Protocol

Determination of Gross Chromosomal Rearrangement Rates

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[Supplemental material is available at http://www.cshprotocols.org/supplemental/.]

INTRODUCTION

Cells devote a significant amount of metabolism to maintaining the stability of their genome and to preventing inappropriate chromosomal rearrangements that are characteristic of many cancers. A simple genetic assay using haploid derivatives of the yeast *Saccharomyces cerevisiae* provides a means to quantitatively measure the rate at which gross chromosomal rearrangements (GCRs) accumulate in different genetic backgrounds. This assay measures the rate of simultaneous inactivation of *CAN1* and *URA3* markers placed on a nonessential end of a yeast chromosome and in principle can be implemented in any haploid strain. Rearrangements detected with this assay include broken chromosomes healed by de novo telomere additions and a spectrum of inter- and intrachromosomal fusion events. The GCR assay allows for detailed analysis of the contributions of individual genes and different pathways in the suppression of genomic instability.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**><**!**>5-FOA Can agar plate media

S. cerevisiae strains appropriate for GCR measurements (e.g., RDKY3615 [*MATa ura3-52 leu2Δ1, trp1Δ63 his3Δ200 lys2ΔBgl hom3-10 ade2Δ1 ade8 hxt13::URA3*]; RDKY6677 [*MATa ura3-52 leu2Δ1, trp1Δ63 his3Δ200 lys2ΔBgl hom3-10 ade2Δ1 ade8 yel068c::CAN1/URA3 iYEL072W::hph*]; and RDKY6678 [*MATa ura3-52 leu2Δ1, trp1Δ63 his3Δ200 lys2ΔBgl hom3-10 ade2Δ1 ade8 yel072w::CAN1/URA3 iYEL072W::hph*])

<**R**>YPD media

Prepare both liquid media and agar plates.

Equipment

Centrifuge

Culture flasks, sterile (of at least twice the volume of the culture; see Step 2)

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Incubator, shaking, preset to 30°C
Scalpel blades, sterile
Tubes, microcentrifuge, sterile, 1.5-mL
Vortex mixer

METHOD

GCR Fluctuation Analysis

1. Streak the yeast strains of interest onto YPD agar plates. Use at least two independent biological isolates per strain.

   Colonies should be visible in 2-3 d if the strains have no growth defects and are grown on rich media. It is important to have well-separated single colonies. Strains with variable-sized colonies should be carefully investigated. Smaller colonies can indicate the spontaneous generation of respiratory-defective petites, whereas larger colonies can indicate the presence of spontaneous mutations that suppress growth defects caused by the desired genotype. Ideally, similar-sized colonies that lack additional genetic changes should be analyzed.

2. Depending on the expected GCR rate, choose an appropriate volume of YPD liquid medium for growing liquid cultures:

   i. For strains with rates similar to wild type (~10^{-10} mutations per cell per generation), use 50-mL liquid cultures.

   ii. For moderately increased GCR rates (~10^{-8} mutations per cell per generation), use 10-mL liquid cultures.

   iii. For very high GCR rates (~10^{-6} mutations per cell per generation), use 1- to 5-mL liquid cultures.

   iv. For unknown rates, initially try 10-mL liquid cultures.

3. For each culture, use a sterile scalpel blade to cut out one colony from the Petri dish. Inoculate each liquid culture with a single colony/agar plug.

   Ensure that the entire colony is used in the inoculation and that each colony/agar plug is not contaminated with cells from other colonies. Grow at least seven cultures for each independent biological isolate; larger numbers of cultures allow more accurate determination of rates.

4. Grow cultures at 30°C with vigorous shaking until they reach saturation.

   In rich media, saturation typically occurs after 24-36 h of growth for strains with wild-type growth rates. Sicker strains can take longer to reach saturation and can reach saturation at lower viable cell densities than wild-type strains; determination of experimental growth curves for these types of strains can be helpful.

5. For each culture, label five sterile microcentrifuge tubes A-E. Add 90 µL of sterile water to each.

6. Prepare a 10^{-5} dilution for each culture as follows:

   i. Transfer 10 µL of the culture to tube A and vortex.

   ii. Transfer 10 µL of A to B and vortex.

   iii. Transfer 10 µL of B to C and vortex.

   iv. Transfer 10 µL of C to D and vortex.

   v. Transfer 10 µL of D to E and vortex.

7. Spread all 100 µL of each tube E (i.e., the 10^{-5} dilution) on 9-cm YPD agar plates.

   This provides a final effective dilution of 10^{-6} viable cells per milliliter, relative to the concentration of the culture.

8. Centrifuge the remainder of the saturated culture at 2000*g* for 10 min at room temperature. Carefully decant the supernatant.

9. Resuspend the pellet in 0.2 mL of sterile water per 10 mL of original culture.

10. Spread the resuspended culture onto 14.5-cm 5-FOA Can plates.

    For up to 10 mL of culture (~10^9 cells), one 14.5-cm plate can be used. For larger cultures, divide each culture evenly between multiple plates, plating no more than 10^9 cells/plate for each culture.

11. Place the YPD and 5-FOA Can plates in the incubator at 30°C.

    Colonies with wild-type growth rates should take 3-5 d to appear on 5-FOA Can plates.

12. Count the number of colonies on the YPD and 5-FOA Can plates.

    In rich media, wild-type cultures at saturation have ~10^8 cells/mL, yielding ~100 colonies/YPD plate.

    See Troubleshooting.

GCR Rate Calculations

13. To calculate the number of viable cells per milliliter (D) for each culture, multiply the number of colonies on the YPD plate by the total dilution factor (in this case, 10^6; see Steps 6 and 7).

14. For each culture, calculate the number of viable cells in the culture (N) using the formula $N = D \times V$, where V is the volume of the culture (in milliliters), and D is the viable cells per milliliter (from Step 13).

15. For each culture, estimate the most likely number of mutational events (m) using the observed number of colonies on the 5-FOA Can plates (r) and the equation $m(1.24 + \ln[m]) - r = 0$.

    This equation was originally derived by Lea and Coulson (1949). Values of r *as a function of* m *can readily be tabulated in spreadsheets (Table 1, or* m *can be calculated from* r *by iterative methods (e.g., bisection, Newton-Raphson). A Visual Basic macro for calculating* m *from* r *is available as Supplemental Material (Suppl_RtoM_Conversion_Macro.doc).*

16. For each culture, calculate P, the most likely rate (in events per cell per generation) using the formula $P = m/N$, where N is the number of cells in the culture (see Step 14) and m is the most likely number of events (from Step 15).

17. For each biological isolate, order all the most likely rates (P, from Step 16) from smallest to largest, including all zero rates.

18. Determine the median rate of the cultures, R.

    The median is the value of the middle element in the ordered list (or the average of the two middle values if there are an even number of elements in the list).

19. If $R = 0$, calculate an upper limit for the rate (R') by replacing all cultures with $r = 0$ with $r = 1$ and determining the new median of these rates. Report the resulting rate as being less than R'. For example, if $R = 0$ and $R' = 1.75 \times 10^{-9}$, the reported rate should be $<1.75 \times 10^{-9}$.

20. Determine the 95% confidence intervals for the median (see, e.g., http://www.math.unb.ca/~knight/utility/MedInt95.htm). For six to 283 cultures, calculate the lower (L) and upper (U) 95% confidence interval limits using the equations $L = \text{floor}[(M+1) / 2 - 0.9789\sqrt{M}]$ and $U = (M + 1) - L$, where M is the number of cultures.

    Briefly, the lower and upper 95% confidence interval limits correspond to the L-th and U-th positions in the ordered list of individual rates. For example, if the number of cultures (M) 14, L = $\text{floor}[15 / 2 - 0.9789\sqrt{14}] = 3$; $U = (14 + 1) - 3 = 12$. Thus, the 95% confidence interval is defined by the third and 12th lowest mutation rates.

    See Troubleshooting.

21. If the median rates for the different biological isolates are within the 95% confidence intervals, merge all rates from the different biological isolates. Determine the median rate and the 95% confidence intervals for the combined list of rates (i.e., repeat Steps 17-20 for the entire set of rates from all biological isolates of a given genotype).

Table 1. *r* and *m* values

r	*m*	*r*	*m*
0	0.000000	51	13.318792
1	0.890054	52	13.525536
2	1.318699	53	13.731630
3	1.696365	54	13.937089
4	2.045410	55	14.141929
5	2.375203	56	14.346164
6	2.690785	57	14.549807
7	2.995255	58	14.752872
8	3.290694	59	14.955372
9	3.578578	60	15.157320
10	3.860007	61	15.358725
11	4.135825	62	15.559601
12	4.406700	63	15.759959
13	4.673170	64	15.959807
14	4.935678	65	16.159158
15	5.194593	66	16.358019
16	5.450227	67	16.556402
17	5.702849	68	16.754315
18	5.952690	69	16.951767
19	6.199949	70	17.148766
20	6.444805	71	17.345321
21	6.687414	72	17.541440
22	6.927914	73	17.737130
23	7.166431	74	17.932399
24	7.403076	75	18.127254
25	7.637951	76	18.321702
26	7.871146	77	18.515750
27	8.102747	78	18.709405
28	8.332830	79	18.902672
29	8.561464	80	19.095559
30	8.788715	81	19.288071
31	9.014641	82	19.480214
32	9.239299	83	19.671994
33	9.462740	84	19.863417
34	9.685011	85	20.054487
35	9.906156	86	20.245211
36	10.126218	87	20.435592
37	10.345235	88	20.625637
38	10.563242	89	20.815350
39	10.780276	90	21.004736
40	10.996367	91	21.193799
41	11.211547	92	21.382545
42	11.425843	93	21.570976
43	11.639283	94	21.759098
44	11.851892	95	21.946915
45	12.063695	96	22.134431
46	12.274714	97	22.321649
47	12.484972	98	22.508575
48	12.694490	99	22.695210
49	12.903287	100	22.881560
50	13.111382		

TROUBLESHOOTING

Problem: No colonies are observed on the 5-FOA Can plates.
[Step 12]
Solution: Use larger culture sizes and/or test the plates to ensure they support the growth of a *can1 ura3* double mutant strain.

Problem: The biological isolates are significantly different on the basis of the 95% confidence intervals.
[Step 20]
Solution: Troubleshooting will depend on the precise genotypes of the strains involved. Consider the following:

1. Test additional biological isolates.
2. Verify strain genotypes.
3. Carefully examine growth rates and drug sensitivities.
4. If need be, construct new isolates for testing.

DISCUSSION

The genetic assay described here measures the rate of simultaneous inactivation of the *CAN1* and *URA3* genes in haploid cells of *S. cerevisiae* using fluctuation analysis (Lea and Coulson 1949). For wild-type cells, this 5-FOA^R CAN^R rate is the result of chromosomal rearrangements and not simultaneous inactivation of *URA3* and *CAN1* by point mutations. However, simultaneous inactivation of *URA3* and *CAN1* by point mutations has been observed in mismatch repair-defective strains (Myung et al. 2001a). The original assay strain (RDKY3615) contains a *hxt13::URA3* insertion telomeric to *CAN1* on the nonessential region of the left arm of chromosome V (Chen and Kolodner 1999). Several modified versions of the assay have since been developed, including those with a *CAN1*/*URA3* cassette placed at a number of different chromosomal locations (RDKY6677, RDKY6678) (Putnam et al. 2009), as well as other variant GCR assays using the loss of *URA3* and *HIS3* markers on chromosome III (Myung et al. 2001b) or loss of *CAN1*, *URA3*, and *ADE2* on chromosome XV (Hackett et al. 2001).

The observed range of chromosome V alterations includes broken chromosomes healed by de novo telomere addition and various forms of translocations and other genome rearrangements that are similar to many of the rearrangements observed in human cancers (Mitelman 1991). These translocations include monocentric translocations, telomere capture events, and interstitial deletions as well as dicentric inter- and intrachromosomal fusions; the dicentric products appear to be unstable and undergo additional rearrangements resulting in stable monocentric products (Pennaneach and Kolodner 2009). Analysis of products can involve polymerase chain reaction (PCR)-based mapping and breakpoint sequencing (Chen and Kolodner 1999; Smith et al. 2004; Schmidt et al. 2006a), PCR amplification of predicted breakpoint junctions (Schmidt et al. 2006b; Motegi and Myung 2007; Putnam et al. 2009), linker-mediated PCR (Motegi and Myung 2007), clamped homogeneous electric field gel analysis (Pennaneach and Kolodner 2009), and array competitive genomic hybridization experiments (Pennaneach and Kolodner 2009; Putnam et al. 2009).

The GCR rates and the resulting chromosomal rearrangement products observed are dependent on a number of factors, including the genomic features present between the counter-selectable markers and the nearest essential genes (Putnam et al. 2009) and the strain genotypes (Chen and Kolodner 1999; Kolodner et al. 2002). This simple protocol for determining GCR mutation rates in a variety of genetic backgrounds coupled with a diversity of modified GCR assays has provided tremendous insight into the large numbers of pathways that suppress genomic instability in *S. cerevisiae* and appear to be relevant to cancer suppression pathways in humans (Kolodner et al. 2002; Wang et al. 2005).

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