

IMPROVED METHODS FOR ANALYSIS OF TELOMERE-INITIATED CELLULAR
SENESCENCE AND TELOMERE SHORTENING
IN *SACCHAROMYCES CEREVISIAE*

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
CHAPTER	
I. INTRODUCTION.....	1
II. MATERIALS AND METHODS.....	13
III. RESULTS AND DISCUSSION.....	22
REFERENCES.....	65

LIST OF TABLES

Table	Page
1a. Analysis of cell senescence frequencies of <i>est2⁻ctt1⁻gpx3⁻</i> -BY4742.....	34
1b. Analysis of cell senescence frequencies of <i>est2⁻yap1⁻gpx3⁻</i> -BY4741.....	34
2. Effects of changing the centrifugation times.....	47
3. Changes in current at different voltages and different concentrations of TB buffer....	58
4. Changes in current in 0.5 x TA buffer.....	60

LIST OF FIGURES

Figure	Page
1. Schematic diagram of telomere replication by telomerase.....	3
2. Relationships among DNA damage, cellular senescence and organismal aging.....	6
3. Yeast cells and bud sizes for each phase of the cell cycle.....	9
4. Comparison between the <i>S. cerevisiae</i> and human telomerase complexes.....	10
5. Randomness of cell senescence rates during the aging process.....	23
6. <i>EST2</i> polymerase expression system.....	24
7A. Solid media-based senescence assay involving a double-column streaking method.....	26
7B. Variations in yeast phenotype on the 4 th streak because of the stochastic nature of cellular senescence.....	26
8A. Comparison between the old and new senescence assay methods.....	28
8B. Plate pictures of the new method.....	28
8C. Total number of assays for each streak.....	28
9. Mechanisms of antioxidant enzymes.....	30
10. Examples of the new senescence assay (<i>est2</i> ⁻ BY4742 and <i>est2</i> ⁻ <i>gpx3</i> ⁻ <i>ctl1</i> ⁻).....	32
11. Southern blot picture	36
12. Basic yeast chromosomal purification protocol developed by Christopher Lee.....	38
13. Average DNA concentrations after different protein precipitation reagents were used for DNA purification using the standard protocol.....	40

14. Average DNA yields after different concentrations of Tris buffer were added to the 3% SDS lysis solution	41
15. Average DNA concentrations produced by varying incubation times from 10, 15, 20, 25, and 30 min at 65 °C	42
16. Average DNA concentrations produced after increasing temperature to 75 °C.....	43
17. A 0.8% agarose gel showing fluorescent bands of yeast chromosomal DNA purified using the standard protocol with incubation for 15 min at 65 °C or 75 °C for 5 min.....	44
18. A 0.8% agarose gel showing fluorescent bands of yeast chromosomal DNA amplified using <i>MRE11</i> gene primers	45
19. Average DNA yields produced with different SDS concentrations in the cell lysis solution	46
20. Comparison of DNA purification performance using the new protocol versus a commercial kit for yeast chromosomal DNA purification.....	48
21. Poor DNA resolution in a gel run at 220 V in conventional 1 x TBE buffer.....	49
22. Chemical structure of disodium EDTA.....	50
23A. Effects of EDTA on electrical currents in standard 1 x TBE	52
23B. Effects of EDTA on electrical currents in standard 1 x TAE	52
24. Comparison of electrical currents at different voltages with various disodium EDTA concentrations in 1 x TBE.....	53
25. Comparison of currents at different voltages using 1 x, 0.5 x and 0.3 x TB buffers.....	54
26. Resolution of DNA fragments in 1% agarose gels containing Tris-boric acid buffers (A) with 2 mM EDTA (B) without EDTA at 1 x (C) at 0.5 x or (D) at 0.3 x concentrations.....	55,56
27. Times required to complete electrophoresis of DNA samples in 1 x TB gels containing 1% agarose	58
28A. Resolution of DNA fragments in 1% agarose gels containing Tris-acetate buffers. Comparison between 1x TB and 1x TA.....	60
28B. Resolution of DNA fragments in 1% agarose gels containing Tris-acetate buffers. 0.5x TA without EDTA.....	60

CHAPTER I

INTRODUCTION

All information needed to build and function as living organisms is written into deoxyribonucleic acid, DNA. Amazingly, the genetic information for each organism is coded by the combination of only four bases, adenine (A), cytosine (C), guanine (G), and thymine (T) formed into a long chain, and the enormous information is tightly packed into cells with proteins as chromosomes. Eukaryotes have linear chromosomes and there are specialized structures at both chromosome ends called telomeres. Telomeres have repetitive G-rich sequences whose specific base sequences are organism-specific. Human chromosomes, for example, contain thousands of TTAGGG sequences at the ends (1). Telomeres are bound by telomere- and chromatin-associated proteins and these protective protein caps help stabilize chromosomes. These unique structures make it easy to distinguish damaged or broken DNA from normal DNA and protect ends from being acted on by DNA repair systems such as homologous recombination and non-homologous DNA end-joining (2).

The linear DNA forms observed in all eukaryotes and some prokaryotes have the “end-replication problem” during replication. While the leading strand can be replicated continuously with a single RNA primer in the 5’ to 3’ direction, on the lagging strand the

DNA polymerases move in the opposite direction of the replication fork, creating a collection of Okazaki fragments. Each Okazaki fragment needs an RNA primer for initiation of replication. The problem of the lagging strand is that the last RNA primer does not get placed at the very ends of the template DNA. This makes the very end of the sequence left out from replication. Furthermore, the last RNA primer is not replaced with DNA by a DNA polymerase after degradation by RNase. As a result of this “end-replication problem,” a linear chromosome gets shorter and shorter from both telomeric ends every DNA replication cycle. However, telomeres and telomerase solve this problem and protect against the loss of essential genetic information.

The discovery of the ribonucleoprotein reverse transcriptase enzyme telomerase by Carol Greider and Elizabeth Blackburn in 1985 was the beginning of the study of cell aging (3). Telomerase includes both protein and RNA components. During the synthesis phase (S phase) of the cell cycle, telomerase detects the specialized repeated sequences of telomeres and extends the 3' end of a chromosome using the RNA component as a template (Figure 1). Then, the newly synthesized single-stranded DNA by telomerase acts as an additional template for new Okazaki fragments, and the telomere is extended by conventional DNA polymerase though it still has a 3' overhang (4). This function of telomerase is a key to maintaining the lengths of telomeric ends.

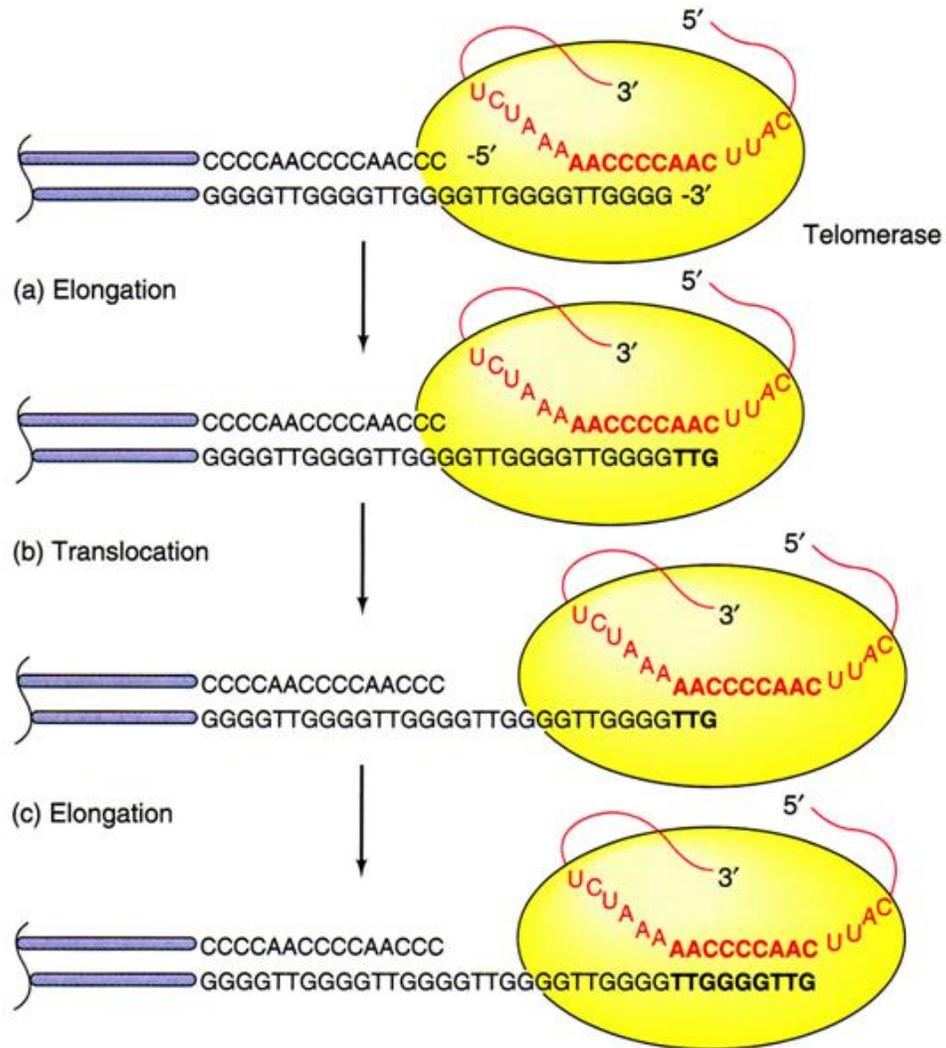


Figure 1. Schematic diagram of telomere replication by telomerase (5). Telomerase detects the specialized repeated sequences at chromosomal ends and extends the 3' end using the RNA component of telomerase as a template.

Telomerase is expressed during early development in all cells to maintain telomere length. However, though telomerase stays active in highly proliferative cells like germ cells, its activity is not detected in most normal somatic cells in adult humans and in many animals (1, 6). Therefore, most cells within older humans have shorter telomeres than those of younger individuals. Cells without telomerase lose approximately

100 base pairs (bp) per cell division over time (7). When the telomere gets short enough, or hits a critical level, the cells stop dividing. This replicative limit is known as the Hayflick limit and represents a type of cell aging called cellular senescence (8). Though these cells are still alive initially, they ultimately undergo death. After human fibroblasts grow for approximately 50 cell cycles in culture under optimal conditions, they undergo senescence (1). Despite the limitless proliferative potential offered cells by telomerase, most human cells don't express telomerase, and this might be because of telomerase promoting formation of cancer cells. Telomerase is up-regulated in approximately 90% of cancer cells (9). That is why cancer cells are mechanically "immortal," without a finite limit.

There are two ways that cells senesce, telomere-dependent or –independent. Telomere-dependent senescence is known as replicative senescence (also called telomere-initiated cellular senescence), due to telomere shortening with age. On the other hand, telomere-independent senescence is premature senescence, caused by various stressors in early passage cells grown in culture. In replicative senescence, when telomeres get critically short, the protective protein caps on telomeres are lost and chromosome ends become highly reactive, resulting in senescence. In premature senescence, stressors such as reactive oxygen species (ROS) cause DNA damage/mutations and even if telomeres do not get short, the cells may go through a type of senescence. In both pathways, cell senescence is a response to DNA damage.

In cells of the budding yeast *Saccharomyces cerevisiae*, senescence of telomerase-deficient cells results in cell cycle arrest in G₂ phase. This cell cycle delay response to senescence requires the same genes as the normal DNA damage response

checkpoints (*MEC3*, *MEC1*, and *DDC2*), although general DNA damage checkpoints were distinguished from the other by the requirement for other extra specific genes (10).

Cellular senescence is suspected of being closely related to organismal aging. With age, production of reactive oxygen species increases and DNA repair capacity decreases, resulting in accumulation of DNA damage. Herbig *et al.* demonstrated that senescent cells exist *in vivo*, and more than 15% of the cell population in various organs can be senescent cells in aged animals (11). Also, higher mortality rates and accelerated aging in individuals with shorter telomeres rather than with longer telomeres was shown by Cawthon *et al.* (12). Tomas-Loba *et al.* demonstrated that constitutive expression of telomerase in cancer-resistant mice extended median life span (13). In Figure 2, major components and steps that lead to aging are shown. Two major components that cause DNA damage are an increase of reactive oxygen species (ROS) production and decline of DNA repair capacity during aging. Also, antioxidant systems are suspected to influence accumulation of DNA damage. Accumulation of DNA damage can result in senescence and/or apoptosis at the cellular level. This causes tissue stem depletion and/or disrupted tissue structure, and ultimately leads to organ dysfunction and organismal aging (Figure 2). From these observations, it seems important to investigate organismal aging at the cellular and/or molecular level.

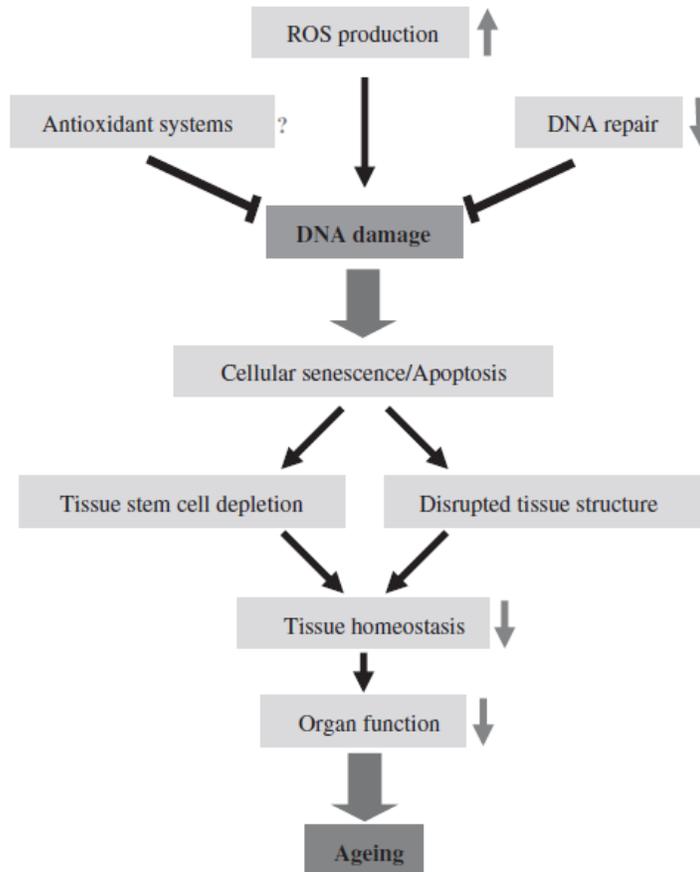


Figure 2. Relationships among DNA damage, cellular senescence and organismal aging (14).

Caloric restriction leads to preservation of cells because of a reduction in oxidative damage to cellular macromolecules. McCay *et al.* demonstrated for the first time in 1935 that caloric restriction, feeding approximately 30 to 40% fewer calories than normal each day, led to longer lifespan in rats (15). Since this first report, hundreds of studies of caloric restriction have been done in mice, fruit flies, yeast, dogs and worms, and showed that reduced calories extended the lifespan (16-22). In the most recent research, Rhesus monkeys which had eaten a low-calorie diet for 20 years had fewer aged-related diseases than Rhesus monkeys which had eaten the normal diet for 20 years.

Also, by magnetic resonance imaging (MRI), the brains, which mostly control thinking or movement, showed slower shrinking with age in calorie-restricted monkeys (23).

Because preservation of cells by caloric restriction was successfully demonstrated in monkeys closely related with humans, the same effects on humans of a low-calorie diet are highly likely as well.

In addition to caloric restriction studies, resveratrol, a chemical found in wine, grape juice and peanuts, and closely related to polyphenols, has been studied extensively, and the development of rejuvenation pills using resveratrol might be possible in the near future. This is because recent research has reported that the pathway activated by resveratrol is the same pathway promoted by calorie restriction, and resveratrol seems to enhance health at the cell level by affecting expression of multiple genes (24). Both resveratrol and caloric restriction promote antioxidant activities through activated intracellular pathways so cells are protected from oxidative damage caused by reactive oxygen species (ROS). These studies demonstrate that antioxidant activities are a key for cellular aging.

In the case of premature senescence, exposure to ROS is a major stressor that causes DNA damage. ROS causes cellular oxidation, primarily because of three common ROS within cells: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$). These ROS are byproducts of metabolism, and are mostly produced in the electron transport chain, where ATP is synthesized by passing electrons to oxygen. The ROS produced in cells lead to undesirable reactions at the molecular level and cause damage to fatty acids, proteins, amino acids as well as DNA (25). In order to protect cells from DNA damage by ROS, organisms have developed defense systems, and one of them

is antioxidant enzymes. The most well-known antioxidants are superoxide dismutases, catalases, and glutathione peroxidases, and the roles of these antioxidant enzymes in protecting against cell aging needs more investigation.

Saccharomyces cerevisiae, generally known as budding yeast, has been studied extensively because it is an excellent experimental model. Even though *S. cerevisiae* is a simple unicellular eukaryote, all central eukaryotic characteristics are found in these simple cells, and many homologous human genes are found. *S. cerevisiae* has a small number of genes, approximately 6,300 genes on 16-haploid chromosomes. In contrast, humans have approximately 22,000 genes on 23-haploid chromosomes (26). Also, yeast cells grow rapidly under rich conditions and double every 90 min at 30 °C. In addition to these benefits, *S. cerevisiae* cells are observed in two states, either a haploid or diploid, depending on nutrient conditions. The existence of two states is an advantage for gene analysis. Haploid cells have only a single copy of genes unlike diploid cells, and it is not as difficult to work with a single copy of a gene as with two copies. Also, genes can be deleted easily in haploid yeast cells if they are non-essential for cell growth, and most genes, ~5,000 genes, are considered non-essential. The other advantage in yeast is homologous recombination, or genetic exchange, which can be caused precisely and rapidly. Any particular gene can be deleted by recombination with a homologous chromosome. Therefore, the establishment of a knocked out gene library with non-essential genes inactivated is possible. Also, the growth stages of *S. cerevisiae* cells can be distinguished simply using microscopy. Cells without a bud are in G₁ phase and cells with a small bud are in the DNA synthesis phase (S phase) of the cell cycle. Cells with a large bud are in G₂ or M phase of cell cycle (Figure 3).

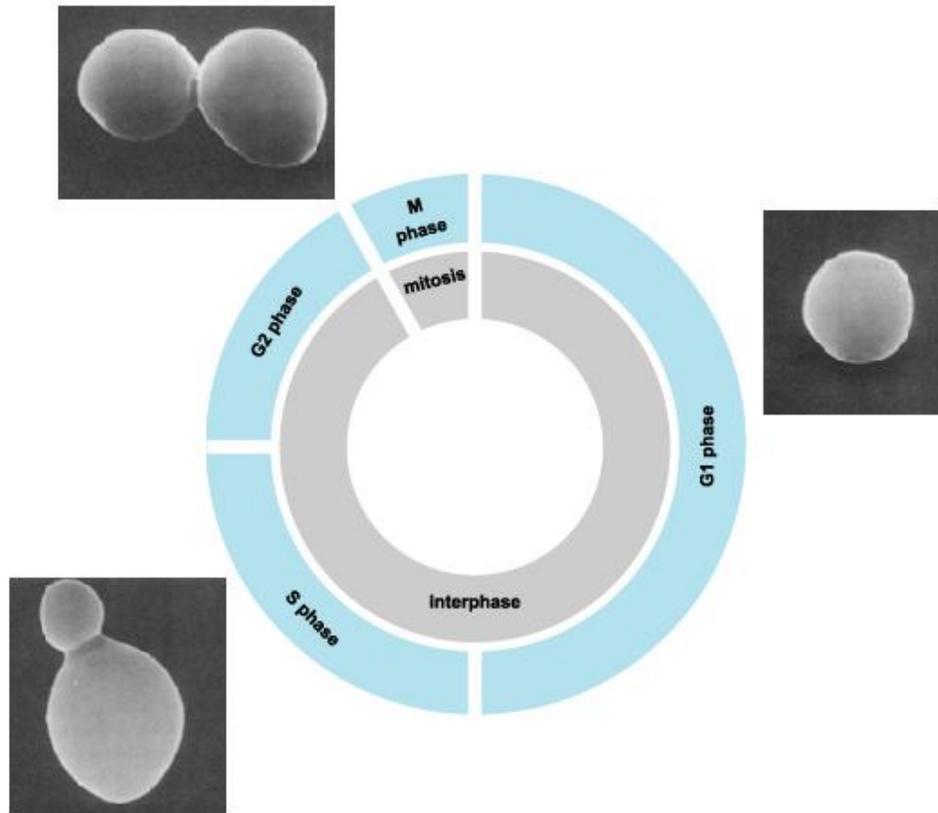


Figure 3. Yeast cells and bud sizes for each phase of the cell cycle.

Like human telomerase, the yeast telomerase complex consists of protein subunits (Est1, Est2, Est3 and Cdc13) and an RNA subunit (*TLC1*). Unlike human telomerase, yeast telomerase is normally expressed and the cells are immortal. Est1 binds to the RNA subunit *TLC1*, and it recruits the telomerase components to chromosome ends with the single strand DNA binding protein Cdc13. Est2 is the polymerase enzyme subunit and Est3 is also important for telomerase activities, but the function of Est3 is not known yet (27, 28) (Figure 4). Telomerase-deficient yeast cells (with an inactivated *est2* gene) created for cell aging studies experience telomere shortening, and cells senesce in culture after ~60-70 cell cycles. The senescence process is highly stochastic, however, with some

cells senescing early (40-50 generations) and other cells very late (80-90 generations). A very small number of cells acquire a mutation that causes telomeres to become more stable, even though there is no telomerase being expressed in the cell. The mutation causes an increase in homologous recombination (strand exchange) at the telomeres and the mutant cells restore the telomeres by unequal exchange events. These mutants are called “survivors” of senescence.

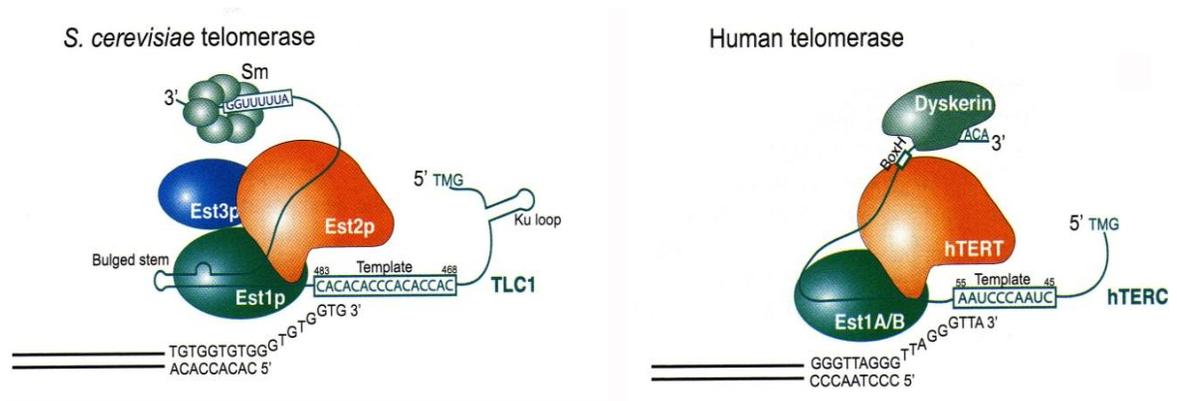


Figure 4. Comparison between the *S. cerevisiae* and human telomerase complexes (26).

A major aim of the current thesis project was to develop a new senescence assay that takes into account the stochastic nature, or random rates, of cellular senescence. The new assay method was used to demonstrate that a specific combination of inactivated antioxidant enzyme genes causes acceleration of cellular senescence. In order to perform solid media-based senescence assays, our laboratory previously developed an *EST2* polymerase expression system (29). A plasmid that has a galactose-inducible promoter *GALI-V10p::EST2* was transformed into yeast cells in which the *EST2* polymerase gene was knocked out. The *GALI-V10p::EST2* promoter is “on” in galactose media,

telomerase is expressed and cells are immortal. On the other hand, the *GALI-VI0p::EST2* promoter is “off” in glucose media and cells go through senescence after ~60-70 generations because of lack of telomerase. Previous studies using a plate- or solid media-based senescence assay have used a double-column streaking method (30). This thesis work has developed a new approach that takes into account the fact that some telomere-deficient cells senesce after 50-60 generations, but others don't senesce until 70-80 generations or more. The new assay method also produced better statistics than previous approaches.

The second objective of this project was to improve Southern blot analysis methods, which are used to evaluate telomere lengths in senescent cells. There were two problems that needed to be addressed. The first problem was the method used to isolate chromosomal DNA from yeast cells. Yeast cells have a complex cell wall and the cell wall is often resistant to traditional methods used to isolate DNA from other organisms. Some previously published methods of yeast chromosomal DNA purification involved use of cell wall degrading enzymes like zymolyase or lyticase (31). These methods can take a long time and are expensive in large quantities. Other methods require harsh chemicals like phenol, chloroform, or sodium hydroxide (32). Also, cell walls and cell membranes have been disrupted by vortexing with glass beads, but this leads to shearing of chromosomal DNA and lower molecular weight fragments (32, 33). Alternatively, commercial purification kits whose contents are unknown are available, but they are expensive. The strategy to address this problem was the development of a new chemical-based method for purifying yeast chromosomal DNA that is simpler than other published protocols and cheaper than commercial purification kits. A recent graduate student in this

lab, Christopher Lee, developed a preliminary chemical-based protocol (34), but a number of important questions were not answered in that work. In the current thesis project, several remaining questions were investigated and a new, final DNA purification protocol was developed.

The second problem encountered in Southern blot analysis is the thickness of the DNA bands produced by gel electrophoresis. In order to evaluate telomere lengths, thinner bands make it possible to do more precise analysis. The most common gel running buffers are TBE and TAE, and they are usually run at 100-150 volts to avoid heat generation. If higher voltages could be used without excess heat production, they would reduce running time and therefore band diffusion could be minimized. The approach for the second problem was a detailed investigation of ways to improve standard TBE and TAE running buffers to allow higher voltages to be used without heat or band distortion. This new approach allows gels in general, and Southern blots in particular, to be run at twice the voltage normally used, resulting in faster run times and thinner bands.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General reagents

Sodium chloride was purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EM Science (Darmstadt, Germany). Ethidium bromide (EtBr) and Tris-HCl were obtained from Shelton Scientific, Incorporated (Shelton, CT). Glacial acetic acid was purchased from Mallinckrodt chemicals (Phillipsburg, NJ). Ethylenediaminetetraacetic acid (EDTA), boric acid, and agarose were obtained from EMD Chemicals, Inc. (Darmstadt, Germany). TE buffer and RNase A were obtained from Epicentre Biotechnologies (Madison, WI). Tris base was purchased from VWR International (West Chester, PA). Hoechst 33258 and potassium acetate were obtained from Sigma Chemical Company (St. Louis, MO). 2-Log DNA ladder and 1 Kb standard DNA ladder were purchased from New England Biolabs (Beverly, MA). Ex Taq polymerase and Ex Taq reaction buffer were obtained from Takara (Madison, WI). MasterPure Yeast DNA Purification Kits was purchased from Epicentre (Madison, WI).

Yeast growth media

All amino acids, D-(+)-galactose and ampicillin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bacto peptone, bacto yeast extract and bacto agar were purchased from Becton Dickinson and Co. Microbiological Systems (Sparks, MD). Anhydrous D-glucose (dextrose) was purchased from Mallinckrodt (Paris, KY).

Cell culture solutions and media

For general nonselective growth, yeast cells were grown on YPDA (rich) media (1% bacto yeast extract, 2% bacto peptone, 2% dextrose, 2% bacto agar and 0.002% adenine). For plasmid selection, yeast cells were grown on synthetic media (2% glucose or galactose, bacto agar, and all essential amino acids minus the amino acids for selection). All quantities listed here as “%” are w/v. Liquid media was prepared as plate media minus agar.

Yeast strains and plasmids

The parent strains used for these studies were derived from BY4742 (*MAT α his3 Δ 1 leu2 Δ 10 lys2 Δ 10 ura3 Δ 10*). BY4742 cells with a deletion of the *EST2* gene (BY4742, *est2 Δ ::HygB^r*) and containing plasmid pLKL82Y [*CEN/ARS URA3 GAL1-VI0p::EST2*] were designated as YLKL803. Antioxidant gene mutants included YLKL829 (BY4742, *est2 Δ gpx3 Δ ctt1 Δ*), and YLKL838 (BY4741, *est2 Δ yap1 Δ gpx3 Δ*) (38). As a control, YLKL836 (BY4741, *est2 Δ*) was also prepared. The genotype of BY4741 is *MAT α his3 Δ 1 leu2 Δ 10 met15 Δ 10 ura3 Δ 10*.

Southern blot assays

DIG High Prime DNA Labeling and Detection Kit II (including blocking agent, DIG easy hyb granules, anti-digoxigenin-AP Fab fragments and CSPD), PCR DIG labeling mix and DNA Molecular Weight Marker III-DIG labeled were obtained from Roche Diagnostics (Indianapolis, IN). Restriction enzymes XhoI, DNA Taq polymerase, and standard Taq reaction buffer were purchased from New England Biolabs (Beverly, MA). MgCl₂ (25 mM) was purchased from Fermentas, Inc. (Glen Burnie, MD). Sodium chloride and sodium citrate dehydrate were obtained from Fisher Scientific (Fair Lawn, NJ). Tween 20 and Sarkosyl (N-lauryl-sarcosine) were purchased from Sigma-Aldrich Chemical Co. (St. Louise, MO). Formamide was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ).

II. METHODS

Solid media-based senescence assays

Cell senescence was analyzed by creation of a series of streaks of double-columns and single-columns of cells onto synthetic glucose complete (glu-com) plates. Telomerase-deficient strains, YLKL803 (BY4742, *est2Δ*) and YLKL836 (BY4741, *est2Δ*) were used as controls and the following antioxidant gene mutants were tested: YLKL829 (BY4742, *est2Δ gpx3Δ ctt1Δ*), and YLKL838 (BY4741, *est2Δ yap1Δ gpx3Δ*).

First, a swath of individual colonies was picked from a fresh galactose minus uracil (gal-ura) stock plate using sterile toothpicks and then streaked as double-columns

on glu-ura plates. The cells were grown for 4 days at 30 °C. After 4 days growth, moderate-sized individual colonies were picked and streaked onto a fresh glu-ura plate and grown 4 days at 30 °C as before. On the 3rd streak, the streaking method was switched from double-columns to single-columns. This process was repeated for the 4th streak when using the BY4742 background strains or for up to the 6th streak when using BY4741 background strains. On the 3rd streak, 16 single-columns were streaked from separate colonies, and after the 4th streak, 32 single-columns were streaked from individual colonies. Each streak involved approximately 20 generations of cell growth and the senescent phenotype appeared at around 60-70 generations for BY4742 and around 90-100 generations for BY4741. Images of each streak were captured using a Canon Powershot G3 digital camera and pictures were saved as .jpg formatted files.

SDS/EDTA method for yeast chromosomal DNA purification

The lab strain BY4742 was spread on YPDA plates and then grown at 30 °C for 2-3 days. A wide swath of cells was harvested from the stock plate using a sterile toothpick and transferred to 1 ml of YPDA broth in a 1.5 ml microfuge tube. The cell suspension was vortexed briefly and then transferred into 50 ml of YPDA liquid media in a 250 ml Erlenmeyer flask. Cells in the flask were grown overnight at 30 °C using a Barnstead MAX Q 4000 benchtop shaker. After overnight culturing, 1.5 ml aliquots of the culture were transferred to 1.5 ml microfuge tubes and then spun at 16,100 x g for 0.5 min using an Eppendorf 5415 D microcentrifuge. The supernatant was removed and an additional 1.5 ml aliquot of the culture was added to each sample. The sample was spun

as before and the supernatant was removed, producing a cell pellet that was derived from 3 ml of cells.

The first step was cell lysis. A cell lysis solution containing specific dilutions of SDS and EDTA with or without Tris (pH 8.0) was prepared and 300 μ l of the solution was added to the cells. The pellets were suspended by scraping and vortexing. The suspended cells were incubated at 65-75 °C for 5-30 min and then transferred to wet ice for 5 min. The next step was protein precipitation. A cold 3 M NaOAc or 3 M KOAc solution was prepared and 150 μ l of the solution was added. The sample was inverted well for 10 sec and then centrifuged at 16,100 x g for 10-15 min. The supernatant was transferred to a new 1.5 ml microfuge tube. The next step was DNA precipitation. To the supernatant, 400-500 μ l of isopropanol was added, and the tube was mixed thoroughly by inverting vigorously. The solution was centrifuged for 5-10 min at full speed and the supernatant was removed. The DNA pellets were washed by adding 500 μ l cold 70 % ethanol for 1 min and the supernatant was removed. The pellets were dried for ~10 min in a Savant Speed Vac on low heat and then resuspended in 50 μ l TE (10 mM Tris and 1.0 mM EDTA). To degrade RNA, 1 μ l of 1 mg/ml RNase A was added to each sample. After gentle vortexing, each sample was placed in a 37 °C waterbath for 10-15 min. The samples were stored at -20 °C.

Fluorometry assays

The concentrations of each purified chromosomal DNA were quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech). Assay solution A

(0.1 µg/ml Hoechst 33258 and 1 x TNE [0.2 M NaCl, 10 mM Tris-HCl and 1 mM EDTA at pH 7.4]) and calf thymus DNA (100 µg/ml) standard were used for the protocol.

Protocol: SDS/EDTA method for yeast chromosomal DNA purification

Several experimental variables were tested, and the following optimum protocol for extraction of yeast chromosomal DNA was developed.

1. Start a 4-5 ml culture of yeast cells in YPDA broth and shake overnight at 30 °C.
2. Transfer 1.5 ml of the culture to a microfuge tube, spin at full speed in a microcentrifuge for 0.5 min, pour off supernatant, add 1.5 ml more cells to the tube, re-spin for 0.5 min and pour off the supernatant again. Add 300 µl SET* (SDS + EDTA + Tris) solution and scrape the tube across the bottom of a test tube rack several times to resuspend the cells.
3. Incubate the suspended cells at 65 °C for 15 min and then transfer to wet ice for 5 min. Note: 75 °C for 5 min produced similar results.
4. Add 150 µl cold 3 M NaOAc** solution. Invert/mix well for 10 sec.
Note: 3 M KOAc*** produced similar results.
5. Spin 10 min at full speed. Transfer the supernatant to a 1.5 ml tube.
6. Add 500 µl isopropanol and mix thoroughly by inverting vigorously.
7. Spin 10 min at full speed and remove the supernatant.
8. Wash DNA pellet by slowly adding 200-500 µl cold 70% EtOH. Wait 1 min and remove the liquid.
9. Dry in speedvac for ~10 min on low heat until pellet is dry.

10. Suspend DNA pellet in 50 μ l TE.
11. Add 1 μ l 1 mg/ml RNase A. Vortex gently and incubate at 37 $^{\circ}$ C for ~10 min.
12. Allow DNA to dissolve at 4 $^{\circ}$ C. (Occasionally insoluble material is visible in the TE later. Remove it by spinning for 2 min and transferring DNA to a new microfuge tube.)

* 6% SET solution: (5 ml final)

1500 μ l 20 % SDS (6% final)

100 μ l 0.5 M EDTA (10 mM final)

150 μ l 1 M Tris (pH 8.0) [30 mM final]

3250 μ l ddH₂O

**NaOAc solution: (50 ml final)

20.4 g Sodium acetate (3 M final)

Adjust volume to 50 ml with ddH₂O

***KOAc solution: (50 ml final)

30.00 ml 5 M KOAc (3 M final)

5.75 ml glacial acetic acid (2 M final)

14.25 ml ddH₂O

PCR and gel electrophoresis

The purified DNA was amplified by PCR (an Applied Biosystems 2720 Thermal Cycler) using primers that specifically amplified the yeast *MRE11* gene. Two μ l of DNA, 5 μ l primer MRE11 forward (5' - CGGGGTACCATGAGTACTGCAGATGCACTT -3'), 5 μ l primer MRE11 reverse (5' - TCCCCGGGTTATCTTCTATTTCTTCTT -3'), 5 μ l 10 x Ex Taq polymerase buffer, 5 μ l 2.5 mM dNTPs, 1 μ l 25 mM MgCl₂, and 1 μ l Ex Taq polymerases (Takara) were combined with sterile ddH₂O to bring each reaction to a final volume of 50 μ l. The reactions were then exposed to the following thermocycler conditions: 94 $^{\circ}$ C, 2 min and then 32 cycles: 94 $^{\circ}$ C , 30 sec, 49 $^{\circ}$ C , 40 sec, 72 $^{\circ}$ C , 1.5 min followed by a final extension period of 72 $^{\circ}$ C for 7 min.

Gel electrophoresis was performed using 0.8% agarose gels in 1x TBE (89 mM Tris-borate, 2 mM EDTA) running buffer in a Life Technologies Horizon 11-14 gel rig. After the gels were run at a voltage of 120 V, they were stained with ethidium bromide (EtBr) and the gel images were captured by an Alpha Innotech RED gel imager.

Running buffers for agarose gel electrophoresis

Quality of gel images, mAmps, running time, pH and heat generation during gel electrophoresis were compared under various conditions such as different running buffers, concentrations of buffers, and/or voltages, using 1.0% agarose gels in a mini-sub cell GT 8 gel rig (BIO-RAD). All gels were allowed to solidify for at least 20 min. After gels were run, they were stained with EtBr and the gel images were captured by an Alpha Innotech RED gel imager.

Southern blot assays

A non-radioactive digoxigenin (DIG)-based system from Roche Diagnostics (DIG High Prime DNA Labeling and Detection Kit II) was used.

PCR amplification of nonradioactive DIG probe

Telomere-specific DNA probes were synthesized by PCR using an Applied Biosystems 2720 Thermal Cycler. Two μ l of plasmid YTCA-1 (35) that was diluted 1/100, 5 μ l primer M13 forward (5'-AGCGCGCAATTAACCCTCACTAAAG-3'), 5 μ l primer M13 reverse (5'-CAGGAAACAGCTATGACC-3'), 5 μ l 10 x Taq polymerase

buffer with MgCl₂, 5 µl DIG labeling mix (2 mM dAMP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM Digoxigenin-11-dUTP), 1 µl 25 mM MgCl₂, and 1 µl Taq polymerase (New England Biolabs) were combined with sterile ddH₂O to bring each reaction to a final volume of 50 µl. The reactions were then exposed to the following thermocycler conditions: 94 °C, 2 min and then 34 cycles: 94 °C , 30 sec, 45 °C , 30 sec, 72 °C , 1 min followed by a final extension period of 72 °C for 7 min.

Probe hybridization and detection

Purified chromosomal DNA was digested with XhoI and 1 µg of the sample was separated on a 1.2% agarose gel in 1x TBE buffer at 110 V. After gel electrophoresis, DNAs were transferred overnight from the gel onto an N+ Hybond membrane (Amersham Pharmacia) and crosslinked with a UV-Stratalinker 2400 (Stratagene) at 120,000 microjoules for 20 sec. The digoxigenin (DIG)-labeled DNA probe was hybridized overnight in the hybridization oven. After the excess probe was thoroughly washed out, enzyme-linked antibody (anti-DIG-AP conjugate) was added. Detection was performed using disodium 3-(4-methoxysprio(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.13.7]decan)-4-yl)phenyl phosphate (CSPD) for chemiluminescence. For imaging, the membrane was placed in a Spectroline Monotec X-ray cassette with MidSic Classic Autoradiography film. Intensifying screens were used and exposure time was adjusted to achieve optimum development.

CHAPTER III

RESULTS AND DISCUSSION

A major focus of this project was to develop a new senescence assay that takes into account the random rates of cellular senescence. In typical mammalian somatic cells, telomerase is not expressed and telomeres get shorter during every round of mitosis. When the telomere length hits a critical level, the cells stop dividing. This replicative limit is known as cell senescence and the cells that stop dividing ultimately die. In earlier studies using telomerase-deficient BY4742 yeast cells in the Lewis lab, most telomerase-deficient BY4742 yeast cells showed cell senescence after approximately 60-70 generations. However, when the same experiments were repeated multiple times, it was common to get variability because some cells took longer or shorter to senesce. There exists a randomness to cell senescence. As shown Figure 5, most cells stop dividing after ~65 cell cycles but a large fraction of them senesce at earlier or later times. In order to take into account the randomness of senescence and investigate the effect of different parameters on senescence rates, a new assay was developed.

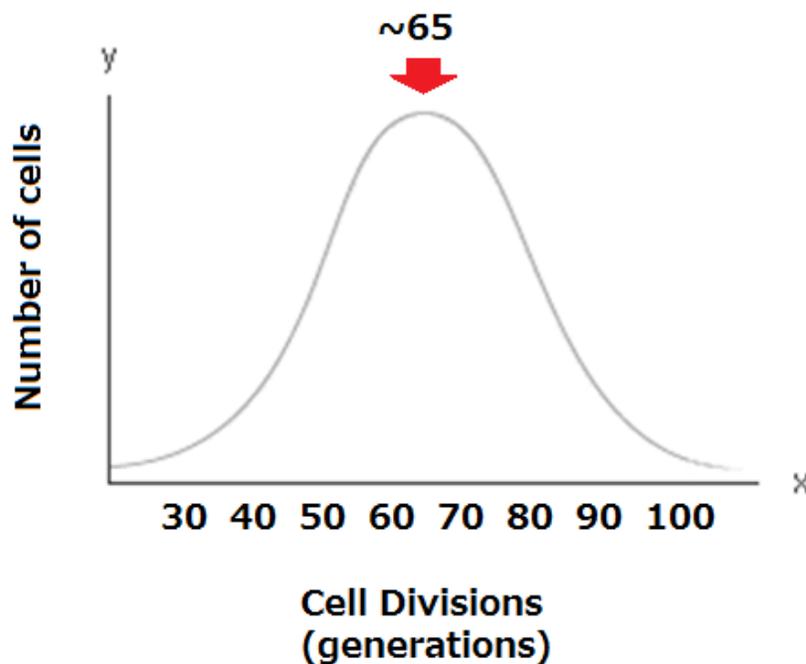


Figure 5. Randomness of cell senescence rates during the aging process. Most cells senesce after around 65 generations.

Graduate student Hiranthi Thambugala in the lab developed a regulatable *EST2* polymerase expression system in yeast (29). Unlike most human cells, telomerase is expressed in normal yeast cells. Therefore, to create an *EST2* expression system, the *EST2* gene which works as the catalytic subunit of telomerase was deleted from BY4742 yeast cells, and then plasmid pLKL82Y, which contains the *EST2* polymerase under the control of a modified galactose-inducible *GAL1* promoter (*GAL1-V10*), was transformed into the telomerase-deficient BY4742 cells. The *GAL1-V10* promoter is regulated by galactose, and *EST2* polymerase is expressed in galactose media while the expression is strongly limited in glucose media. As shown in Figure 6, when cells are grown on glucose plates, no *EST2* polymerase is expressed, resulting in cell senescence after approximately 60-70 generations.

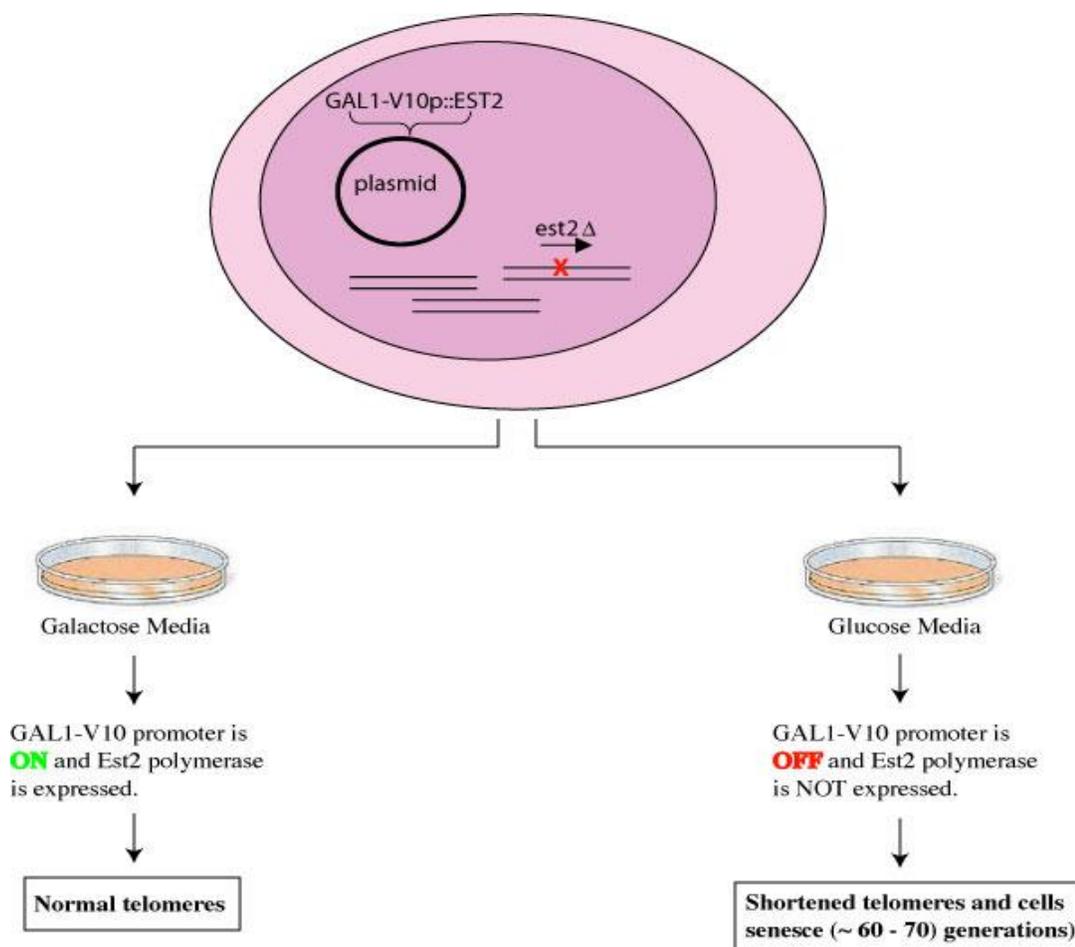


Figure 6. *EST2* polymerase expression system. *EST2* polymerase is expressed on galactose media, but not expressed on glucose media.

A solid media-based senescence assay was developed previously that involves a double-column streaking method (30). To perform this assay using the *est2⁻*-BY4742 cells described earlier, two isolated colonies were picked and streaked as double-columns on a 1st glu-ura plate. The double-columns were created by streaking the 1st column with a sterile toothpick, flipping the toothpick and streaking 2 times into the other side, and then finishing the 2nd column with the same side of the toothpick. The plate was incubated for 3 days at 30 °C, during which the cells experienced ~20 generations of growth and formed two full columns of growth (See “1st streak” in Figure 7A). These

steps were repeated for the 2nd streak by touching individual colonies from the 1st plate with a toothpick and streaking them to a new plate, which resulted in another 20 generations of growth. For the 3rd and 4th streaks, moderate-sized individual colonies were picked and streaked as before. Yeast cells divide approximately twenty times while forming colonies during each of the 1st, 2nd and 3rd streaks. Most telomerase-deficient BY4742 cells stop dividing after the 4th streak, at about ~60-70 generations, while wild type cells show 2 full columns of growth and can be streaked again and again (Figure 7A).

It has proven difficult to evaluate senescence rates quantitatively by this method because of the randomness of cell senescence in different cells. Sometimes the 4th streak plate for telomerase-deficient cells will show no apparent senescence (2 full columns of growth), 1½ columns of growth, only 1 column, less than 1 column, and sometimes no growth (shown schematically in Figure 7B). For example, the 4th streak shown in Figure 7A exhibited less than 1 column of growth, but this does not occur every time the assay is performed. To properly measure senescence kinetics in a particular yeast strain, it is necessary to introduce statistics. To apply statistics, sample numbers were increased by changing the streaking method and a new solid media-based senescence assay was designed.

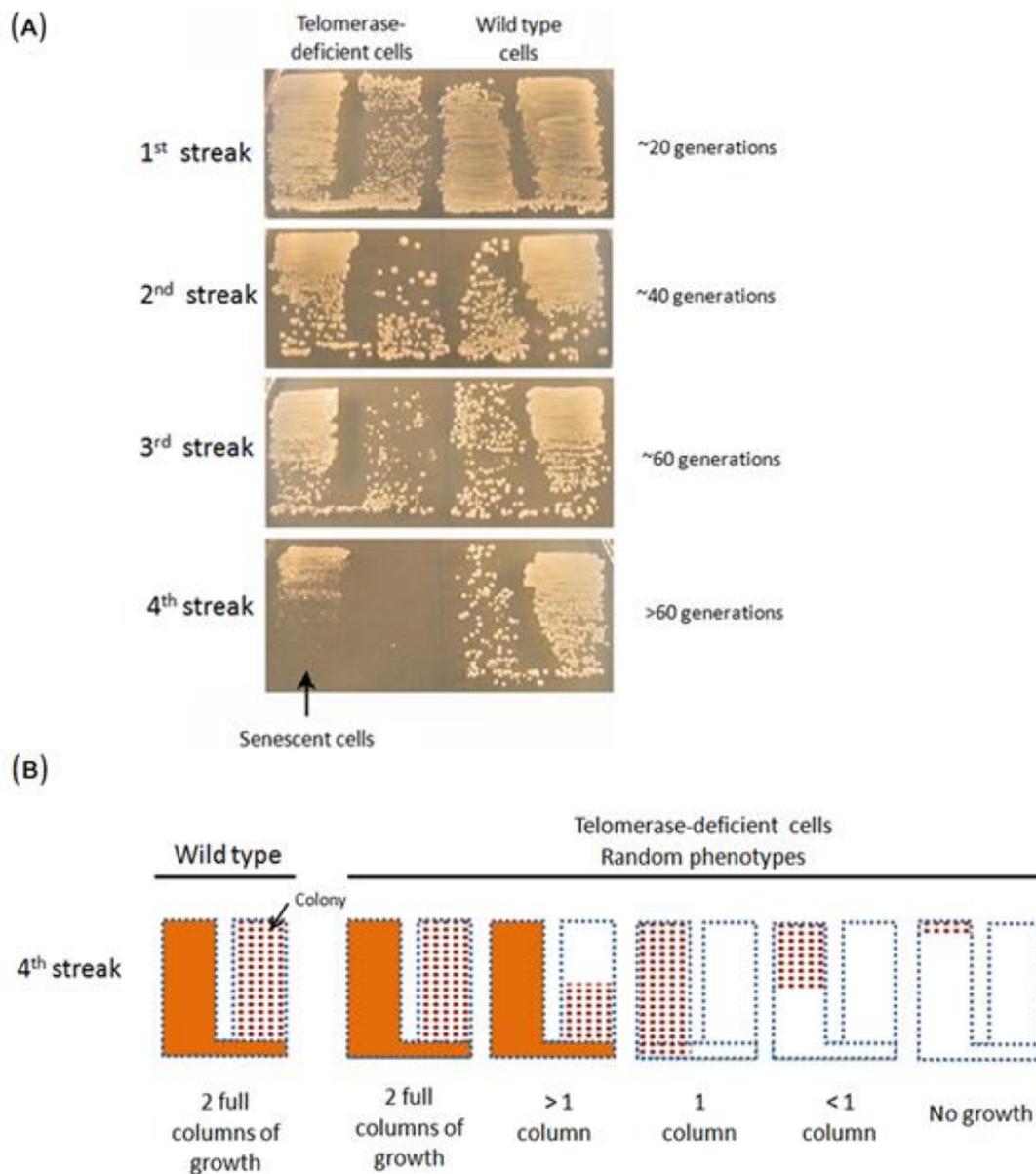


Figure 7. (A) Solid media-based senescence assay involving a double-column streaking method. (B) Variations in yeast phenotype on the 4th streak because of the stochastic nature of cellular senescence.

In the new solid media-based senescence assay, the double-column streaking method was switched to a single-column streaking method beginning on the 3rd streak, resulting in twice as much collected sample data compared with the previous method. On the 3rd streak, the colony density was reduced enough to pick individual isolated colonies, and one could switch to a single-column streaking method. On each 3rd streak, 16 new single-columns were streaked, and for the 4th streak, 32 single-columns were created (Figure 8A and 8B). Several different incubation times, 3-3-3-3 days, 4-4-3-3 days, and 4-4-4-3 days, where the numbers indicate the number of days each streak plate was incubated at 30 °C, were tested. The 4-4-4-3 days incubation showed the highest cell senescence (the least growth) on the 4th streak of synthetic plates (data not shown). Therefore, plates were incubated for 4 days instead of 3 days, which was found to be important in order to give enough time for full growth of each streak. As the parameters for measuring cellular senescence in this project, a reduced ability to form colonies and reduced colony sizes on the last streak plate were counted as cell senescence. An important advantage of the new method is that 8 single column streaks can easily be performed on one plate, and 2 plates were used for the 3rd streak and 4 plates were used for the 4th streak. This means that 16 separate assays (2 plates x 8) and 32 separate assays (4 plates x 8) were employed for every strain.

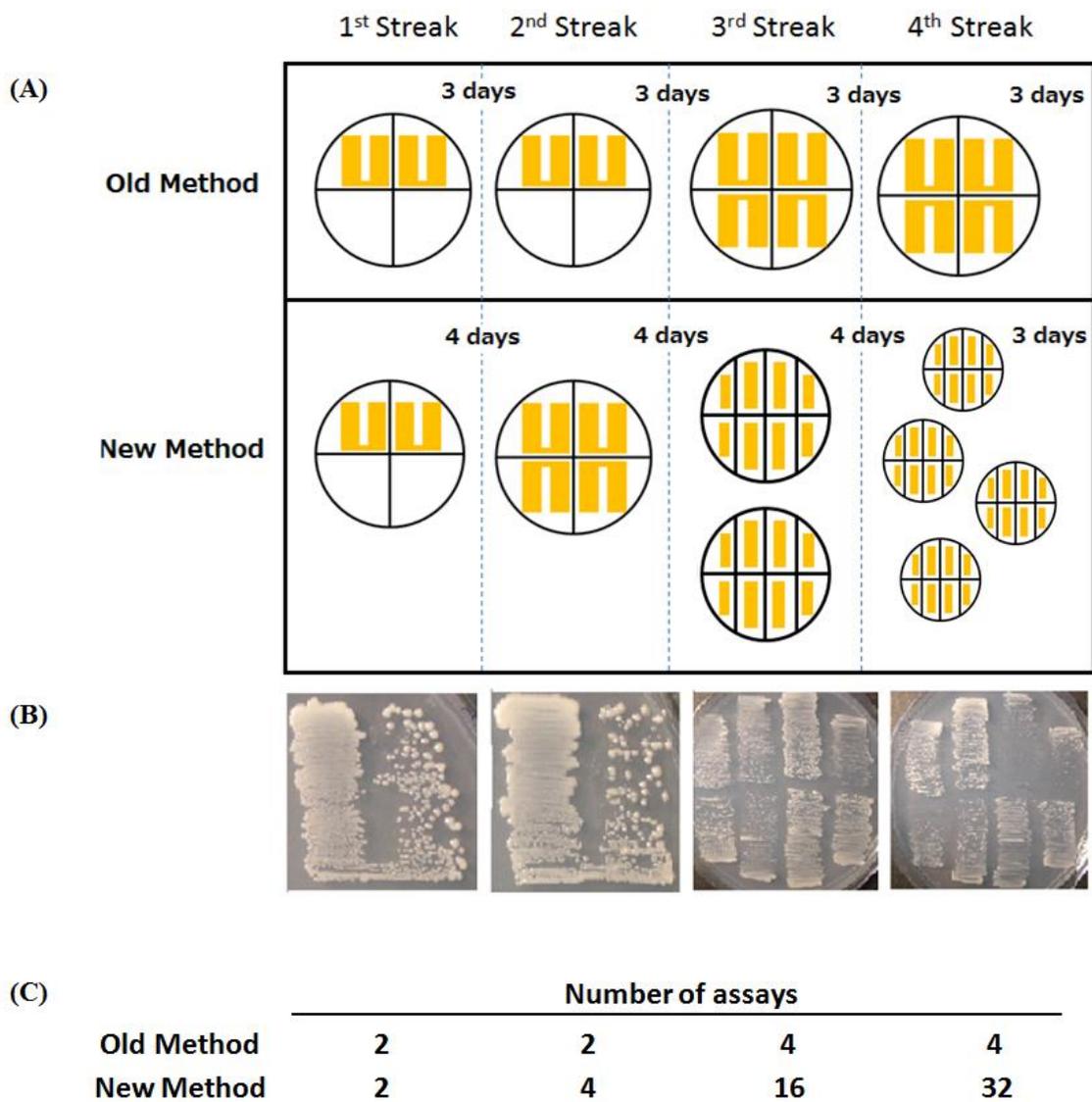


Figure 8. (A) Comparison between the old and new senescence assay methods. (B) Plate pictures of the new method. (C) Total number of assays for each streak.

Previous work with human fibroblasts has demonstrated that telomere shortening and senescence are delayed by addition of certain antioxidant chemicals to the growth medium such as ascorbic acid and α -phenyl-*t*-butyl nitron (PBN) (36, 37). Reactive oxygen species (ROS), which cause oxidative damage to DNA and other biomolecules, have been suspected as a major aging factor at the cellular level. Cells have antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and the antioxidant peptide glutathione (GSH), plus vitamins C and E as effective defense mechanisms against ROS. These enzymes neutralize ROS that damage chromosomal DNA and cell membranes, and help minimize oxidative stress *in vivo*. As shown in Figure 9A, SOD converts superoxide radicals to oxygen and hydrogen peroxide. As shown in Figure 9B and 9C, catalase and GPX break down hydrogen peroxide into water.

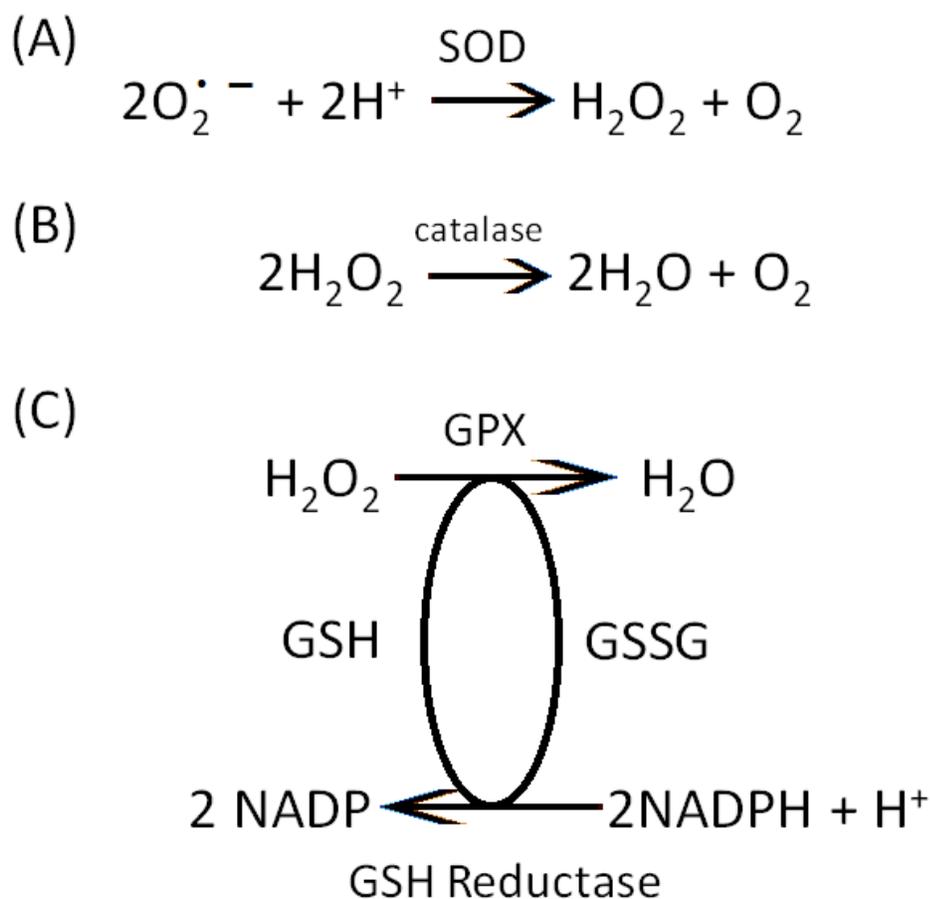


Figure 9. Mechanisms of antioxidant enzymes. (A) Superoxide dismutase (SOD) catalyzes the cleavage of superoxide. (B) Catalase converts hydrogen peroxide into water and oxygen. (C) Antioxidant glutathione (GSH) is oxidized to glutathione disulfide (GSSG) by reacting with hydrogen peroxide catalyzed by glutathione peroxidase (GPX). GSH is regenerated in an NADPH-dependent reaction catalyzed by GSH reductase (25).

In previous studies done by a former graduate student in the Lewis lab, Alison Russell, the gene for the major catalase enzyme, *CTT1*, or the major glutathione peroxidase enzyme, *GPX3*, was deleted in telomerase-deficient BY4742 cells and acceleration of cell senescence rates by inactivating these antioxidant genes was investigated (38). *Saccharomyces cerevisiae* has three genes (*GPX1*, *GPX2*, and *GPX3*), for glutathione peroxidase, and *GPX3* is more important than the other two genes. Also,

two genes, *CTT1* and *CTA1*, have been identified as catalase enzymes, and *CTT1* has most of the cell's catalase activity (39). In Alison's experiments, antioxidant and telomerase-deficient strains, *est2⁻ gpx3⁻* and *est2⁻ ctt1⁻*, were predicted to have higher oxidative stress and undergo senescence earlier than normal *est2⁻* cells. However, significant acceleration of cell senescence rates was not observed in either the *ctt1⁻* or *gpx3⁻* single antioxidant gene mutant (using the old assay method). In addition to two antioxidant single-mutants, Alison obtained a *yap1* mutant strain from Simon Avery's lab at the University of Nottingham, *EST2* was deleted, and the same experiment was performed. Yap1 is a transcription factor that up-regulates antioxidant genes under high oxidative stress in yeast and is considered to be important under accumulated oxidative conditions (40). The parent strain for the *yap1* mutant from Simon Avery's lab was BY4741. This strain required 5 streaks instead of 4 streaks before the cells senesced, but it did not appear to change cell senescence acceleration rates between normal *est2⁻* cells and *est2⁻ yap1⁻* mutant cells. Many more large survivor mutants were observed on the 4th streak in *est2⁻ yap1⁻* cells than in normal *est2⁻* cells, however (38).

In the current project, Alison's work with single antioxidant gene mutants was expanded into specific combinations of multiple inactivated antioxidant enzyme genes. Both the *GPX3* and *CTT1* genes were deleted at the same time in telomerase-deficient yeast cells, and whether senescence can be accelerated in cells was investigated by the new senescence assay. An example of the results seen in these experiments is shown in Figure 10. The variability of senescence was observed in the 4th streak plates. Some isolates showed strong senescence, some showed modest senescence, and some had one full column of growth (no senescence).

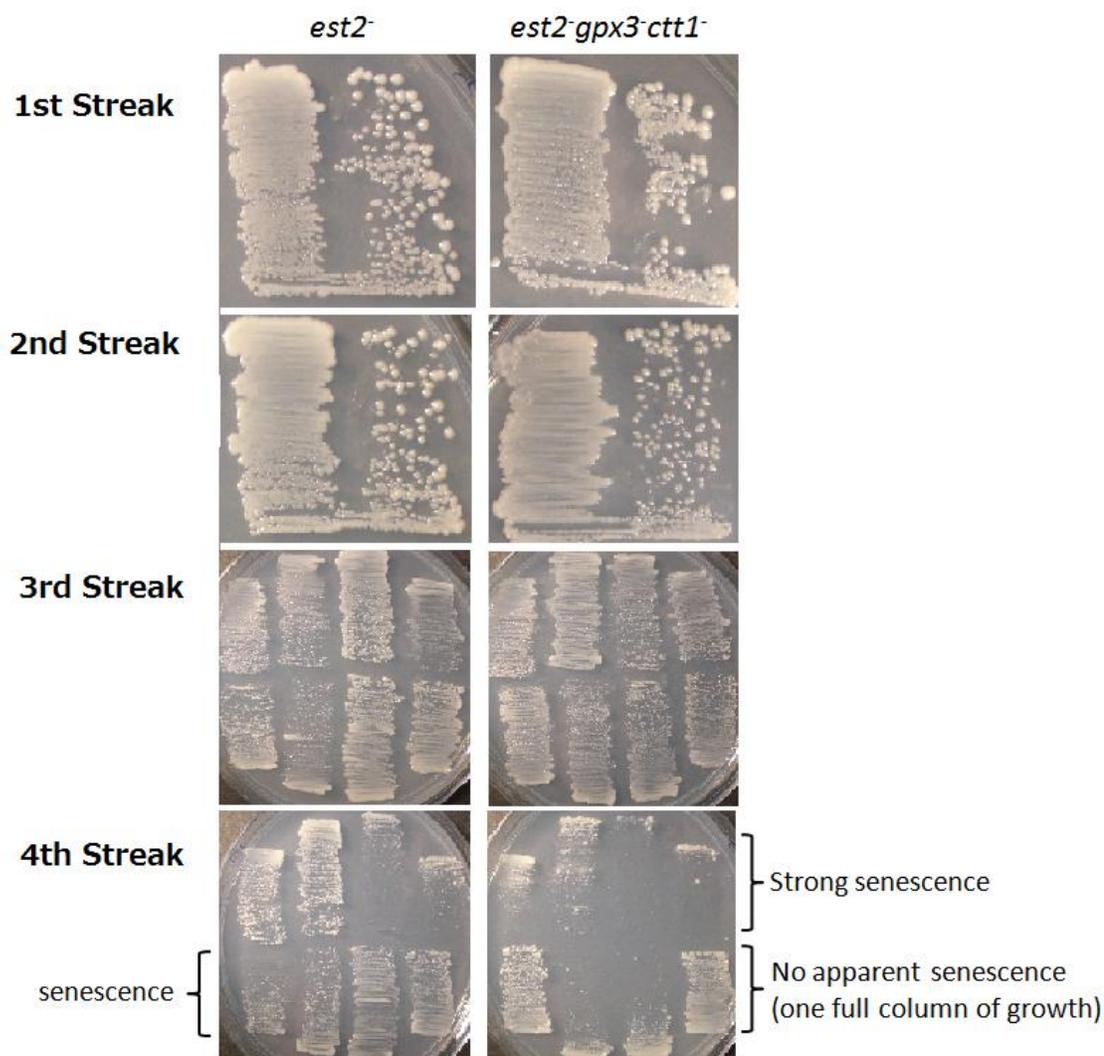


Figure 10. Examples of the new senescence assay. *est2*⁻-BY4742 and *est2*⁻*gpx3*⁻*ctt1*⁻-BY4742 cells were streaked onto synthetic glucose plates at 30 °C.

Two trials were performed and the data is shown in Table 1a. The cells with both *CTT1* and *GPX3* inactivated grew at near normal rates compared to normal telomerase-deficient cell on the 1st, 2nd and 3rd streaks. However, on the 4th streak, the cells showed a moderate increase in isolates that exhibited cell senescence. The number of isolates that senesced (did not achieve a full column of growth) was 26 out of 32 in the double-mutant

cells, but 20 out of 32 in the normal telomerase-deficient cells in the first trial. In the second trial, senescence was seen in 14 out of 32 in the double-mutant cells, but only 7 out of 32 in the normal telomerase deficient cells. Though the same mutant was also tested at a higher temperature, 38 °C, the cells did not grow fully on any columns in the 4th streaks on either wild type or the mutant (not shown). Therefore, the double-mutant cells and normal telomerase deficient cells could not be compared at elevated temperatures. These results suggest that more cells senesced by the 4th streak because oxidative stress is raised and more DNA damage is induced at higher temperatures.

In addition to this combination of inactivated antioxidant enzyme genes, both the *yap1* and *gpx3* genes were inactivated in BY4741 telomerase-deficient cells and tested. For unknown reasons, this strain background required 6 streaks before more than half of the isolates senesced. On the 4th streak, most isolates in both normal telomerase-deficient and antioxidant double mutants did not show senescence (Table 1b). On the 5th streak of *est2⁻ yap1⁻ gpx3⁻* cells, 14/32 in the first trial and 7/32 in the second trial did not produce full columns. In contrast, full growth in all columns was observed in the normal telomerase-deficient cells (i.e., 0/32 isolates had senesced in the *est2⁻* cells). On the 6th streak of the first trial, all *est2⁻* and *est2⁻ yap1⁻ gpx3⁻* isolates senesced. In the second trial, 27/32 isolates senesced in the antioxidant mutant cells, while only 19/32 senesced in the normal telomerase-deficient cells (Table 1). These data suggest that the cell senescence frequency was moderately accelerated in the *yap1⁻ gpx3⁻* mutant cells compared with normal telomerase-deficient cells, which have no antioxidant genes inactivated, especially based on results in the 5th streak.

Table 1. Analysis of cell senescence frequencies of (a) *est2⁻ctt1⁻gpx3⁻*-BY4742 double mutant cells, (b) *est2⁻yap1⁻gpx3⁻*-BY4741 double mutant cells.

(a)	Streak	3	4	
	Strains /BY4742	Senescence (<1 column of growth)		
<i>Trial 1</i>				
	YLKL803 (<i>est2⁻</i>)	0/16	20/32	
	YLKL829 (<i>est2⁻ctt1⁻gpx3⁻</i>)	2/16	26/32	
<i>Trial 2</i>				
	YLKL803 (<i>est2⁻</i>)	1/16	7/32	
	YLKL829 (<i>est2⁻ctt1⁻gpx3⁻</i>)	0/16	14/32	
(b)	Streak	4	5	6
	Strains /BY4741	Senescence (<1 column of growth)		
<i>Trial 1</i>				
	YLKL836 (<i>est2⁻</i>)	0/32	0/32	8/8*
	YLKL838 (<i>est2⁻yap1⁻gpx3⁻</i>)	3/32	14/32	8/8*
<i>Trial 2</i>				
	YLKL836 (<i>est2⁻</i>)	0/16*	0/32	19/32
	YLKL838 (<i>est2⁻yap1⁻gpx3⁻</i>)	0/16*	7/32	27/32

*Some plates were wet. Therefore, the total of 32 sample data could not be collected.

Improvement of Southern blot analysis was the second aim of my thesis project. While loss of cell growth capability is useful to evaluate cell senescence rates of yeast cells on solid media, Southern blot analyses are useful to assess telomere length changes occurring during the senescence process. In the Lewis lab, a non-radioactive digoxigenin (DIG)-based system from Roche Diagnostics (DIG High Prime DNA Labeling and Detection Kit II) has been used for Southern blot analysis. To observe telomere lengths of *S. cerevisiae* in Southern blotting, first of all, yeast chromosomal DNA is purified and digested with the restriction endonuclease XhoI and the digested DNA is separated in a 1.2% agarose gel using gel electrophoresis. XhoI is used because an XhoI restriction site is found within the subtelomere region on a majority of the yeast chromosomes. After DNA is transferred from the gel onto a nylon Hybond membrane, telomere-specific fragments are hybridized with a digoxigenin (DIG) labeled probe, which is synthesized using PCR. DIG is attached to dUTP, and DIG-dUTPs are incorporated into the probe during the amplification reaction. Then, the attached DIG labels are recognized by an alkaline phosphatase-conjugated antibody, and this enzyme-linked antibody produces chemiluminescence after addition of the substrate CSPD (disodium 3-(4-methoxysprio (1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3,3.1.13.7]decan)-4-yl)phenyl phosphate). In Figure 11, the bands represent telomere fragments from both the right and left ends of the yeast chromosomes because chemiluminescence is only detected on telomeric DNAs (where the DIG labeled probe was attached) in the digested yeast chromosomal DNA. The lowest band in each lane is usually broad because 15 different chromosomal DNA end fragments co-migrate at ~1100 bp.

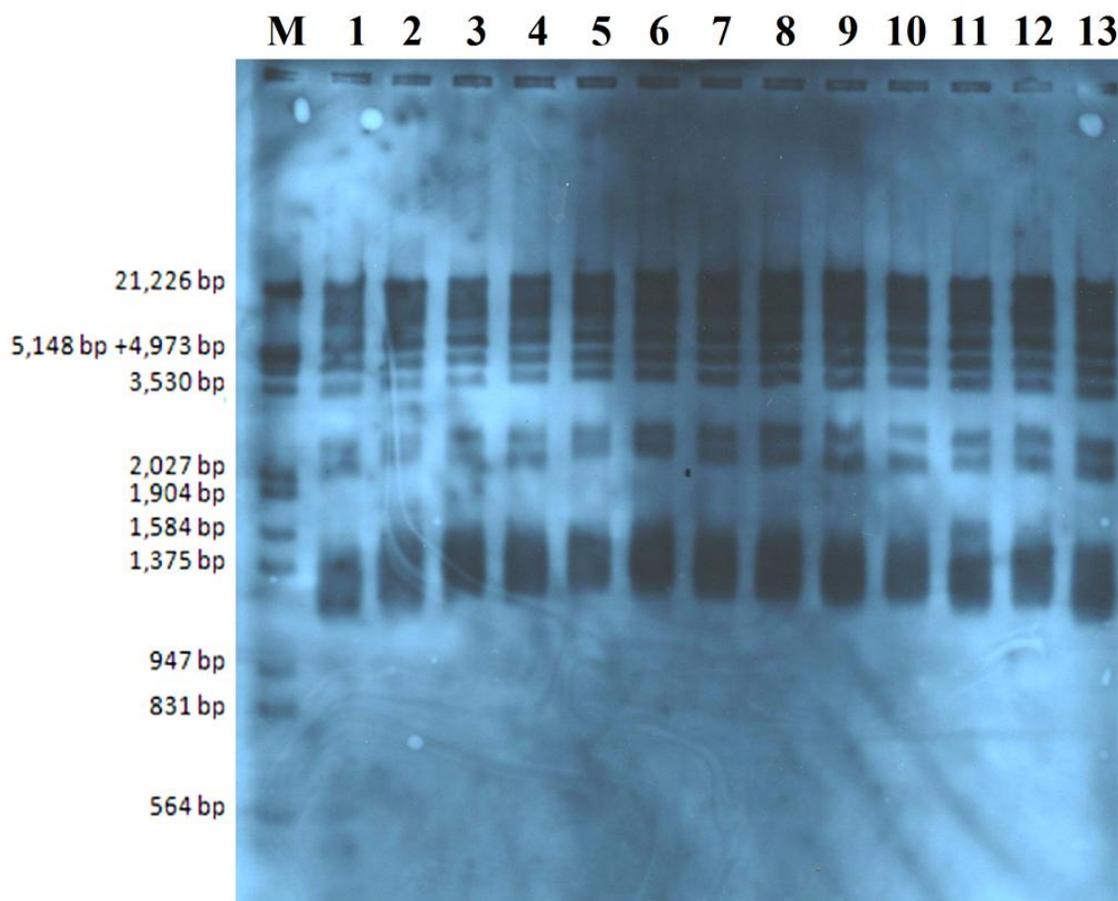


Figure 11. Southern blot picture. Lane M contained DIG labeled DNA molecular weight marker and lanes 1-13 contained XhoI digested yeast chromosomal DNA.

Before a Southern blot is performed, it is necessary to isolate chromosomal DNA from cells. Although a multitude of molecular techniques including Southern blots require the purification of genomic DNA, only a limited number of scientific articles describing yeast chromosomal DNA purification methods have been published. Because yeast cells have a complex cell wall, it is more difficult to lyse the cells than many other eukaryotic cells. There are three major ways to disrupt cell walls; enzymatically, physically, or chemically. When an enzyme such as zymolyase is used to disrupt cell walls, the performance is variable and regular purchase of the enzyme is expensive (31).

In physical disruption, glass beads and vortexing are used but this method causes shearing of the large chromosomal DNA, so it is of lower quality (32, 33). Though chemical-based purification methods are the simplest, most published protocols have used harsh chemicals like phenol, chloroform, and/or sodium hydroxide (32). For these reasons, many laboratories are dependent on expensive commercial purification kits whose contents are proprietary and therefore unknown. The goal of this part of the project was to complete development of a new chemical-based method for purifying yeast chromosomal DNA that is simpler than other published protocols and cheaper than commercial purification kits. A former graduate student in the Lewis laboratory, Christopher Lee, started the experiments using a protocol describing a fast procedure for yeast DNA purification published by Polaina and Adam as a model protocol (41). In Figure 12, the basic protocol developed by Lee is described (34). Briefly, the method involves lysis of cells to release the DNA, removal of proteins and cell debris by precipitation, and then precipitation and concentration of the DNA (Figure 12). His work was successful, but a number of important questions were not answered. Therefore, a series of experiments were carried out involving modifying reagents and incubation times in order to get maximal DNA recovery and his work has been completed in this current project.

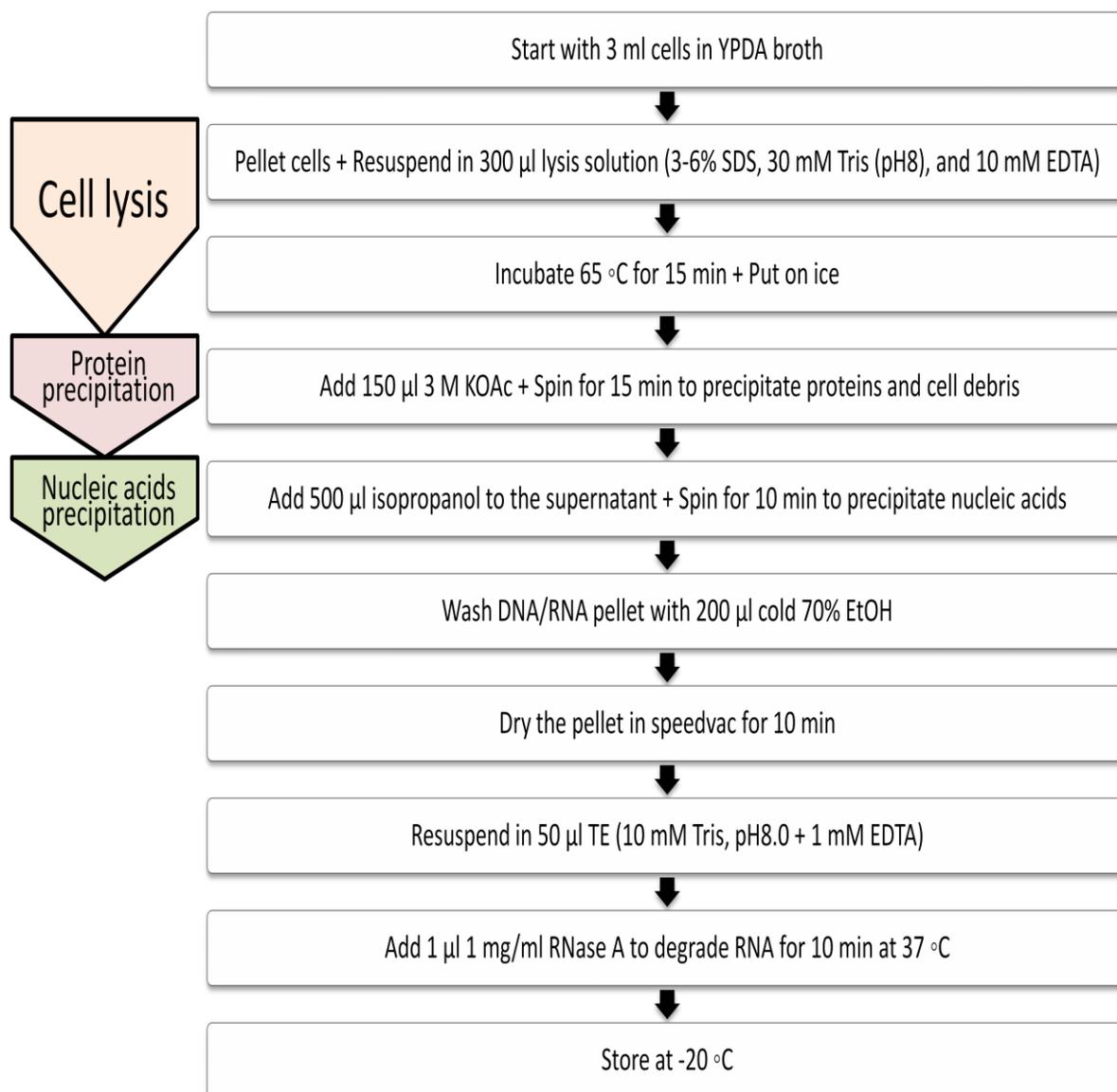


Figure 12. Basic yeast chromosomal purification protocol developed by Christopher Lee (34).

In the initial experiment, the efficiencies of different protein precipitation reagents were evaluated. The basic protocol used for the experiment involved growing 3-4 ml overnight cell cultures. Three ml of cells were lysed in 300 μ l lysis solution which contained 3% SDS and 10 mM EDTA. EDTA was used to inhibit DNase activity by chelating Mg^{2+} ions. The cells in the lysis solution were heated at 65 °C for 15 min and placed on ice. For one sample, 150 μ l of cold 3 M sodium acetate and for another sample, 150 μ l of cold 3 M potassium acetate was added to precipitate proteins and cell debris, followed by 15 min centrifugation and transfer of the supernatant to a new tube. Isopropanol (500 μ l) was added to the supernatant containing nucleic acids, and the DNA was precipitated. The DNA was washed by cold 70% ethanol, dried, resuspended in 50 μ l TE, and 1 μ l 1 mg/ml RNase A was added to degrade RNA. DNA concentrations were quantitated by fluorometry and protocol performances were evaluated based on comparison of relative yields. Four individual samples for each condition were tested.

As shown in Figure 13, protein precipitation with cold 3 M NaOAc produced a 33% increased DNA yield compared to cold KOAc. In a separate experiment, 43% increased product yield was produced using NaOAc (data not shown). From this result, the decision was made to use cold NaOAc instead of cold KOAc to precipitate proteins and cell debris in all following purification experiments.

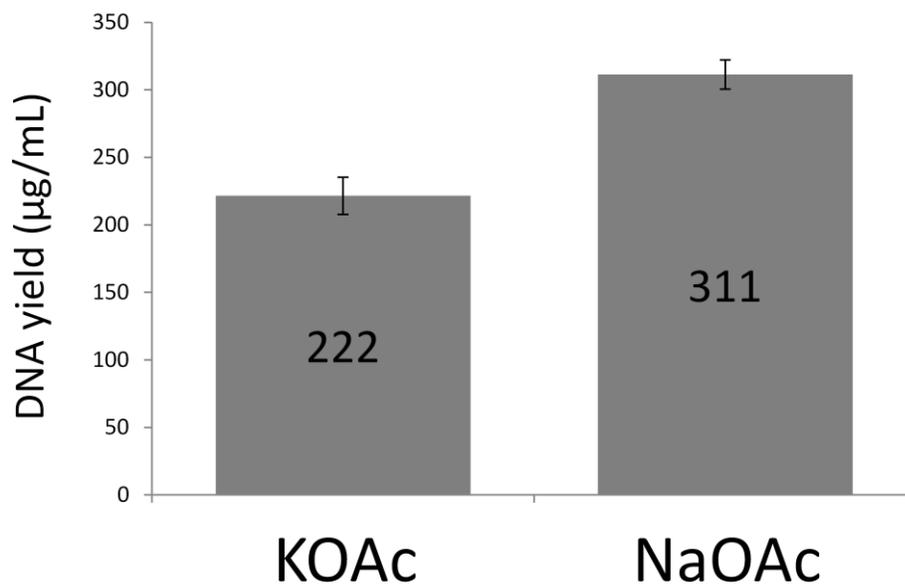


Figure 13. Average DNA concentrations after different protein precipitation reagents were used for DNA purification using the standard protocol. The number within each bar indicates DNA concentrations in µg/ml determined using a DNA fluorometer. Bars indicate standard deviations for 4 individual samples.

In the next experiment, the importance of addition of Tris buffer (pH 8.0) to the lysis solution was tested. The tested conditions were 3% SDS + 10 mM EDTA with (i) no Tris, (ii) 30 mM Tris, (iii) 50 mM Tris, or (iv) 100 mM Tris. The addition of 30 mM and 50 mM Tris to the 3% SDS lysis solution improved the DNA yield compared with the 3% SDS solution without Tris, though standard deviations overlapped so the results indicated a trend but were not statistically significant (Figure 14). Interestingly, Chris Lee also observed that addition of 30 mM Tris increased DNA yields using the older protocol (34). Although it is not clear why the presence of Tris buffer helps to produce more DNA yield, 30 mM Tris (which produced the highest DNA yield) was added to the 3% SDS lysis solution for subsequent yeast DNA purifications.

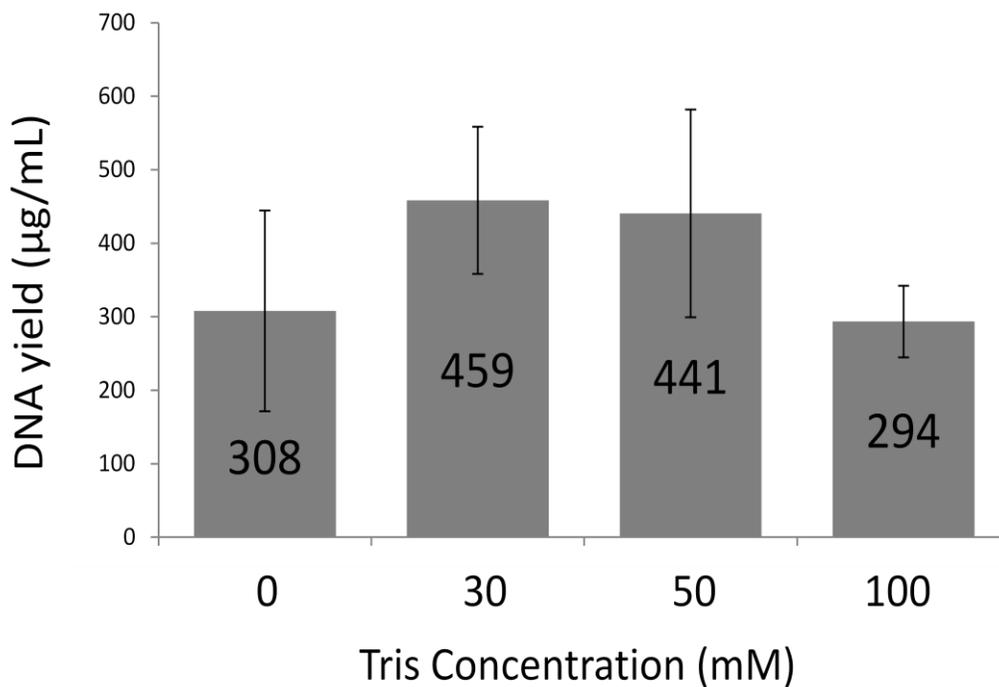


Figure 14. Average DNA yields after different concentrations of Tris buffer were added to the 3% SDS lysis solution. The addition of 30 mM Tris produced the best yield.

In the next experiment, whether longer incubation times at 65 °C increase disruption of the cell wall and cell membrane, and improves DNA yields, was investigated. Ten, 15, 20, 25, and 30 min of incubation time were compared. Although the DNA yield was increased with longer incubation times, overlapping standard deviations indicated that the results were not significant (Figure 15). Therefore, when considering additional time and impact on DNA yields, 15 min incubation time was retained as the standard protocol.

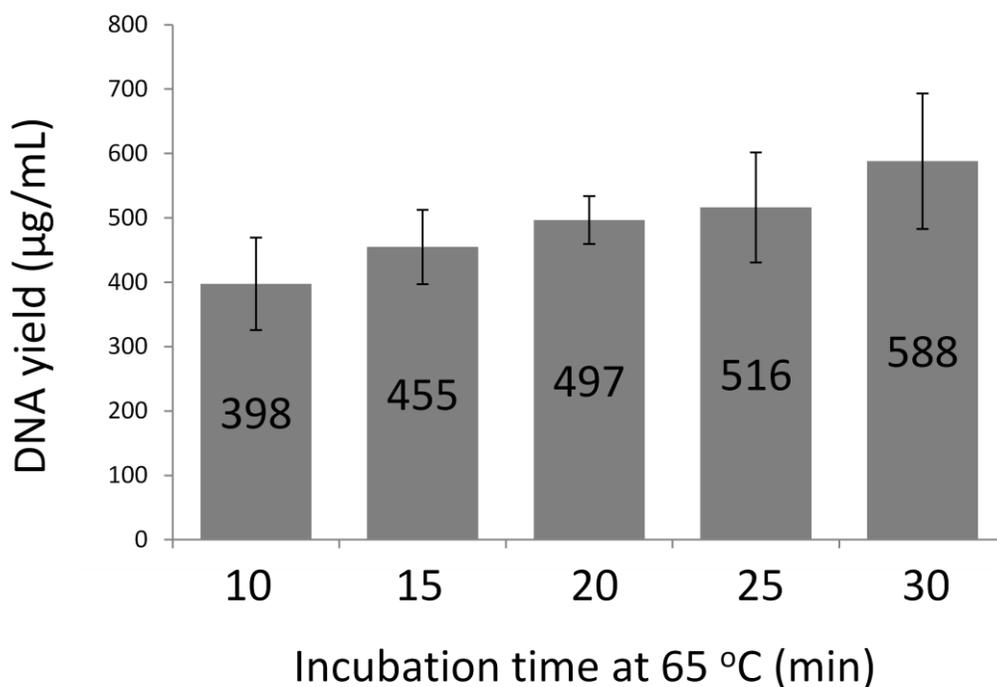


Figure 15. Average DNA concentrations produced by varying incubation times from 10, 15, 20, 25, and 30 min at 65 °C. The DNA yield was improved with longer incubation times, but results were not statistically significant.

The next question asked was, if the temperature is increased (but not so high as to damage the purified DNA), can DNA yield be improved? Incubation at 75 °C for 5, 10, or 15 min was compared to the standard protocol, 15 min at 65 °C. Unlike the previous experiment result, longer incubation at 75 °C did not increase yields. However, both 5 and 10 min at 75 °C gave higher yields than the standard protocol, 15 min at 65 °C. For example, yield was 520 µg/mL for 5 min at 75 °C but only 352 µg/mL for the standard protocol in this experiment (Figure 16). The experiments were repeated with 6% SDS lysis solution instead of 3% SDS, and a similar result was produced using 75 °C and 65 °C temperatures (data not shown).

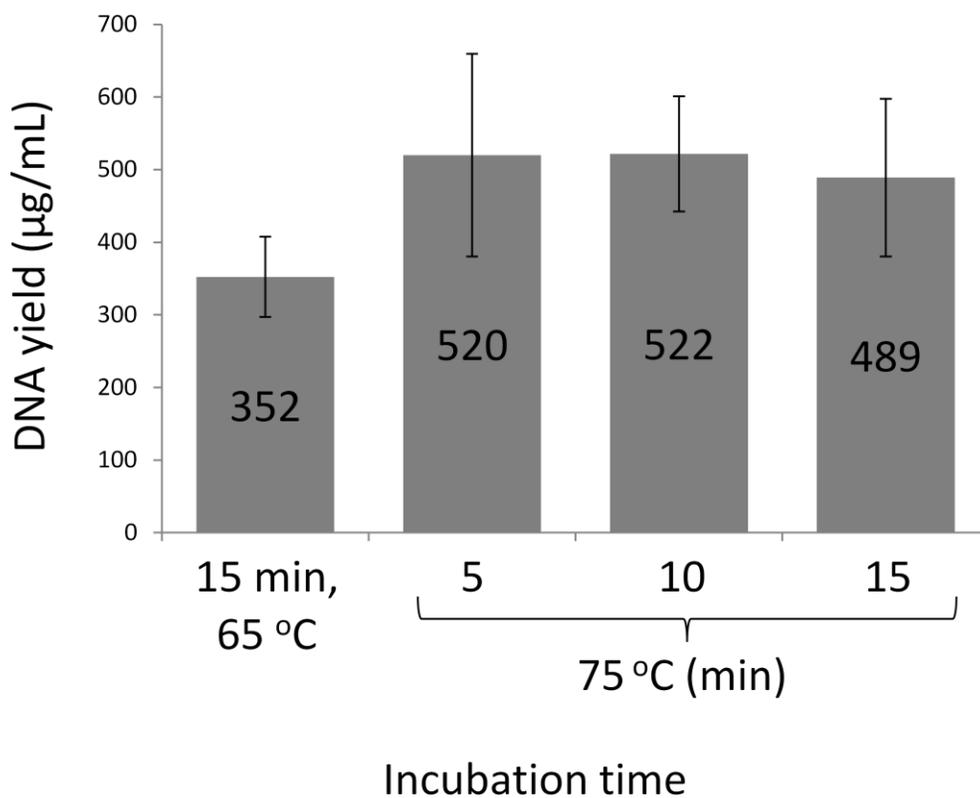


Figure 16. Average DNA concentrations produced after increasing temperature to 75 °C. The DNA yield was enhanced at 75 °C at all three incubation times.

To confirm the purity of the DNA visually, the DNA purified using 6% SDS lysis solution was loaded on a 0.8% gel, and visualized by UV fluorescence after staining with ethidium bromide (Figure 17). Relative band intensities were approximately equal (modest changes in DNA yield could not be detected by the intensities of the gel bands, unlike the more quantitative measurement of concentrations by fluorometer), and bands for purified yeast chromosomal DNA appeared around the expected molecular size of $\geq 50,000$ bp.

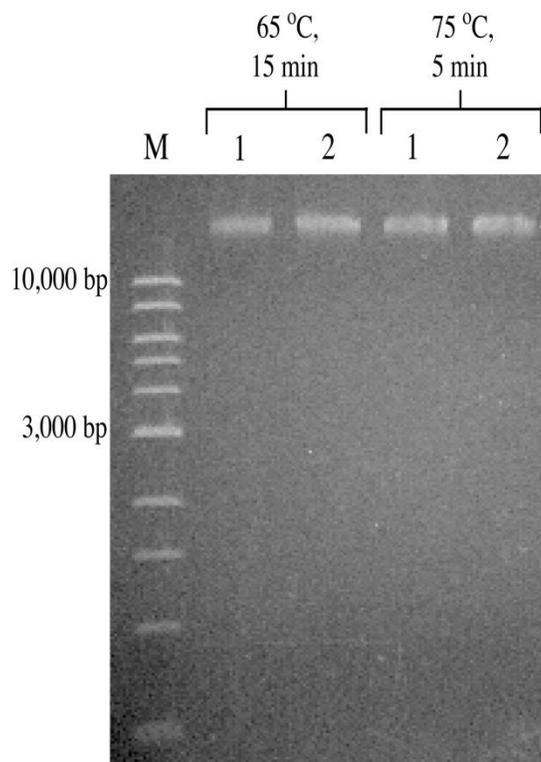


Figure 17. A 0.8% agarose gel showing fluorescent bands of yeast chromosomal DNA purified using the standard protocol with incubation for 15 min at 65 °C or 75 °C for 5 min. The gel was stained with ethidium bromide.

As an additional test of DNA quality, the purified DNA was amplified by PCR using primers that specifically amplified the yeast *MRE11* gene (Figure 18). The expected 2.5 kb *MRE11* gene bands were produced. This proved that DNA prepared using this protocol was pure enough to be amplified, and also that the increase of temperature to 75 °C did not degrade the yeast chromosomal DNA. These results demonstrate that the incubation time can be changed from 15 min at 65 °C to 5 min at 75 °C. However, in the following experiments, samples were incubated for 15 min at 65 °C as in the standard protocol.

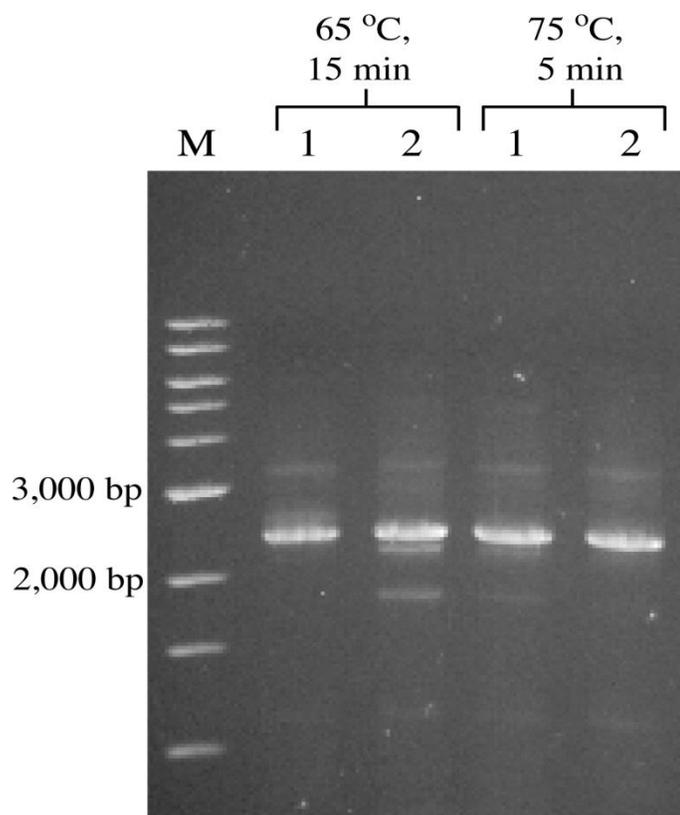


Figure 18. A 0.8% agarose gel showing fluorescent bands of yeast chromosomal DNA amplified using *MRE11* gene primers.

Different concentrations of SDS (3, 4, 5, 6 and 7%) combined with 30 mM Tris and 10 mM EDTA in the lysis solution were tested to optimize the maximal DNA recovery. DNA yield was not enhanced significantly by increasing SDS concentrations. The average yield using 6% SDS was slightly greater than other concentrations of SDS, and 6% SDS was chosen for use in subsequent experiments (Figure 19). A similar, non-statistically significant increase at 6% SDS was also seen by Chris Lee using the old protocol (34).

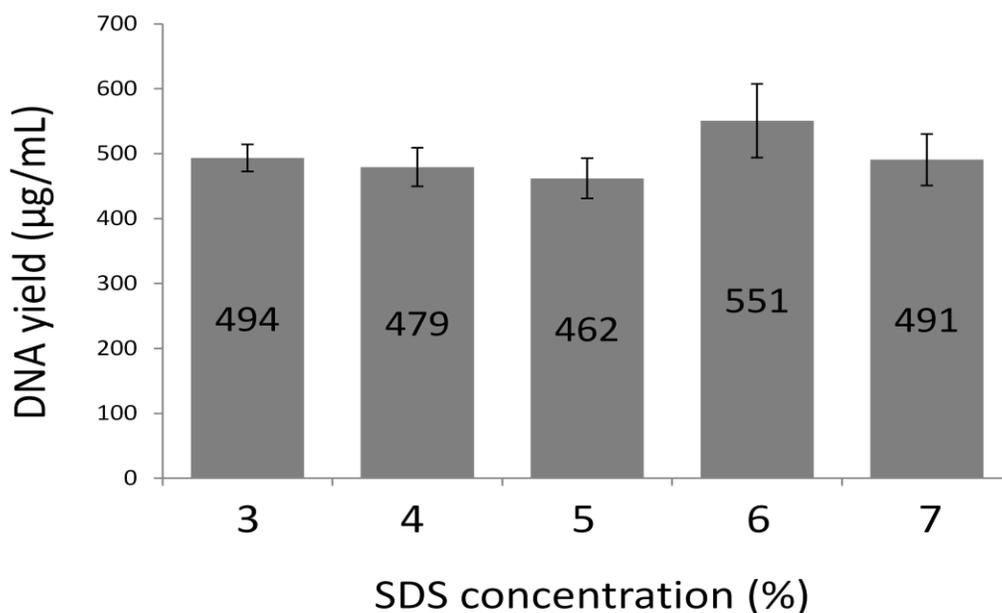


Figure 19. Average DNA yields produced with different SDS concentrations in the cell lysis solution. Tris (30 mM) and EDTA (10 mM) were also present in the lysis solution.

In other experiments, the effects of changing the solution volumes on the efficiency of extraction of DNA from 3 ml cells were assessed. In all cases, reducing volumes of 6% SDS lysis solution (6% SET) from 300 µl to 200 µl, reducing NaOAc from 150 µl to 100 µl, or reducing isopropanol from 500 µl to 400 µl, DNA yield was decreased moderately compared to the normal volumes (data not shown). Therefore, the solution volumes were kept at the same amounts as before.

The effects of changing the centrifugation times on the purified DNA yield were also investigated. The centrifugation time for protein precipitation (after addition of NaOAc) was reduced from 15 min to 10 min. Even after the centrifugation time was reduced, the same DNA yield was observed: 191 µg/ml (± 11.6) for the 15 min spin and 192 µg/ml (± 14.3) for the 10 min spin. In the next experiment, the centrifugation time for

nucleic acid precipitation (after addition of isopropanol) was reduced from 10 min to 5 min. The DNA yield was slightly reduced from 191 $\mu\text{g/ml}$ (± 11.6) for the 10 min spin to 162 $\mu\text{g/ml}$ (± 21.3) for the 5 min spin (Table 2). From these results, we concluded that the centrifugation time for NaOAc can be reduced to 10 min, but the isopropanol spin time should not be reduced.

Table 2. Effects of changing centrifugation times

	Standard protocol (10 min isopropanol 15 min NaOAc)	Isopropanol spin 5 min	NaOAc spin 10 min
DNA yield (S.D)	191 $\mu\text{g/ml}$ (± 11.6)	162 $\mu\text{g/ml}$ (± 21.3)	192 $\mu\text{g/ml}$ (± 14.3)

A new protocol based on these experiments was developed that incorporated use of; cold NaOAc, 6% SDS plus 30 mM Tris, 65 °C for 15 min incubation times and 10 min NaOAc centrifugation times. To test performance of this new protocol, DNA yield was compared with a commercial yeast DNA purification kit (Epicentre) that produces higher DNA yields than any other method tested in the Lewis lab during the past 9 years. Approximately the same amounts of yeast chromosomal DNA were purified: 190 $\mu\text{g/ml}$ for the new protocol and 209 $\mu\text{g/ml}$ for the commercial kit (Figure 20). This kit is expensive and costs \$258 for 200 purifications. In the newly developed protocol, only common laboratory reagents are used, and it is inexpensive, rapid and simple.

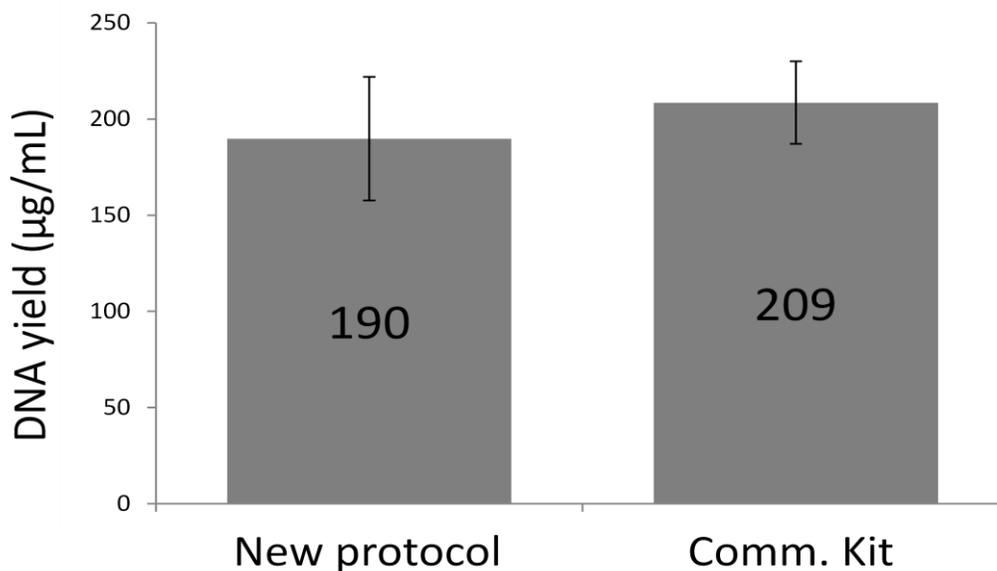


Figure 20. Comparison of average DNA concentrations of purified product using the newly developed protocol versus a commercial kit for yeast chromosomal DNA purification (Kit: MasterPure Yeast DNA Purification Kits).

The second part of the project involving improvement of Southern blot analyses of telomeres was to reduce thickness of bands in agarose gel electrophoresis by investigating ways to improve TBE and TAE running buffers to allow higher voltages. Increasing voltages reduce run times and therefore reduce DNA band diffusion, resulting in sharper (thinner) bands. These bands are highly desirable because they allow one to detect small differences in bands of similar sizes. Gel electrophoresis is one of the most common techniques in biochemistry research, and it is employed when biomolecules are separated and analyzed. Agarose gels are frequently used for analysis of nucleic acids such as DNA and RNA, and there are two common electrophoresis buffers, TBE (Tris, boric acid and EDTA) and TAE (Tris, acetic acid and EDTA) that are used (42). One limitation for agarose gel electrophoresis using these common buffers is that it must be

performed with low voltages (typically 100V-150V), requiring long run times (typically 50-90 minutes). At high voltage, heat is created and causes compression and/or slanting of bands as well as other artifacts. In Figure 21, a conventional 1 x TBE gel that was run at 220 V is shown. A ladder of linear DNA fragments ranging in size from 100 bp to 10,000 bp was loaded into 4 separate lanes. The current started at 115 mA and increased to 200 mA at the end. The temperature changed from 22 °C to 46 °C. At the end of the run, the solution was very hot and the gel was too soft to touch. As a result, bands in the gel were poorly resolved, with many of them slanted and of varying intensities.

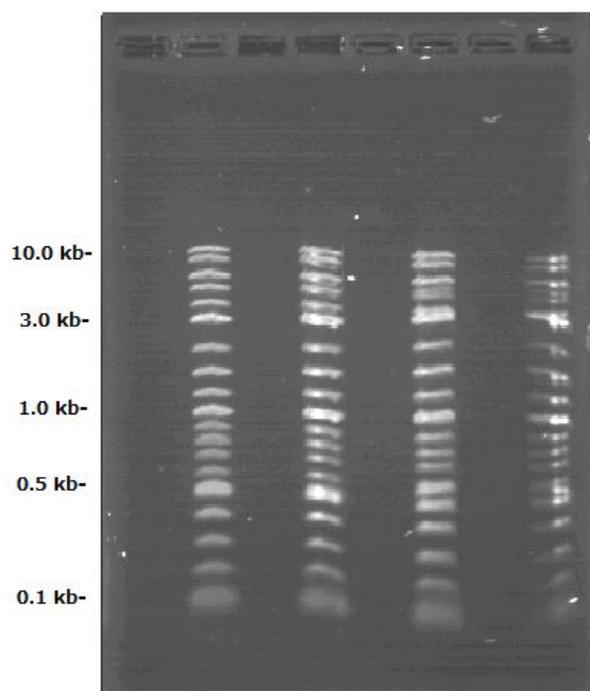


Figure 21. Poor DNA resolution in a gel run at 220 V in conventional 1 x TBE buffer. Lanes contained “2-log” DNA molecular weight standard ladders (New England Biolabs).

The common running buffers contain a weak acid (boric acid or acetic acid) and a weak base (Tris). These can exist in ionic forms, and these ions carry the electrical current, buffer the pH and create a low conductivity medium. EDTA is added to the running buffers because it inactivates any potential contaminating DNA nucleases in the solution by chelating Mg^{2+} ions. Because EDTA is not absolutely essential to the running buffers but can exist in multiple ionic forms (Figure 22), it may contribute to the current. pKa values for the four carboxylic acid groups of disodium EDTA are 1.99, 2.67, 6.16 and 10.26 (<http://openwetware.org>). Since electrophoresis buffers are typically set to ~pH 8, three of these groups are likely to be in their carboxylate ion forms.

Experiments were initiated with the hypothesis that reducing the EDTA concentration in the running buffers might decrease the current. The logic here is that when a gel is run at constant voltage (e.g., 150V), the current (measured in milliamps) will rise over time. This increased current generates heat, which gets distributed asymmetrically in the rig and leads to band distortion and can even melt the gel if the current increases very high.

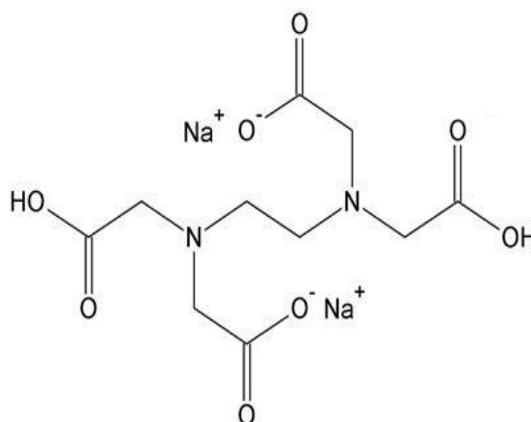


Figure 22. Chemical structure of disodium EDTA.

There are two major commercially available forms of EDTA, the free acid and the disodium salt. The disodium salt is more commonly used in molecular biology labs because its solubility is higher. Standard 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) was prepared with either the free acid or the disodium salt of EDTA, and the currents produced were compared with 1 x TB (without EDTA) at different voltages. Three measurements were collected in a single gel rig and the numbers were averaged. As shown in Figure 23, the current at 300 V for 1 x TBE (EDTA-free acid) was reduced by 34% compared with 1 x TBE (EDTA-disodium salt). This result was not unexpected because the extra sodium ions in disodium EDTA enhance the conductivity and therefore increase the current in the buffer. Moreover, the current at 300 V for 1 x TB (no EDTA) was approximately half of the current compared with 1 x TBE-disodium salt. The 1 x TBE buffer made with free acid EDTA, which does not contain highly conductive Na^+ ions, was expected to have similar current with 1 x TB. However, the current was reduced which suggests that some of the carboxylic acid groups of EDTA were indeed ionized at the pH of the running buffer and the conductivity of the solutions was increased. A more modest current difference was observed with 1 x TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA) versus 1 x TA (no EDTA). Interestingly, the mAmps in TA buffer were >50% higher overall than with TB buffer. For example, at 300 V the currents were 80 and 140 mAmp for 1 x TB and 1 x TA, respectively (Figure 23).

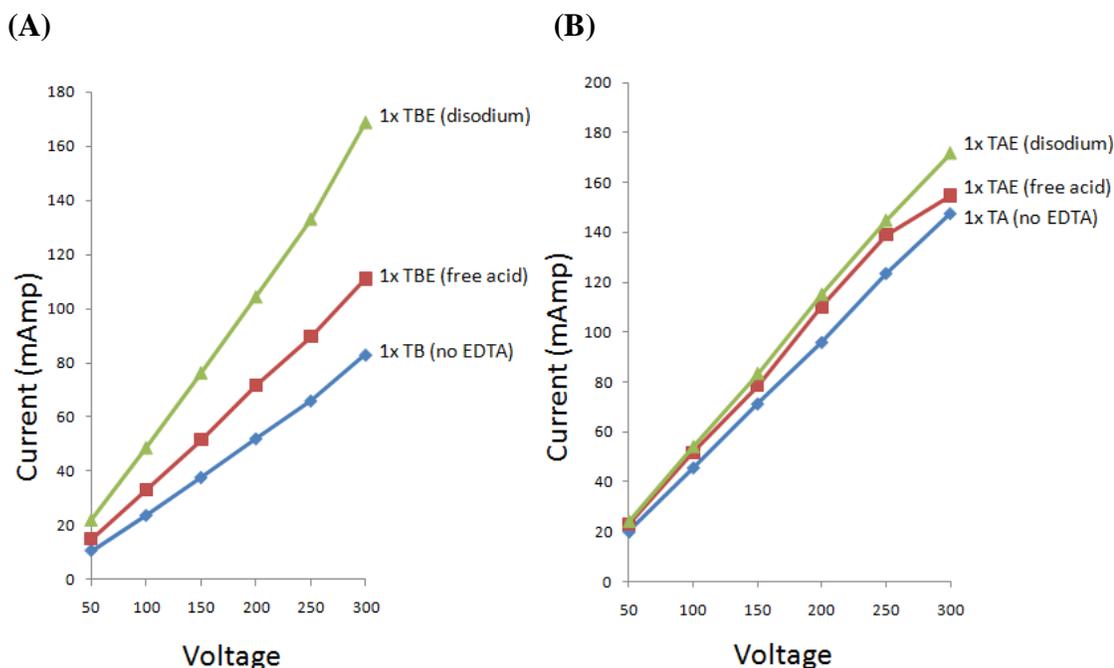


Figure 23. Effects of EDTA on electrical currents (A) in standard 1 x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), and (B) in standard 1 x TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA).

EDTA was revealed to have a strong impact on running buffer current in these experiments. The next question was whether this effect on current could be minimized by reducing the EDTA concentration in the buffer. The concentrations of disodium EDTA in 1 x TBE were reduced to 1, 0.5, 0.1, and 0 mM, and the impact on current was investigated. Concentrations of disodium EDTA of 1 or 2 mM increased the current, but concentrations below 0.5 mM did not increase the current substantially relative to TB buffer without EDTA (Figure 24). Furthermore, reduction or even elimination of EDTA had no effect on DNA resolution or band quality in subsequent experiments (shown below).

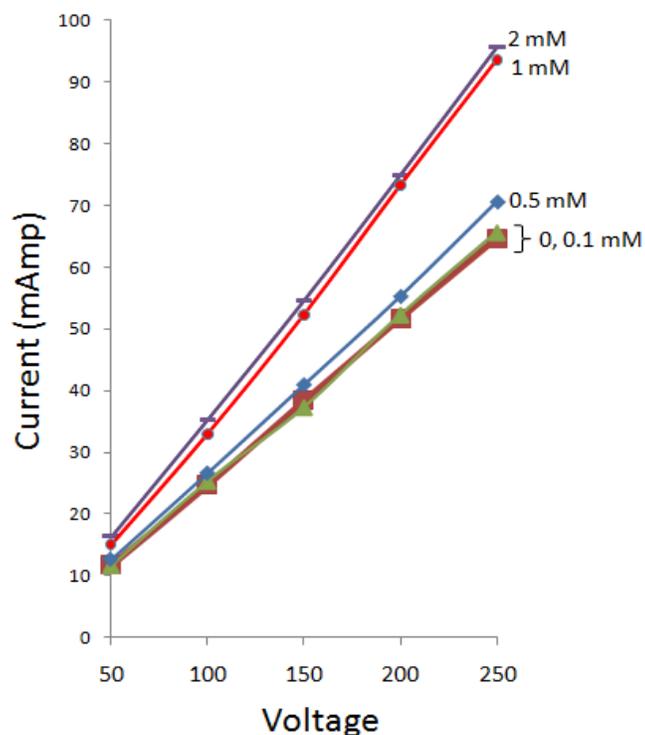


Figure 24. Comparison of electrical currents at different voltages with various disodium EDTA concentrations in 1 x TBE.

In the next experiment, the standard 1 x TB buffer was diluted to 0.5 x or 0.3 x concentrations of Tris and boric acid and the effects on band qualities and current were tested. Current was reduced in 0.5 x and 0.3 x TB relative to 1 x TB (Figure 25). These results makes sense because reducing the concentrations of electrolytes in a solution should reduce its conductivity and therefore its current. The results also suggest that one can run gels with 0.5 x or 0.3 x TB at higher voltage than normal, potentially causing faster migration of bands down the gel. However, lowering the electrolyte concentration may reduce band quality and separation compared to the 1 x buffer.

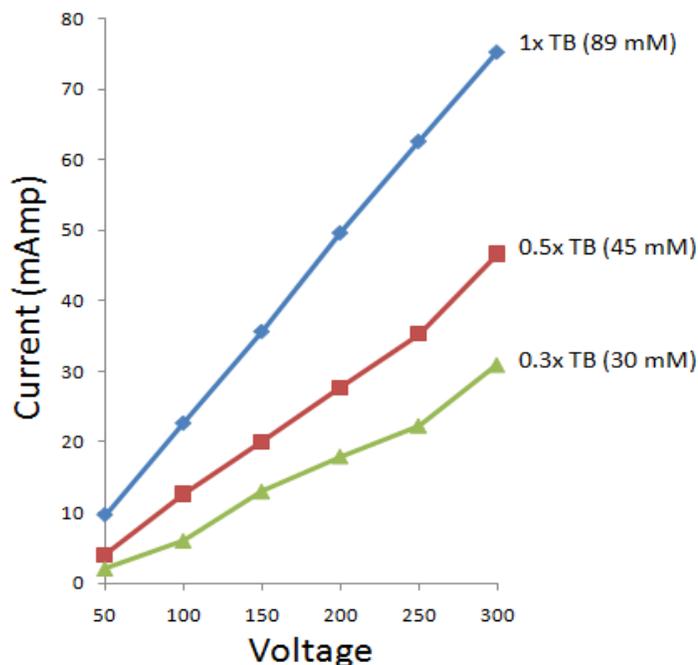


Figure 25. Comparison of currents at different voltages using 1 x, 0.5 x and 0.3 x TB buffers.

Because the current could be reduced (preventing heat generation) by removing EDTA and also by reducing the buffer concentration, gel electrophoresis using 1 x, 0.5 x or 0.3 x TB buffer can be performed at higher voltages now without such a strong current problem. However, band quality must be tested. A series of agarose gel electrophoresis experiments using 1 x, 0.5 x and 0.3 x TB buffers were performed. DNA band quality in gels run at 150 V, 200 V, and 250 V using 1 x TB and 0.5 x TB buffer remained high, but strong compression effects were seen at 300 V (indicated by the letter “C” in Figure 26B, C). In 0.3 x TB buffer, DNA band resolution became poor (compression and broadening of bands) even at 150 V, suggesting that 0.3 x TB cannot be used as a running buffer (Figure 26D).

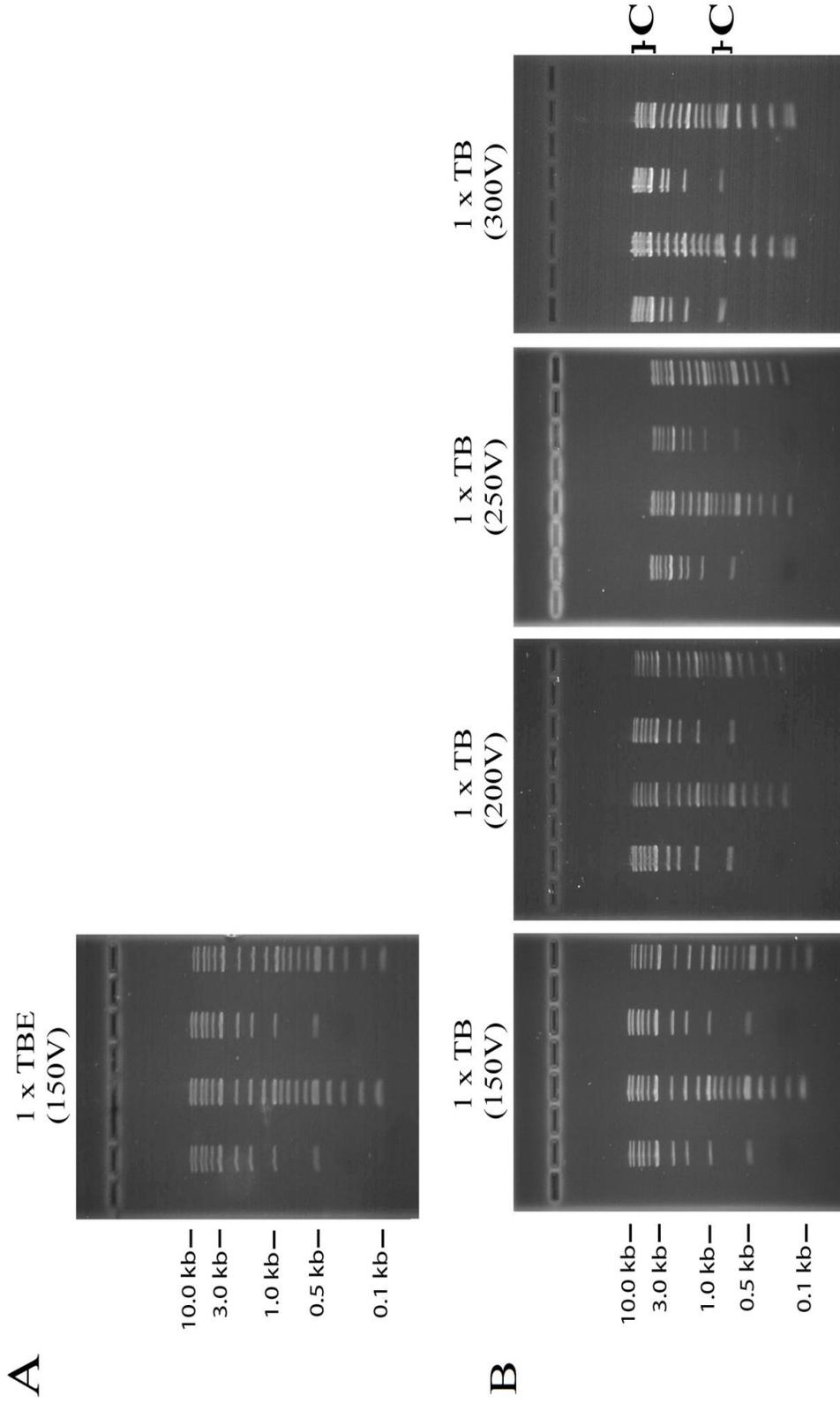


Figure 26. Resolution of DNA fragments in 1% agarose gels containing Tris-boric acid buffers (A) with 2 mM EDTA (B) without EDTA at 1 x (C) at 0.5 x or (D) at 0.3 x concentrations. All gels (10 cm) were run until bromophenol blue dye had migrated 7.5 cm and were stained with ethidium bromide. Lanes contained “1 kb” and “2-log” DNA molecular weight standard ladders (New England Biolabs). The letter “C” indicates strong band compression.

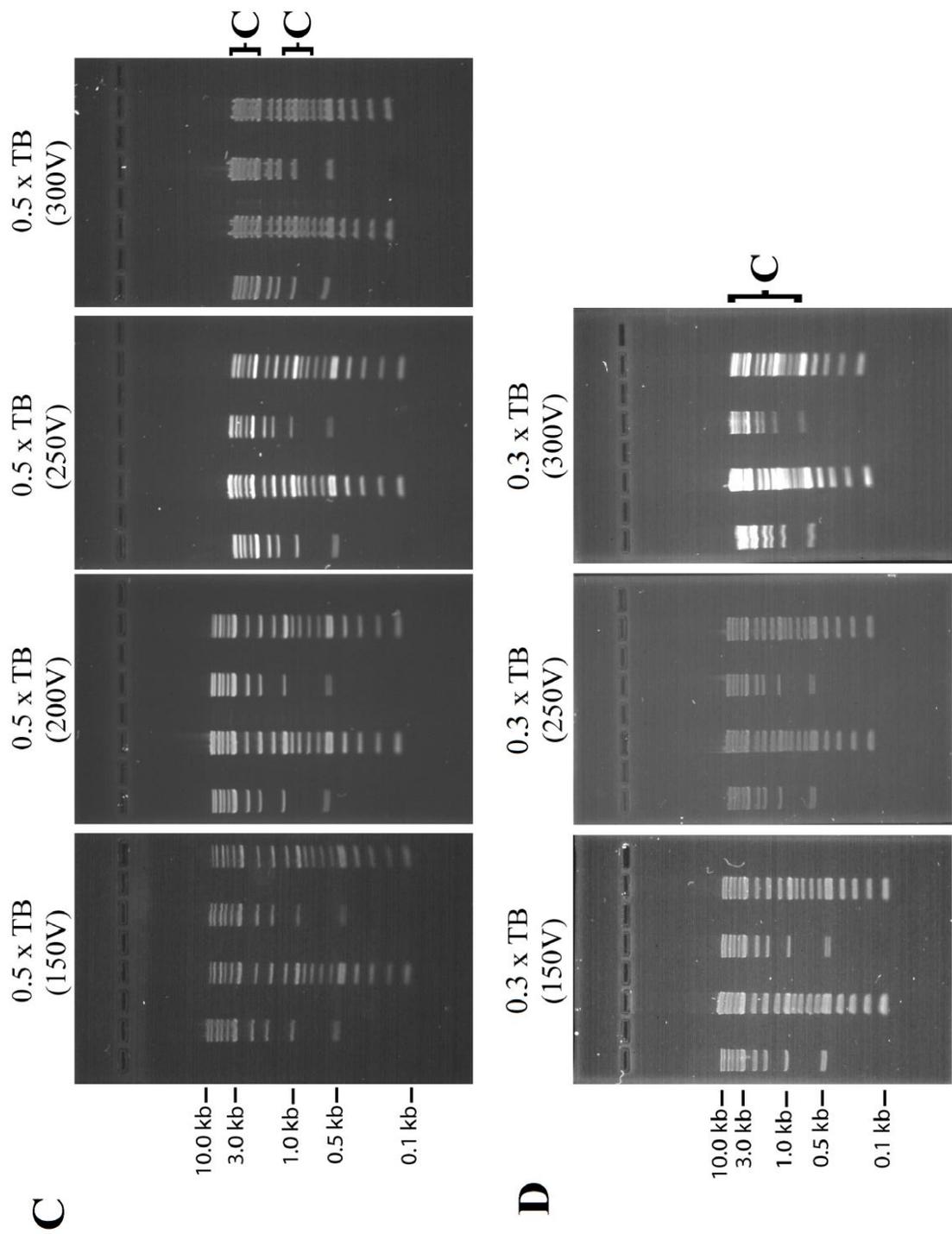


Figure 26. Cont.

The beginning and ending electrical currents were measured for each gel and are shown in Table 3. The TB buffers without EDTA kept lower current than TBE throughout the run except at 300 V in 1 x TB buffer. Currents above 100 mAmps have been shown to produce unacceptable heating in the gel electrophoresis rigs used here. Thus, all of the TB buffers lacking EDTA can be potentially used at up to 250 V (Table 3). However, even though the current was kept low, band qualities in a gel did not always remain high, as seen with the 0.3 x TB gels (Figure 26D).

As shown above (Figure 23), inclusion of 0.5 mM EDTA did not increase the current in TB buffer. As a result, the standard 1 x TBE buffer can be replaced with 1 x TB plus 0.5 mM EDTA (or 0.5 x TB plus 0.5 mM EDTA). The solution would still have a preventative measure to inhibit DNA nucleases that may have contaminated the solution and also can be run at up to 250 V. By running a gel at 250 V instead of the normal low voltage of 100 V, the running time can be reduced to less than half (Figure 27).

Table 3. Changes in current at different voltages and different concentrations of TB buffer. Currents > 100 mAmps generate excessive heating.

		Voltage								
		150		200		250		300		
Current (mAmp)	Buffers	<u>Start</u>	<u>End</u>	<u>Start</u>	<u>End</u>	<u>Start</u>	<u>End</u>	<u>Start</u>	<u>End</u>	
		1x TBE	69	101	112	200	113	202	---	---
		1x TB	37	46	56	83	63	93	72	119
		0.5x TB	21	24	26	32	41	49	42	58
		0.3x TB	14	16	18	20	28	33	32	39

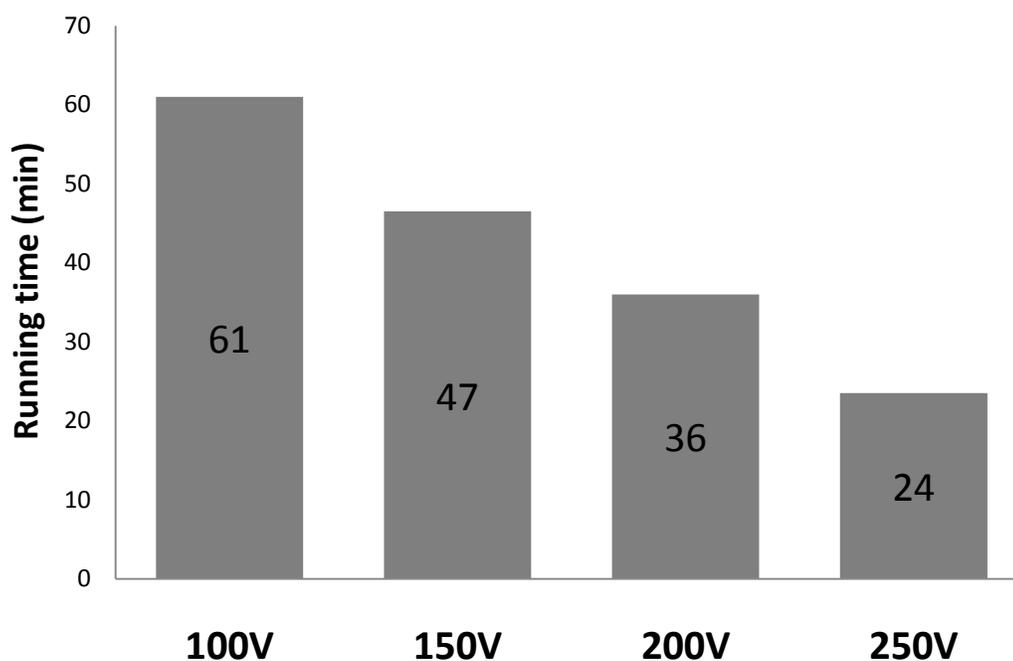


Figure 27. Times required to complete electrophoresis of DNA samples in 1 x TB gels containing 1% agarose. Electrophoresis was performed until the tracking dye, bromophenol blue, had migrated 7.5 cm down the 10 cm gel.

Gels containing 1 x TAE or even 1 x TA (without EDTA) exhibited much higher currents than TB-based gels (Figure 22). Therefore, gels cannot be run above 150 V using 1 x TA-based buffer, unlike 1 x TB-based buffer. On the other hand, the current was reduced using 0.5 x TA buffer and the voltage can be increased up to 250 V without high current or heat generation (Table 4). However, the bottom half of DNA fragments had strong smearing at 250 V using 0.5 x TA buffer (Figure 28B). Interestingly, TA-based buffer had better resolution of the upper bands (10.0 kb - 3.0 kb) than the TB-based buffer. On the other hand, the bottom half of DNA fragments (1.0 kb - 0.1 kb) were separated much better in TB-based than TA-based buffer (Figure 28A).

In theory, band diffusion is reduced to a minimum using the improved running buffer because running time is reduced with higher voltage. It is likely that this will also produce thinner bands on Southern blot analyses and improve analysis.

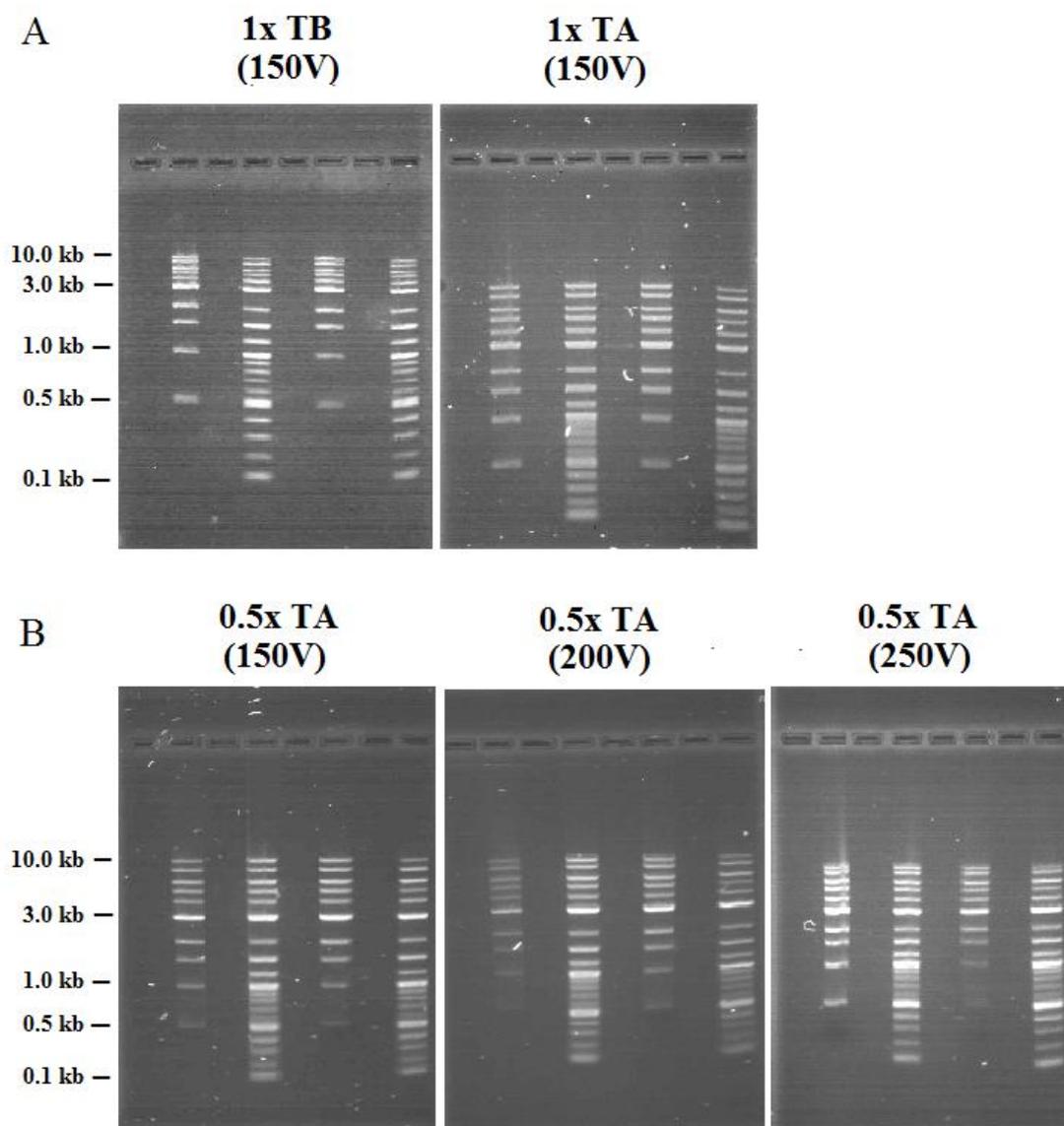


Figure 28. Resolution of DNA fragments in 1% agarose gels containing Tris-acetate buffers. (A) Comparison between 1x TB and 1x TA. (B) 0.5x TA without EDTA.

Table 4. Changes in current in 0.5 x TA buffer.

		Voltage					
		150		200		250	
	Buffer	<u>Start</u>	<u>End</u>	<u>Start</u>	<u>End</u>	<u>Start</u>	<u>End</u>
Current (mAmp)	0.5 x TA	39	41	64	76	80	107

Summary

The current thesis project involved the development of a new senescence assay that takes into account the randomness of cellular senescence. Because of the stochastic nature of cellular senescence, phenotype variations in yeast cells were observed at 60-70 generations (at the 4th streak) when solid media-based senescence assays were used. For example, most telomerase-deficient BY4742 yeast cells showed cell senescence after approximately 60-70 generations, but the level of growth on the 4th streak plate was highly variable. The newly developed solid media-based senescence assay involved streaking a large number of single-columns of cells beginning on the 3rd streak and using 4 days incubation instead of 3 days. A total of 32 single-column isolates were analyzed on the last streak in the new cell senescence assay, which permits, for the first time, application of statistics to the study of cellular senescence.

Using the new cell senescence assay method, the effects of specific combinations of inactivated antioxidant enzyme genes on cell senescence rates were assessed. Antioxidant enzymes are an important defense mechanism against reactive oxygen species *in vivo*. Both the glutathione peroxidase enzyme gene, *GPX3*, and the catalase enzyme gene, *CTT1*, were deleted at the same time in telomerase-deficient yeast cells, and senescence was investigated using the new senescence assay. The cells with both *CTT1* and *GPX3* genes inactivated showed moderate acceleration of cell senescence compared to normal telomerase deficient cells, which have no antioxidant genes inactivated. Yap1 is a transcription factor that up-regulates antioxidant genes under high oxidative stress in yeast. Both the *YAP1* and *GPX3* genes were inactivated in telomerase-deficient cells and tested. Cell senescence was moderately increased in the *est2⁻yap1⁻*

gpx3⁻ mutant cells compared to normal telomerase-deficient cells. These results suggest that these antioxidant activities have a modest role in cellular senescence, possibly by affecting rates of telomere shortening (36, 43).

Improvement of Southern blot analysis was the second aim of my thesis project. While loss of cell growth capability is useful to evaluate cell senescence rates of yeast cells on solid media, Southern blot analyses can be used to assess telomere length changes occurring during the cell senescence process.

Before a Southern blot is performed, it is necessary to isolate chromosomal DNA from cells. However, only a limited number of scientific articles describing yeast chromosomal DNA purification methods are available (31-32, 41), and commercial purification kits are expensive. The goal of this part of the project was to complete the development of a new chemical-based method for purifying yeast chromosomal DNA, which was started by a former graduated student, Christopher Lee (34). In this project, use of cold sodium acetate improved DNA yield compared to cold potassium acetate for protein and cell debris precipitation. Cell lysis solution containing 6% SDS, 10 mM EDTA and 30 mM Tris buffer (6% SET), produced the best DNA yields. Also, these experiments established that the centrifugation time for protein precipitation could be reduced from 15 min to 10 min, and the incubation time could be changed from 15 min at 65 °C to 5 min at 75 °C as well. The newly developed protocol had approximately the same performance as a high yielding commercially available DNA purification kit. Moreover, the new protocol uses inexpensive, readily available chemicals in labs and is very simple and rapid.

The second part of the project involving improvement of Southern blot analyses was to increase resolution of DNA bands in agarose gel electrophoresis. The most common running buffers, TBE and TAE, were modified to allow higher voltages without band distortion. High voltage makes the running time short and a shorter running time reduces band diffusion, resulting in thinner bands. Conventional 1 x TBE or 1 x TAE buffer have a limitation on current in that they cannot be run at more than 150 V because high heat is built up. Standard running buffers contain a weak acid and a weak base, which create a low conductivity medium and buffer the pH, plus EDTA, which prevents DNA degradation by chelating Mg^{2+} ions. For the first time, effects on current using different forms of EDTA were evaluated by Brody *et al.* (44), and EDTA was revealed to enhance conductivity in the buffer and raised current strongly in my experiments. Therefore, a series of experiments were performed using buffers without EDTA. Current was reduced in 0.5 x and 0.3 x TB buffers relative to 1 x TB, so the effects on gel band qualities and currents were tested in 1 x, 0.5 x, and 0.3 x TB buffers at different voltages. Currents remained low up to 300 V in the buffers. However, DNA band qualities remained high only in gels run at 150 V-250 V and only in gels containing 1 x TB and 0.5 x TB buffer. Addition of up to 0.5 mM EDTA into the buffer did not affect the current significantly. Therefore, the standard 1 x TBE buffer can be replaced with 1 x TB plus 0.5 mM EDTA (or 0.5 x TB plus 0.5 mM EDTA). This buffer improvement makes it possible to run a gel at 250 V, shortening the run time to half that of a gel run at 100 V. Also, it is likely to produce thinner bands on Southern blot analyses and therefore improve resolution. Tris-acetate buffers exhibited higher current than Tris-borate buffers, with or without EDTA. Analysis of 0.5 x and 1 x TA gels suggested that 0.5 x TA could

be employed at higher voltages (200-250 V) without excessive heating. However, resolution of smaller DNA fragments (0.1-1.0 Kb) was reduced in the TA buffers.

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