Identifying CKS2 protein interactions with CDK1 and CDK2 by the use of Yeast two-hybrid technology

Thesis submitted for Master's degree in Pharmacy

Irene Sunnvoll
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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

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Abbreviations and gene symbols

5-FOA	5-fluoroorotic acid
A	Adenine
AD	activating domain
Ade	adenine
APC	anaphase promoting complex
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BD	binding domain (also termed DBD)
BLAST	basic local alignment search tool
BSA	bovine serum albumin
C	cytosine
CAK	CDK-activating kinase
CCNA	cyclin A
CCNB	cyclin B
CCNE	cyclin E
CDC25	cell division cycle 25
CDK1	cyclin-dependent protein kinase 1 (alias: CDC2)
CDK1AF	double mutated CDK1 (T14A, Y15F)
CDK2	cyclin-dependent protein kinase 2
CDK2AF	double mutated CDK2 (T14A, Y15F)
CDK2F	single mutated CDK2 (Y15F)
CDKN1A	cyclin-dependent kinase inhibitor 1A (alias: p21)
cDNA	complimentary DNA
CHEK1	CHK1 protein kinase
CHEK2	CHK2 protein kinase
CKI	cyclin-dependent kinase inhibitor
CKS2	CDC28 protein kinase regulatory subunit 2
dATP	deoxyriboadenosine triphosphate
dCTP	deoxyribocytidine triphosphate
dGTP	deoxyriboguanosine triphosphate
DIP	The Database of Interacting Proteins
dTTP	deoxyribothymidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
G_1	gap 1 phase
G_2	gap 2 phase
G	guanine
GAL4	galactose-gene activating transcription factor
HF	high fidelity
His	histidine
IPTG	isopropyl- β-$_D$-galactosidase
LB	Luria-Broth medium
Leu	leucine
M	mitosis
MCS	multiple cloning site

MPF	mitosis promoting factor
mRNA	messenger RNA
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
OD	optical density
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PKMYT1	protein kinase membrane associated tyrosine/threonine 1 (alias: Myt1)
PPI	protein-protein interaction
PRB1	retinoblastoma protein
RNA	ribo nucleic acid
rY2H	reverse yeast two-hybrid
S	synthesis
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
T14	threonine 14
T160	threonine 160
T161	threonine 161
T	thymine
TAE	Tris-acetate and EDTA
TBE	Tris-borate and EDTA
T_m	melting temperature of primer
TP53	tumor protein 53 (alias: p53)
UAS	upstream activating sequences
WEE1	protein kinase
Xgal	5-bromo-4-chloro-3-indolyl-β-$_D$-galactoside
Y1H	yeast one-hybrid
Y15	tyrosine 15
Y2H	yeast two-hybrid
Y3H	yeast three-hybrid

Abstract

The function of CKS2 proteins is not clarified, but they are assumed to play a direct role in cell regulation because of interactions with CDKs. The aim of this project was to establish the Y2H method for assessing the CKS2 interactions with CDK1 and CDK2. In order to simulate the dephosphorylated state of CDK *in vivo*, CDK1AF and CDK2AF sequences containing T14A and Y15F mutations, were used as templates for the CDKs. CDK2F, a template with only a single mutation (Y15F) was also tested. For CKS2, normal cDNA was used as template. Primers were designed and used in PCR to amplify cDNA of *CKS2*, *CDK1AF, CDK2AF* and *CDK2F*. Fusion plasmids were constructed by ligating each gene sequence into both yeast expression vectors pGBKT7 and pGADT7. The plasmid constructs were transformed into XL 10-Gold Ultracompetent *E. coli* cells to produce many replicates of the plasmids. For CKS2 and CDK1AF, direct cloning into pGBKT7 and pGADT7 was not successful, and subcloning into pGEM-T Easy vectors was performed. Plasmid constructs were verified by sequencing, and different combinations of plasmids were transformed into AH109 *S. cerevisiae* cells. CKS2 interactions with CDK1AF, CDK2AF and CDK2F were measured by growth in *S. cerevisiae* two-hybrid analyses. For each PPI, two parallels were tested both when CKS2 was used as bait (cloned in pGBKT7) and prey (cloned in pGADT7). The results showed that CKS2 was interacting with CDK1AF, CDK2AF and CDK2F in the Y2H system, regardless of whether CKS2 or the CDK was cloned as the bait protein. We therefore conclude that the Y2H system can be used to assess CKS2 interactions with CDKs. This is the first step towards establishment of Y2H screens for CKS2. Such screens can either contain a cDNA library to identify other CKS2 interacting proteins, or a library of small molecule compounds to find inhibitors of the CKS2 interactions.

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Introduction

Protein-protein interactions (PPIs) are essential in all cellular processes, for example cell signalling and regulation [1, 2]. Mapping of interaction networks can predict the functional relationship between the interacting proteins, and be of importance for understanding the cellular proteome. Knowledge about PPIs will also have major implications in the understanding of diseases and for drug discovery. The yeast two-hybrid (Y2H) system is the most widely used methodology to identify PPIs. The technique is rapid, efficient and convenient, and detects *in vivo* interactions [1-4].

Proliferating cells go through a defined sequence of molecular processes known as the cell cycle. Throughout the cell cycle, different proteins contribute with regulation of the different events, for example entry into S or M phase. Key regulatory proteins are the cyclin-dependent kinases (CDKs) [5, 6]. The CDKs are activated by cyclins which bind to the catalytic subunit of CDKs [7]. Mutations in genes important for cell cycle regulation can result in uncontrolled proliferation, which is associated with cancer [6].

Overexpression of CDC28 protein kinase regulatory subunit 2 (CKS2), a protein assumed to play a direct role in cell regulation because of its interaction with CDKs [8], is associated with aggressive tumors and poor survival in many malignancies including prostate, gastric, hepatocellular, breast and cervical carcinomas [9-13]. The function of CKS2 has not been fully clarified, but the protein is known to interact with both CDK1 and CDK2 [8, 14-18]. The information of CKS2 overexpression in many malignancies, and acquired knowledge about its interacting partners can be used in development of new drugs. Therapeutic strategies based on CDK inhibition have been proposed. However, current CDK inhibitors like Flavopiridol and CY-202 have shown limited success in clinical trials [19]. Design of small molecules that inhibit PPIs have emerged as new potential options for targeted cancer therapy [20, 21]. A totally new approach for inhibiting cell proliferation in cancers would be to target the interaction between CKS2 and CDKs. It is therefore of interest to develop a Y2H screen with a library of small molecular compounds. Such a Y2H screen with CKS2/CDKs interactions has not been presented before. This project is a step towards the development of a Y2H screen by establishing the Y2H method for assessing the interaction between CKS2 and CDKs.

The purpose of the experiments carried out, was to investigate whether the Y2H system could be used to show CKS2 interaction with CDK1 and CDK2. Phosphorylations on threonine 14 (T14) and tyrosine 15 (Y15) inactivates the CDKs, and these sites must be dephosphorylated in order for the CDKs to be active [22, 23]. To simulate *in vivo* conditions of dephosphorylated sites, CDKs based on T14 and Y15 mutated cDNA sequences were used; i.e., CDK1AF, CDK2AF, and CDK2F.

1 Biological Background

1.1 The Cell Cycle

When cells proliferate, they go through a defined sequence of molecular processes [24]. This essential mechanism by which all living things reproduce is known as the cell cycle. The cell cycle is traditionally divided into four phases known as, G_1 (gap 1), S (synthesis), G_2 (gap 2), and M (mitosis) (figure 1.1). The S phase is the interval where DNA replication (duplication) occurs, and mitosis is the process where the entire duplicated DNA is segregated accurately into two genetically identical daughter cells. A cell needs approximately 24 hours to complete the cell cycle, of which S takes 10-12 hours and M only requires about one hour. The rest of the time, the cells need to grow and increase their mass of proteins and organelles [25, 26].

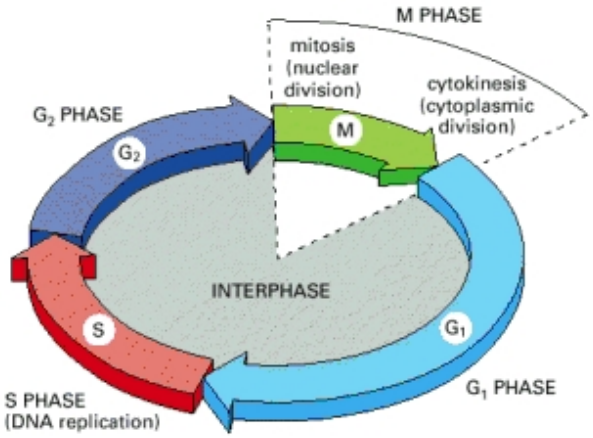

Figure 1.1: The phases of the cell cycle [25]. The cell cycle is traditionally divided into the four phases G_1 (gap 1), S (synthesis), G_2 (gap 2), and M (mitosis). Interphase is the process between two M phases, and consists of G_1, S and G_2. M is further divided into mitosis (division of the nucleus) and cytokinesis (division of the cytoplasm).

G_1 and G_2 will also ensure that external and internal conditions are suitable for the cell cycle to proceed, and the time of these gap-phases will vary in time according to how favourable external conditions and extracellular signals from other cells are. When the cell first has passed through the restriction point (R) in G_1, it has committed

to DNA replication and will continue even if the outer cell growth stimulation is taken away [25].

Chromosomes are structures composed of DNA, carrying the genetic information of an organism. After replication, the chromosomes of the cell will condensate, and this is a visible sign that the cell is ready to enter M. In a light microscope, the chromosomes can first be seen as long threads, and as they condensate, become thicker and shorter. The condensation progress makes the chromosomes easier to segregate later in mitosis [26].

The process between two M phases is called interphase (consisting of G_1, S and G_2). More detailed, M is divided into mitosis (nuclear division) and cytokinesis (cytoplasmic division). Mitosis is again divided into five stages; prophase, prometaphase, metaphase, anaphase and telophase. Figure 1.2 shows the different phases of mitosis [25].

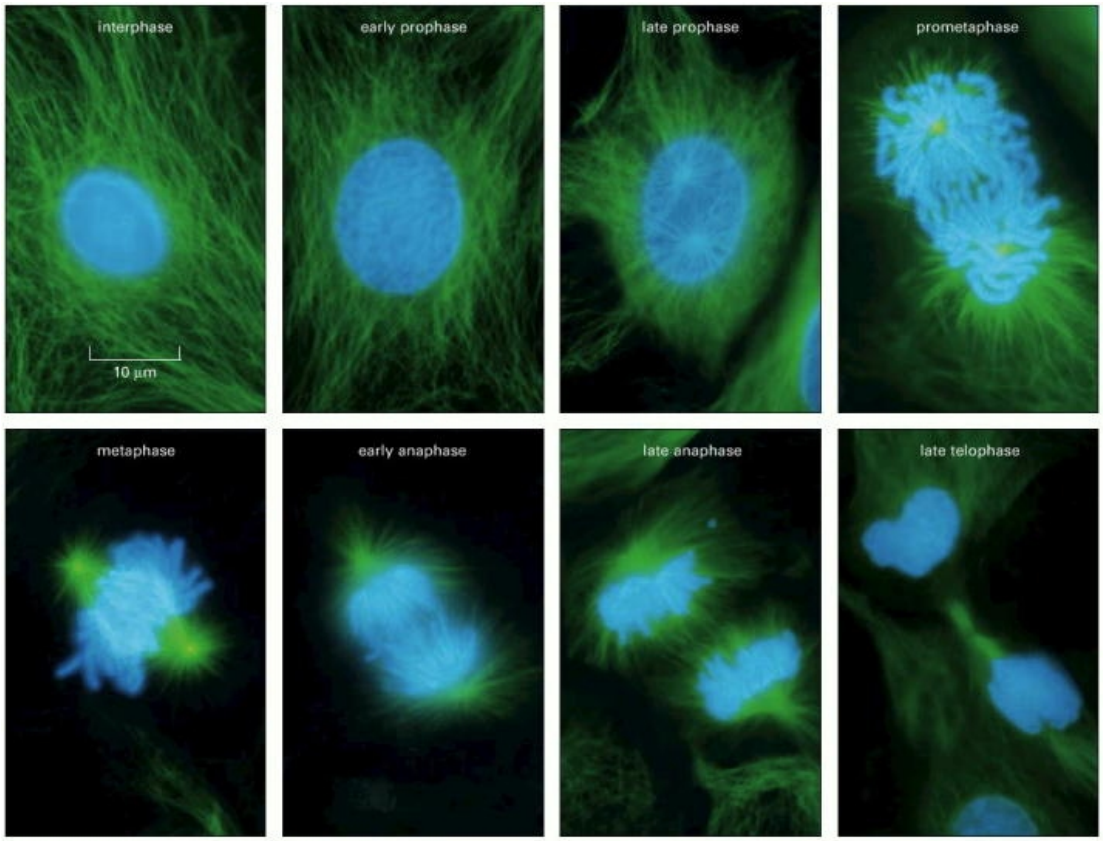

Figure 1.2: The course of mitosis in a typical animal cell [25]. Mitosis is further divided into five stages; prophase, prometaphase, metaphase, anaphase and telophase. DNA is colored with blue and the microtubules are colored with green.

In prophase the replicated chromosomes, now consisting of two sister chromatids, start to condense. Outside the nucleus, the centrosomes have started to move apart forming the mitotic spindle. The mitotic spindle is composed of microtubules, and performs the separation of the chromosomes. At prometaphase, the nuclear envelope breaks down and the chromosomes attach to the mitotic spindle. The chromosomes then align in a plane, midway between the spindle poles in metaphase, and are separated in anaphase, forming two sets of daughter chromosomes. During telophase, a new nuclear envelope reassembles around each set. Formation of two nuclei is completed, and this marks the end of mitosis. In the final stage of M, the cytokinesis, the cytoplasm is divided in two, creating two daughter cells each with one nucleus [25].

1.2 Regulation of the cell cycle

Throughout the process of the cell cycle, different proteins contribute to the regulation of the different events, for example entry into S or M. This is to ensure that the cell does not enter the next stage unless it is prepared, and then triggers the next stage when the cell is ready. Two important checkpoints occur in G_1 and G_2. The G_1 checkpoint ensures that environmental conditions are favourable for cell proliferation and that the cell's DNA is intact before committing to S. If these conditions are not in order, the cell will delay progress through G_1, and may even enter a resting state known as G_0 [26]. In G_0, the cells no longer proliferate [6].The G_2 checkpoint ensures that the cell does not enter mitosis if replication is not complete. Entry into M will also be stopped if the cell has DNA damage. When DNA is repaired and replication is complete, the cell is allowed to enter M [26].

1.2.1 Key proteins involved in regulation

Key regulatory proteins are the cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases [5, 6]. They are present in cells throughout the cell cycle, but are only activated and deactivated at appropriate times. Cyclins, a set of proteins also important in the cell cycle control system, are partly responsible for switching the CDKs on and off [26]. They do so by binding to the catalytic subunit of

CDKs, and thereby activating them [7]. The cyclins, in contrast to the CDKs, are synthesized during progression through the cell cycle, and are quickly proteolysed after ubiquitination, and replaced by other cyclins. In this way, they periodically activate CDKs [24, 27]. When a CDK is activated, it induces downstream processes by phosphorylating certain proteins [6]. In this context, CDK1 and CDK2 will be the main focus (figure 1.3).

1.2.2 Inhibition of CDKs

CDK inhibitors (CKI) can counteract CDK activity by binding to CDKs alone and prevent association with cyclins, or directly to the CDK-cyclin complex [6]. One type of CKI is cyclin-dependent kinase inhibitor 1A (CDKN1A), an enzyme which inhibits CDK by binding to CDK-cyclin complexes, thereby preventing CDK-cyclin complexes from phosphorylating their target substrates. CDKN1A also inhibits DNA synthesis by binding to and inhibiting the proliferating cell nuclear antigen (PCNA). CDKN1A is activated by tumor protein 53 (TP53), a tumor suppressor gene. At the G_1-checkpoint, cell cycle arrest induced by DNA damage is TP53-dependent. The cellular level of TP53 is usually low, but can have rapidly induced activity if DNA damage occurs. This will lead to an induction of CDKN1A followed by G_1-arrest, thereby preventing replication of damaged DNA. If the cells are severely damaged, TP53 activates genes leading to apoptosis (cell death) [6]. When DNA damage occurs in G_2, the kinase ataxia telangiectasia mutated (ATM) is activated. ATM can induce G_2-arrest by two signal paths. ATM can stimulate the kinase CHEK2 which directly inhibits the phosphatase cell division cycle 25 (CDC25). Alternatively; ATM will stimulate the tumor suppressor gene BRCA1 which will then activate the kinase CHEK1. CHEK1 will directly inhibit CDC25, leading to G_2 arrest [24].

1.2.3 Deregulation of the cell cycle and cancer

Mutation of genes important for the regulation of cell cycle can lead to uncontrolled proliferation, and is associated with cancer. Uncontrolled cell division results in unrestrained cell proliferation. Mutations mainly occur in two classes of genes: proto-oncogenes and tumour suppressor genes. In normal cells, proto-oncogenes contribute to stimulation of cell proliferation. When mutations occur, proto-oncogenes or

oncogenes can promote tumour growth. Mutations in tumour suppressor genes like *TP53*, results in dysfunction of proteins that normally inhibit cell cycle progression, and proliferation can continue in damaged cells. The *TP53* gene is the most frequently mutated gene in human cancer. Inactivated TP53 results in loss of regulation of CDKN1A, in response to DNA damage. Mutations of checkpoint proteins are frequent in all types of cancers [6].

1.3 Regulation of CDKs

CDK activity is not only regulated by cyclins, but also by phosphorylation on conserved threonine and tyrosine residues. Amino acid sequences of human CDK1 and CDK2 are 65 % identical, and major phosphorylation sites are equally conserved for both [23]. In order for CDK1 and CDK2 to be fully activated, phosphorylations of T161 and T160, respectively, are required [6, 22, 23]. The conserved region in cyclins, important for binding to CDKs, contains 5 α-helices often referred to as a cyclin box [28]. The equally conserved region for CDKs is often termed the PSTAIR helix and contains this single-letter amino acid code, or variants of it. The PSTAIR helix is adjacent to the catalytic loop [27]. The catalytic loop (also termed T-loop) blocks the substrate binding site, and side chains in the ATP-binding site block phosphotransfer [7]. Binding of cyclins to a CDK results in a conformational change which aligns the PSTAIR helix and T-loop [29]. The crystal structure of CDK2/CCNA, reveals that the three-dimensional structure of the T-loop is changed from “closed” to “open”, thereby exposing T160 of CDK2 [30]. T160 can now be phosphorylated, which is carried out by the CDK-activating kinase (CAK) [31]. Because of structural identity among CDKs, it is predicted that conformational changes for CDK2/CCNA will be maintained in CDK1/CCNB. This is also supported by the structure of CDK2/CCNE [28]. McGrath *et al.* [32] refined a homology model of CDK1/CCNB that supports similar findings reported for CDK2/CCNA, such as conformational change in CDK1 induced by CCNB binding. Several studies have explored the binding of CCNB to CDK1 and found that the complex could form in the absence of phosphorylation, indicating that CAK phosphorylation of T161 occurs after cyclin binding [22, 31].

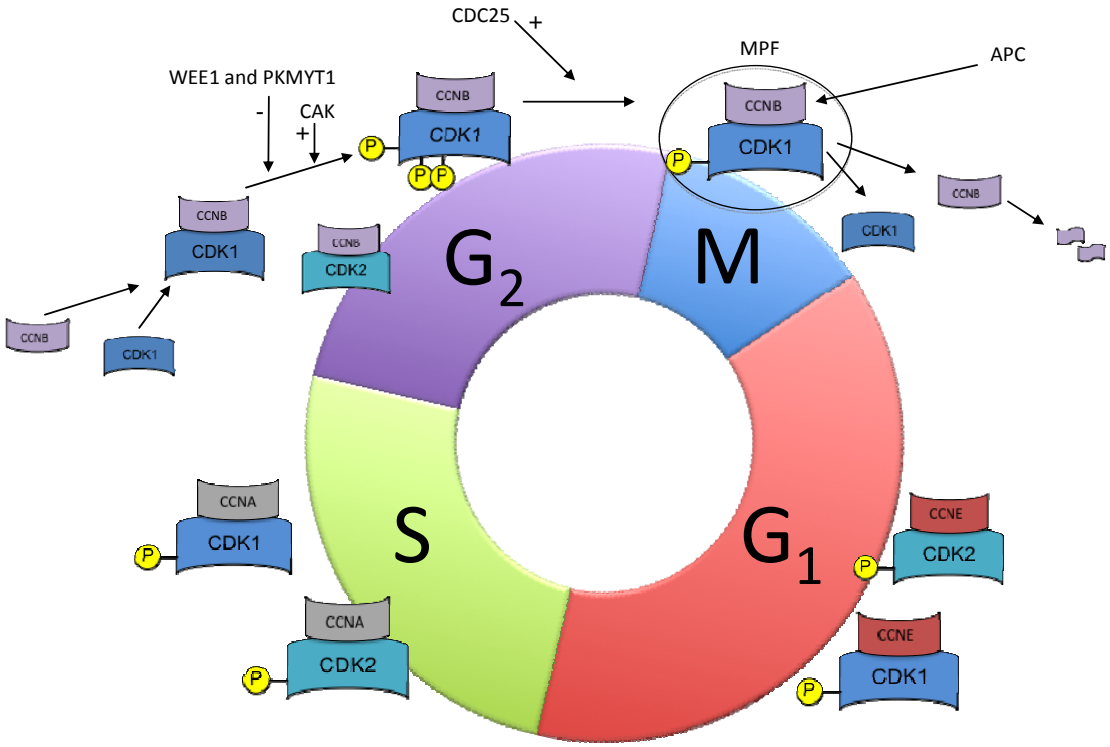

Figure 1.3: Cell cycle regulation. CDK1 and CDK2 form complexes with CCNE in G_1, with CCNA in S, and with CCNB in G_2. The active phosphorylation on T161 and T160 for CDK1 and CDK2 respectively, is indicated by the yellow symbol P. Details on activating phosphorylation by CAK, deactivating phosphorylation by WEE1 and PKMYT1, and activating dephosphorylation by CDC25 is shown for CDK1/CCNB. This complex form the mitosis promoting factor (MPF) in M, and the complex in inactivated by the anaphase promoting complex (APC) which leads to exit from mitosis. APC mediates ubiquitination of CCNB which is then proteolysed.

Inactivating phosphorylation of CDK1 is carried out by the kinases WEE1 and protein kinase membrane associated tyrosine/threonine 1 (PKMYT1) at tyrosine 15 (Y15) [33, 34]. An additional phosphorylation by PKMYT1, when Y15 is also phosphorylated, occurs on threonine 14 (T14) [23, 34]. CDK2 is also phosphorylated on T14 and Y15, and these sites need to be dephosphorylated in order for CDK1 and CDK2 to be active and further progress through the cell cycle. Dephosphorylation is carried out by the phosphatase CDC25 [23, 35].

1.3.1 CDKs and their cyclin-binding partners

CDK1 and CDK2 can both bind to CCNA, CCNE and CCNB [36, 37]. In early G_1, CDK4/6 in complex with CCND (cyclin D) will increase phosphorylation of retinoblastoma protein (PRB1). CDK2/CCNE completes phosphorylation of PRB1 which releases the transcription factor E2F. E2F then controls the expression of different genes necessary for the progression through S [24, 37, 38]. G_1/S transition also involves CDK1/CCNE and CDK2/CCNE. Kaldis *et al.* [37] demonstrated that CDK1 can substitute for CDK2 in this transition. It has been shown that *CDK2*$^{-/-}$ mice were viable, but both male and female were sterile due to meiotic defects [36, 37, 39]. DNA replication is controlled by the CDK2/CCNA and CDK1/CCNA, which also allow the cells to proceed to G_2 [24, 37]. Levels of CCNB accumulate in the cytoplasm during S and G_2, and are in complex with CDK1 and CDK2 in G_2, but CDK1/CCNB activity is still low [23, 37]. At the onset of mitosis, CDC25 activity increases, thereby increasing dephosphorylation of T14/Y15 for both CDK1 and CDK2. At the same time, CCNB levels rise and T161 phosphorylatio increases, leading to an upsurge and peak in CDK1 activity at M [23]. The CDK1/CCNB complex is required for the G_2/M transition [15], and the complex enters the nucleus at the beginning of mitosis [40, 41]. During mitosis, CDK1/CCNB forms the active mitosis promoting factor (MPF) [22]. Inactivation of CDK1 and exit from mitosis require the ubiquitin-dependent proteolysis of CCNB. Ubiquitination of CCNB is mediated by the anaphase-promoting complex (APC) [15, 42, 43]. Although CDK2 also binds to CCNB, the functional significance of the complex is unclear, and CDK2 cannot compensate for the loss of CDK1 [36, 37]. CDK2 activity peaks in G_1/S, and although CDK1 activity is much lower here than in G_2/M, it seems possible that CDK1 activity in this transition approximately equals maximum CDK2 activity [36].

1.3.2 CDKs as targets in cancer therapy

Deregulation of CDKs mediates cell cycle defects such as unscheduled proliferation, genomic instability and chromosomal instability. These cell cycle defects contribute to tumor progression and acquisition of more aggressive phenotypes. Because of the CDKs essential role in driving the cell cycle progression, selective CDK inhibition has been proposed as a therapeutic strategy [44]. A search for CDK inhibitors in the

registry of clinical trials conducted in the Unites States and around the world, yields 28 matches, of which 21 trials are active (may be recruiting volunteers or not), five trials were completed and two trials were terminated [45]. Although the first-generation CDK inhibitors only showed modest activity in the clinic, several second-generation CDK inhibitors are now in clinical trials. However, the effect of such inhibitors are still uncertain, and therefore, development of new targets are needed.

1.4 The role of CKS2

The CKS proteins are essential components of the mitotic CDKs, but their precise function has remained unclear. Martinsson-Ahlzén *et al.* [8] showed in a study that deleting both the *CKS1* and *CKS2* genes in mice, leads to embryonic lethality. It has also been reported that CKS2 is essential for the first metaphase/anaphase transition of mammalian meiosis. *CKS2*$^{-/-}$ animals were viable but sterile, due to an arrest of the germ cells at metaphase I of meiosis [46]. Although not essential for viability, it has been shown that CKS proteins are required for optimal preanaphase ubiquitylation and degradation of cyclin A in mammalian cells [47]. Another possibility is that CKS2 targets CDK/cyclin complexes to phosphoproteins [48]. A targeting function has also been proposed in other studies, and that CKS proteins govern the interactions of CDK1/CCNB with both positive and negative regulators [15, 49, 50]. Targets proposed by Pines [17] are CDC25 in G_2, and the proteolysis machinery in mitosis which inactivates CDK1/CCNB. After determining the atomic structure of CKS2, Parge *et al.* [51] proposed that the role of hexameric CKS2 was to serve as a hub for assembling kinase molecules. John *et al.* [27] reported that CKS proteins binds to CDC25 just prior to mitosis, and participates in a positive feedback loop in which CDC25 activates CDK1 as described previously. Activated CDK1 then activates CDC25 by multiple phosphorylations.

1.4.1 CKS2 and interactions with CDKs

CKS proteins are assumed to play a direct role in cell regulation because of association with CDKs [8]. In humans there are two homologues, CKS1 and CKS2 [52], and their sequences are 81 % identical [53]. The CKS proteins consist of a four-

stranded β-sheet capped at one end by two α-helices. Crystal structure of CKS2 suggests monomer, dimer and hexamer forms for the proteins. The hexamer is formed by three interlocked dimers [18, 51, 53]. A β-hinge in CKS, containing the residues histidine-any-proline-glutamic acid-proline-histidine, can fold back on itself forming a monomer. When the β-hinge is extended, the proteins form a dimer. Molecular modelling predicts that dimerized CKS proteins would not be able to bind to CDKs because residues important for binding would not be able to contact the CDK. A possibility is thus that there is equilibrium in the cell between monomer and dimer forms, where only monomers can bind to CDKs [17]. Watson *et al.* [18] investigated a mutated CKS2, with replacement of glutamine 63 for glutamate (E63Q). The binding to CDKs was drastically impared for mutated CKS2, suggesting that glutamine 63 in CKS2 might be directly involved in interaction with CDKs [18]. Crystal structure of the CDK2/CKS1 complex revealed that CKS1 interaction with CDK2 involved binding of all four β-strands to the C-terminal lobe of CDK2. This binding site is far from the N-terminal lobe, cyclin, and regulatory phosphorylations sites of CDK2 [49].

Studies have shown that CKS2 interacts with the CDK1/CCNB complex [8, 14, 15, 17]. Egan and Solomon [15] demonstrated that CKS2 exhibited cyclin-stimulated binding to CDK1. The study indicated that the presence of cyclins increased CKS binding to CDK1, but it did not exclude a weak interaction of CKS proteins to monomeric CDKs. Also, they showed that phosphorylation of T161 stimulated binding of CKS2 to CDK1. The phosphorylated, cyclin-bound CDK complex is preferentially associated with CKS proteins [15]. The CDK1/CCNB/CKS2 complex is shown in figure 1.4. Interactions of CKS2 to CDK2 have also been shown in several studies [15, 16, 18].

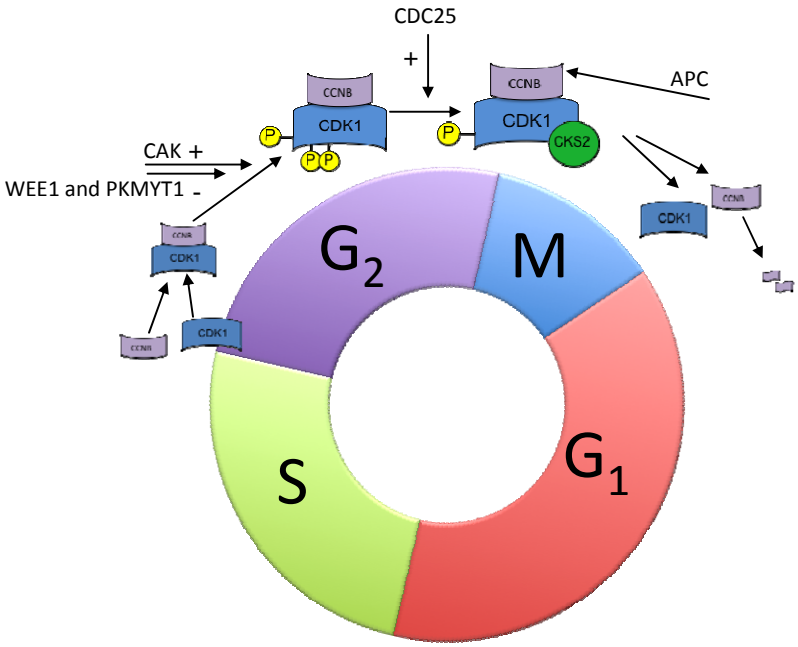

Figure 1.4: Binding of CKS2 to CDK1/CCNB. CKS2 is interacting with the phosphorylated, CCNB-bound CDK1 complex.

1.4.2 CKS2 and cancer

Although the functions of CKS proteins have remained elusive, tumor profiling has revealed that both CKS proteins are frequently overexpressed in a variety of human malignancies. One study identified gene expressions associated with metastatic phenotypes of locally advanced cervical carcinomas. *CKS2* was identified as one of the genes, and was upregulated in node positive compared to node negative tumors. High expression of *CKS2* was associated with poor survival [11]. Van't Veer and co-workers [13] used gene expression profiling to predict clinical outcome of breast cancer, and *CKS2* was identified as one of the genes significantly upregulated in the poor prognosis signature. Overexpression of CKS2 has also been reported to be associated with high aggressiveness and a poor prognosis in various other malignancies, including prostate, gastric and hepatocellular carcinomas [9, 10, 12]. Based on this knowledge, it would be of interest to investigate CKS2 activity. By targeting interactions of CKS2 with CDKs, a new concept for inhibiting cell proliferation is proposed. This project assesses the interaction between CKS2 and CDKs by establishing the use of Y2H, and is a step towards the development of targeted therapeutics.

2 Methodical background

2.1 Yeast two-hybrid (Y2H)

In 1989, Ok-kyo Song and Stanley Fields created the Y2H system, which is a simple robust assay for monitoring protein-protein interactions (PPIs). Before the Y2H method, mammalian PPIs were generally studied by using techniques such as crosslinking, co-immunoprecipitation, and co-fractionation by chromatography [54, 55]. Today, Y2H is widely used to determine PPIs [2], and Xenarios *et al.* [56] reports that the majority of interactions in the Database of Interacting Proteins (DIP) have been detected by the Y2H method. They also hypothesize that many PPIs are first observed in Y2H, and then later confirmed by other methods like co-immunoprecipitation.

The Y2H system takes advantage of the galactose-gene activating transcription factor (GAL4) of the yeast *Saccharomyces cerevisiae*. GAL4 is required for the expression of genes encoding enzymes of galactose utilization. The protein consists of two separable domains; an N-terminal domain (amino acids 1-147), and a C-terminal domain (amino acids 768-881) [54]. The N-terminal domain binds to upstream activating sequences (UAS) that are specific DNA sequences, thereby termed DNA-binding domain (BD or DBD). The C-terminal domain is necessary for activation of transcription, and is termed activating domain (AD). Fields and Song [54] generated a system of two hybrid proteins containing parts of the GAL4 protein. The GAL4 BD was fused to a protein 'X', and the AD of GAL4 was fused to a protein 'Y'. The X and Y proteins are also termed 'bait' and 'prey' respectively (figure 2.1 A and B) [2]. If X and Y interact, the GAL4 domain gets reconstituted resulting in activation of a reporter gene (figure 2.1 C) [3, 54]. The output of the reporter gene can be measured, for example as growth of yeast clones on selective medium or by a color signal [2, 3].

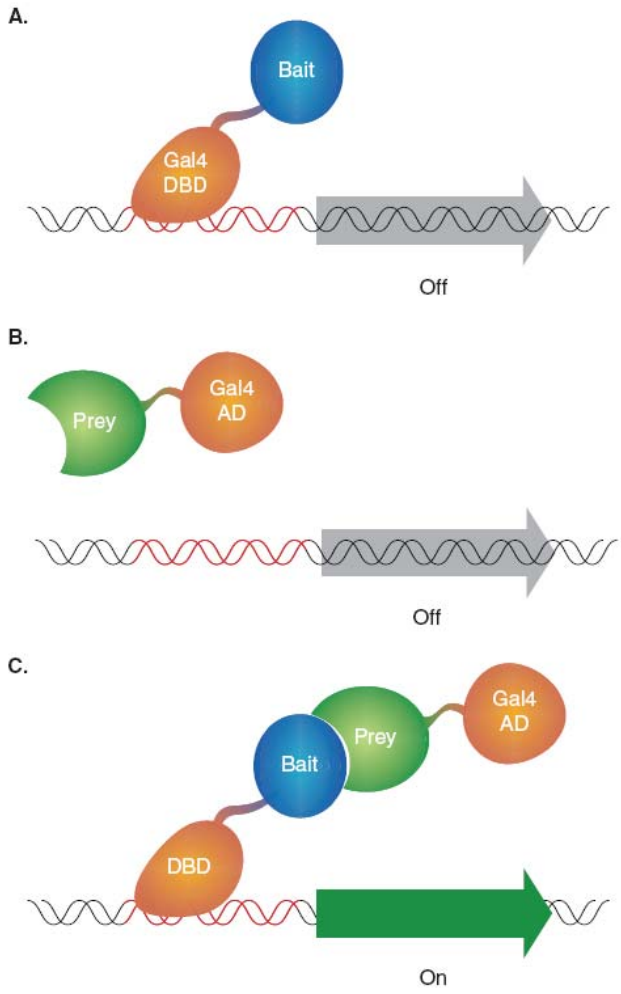

Figure 2.1: The principle of Y2H [3]. **A**: A protein to be investigated (bait) is fused to the DNA-binding domain (DBD) of GAL4. **B**: the other protein to be investigated (prey) is fused to the activating domain (AD) of GAL4. When expressed in isolation, neither the bait nor the prey is able to activate the reporter gene since each fusion protein lacks a domain that is necessary for proper transcriptional activation. **C**: GAL4 is reconstituted because of interacting proteins, and the reporter gene is activated. Output of the reporter gene is used to detect the PPI. Common ways of measuring output is by growth or a color marker.

The Y2H technique involves construction of the fusion plasmids and use of different molecular biological methods. In constructing the fusion plasmids, the proteins of interest must be cloned into yeast expression vectors. Firstly, templates and primers for polymerase chain reaction (PCR) are prepared. PCR is performed to amplify the cDNAs of the fragments to be investigated. Appropriate plasmid constructs are transformed into bacteria in order to produce many replicates of the plasmids. Verified plasmid constructs are co-transformed into a yeast strain. Finally, a two-hybrid analysis is set up to score for PPIs. The different methods will be described below, and an overview is given in figure 2.2.

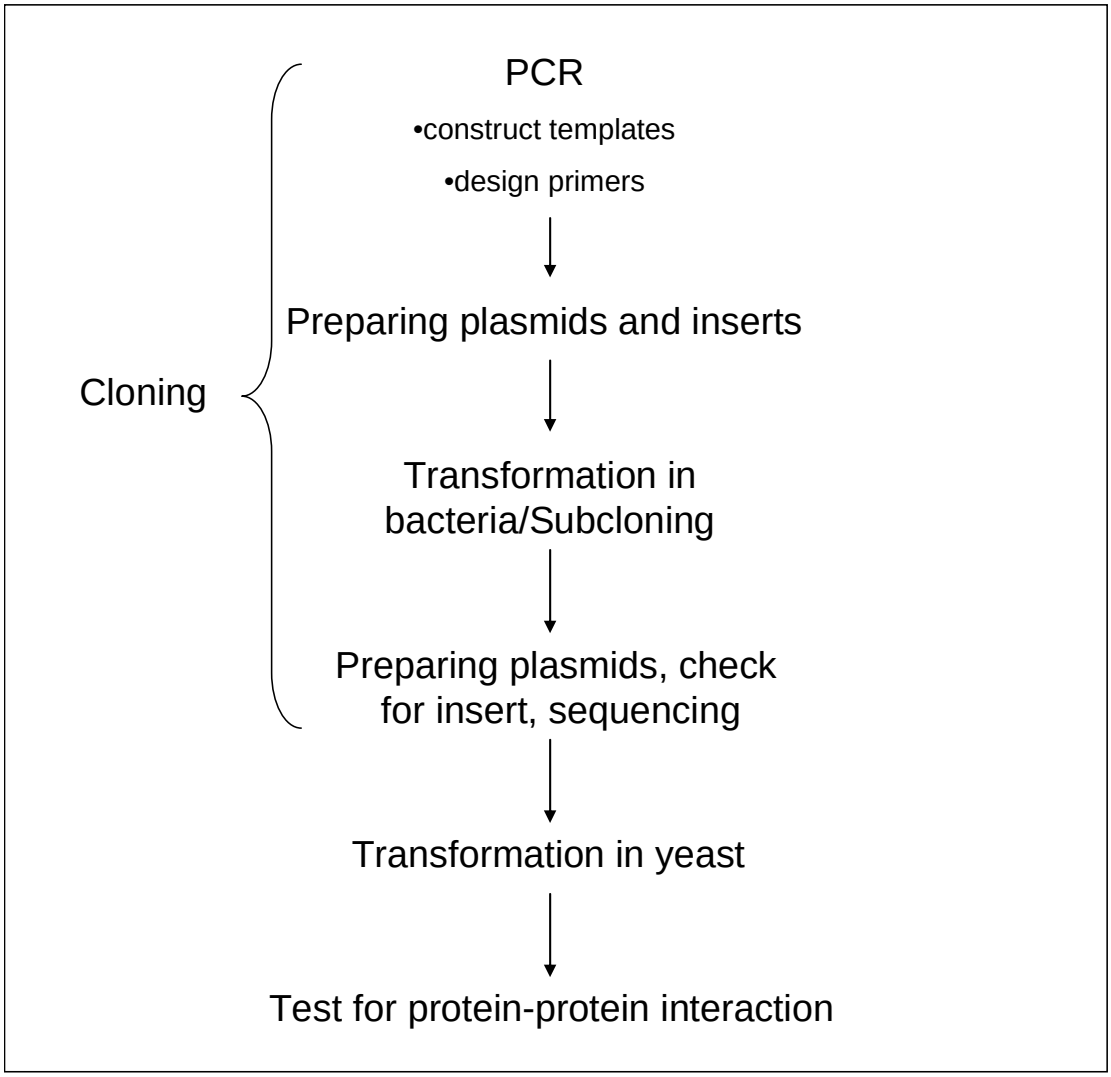

Figure 2.2: An overview of the biological methods included in the Y2H technique. Templates are constructed and primers are designed in order to amplify the cDNA (fragments) of the proteins of interest by PCR. Plasmids and fragments are prepared and ligated together. These constructs are transformed in bacteria cells in order to get high copy replications of the plasmid constructs. The plasmids are screened for inserts and sequenced in order to verify the constructs. Different combinations of constructs are transformed in yeast cells, and the assay for scoring PPI is performed.

2.1.1 Applications of Y2H system in drug discovery and development

In recent years, the Y2H technique has seen extensive modifications. The system was originally invented to identify interactions between two defined proteins, but has been adjusted and can now be used for screening. By replacing the prey protein with cDNA or genomic libraries, interactions can be screened against the libraries and yield a set of proteins interacting with the bait protein. This technique can be used to discover novel interacting proteins, and structures that bind the bait with high affinity may represent important clinical targets [3].

One adaptation, 'reverse' Y2H (rY2H), uses a Y2H setup to screen for small molecule-protein interaction instead of PPIs. Targets can be detected from such a screen with the clinical purpose of blocking PPIs by using small molecules [3]. This system is designed so that the interaction of two proteins activates a reporter gene whose product is toxic to growing cells. By screening for small molecules that block the PPI, activation of the toxic gene is prevented and the output is measured by growth. One version of this screen is to incorporate the *URA3* gene in the yeast genome which encodes the enzyme orotidine-5-phosphate decarboxylase. This enzyme is involved in uracil biosynthesis, and catalyzes the transformation of pro-toxin 5-fluoroorotic acid (5-FOA) into a toxic compound causing cell death. Yeast cells will not grow on medium containing 5-FOA if PPI occur, since the enzyme encoded by *URA3* then will be transcribed. On the other hand, if a test compound is able to block PPI, GAL4 will not be reconstituted, allowing growth of yeast cells on medium containing 5-FOA [3, 4].

2.1.2. Limitations of the Y2H system

Frequent occurrence of false positives and false negatives can be a serious challenge, especially to large-scale Y2H [2, 3]. False positives are spurious interactions (no biological significance) detected in the screening in yeast which are not reproducible in another assay [1, 3]. In order to increase the stringency, more than one reporter gene can be used in parallel. This requires a more solid transcriptional activation and can therefore at the same time penalize detections of weak interactions. Major sources for false positives are non-relevant PPIs forced by the overexpression of heterologous proteins and self-activation of the reporter gene by the bait protein [2, 3]. Auto activation can be avoided by partially inhibiting the enzymatic activity encoded by the reporter gene. For example, imidazole glycerol phosphate dehydrase (the product of *HIS3* reporter), is competitively inhibited by increasing concentrations of 3-aminotriazole (3-AT) [1].

False negatives are occurring PPIs that are undetectable in the Y2H system. Membrane proteins are mostly undetectable, so in working with such proteins, variants of the classical Y2H should be used. Bait or prey protein can sterically hinder the interaction, thereby producing false negatives. Other sources are proteins

depending on post-translational modifications. The modifying enzyme can be different or lacking in yeast when analyzing PPIs of higher eukaryotes. Coexpression of the modifying enzyme in yeast together with bait and prey can solve this. Improper folding of the fusion proteins, and very transient PPIs, may escape detection, and are also sources of false negatives [2, 3].

Another limitation of the Y2H system is the requirement that the bait and prey must be able to enter the nucleus of the yeast cell to activate transcription. Also, the system is not suited for analysis of interaction with bait proteins that are potent activators themselves. Such transactive proteins would trigger transcription in absence of any interaction with a prey [1, 54].

2.2 Cloning

Cloning refers to the process of making many identical copies of a DNA molecule. Cloning also describes the process of isolating a DNA fragment from the rest of the cell's DNA because the process involves making many identical copies of the fragment of interest [26]. Further process involves making recombinant DNA by using plasmid DNA, restriction enzymes, ligation mixture and transformation into an appropriate bacterial strain, usually an *Escherichia coli* strain. Recombinant DNA is produced when two or more DNA fragments from different sources are joined together [25]. The transformants are screened to identify those that carry the desired fragment. Different screens can be used, for example hybridization, PCR or digestion with restriction enzymes [57].

2.2.1 Polymerase chain reaction (PCR)

PCR was invented in the 1980's, and can be carried out entirely *in vitro* without using cells. The technique is rapid and effective in producing large amounts of the gene of interest. PCR is extremely sensitive, and the process is able to detect a single copy of a DNA sequence in a sample. The sequence is then amplified so many times that it can be detected by for example staining after separation by gel electrophoresis. Input template for PCR can be DNA or RNA, so PCR can be used to obtain either a full

genomic copy or a cDNA copy of the gene. There are several useful applications of PCR, for example for cloning short DNA fragments (below 10 000 nucleotide pairs) from a cell. [26].

PCR is performed in a thermal cycler, and the process consists of three steps. The first step is denaturation, where heat is added in order to separate the strands of double-stranded DNA. Typical temperature is about 93-95 °C. The process is followed by cooling where a forward and reverse primer is hybridized to complimentary sequences in each of the two strands. This is the second step, and is termed annealing. The temperature is often about 5 °C below the calculated T_m (melting temperature of the primer). In the third step, the polymerase starts synthesizing DNA by adding deoxyribonucleotide triphosphates (dNTPs). A standard mixture of dNTPs contains equimolar amounts of deoxyriboadenosine triphosphate (dATP), deoxyribocytidine triphosphate (dCTP), deoxyriboguanosine triphosphate (dGTP), and deoxyribothymidine triphosphate (dTTP) [58]. DNA synthesis is typically carried out at 70-75 °C. In the first cycle of the PCR process, two double-stranded DNA molecules are produced. The second cycle then starts with heat treatment, repeating the three steps from the first cycle, now producing four double-stranded DNA molecules. The third cycle produces eight double-stranded DNA molecules and so doubles the amount of DNA from each previous cycle. Usually, 20-30 cycles are required for useful DNA amplification. The newly synthesized strands serve as templates for the next cycle [26, 59]. DNA polymerases for PCR in current use have been isolated from thermophilic bacteria, and will therefore not denature at high temperatures. The polymerase can therefore be used again in all cycles. Many different polymerases are commercially available, and for routine PCR, the *Taq* polymerase is often used. Several of these polymerases will add a single, unpaired nucleotide at the 3’ ends of the amplified DNA fragments, thereby creating a cohesive end [58]. A cohesive end can leave the 5’ end and/or 3’ end with short single-stranded tails [25].

Preparing PCR-template by synthesizing cDNA from RNA. cDNA differs from DNA in that sense that is does not contain genomic DNA (chromosomal DNA), but instead the DNA is copied from the mRNA present in that cell culture sample. The enzyme, reverse transcriptase, makes DNA copies of the mRNA molecules. A very important

advantage of cDNA clones is that they contain the uninterrupted coding sequence of the gene of interest, which is crucial when the aim is to express the cloned gene in bacterial or yeast cells. Neither bacteria nor yeast cells are able to remove introns from mammalian RNA transcripts. The first step is hybridization of a short oligonucleotide complementary to the poly-A tail of the 3' end of the mRNA. This functions as a primer for the reverse transcriptase, which in the second step makes DNA copies complementary to the RNA. The helix is now made up of one DNA strand and one RNA strand. In the final step, the enzyme RNase H will then degrade the RNA strand, and so the single stranded cDNA can be copied into double stranded cDNA by DNA polymerase [26].

Primers and primerdesign. A primer is a short oligonucleotide, which base-pairs specifically to a target sequence to allow a polymerase to initiate synthesis of a complimentary strand [59]. When designing primers, several aspects must be taken into consideration. For optimal effect, the GC-content should be between 40 % and 60 %, the primer length 20-30 nucleotides long, and the 3' end should be a G or a C. [58, 60]. Also, the calculated T_m should not differ between the forward and reverse primers by more than 5 °C. Primer dimers can form if the 3' terminal sequence of one primer can bind to any site on the other primer, and will compete for DNA polymerase, primers, and nucleotides. This will greatly affect the amplification yield, and should therefore be avoided.

2.2.2 Plasmids

Plasmids are extrachromosomal elements of DNA, mostly double stranded, circular molecules that can be isolated from bacterial cells in their natural, supercoiled form. They contain a replication origo and can therefore replicate independently of their host's chromosome [26]. Plasmids often contain genes that are advantageous to the host, for example genes encoding production of enterotoxins and antibiotics resistance (for resistance to example kanamycins, ampicillin, and tetracyclins). The genes that provide resistance to antibiotics can also be used as selective markers. DNA introduced to bacteria is often incorporated into the bacterial genome, and this can happen naturally by the bacteria taking up DNA molecules from its surroundings. It is usually though, easier to manipulate, copy and purify the recombinant DNA when it is

maintained in a vector, separate from the bacterial chromosome [26, 57]. A plasmid vector is therefore used to carry a fragment of DNA and then transformed into a recipient cell for the purpose of gene cloning. The development of plasmids from the early 1970's to present has been through many different phases. Plasmid vectors in current use are small, they replicate fast, and contain one or more selective markers and a multiple cloning site (MCS). The MCS consists of a bank of sequences recognized by restriction enzymes (described below) [57].

Yeast expression vectors. pGBKT7 BD and pGADT7 AD (figure 2.3, A and B respectively) are yeast expression vectors from Clontech. The vectors are designed to express a protein of interest fused to the GAL4 binding domain and activating domain respectively. The constitutive promoter ADH1 drives the expression of the fusion proteins [61, 62]. Both vectors replicates autonomously in *E. coli* from the pUC ori, and in *S. cerevisiae* from the 2 μ ori.

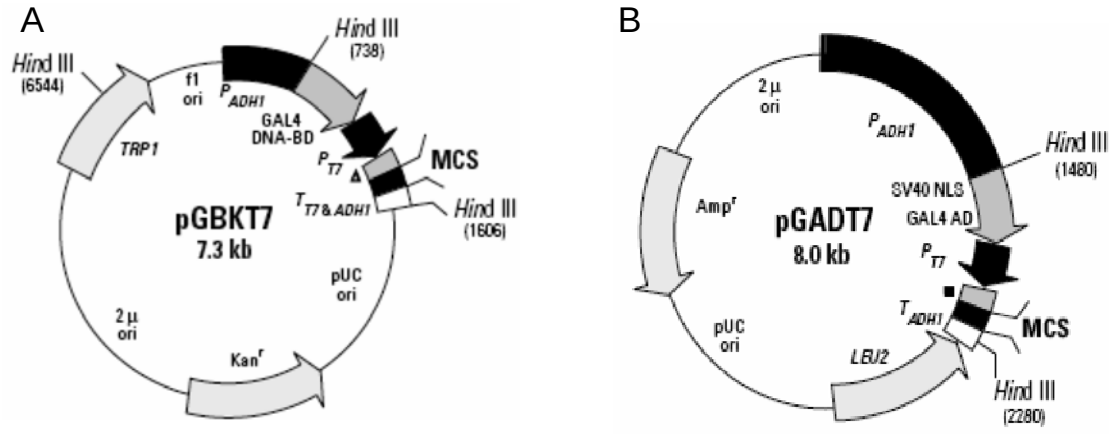

Figure 2.3: The yeast expression vectors pGBKT7 (A) and pGADT7 (B) [61, 62]. pGBKT7 can express a fragment of interest fused to amino acids 1-147 of the GAL4 binding domain (GAL4 DNA-BD). pGADT7 can express a fragment of interest fused to amino acids 768-881 of the GAL4 activating domain (GAL4 AD).

pGBKT7 contains a kanamycin resistance gene which enables selection in *E. coli*, and a *TRP1* nutritional marker for selection in yeast. pGADT7 contains an ampicillin resistance gene enabling selection in *E .coli*, and a *LEU2* nutritional marker for selection in yeast. Both vectors are equipped with a MCS containing several unique cutting sites for different restriction enzymes [61, 62].

Restriction endonucleases. Restriction endonucleases are enzymes that have been purified from many different bacteria species, and they cleave double stranded DNA at specific nucleotide sequences. An enzyme will always cut a given DNA molecule at the same site, and for the most, different enzymes each have their unique cutting site [26]. A few nucleotides, not complementary to the target DNA should be added to the 5' end of the primers, to improve the efficiency of cleavage of restriction enzymes [58]. Bacteria use their restriction endonucleases as protection from viral and other foreign DNA, but are themselves protected by methylation in the genome at an A or a C residue. DNA fragments with cohesive ends are the easiest to clone. These protruding ends can very easily be created by digesting both vector and fragment with a restriction enzyme that cut asymmetrically within the recognition site. By using two restriction enzymes with different recognition sites, the recombinant yield will increase and this is known as forced ligation or directional cloning [25, 57]. When selecting which restriction enzymes to use, possible enzymes are checked for whether they cleave within the sequence of the fragments of interest or not. This can be carried out by the use of different programs available on the internet. When protruding ends are complimentary, the fragment is easily ligated with the plasmid.

Under non-standard conditions, some enzymes can alter specificity and cleave sequences that are similar but not identical to their defined cutting site, and this is known as star activity. Some examples of non-standard conditions are too high glycerol concentration (> 5 % v/v) or use of non-optimal buffer. High fidelity (HF) enzymes bind tighter to the DNA. Sodium dodecyl sulfate (SDS) must therefore be added to disrupt binding before gel electrophoresis is conducted [63]. Digested fragments and vectors must be purified either by gel electrophoresis, or by the use of commercial kits [64]. Both purifying methods were used in this project.

Gel electrophoresis. The purpose of gel electrophoresis is to separate, identify and purify DNA fragments of different lengths. The gel, a matrix usually made from agarose or polyacrylamide, contains a microscopic network of pores. Polyacrylamide gels are more effective for separating small fragments of DNA, and the resolving power is very high. Agarose is a linear polymer composed of alternating residues of D- and L-galactose. In chains, they form helical fibers that aggregate into supercoiled structures. Agarose gels have a lower resolving power, but greater range of

separation. The gel is placed in a buffer-filled tank. Different buffers are available, for example Tris-acetate and EDTA (ethylenediaminetetraacetic acid) (TAE), and Tris-borate and EDTA (TBE). Both works well but with slightly different characteristics, and the choice of buffer is largely a matter of personal preference [57].

Before samples are loaded in the wells, they are mixed with gel-loading buffers. These buffers increase the density of the sample so the DNA sinks evenly into the well, they color the sample so they can easily be loaded and will migrate with the sample through the gel. The samples are then loaded in wells on the negatively charged end, and voltage is applied producing an electric field. DNA is negatively charged and will migrate towards the positively charged end of the gel. A size standard (ladders of different sizes are commercially available) is also loaded to easily identify the fragment of interest. Larger fragments move at slower speed than smaller fragments, because they are more impeded by the matrix. Other factors will also determine the rate of migration, for example the concentration of agarose, the conformation of the DNA, and the applied voltage. After some time, the fragments are spread out across the gel forming a ladder, where each band contains DNA of equal length. The bands are invisible, so the gel is stained, for example with dye that fluoresces when exposed to ultraviolet light [26, 57].

2.2.3 Transformation

Eukaryotes and prokaryotes pass on genes to the next generation by vertical gene transfer. Many prokaryotes can also acquire genes from other microbes – horizontal gene transfer [65]. Transformation is one type of horizontal transfer, and is a process where cells take up DNA molecules from the surroundings and express the genes on that DNA. These cells are said to be competent, which means there has been an alteration in the cell wall and cytoplasmic membrane that allow DNA to enter the cell. Bacteria can also be made competent artificially, and this was the first method developed for transferring genetic elements. It has become an important tool in recombinant DNA technology, since competent cells take up DNA from any donor genome. Other methods of horizontal gene transfer are conjugation (DNA is transferred from one cell to another via cell-cell contact) and transduction (transfer of DNA by viral infection) [65, 66].

Subcloning. PCR products of the fragments of interest may not be directly cloned into the appropriate vector. The pGEM®-T Easy Vector System (Promega), is a system that makes the cloning of PCR products more convenient. The vector is cut with the restriction enzyme EcoRV, which adds single 3'-terminal thymidine (T) overhangs at both ends of the insertion site. By A-tailing the blunt-ended fragments to be inserted, the efficiency of ligation will greatly improve [67]. The MCS of pGEM-T Easy lies within the coding region of β-galactosidase (*lacZ*). Expression of *lacZ* is induced by the synthetic compound isopropyl- β-$_D$-galactosidase (IPTG). 5-bromo-4-chloro-3-indolyl-β-$_D$-galactoside (Xgal) is a histochemical indicator. It is cleaved by β-galactosidase to yield the deep blue dye 5-bromo-4-chloro-indigo, and this can be used for identification by color screening on indicator plates. When an insert is cloned into the vector, the coding sequence of β-galactosidase is interrupted. This will, in most cases, produce white colonies. If the vector is without insert, β-galactosidase will be expressed, Xgal will be cleaved, and blue colonies are formed [66].

The vector also contains an ampicillin resistance gene, and multiple restriction sites within the MCS. To release the insert from the vector, double digestion can be used or single digestion with EcoRI, BstZI or NotI. The MCS is flanked by these three recognition sites, and also by the RNA polymerases T7 and SP6. Inserts can be sequenced using the SP6 and T7 Promoter Primers (both Promega) (described in appendix 14) [67]. pGEM®-T Easy Vector map is shown in appendix 1.

2.3 Detection of PPI

2.3.1 Transformation in *S. cerevisiae* AH 109 cells

The *S. cerevisiae* strain AH109 (Clontech) is designed for detecting protein interactions during a two-hybrid screen. Strain AH109 carries mutations in four genes, and is not able to synthesize adenine (ade), histidine (his), leucine (leu), and tryptophan (trp). AH109 must therefore be grown on medium containing these auxotrophic markers. The strain is *gal4*$^-$, and contains the four distinct reporter constructs *ADE2*, *HIS3*, *lacZ*, and *MEL1*, that are only expressed if PPI occur in the GAL4-based hybrid system. *ADE2* encodes an enzyme in the biosynthesis of adenine,

and expression of the gene will give growth on medium lacking adenine. *HIS3* encodes an enzyme in the biosynthesis of histidine, and expression of the gene will give growth on medium lacking histidine. *LacZ* and *MEL1* encodes β-galactosidase and α- galactosidase respectively, and expression of the genes can both be monitored with a color screen [64].

2.3.2 The *S. cerevisiae* two-hybrid test

The *S. cerevisiae* two-hybrid test is an assay that can be used to score PPIs. The test is carried out by transforming AH109 cells with the plasmid constructs of interest. Aliquots of the cells containing the plasmid constructs to be investigated, are spread onto selective plates, and then incubated at appropriate conditions. PPI is monitored by growth when media lacking adenine or media lacking histidine is used.

3 Methods

3.1 Amplification of fragments by PCR

PCR was used to amplify cDNA of *CKS2, CDK1* and *CDK2*. Before PCR could be carried out input templates had to be constructed, and primers had to be designed and ordered.

3.1.1 Preparation of templates

In order to have the correct template for CKS2, cDNA was synthesized from RNA by using the Illumina® TotalPrep RNA Amplification Kit (Ambion). The control RNA, consisting of 1 mg/ml HeLa cell total RNA, from the kit was used as input. All procedures were carried out according to the producer's protocol (appendix 2).

For CDK1 and CDK2, the gene sequences were already present in vectors and these plasmids could be used directly as templates. CDK1 was fused in the vector pCMV-HA, a gift from R. Syljuåsen (Department of Radiation Biology, the Norwegian Radium Hospital), and CDK2 was present in the vector pSM, a gift from Professor D. Morgan (Department of Physiology, University of California, San Francisco, USA). The templates used for CDK1 and CDK2 contained two substitutions: amino acid 14 was changed from threonine to alanine, and amino acid 15 was changed from tyrosine to phenylalanine. The templates will from now on be referred to as CDK1AF and CDK2AF. For CDK2, a templat containing mutation in the gene sequence only in amino acid 15 (changing tyrosine to phenylalanine) was also tested (gift from R. Syljuåsen). This template, also fused in a pCMV-HA vector, will from now on be referred to as CDK2F. The vectors are described in appendix 14.

3.1.2 Primer and layout for PCR

Six primers were designed; one forward (F) and one reverse (Rev) for each of *CKS2, CDK1* and *CDK2*. Primers were purchased from Eurogentec S.A. (Belgium). Forward and reverse primers contain an NdeI (upstream) and BamHI (downstream) restriction digestion site respectively, to facilitate their subsequent cloning into pGBKT7 and pGADT7. The primers that were designed are listed in table 3.1. The length of the

primers does not go beyond the mutations in the gene-sequences, so the same primers were used for both CDK2AF and CDK2F.

Table 3.1: Primers designed for use in PCR amplification of cDNA for *CKS2, CDK1AF, CDK2AF* and *CDK2F*.

Gene	Primer sequence
CKS2 F	5'-GGAATT**CATATG**GCCCACAAGCAGATCTACTAC-3'
CKS2 Rev	5'-GGAATT**GGATCC**TCATTTTTGTTGATCTTTTGGAAGAGG-3'
CDK1 F	5'-GGAATT**CATATG**GAAGATTATACCAAAATAGAGAAAATTGG-3'
CDK1 Rev	5'-GGAATT**GGATCC**CTACATCTTCTTAATCTGATTG-3'
CDK2 F	5'-GGAATT**CATATG**GAGAACTTCCAAAAGGTGG-3'
CDK2 Rev	5'-GGAATT**GGATCC**TCAGAGTCGAAGATGG-3'

The underlined nucleotides represent start and end of the sequence encoding the gene, and bold nucleotides represent the cutting sites recognized by NdeI and BamHI in forward and reverse primers, respectively.

The nucleotides in bold, represent the cutting sites of NdeI and BamHI. Underlined nucleotides mark the start and end of the coding sequence for the genes. The forward primer will attach to the template strand at the 5' end, and the reverse primer will attach to the complimentary strand at the 5' end. In this position, the primer's 3' ends will point to each other. The forward primer, corresponding to the sequences upstream, and the reverse primer, corresponding to the sequences downstream from the region to be amplified, flanks the target DNA [68].

T_m and GC-content were calculated by using the program OligoCalc (version 3.26) [69]. The gene-sequences were obtained from internet resources of the National Center for Biotechnology Information (NCBI) [70]. Primer lengths were between 16 and 29 nucleotides (complimentary to the coding sequence), and all 3'ends were a G or a C. The GC content varied from 29-50 % for the six primers. Variation in T_m between forward and reverse primers did not exceed 1 °C. T_m for the CKS2 primers was a few degrees higher than T_ms for the CDK1 and CDK2 primers. Because of equal value of T_m for CDK1- and CDK2 primers, PCR could be executed for both fragments with the same program. PCR for the CKS2 fragment was executed under different conditions. Reaction mixtures and cycling conditions are described in

appendix 3. The *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene) was used. This polymerase minimizes potential misincorporations, and generates blunt-ended products [71]. By adding restriction sites to the 5' terminal of the primers, cohesive ends can be generated later by cutting the PCR-products with restriction enzymes [58]. To facilitate restriction enzyme cleavage, all primers were extended at the 5' end with the same randomly chosen sequence GGAATT.

3.2 Preparing plasmids

DH5α/pGADT7 and DH5α/pGBKT7 (provided by Ingvild Flåtten) were cultivated over night in liquid Luria-Broth (LB) medium (appendix 4), and with appropriate antibiotics as described in appendix 5. In order to retrieve the purified plasmids, overnight cultures were prepared the next day by using the kit JETSTAR (Genomed), as described in the producer's protocol (appendix 6). The concentrations were measured by the use of Nanodrop, and the purified plasmids were cut with two restriction enzymes simultaneously (double digestion).

3.2.1 Restriction endonucleases

NdeI and BamHI (New England Biolabs) were selected as restriction endonucleases because both plasmids contain their restriction sites [61, 62]. To be sure that the restriction enzymes did not cut within the fragments of interest, the program Webcutter 2.0 [72] was used (appendix 7). In the latest experiments, BamHI was changed to BamHI-HF™,, which has the same specificity but reduced star activity.

NdeI was originally isolated from *Neisseria denitrificans* and its recognition site is shown in figure 3.1.

Figure 3.1: Recognition site NdeI [63].

BamHI was originally isolated from *Bacillus amyloliquefaciens*. The recognition site of both BamHI and BamHI-HF™ is shown in figure 3.2.

Figure 3.2: Recognition site BamHI and BamHI-HF™ [63].

Two restrictions enzymes can have different activity in the same buffer. The New England Biolabs internet resource [63], provides a tool (Double Digest Finder) that can be used to find the optimal reaction conditions for digestion. The tool will state the recommended buffer, and if bovine serum albumin (BSA) is required or not. Since NdeI only has 75 % activity compared to BamHI in the recommended buffer, NdeI needs more incubation time. NdeI and BamHI-HF on the other hand, have the same activity in the recommended buffer and can be added at the same time. Reaction conditions for double digestion with BamHI and NdeI, and BamHI-HF and NdeI are described in appendix 8.

3.3 Analysis of products

Gel electrophoresis was used to separate DNA fragments amplified by PCR and after digestion of plasmids, and to check for inserts after transformation in *E. coli* (3.5). In the first experiments, 0.5 x TBE buffer (appendix 4) was used and the gel was stained with ethidium bromide (Merck). This was later changed to 1 x TAE buffer (appendix 4), and staining with GelRed (Biotium). After electrophoresis, the gel was immersed in the stain, examined by UV-light, and photographed by using ChemiGenius Bio Imaging System (VWR). GeneSnap (Syngene) was used as image-editing program. Gel electrophoresis and staining was carried out as described in appendix 9.

In order to retrieve the PCR-products and digested plasmids, the bands of interest were cut out from the gel by using a scalpel. The gel pieces were then rinsed by using the kit Wizard® SV Gel and PCR Clean-Up System (Promega) as described in the producer's protocol (appendix 10). The kit consists of reaction mixtures and disposable chromatography columns that absorb the DNA, and can after washing be eluted out.

Digested fragments were not rinsed by gel electrophoresis, but purified by using the kit QIAquick PCR Purification (QIAgen) as described in the producer's protocol (appendix 11).

3.4 Transformation

CKS2 and CDK1AF could not be cloned directly into pGADT7 and pGBKT7, and was therefore subcloned as described in 3.4.1. The cloning of CDK2AF and CDK2F into pGADT7 and pGBKT7 could be done without subcloning.

XL 10-Gold® Ultracompetent Cells (Stratagene) were used for transformation. These cells are tetracycline and chloramphenicol resistant [71]. Ligation mixtures were set up and the competent cells were transformed with the ligation mixtures. The procedures are described in appendix 12. The transformation mixtures were plated on solid LB plates containing the appropriate antibiotics (appendix 4), and incubated at 37 °C overnight. Representative transformants were cultured over night in liquid LB medium with appropriate antibiotics. The next day, purified plasmids were prepared by using the kit Wizard Plus SV Minipreps DNA Purification System (Promega) as described in the producer's protocol (appendix 13). To verify that cloning of fragments in respective plasmids was successful, purified plasmids were digested, and gel electrophoresis was performed (appendix 9). Gel profiles were analyzed, and appropriate clones were subjected to DNA sequence analysis at GATC Biotech (Germany). The clones subjected to DNA sequence analysis were cultured again overnight in liquid LB medium with appropriate antibiotics. Aliquots (1 ml) of each overnight bacterial culture were mixed with 300 µl of 87 % glycerol (Merck) and stored in Cryo tubes at -80 °C.

3.4.1 pGEM®-T Easy Vector System

The pGEM®-T Easy Vector System (Promega) was used for subcloning of the fragments CKS2 and CDK1AF. Before transformation, the blunt-ended fragments from PCR amplification had to be modified. By A-tailing the fragments, only one insert will be ligated into the vector as opposed to multiple insertions that can occur

with blunt-ended cloning [67]. Ligation mixtures were set up and transformed into JM109 High Efficiency Competent Cells (Promega). Indicator LB plates were prepared with ampicillin, IPTG and X-gal. The transformation mixtures were then plated and incubated at 37 °C overnight. All procedures were carried out by following the protocol in the technical manual provided by Promega. The protocol, reaction mixtures, and preparation of solutions and plates are described in appendix 1. Representative transformants were cultured overnight in liquid LB medium with ampicillin to a final concentration of 100 µg/ml. The overnight cultures were then prepared by using the kit Wizard Plus SV Minipreps DNA Purification System (Promega) as described in the producer's protocol (appendix 13). To verify that cloning of fragments in respective plasmids was successful, purified plasmids were digested with EcoRI (New England Biolabs), and gel electrophoresis was performed. Gel profiles were analyzed, and appropriate clones were subjected to DNA sequence analysis at GATC Biotech. Aliquots (1 ml) of each overnight bacterial culture were mixed with 300 µl of 87 % glycerol and stored at -80 °C. After verification of inserted fragment and orientation of it, the fragments were cut from the pGEM-T Easy vectors by double digestion with NdeI and BamHI. The MCS of pGEM-T Easy vector contains, among others, the recognition site of NdeI. Because the recognition sites of NdeI and BamHI were added to the primers, a few possible fragments can result from the digestion. Still, only the fragment of interest will match the digested plasmids pGBKT7 and pGADT7 when ligation mixtures are set up.

3.4.2 Sequencing

The appropriate clones from transformations of pGBKT7 and pGADT7 with inserts, and subcloning of fragments into pGEM-T Easy vectors, were sent to GATC Biotech in Germany to be sequenced. The sequences from GATC Biotech were then compared to the original sequences by the use of Basic Local Alignment Search Tool (BLAST) on NCBI's internet resources [70]. Appropriate primers were included in the shipment to GATC when necessary. These primers are described in appendix 14.

3.5 The *S. cerevisiae* two-hybrid analysis

A small amount of *S. cerevisiae* AH 109 (Clontech) from the -80 °C glycerol stock was resuspended in 1 ml sterile 0.8 % saline. 100 µl of 10^{-1} and 10^{-2} dilutions were plated on solid medium containing a blend of yeast extract, peptone and dextrose (YPD) (appendix 4), and incubated at 30 °C for 3-4 days. After confirmation of the structures of the various plasmid constructs, the two-hybrid analysis was set up to score PPIs between CKS2 and the CDKs. Appropriate combinations of the plasmids isolated from *E. coli* were transformed into the yeast strain by a modified lithium acetate (LiAc) method [64, 73] as described in appendix 15. For each *S. cerevisiae* two-hybrid analysis, eight transformations were conducted. An overview of the transformation combinations is outlined in figure 3.3.

As a positive control, the plasmids pGADT7/hda and pGBKT7/dnaN (provided by Line Johnsen) were used. Previous work by Kurz *et al.* [74] has shown that the *E. coli* proteins hda (DnaA related protein) and dnaN (β-sliding clamp) directly interacts *in vitro*. For negative control, the plasmids pGADT7 and pGBKT7 without inserts were used. As seen in figure 3.3, a transformation of pGADT7 with insert and pBGKT7 without insert, and opposite, was performed. These transformations were used as controls for toxicity and to screen out constructs that could auto activate the *his*-reporter gene. The last two transformations were carried out with the intention to score PPI between CKS2 and the CDKs (CDK1 and CDK2 with two mutations, and CDK2 with the single mutation). To examine the three PPIs of interest, all four fragments were cloned as both bait and prey so the interactions could be tested in both directions. The same outline was performed for all three PPIs.

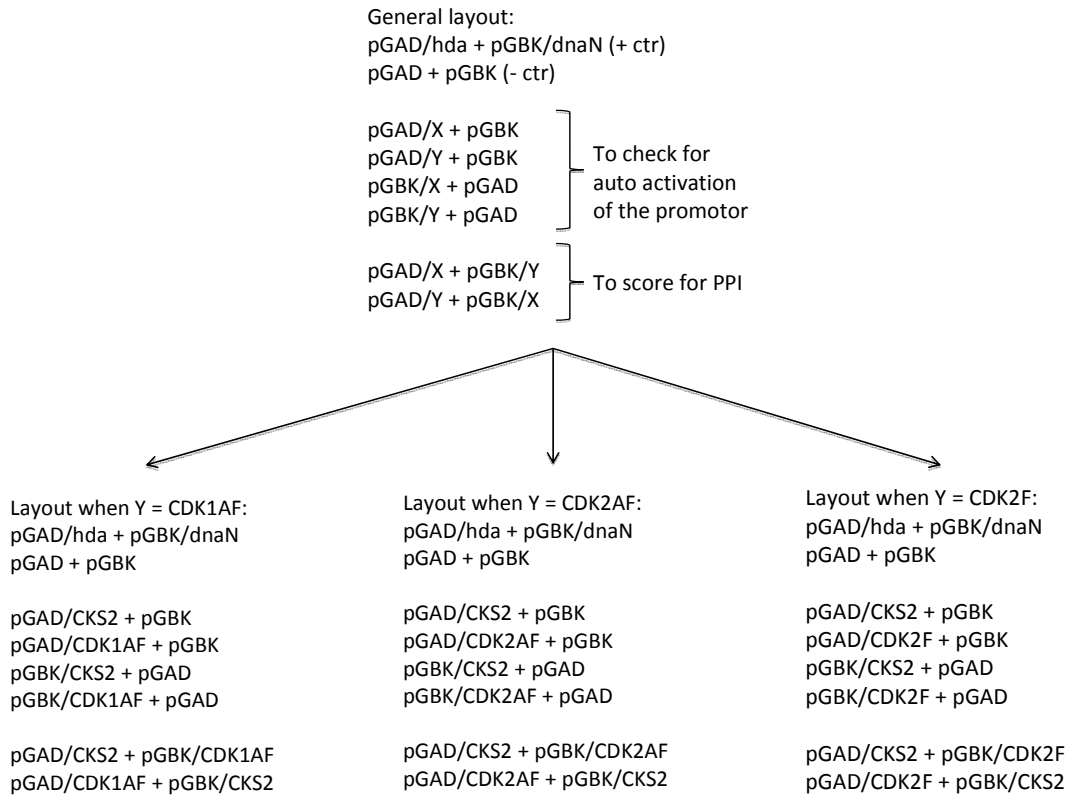

Figure 3.3: An overview of the combinations of the various plasmid constructs transformed into *S. cerevisiae* AH 109 (Clontech). The upper layout shows the transformation combinations in general. As a positive (+ ctr) and negative (- ctr) control, pGAD/hda + pGBK/dnaN, and pGAD + pGBK respectively, were used. The same plasmids for + ctr and - ctr were used for all three *S. cerevisiae* two-hybrid analysis. Protein X = CKS2, and protein Y = CDKAF, CDK2AF or CDK2F. All four transformations with the combination plasmid/insert + plasmid (empty) were controls for toxicity and to screen out constructs that could auto activate the *his*-reporter gene. The last two transformations were used to score for PPI. To examine all the PPIs of interest, all four fragments were cloned as both bait and prey.

Transformation mixtures were plated on solid synthetic dropout medium. The medium contained Minimal SD Base (SD) with added dropout supplement, DO Supplement (SD-DO) (both Clontech) (appendix 4). Plates were incubated at 30 °C until transformants appeared (3-5 days). Appropriate transformants were grown in liquid SD-DO medium lacking leucine and tryptophan (SD-DO –leu/-trp), supplemented with adenine and histidine (both Sigma-Aldrich) to final concentrations of 20 µg/ml, at 30 °C overnight. To test for interactions, overnight cell cultures were collected by centrifugation, washed twice with sterile 0.8 % saline, and adjusted to an optical density at 600 nm (OD_{600}) of ~ 1.0. This was diluted 10, 100 and 1000 times for each sample. The *S. cerevisiae* two-hybrid analysis (appendix 16) was set up by

spotting 10 µl aliquots of each dilution (OD = 1, 10^{-1}, 10^{-2}, 10^{-3}) onto solid SD-DO –leu/-trp (control plate) or lacking leucine, tryptophan, and histidine (SD-DO –leu/-trp/-his) (to score for PPI) (appendix 4). Plates were incubated at 30 °C for 3 days. AH109 cells transformed with both pGBKT7 (provides the cells with trp) and pGADT7 (provides the cells with leu) will grow on SD-DO –leu/-trp medium. Cells where PPI occur will activate the *his*-reporter gene, and grow on SD-DO –leu/-trp/-his medium. A general layout of the two-hybrid analysis is outlined in figure 3.4. For each of the three PPIs tested, the two-hybrid analyses were performed both ways, and two parallels were tested. The two-hybrid analyses were carried out at least twice for each PPI.

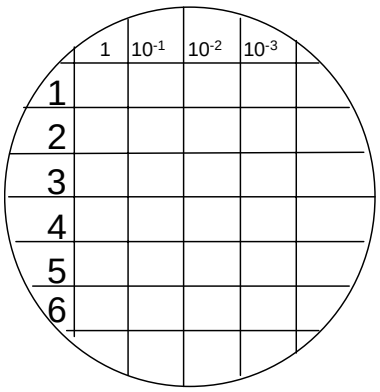

Figure 3.4: An overview of the *S. cerevisiae* two-hybrid analysis. The analysis was set up by spotting 10 µl aliquots of each dilution (OD = 1, 10^{-1}, 10^{-2}, 10^{-3}) of each sample onto solid SD-DO medium lacking leucine and tryptophan (SD-DO –leu/-trp) (control plate) or lacking leucine, tryptophan, and histidine (SD-DO –leu/-trp/-his) (to score for PPI). **1**: positive control, **2**: negative control, **3** and **4**: test for auto activation, **5** and **6**: screen for PPI (two parallels were screened from the same plate of transformants).

4 Results

4.1 PCR and cloning of the *CKS2* gene

cDNA of *CKS2* was amplified by PCR (see Methods section 3.1) using the primers CKS2 F and CKS2 Rev (table 3.1). CKS2 DNA fragments were purified by gel electrophoresis (figure 4.1). The fragments could be seen at around 300 bp on the gel, which is consistent with the size of CKS2 (240 bp, [70]). All the bands (marked in red) were cut out from the gel and purified as described in chapter 3.3. The PCR-products of CKS2 were then digested with NdeI and Bam HI, purified, and included in ligation mixtures with pGBKT7 and pGADT7, also digested with NdeI and BamHI (3.4).

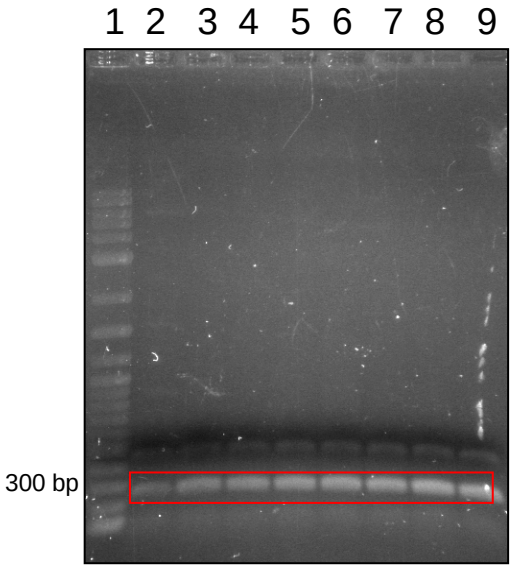

Figure 4.1: Gel electrophoresis of PCR-products of CKS2.
1: 2 log ladder, **2-9**: PCR-products of CKS2.

Clones containing CKS2 could not be found after transformation of the ligation mixtures into XL 10-Gold® cells. Since CKS2 could not be cloned directly into pGBKT7 and pGADT7, subcloning of CKS2 into pGEM-T Easy Vector was performed (see chapter 3.4 for details on subcloning). Different clones were checked for inserts after subcloning by gel electrophoresis (3.3), and the result is shown in figure 4.2 **A**. Two and two lanes contained a sample from the same clone. The leftmost lane of the two contained plasmid digested with EcoRI, and was expected to yield two bands. The rightmost lane of the two contained the same plasmid but uncut.

The bands yielded by the two samples were compared, and so the uncut plasmid served as a control to determine whether the other plasmid had been cut or not. Bands for the CKS2 fragment should have appeared around 300 bp, but no clear bands could be seen, most likely because the concentration of CKS2 fragments were too low to be detected. All the bands for uncut plasmids had about the right size, which is 3015 bp for the pGEM-T Easy vector [67]. When compared to the respective, digested clone in the adjacent lane, neither of the clones seemed to be completely cut. The digested plasmids yielded several bands, most likely due to nicked circle, super coiled, undigested and some digested forms. Either of the clones could therefore contain the CKS2 fragment, and clone #4 was arbitrarily selected and sent to GATC Biotech to be sequenced. Comparison using BLAST computer software (appendix 17) of the original sequence and the sequence obtained from GATC Biotech showed that the plasmid in clone #4 contained the *CKS2* gene in the right orientation.

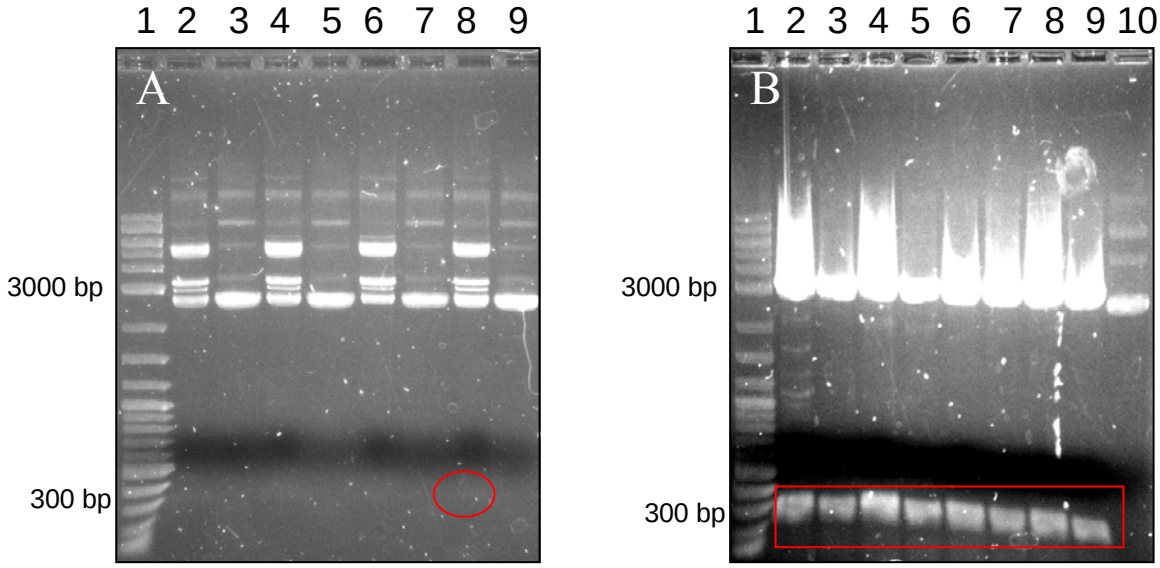

Figure 4.2: Analysis of subcloning of CKS2 by gel electrophoresis.
A: Four clones were tested for CKS2 inserts in pGEM-T Easy vector. **1**: 2 log ladder, **2**: Clone#1 cut, **3**: Clone#1 uncut, **4**: Clone#2 cut, **5**: Clone#2 uncut, **6**: Clone#3 cut, **7**: Clone#3 uncut, **8**: Clone#4 cut (selected for sequencing), **9**: Clone#4 uncut.
B: After verification of Clone#4, CKS2 was cut out from pGEM-T Easy and analyzed by gel electrophoresis. **1**: 2 log ladder, **2-9**: digested pGEM-T Easy/CKS2, **10**: pGEM-T Easy (empty).

Figure 4.2 **B** shows gel electrophoresis of CKS2 cut from the pGEM-T Easy vector. Large amounts of clone #4 were digested with NdeI and BamHI, and the bands could therefore clearly be seen on the gel. Bands appeared two places on the gel, consistent

with the sizes of CKS2 and pGEM-T Easy vector. The bands for CKS2 (marked in red) were cut out from the gel and used further. pGEM-T Easy vector without insert was applied in lane 10 to compare the size of this band to bands yielded by the cut plasmids with fragments.

pGBKT7 and pGADT7 were digested with NdeI and Bam HI, and purified by gel electrophoresis. The results from gel electrophoresis are shown for pGBKT7 and pGADT7 in figure 4.3 **A** and **B** respectively. pGBKT7 have a size of 7300 bp and pGADT7 have a size of 8000 bp [61, 62]. The bands that could be seen on the gel for both plasmids indicate that they have been cut. For each analysis, to give an indication of the size of the plasmid, an uncut sample of the plasmid was applied in lane 1. For pGBKT7, the uncut sample showed very weak bands. This was most likely due to too low amount of plasmid DNA, and could not be detected. The bands of interest (marked in red for both plasmids) were cut out and purified as described in section 3.3. Ligation mixtures with digested and purified CKS2 fragment, pGBKT7 and pGADT7, were set up as described in 3.4.

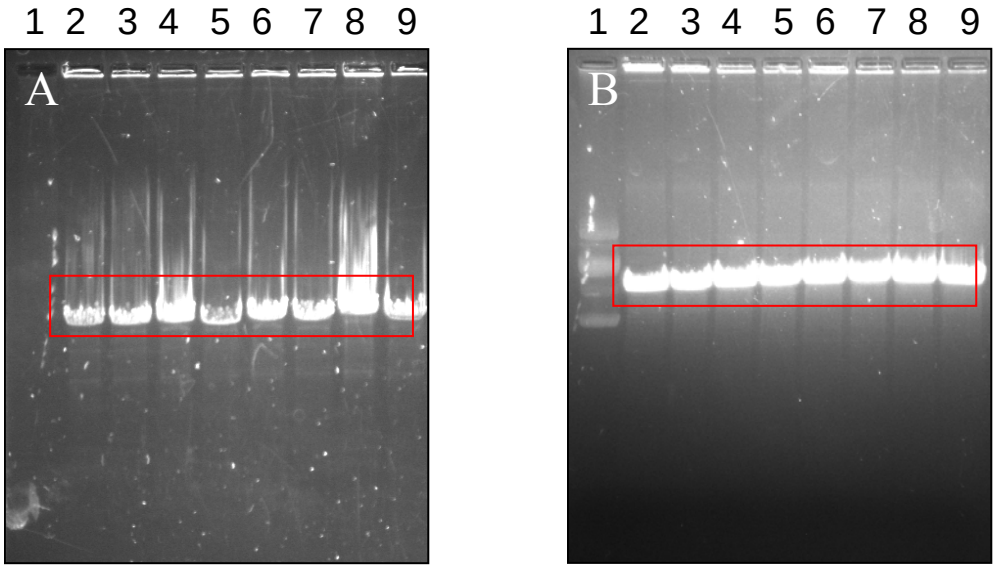

Figure 4.3: Digested pGBKT7 and pGADT7 analysed by gel electrophoresis.
A: pGBKT7 was double digested with restriction enzymes. **1**: Uncut pGBKT7. **2-9**: Cut pGBKT7.
B: pGADT7 was double digested with restriction enzymes. **1**: Uncut pGADT7. **2-9**: Cut pGADT7.

Different clones were tested for CKS2 inserts into pGBKT7 and pGADT7 by digestion with NdeI and BamHI (3.2.1), and analysis by gel electrophoresis (3.3). The results of gel electrophoresis is shown in figure 4.4 **A** and **B**. Figure 4.4 **A** shows that all clones seemed to contain the right fragment, and from figure 4.4 **B** one can see that only clone#3 and clone#6 of the tested clones seemed to contain a fragment of the right size. Even though the CKS2 fragments were sequenced and verified when inserted in the pGEM-T Easy vector, appropriate clones were also sequenced for inserts in pGBKT7 and pGADT7 as an additional control. Clone #1 (figure 4.4.**A**) and clone #3 (figure 4.4 **B**) were selected for verification of CKS2 insert in pGBKT7 and pGADT7, respectively (both marked in red). For the selected clones, the bands of the respective plasmids were consistent with the known sizes. Comparison of sequences by using BLAST (appendix 17), showed that the clones tested contained the right fragment. The plasmids with fragment (constructs) are from here on referred to as pGBK/CKS2 and pGAD/CKS2 and were used in the further analyses to test the interactions with CDK1AF, CDK2AF and CDK2F.

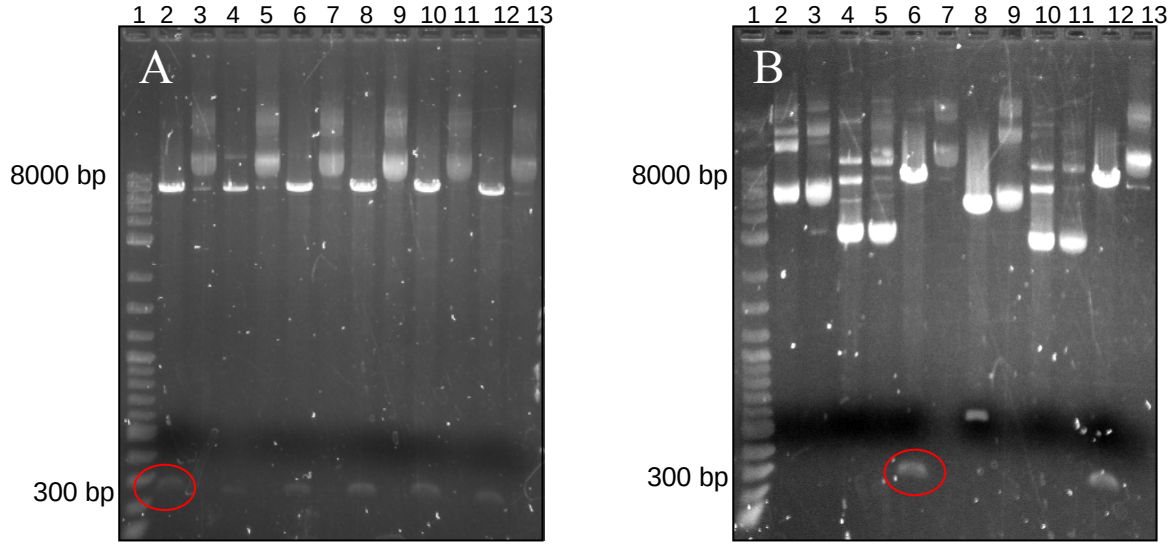

Figure 4.4: Analysis of cloning of CKS2 by gel electrophoresis.
A: Six clones were tested for CKS2 inserts in pGBKT7. **1**: 2 log ladder, **2**: Clone#1 cut (selected for sequencing), **3**: Clone#1 uncut, **4**: Clone#2 cut, **5**: Clone#2 uncut, **6**: Clone#3 cut, **7**: Clone#3 uncut, **8**: Clone#4 cut, **9**: Clone#4 uncut, **10**: Clone#5 cut, **11**: Clone#5 uncut, **12**: Clone#6 cut, **13**: Clone#6 uncut.
B: Six clones were tested for CKS2 inserts in pGADT7. **1**: 2 log ladder, **2**: Clone#1 cut, **3**: Clone#1 uncut, **4**: Clone#2 cut, **5**: Clone#2 uncut, **6**: Clone#3 cut (selected for sequencing), **7**: Clone#3 uncut, **8**: Clone#4 cut, **9**: Clone#4 uncut, **10**: Clone#5 cut, **11**: Clone#5 uncut, **12**: Clone#6 cut, **13**: Clone#6 uncut.
The uncut clones serve as controls for the cut clones.

4.2 PPIs of CKS2 and CDK1AF

4.2.1 PCR and cloning of the *CDK1AF* gene

The primers CDK1 F and CDK1 Rev (table 3.1) were used to amplify cDNA of *CDK1AF* by PCR (3.1). Fragments were purified by gel electrophoresis, and the result is shown in figure 4.5. The fragments could be seen at around 900 bp on the gel, which is consistent with the size of CDK1 (894 bp, [70]). All the bands (marked in red) were cut out. Directly cloning of CDK1AF into pGBKT7 and pGADT7 was attempted but did not succeed. Therefore, subcloning into pGEM-T Easy Vector was also performed for CDK1AF, as for CKS2 (see Methods section 3.4).

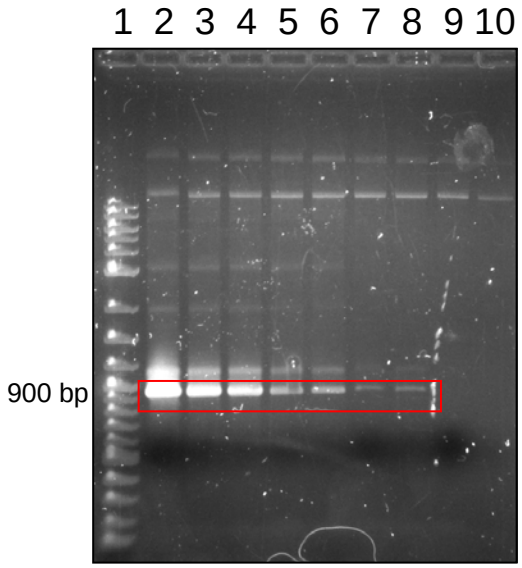

Figure 4.5: Gel electrophoresis of PCR-products of CDK1AF.
1: 2 log ladder, **2-10**: PCR-products of CDK1AF.

Gel electrophoresis for different clones tested after subcloning of CDK1AF in pGEM-T Easy, is shown in figure 4.6 **A**. Two adjacent lanes contained sample from the same clone. Lanes 2, 4, 6 and 8 contained plasmid cut with the restriction enzyme EcoRI. Lanes 3, 5, 7, and 9 contained the respective uncut plasmid so the sizes of cut and uncut plasmid could be compared. Two bands could barely be seen in the gel around 900 bp for clone#8. This was consistent with the size of CDK1. Clone#8 was sent to GATC Biotech to be sequenced. As for the other sequence results, the tool BLAST (appendix 17) was used for comparison of the sequences. The result showed that the plasmid in clone #8 contained CDK1AF in the right orientation.

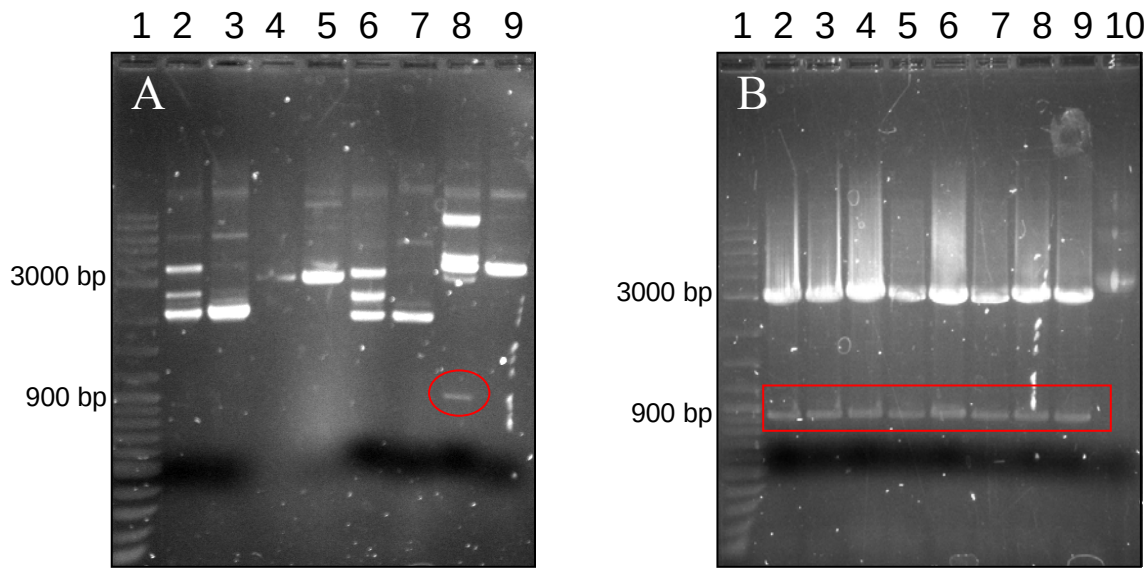

Figure 4.6: Analysis of subcloning of CDK1AF by gel electrophoresis.
A: Four clones were tested for CDK1AF inserts in pGEM-T Easy vector. **1**: 2 log ladder, **2**: Clone#5 cut, **3**: Clone#5 uncut, **4**: Clone#6 cut, **5**: Clone#6 uncut, **6**: Clone#7 cut, **7**: Clone#7 uncut, **8**: Clone#8 cut (selected for sequencing), **9**: Clone#8 uncut.
B: After verification of Clone#8, CDK1AF was cut out from pGem-T Easy. **1**: 2 log ladder, **2-9**: digested pGEM-T Easy/CDK1AF, **10**: pGEM-T Easy uncut.

Figure 4.6 **B** shows gel electrophoresis of CDK1AF cut from the pGEM-T Easy vector with NdeI and BamHI. Bands appeared two places on the gel, consistent with the sizes of CDK1 and pGEM-T Easy vector. The bands for CDK1AF (marked in red) were cut out from the gel and purified. A sample of pGEM-T Easy vector without insert was applied in lane 10 for comparison of band sizes.

Ligation mixtures with digested and purified CDK1 fragment, pGBKT7 and pGADT7, were set up and transformed into XL 10-Gold® cells as described in 3.4. Figure 4.7 **A** and **B** show the results of gel electrophoresis of different clones tested for CDK1AF inserts into pGBKT7 and pGADT7, respectively. Figure 4.7 **A** shows that only clone#11 seemed to contain a fragment of the right size in pGBKT7, and was sent to GATC Biotech for sequencing (marked in red). From figure 4.7 **B**, one can see that all but one of the tested clones seemed to contain the right fragment in pGADT7, and clone #7 was selected to be sequenced (marked in red). This sequencing, as for CKS2 in pGBKT7 and pGADT7, served as an additional control. For the selected clones, the bands for the respective plasmids were consistent with the known sizes. Sequences were compared by the use of BLAST (appendix 17), and

showed that the clones tested contained the right fragment. The constructs are from here on referred to as pGBK/CDK1AF and pGAD/CDK1AF.

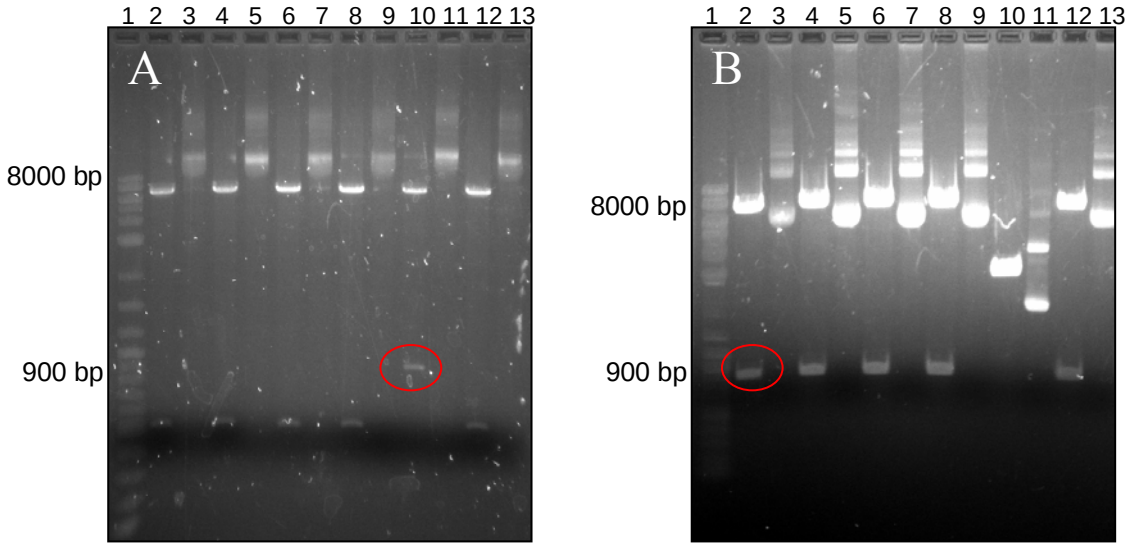

Figure 4.7: Analysis of cloning of CDK1AF by gel electrophoresis.
A: Six clones were tested for CDK1AF inserts in pGBKT7. **1**: 2 log ladder, **2**: Clone#7 cut, **3**: Clone#7 uncut, **4**: Clone#8 cut, **5**: Clone#8 uncut, **6**: Clone#9 cut, **7**: Clone#9 uncut, **8**: Clone#10 cut, **9**: Clone#10 uncut, **10**: Clone#11 cut (selected for sequencing), **11**: Clone#11 uncut, **12**: Clone#12 cut, **13**: Clone#12 uncut.
B: Six clones were tested for CDK1AF inserts in pGADT7. **1**: 2 log ladder, **2**: Clone#7 cut (selected for sequencing), **3**: Clone#7 uncut, **4**: Clone#8 cut, **5**: Clone#8 uncut, **6**: Clone#9 cut, **7**: Clone#9 uncut, **8**: Clone#10 cut, **9**: Clone#10 uncut, **10**: Clone#11 cut, **11**: Clone#11 uncut, **12**: Clone#12 cut, **13**: Clone#12 uncut.
The uncut clones serve as controls for the cut clones.

4.2.2 *S. cerevisiae* two-hybrid analysis for PPIs of CKS2 and CDK1AF

The transformations of plasmid constructs and the *S. cerevisiae* two-hybrid analyses for pGBK/CKS2 and pGAD/CDK1AF, and pGAD/CKS2 and pGBK/CDK1AF were carried out as described in 3.5. The results are shown figure 4.8 and 4.9. Similar results were obtained for all analyses of PPI between CKS2 and CDK1AF.

All samples should grow on the control plate (-leu/-trp) indicating that the cells have taken up both pGBKT7 and pGADT7. The plate to the left (-leu/-trp/-his) scores for PPIs and should only show growth if cells contained plasmids with protein inserts that interacted, thereby were able themselves to produce histidine. Row 1 is the positive control and showed growth on both plates as expected, because the proteins hda and dnaN are known to interact in Y2H. The negative control in row 2 only showed

growth on the control plate. This was also as expected because the plasmids were empty. Rows 3 and 4 tests for auto activation, and therefore growth was only wanted on the control plate. The tests showed no growth on the -leu/-trp/-his plate, indicating that neither of the plasmids contained constructs that were able to auto-activate the promoter. The two rows at the bottom are tests scoring for PPI (in two parallels), and growth on the -leu/-trp/-his plate indicates PPI between the fused proteins. The test showed growth, indicating that CKS2 was interacting with CDK1AF when CKS2 was cloned as the bait protein (figure 4.8). Similar results were obtained when CDK1AF was cloned as the bait protein (figure 4.9).

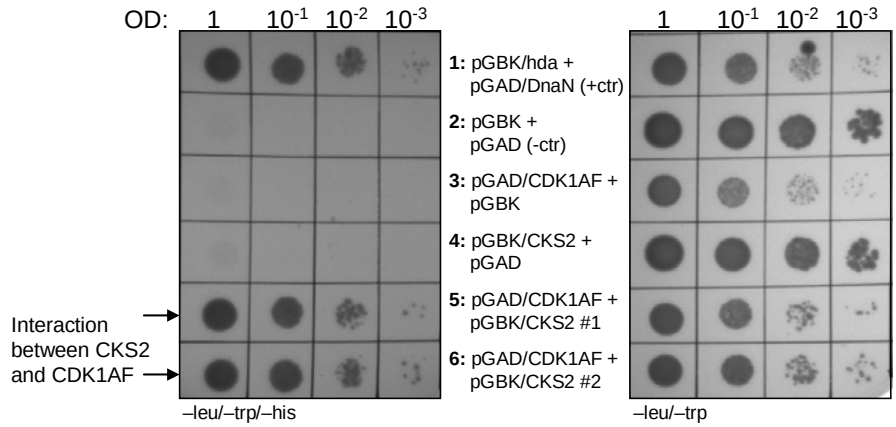

Figure 4.8: *S. cerevisiae* two-hybrid analysis of pGBK/CKS2 and pGAD/CDK1AF. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CDK1AF and pGBK/CKS2, **6**: Score for PPI #2, pGAD/CDK1AF and pGBK/CKS2. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

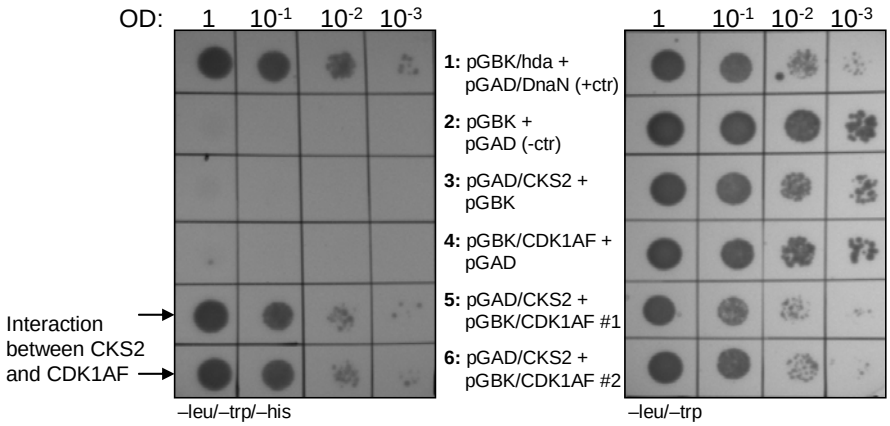

Figure 4.9: *S. cerevisiae* two-hybrid analysis of pGAD/CKS2 and pGBK/CDK1AF. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CKS2 and pGBK/CDK1AF, **6**: Score for PPI #2, pGAD/CKS2 and pGBK/CDK1AF. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

4.3 PPIs of CKS2 and CDK2AF

4.3.1 PCR and cloning of the CDK2AF gene

Amplification of cDNA of *CDK2AF* was given by the primers CDK2 F and CDK2 Rev (table 3.1) and the use of PCR. Fragments were purified by gel electrophoresis, and the result is shown in figure 4.10. The fragments that could be seen on the gel were consistent with the size of CDK2 (897 bp, [70]). The bands of interest (marked in red) were cut out from the gel, purified and digested with NdeI and BamHI-HF, then included in ligation mixtures with cut pGBKT7 and pGADT7. Ligation mixtures were transformed into XL 10-Gold® cells (see sections 3.2-3.4 for details).

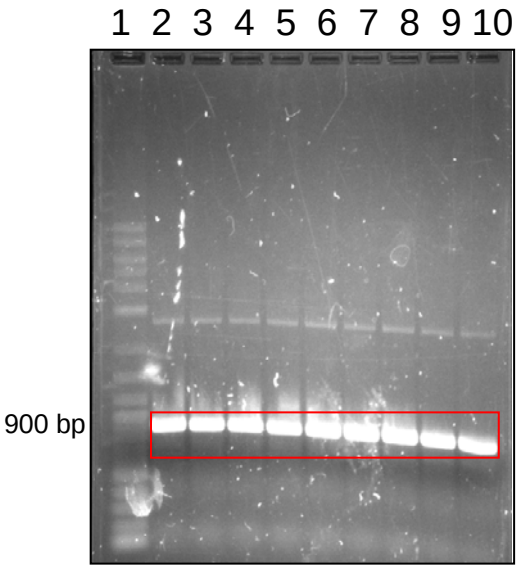

Figure 4.10: Gel electrophoresis of PCR-products of CDK2AF.
1: 2 log ladder, **2-10**: PCR-products of CDK2AF.

Figure 4.11 **A** and **B** show the results of gel electrophoresis of different clones tested for CDK2AF inserts in pGBKT7 and pGADT7 respectively. Figure 4.11 **A** shows that all but clone #1 seemed to contain a fragment of the right size. Clone #4 was selected for sequencing (marked in red). From figure 4.11 **B**, one can see that clones #7, #9, #10 and #12 contained a fragment of the appropriate size. Clone #7 was sequenced (marked in red). For the selected clones, the bands of the respective plasmid were consistent with the known sizes. BLAST (appendix 17) was used to compare the sequences, and showed that the clones tested contained the right fragment. The constructs are from here on referred to as pGBK/CDK2AF and pGAD/CDK2AF.

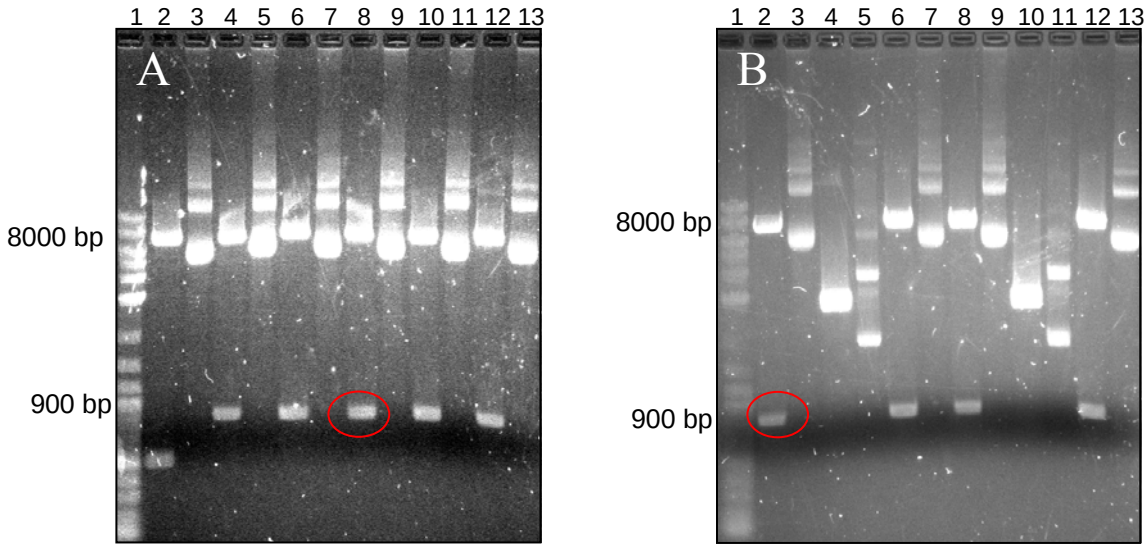

Figure 4.11: Analysis of cloning of CDK2AF by gel electrophoresis.
A: Six clones tested for CDK2AF inserts in pGBKT7. **1**: 2 log ladder, **2**: Clone#1 cut, **3**: Clone#1 uncut, **4**: Clone#2 cut, **5**: Clone#2 uncut, **6**: Clone#3 cut, **7**: Clone#3 uncut, **8**: Clone#4 cut (selected for sequencing), **9**: Clone#4 uncut, **10**: Clone#5 cut, **11**: Clone#5 uncut, **12**: Clone#6 cut, **13**: Clone#6 uncut.
B: Six clones were tested for CDK2AF inserts in pGADT7. **1**: 2 log ladder, **2**: Clone#7 cut (selected for sequencing), **3**: Clone#7 uncut, **4**: Clone#8 cut, **5**: Clone#8 uncut, **6**: Clone#9 cut, **7**: Clone#9 uncut, **8**: Clone#10 cut, **9**: Clone#10 uncut, **10**: Clone#11 cut, **11**: Clone#11 uncut, **12**: Clone#12 cut, **13**: Clone#12 uncut.
The uncut clones serve as controls for the cut clones.

4.3.2 *S. cerevisiae* two-hybrid analysis for PPIs of CKS2 and CDK2AF

Figure 4.12 and 4.13 shows the results of the *S. cerevisiae* two-hybrid analysis for pGBK/CKS2 and pGAD/CDK2AF, and pGAD/CKS2 and pGBK/CDK2AF respectively (see 3.5 for details). The results obtained were similar to the results described for CKS2 interaction with CDK1AF. The control plate showed growth for all samples. The plate scoring for PPIs (-leu/-trp/-his) showed growth for the positive control and for the two parallels testing whether CKS2 interacted with CDK2AF or not. Rows 2, 3 and 4 showed no growth, indicating that neither of the constructs were able to auto-activate the promoter when not interacting with a protein. Both two hybrid analyses show that CKS2 interacts with CDK2AF regardless of whether CKS2 (figure 4.12) or CDK2AF (figure 4.13) is cloned as the bait protein.

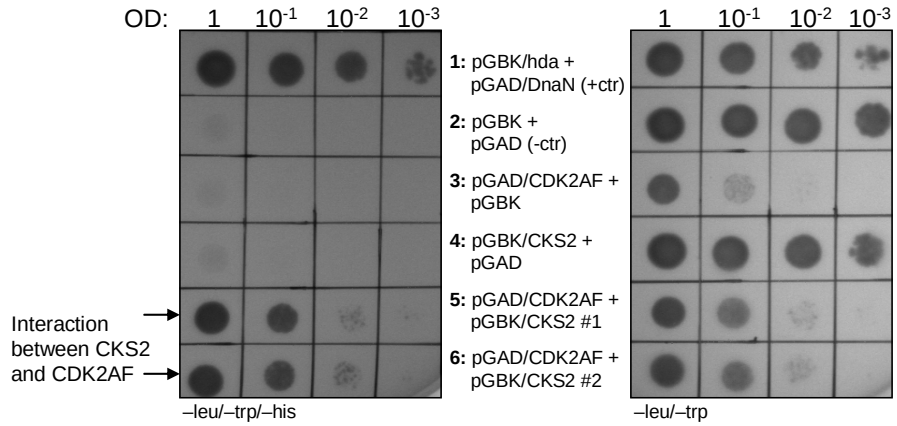

Figure 4.12: *S. cerevisiae* two-hybrid analysis of pGBK/CKS2 and pGAD/CDK2AF. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CDK2AF and pGBK/CKS2, **6**: Score for PPI #2, pGAD/CDK2AF and pGBK/CKS2. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

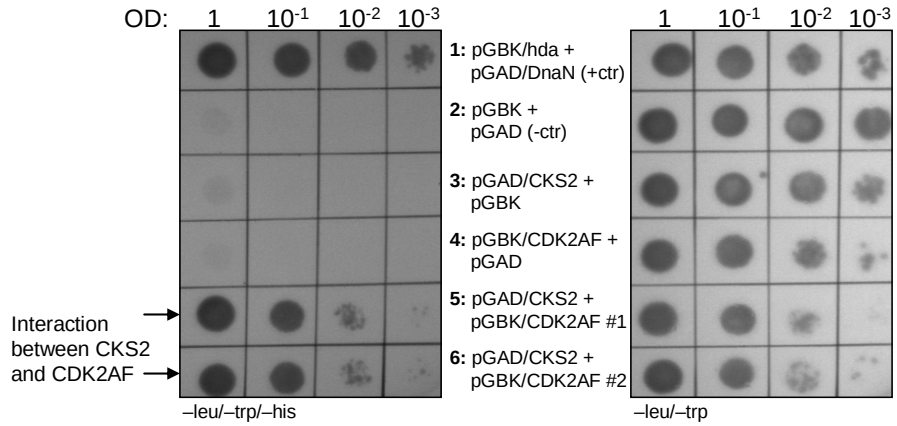

Figure 4.13: *S. cerevisiae* two-hybrid analysis of pGAD/CKS2 and pGBK/CDK2AF. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CKS2 and pGBK/CDK2AF, **6**: Score for PPI #2, pGAD/CKS2 and pGBK/CDK2AF. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

4.4 PPIs of CKS2 and CDK2F

4.4.1 PCR and cloning of the CDK2F gene

cDNA of *CDK2F* was amplified by PCR. The same primers as for CDK2AF were used (CDK2 F and CDK2 Rev, table 3.1). Fragments were purified by gel electrophoresis, and the result is shown in figure 4.14. The fragments that could be seen on the gel were consistent with the size of CDK2 (897bp, [70]). The bands of interest (marked in red), were used further as described for CDK2AF.

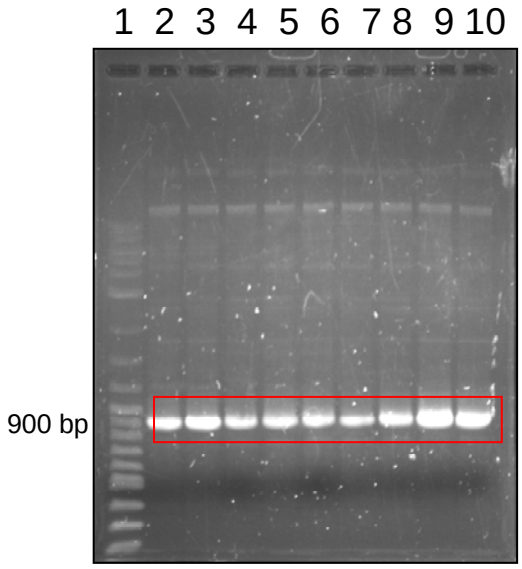

Figure 4.14: PCR-products of CDK2F.
1: 2 log ladder, **2-10**: PCR-products of CDK2F.

Different clones were tested for CDK2F inserts in pGBKT7 and pGADT7, and analyzed by gel electrophoresis. The results are shown in figure 4.15 **A** and **B** respectively. Figure 4.15 **A** shows that clone #7 and #9 seemed to contain the right fragment. Figure 4.15 **B** shows that clone #1, #2 and #5 of the six clones tested, seemed to contain a fragment of the right size. Clone # 7 and clone #1 (both marked in red) were sequenced. Comparison of seqences by the use of BLAST (appendix 17) showed that the clones tested contained the right fragment. The constructs are from here on referred to as pGBK/CDK2F and pGAD/CDK2F.

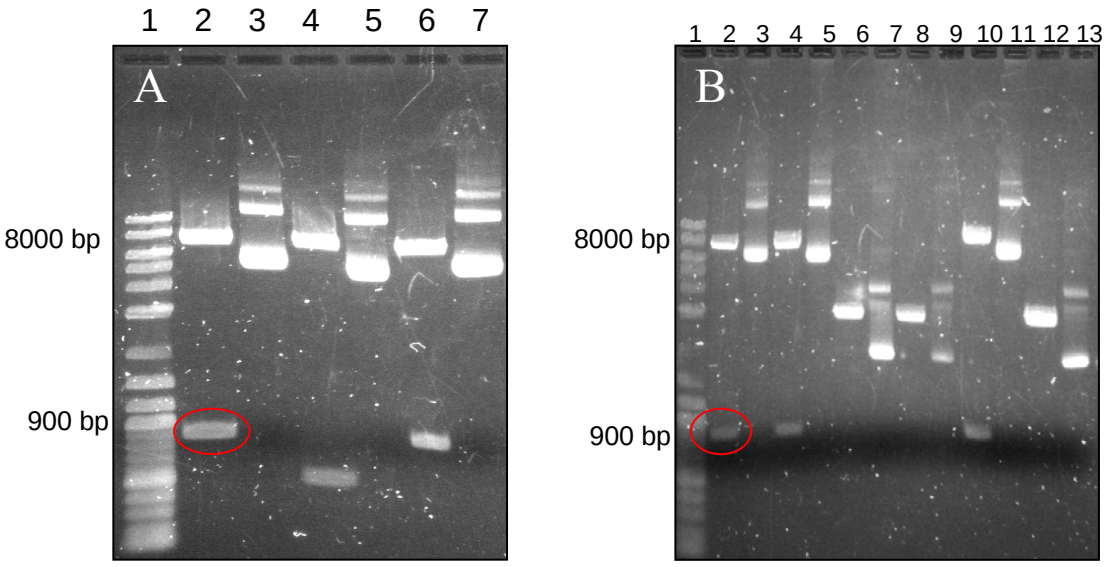

Figure 4.15: Analysis of cloning of CDK2F by gel electrophoresis.
A: Three clones were tested for CDK2F inserts in pGBKT7. **1**: 2 log ladder, **2**: Clone#7 cut (selected for sequencing), **3**: Clone#7 uncut, **4**: Clone#8 cut, **5**: Clone#8 uncut, **6**: Clone#9 cut, **7**: Clone#9 uncut, **8**: Clone#10 cut, **9**: Clone#10 uncut.
B: Six clones were tested for CDK2F inserts in pGADT7. **1**: 2 log ladder, **2**: Clone#1 cut (selected for sequencing), **3**: Clone#1 uncut, **4**: Clone#2 cut, **5**: Clone#2 uncut, **6**: Clone#3 cut, **7**: Clone#3 uncut, **8**: Clone#4 cut, **9**: Clone#4 uncut, **10**: Clone#5 cut, **11**: Clone#5 uncut, **12**: Clone#6 cut, **13**: Clone#6 uncut.
The uncut clones serve as controls for the cut clones.

4.4.2 *S. cerevisiae* two-hybrid analysis for interaction of CKS2 with CDK2F

The results of the *S. cerevisiae* two-hybrid analysis for pGBK/CKS2 and pGAD/CDK2F, and pGAD/CKS2 and pGBK/CDK2F are shown in figures 4.16 and 4.17, respectively. The results are similar to the results already described for CDK1AF and CDK2AF, but the control plate shows poor growth for pGAD/CDK2F. This indicates that CDK2F was mildly toxic to the yeast cells. However, the results show that CKS2 and CDK2F interacted regardless of whether CKS2 (figure 4.16) or CDK2F (figure 4.17) was cloned as the bait protein.

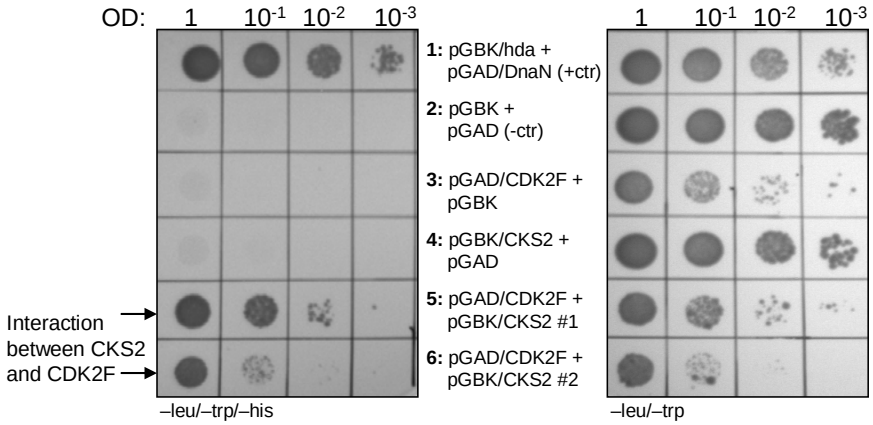

Figure 4.16: *S. cerevisiae* two-hybrid analysis of pGBK/CKS2 and pGAD/CDK2F. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CDK2F and pGBK/CKS2, **6**: Score for PPI #2, pGAD/CDK2F and pGBK/CKS2. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

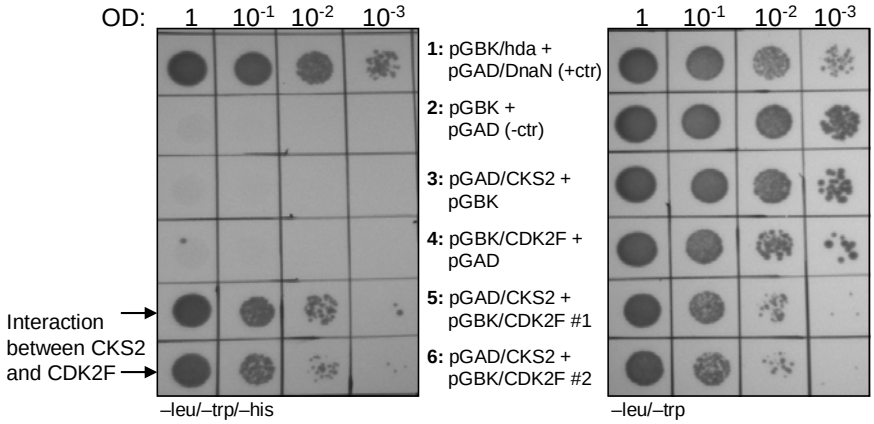

Figure 4.17: *S. cerevisiae* two-hybrid analysis of pGAD/CKS2 and pGBK/CDK2F. The following construct combinations were transformed into AH109: **1**: Positive control, pGBK/hda and pGAD/DnaN, **2**: Negative control, pGBK and pGAD without inserts, **3**: Test for auto-activation, pGAD with insert and pGBK without insert, **4**: Test for auto-activation, pGBK with insert and pGAD without insert, **5**: Score for PPI #1, pGAD/CKS2 and pGBK/CDK2F, **6**: Score for PPI #2, pGAD/CKS2 and pGBK/CDK2F. Right panel shows the control plate (-leu/-trp). Left panel shows plate that scores for PPIs (-leu/-trp/-his).

4.5 Summary of the S. cerevisiae two-hybrid analyses

Table 4.1 summarizes the different PPI's assessed with the *S. cerevisiae* two-hybrid analyses. The interaction of CKS2 with CDK1AF, CDK2AF and CDK2F could be shown both ways by the use of the Y2H system.

Table 4.1: Overview of the PPI's shown with the *S. cerevisiae* two-hybrid analysis.

Plasmid	pGBK/CDK1AF	pGBK/CDK2AF	pGBK/CDK2F
pGAD/CKS2	+	+	+

Plasmid	pGAD/CDK1AF	pGAD/CDK2AF	pGAD/CDK2F
pGBK/CKS2	+	+	+

The positive symbol indicates that the interaction could be shown in the Y2H system.

5 Discussion

5.1 Y2H can be used to identify CKS2 interactions with CDKs

Egan and Solomon [15] explored the binding of CKS2 to CDKs. They found that CDKs with mutations in the gene sequence of the inhibitory phosphorylation sites, changing T14 to alanine and Y15 to phenylalanine, did not affect the binding of CKS2 to CDK1/CCNB. This was tested for both mutations individually (T14A and Y15F), or in combination (AF). This indicates that the dephosphorylated sites are only important for activation of CDKs [23, 35], and not for binding of CKS2. In this project, the CDK gene sequences used to investigate the binding to CKS2 had mutations in the inhibitory phosphorylation sites in order to simulate the dephosphorylated state *in vivo*. Binding was observed regardless of whether the T14A and Y15F mutations or the single Y15F mutation were used, indicating that the T14A mutation was not necessary for CKS2 binding, which is in agreement with findings by Egan and Solomon [15]. Egan and Solomon [15] also tested binding of CKS2 to CDK1 with a changed amino acid at the activating phosphorylation site, T161. They found that phosphorylation of T161 stimulated binding of CKS2. We used no mutations of T161 to simulate activating phosphorylation in this project, indicating that the yeast cells carried out the phosphorylations at T161 in the CDKs. Alternatively, T161 is not necessary for CKS2 binding.

In a previous attempted Y2H study, where a different yeast strain and plasmids were used, yeast cells were not viable [18]. Others have also reported that CDK2 was mildly toxic in yeast cells [75]. In this study, plates with cells that were transformed with pGADT7 containing CDK1AF, CDK2AF or CDK2F often had to be incubated for 1-2 days longer than the other plates, indicating that some toxicity of the CDKs occurred. However, to avoid growth of spontaneously mutated yeast cells, incubation time never exceeded 5 days. With this incubation time, yeast cells grew sufficiently after transformations, so that PPIs could be measured.

Generation of false positive and false negative PPIs is a common problem in Y2H. The use of two reporter genes in parallel can be used to increase the stringency of the analysis and avoid appearance of false positive signals [1]. In the conducted *S.*

cerevisiae two-hybrid analyses, the *HIS3* reporter was used, which is of medium stringency [64]. Transformants tested in the analyses were carefully selected among homogenous colonies. Two parallels were tested for each PPI in each experiment. In some tests only one parallel produced growth. This was most likely due to false negatives. However, the majority of the tests produced growth, suggesting that the analyses were reliable.

The *S. cerevisiae* two-hybrid analyses showed that CKS2 interacts with both CDK1 and CDK2. These results are in agreement with previous studies, which have identified these interactions by the use of other methods, such as immunoprecipitation [8, 14, 15, 17]. The results show that Y2H can be used for assessing CKS2 interactions with both CDK1 and CDK2, and it is likely that the established interactions can be used in further screens.

5.2 Future prospects for use of Y2H

The development of different screens with CKS2 would be a next step towards developing targets of the CKS2/CDK interactions. The established CKS2/CDK1 and/or CKS2/CDK2 interaction can be tested in a reverse screen to detect small molecule compounds able to break the interaction. The *URA3* reporter, often used in reverse screens, is more difficult to activate than the *HIS3* reporter, leading to a higher risk of missing the PPIs (false negatives) [1, 64]. Therefore, the robustness of the CKS2 interactions have to be further investigated for a rY2H screen. However, we conclude that the obtained results are promising and indicate that the CKS2/CDKs interactions may be suited for such a screen. Small molecule compounds detected in a rY2H screen would be candidates for possible drugs in CKS2 interaction targeted therapy.

If the established interactions proved difficult to use in a rY2H screen, other interactions obtained from a cDNA-library screen could then be investigated. The cDNA-library (cloned as prey) screened for interactions with CKS2 (cloned as bait) would reveal possible CKS2 interacting partners present in the library [2, 3, 55]. Such a screen would also contribute to a better understanding of CKS2 function, which is

not fully clarified, and would be of importance for interpretation of the results from the clinical CKS2 studies.

Since our results showed that CKS2 interacted with both CDK1 and CDK2, it would be of interest to identify the CDK/cyclin complex that binds CKS2, and the cell cycle phase in which CKS2 is active. The cyclins are synthesized and degraded at specific phases in the cell cycle, and CDK1 and CDK2 are known to bind to cyclins A, B and E [6, 36, 37]. It has previously been shown that CKS2 is present in all phases of the cell cycle [76]. It should therefore be possible for CKS2 to bind CDK1 and CDK2 in all cell cycle phases, although further investigations are necessary to clarify this. Such knowledge would be of importance for understanding the function of CKS2, and why overexpression of the protein is associated with aggressive tumors.

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Appendices

1. Procedure for subcloning
2. Protocol for reverse transcription to synthesize cDNA
3. PCR-protocols for CDK1, CDK2 and CKS2
4. Recipes for growth media, buffers and solutions
5. Protocol for preparing bacterial culture
6. Protocol for plasmid purification, midi
7. Restriction enzymes that cut and do not cut the CDK1, CDK2 and CKS2 nucleotide seuences
8. Protocol for double digestion with restriction enzymes NdeI and BamHI, and NdeI and BamHI-HF
9. Protocol for analysis by gel electrophoresis
10. Protocol for purification of PCR products
11. Protocol for purification of DNA fragments from PCR and other enzymatic reactions
12. Protocols for ligation mixtures and transformation of XL 10-Gold® Ultracompetent *E. coli* cells
13. Protocol for plasmid purification, mini
14. List of materials used
15. Protocol for transformation in *S. cerevisiae* AH109 by the one-step method
16. Protocol for *S. cerevisiae* two-hybrid analysis
17. Sequencing

Appendix 1
Procedure for subcloning

pGEM®-T Easy Vector System from Promega.
Modified protocol from technical manual, 2007.

The vector map of pGEM®-T Easy Vector is shown in figure A1.1.

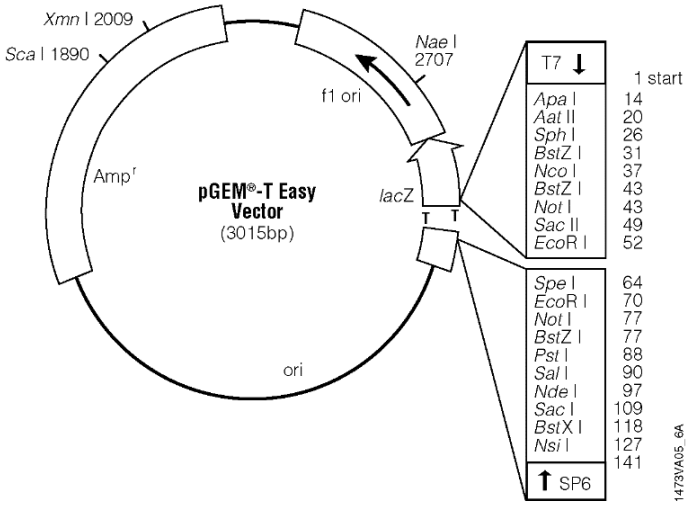

Figure A1.1: pGEM®-T Easy Vector map. The MCS lies within the coding region of β-galactosidase, contains several recognition sites for restriction enzymes, and is flanked by the RNA polymerases T7 and SP6. The vector also contains an ampicillin resistance gene.

An overview of A-tailing is outlined in figure A1.2.

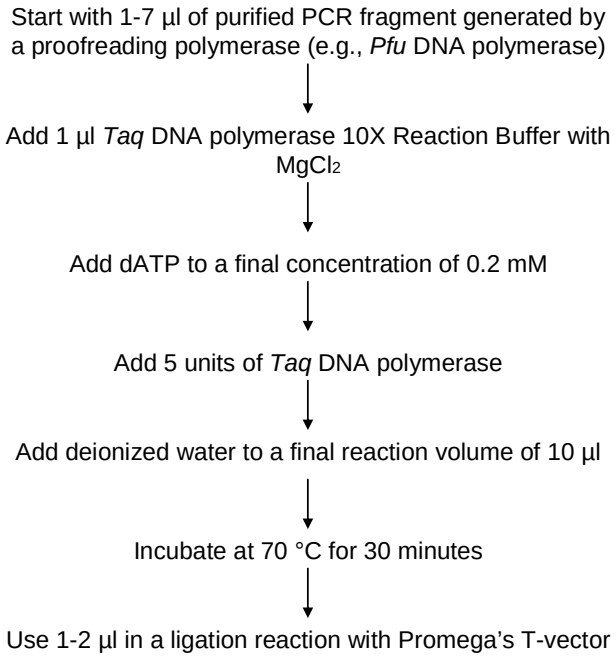

Figure A1.2: A-tailing procedure for blunt-ended PCR fragments. The pGEM-T Easy vector is equipped with single 3'-T overhangs at the insertion site, and this will greatly improve the efficiency of ligation.

Calculating Insert:Vector Molar Ratios:

Ratios from 3:1 to 1:3 provide good initial parameters. The T-vector is approximately 3 kb and is supplied at 50 ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

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

For CKS2 (16 ng/µl), the following parameters were used:

$$\frac{50 \text{ ng} \times 0.240 \text{ kbp}}{3.0 \text{ kb}} \times \frac{2}{1} = 8 \text{ ng}$$

Total volume is 10 µl:
8 ng/µl × 10 µl = 80 ng

16 ng/µl × X µl = 80 ng
X = 5 µl

For CDK1 (48.7 ng/µl), the following parameters were used:

$$\frac{50 \text{ ng} \times 0.90 \text{ kbp}}{3.0 \text{ kb}} \times \frac{2}{1} = 30 \text{ ng}$$

Total volume is 10 µl:
30 ng/µl × 10 µl = 300 ng

30 ng/µl × X µl = 300 ng
X = 6.2 µl

Reaction mixtures for A-tailing:

CKS2		CDK1	
CKS2 PCR product	5.0 µl	CKS2 PCR product	6.2 µl
dATP 2 mM	1.0 µl	dATP 2 mM	1.0 µl
Taq DNA polymerase (5 u/µl)	1.0 µl	*Taq* DNA polymerase (5 u/µl)	1.0 µl
Taq DNA polymerase rxn buffer (10×)	1.0 µl	*Taq* DNA polymerase rxn buffer (10×)	1.0 µl
Deionized water	2.0 µl	Deionized water	0.8 µl
Total	10.0 µl	Total	10.0 µl

Incubate at 70 °C for 30 minutes.

Protocol for ligation of fragment into pGEM-T Easy vector

1. Briefly centrifuge T-vector to collect content at the bottom of the tube.
2. Set up ligation reactions as described below:

2× Rapid Ligation Buffer, T4 DNA Ligase	5.0 µl
pGEM®-T Easy Vector (50 ng)	1.0 µl
PCR product	µl
T4 DNA Ligase (3 Weiss units/µl)	1.0 µl
Deionized water	to 10.0 µl

   Note: vortex the 2× Rapid Ligation Buffer vigorously before each use.
3. Mix the reactions by pipetting. Incubate the reactions overnight at 4 °C.

Protocol for transformation of JM109 High Efficiency Competent *E. coli* cells

1. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction (see below). Equilibrate the plates to room temperature prior to plating (Step 10).
2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2 µl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice.
3. Remove tubes of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. **Note**: avoid excessive pipetting, as the competent cells are extremely fragile.
4. Carefully transfer 50 µl of cells into each tube prepared in step 2.
5. Gently flick the tubes to mix and place them on ice for 20 minutes.
6. Heat-shock the cells for 45-50 seconds in a water bath at exactly 42 °C (Do not shake).
7. Immediately return the tubes to ice for 2 minutes.
8. Add 950 µl room temperature LB medium to the tubes.
9. Incubate for 1.5 hours at 37 °C with shaking.
10. Plate 100 µl of each transformation culture onto one LB/ampicillin/IPTG/ X-Gal plate.
11. Spin down the rest of the transformation culture at 13 000 rpm for 2 minutes, remove the supernatant and resuspend in 100 µl LB. Repeat step 10.
12. Incubate the plates overnight at 37 °C.

White colonies generally contain inserts; however, inserts may also be present in blue colonies.

Composition of Buffers and Solutions:

IPTG stock solution (0.1 M)		X-Gal (50 mg/ml)	
IPTG	1.2 g	X-Gal	100 mg
dH_2O	to 50 ml	N,N´-dimethyl-formamide	2 ml
Filtersterilize and store at 4°C		Dissolve, cover with aluminum foil and store at -20 °C	

LB plates with ampicillin/IPTG/X-Gal:

Melt LB agar in the microwave, and pour onto a 50 ml tube (gives 2 plates). Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100 µg/ml. Supplement with 0.5 mM IPTG and 80 µg/ml X-Gal. Pour 25 ml of medium into 90 mm petri dishes. Let the agar harden. Store at 4 °C for up to 1 month, or at room temperature for up to 1 week.

Appendix 2
Protocol for reverse transcription to synthesize cDNA

Illumina® TotalPrep RNA Amplification Kit from Ambion
Modified from protocol following steps II.C. – II.E.*

C. Reverse Transcription to Synthesize First Strand cDNA

1. Bring RNA samples to 11 µl with nuclease-free water in a 0.5 ml microcentrifuge tube.
2. Add 9 µl of Reverse Transcription Master Mix (table A2.1) to the RNA sample. Mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

Table A2.1: Reverse Transcription Master Mix (for one single 20 µl reaction)

Amount	Component
1 µl	T7 Oligo(dT) Primer
2 µl	10X First Strand Buffer
4 µl	dNTP Mix
1 µl	RNase Inhibitor
1 µl	ArrayScript

3. Place the sample in the thermal cycler, and incubate for 2 hours at 42 °C.
4. After incubation, centrifuge for about 5 seconds to collect the reaction mixture at the bottom of the tube. Place the tubes on ice and immediately proceed to section D.

D. Second Strand cDNA Synthesis

5. Add 80 µl of Second Strand Master Mix (table A2.2) to the sample, mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.

Table A2.2: Second Strand Master Mix (for one single 100 µl reaction)

Amount	Component
63 µl	Nuclease-free Water
10 µl	10X Second Strand Buffer
4 µl	dNTP Mix
2 µl	DNA Polymerase
1 µl	RNase H

Note: to be prepared on ice

6. Place the sample in the thermal cycler, and incubate for 2 hours at 16 °C.
7. Place the sample tube on ice and freeze immediately or proceed to section E.

E. cDNA Purification

8. Preheat a minimum of 20 µl of nuclease-free water to 55 °C.
9. Add 250 µl cDNA Binding Buffer to the sample, and mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times. Centrifuge the sample briefly to collect the mixture in the bottom of the tube.
10. Put the cDNA Filter Cartridge in the wash tube and pass the sample from step 9 onto to the centre of the filter.
11. Centrifuge for 1 minute at 10 000 rpm.
12. Discard the flow-through and replace the filter in the wash tube.
13. Apply 500 µl Wash Buffer onto the filter (make sure that ethanol has been added to the Wash Buffer).
14. Centrifuge for 1 minute at 10 000 rpm.
15. Discard the flow-through and centrifuge the filter for an additional minute to remove trace amounts of Wash Buffer.
16. Transfer the filter to a clean tube.
17. Apply 20 µl of nuclease-free water holding about 55 °C (colder water or more than 58 °C may result in reduced cDNA yield) to the centre of the filter.
18. Incubate at room temperature for 2 minutes and then centrifuge for 1 minute at 10 000 rpm. The double stranded cDNA will now be in the eluate (approximately 17,5 µl).
19. Measure the concentration by using the NanoDrop, and store at – 20 °C.

* The protocol proceeds with In Vitro Transcription to Synthesize cRNA and cRNA Purification. The purpose here was to synthesize cDNA, and therefore the procedure was stopped after cDNA Purification.

Appendix 3
PCR-protocols for CDK1, CDK2 and CKS2

Materials provided by Stratagene:
PfuUltra™ II Fusion HS DNA Polymerase
10× *PfuUltra*™ II Reaction Buffer

Mastermix for CDK1:

Component	1 reaction	5 reactions
Distilled water	40,5 µl	202.5 µl
10× *PfuUltra*™ II Reaction Buffer*	5,0 µl	25.0 µl
dNTP mix (25 mM each dNTP)	0,5 µl	2.5 µl
DNA template, CDK1AF (100 ng/µl)	1,0 µl**	5.0 µl
Primer CDK1F (10 µM)	1,0 µl	5.0 µl
Primer CDK1Rev (10 µM)	1,0 µl	5.0 µl
PfuUltra™ II Fusion HS DNA Polymerase	1,0 µl	5.0 µl
Total reaction volume	50,0 µl	250.0 µl

Mastermix for CDK2:

Component	1 reaction	5 reactions
Distilled water	40,5 µl	202.5 µl
10× *PfuUltra*™ II Reaction Buffer*	5,0 µl	25.0 µl
dNTP mix (25 mM each dNTP)	0,5 µl	2.5 µl
DNA template, CDK2F or CDK2AF	1,0 µl**	5.0 µl
Primer CDK2F (10 µM)	1,0 µl	5.0 µl
Primer CDK2Rev (10 µM)	1,0 µl	5.0 µl
PfuUltra™ II Fusion HS DNA Polymerase	1,0 µl	5.0 µl
Total reaction volume	50,0 µl	250.0 µl

Mastermix for CKS2:

Component	1 reaction	5 reactions
Distilled water	39,5 µl	197.5 µl
10× *PfuUltra*™ II Reaction Buffer*	5,0 µl	25.0 µl
dNTP mix (25 mM each dNTP)	0,5 µl	2.5 µl
DNA template, cDNA CKS2 (15 ng/µl)	2,0 µl**	10.0 µl
Primer CKS2F (10 µM)	1,0 µl	5.0 µl
Primer CKS2Rev (10 µM)	1,0 µl	5.0 µl
PfuUltra™ II Fusion HS DNA Polymerase	1,0 µl	5.0 µl
Total reaction volume	50,0 µl	250.0 µl

Transfer 50 µl mastermix to each PCR tube and run the appropriate programme.

Cycling Parameters for CDK1 and CDK1:

Segment	Number of cycles	Temperature	Duration (cDNA)
1	1	95 °C	2 minutes
2	30	95 °C	20 seconds
		Primer T_m-5 °C	20 seconds
		72 °C	15 seconds for targets ≤ 1 kb
3	1	72 °C	3 minutes

Primer T_m CDK1 = CDK2 = 50 °C
CDK1 = 894 bp
CDK2 = 897 bp

PCR cycling parameters for CKS2:

Segment	Number of cycles	Temperature	Duration (cDNA)
1	1	95 °C	1 minute
2	40	95 °C	20 seconds
		Primer T_m-5 °C	20 seconds
		72 °C	30 seconds for targets ≤ 1 kb
3	1	72 °C	3 minutes

Primer T_m CKS2 = 53 °C
CKS2 = 240 bp

Analyze the PCR amplification products on a 0.8 % agarose gel.

* The 10× buffer provides a final 1 × Mg^{2+} concentration of 2mM
** The amount of DNA template required varies depending on the type of DNA being amplified. Generally 100 ng of genomic DNA template is recommended. Less DNA template should be used for amplification of lambda or vector (5-30 ng) PCR targets.

Appendix 4
Growth media, buffers and solutions

Table A4.1: Growth media for *E. coli*

LB (Luria Broth)	
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH_2O	to 1000 ml
Autoclave	

LB agar
LB medium with 0.5 % Bacto agar
Autoclave

LB medium to plate
LB agar with ampicillin and chloramphenicol to final concentrations of 100 µg/ml and 30 µg/ml, respectively (pGADT7)

LB agar with kanamycin and chloramphenicol to final concentrations of 50 µg/ml and 30 µg/ml, respectively (pGBKT7)

LB agar with ampicillin, IPTG and X-Gal to final concentrations of 100 µg/ml, 0.5 mM, and 80 µg/ml, respectively (pGEM-T Easy Vector)

Table A4.2: Buffers and solutions

5 × TBE buffer	
Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
dH_2O	to 1000 ml
The agarose gels were run in 0.5 × TBE	

50 × TAE buffer	
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
	to 1000 ml

0.8 % NaCl	
Sodium chloride	1.6 g
dH_2O	to 200 ml

Table A4.3: Growth media for *S. cerevisiae*

YPD

YPD	20 g
dH_2O	to 400 ml

Autoclave

YPD agar

YPD	20 g

Add some dH_2O and let it dissolve under stirring
Adjust pH ~ 6.0 with NaOH

Bacto agar	8 g
Complete with dH_2O	to 400 ml

Autoclave

SD-DO

Minimal SD Base	10.7 g
DO supplement (-ade/-his/-leu/-trp)	0.24 g

Add some dH_2O and let it dissolve under stirring
Adjust pH ~ 6.0 with NaOH

Complete with dH_2O	to 400 ml

Autoclave

SD-DO medium to plate

SD-DO medium supplemented with adenine to a final concentration of 20 µg/ml (to score for PPI)

SD-DO medium supplemented with adenine and histidine to final concentrations of 20 µg/ml (control)

Pour plates in aseptic conditions

One-step buffer

Lithium Acetate 1M (pH 7.5)	20 ml
PEG 3350 (or PEG 4000)	40 g

Add some dH_2O and let it dissolve under stirring

Complete with dH_2O	to 100 ml

Sterilize the solution by 3 cycles of warming for 30 minutes at 65 °C (one cycle per day, let the solution at room temperature between each cycle)
Store the sterilized solution at 4 °C

Appendix 5
Protocol for preparing bacterial culture

1. In a 250 ml tubes, add 50 ml LB medium.
2. Add appropriate antibiotics (table A5.1).
3. Add a small amount of the appropriate -80 stock of bacterial culture to the tube.
4. Incubate at 37 °C overnight in a swirling water bath.

Table A5.1: Type and final concentration of antibiotics added to bacterial culture before incubation overnight.

	Plasmid	
	pGBKT7	pGADT7
Antibiotics	Kanamycin (50 mg/ml) 50 µg/ml	Ampicillin (100 mg/ml) 100 µg/ml

Concentrations of stock-solutions are shown in brackets.

Appendix 6
Protocol for plasmid purification, midi

JETSTAR Plasmid Purification Kit from Genomed.

Modified from JETSTAR 2.0 Plasmid Mini/Midi/ Maxi-Protocol
Follow the steps stated for **Midi** which is suitable for low copy plasmids (0.2-1 µg DNA/ml LB medium). Culture volume is 25-100 ml and gives DNA yield of 5-100 µg

1. Column equilibration: add 10 ml of solution E4. Allow the column to empty by gravity flow, do not force out remaining solution.
2. Harvesting bacterial cells: spin down the cell culture (centrifuging at 4 °C, 850×10 rpm for 10 minutes). Remove all traces of LB medium.
3. Cell resuspension: dissolve the pellet with 4 ml of solution E1.
4. Cell lysis: add 4 ml of solution E2. Do not vortex, just gently mix 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. Do not vortex. Then centrifuge at room temperature, 850×10 rpm for 10 minutes.
6. Column loading: apply the supernatant from step 5 to the column, and allow the lysate to run by gravity flow.
7. Column washing: add 10 ml of solution E5, and allow to empty by gravity flow. Repeat step 7. Do not force out remaining solution.
8. Plasmid elution: switch tube under the column. Add 5 ml of solution E6 to the column and allow to run by gravity flow. Do not force out remaining solution.
9. Plasmid precipitation: precipitate the DNA with 3.5 ml isopropanol and centrifuge at 4 °C, 850×10 rpm for 30 minutes. Remove the supernatant.
10. Wash the plasmid DNA with 70 % ethanol and transfer everything to a eppendorf tube. Centrifuge at 14 000 rpm for 30 minutes. Remove the ethanol by using a pipette connected to water suction.
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 and store at -20 °C.

Solutions supplied with the 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 7

Restriction enzymes that cut and do not cut the CDK1, CDK2 and CKS2 nucleotide seuences. The analysis was performed using Webcutter.

CKS2

240 base pairs

Graphic map | Table by enzyme name

```
             XhoII
             MflI              Eco255I             Bse118I
             BstYI             Acc113I             BssAI   NspI
atggcccacaagcagatctactactcggacaagtacttcgacgaacactacgagtaccggcatgttatgttaccc base pairs
taccgggtgttcgtctagatgatgagcctgttcatgaagctgcttgtgatgctcatggccgtacaatacaatggg 1 to 75
             BstX2I            ScaI                BsrFI
             BglII                                 Cfr10I


                                                BseRI       AspEI
                                             Ksp632I        EclHKI
                                           Eco57I      BseRI
agagaactttccaaacaagtacctaaaactcatctgatgtctgaagaggagtggaggagacttggtgtccaacag base pairs
tctcttgaaaggtttgttcatggattttgagtagactacagacttctcctcacctcctctgaaccacaggttgtc 76 to 150
                                             Eam1104I       AhdI
                                             EarI           Eam1105I


                                                                        EarI
                          BspHI                                         Eam11
agtctaggctgggttcattacatgattcatgagccagaaccacatattcttctctttagacgacctcttccaaaa base pairs
tcagatccgacccaagtaatgtactaagtactcggtcttggtgtataagaagagaaatctgctggagaaggtttt 151 to 225
                          RcaI                                          Ksp63




04I
gatcaacaaaaatga  base pairs
ctagttgtttttact  226 to 240
2I
```

The following endonucleases were selected but don't cut this sequence:

AatI, AatII, Acc16I, Acc65I, AccB1I, AccB7I, AccBSI, AccI, AccIII, AclNI,
AcsI, AcyI, AfeI, AflII, AflIII, AgeI, Alw21I, Alw44I, AlwNI, Ama87I, AocI,
Aor51HI, ApaI, ApaLI, ApoI, AscI, AseI, AsnI, Asp700I, Asp718I, AspHI,
AspI, AtsI, AvaI, AviII, AvrII, BalI, BamHI, BanI, BanII, BanIII, BbeI,
BbiII, BbrPI, BbsI, BbuI, Bbv12I, Bbv16II, BcgI, BclI, BcoI, BfrI, BglI,
BlnI, BlpI, BpiI, BpmI, Bpu1102I, Bpu14I, BpuAI, Bsa29I, BsaAI, BsaBI,
BsaHI, BsaI, BsaMI, BsaOI, BsaWI, BscI, Bse21I, Bse8I, BseAI, BseCI, BsePI,
BsgI, Bsh1285I, Bsh1365I, BshNI, BsiEI, BsiHKAI, BsiI, BsiMI, BsiWI, BsmBI,
BsmI, BsoBI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp1720I,
Bsp19I, Bsp68I, BspCI, BspDI, BspEI, BspLU11I, BspMI, BspTI, BspXI, BsrBI,
BsrBRI, BsrDI, BsrGI, BssHII, BssSI, BssT1I, Bst1107I, Bst98I, BstBI, BstD102I,
BstDSI, BstEII, BstH2I, BstI, BstMCI, BstPI, BstSFI, BstSNI, BstXI, BstZI,
Bsu15I, Bsu36I, CciNI, CelII, Cfr42I, Cfr9I, CfrI, ClaI, CpoI, Csp45I,
CspI, CvnI, DraI, DraII, DraIII, DrdI, DsaI, EaeI, EagI, Ecl136II, EclXI,
Eco105I, Eco130I, Eco147I, Eco24I, Eco31I, Eco32I, Eco47III, Eco52I, Eco64I,
Eco72I, Eco81I, Eco88I, Eco91I, EcoICRI, EcoNI, EcoO109I, EcoO65I, EcoRI,
EcoRV, EcoT14I, EcoT22I, EheI, ErhI, Esp1396I, Esp3I, FauNDI, FbaI, FriOI,
FseI, FspI, GsuI, HaeII, Hin1I, HincII, HindII, HindIII, HpaI, Hsp92I,
KasI, Kpn2I, KpnI, Ksp22I, KspI, LspI, MamI, MfeI, MluI, MluNI, Mph1103I,
MroI, MroNI, MscI, MslI, Msp17I, MspA1I, MspCI, MunI, Mva1269I, NaeI, NarI,
NcoI, NdeI, NgoAIV, NgoMI, NheI, NotI, NruI, NsiI, NspBII, NspV, PacI,
PaeI, PaeR7I, Pfl23II, PflMI, PinAI, Ple19I, PmaCI, Pme55I, PmeI, PmlI,
Ppu10I, PpuMI, PshAI, PshBI, Psp124BI, Psp1406I, Psp5II, PspAI, PspALI,
PspEI, PspLI, PspOMI, PstI, PstNHI, PvuI, PvuII, RsrII, SacI, SacII, SalI,
SapI, SbfI, SexAI, SfcI, SfiI, Sfr274I, Sfr303I, SfuI, SgfI, SgrAI, SmaI,
SmiI, SnaBI, SpeI, SphI, SplI, SrfI, Sse8387I, SseBI, SspBI, SspI, SstI,
SstII, StuI, StyI, SunI, SwaI, Tth111I, Van91I, Vha464I, VneI, VspI, XbaI,
XcmI, XhoI, XmaI, XmaIII, XmnI, Zsp2I

CDK1

894 base pairs

Graphic map | Table by enzyme name

```
                                    BshNI
                                    Asp718I
                                    Eco64I                    AccI       SfcI
atggaagattataccaaaatagagaaaattggagaaggtacctatggagttgtgtataagggtagacacaaaact base pairs
taccttctaatatggttttatctcttttaacctcttccatggatacctcaacacatattcccatctgtgttttga 1 to 75
                                    BanI KpnI                             BstSFI
                                    Acc65I
                                    AccB1I


                                                 EarI Asp700I   Eco255I
                                                 Eam1104I       Acc113I
acaggtcaagtggtagccatgaaaaaaatcagactagaaagtgaagaggaaggggttcctagtactgcaattcgg base pairs
tgtccagttcaccatcggtacttttttagtctgatctttcacttctccttccccaaggatcatgacgttaagcc 76 to 150
                                                 Ksp632I        ScaI
                                                      XmnI


                                                   Eco57I
                                                  BbsI
  AcsI                                            BpuAI
gaaatttctctattaaaggaacttcgtcatccaaatatagtcagtcttcaggatgtgcttatgcaggattccagg base pairs
ctttaaagagataatttccttgaagcagtaggtttatatcagtcagaagtcctacacgaatacgtcctaaggtcc 151 to 225
  ApoI                                            Bbv16II
                                                  BpiI


                         NcoI Bsp19I
                         StyI DsaI XhoII
                         Eco130I BstX2I
ttatatctcatctttgagtttctttccatggatctgaagaaatacttggattctatccctcctggtcagtacatg base pairs
aatatagagtagaaactcaaagaaaggtacctagacttctttatgaacctaagatagggaggaccagtcatgtac 226 to 300
                         ErhI BstDSI  Eco57I
                         BssT1I BstYI
                         EcoT14I MflI


                                                                  EarI
                                    SfcI                   XbaI  Eam11
gattcttcacttgttaagagttatttataccaaatcctacaggggattgtgttttgtcactctagaagagttctt base pairs
ctaagaagtgaacaattctcaataaatatggtttaggatgtcccctaacacaaaacagtgagatcttctcaagaa 301 to 375
                                    BstSFI                        Ksp63




04I
cacagagacttaaaacctcaaaatctcttgattgatgacaaaggaacaattaaactggctgattttggccttgcc base pairs
gtgtctctgaattttggagttttagagaactaactactgtttccttgttaatttgaccgactaaaaccggaacgg 376 to 450
2I


                                                        XhoII
                                                        MflI
                                                        BstYI    GsuI
agagcttttggaatacctatcagagtatatacacatgaggtagtaacactctggtacagatctccagaagtattg base pairs
tctcgaaaaccttatggatagtctcatatatgtgtactccatcattgtgagaccatgtctagaggtcttcataac 451 to 525
                                                        BstX2I   BpmI
                                                        BglII


                              XcmI
                            HincII          BshNI
                           GsuI             Eco64I
ctggggtcagctcgttactcaactccagttgacatttggagtataggcaccatatttgctgaactagcaactaag base pairs
gaccccagtcgagcaatgagttgaggtcaactgtaaacctcatatccgtggtataaacgacttgatcgttgattc 526 to 600
                           BpmI             BanI
                             HindII         AccB1I
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```
            NcoI Bsp19I
            StyI DsaI         Ksp22I      EarI
            Eco130I           FbaI        Eam1104I
aaaccacttttccatggggattcagaaattgatcaactcttcaggattttcagagctttgggcactcccaataat base pairs
tttggtgaaaaggtacccctaagtctttaactagttgagaagtcctaaaagtctcgaaacccgtgagggttatta 601 to 675
            ErhI BstDSI       BclI        Ksp632I
            BssT1I                          Eco57I
            EcoT14I

          BalI
          MscI
        EaeI
gaagtgtggccagaagtggaatctttacaggactataagaatacatttcccaaatggaaaccaggaagcctagca base pairs
cttcacaccggtcttcaccttagaaatgtcctgatattcttatgtaaagggtttacctttggtccttcggatcgt 676 to 750
        CfrI
          MluNI




                                                                          AcsI
tcccatgtcaaaaacttggatgaaaatggcttggatttgctctcgaaaatgttaatctatgatccagccaaacga base pairs
agggtacagttttgaacctacttttaccgaacctaaacgagagctttacaattagatactaggtcggtttgct 751 to 825
                                                                          ApoI




atttctggcaaaatggcactgaatcatccatattttaatgatttggacaatcagattaagaagatgtag  base pairs
taaagaccgttttaccgtgacttagtaggtataaaattactaaacctgttagtctaattcttctacatc  826 to 894
```

The following endonucleases were selected but don't cut this sequence:

AatI, AatII, Acc16I, AccB7I, AccBSI, AccIII, AclNI, AcyI, AfeI, AflII,
AflIII, AgeI, AhdI, Alw21I, Alw44I, AlwNI, Ama87I, AocI, Aor51HI, ApaI,
ApaLI, AscI, AseI, AsnI, AspEI, AspHI, AspI, AtsI, AvaI, AviII, AvrII,
BamHI, BanII, BanIII, BbeI, BbiII, BbrPI, BbuI, Bbv12I, BcgI, BcoI, BfrI,
BglI, BlnI, BlpI, Bpu1102I, Bpu14I, Bsa29I, BsaAI, BsaBI, BsaHI, BsaI,
BsaMI, BsaOI, BsaWI, BscI, Bse118I, Bse21I, Bse8I, BseAI, BseCI, BsePI,
BseRI, BsgI, Bsh1285I, Bsh1365I, BsiEI, BsiHKAI, BsiI, BsiMI, BsiWI, BsmBI,
BsmI, BsoBI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp1720I,
Bsp68I, BspCI, BspDI, BspEI, BspHI, BspLU11I, BspMI, BspTI, BspXI, BsrBI,
BsrBRI, BsrDI, BsrFI, BsrGI, BssAI, BssHII, BssSI, Bst1107I, Bst98I, BstBI,
BstD102I, BstEII, BstH2I, BstI, BstMCI, BstPI, BstSNI, BstXI, BstZI, Bsu15I,
Bsu36I, CciNI, CelII, Cfr10I, Cfr42I, Cfr9I, ClaI, CpoI, Csp45I, CspI,
CvnI, DraI, DraII, DraIII, DrdI, EagI, Eam1105I, Ecl136II, EclHKI, EclXI,
Eco105I, Eco147I, Eco24I, Eco31I, Eco32I, Eco47III, Eco52I, Eco72I, Eco81I,
Eco88I, Eco91I, EcoICRI, EcoNI, EcoO109I, EcoO65I, EcoRI, EcoRV, EcoT22I,
EheI, Esp1396I, Esp3I, FauNDI, FriOI, FseI, FspI, HaeII, Hin1I, HindIII,
HpaI, Hsp92I, KasI, Kpn2I, KspI, LspI, MamI, MfeI, MluI, Mph1103I, MroI,
MroNI, MslI, Msp17I, MspA1I, MspCI, MunI, Mva1269I, NaeI, NarI, NdeI, NgoAIV,
NgoMI, NheI, NotI, NruI, NsiI, NspBII, NspI, NspV, PacI, PaeI, PaeR7I,
Pfl23II, PflMI, PinAI, Ple19I, PmaCI, Pme55I, PmeI, PmlI, Ppu10I, PpuMI,
PshAI, PshBI, Psp124BI, Psp1406I, Psp5II, PspAI, PspALI, PspEI, PspLI,
PspOMI, PstI, PstNHI, PvuI, PvuII, RcaI, RsrII, SacI, SacII, SalI, SapI,
SbfI, SexAI, SfiI, Sfr274I, Sfr303I, SfuI, SgfI, SgrAI, SmaI, SmiI, SnaBI,
SpeI, SphI, SplI, SrfI, Sse8387I, SseBI, SspBI, SspI, SstI, SstII, StuI,
SunI, SwaI, Tth111I, Van91I, Vha464I, VneI, VspI, XhoI, XmaI, XmaIII, Zsp2I

CDK2

897 base pairs

Graphic map | Table by enzyme name

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                                    BsiWI
                 Esp1396I           PspLI       BsrGI
                 AccB7I             SplI        SspBI              HincII
atggagaacttccaaaaggtggaaaagatcggagagggcacgtacggagttgtgtacaaagccagaaacaagttg base pairs
tacctcttgaaggttttccacctttttctagcctctcccgtgcatgcctcaacacatgtttcggtctttgttcaac 1 to 75
                 PflMI              BsaAI       Bsp1407I           HindII
                 Van91I             Pfl23II
                                    SunI

                  AflII BfrI
                  BspTI MspCI                                Eco255I
                  Bsp143II                                   Acc113I
acgggagaggtggtggcgcttaagaaaatccgcctggacactgagactgagggtgtgcccagtactgccatccga base pairs
tgccctctccaccaccgcgaattcttttaggcggacctgtgactctgactcccacacgggtcatgacggtaggct 76 to 150
                  HaeII Vha464I                              ScaI
                  BstH2I
                  Bst98I

  XhoII      MspCI
  MflI       Bst98I
  BstYI      BspTI              SspI
gagatctctctgcttaaggagcttaaccatcctaatattgtcaagctgctggatgtcattcacacagaaaataaa base pairs
ctctagagagacgaattcctcgaattggtaggattataacagttcgacgacctacagtaagtgtgtcttttattt 151 to 225
  BstX2I     AflII
  BglII      Vha464I
             BfrI

                            BglII
                            BstX2I                                  BsaMI
     SexAI      AcsI       BsgI         AcsI                        BsmI
ctctacctggtttttgaatttctgcaccaagatctcaagaaattcatggatgcctctgctctcactggcattcct base pairs
gagatggaccaaaaacttaaagacgtggttctagagttctttaagtacctacggagacgagagtgaccgtaagga 226 to 300
                 ApoI          BstYI     ApoI                        Mva1269I
                               MflI
                               XhoII


    Ksp632I                    MspA1I   BpmI                    DraII
    Eam1104I                   PvuII    EcoO109I                PpuMI
cttcccctcatcaagagctatctgttccagctgctccagggcctagctttctgccattctcatcgggtcctccac base pairs
gaaggggagtagttctcgatagacaaggtcgacgaggtcccggatcgaaagacggtaagagtagcccaggaggtg 301 to 375
                               NspBII   GsuI                    EcoO109I
    EarI                               DraII                   Psp5II




       Eco31I        AlwNI                       NheI
cgagaccttaaacctcagaatctgcttattaacacagaggggccatcaagctagcagactttggactagccaga base pairs
gctctggaatttggagtcttagacgaataattgtgtctcccccggtagttcgatcgtctgaaacctgatcggtct 376 to 450
       BsaI                                     PstNHI


                                     Eco91I     Acc65I    Eco24I SstI
                                  Esp1396I      BanI KpnI AspHI FriOI
                                  AccB7I EcoO65I AccB1I Ecl136II BanII
gcttttggagtccctgttcgtacttacacccatgaggtggtgaccctgtggtaccgagctcctgaaatcctcctg base pairs
cgaaaacctcagggacaagcatgaatgtgggtactccaccactgggacaccatggctcgaggactttaggaggac 451 to 525
                                  PflMI BstPI   Eco64I  EcoICRI SacI
                                  Van91I PspEI  Asp718I   Bbv12I Alw21I
                                     BstEII     BshNI     Psp124BI BsiHKAI

                       BstXI
                     NspBII
          SspI       PvuII          GsuI                            Bsp12
ggctgcaaatattattccacagctgtggacatctggagcctgggctgcatctttgctgagatggtgactcgccgg base pairs
ccgacgtttataataaggtgtcgacacctgtagacctcggacccgacgtagaaacgactctaccactgagcggcc 526 to 600
                     MslI           BpmI                            PspOM
                     MspA1I
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   Eco24I                              BsaWI SapI BstYI
EcoO109I                               MroI Bsp13I XhoII    DraII
    0I          GsuI                   BspEI BsiMI BstX2I   PpuMI
gccctattccctggagattctgagattgaccagctcttccggatctttcggactctggggaccccagatgaggtg base pairs
cgggataagggacctctaagactctaactggtcgagaaggcctagaaagcctgagacccctggggtctactccac 601 to 675
    I BanII     BpmI                   AccIII EarI MflI     EcoO109I
DraII ApaI                             Kpn2I Eam1104I       Psp5II
   FriOI                               BseAI Ksp632I

      BalI                                                  BanII
      MscI                                                  Eco24I
    EaeI                                                 Bsp120I
gtgtggccaggagttacttctatgcctgattacaagccaagtttccccaagtgggcccggcaagattttagtaaa base pairs
cacaccggtcctcaatgaagatacggactaatgttcggttcaaaggggttcacccgggccgttctaaaatcattt 676 to 750
    CfrI                                                 PspOMI
      MluNI                                                 FriOI
                                                            ApaI



                                                          BsgI
gttgtacctcccctggatgaagatggacggagcttgttatcgcaaatgctgcactacgaccctaacaagcggatt base pairs
caacatggaggggacctacttctacctgcctcgaacaatagcgtttacgacgtgatgctgggattgttcgcctaa 751 to 825



     StyI
     Eco130I
  EaeI EcoT14I
tcggccaaggcagccctggctcacccttttcttccaggatgtgaccaagccagtaccccatcttcgactctga  base pairs
agccggttccgtcgggaccgagtgggaaagaaggtcctacactggttcggtcatggggtagaagctgagact  826 to 897
  CfrI      XcmI
     ErhI
     BssT1I
```

The following endonucleases were selected but don't cut this sequence:

AatI, AatII, Acc16I, AccBSI, AccI, AclNI, AcyI, AfeI, AflIII, AgeI, AhdI,
Alw44I, Ama87I, AocI, Aor51HI, ApaLI, AscI, AseI, AsnI, Asp700I, AspEI,
AspI, AtsI, AvaI, AviII, AvrII, BamHI, BanIII, BbeI, BbiII, BbrPI, BbsI,
BbuI, Bbv16II, BcgI, BclI, BcoI, BglI, BlnI, BlpI, BpiI, Bpu1102I, Bpu14I,
BpuAI, Bsa29I, BsaBI, BsaHI, BsaOI, BscI, Bse118I, Bse21I, Bse8I, BseCI,
BsePI, BseRI, Bsh1285I, Bsh1365I, BsiEI, BsiI, BsmBI, BsoBI, Bsp106I, Bsp119I,
Bsp1720I, Bsp19I, Bsp68I, BspCI, BspDI, BspHI, BspLU11I, BspMI, BspXI,
BsrBI, BsrBRI, BsrDI, BsrFI, BssAI, BssHII, BssSI, Bst1107I, BstBI, BstD102I,
BstDSI, BstI, BstMCI, BstSFI, BstSNI, BstZI, Bsu15I, Bsu36I, CciNI, CelII,
Cfr10I, Cfr42I, Cfr9I, ClaI, CpoI, Csp45I, CspI, CvnI, DraI, DraIII, DrdI,
DsaI, EagI, Eam1105I, EclHKI, EclXI, Eco105I, Eco147I, Eco32I, Eco47III,
Eco52I, Eco57I, Eco72I, Eco81I, Eco88I, EcoNI, EcoRI, EcoRV, EcoT22I, EheI,
Esp3I, FauNDI, FbaI, FseI, FspI, Hin1I, HindIII, HpaI, Hsp92I, KasI, Ksp22I,
KspI, LspI, MamI, MfeI, MluI, Mph1103I, MroNI, Msp17I, MunI, NaeI, NarI,
NcoI, NdeI, NgoAIV, NgoMI, NotI, NruI, NsiI, NspI, NspV, PacI, PaeI, PaeR7I,
PinAI, Ple19I, PmaCI, Pme55I, PmeI, PmlI, Ppu10I, PshAI, PshBI, Psp1406I,
PspAI, PspALI, PstI, PvuI, RcaI, RsrII, SacII, SalI, SbfI, SfcI, SfiI,
Sfr274I, Sfr303I, SfuI, SgfI, SgrAI, SmaI, SmiI, SnaBI, SpeI, SphI, SrfI,
Sse8387I, SseBI, SstII, StuI, SwaI, Tth111I, VneI, VspI, XbaI, XhoI, XmaI,
XmaIII, XmnI, Zsp2I

Appendix 8

Protocol for double digestion with restriction enzymes NdeI and BamHI, and NdeI and BamHI-HF

Table A8.1 and table A8.2 show reaction conditions for double digestion with NdeI and BamHI, and NdeI and BamHI-HF respectively.

Table A8.1: Double digestion with NdeI and BamHI

Component	Amount per reaction
DNA	4 µg* or 1 µg**
Buffer 3	5 µl
BSA (10 × dilution)	5 µl
Nuclease-free water (ad 45 µl)	µl
NdeI	3 µl → incubate at 37 °C for 2 hours
BamHI	2 µl → incubate at 37 °C for 1.5 hour
Total volume	50 µl

* when digesting plasmids without inserted fragment.
** when digesting plasmids with inserted fragment.

Table A8.2: Double digestion with NdeI and BamHI-HF

Component	Amount per reaction
DNA	4 µg* or 1 µg**
Buffer 4	5 µl
Nuclease-free water (ad 46µl)	µl
NdeI	2 µl
BamHI-HF	2 µl → incubate at 37 °C for 2 hours
Total volume	50 µl

Before gel electrophoresis, add 2.5 µl 10 % SDS to each tube

* when digesting plasmids without inserted fragment.
** when digesting plasmids with inserted fragment.

After double digestion, the cutting mixtures were either used further or stored at -20 °C.

Appendix 9
Protocol for analysis by gel electrophoresis

To all DNA samples including the DNA marker, 2 log ladder (New England Biolabs Inc.), add Loading Dye Blue (6×, New England Biolabs Inc.).

1. Add 0.5 g agarose and 60 ml buffer* to a 250 ml bottle. Heat the mixture in the microwave to the boiling point until a clear, transparent solution is achieved.
2. Let the mixture cool down and pour it into a gel mold.
3. Apply an appropriate comb for forming the sample wells in the gel.
4. Allow the gel to set completely at room temperature (25-30 minutes).
5. Remove the comb and transfer the gel to an electrophoresis tank containing enough buffer* to cover the gel.
6. Apply the DNA marker and the DNA samples to separate wells.
7. Apply a voltage of 100 V and run for 1.5 to 2.5 hours, depending on the size of the samples.
8. Immerse the gel in stain** and allow to stain for 15-30 minutes.
9. Examine the gel by UV light and photograph the gel (when appropriate, cut out the gel pieces with a scalpel).

* 0.5 x TBE or 1 x TAE
** 100 ml dH_2O and 10 µl 1 % ethidium bromide, or 100 ml dH_2O and 30 µl GelRed

Appendix 10
Protocol for purification of PCR products

Wizard® SV Gel and PCR Clean-Up System from Promega.

Modified from Quick Protocol, following the steps for DNA purification by centrifugation.

Gel slice preparation
Dissolving the gel slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 ml microcentrifuge tube.
2. Add 10 µl membrane binding solution per 10 mg of gel slice. Vortex and incubate at 50-65 °C until gel slice is completely dissolved. From time to time, invert tube.

Binding of DNA

1. Insert the minicolumn into the collection tube.
2. Transfer dissolved gel mixture to the minicolumn assembly. Incubate at room temperature for 1 minute (the column can only take 700 µl, if there is not room for all the gel mixture, do steps 2 and 3 again).
3. Centrifuge at 13 000 rpm for 1 minute. Discard the flow-through and reinsert the minicolumn into the collection tube.

Washing

4. Add 700 µl membrane wash solution (ethanol added). Centrifuge at 13 000 rpm for 1 minute. Discard flow-through and reinsert the minicolumn into the collection tube.
5. Repeat step 4 with 500 µl membrane wash solution. Centrifuge at 13 000 rpm for 5 minutes.
6. Empty the collection tube and recentrifuge the column assembly at 13 000 rpm for an additional 1 minute.

Elution

7. Carefully transfer the minicolumn to a clean 1.5 ml microcentrifuge tube.
8. Add 30 µl of nuclease-free water to the minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 13 000 rpm for 1 minute
9. Discard the minicolumn, measure the concentration of the DNA. Store at 4°C or – 20 °C

Appendix 11
Protocol for purification of DNA fragments from PCR and other enzymatic reactions

QIAquick PCR Purification Kit from QIAgen

Modified from Bench Protocol: QIAquick PCR Purification, follow the steps for microcentrifuge

1. Add 5 volumes of Buffer PB to 1 volume of the sample and mix.
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge at 13 000 rpm for 60 seconds. Discard flow-through and place the QIAquick column back into the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge at 13 000 rpm for 60 seconds. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the column for an additional 60 seconds (13 000 rpm).
6. Place the QIAquick column in a clean 1.5 ml microcentrifuge tube
7. To elute DNA, add 30 µl nuclease-free water to the center of the QIAquick membrane. Incubate for 60 seconds and centrifuge the column at 13 000 rpm for 60 seconds
8. Measure the concentration and store at -20 °C

Appendix 12
Protocols for ligation mixtures and transfomations of XL 10-Gold® Ultracompetent *E. coli* cells

Protocol for ligation of fragment into plasmid

1. Add appropriate amount of digested fragment to a 1.5 ml tube.
2. Add appropriate amount of digested plasmid.
3. Add 2 µl of T4 DNA Ligase Buffer (New England Biolabs Inc).
4. Add 1 µl of T4 DNA Ligase (New England Biolabs Inc).
5. Incubate tubes at 16 °C overnight, and store at -20 °C.

Total DNA should be 1-10 ng/µl. The insert:vector molar ratio formula is used to calculate the appropriate amount of digested PCR product (insert) and plasmid to include in the ligation reaction:

$$\frac{\frac{\text{ng insert}}{\text{bp insert}}}{\frac{\text{ng vector}}{\text{bp vector}}} = 2\text{-}6 \quad \text{Optimal ratio}$$

The optimal amount of DNA give insert:vector molar ratios between two and six. Ratios below 2:1 gives poorer ligation efficiency and ratios above 6:1 promote multiple inserts (product information, New England Biolabs Inc).

Protocol for transformation of XL 10-Gold® Ultracompetent *E. coli* (modified protocol provided by Stratagene)

1. Pre-chill tubes on ice. Preheat LB medium to 42 °C.
2. Thaw the XL 10 Gold® cells on ice.
3. When thawed, gently mix the cells by pipetting, and aliquot 100 µl to each tube.
4. Add 4 µl β-Mercaptoethanol (β-ME, Strategene) to each tube and incubate on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 4 µl of ligation mixture to the appropriate tube, swirl gently and incubate on ice for 30 minutes.
6. Heat-shock the tubes at 42 °C for 30 seconds (duration is critical).
7. Incubate the tubes on ice for 2 minutes.
8. Add 900 µl preheated (42 °C) LB to each tube, and incubate at 37 °C for 1 hour with shaking.
9. Plate 100 µl of the transformation mixture on LB plates containing appropriate antibiotics.
10. Pellet the rest of the transformation mixture at 13 000 rpm for 2 minutes. Remove the supernatant and resuspend the cell pellet in 100 µl LB. Repeat step 9.
11. Incubate the plates at 37 °C overnight.

Appendix 13
Protocol for plasmid purification, mini

Wizard® *Plus* SV Minipreps DNA Purification System from Promega

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 13 000 rpm for 3 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.
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 13 000 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 13 000 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 13 000 rpm for 1 minute. Discard flowthrough and reinsert column into collection tube.
11. Repeat step 10 with 250 µl wash solution.
12. Centrifuge at 13 000 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 13 000 rpm for 1 minute.
15. Discard column, measure the concentration of DNA and store at -20 °C.

Appendix 14
List of materials used

Table A14.1: Bacterial strains and plasmids

Strain name	Relevant features	Plasmid	Source
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
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 *(r_k–, m_k+)*, *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*IqZΔM15]	-	Promega
DH5α	*supE*44, Δ*lac*U169(φ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	-	Walter Messer
Y2H 01	DH5α	pGADT7	Ingvild Flåtten
Y2H 03	DH5α	pGBKT7	Ingvild Flåtten

Plasmid	Relevant features	Selection/ nutritional marker	Source
pCMV-HA/CDK1AF	$P_{CMV\ IE}$, pUC ori, SV 40 splice donor/splice acceptor, HA epitope tag, bearing CDK1AF	Amp	Randi Syljuåsen
pCMV-HA/CDK2F	$P_{CMV\ IE}$, pUC ori, SV 40 splice donor/splice acceptor, HA epitope tag, bearing CDK2F	Amp	Randi Syljuåsen
pGADT7	P_{ADH1}, pUC ori, 2 µ ori; yeast two-hybrid vector bearing the GAL4 activation domain	Amp/ *LEU2*	Clontech
pGAD/CDK1AF	pGADT7 bearing *CDK1AF*	Amp/ *LEU2*	This work
pGAD/CDK2F	pGADT7 bearing *CDK2F*	Amp/ *LEU2*	This work
pGAD/CDK2AF	pGADT7 bearing *CDK2AF*	Amp/ *LEU2*	This work
pGAD/CKS2	pGADT7 bearing *CKS2*	Amp/ *LEU2*	This work
pGAD/hda	pGADT7 bearing *hda*	Amp/ *LEU2*	Line Johnsen
pGBKT7	P_{ADH1}, pUC ori, 2 µ ori; yeast two-hybrid vector bearing the GAL4 DNA binding domain	Kan/ *TRP1*	Clontech
pGBK/CDK1AF	pGBKT7 bearing *CDK1AF*	Kan/ *TRP1*	This work
pGBK/CDK2F	pGBKT7 bearing *CDK2F*	Kan/ *TRP1*	This work
pGBK/CDK2AF	pGBKT7 bearing *CDK2AF*	Kan/ *TRP1*	This work
pGBK/CKS2	pGBKT7 bearing *CKS2*	Kan/ *TRP1*	This work
pGBK/dnaN	pGBKT7 bearing dnaN	Kan/ *TRP1*	Line Johnsen
pGEM-T Easy Vector	T7 and SP6 RNA Polymerase promotors, ori, f1 ori, *lacZ*; vector used for subcloningx	Amp	Promega
pGEM/CDK1AF	pGEM bearing *CDK1AF*	Amp	This work
pGEM/CKS2	pGEM bearing *CKS2*	Amp	This work
pSM/CDK2AF	Unknown vector bearing CDK2AF	Amp	David Morgan

Table A14.2: Primers

Primers	Nucleotide sequence	Producer
Primer AD	5'-AGATGGTGCACGATGCACAG-3'	Eurogentec S.A.
Primer BD	5'-TTTTCGTTTTAAAACCTAAGAGTC-3'	Eurogentec S.A.
CDK1F	5'-GGAATTCATATGGAAGATTATACCAAAATAGAGAAAATTGG-3'	Eurogentec S.A.
CDK1Rev	5'-GGAATTGGATCCCTACATCTTCTTAATCTGATTG-3'	Eurogentec S.A.
CDK2F	5'-GGAATTCATATGGAGAACTTCCAAAAGGTGG-3'	Eurogentec S.A.
CDK2Rev	5'-GGAATTGGATCCTCAGAGTCGAAGATGG-3'	Eurogentec S.A.
CKS2F	5'-GGAATTCATATGGCCCACAAGCAGATCTACTAC-3'	Eurogentec S.A.
CKS2Rev	5'-GGAATTGGATCCTCATTTTTGTTGATCTTTTGGAAGAGG-3'	Eurogentec S.A.
Primer T7	5'-TAATACGACTCACTATAGGG-3'	GATC Biotech
Primer SP6	5'-ATTTAGGTGACACTATAGAA-3'	GATC Biotech

Table A14.3: Restriction enzymes

Enzyme	Restriction site	Buffer	Producer
Bam HI (20 000 u/ml)	5'…GGATCC…3'	NEBuffer3 + BSA	New England Biolabs
Bam HI-HF (20 000 u/ml)	5'…GGATCC…3'	NEBuffer 4	New England Biolabs
EcoRI (20 000 u/ml)	5'…GAATTC…3'	NEBuffer EcoRI	New England Biolabs
NdeI (20 000 u/ml)	5'…CATATG…3'	NEBuffer 4	New England Biolabs

Table A14.4: Other enzymes and proteins

Enzyme/protein	Buffer	Producer
BSA (10 mg/ml)		New England Biolabs
PfuUltra™ II fusion HS DNA Polymerase	10 × *PfuUltra*™ II reaction buffer	Stratagene
T4 DNA Ligase	T4 DNA Ligase Reaction Buffer	New England Biolabs
T4 DNA Ligase	2× Rapid Ligation Buffer	Promega
Taq DNA Polymerase	*Taq* DNA Polymerase 10× Reaction Buffer	Promega

Table A14.5: Stock solutions of antibiotics

Antibiotic	Stock solution	Producer
Ampicillin	100 mg/ml in dH_2O	Bristol-Meyers Squibb
Cloramphenicol	30 mg/ml in dH_2O	Sigma-Aldrich
Kanamycin	50 mg/ml in dH_2O	Sigma-Aldrich

Table A14.6: Other chemicals and reagents for molecular biology

Chemical/Reagent	Concentration	Producer
2-Log DNA Ladder (marker)	1000 µg/ml	New England Biolabs
5-bromo-4-chloro-3-indolyl-β-$_D$-galactoside (X-Gal)	50 mg/ml	Sigma-Aldrich
Adenine	2 mg/ml in dH_2O	Sigma-Aldrich
Agarose, Type I		Sigma-Aldrich
β-mercaptoethanol		Stratagene
cDNA ref RNA (for CKS2)	100 ng/µl	This work
dATP	100 mM	Promega
Dithiothreitol (DTT)	1 M	Sigma-ALdrich
dNTP mix	25 mM each dNTP	AB
Ethidium Bromide	1 %	Merck
Gel Loading Dye, Blue	6×	New England Biolabs
GelRed		Biotium
Glyserol	99 %	Merck
Histidine	2 mg/ml in dH_2O	Sigma-Aldrich
Isopropyl- β-$_D$-galactosidase (IPTG)	100 mM	Sigma-Aldrich
Nuclease-Free Water		QIAgen
Sodium Chloride	0.8 % in dH_2O	Merck
Sodium dodecyl sulfate (SDS)	10 %	VWR International

Table A14.7: Commercial kits

Kit	Producer
Illumina® TotalPrep RNA Amplification Kit	Ambion Inc
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

Table A14.8: List of materials and reagents used for Y2H

Name	Relevant features	Producer
AH 109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1UAS-Mel1$_{TATA}$-lacZ*	Clontech
Bacto agar		BD
DO Supplement		Clontech
Glass beads	4 mm	Merck
Herring Testes Carrier DNA		Clontech
Minimal SD Base		Clontech
YPD		Clontech

Table A14.9: Equipment

Equipment	Model	Producer
Centrifues	5900	Kubota
	MIKRO 200R	Hettich Zentrifugen
Electric power supply	Power Pac 300	BioRad
Gel elctrophoresis tank		OWI
Heating block	QBD2	Grant
Imaging system	Chemi Genius	VWR International
Incubation cabinet		Fermaks
Nanodrop	1000	Thermo Scientific
PCR machine	PTC-100	MJ Research, Inc.
Pipettes	Pipetboy comfort	IBS Integra Biosciences
	100-1000, 10-100, 10-20 and 0.5-5 µl	Termo Scientific
Shaking incubator	Thermomixer comfort 2 ml	Eppendorf
Vortexer	MS3 digital	IKA
Water bath shaker	Innova 3100	New Brunswick Scientific
UV spectrophotometer	UV-1800	Shimadzu

Appendix 15

Protocol for transformation in *S. cerevisiae* AH109 by the LiAc method (modified)

1. Mix 100 µl of one-step buffer (appendix 4) with 10 µl of sterile 1M dithiothreitol (DTT) (vortex DTT if cloudy).
2. Resuspend three AH109 colonies from the YPD plate in this solution by gently vortexing (if colonies are < 2 mm, use several colonies).
3. In separate tubes, add ~ 500 ng of each plasmid DNA and 1 µl Herring Testes Carrier DNA (Clontech). The total volume DNA can be max 10 µl and approximately 1 µg.
4. Add one tube of mixture from step 2 to each tube containing the DNA (from step 3).
5. Vortex gently.
6. Incubate for 30 minutes at 45 °C.
7. Apply all the cells in each tube onto solid SD-DO –leu/-trp medium and use 5-7 glass beads to promote even spreading of the cells.
8. Shake the plate back and forth until the transformation mixture is spread over the agar surface and has been absorbed. Incubate the plates at 30 °C until transformants appear (3-5 days).

When transforming simultaneous cotransformations, the bait plasmid (pGBKT7) must be used in excess and the pGADT7 plasmid must be limiting.

Appendix 16

Protocol for *S. cerevisiae* two-hybrid analysis

1. Transfer appropriate transformants from their solid SD-DO –leu/-trp plate to a separate Falcon tube containing 2 ml liquid SD-DO –leu/-trp medium.
2. Incubate at 30 °C overnight in a swirling water bath.
3. Mix aliquots (500 µl) of each overnight yeast culture with 200 µl of 87 % glycerol and store at -80 °C.
4. Pellet the rest from each culture from 3 at 13 000 rpm for 3 minutes, and remove the supernatant.
5. Resuspend the cell pellet in 1 ml sterile 0.8 % saline, pellet at 13 000 rpm and remove the supernatant.
6. Repeat step 5.
7. Resuspend the cell pellet in an appropriate amount of sterile 0.8 % saline (depending on the size of the pellet).
8. Take out 100 µl from each culture in step 7, and dilute with 900 µl of sterile 0.8 % saline.
9. Measure optical density (OD) at 600 nm and adjust with sterile 0.8 % saline to OD ~ 1.0.
10. Prepare 10^{-1}, 10^{-2}, and 10^{-3} dilutions with sterile 0.8 % saline for each sample.
11. Spot 10 µl aliquots of each dilution (OD = 1, 10^{-1}, 10^{-2}, 10^{-3}) onto solid SD-DO –leu/-trp medium (control plate) or SD-DO –leu/-trp/-his medium (to score for PPI).
12. Allow the plates to dry completely and incubate at 30 °C for 3 days.

Appendix 17

Sequencing. Comparisons of all cloned fragments sequenced by GATC Biotech with the known gene sequence. The computer tool BLAST was used for all the cloned fragments. T7 is forward primer, and SP6, BD and AD are revers primers used for sequencing.

BLAST for CKS2 cloned into pGEM-T Easy-T7

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 Identities = 240/240 (100%), Gaps = 0/240 (0%)
 Strand=Plus/Plus

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Query  61   CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  120
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Sbjct  105  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  164

Query  121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  180
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Sbjct  165  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  224

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Sbjct  225  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  284
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BLAST for CKS2 cloned into pGEM-T Easy-SP6

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 Identities = 240/240 (100%), Gaps = 0/240 (0%)
 Strand=Plus/Minus

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Sbjct  313  ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  254

Query  61   CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  194

Query  121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  134

Query  181  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  240
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Sbjct  133  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  74
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BLAST for CDK1AF cloned into pGEM-T Easy-T7

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 Identities = 887/892 (99%), Gaps = 0/892 (0%)
 Strand=Plus/Plus

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Sbjct  45   ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTGCCTTTGGAGTTGTGTATAAG  104

Query  61   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
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Sbjct  105  GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  164

Query  121  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
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Sbjct  165  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  224

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Sbjct  345  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  404

Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
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Sbjct  405  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  464

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Sbjct  465  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  524

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Sbjct  585  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  644

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Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
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Sbjct  765  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  824

Query  781  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  840
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Sbjct  825  TTGNATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  884

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BLAST for CDK1AF cloned into pGEM-T Easy-SP6

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 Strand=Plus/Minus

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Query  241  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
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Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
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Sbjct  544  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  485

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BLAST for CKS2 cloned into pGADT7 #3-T7

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 Identities = 239/240 (99%), Gaps = 0/240 (0%)
 Strand=Plus/Plus

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BLAST for CKS2 cloned into pGBKT7 #1-T7

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  ATGGCCCACAAGCAGATCTACTACTCGGACAAGTACTTCGACGAACACTACGAGTACCGG  339

Query  61   CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338  CATGTTATGTTACCCAGAGAACTTTCCAAACAAGTACCTAAAACTCATCTGATGTCTGAA  279

Query  121  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278  GAGGAGTGGAGGAGACTTGGTGTCCAACAGAGTCTAGGCTGGGTTCATTACATGATTCAT  219

Query  181  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  218  GAGCCAGAACCACATATTCTTCTCTTTAGACGACCTCTTCCAAAAGATCAACAAAAATGA  159
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BLAST for CDK1AF cloned into pGADT7 #1-T7

```
>lcl|9073
Length=1023

 Score = 1629 bits (882),  Expect = 0.0
 Identities = 889/894 (99%), Gaps = 0/894 (0%)
 Strand=Plus/Plus

Query  1    ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
            |||| |||||||||||| |||||||||||||||| || ||| ||||||||||||||||
Sbjct  7    ATGGNAGATTATACCAAANTAGAGAAAATTGGAGAANGTGCCTTTGGAGTTGTGTATAAG  66

Query  61   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  126

Query  121  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  186

Query  181  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  246

Query  241  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  306

Query  301  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  366

Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  426

Query  421  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  486

Query  481  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  546

Query  541  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  606

Query  601  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  666

Query  661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  726

Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  727  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  786

Query  781  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  787  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  846

Query  841  GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  894
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  847  GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  900
```

BLAST for CDK1AF cloned into pGADT7 #1-AD

```
>lcl|12121
Length=1002

 Score = 1618 bits (876),  Expect = 0.0
 Identities = 888/895 (99%), Gaps = 1/895 (0%)
 Strand=Plus/Minus

Query  1    ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
            |||||||||||||||||||||||||||||||||||| ||| ||||||||||||||||
Sbjct  924  ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTGCCTTTGGAGTTGTGTATAAG  865

Query  61   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  864  GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  805

Query  121  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  745

Query  181  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  685

Query  241  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  684  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  625

Query  301  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  624  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  565

Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  505

Query  421  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  445

Query  481  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  385

Query  541  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  325

Query  601  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  265

Query  661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  205

Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  145

Query  781  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  144  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  85

Query  841  GCACTGAATCATCCATATTTTAATGATTTGGACAATCA-GATTAAGAAGATGTAG  894
            |||||||||||||||||||||||||||||| |||    ||||||||||||||||
Sbjct  84   GCACTGAATCATCCATATTTTAATGATTTGGNCAANGNNGATTAAGAAGATGTAG  30
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BLAST for CDK1AF cloned into pGBKT7 #11-T7

```
>lcl|59131
Length=1156

 Score = 1629 bits (882),  Expect = 0.0
 Identities = 889/894 (99%), Gaps = 0/894 (0%)
 Strand=Plus/Plus

Query  1    ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
            ||||  ||||||||||||||||||||||||  ||||||  ||| ||||||||||||||||
Sbjct  7    ATGGNAGATTATACCAAAATAGAGAAAATTGNNGAAGGTGCCTTTGGAGTTGTGTATAAG  66

Query  61   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  126

Query  121  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  186

Query  181  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  246

Query  241  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  306

Query  301  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  366

Query  361  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  426

Query  421  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  486

Query  481  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  546

Query  541  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  606

Query  601  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  607  AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  666

Query  661  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  667  GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  726

Query  721  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  727  TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  786

Query  781  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  787  TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  846

Query  841  GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  894
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  847  GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  900
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BLAST for CDK1AF cloned into pGBKT7 #11-BD

```
>lcl|62571
Length=1152

 Score = 1640 bits (888),  Expect = 0.0
 Identities = 892/894 (99%), Gaps = 0/894 (0%)
 Strand=Plus/Minus

Query  1     ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTACCTATGGAGTTGTGTATAAG  60
             ||||||||||||||||||||||||||||||||||||||| ||| ||||||||||||||||
Sbjct  1053  ATGGAAGATTATACCAAAATAGAGAAAATTGGAGAAGGTGCCTTTGGAGTTGTGTATAAG  994

Query  61    GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGaaaaaaaTCAGACTAGAAAGTGAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  993   GGTAGACACAAAACTACAGGTCAAGTGGTAGCCATGAAAAAAATCAGACTAGAAAGTGAA  934

Query  121   GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  933   GAGGAAGGGGTTCCTAGTACTGCAATTCGGGAAATTTCTCTATTAAAGGAACTTCGTCAT  874

Query  181   CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  873   CCAAATATAGTCAGTCTTCAGGATGTGCTTATGCAGGATTCCAGGTTATATCTCATCTTT  814

Query  241   GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813   GAGTTTCTTTCCATGGATCTGAAGAAATACTTGGATTCTATCCCTCCTGGTCAGTACATG  754

Query  301   GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753   GATTCTTCACTTGTTAAGAGTTATTTATACCAAATCCTACAGGGGATTGTGTTTTGTCAC  694

Query  361   TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693   TCTAGAAGAGTTCTTCACAGAGACTTAAAACCTCAAAATCTCTTGATTGATGACAAAGGA  634

Query  421   ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633   ACAATTAAACTGGCTGATTTTGGCCTTGCCAGAGCTTTTGGAATACCTATCAGAGTATAT  574

Query  481   ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573   ACACATGAGGTAGTAACACTCTGGTACAGATCTCCAGAAGTATTGCTGGGGTCAGCTCGT  514

Query  541   TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513   TACTCAACTCCAGTTGACATTTGGAGTATAGGCACCATATTTGCTGAACTAGCAACTAAG  454

Query  601   AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453   AAACCACTTTTCCATGGGGATTCAGAAATTGATCAACTCTTCAGGATTTTCAGAGCTTTG  394

Query  661   GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  393   GGCACTCCCAATAATGAAGTGTGGCCAGAAGTGGAATCTTTACAGGACTATAAGAATACA  334

Query  721   TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  333   TTTCCCAAATGGAAACCAGGAAGCCTAGCATCCCATGTCAAAAACTTGGATGAAAATGGC  274

Query  781   TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273   TTGGATTTGCTCTCGAAAATGTTAATCTATGATCCAGCCAAACGAATTTCTGGCAAAATG  214

Query  841   GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  894
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213   GCACTGAATCATCCATATTTTAATGATTTGGACAATCAGATTAAGAAGATGTAG  160
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BLAST for CDK2AF cloned into pGADT7 #7-T7

```
>lcl|22439
Length=1152

 Score = 1635 bits (885),  Expect = 0.0
 Identities = 892/897 (99%), Gaps = 0/897 (0%)
 Strand=Plus/Plus

Query  1    ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
            |||||| |||||| |||||||||||||||||||||| | | ||||||||||||||||||
Sbjct  18   ATGGAGNACTTCCNAAAGGTGGAAAAGATCGGAGAGGGCNCCTTCGGAGTTGTGTACAAA  77

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  137

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  138  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  197

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  257

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  317

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  318  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  377

Query  361  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  378  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  437

Query  421  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  497

Query  481  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  557

Query  541  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  617

Query  601  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  618  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  677

Query  661  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  678  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  737

Query  721  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  738  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  797

Query  781  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  798  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  857

Query  841  CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  858  CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  914
```

BLAST for CDK2AF cloned into pGADT7 #7-AD

```
>lcl|14991
Length=1048

 Score = 1637 bits (886),  Expect = 0.0
 Identities = 893/897 (99%), Gaps = 0/897 (0%)
 Strand=Plus/Minus

Query  1    ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
            |||||||||||||||||||||||||||||||||||||| | | ||||||||||||||||
Sbjct  928  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  869

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  868  GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  809

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  808  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  749

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  748  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  689

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  688  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  629

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  628  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  569

Query  361  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  509

Query  421  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  449

Query  481  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  389

Query  541  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  329

Query  601  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  269

Query  661  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  209

Query  721  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  149

Query  781  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  89

Query  841  CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  88   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGNACCCCATCTTCGACTCTGA  32
```

BLAST for CDK2AF cloned into pGBKT7 #4-T7

```
>lcl|38089
Length=1107

 Score = 1613 bits (873),  Expect = 0.0
 Identities = 888/897 (98%), Gaps = 1/897 (0%)
 Strand=Plus/Plus

Query  1    ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
            |||||| ||||||  ||||||||||||||||||   ||| | | ||||||||||||||||
Sbjct  6    ATGGAGNACTTCC-NAAGGTGGAAAAGATCGGACNNGGCGCCTTCGGAGTTGTGTACAAA  64

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  124

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  184

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  244

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  304

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  364

Query  361  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  424

Query  421  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  484

Query  481  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  544

Query  541  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  604

Query  601  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  664

Query  661  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  665  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  724

Query  721  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  725  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  784

Query  781  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  785  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  844

Query  841  CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  845  CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  901
```

BLAST for CDK2AF cloned into pGBKT7 #4-BD

```
>lcl|63797
Length=1162

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

Query  1     ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
             ||||||||||||||||||||||||||||||||||||||| | | ||||||||||||||||
Sbjct  1055  ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCGCCTTCGGAGTTGTGTACAAA  996

Query  61    GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  995   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  936

Query  121   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  935   ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  876

Query  181   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  875   CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  816

Query  241   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  815   GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  756

Query  301   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  755   CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  696

Query  361   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  695   CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  636

Query  421   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  635   ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  576

Query  481   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  575   CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  516

Query  541   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  456

Query  601   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  396

Query  661   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395   ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  336

Query  721   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335   CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  276

Query  781   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275   AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  216

Query  841   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215   CTGGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  159
```

BLAST for CDK2F cloned into pGADT7 #1-T7

```
>lcl|8891
Length=776

 Score = 1354 bits (733),  Expect = 0.0
 Identities = 759/776 (97%), Gaps = 3/776 (0%)
 Strand=Plus/Plus

Query  2    TGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGG--CACGTACGGAGTTGTGTACAA  59
            ||||| ||||||||||||||||||||||||||||||  | | |    ||||||||||||
Sbjct  1    TGGAGNACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCCCCCTTNNANAGTTGTGTACAA  60

Query  60   AGCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGA  120

Query  120  GACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCA  180

Query  180  TCCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTT  240

Query  240  TGAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCC  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TGAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCC  300

Query  300  TCTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTC  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TCTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTC  360

Query  360  TCATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TCATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGC  420

Query  420  CATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACAC  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACAC  480

Query  480  CCATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTA  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CCATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTA  540

Query  540  TTCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCG  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TTCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCG  600

Query  600  GGCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGG  659
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GGCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGG  660

Query  660  GACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTT  719
            ||| ||||||||||||||||||||||||||||||||||||||||||||||  ||   ||
Sbjct  661  GACNCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAANNCANNNTT  720

Query  720  CCCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCC-TGGATGAAGAT  774
            ||||||||||||||||||||||||||||||||||||||||||| || ||||||||
Sbjct  721  CCCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCCTGAATGAAGAT  776
```

GATC Biotech could not provide the reverse sequence, but the ab1 and fas files were analyzed. The inserted fragment in pGADT7 was CDK2F.

BLAST for CDK2F cloned into pGBKT7 #7-T7

```
>lcl|30645
Length=891

 Score = 1578 bits (854),  Expect = 0.0
 Identities = 860/865 (99%), Gaps = 0/865 (0%)
 Strand=Plus/Plus

Query  1    ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTACGGAGTTGTGTACAAA  60
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  25   ATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGCACGTTCGGAGTTGTGTACAAA  84

Query  61   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   GCCAGAAACAAGTTGACGGGAGAGGTGGTGGCGCTTAAGAAAATCCGCCTGGACACTGAG  144

Query  121  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  ACTGAGGGTGTGCCCAGTACTGCCATCCGAGAGATCTCTCTGCTTAAGGAGCTTAACCAT  204

Query  181  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  CCTAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTT  264

Query  241  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  GAATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCT  324

Query  301  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  CTTCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCT  384

Query  361  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  CATCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCC  444

Query  421  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  ATCAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACC  504

Query  481  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  CATGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTAT  564

Query  541  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  TCCACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGG  624

Query  601  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  GCCCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGG  684

Query  661  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  685  ACCCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTC  744

Query  721  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||   ||||||||||||
Sbjct  745  CCCAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGNNNGAAGATGGACGG  804

Query  781  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  805  AGCTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCC  864

Query  841  CTGGCTCACCCTTTCTTCCAGGATG  865
            |||| ||||||||||||||||||||
Sbjct  865  CTGGNTCACCCTTTCTTCCAGGATG  889
```

BLAST for CDK2F cloned into pGBKT7 #7-BD

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>lcl|35035
Length=873

 Score = 1317 bits (713),  Expect = 0.0
 Identities = 714/715 (99%), Gaps = 0/715 (0%)
 Strand=Plus/Minus

Query  183  TAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTTGA  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  873  TAATATTGTCAAGCTGCTGGATGTCATTCACACAGAAAATAAACTCTACCTGGTTTTTGA  814

Query  243  ATTTCTGCACCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCTCT  302
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813  ATTTCTGCNCCAAGATCTCAAGAAATTCATGGATGCCTCTGCTCTCACTGGCATTCCTCT  754

Query  303  TCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCTCA  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  TCCCCTCATCAAGAGCTATCTGTTCCAGCTGCTCCAGGGCCTAGCTTTCTGCCATTCTCA  694

Query  363  TCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCCAT  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693  TCGGGTCCTCCACCGAGACCTTAAACCTCAGAATCTGCTTATTAACACAGAGGGGGCCAT  634

Query  423  CAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACCCA  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633  CAAGCTAGCAGACTTTGGACTAGCCAGAGCTTTTGGAGTCCCTGTTCGTACTTACACCCA  574

Query  483  TGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTATTC  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573  TGAGGTGGTGACCCTGTGGTACCGAGCTCCTGAAATCCTCCTGGGCTGCAAATATTATTC  514

Query  543  CACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGGGC  602
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  CACAGCTGTGGACATCTGGAGCCTGGGCTGCATCTTTGCTGAGATGGTGACTCGCCGGGC  454

Query  603  CCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGGAC  662
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453  CCTATTCCCTGGAGATTCTGAGATTGACCAGCTCTTCCGGATCTTTCGGACTCTGGGGAC  394

Query  663  CCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTCCC  722
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  393  CCCAGATGAGGTGGTGTGGCCAGGAGTTACTTCTATGCCTGATTACAAGCCAAGTTTCCC  334

Query  723  CAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGGAG  782
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  333  CAAGTGGGCCCGGCAAGATTTTAGTAAAGTTGTACCTCCCCTGGATGAAGATGGACGGAG  274

Query  783  CTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCCCT  842
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  CTTGTTATCGCAAATGCTGCACTACGACCCTAACAAGCGGATTTCGGCCAAGGCAGCCCT  214

Query  843  GGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  897
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  GGCTCACCCTTTCTTCCAGGATGTGACCAAGCCAGTACCCCATCTTCGACTCTGA  159
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