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Purification of Restriction Fragments Containing Replication Intermediates from Complex Genomes

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Abstract

In order to perform 2-D gel analyses on restriction fragments from higher eukaryotic genomes, it is necessary to remove most of the linear, non-replicating, fragments from the starting DNA preparation. This is because the replication intermediates in a single-copy locus constitute such a minute fraction of all of the restriction fragments in a standard DNA preparation - whether isolated from synchronized or asynchronous cultures. Furthermore, the very long linear DNA strands that characterize higher eukaryotic genomes are inordinately subject to branch migration and shear. We have developed a method that results in significant enrichment of replicating fragments that largely maintain their branched intermediates. The method depends upon two important factors: 1) replicating fragments in higher eukaryotic nuclei appear to be attached to the nuclear matrix in a supercoiled fashion, and 2) partially single-stranded fragments (e.g., those containing replication forks) are selectively adsorbed to BND-cellulose in high salt concentrations. By combining matrix-enrichment and BND-cellulose chromatography, it is possible to obtain preparations that are enriched 200–300-fold over the starting genomic DNA, and are thus suitable for analysis on 2-D gels.

Keywords

Replication intermediates; nuclear matrix; BND-cellulose chromatography; 2-D gel replicon mapping

1. Introduction

The neutral/neutral and neutral/alkaline two-dimensional (2-D) gel methods were originally introduced more than 20 years ago (1,2), and were utilized initially to examine the characteristics of origins of replication in *S. cerevisiae*. The starting material in each case was a preparation of yeast DNA from synchronized or asynchronous cultures prepared by standard CsCl banding techniques. Together, these two techniques for mapping origins and their corresponding replicons are tremendously powerful: among all the other methods for detecting origins of replication, they still afford the most detailed view of the replication intermediates inhabiting a given restriction fragment. However, the 2-D gel mapping methods were not readily applicable to replicons in the genomes of organisms more evolved than *Physarum* and *D. melanogaster* (e.g., 3,4). This limitation derived primarily from the great complexity of higher eukaryotic genomes, the much longer cell cycle times, and the resulting very low signal-to-noise ratio of replicating to non-replicating DNA. For example, the genome of *S. cerevisiae* is ~300-fold less complex than the genome of a mammalian cell. Therefore, correspondingly more mammalian DNA (most of it non-replicating linear fragments) would

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have to be loaded into the well of a 2-D gel in order to be able to detect the intermediates in a single-copy restriction fragment. Unfortunately, it is simply not possible to effectively separate this much DNA ~1.5 mg on a 2-D gel. Furthermore, the methods routinely used to isolate and purify genomic DNA had to be modified to prevent branch migration and shear, which is a major problem with the long linear chromosomal DNA that characterizes higher eukaryotic genomes. What was needed was a method for removing the vast excess of non-replicating DNA from the fragments containing replication intermediates. With sufficient numbers of starting cells, such an enrichment step therefore would make it possible to search for origins even in mammalian genomes.

Our laboratory developed such a method, which depends upon two older observations. In the first of these, it was shown that DNA is attached at ~100 kb intervals to a proteinaceous nuclear substructure or matrix (reviewed in 5), which renders the DNA less susceptible to both branch migration and shear. The general approach is to extract nuclei with buffers containing either high salt concentrations (6) or a detergent such as lithium diiodosalicylate (LIS; 7). This treatment removes soluble nuclear proteins, histones, and most of the non-histone proteins from DNA, leaving a residual nuclear matrix to which the genomic DNA is attached. This DNA “halo” is essentially protein-free and can be digested with an appropriate restriction enzyme while still attached to the matrix.

Importantly, it also was shown that >90% of restriction fragments containing replication forks preferentially associate with the 4–5% of DNA that remains when a matrix/DNA halo preparation is digested to completion with a six-mer restriction enzyme (8,9). Therefore, by isolating the matrix-attached DNA fraction, an initial 10–20-fold enrichment of replication intermediates is obtained. A second critical observation was that partially single-stranded DNA (such as in a replication fork) is selectively adsorbed to BND-cellulose in the presence of high salt, and can subsequently be eluted with a caffeine wash (10). In practice, this second step eliminates most of the remaining linear fragments from the matrix-attached DNA fraction described above, and affords an additional 5–10-fold enrichment of RIs over linear fragments (9). Together, these two steps constitute the enrichment scheme that has allowed analyses of single-copy loci in mammalian cells by 2-D gel replicon mapping techniques on a routine basis (e.g., 11–14).

Although 2-D gel analysis of mammalian replicons has largely been supplanted by the nascent strand abundance assay, which is somewhat easier and requires less starting material owing to the sensitivity of PCR amplification (15,16), we believe that 2-D gels still afford the most comprehensive view of origin behavior. An important limitation, however, is that it is extremely difficult to detect replication bubble arcs in neutral/neutral 2-D gels in replication intermediates isolated from *asynchronous* cultures of mammalian cells. This is because most mammalian origins are zones of inefficient sites, and the zones themselves are inefficient. Thus, a fragment from an initiation zone will usually be replicated passively from a start site in some neighboring fragment in the zone, resulting in a strong single fork arc and a very weak bubble arc that cannot be detected on film.

Therefore, the majority of our studies have been performed on cells synchronized at the G₁/S boundary, released into the S-period, and sampled at selected times thereafter. In the interest of describing the protocol from start to finish, we will detail the method of synchronizing and preparing matrices from Chinese hamster ovary (CHO) cells, which are grown in monolayers, and human lymphoid cells, which grow in suspension. These are the cells with which we have the most experience. However, the matrix enrichment method has been applied successfully to both Chinese and Syrian hamster cells (17), African Green Monkey cells (P.A. Dijkwel, unpublished), and human HeLa (L.D. Mesner, unpublished), lymphoblastoid (L.D. Mesner, unpublished), and immunoglobulin-producing, cells (14). For the latter cell types, we have not

had success in arresting the population in G₁ by serum or amino acid deprivation. Therefore, double thymidine blocks or a single thymidine block followed by arrest in mimosine was used to prepare cell populations arrested at the G₁/S boundary (described below for lymphoblastoid cells). For other cell types, arrest in mitosis with nocodazole followed by release into medium containing mimosine might be an option. For the preparation of origin libraries by trapping bubbles in agarose, we have routinely used asynchronous cultures, but then have assessed the efficacy of the procedure by 2-D gel analysis of DNA from synchronized cells (see Chapter __, Mesner and Hamlin). We also briefly describe the modifications we have made to the neutral/neutral 2-D gel method to accommodate the larger amounts of DNA loaded onto these gels compared to experiments with yeast DNA.

With attention to the detail provided below, it will be possible for anyone familiar with the preparation of minimally-sheared DNA from mammalian cells to perfect this enrichment technique on synchronized cells. Although we describe the general method for mammalian cells, it should be applicable, in theory, to other cultured cells (e.g., insect).

Materials

2.1. Cell culture and synchrony

1. Complete Minimal Essential Medium (complete MEM) supplemented with non-essential amino acids, 2 mM glutamine, 50 µg/ml Gentamicin, and 10% Fetal Clone II serum (Hyclone)
2. Starvation medium (isoleucine-free MEM) supplemented with non-essential amino acids, 2 mM glutamine, and 10% dialyzed fetal bovine serum (Hyclone)
- 3A. Complete MEM containing 200 µg/ml mimosine (Sigma Chem. Corp.)
- 3B. Complete MEM containing 2 mM thymidine (Sigma Chem. Corp.)
4. Serum-free MEM (2.1.1 above without serum)
5. 15 cm plastic culture dishes (Sarstadt)

2.2. Isolation of nuclei and matrix/halo structures

1A. *CHO cells*: For single-copy loci, eight 15-cm plates of synchronized cells at a density of $\sim 3 \times 10^7$ cells/dish ($\sim 2.4 \times 10^8$ total) for a single time point (e.g., early S-phase). These cells double in 18–20 hr; proportionately larger numbers of plates are required for cell lines with significantly longer cycle times, or when isolating intermediates from asynchronous cultures.

1B. *Human lymphoblastoid cells*: 500 ml of cells in spinner bottles or flasks at a cell density of $\sim 5 \times 10^5$ /ml. These cells double in 20–24 hr; more slowly growing cells may require larger volumes of culture, as will asynchronous cultures.

2. *Cell wash buffer* (CWB: 50 mM KCl, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.5% thiodiglycol, 0.1 mM phenyl methylsulfonyl fluoride, 5 mM Tris-HCl, pH 7.4)

3. *Cell lysis buffer* (CLB; CWB supplemented with 0.05% digitonin)

4. Plastic policemen for scraping cells from plates

5. 15 ml screw-cap polypropylene conical centrifuge tubes (Sarstedt)

5. 5 ml hypodermic syringes fitted with 21-gauge needles

6. 12.5% glycerol in CLB

7. 1.25X *stabilization buffer* (50 mM KCl, 0.625 ml CuSO₄, 0.05 mM spermine, 0.125 mM spermidine, 0.5% thioglycol, 0.05% digitonin, 0.1 mM PMSF, 5 mM Tris-HCl, pH 7.4)
8. *LIS buffer* (11 mM lithium diiodosalicylate, 110 mM lithium acetate, 0.05 mM spermine, 0.125 mM spermidine, 0.05% digitonin, 0.1 mM PMSF, 20 mM Hepes-KOH, pH 7.4)
9. 50 ml round polycarbonate centrifuge tubes (Nalgene)
10. Sorvall preparative centrifuge and HB-6 rotor (or equivalent)
11. *Matrix wash buffer* (MWB: 20 mM KCl, 70 mM NaCl, 10 mM MgCl₂, 20 mM Tris-HCl, pH 7.4)

2.3. Restriction enzyme digestion of matrix/halo structures and isolation of matrix-affixed DNA

1. Restriction enzyme and appropriate 10X and 1X buffer
2. DNase-free RNase (500 µg/ml; Roche)
3. 0.25 M EDTA
4. TEN (10 mM Tris-HCl, pH 7.4, 1.0 mM EDTA, 10 mM NaCl)
5. 5 M NaCl
6. *Proteinase K (PK) buffer* (1% sodium lauroyl sarkosine, 450 ml NaCl, 45 ml EDTA, 60 ml Tris-HCl, pH 7.4)
7. *PK stock solution* (20 mg/ml; AMRESCO)
8. *Dialysis buffer* (0.3 M NaCl, 2.5 mM EDTA, 10 mM Tris-HCl, pH 7.4)

2.4. Purification of replication intermediates on BND-cellulose

1. Scalpel blades
2. BND-cellulose (Sigma)
3. Disposable 2 ml columns (BioRad Polyprep)
4. *Wash buffer* (1 M NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4)
5. *Loading buffer* (0.3 M NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4)
6. *Caffeine wash buffer* (1.8% caffeine, 1 M NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4)
7. Absolute ethanol and isopropanol
8. 40 ml polypropylene screw-cap round-bottomed centrifuge tubes (Oak Ridge High-Speed Teflon; Nalgene)

2.5. Analyzing enriched material for purity

1. 20 µg of enriched replication intermediates (an aliquot of the material from Step 3.4.5 below)
2. *Electrophoresis buffer* (0.5XTBE: 89 mM Tris-HCl, pH 8.0/89 mM boric acid/2 mM EDTA)
3. *Loading dye solution* (35% Ficoll [Type 400], 0.5% Bromophenol blue, 40 mM Tris, 5 mM EDTA, 5 mM sodium acetate, pH 7.4)

4. Materials and equipment for running neutral/neutral 2-D gels as described in Chapter ___ (Brewer)

3. Methods

The method described here is for a single time point (usually early S-phase). It would be scaled up for multiple time points or for non-synchronized cells containing fewer replication intermediates.

3.1.A. Cell culture and synchrony: monolayer cultures (e.g., CHO, HeLa)

1. CHO cells are propagated as monolayers on 15 cm plates in MEM complete medium. Standard protocol is to plate cells at $\sim 5 \times 10^6$ /plate in the early afternoon, to feed them ~ 30 hr later, and to begin the synchronizing regimen 12–18 hr later.
2. Plating medium is replaced with prewarmed isoleucine-free MEM for 36 hr to induce G₀ arrest (equivalent to about twice the average cell cycle time for virtually all of our CHO cell lines).
3. Starvation medium is replaced with prewarmed MEM complete medium containing 200 μ M mimosine (a replication inhibitor; 18).
4. 12 hr later, when the population is arrested at the beginning of S-phase but prior to the establishment of active replication forks (18), the plates are rinsed once with prewarmed serum-free MEM, drained well, and released into warm drug-free complete medium to allow S-phase entry.
5. CHO cells are usually harvested for 2-D gel analysis 80, 160, 320, and sometimes 540 min later, which correspond to early-, early-mid-, mid-late, and late-S-phase, respectively. The peak initiation period for early-firing CHO origins such as DHFR or rhodopsin is between 80 and 90 min after mimosine removal, but this value will have to be established for each individual cell line in pilot pulse-labeling experiments with ³H-thymidine to determine when cells first enter S-phase, followed by a 2-D gel time-course analysis at 15 min intervals over a period that brackets the time of entry and the early part of S-phase.
6. The efficacy of the synchronization protocol is assessed by fluorescence-activated cell sorter (FACS) analysis of companion plates collected before and after starvation, after exposure to mimosine, and at the time that cells are harvested for analysis. If this can be performed soon after harvest, it can truncate experiments in which the cells are clearly not synchronized, thus saving valuable time and supplies.

3.1.B. Cell culture and synchrony: suspension cultures (e.g., human lymphoblastoid, HL60, etc., with cell cycle times of 18–24 hr)

1. Cells are seeded into MEM in 1 liter spinner bottles or in flasks at an appropriate density and are allowed to grow to a density of $\sim 2 \times 10^5$ /ml (a total of 500 ml for each time point). It might be necessary to pellet the cells and resuspend in fresh medium 8–10 hr prior to harvest in order to ensure that the population is genuinely asynchronous (determined by FACS analysis).
2. 500 ml of cells are collected by centrifugation in 40 ml conical polypropylene tubes at $\sim 600 \times g$ for 10 min, and are resuspended in 500 ml MEM complete medium containing 2 mM thymidine for 12–14 hr; this arrests S-phase cells and allows non-S-phase cells to reach the G₁/S boundary.

3. The high thymidine block is reversed by pelleting again and resuspending in fresh complete medium for 10–12 hr; this allows G₁/S and S-phase cells to move into G₂, M, and early G₁, but not to reach the next S-phase.
4. The medium is then replaced with an equal volume of fresh medium containing 200 μ M mimosine to collect them at the beginning of S-phase.
5. 13–14 hr later, cells are again centrifuged, washed once in pre-warmed serum-free MEM, and finally suspended in warm drug-free complete medium to allow entry into the S-phase.
6. Replicate cultures are then harvested for 2-D gel analysis in early S-phase and at appropriate times thereafter in a time-course study (go to Step 3.2.B below).
7. The efficacy of the synchronization protocol is assessed by FACS analysis of aliquots ($\sim 5 \times 10^6$ cells) collected before and after thymidine block, after the 10–12 hr incubation in fresh medium, after 13–14 hr in mimosine, and 2–3 hr after release into complete medium in order to assess the efficacy of each block and whether most cells enter S-phase relatively synchronously.

3.2.A. Isolation of nuclei and matrix/halo structures from monolayer cultures

1. For neutral/neutral 2-D gel analysis of single copy loci in CHO or HeLa cells, $\sim 2.5 \times 10^8$ cells are required per gel (eight 15-cm dishes at a density of $\sim 3 \times 10^7$ cells/dish). The method for preparing matrices is performed entirely in the cold room through Step 3.2.A.5 below.
2. Culture dishes are washed twice with 50 ml CWB. The plates are drained well, divided into pairs, and 5 ml of cold CLB is added to one plate of each pair. The cells are scraped from the first plate with a plastic policeman, transferred to the second plate, and again scraped off.
3. Cell suspensions are forcefully drawn into and out of a 21-gauge hypodermic needle three times to effect cell lysis and liberate the cell nuclei. Each pair of plates ($\sim 6 \times 10^7$ cells) is rinsed with 5 ml of CLB, this wash is forced through the needle three times, and is added to the first 5 ml suspension. Nuclei are monitored by phase contrast microscopy, and should be smooth-surfaced, gray, and not clumped. You should now have four tubes.
4. The 10 ml suspension from each pair of plates ($\sim 6 \times 10^7$ nuclei) is layered over 4 ml 12.5% glycerol in cell lysis buffer (CLB) in 15-ml conical plastic tubes. The nuclei are pelleted in a clinical benchtop centrifuge ($\sim 600 \times g$) for 15 min, and CLB and the glycerol pad are removed by aspiration.
5. Nuclei are resuspended by forcefully ejecting 5 ml of CLB into each of the four pellets from a 5 ml syringe fitted with a 21-gauge needle and forcing the suspension into and out of the needle once. Two 5 ml suspensions ($\sim 1.2 \times 10^8$ nuclei) are combined into one 15-ml tube and the nuclei are pelleted again by centrifugation at $600 \times g$ for 5 min. The supernates are removed by aspiration. Each of the two nuclear pellets are resuspended by forcefully ejecting 2 ml CLB into the tube.
6. Each of the two suspensions is drawn up into a syringe through a 21-gauge needle and ejected into 8 ml of 1.25X stabilization buffer. The tube is then placed on ice for 20 min, after which each suspension is drawn into the syringe through the 21-gauge needle and ejected into 90 ml LIS buffer at *room temperature*. You should now have two 100 ml suspensions for each sample.

7. After 5–10 min to allow extraction of histones and other soluble nuclear proteins, the suspensions are transferred by distributing into four 50 ml round-bottom polycarbonate centrifuge tubes (note: the matrix pellet does not adhere sufficiently to polyethylene or polypropylene).
8. Nuclear matrix/halos are pelleted at 4,000 RPM (2600×g) for 20 min at 4° C in an HB-6 rotor in the Sorvall. The supernatant fluids are decanted and the pellets dislodged by the forceful ejection of 3 ml of cold MWB into each tube. The tubes are filled to the top with MWB and matrices are repelleted for 5 min. You should now have four tubes per sample.
9. The pellets are dislodged as above, 5 ml of 1X cold restriction enzyme buffer is added and swirled to release the cottony pellet from the tube bottom, and the pellet is washed by filling the tube with 1X buffer. After centrifugation at 4,000 RPM (2600×g) for 5 min, supernates are decanted, the 1X buffer wash is repeated, yielding material ready for restriction enzyme digestion (Step 3.3).

3.2.B. Isolation of nuclei and matrix/halo structures from suspension cultures

1. For suspension cultures at $\sim 5 \times 10^5$ cells/ml, make sure pellet from Step 3.1.B.6 above is uniformly suspended ($\sim 2.5 \times 10^8$ cells total) in the small amount of fluid remaining after the medium is decanted; then gently swirl tube while slowly adding 4 vol CWB. Centrifuge at 600×g for 10 min and aspirate 95% of supernatant fluid. Resuspend pellet in remaining CWB by gently flicking the tube bottom.
2. Gradually add 20 vol CWB while gently swirling the cell suspension, and pellet cells.
3. Remove supernate by aspiration and resuspend pellet by the gradual addition of CLB to a final cell density of $\sim 6 \times 10^6$ /ml.
4. Pass cells through a 21-gauge needle three times and continue as outlined above for monolayer cells (Step 3.2.A.3 above).

3.3. Digestion of matrices with restriction enzyme and isolation of matrix-affixed DNA

1. Three ml 1X restriction buffer are added to each of the four matrix pellets ($\sim 6 \times 10^7$ cell equivalents per pellet), and the pellets are broken up into relatively large clumps by trituration through the cut micropipette tip (~ 2 mm bore) using a P-1000 pipettor. The volumes are adjusted to 10 ml each, $\sim 1,500$ units of the appropriate restriction enzyme are added to each tube, and digestion is allowed to proceed for 30–60 min at 37 °C. During this incubation, the clumps should disintegrate and the suspensions should start to become cloudy.
2. After 30–60 min, two digests are combined (two digests total) and the matrices are collected by centrifugation at 4 °C in a Sorvall HB-6 rotor for 10 min at 4,000 rpm (2,600×g). The supernate fluid in each, which contains the DNA loop fraction, is decanted into a flask and placed at 37 °C to ensure complete digestion of the DNA until it is combined with the supernatant from the second digestion (see below).
3. The matrix pellets ($\sim 1.2 \times 10^8$ cell equivalents/pellet) are each resuspended in 10 ml fresh restriction buffer by trituration through the cut tip (~ 1 mm bore) of a P-1000. This can be done relatively vigorously since the DNA should be digested to a considerable extent at this stage. An additional 1,500 units of enzyme are added to each of the two tubes, which are then incubated at 37 °C for 15 min.
4. RNaseA is added to each tube (2.5 µg/ml) and the incubation is continued for another 15–45 min before it is terminated by addition of 1 ml 0.25 M EDTA per tube. Two

reactions are combined into one tube, and matrices are pelleted by centrifugation in the Sorvall HB-6 rotor for 10 min at 5,000 rpm (4,000×g) and 4 °.

5. The supernatant is added to the supernatant of the first digestion, and a 10 ml aliquot of this loop fraction is precipitated with two volumes of room temperature ethanol. The DNA is collected by centrifugation (60 min at 10,000 rpm [16,000×g] in the Sorvall HB-6 rotor), washed with 15 ml 70% ethanol, and dissolved in 500 µl of TEN.
6. The matrix pellet is resuspended in 1.4 ml *dialysis buffer* by trituration through the cut tip of a P-1000, after which 4 ml of *PK buffer* and 0.6 ml of the *PK stock solution* are added (total volume of ~6 ml). Digestion is allowed to proceed for a minimum of 3 hr at room temperature, after which the sample is dialyzed overnight twice against 5 liters of 4 °C *dialysis buffer* (10 liters total).

3.4. Purification of replication intermediates on BND-cellulose

1. With a scalpel blade, the clumps of BND-cellulose (Sigma) are first reduced to a fine powder, and are then wetted with *loading buffer* overnight (e.g., ~5 grams are wetted for an experiment with four time points). The suspension is loaded into a disposable 2 ml Polyprep column to a final bed volume of 2.0 ml. The column is subsequently conditioned with 10 ml *wash buffer*, and equilibrated with 6 ml of *loading buffer*.
2. The dialyzed matrix-associated DNA from Step 3.3.6 above is clarified by centrifugation in the HB-6 rotor at 5,000 RPM (~4,000×g) for 10 min at 4 °C. The supernate is loaded onto the column by gravity, and the non-replicating DNA is eluted by gravity flow with 10 ml *wash buffer* at room temperature.
3. Replication intermediates are recovered from the column by elution with 7 ml *caffeine wash*. Occasionally, the loop fraction is also fractionated over BND-cellulose to determine its content of replicating DNA by subsequent 2D gel analysis. Generally, less than 20% of the replication intermediates are found in this fraction (predominantly forks).
4. Both the salt and caffeine washes are precipitated with 2 volumes absolute ethanol (or with one volume of isopropanol if the volume is large) in 40 ml polypropylene tubes, and the DNA of both fractions is collected by centrifugation (10,000 rpm [16,000×g] for 1 h at 4 °C in a Sorvall HB-6 rotor). The precipitates are resuspended in 500 µl of ice-cold TEN, 30 µl 5 M NaCl are added after 10 min, and the contents are transferred to a 1.8 ml microfuge tube; 1 ml of room temperature absolute ethanol is added, the contents are mixed, and the DNA is collected by centrifugation.
5. The pellets are dissolved in 100 µl TEN. Of this solution, 2 µl is used to determine the DNA concentration, using a fluorimeter after staining with Hoechst 33258 dye (19). The remainder of the caffeine wash and an equivalent amount of the salt wash are then applied to the agarose gels.

3.5. Analyzing preparations for purity on 2-D gels (as in Chapter __ [Brewer], with minor modifications; 17)

1. For the first dimension, a 400 ml 0.3–0.4% agarose gel (depending upon sizes of fragments of interest; see Table 1) is cast in a 20×25 cm tray using a 17-well comb (well capacity ~120 µl).
2. After solidification at 4 °C, the comb is removed, and the gel is placed in the electrophoresis tank, which is partially filled with *electrophoresis buffer*; the buffer meniscus should be level with the top of the gel; avoid submerging gel at this stage).

3. The samples, as well as a 1-kb ladder (BRL), are mixed with 0.1 volumes of *loading dye solution*, are loaded into the wells, and the gel is run at room temperature for 6 hr at 0.5 V/cm.
4. Enough *electrophoresis buffer* is then added to completely submerge the gel and electrophoresis is continued for an appropriate time (see Table 1).
5. The gel is stained with ethidium bromide (0.1 µg/ml in 0.5XTBE) for 60 min and the lanes containing the samples are neatly excised with a scalpel, using a ruler as guide between lanes. The lanes are trimmed to a length that spans the fragment sizes of interest (e.g., a strip migrating between the 2–20 kb markers should encompass all of the replication intermediates in fragments 3–7 in length and will include the entire 1n spot). Each agarose strip is then rotated 90° and two 10-cm strips are placed at the top of a 20×25 cm electrophoresis tray.
6. For the *second dimension*, 400 ml of a 0.6–1.6% agarose solution in 0.5XTBE supplemented with 0.1 µg/ml ethidium bromide is prepared, cooled to 50 °C, and poured into the tray. The actual percentage of agarose will depend upon the size(s) of the fragments that will be queried (see Table 1).
7. After solidification, the gel is run at an appropriate voltage at 4 °C in 0.5XTBE containing 0.1 µg/ml ethidium (see Table 1 for conditions).
8. The DNA is transferred to a positively charged nylon membrane (Hybond N+ or Zetaprobe; BioRad) using an alkaline transfer method (20). The membrane is hybridized successively with the appropriate radiolabeled probes and replication intermediates in the restriction fragments of interest are revealed by autoradiography.

4.0 Notes

1. Unless the region being analyzed is amplified (such as the DHFR and rDNA loci; 21,22), it will not be possible to detect replication bubble arcs in asynchronous cultures.
2. While minimally transformed hamster and murine cell lines can be arrested in G₀ by isoleucine or serum deprivation, respectively, most human cell types cannot be. We described a double block method for synchronizing a human lymphoblastoid cell line in suspension culture, which can be applied to HeLa cells and many other cells growing on plates. An alternative possibility is to collect cells in mitosis with nocodazole followed by release into mimosine. Finally, centrifugal elutriation based on cell size has been used with great success for some cell lines to sort them into different S-phase compartments (23). Great attention to the synchronizing regimen up front will yield more reliable results from the 2-D gels themselves.
3. Since cultures will normally be synchronized, it is important to make sure that the cells are well-fed and cycling prior to application of the first synchronizing regimen, be it deprivation of isoleucine or serum, or administration of a blocking agent such as high concentrations of thymidine. It also seems that cells that spend too much time attached to plates at relatively high densities secrete unknown substance that can confound subsequent attempts to isolate clean matrix preparations.
4. Drain plates well when changing media in the cell synchronization protocols.
5. All buffers are freshly-prepared, and electrophoresis tanks must be scrupulously clean.
6. It is truly important to remember that the product you are attempting to purify (replication intermediates) are fragile branched structures that will be easily sheared

and/or destroyed by branch migration. Therefore, handle with care and store DNA samples at 4 °C when not manipulating during the steps described above.

7. When cut pipettor tips are utilized to triturate or transfer samples, the resulting bore should be 2 mm or more in diameter.
8. More than 80% of replication intermediates should be recovered in the matrix DNA fraction. The primary cause of loss of replicating DNA from this fraction is disintegration of the matrices themselves. Analysis of different cell lines will probably require changes of either the nuclear isolation procedure or of the stabilization step or both. In some cases, a 23-gauge needle might be required to efficiently remove cytoplasmic contaminants from the nuclei.
9. The amount of restriction enzyme required to release the DNA loops will vary depending on the enzyme used; a third digestion of matrix-associated DNA may be required.
10. Use a long wavelength UV light box and minimize exposure when excising the sample-containing lane of the first-dimension gel. Alternatively, the lane can be excised blindly (i.e. without visualizing the DNA).

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Table 1

Conditions for 2-D gel separations for different fragment sizes

For analysis of fragments in the indicated size range, the table indicates the percentages of agarose, the voltages, and run times for both the first and second dimension gels.

Fragment size	1st Dimension		2nd Dimension	
	Agarose	Voltage	Agarose	Voltage
1–2.5 kb	1.45%	0.5V/cm	1.60%	4.0V/cm
3–6 kb	0.40%	0.5V/cm	1.00%	3.0V/cm
7–10 kb	0.35%	0.5V/cm	0.85%	2.5V/cm
10–18 kb	0.30%	0.5V/cm	0.60%	1.5V/cm