Analysis of chromosome/allele loss in genetically unstable yeast by quantitative real-time PCR

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Genome instability is a hallmark of cancer that ranges from small-scale changes, such as point mutations or di- and trinucleotide repeat expansions/contractions, to chromosomal instability resulting in translocations and aneuploidy (1). Chromosome-level changes can result from unrepaired or misrepaired DNA damage including double-strand breaks (DSBs) or from segregation defects. In haploid yeast cells, chromosome loss is lethal due to the loss of essential genes. Chromosome truncations are viable if no essential genes are lost. In diploid cells, loss of part or all of a chromosome is generally tolerated. Chromosome loss can be detected by the loss of a heterozygous nutritional marker. However, marker loss can also result from other genetic alterations, such as gene conversion, crossovers, or break-induced replication (1). In our prior studies of DSB repair in yeast, we distinguished chromosome loss from these other events by using a time-consuming and technically demanding quantitative Southern hybridization procedure (2). Here we describe a rapid, quantitative real-time PCR (qPCR) procedure for analysis of chromosome or allele loss in diploid *Saccharomyces cerevisiae*.

Yeast strains were constructed and cultured as described (2). DSBs were created at an engineered HO nuclease recognition site in *ura3* in diploid yeast strain DY3515 (*MATa-inc*/*MAT*α, *ade2-101*/*ade2-101*, *lys2-801*/*lys2-801*::pHSS*GALHO*::*LYS2*, *trp1-Δ1*/*trp1-Δ1*, *leu2-Δ1*/*leu2-Δ1*, *ura3*-X764::*LEU2*/*ura3*R-HO432-*LEU2*) and related strains by induction of a *GAL1*-promoter regulated HO gene as described (2). Strain JC3517 is identical to DY3515, except it has *HIS3* near the chromosome V telomere (*HIS3*:telV) 108 kb from *ura3* carrying the HO site (Figure 1A). *sgs1Δ* and *rad9Δ* mutations were created by replacing the wild-type genes with *hisG* using a *hisG-URA3-hisG* cassette (3). Genomic DNA was prepared for quantitative Southern hybridization by polyethylene glycol (PEG) precipitation as described (4). Genomic DNA for qPCR was additionally extracted once with phenol:$CHCl_3$:isoamyl-OH (25:24:1), once with $CHCl_3$:isoamyl-OH (24:1), and ethanol precipitated. Quantitative Southern hybridization was performed as described previously (2) using two ^{32}P-labeled probes. A 1453-bp fragment near telV was amplified with primers 5′-AGCAATGAAAGAGCAGACCG-3′ and 5′-CAGCAATAAACCAGCCAGCC-3′, and an 873-bp *NDC1* fragment was amplified with primers 5′-ACAACATCATGTCACTGACGC-3′ and 5′-TGGGTGAAAATAGAGCCGTT-3′. Signals were measured with a Storm® 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). DY3515 has two copies of telV, and the ratio of the telV:*NDC1* signals in DY3515 was normalized to 1.0. Ratios of test samples were typically near 1.0 or 0.5, the latter indicating chromosome loss.

qPCR was performed in 25-µL reactions with 0.5 ng genomic DNA, 0.3 µM primers (5′-ACATACCCTAGCAACCATCGG-3′ and 5′-CACTTATGAGACCGCATCA-3′ amplifying a 160-bp *RMD6* fragment; 5′-AGGCCCCACAAGACTACGTAT-3′ and 5′-CCCTTGTGCAGACAACCTATG-3′ amplifying a 250-bp *NDC1* fragment), and 12.5 µL 2× DyNAmo™ HS SYBR® Green master mix (MJ Research, Waltham, MA, USA). PCR was performed with a DNA Engine Opticon™ System (MJ Research) with an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s. All qPCRs were performed in triplicate.

qPCR measures PCR product accumulation during the exponential phase of the reaction and is widely used to quantify gene expression (5). For chromosome or allele loss in diploid yeast there is at most a 2-fold difference in template concentration. Although qPCR can easily detect 2-fold differences in template concentrations in an internally controlled sample set, such as a dilution series of a single DNA preparation, reliable comparisons of independent samples require the use of a second locus as an internal control. We first used sequences near *MAT* for this purpose, because it is relatively easy to confirm that two copies are present (*MATa* and *MAT*α) by mating tests or by PCR (6). In our system, mutations are present in both *MATa* and *MAT*α to prevent cleavage of these loci by HO nuclease and subsequent conversion, so *MAT* is expected to remain heterozygous, and sequences near *MAT* should be effective internal controls. However, mutations in DNA repair genes often produce severe genome instability phenotypes (7,8). For example, DSB-induced chromosome loss is elevated in diploid cells carrying *mre11Δ*, *rad51Δ*, *sgs1Δ*, or *rad9Δ* mutations, and we observed frequent loss of heterozygosity at *MAT* in an *sgs1Δ rad9Δ* double mutant (unpublished results). Because *MAT* conversion is unlikely, the loss of heterozygosity at *MAT* probably reflects spontaneous loss of all or part of one copy of chromosome III. Thus, *MAT* was not an appropriate two-copy internal control for qPCR, and we turned instead to *NDC1* on chromosome XIII, because *NDC1* is haplo-insufficient (i.e., diploid cells with only one copy of *NDC1* are inviable) (9).

We induced DSBs at *ura3* in our *sgs1Δ rad9Δ* diploid, identified

potential chromosome loss candidates (Ura⁻ His⁻ phenotype), and analyzed these by quantitative Southern hybridization. We identified a chromosome loss product (Figure 1B), hereafter denoted CL. We used CL and the wild-type (denoted WT) parent strain JC3598 to develop the qPCR method. Our initial tests of qPCR used one pair of primers targeted to *NDC1* and several sets of primer pairs targeted to the same intergenic region near *HIS3*:telV used in the quantitative Southern hybridization procedure. However, all of the intergenic primer pairs yielded nonspecific amplification products, perhaps because of similarities between the target sequences and other noncoding regions of the genome. We then designed primers targeted to *RMD6* located 6 kb centromere-proximal to *HIS3*:telV. By using serial dilutions of genomic DNA, standard curves were established, and cycle threshold values (C_t) were determined. The C_t value was set at 10 standard deviations above the mean fluorescence value of the first 3–7 cycles for each sample. C_t values represent the number of amplification cycles required to produce a specific amount of amplification product during the exponential phase of qPCR and hence are inversely proportional to the starting concentration of the template being amplified. C_t values decreased linearly with increasing input DNA at the *NDC1* and *RMD6* targets (Figure 2A). Amplification specificity for both targets was confirmed by agarose gel electrophoresis (Figure 2B) and by determining melting curves (data not shown).

We measured signals from *RMD6* and *NDC1* in the wild-type cells and normalized this ratio to 1.0. Parallel measurements with the CL cells gave *RMD6*/*NDC1* ratios consistently 2-fold lower than the wild-type (Figures 3, panels A–E, left two bars). Thus, the qPCR procedure reproducibly detected the 2-fold difference in chromosome V copy number between the wild-type and CL. We tested 37 Ura⁻ His⁻ products from the *sgs1Δ rad9Δ* double mutant and found 15 with ratios at or above 1.0 and 20 with ratios near 0.5, reflecting loss of chromosome V; the two remaining samples were ambiguous, as *RMD6*/*NDC1* ratios were near 0.75 (Figure 3, B and D, black bars). Although ratios of 0.75 could reflect system variability, they may result from mixed colonies. Thus, if two DSBs were introduced into chromosome V in a G2 cell, and one DSB was repaired by gene conversion while the other led to chromosome loss, the resulting mixed colony would yield an *RMD6*/*NDC1* ratio of 0.75. Subclones of mixed colonies would yield pure conversion and pure loss products. To test this, we performed qPCR on three subclones of one such product and found two subclones that gave ratios near 1.0, and one subclone that gave a ratio near 0.5 (Figure 3E). In other cases, samples that gave ratios near 0.75 gave normal (1.0 or 0.5) ratios upon retesting (data not shown). We have analyzed chromosome loss in greater than 200 products from various wild-type and mutant strains by quantitative Southern hybridization and obtained ambiguous results at similar low frequencies (approximately 5%; unpublished results). Thus, this limitation is common to both qPCR and Southern blot approaches.

To determine the reliability of qPCR, we tested 18 samples by both qPCR and Southern blot analysis, including parental (two-copy) and CL (single-copy) controls, and 16 potential chromosome loss (Ura⁻ His⁻) candidates

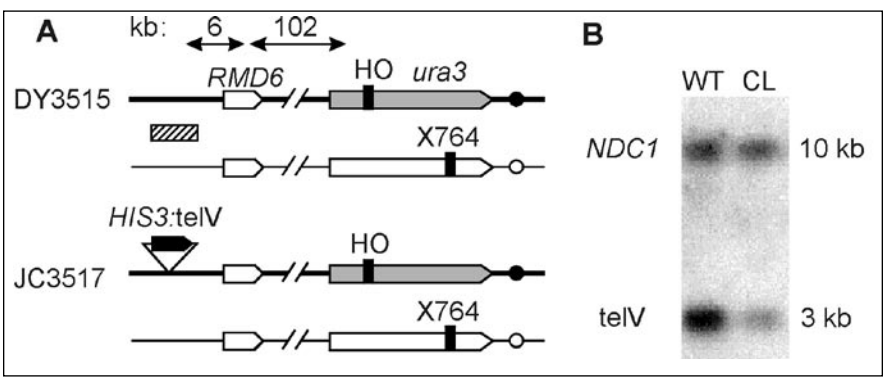

Figure 1. Double-strand break (DSB) repair substrates. (A) Parent strains carry *ura3* inactivated by insertion of an HO recognition sequence (HO) or a +1 frameshift mutation (X764), and a galactose-regulated HO nuclease gene. DSBs at HO sites lead to gene conversion with or without crossovers or loss of all or part of the broken chromosome. *HIS3*:telV can be lost through crossovers or by full or partial loss of the broken chromosome; crossovers are conservative, and two full-length copies of chromosome V are retained. DY3515 was used as a control for quantitative Southern analysis because the chromosome V probe (hatched bar) detects sequences on both sides of the *HIS3*:telV locus in JC3517. Either DY3515 or JC3517 can be used as two-copy controls for quantitative real-time PCR (qPCR) assays using the *RMD6* target. (B) Genomic DNAs from DY3515 (wild-type; WT) and an *sgs1Δ rad9Δ* Ura⁻ His⁻ product (chromosome loss product; CL) were digested with *Hin*dIII, separated on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labled *NDC1* and telV fragments. Signals were detected with a Storm 860 PhosphorImager.

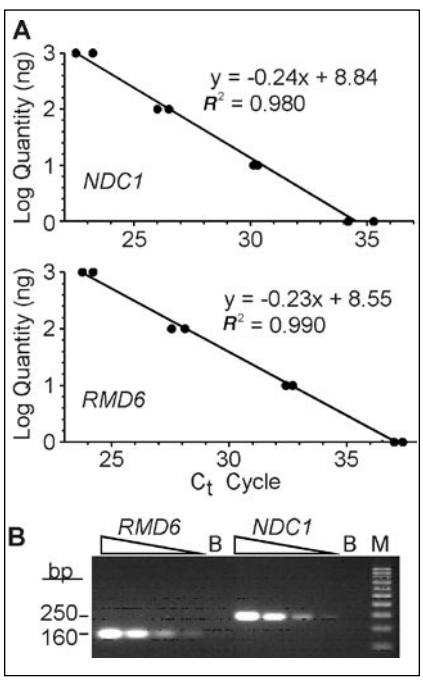

Figure 2. Validation of quantitative real-time PCR (qPCR) primer sets. (A) Dilutions (10-fold) of genomic DNA were amplified by qPCR in duplicate reactions, and standard curves were created by plotting the log of the input DNA quantity in nanograms (Log Quantity) versus cycle threshold (C_t) values for *NDC1* or *RMD6*. (B) Amplification products from 10-fold dilutions of genomic DNA are shown for *NDC1* and *RMD6*. Lanes marked B are blank controls lacking template DNA; lane M indicates the 100-bp DNA Ladder markers (New England Biolabs, Beverly, MA, USA).

from *sgs1Δ rad9Δ* cells. In most cases, the two methods gave the same result. In four samples, the Southern blot analysis indicated that there were two copies of chromosome V, but qPCR indicated a single copy was present; a fifth sample suggested a mixed population by Southern blot analysis, but only one copy by qPCR. Pulse-field gel electrophoresis of these five samples revealed three with an extra band approximately 100 kb shorter than chromosome V, indicative of loss of most of the left arm of one copy of chromosome V (Figure 3F). These "arm loss" products were therefore correctly identified as single-copy by qPCR. The other two samples produced normal patterns, perhaps because the entire chromosome was lost or the truncated chromosome co-migrated with another chromosome. (We cannot reliably detect 2-fold differences in band intensity in ethidium bromide-stained pulse-field gels.) Thus, although there was generally good agreement between qPCR and Southern blot analyses, three of three cases confirmed as arm loss by the independent pulse-field gel assay were correctly scored by qPCR but not by Southern blot analysis. We conclude that qPCR is the more accurate method.

The inconsistent results with quantitative Southern blot analyses may be traced to uneven DNA transfer from gels to membranes and/or uneven background radioactivity due to inefficient or nonhomogeneous washing after hybridization.

In addition to its greater accuracy, qPCR has several other advantages over Southern blot analyses. Three qPCR runs (10 test samples plus controls per run) can be performed in 1 day on a single machine, whereas Southern blot analyses typically require 4–6 days. qPCR does not require restriction digestion, probe preparation, or radioactive materials. qPCR requires only nanogram quantities of DNA versus 2–5 µg for Southern blot analysis. Because of the speed of qPCR, it is reasonable to perform multiple determinations on each sample. Quantitative Southern blot analyses can be performed without expensive equipment (e.g., by using X-ray film and a densitometer), but the procedure is more robust with a phosphorimager, which is typically more expensive than a qPCR machine. The reagents for qPCR are more expensive than those used for Southern blot analysis, but this cost is offset by the decreased labor costs.

There are several other factors to consider when using qPCR with SYBR Green to detect chromosome or allele loss. An internal control locus is required, and it is important that this locus be refractory to genetic change. Amplification specificity must be carefully monitored, because SYBR Green binds to any double-stranded DNA. Primers should be carefully selected, and reaction conditions should be optimized to prevent primer-dimer formation and nonspecific amplification, both of which contribute to background fluorescence. The DyNAmo HS SYBR Green reagent kit includes a hot-start DNA polymerase to reduce primer-dimers and nonspecific amplification. In summary, qPCR is a rapid and accurate method for detecting 2-fold signal differences characteristic of chromosome or allele loss associated with genome instability. qPCR is also useful for determining transgene copy number (10), quantitating rare chromosomal rearrangements (11), and for diagnosis of cancer and other diseases characterized by loss of specific alleles (12,13).

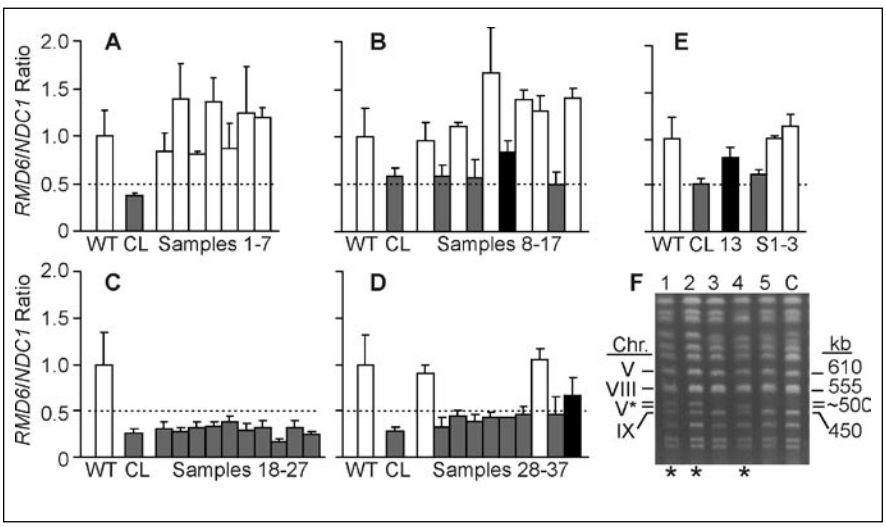

Figure 3. Chromosome/arm loss determined by quantitative real-time PCR (qPCR). (A–D) The ratio of qPCR signals from *RMD6* and *NDC1* in DY3515 (wild-type; WT) was normalized to 1.0; this ratio for the chromosome loss product (CL) control sample was approximately 0.5 in four separate qPCR runs that included 7–10 additional Ura$^-$ His$^-$ samples from the *sgs1Δ rad9Δ* strain. White bars indicate samples with two copies of chromosome V, and gray bars indicate one copy of chromosome V. Sample nos. 13 and 37 gave ambiguous results (black bars). Data are averages ± SD for three determinations per sample. (E) Three subclones of sample no. 13 were tested by qPCR (S1–3), with WT, CL, and the original sample no. 13 as controls. (F) Pulse-field gel electrophoresis reveals loss of approximately 100 kb arm from chromosome V (samples marked by *).

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COMPETING INTERESTS STATEMENT

Y.-C.L. and J.A.N declare no competing interests. R.B.K. is employed by M.J. Research, the manufacturer of the DyNAmo HS SYBR Green qPCR kits and the DNA Engine Opticon System used in this study.

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End-linked amino-modified 50-mer oligonucleotides as RNA profiling probes on nylon arrays: comparison to UV cross-linked DNA probes

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Long oligonucleotides on nylon membrane macroarrays have been investigated as probes for RNA profiling (1), but UV cross-linked double-stranded PCR products are most frequently employed. Double-stranded DNA may be problematic in terms of probe specificity and provenance (2–5). Oligonucleotides circumvent such problems and have been used as end-linked probes in genotyping experiments in macroarray formats (6–9). We evaluated specificity, fractional sensitivity, and reproducibility of hybridization signals obtained from 5′-amino-modified synthetic 50-mer oligonucleotides chemically coupled to negatively charged nylon membranes to determine their suitability relative to UV linkage for focused RNA profiling experiments.

Two amino-modified oligonucleotides representing contiguous segments of the murine proliferin (*plf*) gene family messenger RNA (mRNA) were linked by carbodiimide-mediated amide bond formation to carboxyl groups of Biodyne® C nylon membrane (Pall, East Hills, NY, USA) (6). For comparison, unmodified oligonucleotides or a 470-bp PCR product encompassing the sequence of the oligonucleotides were UV cross-linked (125 mJ/cm^2, Zeta-Probe® membrane; Bio-Rad Laboratories, Hercules, CA, USA). Prehybridization (6 h) and hybridization (18 h) were conducted in roller bottles in 2× SSPE (36 mM NaCl, 2 mM NaH_2PO_4, and 0.2 mM EDTA) with 0.1% sodium dodecyl sulfate (SDS), 100 µg/mL denatured salmon sperm DNA, and 5× Denhardt's solution. Radiolabeled target cDNA was prepared using oligo(dT) priming total RNA (up to 5 µg) extracted (10) from nickel sulfate-induced murine C3H/10T1/2 cells (11), human HepG2 cells, or buffy-coat leukocytes. Washes were in 0.1× SSPE, 0.1% SDS, at 50°C. Images were acquired with a Molecular Dynamics Storm® PhosphorImager system (18 h exposures) and ImageQuant® software (both from Molecular Dynamics, Sunnyvale, CA, USA; see the Supplementary Materials at the *BioTechniques*' web site at www.BioTechniques.com for details).

The dependence of signal performance on the oligonucleotide attachment mode was compared with UV cross-linked cDNA. Both end-linked amino-modified 50-mer probes were indistinguishable, binding approximately 10-fold less ^{32}P-labeled cDNA (target) from induced C3H/10T1/2 cells than did the PCR product probe (probably reflective of the relative difference in probe lengths) when compared at 50 ng/spot (3.2 mm diameter, 6.2 ng/mm^2, suitably spaced to reduce "flare" artifacts) (Figure 1). UV-linked unmodified oligomers captured approximately 400-fold fewer reverse transcripts. Immobilization efficiencies, at 30% and 48% of the end-attached amino-modified and UV cross-linked unmodified 50-mers, respectively (data not shown), were unlikely causes of the reduced signal. UV cross-linking occurs throughout the oligonucleotide, while end-linked molecules were likely to have provided