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Mapping Replication Origin Sequences in Eukaryotic Chromosomes

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Abstract

Recent advances in genome sequencing technology have led towards the complete mapping of DNA replication initiation sites in the human genome. This thorough origin mapping facilitates the understanding of the relationship between replication initiation events, transcription and chromatin modifications and allows the characterization of consensus sequences of potential replication origins. This unit provides a detailed protocol for isolation and sequence analyses of nascent DNA strands. Two variations of the protocol based on non-overlapping assumptions are described below, addressing potential bias issues for whole genome analyses.

Keywords

DNA replication; Replication Origins; Next Generation Sequencing (NGS)

INTRODUCTION

DNA replication initiates at distinct genomic sites called origins of replication (or replication initiation sites). Replication forks, which originate from replication origins, are symmetric and bidirectional and present a different mode of DNA synthesis according to the DNA strand. The enzyme that synthesizes DNA, DNA polymerase, starts DNA synthesis with an RNA primer at the 5' end of the newly replicated DNA strand. At the 5' to 3' direction, termed the leading strand, DNA polymerase will synthesize DNA in a continuous manner from a single RNA primer; on the 3' to 5' direction, termed the lagging strand, DNA is synthesized in a discontinuous way from 100-200 nucleotides long RNA-primed DNA fragments called Okazaki fragments. Newly replicated (nascent) DNA strands are synthesized symmetrically and bi-directionally from the origins of replication. Short Nascent DNA Strands (SNS) are thus expected to straddle and center on replication origins and to

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correspond to genomic regions where DNA replication initiates. Isolation of SNS, therefore, allows identifying the position of replication origins.

A detailed discussion of the experimental approaches utilized in the last few decades to map replication initiation sites is provided in the accompanying Overview (Best Practices For Mapping Replication Origins In Eukaryotic Chromosomes). As described, massively parallel sequencing of isolated SNS was recently used to map the complete profile of replication initiation sites in several mammalian cell lines (Besnard et al, 2012; Martin et al, 2011). Two protocols for purification of nascent strands are described below. Both protocols select single-stranded DNA fragments of 0.5–2.5 kb size derived from replicating cells and involve size fractionation as a first step. Further purification steps of these short newly replicated DNA follow, utilizing two independent properties.

The first method exploits the requirement of a short RNA primer by the DNA polymerase to initiate the DNA synthesis. The short nascent strands, which are primed by an RNA primer, are exposed to lambda exonuclease, which selectively digests DNA without RNA primers from the 5' end and only leaves RNA-primed DNA, corresponding to nascent DNA strands. Thus digestion with lambda exonuclease eliminates contaminating broken DNA and leaves newly replicated DNA intact to create a population of purified SNS (Bielinsky & Gerbi, 1998). Finally, selection of 0.5-2.5 kb SNS allows to keep only the short nascent leading-strand DNA located adjacent to the replication origins and exclude Okazaki fragments that are located on lagging strand throughout the genome.

The second method consists in labeling the replicating DNA with a synthetic nucleoside, BrdU (5-bromo-2'-deoxyuridine), which incorporates into the replicating genomic DNA. Short genomic DNA fragments are isolated and size fractionated from replicating cells that have been pulse-labeled with BrdU, followed by immunoprecipitation with BrdU-specific antibodies.

The two variant techniques result in a population of enriched short, newly replicated DNA fragments or SNS corresponding to origins of replication. Sequencing these populations of DNA fragments involves the construction of libraries and their sequencing followed by their alignment of the resulting sequences to the genome (Besnard et al, 2012; Martin et al, 2011) providing a detailed profile of replication initiation sites throughout the non-repetitive genome. Both protocols are described below starting by the lambda exonuclease-mediated (or exonuclease-facilitated) enrichment of Short Nascent DNA Strands (SNS) and followed by the alternative protocol utilizing BrdU incorporation.

GENERAL CONSIDERATIONS

SNS sequencing is the most commonly used method to map replication origins genome-wide. SNS were successfully obtained and sequenced from human tissue culture cell lines (Besnard et al, 2012; Martin et al, 2011) and primary erythroblasts (Mukhopadhyay et al, 2014). Ideally, $1-3 \times 10^8$ cells cultured in mid-logarithmic growth phase are harvested for an analysis. Although no sequencing with other primary tissues was yet reported, successful SNS abundance analyses were also reported in primary cells from excised tissues (Cleary et al, 2010).

While the two alternative SNS protocols rely on non-overlapping assumption for isolation of newly replicated DNA, our experience suggests that they yield similar results. Since each method might be subject to different experimental limitations, ideally it would be advisable to utilize both methods. Each of the two methods necessitates attention to different critical experimental issues. For example, insuring complete digestion by lambda exonuclease is critical for the exonuclease-facilitated SNS enrichment; incomplete digestion with lambda exonuclease might leave some undigested contaminating DNA. For the BrdU-incorporation method, BrdU will incorporate at the leading strand and the lagging strand at replication origins as well as nascent DNA located in regions distant from the replication origins as inter-origin distance in human cells is in average 100-150kb. Size fractionation and avoidance of DNA breakage, therefore, is critical. It should be noted, however, that in some cases isolation of BrdU-labeled nascent strands might be a challenge. Indeed, tissues in live animal might not be amenable to efficient live labeling and some tissue culture cells might lower levels of nucleotide incorporation (most likely reflecting variations in nucleotide pools).

BASIC PROTOCOL 1

EXONUCLEASE-FACILITATED SNS ABUNDANCE ASSAY

This assay isolates nascent DNA fragment derived from replicating leading strands based on size ranging from 0.5 to 2.5 kb and the presence of an RNA primer. Since DNA replication of the lagging strand is discontinuous and each new fragment contains an RNA primer, smaller DNA strands should be avoided to prevent contamination with Okazaki fragments. The procedure below utilizes asynchronously growing, unperturbed cultured cells.

Materials

2X SDS lysis buffer (see recipe)

DNAzol reagent (Life Technologies, 10503-027)

Phosphate-Buffer Saline (PBS)

Phenol:Chloroform:Isoamyl alcohol 25:24:1 (Phenol/Chloroform)

3M Sodium Acetate pH=5.2

2M Sodium Chloride

100% Ethanol (EtOH)

TE buffer (see recipe)

RNasin ribonuclease inhibitor (Promega)

TNE buffer (see recipe)

5% Sucrose (see recipe)

30% Sucrose (see recipe)

2.5X lambda exonuclease buffer (see recipe)

Day 1: Genomic DNA extraction from cultured cells

Cell harvest: If possible, about $1-3 \times 10^8$ cells should be harvested during the logarithmic growth (24- 48 hours after seeding) to optimize the fraction of cells in S phase. Two lysis processes can be used. The first one uses the 2X SDS buffer and the second one uses DNazol, the two methods are described in the part A and in the part B.

Cell lysis

• In the case of cultures growing in suspension

1. Spin down the cells at 1000 rpm for 5 min.
2. Wash the cell pellet with PBS twice.

• In the case of adherent cells

1. Remove media. Wash the cells with PBS once. Add trypsin to detach the cells from dishes. Harvest cells with media. Spin down the cells at 1000 rpm for 5 min.
2. Wash the cells twice with PBS twice.
3. Resuspend cell pellets at minimal volume of PBS and lyse the cells by adding 15 ml 2x SDS. (Care should be taken to insure that cell lysis is complete and to avoid breaking DNA).
4. Add Proteinase K to 200 µg/ml. Incubate at 37°C O/N or at 50°C for 4 hours. (Samples can be kept at -80°C until ready for the next step.)
5. Extract DNA with Phenol/Chloroform (pH8.0), then with Chloroform once. For each step, rotate the samples with phenol or chloroform for 30 minutes, then centrifuge for 10 minutes. Use wide open pipette tips to transfer DNA to new tubes or remove Phenol/Chloroform from the bottom to avoid breaking DNA.
6. Add 2 volumes ethanol (R.T.) and 0.1 volume 3M Sodium Acetate to precipitate the DNA. Centrifuge at 8000 rpm for 1h or 12000 rpm for 20 min; wash the pellet with 5 ml 70% Ethanol; Centrifuge again; air dry the pellet.
7. Dissolve the DNA pellet in 1 ml DEPC water. Keep the samples in a refrigerator for a few hours to overnight. For long time storage, keep at -80°C.
8. An aliquot should be taken at this point corresponding to the genomic DNA (gDNA) control sample: take 80 µl and keep the gDNA sample at 4°C.
3. Add 12 ml DNazol for 1.2×10^8 cells. Once DNazol is added, resuspend cells very gently by tapping the tube. Do not pipet up and down, as it may break nascent strands.
4. Transfer the resuspended cells to a *Dounce* Homogenizer. Use the loose pestle to homogenize the cells. Make 5-6 movements at the most by turning the pestle

down the dounce to scratch the glass side of the dounce. Split the DNazol containing the homogenized cells into 1.5 ml Eppendorf with a 10 ml pipette (1ml per tube).

- 5 Tubes should be centrifuged: 15 min - 16400 rcf - 4°C, remove the supernatant carefully without disturbing the pellet (containing proteins and RNA), and transfer the supernatant in a 50ml falcon tube.
- 6 Add half the final sample volume of 100% EtOH to the falcon tube (i.e. add 6ml of 100% EtOH to 12mL of sample). Samples can then be kept if necessary at -20°C. Mix by inverting the tube slowly. Leave it at R.T. for 1-3 minutes. Genomic DNA can be isolated by using a p200 tip on a 1000 ml filter tip and turning the 200µL tip on the top of the liquid. Alternatively use a Pasteur pipette with a closed and smoothed extremity to catch the genomic DNA pellet, then to rinse it twice in different 75 % ethanol solutions and to dry well for 15 min.
- 7 Once dry, the pellet should be dissolved in 975 µl TE, 25 µl of RNasin ribonuclease inhibitor at 40 U/µl (RNasin 1U/ml final). Sample should be left at least 2h at 4°C in order to be completely dissolved.
- 8 An aliquot should be taken at this point corresponding to the genomic DNA (gDNA) control sample: take 80 µl and keep the gDNA sample at 4°C.

Day 2 : Separation of DNA fragments according to their size by sucrose gradient centrifugation

1. Use DNA extracted by either procedure on Day 1.
2. Prepare a 5-30% sucrose gradient by a gradient maker. If using a gradient master, use as a parameter SW28 – Long, 5%-30%, sucrose, Time: 1 min 18°C , angle 74°, speed : 24. Using a 25 ml pipette, add 18 ml of 5 % sucrose solution in the Beckman SW28 tubes. Using a syringe, add slowly 17 ml of 30 % sucrose solution underneath of the 5 % sucrose. Place the tube in the gradient master.
3. Heat the DNA sample to 95°C for 5-10 min; rapidly cool down on ice for 5-10 min.
4. Add the sample slowly on the top of the sucrose gradient. Use a wide-open pipette tip to apply sample slowly onto a 5% to 30% sucrose gradient. Apply 0.2 ml of 1 kb DNA ladder to a second gradient as a control.
5. Centrifuge tubes containing the sample should be balanced two by two by adding 5% sucrose solution.
6. Centrifugation at 4°C for 17h at 26000 rpm with a SW28 rotor, 26700 rpm with a SW32i rotor, or 18000 rpm with an SW40 swing bucket rotor.
7. Prepare a large 1.2-1.5% agarose gel (without Ethidium Bromide or other intercalating agent) to check the size of the fractions of DNA samples that will be harvested the next day. This gel can be prepared in denaturing or neutral conditions. Prepare the power supply and keep it in the cold room until use.

- For **denaturing condition**, prepare a 1.2% agarose gel in H₂O; prepare a denaturing buffer containing 50 mM NaOH and 1 mM EDTA in H₂O. Keep the gel in the denaturing buffer at 4°C in the electrophoresis chamber.

- For **non denaturing condition**, prepare a large 1.5% agarose gel in 1X TBE (20 cm X 25 cm, make 350 ml 1.5% agarose and 2L 1X TBE. Non denaturing gel can be prepared before use).

-

Day 3 : Selection of gradient fractions containing the SNS

- 8 At the end of the centrifugation, remove the tubes carefully from the centrifuge without disturbing the newly formed sucrose gradient. 36 fractions of 1ml in total will be successively harvested from SW28 tube gradients starting from the top of the tube (fraction 1), by pipetting 1ml by 1ml very carefully using a pipetman. Short Nascent Strands should have migrated between fractions 5 and 28. The fractions 1-4 will be pooled in a 15 ml tube. The fractions 5-28 will be harvested in individual labeled microtubes. The fractions 29 to 36 will be pooled in a second 15 ml tube. All the fractions should be kept at 4°C.
- 9 Migration of an aliquot of each fraction between 5 and 28 on the agarose gel prepared as described in step 7 allows checking the size of the collected DNA fragments. From our experience, the SNS should have migrated in fractions between 16 and 23. As SNS are not very abundant, fractions can be pooled to concentrate the samples in SNS for further experiments (i.e., fractions 16 and 17 can be pooled together in a new falcon tube, similarly for 18 and 19, 20 and 21, 22 and 23).
- 10 For two pooled fractions containing 0.5-2.5kb SNS, add in the following order (volumes are given for 2ml samples corresponding to 2 pooled fractions):

- 600 µl (1/10 of total volume)	3M Sodium Acetate
- 2 µl	glycogene at 20mg/ml
- 4 mL (2 times the initial fraction volume)	100% ethanol.

Invert the tube slowly. Then split the mix in 4 low retention tubes to limit losing SNS samples by distributing 1ml per tube.

- 11 Keep the tubes for at least 3 or 4 hours to overnight at -20°C.

Agarose gel

- **For denaturing condition:** Load in each well 10 µl of each fraction and 2 µl of alkaline loading buffer (see solution), and on each side of the gel, 10 µl of Molecular Weight ladder (see solution).

Gels should migrate overnight at 4°C and 20 Volts.

Gel should be taken out of the cold room and should be moved into a new tray containing 0.5X TBE with diluted GelRed Nucleic Acid Gel Stain (10 000 X). Stain the gel for 30 min with slow agitation (example of a gel typically obtained, see **Figure 1**)

A smear should be seen in each of the sample taken out after the sucrose gradient. Smear below 500 bp correspond to Okazaki fragments, very short nascent leading-strand and/or degraded DNA. Only the fractions or samples showing a smear with a size range between 500 and 2500 bp, contain the short nascent strands. These fractions also contain broken DNA fragments that will be digested by the lambda exonuclease in a further step.

- ***For non denaturing condition:*** Load 30 μ l of every other fraction on the gel. Also load 5 μ l of a 1kb and 100 bp DNA ladders. Run at 200 V for 1.5 to 2 hours (see **Figure 2** for a typical gel).

Day 4-5 : T4 kinase treatment and lambda exonuclease digestion to further enrich SNS

Sample treatment with polynucleotide kinase: As lambda-exonuclease preferentially digests double-strand as well as single-strand DNA fragments that are phosphorylated, the fractions will be treated with the polynucleotide kinase to phosphorylate the contaminating DNA.

- 12 After having stored the samples for at least for 3-4 h at -20°C (step 10), centrifuge them at maximum speed (25000 rcf) for 20 min at 4°C .
- 13 Remove the supernatant carefully (using a p200 tip on a 1000 ml filter tip without disturbing the pellet). Add 150 μ l 75% EtOH to the pellet. Centrifuge at maximum speed (25000 rcf) for 5 min at 4°C .
- 14 Remove supernatant and leave the tube to air dry at RT for 15 min.
- 15 **NOTE:** A parallel test of exonuclease efficiency using a plasmid control can be included. (See **SUPPORT PROTOCOL 2**). For each sample previously split into 4 eppendorf tubes (step 10), add 25 μ l of the following mix per tube:
 - 2.5 μ l 10x **polynucleotide kinase** buffer
 - 22.5 μ l H_2O
- 16 Leave the tube 15 min at R.T. in order to resuspend the DNA.
- 17 Each set of four tubes should be pooled together for a total volume of 100 μ l.
- 18 Sample should be heated at 95°C for 5 minutes and cooled on ice for 5 min.
- 19 Add the following mix:
 - 10 μ l 10X PNK buffer.
 - 10 μ l of PNKinase (10u/ml final)
 - 2 μ l 100mM ATP
 - 1 μ l RNase inhibitor (40u/ml final)

- 77 μ l H₂O pipet up and down and incubate 30 min at 37°C

20 Add the following mix :

- 5 μ l 5% sarkosyl

- 5 μ l 0.5M EDTA

- 2 μ l Proteinase K (25mg/ml) Incubate 30 min at 50°C

Sample treatment with lambda exonuclease

21 Centrifuge the tubes quickly and proceed to the phenol chloroform extraction under a chemical hood (see *SUPPORT PROTOCOL 1* for details). Resuspend the DNA pellet with:

- 38 μ l of H₂O,

- 32 μ l lambda-exonuclease buffer 2.5X

22 Samples can be kept at 4°C overnight or 5-10 min at RT.

23 Incubate samples 5 min at 95°C and immediately put the tubes on ice for 20 min.

24 Add 6 μ l Lambda-exonuclease (50U/ml final) and 2 μ l RNase inhibitor (40U/ml final). Pipet up and down gently to homogenize. Transfer the mix to RNase-free PCR tubes.

25 Incubation the tubes in a PCR machine (at 37°C for 12h then 75°C for 10min and 4°C until purification).

26 Proceed to a phenol-chloroform extraction (see *SUPPORT PROTOCOL 1*) and resuspend DNA in 100 μ l H₂O.

Day 6 : Quality control of the SNS samples using q-PCR and second strand synthesis

Quality control of the SNS samples

27 PCR reactions

- 12 μ l of master mix (4.5 μ l H₂O and 7.5 μ l SYBR green)

- 1 μ l of 10 μ M of Forward and Reverse primers (see the primers sequence above)

- 2 μ l sample (diluted at 1/5 with H₂O)

Standard Curve preparation: Genomic DNA of the studied cells is used as a control for SNS abundance, and should be fragmented – for example, using a bioruptor. Genomic DNA (from the aliquot saved at Day 1, step 8 of the SDS lysis protocol or step 8 of the DNazol protocol) in 0.5 ml tube (low retention tubes) should be sonicated in a volume of 100 μ l at 250 ng/ μ l with 3 pulses of 30 sec (1 min between each) at medium intensity. The range of size of fragmented genomic DNA should be checked by gel migration and be between 500-2500 bp (as the SNS size).

A typical Standard curve contains DNA at concentration ranges from 10 ng/μl to 0.016 ng/μl.

PCR Conditions: On a light Cycler 480 (Roche), for C-Myc primers:

Activation	15 min 95°C	4.4°C/s	none	X1
Amplification	15 sec 94°C	2.2°C/s	none	X50
		2.2°C/s	single	
		4.4°C/s	none	
Meting curve	65°C to 95°C	2.2°C/s	Continue	X1
Cooling	37°C	keep		X1

For the DBF4 and JunB loci with Applied Biosystems 7900HT Real-Time PCR System.

Step 1: 50 degree for 2 minutes;

Step 2: 95 degree for 10 minutes;

Step 3: 95 degree for 15 seconds; 60 degree for 1 minute.

Repeat **step 3** for 40 times.

28 Analyze sample: quantify the enrichment ratio for nascent strand sequences.

(For example, to calculate the initiation ratio at the C-Myc origin of replication, measure DNA abundance with the primers C-Myc II and the background with primers located near but not overlapping the origin of replication: C-Myc I and C-Myc III)

29 Calculate of the background level is made using the following equation:

$((\text{Concentration calculated with C-Myc I} + \text{Concentration calculated with C-Myc III})/2) * \text{Volume}$

Second Strand Synthesis

30 Take 300-500ng of sample from the previous step, add 20μl of 2.5X Random Primer Solution (e.g. Y01393, Invitrogen), water to 48 μl. D

31 Denature by heating for 5 min in a boiling water bath; immediately cool on ice.

32 Add 1 μl of 10mM dNTP, 1 μl of Klenow Fragment (e.g. Y01396, Invitrogen).

33 Incubate at 37°C for 30-45 minutes.

34 Purify DNA by QIAquick PCR purification kit (28104, Qiagen).

Quality control should be performed before and after second strand synthesis to insure that DNA synthesis did not introduce bias (Figure 4).

Expected results: bona fide nascent strands preparations typically exhibit enrichment in amplification of primer/probe combinations from origin-proximal regions when compared to amplification with primer/probe combinations from adjacent, origin-distal regions (Figure 4).

ALTERNATE PROTOCOL 1

VARIATION: BRDU LABELING AND IMMUNOPRECIPITATION

This protocol involves labeling the replicating DNA with a synthetic nucleoside, BrdU (5-bromo-2'-deoxyuridine), which incorporates into the replicating genomic DNA. Short genomic DNA fragments are isolated and size fractionated from replicating cells that have been pulse-labeled with BrdU, followed by immunoprecipitation with BrdU-specific antibodies. Since this protocol relies on non-overlapping assumptions, it can complement and validate data obtained from lambda exonuclease-mediated SNS preparations.

A detailed discussion of protocol choices in the analyses of replication initiation events is provided above and in the accompanying Overview (Best Practices For Mapping Replication Origins In Eukaryotic Chromosomes).

Materials—50 uM of BrdU solution

sucrose solutions, phenol-chloroform and ethanol as described in the protocol for lambda-exonuclease mediated SNS isolation

0.5 mg/mL anti-BrdU antibody (e.g. BD Pharmingen Cat. #555627)

Rabbit anti-mouse IgG

Proteinase K buffer (50mM Tris-HCl, 10 mM EDTA, 0.5% SDS, 0.2mg/ml proteinase K)

protein G beads

Note: Protect BrdU labeled cells and DNA from light.

1. Label cells with 50uM of BrdU for 10-20 minutes.
2. Harvest cells as described in the main protocol. Purify genomic DNA and fractionate on a sucrose gradient as described in the lambda exonuclease-mediated enrichment of SNS protocol. No RNase-free precautions are necessary, however it is essential to protect BrdU-labeled nascent strands from light.
3. Following sucrose gradient fractionation, boil the pooled size-selected DNA fragments for 5 min, put on ice immediately. Adjust to 1X IP buffer with 10X IP buffer (10X PBS, 0.5% Triton X-100).
4. Add 10µl (you may optimize BrdU antibody concentration according to your DNA amount) of 0.5 mg/mL anti-BrdU antibody (e.g. BD Pharmingen Cat. #555627). Incubate with rocking 60 min at R.T.

5. Add 87 ug (15 μ l, 5.8 mg/ml) Rabbit anti-mouse IgG; shaking 20 min. Centrifuge at 14000 rpm for 15 min. Remove the supernatant. Wash the pellet once with 0.75 ml of 1XPBS/0.1% Triton.
6. Alternatively, add protein G beads. Wash 5 min, three times with 1 ml of 1X PBS/0.1% Triton.
7. Add 100-200 μ l proteinase K buffer to each sample (50mM Tris-HCl, 10 mM EDTA, 0.5% SDS, 0.2mg/ml proteinase K).
8. Incubate at 50°C for 2 hours.
9. Extract with phenol/chloroform and precipitate with ethanol.
10. Resuspend the DNA in water as in step 26 above and proceed to quality control and sequencing as above.

SUPPORT PROTOCOL 1: Phenol Chloroform extraction

This support procedure yields purified, protein-free DNA and avoids shearing and breaking DNA.

Phenol/chloroform extraction

- 1 volume of the mix phenol-chloroform = sample volume (here 200 μ l)
- Inverse tubes 4-5 times
- Centrifuge 5 min - 10000 rcf at **R.T.**
- Recover the upper phase and transfer to a new tube
- Add 1 volume of chloroform (here 200 μ l)
- Inverse tubes 4-5 times
- Centrifuge 5 min - 10000 rcf at **R.T.**
- Recover the upper phase and transfer to a new tube

Ethanol precipitation (we advise to not use glycogen)

- Add 1/10 volume of 3 M Sodium Acetate Solution or of 2 M sodium chloride.
- Add 2 volumes of 100% ethanol (here 400 μ l)
- Keep these sample at -20°C Overnight or 1h at -80°C
- Centrifuge 30 min - 25,000 rcf at -4°C
- Remove supernatant
- Add 150 μ l 75% EtOH to the pellet.
- Centrifuge 5 min 25000 rcf at -4°C
- Remove supernatant
- Air dry sample for 20 min.

- Resuspend the DNA pellet as indicated in the protocol.

SUPPORT PROTOCOL 2: Lambda exonuclease digestion with a plasmid control

This procedure serves to evaluate exonuclease digestion efficiency and should be performed in parallel with steps 15-25 of the main protocol. Plasmid control samples (step #1 below) can be prepared in advance.

1. Digest 20 µg of pBlueScript plasmid or similar with EcoRV or a similar single-cutter enzyme, treat with CIP, inactivate the enzyme in 65°C for 20 minutes, purified with Qiagen PCR purification kit.
2. T4 kinase: Use 40 µl of dissolved DNA in water from step #14 of the main protocol: add 1 µl (about 0.4 µg) of digested control plasmid prepared in step #1 above to the nascent strand sample (Do not add this control plasmid to the nascent strand samples that will **be used for sequencing**). Boil the sample for 10 minutes; cool immediately on ice for 10 minutes.
3. Add the following to the sample: 0.5 µl 100mM ATP, 5µl 10X T4 kinase buffer, 1 µl T4 kinase (NEB), H₂O to 50 µl. Incubate for 30-45 minutes at 37°C.
4. Extract the sample with an equal volume of TE saturated Phenol /Chloroform (pH8.0), and then chloroform, then Add 0.1 volume of 3M Sodium Acetate and 2 volume of ethanol to precipitate DNA.
5. Wash the pellet with 70% ethanol; spin again.
6. Dissolve the pellet with 55 µl of DEPC water. Save 4 µl to a new tube.
7. Add 40 µl of 2.5X λ exonuclease buffers and 9µl of λ exonuclease to the remaining 51 µl sample. Incubate at 37°C o/n.
8. Check the sample by running a 1.5% agarose mini gel :
 - i. DNA before λ exonuclease treatment (step 6 saved samples, 2 µl);
 - ii. DNA after λ exonuclease treatment (5 µl);
 - iii. DNA ladder.

If the plasmid DNA control is not visible in sample (ii), proceed to purify nascent strands as described in the Main Protocol, Day 5, step 26. Otherwise, re-precipitate the DNA and re-digest with λ exonuclease again. (For an example of expected results, see Figure 3)

REAGENTS AND SOLUTIONS

Buffers—2X SDS: 1% SDS, 20 mM Tris-HCl, 10mM EDTA and 600mM NaCl Prepare from:

10% SDS	20 ml
1M Tris-HCl pH 8.0	4 ml

0.5M EDTA	4 ml
5M NaCl	24 ml
H ₂ O	200 ml

TE: 10 mM Tris-HCl, pH8.0, 1 mM EDTA Prepare from:

1M stock Tris-HCl pH8.0	2 ml
0.5M stock EDTA	0.4 ml
H ₂ O	197.6 ml

TNE: 10 mM Tris-HCl pH8.0, 1 mM EDTA, 0.3 M NaCl Prepare from:

1M Tris-HCl pH8.0	2 ml
0.5M EDTA	0.4 ml
5 M NaCl	12 ml
H ₂ O	185.6 ml

0.5% SDS/100mM EDTA

Prepare from:

10% SDS	50 μ l
0.5M EDTA	200 μ l
H ₂ O	750 μ l

2.5X lambda exonuclease buffer

167 mM Glycine-KOH

6.25 mM MgCl₂

125 μ g/ml BSA

pH 8.8 @ 25°C

5% or 30% Sucrose :

5g or 30g sucrose

Completed with TNE buffer (above) to 100 ml

Solution should be autoclaved and kept at 4°C (not longer than 3 months)

Alkaline loading Buffer:

3 volumes of 6X buffer (300 mM NaOH, 6mM EDTA, 18% Ficoll)

1 volume of Bromophenol Blue or Cyanol Xylen 5% (20X)

Molecular Weight ladder:

1 µl of the 1kb DNA ladder

14 µl of 30% sucrose

4 µl of the alkaline buffer

Quality Control Oligonucleotide Sequences (for Human Cells)

C-Myc ORI :

C-Myc non ORI (I):

Forward primer: 5'-CTTATACATTCCTGACCAAGTTGC-3'

Reverse primer: 5'-ATAATAATCAAGAATCGGACGTGA -3'

C-Myc ORI (II):

Forward primer: 5'-TAACGTTGAGGGGCATCG-3'

Reverse primer: 5'-GCACCAAGACCCCTTTAACTC -3'

C-Myc non ORI (III):

Forward primer: 5'-ATCAGCCTACAAGGCTCCTG-3'

Reverse primer: 5'-GTGTCTGATCACTTAGATGCCCTA-3'

DBF4 ORI :

DBF4 ORI :

Forward primer: 5'-GCCATGAGGATCCACAGTAA-3'

Reverse primer: 5'-CGAGGGGAGGAAAGGATTA-3'

Probe : 5'-TCCTCCGCCTGCAGTCCCTT-3'

DBF4 non ORI :

Forward primer : 5'-AAGATTGTGCCACTGCACTC-3'

Reverse primer : 5'-TGAGGATGGGATGGACATAA-3'

Probe : 5'-TCTCGCTCTGTTGCCAGGTG-3'

JunB ORI:

JunB ORI :

Forward primer : 5'-GTGTATCCTGCGTCCGTGT-3'

Reverse primer : 5'-GCCTGCTGTCCTCTGTGA-3'

JunB non ORI :

Forward primer : 5'-CGACACAAGTTAGCCATAGGAA-3'

Reverse primer : 5'-CCCTGGATGCAAAGGTCTAT-3'

COMMENTARY

Background Information

These two complementary methods are used to isolate small newly replicated (nascent) DNA strands to facilitate the identification of replication origins in mammalian cells. The two protocols were originally developed for replication origin mapping using nucleic acid hybridization and PCR. Recently, the two procedures were used in combination with massively parallel sequencing to identify replication origins on a whole-genome scale in human cells (Besnard et al, 2012; Martin et al, 2011 for exonuclease-facilitated SNS; Mukhopadhyay et al., 2014 for BrdU-IP SNS). A detailed discussion of the experimental approaches utilized in the last few decades to map replication initiation sites is provided in the accompanying Overview (Best Practices For Mapping Replication Origins In Eukaryotic Chromosomes).

Critical Parameters and Troubleshooting

The two alternative SNS protocols described below rely on non-overlapping assumption. Although our experience suggests that they yield similar results, each method might be subject to different experimental limitations and ideally it would be advisable to utilize both methods.

Both methodologies involve DNA isolation from proliferating cells. The two methods can be used successfully with asynchronously growing cultures, and a sufficiently high fraction of cells in the S-phase of the cell cycle is critical for insuring a high yield of SNS. The BrdU variation was used successfully on cell-cycle fractionated cells obtained by FACS.

For the lambda exonuclease variation, the most critical parameter is insuring complete digestion by lambda exonuclease. Incomplete digestion with lambda exonuclease might leave some undigested contaminating DNA. Testing for complete exonuclease digestion and typical results are shown in the Anticipated Results below.

For the BrdU-incorporation method, it is critical to obtain a sufficiently high level of BrdU substituted DNA. This method might be hard to implement in cells that exhibit low nucleotide incorporation (most likely reflecting variations in nucleotide pools).

Both methods might be subject to potential biases during the isolation and amplification of the SNS as well as biases reflecting sequencing procedures. To control for such issues, it is advisable to measure the ratio between SNS and genomic DNA using DNA sequences mapping to previously identified origins. Genomic DNA controls from the same cells used for nascent strand isolation should be assayed in parallel. The notes accompanying the protocol provide suggestions for such measurements including several primer pairs corresponding to replication origins that initiate replication in most cells. A comparison between the amplification rates of SNS and genomic DNA using origin-proximal and origin-distal primer pairs should be performed for the original sample obtained directly after SNS isolation and for the amplified sample following second strand synthesis. For a detailed discussion of the potential biases and appropriate controls, please see the accompanying Overview (Best Practices For Mapping Replication Origins In Eukaryotic Chromosomes).

Anticipated Results

Figures 1 through 4 illustrate typical results:

Time Considerations

The protocol takes about 6 days to complete. It incorporates several basic protocols of molecular biology and cell biology including genomic DNA purification, sucrose gradient fraction, T4 kinase, lambda exonuclease digestion, immunoprecipitation, second strand syntheses, Q-PCR, etc. (For details of the time required to perform each technique, please see the individual protocol). Experiments can be paused at any DNA ethanol precipitation step.

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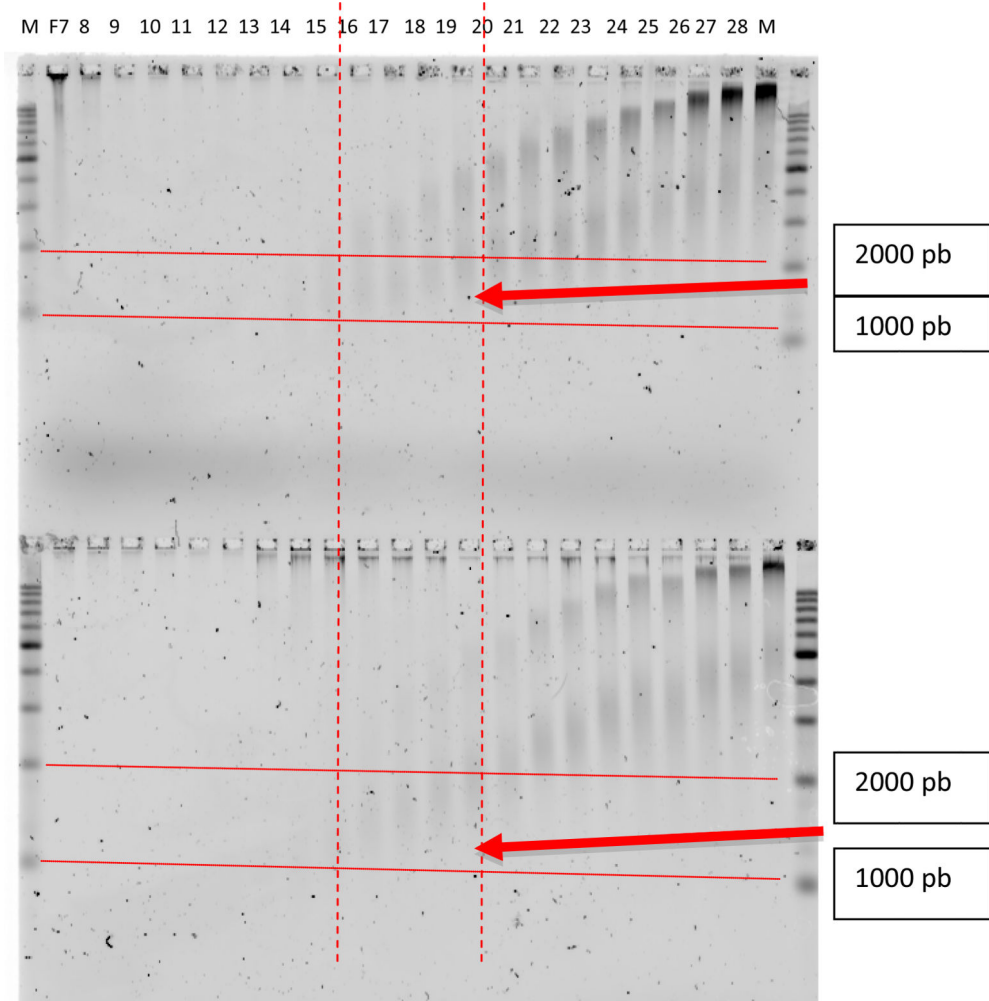


Figure 1. Denaturing gel electrophoresis of samples obtained after the sucrose gradient fractionation

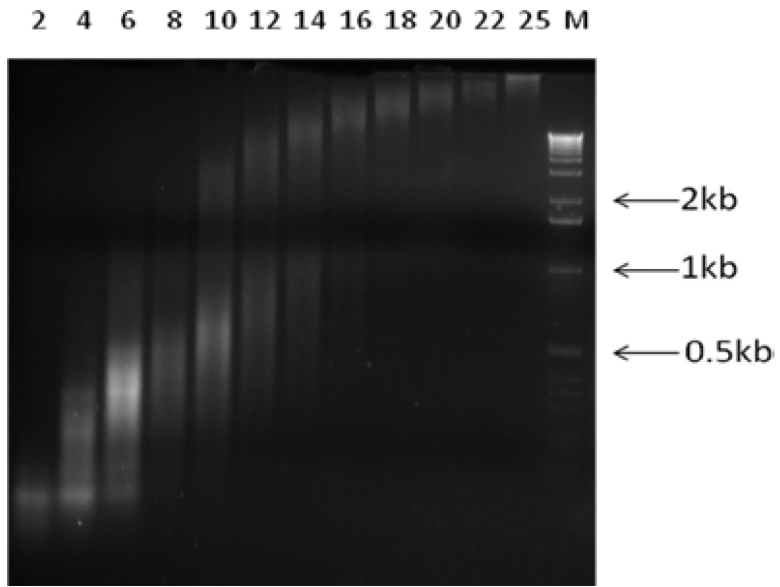


Figure 2.
Non denaturing gel electrophoresis of samples' fractions obtained after the sucrose gradient centrifugation

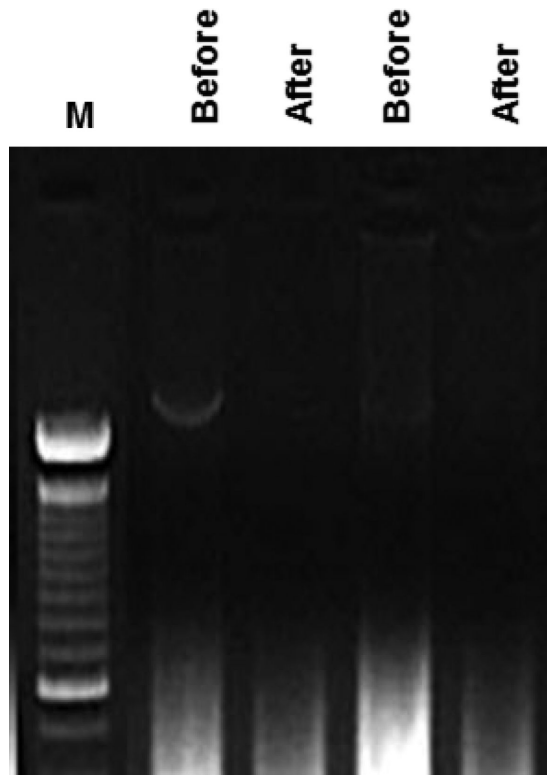


Figure 3. Lambda exonuclease activity test, the disappearance of a plasmid control after lambda exonuclease (lanes marked “After”) suggests that Lambda exonuclease digestion is complete. (For a detailed procedure, see *SUPPORT PROTOCOL 2*).

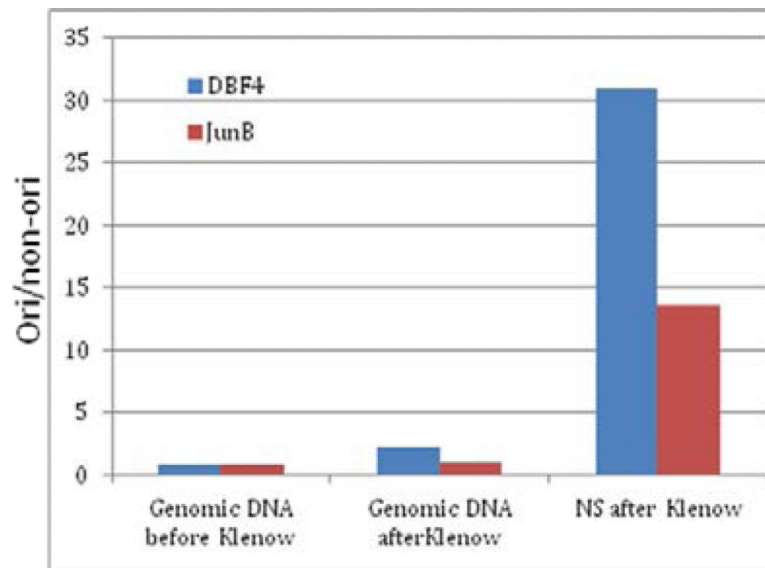


Figure 4. Quality control using RT-PCR prior to sequencing at the *JunB* and *DBF4* loci.