Quantifying Replication Fork Progression at CTG Repeats by 2D Gel Electrophoresis

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Abstract	Physical separation of branched DNA from linear molecules is based on the difference of mobility of linear versus branched DNA during two-dimensional agarose gel electrophoresis. Structured DNA migrates as slower species when compared to linear DNA of similar molecular weight. Metabolic processes such as S phase replication or double strand-break repair may generate branched DNA molecules. Trinucleotide repeats are naturally prone to form secondary structures that can modify their migration through an agarose gel matrix. These structures may also interfere in vivo with replication, by slowing down replication-fork progression, transiently stalling forks, possibly leading to secondary structure such as Holliday junctions or hemicatenanes. Alternatively, reversed replication forks may occur following fork stalling, disrupting replication dynamics and modifying DNA migration on agarose gel. So although two-dimensional agarose gel electrophoresis theoretically allows to resolve a mixture of structured DNA molecules and quantify them by radioactive hybridization, its practical application to trinucleotide repeats faces some serious technical challenges.	
Keywords (separated by " - ")	Trinucleotide repeats - 2D gel - Replication fork - Reversed fork - Recombination - Phosphor screen technology	

Chapter 4

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David Viterbo and Guy-Franck Richard

Abstract

Physical separation of branched DNA from linear molecules is based on the difference of mobility of linear versus branched DNA during two-dimensional agarose gel electrophoresis. Structured DNA migrates as slower species when compared to linear DNA of similar molecular weight. Metabolic processes such as S phase replication or double strand-break repair may generate branched DNA molecules. Trinucleotide repeats are naturally prone to form secondary structures that can modify their migration through an agarose gel matrix. These structures may also interfere in vivo with replication, by slowing down replication-fork progression, transiently stalling forks, possibly leading to secondary structure such as Holliday junctions or hemicatenanes. Alternatively, reversed replication forks may occur following fork stalling, disrupting replication dynamics and modifying DNA migration on agarose gel. So although two-dimensional agarose gel electrophoresis theoretically allows to resolve a mixture of structured DNA molecules and quantify them by radioactive hybridization, its practical application to trinucleotide repeats faces some serious technical challenges.

Key words Trinucleotide repeats, 2D gel, Replication fork, Reversed fork, Recombination, Phosphor screen technology

1 Introduction

Physical separation of branched DNA from linear molecules was developed by Bell and Byers in 1982 [1]. This method was based on the difference of mobility of linear versus branched DNA during two-dimensional agarose gel electrophoresis, in the presence of ethidium bromide. It was shown that structured DNA molecules migrate as slower species when compared to linear DNA of similar molecular weight. Since metabolic processes such as S phase replication or double strand-break repair may generate branched DNA molecules, this method was extensively used to study replication fork progression [2, 3] as well as meiotic recombination intermediates [4, 5]. In order to run a 2D gel, total genomic DNA must be extracted from replicating cells and digested with a restriction enzyme, to generate large DNA fragments, usually more than

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3–4 kb in size, since replication intermediates may be hard to visualize with smaller fragments. Digested DNA is then migrated in the first dimension at low voltage, in the absence of ethidium bromide, so that molecules are separated according to their size. In the second dimension, voltage is increased and ethidium bromide is added in order to rigidify molecules which are then separated according both to their size and to their structure. After the second dimension, the gel is transferred to a nylon membrane and hybridized with a radioactive ^{32}P-labeled probe, whose signal is subsequently quantified using phosphor screen technology (Fig. 1).

Trinucleotide repeat expansions are involved in many neurological pathologies like Huntington disease, myotonic dystrophy type 1 (Steinert disease), fragile-X syndrome, Friedreich's ataxia, or multiple spinocerebellar ataxias. These trinucleotide repeats are located in coding or noncoding sequences, depending on the disease [6–11]. CAG/CTG repeats form imperfect hairpins in vitro (*see* Chapter 1) and these secondary structures may interfere with DNA synthesis during replication, repair or recombination. Therefore, it was early postulated that such repeats may stall replication forks in vivo [12] and subsequently demonstrated by 2D gel

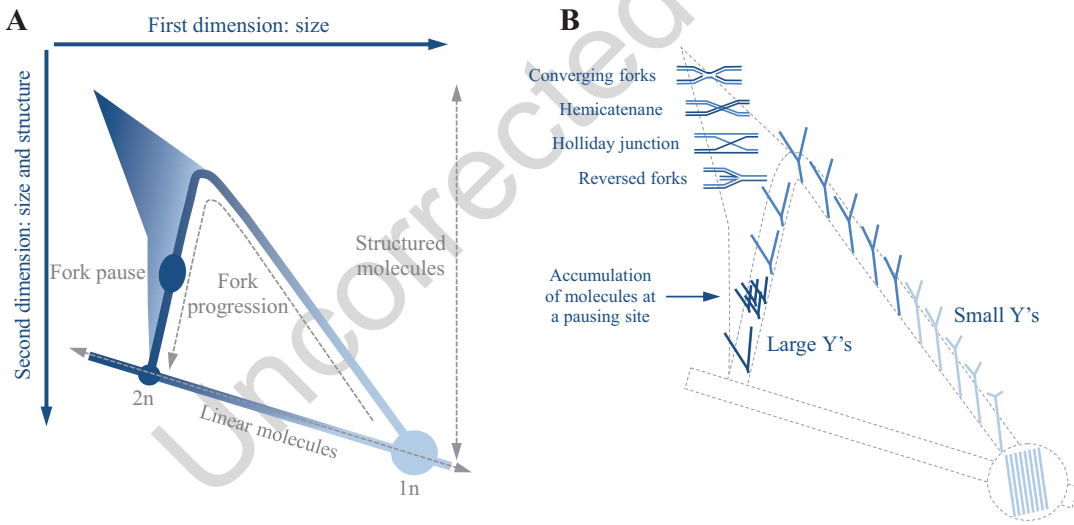

Fig. 1 Different replication and recombination intermediates may be detected by 2D gels. (**a**) Linear molecules migrate relatively to their size only, between *n* and 2*n*, whereas structured molecules migrate according to their size and structure in the second dimension and are therefore detected above the linear ones. The Y arc starts from unreplicated molecules (the *n* spot) to almost completely replicated molecules (the 2*n* spot). A conical shape corresponding to non-Y arc structures is sometimes visible. Fork pausing is detected as a thickening of the Y arc wherever the stalling sequence is located. (**b**) Detailed structured molecules corresponding to Y arc or cone signals. Converging forks, hemicatenanes, Holliday junctions, and reversed forks are hard to discriminate based on their structure and migrate in the same conical area. Note that they are not represented at their expected position in the present figure. Their migration will depend on the time point considered and on the restriction enzyme used to digest genomic DNA. For a comprehensive view of what kind of molecules may be detected and their migration patterns, refer to Schvartzman and colleagues [3]

electrophoresis that it was the case, first in bacteria [13], later on in yeast [14–16]. Secondary structure formation is favored by the presence of extensive single-stranded DNA regions (ssDNA). Therefore, double-strand break repair mechanisms known to generate extensive ssDNA, such as break-induced replication (BIR) [17], single-strand annealing (SSA) [18] or gene conversion should increase trinucleotide repeat instability. Indeed, it was shown in *Saccharomyces cerevisiae* that BIR promotes CAG/CTG repeat expansions [19] and that SSA drives contractions [20], whereas gene conversion induces a high level of both expansions and contractions [21] (reviewed in [22]). In addition, secondary structures may also modify DNA migration in agarose gels, leading to an apparent aberrant mobility [23].

Two-dimensional agarose gel electrophoresis gives a global picture of the replication fork dynamics: replication origin firing, replication fork progression, fork stalling, reversed replication forks, and various replication and recombination intermediates can all be theoretically detected by 2D gels (Fig. 1b) [3, 24, 25]. Variations of this technique were extensively used in *S. cerevisiae* to study replication and recombination intermediates. In order to improve signal quality and signal over background ratio, it is convenient to synchronize yeast cells, either by alpha-factor arrest [18] or by centrifugal elutriation [26]. Cells are subsequently collected at different time points after release into S phase and total genomic DNA is extracted. Different methods can be used to enrich the fraction containing ssDNA, including BND-cellulose columns [27] or extraction in the presence of CTAB (cetyltrimethylammonium bromide), a detergent which stabilizes ssDNA secondary structures [28]. In the present chapter, we describe the 2D gel method on synchronized yeast cells using a simplified CTAB protocol [29] that does not involve a preliminary step of yeast spheroplast preparation. In the following experiments, a $(CAG)_{80}$ or $(CTG)_{80}$ trinucleotide repeat was integrated at the *ARG2* locus on the left arm of yeast chromosome X (strain background BY4741 [30]), 7 kb away from an early replication origin, located in telomeric proximal position as compared to the *ARG2* gene. Maximal replication at this locus occurs 50 min after release from alpha-factor arrest.

2 Materials

Prepare all solutions with deionized water and analytical grade reagents. The quality of the water is crucial in this protocol and changing the water providing source may dramatically alter experimental results. Prepare and store reagents at room temperature, unless otherwise indicated. Follow all waste disposal regulations specific to your institute or country.

2.1 Replication Time Course

1. Alpha-factor (1 mg/mL). Dissolve 10 mg in 10 mL ethanol 100%. Store at 4 °C.
2. Sodium azide. It is kept as a frozen liquid solution at −20 °C, at 100× concentration (10%). It is used as a 0.1% solution to kill yeast cells.
3. 10 mM Tris–HCl, pH 8.0.
4. YPD medium (10 g/L yeast extract, 20 g/L Bacto peptone, 20 g/L dextrose). Autoclave for 20 min at 110 °C. Store at room temperature.
5. 15 mL and 50 mL polypropylene tubes.

2.2 Genomic DNA Extraction

1. Zymolyase 100T (Seikagaku, 30 mg/mL).
2. Extraction solutions.
   Solution I: 2% CTAB (cetyltrimethylammonium bromide), 1.4 M NaCl, 100 mM Tris–HCl pH 7.5, 25 mM EDTA pH 8.0.
   Solution II: 1% CTAB, 50 mM Tris–HCl pH 7.5, 10 mM EDTA.
   Solution III: 1.4 M NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA
3. RNAse A (10 mg/mL).
4. Proteinase K (20 mg/mL).
5. DTT 2 M.
6. Chloroform–isoamyl alcohol 24:1. Order as 250 mL solution, store at 4 °C and use rapidly after opening.

2.3 First Dimension

Use only ultrapure agarose (Lonza Seakem GTG or similar) 0.35% in 300 mL 1× TBE buffer (89 mM Tris–HCl pH 8.0, 89 mM boric acid, 2 mM EDTA pH 8.0). Do *not* add ethidium bromide. Pour the gel in a cold room to quicken polymerization.

2.4 Second Dimension

Use the same agarose. Prepare 300 mL of a 0.9% agarose gel in 1× TBE buffer. Add 9 µL ethidium bromide to the gel *and* 30 µL to 1 L of 1× TBE buffer (0.3 µg/µL final concentration in both gel and buffer).

2.5 Blot

1. Depurination solution, 0.25 N HCl, 1 L.
2. Denaturation solution, 0.5 M NaoH, 1.5 M NaCl, 1 L.
3. Neutralization solution, 0.02 M NaOH, 1 M NH4OAcetetate, 1 L.
4. Six sheets of 3 MM Whatman absorbent paper, cut to the gel dimension.
5. Positively charged nylon membrane cut to the gel dimension (*see* Subheading 3.6).

6. A plastic platform and a plastic tray to set up the transfer (*see* Subheading 3.7).
7. 10× saline sodium citrate (SSC) buffer: 1.5 M NaCl, 150 mM trisodium citrate (adjusted to pH 7.0 with HCl), 1 L.

2.6 Hybridization

1. Hybridization and washes are performed in sodium phosphate buffer ("Church buffer"). Prepare 1 L of 500 mM Na_2HPO_4 adjusted to pH 7.4 with 4 mL orthophosphoric acid.
2. Hybridization: sodium phosphate buffer 250 mM (pH 7.4), SDS 7%, EDTA 1 mM.
3. Washes: 20 mM sodium phosphate buffer (pH 7.4), 1% SDS, 1 mM EDTA.

2.7 Probe Labeling

1. Random Primer (hexadeoxyribonucleotide mix: pd(N)6, 6-mer, 80 nmol), diluted to 120 pmol/μL.
2. 10× Random Priming buffer: 500 mM Tris–HCl pH 7.0, 100 mM $MgSO_4$, 1 mM DTT.
3. [α32P]-dATP, 6000 Ci/mmole.
4. Klenow polymerase 10 U/μL.

3 Methods

3.1 Replication Time Course

Yeast cells respond to the pheromone of the opposite sexual type. Therefore, to perform alpha-factor arrest, cells must be MATa. If working with MATα strains, it is possible to purchase alpha-factor, but its prohibitive price makes this working hypothesis unlikely. If limited amount of alpha-factor are available, it is possible to inactivate the *BAR1* gene, encoding the secreted aspartyl protease that degrades the pheromone, in order to use much lesser amounts of alpha-factor. The amounts indicated here are those recommended for *BAR1* strains, in which the protease is active.

1. Day 1: Grow one colony in 3 mL YDP at 30 °C overnight.
2. Day 2: Transfer in an Erlenmeyer flask, add 200 mL YPD and grow overnight at 23 °C to slow down cell cycle.
3. Day 3: Collect 8×10^9 cells in four 50 mL polypropylene tubes and wash them twice with 20 mL sterile water. Resuspend cells in 800 mL fresh YPD at 9×10^6 cells/mL and incubate 1 h at 23 °C. Add 4 mg alpha-factor (final concentration 5 μg/mL), and incubate cells for 1½ h at 23 °C. Check cell shape under the microscope. They should start to "shmoo" (one end of the cell starts to slightly elongate) in response to the pheromone. If less than 90% of cells are arrested, add an extra 4 mg alpha-factor and incubate cells for an extra 30–45 min. Check cell shape under the microscope. If necessary, an extra 2 mg alpha-factor

may be added to the culture, for a total of 10 mg. When more than 90% of yeast cells are arrested in G1 phase, as shown by microscope observation of "shmoos," centrifuge, wash the culture with 10 mM Tris–HCl (pH 8.0) and resuspend them in fresh YPD at 23 °C. This is the beginning (T_0) of the time course. Progression through cell cycle may be subsequently monitored by microscope observation or followed by FACS, as described in Chapter 5.

4. For each time point, collect 2×10^9 cells (i.e., 200 mL) at 40, 50, 60, and 90 min (*see* **Note 1**).
5. Cells are killed by addition of 2 mL 100× sodium azide (0.1% final concentration). Note that Na-azide is a poison for all eukaryotic cells since it by inhibits mitochondrial oxidative phosphorylations. It is therefore recommended to prepare four flasks containing 2 mL of Na-azide, and to leave them on ice for the duration of the experiment, to avoid repeated manipulations of the stock solution. After incubation with sodium azide, leave cells on ice for 10 min (*see* **Note 2**).
6. Centrifuge cells and discard sodium azide-containing YPD in appropriate waste container. Wash once with 50 mL cold sterile water.
7. Resuspend cells in 50 mL cold sterile water, centrifuge, remove water and freeze dried cell pellet at −80 °C until DNA preparation may take place.

3.2 DNA Extraction, Day 1

1. Thaw the four dried pellets on ice. Resuspend each in 2 mL H_2O, 2.5 mL Solution I, 5 µL DTT (2 M) and 167 µL Zymolyase 100T (Seikagaku, 30 mg/mL). Incubate tube at 30 °C for 1 h. The cell lysate must be viscous. Note that zymolyase digestion of yeast cell wall is performed directly in Solution I, in the presence of CTAB. This is an improvement from former protocols in which spheroplasts had first to be prepared, before Solution I was added.
2. Add 200 µL RNAse (10 mg/mL) in each tube for ½ h at 50 °C.
3. Add 200 µL Proteinase K (20 mg/mL) in each tube for 1.5 h at 50 °C.
4. Add 100 µL Proteinase K (20 mg/mL) in each tube and incubate at 37 °C overnight.

3.3 DNA Extraction, Day 2

Centrifuge tubes 15 min at 3200 × *g* (4000 rpm in our centrifuge). In subsequent steps, pellets and supernatants are independently processed.

3.3.1 Supernatants

1. Gently transfer each supernatant in a 15 mL polypropylene tube.
2. Add 2.5 mL 24:1 chloroform–isoamyl alcohol and gently mix by inversion 5–6 times.
3. Centrifuge 15 min at 3200 × *g*.
4. Gently and slowly transfer each supernatant in a 50 mL polypropylene tube.
5. Add 10 mL of Solution II. Wait 2 to 3 h (*see* **Note 3**).
6. Centrifuge 10 min at 4000 rpm. Discard the supernatant.
7. Add 2.5 mL of Solution III and incubate tubes at 37 °C for 10 min to help dissolve DNA.

3.3.2 Pellets

1. Add 1 mL of Solution III to each pellet. Incubate tubes at 50 °C for 1 h.
2. Add one volume of chloroform–isoamyl alcohol and gently mix by inversion 5–6 times.
3. Centrifuge 10 min at 3200 × *g*.
4. Transfer each supernatant in 15 mL polypropylene tubes containing 2.5 mL of Solution III.
5. Add 5 mL of isopropanol and mix by inversion 5–6 times.
6. Centrifuge 10 min at 4000 rpm.
7. Wash each tube with 2 mL of 70% ethanol.
8. Resuspend each pellet in 250 µL 10 mM Tri–HCl pH 8.0 and incubate tubes at 37 °C for 10 min to help dissolve DNA (*see* **Note 4**).
9. Estimate DNA concentration by loading an aliquot on an agarose gel or using a dedicated setup.
10. Keep DNA at 4 °C (*see* **Note 5**).

3.4 Genomic DNA Restriction Digest

1. Quantify DNA recovered at former step by UV absorbance at 260 nm.
2. Digest 10 µg of DNA for each time point, in a final volume of 100 µL, with 20 units of restriction enzyme (ClaI in our experiments) and 2 µL RNAse A (10 mg/mL) during 6 h. Add 20 more units and leave digestion overnight (*see* **Note 6**).

3.5 First Dimension

1. Pour a 0.35% agarose gel in 300 mL 1× TBE buffer, in a cold room to accelerate polymerization. Do *not* add ethidium bromide. After polymerization, handle the gel with much care because it is *very* fragile.
2. Add 20 µL of 6× loading dye to each digestion and load in wells large enough to accommodate 120 µL. Leave one empty well between each sample for convenient cutting at the next step (Fig. 2a).
3. Run gel overnight at 1 V/cm.

4. With a clean scalpel, cut the lane containing the molecular weight ladder and stain it with ethidium bromide. Measure the distance between which you want to study replication intermediates. For example, if your restriction fragment measures 6 kb, you want to cut first dimension pieces of agarose between ~6 kb and ~12 kb (to catch molecules between *n* and 2*n* in size, *see* **Note 7**).
5. Cut four pieces of similar sizes, one for each time point (Fig. 2b). Watch out at this step because 0.35% agarose is *very* fragile and easily breaks.

3.6 Second Dimension

1. Rotate each piece of DNA by 90° and place them in an empty tray (Fig. 2c).
2. Prepare the volume of agarose 0.9% sufficient to fill the tray up to the height of the four agarose pieces. Add ethidium bromide (0.3 μg/μL final concentration) and let the melted agarose cool down until you may hold the flask (~50 °C).
3. In the meantime, prepare 1× TBE buffer supplemented with ethidium bromide (0.3 μg/μL final concentration) and fill the migration tank in a cold room.
4. Pour the cooled down agarose under a chemical hood, at room temperature and wait for solidification.
5. Take it to the tank in the cold room and migrate the gel at 5 V/cm for ~4.5 h, or until the DNA signal corresponding to linear molecules of *n* size reaches the bottom of the gel. Take a picture under 260 nm UV lamp (Fig. 2d).

3.7 Transfer

1. Soak the gel in 1 L depurination solution (0.25 N HCl) for 10 min.
2. Soak the gel in denaturation solution (0.5 M NaOH/1.5 M NaCl) for 30 min.
3. Soak the gel in neutralization solution (0.02 M NaOH/1 M NH4 Acetate) for 30 min.
4. Cut a positively charged nylon membrane to the size of the gel and soak it in 10× SSC for 5 min (*see* **Note 8**).
5. Transfer the gel by setting up a classical Southern blot apparatus, in a tray filled with 10× SCC buffer: a plastic platform, 3 MM paper soaked in 10× SSC, the gel, the membrane, three layers of 3 MM paper soaked in 10× SSC, finished by a thick layer (20 cm) of absorbent paper. The bottom 3 MM paper must be larger than the gel and soaks into the SSC-containing tray. Leave the transfer overnight.
6. Crosslink DNA to the membrane in a Stratagene Stratalinker at 0.120 J (or a similar UV cross-linker).

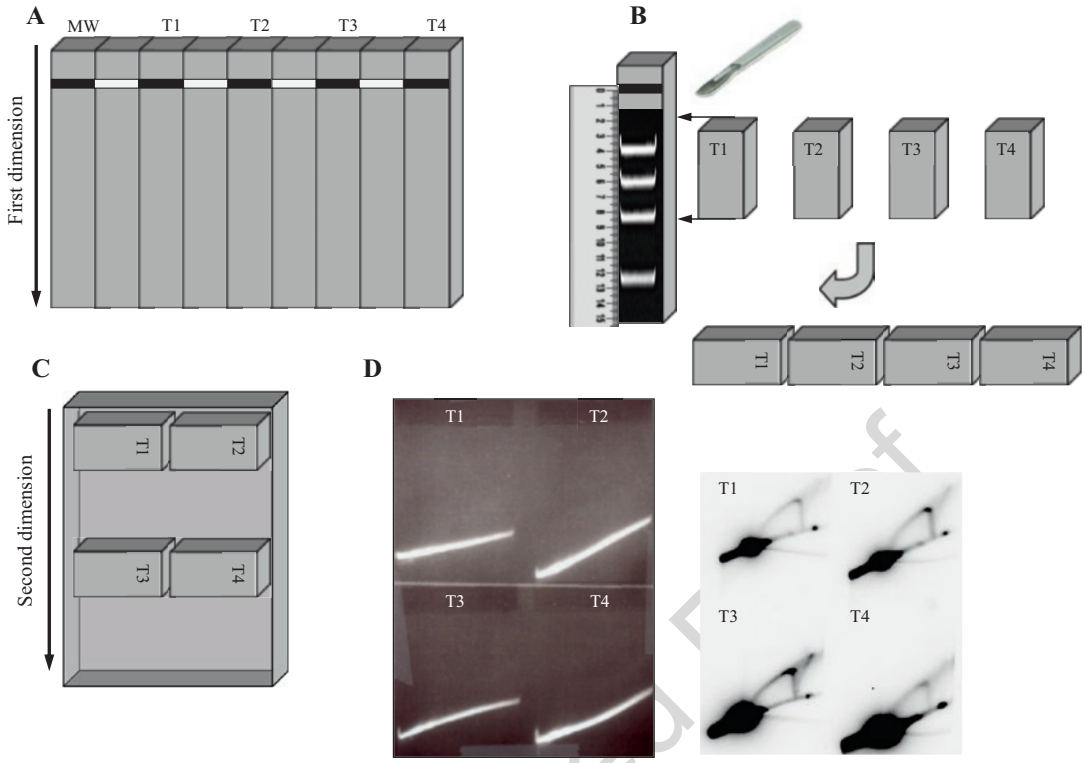

Fig. 2 First and second dimension procedures. (**a**) Each of the four (or more) sample is loaded on an agarose gel, leaving one well free between each sample. Migration is performed at low voltage, in the absence of ethidium bromide. (**b**) The lane containing the molecular weight ladder is cut off and stained to make molecular weight measurements. Each lane containing a DNA sample is subsequently cut between the appropriate molecular weights, as measured on the ladder lane. (**c**) Each cut piece of agarose is turned 90° to be run in the second dimension, at high voltage and in the presence of ethidium bromide. (**d**) Left: Second dimension agarose gel at the end of migration. Linear DNA is stained by ethidium bromide. Structured DNA may sometimes be visible as a weak and fuzzy signal above linear molecules. Right: Hybridization with a radioactive probe specific of the locus studied

3.8 Hybridization

Prehybridization and hybridization are performed at 65 °C in a hybridization oven in a rotating tube.

1. Prehybridize the membrane with 10 mL Church buffer.
2. Prepare the probe by random priming. Use a purified PCR fragment covering the region of interest. Mix 50–60 ng of this PCR product with 120 pmoles hexadeoxyribonucleotide mix (pd(N)6) in 15 µL of 1× random priming buffer (500 mM Tris–HCl (pH 7.0), 100 mM $MgSO_4$, 1 mM DTT).
3. Denaturate DNA for 5 min at 95 °C, then put rapidly on ice.
4. Add 3 µL of α ^{32}P dATP (6000 Ci/mmol), 1 µL of the three remaining dNTP (10 mM each), and 10 units of Klenow polymerase fragment.

5. Incubate at 37 °C for 30–60 min.
6. Purify the probe a ProbeQuant G50 micro sepharose column to quantify the probe. Specific activity must be above 10^8 cpm/μg DNA in order to obtain a clear signal. Do not use lower activity probes.
7. Denaturate probe for 5 min at 95 °C and add to the tube containing the membrane in the hybridization buffer. Hybridization is performed overnight at 65 °C.
8. On the following day, wash twice the membrane with 100 mL of Church washing buffer, for 15 min.
9. Expose 1–4 days on a phosphor screen before quantification. We usually expose 1 day, read the result and possibly reexpose for 2–4 days if the signal is too weak to be accurately quantified.

3.9 Quantification

We routinely use a Fujifilm FLA-9000 to read phosphor screens, but any similar phosphorimaging system could be used.

1. Draw forms surrounding each of the signal to be quantified: the large spot corresponding to *n*, linear DNA, the Y arc, structured molecules migrating as a cone, possibly a pausing signal (Fig. 3 left, *see* **Note 9**).
2. Copy and paste the forms in an area of the membrane containing only background signal (Fig. 3, right).
3. Export data into a spreadsheet. For each signal, subtract the background of the corresponding form.
4. Only signals that are above background by 20% or more were kept for further calculation. Discard other signals.

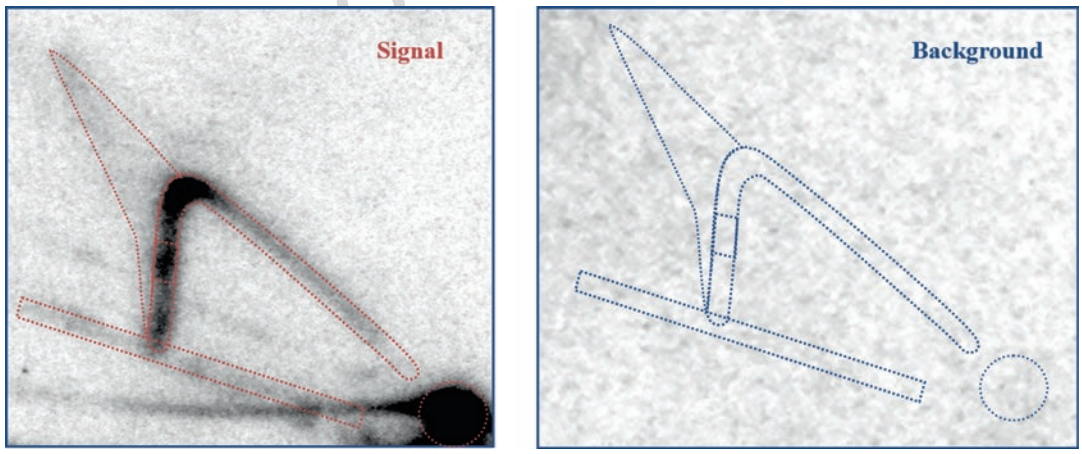

Fig. 3 2D gel quantification. Left: forms of different shapes are drawn around each signal: *n*, linear DNA, Y arc, cone and pausing signal. Right: The same forms are duplicated in another region of the gel in which there is no signal, used as the background to be subtracted from each signal. (Gel originally published by D. Viterbo and G.-F. Richard and reprinted with permission from Elsevier [16])

5. If pausing signals are to be quantified, one may determine a Pausing index (P), as the ratio of pausing signal over Y arc signal, compared to their respective areas. Pausing indexes above one indicate a more intense signal at the pausing locus than on the average of the Y arc [18].

4 Notes

1. In the present experiment, maximum replication timing was known to occur 50 min after alpha-factor release and S phase was finished by 90 min. When working with other yeast strains than S288C (or BY4741) derivatives, or at other genomic loci than *ARG2*, it is advised to experimentally determine the maximum replication timing in preliminary experiments and possibly adjust the above time point values accordingly.
2. Some authors do not use sodium azide, cells are killed by freezing. However, ATP-dependent DNA nucleases may be still active during subsequent steps of DNA isolation, possibly leading to partial DNA degradation, particularly ssDNA. To limit this potential problem, we prefer to shut down ATP metabolism by poisoning the respiratory chain with sodium azide.
3. During this step, the solution must become blurred over time, indicating that DNA is precipitating. If the solution remains crystal clear, it means that the extraction failed or that too little DNA was recovered to form a visible precipitate. If this happens, it is better to stop the experiment at this stage and start over the time course, rather than pursuing through the long and tedious 2D gel protocol with too little DNA to obtain a clear signal at the end.
4. When pipetting DNA use blue tips or yellow tips whose end was cut off with a clean scalpel and slowly pipette to avoid shearing DNA molecules containing single-stranded regions.
5. After extraction, DNA may be kept at 4 °C for weeks. Do not store at −20 °C, to avoid repeated cycles of freezing and thawing, if several experiments are planned.
6. It is very important at this stage that genomic DNA is totally digested. Partial digestion will result in the DNA of interest migrating above its expected size, ending up in missing it when cutting the first dimension gel.
7. Practically, as a security, we recommend cutting agarose a little bit below the n size and a little bit above $2n$ size, to ensure that the entire replication arc will be visible.
8. We used the PerkinElmer charged nylon membrane (NEF1017001PK). However, the experiment may be performed with any other positively charged membrane.

9. Due to the physical properties of a 2D gel, the top of a Y arc always shows more signal than the rest of the arc. Therefore, if planning to study a replication fork pause, choose the restriction enzyme so that the pause will not be exactly in the middle of the restriction fragment, else the pausing signal will be at the top of the Y arc and hard to distinguish from the natural stronger signal at this location (Fig. 3). The enzyme should be chosen so that the pause is in the middle of the ascending or descending Y arc.

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