CHAPTER 7

Determination of Human Telomere Length

Introduction

The goal of this experiment is to determine the length of your telomeric DNA. Telomeres are specialized terminal elements that are present at the ends of most eucaryotic chromosomes. These structures are composed of tandem repetitive sequences that are associated with specific proteins. The length of a telomeric region is a reflection of the "mitotic clock" of normal somatic cells and is therefore age dependent. The length of a telomeric region is inversely related to age: the younger the person, the longer the telomeric region.

In this experiment you will measure the length of your telomere using Southern blot hybridization. First, you will isolate your genomic DNA and digest it with restriction enzymes that do not recognize the telomeric repeated sequence. The chromosomal DNA will be cut into very small fragments except for the telomeric and subtelomeric regions that together comprise the terminal restriction fragment (TRF). The cleaved DNA will be separated by agarose gel electrophoresis, transferred to membrane, and hybridized to probe containing DNA complementary to the telomeric repeat. The TRF will be detected by exposing the blot to antibody against digoxygenin (DIG) conjugated to alkaline phosphatase and chemiluminescent substrate for alkaline phosphatase. You will calculate the average length of your TRF and compare it to standards of "long" (germ line cells) and "short" (old cells) TRFs.

The analysis of telomere DNA length consists of a number of procedures that will be carried out in six laboratory periods. The essential steps are as follows.

1. In this experiment you will purify DNA from your cheek cells. You will learn a new DNA purification procedure that is very quick and does not require the use of organic solvents. In this procedure nucleic acids are purified from cell lysate by precipitating proteins using a "salting out" procedure. This task is described in experiment 1 and will be performed during the first

laboratory period. Next, you will measure the purity and concentration of your DNA. This procedure is described in experiment 2 and will be performed in the second laboratory period.

2. Second, DNA will be digested using two restriction endonuclease enzymes. We will use the *Hinf* I and *Rsa*I enzymes. The *Hinf* I enzyme recognizes GA|NTC sequences and the *Rsa*I enzyme recognizes GT|AC sequences. These sequences do not occur in the TRF region of human telomeres. The DNA fragments resulting from digestion will be separated by size by agarose gel electrophoresis. Experiments 3 and 4 describe these procedures. They will be performed during the second laboratory period.

3. Third, DNA fragments will be transferred to a nylon membrane using the Southern blot technique. The membrane will contain the DNA fragments located in exactly the same positions as they were present in the gel. This procedure will be performed in experiment 5 during the third laboratory period.

4. Fourth, immobilized DNA fragments will be hybridized with labeled DNA probe complementary to telomere repeat sequences and hybridized DNA fragments will be detected by chemiluminescence. The average length of telomeres will be calculated using computer analysis. These procedures will be carried out during the fourth to sixth laboratory periods.

Figure 7.1 presents the overall timetable for these experiments.

Background

Most procaryotic chromosomes are circular, thereby permitting their easy replication and DNA damage repair. In contrast, eucaryotic chromosomes, including humans' chromosomes, are linear structures containing a single linear DNA molecule. The main disadvantages of having linear chromosomes are difficulty in replicating the 5′-end of the lagging DNA strand (Levy et al., 1992) and the presence of free DNA ends that are open to degradation by nuclease and/or to end-joining reactions that fuse two free ends (reviewed in McEachern et al. (2000)). In order to obviate these problems the ends of eucaryotic chromosomes have specialized structures called telomeres. Telomeres consist of a tandem GT-rich repeat that, for humans and many other organisms, has the sequence TTAGGG and associated specific proteins (Brown, 1989; Evans, 2002). These structures form a double-stranded DNA loop named a t-loop (Griffith et al., 1999). The very end of telomeric DNA is single stranded and forms a 3′ overhang. This overhang displaces an upstream telomere repeat and is tacked inside the double-stranded DNA at the t-loop junction. Formation of this structure is dependent on the telomere-binding protein TRF2. Thus, linear chromosomes are indeed capped on both ends by a circular DNA structure. Figure 7.2 presents a schematic view of a telomere.

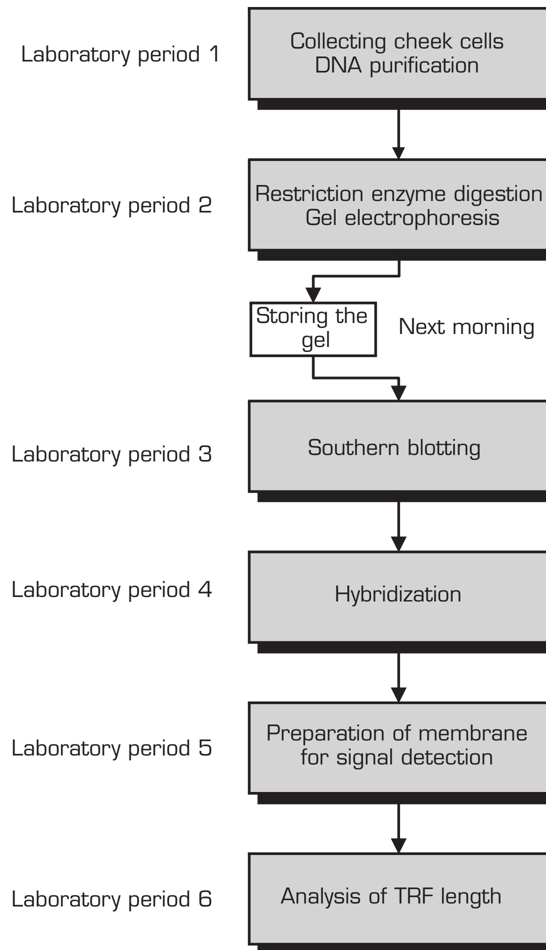


Figure 7.1 Schematic outline of the procedures used in the determination of telomere length.

The length of the telomere region varies considerably among species. The lengths of the simple repeat region can range from 50 bp in *Euplotes* to 300 bp in yeast to over 100 kb in mice. Human telomeres contain 5,000–15,000 nucleotides with a long single-stranded 3′ overhang on at least 80 percent of the telomeres. In addition to the telomere DNA (telomere region), each chromosome contains a subtelomeric region of DNA comprising approximately 4 kb of imperfect tandem-repeated sequences of the original telomeric hexamer.

The length of the telomere region is not stable and depends on cell type and the age of the cell. The telomere region is progressively reduced with each somatic cell division because DNA polymerase α cannot replicate the 5′-end of the lagging strand.

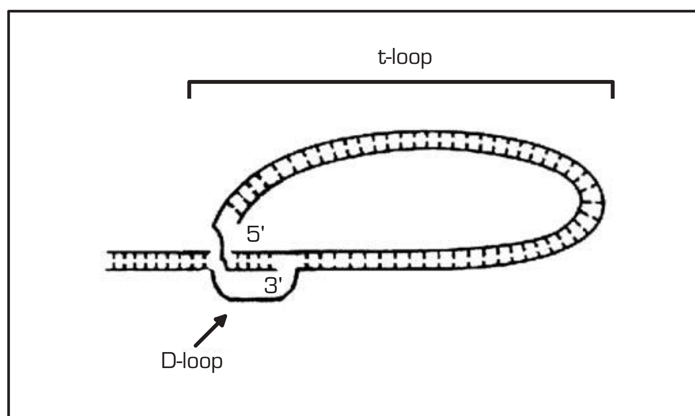


Figure 7.2 Schematic diagram of a telomeric structure on the end of chromosomes.

DNA polymerase α cannot start DNA replication on single-stranded DNA and requires a short double-stranded region with 3′OH in order to start replication (primer). The primer in *in vivo* DNA replication is RNA synthesized by a DNA-dependent RNA polymerase named primase. The RNA primer is subsequently removed, leaving the *de novo*-synthesized strand (and double-stranded DNA) shorter. The next DNA replication will start on the shortened double-stranded DNA and its replication will result in a shorter DNA molecule. The difference in length is the length of the primer. Thus, in every subsequent DNA replication, the chromosome ends are shortened. In humans every DNA replication shortens the chromosome ends by 15–50 bases. Figure 7.3 outlines a schematic of the replication of the ends of linear DNA molecules.

Progressive shortening of the telomere leads to genome instability and aberrant chromosome fusion and rearrangement. Thus, shortening of the telomere region signals the cell to undergo replicative senescence, resulting in cell death. Most normal human cells collected from young individuals can undergo 25 divisions and will enter senescence when their telomeres have been shortened to approximately 5–7 kb. Cells collected from older individuals have much shorter telomeres and will reach a telomere length of 5–7 kb (senescence) after less than 25 cell divisions.

The "end-replication" problem is solved in most eucaryotic cells by activating a specialized reverse transcriptase that is independent of the preexisting parental DNA template. This enzyme, named telomerase or TERT (**Te**lomere **R**everse **T**ranscriptase), can add a repeat sequence to the 3′-chromosome ends using a short region of its associated RNA moiety as a template (Greider and Blackburn, 1985; Blackburn 1992; Linger and Cech, 1998; Nugent and Lundblad, 1998). Thus, the telomerase rebuilds the ends of chromosomes lost during DNA replication. A schematic diagram of the

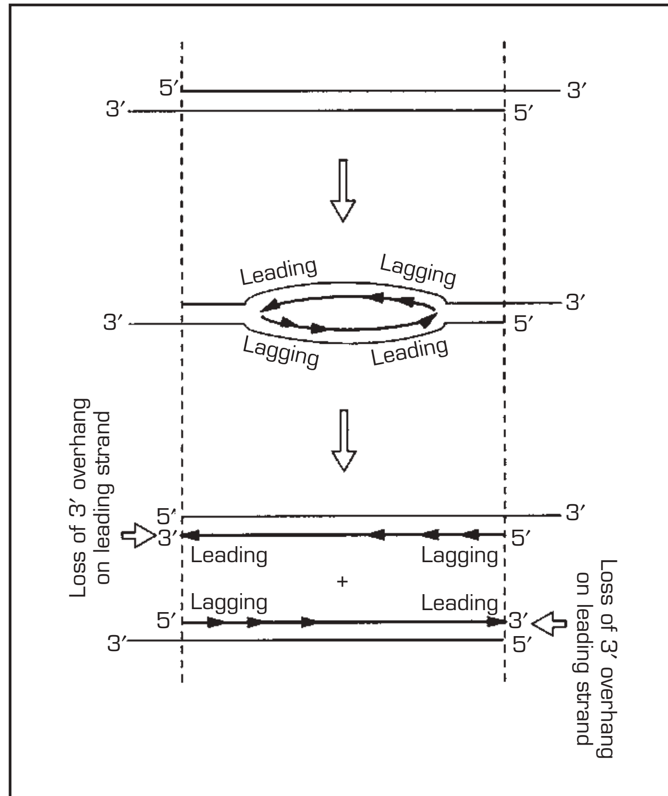


Figure 7.3 DNA replication at the ends of linear molecules.

telomerase reaction that leads to elongation of the single-stranded 3′-end of the telomere is shown in Fig. 7.4.

Telomerase is inactive in most somatic cells and, as a result, the telomere length in these cells is progressively shortened. Thus, the length of the telomere reflects the "replicative history" of the cell, old cells having shorter telomeres than young cells.

Contrary to the situation in somatic cells, in the germ line cell the activity of telomerase is turned on. The restoration of telomere length occurs during the formation of germ line cells. Fusion of gametes creates a zygote with chromosomes containing long telomeres (15–20 kb). Subsequent somatic cell divisions occur without activating telomerase resulting in sequential shortening of telomeres.

Telomerase enzyme is also up-regulated in approximately 85 percent of human tumors leading to continuous division of these cells without senescence (immortalization). Telomerase is activated in most human cancers at the stage when invasive cancer occurs. Thus, during the beginning premalignant stages of cancer development, cells continue to divide in the

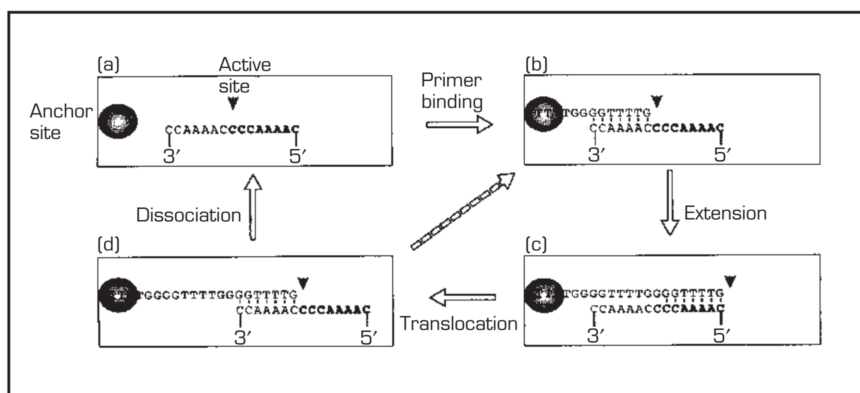


Figure 7.4 Schematic of telomerase activity. Telomerase protein is shown as the rectangle. (a) Active TERT with an RNA template. (b) The 3′-end of DNA binds to an RNA template. (c) Synthesis of 3′ DNA by telomerase using RNA as a template. (d) Telomerase translocated to the end of the newly extended 3′-end of the DNA.

absence of telomerase activity. This results in progressive shortening of telomeres, which leads to genome instability and chromosomal rearrangement in these cells. Persistence of this scrambled genome can result in cell death or in the acquisition of mutations that enhance the capability of the tumor. The activation of telomerase at this stage of cancer development reduces further genomic instability and allows these cells to survive and proliferate. As the result of this process, most cancer cells have highly rearranged genomes and very short telomeres.

The significance of telomerase activity to cell aging and cancer has made telomerase a popular subject of research (reviewed in De Lange and DePinho (1999)).

FIRST LABORATORY PERIOD

Experiment 1: isolation of genomic DNA

Introduction

We will be using a genomic DNA purification kit (the Wizard from Promega Co.) that is designed for quick isolation of DNA without the application of organic solvents. DNA purity and yields are not exceptional, but sufficient for the telomere length assay. You will collect your cheek cells and lyse them in a solution containing detergent. Next you will remove the RNA by RNase digestion and precipitate cellular proteins with ammonium acetate (1.8 *M*). High molecular weight DNA is not precipitated with this salt and is left in the solution. Finally, you will collect and concentrate the genomic DNA by precipitation with isopropyl alcohol. Isopropyl alcohol will be removed by washing pelleted DNA with 70 percent ethanol. You will dissolve your DNA in a small volume of TE buffer and store it for further use.

Background

Removing protein (deproteinization) in the Wizard method is based on the principle of "salting out" of protein, i.e. the phenomenon of decreasing protein solubility at high salt concentrations.

A typical protein in water has hydrophilic and hydrophobic regions (patches) on its surface. The hydrophobic patch forces water molecules to be highly ordered and immobilized when in contact with aqueous solvent. This effectively "freezes" the water around the hydrophobic region. These ordered water molecules are thermodynamically unstable and the water molecules surrounding the hydrophobic patch can be easily removed. When this happens, the hydrophobic regions of proteins interact with each other to form aggregates, causing protein to precipitate out of solution. At high salt concentration, salt ions become solvated, meaning they attract lots of water molecules. When freely available water molecules become scarce the "frozen" water molecules from around hydrophobic patches are pulled off, thereby allowing the hydrophobic regions to interact with other proteins rather than with solvent molecules. Proteins with a large number of or bigger patches will form aggregates sooner and precipitate first, while protein with a few patches will remain in solution even at high salt concentrations. Nucleic acids are fully hydrophilic molecules and cannot be precipitated with salt alone.

In consequence, the deproteinization method used by the Wizard DNA purification kit removes the majority of proteins, in particular those with large numbers of hydrophobic regions but leaves behind highly hydrophilic proteins.

Safety precautions

Each student should work only with his or her own cells. Any student who does not wish to isolate DNA from his or her own cells should be provided with human genomic DNA certified to be free of human immunodeficiency virus. This DNA is commercially available from a number of companies (e.g. Promega Co. and Sigma Co.).

Protocol

Collecting human cheek cells

1. Pour 10 ml of PBS into a 15 ml conical centrifuge tube. Transfer the solution into a paper cup. Pour all the solution into your mouth and swish vigorously for 30–40 seconds. Expel the PBS wash back into the paper cup.
2. Transfer the solution from the paper cup to a 25 ml Corex centrifuge tube and place it on ice.
3. Repeat step 1 one more time with fresh PBS. Expel the mouthwash back into the paper cup and transfer the solution to the same 25 ml Corex tube.
4. Collect the cells by centrifugation at 5,000 r.p.m. for 10 minutes at 4°C.
5. Pour as much supernatant as possible back into the paper cup. Be careful not to disturb the cell pellet. Discard the supernatant from the paper cup into the sink. Invert the Corex centrifuge tube with cells on a paper towel to remove the remaining PBS.

DNA purification

1. Resuspend the cells in 1,000 µl of PBS by gently pipetting the cells up and down. Transfer the cells into a microfuge tube.
2. Centrifuge the cells at maximum speed for 10 seconds in order to pellet them.
3. Remove 700 µl of PBS using a P1000 Pipetman. Resuspend the cells in the remaining 300 µl of PBS by vortexing.
4. Add 600 µl of nucleic lysis solution to the tube. Lyse the cells by pipetting up and down using a blue tip with a cut-off end (P1000). Continue until no visible cell clumps remain. The solution will become viscous indicating that most of the cells have been lysed.
5. Add 3 µl of RNase solution to the lysate and mix the sample by inverting the tube four to five times.
6. Transfer the tube to a 37°C water bath and incubate for 30 minutes.
7. Remove the tube from the water bath and allow the sample to cool to room temperature. This will take approximately 5–10 minutes.
8. Add 200 µl of ammonium acetate solution and mix it with the sample by pipetting up and down using a blue, cut-off tip. The sample should be

uniformly mixed before proceeding to the next step. If the sample cannot be mixed, you can vortex the tube for exactly 20 seconds.

9. Cool the tube on ice for 5 minutes.

10. Centrifuge the tube for 4 minutes at maximum speed. This should precipitate proteins, which will be visible at the bottom of the tube as a tight white pellet.

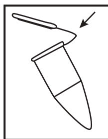
11. Set a P200 Pipetman to 150 µl and remove the supernatant containing DNA to a fresh microfuge tube. Remember to use a yellow tip with a cut-off end for this procedure. Record the volume of the solution transferred. Be very careful not to disturb the pellet. It is better to leave a small amount of the liquid above the pellet than risk transferring some pellet to the fresh tube.

12. Calculate a 0.6 volume of the solution transferred and add this amount of isopropyl alcohol to the tube. This volume should be approximately 600 µl.

13. Precipitate DNA by inverting the tube several times until the white cotton-like clump of DNA becomes visible.

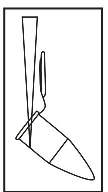
14. Insert the end of a glass hook into the precipitated DNA and swirl the hook in a circular motion to spool out the DNA. The DNA precipitate will adhere to the hook. **Note:** if at this step DNA does not form a clump and instead it forms several smaller fragments, do not try to collect them on a glass hook as described in step 14. Instead collect precipitated DNA by centrifugation as described in steps 16–20.

15. Transfer the hook with DNA into a microfuge tube filled with 1 ml of 70 percent ethanol. Wash the DNA by gently swirling the glass hook. Pour out the 70 percent ethanol and repeat the wash two more times. Proceed to step 21 to resuspend your DNA.



16. Place the tube in a centrifuge, orienting the attached end of the lid away from the center of rotation (see the icon in the margin). Centrifuge the tube at maximum speed for 5 minutes at room temperature.

17. Remove the tubes from the centrifuge. Pour off the ethanol into an Erlenmeyer flask by holding the tube by the open lid and gently inverting the end. Touch the tube edge to the rim of the flask and drain the ethanol. You do not need to remove all the ethanol from the tube. Return the tubes to the centrifuge in the same orientation as before. **Note:** when pouring off ethanol, do not invert the tube more than once because this could disturb the pellet.



18. Wash the pellet with 700 µl of cold 70 percent ethanol. Holding the P1000 Pipetman vertically (see the icon in the margin) slowly deliver the ethanol to the side of the tube opposite the pellet. Do not start the centrifuge. In this step the centrifuge rotor is used as a “tube holder” that keeps the tube at an angle convenient for ethanol washing. Withdraw the tube from the

centrifuge by holding the tube by the lid. Remove the ethanol as described in step 17. Place the tube back into the centrifuge and wash with 70 percent ethanol one more time.

19. After the last ethanol wash, collect the ethanol remaining on the sides of the tube by centrifugation. Place the tubes back into the centrifuge with the side of the tube containing the pellet facing away from the center of rotation and centrifuge for 2–3 seconds. For this centrifugation you do not need to close the lid of the tube. Remove collected ethanol from the bottom of the tube using a P200 Pipetman equipped with a capillary tip.

20. Add 50 µl of TE buffer to the tube and resuspend the pelleted DNA. Use a yellow tip (P200 Pipetman) with a cut-off end for this procedure. Gently pipette the buffer up and down directing the stream of the buffer towards the pellet. If the pellet does not dissolve in several minutes, place the tube in a 65°C water bath and incubate for 10–20 minutes mixing occasionally. Store the tube in a refrigerator until the next laboratory period.

21. To resuspend the DNA collected on the hook immerse the hook in 50 µl of TE buffer and move the hook back and forth until the DNA is dissolved. Store the tube in a refrigerator until the next laboratory period.

In this laboratory period you will carry out three experiments. In the first experiment you will determine the concentration and purity of the DNA purified in experiment 1. In the second experiment you will digest this DNA with enzymes that do not digest telomere DNA. We will use a mixture of two restriction enzymes, *Hinf* I and *Rsa*I. We will use a high concentration of the enzyme mixture in order to shorten the digestion time. In the third experiment you will separate TRF DNA fragments from the remaining digested genomic DNA using agarose gel electrophoresis.

Experiment 2: determination of DNA concentration and purity

The theory of measuring DNA concentration and its purity is described in Chapter 1.

Protocol

1. Determine the concentration of DNA by measuring the absorbance at 260 nm. Initially use a 1 : 20 dilution of the DNA. The absorbance reading should be in the range 0.1–1.5 OD_{260}. Special care must be taken to dilute the viscose solution of DNA when micropipettors are used. Most micropipettes will not measure the volume of a very viscose solution correctly.
2. To prepare a 1 : 20 dilution of DNA, add 100 μl of PBS to a microfuge tube. Prepare a wide-bore, yellow tip by cutting off 5–6 mm from the end of the tip with a razor blade. Withdraw 5 μl of PBS from the tube and mark the level of the liquid with a marking pen. Using the marked tip, draw DNA solution to the 5 μl mark and transfer it to the tube containing PBS. **Note:** DNA concentration should never be measured in water or TE buffer.
3. Determine the absorbance at 260 nm and calculate the DNA concentration using the formula DNA ($\mu g\,ml^{-1}$) = $OD_{260} \times 50 \times$ dilution factor.
4. Determine the purity of DNA by measuring the absorbance at 280 nm and 234 nm. Calculate the 260 nm : 280 nm and 260 nm : 234 nm ratios. Calculate the amount of DNA using equations (1.2) and (1.3) from Chapter 1.
5. Label the tube with your name and group number and indicate the DNA concentration in micrograms per milliliter.

Experiment 3: restriction enzyme digestion

Introduction

In this experiment you will learn how to digest genomic DNA with two

restriction enzymes in a single reaction. The enzymes used will be the *Hinf*I and *Rsa*I restriction endonucleases, which do not recognize DNA sequences present in the telomeric and subtelomeric regions of human chromosomes. The simultaneous digestion reaction using these enzymes is possible because both enzymes are active in the same buffer. The buffer that we will use is *Hinf*I enzyme buffer in which the second enzyme retains 100 percent activity.

These enzymes cleave non-telomeric DNA to low molecular weight fragments of less than 500 bp. These DNA fragments will move with the electrophoresis gel front. The digestion will generate telomere DNA fragments of 1–15 kb in size that are well separated from non-telomeric DNA. This makes it easy to obtain good hybridization results with telomeric probes.

You will use DNA isolated from your cells in this experiment. You will also digest two control DNA samples that will be given to you. One of these samples contains DNA with long telomeres and the other DNA with very short telomeres.

Background

The principles and theory of working with restriction enzymes are described in Chapter 2.

Technical tips

The rules for working with restriction enzymes and preparing digestion reactions are as follows.

1. Store restriction endonuclease at –20°C in a freezer that is not frost free at a concentration of 10 u μl^{-1} or higher.

2. The volume of the digestion reaction should be large enough that the restriction enzyme constitutes no more than 10 percent of the total volume. A 20–30 µl reaction volume should be used.

3. Use a DNA amount no greater than 10 µg added in a volume not to exceed one-third of the reaction volume. Addition of a large volume of DNA dissolved in TE buffer will decrease the Mg^{2+} ion concentration in the reaction, thereby inhibiting restriction enzyme activity. The minimum amount of mammalian genomic DNA that should be used in telomere length analysis is 1.0–1.5 µg per reaction.

4. Use 10–20 units of enzyme per microgram of DNA for telomere analysis. Although this is far more enzyme than is theoretically required, this excess assures complete digestion in the case of impurities in the DNA, decreased enzyme activity from storage, or pipetting errors during enzyme addition. This enzyme concentration will make it possible to complete restriction digestion in 1–2 hours.

Table 7.1 Preparation of restriction reactions

Tube number	Enzyme (10 u μl^{-1})	Add μl	Ten times buffer	Add μl	DNA type (1 μg)	Add μl	Water (μl)
1H	*Hinf*I/ *Rsa*I	1	*Hinf* buffer (ten times)	2	Yours		
2H	*Hinf*I/ *Rsa*I	1	*Hinf* buffer (ten times)	2	Partner's		
3C	*Hinf*I/ *Rsa*I	1	*Hinf* buffer (ten times)	2	Control (long)		
4C	*Hinf*I/ *Rsa*I	1	*Hinf* buffer (ten times)	2	Control (short)		

The reactions should be run using a mixture of the *Rsa*I and *Hinf*I enzymes prepared at a ratio of 1 : 1. The buffer used in the reaction should be buffer for the *Hinf* I enzyme (e.g. NEB buffer 2) rather then for the *Rsa*I enzyme (e.g. NEB buffer 1). The *Rsa*I restriction endonuclease retains 100 percent activity in buffer for the *Hinf*I enzyme while the *Hinf*I enzyme has only 70 percent activity in *Rsa*I buffer.

Protocol

1. Label five sterile 1.5 ml microfuge tubes as MRX, 1H, 2H, 3C, and 4C.

2. Calculate the amounts of DNA necessary to add in order to obtain 2–3 μg of DNA per reaction. Use DNA concentrations determined in the previous experiment for your DNA. The concentration of control DNA will be given to you. Record the amounts in Table 7.1.

3. Calculate the appropriate amount of water to add to each tube in order to bring the final volume to 20 μl. Record these values in Table 7.1. Check your calculations again before proceeding further.

4. Start to assemble the reactions by the addition of water. Remember the rule for reaction mixture assembly: the amount of water is always calculated last, but water is always added first.

5. Next add 2 μl of ten times restriction enzyme buffer to each tube.

6. Add the appropriate amount of DNA to each tube. Consult Table 7.1 for the amount and type of DNA to be added. For the addition of each DNA solution you will need to prepare a wide-bore yellow tip. Prepare each tip by cutting off 5–6 mm from the end of the tip with a razor blade. Set a P20 Pipetman to the required volume for each DNA type. Draw that amount of TE buffer into the tip and mark the level of the liquid with a marking pen. Discard the TE buffer. Using the same tip draw the DNA solution to this mark and transfer it to the reaction mixture. Pipette up and down several times to remove the viscose DNA solution from the inside of the pipette tip. **Note:** it is important to follow the procedure described above in order to prevent DNA shearing during pipetting.

7. Start the reactions by the addition of the enzyme mixture. Do not remove the tube containing the enzymes from the ice bucket.

8. Prepare a fresh wide-bore yellow tip, as described in step 6 and mix the enzyme with the reaction by pipetting up and down several times followed by a 5–10 second centrifugation.

9. Incubate the reactions for 2 hours in a 37°C water bath.

10. Begin preparing the agarose gel as described in the protocol of experiment 4.

11. After 2 hours of incubation, centrifuge each tube for a few seconds in order to remove condensation from the lid and then stop the reactions by adding 5 µl of stop solution to each digest. Mix well by pipetting up and down with using yellow tip and centrifuge for 5–10 seconds.

Experiment 4: agarose gel electrophoresis

Introduction

In this experiment you will use agarose gel electrophoresis for separating telomeric DNA fragments from fragments generated by digestion of the genomic DNA. Separation will be carried out on a large 0.8 percent agarose gel using TAE (Tris–acetate EDTA) buffer. These gels are well suited for Southern blotting. The genomic DNA fragments are small (approximately 500 bp) and will move ahead of the bromophenol blue dye while telomeric DNA will be located in the middle of the gel. To attain higher resolution, electrophoresis will be run at a low voltage gradient of $1\,V\,cm^{-1}$ overnight.

Background

The theory of agarose gel electrophoresis is described in Chapter 2 of this book.

Safety precautions

Ethidium bromide is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Wear gloves when handling ethidium bromide solution and gels containing ethidium bromide.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock, always disconnect the red (positive) lead first.

Technical tips

The size of the gel for telomeric DNA separation should be approximately 20 cm long, 15 cm wide, and approximately 4 mm thick. To obtain maximum

resolution, electrophoresis should be continued until the tracking dye has moved 70–80 percent the length of the gel. The size of the sample well can also affect the resolution of DNA bands. The optimal length of the sample well to use is 1 cm long and 2.0 mm wide. This size of the well will accommodate the whole sample prepared in the digestion experiment. The sample well bottom should be 0.5–1.0 mm above the gel bottom. Most of the commercially available submarine electrophoresis gel boxes fulfill the above requirements.

The molecular weight DNA standard that is suitable for this experiment should have fragments in the range of 1–23 kb. *Hind*III-digested lambda DNA is ideal for this application (e.g. NEB no. 301–2S).

Protocol

1. Seal the opened ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the teeth of the comb are approximately 0.5 mm above the gel bottom. To adjust this height, it is most convenient to place a plastic charge card (e.g. MasterCard) under the comb and adjust the comb height to a position where the card is easily removed from under the comb.

2. Prepare 1,500 ml of one times TAE by adding 30 ml of a 50 times TAE stock solution to a final volume of 1,500 ml of deionized water.

3. Place 150 ml of the buffer into a 500 ml flask and add the appropriate amount of agarose Weigh 1.2 g of agarose for a 0.8 percent agarose gel. Melt the agarose by heating the solution in a microwave oven at full power for approximately 3 minutes. Carefully swirl the agarose solution to ensure that the agarose is dissolved, that is no agarose particles are visible. If evaporation occurs during melting, adjust the volume to 150 ml with deionized water.

4. Cool the agarose solution to approximately 60°C and add 5 μl of ethidium bromide stock solution. Slowly pour the agarose into the gel-casting tray. Remove any air bubbles by trapping them in a 10 ml pipette.

5. Position the comb approximately 1.5 cm from the edge of the gel. Let the agarose solidify for approximately 20–30 minutes. After the agarose has solidified remove the comb with a gentle back and forth motion, taking care not to tear the gel.

6. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the gel box. For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench.

7. Add electrophoresis buffer to the buffer chamber until it reaches a level of 0.5–1 cm above the surface of the gel.

8. Load the samples into the wells using a yellow tip. Place the tip under the surface of the electrophoresis buffer just above the well. Expel the

sample slowly, allowing it to sink to the bottom of the well. Take care not to spill the sample into a neighboring well. During sample loading, it is very important to avoid placing the end of the tip into the sample well or touching the edge of the well with the tip. This can damage the well resulting in uneven or smeared bands. **Note:** samples must be loaded in sequential sample wells. When loading fewer samples than the number of wells it is preferable to leave the wells nearest the edge of the gel empty.

9. First load 8 µl of the lambda DNA standard into the first well. This standard will be given to you ready to be loaded onto the gel. Next load the entire sample (35 µl) using a P200 Pipetman. Load the samples in the following order: lambda DNA standard, 1H, 2H, 3C, 4C, and lambda DNA standard. **Attention:** standard DNA is loaded on both sites of the samples set.

10. Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the edge of the laboratory bench. Turn on the power supply. Adjust the voltage to approximately 1 V cm^{-1}. For example, if the distance between electrodes (not the gel length) is 40 cm, in order to obtain a field strength of 1 V cm^{-1}, the voltage should be set to 40 V.

11. Continue electrophoresis until the tracking dye moves at least half of the gel length. It will take the tracking dye approximately 17 hours to reach this position on a gel 20 cm long.

Next day

1. Turn the power supply off and disconnect the positive (red) lead from the power supply. Remove the gel from the electrophoresis chamber. To avoid electric shock always disconnect the red (positive) lead first.

2. Wrap the gel-casting tray with saran wrap and store in a 4°C refrigerator. Gels can be stored this way for 2–4 days.

THIRD LABORATORY PERIOD

Experiment 5: Southern transfer

Introduction

In Southern transfer, DNA fragments are transferred from the gel to the membrane used for hybridization with a probe. After electrophoresis, the DNA is denatured, transferred to a nylon membrane, and immobilized. The transfer method preserves the separation pattern of DNA on the membrane. Before hybridization DNA will be fixed to the membrane by UV irradiation. The membrane is subsequently used for hybridization with a DIG-labeled telomere probe. DNA of small sizes can easily be transferred directly to the membrane after denaturation. However, large DNA fragments, those bigger than several kilobases, will not transfer efficiently. For this reason, the procedure involves the breakage of large DNA fragments *in situ* before the transfer.

In this experiment you will be using a capillary downward transfer system, which is sometimes called TurboBlotting. This transfer system offers greater speed, target resolution, and convenience than the traditional upward capillary blotting procedure that you learned in the DNA fingerprinting experiment.

The method takes advantage of gravity for accelerating the flow of the transfer solution. Moreover, the downward transfer technique eliminates the need for heavy weights on top of the capillary stack, thus eliminating gel compression that can prevent efficient transfer. The time of transfer can be shortened from 17 hours to approximately 2 hours without lost of transfer efficiency.

Background

The principles and methods of the Southern blotting technique are described in Chapter 2.

Safety precautions

The agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Students should wear gloves when handling these gels. Only powder-free gloves should be used because the procedure uses chemiluminescence for the detection of hybridization. The presence of talcum powder will result in the formation of a “spotted” background. Discard the used gel into the designated container.

When viewing and photographing the gel with a UV transilluminator, gloves, UV-protective glasses, and a face mask should be used all times.

Technical tips

The gel can be photographed using Polaroid film type 667 (ASA 3000) or using a computer imaging system. However, measurement of the telomere length can be made without a photographic image of the gel.

The use of chemiluminescent detection requires a positively charged nylon membrane. These membranes have very low background with chemiluminescent detection. Some producers have developed a chemically optimized, positively charged nylon membrane for chemiluminescent detection. Evaluation of all of the types of commercially available, positively charged nylon membranes showed that the best membrane for chemiluminescent detection is the Magna Graph membrane manufactured by Osmonics Co (Surzycki, 2000).

Instead of the capillary downward transfer system (TurboBlott), the standard upward capillary transfer can be used without seriously affecting the quality of the transfer. The time required for this transfer is 17 hours (overnight) and disassembling of the blot and cross-linking of DNA to the membrane should be done the next day. For overnight blotting, follow the technique described in Chapter 2.

The best signal to noise ratio for the Magna Graph membrane is achieved when DNA is cross-linked by UV light irradiation on both sides of the membrane. Alternatively, the membrane can be wrapped in aluminum foil and baked in an oven at 80°C for 1 hour. The baking step immobilizes DNA on the membrane. Membranes can be stored at room temperature practically indefinitely.

Protocol

1. Transfer the gel to a glass Pyrex dish and trim away any unused areas of the gel with a scalpel. Cut off the gel below the bromophenol blue dye. This part of the gel contains small fragments of genomic DNA. Cut the lower corner of the gel at the bottom of the lane with the first size standards. This will provide a mark for orienting the hybridized bands on the membrane with the bands in the gel.

2. Transfer the gel to a UV transilluminator. Place an acetate sheet on the top of the gel and draw an outline of the gel with a felt-tip pen. Mark the positions of the wells and the position of the cut corner. It is very important that your drawing be as precise as possible. Label the contents of each well on the acetate sheet and mark the bottom left corner of the gel (under well number 1). This will help you locate the positions of the hybridization signals in your Southern blots. Turn on the transilluminator and mark the positions of standard DNA bands on the acetate sheet.

3. Photograph the gel. Use a setting of 1 second at F8 of Polaroid 667 film. One can also use a computer-imaging system to record the results.

Note: this step can be omitted since determination of telomere length does not require photographic imaging of the gel.

4. Transfer the gel back to the Pyrex dish and add enough 0.25 N HCl to allow the gel to move freely in the solution. This will take approximately 150–200 ml of solution for a standard gel size.

5. Place the dish on an orbital shaker and incubate for exactly 10 minutes rotating at 10–20 r.p.m. Decant the acid carefully.

6. Briefly rinse the gel in water (10–20 seconds) and proceed immediately to the next step.

7. Add 200 ml of denaturating solution to the dish and incubate for 20 minutes with gentle agitation.

8. Decant the denaturating solution, holding the gel with the palm of your hand and repeat steps 7 and 8.

9. Rinse the gel once in water to remove most of the denaturating solution trapped on the surface of the gel.

10. Add 100–200 ml of neutralization solution to the dish and treat the gel for 20 minutes with gentle agitation.

11. Discard neutralization solution and repeat step 10.

12. While the gel is being treated, prepare the nylon membrane for transfer. Cut three sheets of Whatman 3MM paper to the size of the gel. Use the drawing of the gel prepared at step 2 as a guide. Then cut the nylon membrane using the 3MM paper cut out as the template. Use gloves and only touch the edges of the membrane. Immerse the membrane in 20 times SSC for 2–3 minutes.

13. Set up the blot. Refer to Fig. 7.5 when setting up the TurboBlotter System.

14. Place the transfer device stack tray on the bench making sure it is leveled.

15. Cut five pieces of Whatman 3MM paper to the size of the GB004 blotting paper provided to you. Place 20 sheets of dry GB004 blotting paper in the stack tray.

16. Dip one sheet of Whatman 3MM paper (prepared in step 15) in 20 times SSC solution and place on the stack of dry GB004 papers.

17. Place the wet transfer membrane on the stack. Remove any air bubbles trapped between the membrane and wet Whatman 3MM paper.

18. Cut away and remove the gel above the wells. Place the agarose gel with **sample wells up** on top of the membrane. Make sure there are no air bubbles between the gel and the membrane. **Note:** because the gel is thinner in the well area, this part of the gel must be removed because the transfer solution may pass preferentially through this part of the gel causing uneven DNA transfer.

19. Wet three sheets of the Whatman 3MM paper prepared in step 15 in 20 times SSC. Wet the top surface of the gel with 2–5 ml of 20 times SSC and place wet Whatman 3MM paper on top of the gel.

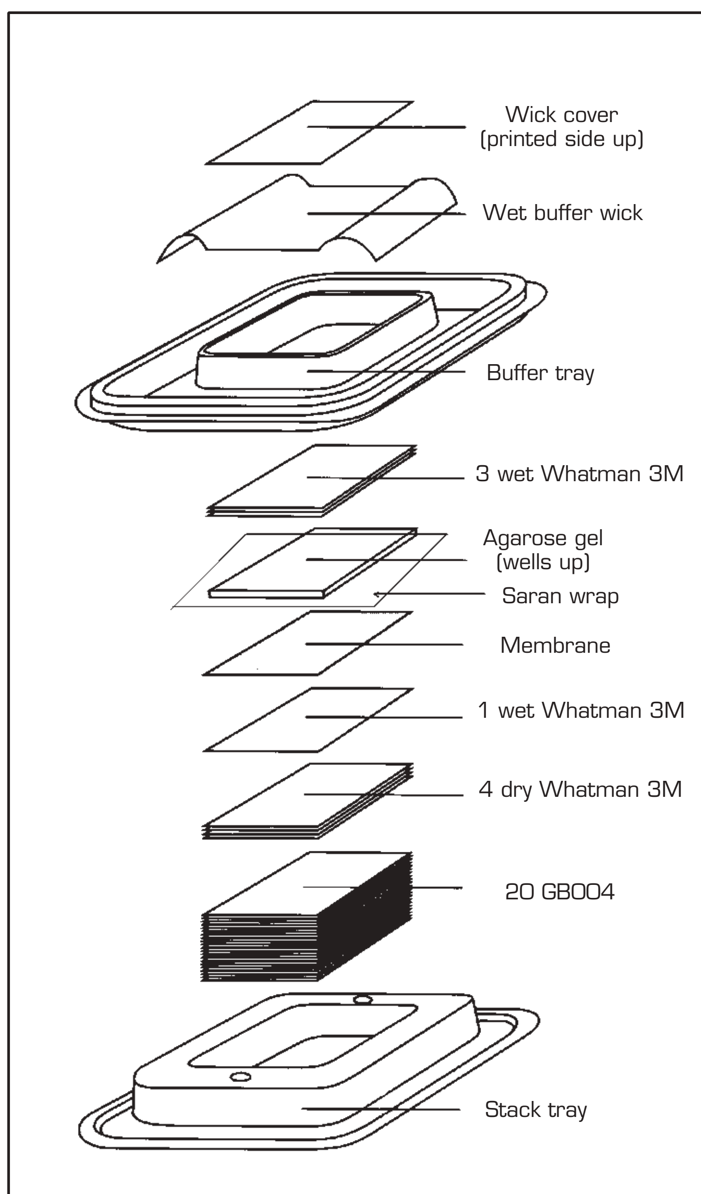


Figure 7.5 Preparation of TurboBlotter for transfer of DNA to a nylon membrane.

20. Cover the entire surface of the gel and surrounding area with saran wrap. With a razor blade "cut away" the saran wrap covering the gel itself. This will leave an opening over the gel.
21. Attach the "buffer tray" of the transfer device to the bottom tray using the circular alignment buttons to align both trays. Fill the buffer tray with 125 ml of 20 times SSC.

22. Start the transfer by connecting the gel stack with the buffer tray using the pre-cut "buffer wick" (included in each blotter stack) pre-soaked in 20 times SSC. Place the wick across the stack so that the short dimension of the wick completely covers the blotting stack and both ends extend into the buffer tray. Place the "wick cover" on top of the stack to prevent evaporation. Make sure the edges of the wick are immersed in the transfer buffer.
23. Continue the transfer for 3 hours. Additional transfer time may be required for gels thicker than 4 mm or larger sized nucleic acids. **Note:** if necessary or for convenience this transfer can be carried out overnight. In this case steps 24–26 should be performed the next morning.
24. Disassemble the blot. Using forceps remove the membrane and place it "DNA side" up (the side that contacted the gel) on a clean sheet of Whatman paper. Write your group number with a pencil on the corner of the membrane.
25. Place the membrane on a sheet of dry Whatman 3MM paper. Do not allow the membrane to dry at any time. Place the membrane into a UV oven. Irradiate the damp membrane to immobilize DNA using the automatic setting of the UV oven. Irradiate both sides of the membrane.
26. Store the membrane at room temperature in a plastic bag.

Experiment 6: DNA hybridization

Introduction

In this experiment you will hybridize DIG-labeled probe that recognizes telomeric and subtelomeric DNA to the membranes prepared in experiment 5. Probe will be given to you. Hybridized probe will be detected with DIG-specific antibody covalently coupled to alkaline phosphatase. The immobilized probe–antibody complex on the membrane will be visualized using chemiluminescent substrate (CDP-Star). Dephosphorylation of the substrate by the enzyme results in the emission of light, which will be captured on photographic film.

Hybridization will be carried out using a hybridization oven in specially designed hybridization roller bottles. The hybridization procedure uses lower stringency conditions that are necessary for hybridizing short tandem repeats of the telomeric probe. The hybridization time is also substantially shorter than in the standard hybridization procedure. This is because telomeric repeat sequences are present at high concentrations and the complexity of the probe is low.

Background

The theory of hybridization is described in Chapter 2.

Technical tips

Hybridization can be performed in glass or plastic dishes or in sealed plastic bags rather than in a hybridization oven and large roller bottles. Using a hybridization oven is the most convenient method for a large class. No more than one membrane should be placed into the roller bottle. Some membrane overlapping will not affect hybridization results.

Using dishes rather then hybridization bottles will substantially increase the volume of reagents used in each step. When using dishes, the minimum volume of the hybridization solution should be 0.04 ml solution per 1 cm^2 of membrane surface area. The volume of all other solutions should be approximately four to five times larger than for hybridization. Care should be taken that the membrane is sufficiently covered with solutions at all the times and that it can float freely in the container.

One of the great advantages of the DIG system is the stability of the labeled probe. The probe can be stored for an indefinite time at −20°C. It is important to collect hybridization solution with probe after hybridization

and store it at –20°C. This probe solution can be reused at least three to four times.

Protocol

Pre-hybridization and hybridization

1. Place the dry membrane into a roller bottle. Make sure that the side of the membrane with DNA is facing away from the glass.
2. Pour 10 ml of Dig Easy solution into a 15 ml plastic centrifuge tube and prewarm it for 10 minutes in a 42°C water bath. Pour it as fast as possible into the roller bottle with a membrane. Close the bottle tightly and label it with your group number.
3. Place the roller bottle into the hybridization oven and allow it to rotate at a slow speed (2–4 r.p.m.) for 30 minutes at 42°C.
4. Ten minutes before the end of pre-hybridization, begin to prepare the probe for hybridization. Add 10 ml of Dig Easy solution into a 15 ml conical centrifuge tube. Add 2 μl of DIG-labeled telomere probe, close the tube tightly, and invert it three to four times to mix the probe with Dig Easy. Place the tube into a 42°C water bath and incubate for 10 minutes.
5. Retrieve the roller bottle from the hybridization oven. Open the roller bottle and pour the pre-hybridization solution into a storage bottle. Pre-hybridization solution can be stored and used again.
6. Remove the tube with the probe from the 42°C water bath and add it to the roller bottle. Return the roller bottle to the oven and allow it to rotate slowly at 42°C for 2 hours.
7. Retrieve your roller bottle from the hybridization oven and pour off the hybridization solution into a 15 ml centrifuge tube. The probe can be stored in a –20°C freezer and reused three to four times.
8. Add 20 ml of washing solution II (two times SSC and 0.1 percent sodium deodecyl sulfate (SDS)) to the roller bottle. Place it into the hybridization oven and allow it to rotate at slow speed at room temperature until the next laboratory period.

FIFTH LABORATORY PERIOD

In this laboratory period, you will continue the hybridization experiment. First, you will remove mismatched hybrids from the membrane using a washing procedure. Second you will prepare your membrane for signal detection by chemiluminescence.

Protocol

Washing reaction

1. Retrieve your roller bottle from the hybridization oven and discard solution II.
2. Add 20 ml of fresh washing solution II to the bottle and place it back into the hybridization oven. Rotate it at maximum speed for 5 minutes.
3. Remove the roller bottle from the hybridization oven and pour off and discard solution II. Drain the liquid well by placing the bottle on end on a paper towel for 1 minute.
4. Add 20 ml of washing solution III prewarmed to 50°C. Place the roller bottle into the hybridization oven preheated to 50°C. Rotate it at a slow speed for 20 minutes.
5. Pour off solution III and discard it. Drain the solution well by placing the roller bottle on end on a paper towel for 1 minute. Repeat the washing with solution III one more time.
6. Remove solution III and drain the roller bottle well as described above.

Preparation of membrane for detection

1. Add 20 ml of buffer A (washing buffer) to the tube. Cool the oven to room temperature and rotate the roller bottle at maximum speed for 2–5 minutes.
2. Retrieve the roller bottle from the oven and discard buffer A. Add 10 ml of blocking solution (buffer B) to the tube. Incubate for 30 minutes, rotating slowly at room temperature.
3. Pour off and discard buffer B. Invert the roller bottle over a paper towel and let it drain well for 1 minute.
4. Add 10 ml of blocking buffer (buffer B) to a plastic conical centrifuge tube and add 1 µl of anti-DIG–alkaline phosphatase solution (antibody solution). Mix well and add the solution to the roller bottle with the membrane.
5. Incubate for 30 minutes, rotating slowly at room temperature. **Note:** the working antibody solution is stable for approximately 12 hours at 4°C.

Do not prolong the incubation with antibody over 30 minutes. This will result in high background.

6. Pour off and discard the antibody solution. Drain the liquid well by placing the roller bottle on end on a paper towel for 2 minutes.

7. Add 30 ml of buffer A to the roller bottle and wash the membrane at room temperature, rotating at slow speed for 15 minutes. Repeat this wash one more time.

8. Retrieve the roller bottle from the oven and move the membrane towards the tube opening by gently shaking the bottle. Do not open the tube for this operation. Open the tube and discard buffer A. Remove the membrane to a Pyrex dish placed on a rotary shaker and add 200 ml of buffer C. Wash the membrane, rotating it slowly for 25 minutes. Discard buffer C and repeat the wash one more time.

9. Place a plastic bag on a sheet of Whatman 3MM paper and add 10 ml of buffer C. Wearing gloves transfer the membrane from the Pyrex dish into the plastic bag. Open the bag and insert the membrane into it. Place the membrane into the pool of buffer and move it with your fingers to the end of the bag. Leave as little space as possible between the membrane and the end of the bag. This will limit the amount of expensive chemiluminescent substrate necessary for filling the bag. Pour off buffer C from the bag. Remove the remaining liquid from the bag by gently pressing it out with a Kimwipe tissue. **Note:** do not press strongly on the membrane because this will increase the background. Most of the liquid should be removed from the bag, leaving the membrane slightly wet. At this time, a very small amount of liquid will be visible at the edge of the membrane.

10. Open the end of the bag slightly, leaving the membrane side that does not contain DNA attached to the side of the bag. Add 0.9–1 ml of CDP-Star solution directing the stream towards the side of the bag. Do not add solution directly onto the membrane.

11. Place the bag on a sheet of Whatman 3MM paper with the DNA side up and distribute the liquid over the surface of the membrane by gently moving the liquid around with a Kimwipe tissue. Make sure that the entire membrane is evenly covered. Do not press on the membrane because this will cause "press marks" on the film. Gently remove excess CDP-Star from the bag by guiding excess solution towards the open end of the bag and onto the Whatman paper with a Kimwipe. Make sure that the membrane remains damp. **Note:** at this point small liquid droplets will be visible on the edge of the membrane, but liquid should not be present on the membrane surface. Seal the bag with a heat sealer.

12. Place the bag in an X-ray film cassette, DNA side of the membrane up. In a darkroom, place X-ray film over the membrane. Expose the film for 5–10 minutes at room temperature. Open the cassette and develop the film using standard procedures for film development. **Note:** maximum light emission for CDP-Star is reached in 20–30 minutes, the light emission

remains constant for approximately 24 hours, and the blot can be exposed to film a number of times during this period. The best results are usually obtained when the membrane is exposed the next day.

13. After exposure, store the bag with membrane at 4°C. The membrane can be stored this way for several weeks.

Experiment 7: analysis of TRF length

Introduction

In this laboratory period you will analyze your hybridization autogram. You will determine the mean length of your telomeres, the most frequently occurring telomere length, and the variability in length of your telomeres set. The film will be scanned and image transferred into a computer. The image will be analyzed manually or using the program Telometric.

Background

The telomere length in human cells can vary over one order of magnitude. This is because not all of the cells that you collected are at the same age and because there is considerable heterogeneity of the telomere length even for cells of the same age. The autogram of the telomere sequences or more precisely of the TRF sequences will not show a single band but a number of overlapping length bands forming a smear. Thus, analyzing the TRF length of a population of cells will provide the average TRF length of this sample but not the length of individual TRFs.

The average TRF length can be estimated using an autogram by visually comparing the size of the signal smear to molecular weight markers. However, this is a very imprecise method, mostly because we assume that the most intense part of the smear represents the most frequent band sizes of the TRFs. This is not always true because even a few very long TRF repeats will generate a strong hybridization signal since they have many copies of the same repeat. The precise determination of the size and distribution of the telomere cannot be carried out using the distribution of the intensities of hybridization of the probe having a single repeat element. The distribution of densities should be converted to the distribution of copy numbers, which is not necessarily proportional to film densities.

There are two ways to correct for this. The first method is to integrate the signal intensity over the entire TRF distribution as a function of TRF length. This can be done using the equation

$$L_{\mathrm{TRF}} = \frac{\sum(\mathrm{OD}_i \times L_{\mathrm{i}})}{\sum \mathrm{OD}_{\mathrm{i}}} \tag{7.1}$$

where OD_i is the chemiluminescent signal intensity and L_i is the length of the TRF fragment (in base pairs) at position i on the gel image, respectively.

This calculation takes into account the higher signal intensity from larger TRF fragments resulting from multiple hybridization of the telomeric-specific probe.

The second method is more sophisticated since it converts the signal intensity at a given point to the relative copy number by adjusting for the number of probes bound to the DNA. This method was recently developed and is performed using the computer program Telometric (Grant et al., 2001). The relative copy number for each DNA is calculated by the equation

$$C_i = \frac{I_i - B_i}{L_i} \qquad (7.2)$$

where C_i is the relative copy number across single autographic line i, I_i is the integrated signal intensity in line i, L_i is the DNA molecular weight (kilobases) in line i, and B_i is the background of the autogram, i.e. the intensity at the position on autogram with no DNA present.

The program generates a plot of the relative copy number of repeats versus molecular weight. This gives a transparent presentation of the actual distribution of the telomere lengths. From this graph a statistic is generated giving the mean, median, and mode of the molecular weight for the telomeric regions. In addition, the variance and semi-interquartile range (heterogeneity for asymmetric distribution) is also calculated.

Technical tips

In order to calculate telomere length successfully, the exposure time of the film should be as short as possible in order to create an autogram having densities of the exposure in the linear range of the film.

Manual determination of telomere length follows the recommendation given in the Roche instruction manual for the Telo TAGGG kit (Roche Molecular Biochemicals Co., 1999). This technique can be carried out on any Wintel or Mac platform. The image should be scanned and transferred to a graphic program capable of measuring the density of the image. The best free program to use is NIH Image, which can run on PC and Macintosh computers. The Macintosh version can be downloaded from //rsb.info.nih.gov/nih-image/ and the PC version (Windows 98 and up) from www.scioncorp.com.

The Telemetric program can only run on Macintosh computers and is written as a macro for the NIH Image program. The macro can be downloaded from www.biotechniques.com. It should be installed into the NIH program before use.

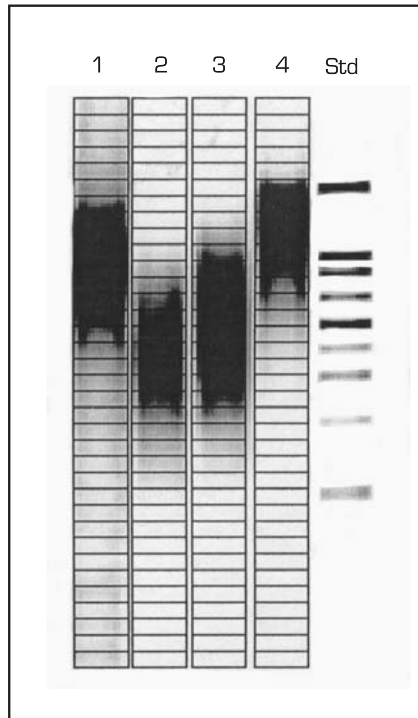


Figure 7.6 Determination of the average length of the TRF region using the manual procedure.

Protocol

Manual method

1. Align the X-ray film with the gel drawing made previously. The outline of the filter should be visible on the film. Transfer the positions of DNA standard bands onto the film. Measure the electrophoretic mobility of each standard band and plot it as a function of the log of fragment size (in kilobases) versus distance traveled. This standard plot will be used to determine the molecular weight of an unknown sample.
2. Divide the scanned image into a grid of columns consisting of each gel lane and rows that cover the entire vertical length of the image. The rows should be 0.5 cm long. See Fig. 7.6 for clarification.
3. Scan the autoradiogram with a computer scanner and save the image into your folder on the hard disk.
4. Determine the size of the TRF present in each square using the standard curve prepared in step 1. The TRF size is taken as the molecular weight (in kilobases) at the middle of each square. Record the size for each square in a table. An example of such a table is shown in Table 7.2.

Table 7.2 Calculation of the TRF average length

Square number	Sample number (line)	OD (mean)	L (size in kb)	OD × L
1	1	185	12.0	2,220
2	1	200	9.0	1,800
3	1	210	6.0	1,260

5. Open the program NIH Image. Import your file into this program. Click on the **file** and **import** buttons. Select your file name and click on it. The image of your autograph with labeled squares should appear on the desktop.
6. Determine the density (OD) for each square. To do this click on the square tool and on **analyze**. Click on **measure** and start outlining each square of your image. The integrated relative density of each square will appear in the result window after clicking on **show results**. The first number is the area of your square and the second the average density (Mean). Record this value in a table opposite the size of the corresponding square.
7. Calculate OD × L and record the result in a table. Calculate the average length of the TRF using equation (7.1). The following is an example of a calculation using data from the table: L_{TRF} = (2220 + 1800 + 1260)/180 + 200 + 210), i.e. L_{TRF} = 5280/590 and, therefore, L_{TRF} = 8.9 kb

Computer method

1. Align the X-ray film with the gel drawing made previously. The outline of the filter should be visible on the film. Transfer the positions of DNA standard bands onto the film.

2. Scan the autoradiogram with a computer scanner and save the image into your folder on the hard disk.

3. Open the program NIH Image. Open the **analyze** menu and select **option**. Select **user1**, **user2**, and **angle**. Select **max measurement** and set it to 2500.

4. Import your file into the program. Click on the **file** and **import** buttons. Select your file name and click on it. The image of your autograph with the labeled position of standards should appear on the desktop.

5. Load the Telometric macro. Go to **special** and click **load macros**. Browse the file system and find Telometric. Load it into the NIH Image program.

6. Click on **special** and start the **calibrate image** macro. Next click the mark on your autogram corresponding to the largest molecular weight standard. Enter the value of it (23 kb) in the window that has appeared and click OK. Click on the remaining standard molecular weight lines one at the time

and enter the corresponding molecular weight values in each window. These values are 9.4, 6.3, 4.3, and 2.3.

7. Press the **option/alt** key to enter these values into the program. You will see a window that states: calibration included five standards. Click the **OK** button.

8. Next you need to subtract the background gray scale. Outline the rectangular area on your image that contains a representative amount of background using **square tool**. Open **special** and click on the **specify background** macro. You should see a window that states: background region has been specified. Click the OK button.

9. Click the arrow on the NIH Image palette and outline the rectangular area that covers the first line of your sample. Adjust the size and the shape of the rectangle to include the entire area of the DNA "smear" in this line. Open **special** and click on the **outline first line** macro. An NIH Image window titled duplicate will appear with the first line outlined and numbered.

10. Click the arrow on the NIH Image and outline the next line as described in step 9. Open **special** and click on **outline next line**. Repeat this procedure for all lines of your autogram.

11. Open **special** and click on the **generate plots** macro. Plot windows that show graphs of a telomere length frequency distribution for each outlined line will appear. The *y*-axis of the graph is a relative telomere copy number and the *x*-axis is telomere length (in kilobases). Print this graph.

12. Open **special** and run the **generate statistic** macro. A table will appear that lists values (in kilobases) for the mean, median, mode, variance, and semi-interquartal for each line. Print this table. The mean value gives the mean telomere length. The mode value describes the most frequently occurring telomere length. The variance describes the variance in telomere length from which the error of the mean value can be calculated. The semi-interquartal and median values indicate the extent of heterogeneity of your telomeres. Large median and semi-interquartal values indicate large length heterogeneity of your telomeres.

13. Compare the values of the mean, median, mode, variance, and semi-interquartal with standard DNA and the DNA of your partner. Answer the following questions. Are your telomeres long or short? What is the molecular weight of the most frequent telomeres in your cells and the cells of your partner? Compare these values to the standard DNA. How homogenous are your telomeres and the telomeres of your partner and the telomeres of the standards given to you?

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