**TITLE:**

**Circle-Seq: Extrachromosomal Circular DNA Purification from Yeast**

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**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 *φ*29 polymerase enriched for circular DNA over linear DNA. Validation of the Circle-Seq method on three *S. cerevisiae* CEN.PK populations of 1010 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 variation1,2. 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 micrographs3, Giemsa staining of metaphase chromosomes4, or two-dimensional gel electrophoresis5. These methods provide little or no information about the sequence of the circular DNA. Targeted techniques such as Southern blotting6,7, inverse PCR8, or fluorescence *in situ* hybridization9 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 arrays10,11. 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 population12,13. 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 line14. However, high-velocity can break or nick the backbone of supercoiled circular DNA structures, altering the sedimentation velocity15 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 cells16,17. 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 species16.

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 method18,19, 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 populations20. 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 cells20. 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 105 cells/ml or an optical density of approximately 0.01 OD600.

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 1010 cells, approximately after 24 to 48 hours or a optical density at OD600 > 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 elements20.

NOTE: In the current dataset, a 7.7 µl plasmid mixture was applied for each sample containing 1010 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 107 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 deleterious21-23 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 H2O-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 *φ*29-amplified genomic DNA from yeast and/or *φ*29-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 *φ*29 DNA polymerase24-26 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 *φ*29 DNA polymerase. Incubate the reaction at 30°C for 16 hours or more (up to 72 hours). Heat inactivate the *φ*29 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 system27,28 and short-read aligner mapping software29.

5.5) Identify reads from regions of putative eccDNAs using contiguous reads, for example, more than seven contiguous reads (>1 kb) without gaps20.

NOTE: Software is available27,28 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 1010 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 previously20 (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, [*HXT6/7circle*] was detected by Circle-Seq in ten S288c populations (each with 1 x 1010 cells) and the circular DNA structure was confirmed by inverse PCR analysis20. The [*HXT6/7circle*] 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 *φ*29polymerase 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 *φ*29*-*amplified sample with only genomic DNA revealed thread-like structures (**Fig. 5C**) similar to the control sample (**Fig. 5E**). The *φ*29*-*amplified sample that had added plasmid revealed foci (**Fig. 5D**), resembling the plasmid control (**Fig. 5F**). The images indicated that *φ*29 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 *φ*29*-*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 *φ*29DNA 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 1010 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 1010 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; [*rDNAcircle*] (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 476genes on 294eccDNA 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 reference20). 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 *φ*29treatment.** A-F, Propidium iodidestaining 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 *φ*29polymerase amplification (EXO 72 h + *φ*29). E, Genomic DNA control after *φ*29polymerase amplification; F, plasmid control (5.5 kb) after *φ*29polymerase 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 + *φ*29; GD and GD+P, after EXO 72 h + *φ*29 (sample as in C-D).See Table S1for 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 described20.

**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 described30. 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 required20. 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* S288c20. 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 [*GAP1circle*] is enriched under nitrogen limited conditions in the CEN.PK background8, though evidence of [*GAP1circle*] 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 [*HXT6/7circle*],[*ASP3-1circle*], [*COS111circle*], and [*CUP1-1 RSC30circle*] 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 *φ*29 DNA polymerase, which uses a rolling-circle amplification method31, 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 elements32-37. 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 variation38-40.

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**DISCLOSURES:**

The authors declare that they have no competing financial interests.

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