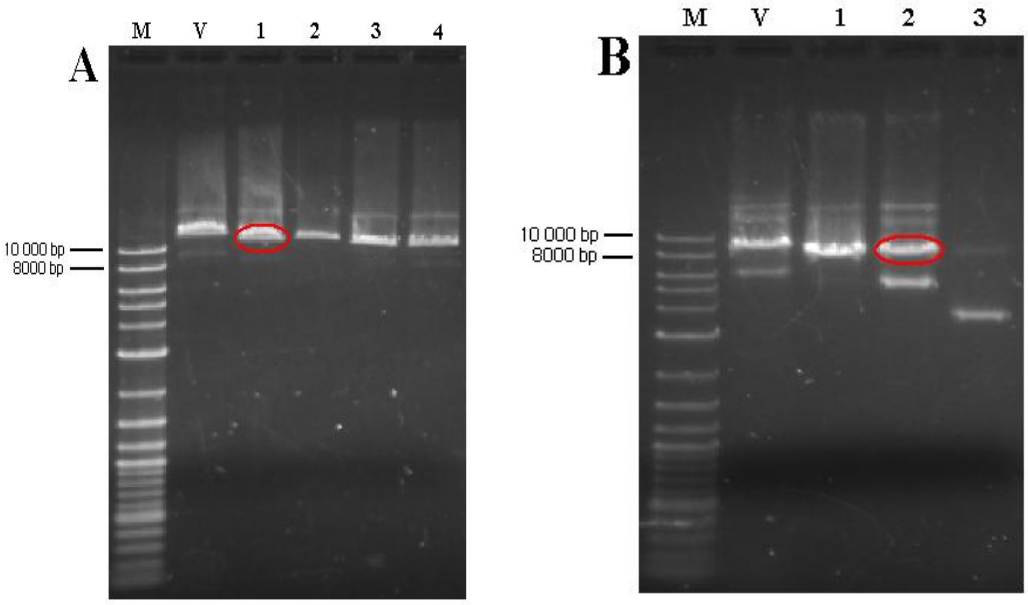


Figure 4.7 Gelelectrophoresis of linearized bait and prey. The leftmost lane shows the DNA marker (2-log ladder), and the next lane was linear control of pDEST32 (marked V). The transformant in lane 1 in panel A and the transformant in lane 2 in panel B, was selected for sequencing,

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).

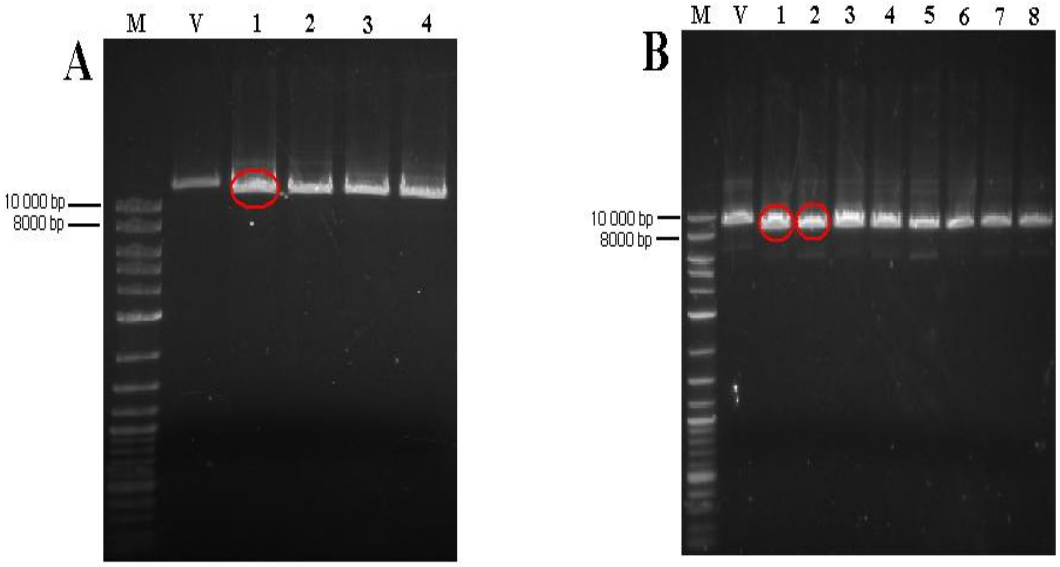


Figure 4.8 Gelelectrophoresis of linearized bait and prey. The leftmost lane shows the DNA marker (2-log ladder), and the next lane was linear control of pDEST32. The transformant in lane 1 in panel A appeared correct and was selected for sequencing. In panel B, all the transformants appeared to be correct. 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, *HIS3* reporter activation was tested. 3-AT was added to reduce auto activation. In plate C and D, *URA3* 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 *HIS3* and *URA3* 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 *HIS3* reporter, but not the *URA3* 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 *HIS3* and *URA3* 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

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

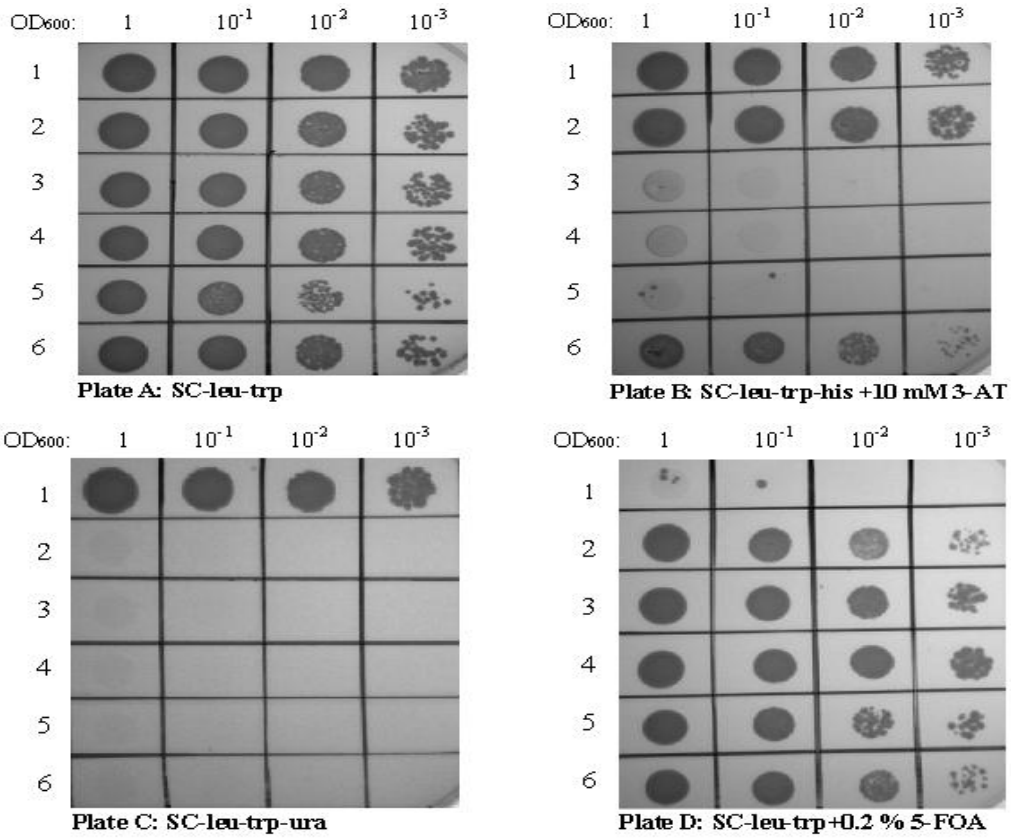


Figure 4.9 *S. cerevisiae* Y2H analysis of CKS2 fused as bait and CDK1 AF fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were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

CDK1 AF as bait, CKS2 as prey

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

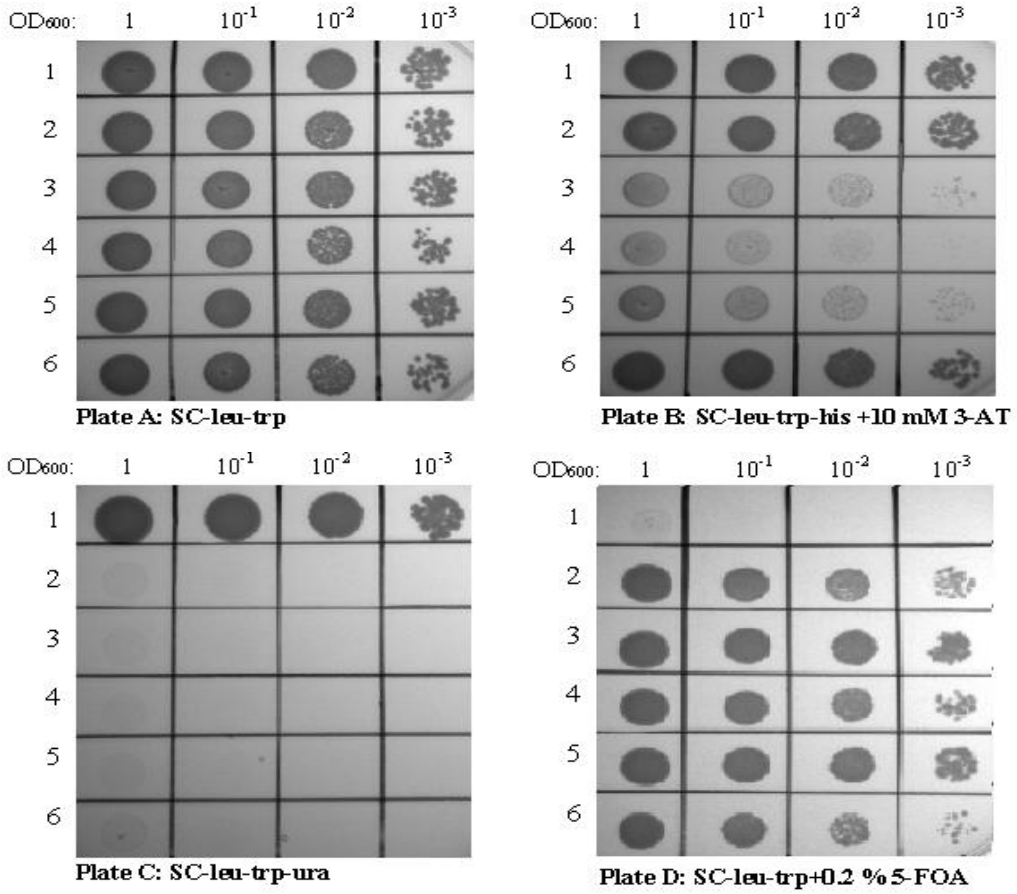


Figure 4.10 *S. cerevisiae* Y2H analysis of CDK1 AF fused as bait and CKS2 fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were spotted in lane 1-5, while the experimental interaction was applied in lane 6.

4.4.2 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

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

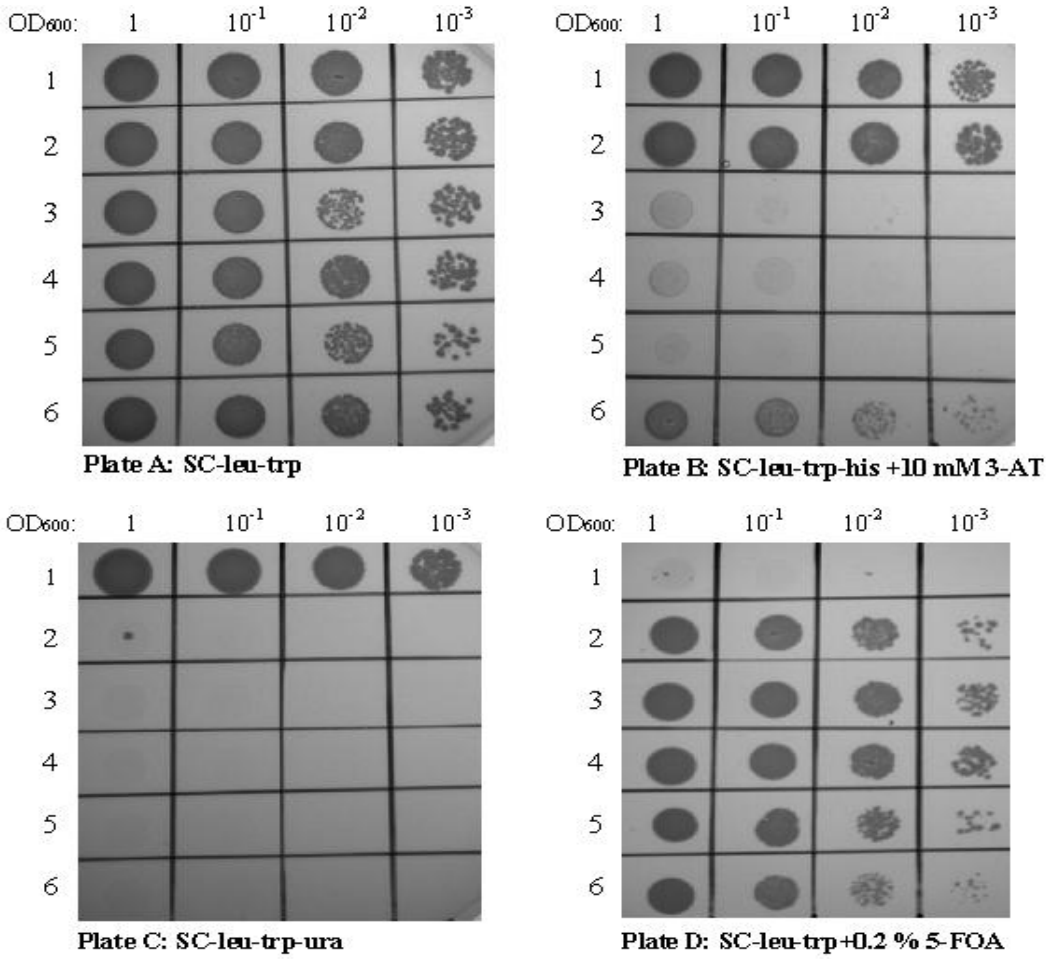


Figure 4.11 *S. cerevisiae* Y2H analysis of CKS2 fused as bait and CDK1 AF fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were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

CDK2 AF as bait, CKS2 as prey

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

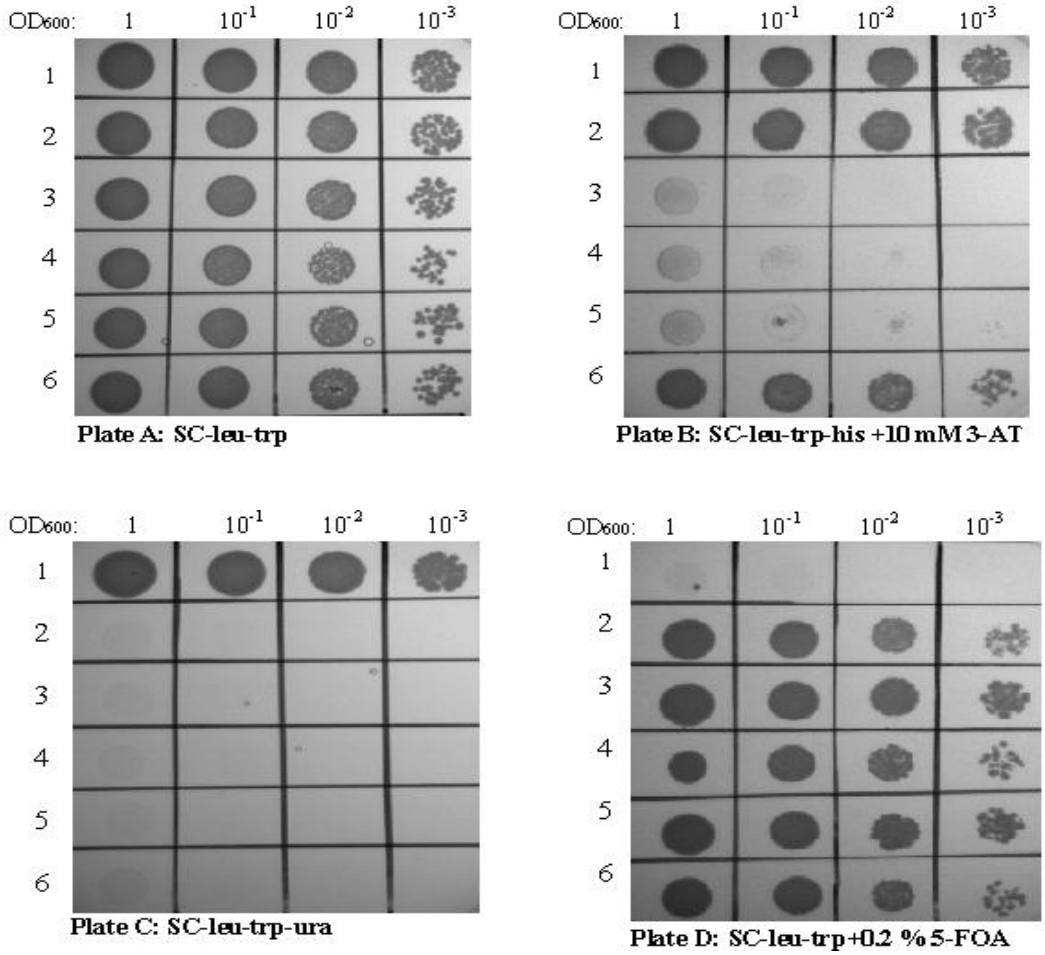


Figure 4.12 *S. cerevisiae* Y2H analysis of CDK2 AF fused as bait and CKS2 fused as prey. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were 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

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

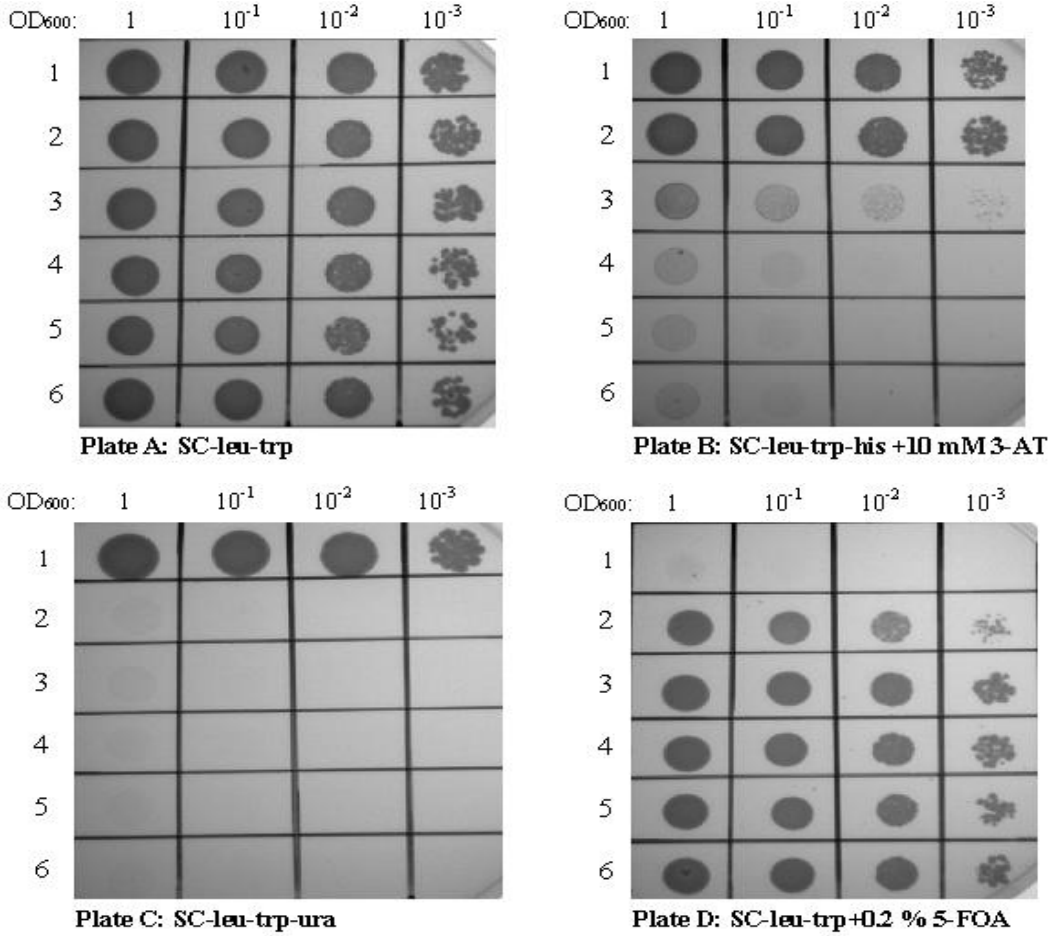


Figure 4.13 *S. cerevisiae* Y2H analysis of the interaction between two CKS2 proteins. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. The control interactions were spotted 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.

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

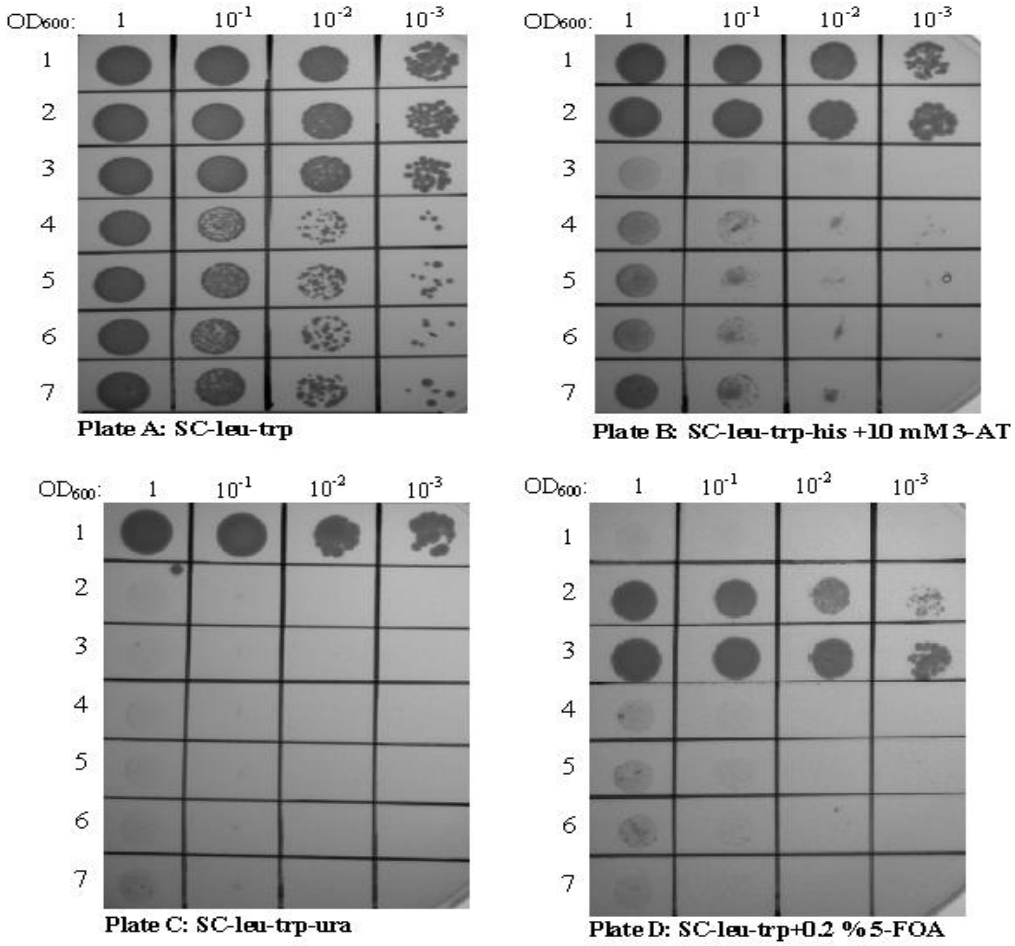


Figure 4.14 *S. cerevisiae* Y2H analysis of the interaction of CKS2 to CDK1 AF and CDK2 AF present in high copy-number vectors. The figure shows yeast growth on selection plates. Plate A: Control plate. Plate B: Test for *HIS3* reporter gene activation. Plate C and D: Test for *URA3* reporter gene activation. 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_{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.

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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*:

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

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

	Direction	Sequence
pDEST^{TM}32	Forward	5’ – CAC-AGA-TAG-ATT-GGC-TTC-AGT-GGA-G – 3’
	Reverse	5’ – AGC-CGA-CAA-CCT-TGA-TTG-GAG –3’
pDEST^{TM}22	Forward	5’– GCG-TAT-AAC-GCG-TTT-GGA-ATC –3’
	Reverse	5’– CCG-ACA-ACC-TTG-ATT-GGA-GAC –3’

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.

	pGBK/CKS2	**pGBK/CDK1 AF**	**pGBK/CDK2 AF**
Distilled water	-	-	-
10x *PfuUltra* II reaction buffer	10	10	10
dNTP mix (25 mM each dNTP)	2,5	2,5	2,5
Vector DNA (10 ng)	-	-	-
Primer FW (10 µM)	2	2	2
Primer RW (10 µM)	2	2	2
Pfu Ultra II fusion HS DNA polymerase	2	2	2
Total reaction volume (µl)	**100**	**100**	**100**

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:

Initial step:	2 min	95 °C	(initial denaturation)
30 cycles:	20 sec	95 °C	(denaturation)
	20 sec	55 °C ± 10 °C	(primer annealing)
	20 sec	72 °C	(elongation)
Final step:	10 min	55 °C	(final elongation)

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:

Initial step:	2 min	95 °C	(initial denaturation)
30 cycles:	20 sec	95 °C	(denaturation)
	20 sec	45 °C	(primer annealing)
	20 sec	72 °C	(elongation)
Final step:	10 min	55 °C	(final elongation)

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

Appendix 3

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 dH_2O 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
pGBKT7	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:

Solution E1 (Cell Resuspending)		Store at RT
50 mM	Tris	
10 mM	EDTA	
	HCl ad pH 8.0	
The RNase-containing solution E1 must be stored at 4°C.		
Solution E2 (Cell Lysis)		Store at RT
200 mM	NaOH	
1.0 %	SDS (w/v)	
Solution E3 (Neutralization)		Store at RT
3.1 M	potassium acetate	
	acetic acid ad pH 5.5	
Solution E4 (Column Equilibration)		Store at RT
600 mM	NaCl	
100 mM	sodium acetate	
0.15 %	TritonX-100	
	acetic acid ad pH 5.0	
Solution E5 (Column Washing)		Store at RT
800 mM	NaCl	
100 mM	sodium acetate	
	acetic acid ad pH 5.0	
Solution E6 (DNA Elution)		Store at RT
1250 mM	NaCl	
100 mM	Tris	
	HCl ad pH 8.5	
		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;

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

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 ratio1:1 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.

PCR product (insert)	0.5 – 4 µl
Salt solution	1 µl
Sterile water	add to a final volume of 5 µl
pENTR™/D-TOPO® vector	1 µl
Final volume:	**6 µl**

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

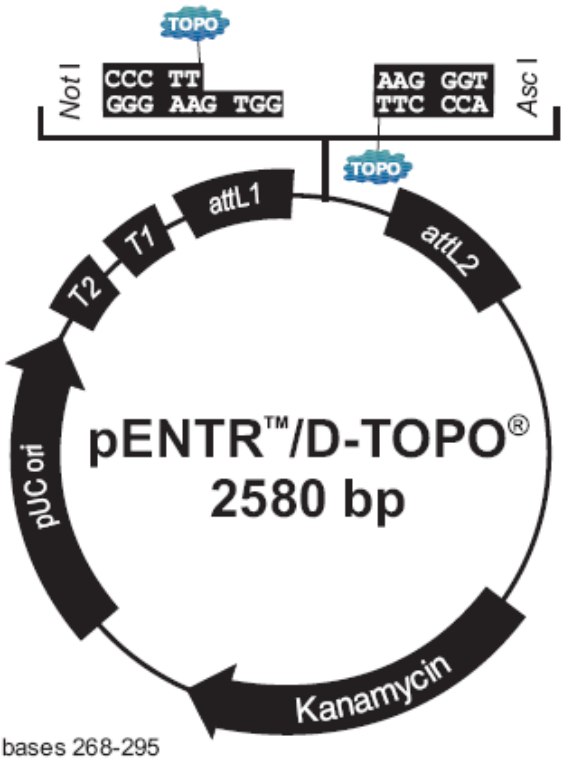
2) Thaw on ice the LR Clonase II enzyme mix for about 2 minutes. Vortex the LR Clonase II enzyme mix briefly twice (2 seconds each time).
3) To each sample (Step 1, above), add 2 µl of LR Clonase II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4) Incubate 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

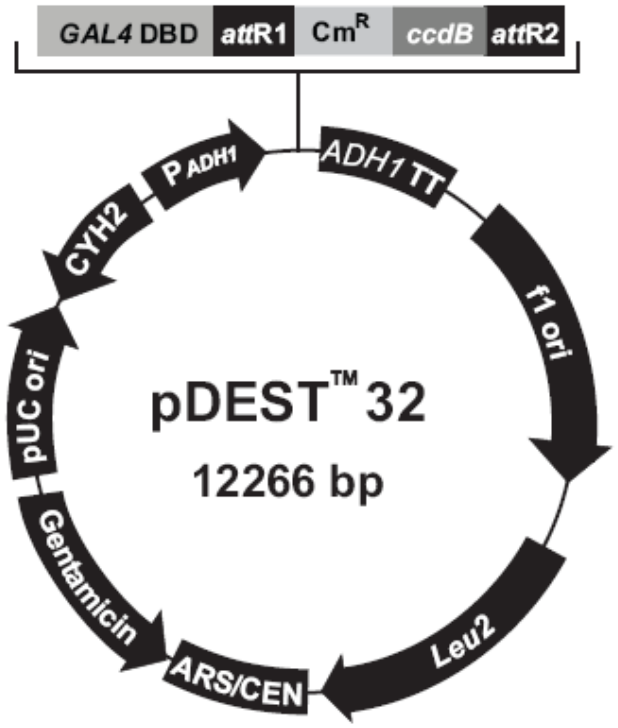
Maps and features of plasmids: pENTR™/D-TOPO®, pDEST™32 and pDEST™22



Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: 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

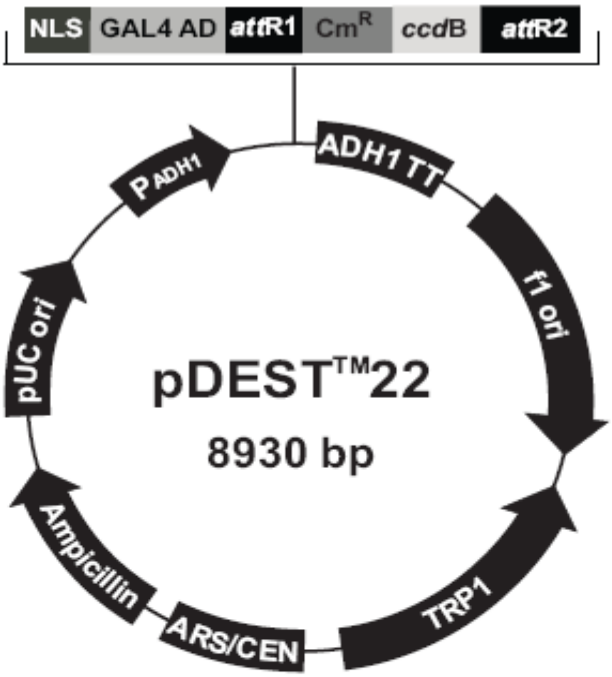
(c) = complementary sequence



Comments for pDEST™ 32
12266 nucleotides

ADH1 promoter: bases 103-1557
GAL4 DNA binding domain: bases 1581-2024
*att*R1 site: bases 2037-2161
Chloramphenicol resistance (Cm^R) gene: bases 2411-3070
*ccd*B gene: bases 3411-3716
*att*R2 site: bases 3757-3881
ADH1 transcription termination region: bases 4119-4276
f1 origin: bases 4603-5058
Leu2 gene: bases 5767-6861
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



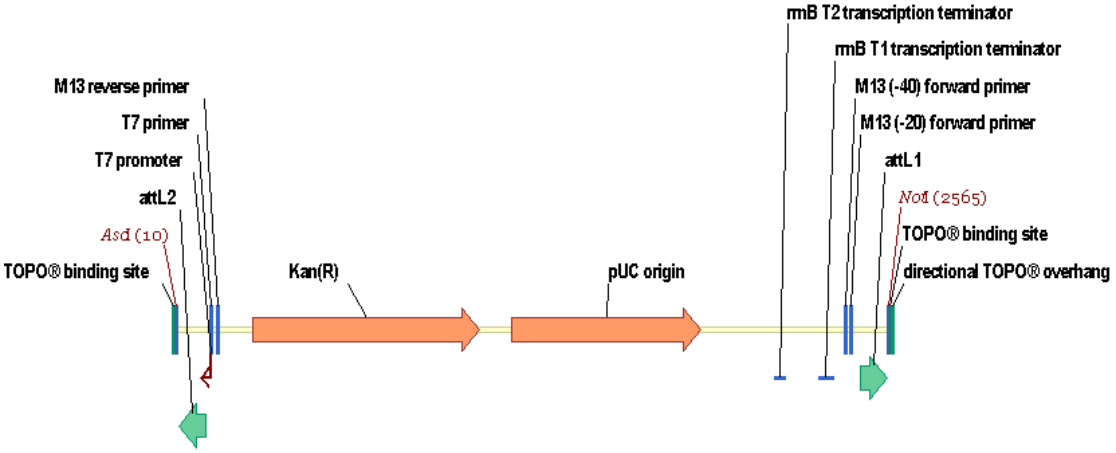
Comments for pDEST™22
8930 nucleotides

ADH1 promoter: bases 272-1726
Nuclear localization signal (NLS): bases 1734-1754
GAL4 DNA activation domain: bases 1761-2105
*att*R1 site: bases 2121-2145
Chloramphenicol resistance (Cm^R) gene: bases 2495-3154
*ccd*B gene: bases 3495-3800
*att*R2 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

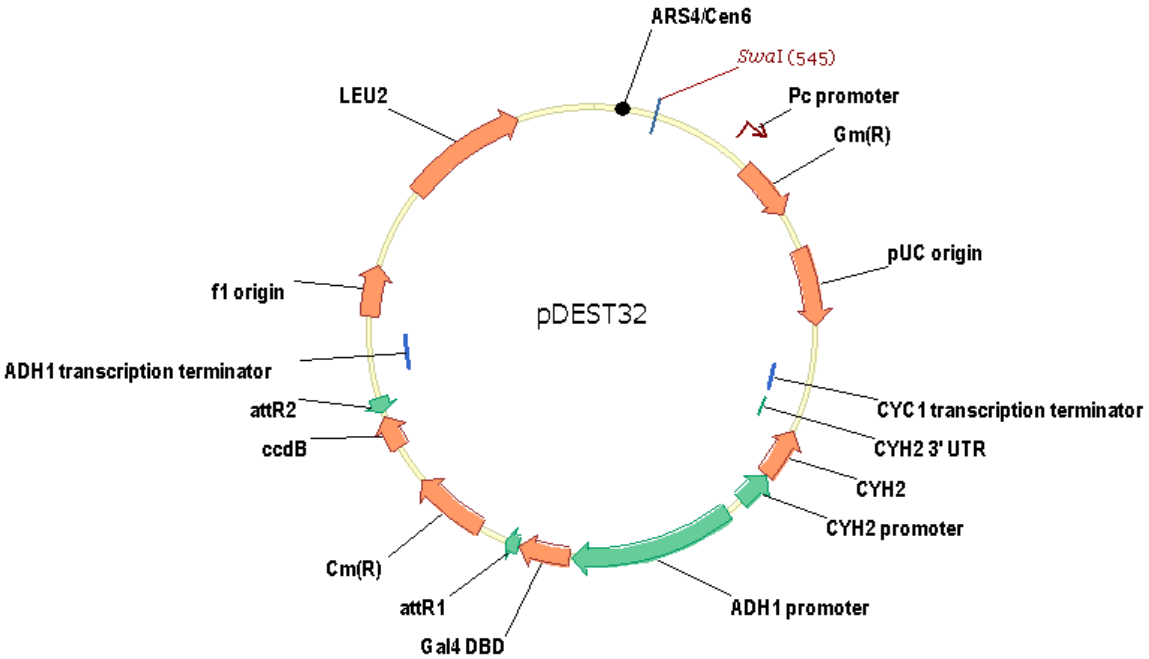
(c) = complementary strand

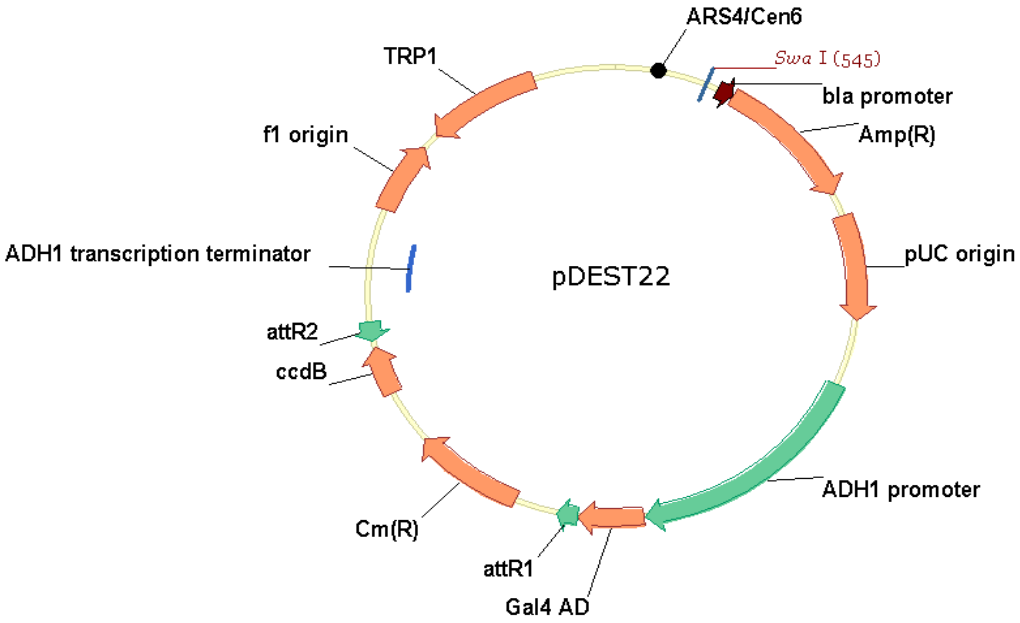
Appendix 13

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



pENTR/D-TOPO




ARS4/Cen6
Swa I (545)
bla promoter
Amp(R)
pUC origin
ADH1 promoter
Gal4 AD
attR1
Cm(R)
ccdB
attR2
ADH1 transcription terminator
f1 origin
TRP1
pDEST22

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):

Nuclease-free water	-
10xNEBuffer4	5
10xBSA	5
0.5 – 1 µg DNA	-
NotI HF enzyme	0.5
AscI enzyme	1
Total volume	**50 µl**

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	5
10xBSA	5
0.5 – 1 µg DNA	-
SwaI enzyme	3
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

ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGGCATGTTATGT

TACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAAGAGGAGTGGAGGAGACTTGG

TGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCATGAGCCAGAACCACATATTCTTCTCTTTAGA

CGACCTCTTCCAAAAGATCAACAAAAATGA

CDK1 (variant 1) 894 bp

ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAGGGTAGACA

CAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAAGAGGAAGGGGTTCCTAGT

ACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTCTTCAGGATGTGC

TTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTC

TATCCCTCCTGGTCAGTACATGGATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATT

GTGTTTTGTCACTCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAG

GAACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATATACACATGA

GGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGTTACTCAACTCCAGTTGAC

ATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAGAAACCACTTTTCCATGGGGATTCAGAAA

TTGATCAACTCTTCAGGATTTTCAGAGCTTTGGGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATC

TTTACAGGACTATAAGAATACATTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTG

GATGAAAATGGCTTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAA

TGGCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG

CDK2 (variant 1) 897 bp

ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAAGCCAGAAACA

AGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAGACTGAGGGTGTGCCCAGTAC

TGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCATCCTAATATTGTCAAGCTGCTGGATGTCATT

CACACAGAAAATAAACTCTACCTGGTTTTTGAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCT

CTGCTCTCACTGGCATTCCTCTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTT

CTGCCATTCTCATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC

ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACCCATGAGGTGG

TGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTATTCCACAGCTGTGGACATCTG

GAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGGGCCCTATTCCCTGGAGATTCTGAGATTGAC

CAGCTCTTCCGGATCTTTCGGACTCTGGGGACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGC

CTGATTACAAGCCAAGTTTCCCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGA

AGATGGACGGAGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC

CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA

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

Query  1    ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  190

Query  61   CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  250

Query  121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  310

Query  181  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  370
```

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

Query  1     ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
             |||||||||||||||||||||||||||||||||||| ||| ||||||||||||||||||
Sbjct  131   ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTGCCTTTGGAGTTGTGTATAAG  190

Query  61    GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  250

Query  121   GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251   GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  310

Query  181   CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311   CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  370

Query  241   GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
```

```
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  430

Query  301  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  490

Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  491  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  550

Query  421  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  551  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  610

Query  481  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  611  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  670

Query  541  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  671  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  730

Query  601  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  731  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  790

Query  661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  791  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACT  850

Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  851  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGN  910

Query  781  -TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAAT  839
             ||||||||||| ||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  911  NTTGGATTTGCTNNCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGNAAAAT  970

Query  840  GGCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAA  884
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  971  GGCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAA  1015
```

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   GAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAGGGTAGACACAAAACTACAGGTCA  83
            |||||||||  ||||| ||| |||||||      ||||||||||||||||  |||||||
Sbjct  981  GAAAATTGGNNAAGGTGCCTTTGGAGTTNNNNNNAAGGGTAGACACAAAACNNCAGGTCA  922

Query  84   AGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAAGAGGAAGGGGTTCCTAGTACTGC  143
            ||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
Sbjct  921  AGTGGTAGCCATGAAAAAAATNNGACTAGAAAGTGAAGAGGAAGGGGTTCCTAGTACTGC  862

Query  144  AATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTCTTCAGGA  203
            ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  861  AATTCGGGAAATTTNTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTCTTCAGGA  802

Query  204  TGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGGATCTGAA  263
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801  TGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGGATCTGAA  742

Query  264  GAAATACTTGGATTCTATCCCTCCTGGTCAGTACATGGATTCTTCACTTGTTAAGAGTTA  323
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741  GAAATACTTGGATTCTATCCCTCCTGGTCAGTACATGGATTCTTCACTTGTTAAGAGTTA  682

Query  324  TTTATACCAAATCCTACAGGGGATTGTGTTTTGTCACTCTAGAAGAGTTCTTCACAGAGA  383
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  681  TTTATACCAAATCCTACAGGGGATTGTGTTTTGTCACTCTAGAAGAGTTCTTCACAGAGA  622

Query  384  CTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGAACAATTAAACTGGCTGATTTTGG  443
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621  CTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGAACAATTAAACTGGCTGATTTTGG  562

Query  444  CCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATATACACATGAGGTAGTAACACTCTG  503
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561  CCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATATACACATGAGGTAGTAACACTCTG  502

Query  504  GTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGTTACTCAACTCCAGTTGACATTTG  563
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  GTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGTTACTCAACTCCAGTTGACATTTG  442

Query  564  GAGTATAGGCACCATATTTGCTGAACTAGCAACTAAGAAACCACTTTTCCATGGGGATTC  623
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  GAGTATAGGCACCATATTTGCTGAACTAGCAACTAAGAAACCACTTTTCCATGGGGATTC  382

Query  624  AGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTGGGCACTCCCAATAATGAAGTGTG  683
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTGGGCACTCCCAATAATGAAGTGTG  322

Query  684  GCCAGAAGTGGAATCTTTACAGGACTATAAGAATACATTTCCCAAATGGAAACCAGGAAG  743
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  GCCAGAAGTGGAATCTTTACAGGACTATAAGAATACATTTCCCAAATGGAAACCAGGAAG  262

Query  744  CCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGCTTGGATTTGCTCTCGAAAATGTT  803
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  CCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGCTTGGATTTGCTCTCGAAAATGTT  202

Query  804  AATCTATGATCCAGCCAAACGAATTTCTGGCAAAATGGCACTGAATCATCCATATTTTAA  863
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  AATCTATGATCCAGCCAAACGAATTTCTGGCAAAATGGCACTGAATCATCCATATTTTAA  142

Query  864  TGATTTGGACAATCAGATTAAGAAGATGTAG  894
            ||||||||||||||||||||||||||||||
Sbjct  141  TGATTTGGACAATCAGATTAAGAAGATGTAG  111
```

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  60
            |||||||||||||||||||||||||||||||||||| | | ||||||||||||||||||
Sbjct  125  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  184

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  244

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  304

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  364

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  424

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  484
```

```
Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  544

Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  604

Query  481   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  664

Query  541   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  665   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  724

Query  601   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  725   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  784

Query  661   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  785   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  844

Query  721   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  845   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGANGAAGATGGACGG  904

Query  781   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  905   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  964

Query  841   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTT  888
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  965   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTT  1012
```

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

Query  1     ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
             |||||||||||||||||||||||||||||||||||| | |  ||||||||||||||||
Sbjct  1009  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTNGGAGTTGTGTACAAA  950

Query  61    GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  949   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  890

Query  121   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  889   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  830

Query  181   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  829   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  770

Query  241   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  769   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  710

Query  301   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  709   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  650

Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  649   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  590
```

```
Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  589   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  530

Query  481   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  529   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  470

Query  541   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  469   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  410

Query  601   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  409   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  350

Query  661   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  349   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  290

Query  721   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  289   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  230

Query  781   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  229   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  170

Query  841   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  169   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  113
```

Results of BLAST search for *CDK2 AF* cloned into pEXP32, FW: #10

```
>lcl|15595
Length=1024

 Score = 1572 bits (851),  Expect = 0.0
 Identities = 879/893 (98%), Gaps = 7/893 (1%)
 Strand=Plus/Plus

Query  1     ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
             |||||||||||||||||||||||||||||||||||| | | ||||||||||||||||||
Sbjct  132   ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  191

Query  61    GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  251

Query  121   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  311

Query  181   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  371

Query  241   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  372   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  431

Query  301   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  491

Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  492   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  551

Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  611
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```
Query  481   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  612   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  671

Query  541   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  672   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  731

Query  601   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  732   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  791

Query  661   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  792   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  851

Query  721   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  852   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  911

Query  781   A-GCTTGTTATCGCAAATGCTGCACTACGACCCTAAC-AAGCGGATTTCGGCCAAGGCAG  838
             | ||||||||||||||||||||||||||||||||  ||||||||||||||||||||||
Sbjct  912   AAGCTTGTTATCGCAAATGCTGCACTACGACCCTAANNAAGCGGATTTCGGCCAAGGCAG  971

Query  839   CCCTGGC-TCACCCTTT-CTTCCAGG-ATGTGACCAAGCC-AGTACCCC-ATC  886
             ||||||| ||||||||| |||||    |||||||||||||| |||||||| |||
Sbjct  972   CCCTGGCCTCACCCTTTTCTTCCNNNNATGTGACCAAGCCCAGTACCCCCATC  1024
```

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  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  954

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   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  714

Query  301   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  654

Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  653   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  594

Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  593   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  534

Query  481   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  533   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  474
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Query  541   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  414

Query  601   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  413   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  354

Query  661   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  353   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  294

Query  721   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  293   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  234

Query  781   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  233   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  174

Query  841   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  173   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  117
```

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    ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  189

Query  61   CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  249

Query  121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  309

Query  181  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  310  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  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    ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
            |||||||||||||||||||||||||||||||||||| ||| ||||||||||||||||||
Sbjct  115  ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTGCCTTTGGAGTTGTGTATAAG  174

Query  61   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175  GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  234
```

```
Query  121  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  294

Query  181  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  354

Query  241  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  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  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  654

Query  541  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  655  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  714

Query  601  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  774

Query  661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  834

Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  894

Query  781  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCC  815
            ||||||||||| | ||||||||||||||||||||
Sbjct  895  TTGGATTTGCTNNCNAAAATGTTAATCTATGATCC  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

Query  77   CAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAAGAGGAAGGGGTTCCTA  136
            |||||||||||||||||||||||||  | ||||||||||||||||||||||||||||||||
Sbjct  924  CAGGTCAAGTGGTAGCCATGAAAAAAATNNGNCTAGAAAGTGAAGAGGAAGGGGTTCCTA  865

Query  137  GTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTC  196
            |||||||||||||||||||| | ||||||||||||||||||||||||||||||||||||
Sbjct  864  GTACTGCAATTCGGGAAATTTNTNTATTAAAGGAACTTCGTCATCCAAATATAGTCAGTC  805

Query  197  TTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGG  256
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804  TTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTTGAGTTTCTTTCCATGG  745

Query  257  ATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATGGATTCTTCACTTGTTA  316
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744  ATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATGGATTCTTCACTTGTTA  685
```

```
Query  317  AGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCACTCTAGAAGAGTTCTTC  376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  684  AGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCACTCTAGAAGAGTTCTTC  625

Query  377  ACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGAACAATTAAACTGGCTG  436
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  624  ACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGAACAATTAAACTGGCTG  565

Query  437  ATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATATACACATGAGGTAGTAA  496
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564  ATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATATACACATGAGGTAGTAA  505

Query  497  CACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGTTACTCAACTCCAGTTG  556
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504  CACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGTTACTCAACTCCAGTTG  445

Query  557  ACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAGAAACCACTTTTCCATG  616
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  ACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAGAAACCACTTTTCCATG  385

Query  617  GGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTGGGCACTCCCAATAATG  676
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  GGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTGGGCACTCCCAATAATG  325

Query  677  AAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACATTTCCCAAATGGAAAC  736
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  AAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACATTTCCCAAATGGAAAC  265

Query  737  CAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGCTTGGATTTGCTCTCGA  796
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  CAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGCTTGGATTTGCTCTCGA  205

Query  797  AAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATGGCACTGAATCATCCAT  856
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  AAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATGGCACTGAATCATCCAT  145

Query  857  ATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  894
            |||||||||||||||||||||||||||||||||||||
Sbjct  144  ATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  107
```

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    ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
            |||||||||||||||||||||||||||||||||||| | | ||||||||||||||||||
Sbjct  118  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  177

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  237

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  297

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  357

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  417

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  477
```

```
Query  361  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  537

Query  421  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  538  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  597

Query  481  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  598  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  657

Query  541  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  658  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  717

Query  601  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  718  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  777

Query  661  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  778  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  837

Query  721  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||  || ||||||||
Sbjct  838  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGANNAANATGGACGG  897

Query  781  AGCTTGTTATC  791
            | |||||||||
Sbjct  898  AACTTGTTATC  908
```

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

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Sbjct  124  TCTTCGACTCTGA  112
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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_2O	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_2O	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.

* Plasmid	Antibiotic resistance gene	Concentration, µg/ml
pGBKT7	Kanamycin	50
pGADT7	Ampicillin	100
pENTR™/D-TOPO®	Kanamycin	50
pDEST™32	Gentamicin	2
pDEST™22	Ampicillin	100

YPD Medium, Agar and Plates:

Rich medium for growth of *S. cerevisiae*

YPD medium

YPD	25 g
dH_2O	to 500 ml

Sterilize by autoclaving: 121° C, 15 min

YPD agar

YPD	20 g

dH_2O to 300 ml and let it dissolve under stirring

Adjust pH to 5.9 – 6.2

Bacto agar	9 g
dH_2O	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.*

Syntetic complete consists of a nitrogen base, a carbon source, and a 'dropout' solution containing essential aminoacids, 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
dH_2O 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
dH_2O 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.

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

* 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
dH_2O to 300 ml and let it dissolve under stirring
Adjust pH 5.9 – 6.2
Complete with dH_2O to 400 ml
Sterilize by autoclaving: 121 °C, 15 min

4 % agar:
Bacto agar 16 g
dH_2O 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
dH_2O to 500 µl

10x loading buffer:
Glycerol, 87 % 500 µl
dH_2O 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.

Appendix 22

List of materials used

Materials and reagents used for Y2H	**Relevant features**	**Source**
MaV203	MATα, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10*::URA*3, *GAL*1::*lacZ*, *HIS*3$_{UAS\ GAL1}$*::HIS*3*@LYS*2, *can*1^{R}, *cyh*2^{R}	Invitrogen
Bacto agar		BD
DO supplement		Clontech
Herring Testes Carrier DNA		Clontech
Minimal SD base		Clontech
YPD		Clontech
5-FOA		Fermentas

Bacterial strains	**Genotype**	**Source**
TOP10 One Shot	F^{-} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G λ-	Invitrogen
XL 10-Gold	Tet^{r}Δ(*mrcA*)*183*Δ(*mrcCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lac1*^{q}*Z* Δ*M15* Tn*10* (Tet^{r}) Amy Cam^{r}]	Stratagene

Nutritional/ selection Plasmids	**Relevant features**	**marker**	**Source**
pGBKT7	P_{ADH1}, pUC ori, 2 μ ori; yeast two-hybrid vector bearing the GAL4 DNA binding domain	Kan/ *TRP1*	Clontech
pGBK/CKS2	pGBKT7 bearing *CKS2*	Kan/ *TRP1*	Irene Sunnvoll
pGBK/CDK1AF	pGBKT7 bearing *CDK1AF*	Kan/ *TRP1*	Irene Sunnvoll
pGBK/CDK2AF	pGBKT7 bearing *CDK2AF*	Kan/ *TRP1*	Irene Sunnvoll
pGADT7	P_{ADH1}, pUC ori, 2 μ ori; yeast two-hybrid vector bearing the GAL4 AD binding domain	Amp/ *LEU2*	Clontech
pGAD/CKS2	pGADT7 bearing *CKS2*	Amp/ *LEU2*	Irene Sunnvoll
pGAD/CDK1 AF	pGADT7 bearing *CDK1 AF*	Amp/ *LEU2*	Irene Sunnvoll
pGAD/CDK2 AF	pGADT7 bearing *CDK2 AF*	Amp/ *LEU2*	Irene Sunnvoll
pENTR™/D-TOPO®	pUC ori, *att*L1, TOPO recognition site 1, *att*L2, TOPO recognition site 2	Kan	Invitrogen
pENTR/CKS2	pENTR™/D-TOPO® bearing *CKS2*	Kan	This work

pENTR/CDK1 AF	pENTR™/D-TOPO® bearing *CDK1 AF*	Kan	This work
pENTR/CDK2 AF	pENTR™/D-TOPO® bearing *CDK2 AF*	Kan	This work
pDEST™32	P_{ADH1}, pUC ori, ARS4/CEN6 ori, *att*R1, *att*R2, *ccdB* gene, Cm^R; yeast two-hybrid vector bearing the GAL4 DNA binding domain	Gent/ *LEU2*	Invitrogen
pEXP™32/Krev1	pDEST™32 bearing Krev1	Gent/ *LEU2*	Invitrogen
pEXP™32/CKS2	pDEST™32 bearing *CKS2*	Gent/ *LEU2*	This work
pEXP™32/CDK1 AF	pDEST™32 bearing *CDK1 AF*	Gent/ *LEU2*	This work
pEXP™32/CDK2 AF	pDEST™32 bearing *CDK2 AF*	Gent/ *LEU2*	This work
pDEST™22	P_{ADH1}, pUC ori, ARS4/CEN6 ori, *att*R1, *att*R2, *ccdB* gene, Cm^R; yeast two-hybrid vector bearing the GAL4 AD binding domain	Amp/ *TRP1*	Invitrogen
pEXP™22/RalGDS-wt	pDEST™22 bearing RalGDS-wt	Amp/ *TRP1*	Invitrogen
pEXP™22/RalGDS-m1	pDEST™22 bearing RalGDS-m1	Amp/ *TRP1*	Invitrogen
pEXP™22/RalGDS-m2	pDEST™22 bearing RalGDS-m2	Amp/ *TRP1*	Invitrogen
pEXP™22/CKS2	pDEST™22 bearing *CKS2*	Amp/ *TRP1*	This work
pEXP™22/CDK1 AF	pDEST™22 bearing *CDK1 AF*	Amp/ *TRP1*	This work
pEXP™22/CDK2 AF	pDEST™22 bearing *CDK2 AF*	Amp/ *TRP1*	This work

Restriction enzymes	**Recognition site**	**Buffer**	**Source**
AscI (10 000 U/ml)	5'…GGCGCGCC…3' 3'…CCGCGCGG…5'	NEBuffer4	New England Biolabs
NotI-HF (20 000 U/ml)	5'…GCGGCCGC…3' 3'…CGCCGGCG…5'	NEBuffer4	New England Biolabs
SwaI (10 000 U/ml)	5'…ATTTAAAT…3' 3'…TAAATTTA…5'	NEBuffer3	New England Biolabs

Other enzymes	**Source**
PfuUltra™ II fusion HS DNA Polymerase	Stratagene

Antibiotics	Stock solution	Source
Ampicillin	100 mg/ml	Bristol-Meyers Squibb
Gentamicin	10 mg/ml	Merck
Kanamycin	50 mg/ml	Sigma-Aldrich

Commercial kits	Source
ProQuest™ Two-Hybrid System	Invitrogen
pENTR™ Directional TOPO® Cloning Kits	Invitrogen
Gateway® Technology with Clonase™ II	Invitrogen
Wizard® SV Gel and PCR Clean-Up System	Promega
QIAquick PCR Purification Kit Protocol	QIAGEN
Wizard® *Plus* SV Minipreps DNA Purification System	Promega
JetStar (Midi-prep)	Genomed

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