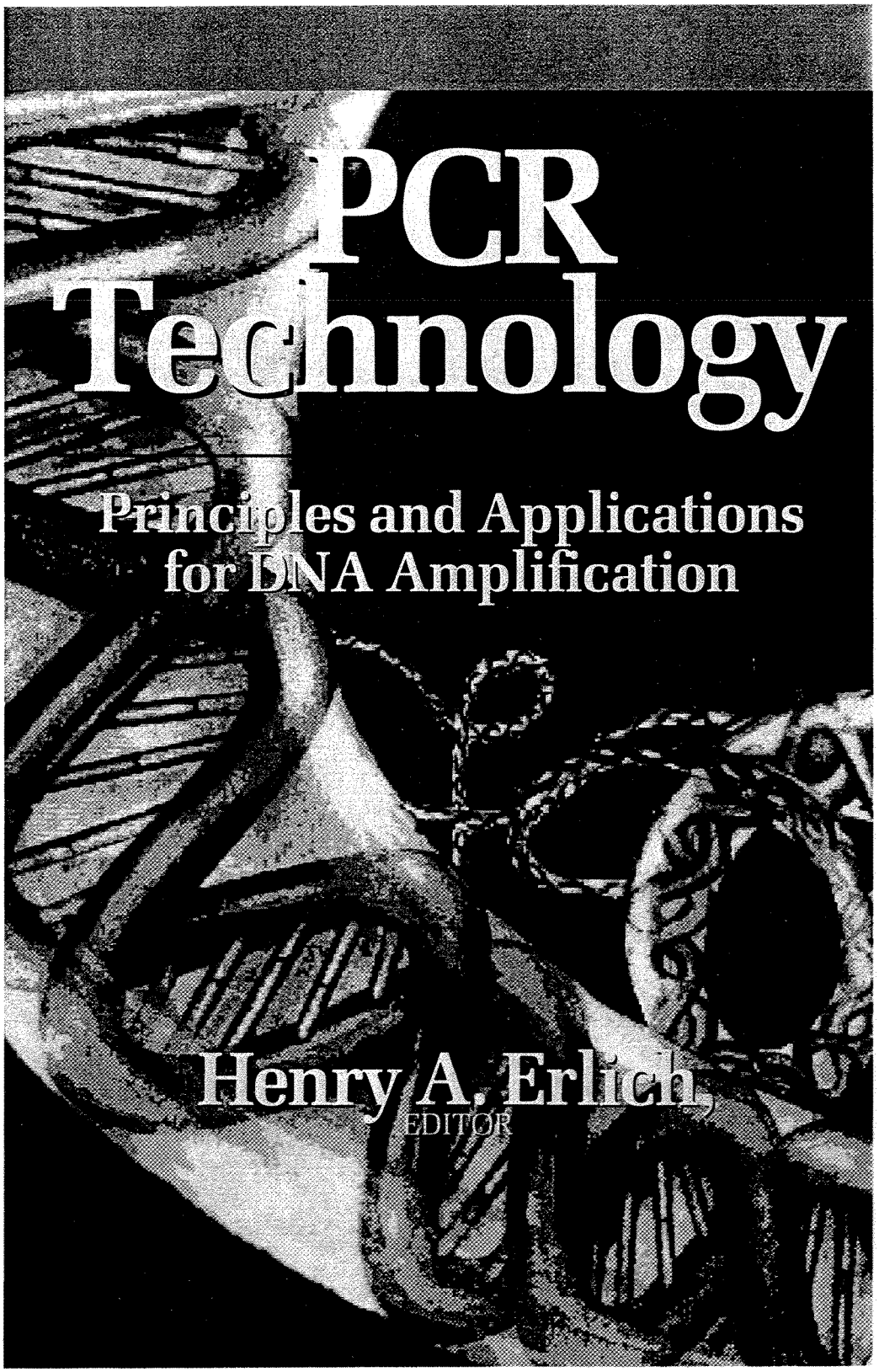

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CHAPTER 4

Simple and Rapid Preparation of Samples for PCR

Russell Higuchi

INTRODUCTION

Sample preparation for PCR can be as simple and rapid as adding cells directly to the PCR.[1] Things are not quite this easy, however, for all samples and applications of PCR. In this chapter, I will discuss what determines the amount of preparation necessary and present example protocols for the rapid preparation for PCR of DNA and RNA from various sources.

In the simplest case cited above, enough of the DNA from a small number of tissue-culture cells was made accessible to PCR merely by the lysis of the cells during the heat-denaturation step. Because of the ability of PCR to produce detectable amounts of product even from a few genome equivalents of DNA,[2] it is not absolutely necessary that making the DNA available for PCR be a particularly efficient process. However, there are several reasons why one would prefer it to be. These are: 1) the more template molecules available, the less likely are false results due to either cross-contamination between samples or "carryover" of analogous PCR

product from earlier amplifications, 2) if the PCR amplification is not particularly specific or efficient, or the target sequences are present infrequently among a large number of cells (see below), the yield of product will be inadequate without enough starting DNA, and 3) it is more difficult to infer target DNA content from PCR product yield when the fraction of the starting DNA available to PCR is uncertain.

SAMPLE PREPARATION

For the direct, cell-lysis protocol, the easiest way to increase the number of templates available to PCR should be to increase the number of cells added to the reaction. As shown in Figure 4 of Saiki *et al.*,[2] however, as the number of cells added goes above 600, the yield of product, unlike the yield from an equivalent amount of purified genomic DNA, does not increase with the addition of more cells, and begins to decline at 4800 cells. Due to the observed "cell-debris," we infer that either more and more DNA is being trapped or that inhibition of the PCR process is occurring or both.

For some applications, this limit on available template is unacceptable. This is true for the screening of samples for infectious agents such as HIV, which occurs in only a few white blood cells out of many thousands. One needs to efficiently screen all the DNA from tens of thousands of cells in order to pick up the HIV DNA from the few infected cells. Other applications where this is important include the screening of transgenic organisms for the transgene,[3] if the transgene is present in only a minority of the cells of a tissue, and the monitoring of bone-marrow transplants for the relative amounts of donor and recipient cells over time.

The problem, then, is to find conditions that simultaneously release DNA and/or RNA from larger numbers of cells in a form suitable for PCR while preserving the activity of *Taq* DNA polymerase. Methods of DNA purification from animal cells often use detergents to solubilize cell components and a proteolytic enzyme to digest away proteins, probably mainly histones, that would otherwise remain strongly bound to the DNA. This procedure is usually followed by extraction with organic solvents to remove residual proteins and membrane components followed by steps, such as precipitation of nucleic acids by ethanol, to remove traces of the organic solvents. Since *Taq* DNA polymerase activity is not significantly affected by certain non-ionic detergents,[4] and since Proteinase K could be inactivated by heat, cells were added directly to a PCR containing non-ionic detergents and Proteinase K, but not yet containing *Taq* DNA polymerase. The Proteinase K was given time to work, and after the residual proteinase activity had been destroyed by incubation at 95°C for 10 min, *Taq* DNA polymerase was added and amplification cycles begun.

As shown in Figure 1, comparative yields of PCR product were obtained from up to 70,000 cells or the equivalent amount of purified DNA using this procedure (see Protocol A, end of chapter). This protocol is routinely used to screen 300,000 cells at a time in an HIV detection assay[5] (S. Kwok, personal communication). Evidently there is little inhibition of PCR by the

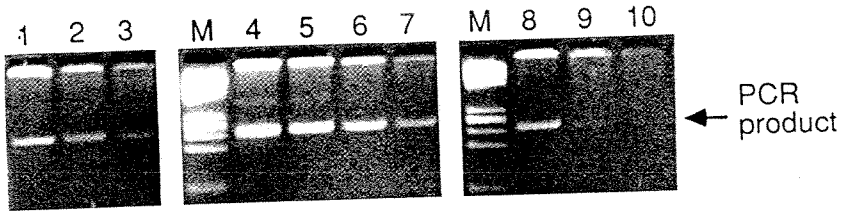

Figure 1. Amplification of class II HLA-DQα sequences[2] from whole blood (lanes 4-7) prepared using Protocol B; density gradient purified mononuclear cells (lanes 8-10) prepared using Protocol A or from purified DNA (lanes 1-3). The number of nucleated cells added were: 250,000, 125,000, 60,000, and 30,000 (lanes 4-7, respectively; 67,000, 33,500, and 16,750 (lanes 8-10, respectively). Purified DNA in amounts equivalent to that from 67,000, 33,500, and 16,750 cells was amplified and run in lanes 1-3. Twenty-five cycles of PCR were performed, 10% of each PCR was electrophoresed on this NuSieve (FMC) agarose gel. M is 0.5 μg of øX 174 DNA cleaved with *Hae*III.

remaining cell components and most of the DNA is available to be amplified. This procedure has worked with washed tissue culture cells and density gradient purified (and washed) peripheral mononuclear cells.

If the same procedure is attempted with whole blood rather than purified mononuclear cells, inhibition of PCR occurs with the addition of as little as 1 μl blood to a 0.1-ml reaction. There is a noticeable precipitate in the reaction tubes and purified DNA "spiked" into these samples is not amplified. The testing of various blood components indicates that porphyrin compounds derived from heme may be the most inhibitory substances found in blood. Hematin has been found to inhibit PCR at as low a level as 0.8 micromolar (Walsh and Higuchi, unpublished observation).

To separate quickly porphyrin compounds from nuclear DNA, Protocol B depends on the osmotic lysis of cells and the pelleting of nuclei and cell debris. Hemoglobin released from RBCs is washed away in several pelleting and washing steps. The final resuspension of the pellet containing nuclear DNA is in a Proteinase K/detergent solution similar to that used in Protocol A; from this point the two protocols are essentially the same.

Figure 2 shows the results of an experiment using Protocol B in which a few fibroblasts containing transfected neomycin resistance genes are added to whole blood and detected at dilutions up to 1 per 10,000 nucleated blood cells; a total of 100,000 nucleated cells in whole blood (about 13 μl) were initially added. 300,000 cells have been successfully screened in this manner.

A simpler protocol has been published that uses boiling to simultaneously lyse cells, release DNA, and precipitate hemoglobin.[6] DNA found in the

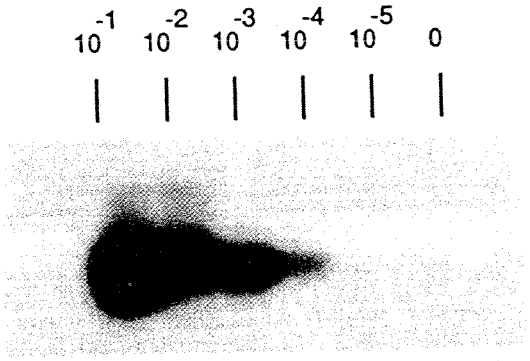

Figure 2. Fibroblast cells transfected with a bacterial neomycin resistance gene (C. Perez, personal communication) were added to whole human blood at a ratio of 1/10, 1/100, 1/1000, 1/10,000, and 1/100,000 nucleated cells. The preparation of the blood was as per Protocol B. The amount of the final lysate sampled was equivalent to the DNA from 100,000 cells. PCR was performed to detect the neomycin resistance gene. Shown is the autoradiograph of a Southern blot performed upon the PCR product and probed with a ^{32}P-labeled neomycin resistance gene probe.

supernatant is added directly to a PCR. However, both the amount of DNA released to the supernatant and the amount of supernatant that can be added to a PCR without inhibition is limited. As discussed above, an upper limit on the amount of template DNA may not be suitable for some applications.

For the preparation of DNA for PCR from tissues other than blood, these same principles apply. Given the amplification potential of PCR, a small amount of DNA can be adequate. Thus, crude lysates can be used by adding small enough amounts that inhibition is avoided. However, if screening for targets that are not present in every cell is required or if the PCR system used is not efficient, then additional effort may be necessary to make available more template DNA. Protocol C below is such a protocol, using non-ionic detergents and Proteinase K as above, that can be used to screen for viral infections in epithelial cells collected as clinical swabs. Protocol D is a protocol for use on single, plucked hairs - a convenient easily obtained source of DNA.

The rapid preparation of mRNA for amplification by PCR has different requirements. The preparation must also be compatible with reverse transcriptase, which is used to make the amplifiable cDNA template, and RNA degradation by endogenous RNAse should be obviated. Protocol E uses DEP to inactivate RNAse and the non-ionic detergent NP40 to lyse cells without disrupting nuclei. mRNA is separated from DNA by centrifugation of the nuclei. DEP can be dissipated by heat and both

reverse transcriptase and *Taq* DNA polymerase activities are compatible with NP40. To further ensure that mRNA, and not genomic DNA sequences, are amplified, primers for PCR are usually chosen that are specific to exons separated by at least one intron, such that the product amplified from a genomic DNA template would be of a much larger size, preferably so large as to not amplify efficiently.

PROTOCOLS

Solutions

PBS

- 0.85% w/v NaCl, 66 mM $NaPO_4$ (pH 7.0)

PCR buffer w/nonionic detergents and Proteinase K

- 50 mM KCl
- 10 mM Tris-HCl (pH 8.3) 2.5 mM $MgCl_2$
- 0.1 mg/ml gelatin
- 0.45% NP40
- 0.45% Tween 20

Autoclave and store frozen. When ready to use, thaw and add 0.6 µl of 10 mg/ml Proteinase K (in H_2O) per 100 µl of solution.

Lysis buffer*

- 0.32 M sucrose
- 10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$
- 1% Triton X-100

Isotonic high pH buffer

- 140 mM NaCl, 10 mM tris-HCl (pH 8.0), 15 mM $MgCl_2$

*from Buffone, G.J. and Darlington, GJ. (1985) *Clin. Chem.* 30(1):164-5.

Protocol A. Mononuclear Cells Purified from Blood on Ficoll-Hypaque Gradients, or Tissue Culture Cells[7]

If RBCs are <10% of cells, use this procedure. If there is >10% RBC contamination, see below.

1. If <5 ml of cells, bring cells to 10 ml with phosphate buffered saline (PBS) in Falcon #2099, 15-ml conical centrifuge tube, or comparable tube.

2. Centrifuge 100 x G for 2-5 min. Remove supernatant with pipet.
3. Resuspend cells in 10 ml PBS and repeat centrifugation/wash
4. Resuspend in a volume of "PCR buffer w/ nonionic detergents and Proteinase K" to give about $6x10^6$ cells per ml. Transfer to 1.5-ml Eppendorf microcentrifuge tube.
5. Incubate at 50-60°C for 1 hr.
6. Incubate at 95°C for 10 min to inactivate the proteinase.
7. Store frozen.

To effect PCR on the lysate produced in step 6, Protocol A, note that 25 µl of this lysate is about equivalent to one microgram of genomic DNA. Add this lysate in up to half the reaction volume of a PCR to a solution that is one-half the volume of the PCR and 1x in PCR buffer, and 2x in triphosphate, primers, and enzyme. Make up any deficiency in volume with water.

With RBC contamination, do one PBS wash as above, then resuspend in 1 ml of "lysis buffer," as in Protocol B below, and transfer to a 1.5-ml Eppendorf centrifuge tube. Do one spin, as below, remove supernatant and resuspend in "PCR buffer w/noninonic detergents and Proteinase K" (to give about $6x10^6$ cells/ml) and proceed as in step 4 above.

Protocol B. Whole Blood[7]

This procedure dissolves the cytoplasmic membrane and pellets nuclei. Therefore, cytoplasmic DNA is lost. Should yield about 20 µg of DNA.

1. Mix 0.5 ml blood with 0.5 ml "lysis buffer" in a 1.5-ml Eppendorf microcentrifuge tube.
2. Centrifuge 13,000 x G for 20 sec.
3. Remove supernatant with pipet and resuspend pellet, using vortex mixer, in 1.0 ml of "lysis buffer."
4. Repeat steps 2 and 3 twice.
5. Centrifuge 13,000 x G for 20 sec, remove supernatant, and resuspend in 0.5 ml of "PCR buffer w/nonionic detergents and Proteinase K."
6. Follow steps 5-7 above.

Protocol C. Clinical Swabs (M. Manos, Cetus Corp.)

1. Collect cervical, vulvar, or penile samples with a pre-wet (PBS) swab or cytobrush.
2. Place the swab into 2 ml PBS (with 2x concentration of Fungibac; Gibco) in 10- to 15-ml conical tube (Falcon #2099) or comparable. Sample can be kept at room temperature for 24 hr; if longer storage required, keep at 4°C.
3. Remove the swab and centrifuge tube for 5' at 2-3,000 rpm in clinical centrifuge to pellet cells. Remove supernatant by aspiration.

4. If red blood cells are present, resuspend in 1 ml lysis buffer, transfer to a 1.5-ml microcentrifuge tube and proceed as in step 2 of Protocol B above. The DNA concentration in the final resuspension depends on the number of cells present.
5. If no red blood cells are present, resuspend pellet in PCR buffer with nonionic detergents and Proteinase K as in Protocol A, step 4 (use 1% Laureth 1Z instead of NP40 and Tween 20 and Proteinase K at 200 µg/ml). Proceed from there through step 7.

Protocol D. Plucked Hairs

1. Cut off 0.5 cm of freshly plucked hair at root end. Use fine-tipped forceps and razor blade. A dissection scope may be helpful.
2. Place this 0.5-cm piece into 0.4 ml of PCR buffer with nonionic detergents and Proteinase K in 1.5-ml microcentrifuge tube.
3. Proceed as in Protocol A, steps 5-7. Use 50 µl lysate to effect PCR.

Protocol E. RNA from Blood Cells (E. Kawasaki, Cetus Corp.)

1. Prepare mononuclear cells from 1-2 ml blood by Ficoll-Hypaque or similar method.
2. Place mononuclear cells in 2-ml screwcap microcentrifuge tube; fill with PBS and pellet cells at 500 xG for 5 min.
3. Meanwhile, prepare DEP (diethylpyrocarbonate; Sigma - warning: causes irritation, take proper precautions) solution by diluting 1:9 in absolute EtOH then 1:999 in IHB that is 0.5% in NP40. Keep ice-cold.
4. Resuspend cell pellet in 200-400 µl of this solution. Vortex to mix.
5. Pellet nuclei by centrifugation at 13,000 xG for 10 sec.
6. Transfer supernatant to new tube and incubate at 37°C for 20 min, then 10 min at 90°C; keep the cap loose to allow gases produced from DEP to escape.
7. Pellet any precipitate that forms and transfer supernatant to new tube. Use 5-10 µl in reverse transcriptase (RT) reaction as described in Chapter 7.
8. If RT reaction fails, it may be because of residual DEP. Reheat sample at 90°C for 5 min and try again.
9. This method can be used for tissue culture cells as well, but use 5-10 x fewer cells as tissue cells have that much more RNA per cytoplasm.

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