Video Article

# Studying Age-dependent Genomic Instability using the *S. cerevisiae* Chronological Lifespan Model

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## Abstract

Studies using the *Saccharomyces cerevisiae* aging model have uncovered life span regulatory pathways that are partially conserved in higher eukaryotes[1-2]. The simplicity and power of the yeast aging model can also be explored to study DNA damage and genome maintenance as well as their contributions to diseases during aging. Here, we describe a system to study age-dependent DNA mutations, including base substitutions, frame-shift mutations, gross chromosomal rearrangements, and homologous/homeologous recombination, as well as nuclear DNA repair activity by combining the yeast chronological life span with simple DNA damage and mutation assays. The methods described here should facilitate the identification of genes/pathways that regulate genomic instability and the mechanisms that underlie age-dependent DNA mutations and cancer in mammals.

## Video Link

The video component of this article can be found at http://www.jove.com/video/3030/

## Protocol

Two lifespan models have being used to study aging in *S. cerevisiae*: RLS and CLS. The replicative (budding) life span (RLS) is based on the observation that yeast mother cells undergo a finite number of divisions[3-6]. We will focus on the chronological lifespan (CLS), a model based on the chronological survival of non-dividing yeast in culture or on plates[7-11].Wild type yeast grows exponentially for 10-12 hours in synthetic dextrose complete (SDC) medium. When glucose level reduces, yeast switches from fermentation to respiration, a process termed diauxic shift. Cells continue to divide slowly during the post-diauxic phase for approximately 48 hours before entering $G_0$ arrest. The metabolic rate of the cells remains high till day 5-6 (day 0 is the inoculation day). Cell viability over time can be accessed by the percentage of the chronologically aging cells, sampled every two days, which can exit $G_0$ arrest and form colonies on rich YPED plates.

## 1. Yeast chronological lifespan (CLS) in liquid culture

1. Inoculate a single colony into 1 ml synthetic complete glucose medium (SDC, Table 1) and incubate overnight with shaking (220 rpm) at 30°C. Three inoculums from independent colonies should be prepared for each strain to provide biological replicas.
2. Dilute the overnight culture into fresh SDC medium (usually 10 ml) to OD=0.05-0.1 (~1:100 dilution) and incubate with shaking (220 rpm) at 30°C. This time point is considered day 0 of the chronological aging. Maintain a 1:5 ratio of culture/flask volume (10 ml culture in 50 ml, or for longer/large experiments 25 ml culture in 125 ml) to ensure proper aeration.
3. Starting from day 3, remove 2 aliquots of 10 µl from the flask, dilute 10,000 times in autoclaved water, 10 µl diluted culture (i.e., $10^4$-10) on YEPD (1% yeast extract, 2% bacto peptone, 2% dextrose, 2% agar) plates, and incubate at 30°C for 48-72 hours before colony forming unite (CFU) is counted.
4. Day 3 CFU is considered 100% survival. Dilution factors of aging culture in the subsequent days should be adjusted accordingly to ensure ~20-100 colonies in the CFU assay (e.g., $10^4$-10, $10^4$-30, $10^3$-10, etc.). For wild type cells in the DBY746 background, the CLS reaches 1% survival around day 11.

Variations of *in situ* viability assay include:

- Calorie restriction (CR) by glucose limitation. Dilution of overnight culture in glucose limited SC medium (such as 0.5 or 0.05% instead of the standard 2% glucose) generally lead to lower population saturation density on day 3, but much extended mean and maximal (10% survival) lifespan[12].
- For extreme CR/starvation, wash the day 3 stationary phase culture (usually 10 ml, as in step 3 above) with autoclaved water (resuspend cells in equal volume of water and spin down at 2500 rpm for 5 min, repeat 3 times). Incubationof cells in water mimics the starvation condition that yeast may encounter in the wild. Every two days subsequently, aging culture should be washed with autoclaved water and resuspended in equal volume of water to remove any nutrients released from lysed dead cells. Perform viability assay by sampling the aging culture as described above. This starvation condition will led to almost a doubling of mean CLS in the wild type DBY746 strain[12].

## 2. *in situ* viability assay

In the liquid culture, a small fraction of the surviving cells may re-entered the cell cycle and grow utilizing remaining nutrients or those released form lysed dead cells in the medium, a phenotype termed regrowth/gasping[13]. We developed a viability-on-a-plate system that utilizes the auxotrophy of the DBY746 strain (trp1)to circumvent the regrowth/gasping problem and also allow the testing of the effect of constant exposure or deprivation of various external nutrients or stimuli on yeast CLS[11]. This *in situ* viability assay also mimics the replicative lifespan model such that cells are constantly exposed to abundant nutrients for the duration of the lifespan analysis.

1. Inoculate a single colony into 1 ml synthetic complete glucose medium (SDC) and incubate overnight with shaking (220 rpm) at 30°C. At least three inoculums from independent colonies should be prepared for each strain to provide biological replicas.
2. Let the culture to grow to ~$10^8$ cells/ml ($OD_{600}$=~10), dilute the culturewith autoclaved water to 100-200 cell/10 µl (usually $10^4$-fold dilution) and 1,000 cells/10 µl ($10^3$-fold dilution).
3. Plate two aliquots of 10-30 µl of diluted culture onto one tryptophan dropout SDC plate. For each strain, prepare a set of 8 to 20 plates and labeled according to plating density(e.g., $10^4$-fold diluted, plate 10 µl or $10^4$-10, $10^4$-30, $10^3$-10, $10^3$-30, etc.), which ensure 10-100 colonies can be counted in anticipation of decreased viability during aging.
4. Incubate the plate set at 30°C for the duration of the lifespan analysis.
5. On the day of plating and every 2 days subsequently, remove one plate and drop-wise add 0.5 ml Tryptophan (2 mg/ml) to allow viable cells to grow. Incubate the plate at 30°C for additional 48-72 hours. Count the CFU for viability on the day of tryptophan addition. The CFU on the day of plating is considered 100% survival.

Variations of *in situ* viability assay include:

- Age cells on agar plates (extreme starvation condition). Add 1 ml 2x YPED instead of tryptophan on subsequent days to allow growth and CFU count[14].
- Age cells on plates with tryptophan dropout synthetic complete medium with various carbon sources, e.g. various concentrations of glucose (calorie restriction by glucose limitation), various carbon sources such as ethanol, glycerol, acetic acid[14]. Add 1 ml glucose/tryptophan solution (20% glucose and 1mg/ml tryptophan) to allow growth and CFU count.
- Other nutrient manipulation, such as nitrogen.

## 3. DNA damage and mutation frequency during chronological aging

Canavanine resistance ($Can^r$) and *can1* sequencing

Spontaneous mutation frequency can be evaluated by measuring the frequency of canavanine resistance ($Can^r$) in chronologically aging cultures. Mutations in the *CAN1 (YEL063)* gene, which encodes the plasma arginine permease, render cells resistant to the arginine analogue L-canavanine.$Can^r$ colonies collected at different time points can also be saved for extraction of genomic DNA and subsequent sequencing of the *CAN1* gene, which can provide mutation spectrum data (with Mutation Surveyor, SoftGenetics).

**Base substitutions ($Trp^+$ reversions)**

Strains with *trp1-289* contain an amber mutation (C403T) in the *TRP1* coding sequence. Measurement of frequency of *trp1-289* to $Trp^+$ reversion[15] allows the estimation of base substitution rate during yeast chronological aging.

**Frame-shift mutations**

The $Lys^-$ strain EH150 (*MATa, lys2ΔBglII, trp1-Δ, his3-Δ200, ura3-52, ade2-1o*) harbors a *lys2ΔBgl*II mutation that was constructed by inserting 4 nucleotides to create a *Bgl*II restriction enzyme site in the *LYS2* gene. The resulting +4 shift in the open reading frame results in auxotrophy for lysine that can be reversed by small insertion/deletion mutations[16-17].

**Gross chromosomal rearrangements (GCRs)**

To detect gross chromosomal rearrangements (GCRs), we generated a mutant strain, in which *HXT13* (YEL069), encoding a highly redundant hexose transporter, was disrupted by a *URA3* cassette[18]. *HXT13* is located 7.5 kb telomeric to *CAN1* on chromosome V. Mutations in both *CAN1* and *URA3* genes render cells resistance to L-canavanine and 5-fluoroorotic acid (5FOA), respectively. Considering the low frequency of point mutations that occur in both genes, analysis of the $Can^r$ $5FOA^r$ frequency provides an estimation of GCRs that result in the loss of both genes.

**Homologous and homeologous recombination**

To monitor the level of homologous (100%) and homeologous recombination (91%) during chronological aging, we generated mutants in which linearized plasmids (*HIS3*::intron-IR-URA3) carrying either 100% homologous inverted repeats (IRs) (pSR406) or 91% homeologous IRs (pSR407) at the *HIS3* locus[19]. Recombination between the IRs allows the expression of functional His3 protein.

1. In parallel to normal viability assay (as described above), remove an appropriate amount of cells from the aging culture,
    1. for $Can^r$ mutation, start with ~$2x10^7$ cells (200 µl of day 3 culture);
    2. for $Trp^+$ reversion, start with ~$10^8$ cells (500-1000 µl of day 3 culture);
    3. for $Lys^+$ frame-shift mutation, start with ~$10^8$ cells (500-1000 µl of day 3 culture);
    4. for GCRs, start with 2-3x$10^8$ cells (2-3 ml of day 3 culture);
    5. for homologous and homeologous recombination, start with ~$5x10^7$ cells (200-500 µl of day 3 culture);

    adjust the plating amount according to the decrease in total viability and the increase in mutation frequency during chronological aging (**Table 2**). In case of strains with hypermutator phenotype (such as those with deficiencies in DNA repair), reduce the sampling amount accordingly.

2. Pellet the cells with bench-top centrifuge machine (6000 rpm for 5 min).
3. Resuspend cells in 1 ml autoclaved water, and pellet the cells again.
4. Resuspend cell pellet in 100 µl of water. Plate cells,
    1. for $Can^r$ mutation, on arginine-dropout synthetic complete medium plates (SDC-Arg, supplemented with 60 µg/ml L-canavanine sulfate);
    2. for $Trp^+$ reversion, on tryptophan-dropout plates (SDC-Trp);
    3. for $Lys^+$ frame-shift mutation, on lysine-dropout plates (SDC-Lys);
    4. for GCRs, on arginine-dropout plates (SDC-Arg)supplemented with 5FOA (1 mg/ml) and L-canavanine sulfate (60 µg/ml);
    5. for homologous and homeologous recombination, on histidine-dropout plates supplemented with galactose (2%).
5. Count CFUs after 3-4 days' incubation at 30°C. The mutation frequency is normalized to the number of viable cells.

Complementary to the *in situ* viability assay, age-dependent $Trp^+$ reversion, $Lys^+$ frame-shift mutation, recombination, or $Can^r$ can also be studied in cells aged on plate.

1. Plate cells (~$10^8$ cells/plate) on to Trp-, Lys-, His-dropout, or L-canavanine synthetic complete medium plates (as described above).
2. Every two days (or at the time point of interest), score the newly emerging colonies.
3. Mutation frequency is estimated by normalizing newly emerging colonies to the total number of viable cells of the specific day.

## 4. Translesion synthesis (TLS)

The age-dependent mutation frequency increase may involve increased macromolecule damage, diminished cellular protection/repair, and increased erroneous DNA repair (such as Polζ-dependent translesion synthesis, TLS) during aging. For example, the long-lived *sch9*Δ mutants exhibit elevated expression of *SOD2*, and decreased expression of the error-prone DNA repair enzyme *Rev1*[20]. Here we describe an assay combining damaged DNA template and whole nuclear extract to evaluate the TLS *in vitro*.

1. Prepare nuclear extract as described by Wang *et al*.[21]; quantify the protein concentration by BCA assay (Pierce).
2. Add 0, 5, 10 or 20 µg of nuclear extracts to 50 µl (final volume) reactions containing TLS Buffer (20 mM HEPES, 7 mM $MgCl_2$, 1 mM DTT, 25 mM NaCl, pH 7.8), 200 µMdNTPs and 100 nM 5'-$^{32}$P-labelled 12-mer primer (5'-CGA TGG TAC GGA-3') annealed to a 48-mer template containing DNA damage of interest (5'-TCG ATA CTG GTA CTA ATG ATT AAC GAC TXA AGC ACG TCC GTA CCA TCG-3', in which X is the damaged site, e.g., abasic site, 8-oxo-G, etc.).
3. Incubate the mixture at 30°C for 30 min, terminate the reaction with the addition of 2.5 µl EDTA (20 mM) and 2 µl of proteinase K (10 mg/mL).
4. Purify DNA with phenol extraction and ethanol precipitation.
5. Resuspend DNA in 50 µl of gel loading buffer (98% formamide, 20 mM EDTA, and dye).
6. Resolve the translesion synthesis products on 19% polyacrylamide gels.
7. Quantify the TLS using phosphorimaging (**Figure 1**).

## 5. Representative Results

We typically used the CFU counts on day 3 as 100 percent survival in the standard liquid CLS analysis. The percentage survival in the subsequent days can be fitted to calculate the mean (50% survival) and maximum (10% survival) life spans[12]. Life span results obtained from the *in situ* viability assay are generally consistent with those using the liquid CLS assay, but with reduced the mean life span, which is due in part to the fact that cells are constantly exposed to nutrients. Presence of glucose inhibits the activity of cellular protection as showed by the reduced transactivation of stress response transcription factor such as Msn2/4 and Gis1[12].

Age-dependent mutation frequency varies greatly depending on the strain background, genetic manipulation, culture conditions, and age. Table 2 shows the typical results obtained in the wild-type strain (DBY746).

| Component | g/L |
|---|---|
| D-glucose | 20 |
| Ammonium sulfate | 5 |
| Nitrogen base (-AS/-AA) | 1.8 |
| NaH2PO4 | 1.4 |
| | **mg/L** |
| Adenine | 80 |
| L-Arginine | 40 |
| L-Aspartic acid | 100 |
| L-Glutamic acid | 100 |
| L-Histidine | 80 |
| L-Isoleucine | 60 |
| L-Leucine | 120 |
| L-Lysine | 60 |
| L-Methionine | 80 |
| L-Phenylalanine | 60 |
| L-Serine | 400 |
| L-Threonine | 200 |
| L-Tryptophan | 80 |
| L-Tyrosine | 40 |
| L-Valine | 150 |
| Uracil | 80 |

**Table 1.** Synthetic complete glucose medium, SDC (adjust to pH 6.0 with NaOH). 4-fold excess of histidine, leucine, tryptophan, and uracil are included to compensate the auxotrophy of the DBY746 strain.

| | Day 3 (mean ± SEM) | Up to (during aging) |
|---|---|---|
| ***Can$^r$*** | 1.76 ± 0.12 x10$^{-6}$ | 6-8 x10$^{-6}$ |
| ***Trp$^+$ reversion*** | 6.60 ± 1.70 x10$^{-8}$ | 1.60 x10$^{-6}$ |
| ***Lys$^+$ frame-shift mutation*** | 3.00 ± 0.78 x10$^{-8}$ | 0.70 x10$^{-6}$ |
| ***GCRs*** | 0.62 ± 0.10 x10$^{-8}$ | 0.30 x10$^{-6}$ |
| ***Homologous recombination*** | 5.55 ± 2.74 x10$^{-6}$ | 27.00 x10$^{-6}$ |
| ***Homeologous recombination*** | 0.12 ± 0.04 x10$^{-6}$ | 0.48 x10$^{-6}$ |

**Table 2**.Typical mutation frequencies of wild type cells (DBY746) during chronological aging.

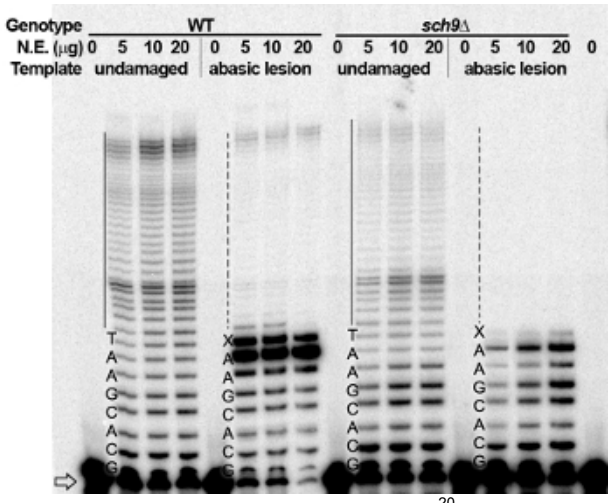


**Figure 1.** Result of translesion synthesis (TLS)[20]. Nuclear extracts from 3 day-old stationary phase wild-type (DBY746) and *sch9*Δ mutant cells were incubated with undamaged or abasic site-containing DNA templates for 30 min at 30°C. TLS products are indicated by solid (with undamaged template) or dotted (with damaged template) lines. There was no translesion synthesis observed in nuclear extract from *sch9*Δ mutants. Free primers are indicated by the open arrow.

## Discussion

Liquid aging cultures sometime exhibit regrowth/gasping phenotype[13], which complicates the CLS analysis. Regrowth typically occurs when more than 90-99% of the population has lost viability. This phenotype is often associated with increased oxidative stress and/or decreased protection in the cell. For example, the frequency of this phenotype more than doubles in cells lacking cytosolic superoxide dismutase and greatly reduces in long-lived mutants (e.g., *sch9*Δ or *ras2*Δ) or mutants overexpressing superoxide dismutases[22]. In practice, we define regrowth as an increase in viability or stabilization in viability for 3 consecutive samplings in the high mortality phase during chronological aging.

Age-dependent frequencies of different DNA mutations vary greatly depending on the strain background, genetic manipulation, culture conditions, as well as regrowth/gasping and extremely low survival. Pre-experiments should be carried out at time points such as the mean and maximum survival with various plating density for mutation assays to determine mutation frequency range before performingthe full scale life span analysis. The wild type strain should always be included in a life span or mutation frequency study in parallel to any treatment or genetic mutant, such that inter-experiment-variations can be accounted for. Multiple biological replicas should be included in the study; and, both liquid and *in situ* viability/mutation assays should be carried out to corroborate the results.

Instead of focusing on one specific type of DNA mutation, profiling of age-dependent genomic instability using multiple assays in combination, may shed light on specific DNA damage and DNA damage repair system(s) that contribute to age-dependant genomic instability. For example, a significant increase in gross chromosomal rearrangements (GCRs), compared to those of other DNA mutations, is observed in wild type yeast suggesting an elevationof double strand break and/or an impairment in non-homologous end joining (NHEJ) during yeast chronological aging (Table 2). The *can1* mutation spectrum (sequencing of the *CAN1* gene) obtained in the wild-type aging yeast suggested an increase in oxidative damage during chronological aging and error-prone DNA repair; whereas, in long-lived *sch9*Δ mutants, less oxidative DNA damage and much reduced error-prone translesion synthesis were observed[20].

Methods described here can be further expended to study age-dependant genomic instability. For example, quiescent and non-quiescent cells can be isolated from yeast stationary-phase cultures using the density gradient method described by Allen *et al.*[23]. Combined with the mutation assays described here, we have reported that a large portion of age-dependent mutations arises from quiescent cells, rather than the dividing, damaged or apoptotic cells[20,24].

## Disclosures

No conflicts of interest declared.

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