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ANALYSIS OF TRIPLET REPEAT DNAs AND RNAs

Analysis of Triplet Repeat Replication by Two-Dimensional Gel Electrophoresis

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Summary

Expansions of triplet repeats are responsible for more than 15 hereditary neurological disorders in humans (*1,2*). Triplet repeats are fairly stable when the number of elementary units is under approx 30, but become polymorphic in length with a clear bias for expansions when this threshold is exceeded. This results in the rapid addition of hundreds or even thousands of extra repeats and, ultimately, disease. The mechanisms of triplet repeat expansions are not yet understood. The role of several genetic processes, including replication (*3*), recombination (*4,5*), and repair (*6*), was suggested. However, given the swift accumulation of extra DNA material, DNA replication seems to be an intuitive candidate for generating expansions. Numerous data point to the aberrant replication of triplet repeats as a cause of triplet repeat expansions (*3,7–16*). Direct experimental proof of aberrant replication through triplet repeats was lacking. This encouraged us to study the mode of replication fork progression through triplet repeats in vivo. We analyzed the effects of triplet repeats on replication of bacterial or yeast plasmids using an approach called two-dimensional neutral/neutral gel electrophoresis of replication intermediates. This technique, originally developed for mapping replication origins (*17,18*), is also instrumental in defining replication pause sites (*19*). Using this technique, we were able to unambiguously demonstrate that expandable triplet repeats attenuate replication fork progression in vivo and get some insights into the mechanisms of repeat expansions (*20,21*).

Key Words: Triplet repeats; 2-D gel electrophoresis; replication fork; replication attenuation; bacterial plasmid; yeast plasmid.

1. Introduction

In our studies, we used two model systems based on cloning triplet repeats into standard *Escherichia coli* and *Saccharomyces cerevisiae* vectors (*20,21*). These multicopy plasmids replicate very efficiently, which allowed us to isolate and analyze

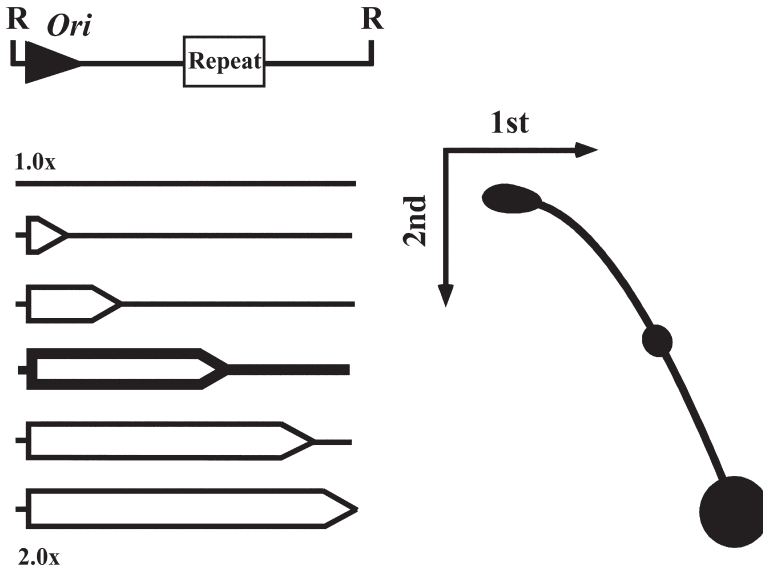


Fig. 1. Schematic representation of electrophoretic separation of replication products of bacterial plasmid containing repeats. Upper left panel shows the structure of the plasmid. The restriction enzyme R cleaves the plasmid upstream of the replication origin. The lower left panel shows the set of intermediates generated during replication. Stalling of the replication fork at a repeat results in the accumulation of a specific bubble intermediate (shown in bold). The right panel diagrams the separation of bubblelike intermediates by two-dimensional neutral/neutral gel electrophoresis. They are resolved by mass in the first dimension and by mass and shape in the second dimension. Stalling of the replication fork at a repeat leads to the appearance of a bulge on the bubble arc.

replication intermediates with a relative ease. The important difference between these two systems from the replication point of view is that replication origins are unidirectional in bacterial vectors and bidirectional in yeast. Consequently, only one replication fork replicates the whole bacterial plasmid, whereas the yeast plasmid is replicated by two forks moving in opposite directions.

The principles of electrophoretic analysis of replication intermediates were first applied to unidirectional replication in **ref. 22**. Intermediate products of plasmid replication are Θ shaped. Upon cleaving these intermediates with a restriction enzyme upstream of the replication origin, they convert into bubble-shaped molecules, where the size of the bubble correlates with the duration of replication (*see Fig. 1*). Bubble intermediates differ in their molecular mass (ranging from one to two plasmid masses) and shape. They are separated in two dimensions: first by mass (low-percentage agarose) and second by mass and shape (high-percentage agarose with ethidium bromide). Southern blotting hybridization with the radioactive plasmid probe reveals a so-called bubble arc (*see Fig. 1*).

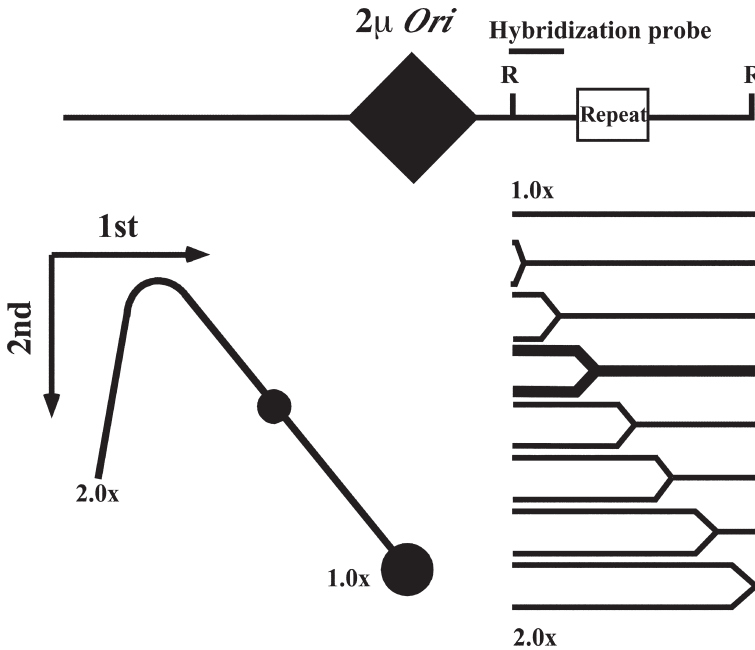


Fig. 2. Electrophoretic analysis of replication intermediates in yeast. Cleavage of replication intermediates with restriction enzyme R generates Y-shaped DNA molecules differing in size and shape that can be resolved by two-dimensional gel electrophoresis. Replication stalling at a repeat leads to the accumulation of a replication intermediate of a given size and shape (right panel), resulting in the appearance of a bulge on the Y arc.

Analysis of replication intermediates in the case of bidirectional replication (17,18) is schematically presented in Fig. 2. Because of the bidirectionality, the plasmid is separated into two domains replicated by different forks. In this diagram, triplet repeats are positioned in the path of the left-to-right replication fork. Replication intermediates generated by this fork convert into Y-shaped structures upon restriction digest downstream of *ori* and in the middle of the plasmid. These Y-shaped intermediates can be resolved in two-dimensional agarose gel and detected by hybridization with the corresponding restriction fragment (see Fig. 2).

For both unidirectional and bidirectional replication, stalling of the replication fork by a triplet repeat should result in the appearance of a bulge on the otherwise smooth bubble or Y arc, respectively, as a result of the preferential accumulation of replication intermediates of a specific size and shape.

Using this approach, we found that all expandable triplet repeats— $d(\text{CGG})_n \bullet d(\text{CCG})_n$, $d(\text{CTG})_n \bullet d(\text{CAG})_n$, and $d(\text{GAA})_n \bullet d(\text{TTC})_n$ —attenuate replication fork progression in both bacteria and yeast (20,21). Characteristic results for $(\text{CGG})_n$ -caused replication blockage in bacteria and yeast are shown in Fig. 3. One can clearly see a bulge on either bubble or Y arcs in replication intermediates isolated from bacteria and yeast, respectively.

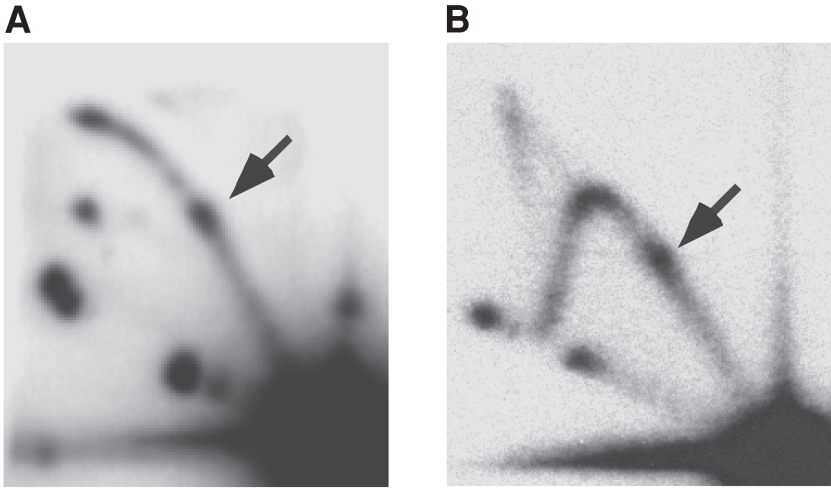


Fig. 3. Experimental data demonstrating replication stalling at the GGC_{70} repeat in *E. coli* (A) and GGC_{40} repeat in *S. cerevisiae* (B). Arrows show replication stall sites.

The ratio of the radioactive signal of this bulge to that of the corresponding area of a smooth arc reflects the efficiency of replication fork stalling by a repeat. **Figure 4** shows an example of the quantitative analysis of the data for a $(\text{CGG})_{40}$ repeat in yeast using a phosphoimager. The ratio of radioactivity in the peak area (areas 1+2) to that in the corresponding area of a smooth replication arc (area 2) represents the extent of replication slowing. In this particular case, it corresponds to 1.8-fold; that is progression of the replication through the repetitive is slowed down about 2-fold.

In both bacteria and yeast, the inhibitory effects of triplet repeats on replication increased with their length. The strength of inhibition in both systems depended on the repeat's base composition in the following order: $(\text{CGG})_n \bullet (\text{CCG})_n > (\text{GAA})_n \bullet (\text{TTC})_n > (\text{CAG})_n \bullet (\text{CTG})_n$. However, even in the strongest cases, replication was only moderately (several-fold) slowed down. Thus, it is exceptionally high resolution of the electrophoretic analysis of replication intermediates that allowed us to detect these effects.

The question still remains: Is there a link between the replication attenuation at triplet repeats and their propensity to expand? It is plausible to speculate that extra repeats are added while the replication fork is trying to bypass the stall site. Although this hypothesis could not be verified by electrophoretic analysis of replication intermediates alone, the approach has already provided important clues to the mysteries of triplet repeat expansions.

2. Materials

2.1. Isolation of Replication Intermediates From *E. coli*

1. Resuspension solution: 25% (w/v) sucrose, 0.25 M Tris-HCl, pH 8.0, store at 4°C.
2. Lysozyme stock solution: 50 mg/mL in water. Store in small aliquots at -20°C.

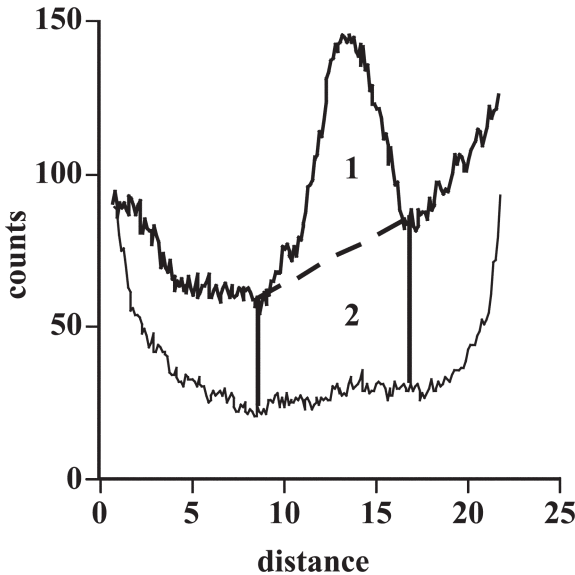


Fig. 4. Densitogram showing a quantitative analysis of the data presented in Fig. 3B. One can see a peak on the portion of the Y arc containing the GGC₄₀ repeat. Thick line = Y arc; thin line = background noise.

3. RNase A stock solution: 10 mg/mL in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl. Incubate in boiling water bath for 5 min to destroy DNase. Store in small aliquots at -20°C .
4. Lysis buffer: 1% (v/v) Brij-58, 0.4% (w/v) sodium deoxycholate, 0.063 M EDTA, pH 8.0, 50 mM Tris-HCl, pH 8.0, store at 4°C .
5. PEG+NaCl: 25% (w/v) PEG 6000–8000, 1.5 M NaCl. Store at 4°C .
6. Deproteinization solution: 1 M NaCl, 10 mM Tris-HCl (pH 9.0), 1 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
7. Proteinase K stock solution: 20 mg/mL in H₂O. Store in small aliquots at -20°C .
8. Tris-buffered phenol, pH 8.0; store at 4°C .
9. Chloroform.
10. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

2.2. Isolation of Replication Intermediates From *S. cerevisiae*

1. NIB buffer: 17% glycerol, 50 mM MOPS, 150 mM NaOAc, 2 mM MgCl₂, 0.5 mM spermidine, 0.15 mM spermine, pH 7.2; store at 4°C .
2. YSTE buffer: 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 20 mM EDTA; store at 4°C .
3. Sarkosyl stock solution: 10% (w/v) solution; store at room temperature.
4. Proteinase K stock solution: 20 mg/mL in water. Store in small aliquots at -20°C .
5. Hoechst 33258 trihydrochloride stock solution: 2 mg/mL in water. Store in a dark bottle at 4°C .
6. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA; store at 4°C .

2.3. Electrophoretic Separation of Replication Intermediates

1. 10X TBE: 450 mM Tris-borate, 10 mM EDTA; store at room temperature.
2. Ethidium bromide stock solution: 10 mg/mL in water; store in a dark bottle at 4°C .

3. Methods

3.1. Isolation of Replication Intermediates From *E. coli*

1. Grow an overnight culture in 2 mL of selective media.
2. Inoculate 2 mL of an overnight culture into 200 mL of fresh selective media.
3. Grow cells until $OD_{600} = 0.6$ (about 3.5 h).
4. While the cells are growing, make sure that the centrifuge and rotor for pelleting the cells are cold.
5. Mix 100 g of ice with 3 mL NaCl in a 500-mL centrifuge tube, shake intensively.
6. When the culture reaches $OD_{600} = 0.6$, transfer it into a centrifuge tube with ice, mix, centrifuge for 7 min at 2500g at 0°C, decant the supernatant, and put the tubes on ice (*see Note 1*).
7. Resuspend cells in 2.5 mL of ice-cold resuspension solution and transfer suspension into a cold 50-mL centrifuge tube. Add 400 μ L of lysozyme stock solution and 25 μ L of RNase A stock solution, mix gently, and incubate on ice for 5 min.
8. Add 1 mL of 0.25 M EDTA, mix gently, and incubate for 5 min on ice.
9. Add 4 mL of lysis buffer, mix by inverting the tube gently, and incubate on ice for 10–20 min, inverting the tube every 5 min.
10. Centrifuge at 14,000g for 1 h at 4°C (*see Note 2*).
11. Transfer supernatant into a 50-mL centrifuge tube, add 5 mL of cold PEG+NaCl solution, vortex, and incubate on ice for about 1–2 h (*see Note 3*).
12. Centrifuge for 20 min at 14,000g at 4°C; decant the supernatant.
13. Dissolve the pellet in 400 μ L of deproteinization solution, add 2 μ L of Proteinase K stock solution, and incubate for 20 min at 65°C (*see Note 4*).
14. Extract DNA two times with an equal volume of tris-buffered phenol, pH 8.0.
15. Extract DNA one time with an equal volume of chloroform.
16. Add 2.5 vol of 100% ethanol, cool at –70°C for 20 min, centrifuge at 14,000g for 15 min, and decant the supernatant.
17. Wash the pellet with 70% ethanol.
18. Dry the pellet in a SpeedVac centrifuge.
19. Dissolve in 30–50 μ L of TE, depending on the pellet size.
20. Check the amount of isolated plasmid DNA using 0.8% agarose gel electrophoresis (*see Note 5*).

3.2. Isolation of Replication Intermediates From *S.cerevisiae*

1. Grow an overnight culture in 10 mL of selective media.
2. Inoculate an overnight culture into 400 mL of fresh selective media to reach $OD_{600} = 0.1$. Grow cells until $OD_{600} = 2.0$.
3. Put 80 mL of 0.2 M EDTA into a 500-mL centrifuge tube and freeze at –20°C.
4. Stop cell growth by adding 4 mL of 10% NaN_3 .
5. Pour the culture into the centrifuge tube with 0.2 M frozen EDTA; mix until the ice comes off the wall.
6. Pellet cells in a cold centrifuge for 5 min at 2500g.
7. Resuspend the pellet in 50 mL of ice-cold water and transfer into a 50-mL centrifuge tube. Centrifuge for 5 min at 2500g.
8. Resuspend the pellet in 4 mL of NIB buffer. Add an equal volume of glass beads and disrupt cells by vortexing for 10 min; chill on ice for 30 s after every 30 s of vortexing.
9. Allow beads to settle out and collect the supernatant. Add another 4 mL of NIB buffer to the beads, vortex, and collect the supernatant again.

10. Combine all the supernatant and centrifuge at 13,000g for 25 min.
11. Resuspend the pellet in 1.5 mL of the YSTE buffer by spreading it along the wall with the pipet. Add 0.225 mL of 10% sarkosyl and 30 μ L of 20 mg/mL proteinase K stock solution, mix gently, and incubate for 1 h at 37°C.
12. Centrifuge at 12,000g for 5 min. Transfer the supernatant into a 15-mL tube.
13. Add 4.5 g of CsCl and 125 μ L of Hoechst 33258 trihydrochloride stock solution and adjust the volume to 5.4 mL with distilled H₂O. Transfer into a Beckman Quick-Seal tube for a VTi65 rotor; seal the tube.
14. Centrifuge the density gradient in a Beckman ultracentrifuge (rotor VTi65) overnight at 55,000 rpm, 25°C.
15. Collect the lower DNA band under ultraviolet (UV) light (*see Note 6*). Adjust the volume to 1 mL with TE.
16. Purify DNA from Hoechst 33258 by four consequent butanol extractions (*see Note 7*).
17. Add 2 vol of 80% ethanol. Invert the tube until chromosomal DNA forms a visible coil. Centrifuge for 1 min at 13,000g; wash the pellet with 70% ethanol. Remove ethanol without drying the pellet.
18. Dissolve the pellet in 30–50 μ L of TE.

3.3. Gel Electrophoretic Separation of Replication Intermediates

3.3.1. Restriction Digestion

1. Digest 2–5 μ L of a replication intermediates sample (approx 0.5–1 μ g of plasmid DNA isolated from bacterial cells, or approx 7–15 μ g of DNA isolated from yeast) with a restriction endonuclease(s) in 50 μ L for 1–3 h (*see Note 8*).
2. Add 50 μ L of TE, extract DNA with 100 μ L of phenol, followed by 100 μ L of chloroform.
3. Add 15 μ L of 3 M NaOAc, pH 7.0, and 300 μ L of ethanol, cool for 10 min at –70°C and centrifuge for 6 min at 13,000g. Remove supernatant with a pipet, spin for 20 s, and remove the rest of supernatant.
4. Add 300 μ L of 70% ethanol, vortex for 3 min, and remove the supernatant completely as after precipitation. Dry the pellets for about 3 min in a SpeedVac.
5. Dissolve in 12 μ L of TE. Add 2 μ L of glycerol dye.

3.3.2. First Dimension of Electrophoresis

1. Prepare 0.4% agarose gel with 1X TBE (*see Note 9*).
2. Load the sample on the gel.
3. Load a 1-kb ladder into the last two wells. Load 10 μ L of concentrated bromophenol blue in one of the wells.
4. Run electrophoresis at 1 V/cm for about 15 h, until bromophenol blue, which runs approximately as a 2-kb-length fragment, reaches the end of the gel.
5. After electrophoresis is completed, cut one of the ladders from the gel and stain it with ethidium bromide to assure that a nonreplicated DNA fragment is at the very end of the gel. If not, continue electrophoresis until the nonreplicated fragment reaches the end of the gel (12 cm from the start).

3.3.3. Second Dimension of Electrophoresis

1. Prepare 1X TBE with 0.3 μ g/mL of ethidium bromide for the second dimension and cool it to 4°C.
2. Stain the first-dimension gel in 400 mL of 1X TBE with 0.3 μ g/mL of ethidium bromide for 20 min (*see Note 10*).

3. Set the second-dimension electrophoresis apparatus at 4–10°C (*see Note 11*).
4. Cut the lane with intermediates from the first-dimension gel under UV light using a razor blade (*see Note 12*). Cut out a portion of the lane corresponding to 1X to 2X sizes of intermediates, according to the ladder; add an extra 5 mm from each end.
5. Place the resultant piece of the gel onto the second-dimension tray on its side in a perpendicular direction. The bottom surface of the gel fragment should be positioned in the direction of electrophoresis.
6. Prepare 1% agarose in 1X TBE with 0.3 µg/mL of ethidium bromide stock solution.
7. Slowly pour the agarose onto the second-dimension tray to cover a piece of the gel from the first dimension.
8. Run electrophoresis with buffer circulation at 5 V/cm at 4–10°C until nonreplicated DNA migrates 7–8 cm from the start (*see Note 13*).
9. Blot-transfer the gel to the positively charged Nylon membrane.
10. Hybridize the blot to radiolabeled probe using a standard protocol.
11. Analyze the radiolabeled membrane on phosphoimager or expose it with an X-ray film (*see Note 14*).

4. Notes

1. All of the steps prior to cell lysis should be performed rapidly without delays. Once chilled, cells should stay cold.
2. The longer the centrifugation step is, the cleaner are the replication intermediates from chromosomal DNA.
3. If a low yield of plasmid is expected (low-copy plasmid, insufficient amount of cells), then overnight incubation is recommended.
4. The pellet will dissolve better if its not overdried. Warming deproteinization solution at 65°C before dissolving the pellet also helps to dissolve it better.
5. Replication intermediates samples are also going to contain variable amount of chromosomal DNA. Equal amounts of plasmid DNA should be taken for electrophoretic analysis.
6. The lower band containing chromosomal and plasmid DNA, along with the thin ribosomal DNA band just below it, should be pooled out with a 1-mL syringe. It usually comes out in a 0.25- to 0.5-mL volume.
7. If CsCl starts to precipitate at any step, add several drops of TE and mix until it dissolves.
8. Choosing restriction enzymes, it is important to remember that to achieve the best electrophoretic resolution, a triplet repeat should be positioned around the center (for the bubble arc) or first third (for the Y arc) of a 2- to 4-kb restriction fragment relative to the direction of replication.
9. The electrophoretic device should be free of ethidium bromide. If it was ever used for ethidium bromide-containing gels, it is necessary to wash it carefully with butanol. Use the tray and electrophoretic apparatus suitable for a 12- to 15-cm tray.
10. The 0.4% agarose gels are very easy to break; thus, staining on the tray helps to keep them intact. Slide the first-dimension gel onto another 12- to 15-cm tray that is specifically reserved for ethidium bromide staining. Staining on the same tray that was used for electrophoresis in the first dimension is not recommended to avoid ethidium bromide contamination. The 1X TBE buffer that was used for the first-dimension electrophoresis can be reused for staining the gel.
11. The second dimension requires an electrophoretic apparatus with a buffer circulation system. It should be different from the first-dimension device to avoid subsequent ethidium

bromide contamination of the first-dimension apparatus. A large device suitable for a 20 × 25-cm tray allows one to process up to nine samples simultaneously.

12. Make cuts as close to the lane as possible.
13. Chromosomal arcs (for yeast) or nonreplicated DNA spots (for bacteria) are easy to visualize under UV light.
14. Exposure times may vary. The usual exposure time for a blue-based film is overnight. For low-copy plasmids, a supersensitive film (Kodak Biomax MS) may be helpful.

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