Extraction of DNA from Whole Blood and White Blood Cells
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PRINCIPLE:

White blood cells (WBCs) are separated from a specimen of whole human blood (or from a "buffy coat" specimen that has been separated from a whole blood sample) by mixing the specimen with a hypotonic EDTA solution. The hypotonic solution lyses red blood cells, but leaves WBCs intact. WBCs are separated by centrifugation, forming a WBC "pellet" in the bottom of the centrifuge tube. The supernatant, containing hemoglobin, plasma proteins, and other soluble components from the lysed red cells or plasma is poured off leaving a relatively clean WBC pellet. The pellet is resuspended in the same hypotonic EDTA solution, and the tube is again centrifuged and decanted, in order to further wash away contaminating proteins and hemoglobin. The WBC pellet is resuspended in a small amount of NaCl solution, which helps break up the pellet and disperse the cells. An SDS solution is added to the WBC/NaCl suspension, which lyses the WBCs, releasing the DNA from the cell. The SDS also dissociates protein/DNA complexes. RNase is added to the solution to destroy RNA. Remaining proteins (including the RNase added in the previous step) are precipitated by phenol:chlorofrom:isoamyl alcohol wash. The DNA is precipitated from the clean aqueous phase with isopropyl alcohol, washed with ethanol, and then dissolved in Tris-EDTA solution. At this point the sample is ready for quantitation, storage, and/or experimental use.

SPECIMEN:

Either whole blood or white blood cells previously separated from whole blood can be used. The blood should be collected in vacutainers containing either ACD or EDTA to prevent DNA degradation, and should be refrigerated until processed. Up to 20 mL of blood may be processed in one tube in this protocol. The approximate yields are 16-50 ug DNA/mL whole blood. Optimum yields of DNA are achieved when the blood samples are processed within five days of being obtained from the patient. Usable DNA may be obtained from samples collected in heparin or without anti-coagulants (requires homogenization of clot), and/or samples that have been stored for longer than five days, but the quality and yield may be reduced.

REAGENTS AND MATERIALS:

Reagents needed to prepare Working Reagent solutions:

- **EDTA 0.5 M Solution, pH 8.0**, prepared with molecular biology grade EDTA disodium salt dihydrate ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$, FW 372.2). Sigma E7889 or equivalent, 100 mL ($22) is sufficient for approximately 250 samples. Store at room temperature.
- **Sodium Chloride**, **5 M Solution**. Prepared with molecular biology grade (DNase, RNase, protease free) NaCl, FW 58.44. Sigma S5150 or equivalent, 1

liter ($28.60) is sufficient for 5000 samples. Store at room temperature.

- **Lauryl Sulfate, 10% Solution.** (10% SDS, 10% sodium dodecyl sulfate). Prepared with molecular biology grade (DNase, RNase free) lauryl sulfate, sodium salt, $C_{12}H_{25}O_4SNa$, FW 288.4. Sigma L4522 or equivalent, 100 mL ($26) is sufficient for 285 samples. Store at room temperature.
- **Tris-EDTA Buffer 100X Concentrate**, (1 M Tris•HCl, 0.1 M EDTA, pH approximately 8.0) prepared with molecular biology grade reagents. Sigma T9285 or equivalent, 100 mL ($11) is sufficient for approximately 1400 samples. Store at room temperature.
- **Ribonuclease A,** free of DNase activity, Sigma R6513 or equivalent, 10 mg ($21) sufficient for 125 samples. (When the Sigma product is purchased in 10 mg amounts, the working solution can be prepared and stored in the vial supplied, rather than having to prepare a separate container.)
- **Sodium Acetate, 3 M Solution, pH 5.2**. Prepared with molecular biology grade (DNase, RNase, protease free) C2H3O2Na, FW 82.03. Sigma S7899 or equivalent, 500 mL ($20) is sufficient for 500 samples.
- **Phenol:chloroform:isoamyl alcohol 25:24:1, saturated with 10 mM Tris, pH 8.0, 1mM EDTA.** Sigma P3803 or equivalent, 400 mL ($98) is sufficient for 400 samples.
- **Isopropanol (isopropyl alcohol), 100%.** Sigma I9516, EM Science A426P-4, or equivalent. 4 L ($22) is sufficient for 570 samples.
- **Ethanol (ethyl alcohol), 95%.** Sigma E7148, AAPER Ethyl Alcohol USP, or equivalent. 1 gallon (3.8 L) ($8) is sufficient for 542 samples.

Preparation of Working Reagents:

- **RBC Lysis Solution** (1 mM EDTA). Dilute 2 mL of EDTA 0.5 M Solution in 998 mL deionized water. Final pH should be approximately 8.0. Store at room temperature.
- **NaCl Solution, 1 M.** Dilute 200 mL of Sodium Chloride, 5 M Solution in 800 mL deionized water. Store at room temperature.
- **Cell Lysis Solution** (10 mM Tris•HCl, 26 mM EDTA, 17.3 mM (0.5%) SDS). Dilute 10 mL Tris-EDTA Buffer 100X Concentrate and 50 mL EDTA 0.5 M Solution and 50 mL Lauryl Sulfate, 10% Solution in 890 mL deionized water. Final pH should be approximately 7.3. Store at room temperature.
- **Tris-EDTA Buffer Solution (TE Buffer).** (10 mM Tris•HCl, 1 mM EDTA, pH 8.0). Dilute 10 mL Tris-EDTA Buffer 100X Concentrate in 990 mL deionized water. Final pH should be approximately 8.0. Store at room temperature.
- **RNase A Solution**, (4 mg/mL). Dissolve 10 mg Ribonuclease A in 2.5 mL TE Buffer. Store at 2-8 degrees C.
- **Sodium Acetate Solution, 3 M.** This solution is used in the form it is purchased, no preparation is needed. Store at 2-8 degrees C.
- **Phenol:chloroform:isoamyl alcohol 25:24:1, saturated with 10 mM Tris, pH 8.0, 1mM EDTA.** This solution is used in the form it is purchased, no preparation is needed. Store at 2-8 degrees C.

- **Isopropanol (isopropyl alcohol), 100%.** This reagent is used in the form it is purchased, no preparation is needed. Store at room temperature.
- **Ethanol (ethyl alcohol), 70%.** Add 737 mL of Ethanol (ethyl alcohol), 95% to a 1000 mL graduated cylinder, and then add deionized water to bring the total volume to 1000 mL. Mix well. Store at 2-8 degrees C.

Other Equipment/Supplies needed:

Centrifuge capable of centrifuging 16mm blood collection tubes, 15 mL conical tubes and 50 mL conical tubes at 2000 x g.
Incubator capable of maintaining a temperature of 37 degrees C.
Vortex Mixer.
Transfer Pipets capable of transferring 2-3mL. Fisher cat. # 13-711-7 or equivalent.
Micropipet capable of dispensing 50 uL of solution accurately.
Pipets or Dispensers capable of dispensing 1-20 mL of solution.
15 mL Conical Centrifuge Tubes, Sarstedt cat. # 62.554.002 PP or equivalent.
50 mL Conical Centrifuge Tubes, Sarstedt cat. # 62.547.004 PP or equivalent.

PROCEDURE:

Caution:

Human blood is potentially infectious for hepatitis B, AIDS, or other bloodborne pathogens. Wear gloves and lab coat. Use face or eye protection from splash. Only persons trained in Bloodborne Pathogen procedures are allowed to perform this procedure, see Safety Officer.

Phenol is corrosive and can cause serious damage to eyes, skin and lungs by exposure from contact or inhalation. Wear gloves and lab coat. Use face or eye protection from splash. Use in ventilated enclosure or fume hood. Flush eyes or skin immediately if exposure occurs.

For whole blood specimens with a volume up to 20 mL or buffy coat specimens separated from up to 20 mL whole blood:

1. Mix the specimen well by inversion.

2. Pour the specimen into a 50 mL conical tube containing 20 mL of RBC Lysis Solution.

    - *If transferring a buffy coat specimen from a small tube with a transfer pipet, rinse the transfer pipet and tube with the RBC Lysis Solution to maximize yield.*

3. Add sufficient RBC Lysis Solution to bring the total volume in the tube to 45 mL.

4. Cap tube and mix well by inversion (>5 times). Incubate for 2 minutes at room temperature. Mix tube once more by inversion (>5 times).

5. Centrifuge tube at 2000 x g for 10 minutes at room temperature.

    - *If using a Beckman J6 centrifuge with a JS4.2 rotor**, 2000 x g = 2650 rpm***.

6. Carefully pour off supernatant into an appropriate biohazard container or a 1:1 solution of water and bleach, leaving the white blood cell pellet and a small amount of residual liquid in the bottom of the tube.

    - *To avoid losing white blood cells, don't invert the tube any more than is necessary to pour off most of the supernatant. Don't attempt to completely pour off every last drop of supernatant.*

7. Vortex tube to resuspend the white blood cells in the residual fluid.

8. Add 20 mL RBC Lysis Solution, cap tube, mix by inversion, and centrifuge again at 2000 x g.

9. Carefully pour off supernatant into an appropriate biohazard container or a 1:1 solution of water and bleach, leaving the white blood cell pellet in the bottom of the tube.

10. Add 1.0 mL NaCl Solution, 1 M.

11. Vortex tube **vigorously** to resuspend and disperse the white blood cells.

    - *There should be no cell clumps. Let tubes that contain stubborn clumps stand a minute or two and vortex again. Repeat until cell clump is well dispersed. This is a crucial step for good recovery of clean DNA.*
    - *Better mixing is achieved by multiple interrupted vortexing than is achieved by one continuous vortex. In any step where vigorous vortexing is used, it is desirable to vortex for a few seconds, then stop briefly to allow the vortex to collapse inside the tube, and then repeat this process two or more times.*

12. Add 6.0 mL of Cell Lysis Solution to the tube.

13. Add 50 uL RNase A Solution to the tube.

14. Cap and vortex gently to mix. Incubate at 37 degrees C. for at least 60 minutes to allow complete lysis of the WBCs and degradation of RNA.

    - *The minimum time needed to lyse the cells is until the solution is free of visible cell clumps and is homogeneous. This will require only a few minutes for most*

samples, particularly if the WBCs have been well dispersed in the previous step. Continuous mixing of the samples can enhance lysis, so the use of an orbital shaker or rotating mixer in the incubator is desirable if available.

- *Samples are stable in Cell Lysis Solution for at least 18 months at room temperature. Therefore this step can be a good stopping point for a day's work, and the incubation allowed to go overnight or over a weekend.*

15. Add 1.0 mL Sodium Acetate Solution, 3 M to each tube. Vortex briefly to mix.

16. Add 1.0 mL of Phenol:chloroform:isoamyl alcohol 25:24:1 to each tube. Cap the tube tightly and vortex vigorously for at least 10 seconds, periodically interrupting the vortex and inverting the tubes to mix the reagent with the sample.

17. Centrifuge tube at 2000 x g for 10 minutes at room temperature to separate phases.

    - *The phenol:chloroform:isoamyl is the lower phase, the interface boundary may contain some protein precipitate visible as a layer on top of the phenol phase, and the DNA is in the lysate solution, which is the upper phase.*

18. Allow samples to stand until the upper layer turns clear or 15 minutes which ever comes first. Transfer the upper phase, using a transfer pipet, to a 15 mL conical centrifuge tube containing 7.0 mL of Isopropanol, 100%. Avoid transferring any of the middle or lower phases. Cap and discard the 50 mL tube into a biohazard waste container.

    - *To avoid contaminating the DNA with phenol solution or proteins, it is necessary to leave behind some of the aqueous phase (usually about 1 mL).*

19. Cap the 15 mL tube tightly and invert it 25 times slowly to facilitate precipitation of the DNA. If DNA precipitation is visible, you may proceed directly to the next step. If no DNA is visible upon inversion, finish the 25 repetitions and allow the sample to stand overnight at 4 degrees C.

20. Centrifuge sample at 2000 x g for 5 minutes at room temperature.

21. Gently pour off the supernatant leaving the DNA pellet at the bottom of the tube. Add 10 mL of Ethanol, 70% to the sample. Cap the tube tightly and invert several times to wash the DNA pellet.

22. Centrifuge at 2000 x g for 3 minutes at room temperature.

23. Pour off ethanol being careful to keep the DNA pellet at the bottom of the tube. Keeping the tube inverted, carefully set the tube in a rack, making sure that the DNA pellet remains at the bottom of the tube. Allow the pellet to air dry for 10 - 15 minutes or until no droplets of moisture are visible in the tube.

- *The pellet may be loose at this step so pour carefully and watch pellet, tilting the tube in a slow, continuous motion. If the pellet begins to slide away and doesn't adhere to the wall of the tube, stop pouring, re-centrifuge, and pour off carefully again.*

24. Add 1000 uL of TE Buffer to the tube. (If the original sample volume was 10 mL or less of blood, add only 500 uL TE Buffer). Cap tightly. Vortex gently for 5-10 seconds. Allow the sample to stand overnight at 37 degrees C to dissolve the DNA pellet completely. Vortex gently to mix. Once the DNA pellet is dissolved fully the sample is ready for quantitation, storage, or testing.

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DNA Extraction Summary

Day 1:

1. Pour blood sample into 50 mL tube containing 20 mL RBC Lysis Solution.
2. Add RBC Lysis Solution to bring total volume to 45 mL.
3. Centrifuge 2650 RPM, 10 minutes, room temperature.
4. Pour off supernatant fluid carefully.
5. Add 20 mL RBC Lysis Solution, mix well.
6. Centrifuge 2650 RPM, 10 minutes, room temperature.
7. Pour off supernatant fluid carefully.
8. Add 1.0 mL NaCl Solution, 1.0 M.
9. Vortex vigorously, break up cell clump.
10. Add 6.0 mL Cell Lysis Solution.
11. Add 50 uL RNase A Solution.
12. Mix well, incubate at 37 degrees C. overnight on shaker.

Day 2:

13. Add 1.0 mL Sodium Acetate Solution, 3.0 M.
14. Add 1.0 mL Phenol:chloroform:isoamyl alcohol.
15. Mix thoroughly by vortex and inversion.
16. Centrifuge 2650 RPM, 10 minutes, room temperature.
17. Transfer upper phase to 15 mL tube containing 7.0 mL Isopropanol, 100%.
18. Centrifuge 2650 RPM, 5 minutes, room temperature.
19. Pour off supernatant.
20. Add 10 mL Ethanol, 70%.
21. Mix by inversion.
22. Centrifuge 2650 RPM, 3 minutes, room temperature.
23. Pour off supernatant **carefully**.
24. Add 1.0 mL TE Buffer.
25. Vortex gently, incubate at 37 degrees C. overnight on shaker.