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Circle-Seq: Extrachromosomal Circular DNA Purification from Yeast

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Abstract:	Extrachromosomal circular DNAs (eccDNAs) are common genetic elements in Saccharomyces cerevisiae and are found in other eukaryotes. EccDNAs contribute to genetic variation among somatic cells in multicellular organisms and to evolution of unicellular eukaryotes. Sensitive methods for detecting eccDNA are needed to clarify how these elements affect genome stability and environmental and biological factors that induce their formation in eukaryotic cells. This video presents a sensitive eccDNA-purification method called Circle-Seq. The method encompasses column purification of circular DNA, removal of remaining linear chromosomal DNA, rolling-circle amplification of eccDNA, high-throughput sequencing, and mapping. Extensive exonuclease treatment (more than 100 units) was typically required for sufficient linear chromosomal DNA degradation. The rolling-circle amplification step by φ29 polymerase enriched for circular DNA over linear DNA. Validation of the Circle-Seq method on three S. cerevisiae CEN.PK populations of 10^10 cells detected hundreds of eccDNA profiles in sizes larger than 1 kilobase. Repeated findings of ASP3-1, COS111, CUP1, RSC30, HXT6, HXT7 genes on circular DNA in both S288c and CEN.PK suggests that DNA circularization is conserved between strains at these loci. In sum, the Circle-Seq method has broad applicability for genome-scale screening for eccDNA in eukaryotes as well as for detecting specific eccDNA types.
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Circle-Seq: Extrachromosomal Circular DNA Purification from Yeast

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Circle-Seq; deletion; eccDNA; rDNA; ERC; ECE; microDNA; minichromosomes; small polydispersed circular DNA; spcDNA; double minute; amplification.

SHORT ABSTRACT:
This paper presents a sensitive method called Circle-Seq for purifying extrachromosomal circular DNA (eccDNA). The method encompasses column purification, removal of remaining linear chromosomal DNA, rolling-circle amplification and high-throughput sequencing. Circle-Seq is applicable to genome-scale screening of eukaryotic eccDNA and studying genome instability and copy-number variation.

LONG ABSTRACT:
Extrachromosomal circular DNAs (eccDNAs) are common genetic elements in *Saccharomyces cerevisiae* and are found in other eukaryotes. EccDNAs contribute to genetic variation among somatic cells in multicellular organisms and to evolution of unicellular eukaryotes. Sensitive methods for detecting eccDNA are needed to clarify how these elements affect genome stability and environmental and biological factors that induce their formation in eukaryotic cells. This video presents a sensitive eccDNA-purification method called Circle-Seq. The method encompasses column purification of circular DNA, removal of remaining linear chromosomal DNA, rolling-circle amplification of eccDNA, high-throughput sequencing, and mapping. Extensive exonuclease treatment (more than 100 units) was typically required for sufficient linear chromosomal DNA degradation. The rolling-circle amplification step by ϕ29 polymerase enriched for circular DNA over linear DNA. Validation of the Circle-Seq method on three *S. cerevisiae* CEN.PK populations of 10^{10} cells detected hundreds of eccDNA profiles in sizes larger than 1 kilobase. Repeated findings of *ASP3-1*, *COS111*, *CUP1*, *RSC30*, *HXT6*, *HXT7* genes on circular DNA in both S288c and CEN.PK suggests that DNA circularization is conserved between strains at these loci. In sum, the Circle-Seq method has broad applicability for genome-scale screening for eccDNA in eukaryotes as well as for detecting specific eccDNA types.

INTRODUCTION:
Detecting early or transient chromosomal amplification is difficult because it requires identifying alterations in single DNA molecules in large populations of cells. Chromosomal copy-number variations (CNVs) are generally detected well after their establishment, leaving only the final CNV structure as evidence of the mechanism that generated the variation[1,2]. Detecting and recovering extrachromosomal circular DNA (eccDNA) in earlier stages of CNV formation might elucidate ongoing processes in genomic rearrangements.

Previously, *de novo* discovery of eccDNA was by electron micrographs[3], Giemsa staining of metaphase chromosomes[4], or two-dimensional gel electrophoresis[5]. These methods provide little or no information about the sequence of the circular DNA. Targeted techniques such as Southern blotting[6,7], inverse PCR[8], or fluorescence *in situ* hybridization[9] provide evidence only about specific eccDNA elements. None of these methods provide the sequence of all existing eccDNA types in a cell population.

Genomic divergence in a pool of cells can be characterized by genome sequencing and/or tiling arrays[10,11]. Detecting a deletion or amplification by conventional DNA purification methods usually requires that a mutated allele represent at least 0.1–1% of the cell population[12,13]. Acentric eccDNAs are expected to be even more transient in a cell culture due to their lack of centromeres and potential absence of DNA synthesis at replication. Thus, since most eccDNAs presumably are in low amounts and their sequences resemble the genome, alternative DNA extraction methods are needed to detect eccDNAs.

Several circular DNA purification techniques exploit the structural differences between chromosomes and circular DNA. For instance, high-speed ultracentrifugation in cesium-chloride gradients is used to isolate 350–3000 basepairs (bp) large eccDNAs from the human HeLa cancer cell line[14]. However, high-velocity can break or nick the backbone of supercoiled circular DNA structures, altering the sedimentation velocity[15] and the eccDNA yield. Dutta and coworkers developed a method for *de novo*, genome-scale identification of circular DNA from mouse tissues as well as from cultures of chicken and human cells[16,17]. Their method is extraction of nuclei from homogenized tissue by sucrose ultracentrifugation followed by plasmid purification and several rounds of enzymatic reactions and DNA extractions. Their protocol primarily identifies 200–400 bp eccDNAs, called microDNAs. Dutta and coworkers also attempted purification of microDNAs from *Saccharomyces cerevisiae* but were unable to record microDNA from this yeast species[16].

We have developed a novel method for *de novo* detection of eccDNA from yeast called Circle-Seq. This method enables genome-scale surveys for circular DNA molecules large enough to carry whole genes and as large as the 86 kilobase (kb) mitochondrial DNA (mtDNA). The Circle-Seq method was developed from a well-established prokaryotic plasmid purification method[18,19], optimized for eukaryotic yeast cells and combined with deep-sequencing technology. Using the Circle-Seq approach, 1756 different eccDNAs, all larger than 1 kb, were detected from ten *S. cerevisiae* S288c populations[20]. A size cut-off was chosen to focus on eccDNA that are large enough to carry whole genes. Circle-Seq was highly sensitive; it detected a single eccDNA within thousands of cells[20]. In the current study, Circle-Seq was used to isolate and identify 294 eccDNAs from three biological replicates of another *S. cerevisiae* yeast strain, CEN.PK. The data suggested that eccDNA is a common genetic element in *S. cerevisiae* strains and possibly also related eukaryotic species.

PROTOCOL:

NOTE: An overview of the circular DNA purification and sequencing method (Circle-Seq) is illustrated in figure 1.

1. Cultivation, cell harvest and plasma membrane disruption

1.1) Inoculate yeast cells (for example *Saccharomyces cerevisiae*) from an overnight culture into 50 ml complete nutrient medium of yeast peptone dextrose (YPD). Inoculate at a low initial cell density of 1–3 x 10^5 cells/ml or an optical density of approximately 0.01 OD_{600}.

1.1.1) Incubate the cells at 30°C with agitation at 150 rounds per minutes (rpm) until cells reach maximum cell density of approximately 1 x 10^{10} cells, approximately after 24 to 48 hours or a optical density at OD_{600} > 10.0.

NOTE: The cultivation time is not crucial as lower cell concentrations can be used.

1.2) Transfer the outgrown culture to a 50 ml conical tube, pellet the cells by centrifugation at 800 x g for 3 minutes (min) and discard the supernatant.

1.3) Wash the pellet with 25 ml buffer solution of 10 mM Tris-Cl, 1 mM EDTA, pH 8.0, re-pellet the cells by centrifugation at 800 x g for 3 min and discard the supernatant.

1.4) Resuspend the cell pellet in 1.2 ml resuspension buffer supplied from a plasmid column-purification kit.

1.5) Optional step: Add highly diluted plasmids as controls for purification of circular DNA elements[20].

NOTE: In the current dataset, a 7.7 µl plasmid mixture was applied for each sample containing 10^{10} cells. The plasmid stock mixture consisted of three plasmids in different concentrations; pBR322 at 38 ng/sample, pUC19 at 0.5 ng/sample, and pUG72 at 0.01 ng/sample.

1.6) Transfer the cell suspension into two 2 ml micro-centrifuge tubes, each supplemented with 0.5 mm glass beads at a 1:3 ratio of the total suspension volume.

1.7) Vortex each tube at maximum speed for 10 min to disrupt plasma cell membranes. Pellet the beads by centrifugation at 268 x g for 30 seconds (sec) and transfer the 1.2 ml combined supernatant from the two microcentrifuge tubes to a new tube.

NOTE: Alternative to step 1.6-1.7, use zymolyase to disrupt cells in 0.6 ml resuspension buffer solution. Ten units of zymolyase can disrupt 5 x 10^7 cells within 1.5 hours at 35°C.

2. EccDNA enrichment by column chromatography

2.1) Follow the protocol from a kit for column purification of plasmids. In brief, treat each sample with 1.2 ml alkaline solution, mix gently and incubate 3 min at room temperature.

2.2) Add 1.2 ml neutralization buffer, mix gently and centrifuge at 9650 x g for 5 min.

2.3) Load the solution onto a column equilibrated with 1 ml equilibration solution and allow the liquid to flow through the column by gravity.

2.4) Wash the column with 4 ml washing solution. When the solution has passed through the resin, carefully add 0.3 ml elution solution to replace most of the 0.35 ml column void volume.

2.5) Elute DNA into a new collection tube with 1 ml elution solution and precipitate the DNA by adding 0.8 ml precipitation mixture. Centrifuge at 9650 x g for 10 min.

2.6) Wash the DNA pellet with 0.5 ml 70% ethanol, centrifuge at 9650 x g for 5 min, air dry for 5 to 15 min and dissolve the purified DNA in 25 µl sterile water.

NOTE: Only short term storage of DNA in water is recommended. Preferentially, proceed directly to step 3.

3. Digestion of remaining linear chromosomal DNA

3.1) Optional step: To facilitate specific digestion of linear DNA by exonuclease, treat the purified DNA with a rare-cutting endonuclease such as *Not*I. For 5 µg DNA, use 1 unit *Not*I, 5 µl 10x digestion buffer and sterile water to a total volume of 50 µl. Incubate the reaction at 37°C for 16 hours and heat inactivate the endonuclease at 80°C for 5 min.

3.2) Add 20 units exonuclease (2 µl), 4 µl ATP (25 mM), 34 µl sterile water and 10 µl 10x reaction buffer directly to the 50 µl endonuclease-cleaved DNA to reach a 1x reaction volume of 100 µl, using the ATP-dependent exonuclease kit.

3.3) Perform hydrolysis of linear single-stranded and double-stranded DNA at 37°C for 5 days or more. Add an additional 4 µl ATP (25 mM), 0.6 µl 10x reaction buffer and 20 units exonuclease every 24 hours to continue the enzymatic DNA digestion at a 1x reaction volume.

3.4) After removal of linear DNA, sample 2 µl from the exonuclease treated solution to confirm elimination of chromosomal linear DNA by quantitative polymerase chain reaction (qPCR), using a chromosomal marker such as the actin gene *ACT1*[20].

3.4.1) Each 20 µl qPCR reaction volume contains 2 µl exonuclease-treated sample, 150 nM *ACT1* primers 5′-TCCGTCTGGATTGGTGGTTCTA-3′ and 5′-TGGACCACTTTCGTCGTATTC-3′, 2% (volume/volume) dimethyl sulfoxide, and 10 µl green fluorescent master mix.

3.4.2) Use the reaction condition; 3 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 30 sec at 60°C.

NOTE: *ACT1* is a particularly suitable marker for linear DNA since copy number variations in this gene are deleterious[21-23] so eccDNA should not carry *ACT1*.

3.4.3) Evaluate DNA digestion by standard PCR.

3.4.3.1) Use 2 µl exonuclease-treated sample as PCR template with *ACT1* primers 5'-TGGATTCTGGTATGTTCTAGC-3' and 5'-GAACGACGTGAGTAACACC-3'. As positive *ACT1* control, use 50-100 ng genomic *S. cerevisiae* DNA as template. PCR reaction conditions; 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 56°C and 1 min at 72°C.

3.4.3.2) Run PCR reactions by gel electrophoresis on 1% agarose with 0.5 µg/ml ethidium bromide. Look for a 0.8 kilo base *ACT1* band.

NOTE: The absence or presence of linear DNA can also be examined by propidium iodide staining before and after DNA amplification (step 4).

3.4.3.3) Mix each DNA sample in a 1:1 volume with a 1:1000 H_2O-diluted solution of 20 mM propidium iodide stock. Leave solution in darkness for 10–20 minutes at room temperature and analyze DNA staining by fluorescence microscopy at 100x magnification using a red excitation fluorescence filter at 663–738 nm and 5 to 30 sec exposure. As a DNA-staining control, use ϕ29-amplified genomic DNA from yeast and/or ϕ29-amplified plasmid.

3.5) Heat inactivate the exonuclease solution at 70°C for 30 min.

4. DNA amplification

4.1) Amplify the purified and enriched eccDNA from step 3.5) with ϕ29 DNA polymerase[24-26] according to the protocol of the polymerase manufacturer. In brief, mix 5 µl enriched eccDNA with 5 µl denaturing buffer.

4.1.1) After 3 min at room temperature, add 10 µl neutralization buffer. Mix gently and add 30 µl master mix containing 29 µl reaction buffer and 1 µl ϕ29 DNA polymerase. Incubate

the reaction at 30°C for 16 hours or more (up to 72 hours). Heat inactivate the ϕ29 DNA polymerase at 65°C for 3 min.

5. Sequencing and data analysis

5.1) Shear the amplified eccDNA with a focused ultrasonicator to an average target peak size of 300 bp. Use the following settings for a 130 µl DNA sample: 450W peak intensity power, 60 sec treatment, 30% duty factor, 200 cycles per burst, temperature 7°C.

5.2) Add barcode index labels and adapters to the fragmented reads for synthesis of libraries for sequencing, using an appropriate method for library preparation.

5.3) Run high-throughput sequencing, for example as 141-nucleotide single-end reads on a sequencing platform.

5.4) Map reads to the yeast reference genome under investigation and allow reads to map to multiple regions. For example, use a freely available workflow system[27,28] and short-read aligner mapping software[29].

5.5) Identify reads from regions of putative eccDNAs using contiguous reads, for example, more than seven contiguous reads (>1 kb) without gaps[20].

NOTE: Software is available[27,28] for exploring mapped reads at genomic regions of interest.

REPRESENTATIVE RESULTS:

To validate the Circle-Seq method, three *S. cerevisiae* CEN.PK populations of 1 x 10^{10} cells were screened after cells were grown separately in YPD for ten generations. Chromosomal linear DNA elimination was confirmed by the absence of a qPCR *ACT1* signal as described previously[20] (data not shown). Purified and enriched eccDNA was sequenced up to 68 million reads (141-nucleotide single-end reads) and mapped to the CEN.PK113-7D reference genome (version 19 June 2012). Recordings of putative eccDNAs from the three samples named C1, C2 and C4 were assigned to genomic regions mapped by contiguous reads longer than 1 kb. Based on 10,000 Monte Carlo simulations, the significance of each region mapped by contiguous reads longer than 1 kb was estimated. From this 79, 159 and 56 regions were annotated as likely eccDNA sequences ($p < 0.1$, **Dataset 1**). The number of recorded contiguous reads ≥ 1 kb increased as a function of sequence depth suggesting that even more eccDNA elements would have been recorded if samples had been sequenced further (**Fig. 2**). As expected, the Circle-Seq method extracted numerous reads from a number of known circular DNA elements including the 2µ plasmid, mitochondrial DNA, ribosomal RNA genes on chromosome XII, and the three internal control plasmids pBR322, pUC19 and pUG72 that were spiked into samples just before column purification (**Fig. 3**).

The video shows an example of contiguous reads that mapped to the *HXT7*_ARS432_*HXT6* locus on chromosome IV. Previously, [*HXT6/7*^{circle}] was detected by Circle-Seq in ten S288c populations (each with 1 x 10^{10} cells) and the circular DNA structure was confirmed by inverse PCR analysis[20]. The [*HXT6/7*^{circle}] was also recorded in each of the three CEN.PK populations (**Fig. 4A**). Moreover, most of the common eccDNA genes among replicate samples of CEN.PK overlapped eccDNA genes from S288c datasets (**Fig. 4B**).

To test the specificity of the Circle-Seq protocol for circular DNA purification, two samples, each with 30 µg genomic DNA, were tested. One sample was supplemented with 100 ng

plasmid DNA and both samples were purified by the Circle-Seq protocol. After column separation, the DNA yield was 1.27% (380 ng) for the sample without plasmid (GD) and 1.60% (480 ng) for the sample with plasmid (GD+P). The efficiency of exonuclease treatment was tested after 29 and 72 hours by analyzing the samples for linear DNA content using PCR against *ACT1*. No samples contained amplified *ACT1* (data not shown). A fraction of each exonuclease-treated sample was further amplified by the ϕ29 polymerase and the products of enzymatic reactions were analyzed by propidium iodide staining (**Fig. 5A-F**) and agarose gel electrophoresis (**Fig. 5G**). Samples after exonuclease treatment showed minimal propidium iodine-stain (**Fig. 5A-B**). The ϕ29-amplified sample with only genomic DNA revealed thread-like structures (**Fig. 5C**) similar to the control sample (**Fig. 5E**). The ϕ29-amplified sample that had added plasmid revealed foci (**Fig. 5D**), resembling the plasmid control (**Fig. 5F**). The images indicated that ϕ29 polymerase enriched for circular DNA over linear DNA. Most linear chromosomal DNA was removed from samples after 29-hour exonuclease treatment (**Fig. 5A-B, G**). However, extensive exonuclease treatment for several days, using more than 100 units, was needed to remove all chromosomal linear DNA, as ϕ29-amplification of samples treated with exonuclease for 72 hours still showed a background of thread-like structures (**Fig. 5C-D**).

FIGURE LEGENDS:

Figure 1. Outline of the Circle-Seq method. The protocol has 5 steps: 1) cell culturing, 2) purification and enrichment of eccDNA by column chromatography, 3) digestion of remaining linear chromosomal DNA in the eluate fraction, 4) amplification of DNA by ϕ29 DNA polymerase, and 5) sequencing of highly enriched eccDNA and mapping of reads to the *S. cerevisiae* reference genome.

Figure 2. Contiguous reads >1 kb as function of sequence depth. EccDNA from 1 x 10^{10} cells increase as a function of sequence depth (in millions of mapped reads). Shown: biological triplicates from haploid CEN.PK *S. cerevisiae* populations (C1, C2, C4) separated by 10^{10} cell divisions.

Figure 3. Detection of known circular DNA elements. A-B) Scatter plots of read coverage (read density) in percent for plasmids in CEN.PK biological replicates C1, C2 and C4. A) Mapped reads to the endogenous yeast plasmids were: 2µ; [$rDNA^{circle}$] (ribosomal RNA genes from chromosome XII); and mtDNA (the mitochondrial DNA), shown as percent. B) Unique reads mapped to control plasmids in percent. Control plasmids were spiked into samples before column purification. Plasmid ratios per cell were: pBR322 (plus signs) 1:1, pUC19 (circles) 1:50, and pUG72 (triangles) 1:2500.

Figure 4. Common eccDNA elements in CEN.PK and S288c. A) Venn diagram displaying overlap among the 476 genes on 294 eccDNA elements in the three CEN.PK samples (C1, C2, C4). The 16 common overlapping eccDNA genes/plasmids are annotated (all gene names are in **Dataset 1**). B) Venn diagram of all recorded genes on putative eccDNAs from the three CEN.PK samples (C1, C2, C4), compared to all recorded genes on putative eccDNAs from 10 S288c samples: S1-S2, R1-R4, Z1-Z4 (see reference[20]). Shown are 13 biological replicates (S1-S2, R1-R4, Z1-Z4, C1-C3) with genes/plasmids and putative eccDNA regions that overlapped a minimum of 2 strain backgrounds and either 3 or more experimental setups. C samples, CEN.PK; R and Z samples, S288c BY4741; S samples, S288c M3750.

Figure 5. Visualization of DNA samples after exonuclease and ϕ29 treatment. A-F, Propidium iodide staining of DNA. Scale bar, 10 µm. A, C and E, samples with genomic

DNA (GD); B and D, samples with GD plus plasmid (GD+P). A-B, after 29-hour exonuclease treatment (EXO 29 h); C-D, after 72-hour exonuclease treatment followed by ϕ29 polymerase amplification (EXO 72 h + ϕ29). E, Genomic DNA control after ϕ29 polymerase amplification; F, plasmid control (5.5 kb) after ϕ29 polymerase amplification; G, agarose gel-eletrophoresis. From left: L, 1 kilobase (kb) markers; P, plasmid control (5.5 kb) after EXO 29 h; GD, after EXO 29 h (sample as in A); GD+P, after EXO 29 h (sample as B); GD and GD+P, after EXO 29 h + ϕ29; GD and GD+P, after EXO 72 h + ϕ29 (sample as in C-D). See Table S1 for extra details.

Dataset 1. Potential DNA circularization regions in CEN.PK.
Shown are sequence data and analyses for 348 regions. Columns are A-D, eccDNA mapping. A (first column from left), sample from which putative eccDNA was identified; B, chromosome; C-D, start and end coordinates of putative eccDNAs. E-H, eccDNA content. E, autonomously replicating sequence (ARS) in the region; F, complete gene in the region; G, part of gene included in the region; H, BLASTN-identified gene. I-O, EccDNA coverage and p-values. I, longest region with a uniquely annotated sequence in bp; J, number of all mapped reads; K, coverage of all mapped reads by fragments per kb from a million mapped reads (FPKM); L, p-value for putative eccDNA compared to occurrence by chance from Monte Carlo simulations; M, number of uniquely mapped reads; N-O; as K and L using only uniquely mapped reads (UFPKM). Parameters for mapping of reads and Monte Carlo simulations were as described[20].

DISCUSSION:
The Circle-Seq method allows genome-scale detection of eccDNA from yeast cells with sequence-level resolution. The method is a mild eccDNA purification that does not require intensive vortex or pipetting and uses column separation by gravity to limit eccDNA breakage that would lead to exonuclease digestion in a subsequent step. These features of the method may be crucial for detecting large eccDNAs that contain gene sequences. Circle-Seq detected numerous eccDNAs including full genes (**Dataset 1**). It also detected the 86-kb yeast mitochondrial DNA. Thus, this protocol appeared to facilitate purification of large circular DNA elements. Keeping the number of DNA extraction steps to a minimum reduced the risk of eccDNA loss and maximized yield. Based on results for control, spiked-in plasmids, Circle-Seq is highly sensitive, detecting a single circular DNA from 2500 cells. Furthermore, removing abundant endogenous plasmids such as 2µ plasmid or mitochondrial DNA can significantly enhance sensitivity. Targeted removal of 2µ from yeast cultures has been described[30]. Use of a rare-cutting endonuclease such as *Swa*I, to cleave 2µ and mitochondrial DNA in *S. cerevisiae* S288c, substantially reduced their abundance. However, the restriction enzyme step could target other eccDNAs of interest and limit the total eccDNA yield.

Critical steps for eccDNA detection were removal of linear DNA (step 3) and DNA sequencing (step 5) to a proper depth. To record the majority of eccDNAs from a cell population, deep-sequencing might be required[20]. Paired-end sequencing provides even greater confidence of eccDNA detection. Circular DNA junctions will yield paired-end reads that map discordantly; these discrepancies support the discovery of circular DNA structures and can potentially be used as an additional eccDNA-detection filter.

The Circle-Seq method was validated using three independent *S. cerevisiae* CEN.PK populations. Detected sequences included previously reported eccDNAs, endogenous plasmids and spiked-in plasmids and hundreds of putative eccDNAs (**Dataset 1**). These

findings support previous Circle-Seq datasets from *S. cerevisiae* S288c[20]. The discovery of several eccDNAs common to CEN.PK and S288c populations indicate that these loci have a propensity to exist as circular elements (**Fig. 4**). We have previously shown that the [*GAP1*^{circle}] is enriched under nitrogen limited conditions in the CEN.PK background[8], though evidence of [*GAP1*^{circle}] in other strain backgrounds has not been found. Finding of the eccDNA from the *CUP1-1 RSC30*, *ASP3-1*, *COS111*, and *HXT6 HXT7* loci in both S288c and CEN.PK suggests that a predisposition for DNA circularization is conserved between the yeast strains. It remains to be shown if [*HXT6/7*^{circle}], [*ASP3-1*^{circle}], [*COS111*^{circle}], and [*CUP1-1 RSC30*^{circle}] confer selective advantages to the cells carrying them or if their existence is merely an effect of high rates of DNA circularization.

Taken together, the results indicate that Circle-Seq is well suited for detecting kilobase-sized eccDNAs and has advantages for identifying eccDNAs with complete genes. Circle-Seq is a highly sensitive method that enables whole genome-scale screens of eccDNAs from yeast. The Circle-Seq method could open a new field of research aimed at elucidating the role of eccDNA in generating gene deletions and amplifications. Given that DNA architecture and structure are largely conserved from eukaryotic yeast to higher eukaryotes, the Circle-Seq method should, in principle, be applicable to all eukaryotic cells, with slight modifications. At present, the method does not appear to have any limitations, although its ability to purify megabase-sized eccDNAs has yet to be shown. In addition, the use of ϕ29 DNA polymerase, which uses a rolling-circle amplification method[31], creates a bias towards smaller eccDNAs making eccDNA quantification more difficult.

Circle-Seq detects eccDNAs large enough to carry full genes, making it suitable for studies on double minutes—circular DNA from human somatic cells. Double minutes can contribute to cancer when proto-oncogenes are amplified by being present on multiple copies of these elements[32-37]. Studies of eccDNAs in germline cells could be used to measure germline mutation rates and assess sperm quality, for example in livestock. Thus, Circle-Seq has the potential to yield insights into the rate at which genetic variation arises in the form of copy number variation, and lead to a novel understanding of diseases that involve gene copy-number variation[38-40].

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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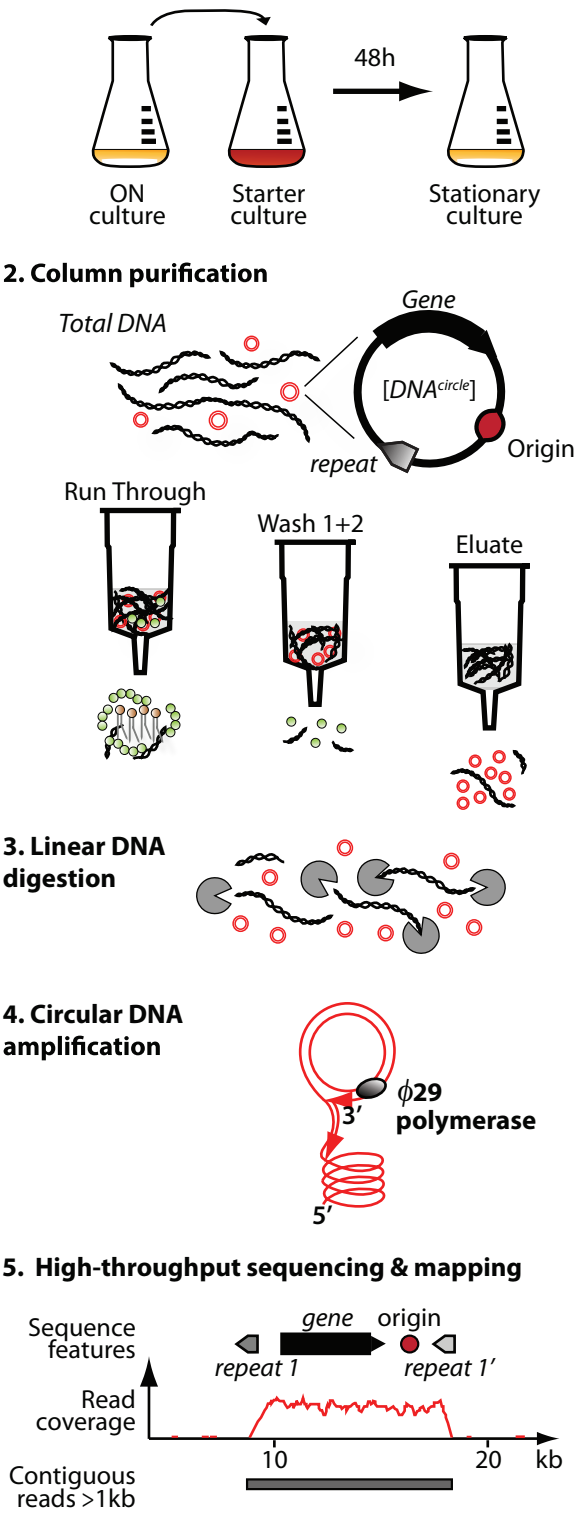

Figure 2

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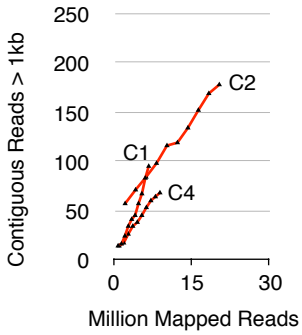

Figure 3

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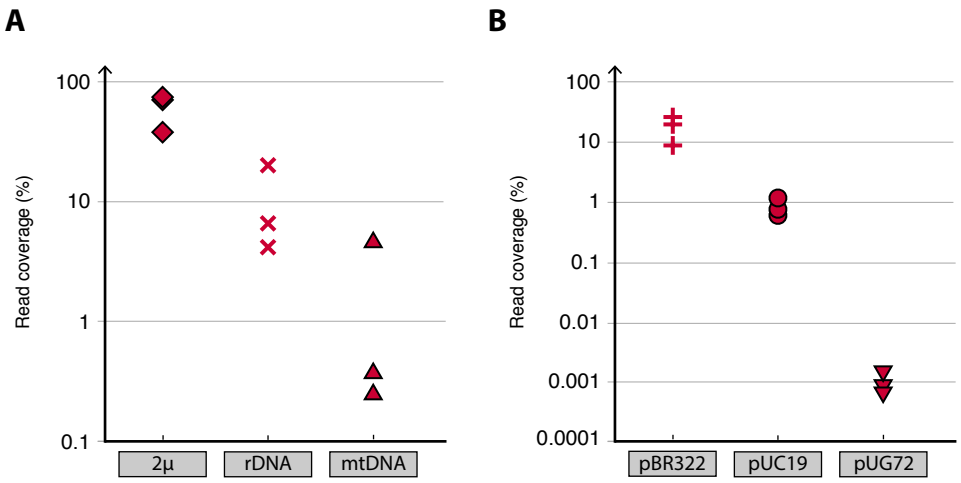
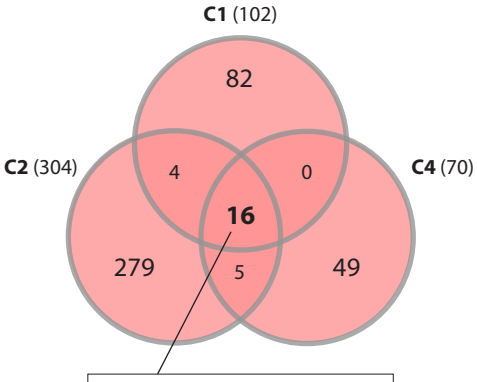


Figure 4

A)

EccDNA genes in CEN.PK

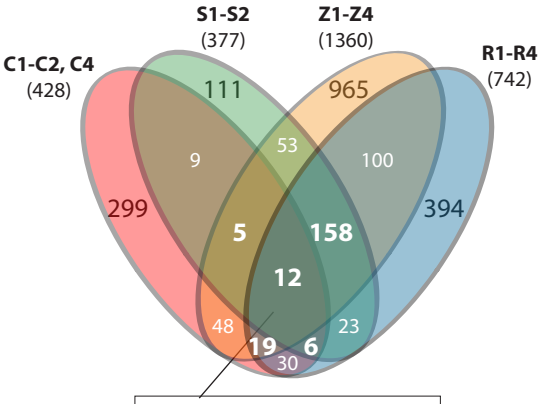


3% of genes/plasmids in all CEN.PK samples

16 = 2μ, *ASP3-1*, *CUP1-1*, *HXT6*, *HXT7*, *KEX1*, mtDNA, pBR322, pUC19, pUG72 rDNA_chrXII, *RSC30*. *YFR1-7*.

B)

Venn diagram of genes/plasmids on putative eccDNAs



0.4 % of genes/plasmids in all 4 experimental setups

12 = 2μ, *ASP3-1*, *COS111*, *CUP1-1*, *HXT6*, *HXT7*, mtDNA, pBR322, pUC19, pUG72 rDNA_chrXII, *RSC30*.

7% of genes/plasmids in 3 experimental setups

5 = *CDC23*, *ERC1*, *FMP40*, *SHR5*, *YCF1*.

6 = *BRR2*, *ENT4*, *OSH2*, *PRP19*, *SOD1*, *URA8*.

19 = *ASK10*, *CDC34*, *FAS2*, *HSP104*, *HXT3*, *ISA1*, *LYS2*, *MCM7*, *MHP1*, *MOD5*, *PAU17*, *PET122*, *PTK1*, *PUT4*, *PYC1*, *RIM20*, YER152, YER158C, YJL043W.

158 = *CAC2*, *CSM3*, *CUP1-2*, *DCP2*, *ENA1*, *ENA2*, *ENA5*, *ERB1*, *FAS1*, *GAT1*, *HAP1*, *LOT5*, *MLS1*, *NUP116*, rDNA ORFs_chrXII (~20), *RRT15*, YDR344C, YFL021C-A, YHR052W-A, YHR054C, YHR054W-A, YNL338W, Ty ORF elements (66), Telomere ORFs (61), *ZWF1*.

Figure 5

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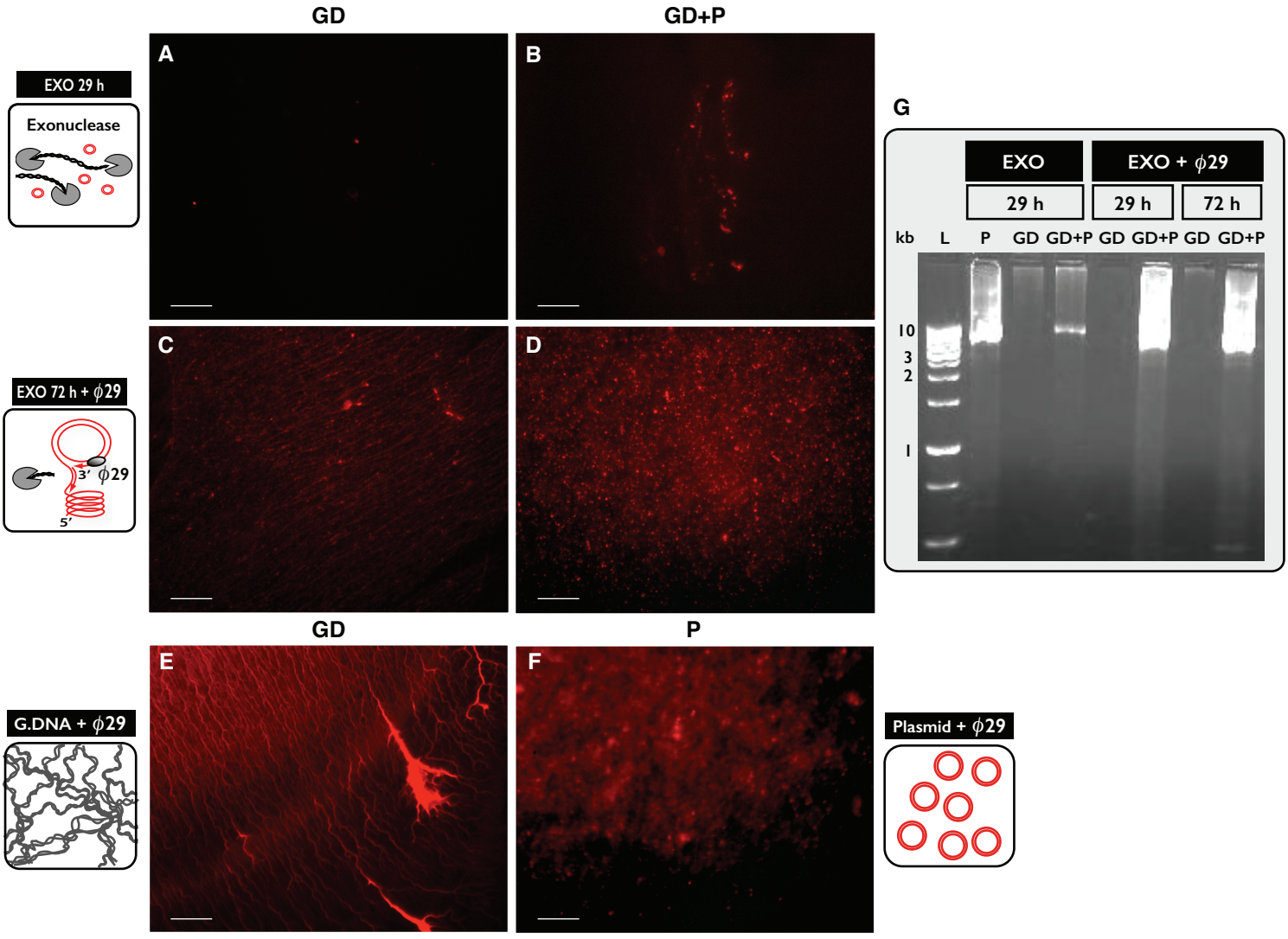

Dataset1 Click here to download Table DATASET1 - CENPK.xlsx

EccDNA mapping					EccDNA content	
Sample	CHROM	START	END	ARS	complete_ge	partial_gene_
C4	2-micron	0	6318	0	.	.
C2	2-micron	0	6318	0	.	.
C1	2-micron	0	6318	0	.	.
C2	chrI	149318	150702	0	.	YAL001C
C2	chrI	152418	154262	0	.	YAR002C-A,YA
C2	chrI	161480	163807	0	.	YAR014C,YAR
C2	chrII	277904	279228	0	.	YBR022W,YBF
C2	chrII	279284	280498	0	.	YBR023C
C2	chrII	292835	293989	0	.	YBR030W
C1	chrII	361914	363138	0	.	YBR063C,YBR
C1	chrII	363831	365967	0	.	YBR066C,YBR
C4	chrII	460474	463012	0	.	YBR114W,YBF
C1	chrII	620603	622082	0	.	YBR202W
C1	chrII	622118	623327	1	.	YBR203W
C2	chrII	639220	640260	0	.,YBR210W	.,YBR211C
C2	chrII	651834	652997	0	.,YBR218C	.,YBR217W
C2	chrII	749141	750412	0	.	YBR275C
C1	chrII	787665	789550	0	.	YBR295W
C1	chrIII	51334	52512	0	.	YCL039W,YCL
C2	chrIII	73063	76109	1	.,YCL026C-A	.,YCL025C,YCL
C4	chrIII	82617	83644	0	YCL021W-A	.
C1	chrIII	91033	92287	0	.,YCL018W	.,YCL018W
C2	chrIII	91578	92741	0	.,YCL018W	.,YCL018W
C1	chrIII	164680	166532	1	.	YCR026C
C2	chrIII	165746	167890	1	.,YCR027C	.,YCR026C
C1	chrIII	177643	180778	0	.	.
C2	chrIV	146313	147402	0	.	YDL173W
C2	chrIV	153522	155119	0	.	YDL170W,YDL
C2	chrIV	176699	177883	0	.	YDL154W,YDL
C1	chrIV	204522	208040	0	.	YDL140C
C1	chrIV	234920	236663	0	.	YDL126C
C2	chrIV	282677	283791	0	.	YDL098C,YDL
C2	chrIV	416224	417785	0	.	YDL019C
C2	chrIV	491917	494979	0	.	YDR026C
C2	chrIV	495121	499283	0	.	YDR028C
C2	chrIV	529295	532063	0	.,YDR036C	.,YDR035W
C2	chrIV	543748	545284	0	.	.
C2	chrIV	552789	554492	1	.	YDR049W
C2	chrIV	560829	563600	0	.	YDR054C,YDR
C2	chrIV	715405	716566	0	.	YDR135C
C2	chrIV	727965	732525	0	.	YDR141C
C1	chrIV	786537	787577	0	.	YDR165W,YDL

C2	chrIV	806954	810441	1	.,YDR172W	.,YDR173C
C4	chrIV	818177	820131	0	.,YDR179W-A	.,YDR180W
C2	chrIV	842325	844159	0	YDR192C	.
C4	chrIV	886401	891481	1	.,YDR213W	.,YDR212W
C4	chrIV	949250	952583	0	.,YDR244W	.,YDR245W
C2	chrIV	973093	974298	0	.	YDR258C,YDR
C2	chrIV	975051	976138	0	.	YDR260C
C2	chrIV	1068350	1069875	0	.	YDR303C
C2	chrIV	1079140	1081658	0	.	YDR310C
C1	chrIV	1146922	1148018	0	.	YDR338C
C2	chrIV	1147297	1148389	0	.	YDR338C
C1	chrIV	1150927	1152586	0	.	YDR341C
C2	chrIV	1151631	1152651	0	.	YDR341C
C4	chrIV	1154660	1158337	0	YDR342C	.
C2	chrIV	1154660	1158337	0	YDR342C	.
C1	chrIV	1154660	1158337	0	YDR342C	.
C4	chrIV	1160054	1161559	0	YDR343C	.
C2	chrIV	1160164	1161505	0	YDR343C	.
C2	chrIV	1161566	1162800	0	.	YDR345C
C2	chrIV	1220285	1224519	0	.,YDR373W,YI	.,YDR372C,YD
C2	chrIV	1269164	1270454	0	.	YDR399W,YDI
C1	chrIV	1476218	1477284	0	.	YDR517W
C1	chrIX	93907	95088	0	.	YIL130W,YIL1
C2	chrIX	98872	100430	0	.	YIL129C
C1	chrIX	117820	119544	0	YIL124W	.
C4	chrIX	123366	126550	0	.,YIL121W	.,YIL120W
C2	chrIX	154976	156615	0	.	YIL107C
C2	chrIX	271403	287713	1	.,YIL031W,YIL	.,YIL039W
C4	chrIX	279897	281023	0	.	YIL035C
C4	chrIX	282927	286069	0	.	YIL031W,YIL0
C2	chrIX	387512	388833	0	.	.
C1	chrIX	397947	399235	0	.	YIR027C
C4	chrMito	0	4535	0	.	.
C2	chrMito	0	13756	0	.	.
C1	chrMito	0	6985	0	.	.
C4	chrMito	4576	12870	0	.	.
C1	chrMito	6986	35874	0	.	.
C4	chrMito	13757	21519	0	.	.
C2	chrMito	13757	35906	0	.	.
C4	chrMito	21520	35873	0	.	.
C2	chrMito	35926	47727	0	.	.
C4	chrMito	35931	45444	0	.	.
C1	chrMito	35931	47718	0	.	.
C4	chrMito	45447	47715	0	.	.

C2	chrMito	47739	57882	0	.	.
C1	chrMito	47757	57831	0	.	.
C4	chrMito	47767	57261	0	.	.
C4	chrMito	57263	62458	0	.	.
C2	chrMito	57883	62486	0	.	.
C1	chrMito	57933	62458	0	.	.
C4	chrMito	64558	65701	0	.	.
C2	chrMito	64558	68689	0	.	.
C1	chrMito	64558	68599	0	.	.
C4	chrMito	66578	67639	0	.	.
C1	chrMito	69093	70545	0	.	.
C4	chrMito	69094	70373	0	.	.
C2	chrMito	69094	73114	0	.	.
C4	chrMito	72005	73098	0	.	.
C2	chrMito	73516	85779	0	.	.
C1	chrMito	73516	85259	0	.	.
C4	chrMito	73646	85259	0	.	.
C2	chrV	17021	20227	1	YEL069C,YELC	.
C1	chrV	32607	33886	0	.	YEL062W
C1	chrV	33906	35120	0	.	YEL061C,YEL0
C1	chrV	35158	36856	0	.	YEL061C
C1	chrV	73088	74730	0	.	YEL041W,YEL
C2	chrV	348766	349773	0	.	YER095W,YER
C2	chrV	349948	351155	1	.	YER096W
C1	chrV	466273	467480	0	.	YER150W,YER
C1	chrV	467493	469077	0	.	YER151C
C1	chrV	469483	471233	0	.	YER151C,YER1
C1	chrV	471557	472764	0	.,YER153C	.,YER152C
C2	chrV	486805	490489	0	.,YER159C	.,YER158C
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C4	chrV	527672	531170	0	.	YER172C
C1	chrVI	28291	29561	0	.	.
C4	chrVI	46174	47274	0	.	YFL041W
C4	chrVI	51065	52309	0	.	YFL038C
C2	chrVI	182677	183950	0	.	YFR019W
C2	chrVI	246849	250300	0	.,YFR052W	.,YFR051C,YFF
C1	chrVI	263357	264647	0	.	.
C2	chrVII	79601	80650	0	.	YGL222C,YGL2
C2	chrVII	80696	83050	0	.,YGL220W,YC	.,YGL219C,YG
C4	chrVII	111259	113355	2	.	YGL203C
C1	chrVII	111291	113679	2	.	YGL203C
C2	chrVII	111966	115279	3	YGL203C	.
C4	chrVII	113358	115279	1	.	YGL203C
C1	chrVII	113695	115279	1	.	YGL203C

C2	chrVII	118552	120951	0	.	YGL201C
C2	chrVII	136085	137160	0	.	YGL195W
C2	chrVII	137195	139273	0	.	YGL195W
C4	chrVII	385335	386723	0	.	YGL062W
C2	chrVII	491840	496365	0	YGL001C,YGL	.
C2	chrVII	532062	535053	0	YGR026W,YG	.
C4	chrVII	567599	568599	1	.	.
C2	chrVII	567599	568599	1	.	.
C1	chrVII	567599	568599	1	.	.
C4	chrVII	570566	574131	1	.	.
C2	chrVII	570566	574131	1	.	.
C1	chrVII	570566	574131	1	.	.
C2	chrVII	653315	666957	3	.,YGR088W,Y	.,YGR091W
C2	chrVII	679383	682812	0	.	YGR097W,YG
C4	chrVII	698698	699704	0	.	YGR106C
C4	chrVII	699716	700790	0	.	.
C4	chrVII	700798	702666	0	.	.
C2	chrVII	707247	711944	0	CENPK1137D	.
C2	chrVII	828660	830151	0	YGR165W	.
C2	chrVII	919092	924115	0	.,YGR214W,Y	.,YGR213C,YG
C1	chrVII	954997	956101	0	.	YGR233C
C2	chrVII	1005956	1007740	0	.	YGR257C,YGR
C2	chrVII	1035077	1037004	0	.	YGR271W
C2	chrVII	1045621	1047457	0	.	YGR277C,YGR
C1	chrVII	1062395	1063458	0	.	YGR285C
C4	chrVIII	14528	16340	0	.	YHL039W
C4	chrVIII	16378	17548	0	.	YHL038C,YHL
C4	chrVIII	18881	21578	0	.,YHL036W	.,YHL035C
C2	chrVIII	66392	67794	0	.	YHL016C
C2	chrVIII	79067	80548	0	CENPK1137D	.
C2	chrVIII	90707	92246	0	.,YHL006C	.,YHL007C
C2	chrVIII	96905	100337	0	.,YHL001W	.,YHL002W,YH
C1	chrVIII	117119	118307	0	.	YHR009C
C1	chrVIII	165049	166226	0	.	YHR031C
C1	chrVIII	166924	167930	1	.	YHR032W
C1	chrVIII	391538	393730	2	.	YHR150W,YH
C2	chrVIII	431149	432281	0	.	YHR166C
C4	chrVIII	448551	449581	0	.	YHR176W
C1	chrVIII	453154	456149	0	.	YHR178W,YH
C1	chrVIII	457228	458393	0	.	CENPK1137D
C1	chrVIII	469190	470414	0	.	YHR186C
C2	chrVIII	517483	518669	0	.	.
C2	chrX	45546	49126	0	.	YJL197W,YJL1
C2	chrX	64660	66600	0	.,YJL184W	.,YJL183W,YJL

C2	chrX	66640	67708	1	.	YJL183W
C2	chrX	119067	120783	0	.	YJL149W,YJL1
C2	chrX	120830	122350	0	.	YJL149W
C2	chrX	123127	125822	0	.,YJL147C	.,YJL146W,YJL
C4	chrX	237484	238855	0	.	YJL093C
C4	chrX	238858	240542	0	.	YJL092W,YJL0
C4	chrX	240556	243144	0	.	YJL092W
C4	chrX	249526	250833	0	.	YJL089W
C1	chrX	309851	310955	0	.	YJL057C,YJL05
C4	chrX	342203	343937	0	.,YJL043W	.,YJL042W
C1	chrX	388089	389764	0	.	YJL016W
C1	chrX	389901	391131	0	.	YJL014W
C2	chrX	440487	443548	2	.,YJR010C-A,Y	.,YJR013W
C1	chrX	480891	482617	0	.	YJR035W
C1	chrX	482623	483730	0	.	YJR035W,YJR(
C1	chrX	592440	594028	0	.	YJR095W,YJR(
C4	chrX	603448	604955	0	.	YJR103W,YJR1
C2	chrX	647018	651379	0	.,YJR130C	.,YJR129C,YJR
C4	chrX	685047	686231	0	.	.
C4	chrX	686271	687357	0	.	YJR147W
C1	chrXI	67829	73767	0	YKL197C,YKL1	.
C2	chrXI	80383	81907	0	.	YKL191W
C2	chrXI	106919	110753	0	.,YKL180W,YK	.,YKL179C
C1	chrXI	130378	131450	0	.,YKL170W	.,YKL168C
C1	chrXI	258082	259266	1	YKL096W-A	.
C2	chrXI	409151	415509	0	.	YKL014C,YKL0
C2	chrXI	422057	426904	0	.,YKL009W	.,YKL010C
C2	chrXI	514577	517284	1	YKR039W	.
C2	chrXI	517443	521683	0	.,YKR041W,YI	.,YKR044W
C2	chrXI	553826	565323	0	.,YKR059W,YI	.,YKR058W,YK
C2	chrXI	576995	580824	0	.,YKR074W,YI	.,YKR072C
C4	chrXII	34746	36051	0	.	YLL043W
C4	chrXII	36325	37801	0	.,YLL042C	.,YLL043W
C4	chrXII	37854	39510	0	.,YLL041C	.,YLL040C
C4	chrXII	39542	40783	0	.	YLL040C
C2	chrXII	50204	55392	1	.,YLL036C,YLL	.,YLL035W
C2	chrXII	72550	77655	1	.,YLL026W	.,YLL027W
C2	chrXII	77678	80305	0	YLL025W	.
C2	chrXII	122360	123663	0	.	YLL005C,YLL0
C2	chrXII	169336	175965	0	.,YLR022C,YLI	.,YLR024C
C1	chrXII	183410	185672	0	.,CENPK1137I	.,YLR028C
C1	chrXII	240998	242047	0	.	YLR060W
C2	chrXII	328062	333473	0	.,YLR103C,YLI	.,YLR102C
C4	chrXII	365997	367164	0	.	YLR116W

C2	chrXII	517889	519032	0	.	YLR189C
C2	chrXII	519498	521084	0	.	YLR190W
C2	chrXII	551872	555947	0	.,YLR214W	.,YLR213C,YLF
C1	chrXII	698814	700718	0	.	YLR288C,YLR2
C2	chrXII	838679	842534	0	.,YLR364W,YL	.,YLR368W
C2	chrXII	976759	978620	0	.	YLR429W,YLR
C2	chrXII	1002134	1004700	0	.,YLR441C	.,YLR440C,YLF
C4	chrXII	1005396	1006438	0	.	YLR442C
C4	chrXII	1006468	1008071	2	.	YLR442C,YLR4
C2	chrXIII	48561	50276	0	.,YML108W	.,YML107C,YN
C4	chrXIII	70799	71842	0	.	YML099C
C2	chrXIII	81756	83122	0	.	YML091C
C2	chrXIII	83620	85334	0	.	YML091C
C2	chrXIII	85591	86689	0	.	.
C4	chrXIII	89106	95011	3	.,YML085C,YN	.,YML083C
C2	chrXIII	218633	225095	0	.,YML022W,Y	.,YML021C
C1	chrXIII	287303	288592	0	.	YMR012W
C2	chrXIII	779019	780107	0	.	YMR259C
C1	chrXIII	783741	786149	0	.,YMR260C	.,YMR261C
C1	chrXIII	786236	788279	1	.	YMR261C,YM
C4	chrXIII	840112	841159	0	.	YMR288W
C2	chrXIII	856759	858855	0	.,YMR298W	.,YMR297W
C1	chrXIII	898290	899295	1	.	YMR315W
C2	chrXIV	77269	83995	0	.,YNL289W,YI	.,YNL292W
C2	chrXIV	102412	112226	0	.,YNL278W,YI	.,YNL277W,YN
C2	chrXIV	155070	156393	0	.	YNL257C
C2	chrXIV	156813	159288	1	.	YNL256W,YNI
C2	chrXIV	159306	163129	0	.,YNL254C,YN	.,YNL256W
C2	chrXIV	164524	166001	0	.	YNL252C,YNL
C2	chrXIV	167124	168192	0	.	YNL251C
C1	chrXIV	296477	298332	0	.,YNL178W	.,YNL177C
C2	chrXIV	510890	513129	0	YNL055C,YNL	.
C2	chrXIV	625348	627008	0	.	YNR002C
C2	chrXIV	654689	656649	1	.	YNR016C,YNR
C2	chrXIV	675598	680458	0	.	YNR031C,YNR
C1	chrXIV	687184	688353	0	.	YNR035C,YNR
C1	chrXV	31112	36121	1	YOL154W	.
C2	chrXV	55527	56885	0	.	YOL141W,YOI
C1	chrXV	74470	76074	0	.	YOL130W
C2	chrXV	105468	112648	1	.,YOL108C,YO	.,YOL107W,YO
C2	chrXV	112649	117642	1	.,YOL104C,YO	.,YOL107W
C4	chrXV	181004	182115	0	.	YOL078W,YOI
C2	chrXV	262765	264351	0	.	YOL033W,YOI
C1	chrXV	340942	342073	0	.	YOR008C

C2	chrXV	357451	363313	0	.,YOR014W,Y	.,YOR017W
C2	chrXV	488678	490639	1	.	YOR087W,YO
C2	chrXV	491097	492173	0	.	YOR090C
C2	chrXV	493988	495038	1	.	YOR091W
C1	chrXV	527024	528559	0	.	YOR109W
C1	chrXV	529227	530590	0	.	YOR110W,YO
C2	chrXV	637250	638333	0	.	YOR161C
C2	chrXV	639688	642044	0	.	YOR162C
C1	chrXV	656870	658471	1	YOR173W	.
C1	chrXV	658481	661234	0	.,YOR174W	.,YOR175C
C2	chrXV	799154	809107	0	.,YOR250C,YC	.,YOR249C,YO
C2	chrXV	838077	839477	0	.	YOR274W,YO
C2	chrXV	839479	840792	0	.	YOR275C
C2	chrXV	842172	843472	0	.,YOR278W	.,YOR279C
C2	chrXV	875128	876693	1	.,YOR298W	.,YOR298W
C2	chrXV	983849	985667	0	.	YOR346W,YO
C4	chrXV	987162	988584	1	.	YOR348C
C4	chrXV	1056506	1059054	0	.	YOR381W
C1	chrXV	1064146	1065812	0	YOR385W	.
C4	chrXVI	0	1424	0	.	.
C2	chrXVI	0	1424	0	.	.
C1	chrXVI	0	1424	0	.	.
C2	chrXVI	34391	38360	0	.,YPL268W	.,YPL267W,YP
C2	chrXVI	53066	54119	0	.	YPL258C
C2	chrXVI	54147	56195	0	.	YPL258C
C4	chrXVI	57974	59481	0	.	.
C2	chrXVI	57974	59481	0	.	.
C1	chrXVI	57974	59481	0	.	.
C4	chrXVI	112437	115317	0	.	YPL230W,YPL
C2	chrXVI	129647	130751	0	.	YPL222W
C2	chrXVI	196421	198345	0	.	YPL184C
C2	chrXVI	198400	199512	0	.,YPL183W-A	.,YPL183C
C2	chrXVI	221555	222686	0	.	YPL174C
C2	chrXVI	232519	235801	0	.	YPL167C
C2	chrXVI	246630	248750	0	.	YPL160W
C2	chrXVI	268897	270525	0	.	YPL150W
C2	chrXVI	283284	301727	2	.,YPL133C,YPI	.,YPL132W
C2	chrXVI	386665	388482	0	.	.
C2	chrXVI	388516	389664	0	.	YPL085W
C2	chrXVI	389776	392632	0	.	YPL085W
C2	chrXVI	392657	393898	0	.	YPL085W
C2	chrXVI	495131	498317	0	.,YPL029W	.,YPL028W,YP
C4	chrXVI	554729	556817	0	.	YPL001W,YPR
C4	chrXVI	556849	558013	0	.	YPR001W

C2	chrXVI	682991	688318	1	.,YPR069C,YP	.,YPR068C,YPI
C1	chrXVI	708524	711028	0	.	YPR085C,YPR(
C1	chrXVI	711080	712156	0	.	YPR086W,YPF
C4	chrXVI	869368	870618	0	.	.
C1	chrXVI	876332	878085	0	YPR166C,YPR	.
C1	chrXVI	879261	880276	0	.	YPR169W
C1	chrXVI	896655	899485	0	.	YPR180W,YPF
C4	pBR322	0	4361	0	.	.
C2	pBR322	0	4361	0	.	.
C1	pBR322	0	4361	0	.	.
C4	pUC19	0	2686	0	.	.
C2	pUC19	0	2686	0	.	.
C1	pUC19	0	2686	0	.	.
C2	pUC72	1458	3973	0	.	.
C1	pUC72	1471	3914	0	.	.
C4	pUC72	1658	3914	0	.	.
C4	scaffold601	0	2519	0	.	.
C2	scaffold601	0	2519	0	.	.
C1	scaffold601	0	2519	0	.	.
C4	scaffold604	0	3534	0	YLR155C,YLR:	.
C2	scaffold604	0	3534	0	YLR155C,YLR:	.
C1	scaffold604	0	3534	0	YLR155C,YLR:	.
C2	scaffold613	199	2458	0	.	.
C2	scaffold617	0	1979	0	YHR056C	.
C1	scaffold617	0	1979	0	YHR056C	.
C4	scaffold617	11	1979	0	.	YHR056C
C2	scaffold618	776	2264	0	.	.
C4	scaffold624	0	1844	0	.	.
C2	scaffold624	0	1844	0	.	.
C1	scaffold624	0	1844	0	.	.
C4	scaffold628	0	1319	0	.	.
C2	scaffold628	0	1319	0	.	.
C1	scaffold628	0	1319	0	.	.
C4	scaffold630	0	2776	0	R0030W	.
C2	scaffold630	0	2776	0	R0030W	.
C1	scaffold630	0	2776	0	R0030W	.
C4	scaffold636	0	2346	0	R0010W	.
C2	scaffold636	0	2346	0	R0010W	.
C1	scaffold636	0	2346	0	R0010W	.
C4	scaffold637	0	1714	0	CENPK1137D	.
C2	scaffold637	0	1714	0	CENPK1137D	.
C1	scaffold637	0	1714	0	CENPK1137D	.

	EccDNA coverage and p-values					
BLASTN ident	UNIQUE_BP	ALL_READ_C(	ALL_FPKM	ALL_PVAL	UNIQUE_REA	UNIQUE_FPK(
	1265	25324816	64237.6285	8.57E-05	3335	299.481788
	1087	11094263	33970.7784	0.0001517	1458	65.9442422
	1292	28351617	68252.1847	0.00013474	4624	540.576271
	1306	42	0.58708348	0.05060679	39	1.46814967
AR002W	1844	56	0.58750798	0.05058406	56	1.49305571
015W	2327	96	0.79810772	0.0431085	91	1.92262202
R023C	1324	30	0.43834891	0.05982667	30	1.11399226
	1208	31	0.49400309	0.05586588	28	1.13956735
	1081	1288	21.5921912	0.00530511	959	43.6155964
065C	1224	27	0.33550631	0.02836102	27	3.33183904
067C	2136	39	0.27770372	0.03493575	39	2.75781434
R115C	2538	80	0.50515097	0.01404933	79	3.53590492
	1414	30	0.30851155	0.03115049	27	2.8841379
	1209	31	0.38999024	0.02418103	28	3.49810948
	1002	36	0.66966226	0.04720679	30	1.47198178
	1163	779	12.9581918	0.00762556	744	31.451555
	1271	21	0.31963947	0.07159087	21	0.81231158
	1860	43	0.34695683	0.02739366	39	3.1670384
040W	1054	17	0.21949366	0.04476627	15	2.14957357
026C-B	3046	170	1.07970642	0.03725339	167	2.69547603
	926	54	0.84264789	0.01021817	41	5.02965243
	1254	31	0.37599537	0.02515787	30	3.61347775
	1163	28	0.46576299	0.05770963	27	1.14138708
	1852	36	0.29565135	0.03272733	36	2.93604823
	2015	59	0.53237039	0.0535102	54	1.31755153
	3135	188	0.91209201	0.01324946	185	8.91324511
	946	45	0.79941317	0.0430602	37	1.92291235
171C	1597	42	0.50878118	0.05490963	41	1.26219903
155W	1184	21	0.3431265	0.06888519	21	0.87200002
	3480	127	0.54906812	0.01805682	121	5.25179533
	1743	46	0.40140131	0.02339471	45	3.89957065
099W	1114	19	0.32995527	0.07040962	19	0.8385275
	1561	37	0.45854872	0.05823804	37	1.16532677
	3062	976	6.16639433	0.01471192	944	15.1570858
	4162	1229	5.71263491	0.01543806	1202	14.1987886
	2768	252	1.76125045	0.02910453	241	4.2805528
	1532	62	0.7808851	0.04358066	61	1.95758204
	1703	38	0.43167372	0.06034769	38	1.09702835
055W	2771	415	2.8973318	0.02225794	398	7.06147536
	1161	19	0.31659791	0.07194712	19	0.80458195
	4560	885	3.75461223	0.01907783	862	9.29375726
R166C	1040	24	0.35099122	0.02706629	23	3.34038222

	3459	146	0.81000476	0.04274713	124	1.76246307
	1954	62	0.50849882	0.01399349	61	3.54625842
	1834	638	6.72989068	0.01398636	592	15.8697936
	5080	250	0.7886769	0.01064284	236	5.27731959
	3333	126	0.60584015	0.0125831	121	4.12396681
259C	1205	20	0.32109209	0.0714295	20	0.81600318
	1087	22	0.3915433	0.06393803	22	0.99504343
	1520	32	0.40594463	0.06261815	30	0.97034589
	2518	2085	16.0190588	0.00641193	1898	37.0586321
	1096	23	0.31917997	0.02999786	22	3.03189246
	1092	14	0.24802306	0.08216984	14	0.63031015
	1659	43	0.3942216	0.0238884	42	3.82388278
	1020	15	0.28449704	0.07635052	15	0.72300282
HXT7	3559	1172	5.10806958	0.00513506	1086	34.6630634
HXT7	3579	3248	17.0887011	0.00615397	2990	41.073186
HXT7	3555	1393	5.7620342	0.00496736	1282	54.4690858
HXT6	1487	32	0.34075034	0.01996621	30	2.29179301
HXT6	1341	26	0.37508633	0.06560508	25	0.91655838
	476	30	0.47031925	0.0573886	5	0.51643058
R375C	4230	142	0.64881989	0.04800281	140	1.62718364
R400W	1290	25	0.37491858	0.06562269	25	0.95279441
	1066	25	0.35669839	0.02664208	25	3.54229291
31C	1181	23	0.29620766	0.03266418	21	2.68578388
	1558	26	0.32284388	0.07117894	26	0.82045506
	1724	39	0.34406912	0.02763366	39	3.41687438
	3169	70	0.35232852	0.01938049	67	2.40169397
	1619	81	0.95607669	0.03943634	77	2.33825988
	16310	10176	12.0700703	0.00842897	9997	30.1345446
	1126	29	0.41274558	0.01659738	29	2.92566578
33C	3142	104	0.53045675	0.01357531	103	3.72388404
	1321	56	0.82010955	0.04248577	52	1.93530504
	1261	24	0.28340906	0.03425786	23	2.75495441
	3931	38714	136.808656	0.00077792	3861	111.573707
	13688	1647809	2317.40194	0.00039205	176344	633.387653
	6518	102927	224.120328	0.00062632	9010	208.791157
	7458	35277	68.1632731	0.00112208	11836	180.280055
	26941	41120	21.6498043	0.00233368	15916	89.2322584
	7658	2560	5.28553739	0.00505973	2349	34.8443815
	21577	84576	73.87197	0.00268182	53257	121.348535
	12742	6311	7.04657888	0.00427792	1738	15.4944896
	11512	204382	335.050675	0.00087273	101980	435.525039
	8985	9470	15.9534753	0.00260649	5500	69.5360013
	11387	24284	31.3354031	0.00173474	11296	149.836296
	2256	8743	61.7789113	0.00115454	3929	197.837237

	9850	208758	398.165259	0.00079432	57694	287.967398
	9303	41850	63.1846903	0.00112105	8678	140.895879
	8313	29305	49.4669713	0.00131428	6767	92.4705628
	4921	1919	5.91987103	0.0046961	1250	28.8550448
	4574	36243	152.324523	0.00165966	27219	292.56671
	4396	992	3.33435191	0.00645473	719	24.7043182
	956	1372	19.2367058	0.00237792	436	51.8076272
	3810	66946	313.513395	0.0009125	9910	127.878514
	3755	12160	45.7681196	0.00137158	4088	164.438161
	889	2860	43.1989779	0.00143376	1062	135.702504
	1264	4047	42.3921002	0.00145895	1048	125.232161
	1144	3907	48.9548461	0.00132078	1011	100.38995
	3880	76039	365.929149	0.00083182	14924	189.104741
	942	1675	24.5593841	0.00194026	207	24.9622971
	11714	72311	114.076004	0.00199318	25544	107.209331
	10869	17624	22.8267335	0.0022421	6042	83.9639379
	10343	12476	17.2168527	0.00250389	4932	54.1678562
	2471	555	3.34900756	0.02051817	392	7.79941849
	1279	32	0.38053778	0.02478945	32	3.77903662
I62W	1214	25	0.31321292	0.03060628	23	2.86161244
	1698	27	0.24184907	0.04061469	27	2.4017497
042W	1642	31	0.28714872	0.03375575	31	2.85161051
096W	1007	25	0.48028298	0.05674542	25	1.22056086
	1183	20	0.32056004	0.07148121	19	0.78961931
151C	1059	30	0.37803528	0.02496208	23	3.2804509
	1584	34	0.3264691	0.02925576	33	3.14673687
152C	1750	32	0.27811875	0.03483996	31	2.67562541
	1207	28	0.35283293	0.02692102	28	3.50390585
	3306	303	1.59114467	0.03071305	255	3.79215633
	2775	74	0.49794403	0.05559145	72	1.27561146
	3494	109	0.49937813	0.0141766	108	3.51128402
	1260	47	0.56287568	0.01783998	45	5.39440607
	1100	23	0.3350873	0.02026361	23	2.37520039
	1197	22	0.2834165	0.02392724	21	1.99292176
	1233	23	0.34953132	0.06824996	20	0.79747269
R053C	3451	97	0.54376774	0.05284429	96	1.36765065
	1290	32	0.37729288	0.02503576	31	3.62972439
223C	1032	16575	305.678372	0.00092386	3912	186.366587
L222C	2352	115	0.94510061	0.03964486	105	2.19482998
	1700	109	0.83340872	0.01027791	74	4.94479058
	1979	65	0.41399718	0.02261156	54	4.12144617
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	1075	34	0.61186712	0.04948406	30	1.37202395
	2035	39	0.36308284	0.06678689	38	0.9180537
	680	16	0.18473677	0.0379532	6	1.00232241
	4514	319	1.36382536	0.03339373	310	3.37636229
	2927	219	1.41649278	0.03270282	189	3.17459248
	849	65	1.04168445	0.00931427	44	5.88721764
	995	1895	36.6602884	0.00405284	1370	67.6934095
	861	87	1.32323688	0.01054736	67	11.7536652
	3560	2448	11.0046112	0.00334415	2196	70.0724726
	3565	41665	226.098934	0.00122273	37299	514.38294
	3556	1941	8.28102978	0.00383157	1727	73.3554274
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R098C	3393	126	0.71086924	0.04577213	122	1.76776639
	980	479	7.63062929	0.00407922	142	16.4599069
	811	28	0.41780783	0.0163779	23	3.22160349
	1691	47	0.40322162	0.0169818	43	2.88861693
	4665	236	0.9720265	0.03914202	227	2.39234115
	1491	38	0.49305188	0.05593065	38	1.25301092
R216C	4424	395	1.52132002	0.03147555	315	3.5006149
	1104	26	0.35819756	0.02650524	24	3.28355152
258C	1756	47	0.5096707	0.05485111	44	1.23190457
	1927	43	0.43169141	0.06034485	41	1.04604663
278W	1836	101	1.06422966	0.03752668	94	2.51712092
	1063	31	0.44355428	0.02112103	31	4.40483957
	1812	42	0.3714616	0.01848569	42	2.63303239
039W	1170	31	0.42461825	0.01620128	31	3.00982286
	2685	58	0.34464332	0.01975971	55	2.32693099
	1402	46	0.6347409	0.04853804	45	1.57802327
	1481	250	3.26566486	0.02090056	229	7.60202557
	1539	66	0.82964438	0.04225452	60	1.91673261
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	1188	33	0.42248943	0.02221998	28	3.55994474
	1171	19	0.24552487	0.04000417	18	2.32175974
	1006	20	0.30237812	0.0319126	20	3.00285029
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	1132	63	1.07666547	0.03731021	57	2.47558208
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_5331	1123	28	0.36555308	0.0258726	26	3.49699699
	1224	15	0.18639239	0.05301152	15	1.85102169
	1186	163	2.65882392	0.02331249	149	6.17661428
98W	3444	129	0.69709721	0.04623747	120	1.71303803
185C	1940	56	0.55843542	0.05207042	56	1.41917254

	1068	34	0.61587748	0.04930111	33	1.51911828
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	1520	53	0.67455745	0.04700679	52	1.68193287
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	1371	33	0.38574411	0.01774413	30	2.4857011
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58C	1052	26	0.35819756	0.02650524	24	3.44585635
	1734	51	0.47135043	0.01479608	48	3.14454091
	1675	33	0.2996522	0.03220628	33	2.97577982
	1230	33	0.40806296	0.0230705	32	3.92958361
	3021	168	1.06177529	0.03756759	159	2.58758903
	1726	32	0.28198599	0.03449575	32	2.80034058
)36C	1107	20	0.27478987	0.03530733	20	2.7288775
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	1088	24	0.32484962	0.02084932	19	1.98376311
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	1524	44	0.55854012	0.05204883	43	1.38717863
	3583	155	0.78210714	0.04354202	120	1.64658191
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ASP3-1_chrXI	3534	4831	21.9075249	0.00220259	4796	154.162141
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Telomere_chı	1444	59	0.50526876	0.05513122	31	1.05546395
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Y'element/YR	1319	38092	462.819664	0.00047662	31988	2754.90988
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0.02587681
0.01479147
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0.04899145
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0.03642612
0.01697156
0.03476873
0.05185168
0.04271929
0.00977661
0.01097401
0.04360452
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0.04644941
0.04896134
0.05111929
0.0076922
0.00879034
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0.0215484
0.02574023
0.05143633
0.02051682
0.00760852
0.01695681
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0.03503365
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0.02048103
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0.02515908
0.02543373
0.03911873
0.0269305

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0.01829366
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0.01732209
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0.02364208
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9.55E-05
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0.00018421
0.00304261
0.00282631
0.00297662
0.00010649
6.31E-05
8.32E-05
0.00143636
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0.0012621
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0.00627402
0.04058464
0.00013247
9.09E-05
0.00010842
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0.00035682
0.00030105
1
0.04640452
1
0.00239091
0.00966818
0.00138105
0.00448181
0.00726022
0.00444526

Materials	Company	Catalog Number
Bacto peptone	BD Difco	211677
Brilliant III SYBR Green PCR Master	Agilent Technologies	600882
Dextrose (D-glucose)	Carl Roth	HN06.4
Disruptor Beads, 0.5 mm	Scientific Industries, Inc.	SI-BG05
Ethidium bromide	Carl Roth	2218.2
GeneJet plasmid miniprep kit	Thermo Fisher	K0502
NotI, FastDigest	Life Technologies - Thermo Fisher Scientific, USA	FD0594
Plasmid Mini AX kit	A&A Biotechnology, Poland	010-50
Plasmid-Safe ATP-dependent DNase kit	Epicentre, USA	E3105K
Propidium iodide	Sigma-Aldrich, USA	81845
pUG6 plasmid	EUROSCARF, Germany	P30114
QIAGEN genomic-tip 100/G	Qiagen, USA	13343
REPLI-g Mini Kit protocol	Qiagen, USA	150023
Yeast extract	BD Difco	210929
Zymolyase 100T (Lyticase, Yeast Lytic Enzyme)	Nordic BioSite, Sweden	Z1004-3
Data access to sequence files	European Nucleotide Archive	

Strains

Saccharomyces cerevisiae CEN.PK113-7D	
Saccharomyces cerevisiae yeast deletion library pool	EUROSCARF, Germany

Equipments

DNA Spectrophotometer	NanoDrop 1000 Spectrophotometer, Thermo Fisher

Fluorescence microscopy	Nikon Optronics Magnafire. Red excitation fluorescence filter, 663-738
Robotic library-build system	Apollo 324, IntegenX Inc.
Sequencing platform	Illumina HiSeq 2000 platform, Illumina Inc.
Ultrasonicator	Covaris LE220, microTUBE AFA Fiber tubes

Methods

2% YPD media

Circle-Seq test on genomic DNA

Mapping software	Bowtie2 aligner, John Hopkins University

Propidium iodide stain	
Workflow bioinformatic system	Galaxy, Open source.

Comment / Description

Alternative product can be used.
For qPCR analysis. Alternative product can be used.
Alternative product can be used.
Glass beads to disrupt plasma cell membranes.
Alternative product can be used.
Agarose gel stain for detecting DNA/RNA.
Plasmid purifcation from bacteria. Alternative product can be used

Endonuclease. Alternative product can be used.
Plasmid purifcation kit used to purify eccDNA.
ATP-dependent exonuclease kit. Alternative product can be used.
Alternative product can be used.
Marker gene: loxP-PAgTEF1-kanMX-TAgTEF1-loxP.
Plasmid requests: Please contact Dr. Peter Philippsen@unibas.ch
Genomic DNA purifcation from yeast. Alternative product can be used.
Amplification of eccDNA by the phi29 polymerase
Alternative product can be used.

Alternative product can be used.

EccDNA dataset from Saccharomyces cerevisiae CEN.PK113-7D. Study accession number PRJEB9684. 2nd accession number is ERP010820. Locus tag prefix is BN2032.

Genotype MATa MAL2-8c SUC2
S288c BY4741 pool of 4400 viable single-gene deletion mutants disrubted by *KanMX* module. Genotypes *MAT* **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 genexxx::KanMX*.

Measuring DNA concentration. Alternative product can be used.

Alternative product can be used.
DNA library preparation. Alternative product can be used.
DNA sequencing. Alternative product can be used.
Alternative product can be used.

Mix 10 g Dextrose, 10 g Yeast extract, 20 g Bacto peptone and add H2O to a total volume of 1000 ml and autoclave.

Genomic DNA was purified (Qiagen) from a pool of the yeast deletion library (Euroscarf). The DNA concentration was measured by nanodrop and 30 µg genomic DNA was pipetted into two micro centrifuge tubes. One micro centrifuge tube was supplemented with 100 nanogram plasmid (pUG6). The DNA samples were purified by Circle-Seq, omitting the protocol steps 1.1-1.3 and 1.5-1.7. The eluted DNA concentrations were measured by nanodrop and the entire DNA yield from sample GD and GD+P was treated with exonuclease for a period of 29 hours. A 10% fraction was collected for phi29-amplification and PCR analysis, while the remaining DNA was subjected to 72 hour exonuclease treatment. The samples were analyzed for linear DNA content by PCR, using the ACT1 gene as chromosomal marker. A 5% fraction of each of the exonuclease treated samples was amplified by the phi29 DNA polymerase for 16 hours (Qiagen). The presence of DNA in each sample was examined by loading an equal amount (7 µl) in wells on an 0.5 µg/ml ethidium-bromide 0.9% agarose gel after running gel-electrophoresis.
Ultrafast short read alignment. Reference: Langmead B, Salzberg S. Fast gapped-read alignment with Bowtie 2. Nature Methods. 2012, 9:357-359.

Images of propidium iodine stained DNA were captured by fluorescence microscopy at 100x magnification (100x/1.30 oil, Nikon) in the RFP channel (red excitation fluorescence filter, 663-738 nm) using identical exposition time (5 seconds).

A free web-based platform for data intensive biomedical research. References: Goecks, J, Nekrutenko, A, Taylor, J and The Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol. 2010 Aug 25;11(8):R86. Blankenberg D, Von Kuster G, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. "Galaxy: a web-based genome analysis tool for experimentalists". Current Protocols in Molecular Biology. 2010 Jan; Chapter 19:Unit 19.10.1-21. Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, Miller W, Kent WJ, Nekrutenko A. "Galaxy: a platform for interactive large-scale genome analysis." Genome Research. 2005 Oct; 15(10):1451-5.

Web Address

http://www.bd.com/ds/productCenter/211677.asp
http://www.genomics.agilent.com/en/SYBR-Master-Mixes/Brilliant-III-SYBR-MM/?cid=AG-PT-179&t
https://www.carlroth.com/de/de/Chemikalien/A-Z-Chemikalien/G/D%28%2B%29-Glucose/D%28%2B%
http://www.scientificindustries.com/disruptorbeads.html

https://www.carlroth.com/de/de/search?text=2218.2
https://www.thermofisher.com/order/catalog/product/K0502

https://www.lifetechnologies.com

http://www.aabiot.home.pl
http://www.epibio.com

http://www.sigmaaldrich.com
http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/Del_plas.html

https://www.qiagen.com

https://www.qiagen.com
http://www.bd.com/ds/productCenter/210929.asp
http://www.nordicbiosite.com

http://www.ebi.ac.uk/ena

http://www.sysbio.se/cenpk/
http://web.uni-frankfurt.de/fb15/mikro/euroscarf/complete.html

http://www.nanodrop.com

http://scopeoptic.biz/industrial-microscopes/nikon/

http://www.wafergen.com/products/apollo-324

http://www.illumina.com/systems/sequencing-platform-comparison.html
http://covarisinc.com/products/afa-tubes-and-vials/

http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

https://galaxyproject.org

abId=prod200005
%29-Glucose/p/000000090001046a00010023_de

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Author(s): Henrik D. Møller, Rasmus Bojsen, Chris Tachibana, Lance Parsons, David Botstein, Birgitte Regenberg.

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18/01/16

Dear Dr. Upponi

Thanks for the feedback.

All comments have now been addressed in a line-by-line response (text in blue) and manuscript edits are marked as tracked changes.

Editorial comments:

•NOTE: Please download this version of the Microsoft word document (File name: 54239_R1_112315) for any subsequent changes.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

The format was maintained.

•Audio issues

• The audio in this version sounds noticeably more compressed than the previous submission. If this was added intentionally, the compression should be removed. If it was done inadvertently due to an export setting, that should be corrected when the next version is exported.

The audio equalizer was altered to improve export of audio. We have made alternative sound versions that we will be happy to supply in case you prefer other settings.

•Commercialization

• 4:51- The shot of the kit insert should be cropped so that the brand name is not visible.

The shot of the isolation protocol was deleted.

• 8:25 - The soundbite naming Illumina HiC and the link to the illumina site should be removed.

The bite was deleted.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include

phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

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Missing DOIs are now included when available.

•***NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.***

A native English speaker has reviewed and edited the manuscript.

Reviewers' comments:

Reviewer #1: *Manuscript Summary:* Recommend for publication after minor revisions.

Major Concerns:

N/A

Minor Concerns:

In the video, the introduction about recombination is not really necessary, as well as showing the growth of cultures.

To maintain focus on the method we moved the model presentation to part 6 (representative results), where it serves as explanation for illegitimate homologous recombination and how some circular DNAs may form. We also made part 1 shorter by excluding video clips of initial growth and absorbance measurement.

It is not clear which type of microscope was used to visualize PI-stained DNA and at which settings/magnification.

The equipment for fluorescence microscopy is mentioned in Table S1 (Nikon

Optronics Magnafire). The method for PI-stain was added to Table S1:
"Images of propidium iodine stained DNA were captured by fluorescence microscopy at 100x magnification (100x/1.30 oil, Nikon) in the RFP channel (red excitation fluorescence filter, 663-738 nm) using identical exposition time (5 seconds)."
As well, to step 3.4.3.3) was added: "at 100x magnification" and to line 358 was added: "See Table S1 for extra details".

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The manuscript by Bojsen et al describes in detail a method to purify extrachromosomal circular DNA from yeast. The method uses glass bead dependent perforation of the S. cerevisiae cell wall, kit based DNA purification, limited digestion of chromosomal DNA, extensive digestion of linear DNA and rolling-circle based amplification of DNA, prior to sequencing. Although the method is designed for yeast it could be adopted to all eukaryotic cells. The method as such has been published, but the video supported detailed description will proof very useful to the scientific community, especially since the protocol describes the reasoning of the method and explains experimental pitfalls. The video is in general of high quality, but the sound quality is poor and should be improved. The video has a nice balance of content to detail and is nicely done. The illustrations are of good quality and help with the explanation. That said, I am not sure why the restriction enzyme digest was not listed in the overview? In summary, I fully support the publication of the video-manuscript.

We thank you for your comments. The sound quality was compressed at export and is improved in the present version of the manuscript. The restriction enzyme digest was not listed in the movie intentionally as this is an optional step in the protocol. We have however listed the restriction enzyme treatment in the written manuscript.

Major Concerns:

I do not have any major concerns with this publication.

Minor Concerns:

-Line113: It would be useful to mention that the 86 kilobase DNA is of mitochondrial origin.
We now mention 86 kilobase DNA is of mitochondrial origin in the introduction. It is

also stated in the discussion as well as in reference 19.

-Why did the authors choose to limit their circular DNA's to a size of >1kb?

The >1kb cut-off was basically a choice to focus on large circular DNAs that could include full genes. We have now explained this in the introduction: "A size cut-off was chosen to focus on eccDNA that are large enough to carry whole genes"

-Line 118: It is stated that the method is sensitive and can detect 1 circle in thousands of cells. Is it possible to estimate how many circle with any given sequence exist per cell?

Currently, the best estimation is obtained by adding control plasmids to a known number of cells. To estimate how many circles exist per cell is very difficult because the phi29 amplification is biased toward small and abundant circular DNA's. Also, we do not expect an even distribution of eccDNAs in cells, why we are reluctant to give an estimate.

-Line 129: yeast peptone (not pentose) dextrose. This has now been corrected.

-Line 130: the movie states 0.012 OD600. This clip was removed and we added "approximately" to line 134.

-Line 139: To spin down yeast at 420 g appears very low - needs checking. Thanks for noticing. The cells were pelleted at 2000 min^{-1} in a swing bucket Sorvall Heraeus Multifuge. radius 18 cm. Line 143+146 were changed to: "800 x g."

-Line 150: Is it useful to indicate the specific volume of plasmid stock mixture? What is the definition of sample?

A stock mix for 20 samples was made (155 µl volume) but we don't find this information important. The note on line 154-157 was revised: "NOTE: In the current dataset, a 7.7 µl plasmid mixture was applied for each sample containing 10^{10} cells. The plasmid stock mixture consisted of three plasmids in different concentrations; pBR322 at 38 ng/sample, pUC19 at 0.5 ng/sample, and pUG72 at 0.01 ng/sample."

-Line 181: Short term storage of DNA in water is OK, but acid hydrolysis limits the long term stability. Would 10 mM Tris pH 8.0 be better? Water was used to avoid potential problems with downstream enzymatic steps. It might work equally well with 10 mM Tris pH 8.0 but we have not tested that. After part 2.6 in the manuscript was added: "NOTE: Only short term storage of DNA in water is recommended. Preferentially, proceed directly to step 3."

-Line 186: Replace "Run" with Incubate. Corrected.

-Line 217: How much genomic DNA was used? Part 3.4.3.1) and 3.4.3.2) was revised to include template amount for control: "3.4.3.1) Use 2 µl exonuclease-treated sample as PCR template with *ACT1* primers 5'-TGGATTCTGGTATGTTCTAGC-3' and 5'-GAACGACGTGAGTAACACC-3'. As positive *ACT1* control, use 50-100 ng genomic *S. cerevisiae* DNA as template. PCR reaction conditions; 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 56°C and 1 min at 72°C.
3.4.3.2) Run PCR reactions by gel electrophoresis on 1% agarose with 0.5 µg/ml ethidium bromide. Look for a 0.8 kilobase *ACT1* band."

-Line 253: In the movie more info was given. Expand? We compared and did not find any crucial information left out from the text relative to the video.

-Line 362: Glass beads have been used to break the yeast cell wall, but they are also known to cause shearing of DNA. How was this balanced? The DNA is protected in the nuclei. In case of DNA shearing, we expect it will mainly target chromosomal DNA. The sheared DNA will facilitate digestion of linear DNA so we are not worried about using glass beads. In any case, we also suggest another disruption approach (line 166-167) by zymolyase, which should cause less DNA shearing than glass beads.

-Lines 362 to 365: It would be useful to compare the new method to the protocol from the Dutta lab and explain in which way the new method works different/better.
Dutta and her lab members tested their method on yeast and did found not circular microDNA from this organism. We speculate that the Dutta set-up might cause degradation of eccDNA or a methodological artifact, such as limited lysis of the yeast cells. Because the yeast protocol is not described in sufficient details, we cannot discuss differences between Duttas set-up and ours. A short sentence summarizing Duttas results on yeast has now been included in the introduction: "Dutta and coworkers also attempted purification of microDNAs from *Saccharomyces cerevisiae* but were unable to record microDNA from this yeast species[16]."

-The inclusion of the tables into the PDF is problematic. Sorry. It was an automated PDF conversion of the Table. It will be solved for the final production.

-Video part 3: The figure is labelled DNase, but it is described as Exo. The label was changed in the video.

-Video part 4: Associate "Professor" is spelled with only 1 f. This error has now been corrected.

Additional Comments to Authors:

N/A