3 MATERIALS AND METHODS

1.1 MATERIALS

1.1.1 MICROSCOPES

Yeast cells were examined for sporulation under phase contrast using a Zeiss Axiostar. Yeast spores were dissected using a Zeiss Axioscop and micromanipulator. Dissecting needles were purchased from Singer Instruments.

1.1.2 SPECTROFOTOMETER

A UV/VIS spectrophotometer was utilized to monitor and analyse yeast cell growth for the senescence experiments.

1.1.3 OLIGONUCLEOTIDES

All oligonucleotides were purchased from Invitrogen Labs. The sequences of oligonucleotides used in the project are shown in Table 1.

1.1.4 YEAST STRAINS

A collection of different geographical diploid isolates of *Saccharomyces cerevisiae* and *S. paradoxus* were used. For the senescence experiments *est2* yeast cells were obtained. For the ALT experiment double mutants were utilized (est2::KANMX/EST2, rad50::HYG/RAD50). Wild type yeast strains were used for the telomere tag experiments and for the variation in telomere length analysis.

1.1.5 PLASMIDS

Several plasmid were utilized for the *knock out* experiments, which contained *URA3*, *KANMX* and *HYG* markers. The transformed pFEP24 plasmid with the artificial telomere was used for the telomere tag experiment. All plasmids were purified with QIAprep Miniprep Kit (QIAGEN)

Figure 6 Plasmid utilized for the selection of *est2::KANMX/EST2 S. cerevisiae* and *S. paradoxus* mutants

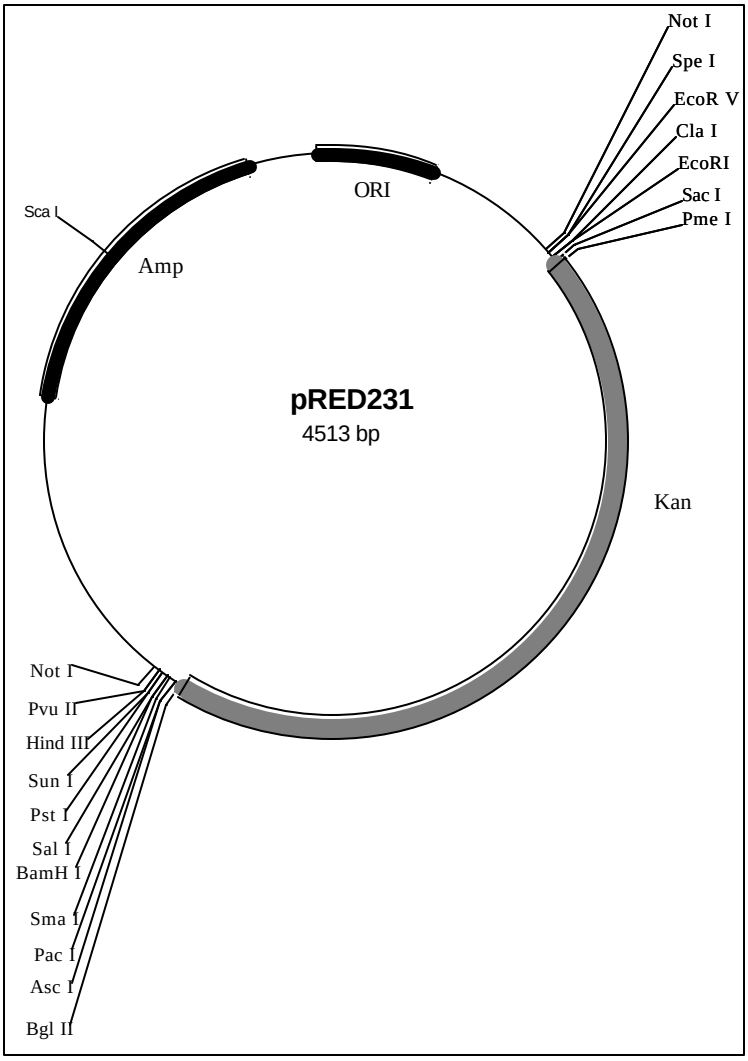
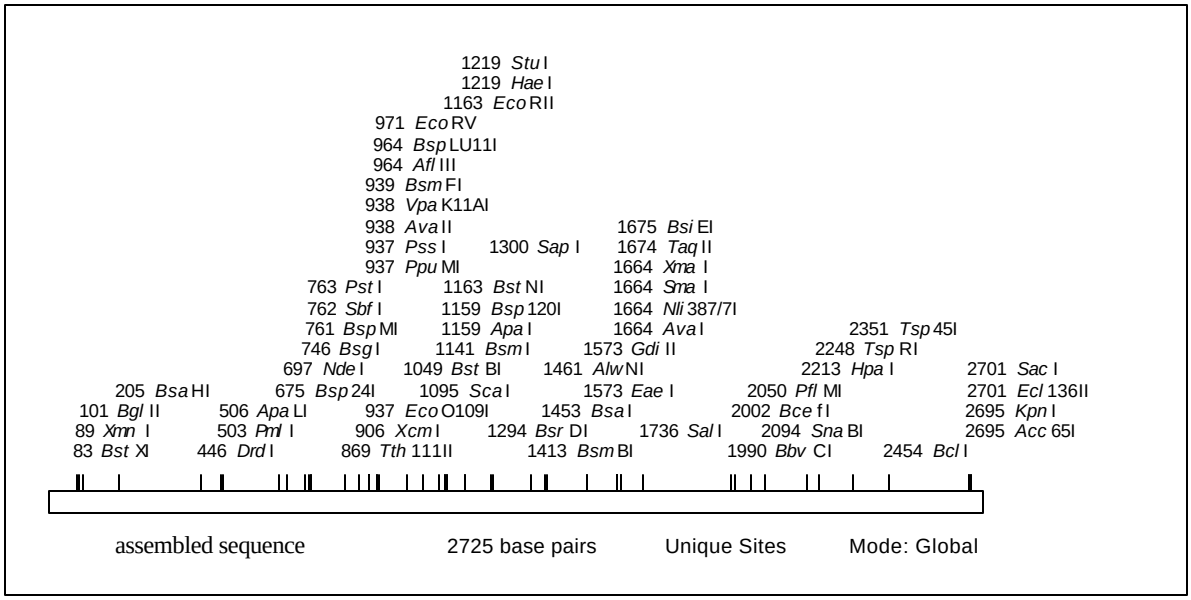


Figure 7 Telomere Tag assembled sequence 2.7 kb long (After PCR of plasmid pFEP24) roughly: *ERR1* 1-540 bp, *URA3* marker 600-1728 bp, Core X 1750-2500 bp, 2448 bp-end telomere.



1.2 METHODS

1.2.1 STANDARD MOLECULAR BIOLOGY TECHNIQUES

The standard techniques used in this project include: agarose gel electrophoresis, restriction enzyme digests and PCR amplification and purification.

1.2.2 SPORULATION AND TETRAD DISSECTION

Diploids were grown on YPD plates overnight before being replica-plated onto Kac media (2% KOAc, 2% bacto-agar) and incubated at room temperature for one week (or until spore formation) to induce sporulation (sporulation is induced by starving cells for nitrogen). After sporulation a small path of cells was transferred to 100 µl dissecting buffer (1M sorbitol, 10mM EDTA, 10mM NaPO4 pH 7.2) and 5 µl zymolyase (5mg/ml in dissecting buffer) and incubated at 37°C with shaking for 30 minutes. A further 400 µl dissecting buffer was added and the cells can be stored at 4°C for uo to four weeks, aliquots of cells were spread onto a YPD plate for dissecting.

Table 2 Sequence of oligonucleotides used in the project

LAB	PRIMER	SEQUENCE 5' to 3'	DESCRPTION
2214	DF RAD50/B	GGTGAATTAGAAGCTGGTAAGGAGACATACGAAAAGAACAGAAGTCACGTACGCTGCAGGTCGAC	primers for amplify MX cassette and disrupt RAD50
2215	DR RAD50/B	AGTTCTTGTAACAACTTACTTTTAAACGATTCATTTTCAAACTTTCTATCGATGAATTCGAGCTCG	
2206	A1 RAD50/B	GCAGTCAGTAGAGCACTTG	primers to check RAD50 disruption
2207	A4 RAD50/B	GGACGAATCTCAGACTCAG	
2232	TEL-URA F	TTCAGATTATCGCAGATGACCTCACGTGCACCAATAGGGCTCGGATCGCCCGTGCTATAAGATACGCAATTAGATCCGAG	used to amplify URA3-CoreX-TG1-3 construct from pFEP24 and add 60 bp of ERR1 to tag chromosome XV-R
2233	TEL-URA R	CTCACTATAGGGCGAATTC	
2251	ERR1 FR	CCC GTG TAA ATG GCT TAG TGC	primers designed for screen XV-R tagging designed in ERR1 (upstream the tagged region) and internal to URA3 respectively
2252	URA3 RV	CAG ATC CTG TAG AGA CCA CAT C	
2230	URA3-DF	ATGTCAAAAGCTACATATAAAGAACGTGCTGCTACTCATCCTAGTCGTACGCTGCAGGTCGAC	primers for amplify MX cassette and disrupt URA3 from START to STOP
2231	URA3-DR	TTAATTTTGCTGGCCGCATCTTCTCAAATACGCCTCCCAGCCCGCATCGATGAATTCGAGCTCG	
2234	URA3 A1	CCT ACG CGT ATT CAT GAA TGA C	primers to check RAD50 deletion
2235	URA3 A4	CCA GAT TAG AGT ACA AAC GC	
2282	FWURA3 INT	CATTGGATGTTCGTACCACC	primers for generate URA3 probe (pLEM3 was used instead)
2283	REVURA3 INT	CTCTACCTTAGCATCCCTTC	
329	EST2 A1	GGA CTT GTC GCA TTT GAG TAG	primers used to amplify a disrupted gene from S. cerevisiae and used to KO EST2 of S. paradoxus
330	EST2 A4	GCA TGA GAA CGG TGT TTG TTG	
1930	EST2-A1	GTGGGTAATAGGTGTAACG	primers used to KO S. paradoxus EST2 (after a deleted strain was obtained using S. cerevisiae construct)
1931	EST2-A4	CATACTCGATTAAAGGATCGC	
1928	HO-A1	GTTATGTGCGCAGATGGCTC	primers for amplify MX cassette and disrupt HO
1929	HO-A4	GTCACAGTAGCAGACATTCC	
1926	HO-DF	AGAGCTTTTGAAGGTGAACCTGGTAGGTTAGACCCTAGGCGTAGAAC CGTACGCTGCAGGTCGAC	primers to check HO disruption
1927	HO-DR	GGCTCTTTTGTTGTACCGTCACCTAGCCATAGACCAAGCATCCAAGCCATATCGATGAATTCGAGCTCG	

Table 3 Yeast Strains Used in the Project

STRAINS USED IN SINGLE TELOMERE TAGGING			
strain	**der.from**	**WT**	**genotype**
FAR EAST			
YGL107		N-44	HO/HO, ura3D::KanMX/URA3 (complete deletion)
YGL108	YGL107	N-44	HO/HO, ura3D::KanMX/ura3D::KanMX(complete deletion)
YGL120	YGL108	N-44	HO/HO, ura3D::HygMX/ura3D::HygMX, err1::URA3-COREX-TG1-3 (at TEL XV)/ERR1
EUROPEAN			
YGL105	OS142	CBS432	HO/HO, ura3D::HYGMX/URA3 (complete deletion)/URA3
YGL114	YGL105	CBS432	HO/HO, ura3D::HygMX/ura3D::HygMX(complete deletion)
YGL119	YGL114	CBS432	HO/HO, ura3D::HygMX/ura3D::HygMX, err1::URA3-COREX-TG1-3 (at TEL XV)/ERR1
YGL139	YGL119	CBS432	HO/HO, ura3D::HygMX/ura3D::HygMX, err1::URA3-COREX-TG1-3 (at TEL XV)/err1::URA3-COREX-TG1-3 (at TEL XV)
YGL140	YGL119	CBS432	HO/HO, ura3D::HygMX/ura3D::HygMX, err1::URA3-COREX-TG1-3 (at TEL XV)/err1::URA3-COREX-TG1-3 (at TEL XV)
AMERICAN			
YGL148	YGL147	OS115	HO/HO, ura3D::KanMX(complete deletion)/ura3D::KanMX(complete deletion)
CROSSES			
YGL149	YGL139xYGL108	CBS432 TAG X N-44	HO/HO, ura3D::KanMX (complete deletion)/ura3D::HYGMX (complete deletion), err1::URA3-COREX-TG1-3 (at TEL XV)/ERR1
YGL150	YGL139xYGL148	CBS432 TAG X OS115	HO/HO, ura3D::KanMX/URA3 (complete deletion)/ura3D::HYGMX(complete deletion), err1::URA3-COREX-TG1-3 (at TEL XV)/ERR1

STRAINS USED IN ALT AND SENESCENCE			
AMERICAN			
YGL143	OS115	YPS138	HO/HO, EST2/est2::KanMX
FAR EAST			
YGL95	YGL54	N-44	HO/HO, a/@, est2::KANMX/EST2, rad50::HYG/RAD50 (complete deletion using pPCR amplified with 2143-4 (A1-A4) from YGL94
YGL96	YGL54	N-44	HO/HO, a/@, est2::KANMX/EST2, rad50::HYG/RAD50 (900bp of internal deletion using primers 2214-5)
YGL54		N-44	HO/HO, est2::KanMX/EST2
YGL55	YGL54	N-44	HO/HO, est2::NATMX/EST2
YGL56	YGL54	N-44	HO/HO, est2::HYGMX/EST2
YGL89	YGL86	N-45	ho::HYG/HO, est2::NATMX/EST2
YGL90	YGL86	N-45	ho::NAT/HO, est2::KanMX/EST2
YGL61		N-45	HO/HO, est2::KanMX/EST2
YGL80	YGL61	N-45	HO/HO, est2::HYGMX/EST2
YGL86	YGL61	N-45	ho::HYG/HO, est2::KanMX/EST2
YGL66		N-46	HO/HO, est2::KanMX/EST2
YGL81	YGL66	N-46	HO/HO, est2::HYGMX/EST2
YGL83	YGL66	N-46	ho::HYG/HO, est2::KanMX/EST2
YGL87	YGL83	N-46	ho::NAT/HO, est2::KanMX/EST2
YGL88	YGL83	N-46	ho::HYG/HO, est2::NATMX/EST2
EUROPEAN			
YGL135	OS142	CBS432	HO/H, OEST2/est2::KanMX
YGL141	OS7	DBVPG6566	HO/HO, EST2/est2::KanMX
YGL144	OS38	Q70.8	HO/HO, EST2/est2::KanMX
CROSSES			
YGL106	CC161	N-44xCBS432	ho::NAT/ho::HYG, est2::HYGMX/EST2

1.2.3 LITHIUM ACETATE TRANSFORMATION OF YEAST

Yeast cells were grown over night in 5 ml YPD at 30 degrees with shaking. The cells should have reached stationary phase by the morning. Then 400 µl of the overnight culture were inoculated into 5 ml of fresh YPD and grown for a further 4 hours using the same conditions as above. The cells were harvested by centrifugation at 2,800 rpm for five minutes, resuspended in 1 ml of sterile H_2O and transferred into two eppendorf tubes. Each culture gives sufficient cells for two transformations. The cells were washed in 1 ml sterile H_2O and then twice in 100mM LiAc. The following components were then added in the following order to the cell pellet: 240 µl PEG (50% w/v), 36 µl LiAc 1M, 25 µl Salmon Sperm DNA (after denaturing at 95°C for 5 min, then put on ice), 20 µl DNA for transformation , 25 µl sterile distilled water. After vortexing to suspension the cells were incubated at 30°C for 30 minutes with shaking and then heat shocked at 42°C for 20 minutes. The transformed cells were washed once in 1 ml sterile H_2O before being plated onto appropriate selective media. For KanMX selection the cells were resuspended in 1ml YPD and incubated for either 4 hours at 30°C or overnight at 4°C to allow expression of the gene prior to plating on G418 plates.

1.2.4 YEAST MEDIA

Strains were grown at 30°C on standard yeast complete medium (YPD), minimal media, media missing one or more amino acids (drop out media) or on G418 plates. YPD contains 1% yeast extract, 2% bacto-peptone, 2% glucose. After autoclaving, 10 ml/l of a 0.5% adenine hemisulphate solution (dissolved in 0.05M HCl, filter sterilised and stored at room temperature) was added. Minimal media is 0.67% yeast nitrogen base (without amino acids) and 2% glucose and is used to select for auxotrophic complementation in diploid strains.

For making Geneticin (G418) media plates, add 300 mg/l of G418 to standard YPD agar. Store the plates for one week at 4°C before using. Ready to use G418 plates are kept at -20°C. Synthetic complete and drop out media is 0.67% nitrogen base (without amino acids), 2% glucose and appropriate amounts of drop out mixture. Drop out mixtures were made by combining amino acid powders in the quantities below (remembering to omit the appropriate supplement).

Table 4 Amino Acid Concentrations for Drop Out Media

AMINO ACIDS	mg/l
Adenine	800
L-arginine	800
L-aspartic acid	4000
L-glutamic acid (monosodium salt)	800
L-histidine	800
L-leucine	1200
L-methionine	800
L-phenylalanine	2000
L-threonine	8000
L-tryptophan	800
L-tyrosine	1200
Uracil	800

Threonine and aspartic acid drop out mixtures are at 540 mg/l, all other amino acids at 870 mg/l. After autoclaving 6 ml of sterile 1% lysine was added per liter of media, and 3 ml each of 1% lysine and threonine (omitted in the appropriate drop outs). Yeast media was adjusted to pH 6.5 prior to autoclaving and solid agar had 2.5% bacto-agar added. 5-fluoro-orotic media was made by supplementing uracil drop out media with 50 mg/l of uracil. After autoclaving 1 g of 5-FOA was added per liter of media. For KAC media add in flask A 10g of potassium acetate and 1g of yeast extract to 500 ml of sterile H_2O. In Flask B disolve 20g of agar in 500 ml of sterile water. After autoclaving add 2 ml of 40% glucose to flask A. Finally mix both flasks and pour onto plates.

1.2.5 STORAGE

For short term storage, yeast were left on plates at 4°C. For longer-term storage (more than 3 weeks) glycerol stocks were made by adding a small patch of cells to 1 ml of 25% glycerol in sterile H_2O and storing at -80°C. DNA is kept at -20°C.

1.2.6 GENOMIC DNA EXTRACTION USING KAC

Yeast cells were grown overnight in 5 ml of liquid YPD and harvested by centrifugation at 3,600 rpm for 5 minutes. The cells were then resuspended in 0.5 ml spheroplasting solution (solution A: 1.2M sorbitol, 200mM Tris-HCL pH 7.5, 20mM EDTA, 0.1% ß-mercaptoethanol) and transferred to eppendorf tubes containing 50µl zymolyase (10µg/ml, made up in the spheroplasting solution minus ß-mercaptoethanol). The cells were spheroplasted for 30 minutes (exact time is strain dependent) at 37°C, then were centrifuged at 13 000 rpm for 30 seconds. The cells were gently resuspended in 50 µl 1M sorbitol and 0.5 ml lysis solution (solution B: 50mM Tris-HCL pH 7.5, 100mM NaCl, 100mM EDTA, 0.5% SDS) to which was added 20 µl of proteinase K (10 mg/ml in 1 x TE buffer pH 7.5) and 50 µl of a 1 mg/ml RNAse solution. After incubation at 65°C for at least 2 hours the samples were put on ice for five minutes then 20 µl of ice cold 5M KOAc was added and the cells incubated for a further 515 minutes on ice. The samples were then centrifugated at 4°C and 13 000 rpm for 20 minutes and the supernatant decanted into fresh eppendorf tubes. An equal volume of isopropanol was added and the DNA was spooled by inversion, precipitating the DNA, then centrifuged at 13 000 rpm for 1 minute and the supernatant removed. DNA was washed with 1 ml 70% ethanol, air-dried briefly and dissolved in 100-200 µl 1 x TE pH 8.0.

1.2.7 GENOMIC PHENOL-CHLOROFORM DNA EXTRACTION

Cells are taken from frozen stocks and inoculated in 5 ml YPD liquid media at 30°C overnight. The cells are resuspended in 0.5 ml of dH_2O, spinned briefly and resuspended in 0.5 ml of solution A (1.2 M sorbitol, 0.2 M Tris-HCl (pH 8.5), 0.1 EDTA, H_20, 0.1% beta mecapto-ethanol). Then 50 µl of Zymolase (10mg/ml) is added. This solution is then incubated at 37°C in

a shaker for 25 minutes or until spheroplasts appear. Gently invert to observe the presence of spheroplasts. Carefully resuspend by flicking in 50 µl of 1 M Sorbitol. Then add 0.5 ml of solution B (50 mM Tris-Hcl (pH 7.5), 100 mM NaCl, 100mM EDTA, 0.5% SDS, H_2O). Additionally, 20 µl of 10 mg/ml of proteinase K and 5 µl of RNAse (10mg/ml) are added. This solution is then incubated at 65°C in a heating block for two hours. Remember to invert tubes every 20 minutes to obtain the complete digestion of cellular components.

In the extraction hood, add 500 µl of Phenol/Chloroform/Isoamylalcohol (25ml/24ml/1ml) solution. Invert tube several times, spin for 5 minuters in a microcentrifuge at 13,000 rpm. After centrifugation two distinct layers are formed, collect only the upper phase and discard the lower phase with the white protein precipitate found between the two phase. This process is generally repeated three times, to obtain very pure DNA. Finally, 1 ml of 100% ethanol is added, the eppendorf is inverted violently several times until a white blob of DNA precipitate is observed. The ethanol is evaporated using a heating block. DNA is then resuspended and frozen for future use.

1.2.8 CHEF GEL

The DNA from individual chromosomes can be purified from an agarose gel following a technique called contour clamped homogeneous electric fields (CHEF). Each band which is generated by the CHEF gel technique is an actual chromosome. DNA is extracted from the yeast cells that have been cultured over night. They are resuspended in 1 ml 50 mM EDTA and centrifuged for 30 seconds. Before making the CHEF plugs, 200 µl 50 mM EDTA is added to the pellet. CHEF plugs are made by adding 100 µl of an SCE solution (1 ml SCE, 9 mg zymolase, 50 µl ßME) with 0.5 ml agarose(1% LMP agarose in 0.125 M EDTA). Mix the pellet, SCE solution and warm agarose and immediately pipette into plug formers which were previously on ice. CHEF plugs are transfered into eppendorfs after they have set for approximately 10 minutes. To the eppendorf tube 0.5 EDTA solution is added (9 ml 0.5 M EDTA, 1 ml 1M TRIS HCL pH 8.0, 0.5 ml ßME. The CHEF plugs are then incubated for a minimum of four hours (over night) at 37°C. The EDTA solution is removed and 0.5 ml of Proteinase solution (10 mg Proteinase K, 9

ml 0.5 M EDTA, 1 ml 10% sodium sakosyl, 0.1 mg /ml) is added for another six hours at 37°C. After incubation the proteinase K solution is pipetted off and the CHEF plugs are washed in 1 x TE for one hour. Finally the CHEF plugs are stored in a TRIS/EDTA solution (9ml 0.5 M EDTA and 1 ml TRIS HCl pH8) at 4°C. When running a CHEF gel, load only 1/3 of the CHEF plug into the 1% agarose gel wells. The settings for running the CHEF gel (time, number of blocks, voltage and degree of angle) are unique to each experiment.

1.2.9 SOUTHERN BLOT

Technique:

Southern transfer results in a membrane that carries a replica of the DNA bands from the agarose gel. If a label probe is applied, hybridization occurs and autoradiography reveals the restriction fragment containing the probed gene. Consecuently, southern transfer and hybridization can be used to locate the position of a gene within a genome.

Experimentally:

In the laboratory, genomic DNA was prepared (isolated and purified) following the phenol-chlorophorm protocol. The DNA from each strain was digested overnight utilizing different enzymes:

For the Telomere Length Variation Analysis, ALT and Senescence experiments:

1. *XhoI* digests
2. Mixture of 5, 4 cutters (*Alu I, Hinf I, Hae III, Msp I, Mbo I*)

For the Telomere Tag experiment:

3. *EcoRV* digest
4. *ScaI* digest
5. *StuI* digest

The genomic DNA digests were run in 25 cm long 0.9 % agarose gels for better resolution of size differences. Southern blotting was performed by using Hybond-N+ membrane (Amersham Biosciences) and UV cross-linked. The membrane was then hybridised with various flourescein-labelled probes (Table 4). Hybridisation was detected using *Gene Images CDP-star* detection kit (American Pharmacia Biotech).

Table 5 Probes used in this study

Name	Gene	Notes and referentes
pEL50	*ERRI*	Enolase-related repeat
pKS4	Y'	TA cloning of 4.6 kbp of internal fragment of Y' shared by the short and long form ending before the 36 bp repeat
pLEM3	*URA3*	Marker inserted into Telomere Tag construct
pKK38	Telomeric repeats	$C_{1-3}A/TG_{1-3}$

Description of Southern Blot protocol follows:

The digests were run at low voltage overnight. The gel was rinsed in water and incubated 30 minutes in denaturation buffer, rinsed and then incubated 2X for 15 minutes in neutralization buffer. The gel was blotted on the Hybond-N+ membrane overnight. The DNA was UV crosslinked in order to bind the DNA to the membrane and then rinsed in 4X SSC. The probe was denatured (boiled 10 minutes and 5 minutes on ice) and added to the hybridization tube carrying the membrane. Hybridization was carried out over night at 60° C.

The non radioactive southern washes were initially carried out at the hybridization oven, adding 2X 200 ml of an (2X SSC, 0.1% SDS) solution for 10 minutes and then, 2X 200 ml of an (0.1X SSC, 0.1% SDS) solution for another 10 minutes. The following washed were carried out at room temperature (25° C): 100ml buffer A solution for 5 minutes, 100 ml 10% block solution for 1 hour, 100 ml buffer A, 0.5% Bovine Serum Albumin (BSA) solution (adding 2 µl anti-probe) for an additional 1 hour and finally 3X 200 ml of a 0.3% Tween 20 solution for 10 minutes. The detection was carried out adding a detection solution to the probed membrane for 5 minutes in the dark. Then the membrane was exposed on photographic paper for 1 hour.